

Flow cytometry: past and future

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ABSTRACT

Flow cytometry is a single-cell technology that measures scatter and fluorescence to establish a set of unique cellular properties. Flow cytometry is used in many areas of science, in particular biotechnology and medicine, but also in industrial applications. Flow cytometry can identify multiple phenotypic subsets from a mixture, select a single cell and even isolate that cell by a process called cell sorting. The field is currently undergoing dramatic changes. We are moving rapidly from the polychromatic flow cytometry that has been the go-to technology for 45 years to spectral flow cytometry, which is now the most significant change in nearly half a century of flow cytometry. With change comes opportunity. Even spectral flow cytometry will morph into second-generation spectral flow cytometry within 5 years. New, exciting features will open up molecular diagnostics and physiology to flow cytometry.

TWEETABLE ABSTRACT

Flow cytometry, a core biotechnology, is changing rapidly. Next-generation instruments will be transformational in biomedicine. Spectral flow cytometry is the new standard, but even it will soon morph into the second generation.

KEYWORDS:

cell function • fluorescence • light scatter • phenotyping • second-generation spectral cytometry • single cell • sorting • spectral flow cytometry

Background

Flow cytometry, as it is currently configured, had its birth with the invention of the cell sorter by Fulwyler in 1965 [1]. However, several developmental attempts established important aspects of the technology, such as the early work of Moldavan, who designed a small cell counter [2]; Gucker and O'Konski, who designed a photomultiplier-based device to count particles [3]; and the concept of hydrodynamic focusing attributed to Crosland-Taylor [4]. Shortly after, Coulter developed the Coulter counter [5], which was followed by early single-cell analysis tools developed by Kamensky *et al.* [6] and their subsequent applications in a multiparametric environment [7].

However, it was not just hardware that drove the evolution of flow cytometry; it was a unique combination of technique and hardware development over the last few decades of the 20th century. It is reasonable to consider that the starting place might be as far back as Ehrlich and Lazarus, who used acidic and basic dyes to identify acidophilic, eosinophilic, basophilic and neutrophilic leukocytes in the 1880s to study the dynamics of ocular fluids, and also used fluorescein [8]. No doubt Kohler's fluorescence microscope developed in 1904 was a key biological enablement tool [9], and Feulgen and Rossenback's early DNA dyes published in 1924 were fundamental discoveries [10]. Caspersson's initial publication that first determined that 'nucleic acids, far from being waste products, were necessary prerequisites for the protein synthesis in the cell' [11] and his subsequent article in 1950 showing that both DNA and RNA increase in actively growing cells [12] accelerated the need for cellular analysis tools.

A critical development, however, was the use of fluorescein conjugated to an antibody. Initially, Coons *et al.* conjugated an antibody to anthracene [13], and Coons and Kaplan then subsequently conjugated an antibody to fluorescein [14]. This could be considered the start of immunologically based fluorescence conjugation to an antibody. Perhaps the most critical molecular development for the future of flow cytometry is best illustrated by Hulett *et al.*, who combined flow and fluorescence [15]. These outstanding scientists created the basis for the flow technology that has matured to be fully integrated into the everyday work environments of clinicians and researchers around the world.

Subsequently, the technology has found applications across almost all aspects of science. Although the majority of applications focus on well-known human medical or biological cell analysis such as immunology, there are numerous publications in veterinary medicine, including sperm analysis [16–20]; biotechnology [21–23]; industrial applications, such as wine [24] and beer [25] production; milk analysis [26,27]; general microbial analysis [28–31] and small particle detection [32]. In addition, cell sorting instruments have been used heavily for single-cell isolation across many fields, including large particle sorting for *Caenorhabditis elegans* [33,34] and other large samples [35].

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Capabilities & features

Most flow cytometers use a laser to excite fluorescence molecules on cells or particles. Incorporation of an array of optical components that focus the laser and subsequent fluorescence signals, combined with light scatter signatures, means that virtually any type of cell or particle in suspension can be analyzed in great detail. It is a powerful technology that can run tens of thousands of particles per second and define subpopulations using multiparameter analysis tools. Flow cytometry operates in clinics to provide diagnostic information on cancer cells, inflammatory conditions and a variety of syndromes; it is used as a core research tool in pharma and biotechnology; and the technology exists in almost every university or research center in the world. It is the go-to technology for analyzing single cells.

Light scatter

Light scatter is one of the wonders of flow cytometry. In the absence of any markers, it is possible to separate particles based on scatter properties. The theory of small particle scatter is founded on theories developed over 100 years ago by Gustav Mie [36]. In flow cytometry, we typically talk about forward scatter, which is essentially in line with the laser beam, and side scatter, which is mostly orthogonal to the beam. At the risk of oversimplifying, we often talk of forward scatter as being related to size, although based on Mie theory, this is strictly limited to spheres. Despite this, if one is looking at blood cells, forward scatter provides an approximation of size, but the approximation is not accurate for other shapes, such as an elongated particle. Side scatter is understood to relate to the complexity or granularity of the cell or particle. A latex bead expresses no complexity, but a granulocyte, which has a highly complex cytoplasm containing multiple nuclear lobes and lots of granules, has significant side scatter properties and is well differentiated from other blood cells. Thus, scatter can be used to create at least a reasonable separation of red and white blood cells and, if combined with polarization, can further dissect granulocytes into subsets in the absence of any labels. Interest in scatter has not expanded much over the history of flow cytometry despite the fact that scatter contains a vast amount of information mostly forgone in flow cytometry.

