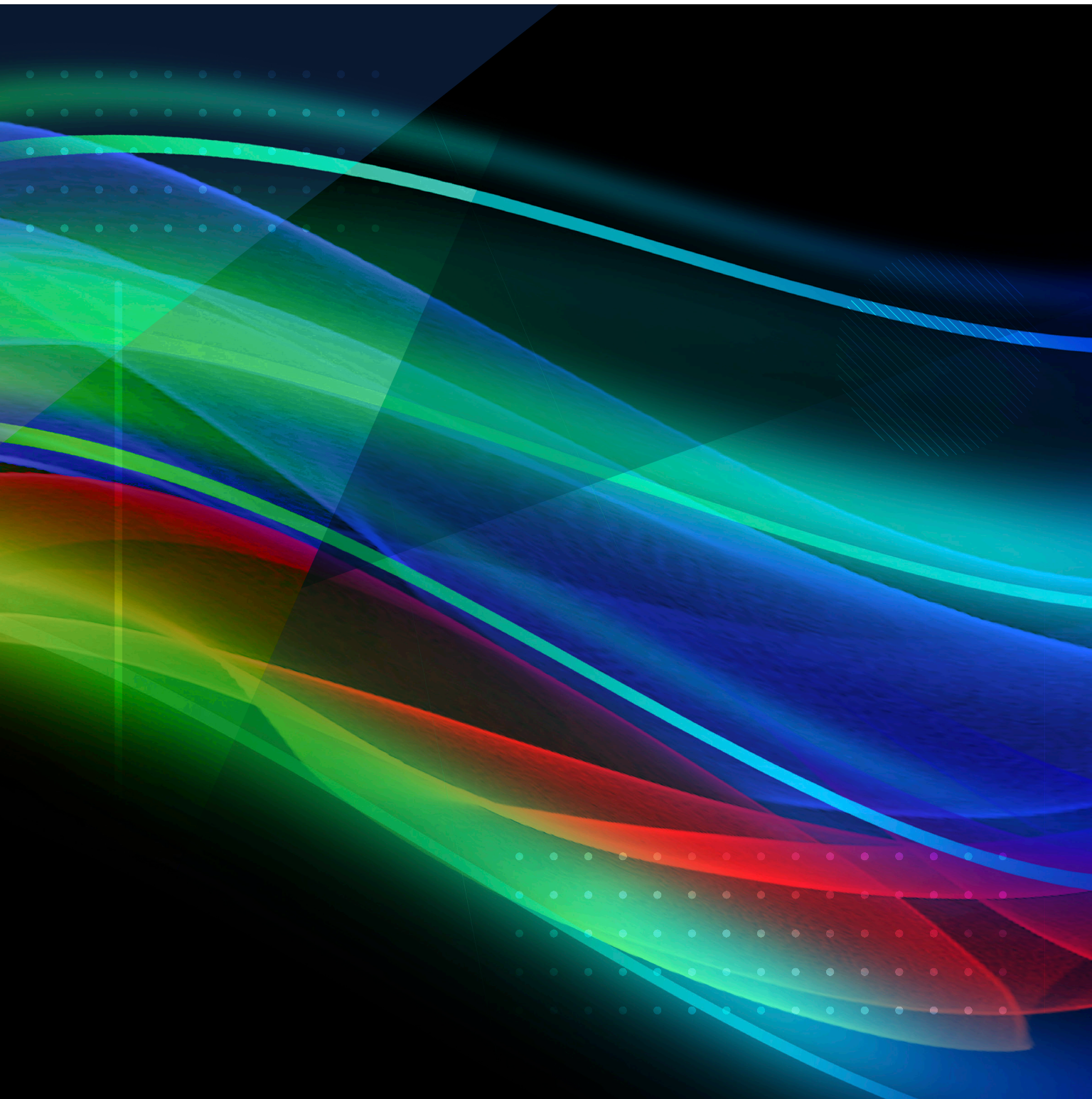
