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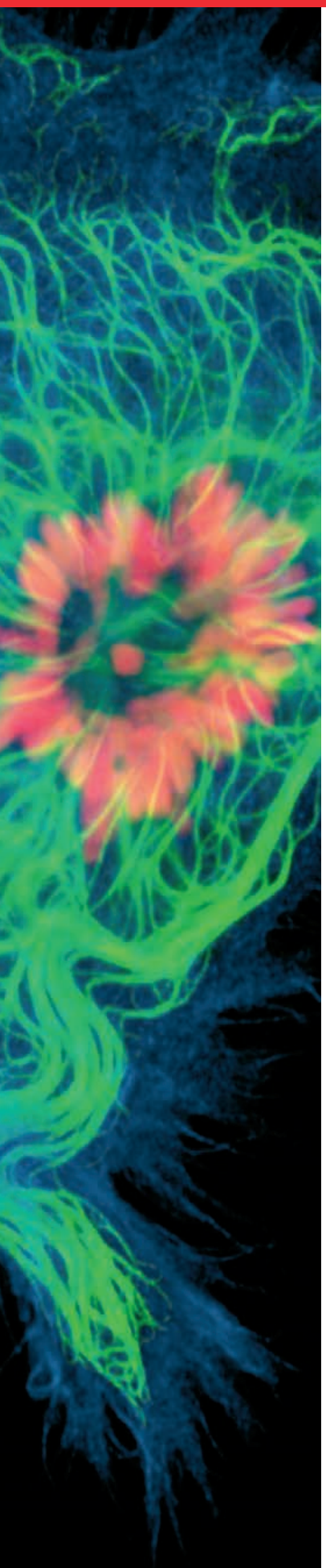

Molecular Probes™ Handbook

A Guide to Fluorescent Probes and Labeling Technologies

11th Edition (2010)

CHAPTER 1

Fluorophores and Their Amine-Reactive Derivatives

Molecular Probes Resources
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Comprehensive guide to fluorescent probes and labeling technologies
thermofisher.com/handbook

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CHAPTER 1

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1.1 Introduction to Amine Modification

The Molecular Probes® Handbook describes a full spectrum of fluorophores and haptens for covalent derivatization of biopolymers and low molecular weight molecules. Chapters 1–5 describe the chemical and spectroscopic properties of the reactive reagents we offer, whereas the remainder of this book is primarily devoted to our diverse collection of fluorescent probes and their applications in cell biology, neurobiology, immunology, molecular biology and biophysics.

Common Applications for Amine-Reactive Probes

Labeling Biomolecules

Amine-reactive probes are widely used to modify proteins, peptides, ligands, synthetic oligonucleotides and other biomolecules. In contrast to our thiol-reactive reagents (Chapter 2), which frequently serve as probes of protein structure and function, amine-reactive dyes are most often used to prepare bioconjugates for immunochemistry, fluorescence *in situ* hybridization (FISH), cell tracing, receptor labeling and fluorescent analog cytochemistry. In these applications, the stability of the chemical bond between the dye and biomolecule is critical. The bioconjugate will typically be applied to a biochemically complex (and sometimes active) specimen. Furthermore, it will often be subjected to a series of post-processing steps such as washing, permeabilization, fixation and mounting. The integrity of the bioconjugate must be maintained throughout these processes if the fluorescence signal generated by the dye is to have any useful relationship to the abundance or localization of the bioconjugate's molecular target.

Our selection of amine-reactive fluorophores for modifying biomolecules covers the entire visible and near-infrared spectrum (Table 1.1, Table 1.2). Up-to-date bibliographies are available for most of our amine-reactive probes at www.invitrogen.com. Also available are other product-specific bibliographies, as well as keyword searches of the over 60,000 literature references in our product application bibliography database.

Chapter 1 discusses the properties of Molecular Probes® fluorophores, including:

- Alexa Fluor® dyes (Section 1.3)
- BODIPY® dyes (Section 1.4)
- Oregon Green® and Rhodamine Green™ dyes (Section 1.5)
- Rhodamine Red™-X and Texas Red® dyes (Section 1.6)
- UV light–excitable Cascade Blue®, Cascade Yellow™, Marina Blue®, Pacific Blue™ and AMCA-X fluorophores (Section 1.7)

Our essentially nonfluorescent QSY® dyes (Section 1.6, Section 1.8) have strong visible absorption, making them excellent acceptors for fluorescence resonance energy transfer (FRET) applications (Fluorescence Resonance Energy Transfer (FRET)—Note 1.2).

Preparing the Optimal Bioconjugate

The preferred bioconjugate usually has a high fluorescence output (or, in the case of a haptenylated conjugate, a suitable degree of labeling) yet retains the critical functional properties of the unlabeled biomolecule, such as solubility, selective binding to a receptor or nucleic acid, activation or inhibition of a particular enzyme or the ability to incorporate into a biological membrane.[1] Frequently, however, conjugates with the highest degree of labeling precipitate out of solution or bind nonspecifically.[2] Thus there is usually a trade-off between degree of labeling and functional properties that must be resolved through experimental optimization. Lysine residues, the primary targets for amine modification of proteins, are relatively abundant. In mammalian proteins, lysine has the fifth highest occurrence frequency of the 20 naturally occurring amino acids. A typical IgG antibody molecule has about 90 lysine residues, of which about 30 at most can be modified under forcing conditions of high acylating reagent concentration and prolonged incubation. However, maintenance of functional properties (more specifically, antigen binding affinity) typically requires a degree of labeling of <10 dyes per IgG, representing a low fractional modification of available targets. A further consequence of low fractional modification is that, with the exception of small peptides or rare proteins with few lysine residues, bioconjugates prepared by amine modification are polydisperse mixtures containing a range of dye:protein stoichiometries.[3,4]

For the most critical assays, we recommend that researchers consider preparing and optimizing their own conjugates. Our amine-reactive dyes are supplied with a detailed protocol that describes how to use them for labeling biomolecules. This procedure is straightforward and requires no special equipment. Following conjugation, it is very important to remove as much unconjugated labeling reagent as possible, usually by gel filtration, dialysis, bioconjugate precipitation and resolubilization, HPLC or a combination of these techniques. The presence of free dye, particularly if it remains chemically reactive, can greatly complicate subsequent experiments with the bioconjugate. The entire process of labeling reaction and conjugate purification can be completed in little more than two hours, and the main prerequisite is a sufficient amount of purified protein or amine-modified nucleic acid.

With the exception of the phycobiliproteins (Section 6.4, Table 6.2), fluorescent microspheres (Section 6.5, Table 6.7), Qdot® nanocrystals (Section 6.6), Zenon® Antibody Labeling Kits (Section 7.3, Table 7.7) and ULYSIS® Nucleic Acid Labeling Kits (Section 8.2, Table 8.6), virtually all reagents used to prepare Molecular Probes® fluorescent bioconjugates are amine-reactive organic fluorophores, and almost all are described in this chapter.

We have also developed convenient kit formats for labeling proteins and nucleic acids with our most important fluorophores, or alternatively with biotin. Section 1.2 and Table 1.3 include a complete description of these kits. Alternatively, we prepare custom fluorescent conjugates for research use; contact Invitrogen Custom Services for more information. Conjugations with phycobiliproteins, fluorescent polystyrene microspheres and Qdot® nanocrystals require specialized procedures that are described in Section 6.4, Section 6.5 and Section 6.6, respectively.

Derivatizing Low Molecular Weight Molecules

Some amine-reactive probes described in this chapter are also important reagents for various bioanalytical applications, including amine quantitation, protein and nucleic acid sequencing and chromatographic and electrophoretic analysis of low molecular weight molecules. Reagents that are particularly useful for derivatizing low molecular weight amines—including fluorescamine, *o*-phthaldialdehyde, ATTO-TAG™ reagents, NBD chloride and dansyl chloride—are

Table 1.1 Molecular Probes® amine-reactive dyes.

Fluorophore	COOH *	SE *	Other *	Abs (nm)	Em (nm)	Notes
Methoxycoumarin	M1420MP	M1410		340	405	• pH-insensitive alternative to 7-hydroxycoumarins
Dansyl		D6104 (X)	D21 (SC)	340	520 ‡	• Environment-sensitive fluorescence • Large Stokes shift
Pyrene		P130 P6114	P24 (SC)	345	378	• Long excited-state lifetime • Spectral shifts due to excimer emission
Alexa Fluor® 350		A10168		346	442	• Higher fluorescence output than AMCA • Optimally detected with DAPI optical filter sets
AMCA		A6118 (X)		349	448	• Widely used blue-fluorescent labeling dye • Compact structure
Marina Blue® dye		M10165		365	460	• Strongly fluorescent at neutral pH • Optimally detected with DAPI optical filter sets
Dapoxyl® dye		D10161	D10160 (SC)	373	551 ‡	• Environment-sensitive fluorescence • Large Stokes shift
Dialkylaminocoumarin	D126 D1421	D374 D1412		375 430	470 § 475 **	• Longer-wavelength alternatives to AMCA
Bimane	B30250			380	458	• Blue-fluorescent dye • Small size
Hydroxycoumarin	H185 H1428	H1193		385	445 ††	• pH-sensitive fluorescence • Compact structure
Cascade Blue® dye			C2284 (AA)	400	420	• Resistant to quenching upon protein conjugation • Water soluble
Pacific Orange™ dye		P30253		400	551	• Excited with the 405 nm violet diode laser • Compatible with the Pacific Blue™ dye for two-color analysis using the violet diode laser
Alexa Fluor® 405		A30000 A30100		402	421	• Cascade Blue® derivative containing a spacer between the fluorophore and the reactive SE • Near-perfect match to the 405 nm violet diode laser
Cascade Yellow™ dye		C10164		402	545	• Large Stokes shift • High molar absorptivity
Pacific Blue™ dye		P10163		410	455	• Recommended for 405 nm violet diode laser excitation
PyMPO		S6110		415	570	• Large Stokes shift
Alexa Fluor® 430		A10169		434	539	• Large Stokes shift
NBD		S1167 (X)	F486 (AH)	465	535	• Environment-sensitive fluorescence • Compact structure
QSY® 35		Q20133		475	none	• Nonfluorescent quencher • An efficient energy transfer acceptor from blue and green fluorophores
Fluorescein	C1359 C1360 C1904 †	C2210 C6164 C1311 † F6106 (X) F2181 (X) † F6129 (X) † F6130 (EX) C20050 (PA)	D16 (DTA) F143 (ITC) F1906 (ITC) F1907 (ITC)	494	518	• Most widely used green-fluorescent labeling dye • Absorption overlaps the 488 nm spectral line of the argon-ion laser • Prone to photobleaching • pH-sensitive fluorescence between pH 5–8 • Fluorescein-5-EX succinimidyl ester (F6130) is the preferred reactive fluorescein for protein conjugation
Alexa Fluor® 488		A20000 † A20100 †	A30005 (TFP) A30052 (SDP)	495	519	• Bright and photostable fluorescein substitute • Fluorescence output unmatched by any other spectrally similar dye • pH-insensitive fluorescence between pH 4 and 10 • Ideal for excitation by the 488 nm spectral line of the argon-ion laser • As compared with the SE, the TFP ester is less susceptible to spontaneous hydrolysis during conjugation reactions
Oregon Green® 488	O6146	O6147 O6149	O6080 (ITC) †	496	524	• Photostable fluorescein substitute • pH-insensitive fluorescence at pH >6
BODIPY® 493/503		D2191		500	506	• pH-insensitive fluorescence • Narrow spectral bandwidth • Higher 488 nm absorptivity than the BODIPY® FL fluorophore
Rhodamine Green dye		R6107 † R6113 (X) †		502	527	• Photostable fluorescein substitute • pH-insensitive fluorescence

The absorption (Abs) and fluorescence emission (Em) maxima listed in this table are for the goat anti–mouse IgG antibody or dextran conjugates in aqueous buffer. Our online Fluorescence SpectraViewer (www.invitrogen.com/handbook/spectraviewer) provides an interactive utility for plotting and comparing fluorescence excitation and emission spectra for over 250 fluorophores (Using the Fluorescence SpectraViewer—Note 23.1). * COOH = carboxylic acid; SE = succinimidyl ester; (AA) = acetyl azide; (AH) = aryl halide; (C_5) = pentanoic acid; (DTA) = dichlorotriazine; (EX) = seven-atom spacer that is more hydrophilic than X; (ITC) = isothiocyanate; (PA) = photoactivatable; (SC) = sulfonyl chloride; (SDP) = sulfodichlorophenol ester; (SSE) = sulfosuccinimidyl ester; (STP) = 4-sulfotetrafluorophenyl ester; (TFP) = tetrafluorophenyl ester; (X) = an aminohexanoyl spacer between the dye and SE. † Mixed isomers. ‡ Emission spectra of dansyl and Dapoxyl® conjugates may vary considerably depending on the dye attachment site and the degree of labeling. § Spectral maxima for D374. ** Spectral maxima for D1412. †† Spectral maxima for H1193. More information on amine-reactive dyes is available at www.invitrogen.com/handbook/labelingchemistry.

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Table 1.1 Molecular Probes® amine-reactive dyes—*continued.*

Fluorophore	COOH *	SE *	Other *	Abs (nm)	Em (nm)	Notes
BODIPY® FL	D2183 ‡ D3834 (C_5)	D2184 D6140 (SSE) D6102 (X) D6184 (C_5)	B10006 (STP)	505	513	• BODIPY® substitute for fluorescein • pH-insensitive fluorescence • Narrow spectral bandwidth
2',7'-Dichloro-fluorescein	C368 †			510	532	• pH-insensitive fluorescence at pH >6
Oregon Green® 514	O6138	O6139		511	530	• Exceptionally photostable • pH-insensitive fluorescence at pH >6
Alexa Fluor® 514		A30002 †		518	540	• Designed to be optically resolved from the Alexa Fluor® 488 dye using spectral imaging instruments with linear-unmixing software • Optimal dye for the 514 nm spectral line of the argon-ion laser
4',5'-Dichloro-2',7'-dimethoxy-fluorescein (JOE)		C6171MP		522	550	• Succinimidyl ester derivative (6-JOE, SE; C6171MP) is widely used for oligonucleotide labeling
Eosin			E18 (ITC)	524	544	• Useful for DAB photoconversion • Phosphorescent
Rhodamine 6G		C6127 C6128 C6157 †		525	555	• Excited by the 514 nm spectral line of the argon-ion laser • Spectra intermediate between those of fluorescein and tetramethylrhodamine
BODIPY® R6G		D6180		528	550	• BODIPY® substitute with spectra similar to rhodamine 6G • pH-insensitive fluorescence • Narrow spectral bandwidth
Alexa Fluor® 532		A20001 A20101MP		531	554	• Bright and photostable dye with spectra intermediate between those of fluorescein and tetramethylrhodamine • Fluorescence output unmatched by any other spectrally similar dye • pH-insensitive fluorescence between pH 4 and 10 • Ideal for excitation by the 532 nm frequency-doubled principal line output of the Nd:YAG laser
BODIPY® 530/550		D2187		534	554	• pH-insensitive fluorescence • Narrow spectral bandwidth
BODIPY® TMR		D6117 (X)		542	574	• BODIPY® substitute for tetramethylrhodamine • pH-insensitive fluorescence • Narrow emission spectral bandwidth
Alexa Fluor® 555		A20009 A20109		555	565	• Red-orange fluorescence • Bright and photostable tetramethylrhodamine or Cy®3 substitute • Spectrally similar to Cy®3
Tetramethyl-rhodamine (TMR)	C6121 C6122 C300 †	C2211 C6123 C1171 † T6105 (X) †	T1480 (ITC) T1481 (ITC) T490 (ITC) †	555	580	• pH-insensitive fluorescence • Good photostability • Prone to aggregation
Alexa Fluor® 546		A20002 † A20102 †		556	575	• Bright and photostable tetramethylrhodamine or Cy®3 substitute • Fluorescence output unmatched by any other spectrally similar dye • pH-insensitive fluorescence between pH 4 and 10 • Less prone to aggregation than tetramethylrhodamine
BODIPY® 558/568		D2219		558	569	• pH-insensitive fluorescence • Narrow spectral bandwidth
QSY® 7		Q10193		560	none	• Nonfluorescent quencher • Broad visible-wavelength absorption • Efficient energy transfer acceptor from green and orange fluorophores
QSY® 9		Q20131		562	none	• Nonfluorescent quencher • Spectrally similar to QSY® 7, but with enhanced water solubility • Efficient energy-transfer acceptor from green and orange fluorophores
BODIPY® 564/570		D2222		565	571	• pH-insensitive fluorescence • Narrow spectral bandwidth
Lissamine rhodamine B			L20 (SC) † L1908 (SC) †	570	590	• Optimal for 568 nm excitation • Photostable
Rhodamine Red dye		R6160 (X) †		570	590	• Conjugates are generally more fluorescent than those of Lissamine rhodamine B sulfonyl chloride, and the succinimidyl ester is more stable in H_2O

The absorption (Abs) and fluorescence emission (Em) maxima listed in this table are for the goat anti–mouse IgG antibody or dextran conjugates in aqueous buffer. Our online Fluorescence SpectraViewer (www.invitrogen.com/handbook/spectraviewer) provides an interactive utility for plotting and comparing fluorescence excitation and emission spectra for over 250 fluorophores (Using the Fluorescence SpectraViewer—Note 23.1). * COOH = carboxylic acid; SE = succinimidyl ester; (AA) = acetyl azide; (AH) = aryl halide; (C_5) = pentanoic acid; (DTA) = dichlorotriazine; (EX) = seven-atom spacer that is more hydrophilic than X; (ITC) = isothiocyanate; (PA) = photoactivatable; (SC) = sulfonyl chloride; (SDP) = sulfodichlorophenol ester; (SSE) = sulfosuccinimidyl ester; (STP) = 4-sulfotetrafluorophenyl ester; (TFP) = tetrafluorophenyl ester; (X) = an aminohexanoyl spacer between the dye and SE. † Mixed isomers. ‡ Emission spectra of dansyl and Dapoxyl® conjugates may vary considerably depending on the dye attachment site and the degree of labeling. § Spectral maxima for D374. ** Spectral maxima for D1412. †† Spectral maxima for H1193. More information on amine-reactive dyes is available at www.invitrogen.com/handbook/labelingchemistry.

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Table 1.1 Molecular Probes® amine-reactive dyes—*continued.*

Fluorophore	COOH *	SE *	Other *	Abs (nm)	Em (nm)	Notes
BODIPY® 576/589		D2225		576	590	• pH-insensitive fluorescence • Narrow spectral bandwidth
Alexa Fluor® 568		A20003 † A20103 †		578	603	• Bright and photostable Lissamine rhodamine B substitute • Fluorescence output unmatched by any other spectrally similar dye • pH-insensitive fluorescence between pH 4 and 10
X-rhodamine	C6124 C6156	C6125 C6126 C1309 †	X491 (ITC) †	580	605	• Succinimidyl ester derivative (6-ROX, SE; C6126) widely used for oligonucleotide labeling
BODIPY® 581/591		D2228		584	592	• pH-insensitive fluorescence • Narrow spectral bandwidth
BODIPY® TR		D6116 (X)		589	617	• BODIPY® substitute for the Texas Red® fluorophore • pH-insensitive fluorescence
Alexa Fluor® 594		A20004 † A20104 †		590	617	• Bright and photostable Texas Red® dye substitute • Fluorescence output unmatched by any other spectrally similar dye • pH-insensitive fluorescence between pH 4 and 10 • Ideal for excitation by the 594 nm spectral line of the He-Ne laser
Texas Red® dye		T6134 (X) † T20175 (X)	T353 (SC) † T1905 (SC) †	595	615	• Good spectral separation from green fluorophores • Texas Red®-X succinimidyl ester typically yields higher fluorescence per attached dye than Texas Red® sulfonyl chloride and is more stable in H_2O
Naphthofluorescein	C652 †	C653 †		605	675	• Very long-wavelength excitation and emission • pH-sensitive fluorescence
Alexa Fluor® 610-X		A30050		612	628	• Bright and photostable Texas Red® dye substitute • Fluorescence output unmatched by any other spectrally similar dye • pH-insensitive fluorescence between pH 4 and 10 • Easily differentiated from green fluorophores • Still visible by eye, unlike longer-wavelength fluorophores
BODIPY® 630/650		D10000 (X)		625	640	• pH-insensitive fluorescence • Ideal for excitation by the 633 nm spectral line of the He-Ne laser
Malachite green			M689 (ITC)	630	none	• Nonfluorescent photosensitizer
Alexa Fluor® 633		A20005 † A20105 †		632	647	• Far-red fluorescence • Good spectral separation from green fluorophores • pH-insensitive fluorescence between pH 4 and 10
Alexa Fluor® 635				633	647	• Far-red fluorescence • Good spectral separation from green fluorophores • pH-insensitive fluorescence between pH 4 and 10
BODIPY® 650/665		D10001 (X)		646	660	• pH-insensitive fluorescence • Longest-wavelength BODIPY® dye currently available
Alexa Fluor® 647		A20006 A20106		650	668	• Far-red fluorescence • Produces conjugates that are brighter than those of the Cy®5 dye • pH-insensitive fluorescence between pH 4 and 10
QSY® 21		Q20132		661	none	• Nonfluorescent quencher • Long-wavelength absorption • An efficient energy transfer acceptor from red and near-infrared fluorophores
Alexa Fluor® 660		A20007 A20107		663	690	• Far-red fluorescence • Good spectral separation from green and red-orange fluorophores • pH-insensitive fluorescence between pH 4 and 10
Alexa Fluor® 680		A20008 A20108		679	702	• Far-red fluorescence • Good separation from red fluorophores—useful for three- and four-color applications • pH-insensitive fluorescence between pH 4 and 10
Alexa Fluor® 700		A20010 A20110		702	723	• Far-red fluorescence • Good separation from red fluorophores—useful for three- and four-color applications • pH-insensitive fluorescence between pH 4 and 10
Alexa Fluor® 750		A20011 A20111		749	775	• Far-red fluorescence • Good separation from red fluorophores—useful for three- and four-color applications • pH-insensitive fluorescence between pH 4 and 10 • Spectrally similar to the Cy®7 dye
Alexa Fluor® 790		A30051		782	805	• Spectrally similar to indocyanine green (ICG) dye

The absorption (Abs) and fluorescence emission (Em) maxima listed in this table are for the goat anti–mouse IgG antibody or dextran conjugates in aqueous buffer. Our online Fluorescence SpectraViewer (www.invitrogen.com/handbook/spectraviewer) provides an interactive utility for plotting and comparing fluorescence excitation and emission spectra for over 250 fluorophores (Using the Fluorescence SpectraViewer—Note 23.1). * COOH = carboxylic acid; SE = succinimidyl ester; (AA) = acetyl azide; (AH) = aryl halide; (C_5) = pentanoic acid; (DTA) = dichlorotriazine; (EX) = seven-atom spacer that is more hydrophilic than X; (ITC) = isothiocyanate; (PA) = photoactivatable; (SC) = sulfonyl chloride; (SDP) = sulfodichlorophenol ester; (SSE) = sulfosuccinimidyl ester; (STP) = 4-sulfotetrafluorophenyl ester; (TFP) = tetrafluorophenyl ester; (X) = an aminohexanoyl spacer between the dye and SE. † Mixed isomers. ‡ Emission spectra of dansyl and Dapoxyl® conjugates may vary considerably depending on the dye attachment site and the degree of labeling. § Spectral maxima for D374. ** Spectral maxima for D1412. †† Spectral maxima for H1193. More information on amine-reactive dyes is available at www.invitrogen.com/handbook/labelingchemistry.

discussed in Section 1.8. However, many of the reactive dyes described in Sections 1.2 to 1.7 can also be used as derivatization reagents; likewise, most of the derivatization reagents in Section 1.8 can be utilized for biomolecule conjugation.

Reactivity of Amino Groups

The amine-reactive organic fluorophores described in this chapter are mostly acylating reagents that form carboxamides, sulfonamides or thioureas upon reaction with amines. The kinetics of the reaction depend on the reactivity and concentration of both the acylating reagent and the amine.[5] Of course, buffers that contain free amines such as Tris and glycine must be avoided when using *any* amine-reactive probe. Ammonium sulfate used for protein precipitation must also be removed before performing dye conjugations. In addition, high concentrations of nucleophilic thiols should be avoided because they may react with the amine-reactive reagent to form an unstable intermediate that could consume the dye. Reagents for reductive alkylation of amines are described in Chapter 2 and Chapter 3.

The most significant factors affecting an amine's reactivity are its class (aliphatic or aromatic) and its basicity. Virtually all proteins have lysine residues, and most have a free amine at the N-terminus. Aliphatic amines such as lysine's ε-amino group are moderately basic and reactive with most acylating reagents. However, the concentration of the free base form of aliphatic amines below pH 8 is very low; thus, the kinetics of amine acylation by isothiocyanates, succinimidyl esters or other reagents are strongly pH dependent. A pH of 8.5 to 9.5 is usually optimal for modifying lysine residues. In contrast, the α-amino group at a protein's N-terminus usually has a pK_a of ~7, so it can sometimes be selectively modified by reaction at near neutral pH. Furthermore, although amine acylation should usually be carried out above pH 8.5, the acylation reagents tend to degrade in the presence of water, with the rate increasing as the pH increases.[5] Protein modification by succinimidyl esters can typically be done at pH 8.3, whereas isothiocyanates usually require a pH >9 for optimal conjugations; this high pH may be a factor when working with base-sensitive proteins. DNA and most polysaccharides can be modified at a relatively basic pH if necessary.

Aromatic amines, which are uncommon in biomolecules, are very weak bases and thus unprotonated at pH 7. Modification of aromatic amines requires a highly reactive reagent, such as an isocyanate, isothiocyanate, sulfonyl chloride or acid halide, but can be done at any pH above ~4. A tyrosine residue can be selectively modified to form an *o*-aminotyrosine aromatic amine, which can then be reacted at a relatively low pH with certain amine-reactive probes[6] (Figure 1.1.1).

In aqueous solution, acylating reagents are virtually unreactive with the amide group of peptide bonds and the side-chain amides of glutamine and asparagine residues, the guanidinium group of arginine, the imidazolium group of histidine and the nonbasic amines, such as adenosine or guanosine, found in nucleotides and nucleic acids.

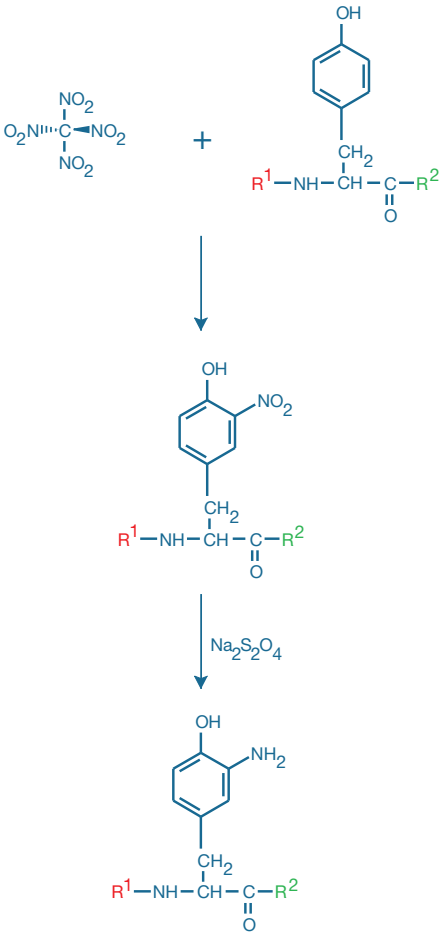

Figure 1.1.1 Nitration of tyrosine by reaction with tetranitromethane, followed by reduction with sodium dithionite, to yield an *o*-aminotyrosine.

Isothiocyanates

Because they are very susceptible to deterioration during storage, we do not sell any isocyanates. Some acyl azides (Section 3.2), however, are readily converted to isocyanates (Figure 1.1.2), which then react with amines to form ureas. As an alternative to the unstable isocyanates, we

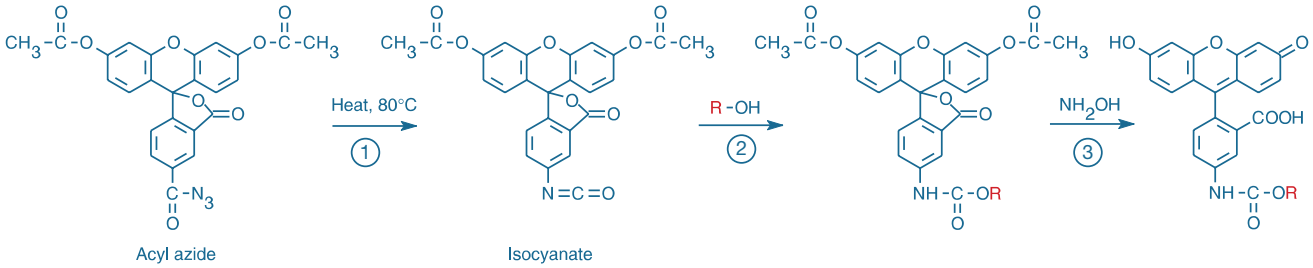

Figure 1.1.2 Derivatization of an alcohol using the diacetate of fluorescein-5-carbonyl azide (F6218). This process consists of three steps: 1) rearrangement of the acyl azide to an isocyanate, 2) reaction of the isocyanate with an alcohol to form a urethane and 3) deprotection of the nonfluorescent urethane derivative using hydroxylamine.

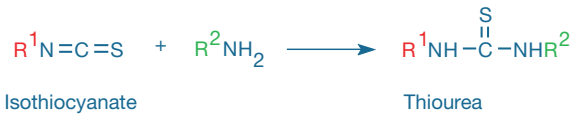

Figure 1.1.3 Reaction of a primary amine with an isothiocyanate.

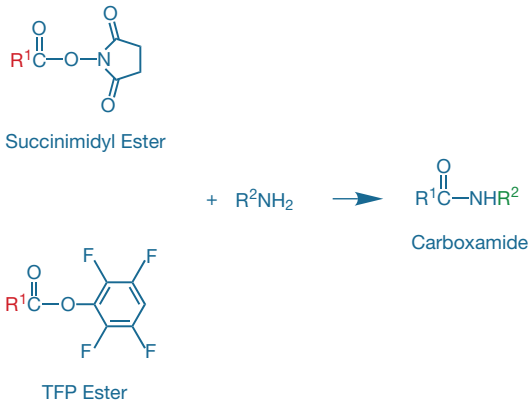

Figure 1.1.4 Reaction of a primary amine with a succinimidyl ester or a tetrafluorophenyl (TFP) ester.

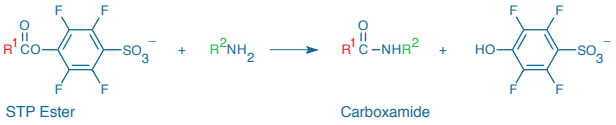

Figure 1.1.6 Reaction of a primary amine with an STP ester.

offer a large selection of isothiocyanates, which are moderately reactive but quite stable in water and most solvents. Isothiocyanates form thioureas upon reaction with amines (Figure 1.1.3). Although the thiourea product is reasonably stable, it has been reported that antibody conjugates prepared from fluorescent isothiocyanates deteriorate over time,[7] prompting us to use fluorescent succinimidyl esters and sulfonyl halides almost exclusively for synthesizing bioconjugates. The thiourea formed by the reaction of fluorescein isothiocyanate with amines is also susceptible to conversion to a guanidine by concentrated ammonia.[8] Despite the growing number of choices in amine-reactive fluorophores, fluorescein isothiocyanate (FITC) and tetramethylrhodamine isothiocyanate (TRITC) are still widely used reactive fluorescent dyes for preparing fluorescent bioconjugates.

Active Esters and Carboxylic Acids

Succinimidyl Esters

Succinimidyl esters are reliable reagents for amine modification because the amide bonds they form (Figure 1.1.4) are as stable as peptide bonds. We provide over 100 succinimidyl esters of fluorescent dyes and nonfluorescent molecules, most of which have been developed within our own laboratories (Table 1.1, Table 1.2). These reagents are generally stable during storage if well desiccated, and they show good reactivity with aliphatic amines and very low reactivity with aromatic amines, alcohols, phenols (including tyrosine) and histidine. Side-reactions of succinimidyl esters with alcohols are generally only observed in applications such as derivatization for mass spectrometry, in which much larger molar excesses of succinimdyl ester reagents and longer reaction times are used than is typically the case in protein labeling for fluorescence detection applications.[9,10] Succinimidyl esters will also react with thiols in organic solvents to form thioesters. If formed in a protein, a thioester may transfer the acyl moiety to a nearby amine. Succinimidyl ester hydrolysis (generating the unreactive carboxylic acid) competes with conjugation, but this side reaction is usually slow below pH 9.

Carboxylic Esters and Their Conversion into Sulfosuccinimidyl Esters and STP Esters

Some succinimidyl esters may not be compatible with a specific application because they can be quite insoluble in aqueous solution. To overcome this limitation, we offer carboxylic acid derivatives of many fluorophores, which can be converted into sulfosuccinimidyl esters or 4-sulfotetrafluorophenyl (STP) esters. These sulfonated esters have higher water solubility than simple succinimidyl esters and sometimes eliminate the need for organic solvents in the conjugation reaction. They are, however, also more polar than succinimidyl esters, which makes them less likely to react with buried amines in proteins or to penetrate cell

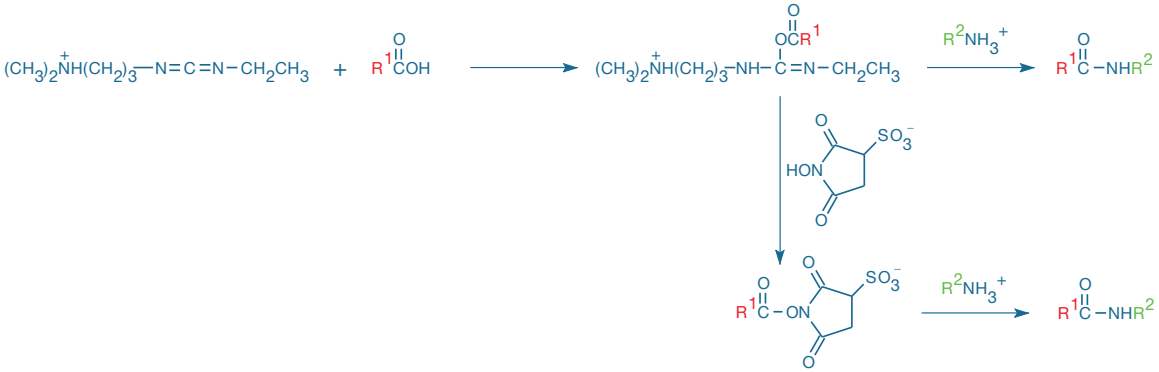

Figure 1.1.5 Stabilization of an unstable *O*-acylisourea intermediate by *N*-hydroxysulfosuccinimide (NHSS, H2249) in a carbodiimide-mediated (EDAC, E2247) modification of a carboxylic acid with a primary amine.

membranes. Because of their combination of reactivity and polarity, sulfosuccinimidyl esters are not easily purified by chromatographic means and thus only a few are currently available. Sulfosuccinimidyl esters can generally be prepared *in situ* simply by dissolving the carboxylic acid dye in an amine-free buffer that contains *N*-hydroxysulfosuccinimide and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (NHSS, H2249; EDAC, E2247; Section 3.4). Addition of NHSS to the buffer has been shown to enhance the yield of carbodiimide-mediated conjugations [11] (Figure 1.1.5). STP esters (Figure 1.1.6) are prepared in the same way from 4-sulfo-2,3,5,6-tetrafluorophenol[12] (S10490, Section 3.4), and we find them to be more readily purified by chromatography than their sulfosuccinimidyl ester counterparts. The carboxylic acid derivatives may also be useful for preparing acid chlorides and anhydrides, which, unlike succinimidyl esters, can be used to modify aromatic amines and alcohols.

Tetrafluorophenyl (TFP) Esters

2,4,5,6-Tetrafluorophenyl (TFP) esters (Figure 1.1.4) are more resistant to nonspecific hydrolysis than either succinimidyl esters (Figure 1.1.7) or sulfosuccinimidyl esters, yet they exhibit equal or superior reactivity with amines.[13] At this time, Alexa Fluor® 488 carboxylic acid is the only fluorescent TFP ester we offer (A30005, Section 1.3).

Sulfodichlorophenol (SDP) Esters

The sulfodicholorphenol (SDP) ester is currently the most hydrolytically stable amine-reactive moiety that we offer. As with TFP esters, Alexa Fluor® 488 carboxylic acid is the only fluorescent SDP ester available (A30052, Section 1.3). Conjugates produced with the Alexa Fluor® 488 5-SDP ester produce the same strong amide bond between the dye and the compound of interest as succinimidyl and tetrafluorophenyl (TFP) esters. Because of its improved stability in water and buffers, however, the SDP ester can potentially offer increased control and consistency in reactions as compared with its succinimidyl ester and TFP ester counterparts.

Carbonyl Azides

Section 3.2 describes coumarin, fluorescein and tetramethylrhodamine carbonyl azides (D1446, M1445, F6218, T6219). Like succinimidyl esters, carbonyl azides are active esters that can react with amines to yield amides; however, a more common application of carbonyl azides is thermal rearrangement to a labile isocyanate (which can then react with both aliphatic and aromatic amines to form ureas) for derivatizing alcohols and phenols (Section 3.2, Figure 1.1.2).

Sulfonyl Chlorides

Sulfonyl chlorides, including the dansyl, pyrene, Lissamine rhodamine B and Texas Red® derivatives, are highly reactive but also quite unstable in water, especially at the higher pH required for reaction with aliphatic amines. For example, we have determined that dilute Texas Red® sulfonyl chloride is totally hydrolyzed within 2–3 minutes in pH 8.3 aqueous solution at room temperature.[14] Protein modification by this reagent is therefore best done at low temperature. Once conjugated, however, the sulfonamides that are formed (Figure 1.1.8) are extremely stable; they even survive complete protein hydrolysis (for example, dansyl end-group analysis[15]).

Sulfonyl chlorides can also react with phenols (including tyrosine), aliphatic alcohols (including polysaccharides), thiols (such as cysteine) and imidazoles (such as histidine), but these reactions are not common in proteins or in aqueous solution. Sulfonyl chloride conjugates of thiols and imidazoles are generally unstable, and conjugates of aliphatic alcohols are subject to nucleophilic displacement.[16] Note that sulfonyl chlorides are unstable in dimethylsulfoxide (DMSO) and should never be used in that solvent.[17]

Other Amine-Reactive Reagents

Aldehydes react with amines to form Schiff bases. Notable aldehyde-containing reagents described in Section 1.8 include *o*-phthaldialdehyde (OPA) and naphthalenedicarboxaldehyde (NDA), as well as the 3-acylquinolinecarboxaldehyde (ATTO-TAG™) reagents CBQCA and FQ devised by Novotny and collaborators.[18–20] All of these reagents are useful for the sensitive quantitation of amines in solution, by HPLC and by capillary electrophoresis. In addition, certain arylating reagents such as NBD chloride, NBD fluoride and dichlorotriazines react with both amines and thiols, forming bonds with amines that are particularly stable.

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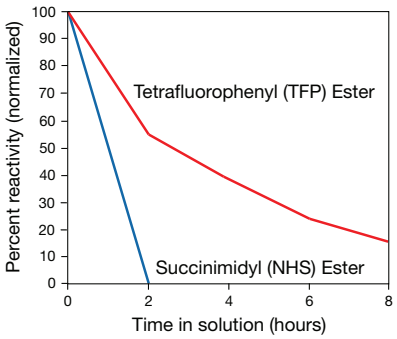

Figure 1.1.7 Stability of the tetrafluorophenyl (TFP) and succinimidyl (NHS) esters at basic pH (8.0–9.0).

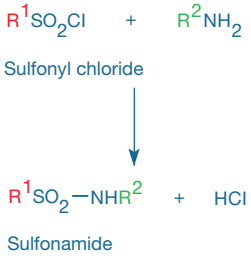

Figure 1.1.8 Reaction of a primary amine with a sulfonyl chloride.

1.2 Kits for Labeling Proteins and Nucleic Acids

We provide a vast number of stand-alone reagents for preparing bioconjugates, most of which are described in detail in other sections of this chapter. This section describes the many specialized kits that we have developed for covalently labeling proteins and nucleic acids with our organic dyes and haptens (Table 1.2, Table 1.3).

As an alternative to direct conjugation of primary antibodies with our reactive dyes and haptens, we recommend using Zenon® technology (Section 7.3) to form labeled antibody complexes. Zenon® labeling can typically be completed in minutes with quantitative yield starting with submicrogram quantities of antibody, and the labeling stoichiometry can be easily adjusted to deliver optimum fluorescence output. Although they do not provide covalent labeling, the Zenon® Antibody Labeling Kits are listed in Table 1.2, as well as in Table 7.7.

Kits for Labeling Proteins with a Fluorescent Dye

APEX® Antibody Labeling Kits

APEX® Antibody Labeling Kits (Table 1.2, Table 1.3) provide a convenient method for covalently labeling small amounts (10–20 µg) of IgG antibody with Alexa Fluor® dyes, Oregon Green® 488 dye or Pacific Blue™ dye. A primary antibody directly labeled with a fluorophore often produces lower background fluorescence and less nonspecific binding than secondary antibodies. Furthermore, multiple primary antibodies of the same isotype or derived from the same species can be used in the same immunostaining experiment if they are directly labeled with compatible fluorophores. Many IgG antibodies, however, are often only available in small quantities and may be mixed with stabilizing proteins, such as BSA, that can react nonproductively with the amine-reactive labeling reagents.

APEX® Antibody Labeling Kits are specifically designed to allow labeling of small amounts of IgG antibody, even in the presence of contaminants. These kits utilize a solid-phase labeling technique that captures the IgG antibody on resin inside an APEX® antibody labeling tip (Figure 1.2.1). Any contaminants, including stabilizing proteins or amine-containing buffers, are eluted through the tip before labeling. After applying the amine-reactive fluorophore to the IgG antibody trapped on the resin, a fluorescent IgG conjugate is formed and subsequently eluted from the resin using elution buffer. The fluorescent IgG conjugate is ready to use in an imaging or flow cytometry assay in as little as 2.5 hours with minimal hands-on time. The typical yield of labeled antibody using this method is between 40 and 80%.

Each APEX® Antibody Labeling Kit provides all reagents required to perform five separate labeling reactions of 10–20 µg IgG antibody, including:

- Five vials of amine-reactive fluorescent dye
- Five APEX® antibody labeling tips
- Wash, labeling, neutralization and elution buffers
- Dimethylsulfoxide (DMSO)
- Labeling protocol

For labeling larger amounts of protein, we recommend the Alexa Fluor® Microscale Protein Labeling Kits, which are optimized for 20–100 µg samples of proteins between 10,000 and 150,000 daltons; the Alexa Fluor® Monoclonal Antibody Labeling Kits, which are optimized for 100 µg samples of mono- or polyclonal antibodies; or the Alexa Fluor® Protein Labeling Kits, which are optimized for 1 mg samples of >30,000-dalton proteins.

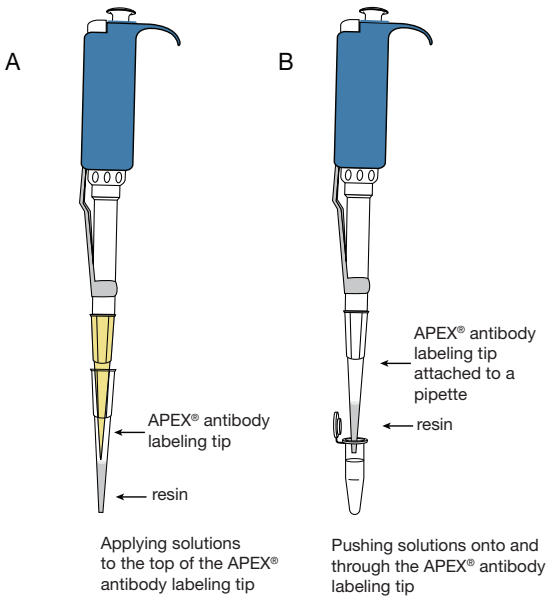

Figure 1.2.1 Illustration of the use of the APEX® antibody labeling tip, provided in APEX® Antibody Labeling Kits. **A**) Applying solutions to the resin in the tip. **B**) Pushing solutions onto the resin in the tip by attaching the APEX® antibody labeling tip to a pipette.

Alexa Fluor® Microscale Protein Labeling Kits

Alexa Fluor® Microscale Protein Labeling Kits (Table 1.2, Table 1.3) provide a convenient means for labeling small amounts (20–100 µg) of purified protein with the highly fluorescent Alexa Fluor® dyes. Spin columns are used to purify the labeled protein, with yields between 60 and 90% depending primarily on the molecular weight of the starting material. Labeling and purification can be completed in as little as 30 minutes.

These kits have been optimized for labeling proteins with molecular weights between 12,000 and 150,000 daltons and contain everything needed to perform three labeling reactions and to separate the resulting conjugates from excess dye. Each Alexa Fluor® Microscale Protein Labeling Kit provides:

- Three vials of amine-reactive fluorescent dye (succinimidyl ester or tetrafluorophenyl ester)
- Sodium bicarbonate
- Reaction tubes
- Purification resin and spin filters
- Detailed protocols for conjugation, purification and determination of the degree of labeling

Table 1.2 Active esters and kits for labeling proteins and nucleic acids.

Label	Fluorescence Color (Abs/Em) *	Succinimidyl Ester, TFP Ester or SDP Ester	Protein Labeling Kits	Zenon® Antibody Labeling Kits	Nucleic Acid Labeling Kits
Alexa Fluor® 350	Blue (346/442)	A10168	A20180 (Mab) A10170 (P)	Z25000 (M IgG_1) Z25100 (M IgG_{2a}) Z25200 (M IgG_{2b}) Z25300 (R IgG) Z25400 (H IgG)	
Marina Blue®	Blue (365/460)	M10165			
Pacific Orange™	Orange (400/551)	P30253	P30014 (Mab) P30016 (P)	Z25256 (M IgG_1) Z25257 (M IgG_{2a})	
Alexa Fluor® 405	Blue (402/421)	A30000 A30100		Z25013 (M IgG_1) Z25113 (M IgG_{2a}) Z25213 (M IgG_{2b}) Z25313 (R IgG)	
Pacific Blue™	Blue (410/455)	P10163	A10478 (APEX®) P30013 (Mab) P30012 (P)	Z25041 (M IgG_1) Z25156 (M IgG_{2a}) Z25341 (R IgG)	
Alexa Fluor® 430	Yellow-green (434/539)	A10169	A10171 (P)	Z25001 (M IgG_1) Z25301 (R IgG)	
Fluorescein-EX	Green (494/518)	F6130	F10240 (P) F6433 (F)	Z25042 (M IgG_1) Z25342 (R IgG)	
FITC	Green (494/518)		F6434 (F)		
Alexa Fluor® 488	Green (495/519)	A20000 † A20100 † A30005 (TFP) A30052 (SDP)	A10468 ‡ (APEX®) A30006 ‡ (Micro) A20181 ‡ (Mab) A10235 ‡ (P)	Z25002 (M IgG_1) Z25090 (TSA™)(M IgG_1) Z25102 (M IgG_{2a}) Z25202 (M IgG_{2b}) Z25302 (R IgG) Z25602 (G IgG) Z25402 (H IgG)	U21650 (ULYSIS®) A21665 (ARES™) F32947 (FT) F32952 (FT) A20191 (Oligo)
Oregon Green® 488	Green (496/524)	O6147 O6149	A10476 (APEX®) O10241 (P) F6153 (F)		U21659 (ULYSIS®)
Oregon Green® 514	Green (511/530)	O6139			
Alexa Fluor® 514	Yellow-green (518/540)	A30002 †			
Alexa Fluor® 532	Yellow (531/554)	A20001 A20101MP	A20182 (Mab) A10236 (P)	Z25003 (M IgG_1) Z25303 (R IgG)	U21651 (ULYSIS®)
Alexa Fluor® 546	Orange (556/573)	A20002 † A20102 †	A20183 (Mab) A10237 (P)	Z25004 (M IgG_1) Z25104 (M IgG_{2a}) Z25204 (M IgG_{2b}) Z25304 (R IgG)	U21652 (ULYSIS®) A21667 (ARES™)
Alexa Fluor® 555	Red-orange (555/565)	A20009 A20109	A10470 (APEX®) A30007 (Micro) A20187 (Mab) A20174 (P)	Z25005 (M IgG_1) Z25105 (M IgG_{2a}) Z25205 (M IgG_{2b}) Z25305 (R IgG) Z25605 (G IgG) Z25405 (H IgG)	A21677 (ARES™) F32948 (FT) F32953 (FT)
Tetramethylrhodamine	Red-orange (555/580)	C2211 C6123 C1171 † T6105 † (X)			
Rhodamine Red™	Red-orange (570/590)	R6160 (X)	F6161 (F)		
Alexa Fluor® 568	Red-orange (578/603)	A20003 † A20103 †	A20184 (Mab) A10238 (P)	Z25006 (M IgG_1) Z25106 (M IgG_{2a}) Z25206 (M IgG_{2b}) Z25306 (R IgG) Z25606 (G IgG)	U21653 (ULYSIS®)
Alexa Fluor® 594	Red (590/617)	A20004 † A20104 †	A10474 (APEX®) A30008 (Micro) A20185 (Mab) A10239 (P)	Z25007 (M IgG_1) Z25107 (M IgG_{2a}) Z25207 (M IgG_{2b}) Z25307 (R IgG) Z25607 (G IgG) Z25407 (H IgG)	U21654 (ULYSIS®) A21669 (ARES™) F32949 (FT) F32954 (FT)

***** Approximate absorption (Abs) and fluorescence emission (Em) maxima for conjugates, in nm. **†** Mixed isomers. **‡** These Alexa Fluor® 488 protein labeling kits contain either the amine-reactive Alexa Fluor® 488 carboxylic acid 5-TFP ester (Micro, Mab, P) or the amine-reactive Alexa Fluor® 488 carboxylic acid 5-SDP ester (APEX®); whereas the Alexa Fluor® 488 nucleic acid labeling kits contain the Alexa Fluor® 488 carboxylic acid succinimidyl ester. **§** Human vision is insensitive to light beyond ~650 nm, and therefore it is not possible to view the far-red– and near-infrared–fluorescent dyes by looking through the eyepiece of a conventional fluorescence microscope. NA = not applicable. (APEX®) = APEX® Antibody Labeling Kit. (ARES™) = ARES™ DNA Labeling Kit. (D) = DSB-X™ Biotin Protein Labeling Kit. (F) = FluoReporter® Protein Labeling Kit. (FB) = FluoReporter® Biotin-XX Protein Labeling Kit. (FMB) = FluoReporter® Mini-Biotin-XX Protein Labeling Kit. (FT) = FISH Tag™ DNA Kit or FISH Tag™ RNA Kit. (G IgG) = Zenon® Goat IgG Labeling Kit. (H IgG) = Zenon® Human IgG Labeling Kit. (M IgG) = Zenon® Mouse IgG Labeling Kit. (Mab) = Monoclonal Antibody Labeling Kit. (Micro) = Alexa Fluor® Microscale Protein Labeling Kit or Biotin-XX Microscale Protein Labeling Kit. (Oligo) = Alexa Fluor® Oligonucleotide Amine Labeling Kit. (P) = Easy-to-Use Protein Labeling Kit. (R IgG) = Zenon® Rabbit IgG Labeling Kit. (SAIVI™) = SAIVI™ Antibody Labeling Kit. (SDP) = Sulfodichlorophenol ester. (TFP) = Tetrafluorophenyl ester. (TSA™) = Enhanced with TSA™ technology. (ULYSIS®) = ULYSIS® Nucleic Acid Labeling Kit. (X) = An aminohexanoyl spacer between the dye and the SE. More information on amine-reactive dyes is available at www.invitrogen.com/handbook/labelingchemistry.

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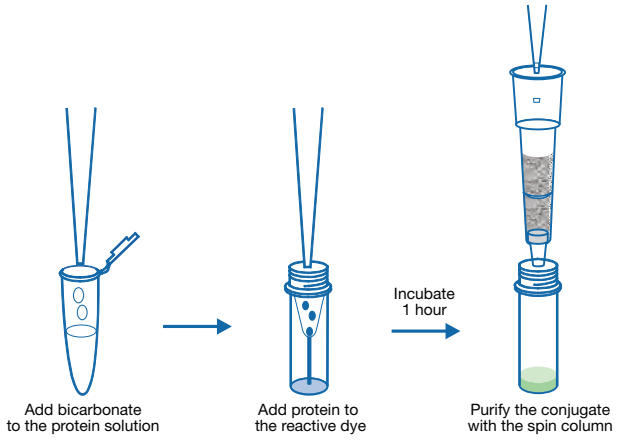

Figure 1.2.2 Illustration of the three simple steps in the protocol for the Monoclonal Antibody Labeling Kits, which provide a convenient method for covalently labeling small amounts of IgG antibodies.

Monoclonal Antibody Labeling Kits

Monoclonal Antibody Labeling Kits (Table 1.2, Table 1.3) provide researchers with a simple yet efficient means of labeling small amounts of IgG antibodies with Alexa Fluor® dyes (Figure 1.2.2). Unlike polyclonal antibodies and most other commercially available proteins, monoclonal antibodies are typically only available in small quantities. These kits contain everything needed to perform five separate labeling reactions containing ~100 µg each of carrier-free monoclonal IgG samples (although other proteins can be labeled).

Each Monoclonal Antibody Labeling Kit provides:

- Five vials of amine-reactive fluorescent dye (succinimidyl ester or tetrafluorophenyl ester)
- Sodium bicarbonate
- Spin columns and collection tubes
- Detailed protocols for conjugation, purification and determination of the degree of labeling

Table 1.2 Active esters and kits for labeling proteins and nucleic acids—*continued.*

Label	Fluorescence Color (Abs/Em) *	Succinimidyl Ester, TFP Ester or SDP Ester	Protein Labeling Kits	Zenon® Antibody Labeling Kits	Nucleic Acid Labeling Kits
Texas Red®	Red (595/615)	T6134 † (X) T20175 (X)	T10244 (P) F6162 (F)	Z25045 (M IgG_1)	
Alexa Fluor® 610	Red (612/628)	A30050 (X)			
Alexa Fluor® 633 §	Far-red (632/647)	A20005 † A20105 †	A20170 (P)		
Alexa Fluor® 635 §	Far-red (633/647)				
Alexa Fluor® 647 §	Far-red (650/668)	A20006 A20106	A10475 (APEX®) A30009 (Micro) A20186 (Mab) S30044 (SAIVI™) A20173 (P)	Z25008 (M IgG_1) Z25108 (M IgG_{2a}) Z25208 (M IgG_{2b}) Z25308 (R IgG) Z25608 (G IgG) Z25408 (H IgG)	U21660 (ULYSIS®) A21676 (ARES™) F32950 (FT) F32955 (FT) A20196 (Oligo)
Alexa Fluor® 660 §	Near-infrared (663/690)	A20007	A20171 (P)	Z25009 (M IgG_1)	
Alexa Fluor® 680 §	Near-infrared (679/702)	A20008 A20108	S30045 (SAIVI™) A20172 (P)	Z25010 (M IgG_1) Z25110 (M IgG_{2a}) Z25210 (M IgG_{2b}) Z25310 (R IgG)	
Alexa Fluor® 700 §	Near-infrared (702/723)	A20010 A20110		Z25011 (M IgG_1)	
Alexa Fluor® 750 §	Near-infrared (749/775)	A20011 A20111	S30046 (SAIVI™)	Z25312 (R IgG)	
Alexa Fluor® 790 §	Near-infrared (782/805)	A30051			
Biotin	NA	B1606 (XX) B6353 (X)	F2610 (FB) F6347 (FMB) B30010 (Micro) B30756 (Micro)	Z25052 (M IgG_1) Z25152 (M IgG_{2a}) Z25252 (M IgG_{2b}) Z25352 (R IgG) Z25452 (H IgG)	
DNP–biotin	NA (364/none)	B2604 (X)	F6348 (F)		
DSB-X™ biotin	NA		D20655 (D)		

***** Approximate absorption (Abs) and fluorescence emission (Em) maxima for conjugates, in nm. **†** Mixed isomers. **‡** These Alexa Fluor® 488 protein labeling kits contain either the amine-reactive Alexa Fluor® 488 carboxylic acid 5-TFP ester (Micro, Mab, P) or the amine-reactive Alexa Fluor® 488 carboxylic acid 5-SDP ester (APEX®); whereas the Alexa Fluor® 488 nucleic acid labeling kits contain the Alexa Fluor® 488 carboxylic acid succinimidyl ester. **§** Human vision is insensitive to light beyond ~650 nm, and therefore it is not possible to view the far-red– and near-infrared–fluorescent dyes by looking through the eyepiece of a conventional fluorescence microscope. NA = not applicable. (APEX®) = APEX® Antibody Labeling Kit. (ARES™) = ARES™ DNA Labeling Kit. (D) = DSB-X™ Biotin Protein Labeling Kit. (F) = FluoReporter® Protein Labeling Kit. (FB) = FluoReporter® Biotin-XX Protein Labeling Kit. (FMB) = FluoReporter® Mini-Biotin-XX Protein Labeling Kit. (FT) = FISH Tag™ DNA Kit or FISH Tag™ RNA Kit. (G IgG) = Zenon® Goat IgG Labeling Kit. (H IgG) = Zenon® Human IgG Labeling Kit. (M IgG) = Zenon® Mouse IgG Labeling Kit. (Mab) = Monoclonal Antibody Labeling Kit. (Micro) = Alexa Fluor® Microscale Protein Labeling Kit or Biotin-XX Microscale Protein Labeling Kit. (Oligo) = Alexa Fluor® Oligonucleotide Amine Labeling Kit. (P) = Easy-to-Use Protein Labeling Kit. (R IgG) = Zenon® Rabbit IgG Labeling Kit. (SAIVI™) = SAIVI™ Antibody Labeling Kit. (SDP) = Sulfodichlorophenol ester. (TFP) = Tetrafluorophenyl ester. (TSA™) = Enhanced with TSA™ technology. (ULYSIS®) = ULYSIS® Nucleic Acid Labeling Kit. (X) = An aminohexanoyl spacer between the dye and the SE. More information on amine-reactive dyes is available at www.invitrogen.com/handbook/labelingchemistry.

Table 1.3 Molecular Probes® kits for protein and nucleic acid labeling.

Kit Name	# Labelings	Kit Components	Features
Kits for Labeling Proteins with Fluorescent Dyes			
APEX® Antibody Labeling Kit	5 labelings of 10–20 µg each of IgG antibody	• 5 vials of amine-reactive fluorescent label • Five APEX® antibody labeling tips • Wash, labeling, neutralization and elution buffers • Dimethylsulfoxide (DMSO) • Labeling protocol	APEX® Antibody Labeling Kits utilize a solid-phase labeling technique that captures the IgG antibody on the resin inside the APEX® antibody labeling tip. Any contaminants, including stabilizing proteins, are eluted through the tip prior to labeling with the amine-reactive fluorescent dye. The fluorescent IgG conjugate is ready to use in 2.5 hours, with ~15 minutes hands-on time.
Alexa Fluor® Microscale Protein Labeling Kit	Three 20–100 µg protein samples of a 12,000- to 150,000-dalton protein	• 3 vials of the succinimidyl ester (or tetrafluorophenyl ester) of the fluorescent dye • Sodium bicarbonate • Reaction tubes • Purification resin and spin filters • Detailed protocols for conjugation, purification and determination of the degree of labeling	Alexa Fluor® Microscale Protein Labeling Kits provide a convenient means for labeling small amounts (20–100 µg) of purified protein with our superior Alexa Fluor® dyes and purifying the resulting conjugate. Convenient spin columns are used to purify the labeled protein, with yields between 60 and 90% depending primarily on the molecular weight of the starting material. Labeling and purification can be completed in as little as 30 minutes. Microscale Protein Labeling Kits are also available for biotinylating proteins.
Monoclonal Antibody Labeling Kit	5 labelings of ~100 µg each of carrier-free monoclonal IgG samples	• 5 vials of the succinimidyl ester (or tetrafluorophenyl ester) of the fluorescent dye • Sodium bicarbonate • Spin columns and collection tubes • Detailed protocols for conjugation, purification and determination of the degree of labeling	A buffered solution of the protein is added to one of the five vials of amine-reactive dye. The reactive dye has a succinimidyl ester (or tetrafluorophenyl ester) moiety that reacts efficiently with primary amines of proteins to form stable dye–protein conjugates. The conjugate can be purified on the included size-exclusion spin columns. Labeling and purification can be completed in less than 2 hours.
SAIVI™ Rapid Antibody Labeling Kit	3 labelings of 0.5–3 mg each of carrier-free antibody solution	• 3 vials of amine-reactive Alexa Fluor® dye • Sodium bicarbonate • Regulator solution • Purification resin and purification columns • Phosphate-buffered saline (PBS) • Syringes, syringe filters, column-loading pipettes and catch tubes • Detailed protocols for conjugation, purification and determination of the degree of labeling	SAIVI™ Antibody Labeling Kits provide a convenient means to label antibodies with an optimal degree of labeling for *in vivo* imaging applications over a 6-fold antibody concentration range with no adjustments in reaction volume, dye concentration or antibody concentration necessary. Purification of the dye-labeled antibody is achieved with a simple protocol that can be completed in less than 10 minutes. These optimally labeled antibodies are ready for applications that require azide-free reagents, such as live-cell imaging or direct injection into animals.
Easy-to-Use Protein Labeling Kit	Three ~1 mg protein samples of a 150,000-dalton protein, such as an IgG	• 3 vials of the succinimidyl ester (or tetrafluorophenyl ester) of the fluorescent dye, each containing a magnetic stir bar • Sodium bicarbonate • Gravity-feed columns, a size-exclusion resin and concentrated elution buffer • Column funnels, foam column holders, disposable pipettes and collection tubes • Detailed protocols for conjugation, purification and determination of the degree of labeling	A buffered solution of the protein is added to one of the three vials of the amine-reactive dye. The reactive dye has a succinimidyl ester (or tetrafluorophenyl ester) moiety that reacts efficiently with primary amines of proteins to form stable dye–protein conjugates. Purification of the conjugate can be accomplished on the included gravity-feed size-exclusion columns.
FluoReporter® Protein Labeling Kit	5 to 10 protein samples of 0.2–2 mg each in 200 µL volumes	• 5 vials of the amine-reactive dye • Anhydrous DMSO • Reaction tubes, each containing a stir bar • Spin columns and collection tubes • Detailed protocols for conjugation, purification and determination of the degree of labeling	The amount of dye necessary for the desired protein sample is calculated using the guidelines outlined in the kit protocol. The reactive dye has a succinimidyl ester moiety that reacts efficiently with primary amines of proteins to form stable dye–protein conjugates. Purification of the conjugate can be easily accomplished using the included spin columns.
Qdot® Antibody Conjugation Kit—see Section 6.6			
Zenon® Antibody Labeling Kit—see Section 7.3			
Kits for Labeling Proteins with Biotin or Dinitrophenyl (DNP)			
Biotin-XX Microscale Protein Labeling Kit	Three 20–100 µg protein samples of a 12,000- to 150,000-dalton protein	• 3 vials of biotin-XX sulfosuccinimidyl ester • Sodium bicarbonate • Reaction tubes • Purification resin and spin filters • Detailed protocols for conjugation and purification	The Biotin-XX Microscale Protein Labeling Kit provides a convenient means for biotinylating small amounts (20–100 µg) of purified protein. Convenient spin columns are used to purify the labeled protein with yields between 60 and 90%, depending primarily on the molecular weight of the starting material. Labeling and purification can be completed in as little as 30 minutes. For determining the degree of labeling, the FluoReporter® Biotin Quantitation Assay Kit for proteins is available separately or in combination with the Biotin-XX Microscale Protein Labeling Kit.
FluoReporter® Mini-Biotin-XX Protein Labeling Kit	5 biotinylation reactions of 0.1–3 mg each	• 5 vials of biotin-XX sulfosuccinimidyl ester • Reaction tubes, each containing a stir bar • Purification resin, spin columns and collection tubes • Dialysis tubing • Detailed protocols for conjugation and purification	The biotin-XX sulfosuccinimidyl ester (SSE) is water soluble and reacts with primary amines of proteins or other biomolecules to form stable biotin conjugates. The biotin-XX SSE has a 14-atom spacer that enhances the binding of biotin derivatives to avidin's relatively deep binding sites. Ready-to-use spin columns are included for purification of the biotinylated protein from excess reagents.

