The Future of Flow Cytometry

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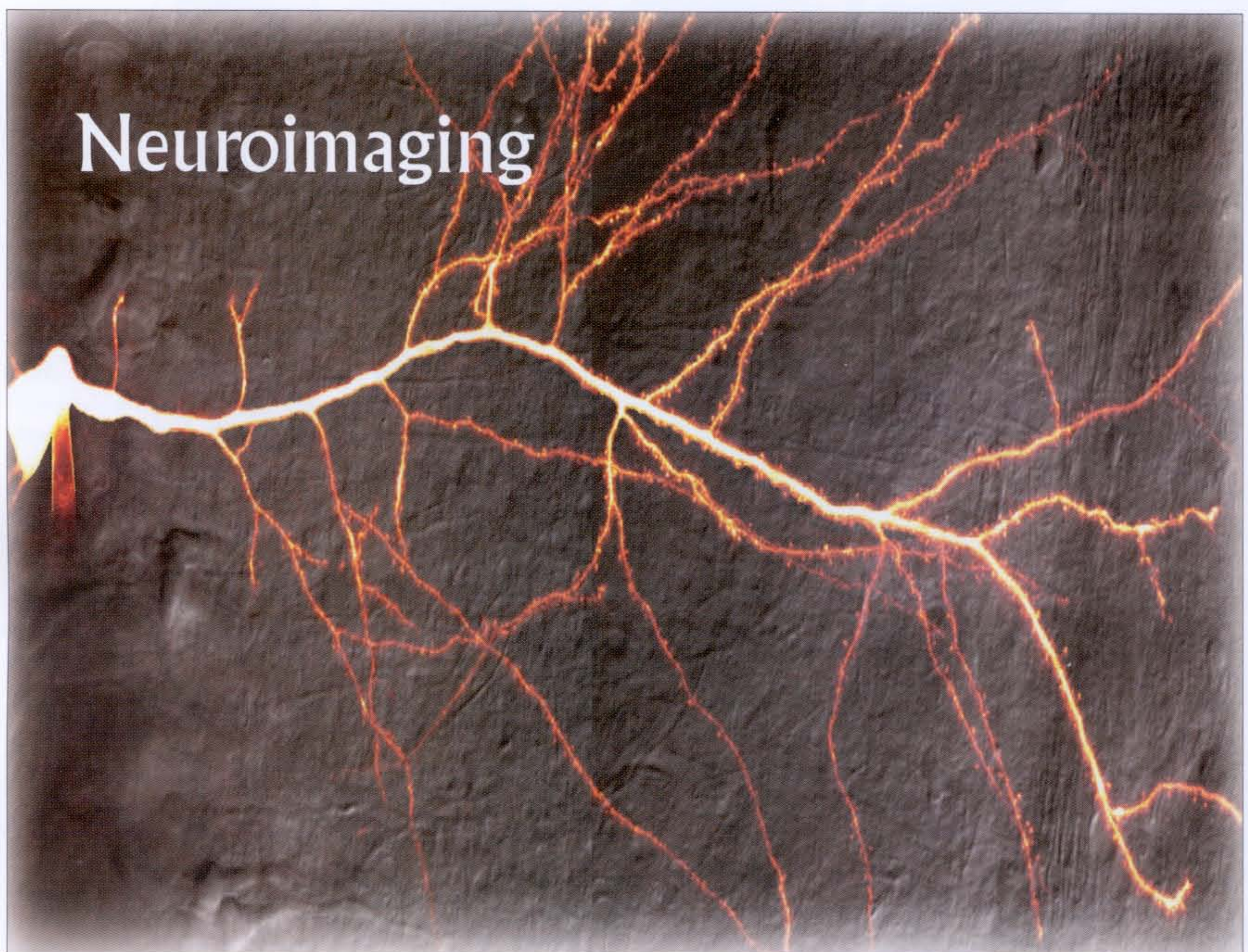


Telemedicine Reaches Out

Multispectral Cytometry: The Next Generation

by Dr. J. Paul Robinson, Purdue University Cytometry Laboratories

Developments in technology and a reawakening of interest in automated classification may make flow cytometry crucial in the next decade.

Flow cytometry, which has had a nearly 40-year track record of being the most accurate and well-defined technology for measuring properties of single cells, is poised to tackle drug discovery, which is being driven by the new field of cytomics. The study of molecular single-cell phenotypes resulting from genotype and exposure in combination with exhaustive bioinformatics knowledge extraction, cytomics demands both high-content screening and high-throughput capabilities. No other technology can analyze 3 million cells a minute with every cell receiving identical attention and carefully standardized measurement.

