

# Multispectral cytometry of single bio-particles using a 32-channel detector

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## ABSTRACT

Detecting biological particles and subsequently identifying them in a very short period of time is highly desirable, but a very difficult task. There are several pathways for developing rapid detection systems. For example, one can reduce sample size to a very small volume, and amplify cellular components by PCR technology with a view to identifying antigen-specific molecules. Alternatively, antibody-based assays allow for detection and identification of a variety of well-characterized pathogens. The system we propose utilizes flow cytometry technology to rapidly detect spectral fingerprints or organisms. However, the current limit for simultaneously detectable fluorescence signals in flow cytometry is around 12-15. Making these measurements is very complex and the necessity for advanced spectral overlap calculations creates a number of difficult problems to solve in a short period of time. Next-generation instruments can either increase the number of detectors or modify the principles of collection. If the detector system were simplified, the overall cost and complexity of single-cell analytical systems might be reduced. This requires changes in both hardware and software that allow for the analysis of 30 or more spectral signals. Further, analysis of complex data sets requires some completely new approaches, particularly in the area of multispectral analysis. This presentation describes the key components and principles involved in building a next-generation instrument which can collect simultaneously 32 bands of fluorescence from a particle in less than 5 microseconds. This would allow the analysis of several thousand bioparticles per second. The flow cytometry system based on our new detector would be designed to be portable and low cost.

**Keywords:** multispectral detection, classification, flow cytometry, biological systems

## 1. INTRODUCTION

New developments in flow cytometry technology and a reawakening of interest in automated classification may rekindle flow cytometry technology as a killer application in the next decade. For nearly 40 years flow cytometry has had a track record of being the most accurate and well-defined technology for measuring properties of single cells. Nonetheless, it is poised to accept the drug discovery challenge – a challenge currently being driven by the new field of Cytomics – which demands both high-content screening and high-throughput capabilities. No other technology can analyze 3 million cells a minute with every single cell getting identical attention and carefully standardized measurements. Flow cytometry is a mature technology that is used in many laboratories, but it has most certainly not reached the pinnacle of its capability. With new lasers and detectors in the marketplace, there are clearly many opportunities for taking flow cytometry to the next level.

The earliest flow cytometers could measure only light scatter and Coulter volume, and had only a minimal ability to measure fluorescence. The technology was initially driven from the perspective of image analysis because of the interest in discovering the differences between normal and cancerous cells. These same goals are still difficult to achieve today. Although the most desirable direction in the early days of cytometry was advanced image analysis, the lack of any substantial computational power from the 1950s to 1980s made the analytical task very difficult indeed. Louis Kametsky was one of the first to pursue the flow aspect more seriously (personal communication). Because of the initial difficulty in designing functional image-based systems, scientists such as Kametsky moved toward a single-cell flow-based system that was a functional predecessor of current-day instruments<sup>1,2</sup>. Others had also been going in this direction, including Mack Fulwyler, who had observed the developments of Richard Sweet in 1963-1965 in his design of the high-speed inkjet printer<sup>3</sup>, which heavily influenced Fulwyler's own design of the first electrostatic-based cell sorter<sup>4</sup> in 1965. Further, Wolfgang Gohde<sup>5</sup> in Germany was one of the earliest inventors to develop advanced cytometric tools. Today, however, the technology of flow cytometry is quite standard and the instrument is an essential workhorse in the fields of immunology and cell biology. The real problem facing us is how do we advance to a really essential change in technology when the current operational models are so ingrained in research and clinical laboratories? The

answer is that we need to make some fundamental changes in photon-collection systems and simultaneously advance the state of multivariate data analysis to provide serious classification systems.

