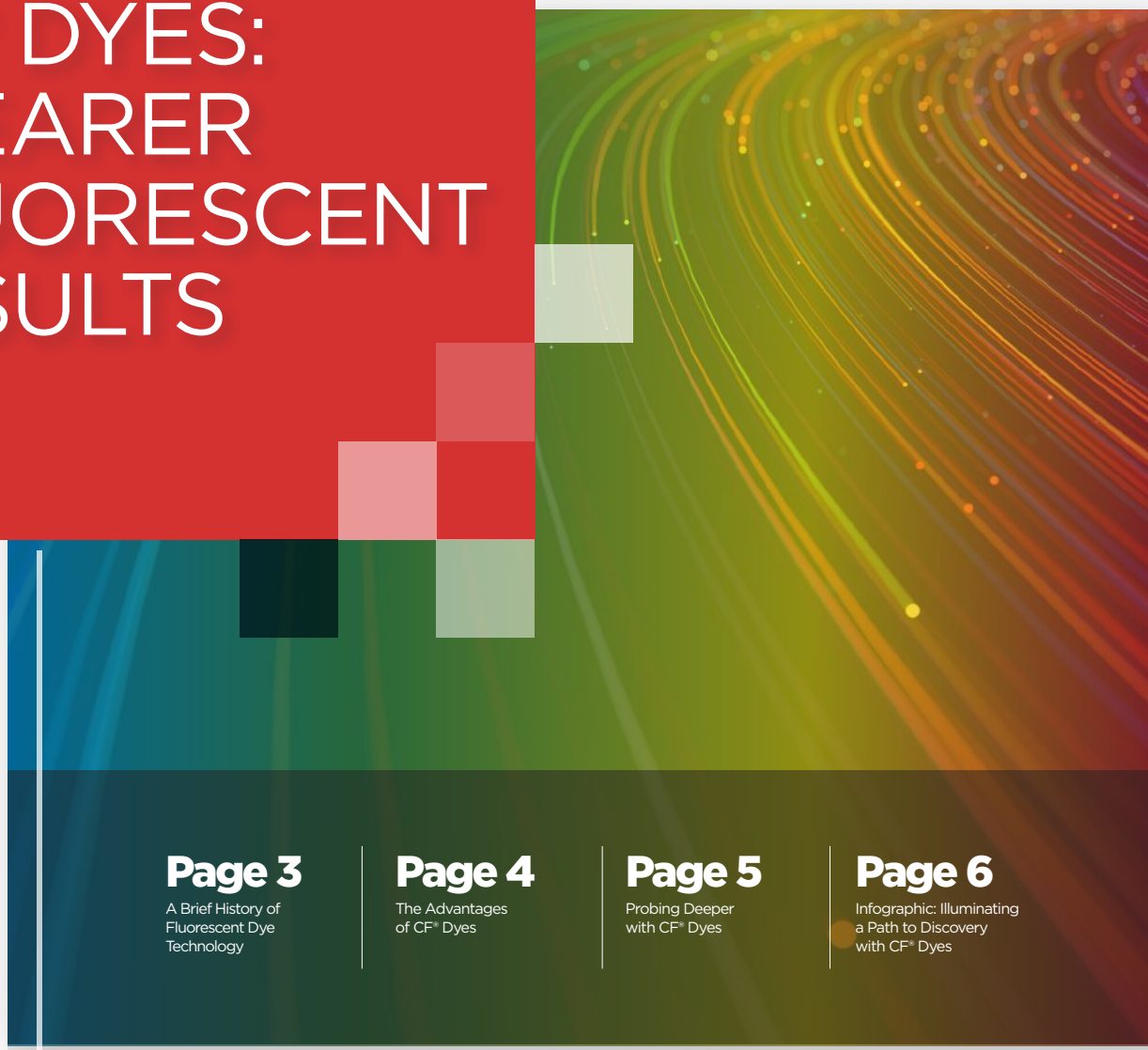
CF® DYES: CLEARER FLUORESCENT RESULTS



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A BRIEF HISTORY OF FLUORESCENT DYE TECHNOLOGY

Fluorescent dye technology has generally focused on fluorophores—small molecules that emit light observed by scientists' eyes and captured by their instruments' sensors.[1] The first recorded observation of fluorescence came in 1845, when Sir Frederik William Herschel noted that quinine, normally colorless, exhibited a "vivid and beautiful celestial blue" when illuminated by sunlight.[2] Roughly twenty-five years later, Adolf von Baeyer produced the first synthetic fluorophore dye, known today as fluorescein.[1] However, it was not until the 1940s that biologists truly recognized the utility of fluorophores in their field.

Fluorescence for Probing Proteins

In 1940, Albert Coons, a new research fellow at Harvard University, realized that labeled antibodies could help scientists localize specific proteins. Because chromogenic dyes offered insufficiently strong pigmentation when deposited in immunohistochemical samples, Coons looked towards fluorescent molecules.[3] In 1941, Coons, with the help of Harvard colleagues Hugh Creech and Norman Jones, successfully created a fluorescein isocyanate compound and conjugated it to an antibody. He used it to visualize pneumococcal antigens in infected tissue samples, thus giving rise to immunofluorescence staining. Although the technique has undergone countless adaptations, improvements, and enhancements in the 70 years since, its core underlying principle remains the same.[3,4]

Overcoming Obstacles

At the beginning, the biggest obstacle faced by practitioners of immunofluorescence staining was autofluorescence. Many biological objects, whether cells (e.g., red blood cells), proteins (e.g., collagen), or molecules (e.g., NAD(P)H and FAD) are fluorescent, and this can make it difficult or impossible to distinguish the signal being emitted by the tagged antibody.[5,6] Processes commonly applied by scientists, such as fixation, can also introduce or enhance autofluorescence. Indeed, Coons himself developed fluorescein because his initial choice, anthracene, was completely masked by autofluorescence.[3]

