Innovations in Multiparametric Flow Cytometry

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Conventional Detection

“Conventional” detection optical design is that which is has been utilized since the technique’s conception and which drives the majority of the systems in labs. In this kind of instrument, each detector is assigned a unique spectral band, determined by the optical mirror and filter combination in front of it, which dedicates that detector for the measurement of a particular fluorophore. In other words, on any given instrument, there is only one Brilliant Violet™ detector, only one Alexa 488 detector, and only one Brilliant™ Blue 515 detector, to give a few examples. Multiple fluorophores with the essentially the same emission spectra (e.g. PE-Cy7 and APC-Cy7) can be detected distinctly by separating their two excitation lasers (561 and 640 nm, respectively) and the associated detection paths in space and time. Two cutting-edge high-parameter conventional cytometers are the BD FACSymphony and the Bio-Rad ZE5.

Becton Dickinson FACSSymphony

The FACSymphony is BD’s newest analyzer, which not only continues the tradition of success of the LSR II, LSRFortessa, and LSRFortessa X-20 but takes it to a new level. This instrument can be configured to detect up to 50 parameters (including forward and side scatter) simultaneously from up to 10 lasers, which can be chosen from 25 available wavelengths during the initial specification process. Currently, experiments measuring at least 30 colors are being performed on this instrument, but this number is bound to grow before too long.

Bio-Rad ZE5

The Bio-Rad ZE5 is a novel high-parameter instrument that excels in a unique way: its unparalleled automation and flexibility coupled to its high sensitivity. The ZE5 was designed by Propel Labs, an incredibly productive engineering company located in Fort Collins, CO. The Propel Labs team has involved in cutting edge flow cytometry projects for many years, beginning with Dako Cytomation MoFlo cell sorter and then the Beckman Coulter MoFlo XDP and MoFlo Astrios cell sorters before joining forces at Propel Labs. The ZE5 can be equipped with five lasers and to measure 30 parameters - two forward scatter signals (one detector is dedicated for small particle measurement), side scatter, and 27 fluorescence channels. The system’s universal loader allows it to sample from virtually any kind of input media including 96 and 384-well plates. This instrument is fast - the electronics are powerful enough to process events at many tens of thousands of events per second. A host of other neat features - including fully-automated and hands-off acquisition, automated QC with on-

board beads, automated startup, shutdown, and cleaning, and a built-in probe crash protection mechanism, to name a few - contribute to this instrument's uniqueness. Currently, experiments measuring at least 21 colors are being performed on the ZE5.

Spectral Detection

Spectral cytometry is a new technology, and it's a fascinating one with a promising future. As opposed to conventional detection, spectral cytometers lack individual detectors for the measurement of a dedicated fluorophore. Rather, a "spectral signature" is registered for each particle or cell that is interrogated, and this spectral signature is deconvoluted in order to extract intensity data for each dye. The beauty of these kinds of systems is that while we usually think of a fluorophore as being a certain color ("Alexa 488 is green), in actuality, the emission spectra - or the range of wavelengths over which a particular fluorophore will generate light upon excitation - contains more than just the nominal color. Therefore, because the bandpass filters that we use in conventional flow cytometry capture only the essence color of the fluorophore, many photons that this fluorophore produces are lost. That loss is vastly mitigated in spectral cytometry.

Example 1: GFP and YFP

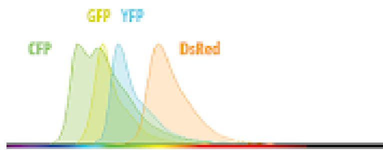

Figure 1: Spectrum plots from a conventional spectrum viewer shows heavy overlap between GFP and YFP.

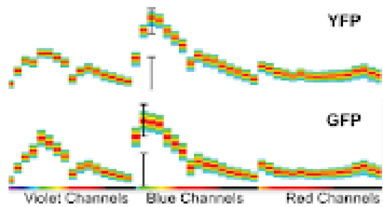

Figure 2: Spectrum plots from Aurora show distinct signatures across three lasers.

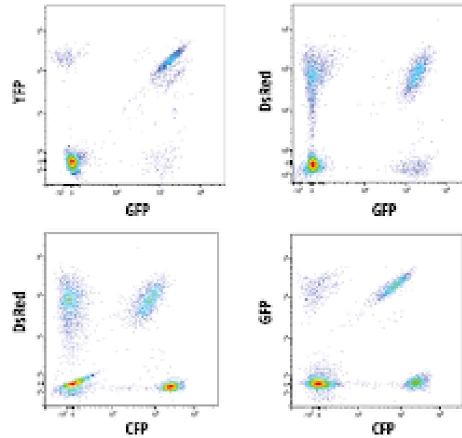

Figure 3: Sp2/0 cells were transfected with GFP, YFP, CFP and/or DsRed (alone or in combination) and run on the Aurora (plots are gated on FSC vs SSC). Each population is clearly identified.

