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Methods of Cell Separation

Volume 3

Edited by Nicholas Catsimpoolas

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Preface

Presently, the need for methods involving separation, identification, and characterization of different kinds of cells is amply realized among immunologists, hematologists, cell biologists, clinical pathologists, and cancer researchers. Unless cells exhibiting different functions and stages of differentiation are separated from one another, it will be exceedingly difficult to study some of the molecular mechanisms involved in cell recognition, specialization, interactions, cytotoxicity, and transformation. Clinical diagnosis of diseased states and use of isolated cells for therapeutic (e.g., immunotherapy) or survival (e.g, transfusion) purposes are some of the pressing areas where immediate practical benefits can be obtained by applying cell separation techniques. However, the development of such useful methods is still in its infancy. A number of good techniques exist based either on the physical or biological properties of the cells, and these have produced some valuable results. Still others are to be discovered. Therefore, the purpose of this open-ended treatise is to acquaint the reader with some of the basic principles, instrumentation, and procedures presently in practice at various laboratories around the world and to present some typical applications of each technique to particular biological problems. To this end, I was fortunate to obtain the contribution of certain leading scientists in the field of cell separation, people who in their pioneering work have struggled with the particular problems involved in separating living cells and who in some way have won. It is hoped that new workers with fresh ideas will join us in the near future to achieve further and much needed progress in this important area of biological research.

Nicholas Catsimpoolas

Cambridge, Massachusetts

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Contents

Chapter 1

Computer Analysis of Lymphocyte Images

Peter H. Bartels and George B. Olson

I. Introduction 1
  A. Computable Image Information 2
  B. Chromatin Distribution Patterns 3
II. Selection of Equipment 4
  A. The Microscope Photometer 4
  B. Interface 7
  C. Computer 9
  D. Photomicrography and Video-Recording 11
III. Computer Programs 11
  A. Design Considerations 11
  B. Data Staging and File Handling 14
IV. Analytical Tasks 16
  A. Cell Image Features 20
  B. Statistical Evaluation of the Data 36
  C. Feature Evaluation and Selection 38
  D. Supervised Learning Algorithms 48
  E. Unsupervised Learning Algorithms 62
V. Application of Image Analysis to Biological Problems 67
  A. X-Ray Radiation: Study One 69
  B. X-Ray Radiation: Study Two 73
  C. X-Ray Radiation: Study Three 74
  D. Virus-Altered Cells: Study Four 81
  E. Detection of T and B Cells: Study Five 88
  References 94

Chapter 2

Principles of Continuous Flow Cell Separation in a Circumferential Flow Disposable Channel

Jeane P. Hester, Robert M. Kellogg, Alfred Mulzet, Kenneth B. McCredie, and Emil J Freireich

I. Introduction 101
II. Design Concepts 102
III. Channel Description 107
IV. Channel Operation 107
  A. Results 109
V. Channel Performance 109
  A. Granulocyte Collection 109
  B. Mononuclear Cell Collection 111
  C. Plasma Exchange 111
VI. Summary 112
  References 112

Chapter 3

Magnetic Microspheres in Cell Separation

Paul L. Kronick

I. Background 115
  A. Physics of Magnetophoresis 115
  B. Previous Examples 119
II. Procedures 123
  A. Preliminary 123
  B. Preparation of Single-Domain Magnetic Affinity Beads 126
III. Examples 129
  A. Toxic Lectins 129
  B. Antibody Ligands 137
  References 137

Chapter 4

Isolation of Human Blood Phagocytes by Counterflow Centrifugation Elutriation

Fabian J. Lionetti, Stephen M. Hunt, and C. Robert Valeri

I. Introduction 141
II. Isolation of Granulocytes from Human Blood 142

III. Isolation of Monocytes of Human Blood 147
A. Isolation of Monocytes by Incremental Flow Elutriation 148
IV. Appraisal of Counterflow Centrifugation for the Isolation of Leukocytes 151
A. Who Needs 1 × 10^8 Granulocytes? 151
B. Scaling the Numbers to 1 × 10^{10} Granulocytes 152
C. Some Technical Considerations 153
References 154

