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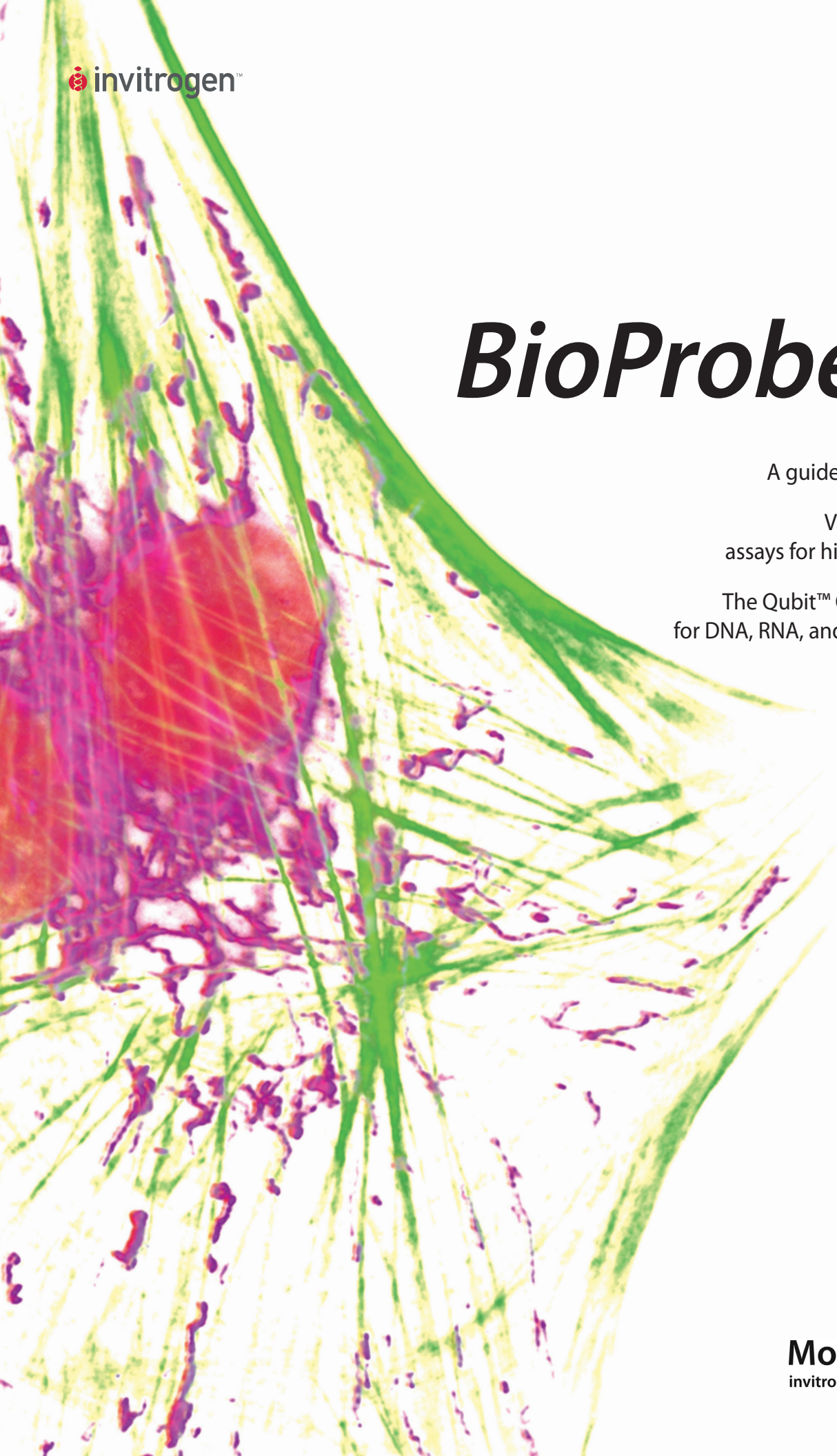


August 2006

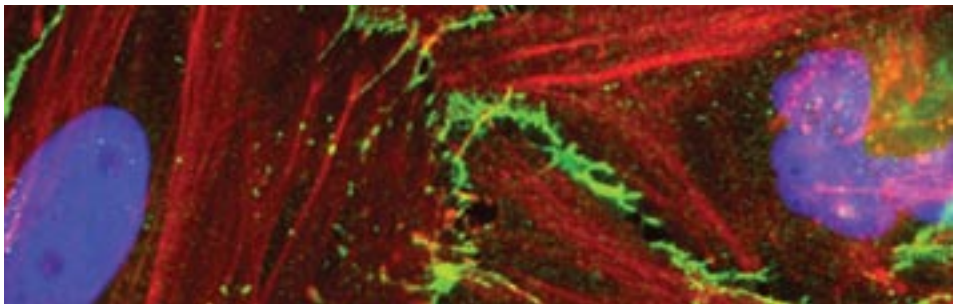
BioProbes 51

A guide to immunodetection

Validated reagents and
assays for high-content screening

The Qubit™ Quantitation Platform
for DNA, RNA, and protein quantitation

Molecular Probes®
invitrogen detection technologies



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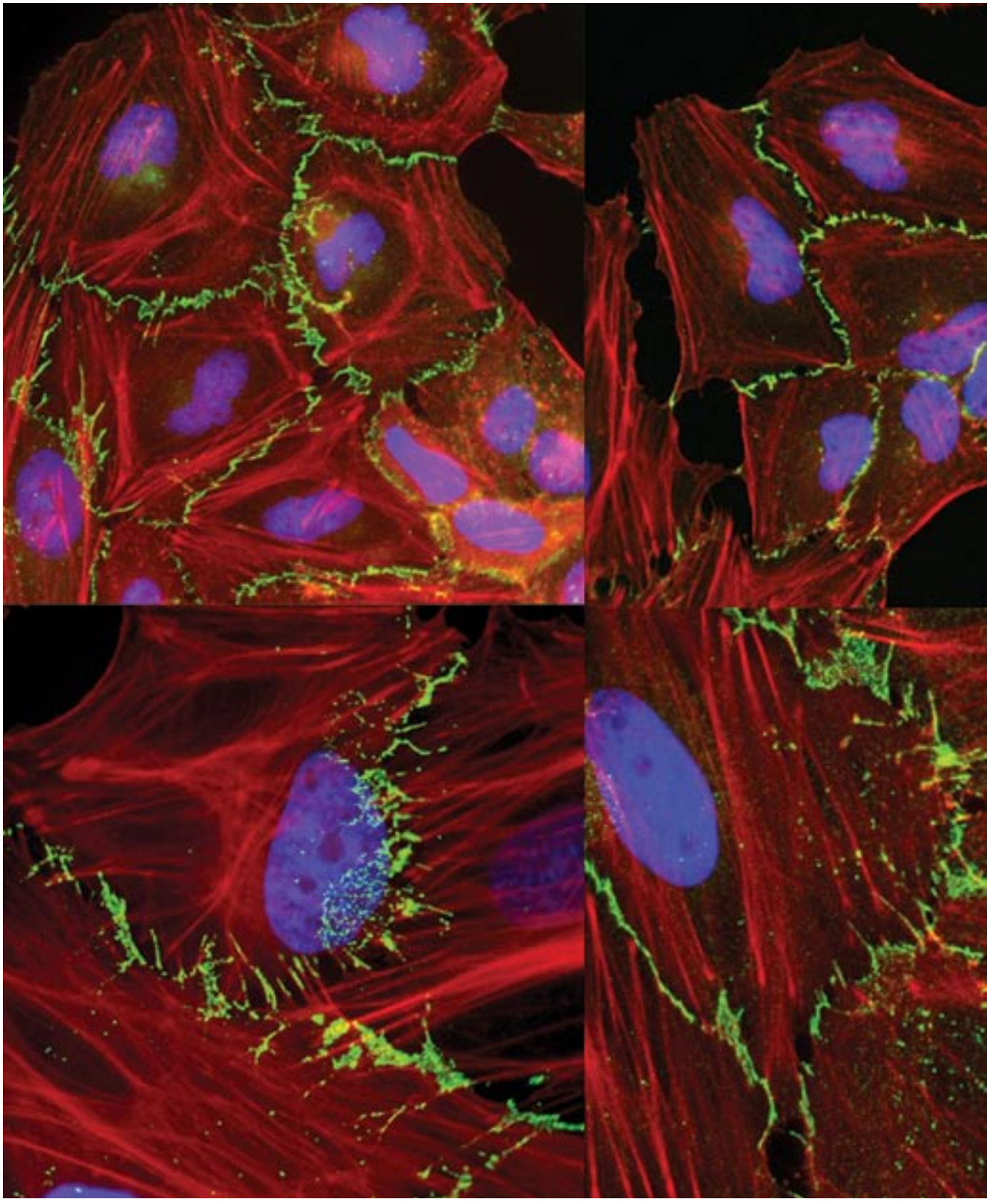

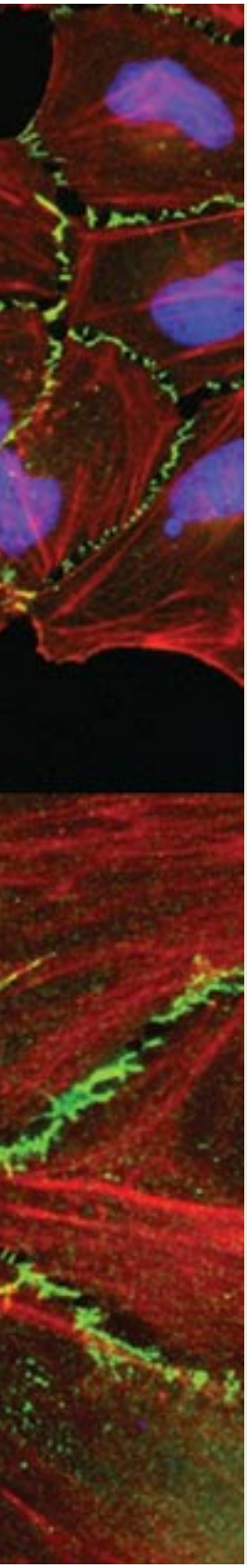

A guide to antibody labeling and detection

CHOICE. BECAUSE THE ONLY PRODUCT YOU REALLY NEED IS THE RIGHT ONE.

Each immunolabeling experiment presents its own challenges. From the initial binding of the primary antibody to the final detection of the fluorophore label—and at every wash step in between—you face optimization protocols that will determine the sensitivity and selectivity of your experiment. A primary antibody present in only small amounts requires specialized techniques for labeling. Likewise, immunodetection of a low-abundance target often entails significant signal amplification steps and extremely low background levels. And because the localization and abundance of the target molecule are critical pieces of information, the fluorophore label you choose for immunodetection often needs to be compatible with fluorescent probes for other cellular structures in a multiplexing protocol. This guide to antibody labeling can help you sift through the options for primary and secondary antibody detection and select the solution that best fits your experiments. →

←

Figure 1—Indirect immunofluorescence staining of adherens junctions. Adherens junctions between highly confluent HeLa cells were labeled using Zymed® mouse anti–α-catenin antibody in conjunction with Alexa Fluor® 488 goat anti–mouse IgG antibody (green). F-actin was labeled with Alexa Fluor® 635 phalloidin, and nuclei were stained with DAPI (blue). After staining, the sample was mounted in ProLong® Gold Antifade Reagent.

finding the primary antibody you need

Achieving quality results in immunodetection begins with primary antibodies that are highly specific for your protein of interest, regardless of your detection methodology. Immunofluorescence microscopy, flow cytometry, immunoprecipitation, western blotting—each of these techniques relies on the specific, high-affinity binding of the primary antibody to its antigen. A misstep here, in the form of a poorly characterized or low-titer antibody, translates into high background levels that only tend to increase through subsequent detection and amplification steps.

With well over 10,000 citations in scientific journal articles, Zymed® antibodies are established reagents for research at the leading edge (Figure 1). Widely published Zymed® primary antibodies include:

- anti–amyloid-β precursor protein [1,2]
- anti–E-cadherin [3,4]
- anti–connexin 43 [5,6]
- anti-occludin [7,8]
- anti–SUMO-1 (GMP-1) [9,10]
- anti–transferrin receptor [11,12]
- anti-ubiquitin [13,14]
- anti–ZO-1 [15,16]

These high-quality antibodies have earned a reputation for highly specific, reproducible binding to their targets and have been validated in multiple research applications. Now that Zymed Laboratories is fully integrated into Invitrogen, you can find these antibodies along with the rest of our extensive portfolio of more than 3,700 primary antibodies online at www.invitrogen.com/antibodies.

options for detecting your primary antibody

With a suitable primary antibody in hand, you must next determine the detection methodology that best fits your application. The multiplexing parameters and instrument platform, as well as the abundance of both the primary antibody and the protein target, will be important in determining whether you should fluorescently label your primary antibody or use a fluorescently labeled secondary antibody for detection. Directly labeled primary antibodies are typically used in flow cytometry applications and secondary detection reagents are more common in imaging experiments; however, there is nothing inherent in either detection method to limit their use with these particular instrument platforms.

