Cell Separation

A PRACTICAL APPROACH

Edited by
D. FISHER, G. E. FRANCIS, and D. RICKWOOD

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Cell Separation

The Practical Approach Series

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Edited by

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Preface

Isolating specific cells or fractionating viable cell populations is an essential step in many techniques used in a wide area of biology and related disciplines. Whether you are a haematologist, cell biologist, or immunologist you will at some stage in your work need to separate cell populations and in the process you will often encounter problems even with the simplest of methods. Hence it is essential that everyone working in all areas of biological sciences knows what methods are available and even more importantly which method is likely to be most useful to them. The chapters in this book provide a comprehensive guide to the methods used to prepare cell suspensions and to separate viable cells depending on their different characteristics such as size, density, surface charge and immunological identity. Each chapter provides direct practical guidance for the various techniques and describes the advantages and limitations of each. The correct choice of method will determine whether or not the procedure is successful; readers should consider all of the options available before deciding which method would be the most appropriate. The editors have sought to ensure that this book will provide the reader with a comprehensive guide to cell separations and we would like to thank our authors for helping us to achieve this aim

London
January 1998

D.F.
G.E.F.
D.R.

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Abbreviations

A	plasma autologous plasma
A.I.	artificial insemination
BSA	bovine serum albumin
CCD	countercurrent distribution
CD	cluster of differentiation
CFES	continuous flow electrophoresis system
CMFSW	calcium- and magnesium-free sea water
DMEM	Dulbecco's modified Eagle's medium
DTT	dithiothreitol
EM	electrophoretic mobility
fa-free BSA	fatty acid-free bovine serum albumin
FCS	fetal calf serum
FFE	free flow electrophoresis
GBSS	Gey's balanced salt solution
HBSS	Hank's balanced salt solution
HI-FCS	heat inactivated fetal calf serum
HIHS	heat inactivated horse serum
HSA	human serum albumin
IMS	immunomagnetic separation
MES	2-(*N*-morpholino)ethanesulfonic acid
MN	mononuclear cells
MOPS	hydroxymethyl-amino methane 2-(*N*-morpholine)propane sulfonic acid
NDA	2-naphthol-6,8-disulfonic acid
n.s.	not specified
PBS	phosphate-buffered saline
PE	phycoerythrin
PEG	polyethylene glycol
PIP	4-(2-hydroxylethyl)piperazine ethanesulfonic acid
Plt	platelet
PMN	polymorphonuclear cells
PRBC	packed red blood cells
PVP	polyvinylpyrrolidone
RBC	erythrocytes
r.c.f.	relative centrifugal force
RI	refractive index
SEC	sinusoidal endothelial cells
TLCCD	thin-layer countercurrent distribution
TMPEG	tresyl monomethoxyPEG
UV	ultraviolet

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1

Preparation of single cell suspensions

K. M. CHEETHAM, N. SHUKLA, and B. J. FULLER

1. Introduction

The cell, as the basic functional unit within a tissue or organism, holds the potential for allowing us to probe the numerous interrelated processes which comprise overall function, or to manipulate specific pathways for scientific or commercial gain. As such, the rapid expansion over the last two decades in cell applications in fields such as biochemistry, molecular biology, and biotechnology has led to an equivalent effort in devising methods for cell isolation and handling. Although many important studies have been performed on continuously growing cell lines (which may often be commercially available), many other investigations can only be performed on cells from selected intact tissues, expressing particular differentiated functions. Thus the ability to liberate high yields of viable cells from the structural constraints of their native surroundings is often the first hurdle to overcome in experimental biology. Section 2 describes these methods of dispersal. These are then complemented by the need to ensure that the process has yielded individual cells which have retained metabolic and functional capabilities. Section 3 describes methods of viability assessment. Section 4 provides methods for separating viable and non-viable cells from the isolate, which invariably is a mixture of populations containing the desired cell type. Methods of cell characterization are described in Section 5. In the current chapter, we have addressed these particular problems, giving a variety of examples from currently used techniques.

2. Methods of dispersal

In certain limited situations described below, individual cells can be obtained from an existing suspending medium (for example red blood corpuscles from whole blood) or by simple washing procedures as in lavages. However, in most cases the cells of interest are locked into a three-dimensional arrangement within a particular tissue by cell:cell interactions (tight junctions, gap

junctions, desmosomes, etc.) and by cell:matrix interactions. In animal systems, the matrix consists of various fibrous proteins and glycosaminoglycan chains in an hydrated gel, with collagen forming the most abundant extracellular protein, whilst in higher plants pectins and hemicelluloses of the cell wall act in a similar fashion. A great deal has been learnt about these interactions in animal and plant systems (1), and the importance of divalent cations, particularly calcium, in promoting cell:cell and cell:matrix adhesions are well documented. Overall, this base of knowledge has been used to develop strategies to release cells from their local attachments. These can be divided into three broad categories:

(a) Mechanical, in which tissues are cut, minced, or squashed through filters to yield individual cells or small fragments from which cell outgrowth in culture can be encouraged.

(b) Chemical, in which the 'linking' divalent cations are washed out or chelated.

(c) Digestive, in which enzyme solutions are used to digest components of the extracellular matrix (e.g. proteases to attack the fibre-forming basement layers).

In reality, most current techniques are a mixture of the various approaches.

The overriding principle is that whatever method is proposed, care must be taken that the technique itself does not damage or destroy the very cells being sought for study. In general, in our experiences with animal cells, straightforward mechanical methods for producing cell suspensions result in low yields of poor quality cells, but can, of course, produce excellent results if outgrowth into culture is the desired aim, always taking into account that the latter approach may take several weeks to yield sufficient cell numbers for experimentation. Similarly, chelating agents to break the 'linking' bonds afforded by divalent cations do not of themselves produce large scale release of animal cells from most tissues. If large numbers of cells are required to be isolated rapidly, digestion of the matrix components is the most effective single strategy, but in practice many methods combine aspects of mechanical, chemical, and digestive approaches to maximize cell recovery.

In the following section, we shall give examples of methods for harvesting cells from pre-existing suspensions (most notably whole blood) or lavages (washes), and from animal or plant tissues. Methods have been outlined for specific cells, but they can be modified to provide approaches for most situations. It should always be remembered that use of human tissues imposes the possibility of encountering harmful infections, so that all the essential precautions (which are beyond the scope of this chapter), including use of suitable laminar flow cabinets (class 2 or 3), testing of samples, immunization of staff, proper disposal of wastes, etc., should be carefully thought through before commencing any studies. Likewise with use of animal tissues, each country has

regulations for the maintenance, care, and handling of laboratory animals with which the investigator must conform. In the UK this is covered by the Animals (Scientific Procedures) Act 1986.

2.1 Cell isolation from suspension

When cells are already in suspension, it is often possible to separate sub-populations of cell types based on differences in cell density, employing simple centrifugation methods, or methods enhanced by the use of density gradient techniques. Chapter 2 will give detailed accounts of theory and practice of the various centrifugal methods, but in this section we will list practical procedures for isolating single cell suspensions from blood or lavages, two of the most commonly encountered instances where cells need to be isolated from pre-existing suspensions. In the case of blood cells, methods using commercially available media will be described as illustrations of the methods listed in Chapter 2, *Table 11*, and to complement the protocols described in Chapter 2.

2.1.1 Blood cells

i. Lymphocytes

Protocol 1 describes a method based on that of Boyum (2) using Lymphoprep.

Protocol 1. Isolation of lymphocytes

Equipment and reagents

- 11 ml round-bottomed plastic centrifuge tubes (Gibco)
- Glass Pasteur pipettes (BDH)
- 10 ml pipettes (Sterilin)
- Bench-top centrifuge
- Heparin Injection B.P. (Monoparin) 1000 U/ml (Fisher)
- Lymphoprep™ (Nycomed UK)
- RPMI 1640 medium with L-glutamine (Gibco)
- 1 M Hepes buffer solution pH 7.3 (Gibco)
- Pen/strep solution: 10000 U/ml penicillin and 10000 μg/ml streptomycin in normal saline (Gibco)

Method

1. Collect 21 ml of venous blood into a sterile tube containing 200 U of heparin and mix well.
2. Pipette 3.5 ml of Lymphoprep solution into seven centrifuge tubes and slowly pipette 3 ml of heparinized blood down the side of each centrifuge tube so that it floats on top of the Lymphoprep.
3. Centrifuge at 800 *g* for 20 min at room temperature.
4. With a glass Pasteur pipette take off the interface layer of lymphocytes (middle layer).[a]
5. Wash the cells in RPMI 1640 medium containing 1 ml pen/strep and 1 ml of 1 M Hepes per 100 ml. Use this medium throughout the procedure.

Protocol 1. *Continued*

6. Place the lymphocytes in four centrifuge tubes containing 2 ml of medium per tube. Mix and fill the tubes with medium.
7. Centrifuge at 500 *g* for 15 min at room temperature.
8. Remove the supernatant and resuspend the cells in medium, making two tubes from the four.
9. Centrifuge at 500 *g* for 15 min at room temperature.
10. Remove the supernatant and resuspend the lymphocytes in 1.5 ml of medium per tube.
11. Combine the contents of the two tubes to give the final lymphocyte preparation.[b]
12. Calculate cell yield and viability using an acridine orange/ethidium bromide (Sigma) mixture under fluorescence microscopy.
    (a) Make a 0.001% (w/v) solution of acridine orange and a 0.02% (w/v) ethidium bromide solution in phosphate-buffered saline (PBS).
    (b) Take equal volumes of each and dilute 1/10 with PBS for use.

    Viable cells fluoresce green and non-viable orange.

[a] Once the lymphocytes have been removed the pellet at the bottom of the tube is the erythrocyte fraction. Discard the top 2 mm of the pellet which will contain polymorphonuclear cells.The separation media contains polysaccharides which will cause aggregation of erythrocytes on resuspension. Another method which does not cause aggregation is described in *Protocol 2.*
[b] It is possible to separate the final lymphocyte preparation into its subfractions of T and B lymphocytes using nylon columns. This was described in 1973 (3). More recently it has become possible to purchase pre-packed columns (Nycomed UK) which give consistently reproducible results.

ii. Erythrocytes

A method for the isolation of erythrocytes is described in *Protocol 2* using NycoPrep 1.150.

Protocol 2. Isolation of erythrocytes

Equipment and reagents

- 11 ml round-bottomed plastic centrifuge tubes (Gibco)
- Glass Pasteur pipettes (BDH)
- 20 ml Universal container (Sterilin)
- Bench-top centrifuge
- NycoPrep™ 1.150 (Nycomed UK)
- Isotonic phosphate-buffered saline (PBS) (Gibco)
- 3.8% trisodium citrate solution (w/v) in water (BDH)

Method

1. Add 9 ml of venous blood to a Universal container containing 1 ml of 3.8% trisodium citrate solution.

2. Spin at 100 *g* for 30 min to sediment the erythrocytes, then discard the supernatant and the top 2 mm of the pellet, which will contain platelets and white blood cells.
3. Mix one part NycoPrep 1.150 and one part PBS to form solution A.
4. Mix one part NycoPrep 1.150 and one part solution A to form solution B.
5. Mix two parts solution A and one part solution B to form solution C.
6. Mix one part solution A and two parts solution B to form solution D.
7. Add 1.5 ml solution A to a centrifuge tube. With a glass Pasteur pipette apply 1.5 ml solution C at the bottom of the tube underneath solution A. Next apply 1.5 ml solution D in the same way to the bottom of the tube. Lastly apply 1.5 ml solution B to the bottom of the tube as before. This yields a discontinuous gradient with the least dense at the top of the tube and the most dense at the bottom.
8. Layer 0.25 ml of packed erythrocytes on to the gradient.
9. Centrifuge at 500 *g* for 30 min at room temperature.
10. Harvest the erythrocytes banded at each interface layer using a glass Pasteur pipette. Each band represents erythrocytes of differing ages, the older cells being more dense and therefore banding at higher buoyant densities than younger cells.
11. Wash the cells in isotonic solutions.

iii. Monocytes

Protocol 3 describes a method for the isolation of monocytes that works on the basis that lymphocytes are more sensitive to osmotic change than monocytes, and uses NycoPrep 1.068 (4).

Protocol 3. Isolation of monocytes

Equipment and reagents

- 11 ml round-bottomed plastic centrifuge tubes (Gibco)
- Glass Pasteur pipettes (BDH)
- 10 ml pipettes (Sterilin)
- *Bench-top centrifuge*
- NycoPrep™ 1.068 (Nycomed UK)
- 1 M Hepes buffer solution pH 7.3 (Gibco)
- Heparin Injection B.P. (Monoparin) 1000 U/ml (Fisher)
- RPMI 1640 medium with L-glutamine (Gibco)
- Pen/strep solution: 10000 U/ml penicillin and 10000 μg/ml streptomycin in normal saline (Gibco)

Method

1. Collect venous blood in a sterile tube containing 10 U/ml heparin.
2. Pipette 3 ml NycoPrep 1.068 into a centrifuge tube and slowly pipette

Protocol 3. *Continued*

3 ml of heparinized blood down the side of the tube so that it floats on top of the NycoPrep.

3. Centrifuge at 600 *g* for 15 min at room temperature.[a]
4. Using a glass Pasteur pipette remove the monocyte layer. This appears as a diffuse band at the sample medium interface. It is important to leave the monocytes closest to the pellet behind to reduce lymphocyte contamination.
5. Wash the cells in the same way as for lymphocytes described in *Protocol 1*, steps 5–11.

[a] The time and speed required to produce the monocyte layer is critical and any deviation from it will cause pelleting of the monocytes.

iv. Granulocytes

Protocol 4 describes a method for the isolation of granulocytes using Polymorphprep.

Protocol 4. Isolation of granulocytes

Equipment and reagents

- 11 ml round-bottomed plastic centrifuge tubes (Gibco)
- Glass Pasteur pipettes (BDH)
- 10 ml pipettes (Sterilin)
- Bench-top centrifuge
- Polymorphprep™ (Nycomed UK)
- Heparin Injection B.P. (Monoparin) 1000 U/ml (Fisons)
- 0.9% sodium chloride (w/v)
- 0.45% sodium chloride (w/v)

Method

1. Collect venous blood in a sterile tube containing 10 U/ml heparin.
2. Pipette 5 ml of Polymorphprep into a centrifuge tube and slowly pipette 5 ml of heparinized blood down the side of the tube so that it floats on top of the Polymorphprep.
3. Centrifuge at 600 *g* for 40 min at room temperature.
4. Using a glass Pasteur pipette remove the granulocyte layer. This can be found by first locating the band of mononuclear cells (middle layer). The granulocytes can be found in a band approx. 5 mm below this.[a]
5. Place the cells in a centrifuge tube and add an equal volume of 0.45% sodium chloride to restore the osmotic balance.
6. Fill the centrifuge tube with 0.9% sodium chloride and centrifuge at 250 *g* for 5 min at room temperature.

7. Reconstitute the pellet in an isotonic medium of choice to give the final granulocyte preparation.

[a] Three cell types constitute the granulocyte fraction, i.e. eosinophils, basophils, and neutrophils. These tend to be harder to isolate from each other since they constitute a relatively small proportion of the total white cell population. However, methods have been described for their isolation: eosinophils (5), basophils (6), and neutrophils (7).

v. Platelets

Protocol 5 describes a method using NycoPrep 1.063 and is based on a method described by Ford *et al.* (8).

Protocol 5. Isolation of platelets

Equipment and reagents

- 11 ml round-bottomed plastic centrifuge tubes (Gibco)
- Glass Pasteur pipettes (BDH)
- 10 ml pipettes (Sterilin)
- Bench-top centrifuge with swing-out rotor
- NycoPrep™ 1.063 (Nycomed UK)
- 3.8% trisodium citrate solution (w/v) in water (BDH)

Method

1. Add 4.5 ml of venous blood to 0.5 ml trisodium citrate solution.
2. Pipette 4 ml of NycoPrep 1.063 into a centrifuge tube and slowly pipette 4 ml of citrated blood down the side of the tube so that it floats on top of the NycoPrep 1.063.
3. Centrifuge at 350 *g* for 15 min at room temperature to produce a pellet of erythrocytes at the bottom of the tube with a layer of leucocytes resting on top of the pellet.[a] Immediately above this is the platelet layer.
4. Using a glass Pasteur pipette remove the platelet layer. Since most of the platelets are recovered closest to the sample medium interface, leave the platelets closest to the erythrocyte pellet behind, thereby reducing contamination by leucocytes.[b]

[a] It is essential that the time and speed of centrifugation is strictly adhered to in order to produce high cell yields.
[b] This method produces platelets of sufficient purity for most applications. However, if necessary a further washing procedure is described in *Protocol 6*.

Protocol 6. Platelet washing

Equipment and reagents

- 11 ml round-bottomed plastic centrifuge tubes (Gibco)
- Bench-top centrifuge
- Sodium chloride Analar (BDH)

Protocol 6. *Continued*

- Potassium chloride Analar (BDH)
- Hepes (Aldrich)
- EGTA (Sigma)
- Prostaglandin E1 (Sigma)
- Glucose Analar (BDH)
- Bovine serum albumin (Fraction V, Sigma)
- Disodium hydrogen orthophosphate Analar (BDH)
- Potassium dihydrogen orthophosphate Analar (BDH)
- Magnesium chloride Analar (BDH)
- Tris buffer base Analar (BDH)

Method

1. Make solution A containing (per litre): 140 mmol sodium chloride, 2.7 mmol potassium chloride, 3.8 mmol Hepes, 5 mmol EGTA, 1 μmol prostaglandin E1,[a] 1 g glucose, and 1 g bovine serum albumin, pH to 7.6 with Tris base.
2. Make solution B containing (per litre): 140 mmol sodium chloride, 2.7 mmol potassium chloride, 8 mmol disodium hydrogen orthophosphate, 1.5 mmol potassium dihydrogen orthophosphate, 0.1 mmol magnesium chloride, 0.1 mmol Hepes, 7 mmol EGTA, and 1 mmol glucose, pH to 7.6 with Tris base.
3. Place the platelet suspension in a centrifuge tube and fill the tube with solution A, mix gently, and centrifuge at 800 *g* for 10 min at room temperature.
4. Remove and discard the supernatant.
5. Resuspend the platelets in solution B.

[a] The prostaglandin E1 is an essential component of solution A since it prevents platelet clumping during centrifugation.

2.1.2 Lavages

It is known that elevated levels of white blood cells are present in certain inflammatory diseases. A lavage is a flushing technique mainly of the lungs and peritoneum, which allows recovery of all cell types present. Examination of the cell population allows clinicians to assess the status of patients with inflammatory diseases. In cases of trauma peritoneal lavages can be a good indicator of intraperitoneal injury. The technique is a useful research tool to study the various types of cells harvested and their functions. *Protocols 7*and *8* describe methods that provide cell suspensions that contain the following cell types:

- epithelial cells
- mast cells
- lymphocytes
- neutrophils
- eosinophils
- monocytes

Protocol 7. Bronchial alveolar lavage

Equipment and reagents

- 50 ml polypropylene centrifuge tubes (Gibco)
- Bench-top centrifuge
- RPMI 1640 with L-glutamine (Gibco)
- Pen/strep solution: 10000 U/ml penicillin and 10000 μg/ml streptomycin in normal saline (Gibco)
- 0.9% (w/v) sterile normal saline (Gibco)

Method

1. Insert a bronchoscope using the standard clinical technique.
2. Flush the lung with 60 ml of normal saline pH 7.3 at 37°C three times via the bronchoscope.
3. After each addition gently suck out the fluid and transfer to 50 ml centrifuge tubes.
4. Centrifuge at 450 *g* for 7 min at room temperature.
5. Resuspend the cell pellet in 10 ml of RPMI 1640 containing 100 μg/ml streptomycin and 100 U/ml penicillin, then fill the tubes with medium.
6. Centrifuge at 450 *g* for 7 min at room temperature.
7. Resuspend the cells in 5 ml of RPMI medium supplemented with penicillin and streptomycin.

Protocol 8. Peritoneal lavage

Equipment and reagents

- See *Protocol 7*

Method

1. Insert a drain catheter into the peritoneum using the standard clinical technique.
2. Infuse a suitable volume of normal saline at 37°C.[a]
3. Massage the abdomen gently.[b]
4. Allow the fluid to exit under gravity whilst applying gentle pressure to the abdomen.
5. Transfer the fluid to centrifuge tubes and centrifuge at 450 *g* for 7 min at room temperature.
6. Resuspend the pellet in 10 ml RPMI 1640 containing 100 μg/ml streptomycin and 100 U/ml penicillin, then fill the tubes with medium.
7. Centrifuge at 450 *g* for 7 min at room temperature.

Protocol 8. *Continued*

8. Resuspend the cells in 5 ml RPMI medium supplemented with penicillin and streptomycin.

[a] Average volumes used and recovered for the human are 500–1000 ml and for the rat/guinea-pig are 50–100 ml.
[b] The lavage fluid must not be left in the abdomen for more than 3 min or absorption will occur.

2.2 Isolation of cells from solid tissues

2.2.1 Mechanical tissue disruption and outgrowth in culture: vascular smooth muscle cells

Protocol 9 describes isolation of vascular smooth muscle cells which depends largely on mechanical tissue disruption, supplemented by a period of outgrowth into culture. Cell culture is an area of study in its own right, with requirement for specialized equipment, substrates, and procedures. Comprehensive descriptions can be found in ref. 9. *Protocol 9* is based on the method reported by Chamley-Campbell *et al.* (10).

Protocol 9. Preparation of vascular smooth muscle cells

Equipment and reagents

- Laminar flow-hood[a]
- Large sterile plastic Petri dish (200 ml)
- Sterile surgical instruments and scalpel
- Cell incubator with CO_2 control
- Dulbecco's minimal essential medium (MEM) plus glucose (Sigma)
- Fetal bovine serum (FBS) (Sigma) for inclusion at 10% (v/v)

Method

1. Whenever possible carry out all procedures in the laminar flow-hood.
2. Collect a specimen of saphenous vein, immersed in sterile Dulbecco's MEM.[b]
3. Place the vein in a Petri dish and grip the end of the vein with forceps, strip downwards with a scalpel to remove the adventitia.
4. Using scissors, open the lumen of the vein and cut into 0.5 cm lengths. Take each segment, grip one corner with forceps, and scrape across the surface with a scalpel (five to ten strokes) to remove the remaining connective tissue. Invert the segment and repeat to remove the endothelial layer.
5. Cut the remaining vein segments into small pieces (1–2 mm^2) with the scalpel.

6. Place 20–30 pieces in 25 cm^2 flasks. Add 2–3 ml Dulbecco's MEM plus glucose and 10% fetal bovine serum. Change the medium, observing sterile techniques, every three to four days. Cell outgrowth takes two to four weeks.

[a] For human tissues, an appropriate laminar flow-hood with safety features (class 2) must be used.
[b] As always when using human tissues, local ethical approval should be obtained. A similar protocol can be used for vessels from animal sources.

2.2.2 Mechanical and digestive dispersal: microvascular endothelial cells

Protocol 10 describes a combination of mechanical and digestive dispersion in the isolation of microvascular endothelial cells from omental fat. Fat is richly supplied by a network of small blood vessels, and as such is an ideal source of endothelial cells from a microvascular environment, which may differ phenotypically from endothelial cells in large blood vessels or the commonly used umbilical vein. The method is based on that described by Kern *et al.* (11).

Protocol 10. Preparation of endothelial cells from omental fat

Equipment and reagents

- Laminar flow-hood suitable for human material (class 2)
- Cell incubator with CO_2 control
- Bench centrifuge
- Sterile surgical instruments and scalpel
- 30 ml sterile plastic pots and wide-bore 10 ml pipettes
- Nylon mesh sieves (pore sizes 250 μm and 30 μm)
- Large (200 ml) sterile plastic Petri dishes
- 25 cm^2 tissue culture flasks (Falcon)
- 37 °C cabinet or hot-box
- Mechanical bottle roller or rotary shaker
- Sterile Dulbecco's phosphate-buffered saline (Sigma)
- Sample of omental fat (30–50 g) collected in a sterile pot
- Medium 199 and fetal bovine serum (Gibco)
- Collagenase (Type IV, Sigma)

Method

1. Whenever possible carry out all procedures in a laminar flow-hood.
2. Wash the fat sample with PBS and trim away large blood vessels with scalpel and forceps.
3. Place the washed fat into the large Petri dish and mince to a slurry using two scalpels cross-slicing in opposite directions.
4. Add an equal volume of 0.1% (w/v) collagenase dissolved in M199 medium and pre-warmed. Place in sterile 30 ml pots on a mechanical roller in a cabinet at 37 °C for up to 30 min. Check every 5 min and shake vigorously. Digestion to a yellow translucent solution signals the end-point.

Protocol 10. *Continued*

5. Add an equal volume of M199 medium containing 20% fetal bovine serum albumin to neutralize the collagenase, and filter through the 250 μm mesh.
6. Allow the filtrate to stand in a sterile pot in the laminar flow-hood. A two-phase system develops over 10–20 min.
7. Remove the top layer (containing adipose cells) and discard. Spin the remaining solution at 400 *g* for 5 min.
8. Resuspend the pellet in 1 ml of M199 medium, and pass through the 30 μm mesh. The microvascular blood vessel fragments collect on the mesh.
9. Wash the mesh filter thoroughly with 5 ml M199 medium plus 5% FBS to resuspend the vessel fragments. Add 25 ml of M199 plus 5% FBS into a 30 ml sterile plastic pot, and carefully layer on the 5 ml suspension with a wide-bore plastic pipette. Leave to stand for 10 min and discard the top 10 ml.
10. Spin the remaining supernatant at 400 *g* for 5 min.
11. Discard the supernatant and resuspend vessel fragments in 5 ml M199 plus 10% FBS.
12. Add to a 25 cm^2 sterile tissue culture flask. Change the medium every two to three days. Endothelial cells with a typical 'cobblestone' morphology grow out from the fragments over one to two weeks.

2.2.3 Mechanical disruption and digestive dispersion: peripheral neuronal cells

Other isolation methods combining mechanical disruption and enzymatic digestion have been developed for a variety of animal cell types. For example, in neurobiology methods of isolation and culture of specific cell types derived from the largely heterogeneous nervous system. One such method, for isolation of peripheral neurones from dorsal root ganglia, has been described (12), and is the basis for *Protocol 11*.

Protocol 11. Isolation of neurones from rat dorsal root ganglia

Equipment and reagents

- Bench-top centrifuge
- Laminar flow-hood
- Dissecting microscope
- Sterile surgical instruments including scalpels, toothed forceps, fine-pointed scissors, curved microsurgical scissors, and watchmaker's forceps
- Cell incubator with CO_2 control
- Autoclave
- Sterile plastic tissue culture dishes (150 mm and 35 mm diameters)
- Conical 15 ml plastic centrifuge tubes (Costar)
- Collagenase (Type IV, Sigma)

- Sterile glass fire-polished Pasteur pipettes (BDH) treated with siliconization solution (Sigma)
- Sterile Dulbecco's phosphate-buffered saline without Ca^{2+} and Mg^{2+} (Gibco)
- Trypsin (2 × crystallized, Worthington)
- Soybean trypsin inhibitor and DNase (Sigma)
- Sterile tissue culture medium: a variety of media can be used, including Eagle's minimal essential medium (MEM), Dulbecco's modified Eagle's medium (DMEM), Ham's F12 or F14 (12) media, with 10% heat inactivated horse serum (Gibco)
- 70% (v/v) alcohol solution

Method

1. Sacrifice an adult rat by decapitation, and swab the dorsal surface liberally with 70% alcohol as a disinfectant. Using a sterile scalpel with a No. 22 blade, make a single incision along the entire dorsal midline (over the vertebral column) working down from the neck end. Peel back the skin to expose the vertebral column, free the column by cutting along either side (at a distance of 5–10 mm from the column). Working down from the neck, lift the vertebral column free and place it in a large plastic tissue culture dish.
2. Working in the laminar flow-hood, grasp the vertebral column firmly with toothed forceps, and cut out a 3–4 mm wide strip of bone from the dorsal roof of the column using sharp fine-pointed scissors. Advance the cut down from the neck until the column becomes too narrow for the scissors. Keep this 'opening' along the centre of the column to avoid damaging the ganglia (which lie on either side of the spinal cord when looking down on the dorsal surface).
3. Without removing the spinal cord, use the fine-pointed scissors to make a single cut along the midline of the ventral (underneath) aspect of the vertebral column, effectively now dividing it into two 'halves'.
4. Under the dissecting microscope, gently displace the spinal cord from each 'half' of the coulmn, working 1–2 cm at a time down from the neck. This exposes the dorsal root ganglia from their protective bony cavities, and they are visually identified as the pronounced bulbous swellings on each nerve trunk, slightly more translucent in appearance than the white nerve element. Carefully excise ganglia by cutting the nerve trunk on either side, using microsurgery scissors and watchmaker's forceps. With practice, up to about 40 ganglia can be recovered from a single adult rat.
5. Collect the ganglia into 2 ml of medium plus 10% horse serum in a 35 mm plastic Petri dish. Continuing under the dissecting microscope and using the microsurgical instruments, clean the ganglia free from residual nerve trunk and capsular tissues. This 'cleaning' procedure is important because it aids enzymatic digestion and ultimately reduces the severity of the mechanical shearing required to dissociate the tissue (which improves cell yields).
6. Transfer the ganglia into fresh pre-warmed medium containing the col-

Protocol 11. *Continued*

lagenase in a clean 35 mm plastic Petri dish. The collagenase concentration should be of the order of 0.125% (w/v), but this will depend on the activity of a particular batch of the enzyme, so this should be tested in pilot experiments. Expose the ganglia for 1.5 h at 37 °C to collagenase solution, carefully remove the solution, and add another 2–3 ml of fresh enzyme solution. Incubate for a further 1.5 h.

7. Transfer the ganglia to a sterile 15 ml plastic conical centrifuge tube, pellet the ganglia by low speed centrifugation (200 *g* for 4 min), and wash twice with sterile Ca^{2+}- and Mg^{2+}-free PBS. Resuspend the ganglia in 2–3 ml of PBS containing 0.25% (w/v) trypsin and expose for 30 min. Wash three times in medium plus 10% horse serum. Finally resuspend in 2 ml medium plus horse serum, DNase (80 μg/ml), and soybean trypsin inhibitor (100 μg/ml).[a]
8. Produce a single cell suspension by trituration of the digested and softened ganglia by sucking up the mixture and expelling it six to ten times through the barrel of a sterile fire-polished siliconized Pasteur pipette. The ganglia should break up fairly readily, and a cloudy suspension of cells should appear. Excessive mechanical force will reduce the yield of viable neurones and indicates that adjustments to the digestion process may be required.

[a] Use good quality trypsin for digestion. DNase and soybean trypsin inhibitor may not be essential; this depends on the severity of enzymatic digestion and the quality of cells liberated, which can be evaluated in pilot experiments.

In practiced hands between 1.5–2.5 $\times$ 10^5 neurones can be produced from a single adult donor. The isolated cell suspension can be used for immediate culture, which will yield a mixed culture of neuronal and non-neuronal cells; further enrichment of neurones can be achieved using a combination of selective adhesion (of non-neuronal cells) and differential centrifugation (12).

2.2.4 Tissue swelling and digestive dispersion: renal proximal tubule cells

For larger tissues or organs, dispersal is only poorly achieved by placing fragments into enzyme solutions. By far the most efficient digestion results from introducing the enzyme solution into the vascular bed, so that it reaches as much of the peri- and intercellular space as possible. Good digestion is also often achieved by producing 'tissue swelling' during introduction of the enzyme solution by increasing hydrostatic pressure (e.g. by infusion rapidly from a syringe or pump). Such procedures are employed in a method for isolating renal proximal tubule cells (13), which also depends upon the spatial localization of desired cells in one region (the cortex) of the kidney (*Protocol*

12). Again, the digestion is reinforced by mechanical homogenization and final outgrowth of cells in culture.

Protocol 12. Separation of renal proximal tubule cells

Equipment and reagents

- Bench-top centrifuge
- Laminar flow-hood
- Magnetic stirrer
- Glass Dounce homogenizer and pestle, loose bore
- Plastic-coated magnetic stirrer bars and stirrer plate
- Hot plate
- Autoclave
- Cell incubator with CO_2 control
- Sterile plastic culture flasks (Falcon, 50 cm^2)
- Glass beakers sterilized by dry heat
- Sterile 50 ml syringe and polished smooth stainless steel cannula (o.d. 3 mm)
- Surgical instruments, scalpel, etc.
- Box of ice
- Nylon screening fabric (250 μm and 85 μm pore sizes)
- Culture medium (CSFM): to ISFM add bovine insulin (5 μg/ml), human transferrin (5 μg/ml), and hydrocortisone (5×10^{-8} M) on day of culture
- Serum-free medium (SFM): for 6 litres use 31.8 g powdered Ham's F12 medium (Gibco), 40.1 g powdered Dulbecco's MEM medium (Gibco), 21.5 g Hepes, and 0.0072 g $NaHCO_3$, stored frozen. On the day of use, thaw and add (for 2 litres) 2.2 g penicillin and 0.24 g streptomycin (Gibco) to form isolation medium (ISFM).
- Hypnorm (Janssen)
- Iron oxide for perfusion: dissolve 2.6 g NaOH and 20 g KNO_3 in 100 ml sterile, oxygen-saturated water, and dissolve 9 g $FeSO_4$ in 100 ml sterile, oxygen-saturated water. Mix the solutions and boil for 20 min. Stand on magnetic plate to collect the precipitate, remove the supernatant, and wash with fresh sterile water. Repeat five times. Resuspend in 2 litres of sterile 0.9% NaCl solution, and autoclave. On day of use, dilute 5 ml to 100 ml sterile saline.
- Collagenase (Type IV, Sigma) and soybean trypsin inhibitor (Sigma): add 0.05 g of each to 100 ml ISFM medium, warm to 37 °C

Method

1. Anaesthetize male albino rabbit with intravenous injection[a] of barbiturates (e.g. Hypnorm). Shave the abdomen, expose the kidneys via a midline incision. Remove kidneys with 1 cm of renal artery attached; administer terminal anaesthetic dose.
2. Place the kidneys in a beaker of chilled ISFM and transfer to the laminar flow-hood.
3. Cannulate each renal artery with the polished cannula attached to a 50 ml syringe containing iron oxide solution; flush through each kidney with 30 ml iron oxide solution by slow injection (over 1–2 min).
4. Wash kidneys in fresh ISFM, peel off the outer capsule with forceps, and cut the kidneys transversely into disks (0.5–1 cm thick). Dissect out the cortex.[b]
5. Homogenize the pieces of cortex in 10 ml ISFM (four strokes of the Dounce homogenizer).
6. Sieve the homogenate, first through the 250 μm mesh. 'Massage' gently with a glass rod on the filter. Pass the filtrate through the 85 μm mesh to collect the tubule fragments (on top). Wash through with

Protocol 12. *Continued*

50 ml ISFM. Scrape off the pellet and resuspend in 50 ml ISFM in a sterile plastic pot.

7. Add a sterile magnetic stirrer bar to collect iron-laden glomeruli. Remove gently after 2–3 min. Repeat with a second magnetic bar.
8. Add soybean trypsin inhibitor (0.05 mg/ml) and collagenase (0.05 mg/ml). Incubate for 5 min at 37 °C with occasional agitation of the suspension.
9. Centrifuge (200 *g*) for 5 min, remove the supernatant, add fresh ISFM, and repeat the washing step.
10. Resuspend final pellet in 400 ml CSFM. Agitate to keep in suspension, and transfer to sterile culture flasks in a cell incubator with CO_2 control. Change medium after two days, then every three to four days. Primary cultures of tubule cells with 'domed' morphology develop over several days.

[a] Rabbits (2–3 kg in weight) may be sedated by intramuscular injection of 0.4 ml Hypnorm, followed after a period of 15–20 min by intravenous injection of barbiturates via a butterfly needle (25 gauge) placed in an ear vein.
[b] The cortex can be easily seen as a pale coloured rim of tissue around the outside of each tissue disk.

2.2.5 Continuous perfusion of digestive enzymes: hepatocytes

In some cases, excellent tissue dispersal can be achieved by continuously perfusing the vascular bed with digestive enzyme solution, which ensures good exposure of all intercellular spaces to the enzyme. Such an approach is frequently used to isolate hepatocytes from animal or human liver samples, combined with a prior chemical step to perfuse out the 'linking' divalent cations, and final mechanical disruption. *Protocol 13* describes a method based on that described by Seglen (14). The liver is also an appropriate organ for this approach because the main cell type of interest (hepatocytes) are by far the most numerous cells and are distributed in equal numbers throughout the whole organ. It is possible, however, to isolate the other, less numerous, cell types (endothelial cells and Kupffer cells) by modifications of the same technique (15).

Protocol 13. Preparation of rat hepatocytes

Equipment and reagents

- Laminar flow-hood
- Bench-top centrifuge
- Cylinder of oxygen gas
- A stainless steel sieve (approx. pore size 1 mm), sterilized by autoclaving
- Nylon mesh sieve (100 μm pore size)
- Sterile 30 ml plastic pots and wide-bore plastic pipettes
- Sterile surgical instruments and a sterile nylon cannula (o.d. 1.5 mm)

- Roller pump and sterile circuit made from nylon/silicone tubing (sterilized by washing or autoclaving): a variety of combinations can be used, as long as the system can recirculate medium at flow rates of 40–60 ml/min, usually perfusing the liver from a hydrostatic pressure head of 30–50 cm H_2O
- Water-bath at 37–40°C
- Laboratory glassware sterilized by dry heat
- Haemocytometer slide for cell counting
- Hypnorm (Janssen)
- 500 ml Ca^{2+}- and Mg^{2+}-free Hank's balanced salts solution (Gibco)
- Leibovitz L15 medium, 1.19 g Hepes buffer, and 1 ml insulin (100 U/ml), pH to 7.4 with 1 M NaOH

Method

1. Anaesthetize a rat (200–300 g body weight) by inhalation anaesthetic and intramuscular injection of barbiturates (e.g. Hypnorm).
2. Warm the bottles of Hank's BSS and L15 medium in the water-bath at 37°C. Bubble 100% oxygen gas into both for 15–20 min. Place the pump input tubing into the L15 medium and fill the circuit plus attached cannula.
3. Shave the abdomen, expose the liver via a midline incision, and insert the cannula into the portal vein.
4. Switch on the pump at flow rate of 20–30 ml/min, and cut the vena cavae below the liver to allow outflow.[a] Sacrifice the animal by cardiac section.
5. Pump 150 ml of L15 medium through the liver to wash out the blood.[b] Stop the pump briefly, switch the pump input tubing into the Hank's BSS solution, and pump 500 ml of warmed solution through the liver at a flow rate of 40–50 ml/min.[c] During this time, dissolve collagenase (0.1 U/ml) in the remaining L15 medium and add to the reservoir of the recirculating system.
6. Stop the pump, remove the liver (with the cannula still tied in place) from the carcass. Attach the cannula to the recirculating system and perfuse for up to 20 min with the collagenase solution.
7. Periodically, check the progression of digestion by pressing the liver lobes with a smooth-ended probe or glass rod. The appearance of fluid-filled indentations under the probe (as the cells 'float apart') signals the end-point.
8. Remove the liver with 20–30 ml of collagenase solution into a sterile 200 ml plastic Petri dish and place in the laminar flow-hood. Gently tease apart the liver lobes using sterile scalpel and forceps. Pass the slurry through the sterile stainless steel filter into a sterile plastic pot. Pipette the slurry up and down two or three times with a wide-bored plastic pipette to enhance cell separation, and divide the slurry into two 30 ml plastic pots.
9. Centrifuge at 50 *g* for 3 min.
10. Remove and discard the supernatant, add fresh medium, and mix with

Protocol 13. *Continued*

a wide-bore pipette. Repeat this washing procedure twice more re-suspending the final pellet in 20 ml of L15 medium, pass through the 100 μm nylon mesh to give the final cell preparation.

11. Determine the cell yield and viability using a haemocytometer.[d]

[a] Venous outflow should be allowed to drain freely immediately perfusion is started, otherwise poor clearance of blood may result. In practice, it is convenient to arrange outflow drain into a sink or plastic bowl to avoid flooding the bench.
[b] Some investigators have used heparin injection to prevent blood clotting during the operation. In our experience, if the cannulation and perfusion are performed in a well-practised manner, heparin is not necessary.
[c] This is a high flow rate compared with that of normal blood, but this rate causes distension of the liver which aids eventual digestion.
[d] Trypan blue dye can be used at this point to gauge cell viability (see Section 3.1 for methods of assessing viability).

2.2.6 Liberation of cultured cells

In a variety of situations it is necessary to subculture cells produced by the types of protocols listed above. Since many animal cell types adhere to the substratum during culture and reform cell:cell junctions (indeed these are often essential to maintenance of function and viability), one step in the procedure is to liberate individual cells from these constraints. The rationale for the approaches is similar to that described above for releasing cells from tissues—removal of divalent cations from the medium to 'break' cell:cell contacts, and a proteolytic digestion to dissolve the extracellular matrix which the cells have synthesized during attachment. We routinely use the following method (*Protocol 14*) to subculture tissue-derived cells such as endothelial cells. Small adjustments in enzyme concentration and time of exposure may be required for other cell types.

Protocol 14. Liberation of cultured cells

Equipment and reagents

- Laminar flow-hood
- Bench centrifuge
- Sterile plastic pipettes and capped pots
- Dulbecco's phosphate-buffered saline (PBS) without Ca^{2+} and Mg^{2+} (Gibco)
- Trypsin/EDTA solution: 5 g porcine trypsin and 2 g EDTA per litre (Sigma)
- Dulbecco's MEM medium plus 20% newborn calf serum (Gibco)

Method

1. Wherever possible, perform procedures in a laminar flow-hood.
2. Dilute the trypsin/EDTA solution 1/10 with pre-warmed PBS minus Ca^{2+} and Mg^{2+}.

3. Remove the cell culture supernatants with a sterile pipette and wash the flasks[a] with 3 ml of PBS minus Ca^{2+} and Mg^{2+}.
4. Add 3 ml of diluted trypsin/EDTA to each flask, and place in the incubator at 37°C. Check the digestion every 1–2 min[b] by gently rocking the flasks, and as soon as it can be seen that the cells have been liberated into suspension,[c] remove the flasks from the incubator.
5. Add an excess (approx 2:1) of pre-warmed MEM plus 20% NBCS to inhibit the proteolytic action.
6. Transfer the suspension to sterile plastic tubes and spin at 400 *g* for 5 min to pellet the cells. Resuspend the pellets in fresh MEM, spin again, and remove the supernatant.
7. Resuspend the cells in the tissue culture medium of choice, count the cells using a haemocytometer (see Section 3.1), and introduce the cells at the required concentration into fresh flasks.

[a] These volumes are used for 25 cm^2 flasks.
[b] Trypsin has a well-recognized digestive effect on cell membrane components and prolonged exposure will often prove lethal.
[c] There may be a need to study cells in synchrony after isolation and culture. One method is to use mitotic release; cells undergoing mitosis are often less strongly adhered to the tissue culture substratum, and can be dislodged from the surface by agitating the plate without adding trypsin. However, for each cell type, it is necessary to determine by prior microscopic examination the time after plating at which most mitotic figures are present, and to harvest cells at this time. Yields of mitotic cells up to 5% of the total cell number plated have been reported (16), although this will vary with cell type and culture conditions.

2.2.7 Plant protoplasts

In order to study certain aspects of plant growth, for example regeneration of the cell wall, hybridization, and ingestion of substances into the cytoplasm, it is necessary to remove the plant cell wall producing a protoplast. This can either be done enzymatically or mechanically. The mechanical method is difficult to perform and produces low protoplast yields with poor viability. Thus *Protocol 15*, based on a method by Dodds and Roberts (17), concentrates solely on the enzymatic method. The cell wall is made of cellulose, pectin, and hemicellulose with small amounts of protein and lipid—it is necessary therefore to use a mixture of enzymes to break down the cell wall. A drawback to this method is that the enzymes contain impurities which may be harmful to the protoplasts. It is possible if necessary to purify the enzymes (18).

Protocol 15. Isolation and purification of plant protoplasts

Equipment and reagents

- Scalpel
- Pointed forceps
- 45 μm pore size nylon mesh
- 0.45 μm pore size syringe filter (Millipore)
- 10 ml pipettes wide-bore (Sterilin)
- Wide-bore Pasteur pipettes (BDH)

Protocol 15. *Continued*

- 11 ml centrifuge tubes capped with aluminium foil (Gibco)
- Parafilm (BDH)
- Aluminium foil
- Bench-top centrifuge with swing-out rotor
- Mature leaves from a suitable plant[a]
- 250 ml sodium hypochlorite solution (2.5%, v/v), with 2 ml of Triton X-100 (Sigma)
- Mannitol Analar (BDH)
- 10 ml enzyme mixture containing: Macerozyme R-10 (0.5%, w/v) plus cellulase Onozuka R-10 (2.0%, w/v) (R. W. Unwin & Company) dissolved in 13% (w/v) mannitol, pH 5.4[b]
- 800 ml Murashige and Skoog's medium (MS) (Gibco) containing 13% (w/v) mannitol pH 5.4 (it is important that the osmolarity and pH are the same as enzyme solution)[b]

Method

1. Sterilize medium and reagents as required making sure not to denature the enzymes.
2. Whenever possible carry out the procedure under aseptic conditions.
3. Rinse the leaves (1 g) in tap-water.
4. Immerse the leaves in the hypochlorite/Triton solution for 10 min.
5. Rinse the leaves three times in MS medium containing 13% mannitol pH 5.4.
6. During the final rinse, remove the lower epidermis with pointed forceps.This is best achieved by inserting the point of the forceps at the junction of the main vein and stripping the epidermal layer towards the edge of the lamina. If this fails allow the leaf to wilt and try again. If this fails then score the epidermis several times with a scalpel blade to aid entry of the enzymes.
7. Cut the leaves into sections and immerse the peeled leaf sections in a Petri dish containing 10 ml sterile enzyme solution. Sterilization is achieved by membrane filtration through a 0.45 μm filter.
8. Seal the Petri dish with Parafilm and wrap in aluminium foil. Leave overnight at room temperature.
9. Tease the leaf strips gently to release the protoplasts.
10. Filter the enzyme solution containing the protoplasts through a 45 μm nylon mesh to remove debris.
11. Centrifuge the filtrate at 75 *g* for 5 min.
12. Remove the supernatant using a Pasteur pipette and resuspend the pellet in 10 ml of MS culture medium (with 13% mannitol).[c]
13. Wash three times with medium.

[a] Plant selection is important, two most commonly used are the *Petunia sp.* and *Hyoscyamus niger* (henbane). Avoid use of any fungicides or insecticides during the growth of the plants.
[b] In developing a technique for each plant species it may be necessary to experiment with various enzyme mixtures and mannitol concentrations (typically 8–15%). Lower concentrations of mannitol will cause fusion of protoplasts.
[c] Resuspension of the protoplasts must be done gently to avoid damage. A typical yield of mesophyll protoplasts is 2–5 × 10^6/g of leaf tissue.

2.2.8 Yeast protoplasts (spheroplasts)

In plant cell biology it is often necessary to isolate and study cell organelles. In the case of yeasts these organelles are fragile and easily broken by the shear forces needed to release them. One way to reduce the damage is to remove the cell wall generating a protoplast (spheroplast) which can be easily broken under conditions which preserve the integrity of the subcellular organelles. Protoplasts have also been widely used in molecular biology, since they have no cell wall they are an excellent tool to study DNA manipulations both on research and commercial levels. *Protocol 16* describes a method used to produce protoplasts from yeast cells, but it can be modified for other types of fungi and bacteria.

Protocol 16. Isolation of yeast protoplasts

Equipment and reagents

- Bench-top centrifuge
- Appropriate centrifuge tubes
- Spectrophotometer for cell number assessment
- Yeast extract (Sigma)
- Bacto peptone (Difco Laboratories)
- Glucose Analar (BDH)
- Tris buffer (BDH)
- Dithiothreitol (DTT) (Sigma)
- Lyticase (Sigma)
- Sorbitol (Sigma)
- Potassium phosphate (BDH)
- Sucrose (BDH)
- Ficoll 400 (Pharmacia)
- 2-(*N*-morpholino)ethanesulfonic acid (MES) (Sigma)

Method

1. Grow yeast cells to their early/mid logarithmic phase in growth medium containing 1% (w/v) yeast extract, 2% (w/v) Bacto peptone, and 2–5% (w/v) glucose.
2. Centrifuge at 3000 *g* for 5 min at room temperature.
3. Wash cells once in distilled water and resuspend at 20 U/ml (OD_{600}) in 0.1 M Tris sulfate pH 9.4, 10 mM DTT for 10 min.This loosens the outer mannoprotein layer to allow attack of the underlying β(1–3) glucan layer by the Lyticase.
4. Wash the cells in 1.2 M sorbitol and resuspend to 50 U/ml (OD_{600}) in buffer containing 1.2 M sorbitol, 10 mM potassium phosphate pH 7.2, then add Lyticase (10–25 U/OD_{600} unit of cells).
5. In order that metabolically active protoplasts are obtained add 0.5% yeast extract and 1% Bacto peptone. Incubate for 45 min with gentle shaking at 25°C, by which time protoplast formation should be completed.
6. To recover the protoplasts centrifuge at 4000 *g* for 10 min at 4°C through a cushion of 0.8 M sucrose, 1.5% (w/v) Ficoll 400, 20 mM MES pH 6.5.

3. Viability measurements

The term viability is one which is frequently used in cell biology, and which everyone believes they understand. However, a closer inspection of what each investigator perceives by their own definition of viability exposes a wide range of physiological and biochemical processes which are deemed to dictate life processes within a cell. In reality, there is no simple single test which can evaluate whether a particular population of cells is performing the many processes that constitute their normal function, and the investigator is left to compromise between the practicality of a given test, its sensitivity, and relevance to the study. For purposes of isolating and handling cells, rapid tests are obviously advantageous, and these by and large are centred on direct microscopic counting allied to the use of various dye chemicals which give information on the integrity of the plasma membrane as a fundamental indication of cell survival (19). The sensitivity of detection may be increased by using chemicals which are enzymatically cleaved in living cells to produce fluorescent stains (20), or fluorescent dyes which bind to intracellular components (21). However, it cannot be stressed too strongly that good results from these tests do not always correlate with more demanding metabolic tests on the same cells. Such informative functional tests often require measurement of specific biochemical processes using radiolabelling, fluorimetry, etc., and may take several hours to complete. Perhaps the most demanding test of viability is the ability of a cell population to replicate itself whilst still maintaining specific functions, but with mammalian cells this usually increases the time of investigation to several days. Also, some cell types, such as mammalian hepatocytes, do not readily divide in culture. In the context of this chapter, viability is discussed in relation to assessing the quality of a particular cell isolate; alternative uses of morphological or biochemical viability tests in studies on cell physiology, pathology, or toxicology can be found in other texts (9, 22). In the following section will be given methods for two of the most frequently used visual tests and one method for metabolic assessment based on incorporation of radiolabelled amino acids into cell proteins. In addition, two methods of measuring cell growth in culture are described, for those cell types for which this is appropriate.

3.1 Viability counting with trypan blue

A method for determining cell viability using trypan blue is described in *Protocol 17.*

Protocol 17. Cell viability using trypan blue

Equipment and reagents

- Light microscope with suitable magnification (usually × 10 to × 40 depending on the cell type)
- Haemocytometer counting chamber and coverslip
- Bench centrifuge

- Trypan blue dye solution: add 0.4 g of trypan blue dye, 0.81 g NaCl, 0.006 g KH_2PO_4, and 0.05 g methyl hydroxybenzoate (preservative) to 95 ml double distilled water. Heat to boiling in a glass beaker and allow to cool. Adjust the pH to 7.2–7.3 with NaOH solution, and adjust the volume to 100 ml. Finally pass through a 5 μm filter.
- Glassware, pipettes, Pasteur pipettes
- Cell suspension
- Dulbecco's phosphate-buffered saline (PBS) or similar medium

Method

1. Fix the coverslip with firm and even pressure on to the haemocytometer chamber (achieved by breathing lightly on the chamber to deposit a thin film of moisture, and pushing down on the coverslip with each thumb on opposite sides of the chamber).
2. Centrifuge the cell suspension (200–500 *g* depending on cell size) to produce a compact pellet. Remove the supernatant and resuspend the cells carefully with a wide-bore Pasteur pipette in the chosen volume of PBS.[a]
3. Add 0.05 ml of cell suspension to 0.95 ml trypan blue solution in a small plastic pot.[b] Allow to equilibrate at room temperature for 5 min.[c]
4. Mix the cell suspension by agitating the pot,[d] and transfer a sample with a Pasteur pipette into the haemocytometer chamber, and under the microscope count the numbers of clear, unstained cells, and total cell numbers within the squares of the chamber.
5. Calculate viability as per cent unstained cells in the total population. This can be related back to the original suspension knowing the volume of the chamber (usually 0.1 mm^3 per large square), the dilution factor (in this case × 20), and the volume of original suspension.

[a] Dissolved proteins in the medium can cause a high background staining, and thus protein-free resuspension medium is recommended.
[b] These can be stored frozen as aliquots ready for use on the day.
[c] The time chosen is arbitrary but commonly used; during prolonged exposure to trypan blue dye (> 30 min), progressively more cells may accumulate the dye (19).
[d] If large cells are under study, they may sediment if left to stand in the dye and thus errors in counting will follow.

3.2 Viability counting by fluorescent dye uptake

An alternative approach to the simple dye exclusion test is that based on fluorescent staining of intracellular components. Some methods combine dyes, such as acridine orange and propidium iodide (see *Protocol 18*). Acridine orange can cross the plasma membrane and stain intracellular nucleic acids, producing a green fluorescence at low dye concentrations. Propidium iodide cannot cross intact plasma membranes, but can penetrate membranes of damaged cell, again staining intracellular nucleic acids, this time with a bright red fluorescence. The dye also competitively excludes acridine orange from

the nuclei of damaged cells. Thus after double staining, it is possible to identify and count all intact cells and damaged cells within the field of view, the cell nuclei displaying the most intense fluorescence (23).

Protocol 18. Viability by fluorescence staining

Equipment and reagents

- Fluorescent microscope with FITC filter set at excitation 490 nm, emission 510 nm (manipulations are generally easier on an inverted microscope)
- Bench centrifuge
- Glassware, microscope slides, pipettes, and Pasteur pipettes
- Acridine orange and propidium iodide dyes (Sigma), dissolved in phosphate-buffered saline (PBS) or similar medium. Acridine orange concentrations in the range 0.5–5 μmol/litre,[a] and propidium iodide concentrations in the range 50–100 μmol/litre usually give good cell staining while avoiding intense background interference, but trial runs should be performed to optimize dye concentrations for particular cell types.

Method

1. Centrifuge the cell suspension (200–500 *g*), remove the supernatant from the pellet, and resuspend the cells in fresh protein-free medium to reduce background fluorescence.
2. On to a microscope slide mix equal volumes (20–50 μl) of resuspended cells and dye solution, ensure good mixing, cover with a coverslip, and leave at room temperature for 10 min.
3. Transfer the slide to the microscope, illuminate with appropriate filter, and count the cells (minimum of 500 for good sample size) that have either red or green fluorescent nuclei.

[a] At concentrations above about 20 μmol/litre, acridine orange also produces a red fluorescence following a metachromatic shift.

3.3 Assessment of metabolic functions: protein synthesis

Moving to assays of biochemical activities which may be related to overall cell function and viability, as already stressed these are most often restricted by the time required to identify and measure the particular biochemical event. For example, in studies on gluconeogenesis, glucose synthesis has to be measured by spectrophotometric or radiotracer methods, both of which assays are time-consuming. Some assays appear deceptively straightforward, such as direct measurement of oxygen uptake by cell suspensions, but the investigator must always be aware of the limitations (in this case, the possibility that respiration may be uncoupled in damaged cells, increasing apparent oxygen consumption in the sample). Nevertheless, after initial experiments to isolate and identify particular cell types, it is useful to consider some of the more demanding tests to gain a better understanding of the quality of the cells produced. One com-

monly studied aspect of cell metabolism which appears to be a sensitive index of viability, is the *de novo* synthesis of proteins, and this can be assayed by incorporation of radiolabelled [^{14}C] or [^{3}H] amino acids into precipitable cell proteins. We have used the technique described to measure protein synthesis in cells and isolated organelles such as mitochondria (24), and it is based as described in *Protocol 19* on the method for isolated hepatocytes, but it can be modified to suit any cell suspension.

Protocol 19. Protein synthesis as a viability index

Equipment and reagents

- Bench centrifuge
- Shaking water-bath at 37 °C
- Scintillation counter
- Gas cylinder (95% O_2:5% CO_2)
- Disposable plastic bottles (20 ml) and appropriate rubber bungs
- Glass bottles (20 ml) with screw-caps
- Scintillation vials, pipettes, and Pasteur pipettes (glass)
- Plastic trays with adsorbent paper lining
- Trichloroacetic acid (TCA) solution: 10% (w/v) in distilled water
- Sodium hydroxide (NaOH) solution: 20% (w/v) in distilled water
- ^{14}C-labelled amino acid: [^{14}C]L-leucine (Amersham International)
- Scintillation fluid (e.g. Instafluor, Packard)
- Solubilizing solution (e.g. Soluene, Packard)
- Krebs Ringer bicarbonate (KRB). Make up stock solutions in (g/100 ml) distilled water as follows: (a) 0.9 g NaCl, (b) 1.15 g KCl, (c) 2.4 g $CaCl_2$ (hydrated), (d) 2.11 g KH_2PO_4, (e) 3.82 g $MgSO_4$ (hydrated), (f) 1.3 g $NaHCO_3$. Store the solutions in a fridge and combine *when needed as follows: 100 parts* (a), 4 parts (b), 5 parts (c), 1 part (d), 1 part (e), and 21 parts (f). Place the KRB on ice and gas for 20 min with 95% O_2:5% CO_2 before use.
- Amino acid supplement for enhancing protein synthesis: e.g. Vamin, Vitrum (Sweden) at 5 ml per 100 ml KRB, or add 1 part full nutrient medium (e.g. Leibovitz L15 medium) to 4 parts KRB before use
- Reagents for protein measurement (e.g. Folin–Ciocalteau reagents)

Method

1. Prepare the gassed KRB solution, and add trace quantities of [^{14}C]L-leucine (0.5 μCi/100 ml buffer gives adequate activity for hepatocyte studies).
2. Centrifuge the cell suspension, resuspend the pellet in fresh protein-free culture medium, and count. Adjust the volume of medium as required (for hepatocytes 5–10 × 10^6 cells/ml are suitable).
3. Place sufficient plastic pots on ice,[a] and pipette into each 2 ml KRB containing [^{14}C]L-leucine and 0.5 ml cell suspension.
4. Blow O_2:CO_2 gas mixture onto the surface of the solutions for 1 min, and stopper tightly with rubber bungs.[b]
5. Place the tubes in a shaking water-bath at 37 °C, at a shaking speed of 50–80 cycles per min.
6. At time intervals, remove tubes, place on ice, and immediately add 5 ml of cold 10% TCA. (For most studies 15 min intervals over 2 h are adequate.) Stand tubes on ice for 30 min.
7. At the end of the time course, spin down the precipitated proteins,

Protocol 19. *Continued*

remove the supernatant, and add to each tube 5 ml fresh 10% TCA. Agitate the tubes to resuspend the pellet, recentrifuge, and discard the supernatant.

8. Add 2 ml 1 M NaOH to each tube and stopper, stand in a water-bath at 70 °C to dissolve the precipitate (2–3 h).
9. Transfer the dissolved proteins with glass Pasteur pipettes into the glass bottles, add 5 ml 10% TCA, and stand on ice for 30 min.[c]
10. Centrifuge the precipitated proteins and remove the supernatant. Allow the pellets to drain of moisture by inverting the bottles on blotting paper in plastic trays for a few hours.
11. Add 1 ml of solubilizing agent to each tube, tightly cap, and leave for several hours (or overnight).
12. Add aliquots (50–100 μl) of dissolved proteins to 10 ml scintillant solution in scintillation vials, and count in the scintillation counter using appropriate times and external/internal standards to correct for quench effects.
13. Relate incorporation of [^{14}C]leucine into protein over time per unit number of cells (derived from the original count).

[a] Keeping the solutions on ice has the benefit that protein synthesis will be inhibited until the samples are all warmed up together, and the solubility of the dissolved gas remains high.
[b] This length of gassing in this head space (in the 20 ml tube) is sufficient for incubations of 2–3 h.
[c] The transfer to glass is necessary because many of the solubilizing solutions will attack plastic tubes. The second dissolution/precipitation step also enhances purification of precipitated proteins from any residual free labelled amino acid.

3.4 Assessment of cell growth as a viability index

In experiments isolating cell types which retain the inherent capacity for cell division in culture (for example, vascular endothelial cells), it is often instructive to measure the potential for replication of a particular isolate, and the response of the cells to a known mitogenic stimulus, before going on to use the the batch of cells in planned physiological or biochemical studies. The time required for such studies is obviously dictated by the replication time of the particular cell type, in addition to the time required for the selected growth assay. In mammalian cells these considerations usually combine to require investigation times of one to two days.

3.4.1 Cell proliferation measured by thymidine incorporation

One commonly used assay is the measurement of incorporation of radiolabelled thymidine into cell DNA; the method described in *Protocol 20* is one we have used to measure replication in cultured human vascular smooth muscle cells (VSMC; see Section 2.2.1).

Protocol 20. Uptake of radiolabelled thymidine

Equipment and reagents

- Laminar flow-hood
- Scintillation counter
- Tissue culture incubator with gaseous atmosphere set at 95% air:5% CO_2
- 96-well tissue culture plates (Costar)
- Vacuum aspirator with sterile Pasteur pipettes
- Dulbecco's modified Eagle's medium (DMEM) with added fetal bovine serum (FBS) at 0.4% (v/v) and 5% (v/v) (Gibco)
- Dulbecco's phosphate-buffered saline (PBS)
- Methyl [^{3}H]thymidine (aqueous solution, 1 mCi; Amersham International)
- Scintillation fluid (e.g. Instafluor, Packard)
- Scintillation vials
- Trichloroacetic acid (TCA) solution: 7.5% (w/v) in distilled water
- Sodium hydroxide (NaOH) solution and hydrochloric acid (HCl) solution: both 4% (w/v) in distilled water
- Human vascular smooth muscle cells (VSMC) at passage 2/3

Method

1. Liberate the VSMC from culture using trypsinization (see Section 2.2.1), count the cells with a haemocytometer, and plate at a concentration of 1–2000 cells/well[a] in 200 μl DMEM plus 5% FBS. Perform this and subsequent steps using sterile techniques in the laminar flow-hood. Culture for 24 h in the incubator to ensure cell attachment. Assign some wells (12–16) as controls for assay of total DNA content.[b]
2. Remove the supernatants by vacuum aspiration, wash the wells with fresh pre-warmed DMEM, add 200 μl of DMEM plus 0.4% FBS,[c] and culture in the incubator for a further 24 h.
3. Remove the supernatants by vacuum aspiration, wash the wells with fresh pre-warmed DMEM, add 200 μl DMEM plus 0.4% FBS to half the controls and half test wells, and to the remainder add 200 μl DMEM plus 5% FBS. To all test wells, add 1 μCi of [^{3}H]thymidine. Transfer the plates to the incubator for a further 24 h.
4. Remove the supernatants by vacuum aspiration, wash the wells twice with pre-warmed PBS, and to the test wells add 200 μl 7.5% TCA. To the control wells, add 200 μl PBS, dislodge the cells by scraping with a sterile pipette, harvest, and use for total DNA measurements.[c] Place the plate on ice for 1 h to ensure precipitation of cell components.
5. Remove the supernatants by vacuum aspiration, wash the wells with 200 μl fresh cold TCA, add 200 μl of 4% NaOH, cover, and leave overnight at room temperature.
6. Remove the NaOH digests and add to 10 ml of scintillation fluid in scintillation vials. Wash each test well with 200 μl of 4% HCl, and add this to the respective scintillation vial. Count the vials in a scintillation counter using appropriate times and external/internal standards to correct for quench effects.

Protocol 20. *Continued*

7. Relate the incorporation of [^{3}H]thymidine to DNA content of the control wells, and assess the replicative stimulus provided by exposure to high (5%) FBS.[d]

[a] Low cell numbers are plated initially to ensure sufficient surface area in the wells to allow continued growth over several days.
[b] Total DNA may be assayed by a fluorimetric technique (25). However, with most cell types there are insufficient cell numbers in a single well of a 96-well plate; this can be overcome by pooling the contents of two or more wells.
[c] Culture in the presence of low FBS for VSMC induces quiescence and synchronizes the growth phase.
[d] Measurement of thymidine incorporation in low (0.4%) and high (5%) FBS allows assessment of promotion of growth in a particular cell isolate and thus comparison between different VSMC preparations.

3.4.2 Cell proliferation measured by enzyme activity

Cell proliferation in culture, as an index of viability, can also be monitored by measuring the concentration of a cell-specific marker, such as the activity of an enzyme. Such methods have the advantages of being relatively simple and rapid to perform, and can be easily applied to a range of different conditions including cell suspensions, cells adhered to culture dishes, or microcarrier beads. However, caution in interpretation is necessary, since enzyme activity may be induced or down-regulated by conditions of isolation and culture, and appropriate controls should be performed to be able to compare enzyme activity with other indices of cell replication such as direct cell counting. Nevertheless, in situations where a particular cell type is routinely isolated and cultured under constant conditions, increases in enzyme activity can be used as a measure of population growth. The method described in *Protocol 21* is based on a measurement of acid phosphatase activity (26) for endothelial cells in culture in multiwell plates.

Protocol 21. Enzyme activity as a growth index

Equipment and reagents

- Tissue culture incubator with gaseous atmosphere set at 95% air:5% CO_2
- Laminar flow-hood
- Vacuum aspirator
- Pipettes and Pasteur pipettes: for multiple determinations, a repeating pipette (e.g. Eppendorf Brinkman) and multichannel pipettor (Flow Laboratories) are useful aids
- Microplate spectrophotometer (Dynatech)
- 96-well culture plates (Costar)
- Sodium hydroxide (NaOH) solution: 4% (w/v) in distilled water
- Dulbecco's modified Eagle's medium (DMEM) with added 10% fetal bovine serum (FBS) (Gibco)
- Dulbecco's phosphate-buffered saline (PBS) (Gibco)
- Reaction medium: 0.1 M sodium acetate buffer pH 5.5, containing 0.1% (v/v) Triton X-100 and 10 mM *p*-nitrophenyl phosphate (Sigma, 104 phosphatase substrate)
- Endothelial cells (see Section 2.2.1), passage 2/3

Method

1. Liberate the cells from culture using trypsinization (see Section 2.2.1), count with a haemocytometer, and plate at a cell concentration of 1–2000/well[a] in 200 μl DMEM plus 10% FBS. Leave the first three wells empty to accommodate appropriate blanks for the assay. Perform all procedures using sterile techniques in the laminar flow-hood. Culture the cells for 24 h in the incubator to ensure attachment.
2. On subsequent days, select replicate wells for assay.[b] Remove the medium by vacuum aspiration, and wash the well with 200 μl pre-warmed PBS.
3. Add to each test well 100 μl of rection mixture. In the first empty well, add 100 μl of PBS, and in the next two wells add 100 μl of reaction mixture. Cover the plate and return to the incubator for 2 h.
4. Remove the plate and add 10 μl of 4% (w/v) NaOH to stop the reaction and develop the colour.
5. After 5 min, read the colour developed in the microplate reader against the blank values. Subtract any non-specific substrate hydrolysis (absorbance of wells with reaction medium containing no cells).

[a] Low cell numbers are plated initially to ensure sufficient surface area in the wells to allow continued growth over several days. The optimal seeding density may vary for different cell types.