Chapter 5

Biological Methods for the Separation of Lymphoid Cells

Chris D. Platsoucas and Nicholas Catsimpoolas

I. General Considerations 157
II. Rosetting Methods 160
A. Introduction 160
B. Spontaneous Rosettes of Sheep Erythrocytes with Human T Lymphocytes 160
C. Separation of Human Lymphocyte Subpopulations Bearing Fc Receptors 165
D. Separation of Lymphocyte Subpopulations Bearing Complement Receptors 169
E. Separation of Human B Lymphocyte Subpopulations by Rosetting Methods 171
F. Separation of Rodent T and B Lymphocytes by Rosetting Methods 171
G. Other Spontaneous Erythrocyte Rosettes 172
III. Affinity Chromatography of Cells and Cellular Immunoabsorbent Methods 174
A. Introduction 174
B. Separation of Immunoglobulin-Positive and Immunoglobulin-Negative Lymphocytes by Affinity Chromatography Methods 174
C. Separation of Fc-Receptor-Bearing Lymphocytes by Affinity Methods 178
D. Separation of Specific Antigen-Binding Lymphocytes by Affinity Methods 179
E. Separation of Cytotoxic T Lymphocytes by Cellular Immunoabsorbent Methods 182
IV. Elimination of Lymphocyte Subpopulations by Antisera Plus Complement Treatment 184

V. Elimination of Specific Lymphocyte Subpopulations by Selective Incorporation of High-Specific-Activity Radioisotopes ("Suicide" Methods) 185
VI. Separation of Lymphocyte Subpopulations by the Use of Lectins 185
VII. Conclusions 187
References 188

Index 201

1

Computer Analysis of Lymphocyte Images

PETER H. BARTELS AND GEORGE B. OLSON

I. INTRODUCTION

Research on computer analysis of microscopic images of cells concentrates at this time on three major applications. These are the automated recognition of cells from the hematopoietic system, the analysis of epithelial cells and the study of lymphocyte populations. Of these, the first two are clearly related to the immediate needs of the clinical laboratory. White blood cell differential counts are carried out at a rate of more than 100 million per annum in the United States alone. Research and development here has progressed from the first feasibility studies accomplished in the 1960s by Preston (1961) and Preston (1962), Prewitt and Mendelsohn (1966), Ingram *et al.* (1968), Young (1969), and Bacus (1970) to the commercially available automated white blood cell differential counting devices. For the analysis of epithelial cells, the major effort has concentrated on the clinical cytology of the female reproductive tract (Wied *et al.*, 1976). Here, research toward the improvement of the diagnostic characterization of the material and extensive efforts to automate the prescreening for cervical cancer are underway. Also directed toward early detection and diagnosis of malignant disease, and particularly toward the extraction of prognostic clues, are research projects on image analysis of urothelial cells (Koss *et al.*, 1975, 1977a,b, 1978a; Bartels *et al.*, 1977c) and cells from the respiratory tract

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(Reale *et al.,* 1978; Wied *et al.,* 1979). The analysis of digitized images of lymphocytes at this time does not have such immediate clinical applications. Rather, image analysis has been applied as a research tool to (a) provide quantitative data for the assessment of the effects of biochemicals, immunobiologic agents, and ionizing radiation on lymphocyte populations (Bartels *et al.,* 1969a; Kiehn, 1972; McKee, 1975; Anderson, 1975a,b), (b) sharpen the discriminatory capabilities of the analytical computer software, and (c) to develop image analysis as a methodology (Bartels *et al.,* 1974).

A. Computable Image Information

One of the early and fascinating results of cell image analysis by the computer has been the realization that computation can extract information which the human eye does not perceive (Bartels *et al.,* 1972, 1974b; Bartels and Wied, 1975a; Wied *et al.,* 1968). Such information is contained in the distribution pattern of the nuclear chromatin. For example, the value of the conditional probability with which a chromatin granule of a certain optical density (OD) occurs at a given location in the nucleus—provided that a granule of another OD had been observed at a certain other location—is not consciously perceived. Human visual perception appears to be insensitive to higher-order statistical textures (Julesz, 1962, 1975; Julesz *et al.,* 1973). Mutual dependencies between numerical values in the digitized cell image, particularly when an extended local neighborhood is considered, are not perceived to a degree that would allow small differences to be clearly recognized. Furthermore, beyond the mutual dependencies of the OD values in the digitized image, the human visual sense provides no clues to the descriptive statistics of the underlying mutual dependence scheme. For example, if one described the mutual dependence by a multivariate process, visual observation will not provide an estimate for the value of a coefficient of one of the eigenvectors of that process. "Computable image information" of this kind has up to now never been used for diagnostic decision making, or even the objective characterization of cell images. The discriminatory power of computation may thus be used to expand our ability to perceive small differences (Bartels and Subach, 1976). Computable image information may not only be used to describe the chromatin distribution pattern of cells of known type in objective, numerical terms. In addition, computed image information may be used to detect differences between cells which appear identical to the eye and to detect minute and early changes in cells.