Fluorescence

Fluorescence is the essence of detection in flow cytometry. Basic quantum mechanics requires that molecules absorb energy as quanta (photons) based on a criterion specific to each molecular structure. Absorption of a photon raises the molecule from its ground state to an excited state. The structure of the molecule dictates the likelihood of absorption of energy to raise the energy state to an excited one. Total energy is the sum of all components (electronic, vibrational, rotational, translational, spin-orientational), and when the excited electrons return to their ground state, they release some of that energy as photons of a higher wavelength. We call these molecules fluorophores, chromophores and fluorochromes since they have this unique capacity to absorb and release energy. Absorption associated with electronic transitions (electrons changing states) occurs in about 1 fs (10^{-15} s) since many of the fluorochromes used in flow cytometry have emission lifetimes from 1 to 10 ns. The extinction coefficient ϵ of a molecule applies to a single wavelength (usually the absorption maximum) where the cross-sectional area of a molecule determines how efficiently it will absorb a photon. The efficiency is defined as the quantum yield (Qf), which is a measure of the integrated photon emission over the fluorophore spectral band. These determinants are important ones that define the usefulness of molecules selected to be used as fluorochromes in flow cytometry.

The number of desired individual measurements, in terms of fluorescent markers, also impacts the type of fluorochromes to be used. Regardless, all polychromatic flow cytometry systems require a series of bandpass filters to isolate as best they can the spectral window that most favors each particular fluorochrome being sensed, as demonstrated in Figure 1. This demands both design and implementation of special bandpass filters for each fluorochrome as well as a complex process of compensation to accommodate spectral overlap between each pair of bands. Doing so allows the collection of bands of fluorescence that are associated with each individual fluorochrome but this scheme also requires a single detector linked to each fluorochrome.

From an immunological perspective, this is termed the number of phenotypic markers desired, which might range from just a couple to 30–70 individual subsets of cells. Currently, in a single assay, around 30 subsets can be defined by polychromatic cytometry, and so far we have about 40 for spectral cytometry. This is an ever-increasing target and is likely to reach over 100 within the next 5 years.

Fluidics operations

Flow cytometers operate within a liquid environment because all cells or particles to be analyzed must be suspended in a liquid. Typically, this can be saline or water, depending on the nature of the particles, but it could also be other fluids, such as milk or beer. Particles are literally fired through one or more laser beams at very high rates in a process that establishes a single, highly focused stream of particles. The focusing process is called hydrodynamic focusing and aligns cells so that just a single cell is passing through the laser at one time. In his excellent article on fluidics, Watson gives Bernoulli the original credit for hydrodynamic focusing [37], but the most recent implementation was, of course, by Crosland-Taylor *et al.* [38]. Alternative approaches are also useful, such as acoustic focusing [39], although, in some instruments, no sheath is used at all. As each cell passes through the laser beam, the laser scatter is often collected at two angles: 1 or 2 degrees within the laser direction and 90 degrees or orthogonal to the laser. The former is called forward scatter and the latter side scatter, orthogonal scatter or 90-degree scatter, as discussed earlier.

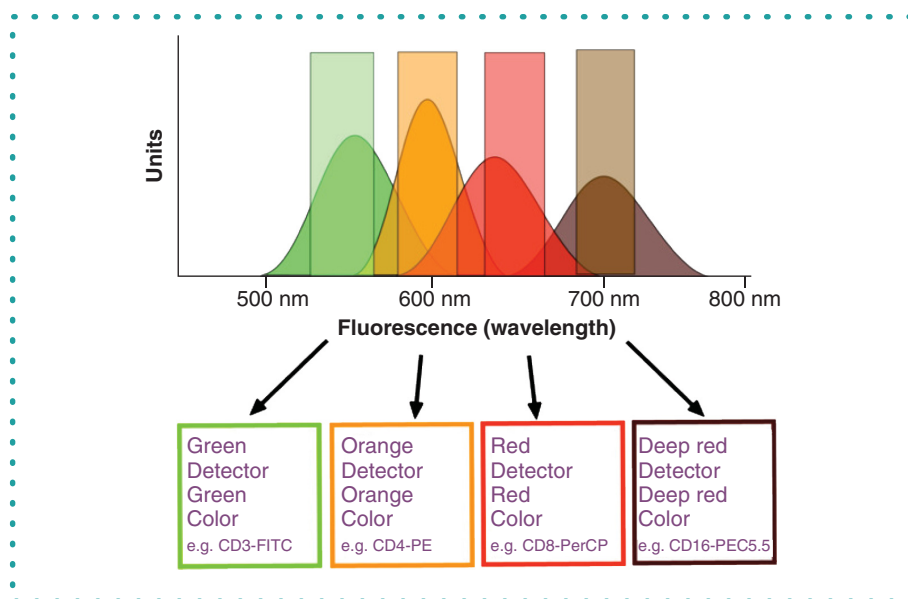


Figure 1. In polychromatic flow cytometry, we require bandpass filters to isolate bands of fluorescence that most reflect the emission of each fluorescence. Each 'color' requires one detector and a bandpass filter to narrow the bandwidth of each window of bandwidth required. Thus, we have one color—one detector technology, which is the basis for polychromatic cytometry. Reproduced with permission from cyto.purdue.edu.