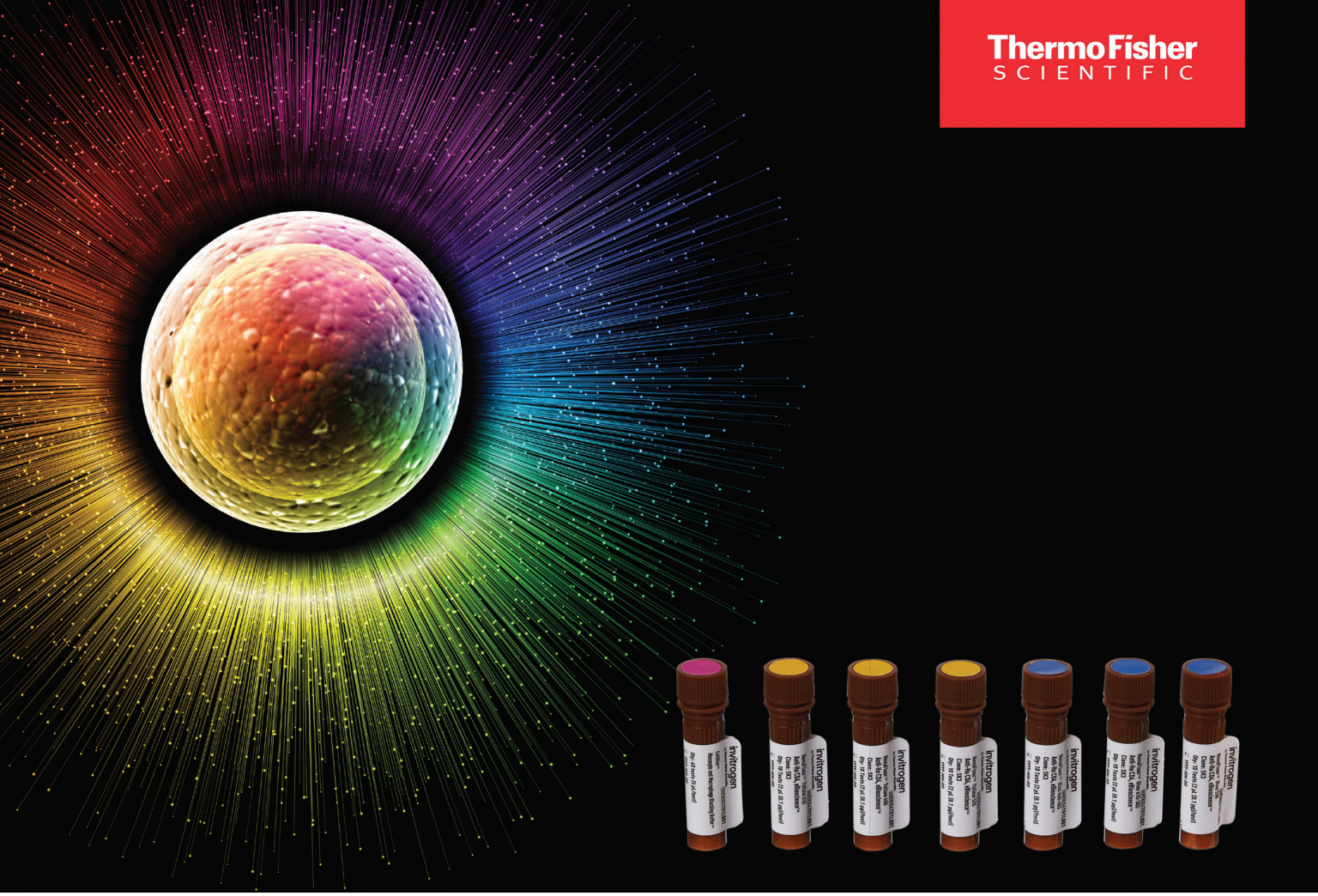
ThermoFisher SCIENTIFIC