[b] The response of particular cell types to growth-promoting agents can also be studied using this method; see also *Protocol 19.* Cultures can be synchronized by serum deprivation, and effects of agents such as endothelial cell growth factor investigated.

4. Separation of viable and non-viable cells

To date there is a no simple or straightforward method to isolate viable from non-viable cells, but two approaches have been most commonly used. These are:

- centrifugation methods
- adherence to a substratum

Most cell suspensions are obtained by enzymic digestion, which may result in damage or cell death. Enzymic digestion also creates a heterogeneous cell population which needs to be further purified without causing further damage or cell loss. For example, enzymatic digestion of the liver yields a mixed population of hepatocytes, endothelial cells, and Kupffer cells (27) (see also Section 2.2.5). Centrifugation has been used to partially purify a cell population but the washing procedures involved may cause further damage. Over the years density centrifugation using gradient medium has been developed for the separation and purification of cells, viruses, and subcellular particles. Density

gradient media have been used to separate cells in an intact state without changing their function (see Chapters 2 and 3). In terms of separating viable and non-viable cells, centrifugation techniques offer the possibility of separating out cells which have been grossly damaged, for example after rupture of the plasma membrane and leakage out of intracellular components, which will alter buoyant density. Density gradient media provide an ideal environment for the debris and layers of ruptured non-viable cells to form close to the top of gradient, whereas the viable cells form lower bands according to their densities. For example, we have used density gradient centrifugation to enrich the population of dye-excluding cells after enzymatic digestion of liver for hepatocyte preparation (28). However, it should be pointed out that in many other situations where cells are rendered non-viable by treatments such as exposure to toxic agents, cell death may not be accompanied by gross disruption (at least, not in the early stages), and in such conditions centrifugation methods are not so successful in separating out non-viable cells. There are a number of commercially available density gradient media which can be used for separating different cell types. They are made from different materials but have essentially the same properties and uses:

- Percoll
- Nycodenz
- Ficoll

Separation by buoyant density can be carried out using continuous or discontinuous gradients. In continuous gradients the density of the solution increases gradually from the top to the bottom of the gradient while discontinuous gradients consist of discrete layers of different densities (see Chapters 2 and 3). In both the principle is the same, during centrifugation the cells will separate at a point where the density of medium is equal to the density of the cell.

As a second approach, if the cells concerned will adhere to tissue culture flasks, a simple method to separate viable cells from non-viable cells is to plate the purified cell suspension on to a tissue culture flask and leave in culture conditions optimized to the cell type to be purified. The majority of viable cells will adhere to the surface by processes which include synthesis of an extracellular matrix, whilst non-viable cells cannot produce this. The non-viable cells will be discarded during subsequent washing procedures. *Protocol 10* describes an example of this.

5. Cell characterization

There are various types of analytical methods for characterizing cells. Light microscopy is an obvious starting point, but the fact that cells may change in gross morphology after being isolated from their natural environment or

during time in culture means that other confirmatory methods are desirable. The increased power of resolution in electron microscopy can be used to identify specific ultrastructural characteristics, such as glycogen storage rosettes in hepatocytes. The introduction of monoclonal antibody technology has meant that components of particular cell types can be identified with a high degree of specificity, and by combining this to secondary antibody reactions carrying fluorescent or peroxidase-linked markers, the sensitivity of detection can also be high. The technology can additionally be used in a variety of different approaches, listed below, which can be adapted to suit specific requirements (e.g. antibody reaction can be combined with magnetic bead technique to both characterize and purify and isolate for a specific cell type).

Several different types of analytical methods can be used for characterizing cells and these include:

- light microscopy
- phase-contrast microscopy
- electron microscopy
- flow cytometry
- magnetic beads
- immunohistochemistry
- metabolic characteristics

5.1 Light microscopy

Once the cells are in culture, light microscopy is usually the first technique used to identify the cell types. As most cells are plated it is advantageous to use an inverted microscope. Although this is an accurate technique, for most cells in culture it must be used in conjunction with other analytical techniques, before a final decision is made on the cell type. For example:

(a) Endothelial cells have a typical 'cobblestone' morphology (see *Figure 1A*).

(b) Fibroblasts have a typical 'spindle' morphology (see *Figure 1B*).

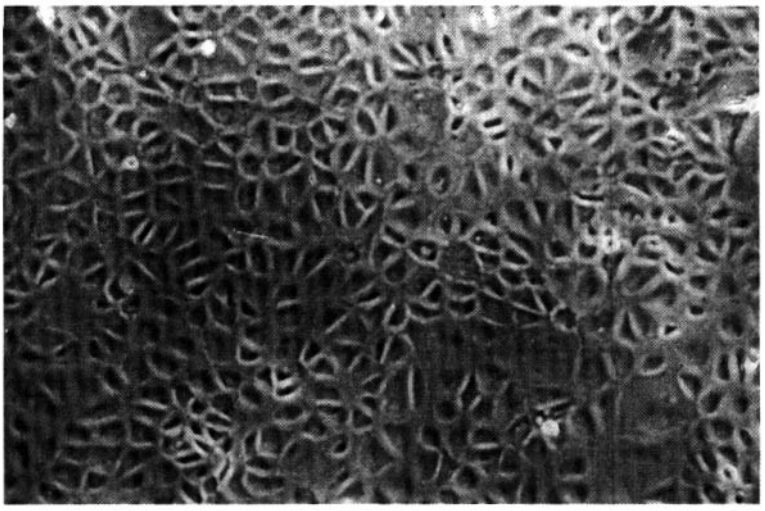
For most cell types a magnification of $\times$ 20 is sufficient. (Higher magnification can be used to observe cell organelles which may be useful in identifying certain cell types.)

5.2 Phase-contrast microscopy

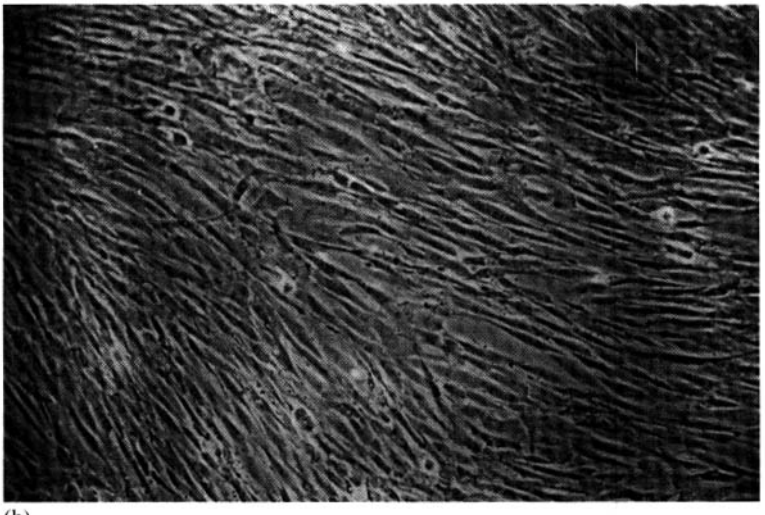
Sometimes it is not possible to distinguish the cell type using light microscopy. Phase-contrast microscopy is used in parallel with light microscopy and it allows you to observe the cell surface in more detail.

5.3 Electron microscopy

Further characterization can be carried out using scanning (SEM) and transmission (TEM) electron microscopy. SEM allows close observation of the cell



(a)



(b)

Figure 1. Morphology by light microscopy used to characterize cells once they become established in culture. (A) Endothelial cells isolated from omental fat display a typical 'cobblestone' appearance. (B) Smooth muscle cells isolated from peripheral vein are spindle-shaped and present in whorls. Phase-contrast microscopy performed on a Nikon Diaphot inverted microscope at magnification × 100.

surface and nature of cell to cell interactions. TEM allows inspection of cross-sections of a cell and is a useful technique to accurately examine cell organelles or inclusions present which may aid characterization. For example, most endothelial cells show presence of Weibel Palade bodies associated with secretory processes.

Both TEM and SEM are specialized time-consuming techniques, and not all institutions will have access to such equipment or the expertise required to interpret the photomicrographs.

5.4 Flow cytometry

Flow cytometry is another technique which over the years has become very popular for the analysis of cells. There are different types of cytometers and cell sorters (see Chapter 5). The FACScan (fluorescence-activated cell sorter) can be used to quantify and characterize cells. The basic principle of the FACScan involves fluorescently labelled cells moving in a liquid stream through a sensing region where they are struck by a focused laser beam and will emit fluorescent light. Either the cell surface or the DNA can be labelled with a specific antibody conjugated to an immunofluorescence dye. The cytometer will identify the cell according to the fluorescence intensity and cell size. Fluorescein is a commonly used dye which is readily excited by an argon or mercury lamp. For plant protoplast flourescein diacetate flurochromasia is used (29).

The advantages of using flow cytometry include:

(a) Rapid cell count, ranging from 10^2 to 10^3 per second.

(b) Automated sampling.

(c) Data can be accumulated, stored, and analysed quantitatively.

The disadvantages of using flow cytometry include:

(a) Costly.

(b) Technical expertise needed in handling the equipment and interpreting data.

5.5 Magnetic beads

A more recent method of isolating and characterizing cell types is by the use of magnetic polystyrene beads (Dynabeads^R) (see also Chapter 6). The surface of the beads are coated using various antibodies or other ligands specific to a certain cell type. A mixed population of cells is incubated with the coated beads in a test-tube. As the core of the beads are ferrous, a magnet is applied to the test-tube, the beads with the cells attached will sediment. The supernatant is discarded, the cells attached to the beads are resuspended, and can be either plated into culture or can be detached by vigorous pipetting, and in some cases the beads can be enzymically detached. The cell suspension can be further characterized by cytocentrifugation and immunostaining. Dynabeads^R

enable many cell types to be isolated and characterized either by positive or negative selection. For example:

(a) Endothelial cells can be positively isolated and identified by specific monoclonal antibodies, for example S-Endo1 (30).

(b) Separation of eosinophilic, granulocytes can be carried out by negative selection using Dynabeads coated with CD16 antibody (31).

5.6 Immunohistochemistry

The most widely and adaptable method of characterizing cell types is by immunohistochemistry. This involves using a labelled antibody which will react with a specific antigen on that particular cell type. Consideration must be given whether to choose the direct method or the indirect method. The direct method involves the primary antibody already labelled with a fluorescent molecule. The indirect method which is more commonly used involves the unlabelled primary antibody, identified by and attaching to secondary antibody, which is labelled with a fluorescent molecule (32). The most common fluorescent molecules used are fluorescein and rhodamine isothiocyanate.

Prior to cell staining it is important to consider the following:

- cell preparation
- type of fixative
- choice of antibody and labelling method
- mounting media
- detection method

The procedures for immunohistochemical analysis involve specialized steps for preparing, fixing, and staining cell populations. In the following sections, methods will be described for cell preparation by cytospin, for staining endothelial cells for von Willebrand factor, and smooth muscle cells for α-actin (*Protocols 22–24*).

5.6.1 Cell preparation

Cells are prepared for immunostaining either by cytocentrifuge (which involves a cell suspension being attached to a microscope slide by centrifugal force), or following growth of cells on either sterile glass or plastic microscope slides, coverslips, or eight-chambered wells. For a successful cytospin 1×10^6 cells/ml in culture medium containing 10% fetal bovine serum are required (*Protocol 22*). For every immunostaining run it is necessary to have a positive and a negative cell control (i.e. cell types which are expected to stain, and others which are not). This is to ensure that all the reagents and antibodies are working optimally.

Protocol 22. Cell preparation by cytocentrifuge for immunostaining

Equipment and reagents

- Cytocentrifuge (Shandon Elliot)
- Filter cards (Marathon)
- Double frosted microscope slides (Marathon)
- Cling film
- Adjustable Eppendorf pipette (200–1000 μl) (Fisher)
- M199 (Gibco)
- Fetal bovine serum (Gibco)

Method

1. After trypsinization resuspend the cells in culture medium (e.g. M199 with 10% fetal bovine serum) at a concentration of 1×10^6 cells/ml.[a]
2. Mount the microscope slides in the centrifuge with the filter papers. Add 100–300 μl of cell suspension into each of the wells.
3. Spin for 10 min at 45 *g*.
4. Remove the slides, 'cytospins', and leave to air dry for 10–15 min at room temperature.
5. Slides can be protected by wrapping in cling film and storing at –20°C[b] for later use, or use straight away.

[a] When characterizing cells it is important to include positive and negative cell controls.
[b] If stored at –20°C prior to use, it is necessary to leave for 10–15 min at room temperature before unwrapping to avoid excessive ice recrystallization.

5.6.2 Fixatives

Fixatives are used to preserve cell structure. Choose a fixative which does not alter the epitope on the antigen or its structure. Information on the type of fixative used can be obtained from the supplier of the antibody. A wide range of fixatives are available. Three of the most common used are:

- acetone
- formaldehyde
- methanol

Protocol 23 gives an example of acetone fixation.

5.6.3 Choice of antibodies

The choice of antibodies available is extensive. There is usually more than one antibody available for each cell type. When determining the working dilutions of the antibody a titre test can be carried out on the antibody. Guidelines are given by commercial suppliers of the antibody. The incubation time and temperature must also be considered as these will affect the quality of the stains.

There are two principal ways to demonstrate the bound antibody. First by conjugating with fluorescent molecules (*Protocol 23*) and secondly by the use of enzymes.

Two of the most common fluorescent molecules are:

- fluorescein isothiocyanate (green excitation at 490 nm)
- rhodamine isothiocyanate (red excitation at 530 nm)

Other fluorescent molecules not so commonly used include:

- Texas Red
- phycoerytherin

Commonly used enzymes (31) are:

- horse-radish peroxidase
- alkaline phosphatase
- β-galactosidase

Enzyme staining methods involve enzyme:substrate reactions to transform colourless chromogens into coloured products. For example DAB (3'-diaminobenzidine tetrahydrochloride) is the most commonly used substrate and one of the most sensitive for horse-radish peroxidase staining.

One of the advantages of using immunoenzymatic staining is that counterstaining can be carried out using, for example, Mayer's Hemulum or haematoxylin. Counterstain is useful to differentiate the various cell types or subcellular structures. It cannot be used in conjunction with fluorescence staining as the counterstains autofluoresce.

5.6.4 Mounting media

This must be compatible with the labelling media. Mounting media fall into two basic categories:

(a) Aqueous (e.g. glycerol), which is non-permanent and is used in immunofluorescence staining.

(b) Non-aqueous (e.g. DPX, commercially available), which is permanent and is used, for example, in peroxidase staining.

Guidelines are given with the antibody specification sheet. Problems, such as fading of the immunofluorescence stain can be reduced by limiting the exposure of the specimen to excitation radiation. It is also advisable to use a mounting media with an anti-fade component. *Protocols 23* and *24* give examples of mounting media.

5.6.5 Detection method

For the fluorescence immunostaining a microscope with an epifluorescence attachment is required. For the peroxidase staining any ordinary light micro-

scope with suitable objective is sufficient. Photographs should be taken as soon as possible as fading will occur with time. *Protocol 23* gives an example of fluorescence.

If fluorescence microscopy is not available, immunohistochemical methods can be modified to suit direct light microscopy by using a stain such as DAB and horse-radish peroxidase, as described in *Protocol 24.*

Protocol 23. Characterization of endothelial cells by indirect immunofluorescence staining

Equipment and reagents

- Humidified chamber
- Waxpen (DAKOPatts)
- Coplin jar (Philip Harris)
- Slide rack (Philip Harris)
- Coverslips (Marathon)
- Adjustable Eppendorf (200–1000 μl) (Fisons)
- Cytospins (positive and negative cell types)
- Phosphate-buffered saline (PBS) (Dulbecco's A) (Gibco)
- Acetone (Analar BDH)
- Primary antibody (e.g. vWF rabbit Ig to human vWF) (DAKOPatts)
- Secondary antibody (e.g. FITC conjugated to swine Ig to rabbit) (DAKOPatts)
- Normal serum (e.g. swine serum) (DAKOPatts)
- Bovine serum albumin (BSA) (Fraction V, Sigma)
- Fluorescence mounting fluid (DAKOPatts)

Method

1. Fix the cytospins in acetone for 10 min at room temperature. Using a pencil label the appropriate slides in duplicates. Carry out all steps at room temperature unless otherwise stated.
2. Using a waxpen carefully circle around the edge of the cytospins. This is not essential, however it contains the solution to the area within the circle.
3. Wash gently twice in PBS for 5 min.
4. Ensure from this stage, the slides *do not dry out.* The slides can be left in PBS.
5. Dilute the serum (1:20) in PBS containing 0.1% BSA.
6. Cover the cytospin by adding (60–80 μl) of the serum solution using a pipette.
7. Cover the lid of the humidified chamber and leave for 30 min.
8. *Do not wash,* but tap off the serum solution and add (60–80 μl) of the primary antibody diluted to (1:500) in PBS containing 0.1% BSA. Leave for 60 min.
9. Wash again, twice in PBS for 5 min, tapping off the excess fluid.
10. Add the secondary antibody diluted to (1:40) in PBS containing 0.1% BSA.
11. Leave in the humidified chamber for further 60 min.

Protocol 23. *Continued*

12. Wash twice in PBS for 5 min, tapping off the excess.
13. Mount using fluorescence mounting fluid.
14. Initially view through the phase-contrast microscope to locate the cells and then with the fluorescein excitation filter. Positive cells fluoresce green and negative cells show no fluorescence. Take photographs as soon as possible.
15. Store in the dark at 4°C.

Protocol 24. Indirect (DAB) immunperoxidase staining using for smooth muscle cell α-actin

Equipment and reagents

- Humidified chamber
- Waxpen (DAKOPatts)
- Coplin jar (Philip Harris)
- Slide rack (Philip Harris)
- Coverslips (Marathon)
- Light microscope
- DAB (3'-diaminobenzidine tetrahydrochloride, Sigma)
- 0.01 M hydrochloric acid (Sigma)
- Imidazole buffer (Sigma)
- Tris base
- Mayer's Hemalum (Sigma)
- 30% hydrogen peroxide (Analar BDH)
- Phosphate-buffered saline (PBS) (Dulbecco's A) (Gibco)
- 10% neutral-buffered formal saline (Sigma)
- 0.01 M sodium hydroxide (Sigma)
- Glycergel (DAKOPatts)
- Cytospins (positive and negative cells)
- Primary antibody (e.g. alpha smooth muscle actin) (DAKOPatts)
- Secondary antibody (e.g. rabbit anti-mouse alpha Ig/horse-radish peroxidase) (DAKOPatts)

Method

1. To 50 ml of PBS add 1.5 ml of hydrogen peroxide. (This is used to quench the endogenous peroxidase activity, step 5.)
2. Fix the cytospins in formalin for 5 min at room temperature. Carry out all steps at room temperature, unless otherwise stated.
3. Wash in PBS for 5 min, gently.
4. Using a waxpen circle around the cytospins. This is not essential, however, it prevents any running of the solution over the slides.
5. Quench the endogenous peroxidase activity by adding a drop of PBS plus hydrogen peroxide and leaving for 10–15 min.
6. Wash twice in PBS for 5 min.
7. Using an Eppendorf add (60–80 μl) of the primary antibody diluted (1:40) in PBS containing 0.1% BSA.
8. Leave for 60 min in the humidified chamber with the lid covered.

9. Tap off the antibody and wash twice in PBS for 5 min.
10. Prepare the secondary antibody as follows: to 900 μl of PBS add 100 μl of normal human serum, and 20 μl of the secondary antibody. Using an Eppendorf add (60–80 μl) of this to each cytospin.
11. Leave for 60 min in the humidified chamber.
12. Prepare DAB solution:[a] add 0.6075 g Tris base to 63 ml of distilled water. Dissolve, then add 38 ml of 0.1 M HCl, and bring the pH to 7.4–7.6 using either sodium hydroxide or hydrochloric acid. To the above add 60 mg of DAB, 40 μl of hydrogen peroxide, and 1 ml of 0.1 M imidazole.
13. Tap off the secondary antibody and wash with PBS for 5 min.
14. Take an aliquot of secondary antibody solution (40 μl) and add an equal amount of DAB solution. This should go brown. If not there is an error in making up of one of the solutions. Immerse the slides in the DAB solution for 5 min.
15. Wash slides with PBS for 5 min.
16. It is possible to counterstain using Mayer's Hemalum for 5–6 min. This is a nuclear stain which will turn blue.
17. Wash in PBS for 5 min.
18. Mount adding a drop of Glycergel on a coverslip, avoiding air bubbles.
19. View using a light microscope. Positive cells stain intense brown. Negative cells and nuclei do not stain at all, unless counterstained.

[a] All laboratory safety procedures must be followed when handling DAB, as this may be carcinogenic. It is important that the solution is made up fresh for each procedure.

5.7 Metabolic characterization of cells

Often, particular cell types will perform specific metabolic functions which relate to their normal physiological role. For example, isolated hepatocytes will synthesize albumin as a reflection of the normal role of liver in plasma protein metabolism. Such activities can be investigated by standard biochemical methods such ELISA, radioimmunoassay, or fluorescent antibody techniques. These tests are more protracted than simple microscopic methods, but can substantially increase the sensitivity of chracterization regimes.

In *Protocol 25*, specific uptake of acetylated low-density lipoprotein is studied in endothelial cells by using a lipoprotein moiety labelled with a fluorescent tag. This is based on the method by Voyta and colleagues (33).

Protocol 25. Characterizing of endothelial cells using Dil-Ac-LDL[a]

Equipment and reagents

- 8-chambered wells (Falcon)
- Fluorescent microscope with rhodamine filter
- Dil-acetylated LDL[a] (Biogenesis)
- 3% formaldehyde
- M199 (Gibco)
- Fetal bovine serum (Gibco)
- 0.1% gelatin (w/v) in PBS (Sigma)
- Phosphate-buffered saline (PBS) without calcium and magnesium (Gibco)

Method

1. Prepare 0.1% gelatin in PBS and autoclave. Coat the 8-chambered wells with 200 μl of 0.1% gelatin and leave overnight at 37°C.
2. Plate cells, positive cells (endothelial cells) and negative (e.g. fibroblasts or smooth muscle cells) onto the 8-chambered wells.
3. Leave overnight in a 5% CO_2 incubator at 37°C and allow the cells to grow until semi-confluent.
4. Prepare dil-acetylated LDL by adding 10 μg/ml in culture medium.
5. Add 300 μl to the cells and incubate for 4–6 h at 37°C in a CO_2 incubator.
6. Discard the media and wash with warm PBS twice.
7. Observe under the microscope using the rhodamine excitation filter. Take photographs as soon as possible. Positive cells fluoresce red.
8. For fixation use 3% formaldehyde in PBS[b] for 20 min at room temperature.
9. Rinse in PBS for a few seconds.
10. Discard the fixative and mount slides by adding a drop of 90% glycerol and 10% PBS.

[a] Acetylated low-density lipoprotein labelled with 1,1 dioctadecyl-1-3,3,3,3-teramethyl-indo-carbocyanine perchlorate.
[b] Do not use methanol or acetone fixation as dil-acetylated LDL is soluble in organic solvents.

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2

Fractionation of cells by sedimentation methods

D. PATEL, T. C. FORD, and D. RICKWOOD

1. Introduction

Cells can be separated and fractionated by a number of techniques using the different properties of the individual cell types. One of the most common methods used for the isolation and purification of cells is sedimentation, usually using centrifugation. Cells may be separated by size using simple differential pelleting procedures or separated on the basis of size and/or density using density gradient centrifugation. Nearly all cell separation procedures involve the pelleting of cells as part of the procedure for harvesting or concentrating cells prior to most fractionation procedures. However, differential pelleting is seldom used for separating different cell types, because the required difference in size, about tenfold, is rarely seen and so this technique will not be described in detail. Instead this chapter will concentrate on cell separations using density gradients with cells sedimenting either under unit gravity or in a centrifugal field. Aspects of cell separations that involve elutriation are described in Chapter 3 and will not be discussed here.

The actual choice of technique will depend on the nature of the cell and the type of fractionation that is required. Cells are extremely diverse in terms of their morphologies but they can be broadly classified into two major groups: those with a rigid cell wall and those without. The former include bacterial, fungal, and plant cells, while the latter are mainly animal cells and protoplasts. Cells that do not possess a rigid cell wall are more difficult to fractionate because any significant variation in osmolality of the medium will cause the volume of the cell to change and so alter its sedimentation properties. Gradients that are used for osmotically-sensitive cells should also be suitable for other types of cells although the presence of a rigid cell wall tends to make cells denser. This chapter will concentrate on the fractionation of animal cells and protoplasts making reference where appropriate to other types of cells.

2. The theory of sedimentation

When cells are suspended in a medium they experience a force. The magnitude and direction of the force depends upon: the effective density and size of the cells, the density and viscosity of the medium in which the cells are suspended, and the force field. In the case of unit gravity separations the force on the cells is that of the Earth's gravity while, in the case of centrifugation, the spinning rotor generates a centrifugal force that is dependent on speed of rotation and the distance from the axis of rotation. Generally speaking, the centrifugal forces used to separate cells are at least several hundred times greater than the Earth's gravity. The further a particle is from the axis, the greater the force it experiences. The force exerted over a particle is called relative centrifugal force (r.c.f.) and is expressed as multiples of the Earth's gravitational field '*g*', thus 100 *g*, 1000 *g*, and so on. The r.c.f. generated by a spinning rotor is related to its speed of rotation in r.p.m. (revolutions per minute) and the distance of the particle from the axis in centimetres by the equation:

$$\text{r.c.f.} = 11.18 \times r \times (\text{r.p.m.}/1000)^2 \qquad [1]$$

and the speed required to achieve the required centrifugal force can be calculated from the equation:

$$\text{r.p.m.} = 299.07/=\sqrt{\text{r.c.f.}/r} \qquad [2]$$

where r is the radius of the particle from the centre of rotation in centimetres.

The rate at which a particle migrates depends upon the force generated and a number of other parameters, as shown in the equation:

$$\upsilon = \frac{d^2 (\rho_p - \rho_m) g}{18\mu} \qquad [3]$$

where υ is the velocity of sedimentation (cm/sec), d is the diameter of the particle (cm), ρ_p is the density of the particle (g/cm^3), ρ_m is the density of the medium (g/cm^3), μ is the viscosity of the medium (poise), and g is the centrifugal force (dyne).

Equation 3 shows that, as previously stated, the sedimentation rate is proportional to the size of the particle and to the difference between the density of the medium and that of the particle. When the densities of the cell and medium are equal, the sedimentation rate becomes zero and the cell stops migrating. When the density of the medium exceeds that of the cell, the rate of sedimentation is negative, and the cell will migrate against the direction of the centrifugal force, moving towards the top of the centrifuge tube.

The equation also implies that cells can be separated either on the basis of their buoyant densities or on the basis of their sedimentation rates, the latter of which is determined primarily by differences in size. Centrifugal techniques that rely on differences in the sizes of cells are differential pelleting and rate-

zonal centrifugation. Techniques relying upon density differences are isopycnic centrifugation and techniques using density barriers. While simple pelleting procedures, such as are involved in differential pelleting, can only give a limited resolution of different types of cells, rate-zonal, isopycnic techniques, and density barriers can give a high degree of resolution.

3. Separation media

3.1 Criteria for cell separation media

The separation of intact cell populations by sedimentation requires careful choice of the separation conditions. For example, animal cells are very sensitive to changes in their osmotic environments: taking up water and swelling in hypo-osmotic conditions, losing water and shrinking in hyperosmotic conditions. In very hypo-osmotic conditions, the swelling of the cells can be sufficient to lyse them, while, in general, cells are less liable to damage by hyperosmotic environments. In either case, the changes in volume brought about by non-physiological osmotic environments cause changes in the buoyant densities of the cells. Small changes in buoyant density, induced by controlled changes in the osmolality of the medium, can sometimes be used to improve the resolution of some cells, while under physiological conditions, their buoyant densities are very close. The ability to control the osmolality of gradients within strict limits, is therefore an advantage for any gradient medium used for separating cells. As shown in *Equation 3*, the viscosity of a medium is also important in determining the migration rates of cells in that medium and this in turn can affect the resolution of bands. The medium itself should, of course, be totally non-toxic to the cells, its presence should not affect the function or morphology of the cells, and it should be easy to remove after fractionation. The presence of the medium should not interfere with any assays, chemical, or enzyme marker. It is also very useful to have a simple, efficient method of determining the density of the gradient solutions and gradient fractions.

The compounds which meet all the requirements for gradient separations of cells are quite limited (*Table 1*). Some solutes that are thought of as being relatively harmless can be toxic to cells. For example, while sucrose is widely used for organelle separations it tends to be toxic to cells, and similarly salts that are used in balanced salt solutions can be toxic at higher concentrations. Three of the original media used for cell separations on gradients were bovine serum albumin (BSA), Ficoll (a synthetic polysaccharide), and for isopycnic separations, a colloidal silica preparation known as Ludox. Although none of these three proved to be ideal, in that they could be toxic to cells and exhibited other disadvantageous properties, Ficoll and BSA are still used to a limited extent. The colloidal silica, Ludox, has been completely replaced by Percoll (Pharmacia Biosystems) which is less toxic as a result of the colloidal particles being coated with polyvinylpyrrolidone (PVP). As the colloidal particles themselves

Table 1. Properties of gradient media for separating cells

Medium	Molecular weight	Osmolality	Comments
Ficoll	400 000	Low at low concentrations	Can be toxic at high concentrations
Bovine serum albumin	68 000	Low–medium	High UV absorption
Percoll	Colloidal particles 15 nm diameter	Very low	Silica particles may stick to cells and be ingested
Metrizamide	789	Medium	High UV absorption
Nycodenz	821	Medium	High UV absorption
OptiPrep	1550	Low–medium	Dimeric structure reduces osmolality

exert no osmotic pressure, the osmolality of the suspension is controlled by adding the required osmotic balancers during the preparation of its gradients. Being a colloidal suspension, self-generated Percoll gradients can be formed very quickly during centrifugation.

While Percoll has become the most widely used medium for isopycnic cell separations, iodinated gradient media have also proved useful for both rate-zonal and isopycnic separations. The ionic, iodinated media, such as sodium metrizoate and sodium diatrizoate, were originally used for cell separations, but their osmolalities are relatively high and they can have toxic effects on cells. However, they are still widely used, very successfully, for some applications, notably for ready prepared solutions for the routine separation of blood cells. The non-ionic, iodinated media, metrizamide, Nycodenz, and OptiPrep, have properties which come close to those of an ideal density gradient medium for cell separations: providing autoclavable, non-toxic, and non-ionic solutions of relatively high densities, and low osmolality and ionic strength.

3.2 Colloidal silica media

Percoll is the best known and most widely described of the colloidal silica media. It is supplied as a prepared suspension of 15 nm diameter silica particles coated with PVP. The suspension supplied has a density of 1.13 g/ml and an osmolality of about 15–20 mOsm. Stock iso-osmotic working solutions should be prepared by adding solutions of either culture medium, sodium chloride, or glucose to the suspension. The working solutions are then further diluted as required for the gradients. Due to the colloidal nature of this medium, density gradients can be generated very rapidly by centrifugation of the Percoll suspension at 20 000–30 000 *g* for 30 min. Gradients form most quickly in vertical and shallow fixed-angle rotors, the use of swing-out rotors is not recommended. As the osmolality is almost entirely due to the osmolality of the diluents, which do not sediment during the period of centrifugation for generating the density gradients and separation of the cells, the osmolality of the gradient can be maintained within narrow limits throughout.

Percoll gradients, in spite of their wide use and popularity, do have a number of disadvantages. While the suspension is sterile as supplied, the solutions used as diluents must be autoclaved separately before adding them to the Percoll stock solution. Percoll cannot be autoclaved in the presence of osmotic balancers. Another problem is caused by the adherence of silica particles to membranes, which prove difficult to remove even after several washes. Such multiple washes tend to damage the cells, causing reduced recovery of the cells of interest. Furthermore, the silica particles have been shown to be ingested by cells at temperatures above 5°C, producing artefactual bands. Percoll absorbs in the UV and is also incompatible with most protein assays.

3.3 Iodinated gradient media

Metrizamide and Nycodenz (*Figure 1a* and *b*) have very similar physical and chemical properties, the important difference being the absence of a sugar group on the Nycodenz molecule. Because of the absence of the sugar group, the presence of Nycodenz does not interfere with a number of the common chemical assays. Furthermore, Nycodenz solutions can be autoclaved while

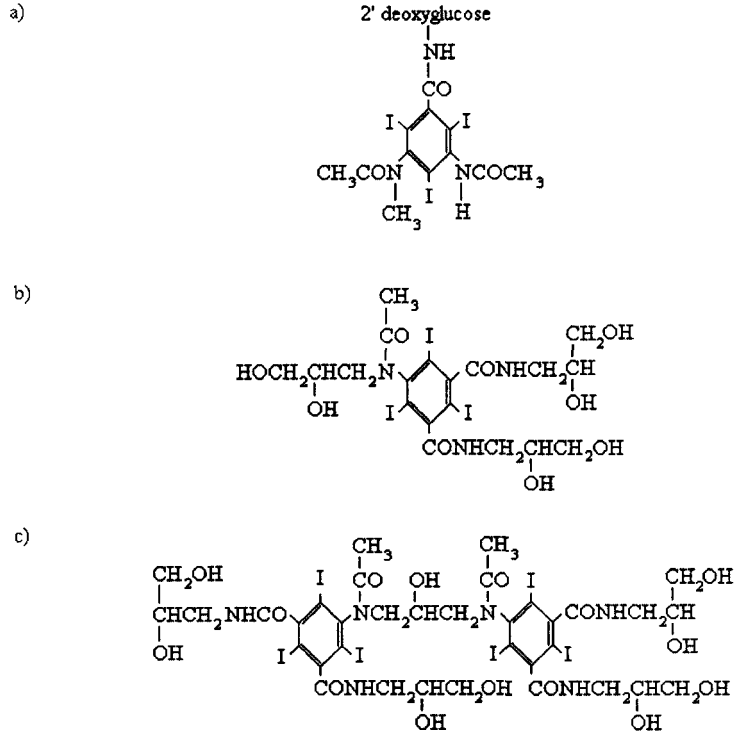

Figure 1. Structures of (a) Metrizamide, (b) Nycodenz, and (c) Iodixanol (OptiPrep).

metrizamide cannot. Both metrizamide and Nycodenz solutions can provide iso-osmotic solutions (approx. 300 mOsm) for cell separations up to densities of 1.16 g/ml, sufficient to band most mammalian cells. Although Nycodenz does not interfere with most biochemical assays, it does interfere with Folin–phenol and microbiuret assays for protein, and the anthrone assay for polysaccharides. It also produces quenching of radiolabel scintillation. Nycodenz is available as a prepared solution of 27.6% (w/v) Nycodenz with an osmolality of 295 mOsm, under the trade name NycoPrep 1.150.

Iodixanol (*Figure 1c*) is essentially a dimer of Nycodenz (1) and is available as a ready to use solution under the trade name OptiPrep. OptiPrep is a 60% (w/v) solution of Iodixanol, made up in water without any other additives. The OptiPrep solution as supplied has a density of 1.32 g/ml and an osmolality of 260 mOsm. The low osmolality, less than half that of Nycodenz or metrizamide solutions of the same density range, is due to the molecular weight of Iodixanol being about twice that of the other two compounds. The low osmolality combined with the high density of OptiPrep has widened the range of applications that can be carried out under iso-osmotic conditions, so that dense cells, such as mammalian sperm, can also be separated without exposing them to high osmolalities.

4. Solutions for iso-osmotic gradients

For separating cells it is always desirable, and sometimes obligatory, to use media that are iso-osmotic with the cells to be separated. In the case of animal cells that have no rigid cell walls it is essential that the osmolality is as close to physiological as possible as otherwise the cells can be damaged. However, as mentioned previously (Section 3.1) it is possible to deviate to some extent from iso-osmotic conditions in order to optimize separations.

4.1 Percoll gradients

The colloidal silica medium, Percoll, is supplied as a 23% (w/w) colloid with a density of 1.13 ± 0.005 g/ml. The silica particles themselves do not contribute to the osmolality or the refractive index of the suspension, so the given osmolality (< 25 mOsm) and refractive index (1.3540) of the suspension is probably due to free PVP.

The Percoll suspension as supplied has to be diluted to a working stock solution using as diluents either 1.5 M NaCl or a ten times concentrated nutrient/culture medium. The working stock and subsequent dilutions with either salt or culture medium diluents will therefore have the osmolalities and refractive indices mainly determined by those of the diluent used.

As stated previously, the colloidal nature of Percoll allows the formation of self-generated gradients in fixed-angle rotors, in relatively short centrifugation times, using centrifugal forces of about 20 000–30 000 *g*, depending on whether

the diluent solution is ionic. The higher ionic strength of salt solutions increase the sedimentation rates of silica particles, thus shorter centrifugation times, or lower rotor speeds are required to achieve the same density gradient, as compared with the non-ionic (e.g. glucose) diluents. The density profiles of Percoll gradients will change continuously during centrifugation as the particles sediment; prolonged centrifugation at high speed will result in all the silica pelleting. This is not a problem with gradients designed for cell separations, as the large size of the cells and their relatively rapid sedimentation rates require short centrifugation times and relatively low centrifugal forces.

The stock working suspensions of Percoll using NaCl as the osmotic balancer should be prepared as follows:

(a) 1.5 M NaCl has a refractive index of 1.3475 and a density of 1.06 g/ml.

(b) Mix 1 vol. of 1.5 M NaCl with 9 vol. of Percoll suspension to obtain a refractive index of 1.3533 and a density of 1.123 g/ml.

The above stock working solution now contains 0.15 M NaCl and has an osmolality of about 290 mOsm. Dilution of this working solution to the densities required for iso-osmotic gradient formation is carried out using 0.15 M NaCl solutions (*Table 2*). The osmolality of the diluted fractions, using 0.15 M NaCl, is approx. 290 mOsm.

The density of the solutions can be calculated by:

$$(A \times \text{density of Percoll}) + (B \times \text{density of diluent}) / (A + B) \qquad [4]$$

where A is the number of parts of Percoll and B is the number of parts of diluent. Using *Table 2*, intermediate densities can be easily calculated.

The density of fractions from a Percoll gradient can best be determined by running an identical gradient, loaded with density marker beads, in place of the sample. After fractionation of both gradients, the position of the marker beads in each fraction indicate the density of the equivalent fraction of the

Table 2. Dilutions for Percoll diluted with sodium chloride

Parts Percoll	Part diluent	Density (g/ml)
1.0	9.0	1.018
2.0	8.0	1.029
3.0	7.0	1.041
4.0	6.0	1.053
5.0	5.0	1.065
6.0	4.0	1.076
7.0	3.0	1.088
8.0	2.0	1.100
9.0	1.0	1.111
10.0	0.0	1.123

sample gradient. The Percoll handbook, available from Pharmacia Biosystems, gives details of the densities of the various marker beads.

4.2 Non-ionic iodinated media

A number of different compounds have been developed for X-ray contrast studies, but only Nycomed Pharma AS and its distributors make their compounds available for use as centrifugation media. This section will concentrate on two of the most useful of these media, Nycodenz and OptiPrep.

Stock solutions of these media can be prepared which are iso-osmotic with mammalian cells. Nycodenz can be obtained in dry powder form or as a ready prepared, iso-osmotic solution, under the trade name NycoPrep 1.150. OptiPrep is obtainable only as an iso-osmotic solution, while metrizamide solutions have to be prepared from dry powder and filter sterilized. The stock solutions can be diluted using diluent solutions designed to maintain the osmolality of the diluted fractions. A selection of suitable diluent solutions is shown for each of these media as described later, but other solutions for specific purposes can be designed as required.

A linear relationship between refractive index and the density of solutions of these media allows an equation to be derived from which the density of gradient fractions can be easily determined in the form:

$$\text{Density} = (\text{RI} \times A) - B \qquad [5]$$

where RI is the refractive index at 20°C and A and B are the derived constants.

This determination of density is very accurate when the diluent is water, but less accurate when used with diluents containing salts and buffers, as the distribution of such salts changes with diffusion and their contribution to the refractive index can only be estimated. Where buffers are used the following equation should be used:

$$\text{Refractive index (RI)} = \text{RI}_{\text{observed}} - (\text{RI}_{\text{buffer}} - \text{RI}_{\text{water}}). \qquad [6]$$

However, for practical, day to day purposes, it is possible to use the approximate constants for the diluents given, which should be sufficiently close and reproducible for most purposes. The derived constants A and B for gradient solutions made up using the various diluents are given with each table of dilutions. For a more precise determination of density, a pycnometer should be used.

4.2.1 Nycodenz

Although Nycodenz is available as a stock iso-osmotic solution, NycoPrep 1.150, the preparation of the solution from the dry powder is described. The inclusion of EDTA in the solution is necessary to stabilize the Nycodenz molecule during autoclaving. Tricine rather than Tris should be used for buffering solutions as the latter is toxic to some cells. Solutions for the preparation of stock Nycodenz solution are shown in *Table 3*. *Tables 4* and *5* show the properties of iso-osmotic Nycodenz solution diluted with diluents.

Table 3. Composition of iso-osmotic Nycodenz solution

Solution	Constituents	Properties
Buffered medium	5 mM Tricine–NaOH pH 7.5, 3 mM KCl, 0.3 mM CaEDTA	Refractive index (20°C) 1.3332; osmolality 20 ± 1 mOsm
Nycodenz solution	27.6 g of solid Nycodenz dissolved and made up to 100 ml with buffered medium	Refractive index (20°C) 1.3784; density 1.148 ± 0.002 g/ml; osmolality 290 ± 5 mOsm
Sodium chloride diluent	0.75 g NaCl made up to 100 ml in the buffered medium	Refractive index (20°C) 1.3345; density 1.003 g/ml; osmolality 250 ± 1 mOsm
D-glucose diluent	4.1 g D-glucose made up to 100 ml in the buffered medium	Refractive index (20°C) 1.3449; density 1.014 g/ml; osmolality 250 ± 1 mOsm

Table 4. Dilutions for Nycodenz diluted with sodium chloride

Parts Nycodenz	Parts diluent	Density (g/ml)[a]	Refractive index
1.0	9.0	1.017	1.3389
2.0	8.0	1.032	1.3433
3.0	7.0	1.046	1.3477
4.0	6.0	1.060	1.3521
5.0	5.0	1.074	1.3564
6.0	4.0	1.089	1.3608
7.0	3.0	1.103	1.3652
8.0	2.0	1.117	1.3696
9.0	1.0	1.132	1.3740
10.0	0.0	1.146	1.3784

[a] In the equation for density (*Equation 5*), $A = 3.2574$ and $B = 3.3440$.

Table 5. Dilutions for Nycodenz diluted with D-glucose

Parts Nycodenz	Parts diluent	Density (g/ml)[a]	Refractive index
1.0	9.0	1.027	1.3483
2.0	8.0	1.040	1.3516
3.0	7.0	1.054	1.3549
4.0	6.0	1.067	1.3583
5.0	5.0	1.080	1.3616
6.0	4.0	1.093	1.3650
7.0	3.0	1.106	1.3684
8.0	2.0	1.120	1.3717
9.0	1.0	1.133	1.3750
10.0	0.0	1.146	1.3784

[a] In the equation for density (*Equation 5*), $A = 3.9403$ and $B = 4.2853$.

4.2.2 OptiPrep

OptiPrep is available only as a prepared solution consisting of 60% (w/v) Iodixanol in water, with no other additives. The solution has a density of 1.32 g/ml, an osmolality of 260 mOsm, and a refractive index of 1.4287. The high density of this solution while maintaining an osmolality compatible with all mammalian cells will be an advantage in some separation methods, as will be shown in the protocols. Working solutions of OptiPrep are prepared from the stock solution using various diluents as shown in *Table 6. Tables 7–10* show the properties of OptiPrep in each of the given diluents.

4.3 Other types of gradient media

4.3.1 Ficoll

Ficoll (Ficoll 400) is a synthetic high molecular weight polymer (M_r 400 000)

Table 6. Diluent solutions for OptiPrep

Diluent	Preparation	Properties
Sodium chloride	0.8% (w/v) NaCl containing 20 mM Tricine–NaOH pH 7.8	Refractive index (20°C) 1.3350; density 1.005 g/ml; osmolality 295 mOsm
D-glucose	4.4% (w/v) D-glucose containing 10 mM Tricine–NaOH pH 7.8	Refractive index (20°C) 1.3398; density 1.0153 g/ml; osmolality 270 mOsm
Mannitol	4.4% (w/v) mannitol containing 10 mM Tricine–NaOH pH 7.8	Refractive index (20°C) 1.3400; density 1.0146 g/ml; osmolality 270 mOsm
Sorbitol	4.4% (w/v) sorbitol containing 10 mM Tricine–NaOH pH 7.8	Refractive index (20°C) 1.3398; density 1.0138 g/ml; osmolality 265 mOsm

Table 7. Dilutions for OptiPrep diluted with sodium chloride

Parts OptiPrep	Parts diluent	Density (g/ml)[a]	Refractive index
1.0	9.0	1.036	1.3444
2.0	8.0	1.068	1.3537
3.0	7.0	1.099	1.3631
4.0	6.0	1.131	1.3725
5.0	5.0	1.163	1.3819
6.0	4.0	1.194	1.3912
7.0	3.0	1.225	1.4006
8.0	2.0	1.257	1.4100
9.0	1.0	1.289	1.4193
10.0	0.0	1.320	1.4287

[a] In the equation for density (*Equation 5*), $A = 3.3618$ and $B = 3.4830$.

Table 8. Dilutions for OptiPrep diluted with D-glucose

Parts OptiPrep	Parts diluent	Density (g/ml)[a]	Refractive index
1.0	9.0	1.046	1.3487
2.0	8.0	1.076	1.3576
3.0	7.0	1.107	1.3665
4.0	6.0	1.137	1.3754
5.0	5.0	1.168	1.3842
6.0	4.0	1.198	1.3931
7.0	3.0	1.229	1.4020
8.0	2.0	1.259	1.4109
9.0	1.0	1.290	1.4198
10.0	0.0	1.320	1.4287

[a] In the equation for density (*Equation 5*), $A = 3.4274$ and $B = 3.5768$.

Table 9. Dilutions for OptiPrep diluted with mannitol

Parts OptiPrep	Parts diluent	Density (g/ml)[a]	Refractive index
1.0	9.0	1.045	1.3487
2.0	8.0	1.076	1.3576
3.0	7.0	1.106	1.3665
4.0	6.0	1.137	1.3754
5.0	5.0	1.167	1.3842
6.0	4.0	1.198	1.3931
7.0	3.0	1.228	1.4020
8.0	2.0	1.259	1.4109
9.0	1.0	1.289	1.4198
10.0	0.0	1.320	1.4287

[a] In the equation for density (*Equation 5*), $A = 3.4353$ and $B = 3.5880$.

Table 10. Dilutions for OptiPrep diluted with sorbitol

Parts OptiPrep	Parts diluent	Density (g/ml)[a]	Refractive index
1.0	9.0	1.044	1.3487
2.0	8.0	1.075	1.3576
3.0	7.0	1.106	1.3665
4.0	6.0	1.136	1.3754
5.0	5.0	1.167	1.3842
6.0	4.0	1.198	1.3931
7.0	3.0	1.228	1.4020
8.0	2.0	1.259	1.4109
9.0	1.0	1.289	1.4198
10.0	0.0	1.320	1.4287

[a] In the equation for density (*Equation 5*), $A = 3.4443$ and $B = 3.6009$.

made by polymerization of sucrose with epichlorohydrin. Ficoll solutions can only be produced to give fairly narrow density ranges between 1.00–1.15 g/ml. Solutions below 20% (w/v) (1.07 g/ml) are osmotically inert but at concentrations above this there is a sharp increase in osmotic strength. Ficoll solutions are very viscous; although the high viscosity is not ideal for cell separation, the gradients are very stable. Ficoll is non-toxic to cells and does not interfere with most biological assays although some quenching of radiolabel scintillation counting can occur. Ficoll in aqueous solution is autoclavable but may caramelize if phosphate is present. Ficoll solutions can be sterilized by filtration or UV irradiation. Ficoll solutions need to be preformed into a gradient, they do not self-generate as Percoll does. The gradients produced can be analysed by measuring the refractive indices of the fractions, at 20°C. The density of the fractions can be determined using *Equation 5*, where A is 2.381 and B is 2.175.

Removal of Ficoll from separated cells is best achieved by repeated washings. Although fibroblasts, hepatocytes, and tumour cells have been fractionated using Ficoll gradients (2–5), the high viscosity and high osmolality found with Ficoll solutions limits their use for cell separation. Another disadvantage of Ficoll is its high cost. Combined with metrizoate or diatrizoate it has proven particularly useful for separating lymphocytes from whole blood where it is used primarily to aggregate the red blood cells (see Section 8).

4.3.2 Bovine serum albumin

Concentrated solutions of bovine serum albumin (BSA) were first used for gradient centrifugation of cells by Leif and Vinograd (6) and Shortman (7). BSA Fraction V is generally used; it is readily soluble in aqueous media but concentrated solutions absorb very strongly in the ultraviolet region of the spectrum and are very viscous. On the other hand, protein solutions protect enzymic activities and help to preserve cell integrity. Furthermore, the high molecular weight of the protein makes it relatively inert osmotically. Continuous gradients are best preformed using a gradient making device (see Section 5.2.2). After fractionation the density of the gradient fractions are calculated using the following equation:

$$\text{Density} = (\text{RI} \times 1.4129) - 0.8814 \qquad [7]$$

where RI = refractive index at 24°C.

Cells can be separated in 10–35% (w/v) gradients of BSA in balanced salt solutions. For isopycnic separations, the cells can be either dispersed throughout the gradient or layered on top or into the bottom of the gradient, and centrifuged at about 13 000 *g* for 30 min at 4°C. Alternatively, cells have been separated in 1–3% (w/v) gradients of BSA using unit gravity sedimentation (1 *g*); unit gravity sedimentation is described in Section 7.2.1. The distribution of cells can be determined by either direct particle counting, sizing apparatus, or by scintillation counting where the cells have been radiolabelled. However,

drawbacks with using BSA as a gradient medium include the great variability between batches of BSA, some of which cause cells to aggregate. As previously stated, concentrated solutions of BSA are very viscous and are unsuitable for the densities required for cell separation. Also, albumin appears to promote pinocytosis by cells, resulting in the formation of cell vacuoles, but this can be minimized by fractionating the cells in the cold.

5. Preparation of iso-osmotic gradients

Iso-osmotic density gradients can be prepared from any of the colloidal silica, non-ionic, iodinated gradient media, Ficoll or BSA solutions, using diluent solutions described for each medium. The colloidal silica media, such as Percoll, can provide useful preformed, self-generated, iso-osmotic gradients for cell separations, but can only be used for isopycnic separations. Preformed continuous or discontinuous iso-osmotic gradients of iodinated media can be prepared by a number of simple methods and they can be used for both rate-zonal and isopycnic separations.

Generally, a discontinuous gradient is only useful for isopycnic separations and in cases where the cell type of interest has a clear difference in buoyant density from the rest of the cells. The number of steps in the gradient, and the layer in which the sample is mixed, will depend upon the distributions of buoyant densities among the cell types present in the sample.

For almost all rate-zonal separations and where the cell type of interest has a buoyant density close to, or overlapping with, other cell types, a continuous gradient should be the gradient of choice. The density profile of a continuous gradient can be controlled to provide very shallow gradients within the density range required, in order to enhance the resolution of the bands of cells.

5.1 Preparation of discontinuous iso-osmotic gradients

Discontinuous gradients are prepared simply by diluting a number of aliquots of a stock solution, with a suitable diluent, to the densities required and underlayering them into a centrifuge tube. The sample solution may be used as a diluent for one of the aliquots, thus the sample may be placed in the gradient in any position, top, middle, or bottom. Once prepared, discontinuous gradients must be used immediately before significant diffusion, which smoothes out the interfaces, can take place.

5.2 Preparation of continuous iso-osmotic gradients

Percoll, because of its colloidal nature, forms density gradients very quickly during centrifugation, while, in the relatively short spin times required, the salt or other diluent does not sediment to any significant degree, thus the gradient remains iso-osmotic throughout. For the purposes of cell separations, continuous gradients of the iodinated media cannot be self-generated.

5.2.1 Preparing continuous gradients by the diffusion method

Two to four aliquots of different densities can be made up and layered into a centrifuge tube, as for a discontinuous gradient; an initial four step gradient will form a better continuous gradient by diffusion than if only two steps are used. The tube is then securely capped and laid in a horizontal position (for a 10 mm diameter tube leave it undisturbed for 45–60 min at room temperature) to let a smooth gradient form by diffusion across the interfaces.

5.2.2 Mechanical gradient mixers

Other methods use mechanical gradient mixers, either the simple two-chamber type (*Figure 2a*), or a more sophisticated type of mixer, such as the Gradient Master, made by Biocomp (*Figure 2b*).

i. Two-chamber type gradient-mixers

Some of the two-chamber devices can be used to prepare two or three gradients at the same time. The two chambers are loaded with the gradient medium, diluted to the two appropriate densities representing the higher and lower ends of the density range required. The chambers are connected at the bottom by a short tube, and the chamber containing the lighter medium connects to the outlet to the centrifuge tubes. A magnetic stirring bar is present in the chamber with the lighter medium. The stirring bar is set spinning and the connecting tube opened to allow passage of the denser medium into the chamber of the lighter. A peristaltic pump draws the mixed medium from the outlet, and delivers it to the bottom of the centrifuge tube. As the process continues, more of the denser medium is mixed with the lighter, so a denser mixture enters the centrifuge tube, underlayering the medium already present. Thus, a continuous gradient is formed, almost linear in profile. The profile can be manipulated to some extent by varying the initial relative heights of the medium in each chamber, so varying the amount and rate of mixing. Some of these devices have the outlet split to enable two or three gradients to be formed simultaneously. Iso-osmotic gradients formed in this way tend to deviate less from isotonicity across the gradient compared with gradients formed by diffusion.

A disadvantage with this type of gradient mixer is that the whole device, chambers and tubes, has to be cleaned and, if necessary, sterilized between operations. To prepare a sterile gradient, the device would have to be used under a sterile hood.

ii. The Gradient Master

The Gradient Master, and similar types of device, operates on a different principle. The centrifuge tubes are loaded with the two densities of gradient solutions, the denser layered under the lighter. The tube is capped and placed into the rotating barrel (see *Figure 2b*). Depending upon the size barrel used, it will take three to six centrifuge tubes at one time. The barrel is then tilted to a

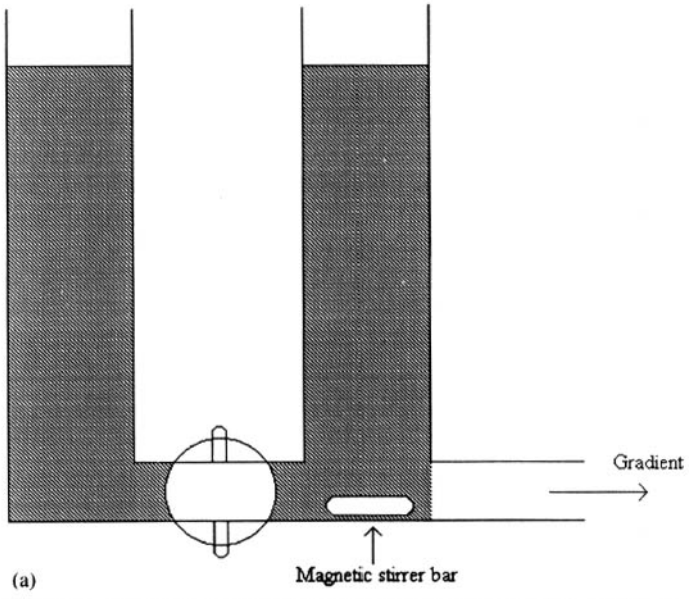

Figure 2. (a) A simple two pot gradient mixer for the preparation of linear gradients. (b) Gradient Master gradient maker uses tube rotation to enhance diffusion and create linear gradients.

given angle from the vertical, about 75–80°, and the barrel is rotated about its own axis for a given time, usually about 1.5–2.5 min. During rotation a continuous gradient is formed by enhanced diffusion of the two solutions across the interface. The density profile of the gradient can be manipulated by variation of the angle of rotation, the time of rotation, and by variation of the densities of the aliquots of medium.

The advantage of this type of mixer is that the gradient medium is completely contained within the centrifuge tubes. No cleaning of the device is needed. Sterile gradients can be used; the dilution of aliquots and the loading of the centrifuge tubes can be carried out under a sterile hood, the tubes capped, and then removed from the hood to the device. It also allows hazardous samples to be mixed with the gradient aliquots and distributed throughout the gradients in a safely contained manner. The disadvantage of this apparatus is that it can only be used for gradients in tubes.

Irrespective of whether a continuous gradient is formed by mechanical means or by diffusion, the sample may be distributed throughout the gradient if one of the aliquots is diluted using the sample solution. Alternatively, after the gradient is formed, the sample can be mixed with the gradient medium to a final density a little denser than that of the bottom of the gradient. The sample may then be underlayered to the bottom of the gradient. Loading samples within, or at the bottom of a gradient has the advantage of minimizing the formation of artefactual bands, that is, bands which contain trapped material which should have passed on to another banding area.

6. Choice of separation method

As described in Section 2, cells can be separated on the basis of size or density. In most cases it is possible to follow an existing published protocol but when working with new types of cell it may be necessary to devise a new separation procedure. As a general rule, isopycnic centrifugation (see Section 8) is sufficient to obtain an adequate fractionation but it is better to confirm this by carrying out an s-ρ analysis (8). In order to do this the cell sample is separated first on the basis of size by rate-zonal centrifugation (see Section 7.2.2), and then on the basis of density by isopycnic centrifugation. The relative purification obtained using each method can then be compared and a decision taken as to which is the best approach to take depending on the nature of the other material present in the sample. Note that in the case of isopycnic centrifugation, separations can be enhanced by density perturbation if the exact nature of the cells of interest are known (see Section 8.8).

7. Separation of cells on the basis of size

7.1 Differential pelleting

The number of cells in the pellet for any given centrifugal force is dependent on their size and the time of centrifugation; if allowed to spin for long enough all the cells will collect in the pellet. In order to obtain good separations between particles by differential pelleting it is necessary to have a size difference of at least tenfold. In practice, very few cells exhibit such large differences in size and so this method is seldom used. One exception to this general rule

are platelets as platelets are much smaller than other blood cells. Centrifugation of anticoagulated peripheral blood at 200 *g* for 10 min leaves the bulk of the platelets in the supernatant plasma. This separation can be improved by increasing the density of the medium (see Section 8.1.1).

Differential pelleting is often used as a first step in a separation procedure of several different cell types, for example, rat liver cells. The first step in the separation of these cells is to separate the parenchymal cells from the other (non-parenchymal) cells, see Section 8.6 by differential pelleting. Pelleting, resuspension, and repelleting can give further enriched fractions but usually at the expense of yields and cell damage.

7.2 Velocity sedimentation

The majority of cells do not have major differences in density but do vary considerably in their size. This attribute is utilized in the following separation methods.

7.2.1 Separation of cells at unit gravity

The principle of unit gravity sedimentation (velocity sedimentation at 1 *g*) is that sedimentation is allowed to proceed under the influence of the Earth's gravity (1 *g*), in a very shallow gradient. The cells in a medium of low density sediment principally on the basis of differences in size. The sample is loaded as a narrow band on top of a continuous gradient of a medium usually of BSA, Ficoll, or Nycodenz. The density of the gradient increases down the tube or chamber but is such that it does not exceed the buoyant densities of the particles in the sample. For osmotically-sensitive animal cells an osmotic balancer will be required for media such as Nycodenz to ensure that cells are not modified as they move down the gradient. The cells can therefore continue to sediment until they reach the bottom of the tube or chamber, given sufficient time. As the cells start out from almost the same starting point, as they sediment the larger cells sediment ahead of the smaller ones, forming zones each containing particles of similar sizes. As they sediment, the space between the zones (the resolution) will increase.

The major attraction of this technique is that it avoids imposing on cells the stress of centrifugation which is known to disrupt the normal functioning of cells (9). However, one of the major problems of these separations is the need to prepare a concentrated suspension of cells and the subsequent interaction of cells in the sample layer. As the density of the cells is greater than the density at any point in the gradient, if left for long enough, all the cells will form a pellet. Moreover, the Earth's gravitational field is fairly weak compared with the forces developed in a centrifuge and so separations take much longer than centrifugal separations.

Several different types of apparatus have been developed for velocity sedimentation at unit gravity. They include the STATUP, Buchler Poly-Prep 200 column, LACS, and the Celsep apparatus. In each of the different forms of

apparatus, cells start as a narrow band near the top of a shallow gradient through which they sediment. The gradient reduces convection and mixing of the different layers of separated cells during unloading. The Buchler Poly-Prep 200 column and the Celsep apparatus are described in *Figures 3* and *6*, respectively.

The following protocol describes the separation of peripheral blood by velocity sedimentation at unit gravity using a Buchler Poly-Prep 200 column.

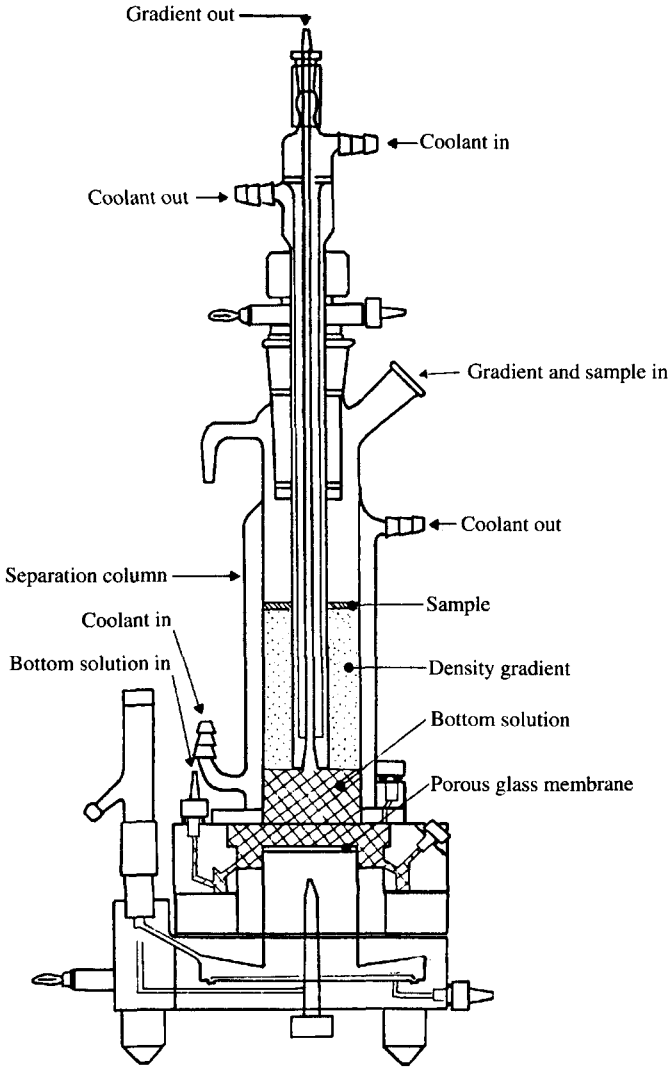

Figure 3. A schematic diagram of a Buchler Poly-Prep preparative electrophoresis column as used for unit gravity separations.

Protocol 1. Rate-zonal separation of cells on a BSA gradient at unit gravity using a Buchler Poly-Prep 200 column (10)

Equipment and reagents

- Buchler Poly-Prep 200 column
- Two-chamber type gradient mixer
- Bovine serum albumin (BSA)
- Whole blood
- Phosphate-buffered saline (PBS): 8 mM Na_2HPO_4, 2 mM $NaH_2PO_4.H_2O$, 0.15 M NaCl pH 7.4

A. *Preparation of BSA*

1. Dissolve 7.5 g BSA (Fraction V) in 80 ml PBS, and dialyse overnight at 4°C against 5 litres of PBS.
2. Make up the dialysed BSA to 250 ml with PBS; this is used as a 3% (w/v) stock solution.
3. Prepare 0.5%, 1%, and 2% solutions of BSA in PBS by appropriate dilution of stock BSA.

B. *Sedimentation procedure*

1. Introduce 50 ml bottom solution (3% BSA) through bottom solution in-port of column.
2. Using a two-chamber type gradient mixer, overlayer bottom solution with 100 ml gradient. Use 50 ml light solution (1% BSA) and 50 ml dense solution (2% BSA) to give a density range of 1.0099–1.0123 g/ml; pump gradient into column at a rate of 1 ml/min through gradient in-port.
3. Overlayer gradient with 10 ml sample solution (0.5% BSA) containing not more than 5×10^6 cells/ml, at a rate of 1 ml/min; sample solution must be cooled to 4°C before applying to gradient.
4. Overlayer with 10 ml PBS at a rate of 1 ml/min.
5. Time zero is taken as that time at which one-half of the sample solution has been layered on the gradient.

C. *Fractionation of gradient*

1. After the desired time of velocity sedimentation, fractionate gradient.
2. Pump bottom solution at a rate of 1 ml/min into the bottom solution in-port of Buchler column.
3. Pump gradient out through the gradient out-port of the column at a rate of 2.9 ml/min. The denser part of the gradient is collected first. Collect 1.5 ml fractions.
4. Record cell number versus fraction number collected and estimate the mean sedimentation velocity of recognizable subpeaks.

Figure 4 shows a schematic diagram of the gradient prepared. A typical fractionation of human blood cells by this method is shown in *Figure 5*. The curves were normalized by computer to the highest peak of each distribution. The polymorphonuclear cells were distinctly separated from the mononuclear cells (lymphocytes and monocytes), erythrocytes, and platelets. However,

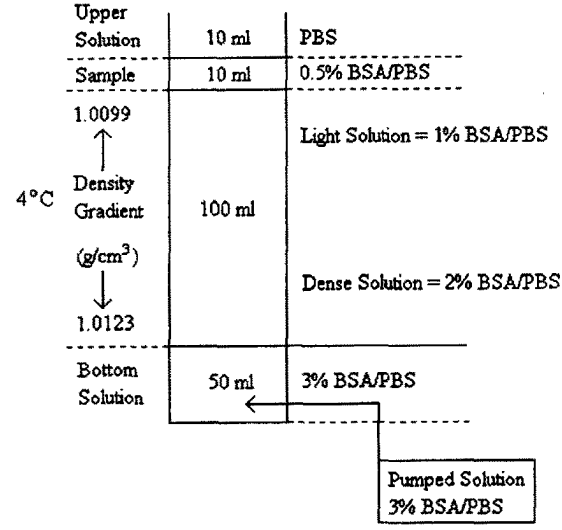

Figure 4. A schematic diagram of the solutions used in conjunction with a Buchler Poly-Prep column for unit gravity separations in BSA gradients.

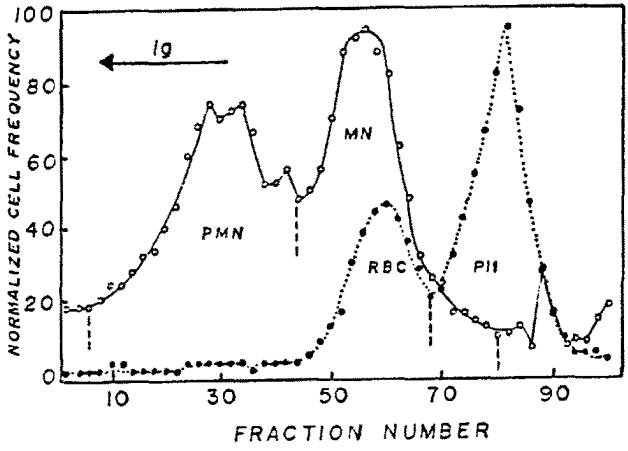

Figure 5. Rate-zonal sedimentation of human blood cells at unit gravity. The position of the bands of polymorphonuclear cells PMN, mononuclear cells MN, erythrocytes RBC, and platelets Plt are shown.

