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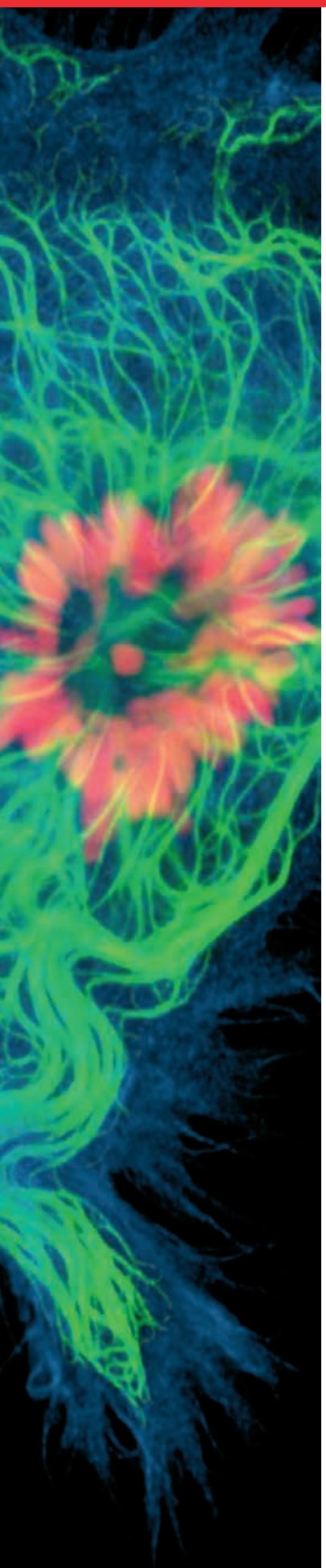

Molecular Probes™ Handbook

A Guide to Fluorescent Probes and Labeling Technologies

11th Edition (2010)

CHAPTER 2

Thiol-Reactive Probes

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CHAPTER 2

Thiol-Reactive Probes

2.1 Introduction to Thiol Modification and Detection

Common Applications for Thiol-Reactive Probes

Labeling Proteins and Nucleic Acids

Thiol-reactive dyes are principally used to label proteins for the detection of conformational changes, assembly of multisubunit complexes and ligand-binding processes[1] In the case of proteins and peptides, the primary targets of thiol-reactive probes are cysteine residues. In mammalian proteins, the occurrence frequency of cysteine targets (3.3%) is less than half that of lysine targets (7.2%), which are labeled by the amine-reactive reagents described in Chapter 1. Some proteins and many peptides have only a single cysteine residue, enabling site-specific labeling with thiol-reactive probes.[2,3] In proteins with multiple cysteine residues, the multiplicity is often small enough that it is practicable to obtain single-cysteine variants by site-directed mutagenesis without significant disruption of the structure or function of the native protein. Site-specific modification is particularly important for labeling small proteins in applications where the activity or binding affinity of the conjugate is paramount;[3,4] thiol-reactive labeling is the preferred approach over amine-reactive labeling in such cases.

The relatively low abundance of cysteine residues also makes it possible to obtain saturating modification with less risk of incurring the penalties of protein precipitation and fluorescence self-quenching interactions that make high-percentage amine-reactive modification largely impracticable.[5,6] In proteins with multiple cysteine residues, however, the reactivity of an individual cysteine can be very dependent on both its local environment and the hydrophobicity of the reactive dye.[7–10] Site-specific modification strategies involving site-directed cysteine mutagenesis, site-dependent variations in thiol reactivity and functional group protection/deprotection have been developed to double-label proteins with donor and acceptor dyes for fluorescence resonance energy transfer (FRET) applications[2,11–16] (Fluorescence Resonance Energy Transfer (FRET)—Note 1.2). Thiol-reactive dyes can also be reacted with thiolated oligonucleotides for hybridization- or ligation-based nucleic acid detection applications[17] and with thiouridine-modified tRNA for studying its association with protein synthesis machinery.[18,19]

Derivatizing Low Molecular Weight Molecules

Several of the thiol-reactive probes described in this chapter are also useful for derivatizing low molecular weight thiols for various analytical assays that employ chromatographic and electrophoretic separation. An extensive review by Shimada and Mitamura describes the use of several of our thiol-reactive reagents for derivatizing thiol-containing compounds.[20]

Quantitating Thiols

Thiols play a principal role in maintaining the appropriate oxidation–reduction state of proteins, cells and organisms. The susceptibility of thiols to oxidation, however, can lead to the formation of disulfides and higher oxidation products, often with loss of biological activity. Measuring the oxidation state of thiols within live cells is complicated by the high concentration of reduced glutathione in cells, which makes them difficult to assay with reagents that stoichiometrically react with the thiol. Nonetheless, many useful reagents and methods have been developed for the quantitative assay of thiols and disulfides.[21]

Reactivity of Thiol Groups

Reducing Disulfides with DTT or TCEP

In proteins, thiol groups (also called mercaptans or sulfhydryls) are present in cysteine residues. Thiols can also be generated by selectively reducing cystine disulfides with reagents such as dithiothreitol[22] (DTT, D1532) or 2-mercaptoethanol (β-mercaptoethanol), each of which must then be removed by dialysis or gel filtration before reaction with the thiol-reactive probe.[21]

Unfortunately, removal of DTT or 2-mercaptoethanol is sometimes accompanied by air oxidation of the thiols back to the disulfides. Reformation of the disulfide bond can often be avoided by using the reducing agent tris-(2-carboxyethyl)phosphine[23,24] (TCEP, T2556), which usually does not need to be removed prior to thiol modification because it does not contain thiols (Figure 2.1.1). However, there have been several reports that TCEP can react with haloacetamides or maleimides under certain conditions and that labeling in the presence of TCEP is inhibited.[6,25] Carrying out thiol-reactive labeling on ammonium sulfate–precipitated proteins facilitates efficient and rapid removal of DTT after the preparatory reduction step and inhibits thiol reoxidation during the subsequent labeling reaction.[3]

TCEP is more stable at a higher pH and at higher temperatures[26] than is DTT and for a longer period of time in buffers without metal chelators such as EGTA; DTT is more stable than TCEP in solutions that contain metal chelators.[23] TCEP is also more stable in the presence of Ni^{2+} levels that commonly contaminate proteins eluted from Ni^{2+} affinity columns and that rapidly oxidize DTT.[23] Spin labels in TCEP are two to four times more stable than those in DTT, an advantage for electron paramagnetic resonance (EPR) spectroscopy.[23] In addition, TCEP is used to stabilize solutions of ascorbic acid.[27] TCEP is generally impermeable to cell membranes and to the hydrophobic protein core, permitting its use for the selective reduction of disulfides that have aqueous exposure. It has also been reported that TCEP can be used to deplete high-abundance

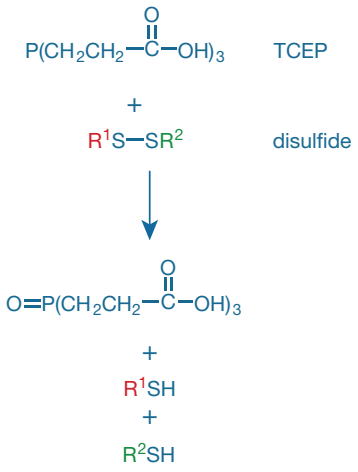

Figure 2.1.1 Reduction of a disulfide using TCEP (tris-(2-carboxyethyl)phosphine, hydrochloride; T2556). Unlike DTT (dithiothreitol, D1532), TCEP does not itself contain thiols, and therefore downstream thiol labeling reactions do not require preliminary removal of the reducing reagent.

plasma proteins (albumins, transferrin, etc.) prior to proteomic analysis because these proteins have a large number of disulfide bridges and are therefore particularly susceptible to reductive denaturation.[28]

Thiol-Reactive Reagents

The primary thiol-reactive reagents, including iodoacetamides, maleimides, benzylic halides and bromomethylketones, react by *S*-alkylation of thiols to generate stable thioether products. Arylating reagents such as NBD halides react with thiols or amines by a similar substitution of the aromatic halide by the nucleophile. Because the thiolate anion is a better nucleophile than the neutral thiol, cysteine is more reactive above its pK_a[21] (~8.3, depending on protein structural context). However, as in the case of amine modification by succinimidyl esters (Chapter 1), reagent stability also decreases with increasing pH (e.g., maleimide hydrolysis to unreactive maleamic acid), and therefore a compromise pH of 7.0–7.5 is typically used for protein modifcation reactions. It has been reported that iodoacetamide and maleimide adducts with intracellular proteins have different degrees of stability and toxicity. Analysis of the intracellular reactivity and toxicity of haloacetyl and maleimido thiol-reactive probes in HEK 293 cells indicates that maleimides are less stable and iodoacetamides are more toxic[29] (putatively because maleimide adducts degrade before they are able to trigger damage-signaling pathways).

Also available are the TS-Link™ series of reagents for reversible thiol modification (Section 2.2). The TS-Link™ reagents are water-soluble thiosulfates that react stoichiometrically with thiols to form mixed disulfides. Thiols also react with many of the amine-reactive reagents described in Chapter 1, including isothiocyanates and succinimidyl esters. However, the reaction products appear to be insufficiently stable to be useful for routine modification of thiols in proteins. Although the thiol–isothiocyanate product (a dithiocarbamate) can react with an adjacent amine to yield a thiourea, the dithiocarbamate is more likely to react with water, consuming the reactive reagent without forming a covalent adduct. In addition to insertion or deletion of cysteine residues by site-directed mutagenesis, several reagents have been developed for introducing thiols into proteins, nucleic acids and lipids. Because the selective introduction of thiols is particularly important for crosslinking two biomolecules, these reagents are discussed in Chapter 5.

Site-Specific Labeling with Thiol-Reactive Reagents

A method for the site-specific double-labeling of a protein containing at least one vicinal diol and another distal thiol has been reported.[11] In this labeling protocol, the vicinal diol is first protected with phenylarsine oxide (PAO) to allow labeling of the unprotected distal thiol with Oregon Green® 488 maleimide (O6034, Section 2.2). The blocked vicinal diol is then deprotected with dithiothreitol (DTT) and labeled with Alexa Fluor® 350 maleimide (A30505, Section 2.3). Target proteins may need to be engineered to contain a vicinal diol and distal thiol in order to employ this labeling strategy.

In a similar double thiol-labeling method, instead of PAO protection/deprotection, the protein's tetracysteine tag was labeled using FlAsH-EDT_2 reagent (T34561, Section 2.2), and the Alexa Fluor® 568 maleimide (A20341, Section 2.2) was used to label a distal cysteine.[30] Fluorescence resonance energy transfer (Fluorescence Resonance Energy Transfer (FRET)—Note 1.2) between the FlAsH label and Alexa Fluor® 568 dye was then used to detect ligand-induced conformational changes in the C-terminal domain of the β_2-adrenoreceptor in SF9 cells.

Iodoacetamides

Iodoacetamides readily react with all thiols, including those found in peptides, proteins and thiolated polynucleotides, to form thioethers (Figure 2.1.2); they are somewhat more reactive than bromoacetamides. When a protein's cysteine residues are blocked or absent, however, iodoacetamides can sometimes react with methionine residues.[13] They may also react with histidine[31] or tyrosine, but generally only if free thiols are absent. Although iodoacetamides can react with the free base form of amines, most aliphatic amines, except the α-amino group at a protein's N-terminus, are protonated and thus relatively unreactive below pH 8.[32] In addition, iodoacetamides react with thiolated oligonucleotide primers, as well as with thiophosphates.[33]

Iodoacetamides are intrinsically unstable in light, especially in solution; reactions should therefore be carried out under subdued light. Adding cysteine, glutathione or mercaptosuccinic acid to the reaction mixture will quench the reaction of thiol-reactive probes, forming highly water-soluble adducts that are easily removed by dialysis or gel filtration. Although the thioether bond formed when an iodoacetamide reacts with a protein thiol is very stable, the bioconjugate loses its fluorophore during amino acid hydrolysis, yielding *S*-carboxymethylcysteine.

Maleimides

Maleimides are excellent reagents for thiol-selective modification, quantitation and analysis. In this reaction, the thiol is added across the double bond of the maleimide to yield a thioether (Figure 2.1.3). Applications of these fluorescent and chromophoric analogs of *N*-ethylmaleimide (NEM) strongly overlap those of iodoacetamides, although maleimides apparently do not react with methionine, histidine or tyrosine. Reaction of maleimides with amines usually requires a higher pH than reaction of maleimides with thiols.

Hydrolysis of the maleimide to an unreactive product can compete significantly with thiol modification, particularly above

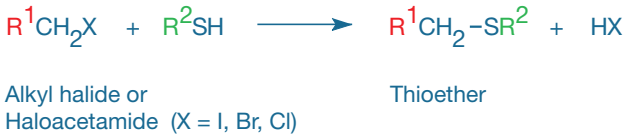

Figure 2.1.2 Reaction of a thiol with an alkyl halide.

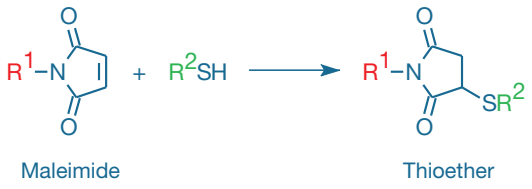

Figure 2.1.3 Reaction of a thiol with a maleimide.

pH 8. Furthermore, once formed, maleimide-derived thioethers can hydrolyze to an isomeric mixture of succinamic acid adducts, or they can undergo cyclization with adjacent amines to yield crosslinked products. This latter reaction is much less frequently encountered than the former.[34] Deliberate acceleration of the hydrolytic succinimide to succinamide acid ring-opening reaction by molybdate or chromate catalysis provides a strategy for decreasing the heterogeneity of bioconjugates derived from maleimide derivatization of thiols.[35]

Reversible Thiol-Reactive Reagents

Several of our thiol-reactive probes can be used to form reversible bonds, including BODIPY® FL L-cystine (B20340), as well as the TS-Link™ BODIPY® thiosulfate and TS-Link™ DSB-X™ biotin C_5-thiosulfate reagents (Section 2.2, Section 4.2).

Symmetric disulfides such as BODIPY® FL L-cystine undergo a thiol–disulfide interchange reaction to yield a new asymmetric disulfide (Figure 2.1.4), a reaction that is freely reversible and thiol-*specific*. This disulfide linkage can be cleaved with reagents such as DTT or TCEP.

Thiosulfates (R–S–SO_3^-), including our water-soluble TS-Link™ reagents, are similar to disulfides in that they stoichiometrically react with thiols to form disulfides (Figure 2.1.5). However, unlike the reaction of the BODIPY® FL cystine probe with a free thiol, no excess of the TS-Link™ reagent is required to drive the equilibrium.

Reagents for Quantitating Thiols

Measure-iT™ Thiol Assay Kit

The Measure-iT™ Thiol Assay Kit (M30550) provides an easy and accurate method for quantitating thiols. This thiol assay has a linear range of 0.05–5 µM thiol (Figure 2.1.6), making it up to 400 times more sensitive than colorimetric methods based on Ellman's reagent.

