Solutions for Detection

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INTRODUCING

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Introduction

Fluorescent techniques are widespread and fast-growing analytical methods used in life science. They allow sensitive and selective investigation of biological processes, diagnostic screening, kinetics and conformational studies. Research is evolving from identification of a large number of target molecules to isolation and investigation at the level of a single molecule. Key innovations for microscopy and nanoscopy in the last decade have been:

• Confocal laser scanning microscopy (CLSM)
• Fluorescence correlation spectroscopy (FCS)
• Total internal reflection of fluorescence (TIRF)
• Stimulated emission depletion (STED) microscopy
• Ground State Depletion (GSD) Microscopy
• Spectral precision distance microscopy (SPDM)
• Scanning nearfield optical microscopy (SNOM)
• Fluorescence photoactivation localization microscopy (FPALM)
• Stochastic optical reconstitution microscopy (STORM)

These super-resolution microscopic and spectroscopic techniques allow a tremendous increase in lateral resolution and enable scientists to have a new perspective of cellular studies. For example, STED microscopy, the revolutionary technique invented by Professor Stefan Hell, enables a microscopic resolution limit below the theoretical limit of resolution and permits more detailed studies in cellular processes. Sigma offers a comprehensive selection of reagents for superior application results including the fluorescent Atto dyes series, CF-Dyes and also anti-Stokes Sunstones® probes developed by Intelligent Material. Sigma has recently launched the new protein detection assay Duolink® developed by Olink Bioscience that allows precise detection and quantification of proteins, protein interactions and protein modifications in fixed cells and tissue samples.

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Accelerate the Signaling Pathway Discovery Process
Detect, quantify and determine cell localization of a specific protein complex in the same experiment. Duolink, based on in situ PLA, which is a proximity ligation assay, enables you to visualize protein interactions in fixed cells and tissue samples, all while under endogenous protein expression.

- Visualize individual interactions without having to overexpress proteins
- Gain high specificity with dual binding of primary antibodies
- Single molecule sensitivity due to signal amplification
- Analyze using standard immunofluorescence instruments

The Duolink kit series of optimized, simple to use reagents, allows the user to combine any pair of immunofluorescence or immunohistochemistry validated antibodies for direct in-cell detection of protein interaction events. Duolink read-out is performed either with a fluorescent label for fluorescence microscopy or HRP for brightfield detection. The resulting distinct spots are derived from individual protein interaction events, which are visualized using a standard microscope.

Discover your pathway at sigma.com/duolink

**Preparation**

**STEP 1.** Fix cells or tissues onto microscope slide or microplate.

**STEP 2.** Wash and add two primary antibodies.

**STEP 3.** Wash and add the PLUS and MINUS PLA probes.

**Detection**

**STEP 4.** Wash and add Ligation solution.

**STEP 5.** Wash and add Amplification solution.

**STEP 6.** Review and capture images.

**Analysis**

**STEP 7.** Single protein interactions visualized using fluorescence and brightfield, respectively.

**STEP 8.** Obtain objective quantification using Duolink Image Tool.

**STEP 9.** Data analysis.

Cells or tissue deposited on slides or in microplates are fixed (Step 1) to preserve activation status and transient interactions. Validated primary antibodies for the targets are added (Step 2) followed by binding of the PLA probes (Step 3). Hybridization of the oligonucleotide arms of the PLA probes will create a template for rolling circle amplification (RCA) only when the epitopes of the target proteins are in close proximity (<40 nm) (Step 4), followed by amplification and labeling of the RCA product by detection probes (Step 5). The result can be detected and visualized by standard microscopy (Step 5, 6 and 7).
Duolink In Situ Product List

To perform a Duolink assay a PLA probe PLUS, a PLA probe MINUS and a Detection Reagent are required. Recommended accessories are Wash Buffers and Mounting Medium.

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<th>Cat. No.</th>
<th>Product Description</th>
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See related accessories below for mounting and washing buffers.

These products are for research use only.

1) Orange – PLA signals detected with the same filters as for e.g. Cy3.
2) Red – PLA signals detected with the same filters as for e.g. TexasRed.
3) Far Red – PLA signals detected with the same filters as for e.g. Cy5, suitable for confocal microscopy.
4) Green – PLA signals detected with the same filters as for e.g. GFP, suitable with red counterstaining e.g. propidium iodide.
5) Brightfield – PLA signals visualized by enzymatic conversion of HRP/NovaRed substrate using brightfield microscope.

To generate a PLA signal, use primary antibodies from Mouse, Goat or Rabbit as shown above.
Alternatively, build your own PLA probe using Probemaker kits DUO92009 and DUO92010.
Prestige Antibodies® Powered by Atlas Antibodies are the Most Highly Characterized in the Industry

- More than 700 IHC, IF and Western blot images for each antibody
- Standardized in detailed universal protocols
- Developed by the Human Protein Atlas (HPA) Project
- Access all data via the Human Protein Atlas website

Since 2008, Sigma has partnered with Atlas Antibodies to provide Prestige Antibodies. Each Prestige Antibody, designed and validated by the Human Protein Atlas Project, is accompanied by 700 immunohistochemistry (IHC), immunofluorescence (IF) and Western blot images. The validation process is supported by publicly available data of the Human Protein Atlas, a part of HUPO's Human Antibody Initiative. To ensure our Prestige Antibody collection has market leading specificity, each antibody is tested by multiple quality assurance steps.

The Human Protein Atlas (proteinatlas.org) displays a collection of more than 13 million images of normal and cancer tissues, cell lines and primary cells. The database delivers visible proof of antibody performance and also provides a knowledge base demonstrating structural and temporal expression of proteins in various cells and tissues.

For more information, visit sigma.com/prestige

**Immunofluorescence**

Anti-TJP1: (Cat. No. HPA001636) Immunofluorescent staining of human cell line U-2 OS shows positivity in cytoplasm & plasma membrane.