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B. Chromatin Distribution Patterns

The chromatin distribution pattern in a cell nucleus is determined by (a) the number and size distribution of the chromatin granules, (b) their staining density, and (c) their spatial arrangement. The chromatin may be described as coarse or fine in granularity as well as uniformly distributed or as aggregated, and one may assess the tendency of the chromatin to concentrate near the nuclear membrane or the center of the nucleus. For cells of a given type or in a given physiologic state, the chromatin distribution pattern is surprisingly consistent and provides for highly reproducible features. Yet, features derived from the chromatin distribution pattern enable one to detect very early responses of a cell to changes in (a) physiologic conditions or (b) to external influences such as ionizing radiation, virus infections, immunologically active agents, and pharmaceutical and toxic substances. Research efforts toward the objective characterization of nuclear chromatin distribution patterns have addressed two major problems. The first is the methodology of introducing a mensuration to what has so far been a strictly subjective procedure; that is, the visual microscopic examination of cells by a cytologist. How can one characterize such chromatin distribution patterns and what measures or features could one define? How can one compute and evaluate these features? How may one apply them for an objective classification or description of a response or a trend? The second problem bears reference to the biologic meaning of these measures. How can one interpret the measures themselves or the observed changes in terms of biologic processes? Of these two problems, the first becomes a prerequisite for a study of the second. The two problems may be addressed independently but it is clear that they are closely related. One can establish a highly successful automated classification procedure for different cell types, e.g., normal and malignant cells. The classifier may use features which consistently render excellent discrimination. To accomplish this, one does not have to have any insight into the biologic reasons as to why these features assume certain values in malignant cells and markedly different values in normal cells. On the other hand, a more satisfactory situation exists whenever a known biologic process can be closely correlated with the value distribution for a given feature and a biologic interpretation is possible. Under these conditions one can measure a biologic response directly. It is well known that the degree of condensation of the nuclear chromatin as heterochromatin or euchromatin reflects different levels of functional activity. Heterochromatin is associated with a lower level of genetic activity. The relationship between heterochromatin and euchromatin and the functional state of cells was explored in several studies by

Kiefer and Sandritter (Sandritter *et al.*, 1967; Sandritter and Kiefer, 1970; Kiefer *et al.*, 1971, 1973, 1974). Increasing differentiation or maturation leads to larger proportions of the nuclear chromatin assuming a condensed form. The ratio of heterochromatin to euchromatin is well defined and varies only within narrow tolerances in cells of a given cell type and in a given physiologic state. Sandritter *et al.* (1967) determined the proportion of heterochromatin in peripheral lymphocytes as 80% whereas lymphoblasts contain 40–60% heterochromatin. Dedifferentiating cells are characterized by a predominance of euchromatic material. The increase in heterochromatin and the loss of genetic information has been described as a pathogenic principle by Sandritter and others (Harbers and Sandritter, 1968; Harbers *et al.*, 1968). Marked changes in the ratio of heterochromatin to euchromatin were observed in transforming lymphocytes (Bartels *et al.*, 1969; Rowinski *et al.*, 1972). For a survey of research efforts aimed at the methodology of cell-image analysis, the reader is referred to the monographs by Wied *et al.* (1970a, 1976), to the proceedings of the First Life Sciences Conference at Los Alamos (Richmond *et al.*, 1975), and to the literature survey given by Preston (1976) and Prewitt (1972). Papers reporting descriptions of cell image features which are suitable for an assessment of the chromatin distribution pattern or of the methodology of classification by supervised learning and unsupervised learning procedures are referenced in this text.

II. SELECTION OF EQUIPMENT

The biomedical researcher who wishes to install an image-analysis facility has to make a number of decisions about the configuration of the system and about specific components. At this stage of the planning process it is very worthwhile to discuss one's plans with researchers who are now operating such facilities, and with personnel from one's own institution to determine what support in digital electronics design, engineering, and computer programming is locally available. One of the major systems components is an optical microscope photometer.