To overcome autofluorescence, scientists needed access to probes that covered different parts of the light spectrum. The natural world provided some opportunities, with the far red-emitting allophycocyanine (APC) and red-emitting phycoerythrin (PE) isolated in the mid-1980s.[7] Scientists also looked more closely at other xanthene-derivative compounds, particularly homing in on rhodamine. Rhodamine's emission peak falls within the visible-red portion of the spectrum, allowing researchers to mitigate tissue autofluorescence and enjoy improved photostability. As a result, not only did rhodamine gain popularity, but scientists developed several derivatives, including carboxytetramethylrhodamine (TAMRA), tetramethylrhodamine (TRITC), and sulforhodamine 101 acid chloride (Texas Red®).

A New Nineties Generation

Unfortunately, rhodamine dyes displayed poor solubility. This drove Alan Waggoner at Carnegie Mellon University to investigate potential alternatives. As an amateur photographer, Waggoner was familiar with using cyanine dyes to produce colors on photographic film. He thus worked in the 1990s to functionalize these dyes as fluorescent probes.[8,9] Not only were these dyes more soluble, they also allowed for greater biochemical flexibility and less dye-to-dye quenching. The excitation/emission spectrum of a fluorophore could be modified by simply altering the length of the polymethine chain present within the molecular structure, and cyanine dyes thus presented a broad emission range extending into the red and near-infrared (NIR) portions of the spectrum.

However, both xanthene- and cyanine-based dyes still suffered from a significant fluorescence decrease post-conjugation. In response, Waggoner introduced negatively charged sulfonic acid groups to cyanine dye molecular structures—a process known as sulfonation. Sulfonation reduced interactions between dye molecules post-conjugation and limited the formation of self-quenching aggregates. It also made fluorophores more hydrophilic and soluble, allowing for greater biocompatibility at longer wavelengths. Within a decade, researchers were able to apply Waggoner's innovative strategy to xanthene-based dyes as well.[10,11]

Today and Tomorrow

Today, fluorescent probes are nearly ubiquitous in the life sciences, used to label cells and study their behaviors, quantify and characterize cell activity during systemic responses, investigate protein-protein interactions, track signaling pathways, and better understand tissue structure and composition, just to name a few applications. Nonetheless, probe technology is far from finished, with scientists constantly looking for improvements—whether that means modifying existing probes or developing new ones—to accommodate constantly changing research needs.