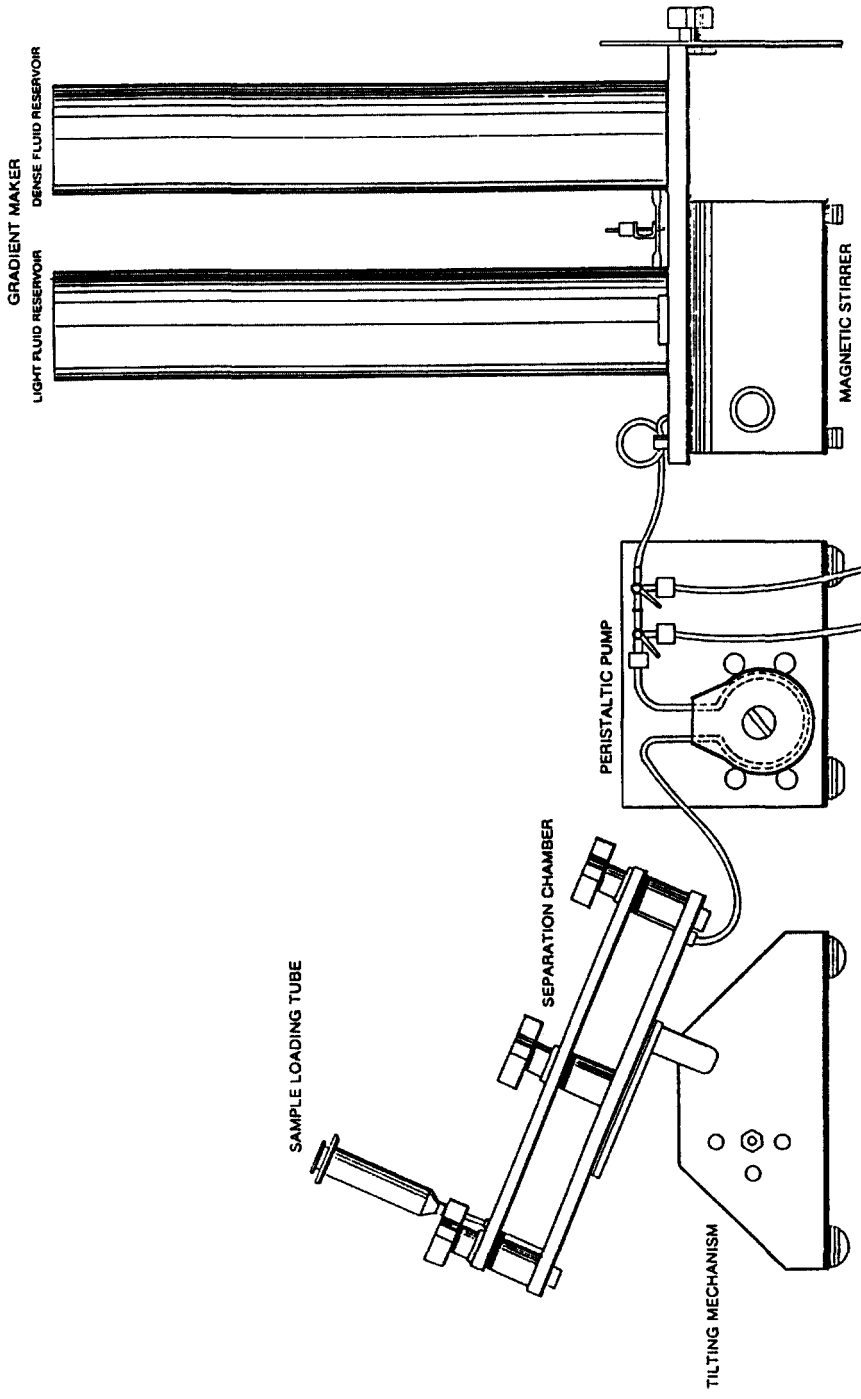

Figure 6. The Celsep unit gravity cell separation system.

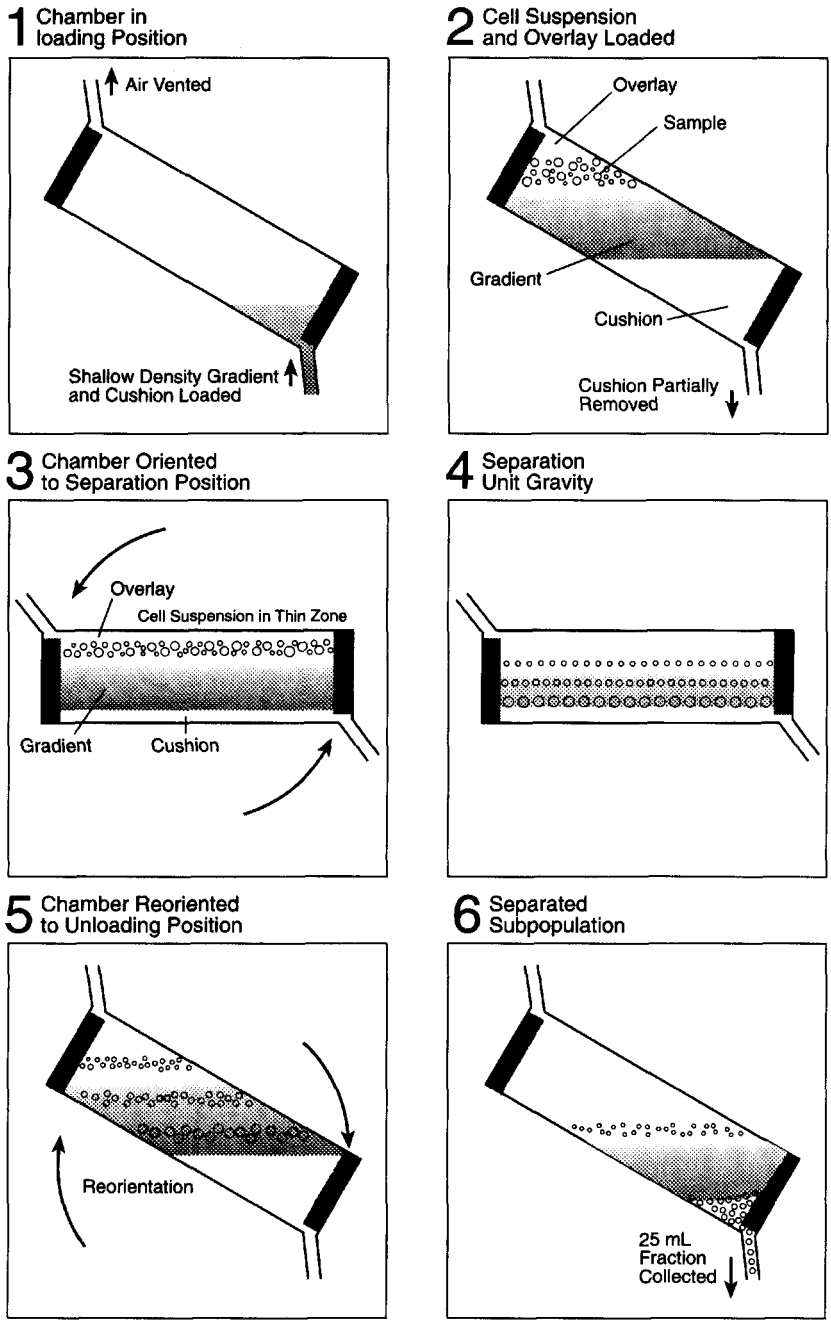

Figure 7. Procedures used for the loading, separation, and unloading of fractions using the Celsep system.

separation of mononuclear cells from erythrocytes was not possible. Essentially, small volume cells (e.g. platelets) are found in the lighter part of the column, the large volume cells (e.g. polymorphonuclear cells) in the denser part of the gradient, and intermediate sized cells are present in the middle fractions. The transition from small to intermediate to large volume cells is gradual, which indicates overlapping of distributions in certain fractions and thus optimal resolution is not achieved. Despite the limited resolution, the described method supplies highly viable and functional cells.

The Celsep apparatus comprises of a cylindrical chamber mounted on a tilting assembly (*Figure 7*). The chamber is formed by two plastic plates which are clamped to a cylindrical section, the chamber holds 1 litre. The tilting assembly is motor driven and tilts the chamber from the horizontal separation position through an angle of 30° to the filling and unloading position. The apparatus can be sterilized and the separation procedure performed in a tissue culture hood if required. The temperature must be fairly well controlled to prevent convection currents; room temperature or a cold room are satisfactory but heating vents and air conditioning should be avoided. The following protocol describes the separation of mononuclear cells by velocity sedimentation at unit gravity using the Celsep apparatus.

Protocol 2. Velocity sedimentation of cells on a Ficoll gradient at unit gravity using the Celsep apparatus (11)

Equipment and reagents

- Celsep apparatus
- Two-chamber type gradient mixer and peristaltic pump
- Hank's balanced salt solution (HBSS)
- 0.9% (w/v) NaCl or culture media
- Ficoll 400
- Human serum
- Mononuclear cells isolated from peripheral blood

A. *Preparation of Ficoll solutions*

1. Prepare cell sample solution, 1% (w/w) Ficoll in HBSS supplemented with 1% serum.
2. Prepare light solution for gradient, 2% (w/w) Ficoll in HBSS; adjust to pH 7.2 with 7.5% sodium bicarbonate. Supplement with 1% serum and filter through a 0.45 μm filter. Solution has density 1.009 ± 0.0014 g/ml and osmolality 275 ± 3 mOsm.
3. Prepare dense solution for gradient, 4% (w/w) Ficoll in HBSS; adjust pH, supplement with serum, and filter as in step 2. Solution has density 1.015 ± 0.0014 g/ml and osmolality 272 ± 4 mOsm.
4. Prepare dense cushion, 10% (w/w) Ficoll in HBSS.

Protocol 2. *Continued*

B. *Sedimentation procedure*

1. Using a two-chamber type gradient mixer and peristaltic pump, pump a continuous gradient (2–4% Ficoll) into the chamber, light end first at a rate of 35–45 ml/min; the chamber is held in the tilted position during this operation.
2. Pump dense cushion (10% Ficoll) underneath the gradient until chamber is completely full at a rate of 35–45 ml/min.
3. Isolate mononuclear cells from peripheral blood (see *Protocol 5*) and suspend cells (1–1.2 × 10^6 cells/ml) in 1% Ficoll in HBSS. Load the cell suspension through the top inlet by reversing the pump and removing a portion of the cushion, at a rate of 15–20 ml/min.
4. Overlay cell suspension with 0.9% (w/v) NaCl or culture media at a rate of 15–20 ml/min.
5. Bring chamber to horizontal position and allow sedimentation to proceed for 2 h at room temperature.

C. *Fractionation and analysis of gradient separation*

1. After sedimentation, reorientate chamber to the tilted position and unload fractions through the bottom port of apparatus at a rate of 30–40 ml/min.
2. Count the number of cells in each fraction.

Monocytes can be identified by non-specific esterase staining and morphology; lymphoid cells and basophils are identified by morphology and Giemsa staining. *Figure 8* shows a schematic diagram of the separation procedure. The chamber contains about 820–845 ml of gradient, 25–50 ml of cushion, 25–53 ml cell suspension, and 65–75 ml of sample overlay. Monocytes are recovered at a relatively high purity as are lymphocytes.

7.2.2 Separation of cells using rate-zonal centrifugation

The method for rate-zonal centrifugation is essentially the same at that for unit gravity separations, but employs centrifugation to decrease the time required to achieve the separation. In rate-zonal centrifugation, the cells are centrifuged through a gradient the maximum density of which is less than that of the cells so that cells can continue to sediment until they reach the bottom of the tube, given sufficient time and speed of centrifugation. Rate-zonal separations are thus time-dependent and use lower centrifugal forces than isopycnic separations. The usual gradient media used for rate-zonal separations have been Ficoll, and to a lesser extent BSA and fetal serum albumin; theoretically, non-ionic gradient media such as Nycodenz and OptiPrep should also prove suitable for rate-zonal separations. A great deal of work has been done on developing isokinetic Ficoll gradients (12) which have the advantage that cells sediment down the

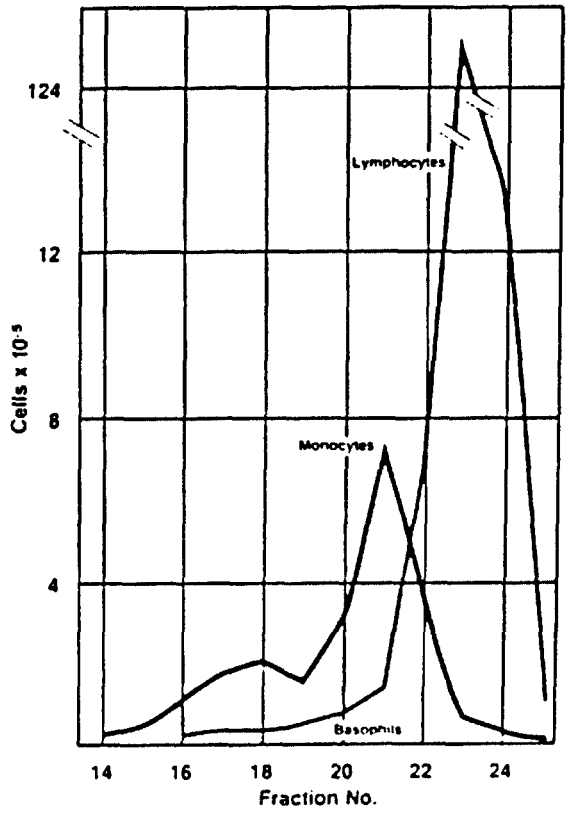

Figure 8. Separation of human monocytes and lymphocytes on a Ficoll gradient using the Celsep system.

gradient at a constant rate. Centrifugation can be performed either in a normal swing-out rotor or in a reorientating zonal rotor. The main problem to be aware of is that there is a tendency for cell clumping or aggregation to occur, particularly when loading gradients with concentrated cell suspensions, and this can limit the use of this method. An example of the rate-zonal separation of proliferative epithelial cells from contaminating cells from a rat cecum and proximal colon cell preparation is given in *Protocol 3* (13).

Protocol 3. Rate-zonal separation of proliferative epithelial cells from rat cecum and proximal colon cell preparation using an isokinetic Ficoll gradient (13)

Equipment and reagents

- Refrigerated centrifuge with swinging-bucket rotor
- 250 ml glass Erlenmeyer flasks
- Metabolic shaker apparatus
- Two-chamber type gradient mixer
- Abbé refractometer

Protocol 3. *Continued*

- Plastic centrifuge tubes
- Nitex (pore diameter 100 μm)
- NaCl, 0.014 M and 0.015 M
- 0.001 M dithiothreitol (DTT)
- Joklik's modified minimal essential medium (MEM)
- Citrate buffer: 0.27 M sodium citrate, 0.0015 M KCl, 0.096 M NaCl, 0.008 M KH_2PO_4, 0.0056 M Na_2HPO_4 pH 7.3
- Ca^{2+}- and Mg^{2+}-free Hank's buffer pH 7.3
- Ficoll
- Rat cecum and proximal colon cell suspension

A. *Preparation of cell suspensions*

1. Rats weighing 250–300 g are fasted 18 h with water *ad libitum*. Kill by cervical dislocation.
2. Excise their cecum and proximal colon, rinse with 0.014 M NaCl and 0.001 M DTT, and evert using a small plastic rod.
3. Tie everted sac at one end, fill with 0.015 M NaCl until it becomes distended, and then tie off the other end.
4. Place into siliconized Erlenmeyer flasks containing pre-heated (37°C) 50 ml solution of 10% FCS in 1.6% Joklik's MEM, and incubate at 37°C for 30 min in Ca^{2+}- and Mg^{2+}-free Hank's buffer pH 7.3.
5. Filter through a single layer of Nitex to remove debris and clumped cells.
6. Centrifuge filtrate at 500 *g* for 10 min. Resuspend pellet in 10% FCS in 1.6% Joklik's MEM at a concentration such that a 7 ml sample suspension contains 2–4 × 10^6 cells.

B. *Gradient preparation*

1. Prepare gradient light solution, 2.7% (w/w) Ficoll in 1.6% Joklik's MEM.
2. Prepare gradient dense solution, 5.5% (w/w) Ficoll in 1.6% Joklik's MEM.
3. Prepare dense cushion solution, 43% (w/w) Ficoll in 1.6% Joklik's MEM.
4. Place 6 ml 43% (w/w) Ficoll dense cushion in 100 ml polycarbonate centrifuge tubes.
5. Using a two-chamber type gradient mixer prepare 76 ml linear density gradient of 2.7–5.5% Ficoll in 1.6% Joklik's MEM, construct on top of dense cushion.

C. *Gradient separation and fractionation*

1. Carefully overlayer 7 ml sample suspension on to isokinetic gradient.
2. Centrifuge for 21 min at 4°C with a centrifugal force of 38 *g* measured at the sample/gradient interface.
3. Fractionate gradient by upward displacement. Collect fractions of 4 ml except for first fraction which will contain 7 ml starting sample layered over the gradient.

D. *Analysis of gradient fractions*

1. Measure refractive index for each fraction using refractometer to confirm linearity of gradient.
2. Count cell numbers in each fraction.
3. Prepare slides of each fraction and stain with haematoxylin and eosin, periodic acid–Schiff (PAS), Wright's, and mucicarmine stains.

The isokinetic gradient in *Protocol 3* varies linearly from 2.7% (w/w) Ficoll at the sample/gradient interface 11 cm from the centre of revolution, to 5.5% (w/w) Ficoll at the gradient/cushion interface 25 cm from the centre of revolution.

Using this approach, Brasitus (13) obtained an enriched subpopulation of viable proliferative epithelial cells. Examination of the starting 7 ml sample suspension revealed a heterogeneous population of cell types. Approximately 70% of these were cuboidal epithelial, 10% columnar epithelial, 15–20% goblet cells, and 1–2% lymphocytes. After rate-zonal centrifugation, over 90% of the cells in fractions 6–18 were found to be cuboidal epithelial cells which became progressively more homogeneous in appearance in successive fractions. The cells in fractions 11–18 were homogeneous and contained 5% goblet cells and an occasional lymphocyte. Fractions 11–18 were highly enriched in proliferative epithelial cells. Fractions 1–5 contained almost all the lymphocytes, columnar epithelial, and goblet cells. The cushion and remaining fractions of the gradient contained debris and numerous clumped cells of various histological types.

The tendency for cell clumping or aggregation with separations using velocity sedimentation limits its use. Therefore, in spite of the wide range of rate-zonal separations of cells described, the preferred method of cell separation is that of separating cells on the basis of their density.

8. Separation of cells on the basis of density

Cells can be separated on the basis of density using either discontinuous or continuous gradients; density barrier methods are included in the former class of procedures. Continuous gradients are ideal for the separation and characterization of cells. The continuous density gradient formed should cover the range of densities of the cells to be separated. The density at the bottom of the gradient is greater than the density of the densest cells to be separated and so however long the cells are centrifuged, the cells will never sediment to the bottom of the tube. The sample may be loaded at the bottom, top, or mixed throughout the gradient.

Isopycnic separations can be carried out either as unit gravity separations or by centrifugation. However, the sedimentation rate of cells in unit gravity is

very slow particularly as cells approach their isopycnic positions (see *Equation 3*) and so isopycnic separations are almost always carried out using centrifugation to ensure that cells reach their isopycnic position in the gradient in a reasonably short time. Generally the centrifugal force used for isopycnic separations is greater than for rate-zonal separations and this may have an adverse effect on the cells (8).

During centrifugation, particles migrate up or down the tube until they reach a point where the density of the medium is equal to the buoyant density of the cells. As in the case of rate-zonal gradients, for osmotically-sensitive animal cells an osmotic balancer will be required for media such as Nycodenz to ensure that cells are not modified as they move down the gradient since changing volumes will modify the effective density of cells.

Once cells have been characterized, it is often more convenient to devise discontinuous gradients or density barrier methods for separating cells for routine or preparative purposes.

8.1 The separation and purification of blood cells using density barrier methods

The diagnostic uses of human peripheral blood has meant that a number of methods have been developed to isolate the different cell types it contains. These usually involve the use of simple density barrier methods and separations may be purely isopycnic or a combination of isopycnic and rate-zonal separations. Because of the routine nature of most of these cell separations which are carried out in hospitals and clinics, ready made solutions have been devised to enable many of these tasks to be carried out in a rapid and simple manner (*Table 11*).

One of the earliest, and still most widely used method for isolating human mononuclear cells, was that published by Bøyum (14). The solution consists of a mixture of sodium metrizoate and Ficoll, which allowed the mononuclear cell fraction to be separated from the erythrocytes and polymorphonuclear cells. This solution, with some commercial modifications, is available under a number of trade names such as Lymphoprep, Ficoll-paque, and Lymphocyte Separation Medium (LSM) (*Table 11*). A number of other simple, routine methods of separating various types of blood cells have since been devised since that first method was published.

8.1.1 Platelets

Platelets are small enucleated cells with functions related to blood clotting. Due to their small size, as discussed in Section 7.1, they will have slower sedimentation rates than the other cells. Simple differential centrifugation can be used to purify platelets but the yield is low (see Section 7.1). A higher yield can be achieved by sedimenting the cells through a density barrier; the erythrocytes and the nucleated cells are able to sediment rapidly through the medium, leaving the platelets sedimenting slowly in a zone above them. This is a type of

Table 11. Commercially available media for the isolation of different types of human blood cells

Medium	Composition (w/v)	Target cells	Supplier
Accu-prep	9.6% sodium metrizoate, 5.6% Ficoll	Mononuclear cells	Accurate Chemical
Ficoll-paque	9.6% sodium diatrizoate, 5.6% Ficoll	Mononuclear cells	Pharmacia Biosystems
Histopaque 1077	9.6% sodium diatrizoate, 5.6% Ficoll	Mononuclear cells	Sigma Chemical
LSM	9.6% sodium metrizoate, 5.6% Ficoll	Mononuclear cells	ICN Pharmaceuticals
Lymphoprep	9.6% sodium metrizoate, 5.6% Ficoll	Mononuclear cells	Nycomed Pharma
1-step 1.077	14.1% Accudenz, 0.44% NaCl	Mononuclear cells	Accurate Scientific
1-step mixer	19% Accudenz, 0.2% NaCl	Mononuclear cells	Accurate Scientific
Nycoprep 1.077	14.1% Nycodenz, 0.44% NaCl	Mononuclear cells	Nycomed Pharma
Nycoprep mixer	19% Nycodenz, 0.2% NaCl	Mononuclear cells	Nycomed Pharma
1-step monocyte	13% Accudenz, 0.58% NaCl	Monocytes	Accurate Chemical
Nycoprep 1.068	13% Nycodenz, 0.58% NaCl	Monocytes	Nycomed Pharma
1-step polymorphs	13.8% sodium metrizoate, 8% dextran 500	Polymorphonuclear cells	Accurate Chemical
Histopaque 1119	16.7% sodium diatrizoate, 6% Ficoll	Polymorphonuclear cells	Sigma Chemical
Mono-poly resolving medium	15.5% sodium diatrizoate, 8.18% Ficoll	Polymorphonuclear cells	ICN Pharmaceuticals
Polymorphprep	13.8% sodium metrizoate, 8% dextran 500	Polymorphonuclear cells	Nycomed Pharma
1-step platelets	12% Accudenz, 0.56% NaCl	Platelets	Accurate Chemical
Nycoprep 1.063	12% Nycodenz, 0.56% NaCl	Platelets	Nycomed Pharma

rate-zonal separation where increasing the density of the medium close to that of platelets greatly reduces their sedimentation rate; yields can be up to four times greater than when using differential pelleting.

Protocol 4. Isolation of platelets from human blood (15)

Equipment and reagents

- Low speed, refrigerated centrifuge with swinging-bucket rotor
- Centrifuge tubes
- Freshly drawn blood with sodium citrate as the anticoagulant
- Separating solution: 12% (w/v) Nycodenz made up in 0.56% (w/v) NaCl solution, containing 5 mM Tricine–NaOH pH 7.0 (the solution has a density of 1.063 g/ml and an osmolality of 320 mOsm)

Method

1. Overlayer 5 ml freshly drawn blood carefully on top of 5 ml medium in a 10–15 ml centrifuge tube at 20°C.
2. Centrifuge in a swinging-bucket rotor at 350 *g* for 12–15 min at 20°C.
3. After centrifugation, a pellet of erythrocytes is found, with the nucleated cells laying on top of the pellet in the form of a 'buffy coat'.

Protocol 4. *Continued*

The platelets are distributed from the sample/medium interface, into the medium. A fairly prominent band occupies the first 1.0–1.5 cm, gradually diffusing towards the pellet.

4. Recover platelets from the prominent band (about 70% of the platelet population can be recovered with less than 1% contamination by other cells).
5. Continue to harvest further towards the pellet (about 95% of the platelets can be recovered, but, the closer to the pellet, the greater the contamination by other cells).

The platelets remain unactivated during fractionation and respond well to functional tests, such as aggregation studies. Keep blood samples and medium at 20 °C and also centrifuge at this temperature.

8.2 Mononuclear cells

The mononuclear fraction of blood consists of lymphocytes, monocytes, and basophils. Fortunately, the buoyant density of these cells in iso-osmotic conditions is significantly less than that of the other cell types: erythrocytes, neutrophils, and eosinophils. This allows the use of a density-barrier type of separation using a layer of medium, dense enough to prevent the mononuclear fraction passing through it, but allowing the other cells to pellet.

Protocol 5. The isolation of mononuclear cells from diluted blood (14)

Equipment and reagents

- Low speed, refrigerated centrifuge with swinging-bucket rotor
- Appropriate centrifuge tubes
- Separating medium: solution of 9.6% (w/v) sodium metrizoate or sodium diatrizoate, and 11% (w/v) polysaccharide (Ficoll 400 or dextran 500) (the solution should have a density of 1.077 g/ml and an osmolality of 290 mOsm)
- Alternatively, ready made separating media can be purchased under various trade names, see *Table 11* for details
- Blood samples, with any of the standard anticoagulants, but there may be variation in the recovery rates depending upon the type of anticoagulant used
- 0.9% (w/v) NaCl

Method

1. Dilute the whole blood with an equal volume of 0.9% (w/v) NaCl.
2. Layer the diluted blood on top of the separating medium in a suitable centrifuge tube, in the ratio of 3 ml medium to 6–7 ml of the diluted blood.
3. Centrifuge at 800 *g* for 20 min at 20 °C.
4. Harvest the band of mononuclear cells found at the sample/medium interface.

The population of total lymphocytes can be further separated into T and B cells by passing the lymphocytes over a column of glass or nylon wool (16) which preferentially binds the B lymphocytes allowing the T cells to pass straight through. Pre-packed, sterile wool columns are available commercially.

Protocol 5 is the standard method for recovering the mononuclear fraction from human blood. It can also be used to recover these cells from disrupted spleen. As there are variations in the make-up and densities of blood cells from mammalian species other than human, the results obtained will vary between species. Sometimes it will be necessary to vary the composition of the medium to obtain maximum recoveries. As explained at the beginning of this section, when faced with a suspension containing cells of unknown densities, initially a continuous or a multilayer discontinuous gradient must be used to determine the banding densities of the cell types contained in it. It may then be possible to devise a single-step solution suitable to deal with the species of interest.

Another solution has been designed for the separation of the mononuclear fraction of blood which avoids the necessity to layer the sample on top of the medium and is particularly suited to the isolation of mononuclear cells from large volumes of blood. In this method, the sample is mixed with an equal volume of the medium before centrifugation (*Protocol 6*).

Protocol 6. The isolation of mononuclear cells from whole blood (17, 18)

Equipment and reagents

- Low speed, refrigerated centrifuge with swinging-bucket rotor
- Appropriate centrifuge tubes
- Separation medium: a solution of 19% (w/v) Nycodenz in 0.2% (w/v) NaCl, 5 mM Tricine–NaOH pH 7.4, density 1.10 ± 0.001 g/ml, osmolality 275 ± 15 mOsm (available as NycoPrep Mixer)

Method

1. Mix the blood sample with an equal volume of the medium by several inversions.
2. Place the mixture in a suitable size centrifuge tube and centrifuge at 800–1000 *g* for 30 min at 20°C.
3. Harvest the mononuclear cells from the top 1.5–2.0 cm of the gradient, making sure to collect all cells around the meniscus.

For a number of procedures it is desirable to separate the two major cell populations of the mononuclear fraction, the monocytes and lymphocytes. Monocytes can be separated from the bulk of the lymphocytes by taking advantage of the differential responses of the two types of cells to hypertonic conditions. Ready made media for the separation of monocytes from lymphocytes are available (*Table 11*).

Protocol 7. The isolation of human mononuclear cells (19)

Equipment and reagents

- Low speed, refrigerated centrifuge with swinging-bucket rotor
- Appropriate centrifuge tubes
- Separation medium: a solution of 13% (w/v) Nycodenz in 0.58% (w/v) NaCl, 5 mM Tricine–NaOH pH 7.4, density 1.068 ± 0.001 g/ml, osmolality 335 ± 5 mOsm

Method

1. Allow the blood (3–5 ml) containing anticoagulant to sediment at room temperature, the actual time required is quite variable depending on the source. Alternatively, the blood can be centrifuged at 200 *g* for 10 min.
2. On top of the pelleted erythrocytes a white top layer will be observed which is called the 'buffy coat'. Using a Pasteur pipette carefully remove as much of the buffy coat layer as possible to give a volume of about 3 ml of leucocyte-rich plasma and place in a 12 ml centrifuge tube.
3. Carefully underlayer the cell suspension with 3 ml of the separating medium and centrifuge at 600 *g* for 15 min at 20°C.
4. The monocyte layer will be seen as a very diffuse, grey band of turbidity centred around the position of the interface. Remove this band using a Pasteur pipette. Immunological staining should show that the population is more than 90% monocytes.

The monocyte separation described in *Protocol 7* does require some skill and experience in that the band of monocytes is not always very apparent because it is so diffuse.

An alternative approach is to separate the monocytes and lymphocytes by rate-zonal flotation which can be carried out using OptiPrep gradients. The basis of this method is that monocytes are marginally less dense than lymphocytes and are significantly larger, therefore they float up faster than lymphocytes (J. Graham, personal communication). Experiments indicate that flotation separations of leucocyte-rich plasma (*Protocol 7*) do indeed separate monocytes and lymphocytes but the quality of the separation can be variable depending on the conditions used.

8.3 Polymorphonuclear cells

The polymorphonuclear cells, neutrophils and eosinophils, can be easily distinguished after staining by their lobed nuclei. Neutrophils are the most numerous fraction of human leucocytes, about 45–70%. Unfortunately, the polymorphonuclear cells have banding densities, in iso-osmotic conditions,

close to and overlapping with the erythrocytes. They can be isolated, with varying degrees of purity and recovery, by a number of methods. A standard, one-step method is described in *Protocol 8.*

Protocol 8. The isolation of polymorphonuclear cells

Equipment and reagents

- Low speed centrifuge, preferably refrigerated, swinging-bucket rotor
- Centrifuge tubes, 10–50 ml, depending upon volumes of blood to be used
- Freshly drawn (less than 4 h) human blood
- Solution of 13.8% sodium metrizoate and 8% Ficoll 400 or dextran 500 (this solution has a density of 1.113 ± 0.001 g/ml and an osmolality of 460 ± 15 mOsm)

Method

1. Use whole, undiluted blood, anticoagulated with EDTA, heparin, or citrate.
2. Place 5 ml of the medium into a 12–15 ml centrifuge tube and layer 5 ml of the undiluted blood on top.
3. Centrifuge at 500 *g* for 30–35 min at 20°C.

After centrifugation, two bands of leucocytes will be found, one at the sample/medium interface, and one about one centimetre below it. The top band is of mononuclear cells, and the lower band consists of neutrophils, together with a few eosinophils that are separated on a rate-zonal basis. The erythrocytes are pelleted. If the separation between the two leucocyte bands is greater or less than required then the length of time of centrifugation must be adjusted. For example, temperature affects the separation; if the temperature of the solutions or centrifuge are higher than 20°C then the time of centrifugation must be shortened.

This method works well because of the selected density, viscosity, and osmolality of the solution. The density is much too high to allow passage of any of the cells in iso-osmotic conditions. The high osmolality causes the erythrocytes to lose water to the surrounding medium, thus they become denser and can sediment into the medium. The loss of water from the erythrocytes at the interface dilutes the medium generating a short density gradient, thus allowing the polymorphonuclear cells to start migrating into this gradient. For this to occur, it is necessary to use undiluted blood in order that sufficient water is lost from the erythrocytes to form the gradient. If only 3 ml of whole blood is used, a similar separation takes place, but the separation of the two leucocyte bands is less than 0.5 cm, making them difficult to harvest without cross-contamination. The gradients can be scaled up, using for example, 20 ml of blood and 10 ml of medium in a centrifuge tube which allows the column heights to be approximately the same as in the smaller scale version.

8.4 The purification of viable spermatozoa from bovine semen

Viable populations of spermatozoa, suitable for use in artificial insemination (A.I.), have been prepared from bovine ejaculates of poor quality, using either OptiPrep or Nycodenz solutions. Poor quality ejaculates, those containing a low percentage of normal, motile sperm and high percentages of dead or abnormal sperm, cannot be used for freezing and storing in aliquots for future A.I. Using the techniques to be described in *Protocol 9*, the motile, normal populations from these ejaculates have been rescued and used successfully for A.I.

Protocol 9. The isolation of viable spermatozoa from bovine semen

Equipment and reagents

- Low speed centrifuge, refrigerated if possible, swinging-bucket rotor
- 30–50 ml centrifuge tubes
- OptiPrep solution, or 40% (w/v) Nycodenz solution made up in distilled water
- Freshly taken bovine ejaculate
- Diluent solution: 11.65 g trisodium citrate, 1.75 g sodium hydrogen carbonate, 5.5 g Tris base, 2.35 g Na_2EDTA, 4.1 g citric acid, 1.0 g lincospectin, 0.75 g potassium chloride, 1 g polyvinyl alcohol, 70 mg cysteine, 16 g sorbitol pH 7.0, made up to 1 litre in glass-distilled water

A. *OptiPrep gradients*

1. Dilute aliquots of OptiPrep with the diluent solution to final densities of 1.11 g/ml (1 vol. OptiPrep to 2 vol. diluent), and 1.13 g/ml (5 vol. OptiPrep to 7 vol. diluent).
2. Mix a freshly taken, undiluted ejaculate with an equal volume of stock OptiPrep, to give a final density of approx. 1.17 g/ml.
3. Prepare a discontinuous gradient by underlayering the aliquots of OptiPrep into a centrifuge tube, 1.11 g/ml first, followed by the 1.13 g/ml aliquot, and finally the sample/OptiPrep mixture.
4. Centrifuge the gradient at 1500 *g* for 25 min at 20°C in a swinging-bucket rotor.
5. A band of viable, motile cells of normal morphology is found at the 1.11/1.13 g/ml interface. A pellet and particulate material consisting of dead cells remains in the loading area, and a rather diffuse band of deformed cells and detached cytoplasmic droplets is found at the top of the gradient.

B. *Nycodenz gradients*

1. Dilute an aliquot of the stock Nycodenz solution to 35% (w/v), using the diluent solution.

2. Mix a freshly taken, undiluted bovine ejaculate with an equal volume of the stock 40% Nycodenz.
3. Place the sample/Nycodenz mixture into a centrifuge tube and underlayer with the 35% Nycodenz aliquot.
4. Centrifuge at 1500 *g* for 25 min at 20°C in a swinging-bucket rotor.
5. A band of viable, motile cells of normal morphology is found at the 35%/40% interface. The pelleted material consists of dead cells, and a band of material at the top of the gradient consists of deformed cells and detached cytoplasmic droplets.

Both the above methods provide a band of motile, viable sperm in a condition suitable for freezing and using for artificial insemination; but there are some important differences.

In the Nycodenz method, the cells are subjected to hyperosmotic conditions, about 370–400 mOsm, which although it does not seem to affect the viability of the cells, does affect their banding densities (20). Keeping the osmolality as low as possible, means that the sample material has to be loaded in the middle of the gradient, with the motile and the dead cells sedimenting. Due to the high concentration of cells necessary in the loading area, during sedimentation, the mass of dead cells tend to sweep up a number of motile cells as they pellet, and the mass of motile cells at the interface tend to trap a number of dead cells within the band. The ability to load the ejaculate under iso-osmotic conditions at the bottom of the gradient when using the OptiPrep method, minimizes this artefactual banding, resulting in a higher recovery of the viable fraction in a highly purified state, generally better than 96% viable, while the Nycodenz method gives a slightly lower recovery, 80–85% viable cells, at a lower concentration.

It has been found that ejaculates from other mammalian species, human, horse, pig, and goat can be separated by the method described, but that some adjustment to the gradient densities and diluent solutions may be required depending on the species.

8.5 Separation of viable and non-viable cells from disaggregated tissues and lavages of body cavities

Naturally occurring single cell suspensions, such as peripheral blood and semen, do not present any special problems other than defining the optimum conditions required to bring about the separation of the various cell types. Cell suspensions obtained by enzymic digestion of tissue (see Chapter 1) and lavages of body cavities, can present difficulties due to the debris from cells broken open during the digestion or lavaging procedure. The macromolecular contents released from these damaged cells, into the suspending medium, especially DNA, causes sticky aggregates to form during centrifugation. Thus,

attempts to separate bands of cells on a density gradient can be frustrated by the presence of long, stringy aggregates throughout the gradient. This not only significantly reduces the recovery of cells from the bands, it can also prevent satisfactory fractionation of the gradients.

The non-ionic, iodinated media offer a method to avoid such difficulties, and also to minimize the damage that can be caused when concentrating the cells by pelleting them. In metrizamide, Nycodenz, and OptiPrep solutions damaged cells assume a higher density than the intact cells. Under iso-osmotic conditions, all intact mammalian cells have been found to have buoyant densities of less than 1.12 g/ml (with the exception of spermatozoa). If a cell suspension is spun down on to a cushion of one of these media, with a density of 1.15 g/ml, the intact cells will stop at the interface, while the damaged cells will sediment through the medium and pellet (*Protocol 10*). Most of the cytoplasmic contents of broken cells which have been released into the suspension will remain in the supernatant. This will allow the band of intact cells at the interface of the suspension and medium to be harvested in a concentrated form, while leaving most of the potentially interfering DNA in the supernatant. The recovered cells can then be separated on a density gradient much more easily.

Protocol 10. Purification and concentration of viable cells prior to further fractionation

Equipment and reagents

- Low speed centrifuge with swinging-bucket rotor
- Centrifuge tubes suitable for the volumes involved
- Suitable diluent solution
- Stock solution of one of the non-ionic, iodinated density gradient media
- Single cell suspension, of either disaggregated tissue, lavage of a body cavity, or cultured cells

Method

1. With the stock solution of the gradient medium and the chosen diluent, make an aliquot with a density of 1.15 g/ml (see *Tables 3–10*).
2. Depending upon the volume of the cell suspension and size of the centrifuge tube to be used, place the suspension into the tube and underlayer with a volume of the diluted medium sufficient to give a layer about 1–2 cm in depth.
3. Centrifuge in a swinging-bucket rotor for 10–15 min at a speed sufficient to allow all the cells to band at the interface with the medium. Generally, 800–1000 *g* is sufficient, but this will vary a little, depending upon the cell suspension and the types of cells it contains.

After centrifugation, all the intact, viable cells should be banded at the sample/medium interface, the dead cells having passed through the medium

and pelleted. Most of the cell debris in the cell suspension will remain in the supernatant, being too small to sediment very far at the low speed and short centrifugation time used. This allows the viable cell populations of the suspension to be harvested at high concentration, and minimizes the formation of sticky aggregates when the cell types are subsequently separated on density gradients. If the presence of stringy aggregates persists then the cell suspension can be treated with a broad range endonuclease to digest the DNA.

8.6 The fractionation of cells from perfused, disaggregated rat liver

The single cell suspension, obtained by perfusion of the whole liver *in situ* (see Chapter 1), will contain a variety of cell types; the greatest proportion (60%) will consist of parenchymal cells, also called hepatocytes. The other cells (non-parenchymal cells) consist primarily of sinusoidal cells, which in turn are composed of endothelial cells (15%), stellate or fat-storing cells (10%), and macrophages (10%), known as Kupffer cells. The percentages account for the proportion of cells of total liver cells. The hepatocytes are significantly larger than the non-parenchymal cells.

From a single rat liver, the suspension of single cells is usually contained in a volume of about 50 ml of incubation medium. The viable cells can be concentrated, while removing the dead cells and macromolecular species, by following *Protocol 10*. The subsequent steps will depend upon the cell population of interest, whether it is the hepatocytes or the non-parenchymal cells that are required. *Protocol 11* describes the separation of rat liver cells using Percoll.

Protocol 11. Isopycnic separation of rat liver cells using continuous Percoll gradients (21)

Equipment and reagents

- Centrifuge tubes
- Centrifuge
- Fixed-angle rotor
- Swing-out rotor with same size tubes as fixed-angle rotor
- Percoll
- Hepes pH 7.0
- 60% (w/v) sucrose
- Rat liver cell preparation

Method

1. Centrifuge 80 ml 10 mM Hepes-buffered Percoll solution in the fixed-angle rotor at 20 000 *g* for 10 min. This generates a continuous gradient of 1.03–1.10 g/ml Percoll.
2. Layer 15 ml of rat liver cell suspension (2–4 $\times$ 10^6 cells/ml) on to the preformed gradient. Transfer the tubes to the swing-out rotor and centrifuge at 800 *g* for 60 min at 4°C.
3. Fractionate gradient by upward displacement using 60% (w/v) sucrose.

Protocol 11. *Continued*

4. Count the number of cells in each fraction.
5. Assay for viability using trypan blue exclusion test, phagocytic activity (by uptake of polystyrene latex particles), and function.

The cells are localized in three regions of the continuous Percoll gradient. Non-viable cells accumulate on top of the gradient, while phagocytic, non-parenchymal cells (Kupffer cells) band between 1.04–1.06 g/ml, and parenchymal cells (hepatocytes) band between 1.07–1.09 g/ml. The distribution of the cells in the gradient is shown in *Figure 9*. The non-parenchymal and parenchymal cells can be distinguished by selective radiolabelling (21).

Alternatively, differential pelleting of cells harvested in *Protocol 10* gives an almost pure suspension of parenchymal cells (hepatocytes). This is possible due to their very large size compared with the non-parenchymal cells and is described in *Protocol 12*.

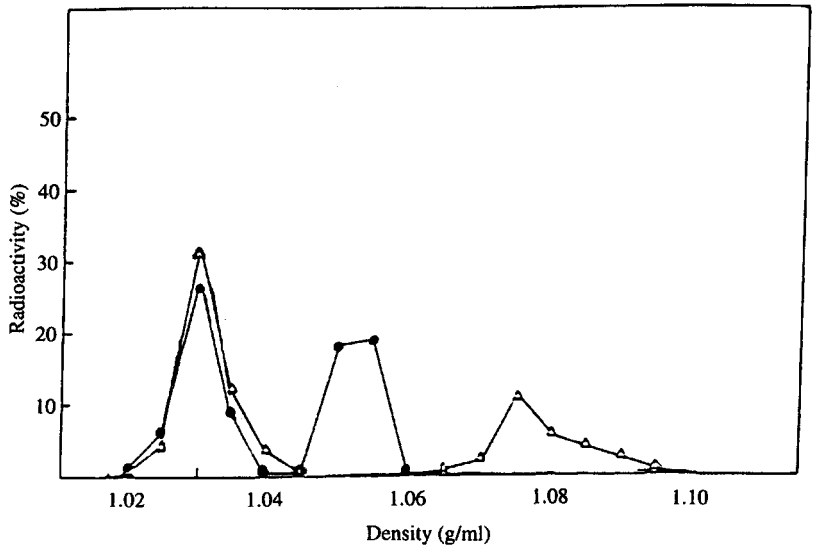

Figure 9. Separation of rat liver cells on a Percoll gradient. The non-parenchymal (●) and parenchymal (△) cells were distinguished by selective radiolabelling (21).

Protocol 12. Isolation of hepatocytes by differential pelleting

Equipment and reagents

- Low speed, refrigerated centrifuge, swinging-bucket rotor
- 30–50 ml centrifuge tubes
- Harvested rat liver cells

Method

1. Dilute the harvested cell band with ice-cold incubation medium to a volume of 40–60 ml.
2. Place the cell suspension in centrifuge tubes and centrifuge at 50 *g* for 1 min at 4°C.
3. Decant supernatants carefully (retain if needed for preparation of non-parenchymal cells) and resuspend pellets in same volume as before of ice-cold incubation medium.
4. Repeat centrifugation step 2.
5. Discard supernatants, resuspend pellets in small volume, and examine for purity. If necessary, wash once more.

This procedure should provide a suspension of almost pure hepatocytes. If necessary, this suspension can be processed following *Protocol 10*, to remove any cells damaged during the washing steps.

The supernatants saved after spinning down the hepatocytes will, of course, be enriched with non-parenchymal cells at low cell concentration in a relatively large volume. The cells may be concentrated as described in *Protocol 10*. However, the supernatant will be contaminated with some parenchymal cells which can be removed by repeated centrifugation at 600 *g* for 4 min at 4°C. The separation of the three populations of non-parenchymal cells can cause some difficulty as their buoyant densities tend to overlap. The yield of non-parenchymal cells obtained is, however, low due mainly to the repeated centrifugation steps. *Protocol 13* describes the separation of liver sinusoidal cells on Nycodenz gradients.

Protocol 13. Separation of sinusoidal cells on continuous Nycodenz gradients (22)

Equipment and reagents

- Two-chamber type gradient mixer
- Gey's balanced salt solution (GBSS): 0.14 M NaCl, 5 mM KCl, 0.3 mM $MgSO_4.7H_2O$, 1 mM $NaH_2PO_4.2H_2O$, 1.5 mM $CaCl_2.2H_2O$, 3 mM $NaHCO_3$, 0.2 mM KH_2PO_4, 1 mM $MgCl_2.6H_2O$, 5.5 mM D-glucose, adjusted to pH 7.4 and 275–285 mOsm
- Abbé refractometer
- Low speed centrifuge with swing-out rotor
- Corex 15 ml centrifuge tubes
- 28.7% (w/v) Nycodenz in GBSS without NaCl, pH 7.4, 285 mOsm
- Non-parenchymal cells

A. *Sample preparation*

1. Use freshly isolated non-parenchymal cells (2–3 × 10^6 cells) from one rat liver.
2. Wash cells three times by suspension in 15 ml of GBSS and pellet at 300 *g* for 5 min at room temperature. This reduces the risk of cell

Protocol 13. *Continued*

clumping and removes most of the contaminating parenchymal cell debris.

3. Resuspend the final pellet of cells in 6 ml GBSS.

B. *Gradient preparation and centrifugation*

1. Dilute the stock solution of 28.7% (w/v) Nycodenz with GBSS to a concentration of 19.1% (w/v). This is the dense solution for preparation of the gradient.
2. Dilute an aliquot of the dense (19.1%, w/v) solution with GBSS to obtain a 7.7% (w/v) light solution for the gradient.
3. Prepare a continuous gradient, 7.7–19.1% (w/v) Nycodenz, using the gradient maker.
4. Centrifuge the gradient at 2000 *g* for 30 min at 4°C in a swing-out rotor, using slow acceleration and deceleration. To avoid cell clumping the time of centrifugation should not exceed 45 min.

C. *Analysis of gradients*

1. Fractionate the gradient using upward displacement, into fractions of 0.5 ml.
2. Determine the refractive index (RI) of each fraction and calculate the corresponding density using the following equation:

$$\text{Density (g/ml)} = (\text{RI} \times 3.242) - 3.323. \quad [8]$$

3. Determine cell number and viability for each fraction.
4. Determine the distribution of Kupffer, endothelial, and other cells by peroxidase staining.

Readers should be aware that Kupffer and endothelial cells of the normal rat liver cannot be completely purified by isopycnic density centrifugation alone. Knook *et al.* (23) have shown that the density separation of these cell types can be improved by pre-loading of the Kupffer cell population with Jectofer® *in vivo*. Jectofer® is an iron–sorbitol–citric acid complex (Astra, Sweden) containing iron (50 mg/ml). Rats are injected intramuscularly with 0.2 ml of Jectofer® per 150 g of body weight four days and one day before isolation of the cells. After this treatment the density distribution of Kupffer cells is shifted towards higher densities and relatively pure fractions of each cell type can be obtained.

8.7 The isolation of protoplasts from digested plant tissue on OptiPrep gradients

Once the cellulose walls are removed from plant cells, they become sensitive to osmotic changes in the environment, shrinking, or swelling and thus varying

in their buoyant densities. Using density gradients to purify the intact protoplasts from the digest of debris from the digested walls and broken protoplasts requires attention to the osmotic strength of the gradient medium.

The osmolality of the digest medium is obviously of importance; Sarhan and Cesar (24) suggested that determining the osmolality of the living plant tissue and using a digest mixture of 1.8 times that measurement gave good results. This procedure is followed in *Protocol 14*, with the osmolality of the gradient solutions also set to that osmolality.

Protocol 14. The isolation and purification of protoplasts from six-day-old barley or wheat using OptiPrep gradients

Equipment and reagents

- Low speed, refrigerated centrifuge, swinging-bucket rotor
- 50 ml centrifuge tubes
- Plasmolysing solution: 5 mM MES (2-(*N*-morpholino)ethanesulfonic acid), 1 mM KH_2PO_4, 0.44 M D-sorbitol, 5 mM $CaCl_2$, 2 mM $MgCl_2$, 2 mM $MnCl_2$, 1 mM L-arginine, 1 mM dithiothreitol (DTT), 0.1% (w/v) polyvinylpyrrolidone-10 (PVP-10), 2 mM glutathione, 2 mM L-ascorbic acid, 0.01% (w/v) trypsin inhibitor soybean S1, 1300 U/ml catalase—pH adjusted to 5.7
- Digesting solution: plasmolysing solution plus 2% (w/v) Cellulysin and 0.5% (w/v) macerase
- 9 cm plastic Petri dishes
- Nylon mesh, pore size 100 μm
- Isolation buffer: plasmolysing solution without catalase or trypsin inhibitor
- OptiPrep solution: as result of the osmolality measurements taken of the leaf tissue, the osmolality of the OptiPrep is increased to 500 mOsm by addition of 0.6% (w/v) KCl (0.6 g added to 100 ml of OptiPrep)
- Seeds of wheat (*Triticum aestivum* L. cv. Mercia) and barley (*Hordeum vulgare* L. cv. Pipkin) sown on Levington potting compost and grown for six days at 25°C with a 16 h photoperiod of 50 $\mu mol/m^2/sec$ in a growth chamber

A. *Determination of leaf osmolality (24)*

1. Powder 3 g of leaf tissue in liquid nitrogen, allow to thaw.
2. Centrifuge at 30 000 *g* for 20 min at 4°C.
3. The osmolality of the supernatant is measured using a depression of freezing point osmometer. The osmolality of all incubating solutions is set at 1.8 times that of the tissue.

B. *Protoplast isolation (24)*

1. Remove leaf blades and surface sterilize in 1% sodium hypochlorite/0.01% (v/v) Tween 80 for 5 min.
2. Rinse the tissue three times with distilled water, transfer to 70% (v/v) ethanol for 2 min, then wash the tissue a further three times with distilled water. This procedure not only surface sterilizes the tissue but also weakens the cuticle thus aiding protoplast release.
3. Place the leaf tissue in the plasmolysing solution (50 ml/g tissue) for 30 min at 20°C.

Protocol 14. *Continued*

4. Remove the leaves from the solution, cut in to 0.5–1 mm pieces, and place in 9 cm Petri plates containing the digesting solution (10 ml/g tissue).
5. Digest the tissue at 20°C, with shaking at 40 r.p.m. for the first and last 30 min.
6. After digestion, filter the contents of each culture plate, containing 2 g of leaf tissue, through nylon mesh (pore size 100 μm). Wash off tissue retained by the mesh in isolation buffer, mash it lightly to release more protoplasts, and again filter.
7. Wash the mesh through with the buffer and make up the volume of filtrate from each plate to 30 ml in 50 ml tubes.

C. *Preparation of gradients*

1. Add 7.5 ml of the prepared OptiPrep to each tube to make up a final density of close to 1.07 g/ml—the density of the diluting medium must be taken into account when calculating the density of the diluted aliquots.
2. Overlayer the mixture with 20 ml of an OptiPrep solution, diluted to about 1.03 g/ml by mixing 20 ml of the isolation buffer with 2 ml of the prepared OptiPrep.
3. Finally, overlayer 2–3 ml of the isolation buffer on top.
4. Centrifuge at 200 *g* for 4 min in a swinging-bucket rotor at 4°C.

After centrifugation, a band of material is found at the top of the medium and in the overlying buffer. There is clear medium from the band down to the 1.03/1.07 g/ml interface. The 1.07 g/ml layer contains particulate material and a pellet is found at the bottom of the tube. The band at the top can best be harvested using a plastic Pasteur pipette with the tip cut off to increase the size of the orifice and reduce damage to the protoplasts during harvesting. This band contains over 95% intact protoplasts, with the remainder just showing signs of lysis and loss of chloroplasts. There are an insignificant number of intact protoplasts in the 1.07 g/ml layer. Recovery of protoplasts when barley was used was about 5×10^6 per gram of tissue; using wheat, the yield was much lower.

The protoplasts are in high concentration and free of any residual enzyme activity, so there is no need to wash the isolated protoplasts, which are harvested in the buffer that contains a small amount of OptiPrep (about 2% Iodixanol) which is not harmful to cells. The practice of washing cells by pellet-

ing and resuspending them several times is very damaging. The method described in this protocol avoids this step.

8.8 Enhanced isopycnic separation of cells by density perturbation

Isopycnic density centrifugation is limited by the differences in density of different cell types. Within a homogeneous population of a particular type of cell, there exists a heterogeneity in cell density (25). Furthermore, heterogeneity exists within a single cell population, such as in the position of cells in the cell cycle (26), or the presence of multiple cell types in the population as a result of cell differentiation (27). The latter have been found to express different cell surface antigens (28). However, a variety of approaches have been developed for modifying selectively the density of cells and subcellular components.

8.8.1 Interaction of cells with dense beads

As previously mentioned, the buoyant densities of lymphocytes and monocytes overlap and so create problems when requiring highly purified lymphocytes. A procedure to overcome this uses the ability of monocytes to engulf colloidal iron particles when incubated *in vitro*. Their density can be increased sufficiently to allow them to sediment through the medium with the erythrocytes and granulocytes, leaving the lymphocytes at the interface (29).

Further density perturbation methods include those of Ghetie *et al.* (30, 31). They designed a cell separation technique based on the interaction between cell surface bound IgG and protein A of *Staphylococcus aureus*. The density of lymphoid cells coated with IgG antibodies against one of the surface markers was increased by adherence of staphylococci. Cells with adhering bacteria were separated from cells without bacteria by density gradient centrifugation (30). Alternatively, lymphocytes were incubated with sheep erythrocytes coated with protein A of *S. aureus*. Rosettes were formed which were then separated from non-rosetted lymphocytes by density gradient centrifugation (31). Many other lymphocyte rosetting techniques and gradient separations exist (32–36).

Recently, the authors demonstrated another density perturbation procedure for the fractionation of cells (37–39). Cells were labelled with antibody coated dense beads and then fractionated on isotonic isopycnic OptiPrep gradients. Cells which had not bound the dense beads were recovered from the top of the gradient while cells associated with the dense beads were found in progressively denser regions of the gradient. Unlabelled cells could be separated from labelled cells. Cells were labelled via antigen–antibody specificity, allowing the isolation of immunologically distinct subpopulations. *Protocol 15* outlines the procedure used.

Protocol 15. Isolation of immunologically distinct cell subpopulations using density perturbation methods with the aid of antibody coated dense beads (37–39)

Equipment and reagents

- Refrigerated bench-top centrifuge with swinging-bucket rotor
- Microcentrifuge
- End-over-end mixer
- Gradient Master
- Sterilin screw-cap centrifuge tubes, 12 ml total capacity
- 1.5 ml microcentrifuge tubes
- Either avidin coated dense polystyrene beads (38, 39) or sheep anti-mouse coated Dynabeads M-450 (Dynal)
- Phosphate-buffered saline (PBS): 0.15 M NaCl, 3 mM KCl, 9 mM Na_2HPO_4, 2 mM KH_2PO_4 pH 7.2, 300 mOsm
- Biotinylated affinity isolated rabbit immunoglobulins to mouse immunoglobulins
- 0.1% (w/v) bovine serum albumin (BSA) in PBS
- Monoclonal mouse antibody to human T cell CD6
- OptiPrep solution: 1.32 g/ml, 260 mOsm
- Diluent: 3 mM KCl, 0.3 mM CaEDTA, 5 mM Tricine–HCl pH 7.2, 0.85% (w/v) NaCl pH 7, 285 mOsm
- MOLT-4 T cells grown in RPMI 1640 supplemented with 10% fetal calf serum

A. *Preparation of beads for labelling cells*

1. Wash 3.9 mg of avidin-coated dense polystyrene beads or 3.6 mg Dynabeads M-450 twice in 250 μl phosphate-buffered saline (PBS). Pellet the beads using a microcentrifuge at 18600 *g* for 15 min at room temperature.
2. Resuspend and incubate 5×10^7 avidin coated dense polystyrene beads with 15 μg biotinylated rabbit anti-mouse immunoglobulins in PBS in a final volume of 125 μl. Mix the suspension on an end-over-end mixer at 15 r.p.m. for 45 min at room temperature.
3. Wash the beads twice with 0.1% (w/v) (BSA) in PBS and once with PBS. Pellet beads by centrifugation as described in step 1.
4. Resuspend and incubate the biotinylated rabbit anti-mouse coated dense polystyrene beads or the Dynabeads M-450 (5×10^7) with 3 μg mouse anti-human CD6 immunoglobulins as described in step 2, mix for 60 min.
5. Wash the antibody coated dense beads as described in step 3.

B. *Bead binding by cells*

1. Resuspend 5×10^7 antibody coated dense beads in 0.1 ml PBS. Mix with viable MOLT-4 T cells (2.5×10^6) in a final volume of 1 ml PBS, in a 1.5 ml microcentrifuge tube. Use a minimum of a 20:1 bead to cell ratio.
2. Mix the suspension on the end-over-end mixer at 15 r.p.m. for 1 h at 25°C.

C. *Fractionation of cells*

1. Prepare 1.03 g/ml and 1.27 g/ml working OptiPrep solutions as follows.
   (a) To prepare 1.03 g/ml solution dilute stock OptiPrep with diluent, 1 part to 11 parts respectively.
   (b) To prepare 1.27 g/ml solution dilute stock OptiPrep with diluent, 5 parts to 1 parts respectively.
2. By gentle inversion of Sterilin tube, mix 1 ml labelled cell suspension with 2.5 ml 1.03 g/ml OptiPrep and 1.5 ml diluent to obtain 1.02 g/ml initial solution.
3. Underlayer with 5 ml 1.27 g/ml OptiPrep.
4. Prepare an isotonic continuous 1.02–1.27 g/ml OptiPrep gradient (osmolality range 290–310 mOsm) by rotation using a Gradient Master (80°, 20 r.p.m., 2 min).
5. Centrifuge the gradient at 220 g_{max} for 90 min at 20°C, using a bench-top centrifuge without applying the brake.
6. Recover cells which have not bound beads from the top of the gradient. Cells associated with antibody coated dense beads are found in denser regions of the gradient.

A typical separation that can be achieved using this density perturbation method is shown in *Figure 10* which shows the fractionation of a culture of MOLT-4 cells into immunologically different subpopulations.

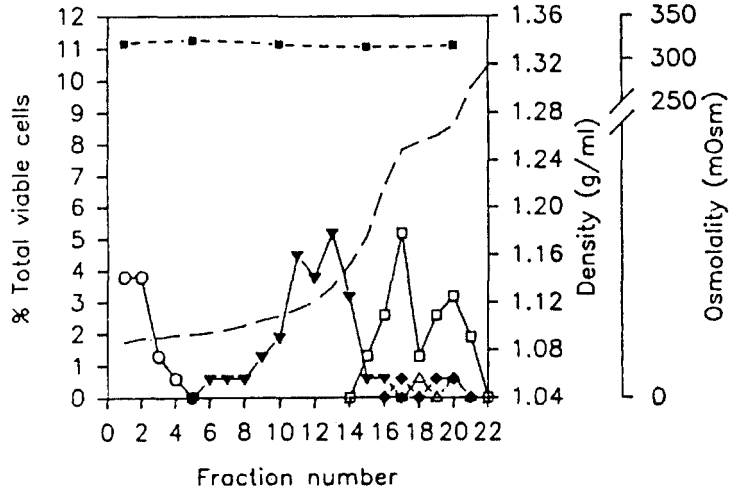

Figure 10. *Fractionation of MOLT-4 cells into different subpopulations using density* perturbation. MOLT-4 cells were mixed with antibody coated Dynabeads as described in *Protocol 15* and separated on a continuous OptiPrep gradient. (○) Cells without beads bound; (▼) cells with 1–3 beads bound; (□) cells with 7–9 beads bound; (◆) cells with 13–15 beads bound; (△) cells with 19+ beads bound; (—) density, g/ml; and (■) osmolality, mOsm.

9. Conclusions

Cell sedimentation separations, particularly centrifugation, offer a very wide range of techniques for separating viable cells from all types of organisms. Using rate-zonal or isopycnic methods, or combinations of the two, it is possible to separate cells which are quite similar. The fact that isopycnic separations can be enhanced by density perturbation methods makes it possible to achieve separations based on the immunological identity of the cell thus greatly enhancing the types of separation that can be carried out.

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3

Centrifugal elutriation

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1. Introduction

Centrifugal elutriation provides a versatile means of separating cells using continuous flow centrifugation. Elutriation is defined as separation by washing and straining or by decanting. Centrifugation provides a means of finely regulating this process. It is a rapid, non-invasive method for the preparation of specific subpopulations of cells from mixed cell types utilizing a balance of two opposing forces; centrifugal force and a fluid counterflow. First described by Lindberg in 1932 (1), its use remained limited until the 1970s when Beckman Instruments first marketed separation chambers and rotors specifically designed for the process. The technique has since become widely used for the separation of cells from an extremely diverse range of sources.

The chief advantage over other techniques is that large numbers of cells can be handled rapidly and relatively gently. In addition, preparations are obtained which possess an improved proportion of viable cells compared to those in the starting suspension. For example, we routinely produce hepatocytes with a viability of over 96% (assessed by trypan blue exclusion) (2) and similarly high viabilities are quoted for pituitary cells (3), monocytes (4), leucocytes (5), and carcinoma cells (6).

With the development of the sophisticated instrumentation required, the applications to which centrifugal elutriation has been applied have increased enormously. These now include fine separation of blood cells into purified populations including platelets, erythrocytes, lymphocytes, and monocytes, separation of cells from solid tissues and tumours, growth synchronization of proliferating cell lines, and fine separation of subpopulations of a single cell type. Centrifugal elutriation has also been applied to the purification of yeast, bacterial, and plant cells.

In this chapter we briefly review the principles behind the process and then using specific examples, provide some applications. There is now a vast and growing literature on the use of centrifugal elutriation for cell separation in a wide array of biological systems. Due to space limitations, the literature cited in this chapter is not exhaustive, but has been selected so as to best illustrate the various concepts and applications presented herein.

2. Principles

The application of Stokes' Law provides the basis for separating cells of varying diameters and densities within a given medium and is the theoretical basis for conventional cell separation by density gradient centrifugation.
Stokes' Law:

$$V_s = \frac{2}{9} \times \frac{r^2(\rho_p - \rho_m)g}{\eta} \quad [1]$$

where V_s = sedimentation velocity, r = radius of the particle, ρ_p = density of the particle, ρ_m = density of the medium, η = viscosity of the medium, and g = gravitational or centrifugal force.

Lindberg (1) first showed that cell fractions could be resolved by 'washing' a cell suspension whilst it was undergoing centrifugation. Alteration of the flow rate of this 'washing' medium provided an elegant and efficient means of modulating this process. Furthermore, it can be employed to subsequently harvest separated fractions during the course of the centrifugation run.

Lindberg devised a conical chamber which was aligned in the centrifuge so that cells or other particles were subjected to two opposing forces:

(a) The centrifugal force generated by the spinning of the rotor.

(b) The fluid counterflow in the opposite (centripetal) direction.

The flow rate of medium perfused through the chamber was such that it was too high to permit the cells to sediment, but was too low to wash the cells out of the chamber. This methodology, originally termed 'counterstreaming centrifugation', was further refined by Lindahl (7) who applied the technique to the separation of a variety of cells including leucocytes, spermatazoa, and yeast cells.

The design of the separation chamber is critical to its performance. By far the most widely available separation chambers are those whose design has been refined and marketed by Beckman Instruments since 1973 and they will be described in some detail here. Other manufacturers produce chambers and centrifuges however (e.g. Hitachi, see Appendix 1) and the principles of the design are the same.

The geometry of the separation chambers is such that there is an increasing cross-sectional area, and therefore a decreasing velocity of flow of the medium, toward the centre of the rotor. Cross-sectional diagrams of the standard chamber are shown in *Figure 1*. The cells within the chamber are subjected to two opposing forces: the centrifugal field generated by the spinning rotor, and the viscous drag of the elutriation buffer or medium flowing at a particular rate (V_f) in the opposite direction. After loading, each cell equilibrates at a position within the chamber where its sedimentation velocity is equal to V_f. Stokes' Law can be rearranged:

$$V_s = V_f = \frac{F}{A} = \frac{d^2(\rho_p - \rho_m)(\omega^2 r)}{18\eta} \quad [2]$$

where F = flow rate of the elutriation buffer or medium (ml/min), A = cross-sectional area (cm^2), d = particle diameter (μm), and $\omega^2 r$ = angular velocity of the rotor. Therefore, if the density of a cell type is known, at a given rotor speed the approximate elutriation flow rate at which the cell will remain in the chamber can be determined.

Because the cross-sectional area of the chamber changes, a flow rate gradient is generated within the chamber and this enables mixtures of cells with a large range of sedimentation velocities to be held in suspension. By increasing

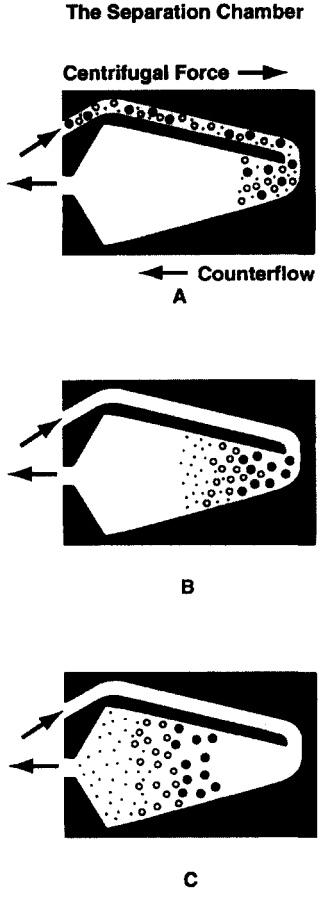

Figure 1. Diagrammatic representation of the standard separation chamber during a typical cell separation. Centrifugal force is opposed by a counterflow of elutriation buffer. (A) A mixed cell population enters the chamber at a given buffer flow rate and rotor speed. (B) The buffer flow rate is increased and the cell subpopulations begin to separate out with the smallest cells leading the elutriation front. (C) The flow rate is again increased and the smallest cells, upon reaching the elutriation boundary, elute from the chamber and can be collected.

the flow rate of the buffer (or by reducing the rotor speed), in small incremental steps successive populations of relatively homogeneous cell size can be eluted from the chamber (see *Figure 1*). Each successive fraction will contain cells that are larger or more dense than those of the previous fraction. Thus with carefully chosen running parameters, accurate fractionation of cells with extremely small differences in size and/or density can be achieved.