Optimizing Fluorescence in Flow Cytometry Panel Design





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CHANGING THE RULES OF FLOW CYTOMETRY

New dye technology revolutionizes the world of flow cytometry by decreasing fluorescent spread and expanding the spectrum of uniquely labeled flow cytometry targets.

The intersection of immunology and virology fascinates Seddon Thomas. Immediately after graduating with a degree in chemistry, Thomas worked in a laboratory developing quantum dot technology. At the time, she was captivated by Laurie Garrett's book, *The Coming Plague*, which inspired her to pursue research on viruses. After completing her graduate degree at Harvard Medical School, where she studied how viruses circumvent the immune response, Thomas delved into the world of immunology and flow cytometry. As a postdoctoral scholar, she quickly became the go-to flow cytometry expert. Today, Thomas is still the go-to person for flow cytometry and specializes in optimal panel design. In 2020, she joined Phitonex, a biotechnology company that was acquired by Thermo Fisher, and used her expertise to help develop novel flow cytometry dyes called NovaFluors, that mitigate problems with large flow cytometry panels and increase the number of dyes that scientists can use to label and identify experimental targets.

What do you find most interesting about your work?

Scientists get used to certain written or unwritten rules that make them believe they cannot do certain experiments. In my work, I like breaking those rules. Poor fluorescence results are usually caused by bad dye performance, not incapable cytometers. For example, having an instrument with lots of detectors will not prevent spillover. I really enjoy seeing the dye-evolution process. I also relish working with a team comprising many backgrounds, from physics to chemistry to computer science. The immunologist in me likes to see how dyes directly impact flow cytometry research. Sometimes scientists talk about discovering new cells or seeing something new phenotypically that they could not see before using these new dyes.

How was NovaFluor technology invented?

Craig LaBoda, one of the founders of Phitonex, was working in the computer science space but had an engineering background. He developed lattice, cruciform DNA structures for nano computing and had an idea to attach molecules to these structures. He started to consider other real-world applications and examined other scientific problems. That is when he came across fluorophores. He asked himself, "If I could design a dye from scratch? What problem would that solve?" One of the great things is the Phitonex founders really stayed focused on solving the problem in flow cytometry that scientists are limited by dye performance. Craig had an engineer's understanding of lasers. He helped flow cytometry researchers understand that the problems they faced were caused by the dyes, not the lasers.

What problems have NovaFluor dyes solved?

Historically, using the older flow cytometers, scientists would have a blue laser and then a red laser, and that was it. Scientists detecting fruit dyes realized they needed a slightly different laser. Around that time, researchers started adding lasers to their instruments. They would move the detectors to yellow-green because with every PE tandem, they could not use the blue laser. It was one or the other, but not both. In contrast, if scientists use two different NovaFluor dyes, such as NovaFluor Blue 610 with NovaFluor Yellow 610, they can expand what they detect because the emission patterns of NovaFluor dyes are so clean they do not cause problems with spectral spread. Scientists can use more detectors and add them to existing panels to look at more parameters.

Seddon Thomas, PhD
Research Application Scientist
Flow Cytometry, Thermo Fisher Scientific

How do you help scientists understand the benefits of the new dyes for spectral cytometry?

Interestingly, a lot of scientists are not aware that cytometers are often built around certain problems that are related to the dyes. We educate researchers through webinars and other outlets and share our data. Spectral flow cytometry is different from conventional flow cytometry because it uses prisms to capture the emitted light from excited fluorophores across a set of detectors. With flow cytometry, emitted photons are collected into individual detectors. Early on, many people were not entirely sure how to use spectral cytometers. Marketing material suggested that spectral signatures were very different from conventional flow cytometry results. I think people thought that they could throw out a lot of the flow cytometry rules. However, like flow cytometry, there is a certain amount of background that will affect the ability to distinguish a positive signal. Scientists are still working on how to design panels for spectral cytometers, and we are figuring out how to help them.