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Simply dissolve the carrier-free monoclonal antibody at ~1 mg/mL in the provided buffer, then add it to one of the five vials of amine-reactive dye; no organic solvents are required. Purification of the fluorescent conjugate is accomplished on a size-exclusion spin column optimized for proteins with molecular weight greater than 30,000 daltons. Labeling and purification can typically be completed in less than 2 hours.

Mouse monoclonal antibodies in serum, in ascites fluid or diluted with carrier proteins should not be labeled with these kits; however, such antibody preparations can be efficiently labeled with the APEX® Antibody Labeling Kits described above or with the Zenon® Mouse IgG Labeling Kits (Section 7.3, Table 7.7) described below.

SAIVI™ Antibody Labeling Kits

The optimal fluorescent antibody conjugate for *in vitro* detection assays produces an intense fluorescent signal yet retains the binding affinity and specificity of the unlabeled antibody. When preparing a fluorescent antibody conjugate for *in vivo* animal imaging, however, the pharmacokinetics of the labeled probe must also be considered.[1] These additional constraints have led to the development of the SAIVI™ Antibody Labeling Kits for small animal *in vivo* imaging applications (Table 1.2, Table 1.3).

SAIVI™ Antibody Labeling Kits feature reactive far-red and near-infrared Alexa Fluor® dyes, along with a labeling protocol specifically designed to produce a suitable degree of labeling (DOL) for *in vivo*

Table 1.3 Molecular Probes® kits for protein and nucleic acid labeling—*continued*.

Kit Name	# Labelings	Kit Components	Features
FluoReporter® Biotin-XX Protein Labeling Kit	5 biotinylation reactions, each with 5–20 mg of protein	• Biotin-XX succinimidyl ester • DMSO • Gel filtration column • Avidin–HABA complex • Biotinylated goat IgG • Detailed protocols for conjugation, purification and determination of the degree of labeling	The biotin-XX succinimidyl ester (SE) reacts with primary amines of proteins or other biomolecules to form stable biotin conjugates. The biotin-XX SE has a 14-atom spacer that enhances the binding of biotin derivatives to avidin's relatively deep binding sites. A gel filtration column is provided for purifying the labeled proteins from excess biotin reagent. After purification, the degree of biotinylation can be estimated using the included avidin–biotin displacement assay.
FluoReporter® Biotin/DNP Protein Labeling Kit	5 to 10 labeling reactions of 0.2–2 mg of protein each	• 5 vials of DNP-X–biocytin-X succinimidyl ester • DMSO • Reaction tubes • Spin columns and collection tubes • Detailed protocols for conjugation, purification and determination of the degree of labeling	The FluoReporter® Biotin/DNP Protein Labeling Kit is similar to other FluoReporter® Protein Labeling Kits, except that it contains DNP-X–biocytin-X succinimidyl ester as the reactive label. When proteins are labeled with this chromophoric biotin derivative, the degree of biotinylation can be readily assessed from the extinction coefficient of DNP (EC_{360} = 15,000 $cm^{-1}M^{-1}$). An additional feature of the conjugates labeled with DNP-X–biocytin-X succinimidyl ester is that they can be recognized by avidin derivatives (or anti-biotin antibodies) and by anti-DNP antibodies, enabling researchers to choose among several detection techniques suitable for fluorescence and electron microscopy.
DSB-X™ Protein Labeling Kit	5 protein conjugations of 0.5–3 mg each	• 5 vials of DSB-X™ biotin succinimidyl ester • DMSO • Reaction tubes • Purification resin, spin columns and collection tubes • Dialysis tubing for larger-scale separations • Detailed protocols for conjugation and purification	DSB-X™ biotin succinimidyl ester, a derivative of desthiobiotin with an additional seven-atom spacer, reacts with amine groups of biomolecules to form stable amides. The DSB-X™ biotin conjugate can be detected with avidin or streptavidin derivatives. Binding is almost totally reversed by addition of free biotin at neutral pH and normal ionic strength. Materials are included for both small- and large-scale preparations.
Kits for Labeling Nucleic Acids with Fluorescent Dyes			
ULYSIS® Nucleic Acid Labeling Kit	20 labelings of 1 µg DNA	• ULS® labeling reagent and appropriate solvent • Labeling buffer • Deoxyribonuclease I (DNase I), for digesting DNA longer than 1000 base pairs prior to labeling • DNase I storage and reaction buffers • Control DNA from calf thymus • Nuclease-free H_2O • Labeling protocol	The ULS® reagent reacts with the *N*-7 position of guanine residues to provide a stable coordination complex between the nucleic acid and the fluorophore label. Separation of the labeled nucleic acids from the unreacted ULS® complex can be accomplished through a simple procedure using a spin column (not provided).
ARES™ DNA Labeling Kit	10 labelings of 1–5 µg DNA	• 5-(3-Aminoallyl)-dUTP • Amine-reactive fluorescent dye and appropriate solvent • Sodium bicarbonate • Nuclease-free H_2O • Detailed protocol for labeling DNA using reverse transcriptase or nick translation	In the first step, an amine-modified nucleotide, 5-(3-aminoallyl)-dUTP, is incorporated into DNA using conventional enzymatic labeling methods. In the second step, the amine-modified DNA is chemically labeled using an amine-reactive fluorescent dye. The amine-modified DNA can be purified using the PureLink™ PCR Purification Kit (K3100-01).
FISH Tag™ DNA Kit—see Section 8.2			
FISH Tag™ RNA Kit—see Section 8.2			
Oligonucleotide Amine Labeling Kit	3 labelings of 50 µg each of an amine-modified oligonucleotide	• 3 vials of the amine-reactive dye • DMSO • Labeling buffer • Labeling protocol	The reactive dye used in the labeling has an amine-reactive succinimidyl ester moiety that reacts efficiently with an amine-modified oligonucleotide. Following the labeling reaction, the conjugate can be purified from the reaction mixture by preparative gel electrophoresis or reverse-phase HPLC.

imaging applications. When optimally labeled, a fluorescent antibody conjugate produces an intense, targeted fluorescent signal that persists throughout the *in vivo* study, without significant redistribution or clearance of the probe.

The conjugation protocol in the SAIVI™ Rapid Antibody Labeling Kits produces an optimal DOL (~2 fluorophores per antibody) over a 6 fold antibody concentration range with no adjustments in reaction volume, dye concentration or antibody concentration necessary. Purification of the dye-labeled antibody is achieved usng a simple protocol that can typically be completed in less than 10 minutes. With these kits, optimally labeled antibodies are ready for applications that require azide-free reagents, such as live-cell imaging or direct injections into animals. SAIVI™ Rapid Antibody Labeling Kits containing either Alexa Fluor® 680 or Alexa Fluor® 750 dye (S30045, S30046) provide sufficient reagents for three labeling reactions of 0.5–3 mg of protein each, including:

- Three vials of amine-reactive Alexa Fluor® 680 or Alexa Fluor® 750 dye
- Sodium bicarbonate
- Regulator solution
- Purification resin and purification columns
- Phosphate-buffered saline (PBS)
- Syringes, syringe filters, column-loading pipettes and catch tubes
- Detailed protocols for conjugation, purification and determination of the degree of labeling

To control the DOL of the antibody conjugate, the SAIVI™ Alexa Fluor® 647 Antibody/Protein Labeling Kit (S30044) includes a DOL modulating reagent and instructions for decreasing the DOL from its intrinsic highest value by adding specific amounts of this reagent to the labeling reaction. With this method, antibody preparations with varying ratios of dye to protein can be quickly and reproducibly obtained without significant alteration of labeling or purification conditions, allowing more efficient optimization for *in vivo* imaging applications. Each kit provides sufficient reagents for three labeling reactions of 1 mg protein each, including:

- Three vials of amine-reactive Alexa Fluor® 647 dye
- Sodium bicarbonate
- DOL modulating reagent
- Purification resin and purification columns
- Wash buffer
- Column funnels, column holders, disposable pipettes and collection tubes
- Detailed protocols for conjugation, purification and determination of the degree of labeling

Easy-to-Use Protein Labeling Kits

Our Easy-to-Use Protein Labeling Kits (Table 1.2, Table 1.3) provide a nearly effortless way to label proteins, especially IgG antibodies, with a fluorescent dye (Figure 1.2.3). Simply add ~1 mg of protein (in a volume of ~500 µL and free of amine-containing buffers such as Tris) to one of the three included vials, which contain a premeasured quantity of amine-reactive dye and a magnetic stir bar. Because the reactive dyes used in these kits are water soluble, no organic solvents are required. Purification is accomplished on a gravity-feed size-exclusion column, which is supplied with the kit. Labeling and purification can typically be completed in about 2 hours, with very little hands-on time.

Each Protein Labeling Kit provides sufficient reagents for labeling three ~1 mg protein samples of a 150,000-dalton protein (such as an IgG), including:

- Three vials of the amine-reactive fluorescent dye (succinimidyl ester or tetrafluorophenyl ester), each containing a magnetic stir bar
- Sodium bicarbonate
- Gravity-feed columns, a size-exclusion resin and concentrated elution buffer
- Column funnels, foam column holders, disposable pipettes and collection tubes
- Detailed protocols for conjugation, purification and determination of the degree of labeling

Researchers have modified α-synuclein using either the Oregon Green® 488 Protein Labeling Kit (O10241) or the Alexa Fluor® 594 Protein Labeling Kit (A10239).[2] They intentionally produced fluorescent conjugates with a low degree of labeling (DOL) so as not to perturb oligomerization. When comparing Oregon Green® 488 synuclein with Alexa Fluor® 594 synuclein, they observed essentially the same results in terms of fibril formation as analyzed by fluorescence polarization. They also detected the Oregon Green® 488 synuclein conjugate with mouse monoclonal anti–fluorescein/Oregon Green® antibody (monoclonal 4-4-20, A6421; Section 7.4) and gold-labeled anti–mouse IgG antibody using electron microscopy.

FluoReporter® Protein Labeling Kits

The FluoReporter® Protein Labeling Kits (Table 1.2, Table 1.3) facilitate research-scale preparation of protein conjugates labeled with some of our brightest fluorescent dyes. Typically, labeling and purifying conjugates with the FluoReporter® Protein Labeling Kits can typically be completed in under 3 hours, with very little hands-on time. First, the amount of dye necessary for the desired protein sample is calculated using the guidelines outlined in the kit protocol. After dissolving the dye in dimethylsulfoxide (DMSO), the calculated amount of dye is added to the protein and the reaction is incubated for 1–1.5 hours. Purification

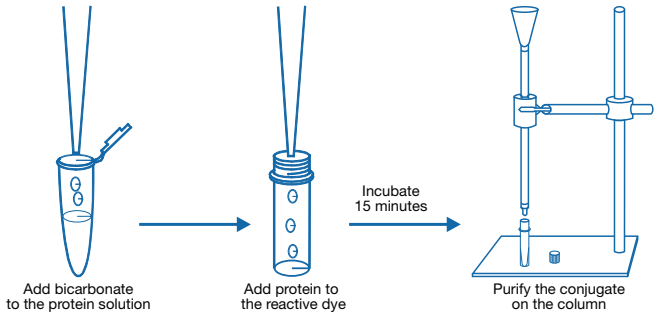

Figure 1.2.3 Illustration of the three simple steps in the protocol for the Easy-to-Use Protein Labeling Kits, which provide a convenient method for covalently labeling most proteins.

is easily accomplished using convenient spin columns designed for use with proteins of molecular weight greater than 30,000 daltons.

Each FluoReporter® Protein Labeling Kits provides sufficient reagents to label 5 to 10 protein samples of 0.2–2 mg each in 200 µL volumes:

- Five vials of the amine-reactive dye
- Dimethylsulfoxide (DMSO)
- Reaction tubes, each containing a stir bar
- Spin columns and collection tubes
- Detailed protocols for conjugation, purification and determination of the degree of labeling

Zenon® Antibody Labeling Kits

The Zenon® Antibody Labeling Kits (Table 1.2, Table 7.7) are useful for the rapid and quantitative labeling of antibodies with dyes (including phycobiliproteins and their tandem conjugates), biotin or enzymes[3] (Figure 1.2.4). Zenon® Antibody Labeling Kits are designed to label intact antibodies in amounts from less than 1 µg to as much as 50 µg, starting with a purified antibody fraction or with a crude antibody preparation such as serum, ascites fluid or a hybridoma supernatant. The Zenon® antibody labeling technology is discussed further in Section 7.3.

Kits for Labeling Proteins with Biotin or Dinitrophenyl (DNP)

Biotin-XX Microscale Protein Labeling Kits

The Biotin-XX Microscale Protein Labeling Kit (B30010; Table 1.2, Table 1.3) provides a convenient means for biotinylating small amounts (20–100 µg) of purified protein. The water-soluble biotin-XX sulfosuccinimidyl ester has a 14-atom spacer (Figure 1.2.5) that enhances the binding of biotin derivatives to avidin's relatively deep binding sites. Spin columns are used to purify the labeled protein, with yields between 60 and 90% depending primarily on the molecular weight of the starting material. Labeling and purification can be completed in as little as 30 minutes.

This kit, which has been optimized for labeling proteins with molecular weights between 12,000 and 150,000 daltons, contains everything needed to perform three labeling reactions and to separate the resulting conjugates from excess reactive biotin. Each Biotin-XX Microscale Protein Labeling Kit provides:

- Three vials of amine-reactive biotin-XX sulfosuccinimidyl ester
- Sodium bicarbonate
- Reaction tubes
- Purification resin and spin filters
- Detailed protocols for conjugation and purification

For determining the degree of labeling, the FluoReporter® Biotin Quantitation Assay Kit for proteins is available separately (F30751) or in combination with the Biotin-XX Microscale Protein Labeling Kit (B30756). When biotinylating larger amounts of protein, we recommend the FluoReporter® Biotin-XX Protein Labeling Kit, which is optimized for 5–20 mg samples, or the FluoReporter® Mini-Biotin-XX Protein Labeling Kit, which is optimized for 0.1–3 mg samples of >30,000-dalton proteins.

FluoReporter® Mini-Biotin-XX Protein Labeling Kit

The FluoReporter® Mini-Biotin-XX Protein Labeling Kit (F6347; Table 1.2, Table 1.3) permits efficient biotinylation of small amounts of antibodies or other proteins. The water-soluble biotin-XX sulfosuccinimidyl ester has a 14-atom spacer (Figure 1.2.5) that enhances the binding of biotin derivatives to avidin's relatively deep binding sites. The ready-to-use spin columns provide a convenient method of purifying the biotinylated protein from excess reagents.

Each FluoReporter® Mini-Biotin-XX Protein Labeling Kit provides sufficient reagents for five labeling reactions of 0.1–3 mg protein each, including:

- Five vials of amine-reactive biotin-XX sulfosuccinimidyl ester
- Reaction tubes, each containing a stir bar
- Purification resin, spin columns and collection tubes
- Dialysis tubing
- Detailed protocols for conjugation and purification

FluoReporter® Biotin-XX Protein Labeling Kit

The FluoReporter® Biotin-XX Protein Labeling Kit (F2610; Table 1.2, Table 1.3) is designed to provide five biotinylation reactions, each containing 5–20 mg of protein. A gel filtration column is used to purify the labeled proteins from excess biotin reagent. Once purified, the degree of biotinylation can be determined using the included avidin–HABA displacement assay; biotinylated goat IgG is provided as a standard.

Each FluoReporter® Biotin-XX Protein Labeling Kit provides sufficient reagents for five labeling reactions of 5–20 mg protein each, including:

- Amine-reactive biotin-XX succinimidyl ester
- Dimethylsulfoxide (DMSO)
- Gel filtration column
- Avidin–HABA complex

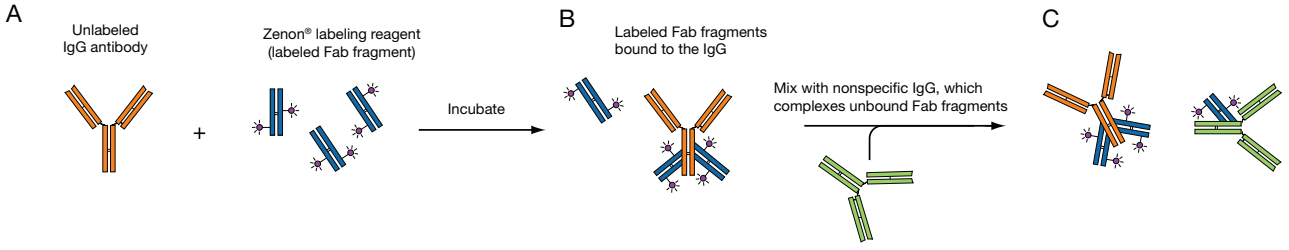

Figure 1.2.4 Labeling scheme utilized in the Zenon® Antibody Labeling Kits. **A**) An unlabeled IgG antibody is incubated with the Zenon® labeling reagent, which contains a fluorophore-labeled, Fc-specific anti-IgG Fab fragment. **B**) This labeled Fab fragment binds to the Fc portion of the IgG antibody. **C**) Excess Fab fragment is then neutralized by the addition of a nonspecific IgG, preventing crosslabeling by the Fab fragment in experiments where primary antibodies of the same type are present. Note that the Fab fragment used for labeling need not be coupled to a fluorophore, but could instead be coupled to an enzyme (such as HRP) or to biotin.

- Biotinylated goat IgG
- Detailed protocols for conjugation, purification and determination of the degree of labeling

FluoReporter® Biotin/DNP Protein Labeling Kit

The FluoReporter® Biotin/DNP Protein Labeling Kit (F6348; Table 1.2, Table 1.3) provides the necessary reagents for labeling proteins with DNP-X–biocytin-X succinimidyl ester (Figure 1.2.6). The degree of biotinylation of proteins labeled with this reagent can be assessed from the optical absorbance of DNP (EC = 15,000 $cm^{-1}M^{-1}$ at ~360 nm). The conjugates are recognized by both avidin derivatives (and anti-biotin antibodies) and by anti-DNP antibodies, permitting a choice of detection techniques.

Each FluoReporter® Biotin/DNP Protein Labeling Kit contains sufficient reagents for 5 to 10 labeling reactions of 0.2–2 mg of protein each, including:

- Five vials of amine-reactive DNP-X–biocytin-X succinimidyl ester
- Dimethylsulfoxide (DMSO) for dissolving the succinimidyl ester
- Reaction tubes
- Spin columns and collection tubes
- Detailed protocols for conjugation, purification and determination of the degree of labeling

DSB-X™ Biotin Protein Labeling Kit

Our unique DSB-X™ biotin technology, which is described in detail in Section 4.1, permits the facile reversal of the biotin–avidin interaction under extremely gentle conditions.[4] DSB-X™ biotin succinimidyl ester, a derivative of desthiobiotin (Figure 1.2.7) with an additional seven-atom spacer, reacts with amine groups of biomolecules to form stable amides. Like biotin conjugates, the DSB-X™ biotin conjugate can be detected with any avidin or streptavidin derivative; with DSB-X™ biotin conjugates, however, this binding is almost totally reversed by addition of free biotin (B1595, B20656; Section 4.2) at neutral pH and normal ionic strength.

The DSB-X™ Biotin Protein Labeling Kit (D20655; Table 1.2, Table 1.3) contains the reagents required for five protein conjugations of 0.5–3 mg each, including:

- Five vials of amine-reactive DSB-X™ biotin succinimidyl ester
- Dimethylsulfoxide (DMSO) for dissolving the succinimidyl ester
- Reaction tubes
- Purification resin, spin columns and collection tubes for small-scale purifications
- Dialysis tubing for larger-scale separations
- Detailed protocols for conjugation and purification

Kits for Labeling Nucleic Acids with a Fluorescent Dye

ULYSIS® Nucleic Acid Labeling Kits

The ULYSIS® Alexa Fluor® Nucleic Acid Labeling Kits (Table 1.2, Table 1.3, Table 8.6) provide a simple, reliable method for producing fluorescent hybridization probes by combining our Alexa Fluor® fluorophores with the versatile, patented Universal Linkage System (ULS®) platinum-based chemistry developed by KREATECH Biotechnology BV. The ULS® technology is based on the use of a platinum dye reagent that forms a stable adduct with the *N*-7 position of guanine and, to a lesser extent, adenine bases in DNA, RNA, peptide–nucleic acid conjugates (PNA) and oligonucleotides (Figure 1.2.8). In protein contexts, ULS® reagents are reactive with cysteine residues and other thiols.[5]

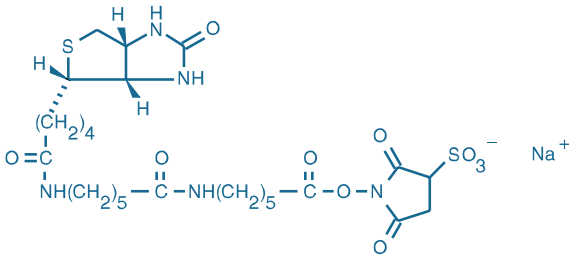

Figure 1.2.5 6-((6-((biotinoyl)amino)hexanoyl)amino)hexanoic acid, sulfosuccinimidyl ester, sodium salt (biotin-XX, SSE; B6352).

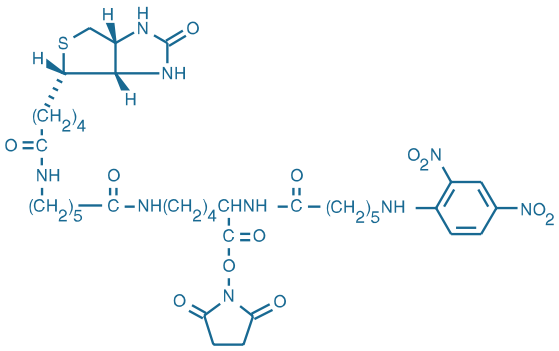

Figure 1.2.6 Biotin-X 2,4-dinitrophenyl-X-L-lysine, succinimidyl ester (DNP-X-biocytin-X, SE; B2604).

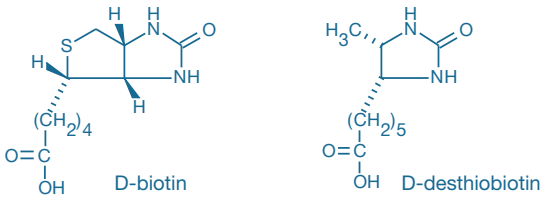

Figure 1.2.7 Comparison of the structures of D-biotin (left) and D-desthiobiotin (right).

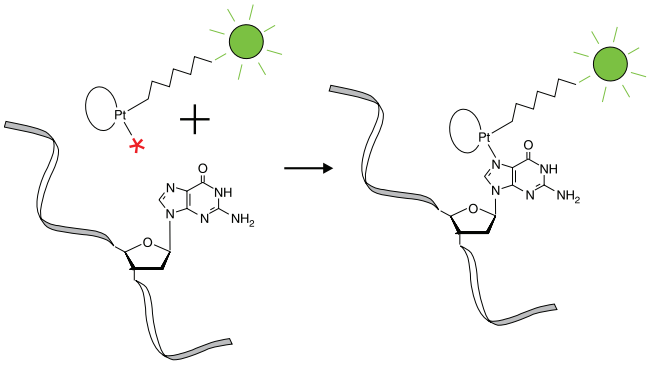

Figure 1.2.8 Schematic diagram of the labeling method provided in our ULYSIS® Nucleic Acid Labeling Kits. The ULS® reagent in the ULYSIS® Nucleic Acid Labeling Kits reacts with the *N*-7 position of guanine residues to provide a stable coordination complex between the nucleic acid and the fluorophore label.

The labeling reaction typically takes only 15 minutes, and separation of the labeled nucleic acids from the unreacted ULS® reagent can be accomplished through use of a simple spin-column procedure (Figure 1.2.9).

In addition to ULYSIS® Alexa Fluor® Nucleic Acid Labeling Kits, we offer the ULYSIS® Oregon Green® 488 Nucleic Acid Labeling Kit (Section 8.2).

Each of these ULYSIS® Kits provides sufficient reagents for 20 labelings of 1 µg DNA each, including:

- ULS® labeling reagent and appropriate solvent
- Labeling buffer
- Deoxyribonuclease I (DNase I), for digesting DNA longer than 1000 base-pairs prior to labeling
- DNase I storage and reaction buffers
- Control DNA from calf thymus
- Nuclease-free H_2O
- Detailed protocols for preparing fluorescent DNA hybridization probes for chromosome *in situ* hybridization and dot-blot hybridization

Probes labeled using the ULYSIS® Kits are stable indefinitely and hybridize effectively to target DNA. The ULS® method has been used to prepare labeled probes for dot, Southern and northern blot analysis, RNA and DNA *in situ* hybridization, multicolor FISH, comparative genome hybridization (CGH) and microarray analysis.

ARES™ DNA Labeling Kits

The ARES™ DNA Labeling Kits (Table 1.2, Table 1.3, Table 8.6) provide a versatile, two-step method for labeling DNA with several of our premier fluorescent dyes[6] (Figure 1.2.10). In the first step, an amine-modified nucleotide, 5-(3-aminoallyl)-dUTP (Figure 1.2.11), is incorporated into DNA using conventional enzymatic labeling methods. This step helps ensure relatively uniform labeling of the probe with primary amine groups. The aminoallyl dUTP substrate used in this reaction is taken up efficiently by reverse transcription or nick translation, for which we provide the protocols; other enzymatic methods are also likely to be compatible. In the second step, the amine-modified DNA is chemically labeled using an amine-reactive fluorescent dye. This chemical reaction varies little in its efficiency from dye to dye, so that it is possible to use any combination of the ARES™ Kits, with their broad selection of the brightest and most photostable dyes, and obtain consistent DNA labeling. The labeling protocols provided generally result in incorporation of about one dye per 12–15 bases, which we have determined to be optimal for fluorescence *in situ* hybridization (FISH) and dot-blot hybridization.

Each ARES™ DNA Labeling Kit provides sufficient reagents for 5 to 10 labelings of 1–5 µg DNA each, including:

- 5-(3-Aminoallyl)-dUTP
- Amine-reactive fluorescent dye and appropriate solvent
- Sodium bicarbonate
- Nuclease-free H_2O
- Detailed protocol for labeling DNA using reverse transcription or nick translation

See Section 8.2 for a complete description of the ARES™ Kits, as well as of the FISH Tag™ DNA and FISH Tag™ RNA Kits, which employ the same aminoallyl nucleotide labeling method but provide a complete workflow solution for fluorescence *in situ* hybridization (FISH) applications. Each FISH Tag™ Kit provides all of the reagents needed for enzymatically incorporating the amine-modified nucleotide (aminoallyl dUTP or aminoallyl UTP) into DNA or RNA, followed by fluorescent labeling with an amine-reactive Alexa Fluor® dye and purification of the labeled probe using PureLink™ nucleic acid purification technology (e.g., PureLink™ PCR Purification Kit, K3100-01). FISH Tag™ DNA and FISH Tag™ RNA Kits are each available as single-color kits containing one of four amine-reactive Alexa Fluor® dyes or in a multicolor kit that contains all four reactive Alexa Fluor® dyes.

Alexa Fluor® Oligonucleotide Amine Labeling Kits

The Alexa Fluor® Oligonucleotide Amine Labeling Kits (Section 8.2; Table 1.2, Table 1.3, Table 8.6) provide the reagents required for labeling synthetic oligonucleotides that have amine groups incorporated at their 5′-terminus. Following purification by standard chromatographic or electrophoretic procedures, these singly labeled oligonucleotides can serve as hybridization or ligation probes for a variety of applications.[7–9]

Each Alexa Fluor® Oligonucleotide Amine Labeling Kit contains sufficient reagents for three labelings of 50 µg each of an amine-modified oligonucleotide, including:

- Three vials of amine-reactive dye
- Dimethylsulfoxide (DMSO)
- Three vials of labeling buffer
- Labeling protocol

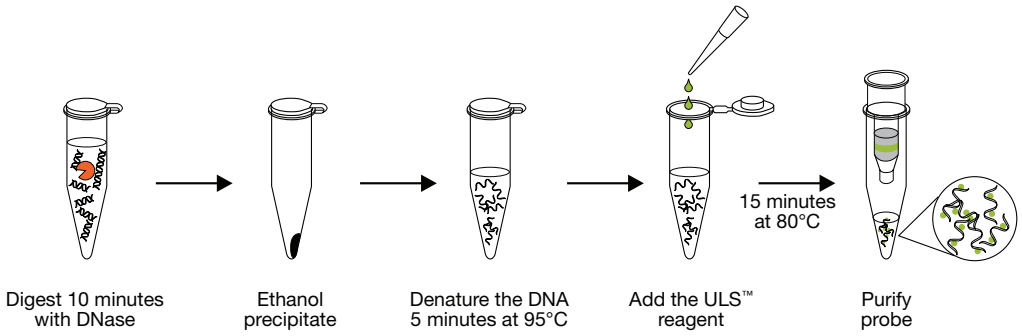

Figure 1.2.9 Nucleic acid labeling method provided in our ULYSIS® Nucleic Acid Labeling Kits.

Biotin Quantitation Assay Kits

FluoReporter® Biotin Quantitation Assay Kit for Biotinylated Proteins

The FluoReporter® Biotin Quantitation Assay Kit for biotinylated proteins (F30751) provides a sensitive fluorometric assay designed to accurately determine the number of biotin labels on a protein. This assay is based on the displacement of a ligand tagged with a quencher dye from the biotin binding sites of Biotective™ Green reagent.[10] The FluoReporter® biotin quantitation assay can detect from 4 to 80 picomoles of biotin in a sample, providing a 50-fold higher sensitivity than the spectrophotometric HABA biotin binding assay. Furthermore, unlike the HABA biotin binding assay, which requires ~1 mg of protein sample, the FluoReporter® biotin quantitation assay requires a minimum of 600 ng of a singly biotinylated IgG with molecular weight 150,000 daltons. For proteins of lower molecular weight or multiple biotin labels, less protein can be used. To expose any biotin groups in multiply labeled proteins that are sterically restricted and inaccessible to the Biotective™ Green reagent, this kit includes protease and an optional protocol for digesting the protein. With this preliminary digestion, biotin assay values agree well with MALDI-TOF determinations. With excitation/emission maxima of 495/519 nm, this assay is compatible with any fluorescence-based microplate reader capable of detecting fluorescein (FITC) or Alexa Fluor® 488 dye; it can also be scaled up for fluorometer-based experiments.

Each FluoReporter® Biotin Quantitation Assay Kit for biotinylated proteins includes:

- Biotective™ Green reagent
- Biocytin standard
- Protease
- Concentrated phosphate-buffered saline (PBS)
- Biotinylated goat anti–mouse IgG antibody for use as a positive control
- Detailed protocols

Sufficient reagents are provided for assaying 5 samples independently using eight wells in triplicate for the standard curve and three dilutions of the sample in triplicate (totaling 33 wells per assay). However, fewer wells may be used to conserve sample and a single standard curve can be used for multiple samples in the same experimental session.

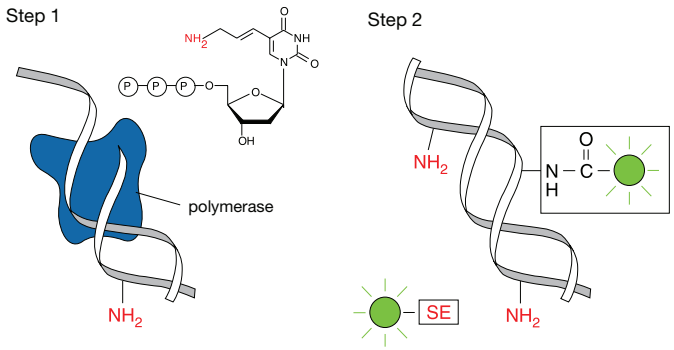

Figure 1.2.10 Schematic diagram of the labeling method provided in our ARES™ DNA Labeling Kits. The ARES™ DNA Labeling Kits use a two-step method to label DNA. Step 1) The aminoallyl dUTP is enzymatically incorporated. Step 2) A reactive fluorophore is used to label the incorporated aminoallyl group.

FluoReporter® Biotin Quantitation Assay Kit for Biotinylated Nucleic Acids

The FluoReporter® Biotin Quantitation Assay Kit for biotinylated nucleic acids (F30755) provides a sensitive fluorometric assay for determining the number of biotin labels on a nucleic acid. This assay is based on the displacement of a quencher dye from the biotin binding sites of Biotective™ Green reagent. The FluoReporter® biotin quantitation assay can detect from 4 to 80 picomoles of biotin in a sample, providing a 50-fold higher sensitivity than the spectrophotometric HABA biotin binding assay. Analysis of multiply biotinylated nucleic acids requires a preliminary nuclease digestion step to avoid underestimation caused by steric restriction of avidin binding. This kit can be applied to as little as 13 ng of biotin-labeled nucleic acid and is ideal for determining the degree of biotinylation of cDNA samples used for microarray expression analysis.

Each FluoReporter® Biotin Quantitation Assay Kit for biotinylated nucleic acids includes:

- Biotective™ Green reagent
- Biotin-dUMP standard
- Nuclease
- Concentrated phosphate-buffered saline (PBS)
- Biotinylated DNA for use as a postive control
- Concentrated nucleic acid digestion buffer
- Detailed protocols

Sufficient reagents are provided for assaying 10 samples independently using eight wells in triplicate for the standard curve and three dilutions of the sample in triplicate (totaling 33 wells per assay). However, fewer wells may be used to conserve sample and a single standard curve can be used for multiple samples in the same experimental session.

REFERENCES

1. Bioconjug Chem (2009) 20:147; **2**. Biochemistry (2007) 46:12522; **3**. J Mol Diagn (2006) 8:246; **4**. Anal Biochem (2002) 308:343; **5**. Clin Chem (2002) 48:1352; **6**. Biotechniques (2004) 36:114; **7**. J Am Chem Soc (2006) 128:11423; **8**. Biochemistry (2006) 45:4164; **9**. Anal Chem (2003) 75:1664; **10**. Biotechniques (2007) 43:503.

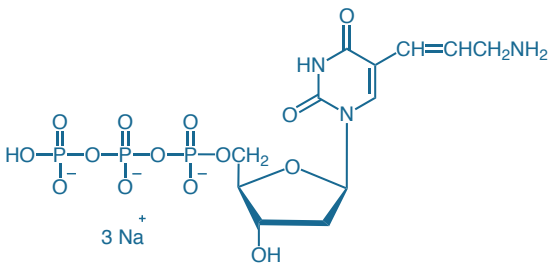

Figure 1.2.11 Aminoallyl-dUTP (5-(3-aminoallyl)-2'-deoxyuridine 5'-triphosphate, trisodium salt, A21664).

PRODUCT LIST 1.2 KITS FOR LABELING PROTEINS AND NUCLEIC ACIDS

Cat. No.	Product	Quantity
A30006	Alexa Fluor® 488 Microscale Protein Labeling Kit *for 20–100 µg protein* *3 labelings*	1 kit
A30007	Alexa Fluor® 555 Microscale Protein Labeling Kit *for 20–100 µg protein* *3 labelings*	1 kit
A30008	Alexa Fluor® 594 Microscale Protein Labeling Kit *for 20–100 µg protein* *3 labelings*	1 kit
A30009	Alexa Fluor® 647 Microscale Protein Labeling Kit *for 20–100 µg protein* *3 labelings*	1 kit
A20180	Alexa Fluor® 350 Monoclonal Antibody Labeling Kit *5 labelings*	1 kit
A20181	Alexa Fluor® 488 Monoclonal Antibody Labeling Kit *5 labelings*	1 kit
A20182	Alexa Fluor® 532 Monoclonal Antibody Labeling Kit *5 labelings*	1 kit
A20183	Alexa Fluor® 546 Monoclonal Antibody Labeling Kit *5 labelings*	1 kit
A20187	Alexa Fluor® 555 Monoclonal Antibody Labeling Kit *5 labelings*	1 kit
A20184	Alexa Fluor® 568 Monoclonal Antibody Labeling Kit *5 labelings*	1 kit
A20185	Alexa Fluor® 594 Monoclonal Antibody Labeling Kit *5 labelings*	1 kit
A20186	Alexa Fluor® 647 Monoclonal Antibody Labeling Kit *5 labelings*	1 kit
A20191	Alexa Fluor® 488 Oligonucleotide Amine Labeling Kit *3 labelings*	1 kit
A20196	Alexa Fluor® 647 Oligonucleotide Amine Labeling Kit *3 labelings*	1 kit
A10170	Alexa Fluor® 350 Protein Labeling Kit *3 labelings*	1 kit
A10171	Alexa Fluor® 430 Protein Labeling Kit *3 labelings*	1 kit
A10235	Alexa Fluor® 488 Protein Labeling Kit *3 labelings*	1 kit
A10236	Alexa Fluor® 532 Protein Labeling Kit *3 labelings*	1 kit
A10237	Alexa Fluor® 546 Protein Labeling Kit *3 labelings*	1 kit
A20174	Alexa Fluor® 555 Protein Labeling Kit *3 labelings*	1 kit
A10238	Alexa Fluor® 568 Protein Labeling Kit *3 labelings*	1 kit
A10239	Alexa Fluor® 594 Protein Labeling Kit *3 labelings*	1 kit
A20170	Alexa Fluor® 633 Protein Labeling Kit *3 labelings*	1 kit
A20173	Alexa Fluor® 647 Protein Labeling Kit *3 labelings*	1 kit
A20171	Alexa Fluor® 660 Protein Labeling Kit *3 labelings*	1 kit
A20172	Alexa Fluor® 680 Protein Labeling Kit *3 labelings*	1 kit
A10468	APEX® Alexa Fluor® 488 Antibody Labeling Kit	1 kit
A10470	APEX® Alexa Fluor® 555 Antibody Labeling Kit	1 kit
A10474	APEX® Alexa Fluor® 594 Antibody Labeling Kit	1 kit
A10475	APEX® Alexa Fluor® 647 Antibody Labeling Kit	1 kit
A10476	APEX® Oregon Green® 488 Antibody Labeling Kit	1 kit
A10478	APEX® Pacific Blue™ Antibody Labeling Kit	1 kit
A21665	ARES™ Alexa Fluor® 488 DNA Labeling Kit *10 labelings*	1 kit
A21667	ARES™ Alexa Fluor® 546 DNA Labeling Kit *10 labelings*	1 kit
A21677	ARES™ Alexa Fluor® 555 DNA Labeling Kit *10 labelings*	1 kit
A21669	ARES™ Alexa Fluor® 594 DNA Labeling Kit *10 labelings*	1 kit
A21676	ARES™ Alexa Fluor® 647 DNA Labeling Kit *10 labelings*	1 kit
B30010	Biotin-XX Microscale Protein Labeling Kit *for 20–100 µg protein* *3 labelings*	1 kit
B30756	Biotin-XX Microscale Protein Labeling Kit with FluoReporter® Biotin Quantitation Assay Kit *includes B30010 and F30751*	1 kit
D20655	DSB-X™ Biotin Protein Labeling Kit *5 labelings*	1 kit
F30755	FluoReporter® Biotin Quantitation Assay Kit *for biotinylated nucleic acids* *10 determinations*	1 kit
F30751	FluoReporter® Biotin Quantitation Assay Kit *for biotinylated proteins* *5 determinations*	1 kit
F6348	FluoReporter® Biotin/DNP Protein Labeling Kit *5–10 labelings*	1 kit
F2610	FluoReporter® Biotin-XX Protein Labeling Kit *5 labelings of 5–20 mg protein each*	1 kit
F6434	FluoReporter® FITC Protein Labeling Kit *5–10 labelings*	1 kit
F6433	FluoReporter® Fluorescein-EX Protein Labeling Kit *5–10 labelings*	1 kit
F6347	FluoReporter® Mini-biotin-XX Protein Labeling Kit *5 labelings of 0.1–3 mg protein each*	1 kit
F6153	FluoReporter® Oregon Green® 488 Protein Labeling Kit *5–10 labelings*	1 kit
F6161	FluoReporter® Rhodamine Red™-X Protein Labeling Kit *5–10 labelings*	1 kit
F6162	FluoReporter® Texas Red®-X Protein Labeling Kit *5–10 labelings*	1 kit
F10240	Fluorescein-EX Protein Labeling Kit *3 labelings*	1 kit
O10241	Oregon Green® 488 Protein Labeling Kit *3 labelings*	1 kit
P30013	Pacific Blue™ Monoclonal Antibody Labeling Kit *5 labelings*	1 kit
P30012	Pacific Blue™ Protein Labeling Kit *3 labelings*	1 kit
P30014	Pacific Orange™ Monoclonal Antibody Labeling Kit *5 labelings*	1 kit
P30016	Pacific Orange™ Protein Labeling Kit *3 labelings*	1 kit
S30044	SAIVI™ Alexa Fluor® 647 Antibody/Protein 1 mg-Labeling Kit *3 labelings*	1 kit
S30045	SAIVI™ Rapid Antibody Labeling Kit, Alexa Fluor® 680 *3 labelings*	1 kit
S30046	SAIVI™ Rapid Antibody Labeling Kit, Alexa Fluor® 750 *3 labelings*	1 kit

PRODUCT LIST 1.2 KITS FOR LABLING PROTEINS AND NUCLEIC ACIDS—*continued*

Cat. No.	Product	Quantity
T10244	Texas Red®-X Protein Labeling Kit *3 labelings*	1 kit
U21650	ULYSIS® Alexa Fluor® 488 Nucleic Acid Labeling Kit *20 labelings*	1 kit
U21651	ULYSIS® Alexa Fluor® 532 Nucleic Acid Labeling Kit *20 labelings*	1 kit
U21652	ULYSIS® Alexa Fluor® 546 Nucleic Acid Labeling Kit *20 labelings*	1 kit
U21653	ULYSIS® Alexa Fluor® 568 Nucleic Acid Labeling Kit *20 labelings*	1 kit
U21654	ULYSIS® Alexa Fluor® 594 Nucleic Acid Labeling Kit *20 labelings*	1 kit
U21660	ULYSIS® Alexa Fluor® 647 Nucleic Acid Labeling Kit *20 labelings*	1 kit
U21659	ULYSIS® Oregon Green® 488 Nucleic Acid Labeling Kit *20 labelings*	1 kit
Z25400	Zenon® Alexa Fluor® 350 Human IgG Labeling Kit *50 labelings*	1 kit
Z25000	Zenon® Alexa Fluor® 350 Mouse IgG_1 Labeling Kit *50 labelings*	1 kit
Z25100	Zenon® Alexa Fluor® 350 Mouse IgG_{2a} Labeling Kit *50 labelings*	1 kit
Z25200	Zenon® Alexa Fluor® 350 Mouse IgG_{2b} Labeling Kit *50 labelings*	1 kit
Z25300	Zenon® Alexa Fluor® 350 Rabbit IgG Labeling Kit *50 labelings*	1 kit
Z25013	Zenon® Alexa Fluor® 405 Mouse IgG_1 Labeling Kit *50 labelings*	1 kit
Z25113	Zenon® Alexa Fluor® 405 Mouse IgG_{2a} Labeling Kit *50 labelings*	1 kit
Z25213	Zenon® Alexa Fluor® 405 Mouse IgG_{2b} Labeling Kit *50 labelings*	1 kit
Z25313	Zenon® Alexa Fluor® 405 Rabbit IgG Labeling Kit *50 labelings*	1 kit
Z25001	Zenon® Alexa Fluor® 430 Mouse IgG_1 Labeling Kit *50 labelings*	1 kit
Z25301	Zenon® Alexa Fluor® 430 Rabbit IgG Labeling Kit *50 labelings*	1 kit
Z25602	Zenon® Alexa Fluor® 488 Goat IgG Labeling Kit *50 labelings*	1 kit
Z25402	Zenon® Alexa Fluor® 488 Human IgG Labeling Kit *50 labelings*	1 kit
Z25002	Zenon® Alexa Fluor® 488 Mouse IgG_1 Labeling Kit *50 labelings*	1 kit
Z25090	Zenon® Alexa Fluor® 488 Mouse IgG_1 Labeling Kit *enhanced with TSA™ technology* *25 labelings*	1 kit
Z25102	Zenon® Alexa Fluor® 488 Mouse IgG_{2a} Labeling Kit *50 labelings*	1 kit
Z25202	Zenon® Alexa Fluor® 488 Mouse IgG_{2b} Labeling Kit *50 labelings*	1 kit
Z25302	Zenon® Alexa Fluor® 488 Rabbit IgG Labeling Kit *50 labelings*	1 kit
Z25003	Zenon® Alexa Fluor® 532 Mouse IgG_1 Labeling Kit *50 labelings*	1 kit
Z25303	Zenon® Alexa Fluor® 532 Rabbit IgG Labeling Kit *50 labelings*	1 kit
Z25004	Zenon® Alexa Fluor® 546 Mouse IgG_1 Labeling Kit *50 labelings*	1 kit
Z25104	Zenon® Alexa Fluor® 546 Mouse IgG_{2a} Labeling Kit *50 labelings*	1 kit
Z25204	Zenon® Alexa Fluor® 546 Mouse IgG_{2b} Labeling Kit *50 labelings*	1 kit
Z25304	Zenon® Alexa Fluor® 546 Rabbit IgG Labeling Kit *50 labelings*	1 kit
Z25605	Zenon® Alexa Fluor® 555 Goat IgG Labeling Kit *50 labelings*	1 kit
Z25405	Zenon® Alexa Fluor® 555 Human IgG Labeling Kit *50 labelings*	1 kit
Z25005	Zenon® Alexa Fluor® 555 Mouse IgG_1 Labeling Kit *50 labelings*	1 kit
Z25105	Zenon® Alexa Fluor® 555 Mouse IgG_{2a} Labeling Kit *50 labelings*	1 kit
Z25205	Zenon® Alexa Fluor® 555 Mouse IgG_{2b} Labeling Kit *50 labelings*	1 kit
Z25305	Zenon® Alexa Fluor® 555 Rabbit IgG Labeling Kit *50 labelings*	1 kit
Z25606	Zenon® Alexa Fluor® 568 Goat IgG Labeling Kit *50 labelings*	1 kit
Z25006	Zenon® Alexa Fluor® 568 Mouse IgG_1 Labeling Kit *50 labelings*	1 kit
Z25106	Zenon® Alexa Fluor® 568 Mouse IgG_{2a} Labeling Kit *50 labelings*	1 kit
Z25206	Zenon® Alexa Fluor® 568 Mouse IgG_{2b} Labeling Kit *50 labelings*	1 kit
Z25306	Zenon® Alexa Fluor® 568 Rabbit IgG Labeling Kit *50 labelings*	1 kit
Z25607	Zenon® Alexa Fluor® 594 Goat IgG Labeling Kit *50 labelings*	1 kit
Z25407	Zenon® Alexa Fluor® 594 Human IgG Labeling Kit *50 labelings*	1 kit
Z25007	Zenon® Alexa Fluor® 594 Mouse IgG_1 Labeling Kit *50 labelings*	1 kit
Z25107	Zenon® Alexa Fluor® 594 Mouse IgG_{2a} Labeling Kit *50 labelings*	1 kit
Z25207	Zenon® Alexa Fluor® 594 Mouse IgG_{2b} Labeling Kit *50 labelings*	1 kit
Z25307	Zenon® Alexa Fluor® 594 Rabbit IgG Labeling Kit *50 labelings*	1 kit
Z25020	Zenon® Alexa Fluor® 610–R-Phycoerythrin Mouse IgG_1 Labeling Kit *10 labelings*	1 kit
Z25608	Zenon® Alexa Fluor® 647 Goat IgG Labeling Kit *50 labelings*	1 kit
Z25408	Zenon® Alexa Fluor® 647 Human IgG Labeling Kit *50 labelings*	1 kit
Z25008	Zenon® Alexa Fluor® 647 Mouse IgG_1 Labeling Kit *50 labelings*	1 kit
Z25108	Zenon® Alexa Fluor® 647 Mouse IgG_{2a} Labeling Kit *50 labelings*	1 kit
Z25208	Zenon® Alexa Fluor® 647 Mouse IgG_{2b} Labeling Kit *50 labelings*	1 kit
Z25308	Zenon® Alexa Fluor® 647 Rabbit IgG Labeling Kit *50 labelings*	1 kit
Z25021	Zenon® Alexa Fluor® 647–R-Phycoerythrin Mouse IgG_1 Labeling Kit *10 labelings*	1 kit
Z25121	Zenon® Alexa Fluor® 647–R-Phycoerythrin Mouse IgG_{2a} Labeling Kit *10 labelings*	1 kit

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PRODUCT LIST 1.2 KITS FOR LABLING PROTEINS AND NUCLEIC ACIDS—*continued*

Cat. No.	Product	Quantity
Z25221	Zenon® Alexa Fluor® 647–R-Phycoerythrin Mouse IgG_{2b} Labeling Kit *10 labelings*	1 kit
Z25009	Zenon® Alexa Fluor® 660 Mouse IgG_1 Labeling Kit *50 labelings*	1 kit
Z25010	Zenon® Alexa Fluor® 680 Mouse IgG_1 Labeling Kit *50 labelings*	1 kit
Z25110	Zenon® Alexa Fluor® 680 Mouse IgG_{2a} Labeling Kit *50 labelings*	1 kit
Z25210	Zenon® Alexa Fluor® 680 Mouse IgG_{2b} Labeling Kit *50 labelings*	1 kit
Z25310	Zenon® Alexa Fluor® 680 Rabbit IgG Labeling Kit *50 labelings*	1 kit
Z25022	Zenon® Alexa Fluor® 680–R-Phycoerythrin Mouse IgG_1 Labeling Kit *10 labelings*	1 kit
Z25011	Zenon® Alexa Fluor® 700 Mouse IgG_1 Labeling Kit *50 labelings*	1 kit
Z25030	Zenon® Alexa Fluor® 700–Allophycocyanin Mouse IgG_1 Labeling Kit *10 labelings*	1 kit
Z25312	Zenon® Alexa Fluor® 750 Rabbit IgG Labeling Kit *50 labelings*	1 kit
Z25031	Zenon® Alexa Fluor® 750–Allophycocyanin Mouse IgG_1 Labeling Kit *10 labelings*	1 kit
Z25350	Zenon® Alkaline Phosphatase Rabbit IgG Labeling Kit *25 labelings*	1 kit
Z25451	Zenon® Allophycocyanin Human IgG Labeling Kit *25 labelings*	1 kit
Z25051	Zenon® Allophycocyanin Mouse IgG_1 Labeling Kit *25 labelings*	1 kit
Z25151	Zenon® Allophycocyanin Mouse IgG_{2a} Labeling Kit *25 labelings*	1 kit
Z25251	Zenon® Allophycocyanin Mouse IgG_{2b} Labeling Kit *25 labelings*	1 kit
Z25351	Zenon® Allophycocyanin Rabbit IgG Labeling Kit *25 labelings*	1 kit
Z25452	Zenon® Biotin-XX Human IgG Labeling Kit *50 labelings*	1 kit
Z25052	Zenon® Biotin-XX Mouse IgG_1 Labeling Kit *50 labelings*	1 kit
Z25152	Zenon® Biotin-XX Mouse IgG_{2a} Labeling Kit *50 labelings*	1 kit
Z25252	Zenon® Biotin-XX Mouse IgG_{2b} Labeling Kit *50 labelings*	1 kit
Z25352	Zenon® Biotin-XX Rabbit IgG Labeling Kit *50 labelings*	1 kit
Z25042	Zenon® Fluorescein Mouse IgG_1 Labeling Kit *50 labelings*	1 kit
Z25342	Zenon® Fluorescein Rabbit IgG Labeling Kit *50 labelings*	1 kit
Z25454	Zenon® Horseradish Peroxidase Human IgG Labeling Kit *25 labelings*	1 kit
Z25054	Zenon® Horseradish Peroxidase Mouse IgG_1 Labeling Kit *25 labelings*	1 kit
Z25154	Zenon® Horseradish Peroxidase Mouse IgG_{2a} Labeling Kit *25 labelings*	1 kit
Z25254	Zenon® Horseradish Peroxidase Mouse IgG_{2b} Labeling Kit *25 labelings*	1 kit
Z25354	Zenon® Horseradish Peroxidase Rabbit IgG Labeling Kit *25 labelings*	1 kit
Z25041	Zenon® Pacific Blue™ Mouse IgG_1 Labeling Kit *50 labelings*	1 kit
Z25156	Zenon® Pacific Blue™ Mouse IgG_{2a} Labeling Kit *50 labelings*	1 kit
Z25341	Zenon® Pacific Blue™ Rabbit IgG Labeling Kit *50 labelings*	1 kit
Z25256	Zenon® Pacific Orange™ Mouse IgG_1 Labeling Kit *50 labelings*	1 kit
Z25257	Zenon® Pacific Orange™ Mouse IgG_{2a} Labeling Kit *50 labelings*	1 kit
Z25455	Zenon® R-Phycoerythrin Human IgG Labeling Kit *25 labelings*	1 kit
Z25055	Zenon® R-Phycoerythrin Mouse IgG_1 Labeling Kit *25 labelings*	1 kit
Z25155	Zenon® R-Phycoerythrin Mouse IgG_{2a} Labeling Kit *25 labelings*	1 kit
Z25255	Zenon® R-Phycoerythrin Mouse IgG_{2b} Labeling Kit *25 labelings*	1 kit
Z25355	Zenon® R-Phycoerythrin Rabbit IgG Labeling Kit *25 labelings*	1 kit
Z25045	Zenon® Texas Red®-X Mouse IgG_1 Labeling Kit *50 labelings*	1 kit
Z25460	Zenon® Tricolor Human IgG Labeling Kit #1 *for green, orange and deep red fluorescence imaging* *3 x 10 labelings*	1 kit
Z25470	Zenon® Tricolor Human IgG Labeling Kit #2 *for blue, green and red fluorescence imaging* *3 x 10 labelings*	1 kit
Z25060	Zenon® Tricolor Mouse IgG_1 Labeling Kit #1 *for green, orange and deep red fluorescence imaging* *3 x 10 labelings*	1 kit
Z25070	Zenon® Tricolor Mouse IgG_1 Labeling Kit #2 *for blue, green and red fluorescence imaging* *3 x 10 labelings*	1 kit
Z25080	Zenon® Tricolor Mouse IgG_1 Labeling Kit #3 *for flow cytometry, 488 nm excitation* *3 x 10 labelings*	1 kit
Z25160	Zenon® Tricolor Mouse IgG_{2a} Labeling Kit #1 *for green, orange and deep red fluorescence imaging* *3 x 10 labelings*	1 kit
Z25170	Zenon® Tricolor Mouse IgG_{2a} Labeling Kit #2 *for blue, green and red fluorescence imaging* *3 x 10 labelings*	1 kit
Z25180	Zenon® Tricolor Mouse IgG_{2a} Labeling Kit #3 *for flow cytometry, 488 nm excitation* *3 x 10 labelings*	1 kit
Z25260	Zenon® Tricolor Mouse IgG_{2b} Labeling Kit #1 *for green, orange and deep red fluorescence imaging* *3 x 10 labelings*	1 kit
Z25270	Zenon® Tricolor Mouse IgG_{2b} Labeling Kit #2 *for blue, green and red fluorescence imaging* *3 x 10 labelings*	1 kit
Z25280	Zenon® Tricolor Mouse IgG_{2b} Labeling Kit #3 *for flow cytometry, 488 nm excitation* *3 x 10 labelings*	1 kit
Z25360	Zenon® Tricolor Rabbit IgG Labeling Kit #1 *for green, orange and deep red fluorescence imaging* *3 x 10 labelings*	1 kit
Z25370	Zenon® Tricolor Rabbit IgG Labeling Kit #2 *for blue, green and red fluorescence imaging* *3 x 10 labelings*	1 kit
Z25380	Zenon® Tricolor Rabbit IgG Labeling Kit #3 *for flow cytometry, 488 nm excitation* *3 x 10 labelings*	1 kit

1.3 Alexa Fluor® Dyes Spanning the Visible and Infrared Spectrum

Overview of the Alexa Fluor® Dyes

The Alexa Fluor® dyes produce exceptionally bright and photostable conjugates (Table 1.4, Note 1.1). The Alexa Fluor® dyes share several significant attributes, including:

- Strong absorption at wavelengths of maximal output of common excitation sources
- Bright and unusually photostable fluorescence of their bioconjugates
- Good water solubility, which makes the reactive dyes easy to conjugate and the conjugates resistant to precipitation and aggregation
- Insensitivity of their absorpion and emission spectra to pH over a broad range
- Well-differentiated spectra, providing many options for multicolor detection and fluorescence resonance energy transfer (Fluorescence Resonance Energy Transfer (FRET)—Note 1.2)
- High quantum yields and long fluorescence lifetimes (Table 1.5)
- Extremely high FRET efficiency, with calculated R_0 values of up to 84 Å between pairs of Alexa Fluor® dyes (Table 1.6) and up to 77 Å between Alexa Fluor® dyes and some nonfluorescent quenchers (Table 1.11)

Alexa Fluor® dyes set new standards for fluorophores and the bioconjugates prepared from them. The absorption spectra (Figure 1.3.1, Figure 1.3.2, Figure 1.3.3) of these superior fluorescent dyes—Alexa Fluor® 350, Alexa Fluor® 405, Alexa Fluor® 430, Alexa Fluor® 488, Alexa Fluor® 514, Alexa Fluor® 532, Alexa Fluor® 546, Alexa Fluor® 555, Alexa Fluor® 568, Alexa Fluor® 594, Alexa Fluor® 610, Alexa Fluor® 633, Alexa Fluor® 635, Alexa Fluor® 647, Alexa Fluor® 660, Alexa Fluor® 680, Alexa Fluor® 700, Alexa Fluor® 750 and Alexa Fluor® 790 dyes—span the visible and infrared spectrum (Table 1.4) and match the principal output wavelengths of common excitation sources.[1,2] Because there are so many different Alexa Fluor® dyes, we have had to develop a systematic strategy for naming them. We identify these dyes with the registered trademark Alexa Fluor® followed by the optimal excitation wavelength in nm; for example, Alexa Fluor® 488 dye is optimally excited by the 488 nm spectral line of the argon-ion laser.

With spectra almost identical to those of fluorescein (Figure 1.3.4), but with far greater conjugate fluorescence and significantly better conjugate photostability, Alexa Fluor® 488 dye is indisputably the best green-fluorescent reactive dye available. Spectra of Alexa Fluor® 555 dye are an almost perfect match to those of Cy®3 dye (Figure 1.3.5), but conjugates of Alexa Fluor® 555 dye are more fluorescent (Figure 1.3.6) and more photostable (Figure 1.3.7) than those of Cy®3 dye. Similarly, spectra of Alexa Fluor® 647 conjugates substantially match those of the Cy®5 dye (Figure 1.3.8) and Alexa Fluor® 680 and Alexa Fluor® 750 dyes exhibit spectral properties similar to those of Cy®5.5 and Cy®7 dyes, respectively (Figure 1.3.9, Figure 1.3.10). Tandem conjugates of the long-wavelength Alexa Fluor® dyes with R-phycoerythrin or allophycocyanin (Section 6.4) further expand the utility of this dye series in multiplex flow cytometry applications[3,4] by shifting the phycobiliprotein emission profile relative to the available detection channels (Figure 1.3.11, Figure 1.3.12).

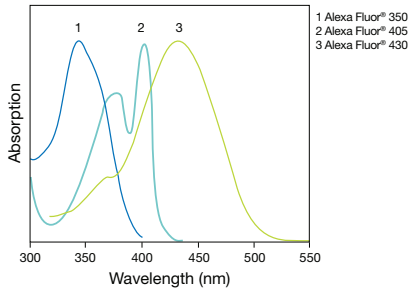

Figure 1.3.1 Absorption spectra of our ultraviolet and blue light–absorbing Alexa Fluor® dyes.

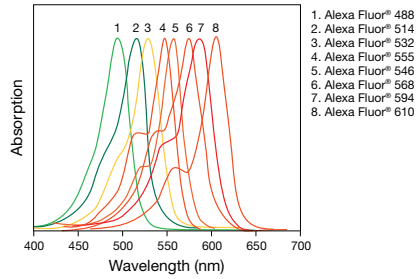

Figure 1.3.2 Absorption spectra of our green, yellow and red light–absorbing Alexa Fluor® dyes.

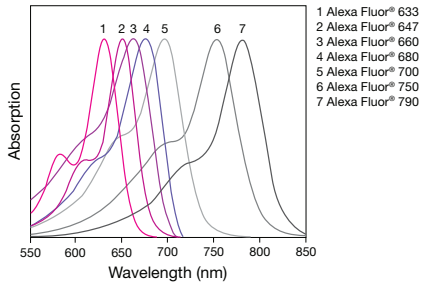

Figure 1.3.3 Absorption spectra of our far-red and infrared light–absorbing Alexa Fluor® dyes. Alexa Fluor® 635 dye, available conjugated to antibodies, streptavidin and phalloidin, is not included here but its absorption spectrum is very similar to that of Alexa Fluor® 633 dye.