3. Equipment

The equipment required for centrifugal elutriation comprises a large capacity slow/medium speed centrifuge, a rotor assembly, and a high-performance variable flow rate pump. Suitable centrifuges, rotor heads, and separation chambers can be obtained from Beckman Instruments or Hitachi. Ascopox epoxy-resin separation chambers for use in Beckman centrifuges are available from Ashland Sudchemie. Cole Parmer Masterflex pumps are obtainable from Barant Corporation. Tygon tubing and silicone tubing can be obtained from Norton Performance Plastics and from Dow Corning Medical Products respectively.

3.1 Separation chamber and rotor head

Over the course of many years, numerous developments and refinements have been made in the production of rotor heads and separation chambers. The JE-6 was the first elutriation system to be mass produced. Further improvements gave rise to the JE-6B rotor (*Figure 2D*), which is now marketed as the JE-6HC. This comprises a single separation chamber with a volume of approximately 5 ml and a bypass chamber housed on the opposite side of the rotor (*Figure 2E*). The chamber is made of transparent synthetic material which allows illumination by a stroboscopic lamp and observation during the elutriation process via a viewing port in the lid of the centrifuge. In order to increase the capacity and therefore the total number of cells which could be handled, a completely redesigned rotor was produced, the JE-10X. This incorporated a larger separation chamber (40 ml) allowing the processing of almost ten times as many cells. More recently, the JE-5.0 system (*Figure 2A–C*) which has greater flexibility and improved ease of handling has superseded the JE-10X system. The JE-5.0 system incorporates interchangeable small and large standard chambers (*Figure 2B* and *C*) as well as the Sanderson chamber (see below for description). This system can be used with two separation chambers in series, but it is more usual to use a single chamber and counterbalance.

Three separation chambers which are used routinely are the standard and Sanderson chambers of the JE-6B and JE-5.0 systems, and the large standard chamber which can only be used in the JE-5.0 rotor assembly. Details of these are given in *Table 1*. The design of the small and large standard chambers is

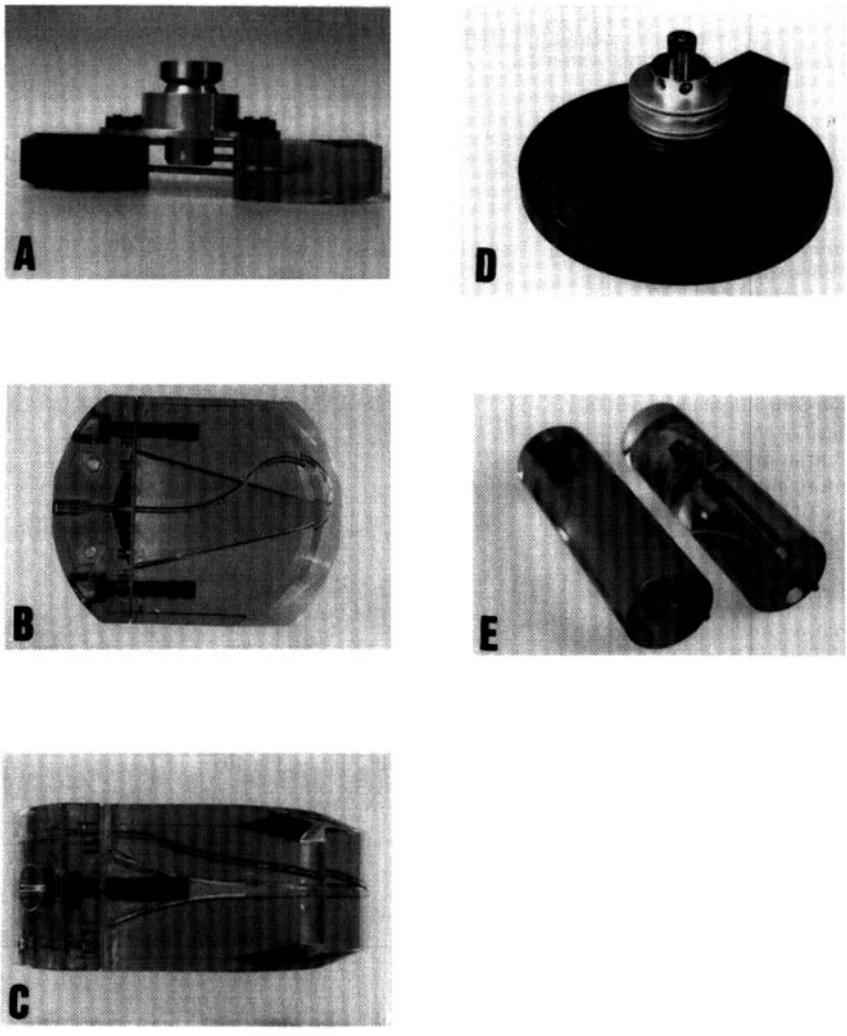

Figure 2. Centrifugal elutriation separation chambers and rotors. (A–C) Beckman JE-5.0 and (D, E) JE-6B systems. (B) and (C) show different aspects of a large standard separation chamber, and (E) shows the standard and bypass chambers.

intrinsically the same and they differ only in their volumes, the large chamber is used merely as a means of increasing the number of cells handled. The Sanderson chamber, however, has been developed for the separation of cells with only very small difference in their physical characteristics. To achieve this, the walls of the chamber diverge rapidly at a point of entry whilst the walls of the upper section are almost parallel (see *Figure 3B*). This produces a wider

Table 1. Specifications on separation chambers and rotor heads used in the JE-5.0 and JE-6B centrifugal elutriation systems

	Chamber		
	Standard	**Sanderson**	**Large standard**
JE-5.0 system			
Maximum rotor speed (r.p.m.)	5000	5000	5000
Chamber capacity (ml)	4.0	5.5	40
Maximum buffer density (g/ml)	3.0	3.0	3.0
Number of cells loaded:			
minimum	$\sim 2 \times 10^7$	$\sim 2 \times 10^6$	$\sim 2 \times 10^8$
maximum	$\sim 10^9$	$\sim 10^9$	$\sim 10^{10}$
Relative centrifugal field at 5000 r.p.m.:			
at base of chamber	3500 *g*	4230 *g*	4700 *g*
at elutriation boundary	2410 *g*	2890 *g*	2410 *g*
Maximum elutriator flow rate (ml/min)	100	100	400
Volume of buffer required to elutriate one fraction of cells (ml)	75–100	75–100	500–1000
JE-6B system			
Maximum rotor speed (r.p.m.)	6000	6000	–
Chamber capacity (ml)	4.2	5.9	
Maximum buffer density (g/ml)	3.0	3.0	
Number of cells loaded:			
minimum	$\sim 1 \times 10^7$	$\sim 1 \times 10^5$	
maximum	$\sim 1 \times 10^9$	$\sim 1 \times 10^7$	
Relative centrifugal field at 6000 r.p.m.:			
at base of chamber	5040 *g*	5080 *g*	
at elutriation boundary	3470 *g*	4270 *g*	
Maximum elutriator flow rate (ml/min)	100	100	
Volume of buffer required to elutriate one fraction of cells (ml)	75–100	75–100	

segregation of the cells in the upper section, produces a very clean separation, and also reduces the likelihood that the cells will clump in the bottom of the separation chamber.

3.2 Centrifuge

Only a limited number of centrifuges are suitable for elutriation. Rotor speed must be finely controlled, as reducing this in small decrements provides a means of separating the cell fractions. If alteration of rotor speed affects a transient decrease in revolution and subsequent 'search' for the exact speed, disturbance of the equilibrium within the chamber and a mixing of cell populations would result. A centrifuge with a precise, linear, and highly reproducible rotor speed is therefore essential. Rotor speed in most modern centrifuges is

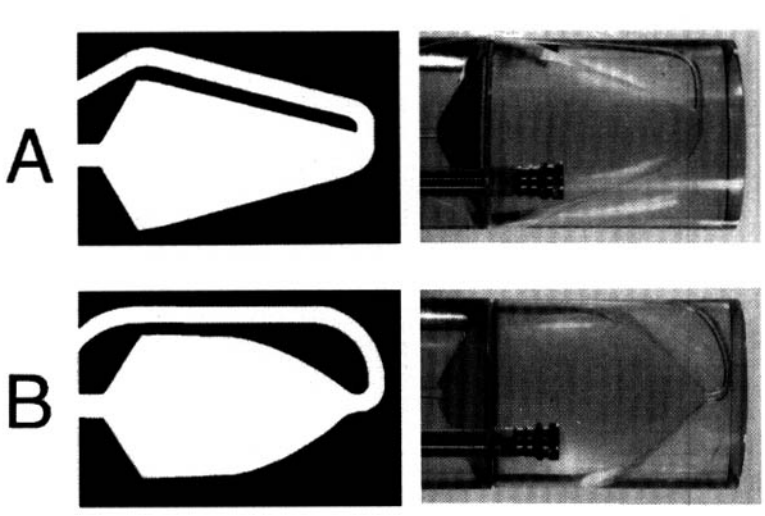

Figure 3. Comparison of the standard (A) and Sanderson (B) chambers. The photographs illustrate the chambers from the JE-6B system.

digitally controlled, but older models with less precise speed control can be modified by the incorporation of a potentiometer, which provides more sensitive modulation. As previously mentioned, centrifuges can be fitted with a stroboscope in the base of the bowl to allow visualization and progression of the cell elutriation boundary within the separation chamber. Although by no means essential, a visual image is beneficial especially in establishing and refining the running conditions for a new procedure.

The JE-6B rotor can run in the J6 and J21 range of centrifuges (maximum running speed 6000 r.p.m. and 21 000 r.p.m. respectively). The more recently developed JE-5.0 system is restricted to the J6 series. *Figure 4A* and *B* show JE-6B and JE-5.0 rotors mounted in a J6 centrifuge. Although it is convenient to have a dedicated centrifuge for elutriation separations, it is feasible to use the machine for conventional purposes also. In experienced hands, conversion from conventional use to one suitable for elutriation takes only five minutes. Additionally, kits are available (from Beckman) to enable conversion of existing centrifuges for elutriation use, thereby reducing the set-up costs for laboratories who already have an appropriate instrument.

3.3 Pump

A high quality pump is an important component of the system. An even, non-pulsatile flow of buffer through the chamber is critical, since even minor fluctuations in the flow rate will adversely influence the equilibrium within the chamber during the run and reduce the efficiency, quality, and reproducibility of the separation. The recommended pump is a Cole Parmer Masterflex (7014

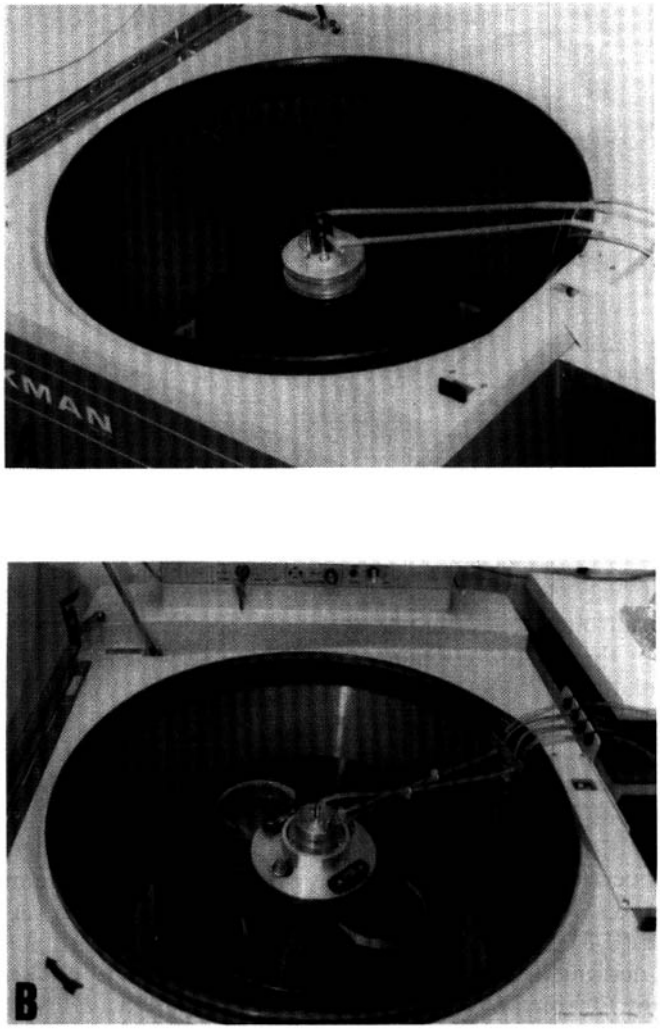

Figure 4. (A) JE-6B and (B) JE-5.0 rotors *in situ* in a J-6 centrifuge set-up for an elutriation separation. Note the inlet and outlet tubing attached to the central housing. The stroboscope lamp assembly lies underneath the rotor head (not seen).

series), which delivers a consistent output. However, some variation in flow rate may still occur. To minimize this, pump tubing should be renewed frequently and the pump should be calibrated before each separation in order that small alterations can be accommodated. Silicone rubber tubing is recommended for the pump head and pressure gauge and Tygon tubing throughout the rest of the system.

4. The elutriation procedure

In this section the basic protocol for setting-up a centrifugal elutriation system will be described (see *Protocol 1*), followed by a number of more specific methodologies. Further details and a more comprehensive range of applications are listed in *Tables 2–5*.

4.1 Setting-up the system

The elutriation system is shown diagrammatically in *Figure 5*. It comprises a buffer reservoir, a loading chamber which can be bypassed using a three-way tap, a pump and pressure gauge, the rotor head and separation chamber itself, and a collection vessel.

Protocol 1. Standard procedure for cell separation by centrifugal elutriation

Equipment and reagents

- Centrifuge rotor (see *Tables 1–5*)
- 20 ml syringe
- Cell suspension
- Elutriation buffer (see *Table 1–5*)
- 70% (v/v) ethanol or 6% (v/v) hydrogen peroxide (if maintenance of sterility is required)

Method

1. Before assembly of the rotor, ensure that the bearings are clean and run smoothly and that the O-rings are in good condition. A light application of silicone grease will ensure that joints remain watertight.
2. If maintenance of sterility is required:
   (a) Flush the system through with 70% (v/v) ethanol (or 6% (v/v) hydrogen peróxide) for 15 min.
   (b) Follow by 500 ml of sterile distilled water.
3. Wash the system through with the elutriation buffer ensuring that the first 150–200 ml runs to waste.
4. Purge the whole system of air:
   (a) Remove any air bubbles in the separation chamber by running the rotor up to speed and down again.
   (b) Check for leaks inside the centrifuge bowl at this stage.
   (c) Regrease the O-rings and joints if leakage has occurred.
   (d) Release bubbles trapped in taps or tubing connectors by pinching off the tubing briefly and releasing after the pressure has increased slightly.
   (e) Remove air pockets present in the pressure gauge by inverting the gauge.

Protocol 1. *Continued*

5. Set the rotor speed, temperature, and pump to the loading rate. Verify calibration of the pump flow rate at this point.
6. Load samples as follows:
   (a) Fill a 20 ml syringe with the concentrated cell suspension ensuring that there are no air bubbles.
   (b) Set the three-way tap to bypass the chamber and carefully inject the cell suspension into the loading chamber via the injection valve so that the cell suspension settles at the bottom of the loading chamber.
7. Invert the loading chamber and open the bypass tap to allow the sample to flow into the separation chamber. The cell suspension can be seen to enter the separation chamber by way of the stroboscope-illuminated viewing port and should form a loose pellet at the bottom of the chamber. Inevitably a proportion of the loading suspension will sediment below the outlet needle of the loading chamber, and it is therefore necessary to *gently* shake the tube a number of times to ensure that all the cells are loaded. It is vital that no air bubbles are allowed to enter the rotor.
8. Set the three-way tap to bypass the loading chamber.
9. After a short equilibration period, increase the flow rate at selected increments and collect 100 ml (small standard and Sanderson chamber) or 800 ml (large standard chamber) eluates, although volumes can be adapted to suit specific requirements.
10. Harvest the cells by conventional centrifugation if required and analyse as appropriate.

Modifications of this basic procedure are used for the many different examples of cell separation as detailed in subsequent sections (see *Tables 2–5*).

4.2 Elutriation buffer

The choice of buffer (or medium) for elutriation is very much dependent upon the cell type to be handled. Serum-containing culture medium may be used but this can be expensive as fractions are collected in volumes of 80–100 ml using the small volume chambers and up to 1000 ml if the large standard chamber is used (see *Table 1*). We routinely use Krebs–Henseleit solution gassed with 95% (v/v) oxygen/5% (v/v) carbon dioxide (pH corrected to 7.45) supplemented with 0.1% (w/v) bovine serum albumin and 0.1% (w/v) glucose immediately prior to use. The buffer selection for a wide variety of cell types is described in *Tables 2–5*.

If clumping of the cells occurs then a number of agents can be added to the

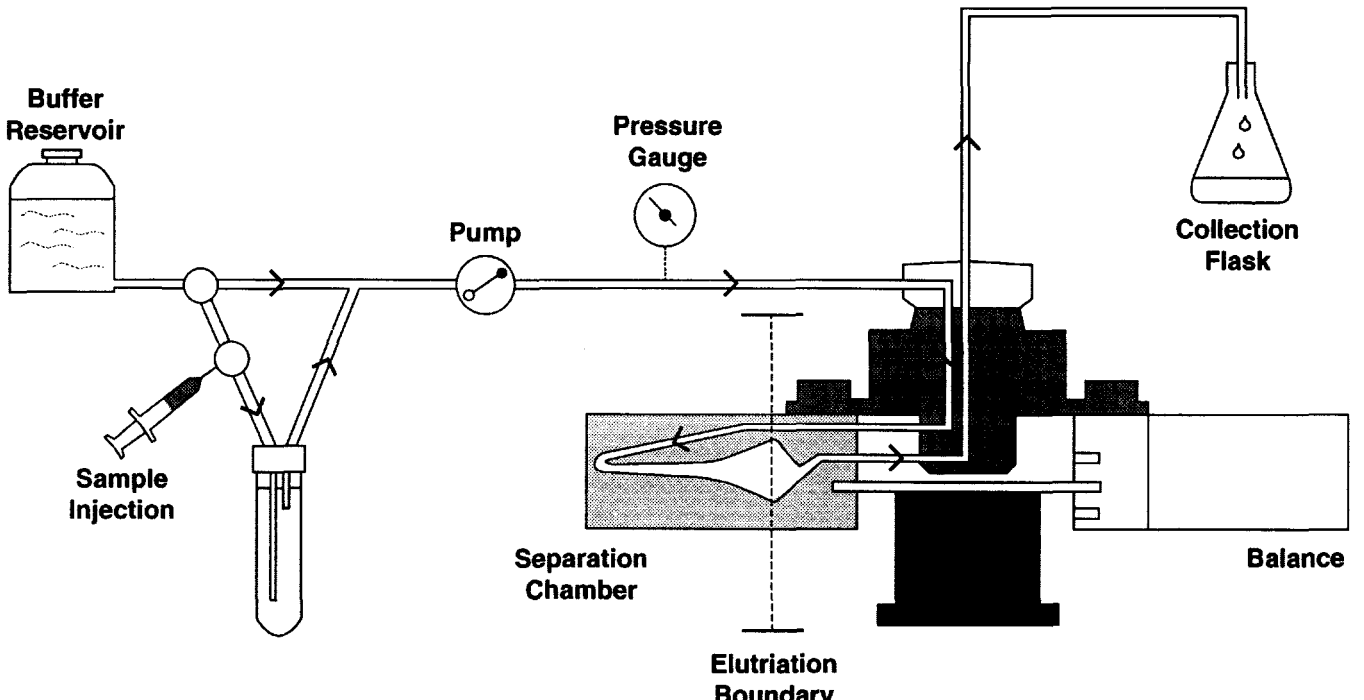

Figure 5. Diagrammatic illustration of the components of the elutriation system. Note that the separation chamber is visualized during the run through a viewing port in the lid of the centrifuge, illuminated by a stroboscopic lamp (not shown). This effectively allows continuous monitoring of the cell suspension as it progresses towards the elutriation boundary in the chamber.

medium to help minimize the problem. DNase I is widely used at a recommended concentration of 0.001% (w/v). Supplementing the elutriation buffer with the divalent cation chelator EDTA may also reduce clumping. Other compounds used to reduce cell clumping in a variety of cell systems include 2-naphthol-6,8-disulfonic acid (8), protease XIV (9), hydroxymethyl-amino methane 2-(*N*-morpholine)propane sulfonic acid (10), and 4-(2-hydroxy ethyl)-1-piperazine ethanesulfonic acid or polyvinylpyrrolidone (11).

4.3 Number of cells loaded

The number and density of the cells loaded into the separation chamber also varies enormously with the cell type used. As a guide, however, up to 10^9 cells can be loaded into the small standard chamber and the Sanderson chamber, whilst the large standard chamber can accommodate up to 10^{10} cells. Overloading the chamber may encourage clumping of the cells or adversely affect the flow equilibrium. If in doubt it is better to run the protocol twice using reduced cell numbers. Conversely, in order to achieve optimal fluid dynamics and cell equilibrium within the separation chamber, there is a lower limit to the number of cells which should be loaded (see *Table 1*).

4.4 Temperature

To ensure good reproducibility, running the process at a constant temperature is important. Although most cells remain stable for longer at lower temperatures and at low population densities, cell membranes are less fluid at such temperatures and are therefore more susceptible to shear forces, which might result in disruption of the plasma membrane and cell damage. At higher temperatures, however, cells are more prone to clumping. It is important to ensure that the chosen temperature is maintained throughout the procedure as it is vital that the viscosity of the medium does not fluctuate.

4.5 Loading suspension

One of the most critical steps in centrifugal elutriation is the preparation of a completely homogeneous single cell suspension. This is relatively easy for cell preparations from some tissues where following disaggregation, repeat-pipetting of the loading suspension through a Pasteur pipette may suffice, but for adherent cell lines growing in monolayers it is crucial to disaggregate both fully and carefully. For this purpose, in addition to gentle trypsin treatment, the addition of DNase to degrade any free DNA released from lysed cells is recommended to prevent cell clumping. This can also be included in the loading suspension or elutriation buffer (see *Table 2* for specific details).

4.6 Cleaning

After each run it is essential to ensure the rigorous removal of all the cellular and proteinaceous material from the system. This can be achieved by several

means. We flush the system through first with 500 ml of distilled water, followed by 500 ml of a weak solution of a neutral detergent (such as Tween 80), then 200 ml of 70% (v/v) ethanol, and finally 2 litres of distilled water.

Maintenance of the O-rings and bearings within the rotor head is critical and the head should be stripped down and thoroughly cleaned frequently. For the JE-6B this should be done at the end of each run. Similarly, if radioactive samples, human cells, or other hazardous substances are used within the chamber, then the head should be dismantled and cleaned after each separation ensuring that the appropriate safety precautions are observed.

5. Applications

The number and variety of biological systems where centrifugal elutriation has been employed to produce enriched or cell-specific populations are now extremely diverse. Inevitably, the specific running conditions will depend upon the size and properties of the cell population in question and upon the particular aims of the study. Some of the major applications in several systems will be surveyed in this section and specific experimental protocols detailed.

5.1 Liver cell populations

The liver is a complex organ composed of several different cell types. Hepatocytes or parenchymal cells make up the largest proportion of the total cell population (approximately 70%), the remainder being comprised of Kupffer cells, cells of Ito (fat-storing cells), sinusoidal endothelial cells, and biliary epithelial cells. Centrifugal elutriation is frequently used to isolate the various cell populations which constitute this tissue including Kupffer cells, biliary epithelial cells, and sinusoidal endothelial cells from a variety of species (see *Table 2* for examples). Additionally, centrifugal elutriation has been used with hepatocyte preparations to fractionate subpopulations of single cells (12, 13) and to purify hepatocyte couplets (pairs of hepatocytes which retain an intact bile canaliculus between adjacent cells) (14) which are used in bile synthesis and secretion studies (see *Protocol 2*).

The graph in *Figure 6* highlights a number of advantages in the use of centrifugal elutriation to subfractionate separate cells such as hepatoctes from a mixed population. First, where the rotor is maintained at a constant speed and the rate of flow of the buffer through the chamber is increased, the small (single) cells elute first, followed sequentially by the couplets, then the triplets, and finally the larger multiples. Secondly, the viable cells elute later than the non-viable cells, and this confers a considerable advantage, as not only is the population collected greatly enriched in the cell type required, but the preparation has a higher percentage cell viability than the initial suspension. Furthermore, separate populations of single cells, couplets, and triplets can be obtained from one animal without compromising the quality of any one group.

Table 2. Summary of protocols adopted in the separation of specific liver cells from mixed populations using centrifugal elutriation

Cell type	Elutriation buffer	Rotor/chamber/ centrifuge	Loading Number	Loading Temp °C	Loading Speed r.p.m.	Loading Flow ml/min	Fraction collection details	Comments	Reference
Murine Kupffer cells	GBSS[a] 0.05% (v/v) FCS[b]	JE-6 n.s.[c] J-21	–	4	2500	21	Collect 200 ml to waste. Collect cells at 40 ml/min.		31
Murine SECs	a-MEM–Hepes 0.0003% (w/v) DNase	JE-6B Standard J2–21	$6–8 \times 10^7$	10	2400	14	Waste for 250ml. Collect 14–45 ml/min.		32
Rat Kupffer cells	GBSS[a] 0.001% (w/v) DNase 0.2% (w/v) pronase	n.s.[c]	–	–	875 *g*	23	Waste to 29 ml/min. Collect small cells at 45 ml/min. Reduce rotor to 1 *g*. Collect remainder (large).	Viability > 98%.	5
Rat liver cells	Leibowitz-15 medium 0.001% (w/v) DNase I 0.2% (w/v) Protease XIV	JE-5.0 Standard J-6M	–	–	2500	12	SECs collected at 12–18 ml/min. Kupffer cells collected at 30–44 ml/min.	9	
Rat Ito cells	HBSS	SCR 20B (Hitachi)	–	–	3250	10	Collect at 16–18 ml/min.	20 mm pore filtrate used for loading.	34
Rat hepatocytes	Krebs–Henseleit pH 7.4 20 mM PIP 5 mM glucose 1% (w/v) PVP 50 mg/ml DNase I	JE-6B	1.25×10^8	8	1700	19	Waste to 27 ml/min. Collect at 33 ml/min, then at 50 ml/min, finally at 60 ml/min.	Diploid cells. Mixed dip- and polyploid. Polyploid cells.	11
Rat BECs	140 mM NaCl 5.4 mM KCl 0.8 mM Na_2HPO_4 25 mM Hepes 3% (v/v) FCS[b] 0.004% (w/v) DNase	JE-6B n.s.[c]	–	–	2500	25	Waste to 30 ml/min. Collect at 60 ml/min.		35

[a] Gey's balanced salt solution.
[b] Fetal calf serum.
[c] Not specified.

Protocol 2. Purification of rat hepatocyte couplets based on a method described by Wilton *et al.* (14)

Reagents

- Hank's balance salt solution (HBSS): 137 mM NaCl, 26 mM $NaHCO_3$, 0.6 mM Na_2HPO_4, 5.4 mM KCl, 0.4 mM KH_2PO_4, 4 mM $CaCl_2$, and 5.6 mM glucose—gas the solution at 19°C with 95% (v/v) O_2/5% (v/v) CO_2 for 20 min, and correct the pH to 7.45
- Calcium-free HBSS: as HBSS but omitting $CaCl_2$
- Collagenase (Type A, Boehringer Mannheim): 0.03% (w/v) in HBSS
- Trypan blue solution: 0.4% (w/v) in saline
- Krebs–Henseleit solution: 118 mM NaCl, 4.8 mM KCl, 1.2 mM $MgSO_4.7H_2O$, 1 mM KH_2PO_4, 24 mM $NaHCO_3$, and 3.2 mM $CaCl_2$—gas the solution at 4°C with 95% (v/v) O_2/5% (v/v) CO_2, correct the pH to 7.45, and store at 4°C
- Elutriation buffer: Krebs–Henseleit solution gassed and stored at 19°C, supplemented with 0.1% (w/v) glucose and 0.1% (w/v) bovine serum albumin (Fraction V, Winlab)
- Leibowitz-15 tissue culture medium (Gibco)

Method

1. Prepare hepatocyte couplets by an incomplete digestion of rat liver using a limited collagenase perfusion (orthograde) *in situ*:
   (a) Pre-perfuse of rat liver for 10 min using calcium-free HBSS followed by a 4 min perfusion in HBSS containing 0.03% (w/v) collagenase.
   (b) Excise liver and plunge into ice-cold Krebs–Henseleit buffer. Chop and strir to release the cells.
   (c) Filter the resulting suspension of cells and debris through 150 μm pore nylon gauze, and allow to settle by gravity sedimentation.
   (d) Wash the cells twice by settling before the elutriation process.
2. Set-up the elutriatior as described in *Protocol 1* using a small standard chamber mounted in a JE-5.0 rotor head and a J6-MB centrifuge. Using a rotor speed of 1100 r.p.m., load the sample at an elutriation buffer flow rate of 10 ml/min. The temperature should be maintained at 19°C throughout the run.
3. Count the cells using phase-contrast microscopy and adjust to an appropriate concentration prior to loading using elutriation buffer. Optimally a volume of approx. 15–20 ml is used to load 2×10^8 cells.
4. Ensure that all the cells are loaded by gently agitating the loading chamber.
5. Once all the cells are loaded, bypass the loading chamber and allow the eluate to run to waste for at least 10 min. Increase the flow rate to 20 ml/min and again discard the eluate which will contain the majority of non-viable cells and cell debris from the loaded suspension.
6. Increase the buffer flow rate in increments of 5 ml/min, and collect 100 ml fractions at each increment. Keep the collected fractions on ice.

Protocol 2. *Continued*

7. Centrifuge the fractions at 85 *g* for 5 min and resuspend in the plating medium (Leibowitz-15). Determine the cell population and assess viability by trypan blue exclusion.

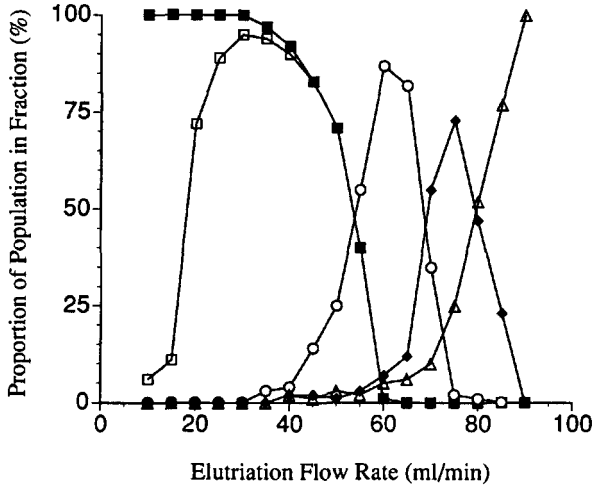

Figure 6. Purification of rat hepatocyte singlets, couplets, and triplets by centrifugal elutriation. Separation profile of total single cells (closed squares), viable single cells (open squares), couplets (open circles), triplets (closed diamonds), and multiples greater than three (open triangles).

It is of course important to verify that the cells fractionated are those of choice. In dealing with a complex organ such as the liver, comprising at least five distinct cell types, cell purity must be critically assessed. As a preliminary check, this can be done morphologically using phase-contrast microscopy. More stringent assessments should be made however, using for instance immuno- or histochemical markers of specific cell types. Ideally this is achieved by using cytospin slides of individual cell fractions. For example, in the liver the following markers are used to specifically identify cell subtypes:

- hepatocytes: the asialoglycoprotein receptor
- biliary epithelial cells: cytokeratin 19
- Kupffer cells: endogenous peroxidase
- fat-storing cells (lipocytes): desmin and ultraviolet autofluorescence
- endothelial cells: factor VIII-related antigen (von Willebrand factor)

Using these various criteria, we found that contaminant (non-parenchymal) cells were restricted to only the early fractions (14) where they represented less than 1% of the total population.

The protocol described above indicates that gross differences in cell size—exemplified here in the isolation of fractions of small hepatocyte multiples from a mixed starting population—allow a clear and highly reproducible separation to be performed. By simply extending the profile of separation it is possible to be even more selective in separating hepatocytes. Hepatocytes in the liver lobule are not uniform. The periportal cells, i.e. those closest to the portal vein, are slightly smaller than the perivenous cells, i.e. those which lie adjacent to the hepatic vein and there is a recognized gradient of cell size between the two locations. We have exploited this property and, by modifying the protocol outlined above, have separated hepatocyte couplets into periportal- and perivenous-enriched subpopulations (15) (*Protocol 3*). Our major interest in this case is in hepatocyte couplets, so we discard the majority of fractions containing single cells and triplets and expand the number of couplet-containing fractions collected.

Protocol 3. Periportal- and perivenous-enriched hepatocyte couplet populations

This protocol is modified from that described in *Protocol 2* as follows:

1. Reduce the rotor speed to 850 r.p.m. and load the cell suspension into the separation chamber at a flow rate of 10 ml/min as in *Protocol 2*, but after loading is complete, increase the buffer flow rate to 22.5 ml/min and discard at least 150 ml eluate.
2. Expel the non-viable cells, cell debris, and the majority of single hepatocytes.
3. Increase the buffer flow rate stepwise at 2.5 ml/min intervals and collect 100 ml fractions at each increment until the flow rate reaches 47.5 ml/min.
4. The couplets used are generated in the flow rates between 25–42.5 ml/min (see *Figure 7A*). Include fractions to either side of this range to ensure collection of the appropriate fractions, even if there are unforeseen alterations in ambient temperature, pump rate, or viscosity of the elutriation medium.

Although *Figure 7B* shows that the couplets yielded in fractions collected between 25–45 ml/min increase in length in a linear fashion, this, however, is not sufficient to categorize the cells with respect to their lobular origin. We therefore assay the activity of an enzyme, glutamine synthetase, which is only present in the perivenous cells. This establishes that the larger cells collected in at flow rates between 37–43 ml/min are perivenous, and the smaller cells collected at 25–30 ml/min are periportal, thus confirming the validity of this separation procedure.

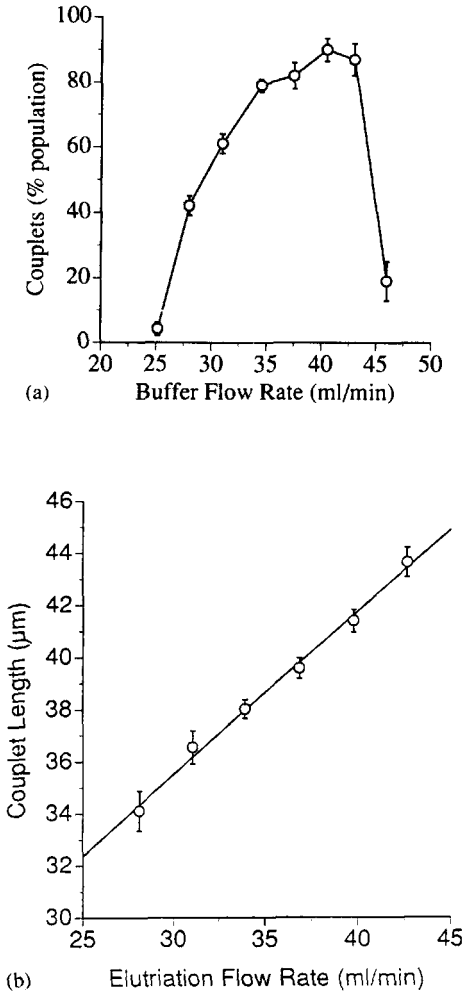

Figure 7. (A) Purification of hepatocyte couplets by centrifugal elutriation. (B) Subfractionation of hepatocyte couplets indicating an increase in overall diameter with increasing buffer flow rate.

5.2 Cell synchrony

As proliferating cells progress through successive phases of the growth cycle, namely G_1, S-phase, and G_2, they increase in size. Following mitosis (M), two smaller daughter cells are produced which then enter a further cycle commencing again with G_1. Centrifugal elutriation has been utilized to exploit, at least with certain cell types, this size differential to enable separation of relatively large yields of growth synchronous cells. This principal has been applied to a wide array of prokaryotic and eukaryotic cells.

In one of the first reports, Miestrich *et al.* (16) separated L-P59 mouse fibroblasts from an exponentially growing population where 36% of the cells were in G_1, 54% in S-phase, and 10% in G_2/M. Cell viability and recovery were high and cell fractions with a purity of approximately 90% G1, 72% S-phase, and 60% G2/M cells were obtained. This degree of success compares favourably with alternative methods of synchronizing cells which involve the use of reagents to reversibly block cells at various stages of the cycle, or require other means of physical separation (17). Centrifugal elutriation, however, yields much higher numbers of cells and full separation is achieved in less than 50 minutes. This time can be further reduced if, for example, only one selected fraction is desired. Other means of synchronizing cells can take much longer, even as much as two to three days (17). *Protocol 4* is based on a method described by Meistrich *et al.* (16) for the preparation of synchronized mouse L-P59 fibroblasts.

Protocol 4. Separation of growth synchronous cells by centrifugal elutriation

Reagents

- Elutriation buffer: McCoy's 5A medium supplemented with 5% (v/v) fetal calf serum and 5 nM 2-naphthol-6,8-disulfonic acid (NDA)
- Confluent cell monolayer
- 0.025% (w/v) trypsin in elutriation buffer
- 0.002% (w/v) DNase I in elutriation buffer
- 10% (v/v) fetal calf serum

Method

1. Remove the cells carefully from the culture flask with 0.025% (w/v) trypsin and 0.002% (w/v) DNase I.
2. Halt the protease action by the addition of medium containing 10% (v/v) fetal calf serum.
3. Examine the cell suspension under a phase-contrast microscope for cell clumping. If this is evident, then disrupt them by vigorous pipetting through a Pasteur pipette.
4. Pellet the cells using low speed centrifugation (150 *g* for 5 min) and resuspend in less than 20 ml of medium containing 10% (v/v) fetal calf serum.
5. Set-up the elutriation system as described in *Protocol 1*. If required, the elutriation chamber, tubing, and rotor head can be sterilized using 70% (v/v) ethanol or 6% (v/v) H_2O_2 as described in *Protocol 1*. Set the rotor speed to 1525 r.p.m., and the loading flow rate at 9.4 ml/min.
6. Once the cells are loaded, run 75–100 ml of the eluate to waste while the cells equilibrate within the chamber.
7. Collect 12 separate fractions of 50–75 ml by increasing the flow rate using 1.4 ml increments.

Protocol 4. *Continued*

8. Stop the centrifuge and collect any cells and aggregates remaining in the chamber as they wash out.
9. Spin down each fraction and remove aliquots for viability checks, Coulter counting, or flow cytometry.

A representative example of a synchronized HeLa cell separation is shown in *Figure 8.*

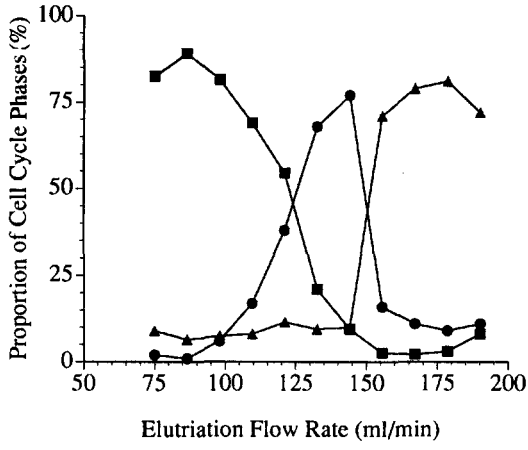

Figure 8. Elutriation profile of a logarithmically proliferating cell population of HeLa cells showing the degree of growth synchrony following separation (see *Protocol 3*). Cells in G_1 phase (squares), S-phase (circles), and G_2/M phase (triangles). After ref. 14.

The number of cell types where centrifugal elutriation has been adopted for synchrony studies is substantial (representative examples are listed in *Table 3*). In prokaryotes, this property has also been exploited to separate growth synchronous populations of bacteria for studies of cell cycle-specific gene expression (19). It has been used extensively in lower eukaryotes, for example, to separate yeast cells (20, 21) subsequently leading to major advances in understanding the genes regulating the control of the cell cycle, and even with plant cells (22).

5.3 Blood cells

Blood cells have the advantage of occurring naturally as non-adherent cells and lend themselves particularly well to the preparation of enriched fractions by centrifugal elutriation.

Platelets are the smallest cells present in blood and will elute first. Red blood cells are the largest and are usually separated by a process of elimina-

Table 3. Summary of protocols adopted to separate blood cells into different cell types using centrifugal elutriation

Cell type	Elutriation buffer	Rotor/ chamber/ centrifuge	Loading: Number	Loading: Temp °C	Loading: Speed r.p.m.	Flow ml/min	Fraction collection details	Comments	Reference
Monocytes	pH 7.4 150 mM NaCl 2.7 mM KCl 2.3 mM phosphate 6 mM glucose 1 mM EDTA 0.1% (w/v) HSA[a]	JE-6B Standard J2.21	–	–	2470	8	Waste to 16.5 ml/min, then collect at 22 ml/min.	Over 90% monocytes, remainder lymphocytes.	36
Porcine neutrophils	PBS[b] 0.2% (w/v) gelatin 0.1% (w/v) glucose	JE-6B Standard n.s.[c]	–	25	2370	4	Waste to 16 ml/min, then collect in 2 ml/min increments to 22 ml/min.	> 95% neutrophils.	37
Human granulocytes	pH 7.45 150 mM NaCl 10 mM Hepes 5 mM KCl 0.1% (w/v) EDTA 2% (w/v) HI-FCS[d]	JE-6B Standard n.s.[c]	2×10^9	15	2000	10	Slow increase to 14.5 ml/min (1 ml/min increments). Run 10 min. Stop. Centrifuge and collect chamber contents.	70% total recovery.	38
Human basophils	25 mM Pipes 110 mM NaCl 5 mM KCl 0.1% (w/v) glucose 0.25% BSA[e]	Beckman n.s.[c]	–	–	2600	20	Reduce speed to 2500 r.p.m. and waste. Reduce to 2300 r.p.m. and collect.		39
Bovine erythrocytes	PBS[b] 2% (w/v) A-plasma[f]	JE-6B Standard n.s.[c]	Same as whole blood	–	2200	7	Waste between 7–14 ml/min. Stop centrifuge and collect chamber contents.		40
Yolk sac and fetal liver erythrocytes (mouse)	PBS[b]	JE-6B Standard J2–21	–	10	2000	3	Reduce rotor speed in 250 r.p.m. 25 increments to 750 r.p.m. waste. Increase flow to 13 ml/min and rotor to 1300 r.p.m. Collect.		
Human erythrocytes	pH 7.4 142 mM NaCl 8.2 mM Na_2HPO_4	JE-6 Standard J-21B	0.5 ml PRBC	10	4200	13	Reduce rotor speed by 100 r.p.m. every 15 min until chamber is empty.		41

Table 3. Continued

Cell type	Elutriation buffer	Rotor/ chamber/ centrifuge	Loading Number	Loading Temp °C	Loading Speed r.p.m.	Loading Flow ml/min	Fraction collection details	Comments	Reference
	2.2 mM KH_2PO_4 5 mM glucose								
Ovine reticulocytes	145 mM $NaNO_3$ 5 mM glucose 10 mM Tris–MOPS[g] pH 7.4	JE-6B Standard J-21B	25% (v/v) suspension	5	3000	5	Collect between 9–24 ml/min.		10
Platelets	105.5 mM NaCl 128 mM Na_2HPO_4 2.8 mM KH_2PO_4 15% (w/v) fa-free BSA[h]	JE-6 n.s.[c] J2–21	5 ml of $5–9 \times 10^9$	22	4000	1	Waste to 2 ml/min, then collect at 4, 6, 8, and 10 ml/min.		42
Monocytes	Mg- and Ca-free HBSS 100 mg/litre EDTA 2% (w/v) FCS[i]	JE-6B n.s.[c] J2–21	–	–	1500	17	Run to waste for 10 min (red blood cells, lymphocytes, and platelets). Stop centrifuge and collect.		43
Monocytes	pH 7.23 Ca-free Krebs Ringer 0.2% (w/v) dextrose 0.05% (v/v) HSA[a]	JE-6B n.s.[c] J2–21	$2.5–3.5 \times 10^8$	25	2500	10	Collect at 16, 21.5, and 28 ml/min.	Last fraction contains 91–94% monocytes. Viability 99%.	4
Monocytes	pH 7.2 PBS[b] 5% (w/v) dextrose 1–2% (v/v) HSA[a]	JE-6 n.s.[c] J-21	1×10^9	–	2080	8	Run at 11 ml/min for 60 min to remove lymphocytes. Remainder monocytes.	Recovery 93%. Viability 96%.	44
Human monocytes	'Lymphocyte separation medium'	JE-5.0 Standard n.s.[c]	–	–	2000		Lymphocyte-rich (> 99%) at 12–16 ml/min. Monocyte-rich (> 90%) at 17–20 ml/min.		45
Lymphocytes		J-21C Sanderson n.s.[c]	1.5×10^9	10	3000	12	Collect at 18 and 28 ml/min.		46
Bovine lymphocytes	HBSS	JE-6 n.s.[c] J-21B	6×10^8	–	2430	10	Collect 9 fractions between 16–81 ml/min.		47

Human megakaryocytes	HBSS 3.5% (v/v) BSA[e] 3.8% Na citrate 2 mM theophyline 1 mM adenosine	JE-6B Standard J2–21	5×10^8	–	1000	7	Run to waste for at least 150 ml. 800 r.p.m. and 15 ml/min. Waste 200 ml. Collect remaining cells.	40–50% megakeryocytes	24, 33
Rat pituitary cells	Ca- and Mg-free Dulbecco's balanced salt solution pH 7.4 1% (w/v) BSA[e] 6 g/litre Hepes 10 mg/litre gentamycin	JE-6B Sanderson J2–21	5–15 $\times 10^6$ (in DNase)	6–8	1920	8	Collect at 15 ml/min (small), 25 ml/min (medium), 35 ml/min (large).	Yield 60–98%. Viability > 96 %.	3, 59
Rat lung cells	HBSS 1000 U/ml DNase I	JE-6B Sanderson	$5–6 \times 10^8$	–	2500	8.5	Reduce spin speed to 2200 r.p.m., waste 100 ml. Collect 10–18 ml/min. 1200 r.p.m. collect 18 ml/min.	Pipette digestion to disaggregate alveolar type I cells. Clara cells.	60
Hamster testes cells	pH 7.2 PBS[b] 0.5% (v/v) BSA[e]	JE-6B Standard J-21B	3–20 $\times 10^9$	4	5000	11	Various fractions. Reduce speed to 2020 r.p.m., flow rate 12–28 ml/min, then 1500 r.p.m. at 17 ml/min and 20 ml/min. Collect 12 fractions.	Table of cell types.	27

[a] Human serum albumin.
[b] Phosphate-buffered saline.
[c] Not specified.
[d] Heat inactivated fetal calf serum.
[e] Bovine serum albumin.
[f] Autologous plasma.
[g] Hydroxymethyl-amino methane 2-(*N*-morpholine)propane sulfonic acid.
[h] Fatty acid-free bovine serum albumin.
[i] Fetal calf serum.

tion, by flushing the smaller cells out of the chamber thereby leaving the erythrocyte fraction. Red blood cells are not a uniform size, they become smaller and more dense as they mature, and therefore can be separated into different age groups (23).

Granulocyte, lymphocyte, leucocyte, and monocyte preparations usually start with an initial step prior to elutriation. This might be lysis of red cells, leukapheresis, density gradient centrifugation, or some other method. The reader is referred to the appropriate literature cited in *Table 4* which details a variety of cell separations from blood using centrifugal elutriation.

Bone marrow has also been used extensively as a starting material for studies of haemopoietic progenitor cells and megakaryocytes (24) and fetal liver has been used to produce embryonic erythrocytes (25). Again, details of some of these protocols are described in *Table 4.*

5.4 Other cell types

We have highlighted three specific applications of centrifugal elutriation. There are however a plethora of experimental systems to which centrifugal elutriation can be beneficial, using various cell types and to various ends. Another tissue which has proven amenable to centrifugal elutriation following disaggregation is the testis. Much of the developmental work on centrifugal elutriation was done by Meistrich and his colleagues using this tissue, and this has been reviewed comprehensively (26). The methods described by Grabske *et al.* (27) are still in use today, although some modifications have been described that significantly improve both yield and quality (28, 29).

Centrifugal elutriation has been shown to be the only adequate method of producing a large collection of highly enriched and viable oocytes by Lazzari *et al.* (30) who used it to produce highly enriched populations of porcine oocytes from newborn piglet ovary. In presenting *Table 5*, we cover some aspects which might help direct the reader to the appropriate literature.

6. Advantages and disadvantages

Centrifugal elutriation clearly offers several advantages over other established methods of cell separation and purification. One major advantage rests in the fact that the choice of medium used for elutriation is adaptable. Cells that are particularly sensitive or fastidious in their culture medium requirements, can be maintained throughout in the optimal medium. Hence, there is no need to expose the cells to reagents of different densities or to compounds that might be potentially toxic. As a consequence, high viability is retained throughout elutriation runs. Sterility can be adequately maintained during the procedure (any contaminating bacteria are usually washed through and discarded whilst the larger cells are loaded onto the elutriator). Furthermore, with refrigerated J6 or J21 centrifuges separations can be performed at any required temperature.

The technique is also a relatively gentle procedure since cells are not subjected to high *g* forces. The stress and shear forces involved in the fluid phase separation of the procedure are much less than those encountered in conventional centrifugation or in the use of density gradients, although this advantage is partly nullified, since each elutriation step generates fractions with relatively large volumes and the subpopulations must often be concentrated. It is generally accepted, however, that even the most fragile cells can withstand these manipulations.

Another attraction is the ability to handle extremely large numbers of cells relatively rapidly. As discussed earlier, with the enlarged separation chamber now available, up to 10^{10} cells can be accommodated in a single run although this obviously depends upon the size of the cell type in question. This exceeds by far the number of cells that can be conveniently run on density gradients or other techniques. Moreover, centrifugal elutriation can be very rapid. Separation of a loaded cell population can be complete in as little as 15–20 minutes although again, this depends upon the specific running conditions and cell properties. Under certain circumstances, for example if particularly labile cell types are used, compromising purity for speed can reduce these times further.

Other benefits of the technique include the dramatic improvement of the viability of cell preparations since dead and dying cells are generally of much lower density than their fully viable counterparts. It can also be used to 'clean-up' cell suspensions with the removal of broken cells and cell debris. Centrifugal elutriation yields more than 90% of those cells loaded. This is an important feature when working with a valuable cellular resource.

As with any specialized technique there are also a number of disadvantages. First it does require sophisticated and specialized equipment with the obvious cost-associated implications. It is also reliant upon having a high quality single cell suspension. Indeed, elutriation was first developed for separation of non-adherent cells such as those of the haemopoietic system for which it is ideal. Although cells growing as adherent monolayers can be elutriated, it is important to disaggregate them as efficiently as possible to yield a homogeneous cell suspension. This is easier to achieve with some cell lines than others. On balance, there is no question that the many advantages considerably outweigh the disadvantages and centrifugal elutriation is now considered an essential component for many experimental systems.

7. Conclusions

Centrifugal elutriation is a powerful and versatile technique for the separation and purification of cell types and subpopulations from an enormous array of cell and tissue sources. It is clearly a valuable alternative to other conventional methods of cell handling covered in this volume, particularly by virtue of the ability to work with large numbers of cells rapidly and relatively gently. The majority of experimental systems where centrifugal elutriation has proven

Table 4. Examples of cell types separated into growth synchronous populations by centrifugal elutriation

Cell type	Elutriation buffer	Rotor/chamber/ centrifuge	Loading				Fraction collection details	Comments	Reference
			Number	Temp °C	Speed r.p.m.	Flow ml/min			
Yeast	Culture medium pH 5.4 (YNB0335–15–9) 2% (w/v) glucose 0.5% (w/v) $(NH_4)_2SO_4$ 4.7 g/litre NaCl	JE-6B Standard n.s.[a]	10^{10}	4	2750	10	Waste for 30 min. Collect 10–14 ml.	Small cells daughters remainder parents.	48
Schizo-saccharomyces pombe	n.s.[a]	JE-6 Sanderson n.s.[a]	7×10^9	33	3700	22	Waste 10 min. Collect at 33 ml/min.		21
Schizo-saccharomyces pombe	n.s.[a]	JE-10X n.s.[a] n.s.[a]	3×10^{10}	25	2100	100	Waste to 120 ml/min. Collect at 120, 130, 1 40 ml/min.		20
Mouse									
L-P59 fibroblasts	McCoys medium 5% (v/v) FCS[b] 5 nM NDA[c]	JE-6 Sanderson	3×10^7	4	1525	9	Collected 12 fractions between 9–25.6 ml/min.		16
Fibrosarcoma	McCoys medium 5% (v/v) FCS[b] 5 nM NDA[c]	JE-6 Sanderson	2×10^8	4	1210	7.4	Collected 12 fractions between 7.4–31.4 ml/min.		49
Erythro-leukaemia cells	DMEM[d] 7.5% (v/v) FCS[b]	JE-6 Sanderson? J-21	10^9	22	2000	15	Waste initially. Collect between 15–35 ml/min.		50
MPC-11 plasmacytoma cells	DMEM[d] 2% (v/v) HIHS[e]	JE-6B Standard J2–2M/E	4×10^8	18	2200	15	Waste for 10 min. Collect at 25 ml/min, then 30–35 ml/min, finally 48–56 ml/min.	G_1 cells, S-phase, G_2/M.	51
Human									
Cervical carcinoma HeLa	PBS[f] 0.3 mM EDTA 1% (v/v) FCS[b] 0.1% (w/v) glucose	JE-10X n.s.[a] n.s.[a]	8×10^{10}	4	1550	65	Waste 500 ml, then 11 fractions collected 75–190 ml/min.	87% G_1, 79% S-phase, and 81% G_2 (see *Figure 7*).	18, 52

Erythro-poietic cells K562	n.s.[a]	JE-5.0 n.s.[a] n.s.[a]	10^{10}	–	3000	65	Fractions collected 75–300 ml/min.		53
Hamster									
Fibroblasts Balb/c 3T3	DMEM[d] 10% (v/v) FCS[b]	JE-9 n.s.[a] n.s.[a]	3×10^8	5	2000	10.2	Waste at 15 ml/min. Collect at 20 ml/min.		54
Bacteria									
E. coli	n.s.[a]	JE-6 n.s.[a] J-2	–	–	5800	2	Waste 100 ml. Collect between 2–4 ml/min.	Fraction determined by cell length.	19

[a] Not specified.
[b] *Fetal calf serum.*
[c] 2-naphthol-6,8-disulfonic acid.
[d] Dulbecco's modified Eagle's medium.
[e] Heat inactivated horse serum.
[f] Phosphate-buffered saline.

Table 5. Examples of cell types from a variety of sources separated by centrifugal elutriation

Cell type	Elutriation buffer	Rotor/chamber/ centrifuge	Number	Loading Temp °C	Speed r.p.m.	Flow ml/min	Fraction collection details	Comments	Reference
Porcine oocytes	PBS[a] 1 mg/ml PVA	JE-6B Sanderson J2–21	–	20	1500	6	300 ml to waste then waste 200 ml at 10 ml/min. Collect remainder.	Purify from 1% in loading suspension to 65–80%.	30
Embryonic blastomeres	CMFSW[b]	JE-6 Standard	4×10^5 to 2×10^7	4	2000	24	50 r.p.m. decrements until 1050 r.p.m. Collect 100 ml between 24–28 ml/min.	Micromeres, mesomeres, and macromeres.	55
REH-6 cell line	Ca- and Mg-free PBS[a] 1% (v/v) FCS[c] 1% (v/v) Pen-strep	JE-6B Large and *Ascopox J-21B	–	–	1600	–	Large chamber: collect at 10–27 ml/min in small variable increments of 1.8–2.7 ml/min. Ascopox: collect at 17–30 ml/min in small variable increments of 2.2–3 ml/min.	Comparison of chambers.	56
Human MANCA	Culture medium (RPMI 1640) 1% (w/v) serum 1% (w/v) glutamine	JE-5.0 Large J6-MC	2×10^9	–	1800	22	Increase flow to 28 ml/min and reduce rotor to 1400 r.p.m. Leave 10 min. Collect 30–45 ml/min.	Collect 5×10^8 cells at 95–99% purity.	57
Cell lines S49.1 WEH 1–3	PBS[a] 1% (v/v) FCS[c] 0.3 mM EDTA	JE-6B n.s. J-6M	2×10^8	–	2400	10	Collect S49.1 between 13–33 ml/min. Collect WEH1–3 at 15–39 ml/min.	WEH 1–3 mouse myelomonocyte. S49.1 mouse T lymphoma.	58

[a] Phosphate-buffered saline.
[b] Calcium- and magnesium-free sea water.
[c] Fetal calf serum.

successful are those where cells are non-adherent (such as yeast cells and blood cells), or where the tissues can be readily dissociated (such as the testis and liver). For many biological research programmes it is now regarded as indespensible. For others it represents a convenient alternative means of achieving similar ends. Although centrifugal elutriation is dependent upon access to specialized equipment, since the diversity of potential applications is considerable, the process lends itself ideally to being run as a shared facility. Thus in the same way as for example ultracentrifugation, fluorescence-activated cell sorting, or confocal microscopy often now is, centrifugal elutriation perhaps should be considered as an essential central resource for any major research department dealing in modern cell biological techiniques.

8. Simulation of centrifugal elutriation for the Beckman JE-6B chamber

S. P. SPRAGG and DAVID RICKWOOD

8.1 Introduction

The isolation of particles having differing sizes and densities using gradient centrifugation is a well established procedure and gives high yields of the individual particles. This procedure has many disadvantages when applied to separating particles enclosed within a semi-permeable membrane because the solvents either have relatively high osmotic pressures or the chemicals that form the gradients have unwanted influences on the biological activities of the particles. These undesirable side-effects meant density gradient centrifugation had a very limited use for the separation of biological cells. In 1948 Lindahl (7) reported on the, then new, technique of counterstreaming centrifugation; this name was subsequently changed to centrifugal elutriation. In this procedure the sample is introduced into a container within the rotor through a tube fixed at the distal end of the container, and while the rotor is running. An exit tube is fixed to the centripetal end of the container and through this is collected the elutriate. The flow rate of the solvent pumped in at the distal entrance is increased while holding the rotor speed constant forcing the particles in the sample to sediment against an opposite bulk flow of the solvent. There is no reason why the rotor speed should be held constant throughout the experiment—instead the flow rate could be kept constant and the rotor speed varied. However, whatever the experimental regime chosen the experimenter needs to run several pilot experiments to define the ideal experimental conditions, varying the rotor speed as well as the flow rates. This imposes many uncertainties on the technique and commits scarce biological material to test runs. It is

for this reason that a simulation of the separation is useful making it easier to explore many regimes before trying out a real experiment.

Lindahl's container was cylindrical in shape which for a given flow rate meant the velocity of the incoming solvent within the cell was constant with respect to the radius. Subsequent commercial containers have modified the shapes to produce cells having profiles varying from sectorial (61) to basically funnel shapes having non-linear sides (61, 62). The narrow entrance to the funnels is at the distal end and the solvent is forced through this. With these containers the flow velocity of the incoming solvent decreases with decreasing radius giving a gradient of velocities within the container for a fixed flow rate. The present simulation is for one of these modern elutriation containers.

Lutz *et al.* (56) have applied an equation to follow the equilibrium between the particle's centrifugal buoyancy and the flow of the solvent which includes an empirical shape factor at a fixed radius. The present simulation is for dynamic separations and includes a model of the container's shape. However, it does not allow for the formation of vortices within the cell caused by flows along the walls and which produce circulation of the particles within the cell; these hydraulic effects have been observed by several workers who have informed us verbally but not recorded them in the literature. Less severe effects occur in all flow experiments and these broaden the boundaries to give results resembling the diffusion of macromolecules. Again, these phenomena have not been included within the simulations.

8.2 Basic differential and integral equations

The velocity of a particle within the container is given by:

$$(\mathrm{d}R/\mathrm{d}t)_\mathrm{T} = (\mathrm{d}R/\mathrm{d}t)_\mathrm{C} + (\mathrm{d}R/\mathrm{d}t)_\mathrm{E} \qquad [3]$$

where R = radius from the centre of rotation, t = time (sec), and subscripts T, C, and E refer to total, centrifugal, and elutriation respectively. The centrifugal velocity is given by:

$$(\mathrm{d}R/\mathrm{d}t)_\mathrm{C} = s \,.\, \omega^2 \,.\, R \qquad [4]$$

where s = sedimentation coefficient (sec), ω = radial rotor velocity (π. r.p.m./30, sec^{-1}, and r.p.m. = rev/min). The elutriation flow rate is given by:

$$(\mathrm{d}R/\mathrm{d}t)_\mathrm{E} = (\mathrm{d}v/\mathrm{d}t) \,.\, (1/A) \qquad [5]$$

where $\mathrm{d}v$ = volume of elutriation solvent flowing in $\mathrm{d}t$, so $(\mathrm{d}v/\mathrm{d}t)$ = flow rate of the incoming solvent (m^3/sec), and A = the area of a plane in the container at radius R. Since A is a function of the container's radius and shape then:

$$(\mathrm{d}v/\mathrm{d}t) \,.\, (1/A) = (\mathrm{d}v/\mathrm{d}t) \,.\, (\mathrm{d}R/\mathrm{d}A) \,.\, (\mathrm{d}A/\mathrm{d}v). \qquad [6]$$

The Beckman container can be considered as having two regions. The peripheral region, extending from R_b (the radius of the bottom of the cell) to R_m (the middle of the cell), and has a shape which approximates to an

exponential horn for the side parallel to the axis of rotation while the other direction (parallel to the plane of the rotor) approximates to a triangle with linear sides (ignoring the curve introduced by the exponential sides). This region holds about 0.8 of the total volume of the container (5 cm^3). The centripetal end, and the one from which the products are collected, approximates to a pyramid having a base with unequal sides, extending from R_e (the exit radius) to R_m. The exponential sides can be described by:

$$a \,.\, \exp[-F \,.\, (R - R_m)]$$

where F is the flare constant and a is the height at R_m, while the triangular part of this region and subtending an angle θ_2 at the bottom is given by:

$$2 \,.\, (R_b - R) \,.\, \tan(\theta_2/2).$$

Combining these two descriptions to find the area at any radius (R) gives:

$$A = b \,.\, (R_b - R) \,.\, \exp[-F \,.\, (R - R_m)] \qquad [7]$$

where $b = 2 \,.\, a \,.\, \tan(\theta_2/2)$ and is a constant for the container.

$$dA/dv = (R_b - R)^{-1}$$

and

$$dR/dA = -\{(R_b - R) \,.\, b \,.\, \exp[-F \,.\, (R - R_m)] \,.\, [1 + F \,.\, (R - R_m)]\}^{-1}$$

Combining these equations in *Equation 3* through *Equation 6* gives the final differential equation in which dv/dt, is replaced by the flow rate, V:

$$(1/R) \,.\, (dR/dt)_T = s \,.\, \omega^2 - V/\{R \,.\, (R_b - R) \,.\, b \,.\, \exp[-F \,.\, (R_b - R)] \,.\, [1 + F \,.\, (R - R_{b)}]\}. \qquad [8]$$

A different equation applies to the pyramidal part which, using conventional relationships for pyramids, includes tangents of θ_1 and ϕ (θ_1 is the profile angle and ϕ the top angle, at the exit) as well as a, $\times$ (the width of the cell), and $(R_m - R)$ to give an equation describing the velocity of the boundary for radii less than R_m:

$$dR/dA = (R_m - R) \,.\, [2 \,.\, (A_2 + A_4) - 8 \,.\, A_3 \,.\, (R_m - R)]$$

where $A_2 = \times \,.\, \tan(\phi/2)$, $A_3 = \tan(\phi/2) \,.\, \tan(\theta_1/2)$, and $A_4 = a \,.\, \tan(\theta_1/2)$. Combining as before gives:

$$(1/R) \,.\, (dR/dt)_T = s \,.\, \omega^2 - \{V/\{R \,.\, (R_m - R) \,.\, [2 \,.\, (A_2 + A_4) - 8 \,.\, A_3 \,.\, (R_m - R)]\}\}. \qquad [9]$$

Integrating *Equation 8* between the limits R_1 and R_2, where $(R_1 - R_2)$ is the radial distance travelled in time Δt, gives:

$$\ln(R_2/R_1) = s \,.\, \omega^2 \,.\, \Delta t - \{V \,.\, \Delta t/\{(R_1 - R_2) \,.\, (R_b - R_2) \,.\, b \,.\, \exp[-F \,.\, (R - R_m)] \,.\, [1 + F \,.\, (R - R_m)]\}\} \qquad [10]$$

and for *Equation 9*:

$$\ln(R_2/R_1) = s \,.\, \omega^2 \,.\, \Delta t - \{V \,.\, \Delta t/[(R_m - R_2) \,.\, (R_1 - R_2) \,.\, [2 \,.\, (A_2 + A_4) - 8 \,.\, A_3 \,.\, (R_m - R_2)]]\}. \quad [11]$$

8.3 Computation

The two integral equations (*Equations 10* and *11*) describe the positions of boundaries within the container and have sets of constants which have to be measured. The linear dimensions were measured for an elutriation cell (JE-6B) and the angles calculated from these dimensions. A final test of the accuracy of these measurements were made by calculating the total volume of the container using the denominators of the right-hand sides of *Equations 10* and *11* and summing the volumes. The values of the radii used were: $R_1 = R_b$ or $R_m = 121$ mm or 8.7 mm; $(R_b - R_m) = 35$ mm; $(R_m - R_2) = 9$ mm, which together with the angles given for *Figure 9* gave the total volume of 5.066 cm^3. This compares favourably with Beckman's specification of 5 cm^3; the small difference between theory and calculated may reflect the errors in the measurements or imperfections in the model. The flare constant, F, was estimated to be 53.48 m^{-1} assuming the entrance hole was 2 mm in diameter ($F = \ln(2/13)/35$).

In the elutriation experiments to be simulated (14, 15) the sample was introduced while the rotor was turning at constant speed and using a relatively slow flow rate (6 cm^3/min). This was followed by increasing the flow rates incrementally while collected fixed volumes of the elutriate; Wilton *et al.* (14, 15) collected fractions of 100 cm^3. Within the container two boundaries form for each cell type, the leading one at the centripetal end which eventually exits first, and the trailing boundary at the centrifugal end. When the trailing boundary reached R_e (the exit point near the centre of rotation) this signals the exit of the particular type of biological cell. Simulating this process using *Equations 10* and *11* means keeping records of the radial positions of both boundaries for each cell type. The calculation of the centrifugation requires a knowledge of sedimentation coefficients (s) of the cells, which if non-ideal thermodynamic and hydrodynamic are ignored, this can be calculated from the biological cell's radii (r) and density (d_p) using established Svedberg's and Stoke's equations, giving:

$$s = 0.22222 \,.\, r^2 \,.\, (d_p - d_s)/N \quad [12]$$

where d_s = density of the solvent and N = its viscosity.