Limitations of current technology

Polychromatic flow cytometry

Polychromatic flow cytometry is based on the principle of one detector, one color. Here the measurements of scatter and fluorescence are based on bandpass and dichroic filters that collect individual bands that do not cover the entire spectrum. Moreover, individual detectors may accept multiple fluorochromes; however, each detector is focused toward collecting a single fluorochrome. Because a single detector is associated with a single fluorochrome, only a portion of that fluorochrome signal will be collected and analyzed.

The fundamental principle of polychromatic cytometry is separation of a specific bandwidth based on the best fit of the emission spectra. As the technology has advanced, users have striven to increase the number of possible 'colors,' the term frequently used to represent individual fluorescence probes. By including several lasers of different wavelengths, we are able to end up with five to ten specifically convenient wavebands per excitation laser to reflect each fluorescence probe that represents a phenotypically diverse population of cells. However, there are limitations to polychromatic flow cytometry. As shown in Figure 2, bands of defined wavelength collect light for an individual detector; however, when considering the entire signal produced by a fluorochrome, it can be seen in Figure 2B that only a portion of that signal can be collected, and this portion contains a 'contaminated' signal that arises from other fluorochromes. This situation is managed by the use of signal compensation: it is possible to define the relative contributions from contaminating signals and remove them successfully. However, compensation does not produce a totally accurate analysis of the signal because, as shown in Figure 2C, none of the signal outside the bandpass filters is collected, and this can amount to a significant amount of valuable signal (e.g., information).

In the end, we can analyze 20–30 populations based on the available array of fluorescence probes. Extending this is both costly and difficult. Polychromatic cytometry most likely will not add more fluorescence parameters.

The problem with this approach, of course, is that the available probes are a restricted subset based on each specific laser excitation. Because of this limitation, from the beginning of the field of flow cytometry, chemists have been trying to design new fluorescent molecules that have narrow emissions or at least narrowly defined peaks. Although the first fluorochromes used were well-known UV dyes such as Hoechst [40] and DAPI [41] or common dyes like fluorescein diacetate [15], it was evident that broadening the wavelength range was going to be necessary. One of the first customized probes to be created was Texas Red [42]. The technology moved from single-color [15] to two-color [43] to five-, eight-, 18- and eventually 20- to 30-color flow cytometry. This, however, appears to be the realistic limit of polychromatic flow cytometry. A key feature in the development of polychromatic cytometry was that the intensity of the fluorochrome signal was the most critical parameter, so driving intensity often required raising laser power. This increased instrument cost, to say the least, without considering the potential impact of high laser power on the fluorochrome itself or on cell function. We were restricted by the number of individual spectral bands based on the available fluorochromes and their spectral overlaps. A new solution was needed to expand the field.

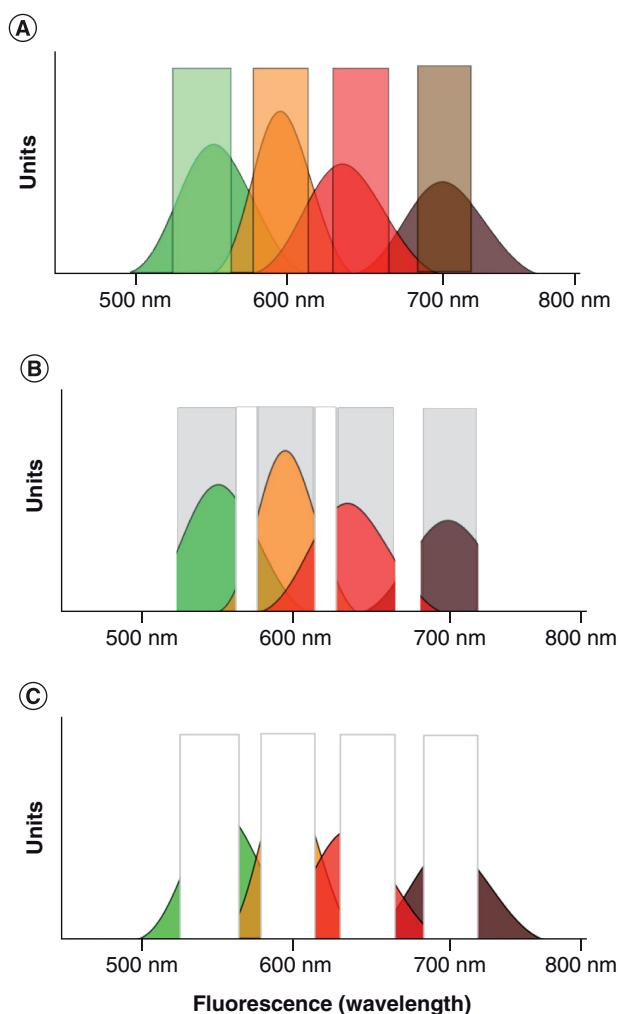


Figure 2. Polychromatic fluorescence signals. (A) The fundamental process of polychromatic cytometry is that bandpass filters create unique bands of light for each fluorochrome. (B) One of the problems is that there is spectral overlap: multiple fluorescence emissions may be measured on any particular detector. (C) An additional problem is that only fluorescence signals within the bandpass filters are collected all the rest of the signal is lost. Reproduced with permission from cyto.purdue.edu.