**Immunohistochemistry**

Anti-MTHFD1: (Cat. No. HPA000704) Immunoperoxidase staining of formalin-fixed, paraffin-embedded human liver tissue shows strong cytoplasmic and/or membranous staining of hepatocytes.
Low Background Detection in Life Science Applications using Sunstone® Luminescent UCP Nanocrystals

Upconversion Imaging Background
While fluorescent imaging applications are commonly used in life science research, inherent limitations to fluorescence, especially for cellular applications, have encouraged development of alternative techniques and compounds. Upconversion luminescence using rare-earth doped nanocrystals is increasingly being used in commercial and industrial applications and more recently has been used in life science applications to overcome these limitations found with quantum dots and other normal fluorophores (see Figure 1).

Normal fluorescence converts higher-energy (shorter wavelength) light to lower-energy (longer wavelength) emitted light. Upconversion luminescence is based on the absorption of two or more low-energy (longer wavelength, typically infrared) photons by a nanocrystal followed by the emission of a single higher-energy (shorter wavelength) photon (see Figure 2).

Sunstone Upconverting Nanocrystals
Sunstone Nanocrystals from Intelligent Material Solutions® Inc. are a proprietary, novel series of rare earth-doped nanocrystals of small size, high quantum efficiency, and high photoluminescent intensity functionalized for use in industrial and life sciences applications. These patented materials possess unique and inherent atomic states that allow the conversion of various wavelengths of light energy up and down the electromagnetic spectrum.

Eliminate Autofluorescence in Biological Samples
Upconversion bioimaging has significantly lower autofluorescence (see Figure 3) and a higher signal to noise ratio when compared to single-photon excitation for fluorescence detection.
Large Anti-Stokes Shift for Discrete Emission Signals in Multiplex Techniques

A large anti-Stokes shift (up to 500 nm) with upconversion phosphorescence means well-separated and discrete emission peaks from the infrared excitation source. For NIR emitting nanoparticles, the longer wavelength allows deeper penetration of the exciting light into biological tissues. Furthermore, by varying the concentration and ratio of lanthanides, the decay time of Sunstone nanocrystals can be modified from less than a femtosecond to a millisecond or longer, and these temporal properties are reproducible for a specific formulation, ensuring consistency in replicate tests. Standard detectors can easily differentiate these temporal signatures. A combination of Sunstone Nanocrystals can be applied in an assay using a single excitation source to produce different emission signals for multiplex applications.

Small Particle Size for Life Science Applications

Sunstone Nanocrystals available through Sigma-Aldrich are 30 nm rods, which are small enough for life science applications without further processing (see Figure 4). Sunstone Nanocrystals have a high degree of structural homogeneity, allowing for finely tuned temporal properties and narrow spectral emission. The nanocrystals are synthesized in the β crystal phase (β-NaYF4), requiring low phonon energy for upconversion1. Sunstone Nanocrystals exhibit excellent size distribution, uniformity in shape, and high monodispersity. Additional sizes and morphologies of Sunstone Nanocrystals will be made available in the future to support life science research.

Resistant to Photobleaching and Fading

Sunstone Upconverting Nanocrystals do not photobleach and allow permanent excitation with simultaneous signal integration. They can be stored indefinitely without a decrease in light emitting efficiency for repeated irradiation and analysis.

Biocompatible and Non-Toxic

Sunstone Nanocrystals are biocompatible and non-toxic, with no cytotoxicity against human osteosarcoma cells2. Upconverting nanocrystals have been used in in vivo experiments with C. elegans3 and mice4 without demonstrating toxicity.

Life Science Applications

Upconverting materials have been used in a broad variety of life science applications including:
- Immunohistochemistry2,5-7
- Immunocytochemistry5-7
- Multiplex immunoassays5,6
- Nucleic acid microarrays6,8
- In vivo, in situ, and ex situ biomedical imaging2,3,5,10,11
- Flow cytometry8,12
- Enzymatic assays6,8,12
- Fluorescence resonance energy transfer (FRET) bioanalytical assays12

Sunstone Nanocrystals are ideal for life science detection and imaging with broad applicability in many in vitro and in vivo techniques. Sunstone Nanocrystals are extremely robust and can be incorporated into many chemical and biochemical compositions without losing their phosphorescence as long as the medium in which they are dispersed is optically transparent to both the absorbed and emitted radiation. With sharp emission bands and large anti-Stokes shifts, Sunstone Nanocrystals may be used in multiplex techniques allowing identification of multiple emission spectra from a single sample (see Figure 5). The nanocrystals are also suitable for FRET based assay systems.

Figure 4. Transmission electron microscope ~30-nm images of rod-shaped NaYF4 Sunstone Upconverting Nanocrystals. Sunstone Nanocrystals exhibit excellent size distribution, uniformity in shape, and high monodispersity.

Figure 5. (green) CD4+ lymphocytes labeled with CD4 UCP-conjugated antibody. (blue) calcein dye.
Sunstone Nanocrystals have been used for \textit{in vivo} and \textit{in situ} macroscopic lymphatic imaging in mice (see \textbf{Figure 6}). Multiplex analysis using \(980\) nm excitation was performed by using a combination of green-emitting and red-emitting upconversion particles.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure6.png}
\caption{Luminescent \textit{in vivo} and \textit{in situ} lymphatic imaging with infrared Sunstone Upconverting nanocrystals obtained with both spectral and single shot imaging. The nanocrystals depicted the draining lymph nodes during \textit{in vivo} and \textit{in situ} imaging in mice. Due to the minimal background, single shot luminescence images obtained at \(800\) nm were comparable to the spectrally unmixed images which were post processed to remove autofluorescence. (Dr. Hisataka Kobayashi, National Institutes of Health)}
\end{figure}

Carboxylated Sunstone Nanocrystals may be attached to biomolecules via the carboxyl group after EDC activation. Carboxylated nanocrystals can also be conjugated via electrostatic binding. Carboxylated Sunstone Nanocrystals can be conjugated to a variety of biomolecules including:

- Antibodies
- Enzymes
- Substrates
- Small molecules and inhibitors

Avidin conjugated Sunstone Nanocrystals can be used in common biotin-avidin/streptavidin reporter systems.

