INTRODUCTION TO FLOW CYTOMETRY

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GROFF M. SCHROEDER
DOUGLAS E. SWARTZENDRUBER

PREFACE

Flow cytometry is a growing field of instrumental analysis with a range of applications in the biomedical sciences. Cytometry performs rapid, quantitative analysis of cell populations through the characterization of individual cells. Although new advancements make modern cytometers easy to operate, operators need to understand the theory, structure, and function of cytometers to get the most from their instruments. This book provides an introduction to flow cytometers and their applications, systems, operation, and safety.

This book is an introduction to the principles and techniques of flow cytometry and not an all inclusive, definitive text on the subject. Included are chapters on cytometer basics, fluidics, illumination sources, fluorescence, photosensing, electronics, data analysis, sorting, and practical use.

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Typographical Conventions

Throughout this book, the word **cell defines** the subjects of cytometric study unless an example specifically refers to another type of particle. Most cytometers use lasers to illuminate cells so this book often refers to illumination sources as **lasers**. The word **component** defines an individual part of a cytometer such as a laser or flow chamber. The word **system** defines a group of components that work together to perform a function such as sorting. The word **characteristic** defines an attribute of an individual cell, while the word **property** defines an attribute of a population.

The use of **bold** defines important terms and concepts throughout the text, and the Appendix contains definitions for most of the terms emphasized with underlining. The **use of the letter "E"** (for exponential) defines scientific notation in the book. For example, 3E8 indicates three times ten to the eighth and 4E-5 indicates four times ten to the minus five.

INTRODUCTION

Flow cytometry is a growing field of instrumental analysis with a range of applications in the biomedical sciences. Cytometry performs rapid, quantitative analysis of cell populations through the characterization of individual cells. Although new advancements make modern cytometers easy to operate, operators need to understand the theory, structure, and function of cytometers to get the most from their instruments. This book provides an introduction to flow cytometers and their applications, systems, operation, and safety.

Throughout this book, the word **cell** defines the subjects of cytometric study unless an example specifically refers to another type of particle. Most cytometers use **lasers** to illuminate cells so this book often refers to illumination sources as lasers. The word **component** defines an individual part of a cytometer such as a laser or flow chamber. The word **system** defines a group of components that work together to perform a function such as sorting. The word **characteristic** defines an attribute of an individual cell, while the word **property** defines an attribute of a population.

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The book is divided into four parts that correspond to the four basic systems of flow cytometry:

* Part One, the delivery system,
* Part Two, the illumination and detection system,
* Part Three, the data collection and analysis system, and
* Part Four, the sorting system.

Each part consists of a group of chapters that examine the structure and function of the components of a system. The sequence of the parts and chapters correspond to the chronological utilization of systems and components in flow cytometric analyses. The division of cytometric function into systems assists with the discussion and instruction of a complex instrumental method. Although some cytometers may be divided into systems such as these, the book's division of the cytometer into these systems is an educational tool.

The last chapter of the book (Chapter Ten) discusses practical flow cytometer operation and safety. The appendix defining important terms follows Chapter Ten. The index helps readers locate specific information in the book, and references for further study are found at the end of each chapter.

CHAPTER ONE: INTRODUCTION TO FLOW CYTOMETRY

Flow cytometry is an instrumental method for the **quantitative analysis** of populations of particles. Although cytometers can analyze virtually any population of microscopic particles, the vast majority of **cytometric studies are performed on biological cells**. Also known as high speed single cell analysis, cytometry exploits a wide variety of technologies to study biological populations through the analysis of individual cells. Flow cytometric studies have great value in immunology, cell culture, oncology, biological research, and clinical medicine.

The cytometer detects the characteristics of each member of a population by carrying individual cells in a flow of liquid past an illumination source. The illuminating light interacts with the cell and photoactive labels attached to the cell. A group of sensors measure the light scattered by the cell, and the fluorescence emission of the cell. A computer stores the signals from the sensors, making a set of data points that correspond to the measured characteristic for each member of the population. Mathematical and non-mathematical analyses of the data points provides detailed information about the population. Most cytometers also sort cells with desired characteristics from the population for further analysis.

Flow cytometer operators often design cytometric studies, interpret cytometric data, and recognize improper operation of cytometric instrumentation. Operators understand the technologies that form the basis of cytometric instrumentation, how these technologies interact with the natural world to yield data, and the basic construction and operation of cytometric instrumentation.

1.1 WHAT IS FLOW CYTOMETRY?

The process of flow cytometry is a synthesis of many technologies. **Chemical tissue disruption** and **fluorescent labels** provide the ability to separate and label cells and their characteristics for study. **Fluid dynamics** provides rapid delivery of individual cells into a small region for sensing. **Laser light sources and optics** illuminate the cells and excite their photochemical labels. **Photosensors and electronics** create electronic signals that are proportional to the characteristics of each cell. **Computer data collection and software data analysis** perform statistical analysis and display the population's properties revealed by the characteristics of each of its members. Finally, computer and electronic control systems use the physical principles of piezoelectricity, fluid dynamics, and electricity and magnetism to **sort subpopulations**. A block diagram of a flow cytometer is shown in Figure 1.1.

Flow cytometry is a process that senses the characteristics of each cell in the population, stores data about each cell, and displays data about the population as a histogram. This process allows the rapid creation of an accurate graph of population data in an afternoon that might take months with conventional microscopic or biochemical methods. This is especially important in the analysis of tissues such as tumors and blood that contain large numbers of cells. In addition, the study of a wide variety of physical and chemical characteristics of the cells is possible.

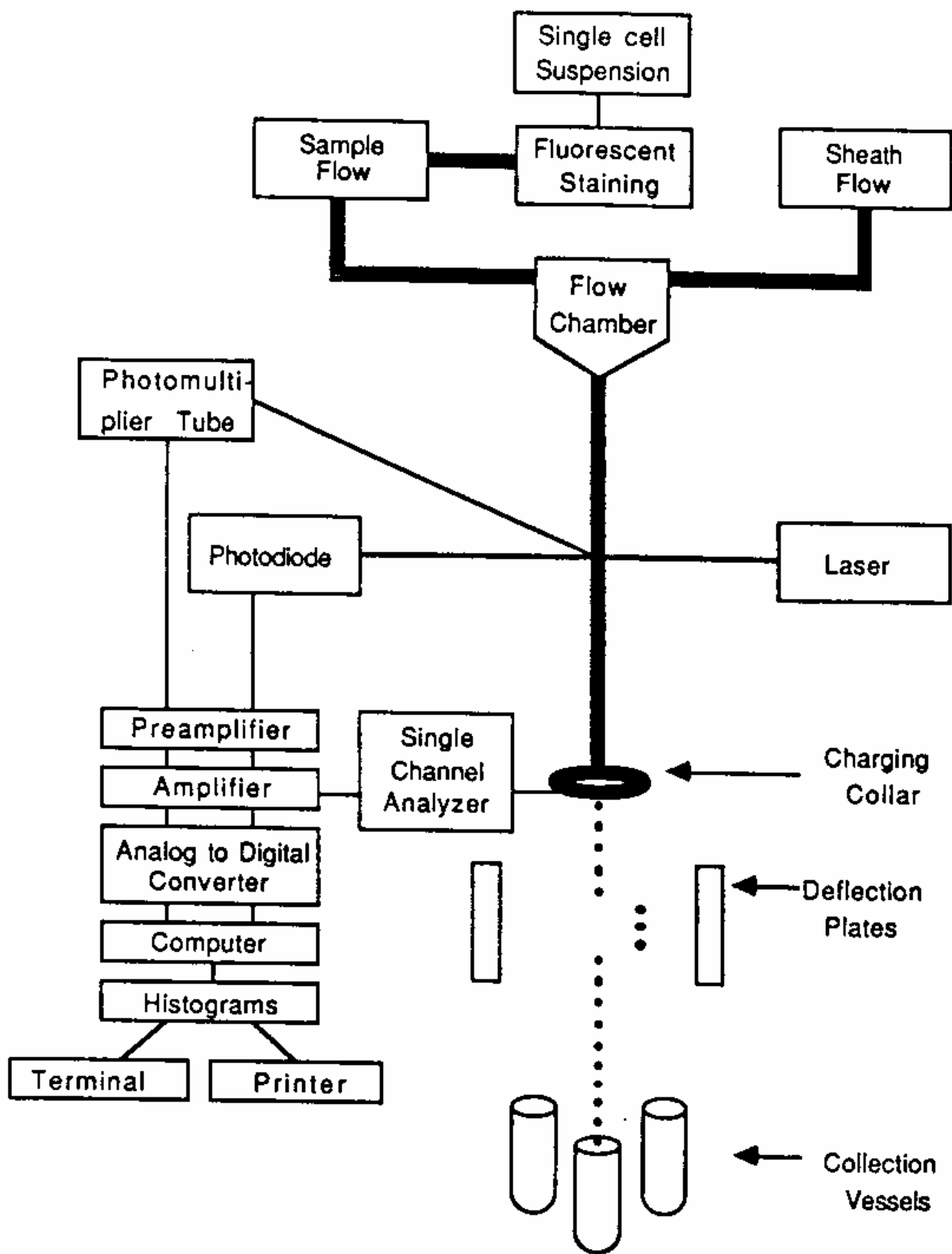

FIGURE 1.1: Block Diagram of a Stream in Air, Laser Based Flow Cytometer. Heavy lines represent liquid flow and medium lines represent light paths. Lines between boxes represent electronic or signal flow. Dots represent droplets.

The diversity of data collection ability makes flow cytometry **a highly adaptable biomedical research method**. Because flow cytometry allows rapid analysis of the individual members of virtually any population, it is an important analytical tool for research, medicine, and the clinical laboratory.

1.2 WHY FLOW CYTOMETRY?

Traditionally, the study of cell biology and biochemistry involves "grind and bind" analytical techniques or microscopic observation of cells. These traditional techniques analyze large numbers of cells as in biochemical studies, or individually as in microscopic studies. The limitations of these approaches are due to the loss of the characteristics of individual population members by studying the population as a whole, or the loss of the properties of the population through the study of individuals.

Biochemistry studies a large population of cells and draws conclusions based on data that represents an average of all of the cells in the population. **Microscopy** draws conclusions based upon the observation of just a few cells that may or may not represent the complete population. Specialized populations, such as complete tumors, may contain hundreds of thousands of cells only 15 micrometers in diameter. The biochemist learns only about the properties of the population as a whole, while the microscopist learns only about the characteristics of a few members of the population. Cytometry fills the gap between the two traditional methods.

The flow cytometer collects data sets from populations based on the measurement of each individual in the population. The cytometer creates a histogram of the cells population under study by placing cells with common characteristics into groups. The diversity of cell types within any given population and the difficulties associated with traditional methods of analysis make flow cytometry an attractive method for the analysis of populations of cells.

Figure 1.2 A presents histograms for three populations analyzed by flow cytometry, while Figure 1.2 B presents the data that might be obtained through traditional biochemical studies of the same populations. Although the populations are very different from one another and contain large variations, biochemical analyses are unable to detect differences between the populations. This inability to detect the differences between the populations in biochemical studies is due to the need to analyze large numbers of cells to obtain macroscopic results. Thus biochemical analyses result in the
detection of averages that represent the populations. A microscopic examination of the same populations might detect qualitative differences between the populations, but still be unable to detect the presence of subpopulations due to the small number of cells observed by the microscopist.

In contrast, flow cytometry collects data about the population based on quantitative analysis of each cell. Each data point plotted on the histogram represents a characteristic of one member of the population. These **data points are objective, quantitative measurements that can be repeated and reproduced**. Flow cytometry presents an advantage over biochemical and microscopic methods because each member of the population contributes to the population data set, and no member of the population escapes analysis.

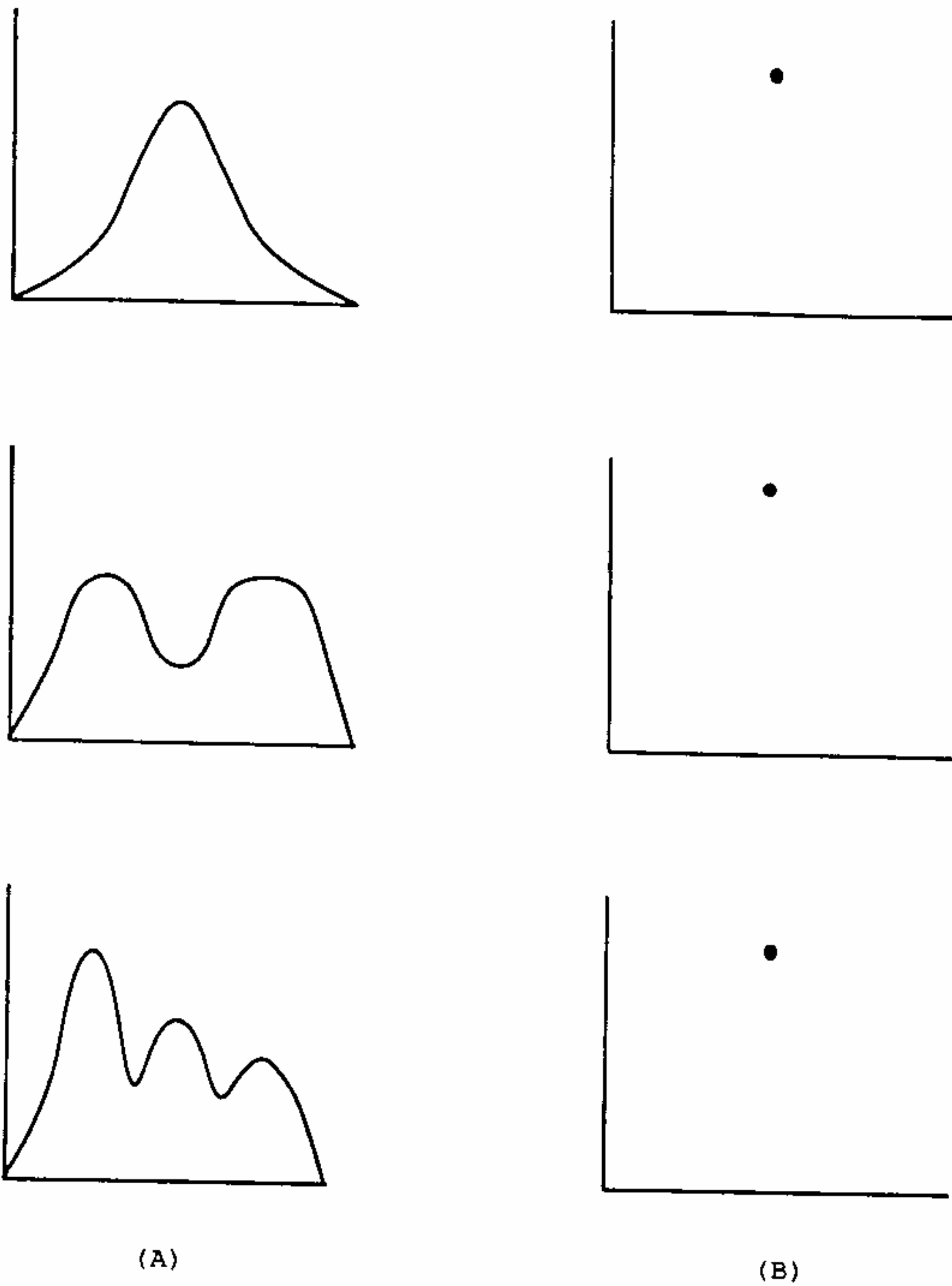

FIGURE 1.2: Population distributions in flow cytometry and biochemistry. (A) Flow cytometry. (B) "Grind and bind" biochemical averages. The result of biochemical studies is an average that may not represent even one member of the population. The flow cytometric studies display properties of the population based on the characteristics of individual members of the population, and each member of the population appears on the graph.

Flow cytometry also allows the detection, display, separation, and analysis of subpopulations. The flow cytometer digitizes and stores the collected data on electronic media, so it can be easily retrieved and reanalyzed with a wide variety of display and analytical methods. The separation of subpopulations from the parent population for further study is another advantage of flow cytometry that cannot be accomplished with traditional methods. These improvements in quantitative analysis of cell populations allow researchers and clinical scientists to make rapid, objective, accurate, and repeatable records of the characteristics of large populations of cells.

In addition to the advantages of quantitative analysis, repeatability, detection of subpopulations, and isolation of subpopulations, the cytometer can perform a wide variety of analyses with and without photoactive labels. Cell characteristics detectable without photoactive labels such as size and internal complexity are called **intrinsic** characteristics. While cellular characteristics that require a photoactive label are called **extrinsic** characteristics. Table 1.1 is a partial list of cytometric analyses.

The ability to study populations of cells and detect small differences between individuals makes flow cytometric instrumentation an **important diagnostic tool** in clinical oncology and immunology. The cytometer's ability to analyze large numbers of cells, detect a wide variety of intrinsic and extrinsic characteristics, and sort individual cells of interest adds to the value of flow cytometry for general biological research.

1.3 HOW FLOW CYTOMETERS WORK

Flow cytometry is laser and computer-based quantitative analysis and sorting of populations of biological cells. "Flow" describes the use of streams of liquid to transport populations of cells, while "cytometry" is a combination of two Greek word stems meaning hollow vessel (cyto-) and the science of measuring (-metry).

Flow cytometers perform quantitative analysis of biological cells, nuclei, chromosomes, and almost any type of microscopic particle. Although flow cytometers perform many tasks as a complete system, the student can consider the instrument as a group of four distinct systems, the delivery system, the illumination and detection system, the data analysis system, and the sorting system.

The **delivery system** prepares the sample for analysis and uses concentric flows of liquid to transport and isolate individual cells. The delivery system performs the following tasks:

a) separation of the population into individual cells,

b) preparation of the cells for analysis,

c) transportation of the cells to the site of analysis, and

d) correct placement of the cells at the site of analysis.

TABLE 1.1: PARTIAL LIST OF CYTOMETRIC ANALYSES

UNLABELED CELLS	LABELED CELLS
Autofluorescence	DNA content
Cell shape	DNA base ratio
Cell size	Endocytosis
Cytoplasmic granularity	Intracellular receptors
Pigment content	Membrane fluidity
Protein fluorescence	Membrane integrity
Redox state	Membrane permeability
	Nucleic acid content
	Protein content
	RNA content

TABLE 1.1: Partial List of Cytometric Analyses. Intrinsic analyses require no specific labeling while extrinsic analyses require specific fluorescence labeling. After Shapiro, H.M., *Practical Flow Cytometry*, Alan R. Liss, Inc., New York, 1988.

The delivery system transports the complete population as single cells to the site of analysis, provides labels that allow the detection of cellular characteristics, and prevents the collection of incorrect data due to clumps of cells or incomplete illumination of cells.

Once the delivery system transports the cells to the site of analysis, an illumination source interacts with the individual cells. Photosensors capture light signals as the cells pass through the illumination source. The optical systems from both the illumination source and the photosensors converge on a single point in the liquid flow called the interrogation point. At the interrogation point, each cell is analyzed as it passes through in single file. The group of components that accomplishes these functions is called the illumination and detection system.

The **illumination and detection system** uses individual photometric analyses to generate electrical signals proportional to the physical or chemical characteristics of each cell. The illumination and detection system performs the following tasks:

a) illumination of the cells with intense laser light, and

b) sensing the interaction of the light and the cells.

The illumination and detection system collects data about the population by detecting the characteristics of each member of the population. A laser illuminates each cell as it passes through the interrogation point, and photosensors detect the interaction of light with each cell. The photosensors emit analog signals corresponding to the amount of light received from each cell. The illumination and detection system sends the analog electrical signals from the photosensors to the data collection and analysis system.

The data collection and analysis system performs electronic amplification, digitization, and storage of the analog signals provided by the illumination and detection system. A dedicated computer stores, analyzes, and displays information about the population. The data collection and analysis system performs the following tasks:

a) amplification and digitization of sensor signals,

b) storage of digitized signals in data sets,

c) mathematical or non-mathematical analysis of the data, and

d) display or printing of data for interpretation.

The **data collection and analysis system** converts the analog light signals from each cell in the population into digital signals and stores them in a data set. The computer displays these data sets as histograms that represent the properties of the population as a frequency distribution of cellular characteristics.

The operator may also wish to separate cells with certain characteristics from the population. The selection and sorting of cells with desired characteristics requires the sorting system, and another pass through the delivery system and the illumination and detection system.

The **sorting system** uses electronic pulses to recognize and sort individual cells from the population. The sorting system performs the following tasks:

a) identification of individual cells for sorting,

b) breaking the flow into droplets containing a single cell,

c) charging the droplets as they break off of the flow,

d) deflection of the droplets with charged plates, and

e) capture of the separated cells in different containers.

The operator sets the cytometer to sort desired cell(s) and replaces the sample into the delivery system. The sorting system compares photosensor signals with the signals that represent the cell(s) chosen for sorting. A piezoelectric element vibrates the flow into droplets, and a charging collar charges the droplets as they break off. An electromagnetic field deflects the droplets that contain the desired cells into a separate container.

These four systems work together as parts of a complex instrument, providing a method of quantitative analysis for populations of particles. The value of cytometric analyses also include the determination of the properties of populations, and the ability to identify and separate individuals with desired characteristics from a population. These capabilities give the cytometer great utility over the traditional methods of population analysis, and provide a method for the isolation of individuals within a population for further analysis or culture.

1.4 THE FOUR SYSTEMS OF FLOW CYTOMETRY

Construction of flow cytometers varies with each manufacturer, but all flow cytometers have a group of common functions. This section discusses these common functions in the context of four interdependent systems described above: the **delivery system**, the **illumination and detection system**, the **data collection and analysis system**, and the **sorting system**. Block diagrams of each of these systems are found at the beginning of each part of this book.

1.4.1 THE COMPLETE CYTOMETER

Modern flow cytometers are complex instruments consisting of a **synthesis of many individual components**. A block diagram of a laser based flow cytometer is shown in Figure 1.1. Successful operation of flow cytometers involves a variety of complex tasks including the correct design of cytometric studies, actual operation of the instrument, the collection of data, and the interpretation of data. To accomplish these tasks, the operator must understand how

physical processes, biochemical techniques, and cellular morphology interact with the hardware components of the cytometer to yield data.

1.4.2 THE DELIVERY SYSTEM

To prepare a population for analysis, each cell must be separated from other cells in the population through the creation of a **single cell suspension**. A single cell suspension is a liquid containing only individual cells. In a fluid tissue such as blood, the natural state of the sample is a single cell suspension. In this context, the operator usually separates the unwanted cells (such as red cells) from desired cells (such as white cells) prior to analysis. In contrast, the **separation of cells from a solid tissue** such as a tumor requires filtration, enzymatic digestion, or mechanical disruption. When tissues are solid, the addition of a liquid aids in the analysis of the population by providing a medium for transportation.

Usually, the liquids that suspend and transport cells in flow cytometry are **isotonic**. The isotonic liquid provides a chemically neutral media for the transportation of cells. In addition, the presence of dissociated ions such as sodium and chloride allow the sorting system to impart an electrical charge to droplets of the liquid.

If the cells are to undergo an intrinsic analysis, the creation of a single cell suspension is the only requirement before analysis. If the cells are to undergo an extrinsic analysis, labeling of the cells occurs before placement in the flow cytometer. Often, the correct concentration of the **fluorescent label** is simply added to the single cell suspension.

The flow cytometer transports cells to the flow chamber in a liquid flow called the sample flow. The sample flow is injected into the center of the sheath flow in the **flow chamber**. The concentric liquids allow rapid transportation of the cells and provide a method for accurate and precise localization of the cells during measurement at the **interrogation point**.

1.4.3 THE ILLUMINATION AND DETECTION SYSTEM

The illumination and detection system illuminates the cells with a very bright light and detects each cell's interaction with that light. Sensors detect light scattered from the surfaces and inner structure of the cell, and fluorescence signals emitted from the labeled characteristics of the cell. These tasks are accomplished with lasers, photodiodes, and photomultiplier tubes.

At the interrogation point, a tightly focused beam of very bright light illuminates the cells. Usually, cytometric illumination sources are **lasers**. The light scatters from the surface of each cell and from structures inside the cell. The light also excites any fluorescent stains in the cell and causes them to fluoresce.

A photodiode detects the light scattered from the cell surfaces and a series of **photomultiplier tubes** detects the various fluorescence wavelengths emitted from labeled cellular components. In some types of analyses, photomultiplier tubes also detect scattered light from cellular components.

A series of **optical filters** and **dichroic mirrors** guide illumination source light, scattered light, and the fluorescence emission from labeled components into the photomultiplier tubes. The filters and mirrors separate and direct different wavelengths of light into separate photomultiplier tubes. This separation of individual wavelengths into dedicated photosensors allows the cytometer to detect the amplitude of each wavelength, and thus, the relative quantity of the labeled characteristic in each cell.

The light emitted from the interrogation point may be light scattered from the edge of the cell, light scattered from cellular components within the cell, or fluorescence emitted from labeled cellular components. Both photodiodes and photomultiplier tubes emit **analog electrical signals** that are proportional to the light received from the interrogation point.

1.4.4 THE DATA COLLECTION AND ANALYSIS SYSTEM

In the data collection and analysis system, the electrical signals from the photosensors undergo amplification, digitization, and storage on floppy or hard disk. A dedicated computer then presents a frequency distribution of the data for interpretation.

Transampedance amplification converts the electrical current output of the sensors into electrical voltage in preparation for digitization. **Analog to digital converters convert** the analog voltage signals to digital representations of the signals. The signals are sent to a **computer**, where tape, floppy disk, or hard disk media store these digital representations of the analog signals for future reference and analysis.

Interpretation of the data employs statistical analysis or a graphical display called a **histogram**. The computer retrieves the data stored in the cytometer's memory and performs mathematical or non-mathematical analyses for redisplay or printing. This allows the operator to reproduce analyses or perform new analyses on old data.

1.4.5 THE SORTING SYSTEM

The sorting system uses the population data collected by the data collection and analysis system to isolate subpopulations of cells. Selection of a subpopulation for sorting **occurs after cytometric analysis** of the complete population.

A **single channel pulse height analyzer** is set to match the waveform of the cells desired for sorting. The operator replaces the population in the delivery system which returns the cells to the interrogation point. As each cell passes through the interrogation point, the waveform it generates is electronically compared with the waveform in the single channel pulse height analyzer. If the waveforms match, the cell is selected for sorting.

As the cells pass out of the interrogation point, a piezoelectric material vibrates the flow, breaking it into droplets. A charging collar charges the droplets that contain cells selected for sorting with an electrostatic charge as they break off of the flow. Finally, pair of electrostatically charged plates deflects the charged droplets that contain selected cells into separate collection vessels.

1.5 APPLICATIONS OF FLOW CYTOMETRY

Flow cytometry has many applications in clinical and laboratory research. Perhaps the most important clinical applications are in the medical specialties of oncology and immunology. Clinical oncologists use **flow cytometric data as a prognostic indicator in certain types of cancer**. Clinical immunologists can use flow cytometric data to monitor immune disorders such as Acquired Immunodeficiency Syndrome. In the research setting, cytometry has applications in a wide variety of protocols including genetics, tissue culture, and the study of non-biological microscopic particles.

1.5.1 FLOW CYTOMETRY IN CANCER RESEARCH

The **traditional diagnosis of tumor malignancy** is microscopic examination of cellular characteristics. Visual observation of degree of tissue and cellular differentiation, nuclear morphology, size of the nucleus, and presence or absence of nucleoli is the most common diagnostic method. The detection of these characteristics relies upon the observation of only a few cells, subjectively indicates if a tumor is malignant, and suggest how aggressive the tumor will be.

Flow cytometry offers a method for extensive and detailed analysis of complete tumor cell populations. One of the most important cytometric analyses of tumors is a measure of the amount of DNA that the tumor cells contain. **Flow cytometry determines the DNA content of each cell in a tumor using a label that binds stoichiometrically to DNA**. By recording the DNA content of normal and abnormal cells, the cytometer can provide an objective measure of the differentiation of the cancerous tissue. Often, tumor cells contain only minute differences from normal cells.

The DNA content of the abnormal (cancer) cells is divided by the DNA content of the normal cells (usually leukocytes), providing a number called the **DNA Index (DI)**. In normal cells, the DNA Index is one. If the DNA content of the tumor cells is abnormal, the DNA Index will be greater than or less than one. The DNA Index may be indicative of the degree of abnormality of the cancerous tissue in an objective, quantitative fashion. The DNA Index often correlates with the aggressiveness of a tumor, and in malignancies such as breast cancer, the DNA Index appears to have important diagnostic and prognostic significance.

1.5.1.1 EFFICACY OF FLOW CYTOMETRY IN BREAST CANCER

Chemotherapy and radiation treatments are risky, expensive, uncomfortable, and often involve serious complications and unwanted side effects. Clinical studies (e.g., Coulson et. al., 1984) have shown that (when combined with estrogen receptor and staging studies) **the DNA Index is of prognostic significance** in breast cancer therapy. This means that a DNA Index study suggesting that a tumor will be aggressive is probably correct.

Interpretation of flow cytometric DNA indexes in conjunction with other studies assists the physician and the patient by suggesting the probable future course of the malignancy. The DNA Index allows the patient to avoid high risk treatments when the prognosis is good, or to employ high risk treatments when the prognosis is bad.

The detection of minute differences between cells is also very important in other medical situations where minor changes in cells have major consequences in the clinical setting. A good example of such a situation is the study and tracking of immunological diseases.

1.5.2 IMMUNOLOGICAL RESEARCH

The value of flow cytometry in the study and diagnosis of immune function and disease relies on the availability of immunofluorescent probes such as **fluorescent monoclonal antibodies**. Fluorescent monoclonal antibodies label immune cell surface markers, receptors, and secretions. Although immunofluorescence is characteristically very weak, the flow cytometer detects minute differences between cells with immunofluorescence analyses, determining leukocyte subset ratios.

1.5.2.1 MONOCLONAL ANTIBODIES IN CYTOMETRY

Advancements in cell culture techniques allow the production of
biologically active moieties that specifically bind to a chosen antigenic site. These moieties are called monoclonal antibodies because they are produced from the clone of a single cell. Laboratory preparation of monoclonal antibodies allow the researcher to create labels that bind to virtually any biochemical.

Attaching fluorescent molecules to a monoclonal antibody makes the antibody detectable to the illumination and detection systems of flow cytometers. The resulting fluorescent probe allows the flow cytometer to **quantify virtually any cell population**. It is possible to create monoclonal antibodies to a variety of biological components, so fluorescent monoclonal antibodies can label almost any cell characteristic. This capability is essential to the cytometric study of a wide variety of biological processes, especially immunological cell surface markers.

1.5.3 GENETICS RESEARCH

In contrast to monoclonal antibodies, intercalating fluorescent chemicals label nucleic acids by fitting stoichiometrically into the spaces between the bases of double stranded polynucleotide chains. These dyes allow flow cytometric determination of the nucleic acid content of many types of biological particles including cells, chromosomes, and double stranded nucleic acids. Labeled cells and chromosomes are studied directly, while smaller sections of nucleic acids are attached to microbeads for transportation and analysis.

The stoichiometric binding of these labels and the high sensitivity of cytometric photosensors allow the detection of small differences in nucleic acid quantity. This property of intercalating fluorescent labels allows the **analysis and sorting of any population that contains double stranded nucleic acids**, even those with only small differences in numbers of base pairs.

In another application, fluorescently labeled DNA identifies bacterial transformants or eukaryotic transfectants by detecting the cells containing the labeled DNA. The sorting system then separates cells that contain labeled DNA for culture or further analysis. With this method, the researcher can be absolutely sure that the sorted population contains only transformants or transfectants.

1.5.4 TISSUE CULTURE

Tissue culture often requires the isolation of a homogeneous population of cells. The sorting capability of the flow cytometer makes it an ideal tool for the **creation of homogeneous cell populations for growth in culture**.

In this application, the researcher performs a cytometric analysis on a population of cells and chooses a subpopulation for culture. The researcher sets the cytometer to recognize and sort that subpopulation and the same population is analyzed with the cytometer. The cytometer separates cells that match the preset conditions, resulting in a sorted subpopulation that is homogeneous. Once the cells are growing in culture, cytometric monitoring of the tissue culture population provides quantitative data about each cell in the population.

1.5.5 NON-BIOLOGICAL SAMPLES

Flow cytometry makes the study of virtually any population of microscopic particles possible. The analysis of intrinsic characteristics such as particle size and surface complexity is easy to perform. In addition, the data obtained by the cytometer is more quantitative than data obtained through other methods of analysis. Such studies are helpful in materials science or other branches of science requiring the quantitative analysis of microscopic particles.

1.6 SUMMARY

The flow cytometer is a complex instrument that **characterizes and sorts populations of biological particles** including cells, chromosomes, organelles, and other microscopic particles. Flow cytometry has important **applications in biophysics, biochemistry, immunology, oncology, genetics, tissue culture, and non-biological particle characterization**. Flow cytometers consist of four interdependent systems.

THE DELIVERY SYSTEM
Preparation of the cells for cytometric study includes the separation of the tissue into a **single cell suspension** and the labeling of the individual cells in the population with **fluorescent molecules**. Two **concentric liquid flows** (the **sample** and **sheath** flows) transport and localize the cells for precise measurement at the **interrogation point**.

THE ILLUMINATION AND DETECTION SYSTEM
The flow cytometer uses a bright beam of light such as a laser to illuminate each cell as it passes through the interrogation point. The cytometer measures cell **size** by detecting the **light scattered** as the cell passes through the interrogation point. **Photomultiplier tubes** measure the **fluorescence** emitted by each cell.

THE DATA COLLECTION AND ANALYSIS SYSTEM
Analog electrical signals from light scatter and fluorescence photodetectors receive **amplification** and **analog to digital conversion** before being sent to a computer as digital signals. The computer stores the **digital signals** on floppy or hard disk where they can be retrieved for later analysis. The computer generates **histograms** of the data for review and interpretation.

THE SORTING SYSTEM
A **single channel pulse height analyzer** matches the analog output pulse of cells with desired characteristics and **directs cell sorting**. The flow is broken into **charged droplets** and an electrostatic charge deflects **droplets** that contain cells matching the preset waveform into a collection vessel.

CONCLUSION
Flow cytometry is a tool for the **quantitative analysis** of biological particles. The instrument is capable of rapid determination of a wide variety of morphological, biochemical, biophysical, and population characteristics. The flow cytometer has many uses in the fields of biomedical research because of it's capability of rapid characterization and sorting of populations of cells.

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CHAPTER 2: FLOW CYTOMETRY IN BREAST CANCER

Pathologists often use optical microscopy to determine the malignancy of cells. The pathologist observes a section of the tumor that has been affixed to a slide. By observing the degree of tissue and cellular differentiation, nuclear morphology, size of the nucleus, and presence or absence of nucleoli, the pathologist is able to diagnose the presence of cancer. This diagnosis relies upon the observation of a limited number of cells, and is a qualitative measure of tumor aggressiveness. In contrast, cytometric analyses of tumor cell populations rely on the analysis of every cell in the tumor, and provide quantitative data about the aggressiveness of the tumor.

2.1 FLOW CYTOMETRY IN ONCOLOGY

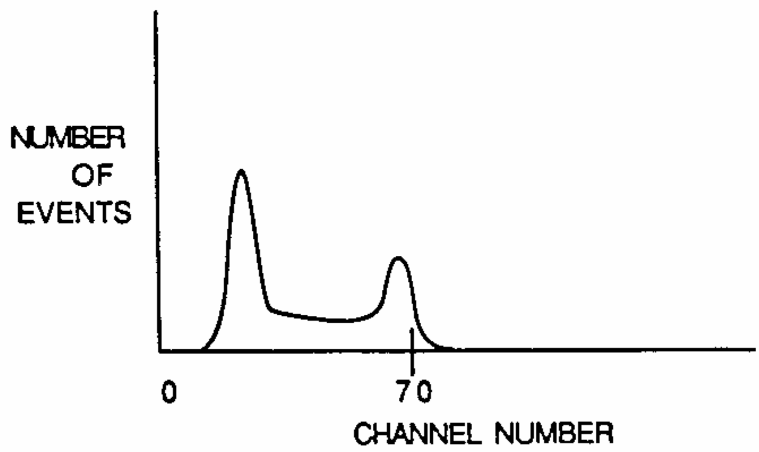
One of the most common uses of cytometric instrumentation is **the determination of DNA indices** for cancerous tissues. These indices, which are a ratio of DNA present in the cancer cells to the amount of DNA present in normal cells, can be an important prognostic indicator. This section describes a cytometric analysis of DNA index and introduces the process of flow cytometry.

2.2 CALIBRATION OF THE CYTOMETER

The collection of correct and repeatable data requires careful calibration of the cytometer before each use. It is important to **document the calibration** of some cytometers by writing down component settings for future reference. Other cytometers may provide this information on a computer printout. The documentation of settings from the first analysis is essential when repeating the analysis. If the settings on the cytometer are different, it will be almost impossible to obtain the same data from a repeat analysis of the same sample.

The first task of calibration is the **alignment of the optics** of the illumination and detection system and the fluid stream that contains the cells. When this alignment is complete, the operator performs an **analysis of fluorescently stained leukocytes** from the individual with the tumor. This analysis creates a baseline of normal DNA content for use in the determination of the DNA index. The operator then places the histogram of the normal cells at some arbitrary point on the histogram.

For this example, the normal G0/G1 peak (representing non-dividing cells with diploid DNA) is set at channel number 280 as shown in Figure 2.1. The horizontal axis of the histogram uses channel numbers because of the output of analog to digital converters occurs in 1024 channels. The vertical axis represents the number of events, in this case cells, that posses the characteristic being measured. See Section 10.7 (page 156) for a discussion of flow cytometer calibration.



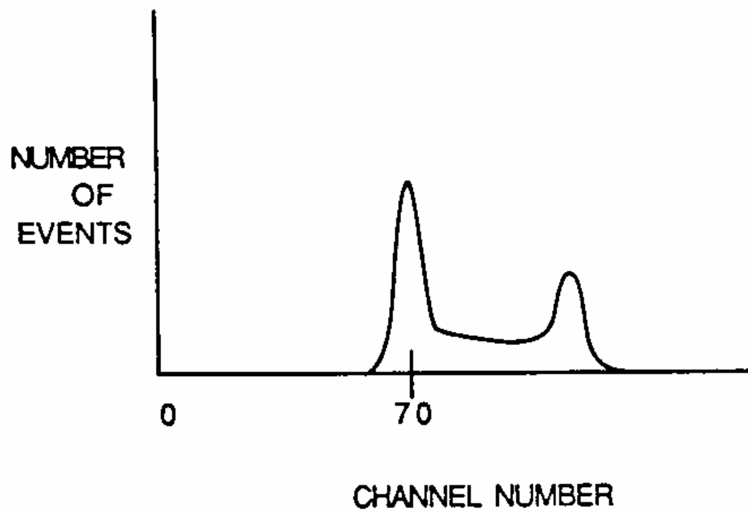


FIGURE 2.1: Histograms illustrating placement of normal cell G peak in channel 70 during calibration of the flow cytometer. Random placement is shown in (A). Placement at channel 70 is shown in (B).

2.3 SAMPLE PREPARATION

The next step in flow cytometric analysis is the **preparation of a single cell suspension**. The operator creates a suspension of single cells using mechanical or chemical disruption of the tumor tissue as described in Section 3.3. The creation of a single cell suspension allows the cytometer to transport the individual cells, and improves the uptake of stain by the tumor cells.

Once the separation of the tissue is complete, the nucleic acid of the cells is stained so they can be detected with the cytometer. Nucleic acid stains usually employ **intercalating fluorescent molecules** such as propidium iodide. These molecules fit into the spaces between the base pairs of double stranded RNA or DNA. Tumor cells contain both DNA and RNA, so the operator treats the cells with RNAse prior to staining with an intercalating stain. The RNAse destroys the double stranded RNA, preventing the RNA from becoming stained. Without RNAse treatment, the analysis yields the DNA plus RNA index or total double stranded nucleic acid content of the cells.

The collection of correct data regarding DNA content requires that the propidium iodide accomplish **stoichiometric** saturation of the DNA. Propidium iodide binding is said to be stoichiometric when the number of dye molecules bound to the DNA is proportional to the number of base pairs. See Chapter 3 for a discussion of fluorescence labeling.

2.4 FLUIDICS

Once sample preparation is complete, the operator places the labeled single cell suspension in the sample container of the flow cytometer. A pressurized gas forces the suspension from the container into the first of two liquid flows called the **sample flow.** The cells move down the center of the tube in single file. The friction due to the walls of the tube is lowest in the center of the tube, so the cells move into the center of the tube, where friction is low and the flow is fast.

The sample flow then enters the **flow chamber** and injects into the center of a second liquid flow called the **sheath flow.** Once again, the cells localize at the very center of the flows, giving them a very predictable location. The flow chamber narrows at the bottom so the velocity of the flow increases, the distance between the cells increases, and the cells become even more localized in the center of the flow. The flow chamber "focuses" the flow of liquid and the cells so that they are at a precise location for illumination and sensing at the interrogation point.

This process creates **positional certainty**, precisely placing each cell in the center of the flow as it leaves the flow chamber and passes through the interrogation point. Figure 2.2 illustrates the creation of positional certainty by the flow chamber. The correct placement of the single file progression of cells in the interrogation point is essential to the collection of good data about the population. See Chapter 4 for a complete discussion of fluidics.

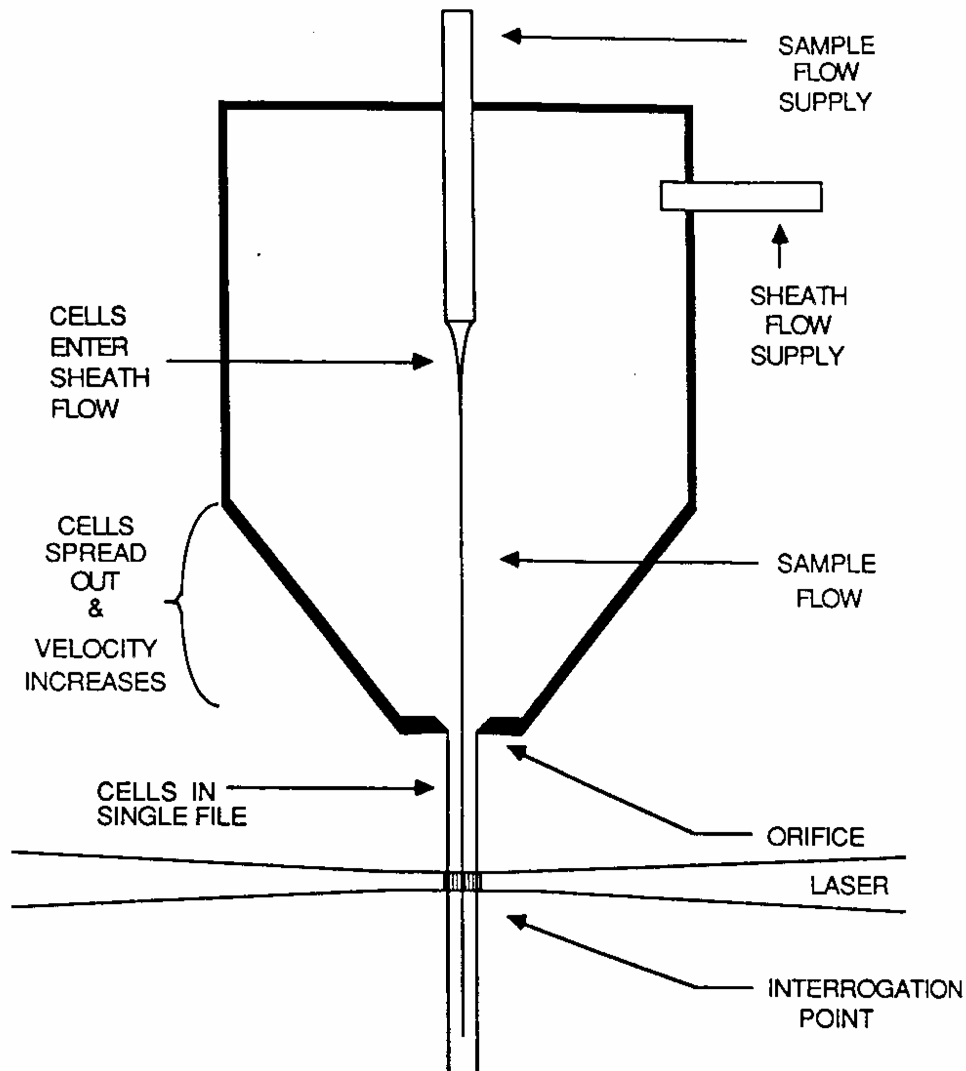


FIGURE 2.2: The flow chamber. In the flow chamber, the sample fluid injects into the center of the sheath flow. As the chamber narrows, the sample flow and cells "focus" into a stream of widely spaced single cells in the center of the sheath/sample flow.

2.5 THE INTERROGATION POINT

The flow chamber creates a stream of moving liquid that carries the cells in single file, precisely localizing the cells at the center of the flow. The liquid and the cells it carries exits the flow chamber and enters the interrogation point. At the interrogation point, the liquid flow coincides orthogonally (at right angles) with the focal beam of the laser and the focal points of the photodetector optics. Chapter 5 discusses illumination sources.

As the cells enter the illuminating beam, **the light interacts with each cell**, creating light signals that correspond to cellular characteristics. The edges and interior structures of the cells scatter the light in all directions producing signals that correspond to the size and complexity of the cell. The propidium iodide bound to the DNA of the cell absorbs the illuminating light and fluoresces, creating a light signal that corresponds to the quantity of DNA present in the cell. Figure 2.3 is an illustration of the interrogation point.