Spectral flow cytometry

The solution to the limitations of polychromatic flow cytometry was first demonstrated by the author's group in 2004 [44–48]. We hypothesized that by evaluating the full spectrum available, we could utilize well-known principles of spectral unmixing developed for satellite remote sensing [49] to achieve far better definition than polychromatic cytometry could achieve. We also hypothesized that by using the spectral approach we designed, we could utilize more fluorescences that were not ideal for polychromatic cytometry. We initially developed spectral flow cytometry using a 32-channel full-spectral detector system as well as the multiparameter electronics necessary for collecting a full spectrum for every cell. By so doing, we created a new approach for flow cytometry, and the spectrum itself became the key parameter (Figure 3). Furthermore, as the inventor of the technology, the author notes that there are many abbreviations for spectral flow cytometry, but let us focus on just one – SFC. It is logical, and frankly, as the author's group developed it, I think 'SFC' is the way it should be.

The second important aspect of this approach was that the intensity of an individual fluorochrome was no longer the most critical parameter for a fluorescence measurement. An example of this approach is shown in Figure 4. As noted earlier, the concept of fluorochrome signal intensity was the central driving force for almost 50 years, but with spectral flow cytometry, signal intensity at any particular wavelength is still important but far less critical. It is the differences in spectral signatures between different fluorochromes that give spectral flow cytometry its huge advantage as well as the critical association of the respective spectral signature with a biologically important event. Figure 4 shows the full spectral signature of a combination of fluorochromes. It is the unmixing of this signature to derive the individual components that allows spectral flow cytometry to capture the additional information that can define the phe-

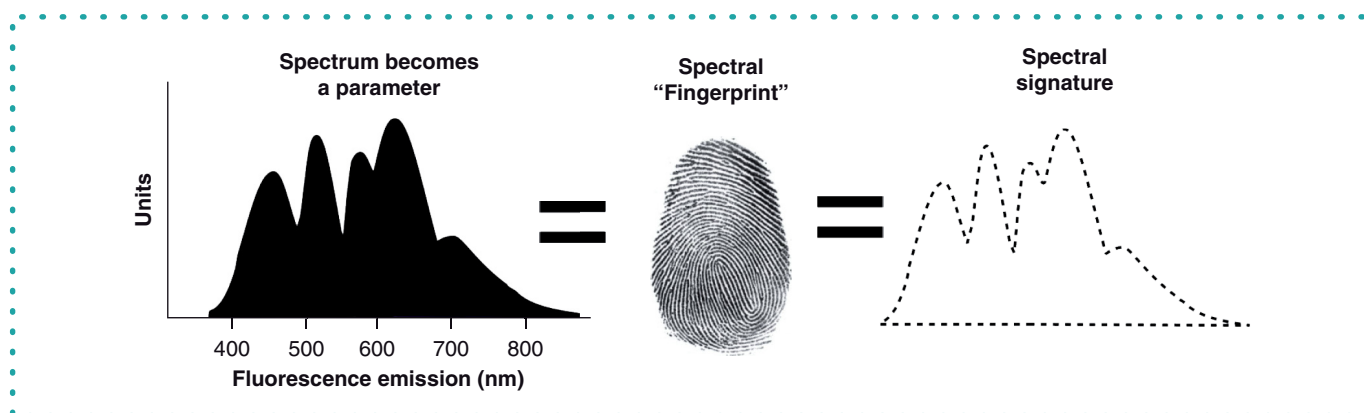


Figure 3. The spectrum becomes the key parameter in spectral flow cytometry. In the same way a fingerprint identifies an individual, so too does a spectral signature identify a particular spectral entity. Reproduced with permission from cyto.purdue.edu.