\textbf{Ordering Information}

<table>
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<th>Product No.</th>
<th>Description</th>
<th>Functional group</th>
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<th>(\lambda_m) max. (nm)*</th>
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<td>804</td>
<td>10 - 60 nm, rods</td>
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*Refers to strongest signal.

Sunstone is a registered trademark of Intelligent Material® Solutions Inc. Sunstone upconverting nanocrystals are produced under license from SRI International.
References
cally controlled synthesis of colloidal upconversion nanophosphors and their shape-
2. Shan, J., Chen, J., Meng, J., Collins, J., Soboyejo, W., Friedberg, J.S., and Ju, Y. Biofunction-
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and scanning electron microscopy imaging of upconverting nanophosphors in Cae-
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6. Corstjens, P., Chen, Z., Zuidervijk, M., Bau, H.H., Abrams, W.R., Malamud, D., Sam Nied-
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responses to infectious disease pathogens (HIV, HCV, and TB). Ann. N.Y. Acad. Sci., 1098,
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R., Malamud, D., and Bau, H. Development of a microfluidic device for detection of patho-
H.J. Infrared up-converting phosphors for bioassays. IEE Proc. Nanobiotechnol., 152, 64-72
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14. Kokko, T., Liljenback, T., Peltola, M.T., Kokko, L., and Soukka, T. Homogeneous dual-
parameter assay for prostate-specific antigen based on fluorescence resonance energy
15. Soukka, T., Rantanen, T., and Kuningas, K. Photon upconversion in homogeneous
H.J. Detection of cell and tissue surface antigens using up-converting phosphors: A new

For more information, visit sigma.com/upconversion
**CF™ Dyes**

Achieve greater sensitivity, brighter results, and better photostability with enhanced CF™ dye technology.

CF dyes are a series of highly water-soluble fluorescent dyes spanning the visible and near-infrared (near-IR) spectrum (Figure 1A and 1B) 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.

Sigma® Life Science 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 Life Science’s broad range of carefully selected secondary antibodies and conjugates, allowing scientists to achieve greater sensitivity, brighter results, and better photostability in immunooassays.

The CF dye quick reference guide will help you choose the correct dye for your application.

---

**Absorption/Emission Spectra of Goat Anti-Mouse Antibody**

**Figure 1A**

![Absorption spectra of CF dyes conjugated to goat anti-mouse IgG](image1)

**Figure 1B**

![Emission spectra of CF dyes conjugated to goat anti-mouse IgG](image2)

**Brightness, Photostability and Conjugate Specificity of CF Dyes Compared to Other Commercial Dyes**

**Figure 2A**

Relative fluorescence of CF543 and Alexa Fluor 546 (AF546) goat anti-mouse conjugates as a function of the number of dye molecules per protein (degree of labeling).

**Figure 2B**

Relative photostability of CF633 and Alexa Fluor 647 (AF647) goat anti-mouse conjugates. Jurkat cells were fixed, permeabilized and stained with rabbit anti-CD3 followed by CF633 or Alexa Fluor 647 goat anti-rabbit IgG conjugates. Cells were imaged using a mercury arc lamp microscope equipped with a Cy5 filter set and CCD camera. Sequential images were captured at 0, 1, and 5 minutes.

**Figure 2C**

Near-IR CF dyes are highly water soluble without carrying excessive negative charge, which can increase non-specific binding of antibody conjugates. Near-IR Western blots imaged using the Odyssey system (Li-COR Biosciences). Two dilutions of HeLa cell lysate were probed with mouse anti-tubulin antibody followed by goat anti-mouse conjugated to Alexa Fluor 790 (AF790) (2, 3) or CF790 (5, 6).

For more information, visit [sigma.com/cfdyes](http://sigma.com/cfdyes)
Mix-n-Stain™ Antibody Labeling Kit

Label 5 to 100 μg of an Antibody with the Brightest Dyes Available in Just 30 Minutes

Mix-n-Stain™ CF™ dye antibody labeling kits dramatically simplify the process of preparing fluorescently labeled antibodies, particularly primary antibodies. Simply mix your antibody with the CF dye of your choice in the buffer provided, a step that takes less than 30 seconds of hands-on time. After a quick 30-minute incubation, you will have a ready-to-use fluorescent antibody conjugate.

Advantages for Using Mix-n-Stain™ Labeling Kits

• No need to calculate how much dye you should use - just mix your antibody with the entire amount of dye provided
• No purification of conjugate necessary
• The labeling reaction can tolerate the presence of common stabilizers, such as sodium azide, Tris, and low levels of glycerol, BSA or gelatin.
• Choose from more than 20 of the brightest and most photostable dyes commercially available.

Simple Antibody Labeling Protocol – 3 Easy Steps

1. Mix antibody and dye in buffer – 30 seconds hands-on time
2. Incubate antibody/dye for 30 minutes
3. Conjugate is ready-to-use — no purification necessary!