Although secondary detection methods can provide both significant signal amplification and additional flexibility for multicolor applications, a primary antibody directly labeled with a fluorophore often produces lower background fluorescence and less nonspecific binding (Figure 2). Furthermore, multiple primary antibodies of the same isotype or derived from the same species can easily be used in the same experiment if they have been directly labeled with compatible fluorophores. However, without the signal amplification step provided by secondary detection methods, it is more important than ever to label the primary antibody with the brightest and most photostable fluorophores available.

And that's where our fluorophores come in. When it comes to choosing a fluorophore for antibody labeling, we offer two very different but equally important options: fluorescent organic dyes, including our Alexa Fluor® dye series (Figure 3) and violet-excited Pacific Blue™ and Pacific Orange™ dyes, and Qdot® semiconductor nanocrystals. With over 12,000 citations in scientific journals, Alexa Fluor® dyes have proven to exhibit superior fluorescence properties in a diverse range of applications. Quantum dots, developed more recently for biological applications,[17–20] are useful when extremely high photostability is paramount, or when you need to perform multicolor analysis using one excitation source.

Your fluorophore choice will ultimately depend on the requirements of your application: What quantity of unlabeled antibody is available for labeling? Do you need to resolve

low-abundance targets? Will the labeled cell or tissue samples be exposed to extraordinary levels of excitation light or archived for long periods of time? Do you need compatible fluorophores for multiplex analysis? The descriptions below of each type of fluorophore can help you find the best match for your experiment.

covalent antibody labeling with Alexa Fluor® dyes

Fluorescent organic dyes, and the Alexa Fluor® dye series in particular, remain the primary workhorses for immunodetection, consistently providing high-quality results in imaging and flow cytometry applications. Tried-and-true Alexa Fluor® dye–labeled primary antibodies have proven superior to antibodies labeled with traditional organic fluorophores such as fluorescein (FITC), tetramethylrhodamine (TRITC), Cy®3, or Cy®5 for immunodetection. Compared to our Qdot® nanocrystals, Alexa Fluor® dyes are significantly smaller in size, which may be an important advantage in some applications where accessibility of the antigen is an issue. In addition, Alexa Fluor® dyes are available with blue emission, a part of the spectrum that Qdot® nanocrystals currently do not cover.

Alexa Fluor® dyes are a series of extremely bright, photostable, pH-insensitive, organic fluorophores that span the spectrum from blue to infrared fluorescence (emission maxima from 421 to 775 nm, Figure 3). These dyes have been carefully selected and optimized to be very water soluble, minimizing dye-to-dye interactions and allowing a higher degree of labeling of a monoclonal or polyclonal primary antibody. If you choose to optimize your own antibody labeling and purification reactions, seventeen Alexa Fluor® dyes are available as amine-reactive fluorophores. We also offer three types of kits for covalently labeling antibodies with Alexa Fluor® dyes—Alexa Fluor® Microscale Protein Labeling Kits, Alexa Fluor® Monoclonal Antibody Labeling Kits, and Alexa Fluor® Protein Labeling Kits, each of which is optimized for labeling a different amount of antibody, from 20 µg to 1 mg (Tables 1 and 2). These labeling kits contain premeasured amine-reactive Alexa Fluor® dyes, along with the reagents and materials needed to quickly and easily purify the resulting antibody conjugates from any unreacted label. Alternatively, custom antibody labeling services are available at probes.invitrogen.com/customantibodies.

covalent antibody labeling with violet-excited Pacific Blue™ and Pacific Orange™ dyes

When excited by the 405 nm spectral line of the violet diode laser, the Pacific Blue™ and Pacific Orange™ dyes brightly fluoresce at 455 and 551 nm, respectively. The nonoverlapping emission spectra of these dyes facilitate two-color analysis using a violet laser–equipped flow cytometer or fluorescence microscope and multiparameter analysis using, for example, green and

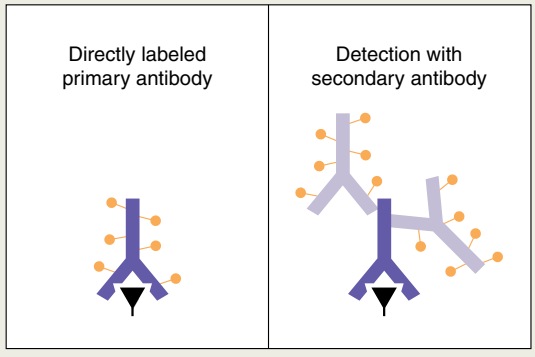

Figure 2—Schematic comparison of directly labeled primary antibody detection and secondary detection. On the left, the primary antibody is directly labeled with fluorophores, then binds to the target. On the right, the primary antibody finds its target and is then recognized by fluorophore-labeled secondary antibodies. Secondary detection offers a greater degree of signal amplification, but at the risk of increased background signal.

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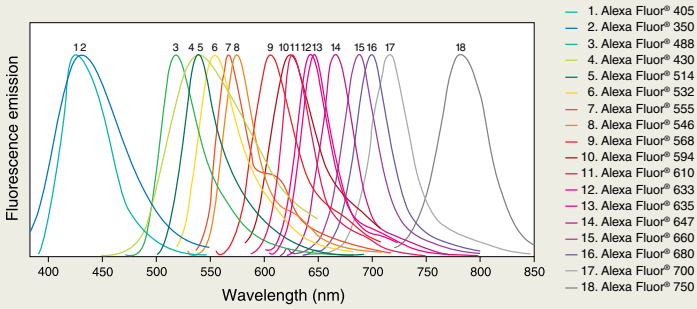

Figure 3—Emission spectra for Alexa Fluor® dyes.

red fluorophores in the other flow cytometer channels. Furthermore, with its strong blue fluorescence, Pacific Blue™ dye is fully compatible with longer-wavelength members of the Alexa Fluor® dye series. We offer the amine-reactive succinimidyl ester of the Pacific Blue™ and Pacific Orange™ dyes, as well as protein labeling kits designed for covalently labeling either 100 µg or 1 mg of IgG antibody and purifying the resulting Pacific Blue™ or Pacific Orange™ conjugate.

10-minute antibody labeling with Zenon® technology

As an alternative to direct chemical labeling of your primary antibody, Zenon® technology provides a versatile, easy-to-use method for noncovalently labeling mouse, rabbit, goat, and human IgG with Alexa Fluor® dyes, Pacific Blue™ dye, or Pacific Orange™ dye, even with submicrogram amounts of starting material. Zenon® technology takes advantage of the immunoselectivity of the antibody binding reaction by forming a complex between an intact primary IgG antibody and a fluorophore-labeled Fab fragment directed against the Fc portion of the IgG. Once prepared, this labeled primary antibody is ready to stain cells or other targets in the same manner as a covalently labeled primary antibody (Figure 4).

As with other directly labeled primary antibodies, Zenon® antibody labeling greatly simplifies time-consuming immunocytochemical

Table 1—Comparison of antibody labeling kits.

Kit	Sample requirements	Number of labelings	Covalent attachment?	Total time	Use
Alexa Fluor® Microscale Protein Labeling Kits	20–100 µg of protein; stabilizing proteins must be removed before labeling	3	Yes	2–3 hours	Optimized for proteins between 10 and 150 kDa, including IgG antibodies
Alexa Fluor® Monoclonal Antibody Labeling Kits	100 µg of IgG; stabilizing proteins must be removed before labeling	5	Yes	1.5–2 hours	Optimized for IgG antibodies
Alexa Fluor® Protein Labeling Kits	1 mg of IgG; stabilizing proteins must be removed before labeling	3	Yes	2–3 hours	Optimized for IgG antibodies
Pacific Blue™ and Pacific Orange™ Monoclonal Antibody Labeling Kits	100 µg of IgG; stabilizing proteins must be removed before labeling	5	Yes	1.5–2 hours	Optimized for IgG antibodies
Pacific Blue™ and Pacific Orange™ Protein Labeling Kits	1 mg of IgG; stabilizing proteins must be removed before labeling	3	Yes	2–3 hours	Optimized for IgG antibodies
Qdot® Antibody Conjugation Kit	600 µg of IgG; stabilizing proteins must be removed before labeling	2	Yes	4–5 hours	Optimized for IgG antibodies
Zenon® IgG Labeling Kit	<1 µg of IgG; compatible with stabilizing proteins such as BSA	10–50	No	10 minutes	Small amounts of IgG; isotype specific; fast noncovalent labeling

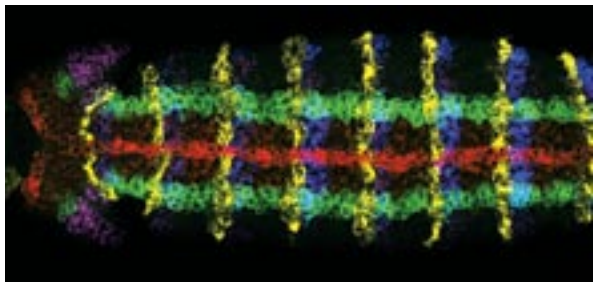

Figure 4—Simultaneous detection of expression of five genes in a whole-mount *Drosophila* embryo by fluorescence *in situ* hybridization (FISH) with five RNA probes. Red: *sog* labeled using aminoallyl UTP and Alexa Fluor® 647 succinimidyl ester. Green: *ind* labeled with DNP, followed by rabbit IgG anti–dinitrophenyl-KLH antibody prelabeled with the Zenon™ Alexa Fluor® 555 Rabbit IgG Labeling Kit. Blue: *en* labeled with biotin and detected with HRP streptavidin and Alexa Fluor® 405 tyramide (TSA Kit #39). Yellow: *wg* labeled with digoxigenin and detected with sheep IgG anti-digoxigenin antibody and Alexa Fluor® 594 donkey anti–sheep IgG antibody. Magenta: *msh* labeled with fluorescein and detected with mouse anti–fluorescein/Oregon Green® IgG2a antibody and Alexa Fluor® 488 goat anti–mouse IgG antibody. Image contributed by Dave Kosman and Ethan Bier, University of California, San Diego.

applications such as the simultaneous use of multiple primary antibodies derived from the same species. For example, Rastaldi and coworkers used the Zenon® Alexa Fluor® 488 and Zenon® Alexa Fluor® 568 Mouse IgG Labeling Kits to allow double-staining of mouse sections with two labeled monoclonal primary antibodies.[21] For simultaneous labeling of tissue sections with two monoclonal or two polyclonal antibodies, Rissman and coworkers used a standard fluorescent secondary antibody to detect one primary antibody and Zenon® labeling to detect the second primary antibody.[22] Additionally, because of the ease of labeling and the small antibody sample requirements, Zenon® antibody labeling provides a means of experimenting with many different dye–antibody combinations in order to find the right one for a particular multicolor flow cytometry or imaging experiment.

Each Zenon® Antibody Labeling Kit (Tables 1 and 2) provides an isotype-specific labeled Fab fragment as well as a blocking reagent to quantitatively prepare labeled primary IgG from less than 1 µg of starting material in under 10 minutes. No pre- or post-labeling purification steps are required. Several of our most popular Zenon® Antibody Labeling Kits for labeling mouse IgG1 antibodies are listed in Table 2; please check our Zenon® product portal at probes.invitrogen.com/zenon for a complete listing of all available Zenon® Antibody Labeling Kits.

Table 2—Alexa Fluor® and Pacific Blue™ Antibody Labeling Kits.

Dye emission color*	Fluorophore	Ex/Em †	Microscale Protein Labeling Kit	Protein Labeling Kit	Monoclonal Antibody Labeling Kit	Zenon® Mouse IgG1 Labeling Kit
Blue	Alexa Fluor® 350	346/442		A10170	A20180	Z25000
Blue	Pacific Blue™	410/455		P30012	P30013	Z25041
Green	Alexa Fluor® 488	495/519	A30006	A10235	A20181	Z25002
Yellow	Pacific Orange™	400/551		P30016	P30014	Z25256
Yellow	Alexa Fluor® 532	531/554		A10236	A20182	Z25003
Orange	Alexa Fluor® 555	555/565	A30007	A20174	A20187	Z25005
Red-orange	Alexa Fluor® 568	578/603		A10238	A20184	Z25006
Red	Alexa Fluor® 594	590/617	A30008	A10239	A20185	Z25007
Far-red	Alexa Fluor® 647	650/668	A30009	A20173	A20186	Z25008
Far-red	Alexa Fluor® 680	679/702		A20172	S30041 ‡	Z25010
Infrared	Alexa Fluor® 750	749/775		S30040 ‡	S30042 ‡	

* The observed fluorescent color depends not only on the emission maximum, but also on the shape of the emission spectrum and the optical filters used. Compared with the Qdot® nanocrystals, the organic dyes generally have broader emission spectra with longer tails, leading to slightly red-shifted colors when observed through longpass optical filters. † Approximate excitation (Ex) and emission (Em) maxima, in nm. ‡ These kits are specifically designed to label antibodies and proteins for use in small animal *in vivo* imaging.

covalent antibody labeling with Qdot® nanocrystals

Qdot® semiconductor nanocrystals (also called quantum dots) are protein-sized particles that generate "tunable" and photostable fluorescence (Figure 5). Their intrinsic brightness is often many times that observed for other classes of fluorophores, and their photostability is many orders of magnitude greater than that associated with traditional fluorescent molecules. These properties enable real-time imaging of low-abundance molecules—even individual receptor molecules on live neurons[23]—as well as long-term imaging under conditions that would lead to the photo-induced deterioration of organic fluorophores. In fact, cells and tissues stained with Qdot® nanocrystals can be archived permanently and re-analyzed with the same level of sensitivity achieved in the first assay.