2.6 LIGHT SCATTER

When cells pass through the laser at the interrogation point, light scatters in all directions from each cell's surface and internal components. The measurement of the scattered light occurs in three ways, detecting three different types of information about the cell that scattered the light.

A **photodiode** in the axis of the illuminating beam performs the primary scattered light measurement. Figure 2.4 illustrates this **Forward Angle Light Scatter (FALS)** measurement that provides information concerning the size of the cell. A more accurate measurement of the cell size uses narrow angle light (NALS) scatter in the axis of the illuminating beam.

Side scatter (also known as 90 degree light scatter) is another method of light scatter detection. Side scatter measurements involve measuring the light scattered at right angles (orthogonally) to the illuminating beam. Side scatter data is related to the internal complexity of the cell. Side scatter measurements are captured by a photosensor called a photomultiplier tube. Chapter 6 discusses each of these scatter measurements in detail.

2.7 FLUORESCENCE MEASUREMENTS

The illumination beam also excites the propidium iodide molecules intercalated into the cell's DNA, causing them to fluoresce. The fluorescence occurs at a slightly longer (lower energy) wavelength than the illuminating wavelength and continues as long as the cell is in the illuminating beam. Once the cell leaves the illuminating beam, fluorescence ceases within 1E-7 seconds. Chapter 3 discusses the basics of fluorescence emission.

A **photomultiplier tube (PMT)** detects fluorescence emission while the cell is in the interrogation point. The photomultiplier tubes are perpendicular to the illuminating beam as shown in Figure 2.5. Propidium iodide binds stoichiometrically to the DNA so the amount of light emitted by the propidium iodide is proportional to the amount of DNA present in the tumor cells.

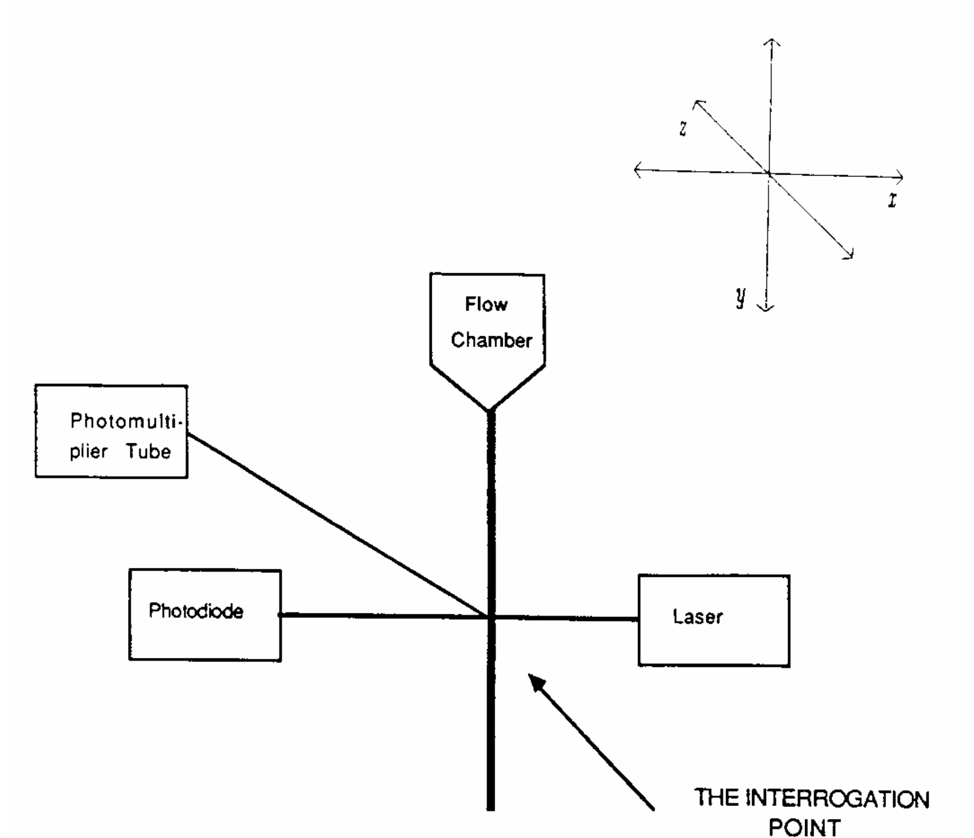


FIGURE 2.3: The interrogation point. At the interrogation point, the sheath/sample flow and the focal points of illumination and detection optics coincide. As the cells pass through the interrogation point, photosensors detect fluorescence and light scatter signals.

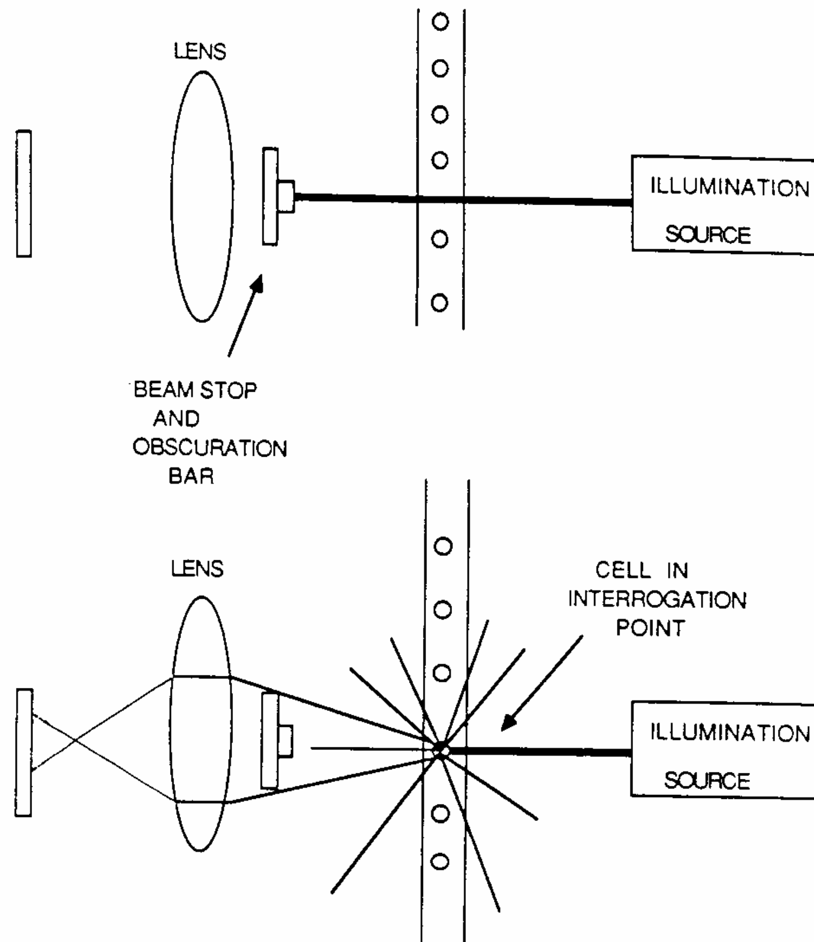


FIGURE 2.4: Forward angle light scatter. When the cell passes through the illuminating light, a photodiode measures the angle of the light scattered by the cell. When no cells pass through the interrogation point, the beam falls on the beam stop and no signals are generated. When no cell is in the interrogation point, the liquid flow diffracts the illuminating beam and the diffracted light falls on the obscuration bar.

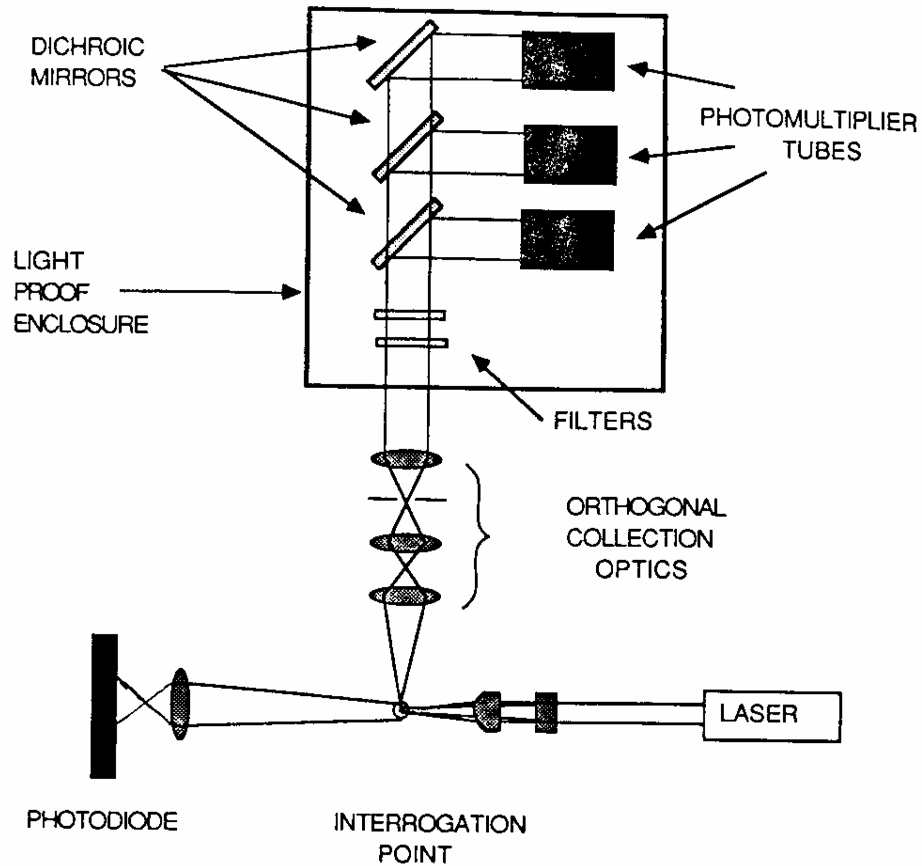


FIGURE 2.5: Orthogonal fluorescence measurement with a photomultiplier tube (PMT). Fluorescence measurement occurs orthogonally (perpendicularly) to the illuminating beam. The PMT detects a light pulse that is proportional to the amount of fluorescent material present in the cell. The photomultiplier tube also detects orthogonal light scatter from the cell.

Normal cells are diploid (2n DNA content), while abnormal cells may be aneuploid if they are hyperdiploid (more DNA than diploid), hypodiploid, (less DNA than diploid), or multiploid (more than one abnormal population). The prognosis for tumors with DNA Indices that are very close to diploid are much better than for tumors with strongly aneuploid or multiploid cell lines.

2.8 DATA ACQUISITION

The **optical sensors** in the cytometer (photodiodes and photomultiplier tubes) convert analog light signals from the cells to analog electrical signals. Electronic amplifiers increase the amplitude of the electrical signals and **analog to digital converters (ADC)s** convert the signals into digital signals. Each detector or amplifier output requires one analog to digital converter. The analog to digital converters send the signals to a computer for storage and analysis. Digitalization of analog signals is essential because computers require digital input while sensors emit analog signals. Chapter 7 discusses electronics and signal processing in flow cytometry.

2.9 DATA ANALYSIS

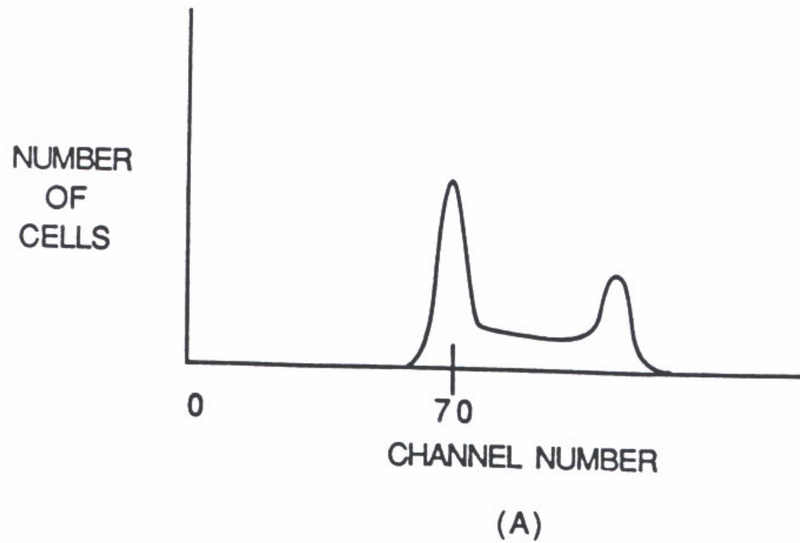
A computer stores, records, and displays the digital signals as graphs called **histograms.** Histograms are two, three, or four dimensional plots of the data points received from the analog to digital converters for each cell. The computer allows the operator to plot the digital data in many ways on the histogram, assisting with interpretation. A video terminal or printer displays the histograms for analysis. Figure 2.6 shows the normal cell histogram (A) and the breast tumor histogram (B). See Chapter 8 for a discussion of data acquisition and analysis.

2.10 CELL SORTING

After the creation of histograms for the population, the operator chooses subpopulations for sorting. The operator selects a subpopulation and programs a **single channel pulse height analyzer (SCA)** to control the sorting of cells with desired characteristics.

Placing the population back into the sample container, the operator passes the sample through the interrogation point a second time. A **piezoelectric crystal** vibrates the flow chamber and breaks the sample/sheath flow into groups of droplets that contain selected cells as shown in Figure 2.7.

The sorting system uses electrical signals from the photosensors to choose a series of several drops that contain no more than one cell. The single channel pulse height analyzer recognizes cells that match the characteristics of cells selected for sorting, and charges the end of the stream as the droplets break off with a **charging collar.** The droplets retain the electrostatic charge present on the stream when they separate, and a set of **deflection plates** deflects the charged droplets into sample containers. Chapter 9 discusses sorting.



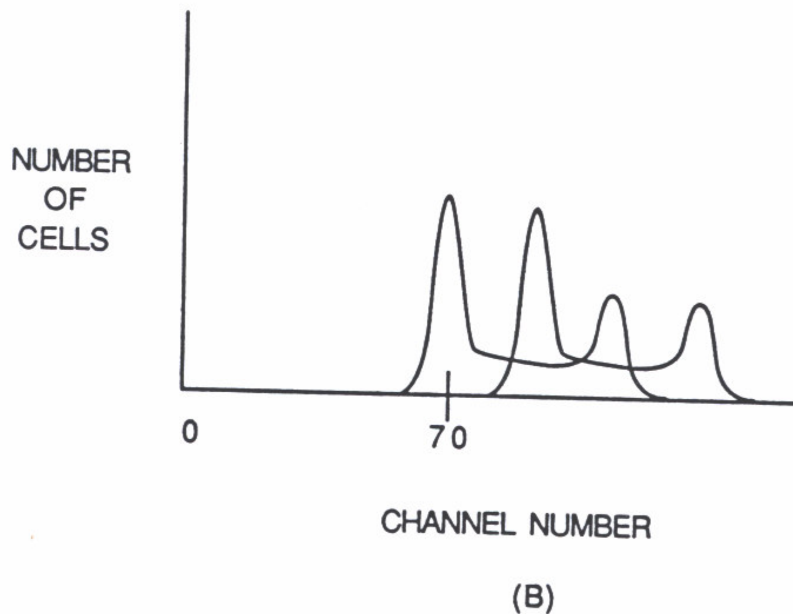


FIGURE 2.6: One parameter histograms of normal breast tissue (A), and an aneuploid breast tumor (B). The digits on the horizontal axis of the histogram are channel numbers that represent analog to digital converter output. The channel number is proportional to the total fluorescence of each cell and the total fluorescence is proportional to the DNA present in each cell. Note the normal G0/G1 reference cells present in channel seventy.

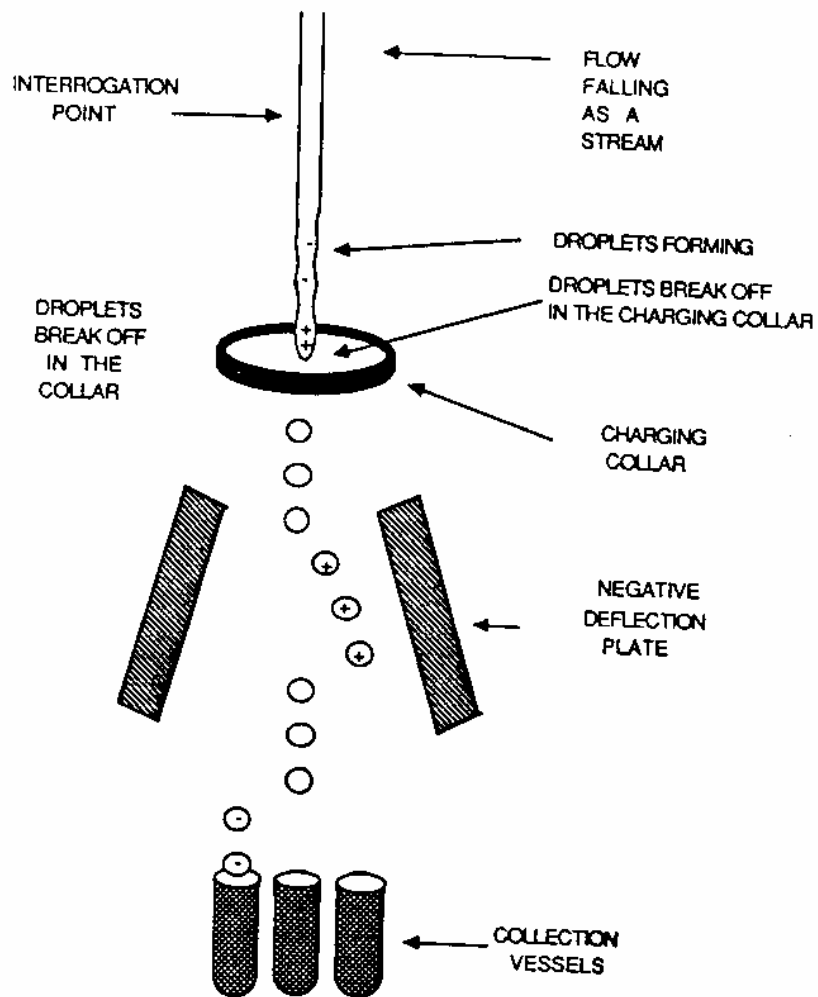


FIGURE 2.7: Charged plate sorting system. A piezoelectric material breaks the flow containing the cells into droplets by vibration. Each droplet for selected sorting receives an electric charge as it passes through a charging collar and breaks off from the rest of the flow. Charged deflection plates deflect the charged droplets into separate collection areas.

2.11 INTERPRETATION

Figure 2.6 illustrates the tumor cell population on the histogram at approximately channel 80. Normal cells are said to be diploid because they contain 2n chromosome number. The normal cells fall into channel 70 so the tumor cells are said to be hyperdiploid because they contain more DNA than the normal, diploid cells. The degree to which the aneuploid cell population differs from the diploid cell population can be used as an indicator of tumor aggressiveness.

2.12 SUMMARY

Flow cytometric studies have significance in the diagnosis and prognosis of certain types of tumor tissues. These diagnostic and prognostic indicators are based on a comparison of the DNA content of normal cells and tumor cells.

THE DELIVERY SYSTEM
The delivery system prepares cells for analysis and delivers them individually, accurately, and precisely to the interrogation point. In the study of tumor cells, preparation includes the **separation of the solid tissue** into individual cells and the staining of their DNA with intercalating fluorescent molecules such as **propidium iodide.** The individual, stained cells are transported in a liquid flow to the flow chamber where a second liquid flow places the cells accurately and precisely in the interrogation point.

THE CELL ILLUMINATION AND DETECTION SYSTEM
The cell illumination and detection system illuminates the stained cells as they pass through the interrogation point. Light scattered from the surfaces of the cells determines the size of the cell while the **fluorescence emission of the fluorescent labels determines the quantity of the labeled characteristic.** In the study of tumor cells, the fluorescent molecule propidium iodide intercalates between the base pairs of each cell's DNA. **The amount of fluorescence emitted by the propidium iodide is proportional to the amount of DNA present in each cell.**

THE DATA COLLECTION AND ANALYSIS SYSTEM
The data collection and analysis system collects the light signals from the cells as they pass through the interrogation point. **Photodiodes collect light scatter data while photomultiplier tubes collect fluorescence data.** These electronic components convert the light signals from the interrogation point into electronic signals that are converted to digital signals and sent to the computer. These signals represent the amount of DNA present in each cell. **The relative amount of the DNA in cancer versus normal cells is of prognostic significance in the treatment of the tumor.**

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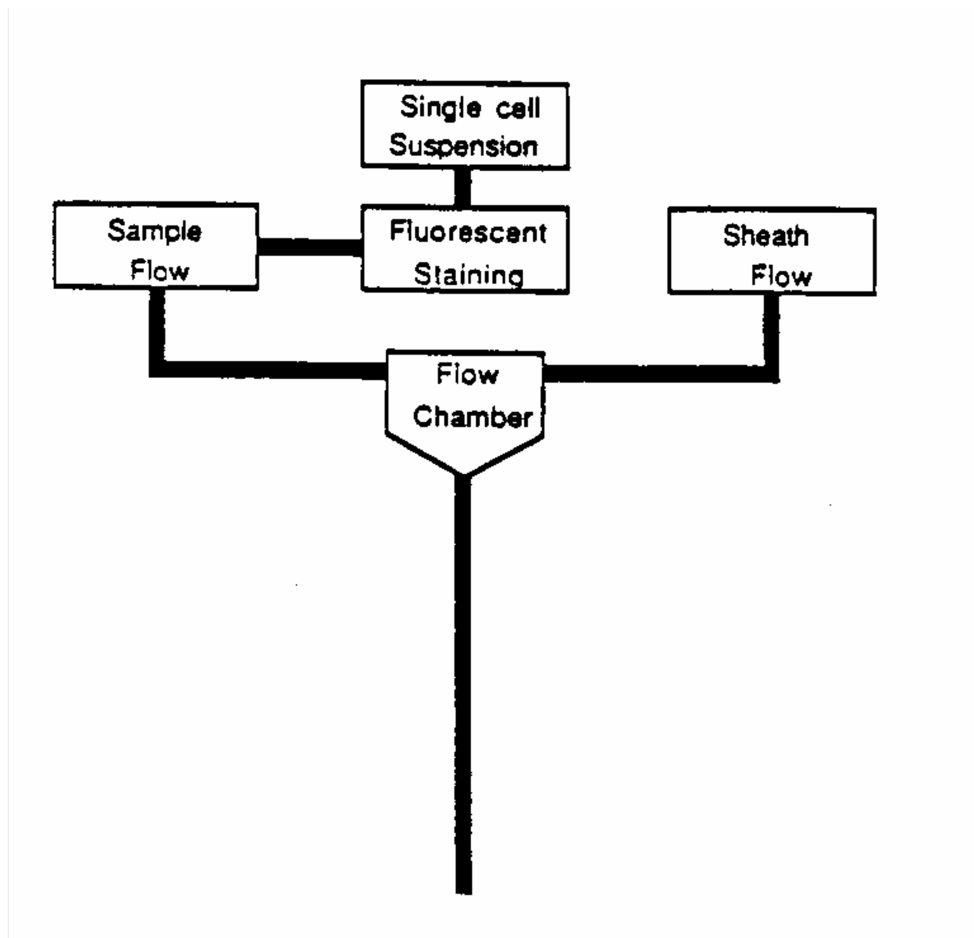
PART ONE
THE DELIVERY SYSTEM



PART ONE:
THE DELIVERY SYSTEM

The flow cytometer is an advanced optical microscope that performs quantitative analysis upon populations of individual cells. As a microscope, the cytometer requires that the cells be accurately placed in the focal point of its optical system, the **interrogation point**. The cytometer analyzes populations of cells so precision is also required for the placement of each cell in the interrogation point.

Unlike optical and electron microscopes that create positional certainty by fixing the sample onto other materials, the flow cytometer requires a complex system of liquid flows, pressurizing gasses, and electronic controls to accurately and precisely place cells in the interrogation point. The cytometer components that perform these functions are referred to as the Delivery System and are the subject of Part One of this book.

The Delivery System includes the preparation of the population for analysis and the transportation of each cell to the interrogation point. **Preparation includes the separation of the population into individual cells, called a single cell suspension, and the labeling of cell characteristics with fluorescent chemicals.** Transportation of the population includes the movement of individual cells in liquid flows and the flow chamber's accurate and precise localization of those cells in the interrogation point.

The Delivery System transports cells in a liquid flow called the **sample flow**. The cells localize in the center of the sample flow which enters the **flow chamber** and is injected into a second flow called the **sheath flow**. The flow chamber narrows, causing the cells to spread out and become even more localized in the center of the two flows. The Delivery System ends when the cells and their transporting flows leave the flow chamber and enter the interrogation point.

Part One focuses on the preparation of cells for flow cytometric studies including the creation of single cell suspensions, the theory and use of fluorescent dyes, the interaction of light with fluorescent materials, and the fluidics that rapidly deliver each cell in the population to the interrogation point.

CHAPTER 3: SAMPLE PREPARATION, LIGHT, AND FLUORESCENCE

All microscopy requires some form of sample preparation. The optical microscope relies upon glass slides that hold a thin slice of the sample under a cover slip. Histological stains color cellular organelles and other characteristics, aiding in their detection and identification. The slide holds the sample in the focal point of the microscope's optics, while stains aid in the detection of structures in the sample by introducing contrast. In flow cytometry, concentric liquid flows place individual cells precisely at the center of the cytometer optics, and fluorescent dyes label cellular characteristics for detection by cytometer sensors.

Sample preparation is arguably the most important step in flow cytometry. To collect correct data about cell populations, each cell must be properly prepared and analyzed. Because cytometry relies on the individual analysis of each member of a population of cells, the separation of the population into a group of individual cells is essential. Once the creation of a single cell suspension is complete, correct labeling of cell components requires an understanding of cell biology and fluorescent labels.

3.1 SAMPLE PREPARATION IN FLOW CYTOMETRY

The first function of sample preparation is the separation of the cells in the population to create a **single cell suspension**. The single cell suspension is a solution of isotonic liquid containing every member of the population as an individual. The single cell suspension allows the transportation and analysis of individual cells.

The second function of sample preparation is the **labeling of cellular characteristics** for data collection. Fluorescent molecules label various characteristics of cells through chemical interaction with cellular components. These labels fluoresce when excited by the illumination beam, and the cytometer detects the wavelength and intensity of the fluorescence emitted. The cytometer's photosensors capture these light signals and convert them to digital signals for storage and interpretation by computer.

The third task of sample preparation is the **prevention of data collection** that does not represent desired cell characteristics. This process removes or inactivates the parts of each cell that could cause the collection of data not related to the characteristics under study. If cellular components that do not represent the characteristic under study are also labeled, they will emit light in the interrogation point and interfere with data collection and interpretation.

It is important for cytometrists to understand light because the cytometer relies upon light signals to determine the characteristics of cells and the properties of populations. An understanding of the processes of fluorescence and phosphorescence is very helpful in the design of cytometric studies and the operation of cytometric equipment.

Sample preparation processes the population for analysis by separating it into individual cells for transportation, labeling cellular characteristics for analysis, and preventing the collection of data that interferes with the analysis. These preparations allow the cytometer to perform rapid,

quantitative analysis of individual cell characteristics and develop histographic representations of the properties of populations.

3.2 SAMPLE PREPARATION

Ideal cytometric samples are homogeneous and representative of the population under study. In the analysis of a population of white blood cells, it is expedient to separate the cells for study (leukocytes) from those cells that are not of interest (erythrocytes). The isolation of the subpopulation under study simplifies and speeds the analysis while eliminating the collection of data points that do not represent the white blood cell population. Although one could analyze a population of white blood cells mixed with red blood cells, such an analysis would be more complex and less informative than an analysis of white blood cells alone.

Preparation of solid tissues such as tumors includes the removal of normal tissue at the borders of the tumor, and disruption of the tissue into a suspension of single cells. The separation of the tissue into a population of single cells allows the cytometer to transport each cell, and facilitates the uptake of fluorescent labels by each member of the population. A homogeneous population of individual cells suspended in a liquid is called a **single cell suspension**.

The cytometer analyzes each cell in a single cell suspension by transporting them through the optical focal point of the cytometer in **concentric liquid flows**. The single cell suspension allows the cytometer to analyze one cell at a time, while concentric liquid flows transport and precisely locate the cells at the focal point of the cytometer's optical systems. The liquid flows cause the cells to localize at the center of the concentric flows, creating **positional certainty** as the cells pass through the interrogation point.

Chemical preparation of cells for flow cytometry employs **fluorescent chemicals** that bind to cellular components. These chemicals fluoresce in response to the light emitted by the illumination source as they pass through the interrogation point. Sensors detect the fluorescence emission from each cell and the amount of light detected is proportional to the amount of fluorescent label in the cell. This process allows the cytometer to collect quantitative data points that correspond to the labeled characteristic of each cell in the population.

The creation of a true single cell suspension and its proper labeling with fluorescent chemicals lays the foundation for all cytometric studies. Errors made in the preparation of the sample for analysis will result in the collection incorrect data for improperly separated or labeled individuals. Cytometric **data analysis depends upon the correct analysis of each individual** in the population so the collection of data from clumps of cells of incorrectly stained cells will make the histograms for the population useless.

3.3 THE SINGLE CELL SUSPENSION

Before the flow cytometer can gather data about any cell in the population, it must transport individual cells through the interrogation point. To accomplish this task, separation of each cell in the population from other cells in the population is essential. When the cells of a population are not thoroughly separated into a single cell suspension, serious problems with data collection and analysis can occur.

A **single cell suspension** is a population of individual cells that is suspended in a liquid. One of the most common problems with sample preparation occurs when the population is not completely separated into individual cells. This problem usually occurs when **clumps** of cells that contain two or more cells remain in the population after preparation. When clumps of cells are analyzed, the data from the clumps cannot be resolved into data points for each individual in the clump. This causes a data point in the population that represents more than one cell and the loss of data points that represent the individuals that adhere to one another in the clump. The presence of clumps of cells in a suspension makes the collection of a data set that accurately represents the properties of the population impossible.

Data collection from **cells that are not members of the population** of interest can also cause problems with data analysis. Fluid tissues such as blood naturally exist as suspensions that are easily separated into subpopulations. When the tissue is a solid, a technique called disruption separates the population into a single cell suspension. To prevent the inclusion of cells that do not belong to the population of interest, these cells should be removed before disruption.

Disruption is the separation of a tissue into a suspension of single cells through mechanical or chemical means. Inadequate disruption of solid tissues results in clumping while overzealous disruption may result in the destruction of individual cells, and the introduction of cellular debris. Both clumping and cellular debris can interfere with data collection.

Mechanical disruption separates cells by mechanically overcoming the forces that hold the cells together. Repeatedly forcing the tissue through a 20 gauge needle, teasing the tissue apart with a sharp probe, or forcing it through a fine wire mesh all result in mechanical disruption. **Chemical disruption** separates the cells through the use of enzymes, ions, or other chemically active substances. Chemical disruption techniques digest the proteins that hold the cells together.

Gentle and thorough disruption of solid tissues is essential for the creation of a single cell suspension. The creation of **the single cell suspension allows transportation of the cells in the concentric flows, proper staining of the cells, and the prevention of the collection of incorrect data**. Once disruption of the population is complete, labeling of the population with fluorescent probes yields a single cell suspension that is ready for cytometric analysis.

3.4 FLUORESCENCE LABELING

Flow cytometers rely upon **photometric methods** for the analysis of cells. The word photometric is a combination of the word stems photo-, meaning light, and -metric, meaning measurement. This word accurately describes the methods flow cytometers use to gather data about cell populations because cytometry relies upon light to measure cell characteristics. These measurements include **light scatter measurements** that do not require labeling, and **fluorescence measurements** that depend on fluorescent labeling of cellular characteristics. In analyses that require labeling, the cytometrist must prevent the collection of data that does not represent the characteristics of the population under study.

3.4.1 PREVENTION OF INCORRECT DATA COLLECTION

One of the most important steps in the labeling of cellular characteristics for flow cytometry is the recognition and prevention of labeling of cellular characteristics that will give an incorrect

result. For example, in the analysis of DNA content, the presence of double stranded RNA in the cytoplasm will cause incorrect data collection. In this case, the use of **RNAase** to break the RNA double strand is essential. If the double stranded RNA is not destroyed, the analysis will yield a DNA plus double stranded RNA index.

3.4.2 FLUORESCENT MATERIALS

Fluorescent materials that label cell characteristics in flow cytometry all **absorb and emit light**. Exposure of a fluorescent material to light of the correct wavelength causes the excitement of electrons in its structure into higher energy levels. When the exciting light is removed, the electrons fall back into their ground states. The return of the electrons to their ground states causes the energy they had while they were in the excited state to be emitted in several forms; one of which is light. Fluorescent materials for use as labels must be excited and fluoresce in response to a wavelength of light provided by the cytometer's illumination source.

The detection of fluorescence emissions quantifies the presence of labeled cellular characteristics. To perform this function, fluorescent materials for the cell interior must be capable of **passing the cell membrane**, while surface labels must attach to selected surface components. Often, fluorescent molecules that do not pass the cell membrane of viable cells, freely pass the membrane after cell death. Labels for the cell exterior generally are not capable of passing the cell membrane. The proper use of fluorescent labels requires that the cytometrist be familiar with the biology of the cells under study, and the interactions of the label with the cell's characteristics.

3.4.3 STOICHIOMETRY OF FLUORESCENT LABELS

Cytometric labels for quantitative analysis must **react stoichiometrically** (bind proportionally to the characteristic being labeled) and must form a **stable complex** once they are bound. Stoichiometric binding of the fluorescent labels provides fluorescence emission from the label that is **proportional** to the amount of the labeled characteristic in the cell. This proportional emission of light means that the light signal emitted from the cell represents the amount of labeled material in the cell. These proportional light emissions form the basis for most types of fluorescence analyses in flow cytometry. If the label does not form a stable stoichiometric complex, the proportionality of the fluorescence emission is lost, and the data collected cannot be correlated with the presence of the characteristic in the cell.

The attachment of fluorescent labels to cell characteristics that undergo change with time allow the changes to be monitored as they occur. To accomplish **time resolved flow cytometry**, the population must be analyzed with the cytometer at intervals for data collection. As the time dependent process occurs, fluorescent materials are either taken up or released by the time dependent process. The amount of label in the cell changes, so the amount of fluorescence emitted in response to illumination with the cytometer also changes. An examination of the histograms at each interval in the study provides information about the time related process.

In summary, illumination of labeled cells occurs as they pass through the interrogation point within concentric liquid flows. The illumination excites the fluorescent molecules in the cell and causes them to fluoresce. The fluorescence emission of properly labeled cells is proportional to the quantity of the labeled cellular characteristic. The light emission that the cytometer detects is

proportional to the quantity of the labeled characteristic in the cell. Photodetectors and electronics within the cytometer convert these proportional fluorescence signals into electrical signals that the computer stores and analyzes.

Formulating an understanding the fluorescent materials that act as labels and the photodetectors that detect their fluorescence requires an understanding of the characteristics of light itself.

3.5 THE CHARACTERISTICS OF LIGHT

Light consists of a stream of **photons** with particle and wave characteristics. **Visible** light is perceived as a spectrum of colors and is only a part of the spectrum of electromagnetic radiation. Most flow cytometric analyses use light in the visible region.

The **spectrum of electromagnetic radiation** includes waves from cosmic rays (wavelength equals 1E-12m) to very low frequency radio waves (1E6m). The visible spectrum of the electromagnetic spectrum falls between violet (350 nanometers (nm)) and red (700 nm). Like the rest of the electromagnetic spectrum, the energy of visible light is due to its wavelength and frequency.

3.5.1 WAVELENGTH, FREQUENCY, AND INTENSITY

All electromagnetic radiation has the characteristics of wavelength, frequency, and intensity. The **wavelength** defines the distance from the peak of one wave to the peak of the next. The wavelength of light is inversely proportional to its frequency so that as wavelength decreases, frequency increases.

The **frequency** of the wave is measured in cycles per second and defines the number of waves passing a point in a second. The **frequency is inversely proportional to the wavelength**. This means that as frequency increases, wavelength decreases and as frequency decreases, wavelength increases. **High energy** light is a stream of photons with **short wavelength** and **high frequency**. X-rays are an example of high energy electromagnetic radiation. **Low energy light** is a stream of photons with **long wavelength** and **low frequency**. Radio waves are an example of low energy electromagnetic radiation. In the visible spectrum, violet light is high energy light, while red light is low energy light. The number of photons per unit time is a measure of the intensity of the light.

3.5.2 RESOLUTION

Resolution is defined as the ability to optically separate two objects and **depends upon the wavelength in relation to the size of the object**. The shorter the wave used for measurement, the higher the resolution of the measurement. If the wavelength of the illuminating light is larger than the objects under study, the objects cannot be resolved. If the wavelength of the illuminating light is shorter than the object, the object may be resolved.

3.5.3 INTERFERENCE

Waves of light may be in phase, where the peaks of the wave move in step with each other, or out of phase, where the peaks of the wave are out of step. Waves of light that are out of phase experience **constructive or destructive interference**. Constructive interference occurs when wave peaks add together, creating a single wave with a larger amplitude. When the peak of one wave corresponds to the valley of a second wave, destructive interference occurs, decreasing the amplitude of the resulting wave. Light that is in phase is called **coherent** light, while light that is out of phase is called **incoherent** light.

3.5.4 PROPERTIES OF LIGHT

Light that consists of a combination of many wavelengths is called **polychromatic** light. Examples of polychromatic light includes light from the sun and light from common incandescent light bulbs. Polychromatic sources emit a wide range of wavelengths simultaneously.

In contrast, light of a single wavelength is called **monochromatic** light. Laser sources emit a series of monochromatic coherent wavelengths (called lines) in the visible region. Monochromatic light can be obtained from polychromatic light through the use of filters, prisms, or dichroic mirrors.

3.5.5 THE VISIBLE REGION

Humans perceive the visible region of the electromagnetic spectrum as **a series of seven colors**. From lowest energy to highest energy the colors of the visible spectrum are: red, orange, yellow, green, blue, indigo, and violet. A convenient mnemonic to remember these colors is the name ROY G. BIV. Light at the edges of the visible spectrum is called infrared light if its wavelength is greater than 700 nm, or ultraviolet light for wavelengths below 300 nm. The spectrum of visible light is shown in Figure 3.1.

3.6 THE ATTRIBUTES OF LIGHT EMISSION

Certain molecules absorb and emit radiation in processes called **fluorescence and phosphorescence**. A subset of these molecules form stable complexes with cellular components and provide labels for cytometry. Some phosphorescent molecules are suitable for cytometric analyses, but most cytometric labels emit fluorescence. Although the detection of certain cellular characteristics do not require the use of a fluorescent label, most cytometric procedures utilize fluorescent staining to specifically label cell characteristics for analysis.

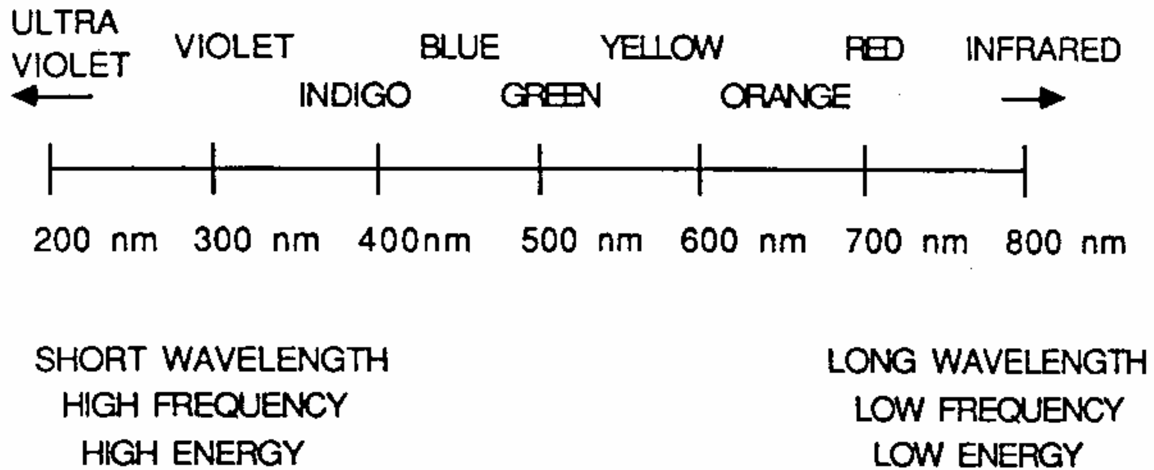


FIGURE 3.1: The visible spectrum of light. Low energy light has a long wavelength and a low frequency. High energy light has a short wavelength and a high frequency.

3.6.1 TYPES OF LIGHT EMISSION

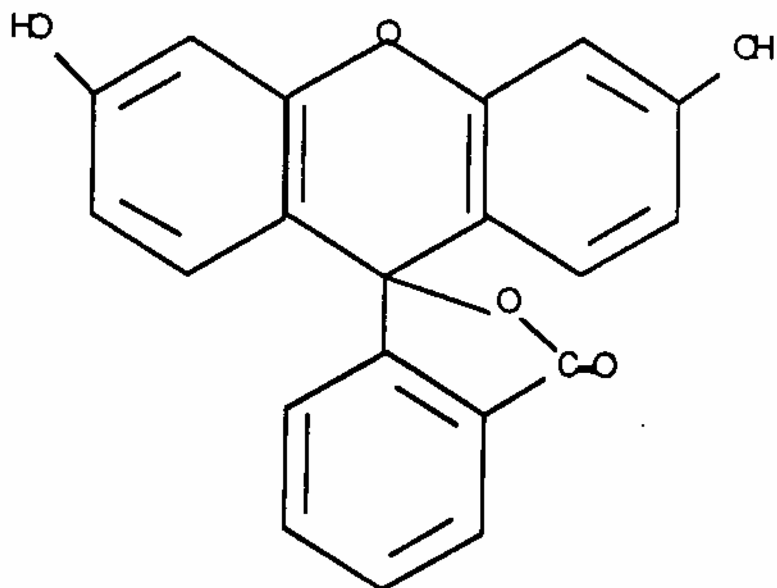
Light emission occurs through the processes of **incandescence and luminescence**. Incandescence is polychromatic light emission due to the physical heating of an object. The filament of a household light bulb is a good example of an incandescent light source. Luminescence is monochromatic or polychromatic light emission due to the excitation of electrons into higher energy states through physical, chemical, or biological processes. Luminescence occurs in atoms or molecules that absorb and emit light, and generally describes all forms of light emission except incandescence.

Luminescence only occurs when some form of energy enters the light emitting system. When the energy is withdrawn, the luminescence ceases. Luminescence occurs in three forms: electroluminescence, bioluminescence, and chemiluminescence. Electroluminescence occurs in gas discharge lamps such as arc lamps, bioluminescence describes light emitted from biological systems, and chemiluminescence occurs in light emitting chemical systems. **Most fluorescent molecules used in flow cytometry exhibit chemiluminescence**. The structures of two fluorescent molecules, fluorescein and propidium iodide, are shown in Figure 3.2.

Chemiluminescence by molecules occurs in two forms, **fluorescence and phosphorescence**. Although fluorescence is of primary interest in flow cytometry, an understanding of phosphorescence is important to the understanding of fluorescence quenching, which interferes with fluorescence.

3.6.2 FLUORESCENCE AND PHOSPHORESCENCE

When a photoactive molecule absorbs light of the appropriate energy, its free electrons move into higher **energy levels** as shown in Figure 3.3. These energy levels exist in two forms, singlet and triplet, designated by the S and T notations respectively. The electrons eventually return to their ground states from these excited states, emitting light. Fluorescence occurs exclusively from the singlet state while phosphorescence occurs exclusively from the triplet state. Fluorescence and phosphorescence emissions have different characteristics of energy, lifetime, and quantum yield.



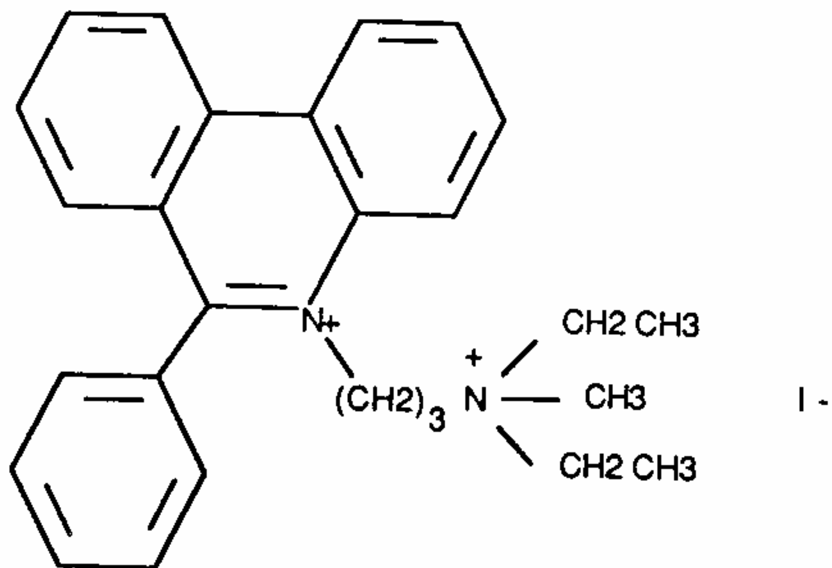


FIGURE 3.2: Structures of fluorescent molecules. Fluorescein (A) and propidium iodide (B). Both molecules have free electrons in the double bonds of their aromatic rings.