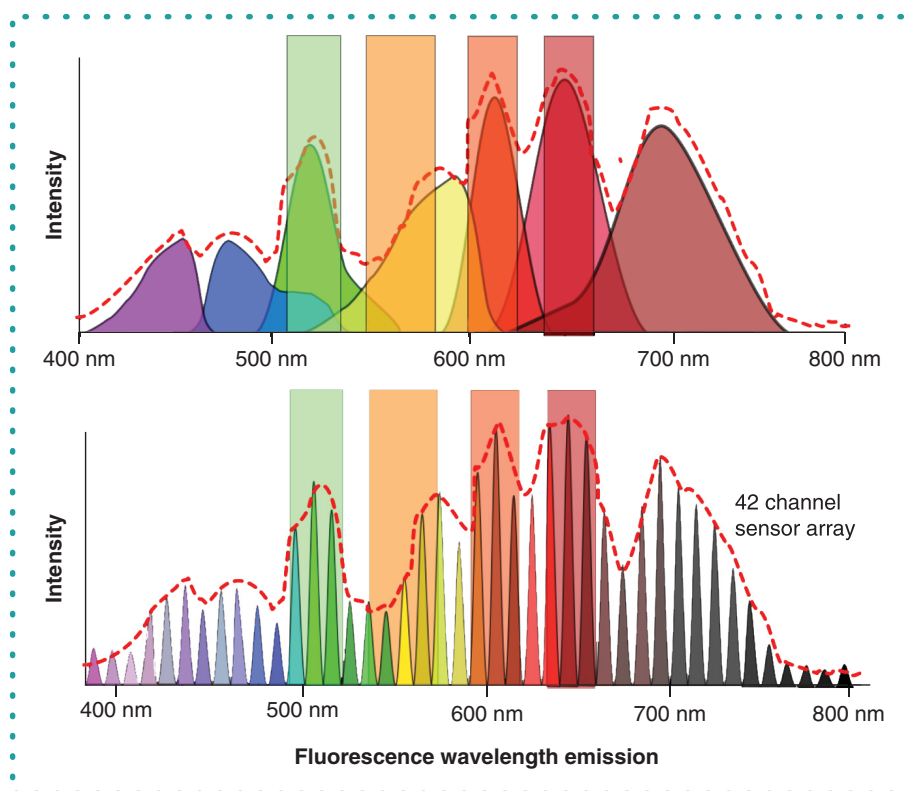


Figure 4. Shown in the upper half is a complex mixture of fluorochromes over the visible spectrum. The added rectangles show the method for collecting specific bands of fluorescence, each representing a single fluorochrome. Below this is an example of a multiarray detector (in this case, 42 channels) used to collect the full spectrum of the fluorescence signal instead of individual bands. The dotted line represents the spectral signature of the fluorescence. By relating this signature to a particular fluorescence, the spectrum of each fluorescence can be unmixed from the mixture. Reproduced with permission from cyto.purdue.edu.

notypic characteristics of the cellular subset of interest. Although the total number of sensors is not critical, there is a balance that provides a near-optimum signal. The author's group initially used a 32-channel photomultiplier tube array; others have used the same array or smaller ones, such as 16- or 24-channel detectors. Having too few detectors limits the ability to capture the full spectrum, and too many may be limiting owing to photon statistics. However, the author's group recently defined a 42-channel detector array that we believe is almost ideal for spectral flow cytometry, as it achieves the goal of having an excess of detectors sufficient to capture enough photons for good signal characterization (Figure 4). Clearly, new approaches to sensor design and capacity will be required for spectral flow cytometry to optimize the maximum potential of this approach.

Table 1. Comparison of polychromatic and spectral cytometry.

Feature	Polychromatic	Spectral
Bandpass filters	Required	Not required
Fluorochromes	Many	All
Fluorochrome intensity	Critical	Useful but not critical
Highly overlapping fluorescences	Not possible	Possible
Total fluorescence signal capture	No	Yes
Hardware	Highly complex	Moderately simple
Compensation	Required	Not required
Spectral unmixing	Not easily	Fully implementable
Over 35 fluorescences	Unlikely	Easily
Over 50 fluorescences	No	Highly likely
Over 100 parameters	No	Likely

There are many advantages to this approach that will literally drive polychromatic cytometry to a long- and well-deserved grave (Table 1), although it may take a few years since the vast majority of the current instruments are polychromatic. No longer does an instrument have to have special hardware configuration to analyze a specific fluorochrome. Fluorochromes that have very similar spectral signatures can now be separated, and we now know that once a fluorochrome is defined and saved in a database, there is no need to continue to collect single-color controls as long as similar assay conditions exist. In addition to all these advantages, spectral flow cytometers are far less complex from an optical perspective and therefore should be, and are in fact, much cheaper to manufacture, particularly for very high parameter detection. The last advantage is that the traditional process for performing fluorescence compensation is no longer needed since spectral unmixing is essentially automated and therefore far more reproducible. This makes spectral flow cytometers perform at least as good as expectations for polychromatic instruments for basic flow cytometry and probably far better than polychromatic cytometry when large, multiplexed assays are designed. Of course, developing large, complex panels is still an intensive process and demands significant knowledge and technical skills, as it always has.

There are limitations to all technologies, and spectral flow cytometry is not exempt from this. As currently designed, all instruments continue to operate in analog space despite the frequent misunderstanding of their actually being 'digital.' The reason for this is that current instruments operate in what is known as the 'current' mode of detection, meaning that detectors, being photomultiplier tubes, avalanche photodiodes or the like, collect analog signals and then use analog-to-digital converters to create a pseudodigital signal from which all of the analysis is accomplished. This can be addressed only by moving to a fully digital technology, which will ultimately be achieved using devices such as silicon photomultiplier detectors operating in single-photon mode [50,51]. This is a huge but necessary step for the field if it is to become a fully quantitative technology, something that has been a desire for decades and only partially achieved some decades ago by creating calibrated particles [52], which does not create truly quantifiable flow cytometry.

One of the biggest limitations of spectral flow cytometry has been that it has been restricted to analysis. However, the recent release of the Bigfoot spectral cell sorter (Thermo Fisher Scientific, MA, USA) changed that dynamic. One of the difficulties that had to be overcome was the necessity to compute spectral unmixing within the short time window between analyzing a particular cell and tagging a droplet containing that cell for sorting. This was solved in Bigfoot by using very high-speed computation in its field-programmable gate array technology. Indeed, we have shown excellent sorting of both blood cells and microbes in our laboratory.