For more information, visit sigma.com/mixnstain
## Fluorescent Dye Comparison

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<th>λ_{em} (nm)</th>
<th>MW</th>
<th>ε</th>
<th>Direct replacement for Reactive forms</th>
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<th>λ_{em} (nm)</th>
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<td>UV</td>
<td></td>
<td>AMCA N HS, sulfo-NHS, hydrazide, HPDP, SE</td>
<td>353 442 330</td>
<td>19,000</td>
<td>- Yields the brightest blue fluorescent antibody conjugates when excited at ~350 nm - Highly water-soluble and insensitive to pH</td>
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<td><strong>CF™ 405S</strong></td>
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<td>405 nm laser</td>
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<td>Alexa Fluor® 405 SE, cadaverine</td>
<td>400 424 1028</td>
<td>35,000</td>
<td>- Much brighter than Alexa Fluor® 405 due to better compatibility with excitation and emission windows on common instruments - Highly water-soluble and insensitive to pH</td>
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<td><strong>CF™ 405M</strong></td>
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<td>408 452 503</td>
<td>41,000</td>
<td>405 nm laser</td>
<td></td>
<td>BD Horizon™ V450 Conjugated</td>
<td>404 448</td>
<td>30,000</td>
<td>- More photostable than Pacific Blue® dye - As bright as Pacific Blue® dye in the blue channel - Less spillover fluorescence in the green channel - Highly water-soluble</td>
<td></td>
<td></td>
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<tr>
<td><strong>CF™ 488A</strong></td>
<td>amine, aminoxy, hydrazide, maleimide, SE</td>
<td>490 515 914</td>
<td>70,000</td>
<td>488 nm laser</td>
<td></td>
<td>Alexa Fluor® 488 S-SDFP, S-TFP, maleimide, alkyne, azide, cadaverine, hydrazide, hydroxylamine, SE</td>
<td>494 517 643</td>
<td>73,000</td>
<td>- Minimal charge compared to Alexa Fluor® 488 reduces nonspecific antibody staining - Less spillover fluorescence in the red channel than Alexa Fluor® 488 - Extremely photostable - Highly water-soluble and pH-insensitive</td>
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<tr>
<td><strong>CF™ 543</strong></td>
<td>amine, hydrazide, maleimide, SE</td>
<td>541 560 870</td>
<td>100,000</td>
<td>532 nm or 543, 546 laser</td>
<td></td>
<td>Alexa Fluor® 546 N/A</td>
<td>556 573 N/A 112,000</td>
<td>- Significantly brighter than Alexa Fluor® 546 - Highly water-soluble and pH-insensitive</td>
<td></td>
<td></td>
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<tr>
<td><strong>CF™ 555</strong></td>
<td>amine, hydrazide, maleimide, SE</td>
<td>555 565 810</td>
<td>150,000</td>
<td>532 nm or 568 nm laser</td>
<td></td>
<td>Alexa Fluor® 555 maleimide, alkyne, azide, cadaverine, hydrazide, hydrazide, SE</td>
<td>555 572 1250</td>
<td>155,000</td>
<td>- Brighter and more photostable than Cy3™ - Minimal charge compared to Alexa Fluor® 555 reduces nonspecific antibody staining</td>
<td></td>
<td></td>
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<tr>
<td><strong>CF™ 568</strong></td>
<td>amine, aminoxy, hydrazide, maleimide, SE</td>
<td>562 583 714</td>
<td>100,000</td>
<td>532 nm or 568 nm laser</td>
<td></td>
<td>Alexa Fluor® 568 maleimide, cadaverine, hydrazide, hydrazide</td>
<td>578 602 792</td>
<td>98,000</td>
<td>- Optimal for the 568 nm line of the Ar-Kr mixed-gas laser - Brighter and more photostable than Alexa Fluor® 568</td>
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<tr>
<td><strong>CF™ 594</strong></td>
<td>amine, aminoxy, hydrazide, maleimide, SE</td>
<td>593 614 729</td>
<td>115,000</td>
<td>532 nm or 594 nm laser</td>
<td></td>
<td>Alexa Fluor® 594 maleimide, alkyne, azide, cadaverine, hydrazide, hydrazide</td>
<td>590 617 820</td>
<td>92,000</td>
<td>- Yields the brightest antibody conjugates among spectrally similar dyes - Extremely photostable</td>
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<tr>
<td><strong>CF™ 620R</strong></td>
<td>maleimide, SE</td>
<td>617 639 738</td>
<td>115,000</td>
<td>633 nm or 635 nm laser</td>
<td></td>
<td>LightCycler® Red 640 NHS</td>
<td>625 640 758</td>
<td>- Excellent energy acceptor for FRET - Highly fluorescent - Extremely photostable - Highly water-soluble</td>
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<td></td>
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<tr>
<td><strong>CF™ 633</strong></td>
<td>amine, aminoxy, hydrazide, maleimide, SE</td>
<td>630 650 821</td>
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<td>633 nm or 635 nm laser</td>
<td></td>
<td>Alexa Fluor® 633 maleimide, hydrazide, SE</td>
<td>621 639 1200</td>
<td>159,000</td>
<td>- Yields the brightest antibody conjugates among spectrally similar dyes - Optimal for 633 nm He-Ne laser or the 635 nm red diode laser - Far more photostable than Alexa Fluor® 647 - Highly water-soluble</td>
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<td><strong>CF™ 640R</strong></td>
<td>amine, aminoxy, hydrazide, maleimide, SE</td>
<td>642 662 832</td>
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<td>Alexa Fluor® 647 maleimide, alkyne, azide, cadaverine, hydrazide, SE</td>
<td>651 672 1250</td>
<td>270,000</td>
<td>- Most photostable among spectrally similar dyes - Yields highly fluorescent protein conjugates - Very water-soluble and pH-insensitive</td>
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<tr>
<td>CF™ dye</td>
<td>Reactive forms</td>
<td>λ&lt;sub&gt;ex&lt;/sub&gt; (nm)</td>
<td>λ&lt;sub&gt;em&lt;/sub&gt; (nm)</td>
<td>MW</td>
<td>ε</td>
<td>Excitation source</td>
<td>Direct replacement for: Reactive forms</td>
<td>λ&lt;sub&gt;ex&lt;/sub&gt; (nm)</td>
<td>λ&lt;sub&gt;em&lt;/sub&gt; (nm)</td>
<td>MW</td>
<td>ε</td>
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<tr>
<td>CF™ 647</td>
<td>hydrazide, maleimide, SE</td>
<td>650</td>
<td>665</td>
<td>836</td>
<td>240,000</td>
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<td>Alexa Fluor® 647 maleimide, alkyne, azide, cadaverine, hydrazide, SE</td>
<td>651</td>
<td>672</td>
<td>1250</td>
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<tr>
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<td>maleimide, SE</td>
<td>667</td>
<td>685</td>
<td>3112</td>
<td>200,000</td>
<td>633 nm, 635 nm, or 640 nm laser</td>
<td>Alexa Fluor® 660 maleimide, SE</td>
<td>668</td>
<td>698</td>
<td>1100</td>
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<tr>
<td>CF™ 660R</td>
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<td>663</td>
<td>682</td>
<td>888</td>
<td>100,000</td>
<td>633 nm, 635 nm, or 640 nm laser</td>
<td>Alexa Fluor® 660 maleimide, SE</td>
<td>668</td>
<td>698</td>
<td>1100</td>
<td>132,000</td>
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<tr>
<td>CF™ 680</td>
<td>maleimide, SE</td>
<td>681</td>
<td>698</td>
<td>3241</td>
<td>210,000</td>
<td>680 nm or 685 nm laser</td>
<td>Alexa Fluor® 680 maleimide, SE</td>
<td>684</td>
<td>707</td>
<td>1150</td>
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<tr>
<td>CF™ 680R</td>
<td>aminooxy, maleimide, SE</td>
<td>680</td>
<td>701</td>
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<td>680 nm or 685 nm laser</td>
<td>Alexa Fluor® 680 maleimide, SE</td>
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<td>707</td>
<td>1150</td>
<td>183,000</td>
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<tr>
<td>CF™ 750</td>
<td>SE</td>
<td>755</td>
<td>777</td>
<td>3009</td>
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<td>680 nm, 685 nm, or 785 nm laser</td>
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<td>753</td>
<td>782</td>
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<td>CF™ 770</td>
<td>SE</td>
<td>770</td>
<td>797</td>
<td>3138</td>
<td>220,000</td>
<td>785 nm laser</td>
<td>DyLight™ 800 NHS, maleimide</td>
<td>777</td>
<td>794</td>
<td>899</td>
<td>270,000</td>
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<td>CF™ 790</td>
<td>SE</td>
<td>784</td>
<td>806</td>
<td>3267</td>
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<td>785 nm laser</td>
<td>Alexa Fluor® 790 SE</td>
<td>785</td>
<td>810</td>
<td>1750</td>
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</tbody>
</table>
Atto™ Dyes for Superior Fluorescent Imaging