→

What's more, the peak emission wavelengths of Qdot® nanocrystals are determined by the engineered physical size of the quantum dots. This "tunability" has been exploited to develop a series of Qdot® products that emit different fluorescent colors for multicolor applications. With their broad excitation and narrow emission properties, Qdot® nanocrystals require only a single excitation source (typically <450 nm), enabling multiplex analysis of multiple targets or events in a sample; simple color filtering can be used to resolve the individual signals. Moreover, because these inorganic nanocrystals are particle-based fluorophores, they are electron dense, delivering powerful multimodality for correlative light and electron microscopy and for imaging studies that utilize both fluorescence and X-ray or computerized tomography (CT).

For applications requiring this high level of sensitivity and photostability, we offer the Qdot® Antibody Conjugation Kits, which are available with reactive Qdot® nanocrystals in six different fluorescent colors. These kits contain all the reagents needed to covalently label 600 µg of antibody and purify the labeled conjugate (Tables 1 and 3). For more information on Qdot® nanocrystal products, visit us at probes.invitrogen.com/products/qdot.

alternatively, amplify your signal with a labeled secondary antibody

If preparing a labeled primary antibody either isn't practical or doesn't provide enough sensitivity, then a high-quality secondary antibody can often be the solution. Secondary detection also offers an additional degree of flexibility by allowing different detection modes for any given primary antibody using different fluorescent, biotinylated, or enzyme-conjugated secondary antibodies. Although background levels may increase due to nonspecific binding of both the primary and secondary antibody, a well-chosen labeled secondary antibody often provides significant signal amplification that can overcome any increased background fluorescence.

However, not all commercially available secondary antibodies are created equal. To prepare Molecular Probes™ secondary antibody conjugates, Invitrogen begins with the highest-quality proteins, then optimizes the degree of labeling to achieve the brightest conjugates, and ends with stringent testing on cell samples to ensure low nonspecific

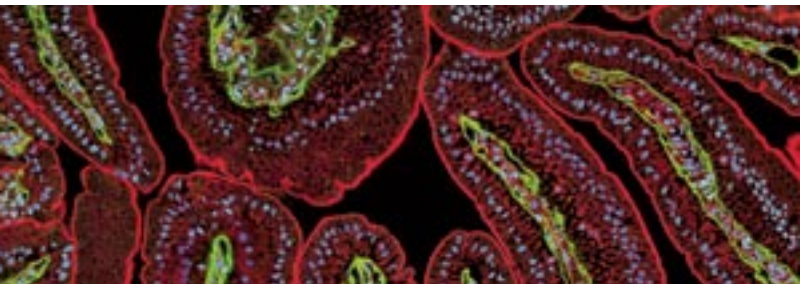

Figure 5—A mouse intestinal section visualized using fluorescent Qdot® nanocrystal conjugates. Actin was labeled with a mouse monoclonal anti-actin antibody and visualized using red-fluorescent Qdot® 655 goat F(ab')$_2$ anti–mouse IgG antibody. Laminin was labeled with a rabbit polyclonal anti-laminin antibody and visualized using green-fluorescent Qdot® 525 goat F(ab')$_2$ anti–rabbit IgG antibody. Nuclei were stained with blue-fluorescent Hoechst 33342. Image contributed by Thomas Deerinck and Mark Ellisman, The National Center for Microscopy and Imaging Research, San Diego, CA.

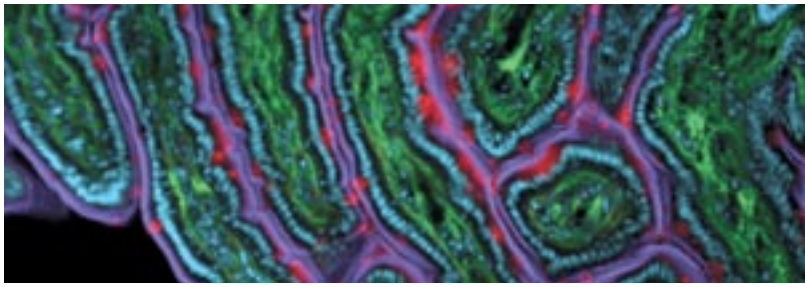

Figure 6—Multicolor fluorescence labeling of a mouse intestine cryosection. Basement membranes were labeled with a chicken IgY anti-fibronectin antibody and Alexa Fluor® 488 goat anti–chicken IgG antibody (green). Goblet cells and crypt cells were labeled with Alexa Fluor® 594 wheat germ agglutinin (red). The microvillar brush border and smooth muscle layers were visualized with Alexa Fluor® 680 phalloidin (pseudocolored purple). The section was counterstained with DAPI (blue).

binding and high specific staining. While all of our secondary antibodies are affinity purified and adsorbed against the sera of a number of species to minimize crossreactivity, we also offer highly cross-adsorbed goat anti–mouse IgG and goat anti–rabbit IgG antibodies for multilabeling experiments in which extremely low background levels are critical.

Fluorophore choice for your labeled secondary antibody is again dependent on the requirements of your application. The same factors discussed previously for choosing a primary antibody label also apply to choosing a secondary antibody label. Invitrogen provides one of the largest selections of fluorescent secondary antibodies available anywhere, including an extensive set of Molecular Probes™ secondary antibodies labeled with an Alexa Fluor® dye, the violet-excited Pacific Blue™ or Pacific Orange™ dyes, or a Qdot® nanocrystal. Alexa Fluor® dye conjugates outperform conventional fluorescent secondary reagents across the spectrum and are rapidly becoming the preferred secondary reagents in all fluorescence-based immunoassays (Figure 6). The brightly fluorescent Pacific Blue™ and Pacific Orange™ dye conjugates not only provide additional color choices but also a means of performing two-color immunochemical analysis using the violet diode laser (see page 23 for a complete product list). Qdot® secondary antibody conjugates are ideal for imaging experiments requiring very long integration times to achieve the desired sensitivity (for example, when detecting very low-abundance targets), and for experiments that call for prolonged storage with repeated analysis of the specimen (Figure 5).

Tables 4 and 5 show a sampling of our most popular secondary antibody conjugates; please see probes.invitrogen.com for a complete list of our species- and isotype-specific anti-IgG and species-specific anti-IgM antibodies, each conjugated to one of more than 40 fluorophores.

Table 3—Qdot® antibody labeling kits.

Dye emission color *	Fluorophore	Ex/Em †	Qdot® Antibody Conjugation Kit
Green	Qdot® 525	<450/525	Q22041MP
Yellow-green	Qdot® 565	<450/565	Q22031MP
Yellow	Qdot® 585	<450/585	Q22011MP
Red-orange	Qdot® 605	<450/605	Q22001MP
Far-red	Qdot® 655	<450/655	Q22021MP
Far-red	Qdot® 705	<450/705	Q22061MP
Infrared	Qdot® 800	<450/800	Q22071MP

***** The observed fluorescent color depends not only on the emission maximum, but also on the shape of the emission spectrum and the optical filters used. Compared with the Qdot® nanocrystals, the organic dyes generally have broader emission spectra with longer tails, leading to slightly red-shifted colors when observed through longpass optical filters.
† Approximate excitation (Ex) and emission (Em) maxima, in nm. Qdot® antibody conjugates absorb efficiently anywhere below approximately 450 nm.

in conclusion

We know that no two immunodetection applications are the same and that the reagents you choose can be critical to your research. If you need more information than that provided in this guide, contact the expert team of scientists in our Technical Support Department to discuss your particular requirements, or visit us at probes.invitrogen.com for more detailed product information. ■

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Table 4—A sampling of available Alexa Fluor® dye–labeled secondary antibody conjugates.

Antibody	Host	Alexa Fluor® 350	Alexa Fluor® 488	Alexa Fluor® 532	Alexa Fluor® 555	Alexa Fluor® 568	Alexa Fluor® 594	Alexa Fluor® 647	Alexa Fluor® 680	Alexa Fluor® 750
Anti–mouse IgG	Goat	A11045 A21049*	A11001 A11029*	A11002	A21422 A21424*	A11004 A11031*	A11005 A11032*	A21235 A21236*	A21057 A21058*	A21037
Anti–mouse IgG	Rabbit	A21062	A11059		A21427	A11061	A11062	A21239	A21065	
Anti–mouse IgG	Donkey		A21202		A31570		A21203	A31571		
Anti–rabbit IgG	Goat	A11046 A21068*	A11008 A11034*	A11009	A21428 A21429*	A11011 A11036*	A11012 A11037*	A21244 A21245*	A21076 A21109*	A21039
Anti–rabbit IgG	Donkey		A21206		A31572		A21207	A31573		
Anti–chicken IgG	Goat		A11039		A21437	A11041	A11042	A21449		
Anti–goat IgG	Rabbit		A11078		A21431	A11079	A11080	A21446	A21088	
Anti–goat IgG	Donkey		A11055		A21432	A11057	A11058	A21447	A21084	
Anti–rat IgG	Goat	A21093	A11006		A21434	A11077	A11007	A21247	A21096	
Anti–sheep IgG	Donkey	A21097	A11015		A21436	A21099	A11016	A21448	A21102	

* These antibodies have been cross-adsorbed against additional species to further reduce the possibility of nonspecific interactions. Don't see what you need? Additional hosts, targets, anti-IgM antibodies, and isotype-specific anti-mouse antibodies can be found at probes.invitrogen.com.

Table 5—Qdot® nanocrystal secondary antibody conjugates.*

Antibody	Qdot® 525	Qdot® 565	Qdot® 585	Qdot® 605	Qdot® 655	Qdot® 705	Qdot® 800
Anti–mouse IgG	Q11041MP	Q11031MP	Q11011MP	Q11001MP	Q11021MP	Q11061MP	Q11071MP
Anti–rabbit IgG	Q11441MP	Q11431MP	Q11411MP	Q11401MP	Q11421MP	Q11461MP	Q11471MP
Anti–rat IgG		Q11631MP		Q11601MP	Q11621MP		
Anti–human IgG		Q11231MP		Q11201MP	Q11221MP		
Anti–goat IgG					Q11821MP		
Anti–chicken IgG		Q14431MP			Q14421MP		

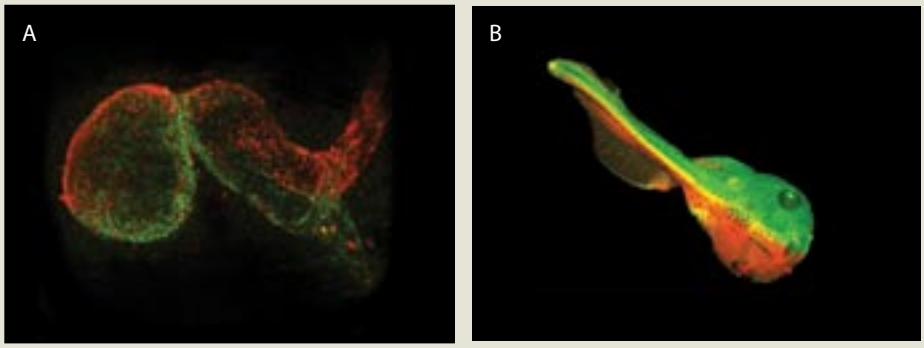
* Except for the Qdot® 655 anti–goat IgG antibody, which is offered as labeled rabbit $F(ab')_2$ fragments, Qdot® nanocrystal secondary antibody conjugates are available as labeled goat $F(ab')_2$ fragments.

JOURNAL HIGHLIGHT

Left–right lineage analysis of the embryonic *Xenopus* heart reveals a novel framework linking congenital cardiac defects and laterality disease

Ramsdell, A.F., Bernanke, J.M., and Trusk, T.C. (2006) *Development* 133:1399 (in press).

How does left–right axis determination affect cardiac development? The dramatic rise in congenital heart defects observed in diseases of body axis symmetry suggests that heart development is strongly influenced by the "handedness" of developmental processes. Cardiac development proceeds from several sets of cell clusters that are arranged bilaterally on either side of the embryonic midline; defective contributions of cells from this bilateral distribution (or "left–right (LR) axial patterning") are strongly associated with heart malformations. Ramsdell and coworkers have proposed that the potential for these cell clusters to correctly develop into cardiac structures is intimately tied to the integrity of their LR axial patterning. The authors investigated this hypothesis using experiments designed to track cardiac development via the administration of fluorescent tracers. *Xenopus* embryos were injected bilaterally with Oregon Green® 488 and Alexa Fluor® 647 dextran conjugates; cardiac development was followed in normal embryos and compared to those in which laterality defects had been induced. Their results revealed clearly defined patterns of lineage marker targeting, and confirmed that certain cardiac structures develop exclusively from red cells, others from green cells, and still others from a mixture of both. Their observations of red–green distribution in the hearts of laterality-defective embryos were consistent with the phenotypic imperfections typically observed in the major types of symmetry disease. Significantly, they observed a strong correlation between left-side defects and malformation of the interatrial septum (IAS), in good agreement with clinical observations of IAS defects in patients with heterotaxy (characterized by a mixture of normal and abnormal organ symmetry). The authors also observed a relationship between expression of *Pitx2c*—a gene normally expressed in left-derived cells but not right-derived—and certain axial pattern defects, pointing to the likelihood that the dose and/or duration of expression this gene may influence at least some cases of abnormal cardiac development.