A. The Microscope Photometer

If the system is intended primarily for the study of lymphocytes, a microscope photometer with scanning stage and a photomultiplier attachment is an excellent choice. Lymphocytes are comparatively small cells and scan fiele sizes from 20 × 20 to 60 × 60 bracket the range for most applications. A spatial sampling interval of 0.5 μm has been found adequate in our studies (Wied *et al.*, 1970a; Bartels and Wied, 1975b); if a sampling interval of 0.25 μm is considered necessary, this is still manageable. The optical microscope employed in microphotometry should be designed for high mechanical stability, especially if the photometer attachment is attached directly to the microscope stand. Most research microscopes follow a modular design principle. This allows for great versatility and is a valid design principle. In microphotometry, however, it has proven to pose serious problems. Every attachment, every adapter ring, every extra observation port becomes a potential source of stray light. The user is well advised to check all such connections and to tape them carefully. In the recording of cell images, use of a 100× oil immersion objective is common. The condenser is usually a 0.30 N.A. microscope objective with an extra long working distance. If a condenser system with an adjustable aperture stop is provided, it is advisable to lock its position permanently to assure reproducibility of results. The condenser centering mounts provided by commercial optical companies are totally inadequate for microphotometry. They do not permit the required precise position control, the control knobs are small, and in several models are all but inaccessible. Their threading is too coarse to allow for smooth and definitive control, and they tend not to hold the condenser in the precisely centered position. In actual use, the operator may have to adjust the condenser centration literally hundreds of times. The user of a scanning microscope photometer may find it necessary to design his own condenser-centering mount. A fixed field stop, restricting the illuminated field in the object plane to an area approximately 5 μm in diameter, is essential to keep stray light low. This field stop should be easily removable so that the operator may see the whole field of view when searching for cells or mapping cell locations. The insertion of the field stop before measurement requires a high degree of positional reproducibility. Its mount should be centerable, with an adequate range. In object-plane scanning microphotometers, as described, the delineation of the actually measured spot is accomplished by means of a small measuring stop mounted in an intermediary image plane. Its area corresponds to the chosen spot size, e.g., 0.5 × 0.5 μm^2 in the object plane. Round and square measuring apertures are employed: results are not directly comparable since the convolution of the image of the corresponding object area by the two types of aperture is different. Adjustable apertures carry the constant risk of change in setting and are not desirable. It is important that the operator can conveniently make frequent checks of the coincidence of the measuring aperture and its position target in the ocular by use of a crosshair or small engraved circle. If an adjustment is needed, this should be possible over a reasonable range with smooth but tightly threaded controls. Scanning stages typically are driven by stepping motors. There are manufacturers who offer scanning stages with a 2 × 2 cm range, with 0.5-μm increments and 200 steps/s. Other manufacturers offer dual stages; a coarse stage with an incremental step of 10 μm spanning a wide range and a 0.5μm-increment stage with a 200 × 200 μm range. The fine stage is mounted on top of the coarse stage. Both arrangements make workable systems but both also pose problems.

The wide-range 0.5-μm-increment stages have frequently been found to suffer from resonance effects when run at full design speed. Scan lines may be out of register, may not have formed rectangular scan fields, or may have skipped points. The dual stage arrangement requires software which will keep track of how many steps were taken in what direction on both the coarse and the fine stage. As a rule, it is not true that twenty 0.5-μm steps taken with the fine stage correspond precisely to a single 10-μm step. Failure to keep count will let the computer get hopelessly lost and unable to relocate mapped objects. A newer scanning stage capable of 10,000 Hz, with 0.25-μm increments and a continuous driven motor, is also available. At the present time, the manufacturer does not supply a software system for its operation.

In recent years the value of spectral information has been appreciated more and more. Photomultiplier attachments with three photomultiplier tubes and a set of dichroic beam splitters appear to be the method of choice to record images in three wavelength bands so that the three images are in fixed registration. One may transfer the three images simultaneously to three A/D converters and sample them sequentially by the data-acquisition software. One may also use a multiplexer and offer the images sequentially to a single A/D converter. The latter operation is more time consuming but more economical. For each photomultiplier tube, a separate operational amplifier has to be provided so that each channel can be adjusted to 100% transmission in the free background. Independent dark current controls are also needed. It is customary to digitize the analog photo signal to 8 bits. This corresponds to a byte, or half a word of the usual 16-bit computer word. Eight bits can represent 256 gray levels. Few microspectrophotometers do indeed reach such photometric precision. For a very few custom-built instruments, the signal to noise ratio is better than 200:1. For most commercial instruments the actual signal to noise ratio varies from 100:1 to 60:1. This corresponds to 1.0–1.5% photometric noise and a precision of from 7 to 6 bits. Results obtained on a given microphotometer are highly reproducible. However, there are substantive differences in the contrast transfer function of different models of microphotometers made by different manufacturers and even within the same company. Qualitative results, such as the kind of features contributing to a discrimination, tend to agree between data recorded on a variety of different instruments. Quantitatively, distinctively different decision rules and feature values are found.