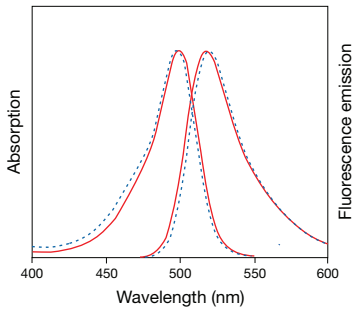

Figure 1.3.4 Absorption and fluorescence emission spectra of fluorescein goat anti–mouse IgG antibody (F2761, (—) and Alexa Fluor® 488 goat anti–mouse IgG antibody (A11001, (- - -)). The fluorescence intensity of the Alexa Fluor® 488 conjugate was significantly higher than that of the fluorescein conjugate. The data are normalized to show the spectral similarity.

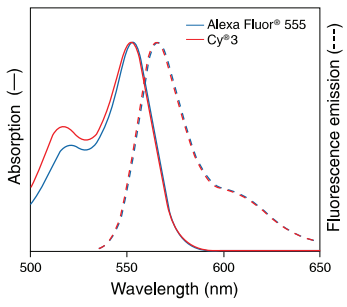

Figure 1.3.5 Comparison of the absorption and fluorescence emission spectra of Alexa Fluor® 555 and Cy®3 dyes. Spectra have been normalized to the same intensity for comparison purposes.

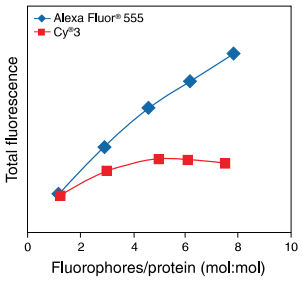

Figure 1.3.6 Comparison of the relative fluorescence of goat anti–rabbit IgG antibody conjugates of Alexa Fluor® 555 and Cy®3 dyes (prepared in our laboratories) at different dye:protein ratios in the conjugate.

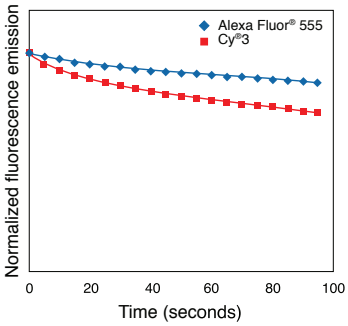

Figure 1.3.7 Photobleaching profiles of Alexa Fluor® 555 and Cy®3 dyes were obtained by placing equal molar concentrations of the free dyes into capillary tubes; the samples were continuously illuminated and data points were collected every 5 seconds. Fluorescence has been normalized to the same initial intensity.

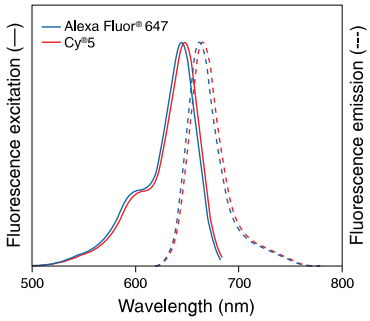

Figure 1.3.8 Comparison of the fluorescence spectra of Alexa Fluor® 647 and Cy®5 dyes. Spectra have been normalized to the same intensity for comparison purposes.

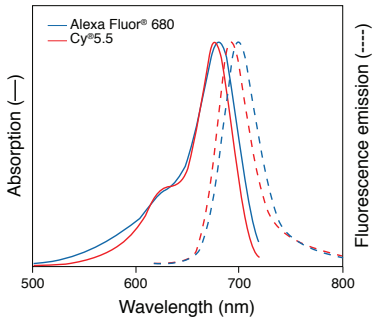

Figure 1.3.9 Comparison of the fluorescence spectra of the unconjugated Alexa Fluor® 680 and Cy®5.5 dyes. Spectra have been normalized to the same intensity for comparison purposes.

Table 1.4 Alexa Fluor® active esters and kits for labeling proteins and nucleic acids.

Alexa Fluor® Dye	Fluorescence Color (Abs/Em) *	Succinimidyl Ester, TFP Ester or SDP Ester	Kits for Labeling Proteins			Kits for Labeling Nucleic Acids and Oligonucleotides
			Protein Labeling Kits	Antibody Labeling Kits	Zenon® Antibody Labeling Kits	
Alexa Fluor® 350	Blue (346/442)	A10168 †	A10170 (P)	A20180 (Mab)	Z25000 (M IgG_1) Z25100 (M IgG_{2a}) Z25200 (M IgG_{2b}) Z25300 (R IgG) Z25400 (H IgG)	
Alexa Fluor® 405	Blue (402/421)	A30000 ‡ A30100 †			Z25013 (M IgG_1) Z25113 (M IgG_{2a}) Z25213 (M IgG_{2b}) Z25313 (R IgG)	
Alexa Fluor® 430	Yellow-green (434/539)	A10169 †	A10171 (P)		Z25001 (M IgG_1) Z25301 (R IgG)	
Alexa Fluor® 488	Green (495/519)	A20000 ‡ § A20100 † § A30005 (TFP) A30052 (SDP)	A10235 (P) ** A30006 (Micro) **	A10468 (APEX®) ** A20181 (Mab) **	Z25002 (M IgG_1) Z25090 TSA™) (M IgG_1) Z25102 (M IgG_{2a}) Z25202 (M IgG_{2b}) Z25302 (R IgG) Z25602 (G IgG) Z25402 (H IgG)	U21650 (ULYSIS®) A21665 (ARES™) F32947 (FT) F32952 (FT) A20191 (Oligo)
Alexa Fluor® 514	Yellow-green (518/540)	A30002 ‡ §				
Alexa Fluor® 532	Yellow (531/554)	A20001 ‡ A20101MP †	A10236 (P)	A20182 (Mab)	Z25003 (M IgG_1) Z25303 (R IgG)	U21651 (ULYSIS®)
Alexa Fluor® 546	Orange (556/573)	A20002 ‡ § A20102 † §	A10237 (P)	A20183 (Mab)	Z25004 (M IgG_1) Z25104 (M IgG_{2a}) Z25204 (M IgG_{2b}) Z25304 (R IgG)	U21652 (ULYSIS®) A21667 (ARES™)
Alexa Fluor® 555	Red-orange (555/565)	A20009 ‡ A20109 †	A20174 (P) A30007 (Micro)	A10470 (APEX®) A20187 (Mab)	Z25005 (M IgG_1) Z25105 (M IgG_{2a}) Z25205 (M IgG_{2b}) Z25305 (R IgG) Z25605 (G IgG) Z25405 (H IgG)	A21677 (ARES™) F32948 (FT) F32953 (FT)
Alexa Fluor® 568	Red-orange (578/603)	A20003 ‡ § A20103 † §	A10238 (P)	A20184 (Mab)	Z25006 (M IgG_1) Z25106 (M IgG_{2a}) Z25206 (M IgG_{2b}) Z25306 (R IgG) Z25606 (G IgG)	U21653 (ULYSIS®)

* Approximate absorption (Abs) and fluorescence emission (Em) maxima for conjugates, in nm. † 5 mg unit size. ‡ 1 mg unit size. § Mixed isomers. ** The APEX® Alexa Fluor® 488 Antibody Labeling Kits contain the Alexa Fluor® 488 carboxylic acid SDP ester, whereas the other Alexa Fluor® 488 protein labeling kits contain the amine-reactive Alexa Fluor® 488 carboxylic acid TFP ester; the Alexa Fluor® 488 kits for labeling nucleic acids and oligonucleotides contain the Alexa Fluor® 488 succinimidyl ester. †† Human vision is insensitive to light beyond ~650 nm, and therefore it is not possible to view the far-red– and near-infrared–fluorescent dyes by looking through the eyepiece of a conventional fluorescence microscope. The Alexa Fluor® Labeling Kits are described in detail in Section 1.2. (APEX®) = APEX® Antibody Labeling Kit. (ARES™) = ARES™ DNA Labeling Kit. (FT) = FISH Tag™ DNA Kit or FISH Tag™ RNA Kit. (G IgG) = Zenon® Goat IgG Labeling Kit. (H IgG) = Zenon® Human IgG Labeling Kit. (M IgG) = Zenon® Mouse IgG Labeling Kit. (Mab) = Monoclonal Antibody Labeling Kit. (Micro) = Alexa Fluor® Microscale Protein Labeling Kit. (Oligo) = Alexa Fluor® Oligonucleotide Amine Labeling Kit. (P) = Easy-to-Use Protein Labeling Kit. (R IgG) = Zenon® Rabbit IgG Labeling Kit. (SAIVI™) = SAIVI™ Antibody Labeling Kit. (SDP) = Sulfodichlorophenol ester. (TFP) = Tetrafluorophenyl ester. (TSA™) = Enhanced with TSA™ technology. (ULYSIS®) = ULYSIS® Nucleic Acid Labeling Kit. (X) = An aminohexanoyl spacer between the dye and the SE. More information on Alexa Fluor® labeling products is available at www.invitrogen.com/handbook/reactivealexa.

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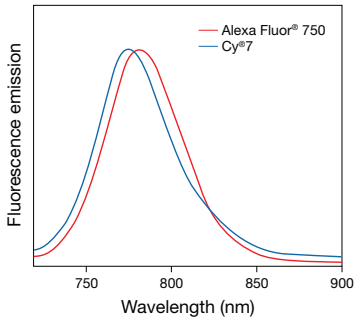

Figure 1.3.10 Comparison of the fluorescence emission spectra of Alexa Fluor® 750 and Cy®7 dyes. Spectra have been normalized to the same intensity for comparison purposes.

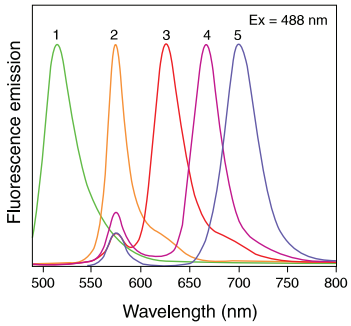

Figure 1.3.11 Normalized fluorescence emission spectra of 1) Alexa Fluor® 488 goat anti–mouse IgG antibody (A11001), 2) R-phycoerythrin goat anti–mouse IgG antibody (P852), 3) Alexa Fluor® 610–R-phycoerythrin goat anti–mouse IgG antibody (A20980), 4) Alexa Fluor® 647–R-phycoerythrin goat anti–mouse IgG antibody (A20990) and 5) Alexa Fluor® 680–R-phycoerythrin goat anti–mouse IgG antibody (A20983). The tandem conjugates permit simultaneous multicolor labeling and detection of up to five targets with excitation by a single excitation source—the 488 nm spectral line of the argon-ion laser.

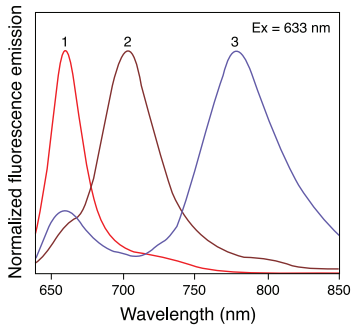

Figure 1.3.12 Normalized fluorescence emission spectra of 1) allophycocyanin goat anti–mouse IgG antibody (A865), 2) Alexa Fluor® 680–allophycocyanin goat anti–mouse IgG antibody (A21000) and 3) Alexa Fluor® 750–allophycocyanin goat anti–mouse IgG antibody (A21006). The tandem conjugates permit simultaneous multicolor labeling and detection of up to three targets with excitation by a single excitation source—the 633 nm spectral line of the He-Ne laser.

Table 1.4 Alexa Fluor® active esters and kits for labeling proteins and nucleic acids—*continued*.

Alexa Fluor® Dye	Fluorescence Color (Abs/Em) *	Succinimidyl Ester, TFP Ester or SDP Ester	Kits for Labeling Proteins			Kits for Labeling Nucleic Acids and Oligonucleotides
			Protein Labeling Kits	Antibody Labeling Kits	Zenon® Antibody Labeling Kits	
Alexa Fluor® 594	Red (590/617)	A20004 ‡ § A20104 † §	A10239 (P) A30008 (Micro)	A10474 (APEX®) A20185 (Mab)	Z25007 (M IgG_1) Z25107 (M IgG_{2a}) Z25207 (M IgG_{2b}) Z25307 (R IgG) Z25607 (G IgG) Z25407 (H IgG)	U21654 (ULYSIS®) A21669 (ARES™) F32949 (FT) F32954 (FT)
Alexa Fluor® 610	Red (612/628)	A30050 (X) ‡				
Alexa Fluor® 633 ††	Far-red (632/647)	A20005 ‡ § A20105 † §	A20170 (P)			
Alexa Fluor® 647 ††	Far-red (650/668)	A20006 ‡ A20106 †	A20173 (P) A30009 (Micro)	A10475 (APEX®) A20186 (Mab) S30044 (SAIVI™)	Z25008 (M IgG_1) Z25108 (M IgG_{2a}) Z25208 (M IgG_{2b}) Z25308 (R IgG) Z25608 (G IgG) Z25408 (H IgG)	U21660 (ULYSIS®) A21676 (ARES™) F32950 (FT) F32955 (FT) A20196 (Oligo)
Alexa Fluor® 660 ††	Near-infrared (663/690)	A20007 ‡	A20171 (P)		Z25009 (M IgG_1)	U21656 (ULYSIS®)
Alexa Fluor® 680 ††	Near-infrared (679/702)	A20008 ‡ A20108 †	A20172 (P)	S30045 (SAIVI™)	Z25010 (M IgG_1) Z25110 (M IgG_{2a}) Z25210 (M IgG_{2b}) Z25310 (R IgG)	
Alexa Fluor® 700 ††	Near-infrared (702/723)	A20010 ‡ A20110 †			Z25011 (M IgG_1)	
Alexa Fluor® 750 ††	Near-infrared (749/775)	A20011 ‡ A20111 †		S30046 (SAIVI™)	Z25312 (R IgG)	
Alexa Fluor® 790 ††	Near-infrared (782/805)	A30051				

* Approximate absorption (Abs) and fluorescence emission (Em) maxima for conjugates, in nm. † 5 mg unit size. ‡ 1 mg unit size. § Mixed isomers. ** The APEX® Alexa Fluor® 488 Antibody Labeling Kits contain the Alexa Fluor® 488 carboxylic acid SDP ester, whereas the other Alexa Fluor® 488 protein labeling kits contain the amine-reactive Alexa Fluor® 488 carboxylic acid TFP ester; the Alexa Fluor® 488 kits for labeling nucleic acids and oligonucleotides contain the Alexa Fluor® 488 succinimidyl ester. †† Human vision is insensitive to light beyond ~650 nm, and therefore it is not possible to view the far-red– and near-infrared–fluorescent dyes by looking through the eyepiece of a conventional fluorescence microscope. The Alexa Fluor® Labeling Kits are described in detail in Section 1.2. (APEX®) = APEX® Antibody Labeling Kit. (ARES™) = ARES™ DNA Labeling Kit. (FT) = FISH Tag™ DNA Kit or FISH Tag™ RNA Kit. (G IgG) = Zenon® Goat IgG Labeling Kit. (H IgG) = Zenon® Human IgG Labeling Kit. (M IgG) = Zenon® Mouse IgG Labeling Kit. (Mab) = Monoclonal Antibody Labeling Kit. (Micro) = Alexa Fluor® Microscale Protein Labeling Kit. (Oligo) = Alexa Fluor® Oligonucleotide Amine Labeling Kit. (P) = Easy-to-Use Protein Labeling Kit. (R IgG) = Zenon® Rabbit IgG Labeling Kit. (SAIVI™) = SAIVI™ Antibody Labeling Kit. (SDP) = Sulfodichlorophenol ester. (TFP) = Tetrafluorophenyl ester. (TSA™) = Enhanced with TSA™ technology. (ULYSIS®) = ULYSIS® Nucleic Acid Labeling Kit. (X) = An aminohexanoyl spacer between the dye and the SE. More information on Alexa Fluor® labeling products is available at www.invitrogen.com/handbook/reactivealexa.

Green-Fluorescent Alexa Fluor® Dyes

Alexa Fluor® 488 Dye: A Superior Fluorescein Substitute

Based on our testing, publications[2,5–7] and results reported by customers, Alexa Fluor® 488 dye is by far the best fluorescein (FITC or FAM) substitute available for most applications (The Alexa Fluor® Dye Series—Note 1.1). It is probably the best dye available for single-molecule detection of bioconjugates, for fluorescence correlation spectroscopy (Fluorescence Correlation Spectroscopy (FCS)—Note 1.3) and for fluorescence polarization measurements[8] (Fluorescence Polarization (FP)—Note 1.4).

This green-fluorescent dye exhibits several unique features:

- Fluorescence spectra almost identical to those of fluorescein, with excitation/emission maxima of 495/519 nm (Figure 1.3.4) and a fluorescence lifetime of ~4.1 nanoseconds (Table 1.5)
- Strong absorption, with an extinction coefficient greater than 65,000 $cm^{-1}M^{-1}$
- Much greater photostability than fluorescein (Figure 1.3.13), allowing more time for observation and image capture (Figure 1.3.14)
- pH-insensitive fluorescence between pH 4 and 10 (Figure 1.3.15)
- Water solubility, with no organic co-solvents required in labeling reactions, suggesting that the succinimidyl ester of Alexa Fluor® 488 carboxylic acid (A20000, A20100) may be the ideal reagent for labeling amines of exposed cell-surface proteins of live cells[9]
- Superior fluorescence output per protein conjugate, surpassing that of any other spectrally similar fluorophore-labeled protein, including fluorescein conjugates (Figure 1.3.16) and Cy®2 conjugates of antibodies (Figure 1.3.17)

NOTE 1.1

The Alexa Fluor® Dye Series

The Alexa Fluor® dyes—a series of superior fluorescent dyes that span the visible spectrum—represent a major breakthrough in the development of fluorescent labeling reagents.[1,2] Benefits of the Alexa Fluor® dyes and their conjugates include:

Brightness—Alexa Fluor® conjugates exhibit more intense fluorescence than other spectrally similar conjugates.

Photostability—Alexa Fluor® conjugates are more photostable than most other fluorescent conjugates, allowing more time for image capture.

Instrument compatibility—Absorption spectra of the Alexa Fluor® conjugates are matched to the principal output wavelengths of common excitation sources.

Color selection—Alexa Fluor® conjugates are available in several distinct fluorescent colors, ranging from blue to red to near-infrared (Figure 1, Table 1).

pH insensitivity—Alexa Fluor® dyes remain highly fluorescent over a broad pH range.

Water solubility—Alexa Fluor® reactive dyes have good water solubility, so protein conjugations can be performed without organic solvents, and the conjugates are relatively resistant to precipitation during storage.

Conventional fluorophores and their conjugates can often be replaced with spectrally similar Alexa Fluor® dyes and conjugates without affecting optical filter choices or other instrumentation considerations (Table 2). Each of the Alexa Fluor® dyes are listed in Table 1 and described in detail in Section 1.3 and in the accompanying Section 1.3 data table.

1. J Histochem Cytochem (2003) 51:1699; **2**. J Histochem Cytochem (1999) 47:1179.

Table 1 Spectral properties of Molecular Probes® Alexa Fluor® dyes.

Alexa Fluor® Dye	Absorption Max (nm) *	Emission Max (nm) *	Emission Color †	Extinction Coefficient ‡
Alexa Fluor® 350	346	442	Blue	19,000
Alexa Fluor® 405	402	421	Blue	35,000
Alexa Fluor® 430	434	539	Yellow-green	15,000
Alexa Fluor® 488	495	519	Green	73,000
Alexa Fluor® 514	518	540	Green	80,000
Alexa Fluor® 532	531	554	Yellow	81,000
Alexa Fluor® 546	556	573	Orange	112,000
Alexa Fluor® 555	555	565	Orange	155,000
Alexa Fluor® 568	578	603	Red-orange	88,000
Alexa Fluor® 594	590	617	Red	92,000
Alexa Fluor® 610	612	628	Red	144,000
Alexa Fluor® 633	632	647	Far-red	159,000
Alexa Fluor® 635	633	647	Far-red	140,000
Alexa Fluor® 647	650	668	Far-red	270,000
Alexa Fluor® 660	663	690	Near-IR §	132,000
Alexa Fluor® 680	679	702	Near-IR §	183,000
Alexa Fluor® 700	702	723	Near-IR §	205,000
Alexa Fluor® 750	749	775	Near-IR §	290,000
Alexa Fluor® 790	782	805	Near-IR §	260,000

* Approximate absorption and emission maxima, in nm, for conjugates. † Typical emission color seen through the eyepiece of a conventional fluorescence microscope with appropriate filters. ‡ Extinction coefficient of the reactive dye at emission maximum in $cm^{-1}M^{-1}$. § Human vision is insensitive to light beyond ~650 nm; it is not possible to directly view far-red– and near-IR–fluorescent dyes.

Table 2 Alexa Fluor® dye alternatives to several common fluorophores.

If you are using...	Try this Alexa Fluor® dye
Coumarin (AMCA)	Alexa Fluor® 350
Cy®2 or Fluorescein (FITC)	Alexa Fluor® 488
Cy®3 or Tetramethyl-rhodamine (TRITC)	Alexa Fluor® 555
Rhodamine Red™	Alexa Fluor® 568
Texas Red®	Alexa Fluor® 594
Cy®5	Alexa Fluor® 647
Cy®5.5	Alexa Fluor® 680
Cy®7	Alexa Fluor® 750

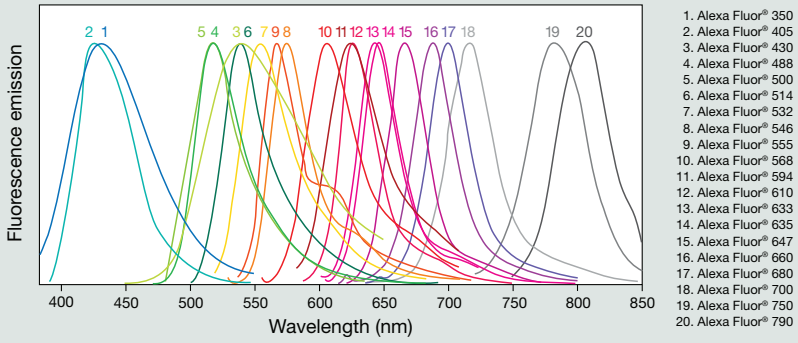

Figure 1 Emission spectra for the Alexa Fluor® dye series.

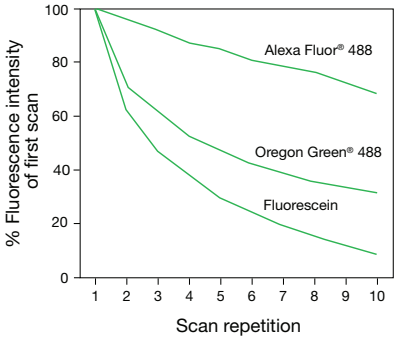

Figure 1.3.13 Photobleaching resistance of the green-fluorescent Alexa Fluor® 488, Oregon Green® 488 and fluorescein dyes, as determined by laser-scanning cytometry. EL4 cells were labeled with biotin-conjugated anti-CD44 antibody and detected by Alexa Fluor® 488 (S11223), Oregon Green® 488 (S6368) or fluorescein (S869) streptavidin. The cells were then fixed in 1% formaldehyde, washed and wet-mounted. After mounting, cells were scanned 10 times on a laser-scanning cytometer; laser power levels were 25 mW for the 488 nm spectral line of the argon-ion laser. Scan durations were approximately 5 minutes, and each repetition was started immediately after completion of the previous scan. Data are expressed as percentages derived from the mean fluorescence intensity (MFI) of each scan divided by the MFI of the first scan. Data contributed by Bill Telford, Experimental Transplantation and Immunology Branch, National Cancer Institute.

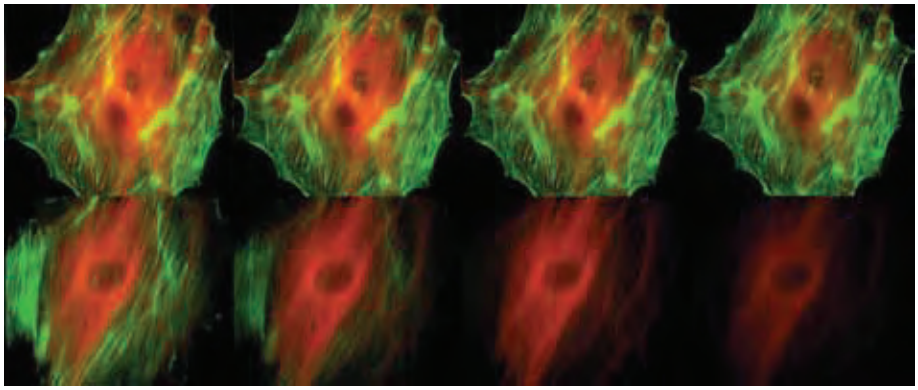

Figure 1.3.14 Comparison of the photobleaching rates of the Alexa Fluor® 488 and Alexa Fluor® 546 dyes and the well-known fluorescein and Cy®3 fluorophores. The cytoskeleton of bovine pulmonary artery endothelial cells (BPAEC) was labeled with (top series) Alexa Fluor® 488 phalloidin (A12379) and mouse monoclonal anti–α-tubulin antibody (A11126) in combination with Alexa Fluor® 546 goat anti–mouse IgG antibody (A11003) or (bottom series) fluorescein phalloidin (F432) and the anti–α-tubulin antibody in combination with a commercially available Cy®3 goat anti–mouse IgG antibody. The pseudocolored images were taken at 30-second intervals (0, 30, 90 and 210 seconds of exposure; left to right). The images were acquired with bandpass filter sets appropriate for fluorescein and rhodamine.

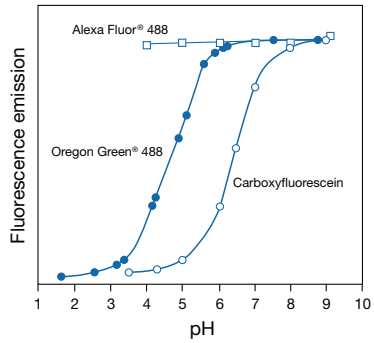

Figure 1.3.15 Comparison of pH-dependent fluorescence of the Oregon Green® 488 (●), carboxyfluorescein (○) and Alexa Fluor® 488 (□) fluorophores. Fluorescence intensities were measured for equal concentrations of the three dyes using excitation/emission at 490/520 nm.

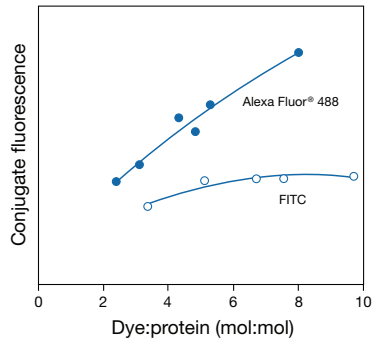

Figure 1.3.16 Comparison of the relative fluorescence of goat anti–mouse IgG antibody conjugates prepared from Alexa Fluor® 488 dye and from fluorescein isothiocyanate (FITC). Conjugate fluorescence is determined by measuring the fluorescence quantum yield of the conjugated dye relative to that of a reference dye and multiplying by the dye:protein labeling ratio.

Table 1.5 Fluorescence quantum yields (QY) and lifetimes (τ) for Alexa Fluor® dyes.

Alexa Fluor® Dye *	QY †	τ (ns) ‡
Alexa Fluor® 488	0.92	4.1 §
Alexa Fluor® 532	0.61	2.5
Alexa Fluor® 546	0.79	4.1
Alexa Fluor® 555	0.10	0.3
Alexa Fluor® 568	0.69	3.6 §
Alexa Fluor® 594	0.66	3.9 §
Alexa Fluor® 647	0.33	1.0
Alexa Fluor® 660	0.37	1.2 **
Alexa Fluor® 680	0.36	1.2
Alexa Fluor® 700	0.25	1.0
Alexa Fluor® 750	0.12	0.7

* Measurements were made on free succinimidyl ester derivatives in aqueous solutiuons. † For Alexa Fluor® 488, Alexa Fluor® 532, Alexa Fluor® 546, Alexa Fluor® 555, Alexa Fluor® 568, Alexa Fluor® 594 and Alexa Fluor® 647 dyes, QY measurements were made in PBS (50 mM potassium phosphate, 150 mM NaCl, pH 7.2) at 22°C relative to fluorescein in 0.01 M NaOH (QY = 0.92). For Alexa Fluor® 660, Alexa Fluor® 680, Alexa Fluor® 700 and Alexa Fluor® 750 dyes, QY measurements were made in PBS (50 mM potassium phosphate, 150 mM NaCl, pH 7.2) at 22°C relative to Alexa Fluor® 647 succinimidyl ester in PBS (QY = 0.33). ‡ Except for the footnoted values, lifetime measurements were made in water at 22°C, data provided by ISS Inc. (Champaign, IL). § Lifetime measurements were provided by the SPEX Fluorescence Group, Horiba Jobin Yvon, Inc. ** Lifetime measurement was made in pH 7.5 buffer at 20°C by Pierre-Alain Muller, Max Planck Institute for Biophysical Chemistry, Göttingen.

Table 1.6 R_0 values for some Alexa Fluor® dyes.

	Acceptor					
Donor	Alexa Fluor® 488	Alexa Fluor® 546	Alexa Fluor® 555	Alexa Fluor® 568	Alexa Fluor® 594	Alexa Fluor® 647
Alexa Fluor® 350	50					
Alexa Fluor® 488	NA	64	70	62	60	56
Alexa Fluor® 546		NA		70	71	74
Alexa Fluor® 555			NA		47	51
Alexa Fluor® 568				NA		82
Alexa Fluor® 594					NA	85
Alexa Fluor® 647						NA

R_0 values in angstroms (Å) represent the distance at which fluorescence resonance energy transfer from the donor dye to the acceptor dye is 50% efficient (Förster radius). Values were calculated from spectroscopic data as outlined (Fluorescence Resonance Energy Transfer (FRET)—Note 1.2). NA = Not applicable.

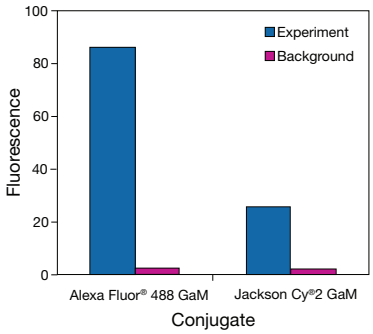

Figure 1.3.17 Brightness comparison of Molecular Probes® Alexa Fluor® 488 goat anti–mouse IgG antibody with Cy®2 goat anti–mouse IgG antibody from Jackson ImmunoResearch. Human blood was blocked with normal goat serum and incubated with an anti-CD3 mouse monoclonal antibody; cells were washed, resuspended and incubated with either Alexa Fluor® 488 or Cy®2 goat anti–mouse IgG antibody at equal concentration. Red blood cells were lysed, and the samples were analyzed with a flow cytometer equipped with a 488 nm argon-ion laser and a 525 ± 10 nm bandpass emission filter.

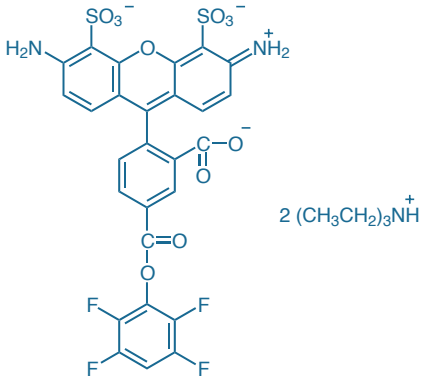

Figure 1.3.18 Alexa Fluor® 488 carboxylic acid, 2,3,5,6-tetrafluorophenyl ester (Alexa Fluor® 488 5-TFP, A30005).

The monosuccinimidyl ester of Alexa Fluor® 488 carboxylic acid is a mixture of two isomers and is available in a 1 mg or 5 mg unit size (A20000, A20100). The isomerically pure 5-isomer of Alexa Fluor® 488 dye is also available as the more hydrolytically stable tetrafluorophenyl (TFP) ester (A30005, Figure 1.3.18) and sulfodichlorophenol (SDP) ester (A30052, Figure 1.3.19).

TFP and SDP esters are an improvement over the succinimidyl ester (NHS ester or SE) chemistry typically used to attach fluorophores or haptens to the primary amines of biomolecules. All three reactive chemistries produce the same strong amide bond between the dye or hapten and the compound of interest (see reaction schemes in Section 1.1), but TFP and SDP esters are less susceptible to spontaneous hydrolysis during conjugation reactions. Both Alexa Fluor® 488 carboxylic

NOTE 1.2

Fluorescence Resonance Energy Transfer (FRET)

Fluorescence resonance energy transfer (FRET) is a distance-dependent interaction between the electronic excited states of two dye molecules in which excitation is transferred from a donor molecule to an acceptor molecule *without emission of a photon*. The efficiency of FRET is dependent on the inverse sixth power of the intermolecular separation,[1] making it useful over distances comparable to the dimensions of biological macromolecules. Thus, FRET is an important technique for investigating a variety of biological phenomena that produce changes in molecular proximity.[2–11] When FRET is used as a contrast mechanism, colocalization of proteins and other molecules can be imaged with spatial resolution beyond the limits of conventional optical microscopy.[12,13]

Primary Conditions for FRET

- Donor and acceptor molecules must be in close proximity (typically 10–100 Å).
- The absorption spectrum of the acceptor must overlap the fluorescence emission spectrum of the donor (Figure 1).
- Donor and acceptor transition dipole orientations must be approximately parallel.

Förster Radius

The distance at which energy transfer is 50% efficient (i.e., 50% of excited donors are deactivated by FRET) is defined by the Förster radius (R_0). The magnitude of R_0 is dependent on the spectral properties of the donor and acceptor dyes (Table 1):

$$R_o = [8.8 \times 10^{23} \cdot \kappa^2 \cdot n^{-4} \, QY_D \cdot J(\lambda)]^{1/6} \text{ Å}$$

where κ^2 = dipole orientation factor (range 0–4; $\kappa^2 = 2/3$ for randomly oriented donors and acceptors)
QY_D = fluorescence quantum yield of the donor in the absence of the acceptor
n = refractive index
$J(\lambda)$ = spectral overlap integral (see figure) $= \int \varepsilon_A(\lambda) \cdot F_D(\lambda) \cdot \lambda^4 d\lambda \text{ cm}^3\text{M}^{-1}$

where ε_A = extinction coefficient of acceptor
F_D = fluorescence emission intensity of donor as a fraction of the total integrated intensity

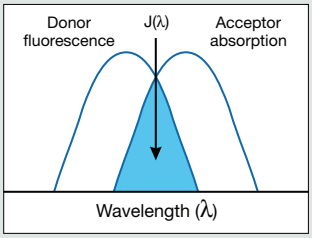

Figure 1 Schematic representation of the FRET spectral overlap integral.

Table 1 Typical Values of R_0.

Donor	Acceptor	R_0 (Å)
Fluorescein	Tetramethylrhodamine	55
IAEDANS	Fluorescein	46
EDANS	Dabcyl	33
Fluorescein	Fluorescein	44
BODIPY® FL	BODIPY® FL	57
Fluorescein	QSY® 7 and QSY® 9 dyes	61

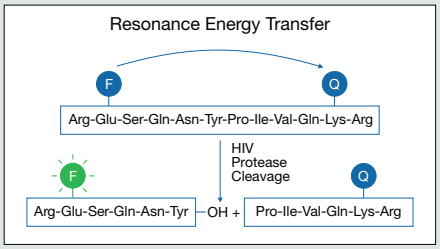

Figure 2 Principle of the fluorogenic response to protease cleavage exhibited by HIV protease substrate 1 (H2930). Quenching of the EDANS fluorophore (**F**) by distance-dependent resonance energy transfer to the dabcyl quencher (**Q**) is eliminated upon cleavage of the intervening peptide linker.

acid TFP ester and Alexa Fluor® 488 SDP ester are stable for several hours at the basic pH typically used for reactions—far outlasting succinimidyl esters. Alexa Fluor® 488 carboxylic acid TFP ester is the amine-reactive dye included in most of our labeling kits for proteins, nucleic acids and oligonucleotides (Section 1.2; Table 1.2, Table 1.4), including the Alexa Fluor® 488 Microscale Protein Labeling Kit (A30006), the Alexa Fluor® 488 Monoclonal Antibody Labeling Kit (A20181), the Alexa Fluor® 488 Protein Labeling Kit (A10235), the ARES™ Alexa Fluor® 488 DNA Labeling Kit (A21665) and the Alexa Fluor® 488 Oligonucleotide Amine Labeling Kit (A20191). Alexa Fluor® 488 carboxylic acid SDP ester is the amine-reactive dye included in the APEX® Alexa Fluor® 488 Antibody Labeling Kit (A10468).

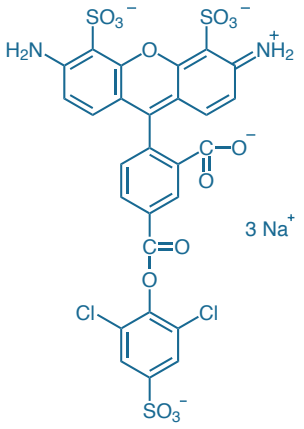

Figure 1.3.19 Alexa Fluor® 488 5-SDP ester (Alexa Fluor® 488 sulfodichlorophenol ester, A30052).

Alexa Fluor® 514 Dye: A Perfect Match to the Argon-Ion Laser

Like our Alexa Fluor® 488 dye, Alexa Fluor® 514 dye (excitation/emission maxima ~518/540 nm) is superior to fluorescein in both brightness and photostability and can be detected with standard fluorescein, Oregon Green® dye or Alexa Fluor® 488 dye filter sets. However, Alexa Fluor® 514 dye is spectrally distinguishable from Alexa Fluor® 488 dye and other green fluorophores using spectral imaging instruments with linear-unmixing software.[10,11] Alexa Fluor® 514 dye is one the brightest and most photostable dyes available for excitation by the 514 nm spectral line of the argon-ion laser. Alexa Fluor® 514 dye is available as a succinimidyl ester (A30002) and as antibody (Section 7.2, Table 7.1) and streptavidin (Section 7.6, Table 7.9) conjugates.

Donor/Acceptor Pairs

In most applications, the donor and acceptor dyes are different, in which case FRET can be detected by the appearance of sensitized fluorescence of the acceptor or by quenching of donor fluorescence. When the donor and acceptor are the same, FRET can be detected by the resulting fluorescence depolarization.[14] Typical values of R_0 for some dye pairs are listed in Table 1 and more extensive compilations are in Table 1.6 and Table 1.11. Note that because the component factors of R_0 are dependent on the environment, the actual value observed in a specific experimental situation is somewhat variable. Extensive compilations of R_0 values can be found in the literature.[3,8,11] Nonfluorescent acceptors such as dabcyl and QSY® dyes (Table 1.10) have the particular advantage of eliminating the potential problem of background fluorescence resulting from direct (i.e., nonsensitized) acceptor excitation. FRET efficiencies from several donor dyes to the QSY® 7 quencher in molecular beacon hybridization probes have been calculated.[15] Probes incorporating fluorescent donor–nonfluorescent acceptor combinations have been developed primarily for detecting proteolysis[16] (Figure 2) and nucleic acid hybridization.

Selected Applications of FRET

- Structure and conformation of proteins [17–22]
- Spatial distribution and assembly of protein complexes [23–27]
- Receptor/ligand interactions [28–32]
- Immunoassays [33,34]
- Probing interactions of single molecules [35]
- Structure and conformation of nucleic acids [36–41]
- Real-time PCR assays and SNP detection [42–47]
- Detection of nucleic acid hybridization [48–53]
- Primer-extension assays for detecting mutations [54]
- Automated DNA sequencing [55–57]
- Distribution and transport of lipids [58–60]
- Membrane fusion assays [61–64] (Lipid-Mixing Assays of Membrane Fusion—Note 13.1)
- Membrane potential sensing [65]
- Fluorogenic protease substrates [16,66–69]
- Indicators for cyclic AMP [70,71] and zinc [72]

1. Proc Natl Acad Sci U S A (1967) 58:719; **2**. Biophys J (2003) 84:3992; **3**. Resonance Energy Transfer: Theory and Data, Van der Meer BW, et al. (1994) p. 133; **4**. J Struct Biol (1995) 115:175; **5**. Photochem Photobiol (1983) 38:487; **6**. Annu Rev Biochem (1978) 47:819; **7**. Methods Enzymol (1995) 246:300; **8**. Anal Biochem (1994) 218:1; **9**. Methods Enzymol (1978) 48:347; **10**. Scanning (1995) 17:72; **11**. J Muscle Res Cell Motil (1987) 8:97; **12**. Methods (2001) 24:289; **13**. Biophys J (1998) 74:2702; **14**. Biophys J (1995) 69:1569; **15**. Nucleic Acids Res (2002) 30:e122; **16**. Science (1990) 247:954; **17**. Biophys J (1998) 74:3111; **18**. Biochemistry (1996) 35:4795; **19**. Biochemistry (1995) 34:8693; **20**. Biochemistry (1995) 34:6475; **21**. J Biol Chem (1998) 273:9119; **22**. J Biol Chem (1993) 268:15588; **23**. Biochemistry (1995) 34:7904; **24**. Biochemistry (1994) 33:13102; **25**. Biochemistry (1994) 33:5539; **26**. J Photochem Photobiol B (1992) 12:323; **27**. J Biol Chem (1989) 264:8699; **28**. J Recept Signal Transduct Res (2002) 22:333; **29**. Biochemistry (1994) 33:11875; **30**. J Cell Physiol (1994) 159:176; **31**. Biophys J (1991) 60:307; **32**. J Biol Chem (1984) 259:5717; **33**. Anal Biochem (1988) 174:101; **34**. Anal Biochem (1980) 108:156; **35**. Proc Natl Acad Sci U S A (1996) 93:6264; **36**. Biochemistry (1998) 37:2979; **37**. Biochemistry (1998) 37:8173; **38**. Anal Biochem (1994) 221:306; **39**. Biophys J (1994) 66:99; **40**. Nucleic Acids Res (1994) 22:920; **41**. Science (1994) 266:785; **42**. Nucleic Acids Res (2000) 28:3752; **43**. Nat Biotechnol (1999) 17:804; **44**. Biotechniques (1999) 27:342; **45**. Genome Res (1996) 6:986; **46**. Nucleic Acids Res (1997) 25:2516; **47**. Genome Res (2001) 11:163; **48**. Nat Biotechnol (1996) 14:303; **49**. Nat Biotechnol (1998) 16:49; **50**. Biochemistry (1995) 34:285; **51**. Nucleic Acids Res (1994) 22:662; **52**. Nonisotopic DNA Probe Techniques, Kricka LR, Ed. (1992) p. 311; **53**. Proc Natl Acad Sci U S A (1988) 85:8790; **54**. Proc Natl Acad Sci U S A (1997) 94:10756; **55**. Anal Biochem (1998) 255:32; **56**. Anal Chem (1995) 67:3676; **57**. Proc Natl Acad Sci U S A (1995) 92:4347; **58**. Biochemistry (1995) 34:4846; **59**. Biochemistry (1992) 31:2865; **60**. J Biol Chem (1983) 258:5368; **61**. Biochemistry (1998) 37:2361; **62**. Biochim Biophys Acta (1994) 1189:175; **63**. Methods Enzymol (1993) 221:239; **64**. Biochemistry (1981) 20:4093; **65**. Biophys J (1995) 69:1272; **66**. FEBS Lett (1997) 413:379; **67**. Techniques in Protein Chemistry V, Crabb JW, Ed. (1994) p. 493; **68**. Biochemistry (1998) 37:11434; **69**. Bioconjug Chem (1993) 4:537; **70**. Nature (1991) 349:694; **71**. Fluorescent and Luminescent Probes for Biological Activity, Mason WT, Ed. (1993) p. 133; **72**. J Am Chem Soc (1996) 118:6514.

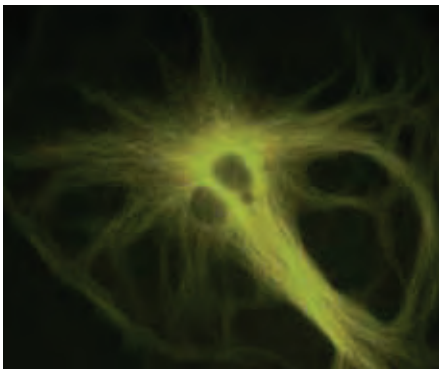

Figure 1.3.20 A bovine pulmonary artery endothelial (BPAE) cell labeled with mouse monoclonal anti–α-tubulin antibody (A11126) in combination with Alexa Fluor® 430 goat anti–mouse IgG antibody (A11063) to stain microtubules. The image was acquired using a longpass filter set allowing excitation at 455 ± 35 nm and emission at wavelengths greater than 515 nm.

Alexa Fluor® 430 Dye: Filling the Spectral Gap Between Green and Yellow

Few reactive dyes that absorb between 400 nm and 450 nm have appreciable fluorescence beyond 500 nm in aqueous solution. Alexa Fluor® 430 dye fills this spectral gap (Figure 1.3.1, Figure 1.3.20). Excitation near its absorption maximum at 431 nm is accompanied by an extremely large Stokes shift and strong yellow-green fluorescence (emission maximum ~541 nm). The coumarin-based amine-reactive succinimidyl ester of Alexa Fluor® 430 carboxylic acid (A10169, Figure 1.3.21) is available, as well as Alexa Fluor® 430 conjugates of secondary antibodies (A11063, A11064; Section 7.2) and streptavidin (S11237, Section 7.6). Alexa Fluor® 430 dye–labeled Fab′ fragments are provided in the Zenon® Alexa Fluor® 430 Antibody Labeling Kits (Section 7.3, Table 7.7). We also offer the Alexa Fluor® 430 Protein Labeling Kit (A10171), which is described in detail in Section 1.2 (Table 1.2, Table 1.4).

Yellow-to-Red–Fluorescent Alexa Fluor® Dyes

As with the green-fluorescent Alexa Fluor® 488 dye, the yellow-, orange- and red-fluorescent Alexa Fluor® dyes exhibit several features that distinguish them from spectrally similar fluorophores:

- Strong absorption, with extinction coefficients greater than 80,000 $cm^{-1}M^{-1}$ for Alexa Fluor® 532, Alexa Fluor® 546, Alexa Fluor® 568 and Alexa Fluor® 594 dyes and greater than 130,000 $cm^{-1}M^{-1}$ for Alexa Fluor® 555 and Alexa Fluor® 610 dyes

NOTE 1.3

Fluorescence Correlation Spectroscopy (FCS)

Fluorescence correlation spectroscopy (FCS) is a technique in which spontaneous fluorescence intensity fluctuations are measured in a microscopic detection volume of about 10^{-15} L (1 femtoliter) defined by a tightly focused laser beam.[1–3] Renewed interest in FCS in recent years has been stimulated by the fact that it is inherently miniaturized and therefore applicable for high-throughput screening applications.[4] Fluorescence intensity fluctuations measured by FCS represent changes in either the number or the fluorescence quantum yield of molecules resident in the detection volume (Figure 1). Small, rapidly diffusing molecules produce rapidly fluctuating intensity patterns, whereas larger molecules produce more sustained bursts of fluorescence.

This situation is in marked contrast to conventional fluorescence photometry carried out in sample volumes of around 0.1–1.0 mL (~10^8 times larger than FCS measurement volumes) that report only the macroscopic average of diffusion-dependent intensity fluctuations. In a typical FCS measurement, fluorescence intensity is recorded for a small number of molecules in the detection volume (e.g., 3 molecules/femtoliter, equivalent to ~5 nM macroscopic concentration) over a time range from about 1 microsecond to 1 second. The time-dependent fluorescence intensity (F(t)) is then analyzed in terms of its temporal autocorrelation function (G (τ)), which compares the fluorescence intensity at time t with the intensity at (t + τ), where τ is a

$$G(\tau) = \frac{\cdot \delta F(t) \sum \delta F(t+\tau) \grave{O}}{\cdot F(t) \grave{O}^2}$$

variable interval, averaged over all data points in the time series:

The autocorrelation function contains information about equilibrium concentrations, reaction kinetics and diffusion rates of molecules in the sample. The initial amplitude of the autocorrelation function is inversely proportional to the number of molecules in the detection volume. The autocorrelation function decays from its initial value with a time-dependence that is determined by molecular diffusion rates. For example, free fluorescent ligands exhibit faster autocorrelation decay than slower-moving complexed ligands (Figure 2).

Probes and Applications for FCS

FCS is applicable for monitoring a multitude of biomolecular association and dissociation processes (Table 1). Because FCS is intrinsically sensitive to the mass changes occurring in these processes, probe design and selection is generally less critical than it is in assays based on macroscopic fluorescence intensity changes generated by dye–dye interactions (FRET, self-quenching etc.) or environment-dependent fluorescence enhancement. Dyes that perform well in confocal laser-scanning microscopy are usually among the best choices for FCS applications. Laser sources used for excitation in FCS include the 488 nm argon-ion spectral line and the 543 nm and 633 nm He-Ne laser spectral lines. Dyes with appreciable rates of triplet state population via intersystem crossing are generally not well suited for FCS measurement because this process results in an additional submillisecond autocorrelation decay component.[5]

Technical Developments in FCS

Two-photon excitation (TPE) has been applied to FCS for reasons similar to those that have motivated its use in fluorescence microscopy—inherent spatial confinement of excitation, diminished photobleaching and phototoxicity, less scattering and better optical penetration in turbid media.[6,7] Dual-color cross-correlation FCS [8,9] measures the cross-correlation of the time-dependent fluorescence intensities of two spectrally distinct dyes, instead of the conventional autocorrelation for a single dye. This approach has the advantage that cross-correlated fluorescence is only generated by molecules or complexes labeled with both dyes, allowing quantitation of interacting molecules without reference to their diffusion characteristics. In practice, discrimination based on mass in conventional FCS requires that the interacting components should have a molecular weight ratio of at least 1:7. FCS measurements using TPE in combination with dual-color cross-correlation have been reported.[10]

- Fluorescence that is more photostable than that of other spectrally similar dyes, allowing more time for observation and image capture
- pH-insensitive fluorescence over a broad range
- Good water solubility, permitting labeling reactions to be performed without organic solvents
- Superior fluorescence output per protein or nucleic acid conjugate, surpassing that of any other spectrally similar fluorophore-labeled protein
- Long fluorescence lifetimes (approximately 4.1, 3.6 and 3.9 nanoseconds for Alexa Fluor® 546, Alexa Fluor® 568 and Alexa Fluor® 594 dyes, respectively (Table 1.5))

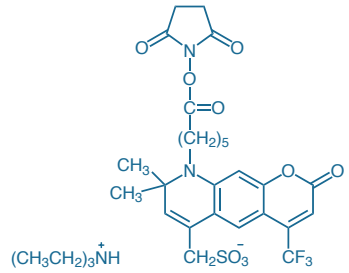

Figure 1.3.21 Alexa Fluor® 430 carboxylic acid, succinimidyl ester (A10169).

Alexa Fluor® 532 Dye: Optimal Dye for 532 nm Diode Lasers

The yellow-fluorescent Alexa Fluor® 532 dye (excitation/emission maxima ~532/554 nm) absorbs in the wavelength between the green-fluorescent Alexa Fluor® 488 dye and orange-fluorescent Alexa Fluor® 546 dye (Figure 1.3.2) and provides strong visible fluorescence that contrasts well with these dyes. Five of our Alexa Fluor® dyes—Alexa Fluor® 488, Alexa Fluor® 532, Alexa Fluor® 546, Alexa Fluor® 568 and Alexa Fluor® 594 dyes—have been utilized for simultaneous seven-color fluorescence imaging in tissue samples.[12] The use of Alexa Fluor® 532 dye–labeled actin (instead of the equivalent tetramethylrhodamine conjugate) has been reported to be crucial for studying the polarity of individual filaments during the formation of actin bundles by total internal reflection fluorescence (TIRF) microscopy.[13] The isomer-free, amine-reactive monosuccinimidyl ester of Alexa Fluor® 532 dye is available in either a 1 mg or 5 mg unit size (A20001, A20101MP) and as a

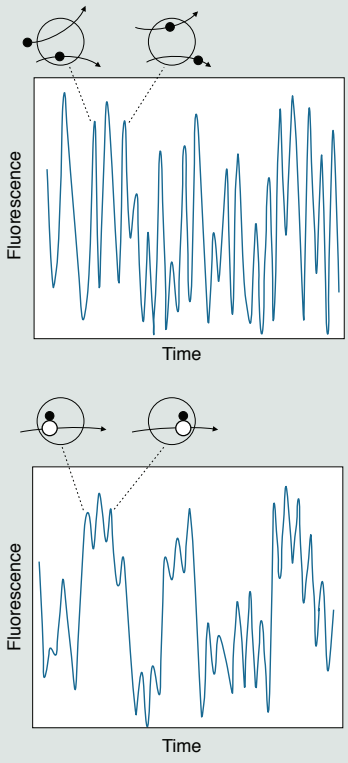

Figure 1 Physical origins of fluorescence correlation spectroscopy data. Free fluorescent ligands move in and out of the detection volume (open circle) and are detected as a series of short, randomized fluorescence bursts (top panel). Macromolecule-bound ligands are less mobile, producing a more slowly fluctuating (i.e., more highly autocorrelated) time-dependent fluorescence pattern (bottom panel).

Table 1 Applications of fluorescence correlation spectroscopy.

Detected Process	References
Nucleic acid fragmentation	Anal Biochem (1998) 260:166; Proc Natl Acad Sci U S A (1998) 95:1416; Proc Natl Acad Sci U S A (1998) 95:1421
Nucleic acid hybridization	Biochemistry (1996) 35:10182; Nucleic Acids Res (1995) 23:1795
PCR product formation	Biochemistry (1998) 37:12971; Biotechniques (1998) 25:706; Proc Natl Acad Sci U S A (1996) 93:12805
Lateral segregation of lipids in bilayer membranes	Cytometry (1999) 36:176; Proc Natl Acad Sci U S A (1999) 96:8461
Molecular diffusion in the nucleus and cytoplasm	Biophys J (1998) 75:2547; Proc Natl Acad Sci U S A (1998) 95:6043
Protein–protein interactions	Biochem Biophys Res Commun (2000) 267:300; Biochemistry (1999) 38:13759; Biochemistry (1999) 38:8402; Chem Biol (1999) 6:53; Cytometry (1999) 36:247; Biophys Chem (1998) 75:151
Binding equilibria for drugs and other low molecular weight ligands	Biochemistry (1999) 38:5082; Biochemistry (1999) 38:8671; J Biomol Screen (1999) 4:355; Biophys J (1997) 73:2195; Biophys Chem (1996) 58:3
Clustering of membrane-bound receptors	Biophys J (1996) 70:2001; Biophys J (1993) 65:1135; Chem Phys Lipids (1989) 50:253

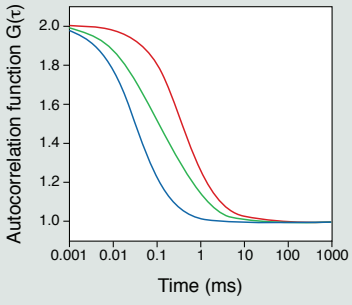

Figure 2 Simulated FCS autocorrelation functions representing a low molecular weight ligand (left curve, blue), macromolecule-bound ligand (right curve, red) and a 1:1 mixture of free and bound ligand (middle curve, green).

1. Biochemistry (2002) 41:697; **2**. Proc Natl Acad Sci U S A (1997) 94:11753; **3**. Ann Rev Biophys Biomol Struct (2007) 36:151; **4**. J Biomol Screen (1999) 4:335; **5**. J Phys Chem (1995) 99:13368; **6**. Biophys J (1999) 77:2251; **7**. Biophys J (1996) 71:410; **8**. Nat Methods (2006) 3:83; **9**. Nat Protoc (2007) 2:2842; **10**. Proc Natl Acad Sci U S A (2000) 97:10377.

NOTE 1.4

Fluorescence Polarization (FP)

Principles

Fluorescence polarization measurements provide information on molecular orientation and mobility and processes that modulate them, including receptor–ligand interactions, protein–DNA interactions, proteolysis, membrane fluidity and muscle contraction (Figure 1).

Because polarization is a general property of fluorescent molecules (with certain exceptions such as lanthanide chelates), polarization-based readouts are somewhat less dye dependent and less susceptible to environmental interferences such as pH changes than assays based on fluorescence intensity measurements. Experimentally, the degree of polarization is determined from measurements of fluorescence intensities parallel and perpendicular with respect to the plane of linearly polarized excitation light, and is expressed in terms of fluorescence polarization (P) or anisotropy (r):

$$P = \frac{(F_\parallel - F_\perp)}{(F_\parallel + F_\perp)} \qquad r = \frac{(F_\parallel - F_\perp)}{(F_\parallel + 2F_\perp)}$$

where $F_\parallel$ = fluorescence intensity parallel to the excitation plane
$F_\perp$ = fluorescence intensity perpendicular to the excitation plane

Note that both P and r are ratio quantities with no nominal dependence on dye concentration. Because of the ratio formulation, fluorescence intensity variations due to the presence of colored sample additives tend to cancel and produce relatively minor inteferences.[1] P has physically possible values ranging from –0.33 to 0.5. In practice, these limiting values are rarely attained. Measured values of P in bioanalytical applications typically range from 0.01 to 0.3 or 10 to 300 mP (mP = P/1000). This measurement range is not as narrow as it might appear to be because very precise measurements (P ± 0.002 or ± 2 mP) are readily obtainable with modern instrumentation.

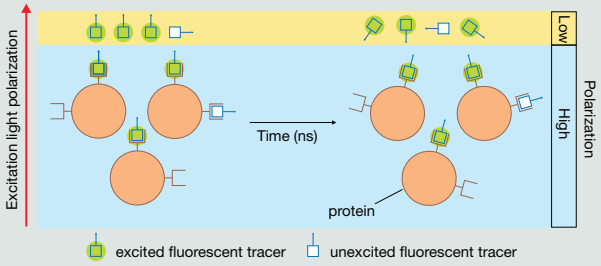

Figure 1 Physical basis of fluorescence polarization assays. Dye molecules with their absorption transition vectors (arrows) aligned parallel to the electric vector of linearly polarized light (along the vertical page axis) are selectively excited. For dyes attached to small, rapidly rotating molecules, the initially photoselected orientational distribution becomes randomized prior to emission, resulting in low fluorescence polarization. Conversely, binding of the low molecular weight tracer to a large, slowly rotating molecule results in high fluorescence polarization. Fluorescence polarization therefore provides a direct readout of the extent of tracer binding to proteins, nucleic acids and other biopolymers.

Dependence of Fluorescence Polarization on Molecular Mobility

Interpretation of the dependence of fluorescence polarization on molecular mobility is usually based on a model derived in 1926 from the physical theory of Brownian motion by Perrin:[2,3]

$$\left(\frac{1}{P} - \frac{1}{3}\right) = \left(\frac{1}{P_0} - \frac{1}{3}\right)\left(1 + \frac{\tau}{\phi}\right)$$

where P_o is the fundamental polarization of the dye (for fluorescein, rhodamine and BODIPY® dyes, P_o is close to the theoretical maximum of 0.5), τ is the excited-state lifetime of the dye and ϕ is the rotational correlation time of the dye or dye conjugate. These relationships can be expressed in terms of fluorescence anisotropy in an equivalent and mathematically simpler manner. For a hydrodynamic sphere, ϕ can be estimated as follows:

$$\phi = \frac{\eta V}{RT}$$

where η = solvent viscosity, T = temperature, R = gas constant and V = molecular volume of the fluorescent dye or dye conjugate. In turn, V can be estimated from the molecular weight of the dye or dye conjugate with appropriate adjustments for hydration. Simulations of these relationships are shown in Figure 2, leading to the following general conclusions:

- Fluorescence polarization increases as molecular weight increases.
- Fluorescence polarization increases as solvent viscosity increases.
- Fluorescence polarization decreases as the excited state lifetime of the dye (τ) increases.

Note that these simulations assume that the dye is rigidly attached to a spherical carrier. When conventional parameter estimates for proteins in aqueous solutions are used, ϕ is found to increase by about 1 ns per 2400 dalton increase of molecular weight.[4]

Dyes for Fluorescence Polarization Assays

Tracers used in fluorescence polarization assays include peptides, drugs and cytokines that are modified by the attachment of a fluorescent dye. Depolarization due to flexibility in the attachment of the dye, sometimes referred to as the "propeller effect," distorts the relationships between P and molecular weight shown in Figure 2. For this reason, it is generally preferable to use reactive dyes without aliphatic linkers between the fluorophore and the reactive group in the preparation of tracers for fluorescence polarization assays.[5,6]

A key factor in the performance of fluorescence polarization assays is the extent to which the biological activity of the tracer is perturbed by the dye modification. BODIPY® dyes generally produce less perturbation of receptor-binding affinity and other activity parameters than conventional dyes such as fluorescein and rhodamine.[7,8] Furthermore, BODIPY® dyes usually have

longer excited-state lifetimes than fluorescein and rhodamine dyes, making their fluorescence polarization sensitive to binding interactions over a larger molecular weight range (Figure 2). The long-wavelength BODIPY® TMR and BODIPY® TR dyes also tend to minimize assay interferences due to intrinsically fluorescent sample contaminants.[7]

Applications

Fluorescence polarization measurements have long been a valuable biophysical research tool for investigating processes such as membrane lipid mobility, myosin reorientation and protein–protein interactions at the molecular level.[9–12] Immunoassays that have been developed and used extensively for clinical diagnostics represent the largest group of bioanalytical applications.[13,14] The more recent advent of microplate readers equipped with polarizing optics has led to the adoption of fluorescence polarization as a readout mode for high-throughput screening.[15–17] Some typical bioanalytical applications of fluorescence polarization–based assays are summarized in Table 1.

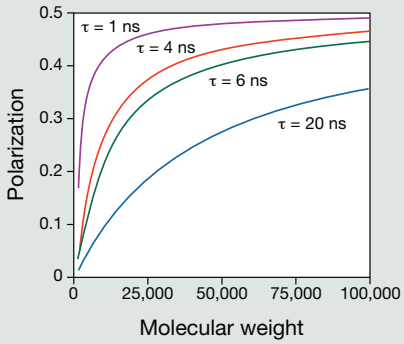

Figure 2 Simulation of the relationship between molecular weight (MW) and fluorescence polarization (P). Simulations are shown for dyes with various fluorescence lifetimes (τ): 1 ns (cyanine dyes) in purple, 4 ns (fluorescein and Alexa Fluor 488 dyes) in red, 6 ns (some BODIPY dyes) in green and 20 ns (dansyl dyes) in blue. At MW = 1000, P = 0.167 for τ = 1 ns, P = 0.056 for τ = 4 ns, P = 0.039 for τ = 6 ns and P = 0.012 for τ = 20 ns. Simulations assume P_o (the fundamental polarization) = 0.5 and rigid attachment of dyes to spherical carriers.

