Blue and Bright:

The introduction of Brilliant Violet 421™, a novel blue-emitting fluorescent polymer for flow cytometry and microscopy applications

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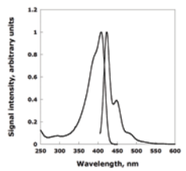
Abstract

Brightness is often a misused term in flow cytometry and microscopy. The brightness of an individual dye is a reflection of its capacity to absorb energy (extinction coefficient) and the efficiency with which it transfers that absorbed energy to an emission (quantum yield). More often, when people refer to brightness they are really referring to sensitivity. Sensitivity is that individual molecule brightness minus the fluorescent background of the biology and instrument noise in the application. Sensitivity in flow cytometry means that when a dye is dim, it becomes more difficult to resolve positive populations where the antigen is lowly expressed or in transition. In microscopy, where background autofluorescence subtracts from signal sensitivity, it often requires the use of secondary detection to amplify the signal. These inherent physical limitations consequentially lead to limitations in the number of antibodies able to be included in a polychromatic flow cytometry panel or multicolor microscopy assay. The few bright fluorophores that exist, like the proteins R-PE and APC with high extinction coefficients and high quantum efficiency, need to be reserved for very lowly abundant antigens in flow cytometry. In microscopy, these fluorescent protein families have extremely poor photostability and photobleach too quickly for practical use as direct conjugates. Other standard organic dye chemistries, like Pacific Blue™ and BD Horizon™ V450 are small structures with limited absorption capacity and photobleach quickly. They are often used only on relatively abundant markers in flow cytometry and require significant amplification to be useful in microscopy.

Introduction

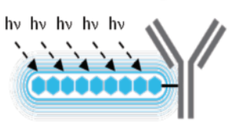
Here we introduce an entirely novel family of fluorescent molecules called Brilliant Violet™. The emission intensity of the Brilliant Violet 421™ is on par with that of R-PE, however this molecule is excited off the 405nm laser and emits into a 450nm bp filter (Figure 1., the Pacific Blue™ channel in flow cytometry). Members of this family of fluorescent molecules are organic polymers. Each monomer subunit absorbs the short wavelength 405nm energy independently but then unlike other fluorescent molecules, those monomers conduct energy to the next, moving energy down the length of the polymer like a lightning rod. This decentralized localization of energy increases the absorptive capacity and transfer efficiency resulting in an overall powerful output intensity (Figure 2).
The brightness of the Brilliant Violet 421™ can enable a wide range of useful titration in addition to its sensitivity detecting lowly expressed populations (Figure 3).

Figure 1.



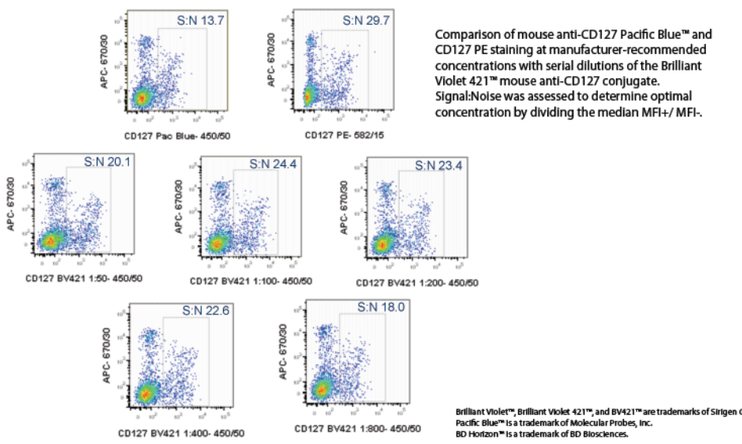
The excitation/emission peaks of Brilliant Violet 421™ are 405nm/ 421nm, suitable for a standard 450/40 emission filter in flow cytometry.

Figure 2.



A conceptual illustration of the Brilliant Violet™ polymer backbone.

Figure 3. Titration of Brilliant Violet 421™ anti-mouse CD127 conjugate

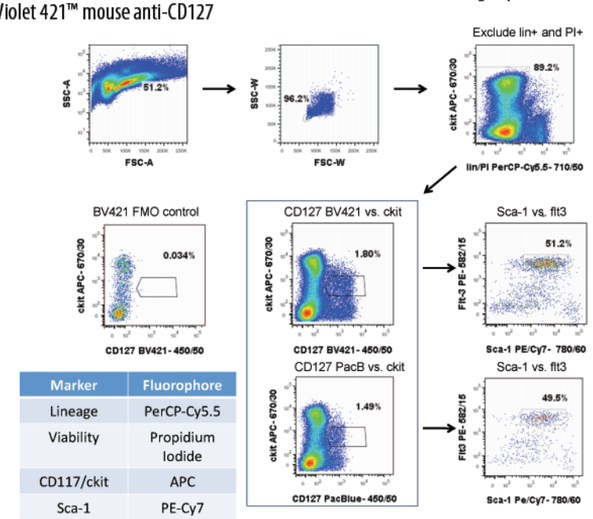


Comparison of mouse anti-CD127 Pacific Blue™ and CD127 PE staining at manufacturer-recommended concentrations with serial dilutions of the Brilliant Violet 421™ mouse anti-CD127 conjugate. Signal:Noise was assessed to determine optimal concentration by dividing the median MFI+/ MFI-.

