CF™ Dyes: Enhanced Fluorophores with Novel Dye Chemistry

On behalf of Sigma® Life Science: Lori M. Roberts, Ph.D., Senior Scientist at Biotium Inc.

CF™ dyes are a series of highly water-soluble fluorescent dyes spanning the visible and near-infrared (near-IR) spectrum (Figure 1) for labeling antibodies, proteins, nucleic acids, and other biomolecules. Developed by scientists using new breakthrough chemistries, the brightness, photostability, and color selection of CF dyes rival or exceed the quality of other commercial dyes as a result of rational dye design.

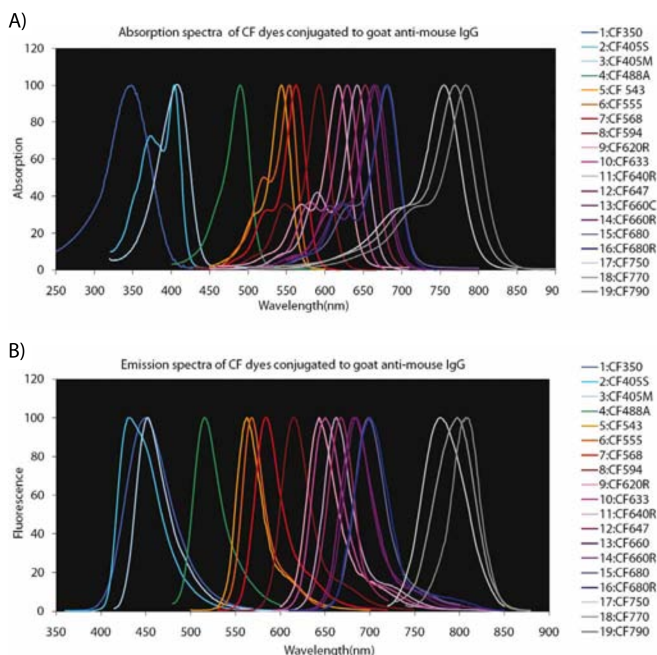


Figure 1. Absorbance and emission spectra of CF Dyes conjugated to goat anti-mouse IgG.

Novel Rhodamine Chemistry

Rhodamine dyes are known for their excellent photostability and good fluorescence quantum yield; consequently several of the Alexa Fluor® dyes bear the rhodamine core structure.[1] Traditional rhodamine chemistry makes it difficult to extend the fluorescence wavelength to the far-red region[2] and even more challenging in the near-IR region, while maintaining water-solubility, a requisite for bioconjugation. A novel rhodamine dye chemistry enabled scientists at Biotium to develop new rhodamine-based dyes with fluorescence ranging from green to near-infrared. As a result, far-red CF dyes are highly fluorescent, water-soluble, and significantly more photostable compared to other far-red dyes (Figure 2).

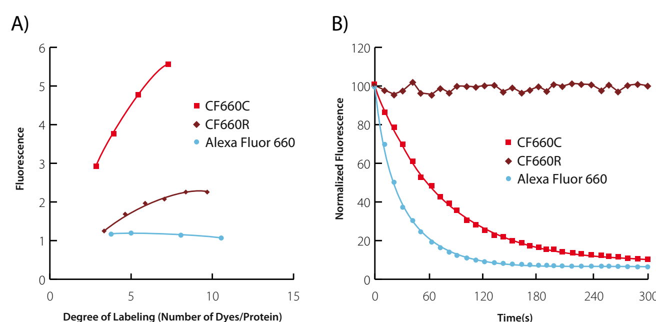
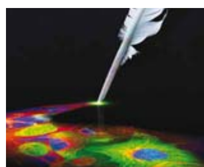


Figure 2. Comparison of fluorescence intensity and photostability for far red CF660C, CF660R, and Alexa Fluor 660 goat anti-mouse IgG conjugates.