Left–right lineage-labeled whole-mount heart and embryo. The left and right halves of 4-cell stage *Xenopus laevis* embryos were microinjected with Oregon Green® 488 dextran (green) and Alexa Fluor® 647 dextran (red), respectively. When the labeled embryos reached stages 45–46 (~5 days), the embryos were fixed and confocal images were collected; a single optical section of the heart (A) and a dorsal view of a labeled embryo (B) are shown. Images contributed by Ann F. Ramsdell, Jayne M. Bernanke, and Thomas C. Trusk, University of South Carolina School of Medicine, and Medical University of South Carolina. Image (B) is reprinted from *Development* 133:1399 (2006) by permission of The Company of Biologists Ltd.

Product	Quantity	Cat. no.
dextran, Oregon Green® 488 *10,000 MW, anionic, lysine fixable*	5 mg	D7171
dextran, Alexa Fluor® 647 *10,000 MW, anionic, fixable*	2 mg	D22914

Reagents and assays for high-content screening

TOOLS FOR IMAGE SEGMENTATION AND ASSAYS FOR CYTOTOXIC EFFECTS ON LIPID METABOLISM.

Beginning with van Leeuwenhoek's first glimpse of bacterial cells in the 17th century, researchers have used microscopes to watch cells grow and divide, differentiate, respond to stimuli, and die. Although new technologies have enabled scientists to tease apart molecular events at cellular and subcellular levels, directly imaging cells remains one of the best methods for examining biological systems. Until recently, optical microscopy was a labor-intensive process; low-throughput, largely qualitative analysis was the norm. But with the advent of high-throughput imaging and analysis—including high-content screening (HCS) applications—researchers can now examine multiple cellular targets in a large number of individually imaged cells and quantitatively assess the data.

applying automation to cellular imaging

Image processing and analysis is about turning images into numeric descriptors, a computation-intensive process. However, with the exponential increase in the processing power of computers, all steps of cellular and tissue image acquisition, processing, and analysis can be automated. HCS (also known as HCA, high-content analysis) allows researchers to examine multiple cellular targets and parameters as well as cellular response to a wide variety of treatments, greatly expanding the capabilities of high-throughput screening (HTS) assays traditionally employed in the drug discovery field. HCS also provides a way to study cellular events occurring in a relatively small portion of the cell population, an advantage that would be difficult, if not impossible, to achieve using other methods.

HCS technology has been adopted by drug discovery researchers and used in toxicological profiling, target validation, secondary screening, and even some primary screening. This technology has emerged as an important tool for the discovery and characterization of cellular events and signaling pathways, and is especially useful when combined with technologies such as recombinant biosensors or RNAi.

new tools for image segmentation and cell identification

Many HCS assays and analysis algorithms rely on the strong blue-fluorescent signals of nuclear stains such as Hoechst 33342, Hoechst 33358, or DAPI for autofocusing and for identifying individual cells in microplates. Though well established in HCS assays, these UV-excitable nuclear stains have two significant limitations. First, the HCS reader is limited by its light-source configuration. For example, laser-based systems without a UV light source cannot efficiently excite the blue-fluorescent Hoechst and DAPI nuclear stains. Second, the software algorithms used in many HCS readers identify cells based on the stained nucleus as a landmark. The cytoplasmic region is then extrapolated from this starting point. For many applications, cell identification based on nuclear staining alone is not sufficient because the cytoplasmic region that is assigned by the algorithm does not match that defined by the actual cell boundaries. The inadequacies of nuclear stains are apparent when the cyto-

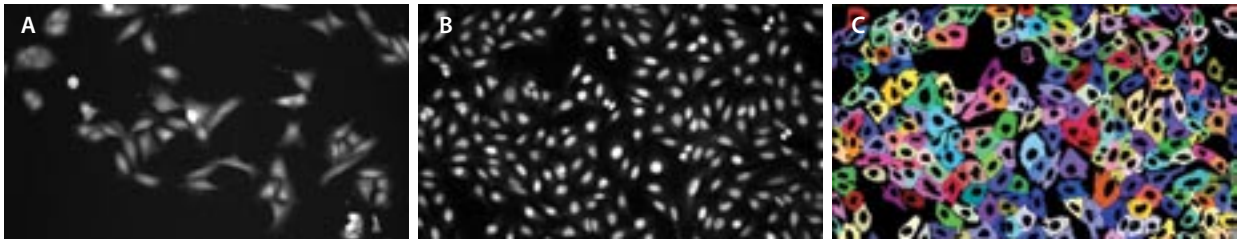


Figure 1—Segmentation of CellMask™ Red cytoplasmic/nuclear stained cells. Image of human osteosarcoma cells (U-2 OS) fixed with formaldehyde and stained with 1 µM (A) and 5 µM (B) HCS CellMask™ Red Cytoplasmic/Nuclear Stain. Image (C) shows the result of segmentation (based on the staining pattern from image B) using a Pathway HT™ imaging system (BD Biosciences). Image (B) is shown at reduced intensity compared to image (A) to illustrate the prominent nuclear staining observed when the stain is used at the higher concentration.

plasm needs to be more accurately identified for detailed analysis of cytoplasmic features revealed in the same or other fluorescence channels.

To meet the need for a cytoplasmic stain–based segmentation tool, Invitrogen has developed HCS CellMask™ Cytoplasmic/Nuclear Stains. Key features include:

- validated reagents for image segmentation on HCS instruments
- staining of the entire cell, including the cytoplasm and nucleus
- choice of red-, blue-, or deep red–fluorescent stains for multiplexing flexibility

a no-wash, red cytoplasmic/nuclear stain

HCS CellMask™ Red Cytoplasmic/Nuclear Stain is designed for use with formaldehyde-fixed mammalian cell lines and produces fluorescent labeling of the entire cell. This red-fluorescent stain (excitation/emission maxima ~622/645 nm) is simply included in the keeping buffer (such as PBS) in the last step of microplate processing; no washing is required. The staining pattern of HCS CellMask™ Red Stain can be modulated by adjusting the concentration used to label cells. This property can easily be exploited to achieve a labeling pattern that better fits the design of particular image analysis algorithms. At low concentrations, such as 0.5 µM, this probe labels the entire cell (Figure 1A). This staining pattern is easily processed by image analysis algorithms that make use of both nuclear labeling and faint cytoplasmic labeling. As the probe concentration is increased (up to 5 µM), the nuclei become more prominently labeled than the cytoplasm (Figure 1B). Figure 1C shows the result of segmentation (based on the staining pattern from Figure 1B).

blue and deep red cytoplasmic/nuclear stains

HCS CellMask™ Blue and HCS CellMask™ Deep Red Stains produce staining of the entire cell, which can be used for autofocusing and cell identification as well as for characterization of overall morphological changes to the cells. The blue- and deep red–fluorescent stains (excitation/emission maxima ~346/442 and ~650/665 nm, respectively) produce similar staining patterns, differing only in their fluorescence excitation and emission properties. Designed for use with formaldehyde-fixed mammalian cells, both dyes are compatible with detergent extraction and can be used to label cells immediately after formaldehyde fixation or in the last step of multiplexing protocols.

image-based assays for cytotoxic effects on lipid metabolism

The detection and understanding of pre-lethal mechanisms in toxicological profiling and compound screening are extremely important components of the drug discovery process. The cationic amphiphilic drugs are among the most prominent examples of drugs that impact lipid metabolism of cells.[1,2] These drugs tend to become enriched in lysosomes to high concentrations and inhibit the normal metabolism of phospholipids. This in turn causes the intracellular accumulation of phospholipids and the formation of lamellar bodies (phospholipidosis). Other drug classes more adversely affect various aspects of neutral lipid metabolism, leading to the cytoplasmic accumulation of neutral lipid as lipid droplets or globules (steatosis).

Invitrogen has released the first in a series of HCS products designed for image-based characterization of drug effects on cellular physiology. The HCS LipidTOX™ Green Phos-

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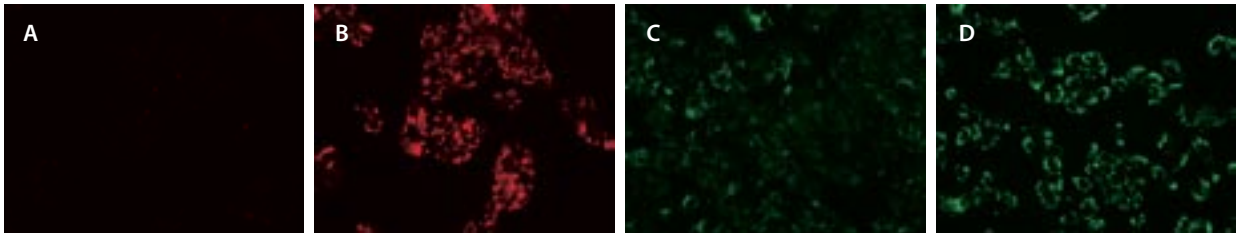


Figure 2—Phospholipidosis and steatosis detection in Hep G2 cells (human hepatocellular carcinoma) using the HCS LipidTOX™ Phospholipidosis and Steatosis Detection Kit. Hep G2 cells were left untreated (A, C), treated with 30 µM propranolol (B), or treated with 30 µM cyclosporin A (D) for 48 hours, then labeled with LipidTOX™ Red Phospholipid Stain (A, B) or LipidTOX™ Green Neutral Lipid Stain (C, D) according to the protocol supplied with the kit. A dramatic increase in phospholipid levels (B) or neutral lipid levels (D) is observed in response to drug treatments. This figure depicts phospholipidosis and steatosis staining as separate experiments. The LipidTOX™ probes can also be used sequentially on the same cell sample.

pholipidosis Detection Kits detect the accumulation of phospholipids in the cytoplasm in response to test-compound incubation, while the HCS LipidTOX™ Phospholipidosis and Steatosis Detection Kits provide simultaneous detection of phospholipidosis and steatosis in the same cell. Key features of the kits include:

- ready-to-use formulations—no organic solvents or sonication required
- designed for fixed end point workflows that incorporate formaldehyde fixation
- validated HCS assays for cytotoxic effects on lipid metabolism

phospholipidosis and steatosis detection kits

The HCS LipidTOX™ Green Phospholipidosis Detection Kit includes LipidTOX™ Green Phospholipid Stain (excitation/emission maxima ~495/525 nm) for detection of phospholipidosis, Hoechst 33342 (excitation/emission ~352/461 nm) for nuclear labeling, and propranolol, which serves as a positive control for the induction of phospholipidosis in mammalian cells.

The HCS LipidTOX™ Phospholipidosis and Steatosis Detection Kit contains LipidTOX™ Red Phospholipid Stain (excitation/emission maxima ~595/615 nm) for detection of phospholipidosis (Figure 2A and B) and LipidTOX™ Green Neutral Lipid Stain (excitation/emission ~495/505 nm) for detection of steatosis (Figure 2C and D). This kit also provides Hoechst 33342 for nuclear labeling, and the positive-control compounds propranolol and cyclosporin A for induction of phospholipidosis and steatosis, respectively. After cells are incubated with the red-fluorescent phospholipid stain and the test compound, cells are fixed with formaldehyde and the green-fluorescent neutral lipid stain is applied. All LipidTOX™ Kits are available in 2-plate or 10-plate sizes, which provide sufficient reagents for 240 or 1,200 assays, respectively.

Invitrogen HCS applications: more content

Invitrogen is committed to providing the research community with tools and validated workflow solutions for high-content screening and analysis. For more information about HCS products and applications, visit us at probes.invitrogen.com. ■

References

1. Halliwell, W.H. (1997) *Toxicol. Pathol.* 25:53.
2. Reasor, M.J. (1989) *Toxicol. Appl. Pharmacol.* 97:47.

Product	Quantity	Cat. no.
HCS CellMask™ Red Cytoplasmic/Nuclear Stain *5 mM solution in DMSO* *for high-content screening* *for cellular imaging*	125 µl	H32711
HCS CellMask™ Blue Cytoplasmic/Nuclear Stain *for high-content screening* *for cellular imaging*	1 set	H34558
HCS CellMask™ Deep Red Cytoplasmic/Nuclear Stain *for high-content screening* *for cellular imaging*	1 set	H34560
HCS LipidTOX™ Green Phospholipidosis Detection Kit *for high-content screening* *for cellular imaging* *2-plate size*	1 kit	H34155
HCS LipidTOX™ Green Phospholipidosis Detection Kit *for high-content screening* *for cellular imaging* *10-plate size*	1 kit	H34156
HCS LipidTOX™ Phospholipidosis and Steatosis Detection Kit *for high-content screening* *for cellular imaging* *2-plate size*	1 kit	H34157
HCS LipidTOX™ Phospholipidosis and Steatosis Detection Kit *for high-content screening* *for cellular imaging* *10-plate size*	1 kit	H34158

Injectable contrast reagents for *in vivo* imaging

SAIVI™ ALEXA FLUOR® INJECTABLE CONTRAST AGENTS ARE POWERFUL TOOLS FOR VASCULAR IMAGING.