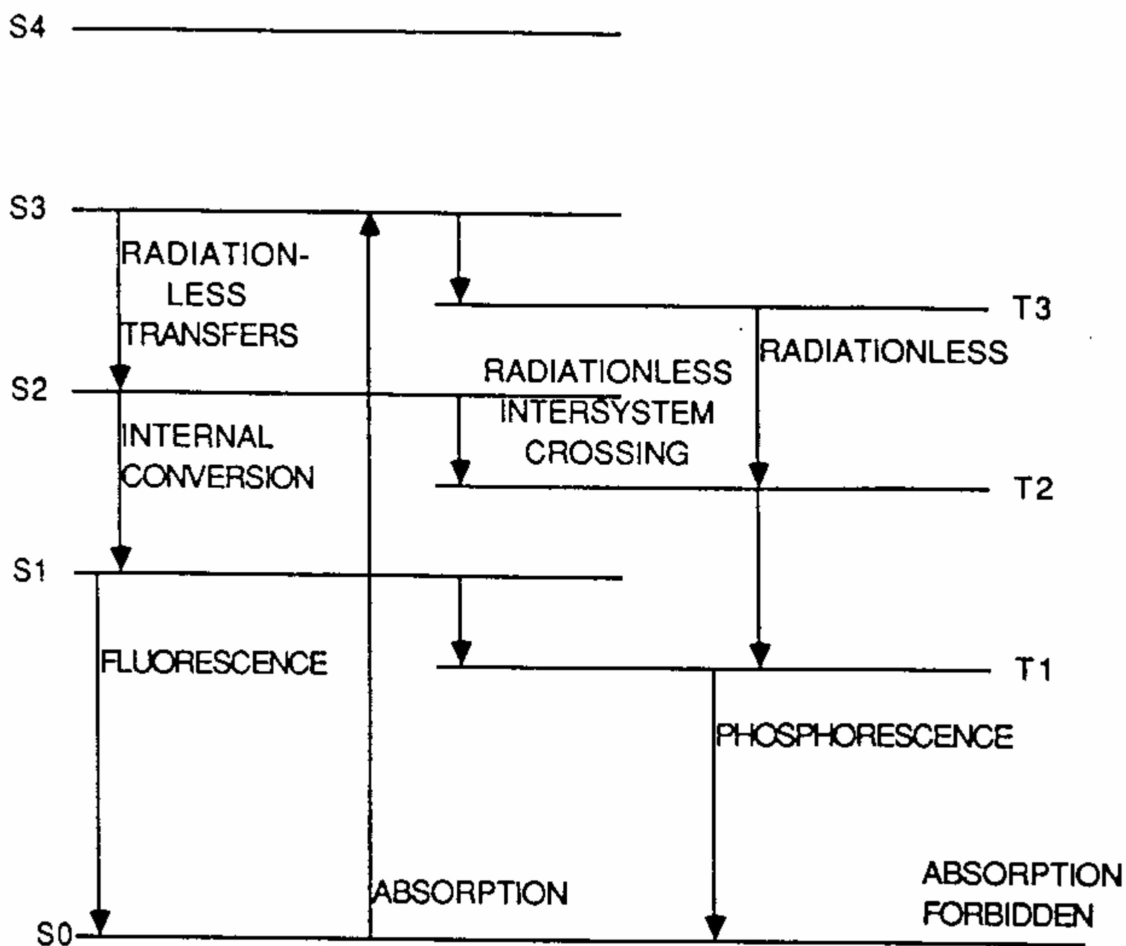


FIGURE 3.3: Energy level diagram of fluorescence and phosphorescence. Note that phosphorescence occurs from the T1 triplet state and fluorescence occurs from the S1 singlet state. The vertical direction of the figure represents increasing energy. After Becker, R.S., *Theory and Interpretation of Fluorescence*, Wiley Interscience, New York, 1969.

3.6.2.1 FLUORESCENCE

Fluorescent molecules commonly contain **free electrons in amino groups, hydroxyl groups, aromatic rings, heterocyclic rings, and double bonds**. When a fluorescent molecule absorbs light of the proper wavelength, the molecule's electrons undergo promotion into the singlet energy level. When exposure to exciting light ceases, the electrons rapidly return to the ground state. Fluorescence occurs as the electrons return to the ground state, and ceases rapidly after the exciting radiation stops. Fluorescence lasts from 1E-10 to 1E-7 seconds after the exciting light is removed.

As shown in Figure 3.3, fluorescence is due to the emission of photons when an electron relaxes from the **S1 excited state** to the **S0 ground state**. The electrons in a molecule may move into higher S states, but fluorescence almost universally occurs between the S1 and S0 states. When electrons enter higher S states, radiationless energy transfers and internal conversion return the electrons to the S1 state prior to fluorescence emission. Although both fluorescent and phosphorescent molecules emit light in response the excitement of electrons into higher energy levels, phosphorescence occurs due to a different mechanism.

3.6.2.2 PHOSPHORESCENCE

Absorption of light by phosphorescent molecules also results in the promotion of electrons into higher energy states. In contrast to fluorescent molecules, electrons move from the **singlet state to the triplet state** in radiationless energy transfers in phosphorescent molecules. The electrons then emit photons as they return to the ground state from the triplet energy level. Phosphorescence emission is characteristically longer than fluorescence emission, lasting from 1E-3 to 10 seconds.

Phosphorescence **occurs when the excited electron relaxes from the triplet state T1 to the ground state S0**. The process by which the electron enters the T1 state from the S1 state is known as intersystem crossing. Since direct excitation of electrons into the T1 state is forbidden (rarely observed in nature), radiationless intersystem crossing is the only entry into the T1 state.

3.6.2.3 PHOSPHORESCENCE VERSUS FLUORESCENCE

In both phosphorescence and fluorescence, the energy of the emitted radiation corresponds to the excited state from which the electron returns to the ground state. Since the lowest triplet state usually has lower energy than the lowest singlet state as shown in Figure 3.3, fluorescence occurs at shorter wavelengths and higher energy than phosphorescence. **Phosphorescence occurs at low energy with a long lifetime while fluorescence occurs at higher energy with a shorter lifetime.** The light emitted from both fluorescent and phosphorescent molecules is of lower energy and longer wavelength than the exciting light the molecules absorb.

3.6.3 LIGHT ABSORPTION AND EMISSION BY FLUORESCENT MOLECULES

The fluorescent molecules that label cellular characteristics in flow cytometry have three characteristic light interaction spectra. The **absorption spectra** defines what wavelengths the dyes absorb. The **excitation spectra** defines what wavelengths excite electrons into higher orbitals. The **emission spectra** corresponds to the wavelengths emitted when the electrons return to the ground state. In many cases, the absorption and excitation spectra are very similar or identical in shape.

When fluorescent molecules absorb light at a given wavelength, the emitted light will almost always have a longer wavelength due to energy loss within the molecule. This fact results in an offset between the excitation spectra and emission spectra. The emission spectrum often resembles a duplicate image of the excitation spectrum as shown in Figure 3.4. The wavelength displacement of the emission spectrum with respect to the excitation spectrum is defined as the **Stokes Shift**.

Most cytometric dyes absorb light in the **middle ultraviolet region (wavelength 200-300 nm)**, the **near ultraviolet region (300-400 nm)**, or the **visible region (400-700nm)**. Although some fluorescent labels require far ultraviolet frequency excitation and may emit ultraviolet or visible radiation, most cytometers operate in the visible range.

3.6.4 FLUORESCENCE QUANTUM YIELD

Fluorescence quantum yield is a measure of the amount of fluorescence that results from the absorption of light by the fluorescent molecule. If all excited electrons in the molecule return to the ground state from the S1 state with the emission of fluorescence, the efficiency of the fluorescence quantum yield is unity. If the excited electrons return to the ground state through some other mechanism, the fluorescence quantum yield will be less than unity. Decreases in the fluorescence quantum yield occur through mechanisms called fluorescence quenching.

3.6.5 FLUORESCENCE QUENCHING

Mechanisms that reduce fluorescence quantum yield are called **quenching mechanisms**. Any functional group that accepts electrons will act as a fluorescence quencher in the presence of a fluorescent molecule. A halide (such as bromine or chlorine) is a good example of an electron accepting fluorescence quencher.

Fluorescence quenching occurs by four mechanisms, **self quenching, energy transfer, charge transfer, and intersystem crossing**. Each of these mechanisms acts in a different way to quench the fluorescence from an excited molecule and reduce its fluorescence quantum yield.

3.6.5.1 SELF QUENCHING

Self quenching occurs through **interactions with unexcited fluorescent molecules**. These interactions between excited and ground state molecules reduce the quantum yield of the excited molecules. It appears that self quenching occurs when the electrons of an excited molecule collide with electrons from an unexcited molecule. These collisions reduce the number of electrons in the excited molecule that return to the ground state from the S1 excited state.

3.6.5.2 ENERGY TRANSFER

In fluorescence quenching by energy transfer, the quenching molecules exist in sufficient concentration to allow **interaction between the singlet state of the fluorescent molecule and the singlet state of the quenching molecule**. Energy transfer between these molecules may be a collisional process as in self quenching, a short

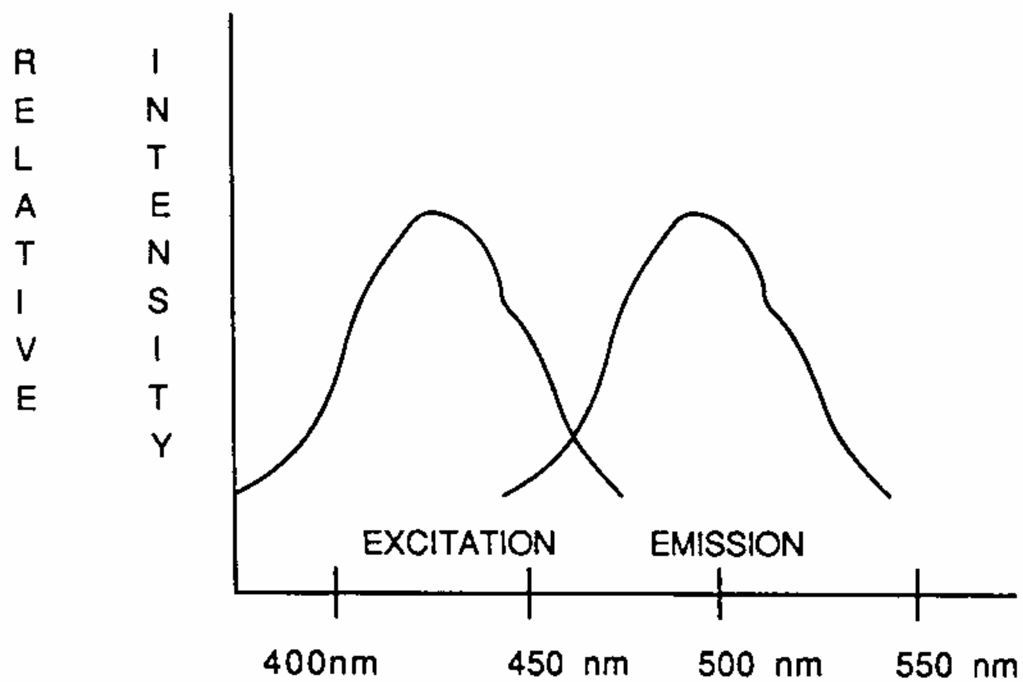


FIGURE 3.4: Relationship of excitation and emission spectra. The displacement of the excitation spectrum with respect to the absorption spectrum is known as the Stokes Shift.

range vibrionic dipole coupling mechanism, or by long range energy transfer. The transfer of energy from the excited molecule to the quenching molecule again results in a decrease in fluorescence quantum yield.

3.6.5.3 CHARGE TRANSFER

When fluorescence quenching occurs by the charge transfer mechanism, an electron moves between the fluorescent molecule and the quenching molecule. The direction of transfer depends on the electron affinities and ionization potentials of the fluorescent and quenching molecules. Once again, this process results in a decrease in the fluorescence quantum yield of the fluorescent molecule.

3.6.5.4 INTERSYSTEM CROSSING

Decrease of fluorescence quantum yield by intersystem crossing occurs when **an electron of the fluorescent molecule enters the triplet state** due to interaction with the quenching molecule. The S1 singlet state of the fluorescent molecule loses its electron to the triplet state and fluorescence does not occur.

In addition to these mechanisms of quenching, the quantum yield of fluorescent molecules can be altered by the physical orientation of the molecule itself. Molecules whose fluorescence depends upon their orientation are called flexible molecules.

3.6.6 FLEXIBLE MOLECULES

The fluorescence emission of some molecules depends upon the flexibility of the molecule. Experiments with rigid and flexible fluorescent molecules shows that the fluorescence of rigid molecules vary little with changing solvent fluidity. In contrast, the **fluorescence of flexible fluorescent molecules varies considerably with solvent fluidity and temperature**.

A good example of a flexible fluorescent molecule is propidium iodide. In solution, **propidium iodide** has a fluorescence quantum yield of 0.05. When propidium iodide is intercalated between the base pairs of double stranded nucleic acids, it has a fluorescence quantum yield of 0.9. The structure of propidium iodide is shown in Figure 3.2. Propidium iodide's high quantum yield when intercalated between the base pairs of double stranded nucleic acids and low quantum yield in solution make it an ideal label for cytometric use.

3.6.7 AUTOFLUORESCENCE

Autofluorescence is the **innate fluorescence of certain biological molecules**. Mammalian cell autofluorescence is primarily due to flavin and pyridine nucleotides, which fluoresce in the blue-excited green and ultraviolet excited blue regions respectively. Other autofluorescent mammalian molecules include blue fluorescent lipofuschins and protoporphyrin. In addition, the amino acids tyrosine and tryptophan fluoresce at 280 nm and phenylalanine fluoresces at 260 nm. Autofluorescence is an important source of interference when measuring cell characteristics with weak fluorescence and should be considered when designing analyses in these regions.

3.6.8 BACKGROUND FLUORESCENCE

Background fluorescence is fluorescence from **extraneous sources** and can radically alter fluorescence data. Phenol phthalene from tissue culture media, debris bound to fluorescent molecules, and fixative-induced cell fluorescence are important considerations in sample preparation and data gathering. Problems with fluorescent binding debris may be resolved with gating (see Section 8.2.4), but highly fluorescent fixatives are best eliminated from the experimental procedure.

The common cytofixative **formaldehyde fluoresces in the visible range** and can be a source of background fluorescence. Paraformaldehyde, with similar fixative properties, is much less fluorescent. Replacement of formaldehyde with paraformaldehyde in flow cytometric procedures is essential for the elimination of background fluorescence.

3.7 SUMMARY

Proper sample preparation is essential to the correct collection and analysis of data in flow cytometry. The creation of single cell suspensions, elimination of sources of error, and proper labeling of cell characteristics laid the foundation for all cytometric analyses.

THE SINGLE CELL SUSPENSION AND LABELING
The first step of sample preparation is the creation of a **single cell suspension**. The single cell suspension permits the transportation of cells in concentric liquid flows that provide positional certainty. The cells may also undergo chemical treatment to label cell characteristics of interest, and to remove any molecules that might cause a spurious result. The fluorescent molecules bind **stoichiometrically** to cellular characteristics so their fluorescence emission is proportional to the quantity of the labeled characteristic. If the fluorescent molecule does not form a stable stoichiometric complex with the characteristic, the proportionality of light emission from the characteristic will be lost.

LIGHT AND LUMINESCENCE
Light has **higher energy** when the **wavelength is short and the frequency is high**. **Coherent** light is **in phase and monochromatic** light of a single wavelength. **Luminescence** describes all light emission except incandescence (light emitted from a heated object), and **chemiluminescent** molecules emit fluorescence or phosphorescence when they absorb light of the proper wavelength.

FLUORESCENCE AND PHOSPHORESCENCE
Certain molecules with free electrons in double bonds, amino groups, hydroxyl groups, aromatic rings, or heterocyclic rings absorb light, causing their electrons to move into higher energy levels. **Fluorescence** is rapid, high energy emission of light as electrons move from the S1 energy level to the S0 ground state. **Phosphorescence** is slow, low energy emission of light as electrons move from the T1 triplet energy level to the S0 ground state. Phosphorescence may continue for as long as ten minutes after the exciting radiation has ceased, while fluorescence ceases within 10E-7 seconds.

FLUORESCENCE QUANTUM YIELD
The amount of fluorescence a molecule emits in response to radiation absorption is its

fluorescence quantum yield. If a molecule fluoresces without electrons proceeding through radiationless transitions or intersystem crossing, the quantum efficiency is 1. If energy is lost to quenching mechanisms, the quantum efficiency is less than 1. Flexible fluorescent molecules such as propidium iodide, exhibit an increase in quantum efficiency when bound to a cell characteristic.

FLUORESCENCE QUENCHING

Quenching occurs when molecules lose their energy to other molecules through one of four mechanisms: self quenching, energy transfer, charge transfer, or intersystem crossing.

BACKGROUND FLUORESCENCE AND AUTOFLUORESCENCE

Spurious data collection may be due to background fluorescence or cell characteristics that are autofluorescent. Background fluorescence may occur as a result of fluorescent molecules binding debris or from fluorescent fixatives. Fixatives with very low fluorescence such as paraformaldehyde can decrease background fluorescence.

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CHAPTER 4: FLUIDICS

Fluidics is general term describing the nature of fluids. In flow cytometry, fluidics describes the systems used to **transport and separate cells** in preparation for photometric analysis at the interrogation point. Fluidics systems vary by manufacturer, but all rely on the creation of **positional certainty** in a flow chamber. The flow chamber's creation of positional certainty holds the sample for analysis as does the fixation of samples to slide in light microscopy, or the creation of replicas for scanning electron microscopy. Most cytometers use either the **stream-in-air** flow chamber design that releases the liquid flow into the air for interrogation, or the **stream in cuvette** design, where the flow is contained in a cuvette for interrogation.

4.1 FLUIDICS IN CYTOMETRY

Population analysis relies upon the transportation of individual cells to the interrogation point where cell sensing occurs. The transportation of cells in flow cytometry exploits the basic laws of flow in tubes described by Daniel Bernoulli and Giovanni Venturi during the eighteenth century.

Both **Bernoulli** and **Venturi** showed that fluid moves fastest at the center of a tube, and that narrowing of the tube causes the center of the flow to narrow and move faster. Bernoulli showed that velocity increases and pressure decreases in the vicinity of a constriction in a tube. Venturi showed that flow is induced in an unoccluded tube immersed in a flowing liquid. These principles form the basis of the fluidics systems found in modern flow cytometers.

Flow cytometers utilize flow chambers to create concentric, non-turbulent flows of liquid that transport and localize the cells under investigation. The **sample flow** contains the cells under analysis, while the **sheath flow** surrounds and controls the size of the sample flow. The transportation of cells in flowing liquids allows rapid **cell counting, sizing, and characterization** of the cell population. In addition, fluid stream transportation of cells allows the use of **liquid droplets as a sorting mechanism**, and the ability to study living cells.

4.2 LAMINAR AND TURBULENT FLOW

Movement of fluids such as air and water may be turbulent or laminar. **Turbulent flow** is disordered and inefficient, **consisting of disorganized movement of the fluid molecules** that mixes the fluid. The injection of ink into the center of a turbulent flow of water results in complete mixing of the water and ink.

In contrast, **laminar flow is smooth and efficient**, consisting of rapid movement of the liquid with very little mixing. The **injection of ink into the center of a laminar flow of water produces a concentrated central flow of ink** that is carried in the center of the water flow without mixing. Streamlines are preserved in laminar flow, while in turbulent flow, streamlines are destroyed. The goal of fluidic systems in flow cytometry is the efficient transportation of sample particles into a very small region for analysis, a task that only laminar flows can accomplish.

When liquids experience laminar flow in tubes, the molecules near the wall of the tube move slowly due to the effects of friction. Molecules a little closer to the center of the tube move faster because the friction from the walls is reduced. In the very center of the tube, the friction from the walls of the tube is lowest, and the liquid moves at its fastest rate. Figure 4.1 (A) illustrates the development of a rapidly moving laminar flow in the center of a tube due to friction.

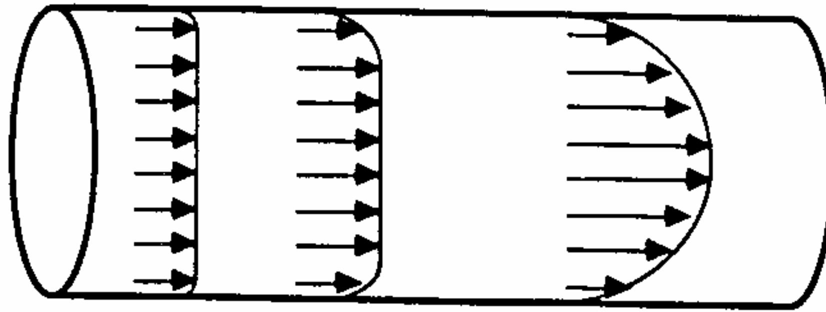
Laminar flow allows cytometers to transport the sample population to the site of analysis, and causes each individual in the population to move to the center of the liquid flow. A single laminar flow is adequate for the transportation of individuals to the interrogation point. However, the analysis of individual cells requires that they pass through the exact center of the focal points of the illumination source and the sensor optics. If the cells do not pass accurately through the focal points of the illumination and sensor optics, the cytometer collects spurious data for that cell. If the sample is not transported through the interrogation point precisely, the cytometer collects spurious data for the population. The relative accuracy and precision of the cell position in the illumination and sensor optics is called **positional certainty**.

4.3 POSITIONAL CERTAINTY

All forms of microanalysis require positional certainty to stabilize the object of study and position it for visualization. In light microscopy, positional certainty takes the form of wet mounts, where the object is trapped in a liquid under a cover slip, or fixation, where the object is chemically cross-linked, sectioned, and permanently mounted on a slide. In scanning electron microscopy, positional certainty requires that the object be fixed, attached to a conductive stage, and coated with a conductive material that creates a "replica" of the specimen. Transmission electron microscopy requires that the object be embedded in a polymer that is very thinly sliced before placement on the stage. Other forms of microscopy employ similar techniques to position the sample for study.

The flow cytometer creates positional certainty through the use of the hydrodynamic principles of liquid flow first described by Bernoulli and Venturi. This technique creates positional certainty by utilizing rapid flow at the center of a tube, increasing the speed of the flow by decreasing the diameter of the tube (the **Bernoulli Effect**), and the initiation of flow in the sample tube when it is placed in the moving sheath flow (the **Venturi Effect**). The properties of flow in tubes are shown in Figures 4.1 and 4.2.

In flow cytometry, the study of population properties by collecting data about the characteristics of individuals requires that each individual move through the interrogation point. While other forms of microscopy allow the fixation of samples in three dimensions, cytometry's requirement that sample cells move through the interrogation point means that the cells must be stable in two dimensions while moving through a third dimension. Two dimensional positional certainty is **accomplished with concentric laminar flows**.



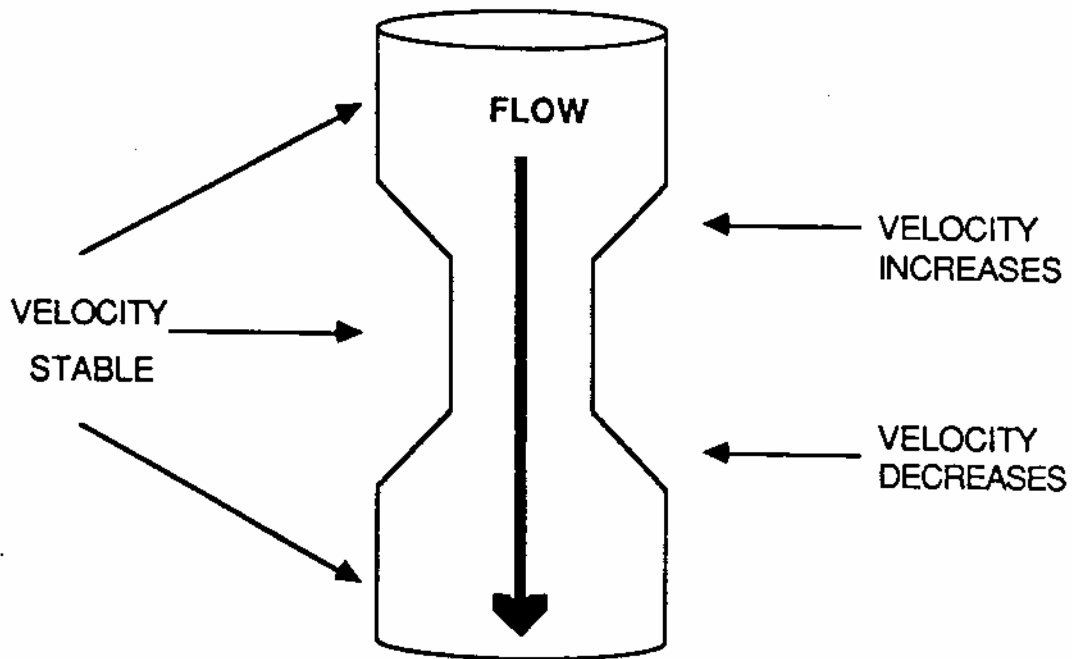


FIGURE 4.1: Flow in tubes. The velocity of the liquid on the inside of a tube is slowest closest to the walls of the tube where the friction is highest, and fastest in the center of the tube where the friction is lowest (A). Velocity increases in regions where the diameter of the tube narrows and decreases in regions where the diameter of the tube widens (B).

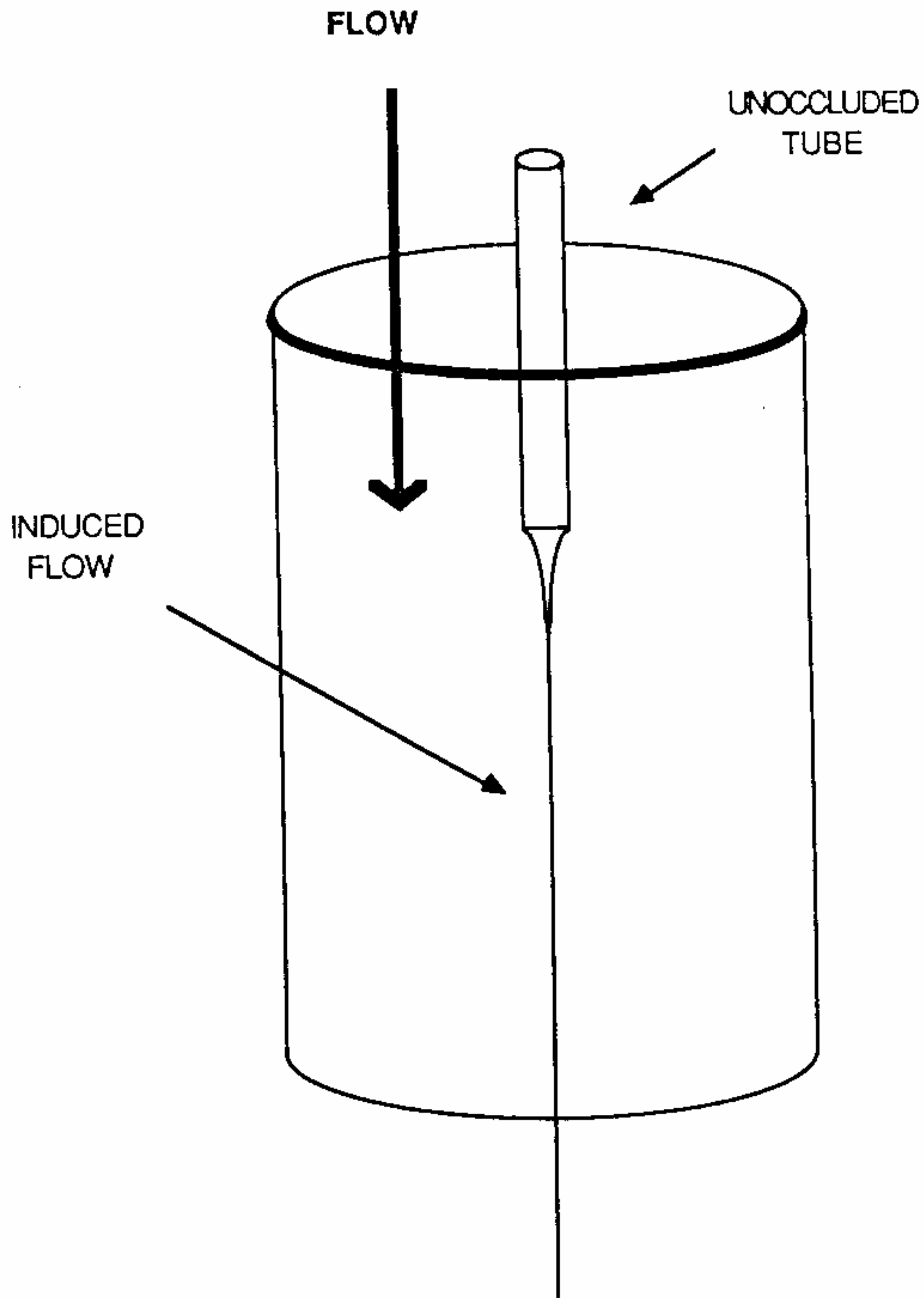


FIGURE 4.2: Induction of flow in an unoccluded tube. When liquid flows past the unoccluded tube in the center of the larger tube, flow is induced in the smaller tube.

The use of a single laminar flow is sufficient for the transportation of cells. The injection of the sample flow into the center of a second "sheath" flow creates two concentric flows that increase the accuracy and precision of the location of the cells at the center of the flow. These concentric flows increase the probability that the cells will be at the very center of the flow. Both flows are then placed in a position where they pass through the focal point of the illumination and detection optical systems. This use of the properties of flow in tubes to create positional certainty for flow cytometry is called **hydrodynamic focusing**.

4.4 HYDRODYNAMIC FOCUSING

Hydrodynamic focusing occurs whenever the properties of flow in tubes control the position of the cells in a liquid flow. The creation of positional certainty by **injecting the sample flow into the fastest moving part (center) of the sheath flow** is a good example of hydrodynamic focusing.

Usually, cytometers accomplish hydrodynamic focusing in a **flow chamber**. The sample flow is injected into the sheath flow in the flow chamber, and **positional certainty increases as the inside diameter of the flow chamber decreases**. As the chamber narrows, the flow velocity in the tube increases, **increasing the distance between the cells and causing them to move into the very center of the flow**. The process of hydrodynamic focusing is illustrated in Figure 4.3.

When the inside diameter of the flow chamber is narrowed, the velocity of the flow at the center of the tube increases in a process called **jetting**. Since there is very little mixing of sheath and sample flow, the increased velocity at the center of the tube causes the distance between the cells to increase.

Changing the diameter of the tube also makes it possible to alter the amount of time that each cell spends in the interrogation point. When the diameter of the tube is decreased, the flow speeds up and the distance between the cells increases. When the diameter of the tube increases, the flow slows down and the distance between the cells decreases. When the speed of the flow is reduced due to an increase in the diameter of the tube, each cell spends more time in the interrogation point, a capability that improves the accuracy of weak fluorescence measurements. The construction of flow chambers rarely allow the operator to change the diameter of the chamber so most cytometers use pressure to control hydrodynamic focusing.

4.5 CONSTRUCTION OF FLOW CHAMBERS

Nearly all flow chambers share the same basic design. A laminar sample flow is injected into the center of a laminar sheath flow with the utilization of the Venturi Effect. The flow is allowed to stabilize briefly before a narrowed region in the flow chamber increases the distance between the cells by increasing the velocity of the flow. This narrowing of the flow chamber increases the distance between the cells, and forces the cells to localize in the very center of the fluid flow.

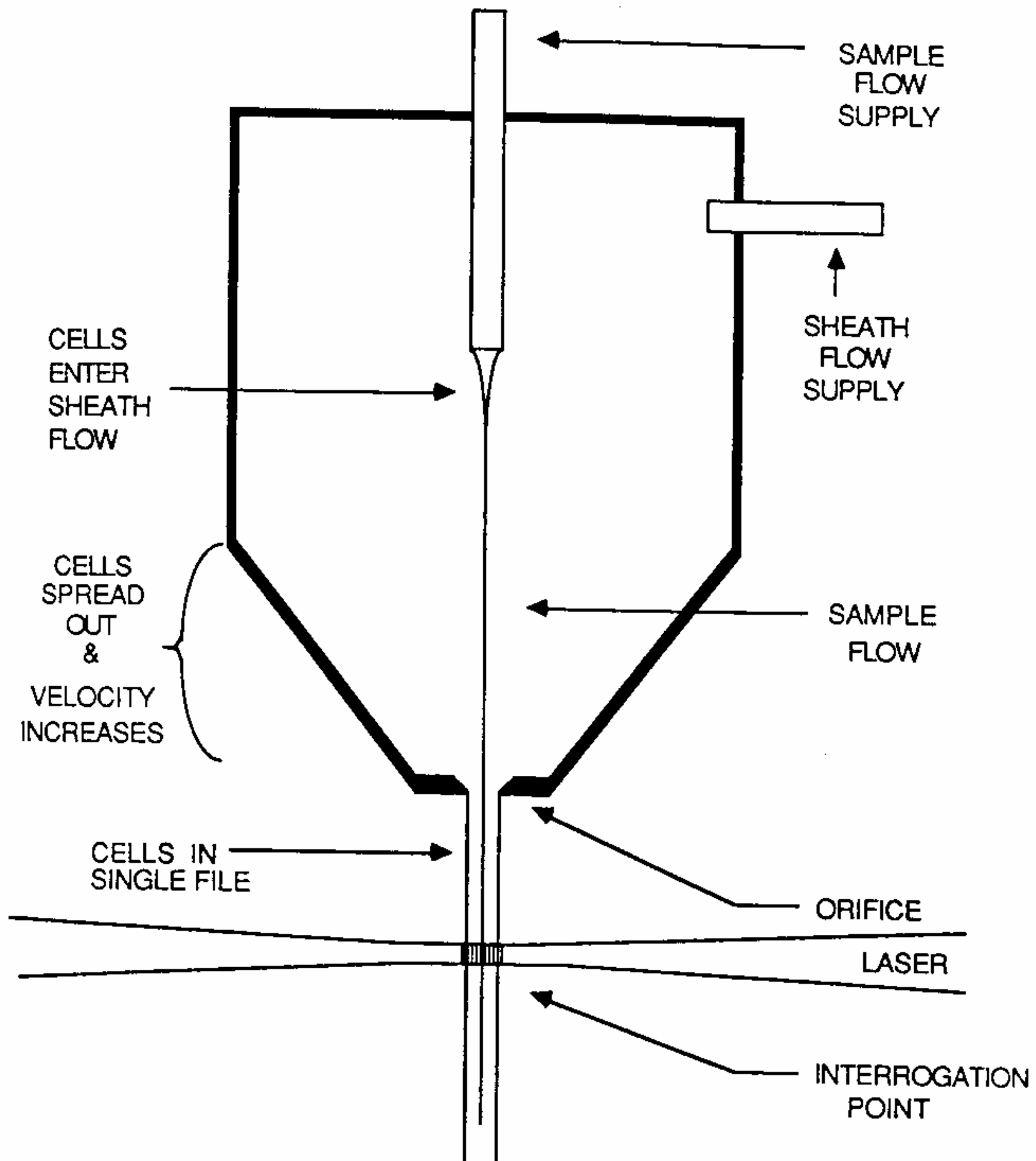


FIGURE 4.3: Hydrodynamic focusing. The cells are injected into the fastest part of the fluid flow. The chamber narrows and moves the cells into single file at the center of the flow in preparation for measurement at the interrogation point. As the chamber narrows, the distance between the cells increases and the cells move into the very center of the liquid flow.

4.5.1 TYPES OF FLOW CHAMBERS

Cytometers usually employ one of **two types of flow chambers**. Flow chambers are designed for stream-in-air sensing or stream-in-cuvette sensing. Stream-in-air chambers are useful for sorting the sample population after analysis. Stream-in-cuvette chambers improve data collection by reducing the amount of light deflected from by the cylindrical flow of liquid as it exits the flow chambers.

For **stream-in-air** measurements, the fluid exits the **orifice** of the chamber after jetting and falls in a laminar stream. The stream passes through the interrogation point and part of the light incident on the stream is lost due to reflection from the curved surface of the stream. Stream-in-air chambers are useful for sorting because the interrogation point can be placed closer to the charging collar, decreasing timing errors in sorting.

For **stream-in-cuvette** measurements, the flow enters a **cuvette** after jetting and passes through the interrogation point before exiting the cuvette. Stream-in-cuvette chambers provide **optically flat surfaces** that minimize diffraction of the illumination source and provide more accurate data about the cell. Stream-in-cuvette chambers also require larger distances between the interrogation point and the charging collar, inducing sorting errors. See Figure 4.4 for examples of both designs.

4.6 DIFFERENTIAL PRESSURE

Although cytometers can induce flow in the sample tube with the Venturi Effect alone, most cytometers also allow the **pressurization of the sample flow**. Pressurization of the sample flow allows the control the diameter of the sample flow within the sheath flow. The pressurization of the sample flow is often accomplished through the pressurization of the sample container.

The sample container is a sealed vessel with a pressure input and a sample flow tube. The sample flow tube extends into the sample container where it is immersed in the single cell suspension. **Pressurization of the sample container causes the single cell suspension to move out of the container in the sample flow tube**. A similar mechanism induces flow in the sheath flow tube.

In many flow cytometers the pressure on the **sheath flow is a constant 13 pounds per square inch (psi)** and the **sample flow operates at pressures from 0 to 13 psi**. The relative size of the sample flow within the sheath flow depends upon the relative pressure difference between the two flows.

The pressure on the sample stream determines the **size of the sample stream** within the sheath flow. When the pressure on the sample flow is high, the size of the sample flow in the sheath flow will be large. When the pressure on the sample flow is very low, the size of the sample flow in the sheath flow will be small. Increasing the pressure on the sample stream causes the sample flow diameter to become larger within the sheath flow. Decreasing the pressure on the sample stream causes the sample stream diameter to become smaller in the sheath flow. As the diameter of the sample flow in the sheath flow decreases, the positional certainty of the flow increases.

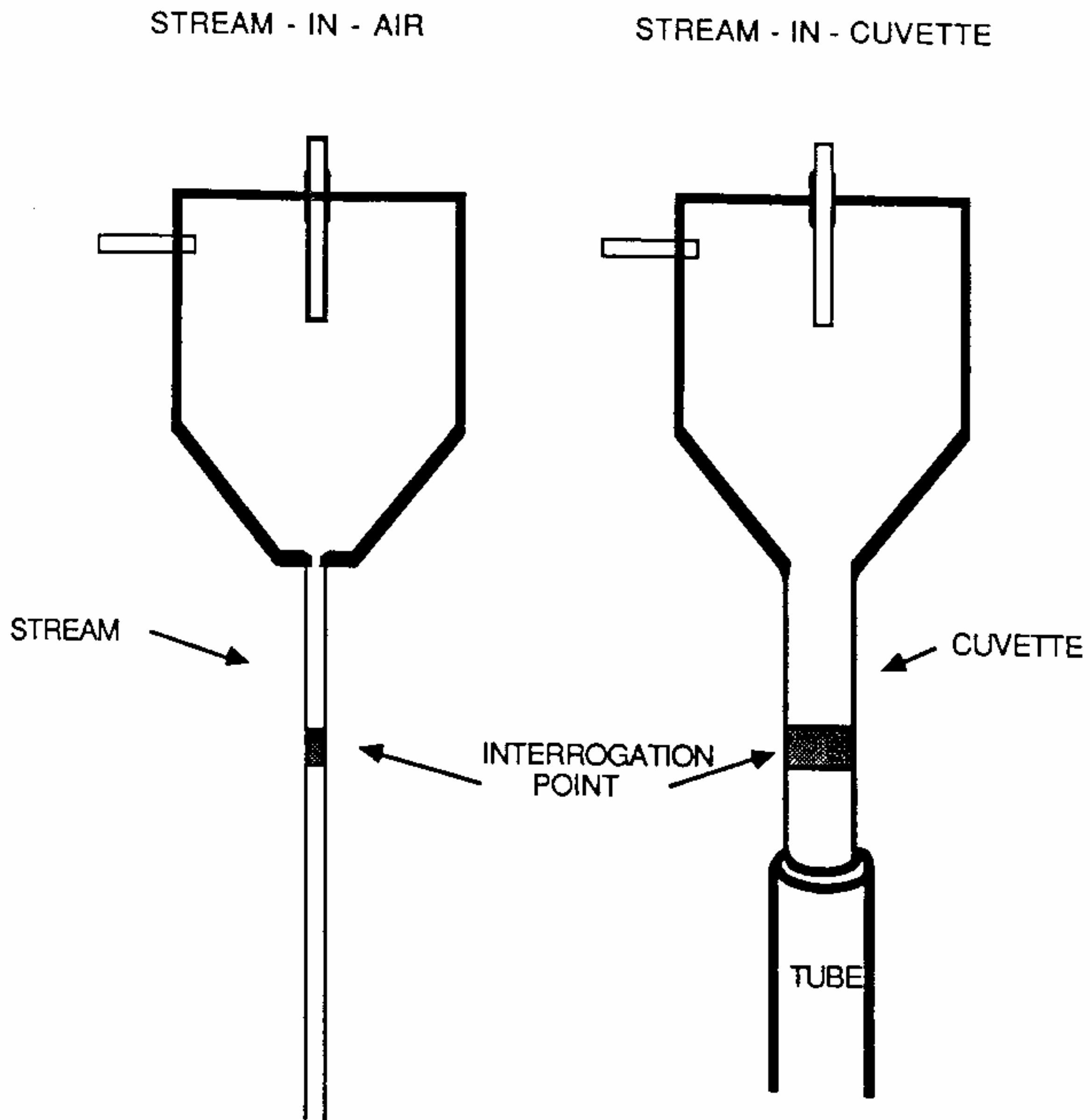


FIGURE 4.4: Flow chamber construction. The flow chamber on the right is a stream-in-cuvette flow chamber. The chamber on the left is a stream-in-air type.

The size of the sample flow within the sheath flow is due to the difference in the pressure between the two flows. Manipulation of the pressure difference between the flows is a convenient way to control positional certainty and is called the **differential pressure**. Subtracting the sample pressure from the sheath pressure results in a numerical value for the differential pressure.

SHEATH PRESSURE - SAMPLE PRESSURE = DIFFERENTIAL PRESSURE

If the sheath pressure is 13 and the pressure on the sample flow is 0, the differential pressure is:

$$13 - 0 = 13.$$

If the sheath pressure is 13 and the pressure on the sample flow is 5, the differential pressure is:

$$13 - 5 = 8.$$

The **typical sheath flow of 13 pounds per square inch creates a chamber exit velocity of about 10 meters per second (m/s)**. The use of a sample pressure of 0 psi (differential pressure equals 13) creates a minimal sample flow with high positional certainty. Use of a sample pressure of 9 psi (differential pressure equals 4 psi) causes a much larger volume of sample flow to enter the chamber.

Thus, an **increase in the differential pressure reading results in an increase in positional certainty** while a decrease in the differential pressure reading results in a decrease in positional certainty. The relationship of differential pressure and sample flow size is shown in Figure 4.5.

Low sample pressures keep the diameter of the sample flow to a minimum and decrease the chance of the creation of a sample flow blossom at the injection point. The blossom effect occurs when sample pressure is too high. The sample flow enters the sheath flow faster than it is carried away, allowing it to collect at the end of the sample injection tube. Sample **blossoming can be responsible for decreased positional certainty**, leading to the collection of inconsistent or incorrect data.