1. Anal Biochem (1997) 247:83; **2**. J Phys Radium (1926) 7:390; **3**. Fluorescence and Phosphorescence Analysis, Hercules DM, Ed. (1966) p. 217; **4**. Biophysical Chemistry, Part 2, Cantor CR, Schimmel PR, Eds (1980) p. 454; **5**. Anal Biochem (1997) 249:29; **6**. Anal Biochem (1997) 247:77; **7**. J Biomol Screen (2000) 5:329; **8**. Endocrinology (1997) 138:296; **9**. Methods (1999) 19:222; **10**. Biophys J (1996) 71:3330; **11**. Chem Phys Lipids (1993) 64:99; **12**. Methods Enzymol (1995) 246:283; **13**. Immunochemistry (1970) 7:799; **14**. Immunochemistry (1973) 10:219; **15**. J Biomol Screen (2000) 5:297; **16**. High Throughput Screening: The Discovery of Bioactive Substances, Devlin J, Ed. (1997) p. 389; **17**. J Biomol Screen (2001) 6:275.

Table 1 Examples of fluorescence polarization[endash]based assays.

Assay Target	Tracer	References
Ligand binding to neurokinin 1 (NK1) receptor	Fluorescein-labeled substance P	Biochemistry (1994) 33:13079
Ligand binding to melanocortin G-protein–coupled receptors	BODIPY® TMR dye–labeled NDP-αMSH	J Biomol Screen (2000) 5:329
Ligand binding to B2 bradykinin receptor, a G-protein–coupled receptor	BODIPY® TMR dye–labeled HOE140	J Biomol Screen (2002) 7:111
Ligand binding to estrogen receptors	Fluorescein-labeled estradiol	J Biomol Screen (2000) 5:77
Ligand binding to tyrosine kinase Src homology domains	Fluorescein- and BODIPY® TR dye–labeled phosphopeptides	Anal Biochem (1999) 275:62; Anal Biochem (1997) 247:77
Substrate binding to protein farnesyltransferase	Oregon Green® 488 dye–labeled peptide	Biochemistry (1999) 38:13138
β-Lactam antibiotic binding to penicillin-binding proteins	BODIPY® FL dye–labeled penicillin V	Antimicrob Agents Chemother (1999) 43:1124
Protein kinase activity	Fluorescently labeled phosphopeptide	Anal Biochem (2000) 278:206; Methods (2000) 22:61
Nonspecific protease activity	BODIPY® FL dye–labeled casein	Anal Biochem (1996) 243:1
Detection of specific PCR products	Fluorescein-labeled oligonucleotide	Gene (2000) 259:123
Ligation and cleavage of RNA by ribozymes	Fluorescein- or tetramethylrhodamine-labeled oligoribonucleotide	Biotechniques (2000) 29:344
SNP detection by allele-specific primer extension	Fluorescent ddNTP	Genome Res (1999) 9:492
Protein–protein and protein–nucleic acid interactions	Alexa Fluor® 488 dye–labeled human Factor VIIa, Oregon Green® 488 dye–labeled soluble human tissue factor and Oregon Green® 514 dye–labeled oligonucleotide	Anal Biochem (2002) 308:18
Oligomerization and fibril formation of α-synuclein	Oregon Green® 488 dye– and Alexa Fluor® 594 dye–labeled α-synuclein	Biochemistry (2007) 46:12522

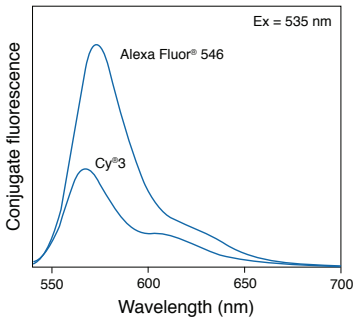

Figure 1.3.22 Fluorescence output from an Alexa Fluor® 546 goat anti–mouse IgG antibody (dye:protein ratio = 5.7) and a commercially available Cy®3 goat anti–mouse IgG antibody (dye:protein ratio = 3.8). Antibody concentrations were adjusted to give equal absorbance at the excitation wavelength (535 nm). The relative fluorescence quantum yield of Alexa Fluor® 546 conjugates is higher than that of Cy®3 conjugates, even at high dye:protein ratios that would typically result in self-quenching effects with most other protein-labeling dyes.

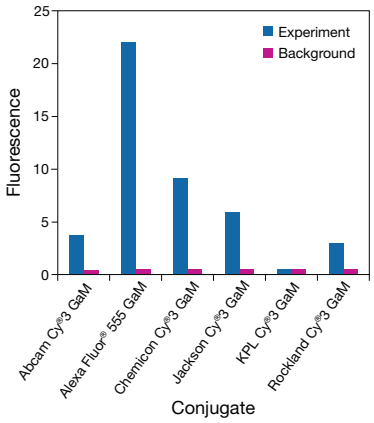

Figure 1.3.23 Brightness comparison of Alexa Fluor® 555 goat anti–mouse IgG antibody with commercially available Cy®3 goat anti–mouse IgG antibody conjugates. Human blood was blocked with normal goat serum and incubated with a mouse monoclonal anti-CD3 antibody; cells were washed, resuspended and incubated with either Alexa Fluor® 555 or Cy®3 goat anti–mouse IgG antibody at equal concentrations. Red blood cells were lysed and the samples were analyzed with a flow cytometer equipped with a 488 nm argon-ion laser and a 585 ± 21 nm bandpass emission filter.

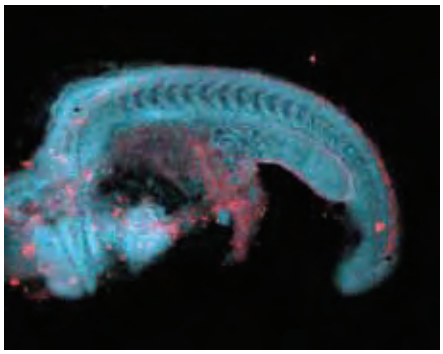

Figure 1.3.24 Neuronal cells in a 22-hour zebrafish embryo were identified with anti–HuC/HuD mouse monoclonal antibody (A21271) and visualized with red-orange–fluorescent Alexa Fluor® 568 goat anti–mouse IgG antibody (A11004). The nuclei were stained with blue-fluorescent DAPI (D1306, D3571, D21490).

component of several labeling kits (Table 1.2, Table 1.4). The contents and utility of these protein and nucleic acid labeling kits are discussed in detail in Section 1.2.

Alexa Fluor® 546 and Alexa Fluor® 555 Dyes: Superior Alternatives to Cy®3 and Tetramethylrhodamine

The orange-fluorescent Alexa Fluor® 546 (excitation/emission maxima ~556/573 nm) and Alexa Fluor® 555 (excitation/emission maxima ~555/565 nm) dyes have spectra that are similar to tetramethylrhodamine and the Cy®3 dye. The spectra of Alexa Fluor® 555 dye are an almost exact match to those of the Cy®3 dye (Figure 1.3.5), and therefore optical filters designed for Cy®3 dye also work with Alexa Fluor® 555 dye. Conjugates of Alexa Fluor® 546 and Alexa Fluor® 555 dyes typically outperfom tetramethylrhodamine (TRITC and TAMRA) and Cy®3 conjugates (Figure 1.3.22, Figure 1.3.23), and Alexa Fluor® 555 conjugates are more fluorescent at a higher degree of substitution (DOS) than are Cy®3 conjugates (Figure 1.3.6). Alexa Fluor® 555 dye is also more photostable than Cy®3 dye (Figure 1.3.7), providing more time for image capture.

We have observed that, unlike most other Alexa Fluor® dyes, antifade reagents provide little protective effect for conjugates of Alexa Fluor® 546 dye; if photobleaching is a limitation, the spectrally similar Alexa Fluor® 555 dye should be used in place of Alexa Fluor® 546 dye. The isomeric mixture of the amine-reactive monosuccinimidyl ester of Alexa Fluor® 546 dye (A20002, A20102) and the isomer-free monosuccinimidyl ester of Alexa Fluor® 555 dye (A20009, A20109) are available in either a 1 mg or 5 mg unit size and as components of several labeling kits (Table 1.2, Table 1.4). The contents and utility of these protein and nucleic acid labeling kits are discussed in detail in Section 1.2.

Alexa Fluor® 568 Dye: A Perfect Match to 561 nm Diode Lasers

The red-orange–fluorescent Alexa Fluor® 568 dye (excitation/emission maxima ~578/603 nm, Figure 1.3.24) is optimally excited by the 561 nm diode lasers used in many confocal laser-scanning microscopes. Although Alexa Fluor® 568 conjugates exhibit absorption and fluorescence emission maxima similar to those of Lissamine rhodamine B conjugates, they are considerably brighter. The isomeric mixture of the amine-reactive monosuccinimidyl ester of Alexa Fluor® 568 dye is available in either a 1 mg or 5 mg unit size (A20003, A20103) and as a component of several labeling kits (Table 1.2, Table 1.4). The contents and utility of these protein and nucleic acid labeling kits are discussed in detail in Section 1.2.

Alexa Fluor® 594 and Alexa Fluor® 610 Dyes: Brighter Red-Fluorescent Dyes

The red-fluorescent Alexa Fluor® 594 dye (excitation/emission maxima ~590/617 nm) has absorption and fluorescence emission maxima similar to those of Texas Red® dye, making it particularly useful for multilabeling experiments in combination with green-fluorescent probes. Alexa Fluor® 594 conjugates are brighter than similarly labeled Texas Red® conjugates, can be labeled to a higher degree of substitution (DOS) (Figure 1.3.25), and are efficiently excited by 561 nm diode lasers and the 594 nm line of the orange He-Ne laser.

The bright and photostable Alexa Fluor® 610 dye (excitation/emission maxima ~612/628 nm) emits an intense red fluorescence that is easily distinguished from green fluorescence and can be visualized with the same optics used for Texas Red® and Alexa Fluor® 594 dyes. Unlike the fluorescence of Alexa Fluor® 633 dye and longer-wavelength fluorophores, Alexa Fluor® 610 fluorescence can still be seen with the human eye.

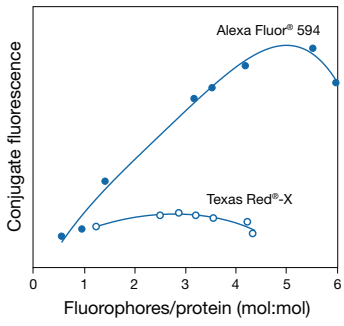

Figure 1.3.25 Comparison of the relative fluorescence of Alexa Fluor® 594 and Texas Red®-X goat anti–mouse IgG antibody F(ab')$_2$ fragment conjugates at different dye:protein ratios.

The isomeric mixture of the amine-reactive monosuccinimidyl ester of Alexa Fluor® 594 dye is available in either a 1 mg or 5 mg unit size (A20004, A20104) and the 6-isomer of the monosuccinimidyl ester of Alexa Fluor® 610-X dye is available in a 1 mg unit size (A30050). These red-fluorescent Alexa Fluor® dyes are also available as components of several labeling kits (Table 1.2, Table 1.4); the contents and utility of these protein and nucleic acid labeling kits are discussed in detail in Section 1.2.

Far-Red– and Near-Infrared–Fluorescent Alexa Fluor® Dyes

One of our long-term goals has been to develop superior dyes that can be excited by long-wavelength excitation sources, including the red He-Ne laser (at 633 nm), krypton-ion laser (at 647 nm) and diode lasers. Long-wavelength excitation generally provides increased optical penetration of tissues and decreased autofluorescence background, both key enabling prerequisites for *in vivo* imaging applications.[14–16] It has been particularly challenging to prepare long-wavelength reactive dyes whose fluorescence is not significantly quenched upon conjugation. Our far-red– and near-infrared–fluorescent Alexa Fluor® dyes (Figure 1.3.3) meet our goals in several ways:[1,17]

- Very high extinction coefficients—typically >165,000 $cm^{-1}M^{-1}$ but up to 290,000 $cm^{-1}M^{-1}$ for Alexa Fluor® 750 dye
- Excellent spectral match to common long-wavelength excitation sources
- Spectra of Alexa Fluor® 647, Alexa Fluor® 680 and Alexa Fluor® 750 conjugates that virtually match those of the Cy®5 dye (Figure 1.3.8), Cy®5.5 dye (Figure 1.3.9) and Cy®7 dye (Figure 1.3.10), respectively, resulting in an optimal match to optical filters designed for these dyes
- Photostability of Alexa Fluor® 633 and Alexa Fluor® 647 conjugates that exceeds that of Cy®5, allophycocyanin and PBXL-3 conjugates (Figure 1.3.26)
- Unusually low fluorescence quenching upon conjugation to proteins, even at relatively high degrees of substitution (Figure 1.3.27), resulting in protein conjugates that are typically at least three to four times brighter than those of Cy®5, Cy®5.5, Cy®7 and similar dyes [18,19] but that are, in some cases, as much as 40-fold brighter at equal antibody concentrations (Figure 1.3.28, Figure 1.3.29)
- Fluorescence of the nucleotide, oligonucleotide and nucleic acid conjugates of Alexa Fluor® 647 dye that usually exceeds that of the Cy®5 dye conjugates (Section 8.2)

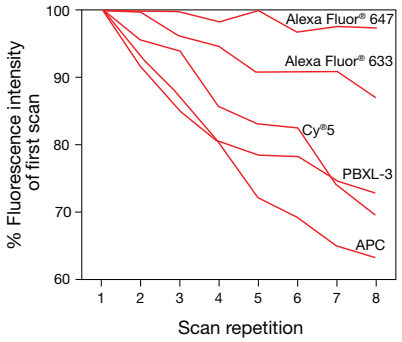

Figure 1.3.26 Photobleaching resistance of the far-red–fluorescent Alexa Fluor® 647, Alexa Fluor® 633, PBXL-3 and Cy®5 dyes and the allophycocyanin fluorescent protein, as determined by laser-scanning cytometry. EL4 cells were labeled with biotin-conjugated anti-CD44 antibody and detected by Alexa Fluor® 647 (S21374), Alexa Fluor® 633 (S21375), PBXL-3, Cy®5 or allophycocyanin (APC, S868) streptavidin. The cells were then fixed in 1% formaldehyde, washed and wet-mounted. After mounting, cells were scanned eight times on a laser-scanning cytometer; laser power levels were 18 mW for the 633 nm spectral line of the He-Ne laser. Scan durations were approximately 5 minutes apiece, and each repetition was started immediately after completion of the previous scan. Data are expressed as percentages derived from the mean fluorescence intensity (MFI) of each scan divided by the MFI of the first scan. Data contributed by Bill Telford, Experimental Transplantation and Immunology Branch, National Cancer Institute.

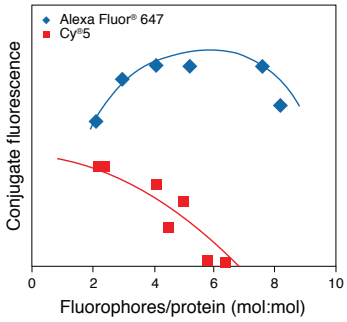

Figure 1.3.27 Comparison of the brightness of Alexa Fluor® 647 and Cy®5 dye antibody conjugates (prepared in our laboratories). More Alexa Fluor® 647 dye molecules can be attached to proteins and nucleic acids without significant quenching, thus yielding conjugates that are much brighter than those possible using the Cy®5 dye.

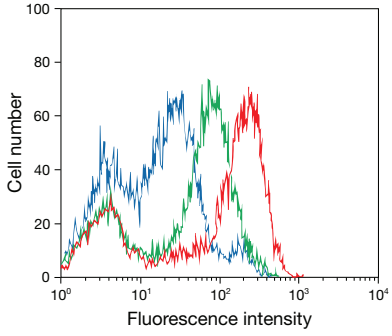

Figure 1.3.28 Flow cytometry was used to compare the brightness of Molecular Probes® Alexa Fluor® 647 goat anti–mouse IgG antibody (red, A21235) with commercially available Cy®5 goat anti–mouse IgG antibody from Jackson ImmunoResearch Laboratories (green) and Amersham® Biosciences Biotech (blue). Human blood was blocked with normal goat serum and incubated with an anti-CD3 mouse monoclonal antibody; cells were washed, resuspended and incubated with either an Alexa Fluor® 647 or Cy®5 goat anti–mouse IgG secondary antibody at equal concentration. Red blood cells were lysed and the samples were analyzed on a flow cytometer equipped with a 633 nm He-Ne laser and a longpass emission filter (>650 nm).

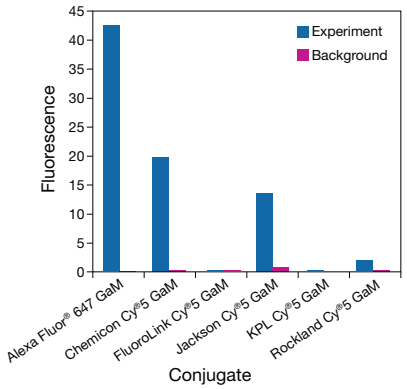

Figure 1.3.29 Brightness comparison of Molecular Probes® Alexa Fluor® 647 goat anti–mouse IgG antibody with Cy®5 goat anti–mouse IgG antibody conjugates commercially available from other companies. Human blood was blocked with normal goat serum and incubated with an anti-CD3 mouse monoclonal antibody; cells were washed, resuspended and incubated with either Alexa Fluor® 647 or Cy®5 goat anti–mouse IgG antibody at an equal concentration. Red blood cells were lysed and the samples were analyzed with a flow cytometer equipped with a 633 nm He-Ne laser and a longpass emission filter (>650 nm).

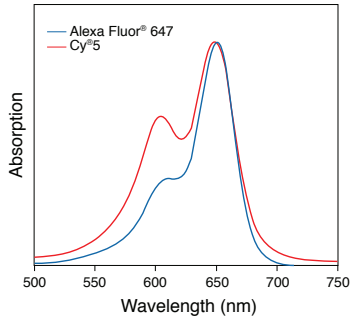

Figure 1.3.30 The absorption spectra of Cy®5 dye conjugates of both proteins and nucleic acids show an additional peak at about 600 nm when compared to the spectrum of the free dye. However, the light absorbed by Cy®5 conjugates at this wavelength does not result in fluroescence. Alexa Fluor® 647 protein conjugates do not exhibit this spectral anomaly. Spectra have been normalized to the same peak intensity for comparison purposes.

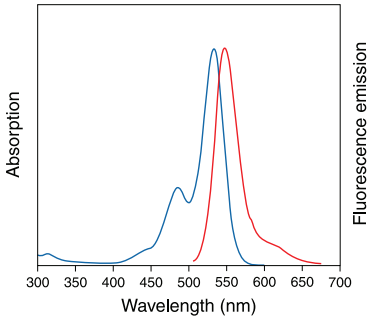

Figure 1.3.31 Absorption and fluorescence emission spectra of Alexa Fluor® 635 goat anti–mouse IgG antibody in pH 7.2 buffer.

- Unlike the Cy®5 dye, very little change in absorption or fluorescence spectra when conjugated to most proteins, oligonucleotides and nucleic acids (Figure 1.3.30), thus yielding significantly greater total fluorescence at the same degrees of substitution (Figure 1.3.27, Figure 1.3.28, Figure 1.3.29)
- Reasonable water solubility of their succinimidyl esters, permitting conjugations to be done without addition of organic solvents if desired
- Chemistry that permits synthesis of pure, monofunctional reactive dyes, thus avoiding crosslinking reactions

The lifetimes and fluorescence quantum yields of far-red and near-infrared Alexa Fluor® dyes are generally shorter and lower, respectively, than the their counterparts in the green-orange-red visible wavelength (488–594 nm) excitation range (Table 1.5). However, the lower quantum yields are compensated by larger extinction coefficients in terms of total fluorescence output. Fluorescence of these long-wavelength Alexa Fluor® dyes is not visible to the human eye but is readily detected by most imaging systems. Pictures of these dyes throughout *The Molecular Probes® Handbook* have been pseudocolored to represent the staining that is observed with sensitive detection equipment.

Alexa Fluor® 633 and Alexa Fluor® 635 Dyes: Optimal Excitation with the He-Ne Laser

These far-red–fluorescent Alexa Fluor® dyes are important labels for fluorescence imaging because their spectra are beyond the range of most sample autofluorescence. With an excitation maximum of 633 nm and 635 nm, respectively, Alexa Fluor® 633 and Alexa Fluor® 635 dyes are a perfect match to the 633 nm spectral line of the He-Ne laser and the 635 nm spectral line of red diode lasers. Although their fluorescence is not visible to the human eye, Alexa Fluor® 633 and Alexa Fluor® 635 conjugates are bright and photostable (Figure 1.3.26), with peak emission at 647 nm.

An isomeric mixture of the amine-reactive succinimidyl ester of Alexa Fluor® 633 dye is available as a stand-alone reagent in either a 1 mg or 5 mg unit size (A20005, A20105) and as a component of several labeling kits (Table 1.2, Table 1.4), which are described in detail in Section 1.2. Alexa Fluor® 635 dye, which is currently only available as antibody (Table 7.1, Figure 1.3.31) and streptavidin (Table 7.9) conjugates, typically produces brighter protein conjugates than does Alexa Fluor® 633 dye because it is less susceptible to self-quenching.

Alexa Fluor® 647 Dye: A Superior Alternative to Cy®5 Dye

Spectra of Alexa Fluor® 647 conjugates are virtually identical to those of the Cy®5 dye (Figure 1.3.8), resulting in an optimal match to optical filters designed for that dye. Total fluorescence of Alexa Fluor® 647 secondary antibody conjugates, however, is significantly higher that that of Cy®5 conjugates commercially available from other suppliers (Figure 1.3.27, Figure 1.3.28, Figure 1.3.29). Also, unlike Cy®5 dye, Alexa Fluor® 647 dye has very little change in absorption or fluorescence spectra upon conjugation to most proteins and nucleic acids (Figure 1.3.30), thus yielding greater total fluorescence at the same degree of substitution.[1] The spectral characteristics of thirteen different red-fluorescent fluorophores, including Alexa Fluor® 647 and BODIPY® 630/660 (Section 1.4) dyes, have been evaluated in different surrounding media to assess the influence of polarity, viscosity and detergent concentration and to facilitate probe choice in fluorescence-based assays.[17] The monosuccinimidyl ester of Alexa Fluor® 647 dye is available as a stand-alone reagent in either a 1 mg or 5 mg unit size (A20006, A20106) and as a component of several labeling kits (Table 1.2, Table 1.4), which are described in detail in Section 1.2.

Alexa Fluor® 660 Dye: A Match for the Krypton-Ion Laser

Alexa Fluor® 660 is optimally excited by the 647 nm spectral line of the krypton-ion laser and well excited by the 633 nm spectral line of the He-Ne laser. Protein conjugates of Alexa Fluor® 660 dye produce bright near-infrared fluorescence, with a peak at 690 nm. This long-wavelength emission is well separated from that of other fluorophores, including Alexa Fluor® 546 and Cy®3 dyes and phycoerythrin conjugates. Alexa Fluor® 660 dye is also the dye of choice as a second label for use with allophycocyanin (APC) conjugates in flow cytometry applications. The monosuccinimidyl ester of Alexa Fluor® 660 dye is available as a 1 mg stand-alone reagent (A20007) and as a component of several labeling kits (Table 1.2, Table 1.4), which are described in detail in Section 1.2.

Alexa Fluor® 680 Dye: An Alternative to the Cy®5.5 Dye

With a peak excitation at 679 nm and maximum emission at 702 nm, Alexa Fluor® 680 dye is spectrally similar to the Cy®5.5 dye[14] (Figure 1.3.9). Fluorescence emission of Alexa Fluor® 680 dye is well separated from that of other commonly used red fluorophores, such as the tetramethylrhodamine, Texas Red®, R-phycoerythrin, Alexa Fluor® 594 and Alexa Fluor® 647 dyes, making it ideal for three- and four-color labeling. The monosuccinimidyl ester of Alexa Fluor® 680 dye is available as a stand-alone reagent in either a 1 mg or 5 mg unit size (A20008, A20108) and as a component of several labeling kits (Table 1.2, Table 1.4), which are described in detail in Section 1.2.

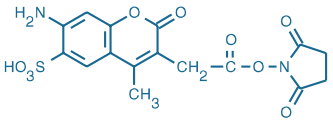

Figure 1.3.32 A10168 Alexa Fluor® 350 carboxylic acid, succinimidyl ester

Alexa Fluor® 700, Alexa Fluor® 750 and Alexa Fluor® 790 Dyes: Our Longest-Wavelength Dyes

With an absorption maximum at 702 nm, Alexa Fluor® 700 dye can be excited with a xenon-arc lamp, far-red diode lasers or dye-pumped lasers operating in the 675–700 nm range. Alexa Fluor® 700 dye provides near-infrared fluorescence emission, with a peak at 723 nm. Alexa Fluor® 750 dye exhibits fluorescence spectra that are very similar to those of Cy®7 dye (Figure 1.3.10). Its fluorescence emission maximum at 775 nm is well separated from commonly used far-red fluorophores such as Alexa Fluor® 647, Alexa Fluor® 660 or allophycocyanin (APC), facilitating multicolor analysis. With a peak excitation at 749 nm, conjugates of Alexa Fluor® 750 dye are well excited by a xenon-arc lamp or dye-pumped lasers operating in the 720–750 nm range. The monosuccinimidyl esters of Alexa Fluor® 700 (A20010, A20110) and Alexa Fluor® 750 (A20011, A20111) dyes are available as stand-alone reagents in either a 1 mg or 5 mg unit size, and as components of labeling kit (Table 1.2, Table 1.4), which are described in detail in Section 1.2.

Alexa Fluor® 790 dye is the longest-wavelength Alexa Fluor® dye available. With excitation/emission maxima of 784/814 nm, Alexa Fluor® 790 dye has spectral properties similar to those of indocyanine green (ICG) and IRDye® 800 dyes (LI-COR Biosciences). This fluorophore is especially useful for researchers who require an amine-reactive, near-infrared label for small animal *in vivo* imaging (SAIVI) applications, as well as for multicolor analysis with Alexa Fluor® 680 dye and the LI-COR Odyssey® infrared imaging system.[20,21] The monosuccinimidyl ester of Alexa Fluor® 790 dye is supplied in a 100 µg unit size (A30051), enough to label ~1 mg of IgG antibody.

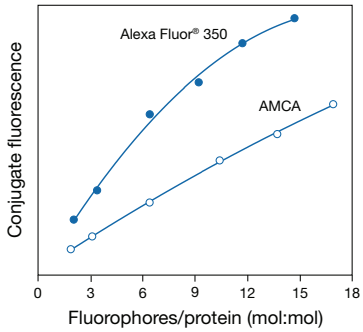

Figure 1.3.33 Comparison of the relative fluorescence of 7-amino-4-methylcoumarin-3-acetic acid (AMCA) streptavidin (○) and Alexa Fluor® 350 streptavidin, a sulfonated AMCA derivative (S11249, ●). Conjugate fluorescence is determined by measuring the fluorescence quantum yield of the conjugated dye relative to that of the free dye and multiplying by the number of fluorophores per protein.

Blue-Fluorescent Alexa Fluor® Dyes

Because their structures are closely related to those of the coumarins and pyrenes, the blue-fluorescent Alexa Fluor® 350 and Alexa Fluor® 405 dyes, as well as the yellow-green–fluorescent Alexa Fluor® 430 dye described above, are also included in Section 1.7. We summarize their properties here to complete our discussion of the Alexa Fluor® dye series.

Alexa Fluor® 350 Dye: Brighter Blue Fluorescence

The blue-fluorescent Alexa Fluor® 350 carboxylic acid succinimidyl ester (A10168, Figure 1.3.32) is a sulfonated coumarin derivative that is more water soluble than either AMCA succinimidyl ester or AMCA-X succinimidyl ester (A6118, Section 1.7) and yields protein conjugates that are typically 50% more fluorescent than those prepared from its nonsulfonated analog (Figure 1.3.33). Alexa Fluor® 350 protein conjugates are optimally excited at 346 nm and exhibit bright blue fluorescence at wavelengths slightly shorter than AMCA or AMCA-X conjugates (442 nm versus 448 nm), which reduces the dye's spectral overlap with the emission of fluorescein. We also prepare Alexa Fluor® 350 conjugates of secondary antibodies (Section 7.2, Table 7.1) and streptavidin (Section 7.6, Table 7.9), as well as several Alexa Fluor® 350 protein labeling kits, which are described in detail in Section 1.2 (Table 1.2, Table 1.4).

Alexa Fluor® 405 Dye: Near-Perfect Match to the Violet Diode Laser

With excitation/emission maxima of 402/421 nm (Figure 1.3.1), the blue-fluorescent Alexa Fluor® 405 dye is well matched to the 405 nm spectral line of violet diode lasers now widely implemented in fluorescence microscopy and flow cytometry. Alexa Fluor® 405 succinimidyl ester is an amine-reactive derivative of our Cascade Blue® dye, which was previously available in amine-reactive form only as its acetyl azide (C2284, Section 1.7). Not only is it offered at higher purity than the alternative Cascade Blue® acetyl azide, but Alexa Fluor® 405 succinimidyl ester also contains a 4-piperidinecarboxylic acid spacer that separates the fluorophore from its reactive moiety

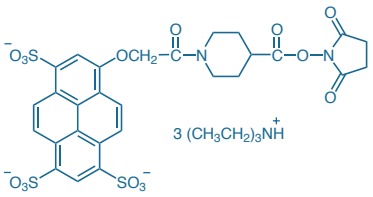

Figure 1.3.34 Alexa Fluor® 405 carboxylic acid, succinimidyl ester (A30000).

(Figure 1.3.34). This spacer enhances the reactivity of the succinimidyl ester and minimizes any interactions between the fluorophore and the biomolecule to which it is conjugated.

As with Cascade Blue® acetyl azide, Alexa Fluor® 405 dye shows minimal spectral overlap with green fluorophores, making it ideal for multicolor applications.[3,22] Alexa Fluor® 405 dye is available as a succinimidyl ester (A30000, A30100) and a cadaverine derivative (A30675, Section 3.4), as well as conjugated to secondary antibodies (Section 7.2, Table 7.1) and streptavidin (Section 7.6, Table 7.9).

Alexa Fluor® 405 dye is also recognized by our anti–Alexa Fluor® 405/Cascade Blue® dye antibody (A5760, Section 7.4). In addition, Alexa Fluor® 405 dye–labeled Fab′ fragments are provided in the Zenon® Alexa Fluor® 405 Antibody Labeling Kits (Section 7.3, Table 7.7).

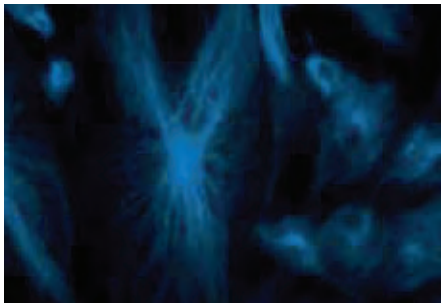

Figure 1.3.36 Fixed and permeabilized bovine pulmonary artery endothelial (BPAE) cells labeled with mouse monoclonal anti–α-tubulin antibody (A11126) and detected using TSA™ Kit #7 with the HRP conjugate of goat anti–mouse IgG antibody and Alexa Fluor® 350 tyramide (T20912).

Alexa Fluor® Protein and Nucleic Acid Labeling Kits

Alexa Fluor® Labeling Kits

As described above, all of our Alexa Fluor® dyes are available as amine-reactive succinimidyl esters (Table 1.2, Table 1.4), and the Alexa Fluor® 488 dye is additionally available as its single-isomer, hydrolysis-resistant tetrafluorophenyl (TFP) ester (A30005). Most of these amine-reactive Alexa Fluor® dyes are also offered as components of several protein and nucleic acid labeling kits (Table 1.2), which are described thoroughly in Section 1.2 and include:

- APEX® Antibody Labeling Kits
- Microscale Protein Labeling Kits
- Monoclonal Antibody Labeling Kits
- SAIVI™ Rapid Antibody Labeling Kits
- Alexa Fluor® Protein Labeling Kits
- Zenon® Antibody Labeling Kits (Section 7.3, Table 7.7)
- ARES™ DNA Labeling Kits (Section 8.2)
- FISH Tag™ DNA Kits and FISH Tag™ RNA Kits (Section 8.2)
- Alexa Fluor® Oligonucleotide Amine Labeling Kits (Section 8.2, Table 8.6)
- ULYSIS® Nucleic Acid Labeling Kits (Section 8.2, Table 8.6)

The purity of the Alexa Fluor® carboxylic acid succinimidyl esters dyes when prepared and packaged in a 5 mg unit size is usually >80–95% by HPLC. However, Alexa Fluor® dyes tenaciously bind water, and packaging of these products in smaller unit sizes—such as the multiple vials used in the Alexa Fluor® labeling kits and the 1 mg stand-alone reagents—may result in some loss of reactivity. Our specifications for Alexa Fluor® carboxylic acid succinimidyl esters provided as kit components or as stand-alone reagents require that the product has ≥50% reactivity after packaging.

Alexa Fluor® Decapacks for Labeling Amine-Modified DNA or RNA

For labeling amine-modified DNA or RNA probes in microarray-based experiments, we offer the Alexa Fluor® 488 reactive dye decapack (A32750), Alexa Fluor® 555 reactive dye decapack

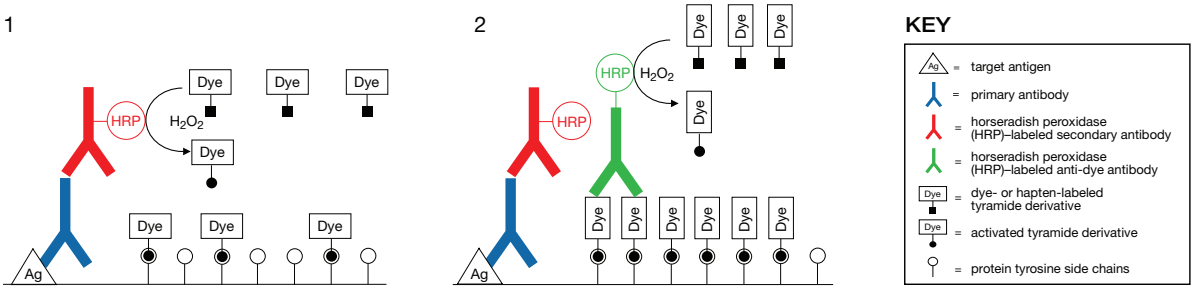

Figure 1.3.35 Schematic representation of TSA™ detection methods applied to immunolabeling of an antigen. The antigen is detected by a primary antibody, followed by a horseradish peroxidase–labeled secondary antibody in conjunction with a dye-labeled (or hapten-labeled) tyramide, resulting in localized deposition of the activated tyramide derivative (Stage 1). Further dye deposition, and therefore higher levels of signal amplification, can be generated by detecting dye deposited in Stage 1 with a horseradish peroxidase–labeled anti-dye antibody in conjunction with a dye-labeled tyramide (Stage 2).

(A32756) and Alexa Fluor® 647 reactive dye decapack (A32757), which provide the corresponding Alexa Fluor® succinimidyl ester conveniently packaged in 10 single-use vials. These specially packaged amine-reactive Alexa Fluor® dyes can be used in conjunction with the aminoallyl dUTP (A21664, Section 8.2) nucleotide[23] or with commercially available aminoallyl nucleotide–based nucleic acid labeling kits.

Each single-use vial contains sufficient Alexa Fluor® succinimidyl ester to optimally label the amount of cDNA produced from reverse transcription of either 20 µg of total RNA or 1–5 µg of poly(A)+ RNA, in the presence of aminoallyl dUTP. For added convenience, we offer a combination set of the Alexa Fluor® 555 and Alexa Fluor® 647 reactive dye decapacks (A32755) that contains 10 vials of each succinimidyl ester and is sufficient for 10 two-color labeling reactions. The Alexa Fluor® 555/Alexa Fluor® 647 dye pair has been shown to display higher signal correlation coefficients than the Cy®3/Cy®5 dye pair in two-color DNA microarray assays.[24–26]

Other Reactive Alexa Fluor® Derivatives

Several Alexa Fluor® dyes are also available as thiol-reactive maleimides (Section 2.2, Table 2.1), as alkynes and azides for bioorthogonal "click" reactions (Section 3.1) and as aldehyde- and ketone-reactive hydrazides and hydroxylamines (Section 3.3, Table 3.2). The Alexa Fluor® hydrazides and hydroxylamines are also important probes for intracellular tracing (Section 14.3). Although some of the Alexa Fluor® dyes are mixtures of two isomers, all the reactive Alexa Fluor® dyes have only a single reactive substituent linked to each fluorophore.

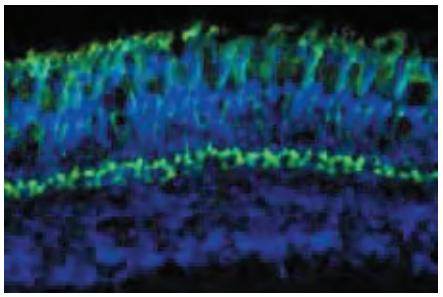

Figure 1.3.37 A zebrafish retina cryosection labeled with the mouse monoclonal antibody FRet 43 and detected using TSA™ Kit #2 with the HRP conjugate of goat anti–mouse IgG antibody and green-fluorescent Alexa Fluor® 488 tyramide (T20912). The nuclei were counterstained with blue-fluorescent Hoechst 33258 (H1398, H3569, H21491).

Alexa Fluor® Tandem Conjugates and Other Bioconjugates

Alexa Fluor® Dye–Phycobiliprotein Tandem Conjugates

We have conjugated R-phycoerythrin with Alexa Fluor® 610, Alexa Fluor® 647 or Alexa Fluor® 680 dye—and in turn conjugated these fluorescent proteins to antibodies or streptavidin, yielding tandem conjugates that permit simultaneous multicolor labeling and detection of multiple targets using a single excitation source (the 488 nm spectral line of the argon-ion laser) and monitoring emission at 628 nm, 668 nm or 702 nm, respectively[3,4] (Section 6.4, Figure 1.3.11). Additionally, we have conjugated allophycocyanin to Alexa Fluor® 680, Alexa Fluor® 700 or Alexa Fluor® 750 dyes to create tandem conjugates for multicolor measurements using excitation sources in the 633 to 650 nm range (Figure 1.3.12). Zenon® Antibody Labeling Kits for the rapid and quantitative labeling of antibodies with the phycobiliprotein tandem dyes are also available (Section 7.3, Table 7.7).

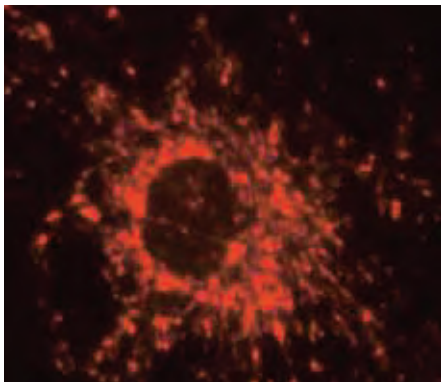

Figure 1.3.38 Fixed and permeabilized bovine pulmonary artery endothelial cell (BPAEC) labeled with anti–OxPhos Complex IV subunit I (human) antibody (anti–cytochrome oxidase subunit I) and detected using TSA™ Kit #4 with the HRP conjugate of goat anti–mouse IgG antibody and Alexa Fluor® 568 tyramide (T20914).

Other Alexa Fluor® Bioconjugates

For immunofluorescence, receptor labeling, nucleic acid synthesis, cell tracing and many other applications, we offer a wide variety of Alexa Fluor® conjugates, including labeled antibodies (Section 7.2, Table 7.1), streptavidin (Section 7.6, Table 7.9) and many other proteins, ligands and nucleotides.

Signal Amplification with Alexa Fluor® Dyes

Tyramide Signal Amplification

Tyramide signal amplification (TSA™) technology, which was developed by NEN (now a part of PerkinElmer Corporation) and licensed for in-cell and in-tissue applications, permits significant amplification of cellular targets by a horseradish peroxidase (HRP)–mediated scheme (Figure 1.3.35). We have introduced several TSA™ Kits (Section 6.2, Table 6.1), including kits that utilize one of the following Alexa Fluor® tyramides:

- Alexa Fluor® 350 tyramide (Figure 1.3.36)
- Alexa Fluor® 488 tyramide (Figure 1.3.37)
- Alexa Fluor® 546 tyramide
- Alexa Fluor® 555 tyramide
- Alexa Fluor® 568 tyramide (Figure 1.3.38)
- Alexa Fluor® 594 tyramide
- Alexa Fluor® 647 tyramide (Figure 1.3.39)

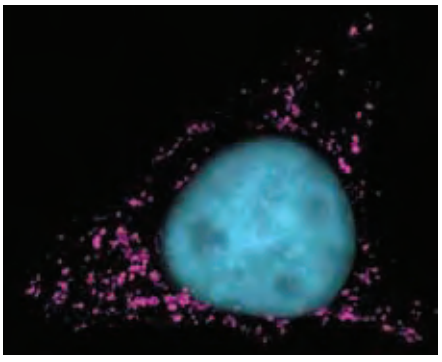

Figure 1.3.39 Fixed and permeabilized bovine pulmonary artery endothelial cell (BPAEC) labeled with anti–OxPhos Complex IV subunit I (human) antibody (anti–cytochrome oxidase subunit I) and detected using TSA™ Kit #6 with the HRP conjugate of goat anti–mouse IgG antibody and Alexa Fluor® 647 tyramide (T20916). The image was deconvolved using Huygens software (Scientific Volume Imaging, http://www.svi.nl/).

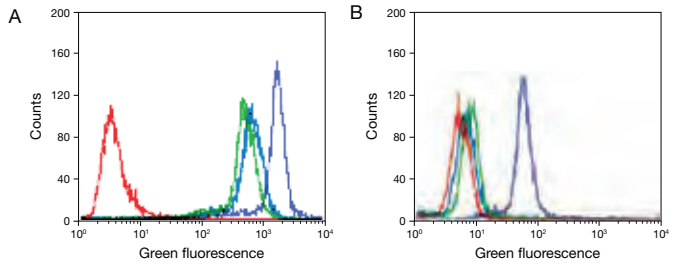

Figure 1.3.40 Detection of epidermal growth factor (EGF) receptors directly or with signal amplification. Cells expressing high (A431 cells, panel **A**) and low (NIH 3T3 cells, panel **B**) levels of EGF receptors were either directly labeled with the preformed Alexa Fluor® 488 complex of biotinylated epidermal growth factor (E13345, blue) or indirectly labeled with biotinylated EGF (E3477) followed by either Alexa Fluor® 488 streptavidin (S11223, green) or HRP-conjugated streptavidin and Alexa Fluor® 488 tyramide (purple), components of our TSA™ Kit #22 (T20932).

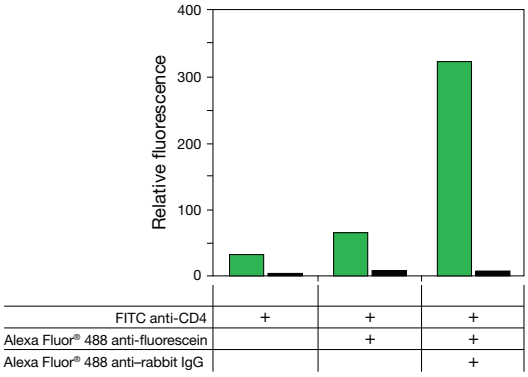

Figure 1.3.41 An example of flow cytometry results obtained using the Alexa Fluor® 488 Signal Amplification Kit for Fluorescein- and Oregon Green® Dye–Conjugated Probes (A11053). Human T cell leukemia cells (Jurkat) were stained with fluorescein (FITC) mouse anti-CD4 antibody and, as indicated, with Alexa Fluor® 488 rabbit anti–fluorescein/Oregon Green® antibody (A11090) and Alexa Fluor® 488 goat anti–rabbit IgG antibody (A11008). The fluorescence values of the negative controls, in which the FITC anti-CD4 antibody was omitted, are shown (black) together with the fluorescence values of the experimental samples (green). The fluorescence values represent the average signals from the population of cells analyzed.

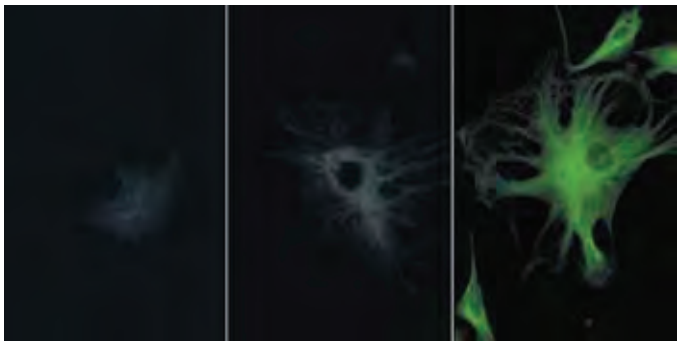

Figure 1.3.42 Demonstration of the amplification obtained with the Alexa Fluor® 488 Signal Amplification Kit for Fluorescein- and Oregon Green® Dye–Conjugated Probes (A11053). Bovine pulmonary artery endothelial cells were labeled with anti–α-tubulin antibody (A11126) in combination with fluorescein goat anti–mouse IgG antibody (F2761) (left panel). The center panel shows the cells after treatment with Alexa Fluor® 488 rabbit anti–fluorescein/Oregon Green® antibody (A11090), and the right panel shows the cells after additional labeling with Alexa Fluor® 488 goat anti–rabbit IgG antibody (A11008). The images were acquired using identical exposure times, and a bandpass filter set appropriate for fluorescein.

The HRP-catalyzed immobilization of a fluorescent tyramide can yield far greater total fluorescence than would ever be possible with direct labeling of the target, enabling detection of very low-abundance receptors (Figure 1.3.40). Furthermore, TSA™ can be used in either live- or fixed-cell preparations, and the increased sensitivity of this signal amplification method often permits use of greatly decreased quantities of antibodies or nucleic acid probes. Our complete selection of TSA™ Kits are listed in Table 6.1 and are extensively discussed in Section 6.2.

Antibody-Based Signal Amplification Kits

Although the direct fluorescence signal of Alexa Fluor® conjugates tends to be significantly greater than that of other dyes with comparable spectra, we have also developed two kits that take further advantage of the superior brightness and photostability of Alexa Fluor® 488 dye– and Alexa Fluor® 594 dye–labeled reagents. These Alexa Fluor® Signal Amplification Kits are designed to substantially increase the signals obtained by immunofluorescence techniques (Figure 1.3.41), thus permitting detection of low-abundance targets.[27] The Alexa Fluor® 488 Signal Amplification Kit for Fluorescein-Conjugated Probes (A11053) provides methods and reagents for two-stage amplification of fluorescein (FITC)–labeled primary antibodies and also overwrites the fluorescein fluorescence signal with that of the brighter and more photostable Alexa Fluor® 488 dye (Figure 1.3.42). The Alexa Fluor® 488 Signal Amplification Kit for Mouse Antibodies (A11054) provides two-stage amplification of imunocytochemical labeling generated by mouse primary antibodies. The similar Alexa Fluor® 568 and Alexa Fluor® 594 Signal Amplification Kits for Mouse Antibodies (A11066, A11067) provide ultrasensitive immunofluorescent detection at longer wavelengths. For additional details about these kits, see Section 7.2.

Alexa Fluor® Conjugates of Anti–Fluorescein/ Oregon Green® Antibody

Alexa Fluor® 488 dye–labeled rabbit anti–fluorescein/Oregon Green® antibody (A11090, Section 7.4) can be used to enhance the green-fluorescent signal of the fluorescein (or Oregon Green®) hapten without changing its fluorescence color. Thus, this conjugate allows researchers to take advantage of the superior photostability of Alexa Fluor® 488 dye, while utilizing existing fluorescein- or Oregon Green® dye–labeled probes and fluorescein-compatible optics. Alexa Fluor® 594 dye–labeled rabbit anti–fluorescein/Oregon Green® antibody (A11091) can be used to convert the green fluorescence of fluorescein or Oregon Green® conjugates into exceptionally photostable red fluorescence (Figure 1.3.43), and to amplify the signal from fluorescein and Oregon Green® conjugates by as much as 100-fold (Figure 1.3.44).

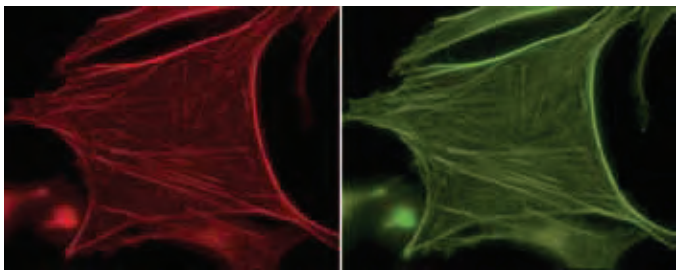

Figure 1.3.43 Fixed and permeabilized bovine pulmonary artery endothelial cells were labeled with the filamentous actin (F-actin) stain, fluorescein phalloidin (F432, right). An Alexa Fluor® 594 anti–fluorescein/Oregon Green® rabbit IgG antibody (A11091) converted the green fluorescence to red (left).

Antibodies to Alexa Fluor® 488 and Alexa Fluor® 405 Dyes

We offer a rabbit polyclonal antibody to Alexa Fluor® 488 dye (A11094, Section 7.4) that quenches the dye's fluorescence and can be used in various signal amplification schemes, including further amplification of the signal from Alexa Fluor® conjugates of proteins or nucleic acids or potentially from Alexa Fluor® 488 tyramide in the corresponding TSA™ Kits (T20912, T20922, T20932; Section 6.2).

As expected, the rabbit polyclonal antibody to the Cascade Blue® dye (A5760, Section 7.4) strongly interacts with Alexa Fluor® 405 dye, making it useful for various fluorescence quenching and amplification schemes. Our Zenon® Rabbit IgG Labeling Kits (Section 7.3, Table 7.7) can also be used to prepare fluorescent dye–, biotin- or enzyme-labeled complexes of these rabbit IgG antibodies for use in various detection and amplification schemes.

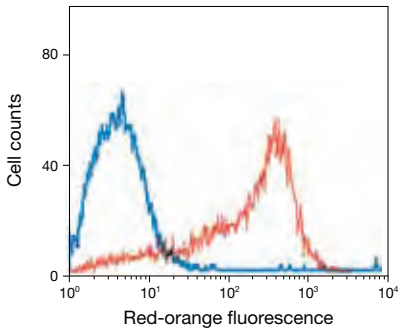

Figure 1.3.44 Color-shifting using a labeled anti–fluorescein/Oregon Green® antibody. Jurkat cells were first stained with a primary mouse anti–human CD3 antibody, followed by fluorescein goat anti–mouse IgG antibody (F2761), with the resultant fluorescence detected in the R-phycoerythrin (red-orange fluorescence) channel of a flow cytometer (blue curve). The weak signal was then shifted to better suit the R-phycoerythrin channel by the addition of an R-phycoerythrin conjugate of anti–fluorescein/Oregon Green® antibody (A21250). The resulting signal intensity is approximately two orders of magnitude greater (red curve) than the direct fluorescence from the first staining step (blue curve).

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1. J Histochem Cytochem (2003) 51:1699; **2**. J Histochem Cytochem (1999) 47:1179; **3**. Nat Protoc (2009) 4:372; **4**. Genes Dev (2008) 22:463; **5**. Cytometry (2000) 41:316; **6**. J Bacteriol (2000) 182:2793; **7**. J Histochem Cytochem (1999) 47:1213; **8**. Anal Biochem (2002) 308:18; **9**. Electrophoresis (2004) 25:779; **10**. Cytometry A (2007) 71:174; **11**. Microsc Res Tech (2005) 68:307; **12**. J Histochem Cytochem (2000) 48:653; **13**. Curr Biol (2006) 16:1924; **14**. Mol Cancer Ther (2009) 8:232; **15**. Clin Cancer Res (2008) 14:4146; **16**. Bioconjug Chem (2008) 19:1186; **17**. Bioconjug Chem (2003) 14:195; **18**. J Immunol Methods (2002) 271:17; **19**. Bioconjug Chem (2000) 11:696; **20**. J Biol Chem (2007) 282:7777; **21**. Proc Natl Acad Sci U S A (2006) 103:15479; **22**. Proc Natl Acad Sci U S A (2005) 102:5346; **23**. Biotechniques (2004) 36:114; **24**. Proteomics (2007) 7:3055; **25**. Nucleic Acids Res (2005) 33:2952; **26**. Anal Biochem (2004) 331:243; **27**. Methods (2003) 30:191.

DATA TABLE 1.3 ALEXA FLUOR® DYES SPANNING THE VISIBLE AND INFRARED SPECTRUM

Cat. No.	MW	Storage	Soluble	Abs	EC	Em	Solvent	Notes
A10168	410.35	F,D,L	H_2O, DMSO	346	19,000	445	pH 7	1
A10169	701.75	F,D,L	H_2O, DMSO	430	15,000	545	pH 7	1
A20000	643.41	F,DD,L	H_2O, DMSO	494	73,000	517	pH 7	1, 2, 3, 4
A20001	723.77	F,DD,L	H_2O, DMSO	530	81,000	555	pH 7	1, 5
A20002	1079.39	F,DD,L	H_2O, DMSO	554	112,000	570	pH 7	1, 6
A20003	791.80	F,DD,L	H_2O, DMSO	578	88,000	602	pH 7	1, 7, 8
A20004	819.85	F,DD,L	H_2O, DMSO	590	92,000	617	pH 7	1, 9, 10
A20005	~1200	F,DD,L	H_2O, DMSO	621	159,000	639	MeOH	1, 11, 12
A20006	~1250	F,DD,L	H_2O, DMSO	651	270,000	672	MeOH	1, 13, 14
A20007	~1100	F,DD,L	H_2O, DMSO	668	132,000	698	MeOH	1, 15, 16
A20008	~1150	F,DD,L	H_2O, DMSO	684	183,000	707	MeOH	1, 17, 18
A20009	~1250	F,DD,L	H_2O, DMSO	555	155,000	572	MeOH	1, 19
A20010	~1400	F,DD,L	H_2O, DMSO	702	205,000	723	MeOH	1, 20, 21
A20011	~1300	F,DD,L	H_2O, DMSO	753	290,000	782	MeOH	1, 22, 23
A30000	1028.26	F,DD,L	H_2O, DMSO	400	35,000	424	pH 7	1, 24
A30002	713.69	F,DD,L	H_2O, DMSO	517	80,000	542	pH 7	1
A30005	884.91	F,DD,L	H_2O, DMSO	494	72,000	520	pH 7	2, 4, 25
A30050	1284.82	F,DD,L	H_2O, DMSO	603	144,000	623	MeOH	1
A30051	~1750	F,DD,L	H_2O, DMSO	784	260,000	814	MeOH	1
A30052	825.46	F,DD,L	H_2O, DMSO	493	73,000	520	pH 7	2, 4, 25

For definitions of the contents of this data table, see "Using *The Molecular Probes® Handbook*" in the introductory pages.

Notes

1. This sulfonated succinimidyl ester derivative is water soluble and may be dissolved in buffer at ~pH 8 for reaction with amines. Long-term storage in water is NOT recommended due to hydrolysis.
2. The fluorescence lifetime (τ) of the Alexa Fluor® 488 dye in pH 7.4 buffer at 20°C is 4.1 nanoseconds. Data provided by the SPEX Fluorescence Group, Horiba Jobin Yvon Inc.
3. The fluorescence quantum yield of Alexa Fluor® 488 carboxylic acid, succinimidyl ester in 50 mM potassium phosphate, 150 mM NaCl pH 7.2 at 22°C is 0.92.
4. Abs and Em of the Alexa Fluor® 488 dye are red-shifted by as much as 16 nm and 25 nm, respectively, on microarrays relative to aqueous solution values. The magnitude of the spectral shift depends on the array substrate material. (Biotechniques (2005) 38:127)
5. The fluorescence quantum yield of Alexa Fluor® 532 carboxylic acid, succinimidyl ester in 50 mM potassium phosphate, 150 mM NaCl pH 7.2 at 22°C is 0.61.
6. The fluorescence quantum yield of Alexa Fluor® 546 carboxylic acid, succinimidyl ester in 50 mM potassium phosphate, 150 mM NaCl pH 7.2 at 22°C is 0.79.
7. The fluorescence lifetime (τ) of the Alexa Fluor® 568 dye in pH 7.4 buffer at 20°C is 3.6 nanoseconds. Data provided by the SPEX Fluorescence Group, Horiba Jobin Yvon Inc.
8. The fluorescence quantum yield of Alexa Fluor® 568 carboxylic acid, succinimidyl ester in 50 mM potassium phosphate, 150 mM NaCl pH 7.2 at 22°C is 0.69.
9. The fluorescence lifetime (τ) of the Alexa Fluor® 594 dye in pH 7.4 buffer at 20°C is 3.9 nanoseconds. Data provided by the SPEX Fluorescence Group, Horiba Jobin Yvon Inc.
10. The fluorescence quantum yield of Alexa Fluor® 594 carboxylic acid, succinimidyl ester in 50 mM potassium phosphate, 150 mM NaCl pH 7.2 at 22°C is 0.66.
11. Alexa Fluor® 633 dye–labeled proteins typically exhibit two absorption peaks at about ~580 and ~630 nm. Fluorescence excitation is more efficient at the 630 nm absorption peak.
12. The fluorescence lifetime (τ) of the Alexa Fluor® 633 dye in H_2O at 20°C is 3.2 nanoseconds. Data provided by LJL BioSystems/Molecular Devices Corporation.
13. The fluorescence lifetime (τ) of the Alexa Fluor® 647 dye in H_2O at 20°C is 1.0 nanoseconds and 1.5 nanoseconds in EtOH. (Bioconjug Chem (2003) 14:195)

continued on next page

DATA TABLE 1.3 ALEXA FLUOR® DYES SPANNING THE VISIBLE AND INFRARED SPECTRUM—*continued*

14. The fluorescence quantum yield of Alexa Fluor® 647 carboxylic acid, succinimidyl ester in 50 mM potassium phosphate, 150 mM NaCl pH 7.2 at 22°C is 0.33.
15. The fluorescence lifetime (τ) of the Alexa Fluor® 660 dye in pH 7.5 buffer at 20°C is 1.2 nanoseconds. Data provided by Pierre-Alain Muller, Max Planck Institute for Biophysical Chemistry, Göttingen.
16. The fluorescence quantum yield of Alexa Fluor® 660 carboxylic acid, succinimidyl ester in 50 mM potassium phosphate, 150 mM NaCl pH 7.2 at 22°C is 0.37.
17. The fluorescence lifetime (τ) of the Alexa Fluor® 680 dye in pH 7.5 buffer at 20°C is 1.2 nanoseconds. Data provided by Pierre-Alain Muller, Max Planck Institute for Biophysical Chemistry, Göttingen.
18. The fluorescence quantum yield of Alexa Fluor® 680 carboxylic acid, succinimidyl ester in 50 mM potassium phosphate, 150 mM NaCl pH 7.2 at 22°C is 0.36.
19. The fluorescence quantum yield of Alexa Fluor® 555 carboxylic acid, succinimidyl ester in 50 mM potassium phosphate, 150 mM NaCl pH 7.2 at 22°C is 0.10.
20. The fluorescence lifetime (τ) of the Alexa Fluor® 700 dye in H_2O at 22°C is 1.0 nanoseconds. Data provided by ISS Inc. (Champaign, IL).
21. The fluorescence quantum yield of Alexa Fluor® 700 carboxylic acid, succinimidyl ester in 50 mM potassium phosphate, 150 mM NaCl pH 7.2 at 22°C is 0.25.
22. The fluorescence lifetime (τ) of the Alexa Fluor® 750 dye in H_2O at 22°C is 0.7 nanoseconds. Data provided by ISS Inc. (Champaign, IL).
23. The fluorescence quantum yield of Alexa Fluor® 750 carboxylic acid, succinimidyl ester in 50 mM potassium phosphate, 150 mM NaCl pH 7.2 at 22°C is 0.12.
24. The Alexa Fluor® 405 and Cascade Blue® dyes have a second absorption peak at about 376 nm with EC ~80% of the 395–400 nm peak.
25. TFP and SDP ester derivatives are water soluble and may be dissolved in buffer at ~pH 8 for reaction with amines. Long-term storage in water is NOT recommended due to hydrolysis.