See references on page 7.

THE ADVANTAGES OF CF® DYES

Fluorescent dye technology has come a long way since the 1940s. However, while sulfonated dyes allow for greater biocompatibility by making fluorophores more hydrophilic and soluble, they promote non-specific binding to positively charged biomolecule moieties, leading to decreased specificity, elevated background staining, and decreased image contrast.

Creating CF® Dyes

In response to this problem, Biotium co-founder Fei Mao and his colleagues modified sulfonated fluorescent dyes by attaching polyethyleneglycol (PEG) polymers. This process, known as pegylation or PEG modification, physically impeded interactions with the negatively charged sulfonate groups. Pegylated dyes presented significantly diminished background fluorescence and enhanced signal-to-noise ratio. Moreover, the bulky polymers further reduced dye aggregation and improved solubility, leading to increased quantum yield and biocompatibility. The benefits of tandem pegylation and sulfonation led to the creation of CF® Dyes, which offer the advantages of both chemical modifications without compromising specificity.

Although the "CF" initially stood for "cyanine-based fluorescent" dyes, the CF® Dye portfolio quickly expanded beyond cyanine-based molecular structures. Mao and his Biotium team discovered that replacing the benzene ring present in rhodamine's core structure with an imidazole group shifted the emission wavelength by 30 to 60 nm towards near-IR. This made rhodamine-based near-IR dyes possible, finally creating a solution that combined rhodamine's photo- and chemical stability and improved solubility with the spectral flexibility and autofluorescence avoidance offered by near-IR emission.

Function and Flexibility

Since their invention, the CF® Dyes portfolio has expanded to over 40 dyes, spanning the full spectrum from 350 nm (blue) to 870 nm (near-IR). This expansive portfolio is designed with multiplexing in mind, offering exceptional flexibility for panel building and other applications where simultaneously probing multiple targets is imperative. For example, scientists use fluorescence microscopy to investigate protein distribution and interaction within a spatial context—relying on co-localization of signals from two or more fluorophores to detect protein-protein interactions or to confirm the identities of the cells expressing the target of interest. Researchers use a similar strategy in flow cytometry, using cell marker expression as a means of characterizing cellular phenotypes.

When analyzing co-localization, superior probe brightness limits false negatives, while probe specificity helps prevent false positives. Additionally, researchers must plan carefully so that the probes do not generate false positives by spectrally overlapping with each other or with autofluorescence emission spectra. The CF® Dye portfolio gives scientists the best of both worlds, combining specificity and brightness with flexibility. Researchers can choose from over 2,000 primary monoclonal antibodies, each available in six CF® Dye colors, as well as more than twenty colors for their secondary antibody needs. CF® Dyes are also available as bioconjugates with widely used non-antibody probes such as lectins, Annexin V, and dextran. Finally, CF® Dyes include spectrally-unique options that are useful for spectral flow cytometry, and include many dyes validated for—or even designed specifically for—super-resolution techniques such as stochastic optical reconstruction microscopy (STORM) (see page 5 for more information).

New Tricks with Near-IR

The ability to make bright conjugates of near-infrared dyes have led scientists to incorporate fluorescence into established techniques. For example, fluorescent western blotting in the near-IR range delivers a quantifiable linear signal profile and is multiplex-capable.[1] The unique chemistry of certain CF® Dyes, particularly CF®680 and CF®770, offers superior solubility, brightness, and signal-to-noise for western blotting compared to other fluorescent dyes. Additionally, near-infrared dyes possess greater tissue penetration for in vivo imaging, and there is less endogenous autofluorescence in the near-IR portion of the spectrum, allowing for a clearer signal. Finally, near-IR CF® Dyes now have emission as high as ~900 nm, thereby giving researchers more multiplexing flexibility for microscopy, flow cytometry, and immunofluorochemistry by expanding their options within the near-IR range.