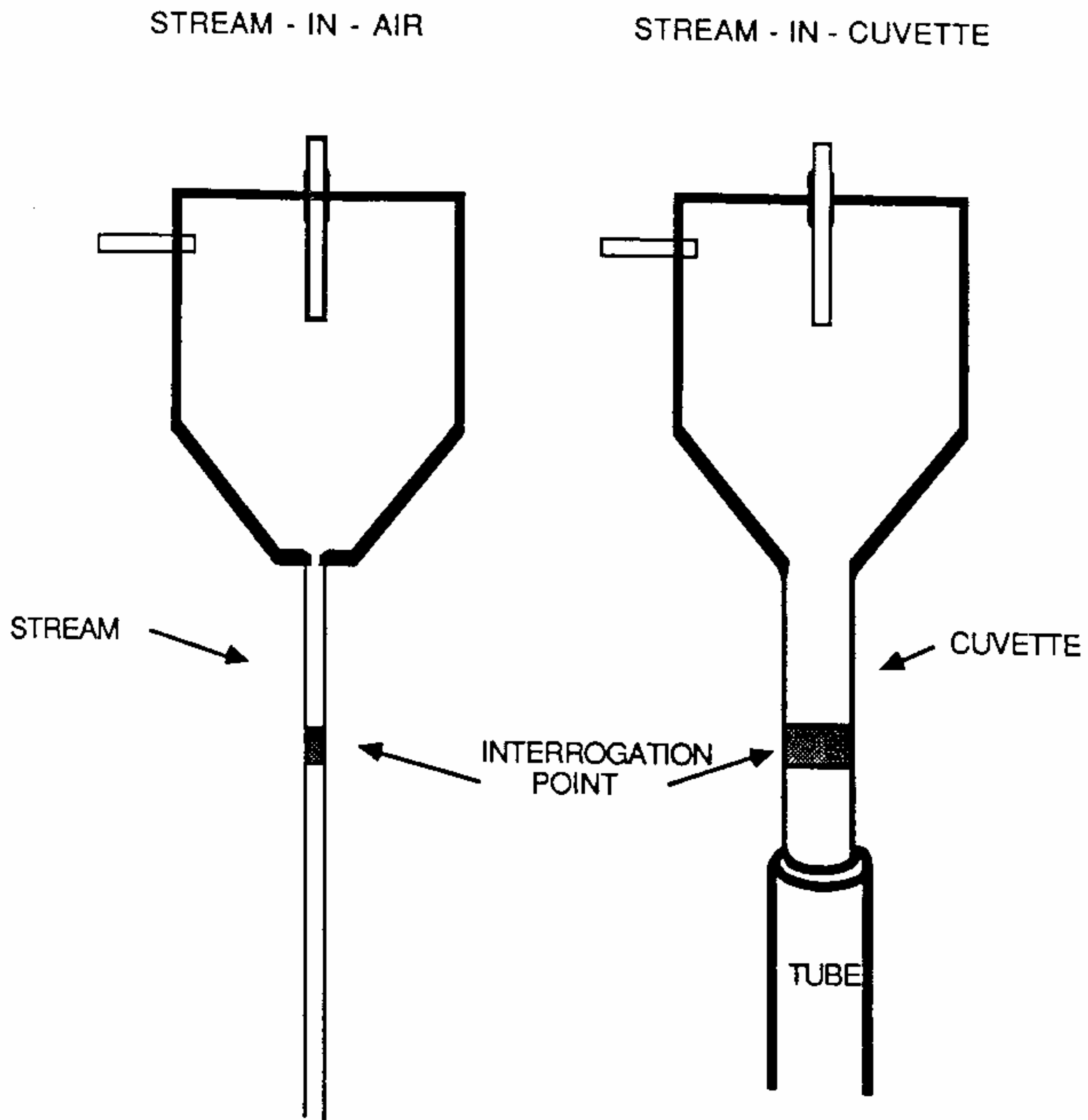


FIGURE 4.5: Differential pressure and sample flow diameter. Sample flow is shaded in the drawings. Increase in sample pressure causes an increase in the diameter of the sample flow and a decrease in positional certainty of the cells.

4.7 SUMMARY

THE BERNOULLI AND VENTURI EFFECTS
The flow chamber of the flow cytometer utilizes the principles of fluid flow first described by **Daniel Bernoulli** and **Giovanni Venturi**. Both showed that **the fastest movement in a flowing liquid occurs at the center of the flow** where the friction is least. Bernoulli showed that fluid **speed increases as diameter decreases** and Venturi showed that **a fluid stream will induce flow in an unoccluded tube**.

POSITIONAL CERTAINTY AND HYDRODYNAMIC FOCUSING
The flow chamber achieves **positional certainty** through **hydrodynamic focusing**. Positional certainty implies accurate and precise localization of the cell in the interrogation point for photometric analysis. Hydrodynamic focusing occurs when the sample flow is drawn into the center of the sheath flow, where the flows are allowed to stabilize before the flow chamber narrows. As the chamber narrows, the distance between the cells increases and the cells are forced into the center of the fluid flow. This process places the cells precisely at the center of the fluid flow for measurement at the interrogation point.

TYPES OF FLOW CHAMBERS
Chambers that contain the flow in a cuvette or other optically flat area to reduce diffraction of the illuminating beam at the interrogation point are called **stream-in-cuvette** flow chambers. Flow chambers that release the flow into the air through an orifice before the interrogation point are called **stream-in-air** flow chambers. Both types of flow chambers share the same construction for hydrodynamic focusing but stream-in-cuvette chambers yield more accurate data bout the population while stream-in-air chambers are better for sorting.

DIFFERENTIAL PRESSURE
The diameter of the sample flow within the sheath flow (and thus the positional certainty) is controlled by the **differential pressure**. Differential pressure is the **sheath pressure minus the sample pressure**. The greatest positional certainty is achieved when the differential pressure is as high as possible, resulting in a sample flow with the smallest possible diameter.

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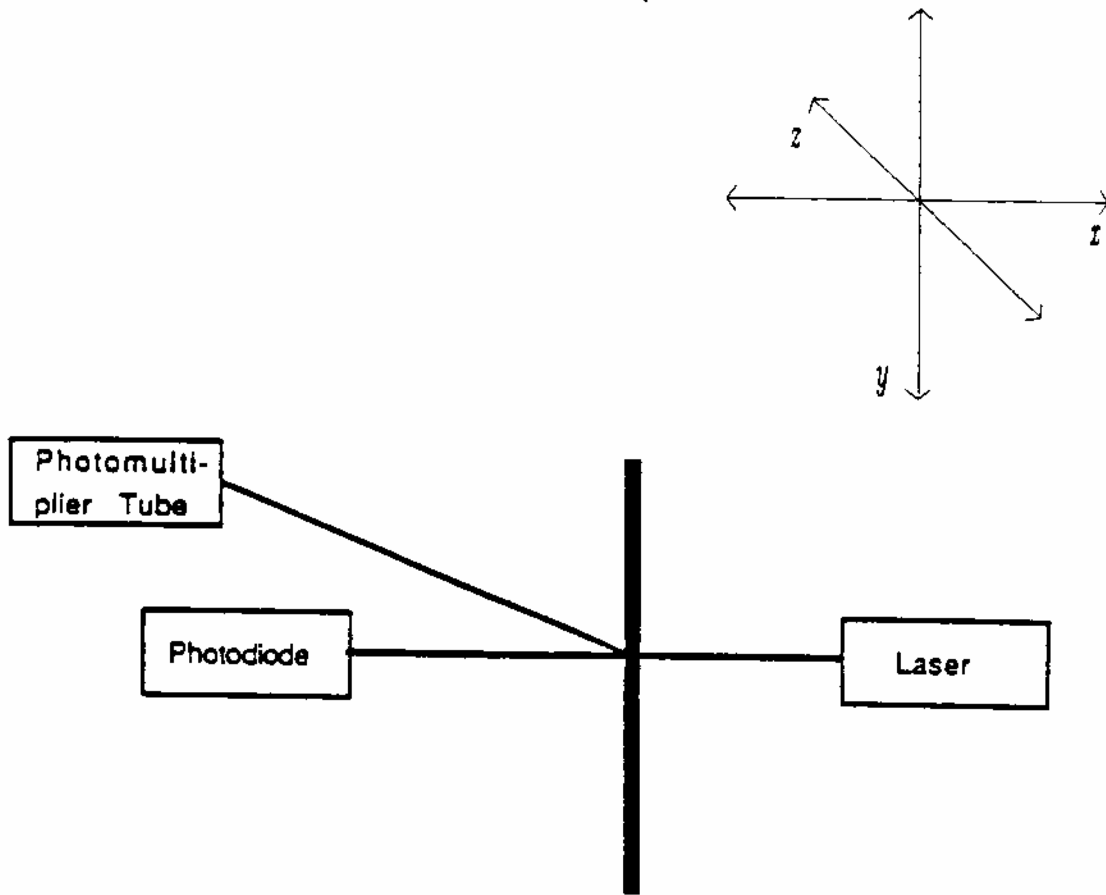
PART TWO: THE CELL DETECTION AND ILLUMINATION SYSTEM



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PART TWO: THE CELL ILLUMINATION AND DETECTION SYSTEM

The flow cytometer performs quantitative analysis on population of cells by analyzing the individuals in the population with light. The cytometer detects light scattered from the surface of the cell and light emitted from fluorescent labels in, or on, the cell.

An extremely **bright and tightly focused light source** illuminates the cells as they pass through the interrogation point. A **photodiode** detects the light scattered from the surfaces of the cells, while **photomultiplier tubes** detect light scattered from the internal components of the cells. Photomultiplier tubes also detect fluorescence emissions from labels attached to the cells.

The Illumination and Detection System performs the illumination of the cell in the interrogation point, and detects each cell's interaction with the illuminating beam. The illumination of the cells in the interrogation point includes the generation of the illuminating light and the optical methods that direct the beam into the interrogation point. The detection of light scatter and fluorescence includes the **optical systems** that direct the light signals into the sensors, and the function of the sensors that convert those light signals into electrical signals.

Part Two concentrates on the components and processes that generate light, the optical systems that focus that light into the interrogation point, the optical systems that collect light signals, and the photosensors that convert light signals into electrical signals.

CHAPTER 5: ILLUMINATION SOURCES

All forms of microscopy require bright, finely focused illumination sources. Light microscopy utilizes light from an incandescent lamp, while electron microscopy uses light from an electron source. These sources must provide enough focused light that the sample is properly illuminated for visualization. Both light and electron microscopy provide illumination that continually bathes the sample, while the cytometer only illuminates the cell during the time it is under analysis. To properly illuminate these cells, cytometric illumination sources must share the brightness and finely focused characteristics of other microscopic light sources. In addition, sources for cytometry must also emit wavelengths suitable for the excitation of common fluorescent molecules.

5.1 ILLUMINATION SOURCES IN FLOW CYTOMETRY

Illumination sources in cytometry are primarily **lasers and arc lamps**. These sources provide the brightness required for illumination of the cell during the short time that it is in the interrogation point. Lasers provide specific wavelengths needed to excite fluorescent molecules used as labels, while arc sources provide a spectrum of light that includes useful wavelengths. To collect usable data about the population, the operator must select fluorescent probes that are excited by the illumination source, and correctly capture fluorescence emission signals from the fluorescent molecules in or on the cell.

The brightness, fine focus, and spectral output of cytometric illumination sources can cause damage to human tissues, especially the tissue of the retina. These and other hazards associated with the use of cytometric illumination sources are discussed in Chapter 10.

5.2 LIGHT REQUIREMENTS IN FLOW CYTOMETRY

Flow cytometry requires intense, finely focused light in a wide range of wavelengths. The light must be intense to illuminate very small objects during very short exposure times. The light must be easy to focus into a small diameter beam (about 15 micrometers) for the illumination of the microscopic particles in the sample flow. Ideal cytometric light sources also **emit a wide range of wavelengths causing the emission of fluorescence from many different fluorescent molecules.**

These requirements limit the choices for illumination sources to **arc lamps and lasers**. The output from these light sources have intensities and spectral characteristics suitable for the excitation of many fluorescent molecules. Arc sources and lasers also focus into very narrow beams for the illumination of very small particles at the interrogation point. These sources are intense, focusable, and have a wide range of wavelengths.

5.2.1 POLYCHROMATIC VERSUS MONOCHROMATIC SOURCES

Arc sources emit light consisting of many wavelengths. The light appears white and is called **polychromatic** light. Lasers emit single wavelengths (**lines**) of light that are called

monochromatic light. Lasers may also be tuned to emit several lines simultaneously. Each of these sources have advantages and disadvantages when employed in cytometric instrumentation.

The advantage of polychromatic sources in cytometry include the ability to excite most fluorescent molecules. A disadvantage of polychromatic sources is that common fluorescent molecules present in the cell may also fluoresce, causing a problem called **background fluorescence**. Monochromatic sources eliminate background fluorescence because only one wavelength of light is emitted from the illumination source. When only one wavelength is emitted, only fluorescent molecules with an absorption spectra that corresponds to the emitted wavelength will fluoresce. Both types of light sources find important applications in flow cytometry.

5.3 ARC SOURCES

Arc sources **provide a spectrum of light** with peaks of wavelength intensities that excite a wide variety of fluorescent molecules. Arc sources consist of an anode, a cathode, and an ionic gas contained in a strong glass tube. When an **electric discharge** passes through the ionic gas, the gas emits polychromatic light. Arc sources emit a combination of incoherent (out of phase) wavelengths throughout a polychromatic spectrum, and are less expensive than laser sources.

The output characteristics of any arc source corresponds to the spectral radiance of the gas ion used. Mercury and xenon gas ion lamps are the most common arc sources in flow cytometry because of their wide spectral output and low cost. **Mercury arc lamps** are popular because of a wide variety of output peaks and a small background continuum. Xenon arc lamps provide an essentially continuous spectrum of light throughout the visible range.

5.3.1 MERCURY ARC LAMPS

Mercury arc lamps have **strong output lines between 240 and 600 nm** and a weak background continuum from 240 nm to 2600 nm. The spectral distribution of mercury arc lamps, shown in Figure 5.1, makes them well suited for the excitation of many fluorescent molecules. This property, in addition to the low cost of the mercury arc lamp, make it an economic source of illumination for a wide range of cytometric studies.

5.3.2 DISADVANTAGES OF MERCURY ARC LAMPS

In spite of relatively low cost and convenient output characteristics, mercury arc sources have several disadvantages. Arc sources are subject to **wander**, a situation where the most intense part of the source moves slightly within the lamp. Wander makes it difficult to align the brightest part of the source with the cytometer illumination optics. Another disadvantage of the mercury arc lamp occurs when these sources are used to perform immunofluorescence measurements. The illumination intensity of mercury arc sources is often inadequate for weak immunofluorescence measurements.

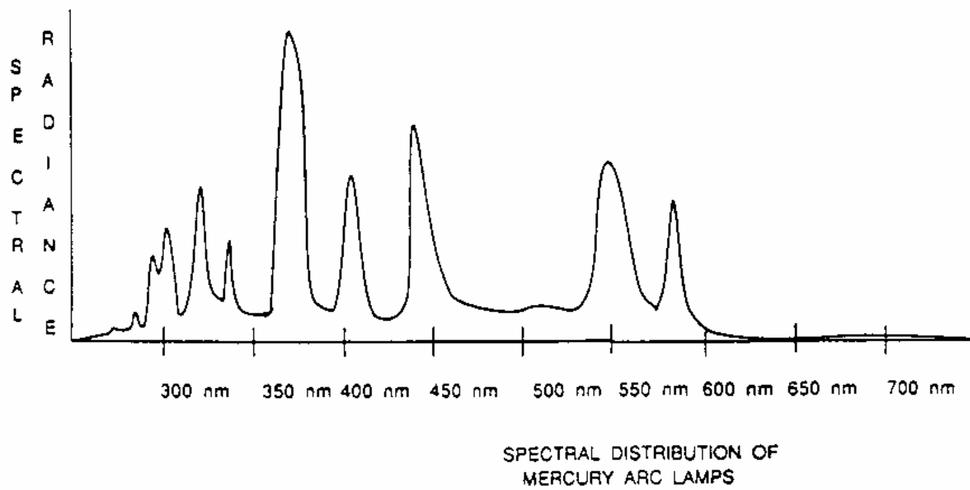


FIGURE 5.1: Spectral distribution of mercury arc lamps. After Van Dilla, M.A., Dean, P.N., Laerum, O.D., Melamed, M.R., *Flow Cytometry: Instrumentation and Data Analysis*, Academic Press, New York, 1985.

Mercury arc source output changes as conditions in the tube change. This fact causes the output of mercury arc sources to vary with changes in temperature and pressure within the lamp. For this reason, most flow cytometric applications require the use of a regulating wattage dc power source to maintain constant wattage as the lamp impedance changes. The **regulating wattage power supply** provides constant light output in the presence of changing physical conditions inside the lamp.

Another disadvantage of mercury arc lamps is their requirement for shielding. Operation of mercury arc lamps causes an internal pressure of up to 40 atmospheres. These high pressures within the lamp mean that **arc tubes must be shielded** because of the possibility of explosion. Arc lamps also emit UV light that is damaging to human vision. Shielding also contains the UV emission so that the lamp may be used safely. Arc sources should never be handled when hot and must always be operated with the anode (positive terminal) down. Arc sources also generate large amounts of heat which must be dissipated with water or infrared filters.

Another disadvantage of mercury arc lamps is that they are difficult to start when hot. This problem may be overcome with a special starting electrode and a 50 kV pulse. An electromagnetic pulse of this magnitude is sufficient to **adversely affect computers** in the immediate vicinity of the mercury arc lamp.

5.3.3 XENON ARC SOURCES

Xenon arc sources are similar to mercury arc sources in many ways. Like mercury sources, xenon lamps require a **constant wattage power supply** and require shielding to protect the user from explosion and UV radiation hazards. Like mercury sources, xenon sources also require the use of water filters or infrared filters to remove heat generated by the infrared lines. Unlike mercury arc sources, xenon sources are **easy to restart when hot** with a 10 to 50 kV pulse.

In contrast to the mercury lamp which provides strong spectral lines and a background continuum, xenon lamps provide a nearly **continuous spectrum** of light. The radiance spectrum of xenon sources occurs between 190 nm and 2600 nm as shown in Figure 5.2. Xenon sources are most useful when a continuum of radiation is needed. The spectral continuum of the mercury lamp is almost as strong as that of xenon. Because of the advantages of a wide spectral output and mercury lines in the UV, green, and blue regions, mercury arc lamps are more commonly used as arc sources in flow cytometry.

5.4 OPTICAL SYSTEMS FOR ARC SOURCES

Arc sources require optical systems significantly different from those employed in laser illumination. Optical systems for arc sources are based on **critical**, **Koehler**, or **epi-illumination** optical configurations.

5.4.1 FOCUSING THE ARC SOURCE

The arc source delivers a non-uniform luminance distribution that is brightest at the tip of the cathode. Flow cytometry requires a collimated (parallel), focused beam of light about five to ten micrometers in diameter. Cytometric **optical systems for the arc source create a collimated,**

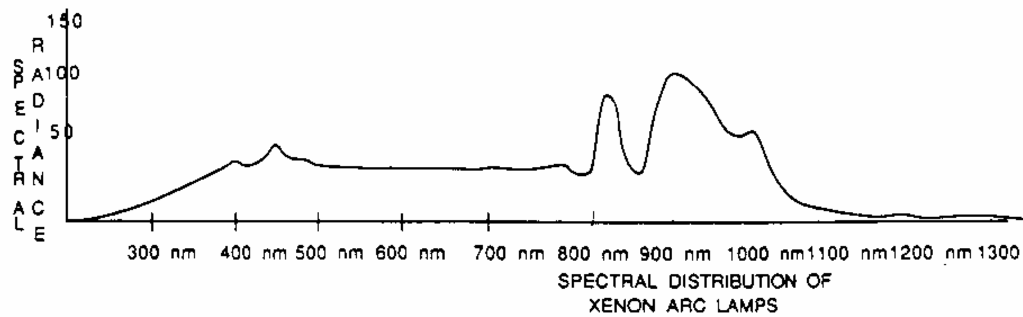


FIGURE 5.2: The spectral distribution of xenon arc lamps. Note the continuous nature of the output and the strong spectral lines in the 750 and 1000 nm regions. After Van Dilla, M.A., Dean, P.N., Laerum, O.D., Melamed, M.R., *Flow Cytometry: Instrumentation and Data Analysis*, Academic Press, New York, 1985.

focused beam with a spherical focusing mirror and lenses that transmit the focused radiation to the interrogation point.

The three types of illumination systems found in cytometers, **critical**, **Koehler**, and **epi-illumination,** focus the non-uniform light distribution found at the tip of the cathode through direct focusing, use of an intermediate plane, or the use of collection optics placed on the same side of the sample as the illumination optics.

5.4.2 CRITICAL ILLUMINATION

Critical illumination focuses the brightest point of an illumination source into the interrogation point through a lens or lens system. The lens system **focuses the source directly** into the interrogation point without the use of intermediate apertures (see Figure 5.3).

5.4.3 KOEHLER ILLUMINATION

Koehler illumination provides more uniform radiance from arc sources than critical illumination. Koehler illumination **focuses the source through an intermediate plane and an aperture** that excludes all but the center of the illuminating light. The focused light that passes through the aperture is focused by a second lens onto the interrogation point. Figure 5.4 is an illustration of Koehler illumination.

5.4.4 EPI-ILLUMINATION SYSTEMS

Epi-illumination systems provide optics for **illumination and data collection on the same side of the interrogation point**. The illumination beam is separated from the fluorescence emission with optical components called dichroic mirrors or filters. Chapter 6 discusses these optical components in detail.

Epi-illumination systems **reduce cytometer costs** because the flow chamber may be simple in construction and by reducing the need for expensive optical components. Flow cytometer epi-illumination systems are similar to epi-illumination systems in common use for standard optical fluorescence microscopy. Figure 5.5 illustrates an epi-illumination system.

Despite the low cost and functional capabilities of arc sources, **laser illumination sources provide important advantages** for flow cytometry.

5.5 LASER ILLUMINATION SOURCES

Laser is an acronym for (L)ight (A)mplification by (S)timulated (E)mission of (R)adiation. Just as the acronym suggests, light is amplified by stimulating the emission of radiation. This process is dependent upon the creation of a **population inversion** in an active medium, which is usually a fluorescent or luminescent material.

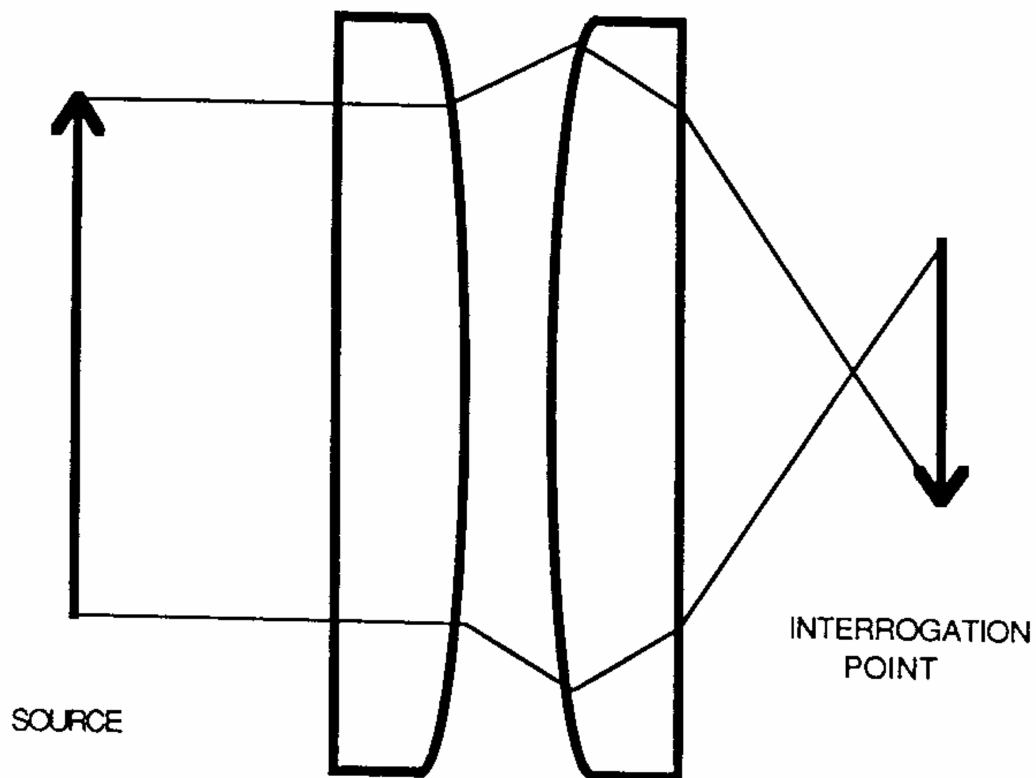


FIGURE 5.3: A critical illumination system. Note that the focal point of the image of the source is focused directly onto the interrogation point. After Van Dilla, M.A., Dean, P.N., Laerum, O.D., Melamed, M.R., *Flow Cytometry: Instrumentation and Data Analysis*, Academic Press, New York, 1985.

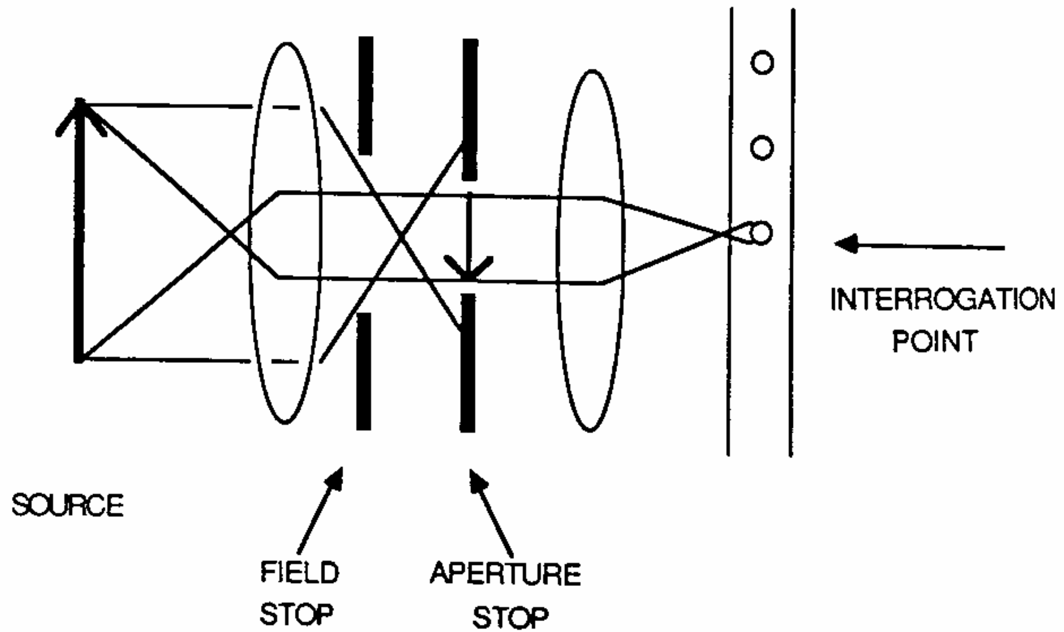


FIGURE 5.4: A Koehler illumination system. Note that the image of the source is focused into an intermediate plane and then focused through another set of lenses onto the interrogation point. After Van Dilla, M.A., Dean, P.N., Laerum, O.D., Melamed, M.R., *Flow Cytometry: Instrumentation and Data Analysis*, Academic Press, New York, 1985.

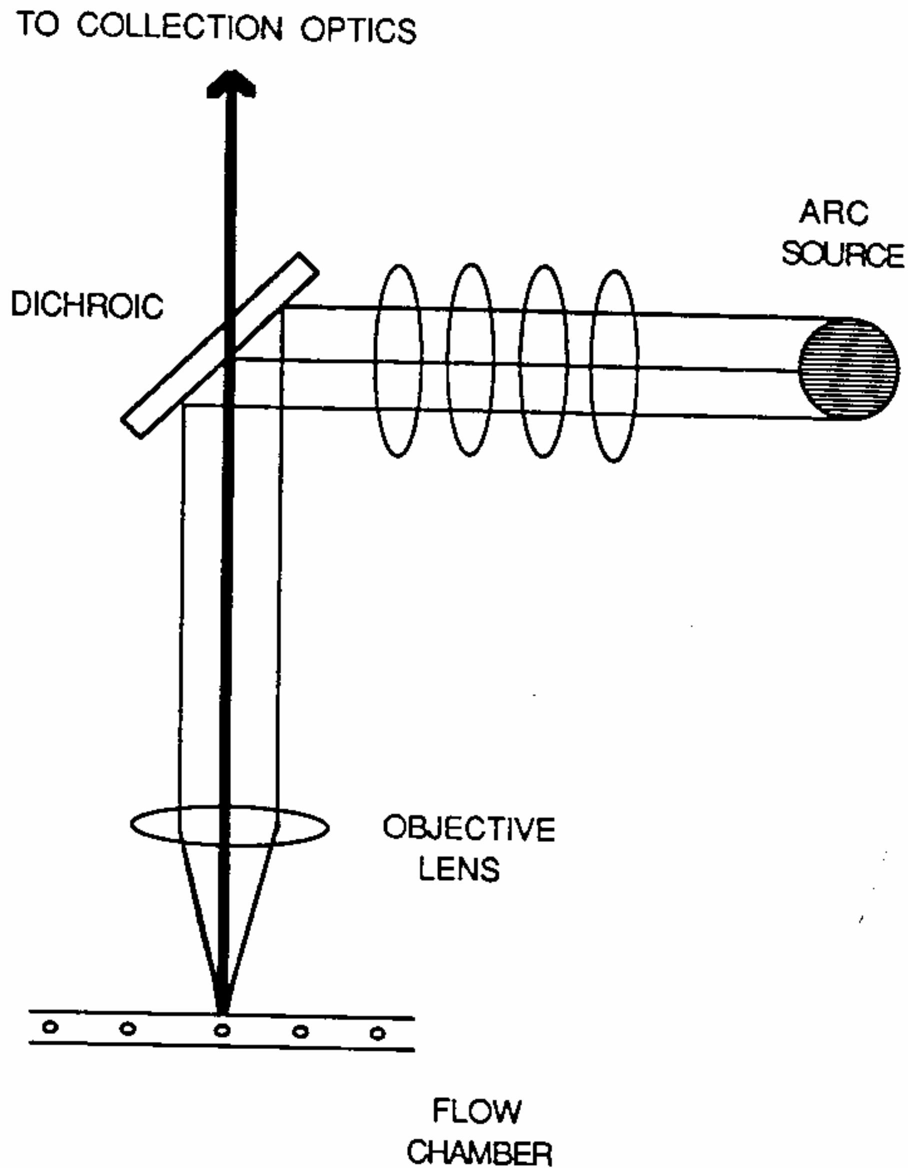


FIGURE 5.5: An epi-illumination system. Note that illumination beam source and data collection optics are located on the same side of the sample. After Van Dilla, M.A., Dean, P.N., Laerum, O.D., Melamed, M.R., *Flow Cytometry: Instrumentation and Data Analysis*, Academic Press, New York, 1985.

A population inversion is generated by applying an electric, thermal, or chemical discharge to an active medium. Most lasers in cytometry provide an electric discharge to a gas ion confined in an oblong cavity with mirrors on both ends. The discharge causes the electrons of the gas ion to be excited into higher atomic orbitals. This **pumping process creates a population inversion** in the gas ion.

Normally, the electrons in a molecule are in thermodynamic equilibrium, with most electrons in the ground state, fewer electrons in the first excited state, and the fewest electrons in subsequent excited states. The pumping process excites more electrons into the excited states than remain in the ground state, creating a **population inversion**. A population inversion is essential to the function of a laser because more electrons must be in the excited state than the ground state for light amplification to occur.

Once a population inversion exists in a laser medium, the presence of an incident electromagnetic beam forces the electrons in higher energy levels to return to the ground state. When the electrons return to the ground state, they emit a photon of the same wavelength as the incident beam, reinforcing the incident beam. This **stimulated emission** of a photon amplifies the incident electromagnetic beam, creating a monochromatic, coherent beam of light.

The light output of laser illumination sources is preferred over arc sources because of **stability, high radiance, spectral purity, and generation as a beam**. Unlike the arc source, the laser beam does not wander in the optical field but remains stable, easing the alignment of cytometer optical systems. The laser can be tuned to emit one wavelength (single line output) or several wavelengths (multi line output) and the beam may be focused to the size of a cell or smaller. These advantages make the laser the most common cytometric illumination source.

The two types of laser sources widely used in flow cytometry are **gas ion lasers**, and **dye lasers**.

Gas ion lasers generate laser radiation by the discharge of a electric potential in a cavity filled with an ionized gas (see Figure 5.6). The multi-line output of the **argon ion laser** makes it the most common laser illumination source. Some systems use a krypton gas ion laser either as the primary illumination source or as a secondary illumination source when more than one illumination source is needed. Gas ion lasers are also employed to pump dye lasers.

Dye lasers use a dye filled cavity to create a highly tunable illumination source. Dye lasers usually use gas ion lasers to pump a dye stored in a special cavity. A simple exchange of the dye in the cavity changes the spectral output characteristics of the laser. This ability to change the spectral output capability of the laser is called **tunability**. Dye lasers can be tuned to emit a very wide range of wavelengths, but are more difficult to work with than gas-ion systems. Low cost helium-neon gas ion lasers are often used to pump dye lasers.

5.5.1 GAS ION LASERS

The gas ion laser is common in flow cytometric use because of its **visible line output, high radiance, and tunability**. In the past, disadvantages of gas ion laser sources included requirements for extensive liquid cooling systems and large amounts of three phase electrical power. Air cooled lasers promise to change the cost effectiveness of laser illumination sources by reducing cooling requirements and permitting operation on a standard 110 volt line.

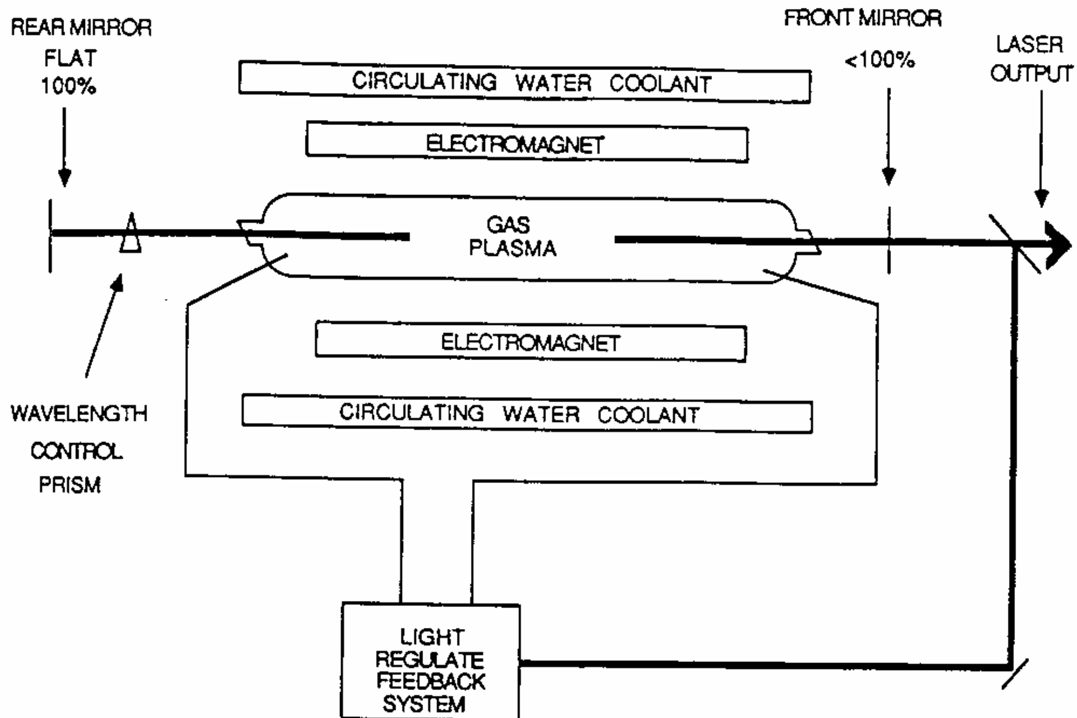


FIGURE 5.6: A gas ion laser. Note the electromagnet surrounding the plasma tube. This magnet keeps the electrons away from the side of the tube and allows the creation of transverse electric and magnetic field (TEM) modes. The mirrors on either end of the tube reflect the amplified beam, and allow it to escape the tube in only one direction.

5.5.2 ARGON ION LASERS

The argon ion laser is the most commonly used light source in flow cytometry. Argon lasers have **single line outputs from 413.1 nm to 514.5 nm and multi line outputs in the 457.9 nm to 514.5 nm and 351.1 nm to 363.8 nm ranges**. Multi line output occurs when the laser output is released through the mirror without modification. Single line output occurs when a prism is placed in the path of the laser output and rotated until only a single line is emitted from the laser system. UV lines are obtained with a special high magnetic field setting. The multi-line output in combination with a prism results in the argon ion laser's limited tunability. Argon lasers are capable of exciting many fluorescent molecules, and are intense enough for very weakly fluorescing studies such as immunofluorescence.

Argon lasers offer the **advantages** of high power visible line output, significant UV output, spectral purity, high radiance, and the ability to be focused to the size of a cell. Argon lasers are operated at the 488 nm line in most applications. The 488 nm wavelength is useful for the excitation of many fluorescent molecules including fluorescein, phycoerythrin, propidium iodide, acridine orange, rhodamine, and cyanine dyes. The **disadvantages** of high output argon lasers include high price and extensive cooling and power requirements.

5.5.3 KRYPTON LASERS

The krypton laser has a lower power output, is more expensive, and is harder to operate than the argon laser. Krypton light sources are useful because they **emit over a wide spectral range from 520 to 799 nm**. Some flow cytometers are equipped with krypton lasers as a second light source for multiparameter analysis.

5.5.4 DYE LASERS

Dye lasers are **highly tunable** light sources that are pumped by gas ion lasers. Tuning of dye lasers is accomplished by replacing the dye in the laser cavity with a dye that has different output characteristics.

A gas ion laser such as a helium-neon laser, creates the population inversion required for the lasing of the dye. Dye lasers are **less stable** than gas ion sources and **require more optical and laser adjustment** by the cytometer operator. The main advantage of dye laser sources is their high degree of tunability.

5.5.5 MULTIPLE LASER SYSTEMS

Some cytometric systems use multiple lasers and multiple stations. **With multiple lasers, the cells are illuminated several times at different wavelengths** as they travel through the interrogation point. The use of several fluorescent stains with differing excitation and emission spectra allows the collection of more data from a single sample run. The use of **multiple**

stations allows the detection of several fluorescence probes simultaneously eliminating the need for repeated analyses of the same sample.

Multistation cytometers focus the illumination beam of the second laser on the interrogation point just below the focal point of the primary laser. This process requires a second optical system to focus the beam from the second laser. A **single photosensor system** detects the light emissions from the sample as it passes through both stations. A time delay separates the signals from each station. The time delay is associated with the distance the cell travels before being illuminated by the second laser.

5.5.6 MODULATION OF LASER OUTPUT

Laser output can be modulated (controlled) in several ways. The power of the beam may be regulated by adjusting the input current or filtering the light emitted from the laser. The **wavelength** of the output line can be adjusted optically, and the **configuration** of the beam itself can be chosen. These modulations allow the operator to configure the laser for a wide variety of analyses.

5.5.6.1 CURRENT REGULATE MODE

The simplest way to regulate a laser (called current regulate mode) allows the current entering the laser from the power line to determine power output. In this mode, the **power output of the beam will vary with changes in line current**. Since input current constantly fluctuates, gathering data in current regulate mode may cause inaccuracies in the data due to fluctuations in laser intensity. To alleviate these problems, flow cytometer lasers often contain output feedback systems to regulate laser output independently of line current. Operation of a laser so that it's power output remains stable in spite of variations in power line current is called light regulate mode.

5.5.6.2 LIGHT REGULATE MODE

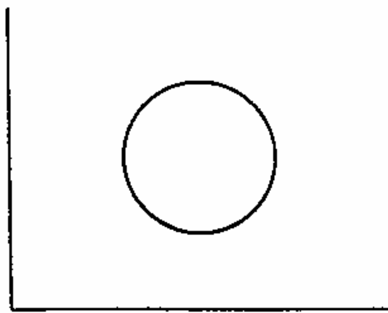
The light regulate mode **utilizes feedback circuitry to stabilize the power output of the laser**. In light regulate mode, the feedback circuit varies the current input into the discharge electrodes of the laser such that the light emitted from the laser retains stable power characteristics. Figure 5.6 includes the elements of a light regulate system.

Light regulate mode enables the operator to stabilize the power output of the laser during cytometric analyses, creating a constant illumination intensity. Without light regulate mode, line current variations may render cytometric data inaccurate or useless because the data collected will include changes in fluorescence due to line current fluctuations.

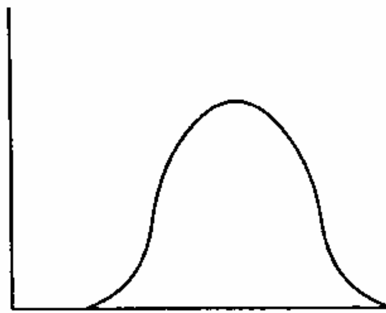
5.5.6.3 TRANSVERSE ELECTRIC & MAGNETIC FIELD MODES

Gas ion lasers have the unique capability of emitting spatial irradiance distributions called **transverse electric and magnetic field modes (TEM)**. These modes are created as the light reflecting between the two mirrors in the laser cavity stabilizes into a pattern. See Figure 5.7 for examples of transverse electric and magnetic field modes and their intensity distributions.

TEM 00



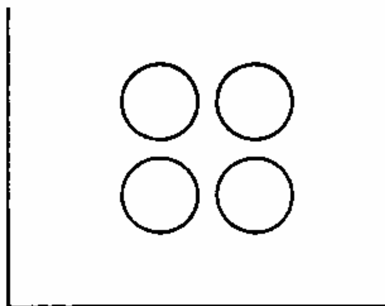
OUTPUT
CONFIGURATION



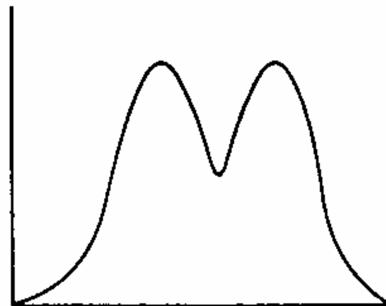
INTENSITY
PROFILE

(A)

TEM 01



OUTPUT
CONFIGURATION



INTENSITY
PROFILE

(B)

FIGURE 5.7: Transverse emission modes and intensity distributions. TEM 00 (A), TEM01 (B).

TEM01 provides an output configuration with four regions of brightness and an intensity profile with two peaks. The TEM00 mode provides a circular area of light intensity with a Gaussian power distribution across the beam diameter. The **TEM00 mode can be focused to the smallest spot and is the mode required for cytometric analysis**. If the laser is set in a mode other than TEM00, all data collected will reflect the light intensity distribution of the mode selected. Usually, data collected in modes other than TEM00 have little value for cytometric studies.

5.5.6.4 WAVELENGTH CONTROL

Tunable gas ion lasers utilize a **prism in the beam path to select available laser lines**. By changing the attitude of the prism, the different wavelengths of the laser output can be selected. See Figure 5.6 and Section 5.5.2 for an illustration and description of this process.

5.6 LASER FOCUSING OPTICS

At the interrogation point, the sample, illuminating beam, and the focal point of the emission and collection optics converge. The optical system uses **crossed cylindrical lenses** to focus the beam into a small, uniform ellipse, and direct it into the interrogation point. The light beam must be large enough to illuminate individual cells, but small enough to illuminate only one cell at a time. When a cell passes through the interrogation point, the light is scattered by the cell's surface and internal characteristics and simultaneously excites any fluorescent molecules present in, or on, the cell.

The beam focusing optics of the flow cytometer reduce the laser's circular output beam to an **elliptical focal point** in the micrometer range without loss of intensity. Moving the crossed cylindrical lenses on a stage focuses the beam at the interrogation point. These crossed cylindrical lenses change the configuration of the laser beam so that it correctly illuminates the cells in the interrogation point.

5.6.1 CROSSED CYLINDRICAL LENSES

Crossed cylindrical lenses focus the laser beam into an **elliptical beam spot**. The cylindrical shape of each lens concentrates the beam into a focal line. One lens focuses the beam horizontally and the second lens focuses the beam vertically. By crossing the two cylindrical lenses at an angle of 90 degrees, the beam may be focused into an elliptical beam spot with a Gaussian energy distribution. This elliptical zone of light is the focal point of the laser output and coincides with the sample flow and the focal point of the detection optics at the interrogation point.

The size and shape of the beam spot can be controlled by placing the crossed lenses at **different lengths from the interrogation point**. Cytometers generally use one of two configurations, confocal or non-confocal. In **confocal systems, the crossed cylindrical lenses are placed such that both lenses share a common focal point**.

In **non-confocal systems the focal point of one lens focuses just in front of or beyond the focal point of the second lens, resulting in a larger beam spot**. A confocal system generates a beam spot about 15 by 40 micrometers while a non-confocal optical system generates a beam spot about 15 by 130 micrometers.