Although the technology might appear to be commonplace and mature, it has most certainly not reached the pinnacle of its capability. Next-generation instruments will be heavily involved in high-content screening and also will affect clinical diagnostics through the use of advanced hyperspectral analysis.

Flow cytometry quickly analyzes the optical characteristics of each particle or cell in a suspension. As the particles or cells pass through a liquid-handling system, hydrodynamic forces in the flow chamber organize them into single file (Figure 1). The line of particles/cells passes through a very narrow beam of intense laser light, and a detector collects a large number of variables such as light scatter and spectral signatures. Some of the light scatter helps to establish physical characteristics of the cells, such as size, shape or refractivity.

The flow cytometer also can collect from each cell a fluorescence signature that can be multiple bands of a subset of wavelengths, depending on the excitation sources and the nature of the fluorochromes. Multivariate software can deduce population information from these cells, so that the instrument can easily separate complex mixtures of cells into distinct electronic populations.

Separating cells

If required, more advanced flow cytometers (cell sorters) can physically separate individual cells by depositing a cell of interest into a single well or by collecting entire purified populations. This allows further biochemical, proteomic or genomic characterization of the cells.

It is otherwise difficult, if not impossible, to physically separate many types of

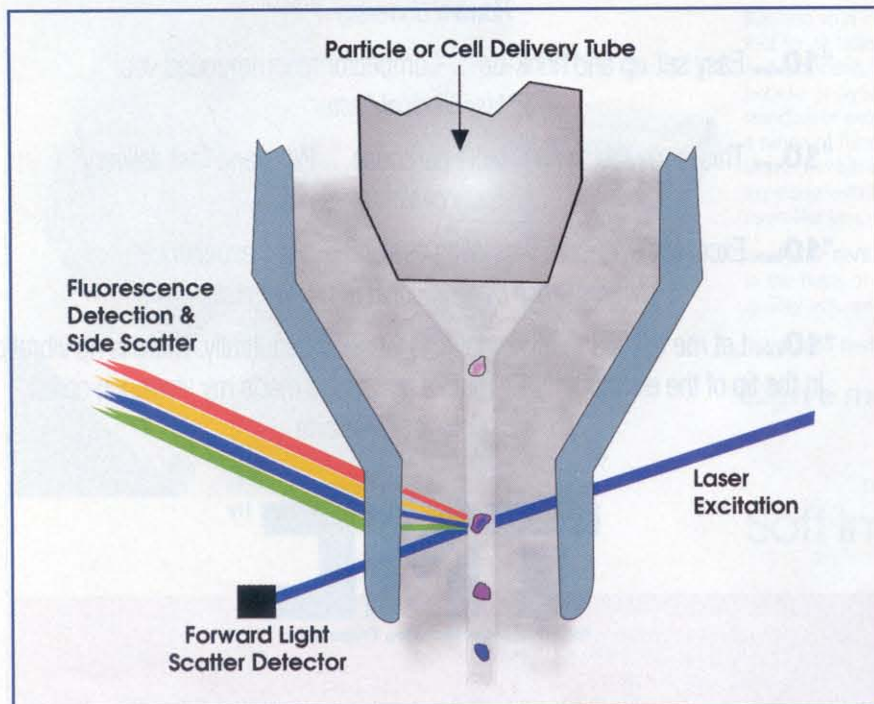
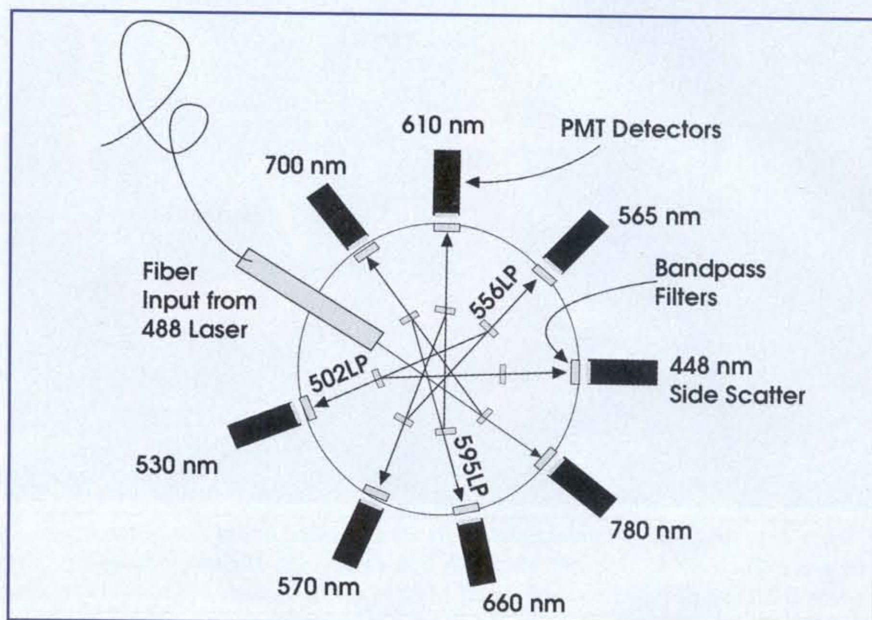