PRODUCT LIST 1.3 ALEXA FLUOR® DYES SPANNING THE VISIBLE AND INFRARED SPECTRUM

Cat. No.	Product	Quantity
A10168	Alexa Fluor® 350 carboxylic acid, succinimidyl ester	5 mg
A30000	Alexa Fluor® 405 carboxylic acid, succinimidyl ester	1 mg
A30100	Alexa Fluor® 405 carboxylic acid, succinimidyl ester	5 mg
A10169	Alexa Fluor® 430 carboxylic acid, succinimidyl ester	5 mg
A20000	Alexa Fluor® 488 carboxylic acid, succinimidyl ester *mixed isomers*	1 mg
A20100	Alexa Fluor® 488 carboxylic acid, succinimidyl ester *mixed isomers*	5 mg
A30005	Alexa Fluor® 488 carboxylic acid, 2,3,5,6-tetrafluorophenyl ester (Alexa Fluor® 488 5-TFP) *5-isomer*	1 mg
A30002	Alexa Fluor® 514 carboxylic acid, succinimidyl ester *mixed isomers*	1 mg
A20001	Alexa Fluor® 532 carboxylic acid, succinimidyl ester	1 mg
A20101MP	Alexa Fluor® 532 carboxylic acid, succinimidyl ester	5 mg
A20002	Alexa Fluor® 546 carboxylic acid, succinimidyl ester	1 mg
A20102	Alexa Fluor® 546 carboxylic acid, succinimidyl ester	5 mg
A20009	Alexa Fluor® 555 carboxylic acid, succinimidyl ester	1 mg
A20109	Alexa Fluor® 555 carboxylic acid, succinimidyl ester	5 mg
A20003	Alexa Fluor® 568 carboxylic acid, succinimidyl ester *mixed isomers*	1 mg
A20103	Alexa Fluor® 568 carboxylic acid, succinimidyl ester *mixed isomers*	5 mg
A20004	Alexa Fluor® 594 carboxylic acid, succinimidyl ester *mixed isomers*	1 mg
A20104	Alexa Fluor® 594 carboxylic acid, succinimidyl ester *mixed isomers*	5 mg
A30050	Alexa Fluor® 610-X, succinimidyl ester, bis(triethylammonium salt) *6-isomer*	1 mg
A20005	Alexa Fluor® 633 carboxylic acid, succinimidyl ester	1 mg
A20105	Alexa Fluor® 633 carboxylic acid, succinimidyl ester	5 mg
A20006	Alexa Fluor® 647 carboxylic acid, succinimidyl ester	1 mg
A20106	Alexa Fluor® 647 carboxylic acid, succinimidyl ester	5 mg
A20007	Alexa Fluor® 660 carboxylic acid, succinimidyl ester	1 mg
A20008	Alexa Fluor® 680 carboxylic acid, succinimidyl ester	1 mg
A20108	Alexa Fluor® 680 carboxylic acid, succinimidyl ester	5 mg
A20010	Alexa Fluor® 700 carboxylic acid, succinimidyl ester	1 mg
A20110	Alexa Fluor® 700 carboxylic acid, succinimidyl ester	5 mg
A20011	Alexa Fluor® 750 carboxylic acid, succinimidyl ester	1 mg
A20111	Alexa Fluor® 750 carboxylic acid, succinimidyl ester	5 mg
A30051	Alexa Fluor® 790 carboxylic acid, succinimidyl ester, penta(triethylammonium) salt	100 µg
A30006	Alexa Fluor® 488 Microscale Protein Labeling Kit *for 20–100 µg protein* *3 labelings*	1 kit
A30007	Alexa Fluor® 555 Microscale Protein Labeling Kit *for 20–100 µg protein* *3 labelings*	1 kit
A30008	Alexa Fluor® 594 Microscale Protein Labeling Kit *for 20–100 µg protein* *3 labelings*	1 kit
A30009	Alexa Fluor® 647 Microscale Protein Labeling Kit *for 20–100 µg protein* *3 labelings*	1 kit
A20180	Alexa Fluor® 350 Monoclonal Antibody Labeling Kit *5 labelings*	1 kit
A20181	Alexa Fluor® 488 Monoclonal Antibody Labeling Kit *5 labelings*	1 kit
A20182	Alexa Fluor® 532 Monoclonal Antibody Labeling Kit *5 labelings*	1 kit
A20183	Alexa Fluor® 546 Monoclonal Antibody Labeling Kit *5 labelings*	1 kit
A20187	Alexa Fluor® 555 Monoclonal Antibody Labeling Kit *5 labelings*	1 kit
A20184	Alexa Fluor® 568 Monoclonal Antibody Labeling Kit *5 labelings*	1 kit
A20185	Alexa Fluor® 594 Monoclonal Antibody Labeling Kit *5 labelings*	1 kit

PRODUCT LIST 1.3 ALEXA FLUOR® DYES SPANNING THE VISIBLE AND INFRARED SPECTRUM—*continued*

Cat. No.	Product	Quantity
A20186	Alexa Fluor® 647 Monoclonal Antibody Labeling Kit *5 labelings*	1 kit
A20191	Alexa Fluor® 488 Oligonucleotide Amine Labeling Kit *3 labelings*	1 kit
A20196	Alexa Fluor® 647 Oligonucleotide Amine Labeling Kit *3 labelings*	1 kit
A10170	Alexa Fluor® 350 Protein Labeling Kit *3 labelings*	1 kit
A10171	Alexa Fluor® 430 Protein Labeling Kit *3 labelings*	1 kit
A10235	Alexa Fluor® 488 Protein Labeling Kit *3 labelings*	1 kit
A10236	Alexa Fluor® 532 Protein Labeling Kit *3 labelings*	1 kit
A10237	Alexa Fluor® 546 Protein Labeling Kit *3 labelings*	1 kit
A20174	Alexa Fluor® 555 Protein Labeling Kit *3 labelings*	1 kit
A10238	Alexa Fluor® 568 Protein Labeling Kit *3 labelings*	1 kit
A10239	Alexa Fluor® 594 Protein Labeling Kit *3 labelings*	1 kit
A20170	Alexa Fluor® 633 Protein Labeling Kit *3 labelings*	1 kit
A20173	Alexa Fluor® 647 Protein Labeling Kit *3 labelings*	1 kit
A20171	Alexa Fluor® 660 Protein Labeling Kit *3 labelings*	1 kit
A20172	Alexa Fluor® 680 Protein Labeling Kit *3 labelings*	1 kit
A32750	Alexa Fluor® 488 reactive dye decapack *for microarrays* *set of 10 vials*	1 set
A32756	Alexa Fluor® 555 reactive dye decapack *for microarrays* *set of 10 vials*	1 set
A32757	Alexa Fluor® 647 reactive dye decapack *for microarrays* *set of 10 vials*	1 set
A32755	Alexa Fluor® 555 and Alexa Fluor® 647 reactive dye decapacks *for microarrays* *set of 2 x 10 vials* *includes A32756 and A32757 decapacks*	1 set
A30052	Alexa Fluor® 488 5-SDP ester (Alexa Fluor® 488 sulfodichlorophenol ester)	1 mg
A10468	APEX® Alexa Fluor® 488 Antibody Labeling Kit	1 kit
A10470	APEX® Alexa Fluor® 555 Antibody Labeling Kit	1 kit
A10474	APEX® Alexa Fluor® 594 Antibody Labeling Kit	1 kit
A10475	APEX® Alexa Fluor® 647 Antibody Labeling Kit	1 kit
A21665	ARES™ Alexa Fluor® 488 DNA Labeling Kit *10 labelings*	1 kit
A21667	ARES™ Alexa Fluor® 546 DNA Labeling Kit *10 labelings*	1 kit
A21677	ARES™ Alexa Fluor® 555 DNA Labeling Kit *10 labelings*	1 kit
A21669	ARES™ Alexa Fluor® 594 DNA Labeling Kit *10 labelings*	1 kit
A21676	ARES™ Alexa Fluor® 647 DNA Labeling Kit *10 labelings*	1 kit
F32950	FISH Tag™ DNA Far Red Kit *with Alexa Fluor® 647 dye* *10 reactions*	1 kit
F32947	FISH Tag™ DNA Green Kit *with Alexa Fluor® 488 dye* *10 reactions*	1 kit
F32951	FISH Tag™ DNA Multicolor Kit *Alexa Fluor® dye combination* *10 reactions*	1 kit
F32948	FISH Tag™ DNA Orange Kit *with Alexa Fluor® 555 dye* *10 reactions*	1 kit
F32949	FISH Tag™ DNA Red Kit *with Alexa Fluor® 594 dye* *10 reactions*	1 kit
F32955	FISH Tag™ RNA Far Red Kit *with Alexa Fluor® 647 dye* *10 reactions*	1 kit
F32952	FISH Tag™ RNA Green Kit *with Alexa Fluor® 488 dye* *10 reactions*	1 kit
F32956	FISH Tag™ RNA Multicolor Kit *Alexa Fluor® dye combination* *10 reactions*	1 kit
F32953	FISH Tag™ RNA Orange Kit *with Alexa Fluor® 555 dye* *10 reactions*	1 kit
F32954	FISH Tag™ RNA Red Kit *with Alexa Fluor® 594 dye* *10 reactions*	1 kit
S30044	SAIVI™ Alexa Fluor® 647 Antibody/Protein 1 mg-Labeling Kit *3 labelings*	1 kit
S30045	SAIVI™ Rapid Antibody Labeling Kit, Alexa Fluor® 680 *3 labelings*	1 kit
S30046	SAIVI™ Rapid Antibody Labeling Kit, Alexa Fluor® 750 *3 labelings*	1 kit
U21650	ULYSIS® Alexa Fluor® 488 Nucleic Acid Labeling Kit *20 labelings*	1 kit
U21651	ULYSIS® Alexa Fluor® 532 Nucleic Acid Labeling Kit *20 labelings*	1 kit
U21652	ULYSIS® Alexa Fluor® 546 Nucleic Acid Labeling Kit *20 labelings*	1 kit
U21653	ULYSIS® Alexa Fluor® 568 Nucleic Acid Labeling Kit *20 labelings*	1 kit
U21654	ULYSIS® Alexa Fluor® 594 Nucleic Acid Labeling Kit *20 labelings*	1 kit
U21660	ULYSIS® Alexa Fluor® 647 Nucleic Acid Labeling Kit *20 labelings*	1 kit
Z25400	Zenon® Alexa Fluor® 350 Human IgG Labeling Kit *50 labelings*	1 kit
Z25000	Zenon® Alexa Fluor® 350 Mouse IgG_1 Labeling Kit *50 labelings*	1 kit
Z25100	Zenon® Alexa Fluor® 350 Mouse IgG_{2a} Labeling Kit *50 labelings*	1 kit
Z25200	Zenon® Alexa Fluor® 350 Mouse IgG_{2b} Labeling Kit *50 labelings*	1 kit
Z25300	Zenon® Alexa Fluor® 350 Rabbit IgG Labeling Kit *50 labelings*	1 kit
Z25013	Zenon® Alexa Fluor® 405 Mouse IgG_1 Labeling Kit *50 labelings*	1 kit
Z25113	Zenon® Alexa Fluor® 405 Mouse IgG_{2a} Labeling Kit *50 labelings*	1 kit
Z25213	Zenon® Alexa Fluor® 405 Mouse IgG_{2b} Labeling Kit *50 labelings*	1 kit
Z25313	Zenon® Alexa Fluor® 405 Rabbit IgG Labeling Kit *50 labelings*	1 kit
Z25001	Zenon® Alexa Fluor® 430 Mouse IgG_1 Labeling Kit *50 labelings*	1 kit

continued on next page

PRODUCT LIST 1.3 ALEXA FLUOR® DYES SPANNING THE VISIBLE AND INFRARED SPECTRUM—*continued*

Cat. No.	Product	Quantity
Z25301	Zenon® Alexa Fluor® 430 Rabbit IgG Labeling Kit *50 labelings*	1 kit
Z25602	Zenon® Alexa Fluor® 488 Goat IgG Labeling Kit *50 labelings*	1 kit
Z25402	Zenon® Alexa Fluor® 488 Human IgG Labeling Kit *50 labelings*	1 kit
Z25002	Zenon® Alexa Fluor® 488 Mouse IgG_1 Labeling Kit *50 labelings*	1 kit
Z25090	Zenon® Alexa Fluor® 488 Mouse IgG_1 Labeling Kit *enhanced with TSA™ technology* *25 labelings*	1 kit
Z25102	Zenon® Alexa Fluor® 488 Mouse IgG_{2a} Labeling Kit *50 labelings*	1 kit
Z25202	Zenon® Alexa Fluor® 488 Mouse IgG_{2b} Labeling Kit *50 labelings*	1 kit
Z25302	Zenon® Alexa Fluor® 488 Rabbit IgG Labeling Kit *50 labelings*	1 kit
Z25003	Zenon® Alexa Fluor® 532 Mouse IgG_1 Labeling Kit *50 labelings*	1 kit
Z25303	Zenon® Alexa Fluor® 532 Rabbit IgG Labeling Kit *50 labelings*	1 kit
Z25004	Zenon® Alexa Fluor® 546 Mouse IgG_1 Labeling Kit *50 labelings*	1 kit
Z25104	Zenon® Alexa Fluor® 546 Mouse IgG_{2a} Labeling Kit *50 labelings*	1 kit
Z25204	Zenon® Alexa Fluor® 546 Mouse IgG_{2b} Labeling Kit *50 labelings*	1 kit
Z25304	Zenon® Alexa Fluor® 546 Rabbit IgG Labeling Kit *50 labelings*	1 kit
Z25605	Zenon® Alexa Fluor® 555 Goat IgG Labeling Kit *50 labelings*	1 kit
Z25405	Zenon® Alexa Fluor® 555 Human IgG Labeling Kit *50 labelings*	1 kit
Z25005	Zenon® Alexa Fluor® 555 Mouse IgG_1 Labeling Kit *50 labelings*	1 kit
Z25105	Zenon® Alexa Fluor® 555 Mouse IgG_{2a} Labeling Kit *50 labelings*	1 kit
Z25205	Zenon® Alexa Fluor® 555 Mouse IgG_{2b} Labeling Kit *50 labelings*	1 kit
Z25305	Zenon® Alexa Fluor® 555 Rabbit IgG Labeling Kit *50 labelings*	1 kit
Z25606	Zenon® Alexa Fluor® 568 Goat IgG Labeling Kit *50 labelings*	1 kit
Z25006	Zenon® Alexa Fluor® 568 Mouse IgG_1 Labeling Kit *50 labelings*	1 kit
Z25106	Zenon® Alexa Fluor® 568 Mouse IgG_{2a} Labeling Kit *50 labelings*	1 kit
Z25206	Zenon® Alexa Fluor® 568 Mouse IgG_{2b} Labeling Kit *50 labelings*	1 kit
Z25306	Zenon® Alexa Fluor® 568 Rabbit IgG Labeling Kit *50 labelings*	1 kit
Z25607	Zenon® Alexa Fluor® 594 Goat IgG Labeling Kit *50 labelings*	1 kit
Z25407	Zenon® Alexa Fluor® 594 Human IgG Labeling Kit *50 labelings*	1 kit
Z25007	Zenon® Alexa Fluor® 594 Mouse IgG_1 Labeling Kit *50 labelings*	1 kit
Z25107	Zenon® Alexa Fluor® 594 Mouse IgG_{2a} Labeling Kit *50 labelings*	1 kit
Z25207	Zenon® Alexa Fluor® 594 Mouse IgG_{2b} Labeling Kit *50 labelings*	1 kit
Z25307	Zenon® Alexa Fluor® 594 Rabbit IgG Labeling Kit *50 labelings*	1 kit
Z25020	Zenon® Alexa Fluor® 610–R-Phycoerythrin Mouse IgG_1 Labeling Kit *10 labelings*	1 kit
Z25608	Zenon® Alexa Fluor® 647 Goat IgG Labeling Kit *50 labelings*	1 kit
Z25408	Zenon® Alexa Fluor® 647 Human IgG Labeling Kit *50 labelings*	1 kit
Z25008	Zenon® Alexa Fluor® 647 Mouse IgG_1 Labeling Kit *50 labelings*	1 kit
Z25108	Zenon® Alexa Fluor® 647 Mouse IgG_{2a} Labeling Kit *50 labelings*	1 kit
Z25208	Zenon® Alexa Fluor® 647 Mouse IgG_{2b} Labeling Kit *50 labelings*	1 kit
Z25308	Zenon® Alexa Fluor® 647 Rabbit IgG Labeling Kit *50 labelings*	1 kit
Z25021	Zenon® Alexa Fluor® 647–R-Phycoerythrin Mouse IgG_1 Labeling Kit *10 labelings*	1 kit
Z25121	Zenon® Alexa Fluor® 647–R-Phycoerythrin Mouse IgG_{2a} Labeling Kit *10 labelings*	1 kit
Z25221	Zenon® Alexa Fluor® 647–R-Phycoerythrin Mouse IgG_{2b} Labeling Kit *10 labelings*	1 kit
Z25009	Zenon® Alexa Fluor® 660 Mouse IgG_1 Labeling Kit *50 labelings*	1 kit
Z25010	Zenon® Alexa Fluor® 680 Mouse IgG_1 Labeling Kit *50 labelings*	1 kit
Z25110	Zenon® Alexa Fluor® 680 Mouse IgG_{2a} Labeling Kit *50 labelings*	1 kit
Z25210	Zenon® Alexa Fluor® 680 Mouse IgG_{2b} Labeling Kit *50 labelings*	1 kit
Z25310	Zenon® Alexa Fluor® 680 Rabbit IgG Labeling Kit *50 labelings*	1 kit
Z25022	Zenon® Alexa Fluor® 680–R-Phycoerythrin Mouse IgG_1 Labeling Kit *10 labelings*	1 kit
Z25011	Zenon® Alexa Fluor® 700 Mouse IgG_1 Labeling Kit *50 labelings*	1 kit
Z25030	Zenon® Alexa Fluor® 700–Allophycocyanin Mouse IgG_1 Labeling Kit *10 labelings*	1 kit
Z25312	Zenon® Alexa Fluor® 750 Rabbit IgG Labeling Kit *50 labelings*	1 kit
Z25031	Zenon® Alexa Fluor® 750–Allophycocyanin Mouse IgG_1 Labeling Kit *10 labelings*	1 kit

1.4 BODIPY® Dye Series

Overview of the BODIPY® Fluorophores

The BODIPY® fluorophores have spectral characteristics that are often superior to those of fluorescein, tetramethylrhodamine, Texas Red® and longer-wavelength dyes. With derivatives that span the visible spectrum (Figure 1.4.1), BODIPY® dyes are extremely versatile.[1] We use them to generate fluorescent conjugates of proteins, nucleotides, oligonucleotides and dextrans, as well as to prepare fluorescent enzyme substrates, fatty acids, phospholipids, lipopolysaccharides, receptor ligands and polystyrene microspheres.

BODIPY® dyes are unusual in that they are relatively nonpolar and the chromophore is electrically neutral (Figure 1.4.2). These properties tend to minimize dye-induced perturbation of conjugate functional properties. BODIPY® dyes are therefore often the preferred choice for labeling nucleotides, amino acids and other low molecular weight ligands.[2–4] BODIPY® dye conjugates of low molecular weight molecules also tend to be more permeant to live cells than are conjugates of charged fluorophores (Section 14.2). With their high peak intensity, reactive BODIPY® dyes are among the most detectable amine-derivatization reagents available for HPLC and capillary electrophoresis.[5–8] BODIPY® dyes are also more useful than most other long-wavelength dyes, including fluoresceins and carbocyanines, for assays that measure fluorescence polarization[9–12] (Fluorescence Polarization (FP)—Note 1.4), and they have large cross-sections for excitation by multiphoton excitation sources [13,14] (Fluorescent Probes for Two-Photon Microscopy—Note 1.5).

The core structure of the BODIPY® fluorophore is shown in Figure 1.4.2. Solutions of the alkyl-substituted derivatives have a green, fluorescein-like fluorescence. However, when substituents that yield additional conjugation are added to the parent molecule, both the absorption and emission spectra of the resulting derivatives can shift to significantly longer wavelengths, with emission maxima of greater than 750 nm now possible with some BODIPY® derivatives. Our goal has been to develop BODIPY® dyes that are optimal for the major excitation sources and that match the common optical filter sets. Accordingly, our recommended BODIPY® substitutes for the fluorescein, rhodamine 6G, tetramethylrhodamine and Texas Red® fluorophores are named BODIPY® FL, BODIPY® R6G, BODIPY® TMR and BODIPY® TR, respectively (Figure 1.4.3). Because there are so many different BODIPY® dyes, we have had to develop a systematic strategy for naming them. Except for BODIPY® FL, BODIPY® R6G, BODIPY® TMR and BODIPY® TR, we identify these dyes with the registered trademark BODIPY® followed by the approximate absorption/emission maxima in nm (determined in methanol); for example, the BODIPY® 581/591 dye.

Amine-reactive BODIPY® dyes (Table 1.7) are discussed below; thiol-reactive BODIPY® dyes are included in Section 2.2. Other reactive BODIPY® dyes useful for derivatizing aldehydes, ketones and carboxylic acids are described in Section 3.3 and Section 3.4. Applications of some thiol-reactive BODIPY® dyes for cell tracing are discussed in Section 14.2.

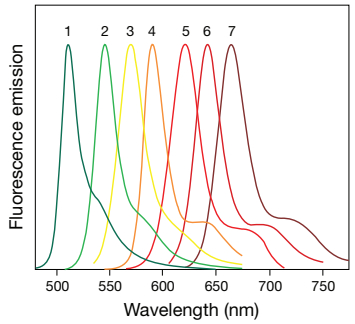

Figure 1.4.1 Normalized fluorescence emission spectra of 1) BODIPY® FL, 2) BODIPY® R6G, 3) BODIPY® TMR, 4) BODIPY® 581/591, 5) BODIPY® TR, 6) BODIPY® 630/650 and 7) BODIPY® 650/665 fluorophores in methanol.

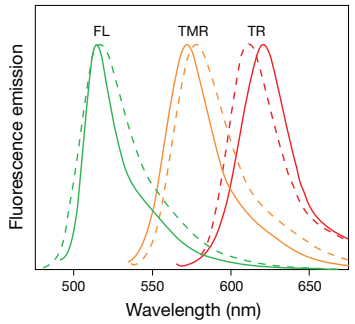

Figure 1.4.3 Normalized fluorescence emission spectra of goat anti–mouse IgG antibody conjugates of fluorescein (FL), tetramethylrhodamine (TMR) and the Texas Red® (TR) dyes, shown by dashed lines (- - -), as compared with goat anti–mouse IgG antibody conjugates of BODIPY® FL, BODIPY® TMR and BODIPY® TR dyes, respectively, shown by solid lines (—).

BODIPY® FL Dye: A Substitute for Fluorescein

With the most fluorescein-like spectra of the BODIPY® dyes, the green-fluorescent BODIPY® FL fluorophore (Figure 1.4.4) (excitation/emission maxima ~503/512 nm) has several characteristics[15,16] that make it potentially superior to fluorescein in some applications. These include:

- High extinction coefficient (EC >80,000 $cm^{-1}M^{-1}$) and high fluorescence quantum yield (often approaching 1.0, even in water)
- Lack of ionic charge and spectra that are relatively insensitive to solvent polarity and pH[15]
- Narrow emission bandwidth (Figure 1.4.3), resulting in a higher peak intensity than that of fluorescein

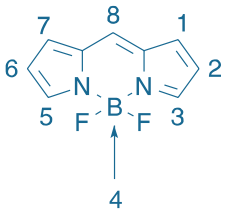

Figure 1.4.2 The structure and numbering of the BODIPY® fluorophore, 4,4-difluoro-4-bora-3a,4a-diaza-*s*-indacene.

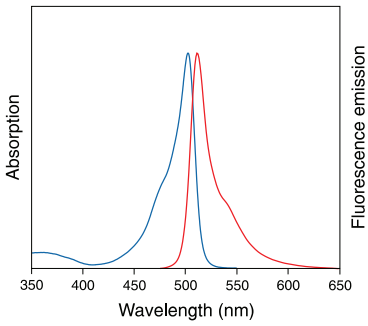

Figure 1.4.4 Absorption and fluorescence emission spectra of BODIPY® FL propionic acid, succinimidyl ester in methanol.

- Red shift in fluorescence emission at high dye concentrations—a property that can be used to detect regions of high probe density[17] (Figure 1.4.5)
- Relatively long excited-state lifetime (typically 5 nanoseconds or longer), which is useful for fluorescence polarization–based assays (Fluorescence Polarization (FP)—Note 1.4)
- Little or no spectral overlap with longer-wavelength dyes such as tetramethylrhodamine and Texas Red® dye (Figure 1.4.3), making BODIPY® FL one of the preferred green-fluorescent dyes for multicolor applications[18]
- Greater photostability than fluorescein in some environments[19]
- Large two-photon cross-section for multiphoton excitation[13,14] (Fluorescent Probes for Two-Photon Microscopy—Note 1.5)

Longer-Wavelength BODIPY® Dyes

It is possible to synthesize BODIPY® fluorophores with altered spectral properties by simply changing the substituents on the parent molecule. This discovery has led to creation of a series of longer-wavelength BODIPY® dyes with fluorescence spectra that span the visible spectrum (Figure 1.4.1). The BODIPY® R6G (excitation/emission maxima ~528/547 nm), BODIPY® TMR (excitation/emission maxima ~543/569 nm) and BODIPY® TR (excitation/emission maxima ~592/618 nm) fluorophores are spectrally similar to the rhodamine 6G, tetramethylrhodamine and Texas Red® fluorophores, respectively, and are thus compatible with standard optical filter sets designed for these important dyes.

The BODIPY® 630/650-X and BODIPY® 650/665-X fluorophores are the longest-wavelength amine-reactive BODIPY® fluorophores currently available. The spectral properties of these longer-wavelength BODIPY® derivatives retain most of the advantages of the BODIPY® FL fluorophore, including narrow bandwidths, high extinction coefficients, good fluorescence quantum yields and relatively long excited-state lifetimes (>3 nanoseconds for the BODIPY® 630/650 dye[20,21]). Like the BODIPY® FL fluorophore, however, most of these dyes have a small Stokes shift, which may require that they be excited or detected at suboptimal wavelengths. The spectral characteristics of 13 different red-fluorescent fluorophores, including the Alexa Fluor® 647 (Section 1.3) and BODIPY® 630/650 dyes, have been evaluated in different surrounding media to assess the influence of polarity, viscosity and detergent concentration and to facilitate probe choice in fluorescence-based assays.[20]

Amine-Reactive BODIPY® Dyes

BODIPY® Succinimidyl Esters

We offer an extensive selection of amine-reactive BODIPY® dyes (Table 1.7). These include succinimidyl esters of several BODIPY® propionic acids and of BODIPY® FL pentanoic acid:

- BODIPY® FL C_3 succinimidyl ester (D2184)
- BODIPY® FL C_5 succinimidyl ester (D6184)
- BODIPY® R6G C_3 succinimidyl ester (D6180)
- BODIPY® 493/503 C_3 succinimidyl ester (D2191)
- BODIPY® 530/550 C_3 succinimidyl ester (D2187)
- BODIPY® 558/568 C_3 succinimidyl ester (D2219)
- BODIPY® 564/570 C_3 succinimidyl ester (D2222)
- BODIPY® 576/589 C_3 succinimidyl ester (D2225)
- BODIPY® 581/591 C_3 succinimidyl ester (D2228)

We have also prepared reactive BODIPY® X succinimidyl esters that contain an additional seven-atom aminohexanoyl spacer ("X") between the fluorophore and the succinimidyl ester group. This spacer helps to separate the fluorophore from its point of attachment, potentially reducing the interaction of the fluorophore with the biomolecule to which it is conjugated and making it more accessible to secondary detection reagents such as anti-dye antibodies.[22–24] These BODIPY® X succinimidyl esters include:

Table 1.7 Amine-reactive BODIPY® dyes.

BODIPY® Dye	Abs *	Em *	COOH	STP	Succinimidyl Ester
BODIPY® 493/503	500	506			D2191
BODIPY® FL	505	513	D2183 (C_3) D3834 (C_5)	B10006	D2184 (C_3) D6140 (SSE) D6102 (X) D6184 (C_5)
BODIPY® R6G	528	550			D6180
BODIPY® 530/550	534	554			D2187
BODIPY® TMR	542	574			D6117 (X)
BODIPY® 558/568	558	569			D2219
BODIPY® 564/570	565	571			D2222
BODIPY® 576/589	576	590			D2225
BODIPY® 581/591	584	592			D2228
BODIPY® TR	589	617			D6116 (X)
BODIPY® 630/650-X †	625	640			D10000
BODIPY® 650/655-X †	646	660			D10001

* Approximate absorption (Abs) and fluorescence (Em) maxima, in nm, for the goat anti–mouse IgG antibody or dextran conjugates in aqueous buffer. † Not recommended for derivatizing proteins. (C_3) = Propionic acid. (C_5) = Pentanoic acid. (COOH) = Carboxylic acid. (SSE) = Sulfosuccinimidyl ester. (STP) = 4-Sulfotetrafluorophenyl ester. (X) = Aminohexanoyl spacer separating the dye and the SE.

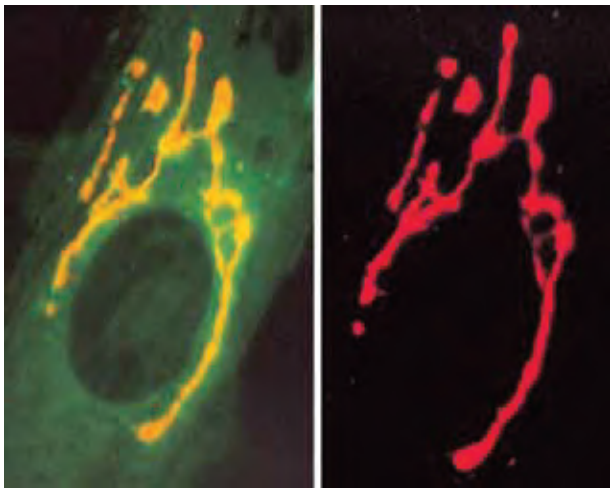

Figure 1.4.5 Selective staining of the Golgi apparatus using the green-fluorescent BODIPY® FL C_5-ceramide (D3521) (left). At high concentrations, the BODIPY® FL fluorophore forms excimers that can be visualized using a red longpass optical filter (right). The BODIPY® FL C_5-ceramide accumulation in the trans-Golgi is sufficient for excimer formation (J Cell Biol (1991) 113:1267). Images contributed by Richard Pagano, Mayo Foundation.

- BODIPY® FL-X succinimidyl ester (D6102)
- BODIPY® TMR-X succinimidyl ester (D6117)
- BODIPY® TR-X succinimidyl ester (D6116)
- BODIPY® 630/650-X succinimidyl ester (D10000)
- BODIPY® 650/665-X succinimidyl ester (D10001)

BODIPY® succinimidyl esters are particularly useful for preparing conjugates of peptides, nucleotides, oligonucleotides, drugs, toxins, sphingolipids and other low molecular weight ligands that contain aliphatic amines.[2,25] Several BODIPY® succinimidyl esters have been conjugated to aminoacyl tRNAs for metabolic incorporation into proteins through *in vitro* translation.[26–29]

BODIPY® TMR-X SE has been reacted with a series of peptide ligands for use in a high-throughput fluorescence polarization assay of ligand binding to G protein–coupled receptors.[30] The red fluorescence of the BODIPY® 581/591 fluorophore shifts to green fluorescence upon peroxidation, a unique feature that has been exploited for ratiometric measurements of lipid oxidation in live cells (Section 18.2). BODIPY® 630/650-X and BODIPY® 650/665-X succinimidyl esters (D10000, D10001) are quite fluorescent when conjugated to nucleotides[31] and oligonucleotides and can be excited with near-infrared excitation sources.

For amplifying the BODIPY® FL dye signal or converting it into an electron-dense signal, we offer an unlabeled anti–BODIPY® FL rabbit polyclonal antibody (A5770, Section 7.4). This antibody cross-reacts with some other BODIPY® dyes, but not with other fluorophores, and therefore should not be used for simultaneous detection of more than one dye based on the BODIPY® fluorophore.

Water-Soluble BODIPY® Sulfonated Succinimidyl Esters

The moderate lipophilicity of the BODIPY® propionic acid succinimidyl esters discussed above requires their dissolution in an organic solvent before use in conjugations. Although these reactive dyes are very useful for preparing conjugates of amines in organic solvents, they are less suitable for reaction with proteins.

To address the solubility in aqueous solution, we have prepared the sulfosuccinimidyl ester of BODIPY® FL propionic acid (BODIPY® FL, SSE; D6140), as well as the STP ester of BODIPY® FL propionic acid (B10006). STP esters,[32] which are prepared by coupling a carboxylic acid and 4-sulfo-2,3,5,6-tetrafluorophenol (S10490, Section 3.4, Figure 1.4.6), are more readily purified than sulfosuccinimidyl esters but equally amine reactive. BODIPY® FL SSE and BODIPY® FL STP ester are quite soluble in water and more suitable than the corresponding BODIPY® succinimidyl esters for amine conjugation in aqueous solution. These sulfonated succinimidyl esters are useful for preparing conjugates of proteins, amine-modified oligonucleotides and other biomolecules.[33]

BODIPY® Carboxylic Acids

Two green-fluorescent BODIPY® carboxylic acids (D2183, D3834) are available. These carboxylic acid derivatives can be converted to fluorescent esters,[34] acid halides or amides using standard chemical techniques.

BODIPY® Dye Conjugates and Their Applications

The versatility of the BODIPY® fluorophore is demonstrated by its incorporation into literally hundreds of products listed in this *Handbook*, including many of our FluoSpheres® and TransFluoSpheres® microspheres (Section 6.5), enzyme substrates (Chapter 10) and several of our imaging and flow cytometry standards (Section 23.1, Section 23.2). Some examples of our BODIPY® dye conjugates are described below.

BODIPY® Peptide, Protein and Polysaccharide Conjugates

As is common with many fluorescent dyes, conjugation of BODIPY® dyes to proteins is sometimes accompanied by significant fluorescence quenching.[35] Because of this potential problem, we do *not* recommend using the simple BODIPY® propionic acid succinimidyl esters discussed above for preparing most protein conjugates, although peptides labeled with a single BODIPY® dye can be quite fluorescent and are quite useful for fluorescence polarization–based assays[36,37] (Labeling Small Peptides with Amine Reactive Dyes in Organic Solvents—Note 9.2). We prepare conjugates of its BODIPY® dyes with an exceptionally wide variety of peptides, proteins and polysaccharides, including:

- Pepstatin A, a membrane-permeant analog of this important cathepsin D inhibitor (P12271, Section 10.4)
- Antibodies (Section 7.4; Table 7.1)
- Phallacidin and phalloidin for staining F-actin filaments (B607, B3475, B12382; Section 11.1; Table 11.2)
- Bovine serum albumin, for use as a tracer (A2750, Section 14.7)
- Acetylated and non-acetylated low-density lipoproteins (L3485, L3483; Section 16.1)
- *Escherichia coli* and *Staphylococcus aureus* BioParticles® conjugates (E2864, S2854; Section 16.1; Table 16.3)
- Lipopolysaccharide (L23350, Section 16.1; Table 16.1)
- Dextran (D7168, Section 14.5)

In addition, we prepare conjugates of proteins (and of starch) that are so heavily labeled that they are almost nonfluorescent (Figure 1.4.7). Use of the EnzChek® Kits and DQ™ reagents that incorporate these bioconjugates as fluorogenic enzyme substrates is described later in this section and in Section 10.4.

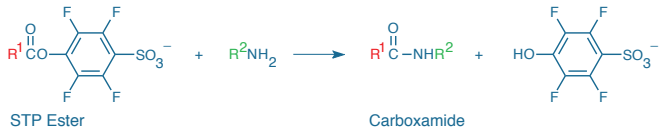

Figure 1.4.6 Reaction of a primary amine with an STP ester.

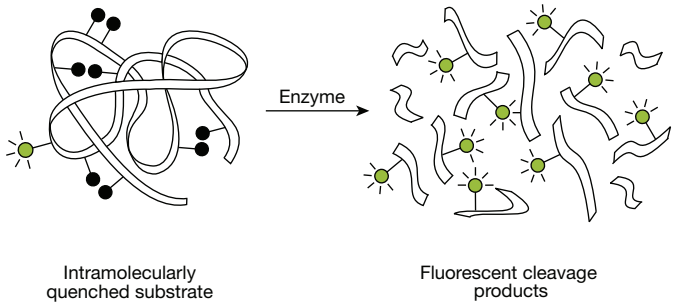

Figure 1.4.7 Principle of enzyme detection via the disruption of intramolecular self-quenching. Enzyme-catalyzed hydrolysis of the heavily labeled and almost totally quenched substrates provided in our EnzChek® Protease Assay Kits relieves the intramolecular self-quenching, yielding brightly fluorescent reaction products.

NOTE 1.5

Fluorescent Probes for Two-Photon Microscopy

Defining Characteristics of Two-Photon Excitation

Two-photon excitation (TPE) is a nonlinear optical process first predicted theoretically by Maria Göppert-Mayer in 1931.[1] Its application to fluorescence microscopy was pioneered much more recently by Denk, Strickler and Webb.[2] In TPE, a fluorophore is excited via near simultaneous absorption of two photons, each having half the energy (twice the wavelength) required for the transition from the ground to the first singlet excited state (Figure 1). The prerequisite for near-simultaneous absorption and the timescale of molecular light absorption (~10–16 seconds) dictates the use of specialized excitation sources; in current instruments, this is typically a mode-locked Ti-Sapphire,Ñ¢ laser delivering infrared light pulses of femtosecond duration at high repetition rates.[3] Two-photon excited fluorescence has a characteristic dependence on the square of the excitation light intensity; doubling the excitation intensity quadruples the fluorescence signal. In contrast, fluorescence derived from conventional one-photon absorption exhibits linear dependence on excitation light intensity.

There are many practical benefits to using TPE, given the transparency of tissues to infrared excitation light:

- Spatial confinement of fluorescence to a very small volume (~0.1 μm^3) defined by the focused excitation light, providing inherent 3D imaging capability (Figure 2)
- Capacity for imaging at increased depths in tissues [4]
- Confinement of photodamage and photobleaching effects to the excitation volume, resulting in increased viability of live specimens.[5,6]

However, in addition to requiring specialized (and therefore fairly expensive) excitation sources, TPE produces photodamage and photobleaching effects within the confined excitation volume that are often more acute than those produced by laser scanning confocal microscopy.[7–10] The advantages of TPE primarily relate to imaging of live specimens. Accordingly, neuroscience—specifically structural and functional imaging of the nervous system—is the largest field of current applications (Table 1). In addition to providing benefits for fluorescence microscopy, TPE offers advantages in other biophotonic techniques such as fluorescence correlation spectroscopy,[11] controlled photoablation,[12] photodynamic therapy [13] and activation of "caged" compounds.[14] To learn more about the technical foundations and applications of TPE microscopy, researchers should consult the growing collection of available review literature.[15–20]

Fluorescence Excitation and Emission Spectra

One-photon and two-photon excitation of a given fluorophore generally result in identical fluorescence emission spectra, as the originating excited state and the photon emission process are the same (Figure 1.1). However, two-photon excitation spectra differ from their one-photon counterparts to an extent that depends on the molecular orbital symmetry of the fluorophore (greater difference for higher symmetry fluorophores).[18] Consequently, most two-photon excitation spectra are blue-shifted and broader compared to the corresponding one-photon spectra plotted on a doubled wavelength axis. Simply stated, a fluorophore with a one-photon excitation peak at 500 nm will probably have a two-photon excitation maximum at <1,000 nm (Figure 3).

Because two-photon excitation spectra are relatively broad, multiplex detection schemes in which two or more fluorophores are excited at a single wavelength and discriminated on the basis of different emission spectra are relatively easy to implement (some examples are included in Table 1). The two-photon absorption cross-section (σ) in units of GM (for Göppert-Mayer; 1 GM = 10^{-50} cm^4 seconds) quantifies the efficiency of TPE for different fluorophores and is plotted on the y-axis of excitation spectra (Figure 3). There are several published collections of two-photon excitation spectra and cross-sections that provide guidance on compatibility of dyes and probes with excitation sources.[21–25] Excitation wavelengths used in selected published TPE microscopy applications are listed in Table 1.

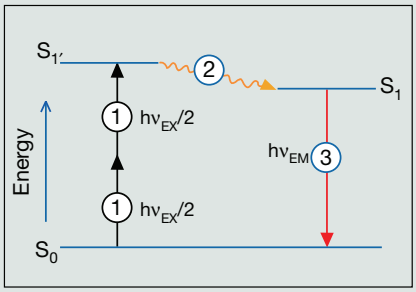

Figure 1 Excited-state energy diagram showing two-photon excitation (**1**), followed by nonradiative vibrational relaxation (**2**) and spontaneous fluorescence photon emission (**3**). In conventional fluorescence detection systems, excitation is achieved by absorption of a single photon of energy ($h\nu_{EX}$); processes (**2**) and (**3**) are essentially the same.

Figure 2 An experiment illustrating ordinary (single-photon) excitation of fluorescence and two-photon excitation. The cuvette contains a solution of the dye safranin O, which normally emits yellow light when excited by green light. The upper lens focuses green (543 nm) light from a CW helium–neon laser into the cuvette, producing the expected conical pattern of excitation (fading to the left). The lower lens focuses pulsed infrared (1046 nm) light from a neodymium–YLF laser. In two-photon absorption, the excitation is proportional to the square of the intensity; thus, the emission is confined to a small point focus (see arrow), which can be positioned anywhere in the cuvette by moving the illuminating beam. Image contributed by Brad Amos, Science Photo Library, London.

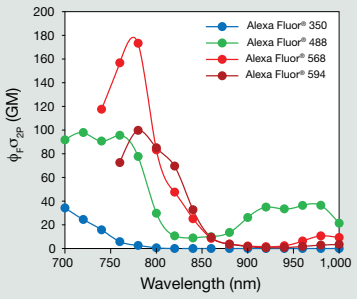

Figure 3 Two-photon excitation spectra of Alexa Fluor® 350, Alexa Fluor® 488, Alexa Fluor® 568 and Alexa Fluor® 594 dyes. The y-axis units are products of fluorescence quantum yields (Φ_F) and two-photon absorption cross-sections (σ). Data courtesy of Warren Zipfel, Cornell University.

Fluorescent Probes for TPE Microscopy

TPE has added a new spectral dimension to fluorescence microscopy. Probes such as fura-2 (for Ca^{2+}), SBFI (for Na^{+}), monochlorobimane (for glutathione), and DAPI (for nuclear DNA) were previously of limited utility in confocal microscopy due to their requirements for ultraviolet excitation; now, however, these probes have a new lease on life (Table 1). Furthermore, *in situ* imaging of small endogenous fluorophores, such as serotonin and NADH, that are almost inaccessible to one-photon excitation has now become practicable.[26] Organic fluorophores and fluorescent proteins typically have two-photon absorption cross-sections in the range 1–100 GM. However, fluorophores with smaller cross-sections (e.g., NADH, $\sigma < 0.1$ GM) can still generate sufficient TPE fluorescence for imaging purposes.[26] At the opposite extreme, Qdot® nanocrystals have cross-sections exceeding 10,000 GM, promising even further expansion of TPE imaging, particularly in the area of *in vivo* applications.[27,28]

1. Ann Phys (1931) 9:273; **2**. Science (1990) 248:73; **3**. Micros Res Tech (2005) 67:8; **4**. Nat Methods (2005) 2:932; **5**. Nature Biotech (1999) 17: 763; **6**. Real-Time Imaging (2002) 8:175; **7**. Chemphyschem (2005) 6:791; **8**. Biophys J (2001) 80:2029; **9**. Biophys J (2000) 78:2159; **10**. Appl Phys B (2001) 73:829; **11**. J Cell Biol (2005) 171:527; **12**. Mol Biol Cell (2003) 14:1808; **13**. Photochem Photobiol (2006) 82:443; **14**. Methods Enzymol (1998) 291:356; **15**. Neuron (2006) 50:823; **16**. Q Rev Biophys (2005) 38:97; **17**. Circ Res (2004) 95:1154; **18**. Nature Biotech (2003) 21:1369; **19**. Nat Methods (2005) 2:932; **20**. Handbook of Biological Confocal Microscopy, Third Edition (2006) p. 535; **21**. Proc Natl Acad Sci U S A (1996) 93:10763; **22**. J Microscopy (2002) 208: 108; **23**. J Biomed Opt (2003) 8: 329; **24**. J Neurosci Methods (2005) 148: 94; **25**. Biophys J (2004) 86:1726; **26**. Proc Natl Acad Sci U S A (2003) 100:7075; **27**. Science (2003) 300:1434; **28**. Science (2005) 307:538.

Table 1 Selected applications of fluorescent probes for two-photon excitation (TPE) microscopy.

Probe	TPE Excitation Wavelength	Application	References	Cat. No.
Alexa Fluor® 488 phalloidin	720 nm or 830 nm	Imaging F-actin organization in pancreatic acinar cells	J Biol Chem (2004) 279:37544	A12379
Alexa Fluor® 594 hydrazide	810 nm	Ca^{2+}-insensitive, neuronal tracer *	Neuron (2002) 33:439; www.stke.org/cgi/content/full/sigtrans; 2004/219/pl5	A10438, A10442
Amplex® Red reagent	750 nm or 800 nm	Detection of reactive oxygen species (ROS) associated with amyloid plaques	J Neurosci (2003) 23:2212	A12222, A22177
CFSE, CMTMR	820 nm	Tracking T and B lymphocytes and dendritic cell motility patterns in intact mouse lymph nodes †	Science (2002) 296:1869; Proc Natl Acad Sci U S A (2004) 101:998	C1157, C2927
CM-H_2DCFDA	740 nm	Detection of localized reactive oxygen species release in cardiomyocytes ‡	J Biol Chem (2003) 278: 44735	C6827
DAPI, Hoechst 33342	740 nm	Imaging DNA in nuclei and isolated chromosomes	Micron (2001) 32:679; Histochem Cell Biol (2000) 114:337	D1306, D3571, D21490, H1399, H3570, H21492
DiD	817 nm	Intravital imaging of mouse erythrocytes	Proc Natl Acad Sci U S A (2005) 102:16807	D307, D7757
FM® 1-43	840 nm	Monitoring synaptic vesicle recycling in rat brain slices	Biotechniques (2006) 40:343	T3163, T35356
Fluo-5F §	810 nm	Imaging Ca^{2+} concentration dynamics in dendrites and dendritic spines	Neuron (2002) 33:439; www.stke.org/cgi/content/full/sigtrans; 2004/219/pl5	F14221, F14222
Fura-2	780 nm	Detection of GABA-mediated Ca^{2+} transients in rat cerebellar Purkinje neurons	J Physiol (2001) 536:429	F1200, F1201, F1221, F1225, F6799, F14185
Lucifer yellow CH	850 nm	Identification of gap junctions in rat brain slices	J Neurosci (2003) 23:9254	L453, L682, L1177
Laurdan	800 nm	Detection of ordered membrane lipid domains	Proc Natl Acad Sci U S A (2003) 100:15554; J Cell Biol (2006) 174:725	D250
Monochloro-bimane	780 nm	Imaging glutathione levels in rat brain slices and intact mouse brain	J Biol Chem (2006) 281:17420	M1381MP
MQAE	750 nm	Fluorescence lifetime imaging (FLIM) of intracellular Cl^- concentrations in olfactory sensory neurons	J Neurosci (2004) 24:7931	E3101
Oregon Green® 488 BAPTA-1	880 nm	Imaging spatiotemporal relationships of Ca^{2+} signals among cell populations in rat brain cortex	Proc Natl Acad Sci U S A (2005) 102:14063	O6806, O6807
Qdot® 525, Qdot® 585, Qdot® 655 nanocrystals	750 nm	Multiplexed immunohistochemical analysis of arterial walls **	Am J Physiol (2006) 290:R114	Q11441MP, Q10111MP, Q11621MP, Q11421MP
SBFI	760 nm	Imaging of intracellular Na^+ gradients in rat cardiomyocytes	Biophys J (2004) 87:1360	S1262, S1263, S1264
TMRE	740 nm	Mitochondrial membrane potential sensor ‡	J Biol Chem (2003) 278:44735; Circulation (2006) 114:1497	T669
X-rhod-1	900 nm	Simultaneous imaging of GFP-PHD translocation and Ca^{2+} dynamics in cerebellar purkinje cells	J Neurosci (2004) 24:9513	X14210

* Used in combination with fluo-4, fluo-5F or fluo-4FF to obtain ratio signals that are insensitive to small changes in resting Ca^{2+} and are independent of subcellular compartment volume. † Multiplexed (single excitation/dual channel emission) combination of CFSE and CMTMR. § Techniques also applicable to fluo-4 and fluo-4FF indicators. ‡ Multiplexed (single excitation/dual channel emission) combination of TMRE and CM-H_2DCFDA. ** Multiplexed (single excitation/dual channel emission) combination of Qdot® 585 and Qdot® 655 nanocrystals. PHD = pleckstrin homology domain.

BODIPY® Nucleotide and Oligonucleotide Conjugates

With the exception of guanosine nucleotides, fluorescence quenching is usually not a problem if the BODIPY® derivative is conjugated to nucleotides, oligonucleotides, peptides or low molecular weight amines in which the stoichiometry of modification is 1:1. BODIPY® FL dye–labeled oligonucleotide primers also have lower photodestruction rates than fluorescein-labeled primers, improving the detectability of labeled DNA in sequencing gels.[38] Oligonucleotide conjugates of several of our BODIPY® dyes have been shown to be useful for DNA sequencing[39,40] (Section 8.2, Table 8.7), in part because the dye exhibits minimal effect on the mobility of the fragment during electrophoresis.[41] We also offer a ChromaTide® BODIPY® FL-14-dUTP for enzyme-mediated incorporation into nucleic acids (C7614, Section 8.2, Table 8.5).

In addition, we have prepared BODIPY® FL conjugates of ATP and GTP that are labeled through the ribose moieties and serve as structural probes of nucleotide-binding proteins (A12410, G12411; Section 17.3). The fluorescence of BODIPY® dyes is quenched by photoinduced electron transfer from proximal guanosine bases.[42,43] BODIPY® FL GTP therefore shows significant fluorescence quenching (Figure 1.4.8) that is relieved by binding to GTP-binding proteins (G-proteins). Longer-wavelength BODIPY® TR conjugates of ATP and GTP are also available (A22352, G22351; Section 17.3).

For protein-binding studies that require nonhydrolyzable nucleotides, we offer the BODIPY® FL fluorophore linked through the γ-thiol of ATP-γ-*S* (A22184, Figure 1.4.9) and the BODIPY® FL, BODIPY® 515/530 and BODIPY® TR fluorophores linked through the γ-thiol of GTP-γ-*S*[44] (G22183, G35779, G35780; Section 17.3). Like BODIPY® FL GTP, the fluorescence of the BODIPY® GTP-γ-*S* thioesters is quenched ~90% relative to that of the free dye but is recovered upon protein binding to at least some G-proteins.[44] The green-fluorescent BODIPY® FL GTP-γ-*S* has been used to detect GTP-binding proteins separated by capillary electrophoresis.[45] BODIPY® 515/530 GTP-γ-*S* thioester also exhibits green fluorescence and has a greater fluorescence increase upon protein binding, as compared with the BODIPY® FL GTP-γ-*S* thioester. The BODIPY® TR GTP-γ-*S* thioester is a red-fluorescent analog with spectral properties similar to those of the Texas Red® dye.

We also offer the green-fluorescent BODIPY® FL GTP-γ-*NH* amide (G35778, Section 17.3) as another choice for protein-binding studies. Although this analog exhibits less fluorescence enhancement upon protein binding, it is reportedly the best of the three green-fluorescent GTP-γ analogs for directly monitoring nucleotide exchange.[46] The different linker lengths of the green-fluorescent GTP-γ analogs (six-carbon for BODIPY® FL GTP-γ-*NH* amide, four-carbon for BODIPY® FL GTP-γ-*S* and one-carbon for BODIPY® 515/530 GTP-γ-*S*) may be useful for understanding protein active-site geometries.

In addition to their potential use for binding studies, the BODIPY® FL ATP-γ-*S* and BODIPY® FL GTP-γ-*S* thioesters are important substrates for Fhit (Figure 1.4.10), a member of the histidine triad superfamily of nucleotide-binding proteins that bind and cleave diadenosine polyphosphates.[47–49] Fhit, one of the most frequently inactivated proteins in lung cancer, functions as a tumor suppressor by inducing apoptosis.[48,50,51] These BODIPY® nucleotides should be especially useful for screening potential Fhit inhibitors and activators.

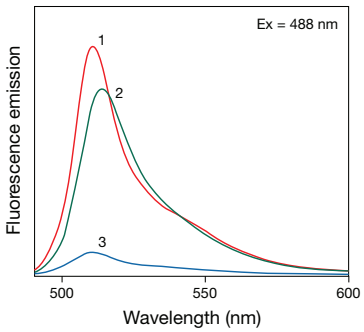

Figure 1.4.8 Fluorescence emission spectra of (1) free BODIPY® FL dye in phosphate-buffered saline, pH 7.2; (2) BODIPY® FL ATP (A12410); and (3) BODIPY® FL GTP (G12411). Samples were prepared with equal absorbance at the excitation wavelength (488 nm). The areas under the curves are therefore proportional to the relative fluorescence quantum yields, clearly showing the quenching effect caused by interaction of the BODIPY® FL fluorophore with the guanine base of GTP.

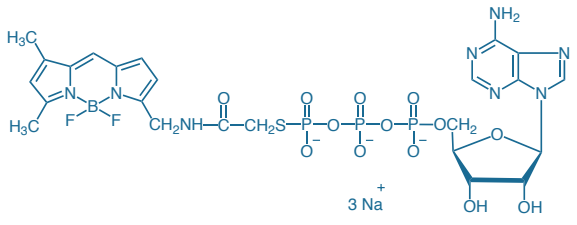

Figure 1.4.9 Adenosine 5'-O-(3-thiotriphosphate), BODIPY® FL thioester, sodium salt (BODIPY® FL ATP-γ-S, thioester, A22184).

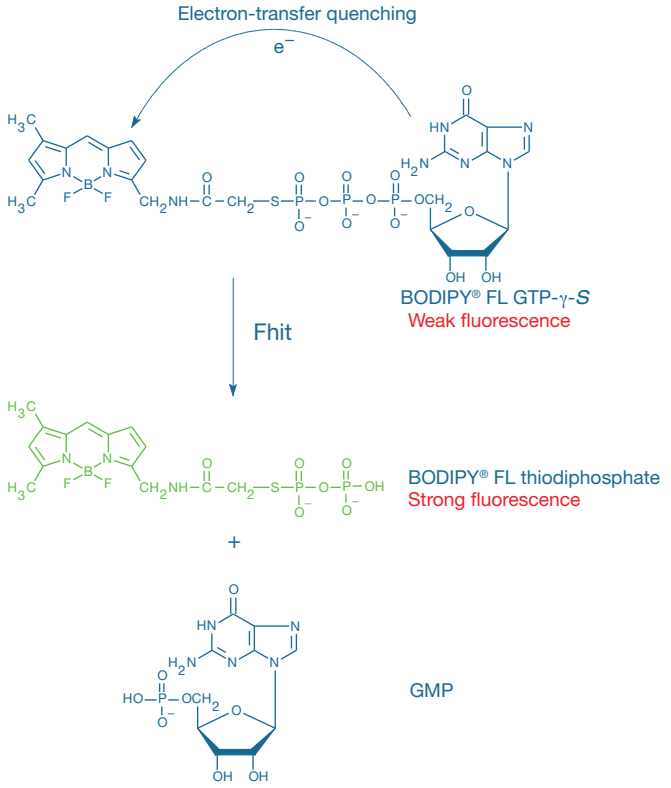

Figure 1.4.10 Principle of fluorescence-based detection of the diadenosine triphosphate hydrolase activity of Fhit using BODIPY® FL GTP-γ-*S* thioester (G22183) as a substrate analog.

BODIPY® Lipids and BODIPY® Receptor Ligand Conjugates

BODIPY® dye conjugates of lipids, toxins, steroids, drug analogs and receptor probes typically have quantum yields approaching unity, especially in organic solvents. The low polarity of the BODIPY® fluorophore makes probes containing these dyes excellent analogs of biological lipids (Chapter 13). Consequently, these probes are well tolerated by enzymes that metabolize lipids, including phospholipases and sphingomyelinases (Section 17.4, Table 17.3). In most cases, lack of a spectral shift in the metabolic product's fluorescence requires use of an easy extraction and chromatographic separation step to detect product formation, with quantitation possible by photography or with a fluorescence- or absorption-based scanner. BODIPY® derivatives of lipids and low molecular weight ligands include analogs of:

- Phospholipids and fatty acids, numerous versions of which are described in Section 13.2
- Sphingolipids, including several ceramide derivatives (Section 13.3)
- Cholesterol (C3927MP, Section 13.3)
- Cytochalasin D, an actin-polymerization inhibitor (C12377, Section 11.1)
- Paclitaxel (Taxol®), for staining tubulin in isolated preparations (P7500, P7501; Section 11.2)
- Vinblastine, a microtubule-disrupting agent (V12390, Section 11.2)
- Vancomycin, an antibiotic (V34850, Section 15.2)
- Penicillin V (B13233, B13234; Section 15.2)
- Verapamil, for investigating multidrug resistance (B7431, Section 15.6)
- Forskolin, an adenylate cyclase activator (B7469, Section 15.6)
- Prazosin, an α_1-adrenergic receptor probe (B7433, Section 16.2)
- Ouabain, a cardiac glycoside (B23461, Section 16.3)
- Glibenclamide, a probe for potassium channels (E34250, E34251; Section 16.3)
- Dihydropyridine derivative that is selective for Ca^{2+} channels (D7443, S7445; Section 16.3)
- Thapsigargin, which promotes Ca^{2+} release by inhibiting the endoplasmic reticulum Ca^{2+}-ATPase (B7487, B13800; Section 17.2)

In addition to the BODIPY® dye conjugates of receptor ligands in the list above, we have utilized BODIPY® dyes for synthesis of several LysoTracker® and LysoSensor™ dyes, as well as BODIPY® FL histamine (B22461, Figure 1.4.11), which are extremely useful probes for labeling acidic organelles in live cells. These products are discussed in Section 12.3.

DQ™ Reagents: Heavily Labeled BODIPY® Dye Conjugates as Fluorogenic Enzyme Substrates

We have found BODIPY® dye conjugates to be very useful reagents for numerous bioanalytical screening applications. In particular, we have utilized the tendency of BODIPY® dyes to quench their fluorescence on conjugation to certain biopolymers to our advantage (Figure 1.4.7) in the following enzyme-assay kits and reagents:

- EnzChek® Protease Assay Kits, which contain almost nonfluorescent casein derivatives that are heavily labeled with either the green-fluorescent BODIPY® FL dye (E6638; Section 10.4) or red-fluorescent BODIPY® TR-X dye (E6639, R22132; Section 10.4)
- EnzChek® Elastase Assay Kit (E12056, Section 10.4), with DQ™ elastin, a quenched BODIPY® FL conjugate
- EnzChek® *Ultra* Amylase Assay Kit (E33651, Section 10.2), containing a highly quenched BODIPY® FL starch derivative
- DQ™ Green BSA (D12050, Section 10.4) and DQ™ Red BSA (D12051, Section 10.4), heavily labeled and almost nonfluorescent BODIPY® BSA conjugates that yield intense green or red fluorescence upon proteolytic digestion
- DQ™ ovalbumin (D12053, Section 10.4,), a heavily labeled and almost nonfluorescent BODIPY® FL ovalbumin conjugate

Conjugation of either the BODIPY® FL dye (excitation/emission maxima ~500/506 nm) or BODIPY® TR dye (excitation/emission maxima ~589/617 nm) to a biopolymer at high degrees of substitution (DOS) results in almost total quenching of the conjugate's fluorescence; they typically exhibit <3% of the fluorescence of the corresponding free dyes. Enzyme-catalyzed hydrolysis relieves this quenching, yielding brightly fluorescent BODIPY® FL dye– or BODIPY® TR-X dye–labeled peptides (Figure 1.4.7), or, in the case of the BODIPY® FL amylase substrate in the EnzChek® *Ultra* Amylase Assay Kit, BODIPY® FL dye–labeled carbohydrates. The increase in fluorescence, which can be measured with a spectrofluorometer, minifluorometer or fluorescence microplate reader, is proportional to enzymatic activity. The DQ™ BSA and DQ™ ovalbumin substrates are particularly suitable for the study of receptor labeling and antigen processing. DQ™ BSA conjugates can be targeted to Fc receptors after they are complexed with our anti-BSA antibody (A11133, Section 7.5). Ovalbumin is efficiently processed through mannose receptor–mediated endocytosis by antigen-presenting cells and is widely used for studying antigen processing. Upon endocytosis and proteolysis, highly fluorescent peptides are released within intracellular vacuoles. DQ™ ovalbumin appears to be an excellent indicator of macrophage-mediated antigen processing in flow cytometry and microscopy assays.

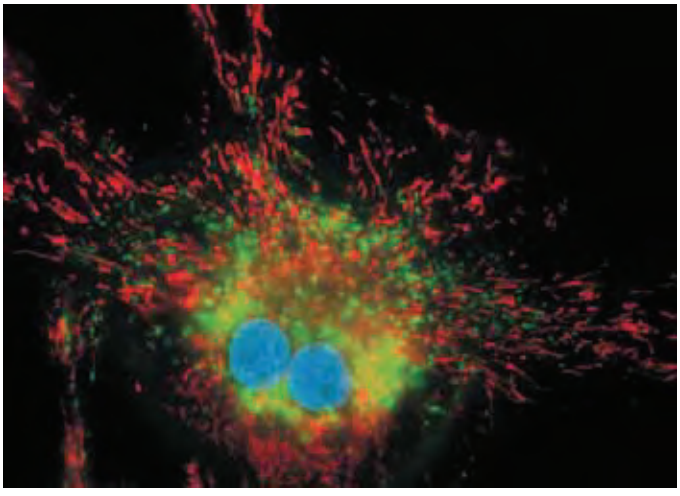

Figure 1.4.11 Viable bovine pulmonary artery endothelial cells simultaneously stained with BODIPY® FL histamine (B22461), MitoTracker® Red CMXRos (M7512) and Hoechst 33342 (H1399, H3570, H21492). Green-fluorescent BODIPY® FL histamine localized to lysosomes, red-fluorescent MitoTracker® Red CMXRos accumulated in the mitochondria and the blue-fluorescent Hoechst 33342 dye stained the nuclei. This multiple-exposure image was acquired with band-pass filters appropriate for fluorescein, the Texas Red® dye and DAPI.

BODIPY® Dye Conjugates for Fluorescence Polarization–Based Assays

When a fluorescent molecule tethered to a protein is excited by polarized fluorescent light, the polarization of fluorescence emission is dependent on the rate of molecular tumbling. Upon proteolytic cleavage of the fluorescently labeled protein, the smaller peptides that result tumble faster and the emitted light is depolarized relative to the light measured from the intact conjugate (Fluorescence Polarization (FP)—Note 1.4). Fluorescence polarization technology is more sensitive than many other nonradioactive assays for proteases and allows measurements to be taken in real time, permitting the collection of kinetic data.

The relatively long fluorescence lifetimes (typically >5 nanoseconds) at visible wavelengths, good anisotropy properties, high molar absorptivity and fluorescence intensity and lack of pH sensitivity in the spectra of the BODIPY® dyes have been shown to make these dyes the preferred fluorophores for high-throughput fluorescence polarization–based assays. The EnzChek® Polarization Assay Kit for Proteases (E6658, Section 10.4) contains green-fluorescent BODIPY® FL casein with an optimal degree of labeling for fluorescence polarization–based protease assays. BODIPY® dye conjugates of nucleotides, peptides and drug analogs are available or are readily prepared from the chemically reactive BODIPY® dyes. Fluorescence polarization–based assays for G-protein–coupled receptors, kinases and phosphatases and for high-affinity receptors are particularly important when screening for new drug candidates.