Macromolecules that accumulate in tumors via the enhanced permeability and retention (EPR) effect are useful for detecting cancerous tissue in *in vivo* imaging applications. The serum proteins transferrin and albumin are widely employed as macromolecular carriers for tumor detection,[1,2] and have been effectively used to transport cytostatic agents into tumors. By conjugating these macromolecules to Molecular Probes™ long-wavelength Alexa Fluor® 680 and Alexa Fluor® 750 dyes, Invitrogen has provided optical imaging researchers with powerful tools for cancer research.

Injectable SAIVI™ Alexa Fluor® 680 and Alexa Fluor® 750 conjugates of bovine serum albumin and human serum transferrin accumulate in tumors, increasing tumor visibility in small animal *in vivo* imaging applications. These imaging reagents are also useful for detecting the vascular changes associated with inflammatory disease (e.g., arthritis). SAIVI™ Injectable Contrast Agents have been optimized for emission intensity and tested by *in vivo* imaging after injection in disease models established in mice; no signs of acute or long-term toxicity were observed. These agents offer significant performance benefits, including:

- bright and photostable near-infrared Alexa Fluor® dye fluorescence
- choice of two serum proteins with known efficacy in tumor targeting
- simple injectable delivery format ■

References

1. Matsumura, Y. and Maeda, H. (1986) *Cancer Res.* 46:6387.
2. Peters, Theodore, Jr. *All About Albumin: Biochemistry, Genetics, and Medical Applications*, Academic Press, New York (1996).

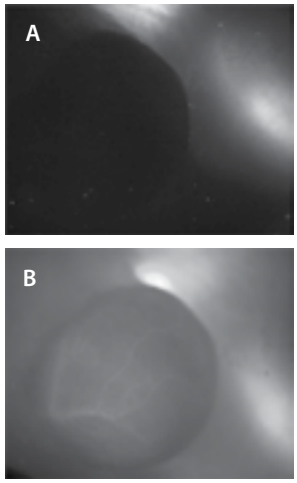


Figure 1—Highlighting tumor vasculature in a human cancer xenograft using *in vivo* imaging. Prior to injection of SAIVI™ Alexa Fluor® 680 Injectable Contrast Agent, the vasculature is not visible in the near infrared (A). 60 minutes after injection, the agent clearly delineates the blood vessels (B). The animal was imaged using the Maestro™ In-Vivo Fluorescence Imaging System (Cambridge Research and Instrumentation) with 687 nm excitation and 740–950 nm bandpass emission.

Product	Quantity	Cat. no.
SAIVI™ Alexa Fluor® 680 Injectable Contrast Agent *bovine serum albumin*	1 ml	S34788
SAIVI™ Alexa Fluor® 750 Injectable Contrast Agent *bovine serum albumin*	1 ml	S34789
SAIVI™ Alexa Fluor® 680 Injectable Contrast Agent *human serum transferrin*	1 ml	S34790
SAIVI™ Alexa Fluor® 750 Injectable Contrast Agent *human serum transferrin*	1 ml	S34791

The Qubit™ Quantitation Platform

A COMPLETE, INTEGRATED SOLUTION FOR ALL YOUR LAB QUANTITATION NEEDS.

The Qubit™ Quantitation Platform pairs the power and simplicity of the Qubit™ Fluorometer with the unmatched performance of Quant-iT™ Assay Kits (Table 1); together, they can eliminate a major source of uncertainty from your workflow. This powerful pairing offers:

- selective quantitation—more accurate than UV absorbance readings
- high sensitivity—use as little as 1 µl of sample for quantitation
- intuitive integrated platform—sophisticated quantitation in 5 minutes or less

the best reagents

Quant-iT™ Assay Kits use advanced Molecular Probes™ fluorophores that become fluorescent upon binding to DNA, RNA, or protein. The selectivity of these interactions ensures more accurate results than can be obtained with UV absorbance readings; Quant-iT™ Assay Kits only report the concentration of the molecule of interest, not sample contaminants (Figure 1). More accurate quantitation means a better chance for success in downstream applications. Quant-iT™ Assay Kits are up to 1000 times as sensitive as UV absorbance readings, so as little as 1 µl of your sample is all that's needed to get accurate, reliable quantitation. There are Quant-iT™ Assay Kits for all your quantitation needs:

- Quant-iT™ dsDNA Kits—for sequencing samples, genomic DNA samples, and routine cloning experiments
- Quant-iT™ RNA Kit—for microarray experiments, real-time PCR samples, and northern blots

Table 1—Quant-iT™ Assay Kits.

Product	Features	Concentration †	Applications
Quant-iT™ dsDNA Assay Kit, High Sensitivity, 1000 assays *0.2–100 ng*	Selective for low concentration dsDNA; can be used to quantitate dsDNA in the presence of free nucleotides, RNA, and protein	10 pg/µl to 100 ng/µl dsDNA	Genomic DNA, viral dsDNA, PCR products, genotyping
Quant-iT™ dsDNA Assay Kit, Broad Range, 1000 assays *2–1000 ng*	Selective for high concentration dsDNA; can be used to quantitate dsDNA in the presence of free nucleotides, RNA, and protein	100 ng/µl to 1 µg/µl dsDNA	Plasmid preps. subcloning fragments, DNA for transfection
Quant-iT™ RNA Assay Kit, 1000 assays *5–100 ng*	Selective for RNA; can quantitate RNA in the presence of free nucleotides, DNA, and proteins	250 pg/µl to 100 ng/µl RNA	Microarray samples, real-time PCR samples, *in vitro* transcription
Quant-iT™ Protein Assay Kit, 1000 assays *0.25–5 µg*	Selective for proteins; unaffected by DTT, β-mercaptoethanol, amino acids, and DNA contamination	12.5 µg/ml to 5 mg/ml protein	PAGE gels, western blots, activity assays

† Sample concentration assumes a 1–20 µl sample volume in a 200 µl assay.

Figure 2—The Qubit™ Fluorometer. The Qubit™ Quantitation Platform is an efficient combination of sophisticated, accurate, and highly sensitive fluorescence-based quantitation assays and the most user-friendly fluorometer available.

- Quant-iT™ Protein Kit—for western blotting, activity assays, and SDS-PAGE or 2D gel electrophoresis

the ideal fluorometer

The Qubit™ Fluorometer (Figure 2) is designed to work seamlessly with the Quant-iT™ Assay Kits. Together, they are a completely integrated solution that sets the new standard for quantitation confidence. Just choose the assay, open the kit, and go. All settings and calculations are performed for you. In addition to providing quantitation results that are fast, easy, and reliable, the Qubit™ Fluorometer features software that is fully upgradable. As we develop and offer new quantitation solutions, you'll be able to download new software to make sure your Qubit™ Fluorometer stays as state-of-the-art as the day you bought it. The Qubit™ Fluorometer offers:

- power—sophisticated data analysis algorithms for high accuracy
- simplicity—an intuitive user interface for easy use the first time and every time
- flexibility—seamless integration with the full range of Quant-iT™ Assay Kits ■

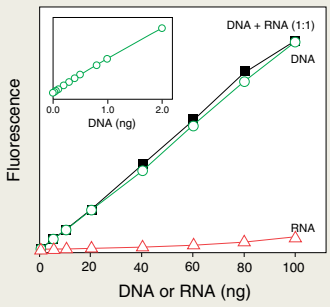


Figure 1—Performance of the Quant-iT™ dsDNA HS assay. The Quant-iT™ dsDNA HS assay has a linear detection range of 0.2–100 ng and is selective for dsDNA, even in the presence of an equal mass of RNA.

Product	Quantity	Cat. no.
Qubit™ Fluorometer	each	Q32857
Quant-iT™ dsDNA HS Assay Kit, 100 assays *0.2–100 ng* *for use with the Qubit™ Fluorometer*	1 kit	Q32851
Quant-iT™ dsDNA HS Assay Kit, 500 assays *0.2–100 ng* *for use with the Qubit™ Fluorometer*	1 kit	Q32854
Quant-iT™ dsDNA BR Assay Kit, 100 assays *2–1000 ng* *for use with the Qubit™ Fluorometer*	1 kit	Q32850
Quant-iT™ dsDNA BR Assay Kit, 500 assays *2–1000 ng* *for use with the Qubit™ Fluorometer*	1 kit	Q32853
Quant-iT™ RNA Assay Kit, 100 assays *5–100 ng* *for use with the Qubit™ Fluorometer*	1 kit	Q32852
Quant-iT™ RNA Assay Kit, 500 assays *5–100 ng* *for use with the Qubit™ Fluorometer*	1 kit	Q32855
Quant-iT™ Protein Assay Kit, 100 assays *0.25–5 µg* *for use with the Qubit™ Fluorometer*	1 kit	Q33211
Quant-iT™ Protein Assay Kit, 500 assays *0.25–5 µg* *for use with the Qubit™ Fluorometer*	1 kit	Q33212
Qubit™ Quantitation Starter Kit (1 Qubit™ Fluorometer and 4 Quant-iT™ assays)	1 kit	Q32860
Qubit™ Quantitation Lab Starter Kit (5 Qubit™ Fluorometers and 4 Quant-iT™ assays)	1 kit	Q32861

Develop your own fluorescence-based ELISA

YOU PROVIDE THE PRIMARY CAPTURE AND DETECTION ANTIBODIES—WE SUPPLY THE REST.

ELISAs (enzyme-linked immunosorbent assays) are powerful tools for detecting low levels of metabolites, including proteins of interest. Fluorescence-based ELISAs take the level of detection even lower. Molecular Probes™ ELISA Development Kits provide everything you need to develop your own fluorescence-based sandwich ELISA except the primary capture and detection antibodies. Amplex® ELISA Development Kits and SensiFlex™ ELISA Development Kits give you two options to fit your needs and instrumentation.

HRP-based ELISAs

Amplex® ELISA Development Kits allow you to create a highly sensitive fluorescence-based ELISA using mouse or rabbit primary detection antibodies. The assay employs Amplex® UltraRed Reagent, a fluorogenic substrate for horseradish peroxidase (HRP) that reacts with H_2O_2 in a 1:1 stoichiometric ratio to produce a brightly fluorescent and strongly absorbing reaction product (excitation/emission maxima ~568/581 nm). In contrast to commonly used reagents such as ABTS and TMB, Amplex® UltraRed Reagent is exceptionally resistant to auto-oxidation, making it a superior alternative for HRP-amplified ELISAs. With a high extinction coefficient and good quantum efficiency, the fluorescence-based Amplex® UltraRed Reagent is more sensitive than standard colorimetric reagents (Table 1) and provides a broader measurement range for your ELISAs.

The reaction product of this versatile reagent can be detected using either fluorescence- or absorbance-based instrumentation. No stop or signal development reagents are required; however, Amplex® Red/UltraRed Stop Reagent, which provides a stable signal for several hours after the reaction is terminated, is included in the kit for convenience. Amplex® Red/UltraRed Stop Reagent is also available as a stand-alone product that should be effective in other HRP-coupled nonradioactive detection systems.

Table 1—Advantages of Amplex® UltraRed Reagent over chromogenic reagents.

Peroxidase detection reagent	Ex*	Em*	Notes
Amplex® UltraRed Reagent	568	581	Resistant to auto-oxidation More sensitive Provides rapid results
ABTS (2′-azino-*bis*-(3-ethylbenzothiazoline-6-sulfonic acid))	405	NA	Readily oxidized Slow color development
OPD (*o*-phenylenediamine dihydrochloride	492	NA	Light sensitive
TMB (3,3′,5,5′-tetramethylbenzidine)	450 (stopped reaction) 653 (kinetic assay)	NA	Precipitates easily due to low water solubility Readily oxidized

* Fluorescence excitation (Ex) and emission (Em) maxima, in nm. NA = Not applicable.

β-lactamase–based ELISAs

SensiFlex™ ELISA Development Kits are based on the robust reaction between β-lactamase and Fluorocillin™ Green Reagent, a novel fluorescence-based substrate. The reaction is extremely sensitive and consistent, making assay development easier than for protocols that employ an enzyme with faster kinetics, such as HRP or alkaline phosphatase, which often require fine-tuning to obtain a low limit of detection and broad detection range. SensiFlex™ Kits provide an optimized fluorescence detection method, giving you excellent results right out of the box. Fluorocillin™ Green Reagent has a broad measurement range and is more sensitive than common colorimetric substrates. Following cleavage by β-lactamase, Fluorocillin™ Green Reagent exhibits green fluorescence (emission maximum ~520 nm) and can be detected with standard fluorescein (FITC) optics.

two options for making your own ELISAs

Both the Amplex® and SensiFlex™ ELISA Development Kits are available with either enzyme-labeled goat anti–mouse IgG or goat anti–rabbit IgG secondary detection antibodies, and provide sufficient materials to develop and perform 500 assays using a 100 µl assay volume. ■

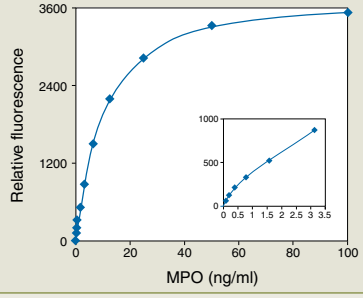
PRODUCT HIGHLIGHT

Zen™ Microplates for ELISAs

Take your ELISAs to the next level with Zen™ Microplates. These microplates give you:

- ELISAs in 2 hours or less
- compatibility with pure antibody, crude ascites, or hybridoma fluids
- binding capacity at least 10 times greater than passively adsorbed antibodies
- better CVs at the limit of detection
- a need for ~10 times less capture antibody than passive adsorption

This microplate technology is available in the Zen™ Myeloperoxidase (MPO) ELISA Kit, providing a very low limit of detection and a broad measurement range (0.2–100 ng/ml). Zen™ Microplates are also available as custom products; to learn more, send an email to amplexred@invitrogen.com with "Zen™ ELISA" in the subject line.