What is the importance of panel design, and how do you help scientists design a good panel?

If it is possible to build a good 20-color panel, it is important to remember that it is also possible to build a bad 20-color panel. Sometimes researchers focus a lot on getting a specific number of colors, but then I look at their data and realize that they cannot use certain dyes together because they will cause background problems or spectral spread, even on a spectral instrument. You need to make sure that you are not building too much spread into your panel. This can happen cumulatively from one particular dye or due to using certain dyes together. Bad panels can create false positives.

When I was in graduate school, members of my laboratory published a paper to show that a certain result published by another group was an artifact caused by poor panel design. At the time, the researchers were using the bad data to argue that a certain cell type was more important from a therapeutic perspective. They shifted focus and money towards working on these pretty rare cells. We brought it to their attention and luckily managed to prevent more resources from being wasted. This shows that panel design, as well as other factors, has huge implications for therapeutics and other practical applications. This has happened before with cell markers as well. For a long time, the literature suggested that CCR3 was expressed on Th2 cells. However, over time, people realized that it was an experimental artifact. Bad results can influence the entire conversation in a field so much so that scientists struggle to publish the true results because they are not believed. Designing a good panel is sometimes a balancing act in matching the brightness required with spread. Scientists have to consider that some cells, such as alveolar macrophages, have some amount of autofluorescence, and others have nonspecific binding. When I'm helping people design a panel, I point out areas that they can gain an extra marker if they are willing to use two NovaFluors, with the caveat that they might need the brightness of a different dye if something has low expression. To help scientists make the best choices when they are designing panels, we have made information about NovaFluor dyes, such as separation index and spread data, easily available online.

What are you working on next?

One problem that we are trying to solve is intercalating viability dyes. They bind to DNA and either quench or alter the fluorescence of the NovaFluor dyes, so we are trying to find a way to protect them. Another area of active work is developing violet-light excitable dyes, which is something we do not yet have. We are also trying to develop dyes that have longer Stokes shifts, so more red-shifted dyes. We are asking, "Can we use this backbone to engineer something that does not exist yet?" Finally, we are expanding our antibody portfolio, getting NovaFluors onto more antibodies. Scientists will be able to use NovaFluors for additional markers without using conjugation kits, which should be really helpful.