Both *Equations 10* and *11* have two boundary radii, R_1 and R_2, but to find one it is necessary to know the other. For the present discussion R_2 is calculated assuming R_1 is known for each time increment, Δt. This calculation requires an iterative procedure and it is important to realize that both equations have two conditions for instability; when $R_1 = R_2$ or $R_2 = R_b$ (*Equation 10*) or $R_2 = R_m$ (*Equation 11*), otherwise the equations are well behaved. The most economical method for calculating R_2 is the Newton–Raphson procedure

using first derivatives to improve the convergence; the basic Newton–Raphson equation is:

$$R_2^{(n+1)} = R_2^{(n)} - f(R_2)/f'(R_2). \qquad [13]$$

Superscript n identifies the nth iterative cycle at Δt, while $f(R_2)$ is defined in *Equation 14* and $f'(R_2)$, the first derivative, is given by *Equation 15*. (In the equations part a refers to the exponential region and part b the pyramidal part.)

$$f(R_2) = s \,.\, \omega^2 \,.\, \Delta t - [V \,.\, \Delta t/\{(R_1 - R_2) \,.\, (R_b - R_2) \,.\, b \,.\, \exp[-F \,.\, (R - R_m)] \,.\, [1 + F \,.\, (R - R_m)]\}] - \ln (R_2/R_1) \qquad [14a]$$

$$f(R_2) = s \,.\, \omega^2 \,.\, \Delta t - [V \,.\, \Delta t/\{\{(R_m - R_2) \,.\, (R_1 - R_2) \,.\, [2 \,.\, (A_2 + A_4) - 8 \,.\, A_3 \,.\, (R_m - R_2)]\}] - \ln (R_2/R_1) \qquad [14b]$$

$$f'(R_2) = \{[-V \,.\, \Delta t/D_e] \,.\, \{(R_1 - R_2)^{-1} + (R_b - R_2)^{-1} + F \,.\, [(1 + m \,.\, (R - R_m))^{-1} - 1]\}\} - (1/R_2) \qquad [15a]$$

where $D_e = (R_1 - R_2) \,.\, (R_b - R_2) \,.\, b \,.\, \exp[-F \,.\, (R - R_m)] \,.\, [1 + m \,.\, (R - R_m)]$.

$$f'(R_2) = \{[-V \,.\, \Delta t/D_p] \,.\, \{(R_m - R_2)^{-1} + (R_1 - R_2)^{-1} - [8 \,.\, A_3/[2 \,.\, (A_2 + A_4) - 8 \,.\, A_3 \,.\, (R_m - R_2)]]\} - (1/R_2) \qquad [15b]$$

where $D_p = (R_m - R_2) \,.\, (R_1 - R_2) \,.\, [2 \,.\, (A_2 + A_4) - 8 \,.\, A_3 \,.\, (R_m - R_2)]$.

These equations contain both R_1 and R_2 and for present purposes it is assumed at each stage R_1 is known and R_2 is to be estimated using *Equations 14* and *15*. Since the elutriation experiment starts while the sample is being loaded the simulation must also begin with this stage. Thus, at the start $R_1 = R_b$ for the trailing boundary and $R_1 = R_e$ for the exit boundary. Following this first calculation of R_2 for the two boundaries and for each simulated cell type, subsequent steps with each new flow rate puts R_1 equal to the previous R_2 before the new estimates are made. During all these calculations the following checks must be made:

(a) When the new R_2 crosses R_m then the model must be changed.

(b) If $R_2 < R_e$, then the boundary has left the cell.

(c) If $R_2 > R_b$, then R_2 must be replaced by R_b.

The variable $V \,.\, \Delta t$ = volume of the fractions collected at each flow rate; when calculating the boundary positions at the start and during loading this can be assumed to equal the volume of the container. The calculations were finished when the last flow rate is processed.

Presentation of the results must take into account the following extreme outcomes. It is possible for the trial flow rates to be too high for the chosen rotor speed and all the cell types are pushed out of the container during loading of the sample. A second extreme occurs when the flow rates are all too low when no separation of the cells occur, or worse, they centrifuge out of the loading port. These cases must be tested for by, for example, in the first case

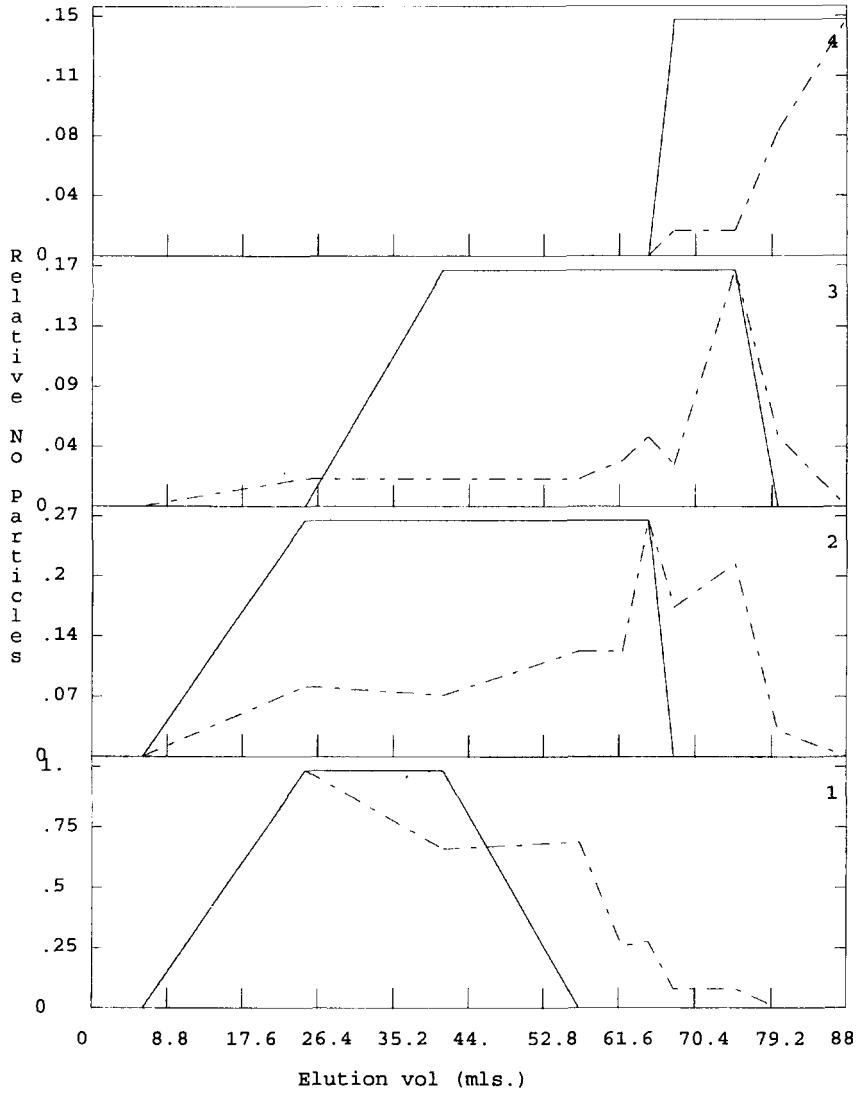

Figure 9. A comparison between theoretical (full line) and experimental (broken line) results for the separation of hepatocyte cells in a Beckman elutriation cell JE-6B. The values measured from an elutriation cell were: R_b = 121 mm; R_m = 86 mm; R_e = 77 mm; a = 13 mm; $\times$ = 25 mm; $\theta_2/2$ = 0.254476 radians; $\theta_1/2$ = 0.79106 radians; $\xi/2$ = 0.5281 radians. The theoretical results were calculated using: rotor speed = 1100 r.p.m.; particle radii were single cell (1) = 15 nm; couplet (2) = 30 nm; triplet (3) = 37 nm; aggregate (4) = 100 nm (labelled at right-hand corner of each plot). The density of the cells was assumed constant for all and equal 1.05 g/cm^3. The experimental results were taken from Wilton *et al.* (65).

putting all the R_2s equal to R_e when the situation occurs, similarly for the second case put R_2s equal to R_b if $R_2 > R_b$. Often the simplest presentation is through a list but the present test of the model presented them graphically (*Figures 9* and *10*) and for the boundary positions within the elutriation cell.

8.4 Correction for wall collisions

The formulation of the sedimentation of the particles only applies to a container having a sector shape since only then does the centrifugal vector follow the cell's geometry. Clearly this is not the case in the elutriation cell and centrifugation resembles that in a cylindrical tube in a fixed-angle rotor. In the elutriation cell this effect is exaggerated because the area of a plane in the main part decreases with increasing radius and particles encounter walls on four sides of the elutriation cell. An approximate allowance can be made to compensate for collision with the walls by assuming the collisions are elastic, i.e. the particulate kinetic energy on impact equals that after leaving the vicinity of the wall. Physical conservation laws show that for this situation the radial position of the particle after rebounding from the wall is greater than for a sectorial container. This can be allowed for using the reciprocal of the sine of the angle of incidence of the particle if calculating s from data or cosine if converting a given s to its operational value, as in the simulation. In the elutriation cell this impact angle changes with changing radial position. There are two components to the correction factor:

(a) That from the exponential sides and equal to:

$$2/\{\sin(\pi \,..\, 5 - \alpha)\}$$

where $\alpha = A\tan\,[-F \,.\, a \,.\, \exp(-F \,.\, (R - R_m))]$.

(b) That from the other sides modelled as triangles giving:

$$2/\{\sin(\pi \,..\, .5 - .5 \,.\, \theta_2)\}.$$

Thus, combining these gives the multiplier for the sedimentation coefficient calculated from *Equation 12*. Unfortunately this correction is necessary every time a new value for R_2 is calculated in the iteration. The derivatives used in the Newton–Raphson could have included the correction but for clarity in presentation this has been omitted here. It may appear that the correction need only be applied during sedimentation in the main part of the container but in order to conserve mass within the volume between the two boundaries for each biological cell type the correction calculated for each estimated R_2 when in the exponential part must also be applied in the pyramidal part . For the Beckman cell the correction varies between 4 and 5 meaning the particles appear to sediment with rates similar to particles having twice the microscopic radius.

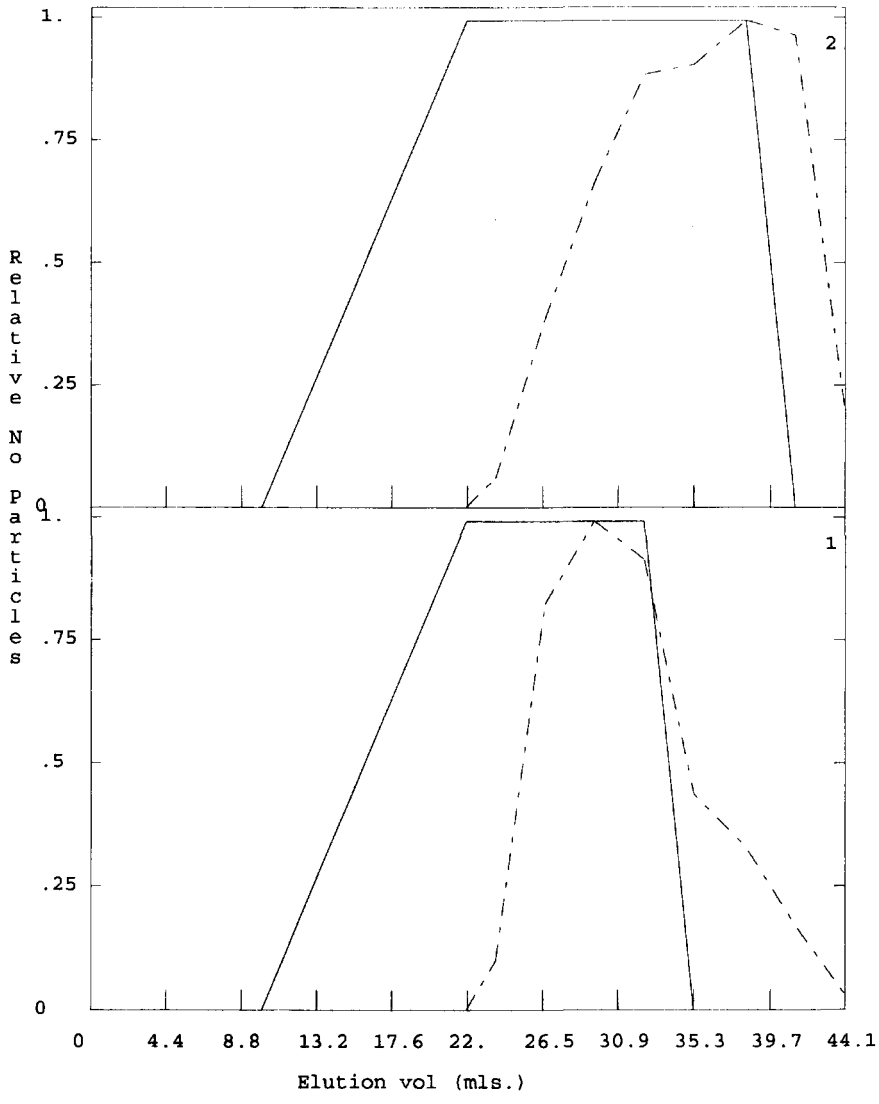

Figure 10. A comparison between theoretical (full line) and experimental (broken line) results for rat hepatocyte cells. Graph 1 is for cells treated with CCl_4, and graph 2 for untreated cells (from ref. 66). The radii of the cells were (1) 30 nm and (2) 40 nm (labelled 1 and 2 in right-hand corner of each plot), while the density was assumed to be the same for both types and equal to 1.05 g/cm^3. The rotor speed was 850 r.p.m.

8.5 Comparison of theoretical with experimental results

A suitable set of experimental results in which hepatocyte cells were separated by elutriation are given by Wilton *et al.* (14, 15). The flow rates and rotor speeds were taken from these publications while the radii of an individual cell was assumed to be 15 nm, and all cell types had densities of 1.05 g/cm^3. In the experiment (*Figure 9*) there occurred a distribution of the eluted cell types which could have been caused by small variations in their radii, or densities, or turbulence within the cell, or all these factors and which are not simulated in this simple model. Within this limitation the theoretical eluates resembled the experimental results for the peaks in the cell numbers (these are plotted as relative numbers in both the experimental and theoretical plots).

In the second set of results in which hepatocyte cells treated with CCl_4 were compared with untreated cells (15) simulations were made assuming the cells were couplets, as observed microscopically (*Figure 10*). The radius of the treated couplets was assumed to be 30 nm, but that of the untreated cells was unknown so several values were tried and the best fit between the theoretical and experimental was found for a radius of 40 nm; the density was assumed constant at 1.05 g/cm^3 for all cells. It was found from the simulation that untreated cells needed to be larger than the treated. Similar fits could be obtained by keeping the radii constant and increasing the density of the treated cells but the width of the simulated rectangle did not match that of experimental spread so well.

8.6 Conclusions

The comparisons given in *Figures 9* and *10* show that *Equations 10* and *11* reflect experimental results within the limitations set by the unknown distributions of cellular physical properties and any turbulence. This gives confidence that proposed experimental regimes can be explored theoretically before sacrificing valuable biological material to an experiment. A further bonus of the simulation when compared with the experiment is to give estimates of the physical properties of the cell types in the experiment. These can only be tentative since the radius and density are correlated and can only be guessed.

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4

Separation and fractionation of cells by partitioning in aqueous two-phase systems

D. FISHER

1. Introduction

In the technique of cell partitioning, cells are added to the aqueous two-phase systems that are formed when aqueous solutions of certain polymers (typically dextran and polyethylene glycol) are mixed above critical concentrations. The cells distribute (partition) between the top phase, the bottom phase, and the interface between them (frequently termed the bulk interface). By appropriate choice of the composition of the phase system (see below) this partitioning can be selected to be dominated by different cell surface properties:

- cell surface charge
- non-charge surface properties
- surface antigen/receptor status

With the exception of some examples of affinity cell partitioning (see below), cells will not show sufficiently marked differences in partitioning behaviour to be separated in a single step; multiple partitions will be required for separations and these are carried out by countercurrent distribution (CCD). Because two-polymer aqueous phase systems separate much more slowly than the aqueous–organic two-phase systems employed in countercurrent chromatography, thin layers of phase are used in the settling step of the CCD procedure and specifically designed thin-layer countercurrent distribution (TLCCD) apparatus is employed.

A particular application of CCD lies in its providing information on the heterogeneity of cell populations. The partitioning behaviour of cells (expressed as the partition coefficient, K), is measured through the CCD profile. A progressive change in K through the profile indicates heterogeneity with respect to the cell surface properties selected for, whereas a constant K indicates that the cell population is homogeneous in these surface properties.

Cell partitioning depends very sensitively (often exponentially) on the cells'

surface properties and/or on the phase compositions. Although this provides the opportunity for sensitive separations and fractionations of cell populations, it does provide an experimental challenge to the user; *without careful attention to the preparation and use of the phase systems, reproducible results will not be obtained.* This is, however, not a difficult task to achieve, provided the user is aware of some important basic aspects of the methodology. These are described in Section 2. Detailed treatments of phase partitioning are available (1–4).

2. Theoretical background and its impact on methodology

2.1 The influence of polymer concentration: phase separation and phase diagrams

Certain water soluble polymers when mixed together above critical concentrations separate into two immiscible aqueous phases, each enriched with one of the polymers. Although many pairs of polymers show this behaviour, for the purpose of cell separations dextrans and polyethylene glycols (PEG) have generally been used. Dextran–PEG systems form a PEG-rich top phase and a dextran-rich bottom phase. Both phases have very high water contents (typically about 90%, w/w) and hence these two-phase systems have a very low interfacial tension (0.1–100 μN/m), orders of magnitude less than aqueous–organic two-phase systems. This low interfacial tension, and the fact that phase systems can readily be rendered isotonic and buffered by the addition of appropriate small molecules, leads to two-phase systems being very mild for biological materials including cells.

With increasing concentration of the polymers the two phases are increasingly dissimilar in polymer composition, thus an infinitely variable pair of aqueous solvents is possible by simple alteration of the polymer composition. This provides considerable flexibility in selection of phase compositions to optimize separations. The compositions of the two-phase systems are described by the phase diagram (see *Figure 1*). There is generally no need to construct a phase diagram (although this is described elsewhere) (1, 6), but there are some important features of phase diagrams that it is useful to appreciate:

(a) Separation into two phases occurs only above certain concentrations of the two polymers, defined by the curve called the binodal. In *Figure 1* the mixture A_1 separates into a top phase of composition T_1 and a bottom phase of composition B_1. The top phase is PEG-rich and the bottom phase is dextran-rich. The line T_1B_1 passes through A_1 and is called a tie line.

(b) Two-polymer phase systems are prepared on a weight for weight basis and a typical phase system of 5% (w/w) Dextran T500 and 4.5% (w/w) PEG 6000 separates at 25°C into top and bottom phases with the following compositions (7):

	PEG	Dextran	Water
Top phase	6.2% (w/w)	1.3% (w/w)	92.5% (w/w)
Bottom phase	2.4% (w/w)	9.9% (w/w)	87.7% (w/w)

with a tie line length of 9.2 (w/w).

(c) The relative proportions of the top and bottom phases are given by the ratio A_1B_1:T_1A_1. Thus in this particular sample there is about three times more top phase than bottom phase. Strictly the ratio A_1B_1:T_1A_1 is the weight ratio of top:bottom phases, but it approximates to the volume ratio. Any phase composition that falls on this particular tie line will separate into top and bottom phases of identical concentrations, however the volume ratios will differ. The practical consequence of this is that a phase system can be prepared and the two phases separated. The phase system can then be reconstituted in any volume ratio required and the top and bottom phases will still have the same compositions as they had when initially separated; the two phases can be treated just like the pairs of immiscible liquids encountered in organic chemistry, such as benzene and water.

(d) Point O in *Figure 1* is a unique composition, called the critical point, where the tie line is vanishingly small. Frequently phase systems of increasing polymer compositions are referred to as being of increasing distance from the critical point. As the tie line length increases, the top and bottom phases are increasingly different in composition.

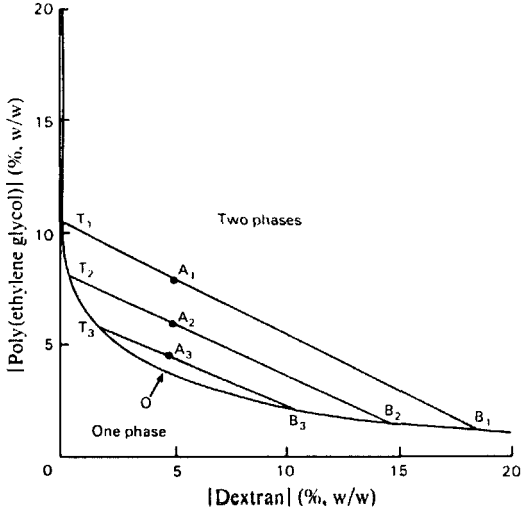

Figure 1. Phase diagram for Dextran T500 and PEG 6000 in 0.15 M NaCl buffered with 0.01 M sodium phosphate pH 6.8 at 25°C. The curved line is the binodal curve. Three compositions, A_1, A_2, A_3 are shown separating into top phase compositions of T_1, T_2, T_3 and bottom phase compositions of B_1, B_2, B_3 respectively. Lines T_1B_1, T_2B_2, and T_3B_3 are tie lines of decreasing length. O is the critical point. Taken from ref. 5.

A number of practical consequences arise from this phase behaviour:

(a) Phase diagrams vary with the molecular weight of the polymers: essentially the larger the polymer the more readily will phase separation occur. Thus an increase in the molecular weight of a polymer will shift the binodal to the left and increase the dissimilarity of the phases that separate for any given composition. Since batches of polymers as purchased will show variations in their molecular weight distributions, even when purchased from the same distributor, it has to be expected that the behaviour of phase systems will vary with polymer batches. Practically this means that it is an advantage to perform a series of separations with the same polymer batch. Furthermore, in following recipes from the literature it must be expected that some variation in partitioning behaviour will be obtained; it may be necessary to modify the phase system slightly to reproduce the conditions of the published separations. Clearly, if you do not appreciate this, then your first experience of phase separations might even be as bad as 'the system did not form two phases'. The most frequently used dextrans have molecular weights of 500 000 (Dextran T500) and 40 000 (Dextran T40). PEGs are available in a range of sizes, and most frequently PEG 6000 and PEG 8000 (molecular weight 6000 and 8000 respectively) are used. It should be noted that PEG designated as PEG 6000 by Union Carbide up to the early 1980s, subsequently was designated PEG 8000.

(b) Temperature influences the phase diagram. Thus good temperature control is required. Phase systems are made at the temperature at which they are to be used and the top and bottom phases are separated at this temperature. These can then be stored in the fridge, to minimize bacterial contamination. They will then, of course, phase separate further because of the lower temperature, forming a two-phase system. It is therefore essential that before use they are brought back to the temperature of preparation and thoroughly mixed. Standard procedure when dispensing separated phase systems should always be to ensure appropriate temperature equilibration followed by thorough mixing before dispensing any aliquot.

(c) The phase have specific gravities just above 1, the dextran-rich phase is clearly a little denser as it forms the bottom phase. The dextran-rich phase has, however, a much higher viscosity that the PEG-rich phase and this places some constraint on how it should be dispensed. In making up phase systems from concentrated polymer solutions, volume measurements should not be used; all solutions should be made up on the balance by weight. The final polymer concentrations in formed phase systems is lower than in the concentrated stock solutions and this does permit the use of pipettes but the operator must be aware that dextran-rich bottom phases are still somewhat viscous and positive displacement pipettes are recommended for the sampling of the phases.

(d) The polymers contribute little to the osmolarity of the phase systems; typically this is 10–20 mOsm, the contribution of the PEG being about ten times greater (6). Tonicity must be provided by salts or sucrose. For osmotically-sensitive cells it may be necessary to measure the osmotic pressure, but a freezing point osmometer cannot be used because of the complex freezing behaviour of polymer solutions; a vapour pressure osmometer must be used. Examining the effect of the phase system on the size of cells (determined by a size analyser after fixation) is a direct way to optimize the tonicity.

2.2 The influence of ionic composition: charge-sensitive and non-charge-sensitive cell partitioning

The phase systems can be buffered and rendered isotonic by the incorporation of salts. However, salts can have important effects on the properties of phase systems that can be exploited for separation purposes.

Certain ions (e.g. NaCl) distribute evenly between the phases whereas other ions do not. In particular, phosphate favours the bottom phase and its uneven partitioning gives rise to an electrostatic potential difference between the phase ($\Delta\psi$); the top phase behaves as if it is positively charged with respect to the bottom phase. By preparing isotonic NaCl–phosphate phase systems with increasing phosphate compositions, phase systems with increasing $\Delta\psi$ are obtained. The partition of negatively charged cells is heavily influenced by $\Delta\psi$; cells favour the top phase on the basis of their charge status and the magnitude of $\Delta\psi$. Phase systems with low $\Delta\psi$ are termed 'non-charge-sensitive' phase systems as in the absence of any appreciable $\Delta\psi$, the cell partitioning is not determined by the interaction between the $\Delta\psi$ and the cell charge; it is solely determined by the interactions of the cell surface with the phases, which is dependent on the polymer concentrations. Phases where $\Delta\psi$–cell charge interactions do influence partition are termed 'charge-sensitive phase systems'. Although correlations between top phase partition and electrophoretic mobilty have frequently been observed (8), there are exceptions, so it must not be considered that cell separations by charge-sensitive phase partition and by cell electrophoresis (described in Chapter 7) will produce identical results. The reason for this is that in the partition behaviour of cells in charge-sensitive phases there is always present a component arising from the interactions with the polymer phases (a non-charge-sensitive component).

2.3 The influence of polymer ligands: affinity cell partitioning

Ligands can be covalently attached to either of the phase-forming polymers. Generally PEG–ligands have been made; the coupling chemistry to PEG is simpler than to dextran. PEG–ligands incorporated into phase systems will associate with the cell, making it 'PEG-like' and thereby increasing its

partition into the PEG-rich top phase. Non-charge-sensitive phases are used and adjusted so that in the absence of the PEG–ligand top phase partition is very low. By incorporating the PEG–ligand in the phase system, only those cells with affinity for the ligand will be partitioned into the top phase, thereby providing a selective extraction. However, it is important to appreciate that if the PEG–ligand has relatively similar affinity for all the cells in the population, all cells will be relatively similarly 'coated' with PEG and favour the PEG-rich phase. It is still likely that different partitions will be obtained, and separations made possible, because this relatively similar affinity for the PEG-rich phase will be balanced by the underlying differences in affinity for the dextran-rich phase. The significance of this is that difference in partition in affinity partitioning systems do not necessarily imply difference in affinity for the PEG–ligand. Thus it must be appreciated that affinity partitioning of cells will arise from 'non-specific' parameters as well as 'specific' parameters.

2.3.1 Charged PEG–ligands

Positively charged trimethylamino–PEG (TMA-PEG) and the negatively charged PEG sulfonate (S-PEG) can be used to provide charge-sensitive cell partitioning. Low ionic strength medium is required. This application has been particularly successful with bacterial surfaces (9, 10).

2.3.2 Hydrophobic PEG–ligands

PEG–esters formed with long chain fatty acids interact hydrophobically with cells, providing hydrophobic affinity cell partitioning. The method is very sensitive, and very little PEG–palmitate, for example, has to be added (e.g. 0.001%, w/w) to extract cells into the top phase. At such low concentration the PEG–ligand makes little contribution to the total PEG concentration in the phase diagram. When used in phase systems supplemented with serum, it is necessary to use higher concentrations of PEG–palmitate as fatty acids bind to serum, and a large proportion of the PEG–palmitate will be neutralized. For example in a 5.8% (w/w) Dextran T500–5.8% (w/w) PEG 4000 system containing 5% fetal calf serum, Sharpe recommends 0.0041% PEG–palmitate (11).

Although PEG–esters have been used extensively in analytical applications to measure cell surface hydrophobicity, particularly of bacterial surfaces (9, 10), applications for the separation and recovery of cells have been scant.

2.3.3 PEG–antibodies

By coupling PEG to antibodies, specific immunoaffinity extractions and separations of cells can be achieved. This clearly has the possibility of providing selective cell purifications either in single-step extractions or in CCD. Immunoaffinity cell partitioning is described in Section 4.1, *Protocol 4* and elsewhere (12–22).

2.3.4 PEG–metal chelates

Metal chelates attached to PEG, such as PEG iminodiacetate/CuII, called PEG-IDA-Cu, binds to cell surface histidine groups and causes cells to partition into the upper, PEG-rich phase in PEG–dextran systems. The presence of the PEG–ligand raises the partition coefficient in both charge-sensitive and charge-insensitive phases. When a series of erythrocytes was examined, similarities in the relative partitioning behaviour with and without the PEG-IDA-Cu were observed (23, 24). This suggests that all that the PEG–ligand is doing is increasing partition overall for all cells and that its affinity partitioning effect is not specific. However, some examples of difference have been seen that indicate that specific parameters are also contributing. A problem with the use of such ligands is that considerably high concentrations of the ligands are required, about 10% of the PEG concentration. Since the PEG–ligands will necessarily be made from PEGs different from those used for phase formations, there are additional problems (23, 24).

2.4 How cell partitioning arises and the practical consequences

Cell partitioning is a highly dynamic process and separations are achieved by exploiting the difference in the dynamics of different cell populations (25–28). There is no need to understand the details of this, but the principles have an impact on how to achieve good separations and give rise to some practical constraints (25–27).

When phase systems are mixed, they break down to very small droplets of both top and bottom phases, essentially similar to an emulsion. Once mixing has ended these droplets fuse with similar droplets and the larger droplets begin to move upwards (top phase-rich droplets) and downwards (bottom phase-rich droplets) under the influence of gravity. At the bulk interface that forms between the developing top and bottom phases, the droplets fuse and deliver their contents into the developing bulk phases. Phase separation thus takes an appreciable time as these processes occur. Typically a 'test-tube' of phase will take about 20 minutes for a distinct interface to form.

When cells are present in cell partitioning, they associate with these droplets and move with them as phase separation proceeds. By the time phase separation has proceeded extensively, cells can be found in three positions: in the top phase on the surface of small droplets that are settling slowly to the bulk interface (as the droplets are small); in the bottom phase (associated with droplets that are rising to the bulk interface); and at the bulk interface, to which they have been delivered on the surfaces of bigger, faster clearing droplets. How cells are distributed in these three positions is determined by the strength of attachment of the cells to the droplets. For example, weakly attached cells will shear off rapidly moving droplets and attach to smaller, more slowly moving droplets. Consequently, their partition in the top phase will be higher than that

of cells that attach more strongly and are cleared from the top phase more rapidly. This attachment is determined by the nature of the cell surface and properties of the phase system, e.g. $\Delta\psi$ and the interfacial tension (28).

As it takes time for phase separation to proceed, the partitioning of cells between these three positions will change after mixing. Nevertheless, when phase separation is almost complete, the distributions will be changing only slowly and therefore the cells can be regarded as having a defined partition. However, when phase separation is complete, the cells will all have been delivered to the bulk interface on the surfaces of the droplets and no differences will be apparent between different cell populations. Thus partitioning in a non-equilibrium process, and the partitions used for separations are not equilibrium values (25). *Practically, this means that it is important to control the time of phase separation.*

Furthermore, how the phase separation step is performed will influence the kinetics of phase separation and hence the kinetics of cell partition. Thus the volumes of top and bottom phases used, and the size and shape of the settling vessel (CCD chamber, or test-tube) needs to be controlled. A simple way to alter the settling chamber in a single tube partition is to place the (stoppered) tube on its side, so that the phases are much thinner. Bringing the tube back to the vertical permits the contents to be sampled. Although reducing the phase column height speeds up phase separation there is a reduction in the efficiency of separation of cell mixtures (29).

In summary, provided procedures are controlled and are not varied, reproducible results are obtained. Differences are to be expected between laboratories with respect to absolute values of partition behaviour, but the relative behaviour of cells should be the same.

3. Practical aspects of phase partitioning

3.1 Selection of phase systems

Phase systems for non-charge-sensitive phase partitioning are typically prepared in 0.15 M NaCl with 0.01 M sodium phosphate buffer. Tonicity can be controlled by varying the NaCl or adding sucrose. The partition coefficient can be adjusted by selection of the polymer concentrations. This is performed systematically, for example, using phase systems of 5% (w/w) Dextran T500 with increasing concentrations of PEG 6000: 3.5%, 4%, 4.5%, and 5% (w/w). Subfractionation by CCD of a cell population is best achieved when about 50% of the cells are in the top phase (30).

For charge-sensitive phase partitioning it is first important to select a non-charge phase system in which the cells show little partition to the top phase but, if the polymer concentrations were lowered, would begin to go into the top phase. Mechanistically, this can be viewed as selecting interfacial conditions such that the cells are just sufficiently attached to be delivered to the

bulk interface by the time partition is measured. Any weakening of the interaction with the interface will increase top phase partition. Thus using this polymer composition, but replacing the buffer with 0.11 M sodium phosphate will provide a phase system with an electrostatic potential difference; the partition that now occurs into the top phase will be determined, at least to some extent, by the charge properties of the cells. If partition into the top phase is too great, isotonic NaCl–phosphate systems with lower phosphate components can be used to produce 'semi-charged' phase systems.

For affinity partitioning, a non-charge-sensitive phase system is similarly selected in which the top phase partition is low; incorporation of the affinity ligand will then increase top phase partition as a consequence of the affinity interaction, whatever the nature of this interaction might be.

3.2 Preparation of phase systems

Phase systems are made on a weight for weight basis from stock aqueous solutions of solutions of dextran, PEG, and buffer. As dextran samples can be variably hydrated, it is preferable to prepare a stock solution about 10% higher than the desired concentration of 20% (w/w) (i.e. about 22%, w/w) and calibrate its dextran content from its optical rotation. This ensures that other solutions prepared from the same polymer batch will be reproducibly the same. However, if a polarimeter is not available, simply prepare sufficient stock dextran for the experimental series required, aliquot these, and freeze them. The compositions prepared from these stocks may not be exactly known, but will be reproducible for the experiments undertaken. PEG samples are treated as if they are dry although they contain some water.

A wide range of compositions has been reported, but a system of general applicability comprises PEG 6000, Dextran T500, and sodium phosphate-buffered saline. If serum is required it can be incorporated by replacing some of the water. Typically 5% or 10% fetal calf serum is used. Stock solutions and isolated phases can be sterilized by filtration through a 0.22 μm Millipore filter.

Protocol 1. Preparation of stock polymer and buffer solutions

Reagents

- Dextran T500 (Pharmacia Fine Chemicals)
- PEG 6000 (BDH Chemicals)
- Analar sodium chloride
- Analar disodium hydrogen phosphate
- Analar dihydrogen sodium phosphate
- Water: sterile, endotoxin-free water, pH 6.8 (Travenol Laboratories), particularly if dealing with neutrophils

A. *Dextran T500 solutions (about 22%, w/w)*

1. Weigh out 220 g of Dextran T500 into a glass beaker.
2. Into a separate beaker weigh out 720 g of water and gently heat on a hot plate with magnetic stirring.

Protocol 1. Continued

3. Pour the dextran slowly into the stirred water, cover with cling film to minimize evaporation and contamination, and stir until all dextran is dissolved. This may take overnight.
4. Dispense the dextran solution as 50 ml aliquots in sterile plastic containers and store at –20 °C.
5. If a polarimeter is available, measure the optical density of a sample (5 g diluted to 25 ml in a graduated flask). The dextran concentration of the diluted sample is given by:

$$\text{dextran (g/100 ml)} = \text{measured rotation}/l[\alpha]$$

where l is the path length of the sample measured in decimetres and $[\alpha]$ is the specific rotation of dextran, which is 199°. Multiply this value by five to obtain the dextran concentration of the stock, expressed as g/100 g.

B. *40% (w/w) PEG solution*

1. Add 400 g of PEG 6000 to 600 g of water in a glass beaker. Stir magnetically for several hours to dissolve.
2. Dispense the PEG solution as 50 ml aliquots in sterile plastic containers and store at –20 °C.

C. *0.44 M phosphate buffer*

1. Prepare 0.44 M disodium hydrogen phosphate and 0.44 M sodium dihydrogen phosphate in water.
2. Adjust pH of the disodium hydrogen phosphate to desired pH (e.g. pH 7.2) by addition of sodium dihydrogen phosphate.
3. Sterilize by Millipore filtration and store at 4 °C in plastic bottles.

D. *0.6 M sodium chloride*

1. Prepare 0.6 M NaCl in water.
2. Sterilize by Millipore filtration and store at 4 °C in plastic bottles.

E. *Preparation of phase systems*

1. Bring all stock solutions to room temperature and thoroughly mix before weighing out.
2. Into a glass centrifuge bottle weigh out the components for the selected phase systems in order of their densities: dextran, PEG, salts, and water. Add each component so as not to disturb the one below. This permits removal of the components being weighed out if too much is added.

3. Cover the bottle with plastic film or stopper and equilibrate to the temperature that the phase system will be used at. It is preferable to place at 25°C in a water-bath than to use an ill-defined room temperature.
4. Thoroughly mix and allow the phases to separate, either in a water-bath or by centrifuging (at the temperature required) (3000 *g* for 30 min).
5. Remove the top and bottom phases by using a large syringe with narrow plastic tubing attached. Suck off the top phase until the interface is approached; leave a layer of top phase above the interface. Place the top phase in plastic tubes. Using a new syringe and tubing, pass the tubing through the interface to the bottom of the bottom phase and suck off the bottom phase, until the interface approaches the bottom of the tube. Do not attempt to collect the residue of the top and bottom phases. Place the bottom phase in plastic tubes. If required, sterilize both phases by filtration through a 0.22 μm Millipore filter.
6. Store the separated top and bottom phases at 4°C until required.
7. Reconstitute the phase systems for use by first bringing both top and bottom phases to the temperature at which they were prepared and are to be used. Thoroughly mix the tubes before taking aliquots for the application in hand.

3.3 Preparation of cells

Single cell suspensions are required for cell partitioning. As partitioning is principally determined by cell surface composition, isolation of cells that alter the cell surface will influence the partitioning. Nevertheless, separations of cells isolated by trypsinization have been achieved. However, it has been reported that trypsinization of osteoblasts progressively removes surface proteins producing a more hydrophobic cell surface, seen as a population with a higher partition in non-charge-sensitive phases in CCD (31). Moreover, slower growing cells in the population are more sensitive to trypsin (32) whereas rapidly growing cells are less affected, and have a lower *K*. Mild trypsin treatment has been reported to not alter surface properties as detected by CCD (33).

Cells are recovered from phase systems with good viability. There are numerous studies in which cells after TLCCD perform biological functions. Sharpe (34) reports recoveries of slime moulds after CCD with viabilities in the range 90–95%. It should be noted that non-viable cells can be separated from viable cell by TLCCD. In charge-sensitive phases non-viable cells form a peak of low partition; the gradual shift of the partitioning of cell populations as a whole to lower partition which has been observed for a variety of cultured cell lines (35–37) is now attributed to the formation of non-viable cells which increases with time in culture.

3.4 Single tube partitioning

Generally in PEG–Dextran T500 systems cells partition between the top phase and the bulk interface. The partition ratio, also termed the partition coefficient, K, is defined as the ratio of the number of cells in the top phase to the number of cells in the bottom phase and in the interface. Partition behaviour is also frequently described by P, the number of cells in the top phase expressed as a percentage of the total cells present.

The proportion of cells in the top phase is independent of the volume ratio of the phases used. This is in contrast to the partitioning behaviour of soluble material, such as protein, where the partition coefficient (defined as the ratio of concentrations in each phase, as there is no interface partitioning for proteins) is independent of volume ratio. This is most probably a consequence of cell partitioning being between the interface and one phase (as described in Section 2.4).

Generally equal volumes of top and bottom phases are used, as the volume ratio influences the rate of phase separation. Phase systems can be set up by pipetting previously prepared top and bottom phases into glass tubes. Automatic pipettes can be used, provided phases are taken up carefully; the bottom phase is a little viscous. Since the shape of the settling chamber can influence the partition behaviour, it is sensible to keep to a standard size tube. Typical size tubes we have used for systems of 1 ml of each phase (2 ml systems) have been flat-bottomed glass tubes, 48 mm × 8 mm.

Cells can be added as packed cells. However, it may be useful to resuspend the cells in one phase, the PEG-rich top phase is preferable, and add to a volume of bottom phase. There is an upper limit to the cell number that can be loaded, and we have found 10^8 to be about the limit for the 2 ml systems. For very low number of cells, the addition of serum is suggested (38).

It is generally useful to cover the tubes with plastic film for mixing the contents, rather than use a plastic bung, as with the latter drops of phase and cells can remain trapped in the bung when the tube and contents are set to stand for phase separation to occur. These can drop down into the bulk phase at indeterminate times and alter the apparent partition coefficient.

If the partition is being used analytically to select partitioning conditions small samples of the top and bottom phases can be withdrawn for analysis. Because the dynamics of partitioning give rise to some heterogeneity in the phases, it is important that samples are taken from the same place in the tube; the centre of each phase. It is also advisable to use Hamilton syringes to remove samples as these do not perturb the phases system and easily pass through the interface. Positive displacement pipettes can also be used. If the partitioning is being used preparatively most of the top phase can be removed.

Protocol 2. Single tube partitioning

Equipment and reagents

- Flat-bottomed glass tubes 48 mm × 9 mm
- Hamilton syringe (50 μl)
- Water-bath set to 25°C
- Isolated top and bottom phases
- Washed packed cells

Method

1. Place the separated top and bottom phases in the water-bath and check that they reach 25°C.
2. Mix the phases thoroughly and replace them in the bath.
3. Pipette 1 ml of mixed bottom phase into each of three glass tubes using an automatic pipette.
4. Pipette just 3.5 ml of mixed top phase into a test-tube and add 35 μl of packed cells (10 μl packed cells/ml of phase) and mix gently. This is termed the load mix.
5. Add 1 ml of cell suspension in top phase to each of the three tubes containing bottom phase.
6. Cap with Parafilm and place in water-bath for 10 min.
7. At 2 min intervals, mix the tubes by gentle inversion (up to 60 times) for 1 min and return to water-bath.
8. After phase separation has proceeded for 20 min, remove from each tube triplicate 25 μl samples with a Hamilton syringe from the centres of the top and bottom phases and add to 10 ml of Isoton.
9. Add 25 μl from the cell suspension in top phase to 10 ml Isoton. Perform this in triplicate.
10. Count cells with a Coulter counter and calculate the mean of the triplicate samples.
11. Calculate percentage distribution of cells in top and bottom phases in each tube from:

$$\text{Cells in top phase (\%)} = \frac{(\text{number of cells in top phase sample}) \times 100}{(\text{number of cells in load mix}) \times 2}$$

$$\text{Cells in bottom phase (\%)} = \frac{(\text{number of cells in bottom phase sample}) \times 100}{(\text{number of cells in load mix}) \times 2}$$

12. Obtain the percentage of cells present at the interface by difference.
13. Calculate the mean ± SD of the triplicate partitions.

3.5 Countercurrent distribution

3.5.1 Conventional thin-layer CCD

i. Basic method

As polymer two-phase systems take time to separate, to be able to undertake multiple partition steps in an acceptable time, thin layers of phases are required. Conventional thin-layer CCD machines are based on the original design made by Albertsson in 1965 (39). Machines are manufactured by the University of Lund, Sweden (40) and the University of Sheffield, UK (41).

TLCCD machines consist of the rotors, in which the multiple partitions are performed, and a mechanical system that shakes the phases and performs the 'transfer' steps (see below). The rotors consist of two circular plates made of acrylic plastic, with 60 or 120 concentric cavities (*Figure 2*). The bottom plate remains stationary in the apparatus and the upper plate can rotate, so that its contents are transferred to be in contact with the contents of the adjacent bottom chamber. The upper plate has a hole for emptying and filling, and in operation this is closed by a plastic plate placed over the whole rotor.

In outline, the procedure is to load all the chambers with bottom phase (the volume required is discussed below), then add upper phase to all chambers except the early chambers which receive sample prepared in upper phase. Multiple partition steps are carried out automatically by the unit performing a repeated sequence of steps on the rotor:

(a) Shaking; to mix the phases.

(b) Settling; a period of no shaking during which the phases separate and the cells undergo partition between the phases and the interface.

(c) Transfer; each top phase is moved to the next bottom phase by the top plate rotating.

This cycle is continued for as many transfers as are required up to the maximum determined by the number of cavities in the rotor.

The rotor is unloaded (see below) by attaching a fraction unloader to the plates, which consists of plastic tubes that sit over the holes in the top rotor. Inverting the rotor permits the contents of each chamber to drain into the tubes. Each fraction can then be analysed for contents. A CCD profile is obtained by plotting the number of cell in each fraction against the cavity (fraction) number.

The separation required can then be optimized by adjustment of the CCD conditions and/or the phase composition. Single tube partitions give some indication of how the bulk population of cells will behave in CCD. For example, cells that favour the top phase will, naturally, appear in the fractions of highest number, to the right in the profile. However, since parameters such as phase volume and chamber shape can influence partitioning, optimization of CCD separations is achieved in the CCD machine itself, by performing several CCD runs.

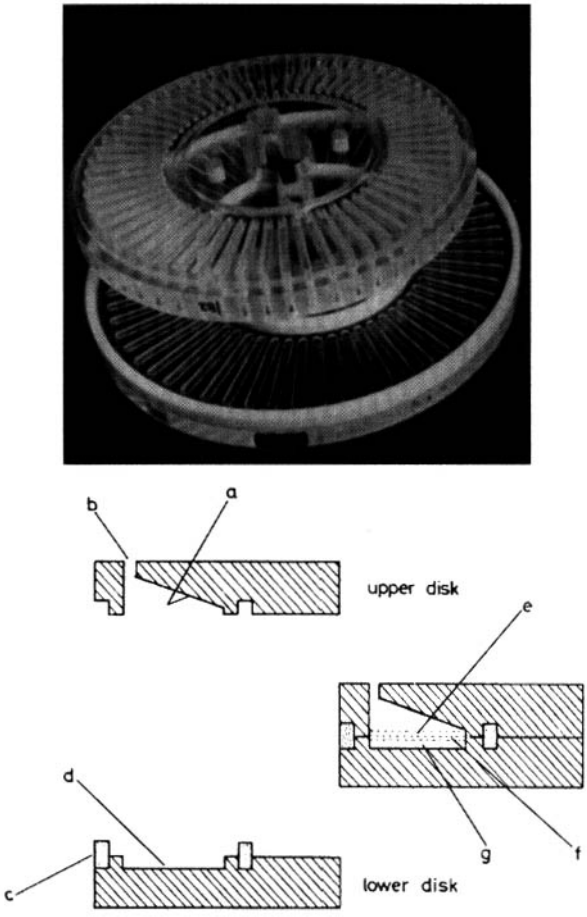

Figure 2. TLCCD rotor. The photograph shows a 60 chamber rotor (Bioshef), with the upper half of the rotor inverted to show the cavities. The cross sections show: (a) sloped upper roof of chamber to facilitate emptying; (b) filling and removal hole; (c) removable nylon rings to allow contact surfaces to be lapped on a granite surface plate as required; (d) lower disk cavities. In the assembled unit in operation with phase (e) top phase; (f) position of the interface; (g) bottom phase. Taken from ref. 41.

If in a cell population there is a low partitioning subpopulation of interest (i.e. on the left-hand side of the profile), the position of the main peak can be adjusted to be to the right (high partitioning side) of the profile, so that the low partition components are spread over a greater range of fractions. If there were two major components to separate, the optimum would be achieved by having them located symmetrically on opposite sides of the centre of the profile.

For cells that require sterile conditions the plate can be wiped with alcohol (but not left standing in alcohol). The phase systems can be sterilized by filtration.

The unit generally operates in a cold room, or in a cold cabinet, although runs at room temperature can be undertaken if required.

ii. Important points of choice

Position of the interface

The volume of the bottom phase used is a very important selection. The volume of the chambers of the bottom rotor are about 0.7–0.9 ml depending on the source of manufacture. You must expect that each rotor will be different and take account of this. Suppliers provide the volumes of bottom chambers.

If the volume of bottom phase added to the rotor is equal to the bottom chamber volume, the interface between the phases once they have settled at the end of the settling stage will coincide with the junction between the two plates. Consequently at transfer all the top phase will be moved to the next chamber, and, most importantly, any cells that had accumulated at the interface on the top phase side will also be transferred. Since partition as frequently encountered is between the top phase and the interface, such an arrangement will give little net partition; the peak of cells obtained will be very much to the right of the profile (in fractions of high number). It is therefore important not to fill the bottom chamber completely with bottom phase. You can select to fill the bottom chamber 90% (or some other proportion) with bottom phase. At the transfer step some of the top phase will be left behind but this will permit cells at the interface not to be transferred. The volume conditions selected, both the volume of the bottom and the top phases, will be an important parameter of the CCD conditions.

Cell load

Typically 10^8 cells of diameter 10 μm can be loaded into one cavity. For larger cell loads, more of the early cavities can be loaded; for example, the first six cavities in a 60 chamber rotor have frequently been loaded with cells in the extensive CCD studies performed by Walter and colleagues (8, 42, 43).

Unloading

To unload the cells an appropriate buffer can be added to all the chambers. This will dilute the polymers so that a single phase system is obtained (this is referred to as 'breaking the phase system'). It is then much easier to unload the chambers.

However, unloading the chambers without breaking the phases can be of use if you want to check the partition coefficient through the profile as an index of homogeneity/heterogeneity. To do this, the phases are emptied directly into the test-tubes without any buffer being added. The K of the cells

in each fraction is then determined by remixing the fractions, taking a sample for total cells, and after permitting phase separation, sampling both top and bottom phases to determine K.

iii. Distribution curves

The distribution of material between the phases is described by K, the partition coefficient, where:

$$K = c_t/c_b$$

where c_t is concentration in top phase and c_b is concentration in the bottom phase.

The partition ratio G is the ratio of the amount of material in the top phase to the amount in the bottom phase:

$$G = \frac{c_t \cdot V_t}{c_b \cdot V_b} = K \cdot \frac{V_t}{V_b}$$

where V_t and V_b are the volumes of the top and bottom phases respectively.

In describing the partition behaviour of material in CCD by theoretical CCD curves (41), a parameter P is used. P is the fraction of the total amount of material in a chamber that appears in the top phase (and is transferred at each step):

$$P = G/(G + 1)$$

and the fraction of the total population appearing in the rth cavity, $F(r)$ is given by the binomial distribution:

$$F(r) = \frac{n!}{r!(n-r)!} Pr(1 - P)^{n-r}$$

where n distributions are carried out (i.e. $n - 1$ transfers).

CCD profiles of 60 distributions are shown in *Figure 3*. The position in the profile is determined by K and the volume ratio. The location of a peak r_{max} is given by:

$$r_{max} = nG/(G + 1)$$

for n fractions, obtained by $(n - 1)$ transfer steps.

Conversely the position of a peak in the CCD profile can be used to calculate G from:

$$G = r_{max}/(n - r_{max}).$$

For a homogeneous population of cells, the peak should be described by the calculated theoretical curve; any broadening indicates lack of homogeneity. As a check, the curve can be compared with that obtained by running a soluble dye, which will behave ideally (41). A simpler test for homogeneity is to measure K through the profile, by remixing the unbroken phases in each fraction and determining K.

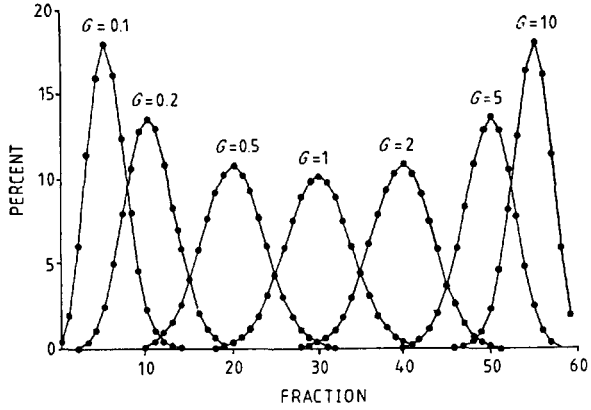

Figure 3. Theoretical curves for a 60 transfer CCD having different values of partition ratio *G*. Calculation has been made for a phase ratio of one, so $G = K$. Note that materials with *G* values other than one produce asymmetric peaks to the left or right of the centre, which are skewed towards the centre. Taken from ref. 41.

A computer program developed by Blomquist and Wold (44) has been used to resolve profiles into component curves (11, 33). More recently simulated CCD curves have been used to determine the feasibility of cell separations by CCD based on values of *K* determined in single tube partitions (17).

The most extensive TLCCD studies with non-charge-sensitive phase systems have been performed on slime mould populations (see Section 4.2.1). With charge-sensitive phase systems, apart from the extensive studies by Walter on erythrocytes (8, 43), the lymphocyte subpopulations have been most extensively examined (Section 4.2.4). *Figures 5–7* in Section 4.2 illustrate how each of the types of phase systems can provide broad cell distributions which reveal the heterogeneity of the populations. Spreading the profiles across the fractions is important if subpopulations are to be revealed.

Protocol 3. TLCCD of haematopoietic cells from bone marrow (45)

Equipment and reagents

- Biosheff TLCCD machine with 60 chamber rotor (bottom chamber volume 0.6 ml)
- Repeat pipette (Gilson Repetman, Anachem)
- Mononuclear cells isolated from bone marrow with Lymphoprep (density 1.077)
- Isolated top and bottom phases of a 5% Dextran T500–3.4% PEG 8000 phase system prepared in 0.01 M sodium phosphate buffer pH 7.4, 0.12 M NaCl, 5% fetal calf serum (FCS) at 4°C

Method

1. Isolate mononuclear cells free of the majority of mature granulocytes and erythrocytes from human bone marrow (10 ml) by overlaying

Lymphoprep (7 ml), centrifuging at 400 *g* for 40 min at room temperature, and collecting the cells from the interface.

2. Wash cells twice in 0.12 M NaCl, 0.01 M sodium phosphate buffer pH 7.4 supplemented with 10% FCS and 1 U/ml heparin by centrifuging cells (3000 *g* for 6 min) at room temperature. Resuspend pellet using a syringe fitted with a 19 gauge needle to disperse any cell aggregates.
3. Resuspend in top phase at 1–5 $\times$ 10^7 cells/ml and pass cell suspension through a 0.22 μm filter needle as an additional precaution against cell clumping.
4. Pipe a thin layer of paraffin wax around the external and internal rims of the top CCD rotor to aid sealing, place rotors on CCD machine at 4°C, and complete assembly.
5. Pipette 0.6 ml of bottom phase into all the chambers via the entry port on the top rotor using the repeat pipette. Take care, particularly with the more viscous bottom phase, to thoroughly pump through each phase before dispensing to ensure the removal of air bubbles within the repeat pipette.
6. Pipette 0.6 ml of cell suspension into chambers 1–3 and 0.8 ml of top phase into chambers 4–60.
7. Close the top rotor entry ports by clamping the plastic clamping ring on to the top rotor.
8. Set controls for a shaking time of 25 sec and settling time of 7 min; perform CCD operation (about 8 h).
9. Add 1 ml PBS to each chamber. Shake rotor on the machine for 1 min to mix and break phase system.
10. Unload fractions by clamping on the fraction collector (plastic ring holding 60 plastic LP4 tubes) and invert the rotor so that the chamber contents drain into the tubes.
11. Count cells in the fractions with a Coulter counter.

3.5.2 Manually operated CCD

Standard TLCCD techniques for cells involve exposure of cells to phase for up to six hours. This might be a problem for certain cell types. For example neutrophils are short-lived cells that are very sensitive to excessive manipulation and environmental change. Exposure of isolated neutrophils to phase reagents for four to five hours led to gradual morphological deterioration of the cells with evidence of degranulation in many cells (46). We therefore developed a simple manual CCD method which allows neutrophils to be fractionated by CCD within two hours, with retention of morphology and function (46–48). The apparatus can be made in the workshop relatively easily, in contrast to conventional TLCCD apparatus which requires considerable skill, experience,

and cost. It is described in Section 6 together with a protocol for its use with neutrophils (*Protocol 5*). Results of cell fractionation studies using this apparatus are described in Section 4.2.3.

3.5.3 Centrifugal CCD

A fully automated CCD apparatus has been developed in which the speed of phase separation is increased by a centrifugal stage being employed (49–51). This has proved useful for sperm cell separations as CCD separations and subfractionations can be achieved rapidly (see Section 4.2.5).

4. Selected applications

4.1 Immunoaffinity cell extractions

The principle of immunoaffinity cell partitioning is that a PEG–antibody interacts specifically with a target cell population and these PEG–antibody coated cells are then selectively removed from the bulk population by their preferential partition to the PEG-rich phase of a PEG–dextran biphasic system.

The steps involved are shown schematically in *Figure 4* (17) and consist of:

(a) Production of the PEG-modified antibody.

(b) Incubation of the PEG–antibody with the cell mixture.

(c) Positive selection of the target cells into the PEG-rich phase by partitioning in an appropriate two-phase system:

   i. By single tube extractions.

   ii. By CCD.

(d) Recovery of cells.

A suitable non-charge-sensitive phase system is first selected in which cells 'just partition to the interface'. Addition of PEG–antibody will move the antigen-bearing cells into the top phase. Increasing the polymer concentrations will ensure that all cells in the population are 'tightly associated with the interface'. Although this will decrease the top phase partition of 'unwanted' cells, it will also require the binding of more PEG–antibody to extract antigen binding cells into the top phase. Thus a balance needs to be achieved in selecting the polymer composition of the phase system. Furthermore, although virtually all the candidate cells can be extracted into the top phase by this method, and the contaminating cells can be adjusted to have very low partition (e.g. 1%), it is important to appreciate that when a population of very low abundance is being extracted, these will still have substantial contamination with the bulk cells, albeit very much less than originally. A CCD step will then completely purge this preparation of the contaminating cells.

The PEG–antibody is readily prepared *in situ*, but it is necessary to quench

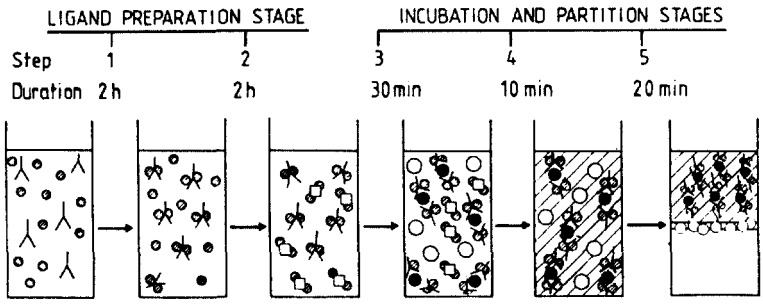

Figure 4. Procedure for immunoaffinity cell partitioning by bulk extraction. Step 1: monomethoxyPEG is linked to antibody using tresylated monomethoxyPEG (TMPEG). Step 2: excess TMPEG is inactivated by incubation with bovine serum albumin. Step 3: cell mixture is added and incubated for 30 min. Step 4: cells are isolated by centrifugation and resuspended in top phase. Step 5: bottom phase is added and the system mixed and allowed to phase separate for 20 min. The cells that do not interact with the PEG–antibody remain at the interface whereas cells that do interact are recovered in the PEG-rich top phase. Taken from ref. 17.

the excess PEGylating agent, for example by adding BSA to provide a molar excess of lysine per molecule of activated PEG. There are many activated PEG derivatives to couple PEG to proteins (52), and many involve reaction with primary nitrogens at the N terminus and on lysyl side chains. We have favoured the tresylate of monomethoxyPEG (TMPEG) (53). A range of activated PEGs is available commercially from Shearwater Polymers, Inc., and from Sigma. *Protocol 4* shows a procedure for extracting cells in a model system of human red cells present at 1% abundance with HL-60 cells which serves to illustrate the application of this method (15, 18).

Protocol 4. Immunoaffinity cell extraction (17, 20)

Reagents

- Cell mixture of human red cells (1%) and HL-60 cell (99%)
- Anti-human red blood cell IgG (α-HRBC) (Dako)
- Tresyl monomethoxyPEG (TMPEG) (Shearwater Polymers, Inc., or Sigma)
- Isolated top and bottom phase of 4.75% Dextran T500 and 4.75% PEG 6000 phase system in 0.15 M NaCl buffered with 0.01 M sodium phosphate pH 6.8
- PBS: 50 mM sodium phosphate buffer pH 7.5, containing 125 mM NaCl

Method

1. Prepare TMPEG in PBS (86 mg/ml) immediately before use to minimize hydrolysis.
2. Mix 0.5 ml of TMPEG solution with 0.5 ml α-HRBC (3.25 mg/ml in PBS) and incubate for 2 h at room temperature with gentle mixing on a rotary mixer. The PEG–antibody can be stored at 4°C until required.

Protocol 4. Continued

3. Prior to use, 'quench' the excess TMPEG by adding 1 ml of BSA in PBS (84 mg/ml) and incubate for a further 2 h.
4. Dilute 0.5 ml of this preparation with 0.5 ml of PBS to provide a solution of 5.5×10^{-9} mol of PEG–α-HRBC.
5. Incubate 1 ml of cell suspension in PBS (2.75×10^7 cells) with 1 ml of PEG–α-HRBC (5.5×10^{-9} mol) for 30 min at 37 °C.
6. Collect the cells by centrifugation (400 *g* for 10 min) at 4 °C, and resuspend in 5 ml of top phase.
7. Mix with 5 ml of bottom phase in a Universal tube. Invert gently 60 times and then allow to phase separate for 20 min at 25 °C in a water-bath.
8. Remove top phase and retain. (It contains about 50% of the red cells and about 3% of the HL-60 cells.)
9. Add 5 ml top phase to bottom phase, mix, phase separate, and remove top phase.
10. Repeat step 9.
11. Unite top phases and isolate cells by centrifugation. About 88% of the red cells will have been recovered with only about 9% of the original contaminating cell population giving a purity of about 50%.
12. Use 30 transfer CCD to completely separate erythrocytes from HL-60 cells (yield 88% with 100% purity). In the absence of CCD resuspend cells in 5 ml of top phase and repeat sequence of bulk extraction (steps 7–11) two more times. (Yield of red cells is about 67% with a 91% purity.)

Using a primary antibody positive selection of human erythrocytes from model mixtures with sheep (12) and rabbit (13) erythrocytes using CCD have been obtained. Double layer systems of secondary antibodies (16) and protein A (15, 21) linked to PEG, and PEG–avidin and biotinylated antibodies (22) have been used as general affinity ligands for the extraction of cells selectively coated with primary antibody. Strategies for bulk extraction of cells present at low abundance have been devised (17). However, most examples of immunoaffinity cell partitioning reported have used target antigens present at about 500 000 copies per cell. It appears that 80 000 is the minimum number of receptors needed to provide a sufficient PEG coat to be of use (54). Covalent attachment of polylysine to the antibody will increase the number of sites that can be PEGylated and this has been suggested as a means of increasing sensitivity (20). Polyacrylamide-derivatized antibodies, which affect immunoaffinity partition to the dextran-rich bottom phase have been used to improve the partitioning change per bound antibody (19).

4.2 Fractionations of cells into subpopulations

Although CCD can be used to separate cell populations, by far the most interesting and useful aspect of CCD and cell partitioning is the ability of the method to subfractionate cell populations, which may by other methods appear homogeneous. In addition, the advantage of the partitioning technique lies in the fact that it does not require any specific knowledge on the nature of the cell surface changes; for example much has been learned of the surface changes that accompany the growth and development of slime moulds despite any of the surface molecules involved in the changes being identified (34). Furthermore TLCCD has been used to detect small differences in the surfaces of intact cells that arise from the ectopic expression of the murine *Hox-3.3* gene (55).

Simple tests can reveal heterogeneity in the cell populations:

(a) Measurement of the partition ratios of the fractions comprising the peak is informative. The contents of the CCD cavities are emptied directly into tubes without the addition of isotonic medium to 'break' the phase system. The tubes are then mixed and the cell partition determined. An increase in the partition ratio through the profile indicates that the population has been subfractionated whereas if it does not change through the profile the population is homogeneous, and has not been subfractionated, under these conditions.

(b) The fractions from the left of the CCD profile are pooled and subjected to a second CCD. Similarly fractions from the right of the profiles are pooled and rechromatographed. If these new profiles are still to the left and right of each other respectively, then the population was heterogeneous. If, however, the peaks on second CCD coincide, then clearly the fractions from each side of the first CCD do not differ in partition behaviour, and hence the population is not heterogeneous under these conditions.

(c) Differences in functional properties of the cells through the profile also serve to show that the population is heterogeneous.

(d) Very small differences in the partitioning behaviour of two closely related cell populations (such as cultured cell sublines, red blood cells from different individuals) can be detected by a modified method of CCD. One population of cells is labelled with an appropriate radioactive isotope, mixed with an excess of the second population, and the sample subjected to CCD. A displacement in the profile of radioactivity from the profile of the main population indicates that the two populations have different surface properties. Control experiments are required to demonstrate that each population, labelled and unlabelled, has CCD curves which are coincident. Using this method, surface differences both charge associated and non-charge related, between any two sublines of K-562 cells, a human leukaemia cell line, have been observed (56).

Partitioning has been used to subfractionate cell populations and to trace surface alterations that occur as a function of normal or abnormal *in vivo* processes or *in vitro* treatments. When reviewed in 1985 (42) these included:

(a) Reticulocyte maturation and erythrocyte aging in the peripheral blood.

(b) Erythrocytes from disease states.

(c) Subfractionations of lymphocytes into subsets having different biological activities and the heterogeneity of these subpopulations.

(d) Subfractionation of peripheral blood monocytes having different phagocytic properties.

(e) Surface heterogeneity associated with differentiation and growth of cells in culture.

(f) Fractionation of metastatic cell populations into metastatic variants.

Since then there have been a number of developments which are discussed below. *Table 1* summarizes cell types that have been examined by partitioning.

4.2.1 Surface changes during the growth and development

An extensive study by Sharpe and colleagues on the slime moulds (*Dictyostelium discoideum* and *Physarum polycephalum*), principally by TLCCD

Table 1. Cell types separated in aqueous two-phase systems

Cell	Publications prior to 1984/85[a]	Publications since 1985[b]
Red cells	8, 42, 90	12–18, 43, 91–104
Reticulocytes	8, 42, 90	43, 54, 98, 105
Bone marrow cells	8, 42, 90	106–109
Neutrophils	8, 42, 90	46–48, 69
Spleen cells	8, 42, 90	
Lymphocytes	8, 42, 70–77, 90	78–80
Monocytes	8, 42, 90	43
Liver cells	8, 42, 90	
Epithelial cells	8, 42, 90	43
Mast cells	8, 42, 90	
Cell lines	8, 42, 90	43, 56, 65, 110
Metastatic cell lines	8, 42, 67, 68, 90	
Testis cells	8, 42, 90	
Spermatozoa		51, 84–89, 111
Chlorella	8, 42, 90	112
Bacteria	8, 10, 42, 90	113
Algae	8, 42, 90	
Slime moulds	8, 31, 42, 57–59, 64, 90, 114, 115	11, 61, 116
Pollen	117, 118	

[a] Over 70 papers cited before 1984/85 can be found in the 1985 reviews (8, 42, 90) and papers cited in this chapter.
[b] Over 50 papers have been published since 1985.