Typical standard curve for detection of MPO using the Zen™ Myeloperoxidase (MPO) ELISA Kit. The sandwich ELISA was carried out using a mouse anti-MPO primary capture antibody, MPO standards ranging from 0.2 ng/ml to 100 ng/ml, and a rabbit anti-MPO primary detection antibody.

Product	Quantity	Cat. no.
Zen™ Myeloperoxidase (MPO) ELISA Kit *200 assays*	1 kit	Z33857

Product	Quantity	Cat. no.
Amplex® ELISA Development Kit for Mouse IgG *with Amplex® UltraRed reagent* *500 assays*	1 kit	A33851
Amplex® ELISA Development Kit for Rabbit IgG *with Amplex® UltraRed reagent* *500 assays*	1 kit	A33852
SensiFlex™ ELISA Development Kit for Mouse IgG *with Fluorocillin™ Green 495/525* *500 assays*	1 kit	S33853
SensiFlex™ ELISA Development Kit for Rabbit IgG *with Fluorocillin™ Green 495/525* *500 assays*	1 kit	S33854
Amplex® Red/UltraRed Stop Reagent *500 tests* *set of 5 vials*	1 set	A33855

Analyzing cell culture health and concentration

MIX, INCUBATE, AND READ REAGENTS ALLOW YOU TO IDENTIFY AND ENUMERATE STRESSED CELL POPULATIONS BY FLOW CYTOMETRY.

Bioreactors and other mass cell cultures are normally monitored for gross parameters such as dissolved oxygen levels, pH, and lactate concentration. Vitality assays aimed at identifying early signs of stress in such cultures are less straightforward and generally not available in user-friendly formats. Ideally, assays for cell health should be rapid, require little manipulation of the sample, and include data on cell concentration so that overall cell mass as well as level of cell stress can be determined.

Two Molecular Probes™ vitality assays can be used in conjunction with CountBright™ Absolute Counting Beads for identifying and enumerating stressed populations of cultured cells. For each of the assays, a known volume of cells is taken from culture without washing, the vitality reagents are added directly to the sample, and the sample is incubated. Just before analysis, a known volume of CountBright™ Beads is added to the sample, which is then analyzed, without washing, on a flow cytometer.

vitality and cell counts

The LIVE/DEAD® Viability/Cytotoxicity Assay Kit provides a two-color readout that is based on the simultaneous determination of live and dead cells using fluorescent probes that measure recognized indicators of cell viability—intracellular esterase activity and plasma membrane integrity using calcein AM and ethidium homodimer (EthD-1), respectively.[1–5]

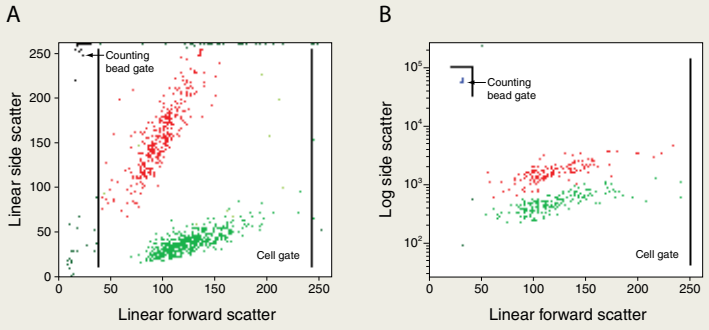


Figure 1—Gating options to include CountBright™ Counting Beads and both live and dead cells. The sample is a mixture of live and heat-killed Jurkat cells (human T-cell leukemia) containing CountBright™ Counting Beads. More traditional gating uses linear forward and side scatter and the cell gate is drawn to include all cells except debris close to the origin. The CountBright™ bead gate is adjusted to include the last channel in side scatter (A). The same gating can be done with linear forward scatter versus logarithmic side scatter, allowing the CountBright™ Beads to appear entirely within the analysis region (B).

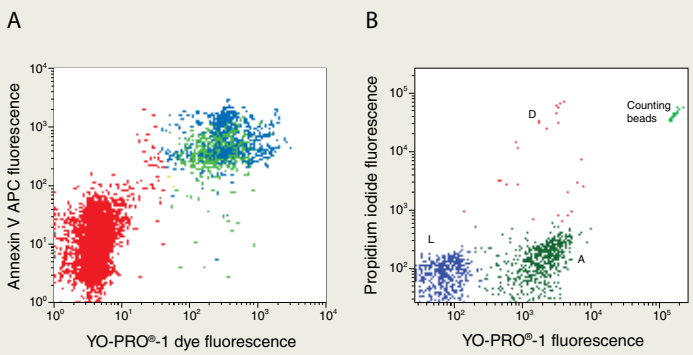


Figure 3—YO-PRO®-1 dye and propidium iodide (PI) staining of camptothecin-induced Jurkat cells. (A) Cells stained with YO-PRO®-1 dye, allophycocyanin annexin V, and PI show co-staining of cells with annexin V and YO-PRO®-1 dye. Live cells are shown in red, apoptotic cells in green, and dead cells in blue, based on annexin V and impermeant dye staining. (B) Cells stained with YO-PRO®-1 dye and PI can be resolved into apoptotic (A), live (L), and dead (D) populations. CountBright™ Absolute Counting Beads were added before data acquisition on the flow cytometer. The sample was analyzed using 488 nm excitation and collected using a 530/30 nm bandpass filter (YO-PRO®-1) and a 585/42 nm bandpass filter (PI).

For analysis, one gate must be used for live, dead, and apoptotic cells and another gate for the counting beads (Figure 1). Live cells are distinguished by the presence of ubiquitous intracellular esterase activity (Figure 2), determined by the enzymatic conversion of the virtually nonfluorescent cell-permeant calcein AM to the intensely fluorescent calcein (excitation/emission ~495/515 nm). EthD-1 enters cells with damaged membranes and undergoes a 40-fold enhancement of fluorescence upon binding to nucleic acids, producing bright red fluorescence (excitation/emission ~495/635 nm) in dead cells. The counting beads let you express both populations in terms of cells per ml of culture.

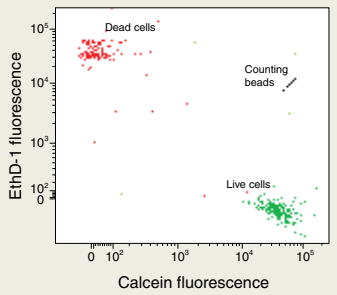


Figure 2—Calcein and EthD-1 staining of live and dead Jurkat cells. A mixture of live and heat-killed Jurkat cells was stained with calcein AM and ethidium homodimer-1 (included in the LIVE/DEAD® Viability/Cytotoxicity Kit). CountBright™ Absolute Counting Beads were added prior to data acquisition on the flow cytometer using 488 nm excitation. Calcein fluorescence, collected through a 530/30 nm bandpass filter, vs. ethidium homodimer-1 fluorescence, collected through a 610/20 nm bandpass filter, shows clear separation of live and dead cells.

apoptosis and cell counts

Apoptosis is a carefully regulated process of cell death that can occur in cell populations as a culture becomes stressed. During apoptosis the cytoplasmic membrane becomes slightly permeant. Certain dyes, such as green-fluorescent YO-PRO®-1 dye, can enter apoptotic cells, whereas other dyes, such as red-fluorescent propidium iodide (PI), cannot. YO-PRO®-1 dye has been shown to enter cells that have undergone small changes in membrane permeability associated with annexin V staining,[6] and the two reagents have been shown to co-stain apoptotic cells (Figure 3A).

After staining a sample with YO-PRO®-1 dye and PI, apoptotic cells show green fluorescence, dead cells show red and green fluorescence, and live cells show little or no fluorescence (Figure 3B). Thus, using YO-PRO®-1 dye and PI together (Vybrant® Apoptosis Assay Kit #4) provides a sensitive indicator for apoptosis.[6–9] As above, counting beads allow the cell populations to be expressed in terms of cell concentration. ■

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Product	Quantity	Cat. no.
LIVE/DEAD® Viability/Cytotoxicity Kit *for mammalian cells*	1 kit	L3224
Vybrant® Apoptosis Assay Kit #4 *YO-PRO®-1/propidium iodide* *200 assays*	1 kit	V13243
CountBright™ Absolute Counting Beads *for flow cytometry* *100 tests*	5 ml	C36950

Multicolor immunodetection using the violet laser

VIOLET-EXCITED DYES AND QDOT® REAGENTS FOR MULTICOLOR APPLICATIONS.

Violet diode lasers are becoming more common in flow cytometers, allowing many applications to be extended to this excitation source as reagents become available. Invitrogen offers two violet-excited organic dyes, Pacific Blue™ and Pacific Orange™ dyes, as well as a range of Qdot® reagents (Table 1).

new fluorophores for your violet laser

The Pacific Blue™ and Pacific Orange™ organic dyes are spectrally resolved and available on a range of Caltag™ and Molecular Probes™ primary antibodies and secondary reagents (visit probes.invitrogen.com/flowcytometry for a full list). Pacific Orange™ dye is at least twice as bright as the other green-emitting, violet-excitable dyes, Cascade Yellow™ and Alexa Fluor® 430 dyes, and can be used to visualize densely expressed antigens such as CD8 and CD45. Figure 1 shows an example of Pacific Blue™ and Pacific Orange™ dyes used for an immunophenotyping application.

Qdot® reagents are semiconductor nanocrystals with unique spectral properties. They are excited to a much greater extent by UV and violet wavelengths than by blue to yellow;

Table 1—Spectral properties of selected reagents.

Fluorescent reagent	Ex*	Em*	Suggested bandpass filter†
Pacific Blue™	410	455	450/50
Pacific Orange™	400	551	575/20 or 585/42
Qdot® 565	‡	565	575/20 or 585/42
Qdot® 605	‡	605	605/20
Qdot® 655	‡	655	655/20
Qdot® 705	‡	705	720/20
Qdot® 800	‡	800	787/42

* Fluorescence excitation (Ex) and emission (Em) maxima, in nm. † In nm. ‡ Qdot® reagents excite efficiently at wavelengths between UV and green, but excite best in the UV to violet range.

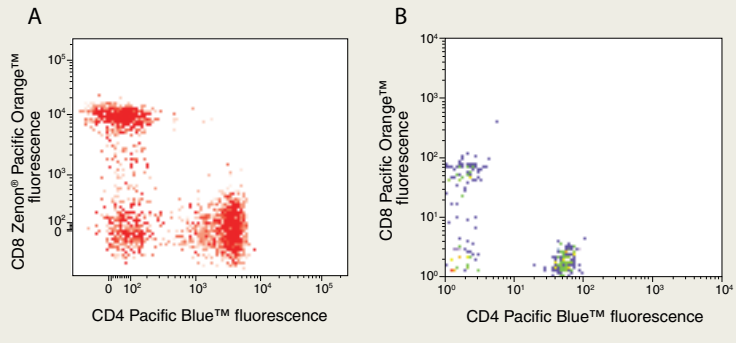

Figure 1—Staining human leukocytes using Pacific Blue™ dye for CD4 and Pacific Orange™ dye for CD8. (A) Zenon® Pacific Orange™ IgG1 Labeling Reagent complexed with mouse anti–human CD8 and Pacific Blue™ mouse anti–human CD4 antibodies (Caltag). (B) Pacific Orange™ mouse anti–human CD8 and Pacific Blue™ mouse anti–human CD4 antibodies (Caltag). Optimizing the ratio of Zenon™ Labeling Reagent to primary antibodies produces a brighter signal (A). Samples were analyzed on a BD™ LSR II flow cytometer using a 25 mW violet diode laser with 450/50 nm and 565/40 nm bandpass filters.