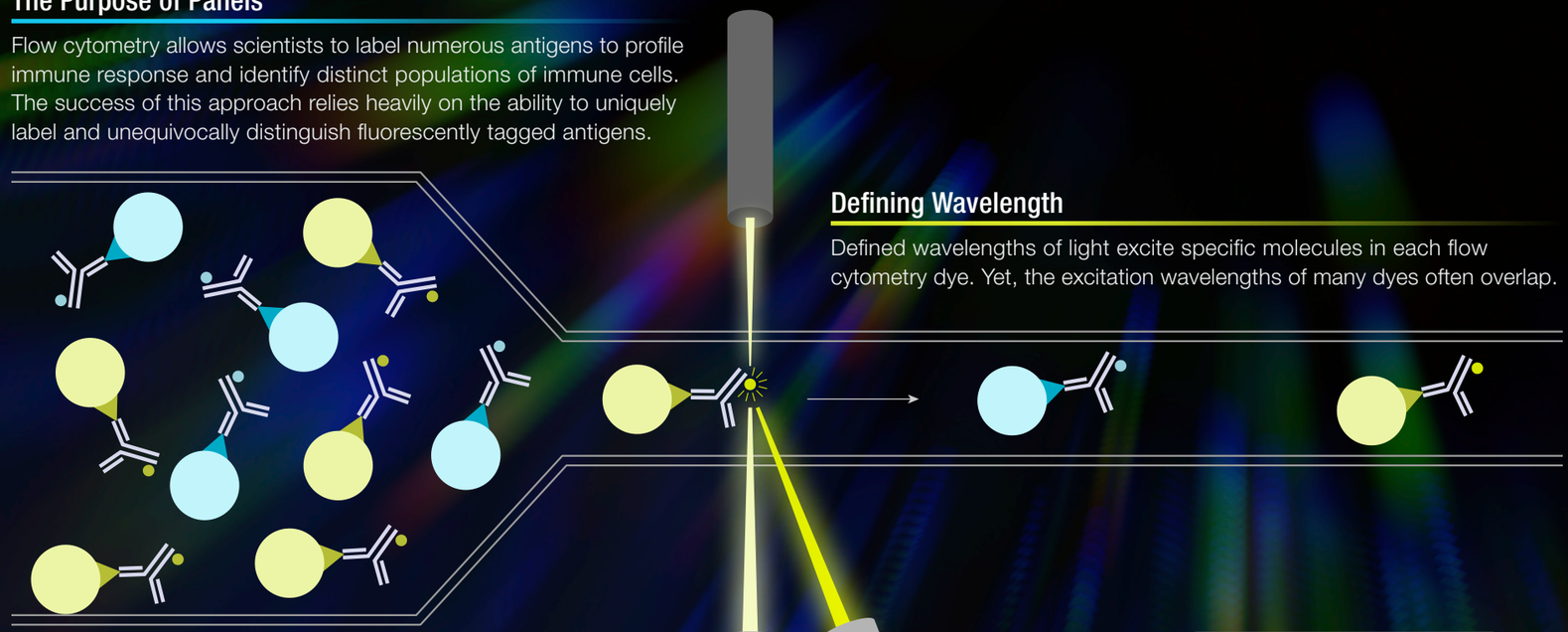
Help me with my panel design

Designing the Perfect Flow Cytometry Panel for Immunoprofiling

Building the ideal multi-color immunology panel relies on optimal dye selection.

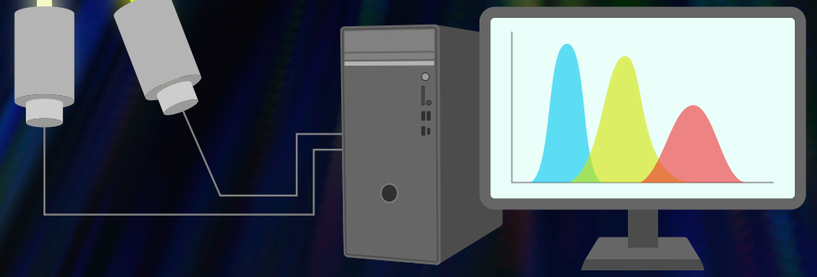
The Purpose of Panels

Flow cytometry allows scientists to label numerous antigens to profile immune response and identify distinct populations of immune cells. The success of this approach relies heavily on the ability to uniquely label and unequivocally distinguish fluorescently tagged antigens.



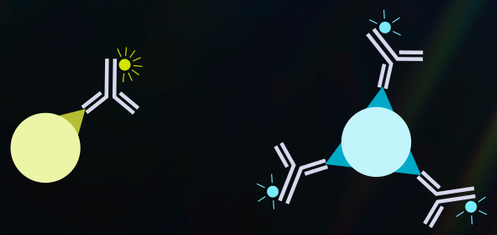
Defining Wavelength

Defined wavelengths of light excite specific molecules in each flow cytometry dye. Yet, the excitation wavelengths of many dyes often overlap.



Flow Cytometer Configurations

Instrument configuration affects dye performance. Some instruments use photomultiplier tubes (PMTs) that detect better in the UV and violet spectrum, whereas others use avalanche photodiodes (APDs) that detect better in red.



Consider Cell Characteristics

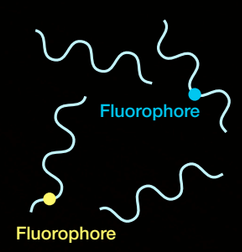
Cell types with low frequency or low amounts of an antigen may require **brighter** fluorophores. Higher frequency cells or cells expressing a higher amount of an antigen may require **dimmer** fluorophores.