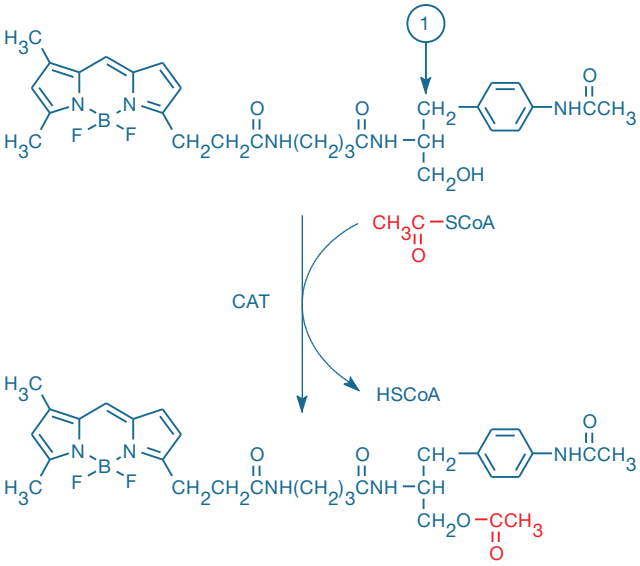

Figure 1.4.12 The green-fluorescent BODIPY® FL 1-deoxychloramphenicol substrate in our *FAST* CAT® Green (deoxy) Chloramphenicol Acetyltransferase Assay Kit (F6616). CAT-mediated acetylation of this substrate and of the BODIPY® TMR 1-deoxychloramphenicol in our *FAST* CAT® Yellow (deoxy) Chloramphenicol Acetyltransferase Assay Kit (F6617) results in single fluorescent products because these substrates contain only one hydroxyl group that can be acetylated. In contrast, the BODIPY® FL chloramphenicol substrate in our original *FAST* CAT® Kit (F2900) contains a second hydroxyl group at the 1-position (indicated by the labeled arrow). This hydroxyl group undergoes a nonenzymatic transacetylation step, restoring the original hydroxyl for a second acetylation. CAT-mediated acetylation of this chloramphenicol substrate produces three fluorescent products, thus complicating the analysis.

BODIPY® Substrates for Chloramphenicol Acetyltransferase

Chloramphenicol acetyltransferase (CAT), an enzyme that is encoded by an important reporter gene, can acetylate chloramphenicol derivatives that incorporate the BODIPY® fluorophore (Figure 1.4.12). The acetylated products are readily separated from the substrate by thin-layer chromatography (Figure 1.4.13) and quantitated by photography, fluorometry or with a plate scanner. Our original *FAST* CAT® Chloramphenicol Acetyltransferase Assay Kit[52,53] (F2900) and improved *FAST* CAT® (deoxy) Chloramphenicol Acetyltransferase Assay Kit (F6616) utilize a green-fluorescent BODIPY® FL substrate, and the *FAST* CAT® Yellow (deoxy) Chloramphenicol Acetyltransferase Assay Kit (F6617) employs a yellow-fluorescent BODIPY® TMR 1-deoxychloramphenicol substrate. These products are described in detail in Section 10.6.

Additional Methods of Analysis Using BODIPY® Dye Conjugates

In addition to their general utility for the intensity-based and fluorescence polarization–based assays described above, the BODIPY® dyes are near optimal for a variety of other bioanalytical techniques:

- The spectral variety and high absorbance of the BODIPY® dyes (Figure 1.4.1) permits their use as efficient donor or acceptor dyes for numerous assays that use fluorescence resonance energy transfer, including internally quenched endopeptidase substrates[54] (Section 10.4), nucleic acid hybridization assays and receptor-binding assays (Fluorescence Resonance Energy Transfer (FRET)—Note 1.2).
- BODIPY® dye conjugates of peptides are readily separated by chromatographic means and can be used to detect the activity of enzymes that catalyze secondary modifications, such as phosphorylation/dephosphorylation, glycosylation/deglycosylation, oxidation/reduction, myristoylation, farnesylation and peptide–peptide crosslinking.
- Hydrolysis of peptides that are singly labeled with BODIPY® dyes to smaller peptides can be detected chromatographically with extremely high sensitivity.
- With their high peak intensity and narrow emission spectra, reactive BODIPY® dyes are among the most detectable amine-derivatization reagents available for HPLC and capillary electrophoresis; thus, amine-containing metabolites can be derivatized with succinimidyl esters of the BODIPY® dyes (Table 1.7) for ultrasensitive analysis.[7,55]

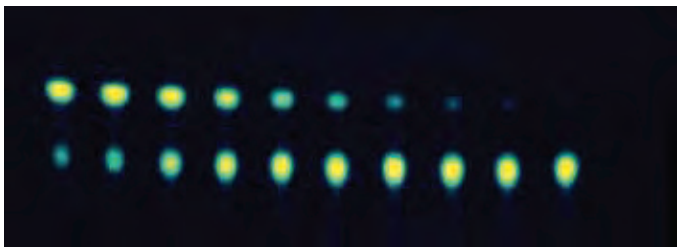

Figure 1.4.13 Chloramphenicol acetyltransferase (CAT) assays using our *FAST* CAT® Yellow (deoxy) Chloramphenicol Acetyltransferase Assay Kit (F6617). Decreasing amounts of purified CAT enzyme (2-fold dilutions) were incubated with the corresponding deoxy substrate in the presence of acetyl CoA; the reaction mixture was then separated with standard thin-layer chromatography (TLC) methods and visualized with 366 nm epi-illumination. The bottom row of fluorescent spots in each TLC represents the substrate; the top, the monoacetylated reaction product.

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1. Angew Chem Int Ed Engl (2008) 47:1184; **2**. Bioorg Med Chem Lett (2006) 16:3886; **3**. J Med Chem (2007) 50:782; **4**. Pharmacol Ther (2003) 100:101; **5**. Electrophoresis (2007) 28:3901; **6**. Electrophoresis (2005) 26:2599; **7**. Anal Chem (2007) 79:345; **8**. Electrophoresis (2004) 25:839; **9**. J Biochem Biophys Methods (2000) 42:137; **10**. Anal Biochem (1999) 275:62; **11**. Jpn J Physiol (1995) 45:673; **12**. Anal Biochem (1996) 243:1; **13**. Diabetes (2002) 51 Suppl 1:S25; **14**. J Opt Soc Am B (1996) 13:481; **15**. J Am Chem Soc (1994) 116:7801; **16**. Optical Microscopy for Biology, Herman B, Jacobson K, Eds. 1990; p. 143; **17**. J Am Chem Soc (2002) 124:196; **18**. J Microsc (1992) 168:219; **19**. Chemphyschem (2008) 9:2019; **20**. Bioconjug Chem (2003) 14:195; **21**. Biophys J (2002) 83:605; **22**. Biochim Biophys Acta (1992) 1104:9; **23**. Biochim Biophys Acta (1984) 776:217; **24**. Biochemistry (1982) 21:978; **25**. J Am Chem Soc (2005) 127:12766; **26**. Biophys J (2009) 96:226; **27**. ACS Chem Biol (2008) 3:555; **28**. Nat Methods (2006) 3:923; **29**. Nat Biotechnol (2003) 21:1093; **30**. J Recept Signal Transduct Res (2002) 22:333; **31**. Proc Natl Acad Sci U S A (2004) 101:5488; **32**. Tetrahedron Lett (1999) 40:1471; **33**. Bioconjug Chem (2006) 17:1612; **34**. Anal Biochem (1986) 156:220; **35**. Bioconjug Chem (2003) 14:1133; **36**. Endocrinology (1997) 138:296; **37**. Lett Pept Sci (1995) 1:235; **38**. Electrophoresis (1992) 13:542; **39**. Proc Natl Acad Sci U S A (2005) 102:5926; **40**. Proc Natl Acad Sci U S A (2005) 102:5346; **41**. Electrophoresis (1997) 18:2893; **42**. Anal Sci (2001) 17:155; **43**. Nucleic Acids Res (2001) 29:E34; **44**. J Biol Chem (2001) 276:29275; **45**. Anal Chem (2003) 75:4297; **46**. Proc Natl Acad Sci U S A (2004) 101:2800; **47**. Proc Natl Acad Sci U S A (2003) 100:1592; **48**. Curr Biol (2000) 10:907; **49**. J Biol Chem (2000) 275:4555; **50**. Am J Pathol (2000) 156:419; **51**. J Natl Cancer Inst (2000) 92:338; **52**. Biotechniques (1990) 8:170; **53**. Anal Biochem (1991) 197:401; **54**. Nat Biotechnol (2000) 18:1071; **55**. Electrophoresis (2008) 29:4900.

DATA TABLE 1.4 BODIPY® DYE SERIES

Cat. No.	MW	Storage	Soluble	Abs	EC	Em	Solvent	Notes
B10006	542.19	F,D,L	H_2O, DMSO	502	80,000	510	MeOH	1, 2
D2183	292.09	F,L	DMSO, MeCN	505	91,000	511	MeOH	1
D2184	389.16	F,D,L	DMSO, MeCN	502	82,000	510	MeOH	1, 3
D2187	513.31	F,D,L	DMSO, MeCN	534	77,000	551	MeOH	1
D2191	417.22	F,D,L	DMSO, MeCN	500	79,000	509	MeOH	1
D2219	443.23	F,D,L	DMSO, MeCN	559	97,000	568	MeOH	1
D2222	463.25	F,D,L	DMSO, MeCN	563	142,000	569	MeOH	1
D2225	426.19	F,D,L	DMSO, MeCN	575	83,000	588	MeOH	1
D2228	489.28	F,D,L	DMSO, MeCN	581	136,000	591	MeOH	4
D3834	320.15	F,L	DMSO, MeCN	505	96,000	511	MeOH	1
D6102	502.32	F,D,L	DMSO, MeCN	504	85,000	510	MeOH	1
D6116	634.46	F,D,L	DMSO, MeCN	588	68,000	616	MeOH	1, 5
D6117	608.45	F,D,L	DMSO, MeCN	544	60,000	570	MeOH	1
D6140	491.20	F,D,L	H_2O, DMSO	502	75,000	510	MeOH	1, 6
D6180	437.21	F,D,L	DMSO, MeCN	528	70,000	547	MeOH	1
D6184	417.22	F,D,L	DMSO, MeCN	504	87,000	511	MeOH	1
D10000	660.50	F,D,L	DMSO, MeCN	625	101,000	640	MeOH	1, 7
D10001	643.45	F,D,L	DMSO, MeCN	646	102,000	660	MeOH	1

For definitions of the contents of this data table, see "Using *The Molecular Probes® Handbook*" in the introductory pages.

Notes

1. The absorption and fluorescence spectra of BODIPY® derivatives are relatively insensitive to the solvent.
2. This sulfotetrafluorophenyl (STP) ester derivative is water soluble and may be dissolved in buffer at ~pH 8 for reaction with amines. Long-term storage in water is NOT recommended due to hydrolysis.
3. The fluorescence lifetime (τ) of D2184 in MeOH at 20°C is 5.7 nanoseconds. Data provided by the SPEX Fluorescence Group, Horiba Jobin Yvon Inc.
4. Oxidation of the polyunsaturated butadienyl portion of the BODIPY® 581/591 dye results in a shift of the fluorescence emission peak from ~590 nm to ~510 nm. (Methods Enzymol (2000) 319:603, FEBS Lett (1999) 453:278)
5. The fluorescence lifetime (τ) of D6116 in MeOH at 20°C is 5.4 nanoseconds. Data provided by the SPEX Fluorescence Group, Horiba Jobin Yvon Inc.
6. This sulfonated succinimidyl ester derivative is water soluble and may be dissolved in buffer at ~pH 8 for reaction with amines. Long-term storage in water is NOT recommended due to hydrolysis.
7. The fluorescence lifetime (τ) of the BODIPY® 630/650 dye at 20°C is 3.9 nanoseconds in H_2O and 4.4 nanoseconds in EtOH. (Bioconjug Chem (2003) 14:195)

PRODUCT LIST 1.4 BODIPY® DYE SERIES

Cat. No.	Product	Quantity
B10006	BODIPY® FL, STP ester, sodium salt	5 mg
D3834	4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-pentanoic acid (BODIPY® FL C_5)	1 mg
D6184	4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-pentanoic acid, succinimidyl ester (BODIPY® FL C_5, SE)	5 mg
D2183	4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionic acid (BODIPY® FL)	5 mg
D2184	4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionic acid, succinimidyl ester (BODIPY® FL, SE)	5 mg
D6140	4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionic acid, sulfosuccinimidyl ester, sodium salt (BODIPY® FL, SSE)	5 mg
D6102	6-((4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionyl)amino)hexanoic acid, succinimidyl ester (BODIPY® FL-X, SE)	5 mg
D6117	6-((4,4-difluoro-1,3-dimethyl-5-(4-methoxyphenyl)-4-bora-3a,4a-diaza-s-indacene-2-propionyl)amino)hexanoic acid, succinimidyl ester (BODIPY® TMR-X, SE)	5 mg
D2187	4,4-difluoro-5,7-diphenyl-4-bora-3a,4a-diaza-s-indacene-3-propionic acid, succinimidyl ester (BODIPY® 530/550, SE)	5 mg
D6180	4,4-difluoro-5-phenyl-4-bora-3a,4a-diaza-s-indacene-3-propionic acid, succinimidyl ester (BODIPY® R6G, SE)	5 mg
D2228	4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene-3-propionic acid, succinimidyl ester (BODIPY® 581/591, SE)	5 mg
D2225	4,4-difluoro-5-(2-pyrrolyl)-4-bora-3a,4a-diaza-s-indacene-3-propionic acid, succinimidyl ester (BODIPY® 576/589, SE)	5 mg
D10001	6-(((4,4-difluoro-5-(2-pyrrolyl)-4-bora-3a,4a-diaza-s-indacene-3-yl)styryloxy)acetyl)aminohexanoic acid, succinimidyl ester (BODIPY® 650/665-X, SE)	5 mg
D2222	4,4-difluoro-5-styryl-4-bora-3a,4a-diaza-s-indacene-3-propionic acid, succinimidyl ester (BODIPY® 564/570, SE)	5 mg
D2191	4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-s-indacene-8-propionic acid, succinimidyl ester (BODIPY® 493/503, SE)	5 mg
D2219	4,4-difluoro-5-(2-thienyl)-4-bora-3a,4a-diaza-s-indacene-3-propionic acid, succinimidyl ester (BODIPY® 558/568, SE)	5 mg
D6116	6-(((4-(4,4-difluoro-5-(2-thienyl)-4-bora-3a,4a-diaza-s-indacene-3-yl)phenoxy)acetyl)amino)hexanoic acid, succinimidyl ester (BODIPY® TR-X, SE)	5 mg
D10000	6-(((4,4-difluoro-5-(2-thienyl)-4-bora-3a,4a-diaza-s-indacene-3-yl)styryloxy)acetyl)aminohexanoic acid, succinimidyl ester (BODIPY® 630/650-X, SE)	5 mg

1.5 Fluorescein, Oregon Green® and Rhodamine Green™ Dyes

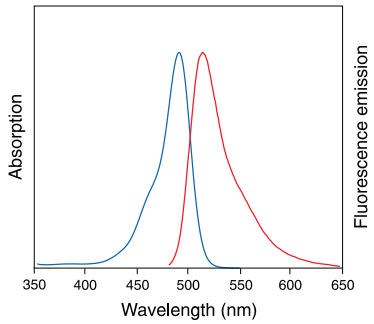

Figure 1.5.1 Absorption and fluorescence emission spectra of fluorescein in pH 9.0 buffer.

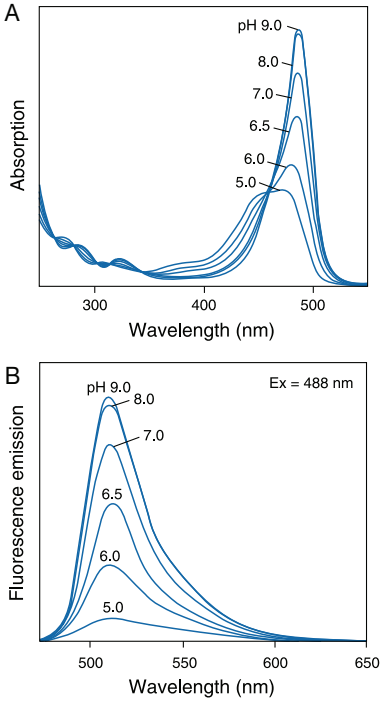

Figure 1.5.2 The pH-dependent spectra of fluorescein (F1300): **A**) absorption spectra, **B**) emission spectra.

Fluorescein

The amine-reactive fluorescein derivatives (Table 1.8) have been the most common fluorescent derivatization reagents for covalently labeling proteins. In addition to its relatively high absorptivity, excellent fluorescence quantum yield and good water solubility, fluorescein (F1300, Figure 1.5.1) has an excitation maximum (494 nm) that closely matches the 488 nm spectral line of the argon-ion laser, making it an important fluorophore for confocal laser-scanning microscopy[1] and flow cytometry applications. In addition, fluorescein's protein conjugates are not inordinately susceptible to precipitation. Because it can be prepared in high purity, fluorescein is one of the five dyes in the Reference Dye Sampler Kit[2] (R14782, Section 23.1). We are also the source of the NIST-traceable fluorescein standard (F36915) described below.

NIST-Traceable Fluorescein Standard

The National Institute of Standards and Technology (NIST) chose a high-grade fluorescein synthesized in our laboratories to create Standard Reference Material 1932 (SRM® 1932), a certified fluorescein solution. We now offer a NIST-traceable fluorescein standard (F36915) that not only meets the stringent criteria established by NIST, but is also directly traceable to SRM® 1932. We supply our NIST-traceable fluorescein standard as a calibrated 50 µM solution of fluorescein in 100 mM sodium borate buffer, pH 9.5; under these conditions, fluorescein is completely ionized[3] and is therefore in its most fluorescent form (Figure 1.5.2), exhibiting an extremely high quantum yield of 0.93 (Section 20.2).

Table 1.8 Amine-reactive xanthene derivatives in this section.

Fluorophore (Abs/Em) *	COOH	Succinimidyl Ester	Other	Protein and Nucleic Acid Labeling Kits	Notes
Eosin (524/544)			E18 (ITC)[5]		• Useful for DAB photoconversion • Phosphorescent
Fluorescein (494/518)	C1359[5] C1360[6] C1904[M]	C2210[5] C6164[6] C1311[M] F6106 (X)[6] F2181 (X)[M] F6129 (X)[M] F6130 (EX)[5] C20050 (C)[5]	F143 (ITC)[5] F1906 (ITC)[5] F1907 (ITC)[5] D16 (DTA)[5]	F6433 (F) F6434 (F) F10240 (P) Z25042 (Z) Z25342 (Z)	• Most widely used green-fluorescent labeling dye • Absorption overlaps the 488 nm spectral line of the argon-ion laser • Prone to photobleaching • pH-sensitive spectra between pH 5 and pH 8 • Common donor in FRET applications that utilize tetramethylrhodamine as the acceptor
JOE (520/548)		C6171MP[6]			• Traditional fluorophore used in automated DNA sequencing • pH-insensitive spectra at pH >6
Oregon Green® 488 (496/524)	O6146[5]	O6147[5] O6149[6] O6185 (X)[6]	O6080[M]	F6153 (F) O10241 (P) A10476 (APEX®) U21659 (U)	• Photostable fluorescein substitute • pH-insensitive spectra at pH >6 • Recognized by anti-fluorescein antibodies
Oregon Green® 514 (511/530)	O6138[M]	O6139[6]			• Photostable fluorescein substitute • pH-insensitive spectra at pH >6
Rhodamine Green™ (502/527)		R6107[M] R6113 (X)[M]			• Photostable fluorescein substitute • pH-insensitive spectra

* The numbers in parentheses reflect the absorption (Abs) and fluorescence emission (Em) maxima, in nm, of the goat anti–mouse IgG antibody or dextran conjugates in aqueous buffer. (APEX®) = APEX® Antibody Labeling Kit (Section 1.2). (C) = Caged; the probe is nonfluorescent until the caging group is removed by UV illumination. (COOH) = Carboxylic acid. (DTA) = Dichlorotriazine. (EX) = A seven-atom spacer that is more hydrophilic than X. (F) = FluoReporter® Protein Labeling Kit (Section 1.2). (ITC) = Isothiocyanate. (5) = 5-Isomer. (6) = 6-Isomer. (M) = Mixture of 5- and 6-isomers. (P) = Easy-to-Use Protein Labeling Kit (Section 1.2). (U) = ULYSIS® Nucleic Acid Labeling Kit (Section 8.2). (X) = Aminohexanoyl spacer separating the dye and SE. (Z) = Zenon® Antibody Labeling Kit (Section 7.3).

Academic researchers and industry scientists alike can use our NIST-traceable fluorescein standard to assess day-to-day or experiment-to-experiment variation in fluorescence-based instrumentation, as well as to determine the Molecules of Equivalent Soluble Fluorophore (MESF) value for an experimental solution. The MESF value is defined not as the actual number of dye molecules present, but rather as the number of fluorophores that would yield a fluorescence intensity equivalent to that of the experimental solution when analyzed on the same instrument under the same conditions.[4–6] Consequently, the MESF value is an important tool for characterizing the fluorescence intensity of a solution containing spectrally similar dye molecules attached to antibodies, nucleic acids, microspheres or other substrates that might enhance or diminish the fluorescence. When its pH is carefully matched with that of the experimental solution, our NIST-traceable fluorescein standard can be used for accurate MESF determinations of a wide range of green-fluorescent dye solutions and on an assortment of fluorescence-based instruments.

Limitations of Fluorescein

Even though fluorescein has been used to derivatize biomolecules for decades, fluorescein-based dyes and their conjugates have several significant drawbacks, including:

- A relatively high rate of photobleaching[7–9] (Figure 1.5.3, Figure 1.5.4)
- pH-sensitive fluorescence[10,11] (pK_a ~6.4) that is significantly reduced below pH 7 (Figure 1.5.5)
- A relatively broad fluorescence emission spectrum, limiting their utility in some multicolor applications
- A tendency toward quenching of their fluorescence on conjugation to biopolymers, particularly at high degrees of labeling (Figure 1.5.6)

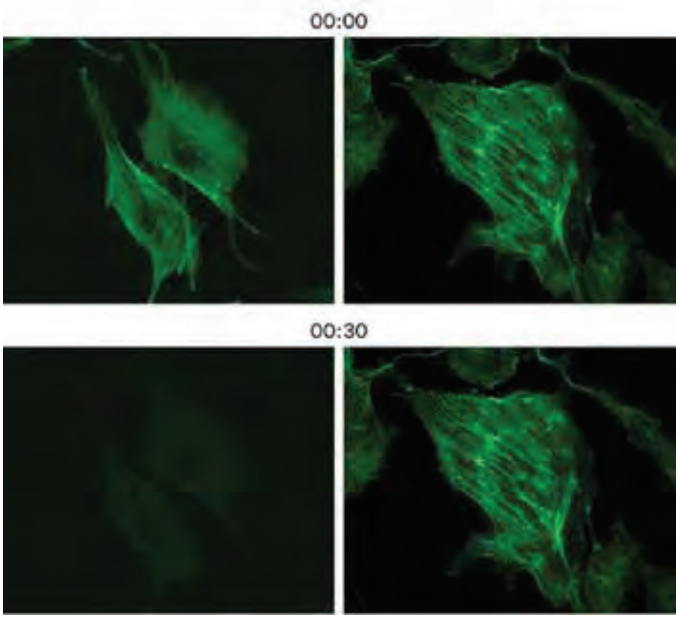

Figure 1.5.4 Bovine pulmonary artery endothelial cells (BPAEC) were labeled with fluorescein phalloidin (left panels, F432), or Alexa Fluor® 488 phalloidin (right panels, A12379), which labels filamentous actin, and mounted in PBS. The cells were placed under constant illumination on the microscope with an FITC filter set using a 60× objective. Images were acquired at 1-second intervals for 30 seconds. Under these illumination conditions, fluorescein photobleached to about 20% of its initial value in 30 seconds; the fluorescence of Alexa Fluor® 488 phalloidin stayed at the initial value under the same illumination conditions.

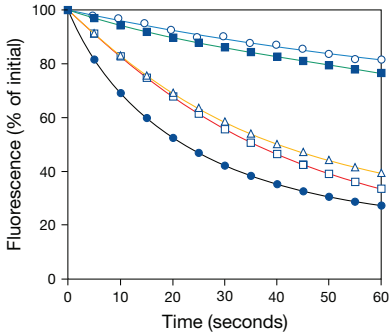

Figure 1.5.3 Comparison of photostability of green-fluorescent antibody conjugates. The following fluorescent goat anti–mouse IgG antibody conjugates were used to detect mouse anti–human IgG antibody labeling of human anti-nuclear antibodies in HEp-2 cells on prefixed test slides (INOVA Diagnostics Corp.): Oregon Green® 514 (O6383, ■), Alexa Fluor® 488 (A11001, ○), BODIPY® FL (B2752, △), Oregon Green® 488 (O6380, □) or fluorescein (F2761, ●). Samples were continuously illuminated and viewed on a fluorescence microscope using a fluorescein longpass filter set. Images were acquired every 5 seconds. For each conjugate, three data sets, representing different fields of view, were averaged and then normalized to the same initial fluorescence intensity value to facilitate comparison.

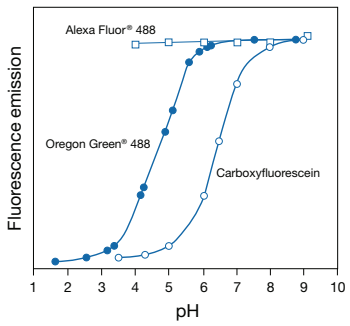

Figure 1.5.5 Comparison of pH-dependent fluorescence of the Oregon Green® 488 (●), carboxyfluorescein (○) and Alexa Fluor® 488 (□) fluorophores. Fluorescence intensities were measured for equal concentrations of the three dyes using excitation/emission at 490/520 nm.

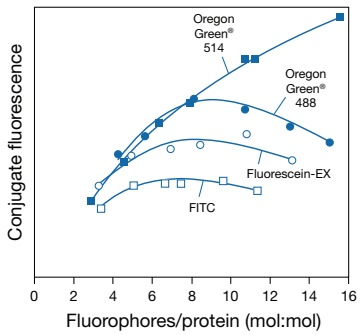

Figure 1.5.6 Comparison of relative fluorescence as a function of the number of fluorophores attached per protein for goat anti–mouse IgG antibody conjugates prepared using Oregon Green® 514 carboxylic acid succinimidyl ester (O6139, ■), Oregon Green® 488 carboxylic acid succinimidyl ester (O6147, ●), fluorescein-5-EX succinimidyl ester (F6130, ○) and fluorescein isothiocyanate (FITC, F143, F1906, F1907, □). Conjugate fluorescence is determined by measuring the fluorescence quantum yield of the conjugated dye relative to that of the free dye and multiplying by the number of fluorophores per protein.

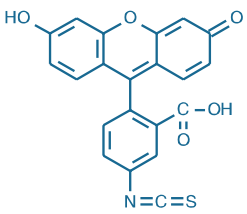

Figure 1.5.7 Fluorescein-5-isothiocyanate (FITC 'Isomer I', F143).

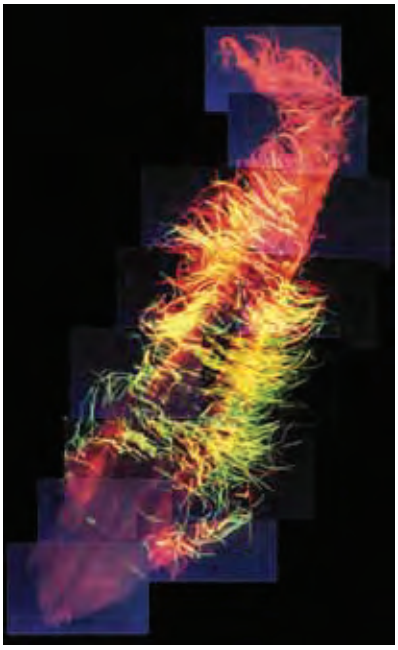

Figure 1.5.8 Two proteobacterial symbionts localized with phylotype-specific 16S rRNA–directed oligonucleotide probes labeled with either fluorescein-5-isothiocyanate (F143, F1906, F1907) or Texas Red® sulfonyl chloride (T353, T1905). The filamentous bacteria are attached to a hair-like structure secreted from a pore on the dorsal surface of the deep-sea hydrothermal vent polychaete *Alvinella pompejana*. Image contributed by M. Cottrell and C. Cary, College of Marine Studies, University of Delaware.

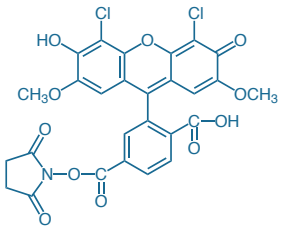

Figure 1.5.9 6-carboxy-4',5'-dichloro-2',7'-dimethoxyfluorescein, succinimidyl ester (6-JOE, SE; C6171MP).

The photobleaching and pH sensitivity of fluorescein make quantitative measurements with this fluorophore problematic. Furthermore, fluorescein's relatively high photobleaching rate limits the sensitivity that can be obtained, a significant disadvantage for applications requiring ultrasensitive detection, such as DNA sequencing, fluorescence *in situ* hybridization and localization of low-abundance receptors. These limitations have encouraged the development of alternative fluorophores.

There are no new dyes available that completely solve fluorescein's photobleaching problems, but we have developed some excellent dyes whose spectra mimic those of fluorescein—the Alexa Fluor® 488 (Section 1.3), BODIPY® FL (Section 1.4), Oregon Green® 488, Oregon Green® 514 and Rhodamine Green™ dyes (this section). These dyes are much more photostable than fluorescein and have less or no pH sensitivity in the physiological pH range. When compared with fluorescein, all of these dyes exhibit the same or slightly longer-wavelength spectra (absorption maxima ~490–515 nm) and comparably high fluorescence quantum yields. Alternatively, where they can be used, our yellow-green fluorescent FluoSpheres® microspheres and our Qdot® nanocrystals (Section 6.5, Section 6.6, respectively) provide a means of preparing bioconjugates that have a combination of fluorescence intensity and photostability far superior to that of any simple dye conjugate.

Single-Isomer Fluorescein Isothiocyanate (FITC) Preparations

Despite the availability of alternative amine-reactive fluorescein derivatives that yield conjugates with superior stability and comparable spectra, fluorescein isothiocyanate (FITC) remains one of the most popular fluorescent labeling reagents.[12] The synthesis of fluorescein isothiocyanate, carboxyfluorescein (FAM) and similar fluorescein-derived reagents yields a mixture of isomers at the 5- and 6-positions of fluorescein's carboxyphenyl ("bottom") ring (Figure 1.5.7). Spectra of the two isomers are almost indistinguishable in both wavelength and intensity. The isomers, however, may differ in the geometry of their binding to proteins, and the conjugates may elute under different chromatographic conditions or migrate differently in an electrophoretic gel. Thus, certain applications may require the single-isomer preparations. Many fluorescein (and rhodamine) probes are available either as a mixture of isomers or as purified single isomers.

The 5-isomer or "isomer I" of FITC (F143, Figure 1.5.7, Figure 1.5.8) is the most widely used FITC isomer, probably because it is easier to isolate in pure form. Because isothiocyanates may deteriorate during storage, we recommend purchasing the 5-isomer of FITC specially packaged in individual vials (F1906, F1907). FITC is readily soluble in aqueous solutions that have a pH above 6. FITC is also available in our FluoReporter® FITC Protein Labeling Kit (F6434, Table 1.2), which is described in Section 1.2.

Mixed-Isomer and Single-Isomer Preparations of FAM and JOE Succinimidyl Esters

Although many other companies still prepare their fluorescein bioconjugates with FITC, we prefer to use amine-reactive succinimidyl esters of carboxyfluorescein (commonly called FAM), which yield carboxamides that are more resistant to hydrolysis. We offer both mixed-isomer and single-isomer preparations of FAM (C1904, C1359, C1360) and FAM succinimidyl esters (C1311, C2210, C6164). A study comparing the relative conjugation rate of several reactive fluorescein derivatives with a protein or L-lysine and the stability of the resulting conjugates concluded that the succinimidyl ester of carboxyfluorescein showed superior performance, followed by fluorescein dichlorotriazine (DTAF). FITC was both the slowest to react and yielded the least stable conjugates;[13] however, the degree of labeling was most easily controlled with FITC.[13] The succinimidyl ester of 5-FAM (C2210) is reported to react much faster than FITC when used to derivatize small biomolecules prior to separation by capillary electrophoresis.[14] We also offer a single-isomer preparation of the succinimidyl ester of 6-carboxy-4′,5′-dichloro-2′,7′-dimethoxyfluorescein (6-JOE, SE; C6171MP; Figure 1.5.9). 6-JOE is one of the four fluorophores (i.e., 5-FAM, 6-JOE, 6-TAMRA and 6-ROX) used in first-generation electrophoretic DNA sequencing instruments.

Succinimidyl Esters of Fluorescein with Spacer Groups

We also prepare succinimidyl esters of fluorescein that contain aliphatic spacers between the fluorophore and the reactive group. These include mixed-isomer (F2181, F6129) and single-isomer

(F6106) preparations of fluorescein-X succinimidyl ester (SFX), which contains a seven-atom aminohexanoyl spacer ("X") between the FAM fluorophore and the succinimidyl ester (Figure 1.5.10). In addition, we offer fluorescein-5-EX succinimidyl ester (F6130), which contains a seven-atom spacer that is somewhat more hydrophilic than is the spacer in SFX (Figure 1.5.11). These spacers separate the fluorophore from the biomolecule to which it is conjugated, potentially reducing the quenching that typically occurs upon conjugation. We have determined that conjugates of some proteins prepared with fluorescein-5-EX succinimidyl ester are up to twice as fluorescent as the corresponding conjugates labeled with FITC at the same degree of labeling (Figure 1.5.6). Consequently, we now recommend this fluorescein derivative as the preferred dye for preparing most fluoresceinated proteins. Fluorescein-5-EX succinimidyl ester is also available in our convenient FluoReporter® Fluorescein-EX Protein Labeling Kit (F6433) and Fluorescein-EX Protein Labeling Kit (F10240). See Section 1.2 and Table 1.3 for more details about these labeling kits.

The spacers in our SFX and fluorescein-5-EX succinimidyl esters may also make the fluorophore more accessible to secondary detection reagents.[15–17] For example, the spacers should make the fluorescein moiety more available for quenching by our polyclonal and monoclonal anti–fluorescein/Oregon Green® antibodies, a technique used to determine the accessibility of the fluorophore in proteins, membranes and cells.[16,18] Fluorescein is frequently used as a hapten on a primary detection reagent that can be either amplified or converted into a longer-wavelength or electron-dense signal with the appropriate secondary detection reagent. Section 7.4 describes our extensive selection of antibodies to fluorescein and other dyes.

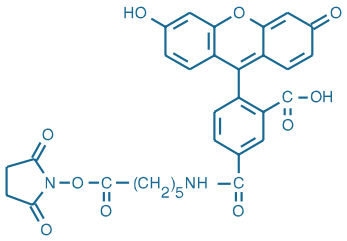

Figure 1.5.10 6-(fluorescein-5-carboxamido)hexanoic acid, succinimidyl ester (5-SFX, F6106).

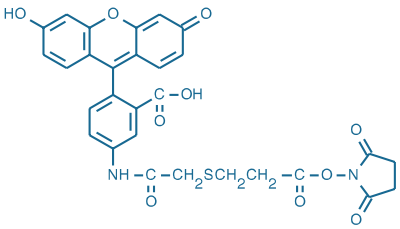

Figure 1.5.11 Fluorescein-5-EX, succinimidyl ester (F6130).

Fluorescein Dichlorotriazine (DTAF)

The 5-isomer of fluorescein dichlorotriazine (5-DTAF, D16) is highly reactive with proteins[19,20] and is commonly used to prepare biologically active fluorescein tubulin.[21] Unlike other reactive fluoresceins, 5-DTAF also reacts directly with polysaccharides and other alcohols in aqueous solution at pH above 9, but cannot be used to modify alcohols in the presence of better nucleophiles such as amines or thiols.[22] Polysaccharides that have been modified by DTAF (or other fluorescein derivatives) are readily radioiodinated.[23]

Caged Fluorescein Succinimidyl Ester

Caged probes are those that can liberate an active species upon illumination with ultraviolet light (Section 5.3). Caged fluorescent dyes can be utilized as polar tracers whose fluorescence can be spatially and temporally activated by illumination. Conjugation of the succinimidyl ester of our water-soluble, caged carboxyfluorescein β-alanine-carboxamide (C20050, Figure 1.5.12) to a biomolecule of interest produces an essentially nonfluorescent probe that yields a green-fluorescent fluorescein-labeled product only after ultraviolet photoactivation. Furthermore, caged fluorescein probes are immunochemically cryptic (i.e., the probe is immunoreactive with anti–fluorescein/Oregon Green® antibodies after but not before photoactivation). Caged fluorescein succinimdyl ester is also a key starting material for preparing probes for super-resolution photoactivation microscopy.[24,25]

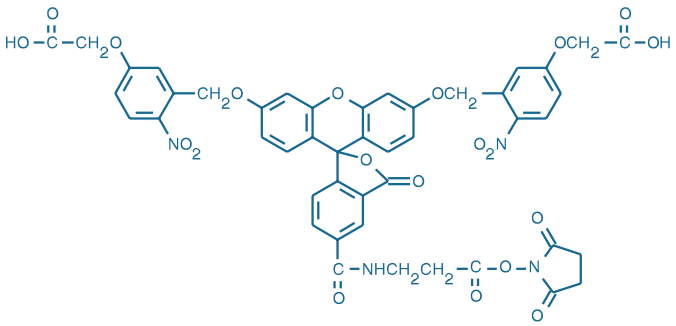

Figure 1.5.12 5-carboxyfluorescein-bis-(5-carboxymethoxy-2-nitrobenzyl) ether, β-alanine-carboxamide, succinimidyl ester (CMNB-caged carboxyfluorescein, SE; C20050).

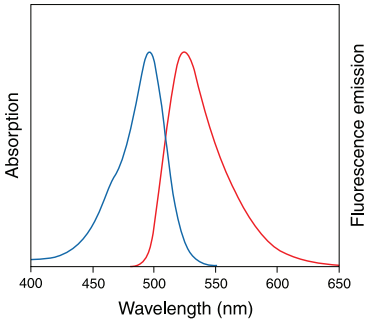

Figure 1.5.13 Absorption and fluorescence emission spectra of Oregon Green® 488 goat anti–mouse IgG antibody in pH 8.0 buffer.

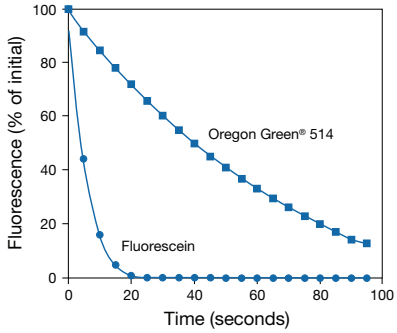

Figure 1.5.14 Photostability comparison for Oregon Green® 514 phalloidin (O7465) and fluorescein phalloidin (F432). CRE BAG 2 fibroblasts were fixed with formaldehyde, permeabilized with acetone and then stained with the fluorescent phallotoxins. Samples were continuously illuminated and images were acquired every 5 seconds using a Star 1 CCD camera (Photometrics®); the average fluorescence intensity in the field of view was calculated with Image-1 software (Universal Imaging Corp.) and expressed as a fraction of the initial intensity. Three data sets, representing different fields of view, were averaged for each labeled phalloidin to obtain the plotted time courses.

Oregon Green® 488 and Oregon Green® 514 Dyes

Oregon Green® 488 and Oregon Green® 514 dyes are fluorinated analogs of fluoresceins.[26] The absorption and emission spectra of Oregon Green® 488 dye (2′,7′-difluorofluorescein; D6145; Figure 1.5.13) is designed to perfectly match those of fluorescein. With additional fluorination of the carboxyphenyl ("bottom") ring of fluorescein, Oregon Green® 514 dye exhibits a moderate shift in its absorption and fluorescence spectra of about 15 nm relative to those of fluorescein or Oregon Green® 488 dye. Because of the near match of their absorption maxima on proteins (~498 nm and ~512 nm) to the strong 488 nm and 514 nm spectral lines of the argon-ion laser, the Oregon Green® 488 and Oregon Green® 514 fluorophores are important dyes for both confocal laser-scanning microscopy and flow cytometry applications. Furthermore, spectral imaging systems with linear-unmixing analysis software[27–29] permit the resolution of Oregon Green® 514 dye from other green-fluorescent dyes.

Bioconjugates prepared from Oregon Green® 488 and Oregon Green® 514 dyes share several advantages over those of other fluorescein dyes. These include:

- Fluorescence of protein conjugates prepared from Oregon Green® 488 and Oregon Green® 514 dyes is not appreciably quenched, even at relatively high degrees of labeling (Figure 1.5.6).
- Conjugates of Oregon Green® 488 and Oregon Green® 514 fluorophores are more photostable than those of fluorescein (Figure 1.5.3, Figure 1.5.14), allowing increased acquisition of photons before photodestruction of the dye and making Oregon Green® dyes particularly useful substitutes for fluoresceins for fluorescence imaging applications (Figure 1.5.15).
- Oregon Green® dyes have a lower pK_a (pK_a = 4.7 versus 6.4 for fluorescein) (Figure 1.5.5), making their fluorescence essentially pH insensitive in the physiological pH range. However, the pH sensitivity of Oregon Green® dyes in the weakly acidic range (pH 4 to 6) also makes these dyes useful as pH indicators for acidic organelles of live cells[30,31] (Section 20.3).
- Oregon Green® dyes are excellent haptens for anti–fluorescein/Oregon Green® antibodies (Section 7.4, Table 4.2), making Oregon Green® bioconjugates useful in a variety of signal amplification schemes.

Both Oregon Green® 488 and Oregon Green® 514 dyes have also proven useful as fluorescence anisotropy probes for measuring protein–protein and protein–nucleic acid interactions.[32]

Reactive Oregon Green® Dyes

We have prepared a variety of amine-reactive derivatives that enable researchers to take advantage of the spectral properties of Oregon Green® 488 and Oregon Green® 514 dyes

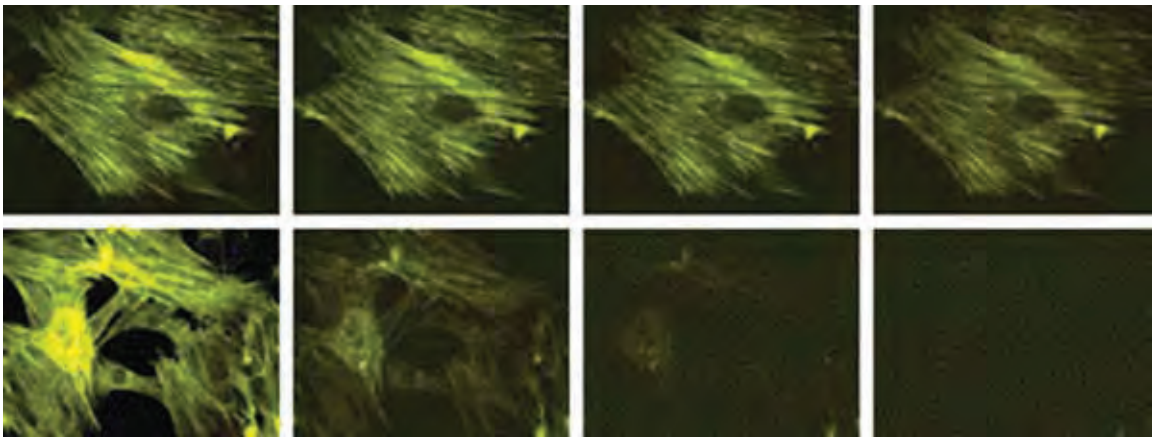

Figure 1.5.15 Photostability comparison of Oregon Green® 514 phalloidin (O7465, upper series) and fluorescein phalloidin (F432, lower series). CRE BAG 2 fibroblasts were fixed with formaldehyde, then permeabilized with acetone and stained with the fluorescent phallotoxin. Samples were illuminated continuously and viewed on a fluorescence microscope equipped with a fluorescein longpass optical filter set. Images acquired at 1, 10, 20 and 30 seconds after the start of illumination (left to right) demonstrate the superior photostability of the Oregon Green® 514 fluorophore.

(Table 1.8). These include the FITC analog, Oregon Green® 488 isothiocyanate (F_2FITC, O6080), and the single-isomer succinimidyl esters of Oregon Green® 488 carboxylic acid (O6147, O6149) and Oregon Green® 514 carboxylic acid (O6139). In addition, we offer the 5-isomer of Oregon Green® 488 carboxylic acid (O6146, Figure 1.5.16) and the mixed-isomer preparation of Oregon Green® 514 carboxylic acid (O6138, Figure 1.5.17). The 6-isomer of Oregon Green® 488-X succinimidyl ester (O6185, Figure 1.5.18) contains a seven-atom aminohexanoyl spacer ("X") between the fluorophore and the succinimidyl ester group. This spacer helps to separate the fluorophore from its point of attachment, reducing the interaction of the fluorophore with the biomolecule to which it is conjugated[33] and making it more accessible to secondary detection reagents.

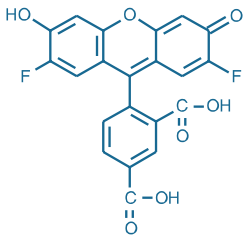

Figure 1.5.16 Oregon Green® 488 carboxylic acid (O6146).

Oregon Green® Protein and Nucleic Acid Labeling Kits

When directly compared with their fluorescein analogs, Oregon Green® 488 and Oregon Green® 514 conjugates typically have higher fluorescence yields and greater resistance to photobleaching. We have used succinimidyl esters of the Oregon Green® 488 and Oregon Green® 514 carboxylic acids to prepare conjugates of antibodies (Section 7.2, Table 7.1), streptavidin (Section 7.6, Table 7.9) and a variety of other proteins and ligands.

To facilitate direct labeling of biomolecules with Oregon Green® dyes, we offer several kits that are easy to use and produce reliable conjugations in minimal time. Our Oregon Green® protein and nucleic acid labeling kits, which are described in detail in the indicated sections, include:

- APEX® Oregon Green® 488 Antibody Labeling Kit (A10476, Section 1.2)
- FluoReporter® Oregon Green® 488 Protein Labeling Kit (F6153, Section 1.2)
- Oregon Green® 488 Protein Labeling Kit (O10241, Section 1.2)
- ULYSIS® Oregon Green® 488 Nucleic Acid Labeling Kit (U21659, Section 8.2)

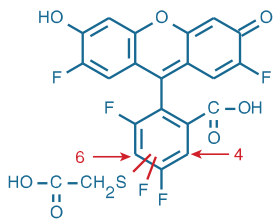

Figure 1.5.17 Oregon Green® 514 carboxylic acid (O6138).

Rhodamine Green™ and Rhodamine Green™-X Dyes

Carboxyrhodamine 110, which we have named Rhodamine Green™ dye, is the nonsulfonated analog of Alexa Fluor® 488 dye. Rhodamine Green™ dye offers a combination of desirable properties, including good photostability, a high extinction coefficient (>75,000 $cm^{-1}M^{-1}$) and a high fluorescence quantum yield, particularly in its nucleotide and nucleic acid conjugates. The Rhodamine Green™ fluorophore is even more photostable than the Oregon Green® 488 dye and about equivalent in photostability to the Oregon Green® 514 dye (Figure 1.5.3). Moreover, the fluorescence of its conjugates is completely insensitive to pH between 4 and 9.

Reactive versions of the Rhodamine Green™ dye (Table 1.8) were originally developed in our laboratories for use in DNA sequencing and other applications. Rhodamine Green™ conjugates can be prepared using the amine-reactive succinimidyl ester of Rhodamine Green™ dye (5(6)-CR 110, SE; R6107) or the succinimidyl ester of the Rhodamine Green™-X dye (R6113), which has an additional seven-atom aminohexanoyl spacer ("X") to reduce interaction of the fluorophore and its reaction site. The absorption and fluorescence emission maxima of Rhodamine Green™ conjugates are red-shifted about 7 nm compared with those of fluorescein; however, they remain compatible with standard fluorescein optical filter sets.

Although the Rhodamine Green™ dye is one of the most photostable of the fluorescein substitutes, its fluorescence when conjugated to proteins is often substantially quenched, and these conjugates also tend to precipitate from solution. Therefore, we do not recommend any of the Rhodamine Green™ succinimidyl esters for preparing protein conjugates. However, when conjugated to dextrans,[34] nucleotides and oligonucleotides,[35] the Rhodamine Green™ fluorophore remains highly fluorescent, and we currently offer two Rhodamine Green™ dextrans (D7153, D7163; Section 14.5, Table 14.4). In addition, Rhodamine Green™ dye–labeled probes have been frequently used for fluorescence correlation spectroscopy[36–38] (Fluorescence Correlation Spectroscopy (FCS)—Note 1.3).

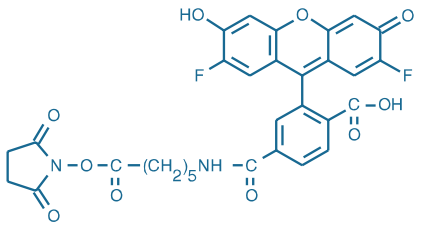

Figure 1.5.18 Oregon Green® 488-X, succinimidyl ester (O6185).

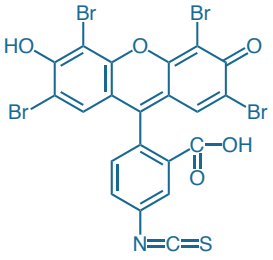

Figure 1.5.19 Eosin-5-isothiocyanate (E18).

Eosin

Eosin (2′,4′,5′,7′-tetrabromofluorescein, Figure 1.5.19) is usually not chosen for its fluorescence properties—the fluorescence quantum yield is typically only about 10–20% that of fluorescein—but rather for its ability to act as phosphorescent probe or as a fluorescence quencher.[39] With its high quantum yield (~0.57) for singlet oxygen generation, eosin and its conjugates can be used as effective photosensitizers of diaminobenzidine (DAB) oxidation in high-resolution electron microscopy studies (Fluorescent Probes for Photoconversion of Diaminobenzidine Reagents—Note 14.2). Like its thiol-reactive maleimide counterpart (E118, Section 2.2), eosin isothiocyanate (E18) is particularly useful as a phosphorescent probe for measuring the rotational properties of proteins, virus particles and other biomolecules in solution and in membranes.[40] In addition, eosin conjugates are employed for fluorescence resonance energy transfer (FRET) studies (Fluorescence Resonance Energy Transfer (FRET)—Note 1.2) and for fluorescence recovery after photobleaching (FRAP) measurements of lateral diffusion.

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DATA TABLE 1.5 FLUORESCEIN, OREGON GREEN® AND RHODAMINE GREEN™ DYES

Cat. No.	MW	Storage	Soluble	Abs	EC	Em	Solvent	Notes
C1311	473.39	F,D,L	DMF, DMSO	495	74,000	519	pH 9	1
C1359	376.32	L	pH >6, DMF	492	79,000	518	pH 9	1
C1360	376.32	L	pH >6, DMF	492	81,000	515	pH 9	1
C1904	376.32	L	pH >6, DMF	492	78,000	517	pH 9	1, 2
C2210	473.39	F,D,L	DMF, DMSO	494	78,000	520	pH 9	1
C6164	473.39	F,D,L	DMF, DMSO	496	83,000	516	pH 9	1
C6171MP	602.34	F,D,L	DMF, DMSO	520	75,000	548	pH 12	3
C20050	962.79	F,D,LL	DMSO	289	9500	none	MeOH	4, 5
D16	495.28	F,D,L	pH >6, DMF	492	83,000	516	pH 9	1, 6
D6145	368.29	L	pH >6, DMF	490	87,000	514	pH 9	7
E18	704.97	F,DD,L	pH >6, DMF	521	95,000	544	pH 9	8, 9
F143	389.38	F,DD,L	pH >6, DMF	494	77,000	519	pH 9	1, 8, 10
F1300	332.31	L	pH >6, DMF	490	93,000	514	pH 9	1
F1906	389.38	F,DD,L	pH >6, DMF	494	77,000	519	pH 9	1, 8, 10
F1907	389.38	F,DD,L	pH >6, DMF	494	77,000	519	pH 9	1, 8, 10
F2181	586.55	F,D,L	DMF, DMSO	494	74,000	520	pH 9	1
F6106	586.55	F,D,L	DMF, DMSO	494	75,000	521	pH 9	1
F6129	586.55	F,D,L	DMF, DMSO	494	74,000	520	pH 9	1
F6130	590.56	F,D,L	DMF, DMSO	491	86,000	515	pH 9	1
F36915	332.31	RO,L	see Notes	490	93,000	514	pH 9.5	1, 11
O6080	425.36	F,DD,L	DMF, DMSO	493	78,000	520	pH 9	7, 8
O6138	512.36	L	pH >6, DMF	506	86,000	526	pH 9	12, 13
O6139	609.43	F,D,L	DMF, DMSO	506	85,000	526	pH 9	12, 13
O6146	412.30	L	pH >6, DMF	492	85,000	518	pH 9	7, 14
O6147	509.38	F,D,L	DMF, DMSO	495	76,000	521	pH 9	7, 14
O6149	509.38	F,D,L	DMF, DMSO	496	82,000	516	pH 9	7, 14
O6185	622.53	F,D,L	DMF, DMSO	494	84,000	517	pH 9	7
R6107	507.89	F,D,L	DMF, DMSO	504	78,000	532	MeOH	
R6113	621.05	F,D,L	DMF, DMSO	503	74,000	528	MeOH	

For definitions of the contents of this data table, see "Using *The Molecular Probes® Handbook*" in the introductory pages.

Notes

1. Absorption and fluorescence of fluorescein derivatives are pH dependent. Extinction coefficients and fluorescence quantum yields decrease markedly at pH <7.
2. This product is specified to equal or exceed 98% analytical purity by HPLC.
3. Absorption and fluorescence of C6171MP are pH dependent (pK_a ~11.5). Fluorescence is maximal at pH >12.
4. All photoactivatable probes are sensitive to light. They should be protected from illumination except when photolysis is intended.
5. This product is colorless and nonfluorescent until it is activated by ultraviolet photolysis. Photoactivation generates a fluorescein derivative with spectral characteristics similar to C1359.
6. Unstable in water. Use immediately.
7. Absorption and fluorescence of Oregon Green® 488 derivatives are pH dependent only in moderately acidic solutions (pH <5).
8. Isothiocyanates are unstable in water and should not be stored in aqueous solution.
9. Eosin derivatives also exhibit phosphorescence with an emission maximum at ~680 nm. The phosphorescence lifetime is ~1 millisecond for eosin. (Biochem J (1979) 183:561, Spectroscopy (1990) 5:20) Fluorescence lifetime (τ) is 1.4 nanoseconds (QY = 0.2) for eosin. (J Am Chem Soc (1977) 99:4306)
10. The extinction coefficient of fluorescein isothiocyanate decreases about 10% on protein conjugation. (J Immunol Methods (1974) 5:103) The fluorescence lifetime (τ) is 3.8 nanoseconds.
11. F36915 consists of a fluorescein solution in 100 mM sodium borate buffer pH 9.5. The concentration of fluorescein is set spectrophotometrically to be equivalent to that of NIST Standard Reference Material (SRM®) 1932.
12. Absorption and fluorescence of Oregon Green® 514 derivatives are pH dependent only in moderately acidic solutions (pH <5).
13. The fluorescence lifetime (τ) of the Oregon Green® 514 dye in pH 9.0 buffer at 20°C is 4.2 nanoseconds. Data provided by the SPEX Fluorescence Group, Horiba Jobin Yvon Inc.
14. The fluorescence lifetime (τ) of the Oregon Green® 488 dye (O6146) in pH 9.0 buffer at 20°C is 4.1 nanoseconds. Data provided by the SPEX Fluorescence Group, Horiba Jobin Yvon Inc.

PRODUCT LIST 1.5 FLUORESCEIN, OREGON GREEN® AND RHODAMINE GREEN™ DYES

Cat. No.	Product	Quantity
A10476	APEX® Oregon Green® 488 Antibody Labeling Kit	1 kit
C6171MP	6-carboxy-4′,5′-dichloro-2′,7′-dimethoxyfluorescein, succinimidyl ester (6-JOE, SE)	5 mg
C1359	5-carboxyfluorescein (5-FAM) *single isomer*	100 mg
C1360	6-carboxyfluorescein (6-FAM) *single isomer*	100 mg
C1904	5-(and-6)-carboxyfluorescein (5(6)-FAM) *FluoroPure™ grade* *mixed isomers*	100 mg
C2210	5-carboxyfluorescein, succinimidyl ester (5-FAM, SE) *single isomer*	10 mg
C6164	6-carboxyfluorescein, succinimidyl ester (6-FAM, SE) *single isomer*	10 mg
C1311	5-(and-6)-carboxyfluorescein, succinimidyl ester (5(6)-FAM, SE) *mixed isomers*	100 mg
C20050	5-carboxyfluorescein-bis-(5-carboxymethoxy-2-nitrobenzyl) ether, β-alanine-carboxamide, succinimidyl ester (CMNB-caged carboxyfluorescein, SE)	1 mg
D16	5-(4,6-dichlorotriazinyl)aminofluorescein (5-DTAF) *single isomer*	100 mg
D6145	2′,7′-difluorofluorescein (Oregon Green® 488)	10 mg
E18	eosin-5-isothiocyanate	100 mg
F6434	FluoReporter® FITC Protein Labeling Kit *5–10 labelings*	1 kit
F6433	FluoReporter® Fluorescein-EX Protein Labeling Kit *5–10 labelings*	1 kit
F6153	FluoReporter® Oregon Green® 488 Protein Labeling Kit *5–10 labelings*	1 kit
F36915	fluorescein *NIST-traceable standard* *nominal concentration 50 µM* *special packaging*	5 x 1 mL
F1300	fluorescein *reference standard*	1 g
F6106	6-(fluorescein-5-carboxamido)hexanoic acid, succinimidyl ester (5-SFX) *single isomer*	5 mg
F2181	6-(fluorescein-5-(and-6)-carboxamido)hexanoic acid, succinimidyl ester (5(6)-SFX) *mixed isomers*	10 mg
F6129	6-(fluorescein-5-(and-6)-carboxamido)hexanoic acid, succinimidyl ester (5(6)-SFX) *mixed isomers* *special packaging*	10 x 1 mg
F10240	Fluorescein-EX Protein Labeling Kit *3 labelings*	1 kit
F6130	fluorescein-5-EX, succinimidyl ester	10 mg
F143	fluorescein-5-isothiocyanate (FITC 'Isomer I')	1 g
F1906	fluorescein-5-isothiocyanate (FITC 'Isomer I') *special packaging*	10 x 10 mg
F1907	fluorescein-5-isothiocyanate (FITC 'Isomer I') *special packaging*	10 x 100 mg
O6146	Oregon Green® 488 carboxylic acid *5-isomer*	5 mg
O6147	Oregon Green® 488 carboxylic acid, succinimidyl ester *5-isomer*	5 mg
O6149	Oregon Green® 488 carboxylic acid, succinimidyl ester *6-isomer*	5 mg
O6080	Oregon Green® 488 isothiocyanate (F_2FITC) *mixed isomers*	5 mg
O10241	Oregon Green® 488 Protein Labeling Kit *3 labelings*	1 kit
O6185	Oregon Green® 488-X, succinimidyl ester *6-isomer*	5 mg
O6138	Oregon Green® 514 carboxylic acid	5 mg
O6139	Oregon Green® 514 carboxylic acid, succinimidyl ester	5 mg
R6107	Rhodamine Green™ carboxylic acid, succinimidyl ester, hydrochloride (5(6)-CR 110, SE) *mixed isomers*	5 mg
R6113	Rhodamine Green™-X, succinimidyl ester, hydrochloride *mixed isomers*	5 mg
U21659	ULYSIS® Oregon Green® 488 Nucleic Acid Labeling Kit *20 labelings*	1 kit
Z25042	Zenon® Fluorescein Mouse IgG_1 Labeling Kit *50 labelings*	1 kit
Z25342	Zenon® Fluorescein Rabbit IgG Labeling Kit *50 labelings*	1 kit

1.6 Long-Wavelength Rhodamines, Texas Red® Dyes and QSY® Quenchers

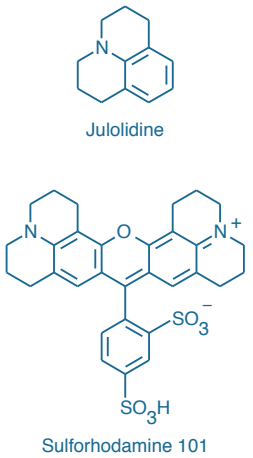

Figure 1.6.1 The amine substituents of X-rhodamine, sulforhodamine 101 and Texas Red® dyes are rigidified in a julolidine ring structure.

This section includes dyes that have absorption maxima beyond about 520 nm, extending to nearly 800 nm. Significant exceptions, however, are the long-wavelength Alexa Fluor® dyes, which are all discussed in Section 1.3, the long-wavelength BODIPY® dyes—BODIPY® TMR, BODIPY® TR, BODIPY® 630/650 and BODIPY® 650/665—which are described in Section 1.4 and the 2′,4′,5′,7′-tetrabromofluorescein (eosin) and JOE dyes, which also absorb maximally beyond 520 nm but are discussed with other fluoresceins in Section 1.5. In many applications, the versatile Alexa Fluor® and BODIPY® dyes provide demonstrably superior performance relative to the dyes in this section.

Rhodamine dyes (Table 1.9) are among the most photostable fluorescent labeling reagents available. Moreover, spectra of most of these dyes are not affected by changes in pH between 4 and 10, an important advantage over the fluoresceins for many biological applications.[1] The most common members of this group have been the tetramethylrhodamines—including the reactive isothiocyanate (TRITC) and carboxylic acid (TAMRA) derivatives—as well as the X-rhodamines. The X prefix of the X-rhodamines, which include Texas Red® derivatives, refers to the fluorophore's extra julolidine rings (Figure 1.6.1). These rings prevent rotation about the nitrogen atoms, resulting in a shift in the fluorophore's spectra to longer wavelengths and usually an increase in its fluorescence quantum yield.

QSY® 7, QSY® 9 and QSY® 21 dyes are essentially nonfluorescent diarylrhodamine chromophores with strong absorption in the visible wavelength region, and they have proven to be extremely effective fluorescence quenchers.[2,3] QSY® 7, QSY® 9 and QSY® 21 dyes complement the QSY® 35 dye, a nonfluorescent quencher based on the NBD fluorophore that absorbs maximally near 475 nm, and the dabcyl quencher, both of which are described in Section 1.8.

Table 1.9 Amine-reactive, orange- and red-fluorescent fluorophores in this section.