## 2. WHAT IS FLOW CYTOMETRY?

Flow cytometry is a technique whereby every single particle or cell in a suspension can be individually analyzed for its optical characteristics very rapidly. As the particles or cells pass through a liquid-handling system, they are organized into a single file via hydrodynamic forces within the flow chamber. Once this hydrodynamic focusing has been accomplished, the particles/cells usually pass through a very narrow beam of intense laser light, during which time a large number of variables such as light scatter and spectral signatures are collected (see Figure 1). Some of the light scatter is used to establish physical characteristics of the cells such as size, shape, or refractivity.

At the same time, a fluorescence signature can be collected from each cell; this signature can be multiple bands of a subset of wavelengths, depending on the excitation sources and the nature of the fluorochromes. By using advanced

multivariate software, population information can be readily deduced from these cells. This allows the possibility that complex mixtures of cells can be electronically separated into distinct populations with ease. Further, if required, more advanced flow cytometers called cell sorters can physically separate individual cells either by depositing a cell of interest into a single well, or by collecting entire purified populations if desired. This powerful capability makes flow cytometry a unique and indispensable technology in the world of advanced cell biology.

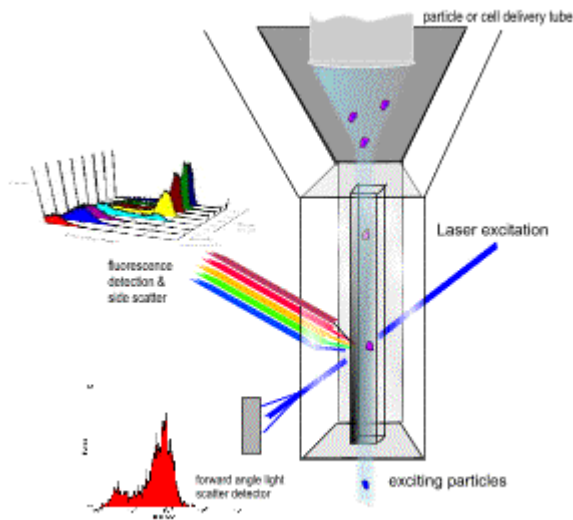


Figure 1: The flow chamber, through which suspended cells are driven through a laser beam, during which time the fluorochromes on the cells or particles are excited and emit fluorescence.

<http://www.cyto.purdue.edu/photonic>

### 2.1. Advantages of the flow cytometry approach

In the absence of flow cytometers it would be very difficult if not impossible to physically separate many types of cells from heterogeneous mixtures. Although there are many cell separation technologies, such as centrifugation, chromatography, and magnetic beads, none of these can match the very high degree of specificity in a multivariate format that flow cytometry can achieve. For example, it is not possible to separate very specific subsets of

certain T cells from a blood cell population by most available cell separation techniques, because of the complexity of the population identification. To achieve such separation, it is necessary first to identify the cells of the desired subset (for example CD4 positive T cells), and then to exclude immunologically different cells that have similar cytochemical characteristics. This requires simultaneous identification of many cellular characteristics in a very short time period ( $\sim 1-5 \times 10^{-5}$  seconds, or 10-50  $\mu$  seconds) so that a sorting decision can be made in real time. The ability to physically separate a specific cell population means that further biochemical, proteomic, or genomic characterization is then a distinct possibility<sup>6</sup>.

### 2.2. Essential problems of current-day instruments

The basic operating principles of flow cytometers have remained almost unchanged. The instruments have used lasers as light sources and photomultipliers for detectors for about 35 years. Initially, only one fluorescent probe could be measured; subsequently this increased to 2, or three colors for the most advanced users. In the 1990s, however, it became clear that three colors were not sufficient to satisfy the demands of the growing applications, particularly in immunology. A gradual move to four or five and even six colors led to a dramatic increase in separation capability, and by 1997 publications were describing the use of eight simultaneous colors<sup>7</sup>; by 2001 this had increased to 11 colors<sup>8</sup> and

more recently to seventeen colors<sup>9</sup>. The use of multiple colors brings up two essential problems: the need for good excitation sources that allow broad spectrum collection, and the ability to analyze the resultant complex data, particularly with regard to the spectral overlap that occurs with multiple fluorochromes.