A) Relative fluorescence of CF660C, CF660R, and Alexa Fluor 660 goat anti-mouse conjugates as a function of number of dyes per protein (degree of labeling). Fluorescence was measured at each dye's emission maximum in PBS using 633 nm excitation.

B) Photostability of far red dyes CF660C, CF660R, and Alexa Fluor 660. HeLa cells were fixed, permeabilized and stained with mouse α-tubulin followed by CF660C, CF660R or Alexa Fluor 660 goat anti-mouse IgG conjugates. Cells were imaged using an Olympus mercury arc lamp microscope equipped with a Cy5 filter set and CCD camera. The graph illustrates the relative fluorescence intensities of sequential images taken every 10 seconds for five minutes.



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Unrivaled Near-IR Dyes

Near-infrared dyes offer important advantages over visible light dyes. Autofluorescence in the visible spectrum can be a serious impediment to fluorescence imaging of tissue specimens. Because cells and tissues generate minimal autofluorescence in the near-IR emission wavelengths,[3] near-IR dyes have the potential to offer highly specific and sensitive detection in complex biological systems. The fluorescence emitted by near-IR dyes can penetrate tissues for up to several centimeters,[4] permitting small animal *in vivo* imaging of tissues and tumors (Figure 3). Near-IR dye-conjugated antibodies are useful for Western blotting and cell-based protein quantitation assays due to the broad linear dynamic range of near-IR fluorescence compared to chemiluminescence,[5] and the ability to perform multiplex Western blotting with near-IR dye conjugates (Figure 4).

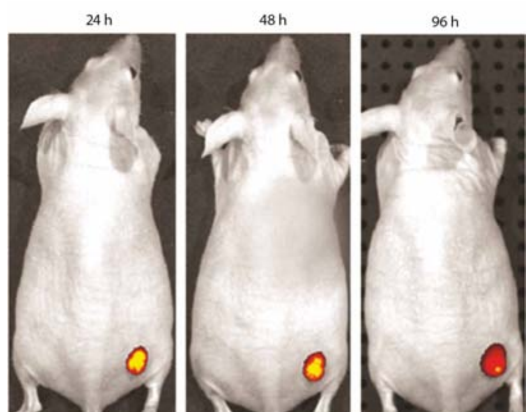


Figure 3. *In vivo* imaging of tumors with a tumor-targeting near-infrared CF750 antibody conjugate.

Tumors in mice were imaged using an IVIS imaging system (Caliper Life Sciences) 24 hours, 48 hours, and 96 hours after i.v. injection of CF750-Avastin conjugate. Images courtesy of Caliper Life Sciences.

Near-IR CF™ dyes are next-generation long wavelength dyes representing a true breakthrough in the field. Near-IR dyes are typically much larger in size than dyes in the visible range. The large size often results in serious problems of low dye solubility, dye aggregation, and poor fluorescence quantum yield. To overcome these problems, many near-IR dyes, such as the near-IR Alexa Fluor® dyes, DyLight® dyes, and IRDyes® incorporate negatively charged sulfonate groups in the dye structure.[6] While sulfonation improves dye solubility and fluorescence quantum yield to some degree, the addition of negative charge can contribute to non-specific binding of bioconjugates prepared from the dyes. For example, conjugation to a highly negatively charged dye can dramatically alter the isoelectric point (iP) of antibodies, which can alter the specificity of antibody-

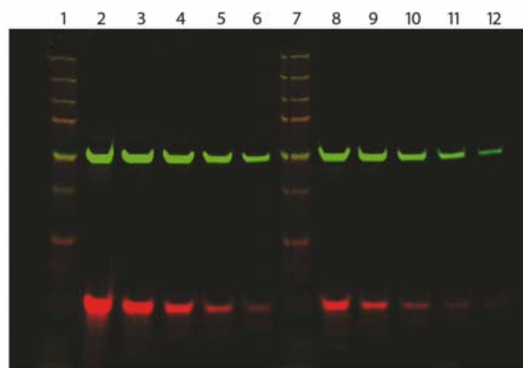
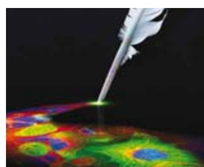


Figure 4. Multiplex Western blotting with near-infrared antibody conjugates.