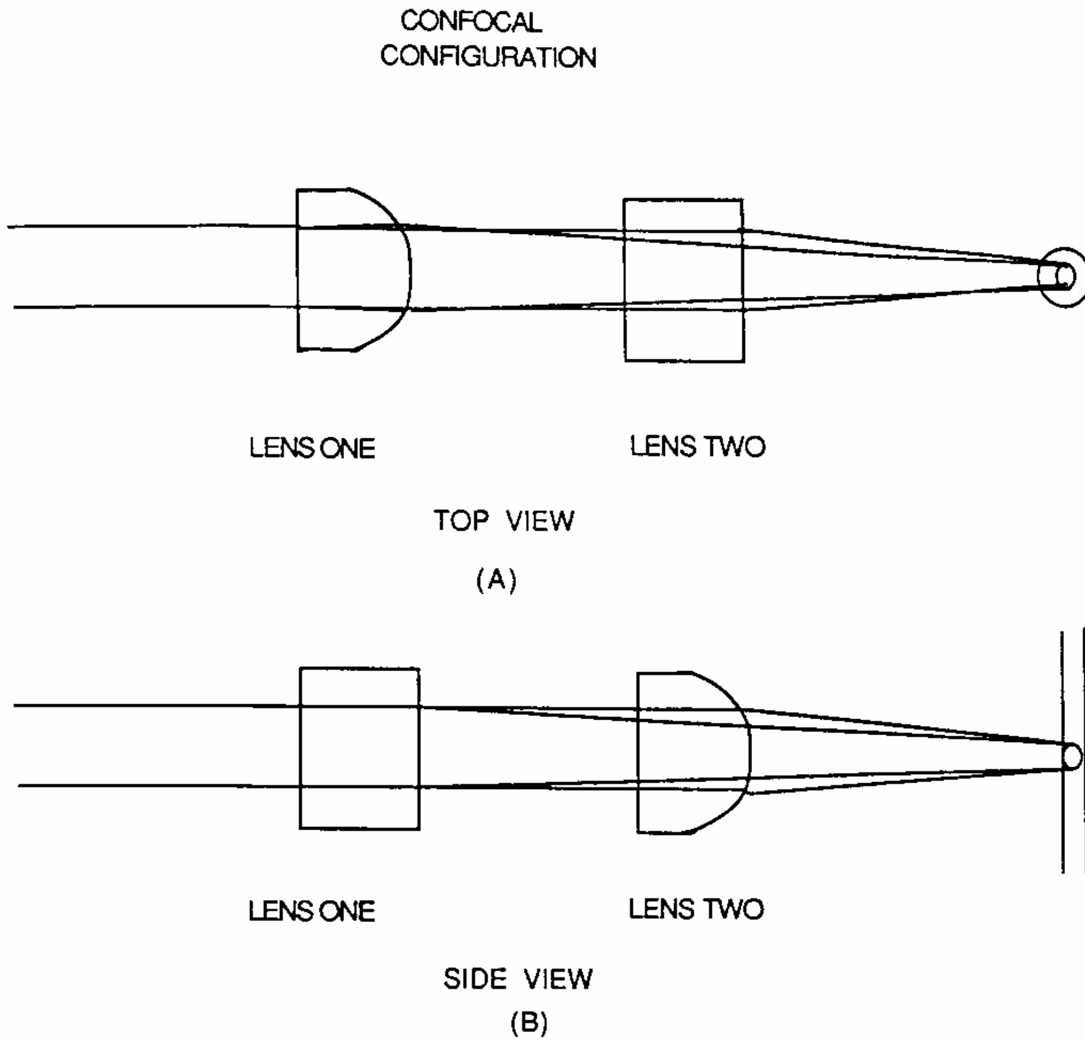
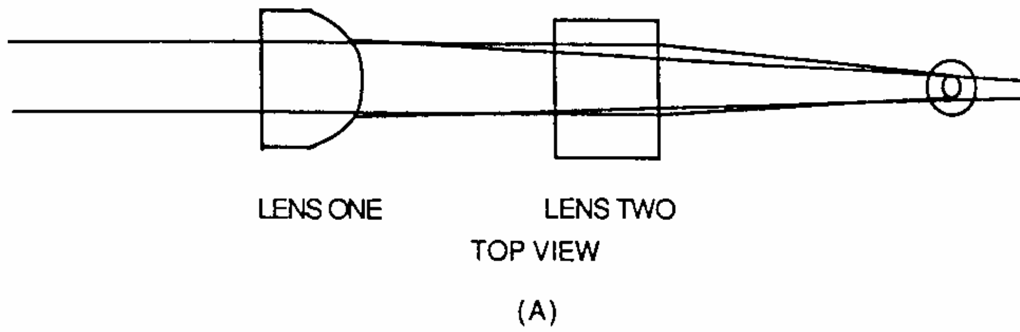


FIGURE 5.8: Confocal optical configuration. Viewed from above (A), viewed from the side (B). Both lenses focus the laser light into a single focal point creating a small elliptical beam spot.

NON - CONFOCAL
CONFIGURATION



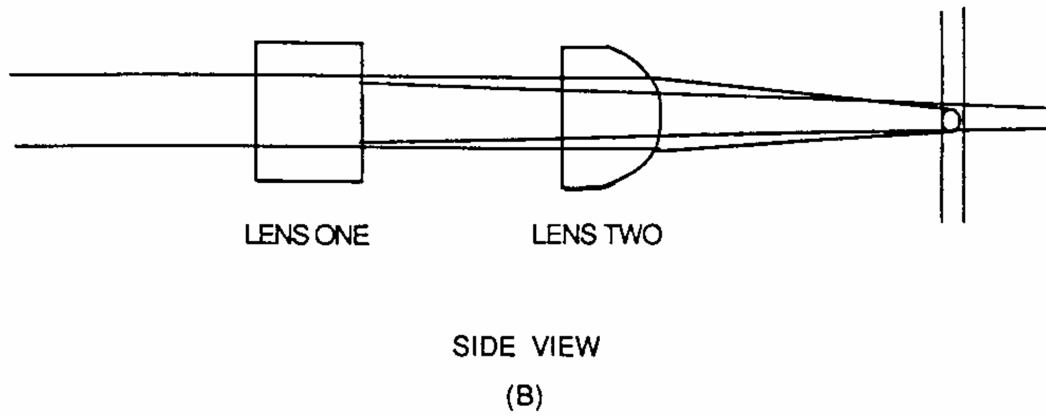


FIGURE 5.9: Non-confocal optical configuration. Viewed from above (A), viewed from the side (B).

Both cylindrical lenses are mounted together on a stage so they move together when the operator focuses their common focal ellipse onto the interrogation point. Correct placement of the elliptical beam spot on the interrogation point is an important part of the **calibration** of the cytometer because it is essential that the beam be exactly focused on the interrogation point. See Figure 5.8 for an illustration of a confocal optical system and Figure 5.9 for an illustration of a non-confocal optical system.

5.7 SUMMARY

The illumination of individual cells for light scatter and fluorescence detection requires an intense, stable light source that can be focused into an ellipse five to ten micrometers in diameter. Cytometric illumination sources include arc sources and laser sources. **Arc sources** are low cost light sources that emit light over a wide spectrum of wavelengths. **Lasers** are expensive light sources that emit extremely bright light in just one, or a small number, of wavelengths. **Lasers are the most common sources of illumination in flow cytometry**.

ARC ILLUMINATION SOURCES
The **cost advantage and wide spectral emission** of arc sources make them valuable for general flow cytometric use. The **mercury arc lamp is the most common** arc illumination source used in cytometry. Some cytometers systems use **xenon arc** sources because of their wide background continuum. The optical systems that focus arc illumination into the interrogation point include **critical, Koehler, and epi-illumination systems**. Arc sources **emit incoherent light** over a wide range of wavelengths (**polychromatic**).

LASER ILLUMINATION SOURCES
Lasers are **widely used as illumination sources** in flow cytometry. Gas ion lasers have higher initial cost and maintenance requirements than arc sources but deliver **spectrally pure light**, are **more intense**, and **excite even weak immunofluorescent labels**. Some cytometers achieve greater illumination wavelength selection with a dye laser. **Dye lasers can be tuned** to emit a wide variety of wavelengths. Gas ion lasers for cytometric use can be tuned to a small number of different wavelengths with a prism between the mirrors of the laser cavity. Some cytometers use more than one laser focused just below the primary laser to allow multiparameter analysis. Laser sources emit **coherent** light over just a few selectable wavelengths (**monochromatic**).

LASER MODULATION
When a gas ion laser operates in the **current regulate mode**, its **output fluctuates with any variation in the input current**. The light regulate mode uses a feedback circuit to maintain a constant laser output in spite of fluctuation in input current. This prevents the collection of inaccurate data due to fluctuations in line current. Gas ion lasers have **transverse electric and magnetic field (TEM)** modes due to the electromagnetic control of the electrons in the laser cavity. These TEM modes may cause analysis difficulties if not properly chosen. TEM00 provides the single beam usually required for cytometric analyses.

LASER OPTICS
Laser light is focused on the interrogation point by two **crossed cylindrical lenses**. These lenses create a **narrow beam ellipse** that is large enough to illuminate individual cells but small enough not to illuminate multiple cells. **Confocal optical systems** place the focal point of the second lens exactly at the focal length of the first to **create a small beam ellipse**. **Non-confocal optical systems** slightly offset the focal point of the two lenses to **create a larger beam ellipse.**

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CHAPTER 6: PHOTOSENSING

In the optical microscope, light rays refract off of structures in the sample, projecting an image of the sample onto the retina. Similarly, the transmission electron microscope projects an image of the sample onto a screen. In scanning electron microscopy, a detector captures secondary electrons emitted as the electron beam scans over the surface of the sample. The detector feeds electronic signals to a monitor, which displays an image of the sample.

Like optical microscopy, cytometry depends on light for the detection of cellular characteristics. Unlike optical microscopy, which carries images to the human eye on diffracted light waves, the cytometer collects digital information about cellular characteristics. Photosensors detect light signals as each cell passes through the interrogation point, and electronic components transfer the signals to the computer.

6.1 PHOTOSENSING IN FLOW CYTOMETRY

Cytometers use sensors to capture information about each cell as it passes through the interrogation point. The illumination of cells at the interrogation point causes **light scatter** from cell surfaces and internal components, and **the excitation of autofluorescent materials and fluorescent labels**. The light emitted from these processes radiate away from the cell in all directions, and is proportional to cellular characteristics.

Light scatter and fluorescence events occurring at the interrogation point are detected by **photodiodes** and **photomultiplier tubes**. These sensors convert light signals into electrical signals. **Analog to digital converters** change the electrical signals from the sensors into digital signals, and electronic busses transfer these digital signals to the computer for storage and analysis.

6.1.1 THE PHOTOELECTRIC EFFECT

The photoelectric effect forms the basis of all modern photosensing devices. In the 19th century, Heinrich Hertz performed experiments that totally contradicted the classical wave theories of light. During experiments with a radio transmitter, Hertz found that the illumination of a charged plate in a vacuum caused a current to flow. Albert Einstein's explanation of the effect in 1905 won him a **Nobel Prize** and opened the door to modern quantum mechanics.

When photons strike the surface of a cathode in a vacuum, they deliver a discrete amount of energy to its surface. If the photon's energy is high enough to overcome the force that binds the electrons to the atoms of the cathode, **one electron will be emitted for each photon that strikes the cathode**. Figure 6.1 illustrates the photoelectric effect.

The photoelectric effect depends on the wavelength (energy) of the light, not the intensity (number of photons per unit time). Both **photodiodes and photomultiplier tubes use the photoelectric effect** to generate electric signals from light signals. The photodiode uses the effect directly, while the photomultiplier tube uses the effect to generate electrons for amplification by structures called dynodes.

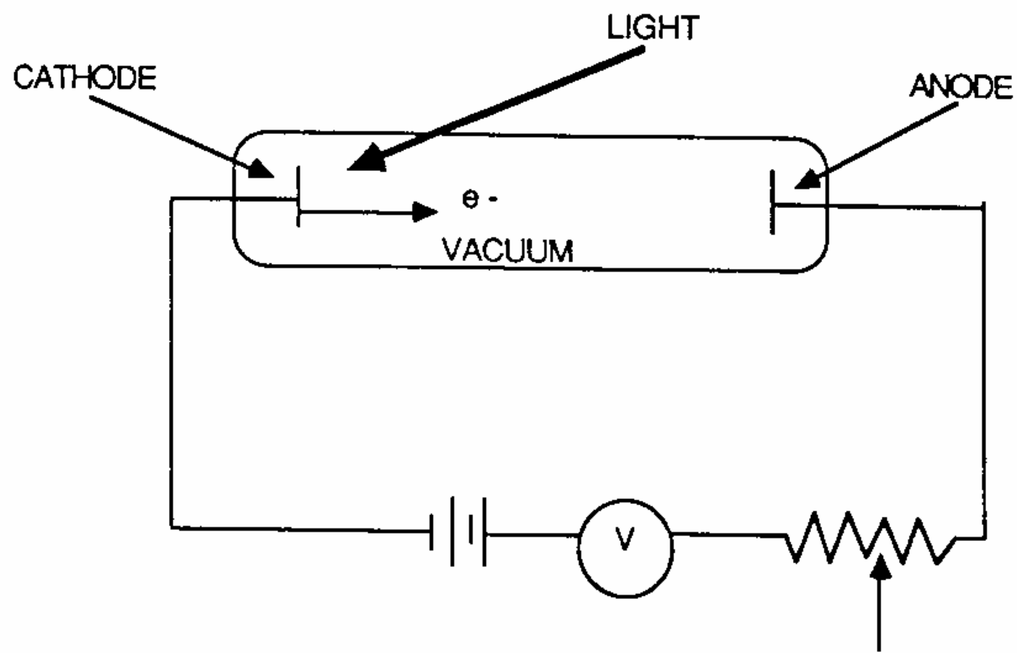

FIGURE 6.1: The photoelectric effect. The incident radiation overcomes the energy binding electrons to the surface of the cathode.

6.2 THE PHOTODIODE

When no cell is in the interrogation point, the illuminating light from the laser does not reach the photodiode, and no electricity is generated. When a cell passes through the interrogation point, light scattered from the surface of the cell strikes the photodiode and generates a current. The current signal acts as a single data point for forward angle light scatter measurements, and each cell passing through the interrogation point generates a new light scatter current signal. These **light scatter current signals also trigger the electronic systems** of the flow cytometer, initiating the electronic processes that result in data collection.

6.2.1 MECHANISM OF FUNCTION

Photodiode sensors are **constructed of semiconducting silicon layers**. In a silicon semiconductor, silicon layers with differing charge characteristics are interfaced. Properties of the silicon materials allow the generation of electron-holes which can carry charge. The electron holes allow the generation of a current when a photon with sufficient energy strikes the photodiode.

The **photodiode functions without the input of external power**, because it utilizes the photoelectric effect to generate an electric current. The input to the photodiode, light, is the only power source, so the output of the photodiode, electric current, must be amplified prior to analog to digital conversion. If a large number of individual photodiodes are connected into an array, the incidence of photons on small sections of the array can be detected and quantified. Photodetector arrays allow precise measurements of cell size by determining the exact angle of light scatter from the surface of the cell.

6.2.2 THE PHOTODIODE IN THE MEASUREMENT OF LIGHT SCATTER

There are three types of light scatter measurements: **forward angle light scatter** (FALS), **narrow angle light scatter** (NALS), and **90 degree light scatter** (90LS). A photodiode detects forward angle light scatter and **narrow angle light scatter**. Photomultiplier tubes detect **90 degree light scatter**. Narrow angle light scatter defines light scatter measurements occurring between 0 and 1 degree, and are proportional to cell size. Forward angle light scatter defines light scatter measurements occurring between 1 and 19 degrees, and are related to a combination of cell size and internal complexity. Ninety degree light scatter defines light scatter measurements occurring orthogonally to the illuminating beam, at 90 degrees, and are proportional to cell **complexity**.

The photodiode measures forward angle light scatter. When the cell passes between the illumination source and the photodiode, the **illuminating light is scattered in the forward direction**. The forward angle light scatter photodiode senses the angle of the scattered light, and triggers the electronic systems that perform data collection. The degree of forward angle light scatter is proportional to the size and complexity of the cell. Similarly, the narrow angle light scatter measurement collects data about particle size from the angle of the scattered light. Most cytometers require photodiode arrays to collect narrow angle light scatter data.

Forward angle and narrow angle photodiodes detect only the light scattered by the particle and not the illuminating light because of the presence of an **obscuration bar** and **beam stop**. The beam stop and obscuration bar prevent the light that travels directly through the cell and the light reflected off the sample/sheath flow from reaching the photodiode. Figure 6.2 illustrates the function of the beam stop and obscuration bar. With this configuration, no signal reaches the photodiode except when a particle passes through the interrogation point.

6.3 THE PHOTOMULTIPLIER TUBE

The photomultiplier tube electronically **amplifies light signals** to sense very dim light. Photomultiplier tubes sense dim fluorescence signals by amplifying an electric current produced by the photoelectric effect. The photomultiplier tube's electronic amplification system allows detection and storage of dim light signals by cytometer electronic and computer systems.

Photomultiplier tubes are available in three types, **bialkali**, with a peak output of 40mA/W at 400 nm, **gallium arsenide** with an output of 50mA/W over the range from 300 - 850 nm, and **multialkali** which are useful only at wavelengths above 750 nm. Light enters the photomultiplier tube through a **window** that may be placed on the end or the side of the tube. Most cytometers use end-window photomultiplier tubes as shown in Figure 6.3.

6.3.1 STRUCTURE AND FUNCTION OF PHOTOMULTIPLIERS

Photomultiplier tubes consist of a negatively charged **cathode**, that **emits electrons in response to light**, a series of individual **dynodes**, **that release more than one electron** for each electron that strikes them, and an **anode that accepts the electrons** from the dynodes, providing the output current signal through an electrode. The photocathode, dynodes, and anode are enclosed in an airtight case that provides a vacuum, allowing the transmission of electrons.

A window on the side or end of the case allows light to strike the cathode, initiating a **cascade of electrons**. When a photon strikes the cathode, the cathode emits an electron that strikes the closest dynode. The dynodes each have a successively stronger positive charge, causing electrons to cascade through the dynodes until they are collected by the anode. The cascade of electrons through the dynodes amplifies the signal generated by the photon. Electrodes on the outside of the case carry the amplified electrons from the anode out of the photomultiplier tube and into the cytometer electronic system. Each photomultiplier tube generates an electronic signal for each cell that emits a photon or photons that strikes the cathode.

Photomultiplier tubes **require external power** to make low level light measurements. The external power provides an increasingly stronger electric potential between the cathode, dynodes, and anode, causing amplification of the weak light signal. Figure 6.3 illustrates an end window photomultiplier tube.

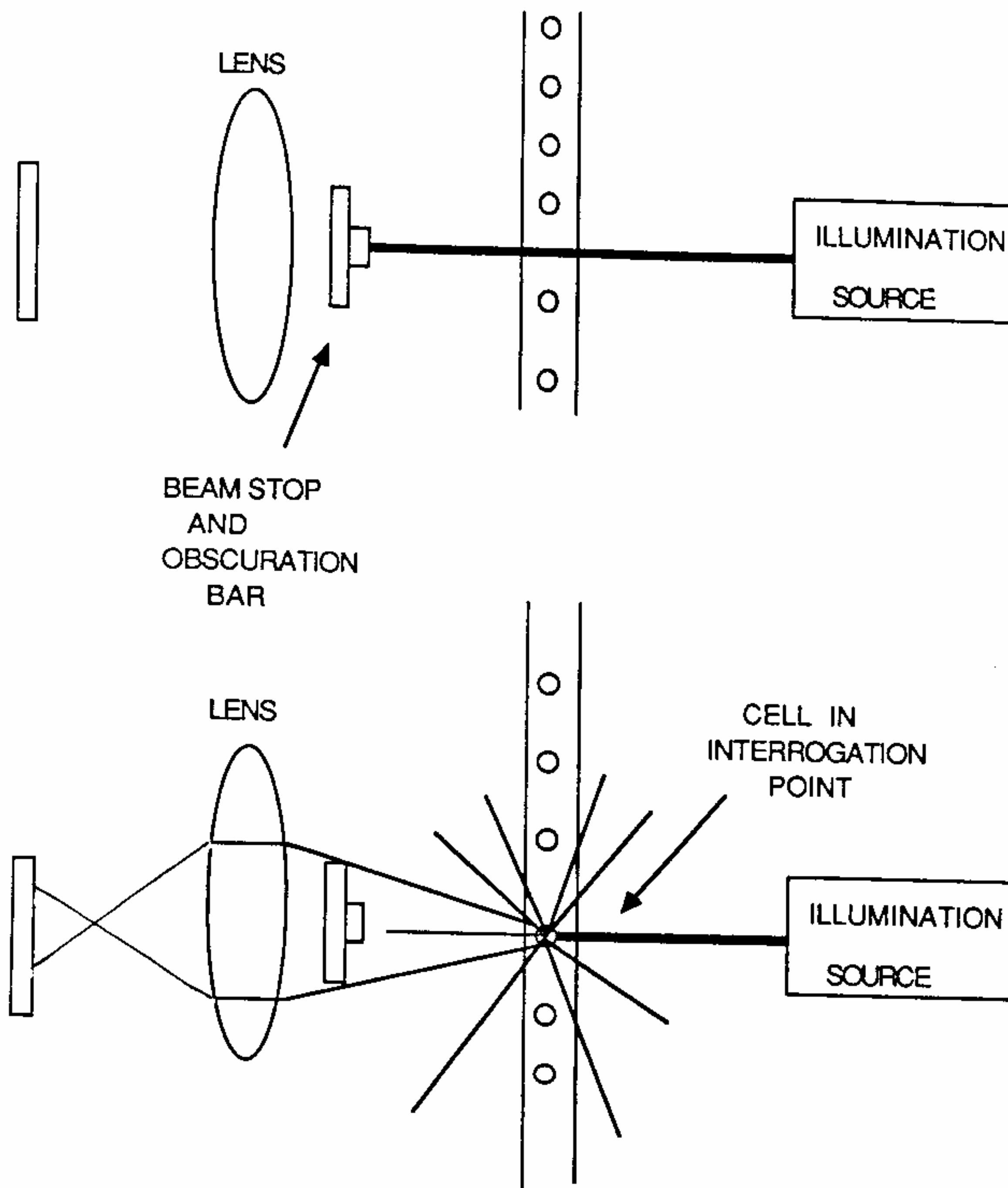

FIGURE 6.2: Beam stop and obscuration bar. The photodiode is not exposed to light except when a cell passes through the interrogation point. (A) No particle in the interrogation point. (B) Forward angle light scatter when a cell passes through the interrogation point.

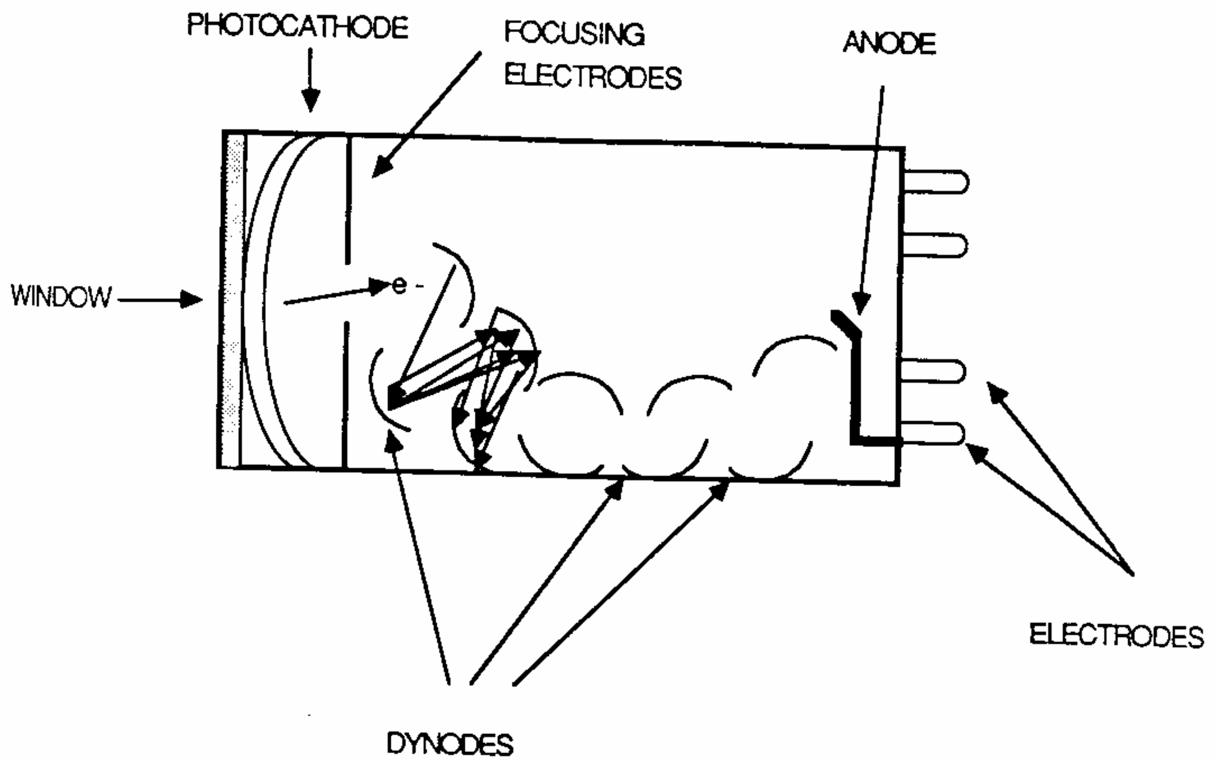

FIGURE 6.3: The photomultiplier tube. The end window design is shown during the amplification of a signal. Note the electrons cascading down the increasingly positive dynodes. As each electron strikes the next dynode, more electrons are generated.

6.3.1.1 THE PHOTOCATHODE

Photons enter the photomultiplier tube through the window, strike the photocathode, and **cause the release of electrons** due to the photoelectric effect. The number of electrons emitted from the photocathode is proportional to the number of photons (intensity) of the light that strikes it. The intensity of light required to cause the release of electrons from the photocathode is called the trigger amplitude and can be adjusted to permit the detection of a wide range of light intensities.

The **high voltage** adjustment of the photomultiplier tube controls the trigger amplitude and the amplification of the signal. Successively higher positive voltages are applied to the dynodes, causing electrons to cascade through the dynodes to the anode. The larger the high voltage setting, the smaller the amount of light required to trigger the tube, and the larger the amplification of the light signal.

The high voltage adjustment of the photomultiplier tube does not trigger data collection as the photodiode does. Increasing the high voltage adjustment of the photomultiplier tube increases the tube's sensitivity to low light intensity and the amplification of the light signal. The lower the intensity of the light to be measured, the higher the high voltage setting on the photomultiplier tube.

Photomultiplier tubes are subject to increasing **noise** as the high voltage setting increases. Noise occurs when the voltage supplied to the photomultiplier tube is large enough to cause electron flow down the dynodes in the absence of electrons generated by the photocathode. The propensity of photomultiplier tubes to produce noise is called the **dark current**, and is defined as the current that the tube will produce when operated in total darkness.

The high voltage adjustment controls the intensity of light that will cause the emission of a signal from the photomultiplier tube. In contrast, the **gain** of the photomultiplier tube controls the amplification of the signal. The gain setting controls a separate amplifier that provides additional amplification to the photomultiplier tube output signal. Increasing the gain increases the amplification of the signal for a given high voltage setting.

6.3.1.2 THE DYNODES

When an electron strikes a dynode, the dynode releases a number of secondary electrons. Each dynode has a slightly higher positive charge, causing the electrons to be pulled toward them in succession as shown in Figure 6.3. **Each dynode releases several secondary electrons for each electron that strikes it**, causing amplification of the signal.

The high voltage adjustment of the photomultiplier tube controls the number of electrons released from the dynodes for each electron that strikes them. **Increasing the high voltage setting causes a non-linear increase in the number of electrons that are released** from each dynode as the electrons cascade toward the anode. In contrast, **increasing the gain setting of the photomultiplier tube causes a linear increase in amplification**.

6.3.1.3 THE ANODE

The final electron acceptor in the photomultiplier tube is the positively charged anode. The anode is **connected directly to the output electrode**, and electrons that strike the anode exit the

photomultiplier tube through the electrode. The amplification produced by the dynodes can deliver a current to the anode that is 1E6 or 1E7 times greater than the current generated by the photons that strike the cathode.

6.3.2 THE PHOTOMULTIPLIER TUBE IN FLOW CYTOMETRY

Photomultiplier tubes perform low level light measurements where photodiodes cannot detect a light signal. The photomultiplier tube detects **cellular fluorescence and orthogonal light scatter** as each cell passes through the illuminating beam in the interrogation point. The photomultiplier tube can be adjusted to produce a strong signal from very dim light, so it is essential for low intensity light measurements such as immunofluorescence. In some systems, a photomultiplier tube replaces the forward angle light scatter photodiode.

6.3.2.1 DETECTION OF FLUORESCENCE

Fluorescence detection occurs in the orthogonal plane. The **orthogonal plane** is perpendicular to the plane of the sample flow and the illuminating light. Flow cytometers often use orthogonal detection systems to measure fluorescence because there is less interference from illuminating wavelengths than in the forward angle. The orthogonal configuration also decreases the need for many sensors in a small region by allowing fluorescence detection at right angles in lieu of the forward angle.

Many flow cytometers use **multiple photomultiplier tubes** to allow multiple simultaneous fluorescence measurements on a single sample population. For example, a three photomultiplier tube system would allow concurrent detection of red fluorescence, green fluorescence, and orthogonal light scatter.

The use of optical filters and dichroic mirrors separates selected fluorescence wavelengths into individual photomultiplier tubes, allowing simultaneous measurement. For orthogonal light scatter measurements, only the wavelength of the illuminating beam enters the photomultiplier tube. The optical separation of wavelengths that represent different characteristics of the cells in the population allows cytometers to rapidly collect data about the complete population.

6.3.2.2 OPTICAL SYSTEMS FOR FLUORESCENCE DETECTION

The goal of fluorescence detection optical systems is to deliver light scattered in the orthogonal plane and light from fluorescence emissions to the photomultiplier tubes. Like the illumination system, the detection optics may use a system of **crossed cylindrical lenses** to focus the light radiating from the interrogation point into the photomultiplier tubes. See Section 5.6.1 for a description of the use of crossed cylindrical lenses in illumination systems. The same principles apply to the use of crossed cylindrical lenses in detection systems.

Most cytometer detection systems use **three aspherical lenses and a pinhole to eliminate extraneous light**. Figure 6.4 illustrates this optical system. The first lens is placed with the focal point coinciding with the interrogation point, and projects a parallel beam onto the second aspherical lens. The focal point of the second aspherical lens coincides with a pinhole. The light

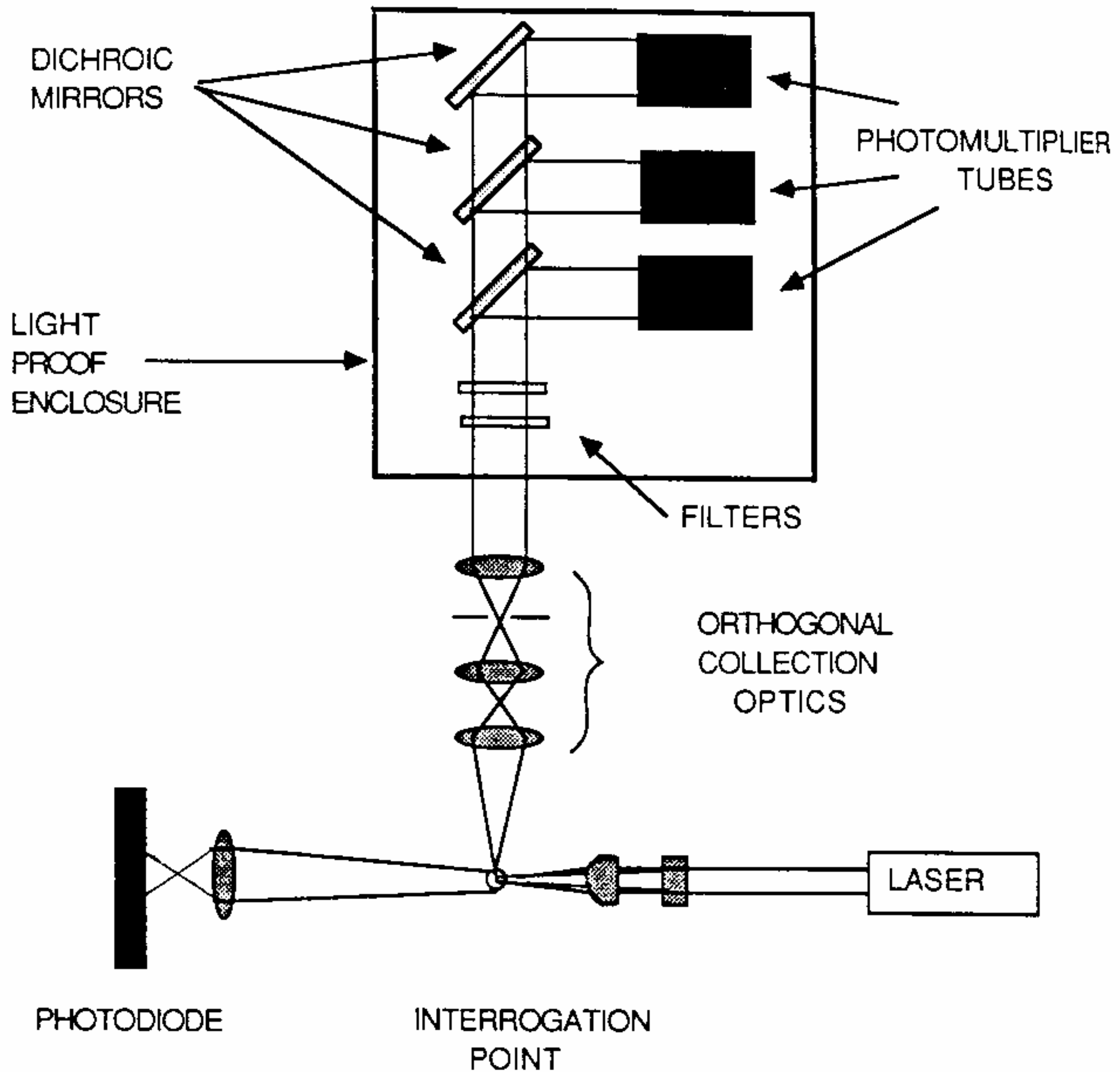

FIGURE 6.4: Fluorescence collection optics viewed from above. Note the placement of the pinhole at the focal point between the first two of the three aspherical lenses. The system reduces the incidence of extraneous light on the fluorescence detection optics. The filters and dichroic mirrors separate the individual wavelengths of light emitted by the cell into different photomultiplier tubes for detection.

collected at the interrogation point passes through both lenses and the pinhole before entering another aspherical lens. Extraneous light is blocked by the opaque region around the pinhole. The third aspherical lens is one focal length away from the pinhole and projects a parallel beam into the detector. Thus, only light originally emitted or scattered from the interrogation point enters the photomultiplier tube.

The detection optical system also allows the placement of **filters and dichroic mirrors** between the interrogation point and the photomultiplier tubes. By placing dichroics and filters in the path of the light, the operator is able to isolate selected wavelengths into individual photomultiplier tubes. This isolation of wavelengths allows the output each photomultiplier tube to represent a single cellular characteristic. Isolation of selected wavelengths that represent certain cellular characteristics into a single sensor allows the interpretation of the sensor's output as a proportional representation of the characteristic being measured. This capability speeds the analysis of populations of cells, and facilitates the electronic and computational functions of the cytometer.

6.4 WAVELENGTH SELECTION

In a system where multiple fluorescence detection is the goal, the **separation of wavelengths** emitted by fluorescent labels into individual photomultiplier tubes is essential. **Filters, dichroic mirrors, and beam splitters** are optical components that isolate wavelengths into individual photomultiplier tubes.

6.4.1 OPTICAL COMPONENTS USED IN FLOW CYTOMETRY

The control of light direction, intensity, and wavelength is an important part of flow cytometry. Flow cytometrists employ a wide variety of optical components to control these attributes of light.

Neutral density filters decrease the intensity of light throughout the spectrum. **Absorption filters** absorb regions of unwanted wavelengths and allow the transmission of desired wavelengths. **Interference filters** interfere constructively with some regions of wavelengths (allowing them to be transmitted) and interfere destructively with others (blocking their transmission). **Beam splitters** divide a beam of light into two distinct beams of equal wavelength. **Dichroic mirrors** reflect specific wavelengths and transmit other wavelengths, dividing a beam into two beams with different wavelength characteristics. Table 6.1 lists the functions of optical components.

6.4.2 FILTERS

Filters absorb or interfere with unwanted wavelengths. **Absorption filters have dyes embedded** in the glass of the filter that absorb unwanted wavelengths. **Interference filters have dielectric waveguides** that constructively interfere with the wavelengths for transmission and destructively interfere with the wavelengths to be blocked.

TABLE 6.1

FILTER	FUNCTION
NEUTRAL DENSITY FILTER	ABSORBS ALL WAVELENGTHS OF LIGHT TO DECREASE THE LIGHT INTENSITY
ABSORPTION FILTERS	ABSORBS WAVELENGTHS TO BE FILTERED OUT
INTERFERENCE FILTERS	CONSTRUCTIVELY INTERFERES WITH TRANSMITTED WAVE LENGTHS AND DESTRUCTIVELY INTERFERES WITH FILTERED WAVELENGTHS
BAND PASS FILTER	TRANSMITS ALL WAVELENGTHS IN A CERTAIN "BAND" OF WAVELENGTHS
HIGH PASS FILTER	TRANSMITS ALL WAVELENGTHS ABOVE A CERTAIN CUTOFF WAVELENGTH
LOW PASS FILTER	TRANSMITS ALL WAVELENGTHS BELOW A CERTAIN CUTOFF WAVELENGTH
BEAM SPLITTER	SPLITS BEAM INTO TWO BEAMS OF EQUIVALENT WAVELENGTHS
DICHROIC	SPLITS BEAM INTO TWO WAVELENGTHS OF DIFFERENT WAVELENGTHS

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TABLE 6.1: Functions of optical filters in flow cytometry.

6.4.2.1 NEUTRAL DENSITY FILTERS

Neutral density filters **reduce the intensity** of a light source. A neutral density filter fits between a strong illumination source and the interrogation point, reducing the intensity of all wavelengths of the light in the illumination beam. Without a neutral density filter, the light incident on the photomultiplier tube would be too intense for an accurate reading. Water filters may also be used to reduce the intensity of a light source while carrying away heat generated by the source.

6.4.2.2 BAND PASS FILTERS

The **band pass** or **band reject** filter transmits or blocks a series of wavelengths. Band filters are characterized in terms of their **bandwidth.** The bandwidth is described as the difference in wavelength between the two points where the transmission (or rejection) is one half of the maximum transmission. The bandwidth of a filter with a 50% rejection at 530 nm and a 50% rejection at 580 nm is 50 nm. Filter bandwidths are often characterized with the term **full width half maximum** or by the abbreviation **FWHM**.

6.4.2.3 SHORT AND LONG PASS FILTERS

Short pass filters allow the transmission of short wavelengths and block the transmission of long wavelengths. Short pass filters are characterized by the **cutoff wavelength** where there is a 50% transmission of the blocked frequency. An idealized 550 nm short pass filter would allow 50% transmission of the 550 nm wavelength, transmit 100% of wavelengths shorter than 550 nm, and block the transmission of 100% of all wavelengths longer than 550 nm. In reality, wavelengths near the cutoff frequency are transmitted at a little more or a little less than 50% as shown in Figure 6.5.

Long pass filters utilize the same principles as short pass filters except that the **long wavelengths are passed and the short wavelengths are blocked**. Thus, a 550 nm long pass filter would allow 50% transmission of the 550 nm wavelength, transmit 100% of wavelengths longer than 550 nm, and block the transmission of 100% of all wavelengths shorter than 550 nm.

6.4.3 BEAM SPLITTERS AND DICHROIC MIRRORS

Regardless of input wavelength, the beam splitter **divides the input beam into two equal beams**. Most beam splitters sit at 45 degree angle to the path of the beam to be split. Figure 6.6 (A) illustrates the function of a beam splitter. The beam splitter transmits 50% of the beam while reflecting 50% of the beam at 90 degrees. Beam splitters are useful for the diversion of one wavelength into more than one sensor.

Beam splitting optical surfaces with selective reflective characteristics are called **dichroic mirrors or filters**. By acting to **transmit some wavelengths and to reflect other wavelengths**, the dichroic directs light with specific wavelengths in the cytometer. For example, short pass dichroic mirrors pass short wavelengths and reflect long wavelengths. Long pass dichroic mirrors pass long wavelengths and reflect short wavelengths. Figure 6.6 (B) illustrates a dichroic mirror.

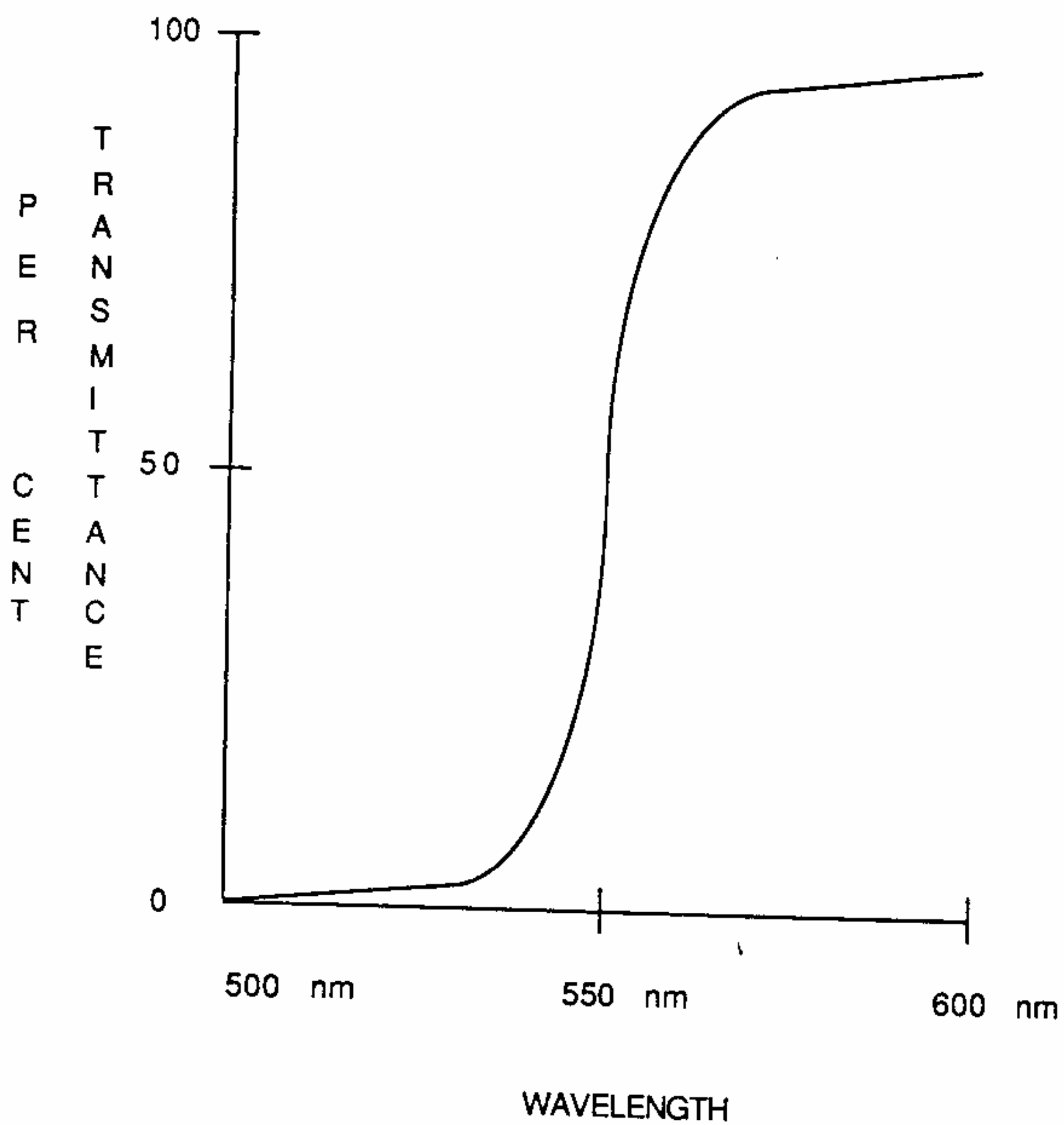
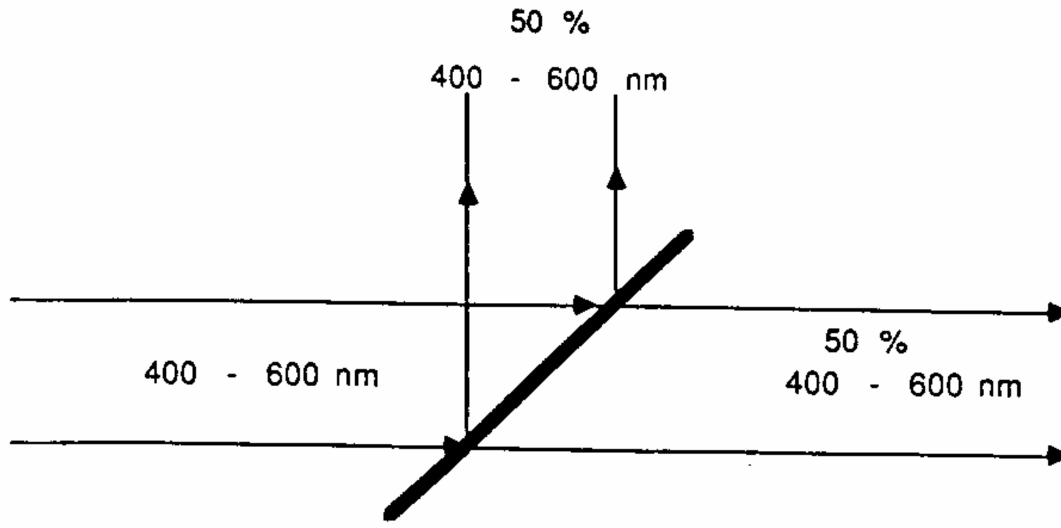


Figure 6.5: Transmission characteristics of a 550 long pass absorption filter. Crystals embedded in the filter scatter or absorb the light below the cutoff frequency. Note that the cutoff frequency is not absolute but changes over a region between 545 and 555 nm.