### 3. COMPONENT PARTS

#### 3.1. Light sources

Lasers are the most frequent light source for virtually all flow cytometers on the market today, although some use mercury arc lamps and diodes. The key issue that drives the decision-making in this area is the optimization of excitation wavelength. From the perspective of analyzers, the driving force has been to identify fluorescent dyes to 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 as long as appropriate fluorochromes are selected. Over the past ten years, literally hundreds of new fluorescent probes have been developed to provide this needed diversity for multicolor analysis.

#### 3.2. Detectors

All flow cytometers operate in almost the same fashion as the instruments built nearly 35 years ago. Signals are collected from photomultiplier tubes (PMTs) that collect photons emitted by fluorescent probes as the cells or beads with these tags literally fly past the excitation point at between 2 and 10 m/s. Over the years, instruments gradually expanded from a single PMT to two, three, and currently 10-14 PMTs<sup>10</sup>. Many PMTs with smaller footprints and increased sensitivity are used in today's instruments. Instruments with 14 PMTs will typically have between 20 and 30 optical filters for excitation blocking and spectral separation and bandpass filters for narrow bands. The most sophisticated current system is the B-D Aria (see figure 2), which has the ability to simultaneously collect 14 fluorescent bands and more recently with modifications 17 bands.

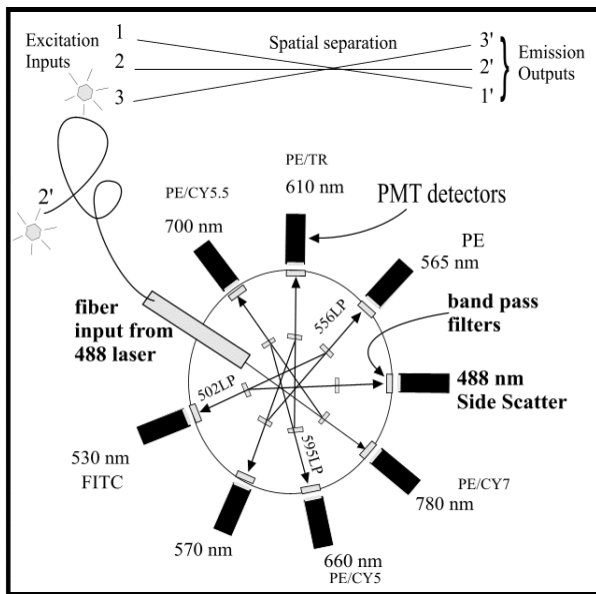


Figure 2: Example of a current advanced-level spectral detection system. This is the optical layout of one of the emission lines from the high-speed cell sorter (Aria) from Becton-Dickinson. Within this component, which is one of 3 optical detection sets, are 8 PMTs and 16 optical filters. A clever use of dichroic filters allowed the designers to gain the maximum sensitivity possible with 8 detectors. However, the system is complex, expensive, and not easily modified.

## 4. THE NEXT GENERATION OF CYTOMETERS

### 4.1. True multispectral analysis

The next generation of flow cytometers will operate on principles slightly different from those of instruments operating today. The driving force for this is the necessity of collecting a significantly increased number of variables. Two essential issues must be solved, both of which are currently under attack in our laboratory: broader spectral photon detection and spectral unmixing including classification. If these instruments are also going to drive cell sorters, an additional complexity that will constrain this already demanding technology will be the speed with which calculations can be made. This will be solved by the never-ending speed increase in computers. For the moment, however, advanced analytical capabilities would make a significant difference in both research and clinical application of flow cytometry. Comparison of the optics of **Figure 2** with that of **Figure 3** reveals a number of important differences. First, the multispectral instrument has the ability to collect more than double the number of spectral bands<sup>11</sup>. Second, the optical design is much smaller and technically simpler, with a vastly reduced number of components. While initial versions of our multispectral cytometer had a restricted wavelength sensitivity (maximum of 650 nm), more recent versions have advanced this to 880 nm, more than adequate to handle all of the