Two-fold dilutions of HeLa cell lysate were run on an acrylamide gel, transferred to a nitrocellulose membrane and probed with mouse anti-tubulin and rabbit anti-COX IV primary antibodies followed by goat anti-mouse CF770 (green, lanes 2-6) or IRDye 800 (green, lanes 8-12) and goat anti-rabbit CF680 (red, lanes 2-6) or IRDye680 (red, lanes 8-12) at the same final concentration. Membranes were scanned using an Odyssey® infrared imaging system (Li-Cor Biosciences). Quantitation of the bands showed approximately a 3.5-fold higher fluorescence intensity of CF dyes compared to IRDye secondary antibodies. Lanes 1 and 7 contain Odyssey Two-Color Molecular Weight Markers (10-250 kDa).

antigen interaction. Non-specific charge-based background has been reported for antibodies conjugated to fluorescein, which carries two negative charges.[7] In comparison, some cyanine-based Alexa Fluor dyes carry up to four negative charges,[6] while the near-IR dye Alexa Fluor 790 carries at least five negative charges based on the number of counter ions associated with the dye.[8] Near-infrared Cy5.5[9] and IRDyes[10-13] also carry multiple negative charges. Excessive negative charge can contribute to non-specific binding of antibody conjugates (Figure 5A). Also, alterations in antibody iP have been shown to affect antibody distribution and pharmacokinetics *in vivo*, with decreased positive charge resulting in decreased retention of labeled antibodies in tissues and increased clearance of antibody conjugates from blood.[14]

With this insight, scientists devised a revolutionary new approach to near-IR dye design that results in superior physical properties of the dyes without introducing an excessive amount of negative charge. Near-IR CF dyes are based on the core structure of either cyanine dyes or rhodamine dyes. Those core structures are modified so the intramolecular mobility of the dye structure is restricted, leading to higher quantum yield and better water solubility without adding excessive charge. As a result, near-IR CF dyes are brighter and more photostable than any other near-IR dyes. Most importantly, antibodies labeled with near-IR CF dyes give far better signal-to-noise ratio in immunostaining compared with antibody conjugates prepared with other commercial near-IR dyes (Figure 5B).



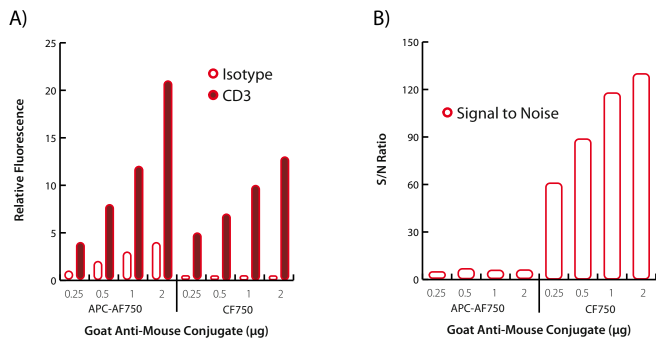


Figure 5. Specificity of CF750 antibody conjugate compared to APC-Alexa Fluor 750 antibody conjugate.

Jurkat cells were stained with isotype or mouse anti-human CD3 antibody followed by goat anti-mouse APC-Alexa Fluor 750 or CF750 using the amount of antibody shown. Fluorescence was analyzed using a BD LSR II flow cytometer.

A) Bars represent the relative fluorescence values of the geometric means.

B) Bars represent the signal to noise ratio (CD3 geometric mean/Isotype geometric mean). Error bars represent standard deviation of the mean, n=2.