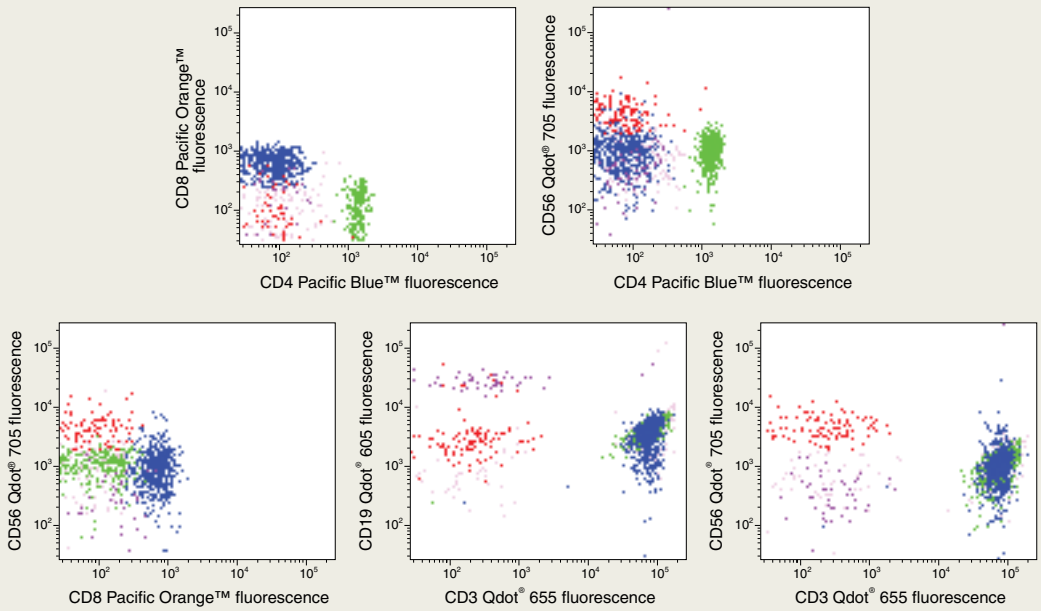

Figure 2—Five-color immunophenotyping of human peripheral blood leukocytes. Cells were labeled in the following order: mouse anti–human CD3 biotin and Qdot® 655 streptavidin, mouse anti–human CD56 biotin and Qdot® 705 streptavidin, mouse anti–human CD19 biotin and Qdot® 605 streptavidin, and Pacific Orange™ mouse anti–human CD8 and Pacific Blue™ mouse anti–human CD4 antibodies. Biotin conjugate and streptavidin steps were done sequentially with washes between; the direct fluorophore conjugates were added together. Cells were analyzed on a BD™ LSR II flow cytometer using violet diode excitation and the following bandpass filters: 450/50, 585/42, 605/20, 655/20, and 720/20 nm. Compensation was performed using autocompensation feature with single-color controls. Note: CD8 stain was dimmer due to the amount of compensation required between Qdot® 605 and Pacific Orange™ dyes.

excitation disappears in the red region. Emission wavelengths have been obtained between 525 and 800 nm by controlling nanocrystal size, and many exhibit very narrow emission peaks.

multiplexing with organic fluors and Qdot® reagents

Pacific Blue™ and Pacific Orange™ dyes can be easily multiplexed with quantum dots using reagent combinations that are spectrally resolved. Figure 2 shows five-color immunostaining with violet laser excitation and relatively easy correction for spectral overlap. Staining was performed using Qdot® streptavidin conjugates, with washes between staining steps. ■

Product	Quantity	Cat. no.
Pacific Blue™ F(ab')$_2$ fragment of goat anti–mouse IgG (H+L) *2 mg/ml*	250 µl	P31581
Pacific Blue™ goat anti–mouse IgG (H+L) *2 mg/ml*	0.5 ml	P10993
Pacific Blue™ goat anti–mouse IgG (H+L) *highly cross-adsorbed* *2 mg/ml*	0.5 ml	P31582
Pacific Blue™ goat anti–rabbit IgG (H+L) *2 mg/ml*	0.5 ml	P10994
Pacific Orange™ F(ab')$_2$ fragment of goat anti–mouse IgG (H+L) *2 mg/ml*	250 µl	P31585
Pacific Orange™ goat anti–mouse IgG (H+L) *2 mg/ml*	0.5 ml	P31583
Pacific Orange™ goat anti–mouse IgG (H+L) *highly cross-adsorbed* *2 mg/ml*	0.5 ml	P31586
Pacific Orange™ goat anti–rabbit IgG (H+L) *2 mg/ml*	0.5 ml	P31584
streptavidin, Pacific Blue™ conjugate	1 mg	S11222
streptavidin, Pacific Orange™ conjugate	1 mg	S32365
Zenon® Pacific Blue™ Mouse IgG1 Labeling Kit *50 labelings*	1 kit	Z25041
Zenon® Pacific Blue™ Mouse IgG2a Labeling Kit *50 labelings*	1 kit	Z25156
Zenon® Pacific Blue™ Rabbit IgG Labeling Kit *50 labelings*	1 kit	Z25341
Zenon® Pacific Orange™ Mouse IgG1 Labeling Kit *50 labelings*	1 kit	Z25256
Zenon® Pacific Orange™ Mouse IgG2a Labeling Kit *50 labelings*	1 kit	Z25257

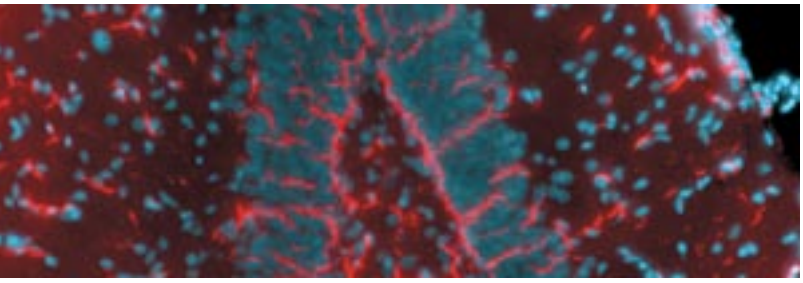

Figure 1—Microglial cells in a rat hippocampus cryosection. Cells were labeled with red-orange–fluorescent DiI acetylated low-density lipoprotein and stained using blue-fluorescent DAPI.

Tools for membrane research

EXPANDING APPLICATIONS FOR LIPID PROBES.

With the recent surge of information detailing the molecular basis of lipid storage diseases and the recognition of the role of lipid metabolites in cell regulation, lipid probes are no longer restricted to structural membrane studies. Invitrogen is keeping pace with these developments by continually updating our selection of Molecular Probes™ lipid probes and expanding the applications for these probes.

finding the probe you need

Table 1 (page 26) summarizes the most widely used fluorescent lipid probes, along with their spectral properties and key applications. You will find two major classes of probes listed here—biologically active, fluorescent lipid analogs and lipophilic organic dyes—both of which are important for studying the plasma and intracellular membranes of live cells, the artificial membranes of liposomes, and the proteins and lipids that interact with them. Many of these probes may be combined with other fluorescent labels for multiplex analyses (Figure 1) and are compatible with several instrument platforms, including fluorescence microscopy, flow cytometry, and high-throughput screening (HTS) and high-content screening (HCS) instrumentation (read about new products for high-content screening on pages 12–14).

fluorescent lipid analogs for probing membrane functions

Fluorescent analogs of naturally occurring phospholipids, sphingolipids, and steroids have proven to be important tools for studying many facets of cell regulation.[1] Phospholipases are responsible for generating critical second messengers such as diacylglycerol, arachidonate, and inositol 1,4,5-triphosphosphate (Ins 1,4,5-P3), and accordingly fluorescence-based phospholipase assays have made significant contributions to the broad field of signal transduction. Similarly, sphingomyelinases, which are functionally analogous to phospholipase C in their chemistry, hydrolyze sphingomyelins into the lipid second messenger ceramide,[2,3] making the fluorescent ceramide analogs popular probes for investigating the relationship between sphingolipid metabolism and protein secretion and for selectively labeling the Golgi apparatus.[4,5]

Fluorescent sphingolipids have also been instrumental in examining the underlying mechanisms of several lipid metabolism and storage diseases:

- Atherosclerosis[6]
- Gaucher (Ashkenazi) disease[7]
- Krabbe disease[8]
- Niemann–Pick disease[9–11]

Moreover, a recent review on cellular lipidomics states that all major human diseases have a lipid component,[12] including Alzheimer disease[13] and cancer,[14,15] and therefore dissecting lipid pathways in cells will likely impact disease diagnosis, treatment, and prevention.

fluorescent dyes for probing membrane structure

With their low cell toxicity and stable retention in membranes, lipophilic fluorophores such as DiI are widely used to label cells, organelles, liposomes, viruses, and lipoproteins in a wide variety of cell tracing applications (Figure 2), including cell transplantation, migration, adhesion, and fusion studies. DiI and its analogs are especially favored for long-term tracing experiments because probe transfer between intact membranes is usually negligible; however, these lipophilic dyes do eventually internalize and stain intracellular membranes.

In contrast, the amphiphilic FM® dyes can be used to selectively stain the plasma membrane, where they bind rapidly and reversibly with strong fluorescence enhancement. When used in conjunction with a fluorescent receptor ligand or other selective, fluorescence-based probe, these general plasma membrane stains can serve as a control for differences in membrane surface area and optical artifacts by employing ratiometric measurements.[16]

other membrane probes

Lipid probes are even more expansive than the lipid analogs and lipophilic dyes described here because lipids themselves are a complex family of molecules that differ in their structure, localization, and concentration in any given cell. For example, the dynamic formation and regulation of lipid rafts can be studied using fluorescent cholera toxin subunit B conjugates, which bind the plasma membrane ganglioside G_{M1} selectively found in these specialized membrane structures. Likewise, fluorescent annexin V conjugates provide a quick and reliable method for detecting the translocation of phosphatidylserine from the inner to the outer leaflet of the plasma membrane, a critical indicator of the intermediate stages of apoptosis. With over 1,000 different species of lipids in a eukaryotic cell, the tools needed to examine their functions will likely continue to expand with our increasing understanding of the structural and regulatory roles of these essential molecules.

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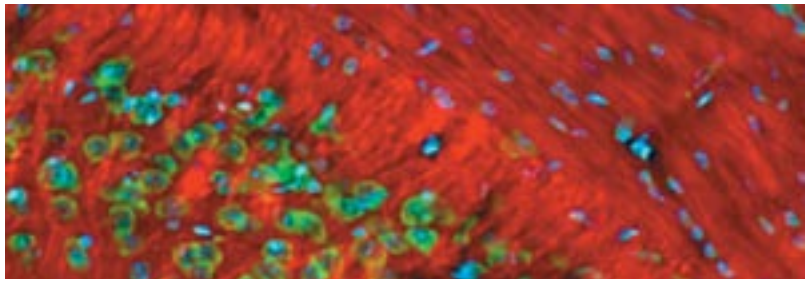

Figure 2—Fluorescently labeled mouse brain cryosection. Axons were labeled with Vybrant® DiI Cell-Labeling Solution, neuron cell bodies were stained with NeuroTrace® 500/525 Green Fluorescent Nissl Stain, and nuclei were stained with DAPI.

Table 1—Fluorescent lipid probes and kits.