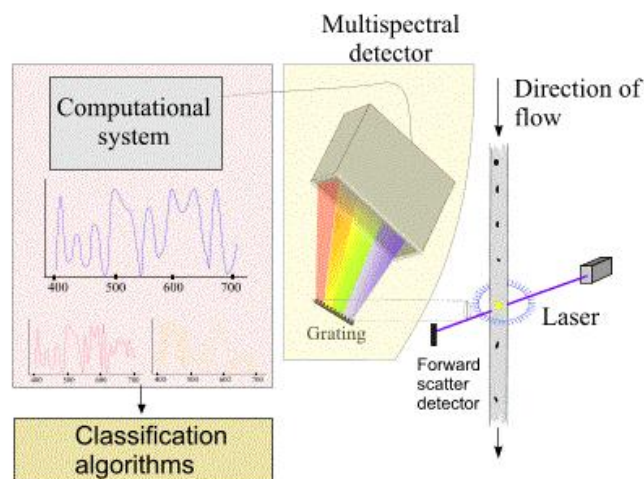


Figure 3: A simplified cartoon showing the operation of the multispectral flow cytometer. Signals piped from the fluorescence collector lens are dispersed by a grating to a 32-channel Hamamatsu PMT. Data from the detectors are submitted to the computational system for processing. Hyperspectral curves are determined for every particle and spectrally unmixed. Color version of these figures are available at <http://www.cyto.purdue.edu/photonic>

currently used probes. Third, the grating is not as efficient as optical filters, but improving the optical train more than compensates for this deficiency. Fourth, while the 32 anodes in the PMT cannot be individually controlled, it is necessary to perform a normalization algorithm on all spectral data collected. Clearly, without spectral unmixing algorithms, this technology is not particularly useful. However there are a number of advantages in performing spectral analysis in this mode. While signal intensity is very important in current technologies, it may be far less significant than the spectral signature itself. Thus, cytometry may no longer retain its dependency on intensity measurements. This is a difficult concept for current instrument users to grasp; however, the ability to move away from this paradigm opens up new methods for classification. It is possible using this technology to identify tiny differences in spectral signature which cannot be differentiated using systems that separate signals based on intensity.

### 4.2. Advanced data processing and analysis

Even if next-generation lasers and detectors were not available, there is still an important need for advanced data processing in new instruments although one can collect 14 simultaneous variables. Instruments designed today essentially ignore the complexity of the analysis of the data sets they collect. For example, when there are no well-defined solutions for data classification. Flow cytometers available today simply collect data in what is known as a listmode file. This is a correlated data set which in the above case contains 14 values for every cell. Plotting these data is a very complex task since it requires multiple sequential analyses using multiple Boolean operators that provide information about each subpopulation identified. Automated classification algorithms have rarely been applied to flow cytometry data, except for some cases using marine microorganisms<sup>12</sup>. There is a tremendous opportunity in the world

of clinical diagnostics to develop automated classification systems. The powerful data collection capabilities of flow cytometers have never been appropriately addressed at the data-processing data-analysis level. This opens up significant opportunities for advanced analytical approaches such as those described in **Figure 4** and **Figure 5**, in which clustering has been achieved using principal component analysis (PCA). Clearly, if one has 30 or more variables, current methods of data analysis will not be useful.