Enhanced Stability of Reactive Dye Derivatives

Reactive dyes for bioconjugation are generally susceptible to hydrolysis, which can result in lower labeling efficiency. Heavily sulfonated dyes, such as the Alexa Fluor® dyes, DyLight® dyes and IRDyes® are particularly hygroscopic, worsening the hydrolysis problem. For example, the percent of active Alexa Fluor 488 succinimidyl ester (SE) could be well below 50% by the time of application.[15, 16] In a number of Alexa Fluor SE reactive dyes, the SE group is derived from an aromatic carboxylic acid, while in all CF™ dyes the SE group is prepared from an aliphatic carboxylic acid. This structural difference reduces the susceptibility of CF dye SE reactive groups to hydrolysis, resulting in relatively stable reactive dyes with consistently higher labeling efficiency compared to other SE derivatives of other fluorescent dyes (supplier data).

Mix-n-Stain™ Antibody Labeling Technology

Direct labeling of primary antibodies is useful for flow cytometry applications and when primary antibodies from different species are not available for multicolor immunofluorescence experiments. Typically, labeling antibodies with reactive dyes requires antibody buffer exchange, long incubation times, and time-consuming purification steps to remove unconjugated dye.[17, 18] Mix-n-Stain antibody labeling kits are a breakthrough technology for simple and rapid small scale antibody labeling. Reaction of antibody with the optimized dye and buffer provided in the kit for 30 minutes yields optimally labeled CF dye-antibody conjugate ready for immunostaining without a post-labeling purification step (Figure 6). Dye labeling of antibody is covalent, generating stable antibody conjugates, and the reaction can tolerate common antibody storage buffer components such as azide. Mix-n-Stain labeling technology provides unprecedented convenience for multicolor immunostaining, especially when pre-labeled primary antibodies are not commercially available, or when indirect staining via pre-labeled fluorescent secondary antibodies is problematic due to cross-interaction among secondary antibodies.

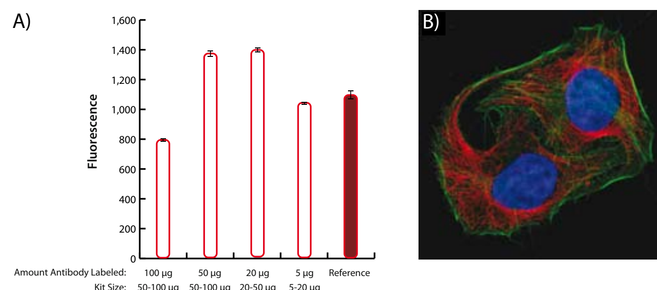


Figure 6. Evaluation of CF Dye Mix-n-Stain labeled antibodies.

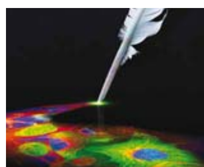
A) Flow cytometry analysis (on BD FACS Calibur) of Jurkat cells stained with CF633 Mix-n-Stain labeled mouse anti-human CD3 antibodies. For reference, cells were stained with commercial Alexa Fluor 647-mouse anti-human CD3. Bars represent the relative fluorescence values of the geometric means. Fluorescence was analyzed using a BD FACSCalibur flow cytometer.

B) HeLa cells were stained with β-tubulin IgM conjugated with CF633 Mix-n-Stain (red), followed by CF488A phalloidin (green) and DAPI (blue). Images were captured on a Zeiss 510 Meta confocal microscope.

Sigma® currently offers 19 CF dyes spanning the visible and near-IR wavelengths, with additional colors in development. The CF dye product line includes reactive CF dyes, labeling kits, CF-labeled secondary antibodies, and other bioconjugates. This collection further expands Sigma's broad range of carefully selected secondary antibodies and conjugates, allowing scientists to achieve greater sensitivity, brighter results, and better photostability in immunoassays.

To help researchers evaluate their secondary antibody products with complete peace of mind, Sigma is offering the Bioguarantee* on all CF dye labeled antibody products. This warranty applies to Sigma's complete collection of over 50,000 world-class primary and secondary antibodies, allowing customers to claim a full credit or replacement product should the purchased antibody product not perform in their application or species of interest.

