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Spectral Flow Cytometry—*Quo Vadimus*?

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FOR 40 years, flow cytometry has been dominated by polychromatic flow cytometry. Despite the fact that Fulwyler's instrument (1) was based on Coulter volume, fluorescence became the dominant technology within a very few years. From the early 1980s until the present day, the focus of technology developers has been on building instruments that have the capacity to measure more spectral bands, what we commonly refer to as "colors." For example, the first published paper on fluorescence flow cytometry was by Göhde (2) in 1968, closely followed by reports by van Dilla (3) at Los Alamos, both of which measured a single fluorescence signal. Within 10 years, Mike Loken determined that there would be a great advantage in being able to measure two parameters (4). Suddenly, the field of flow cytometry emerged from the world of single-parameter detection into multiparameter analysis.

The earliest three-color flow cytometry appeared in the early 1980s (5); within a few years three-color flow cytometry became repeatable (6), and by the mid-1990s, three-fluorescent parameter flow cytometry was commonplace, at which point four-color naturally appeared (7,8). Almost immediately the problem of compensation reared its ugly head (9), because it was clearly an issue that needed resolution; this became a classic case for workshops (10) that have been repeated literally hundreds of times to this day.

During the 1990s, it was clear that increasing the parameter space in flow cytometry was a mechanism for enabling discovery in biology. The ability to show direct and conclusive relationships between cellular receptors and functional roles at the single-cell level became the ultimate goal of flow-cytometry technology. While we already had a unique ability to link forward and side scatter to fluorescence, adding more fluorescent parameters provided directly correlated relationships that could not be identified by generic markers such as light scatter.

Of course, Mario Roederer and his team trumped everyone with 8-color flow cytometry by 1997 (11), 11-color by 2001 (12), and 17-color by 2004 (13). Between 2004 and the present day, there has been a steady increase in fluorescence parameters, and by 2014, it was pronounced that 40 parameters should be an achievable goal (14). To date, it is likely that about 27- to 30-color flow cytometry based on traditional polychromatic technology has been successfully achieved.

But the bottom line is that it has taken 50 years to move flow cytometry from a 1-parameter to a 30-parameter technology. One reason is the complexity of the hardware. An average 12-color flow cytometer would contain 12–14 independent detectors and 40-plus optical filters. Consider the components of a current 30-fluorescence parameter instrument. Thirty-two independent detectors (plus power supplies and preamps) and around 50- to 60-plus additional optical components are required—and this is only on the emission side of the instrument. The sheer size, complexity, and cost of manufacturing such complicated instruments are significant. It is now possible to purchase a commercial flow cytometer with over 50 parameters but with a price of around a million dollars. The problem is why does the continued direction of many manufacturers focus on traditional polychromatic and to a large extent outdated detection approaches? This current focus on old photomultiplier tube (PMT) technology, old filter technology, and worst of all, old compensation technology is not advancing the science of flow cytometry. There is an alternative that can exceed the capabilities of these polychromatic instruments, probably at a fifth of the cost.

It is called spectral flow cytometry. Our group first presented functional spectral flow cytometry at the congress of the International Society for Analytical Cytology (now the Society for Advancement of Cytometry) in 2004 (15); we then

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presented this in a Photonics publication the same year (16), followed by a patent issued to Purdue University in 2007 (17). The principle of the approach at that time was to use a 32-channel PMT array connected to a dispersion element; hardware components were reduced to just a couple of optics and a single detector array. I traveled the world promoting the technology and wrote NIH grants to obtain funds to develop the spectral flow cytometry technology. NIH reviewers thought it a ridiculous idea and refused funding despite clear evidence that spectral flow cytometry was not only feasible but also scientifically useful.

In 2011, Sony licensed the spectral technology patent from Purdue University and pursued the development of a spectral flow cytometer. Within a few years, several publications demonstrated >10-color spectral flow cytometry (18); spectral cytometry has subsequently achieved over 20-color flow cytometry (19) with excellent results using spectral unmixing. Indeed, the very goal of spectral flow cytometry was to transfer the focus of our attention from intensity of signal to spectral signature as the most significant parameter. We have for so long considered "brightness" to be the most important feature of a signal that we have ignored the power of a spectral signature. It is highly relevant that spectral flow cytometry can analyze fluorochromes that cannot be adequately separated by polychromatic flow cytometers.

I was recently asked a question about why spectral flow cytometry was not front and center for the flow-cytometry community. The answer is simple. Polychromatic flow cytometry is so embedded in both the commercial supply and the users that the transformation would appear to be too painful. I constantly ask the question "*Why do we spend so much time and energy focusing on compensation*?"—tutorial after tutorial, paper after paper, endless questions from users, when we could just remove this evil aspect of flow cytometry from the face of the earth. Do users really love the fact that they have to spend countless hours finding matching fluorochromes for their filters, changing filters, and matching fluorochromes with their target antigens? Is pain and anguish a necessary component of good flow cytometry?

Spectral flow cytometry is going to replace polychromatic flow cytometry eventually. It is not a matter of if, but when. No one claims that spectral flow cytometry is without challenges. Every technology has advantages and disadvantages. But the fact remains that flow cytometry is a technology based on spectroscopy. Current flow cytometers are the least advanced technologies in the field of spectroscopy, period. There is so much capacity to expand that it makes one excited for the future. It is time to change. *Quo vadimus*? It is in the tea leaves.

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