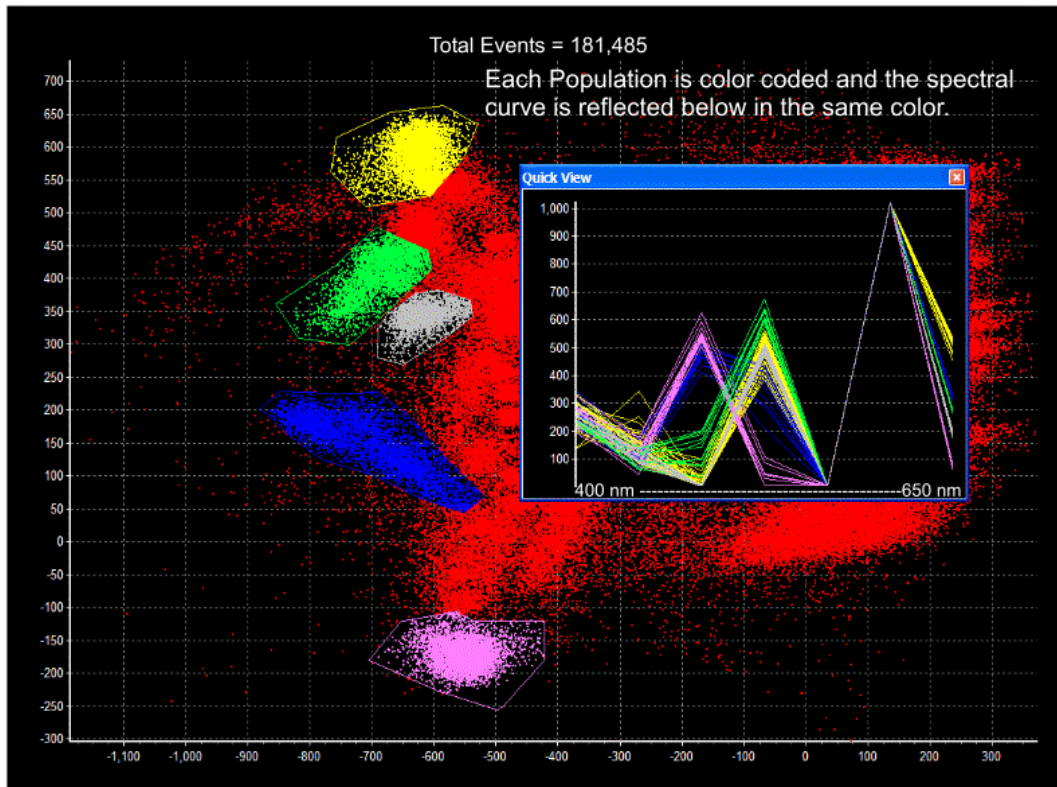


Figure 4: Principal component analysis of flow cytometry data. In this example, 5 populations are identified on the left of the image. The total number of cells analyzed was 181,486 with 7 variables. To demonstrate these characteristics in an alternative way, the inset shows what individual cells from each colored population look like when several of each are plotted as correlation events. (Color is required to see the differences in these curves. Color version of this figure is available at <http://www.cyto.purdue.edu/photonics> )

The next-generation approach has some attractive advantages. For example, one of the most complex issues facing current technologies is that of spectral compensation. When we collect several bands of fluorescence that overlap, a complex compensation matrix must be applied to compensate for the spectral overlap from signal that leaks from one band to the next. It is not uncommon to have to subtract as much as 80% of a signal for one detector from another detector. This is not only complex, but also not particularly accurate. By identifying cells by their spectral classification, we can very likely circumvent most of this issue. While the process of spectral classification is in itself a very complex process, it is one for which there are many already successful approaches in fields outside of biology.