Overlapping Spectrums

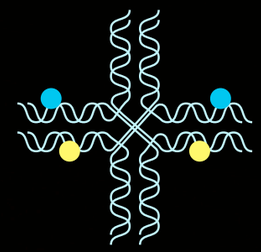
Broad excitation wavelengths cause broad emission patterns, where emitted light from one fluorophore bleeds into the emission profile of other fluorophores. Spectral spread confounds flow cytometry data and prevents the separation of two differently labeled antigens.

Solving Panel Design Problems with Better Dyes

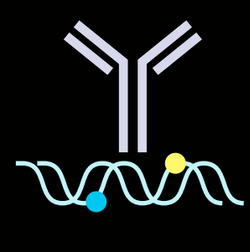
Thermo Fisher Scientific developed the Invitrogen™ NovaFluor™ dyes that maximize the number of antigens scientists can label with better separation and resolution. The NovaFluor technology produces unique fluorescent dyes that are engineered for excitation, emission, brightness and stability.



Small moleucle dyes are precisely positioned on single stranded DNA.



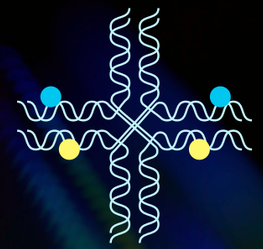
The ssDNA self assembles to from a stable DNA cruciform structure.

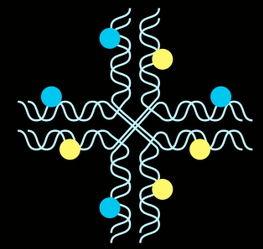


NovaFluor dyes link to antibodies via a linker sequence, allowing the labeling of cellular antigens.

Channeling Fluorescence

The fluorophores are placed at optimal distances on the DNA structure to allow FRET between donor and acceptor molecules.



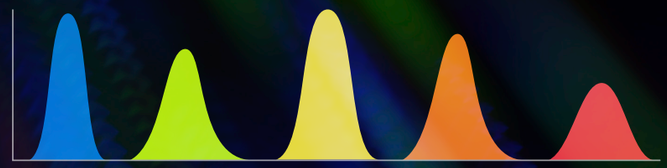


Engineered Excitation and Emission

Changing the pairs of donor and acceptor molecules changes the wavelength at which fluorophores excite and emit.

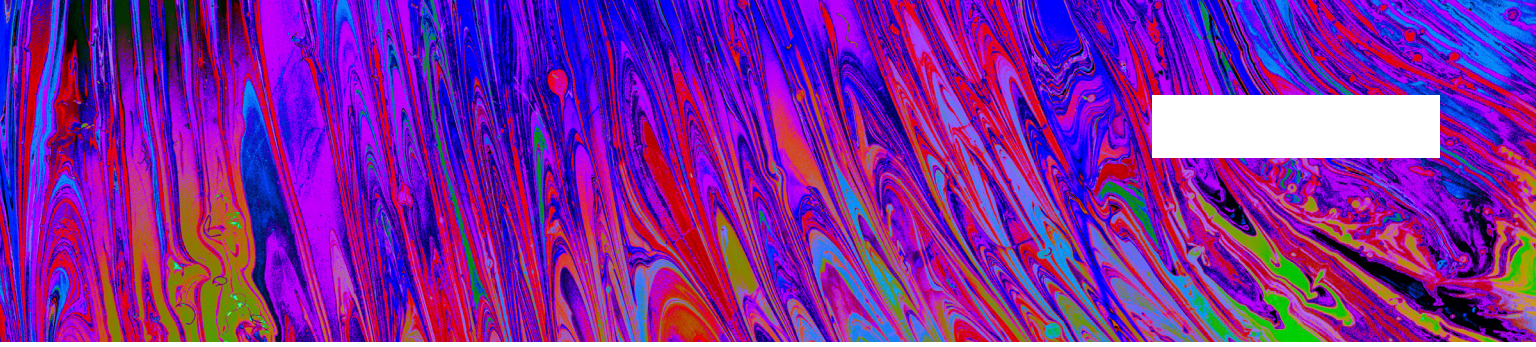
Controlling Brightness

Scientists can moderate NovaFlour brightness by adding additional donor and acceptor molecules to the cruciform DNA structure.