Activated fluorescent dyes are routinely used to tag proteins, nucleic acids, and other biomolecules for use in life science applications including fluorescence microscopy, flow cytometry, fluorescence in situ hybridization (FISH), fluorescence resonance energy transfer (FRET) techniques, receptor binding assays, and enzyme assays. The Atto dyes are a series of fluorescent dyes that meet the critical needs of modern fluorescent technologies:

- Enhanced photostability and ozone resistance
- Long signal lifetimes
- Reduced background for greater sensitivity
- Extensive selection of alternatives to common dyes
- Recommended for multiplex applications

Atto 655 and Atto 647N – Photostable and Ozone Resistant for Microarray Applications

In contrast to fluorescein and cyanine-based dyes, Atto 655 and Atto 647N have a more rigid molecular structure and are more photostable under prolonged light exposure (see Figure 1). Excitation and emission wavelengths and the emission signal decay time are relatively insensitive to pH and other environmental changes such as temperature and atmospheric humidity.

Long Signal Lifetimes

Atto dyes exhibit longer fluorescence signal lifetimes (0.6-3.8 ns) than either carbocyanine dyes or most of the autofluorescence inherent in cells and biomolecules.

Longer Excitation Wavelengths for Reduced Background

Diode laser excitation at 635 nm and red-absorbing fluorescent dyes were shown to reduce autofluorescence of biological samples sufficiently so that individual antigen and antibody molecules could be detected in human serum samples.\(^1\)\(^,\)\(^2\) Excitation in the red spectral region also reduces cell damage when working with live cells.\(^3\)

- Many Atto dyes (Atto 590 and above) can be excited using wavelengths greater than 600 nm.
- Using long-wavelength activated Atto dyes with the appropriate excitation wavelength reduces autofluorescence due to sample, solvent, glass, or polymer support.
- Background due to Rayleigh and Raman scattering can be dramatically reduced.
- Improved overall sensitivity in biological analysis and imaging techniques can be obtained since Atto dyes have less interference from fluorophores with shorter lifetimes.
- Fluorescent signals are stronger with the same molar amount of Atto 655 or Atto 680 since less dye is lost to inactivation. Other Atto dyes also have low triplet formation.

Fluorescence microscopy of paraffin fixed tissue of oesophagus of rat with staining the carbohydrate subunits of the glycoprotein laminin. The target carbohydrate subunit chitotriose [(GlcNAc)_3] is specifically bound to lectin from Phytolacca americana-Atto 488 (Cat. No. 39905, green). Concanavalin A-Atto 564 (Cat. No. 69535, red) is specifically bound to α-D-mannose and α-D-galactose. Nucleus counterstained with DAPI (blue).
**Atto™ Dyes - Superior Tool for Super-Resolution Microscopy Application**

Recent developments in microscopy application, like e.g. STED microscopy enables resolutions down to 10 nm. These applications require fluorescent dyes, that fulfill superior photo-physical criteria. Some Atto-dyes, Atto 488, Atto 647N, and Atto 655, have proven a suitable for techniques such as PALM, dSTORM, STED, etc.

**Atto 655 and Atto 680 — Less Molecular Inactivation for Greater Signal**

Non-fluorescent triplet states and cis-conformations result in fluctuations that interfere with fluorescent signal yield. Dyes such as Cy5, Cy5.5, or Alexa Fluor® 647 may form cis-isomers and triplet states, which precludes their usage in demanding techniques including fluorescence correlation spectroscopy (FCS), single molecule detection (SMD), and as acceptors in fluorescence resonance energy transfer (FRET). Atto 655 and Atto 680 have low intersystem crossing and lack an isomeric bond so they cannot undergo configuration isomerization.

**Recommended for Fluorescent Multiplex Detection**

Atto dyes can be used to conjugate probes and biomolecules for multiplex applications. Selection of two Atto dyes with separated emission signals supports multiple excitation and measurement results from a single experiment.

**Alternatives to Common Fluorophores**

With the extensive selection available, Atto dyes can replace commonly used fluorescent dyes. There are Atto dyes suitable for use with any common excitation light source.