Fluorophore (Abs/Em) *	Succinimidyl Ester	Other	Protein Labeling Kits	Notes
Lissamine rhodamine B (570/590)		L20 (SC)[M] L1908 (SC)[M]		• Optimal for 568 nm excitation • Photostable
Naphthofluorescein (602/672)	C653[M]	C652 (COOH)[M]		• Very long-wavelength excitation and emission • pH-sensitive fluorescence, with a high pK_a (~7.6)
Rhodamine 6G (525/555)	C6127[5] C6128[6] C6157[M]			• Excited by the 514 nm spectral line of the argon-ion laser • Spectra intermediate between those of fluorescein and tetramethylrhodamine
Rhodamine Red™-X (580/590)	R6160 (X)[5]		F6161 (F)	• Conjugates of Rhodamine Red™-X are generally more fluorescent than those of Lissamine rhodamine B, and the succinimidyl ester is more stable in H_2O
Tetramethylrhodamine (555/580)	C2211[5] C6123[6] C1171[M] T6105 (X)[M]	C6121 (COOH)[5] C6122 (COOH)[6] C300 (COOH)[M] T1480 (ITC)[5] T1481 (ITC)[6] T490 (ITC)[M]		• pH-insensitive fluorescence • Good photostability • Conjugates are prone to aggregation • Succinimidyl ester derivative (6-TAMRA, SE; C6123) is widely used for oligonucleotide labeling
Texas Red® dye (595/615)	T6134 (X)[M] T20175 (X)[S]	T353 (SC)[M] T1905 (SC)[M] T30200 (DTA)[M]	F6162 (F) T10244 (P) Z25045 (Z)	• Good spectral separation from green fluorophores • Texas Red®-X succinimidyl ester typically yields greater fluorescence per attached dye than Texas Red® sulfonyl chloride and is more stable in H_2O
X-rhodamine (580/605)	C6125[5] C6126[6] C1309[M]	C6124 (COOH)[5] C6156 (COOH)[6] X491 (ITC)[M]		• Succinimidyl ester derivative (6-ROX, SE; C6126) is widely used for oligonucleotide labeling

* The numbers in parentheses reflect the absorption (Abs) and fluorescence emission (Em) maxima, in nm, of the goat anti–mouse IgG antibody or dextran conjugates in aqueous buffer. (5) = 5-Isomer. (6) = 6-Isomer. (COOH) = Carboxylic acid. (F) = FluoReporter® Protein Labeling Kit (Section 1.2). (ITC) = Isothiocyanate. (M) = Mixed isomers. (P) = Easy-to-Use Protein Labeling Kit (Section 1.2). (S) = Single isomer. (SC) = Sulfonyl chloride. (X) = Aminohexanoyl spacer separating the dye and the SE. (Z) = Zenon® Antibody Labeling Kit (Section 7.3).

Tetramethylrhodamine

Tetramethylrhodamine (TMR) has been an important fluorophore for preparing protein conjugates, especially the fluorescent antibody and avidin derivatives used in immunochemistry. Under the name TAMRA, the carboxylic acid of TMR has also achieved prominence as a dye for oligonucleotide labeling[3,4] (Section 8.2, Table 8.7) and single-molecule detection applications.[5,6] Because it can be prepared in high purity, the 5-isomer of TAMRA (C6121) is one of the five dyes in our Reference Dye Sampler Kit (R14782, Section 23.1). TMR is efficiently excited by the 543 nm spectral line of the green He-Ne laser, which is increasingly being used for analytical instrumentation; diode lasers with 561 nm output[7] are slightly suboptimal but still effective.

TMR dyes such as TAMRA and TRITC are quite hydrophobic (Figure 1.6.2) when compared with their fluorescein counterparts FAM and FITC. As a result, they have a tendency to aggregate in aqueous solutions under conditions where the labeling density is sufficient to permit dye–dye interactions. A further consequence of these interactions is fluorescence self-quenching, which reduces the fluorescence output of the conjugate. Dye–dye interactions and self-quenching are much less prevalent with the more polar and water-soluble Alexa Fluor® dyes.[8] Another indication of intermolecular interactions of TMR dyes is that the absorption spectrum of TMR-labeled proteins is frequently complex (Figure 1.6.3), usually splitting into two absorption peaks at about 520 and 550 nm,[9] so that the actual degree of labeling is difficult to determine. Excitation at wavelengths in the range of the short-wavelength peak fails to yield the expected amount of fluorescence, indicating that it arises from a nonfluorescent dye aggregate. Furthermore, when the TMR-labeled protein conjugate is denatured by guanidine hydrochloride, the long-wavelength absorption increases, the short-wavelength peak mostly disappears and the fluorescence yield almost doubles[10] (Figure 1.6.3). The absorption spectra of TMR-labeled nucleotides and of other probes such as our rhodamine phalloidin (R415, Section 11.1) do *not* split into two peaks, indicating a labeling ratio of one dye molecule per biomolecule. The emission spectrum of TMR conjugates does not vary much with the degree of labeling.[9] An improved method for estimating the degree of substitution of TRITC conjugates has been described.[10]

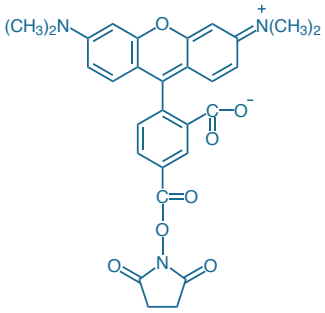

Figure 1.6.2 5-carboxytetramethylrhodamine, succinimidyl ester (5-TAMRA, SE; C2211).

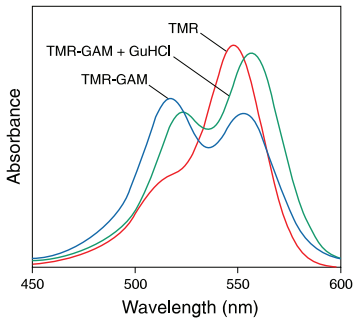

Figure 1.6.3 Effect of protein conjugation on the absorption spectrum of tetramethylrhodamine. The absorption spectrum of tetramethylrhodamine conjugated to goat anti–mouse IgG antibody (TMR-GAM, T2762) shows an additional peak at about 520 nm when compared with the spectrum of the same concentration of the free dye (TMR). Partial unfolding of the protein in the presence of 4.8 M guanidine hydrochloride (TMR-GAM + GuHCl) results in a spectrum more similar to that of the free dye.

Mixed-Isomer and Single-Isomer TRITC Preparations

Our tetramethylrhodamine isothiocyanate (TRITC) is of the highest quality available from any commercial source. Both the mixed-isomer (T490) and single-isomer (T1480, T1481) TRITC preparations typically have extinction coefficients above 80,000 $cm^{-1}M^{-1}$, whereas some competitive sources of TRITC have extinction coefficients reported to be below 50,000 $cm^{-1}M^{-1}$. TRITC is widely used by other companies to prepare most of their so-called "rhodamine" immunoconjugates; however, they also often employ reactive versions of rhodamine B or Lissamine rhodamine B, which have somewhat different spectra, resulting in some confusion in matching the product name to the correct fluorophore.

Succinimidyl Esters of Carboxytetramethylrhodamine

Almost all Molecular Probes® TMR conjugates are prepared using succinimidyl esters of carboxytetramethylrhodamine (TAMRA™ dye), rather than TRITC, because bioconjugates from succinimidyl esters are more stable and often more fluorescent. We offer the mixed-isomer (C300) and single-isomer (C6121, C6122) preparations of carboxymethylrhodamine, as well as the corresponding mixed-isomer (C1171) and single-isomer (C2211, C6123) succinimidyl esters. The single-isomer preparations are most important for high-resolution techniques such as DNA sequencing[11] and separation of labeled carbohydrates by capillary electrophoresis.[12] 6-TAMRA™ dye is one of the traditional fluorophores (5-FAM™, 6-JOE™, 6-TET™, 6-HEX™, 6-TAMRA™ and 6-ROX™ dyes) used in first-generation electrophoresis-based DNA sequencing[11,13–15] (Section 8.2, Table 8.7).

We have also prepared the mixed-isomer TAMRA-X succinimidyl ester (5(6)-TAMRA-X, SE; T6105), which contains a seven-atom aminohexanoyl spacer ("X") between the reactive group and the fluorophore (Figure 1.6.4). This spacer helps to separate the fluorophore from its point of attachment, reducing the interaction of the fluorophore with the biomolecule to which it is conjugated, making it more accessible to secondary detection reagents and facilitating orientational averaging in fluorescence resonance energy transfer (FRET) applications[16] (Fluorescence Resonance Energy Transfer (FRET)—Note 1.2). Polyclonal anti-tetramethylrhodamine and anti–Texas Red® dye antibodies that recognize the tetramethylrhodamine, Rhodamine Red™-X, X-rhodamine and Texas Red® fluorophores are available (Section 7.4).

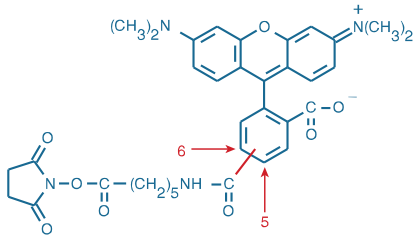

Figure 1.6.4 6-(tetramethylrhodamine-5-(and-6)-carboxamido)hexanoic acid, succinimidyl ester (5(6)-TAMRA-X, SE; T6105).

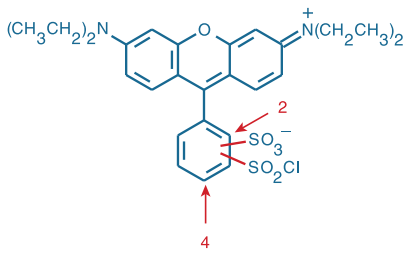

Figure 1.6.5 Lissamine rhodamine B sulfonyl chloride (L20).

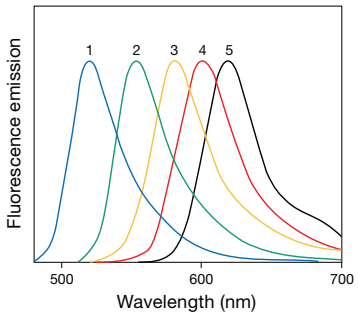

Figure 1.6.6 Normalized fluorescence emission spectra of goat anti–mouse IgG antibody conjugates of 1) fluorescein, 2) rhodamine 6G, 3) tetramethylrhodamine, 4) Lissamine rhodamine B and 5) Texas Red® dyes.

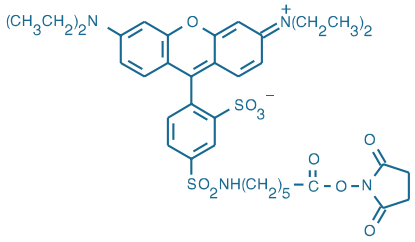

Figure 1.6.7 Rhodamine Red™-X, succinimidyl ester (R6160).

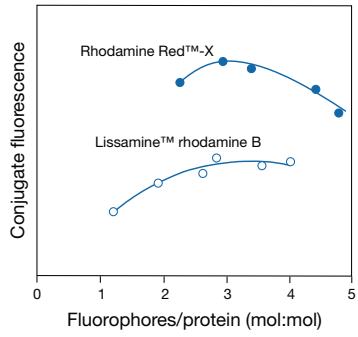

Figure 1.6.8 Comparison of the relative fluorescence of goat anti–mouse IgG antibody conjugates of Rhodamine Red™-X succinimidyl ester (R6160, ●) and Lissamine rhodamine B sulfonyl chloride (L20, L1908, ○). Conjugate fluorescence is determined by measuring the fluorescence quantum yield of the conjugated dye relative to that of the free dye and multiplying by the number of fluorophores per protein. Higher numbers of fluorophores attached per protein are attainable with Rhodamine Red™-X dye due to the lesser tendency of this dye to induce protein precipitation.

Lissamine Rhodamine B and Rhodamine Red™-X Dyes

Lissamine Rhodamine B Sulfonyl Chloride

Lissamine rhodamine B sulfonyl chloride (L20, L1908; Figure 1.6.5) is much less expensive than Texas Red® sulfonyl chloride, and the fluorescence emission spectrum of its protein conjugates lies between those of tetramethylrhodamine and Texas Red® conjugates[17] (Figure 1.6.6). It is more frequently employed as a synthetic precursor for preparing affinity labeling reagents than as a labeling reagent for protein conjugation.[18–20]

Rhodamine Red™-X Succinimidyl Ester

Lissamine rhodamine B sulfonyl chloride is unstable, particularly in aqueous solution, making it somewhat difficult to achieve reproducible conjugations using this dye. Unlike Lissamine rhodamine B sulfonyl chloride, which is a mixture of isomeric sulfonyl chlorides (Figure 1.6.5), Rhodamine Red™-X succinimidyl ester (R6160, Figure 1.6.7) is isomerically pure and is hydrolytically stable for practical purposes at the mild alkaline pH levels typically used for amine-reactive protein conjugation. Rhodamine Red™-X succinimidyl ester incorporates a spacer between the fluorophore and the reactive site, resulting in minimized perturbation of the conjugation partner's functional properties.[21] Moreover, we have found that protein conjugates of Rhodamine Red™-X dye are frequently brighter than those of Lissamine rhodamine B (Figure 1.6.8), and less likely to precipitate during storage.[22] Rhodamine Red™-X succinimidyl ester is used in the FluoReporter® Rhodamine Red™-X Protein Labeling Kit (F6161); see Section 1.2 for further information on preparing red-fluorescent protein conjugates with this kit.

X-Rhodamine

The derivatives of carboxy-X-rhodamine (ROX™ dye)—a dye originally developed in our laboratories in 1986—are widely used for oligonucleotide labeling and DNA sequencing applications (Section 8.2, Table 8.7). Conjugates of this dye and of the similar isothiocyanate (5(6)-XRITC, X491; Figure 1.6.9) have longer-wavelength spectra (Figure 1.6.10) than the spectra of Lissamine rhodamine B, but somewhat shorter-wavelength spectra than those of Texas Red® conjugates. Both the pure 5-isomer (C6124) and 6-isomer (C6156) of carboxy-X-rhodamine are available, as are mixed-isomer (C1309, Figure 1.6.11) and single-isomer (C6125, C6126) preparations of the succinimidyl ester.

Texas Red® and Texas Red®-X Dyes

The Texas Red® fluorophore emits at a longer wavelength than do either tetramethylrhodamine or Lissamine rhodamine B (Figure 1.6.6), making Texas Red® conjugates among the most commonly used long-wavelength "third labels" in fluorescence microscopy (Figure 1.6.12, Figure 1.6.13). Unlike the other rhodamines, the Texas Red® fluorophore exhibits very little spectral overlap with fluorescein (Figure 1.6.6), and its fluorescence can be distinguished from that of

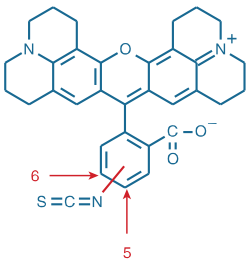

Figure 1.6.9 X-rhodamine-5-(and-6)-isothiocyanate (5(6)-XRITC, X491).

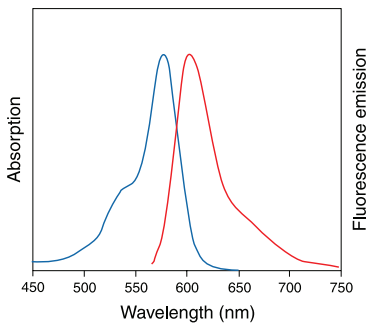

Figure 1.6.10 Absorption and fluorescence emission spectra of 5-carboxy-X-rhodamine (5-ROX) in pH 7.0 buffer.

phycoerythrins. When the correct optical filter sets are used, Texas Red® conjugates are brighter and have lower background than conjugates of the other commonly used red-fluorescent dyes, with the exception of the Alexa Fluor® 594 dye. Texas Red® conjugates are particularly well suited for excitation by the 594 nm spectral line of the orange He-Ne laser; diode laser excitation at 561 nm is also efficient.

Texas Red® Sulfonyl Chloride

Texas Red® sulfonyl chloride is our trademarked mixture of isomeric sulfonyl chlorides (Figure 1.6.14) of sulforhodamine 101.[23,24] This reagent is quite unstable in water, especially at the higher pH required for reaction with aliphatic amines. For example, dilute solutions of Texas Red® sulfonyl chloride are totally hydrolyzed within 2–3 minutes in pH 8.3 aqueous solution at room temperature.[22] Protein modification by this reagent is best done at low temperature. Once conjugated, however, the sulfonamides that are formed (Figure 1.6.15) are extremely stable; they even survive complete protein hydrolysis.

Because Texas Red® sulfonyl chloride rapidly degrades upon exposure to moisture, we offer this reactive dye specially packaged as a set of 10 vials (T1905), each containing approximately 1 mg of Texas Red® sulfonyl chloride for small-scale conjugations. We also offer the 10 mg unit size packaged in a single vial (T353) for larger-scale conjugations. Each milligram of Texas Red® sulfonyl chloride modifies approximately 8–10 mg of protein. Note that sulfonyl chlorides are unstable in dimethylsulfoxide (DMSO) and should never be used in that solvent.[25] Polyclonal anti-tetramethylrhodamine and anti–Texas Red® antibodies that recognize tetramethylrhodamine, Rhodamine Red™, X-rhodamine and Texas Red® fluorophores are available (Section 7.4, Table 4.2).

Texas Red®-X Succinimidyl Ester

Texas Red® sulfonyl chloride's susceptibility to hydrolysis and low solubility in water may complicate its conjugation to some biomolecules. To overcome this difficulty, we have developed Texas Red®-X succinimidyl ester, which contains an additional seven-atom aminohexanoyl spacer ("X") between the fluorophore and its reactive group.[22] The single-isomer preparation of Texas Red®-X succinimidyl ester (T20175, Figure 1.6.16) is preferred over the mixed-isomer product (T6134) when the dye is used to prepare conjugates of low molecular weight peptides, oligonucleotides and receptor ligands that are to be purified by high-resolution techniques. Also, because isomers of a reactive dye may differ in their binding geometry, certain applications such as fluorescence resonance energy transfer (FRET) may benefit from the use of single-isomer reactive dyes[26] (Fluorescence Resonance Energy Transfer (FRET)—Note 1.2). Thiol-reactive Texas Red® derivatives that are based on a similar synthetic approach are described in Section 2.2. Texas

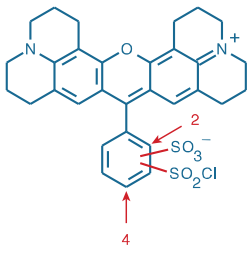

Figure 1.6.14 Texas Red® sulfonyl chloride (T353).

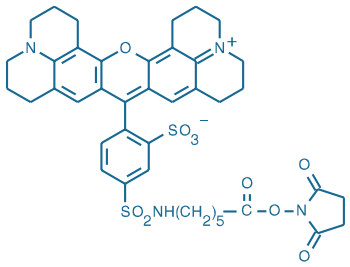

Figure 1.6.16 Texas Red®-X, succinimidyl ester (T20175).

Figure 1.6.15 Reaction of a primary amine with a sulfonyl chloride.

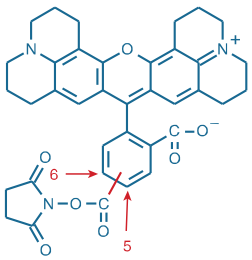

Figure 1.6.11 5-(and-6)-carboxy-X-rhodamine, succinimidyl ester (5(6)-ROX, SE; C1309).

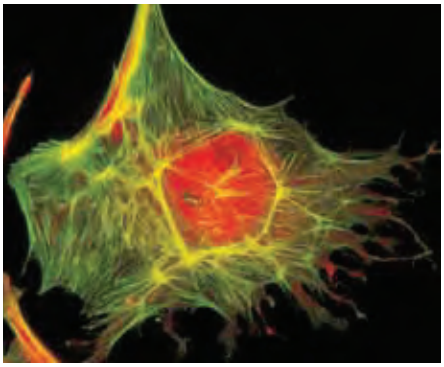

Figure 1.6.12 Simultaneous visualization of F- and G-actin in a bovine pulmonary artery endothelial cell (BPAEC) using F-actin–specific Oregon Green® 488 phalloidin (O7466) and G-actin–specific Texas Red® deoxyribonuclease I. The G-actin appears as diffuse red fluorescence that is more intense in the nuclear region where the cell thickness is greater and stress fibers are less dense. The image was obtained by taking multiple exposures through bandpass optical filter sets appropriate for fluorescein and the Texas Red® dye.

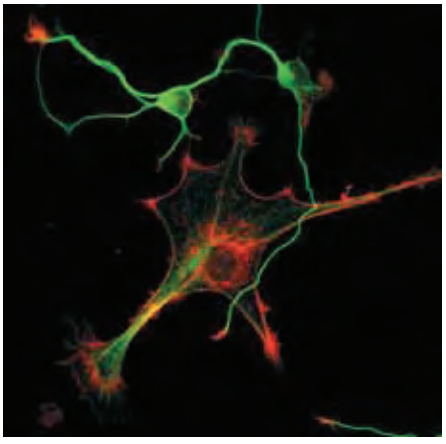

Figure 1.6.13 Confocal micrograph of the cytoskeleton of a mixed population of granule neurons and glial cells. The F-actin was stained with red-fluorescent Texas Red®-X phalloidin (T7471). The microtubules were detected with a mouse monoclonal anti–ß-tubulin primary antibody and subsequently visualized with the green-fluorescent Alexa Fluor® 488 goat anti–mouse IgG antibody (A11001). The image was contributed by Jonathan Zmuda, Immunomatrix, Inc.

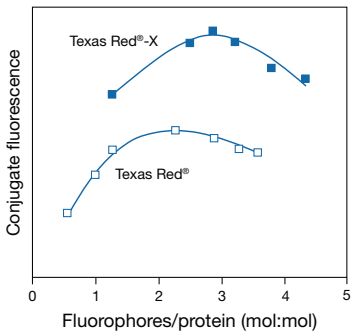

Figure 1.6.17 Comparison of the relative fluorescence of goat anti–mouse IgG antibody conjugates of Texas Red®-X succinimidyl ester (T6134, ■) and Texas Red® sulfonyl chloride (T353, □). Conjugate fluorescence was determined by measuring the fluorescence quantum yield of the conjugated dye relative to that of the free dye and multiplying by the number of fluorophores per protein. Higher numbers of fluorophores attached per protein are attainable with the Texas Red®-X dye due to the lesser tendency of this dye to induce protein precipitation.

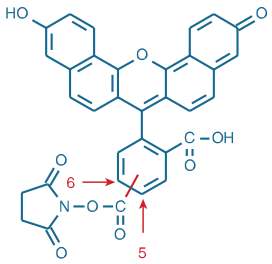

Figure 1.6.18 5-(and-6)-carboxynaphthofluorescein, succinimidyl ester (C653).

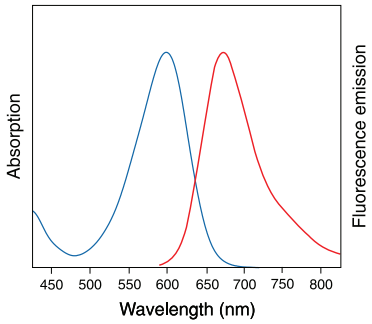

Figure 1.6.19 Absorption and fluorescence emission spectra of 5-(and-6)-carboxynaphthofluorescein in pH 10.0 buffer.

Red®-X succinimidyl ester offers significant advantages over Texas Red® sulfonyl chloride for the preparation of bioconjugates:

- In the absence of amines, greater than 80% of Texas Red®-X succinimidyl ester's reactivity is retained in pH 8.3 solution after one hour at room temperature.[22]
- Much less Texas Red®-X succinimidyl ester (usually half or less of the amount of Texas Red® sulfonyl chloride) is required to yield the same degree of labeling, making the effective costs of these two reagents about the same.
- Conjugations with Texas Red®-X succinimidyl ester are more reproducible.
- Unlike Texas Red® sulfonyl chloride, which can form unstable products with tyrosine, histidine, cysteine and other residues in proteins, the Texas Red®-X succinimidyl ester reacts almost exclusively with amines.
- Protein conjugates prepared with Texas Red®-X succinimidyl ester have a higher fluorescence yield than those with the same labeling ratio prepared with Texas Red® sulfonyl chloride (Figure 1.6.17).
- Texas Red®-X protein conjugates show a decreased tendency to precipitate during the reaction or upon storage.

Texas Red® C_2-Dichlorotriazine

Texas Red® C_2-dichlorotriazine (T30200) is a reactive dye with absorption/emission maxima of ~588/601 nm. Dichlorotriazines readily modify amines in proteins, and they are among the few reactive groups that are reported to react directly with polysaccharides[27] and other alcohols in aqueous solution, provided that the pH is >9 and that other nucleophiles are absent.

Texas Red®-X Conjugates and Texas Red®-X Labeling Kits

Because of the advantages of Texas Red®-X succinimidyl ester, we have converted some of our Texas Red® conjugates to the Texas Red®-X conjugates. We have prepared Texas Red®-X conjugates of:

- Antibodies (Section 7.2, Table 7.1)
- Streptavidin (S6370, Section 7.6, Table 7.9)
- Phalloidin (T7471, Section 11.1, Table 11.2)
- Wheat germ agglutinin (W21405, Section 7.7)
- dUTP (C7631, Section 8.2)

Protein conjugates of the Texas Red®-X dye are readily prepared using our FluoReporter® Texas Red®-X Protein Labeling Kit (F6162) and Texas Red®-X Protein Labeling Kit (T10244); see Section 1.2 for further information on preparing fluorescent protein conjugates with these kits. Zenon® Texas Red®-X Antibody Labeling Kit for mouse IgG_1 antibodies (Z25045, Section 7.3) permits the rapid and quantitative labeling of antibodies from a purified antibody fraction or from a crude antibody preparation such as serum, ascites fluid or a hybridoma supernatant with the Texas Red®-X dye.[28] Polyclonal anti-tetramethylrhodamine and anti–Texas Red® antibodies that recognize tetramethylrhodamine, Rhodamine Red™, X-rhodamine and Texas Red® fluorophores are available (Section 7.4, Table 7.8).

Naphthofluorescein

Naphthofluorescein carboxylic acid and its succinimidyl ester (C652, C653; Figure 1.6.18) have emission maxima of approximately 670 nm in aqueous solution at pH 10 (Figure 1.6.19). However, the fluorescence of naphthofluorescein is pH dependent (pK_a ~7.6), requiring a relatively alkaline pH for maximal fluorescence.

Carboxyrhodamine 6G

The excitation and emission spectra of carboxyrhodamine 6G (CR 6G) fall between those of fluorescein and tetramethylrhodamine (Figure 1.6.6). With a peak absorption at ~520 nm, conjugates prepared from the mixed-isomer (C6157) or single-isomer (C6127, C6128) preparations

of CR 6G succinimidyl esters are an excellent match to the 514 nm spectral line of the argon-ion laser. They also tend to exhibit a higher fluorescence quantum yield than tetramethylrhodamine conjugates, as well as excellent photostability. As with Rhodamine Green™ dyes, carboxyrhodamine 6G dyes are more suitable for preparing nucleotide and oligonucleotide conjugates than for preparing protein conjugates. Oligonucleotide conjugates of CR 6G have spectroscopic and electrophoretic properties that are superior to the JOE dye (C6171MP, Section 1.5) that is often used for DNA sequencing (Section 8.2, Table 8.7).

QSY® Dyes: Fluorescence Quenchers

Dyes that quench the fluorescence of visible light–excited fluorophores are increasingly important for use in fluorescence resonance energy transfer (FRET) proximity assays (Fluorescence Resonance Energy Transfer (FRET)—Note 1.2), such as those based on DNA hybridization.[29–31] Nonfluorescent acceptors are advantageous in FRET assays because they avoid the complications of proximity-independent signals resulting from direct excitation of fluorescent acceptors. Our QSY® 7, QSY® 9 and QSY® 21 dyes (Table 1.10) are diarylrhodamine derivatives that have several properties that make them superior to the commonly used dabcyl chromophore (Section 1.8) when preparing bioconjugates for use in FRET-based assays:

- Broad absorption in the visible-light spectrum, with an absorption maximum near 560 nm for both the QSY® 7 and QSY® 9 dyes and near 660 nm for the QSY® 21 dye (Figure 1.6.20)
- Extinction coefficients that are typically in excess of 90,000 $cm^{-1}M^{-1}$
- Absorption spectra of the conjugates that are insensitive to pH between 4 and 10
- Fluorescence quantum yields typically <0.001 in aqueous solution (in a few isolated cases, we have observed that some QSY® dyes can exhibit fluorescence when placed in a rigidifying environment such as glycerol.)
- Efficient quenching of the fluorescence emission of donor dyes by the QSY® 7 and QSY® 9 dyes, including blue-fluorescent coumarins, green- or orange-fluorescent dyes, and red-fluorescent Texas Red® and Alexa Fluor® 594 conjugates
- Quenching of red-fluorescent dyes, including Alexa Fluor® 647 dye, by the long-wavelength light–absorbing QSY® 21 dye[32] (Table 1.11)
- Quenching of most green and red fluorophores that is more effective at far greater distances than is possible with dabcyl quenchers (Figure 1.6.21)
- Residual fluorescence of the conjugates, at close spatial separations, that is typically lower than in conjugates that use dabcyl as the quencher
- High chemical stability of the conjugates and very good resistance to photobleaching

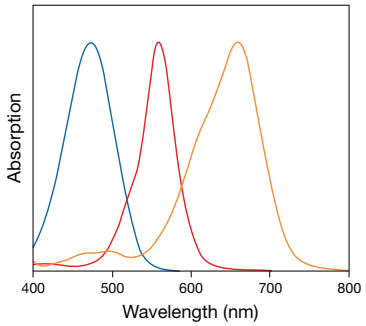

Figure 1.6.20 Normalized absorption spectra of the QSY® 35 (blue), QSY® 7 (red) and QSY® 21 (orange) dyes. The QSY® 7 and QSY® 9 dyes have essentially identical spectra.

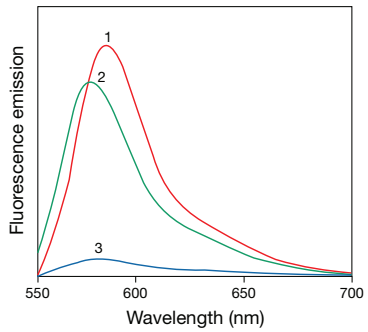

Figure 1.6.21 Fluorescence quenching of 5′-tetramethylrhodamine–labeled M13 primers by nonfluorescent dyes attached at the 3′-end. The comparison represents equal concentrations of oligonucleotides with 1) no 3′-quencher (control), 2) 3′-dabcyl quencer, and 3) 3′-QSY® 7 quencher.

Table 1.10 Molecular Probes® nonfluorescent quenchers and photosensitizers.

Dye	Abs *	Extinction Coefficient †	Amine-reactive Dyes‡	Notes
Dabcyl	453	32,000	D2245 (SE)	• Broad and intense visible-wavelength absorption • Efficient energy transfer acceptor from blue- and green-fluorescent dyes in FRET applications
Malachite green	628	76,000	M689 (ITC)	• Nonfluorescent photosensitizer
QSY® 7	560	90,000	Q10193 (SE)	• Essentially nonfluorescent quencher • Broad visible-wavelength absorption • Efficient energy transfer acceptor from UV light–excited green- and orange-fluorescent dyes in FRET applications
QSY® 9	562	88,000	Q20131 (SE)	• Essentially nonfluorescent quencher • Spectrally similar to QSY® 7, but with enhanced water solubility • Efficient energy transfer acceptor from UV light–excited green- and orange-fluorescent dyes in FRET applications
QSY® 21	661	90,000	Q20132 (SE)	• Essentially nonfluorescent quencher • Long-wavelength absorption • Efficient energy transfer acceptor from red- and near-infrared–fluorescent dyes in FRET applications
QSY® 35	475	23,000	Q20133 (SE) §	• Nonfluorescent quencher • Spectrally similar to dabcyl • Efficient energy transfer acceptor from blue- and green-fluorescent dyes in FRET applications

* Absorption (Abs) maxima, in nm. † Molar extinction coefficient in $cm^{-1}M^{-1}$ determined at the wavelength listed in the column headed Abs. These values may vary with the environment, particularly for the QSY® 35 dye. ‡ (ITC) = Isothiocyanate. (SE) = Succinimidyl ester.

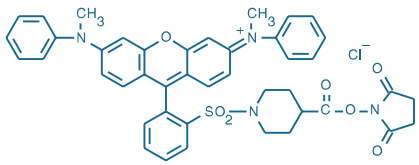

Figure 1.6.22 QSY® 7 carboxylic acid, succinimidyl ester (Q10193).

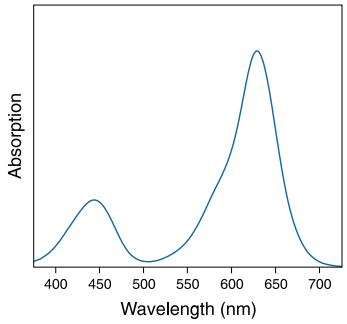

Figure 1.6.23 Absorption spectrum of malachite green isothiocyanate in acetonitrile.

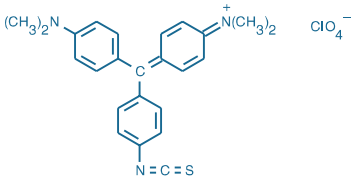

Figure 1.6.24 Malachite green isothiocyanate (M689).

A particularly frequent and effective application of QSY® dyes is as components of fluorogenic protease and peptidase substrates consisting of a fluorescent dye and a QSY® quencher attached to opposite ends of a peptide sequence that is specifically recognized and cleaved by the enzyme.[2,32,33] QSY® 7 dye has been used to create activatable targeted probes comprising a fluorophore–quencher pair and a targeting protein moiety (avidin, which targets the D-galactose receptor, or trastuzumab, a monoclonal antibody that recognizes the human epithelial growth factor receptor type 2 or HER2/neu) for use in *in vivo* tumor imaging. The fluorophore–quencher interaction, in this case between tetramethylrhodamine and QSY® 7 dyes, is disrupted when the probe is internalized in a tumor by receptor-mediated uptake, thereby activating the fluorescence.[34] In this imaging application, QSY® 7 was reported to be superior to azobenzene quenchers (e.g., dabcyl derivatives) because azobenzene dyes are vulnerable to reductive cleavage *in vivo*, producing a false positive signal. Both the amine-reactive and thiol-reactive QSY® 7 derivatives (Q10193, Q10257) have been used to create molecular beacon probes for following the transport of mRNAs in *Drosophila melanogaster* oocytes.[35]

The distance at which energy transfer is 50% efficient (i.e., 50% of excited donors are deactivated by fluorescence resonance energy transfer) is defined by the Förster radius (R_0). The magnitude of R_0 is dependent on the spectral properties of the donor and acceptor dyes. R_0 values calculated for energy transfer from various Alexa Fluor® dyes to QSY® and dabcyl quenchers are listed in Table 1.11. FRET efficiencies from several donor dyes to the QSY® 7 quencher in molecular beacon hybridization probes have also been calculated.[3]

For preparing bioconjugates, we offer several reactive versions of these QSY® dyes:

- Amine-reactive QSY® 7 (Figure 1.6.22), QSY® 9 and QSY® 21 succinimidyl esters (Q10193, Q20131, Q20132)
- Thiol-reactive QSY® 7 C_5-maleimide and QSY® 9 C_5-maleimide (Q10257, Q30457; Section 2.2)
- QSY® 7 aliphatic amine (Q10464, Section 3.4), which can be coupled to carbodiimide-activated carboxylic acids and other functional groups
- α-FMOC-ε-QSY® 7-L-lysine (Q21930, Section 9.5), for automated synthesis of peptides containing the QSY® 7 quencher

In addition to the QSY® 7, QSY® 9 and QSY® 21 dyes, we offer other quenchers that absorb maximally below 500 nm, including the QSY® 35 and dabcyl dyes (Table 1.10). These products are described in Section 1.8.

Nonfluorescent Malachite Green

Malachite green is a nonfluorescent photosensitizer that absorbs at long wavelengths (~630 nm, Figure 1.6.23). Its photosensitizing action can be targeted to particular cellular sites by conjugating malachite green isothiocyanate (M689, Figure 1.6.24) to specific antibodies. Enzymes and other proteins within ~10 Å of the binding site of the malachite green–labeled antibody can then be selectively destroyed upon irradiation with long-wavelength light.[36,37] Studies by Jay and colleagues have demonstrated that this photoinduced destruction of enzymes in the immediate vicinity of the chromophore is apparently the result of localized production of hydroxyl radicals, which have short lifetimes that limit their diffusion from the site of their generation.[38] Earlier studies had supported a thermal mechanism of action.[39–41]

Table 1.11 R_0 values for QSY® and dabcyl quenchers.

	Acceptor			
Donor	QSY® 35	dabcyl	QSY®7and QSY® 9	QSY® 21
Alexa Fluor® 350	47	50		
Alexa Fluor® 488	44	49	64	
Alexa Fluor® 546	25	29	67	
Alexa Fluor® 555			45	
Alexa Fluor® 568			56	75
Alexa Fluor® 594				77
Alexa Fluor® 647				69

R_0 values in angstroms (Å) represent the distance at which fluorescence resonance energy transfer from the donor dye to the acceptor dye is 50% efficient (Förster radius). Values were calculated from spectroscopic data as outlined (see Fluorescence Resonance Energy Transfer (FRET)—Note 1.2).

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1. Bioconjug Chem (2008) 19:1735; **2**. Nat Chem Biol (2007) 3:668; **3**. Nucleic Acids Res (2002) 30:e122; **4**. J Neurosci Methods (2003) 123:31; **5**. Biophys J (2007) 92:303; **6**. Biophys J (2005) 88:2939; **7**. Cytometry A (2005) 68:36; **8**. ACS Chem Biol (2009) 4:535; **9**. Anal Biochem (1977) 80:585; **10**. J Immunol Methods (1991) 143:263; **11**. Nucleic Acids Res (1992) 20:2471; **12**. J Chromatogr B Biomed Appl (1994) 657:307; **13**. Anal Biochem (1996) 238:165; **14**. Anal Biochem (1994) 223:39; **15**. Proc Natl Acad Sci U S A (1989) 86:9178; **16**. Proc Natl Acad Sci U S A (2010) 107:5453; **17**. J Photochem Photobiol A (2001) 139:151; **18**. Bioconjug Chem (2006) 17:1618; **19**. Bioconjug Chem (2006) 17:735; **20**. Bioconjug Chem (2000) 11:51; **21**. J Med Chem (2005) 48:7513; **22**. Bioconjug Chem (1996) 7:482; **23**. Bioconjug Chem (2001) 12:186; **24**. J Immunol Methods (1982) 50:193; **25**. J Org Chem (1966) 31:3880; **26**. Proc Natl Acad Sci U S A (2000) 97:13021; **27**. J Immunol (2009) 182:3573; **28**. J Biol Chem (2006) 281:18156; **29**. J Virol (2004) 78:4330; **30**. Mol Cell Probes (2004) 18:117; **31**. J Forensic Sci (2003) 48:282; **32**. Bioconjug Chem (2009) 20:702; **33**. Anal Biochem (2004) 332:90; **34**. Mol Pharm (2009) 6:386; **35**. Proc Natl Acad Sci U S A (2003) 100:13308; **36**. J Cell Biol (1996) 134:1197; **37**. Nature (1995) 376:686; **38**. Proc Natl Acad Sci U S A (1994) 91:2659; **39**. Biophys J (1992) 61:956; **40**. Biophys J (1992) 61:631; **41**. Proc Natl Acad Sci U S A (1988) 85:5454.

DATA TABLE 1.6 LONG-WAVELENGTH RHODAMINES, TEXAS RED DYES AND QSY QUENCHERS

Cat. No.	MW	Storage	Soluble	Abs	EC	Em	Solvent	Notes
C300	466.92	L	DMF, DMSO	540	95,000	565	MeOH	1
C652	476.44	L	pH >6, DMF	598	49,000	668	pH 10	2
C653	573.51	F,D,L	DMF, DMSO	602	42,000	672	pH 10	2
C1171	527.53	F,D,L	DMF, DMSO	546	95,000	576	MeOH	1, 3
C1309	631.68	F,D,L	DMF, DMSO	576	80,000	601	MeOH	1
C2211	527.53	F,D,L	DMF, DMSO	546	95,000	579	MeOH	1, 3
C6121	430.46	L	pH >6, DMF	542	91,000	568	MeOH	1
C6122	430.46	L	pH >6, DMF	540	103,000	564	MeOH	1
C6123	527.53	F,D,L	DMF, DMSO	547	91,000	573	MeOH	1, 3
C6124	635.80	F,L	pH >6, DMF	567	92,000	591	MeOH	1
C6125	631.68	F,D,L	DMF, DMSO	574	78,000	602	MeOH	1
C6126	631.68	F,D,L	DMF, DMSO	575	82,000	602	MeOH	1
C6127	555.59	F,D,L	pH >6, DMF	524	108,000	557	MeOH	
C6128	555.59	F,D,L	DMF, DMSO	524	102,000	550	MeOH	
C6156	534.61	F,L	pH >6, DMF	570	113,000	590	MeOH	1
C6157	555.59	F,D,L	DMF, DMSO	524	92,000	552	MeOH	
L20	577.11	F,DD,L	DMF, MeCN	568	88,000	583	MeOH	4
L1908	577.11	F,DD,L	DMF, MeCN	568	88,000	583	MeOH	4
M689	485.98	F,DD,L	DMF, DMSO	629	75,000	none	MeCN	5
Q10193	791.32	F,D,L	DMSO	560	90,000	none	MeOH	
Q20131	951.43	F,D,L	H_2O, DMSO	562	88,000	none	MeOH	6
Q20132	815.34	F,D,L	DMSO	661	90,000	none	MeOH	
R6160	768.90	F,D,L	DMF, DMSO	560	129,000	580	MeOH	
T353	625.15	F,DD,L	DMF, MeCN	588	84,000	601	$CHCl_3$	4
T490	443.52	F,DD,L	DMF, DMSO	544	84,000	572	MeOH	3, 5
T1480	443.52	F,DD,L	DMF, DMSO	543	99,000	571	MeOH	3, 5
T1481	443.52	F,DD,L	DMF, DMSO	544	90,000	572	MeOH	3, 5
T1905	625.15	F,DD,L	DMF, MeCN	587	85,000	602	$CHCl_3$	4
T6105	640.69	F,D,L	DMF, DMSO	543	92,000	571	MeOH	1, 3
T6134	816.94	F,D,L	DMF, DMSO	583	112,000	603	MeOH	
T20175	816.94	F,D,L	DMF, DMSO	587	96,000	602	MeOH	
T30200	796.74	F,D,L	DMF, DMSO	583	87,000	604	MeOH	
X491	547.67	F,DD,L	DMF, DMSO	572	92,000	596	MeOH	5

For definitions of the contents of this data table, see "Using *The Molecular Probes® Handbook*" in the introductory pages.

Notes

1. Abs and Em for TAMRA and ROX dyes in pH 8 buffer are red-shifted approximately 8 nm compared to MeOH, with EC lower by ~10%.
2. Absorption and fluorescence of naphthofluorescein derivatives are pH dependent. Both the absorption and emission spectra shift to much shorter wavelengths at pH <8. Fluorescence quantum yield ~0.14 at pH 9.5.
3. Tetramethylrhodamine protein conjugates often exhibit two absorption peaks at about 520 and 545 nm. The 520 nm peak is due to nonfluorescent dye aggregates. (J Immunol Methods (1991) 143:263, J Phys Chem B (1998) 102:1820)
4. Do NOT dissolve in DMSO.
5. Isothiocyanates are unstable in water and should not be stored in aqueous solution.
6. This sulfonated succinimidyl ester derivative is water soluble and may be dissolved in buffer at ~pH 8 for reaction with amines. Long-term storage in water is NOT recommended due to hydrolysis.

PRODUCT LIST 1.6 LONG-WAVELENGTH RHODAMINES, TEXAS RED DYES AND QSY QUENCHERS

Cat No.	Product	Quantity
C652	5-(and-6)-carboxynaphthofluorescein *mixed isomers*	100 mg
C653	5-(and-6)-carboxynaphthofluorescein, succinimidyl ester *mixed isomers*	25 mg
C6127	5-carboxyrhodamine 6G, succinimidyl ester (5-CR 6G, SE) *single isomer*	5 mg
C6128	6-carboxyrhodamine 6G, succinimidyl ester (6-CR 6G, SE) *single isomer*	5 mg
C6157	5-(and-6)-carboxyrhodamine 6G, succinimidyl ester (5(6)-CR 6G, SE) *mixed isomers*	5 mg
C6121	5-carboxytetramethylrhodamine (5-TAMRA) *single isomer*	10 mg
C6122	6-carboxytetramethylrhodamine (6-TAMRA) *single isomer*	10 mg
C300	5-(and-6)-carboxytetramethylrhodamine (5(6)-TAMRA) *mixed isomers*	100 mg
C2211	5-carboxytetramethylrhodamine, succinimidyl ester (5-TAMRA, SE) *single isomer*	5 mg
C6123	6-carboxytetramethylrhodamine, succinimidyl ester (6-TAMRA, SE) *single isomer*	5 mg
C1171	5-(and-6)-carboxytetramethylrhodamine, succinimidyl ester (5(6)-TAMRA, SE) *mixed isomers*	25 mg
C6124	5-carboxy-X-rhodamine, triethylammonium salt (5-ROX) *single isomer*	10 mg
C6156	6-carboxy-X-rhodamine (6-ROX) *single isomer*	10 mg
C6125	5-carboxy-X-rhodamine, succinimidyl ester (5-ROX, SE) *single isomer*	5 mg
C6126	6-carboxy-X-rhodamine, succinimidyl ester (6-ROX, SE) *single isomer*	5 mg
C1309	5-(and-6)-carboxy-X-rhodamine, succinimidyl ester (5(6)-ROX, SE) *mixed isomers*	25 mg
F6161	FluoReporter® Rhodamine Red™-X Protein Labeling Kit *5–10 labelings*	1 kit
F6162	FluoReporter® Texas Red®-X Protein Labeling Kit *5–10 labelings*	1 kit
L20	Lissamine rhodamine B sulfonyl chloride *mixed isomers*	1 g
L1908	Lissamine rhodamine B sulfonyl chloride *mixed isomers* *special packaging*	10 x 10 mg
M689	malachite green isothiocyanate	10 mg
Q10193	QSY® 7 carboxylic acid, succinimidyl ester	5 mg
Q20131	QSY® 9 carboxylic acid, succinimidyl ester	5 mg
Q20132	QSY® 21 carboxylic acid, succinimidyl ester	5 mg
R6160	Rhodamine Red™-X, succinimidyl ester *5-isomer*	5 mg
T6105	6-(tetramethylrhodamine-5-(and-6)-carboxamido)hexanoic acid, succinimidyl ester (5(6)-TAMRA-X, SE) *mixed isomers*	10 mg
T1480	tetramethylrhodamine-5-isothiocyanate (5-TRITC; G isomer)	5 mg
T1481	tetramethylrhodamine-6-isothiocyanate (6-TRITC; R isomer)	5 mg
T490	tetramethylrhodamine-5-(and-6)-isothiocyanate (5(6)-TRITC) *mixed isomers*	10 mg
T30200	Texas Red® C_2-dichlorotriazine	5 mg
T353	Texas Red® sulfonyl chloride *mixed isomers*	10 mg
T1905	Texas Red® sulfonyl chloride *mixed isomers* *special packaging*	10 x ~1 mg
T10244	Texas Red®-X Protein Labeling Kit *3 labelings*	1 kit
T6134	Texas Red®-X, succinimidyl ester *mixed isomers*	5 mg
T20175	Texas Red®-X, succinimidyl ester *single isomer*	2 mg
X491	X-rhodamine-5-(and-6)-isothiocyanate (5(6)-XRITC) *mixed isomers*	10 mg
Z25045	Zenon® Texas Red®-X Mouse IgG_1 Labeling Kit *50 labelings*	1 kit

1.7 Coumarins, Pyrenes and Other Ultraviolet Light–Excitable Fluorophores

Shorter-wavelength amine-reactive fluorophores are less frequently used for preparing bioconjugates because dyes excited with longer wavelengths, and therefore lower energy, are widely available and less likely to cause photodamage to labeled biomolecules. Moreover, many cells and tissues autofluoresce when excited with ultraviolet (UV) light, producing detection-confounding background signals. However, for certain multicolor fluorescence applications—including immunofluorescence, nucleic acid and protein microarrays, *in situ* hybridization and neuronal tracing—a blue-fluorescent probe provides a contrasting color that is clearly resolved from the green, yellow, orange or red fluorescence of the longer-wavelength probes.

The short-wavelength reactive dyes that we recommend for preparing the brightest blue-fluorescent bioconjugates are the Alexa Fluor® 350, Alexa Fluor® 405, AMCA-X, Marina Blue®, Pacific Blue™ and Cascade Blue® derivatives (Table 1.12). Alexa Fluor® 430, Pacific Orange™ and Cascade Yellow™ dyes fill a spectral void because they exhibit the rare combination of absorption between 400 nm and 450 nm and fluorescence emission beyond 500 nm. The amine-reactive naphthalene, pyrene and Dapoxyl® derivatives are important for the production of environment-sensitive probes in protein structure and function studies (Table 1.13); their thiol-reactive counterparts are discussed in Section 2.3. Many of our UV light–excitable reactive dyes are more commonly employed for such bioanalytical techniques as HPLC derivatization, amino acid sequencing and protein determination and are therefore discussed in Section 1.8.

Table 1.12 Amine-reactive, ultraviolet light–excitable fluorophores for labeling proteins and nucleic acids.

Fluorophore	Abs *	Em *	Amine-Reactive Dyes	Protein and Nucleic Acid Labeling Kits	Notes
Alexa Fluor® 350	346	442	A10168 (SE)	A20180 (Mab) A10170 (P) Z25000 (Z) Z25100 (Z) Z25200 (Z) Z25300 (Z) Z25400 (Z)	• Higher fluorescence per attached dye than AMCA • Protein conjugates emit at slightly shorter wavelengths than AMCA or AMCA-X
Alexa Fluor® 405	402	421	A30000 (SE) A30100 (SE)	Z25013 (Z) Z25113 (Z) Z25213 (Z) Z25313 (Z)	• Cascade Blue® derivative containing a spacer between the fluorophore and the reactive SE • Near-perfect match to the violet diode laser (405 nm)
Alexa Fluor® 430	433	539	A10169 (SE)	A10171 (P) Z25001 (Z) Z25301 (Z)	• Extremely large Stokes shift • Fills spectral gap between green- and yellow-fluorescent dyes
AMCA-X	353	442	A6118 (SE)		• Widely used blue-fluorescent labeling dye • Compact structure
Bimane	380	458	B30250 (COOH)		• Blue-fluorescent dye • Small size
Cascade Blue®	400	420	C2284 (AA)		• Resistant to quenching upon protein conjugation • Trisulfonated pyrene
Dialkylaminocoumarin	375 435	470 [1] 475 [2]	D126 (COOH) D1421 (COOH) D374 (SE) D1412 (SE) D10166 (ITC)		• Longer-wavelength alternatives to AMCA
Hydroxycoumarin	385 360	445 [3] 455 [4]	H185 (COOH) H1428 (COOH) H1193 (SE)		• pH-sensitive fluorescence • Compact structure
Marina Blue®	365	460	M10165 (SE)		• Optimal for 365 nm excitation sources
Methoxycoumarin	358	410	M1420MP (COOH) M1410 (SE)		• pH-insensitive fluorescence • Compact structure
Pacific Blue™	410	455	P10163 (SE)	A10478 (APEX®) P30013 (Mab) P30012 (P) Z25041 (Z) Z25341 (Z)	• Longer-wavelength alternative to the Alexa Fluor® 350 and AMCA-X dyes • Excited with violet diode laser (405 nm)
Pacific Orange™	400	551	P30253 (SE)	P30014 (Mab) P30016 (P) Z25256 (Z) Z25257 (Z)	• Excited with the violet diode laser (405 nm) • Compatible with the Pacific Blue™ dye for two-color analysis using the blue diode laser

* The absorption (Abs) and fluorescence emission (Em) maxima, in nm, listed in this table are for the goat anti–mouse IgG antibody or dextran conjugates in aqueous buffer. (APEX®) = APEX® Antibody Labeling Kit (Section 1.2). (AA) = Acetyl azide. (COOH) = Carboxylic acid. (ITC) = Isothiocyanate. (Mab) = Monoclonal Antibody Labeling Kit (Section 1.2). (P) = Easy-to-Use Protein Labeling Kit (Section 1.2). (SE) = Succinimidyl ester. (Z) = Zenon® Antibody Labeling Kit (Section 7.3). **1**. Spectral maxima for D374; **2**. Spectral maxima for D1412; **3**. Spectral maxima for H1193; **4**. Spectral maxima for 7-hydroxy-4-methylcoumarin-3-acetic acid, succinimidyl ester.

Table 1.13 Amine-reactive, environment-sensitive fluorophores.

Fluorophore	Abs *	Em *	Succinimidyl Ester	Other	Notes
Cascade Yellow™	402	545	C10164		• Fluorescence emission spectrum shifts to shorter wavelengths in nonpolar solvents
Dansyl	335	518	D6104 (X)	D21 (SC)	• Sulfonyl chloride is nonfluorescent until it reacts with amines • Weak fluorescence in aqueous solutions
Dapoxyl®	395	601	D10161	D10160 (SC)	• Very low fluorescence in water • Large Stokes shifts (up to ~200 nm) • Large extinction coefficients of Dapoxyl® derivatives in some solvents [1]
NBD	466	535	S1167 (X)	C20260 (AH) F486 (AH)	• NBD amine derivatives have low fluorescence in water; emission spectra and quantum yields in other solvents are variable [2]
PyMPO	415	570	S6110		• Fluorescence emission spectrum shifts to shorter wavelengths in nonpolar solvents
Pyrene	340	376	P130 P6114 (CASE)	P24 (SC)	• Forms excited-state dimers (excimers) that emit at longer wavelengths (~470 nm) than the lone excited fluorophore • Extremely long fluorescence lifetime (can be >100 ns)

Absorption (Abs) and Emission (Em) maxima, in nm, are for conjugates. (AH) = Aryl halide. (CASE) = Cysteic acid separating the dye and the SE. (SC) = Sulfonyl chloride. (X) = Aminohexanoyl spacer separating the dye and the SE. **1**. Photochem Photobiol (1997) 66:424; **2**. Biochemistry (1977) 16:5150.a

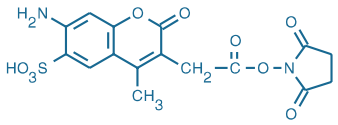


Figure 1.7.1 Alexa Fluor® 350 carboxylic acid, succinimidyl ester (A10168).

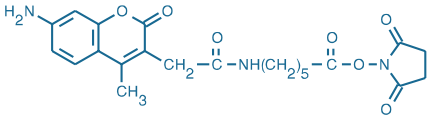


Figure 1.7.2 6-((7-amino-4-methylcoumarin-3-acetyl)amino)hexanoic acid, succinimidyl ester (AMCA-X, SE; A6118).

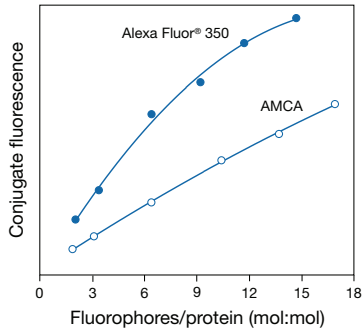


Figure 1.7.3 Comparison of the relative fluorescence of 7-amino-4-methylcoumarin-3-acetic acid (AMCA) streptavidin (○) and Alexa Fluor® 350 streptavidin, a sulfonated AMCA derivative (S11249, ●). Conjugate fluorescence is determined by measuring the fluorescence quantum yield of the conjugated dye relative to that of the free dye and multiplying by the number of fluorophores per protein.

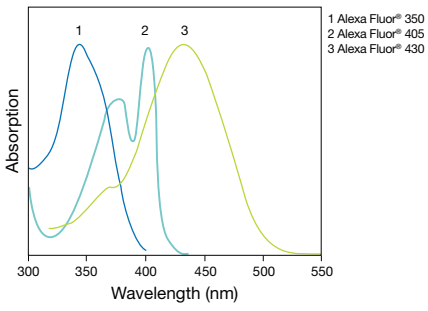


Figure 1.7.4 Absorption spectra of our ultraviolet and blue light–absorbing Alexa Fluor® dyes.

Alexa Fluor® 350 and Other Coumarin Derivatives

Alexa Fluor® 350 and AMCA-X Dyes

Derivatives of 7-aminocoumarin dyes are widely used labeling reagents for preparing protein and nucleic acid conjugates,[1] and we offer two important amine-reactive 7-aminocoumarin derivatives: Alexa Fluor® 350 carboxylic acid succinimidyl ester[2,3] (A10168) and AMCA-X succinimidyl ester (AMCA-X, SE; A6118).

The sulfonated coumarin derivative, Alexa Fluor® 350 carboxylic acid succinimidyl ester (Figure 1.7.1), is more water soluble than either AMCA succinimidyl ester or AMCA-X succinimidyl ester (Figure 1.7.2) and yields protein conjugates that are more fluorescent than those prepared from its nonsulfonated analog (Figure 1.7.3). Alexa Fluor® 350 protein conjugates are optimally excited at 346 nm and have bright blue fluorescence emission (Figure 1.7.4, Figure 1.7.5) at wavelengths slightly shorter than AMCA or AMCA-X conjugates (Figure 1.7.6) (442 nm versus 448 nm), which reduces the dye's spectral overlap with the emission of fluorescein. We offer several reactive versions of Alexa Fluor® 350 dye, including:

- Amine-reactive succinimidyl ester (A10168)
- Thiol-reactive maleimide (A30505, Section 2.3)
- Aldehyde- and ketone-reactive hydrazide and hydroxylamine (A10439, A30627; Section 3.3)
- Aldehyde- and ketone-reactive cadaverine (A30674, Section 3.4)

AMCA-X succinimidyl ester (A6118) contains a seven-atom aminohexanoyl spacer ("X") between the fluorophore and the reactive group. This spacer separates the fluorophore from the biomolecule to which it is conjugated, potentially reducing the quenching that typically occurs upon conjugation and making the dye more available for recognition by secondary detection reagents. Slightly longer-wavelength conjugates can be prepared from the isothiocyanate (DACITC, D10166), succinimidyl esters (D374, D1412) or free acids (D126, D1421) of 7-dialkylaminocoumarins.[4,5]

Alexa Fluor® 430 Dye

Few reactive dyes that absorb between 400 nm and 450 nm have appreciable fluorescence beyond 500 nm in aqueous solution. Alexa Fluor® 430 dye fills this spectral gap.[2] Excitation near its absorption maximum at 431 nm (Figure 1.7.4) is accompanied by strong green fluorescence with an emission maximum at 541 nm. The amine-reactive succinimidyl ester of Alexa Fluor® 430 carboxylic acid (A10169, Figure 1.7.7) is available, as well as Alexa Fluor® 430 conjugates of secondary antibodies (A11063, A11064; Section 7.2, Table 7.1) and streptavidin (S11237, Section 7.6, Table 7.9).

Marina Blue® and Pacific Blue™ Dyes

Marina Blue® and Pacific Blue™ dyes, both of which are based on the 6,8-difluoro-7-hydroxycoumarin fluorophore, exhibit bright blue fluorescence emission near 460 nm[6] (Table 1.12). The Marina Blue® dye is optimally detected using optical filters configured for DAPI, whereas

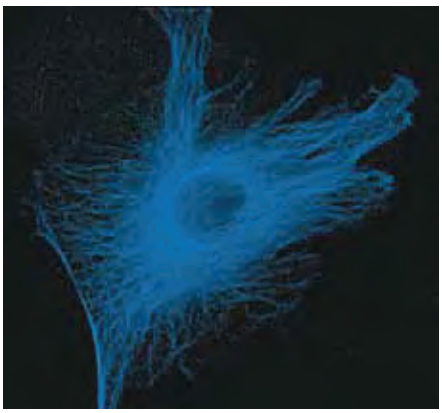


Figure 1.7.5 The microtubules of fixed bovine pulmonary artery endothelial cells (BPAEC) localized with mouse monoclonal anti–α-tubulin antibody (A11126), which was visualized with Alexa Fluor® 350 goat anti–mouse IgG antibody (A11045). The image was acquired using a longpass filter set appropriate for DAPI.

the Pacific Blue™ dye is ideally suited for 405 nm violet diode laser excitation on the Applied Biosystems® Attune™ Acoustic Focusing cytometer and similarly equipped fluorescence microscopes (Figure 1.7.8). Significantly, the pK_a values of these 6,8-difluoro-7-hydroxycoumarin derivatives are 2–3 log units lower than those of the corresponding 7-hydroxycoumarins (Figure 1.7.9). Thus, the Marina Blue® and Pacific Blue™ dyes yield conjugates that are strongly fluorescent, even at neutral pH. For preparing bioconjugates, we offer amine-reactive succinimidyl esters of the Marina Blue® and Pacific Blue™ dyes (M10165, Figure 1.7.10; P10163, Figure 1.7.11).

Other Hydroxycoumarin and Alkoxycoumarin Derivatives

The hydroxycoumarins (H185, H1193, H1428) exhibit pH-sensitive spectral properties, but the methoxycoumarins (M1410, M1420MP) do not.[7,8] Hydroxycoumarins are often used to prepare reactive intermediates for the synthesis of radioiodinated materials.[9] The spectral properties of the hydroxycoumarins allow their quantitation prior to radioiodination.[9]

Alexa Fluor® 350, Alexa Fluor® 430 and Pacific Blue™ Protein Labeling Kits

For easy and trouble-free labeling of proteins with succinimidyl esters of the Alexa Fluor® 350, Alexa Fluor® 430 and Pacific Blue™ dyes, we offer Alexa Fluor® 350, Alexa Fluor® 430 and Pacific Blue™ Protein Labeling Kits (A10170, A10171, A10171; Table 1.2). These kits, which are described in greater detail in Section 1.2, contain everything that is required to perform three separate labeling reactions and to purify the resulting conjugates (Table 1.3). The Alexa Fluor® 350 and Pacific Blue™ Monoclonal Antibody Labeling Kits (A20180, P30013) can be used to prepare blue-fluorescent conjugates of monoclonal antibodies, as well as of other proteins in limited quantities (five labeling reactions of ~100 µg each). The APEX® Pacific Blue™ Antibody Labeling Kit (A10478) utilizes a solid-phase technique to label 10–20 µg IgG antibody, even in the presence of stabilizing proteins or amine-containing buffers.

The Zenon® Alexa Fluor® 350, Zenon® Alexa Fluor® 430 and Zenon® Pacific Blue™ Antibody Labeling Kits (Table 7.7) permit the rapid and quantitative labeling of antibodies—even submicrogram amounts—using a purified antibody fraction or a crude antibody preparation such as serum, ascites fluid or a hybridoma supernatant. These kits, along with Zenon® technology, are described in detail in Section 7.3.