As shown in Figure 5, this spectral sorter moves the technology significantly forward. One of the advantages of spectral flow cytometry is the ability to increase the potential number of subpopulations that can be identified. Until the technology advanced to sorting, it was not possible to physically isolate these unique populations. What is unique about the technology in Bigfoot is the ability to calculate the spectral unmixing matrix in real time and apply sorting instructions to the sorter for particle separation (Figure 6). One major advantage of the Bigfoot spectral sorter is that it is suitable for sorting pathogenic organisms because of its sophisticated safety features. This has become an important aspect of the use of spectral sorting in our laboratory. Future sorting based on some of the new display modes currently used in flow cytometry, such as t-distributed stochastic neighbor embedding [53], will no doubt create completely new opportunities to identify and isolate unique populations of cells or particles.

Advances in data processing & analysis

Flow cytometry is one of several advanced technologies that have been able to take advantage of the vast array of informative tools available. In the early days of flow, users were restricted to histograms and dot plots, which were adequate for two to five parameters. Some early attempts were made to create alternative presentations by combining parameters and displays [54,55] or tools like principal component analysis [56]. Despite the potential, users found using traditional plots adequate until there were literally too many parameters to display, which required the identification of alternative approaches. As the number of parameters increased, so too did the demand to expand analytical approaches. An early approach was the generation of maps that created a unique approach to complex mixtures [57].

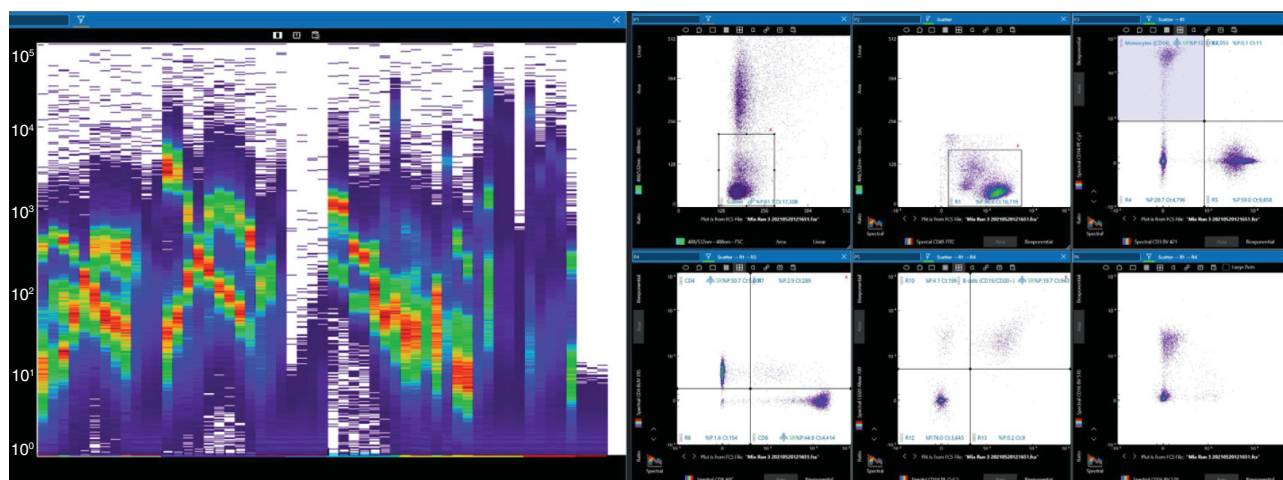


Figure 5. A screenshot from the Bigfoot spectral sorter system showing the combination of nine-color assay spectral signature on the left and individual sorted populations on the right. Reanalysis of each sorted population showed purified sorted cells based on real-time spectral unmixing. Reproduced with permission from cyto.purdue.edu.

Subsequently, a number of alternative approaches were identified from earlier non-cytometry-related publications, such as spanning tree progression of density-normalized events [57] and t-distributed stochastic neighbor embedding [53], which were then applied to flow [58], Uniform Manifold Approximation and Projection [59] and many other approaches that have advanced the field tremendously by literally embedding informatics within the field rather than using informatics as a convenient tool. With the advent of 40-plus parameter studies [60], it is now evident that flow cytometry operates at a much more complex level than it ever did before.

Second-generation spectral flow cytometry

Spectral flow cytometry is most definitely the future of flow cytometry. However, in its present form, there are still many features that can be significantly improved. The next generation of flow cytometers will build upon current spectral flow cytometry but will focus on the opportunities that spectral affords but that are not attainable with current instruments. For example, the majority of spectral cytometry today is focused on immunophenotyping, which, although the major application, is mostly about increasing the number of possible parameters. However, immunophenotyping is by no means the only opportunity for spectral flow cytometry. For example, there is an almost unlimited potential for combining assays not commonly used in flow cytometry with cell cycle, metabolic and structural components of cells (Figure 5). Almost endless possibilities exist, most of which could not be accomplished using polychromatic flow cytometry. The power of spectral cytometry is that it builds on decades of analytical knowledge that began over 40 years ago when Kettic and Landgrebe [61] and others developed multispectral systems for remote sensing. Since that time, thousands of articles have been published on analytical toolsets for spectral analysis across many different application spaces. Spectral flow cytometry can take advantage of this vast knowledge base as the technology advances.