<table>
<thead>
<tr>
<th>Fluorophore</th>
<th>Recommended Atto dye alternative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alexa Fluor® 488</td>
<td></td>
</tr>
<tr>
<td>FITC</td>
<td>Atto 488</td>
</tr>
<tr>
<td>FAM™</td>
<td></td>
</tr>
<tr>
<td>JOE™</td>
<td>Atto 520</td>
</tr>
<tr>
<td>TET™</td>
<td></td>
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<tr>
<td>Alexa Fluor® 532</td>
<td>Atto 532</td>
</tr>
<tr>
<td>HEX™</td>
<td>Atto 532, Atto Rho6G</td>
</tr>
<tr>
<td>TAMRA™</td>
<td>Atto 550</td>
</tr>
<tr>
<td>Cy3</td>
<td>Atto 550</td>
</tr>
<tr>
<td>Cy3.5</td>
<td>Atto 555</td>
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<tr>
<td>ROX™</td>
<td>Atto 565, Atto Rhod11</td>
</tr>
<tr>
<td>Alexa Fluor® 594</td>
<td>Atto 590, Atto 594</td>
</tr>
<tr>
<td>Texas Red®</td>
<td>Atto 590</td>
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<tr>
<td>Alexa Fluor® 633</td>
<td>Atto 633, Atto Rhod14</td>
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<tr>
<td>Cy5</td>
<td>Atto 647, Atto 647N, Atto 655</td>
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<tr>
<td>Alexa Fluor® 647</td>
<td>Atto 647, Atto 647N, Atto 655</td>
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<tr>
<td>Cy5.5</td>
<td>Atto 680, Atto 700</td>
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</table>

<table>
<thead>
<tr>
<th>Light source</th>
<th>Main lines (nm)</th>
<th>Recommended Atto dyes</th>
</tr>
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<tbody>
<tr>
<td>Mercury arc lamp</td>
<td>577</td>
<td>Atto 590, Atto Rhod101, Atto 594, Atto Rhod3, Atto 610, Atto 61x</td>
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<tr>
<td>Xenon arc lamp</td>
<td>Continuum and peaks &gt;800 nm</td>
<td>Atto 610, Atto 620, Atto 647, Atto 647N, Atto 655, Atto 680</td>
</tr>
<tr>
<td>Halogen lamp</td>
<td>Little UV and violet emission; Higher intensity toward longer wavelengths</td>
<td>Atto 610, Atto 620, Atto 647, Atto 647N, Atto 655, Atto 680</td>
</tr>
<tr>
<td>Argon ion laser</td>
<td>488, 514</td>
<td>Atto 488, Atto 520, Atto 532, Atto 550</td>
</tr>
<tr>
<td>Argon-krypton laser</td>
<td>488, 514, 647, 676</td>
<td>Atto 520, Atto 647, Atto 647N, Atto 655, Atto 680</td>
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<tr>
<td>He-Ne laser</td>
<td>633</td>
<td>Atto Rhod14, Atto 633, Atto 647, Atto 647N</td>
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<tr>
<td>Common diode laser</td>
<td>635, 650, 670</td>
<td>Atto 633, Atto 647, Atto 647N, Atto 655, Atto 680</td>
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</table>

Confocal microscopy (CLSM) image of paraffin fixed tissue of rat stomach. Actin stained with mouse anti-smooth muscle α-actin antibody and Atto 488 anti-mouse IgG (Cat. No. 62197, green), cytokeratin stained with polyclonal rabbit anti-cytokeratin and Atto 647N anti-rabbit (igG) (Cat. No. 40839, red).

Immunoblot detection of Protein 1 and Protein 2 using two primary antibodies and two anti-IgG-Atto dye conjugates. Imaging was done sequentially using a FLA-3000 Fuji® laser scanner, first at an excitation wavelength of 532 nm with a 580 nm emission filter to detect Atto 550, then at an excitation wavelength of 633 nm with a 675 nm emission filter to detect Atto 633. The image overlay was done using a software tool.
Reactive Atto Dyes

Atto dyes produce intense fluorescent signals due to strong absorbance and high quantum yields.

- **Strong signal intensity** — Most Atto dyes have $\varepsilon_{\text{max}}$ values $> 100,000$.
- **Ideal for multiplex techniques using visible and near-IR emission wavelengths** — Low excitation/emission overlap and good Stokes’ shift separation.
- **Selection and versatility** — There is an Atto dye suitable to use with any common excitation light source.

Atto dyes are available as:
- **Free acid** dyes for all routine staining applications.
- **NHS-esters** for use in common conjugation protocols.
- **Maleimides** for use in coupling to thiol-containing groups such as cysteine residues and thiol (-SH) tags added during automated synthesis.

Atto 655, Atto 680, and Atto 700 are quenched by guanosine, tryptophan and related compounds through direct contact between the dye and the quenching agent and using an electron transfer process. Fluorescent quenching of dyes by tryptophan residues in proteins has been used to differentiate unbound (non-fluorescent) protein from protein-antibody (fluorescent) interactions.\(^1\)

### Flourescent Signal Information for Atto Dyes

<table>
<thead>
<tr>
<th>Atto Dye</th>
<th>$\lambda_{\text{abs}}$ [nm]</th>
<th>$\varepsilon_{\text{max}}$ [m$^{-1}$ cm$^{-1}$]</th>
<th>$\lambda_{\text{em}}$ [nm]</th>
<th>$\eta_{\text{em}}$ [%]</th>
<th>$\tau_{\text{em}}$ [ns]</th>
<th>Catalog Number</th>
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<tbody>
<tr>
<td>Atto 390</td>
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<td>479</td>
<td>90</td>
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<td>55</td>
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$\lambda_{\text{abs}}$ - longest-wavelength absorption maximum  
$\varepsilon_{\text{max}}$ - molar extinction coefficient at the longest-wavelength absorption maximum  
$\lambda_{\text{em}}$ - fluorescence maximum  
$\eta_{\text{em}}$ - fluorescence quantum yield  
$\tau_{\text{em}}$ - fluorescence decay time
Convenient Atto™ Dye Conjugates

An extensive selection of Atto dye conjugates and kits are available, including:

- **Protein Labeling Kits**
  - **Atto 488** is a superior alternative to fluorescein and Alexa Fluor® 488, producing conjugates with more photostability and brighter fluorescence.
  - **Atto 550** is an alternative to rhodamine dyes, Cy®3, and Alexa Fluor 550, offering more intense brightness and increased photostability.
  - **Atto 594** is an alternative to Alexa Fluor 594 and Texas Red®.
  - **Atto 647N**, an extraordinary highly fluorescent dye, and **Atto 655** are alternatives to Cy5 and Alexa Fluor 647.
  - **Atto 633** is an alternative to Alexa Fluor 633.