Each Measure-iT™ Thiol Assay Kit contains:

- Measure-iT™ thiol quantitation reagent (100X concentrate in 1,2-propanediol)
- Measure-iT™ thiol quantitation buffer (50 mM potassium phosphate buffer)
- Measure-iT™ thiol quantitation standard (reduced glutathione)
- Detailed protocols

Simply dilute the reagent 1:100, load 100 µL into the wells of a microplate, add 1–10 µL sample volumes, mix, then read the fluorescence. Maximum fluorescence signal is attained within 5 minutes and is stable for at least 1 hour. The assay is performed at room temperature, and common contaminants are well tolerated in the assay. The Measure-iT™ Thiol Assay Kit provides sufficient materials for 500 assays, based on a 100 µL assay volume in a 96-well microplate format; this thiol assay can also be adapted for use in cuvettes or 384-well microplates.

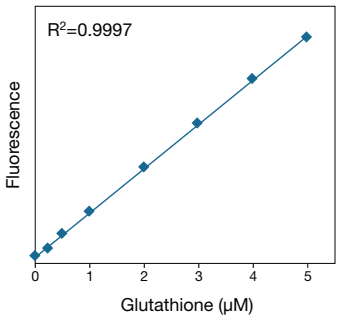

Figure 2.1.6 Linearity and sensitivity of the Measure-iT™ thiol assay. Triplicate 10 µL samples of glutathione were assayed using the Measure-iT™ Thiol Assay Kit (M30550). Fluorescence was measured using excitation/emission of 490/520 nm and plotted versus glutathione concentration. The variation (CV) of replicate samples was <2%.

Figure 2.1.4 Reaction of a thiol with a symmetric disulfide.

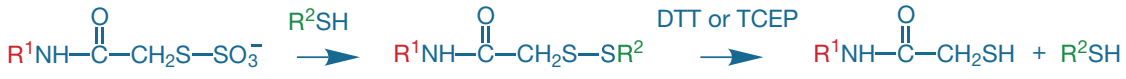

Figure 2.1.5 Reaction of a TS-Link™ reagent with a thiol, followed by removal of the label with a reducing agent.

Thiol and Sulfide Quantitation Kit

Ultrasensitive colorimetric quantitation of both protein and nonprotein thiols can be achieved using the Thiol and Sulfide Quantitation Kit (T6060). In this assay, which is based on a method reported by Singh,[36,37] thiols or sulfides reduce a disulfide-inhibited derivative of papain, stoichiometrically releasing the active enzyme (Figure 2.1.7). Activity of the enzyme is then measured using the chromogenic papain substrate L-BAPNA via spectrophotometric detection of *p*-nitroaniline release at 412 nm.

Although thiols and inorganic sulfides can also be quantitated using 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB or Ellman's reagent, D8451), the enzymatic amplification step in the Thiol and Sulfide Quantitation Kit enables researchers to detect as little as 0.2 nanomoles of thiols or sulfides—a sensitivity that is about 100-fold better than that achieved with DTNB.[38,39] Thiols in proteins can be detected indirectly by incorporating the disulfide cystamine into the reaction mixture. Cystamine undergoes an exchange reaction with protein thiols, yielding 2-mercaptoethylamine (cysteamine), which then releases active papain. Thiols that are alkylated by maleimides, iodoacetamides or other reagents are excluded from detection and can therefore be assayed subtractively.[40]

The Thiol and Sulfide Quantitation Kit contains:

- Papain–$SSCH_3$, the disulfide-inhibited papain derivative
- L-BAPNA, a chromogenic papain substrate
- DTNB (Ellman's reagent), for calibrating the assay
- Cystamine
- L-Cysteine, a thiol standard
- Buffer
- Detailed protocols for measuring thiols, inorganic sulfides and maleimides

Sufficient reagents are provided for approximately 50 assays using standard 1 mL cuvettes or 250 assays using a microplate format.

Ellman's Reagent (DTNB) for Quantitating Thiols

Ellman's reagent[41] (5,5′-dithiobis-(2-nitrobenzoic acid) or DTNB; D8451) remains an important reagent for spectrophotometric quantitation of protein thiols and, by extension, the analysis of thiol–disulfide exchange reactions[42] and oxidative thiol modifications.[43] It readily forms a mixed disulfide with thiols, liberating the chromophore 5-mercapto-2-nitrobenzoic acid[44] (absorption maximum 410 nm, EC ~13,600 $cm^{-1}M^{-1}$). Only protein thiols that are accessible to this water-soluble reagent are modified.[45,46] Inaccessible thiols can usually be quantitated by carrying out the titration in the presence of 6 M guanidinium chloride. DTNB conjugates of glutathione and other thiols can be separated by HPLC and quantitated based on their absorption.[47]

Other Fluorometric Reagents for Quantitating Thiols

Several maleimides—including 7-diethylamino-3-(4′-maleimidylphenyl)-4-methylcoumarin (CPM, D346; Section 2.3) and *N*-(7-dimethylamino-4-methylcoumarin-3-yl)maleimide (DACM, D10251; Section 2.3)—are not appreciably fluorescent until after conjugation with thiols, and are therefore useful for thiol quantitation. Similarly, fluorescein-5-maleimide (F150, Section 2.2) exhibits an analytically useful 10-fold fluorescence enhancement upon reaction with thiols.[48] Monobromobimane (M1378, M20381; Section 2.3) is also essentially nonfluorescent until it reacts with thiols and can be used to determine thiol levels in cells.

In addition, most of the fluorescent thiol-reactive reagents in this chapter can be used as derivatization reagents for analyzing thiols by techniques such as HPLC that utilize a separation step. 5-(Bromomethyl) fluorescein (B1355, Section 2.2) is the reagent with the greatest intrinsic sensitivity for this application. See Section 15.6 for a further discussion of methods to quantitate reduced glutathione in cells.

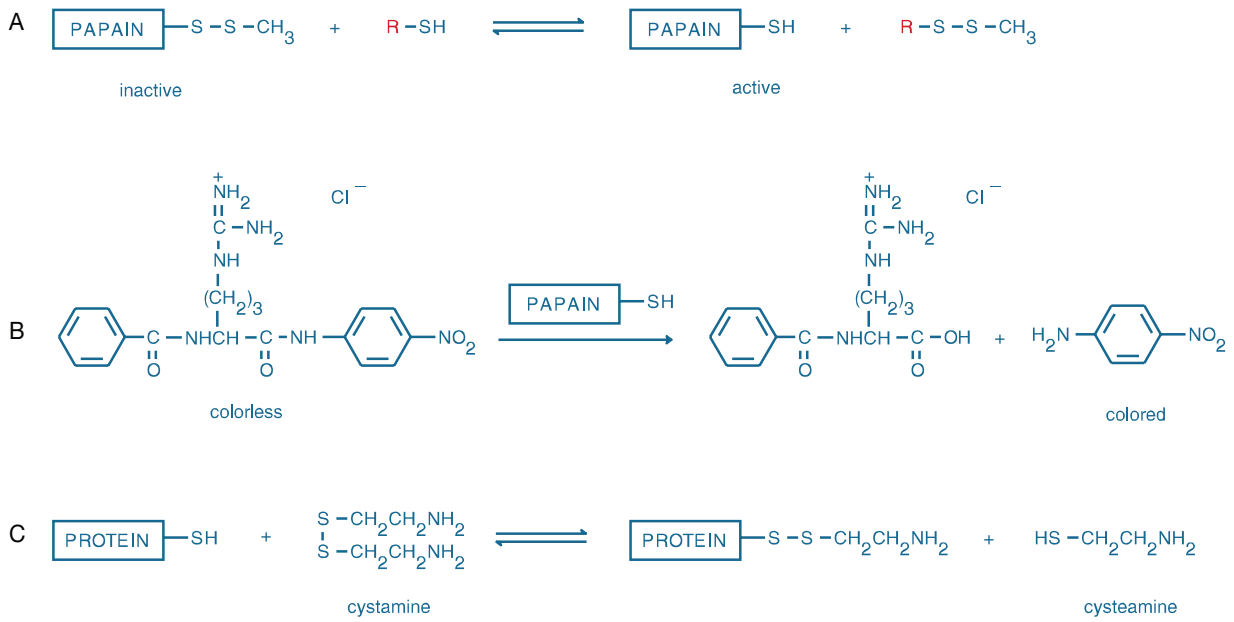

Figure 2.1.7 Chemical basis for thiol detection using the Thiol and Sulfide Quantitation Kit (T6060): **A**) The inactive disulfide derivative of papain, papain–$SSCH_3$, is activated in the presence of thiols; **B**) active papain cleaves the substrate L-BAPNA, releasing the *p*-nitroaniline chromophore; **C**) protein thiols, often poorly accessible, exchange with cystamine to generate 2-mercaptoethylamine (cysteamine), which is functionally equivalent to the thiol R–SH in step **A**.

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DATA TABLE 2.1 INTRODUCTION TO THIOL MODIFICATION AND DETECTION

Cat. No.	MW	Storage	Soluble	Abs	Em
D1532	154.24	D	H_2O	<300	none
D8451	396.35	D	pH >6	324	none
T2556	286.65	D	pH >5	<300	none

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

PRODUCT LIST 2.1 INTRODUCTION TO THIOL MODIFICATION AND DETECTION

Cat. No.	Product	Quantity
D8451	5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB; Ellman's reagent)	10 g
D1532	dithiothreitol (DTT)	1 g
M30550	Measure-iT™ Thiol Assay Kit *500 assays*	1 kit
T6060	Thiol and Sulfide Quantitation Kit *50–250 assays*	1 kit
T2556	tris-(2-carboxyethyl)phosphine, hydrochloride (TCEP)	1 g

2.2 Thiol-Reactive Probes Excited with Visible Light

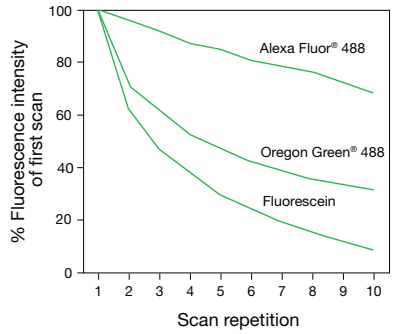

Figure 2.2.1 Photobleaching resistance of the green-fluorescent Alexa Fluor® 488, Oregon Green® 488 and fluorescein dyes, as determined by laser-scanning cytometry. EL4 cells were labeled with biotin-conjugated anti-CD44 antibody and detected by Alexa Fluor® 488 (S11223, S32354), Oregon Green® 488 (S6368) 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 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.

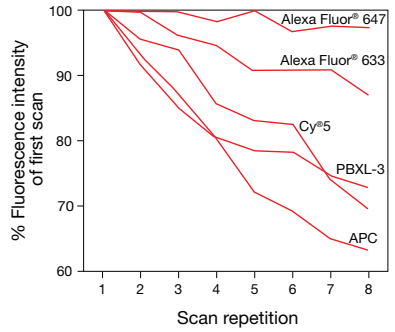

Figure 2.2.2 Photobleaching resistance of the red-fluorescent Alexa Fluor® 647, Alexa Fluor® 633, PBXL-3 and Cy®5 dyes and the allophycocyanin fluorescent protein, as determined by laser-scanning cytometry. EL4 cells were labeled with biotin-conjugated anti-CD44 antibody and detected by Alexa Fluor® 647 (S21374, S32357), 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.

The thiol-reactive Alexa Fluor®, BODIPY®, fluorescein, Oregon Green®, tetramethylrhodamine and Texas Red® derivatives have strong absorptivity and high fluorescence quantum yields. This combination of attributes makes these compounds the preferred reagents for preparing protein and low molecular weight ligand conjugates to study the diffusion, structural properties and interactions of proteins and ligands using techniques such as:

- Fluorescence recovery after photobleaching (FRAP)
- Fluorescence polarization (FP) (Fluorescence Polarization (FP)—Note 1.4)
- Fluorescence correlation spectroscopy (FCS) (Fluorescence Correlation Spectroscopy (FCS)—Note 1.3) and other single-molecule detection techniques
- Fluorescence resonance energy transfer (FRET) (Fluorescence Resonance Energy Transfer (FRET)—Note 1.2)

In this section and in Section 2.3, thiol-reactive reagents with similar spectra, rather than the same reactive group, are generally discussed together. The probes described in this section have visible absorption maxima beyond 410 nm; thiol-reactive probes with peak absorption below 410 nm are described in Section 2.3. Table 2.1 summarizes this section's thiol-reactive probes excited with visible light.

Alexa Fluor® Maleimides

Alexa Fluor® dyes set new standards for fluorescent dyes and the bioconjugates prepared from them (The Alexa Fluor® Dye Series—Note 1.1). Alexa Fluor® dyes exhibit several unique features:

- Strong absorption, with extinction coefficients greater than 65,000 $cm^{-1}M^{-1}$
- Excellent photostability (Figure 2.2.1, Figure 2.2.2), providing more time for observation and image capture than spectrally similar dyes allow (Figure 2.2.3)
- pH-insensitive fluorescence between pH 4 and pH 10
- Superior fluorescence output per protein conjugate, surpassing that of other spectrally similar fluorophore-labeled protein, including fluorescein, tetramethylrhodamine and Texas Red® conjugates, as well as Cy®3 and Cy®5 conjugates [1]

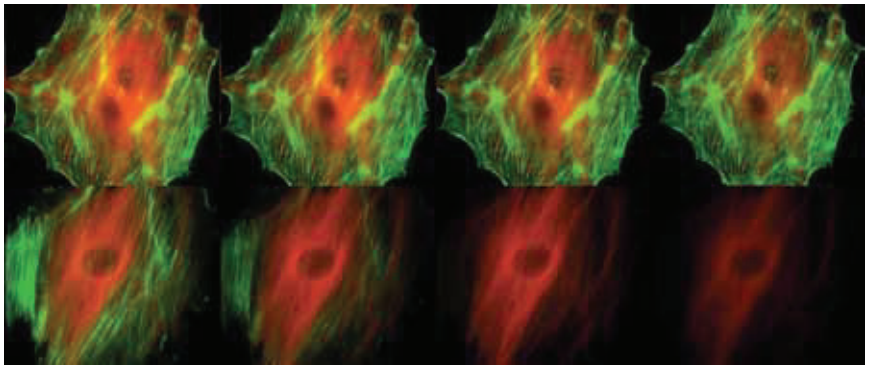

Figure 2.2.3 Comparison of the photobleaching rates of the Alexa Fluor® 488 and Alexa Fluor® 546 dyes and the well-known fluorescein and Cy®3 fluorophores. The cytoskeleton of bovine pulmonary artery endothelial cells (BPAEC) was labeled with (top series) Alexa Fluor® 488 phalloidin (A12379) and mouse 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 from left to right). The images were acquired with bandpass filter sets appropriate for fluorescein and rhodamine.

For labeling thiol groups, we offer thiol-reactive Alexa Fluor® dyes that span the visible spectrum:

- Alexa Fluor® 350 C_5-maleimide (A30505, Section 2.3)
- Alexa Fluor® 488 C_5-maleimide (A10254, Figure 2.2.4)
- Alexa Fluor® 532 C_5-maleimide (A10255)
- Alexa Fluor® 546 C_5-maleimide (A10258)
- Alexa Fluor® 555 C_2 maleimide (A20346)
- Alexa Fluor® 568 C_5-maleimide (A20341)
- Alexa Fluor® 594 C_5-maleimide (A10256)
- Alexa Fluor® 633 C_5-maleimide (A20342)
- Alexa Fluor® 647 C_2-maleimide (A20347)
- Alexa Fluor® 660 C_2-maleimide (A20343)
- Alexa Fluor® 680 C_2-maleimide (A20344)
- Alexa Fluor® 750 C_5-maleimide (A30459)

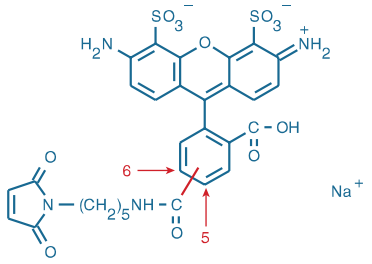

Figure 2.2.4 Alexa Fluor® 488 C_5-maleimide (A10254).