Figure 1. In a flow cytometer, hydrodynamic forces propel individual cells past a laser beam, which excites fluorochromes on the cells to emit fluorescence.

Figure 2. One of the three optical detection sets in the Aria high-speed cell sorter uses eight photomultiplier tubes (PMTs) and 16 optical filters. Dichroic filters enable maximum sensitivity, but the system is complex, expensive and difficult to modify.



cells from mixed or heterogeneous populations. Cell separation technologies such as centrifugation, chromatography and magnetic beads cannot match the very high degree of specificity that flow cytometry can achieve.

For example, most available cell separation techniques cannot separate very specific subsets of certain T cells from a blood cell population, which requires the identification of CD4-positive T cells and the exclusion of immunologically different cells with similar cytochemical characteristics. A real-time sorting decision requires simultaneous identification of a number of characteristics in a very short time (~5×10^{-5} s, or 50 µs).

Excitation and detection

Historically, cytometry technology has been driven more from the perspective of image analysis because of the interest in discovering the differences between normal and cancerous cells. Ironically, we are still attempting to make the same distinctions with subsequent generations of technology.[1,2]

The lack of substantial computational power from the 1950s to the 1970s made the analytical task difficult. Because of the initial difficulty in designing functional image-based systems, scientists such as Louis A. Kamentsky moved toward a single-cell flow-based system that was a functional predecessor of current-day instruments.[3,4] Others also had been heading in this direction, including Mack J. Fulwyler, who had observed the developments of Richard G. Sweet in his 1965 design of the high-speed ink-jet printer,[5] which heavily influenced Fulwyler's own design of the first electrostatic-based cell sorter[6] in the same year.

The basic operating principles of flow cytometers have remained almost unchanged, relying on lasers as light sources and on photomultiplier tubes as detectors for about 35 years. Initial devices could measure no more than three fluorescent probes simultaneously. In the 1990s, however, it became clear that three colors were insufficient to satisfy the demands of a developing field. By 1997, publications were describing the use of eight simultaneous colors[7]; by 2001, this had increased to 11.[8]

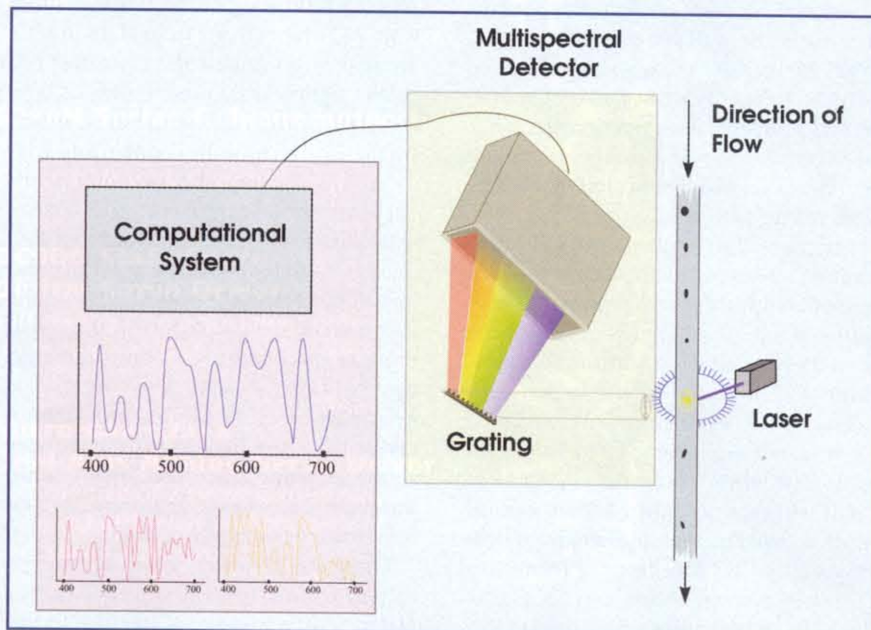


Figure 3. In a multispectral flow cytometer, a grating disperses signals from the fluorescence collector lens to a 32-channel Hamamatsu PMT. Detectors submit the data to the computational system, which determines hyperspectral curves for every particle and performs spectral unmixing.