- **Lectins** for carbohydrate binding studies.

- **Primary and secondary antibodies** for direct and indirect ELISA, immunoblotting, immunohistochemistry, and other protein identification applications.

- **Biotin and Streptavidin** for avidin/streptavidin/biotin conjugation in applications including ELISA, immunohistochemistry, in situ hybridization, and flow cytometry.

- **NTA Nickel conjugates** for direct detection of polyhistidine-tagged recombinant proteins.

For more information and a comprehensive list of products, visit [sigma.com/atto](http://sigma.com/atto)

References

Superior Super-Resolution Microscopy Application using Abberior® Dyes

Microscopic methods in life sciences are of tremendous importance for visualization cellular and tissue structures. In recent years, development has reached a revolution in order to overcome the resolution barrier given by the diffraction limit. New microscopy concepts that enable resolution limits down to about 10 nm and the visualization of cellular structures and molecular interactions reveal new understanding in biological processes.

These super-resolution microscopy principles are based on several technological approaches. Conventional light microscopy enables a resolution limit of about 250 nm in the x- and y-direction and 450 – 700 nm in the z-direction. Super-resolution techniques have overcome the resolution-limit (Point-spread function), by at least a factor of 2. The resolution of super-resolution microscopy depends on the number of points that can be resolved on the structure of interest. Crucial for successful super-resolution imaging is the choice of fluorescent probe. Brightness and high contrast ratio between the states are of great importance. In most super-resolution methods, the states of the probe must be controllable, reversible or irreversible, switchable between a light or a dark state. Depending on the super-resolution method, further photo physical criteria the probe must be fulfilled. Established techniques are:

- STED (Stimulated emission depletion)
- GSDIM (Ground State Depletion)
- PALM (Photoactivated localization microscopy)
- STORM (Stochastic optical reconstruction microscopy)
- RESOLFT [Reversible saturable optical (fluorescence) transitions]

Sigma now offers the superior series of Abberior dyes that are especially designed and tested for super-resolution microscopy such as STED, RESOLFT, PALM, STORM, GSDIM and others. Abberior STAR, Abberior CAGE, Abberior FLIP and Abberior RSFP – the specific requirements of the super-resolution techniques are served with dedicated dye series.

Super-resolution microscopy depends on fluorescent labels more than any other fluorescence imaging technique. Manufactured by Abberior, the STAR, CAGE and FLIP dyes as well as RSFPs are exceptionally bright and photostable and provide optimized photoswitching for RESOLFT and PALM/STORM imaging. They are the only commercially available dyes tailored specifically to the needs of super-resolution microscopy.

Abberior dyes are also exceptionally well suited for confocal microscopy, epifluorescence imaging and single molecule applications. Basically all fluorescence applications which depend on a good signal to noise ratio and low background benefit from the novel Abberior dyes.

Benefits
- Optimized for brightness and very low background
- Optimized switching behavior being the key for super-resolution
- All markers are tested for different super-resolution methods
  - Abberior STAR for STED, confocal and epifluorescence imaging
  - Abberior CAGE & FLIP for PALM, STORM and GSDIM
- Abberior dyes are recommended by renowned microscope vendors
- Proprietary, IP protected products
- Detailed characteristics of the dyes provided, e.g. optimal STED wavelength

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## Overview of Abberior® dyes