Pacific Orange™ Dye

The succinimidyl ester of the Pacific Orange™ dye (P30253) yields conjugates with excitation/emission maxima of ~400/551 nm, making it ideal for use with 405 nm violet diode laser–equipped flow cytometers[10] and fluorescence microscopes. Moreover, Pacific Blue™ and Pacific Orange™ conjugates can be simultaneously excited at 405 nm and emit at 455 and 551 nm, respectively, facilitating two-color analysis.

Several of our kits facilitate protein labeling with the Pacific Orange™ succinimidyl ester, including the Pacific Orange™ Protein Labeling Kit (P30016), the Pacific Orange™ Monoclonal Antibody Labeling Kit (P30014) and the Zenon® Antibody Labeling Kits (Table 7.7), all of which are described in greater detail in Section 1.2.

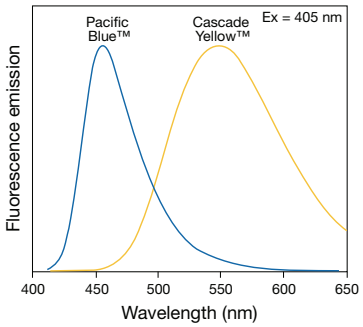


Figure 1.7.8 Normalized fluorescence emission spectra of Pacific Blue™ goat anti–mouse IgG antibody (P10993) and a Cascade Yellow™ goat anti–mouse IgG antibody conjugate prepared with the Cascade Yellow™ succinimidyl ester (C10164). Both fluorescent conjugates are excited at 405 nm. When samples containing equal concentrations of antibody are compared, the peak fluorescence intensity of the Pacific Blue™ conjugate at 456 nm is nine times greater than that of the Cascade Yellow™ conjugate at 548 nm.

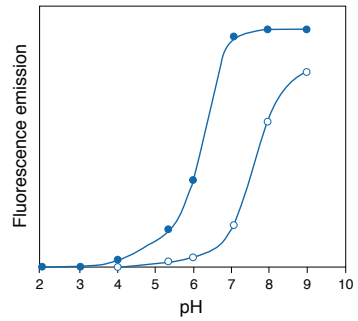


Figure 1.7.9 Comparison of the pH-dependent fluorescence changes produced by attachment of electron-withdrawing fluorine atoms to a hydroxycoumarin. 7-Hydroxy-4-methylcoumarin-3-acetic acid (○, H1428) and 6,8-difluoro-7-hydroxy-4-methylcoumarin (●, D6566). Fluorescence intensities were measured for equal concentrations of the two dyes using excitation/emission at 360/450 nm.

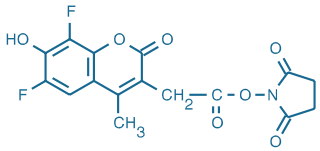


Figure 1.7.10 Marina Blue® succinimidyl ester (M10165).

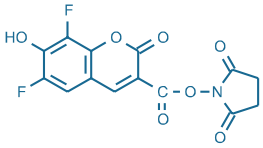


Figure 1.7.11 Pacific Blue™ succinimidyl ester (P10163).

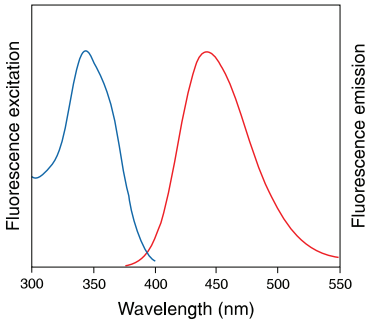


Figure 1.7.6 Fluorescence excitation and emission spectra of Alexa Fluor® 350 goat anti–mouse IgG antibody in pH 8.0 buffer.

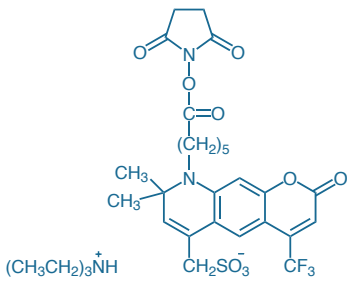


Figure 1.7.7 Alexa Fluor® 430 carboxylic acid, succinimidyl ester (A10169).

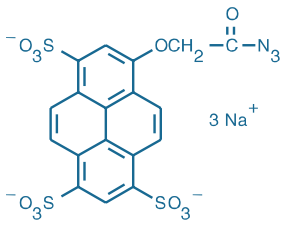


Figure 1.7.12 Cascade Blue® acetyl azide, trisodium salt (C2284).

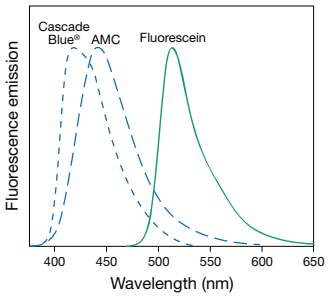


Figure 1.7.13 Normalized fluorescence emission spectra of Cascade Blue® (CB), 7-amino-4-methylcoumarin (AMC) and fluorescein in aqueous solutions.

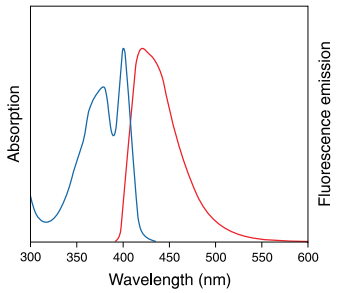


Figure 1.7.14 Absorption and fluorescence emission spectra of Cascade Blue® dye–labeled bovine serum albumin (BSA) in pH 7.0 buffer.

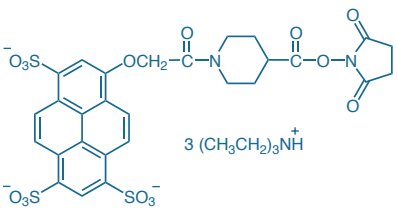


Figure 1.7.16 Alexa Fluor® 405 carboxylic acid, succinimidyl ester (A30000).

Cascade Blue® and Other Pyrene Derivatives

Cascade Blue® Acetyl Azide

Cascade Blue® acetyl azide is the amine-reactive sulfonated pyrene derivative [11] that we use to prepare blue-fluorescent Cascade Blue® dye–labeled proteins and dextrans. The polar nature of this reagent makes it difficult to purify to homogeneity; however, we offer a Cascade Blue® acetyl azide preparation (C2284, Table 1.12, Figure 1.7.12) that is ~60% reactive and packaged according to the net weight of the reactive dye. The remaining constituents are inorganic salts or unreactive forms of the dye that can readily be removed following conjugation.

As compared with the aminocoumarin derivatives, the Cascade Blue® fluorophore shows less spectral overlap with fluorescein (Figure 1.7.13), an important advantage for multicolor applications. In addition, this reactive Cascade Blue® derivative has high absorptivity (Figure 1.7.14), is highly fluorescent and resists quenching upon protein conjugation (Figure 1.7.15). Even at low degrees of labeling, Cascade Blue® conjugates are significantly more fluorescent than are those of 7-amino-4-methylcoumarin-3-acetic acid (AMCA),[11] and they remain preferred reagents for multicolor flow cytometry.[12–14]

Alexa Fluor® 405 Dye

With excitation/emission maxima of 402/421 nm (Figure 1.7.4), Alexa Fluor® 405 dye is well matched to the 405 nm spectral line of violet diode lasers for fluorescence microscopy and flow cytometry.[10,15,16] Alexa Fluor® 405 succinimidyl ester is an amine-reactive derivative of our Cascade Blue® dye. Not only is it offered at higher purity than the alternative Cascade Blue® acetyl azide, but Alexa Fluor® 405 succinimidyl ester also contains a 4-piperidinecarboxylic acid spacer that separates the fluorophore from its reactive moiety (Figure 1.7.16). This spacer enhances the reactivity of the succinimidyl ester and minimizes any interactions between the fluorophore and the biomolecule to which it is conjugated.

As with Cascade Blue® acetyl azide, Alexa Fluor® 405 dye shows minimal spectral overlap with green fluorophores, making it ideal for multicolor applications.[15,17] However, the violet fluorescence of Cascade Blue® and Alexa Fluor® 405 dyes is less visible to the human eye in fluorescence microscopy applications than the blue fluorescence of Alexa Fluor® 350 and AMCA-X dyes. Alexa Fluor® 405 dye is available as:

- Amine-reactive succinimidyl ester (A30000, A30100)
- Aldehyde- and ketone-reactive cadaverine (A30675, Section 3.4)

We also prepare Alexa Fluor® 405 conjugates of secondary antibodies (Section 7.2, Table 7.1) and streptavidin (Section 7.6, Table 7.9). Alexa Fluor® 405 conjugates are recognized by the anti–Alexa Fluor® 405/Cascade Blue® dye antibody (A5760, Section 7.4).

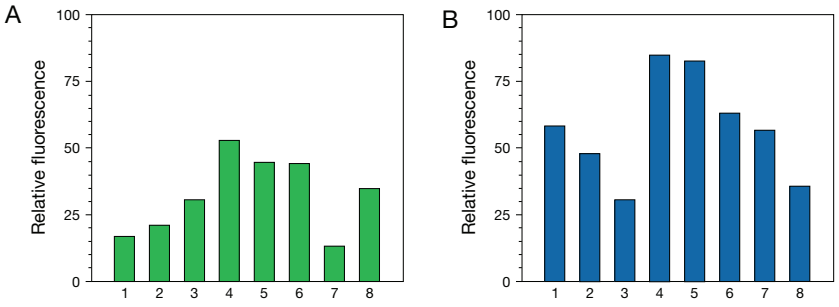


Figure 1.7.15 Histograms showing the fluorescence per fluorophore for **A**) fluorescein and **B**) Cascade Blue® conjugated to various proteins, relative to the fluorescence of the free dye in aqueous solution, represented by 100 on the y-axis. The proteins represented are: **1**) avidin, **2**) bovine serum albumin, **3**) concanavalin A, **4**) goat IgG, **5**) ovalbumin, **6**) protein A, **7**) streptavidin and **8**) wheat germ agglutinin.

Other Pyrenes

Conjugates of the pyrene succinimidyl esters (P130, P6114) have exceptionally long excited-state lifetimes (sometimes >100 nanoseconds), relatively short-wavelength emission and capacity for proximity-dependent excimer formation (Figure 1.7.17). These amine-reactive pyrene derivatives have primarily been used for labeling and detecting oligonucleotides,[18,19] biogenic amines[20] and polyamines.[21] Pyrene binds strongly to carbon nanotubes via pi-stacking interactions. This property makes 1-pyrenebutanoic acid succinimidyl ester (P130) a valuable reagent for functionalizing these remarkable nanomaterials for coupling to proteins.[22,23]

The long fluorescence lifetime of pyrenebutyric acid (1-pyrenebutanoic acid) permits time-gating of the fluorescence, which is a useful technique for discriminating between the dye signal and sample autofluorescence,[24] and has been exploited for fluorescence immunoassays.[25] For preparing pyrene conjugates with long fluorescence lifetimes, we recommend the more water-soluble succinimidyl ester of *N*-(1-pyrenebutanoyl)cysteic acid (P6114, Figure 1.7.18). The amine-reactive 1-pyrenesulfonyl chloride (P24, Section 1.8) has been used to generate a fluorescent ATP sensor via modification of an ATP-binding ribonucleopeptide.[26]

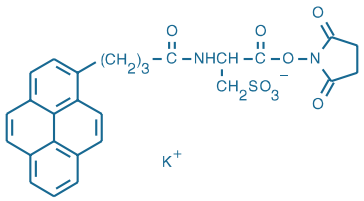


Figure 1.7.18 *N*-(1-pyrenebutanoyl)cysteic acid, succinimidyl ester, potassium salt (P6114).

Cascade Yellow™ and Other Pyridyloxazole Derivatives

Cascade Yellow™ Dye

Like the Alexa Fluor® 430 and Pacific Blue™ dyes described above, the Cascade Yellow™ dye exhibits an excitation maximum that falls between those of the UV light–excited dyes and the fluoresceins. This sulfonated pyridyloxazole (PyMPO) laser dye (Figure 1.7.19) exhibits an absorption maximum near 410 nm and an unusually high Stokes shift, with relatively strong emission at 550–570 nm[27–29] (Figure 1.7.20). The large Stokes shift permits detection at a wavelength well beyond that of most sample autofluorescence, and allows multiple fluorophores to be excited at the same wavelengths and detected at different wavelengths. For example, protein conjugates of Cascade Yellow™ succinimidyl ester (C10164) can be simultaneously excited at 405 nm with Pacific Blue™ conjugates, and then separately detected at longer wavelengths (Figure 1.7.8). Cascade Yellow™ and Cascade Blue® antibody conjugates, along with several phycobiliprotein tandem conjugates, are utilized in an 11-color polychromatic flow cytometry technique.[12,13]

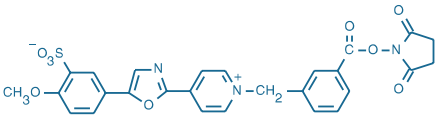


Figure 1.7.19 Cascade Yellow™ succinimidyl ester (C10164).

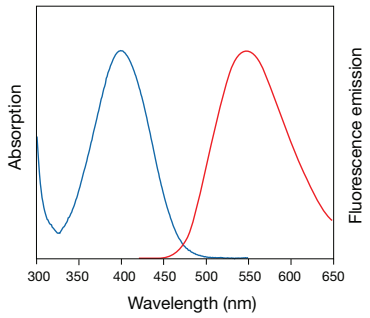


Figure 1.7.20 Absorption and fluorescence emission spectra of Cascade Yellow™ goat anti–mouse IgG antibody in pH 8.0 buffer.

PyMPO Dye

The pyridyloxazole derivatives—including the succinimidyl ester (PyMPO, SE; S6110; Figure 1.7.21) and the thiol-reactive maleimide (M6026, Section 2.2)—fill the spectral gap between UV light–excited dyes and the fluoresceins. These derivatives of the laser dye PyMPO exhibit absorption maxima near 415 nm and unusually high Stokes shifts, with emission at 560–580 nm.[28] Like the naphthalene-based dyes, the pyridyloxazole dyes exhibit environment-sensitive fluorescence spectra. PyMPO SE has been used to synthesize fluorescent gramicidin derivatives for following ion channel–gating processes.[30]

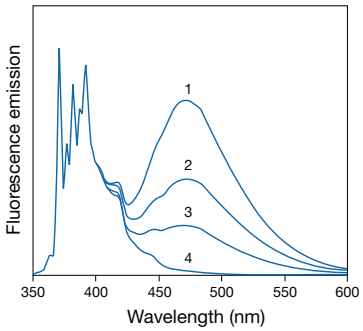


Figure 1.7.17 Excimer formation by pyrene in ethanol. Spectra are normalized to the 371.5 nm peak of the monomer. All spectra are essentially identical below 400 nm after normalization. Spectra are as follows: 1) 2 mM pyrene, purged with argon to remove oxygen; 2) 2 mM pyrene, air-equilibrated; 3) 0.5 mM pyrene (argon-purged); and 4) 2 µM pyrene (argon-purged). The monomer-to-excimer ratio (371.5 nm/470 nm) is dependent on both pyrene concentration and the excited-state lifetime, which is variable because of quenching by oxygen.

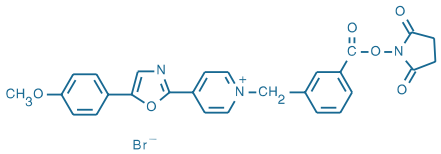


Figure 1.7.21 1-(3-(succinimidyloxycarbonyl)benzyl)-4-(5-(4-methoxyphenyl)oxazol-2-yl)pyridinium bromide (PyMPO, SE; S6110).

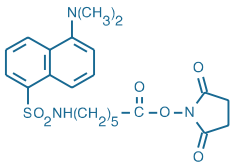


Figure 1.7.22 6-((5-dimethylaminonaphthalene-1-sulfonyl) amino)hexanoic acid, succinimidyl ester (dansyl-X, SE; D6104).

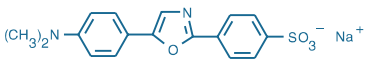


Figure 1.7.23 Dapoxyl® sulfonic acid, sodium salt (D12800).

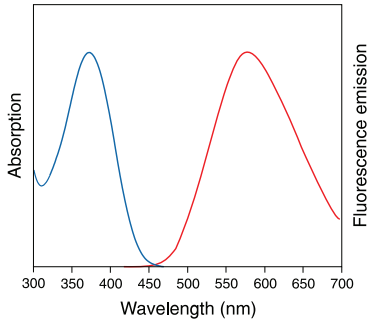


Figure 1.7.24 Absorption and fluorescence emission spectra of Dapoxyl® (2-aminoethyl)sulfonamide in methanol.

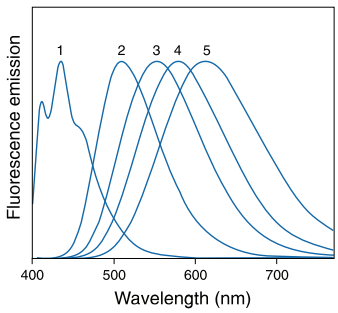


Figure 1.7.25 Normalized fluorescence emission spectra of Dapoxyl® (2-aminoethyl)sulfonamide (D10460) in **1**) hexane, **2**) chloroform, **3**) acetone, **4**) acetonitrile and **5**) 1:1 acetonitrile:water.

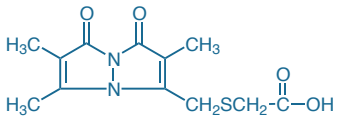


Figure 1.7.26 Bimane mercaptoacetic acid (carboxymethylthiobimane, B30250).

Naphthalenes, Including Dansyl Chloride

Aminonaphthalene-based probes tend to have emission spectra that are sensitive to the environment and to exhibit weak fluorescence in aqueous solution. Spectra of environment-sensitive probes respond to perturbations in the local environment (Table 1.13). For example, changes in solvation that occur because of ligand binding, protein assembly or protein denaturation can often evoke changes in the fluorescence properties of these probes. This property has made dansyl chloride (5-dimethylaminonaphthalene-1-sulfonyl chloride, D21) and other aminonaphthalene-based dyes important tools for protein structural studies.

Dansyl chloride is nonfluorescent until it reacts with amines. The resulting dansyl amides have environment-sensitive fluorescence quantum yields and emission maxima, along with large Stokes shifts. Despite the weak absorptivity (EC ~4000 $cm^{-1}M^{-1}$ at 330–340 nm) and moderate fluorescence quantum yield of dansyl sulfonamides, dansyl chloride is widely used as a derivatization reagent for end-group analysis of proteins, amino acid analysis and HPLC detection (Section 1.8). The succinimidyl ester of dansylaminohexanoic acid (dansyl-X, SE; D6104; Figure 1.7.22) contains a seven-atom spacer ("X") that places the dansyl fluorophore further from its reaction site, potentially reducing the interaction of the fluorophore with the biomolecule to which it is conjugated and enhancing accessibility to antibody binding.[31–33] A rabbit polyclonal antibody to the 1,5-dansyl fluorophore (A6398) that significantly enhances the dye's fluorescence is described in Section 7.4.

Conjugates of two isomers of dansyl chloride (2,5-dansyl chloride, D22; 2,6-dansyl chloride, D23) have smaller Stokes shifts and appreciably longer fluorescence lifetimes (up to ~30 nanoseconds) than conjugates of 1,5-dansyl chloride, making these isomers among the best available probes for fluorescence depolarization studies.[34] These dyes are particularly useful for preparing fluorescent drug or ligand analogs that are expected to bind to hydrophobic sites in proteins or membranes. The lipophilicity of these reagents may also facilitate the labeling of sites within the membrane-spanning portions of cellular proteins.

Dapoxyl® Dye

Dapoxyl® dye (Figure 1.7.23) is a particularly versatile derivatization reagent and precursor to environment-sensitive probes.[35] Like Cascade Yellow™ dye, Dapoxyl® dye exhibits an exceptionally large Stokes shift, with excitation/emission maxima of ~370/580 nm (Figure 1.7.24). Sulfonamides from Dapoxyl® sulfonyl chloride (D10160) have much higher extinction coefficients than those of dansyl chloride (~26,000 $cm^{-1}M^{-1}$ versus about 4000 $cm^{-1}M^{-1}$) and equal or greater quantum yields when dissolved in organic solvents; however, the fluorescence of Dapoxyl® derivatives is very sensitive to the dye environment, and fluorescence in water is very low, making them useful for sensing conformational changes,[36] denaturation and phosphorylation states[37] of proteins.

In addition to Dapoxyl® sulfonyl chloride, we offer the amine-reactive Dapoxyl® succinimidyl ester (D10161) and the carboxylic acid–reactive Dapoxyl® (2-aminoethyl)sulfonamide (D10460, Section 3.4). We have also exploited the environment-sensitive fluorescence of the Dapoxyl® dye (Figure 1.7.25) to develop a highly selective and photostable stain for the endoplasmic reticulum (ER-Tracker™ Blue-White DPX, E12353; Section 12.4).

Bimane Derivative

Bimane mercaptoacetic acid (carboxymethylthiobimane, B30250; Figure 1.7.26) is a blue-fluorescent dye with excitation/emission maxima of ~380/458 nm. It is useful as a reference standard for the fluorogenic monobromobimane and monochlorobimane reagents (Section 2.3) because it is an analog of the thioether product of their reaction with glutathione and other thiols.

REFERENCES

1. Histochem J (1986) 18:497; **2**. J Histochem Cytochem (1999) 47:1179; **3**. Bioorg Med Chem Lett (1999) 9:2229; **4**. J Biol Chem (1996) 271:31160; **5**. Biochemistry (1988) 27:8889; **6**. Bioorg Med Chem Lett (1998) 8:3107; **7**. Anal Chem (1968) 40:803; **8**. Nat Protoc (2007) 2:227; **9**. FEBS Lett (1985) 182:185; **10**. Cytometry A (2006) 69:1153; **11**. Anal Biochem (1991) 198:119; **12**. Nat Med (2001) 7:245; **13**. Nat Biotechnol (2002) 20:155; **14**. Cytometry (1997) 29:328; **15**. Nat Protoc (2009) 4:372; **16**. Proc Natl Acad Sci U S A (2006) 103:12063; **17**. Proc Natl Acad Sci U S A (2005) 102:5346; **18**. Biochemistry (2008) 47:6279; **19**. Nucleic Acids Res (2002) 30:e19; **20**. Anal Sci (2004) 20:557; **21**. J Chromatogr A (2002) 946:265; **22**. Anal Biochem (2008) 381:193; **23**. J Am Chem Soc (2001) 123:3838; **24**. J Biochem Biophys Methods (1994) 29:157; **25**. Anal Biochem (1988) 174:101; **26**. J Am Chem Soc (2006) 128:12932; **27**. Cytometry (1998) 33:435; **28**. IEEE J Quantum Electronics (1980) 16:777; **29**. Cytometry (1999) 36:36; **30**. Bioconjug Chem (2001) 12:594; **31**. Biochim Biophys Acta (1992) 1104:9; **32**. Biochim Biophys Acta (1984) 776:217; **33**. Biochemistry (1982) 21:978; **34**. J Biochem Biophys Methods (1981) 5:1; **35**. Photochem Photobiol (1997) 66:424; **36**. J Am Chem Soc (2006) 128:11736; **37**. Biochim Biophys Acta (2008) 1784:94.

DATA TABLE 1.7 COUMARINS, PYRENES AND OTHER ULTRAVIOLET LIGHT–EXCITABLE FLUOROPHORES

Cat. No.	MW	Storage	Soluble	Abs	EC	Em	Solvent	Notes
A6118	443.46	F,D,L	DMF, DMSO	353	19,000	442	MeOH	
A10168	410.35	F,D,L	H_2O, DMSO	346	19,000	445	pH 7	1
A10169	701.75	F,D,L	H_2O, DMSO	430	15,000	545	pH 7	1
A30000	1028.26	F,DD,L	H_2O, DMSO	400	35,000	424	pH 7	1, 2, 3
B30250	282.31	F,D,L	DMSO	380	5700	458	MeOH	
C2284	607.42	F,D,LL	H_2O, MeOH	396	29,000	410	MeOH	2, 4
C10164	563.54	F,D,L	DMF, DMSO	409	24,000	558	MeOH	5
D21	269.75	F,DD,L	DMF, MeCN	372	3900	none	$CHCl_3$	6, 7
D22	269.75	F,DD,L	DMF, MeCN	403	2900	none	MeOH	7, 8
D23	269.75	F,DD,L	DMF, MeCN	380	16,000	none	$CHCl_3$	7, 8
D126	247.25	L	pH >6, DMF	370	22,000	459	MeOH	
D374	344.32	F,D,L	DMF, MeCN	376	22,000	468	MeOH	
D1412	358.35	F,D,L	DMSO, MeCN	442	64,000	483	pH 9	9
D1421	261.28	L	pH >6, DMF	409	34,000	473	pH 9	
D6104	461.53	F,D,L	DMF, MeCN	335	4200	518	MeOH	
D10160	362.83	F,DD,L	DMF, MeCN	403	22,000	see Notes	MeOH	7, 10
D10161	405.41	F,D,L	DMF, DMSO	395	20,000	601	MeOH	11
D10166	260.31	F,DD,L	DMF, MeCN	400	36,000	476	MeOH	12, 13
H185	206.15	L	pH >6, DMF	386	29,000	448	pH 10	14
H1193	303.23	F,D,L	DMF, MeCN	419	36,000	447	MeOH	
H1428	234.21	L	pH >6, DMF	360	19,000	455	pH 10	
M1410	317.25	F,D,L	DMF, MeCN	358	26,000	410	MeOH	
M1420MP	220.18	L	pH >6, DMF	336	20,000	402	pH 9	
M10165	367.26	F,D,L	DMF, MeCN	362	19,000	459	pH 9	
P24	300.76	F,DD,L	DMF, MeCN	350	28,000	380	MeOH	7, 15
P130	385.42	F,D,L	DMF, DMSO	340	43,000	376	MeOH	16
P6114	574.65	F,D,L	H_2O, DMSO	341	38,000	376	MeOH	1, 16
P10163	339.21	F,D,L	DMF, MeCN	416	46,000	451	pH 9	
P30253	~750	F,D,L	H_2O, DMSO	404	25,000	553	MeOH	1
S6110	564.39	F,D,L	DMF, DMSO	415	26,000	570	MeOH	5

For definitions of the contents of this data table, see"Using *The Molecular Probes® Handbook*" in the introductory pages.

Notes

1. This sulfonated succinimidyl ester derivative is water soluble and may be dissolved in buffer at ~pH 8 for reaction with amines. Long-term storage in water is NOT recommended due to hydrolysis.
2. The Alexa Fluor® 405 and Cascade Blue® dyes have a second absorption peak at about 376 nm with EC ~80% of the 395–400 nm peak.
3. A30100 is an alternative packaging of A30000 but is otherwise identical.
4. Unstable in water. Use immediately.
5. Fluorescence emission spectrum shifts to shorter wavelengths in nonpolar solvents.
6. D21 butylamine derivative has Abs = 337 nm (EC = 5300 $cm^{-1}M^{-1}$), Em = 492 nm in $CHCl_3$. Em and QY are highly solvent dependent: Em = 496 nm (QY = 0.45) in dioxane, 536 nm (QY = 0.28) in MeOH and 557 nm (QY = 0.03) in H_2O. (Biochemistry (1967) 6:3408) EC typically decreases upon conjugation to proteins (EC = 3400 $cm^{-1}M^{-1}$ at 340 nm). (Biochemistry (1986) 25:513) Fluorescence lifetimes (τ) of protein conjugates are typically 12–20 nanoseconds. (Arch Biochem Biophys (1969) 133:263, Arch Biochem Biophys (1968) 128:163)
7. Do NOT dissolve in DMSO.
8. D22 butylamine derivative: Abs = 375 nm (EC = 3100 $cm^{-1}M^{-1}$), Em = 470 nm in MeOH. D23 butylamine derivative: Abs = 375 nm (EC = 13,000 $cm^{-1}M^{-1}$), Em = 419 nm in $CHCl_3$.
9. D1412 reaction product with 1-butylamine has Abs = 427 nm (EC = 48,000 $cm^{-1}M^{-1}$), Em = 478 nm in pH 9 buffer.
10. D10160 fluorescence is very weak. Reaction product with butylamine has Abs = 373 nm (EC = 26,000 $cm^{-1}M^{-1}$), Em = 551 nm.
11. D10161 butylamine derivative: Abs = 367 nm (EC = 25,000 $cm^{-1}M^{-1}$), Em = 574 nm in MeOH. QY of the derivative is approximately 15-fold higher than the unreacted reagent.
12. Isothiocyanates are unstable in water and should not be stored in aqueous solution.
13. D10166 butylamine derivative: Abs = 376 nm (EC = 25,000 $cm^{-1}M^{-1}$), Em = 469 nm in MeOH. QY of the derivative is approximately 6-fold higher than the unreacted reagent.
14. H185 Abs = 339 nm (EC = 19,000 $cm^{-1}M^{-1}$), Em = 448 nm at pH 4.
15. Spectra of the reaction product with butylamine.
16. Pyrene derivatives exhibit structured spectra. The absorption maximum is usually about 340 nm with a subsidiary peak at about 325 nm. There are also strong absorption peaks below 300 nm. The emission maximum is usually about 376 nm with a subsidiary peak at 396 nm. Excimer emission at about 470 nm may be observed at high concentrations.

PRODUCT LIST 1.7 COUMARINS, PYRENES AND OTHER ULTRAVIOLET LIGHT EXCITABLE FLUOROPHORES

Cat. No.	Product	Quantity
A10168	Alexa Fluor® 350 carboxylic acid, succinimidyl ester	5 mg
A30000	Alexa Fluor® 405 carboxylic acid, succinimidyl ester	1 mg
A30100	Alexa Fluor® 405 carboxylic acid, succinimidyl ester	5 mg
A10169	Alexa Fluor® 430 carboxylic acid, succinimidyl ester	5 mg
A20180	Alexa Fluor® 350 Monoclonal Antibody Labeling Kit *5 labelings*	1 kit
A10170	Alexa Fluor® 350 Protein Labeling Kit *3 labelings*	1 kit
A10171	Alexa Fluor® 430 Protein Labeling Kit *3 labelings*	1 kit
A6118	6-((7-amino-4-methylcoumarin-3-acetyl)amino)hexanoic acid, succinimidyl ester (AMCA-X, SE)	10 mg
A10478	APEX® Pacific Blue™ Antibody Labeling Kit	1 kit
B30250	bimane mercaptoacetic acid (carboxymethylthiobimane)	5 mg
C2284	Cascade Blue® acetyl azide, trisodium salt	5 mg
C10164	Cascade Yellow™ succinimidyl ester	5 mg
D10161	Dapoxyl® carboxylic acid, succinimidyl ester	5 mg
D10160	Dapoxyl® sulfonyl chloride	10 mg
D21	5-dimethylaminonaphthalene-1-sulfonyl chloride (dansyl chloride)	1 g
D22	2-dimethylaminonaphthalene-5-sulfonyl chloride	100 mg
D23	2-dimethylaminonaphthalene-6-sulfonyl chloride	100 mg
D6104	6-((5-dimethylaminonaphthalene-1-sulfonyl)amino)hexanoic acid, succinimidyl ester (dansyl-X, SE)	25 mg
D1421	7-diethylaminocoumarin-3-carboxylic acid	100 mg
D1412	7-diethylaminocoumarin-3-carboxylic acid, succinimidyl ester	25 mg
D126	7-dimethylaminocoumarin-4-acetic acid (DMACA)	100 mg
D374	7-dimethylaminocoumarin-4-acetic acid, succinimidyl ester (DMACA, SE)	25 mg
D10166	7-dimethylamino-4-methylcoumarin-3-isothiocyanate (DACITC)	10 mg
H185	7-hydroxycoumarin-3-carboxylic acid *reference standard*	100 mg
H1193	7-hydroxycoumarin-3-carboxylic acid, succinimidyl ester	25 mg
H1428	7-hydroxy-4-methylcoumarin-3-acetic acid	100 mg
M10165	Marina Blue® succinimidyl ester	5 mg
M1420MP	7-methoxycoumarin-3-carboxylic acid	100 mg
M1410	7-methoxycoumarin-3-carboxylic acid, succinimidyl ester	25 mg
P30013	Pacific Blue™ Monoclonal Antibody Labeling Kit *5 labelings*	1 kit
P30012	Pacific Blue™ Protein Labeling Kit *3 labelings*	1 kit
P10163	Pacific Blue™ succinimidyl ester	5 mg
P30014	Pacific Orange™ Monoclonal Antibody Labeling Kit *5 labelings*	1 kit
P30016	Pacific Orange™ Protein Labeling Kit *3 labelings*	1 kit
P30253	Pacific Orange™ succinimidyl ester *triethylammonium salt*	1 mg
P130	1-pyrenebutanoic acid, succinimidyl ester	100 mg
P6114	N-(1-pyrenebutanoyl)cysteic acid, succinimidyl ester, potassium salt	5 mg
P24	1-pyrenesulfonyl chloride	100 mg
S6110	1-(3-(succinimidyloxycarbonyl)benzyl)-4-(5-(4-methoxyphenyl)oxazol-2-yl)pyridinium bromide (PyMPO, SE)	5 mg
Z25400	Zenon® Alexa Fluor® 350 Human IgG Labeling Kit *50 labelings*	1 kit
Z25000	Zenon® Alexa Fluor® 350 Mouse IgG_1 Labeling Kit *50 labelings*	1 kit
Z25100	Zenon® Alexa Fluor® 350 Mouse IgG_{2a} Labeling Kit *50 labelings*	1 kit
Z25200	Zenon® Alexa Fluor® 350 Mouse IgG_{2b} Labeling Kit *50 labelings*	1 kit
Z25300	Zenon® Alexa Fluor® 350 Rabbit IgG Labeling Kit *50 labelings*	1 kit
Z25013	Zenon® Alexa Fluor® 405 Mouse IgG_1 Labeling Kit *50 labelings*	1 kit
Z25113	Zenon® Alexa Fluor® 405 Mouse IgG_{2a} Labeling Kit *50 labelings*	1 kit
Z25213	Zenon® Alexa Fluor® 405 Mouse IgG_{2b} Labeling Kit *50 labelings*	1 kit
Z25313	Zenon® Alexa Fluor® 405 Rabbit IgG Labeling Kit *50 labelings*	1 kit
Z25001	Zenon® Alexa Fluor® 430 Mouse IgG_1 Labeling Kit *50 labelings*	1 kit
Z25301	Zenon® Alexa Fluor® 430 Rabbit IgG Labeling Kit *50 labelings*	1 kit
Z25041	Zenon® Pacific Blue™ Mouse IgG_1 Labeling Kit *50 labelings*	1 kit
Z25341	Zenon® Pacific Blue™ Rabbit IgG Labeling Kit *50 labelings*	1 kit
Z25256	Zenon® Pacific Orange™ Mouse IgG_1 Labeling Kit *50 labelings*	1 kit
Z25257	Zenon® Pacific Orange™ Mouse IgG_{2a} Labeling Kit *50 labelings*	1 kit

1.8 Reagents for Analysis of Low Molecular Weight Amines

Not only are low molecular weight amines abundantly distributed in nature, but numerous drugs, synthetic probes and other molecules of interest also contain amino groups. The sensitive detection, identification and quantitation of amines are important applications of many of the reactive fluorophores in this section. Some of these reagents have also been used to indirectly detect carbohydrates, carboxylic acids, thiols and cyanide.

The preferred reagents for detecting and quantitating amines in solution or on amine-containing polymers are those that are nonfluorescent but form fluorescent conjugates stoichiometrically with amines. It is difficult to compare the sensitivity for amine detection of the different reagents because it depends heavily on the equipment and detection technology used. Many of the assays, however, are rapid, reliable and adaptable to a variety of different sample types and instrumentation.

Fluorescamine

Fluorescamine (F2332, F20261) is intrinsically nonfluorescent but reacts rapidly with primary aliphatic amines, including those in peptides and proteins, to yield a blue-green–fluorescent derivative[1,2] (Figure 1.8.1). Modifications to the reaction protocol permit fluorescamine to be used to detect those amino acids containing secondary amines,[3] such as proline. Excess reagent is rapidly converted to a nonfluorescent product by reaction with water,[4] making fluorescamine useful for determining protein concentrations of solutions.[5,6]

Fluorescamine can also be used to detect proteins in gels and to analyze low molecular weight amines by TLC, HPLC and capillary electrophoresis.[7,8] An optimized procedure that employs fluorescamine for amino acid analysis in microplates has been published.[9] Chiral separation of fluorescamine-labeled amino acids has been optimized using capillary electrophoresis in the presence of hydroxypropyl-β-cyclodextrin, a method designed for use in extraterrestrial exploration on Mars.[10] Furthermore, a 200-fold increase in sensitivity and improved resolution in these measurements has been obtained by replacing fluorescamine with Pacific Blue™ succinimidyl ester[11] (P10163, Section 1.7).

Dialdehydes: OPA and NDA

Analyte Detection with OPA and NDA

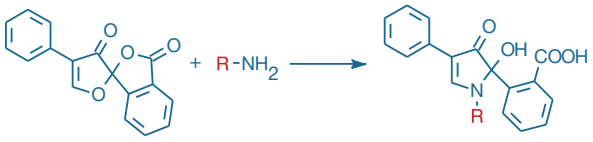
The homologous aromatic dialdehydes *o*-phthaldialdehyde[12] (OPA, P2331MP) and naphthalene-2,3-dicarboxaldehyde[13] (NDA, N1138) are essentially nonfluorescent until reacted with a primary amine in the presence of a thiol such as 2-mercaptoethanol, 3-mercaptopropionic acid or the less obnoxious sulfite,[14] or in the presence of excess cyanide, to yield a fluorescent isoindole (Figure 1.8.2, Figure 1.8.3). Improved detection sensitivity can be obtained by using SAMSA fluorescein (A685, Section 5.2) as the thiol reagent, thereby incorporating fluorescein as the R^2 substituent of the isoindole product[15] (Figure 1.8.2). Modified protocols that use an excess of an amine and limiting amounts of other nucleophiles permit the determination of carboxylic acids[16] and thiols,[17] as well as of cyanide in blood, urine and other samples.[18–21] Without an additional nucleophile, NDA forms fluorescent adducts with both hydrazine and methylated hydrazines[22] (excitation/emission maxima ~403/500 nm).

Sensitivity of OPA and NDA

Amine adducts of NDA have longer-wavelength spectral characteristics and greater sensitivity than the amine adducts of OPA. The stability and detectability of the amine derivatives of NDA are also superior;[23,24] the detection of glycine with NDA and cyanide is reported to be 50-fold more sensitive than with OPA and 2-mercaptoethanol.[13] The limit for electrochemical detection of the NDA adduct of asparagine has been determined to be as low as 36 attomoles[25,26] (36×10^{-18} moles). An optimized procedure that uses NDA for amino acid analysis in microplates has been published.[9]

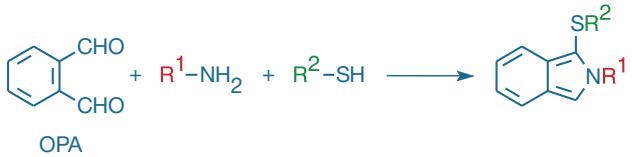
Applications for OPA and NDA

OPA and NDA are used extensively for both pre- and post-column derivatization of amines (and thiols) separated by HPLC[27,28] or by capillary electrophoresis.[27,29] The amines in a single cell have been analyzed by capillary electrophoresis using a sequence of on-capillary lysis, derivatization with NDA and cyanide, and laser-excited detection.[30,31]



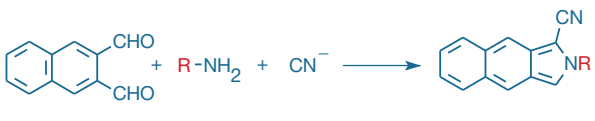
Fluorescamine

Figure 1.8.1 Fluorogenic amine-derivatization reaction of fluorescamine (F2332, F20261).



OPA

Figure 1.8.2 Fluorogenic amine-derivatization reaction of *o*-phthaldialdehyde (OPA, P2331MP).



NDA

Figure 1.8.3 Fluorogenic amine-derivatization reaction of naphthalene-2,3-dicarboxaldehyde (NDA, N1138).

ATTO-TAG™ Reagents

Sensitivity of ATTO-TAG™ CBQCA and ATTO-TAG™ FQ

ATTO-TAG™ CBQCA (A6222, A2333) and ATTO-TAG™ FQ (A10192, A2334) are designed to provide ultrasensitive detection of primary amines, including those in peptides, aminophospholipids and glycoproteins.[32–35] These reagents combine high sensitivity, visible-wavelength excitation and freedom from background fluorescence, making them useful for research, as well as analytical, forensic and clinical[36] applications. Developed by Novotny and collaborators, the ATTO-TAG™ reagents are similar to OPA and NDA in that they rapidly react with amines in the presence of thiols or cyanide to form highly fluorescent isoindoles[37–47] (Figure 1.8.4).

ATTO-TAG™ CBQCA reagent reacts specifically with amines to form charged conjugates that can be analyzed by electrophoresis techniques. Carbohydrates lacking amines can be detected following reductive amination with ammonia and $NaCNBH_3$.[46,48,49] ATTO-TAG™ CBQCA conjugates are maximally excited at ~456 nm or by the 442 nm spectral line of the He-Cd laser, with peak emission at ~550 nm, whereas ATTO-TAG™ FQ conjugates are maximally excited at ~480 nm or by the 488 nm spectral line of the argon-ion laser, with peak emission at ~590 nm. Ultrasensitive detection of CBQCA-derivatized amino sugars, amino acids and low molecular weight peptides by capillary electrophoresis has been reported.[35,50] In capillary electrophoresis, the sensitivity of amine detection of the laser-induced fluorescence is in the subattomole range ($<10^{-18}$ moles) for ATTO-TAG™ CBQCA and subfemtomole range ($<10^{-15}$ moles) for ATTO-TAG™ FQ.[51,52] Detection sensitivity of reductively aminated glucose using ATTO-TAG™ CBQCA is reported to be 75 zeptomoles[53] (75×10^{-21} moles).

ATTO-TAG™ reagents can, of course, be used in HPLC and other modes of chromatography with either absorption or fluorescence detection. The principal limitation to obtaining ultrasensitive detection using the ATTO-TAG™ reagents and all other chemical derivatization reagents is that relatively high concentrations of the derivatizing reagent are required to obtain adequate kinetics and quantitative modification of the analyte.[54,55] A very sensitive assay that uses ATTO-TAG™ CBQCA for rapid quantitation of protein amines in solution (C6667) is described in Section 9.2. Similarly, ATTO-TAG™ CBQCA has proven useful for *in situ* quantitation of proteins attached to microspheres.[56]

ATTO-TAG™ Reagents and Kits

Because cyclodextrins have been reported to amplify the signal from ATTO-TAG™ CBQCA conjugates up to 10-fold,[34,57] we have included β-cyclodextrin in our ATTO-TAG™ Amine Derivatization Kits (A2333, A2334). These kits contain:

- 5 mg of ATTO-TAG™ CBQCA (in Kit A2333) or ATTO-TAG™ FQ (in Kit A2334)
- Potassium cyanide
- β-Cyclodextrin
- Protocol for amine modification

The ATTO-TAG™ CBQCA and ATTO-TAG™ FQ Amine Derivatization Kits supply sufficient reagents for derivatizing approximately 150 and 100 samples, respectively, depending on the amine concentration and sample volume.

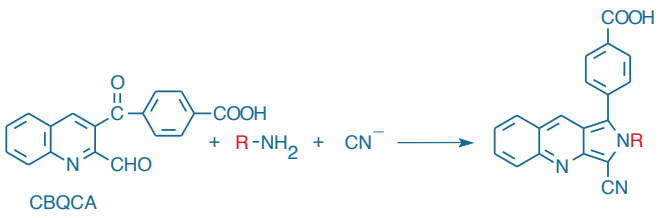


Figure 1.8.4 Fluorogenic amine-derivatization reaction of CBQCA (A6222, A2333).

7-Nitrobenz-2-Oxa-1,3-Diazole (NBD) Derivatives

NBD chloride (C20260, Figure 1.8.5) was first introduced in 1968 as a fluorogenic derivatization reagent for amines.[58] NBD fluoride (F486) usually yields the same products as NBD chloride but is much more reactive;[59] for example, the reaction of NBD fluoride with glycine is reported to be 500 times faster than the reaction of NBD chloride with glycine.[60] Reaction of NBD fluoride with alcohols leads to their utility for derivatizing and detecting lipopolysaccharides[61] (LPS). Unlike OPA and fluorescamine, both NBD chloride and NBD fluoride react with secondary amines and are therefore capable of derivatizing proline and hydroxyproline.[59,62] NBD chloride and NBD fluoride are extensively used as derivatization reagents for chromatographic analysis of amino acids[63] and other low molecular weight amines.[33]

The absorption and fluorescence emission spectra, quantum yields and extinction coefficients of NBD conjugates are all markedly dependent on solvent;[64,65] in particular, the fluorescence quantum yield in water of NBD adducts of amines can be very low (<0.01), particularly of secondary amines. NBD adducts of aromatic amines are essentially nonfluorescent, a property that we have utilized to prepare our QSY® 35 quenchers.

Fluorescence of lysine-modified NBD-labeled actin is sensitive to polymerization.[66] Inactivation of certain ATPases by NBD chloride apparently involves a tyrosine modification followed by intramolecular migration of the label to a lysine residue.[67,68] NBD is also a functional analog of the dinitrophenyl hapten, and its fluorescence is quenched upon binding to anti-dinitrophenyl antibodies[65,69] (Section 7.4).

NBD aminohexanoic acid (NBD-X, N316) and its succinimidyl ester (NBD-X, SE; S1167) are precursors to NBD-labeled phospholipids (Section 13.2), NBD C_6-ceramide (N1154, Section 12.4) and other probes.

Dansyl Chloride and Other Sulfonyl Chlorides

Many of the sulfonyl chlorides described in Section 1.7, including dansyl chloride (D21), 1-pyrenesulfonyl chloride (P24) and Dapoxyl® sulfonyl chloride (D10160), react with amines to yield blue- or blue-green–fluorescent sulfonamides and are particularly useful as chromatographic derivatization reagents. They react with both aliphatic and aromatic amines to yield very stable derivatives. In addition, they are

generally good acceptors for fluorescence resonance energy transfer (FRET) from tryptophan, as well as good donors to longer-wavelength dyes such as QSY® dyes (Section 1.6) (Fluorescence Resonance Energy Transfer (FRET)—Note 1.2). Fluorescence of dansyl conjugates in aqueous solutions can be enhanced by adding cycloheptaamylose.[70]

Although dansyl chloride is the most commonly used of these reagents, the stronger absorption of 1-pyrenesulfonamides and large Stokes shift of Dapoxyl® sulfonamides[71] (Figure 1.8.6) should make these sulfonyl chlorides more sensitive reagents for amine analysis. Note that sulfonyl chlorides are unstable in dimethylsulfoxide (DMSO) and should never be used in that solvent.[72]

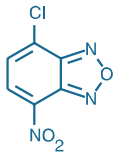


Figure 1.8.5 4-chloro-7-nitrobenz-2-oxa-1,3-diazole (NBD chloride; 4-chloro-7-nitrobenzofurazan, C20260).

Dansyl Chloride

Since its development by Weber in 1951,[73] dansyl chloride (D21, Section 1.7) has been used extensively to determine the N-terminal amino acid residue of proteins and to prepare fluorescent derivatives of drugs, amino acids, oligonucleotides and proteins for detection by numerous chromatographic methods.[74] Nonfluorescent dansyl chloride reacts with amines to form fluorescent dansyl amides that exhibit large Stokes shifts, along with environment-sensitive fluorescence quantum yields and emission maxima.

Pyrene Sulfonyl Chloride

The absorptivity (and therefore ultimate fluorescence output) of dansyl derivatives is weak compared with that of the more strongly UV light–absorbing fluorophores such as pyrene. Thus, 1-pyrenesulfonyl chloride (P24, Section 1.7) should have greater sensitivity for detection of amines. The fluorescence lifetime of pyrenesulfonamides can also be relatively long (up to ~30 nanoseconds), making them useful for fluorescence anisotropy measurements.[75] Fluorescence polarization measurements of DNA probes labeled with 1-pyrenesulfonyl chloride permit homogeneous detection of hybridization.[76]

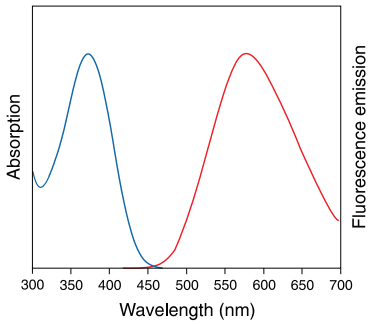


Figure 1.8.6 Absorption and fluorescence emission spectra of Dapoxyl® (2-aminoethyl)sulfonamide in methanol.

Dapoxyl® Sulfonyl Chloride

Sulfonamides derived from Dapoxyl® sulfonyl chloride (D10160, Section 1.7) have much higher extinction coefficients than those of dansyl chloride (~22,000 $cm^{-1}M^{-1}$ versus ~4000 $cm^{-1}M^{-1}$) and equal or greater quantum yields when dissolved in organic solvents; however, Dapoxyl® derivatives have very low fluorescence in water. The huge Stokes shifts (up to ~200 nm) and large extinction coefficients of Dapoxyl® derivatives in some solvents[71] (Figure 1.8.6) make the reactive Dapoxyl® derivatives a good choice for derivatization reagents in chromatographic and electrophoretic analysis.[77]

FITC and Other Isothiocyanates

Isothiocyanates for preparing bioconjugates have been described in several sections of this chapter. However, FITC (F143, F1906, F1907; Section 1.5) can also be used for derivatizing low molecular weight amines[33] and, like phenyl isothiocyanate, for microsequencing of peptides as their thiohydantoins.[78] A method for specific derivatization of the N-terminus of peptides by FITC has been described.[79] FITC-labeled amino acids and peptides have been separated by capillary electrophoresis with a detection limit of fewer than 1000 molecules.[80,81]

Succinimidyl Esters and Carboxylic Acids

Succinimidyl esters have a high selectivity for reaction with aliphatic amines. Most of the succinimidyl ester reagents described elsewhere in this chapter can be used to derivatize low molecular weight amines for subsequent separation by chromatography or capillary electrophoresis. Alexa Fluor®, BODIPY®, Oregon Green® and fluorescein derivatives typically yield the greatest sensitivity, particularly when the conjugate is detected with laser excitation. Use of single isomers of these reactive dyes is essential for all high-resolution analyses. Analysis by capillary electrophoresis shows that carboxyfluorescein succinimidyl ester reacts faster and yields more stable amine conjugates than FITC or DTAF.[82]

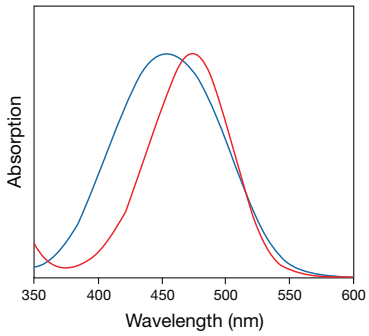


Figure 1.8.7 Normalized absorption spectra of the succinimidyl esters of the dabcyl (D2245, blue) and QSY® 35 (Q20133, red) dyes.

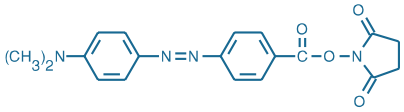


Figure 1.8.8 4-((4-(dimethylamino)phenyl)azo)benzoic acid, succinimidyl ester (dabcyl, SE; D2245).

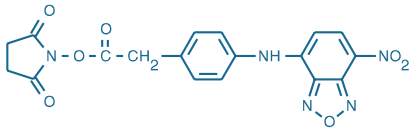


Figure 1.8.10 QSY® 35 acetic acid, succinimidyl ester (Q20133).

The UV light–excitable coumarins described in Section 1.7 have good absorptivity at ~320–420 nm, with purple to bright blue emission at 400–500 nm. Aliphatic polyamines derivatized with 1-pyrenebutanoic acid succinimidyl ester (P130, Section 1.7) have been differentiated from pyrene-labeled monoamines by HPLC using their fluorescent excimer formation.[83]

The Smallest Reactive Fluorophore

N-methylisatoic anhydride (M25) is a useful precursor for preparing esters or amides of the small *N*-methylanthranilic acid fluorophore. The small size of this fluorophore should reduce the likelihood that the label will interfere with the function of the biomolecule, an important advantage when designing site-selective probes. This amine-acylating reagent is often used to prepare fluorescent derivatives of biologically active peptides and toxins[84–87] and, in combination with a quencher, to prepare fluorogenic endoprotease substrates.[88,89]

Chromophoric Succinimidyl Esters: Fluorescence Quenchers

Dabcyl has broad and intense visible absorption (Figure 1.8.7) but no fluorescence, making it useful as an acceptor in FRET applications (Fluorescence Resonance Energy Transfer (FRET)—Note 1.2). Biomolecules double-labeled with dabcyl and the appropriate fluorophore can be used to monitor proteolytic cleavage, conformational changes and other dynamic spatial movements. Dabcyl succinimidyl ester (dabcyl, SE; D2245; Figure 1.8.8) is particularly useful in preparing quenched fluorogenic substrates for proteases, including our HIV protease (Figure 1.8.9) and renin substrates[90–92] (H2930, R2931; Section 10.4), papain,[93,94] Alzheimer disease–associated proteases[95] and others.[96–99] Fluorogenic substrates using this quenching group have also been prepared for interleukin-1β–converting enzyme (ICE),[100] a cysteine protease that is proposed to function in the onset of apoptosis.[101] The dabcyl chromophore has been used as the quencher in donor–acceptor labeled oligonucleotides (molecular beacons);[02–105] unfolding of these probes upon hybridization leads to recovery of the donor dye's fluorescence.

QSY® 35 acetic acid succinimidyl ester (Q20133, Figure 1.8.10) is an essentially nonfluorescent nitrobenzoxadiazole (NBD) derivative. Like the QSY® 7, QSY® 9 and QSY® 21 dyes (Section 1.6), the QSY® 35 dye has absorption at longer wavelengths than does the dabcyl dye (Figure 1.8.7), making it a very good acceptor from most blue-fluorescent dyes. A peptide containing the QSY® 35 quencher paired with the blue-fluorescent 7-hydroxy-4-methyl-3-acetylcoumarin fluorophore has proven useful in a fluorescence resonance energy transfer (FRET) assay for *Bacillus anthracis* lethal factor protease.[106] QSY® 35 iodoacetamide (Q20348, Section 2.2) and an FMOC-protected QSY® 35 amino acid (Q21931, Section 9.5) are available for automated preparation of FRET-based protease substrates.

N-(*t*-BOC)-Aminooxyacetic Acid TFP Ester

The tetrafluorophenyl ester (TFP) of *N*-(*t*-BOC)-aminooxyacetic acid (B30300) is an amine-reactive protected hydroxylamine that is useful for synthesizing new aldehyde- and ketone-reactive probes in an organic solvent. Following coupling to aliphatic amines, the *t*-BOC group can be quantitatively removed with trifluoroacetic acid. The resultant hydroxylamine can then spontaneously react with aldehydes, the reducing ends of saccharides and oligosaccharides and abasic sites in oligonucleotides to form stable adducts (Section 3.3).

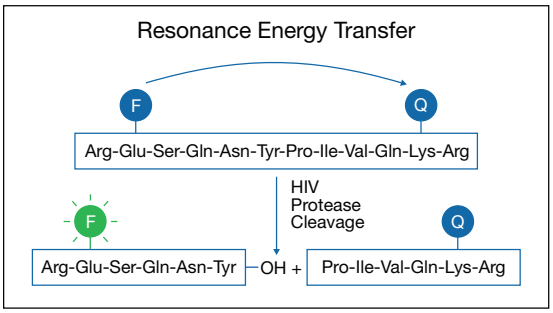


Figure 1.8.9 Principle of the fluorogenic response to protease cleavage exhibited by HIV protease substrate 1 (H2930). Quenching of the EDANS fluorophore (**F**) by distance-dependent resonance energy transfer to the dabcyl quencher (**Q**) is eliminated upon cleavage of the intervening peptide linker.

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DATA TABLE 1.8 REAGENTS FOR ANALYSIS OF LOW MOLECULAR WEIGHT AMINES

Cat. No.	MW	Storage	Soluble	Abs	EC	Em	Solvent	Notes
A2333	305.29	F,D,L	MeOH	465	ND	560	MeOH	1, 2, 3, 4
A2334	251.24	F,D,L	EtOH	486	ND	591	MeOH	4, 5
A6222	305.29	F,D,L	MeOH	465	ND	560	MeOH	1, 2, 3
A10192	251.24	F,L	EtOH	486	ND	591	MeOH	2, 5
B30300	339.24	F,D	DMSO	<300	ND	none		
C20260	199.55	F,D,L	DMF, MeCN	336	9800	none	MeOH	6, 7, 8
D2245	366.38	F,D,L	DMF, DMSO	453	32,000	none	MeOH	9
F486	183.10	F,D,L	MeCN, $CHCl_3$	328	8000	none	MeOH	6, 7
F2332	278.26	F,D,L	MeCN	380	7800	464	MeCN	10
F20261	278.26	F,D,L	MeCN	380	8400	464	MeCN	8, 10
M25	177.16	D	DMF, DMSO	316	3500	386	MeOH	11
N316	294.27	L	DMSO	467	23,000	539	MeOH	7
N1138	184.19	L	DMF, MeCN	419	9400	493	see Notes	12
P2331MP	134.13	L	EtOH	334	5700	455	pH 9	13
Q20133	411.33	F,D,L	DMSO	475	23,000	none	MeOH	
S1167	391.34	F,D,L	DMF, DMSO	466	22,000	535	MeOH	7

For definitions of the contents of this data table, see "Using *The Molecular Probes® Handbook*" in the introductory pages.

Notes

1. Spectral data are for the reaction product with glycine in the presence of cyanide. Unreacted reagent in MeOH: Abs = 254 nm (EC = 46,000 $cm^{-1}M^{-1}$), nonfluorescent.
2. ND = not determined.
3. Solubility in methanol is improved by addition of base (e.g., 1–5% (v/v) 0.2 M KOH).
4. Data represent the reactive dye component of this labeling kit.
5. Spectral data are for the reaction product with glycine in the presence of cyanide. Unreacted reagent in MeOH: Abs = 282 nm (EC = 21,000 $cm^{-1}M^{-1}$), nonfluorescent.
6. Spectra for primary aliphatic amine derivative of NBD chloride in MeOH: Abs = 465 nm (EC = 22,000 $cm^{-1}M^{-1}$), Em = 535 nm (QY = 0.3). Spectra for secondary aliphatic amine derivative in MeOH: Abs = 485 nm (EC = 25,000 $cm^{-1}M^{-1}$), Em = 540 nm (QY <0.1). Aromatic amine derivatives are nonfluorescent. All NBD amine derivatives are almost nonfluorescent in water and have strongly solvent-dependent emission spectra. NBD fluoride yields the same derivatives as NBD chloride but is more reactive.
7. Fluorescence of NBD and its derivatives in water is relatively weak. QY and τ increase and Em decreases in aprotic solvents and other nonpolar environments relative to water. (Biochemistry (1977) 16:5150, Photochem Photobiol (1991) 54:361)
8. This product is specified to equal or exceed 98% analytical purity by HPLC.
9. D2245 is nonfluorescent both before and after reaction with amines. Reaction product with butylamine has Abs = 428 nm (EC = 32,000 $cm^{-1}M^{-1}$) in MeOH.
10. Fluorescamine spectra are for the reaction product with butylamine. The fluorescence quantum yield and lifetime of the butylamine adduct in EtOH are 0.23 and 7.5 nanoseconds, respectively. (Arch Biochem Biophys (1974) 163:390) The unreacted reagent is nonfluorescent (Abs = 234 nm, EC = 28,000 $cm^{-1}M^{-1}$ in MeCN).
11. The amide reaction product of M25 with butylamine has Abs = 353 nm (EC = 5900 $cm^{-1}M^{-1}$), Em = 426 nm in MeOH. Ester reaction products with alcohols have Abs = 350 nm (EC = 5700 $cm^{-1}M^{-1}$), Em = 446 nm in water (pH 8).
12. Spectral data are for the reaction product with glycine in the presence of cyanide, measured in pH 7.0 buffer/MeCN (40:60). (Anal Chem (1987) 59:1102) Unreacted reagent in MeOH: Abs = 279 nm (EC = 5500 $cm^{-1}M^{-1}$), Em = 330 nm.
13. Spectral data are for the reaction product of P2331MP with alanine and 2-mercaptoethanol. The spectra and stability of the adduct depend on the amine and thiol reactants. (Biochim Biophys Acta (1979) 576:440) Unreacted reagent in H_2O: Abs = 257 nm (EC = 1000 $cm^{-1}M^{-1}$).

PRODUCT LIST 1.8 REAGENTS FOR ANALYSIS OF LOW MOLECULAR WEIGHT AMINES

Cat. No.	Product	Quantity
A2333	ATTO-TAG™ CBQCA Amine-Derivatization Kit	1 kit
A6222	ATTO-TAG™ CBQCA derivatization reagent (CBQCA; 3-(4-carboxybenzoyl)quinoline-2-carboxaldehyde)	10 mg
A2334	ATTO-TAG™ FQ Amine-Derivatization Kit	1 kit
A10192	ATTO-TAG™ FQ derivatization reagent (FQ; 3-(2-furoyl)quinoline-2-carboxaldehyde)	10 mg
B30300	N-(t-BOC)-aminooxyacetic acid, tetrafluorophenyl ester	25 mg
C20260	4-chloro-7-nitrobenz-2-oxa-1,3-diazole (NBD chloride; 4-chloro-7-nitrobenzofurazan) *FluoroPure™ grade*	100 mg
D2245	4-((4-(dimethylamino)phenyl)azo)benzoic acid, succinimidyl ester (dabcyl, SE)	100 mg
F2332	fluorescamine	100 mg
F20261	fluorescamine *FluoroPure™ grade*	100 mg
F486	4-fluoro-7-nitrobenz-2-oxa-1,3-diazole (NBD fluoride; 4-fluoro-7-nitrobenzofurazan)	25 mg
M25	N-methylisatoic anhydride *high purity*	1 g
N1138	naphthalene-2,3-dicarboxaldehyde (NDA)	100 mg
N316	NBD-X (6-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)hexanoic acid)	100 mg
P2331MP	*o*-phthaldialdehyde (OPA) *high purity*	1 g
Q20133	QSY® 35 acetic acid, succinimidyl ester	5 mg
S1167	succinimidyl 6-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)hexanoate (NBD-X, SE)	25 mg

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Ordering Information

Product	Cat. No.
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Find out more at **thermofisher.com/furl**

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