The use of multiple colors brings up two essential problems: the need for excitation sources that allow broad-spectrum collection, and the ability to analyze the resultant complex data.

Modern instruments use three types of light sources: lasers, mercury arc lamps and LEDs. The choice of a light source is based upon optimizing the excitation

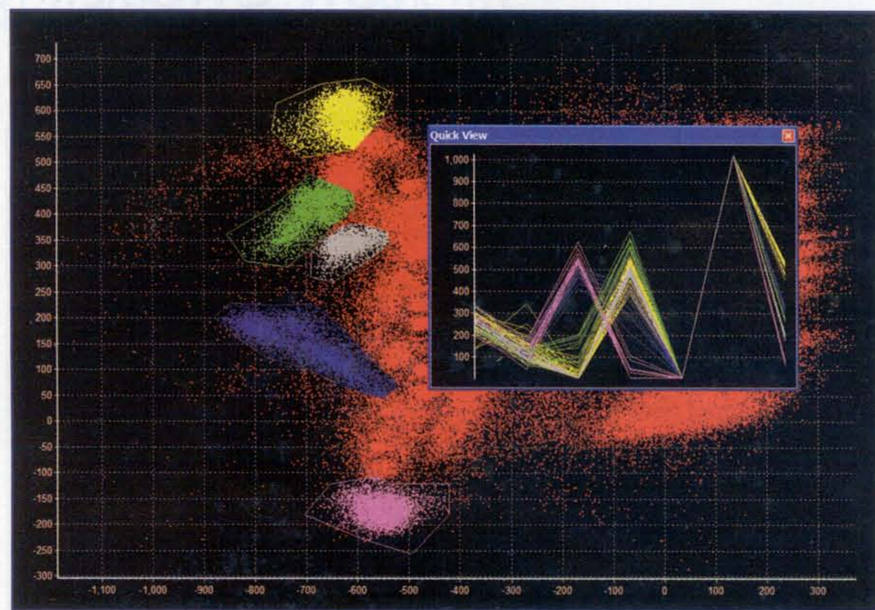


Figure 4. In this example, five populations are identified on the left of the image by color. The cells totaled 181,486, with seven variables. The inset shows the correlation events of several individual cells.

wavelength, or identifying fluorescent dyes that match the available excitation sources. With the increase in inexpensive sources of 375-, 405-, 457-, 488-, 514-, 532-, 568- and 633-nm excitation wavelengths, it has been possible to significantly expand the number of simultaneous colors that can be detected on each cell.

Photomultiplier tubes collect photons emitted by fluorescent probes as the cells or beads with these tags pass by the excitation point. They are essentially those used 35 years ago, but with smaller footprints and increased sensitivity.

Over the years, instruments expanded from one to the current 10 to 14 photomultiplier tubes.[8] Instruments with 14 of them will typically use 20 to 30 optical filters to separate the wavelengths of light (Figure 2). BD Biosciences Immunocytometry Systems of San Jose, Calif., produced the most sophisticated current system, the BD FacsAria, which can simultaneously collect 14 fluorescent bands.

Adding hyperspectral analysis

The next generation of flow cytometers will change slightly so that they can collect a vastly increased amount of data. Purdue's laboratory is working to solve two essential issues: broadening the spectral photon detection and spectral unmixing.

Our multispectral instrumentation approach (Figure 3) replaces the traditional optical filters with a dispersion grating, and the multiple photomultiplier tubes with a 32-channel one from Hamamatsu. These changes enable the instrument to collect significantly more bands of light with an optical design that is much smaller and technically simpler, and with a vastly reduced number of components.

However, the multispectral detector's wavelength sensitivity is restricted to a maximum of 650 nm. The grating is also less efficient than the optical filters in the commercial device, reducing the sensitivity of the instrument compared with the traditional design. Also, the photomultiplier tubes' 32 anodes cannot be individually controlled, so analysis requires a normalization algorithm. Finally, without spectral unmixing algorithms, the system is not particularly useful.

There are, however, some advantages in performing spectral analysis in this fashion. Although signal intensity is important in current technologies, it may be far less significant than the spectral signature itself.

Dropping the dependence on intensity may be a difficult concept for current instrument users to grasp, but it opens up new methods for classification: This technology can identify spectral signature differences that are much smaller than would be required to separate signals based on intensity, if one were restricted to a few broad bands of spectral region alone.