Table 2.1 Molecular Probes® thiol-reactive dyes excited with visible light.

Derivative	Abs *	Em *	Maleimide	Haloacetamide	Bromomethyl	Halide	Cystine [C] or Thiosulfate [T]
Alexa Fluor® 488	495	519	A10254 [M]				
Alexa Fluor® 532	532	553	A10255				
Alexa Fluor® 546	556	575	A10258 [M]				
Alexa Fluor® 555	555	565	A20346				
Alexa Fluor® 568	578	603	A20341 [M]				
Alexa Fluor® 594	590	617	A10256 [M]				
Alexa Fluor® 633	632	647	A20342 [M]				
Alexa Fluor® 647	650	665	A20347				
Alexa Fluor® 660	663	690	A20343				
Alexa Fluor® 680	679	702	A20344				
Alexa Fluor® 750	749	775	A30459				
BODIPY® FL	505	513	B10250	D6003			B20340 [C] T30453 [T]
BODIPY® TMR	542	574	B30466				T30454 [T]
BODIPY® TR	589	617					T30455 [T]
BODIPY® 493/503	493	503			B2103		
BODIPY® 499/508	499	508	D20350				
BODIPY® 507/545	508	543		D6004			
BODIPY® 577/618	577	618	D20351				
BODIPY® 630/650	625	640			B22802		T30456 [T]
4-Dimethylamino phenylazophenyl	419	NA	D1521				
Eosin	524	544	E118 [5]				
Fluorescein	494	518	F150 [5]	I30451 [5] I30452 [6]	B1355 [5]		
Lucifer yellow	426	531		L1338			
NBD	478	541		I9 † D2004		F486 F6053 ‡ C20260	
Oregon Green® 488	496	524	O6034 [5]	O6010 [M]			
PyMPO	415	570	M6026				
QSY® 7	560	NA	Q10257				
QSY® 9	562	NA	Q30457				
QSY® 35	475	NA		Q20348			
Rhodamine Red™	570	590	R6029 [M]				
Sulfonerhodamine	555	580		B10621 §			
Tetramethylrhodamine	555	580	T6027 [5] T6028 [6]	T6006 [5]			
Texas Red®	595	615	T6008 [M]		T6009 [M]		

* Absorption (Abs) and emission (Em) maxima, in nm. † Iodoacetate ester. ‡ Like the NBD probes, ABD-F (F6053) is a benz-2-oxa-1,3-diazole, except that it is sulfonated (i.e., an SBD probe) instead of nitrated (i.e., an NBD probe); its reaction product with dimethylaminoethanethiol has Abs/Em maxima of 376/510 nm. § Bifunctional crosslinker. 5 = 5-Isomer. 6 = 6-Isomer. M = Mixed isomers. C = BODIPY® FL L-cystine. T = TS-Link™ fluorescent thiosulfate. NA = Not applicable. More information on thiol-reactive dyes is available at www.invitrogen.com/handbook/labelingchemistry.

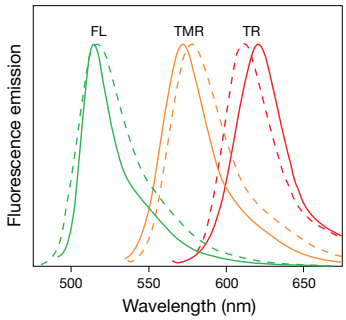

Figure 2.2.5 Normalized fluorescence emission spectra of goat anti–mouse IgG antibody conjugates of fluorescein (FL), tetramethylrhodamine (TMR) and the Texas Red® (TR) dyes, shown by dashed lines (- - -), as compared with goat anti–mouse IgG antibody conjugates of BODIPY® FL, BODIPY® TMR and BODIPY® TR dyes, respectively, shown by solid lines (—).

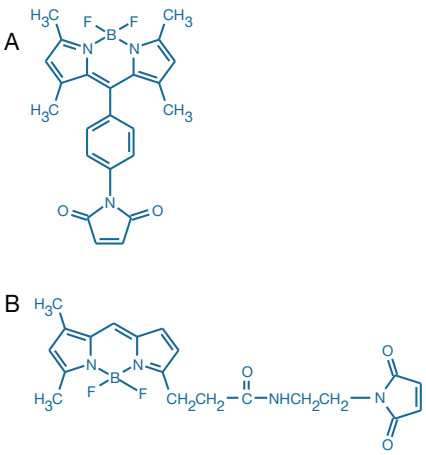

Figure 2.2.6 Comparison of the fluorophore orientation relative to the reactive moiety of two spectrally similar thiol-reactive BODIPY® dyes: **A**) BODIPY® 499/508 maleimide (D20350) and **B**) BODIPY® FL *N*-(2-aminoethyl)maleimide (B10250).

The Alexa Fluor® maleimides are particularly useful for labeling thiol-containing proteins on the surface of live cells, where their polarity permits the sensitive detection of exposed thiols.[2–4] In proteomics applications, Alexa Fluor® protein conjugates can be electrophoretically separated and then detected without additional staining.[5–7] As with their amine-reactive succinimidyl ester counterparts (Section 1.3), Alexa Fluor® 647 maleimide, Alexa Fluor® 750 maleimide and other long-wavelength reactive dyes are frequently used to make conjugates for *in vivo* imaging applications.[8,9] In experiments using Alexa Fluor® 488 maleimide, immunodetection of labeled proteins can be accomplished using our anti–Alexa Fluor® 488 antibody (A11094, Section 7.4).

BODIPY® Derivatives

BODIPY® Iodoacetamides, Maleimides and Methyl Bromides

Like their amine-reactive BODIPY® counterparts (Section 1.4), BODIPY® iodoacetamides, BODIPY® maleimides and BODIPY® methyl bromides yield thiol adducts with several important properties:

- High extinction coefficients (EC >60,000 $cm^{-1}M^{-1}$)
- High fluorescence quantum yields, often approaching 1.0, even in water
- Narrow emission bandwidths (Figure 2.2.5)
- Good photostability[10]
- Spectra that are relatively insensitive to solvent polarity and pH of the medium[11]
- Lack of ionic charge, which is especially useful when preparing membrane probes and cell-permeant reagents

BODIPY® dyes are chemically stable between about pH 3 and pH 10, although they are less stable to extremes of pH than are fluorescein and Alexa Fluor® derivatives. All of the thiol-reactive BODIPY® dyes are suitable for labeling cysteine residues in proteins and thiolated oligonucleotides and for detecting thiol conjugates separated by HPLC and capillary electrophoresis using ultrasensitive laser-scanning techniques.[11] BODIPY® FL iodoacetamide has been shown to be highly selective for cysteine labeling, producing little or no nonspecific labeling even at high dye:thiol ratios; in contrast, tetramethylrhodamine iodoacetamide exhibited nonspecific labeling as dye concentrations increased.[12] Furthermore, actin labeling with BODIPY® FL iodoacetamide (D6003) reportedly does not perturb actin polymerization.[13] BODIPY® FL maleimide is a useful reagent for flow cytometric quantitation and confocal imaging of microparticles released upon agonist-elicited activation of human platelets.[14,15] Labeling can be carried out after activation, avoiding concerns that pre-labeling might interfere with cellular functions involved in the activation process.

Our selection of thiol-reactive BODIPY® reagents includes:

- BODIPY® FL maleimide and BODIPY® FL iodoacetamide (B10250, Figure 2.2.6; D6003), which exhibit spectral characteristics very similar to fluorescein
- BODIPY® 507/545 iodoacetamide (D6004)
- BODIPY® TMR maleimide (B30466)
- BODIPY® 493/503 methyl bromide (B2103)
- BODIPY® 630/650 methyl bromide (B22802), with very long-wavelength spectra

Two additional symmetric maleimidylphenyl BODIPY® derivatives are available with excitation/emission maxima of ~499/508 nm (D20350, Figure 2.2.6) and ~577/618 nm (D20351).

BODIPY® FL L-Cystine

We have attached the BODIPY® FL fluorophore to the amino groups of the disulfide-linked amino acid cystine to create a reagent for reversible, *thiol-specific* labeling of proteins, thiolated oligonucleotides and cells.[16] BODIPY® FL L-cystine (B20340) is virtually nonfluorescent due to interactions between the two fluorophores; however, thiol-specific exchange to form a mixed disulfide results in significant enhancement of the green fluorescence (Figure 2.2.7).

TS-Link™ BODIPY® Thiosulfate Reagents

The TS-Link™ BODIPY® reagents are water-soluble, fluorescent thiosulfates that react readily and selectively with free thiols to form disulfide bonds (Figure 2.2.8). In contrast to the thioether bonds formed by maleimides and iodoacetamides, the disulfide bond formed by the TS-Link™ reagents is reversible; the TS-Link™ BODIPY® fluorophore can easily be removed using a reducing agent such as dithiothreitol or tris-(2-carboxyethyl)phosphine (DTT, D1532; TCEP, T2556; Section 2.1), leaving the molecule of interest unchanged for downstream processing. These TS-Link™ reagents yield the same disulfide products as methanethiosulfonates (MTS reagents), but they are much more polar and water soluble and may therefore selectively react with residues on the surface of a protein or live cell.[17]

We currently offer:

- TS-Link™ BODIPY® FL C_2-thiosulfate (T30453)
- TS-Link™ BODIPY® TMR C_5-thiosulfate (T30454)
- TS-Link™ BODIPY® TR C_5-thiosulfate (T30455)
- TS-Link™ BODIPY® 630/650 C_5-thiosulfate (T30456)

We also offer TS-Link™ DSB-X™ biotin C_5-thiosulfate (TS-Link™ desthiobiotin-X C_5-thiosulfate, T30754), which is described in Section 4.2.

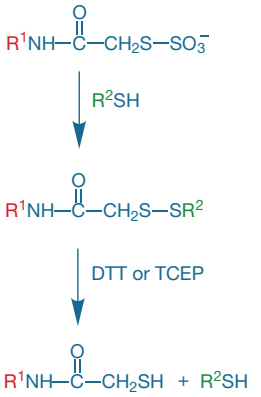

Figure 2.2.8 Reaction of a TS-Link™ reagent (R^1) with a thiol (R^2), followed by removal of the label with a reducing agent.

Fluorescein Derivatives, Including Thiol-Reactive Oregon Green® Dyes

Fluorescein Iodoacetamide, Maleimide and Methyl Bromide

The excellent water solubility of the fluorescein iodoacetamide single isomers (I30451, I30452) and fluorescein-5-maleimide (F150, Figure 2.2.9) at pH 7 makes it easy to prepare green-fluorescent thiol conjugates of biomolecules. Fluorescein maleimide and 5-iodoacetamidofluorescein have been the most extensively used visible wavelength–excitable, thiol-reactive dyes for modifying proteins, nucleic acids and other biomolecules. Following conjugation to thiols, fluorescein-5-maleimide (and other fluoresceins) can be radioiodinated.[18]

When compared with these iodoacetamide and maleimide derivatives, 5-(bromomethyl)fluorescein (B1355, Figure 2.2.10) reacts more slowly with thiols of peptides, proteins and thiolated nucleic acids but forms stronger thioether bonds that are expected to remain stable under the conditions required for complete amino acid analysis. With the possible exception of our Alexa Fluor® maleimides and the thiol-reactive BODIPY® dyes described above, 5-(bromomethyl)fluorescein has the highest intrinsic detectability of all thiol-reactive probes, particularly for capillary electrophoresis instrumentation that uses the 488 nm spectral line of the argon-ion laser.[19]

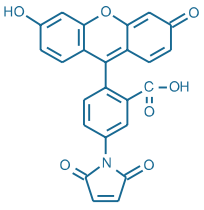

Figure 2.2.9 Fluorescein-5-maleimide (F150).

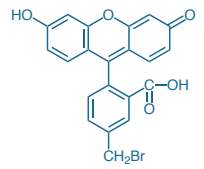

Figure 2.2.10 5-(bromomethyl)fluorescein (B1355).

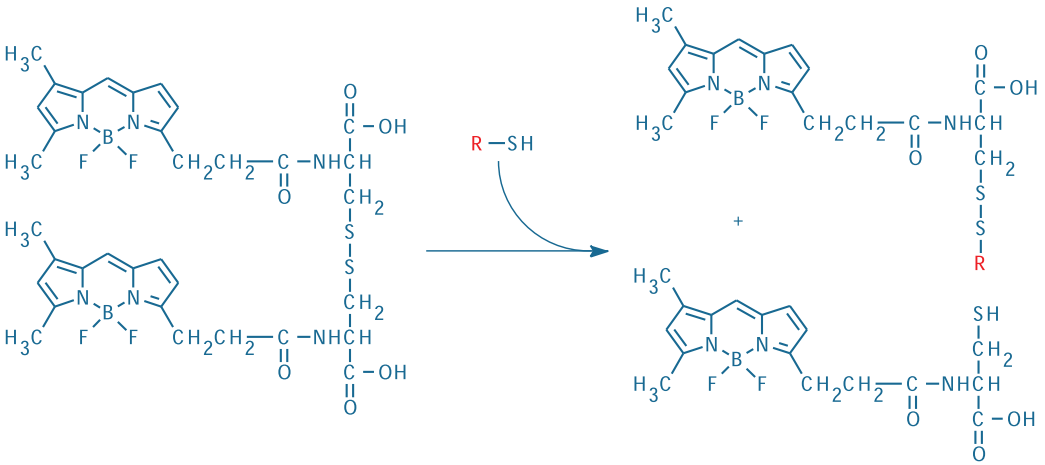

Figure 2.2.7 Reaction of intramolecularly quenched BODIPY® FL L-cystine (B20340) with a thiol, yielding two fluorescent products—a mixed disulfide labeled with the BODIPY® FL dye and a BODIPY® FL cysteine derivative.

Oregon Green® 488 Iodoacetamide and Maleimide

The Oregon Green® 488 dye (2′,7′-difluorofluorescein, D6145; Section 1.5) has absorption and emission spectra that are a perfect match to those of fluorescein. In addition to Oregon Green® 488 isothiocyanate, carboxylic acid and succinimidyl ester derivatives (Section 1.5), we have synthesized the isomeric mixture of Oregon Green® 488 iodoacetamide (O6010) and the single-isomer Oregon Green® 488 maleimide (O6034, Figure 2.2.11). These thiol-reactive probes yield conjugates that have several important advantages when directly compared with fluorescein conjugates, including:

- Greater photostability (Figure 2.2.12)
- A lower pK_a (pK_a of 4.8 for 2′,7′-difluorofluorescein versus 6.4 for fluorescein) (Figure 2.2.13)
- Higher fluorescence and less quenching at comparable degrees of substitution (Figure 2.2.14)
- Utility as fluorescence anisotropy probes for measuring protein–protein and protein–nucleic acid interactions[20] (Fluorescence Polarization (FP)—Note 1.4)

Eosin Maleimide

As compared with the corresponding fluorescein derivative, eosin maleimide (E118, Figure 2.2.15) is less fluorescent but much more phosphorescent and a better photosensitizer.[21] With eosin's high quantum yield of 0.57 for singlet oxygen generation,[22–24] eosin conjugates can be used as effective photooxidizers of diaminobenzidine (DAB) in high-resolution electron microscopy studies[25] (Fluorescent Probes for Photoconversion of Diaminobenzidine Reagents—Note 14.2).