With novel technological approaches, many of these new opportunities will be proved possible. For example, with the newest generation of high-speed single-photon sensors [62], flow cytometry will advance to the most sensitive fluorescence detection systems possible. This will enable new possibilities, such as lifetime fluorescence analysis within a standard spectral instrument, allowing conformational changes to be observed. In addition, with highly cooled, low-noise single-photon sensor technologies being entirely digital, the inherent noise within current analog technologies will be a thing of the past as new systems become entirely digital. This will enable absolute quantitation – a holy grail for flow cytometry for nearly 40 years. It will allow direct comparability among instruments around the world since the readouts will be in standard units, such as the SI units most frequently used across quantitative sciences, and the basis of the measurements will be actual photons, not an average signal generated by an average detector. Is it not time to move beyond the ‘arbitrary units’ we have become so fond of in flow cytometry? This may have a tremendous impact on clinical cytometry as well as pharmacological sciences.

High laser power will no longer be a requirement for such systems. Although increased laser power is often needed in polychromatic instruments to raise the intensity levels of some fluorophores, this will be unnecessary with second-generation spectral instruments. Indeed, high laser power drives high background and fluorophore or cellular damage; reducing the background will increase system sensitivity with single-photon approaches. Another advantage to be seen with next-generation single-photon approaches is the ability to analyze smaller particles. Current attempts to look at particles from 20 to 200 nm in size have proved to be problematic with current instruments. There are many reasons for this, but the primary one is that the fundamental design of flow cytometry fluidic and optical

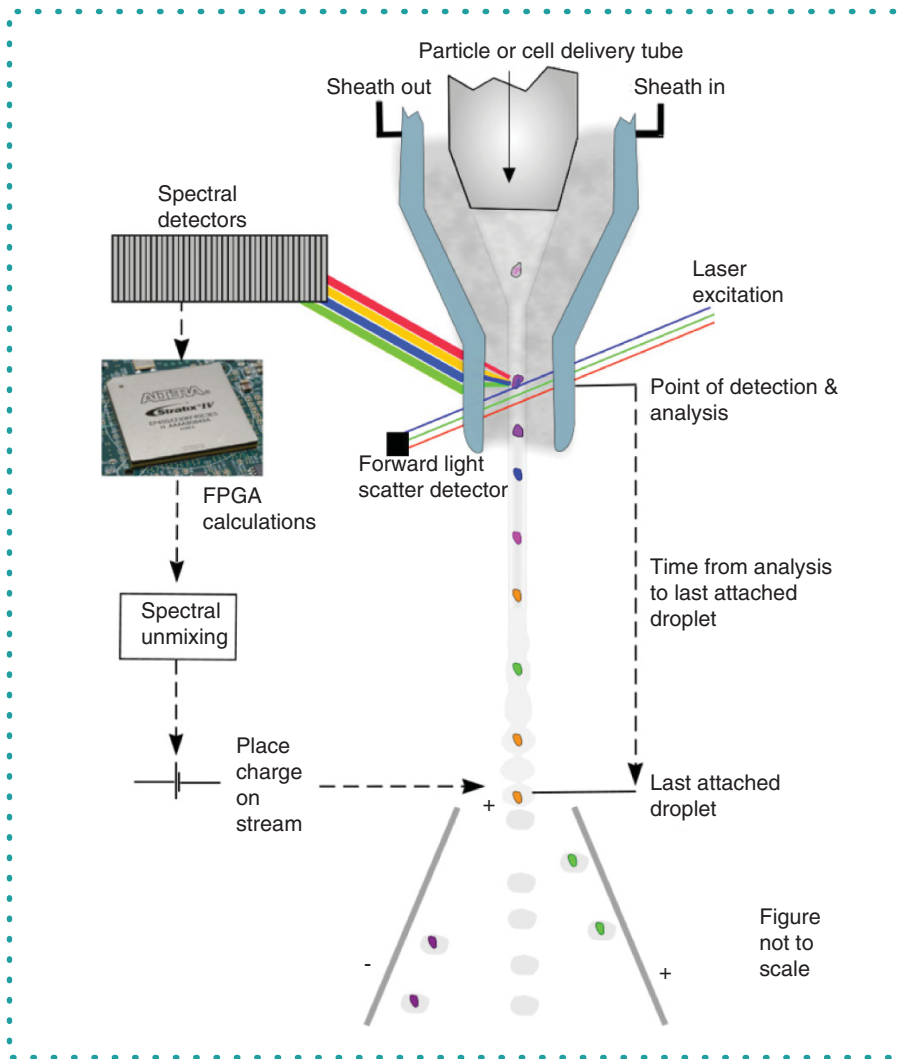


Figure 6. To sort based on spectral flow cytometry, there is a requirement for high-speed field-programmable gate arrays and unique electronics in order to make sorting decisions based on the spectral unmixing matrix created. Shown here is the basic process that operates in a spectral sorter. FPGA: Field programmable gate array. Reproduced with permission from cyto.purdue.edu.

components was optimized for 5- to 15- μm cells or particles. When you move below the diffraction limit, current flow cytometry fails. Although it is true that it is possible to detect fluorescence signals from small particles, even this is complicated by the very large analysis volume created by the focused laser beam. However, alternative designs that focus on single-photon sensing will drive new applications in this arena (Figure 7).