Expand Panels with Maximized Resolution

The defined excitation and emission spectrums of NovaFluor dyes decrease the possibility of spectral overlap, allowing scientists to fill in the gaps in their existing panels, add more target antigens, and confidently distinguish unique cell populations during immune profiling.



BUILT FOR BIOLOGY: CLEANER FLUORESCENT DYES

Plug-and-play dyes created using fluorophore-functionalized, folded, single-stranded DNA allow scientists to answer more biological questions.

Despite immense advances in flow cytometry hardware and dye portfolios in the last decade, researchers have reached a bottleneck where flow cytometry hardware has outpaced dye technology. When scientists use conventional dyes for flow cytometry, they may encounter problems in data resolution caused by spectral spillover and cross-excitation events. Spectral spillover occurs when the emission spectra of two dyes overlap. In contrast, cross-excitation occurs when a single dye is excited by multiple lasers. Due to these issues, scientists struggle to construct large, high-resolution flow cytometry panels using conventional dyes, even when flow cytometers have broad detection capabilities.

NovaFluor™ dyes are different. Scientists designed NovaFluor dyes from the bottom up for better resolution using Phiton™ technology. A Phiton comprises a fluorophore-functionalized, folded, single-stranded DNA (ssDNA). To create each Phiton, scientists precisely position small molecule dyes on synthetically-generated ssDNA, which then self-anneals to form a cruciform structure. The distance between dye donor and acceptor molecules on this structure allows scientists to better control dye excitation and emission. The technology leverages decades of research in DNA chemical modification, well-understood oligo synthesis for consistency, and DNA self-assembly to control fluorescent properties (1–11). Like conventional dyes, NovaFluor dyes can be conjugated to antibodies or other macromolecules for specific downstream applications such as flow cytometry. These uniquely engineered dyes have narrow emission spectra, which exhibit minimal cross-laser excitation with up to 70% less than conventional tandem dyes. NovaFluor dyes diminish the need for compensation, decrease spreading error, and increase opportunities to add new markers.

NovaFluor dyes also open up more channels for detection. The six new fluorescent labels (NovaFluor Blue 530, 610, and 660, and NovaFluor Yellow 570, 610, and 660) function as plug-and-play dyes. Scientists may use them in existing panels to increase the number of colors detected per cell or swap out dyes that are excited by multiple lasers or affected by spillover events. For example, scientists may choose to replace PE-eFluor 610 (which is excited by both the blue and yellow lasers) for NovaFluor Blue 610 and NovaFluor Yellow 610. Cell biologists can then ask more questions in a single experiment and more easily identify populations and clusters, leading to a deeper understanding of biology.

NovaFluor dyes are stable and easy to use. Although NovaFluor dyes should ideally be kept in the dark in the fridge, if someone mistakenly leaves them out, NovaFluor labels are stable at room temperature and will not lose fluorescence for at least three weeks. They even maintain their stability in harsh chemicals, such as formalin, for at least two weeks. Researchers can use them without special buffers and rest assured in their lot-to-lot consistency. According to spectral comparisons, NovaFlour dyes have less than 5% variation between lots for all six dyes. These properties appeal to scientists performing longitudinal and high-throughput studies where consistency, stability, and flexibility are key.

NovaFluor dyes allow scientists to perform broad, deep, high-resolution phenotyping experiments. With NovaFluors, researchers can increase the number of parameters across their flow cytometry panels and on extant instruments with near-zero cross excitation and the lowest spread performance of any blue and yellow-green excited fluorescent labels.