Eosin (excitation/emission maxima ~519/540 nm) derivatives efficiently absorb the fluorescence from fluorescein and other fluorophores such as the BODIPY® FL, Alexa Fluor® 488, Oregon Green® 488, dansyl and coumarin dyes, making them good acceptors in FRET techniques[26] (Fluorescence Resonance Energy Transfer (FRET)—Note 1.2).

Although usually selectively reactive with thiols, eosin maleimide reportedly also reacts with a specific lysine residue of the band-3 protein in human erythrocytes, inhibiting anion exchange in these cells.[27,28] A flow cytometry assay for hereditary spherocytosis (HS), characterized by band-3 protein–deficient erthrocytes, has been developed using this selective binding by eosin maleimide.[29–31] In this assay, HS erythrocytes are identified as the population exhibiting low eosin fluorescence.

Rhodamine Derivatives, Including Thiol-Reactive Texas Red® Dyes

Tetramethylrhodamine Iodoacetamide and Maleimide

Tetramethylrhodamine iodoacetamide (TMRIA) and tetramethylrhodamine maleimide yield photostable, pH-insensitive, red-orange–fluorescent thiol conjugates.[32,33] These iodoacetamide and maleimide derivatives, however, are difficult to prepare in pure form and different batches of our mixed-isomer products have contained variable mixtures of the 5- and 6-isomers. Moreover, certain cytoskeletal proteins

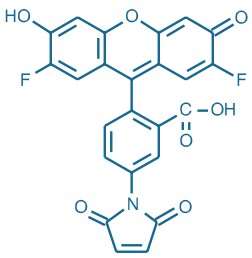

Figure 2.2.11 Oregon Green® 488 maleimide (O6034).

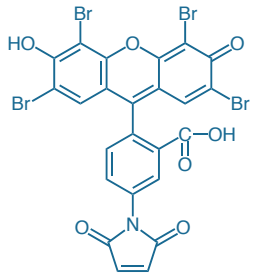

Figure 2.2.15 Eosin-5-maleimide (E118).

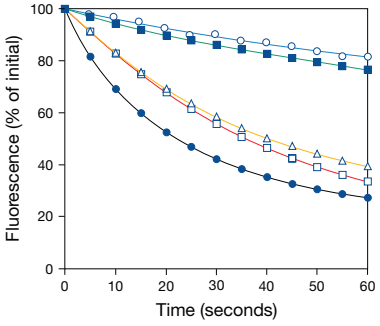

Figure 2.2.12 Comparison of photostability of green-fluorescent antibody conjugates. The following fluorescent goat anti–mouse IgG antibody conjugates were used to detect mouse anti–human IgG antibody labeling of human anti-nuclear antibodies in HEp-2 cells on prefixed test slides (INOVA Diagnostics 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.

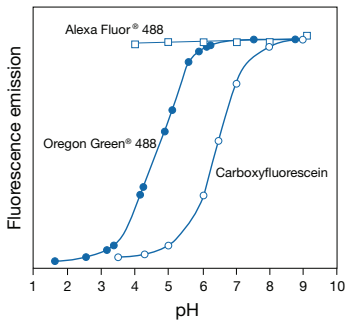

Figure 2.2.13 Comparison of pH-dependent fluorescence of the Oregon Green® 488 (●), carboxyfluorescein (○) and Alexa Fluor® 488 (□) fluorophores. Fluorescence intensities were measured for equal concentrations of the three dyes using excitation/emission at 490/520 nm.

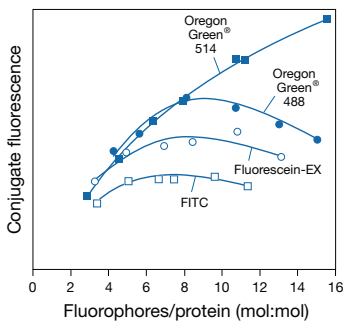

Figure 2.2.14 Comparison of relative fluorescence as a function of the number of fluorophores attached per protein for goat anti–mouse IgG antibody conjugates prepared using Oregon Green® 514 carboxylic acid succinimidyl ester (O6139, ■), Oregon Green® 488 carboxylic acid succinimidyl ester (O6147, ●), 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.

preferentially react with individual isomers, leading to complications in the interpretation of labeling results.[34–37] Consequently, we now prepare the 5-isomer of TMRIA (T6006, Figure 2.2.16) and the 5-isomer (T6027, Figure 2.2.17) and 6-isomer (T6028, Figure 2.2.18) of tetramethylrhodamine maleimide. A fluorogenic ADP biosensor has been described that exploits nucleotide-modulated self-quenching of two TMRIA labels that have been site-specifically attached to *Escherichia coli* ParM nucleotide-binding protein.[38] Tetramethylrhodamine-5-maleimide is often used for voltage-clamp fluorometry,[39] wherein it is attached to cysteine residues in the voltage-sensor domains of ion channels, generating fluorescence signals that are responsive to structural rearrangements associated with channel gating.[40,41] In this context, the dye is sometimes referred to as TMRM, but it should not be confused with tetramethylrhodamine methyl ester (T668, Section 12.2), a structurally similar but functionally quite different dye that is identified by the same acronym.

Rhodamine-Based Crosslinking Reagent

The thiol-reactive, homobifunctional crosslinker bis-((*N*-iodoacetyl)piperazinyl)sulfonerhodamine (B10621, Figure 2.2.19) is derived from a relatively rigid rhodamine dye. It is similar to a thiol-reactive rhodamine-based crosslinking reagent used to label regulatory light-chains of chicken gizzard myosin for fluorescence polarization experiments.[42] Researchers have attached bis-((*N*-iodoacetyl)piperazinyl) sulfonerhodamine to the kinesin motor domain and determined the orientation of kinesin bound to microtubules in the presence of a non-hydrolyzable ATP analog by fluorescence polarization microscopy.[43,44] Images of single molecules of chicken calmodulin crosslinked between two engineered cysteines by bis-((*N*-iodoacetyl)piperazinyl) sulfonerhodamine have been used to generate comparisons of experimental and theoretical super-resolution point-spread functions[45] (PSF). Dibromobimane (D1379, Section 2.3) is a shorter-wavelength alternative for applications requiring a fluorescent homobifunctional thiol crosslinker.

Rhodamine Red™ Maleimide

We offer a maleimide derivative of our Rhodamine Red™ fluorophore (R6029), which is spectrally similar to Lissamine rhodamine B (Figure 2.2.20). The spectral properties of Rhodamine Red™ maleimide have been exploited to improve the light-harvesting efficiency of chlorophyll by site-specific labeling of cysteine residues in the recombinantly expressed apoprotein in order to fill in the "green gap" in the absorption spectrum.[46] Rhodamine Red™ C_2-maleimide is a mixture of two isomeric sulfonamides (Figure 2.2.21).

Texas Red® Bromoacetamide and Maleimide

Conjugates of the bromoacetamide and maleimide derivatives of our Texas Red® fluorophore (T6009, T6008) have very little spectral overlap with fluorescein or Alexa Fluor® 488 conjugates (Figure 2.2.20) and are therefore useful as second labels in multicolor applications or as energy transfer acceptors from green-fluorescent dyes.[47,48] Bromoacetamides are only slightly less reactive with thiols than are iodoacetamides. The Texas Red® bromoacetamide (Figure 2.2.22) and maleimide (Figure 2.2.23) derivatives are mixtures of the corresponding two isomeric sulfonamides.

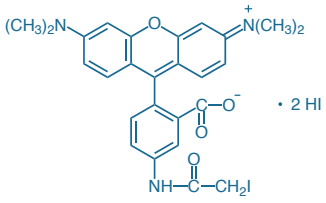

Figure 2.2.16 Tetramethylrhodamine-5-iodoacetamide dihydroiodide (5-TMRIA, T6006)

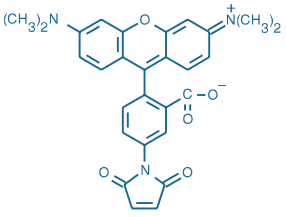

Figure 2.2.17 Tetramethylrhodamine-5-maleimide (T6027).

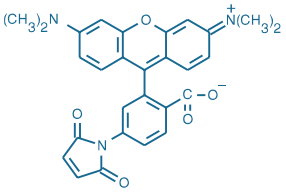

Figure 2.2.18 Tetramethylrhodamine-6-maleimide (T6028).

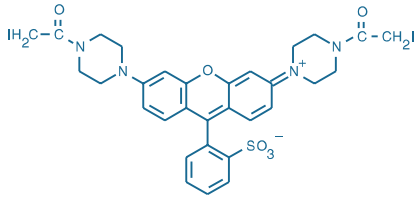

Figure 2.2.19 bis-((*N*-iodoacetyl)piperazinyl)sulfonerhodamine (B10621).

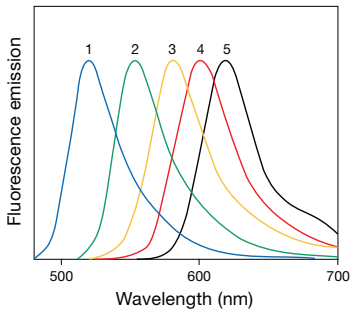

Figure 2.2.20 Normalized fluorescence emission spectra of goat anti–mouse IgG antibody conjugates of 1) fluorescein, 2) rhodamine 6G, 3) tetramethylrhodamine, 4) Lissamine rhodamine B and 5) Texas Red® dyes.

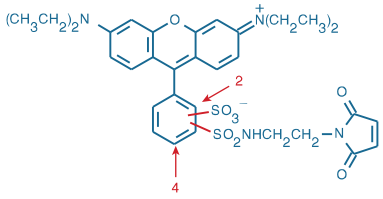

Figure 2.2.21 Rhodamine Red™ C_2-maleimide (R6029).

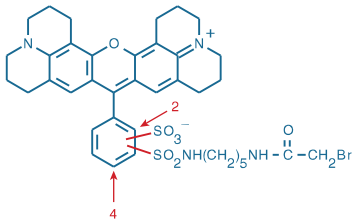

Figure 2.2.22 Texas Red® C_5-bromoacetamide (T6009).

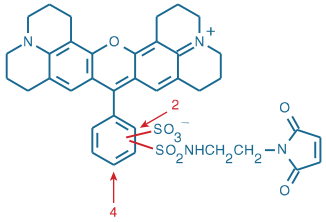

Figure 2.2.23 Texas Red® C_2-maleimide (T6008).

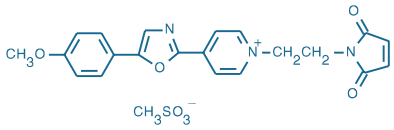

Figure 2.2.24 1-(2-maleimidylethyl)-4-(5-(4-methoxyphenyl)oxazol-2-yl)pyridinium methanesulfonate (PyMPO maleimide, M6026).

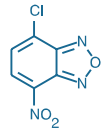

Figure 2.2.25 4-chloro-7-nitrobenz-2-oxa-1,3-diazole (NBD chloride; 4-chloro-7-nitrobenzofurazan, C20260).

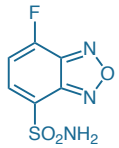

Figure 2.2.26 7-fluorobenz-2-oxa-1,3-diazole-4-sulfonamide (ABD-F, F6053).

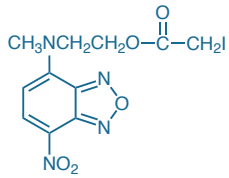

Figure 2.2.27 *N*-((2-(iodoacetoxy)ethyl)-*N*-methyl)amino-7-nitrobenz-2-oxa-1,3-diazole (IANBD ester, I9).

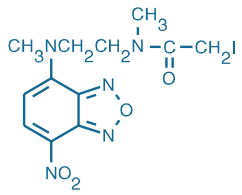

Figure 2.2.28 *N,N′*-dimethyl-*N*-(iodoacetyl)-*N′*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)ethylenediamine (IANBD amide, D2004).

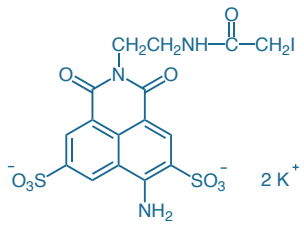

Figure 2.2.29 Lucifer yellow iodoacetamide, dipotassium salt (L1338).

PyMPO Maleimide

PyMPO maleimide (M6026, Figure 2.2.24) is an environment-sensitive thiol-reactive dye with a fluorescence excitation peak near 415 nm and an unusually long Stokes shift (fluorescence emission peak at ~560–580 nm). Its most widespread application is for labeling cysteine residues in the voltage-sensor domains of ion channels, where its fluorescence is exquisitely sensitive to structural rearrangements associated with channel gating.[40,49] This technique is commonly referred to as voltage-clamp fluorometry.[39]

Benzoxadiazole Derivatives, Including NBD Probes

NBD Chloride and NBD Fluoride

NBD chloride (C20260, Figure 2.2.25) and the more reactive NBD fluoride (F486) are common reagents for amine modification (Section 1.8). They also react with thiols [50–52] and cysteine in several proteins [53–57] to yield thioethers. NBD conjugates of thiols usually have much shorter-wavelength absorption and weaker fluorescence than do NBD conjugates of amines.[52] Selective modification of cysteines in the presence of reactive lysines and tyrosines is promoted by carrying out the reaction at pH <7; [58,59] however, NBD conjugates of thiols are often unstable, resulting in time-dependent label migration to adjacent lysine residues.[52,59]

ABD-F

Thiol conjugates of 7-fluorobenz-2-oxa-1,3-diazole-4-sulfonamide [60,61] (ABD-F, F6053; Figure 2.2.26) are much more stable in aqueous solution than are the thiol conjugates prepared from NBD chloride or NBD fluoride.[60] ABD-F is nonfluorescent until reacted with thiols and therefore can be used to quantitate thiols in solution,[62] as well as thiols separated by HPLC [63] or TLC.[64] ABD-F also reportedly reacts slowly with the hydroxy group of some tyrosine residues as well as α-amino groups in some proteins, forming products that are nonfluorescent but can be detected by absorbance at 385 nm.[65] ABD-F labeling is blocked by zinc binding to protein thiols and can therefore be used as an inverse proportionality indicator of bound Zn^{2+}.[66,67] In contrast, the fluorescent zinc indicators described in Section 19.7 primarily detect free Zn^{2+} ions. ABD–cysteine conjugates are very stable to acid hydrolysis, but labeling is partially reversed in basic solution containing DTT [68,69] (D1532; Section 2.1).