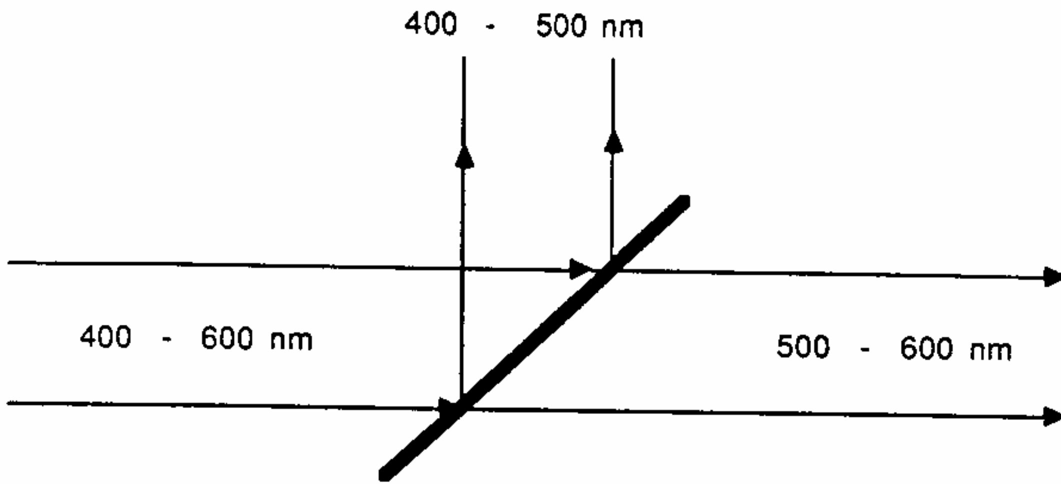


Figure 6.6: Beam splitter and dichroic mirror. The beam splitter (A) divides the beam into two distinct beams with similar wavelength, each with 50% of the intensity of the incident beam. The 500nm long pass dichroic (B) divides the beam into two beams of different wavelengths by reflecting wavelengths less than 500 nm and transmitting wavelengths longer than 500 nm.

6.5 SUMMARY

Photodiodes and photomultiplier tubes are sensors that detect optical signals and convert them to electronic signals. These sensors produce electronic signals that are proportional to the light signals produced by the interaction of the cell in the interrogation point and the illumination beam. Filters and dichroic mirrors direct and separate light signals from the interrogation point into individual photodetectors. The wavelengths directed into the photodetectors correspond to characteristics of the cells in the population, so the each photodetectors emits an electronic signal proportional to a characteristic of each cell. The set of signals collected from a population corresponds to population's properties.

PHOTODIODES AND PHOTOMULTIPLIER TUBES
Photodiodes detect forward angle light scatter (FALS) and narrow angle light scatter (NALS). In most cases, photomultiplier tubes in the orthogonal plane (90 degrees to the axis of the illuminating beam) detect fluorescence signals. Photomultiplier tubes contain a **photocathode** that emits electrons in response to light, **dynodes** that amplify the number of electrons received from the photocathode, and an **anode** that accepts the electrons from the dynodes and delivers them to the electrodes.

FILTERS, BEAM SPLITTERS, AND DICHROICS
Filters control wavelengths and optimize light signals in flow cytometry. **Absorption filters** absorb or scatter unwanted wavelengths with optically active crystals embedded in the glass of the filter. **Interference filters** constructively or destructively interfere with light. **Band pass filters** transmit light over a range of wavelengths called the full width half maximum (FWHM). Band reject filters reject light over a FWHM range. **Long pass filters** transmit 50% of the cutoff frequency and all wavelengths longer than the cutoff frequency. **Short pass filters** transmit 50% of the cutoff frequency and all wavelengths shorter than the cutoff frequency.

Beam splitters divide the beam into two beams of equal wavelengths while **dichroic mirrors** divide the beam into two beams with different wavelengths by reflecting certain wavelengths and transmitting other wavelengths. The use of filters, beam splitters, and dichroic mirrors allow the separation of different wavelengths of light that represent different cellular characteristics into different photodetectors. The photodetectors then emit electronic signals that are proportional to the quantity of the characteristic in each cell. The set of all signals collected from a population as it passes through the interrogation point represents the properties of the population and is displayed as a histogram.

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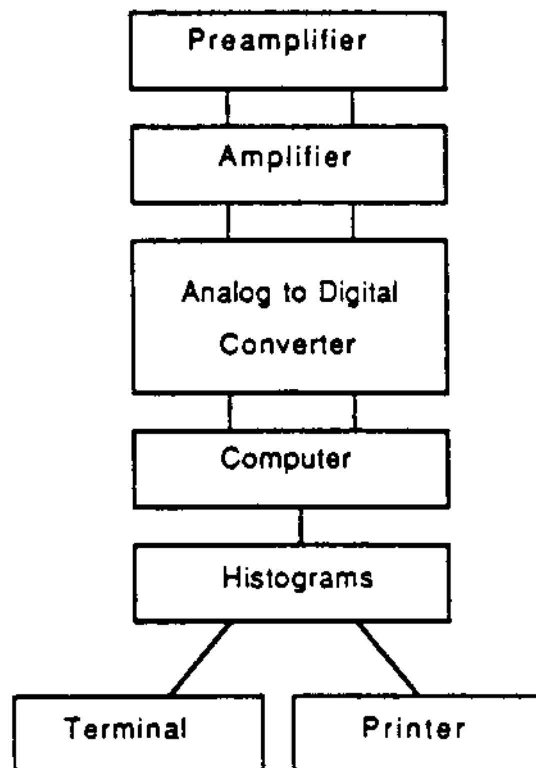
PART THREE: THE DATA COLLECTION AND ANALYSIS SYSTEM



PART THREE: THE DATA COLLECTION AND ANALYSIS SYSTEM

The flow cytometer collects light scattered from the surfaces of cells, the internal structures of cells, and the light emitted from fluorescent labels attached to the cell. Photosensors detect these light signals and convert them into electrical signals. These electrical signals form the basis for data collection and analysis.

The Data Collection and Analysis System converts the electrical signals from the photosensors into digital signals for storage on electronic media such as floppy or hard disk. This conversion relies upon the amplification of the electrical signals and analog to digital conversion. Once the conversion of the analog signals to digital signals is complete, the cytometer's computer stores, retrieves, and performs mathematical and non-mathematical analyses upon the digital data.

Part Three discusses the electronic methods cytometers use to amplify and convert analog signals into digital signals, and the computer methods employed in the analysis of population data.

CHAPTER 7: ELECTRONICS AND SIGNAL PROCESSING

When the optical microscopist switches on her light source, she depends on an electronic system to convert electricity into light. The electron microscope must have even more complex electronic systems to generate, focus, and project the electrons that interact with the sample. Scanning electron microscopy also requires signal processing electronics to convert the secondary electrons from the surface of the sample into an image.

The complexity of these systems pale in comparison to the complexity of the electronic systems found in cytometry. Simple electronic systems in cytometry orchestrate electromechanical functions such as the opening of gas valves to initiate liquid flows, provision of power for intricate illumination sources, and provision of power to components that detect and convert analog signals to digital signals. The electronic signal processing capability of cytometers includes the ability to store, retrieve, manipulate, and display data sets, and computational methods to analyze those data sets.

7.1 ELECTRONICS IN CYTOMETRY

Cytometers rely on electronics for communications between components, transmission of data, collection of data, storage of data, analysis of data, and cell sorting. The electronic systems that allow automated function of cytometric systems are an outgrowth of instrumentation technologies that started with the Coulter Counter and the ink jet printer. Electronics provides the integrated function that allows cytometers to collect, organize, and analyze data about populations.

Flow cytometry uses electronics for power, timing, and electronic signal processing for the collection and analysis of data. An understanding of the electronic power supply systems is not required for the operation of cytometric equipment. However, cytometer **operators must understand the function of photosensors, the signals they emit, and the electronic methods that make those signals suitable for transmission to computer equipment**. Understanding the details of power supplies, component timing, and electromechanical equipment is not required for the operation of cytometric instrumentation. However, operators do need an understanding of electronic systems that capture and analyze data.

Electronic components called photosensors measure the light emissions due to the interaction of light from the illumination source with the cell at the interrogation point. The photosensors convert the light emissions into electronic signals. The most common photosensors used in flow cytometry are photodiodes and photomultiplier tubes.

Cells in the interrogation point emit analog light signals, which are detected by photosensors. The photosensors emit **analog electrical signals that undergo** amplification and conversion to digital signals. Once the conversion of analog light signals to digital electrical signals is complete, a computer can store and analyze the signals. Digital signal processing also allows the cytometer to rapidly choose and sort specific cells by recognizing the signals that they generate.

7.2 TYPES OF SIGNALS

There are three important types of signals in flow cytometry: **analog light signals, analog electronic signals, and digital electronic signals**. Each of these signal types play an important role in cytometer function. Analog light signals allow the direct measurement of cellular characteristics. Analog electronic signals provide a method for the conversion of light signals to digital signals. The cytometer and its computer use digital electronic signals to represent, store, process, and analyze cellular characteristics and the properties of populations.

Analog signals have a magnitude that varies over time. A sine wave is a good example of an analog signal. Analog signals vary smoothly over time an cannot be understood by modern computers that utilize digital logic systems.

Digital systems use binary codes to represent signals. Digital representations of analog signals recreate the analog signal with a series of discrete, numerical values. The advantage of digital signals in flow cytometry stems from the use of binary numbers by computers and electronic components. **Digital values are easy to represent with binary numbers while analog values are not**.

7.2.1 LIGHT SIGNALS

Light signal is a light emission from the cell under study. These emissions include light scattered from the cell, and fluorescence emitted from fluorescent labels attached to cellular components. The light signals emitted from a cell are proportional to certain characteristics of that cell. These signals can represent a wide variety of cellular characteristics such as DNA content, cell surface markers, or cellular complexity. The intensity and form of a light signal represents a **quantitative measure** of a cellular characteristic. Light signals are analog signals.

7.2.2 ANALOG SIGNALS

Analog signals have shape and magnitude that are represented by an amplitude that changes over time. Analog signals represent cellular characteristics with a proportional value. With light signals, **light intensity is proportional to the quantity of a cellular characteristic**. The photosensors produce electronic analog signals, emitting a current that is proportional to the analog light signal and, therefore, the quantity of the labeled cellular characteristic.

7.2.3 ELECTRONIC ANALOG SIGNALS

Photodiodes and photomultiplier tubes convert analog light signals to analog electrical signals. The analog electrical signal is a proportional current representation of the light intensity emitted by a cellular characteristic. For instance, if the peak light signal from a cell measurement is 0.2 of the 10 microamp maximum output current of the photosensor, the analog output signal of the sensor is 2 microamps. Therefore, the output of the photosensor is an analog electrical current that is proportional to the light signal emitted by the cell under study.

7.2.4 DIGITAL ELECTRONIC SIGNALS

There is at least one analog electrical signal emitted from at least one photosensor for each cell in the population. **The electronic components and computer of the cytometer use only digital voltage signals**. To collect and store data on the properties of populations, the cytometer must convert analog current signals into digital voltage signals.

Digital electronics uses **small voltage pulses to represent binary numbers**. For instance, in binary (base 2), the numeral 2 is represented as binary 10. A digital computer uses two voltage states, 1 or 0, to represent binary values. If the computer uses +5 volts as a 1 and 0 volts as a 0, the computer would represent the numeral 2 as the binary number 10 with two voltages in sequence, in this case +5, 0. The +5 voltage state represents a binary 1 and the 0 voltage state represents a binary 0. Similarly, the computer would represent the decimal number 100 as the binary number 1100100 with the voltage states +5, +5, 0, 0, +5, 0, 0. To reduce the size of these numbers, most electronic equipment uses a method of coding decimals in binary called binary coded decimal.

By using binary numbers and a series of numerical representations of letters called an **ASCII table**, digital electronic devices such as the cytometer can exchange, analyze, and display numbers, words, and data. Virtually all functions of a flow cytometer rely on digital electronics.

7.3 ELECTRONICS AND CYTOMETER FUNCTION

Digital electronics is the common language that cytometer components use for communication, data collection, data analysis, and sorting. Digital signals control the internal functions of the cytometer, transmit data collected by the photosensors, analyze that data, choose cells for sorting, and accomplish the sorting process.

Digital **communication** between cytometer components controls the operation of the cytometer by opening flow valves, detecting the presence of a cell in the interrogation point, and orchestrating hardware interactions. Digital communications between components assures that cytometer functions occur on time and in the correct order. In addition to controlling cytometer function, digital communications also transmits digital signals from the sensors to components such as the computer on a dedicated communications line.

The signals that contain data are fundamentally different from the signals used for communication within the cytometer because they contain information about the population under study. In contrast, the signals that the cytometer uses to control its function do not contain data about the population and contain special codes, such as the address of a memory location, to cause complex cytometric functions. Digital communications signals and digital representations of data are separated in the cytometer by special communication lines called **busses**. The address and control busses carry information that the cytometer uses to accomplish its functions, while the data bus carries data collected from the sensors.

In **data collection**, the photosensors emit analog current signals that are proportional to the light signal emitted by the cell and collected by the photosensor. In contrast to digital signals that represent numbers as a series of 1s and 0s to describe a value as a binary number, an analog

electrical signal is equal (analogous) to the value it represents. The analog current signals from the photosensors undergo amplification and analog to digital conversion before transmission to the computer.

Data analysis revolves around the creation of frequency distributions from the digital signals collected from each cell as it passes through the interrogation point. The computer stores the digital signals from the analog to digital converters on a disk drive. For analysis, the computer retrieves all of the signals from a certain population and performs mathematical and non-mathematical analysis upon them. The computer organizes the data as a histogram and displays it on a terminal or printer for human analysis and interpretation.

Digital electronic components called comparators (i.e. single channel analyzers) choose cells fast enough to make **sorting** feasible. The signals created by cells passing through the interrogation point are compared with a signal stored in the analyzer. If the signals match, the cell is sorted from the rest of the population. If the stored signal does not match, the cell is not sorted.

Digital electronic signals are the basis of virtually every aspect flow cytometer function including instrument control, communications, data collection, data analysis, and sorting. In contrast to the signals the cytometer uses to communicate and organize its function, signals that contain data about the population under study may undergo additional electronic techniques called **signal processing**.

Much of flow cytometry is the **collection and processing of signals from individual cells** to produce data sets representing the entire population. Analog electrical signals from the sensors are amplified, converted from analog to digital, passed to the computer as digital signals that are stored, interpreted, and processed. The computer stores and processes peak and integral signal data, accepts data from linear and logarithmic amplification processes, and displays or prints these data as histograms.

7.4 PHOTOSENSORS, AMPLIFIERS, SIGNAL CONVERSION, AND TIMING

Both the sheath flow and the concentric sample flow pass through the illumination beam at the **interrogation point**. If the cytometer is properly calibrated, the cells in the center of the sample flow pass precisely through the illuminating beam in single file.

As each cell passes through the interrogation point, the light from the illuminating beam scatters off its surface and its organelles, creating **light signals** that correspond to specific characteristics of the cell. The light also causes fluorescent labels present in or on the cell to fluoresce, creating another type of light signal. These light signals, due to scattering from the cell or due to the emission of fluorescence, are **analog** light signals that are measured by photosensors.

Two types of photosensors, photodiodes and photomultiplier tubes, convert the light scattered or emitted by the cell into electrical signals. Each of these photosensor types emit an **analog current output proportional to the analog light input** received by the photosensor. The analog current signals from the photosensors are very small and must undergo amplification and conversion to digital signals before a digital computer will accept them.

Electronic components called **amplifiers** increase the electronic output of the sensors, often converting the current signal to a voltage signal. An electronic component called an **analog to digital converter**, translates the analog signals to digital signals. The digital signals from the analog to digital converter are digital representations of the analog signal fed to the converter. The accuracy of the digital representation of an analog waveform depends on the resolution of the analog to digital converter. The cytometer transmits the digital signals to the computer for storage and analysis.

Photosensors, amplifiers, analog to digital converters, computers, sorting components, and other parts of the cytometer often work at different speeds. If the components communicate in real time, some components send their signals before others are ready to receive them. When the slow component is ready, it waits endlessly for a signal that has already been transmitted. These timing problems are resolved by electronic components such as **shift registers** that hold the digital signals until other components are ready. These timekeeping functions would be extremely difficult without the use of digital electronic components.

7.4.1 OUTPUT OF THE PHOTOSENSORS

Both photodiodes and photomultiplier tubes convert light signals into **analog current signals in the range of a few nanoamperes to a few microamperes**. These small currents are rarely large enough for direct routing to an analog to digital converter. To solve the problems associated with the small current output of the photosensors, their signals usually undergo preamplification and amplification prior to analog to digital conversion. Although photomultiplier tube output may be large enough for use without amplification, cytometer systems usually route these signals through the preamplification/amplification system.

7.4.2 PREAMPLIFICATION OF THE OUTPUT CURRENT

Preamplification is an electronic process that prepares the signal for amplification. Both photodiodes and photomultiplier tubes have a current output that cannot interact with the cytometer's voltage based electronic systems. Preamplification **changes the current output of the sensors to a voltage** through a process called **transampedance amplification**.

7.4.3 AMPLIFICATION

After current to voltage conversion in the transampedance preamplifier, an amplifier boosts the **voltage signals from the sensors into the range of 0 to 10 volts**. Amplification into this range enables the analog to digital converter and other cytometer components to recognize and accept the signals. Important characteristics of amplifiers include **rise-time, amplitude accuracy, signal to noise ratio, and recovery time**.

7.4.3.1 RISE TIME

Cells pass through the interrogation point very rapidly during the analysis of a population. To collect data that accurately represents the cell in the interrogation point, the cytometer must be capable of making an accurate measurement of the cell in a very short time. This fact makes an **amplifier's speed of response** to an input signal, called the rise-time, an important consideration

in flow cytometry.

Amplifier rise-time is a measure of the time it takes for the amplifier output to reach 90 to 95% of the input value. Rise-time measurements begin at the time when the amplifier output is 5 to 10% of the input value. A fast rise-time is important because the **amplifier must be able to reach full output before the cell leaves the interrogation point** and the input signal begins to drop off. If the rise-time is too slow, the output signal will not reach the true input value before the input pulse begins to drop off. This situation results in the collection of incorrect data for the cell.

7.4.3.2 AMPLITUDE ACCURACY

Amplitude accuracy is another important characteristic of amplifiers for use in flow cytometry. Amplitude accuracy is a **measure of the accuracy of the output pulse compared to the input pulse**. If the amplifier cannot accurately amplify the input pulse, the cytometer will not provide correct data about individual cells or the population.

7.4.3.3 SIGNAL TO NOISE RATIO

Noise is a term that describes unwanted signals in electrical processing. The signal to noise ratio is a measure of the amount of noise that accompanies the signal after amplification. Noise may be due to the processing itself, radio interference, or other factors such as instruments in the same area or on the same electrical line.

A **high signal to noise ratio** is important for the same reasons described for amplitude accuracy. If the amplifier outputs a high proportion of noise in relation to the input signal, the data may be useless.

7.4.3.4 SHORT RECOVERY TIME

Like rise time, a short recovery time is important because of the rapid analysis of large numbers of cells. Once the amplifier has amplified a signal, it needs time to recover before it receives the next signal for amplification. **The amplifier must be capable of completing the output of one signal before the next signal enters the amplifier input**. If the amplifier recovery time is too slow, the output for successive signals will overlap.

7.4.3.5 LINEAR AMPLIFICATION

Linear amplifiers **increase the amplitude of the output signals of the photodiode and photomultiplier tubes linearly**. This means that the amplified output signal is proportional to the value of the input signal. For example, in a tenfold linear gain amplifier, if 100 millivolts enters the amplifier, the amplifier will emit a one volt signal. If a 500 millivolt signal enters the amplifier, the amplifier will emit a 5 volt signal. Therefore, the dynamic range of this linear amplifier is one order of magnitude.

7.4.3.6 LOGARITHMIC AMPLIFICATION

Logarithmic amplifiers **increase input signals such that the output represents three orders of magnitude** of input voltage (0.01 to 0.1 volts, 0.1 to 1 volts, and 1 to 10 volts). The logarithmic distribution of values through three decades of a logarithmic amplifier is shown in Figure 7.1.

Logarithmic amplification performs **three important functions** in flow cytometry: increasing the dynamic range of the data displayed on a single histogram, preserving histogram shape independent of system gain, and representing logarithmic cell distributions as normal cell

distributions.

The dynamic range of the system increases because the system can display signals from the sensors that would normally exceed the range of the output recording device (histogram, display, or printer). Because histogram shape is independent of amplifier gain, histogram detail can be compared from sample to sample. When approximately logarithmic cell distributions undergo logarithmic amplification, the resulting distributions may be easier to analyze.

7.4.4 PULSE PROCESSING ELECTRONICS

There are two ways to interpret the output signals of the photosensors. The **peak signal** is a proportional representation of the highest voltage of the sensor's output pulse. The **integral signal** is a proportional representation of the total light emitted by the cell while it is in the interrogation point.

Peak signals yield information about cell brightness or fluorescence density while **integral signals yield information about the total fluorescence** of the cell. Integral output signals yield information about the total amount of cellular constituent labeled. Both integrated signals and peak signals may undergo linear or logarithmic amplification. Peak signals, integral signals, and logarithmic and linear histograms are shown in Figure 7.2.

7.4.5 ANALOG TO DIGITAL CONVERTERS

Analog to digital converters **convert analog electrical signals from the amplifiers** to digital signals for use by the computer. Analog to digital converters provide the link from analog devices to digital information processing systems. Eight bit analog to digital converters function by allocating analog values (0 to 10 volts) to discrete digital values (0 to 256 digits) as shown in Figure 7.3. Higher resolution analog to digital converters assign the 0 to 10 volt range over 0 to 512 digits (9 bit) or 0 to 1024 digits (10 bit).

As the number of digits (channels) in an analog to digital converter increases, the resolution of the analog to digital converter increases. The resolution of an analog to digital converter is a measurement its accuracy. **More channels means more information** and higher resolution. Higher resolution produces a digital signal that is a more accurate representation of the analog signal because there are more channels to carry information.

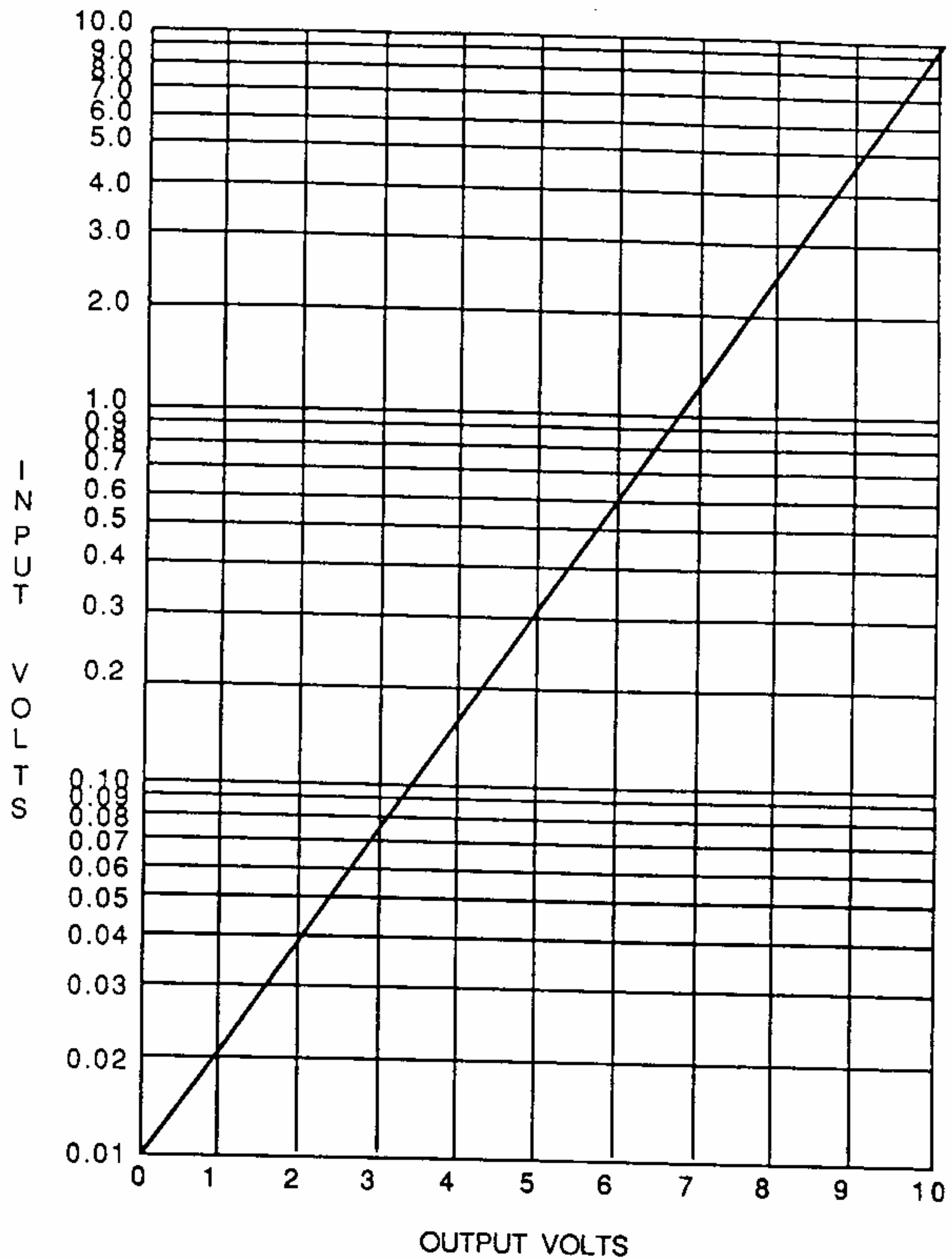
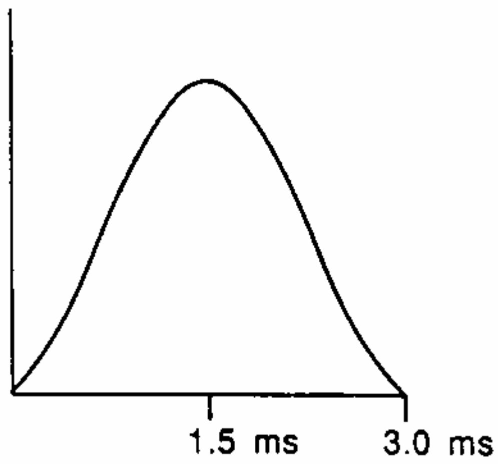
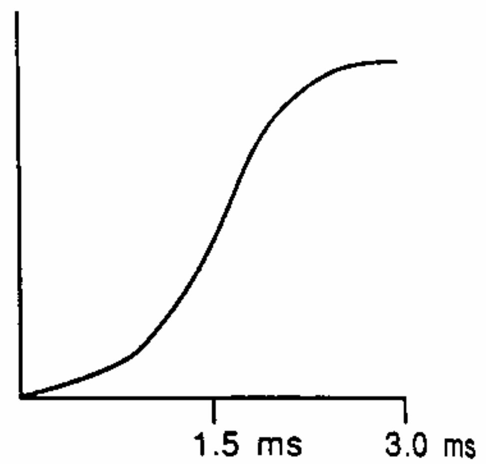


FIGURE 7.1: Logarithmic amplifier output. The logarithmic amplifier includes three decades, 0.0 to 0.01, 0.01 to 1.0, and 1.0 to 10.0, increasing the dynamic range of the amplifier and assisting with comparisons between signals of different amplitudes.



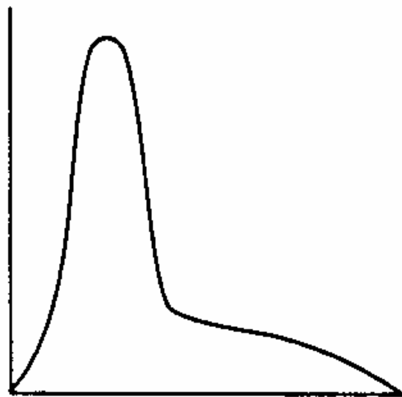
PEAK SIGNAL

(A)



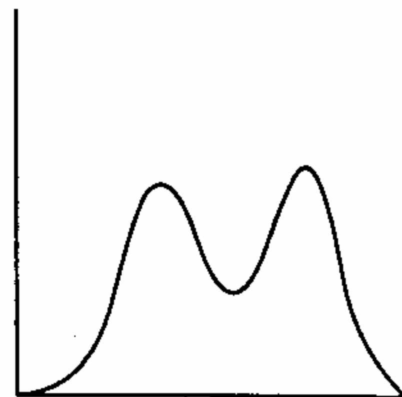
INTEGRAL SIGNAL

(B)



LINEAR HISTOGRAM

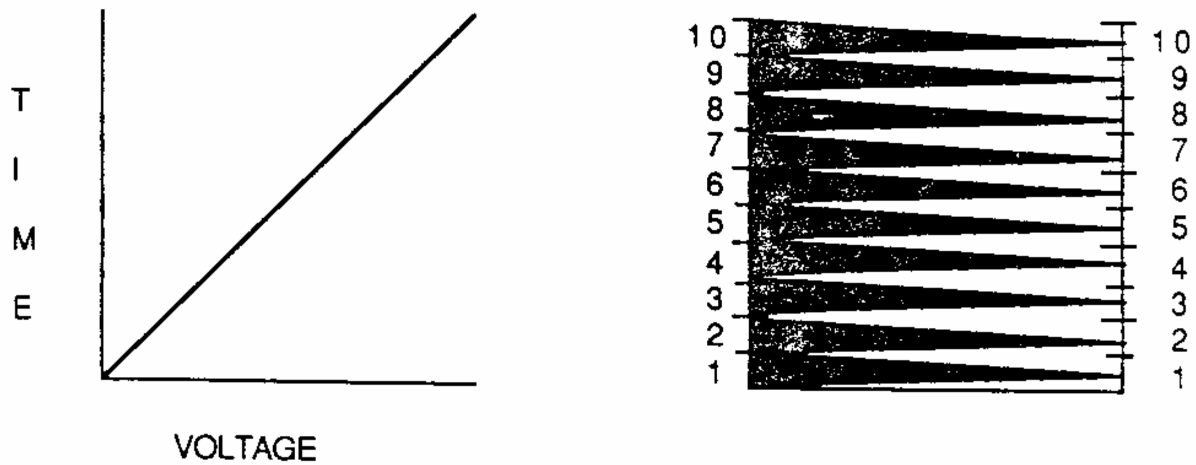
(C)



LOGARITHMIC HISTOGRAM

(D)

FIGURE 7.2: Signal and histogram types. Peak signal (A), integrated signal (B), linear histogram (C), logarithmic histogram (D).



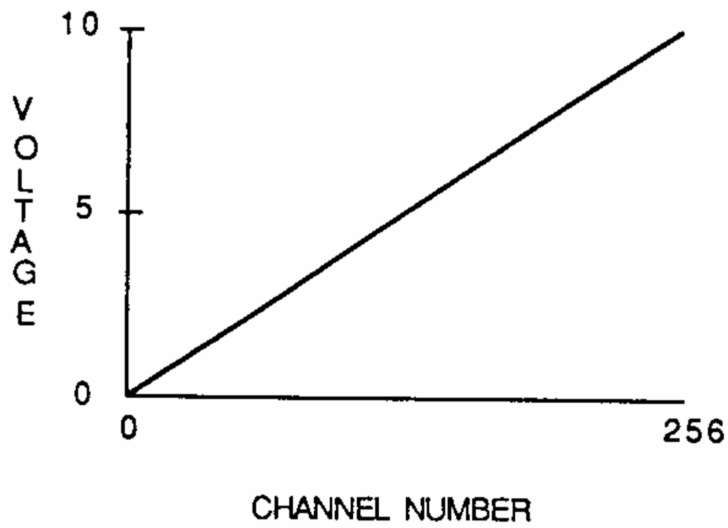


FIGURE 7.3: Allocation of analog values to digital values. The analog to digital converter can allocate the analog voltage (0 to 10 volts) to 0 to 256, 0 to 512, or 0 to 1024 digits depending on fixed analog to digital converter resolution.

Flow cytometers usually employ one of two types of analog to digital converters. The **Wilkinson-type analog to digital converter** charges a capacitor to the amplitude of the peak signal. A constant current drain then discharges the capacitor linearly. During discharge, a gated high frequency clock records the number of cycles it takes to discharge the capacitor. Since the voltage drop is continuous with time and the clock operates at a constant frequency, the time for the voltage drop to zero can be divided into equally spaced channels. Wilkinson type analog to digital converters operate on the same basic principle as single slope analog to digital converters.

The second type of analog to digital converter used in flow cytometry is the **successive approximation type**. The successive approximation analog to digital converter works by generating comparison voltages with a digital to analog converter and comparing the input to the internal voltage generated by the digital to analog converter. The ratio of the input voltage over the internal voltage generates a series of digital values.

Usually, each sensor has a dedicated amplifier and analog to digital converter. This arrangement allows the sensor, amplifier, and analog to digital converter system to rapidly create output pulses for each cell. The output of the analog to digital converters is carried on the data bus to the cytometer's computer for storage and manipulation with software.

7.4.5.1 DISCRIMINATION

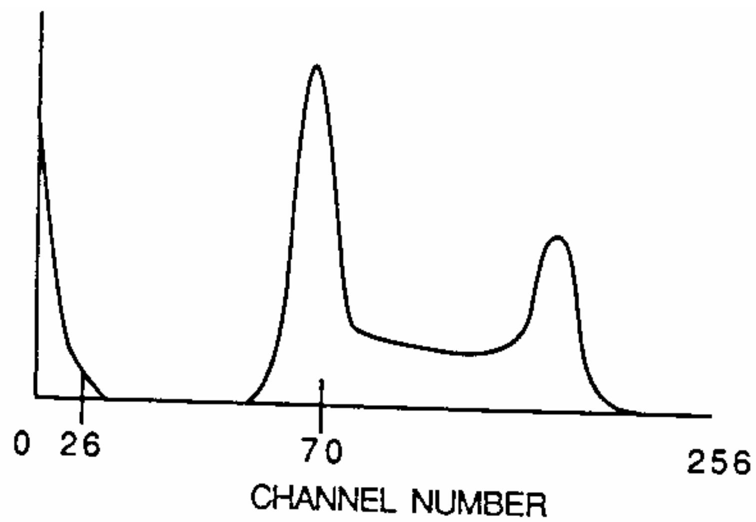
Discrimination describes methods for **removing an unwanted zone of data**. Discrimination is an important capability of the flow cytometer because it allows specific analysis of selected subpopulations by preventing the analysis of unwanted populations such as debris. Discrimination is useful when the sample contains a large amount of debris or when the operator wants to separate a population for analysis. Both hardware techniques and software gating can accomplish discrimination.

7.4.5.2 HARDWARE DISCRIMINATION WITH ANALOG TO DIGITAL CONVERTERS

Hardware discrimination is an analog to digital converter adjustment where the **analog to digital converter reports all channels in the desired discrimination region as zero**. When the analog to digital converter is adjusted in this fashion, no matter what the signal is in the discrimination region, the analog to digital converter will report it as a zero. Figure 7.4 illustrates a discrimination of values in channels from 0 to 26 by utilizing a 0 to 1 volt discrimination in the analog to digital converter. **Discrimination occurs in the preamplification phase prior to analog to digital conversion**.

Although discrimination can be a useful tool to remove unwanted data due to a poor sample, it can also remove useful data. The operator must use caution when employing discrimination to prevent the loss of useful data by discriminating against signals that are relevant to the study.

As with other adjustments to the cytometer, the use of hardware discrimination should be recorded for future reference if the analysis needs to be repeated.



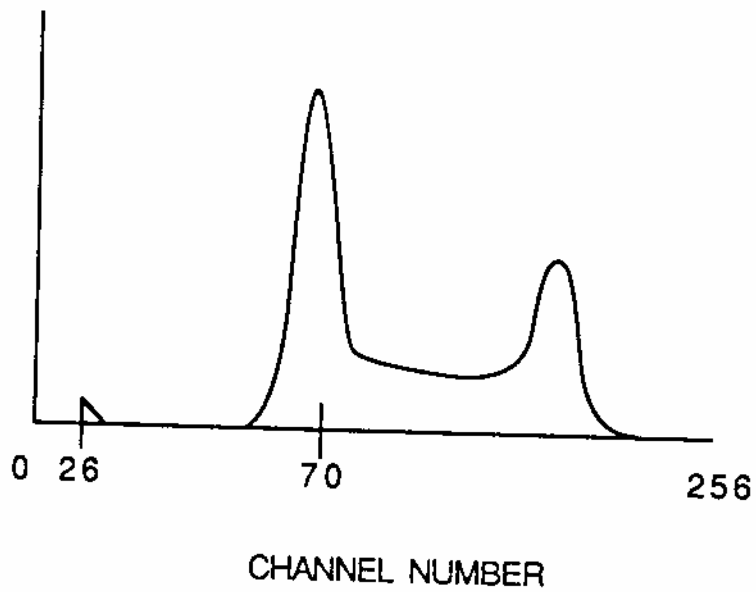


FIGURE 7.4: Hardware discrimination in the analog to digital converter. Signal with debris (A). Discriminated signal (B).

7.3 SUMMARY

Electronic components are essential to the function of the flow cytometer. Photosensors detect analog light scatter and fluorescence signals emitted from the interrogation point and produce **analog electrical signals**. These signals are **currents** in the range of a **few nanoamperes to a few microamperes** and are proportional to the light signals that generate them.

AMPLIFICATION AND DISCRIMINATION

Analog signals from the photosensors are **preamplified and discriminated**. Preamplification converts the current output of the sensors to a small voltage in a process called **transampedance amplification**. After preamplification, the amplifier increases the values of the signals into the range of 0 to 10 volts. Discrimination removes unwanted regions of data by setting the analog to digital converter to report a certain range of voltages as zero. Discrimination is important when debris or other unwanted signals are a problem.

ANALOG TO DIGITAL CONVERSION

Analog to digital converters convert the 0 to 10 volt pulse to a **series of discrete digits by assigning the voltages to a range of channels**. High resolution analog to digital converters assign the 10 volts over ranges from 0 to 512 or 0 to 1024 channels. Analog to digital conversion is essential so that cytometer components can transit the data to the computer for analysis.

TYPES OF ANALOG TO DIGITAL CONVERTERS

Wilkinson-type analog to digital converters convert the analog signal to a digital signal by storing the peak analog signal in a capacitor and measuring the time it takes for the signal to drop to zero. Since the voltage drop is constant, the conversion divides the voltage drop into digits. **Successive approximation** analog to digital converters generate an internal analog waveform and compare the input signal to the internal signal. The ratio of the internal analog signal versus the incoming analog signal results in a series of discrete signals.

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CHAPTER 8: DATA ACQUISITION AND ANALYSIS

In light microscopy, data takes the form of diffracted light waves that are acquired by the eye and analyzed in the brain. Scanning electron microscopes acquire data through an electron detector that captures secondary electrons and displays an image on a screen. Once again, data analysis is left up to the human brain.

In cytometry, photosensors detect light signals and convert them to current signals. The current signals from these sensors are converted to digital voltage signals and routed to the computer on the data bus. The computer stores these signals as data sets which can be analyzed by the computer or displayed as histograms for human interpretation. Unlike the real time images that optical and electron microscopes produce, the data sets collected by flow cytometers remain available on magnetic media and contain more information than a simple image. This allows cytometrists to retrieve and review the data for previously accomplished studies.

8.1 DATA ACQUISITION AND ANALYSIS IN FLOW CYTOMETRY

The cytometer's computer stores **digital data** from the analog to digital converters **on tape, hard disk, or floppy disk**. A data set that represents the output of a photosensor is called a parameter. The computer plots these parameters on graphical displays called **histograms**. If the **analog to digital converter produces 256 discrete digits**, the **histogram** generated from the data **will have 256 channels**. The histogram may be interpreted visually, as a frequency distribution, by comparison with a data base of similar cells, or through mathematical analysis.

Analytical methods are called **parametric** if they rely on mathematical models of the graph to describe the data, or **nonparametric** if they employ simpler methods to analyze the shape of a graph. Parametric and nonparametric methods of data analysis may be applied to univariate (one parameter) or bivariate (two parameter) data.

8.2 DATA ACQUISITION

Individual cells passing through the interrogation point generate flow cytometric data. **Forward angle light scatter, orthogonal light scatter, and fluorescence signals** cause the photomultiplier tubes and photodiodes to emit electrical current signals. As each cell passes through the interrogation point, it creates a set of signals that correspond to the output of the photosensors. The analog electrical signals are converted to digital signals by analog to digital converters. The set of digital signals emitted from the analog to digital converters are sent to the computer for storage. Once the computer has stored the digital sensor signals for each member of the population, the computer displays the population as a graph called a histogram.

8.2.1 COMPUTER DATA ACQUISITION

Computers rely on **binary numbers** to represent characters, decimal numbers, and

communications codes. At the hardware level, these binary numbers take the form of discrete voltages, usually in the range of 0 to 5 volts. A 0 voltage state represents a binary zero, while a +5 voltage state represents a binary 1. Virtually all of the computer's functions utilize these binary voltages.

To communicate with the computer, **cytometric data is converted to digital signals that are represented as binary numbers**. These digital signals are carried from the sensors to the computer on a dedicated communications line called a data bus. The data bus carries the signals from each cell to the computer where they are held until all the cells pass through the interrogation point. Once the signals for all the cells in the population have been collected, the computer either stores all the data points that represent the population, or generates a histogram of the population data for storage. List mode is the process that stores all data points for the population, while gating is the process that stores only a histogram of the population.

8.2.2 HISTOGRAMS

Histograms are **graphs of frequency distributions**, and are the most common form for the delivery of cytometric data to the operator. Although data may also be displayed in a tabular format, the graphical nature of the histogram makes it easier to analyze.

A **single parameter histogram** plots the **number of events** on the vertical axis of a graph **versus channel number** on the horizontal axis of the graph. The channel number represents an analog to digital converter output such as forward angle light scatter or fluorescence. The single parameter histogram may also be referred to as a univariate histogram.

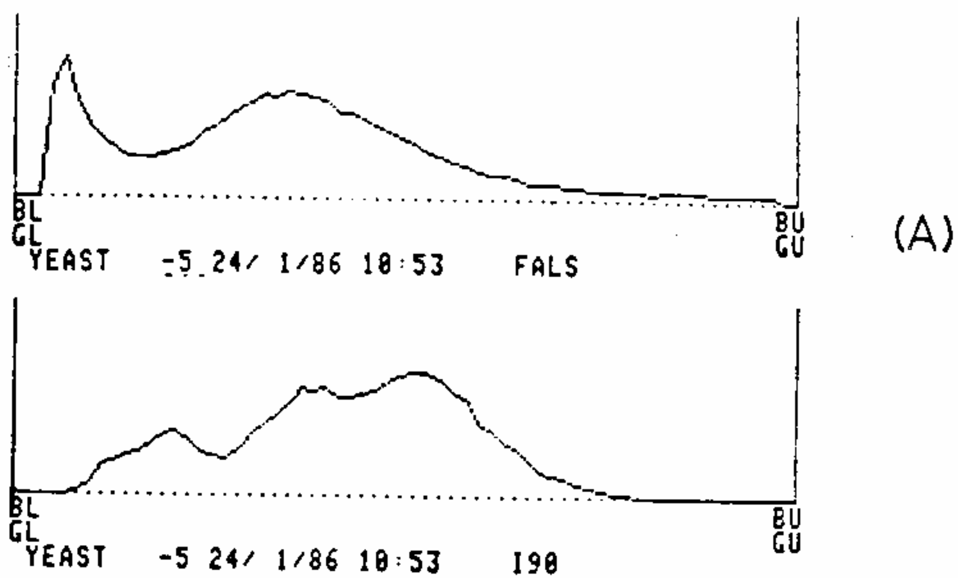
Dual parameter histograms are generated by plotting output of one analog to digital converter on one axis against the output of a second analog to digital converter on a second axis and number of events on a third axis. The dual parameter histogram may also be referred to as a bivariate histogram. Figure 8.1 illustrates single and dual parameter histograms.

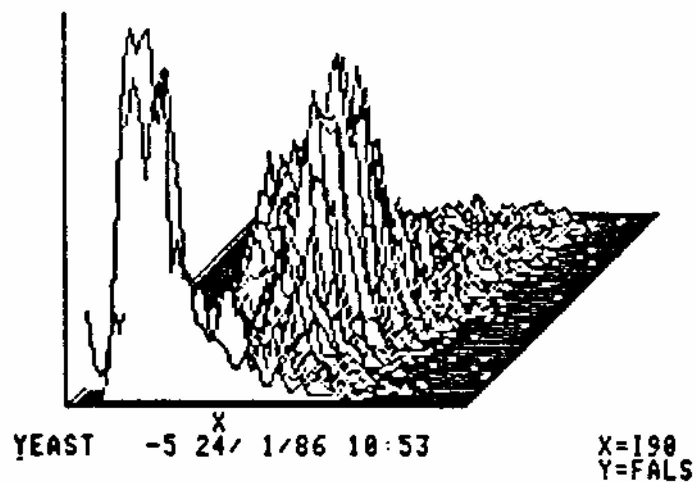
Single and dual parameter histograms give the operator a selection of possible displays of the data collected. If the operator is not sure what parameters she wants on the final histogram, she may collect the data in list mode.

8.2.3 LIST MODE

In list mode, the flow cytometer **stores all the data collected from all the sensors**. List mode allows the operator to collect data about a population without deciding how the data collected will ultimately be displayed. Once data collection is complete, the operator reviews the data with software that allows the display of any combination of sensor data as a histogram. Changing instructions to generate new histograms from list mode data is termed **reprocessing**.

The **list mode** function is a helpful method of data storage when convenience and versatility of analysis are important factors. However, list mode requires a relatively **large amount of memory and storage capability** because data is recorded on a per cell basis rather than a histogram basis. When disk space is not a problem, list mode gives the operator a method of collecting and storing a large amount of data about a population. When disk space is scarce, gating reduces the size of data for storage.





(B)

FIGURE 8.1: Single (A) and dual parameter (B) histograms.