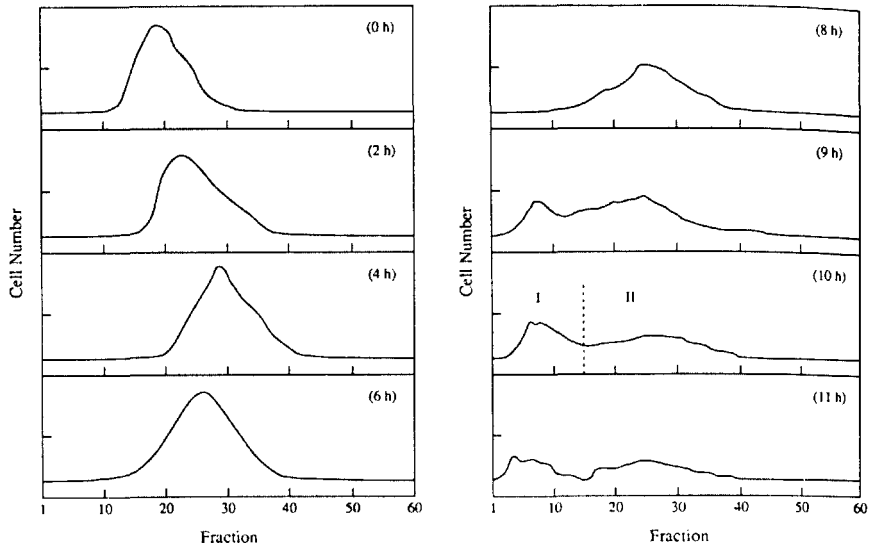

Figure 5. TLCCD of *Dictyostelium discoidem* in a non-charge-sensitive phase system during development. Peaks I and II contain predominantly prespore and prestalk cells respectively. Taken from ref. 57.

in non-charge-sensitive phase systems, has detected surface changes that can be correlated with developmental potential (34, 57–62). *D. discoideum* showed a progressive increase in surface hydrophobicity during growth, which may be due to loss of cell surface carbohydrate residues (58). TLCCD can also fractionate amoebae on the stage of cell cycle (59, 60). Developmental changes have also been detected (59). Non-charge-sensitive phase partitioning has also detected surface differences between *Physarum* amoebae with different mating types (61, 62).

Figure 5 shows how surface changes of *D. discoideum* during development are revealed by TLCCD in non-charge-sensitive phase system (5.5% (w/w) Dextran T500–5.5% (w/w) PEG 4000 in 0.5 M NaCl, 10 mM potassium phosphate buffer pH 7.8). With time the population resolves into two broad peaks (I and II) which contain cells that are predominantly prespore and prestalk respectively (57).

4.2.2 Cell growth

i. Cell cycle

Heterogeneity detected by TLCCD can arise from cells being in different parts of the cell cycle. With charge-sensitive partition there is a tendency for cells in G_0, G_1, and early S to decrease, and cells in late S, G_2, and M to increase, with increasing partition ratio (63). In non-charge-sensitive phases, amoebae on the left side of the profile (lowest partition, lowest hydrophobicity) are predomi-

nantly in G_1, cells in the centre of the peak are mainly in G_2, whilst cells on the right-hand side of the peak (highest K and highest hydrophobicity) are in S (60, 64).

ii. Viable and non-viable cells

CCD can discriminate between viable and non-viable cells. For example charge-sensitive partitioning of K-562 cells grown in suspension culture shows a change from a single peak of viable cells to two peaks, in which the second, lower partitioning peak consists of non-viable cells. Its proportion increased with the culture time (65). In non-charge-sensitive phases sperm that are non-viable appear also in peaks to the left of the CCD profile (66).

iii. Metastatic variants

TLCCD of metastatic variants of cell lines in charge-sensitive phases have higher partitions than non-metastatic parental lines (67, 68) and CCD has been used to monitor the drift in cell surface properties as cells became more metastatic on prolonged cell culture. In addition it has been possible to subfractionate metastatic variants from the parental cell line (67).

4.2.3 Functional subpopulations of human neutrophils

Isolated human blood neutrophils have been separated into fractions by CCD in a manually operated CCD unit using 20 transfer in a charge-sensitive phase system of 5% Dextran T500 and 6.5% PEG 6000 in 0.1 M sodium phosphate buffer pH 7.2 at 21 °C (*Figure 6*). The neutrophils separated as a broad profile, which reflected heterogeneity in surface charge, as there was a progressively increasing cell surface negative charge through the profile, demonstrated by analytical cell electrophoresis on each fraction. Functional assays of chemotaxis, phagocytosis, and respiratory burst showed an inverse relationship between these functions and surface charge, often with a two- to threefold difference in properties across the profile (48, 69).

4.2.4 Lymphocyte subpopulations

CCD in charge-sensitive phase systems has been used to separate the major lymphocyte subpopulations of man (70–80), rats (81, 82), and mice (83). B lymphocytes have a lower affinity for the top phase (low partition ratio), T cells have an intermediate partition ratio, and null cells have a high partition ratio (*Figure 7*).

Using a phase system of lower electrostatic potential, obtained with 150 mOsm sodium phosphate pH 7.4 and 130 mOsm NaCl, the higher partitioning fraction has been examined in more detail (78). It contained virtually all the large granular lymphocytes and the natural killer lymphocytes of the unseparated lymphocytes.

The T cell population has also been examined in detail using a phase system of intermediate potential (200 mOsm sodium phosphate buffer pH 7.4, 70 mOsmol NaCl) (79). The helper/inducer T cell subset (OKT4/Leu3$^+$) had a

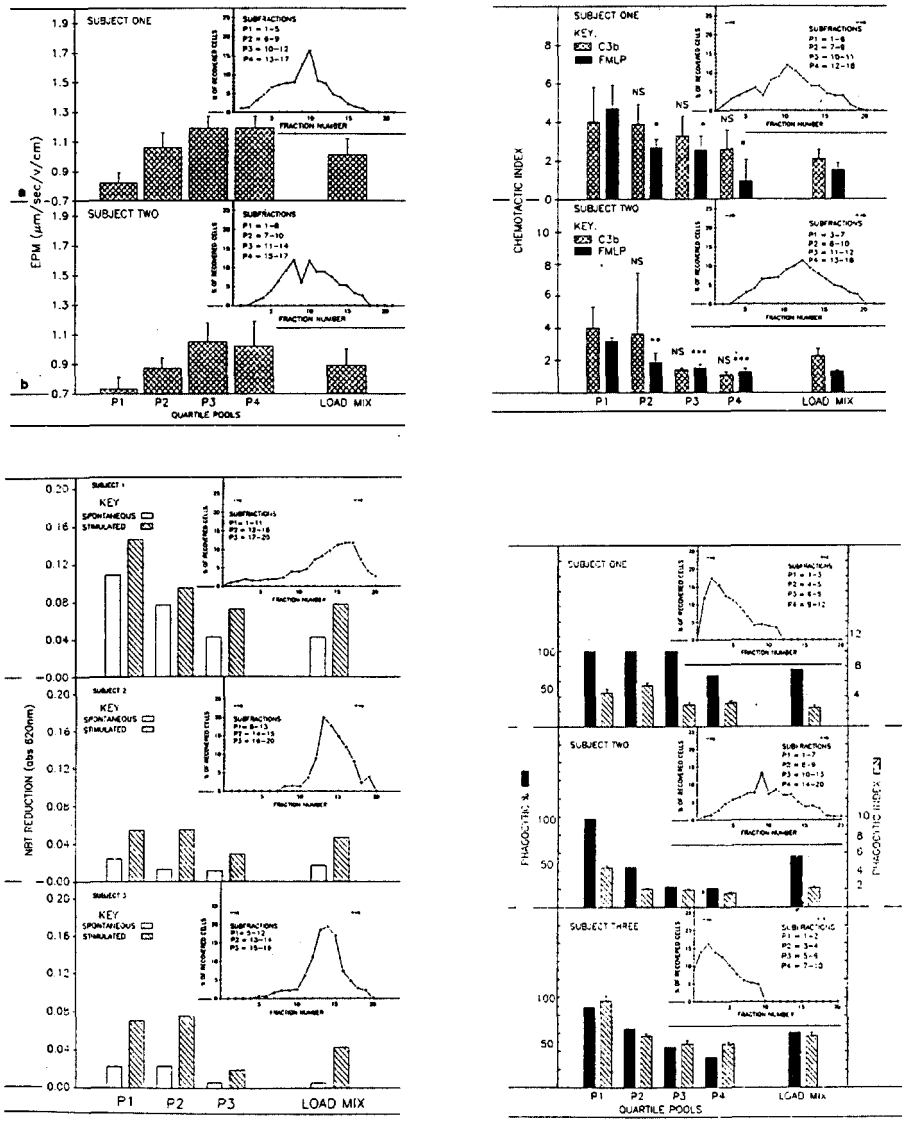

Figure 6. CCD of human neutrophils in a charge-sensitive phase system. Cells were separated by 20 transfer in a manually operated CCD machine and pooled into four subfractions of equal cell number. The insets above each histogram show the complete separation profiles and indicates the pooling. The fractions were examined for electrophoretic mobility (top left); chemotaxis using two chemoattractant (top right); resting and stimulated respiratory burst (bottom left); and phagocytosis (bottom right). Reproduced from ref. 48.

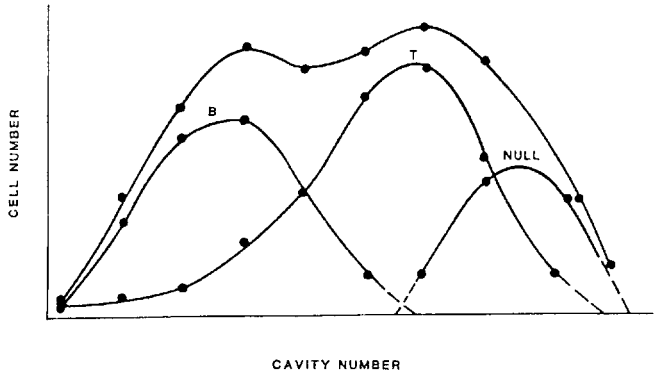

Figure 7. Schematic diagram of the distributions of lymphocyte subpopulations (from young B/W mouse spleens) constitute the CCD curve obtained in a charge-sensitive phase system for the entire cell population. Taken from ref. 83.

single distribution curve with a lower partition ratio, whereas the suppressor/cytotoxic (OKT8/Leu2$^+$) subset was heterogeneous giving two peaks, indicating two major subsets of the OKT8 T cell subpopulation which differ in charge-associated surface properties. One of these had surface properties, as reflected by its partitioning behaviour in the system used, related to those of OKT4/Leu3$^+$ cells, while the other had partitioning characteristics similar to those of NK cells. A majority of the Leu2$^+$ cells with a high partition co-expressed an NK marker whereas the Leu2$^+$ cells with the lower partition ratio were depleted of cells with both markers.

4.2.5 Sperm subpopulations

As sperm pass through the epididymis and into the vas deferens they are known to undergo a number of different surface changes associated with the acquisition of fertilizing capacity. CCD has been used to follow the changes in sperm surface properties as they mature. Heterogeneous surface properties have been revealed in non-charge-sensitive phases (51, 84–89). Some separation of X and Y sperm has been observed (88).

The experimental drawback of CCD of sperm is that the long times required for phase separation at unit gravity may increase cell death during this separation process. Heterogeneity in sperm populations have been associated with different viability states of the fractionated cells; loss of semen viability appears to result in a decrease hydrophobicity of the cell surface (89). Centrifugal CCD has been used to shorten the separation time (51).

5. Conclusions

Although the principles of phase partitioning for cell separations are relatively simple, the technique requires some experience and determination to apply.

Typically the applications have come from groups with a prime interest in the methodology, and collaborations of such groups with groups with experience of particular cell systems. Contact with experienced partitioning groups is advised; this can cut many corners, prevent 'many wheels being re-invented', and access CCD facilities. To this end advantage can now be taken of the extensive, virtually complete bibliography compiled in 1985 (90). This has since been up-dated by Dr Harry Walter and comprehensive bibliographies (1956–1984; 1985–1990; 1991–present) are now on-line in the Internet at:

`http://www.as.ua.edu/rdrogers/aq2phase`

In addition, this internet site has information on conferences and addresses of phase partitioners worldwide, thereby providing ease of contact with experienced workers in the field.

6. Manually operated countercurrent distribution apparatus

I. A. SUTHERLAND, P. EGGLETON, and D. FISHER

6.1 Introduction

To obtain cell separations or fractionations by partitioning in aqueous two-phase systems, multiple partitions are required and these are carried out by countercurrent distribution (CCD) (1–3). Aqueous two-phase systems separate much more slowly than the aqueous–organic two-phase systems employed in countercurrent chromatography and therefore, to obtain settling times for phase separation that are not excessively long, thin layers of phase are used in specifically designed thin-layer countercurrent (TLCCD) apparatus (39, 40).

TLCCD machines consist of the rotors, in which the multiple partitions are performed, and a mechanical system that shakes the phases and performs the 'transfer' steps. The rotors consist of two circular plates made of acrylic plastic, with 60 or 120 concentric cavities into which the phase system is loaded. The shape of these cavities is designed to provide thin layers of phase. In CCD operation the bottom plate remains stationary in the apparatus and the upper plate is rotated so that the contents of each top chamber are transferred to be in contact with the contents of the adjacent bottom chamber. Fuller details of construction and use are given in earlier sections of this chapter.

However, these machines are large, not portable, and require considerable skill, experience, and cost to construct. In addition standard TLCCD techniques involve exposure of cells to phase for up to six hours, and also rather

vigorous mixing (see Section 3.5.2). This might be a problem for certain cell types.

We have therefore developed a simple manual CCD method which allows cells to be fractionated by CCD within two hours under gentle conditions. The apparatus can be made in the workshop relatively easily. It has been used for the fractionation of neutrophils by CCD within two hours into functional subpopulations, with retention of activity and function (46–48, 69).

6.2 Description of the apparatus

The apparatus is shown in *Figure 8*. It consists of a top and bottom circular Perspex rotor (diameter 16 cm) each with 30 holes drilled through them, arranged around the circumference. The bottom rotor is placed on a steel base plate, thereby providing 30 chambers. It is relatively thin (1 cm), and the volume of the chambers is 0.7 ml. The chambers are numbered 1–30. The top rotor is relatively thick (5 cm) and the chambers have a volume of 3.5 ml. The top rotor is sealed over the bottom plate with paraffin wax and is held in place by a central shaft which has an aluminium base and top. The upper ends of the top rotor chambers are closed by a stainless steel plate. This and the top rotor are held in place over the bottom rotor by a bearing and clamp nut connected to the central shaft. The nut is tightened just sufficiently for the top rotor to be rotated by hand over the bottom rotor.

Bottom phase is loaded into all the chambers. Top phase is then added to chambers 2–30, with a cell load in top phase being placed in chamber 1. To ensure that any interface material is not transferred with the top phase, the volume of bottom phase is selected to be less than the bottom chamber volume. Typically 0.65 ml of bottom phase is loaded followed by 0.65 ml of top phase. This allows 0.05 ml of top phase to be left behind in the bottom chambers at each transfer, so that any interface material is not transferred with the top phase.

The settling chamber is very thick and in the vertical position phase separation would take a long time (about 20 min). However, by placing the unit on its side thin layers are achieved which permit shorter settling times to be used. Bringing the unit back to the vertical permits the transfer steps to be performed. The unit is easily unloaded by firmly inserting plastic test-tubes (1.1 × 7.5 cm) into the entry ports and inverting the unit. The tubes may need slight grinding at the top to provide a bevelled lip.

We have used the unit only at room temperature but it could easily be used in a cold room, or with a pre-cooled unit a fridge could be used for the settling period.

The unit has several advantages over the conventional TLCCD:

(a) Mixing is very gentle and this may be of advantage with fragile cells.

(b) The unit is portable. It is easier in collaborative studies to take the unit to a laboratory that has specialized cells and cell assays, rather than establish these in one's own laboratory.

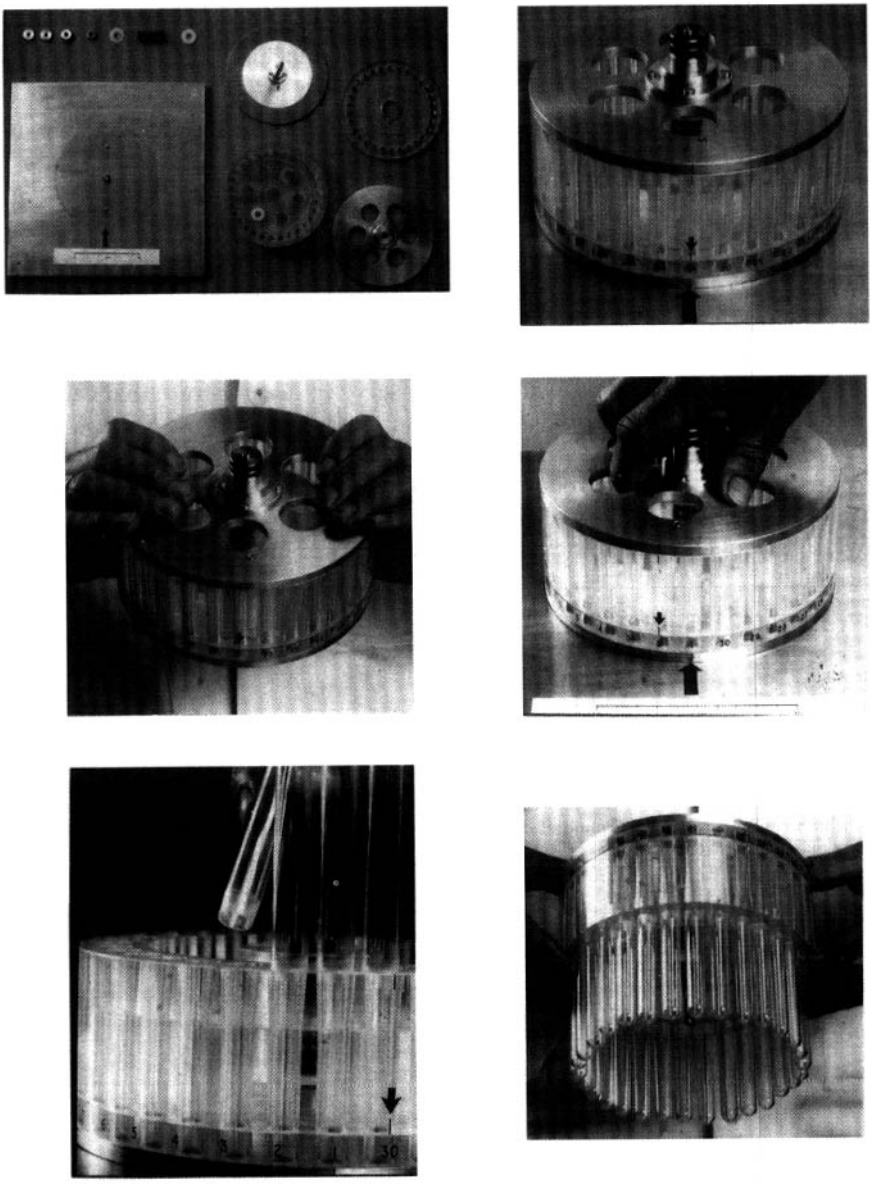

Figure 8. Hand operated CCD apparatus. Top left: the components (the scale is 10 cm). Top right: the assembled unit with the top rotor in the initial position (note arrows). Centre left: the unit with phases is shaken (mixing step). Centre right: after the phases have separated by placing unit on its side (settling step, not shown), the unit is returned to the vertical position and the top rotor turned one chamber (transfer step, note arrows). Bottom left: at the end of the CCD (note arrows), unloading tubes (with bevelled edges) are placed in the top of the top rotor. Bottom right: the phases and fractionated cells are unloaded by inverting the unit.

(c) The unit is very compact and can be manufactured in one's own workshop.

(d) It is cheaper than a conventional TLCCD machine and is suitable for feasibility studies that if successful would justify a TLCCD apparatus.

(e) We have employed much shorter operation times than those used with conventional TLCCD.

6.3 Applications

We have used this apparatus principally for the subfractionation of human neutrophils (46–48, 69). These are short-lived cells that are sensitive to excessive manipulation and environmental change. As such they represent as stringent test of the apparatus. In addition, neutrophils can be examined by a battery of functional assays, which in turn serve to demonstrate that recovery of functional cells from the CCD separation is possible (see also Section 4.2.3). *Protocol 5* describes the use of the apparatus for subfractionating neutrophils at room temperature, which has general application to white cell populations. Details of the preparation of aqueous two-phase systems containing polyethylene glycol (PEG) and dextran are given earlier in this chapter.

Protocol 5. Subfractionation of leucocyte populations in manually operated CCD

Equipment and reagents

- Hand-held CCD unit
- Plastic test-tubes (1.1 × 7.5 cm) suitably ground at end
- Human neutrophils resuspended in top phase at about 10^8 cells/ml
- Isolated top and bottom phases of 5% Dextran T500 and 6.5% PEG 6000 in 0.1 M sodium phosphate buffer pH 7.2, prepared at 21°C
- Hank's buffered salt solution (HBSS)

Method

1. Pipe a thin band of paraffin wax round the external and internal rims of both sides of the bottom rotor and place it on the steel base plate.
2. Fill the bottom chambers with 0.65 ml of bottom phase.
3. Position the top rotor over the bottom rotor.
4. Load 0.65 ml of packed cells resuspended in top phase (10^8 cells/ml) into chamber 1.
5. Pipette 0.65 ml of top phase into chambers 2–30.
6. Place plastic washer, metal spring, and second plastic washer over the central columns and tighten the spring to effectively adhere the rotors.
7. Place steel plate over the top rotor and screw down firmly.
8. Invert the unit gently 15 times to thoroughly mix the phases.
9. Place the unit on its side for 2.5 min.

10. Bring the unit back to the vertical carefully.
11. Rotate the top rotor clockwise one cavity.
12. Repeat the mixing, settling, and transfer steps for the desired number of transfers (maximum is 29).
13. Remove the overlying steel plate to reveal the entry ports.
14. Add 0.6 ml of an appropriate salt solution (e.g. HBSS) to break phase system and permit better cell recovery.
15. Fit small plastic test-tubes into the entry ports and invert the unit so that the fractions drain into the tubes.
16. Count cells in fractions in Coulter counter and perform functional assays on fractions.

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5

Separation of cells by flow cytometry

M. G. ORMEROD

1. Introduction

A flow cytometer uses a flow system to deliver cells in single file past a point of measurement. Light is focused at this point and the fluorescence and the light scattered by the cells is collected and measured.

Flow cytometers measure several parameters on single cells at rates between hundreds to thousands of cells per second. The method can be used to define and to enumerate accurately subpopulations. Once identified, such subpopulations can be physically sorted for further study.

Typically, five parameters might be measured on 20000 cells. Using blue light for excitation, one might record green, orange, and red fluorescence, and blue light scattered in a forward direction and at right angles to the laser beam. The large amount of data generated cannot be adequately processed without a computer which is not an optional extra but an essential part of the instrument.

The advantages of flow sorting are:

(a) High purity can be achieved (> 98%).

(b) Small subpopulations can be selected (1% or fewer of the cells).

(c) Several parameters can be used to select the cells for sorting.

The disadvantage of flow sorting is the low throughput of cells compared to other methods (about 10000 cells/sec is generally the maximum achievable flow rate).

There are two basic types of flow cytometer—those that only analyse cells, and those that analyse cells and can also physically sort them. In this article, we are only interested in the latter. At present, there are four major commercial instruments. The FACS Vantage (Becton Dickinson), the FACSCalibur (Becton Dickinson), the Epics Elite ESP (Coulter), and the Pas III (Partec). Older versions of the Epics range and of the FACS range (FACS IV, FAC-Star, FACStar Plus, FACSort) can be found in many laboratories. Becton Dickinson have used the acronym, FACS (fluorescence-activated cell sorter),

as a trade name for their range of instruments since 1974 when they introduced the first commercial flow cytometer. The FACSCalibur, FACSort and the Partec instruments sort by 'stream-switching'; the other instruments sort by 'droplet deflection'.

In this article, reference will be made to cell sorting only. Most of the article would apply equally to sorting other particles, such as nuclei or individual chromosomes.

2. The basic instrument

2.1 Introductory comments

A basic flow cytometer consists of a source of light, a flow chamber, optical components to focus light of different colours onto the detectors, electronics to amplify and process the resulting signals, and a computer. The major features of a flow cytometer are outlined below. For a brief but more detailed description of the instrument and its optical and electronic components, see refs 1 and 2.

In a laser-based flow cytometer, light scattered by the cells is measured at right angles to the laser beam and over a narrow angle in a forward direction. Forward angle scattered light is related to cell size. Right angle scattered light is also affected by cell size but is more closely related to other factors, such as changes in refractive index and granularity. Measurement of light scatter is an important feature of flow cytometry and is used to select different types of cell and to distinguish single cells from clumps and debris.

The other important measurement is fluorescence. There are a wide variety of dyes which stain different cell components, such as DNA, RNA, protein, and lipids. For cell sorting, antibodies directed at antigens on the cell surface are used extensively. The antibodies are labelled with fluorescent compounds, such as fluorescein. Many instruments can measure light scatter and three or four different fluorescences on each cell.

2.2 Light source

In most instruments, the source of light is an argon-ion laser tuned to produce blue light (488 nm). Small, air-cooled, lasers are fixed at this wavelength but larger, water-cooled, lasers can be tuned to other wavelengths, particularly in the UV at 360–380 nm. Most cell sorters can accommodate a second laser so that cells may be excited at two different wavelengths.

Argon-ion lasers tuned to 488 nm were originally chosen because this wavelength could be used to excite fluorescein—a fluorochrome widely used as an immunofluorescent label. Over the years, many dyes have been produced which can also be excited by blue light. With a single laser, multiple immunofluorescences can be observed (*Table 1*). Dyes which require a UV laser for excitation include the *bis*-benzimidazoles (Hoechst 33342 and 33258) which label DNA, and indo-1 which is used to measure intracellular calcium ions (1, 2).

Table 1. Dyes which can be excited by the blue line from an argon-ion laser (488 nm)

Fluorochromes	Emission maxima (nm)[a]	Colour
To label proteins:		
Fluorescein	520	Green
R-phycoerythrin	576	Orange
Phycoerythrin–Texas Red conjugate (ECD)	620	Red
Phycoerythrin–cyanine5 conjugate (Cy-chrome)	670	Deep red
Conjugated peridinin chlorophyll (PerCP)	677	Deep red
To label DNA:		
Propidium iodide	639	Red

[a] It should be noted that the wavelength of emission may depend on the environment of the fluorochrome.

Mercury arc lamps are used in some instruments, in particular the PAS range sold by Partec. They are useful as an inexpensive source of UV light but do not give the sensitivity for the observation of weak immunofluorescence. With arc lamps, the correct wavelength of excitation must be selected using optical filters.

2.3 The flow chamber

The flow chamber lies at the heart of the instrument. Its purpose is to deliver the cells singly to a specific point at which the source of light is focused (the detection point). The sample is injected into the centre of a stream of liquid—the sheath fluid (usually isotonic saline). The chamber is designed so that the sheath fluid hydrodynamically focuses the sample stream delivering the cells to the point of detection with an accuracy of ± 1 μm or better.

There are three types of flow chamber in general use. One consists of a quartz cuvette (normally rectangular) placed at right angles to the laser beam with a channel cross-section of about 250 μm (*Figure 1*). This design has the advantage of minimizing scattered light from the flow system. Fluorescence and light scatter can be measured over a wide angle at right angles to the laser beam. Light scatter can also be measured in a forward direction with the addition of an obscuration bar to block the main laser beam. A typical speed of a cell through a flow cuvette is 1 m/sec and this speed limits the rate of cell sorting (see Section 3.2.4).

An alternative flow chamber is based on a microscope; in its simplest form, the sheath fluid and sample are squirted across a microscope slide. In a more sophisticated system, a channel is cut in a block replacing the microscope stage, the top surface of the chamber being a glass coverslip. Epi-illumination is used as in a conventional fluorescent microscope, that is, fluorescence is measured along the same optical path as the exciting light (3). It is more difficult to measure light scatter in this type of chamber.

The third design employs 'stream-in-air', sometimes called 'jet-in-air (JIA)

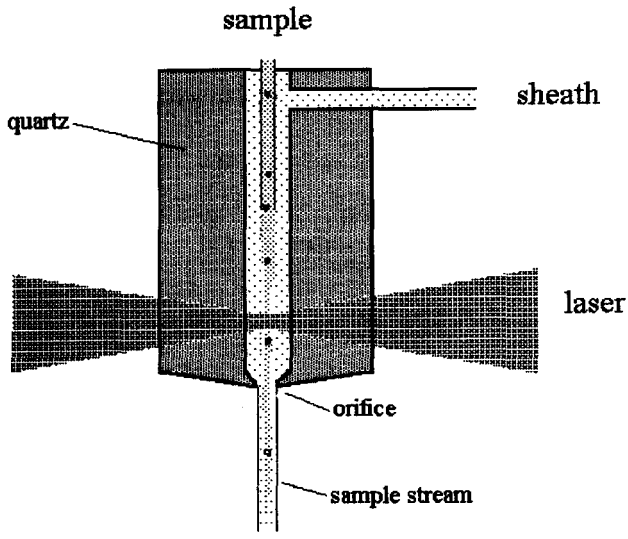

Figure 1. 'Cuvette' flow cell.

(*Figure 2*). The sheath fluid, containing the hydrodynamically focused sample stream, emerges into the air from a nozzle just below which the laser beam is focused. Because the diameter of the exit orifice is smaller than the diameter of the flow chamber, the cells accelerate as they emerge into air and travel at about 10 m/sec. Fluorescence and light scatter are measured as with the quartz chamber except that, because of the additional light scattered by the stream, a second obscuration bar has to be placed in the light collection path orthogonal to the laser beam. This design is frequently used in cell sorters.

2.4 Optics

2.4.1 Focusing the excitation beam

The laser beam is focused onto the sample stream, in the simplest configuration, using a lens giving a beam cross-section of, typically, about 50 μm. Some instruments use a spheral cylindrical lens to produce a 20 × 60 μm elliptical beam. An alternative configuration is a crossed cylindrical pair of lenses which can produce an elliptical spot of, typically, 5 × 120 μm from a laser beam of 1 mm diameter.

Beams with a circular, or near-circular, cross-section are used with stream-in-air systems in which the diameter of the beam has to be less than that of the stream to minimize excessive light scatter from the stream–air interface. The higher speed of the cells in this system gives an acceptably fast signal pulse (5–10 μsec) with the wide laser beam. Wide, flat beams are used with cuvette flow chambers. The small beam height also gives fast electronic pulses (~ 20 μsec) from the more slowly moving cells.

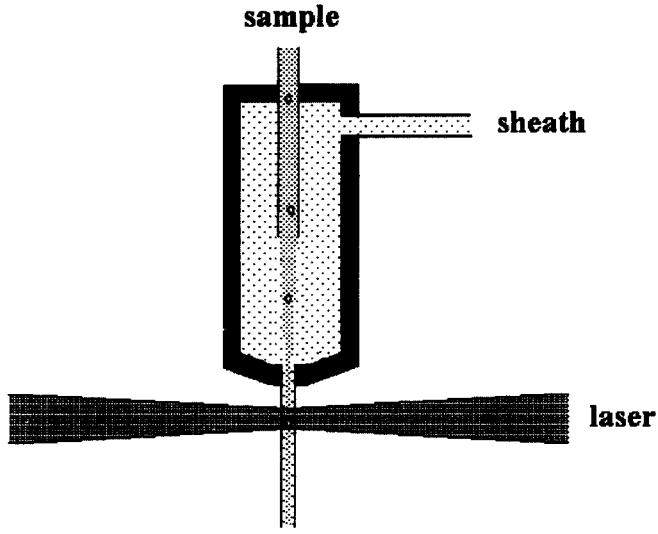

Figure 2. 'Stream-in-air' flow chamber.

2.4.2 Light collection

The orthogonal collection lens has a high numerical aperture in order to collect as much of the fluorescence as possible. For collection of forward scattered light, sensitivity is not a problem and a simple long-working distance lens is satisfactory. Forward scatter is sensitive to the angle over which the scattered light is collected and will, therefore, depend on the geometry of the light collection. Consequently, scatter profiles may differ between instruments.

2.4.3 Optical filters

Lasers give monochromatic light which does not need further filtration. If the excitation source is an arc lamp, optical filters, usually made from coloured glass, are needed to select the correct wavelength of excitation.

The dichroic (beam splitter) and bandpass filters used on the output side are normally interference filters. The dichroic filters are placed at 45° to the emission beam and are used to select the different fluorescence colours. A long pass filter will reflect light below the cut-off wavelength and transmit light above it; a short pass filter reflects light above the cut-off wavelength. Bandpass filters transmit light over a narrow band and are placed immediately in front of the detector.

2.5 Signal detection and processing

Photomultipliers are used for the measurement of fluorescence and orthogonal scatter and a photodiode for measuring forward scattered light. The signals from the detectors are amplified and processed before analogue to

digital conversion and transmission to the computer. A threshold level is usually set on light scatter so that the instrument will ignore electronic noise and small signals from debris, etc. Most cytometers offer a choice between linear and logarithmic amplification. For DNA measurement, linear amplification should always be used. For immunofluorescence, logarithmic amplification increases the dynamic range so that weak and strong signals can be recorded on the same scale. For analogue to digital conversion, a 10 bit converter is used in most instruments to give 1024 channel resolution.

2.6 Data analysis and gating

Data analysis is an important feature of flow sorting. The better the identification of the desired population of cells and the better their separation from the other cells, the purer is the sorted population.

Most flow cytometers are capable of analysing at least five parameters. All the parameters cannot be displayed in a correlated fashion and, to make full use of the information collected, 'gating' is employed. Data are displayed either in univariate histograms or in bivariate histograms (called cytograms). For display in real time, the latter are shown as 'dot plots' (*Figure 3*). Regions of interest (gates) are defined to select desired populations of cells for display of further parameters. For example, *Figure 3* shows data from peripheral blood leucocytes labelled with antibodies to CD4 (fluorescein) and CD8 (phycoerythrin, PE). On a cytogram of right angle versus forward light scatter, a region has been drawn to define the lymphocytes. The second cytogram shows the green (CD4-fluorescein) versus the orange (CD8-PE) fluorescence of the lymphocytes (that is, those cells which fell within the gate set on the cytogram of light scatter). On a cytogram, these regions can be given quite complex shapes. On the final histogram or cytogram, regions are set to define the populations to be sorted.

In the early instruments, the gates for cell sorting were set electronically. This method had the advantage that sorting decisions were made quickly; it had the disadvantage that only a rectangular region could be set and that more complex forms of analysis could not be used. In today's machines, sort decisions are made by a computer which can use multiple gates and permit the setting of complex regions. Modern computers are now so fast that the time taken to analyse a set of signals and to issue a sort decision does not compromise the sorting speed.

3. Cell sorting by droplet deflection

3.1 Introductory comments

The commonest method of sorting cells is by electrostatic deflection of charged droplets. A conductive sheath fluid is used (buffered saline). The flow chamber is vibrated vertically causing the fluid emerging from the exit nozzle

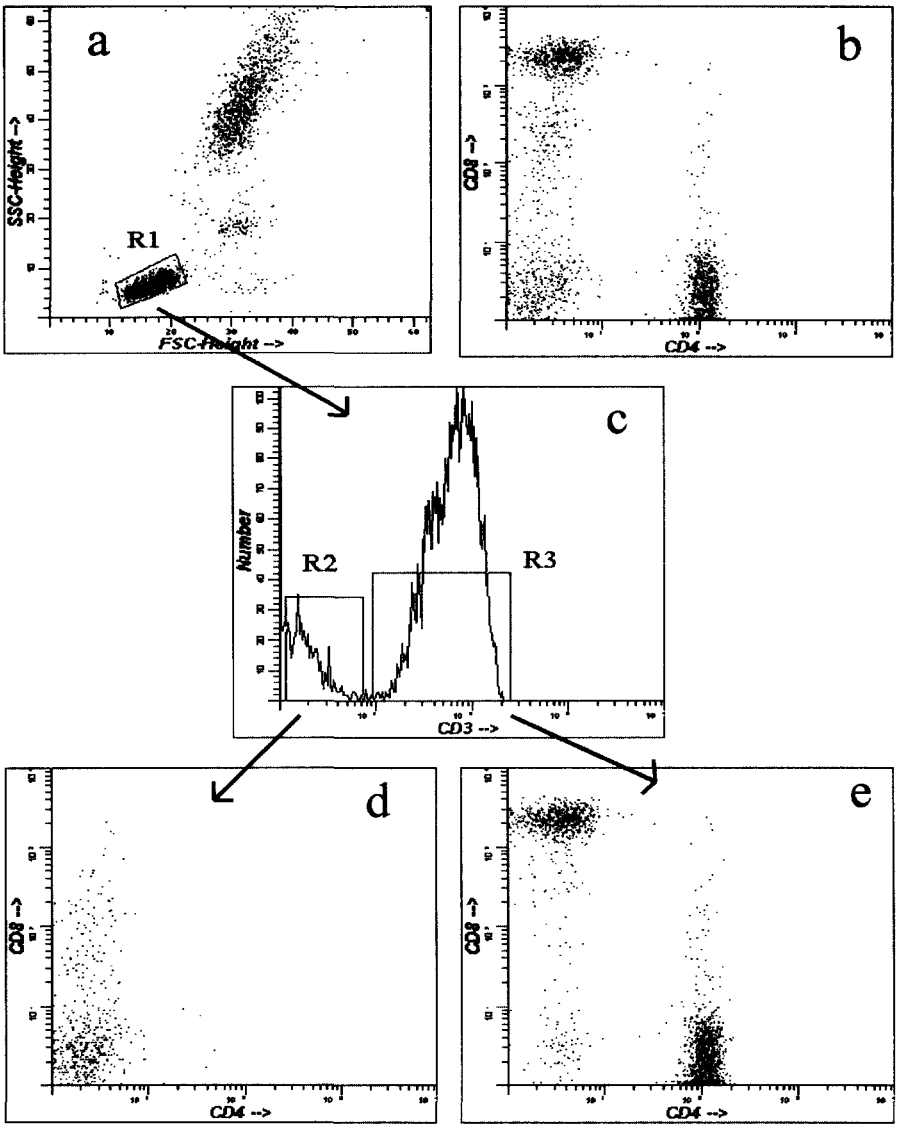

Figure 3. Flow cytometric analysis of human peripheral blood lymphocytes. (a) Cytogram ('dot plot') of right angle (SSC) versus forward (FCS) light scatter; the region (R1) defines the population of lymphocytes. (b) Cytogram of orange (CD8-PE) versus green (CD4-fluorescein) fluorescence of the cells without gating. (c) Histogram of red (CD3-PerCP) fluorescence of the small lymphocytes (gated with R1 on light scatter). (d) Cytogram of CD8 versus CD4 after gating for $CD3^-$ (R2 on red fluorescence); the cells are $CD4^-$ and $CD8^-$ or CD8 weak. (e) Cytogram of CD8 versus CD4 after gating for $CD3^+$ (R3 on red fluorescence); the cells are either $CD4^+/CD8^-$ or $CD4^-/CD8^+$. Data recorded on a Becton Dickinson FACScan by Dr Mark Lowdell, Royal Free Hospital, London.

to break up into droplets. The flow chamber is charged at the moment a cell of interest is inside the droplet currently being formed. The stream of droplets passes through a pair of charged plates so that droplets which are charged are deflected and collected together with the cell contained therein (*Figure 4*).

To ensure that the flow chamber is charged at the correct moment, the time delay between a cell passing through the laser beam and the droplet break-off point has to be determined. Anything that influences the position of the break-off point (a change in temperature, a draught, dirt in the flow chamber orifice) will adversely affect the stability of the sorter.

To obtain the best performance from a cell sorter, it is important to have a good understanding of the principles of the method which are described below. I have drawn heavily on the more detailed descriptions by Pinkel and Stovel (4) and Lindmo *et al.* (5) to which the reader is referred for more information.

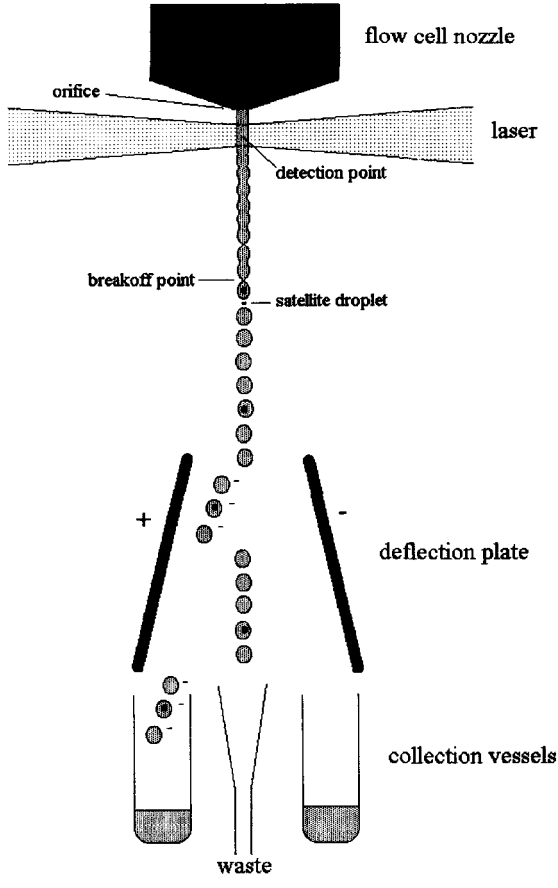

Figure 4. Cell sorting by droplet deflection (stream-in-air system).

3.2 The principles of flow sorting

3.2.1 Droplet formation

Generally, any small instability in a liquid jet will cause it to break into droplets. This process can be precisely controlled by vibrating the flow chamber so that a standing wave is set-up in the jet. The vibration is produced by mounting the chamber in a piezoelectric transducer. Droplet formation was first analysed mathematically by Lord Rayleigh in 1879. Theory predicts that:

(a) For droplet formation, the wavelength of vibration (λ) must be greater than π times the jet diameter (d).

(b) The rate of growth of droplets will depend on the ratio, λ/d.

(c) The maximum growth rate will occur when $\lambda/d = 4.5$.

The wavelength is related to the jet velocity, v, by the equation:

$$v = f\lambda$$

so that the optimum frequency of vibration is:

$$f_{opt} = v/4.5d.$$

For a jet velocity of 10 m/sec and a flow chamber orifice of 75 μm, the transducer frequency should be 30 kHz.

The viscosity of water is temperature-dependent. Any change in viscosity will alter the speed of the jet and hence the distance between the nozzle of the flow chamber and the point at which a droplet is formed (break-off point). For this reason, it is advisable to fit air conditioning in a sorting laboratory to ensure that a constant temperature is maintained.

The droplet break-off point is governed by the ratio λ/d (see above) and by the amplitude of the transducer wave. In a 'stream-in-air' system, vibration of the stream will affect the light scattered at the stream–air interface. The transducer amplitude used is a trade-off between the necessity of having a large enough vibration to give stable droplet formation, the need to minimize any effect on signal detection, and the desire to minimize the distance between the detection and break-off points.

During droplet formation, small satellite droplets are created from the thin neck that connects the larger droplets just before break-off. The satellites have a different speed from that of the main droplets and will usually merge with the parent droplet within a few droplet wavelengths. Satellites which are still separate when they enter the electric field of the deflection plates can cause problems. For example, if they land on a deflection plate, they could alter the electric field and disrupt the sort. A problem with errant satellite droplets can be cured by altering the transducer amplitude.

The presence of cells can affect droplet formation in two ways. The passage of a cell through the orifice momentarily perturbs the stream and this disturbance can make a small change in the break-off point. This effect depends on

the ratio of the diameters of the cell and the jet orifice. The second effect arises when a cell is within or close to the neck when a droplet is formed. The timing of droplet formation may be altered.

3.2.2 Droplet charging

For a cell to be sorted, it must be contained within a charged droplet. Droplets are charged by the application of a charging pulse (± 50–150 V) to the flow chamber while the appropriate droplet is being formed. The time taken for a cell to travel from the detection point to the droplet break-off point must be fed into the computer. Strategies for determining this time are given below (Section 3.3.3).

For accurate deflection, the whole of the charging pulse needs to be applied to the relevant droplet. Therefore the charge has to be applied only during the time the relevant droplet is being formed, a partial charge on a neighbouring droplet must be avoided. Consequently, the charging pulse must have the correct phase relative to transducer wave.

To minimize the influence of small changes in sorting conditions and to ensure that the correct droplet is charged and hence deflected, it is customary to charge more than one droplet, usually two or three. The computer programs in more sophisticated sorters can calculate whether it is more probable that a cell will arrive in the centre or in the leading or trailing ends of a droplet. In the latter cases, if high yield is desired, an extra droplet is charged; if the object is high purity and sorting accuracy (for example, during single cell deposition), the sort is aborted (*Figure 5*). This process is sometimes called 'phase gating'.

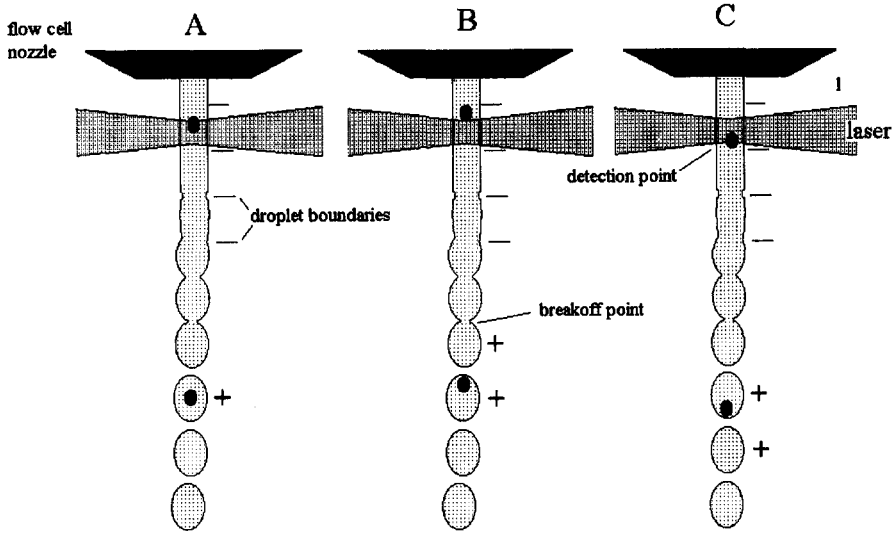

Figure 5. 'Phase' gating. (A) Cell is in the centre of the notional droplet boundary; one droplet charged. (B and C) Cell is in the trailing or leading edge of the notional droplet boundary; two droplets charged.

3.2.3 Coincidence

Cells arrive at the detection point at random. There is a finite probability that two cells will be so close to one another that they might be sorted together. If high yield at the sacrifice of purity is required, the instrument can be set to sort all positive events. If high purity is the aim, a strategy must be adopted to abort a sort when cell coincidences occur.

There are three types of coincidence to be considered:

(a) The cells are so close to one another that they are not recognized as being separate.

(b) The cells are both detected but are too close for the computer to process the signals and make a sorting decision on both cells.

(c) Both cells are detected and the signals processed but they are too close to be independently deflected.

The first condition cannot be resolved during the sorting decision. These coincidences can be minimized by ensuring during sample preparation that the number of clumped cells is reduced to a minimum and by gating on light scatter to exclude larger particles. It should also be noted that debris which is too small to be detected by light scatter will not be recognized by the system and such debris will contaminate the sorted sample. Care should be taken not to set the electronic threshold too high (see Section 2.4). This potential problem again emphasizes the importance of good sample preparation.

The increase in speed of computation of modern computers has reduced the importance of the second source of coincident cells (point b) which will only be a contributory factor at the highest flow rates.

The designer of the electronics of a flow sorter must adapt a strategy to cope with the third type of coincidence (point c). If the droplet deflection envelopes of two cells overlap, if both cells are to be sorted into the same arm, then droplet charging proceeds; if the cells are different, then the sorting decision is aborted and neither cell is sorted. If three droplets are charged for each sort, the instrument can cope with some situations in which two different cells have overlapping deflection envelopes by only charging one or two droplets (*Figure 6*).

3.2.4 Sorting speed

It can be shown that the sorting speed is given by:

$$S = que^{-(1-q)uTn}$$

where q is the fraction of cells in the subpopulation to be sorted, u is the rate at which the cells are measured, T is the droplet frequency, and n is the number of droplets deflected per sort (4).

For reasons stated above (Section 3.2.2), n is usually set to two or three. For maximum rate of sorting, one droplet deflection together with 'phase gating'

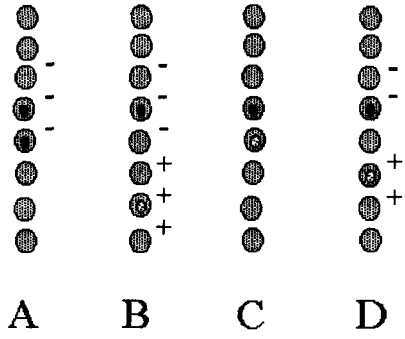

Figure 6. Droplet charging to avoid decreased purity due to coincident cells. (A) Like cells in deflection envelope—sort left. (B) Unlike cells separated—sort one left, other right. (C) Unlike cells too close together—no sort. (D) Unlike cells separted by one drop—leave separating drop uncharged.

can be used. If the computer anticipates that a cell will fall in the centre of the sorted droplet, one droplet is charged; if the cell is destined to be in the leading quarter of the droplet, the droplet ahead is also charged; if it will arrive in the trailing quarter, the following droplet is additionally charged (see *Figure 5*).

'Stream-in-air' sorters will have a higher sorting rate because the cells have a higher flow rate. The slow flow rate in a cuvette system can be partly counteracted by using a narrow waisted laser beam.

The droplet frequency (and in 'stream-in-air' configuration, the flow rate) can be increased by decreasing the diameter of the flow chamber orifice (Section 3.2.1). The minimum size of orifice is determined by the size of the cells being sorted. However, a small diameter is more likely to become blocked and nothing slows down a sort more than having to clear the flow chamber orifice.

Most manufacturers supply a choice of flow chamber orifice ranging from 50–400 μm. Typically, with a 'stream-in-air' system with a flow rate of 10 m/sec, for sorting lymphocytes, one might use an orifice diameter of 50 μm and a transducer frequency of 45 kHz; for epithelial cells, which are larger, an orifice diameter of 75 μm and a transducer frequency of 30 kHz could be selected. It must be stressed that the optimum frequency depends on the flow rate and the manufacturer's recommendation should be followed.

3.3 Practical considerations

3.3.1 Which cells can be sorted?

The first fluorescence-activated cell sorters were primarily used in immunological applications to sort lymphocytes. Since then, flow sorters have been used to sort a wide variety of types of cell. Any cell which can be prepared as a suspension of single cells can be analysed and sorted.

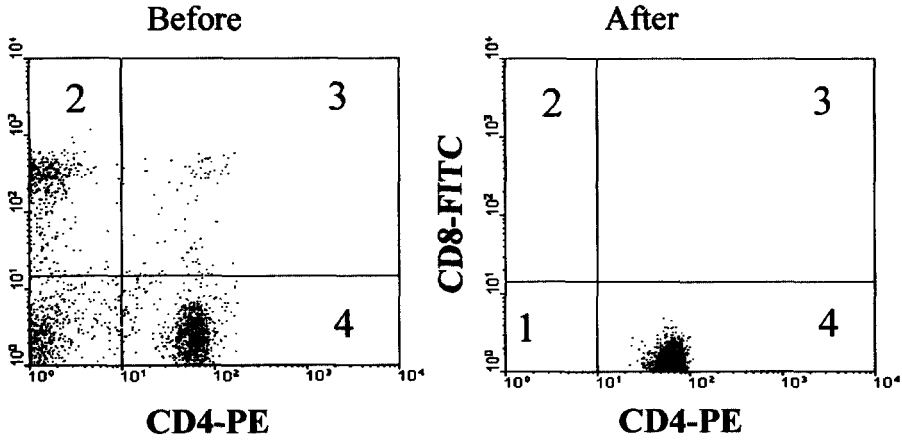

Figure 7. Reanalysis of sorted cells. CD8-FITC versus CD4-PE fluorescence after gating on light scatter to select small lymphocytes (see *Figure 3*), before and after sorting for $CD4^+/CD8^-$ cells. Quadrant 1-UL: 21.7% unsorted, 0% sorted. Quadrant 2-UL: 3.3% unsorted, 0% sorted. Quadrant 3-LL: 33.9% unsorted, 0.1% sorted. Quadrant 4-LR: 41.1% unsorted, 99.9% sorted. Analysis and sort performed on a Coulter Elite ESP by Mark Cheetham, Coulter Ltd., Luton, UK.

The cells to be sorted are most commonly selected by labelling the cells with fluorescent antibodies to cell surface antigens (for example, see *Figure 7*). However, cells may be sorted using any suitable parameter. Clara cells from rat lungs have been sorted on their increased content of glutathione (6). If a suitable substrate is available, cells can be sorted according to an enzyme activity. An interesting application of this approach is the detection of the presence of the *E. coli* β-D-galactosidase gene (*lacZ*) in mammalian cells (7). The *lacZ* gene is used as a 'reporter' gene during the introduction of constructs of cloned DNA into cultured cells. Galactosidase is detected by the production of fluorescein from the substrate, fluorescein β-galactopyranoside. Individual cells which have incorporated the foreign construct can then be sorted.

3.3.2 Sample preparation

A suspension of single cells is the essential element of any flow cytometry and is the key to successful flow sorting. The sample should contain as little debris and as few dead cells and clumps as possible.

Cells obtained from the peripheral blood or from an aspirate of bone marrow are already in the form of single cells. It is usually advisable to remove the contaminating erythrocytes either by differential lysis or by centrifugation in a density gradient.

Generally, other tissues have to be disaggregated, either mechanically or by enzymatic digestion. The heterogeneity of biological tissues has generated a similar diversity of preparation techniques. The best conditions have to be

worked out for each tissue used; a technique that works well with one will not necessarily be successful with another.

I have drawn together a set of protocols for preparing single cells from different tissues (see ref. 1, Chapter 3).

3.3.3 Data analysis

Cells can only be sorted if a property, or properties, can be identified which separates the desired subpopulation. The better the analytical separation the better is the physical separation of the cells. Time spent in selecting the optimum reagents and in analysing the cell population before planning a sort is a good investment. The aim should be to obtain the maximum separation between the subpopulation(s) to be sorted from the other cells by using multiparametric analysis. Particular attention should be paid to setting the gates (see Section 2.6).

3.3.4 Adjusting conditions for droplet formation

The exact protocol to be used in setting-up a flow sorter depends on the instrument and the model. In this chapter generalized protocols are given to supplement the detailed protocols which are supplied by the manufacturers and which should be followed. While setting-up the sorter, droplet formation can be observed using a small strobed light source (normally built into the instrument). In all instruments, to facilitate adjustment of the sorting conditions, a regular charging pulse can be applied to the stream so that a regular number of droplets can be deflected left and right to give two side streams.

Protocol 1. Initial steps for setting-up a flow sorter

1. Check that the sheath fluid reservoir contains saline solution. Switch on the instrument and set-up as for normal analysis. Ensure that the stream is central between the deflection plates. Allow the sheath fluid to run for about 30 min to ensure stable conditions of flow. (Preliminary analysis of the sample to be sorted may be carried out during this time.)
2. Select the correct transducer frequency for the chosen flow chamber orifice diameter.
3. Switch on the transducer. While observing the droplet break-off point, adjust the transducer amplitude to minimize the break-off distance and the number of satellite droplets.
4. Switch on the high voltage on the deflection plates and the test charging pulse. Adjust the phase of the charging pulse (sometimes called the charge control angle) to give two sharp side streams. An incorrectly adjusted phase gives 'fanning' of side streams. Failure to obtain well-

defined side streams is usually caused by dirt in the flow chamber orifice. Adjust the charge pulse amplitude to give the desired amount of deflection.

5. Turn off the test pulse. Set the droplet delay time according the manufacturer's protocol.

3.3.5 Setting the droplet delay time

The time taken for a cell to travel from the detection point in the laser beam and the break-off point is called the droplet delay time. This time must be known by the computer to ensure that the correct droplet is charged. Accurate setting of the droplet delay time is critical to flow sorting cells.

In a 'stream-in-air' system, the time can be measured directly by observing the distance between the detection and break-off points using a low-powered travelling microscope. In a modern system, the computer will be able to compute this time. In a cuvette system, the cells accelerate after they have passed the detection point as they emerge into the air from the flow chamber. The droplet delay time cannot be determined directly and should be determined empirically. In some systems, a good estimate can first be obtained by measuring the distance of travel within and outside the flow chamber using cursors set on an image of the flow stream. In either case, the correct setting should always be checked experimentally.

The droplet delay time is usually measured in units of the transducer wavelength (often expressed as the number of drops).

Protocol 2. Checking the droplet delay time

1. Set-up the cytometer as described in *Protocol 1.*
2. Run a test sample in the cytometer, either the sample to be sorted or some fluorescent beads.
3. Select a gate to sort cells (or beads) to the left.
4. Select one droplet sorting. Decrease the droplet delay time by two.
5. Sort a given number of cells (or beads) (50 or 100) onto a microscope slide. Increase the drop delay time by one unit, move the microscope slide to a fresh area, and repeat the sort. Repeat a further three times so that there are five drops of liquid on the slide. (The drops will be nicely formed if the slide is slightly greasy. To achieve this, run your thumb over the surface of the slide before using it.)
6. Under a microscope, count the number of cells (or beads) in each drop. If the cells are shared between two droplets, either:
   (a) If the drop delay time can be set in fractions of a transducer wavelength, make a small, fractional, change to the drop delay time and repeat step 5.

Protocol 2. *Continued*

(b) If the drop delay can only be set in integers of the drop period, turn off the sample flow and turn on the test pulse, make a small change to the transducer amplitude, adjust the phase of the charging pulse to obtain sharp side streams again, turn off the test pulse, rerun the sample, and repeat step 5.

The aim is to have 90% or more of the cells in a single drop.

7. If necessary, adjust the drop delay time to that of the drop which contained the sorted cells.

The instrument is now ready to sort cells.

3.3.6 Collection vessels

To collect large numbers, cells are usually sorted into centrifuge tubes. If plastic tubes are used, during a long sort, charge can build up on the tubes to such an extent that charged droplets may be deflected, reducing the efficiency of collection. If this problem arises, an earthed platinum wire should be inserted in the collection tubes. Static electricity causes fewer problems with glass collection tubes because the charge is dissipated more efficiently across the glass surface.

Collection tubes can be coated with protein, for example, with fetal bovine serum, before sorting to discourage cells from adhering to the walls of the tube. It is difficult to prevent fixed cells from sticking to the tube. When we sorted ethanol fixed cells for subsequent extraction of DNA, we collected into small glass tubes and extracted the DNA in the same tube.

If cells are required for amplification of their DNA by a polymerase chain reaction (PCR), they can be sorted directly into the PCR reaction tube.

If cells which grow attached to plastic surfaces are being sorted for culture, they can be sorted directly into the wells of a multiwell culture plate. Start with the wells about one-third full of culture medium. After the sort, incubate the cells at 37 °C for 1–2 h to allow the cells to settle and to start to attach to the plastic surface, carefully remove three-quarters of the supernatant, and add fresh medium.

Manufacturers sell special attachments for 96-multiwell plates. Single droplet deflection can be used with 'phase gating' (see Section 3.2.2). After each sort command, the plate is moved by instruction from the computer so that a single cell is sorted into each well. This system is used for cloning cells; it can be used, for example, to sort hybridomas.

Cells sorted in small numbers for identification can be collected directly on a microscope slide. Cells can also be sorted into small wells on specially manufactured slides which are covered with PTFE film with circular holes cut in it. If the slides are pre-coated with polylysine, cells will adhere more easily to the glass. After giving the cells time to settle, the slides can be immersed gently into a fixative and the cells stained for microscopic examination.

If the desired cells are in a low concentration, the rate of sorting onto a microscope slide will be low and the sheath fluid will evaporate during the sort. The salt will crystallize and the sorted cells will shrink and lose their morphology. The problem can be lessened by using a lower salt content in the sheath fluid (diluted 1 in 50) (4).

If the cells are being sorted to use as dot blots in DNA hybridization, they can be collected directly onto a nylon filter. It is not difficult to construct a filter holder mounted in a small chamber to which a slight vacuum is applied, removing the sheath fluid as the droplets land.

3.3.7 Blockages during sorting

The importance of careful preparation of the sample has been emphasized throughout this chapter. Clumps of cells or small pieces of undigested tissue will block the flow chamber orifice and disrupt the sort. Although a blockage can usually be cleared by back-flushing the flow chamber, a severe blockage may necessitate removing the flow chamber and subsequently having to re-adjust the instrument before recommencing the sort (*Protocols 1* and *2*). A partial blockage can cause a deflection of the sample stream emerging from the flow chamber and sheath fluid may be sprayed over the deflection plates. If this happens, switch off the high voltage, carefully clean the plates, and readjust the instrument.

3.3.8 Purity and yield

The purity of the sorted sample can be measured by reanalysing the sorted cells in the flow cytometer (*Figure 7*). The sample delivery system should be flushed through thoroughly before reanalysis in case there are unsorted cells in the sample delivery line. The sorted cells may show slightly weaker immunofluorescence due to bleaching as they pass through the laser beam.

The computer will give the total number of cells analysed, number of sort decisions issued, and how many sorts were aborted because of coincidence. The issue of a sort decision by the computer does not necessarily mean that a cell has been sorted. If the sorter was maladjusted, the collected droplet may not have contained a cell. Also the sorted droplets may not have reached the collection vessel. The yield of cells recorded by the computer should occasionally be checked experimentally to see whether there is reasonable agreement between the two.

Protocol 3. Measuring the yield of sorted cells

1. Before sorting, measure the concentration of cells in the sample, preferably on a Coulter counter—haemocytometers are notoriously inaccurate.
2. Weigh the sample and collection tubes.

Protocol 3. *Continued*

3. During the flow cytometric analysis, record the percentage of cells in the gate selected for sorting.
4. Carry out the sort.
5. Weigh the sample and collection tubes. The difference in weight before and after sorting will determine the volume of cells which passed through the sorter and the volume of cells collected.
6. Calculate the number of selected cells which passed through the sorter (sample volume × concentration × % in gate).
7. Measure the concentration of cells in the sorted sample.
8. Reanalyse some sorted cells to measure the purity.
9. Calculate the number of sorted cells (collected volume × concentration × purity).
10. Divide the figure in step 9 by that obtained at step 6 and multiply by 100. This number is the percentage yield.

3.3.9 Sorting rate

The sorting rate is the number of cells sorted divided by the time taken to sort them and should be recorded by the computer. As the sample flow rate is increased, the number of coincidences will increase. If the object is to produce high yield at the expense of purity, the coincidence circuitry is switched off ('enrich mode'). The sample flow rate is then adjusted to give a compromise between sorting rate and purity—the higher the flow, the lower the purity.

If high purity is desired, as is usually the case, increasing the flow rate will increase the sorting rate but reduce the yield because of the increased number of aborted sorts due to coincidences. If the sample is limited, as could be the situation with cultured cells, use a slower flow rate to maximize the yield. If the sample has more than enough cells, increase the sorting rate and hence decrease the sorting time by boosting the flow rate. As the flow rate is increased, the number of coincidences will eventually become so large that the sorting rate will reach a maximum. Another limiting factor is the degradation in performance caused by the increase in the diameter of the sample core at high flow rates. The latter effect should be minimized by having the cells at a high concentration (about 2×10^6/ml). With a sample of small cells, such as lymphocytes, the maximum usable flow rate in a 'stream-in-air' instrument is 10–15 000 cells/sec and 5–10 000 cells/sec in a cuvette system. Most instruments and operators are performing well if they can achieve half these rates.

3.3.10 Long sorting times

If a sort extends over one to several hours, the cells will settle in the sample container. They should either be stirred gently or the sample tube should be

occasionally agitated. To avoid undesirable changes to the cells, cool both the sample and collection tubes to 0–4 °C.

During the sort, the droplet delay time may drift, particularly if room temperature changes. The position of the droplet break-off point should be monitored and if any change occurs, the sort should be stopped and the droplet delay time checked. On older instruments and during the familiarization period with a new instrument, it can be helpful to monitor the sort while it is in progress. Every half hour, collect 20–50 droplets on a microscope slide and check the cell concentration under the microscope. The concentration of the sorted cells is dependent on droplet size and the number of droplets deflected. For a given flow chamber orifice and transducer frequency, the number of droplets deflected is the only important parameter. An operator soon becomes familiar with the expected cell concentration and a quick glance down the microscope will confirm that the sort is proceeding properly. A better estimate can be obtained by sorting onto a haemocytometer and counting the cells.

3.3.11 Sorting rare events

Rare events present a particular problem because of the time needed to collect enough cells for further study. The sample can be enriched by pre-sorting at a high flow rate in enrich mode (giving high yield but low purity) and then resorting for high purity. If the rare cell is being selected on positive fluorescence (as will undoubtedly be the case), the speed of the preliminary sort can be increased by triggering the electronics on the fluorescent signal and setting the threshold so that negative events are not 'seen' by the system. Using this strategy, the purity will be poor but all the rare events should be detected and sorted since the system is not overloaded by processing data from unwanted cells.

An alternative method of pre-enrichment is to remove a high percentage of unwanted cells by magnetic separation (see Chapter 6). This strategy can only be adopted if there is an antigen expressed on the surface of 90% or more of the unwanted cells and not expressed on the rare cells.

3.3.12 Sterile sorting

Because droplets are deflected in air, truly sterile sorting is not possible without enclosing the whole flow chamber assembly in a sterile hood. However, if a few sensible precautions are taken, it is possible to sort cells which can be maintained in culture for one to two weeks without growth of contaminants.

(a) Sterilize the sheath tubing. Pass a sterilizing fluid (for example, a dilute solution of bleach, such as 1% hypochlorite) through the sheath fluid assembly. Flush out the bleach with sterile, filtered (0.2 μm) water for at least 15 min. Finally run sterile sheath fluid as part of the preparation procedure (*Protocol 1*, step 1).

(b) Clean all surfaces around the sample collection area and then wipe them down with a disinfectant (e.g. 70% ethanol).

(c) Sterilize the sample delivery system. Pass dilute bleach through the sample delivery system. Flush out the system with sterile, filtered water. This can be done after the instrument has been adjusted for correct sorting and immediately before running the sample. Do not allow an unsterile sample (for example, a suspension of beads used for alignment) to contact the sample collection tube before commencing the sort.

(d) Enclose the sample collection area during the sort.

4. Other flow sorters

The Partec PAS III cell sorter and the Becton Dickinson FACSCalibur and FACSort sort cells by deflection of the sample stream. The flow chamber in the PAS III is based on a microscope stage. The sample stream flows horizontally through a channel which bifurcates after the observation point. Unsorted cells traverse one arm and a piezoelectric fluidic valve deflects sorted cells down the other arm. The sorter can be used for sorting large cells, such as protoplasts, or even clusters of cells, because it avoids the limitations of size imposed by the need for accurate droplet formation in the electrostatic sorters. The maximum achievable sort rate is 1000 cells/sec.

The FACSort has a vertical quartz flow chamber with a closed fluidic system. A piezoelectric device deflects a small collector into the centre of the sample stream to gather a cell selected for sorting. The collector sits on the edge of the sample stream so that it collects sheath fluid even when a cell is not being sorted. The position of the deflector is critical; adjustment should only be made by a trained service engineer. The maximum rate of sorting is about 300 cells/sec.

Because of the mechanical nature of the sorting process in these instruments, sort rates are comparatively low and only one subpopulation may be sorted. The other disadvantage is that the sorted cells are heavily diluted.

The advantage of these sorters is that they are simple to operate and that they can be fully enclosed enabling biologically hazardous samples to be sorted.

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6

Immunomethods: magnetic, column, and panning techniques

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1. Introduction

Cell separation techniques mediated by antibody–antigen interactions are now widely used in both research and clinical laboratories. This has largely come about with the advent of monoclonal antibodies and their exquisite specificity for defined antigen epitopes. Cell surface molecules to which monoclonal antibodies have been developed are identified with the internationally recognized nomenclature of cluster of differentiation (CD) antigens. Each CD antigen is assigned a unique number following Leucocyte Typing Workshops (1). Some commonly occurring CD antigens, such as CD4 and CD8 present on T lymphocytes, have been recognized for many years while others, largely those with higher CD numbers, have been defined only recently.