<table>
<thead>
<tr>
<th>Dyes</th>
<th>Description</th>
<th>Absorption Maximum/Extinction Coefficient, ε(λ)</th>
<th>Fluorescence Maximum, λf</th>
<th>Recommended STED</th>
<th>Cat No., NHS activated</th>
<th>Cat No., maleimid activated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abberior® CAGE 500</td>
<td>for single-molecule switching microscopy (e.g. PALM, STORM, GSDIM)</td>
<td>300 nm (caged, pH 7); 501 nm (uncaged, pH 7)</td>
<td>85,000 — 88,000 m⁻¹cm⁻¹ (pH 7, unaged)</td>
<td>524 nm (pH 7), 523 nm (MeOH)</td>
<td>595-615 nm</td>
<td>44254 92546</td>
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<tr>
<td>Abberior® CAGE 532</td>
<td>for single-molecule switching microscopy (e.g. PALM, STORM, GSDIM)</td>
<td>304 nm (caged, PBS, pH 7); 518 nm (uncaged, pH 7)</td>
<td>541 nm (pH 7)</td>
<td>610 - 640 nm</td>
<td>38977 95705</td>
<td></td>
</tr>
<tr>
<td>Abberior® CAGE 552</td>
<td>for single-molecule switching microscopy (e.g. PALM, STORM, GSDIM)</td>
<td>300 nm (caged, pH 7); 552 nm (uncaged, pH 7)</td>
<td>66,000 m⁻¹cm⁻¹ (pH 7, unaged)</td>
<td>574 nm (pH 7)</td>
<td>650-670 nm</td>
<td>94822 92545</td>
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<tr>
<td>Abberior® FLIP 565</td>
<td>for single-molecule switching microscopy (e.g. PALM, STORM, GSDIM)</td>
<td>314 nm (PBS, pH 7.4)</td>
<td>51,000 m⁻¹cm⁻¹ (MeOH)</td>
<td>580 nm (PBS, pH 7.4)</td>
<td>79189 92544</td>
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<tr>
<td>Abberior® STAR 440SX</td>
<td>for long Stokes STED and 2-color STED application</td>
<td>430 nm (MeOH), 437 nm (PBS, pH 7.4)</td>
<td>30,800 m⁻¹cm⁻¹ (MeOH), 22,700 m⁻¹cm⁻¹ (PBS, pH 7.4)</td>
<td>501 nm (MeOH), 515 nm (PBS, pH 7.4)</td>
<td>590-620 nm</td>
<td>68221 38361</td>
</tr>
<tr>
<td>Abberior® STAR 470SX</td>
<td>for long Stokes STED and 2-color STED application</td>
<td>475 nm (MeOH), 477 nm (PBS, pH 7.4)</td>
<td>30,400 m⁻¹cm⁻¹ (MeOH), 22,700 m⁻¹cm⁻¹ (PBS, pH 7.4)</td>
<td>609 nm (MeOH), 627 nm (PBS, pH 7.4)</td>
<td>740 - 770 nm</td>
<td>95348</td>
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<tr>
<td>Abberior® STAR 488</td>
<td>for STED application</td>
<td>501 nm (PBS, pH 7.4)</td>
<td>86,000 m⁻¹cm⁻¹ (MeOH)</td>
<td>524 nm (PBS, pH 7.4)</td>
<td>585 - 605 nm</td>
<td>61048</td>
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<tr>
<td>Abberior® STAR 512</td>
<td>for STED application</td>
<td>517 nm (MeOH), 512 nm (PBS, pH 7.4)</td>
<td>74,000 m⁻¹cm⁻¹ (MeOH)</td>
<td>536 nm (MeOH), 530 nm (PBS, pH 7.4)</td>
<td>590 - 620 nm</td>
<td>38922 03004</td>
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<tr>
<td>Abberior® STAR 580</td>
<td>for STED application</td>
<td>587 nm (MeOH), 583 nm (PBS, pH 7.4)</td>
<td>64,300 m⁻¹cm⁻¹ (MeOH)</td>
<td>609 nm (MeOH), 605 nm (PBS, pH 7.4)</td>
<td>690 - 720 nm</td>
<td>38377</td>
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<tr>
<td>Abberior® STAR 635</td>
<td>for STED application</td>
<td>639 nm (MeOH), 634 nm (PBS, pH 7.4)</td>
<td>69,000 m⁻¹cm⁻¹ (MeOH)</td>
<td>659 nm (MeOH), 654 nm (PBS, pH 7.4)</td>
<td>740 - 770 nm</td>
<td>30558 96013</td>
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<tr>
<td>Abberior® STAR 635P</td>
<td>for STED application</td>
<td>635 nm (MeOH), 634 nm (PBS, pH 7.4)</td>
<td>80,000 m⁻¹cm⁻¹ (water)</td>
<td>655 nm (MeOH), 654 nm (PBS, pH 7.4)</td>
<td>740 - 770 nm</td>
<td>no, only Azide derivative available</td>
</tr>
</tbody>
</table>

### References
2. Diffraction-unlimited all-optical imaging and writing with a photochromic GFP

For a list of Abberior dyes, visit [sigma.com/abberior](http://sigma.com/abberior)
Spectral Fluorescence Standard Kit

The Spectral Fluorescence Standard Kit (Cat. No. 69336) enables a simple characterization of the relative spectral responsivity and the long-term stability of the emission channel of fluorescence instruments under routine measurement conditions and provides the basis for an improved comparability of fluorescence measurement and eventual standardization.

- **CD with Improved Software** LINKCORRWIN V.1.1.0.0. developed by BAM for the data evaluation. Version1.1.0.0 only performs well in conjunction with BAM-F002a. The CD also contains instructions for use of BAM-F001 – BAM-F005 and LINKCORRWIN.
- **Five Spectral Fluorescence Standards** Ready-made from Sigma-Aldrich GmbH, which cover the spectral region of 300 nm to 770 nm as a set.
- **Ethanol, absolute** (Cat. No. 34923) Addition of aliquots of 10 mL of ethanol to each solid dye yields a solution that can be measured without additional dilution steps.
- **BAM Certificate** of the normalized corrected emission spectra of BAM-F001 – BAM-F005 (see also CD).

Application of the Emission Correction Curve

**Bottom:** Certified normalized corrected emission spectra (solid lines; \(I(\lambda_{em})\)) of the kit components and uncorrected, i.e. instrument-dependent emission spectra (dashed lines; \(I_0(\lambda_{em})\)), measured with the instrument to be calibrated.

**Middle:** Individual quotient \(Q^{BAM}_x = I^{BAM}_x(\lambda_{em})/I_0^{BAM}_x(\lambda_{em})\) for each kit dye equaling \(1/s(\lambda)\) of the instrument to be calibrated within the spectral region of the respective fluorescence standard.

**Top:** Combined emission correction curve \(1/s(\lambda)\) (solid pink line) calculated from the statistically weighted \(Q^{BAM}_x\) as well as its reciprocal \(s(\lambda)\) (black solid line).

Corrected emission spectra are obtained by multiplication of measured spectra by the output of LINKCORR, i.e., \(1/s(\lambda)\).

Determination of the relative spectral responsivity \(s(\lambda)\) of a fluorescence instrument with the Spectral Fluorescence Standard Kit and LINKCORRWIN.
Certified Reference Materials

- For the determination of the relative spectral responsivity $s(\lambda)$ of fluorescence instruments. $1/s(\lambda)$ is termed emission correction curve. Both can be determined by LINKCORRWIN.
- For the determination of corrected, i.e., instrument-independent emission spectra that are comparable across instruments.
- For the characterization of the long-term stability of the emission channel of fluorescence instruments. Fluorescence spectra that are corrected accordingly do not contain contributions from aging of optical components in the emission channel.

Certified Properties

Normalized corrected emission spectra of BAM-F001 – BAM-F005 in ethanol for $T=25$ °C. The emission spectra are traceable to the spectral radiance realized and disseminated in Germany by the Physikalisch-Technische Bundesanstalt (PTB).

The Calibration Kit has been tested in an interlaboratory comparison by the National Metrological Institutes NIST (National Institutes of Standards and Technology, USA), NRC (National Research Council, Canada), PTB (Physikalisch-Technische Bundesanstalt, Germany), and BAM (Federal Institute for Material Research and Testing) employing two different dye concentrations and two different measurement geometries, i.e., $0^\circ/90^\circ$ and $45^\circ/0^\circ$.

For more information, please visit sigma.com/spectral

References