*Experimental results must be submitted via the Antibody Bioguarantee Form within 12 months of the date of purchase. All required fields of the Antibody Bioguarantee Form must be completed. Refunds and replacements contingent to claim review by technical service team. Credit covers the cost of antibody. Product replacements depend on product availability. Antibodies purchased in bulk order or for resale purposes are expressly excluded from this Bioguarantee. This Bioguarantee is non-transferable and void upon resale of antibody.



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References

(1) Panchuk-Voloshina N, Haugland RP, Bishop-Stewart J, et al. Alexa dyes, a series of new fluorescent dyes that yield exceptionally bright, photostable conjugates. *J Histochem Cytochem.* 1999;47(9):1179–1188.

(2) Kolmakov K, Belov VN, Bierwagen J, et al. Red-emitting rhodamine dyes for fluorescence microscopy and nanoscopy. *Chemistry.* 2010;16(1):158–166.

(3) Hilderbrand SA, Weissleder R. Near-infrared fluorescence: application to *in vivo* molecular imaging. *Curr Opin Chem Biol.* 2010;14(1):71–79.

(4) Hawrysz DJ, Sevick-Muraca EM. Developments toward diagnostic breast cancer imaging using near-infrared optical measurements and fluorescent contrast agents. *Neoplasia.* 2000;2(5):388–417.

(5) Wang YV, Wade M, Wong E, Li YC, Rodewald LW, Wahl GM. Quantitative analyses reveal the importance of regulated Hdmx degradation for p53 activation. *Proc Natl Acad Sci U S A.* 2007;104(30):12365–12370.

(6) Leung W, Cheung C, Yue S. Modified carbocyanine dyes and their conjugates. United States Patent 6974873. 2005.

(7) Mahmudi-Azer S, Lacy P, Bablitz B, Moqbel R. Inhibition of nonspecific binding of fluorescent-labelled antibodies to human eosinophils. *J Immunol Methods.* 1998;217(1-2):113–119.

(8) Invitrogen. Alexa Fluor® 790 carboxylic acid, succinimidyl ester, penta(triethylammonium) salt, material safety data sheet. http://probes.invitrogen.com/media/msds/30051.html.

(9) Mujumdar SR, Mujumdar RB, Grant CM, Waggoner AS. Cyanine-labeling reagents: sulfobenzindocyanine succinimidyl esters. *Bioconjug Chem.* 1996;7(3):356–362.

(10) LICOR. http://www.licor.com/bio/products/reagents/irdye/irdye680lt_nhsEster.jsp.

(11) LICOR b. http://www.licor.com/bio/products/reagents/irdye/irdye650.jsp.

(12) LICOR c. http://www.licor.com/bio/products/reagents/irdye/irdye750.jsp.

(13) Xu X, Draney DR. Fluorescent imaging with substituted cyanine dyes. United Stated Patent Application 0320089 A1. 2010.

(14) Boswell CA, Tesar DB, Mukhyala K, Theil FP, Fielder PJ, Khawli LA. Effects of charge on antibody tissue distribution and pharmacokinetics. *Bioconjug Chem.* 2010;21(12):2153–2163.

(15) Invitrogen. Technical Tip: Greater Control and More Reaction Time with SDP Ester (X30052). http://probes.invitrogen.com/media/pis/x30052.pdf.

(16) Invitrogen. Alexa Fluor® Succinimidyl Esters (MP10168). http://probes.invitrogen.com/media/pis/mp10168.pdf.

(17) Brinkley MA. brief survey of methods for preparing protein conjugates with dyes, haptens, and cross-linking reagents. *Bioconjug Chem.* 1992;3(1):2–13.

(18) Haugland RP. Coupling of monoclonal antibodies with fluorophores. *Methods Mol Biol.* 1995;45:205–221.

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