Conclusion

Flow cytometry is one of the most important tools biologists, immunologists and physicians have in their armamentarium. It has rightfully claimed a key position as a technological leader for all things single cell. Its powerful analytical capacity allows flow cytometry to collect data from complex mixtures of cells and discern even the rarest of populations without the need for physical separation. However, these rare populations can be physically sorted if needed by the cell sorting versions of flow cytometers, which can operate at rates approaching 100,000 events per second. These separated cells can even be isolated under sterile conditions if desired. Today, there is also a sorting version of spectral flow cytometry that has proved to have analytical capacity fast enough to sort cells based on spectral unmixing. The first example of this technology is the Bigfoot cell sorter. Flow cytometry is a critically important tool in the clinical environment by which aberrant cells can be identified, excess or reduced populations of particular cells can be quantified and patients can be monitored for changes in tracked populations of specific cells. In the research lab, flow cytometry is comfortable analyzing microbial populations, human blood cells and other body fluids as well as cultured cell lines. Plant materials can be analyzed for cell cycle or simply for haploid or polyploid populations, yeast populations in beer can be monitored and, to some extent, metabolic processes

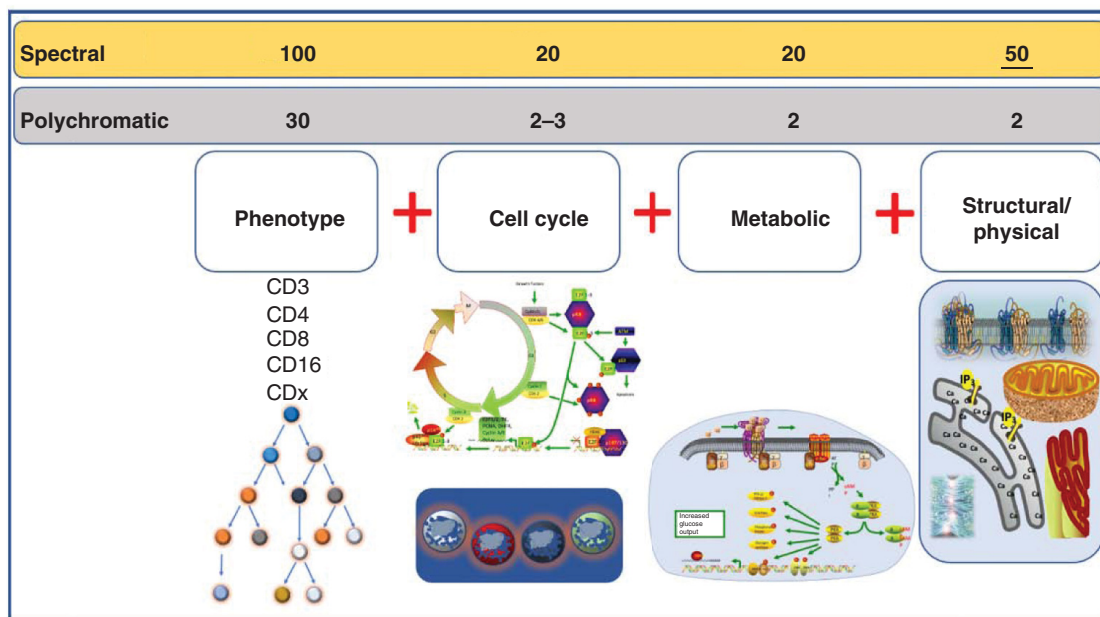


Figure 7. The future of spectral flow cytometry will be increased access to phenotypes, nuclear antigens, metabolic pathways and structural components.

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such as ion fluxes can be measured in real time. Given the broad spectrum of manufacturers and instruments, some instruments are preferred for some applications, but in general, it is fair to say that most flow cytometers on the market can perform most, if not all, of the current applications at a high-quality level.

Future perspective

Flow cytometry has a bright future because it is the only viable technology for comprehensive and multiplexed analysis of single cells. Furthermore, with the latest spectral cell sorting technology, a single cell from a multicellular mixture, identifiable only by a complex algorithm, can be separated for cloning or sequencing. Even in the absence of sorting, which recently arrived, spectral cytometry has revolutionized the field after over 50 years of iterative advances. The clear advantage of being able to use any dye under almost any circumstance is a game-changing feature of spectral flow cytometry. In addition, there is no longer an issue with using dyes with almost overlapping spectra. Even minor differences in spectra allow unmixing of such dyes. What is particularly exciting is the potential of the second generation of spectral flow cytometers, which will have all the advantages of current systems and then some. Current spectral instruments have many advantages over polychromatic instruments but still suffer from some of the same problems, including background noise, lack of true calibration capacity and operation that remains in the analog domain. The next generation will function entirely in the digital domain, have features currently available only on custom-built instruments and have the capacity for potentially 150–200 parameters, which will engage metabolic, structural and functional probes, extending the field for several decades.

Executive summary

- Flow cytometry has become the go-to technology for many in the biotechnology, pharmacology and immunology domains. Its ability to analyze single cells makes it attractive to microbiologists interested in the human biome, for example, or even for more rapid clinical diagnostics. For over 50 years, the technology has been evolving from a single-parameter instrument to a complex multiparameter analytical engine capable of extracting a huge amount of information from complex mixtures. It has evolved to spectral flow cytometry, which has opened huge potential for the field. With the addition of sorting capacity on spectral instruments, flow cytometry is indeed a critical tool in biomedical and biological science.

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