With the increasing use of this methodology, introduction of an industry standard should receive serious consideration.

B. Interface

The microscope and its photometric accessories are linked to the computer by an interface. There are two major functions involved in the control of the scanning microphotometer by the computer. These are the collection of image data from the photometer and the operation of the scanning stage. These two must be rigidly coordinated. A typical sequence of operations during data recording is as follows. The operator looks through the microscope and searches for a cell to be scanned. For this, the stage is controlled manually by means of a joystick. The computer keeps track of the stage's x and y positions. Once a cell is found, the operator presses a "store" button on the interface and the computer stores the x,y coordinates in a location map on a disc file. The operator is now free to search for the next cell. The mapping program is particularly useful when an expert is required to identify the cells to be measured. The program allows the specialist to designate a large number of cells for measurement within a relatively short time. The measurements themselves may then be taken by a technician. The search for cells is frequently conducted so that cell selection is randomized. Even before the first cell is located, the operator uses the joystick to circumscribe the area on the slide where cells are found. This boundary is stored by the computer. A program then generates a pair of random numbers denoting an x and a y coordinate. The generated stage position is checked against the stored boundary. If it falls inside the area containing cells, the stepping motors drive the stage to that location. The cell nearest to the crosshair center in the field of view is chosen for measurement. If the randomly generated stage position falls outside the boundary, a new coordinate pair is generated automatically. Another method for mapping cells on a microscope slide and for the exchange of microscope slides between different institutions employs the use of a glass stage micrometer. With this procedure, it is possible to locate a cell within an accuracy of one micron.

After a set of cells has been mapped, the computer recalls the coordinates of the first cell. The operator enters the number of steps per scan line and the number of scan lines. He positions the scanning stage at the upper left-hand corner of the intended scan field and makes a test scan. The test scan merely drives the stage around the periphery of the outlined scan field. It shows the operator whether the cell is completely enclosed by the area. If no correction is needed, the light transmission is corrected to zero OD. This may be accomplished by several methods. One method is to set the value of the starting background point to zero OD. A second method is to determine the mean value of the light points of one scan line and equate this to zero OD. A third method is to scan the entire scan, checking to be sure all values are within the 256 gray levels, and by use of a software program determine that the maximum values of the first peak of the histogram are declared to be zero OD. Scanning is then initiated.

Scanning proceeds in discrete incremental steps. When the stage has arrived at a point, the program waits for a specified amount of time to allow for settling of mechanical vibrations. The analog signal from the photomultiplier tube, i.e., the photocurrent, is then sent to the A/D converter, sampled, and averaged a number of times. If more than one wavelength is recorded, this is done for all channels. Once the value is digitized, a "ready" signal is issued. The stepping motor takes an incremental step. The step counters are updated. A determination is made to ascertain whether the next scan line has to be started. When the scan is completed, the stage is driven back to the starting point. At this time, the operator is offered the option to discard the recorded scan field and to rescan it. If the scan is accepted, the program assigns a name to the cell image. An ID number and pertinent header information for the data file, such as wavelength, spatial resolution, name of the operator, and date, are automatically entered. The map file is updated and the cell is identified as already scanned. The operator is informed how many cells are still to be scanned. For the use of the manual joystick, it is desirable to have a nonlinear response built into the software. When the joystick is held at a large deflection, the stage should run fast. When one wants an exact positioning, the stepping motors should step very slowly.

It is essential to have some sort of visual display so that the scan field can be edited while the original cell is still in the field of view. "Editing" means the elimination of data points that fall into the scan field but are not part of the cell. Rather, they may be due to debris, uneven background due to staining or portions of other cells. For the editing, one may use an interactive program. One may display a cursor which can be driven by the joystick. This cursor can then be used to draw an outline. Everything outside that outline is discarded. One may also use a cursor box where a small frame is displayed and moved around by the joystick The cursor box is used much like a vacuum cleaner: every point that falls into the cursor box is automatically eliminated.

The editing may be done entirely by software. The display then merely shows the results, which one may accept or reject. In the latter case the algorithm will request the entering of new parameters, such as a new boundary threshold. We have found it practical never to discard any of the originally recorded data points. Edited points are merely set to negative values. They can thus always be restored.

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