See reference on page 7.

PROBING DEEPER WITH CF® DYES

As scientists unravel the finer details of biological systems, they need technology that will enable them to see more precisely, gather more comprehensive data, and do these things within an expedient timeframe. Probe development is done with these needs in mind, and the latest generation of CF® Dyes helps researchers tap into the full potential of high-resolution and high-throughput techniques.

Enabling a Clearer Picture with Spectral Flow Cytometry

Flow cytometry is a prolific technique that provides scientists with highly specific information on individual cells within a sample. However, conventional flow cytometry partitions light into narrow bandwidth ranges for detection, and thus can only measure as many parameters as there are filters. By contrast, spectral flow cytometry uses dispersive optics to capture the entire emission spectrum for each measured fluorophore. This gives scientists with ever-increasing experimental demands the ability to simultaneously measure many more parameters than conventional flow cytometry.[1,2]

The ability to simultaneously probe 30-40 parameters is crucial for researchers investigating complex and dynamic systems, such as the immune response, that would otherwise need to use three or four separate panels to gather the same amount of data.[3] However, to take full advantage, scientists still need access to suitable spectrally unique dyes that permitted unmixing. In a 2021 study, Biotium scientists, together with colleagues from the University of California, Los Angeles and Cytek Inc., showed that CF® Dyes were not only well suited for spectral flow cytometry, but that they outperformed other existing dyes.[3] The spectrally unique colors offered by CF® Dyes provided more multiplexing options, enabling the creation of high-dimensional panels that reach the aforementioned 30-40 colors.

Special Solutions for Super-Resolution Microscopy

Due to the physical limitation of light diffractions, conventional (light) microscopy can only resolve cellular structures to roughly 200 nm. Super-resolution microscopy (SRM) refers to techniques that can break this diffraction limit to achieve higher resolutions.[4] Multiple CF® Dyes have been validated for SRM methods including structured illumination microscopy (SIM), stimulated emission depleted (STED) microscopy, and stochastic optical reconstruction microscopy (STORM). Of these techniques, STORM offers the highest resolution.[5] First pioneered in 2006, STORM constructs fluorescent images by localizing individual fluorescent molecules that are switched on and off using light of different colors. Because only a fraction of the fluorophores in the field of view are switched on during each cycle, they do not overlap with each other and can be resolved positionally with high accuracy. Generating such images across multiple cycles and then using them to reconstruct an overall image can yield an imaging resolution of approximately 20 nm.[4]

The nature of STORM means that extremely precise control over fluorophore excitation, emission, and light capture is necessary. Researchers performing STORM therefore commonly rely on photoswitchable red-excited dyes such as CF®647, as well as the cyanine-based CF®660C and CF®680 and their rhodamine-based counterparts CF®660R and CF®680R. Indeed, when Lehmann and colleagues examined 28 different commercially available dyes for STORM suitability, they found eight candidates with good-to-excellent photochemical switching properties. Of those eight, only three dyes were suitable for high quality 2- or 3-color STORM with low color crosstalk: CF®647, CF®680, and CF®568.[6]

A major obstacle impeding high-quality multicolor STORM is a lack of suitable dyes outside of the red excitation range. However, Biotium is helping to change this landscape. In 2021, the company, together with STORM scientist Ke Xu from the University of California, Berkeley, developed two green-excitation dyes specifically for STORM: CF®583R and CF®597R.[7]

Rhodamine dye photoswitching, when induced under reducing conditions, generates a long-lived photoactivated radical dark state that reverts to a fluorescence emitting state with violet light or the addition of oxygen. Realizing this, Biotium researchers replaced the benzene ring in the rhodamine core with a positively charged 1,3-disubstituted imidazolium. Xu's team then found that the resulting dyes, CF®583R and CF®597R, were markedly sensitized toward photoswitching and outperformed the older generation of green-excited STORM dyes—generating data on par with red-excited dyes.[7] This opens the door for expanded multicolor STORM imaging strategies and the development of other fluorescent dyes for STORM.