IANBD Ester and IANBD Amide

When conjugating the NBD fluorophore to thiols located in hydrophobic sites of proteins, we recommend using the NBD iodoacetate ester (IANBD ester, I9; Figure 2.2.27) or, preferably, the more hydrolytically stable NBD iodoacetamide (IANBD amide, D2004; Figure 2.2.28). These reactive reagents exhibit appreciable fluorescence only after reaction with thiols that are buried or unsolvated, and this fluorescence is highly sensitive to changes in protein conformation and assembly of molecular complexes.[70,71]

Lucifer Yellow Iodoacetamide

Lucifer yellow CH is a well-known polar tracer for neurons (Section 14.3). Its iodoacetamide derivative (L1338, Figure 2.2.29) similarly has high water solubility and visible absorption and emission spectra similar to those of lucifer yellow CH (Figure 2.2.30). As with the polar Alexa Fluor® maleimides and the stilbene iodoacetamide and maleimide (A484, A485; Section 2.3), a principal application of lucifer yellow iodoacetamide is the labeling of exposed thiols of proteins in solution, as well as in the outer membrane of live cells.[72] Lucifer yellow iodoacetamide has also been used as a fluorescence energy acceptor from aequorin in bioluminescence resonance energy transfer (BRET) assays.[73]

TC-FlAsH™ and TC-ReAsH™ Detection of Tetracysteine-Tagged Proteins

TC-FlAsH™ and TC-ReAsH™ Detection Technology

TC-FlAsH™ and TC-ReAsH™ detection technology, based on the tetracysteine tag first described by Griffin, Adams and Tsien in 1998,[74,75] takes advantage of the high-affinity interaction of a biarsenical ligand (FlAsH-EDT$_2$ or ReAsH-EDT$_2$) with the thiols in a tetracysteine (TC) expression tag fused to the protein of interest. The FlAsH-EDT$_2$ ligand is essentially fluorescein that has been modified to contain two arsenic atoms at a set distance from each other, whereas the ReAsH-EDT$_2$ ligand is a similarly modified resorufin (Figure 2.2.31). Virtually nonfluorescent in the ethanedithiol (EDT)-bound state, these reagents become highly fluorescent when bound to the tetracysteine tag Cys-Cys-Xxx-Yyy-Cys-Cys, where Xxx-Yyy is typically Pro-Gly[76] (Figure 2.2.32). Modified tags with additional flanking sequences produce higher-affinity binding of the biarsenical ligand, resulting in improved signal-to-background characteristics.[77,78] Background due to off-target endogenous thiols can be diminished by washing with competitor dithiols such as 2,3-dimercaptopropanol (BAL). Although tetracysteine tag labeling is best suited to reducing intracellular environments, protocols involving co-administration of trialkylphosphine or dithiothreitol (DTT, D1532; Section 2.1) reducing agents have been devised for applications in oxidizing environments, including cell surfaces.[79,80] Photosensitized oxidation of diaminobenzidine (Fluorescent Probes for Photoconversion of Diaminobenzidine Reagents—Note 14.2) by ReAsH enables correlated fluorescence and electron microscopy of tetracysteine-tagged proteins.[80–82]

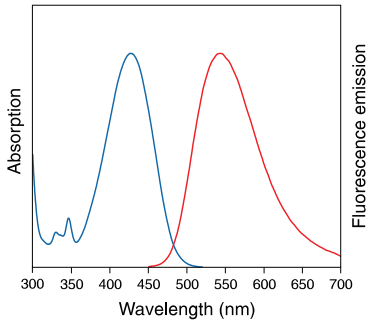

Figure 2.2.30 Absorption and fluorescence emission spectra of lucifer yellow CH in water.

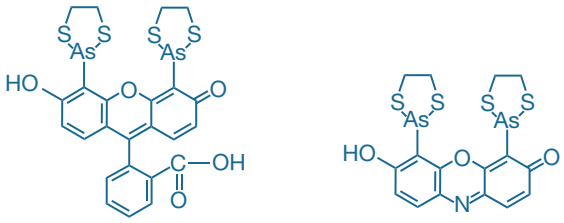

Figure 2.2.31 The structures of **A**) FlAsH-EDT$_2$ ligand and **B**) ReAsH-EDT$_2$ ligand, which are biarsenical labeling reagents provided in the TC-FlAsH™ II and TC-ReAsH™ II In-Cell Tetracysteine Tag Detection Kits (T34561, T34562), respectively.

The six–amino acid tetracysteine tag is less likely to disrupt native protein structure and function than larger tags such as Green Fluorescent Protein[83] (GFP, 238 amino acids). Although the majority of TC-FlAsH™ and TC-ReAsH™ applications have been in mammalian cells (Figure 2.2.33), the reagents and associated methods are also particularly useful for nondisruptive labeling of viral coat proteins[84–88] and successful adaptations for labeling proteins in yeast,[83] bacteria,[89,90] *Dictyostelium discoideum*[91] and plants[92] have been described.

TC-FlAsH™ and TC-ReAsH™ Tetracysteine Tag Detection Kits

Transfecting the host cell line with an expression construct comprising the protein of interest fused to a tetracysteine tag (CCPGCC) is the first step into TC-FlAsH™ TC-ReAsH™ detection. The tagged protein is then detected by the addition of FlAsH-EDT$_2$ reagent or ReAsH-EDT$_2$ reagent, which generates green or red fluorescence, respectively, upon binding the tetracysteine motif. For detection of tetracysteine-tagged proteins expressed in cells, we offer the TC-FlAsH™ II and TC-ReAsH™ II In-Cell Tetracysteine Tag Detection Kits (T34561, T34562), which provide:

- FlAsH-EDT$_2$ or ReAsH-EDT$_2$ reagent (in Kit T34561 or T34562), respectively
- BAL wash buffer
- Detailed protocols

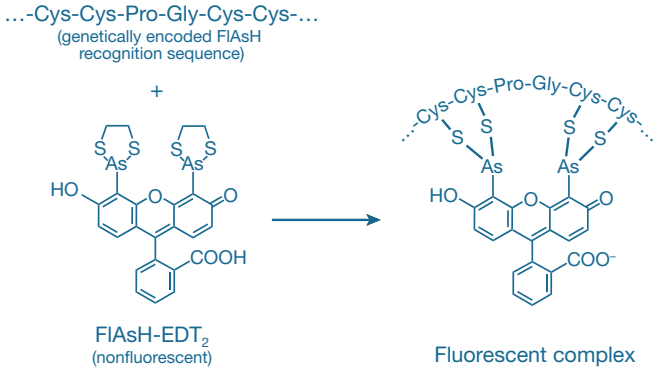

Figure 2.2.32 Binding of the nonfluorescent FlAsH-EDT$_2$ ligand to a recombinantly expressed tetracysteine sequence yields a highly fluorescent complex.

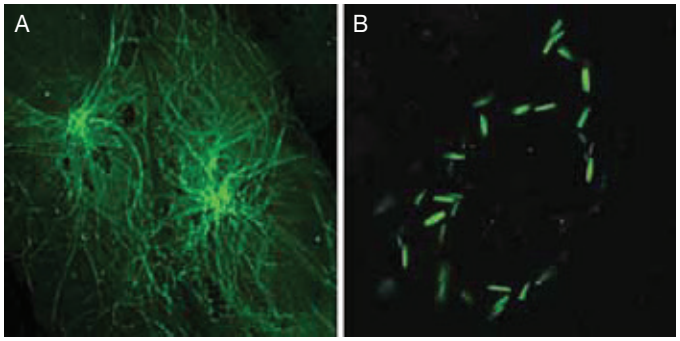

Figure 2.2.33 CHO-k1 cells expressing a tetracysteine-tagged version of β-tubulin labeled with FlAsH-EDT$_2$ reagent, provided in the TC-FlAsH™ II In Cell Tetracysteine Tag Detection Kit (T34561). Upon treatment with vinblastine, a compound known to perturb cytoskeletal structure, tubulin drastially rearranges from **A**) a reticular structure to **B**) rod-shaped structures.

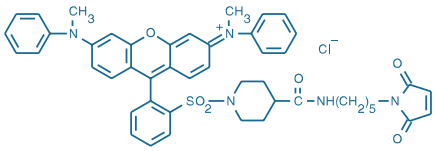

Figure 2.2.35 QSY® 7 C_5-maleimide (Q10257).

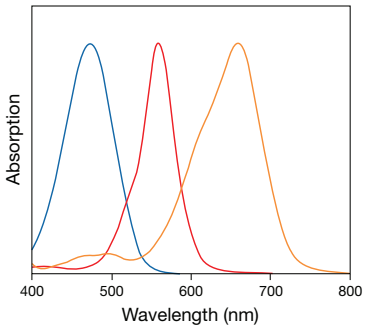

Figure 2.2.36 Normalized absorption spectra of the QSY® 35 (blue), QSY® 7 (red) and QSY® 21 (orange) dyes. The QSY® 7 and QSY® 9 dyes have essentially identical spectra.

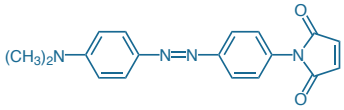

Figure 2.2.37 4-dimethylaminophenylazophenyl-4´-maleimide (DABMI, D1521).

We also offer these TC-FlAsH™ and TC-ReAsH™ detection reagents bundled with Gateway expression vectors for use in cloning the tetracysteine-tagged protein fusion. The TC-FlAsH™ II TC-ReAsH™ II In-Cell Tetracysteine Tag Detection Kit (with mammalian TC-Tag Gateway expression vectors) (T34563) provides:

- FlAsH-EDT_2 and ReAsH-EDT_2 reagents
- BAL wash buffer
- pcDNA 6.2/cTC-Tag-DEST
- pcDNA 6.2/nTC-Tag-DEST
- pcDNA 6.2/nTC-Tag-p64 control plasmid
- Detailed protocols

In addition to these kits for in-cell detection, we offer the TC-FlAsH™ Expression Analysis Detection Kits (A10067, A10068; Section 9.4), which are designed for detecting tetracysteine-tagged proteins in polyacrylamide gels (Figure 2.2.34).

Chromophoric Maleimides and Iodoacetamides

QSY® Maleimides and Iodoacetamide

QSY® 7 C_5-maleimide[93] (Q10257, Figure 2.2.35) and QSY® 9 C_5-maleimide (Q30457) are nonfluorescent, thiol-reactive diarylrhodamines with absorption spectra similar to those of our QSY® 7 and QSY® 9 succinimidyl esters (Q10193, Q20131; Section 1.6; Figure 2.2.36), respectively. Although the QSY® 7 and QSY® 9 chromophores are spectrally similar, QSY® 9 dye exhibits enhanced water solubility. QSY® 35 iodoacetamide (Q20348) is a nonfluorescent thiol-reactive analog of the amine-reactive nitrobenzoxadiazole (NBD) dye.

The principal applications of these thiol-reactive QSY® derivatives are as nonfluorescent acceptor dyes in fluorescence resonance energy transfer (FRET) assays[93–95] (Fluorescence Resonance Energy Transfer (FRET)—Note 1.2). The use of nonfluorescent acceptor dyes avoids the background fluorescence that often results from direct (i.e., nonsensitized) excitation of fluorescent acceptor dyes. The broad and strong absorption of QSY® 7 and QSY® 9 dyes (absorption maximum ~560 nm, EC ~90,000 $cm^{-1}M^{-1}$) yields extraordinarily efficient quenching of donors that have blue, green, orange or red fluorescence. QSY® 35 derivatives absorb light maximally near 470 nm (Figure 2.2.36), making their conjugates excellent FRET acceptors from UV light–excited donor dyes.

DABMI

DABMI (D1521, Figure 2.2.37) is the thiol-reactive analog of dabcyl succinimidyl ester (D2245, Section 1.8) and has similar properties and applications. Its principal application is as a nonfluorescent acceptor dye in fluorescence resonance energy transfer (FRET) assays[96–98] (Fluorescence Resonance Energy Transfer (FRET)—Note 1.2). The donor dyes in these assays typically include IAEDANS (I14) and other dyes described in Section 2.3. DABMI is also a useful derivatization reagent for MALDI-MS fragmentation analysis of cysteine-containing peptides.[99]

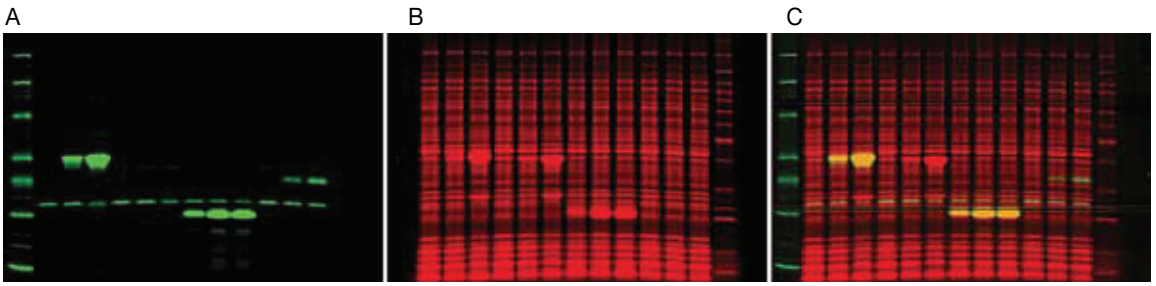

Figure 2.2.34 Protein gel staining using TC-FlAsH™ Expression Analysis Detection Kit (A10068). **A**) Tetracysteine-tagged proteins are labeled with FlAsH-EDT_2 reagent and fluoresce green. **B**) Total proteins are labeled with the Red total-protein stain provided in the kit and fluoresce red. **C**) an overlay of the two images reveals relative amounts of protein.

NANOGOLD® Monomaleimide

In collaboration with Nanoprobes, Inc. (www.nanoprobes.com), we offer thiol-reactive NANOGOLD® monomaleimide (N20345). NANOGOLD® particles are small metal cluster complexes of gold particles for research applications in light or electron microscopy.[100,101] These cluster complexes are discrete chemical compounds, not gold colloids. NANOGOLD® monomaleimide (N20345) permits attachment of these very small (1.4 nm) yet uniformly sized gold particles to accessible thiol groups in biomolecules (Figure 2.2.38, Figure 2.2.39). NANOGOLD® monomaleimide, which is supplied as a set of five vials of a powder lyophilized from pH 7.5 HEPES buffer, is simply resuspended with the thiol-containing protein in deionized water at room temperature or below to form the conjugate, after which any excess NANOGOLD® monomaleimide is removed by gel filtration.[102,103]

In addition to its many uses for light and electron microscopy, NANOGOLD® monomaleimide has been shown to be an extremely efficient quencher for dyes in molecular beacons—probes that can be used for homogeneous fluorescence *in situ* hybridization assays.[104] NANOGOLD® conjugates of antibodies and streptavidin are described in Section 7.2 and Section 7.6, respectively, along with reagents and methods for silver enhancement to amplify electron microscopy detection.[104]

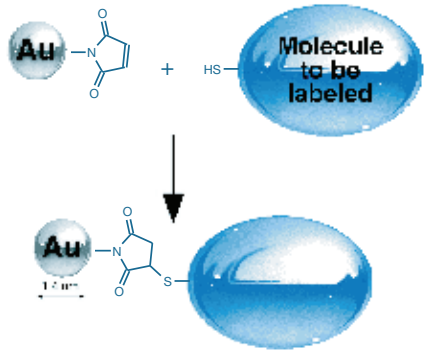

Figure 2.2.38 Reaction of NANOGOLD® monomaleimide (N20345) with a thiol. Image courtesy of Nanoprobes, Inc.

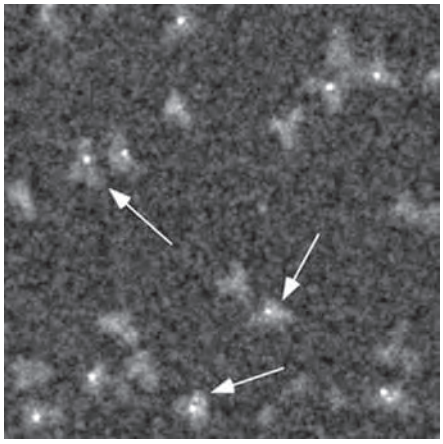

Figure 2.2.39 Scanning transmission electron microscope (STEM) image indicating that labeling with NANOGOLD® monomaleimide (N20345) occurs specifically at a hinge-thiol site on the IgG molecule. Image courtesy of Nanoprobes, Inc.