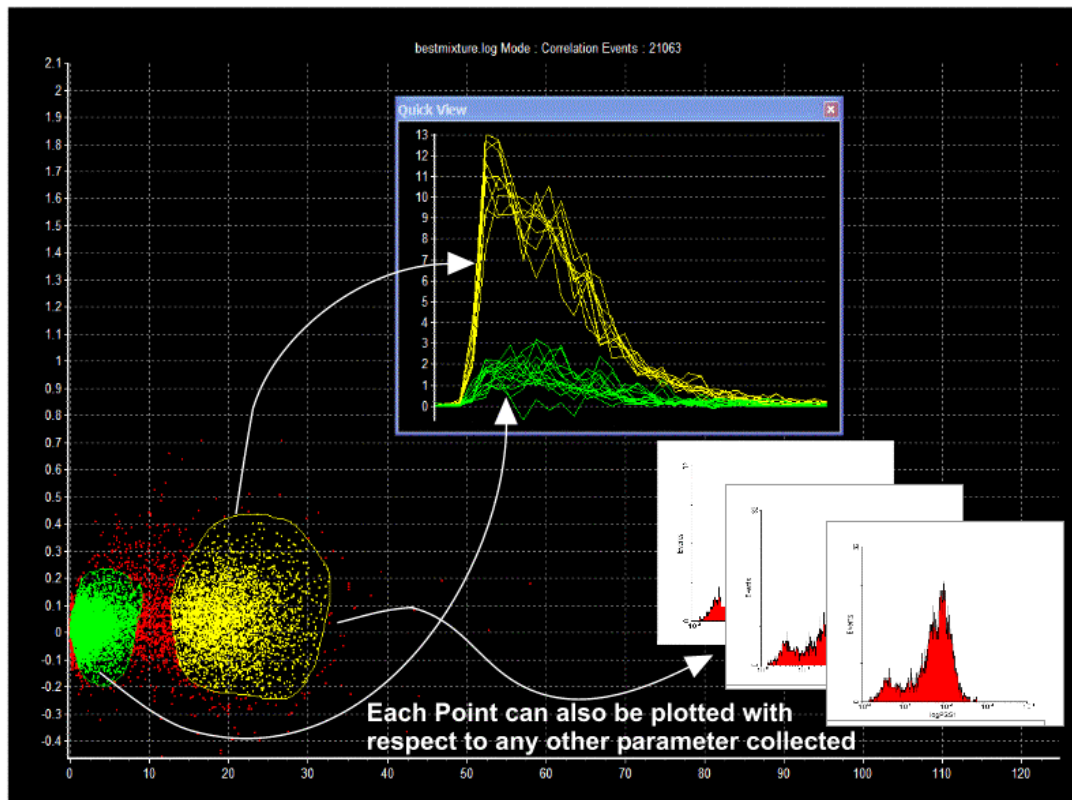


Figure 5: In this simple example of principal component analysis, two different populations were run in a flow cytometry with a 32-channel detector. The signals originate from 2 different colored beads which are shown as green and yellow regions representing principal components on the figure. The insert shows the spectral range of a dozen or so randomly selected beads from either the yellow or green region. The range of the spectral signal in the insert is from 400 to 650 nm in 32 divisions. Beads were 5  $\mu$ m diameter. Color version of this figure is available at <http://www.cvto.purdue.edu/photonics>.

## 5. CONCLUSIONS

In the past couple of decades, flow cytometry was once of interest only to a small group of scientists who used it to sort pure populations of cells, perform cell cycle analysis, or do simple phenotypic classification. However, this is no longer the case because the power of this technology is being better understood and applied in many cross-disciplinary opportunities. Flow cytometry has moved well beyond the field of immunology to influence cell biology, microbiology, food science, and, engineering. For example, use of flow cytometry to monitor microbial contamination within water treatment systems is an excellent application, while there are many more within the fields of bioengineering and biotechnology. Flow cytometry is now recognized as being one of the most critical technologies available for cell biologists and biotechnologists. Its unique ability to collect vast amounts of high-content screening data in a very short time frame, as well as to facilitate statistical analysis of mixed populations without physical separation, is now only just being recognized by groups that were unaware of even the typical blood cell uses of flow cytometry. Next-generation instruments will be heavily involved in the current HCS revolution, and will also impact clinical diagnostics through the use of advanced hyperspectral analysis. High-speed detectors, small compact fluidic devices, and integrated light-source/detector modules will most certainly be on the horizon. A key to the success of this approach, however, is most assuredly the ability to perform fast and accurate classification.

## ACKNOWLEDGMENTS

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