8.2.4 GATING

Software gating manages data during collection by making data collection in one parameter dependent upon data collection in another parameter. **Gating is acquisition of one parameter data based on the cell fulfilling certain requirements of a second parameter**. During gating, the computer stores only data common to both parameters as a histogram. Gating thus reduces the amount of data collected by eliminating data points that are not common to both parameters. Gating can also remove a section of unwanted data in a selected region of channels.

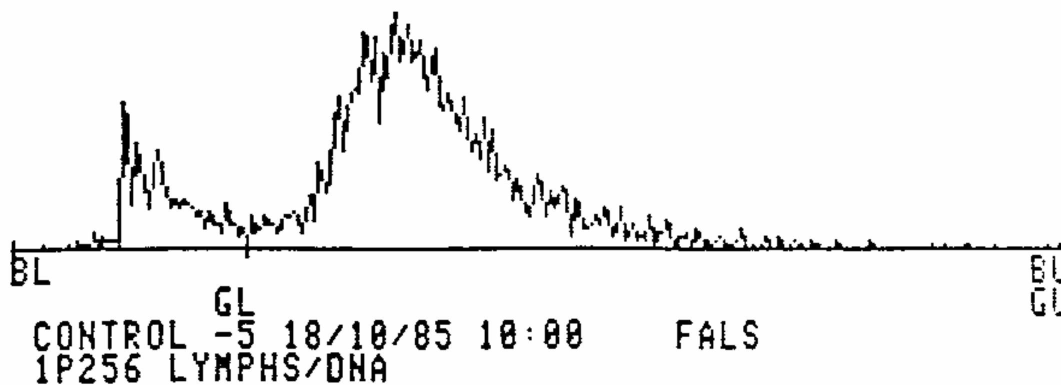
If an operator gates red fluorescence on forward angle light scatter, **only those events common to both data sets will appear on the histogram**. In this case, only the cells causing a forward angle light scatter signal that also have a red fluorescence signal are plotted on the histogram. This gating operation would not plot debris that causes a red fluorescence signal but is not large enough to cause a forward angle light scatter signal. Similarly, particles in the interrogation point that cause a forward angle light scatter signal but not a red fluorescence signal will not appear on the histogram.

Real time gating occurs during data collection and results in a gated histogram of the two parameters. The same histogram can be generated from list mode data through reprocessing. The decision to gate in real time or after the collection of data in list mode depends on the application. However, the collection of data in list mode provides a wide variety of options to the operator and excess data can always be erased. The decision not to collect list mode data means that the data points that are not common to both parameters are not recorded and cannot be reprocessed at a later date. Errors that appear in list mode data may not be obvious in gated data collections.

When debris exists in a sample, it usually appears in channels one through fifty of the univariate forward angle light scatter histogram. Gating red fluorescence acquisition on forward angle light scatter will yield a bivariate light scatter and fluorescence histogram that does not display the fluorescence associated with the debris in channel one through fifty. The forward angle light scatter gated fluorescence histogram does not include debris because gated histograms include only data that are common to both data sets, and the **debris does not cause a forward angle light scatter signal**. Gates can also be placed on univariate histograms to remove the data in a specific range of channel numbers. See Figure 8.2 for examples of gated histograms.

8.3 DATA ANALYSIS

Univariate data collection is the collection of data for a single parameter. The histogram representing univariate data consists of two axes, frequency on the vertical axis and channel number on the horizontal axis. DNA or immunofluorescence histograms are good examples of univariate data. These applications plot fluorescence intensity (as a channel number) on the x axis, versus number of events (cells) on the y axis.



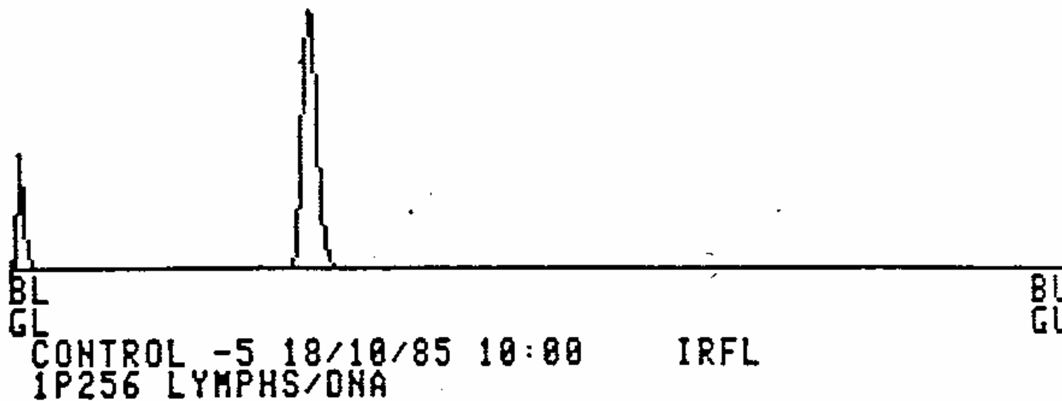


FIGURE 8.2: Gating in data acquisition. (A) forward angle light scatter histogram with debris. Note "GL" mark is lower gate. (B) Integrated red fluorescence histogram with debris gated out. Cells that do not fall above the "GL" gate will not appear on the fluorescence histogram no matter where they would appear on the un-gated fluorescence histogram. Note that the area on far left of histogram A represents discrimination against noise.

In contrast, **multivariate data represents multiple parameters**, such as light scatter (x axis) versus fluorescence (z axis) versus number of cells (y axis). Multivariate data are plotted on **multidimensional histograms**.

Parametric data analysis describes the histogram in terms of a mathematical model. Often, the goal of parametric analysis is to determine the area under a complex curve on the histogram. Parametric data analysis may use the sums of a series of Gaussian curves or Gaussian curves added to other expressions to determine this area, and thus, the properties of the population. Parametric methods of data analysis are computer time and memory intensive, so they are usually applied to only to univariate data.

Nonparametric data analysis avoids complex mathematical models, relying on rectangles, or reflected sections to determine population properties. Nonparametric methods may be as simple as visual inspection of the histogram, and are useful in the analysis of univariate and bivariate histograms. A common method of nonparametric analysis is the coefficient of variation.

The percent **coefficient of variation (%CV)** measurement is defined as the constant 0.425 times the standard deviation divided by the mean times 100. It is difficult to graphically determine the standard of deviation, so the "full width, half maximum" (FWHM) of the peak replaces the standard deviation in the measurement. FWHM is the number of channels in the peak at one half of the peak height.

$$\%CV = 0.425 \frac{FWHM}{mean} \times 100$$

The coefficient of variation can determine how well a flow cytometer is performing. In a properly tuned flow cytometer, **analysis of very uniform microspheres should yield distributions with very low % CV**. In nonparametric data analysis of DNA content, the CV is a measurement of the degree of variation of the DNA content in the population.

8.4 DNA ANALYSIS

An important use of the flow cytometer is the determination of the DNA content of neoplastic cells. Many neoplastic cell populations have abnormal amounts of DNA, a finding that may have prognostic significance. The flow cytometer is capable of very accurately **determining the amount of DNA present in cells**, and the variation of DNA content within a population.

Analysis of DNA distributions requires the generalization of the population into a biological model. The model of **Howard and Pelc** is suitable for this purpose. This model divides the cell cycle into five sections: G0 with constant 2n resting cells, G1 with constant 2n DNA content, S with a continuum of 2n to 4n DNA content due to DNA replication, G2 with constant 4n DNA content, and M where mitosis changes DNA content from 4n to 2n. See Figure 8.3.

The Howard and Pelc model of the cell cycle **allows us to assign peaks on the histogram to sections of the cell cycle**. Once correlated with the sections of the cell cycle, nonparametric and parametric analysis of the histogram determines what proportion of the population is participating in any given section of the cell cycle at the time the data was collected.

8.4.1 NONPARAMETRIC DNA ANALYSIS

Nonparametric methods of DNA content data analysis include the **rectangle method and the peak reflect method**. Both methods rely on a low coefficient of variation and have known error characteristics. Parametric methods of analysis are more accurate but require more computer time than non-parametric methods. See Figure 8.4 for examples of the rectangle and peak reflect methods of data analysis.

The **rectangle method** determines the relative frequencies of the members of the population undergoing DNA synthesis by placing a rectangle over the S phase cells. Figure 8.4 (A) illustrates the rectangle method. The rectangle method consistently overestimates the number of cells in the G1 and G2M peaks while underestimating the S phase population.

The **peak reflect method** (Figure 8.4 B) determines the number of cells with 2n or 4n DNA content by measuring the G1 and G2M peaks in the area where they do not overlap with the S phase. The area of the half of the peak that does not overlap is determined and multiplied by two to determine the area of the whole peak.

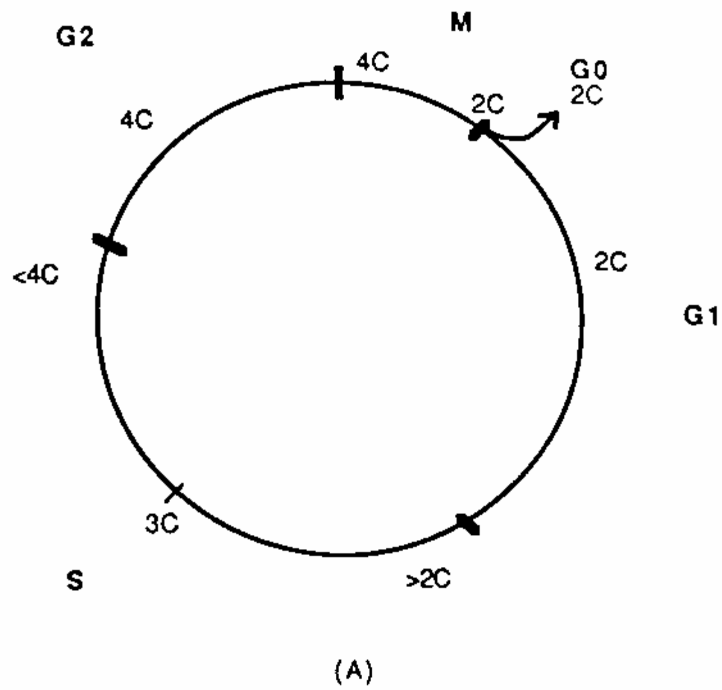
Once the areas of the G1 and G2M peaks are determined, the S phase population is determined by subtraction. The peak reflect method overestimates the G1 and G2M phases and underestimates the S phase but is quite accurate for the determination of G1 populations where the S phase is small.

8.4.2 PARAMETRIC DNA ANALYSIS

Parametric methods of DNA analysis include **broadened polynomial, broadened polynomial plus Gaussian, sum of Gaussians, and sums of broadened rectangles**. Each of these methods uses a mathematical model to determine the area under the curves in a DNA distribution.

The **broadened polynomial method is the sum of two Gaussian functions** (describing the G1 and G2M peaks) and a polynomial that describes the S phase. The sum of Gaussians method utilizes the sum of many Gaussian curves to describe the DNA histogram. Figure 8.5 illustrates the parametric methods of broadened polynomial and sum of Gaussians.

Parametric methods are used when the sample population is asynchronous or perturbed, when there are anomalies in data, when a high proportion of the population is in the S phase, or when CV is large. Parametric data analysis relies on the estimation of small portions of the histogram and the subsequent summing of those estimates to determine the population in each phase.



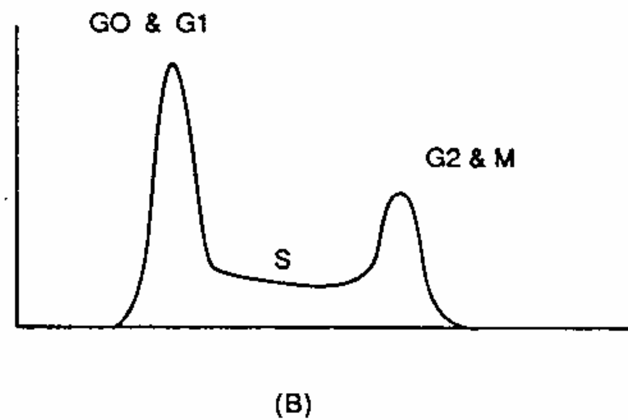
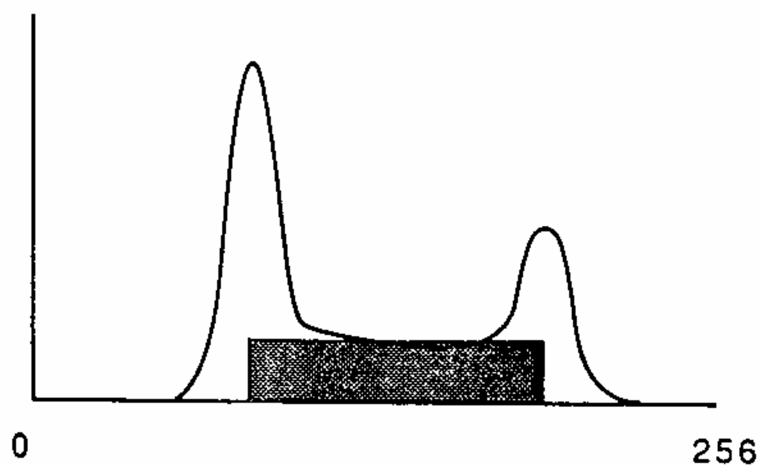


FIGURE 8.3: Howard and Pelc model of the cell cycle. (A) The cell cycle. G1 represents constant DNA content at 2n, S represents a continuum of DNA content from 2n to 4n due to DNA replication, G2 represents constant 4n, and M represents mitosis where DNA content changes from 4n to 2n. (B) Appearance of cell cycle components on DNA histogram.



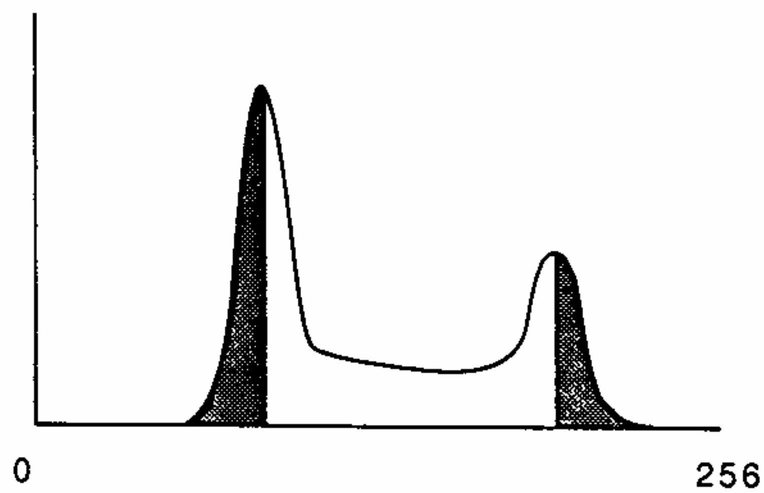
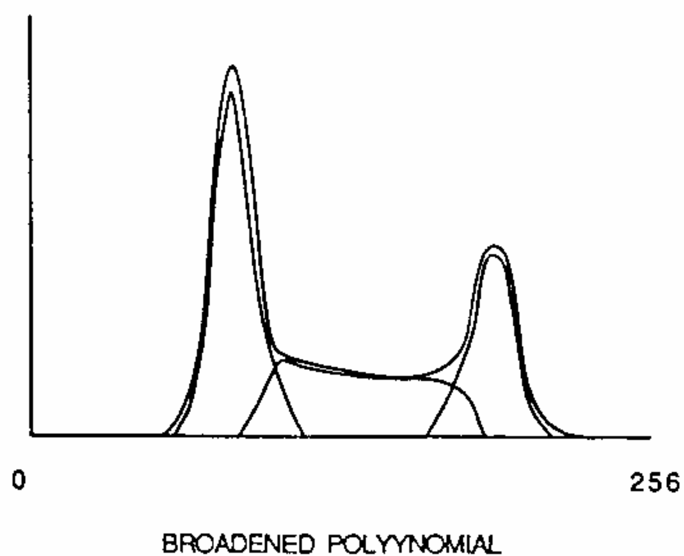


FIGURE 8.4: Nonparametric data analysis. The rectangle (A) and peak reflect (B) methods are shown.



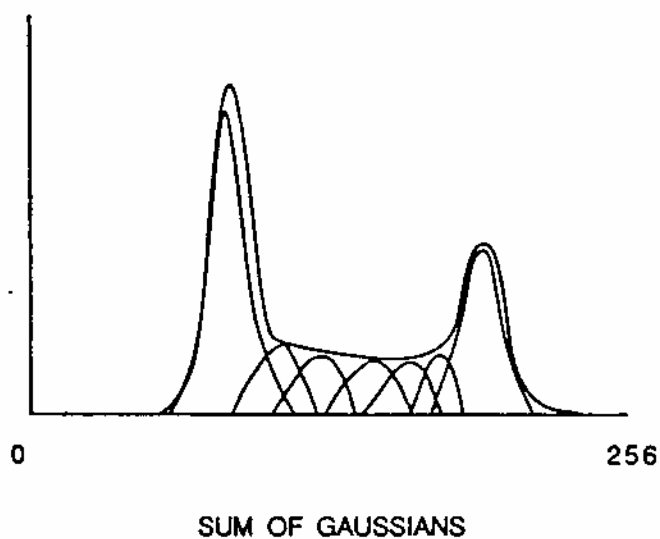


FIGURE 8.5: Methods of parametric data analysis. The broadened polynomial method (A) represents the G1 and G2M peaks with Gaussian curves and the S region with a polynomial. The sum of Gaussians method (B) represents the DNA distribution curve as a sum of many Gaussian functions.

8.5 METHODS OF IMMUNOFLUORESCENCE ANALYSIS

Immunofluorescence analysis is essential for immunological studies with the flow cytometer. Immunofluorescence refers to the use of fluorescent monoclonal antibodies that bind to specific antigenic sites. Fluorescent monoclonal antibodies are very useful in cytometry because they can be used to mark virtually any cellular characteristic.

Monoclonal antibodies are protein molecules with the ability to bind with specific biomolecules. They are produced by cells that result from the fusion of a B lymphocyte with a myeloma cell. When exposed to an antigen, the B cell produces antibodies to that antigen. Virtually any antigen may be presented to the fused B cells, so antibodies for a wide variety of biomolecules can be produced.

When **fluorescent molecules are bound to the antibodies**, their presence can be detected by their fluorescence emissions. These molecular probes allow cytometrists to gather information on virtually any cell characteristic. Because these proteins are very small, the excitation of fluorescent molecules attached to the antibodies results in very low intensity fluorescence emission.

As with DNA analysis techniques, immunofluorescence data can be analyzed with parametric or nonparametric techniques. Immunofluorescence data are generally **amplified logarithmically** because of the low number of fluorescent markers per cell (and thus very low intensity of the fluorescence emission) and because of the larger dynamic range of the distribution of members in the population.

Logarithmically amplified immunofluorescence histograms usually have a **negative peak (low channel numbers) and a positive peak (higher channel numbers)**. The positive peak results from specific binding of the monoclonal antibody to the antigen of choice. Unfortunately, the antibodies also bind nonspecifically to similar antigens on the surfaces of cells.

For example, if a fluorescent mouse antibody to human T-cell receptor is the fluorescent probe, the cells that bind the probe will have greater fluorescence (be brighter) because of the presence of bound antibodies on the surface. But cells without the antigen will also cause nonspecific binding of the antibody. This nonspecific binding is responsible for the "negative" peak. Cytometrists use a process called **histogram subtraction** to remove the data points due to nonspecific binding from the histogram.

8.5.1 HISTOGRAM SUBTRACTION

A more accurate determination of the presence of cell surface antigens employs a control population treated with a non-relevant antibody. The **control histogram is subtracted from the immunofluorescence histogram** that is positive for the surface antigen. The resulting histogram displays only the positive peak because the negative peak has been removed. The control antibody is generally a mouse antihuman antibody. Histogram subtraction is shown in

Figure 8.6.

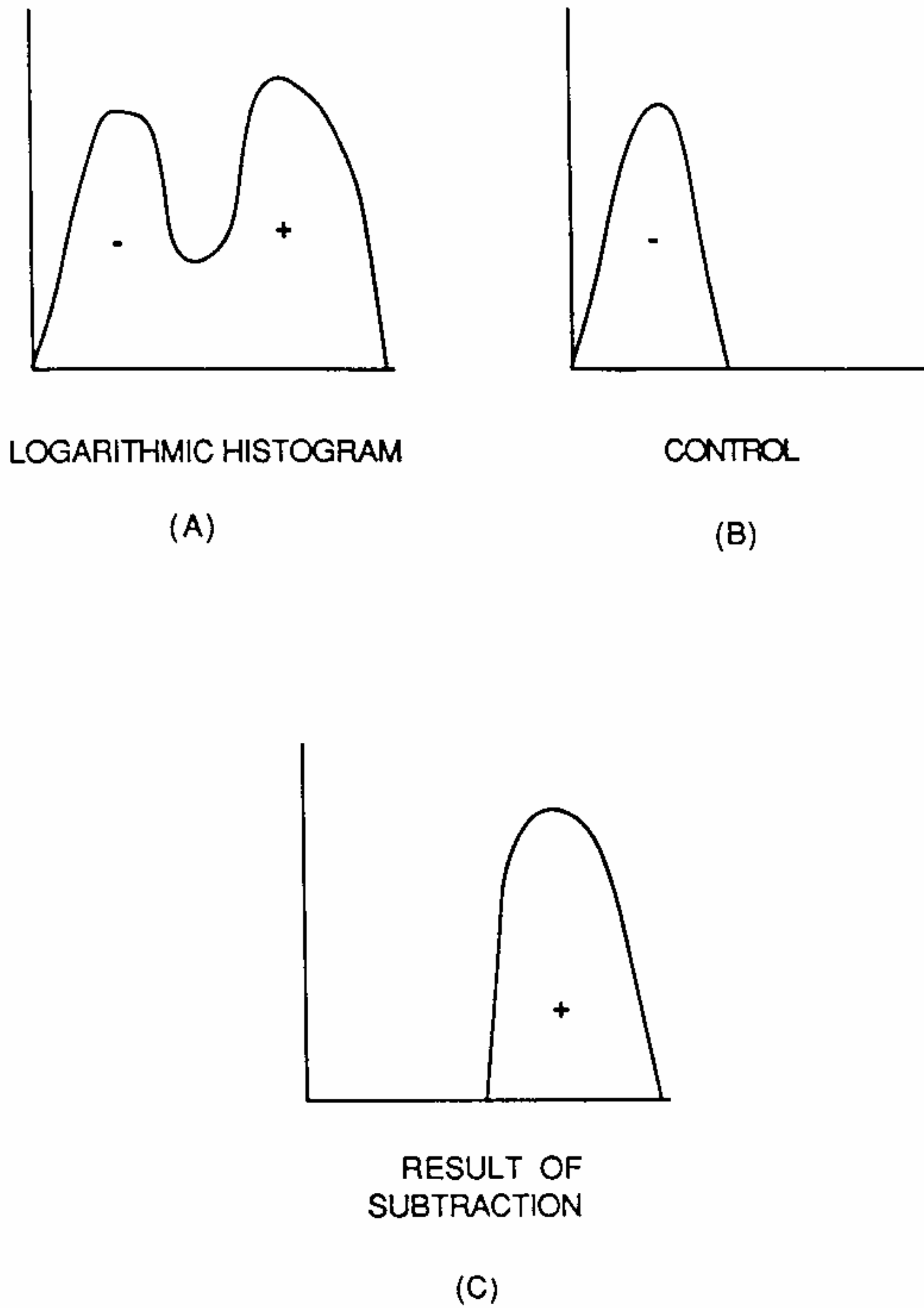


FIGURE 8.6: Immunofluorescence histogram subtraction. Logarithmically amplified immunofluorescence histogram (A). Control histogram (B). Result of subtraction (C).

8.6 MULTIVARIATE DATA ANALYSIS

Histograms with **three or more parameters** require the display of more than two dimensions. One, two, and three dimensional histograms are shown in Figure 8.7. Three parameter analysis can be displayed as two three-variable histograms or three one-variable histograms. Multivariate data analysis is much more complex than univariate or bivariate data analysis and parametric analysis of multivariate data is very time consuming.

In **one parameter histograms**, the vertical axis represents number of events (frequency) and the horizontal axis represents the output of an analog to digital converter as channel numbers. The number of channels in the histogram corresponds to the resolution of the analog to digital converter.

A **two parameter histogram** represents the data from two one dimensional histograms on a single histogram. The two parameter histogram does not contain information regarding the number of events, and like the one dimensional histogram, is displayed in only two dimensions. Only those data points common to both parameters are displayed.

A **three parameter histogram** represents the data from two one dimensional histograms including a dimension displaying the number of events. The number of events is displayed on a third axis for easier viewing. Only data points common to both parameters are displayed.

Time resolved cytometry can also be performed as a method of multivariate data analysis. In time resolved cytometry, the cytometrist collects data from a dynamic population over time. This process creates a series of histograms that represent changes in the properties of the population occurring over the passage of time.

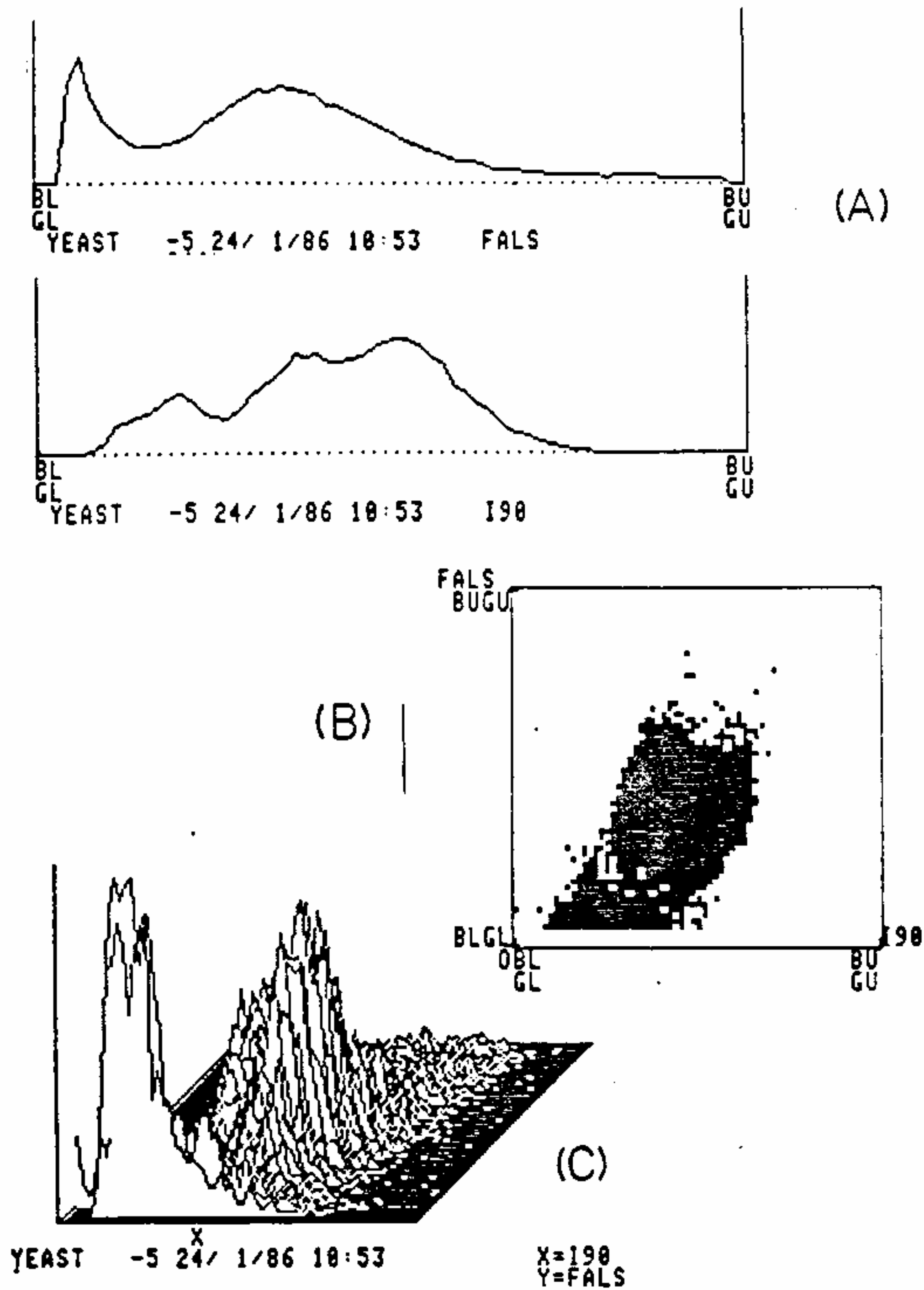


FIGURE 8.7: One, two, and three dimensional histograms. Two one parameter histograms (A) where the x axis represents events (number of cells) and the y axis represents forward angle light scatter and the integral of orthogonal light scatter. (B) A two dimensional histogram from the one parameter histograms. (C) A three-dimensional histogram of the same data where the y axis represents events.

8.7 SUMMARY

Cytometers collect quantitative data from each individual in a population and display that data on a graph of a frequency distribution called a histogram. Histograms may represent univariate, bivariate, or multivariate data. The analysis of **univariate, bivariate, or multivariate** data occurs **parametrically or** non-parametrically. **Parametric data analysis is based on a mathematical model that relates directly to the type of curve found in the histogram. In contrast,** nonparametric data analysis is capable of determining the area under any curve without regard to its shape and is based on the nonmathematical analysis of the curve.

PARAMETRIC DATA ANALYSIS
Parametric data analysis is accomplished by **summing Gaussian curves and other mathematical expressions** that fit the curve of interest. Parametric analysis of univariate histograms is performed when the CV is large, the population is perturbed, the S phase is large, or there are anomalies in the data. The advantage of parametric analysis is increased accuracy.

NONPARAMETRIC DATA ANALYSIS
Nonparametric methods of analysis include analysis of the **coefficient of variation (CV)**, **addition and subtraction of the univariate histograms, the** rectangle method, and the **peak reflect method**. Non-parametric methods may be as simple as visual examination of the histogram and are much more practical for multivariate data.

MULTIVARIATE DATA ANALYSIS
Multivariate analysis is performed as nonparametric visual inspection of three dimensional histograms. Parametric analysis of multivariate data is possible but **time consuming**.

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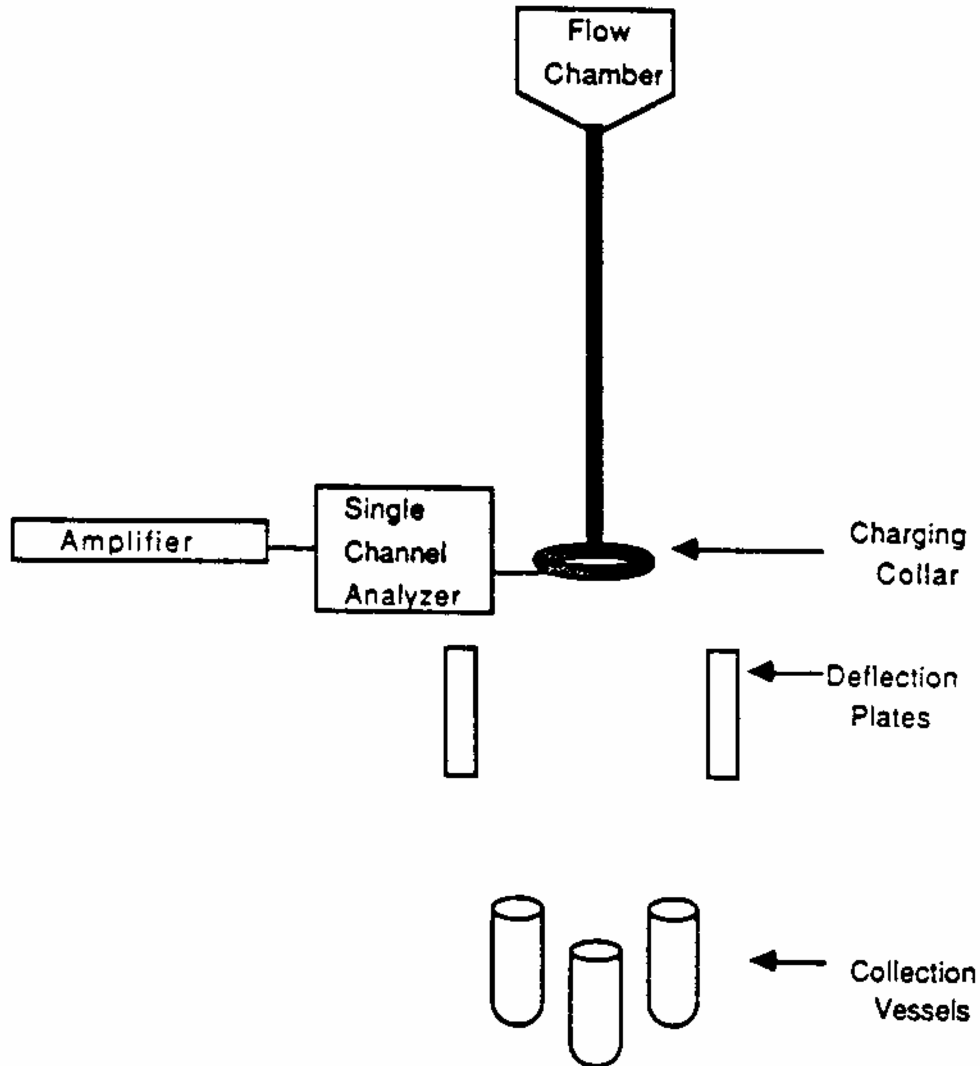
PART FOUR:
THE CELL SORTING SYSTEM



PART FOUR:
THE SORTING SYSTEM

The flow cytometer is an important tool for the analysis of populations of cells. The sensing of individual cells, storage of data sets about populations, and the display of histographic population data provides exciting information about cell populations such as tumors. Researchers and clinicians can study the tumors and other populations, recognizing properties and subpopulations with important clinical and research significance.

Another important feature of flow cytometers is their ability to **sort subpopulations of cells**. This ability allows researchers to separate subpopulations from the cell population under study and perform further analysis on those subpopulations. This important capability of the flow cytometer is dependent upon the Sorting System.

The Sorting System allows the cytometer to separate out cells with desired characteristics through the **principles of electronics, piezoelectric materials, and electromagnetism**. These principles are combined in a system, based on the principles first utilized in ink jet printing, to allow the cytometer to separate cells contained in tiny drops of liquid.

Part Four explores the theory, structure, and function of the Sorting System and the calibration, safe operation, and use of flow cytometers.

CHAPTER 9: SORTING

Biomedical research has traditionally used populations of cells for analysis. Microscopy provides images of individual cells within a population, while biochemical techniques determine the biochemical characteristics of populations. Neither of these techniques can isolate individual cells with desired characteristics for further study. Only flow cytometry is capable of identifying and separating specific, individual cells from populations.

The flow cytometer's sorting capabilities allow the **isolation of selected subpopulations of cells**. Sorters can isolate virtually any subpopulation with characteristics that can be measured with the flow cytometer. The advantages of sorting include individual isolation for cell culture, rare event selection, and selection of specific population members for light microscopy or other studies.

9.1 SORTING IN FLOW CYTOMETRY

Sorting depends on data from the analysis of a population with standard cytometric means. Analysis of the population must precede sorting to identify subpopulations, and to identify the characteristics that will be used to separate the subpopulation. The identification of cells for sorting depends upon **matching** the signals of those **cells with a preset signal** determined in the preliminary analysis. The actual separation of cells from the population occurs through the isolation of cells in droplets, imparting droplets that contain selected cells with an electric charge, and deflecting the charged droplets with an electromagnetic field.

Cell sorting techniques developed as an outgrowth of ink jet printing technology in conjunction with the development of the analytical techniques of flow cytometry. Early attempts to isolate cells by sorting utilized Coulter orifice data regarding cell size. Later, light scatter and fluorescence characteristics selected cells for sorting. Early experimenters explored several sorting systems, but the **deflected droplet system** is the method in most common use today.

9.2 DEFLECTED DROPLET SORTING

High speed single cell sorters commonly use the deflected droplet sorting system. See Figure 9.1. Deflected droplet systems use **electrostatic charges** to deflect charged droplets from the sample/sheath flow into collection vessels.

Deflected droplet sorting systems are based on the principles of **charged droplet deflection** first developed for ink jet printing. In ink jet printing, letters are formed by producing a spray of electrostatically charged ink droplets that are deflected into the shape of letters. By directing the droplets into the correct path, letters can be printed rapidly and cost effectively. In cytometry, the droplets are deflected into collection vessels instead of onto paper. In both systems, droplets are formed with piezoelectric crystals.

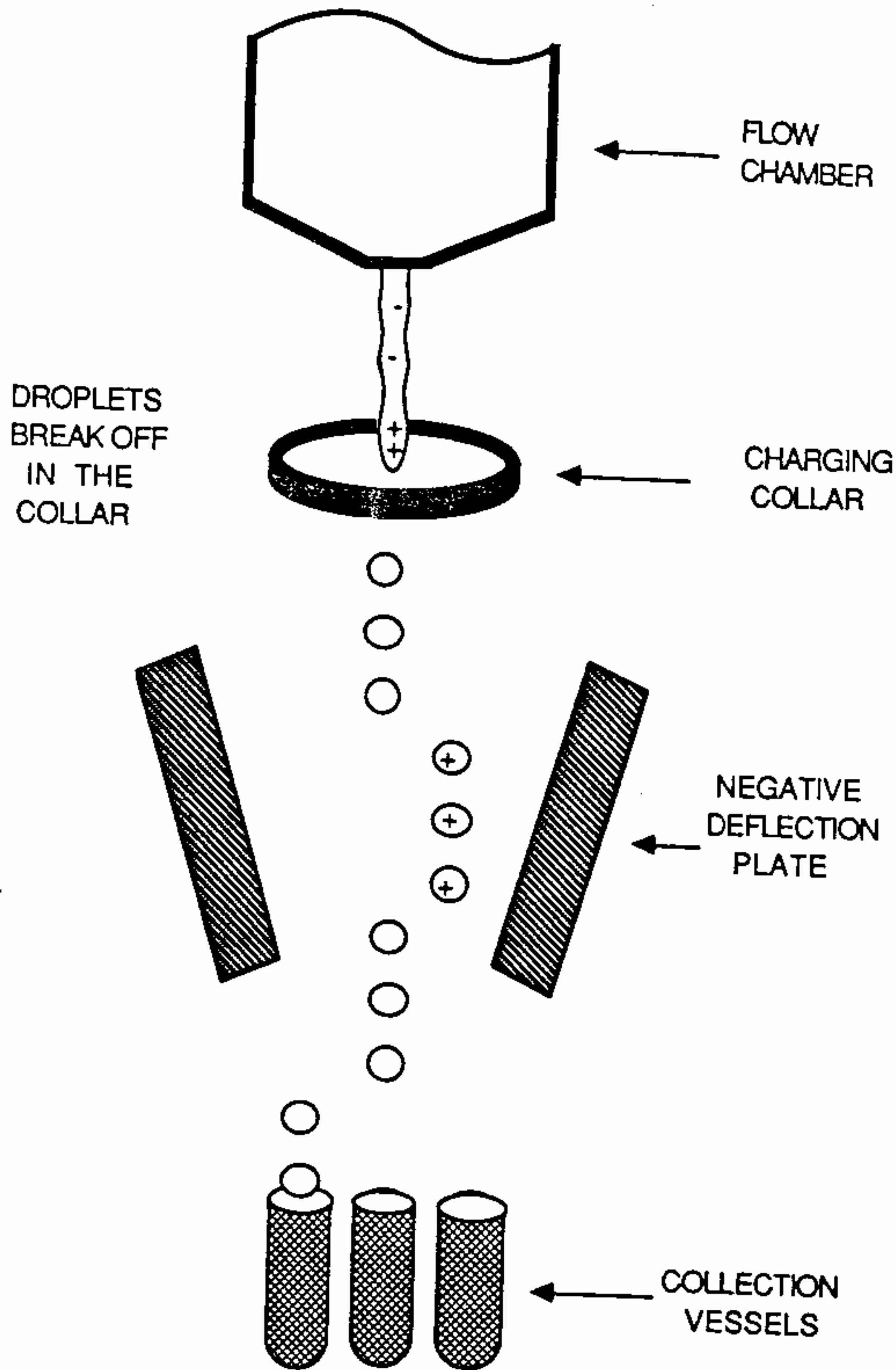


FIGURE 9.1: Deflected droplet sorting system. Note that the stream is charged as the droplet breaks off in the charging collar. Deflection plates are charged with a stable electric potential that deflects the charged droplets.

9.2.1 THE PIEZOELECTRIC EFFECT

Piezoelectric effects are due to the interaction of electrical and mechanical stress-strain variables in certain crystalline materials. When an **electric charge is applied to the crystal interface, the crystals expand or contract**. Piezoelectric materials are an important part of many devices that sense or create vibration, and are in common use as transducers, sensors, microphones, speakers, and vibrators.

In flow cytometric sorting systems, **piezoelectric crystals vibrate the flow chamber** to break the flow into droplets. An alternating current applied to a piezoelectric crystal attached to the flow chamber causes the crystal to vibrate at a frequency equal to the frequency of the alternating current. The vibration of the flow chamber causes the stream to break into droplets at the same rate as the crystal's oscillatory frequency.

The **droplet formation rate is directly proportional to the output frequency of the crystal**. If a 40 kHz crystal is attached to a flow chamber and an alternating electric current is applied, the stream emerging from the flow chamber will be broken into 40,000 droplets per second. **The size of the droplets is proportional to the size of the sample/sheath stream**.

9.2.2 DROPLET FORMATION

Cytometers with sorting systems usually employ **stream-in-air flow chambers**. Stream-in-air chambers discharge the sample/sheath flow from the flow chamber into the air where the stream passes through the interrogation point. Stream-in-cuvette flow chambers contain the flow in a cuvette and are less convenient for sorting. Both flow chamber designs can be modified to allow the stream to be broken into droplets after it leaves the interrogation point. Figure 9.2 illustrates droplet formation due to flow chamber vibration.

9.2.3 DROPLET CHARGING

To sort the droplets that contain selected cells from the other droplets, there must be some **mechanism for altering the droplet's path as they fall**. In cytometry, this is accomplished by charging the droplets as they break off of the stream.

The **charging collar** imparts an **electrostatic charge** to the end of the stream before the droplets break off. As the stream passes through the collar, the collar receives a pulse from the sorting electronics which charges the end of the stream. The stream consists of an ionic solution such as normal saline (0.9% sodium chloride), so ions of the same charge as the collar migrate up the stream. Similarly, ions of opposite charge migrate toward the collar and into the end of the stream. When the droplet breaks off, it contains more ions of charge opposite to the collar and is therefore charged. This process depends on the piezoelectric crystal, charging collar location, and correct localization of the droplet break off point. Figure 9.3 illustrates the droplet charging process.

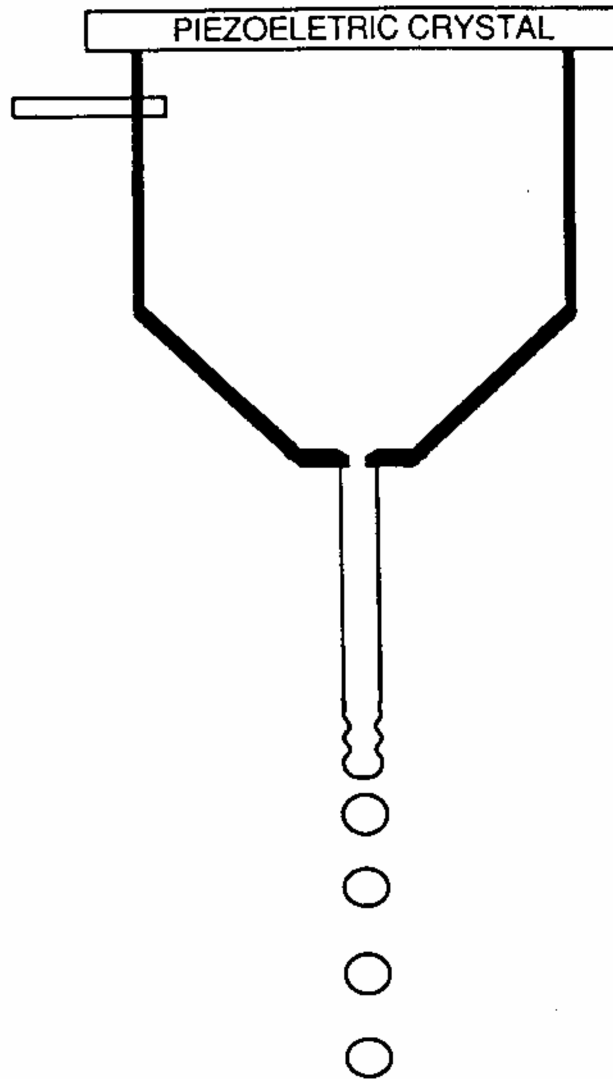


FIGURE 9.2: Droplet formation in a sorting system. If the piezoelectric crystal vibrates the chamber at a 40,000 cycles per second, droplets will be created at a rate of 40,000 per second.