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DATA TABLE 2.2 THIOL-REACTIVE PROBES EXCITED WITH VISIBLE LIGHT

Cat. No.	MW	Storage	Soluble	Abs	EC	Em	Solvent	Notes
A10254	720.66	F,DD,L	H_2O, DMSO	493	72,000	516	pH 7	1, 2, 3
A10255	812.88	F,DD,L	H_2O, DMSO	528	78,000	552	MeOH	1
A10256	908.97	F,DD,L	H_2O, DMSO	588	96,000	612	pH 7	1, 4
A10258	1034.37	F,DD,L	H_2O, DMSO	554	93,000	570	pH 7	1
A20341	880.92	F,DD,L	H_2O, DMSO	575	92,000	600	pH 7	1, 5
A20342	~1300	F,DD,L	H_2O, DMSO	622	143,000	640	MeOH	1
A20343	~900	F,DD,L	H_2O, DMSO	668	112,000	697	MeOH	1, 6
A20344	~1000	F,DD,L	H_2O, DMSO	684	175,000	714	MeOH	1, 7
A20346	~1250	F,DD,L	H_2O, DMSO	556	158,000	572	MeOH	1

continued on next page

DATA TABLE 2.2 THIOL-REACTIVE PROBES EXCITED WITH VISIBLE LIGHT—*continued*

Cat. No.	MW	Storage	Soluble	Abs	EC	Em	Solvent	Notes
A20347	~1300	F,DD,L	H_2O, DMSO	651	265,000	671	MeOH	1, 8
A30459	~1350	F,DD,L	H_2O, DMSO	753	290,000	783	MeOH	1, 24
B1355	425.23	F,D,L	pH >6, DMF	492	81,000	515	pH 9	9
B2103	341.00	F,D,L	DMSO, MeCN	533	62,000	561	$CHCl_3$	10, 11
B10250	414.22	F,D,L	DMSO, MeCN	504	79,000	510	MeOH	11
B10621	840.47	F,D,L	DMSO	549	88,000	575	MeOH	12
B20340	788.44	F,D,L	DMSO	504	132,000	511	MeOH	13
B22802	449.14	F,D,L	DMSO, MeCN	658	73,000	678	$CHCl_3$	14
B30466	562.42	F,DD,L	DMSO, MeCN	544	60,000	570	MeOH	11
C20260	199.55	F,D,L	DMF, MeCN	336	9800	none	MeOH	15, 16
D1521	320.35	F,D,L	DMF, MeCN	419	34,000	none	MeOH	17
D2004	419.18	F,D,L	DMF, DMSO	478	25,000	541	MeOH	12, 17
D6003	417.00	F,D,L	DMSO, MeCN	502	76,000	510	MeOH	11, 12
D6004	431.03	F,D,L	DMSO, MeCN	508	69,000	543	MeOH	11, 12
D20350	419.24	F,D,L	DMSO	499	88,000	508	MeOH	18
D20351	575.38	F,D,L	DMSO	577	60,000	618	MeOH	18
E118	742.95	F,D,L	pH >6, DMF	524	103,000	545	MeOH	1, 19
F150	427.37	F,D,L	pH >6, DMF	492	83,000	515	pH 9	1, 9, 20
F486	183.10	F,D,L	MeCN, $CHCl_3$	328	8000	none	MeOH	15
F6053	217.17	F,D,L	DMF, DMSO	320	4800	none	MeOH	21
I9	406.14	F,D,L	DMF, MeCN	472	23,000	536	MeOH	12, 17
I30451	515.26	F,D,L	pH >6, DMF	492	78,000	515	pH 9	1, 9, 12
I30452	515.26	F,D,L	pH >6, DMF	491	82,000	516	pH 9	1, 9, 12
L1338	659.51	F,D,L	H_2O	426	11,000	531	pH 7	12
M6026	471.48	F,D,L	DMSO	412	23,000	561	MeOH	22
O6010	551.24	F,D,L	pH >6, DMF	491	68,000	516	pH 9	1, 12, 23
O6034	463.35	F,D,L	pH >6, DMF	491	81,000	515	pH 9	1, 23
Q10257	858.45	F,D,L	DMSO	560	92,000	none	MeOH	
Q20348	453.20	F,D,L	DMSO	475	24,000	none	MeOH	12
Q30457	1083.30	F,D,L	H_2O, DMSO	562	90,000	none	MeOH	1
R6029	680.79	F,D,L	DMSO	560	119,000	580	MeOH	
T6006	825.22	F,D,L	DMSO	543	87,000	567	MeOH	12
T6008	728.83	F,D,L	DMSO	582	112,000	600	MeOH	
T6009	811.80	F,D,L	DMSO	583	115,000	603	MeOH	
T6027	481.51	F,D,L	DMSO	541	95,000	567	MeOH	
T6028	481.51	F,D,L	DMSO	541	91,000	567	MeOH	
T30453	510.31	F,D,L	DMSO	503	80,000	510	MeOH	
T30454	658.52	F,D,L	DMSO	544	58,000	570	MeOH	
T30455	684.53	F,D,L	DMSO	589	63,000	617	MeOH	
T30456	710.57	F,D,L	DMSO	625	93,000	640	MeOH	
T34561	664.49	FF,D,L,AA	DMSO	508	70,000	530	pH 7.2	25, 26
T34562	545.37	FF,D,AA	DMSO	596	69,000	608	pH 7.2	25, 27

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

Notes

1. Aqueous stock solutions should be used within 24 hours; long-term storage is NOT recommended.
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. 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)
4. 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.
5. 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.
6. 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.
7. 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.
8. 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)
9. Absorption and fluorescence of fluorescein derivatives are pH dependent. Extinction coefficients and fluorescence quantum yields decrease markedly at pH <7.
10. B2103 spectra are for the unreacted reagent. The thiol adduct has Abs = 493 nm, Em = 503 nm in MeOH.
11. The absorption and fluorescence spectra of BODIPY® derivatives are relatively insensitive to the solvent.
12. Iodoacetamides in solution undergo rapid photodecomposition to unreactive products. Minimize exposure to light prior to reaction.
13. Fluorescence emission of B20340 is relatively weak until the disulfide linkage between its two BODIPY® FL fluorophores is reductively cleaved.
14. B22802 spectral data are for the unreacted reagent. The thiol adduct has Abs = 629 nm, Em = 647 nm in dichloromethane (CH_2Cl_2).
15. Spectra of 2-mercaptoethanol adduct of NBD chloride in MeOH: Abs = 425 nm (EC = 13,000 $cm^{-1}M^{-1}$), Em = 520 nm. NBD fluoride yields the same derivatives as NBD chloride but is more reactive.
16. This product is specified to equal or exceed 98% analytical purity by HPLC.
17. Spectral data of the 2-mercaptoethanol adduct.
18. Spectral data are for the unreacted reagent and are essentially unchanged upon reaction with thiols.
19. Eosin and erythrosin derivatives also exhibit phosphorescence with an emission maximum at ~680 nm. The phosphorescence lifetime is ~1 millisecond for eosin and 0.5 milliseconds for erythrosin. (Biochem J (1979) 183:561, Spectroscopy (1990) 5:20) Fluorescence lifetimes (τ) are 1.4 nanoseconds (QY = 0.2) for eosin and 0.1 nanoseconds (QY = 0.02) for erythrosin. (J Am Chem Soc (1977) 99:4306)
20. QY increases on reaction with thiols; Abs, EC and Em are essentially unchanged. (Anal Biochem (2001) 295:101)
21. F6053 reaction product with dimethylaminoethanethiol has Abs = 376 nm (EC ~8000 $cm^{-1}M^{-1}$), Em ~510 nm in MeOH.
22. Fluorescence emission spectrum shifts to shorter wavelengths in nonpolar solvents.
23. Absorption and fluorescence of Oregon Green® 488 derivatives are pH dependent only in moderately acidic solutions (pH <5).

DATA TABLE 2.2 THIOL-REACTIVE PROBES EXCITED WITH VISIBLE LIGHT—*continued*

24. 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).
25. This product is supplied as a ready-made solution in the solvent indicated under "Soluble."
26. Data for T34561 represents FlAsH complexed with the tetracysteine peptide FLNCCPGCCMEP. (Nat Biotechnol (2005) 23:1308, Nat Protoc (2008) 3:1527) The FlAsH-EDT_2 reagent is essentially nonfluorescent and has Abs = 496 nm (EC = 69,500 $cm^{-1}M^{-1}$) in 0.1 M NaOH. (Nat Protoc (2008) 3:1527)
27. Data for T34562 represents ReAsH complexed with the tetracysteine peptide FLNCCPGCCMEP. (Nat Biotechnol (2005) 23:1308, Nat Protoc (2008) 3:1527) The ReAsH-EDT_2 reagent is essentially nonfluorescent and has Abs = 579 nm (EC = 63,000 $cm^{-1}M^{-1}$) in 0.1 M NaOH. (Nat Protoc (2008) 3:1527)

PRODUCT LIST 2.2 THIOL-REACTIVE PROBES EXCITED WITH VISIBLE LIGHT

Cat. No.	Product	Quantity
A10254	Alexa Fluor® 488 C_5-maleimide	1 mg
A10255	Alexa Fluor® 532 C_5-maleimide	1 mg
A10258	Alexa Fluor® 546 C_5-maleimide	1 mg
A20346	Alexa Fluor® 555 C_2-maleimide	1 mg
A20341	Alexa Fluor® 568 C_5-maleimide	1 mg
A10256	Alexa Fluor® 594 C_5-maleimide	1 mg
A20342	Alexa Fluor® 633 C_5-maleimide	1 mg
A20347	Alexa Fluor® 647 C_2-maleimide	1 mg
A20343	Alexa Fluor® 660 C_2-maleimide	1 mg
A20344	Alexa Fluor® 680 C_2-maleimide	1 mg
A30459	Alexa Fluor® 750 C_5-maleimide	1 mg
B10621	bis-((*N*-iodoacetyl)piperazinyl)sulfonerhodamine	5 mg
B2103	BODIPY® 493/503 methyl bromide (8-bromomethyl-4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-*s*-indacene)	5 mg
B22802	BODIPY® 630/650 methyl bromide (8-bromomethyl-4,4-difluoro-3,5-bis-(2-thienyl)-4-bora-3a,4a-diaza-*s*-indacene)	1 mg
B10250	BODIPY® FL N-(2-aminoethyl)maleimide	5 mg
B20340	BODIPY® FL L-cystine	1 mg
B30466	BODIPY® TMR C_5-maleimide	1 mg
B1355	5-(bromomethyl)fluorescein	10 mg
C20260	4-chloro-7-nitrobenz-2-oxa-1,3-diazole (NBD chloride; 4-chloro-7-nitrobenzofurazan) *FluoroPure™ grade*	100 mg
D20351	4,4-difluoro-3,5-bis(4-methoxyphenyl)-8-(4-maleimidylphenyl)-4-bora-3a,4a-diaza-*s*-indacene (BODIPY® 577/618 maleimide)	5 mg
D6003	*N*-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-yl)methyl)iodoacetamide (BODIPY® FL C_1-IA)	5 mg
D20350	4,4-difluoro-1,3,5,7-tetramethyl-8-(4-maleimidylphenyl)-4-bora-3a,4a-diaza-*s*-indacene (BODIPY® 499/508 maleimide)	5 mg
D6004	*N*-(4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-*s*-indacene-2-yl)iodoacetamide (BODIPY® 507/545 IA)	5 mg
D1521	4-dimethylaminophenylazophenyl-4′-maleimide (DABMI)	100 mg
D2004	*N,N´*-dimethyl-*N*-(iodoacetyl)-*N´*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)ethylenediamine (IANBD amide)	25 mg
E118	eosin-5-maleimide	25 mg
F150	fluorescein-5-maleimide	25 mg
F6053	7-fluorobenz-2-oxa-1,3-diazole-4-sulfonamide (ABD-F)	10 mg
F486	4-fluoro-7-nitrobenz-2-oxa-1,3-diazole (NBD fluoride; 4-fluoro-7-nitrobenzofurazan)	25 mg
I30451	5-iodoacetamidofluorescein (5-IAF)	25 mg
I30452	6-iodoacetamidofluorescein (6-IAF)	25 mg
I9	*N*-((2-(iodoacetoxy)ethyl)-*N*-methyl)amino-7-nitrobenz-2-oxa-1,3-diazole (IANBD ester)	100 mg
L1338	lucifer yellow iodoacetamide, dipotassium salt	25 mg
M6026	1-(2-maleimidylethyl)-4-(5-(4-methoxyphenyl)oxazol-2-yl)pyridinium methanesulfonate (PyMPO maleimide)	5 mg
N20345	NANOGOLD® monomaleimide *special packaging*	5 x 6 nmol
O6010	Oregon Green® 488 iodoacetamide *mixed isomers*	5 mg
O6034	Oregon Green® 488 maleimide	5 mg
Q10257	QSY® 7 C_5-maleimide	5 mg
Q30457	QSY® 9 C_5-maleimide	5 mg
Q20348	QSY® 35 iodoacetamide	5 mg
R6029	Rhodamine Red™ C_2-maleimide	5 mg
T34561	TC-FlAsH™ II In-cell Tetracysteine Tag Detection Kit *green fluorescence* *for live-cell imaging*	1 kit
T34562	TC-ReAsH™ II In-Cell Tetracysteine Tag Detection Kit *red fluorescence* *for live-cell imaging*	1 kit
T34563	TC-FlAsH™ TC-ReAsH™ II In-cell Tetracysteine Tag Detection Kit *with mammalian TC-Tag Gateway® expression vectors* *green fluorescence* *red fluorescence*	1 kit
T6006	tetramethylrhodamine-5-iodoacetamide dihydroiodide (5-TMRIA) *single isomer*	5 mg
T6027	tetramethylrhodamine-5-maleimide *single isomer*	5 mg
T6028	tetramethylrhodamine-6-maleimide *single isomer*	5 mg
T6008	Texas Red® C_2-maleimide	5 mg
T6009	Texas Red® C_5-bromoacetamide	5 mg
T30456	TS-Link™ BODIPY® 630/650 C_5-thiosulfate, sodium salt	5 mg
T30453	TS-Link™ BODIPY® FL C_2-thiosulfate, sodium salt	5 mg
T30454	TS-Link™ BODIPY® TMR C_5-thiosulfate, sodium salt	5 mg
T30455	TS-Link™ BODIPY® TR C_5-thiosulfate, sodium salt	5 mg

2.3 Thiol-Reactive Probes Excited with Ultraviolet Light

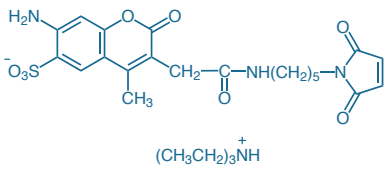

Figure 2.3.1 Alexa Fluor® 350 C_5-maleimide (A30505).

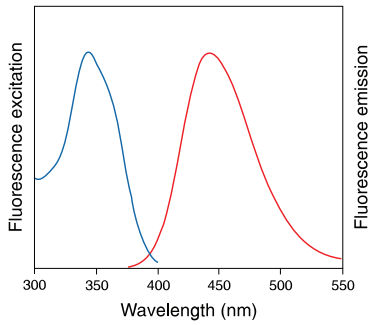

Figure 2.3.2 Fluorescence excitation and emission spectra of Alexa Fluor® 350 goat anti–mouse IgG antibody in pH 8.0 buffer.