See references on page 7.

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ILLUMINATING A PATH TO DISCOVERY WITH CF® DYES

CF® Dyes combine pegylation and sulfonation to create fluorescent dyes with better signal intensity, more solubility, and superior specificity. CF® Dyes are ideal for a wide range of applications, from western blotting to in vivo imaging.

WIDE LINEAR RANGE

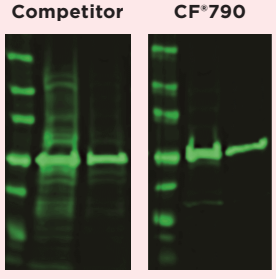
Biotium's bright and photostable near-IR CF® Dyes allow for a strong and stable signal with excellent linearity over a wide range.

MULTIPLEXING

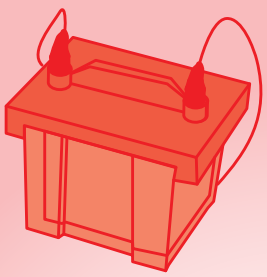
Unlike chemiluminescence, fluorescent western blotting with dyes such as CF® Dyes enables same-blot multiplexing for deeper analysis.

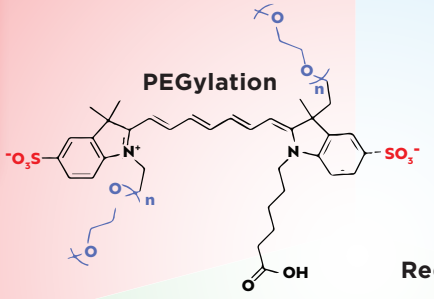
SIGNIFICANTLY LOWER BACKGROUND

PEG modifications significantly reduce nonspecific background from charged fluorescent dyes, allowing clearer results analysis.



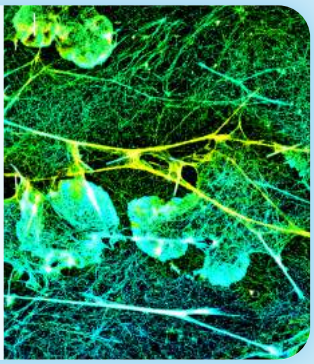
Western Blotting



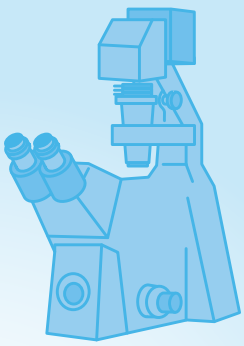


UNIQUE DYES FOR SUPER-RESOLUTION

Multiple CF® Dyes are validated for super-resolution imaging including SIM, STED, and STORM, including best-in-class dye options developed specifically for STORM.

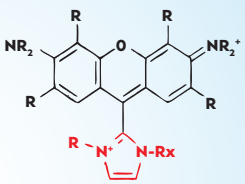


Fluorescence Microscopy



SUPERIOR BRIGHTNESS

Chemical modifications including PEGylation improve biocompatibility, solubility, and brightness and lower non-specific background for improved imaging results.



Red-shifted Rhodamines

UNRIVALED PHOTOSTABILITY

Biotium's red-shifted rhodamine dyes allow for significant improvements to photostability in the far-red emission compared to competitor dyes.

Flow Cytometry

NARROW EMISSION SPECTRA

CF® Dyes have narrower emission spectra than similar commercially available dyes, allowing for less spillover and easier spectral unmixing.

EXCEPTIONAL SIGNAL-TO-NOISE

PEG modifications improve solubility and reduce dye self-quenching, leading to conjugates with exceptional signal-to-noise.

SPECTRALLY UNIQUE

Several CF® Dyes, particularly in the far-red range, have unique spectral profiles, allowing for greater multiplexing by spectral cytometry.

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