Flow Cytometry Applications

An example of the utility of such a bright conjugate is in the detection of low expressing antigens on a lowly abundant cell population like CD127+ common lymphoid progenitor (CLP) population isolated from mouse bone marrow. With an expectation of less than 1.5% abundance of the CLP and CD127+ phenotype, sorting a few hundred million cells is required for sufficient sample for post-sort analysis.

Figure 4. Rare event detection of CD127+ CLP subsets in a 7 color cell sorting experiment using Brilliant Violet 421™ mouse anti-CD127



Marker	Fluorophore
Lineage	PerCP-Cy5.5
Viability	Propidium Iodide
CD117/ckit	APC
Sca-1	PE-Cy7
Flt3	PE
CD127	Brilliant Violet 421™

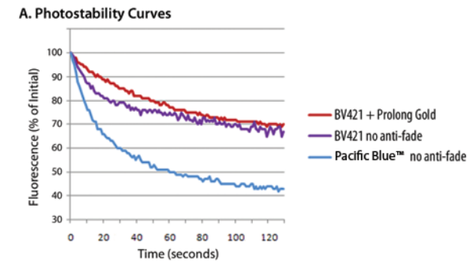
In this analysis, lymphocytes are isolated from FSC vs. SSC. Dead cells (PI+) and lineage+ cells are then excluded from the total lymphocytes. To identify lmphoid progenitors, CD117/ckit + vs CD127+ expression. In addition, expression of flt-3 and Sca-1 used to ID lymphoid subpopulation of interest. Sort was conducted on a 4-laser SORP ARIAII at the University of Chicago Flow Cytometry Facility.

Data in Figures 3 and 4 courtesy of Renee DePooter and David Leclerc, University of Chicago

Microscopy Applications

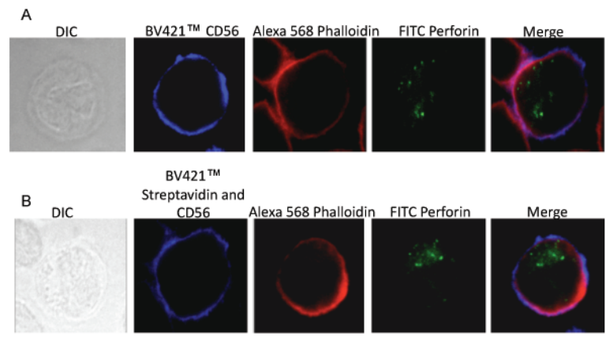
The Brilliant Violet 421™ direct conjugates also enable multicolor microscopy. Blue-emitting fluorophores often photobleach very quickly. Many dye families have been developed specifically to have greater resistance to photobleaching but with little success in the blue emission range of fluorophore. The Brilliant Violet 421™ shows superior photostability, with and without antifade protection as compared with Pacific Blue (Figure 5). In addition to the poor photostability of blue-emitting dyes, using direct conjugates is often insufficient amplification of an already dim fluorescent signal to be sensitive enough for phenotyping with antibodies. Thus, in microscopy we rely heavily on the use of secondary antibodies and streptavidin for amplification. However, since most primary antibodies are raised in rabbit, rat or mouse, there are often limitations as to how many primary antibodies can be detected with secondary antibodies exclusively. For example, detecting mouse primary antibodies on mouse tissue often has undesireable high background due to the non-specificity of the secondary antibody. Also, often two primary antibodies needed for an assay can only be found derived from rabbit. In these instances, even sequential staining will not enable the detection of those primary antibodies by separate secondary antibodies. Brilliant Violet 421™ can be used as both a direct conjugate for markers of moderate abundance or as an amplification method in the form of a streptavidin or secondary antibody (Figure 6).

Figure 5.



Photobleaching is plotted as a loss of a percent of the initial intensity of the fluorescent signal over time. Fluorescence was normalized to 100%, although the actual fluorescence units for Pacific Blue™ was significantly less than that of BV421™. Photobleaching was conducted on a spinning disc confocal, imaging for a total of 120 seconds, 300ms exposures every 1 second.

Figure 6.



DIC (differential interference contrast) image shows the overall boundaries of the cell. A direct CD56 BV421 conjugate was used in figure A and a streptavidin BV421 amplified CD56-biotin was used in figure B. A direct conjugate of FITC-Perforin and Alexa 568 phalloidin for direct actin detection were also used.

Data in Figure 6 courtesy of Emily Mace and Jordan Orange, University of Pennsylvania

Conclusions

- Brilliant Violet 421™ primary antibody conjugates offer an alternative to PE for resolving populations of lowly expressed antigens.
- Brilliant Violet 421™ is superior to the spectral equivalent Pacific Blue™ fluorophore in overall brightness and signal: noise resolution.
- The superior photophysical properties of the Brilliant Violet 421™ enables the detection of a rare event phenotype in flow cytometry.
- Brilliant Violet 421™ is very photostable, increasing it's utility in fluorescence microscopy.
- The brightness of a single molecule of Brilliant Violet 421™ makes it useful for detecting directly conjugated primary antibodies in fluorescence microscopy.

Learn more about Brilliant Violet™ antibodies at
www.biolegend.com/brilliantviolet.