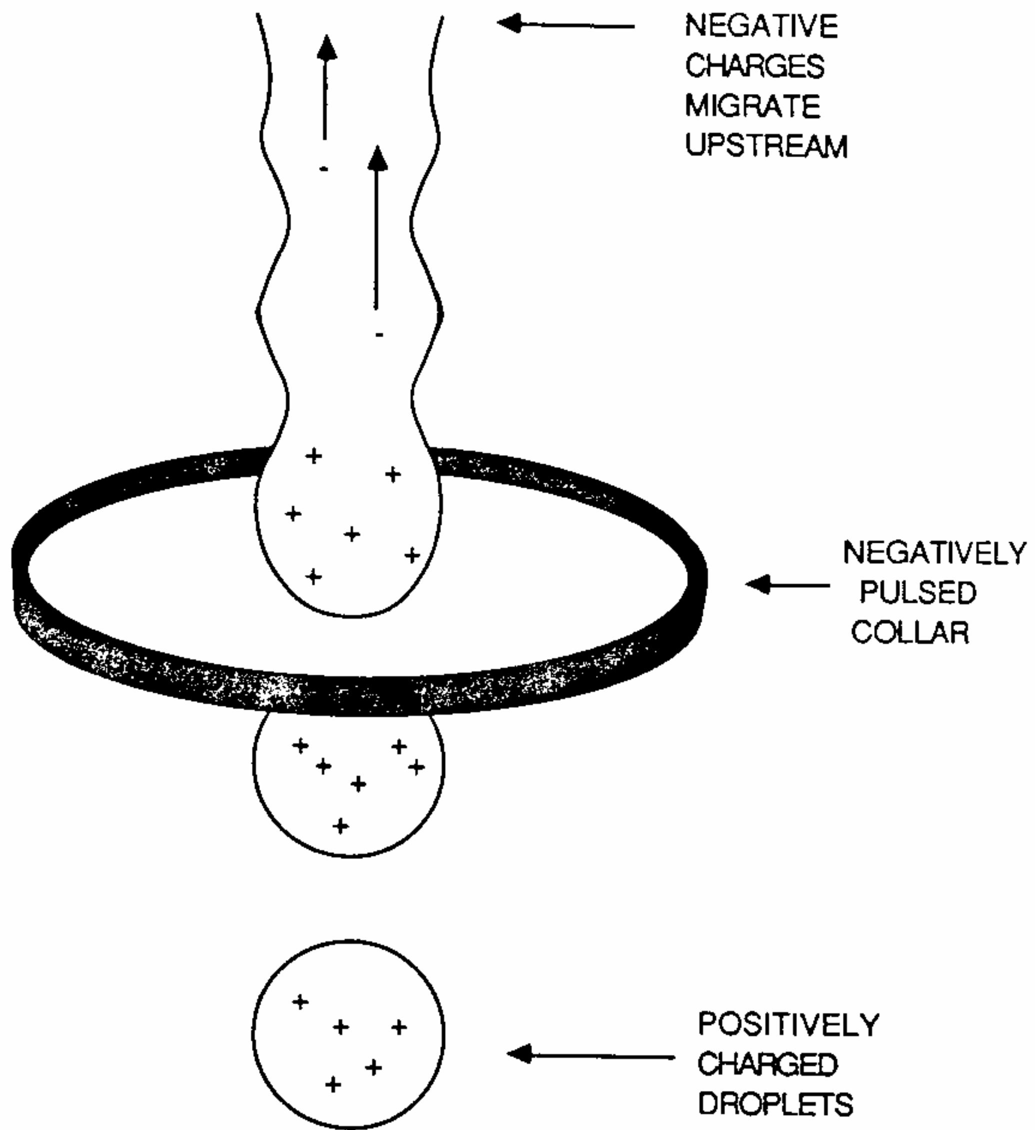


FIGURE 9.3: Droplet break off in the charging collar. Note that the droplet breaks away from the stream in the charging collar. Since the stream is charged, the droplet retains a charge when it separates from the stream. For deflection toward the negative deflection plate, the droplet is given a positive charge. For deflection toward the positive deflection plate, the droplet is given a negative charge.

Droplet break off is not caused by the electrostatic charge or the charging collar. Instead, it is a **function of the vibrating frequency of the piezoelectric crystal and the placement of the charging collar**. Droplet separation **must occur in the collar** for droplet charging. Charging of individual droplets is difficult because of timing and the requirement for a larger charging pulse. For this reason, the cytometer makes multiple droplets for each cell, and charges groups of droplets for sorting.

In most sorting applications, **only one of many droplets contains a cell**. If analysis proceeds at 1,000 cells per second and the piezoelectric crystal vibrates at 40,000 cycles per second, there is one cell in every 40 droplets. Even the fastest charging collar takes time to reach it's full potential, so most cytometers charge about three droplets at a time.

Charging of multiple droplets also has the advantage of decreasing errors in the sorting system. When three droplets are charged, the probability that the droplet containing the cell will be charged is increased. If two cells are close enough to occur in a group of three droplets, the **sorter logic aborts the process** and waits for a group of three droplets with only one cell.

9.2.4 DROPLET DEFLECTION

The charge on the droplets that have been selected for sorting is relatively small due to the small size of the ions that charge the cell. To deflect these droplets, the cytometer relies on a **high voltage electric field**.

A pair of electrodes called deflection plates deflects the charged droplets. These plates maintain a constant charge of about 2000 volts. One plate has a positive 2,000 volt charge and the other plate has a negative 2,000 volt charge. The polarity of the plates remains constant and the interaction of the droplets with the high voltage electric field generated by the plates causes sorting.

When the charged droplets pass between the deflection plates, **positively charged droplets are attracted toward the negatively charged plate** and negatively charged droplets are attracted toward the positively charged plate. Droplets that have not been charged continue in their original trajectory without deflection. The attraction of the plates causes a deflection in the trajectory of the falling droplets, and droplets selected for sorting fall into different collection vessels than the uncharged droplets. Figure 9.4 illustrates droplet deflection.

It is very difficult to change the charge on the plates faster than once every three milliseconds. The utilization of different charges on the droplets and a stable charge on the plates allows sorting systems to **sort more cells** than if the charge on the plates were changed. The droplets in the field have a low charge to mass ratio, so they must spend about three milliseconds in the field to be deflected. Because it would take three milliseconds to change the charge of the plates, cells passing between the plates during charge redistribution could not be sorted. Therefore it is faster to charge the droplets upstream and let them be deflected by a constant charge on the plates.

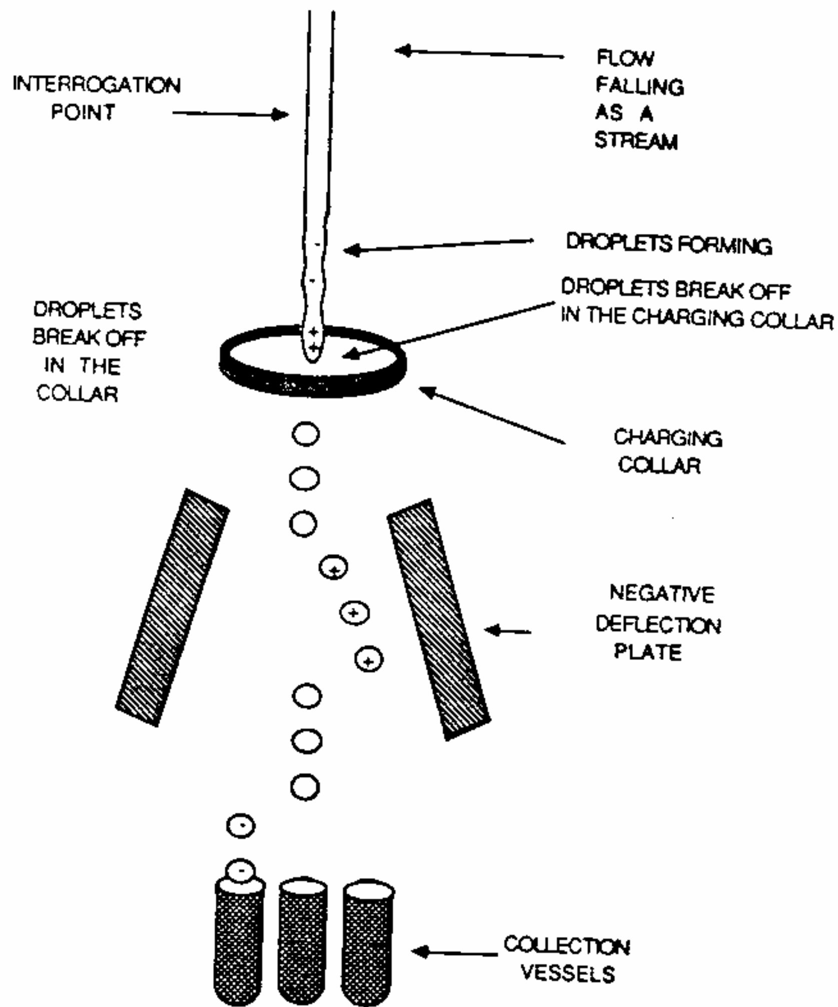


FIGURE 9.4: Droplet deflection. The droplets charged in the charging collar pass between the deflection plates. Positively charged droplets move toward the negatively charged plate, and negatively charged droplets are attracted to the positively charged plate. The charge on the plates is positive or negative 2000 volts. The plates retain constant polarity throughout the sorting process, and the attraction of charged droplets to the plates deflects their trajectory into collection vessels.

9.2.5 DROPLET COLLECTION

The most popular droplet collection systems are the **three well and ninety six well** systems. Three well systems collect cells as described above (right, left, or undeflected) while 96 well systems utilize an additional system to move a 96 well collection plate under the sorting system. The primary applications of 96 well systems are cell culture and cloning applications.

9.2.6 SORTING LOGIC

Most flow cytometer data analysis computers do not drive the sorting logic because computation times are too slow. Instead, sorting systems use a hardware device called a **single channel analyzer (SCA)**.

The operator programs the single channel analyzer with the signal characteristics of the cells desired for sorting. These signal characteristics were obtained during the analysis of the population through normal data acquisition methods. When the population passes through the interrogation point, the single channel analyzer compares the sensor output for each cell with the signal in the single channel analyzer. If the signals match, the cell is selected for sorting. This configuration allows the sorter logic to operate rapidly, precisely, and independently of the data acquisition logic.

The single channel analyzer **compares the amplified signal from each cell with the preprogrammed amplified signal obtained from previous measurements of the same population**. If the pulses match, the single channel analyzer sends a signal to the charging collar that charges a group of droplets containing the selected cell. If the cytometer's computer accomplishes this process, the decision to sort is made long after the cell has already passed through the charging collar. The use of the single channel analyzer produces a similar problem in that the sorting pulse occurs long before the droplet that contains the cell reaches the charging collar.

The **decision to sort the cell is made very rapidly** and the cell does not reach the charging collar until tens to hundreds of microseconds after the sort decision has been made. If the flow velocity is 10 m/s and the distance between the interrogation point and the dropoff point is 2.5 mm, there will be a 25 millisecond delay between the decision and the charging pulse. If the transducer frequency is 40 kHz (droplet period 25 microseconds) 1000 droplets pass through the collar while the single channel analyzer waits for the droplet it has chosen for sorting.

The inefficiency of this timing situation is resolved with the use of **shift registers**. Shift registers **store the binary charging command** until the droplet arrives at the charging collar. Shift registers increase the efficiency of sorters by allowing them to store decisions. Shift registers also allow the sorter to store decisions not to sort a specific droplet.

9.3 SUMMARY

Deflected droplet sorters are in wide use in flow cytometry. Sorting systems allow the operator to isolate cells from populations for further study and analysis. This capability allows cytometrists to identify subpopulations with interesting properties, separate them from the parent population, and study the individuals in the subpopulation.

SELECTION OF CELLS FOR SORTING
The operator selects the subpopulation for sorting from a population that has undergone previous analysis with the flow cytometer. The operator then sets a **single channel analyzer** to recognize the amplified signal of the cells in the subpopulation for sorting.

GENERATION OF DROPLETS
Droplets are generated by vibrating the flow chamber with a **piezoelectric crystal**. As the flow exits the chamber, the vibration causes the flow to break into droplets. The number of droplets formed per minute is equal to the frequency of vibration of the piezoelectric crystal.

CHARGING OF DROPLETS
Droplet charging is accomplished by charging the sample/sheath stream with a **charging collar**. The collar charges the end of the stream with a **positive or negative charge**. The charge of the collar causes ions in the flow to migrate up and down the stream. As the droplets break off in the charging collar, they contain less ions of the same charge as the collar because those ions have migrated up the stream. When the droplet breaks off within the charging collar, it **retains a charge** because the number of ions in the droplet can no longer change. **If the droplet will not be sorted, no charge is imparted to the droplet.**

DEFLECTION OF DROPLETS
Deflection plates deflect the charged droplets. The plates have a constant plus or minus 2,000 volt charge. Uncharged droplets pass through the deflection plates undeflected. Positively charged droplets move toward the negative plate and negatively charged droplets move toward the positive plate. The droplet's trajectory causes them to land in three well or ninety six well collection vessels. The three well systems are stable while the ninety six well systems are moved under the deflected streams.

ELECTRONICS OF SORTING SYSTEMS
Because **sorter logic** relies on decisions that must be made too quickly for low cost computers, sort parameters are programmed into an electronic component called a **single channel analyzer**. The single channel analyzer compares the programmed sort characteristics with the signal from the sensors and sends the appropriate **sorting pulse** to the charging collar. Because of time lag due to distance between the interrogation point and the charging collar, sorting pulses are stored in a **shift register**.

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CHAPTER 10: OPERATION OF FLOW CYTOMETERS

The operation of laboratory and research instrumentation can be a dangerous undertaking. Even the most user friendly equipment often utilizes high voltage, toxic materials, lasers, and other potentially hazardous components. These instruments present a special challenge to those who operate them, especially during emergency operations.

The operation of flow cytometers is both an art and a science. Cytometers contain or employ chemical mutagens, lasers, high voltage electrical fields, and pathogens. Operating flow cytometers in a safe and efficient manner requires attention to health and safety issues, and the ability to understand and apply the physical and chemical processes of flow cytometry.

10.1 INTRODUCTION

Flow cytometers are complex laboratory instruments that meet diverse needs in the areas of medical diagnostics and biomedical research. A number of instrument manufacturers make flow cytometers with differing capabilities and degrees of complexity. Flow cytometers have also been constructed in laboratories by dedicated experimentalists. Because of the diversity of manufacture, capability, function, and complexity of cytometers in current use, **it is difficult to define a set of instructions that will satisfy all situations**. However, all cytometer operations require attention to safety, instrument care, and calibration.

Safety includes protection of vision, prevention of exposure to pathogens, and prevention of exposure to other toxic substances. General care of flow cytometers includes care of lasers and electro-optical devices. Calibration includes setting up the cytometer for research and clinical use. Calibration procedures are different for each instrument but the need to maintain adequate records of cytometer operations is independent of cytometer type or manufacturer.

10.2 ILLUMINATION SOURCE SAFETY

A very important task in any laboratory situation is **safety**. When instrument operators are injured or equipment is damaged, the cost effectiveness of the laboratory is lost. In the cytometry lab, the major **threats to the health and safety of the operator** come from the operation of laser or arc illumination sources, and from exposure to toxic chemicals and pathogens. Flow cytometer operators can prevent injuries through a strong understanding of the dangers associated with cytometry operations. Creation and execution of emergency plans and drills minimize injuries and equipment damage when accidents do occur.

10.2.1 LASER SAFETY

Laser systems **threaten the vision** of personnel who work in the vicinity of the cytometer. Cytometer operators must remember that laser light is a form of radiation that is easily reflected both inside and outside of the lab. Operators must therefore protect themselves and prevent the inadvertent exposure of others.

Laser systems for cytometric use often have power outputs in the range of 4 or 5 Watts. Laser radiation is monochromatic, collimated, and extremely bright. There is little evidence of lasting skin tissue injury due to laser radiation, however, **5 watts of laser output will easily burn tissues**. Although difficult to quantify because of individual differences (eye color, response to light, etc.) direct exposure of the retina to **a 5 Watt beam will permanently damage vision**. Operators should routinely wear laser safety glasses when in the cytometry lab.

10.2.1.1 VISION EXAMINATIONS

One of the first steps in laser safety is the provision of regular vision examinations for all operators. These examinations establish a baseline for operator vision and monitor small changes in vision that the operator might not notice. With regular examinations, detection of vision damage due to laser radiation or other causes is routine. Without regular examinations, **loss of vision may go unnoticed** until the damage is severe.

10.2.1.2 LASER DEMONSTRATIONS

Many laser operators enjoy demonstrating the power of their laser by placing a piece of paper or other material in the path of the beam. To perform this demonstration safely, it is important that participants be warned to **avoid direct observation of the beam or the diffracted beam**. Laser safety glasses are always an important part of laser safety and help to protect vision during demonstrations. Flow cytometer operators can best protect themselves and those unfamiliar with laser radiation by always isolating the beam in a light proof area.

10.2.1.3 THE INTERLOCK AND REFLECTIVE SURFACES

Most flow cytometers have a system called an **interlock** to prevent accidental exposure to laser radiation. An interlock is a locking system that contains the radiation in a light proof area during cytometer operation. To protect the vision of the operator and others in the immediate vicinity of the flow cytometer, **do not defeat the interlock** while the laser is in use.

Operators should also avoid the use of highly reflective surfaces on, and adjacent to, the laser target areas. All personnel near the flow cytometer during operation should always **wear protective eye wear**. Changes in laboratory configuration or operational procedures should include analysis and planning for possible changes in safety precautions.

10.2.2 SAFE UTILIZATION OF ARC SOURCES

Arc sources also **emit light that is damaging to human vision**. Ultraviolet radiation and extremely bright wide spectrum radiation can damage the retina just as easily as laser radiation. As with laser sources, operators and others should not look directly into the beam or the diffracted beam, use of interlocks should be mandatory, and operators should wear protective eye wear and have regular eye examinations.

10.2.2.1 EXPLOSION HAZARD

Arc sources may explode during normal use. It is essential that laboratory personnel be **shielded** from both the UV radiation and possible explosion hazard of the arc source. This is best accomplished by housing the arc source in a light-proof, explosion containing area, and by not defeating the interlock.

10.3 ELECTRICAL HAZARD

Many cytometers can be modified extensively by the operator. Since three phase (220V) electrical power is commonly required for laser systems, **system modification without first unplugging the instrument can be a serious threat to the health of the operator**. Even small voltages can cause lethal cardiac dysrythmias or cardiac arrest.

When operators are exploring, reconfiguring, or repairing the cytometer, **ALL electrical power connections to the cytometer should be disconnected**. Some cytometers have multiple power sources that may not be obvious to the operator. The operator must carefully check each electrical source to be sure it is disconnected prior to removing cytometer panels or engaging in reconfiguration or repair. Ideally, an assistant trained in cardiopulmonary resuscitation should be present during tasks that require disassembly of any laboratory instrument. Even an assistant who can only call 911 in the event of an emergency is better than working alone.

10.4 CHEMICAL HAZARDS

Most cytometer operations utilize a wide variety of chemicals. Chemicals in use in the cytometer lab may range from large bottles of **nitrogen gas to potent mutagens and carcinogens**. Although nitrogen gas may seem relatively harmless, nitrogen gas leaks have caused fatalities.

10.4.1 NITROGEN GAS

Nitrogen is an **odorless, colorless, tasteless gas that displaces oxygen**. When a leak develops in a nitrogen system, the **nitrogen gas can completely replace the air in enclosed spaces**. Humans who enter these spaces are unaware of the lack of oxygen because they are still able to breath normally. It is very easy to be overcome and lose consciousness in such a situation without ever knowing you are in danger. Loss of consciousness in a nitrogen atmosphere can cause death within four to six minutes. These problems can be prevented by ventilating the lab or installing a nitrogen gas monitor.

10.4.2 HAZARDOUS CHEMICALS

Cytometry employs a wide variety of fluorescent chemicals to label cellular components. Many of these chemicals are hazardous. For example, fluorescent materials used to study nucleic acid content form complexes with nucleic acids and are often **potent carcinogens or mutagens**.

To protect themselves and others, cytometer operators must **be aware of the chemical and physical properties** of the chemicals they use. One of the most important aspects of working with hazardous chemicals is the **prevention of contamination** (see Section 10.6.1 below). Once the chemicals have outlived their usefulness in the laboratory, **disposal should be in accordance with local, state, and federal hazardous materials laws**.

Cytometer operators can learn a great deal about the chemical, physical, and hazardous

properties of the chemicals they use by reading the materials safety data sheets (MSDS) issued by the chemical manufacturer. MSDSs contain a wide variety of information about chemicals including physical effects on the human body, toxic dose levels, fire fighting procedures, and storage procedures. **These documents can be indispensable in an accident or emergency**. Cytometer operators should keep MSDSs for each and every chemical in use in the cytometry lab organized and ready for easy access. Federal law mandates that MSDSs for all chemicals in use in the workplace be readily available to chemical workers and emergency personnel at all times.

10.4.3 DISPOSAL OF CHEMICAL WASTES

When use of a chemical is complete or the substance is no longer active, **proper disposal of all chemical wastes is essential**. Most universities, health care providers, and other chemical consumers have specific guidelines and procedures for the disposal of chemical wastes. Often, flow cytometric chemicals qualify as hazardous materials and must be disposed of in accordance with state and federal laws. Contact the public safety office or fire department at your location for details of on site waste disposal.

Modern **hazardous waste laws** demand "cradle to grave" tracking of hazardous materials. The manufacturer of the laboratory chemicals reports the manufacture and sale of these chemicals to the government. The responsibility for these chemicals is transferred from the manufacturer to the cytometry lab (or other consumer) at the time of sale. Only the documentation of correct disposal of the chemicals by a hazardous waste facility relieves the consumer of responsibility for the chemicals.

Failure to properly dispose of hazardous chemicals can result in **criminal prosecution and environmental fines**. Usually, organizations that use chemicals have an internal chemical waste disposal system. Chemical end users need only place their waste chemicals into the system to complete their responsibility under environmental laws.

Before the internal chemical waste disposal system picks up the waste chemicals, the chemical user is usually asked to complete a **manifest** that lists the chemicals, their concentrations, and other information about the waste. Then the wastes are picked up and transported to an on-site accumulation site where they are packed in 55 gallon drums for shipment. The drums are then released to a chemical disposal company that sends them to a **central receiving area** where they are opened and sorted. The hazardous chemicals are **repackaged in drums with similar chemicals and sent to a hazardous waste repository**. Usually, these are relatively remote properties where the drums are stacked and protected from environmental conditions that would cause them to fail.

10.4.3.1 HAZARDOUS MATERIAL RELEASES

Protocols regarding contamination should also contain **procedures for the cleanup of hazardous material or pathogen releases** (spills). Workers should be aware of emergency and cleanup procedures including who to call to report a spill, methods of cleaning up the spill, the location of equipment to clean up the spill, and when to leave a spill for professionals such as a fire department hazardous materials unit. Accidents happen every day, and it is much easier to clean up a large release from scratch than to clean up after a release and an ineffective attempt to clean it up. In addition, chemical releases often create situations that can be life threatening to individuals without protective clothing.

10.5 BIOLOGICAL HAZARDS

In addition to the hazards generated by hardware components, gasses, and flow cytometric chemicals, hazards exist in the **biological particles** studied with the flow cytometer. Contamination of laboratory personnel can occur through ingestion, inhalation, or skin exposure. The most notable pathogen under study with cytometry is the Human Immunodeficiency Virus (HIV). Blood products in the cytometry lab are often contaminated with HIV and other dangerous pathogens such as hepatitis, and other bacterial and viral pathogens.

10.5.1 CONTAMINATION

Contamination may occur with pathogens or hazardous materials. Cytometer **operators have a responsibility to protect themselves** from contamination **as well as those who enter the cytometer area**. Individuals without an understanding of flow cytometry may not be aware of the presence of pathogens and hazardous chemicals. These individuals could easily contaminate themselves, the lab, and the operator without ever knowing they had become contaminated.

10.5.1.1 MECHANISMS OF CONTAMINATION

Because of the many variations in the configurations of flow cytometers, it is impossible to suggest protocols that would fit each situation. However, an understanding of the basic **principles of pathogen containment** are a valuable tool in the prevention of contamination.

Contamination occurs through **touch, air currents, gravity, and capillary action**. Policies for the prevention of the contamination of flow cytometer operators and others must address each of these mechanisms. The inadvertent exposure of visitors, maintenance, and janitorial personnel by these mechanisms should also be considered when devising plans to protect workers.

Although these actions primarily apply to pathogens, hazardous chemicals can also be transferred by these mechanisms. To prevent inadvertent contamination of visitors to the lab, **thoroughly mark all areas where pathogens or hazardous materials are present** with the proper international identification symbols. Individuals with a strong understanding of the pathogenic or hazardous materials should clean contaminated areas. If janitorial personnel perform cleaning tasks in contaminated areas, they must be informed and educated about the pathogens and hazardous materials in the areas that they clean.

10.5.2 PREVENTION OF CONTAMINATION

The most basic step that can be taken to prevent contamination by pathogenic or hazardous materials is to create a **flow cytometer protocol**. The protocol should clearly define the hazards associated with cytometer operations, and detail a sequence of actions for each aspect of cytometer use. Without a clear definition of the actions to be taken, attempts at contamination prevention may be haphazard and inconsistent. Workers should take instruction in the procedures they must follow in the laboratory as well as the **rationale** for those procedures. Regular review and update of laboratory procedures is essential.

10.5.2.1 GENERATION OF AEROSOLS

Stream-in-air systems may produce pathogen-containing aerosols. Sorting also produces aerosols that may contaminate workers and laboratory spaces. The creation of aerosols can be avoided by using stream-in-cuvette systems and by closing the interlock when sorting is in progress. Systems that are completely closed from sample input port to waste bottle are available for analyzing highly pathogenic materials.

10.5.3 DISPOSAL OF BIOLOGICAL WASTES

Disposal of biological wastes, like toxic wastes, is an important part of cytometer lab safety. State, local, and federal organizations maintain guidelines for the proper disposal of biological wastes. To **obtain a complete description of procedures in your area**, contact the public safety office at your site or the local Health Department.

10.6 CYTOMETER CARE

In addition to the expense and inconvenience of a major equipment failure, some **equipment failures can cause serious injury or death**. Poor maintenance or incorrect operation can result in serious threats to the health and safety of flow cytometry personnel. **Proper care and maintenance of the flow cytometer can prevent expensive and dangerous situations** through correct, efficient operation of the flow cytometer.

10.6.1 RUPTURE OF THE LASER CAVITY

The most serious threat to the safety and budget of cytometry laboratories is the rupture of the laser cavity. Most laser cavity ruptures are a result of insufficient cooling. **Explosion, fire, escape of superheated toxic gases, and interruption of daily activities** by firefighters, police officers, and insurance agents can ruin any flow cytometer operator's day.

To prevent cavity rupture it is essential that the laser tube remains **properly cooled** during each and every operation. Safety interlocks that prevent population inversion in the absence of coolant and detailed startup and shutdown check lists are two ways to prevent cavity rupture. However, sudden and unexpected interruption in water flow could occur at any time. This fact makes a **fire extinguisher** and **protective eye wear** essential additions to the cytometry lab.

10.6.2 CHECKLISTS

Checklists are a handy mechanism to assist humans with complex technical procedures. Checklists **provide a guide to operators** during cytometric procedures and operation. Checklists also provide a record of problems, maintenance, and other procedures for future reference.

Startup and shutdown checklists can prevent injury, damage, and frustrating interruptions in studies due to a wide variety of problems including overflowing collection vessels. Flow cytometers may require extensive and complex startup and shutdown procedures, so detailed

checklists should be readily available to the operator at all times. Operational checklists should include procedures that prevent contamination of operators and other personnel by pathogens or hazardous chemicals. Ideally, the checklists should also include telephone numbers of local emergency services and **emergency shutdown procedures in case of fire, loss of water pressure, or other unexpected disruptions.**

10.7 CALIBRATION OF FLOW CYTOMETERS

Like other laboratory instrumentation, flow cytometers require calibration. **Microspheres** of standard size and fluorescence intensities are used in the calibration of flow cytometers. Setting instrument parameters involves adjusting the interrogation point, photomultiplier tubes, and channel number while running a population of microspheres through the cytometer. Do not forget to apply any standards you may require for your analysis.

The alignment of the laser is accomplished through the use of the coefficient of variation measurement. The **coefficient of variation (CV)** is defined as the standard deviation divided by the mean channel number times the constant 0.425. See Section 8.3 for a discussion of CV. Tuning with the coefficient of variation is accomplished by adjusting the laser, detection optics, differential pressure, and sample/sheath stream in the interrogation point until the CV is lowest.

Calibration of the photomultiplier tubes involves setting them to give a desired response to the fluorescent microspheres. The high voltage of the photomultiplier tube sets the trigger amplitude of the tube. Increasing the high voltage adjustment will cause the photomultiplier tube to emit a signal in response to a lower light level. In high light applications, the photomultiplier tube is set to a low high voltage setting. The higher the high voltage setting, the more **thermionic emission** from the tube. The **gain** controls the **amplification of the signal** once the photomultiplier tube has emitted the signal.

Once the cytometer is calibrated, all **pertinent settings including laser power, flow rate, PMT high voltage, gain, and frequency should be recorded prior to data collection**. Proper documentation of illumination source, sensor, flow rate, and sorting parameters assist with repeatability of the collected data.

10.8 SUMMARY

Flow cytometers vary in capability and construction so only a discussion of general guidelines for **cytometer safety, care, and calibration** is possible.

PROTECTION OF VISION IN THE CYTOMETER LABORATORY
Vision **safety** in the cytometer laboratory involves **prevention of damage to the vision** through isolation of laser or arc radiation behind an **interlock**. Even short periods of exposure to the direct emission of a laser or arc source can permanently damage the retina. Regular vision examinations help to protect the operator's vision through the early detection of vision loss.

PREVENTION OF CONTAMINATION
Prevention of contamination involves the understanding and elimination of the **mechanisms of contamination**. Contamination can occur through **touch, gravity, air currents, and capillary motion**.

The use of an **established set of guidelines and protocols** may help to prevent contamination by defining operator actions, precautions, and waste disposal operations. Guidelines should include rationales for the precautions, and directions for handling emergency situations. Emergency drills can help to prevent or ameliorate emergencies by familiarizing personnel with emergency procedures.

Contamination by hazardous chemicals can occur by the same mechanisms as **contamination by pathogens**. Unwanted hazardous waste must be disposed of in a safe and timely manner. Failure to properly dispose of hazardous materials may result in violations of tough federal regulations and painful fines and imprisonment. Contact on site waste disposal officials, local fire departments, the local health department, or the Environmental Protection Agency for more information about proper waste disposal techniques.

CARE OF THE ILLUMINATION SOURCE
Care of the cytometer includes care of the **illumination source**. Insufficient cooling of the laser cavity can cause rupture of the laser cavity that may involve explosion, fire, and the escape of superheated gasses. Rupture and other health and safety problems can be prevented through the use of **startup and shutdown checklists**. Arc sources are also subject to explosion and must be shielded.

CALIBRATION OF THE FLOW CYTOMETER
The cytometer is **calibrated** with **fluorescent microspheres** by measuring the **coefficient of variation**. All **flow, laser, and photomultiplier tube settings should be recorded prior to data collection.**

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APPENDIX A: GLOSSARY

ABSORPTION FILTER: optical filter that removes light from a beam by absorbing unwanted wavelengths.

A.I.D.S.: (A)quired (I)mmuno (D)eficiency (S)yndrome. A usually fatal weakening of the body's immunological defense mechanism that is caused by the Human Immunodeficiency Virus (HIV).

AMPLIFICATION: increasing the amplitude of the output signals of photodetectors.

ANALOG SIGNAL: type of signal characterized by smooth, curving lines. Analog signals have a magnitude that varies over time and are analogous to the value they represent.

ANALOG TO DIGITAL CONVERTER: electronic component that converts electrical (analog) signals into digital signals.

ANODE: provides the output current signal in photomultiplier tubes when electrons from the dynodes strike it.

ARGON ION LASER: type of laser that produces coherent radiation with an electric discharge in an argon ion gas.

BAND PASS AND REJECT FILTERS: band pass filters pass all wavelengths in a certain region. Band reject filters block all wavelengths in a certain region. Band pass and band reject filters may be absorption or interference filters.

BEAM SPLITTER: optical component that divides light beams into two beams of equal wavelength.

BEAM STOP: location where the laser beam is stopped when no cells are in the interrogation point. Prevents the continuous generation of a signal from the photodiode when a cell is not in the interrogation point.

BERNOULLI, DANIEL: described the flow of liquids in tubes in the Hydrodynamica, 1733. Bernoulli showed that velocity increases and pressure decreases in regions of tubes with decreased diameter.

BLOSSOM: a collection of the sample stream at the injection point caused by high sample pressure. See Differential Pressure.

BROADENED POLYNOMIAL: parametric data analysis technique that analyzes a histogram by adding Gaussian curves and a polynomial.

CARCINOGEN: chemical known to have cancer causing activity in animals and humans. Carcinogens used in cytometry often act upon nucleic acids.

CHARGING COLLAR: collar used to charge the end of the stream and the site of droplet breakoff in sorting. As droplets break off in the charging collar they retain the charge that was present on the stream at the time of breakoff. The charge enables sorting.

CHEMILUMINESCENCE: non-incandescent (heated substances are incandescent) light emission due to a chemical process.

COEFFICIENT OF VARIATION: percent coefficient of variation equals 100 times the quantity 0.425, times the full width half maximum divided by the mean channel number.

COLLIMATED: light beam with parallel rays.

CONFOCAL: illumination optics configuration that utilizes two crossed cylindrical lenses placed so the focal points of both lenses are focused on the same spot. Creates the smallest possible Gaussian beam spot at the interrogation point.

CONTAMINATION MECHANISMS: touch, air currents, gravity, and capillary action all transfer pathogens or hazardous materials.

COULTER ORIFICE: cell sizing and counting device that measures changes in electric potential across an opening as cells pass through it.

CRITICAL ILLUMINATION: optical system that focuses the light from an arc source directly onto the interrogation point.

CROSSED CYLINDRICAL LENSES: lenses of illumination and detection optics that are intersected at 90 degree angles to focus light into an elliptical beam spot.

CURRENT REGULATE MODE: a method of laser power regulation where the current present in the line power input regulates laser output. In this mode, the laser output will vary with variations in the input current. See Light Regulate Mode.

CUTOFF WAVELENGTH: frequency at which 50% of the transmitted or rejected wavelength is blocked or transmitted by a filter.

DARK CURRENT: the property of electro-optical sensors (especially photomultiplier tubes) to generate a signal in the absence of an incoming signal. The dark current is often used as a measure of the quality of photomultiplier tubes and represents the signal the tube would emit in total darkness.

DEFLECTION PLATES: charged plates used to deflect electrostatically charged droplets in sorting.

DICHROIC MIRROR: optical component that divides a light beam into two beams with different wavelengths.

DIFFERENTIAL PRESSURE: pressure differential between sample and sheath flow. As sample pressure increases, the diameter of the sample flow increases.

DISCRIMINATION: removing an unwanted zone of data with an amplifier or analog to digital converter. Usually refers to hardware techniques as opposed to software gating.

DNA INDEX: channel number of the aneuploid population divided by the channel number of the diploid population. The DNA index is a measure of tumor cell aneuploidy.
DYE LASER: laser that may be tuned by changing the dye in the laser cavity. Dye lasers rely on gas ion lasers to achieve population inversion (pumping).

DYNODES: positively charged internal components of a photomultiplier tube. Dynodes cause amplification of the input signal because each has a slightly stronger positive charge.

EPI-ILLUMINATION: optical configuration in which data acquisition occurs on the same side of the sample as illumination.

FALS: acronym for forward angle light scatter.

FLUORESCENCE: characteristic of certain chemicals to emit light when exposed to heat, light, or radiation. The emitted light has a longer wavelength (lower energy) than the incident radiation.

FORWARD ANGLE LIGHT SCATTER (FALS): scattering of light by a particle in the sheath/sample flow. Forward angle light scatter is proportional to particle size or complexity.

FULL WIDTH HALF MAXIMUM: measure of the width of a curve at one half of its maximum height.

GAIN: controls amplification of photosensor signals. Increasing gain increases amplification of the signal.

GATING: elimination of unwanted data with software. Gating removes an unwanted zone of data directly or by making histogram output conditional on requirements of a second parameter. When one parameter is gated on another, only data common to both data sets appear on the histogram.

GAS ION LASER: a laser that generates laser output using an electric discharge in a ionized gas.

HIGH VOLTAGE: controls amplitude of optical signal required to trigger a photomultiplier tube. Increasing high voltage decreases brightness of the light signal needed to trigger photomultiplier tube. Increasing high voltage also causes nonlinear amplification of the signal.

HISTOGRAM: graphical representation of a frequency distribution.

HIV: acronym for Human Immunodeficiency Virus. See A.I.D.S..

HYDRODYNAMIC FOCUSING: fluidic manipulation of cells in a liquid flow to achieve positional certainty.

IMMUNOFLUORESCENCE: fluorescence of antibodies or antibodies attached to other materials.

INCANDESCENCE: a type of light emitted from a heated object.

INTEGRAL SIGNAL: the integral of the input voltage.

INTERCALATING DYE: fluorescent dye that fits between the base pairs of nucleic acids.

INTENSITY: a measure of energy per unit time of light. Intensity depends on the number of photons per unit time.

INTERFERENCE FILTER: filters that use dielectric waveguides to constructively interfere with wavelengths to be transmitted and destructively interfere with wavelengths to be blocked.

INTERLOCK: locking safety device to contain radiation from a laser or arc source during use of the laser. Encloses flow chamber and interrogation point in light proof area.

INTERROGATION POINT: the location where the illumination source, sample/sheath flow, and detection optics meet. Data is collected from the light scatter and fluorescence emitted when the particle is illuminated.

ISOTONIC: a liquid with osmotic pressure equal to the cells being studied.

JETTING: increased velocity of the sample flow when the flow chamber narrows.

KOHLER ILLUMINATION: arc source optical system that uses an intermediate plane and a aperture to focus light from the illumination source onto the interrogation point.

LAMINAR FLOW: non-turbulent flow.

LASER: acronym for Light Amplification by Stimulated Emission of Radiation. Laser light is coherent, monochromatic, collimated, and polarized. Laser illumination is especially well suited to cytometry because of extreme brightness and generation as a beam.

LASER CAVITY RUPTURE: expensive and dangerous laser failure caused by insufficient cooling. Explosion, escape of toxic gasses, and fire are health hazards presented by this failure.

LIGHT REGULATE MODE: laser mode where a feedback system uses laser output beam to maintain a stable laser output power in spite of variations in electrical power input.

LIST MODE: configuration for data acquisition in which all data are stored without gating. The data may be subsequently gated and interpreted at a later date. See Reprocessing.

LONG PASS FILTER: a filter that allows transmission of all wavelengths above a certain cutoff frequency.

LUMINESCENCE: a type of light emission from electrical, chemical, or biological processes.

MERCURY ARC LAMP: an illumination source that uses an electric discharge in mercury vapor to create a bright, wide spectrum light emission.

MIE SCATTERING: low angle light scattering. Type of scattering that occurs when the illuminating beam is scattered by the particle in the sheath/sample flow.

MULTI LINE OUTPUT: laser output of more than one wavelength.

MULTIVARIATE DATA: data from more than one variable.

NALS: acronym for narrow angle light scatter.

NARROW ANGLE LIGHT SCATTER: light scatter from the sample cell at angles between 0.1 and 1 degree. The light scattering is proportional to size of the cell.

NEUTRAL DENSITY FILTERS: filters that reduce the intensity of incident light.

NOISE: unwanted signals in photodetection, amplification, or data communications.

NON-CONFOCAL: optical configuration that uses crossed cylindrical lenses focused so their focal points do not coincide. Used to generate a large elliptical focal spot. See Confocal.

NON-PARAMETRIC: a method of data analysis that does not use mathematical models to describe the data.

OBSCURATION BAR: a bar that stops light diffracted from the sample/sheath stream from entering the detection optics.

OLS: acronym for orthogonal light scatter.

ORTHOGONAL: at right angles.

ORTHOGONAL LIGHT SCATTER: (OLS, 90LS, SS) light scatter measured at 90 degrees that increases with the complexity of the cell.

PARAMETRIC: a method of data analysis that utilizes mathematical models of graphs to describe data.

PEAK REFLECT METHOD: a method of nonparametric data analysis that determines the number of cells represented by peak by determining the area of one half of the peak. The area of one half of the peak is doubled and the total is considered to be the area of the entire peak.

PEAK SIGNAL: the highest voltage of the sensor output pulse.

PEC: acronym for piezoelectric crystal.

PHOTOCATHODE: component of photomultiplier tubes that emits electrons in response to incident light.

PHOTODIODE: an electro-optical sensor that uses a semiconducting positive-negative junction to make electrical signals from light incident on the diode. Photodiode output must be amplified before analog to digital conversion. Photodiodes do not require an energy source.

PHOTOMULTIPLIER TUBE: an electro-optical sensor that uses system of dynodes to amplify low level incident light signals and convert them to electrical signals. Photomultiplier tubes amplify the light input signal through the use of a series of increasingly positively charged dynodes. The dynodes cascade electrons into a stronger signal. Photomultiplier tubes require a

power source to operate.

PIEZOELECTRIC CRYSTAL: an electronic component constructed from crystalline materials without a center of symmetry. When an electric charge is applied across the crystal interface, the entire crystal vibrates at a frequency characteristic of the two molecules used.

PMT: acronym for photomultiplier tube.

POPULATION INVERSION: condition of an ionic gas when most atoms have electrons that have been excited into higher energy states.

POSITIONAL CERTAINTY: a condition that exists when cells are focused into a region where their position is known with a high degree of accuracy.

PUMPING: the creation of a population inversion in a laser tube or lasing medium.

QUANTUM EFFICIENCY: the amount of fluorescence resulting from light absorption by a phosphorescent or fluorescent molecule. If all excited electrons return to the ground state with the emission of radiation, the quantum efficiency will be 1. If some other mechanism (such as a quenching mechanism) is involved, quantum efficiency will be less than 1.

RADIANCE: brightness.

RECTANGLE METHOD: a nonparametric method of data analysis that determines the number of S phase cells by covering the area they occupy in a histogram with a rectangle and determining the area of the rectangle.

REGULATING WATTAGE POWER SOURCE: a power source for arc lamps that maintains a constant power output to produce a constant light output from the arc lamp.

SAMPLE FLOW: a stream of liquid containing the sample to be analyzed in flow cytometry. The sample flow is injected into the center of the sheath stream and is carried in the center of the sheath/sample stream with great positional certainty.

SCA: acronym for single channel analyzer.

SHEATH FLOW: flow of liquid used to transport and focus the sample flow into a single file progression of particles. The sample flow is injected into the center of the sheath flow.

SHIFT REGISTER: an electronic component that stores electronic pulses for later use.

SHORT PASS FILTERS: filters that transmit all wavelengths shorter than the cutoff frequency.

SINGLE CELL SUSPENSION: a suspension of individual cells produced by the mechanical or chemical disruption of a tissue.

SINGLE CHANNEL ANALYZER: a hardware component that compares sensor signals with preprogrammed values and outputs sorting pulses when the signal of an individual in the population matches a specific, predefined signal.

SINGLE LINE OUTPUT: laser output of a single wavelength.

SORTING: a method for the physical separation of cells in flow cytometry. Charged plates deflect charged droplets that contain cells.

SPECTRAL PURITY: a property of light composed of only one wavelength.

STATION: a sensing location in a flow cytometer.

STOKES SHIFT: wavelength displacement of the emission spectra with respect to the excitation spectra.

STREAM-IN-AIR: a flow chamber that releases its flow into the air.

STREAM-IN-CUVETTE: a flow chamber that contains the interrogation point in a cuvette.

SUCCESSIVE APPROXIMATION ANALOG TO DIGITAL CONVERTER: an electronic component that converts analog signals to digital signals by comparing the analog voltage from a sensor to the output of a digital to analog converter. The ratio of the input values divided by the output of the digital to analog converter gives a series of digital values.

SUM OF GAUSSIANS: a parametric data analysis technique that describes a peak with a series of Gaussian peaks that are added together.

THERMIONIC EMISSION: the spontaneous emission of electrons from the anode of a photomultiplier tube due to poor construction or large high voltage setting.

TRANSAMPEDANCE AMPLIFIER: an electronic component that converts current output of the photosensors to voltage output for use by the analog to digital converters.

TRANSVERSE ELECTRIC AND MAGNETIC FIELD MODES: types of laser output modes caused by diffraction and magnetic fields.

VENTURI, GIOVANNI BATISTA: Venturi showed that fluid flow will be induced in an unoccluded tube when one end of the tube is introduced into a flowing liquid. Also described the action of fluid flow in narrowing and widening tubes.

ULTRAVIOLET REGION: the region of the electromagnetic spectrum between 200 and 300 nanometers (middle ultraviolet) and 300 - 400 nanometers (near ultraviolet).

UNIVARIATE DATA: data from only one variable.

VIDEO TERMINAL: a computer terminal.

VISIBLE REGION: the region of the electromagnetic spectrum responsible for visible light. The visible region consists of the wavelengths from 400 to 700 nanometers.

WANDER: the tendency of brightest part of illumination to move about in the interior of an arc lamp.

WILKINSON-TYPE ANALOG TO DIGITAL CONVERTER: an electronic component that

converts analog signals to digital signals by comparing the analog signal to a discharging capacitor over time.

XENON ARC LAMP: an Illumination source utilizing the xenon ion as the gas surrounding an electrical discharge arc.

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