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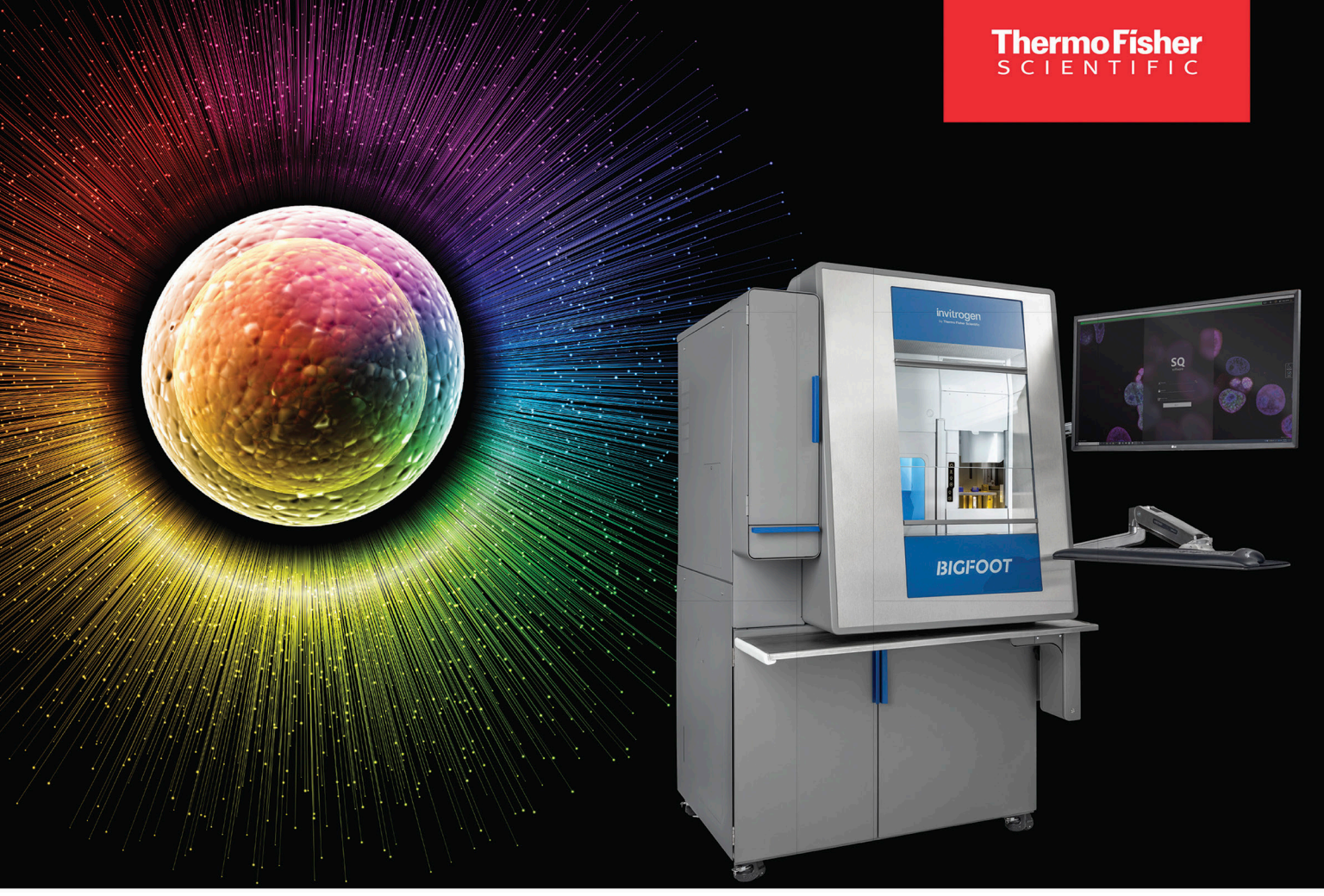

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