The need to separate cells arises because populations are frequently heterogeneous and one of the following conditions exists:

- specific cells are needed for patient treatment
- functional assays are to be performed
- cell populations need to be quantified

In bone marrow transplantation, in the treatment of leukaemia for instance, it is highly desirable that only donor stem cells are introduced to the recipient; if immunocompetent mature donor T cells are transplanted then life threatening graft-versus-host disease is inevitable. Similarly if residual tumour cells are reintroduced the whole therapeutic approach may be compromised. Thus the need for separation of stem cells from other contaminating donor cells is paramount.

The use of pure cell populations in functional assays is probably the widest application of immune-mediated separation methods varying from phenotyping of bacteria for clinical diagnosis, tissue typing for transplantation, to complex assays of *in vitro* immune function such as defining the cell types responsible for specific processes in the immune response. Quantification of

cell types is used in lymphocyte subset analysis, and in immunohistochemistry when occurrence of antigens in tissue sections can be defined precisely using antibodies tagged with fluorochromes, radiolabels, or heavy metals such as gold. In some cases intracellular location of antigens can be defined in this way.

The key to successful immunoseparation methods is usually the antibody. Whilst polyclonal antibodies have been successfully used in separation methods such as depletion by cytotoxicity, by and large the use of monoclonal antibodies is encouraged because of their specificity.

The techniques used for immunoseparation methods may be conveniently divided into the following broad categories:

- rosetting
- cytotoxicity
- flow cytometry
- column and panning

The first category is now largely represented by immunomagnetic separation (IMS) technology where paramagnetic particles coated with specific ligands (antibodies) are used to positively isolate a specific cell population. In cytotoxicity, antibody and complement are used to kill a defined cell population leaving either cells of reduced heterogeneity or a specific cell population. Flow cytometry allows precise immunophenotyping of mixed cell populations and their physical separation is dealt with in Chapter 5. Binding and selection of cells using a solid phase via an antibody is possible using supports such as *Staphylococcus aureus* or plastic plates, but the advent of immunomagnetic beads has made such techniques largely redundant.

Whilst all laboratory techniques are problematic immunoseparation of cells has been improved hugely by the use of immunobeads. Variables which adversely affect all methods for immunoseparation include:

- contamination of cell population
- poor viability of cells
- antibody/antigen affinity or avidity
- concentration of antibody/antigen
- variation between cell populations

Each of these areas must be addressed when initiating novel techniques and the last point is an ongoing issue for routine clinical work.

In this chapter we aim to review the basic principles of cell separation techniques using immunomethods, in particular IMS, which are used routinely in established laboratories or have been published in peer review journals. They are robust techniques which are continually being developed commercially and manufacturers' protocols should be consulted for most recent advances.

2. Column and panning methods

Both column and panning methods were developed as solid phase supports for bound antibody to provide the specificity for cell separation. Column methods use supports such as Sephadex or other similar gel matrices to which antibody may be readily coupled. The cell suspension percolates the column when cells may become attached by matrix-bound antibody and retained. Effluent cells can be collected while bound cells may be released by a change in pH or ionic concentration of the column buffer. A method for the isolation of HLA-DR positive lymphocytes is given in *Protocol 1*.

Protocol 1. Isolation of HLA-DR positive lymphocytes

Reagents

- Monoclonal antibody specific for HLA-DR, e.g. ATCC L243
- Acetate buffer: 0.1 M Na acetate, 0.5 M NaCl pH 4
- Bicarbonate buffer: 0.1 M $NaHCO_3$, 0.5 M NaCl pH 7.7 (in 1 litre distilled water; adjust to pH 7.7 with 1 M HCl)
- CNBr-activated Sepharose 4B (Pharmacia)

A. *Preparation of antibody column*

1. Place 1 g CNBr-activated Sepharose 4B in 12 ml plastic column.
2. Wash column with 20 ml 1 mM HCl.
3. Wash column with 20 ml bicarbonate buffer.
4. Maintain column with 10 ml bicarbonate buffer.
5. Add 6 mg antibody in 1 ml to column.
6. Seal column and rotate end-over-end slowly for 2 h at 22°C.
7. Wash column with 250 ml bicarbonate buffer.
8. Add 10 ml ethanolamine and rotate as in step 6.
9. Wash column with 250 ml bicarbonate buffer followed by acetate buffer.
10. Repeat washes from step 9.
11. Wash column with 50 ml PBS; add 0.01% Na azide.
12. Store column at 4°C.

B. *Isolation of HLA-DR positive lymphocytes*

1. Obtain a suspension of lymphocytes at a maximum of 10×10^6/ml.
2. Suspend lymphocytes in 5 ml PBS and 20% fetal calf serum (FCS).
3. Add lymphocyte suspension to column, seal, and incubate for 20 min at 20°C.
4. Incubate for 20 min at 4°C.
5. Remove Sepharose and cell suspension from column and layer on 10 ml 40% Percoll.

Protocol 1. *Continued*

6. Centrifuge at 700 *g* for 20 min at 20°C.
7. Harvest HLA-DR positive lymphocytes bound to Sepharose from interface.
8. Wash in 5 ml PBS and sediment at 400 *g* for 10 min.
9. Vortex in 5 ml 40% Percoll and centrifuge at 700 *g* for 20 min to remove lymphocytes from Sepharose.
10. Recover HLA-DR positive lymphocytes from layer above Percoll.

In panning methods, antibody is attached to a plastic plate and cell suspensions are subsequently incubated so that plate-bound antibody can bind to cell surface molecules. Non-binding cells are simply poured off while bound cells can be removed mechanically. Cells known to be naturally adherent, such as peripheral blood monocytes, can be isolated as in *Protocol 2*.

Protocol 2. Isolation of monocytes by panning[a]

Reagents

- Leucocytes isolated from whole blood (see Chapters 1 and 2)
- McCoys medium
- Fetal calf serum
- Serum-free RPMI

Method

1. Incubate plastic Petri dish with 1 ml fetal calf serum for 45 min at 37°C in a humid box.
2. Decant FCS and add 2 ml leucocytes at 5×10^6/ml isolated from whole blood.
3. Incubate for 60 min at 37°C.
4. Decant supernatant and wash off non-adherent cells with McCoys medium plus 10% FCS at 37°C.
5. Elute monocytes by incubating Petri dish with serum-free RPMI for 15 min at 22°C.[b]
6. Repeat step 5 twice.
7. Enhance removal of monocytes by scraping Petri dish with rubber 'policeman'.
8. Repeat step 7 twice.
9. Collect monocytes from washings by centrifugation.

[a] An alternative approach is to use glass beads of 450 μm supported in a column of about 15 cm × 2.5 cm as the solid phase.
[b] An alternative method to release adherent cells is to incubate Petri dishes with 30 mM Lidocaine for 15 min at 22°C.

Neither column nor panning techniques are currently in widespread use due to the remarkable success of magnetic beads as a solid phase support for antibody.

3. Reagents for immunoseparation methods

3.1 Antibodies

Monoclonal antibodies can be obtained in several ways:

- in-house production
- a gift from collaborators
- commercial companies

Some research teams have established monoclonal antibody producing units which undertake contract manufacture; the success of this approach is heavily dependent on the immunogen. It is unwise to undertake production of a monoclonal antibody in a single instance because of the considerable effort involved. Often the desired antibody can be obtained as a collaboration with the author of a published paper which describes the desired antibody. In some cases hybridomas producing the antibody are available at a small cost from national and international cell repositories such as the American Type Culture Collection (ATCC). Many companies now offer a very wide range of monoclonal antibodies conveniently defined as being specific for individual CD antigens.

3.2 Immunomagnetic beads

There are several commercial suppliers of immunobeads; most are magnetic to ease separation of attached cells. Suppliers include:

- Biotest 'Lymphobeads'
- Dynal 'Dynabeads'
- Hoefer 'MPG'
- Metachem/Advance Magnetics 'BioMag'
- One Lambda 'Fluorobeads'

Each supplier lists possible applications for their products and there are different sizes of beads available to suit different applications. Some products are available for coating with antibody by the user while others come ready coated for immediate use in separating specified cell populations. It is important to note that a procedure which works well with one product may not be as successful with a different product.

4. General cell separation using immunomagnetic beads

Efficient immunomagnetic separation relies upon the application of a suitable paramagnetic particle coated with antibody or other ligand.

Based on the patented work of Professor John Ugelstad at SINTEF in Norway, Dynabeads® have been available for cell separations since about 1985. They are uniform magnetizable polystyrene beads with a surface suitable for coating with antibodies or other ligands. Beads coated with antibody are used to capture target cells in heterogeneous suspension which are recovered with a magnet, thus separating the attached target cell from the suspension.

4.1 Coating immunomagnetic beads with antibodies

4.1.1 Antibody class

i. IgM antibodies

The choice of antibody class may influence the separation strategy. Monoclonal antibodies can be coated non-covalently directly onto the surface of uncoated and non-activated beads and generally will function efficiently. Possibly because of their size and pentavalent structure they tend not to be affected by steric hinderance. IgM (or any other ligand intended for direct coating) must be first purified to prevent binding of irrelevant proteins or stabilizing agents. This can be achieved by passing the concentrated antibody supernatant down a Sephadex® G50 column and collecting the void volume. This will contain only high molecular weight material including IgM.

Perhaps a better alternative is the use of an immunomagnetic bead coated with a secondary antibody, e.g. rat anti-mouse IgM, which can be used to adsorb IgM directly without the need for initial purification.

Protocol 3. Direct coating of immunomagnetic beads

Equipment and reagents

- Magnetic particle concentrator (e.g. Dynal MPC® 1, Cat. No. 120.01)
- Sample mixer giving rocking and rolling action
- Dynabeads M-450 uncoated (Dynal, Cat. No. 140.01/140.02)
- Antibody or ligand
- Alternative coating buffer (0.01 M phosphate-buffered 0.15 M saline pH 7.4) (PBS): per litre, 0.157 g $NaH_2PO_4.H_2O$, 1.48 g $Na_2HPO_4.H_2O$, 8.1 g NaCl
- Coating buffer (0.17 M borate buffer pH 9.5): 105.1 g citric acid, 30.9 g boric acid, 69 g $NaH_2PO_4.H_2O$, 1.5 litre distilled water—dilute 250 ml of this stock solution to 400 ml with distilled water and adjust to pH 9.5 with conc. NaOH (up to 100 ml may be added without critically affecting the molarity)
- Blocking buffer: 0.01 M PBS containing 0.1% bovine or human serum albumin
- Storage buffer: PBS blocking buffer containing 0.02% sodium azide

Method

1. Make a homogeneous suspension of beads with vigorous mixing; pipette out the desired amount for coating and wash twice in coating buffer.[a]

2. Prepare antibody in coating buffer; sufficient to coat 2–4 μg antibody per 1×10^7 beads.
3. Suspend the washed beads in coating buffer and mix vigorously *immediately before* adding the antibody.[b] The recommended concentration of beads in the final solution is 10^8–10^9/ml.
4. Incubate for 24 h at 4°C.
5. Place the tube in a magnet and remove the supernatant. Generally 40–80% of the added antibody will be bound to the beads.
6. Wash three times, the last overnight at 4°C using blocking buffer.
7. Resuspend in storage buffer and keep at 4°C.

[a]Borate or phosphate buffers in the pH range 7.0–9.5 may be used for coating Dynabeads M-450 uncoated beads. The pH sensitivity of the antibody will determine the upper limit.
[b]The molarity of salt in the final coating solutions should never be less than 0.05 M.

ii. IgG antibodies

IgG antibodies do not perform at their most efficient in cell separation when coupled directly to the surface of a particle. The Fab region may be incorrectly orientated with respect to the target cell and the whole system may suffer through steric hinderance. This is overcome by the use of a secondary antibody coated bead (e.g. sheep anti-mouse IgG). These can be used to specifically adsorb IgG onto the bead without requiring any initial antibody purification step. Further, the secondary antibody will act as a spacer arm to prevent steric hinderance during the cell isolation process, and help to orientate the primary antibody Fab region external facing to the particle.

Further discrimination can be achieved by the use of subclass-specific secondary antibody coated beads (e.g. rat anti-mouse IgG1, 2a, 2b, 3).

Binding of IgG antibodies to secondary coated beads is normally complete within 30 min (*Figure 1*). Typically an antibody concentration of 0.2–2.0 μg offered per 10^7 beads (approx.) is sufficient. 2.0 μg will exceed the binding capacity of 10^7 beads.

Protocol 4. Antibody coating of secondary coated immunomagnetic beads

Equipment and reagents

- Magnetic particle concentrator (e.g. MPC-1, Dynal Cat. No. 120.01)
- Sample mixer giving rocking and rolling action
- Dynabeads M-450 coated with secondary antibody (e.g. Dynal, Cat. No. 110.01/110.20)
- Storage buffer (PBS/BSA/NaN_3): blocking buffer containing 0.02% sodium azide
- Primary antibody
- Phosphate-buffered saline (PBS) pH 7.4: per litre 0.157 g $NaH_2PO_4.H_2O$, 1.48 g $Na_2HPO_4.H_2O$, 8.1 g NaCl
- Blocking buffer (PBS/BSA): 0.01 M PBS containing 0.1% bovine or human serum albumin

Protocol 4. *Continued*

Method

1. Make a homogeneous suspension of beads with vigorous mixing, pipette out the desired amount for coating, and wash twice in coating buffer.
2. Prepare antibody sufficient to coat 0.2–2 μg per 1.3×10^7 beads in coating buffer.
3. Suspend the washed beads in coating buffer and mix vigorously *immediately before* adding the antibody. The recommended concentration of beads in the final solution is 10^8–10^9/ml.
4. Incubate for 30 min at 4°C with gentle rotation. Do not allow the beads to settle during this time.
5. Place the tube in a magnet and remove the supernatant.
6. Wash a minimum of three times for 5 min each at 4°C using PBS/BSA.
7. Store in PBS/BSA buffer containing 0.02% sodium azide at 4°C for several months.

iii. Polyclonal antibodies

Antibodies in polyclonal sera are best adsorbed onto beads coated with secondary antibodies, e.g. sheep anti-rabbit IgG.

iv. Other ligands

Non-antibody ligands such as lectins can be covalently bound to beads activated to allow chemical coupling. Uncoated Dynabeads are also available activated with a tosyl group which will covalently bind with protein.

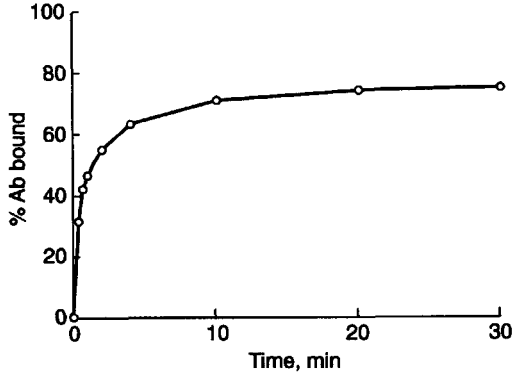

Figure 1. Example of binding kinetics obtained with secondary coated Dynabeads. For this particular lot the binding capacity was 0.75 μg antibody/mg beads when adding 1 μg antibody.

4.2 Immunomagnetic separation strategies

In common with all immunological techniques, factors such as incubation time, temperature, and concentration of reactants have a measurable effect on the efficiency of immunomagnetic separation. Further, the process is also controlled by specific key parameters that will combine to influence the choice of separation strategy:

- nature and state of the target cell
- characteristics of the antigen/antibody binding
- sample type
- bead:cell ratio
- bead concentration
- intended use of the isolated cells

4.2.1 Target cells

Antigen expression on the surface of the target cell at the time of separation is important. Cells with low antigen density can be isolated by immunomagnetic separation but increased numbers of beads may be needed. The amount of antibody and whether to use the direct or indirect method must be tested in each case because of variable binding configurations for different antibodies. After separation when the beads are attached to the cells the availability of the antigen in question will naturally be reduced especially in the indirect technique. In the direct technique few of the antigen sites will be occupied by antibody coated beads.

4.2.2 Antibody/antigen

The specificity of the primary antibody will determine the nature of the isolated cell type and care must be taken to establish the antigen binding characteristics of the antibody used. When undesired reactions occur they can be 'specific', with the primary antibody reacting with the same or similar antigen on another cell, or 'non-specific', e.g. the binding of cells bearing surface immunoglobulin through species cross-reaction with the secondary coated antibody. These effects can usually be overcome by antibody blocking or pre-depletion of the offending cell. Cross-reactions are minimized in the direct technique. If the species cross-reaction cannot be resolved in this way consideration should be given to the use of biotin labelled antibodies and streptavidin coated beads.

Occasionally, certain cell types may be 'sticky' and adhere to the beads. These can be removed by pre-depletion with uncoated beads.

Monoclonal antibodies of any class can be used with the appropriate secondary antibody coated beads directly from culture supernatant since the vast majority of immunoglobulin in the system is specific antibody. Polyclonal sera and IgG fractions are often used directly very successfully as they tend

to have a high titre of the specific antibody. None the less, affinity purified polyclonal antibodies are preferred.

4.2.3 Sample type

i. Unseparated blood and marrow

The physical presentation of the cells in IMS is important in determining the outcome of the separation. Unseparated blood and bone marrow, for example, offer rapid and direct access to the maximum number of target cells that have undergone the minimum amount of interference. They are used very effectively in small scale applications where positive selection yields a set amount of cells for analysis (e.g. class I and class II lymphocyte isolation for tissue typing) or in positive selection or depletions (CD14, CD4, CD8) for lymphocyte subset analysis.

ii. Buffy coat preparations

In some circumstances dense, red blood cell (RBC)-rich samples need further resolving to remove the RBC load, and in the case of bone marrow to remove extraneous DNA. The use of buffy coat preparations is a valuable intermediate between unseparated bloods and density gradient isolated cells. They have the dual advantage of containing the target cell in more physical purity, with respect to both plasma proteins and RBCs, without the cell losses associated with mononuclear cell preparations.

Density gradient isolated cells provide a milieu in which the target cells are relatively pure allowing more cells to be isolated at the same bead concentration. The down side is that there are inevitable cell losses during the physical processing by centrifugation.

iii. Bead:cell ratios

The attachment of between one to three Dynabeads to a target cell provides sufficient magnetic material to ensure proper capture on a magnet. The optimal bead to target cell ratio to achieve this varies in different systems and applications. Dynal recommend a minimum bead to cell ratio of 4:1 for all samples, and a bead concentration of $1–2 \times 10^7$/ml in all cases.

iv. Positive selection

Positive selection is the intentional capture of a desired population of cells for analysis. The remaining non-target cells are usually discarded. The isolated cells may, in many cases, be analysed with the beads remaining attached. 99% purity and 95% viability of the isolated cells are the typical values achieved, with a yield of 80–90%.

Positive selection may be direct or indirect (*Figure 2*). In the direct technique, antibody is attached to the bead either directly onto the bead surface or through a secondary antibody prior to use as a capture reagent. This option is fast and requires very little preparation of the target cell population. Also it

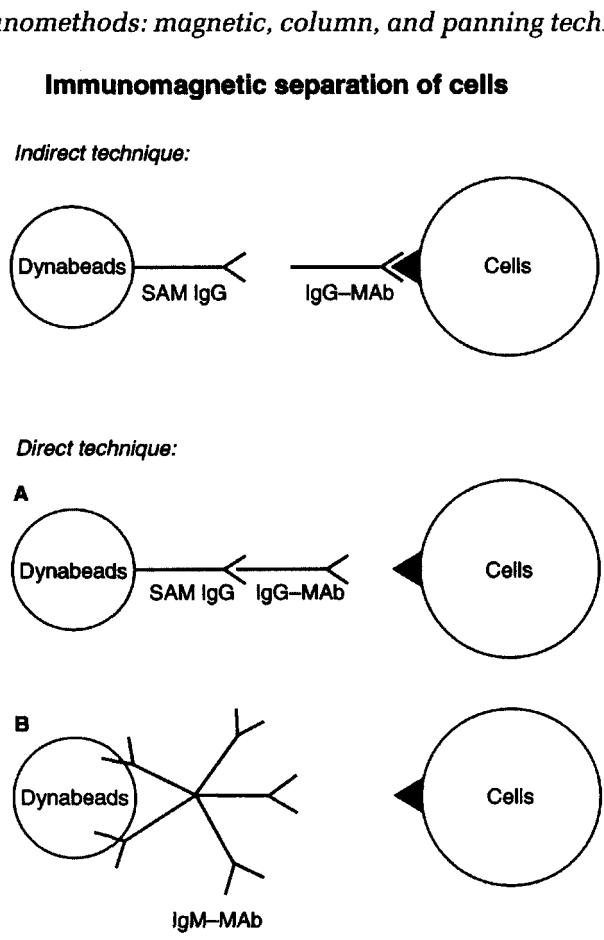

Figure 2. In the indirect technique antibody is first reacted with target cells. In the direct technique, using either (A) secondary antibody coating or (B) direct coating, the selecting antibody is reacted with the immunomagnetic bead first.

allows the preparation of stock amounts of reagent which will further improve the uniformity of the system. *Protocol 5* illustrates this option with Dynabeads M-450 CD4.

In the indirect technique antibody is first coated onto the target cells which are then captured with a secondary antibody coated bead (*Protocol 6*). The advantage of this option is greater sensitivity; particularly useful when epitope density on the target cell is low, and the ability to use cocktails of antibodies targeting the same or multiple cells in the sample. The disadvantage is that cells need to be washed several times by centrifugation to remove unbound primary antibody. Any physical manipulation can result in cell losses.

Protocol 5. Direct technique positive selection of $CD4^+$ T cells from 5 ml whole blood

Equipment and reagents

- Dynabeads M-450 CD4 (Dynal, Cat. No. 111.15/111.16)
- 2 ml EDTA or ACD anticoagulated blood
- 10 ml plastic or glass tissue culture grade round-bottom tubes
- Cooling apparatus (ice water-bath/refrigerator)
- Magnetic device: Dynal MPC-1, MPC-2, or MPC-6 (Dynal)
- Pipettes: 5 ml and 1 ml, Pasteur pipettes, vacuum pump and trap (optional)
- Apparatus for tilting and rotating of tubes
- Phosphate-buffered saline (PBS) pH 7.4: see *Protocol 4*
- PBS/sodium citrate: PBS containing 0.6% (w/v) Na citrate (optionally 100 U heparin/ml)
- PBS/FCS or PBS/BSA: PBS containing 1% fetal calf serum or bovine serum albumin
- Culture media or Tris balanced salt solution (TBSS) pH 7.4: 4 g NaCl, 0.2 g KCl, 0.1 g $MgSO_4.7H_2O$, 29.25 mg Tris

A. *Preparation of washed Dynabeads M-450 CD4*

1. Calculate the volume of Dynabeads required for the application.[a]
2. Mix the contents of the manufacturer's vial very well to fully resuspend the Dynabeads.
3. Transfer the calculated amount to a 10 ml tube.
4. Place the tube on a magnetic particle concentrator (Dynal MPC) for 60 sec (if the amount is small some PBS/FCS may be added to make a workable volume).
5. Pipette off the supernatant and replace with a volume of PBS/FCS.[b]
6. Repeat steps 3 and 4, and finally resuspend back to the original volume with PBS/FCS.

B. *Cell isolation*

1. Collect 5 ml blood in a standard ACD blood collecting tube.
2. Cool blood, buffers, and Dynabeads to 2–4 °C.
3. Add the blood to the Dynabeads directly in the 10 ml tube.
4. Mix *gently* for 30–60 min by tilting and rotation (do not use end-over-end rotation as this may damage the target cells).
5. Add 5 ml PBS pH 7.4 containing 0.6% sodium citrate.
6. Isolate the rosetted cells by placing the tube in the MPC for 2–3 min.
7. Discard the supernatant while the rosetted cells are attracted to the wall of the tube by the magnet.
8. Wash the isolated cells as follows:
   (a) Separate the test-tube and magnet.
   (b) Resuspend the rosetted cells in 5 ml PBS pH 7.4 containing 0.6% sodium citrate by trickling the buffer into the tube on the opposite

side to the line of cells to avoid 'bombing'. Gentle inversion of the tube several times should allow the cells to resuspend completely into the buffer. Vigorous shaking should be avoided.

(c) Replace the tube in the MPC magnet for a minimum of 2 min to collect the rosetted cells.

(d) Discard the supernatant and resuspend the rosetted cells in 5 ml PBS/BSA pH 7.4 without sodium citrate.

(e) Repeat steps 8c and 8d as many times as needed (usually two or three).

(f) Resuspend rosetted cells in the desired volume of culture media or TBSS.[c]

[a] The amount of Dynabeads required in this example is calculated as follows. Normal adult CD4 cell concentration is 1.07×10^6/ml × 5 ml sample = 5.35×10^6 total. Concentration recovered = 1 × 10^7/ml Dynabeads M-450 CD4 at 1.4×10^8/ml (manufacturer's concentration) = 382 μl.

[b] Under normal circumstances the beads will resuspend easily. If, after removing the tube from the magnet, the 'line' of beads does not disperse it is likely that there is an electrostatic interaction between beads and plastic. Pre-incubation of the tube with PBS/FCS or PBS/BSA will prevent this; better still use a better grade of tube!

[c] If the volume for resuspension is small (< 0.5 ml) it may be difficult to collect the cells without loss on the sides of the tube. In such cases resuspend in a larger volume, say 0.5–1 ml, reposition in MPC, and when the line of bead rosettes has formed slowly remove the tube by sliding it vertically across the face of the magnet. The effect is that the bead line concentrates to a button at the edge of the magnet which can be manipulated to the bottom of the tube. Nearly all the supernatant can be removed and the cells resuspended in as little as 10 μl buffer.

Protocol 6. Preparation of antibody pre-coated cells for use in the indirect technique

Equipment and reagents

- 10 ml plastic (or glass) round-bottom tissue culture grade tubes
- Centrifuge
- Target cell
- Antibody
- Phosphate-buffered saline (PBS) pH 7.4: see *Protocol 4*
- Tissue culture media: Hank's balanced salt solution (HBSS) pH 7.4 or similar

Method

1. Incubate the cell sample with sufficient antibody at 4 °C for 30 min.[a]
2. Collect the pre-treated cells by centrifugation at 800 *g* for 10 min and discard the supernatant.
3. Resuspend the cells in HBSS and wash by centrifugation a further two times to remove all unbound antibody.
4. Resuspend the cells in HBSS or similar, and adjust volume to allow the use of 1–2 × 10^7 Dynabeads/ml at a minimum bead:target cell ratio of 4:1.[b]

Protocol 6. *Continued*

5. Isolate the target cells treated with specific antibody with secondary coated immunomagnetic beads, otherwise apply the same technical considerations for direct isolation.

[a] The amount depends on the number of target cells, and the antigen density. Saturating amounts are recommended.
[b] If the target is 10% of the total cell count, the desired bead:target cell ratio is 4:1, and the minimum bead concentration is 1×10^7 beads/ml then: the minimum number of target cells required is $1 \times 10^7/4 = 2.5 \times 10^6$/ml, and the concentration of total cells is 2.5×10^7 total cells/ml.

4.2.4 Detachment of positively selected cells

Many types of analysis (e.g microcytotoxicity) can be performed with the beads still attached to the cell, but in many circumstances it is desirable or necessary to remove them. However, antibody/antigen systems are not easily resolved except under fairly strong conditions which may be harmful to the isolated cell (e.g. acid pH). Passive overnight incubation can lead to detachment of certain cell types; in particular those in which the antigen is likely to cap, and a combination of incubation and shear force through vigorous pipetting can yield good results. Limited proteolysis with enzymes is an alternative but may damage surface markers and compromise further analysis.

Anti-Fab antibodies have been developed which bind to the Fab region of the primary antibody and alter its affinity for antigen producing gentle detachment. The advantage of this system is that after detachment the reacting antibodies remain associated with the beads and unreacted anti-Fab may be washed away, leaving the isolated cell free of original isolating antibody (*Figure 3*).

For all detachment methods the number of beads attached to the isolated cells will influence the efficiency of detachment. Keeping the number of beads per target cell to the minimum will increase the yield of released cells.

Protocol 7. Detachment of beads from positively isolated cells

Equipment and reagents

- DETACHaBEAD (Dynal, Cat. No. 125.03..125.06)
- Apparatus for tilting and rotating of tubes
- Magnetic device: Dynal MPC-1, MPC-2, or MPC-6 (Dynal)
- Pipettes: 5 ml and 1 ml, Pasteur pipettes, vacuum pump and trap (optional)
- $1–10 \times 10^6$ cells isolated with primary coated Dynabeads at a bead:cell ratio of between 4:1 and 10:1
- Tissue culture media (e.g. RPMI 1640) containing 1% FCS
- Phosphate-buffered saline (PBS) pH 7.4: see *Protocol 4*

Method

1. Resuspend the rosetted cells in 100 μl tissue culture medium (e.g. RPMI 1640/FCS) and add 1 U (100 μl) DETACHaBEAD.

2. Incubate for 45–60 min at ambient temperature (22°C) on a tilting and rotation apparatus. Due to the relatively small sample volume, care should be taken that the cells remain in the bottom of the test-tube during agitation.
3. Remove the detached beads by placing the tube on a magnet for 2–3 min.
4. Pipette the cell suspension from the tube while the beads are still attached to the wall of the tube by the magnet.
5. To obtain the residual cells wash the detached beads two or three times in tissue culture media and combine the supernatants.
6. Wash the detached cells two or three times to remove unused DETACHaBEAD.
7. Resuspend the cells in the desired volume of buffer.

4.2.5 Depletion

Depletion is the removal of an unwanted population of cells from a heterologous mixture, leaving behind the desired population for analysis or further purification. Depletion efficiency of 100% and purity of the captured cells (depleting only that which is intended) are the critical factors. Viability of the depleted cell is of less concern. For depletions and applications demanding > 98% yield the optimum bead:cell ratio may be in the range 4–10:1 or greater, and the minimum bead concentration should be 2×10^7/ml. In the example shown in *Figure 4* Dynabeads have been used at a concentration in the range $0–4 \times 10^7$/ml to maximally deplete a cell population in a mononuclear cell preparation. The curves show that, under these conditions, maximum depletion efficiencies are obtained at over 2×10^7/ml. Two or more depletion cycles at a lower ratio are often a good and economical option.

4.3 Human peripheral blood cells isolated using Dynabeads

Table 1 illustrates the use of immunomagnetic separation of human peripheral blood cells. Where known the tissue origin, target antigen, and ligand are stated. The yield or purity are given as per the original referenced texts.

5. Functional approaches

In some instances assays dependent on *in vitro* immune-mediated mechanisms can be used to separate cells. These are often unique to a particular field but may be of use in developing new solutions to a problem.

5.1 Cytotoxicity

Killing of a population of cells is usually dependent on two agents of the immune system, complement and antibody. Complement is not a single entity

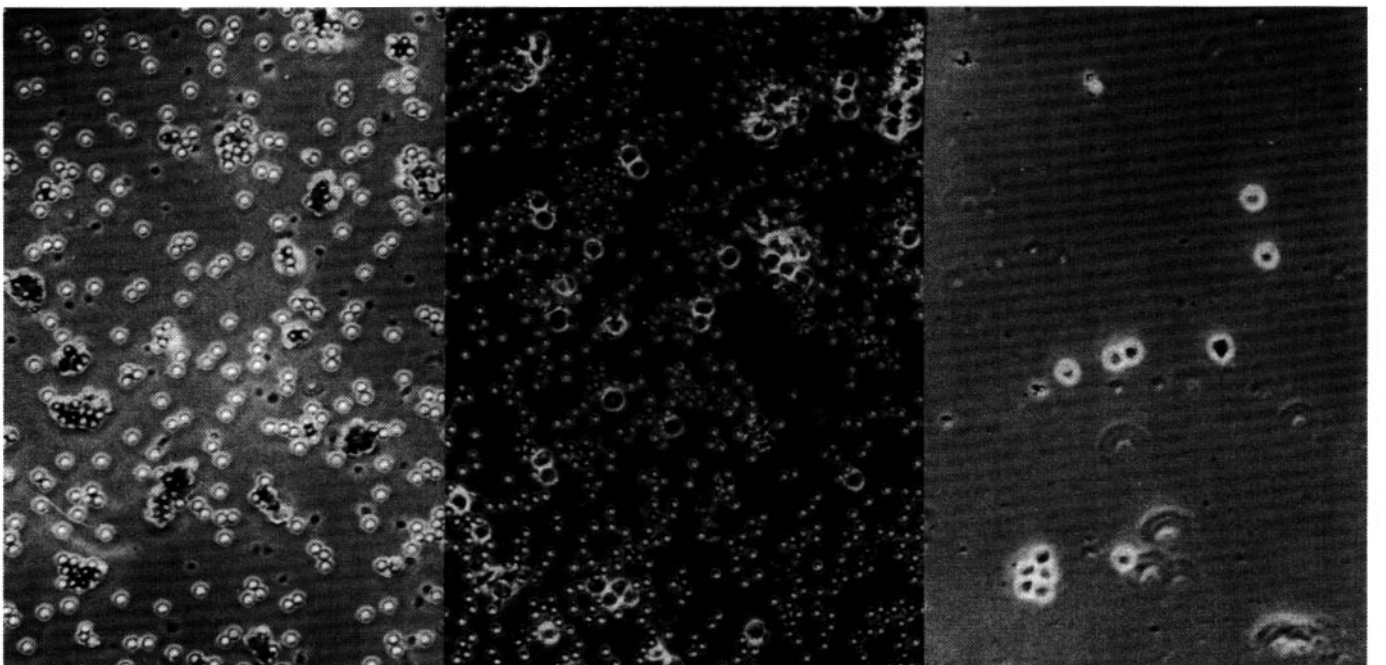

Figure 3. Anti-Fab antibodies (Detachabead, Dynal) are used to release cells from immunomagnetic beads. Left-hand panel: rosetted human CD4 lymphocytes. Centre panel: after the addition of Detachabead the cells are released from the beads. Right-hand panel: applying a magnet removes the beads from the system.

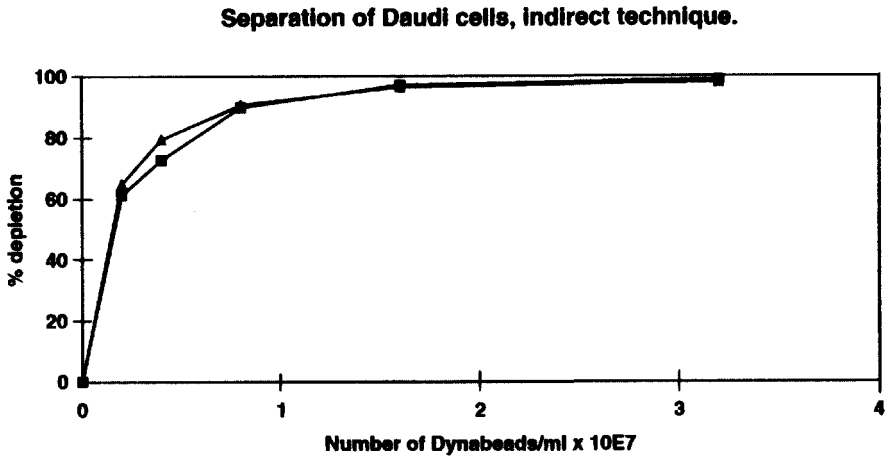

Figure 4. Indirect technique for separation of Daudi cells, titration of Dynabeads concentration. Dynabeads M-450 sheep anti-mouse IgG (squares) and Dynabeads rat anti-mouse IgG (triangles) are compared. Daudi cells pre-treated with IgG2a. Incubation for 10 min at 4°C, and 4 Dynabeads per target cell.

but is a series of molecules present in serum which interact in a cascade effect. The components of the complement system are numbered C1–C9 with associated co-factors. The early molecules initiate activation of complement by binding to receptors on the surface of cells and on antibodies. The *in vivo* function of complement is as an acute phase reactant to non-specifically destroy foreign organisms such as bacteria and virus-infected cells. This mechanism can be exploited to selectively destroy a specific cell population so long as a monoclonal antibody with specificity for that population is available. The monoclonal must also be able to bind complement and is therefore most often of the IgM class. A suitable cytotoxicity assay is shown in *Protocol 8*; several variations of this assay have been developed such as the application to HLA antigen testing or 'tissue typing' (2). Although this method is a microscale assay it is possible to scale up to prepare viable cell populations by negative selection which can be used in further assays.

5.2 Cell phenotyping

There are two reasons why a cell population phenotype may vary: these are due to the presence of subpopulations or to genetic polymorphisms. Immunophenotyping for cell subpopulations is usually performed by flow cytometry for cell suspensions (see Chapter 5) or by immunohistochemistry of tissue sections. The latter technique has been the subject of a comprehensive review (3) and it may be argued that it is a method of differentiating between cell types rather than physically isolating cells. An outline for immunohistochemistry is shown in *Protocol 9*.

Table 1. Human peripheral blood cells isolated using Dynabeads

Cell type	Cell source organ/tissue	Selection mode	Target antigens	Ligand (supplier)	Efficacy yield; purity	Comments	Reference
B lymphocytes	WPB	Positive	CD19	AB1	40–60%; > 95%	Direct; EDTA or ACD collected blood	5
	PBMCs	Negative	CD2, CD14 CD16	(Becton Dickinson) (Medarex, Inc.)	n.d.; > 95%	Indirect	6
	PBMCs	Depletion	CD19 CD20	Leu-12 Leu-16	> 97%; n.d.	Indirect	7
Basophils	PBMCs from Percoll gradient	Negative	CD3, 4, 8, 14, 15, 19	(Dakopatts)	66%; 97%	Direct; PBMC density between 1.070 and 1.080	8
Eosionophils	Leukopheresis	Negative	CD16	3G8	$2–20 \times 10^7$; 95% per donor	Indirect	9
Haematopoietic progenitors	PBMCs, cord blood/marrow leukopheresis	Positive	CD34	ITI-561	35–90%*; 90%	Direct; *35% (PBMCs), 80–95% (cord blood), 50–90% (bone marrow)	5
Large granular lymphocytes	Adherent cell-depeleted PBMCs	Negative	CD14 CD3	Leu-M3 OKT3	n.d.; 80–90%	Indirect	10
Monocytes	E–PBMCs	Negative	CD56 CD19	Leu-19 Leu-12	n.d.; 95%	Direct	11
	PBMCs	Negative	CD2 CD19	BMA 0111 AB1	72%; 85%	Direct; $CD16^+/56^+$ removal may improve purity	12
Megakaryocytes	Filtered bone marrow cells	Positive	GP IIb/IIIa	Plt-1	76%; 98%	Indirect	13
Neutrophils	Leukopheresis	Depletion	CD16	3G8	n.d.; n.d.	Indirect; purifying eosinophils	9
NK cells	PBMCs	Negative	CD4 CD8 CD14 CD19	T4-ATCC RFT8 63D3-ATCC HD37	> 98%; > 80%	Indirect; two cyles of cell removal	14
	PBMCs	(a) Positive—followed by—(b) negative	CD56 CD3	Leu-19 CRL8001-ATCC	5.3%; > 94% PBMCs	Indirect; CD3 depletion after O/N detachment	15

Platelets	PBMCs	Positive	GPIIb/IIIa	ITI-PI-1	n.d.; n.d.	Direct; EDTA as anticoagulant	16
	Platelet-enriched plasma	Negative	CD45	Anti-LCA (Immunotech)	90% depletion of lymphocytes	Direct	17
Reticulocytes	Leucocyte-depleted WPB	Positive	CD71 (Tfr)	ITI-2B4	15–42%; > 98%	Direct; > 99% filter depletion of leucocytes	18
T lymphocytes	WPB	Positive	CD2	BMA 0111	> 90%; > 99%	Direct; EDTA/ACD as anticoagulant	5
T lymphocytes	Adherent cell-depleted PBMCs	Negative	CD14 CD16 CD19 CD56	Leu-M3 Leu-11a Leu-12 Leu-19	n.d.; 97–99%	Indirect	19
– $CD4^+$	WPB	Positive	CD4	66.1	> 80%; > 95%	Direct	5
	Adherent cell-depleted PBMCs	Negative	CD8, 14, 19 CD16, 56	(Dakopatts) (Immunotech)	n.d.; > 96%	Indirect	20
	PBMCs	Depletion	CD4	ST4	> 99% depletion	Direct	5
– $CD8^+$	WPB	Positive	CD8	ITI-5C2	> 80%; > 95%	Direct; EDTA/ACD as anticoagulant	5
	PBMCs	Depletion	CD8	ITI-5C2	> 99% depletion	Direct	5
– $CD4^+$ memory	Lymphocytes isolated from PBMCs by elutriation	Negative	CD8 CD45RA CD11b CD14 CD16 CD19 HLA class II	CLB-T8/4 2H4 OKM1 CLB-CD14 CLB-FcR gran1 CLB-CD19 E-1	n.d.; > 96%	Indirect	21
– activated	PBMCs	Depletion	CD25	anti-Tac	n.d.; n.d.	Direct	22
	Ag stimulated PBMCs	Positive	CD25	anti-Tac	24–74% Ag responding clones*	Direct; *yield varies with antigen used	22
T lymphocytes – antigen-specific	PBMCs	Positive	TCR	Antigent-presenting cells with internalised magnetic beads		APCs allow to phagocytose beads	23
Tumour infiltrating lymphocytes	Density gradient separated glioma digests	Positive	CD2 CD3 α/β TCR	BMA 0110 IOT3 BHA 031	n.d.; n.d.	Direct; each mAb used separately	24

Protocol 8. Separation of specific cell population by cytotoxicity

Equipment and reagents

- Terasaki trays (Robbins Scientific, 1003–00–0)
- Complement from guinea-pig or rabbit: store lyophilized at 4 °C
- Monoclonal antibody with specificity for cell to be killed
- Staining cocktail: 1 vol. ethidium bromide (0.1% (w/v) in PBS; Sigma, E8751), 2 vol. acridine orange (0.1% (w/v) in PBS; Sigma, A6014), 5% EDTA (w/v), 3 vol. black calligraphy ink (10% in PBS; Staedtler, 745)—store in dark at 4 °C and mix immediately before use

A. *Target cell isolation and assessment of viability*

1. Obtain a pure target cell population (see Chapter 1).
2. Resuspend target cells to a concentration of 4×10^6/ml.
3. Assess target cell viability as follows:
   (a) Pre-load Terasaki plate wells with tissue culture paraffin oil. Mix 1 μl volumes target cell suspension and staining cocktail in Terasaki plate well.
   (b) Centrifuge Terasaki plate at 500 *g* for 5 sec.
   (c) Observe staining pattern using an inverted microscope with fluorescence lamp attachment, e.g. quartz-halogen bulb and blue light filter. Viable cells stain green, dead cells stain orange/red. Cell viability must exceed 80%.

B. *Cytotoxicity assay*

1. Establish highest titre of monoclonal antibody which gives 100% target cell killing in a cytotoxic test. Use monoclonal antibody at one dilution lower titre.
2. Dispense 1 μl volumes monoclonal antibody at working dilution into Terasaki plate wells under oil. Pre-prepared plates may be stored frozen.
3. Add 1 μl target cell suspension at 4×10^6 target cells/ml to plate wells.
4. Centrifuge as in part A, step 3b.
5. Incubate for 30 min at 22 °C.
6. Add 4 μl complement to each well. The working dilution of complement should be established in the same manner as the working dilution for monoclonal antibody.
7. Centrifuge as in part A, step 3b.
8. Incubate for 60 min at 22 °C.
9. Add 1 μl staining cocktail. Centrifuge as in part A, step 3b. Assess per cent killing as in part A, step 3c.

Protocol 9. Separation of cells in immunohistochemistry

Reagents

- Monoclonal antibody of known specificity (CD phenotype)
- Frozen tissue sections

Method

1. Cut frozen sections approx. 6 μm thick, air dry to glass slides for 30 min, then fix in acetone for 10 min.
2. Incubate with 50 μl specific monoclonal antibody for 45 min.
3. Wash gently in TBS for 5 min.
4. Incubate with second antibody, usually fluorochrome labelled rabbit anti-mouse immunoglobulin.
5. Repeat steps 2 and 3.
6. View stained section with fluorescence microscope.

Separation of cells on the basis of genetic polymorphisms by immunomethods is exemplified by HLA typing. Histocompatibility laboratories exist to match cadaver organ donors and recipients for genetic polymorphisms coded by the human major histocompatibility complex on chromosome 6; this is the most polymorphic genetic region in man. Products of HLA genes are expressed as cell surface glycoproteins on most body cells; in organ transplants these antigens are recognized by the host immune system as 'foreign' and may be the target of rejection processes. To prevent rejection, organs are allocated when HLA antigens of donor and recipient are similar. Typing for HLA antigens is carried out using a cytotoxic assay in which the antibodies define genetically encoded specificities. Not unexpectedly such assays are being superseded by molecular biological methods which define gene polymorphisms at the DNA level.

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7

Separation of cells using free flow electrophoresis

P. EGGLETON

1. Introduction

The principal physical characteristics that have been exploited for cell separation techniques are their size (volume), density, or surface electrical charge. If cells differ sufficiently in density, then density gradient sedimentation techniques should be used (see Chapter 2). Cells which differ in size or volume can be separated by countercurrent centrifugal elutriation (see Chapter 3). As all mammalian cells carry a net surface negative charge at physiological pH, free flow electrophoresis (FFE) is able to maximize subtle differences in the charge of cell surface molecules leading to the separation of cells from one another. As the electrophoretic separation normally occurs in a continuous flowing buffer which exceeds 1 *g* sedimentation, within certain limits, the size or density of the cell is not normally influential in the separation. Therefore free flow electrophoresis is particularly useful in separating cells which differ only very slightly in size or density from one another. FFE is commonly employed to further separate purified cells into subpopulations which differ in net surface charge characteristics. As shown in *Table 1*, a number of diverse cell types have been separated by FFE. Once separated and collected the cells can undergo further analysis. FFE also can be exploited to monitor changes in the electrophoretic mobility of a single population of cells which have been treated in some way (e.g. metabolically activated, neuraminidase treated, or surface labelled). In this chapter, the isolation of subpopulations of cells by FFE will be described, and ways in which the technique can be used to examine changes in the physicochemical status of separated cells will be discussed.

1.1 Factors governing the electrophoretic mobility of cells during electrophoresis

The cell surface charge characteristics exploited in FFE do not directly reflect the electrochemical composition at the membrane bilayer/environment interface, since in an ionic medium a layer of ions of opposite charge surrounds the cell forming an electrical double layer. Some of these positive ions neutralize

Table 1. Application of free flow electrophoresis to study various cell types

Cell type	Reference	Cell type	Reference
Blood		**Somatic**	
Lymphocytes	16		
Neutrophils	12	Hepatocytes	14
Erythrocytes	15	Fibroblasts	3
Platelets	11	Kidney	4
Monocytes	17	**Germline**	
Bone marrow	18	Spermatozoa	19
Tumour cells		**Prokaryotic**	
Ehrlich ascites	20	*E. coli*	21
Mast cell ascites	5	*Streptococci sp.*	22
Leukaemic	23	*Stap. aureus*	24
Protozoa			
Trypanosomes	25		
Plasmodium sp.	26		

cell membrane charge moieties, and the apparent cell surface charge at the shear plane of the electrokinetic zone and the medium is referred to as the 'zeta potential'.

The extent to which a cell is deflected towards the anode as a function of its surface zeta potential, the applied electrical field, and the rate of buffer flow has been described mathematically. The equation is as follows:

$$\text{Apparent electrophoretic mobility } U = \frac{l \times A \times K}{I \times t}$$

where: l = migration distance (cm), A = cross-sectional area of chamber (cm^2), K = specific conductivity of medium (Siemens cm^{-1}), I = current (A), t = residence time in chamber (sec):

$$t = \frac{2 \times Vk}{3 \times Qk}$$

where: Vk = chamber volume (cm^3) and Qk = chamber flow rate (cm^3/sec).

1.2 The main types of free flow electrophoresis equipment

1.2.1 VAP, ACE, and McDonnell–Douglas instruments

Three types of free flow electrophoresis apparatus have been frequently employed for analytical purposes. The most common type used in cell separation work is the Elphor VAP (Bender Hobein) machines which were originally developed by Kurt Hannig and colleagues at the Max-Plank-Institut for Biochemistry. Several models have been used successfully including the VAP 5, VAP 11, VAP 21, and VAP 22. Another type, the ACE 710 was formally manufactured by Hirschmann (Munich, Germany) and appeared more compact,

with a smaller separation chamber (12 cm high, 3 cm wide, and 0.03 cm thick). Near the base of the separation chamber it had a UV permeable window though which a sensitive video densitometer measuring system connected to a monitor allowed for examination of proteins or cells as they passed from the chamber into collection tubes, thus allowing identification of the location of the cell fractions, as well as detecting the formation of any cell aggregates. A similar optical window for densitometric evaluation of separated protein or cell bands can also be fitted to the more modern VAP machines (e.g. VAP 22). The third type of continuous flow electrophoresis system (CFES), manufactured by McDonnell–Douglas, has a separation chamber which is 6 cm wide allowing 197 discrete cell fractions to be collected after their passage through the chamber.

The VAP instruments have been most frequently used for cell separations and the methods and comments in this chapter specifically apply to machines originally manufactured by Bender Hobein (but now serviced by Dr Weber GmbH, Ismaning, Germany), because these have been in regular use in the authors' laboratory. None the less, the majority of remarks still apply to other manufacturers' machines which employ similar principles. The Elphor VAP 22 instrument will be described in detail (*Figure 1*). There are a number of pieces of apparatus which make up the free flow electrophoresis machine, and these can generally be divided into four separate units.

(a) The control unit, which is the major electronic module of the apparatus, regulates chamber and electrode buffer flow rates, temperatures, and the voltage and current applied across the separation chamber. It also houses reservoirs of electrode buffer media which are transported to the electrode compartments which border both edges of the separation chamber. Continual circulation of the electrode buffer over the electrodes minimizes changes in pH and ionic composition of the buffer. Also housed in the control unit are a number of safety circuits informing the operator of electrical and fluid leaks, overheating, or surges in current or voltage.

(b) The separation chamber consists of a mirrored glass inner and plastic outer chamber wall, forming a chamber 50 cm high, 10 cm wide, and 0.05 cm gap between them, down which flows a continuous film of chamber buffer from a reservoir placed on top of the chamber. The chamber buffer flows down between the two electrode compartments and exits through 96 ports into collection tubes, in a fraction collection chamber that can also be temperature controlled. The flow rate of the chamber buffer is controlled by a peristaltic pump, situated two-thirds of the way down the separation chamber, which can be adjusted by the hexagonal rotary clamp on the front of the chamber.

(c) The sample injection unit situated on top of the chamber unit, incorporates a glass Hamilton syringe within a cooling chamber which also rotates to maintain the cells in suspension during their passage into the chamber. A second syringe connected to it and drawn by a syringe pump, controls

the sample injection rate into the downward flowing chamber buffer. The sample can enter the chamber from one of four desired inlet ports of entry across the chamber and can be readily visualized against the silver rear wall of the chamber.

(d) The cooling system pumps cooler fluid behind the mirrored plate in the sample chamber and the gap through which the sample buffer flows between this and the outer plate is only 0.05 cm, consequently, the heat generated by the electrical current is removed rapidly and efficiently, preventing disturbance of the laminar flow. The temperature can be set between 4 °C and 37 °C.

1.2.2 OCTOPUS-PZE

The OCTOPUS free flow electrophoresis instrument borrows several design features from the older and more established generation of VAP machines, in appearance it is not dissimilar to the VAP 22, but as shown in *Figure 2* it is more compact. The dimensions of the separation chamber are the same as that used in the VAP 22 and still allows for 96 fractions to be collected. However the separation procedure does differ in some unique ways:

(a) The cell sample and chamber buffer are injected into the bottom of the chamber, rather than the top. The separation medium flow upwards is controlled by a manifold pump.

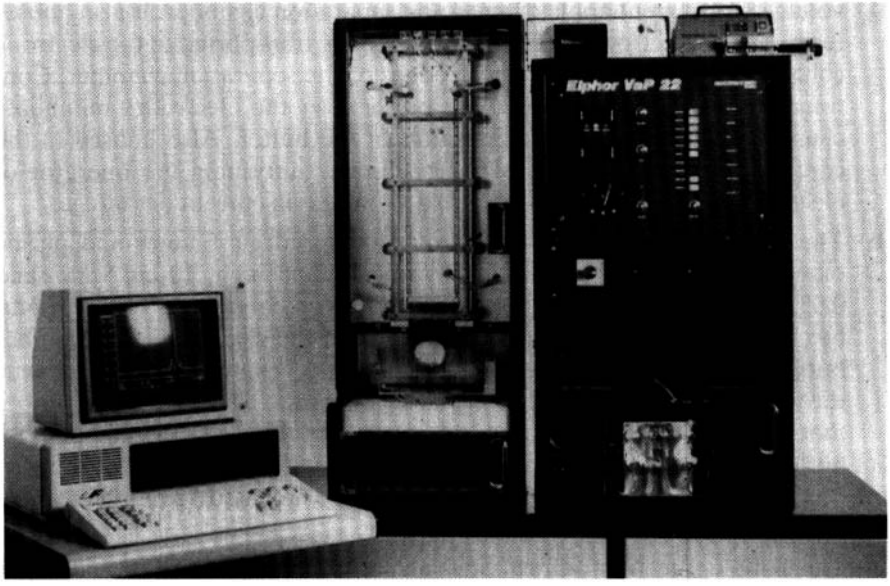

Figure 1. Elphor VAP 22. Complete view of FFE apparatus showing from right to left, the control unit, separation chamber, and visual display unit. The sample injection unit is situated on top of the control unit.

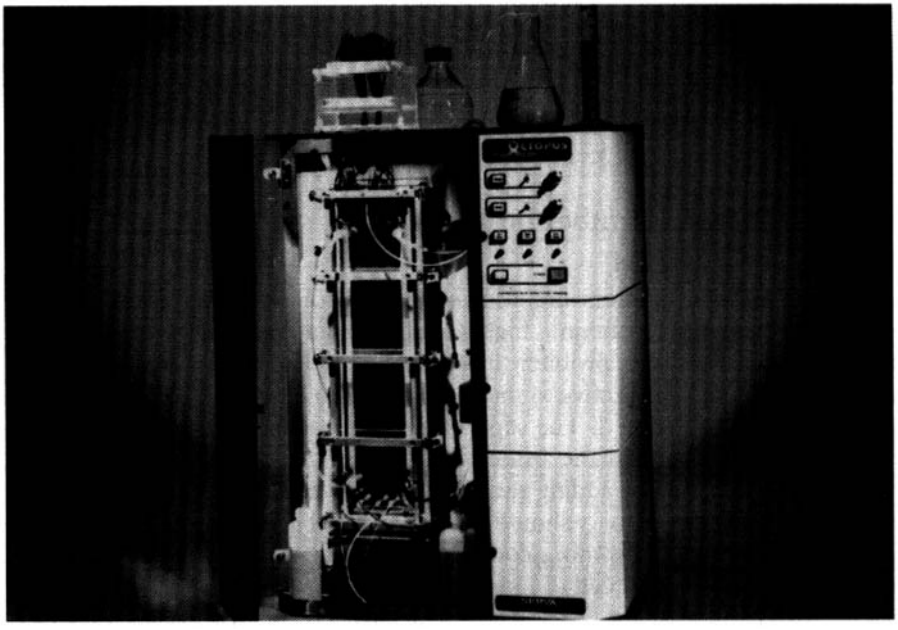

Figure 2. OCTOPUS-PZE apparatus showing separation chamber and control unit.

(b) A counterflow medium is introduced at the top of the chamber to enhance separation.

(c) Cell separation on the basis of differences in surface zeta potential is enhanced still further by the infusion of a high conductivity medium—stabilization medium, which is allowed to run parallel to the electrodes within the separation chamber.

One problem with older versions of free flow electrophoresis apparatus is retaining ions in the vicinity of the electrodes in sufficient consistency and quantity to allow for good separation. Seepage of ions through the electrode membrane into the separation chamber can sometimes lead to inconsistencies in separation. The OCTOPUS-PZE method supplies additional ions in the form of stabilization media actually in the separation chamber nearby the electrodes, ensuring sufficient quantities of ions are present during separation.

2. Buffers

An ongoing concern with most whole cell separation techniques is the effect that the separation media may have on cell viability and function. Free flow electrophoresis is a relatively gentle cell separation technique. A number of buffers have been developed with the aim of maintaining the cells in a viable

state, while enhancing the separation resolution within the constraints of the apparatus design. Some of the most common buffers used to separate cells by FFE are described below. No one buffer is best, and some empirical analysis may have to be performed to optimize the buffer system of choice to suit the cells to be separated.

2.1 Separation chamber buffers

2.1.1 Low ionic media

Low ionic strength buffers are routinely used for cell separation by electrophoresis in order to obtain a high voltage with a low heat production, this latter factor is less of a problem in modern day FFE apparatus which are efficiently cooled. Irrespective of the type of cells to be separated, most buffers are triethanolamine-based. The separation buffer should be of physiological pH between 7.0–7.4, and the osmolarity is normally maintained within the range 0.28–0.33 Osmol by adding glycine, glucose, sucrose, or an alternative non-ionic sugar. The conductivity should be between 800 μS/cm and 200 μS/cm, the lower end of this range is more common. Some empirical experimentation is often required with each cell of interest before a final buffer recipe is decided upon. In particular, if the cells of interest have not been separated by FFE before, they should be suspended in the chosen separation buffer for periods of time and some aspect of their functional or biochemical status monitored, in order to ensure the buffer does not activate or affect any of the cell's metabolic pathways which may wished to be assayed after separation. Generally, sugar-based buffers containing low concentrations of triethanolamine, and a pH of 7.2–7.4 are suitable for most separations, also they have not been reported to impair cell function. Examples of commonly used sample chamber and electrode buffers are listed in *Table 2*. The use of sodium chloride in separation buffers is controversial, several research groups are reluctant to use it because ions such as chloride are changed by electrical current into hypochlorite, which is toxic to cells. Conversely, the inclusion of 50 mM calcium has been suggested to enhance cell separation. The exposure of cells to triethanolamine-based buffers should nevertheless be kept to a minimum. Standard FFE buffers have been shown to buffer poorly at the pH employed in most separation studies and it has been recommended that zwitterionic buffers are used instead. The residency time of cells in the separation chamber can be as little as two minutes, depending on the chamber buffer flow rate chosen. Realistically, the cells once suspended in buffer can be separated, fractionated, washed, and removed from chamber buffer of choice within two hours.

2.1.2 Triethanolamine-free media

There is some debate concerning the use of physiological buffers for cell separation, especially those containing chloride ions. However the use of such media have been designed for use in the new OCTOPUS-PZE process. The

Table 2. Chamber and electrode buffers used in VAP instruments during FFE of various cell types

Cell type	Chamber buffer	mM	Electrode buffer	mM	Machine	Reference
Lymphocytes	Triethanolamine	15	Triethanolamine	75	FFIV	27
	K acetate	4	K acetate	20		
	Glycine	240				
	Glucose	11				
Platelets	Triethanolamine	10	Triethanolamine	100	VAP 5	11
	Glycine	280				
Neutrophils	Triethanolamine	280	Triethanolamine	100	VAP 22	12
	Glucose	30				
Kidney proximal tubule	Sucrose	210	Triethanolamine	100	VAP 21	4
	Glycine	100				
	Na_2HPO_4	4				
	NaH_2PO_4	1				
	Glucose	5				
	$CaCl_2$	1				
	$MgCl_2$	0.025				
	5 mg/litre BSA					
Malarial parasites	Triethanolamine	10	Triethanolamine	110	VAP 5	26
	Acetic acid	10	Acetic acid	110		
	$MgSO_4$	0.1				
	Sucrose	250				

new media, which comprises of a sodium chloride-based buffered saline, combines the use of iso-osmolal glucose, glycine, and sucrose in the volume ratio 1:1.3:1:0.7 respectively and thus avoids altogether the use of triethanolamine. This new buffer in combination with the OCTOPUS-PZE process has been used to successfully separate mixed populations of lymphocytes. As shown in *Figure 3A*, lymphocytes separated by the OCTOPUS-PZE procedure appear as a broad profile separating over eight fractions with two distinct peaks. When each individual fraction is examined as shown in *Figure 3B*, isolated T and B lymphocytes of sufficient purity can be achieved (B. Bondy, B. Bauer, I. Seuffert, and G. Weber, personal communication). In addition, buffer Q is a commercially prepared zwitterionic buffer (Ampholite Technologies, Inc.), which contains glycine, glucose, sorbitol, and Mg^{2+} and Ca^{2+} ions, with an adjustable pH between 7.0–7.8, and adjustable conductivity range of 90–200 μS, and this too has been recommended for cell separation by free flow electrophoresis.

2.2 Electrode chamber buffers

Unlike the separation chamber buffers which come into contact with cells, the electrode buffers are contained within the channels of the electrode chambers behind filter membranes, consequently the conductivity of the buffer is more critical than the osmolarity. Normally, electrode buffers with conductivity

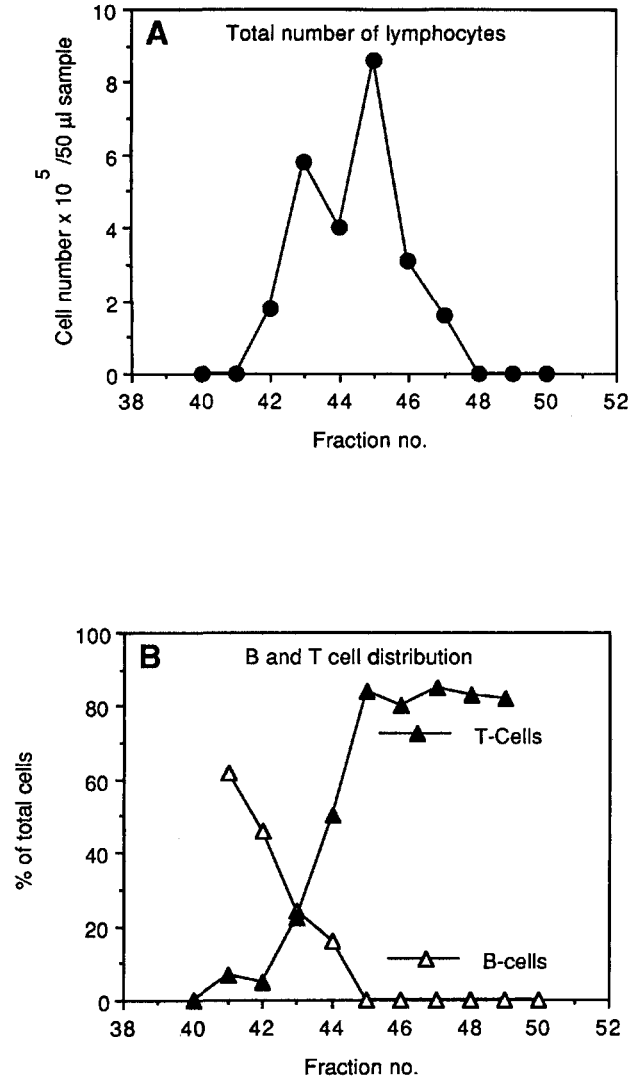

Figure 3. Separation of T and B lymphocytes in a sodium chloride-based buffer using the OCTOPUS-PZE free flow electrophoresis apparatus. (A) Total distribution of lymphocytes. (B) B and T cell distribution.

values five to ten times greater than the separation buffers are used in order to supply sufficient ions to aid separation of the cells as they pass down through the separation chamber. The rate and type of ion supply is dependent somewhat on the membrane system employed (i.e. ion exchange or filter membrane). The quality of the membrane can also affect separation (see Section

Table 3. Composition of electrode and stabilization buffer used in the OCTOPUS instrument for cell separation

	Electrode buffer		Stabilization buffer	
	g/litre	mM	g/litre	mM
Anodal media[a]				
Phosphoric acid	19.6	200	9.8	100
Glycine		–	5.6	75
Sucrose		–	17.1	50
Cathodal media[b]				
Tris–HCl	23.6	150	11.8	75
NaCl	8.8	150	4.4	75
Glycine		–	5.6	75
Sucrose		–	17.1	50

[a] Anodal media are adjusted to pH 7.4 with NaOH.
[b] Cathodal media are adjusted to pH 7.4 with HCl.

6.6). Nevertheless most workers have employed electrode buffers which are triethanolamine-based (see *Table 2*) for use in VAP instruments.

The OCTOPUS-PZE process attempts to improve cell separation by preventing fluctuations in ion concentrations at the intersection between the electrode and cell separation chamber. It achieves this by employing a third buffer called the 'stabilization' buffer. The use of stabilization media allows a more homogeneous supply of ions in the area near the membranes inside the separation chamber, this affords better separation of cells in accordance with the theoretical aspects of cell separation by FFE. As show in *Table 3*, the electrode and stabilization media are custom designed for use with each individual electrode. Furthermore, unlike the electrode buffers used in the former VAP instruments, they do not contain triethanolamine.

3. Preparation of cell samples for free flow electrophoresis

3.1 Types of cells separated by FFE

Many cell types have been successfully separated by free flow electrophoresis (*Table 1*). It is important that the cells are suspended in a medium which allows the cells to remain in a stable state and at a concentration that will prevent or discourage cell aggregation. As with most cell separation techniques, the cells to be separated must be in a good physical and metabolic state. FFE has been used mostly to study blood cells which reflects the initial ease by which these cells can be isolated by standard density gradient techniques.

However, cells from other tissues are often obtained by mechanical disruption, enzymatic treatment, or a combination of both. This can lead to changes in the cell surface charge, receptor status, and overall protein composition which may interfere with the subsequent electrokinetic properties of the cell surface membrane upon separation by FFE. Nevertheless, a number of laboratories have successfully employed FFE to study hepatocytes (2), fibroblasts (3), and kidney cells (4). In addition, protozoa, bacteria, and tumour or immortal cell lines have been studied. Another limiting step in the successful separation of different cell types is the concentration of the sample, the cells should be of sufficient number to detect them in individual fractions by conventional means, and starting concentrations of 2–5 $\times$ 10^7 cells/ml are optimum, but fewer cells have commonly been used.

3.2 Purification and preparation of cell samples before FFE

Most purified cell preparations can be suspended in media commonly used to maintain the cells such as PBS or Hank's balanced salt solution (HBSS) without calcium and magnesium, and they can be placed in sample buffer and adjusted to the appropriate concentration just prior to electrophoresis. However, the following considerations outlined in *Protocol 1* are important for successful separation of specific cell types, particularly suited for separation by FFE. Depending on the cell type and its potential to aggregate due to temperature fluctuations, a temperature should be chosen at which the cells do not undergo thermal shock or activation. The general rule is that most cells which can be prepared at 4 °C should be separated by FFE at a slightly higher temperature, preferably 7–10 °C. Whereas temperature-sensitive cells such as tissue culture cells or platelets should be maintained at a higher temperature before, during, and after FFE. Cells prepared from tissues by enzymatic digestion should be filtered to remove debris. Depending on the objectives of a particular experiment, the choice of a particular cell preparation technique is important. For instance in cell heterogeneity studies, the initial purification step must provide high yields of cells. In such studies where platelet and neutrophil subpopulation heterogeneity have been investigated, standard neutrophil and platelet purification techniques have been modified to enhance cell yields prior to free flow electrophoresis to prevent selective loss of subpopulations of cells. In investigations in which cells differ very little in any physical characteristics, subsequent modification of the surface of the cells is required before FFE is undertaken. An excellent way to achieve this is to pre-label the cells of interest with monoclonal antibodies specific for one subset of cells. As antibodies have a lower net negative charge than cell membranes, labelling cells with immunoglobulins effectively lowers the electrophoretic mobility of the cells, allowing resolution of the two or more populations of cells.