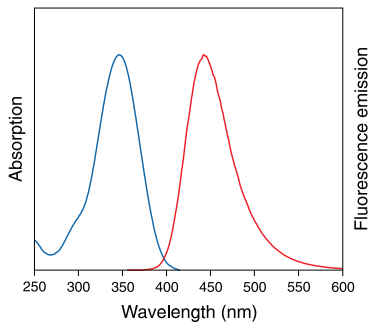

Figure 2.3.3 Absorption and fluorescence emission spectra of 7-amino-4-methylcoumarin in pH 7.0 buffer.

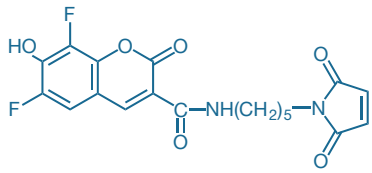

Figure 2.3.4 Pacific Blue™ C_5-maleimide (P30506).

The thiol-reactive dyes described in this section have their longest-wavelength absorption peaks at less than 410 nm (Table 2.2). Typically, these dyes exhibit blue fluorescence and have much weaker absorption than the dyes described in Section 2.2, with extinction coefficients often below 20,000 $cm^{-1}M^{-1}$.

The strong environmental dependence of the emission spectra and quantum yields of several of the dyes—especially the coumarin, benzoxadiazole (NBD probes and ABD-F, Section 2.2), aminonaphthalene (e.g., dansyl) and Dapoxyl® fluorophores—makes some of these thiol-reactive probes useful for investigating protein structure and assembly, following protein transport through membranes and studying ligand binding to receptors.[1]

Coumarin Derivatives

Alexa Fluor® 350 Maleimide

Alexa Fluor® 350 C_5-maleimide (A30505), a thiol-reactive, sulfonated coumarin derivative (Figure 2.3.1), produces protein conjugates that are optimally excited at 346 nm and have bright blue fluorescence emission (Figure 2.3.2) at wavelengths slightly shorter than AMCA or AMCA-X conjugates (Figure 2.3.3) (emission maximum ~442 nm versus 448 nm). The shorter emission maximum of Alexa Fluor® 350 conjugates reduces their spectral overlap with the emission of fluorescein and Oregon Green® 488 dyes.[2] Like our other Alexa Fluor® dyes, Alexa Fluor® 350 C_5-maleimide offers unrivaled brightness and pH-independent fluorescence, as well as water solubility and a low degree of quenching upon conjugation (Section 1.3).

Pacific Blue™ Maleimide

The Pacific Blue™ dye, which is based on the 6,8-difluoro-7-hydroxycoumarin fluorophore (Figure 2.3.4), exhibits bright blue fluorescence, with excitation/emission maxima of ~410/455 nm. Significantly, the pK_a value of this 6,8-difluoro-7-hydroxycoumarin derivative is 2–3 log units lower than that of the corresponding 7-hydroxycoumarin. Thus, the thiol-reactive

Table 2.2 Molecular Probes® thiol-reactive dyes excited with ultraviolet light.

Derivative	Abs *	Em *	Haloacetamide	Maleimide	Other
Alexa Fluor® 350	346	442		A30505	
Anilinonaphthalene †	326	462	IAANS, I7	MIANS, M8	
Benzophenone	282	NA		B1508	
Bimane	375	456	B30500	B30501	
Dibromobimane	394	490			bBBr, D1379 ‡
Diethylaminocoumarin	384	470	DCIA, D404	CPM, D346 ‡ MDCC, D10253	
Dimethylaminocoumarin	376	465	DACIA, D10252	DACM, D10251 ‡	
Dimethylaminonaphthalene †	391	500	badan, B6057		acrylodan, A433
Monobromobimane	394	490			mBBr, M1378 ‡ mBBr, M20381 ‡
Monochlorobimane	394	490			mBCl, M1381MP ‡
Naphthalene †	336	490	IAEDANS, I14		
Pacific Blue™	410	455		P30506	
Pacific Orange™	400	551		P30507	
Phenanthroline	270	NA	P6879		
Pyrene †	339	384	P29 P2007MP	P28	
Stilbene †	329	408	A484	A485	

* Approximate absorption (Abs) and emission (Em) maxima, in nm, for the reagent (if fluorescent) or the fluorescent thiol adduct. † Environment-sensitive fluorophore. ‡ Very weakly fluorescent until reacted with thiols. NA = Not applicable.
More information on thiol-reactive dyes is available at www.invitrogen.com/handbook/labelingchemistry.

Pacific Blue™ C_5-maleimide (P30506) yields conjugates that are strongly fluorescent, even at neutral pH. In addition, Pacific Blue™ conjugates are efficiently excited by the 405 nm spectral line of the violet diode laser developed for fluorescence microscopy and flow cytometry.[3]

Other Coumarin Maleimides and Iodoacetamides

We offer several blue-fluorescent thiol-reactive dialkylcoumarins (Table 2.2), including 7-diethylamino-3-(4′-maleimidylphenyl)-4-methylcoumarin (CPM, D346; Figure 2.3.5) and *N*-(7-dimethylamino-4-methylcoumarin-3-yl)maleimide (DACM, D10251) and the corresponding iodoacetamides DCIA (D404) and DACIA (D10252). The dialkylcoumarin fluorophore is an excellent fluorescence resonance energy acceptor from tryptophan and a good donor to fluorescein, NBD, Alexa Fluor® 488 dye, Green Fluorescent Protein (GFP) and the nonfluorescent QSY® 7, QSY® 9 and QSY® 35 quenchers, making these thiol-reactive coumarins especially valuable for studying protein structure and for detecting protein–membrane interactions.[4–6] Fluorescence emission of the dialkylcoumarin conjugates is moderately sensitive to environment.

Unlike MDCC (described below), which is intrinsically fluorescent, the maleimides CPM and DACM are essentially nonfluorescent until they react with thiols, permitting thiol quantitation without a separation step.[7,8] The environment-sensitive fluorescence of CPM is also a useful indicator of protein folding in thermal-shift assays of ligand- and mutagenesis-dependent protein stability[9] (Monitoring Protein-Folding Processes with Environment-Sensitive Dyes—Note 9.1).

We also offer 7-diethylamino-3-((((2-maleimidyl)ethyl)amino) carbonyl)coumarin (MDCC, D10253; Figure 2.3.6). When conjugated to a mutant phosphate-binding protein, MDCC has proven useful for direct, real-time measurement of inorganic phosphate release during enzymatic reactions.[10,11] Similarly, environment-sensitive fluorescence of MDCC site-specifically attached to *Escherichia coli* ParM nucleotide-binding protein provides the basis for a fluorogenic ADP biosensor.[12]

Pacific Orange™ Maleimide

The thiol-reactive Pacific Orange™ C_5-maleimide (P30507) yields conjugates with excitation/emission maxima of ~400/551 nm, making it ideal for use with violet diode laser–equipped flow cytometers and fluorescence microscopes. Moreover, Pacific Blue™ conjugates (described above with the other coumarins) and Pacific Orange™ conjugates are both excited with the 405 nm spectral line of the violet diode laser and emit at 455 and 551 nm, respectively, facilitating two-color analysis using 405 nm excitation and multiparameter analysis using the other flow cytometer channels.[3]

Pyrene Derivatives

Pyrene Maleimide

Not only is *N*-(1-pyrene)maleimide (pyrene maleimide, P28; Figure 2.3.7) essentially nonfluorescent until it has reacted with thiols, but once excited, pyrene–thiol conjugates can interact to form excited-state dimers (excimers) that emit at longer wavelengths than the excited monomeric fluorophore. Pyrene maleimide conjugates often have very long fluorescence lifetimes (>100 nanoseconds), giving proximal pyrene rings within 6–10 Å of each other ample time to form the spectrally altered excimer (Figure 2.3.8). The excimer-forming capacity of pyrene maleimide can be exploited for detection of conformational changes[13] and subunit assembly[14] of proteins and for analysis of protease activity.[15]

Pyrene Iodoacetamides

Fluorescence of the actin monomer labeled with pyrene iodoacetamide (P29, Figure 2.3.9) has been demonstrated to change upon polymerization, making this probe a widely utilized tool for following the kinetics

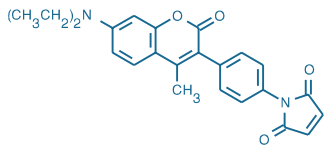

Figure 2.3.5 7-diethylamino-3-(4'-maleimidylphenyl)-4-methylcoumarin (CPM, D346).

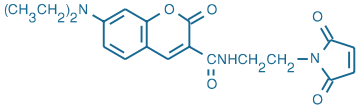

Figure 2.3.6 7-diethylamino-3-((((2-maleimidyl)ethyl) amino)carbonyl)coumarin (MDCC, D10253).

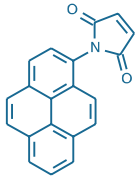

Figure 2.3.7 *N*-(1-pyrene)maleimide (P28).

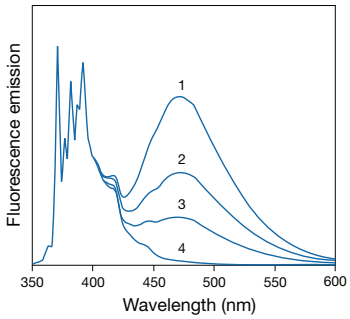

Figure 2.3.8 Excimer formation by pyrene in ethanol. Spectra are normalized to the 371.5 nm peak of the monomer. All spectra are essentially identical below 400 nm after normalization. Spectra are as follows: 1) 2 mM pyrene, purged with argon to remove oxygen; 2) 2 mM pyrene, air-equilibrated; 3) 0.5 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.

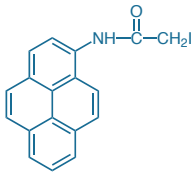

Figure 2.3.9 *N*-(1-pyrene)iodoacetamide (P29).

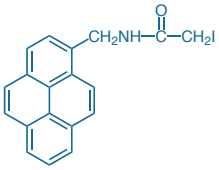

Figure 2.3.10 *N*-(1-pyrenemethyl)iodoacetamide (PMIA amide, P2007MP).

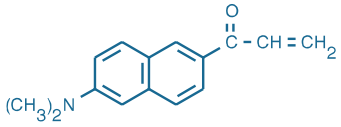

Figure 2.3.11 6-acryloyl-2-dimethylaminonaphthalene (acrylodan, A433).

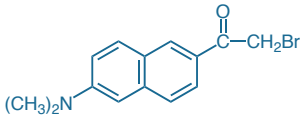

Figure 2.3.12 6-bromoacetyl-2-dimethylaminonaphthalene (badan, B6057).

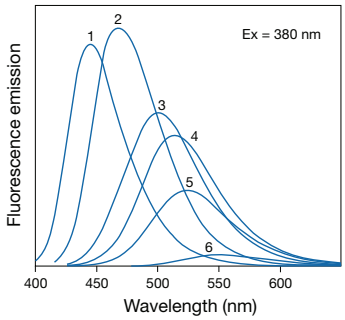

Figure 2.3.13 Fluorescence emission spectra of the 2-mercaptoethanol adduct of badan (B6057) in: 1) toluene, 2) chloroform, 3) acetonitrile, 4) ethanol, 5) methanol and 6) water. Each solution contains the same concentration of the adduct. Excitation of all samples is at 380 nm.

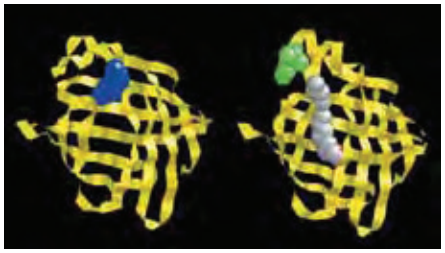

Figure 2.3.14 Ribbon representation of the ADIFAB free fatty acid indicator (A3880). In the left-hand image, the fatty acid binding site of intestinal fatty acid–binding protein (yellow) is occupied by a covalently attached acrylodan fluorophore (blue). In the right-hand image, a fatty acid molecule (gray) binds to the protein, displacing the fluorophore (green) and producing a shift of its fluorescence emission spectrum. Image contributed by Alan Kleinfeld, FFA Sciences LLC, San Diego.

of actin polymerization.[16–19] Conjugates of *N*-(1-pyrenemethyl)iodoacetamide (P2007MP, Figure 2.3.10) have the longest excited-state fluorescence lifetimes (>100 nanoseconds) of all reported thiol-reactive probes. Excimer formation can also be a useful indicator of protein folding.[20]

Naphthalene Derivatives

Acrylodan and Badan

As compared with iodoacetamides or maleimides, acrylodan (A433, Figure 2.3.11) and 6-bromoacetyl-2-dimethylaminonaphthalene (badan, B6057, Figure 2.3.12) generally react with thiols more slowly but they form very strong thioether bonds that are expected to remain stable under conditions required for complete amino acid analysis. The fluorescence emission peak and intensity of these adducts (Figure 2.3.13) are particularly sensitive to conformational changes or ligand binding, making these dyes some of the most useful thiol-reactive probes for protein structure studies.[21–23] For example, the acrylodan conjugate of an intestinal fatty acid–binding protein, ADIFAB (A3880, Section 17.4), is a sensor for free fatty acids [24] (Figure 2.3.14).

IAANS and MIANS

To develop appreciable fluorescence, both the reactive anilinonaphthalenesulfonate iodoacetamide (IAANS, I7; Figure 2.3.15) and maleimide (MIANS, also called Mal-ANS; M8, Figure 2.3.16) must be reacted with thiols that are located in hydrophobic sites. Often, however, buried unsolvated thiol residues are exceptionally reactive, allowing these sites to be selectively modified by these reagents. The environment-sensitive fluorescence properties of the protein conjugates of MIANS and IAANS are similar to those of the structurally related probes 1,8-ANS and 2,6-TNS (A47, T53; Section 13.5). The fluorescence intensity, and to a lesser extent, the emission wavelengths of the conjugates, tend to be very sensitive to substrate binding and folding and unfolding of the protein, as well as the association of the labeled protein with other proteins, membranes or nucleic acids.[25,26]

IAEDANS

The fluorescence of IAEDANS (I14, Figure 2.3.17) is quite dependent upon environment,[27] although less so than that of IAANS and MIANS conjugates. Its conjugates frequently respond to ligand binding by undergoing spectral shifts and changes in fluorescence intensity that are determined by the degree of aqueous solvation. Advantages of this reagent include high water solubility above pH 4 and a relatively long fluorescence lifetime (sometimes >20 nanoseconds, although commonly 10–15 nanoseconds), making the conjugates useful for fluorescence polarization assays [28,29] (Fluorescence Polarization (FP)—Note 1.4). The emission spectrum of IAEDANS overlaps well with the absorption of fluorescein, Alexa Fluor® 488 and Oregon Green® 488 dyes, as well as that of Green Fluorescent Protein (GFP). IAEDANS is an excellent reagent for fluorescence resonance energy transfer (FRET) measurements [30–32] (Fluorescence Resonance Energy Transfer (FRET)—Note 1.2). IAEDANS usually reacts with thiols; however, it has been reported to react with a lysine residue in tropomyosin.[33]

Bimanes for Thiol Derivatization

Monobromobimane and Monochlorobimane

Monobromobimane (M1378, M20381; Figure 2.3.18), which is essentially nonfluorescent until conjugated, readily reacts with low molecular weight thiols,[34] including glutathione.[35] This reagent, originally described by Kosower and colleagues,[36,37] is also useful for detecting the distribution

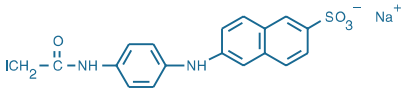

Figure 2.3.15 2-(4′-(iodoacetamido)anilino)naphthalene-6-sulfonic acid, sodium salt (IAANS, I7).