Example 2: Qdot 705 and BV711

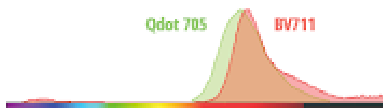

Figure 4: Spectrum plots from conventional spectrum viewer shows heavy overlap between Qdot 705 and BV711.

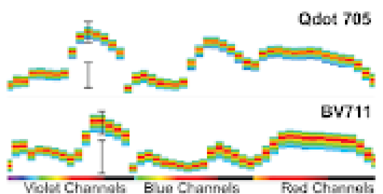

Figure 5: Spectrum plots from Aurora show distinct signatures for BV711 and Qdot 705.

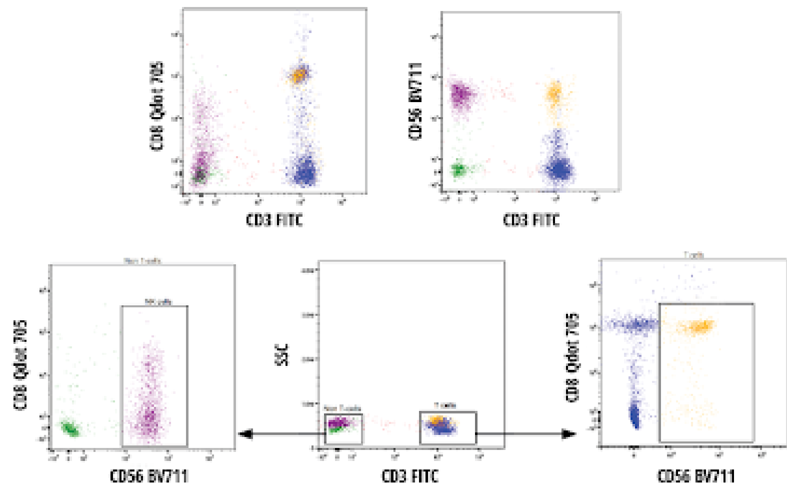

Figure 6: Normal human whole blood was stained, lysed, washed, and analyzed on the Aurora. Subsets of NK and NK T-cells that co-express CD56 BV711 and CD8 Qdot705 were easily identified.

Sony SP6800

The SP6800 was released several years ago and paved the way for spectral detection technology. The optical configuration of this instruments is based on a prism collection system that contains 10 prisms that direct fluorescence-generated photons to specific wavelength-dedicated on a 32-channel PMT. This detector in essence contains 32 independent detectors contained in one device that cover the spectrum from 420nm-800nm. In other words, the prism array and PMT together can measure a spectral profile of any cell or particle from the blue (420 nm) range to the far red (800 nm). The system is impeccably and thoughtfully designed in many other ways including the flow cell design, which is based on a replaceable microfluidics chip with an integrated quartz cuvette. The SP6800 is

somewhat limited in the number of colors it can feasibly measure simultaneously due to the number of lasers and their configuration, but is reported to generate high quality data that reflect the benefits of spectral detection.

Cytek Aurora

The newest player in the spectral arena is the Cytek Aurora, which is generating a lot of excitement. Cytek Development was founded 25 years ago by engineers and has differentiated itself by providing service and technical support primarily for Becton Dickinson cytometers and over the years has engineered innovated cytometer components, software, and eventually its own line of instrumentation. Recently, Cytek Development, in partnership with a venture capital-backed Cytoville, Inc, the fruition of which is the Aurora.

Innovations in Fluorophores

Another aspect of this high-parameter space is the availability of dyes. The color palette has expanded in two ways. First, the Sirigen polymer dyes and associated tandem fluorophores have revolutionized multicolor flow cytometry by offering a host of options, like the Brilliant Violet™ dyes, to maximize the spectral detection space from each laser line. The Brilliant Violet™ dyes have become a standard component of panels and are now offered by multiple vendors, and polymers excitable by other laser lines are being utilized more and more. New laser lines, like deep UV, are even being exploited to expand the possibilities.

Other Advances - Cell Sorters

These advances in high-parameter multicolor flow cytometry highlight only a portion of the innovations of the past several years. Automation in cell sorting is another hot topic, with three benchtop simple cell sorters - the Bio-Rad S3e, the Sony SH800, and the BD FACSMelody, and instruments using microfluidics technologies to sort cells under much lower pressure and gentler conditions than was traditionally possible. The high-end cell sorter realm has seen less innovation, but there are rumors that BD is developing a 50-color, 6-way sorter to be released this year, and a 60-color 10-way sorter to be released after that within the next few years. The pace of the development of new technology in flow cytometry has paralleled developments globally, and this pace will continue for years to come. It’s an exciting time for flow cytometry to be a part of our science.

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Of all of the tools available to biomedical scientists, flow cytometry ranks among the most powerful. Its capacity to interrogate tens of thousands of cells per second and to measure

multiple parameters simultaneously, coupled with its flexibility, has established this technique

as central to the experimental process in many different disciplines. This is all the more true for hematology, whose target cells' size, shape, and nature to exist as a single-cell suspensions makes flow cytometry indispensable for addressing biological questions we ask.