Protocol 1. Purification and preparation of various cell types for FFE

Equipment and reagents

- Bench centrifuge
- Glass beads
- Gauze
- Nitex (36 μm and 48 μm) (TETKO, Inc.)
- Plastic conical-bottom Universal tubes (25 mm × 90 mm)
- Hypaque-Ficoll
- 10% fetal calf serum in minimum essential medium
- 0.25% trypsin in minimum essential medium
- Hank's balanced salt solution (HBSS) with 5 U/ml heparin
- Puck G (Difco)
- MSL® (density 1.077 g/ml, Mediapharm)
- Monoclonal antibody OKT8 (Ortho)
- Rabbit anti-human IgM antiserum (Behringwerke)
- Tetrarhodamine isothiocyanate (TRITC)-conjugated goat anti-rabbit IgG immunoglobulin (Nordic Immunology)
- Rabbit anti-mouse IgG immunoglobulin (Nordic Immunology)
- CDP–adenine (Sigma)
- Citrate buffer: 36 mM citric acid, 5 mM glucose, 5 mM KCl, 90 mM NaCl, 10 mM EDTA, adjusted to pH 6.5 with NaOH
- Taxol (Calbiochem)
- Isotonic ammonium chloride
- Hank's balanced salt solution (HBSS)
- EDTA

A. *Ascites tumour cells (5)*

1. Harvest ascitic tumours from the peritoneal cavity of mice by repeated washing with 3 ml aliquots of 10% fetal calf serum (FCS) in minimum essential medium at 4°C.
2. Centrifuge the cells at 97 *g*, for 8 min at 4°C. Wash three times in FFE sample chamber buffer and adjust cell concentration to 1–2 × 10^7 cells/ml.
3. Filter cells through a layer of Nitex (48 μm) to remove aggregates. Maintain cells at 4°C until separated.

B. *Kidney cells (6)*

1. Perfuse rat kidneys with HBSS containing 5 U/ml heparin, excise, and remove the papillae. Take the remaining tissue and mince to fragments of 1–2 mm. Then agitate them for 10 min each in three changes of minimum essential medium containing 10% fetal calf serum.
2. Discard the supernatants from the wash fluids after allowing the tissue to settle, then disaggregate the kidney fragments in ten times 50 ml changes of minimum essential medium containing 0.25% (v/v) trypsin at room temperature of 20 min durations.
3. Collect the cells from the third to tenth trypsin supernatants, cool to 4°C, and recover the cells at 97 *g* for 8 min.
4. Filter cells through a layer of Nitex (36 μm) to remove aggregates. Adjust cell concentration to 1–2 × 10^7 cells/ml in sample chamber buffer. Maintain cells at 4°C until separated.

Protocol 1. *Continued*

C. *B and T lymphocytes (7)*

1. Collect 200 ml human peripheral blood and defibrinate by gentle shaking in 50 ml tubes with 20 glass beads.
2. Remove the clot by filtration through gauze, centrifuge the cells at 200 *g* for 10 min at room temperature, and discard the platelet containing supernatant.
3. Prepare lymphocyte-rich cells by Ficoll-hypaque density centrifugation. Resuspend the cells in three volumes of Puck G (Difco). Then place two volumes of cells on top of one volume of MSL (1.077 g/ml) and centrifuge for 30 min at 400 *g* at room temperature. Remove the cell band and wash three times in cold Puck G medium by centrifugation for 10 min at 100 *g*. Concentrate the lymphocytes to 5×10^7 cells/ml.
4. Maintain cells at 4°C and incubate for 20 min with the appropriate dilutions of either rabbit anti-human IgM to identify B lymphocytes; mouse anti-human OKT8 to label $CD8^+$ T cells; mouse anti-human OKT4 to label $CD4^+$ cells. Wash twice in Puck G for 7 min at 100 *g*.
5. Second antibody label the appropriate cells using tetrarhodamine isothiocyanate (TRITC)-conjugated goat anti-rabbit and rabbit anti-mouse IgG immunoglobulin for 20 min at 4°C, then wash twice.
6. Adjust the cell concentration to 5×10^7 cells/ml. Maintain the antibody treated cells at 4°C, and place in sample chamber buffer before FFE.

D. *Platelets (8)*

1. Centrifuge 60 ml of CDP–adenine anticoagulated whole blood at 120 *g* for 20 min at room temperature. Remove the platelet-rich plasma.
2. Resuspend the remaining red cell layer in citrate buffer to the original whole blood volume and recentrifuge as before. Collect the supernatant containing additional platelets and add to the platelet-rich plasma pool. Repeat this procedure once more.
3. Resuspend platelets in citrate buffer to a cell volume ratio of 1:3 and wash twice. Ensure yield of platelets is in the order of 90–95%, which is greater than that achieved by conventionally prepared platelet-rich plasma techniques (approx. 70%).
4. Suspend cells in citrate buffer containing 10^{-5} M taxol to help maintain their discoidicity, for 25–30 min. Wash the cells in citrate buffer to remove excess taxol, and then resuspend the platelets in chamber buffer before FFE.

E. *Neutrophils (9)*

1. Mix one volume of EDTA (1.5 mg/ml blood) anticoagulated whole blood with four volumes cold (4°C) isotonic ammonium chloride, mix,

and leave for 15 min to permit erythrocyte haemolysis. Pellet the leucocytes at 160 *g* for 10 min in a plastic Universal tube.

2. Resuspend the leucocytes in 10 ml HBSS and centrifuge at 55 *g* for 10 min. Discard the supernatant and repeat the washing procedure. The yield of resulting neutrophils should be in the order of 70% and approx. 80% pure.[a]
3. Suspend the cells in chamber buffer prior to use.

[a] *This method produces a substantially higher yield of neutrophils than either dextran sedimentation* (50% yield) or dextran sedimentation and Ficoll-hypaque centrifugation combined (15–20% yield) generate.

4. Preparation and application of free flow electrophoresis for separation of whole cells

4.1 Electrode chamber

The electrode chambers are located on either side of the front panel of the separation chamber as narrow canals, which each contain a platinum electrode. Overlaid on top of each of these is a gasket of red silicone rubber on which a 250 μm filter membrane lies and a strip of electrophoresis paper, 0.8–1 mm thickness (*Figure 4*). Filter membranes are used for most cell separation work, rather than ion exchange membranes. Filter membranes are permeable to both anions and cations and accommodate the high currents of 1000 mA or more which are often applied across the separation chamber during cell separation procedures. Cellulose acetate membranes are available which are more resistant to drying out and micro-organism contamination. The electrode buffer reservoirs are housed below the control unit. They consist of two glass chromatography tanks each with a 2 litre capacity. Clear plastic tubing leads from one tank, passes through a filter, and is then pumped at a fixed flow rate through one and then the other electrode channel on either side of the separation chamber at a rate sufficient to dissipate the heat created by the electrodes. The buffer leaves the electrodes via another plastic pipe and finally returns to the second tank of electrode buffer. A second series of pipes transfers buffer from this second tank back to the first, ensuring the electrode buffer is well mixed. The electrode buffer pump is switched on at the control panel and allowed to circulate through the electrode chambers before the separation chamber is allowed to fill with buffer. This is to ensure that there is no visible leakage of electrode buffer into the separation chamber. The preparation and use of a typical electrode buffer acceptable for most cell separations performed in VAP machines is described in *Protocol 2*.

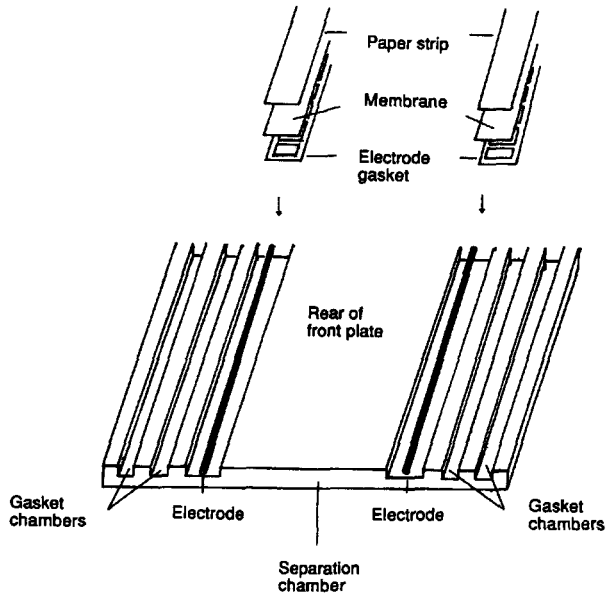

Figure 4. Schematic diagram of the positioning of the membranes over the electrodes within the separation chamber. A rubber gasket is placed over the platinum electrodes, followed by the membrane, and finally a paper strip.

Protocol 2. Preparation of electrode buffer and pre-run check

Equipment and reagents

- Osmometer
- Conductivity meter
- pH meter
- Glacial acetic acid
- 100 mM triethanolamine

A. *Preparation of electrode buffer*

1. Prepare 100 mM triethanolamine by adding 59.68 g triethanolamine to 4 litre distilled water.
2. Check the initial pH is in the range pH 9.8–10.2. Adjust pH to 7.35 with glacial acetic acid.
3. Remove 20 ml and determine the conductivity of the buffer, which should be in the range 0.6–0.65 $\times$ 10^3 μS/cm.
4. Check the osmolarity of the buffer which should be approx. 180 mOsm/kg.

B. *Pre-run of electrode buffer*

1. Place 2 litres of electrode buffer in each electrode buffer reservoir.

2. Press the EL BUFFER switch on the control panel and allow the buffer to circulate for about 10 min. During this time check the following:
   (a) Leakage of electrode buffer into separation chamber.
   (b) Ensure all tubing leading from buffer reservoirs are secure and not leaking.
   (c) Monitor leak safety light, which will come on if a drop in electrode buffer is detected.

4.2 Separation chamber

Free flow electrophoresis separation chamber (*Figure 5*) consists of the chamber itself, an inlet tube, and 96 outlet tubes. None of these items are sterile. The single inlet tubing leads from the sample syringe through the outer plastic chamber and into the 0.5 mm gap between the two plates. The outlet tubes are positioned at the base of the separation chamber and consist of 100 silica rubber small bore tubes (0.5 mm internal diameter) leading from the chamber to a fraction collection unit. These are all sites of possible

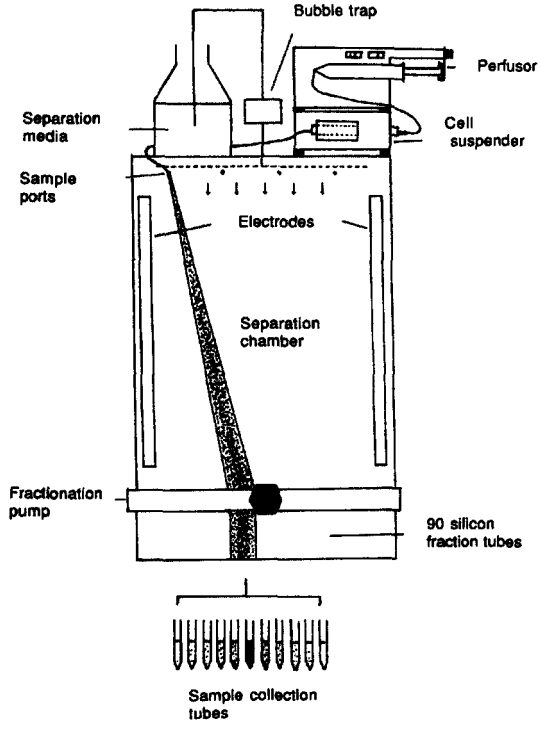

Figure 5. Graphic representation of cells flowing through the separation chamber of a free flow electrophoresis apparatus.

contamination and blockage due to cell aggregation or salt deposits and must be cleaned and kept unblocked during a FFE run. The chamber and tubing is cleaned with a mild detergent, e.g. Teepol, and then the chamber is coated with 3% BSA which prevents cells sticking to the chamber and enhances separation. The protocol for maintaining the separation chamber clean and free of blockages is outlined in *Protocol 3*.

Protocol 3. Cleaning and preparing the separation chamber for use

Equipment and reagents

- 60 ml syringe and blunt-ended 19 gauge needle
- 1 litre 2% (v/v) Teepol in distilled water
- Chamber buffer (see *Table 2*)
- 60 ml 3% (w/v) bovine serum albumin (Sigma)

A. *Cleaning the chamber*

1. Turn the hexagonal rotary clamp situated three-quarters of the way down the separation chamber 20° clockwise to prevent liquid from flowing down through of the separation chamber.
2. Fill a 60 ml syringe with a Teepol and attach the blunt-ended syringe to the T connection separation chamber entry port via a single silica rubber tube at the base of the chamber. Slowly infuse the detergent up through the tubing which feeds into the chamber just above the peristaltic pump region. As the meniscus forms along the base of the chamber, allow any air bubbles to dissipate before allowing the detergent to fill the chamber completely. If air bubbles form while the detergent is slowly moving up between the surface of the two plates comprising the separation chamber, draw some of the detergent back into the syringe several times to dislodge the bubbles.
3. Allow the detergent to pass through the bubble traps, until drips are seen coming out of the sample inlet tube at the top of the chamber, then clamp the syringe tubing. Place the sample inlet tube in a beaker of detergent, release the rotary clamp, and allow detergent to flow through the fraction collection tubes for several seconds. This will reveal any air pockets at the top of chamber. Clamp the fraction tubes once more, release the syringe clamp, and infuse more detergent into the chamber. This will force the bubbles out through the sample inlet tube and into the beaker of detergent. Repeat this procedure several times until you are satisfied that all air bubbles are removed from the chamber.
4. Loosen the rotary clamp by turning it anticlockwise 20°, and allow the detergent to flow freely through the chamber and fraction collection tubes for 30 min.
5. Tighten the rotary clamp once more to stop the flow of liquid and exchange the detergent reservoir for distilled water. Then loosen the

clamp and allow 1.5 litres of distilled water to flow through chamber and fraction tubing.

6. Close the rotary clamp once more to stop the flow of water and insert a 60 ml syringe containing 3% (w/v) BSA solution (bubble-free) in the bottom inlet tube. Slowly infuse 45 ml BSA up through the chamber. Clamp the bottom inlet tube and leave the BSA for at least 2 h, but preferably overnight.

B. *Preparation of chamber buffer*

1. Prepare the chamber buffer of choice (see *Table 2*), and infuse it up through the chamber via the T junction inlet tube at the bottom of the chamber. Place 2 litres of chamber buffer above the separation chamber. Place the upper inlet tubing in the buffer, open the fraction tubing clamp, and allow the buffer to flow through the chamber for 20 min. Then close the fraction tubes clamp.
2. Ensure there is a sufficient sample buffer reservoir above the sample chamber. Then turn on the following switches to flush the chamber with buffer.
   (a) Switch on EL BUFFER, POWER, and FRACTION MEDIA switches, and adjust flow rate dial to an intermediate setting.
   (b) Leave on for a few minutes and check for leaks. If no problems are observed the chamber is ready for sample loading.
3. The sample chamber is now ready for use.

It is difficult to keep all parts of the FFE apparatus completely sterile. However, if sterile conditions are desirable, then the separation chamber, buffer supply bottles, and all tubing can be treated with 3.7% (w/v) formaldehyde solution for 1–2 h. This should be then thoroughly removed by rinsing all formaldehyde-treated apparatus with sterile endotoxin-free distilled water.

4.2.1 Cooling system

During electrophoretic separation, the heat generated between the separation plates is efficiently removed by the liquid cooling system situated behind the separation chamber. The heat generated between the electrodes is transferred to the cooling unit via the 'cooling circuit 1' tubing. This contains a mixture of 50% (v/v) water, 25% (v/v) ethylene glycol, and 25% (v/v) ethanol. 'Cooling circuit 2' maintains the cells in the cell suspender and fraction collector at the desired temperature.

4.2.2 Temperature

The temperature of the separation chamber can be adjusted between 4 °C and 37 °C quite easily by adjusting the temperature dial. A temperature sensor which inserts into the front plate of the separation chamber monitors the actual temperature inside the chamber during the separation.

4.3 Preparation of sample syringe

A 20 ml Hamilton sample syringe which is inserted into a temperature controlled mixing chamber is used to inject the cells in a controlled manner into one of the four entry ports at the top of the separation chamber. The flow of cells is controlled by hydraulic pumping by a second syringe attached to a peristaltic pump. The syringe is assembled and prepared as described in *Protocol 4* and illustrated in *Figure 6*.

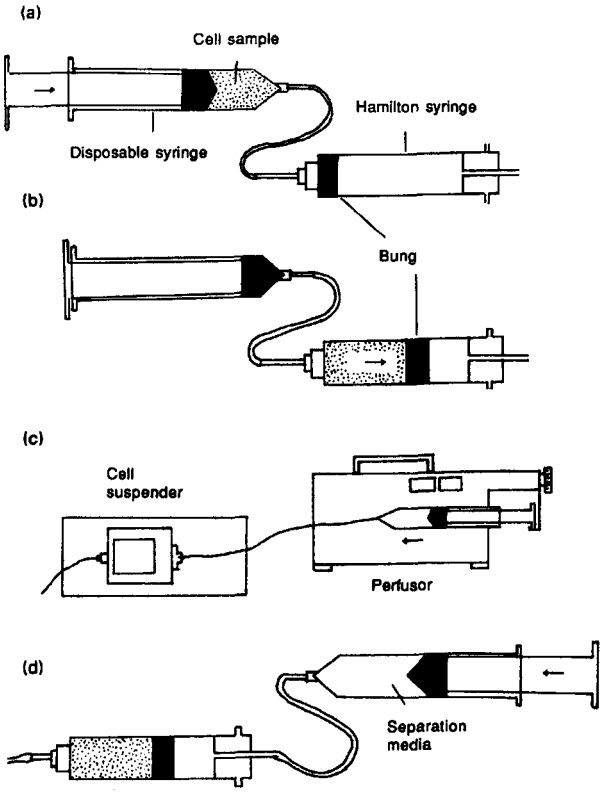

Figure 6. Loading the cell sample in preparation for cell separation. (a) Isolated cells are drawn up into a disposable syringe with a blunt-ended needle. A 20 ml siliconized glass Hamilton syringe is filled with chamber buffer. A bung is forced down the chamber and the area behind the bung is filled with chamber buffer once more and sealed at the end with a bung with a bore tube running though its length. The syringe containing cells is connected to the Hamilton syringe by a silica tubing. (b) The Hamilton syringe is filled by forcing the sample up into the Hamilton syringe. (c) The Hamilton syringe is inserted in the mixing chamber of the cell suspender. A disposable syringe is filled with chamber buffer and inserted into the perfusor device. The needle end of the syringe is connected to the capillary bore extending from the rear of the Hamilton syringe. The sample syringe is connected to the separation chamber inlet tubing. (d) The perfusor is switched on to pump the cells into the chamber.

Protocol 4. Assembly and loading of sample syringe (*Figure 6*)

Equipment and reagents

- Many-fold peristaltic pump
- 20 ml Hamilton syringe (Dr Weber GmbH)
- Tubing (1 mm i.d.)
- Chamber buffer of choice
- Concentrated cell suspension (1–5 × 10^7/ml)
- Replicote (Sigma)

Method

1. Clean 20 ml glass syringe, several small pieces of tubing (1 mm i.d.), and a 60 ml disposable syringe in Teepol detergent. Rinse in distilled water three times and dry in an oven at 80°C for 1 h.
2. Siliconize the glass syringe with 'Replicote', or some other water repellant agent, and rinse thoroughly with distilled water.
3. Fill the 20 ml syringe with chamber buffer and force the piston head (silver side facing out) to the bottom of the syringe.
4. Fill the remaining volume of the syringe with chamber buffer and place a stopper containing a 1 mm stainless steel bore protruding from it in the end of the syringe.
5. Place a blunt-ended needle on the syringe and attach a small piece of clean tubing. Place the other end of the tubing onto a sterile disposable syringe containing the cells of interest.
6. Slowly push the cell into the glass syringe from the needle end, chamber buffer should be displaced out of the top of the syringe as the syringe is filling.
7. Place the syringe in the refrigerated rotary chamber and switch on cell rotor.
8. Insert the 60 ml syringe containing about 30 ml of chamber buffer in the peristaltic pump holder and connect it to the end of the cell sample syringe with a small piece of tubing.
9. Set the pump on 20 ml/h flow rate and watch the sample syringe until the first drop of sample appears at the end of the blunt-ended needle. Then stop the pump.
10. Connect the needle to the sample inlet tubing which allows the sample to enter the separation chamber and set the pump at the appropriate flow rate.

4.4 Running samples in FFE

4.4.1 Running a cell suspension through the VAP 22

Once the electrode buffer and chamber buffer has been prepared and placed in the appropriate sections of the FFE apparatus, after a short cell-free pre-run

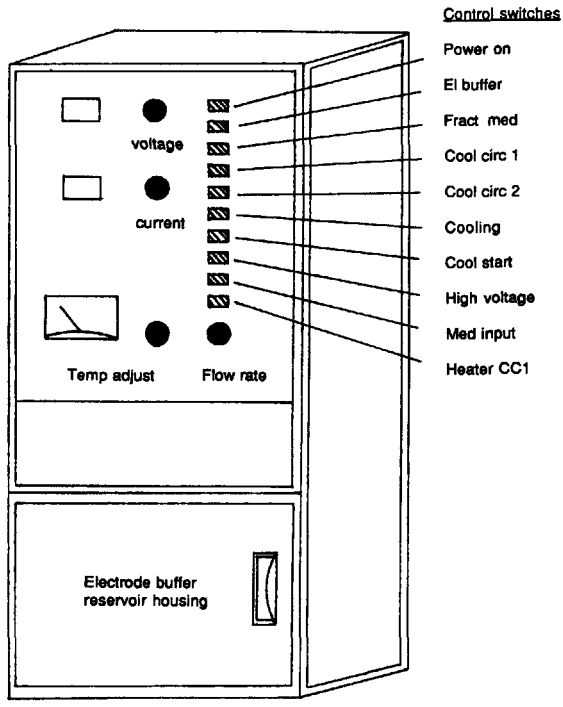

Figure 7. Graphic illustration of the control panel of the Elphor VAP 22 apparatus showing the operation switches.

to check for leaks and power surges, the machine can be prepared for cell separation. A step by step guide is provided in *Protocol 5* and the control switches are illustrated in *Figure 7*. *Table 4* serves as a guide to the settings for chamber buffer flow rates and voltage commonly used for a number of different cell types.

Table 4. Chamber buffer flow rates and voltage commonly used during separation by FFE

Cell type	Flow rates (ml/h)	V/cm	Reference
Lymphocytes	550	118	27
Platelets	500	130	11
Neutrophils	405	90–100	12
Kidney	180	170	4
Malarial parasites	180	135	26
Spermatozoa[a]	Variable	80–120	19

[a] An ACE 710 apparatus was used in this analysis.

Protocol 5. Cell separation by free flow electrophoresis (VAP 22)

Equipment and reagents

- VAP 22 FFE separation chamber, cooling system
- Control unit and sample injection pump
- Electrode buffer
- Chamber buffer

A. *Running the VAP 22*

1. Fill electrode buffer reservoirs, fill separation chamber with chamber buffer, and place a reservoir of buffer on top of the apparatus. Allow buffer to circulate through electrode chambers by pressing EL BUFFER.
2. Ensure separation chamber rotary clamp is closed, then turn on POWER and FRAC MED switches. This will operate the separation chamber peristaltic pump. Set the flow dial to the desired setting (see *Table 4*). Leave for a few minutes so that the flow rate can stabilize.
3. Next turn on COOL CIR 1, COOL CIR 2, and COOLING. Both the separation chamber and sample syringe chamber will begin cooling to the desired temperature (6°C normally). The desired temperature can be adjusted by turning the 'chamber temperature dial' (0.0–5.0 corresponds to 0–25°C). Watch the temperature gauge, make sure the temperature begins to fall to the appropriate setting.
4. Slowly begin to turn up the voltage to the desired V/cm width (see *Table 4*). Do this in 25 V intervals and watch the chamber for problems such as leaks or short circuiting.
5. When the flow rate, voltage, and temperature has equilibrated, place the cell sample in the Hamilton syringe (see *Figure 6*). Insert into refrigerated housing of the cell perfuser and connect the end of the sample syringe to the buffer syringe housed in the manifold pump.
   (a) Set the pump flow rate to 50 ml/h and begin the pump.
   (b) Ensure the EL BUFFER, POWER, FRAC MED, COOL CIR 1 and 2, HIGH VOLTAGE, and IMPUT MEDIA are turned on.
   (c) Allow a drop of sample to pass out from the end of the syringe before attaching the sample syringe to chamber inlet tube. This ensures no air from the barrel of the needle enters the chamber.
   (d) Set the cell perfusor pump to appropriate flow rate (approx. 1–2 ml/h) and allow the sample to enter the chamber.
6. Watch for the sample to enter the chamber and monitor its progress down the chamber by shining a torch at an angle at the mirrored surface of the back plate. The cells should be observed to deflect at an angle away from the cathode (negative electrode).

Protocol 5. *Continued*

7. After leaving the separation chamber the cells will pass through the fraction collection tubes and may be collected in tubes or microtitre plates.

B. *Turning off the VAP 22*

1. Turn off sample perfusor pump.
2. On the control panel, turn off in order INPUT MEDIA, COOLING, and COOL CIRC 1 and 2. Allow the temperature to rise to at least 15°C, to prevent the separation chamber plate breaking.
3. Turn the mAmp dial to zero and then turn off HIGH VOLTAGE switch.
4. Turn off FRAC MED, and replace chamber buffer reservoir with distilled water. Open the rotary clamp on the front of the separation chamber and allow water to freely flush through the chamber. Flush a few millilitres of water through the sample inlet tubing too.
5. Turn off POWER and EL BUFFER, replace electrode buffer reservoirs with distilled water, and rinse the electrode chambers by pressing EL BUFFER for 10 min.

4.4.2 The OCTOPUS-PZE apparatus

The OCTOPUS-PZE shares many common features of the VAP machines and in many regards is an improved version of the older VAP apparatus. Electrode buffer, separation media, sample flow rates are all controlled in a similar way as described for the VAP 22. The separation chamber, the electrode compartments, and 96-fold fractionation capacity has not altered. But unlike the VAP apparatus, both the separation media and cell samples are delivered into the separation chamber from the bottom of the chamber rather than the top. Media is also injected through the top of the chamber, the counterflow action ensures good mixing of the separation medium. The OCTOPUS manual should be consulted for the precise choice of flow rates to ensure both good and reproducible separation of cells.

4.5 Construction of cell fractionation profiles

As the cells separate in the chamber on the basis of differences in electrokinetic properties, they form a broad band covering an area of 10–20 fractions. While leaving the chamber they pass through 10–20 of the 96 silica tubes located at the base of the chamber, and accumulate into plastic collection tubes, or can be alternatively collected in two 48-well microtitre plates. To identify the location of the cells either of two standard techniques can be used:

- haemocytometer counts
- turbidity measurements in a spectrophotometer at either 500 nm or 280 nm

Figure 8 (*top*) shows the relationship between absorbance/fraction and cell count/fraction. Cell profiles appear essentially of the same shape and span the same number of fraction tubes, irrespective of the method of detection engaged. Non-fixed and formaldehyde-fixed cell profiles appear similar after separation by FFE as shown in *Figure 8* (*bottom*).

Biochemical and functional analysis of the separated cells as subpopulations can be achieved by either assaying every individual fraction, or pooling the cells as three of four subpopulations of equal number. Two methods have been commonly used to prepare the cells as subpopulations. As illustrated in *Figure 9* (*top*) a cell profile can be subdivided as three subpopulations, the least electronegative (A), the median (B), and the most electronegative (C). The least and most electronegative subpopulations are selected by bisecting the profile at the point which represents 50% of the total cell population recovered. Lines are drawn perpendicular to these intersecting points and the cells pooled from the selected areas under the curve. Generally the least and most electronegative cells populations each represent about 15–20% of the total cells recovered, while the medium fraction contains approximately 60–70% of the cells. An alternative method is to simply subdivide the total cells recovered into four equal 25% portions as shown in *Figure 9* (*bottom*). This latter method is more crude and some resolution of subtle differences between subpopulations of cells may be lost as a result of greater overlap of cells which may only differ slightly with respect to biochemical or functional divergence.

4.5.1 Recovery and viability of cells

Free flow electrophoresis can achieve a high cell recovery and viability. As long as the cells do not aggregate for any reason during their passage through the separation chamber, 90% cell recovery should be attainable. Cell viability and functional integrity has been reported to be good for neutrophil, T and B lymphocytes, *Plasmodium falciparum*, kidney cells, tumour cells, and platelets, based on functional analysis of FFE separated cells compared to non-electrophoretically separated cells.

4.6 Electrophoretic mobility of cells

The following formula can be used to estimate the relative electrophoretic mobility of cells which are passing through the FFE separation chamber:

$$U = D/F \times t\,(\mathrm{cm}^2 \times \mathrm{volt}^{-1} \times \mathrm{sec}^{-1})$$

where U = electrophoretic mobility, D = deflection in cm, F = field strength/cm, t = time that the sample remained in the electric field in sec.

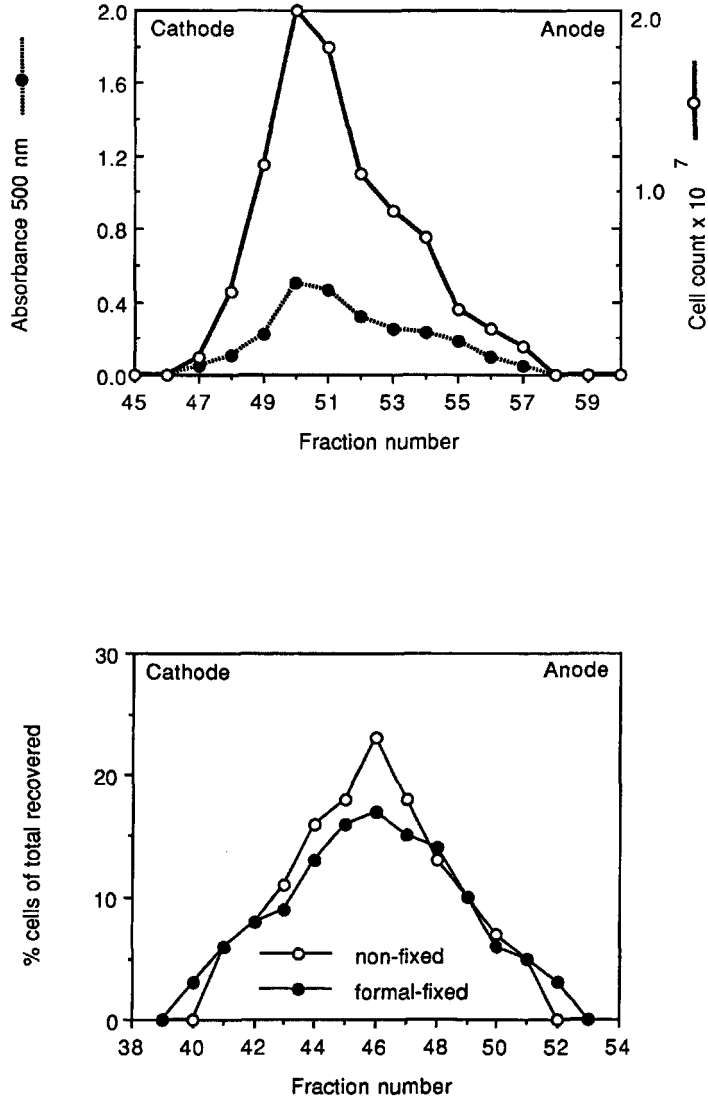

Figure 8. Human neutrophils at a concentration of 2×10^7 cells/ml were resuspended in chamber buffer and separated by continuous flow electrophoresis using a VAP 22 apparatus, in a glycine–glucose–triethanolamine buffer of pH 7.35. Current was set at 100 mA, producing a voltage of approx. 100 V/cm. The sample buffer flow rate was 405 ml/h. *Top*: comparison of free flow electrophoresis profiles of human neutrophils determined by cell counting using a Coulter counter (o) and turbidimetric analysis in a spectrophotometer at an absorbance of 500 nm (Σ). *Bottom*: comparison of FFE separation profiles for non-fixed (o) and formal-fixed (0.4% (v/v) in PBS) neutrophils (Σ), which are expressed as the per cent of cells of total recovered.

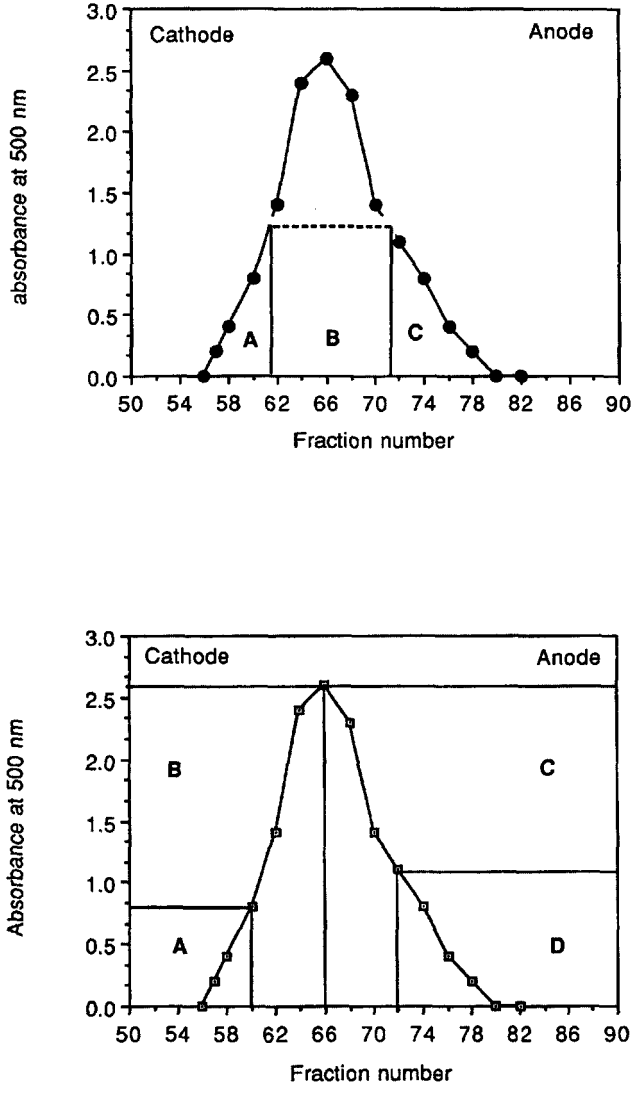

Figure 9. Two different ways of subdividing cells into subpopulations after FFE. *Top*: the cell profile is subdivided into three populations, (A) the least electronegative, (B) the median population, (C) the most electronegative population. *Bottom*: the individual fractions can be pooled into four or more subpopulations of equal cell number based on absorbance values.

5. Evaluation of the electrokinetic properties of cells separated by FFE

5.1 Ability of FFE to separate cells with small differences in electrophoretic mobility

The commercially available free flow electrophoresis separation machines are capable of separating cells which differ by only 5–10% in electrophoretic mobility (EM). Peripheral blood contains a number of different cell types which possess EM values which range only from 0.9–1.1 $\times$ 10^{-4} $cm^2/V/sec$, a difference of 20%. Platelets and B lymphocytes have the lowest EM values which are approximately 0.9 $\times$ 10^{-4} $cm^2/V/sec$; granulocytes and monocytes which an intermediate EM of 0.95 $\times$ 10^{-4} $cm^2/V/sec$; while the most electronegative cells of the peripheral blood are T cells and erythrocytes with EM values of 1.1 $\times$ 10^{-4} $cm^2/V/sec$. *Figure 10* (*top*) shows the distribution curve obtained from whole blood. Despite the limited differences in electrophoretic mobility, a broad distribution in the electrophoretic profile is obtained which separate over 24 fractions. Examination of the individual fractions confirms the ability of FFE to separate cells according to electrophoretic mobility differences. The least electrophoretic cells are comprised mainly of platelets and granulocytes, the most electronegative fractions contain lymphocytes and erythrocytes. When peripheral blood is separated by simple buffy coat separation techniques, and separated by FFE, the profiles shown in *Figure 10* (*middle*) and *Figure 10* (*bottom*) are typically seen. Although these data serve to illustrate the ability of FFE to separate cells solely on the basis of differences in electrophoretic mobility, it is not recommended to use FFE as a sophisticated technique to separate individual cells that possess gross morphological or functional differences, when simpler and less expensive density gradient techniques will suffice. Isolation of cells by gradient density techniques prior to FFE is recommended to remove gross contamination of other cell types. Then FFE can be used to separate cells on the basis of differences in electrokinetic properties alone; as is the case in isolation of subpopulations of human neutrophils and Y- and X-bearing spermatozoa or, in combination with immunological techniques to isolate pure populations of B and T lymphocytes.

Once cells have been separated by FFE a good way of ensuring the cells have indeed been separated on the basis of real and reproducible electrokinetic differences, is to re-electrophorese the pools of isolated cell subpopulations singly or in pairs. Subpopulations of cells collected from each end of the first profile upon re-electrophoresis are mixed and separated once more. Each pool of cells should relocate at a peak position coinciding with the same population of cells in the original separation (*Figure 11*). It is best to perform such separations on fixed cells so as to avoid changes in membrane characteristics during the procedure, which may occur by passing live cells through the chamber more than once.

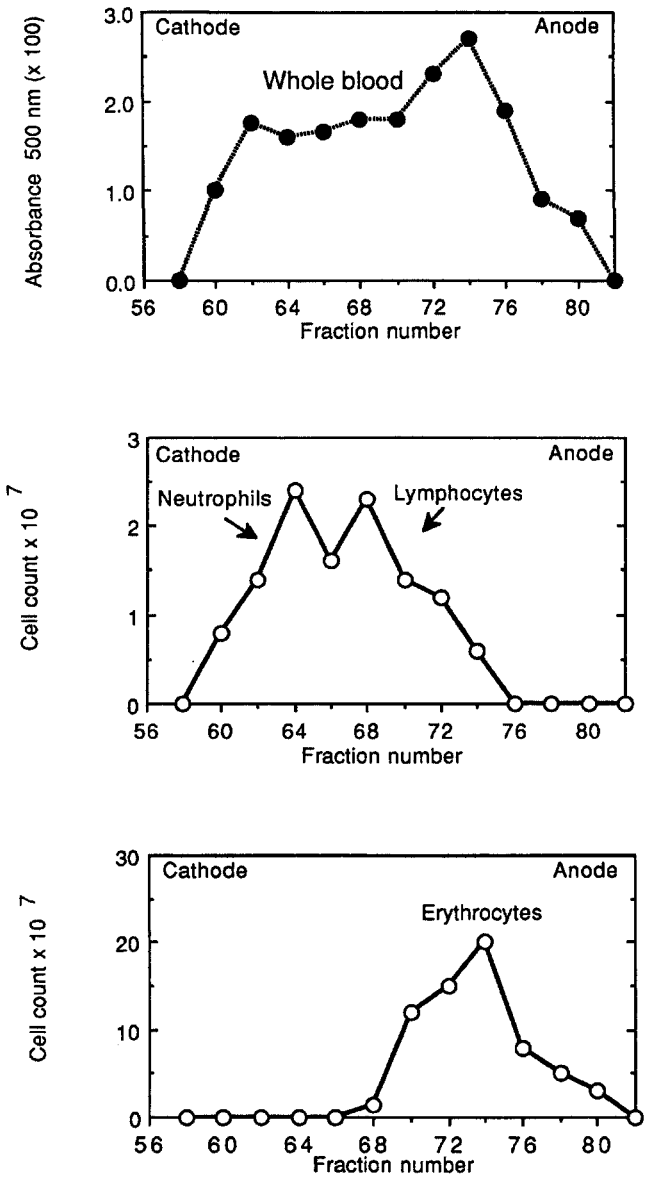

Figure 10. *Top*: VAP 22 FFE distribution curves obtained from human whole blood. *Middle*: white cell fractions produced two predominant peaks, the peak nearest the cathode is made up mainly of granulocytes, while the anodal peak consists of B and T lymphocytes. *Bottom*: the erythrocytes represent the most electrophoretically mobile fractions and appear in the extreme anodal fractions.

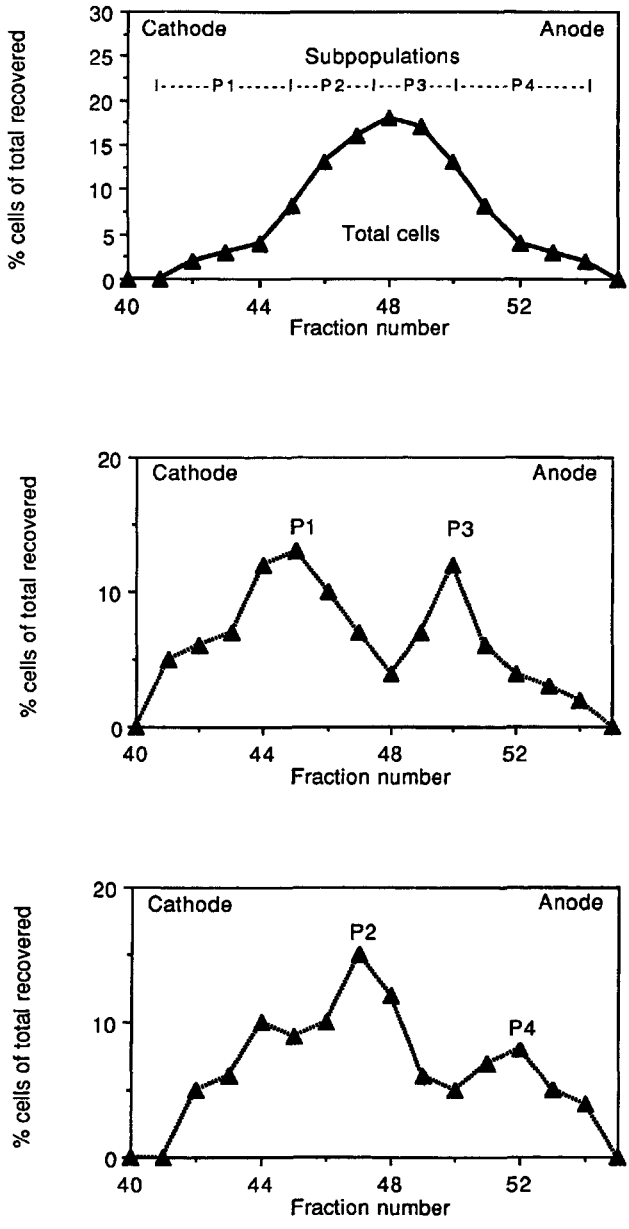

Figure 11. Demonstration of the different electrokinetic nature of subpopulations of cells after separation by FFE. *Top*: neutrophils were formalin-fixed, separated by FFE, and subdivided into four subpopulations of equal number. *Middle*: pool P1 and P3 were mixed together and re-electrophoresed, two distinct peaks were observed. *Bottom*: pool P2 and P4 were similarly remixed and re-electrophoresed under the same conditions. Each subpopulation of cells relocate essentially in their original position.

Protocol 6. Re-electrophoresis of FFE separated cells

Equipment and reagents

- See *Protocol 5*
- 0.4% (v/v) formaldehyde in PBS

Method

1. Take a suspension of cells to be electrophoresed between 10^7–10^8 cells/ml and fix them in PBS containing 0.4% (v/v) formaldehyde.
2. Separate the cells by FFE, employing the buffers, flow rate, and electrical conditions of choice (e.g. *Protocol 5*).
3. Determine which collection tubes contain cells either by cell counting, or spectrophometric analysis absorbance at 500 nm or 280 nm.
4. Construct a graph of the cell separation profile and divide the cells into three or four subpopulations which contain at least 10^7 cells/ml.
5. Take two cell populations differing in electrophoretic mobility, mix them together, and resuspend in a small volume of chamber buffer (1–2 ml), so that the cell concentration is at least 10^7 cells/ml.
6. Re-electrophorese the cells under identical conditions and preferably on the same day as the original cells. Construct a graph of the new cell profile. If the cells have genuinely different electrokinetic properties, two peaks coinciding with the original separation should be observed (see *Figure 11*).

5.2 Removal of sialic acid from cell surface and effects on cell separation

FFE should not be thought of as purely a separation technique. If reasonably pure populations of cells are available as a starting preparation, FFE can be used to study changes in electrokinetic properties of the membranes of whole cells. The major contributor of surface electronegative charge on many cells is believed to be sialic acid moieties associated with the membrane glycoproteins. By incubating the cells with various concentrations of the enzyme neuraminidase (Sigma) (0.1–0.5 U/ml per 10^7 cells), for 60 min at 37 °C, terminal labile sialic acid residues are cleaved from the outer surface from the cell membrane. Cells treated with and without neuraminidase treatment can then be fractionated by FFE. *Figure 12* shows how neuraminidase treatment can lead to a major cathodal shift in the cell fractionation profile of human neutrophils. In addition, such treatment often leads to a narrowing of the actual cell profile, generating a less heterogeneous population of cells.

5.3 Application of FFE to study the dynamic changes in the electrokinetic status of cells

Free flow electrophoresis separates cells into subpopulations by exploiting differences in cell surface charge, but FFE can also be applied to study alteration

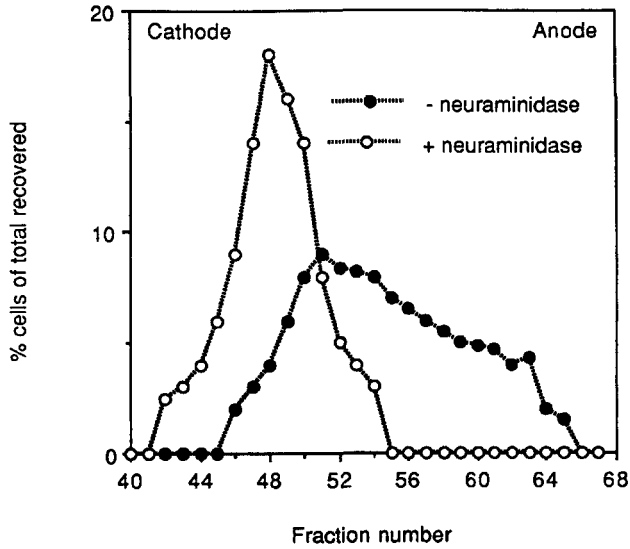

Figure 12. Effect of neuraminidase on electrokinetic characteristics of cells separated by FFE. Typical FFE profile of human neutrophils (Σ). FFE separation profile of cells after treatment with neuraminidase (0.4 U/ml for 1 h at 37°C) (o). Neuraminidase cleaves the labile membrane-associated sialic acid residues resulting in a cathodal shift in the electrophoretic nature of the cell population as a whole.

of membrane electrokinetic properties of whole cell populations in a dynamic fashion. The cell surface of most eukaryotic cells is electronegatively charged; the major contributor of surface charge can be largely accounted for by sialic acid moieties at the cell surface. Other contributors of charge include phosphate groups, or carboxyl groups of glutamate and aspartate in integral membrane proteins. Consequently, changes in cell surface charge often reflect modification or alteration of membrane-associated processes. Blood cells in particular are excellent cell models to study electrokinetic properties during cell activation. As shown in *Figure 13*, when neutrophils are allowed to phagocytose bacteria for 15 minutes in which the ratio of bacteria:cells vary from 0:1, 2:1, and 20:1, the cells substantially shift to the left of the control resting cells. This indicates that after phagocytosis the cells migrate with lower electrophoretic mobilities than control cells.

Free flow electrophoresis can also be used to monitor alterations in membrane-associated cell functional events, particularly those associated with changes in whole cell membrane charge with time. For example, time course experiments of cell surface charge during phagocytosis can be monitored. Neutrophils or other phagocytic cells are permitted to phagocytose bacteria for various lengths of time, washed and fixed, and the cells from each timed experiment placed in chamber buffer and separated by FFE, one sample after the other. Cell separation profiles are created for each timed experiment and

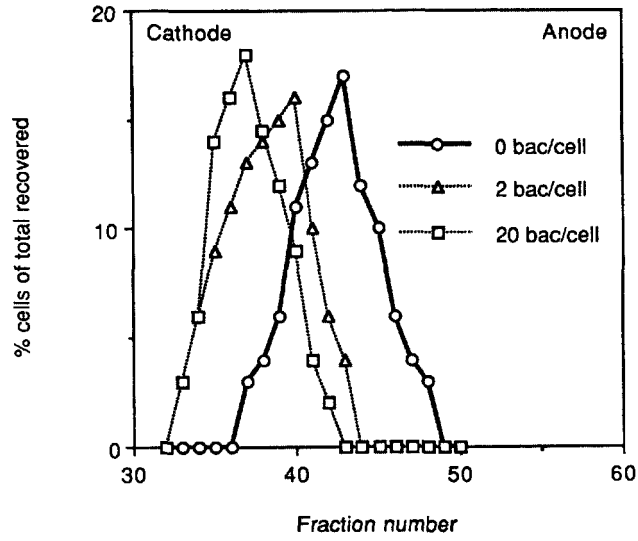

Figure 13. Application of FFE to study the relationship between surface charge characteristics and cell function. Human neutrophils were incubated with various numbers of serum opsonized *Staphylococci aureus* for 15 min at 37°C. The cells were washed, formal-fixed, and subjected to FFE. As the cells took up greater numbers of bacteria, they become less electronegative as indicated by a shift of the whole cell population to a lower electrophoretic mobility.

then superimposed on top of one another. *Figure 14* shows that actively phagocytosing neutrophils display a marked reduction in their cell surface charge properties upon phagocytosis compared with non-phagocytosing cells, but begin to regain their original electrophoretic mobility characteristics, after 60 minutes of such treatment, and eventually relocate in the same fractions as they did prior to phagocytosis. Similar results can be seen when cells are stimulated with soluble stimuli such as cytokines and chemoattractants and may reflect membrane-associated events such as receptor up- and down-regulation by these compounds.

Some of the chemical and physical differences observed between subpopulations of blood cells after separation by FFE are listed in *Table 5* and serve to illustrate the complex make-up of the electrokinetic characteristics of different cell types.

5.4 Application of FFE to monitor haematological disorders

FFE has been used to investigate heterogeneity in the circulating platelets from patients with idiopathic thrombocytopenia purpura (ITP) and essential thrombocythaemia (ET) (10, 11). In idiopathic thrombocytopenia, platelet survival times are shortened and the condition may be an early manifestation of an intrinsic stem cell defect. ITP platelets have a greater sialic acid status

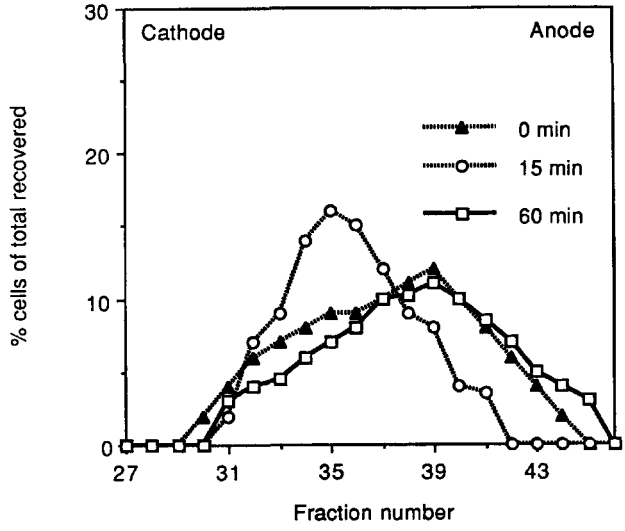

Figure 14. Application of FFE to study dynamic changes in electrophoretic mobility of cells during phagocytosis. FFE patterns of formal-fixed human neutrophils after they were permitted to phagocytose a fixed number of *Staphylococci aureus* for up to 60 min. The electrophoretic mobility of neutrophils is rapidly reduced during the initial phase of phagocytosis. After 60 min, the electrophoretic characteristics of the cells return to their original status prior to phagocytosis.

and consequently FFE profiles of these platelets separate closer to the anode than normal platelets (*Figure 15, top*). Platelets in essential thrombocythaemia are characterized with surface membrane abnormalities such as decreased sialyation, reduction in α_2-adrenergic receptors, and prostaglandin D_2 receptors. FFE has been used to characterize the electrokinetic characteristics of

Table 5. Comparison of the physical and biochemical differences of FFE separated platelets and neutrophil subpopulations

	Neutrophils (10^7/ml)			Platelets[a] (10^9/ml)		
	Total population	**Least negative**	**Most negative**	**Total population**	**Least negative**	**Most negative**
Neuraminidase-labile sialic acid (nmoles)	6.7	6.1	6.1	22.3	21.8	29.1
Surface DTNB-reactive SH groups	11	8	13.3	87.2	97.6	56.7
EPM μm/S/V/cm	1.06	1.04	1.18	0.89	0.83	0.93
Buoyant density	1.061	1.062	1.061	1.055	1.055	1.053
% difference in volume	100%	110%	91%	100%	96.7%	124%

[a] Adapted from ref. 8.

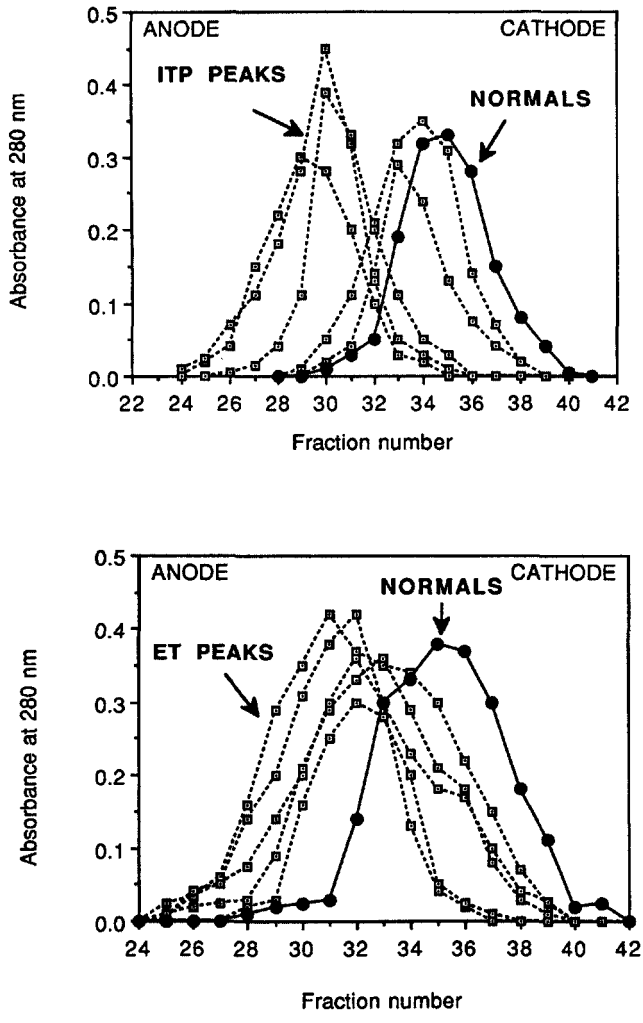

Figure 15. Application of FFE to detect differences in electrokinetic properties of isolated platelets from individuals with platelet disorders. Human platelets at a concentration of 1.8×10^6 cells/ml were resuspended in chamber buffer and separated by continuous flow electrophoresis using a VAP 5 apparatus. These conditions were maintained for all FFE experiments involving platelets. Electrophoretic separation was carried out in a 280 mM glycine and 10 mM triethanolamine buffer of pH 7.4. Current was set at 130 mA. The sample buffer flow rate was 500 ml/h. *Top*: FFE separation profiles of platelets from a typical normal control subject (•) and from five patients with idiopathic thrombocytopenic purpura (ITP) ( ). Taken from ref. 10. *Bottom*: platelet electrophoretic profiles of five patients with essential thrombocythaemia ( ) compared to a profile from a representative normal donor (•). Taken from ref. 11.

this disorder. Platelets from this condition also show significant shifts towards the anode (*Figure 15, bottom*). In both these studies it was concluded that in thrombocytopenic states, a higher proportion of premature platelets enter the circulation originating from megakaryocytes of lower ploidy class, with higher electrophoretic mobility, and more sialic acid than normal platelets.

5.5 Application of FFE to study biochemical differences in cell subpopulations

Free flow electrophoresis has also been used to separate a number of cell types into subpopulations so as to investigate the relationship between their surface membrane electrokinetic properties and various resting and stimulated states. In this respect the neutrophil is an excellent model cell because it possesses many stimulant motile activities, such as adhesion, spreading, chemotactic migration, phagocytosis, and secretion of granule-stored constituents which require reorganization of certain elements of the plasma membrane–cytoskeleton axis. Separation of neutrophils into subpopulations by FFE has revealed that the least and most electronegative cells differ in electrophoretic mobilities that range between 1.04–1.18 μm/sec/V/cm respectively. Biochemical differences are also evident, the least electronegative cells generally phagocytose bacteria and generate superoxide after stimulation at twice the rate of the most electronegative cells separated by FFE (12).

6. Overcoming problems

When FFE is used for the first time by an investigator to separate whole cells, there is often a feeling of apprehension, as there are quite a number of steps which have to be followed in strict order. It is advisable here to stress that the manual and operating instructions provided with each machine should be read thoroughly before attempting to perform FFE. In addition, a number of problems can arise even for the most experienced operator while performing a run. Some of these problems and their remedies are described below.

6.1 Cell aggregates

Free flow electrophoresis is an excellent separation technique for blood cells. Unfortunately, most types of blood cells are prone to aggregation during separation. Alteration in physical parameters such as temperature, pH, isotonicity, and *g* forces may lead to aggregate formation. Most of these factors are not a problem during FFE, as the technique is a gentle one, and most of the physical parameters can be adjusted to maintain the cells in a labile state. However, the maintenance of a highly concentrated suspension of cells in the sample syringe, or their passage through a narrow bore syringe into the separation chamber can sometimes result in cell aggregates forming. The formation of

such aggregates will affect the separation resolution. Consequently, if aggregate formation proves to be a problem it is recommended that the cells are suspended in chamber buffer containing a protease cocktail. In the case of neutrophils, the protease cocktail listed in *Protocol 7* works well.

Protocol 7. Preparation of protease cocktail to inhibit cell aggregation in FFE separation chamber

Reagents

- DNase (Sigma, DN-25)
- Leupeptin: M_r 475.6 (Sigma, I-2884)
- Chamber buffer of choice
- Phenylmethylsulfonyl fluoride(PMSF): M_r 174.2 (Sigma, P-7626)
- Sterile distilled water

Method

1. Prepare a 10000 μg/ml stock concentration of DNase, by adding 10 mg of DNase to 1 ml sterile distilled water.
2. Prepare a 2.1 mM stock concentration of leupeptin, by adding 5 mg of leupeptin to 5 ml sterile distilled water.
3. Prepare a 20 mM stock concentration of PMSF, by adding 35 mg of PMSF to 5 ml 100% methanol. Then slowly add an equal volume of water.
4. To each 1 ml sample of cells resuspended in chamber buffer add 10 μl stock DNase, leupeptin, and PMSF.

6.2 Leaks

Large volumes of liquid run through the FFE apparatus in the form of electrode buffer, separation chamber buffer, and cell sample. Each of these liquids enter the separation chamber area via Teflon or silica rubber tubing. Leaks occur most commonly at sites where the tubes leave and enter the separation chamber. The separation chamber is sealed with double gaskets to prevent leakage from the chamber itself, this is an important safety feature, as leakage of fluid from this area while the chamber is in use, may result in high voltage electrical arching between the electrodes. Another potential site of leakage is between the electrode compartment and the medium in the separation chamber. Leakage of one fluid into the region of the other will lead to voltage fluctuation and cell separation will not be efficient. Before beginning any separation, check that all major tubing are connected correctly. It is good practice to run the machine for at least 15 minutes before addition of the sample, to check for such leaks. If a leak does occur a safety light will come on. The most common leak occurs between the tubing connections between the electrode buffer reservoir and the electrode compartment. If this does occur during separation, quickly turn off the EL BUFFER switch to prevent additional leakage, replenish the electrode containers with buffer, and reconnect the

tubing before turning the EL BUFFER switch back on. If a leak occurs in the separation chamber while an actual separation is in progress, the separation will have to be stopped, the chamber temperature will have to be brought up to approximately 15 °C, before all power can be turned off and the leak repaired. In practice regular maintenance of the apparatus and all tubing will prevent any such leaks occurring during separation.

6.3 Bacterial contamination

By the very nature of the separation media used in FFE, the apparatus and tubing are prone to bacterial contamination. A number of precautions can be performed to eliminate or avoid such contamination:

(a) Regular cleaning with Teepol or similar mild detergent.

(b) Sterilization with formaldehyde, however this will shorten the life of the tubing.

(c) Removal of all cell separation media immediately after use.

(d) Regular flushing of apparatus with sterile distilled water.

6.4 Fluctuations of temperature

For many cell separations it is desirable to maintain the cells and the separation chamber between 4 °C and 6 °C. If for any reason a circuit overload occurs, as indicated by a red indicator lamp, the current is shut off automatically as a safety control. This can lead to a rapid cooling of the separation chamber to an unfavourable temperature; if the drop in temperature is severe, the back plate of the chamber may crack and break. Therefore immediately switch off the cooling circuit button to prevent further refrigeration. Attempt to discover the fault. The most common cause of circuit overload and shut-off is leakage of separation or electrode buffer from their respective chambers. In addition, faulty membranes, thermosensor, or incorrect maintenance of the apparatus can result in such problems. If possible, get the power to come back on and increase the current to help warm the plate. The flow rate can also be reduced to impair heat loss and help warm the plate.

6.5 Fluctuations in separation buffer pH and conductivity

Cell separation by FFE should be reproducible and reliable and ways of checking the reliability of the system have be described in Section 5.1. However, if fluctuations in current or voltage are observed during a cell separation run and the subsequent cell profiles are not as well resolved as usual, there may well be a problem with the separation or electrode buffer. To help ensure reproducibility, always prepare both electrode and separation buffer on the day of use, or the evening before, and store them at 4 °C. It is also important to check the pH, osmolarity, and conductivity of each buffer before use. Furthermore,

Rodkey (1) has shown that both the pH and conductivity of certain triethanolamine-based separation buffers alter during separation. Such instability may alter the ionic balance of the media resulting in a reduction in the resolution of the cell separation profiles. Therefore before performing FFE on cells, the buffer of choice should be electrophoresed alone and its pH and conductivity assessed. Samples should be assayed from the cathodal and anodal end of the separation chamber, as well as several intermediate fractions, and compared with the original buffer. A buffer shown to be stable across the separation chamber within the electrical field in terms of pH and conductivity is optimal.

6.6 Maintenance of filter membranes

The filter membranes for use in the electrode chambers are supplied in rolls and have to be cut to size. Before being placed over the electrodes, the filters should be pre-wetted with distilled water. There are two potential problems associated with the membranes: drying out and bacterial contamination. If the FFE apparatus is not being used routinely, it is often best to wash and drain the separation and electrode chamber of all fluid, to prevent bacterial contamination. However, this will lead to drying out of the filter membranes. Once this occurs the separation properties of the membranes deteriorates. Therefore the membranes should be removed and stored in a mild antibacterial solution, such as a 2–5% (v/v) formaldehyde solution until required. Before use, the membranes should be rinsed in distilled water or a small quantity of 70% ethyl alcohol followed by water.

7. Combination of FFE with other techniques

In certain circumstances when the electrophoretic mobility properties of subsets of cells do not differ substantially, the separation of cell subpopulations by free flow electrophoresis is sometimes limited. This has often been the case while attempting to separate B and T lymphocytes of human origin by FFE. Consequently a number of technical modifications have been developed, some such as the OCTOPUS-PZE method, employ novel flow systems to enhance cell separation. However, two other techniques combine FFE with a second technique in order to enhance separation of such cells and these are described below. The success of these combination techniques in comparison with standard FFE methods to separate T and B lyphocytes which differ very little in electrophoretic mobility is shown in *Table 6*.

7.1 Free flow magnetophoresis

For a number of years now, the Dynal Incorporation of Norway have been manufacturing Dynabeads which are superparamagnetic polystyrene beads containing iron oxide (Fe_3O_4) which can be labelled with fluorescently labelled

Table 6. Comparison of four different electrophoresis techniques to separate mixed suspensions of human T and B lymphocytes

Method	B cell purity	T cell purity	Reference
VAP free flow electrophoresis	Poorly resolved	Poorly resolved	27
OCTOPUS-PZE free flow electrophoresis[a]	60–70%	≥ 80%	
Free flow magnetophoresis	83.9%	88.7%	13
Antigen-specific electrophoresis	92%	91%	7

[a] Personal communication by B. Bondy, B. Bauer, I. Seuffert, and G. Weber.

antibodies directed specifically against various cell types. For instance, magnetic beads pre-coated with CD19 antibodies specific for B lymphocytes or, CD2 antibodies directed against T lymphocytes can be exploited to separate each type of lymphocyte from one another. In some instances, separation of one cell type from another can be achieved by holding a hand-held magnet up against the side of the tube containing the cells. When larger numbers of cells are required, cells can be separated by magnetic filters which are also commercially available. These techniques are described in more detail in Chapter 6.

In 1992, Hartig and workers (13) developed a cell separation system which combines the most beneficial aspects of both electrophoresis and magnetic separation to enhance cell separation. They have called their new technique 'free flow magnetophoresis' (FFM). The FFM technique takes advantage of the established FFE apparatus described in detail already. The major difference is that the electrodes contained within the separation chamber are replaced by solenoids that generate an inhomogeneous magnetic field across the separation chamber. Once the magnetically labelled particles are injected into the chamber, this has the effect of deflecting the magnetically labelled particles towards one solenoid, while the unlabelled particles pass vertically down the chamber. FFM can be used to either concentrate or deplete magnetically sorted cells. However, if the technique is used to isolate or concentrate a rare cell present in the suspension, the cells inevitably undergo a great deal of manipulation, attachment to 3–5 μm polystyrene beads, labelled with antibodies, plus additional manipulation to remove the beads, etc.

The technique is rather more useful in the depletion of unwanted cells. Such cells can be labelled with specific antibody containing beads and electrophoresed. The unlabelled cells can be then collected in fraction tubes in a similar way to standard FFE without any additional manipulation. Isolation of specific subsets of cells in this way can become expensive if the starting preparation of cells are very impure, because a greater number of different antibody

labelled beads will have to be used. One major advantage of this technique is the separation of cells can be performed in phosphate-buffered saline or other innocuous media. In the studies performed by Hartig and colleagues, mixtures containing B and T cells resulted in the isolation of non-labelled T cells of 88.7% purity with only 0.1% contaminating B cells.

7.2 Antigen-specific electrophoresis

While free flow magnetophoresis is an appealing technique to use for large scale preparation of subsets of leucocytes, it is not a commonly available procedure. Hansen and co-workers (7) have instead exploited commercially available FFE apparatus to separate T and B lymphocytes from one another. The principle behind their procedure is to combine the high separation capacity of FFE together with the high specificity of antibody–cell surface interactions. Their technique is called antigen-specific electrophoresis cell separation (ASECS) and utilizes the fact that immunoglobulins bear a lower electronegative charge than cell surfaces. When antibodies against specific cell types are used, the electrophoretic mobility of the cell population of interest can be reduced, allowing their separation from unwanted cells with a similar electrophoretic mobility.

The technique appears relatively straightforward and can be applied to almost any cell preparation in suspension for which there are specific antibodies directed against cell surface antigens. It is, however, important to determine the optimum concentration of antibodies by titration experiments. In most instances, double labelling the cells improves the resolution of separation. The method employs rather high titres between 1:100 and 1:20 dilution of primary antibody, followed by 1:10 to 1:2 of fluorescent labelled secondary antibody/5 $\times$ 10^7 cells. The use of non-capping conditions is essential to prevent internalization as well as capping of antibodies, also all separations should be performed below 5 °C.

ASECS has several advantages over magnetophoresis, in that it combines well established techniques and instrumentation without the need for cell separation in contact with solid surfaces or particles. In some ways it resembles fluorescence-activated cell sorting (FACS) in that it separates cells on the basis of a physical parameter and antibody-dependent component combined. However, ASECS can separate cells at a rate of 100000/sec which is 20 times more rapid than that achieved by FACS.

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