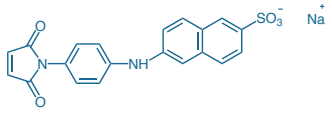

Figure 2.3.16 2-(4′-maleimidylanilino)naphthalene-6-sulfonic acid, sodium salt (MIANS, M8).

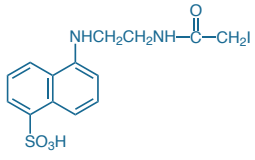

Figure 2.3.17 5-((((2-iodoacetyl)amino)ethyl)amino)naphthalene-1-sulfonic acid (1,5-IAEDANS), I14).

of protein thiols in cells before and after chemical reduction of disulfides.[38] Monobromobimane is reportedly susceptible to inactivation by the disulfide reducing agent TCEP [39] (T2556, Section 2.1). Both monobromobimane and the more thiol-selective monochlorobimane (M1381MP) have been extensively used for detecting glutathione in live cells [40,41] (Section 15.6).

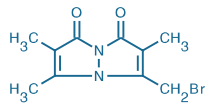

Figure 2.3.18 Monobromobimane (mBBr, M1378).

Dibromobimane

Dibromobimane (D1379, Figure 2.3.19) is an interesting homobifunctional crosslinking reagent for proteins [42–44] because it is unlikely to fluoresce until *both* of its alkylating groups have reacted. It has been used to crosslink thiols in myosin,[45] *Escherichia coli* lactose permease [46] and P-glycoprotein.[47] Despite its short length, dibromobimane is also an effective intermolecular crosslinker in some cases.[48]

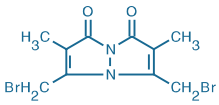

Figure 2.3.19 Dibromobimane (bBBr, D1379).

Bimane Iodoacetamide and Maleimide

Bimane iodoacetamide (B30500, Figure 2.3.20) and bimane C_3-maleimide (B30501, Figure 2.3.21) are blue-fluorescent thiol-reactive fluorophores with excitation/emission maxima of ~375/456 nm. The small size of the bimane fluorophore reduces the likelihood that the label will interfere with the function of the biomolecule, an important advantage for site-selective probes.

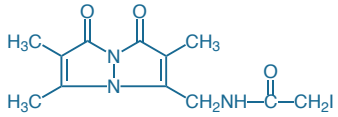

Figure 2.3.20 Bimane iodoacetamide (B30500).

Polar Reagents for Determining Thiol Accessibility

Like IAEDANS (I14), the iodoacetamide and maleimide derivatives of stilbene (A484, A485) have high water solubility and are readily conjugated to thiols. Their combination of high polarity and membrane impermeability makes these polysulfonated dyes useful for determining whether thiol-containing proteins and polypeptide chains are exposed at the extracellular or cytoplasmic membrane surface.

The sulfonated stilbene iodoacetamide (A484, Figure 2.3.22) was used to label single-cysteine mutants of staphylococcal α-hemolysin in order to determine structural changes that occur during oligomerization and pore formation [49] and of the lipid-binding region of *E. coli* pyruvate oxidase in order to detect conformational changes upon substrate binding.[50] Similarly, single-cysteine mutants of *Escherichia coli* Na^+-glutamate transporter GHS have been probed with the sulfonated stilbene maleimide (A485, Figure 2.3.23) to systematically study the topology of this membrane protein.[51]

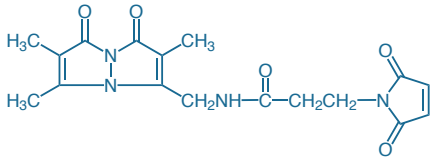

Figure 2.3.21 Bimane C_3-maleimide (B30501).

1,10-Phenanthroline Iodoacetamide for Preparing Metal-Binding Conjugates

Conjugation of *N*-(1,10-phenanthrolin-5-yl)iodoacetamide (P6879, Figure 2.3.24) to thiol-containing ligands confers the metal-binding properties of this important complexing agent on the ligand. For example, the covalent copper–phenanthroline complex of oligonucleotides or nucleic acid–binding molecules in combination with hydrogen peroxide acts as a chemical nuclease to selectively cleave DNA or RNA.[52,53]

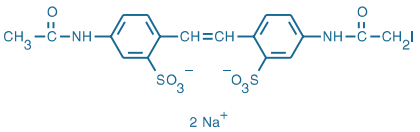

Figure 2.3.22 4-acetamido-4′-((iodoacetyl)amino)stilbene-2,2′-disulfonic acid, disodium salt (A484).

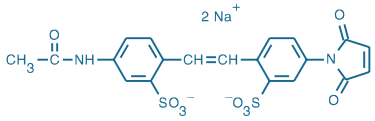

Figure 2.3.23 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid, disodium salt (A485).

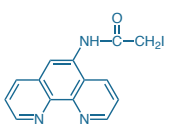

Figure 2.3.24 *N*-(1,10-phenanthrolin-5-yl)iodoacetamide (P6879).

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1. Neuron (2010) 66:170; **2**. Protein Sci (2009) 18:1033; **3**. Cytometry A (2006) 69:1153; **4**. J Biochem (2007) 141:889; **5**. Biophys J (2004) 87:344; **6**. Free Radic Biol Med (2008) 45:971; **7**. Assay Drug Dev Technol (2008) 6:361; **8**. Anal Biochem (1998) 265:8; **9**. Structure (2008) 16:351; **10**. Methods (2005) 37:183; **11**. Biochemistry (1994) 33:8262; **12**. J Biol Chem (2009) 284:33130; **13**. J Biol Chem (2004) 279:945; **14**. Biochemistry (2002) 41:3468; **15**. Anal Biochem (2002) 306:247; **16**. J Biol Chem (2008) 283:7135; **17**. Biochemistry (2007) 46:2707; **18**. Biophys J (2007) 92:2162; **19**. J Biol Chem (2006) 281:10635; **20**. FEBS Lett (1997) 420:63; **21**. J Am Chem Soc (2009) 131:13286; **22**. J Biol Chem (2008) 283:4124; **23**. Biochemistry (2007) 46:106; **24**. J Biol Chem (1992) 267:23495; **25**. J Biol Chem (2008) 283:9060; **26**. Anal Chem (2003) 75:3119; **27**. Biochemistry (1973) 12:4154; **28**. EMBO J (2007) 26:2786; **29**. Biochemistry (1973) 12:2250; **30**. Nat Struct Mol Biol (2008) 15:303; **31**. J Biol Chem (2007) 282:8265; **32**. J Biol Chem (2005) 280:2613; **33**. Eur J Biochem (1990) 187:155; **34**. J Chromatogr B Analyt Technol Biomed Life Sci (2004) 801:359; **35**. J Am Chem Soc (1986) 108:4527; **36**. Methods Enzymol (1987) 143:76; **37**. Proc Natl Acad Sci U S A (1979) 76:3382; **38**. Anal Chem (2006) 78:7959; **39**. Anal Biochem (2003) 318:325; **40**. Nat Protoc (2009) 4:1790; **41**. J Biol Chem (2006) 281:17420; **42**. Anal Biochem (2004) 331:27; **43**. Protein Sci (2001) 10:1293; **44**. Anal Biochem (1995) 225:174; **45**. Proc Natl Acad Sci U S A (2000) 97:1461; **46**. Proc Natl Acad Sci U S A (1996) 93:10123; **47**. J Biol Chem (1999) 274:35388; **48**. J Biol Chem (2006) 281:14163; **49**. FEBS Lett (1994) 356:66; **50**. Biochemistry (1997) 36:11564; **51**. Biochemistry (2007) 46:2326; **52**. Biochemistry (2000) 39:4068; **53**. Bioconjug Chem (1996) 7:413.

DATA TABLE 2.3 THIOL-REACTIVE PROBES EXCITED WITH ULTRAVIOLET LIGHT

Cat. No.	MW	Storage	Soluble	Abs	EC	Em	Solvent	Notes
A433	225.29	L	DMF, MeCN	391	20,000	500	MeOH	1
A484	624.33	F,D,L	H_2O	329	39,000	408	pH 8	2, 3
A485	536.44	F,D	H_2O	322	35,000	411	pH 8	2
A30505	578.68	F,DD,L	H_2O, DMSO	345	17,000	444	pH 7	4
B6057	292.17	F,L	DMF, MeCN	387	21,000	520	MeOH	5
B30500	375.17	F,D,L	DMSO	375	5800	456	MeOH	3
B30501	358.35	F,D,L	DMSO	375	5700	458	MeOH	
D346	402.45	F,D,L	DMSO	384	33,000	469	MeOH	6
D404	490.34	F,D,L	DMSO	384	31,000	470	MeOH	2, 3
D1379	350.01	L	DMF, MeCN	391	6100	see Notes	MeOH	7
D10251	298.30	F,D,L	DMSO	383	27,000	463	MeOH	8
D10252	386.19	F,D,L	DMSO	376	24,000	465	MeOH	3
D10253	383.40	F,D,L	DMSO	419	50,000	466	MeOH	9
I7	504.27	F,D,L	DMF	326	27,000	462	MeOH	2, 3
I14	434.25	F,D,L	pH >6, DMF	336	5700	490	pH 8	3, 10
M8	416.38	F,D,L	DMSO, DMF	322	27,000	417	MeOH	11
M1378	271.11	F,L	DMF, MeCN	398	5000	see Notes	pH 7	7
M1381MP	226.66	F,L	DMSO	380	6000	see Notes	MeOH	7
M20381	271.11	F,L	DMF, MeCN	398	5000	see Notes	pH 7	7, 12
P28	297.31	F,D,L	DMF, DMSO	338	40,000	375	MeOH	13, 14
P29	385.20	F,D,L	DMF, DMSO	339	26,000	384	MeOH	2, 3
P2007MP	399.23	F,D,L	DMSO	341	41,000	377	MeOH	2, 3, 14
P6879	363.16	F,D,L	DMSO	270	28,000	none	$CHCl_3$	3
P30506	406.34	F,DD,L	DMSO	402	40,000	451	pH 9	15
P30507	~800	F,DD,L	DMSO	403	23,000	552	MeOH	

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

Notes

1. Fluorescence of unconjugated A433 is weak, increasing markedly upon reaction with thiols. Em (QY) for the 2-mercaptoethanol adduct are: 540 nm (0.18) in H_2O, 513 nm (0.57) in MeOH, 502 nm (0.79) in EtOH, 468 nm (0.78) in MeCN, 435 nm (0.83) in dioxane. (J Biol Chem (1983) 258:7541)
2. Spectral data of the 2-mercaptoethanol adduct.
3. Iodoacetamides in solution undergo rapid photodecomposition to unreactive products. Minimize exposure to light prior to reaction.
4. Aqueous stock solutions should be used within 24 hours; long-term storage is NOT recommended.
5. Em for 2-mercaptoethanol adduct of B6057: 550 nm in H_2O (pH 7), 523 nm in MeOH, 514 nm in EtOH, 502 nm in MeCN, 469 nm in $CHCl_3$, 457 nm in dioxane, 445 nm in toluene. Abs is relatively independent of solvent.
6. Spectral data are for the 2-mercaptoethanol adduct. The unreacted reagent is nonfluorescent, Abs = 384 nm (EC = 32,000 $cm^{-1}M^{-1}$) in MeOH.
7. Bimanes are almost nonfluorescent until reacted with thiols. For monobromobimane conjugated to glutathione, Abs = 394 nm, Em = 490 nm (QY ~0.1–0.3) in pH 8 buffer. (Methods Enzymol (1987) 143:76, Methods Enzymol (1995) 251:133)
8. Spectral data are for the 2-mercaptoethanol adduct. The unreacted reagent is nonfluorescent, Abs = 381 nm (EC = 27,000 $cm^{-1}M^{-1}$) in MeOH.
9. QY increases on reaction with thiols; Abs, EC and Em are unchanged. (J Chem Soc Perkin Trans I (1994) 2975)
10. The 2-mercaptoethanol adduct of I14 has essentially similar spectral characteristics in aqueous solution. (Biochemistry (1973) 12:4154) Fluorescence lifetime (τ) = 21 nsec when conjugated to myosin subfragment-1. (Biochemistry (1973) 12:2250)
11. Spectral data are for the 2-mercaptoethanol adduct. The unreacted reagent is nonfluorescent, Abs = 443 nm (EC = 13,000 $cm^{-1}M^{-1}$) in MeOH.
12. This product is specified to equal or exceed 98% analytical purity by HPLC.
13. Fluorescence of unreacted P28 is weak. Em data represent the 2-mercaptoethanol adduct.
14. 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.
15. The fluorescence quantum yield of Pacific Blue™ dye in 50 mM potassium phosphate, 150 mM NaCl, pH 7.2, at 22°C is 0.78.

PRODUCT LIST 2.3 THIOL-REACTIVE PROBES EXCITED WITH ULTRAVIOLET LIGHT

Cat. No.	Product	Quantity
A484	4-acetamido-4′-((iodoacetyl)amino)stilbene-2,2′-disulfonic acid, disodium salt	25 mg
A485	4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid, disodium salt	25 mg
A433	6-acryloyl-2-dimethylaminonaphthalene (acrylodan)	25 mg
A30505	Alexa Fluor® 350 C_5-maleimide	1 mg
B30501	bimane C_3-maleimide	5 mg
B30500	bimane iodoacetamide	5 mg
B6057	6-bromoacetyl-2-dimethylaminonaphthalene (badan)	10 mg
D1379	dibromobimane (bBBr)	25 mg
D404	7-diethylamino-3-((4′-(iodoacetyl)amino)phenyl)-4-methylcoumarin (DCIA)	25 mg
D10253	7-diethylamino-3-((((2-maleimidyl)ethyl)amino)carbonyl)coumarin (MDCC)	5 mg
D346	7-diethylamino-3-(4′-maleimidylphenyl)-4-methylcoumarin (CPM)	25 mg
D10252	*N*-(7-dimethylamino-4-methylcoumarin-3-yl)iodoacetamide (DACIA)	10 mg
D10251	*N*-(7-dimethylamino-4-methylcoumarin-3-yl)maleimide (DACM)	10 mg
I7	2-(4′-(iodoacetamido)anilino)naphthalene-6-sulfonic acid, sodium salt (IAANS)	100 mg
I14	5-((((2-iodoacetyl)amino)ethyl)amino)naphthalene-1-sulfonic acid (1,5-IAEDANS)	100 mg
M8	2-(4′-maleimidylanilino)naphthalene-6-sulfonic acid, sodium salt (MIANS)	100 mg
M1378	monobromobimane (mBBr)	25 mg
M20381	monobromobimane (mBBr) *FluoroPure™ grade*	25 mg
M1381MP	monochlorobimane (mBCl)	25 mg
P30506	Pacific Blue™ C_5-maleimide	1 mg
P30507	Pacific Orange™ C_5-maleimide	1 mg
P6879	*N*-(1,10-phenanthrolin-5-yl)iodoacetamide	5 mg
P29	*N*-(1-pyrene)iodoacetamide	100 mg
P28	*N*-(1-pyrene)maleimide	100 mg
P2007MP	*N*-(1-pyrenemethyl)iodoacetamide (PMIA amide)	25 mg