Flow cytometry in some form has been around since the 1940s, when it was used to detect bacteria in aerosols through illumination by a Ford headlight and detected via a photomultiplier tube (Gucker FT et al., 1947; for historical overview on flow cytometry, see Shapiro H, 2003). Since then, the technique's development has been immense, limited essentially only by the technological constraints of the time. As lasers became smaller, so did flow cytometers; as detectors and optical components became more sensitive, so did the instruments; and as electronic processing power became faster, so did the speed at which flow cytometers were capable of analyzing and sorting particles. Over the past several years, however, development has been faster than ever. As flow cytometry has become more relevant to a wider variety of research areas, its market size has become bigger than ever and more and more engineering firms and companies have become involved in the development and marketing of new technologies. The most noteworthy change has been the advent of truly multiparametric fluorescence-based flow cytometry both with regards to instrumentation and reagents.

Almost a decade ago, the advent of high-parameter cytometry was spearheaded by DVS Sciences with their launch of the CyTOF mass cytometer in 2009. The CyTOF, which has undergone further development over the years, uses antibodies conjugated to metals rather than fluorophores. The instrument detects the presence of a metal, and hence a target, from nebulized and through time-of-flight ("TOF") mass spectrometry. Unlike fluorescence-based flow cytometry, overlap between the probes' detection is minimal, which makes mass cytometry an attractive tool when many probes are utilized simultaneously. On the other hand, these assays require specialized reagents that are not as readily available as fluorophore-conjugated antibodies, and the instruments require dedicated infrastructure (due to the operating conditions) and more maintenance than a traditional cytometer.

However, over the past several years, advances in fluorescence-based cytometry have paved the way for the truly feasible high-parameter flow cytometry experiments. Between the development of new high-end instrumentation and novel dyes that take advantage of previously-unused spectral ranges, experiments that measure as many as 30 parameters via fluorescence detection are now possible. This "high-parameter" space is comprised of instruments that are based on two kinds of technologies: so-called "traditional" or "conventional" flow cytometry and "spectral" cytometry, which differ fundamentally in the way that they detect the presence of a fluorophore on a cell or other particle.

Instead of individual PMTs, spectral systems use multichannel detectors or detector arrays, and each channel or element of the array is dedicated to a narrow band of light. For example, the first channel of the detector measures the intensity of light from 500-510 nm, the second channels measures light from 510-520 nm, etc. In this way, the entire emission

profile of the cell is captured. The deconvolution process is another element of the innovation. Using data from single-color controls (like compensation controls that we use in conventional cytometry), software calculate the contribution of each individual fluorophore, based on its spectral signature, to the total signal.

The raw data is collected in “spectral plots,” which graph collection wavelength against signal intensity.

There are three main benefits (among others) of this technology. First, dyes that normally cannot be used together can be on these systems. For example, QDot 700 and BV711 have extremely similar emission spectra and are detected with the same mirror and filter set on a conventional cytometer; they cannot be distinguished. However, because their emission spectra are not exactly the same, a spectral cytometer can distinguish these two dyes (see example from Cytek Aurora below). Therefore, bigger panels can be built with less worry about the effect of spectral overlap. Second, sensitivity resolution between populations is excellent, since more photons (more data) are collected from each fluorescence event, minimizing error and keeping populations tight. Moreover, the spreading of data after compensation (spillover spreading) is mitigated, which also leads to better resolution and sensitivity. Finally, autofluorescence can be extracted as a parameter and subtracted from the total signal. This aspect can be very advantageous when working with cells whose high autofluorescence may compromise the detection of dim fluorescence signal.

The capabilities of spectral cytometers may seem somewhat magical for us that are used to conventional detection, but it’s important to keep in mind that this technology is bound by same controls and good practices that conventional cytometers require: single-color controls, fluorescence minus one controls for gating, and good practices for panel design, to name a few.

There are currently two spectral cytometers on the market: the Sony SP6800 and the Cytek Aurora™.

The SP6800 has proven capability performing a 16-color panel with excellent resolution, measuring challenging dye combinations with high fidelity, as well as the subtraction of cellular autofluorescence to improve resolution.

The detection technology employed by the Aurora consists of “proprietary high sensitivity Coarse Wavelength Division Multiplexing (CWDM) 16-channel semiconductor detector arrays for each laser”. Because the lasers are spatially separated and each laser’s detection path is assigned its own detector array, the color palette is theoretically limited only by the number of dyes available. Currently, a 3-laser (488, 561, 640 nm) is available, and systems with up to 5 lasers are being developed for release later in 2018.

Cytek has shown a 20-color panel with excellent resolution as well as the capability of the instrument to resolve difficult, normally undistinguishable, colors.

Second, panel choices effectively expand when using spectral cytometry, which permits dye combinations that would be difficult or nearly impossible to measure simultaneously using conventional cytometry