One advantage of this next-generation approach is that it ameliorates some of the most complex issues that we face with current technologies. For example, when we collect several overlapping bands of fluorescence, we must perform a complex compensation matrix to account for the spectral overlap (signal that leaks from one band to the next) to determine the contribution of signal to a particular band. It is not uncommon to have to subtract as much as 80 percent of a signal for one detector from another detector. This is complex and somewhat inaccurate.

Identifying cells by their spectral classification can avoid this problem. Of course, the process of spectral classification is, in itself, complex and has its own problems.

Classifying data

Even without new lasers and detectors, instruments will improve through advanced data processing. Today's instruments essentially ignore the complexity of the analysis of the data they collect.

For example, if you collect 14 simultaneous variables, how do you classify the data? Very simply — you don't. Current instruments would simply collect data in what is known as a list-mode file, a correlated data set comprising 14 values for each cell. Plotting these data is complex, requiring multiple sequential analyses that provide information about each subpopulation identified.

A tremendous opportunity exists in the world of clinical diagnostics to develop automated classification systems. Automated classification algorithms have been applied to flow cytometry data only in some cases using marine microorganisms.[9] The powerful data collection capabilities of flow cytometers have never been matched at the data-processing level.

The power of new approaches to analysis can be seen in the data from Figures 4 and 5, in which clustering has been achieved using principal component analysis. Clearly, if one has 20 to 30 variables, it is necessary to move in this direction, as the more simplistic sequential analytical solutions currently being used would fail.

If these instruments also will be used for sorting, additional complexity will constrain this already demanding technology. For the moment, however, advanced data analysis would make a significant difference in both research and clinical application of flow cytometry.

Flow cytometry, once of interest to only a small group of scientists, has moved

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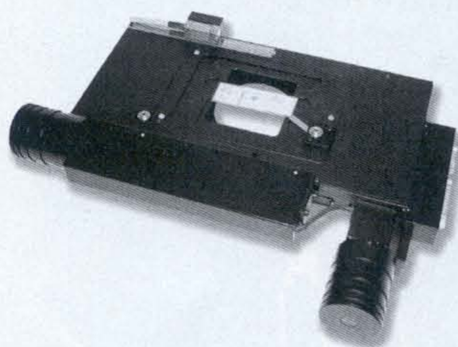

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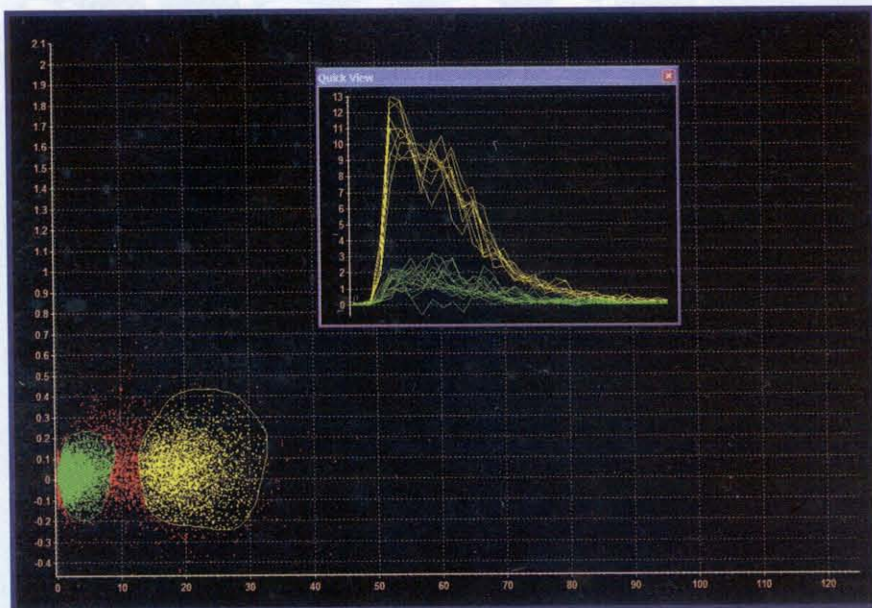

Figure 5. In this simple example of principal component analysis, two populations were run in a 32-channel flow cytometer. The signals originate from beads of two colors, shown as green and yellow regions representing principal components. The inset shows the spectral ranges of some randomly selected beads from either the yellow or green region.

well beyond the field of immunology to influence cell biology, microbiology and food science. The ability to collect high-content screening data on enormous numbers of individual cells in a very short time frame, as well as to facilitate statistical analysis of mixed populations without physical separation, is powerful. Next-generation instruments will be heavily involved in high-content screening and will affect clinical diagnostics through the use of advanced hyperspectral analysis. □

Acknowledgments

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