Probe or kit	Ex/Em *	Cat. no.	Application notes
Phospholipid probes and phospholipase assays for studying membrane structure and metabolism			
Phospholipids with a labeled acyl chain:			Fluorophores in these phospholipids generally remain buried in the hydrophobic interior of the lipid bilayer, making them sensitive to lipid fluidity and structural perturbations by proteins and drugs.[1,2] The hydrophobic BODIPY® FL and pyrene dyes readily localize within membranes and form red-shifted excimers at high concentrations (emission at ~620 and ~470 nm, respectively). The relatively polar, environment-sensitive NBD dye is self-quenched at high concentrations and tends to localize at the lipid–water interface of membranes.
β-BODIPY® FL C_5-HPC	503/512	D3803	
β-BODIPY® FL C_{12}-HPC	506/513	D3792	
β-BODIPY® FL C_5-HPA	504/511	D3805	
β-py C_{10}-HPC	342/376	H361	
NBD C_6-HPC	465/533	N3786	
NBD C_{12}-HPC	465/534	N3787	
Phospholipids with a labeled head group:			Fluorophores in these phospholipids tend to remain at the membrane surface and are accessible to anti-dye antibodies, including those that recognize BODIPY® FL, fluorescein, Oregon Green® 488, tetramethylrhodamine, and Texas Red® dyes. These phospholipids generally stay in the plasma membrane and do not get internalized.[3] With a pK_a of ~6.2, membrane-intercalated fluorescein DHPE can serve as an indicator of lateral proton conduction along membrane surfaces;[4] Oregon Green® 488 DHPE may be similarly useful for acidic environments.
BODIPY® FL DHPE	505/511	D3800	
fluorescein DHPE	496/519	F362	
NBD PE	463/536	N360	
Oregon Green® 488 DHPE	501/526	O12650	
Pacific Blue™ DMPE	411/454	P22652	
TRITC DHPE	540/566	T1391	
Texas Red® DHPE	583/601	T1395MP	
Free fatty acid (FFA) sensor: ADIFAB fatty acid indicator	365/432	A3880	This protein-based FFA sensor is useful for assaying fatty acid transport and phospholipase A_1 and A_2 activity.[5,6] ADIFAB shows an increase in emission ratio at 505/432 nm upon binding FFA, with detection limits in the micro- to submicromolar concentration range.
Phospholipase A_1 or A_2 substrate: bis-BODIPY® FL C_{11}-PC	501/512†	B7701	Phospholipase A_1 or A_2 activity disrupts intramolecular quenching of the two BODIPY® FL dyes, yielding an increase in emission at 512 nm.[7]
Phospholipase A_2 substrate: PED6	505/511†	D23739	Phospholipase A_2 activity disrupts intramolecular quenching of BODIPY® FL dye by DNP, yielding an increase in emission at 511 nm.[8]
Secreted phospholipase A_2 substrates:			Secreted phospholipase A_2 activity disrupts intermolecular excimer formation by the pyrene fluorophores in these acidic phospholipids, yielding an increase in emission at 376 nm.[9,10]
β-py C_{10}-PG	341/376	H3809	
β-py C_{10}-HPM	341/376	H3810	
Amplex® Red PC-Specific Phospholipase C Assay Kit	570/585†	A12218	This enzyme-coupled assay detects ≥0.2 mU/ml PC-specific phospholipase C activity *in vitro* using a fluorescence microplate reader or fluorometer.[11,12]
Amplex® Red Phospholipase D Assay Kit	570/585†	A12219	This enzyme-coupled assay detects ≥10 mU/ml phospholipase D activity *in vitro* using a fluorescence microplate reader or fluorometer.[11,12]
Phosphatidylinositol-specific phospholipase C, from *Bacillus cereus*	NA	P6466	This enzyme cleaves phosphatidylinositol to yield D-*myo*-inositol 1,2-cyclic monophosphate and diacylglycerol.[13]
Phosphoinositide probes for studying phosphoinositide-mediated signal transduction			
Fluorescent phosphoinositides:			These fluorescent analogs of phosphorylated phosphatidylinositol, which can be delivered into live cells using the Shuttle PIP™ carrier-1, are important probes of phosphoinositide-mediated signal transduction.[14,15]
BODIPY® FL C_5-C_6-PtdIns(4,5)P_2	504/511	B22627	
BODIPY® TMR-X C_6-PtdIns(3,4)P_2	544/570	B22635	
Shuttle PIP™ carrier-1 *histone H1*	NA	S23731	This polybasic molecule facilitates passive delivery of phosphatidylinositol di- and triphosphates into live mammalian, yeast, plant, and bacterial cells.
Anti-phosphoinositide monoclonal antibodies:			These monoclonal IgM antibodies are useful for immunocytochemical localization of their cognate phosphoinositides, with only slight crossreactivity with other phosphoinositides and phospholipids.[16]
anti-PtdIns(4,5)P_2	NA	A21327	
anti-PtdIns(3,4,5)P_3	NA	A21328	
Sphingolipid probes and sphingomyelinase assays for studying sphingolipid transport and metabolism			
BODIPY® dye–labeled sphingolipids:			Fluorescent sphingolipids have proven useful for studying sphingolipid metabolism and trafficking, especially as they relate to sphingolipid storage and transport diseases.[17] The NBD sphingolipids do not exhibit the concentration-dependent excimer formation of the BODIPY® FL sphingolipids and are less photostable, but NBD C_6-ceramide is sensitive to the cholesterol content of the Golgi apparatus, a phenomenon that is not observed with BODIPY® FL C_5-ceramide.[18] Several of these fluorescent sphingolipids are available complexed to BSA to facilitate cell labeling by eliminating the need for organic solvents to dissolve the probe.
BODIPY® FL C_5-ceramide	505/511	D3521, B22650	
BODIPY® TR ceramide	589/616	D7540, B34400	
BODIPY® FL C_5-lactosylceramide	505/511	D13951, B34402	
BODIPY® FL C_5-ganglioside G_{M1}	505/512	B13950, B34401	
BODIPY® FL C_{12}-galactocerebroside	505/511	D7519	
BODIPY® FL C_5-sphingomyelin	505/512	D3522	
BODIPY® FL C_{12}-sphingomyelin	505/513	D7711	
NBD-labeled sphingolipids:			
NBD C_6-ceramide	466/536	N1154, N22651	
NBD C_6-sphingomyelin	466/536	N3524	
Amplex® Red Sphingomyelinase Assay Kit	570/585†	A12220	This enzyme-coupled assay detects ≥80 µU/ml sphingomyelinase activity *in vitro* using a fluorescence microplate reader or fluorometer.[19]

Table 1—Fluorescent lipid probes and kits, continued.

Probe or kit	Ex/Em *	Cat. no.	Application notes
Cholesterol probes and cholesterol esterase assays for studying cholesterol transport and metabolism			
NBD cholesterol	469/537	N1148	NBD cholesterol is used to monitor HDL-mediated cholesterol uptake [20] and to screen for inhibitors of acyl CoA:cholesterol acyltransferase [21] (ACAT).
Cholesteryl BODIPY® FL C_{12}	505/511	C3927MP	BODIPY® FL cholesteryl ester can be used to follow cholesterol transport [22] and to measure cholesteryl ester–transfer protein (CETP) activity.[23]
Amplex® Red Cholesterol Assay Kit	570/585 †	A12216	Cholesterol produced by cholesterol esterases is readily quantitated using this enzyme-coupled assay and a fluorescence microplate reader or fluorometer,[24] with a detection limit of ~80 ng/ml.
General lipase substrates			
O-pivaloyloxymethyl umbelliferone (C-POM)	360/460 †	P35901	A substrate for a variety of lipases, C-POM displays excellent stability in solution, making it ideal for general or high-throughput screening.[25]
6,8-difluoro-4-methylumbelliferyl octanoate (DiFMU octanoate)	358/452 †	D12200	Unlike β-methylumbelliferone esters, DiFMU octanoate can be used for the continuous *in vitro* assay of lipases [26] at pH ≥6.
EnzChek® lipase substrate	505/515 †	E33955	In the presence of lipases, this nonfluorescent substrate produces a green-fluorescent product for accurate lipase detection in solution.
Fatty acid analogs for studying lipid peroxidation			
cis-parinaric acid	304/416	P36005	Fluorescence quenching of this fatty acid analog is useful for lipid peroxidation assays, including quantitative assays in live cells.[27,28]
BODIPY 581/591 C_{11}	582/591	D3861	Oxidation of this fatty acid yields a shift in the fluorescence emission peak from ~590 to ~510 nm, allowing ratiometric measurements.[29,30]
Fluorescent low-density lipoproteins (LDL) and lipopolysaccharides (LPS) for following receptor-mediated endocytosis			
Fluorescent and unlabeled LDL from human plasma:			
unlabeled LDL	NA	L3486	The LDL complex delivers cholesterol to cells via receptor-mediated endocytosis. Fluorescent LDL has been used to quantitate LDL receptors, analyze their motion and clustering, and follow their internalization.[31,32]
BODIPY® FL LDL	515/520	L3483	
DiI LDL	554/571	L3482	
Fluorescent and unlabeled acetylated LDL (AcLDL):			
unlabeled AcLDL	NA	L35354	AcLDL no longer binds to the LDL receptor, but instead is taken up by macrophage and endothelial cells that possess scavenger receptors specific for the modified LDL.[33] Using DiI AcLDL, researchers have found that the scavenger receptors on rabbit fibroblasts and smooth muscle cells are up-regulated through activation of the protein kinase C pathway.[34]
Alexa Fluor® 488 AcLDL	495/519	L23380	
Alexa Fluor® 594 AcLDL	590/617	L35353	
BODIPY® FL AcLDL	510/518	L3485	
DiI AcLDL	554/571	L3484	
Fluorescent LPS:			
Alexa Fluor® 488 LPS from *E. coli*	495/519	L23351	Recognition of LPS in the outer cell walls of gram-negative bacteria by the CD14 cell-surface receptor in phagocytes is the key initiation step in the mammalian immune response to infection. Fluorescent LPS have been used to study LPS binding, transport, and cell internalization.[35,36]
Alexa Fluor® 488 LPS from *S. minnesota*	495/519	L23356	
Alexa Fluor® 568 LPS from *E. coli*	578/603	L23352	
Alexa Fluor® 594 LPS from *E. coli*	590/617	L23353	
BODIPY® FL LPS from *E. coli*	505/513	L23350	
Lipophilic membrane probes for staining cell membranes			
DiI NeuroTrace® DiI tissue-labeling paste Vybrant® DiI cell-labeling solution	549/565	D282, D3911 N22880 V22885	The premier probes for labeling cell membranes—DiI, DiO, DiD, and DiR—are weakly fluorescent in water but highly fluorescent and quite photostable when incorporated into membranes. Transfer of the long-chain carbocyanine dyes between intact membranes is usually negligible; however, unlike the FM® dyes described below, they do eventually internalize and stain intracellular membranes. The ready-to-use NeuroTrace® tissue-labeling pastes can be applied directly to tissues using the tip of a needle, allowing dye penetration into bundled neurons for labeling axons both on and below the surface. The Vybrant® cell-labeling solutions are filtered, dye-delivery solutions that can be added directly to normal culture media to uniformly label suspended or attached culture cells.
FAST DiI	549/564	D3899, D7756	
DiO NeuroTrace® DiO tissue-labeling paste Vybrant® DiO cell-labeling solution	484/501	D275 N22881 V22886	
FAST DiO	484/499	D3898	
DiD Vybrant® DiD cell-labeling solution	644/665	D307, D7757 V22887	
DiR	650/670	D12731	
CellTracker™ CM-DiI NeuroTrace® CM-DiI tissue-labeling paste Vybrant® CM-DiI cell-labeling solution	553/570	C7000, C7001 N22883 V22888	CellTracker™ CM-DiI is a fixable DiI analog with a chloromethyl moiety that allows the dye to covalently bind cellular thiols, making it compatible with subsequent immunohistochemical or FISH analysis.

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Table 1—Fluorescent lipid probes and kits, continued.

Probe or kit	Ex/Em*	Cat. no.	Application notes
DiA	457/586	D3883	Because of its faster diffusion rate and better solubility, DiA is often preferred over DiO for multicolor labeling with DiI.
FAST DiA	457/586	D7758	
Membrane probes for selectively staining the plasma membrane			
FM® 1-43 FM® 1-43FX, a fixable analog of FM® 1-43	479/598	T3163, T35356 F35355	Unlike the lipophilic DiI analogs, the amphiphilic FM® dyes are easily applied to cells, where they bind rapidly and reversibly to the plasma membrane with strong fluorescence enhancement. FM® 1-43 has been used to outline membranes in sea urchin eggs and to identify actively firing neurons. The more hydrophobic FM® 4-64 and FM® 5-95 exhibit long-wavelength emission (690–730 nm) in cells that can easily be distinguished from the green fluorescence of GFP reporter fusions.
FM® 4-64 FM® 4-64FX, a fixable analog of FM® 4-64	568/ 690–730	T3166, T13320 F34653	
FM® 5-95	568/ 690–730	T23360	
RH 414	500/635	T1111	RH 414 is commonly used as a general plasma membrane stain.
Image-iT™ LIVE Plasma Membrane and Nuclear Labeling Kit	590/617‡	I34406	This kit provides red-fluorescent Alexa Fluor® 594 wheat germ agglutinin and blue-fluorescent Hoechst 33342 (excitation/emission ~350/461 nm when bound to DNA) for highly selective staining of the plasma membrane and nucleus, respectively, of live GFP-transfected cells.
Membrane probes for labeling lipid rafts			
Vybrant® Lipid Raft Labeling Kits:			Vybrant® Lipid Raft Labeling Kits provide the key reagents for convenient, reliable, and extremely bright fluorescent labeling of lipid rafts in live cells. Cells are first labeled with a fluorescent Alexa Fluor® CT-B conjugate, which binds to ganglioside G_{M1} found selectively in lipid rafts, then an anti–CT-B antibody is added to crosslink the CT-B–labeled lipid rafts into distinct plasma membrane patches that are visible by fluorescence microscopy.[37] Also available are fluorescent, biotinylated, and HRP-labeled CT-B conjugates, as well as the green-fluorescent BODIPY® FL C_5-ganglioside G_{M1} (see page 26).
Vybrant® Alexa Fluor® 488 Lipid Raft Kit	495/519‡	V34403	
Vybrant® Alexa Fluor® 555 Lipid Raft Kit	555/565‡	V34404	
Vybrant® Alexa Fluor® 594 Lipid Raft Kit	590/617‡	V34405	
Membrane probes for detecting the phosphatidylserine externalization, a marker of the intermediate stages of apoptosis			
Vybrant® Apoptosis Assay Kits:			In these Vybrant® Apoptosis Assay Kits, apoptotic cells are detected on the basis of the externalization of phosphatidylserine (PS), an indicator of intermediate stages of apoptosis. In normal viable cells, PS is located on the cytoplasmic surface of the cell membrane. However, in apoptotic cells, PS is translocated from the inner to the outer leaflet of the plasma membrane, exposing PS to the external cellular environment where it can be detected by annexin V conjugates.[38] The difference in fluorescence intensity between apoptotic and nonapoptotic cells stained by our fluorescent annexin V conjugates, as measured by flow cytometry, is typically about 100-fold. Also available are fluorescent and biotinylated annexin V conjugates.
Kit #1 with Alexa Fluor® 488 annexin V	495/519‡	V13240	
Kit #2 with Alexa Fluor® 488 annexin V	495/519‡	V13241	
Kit #3 with FITC annexin V	494/518‡	V13242	
Kit #8 with R-phycoerythrin annexin V	496/578‡	V35112	
Kit #9 with allophycocyanin annexin V	650/660‡	V35113	
Kit #10 with allophycocyanin annexin V	650/660‡	V35114	
Kit #11 with Alexa Fluor® 488 annexin V	495/519‡	V35116	
Kit #14 with Pacific Blue™ annexin V	410/455‡	V35124	

***** Excitation and emission maxima (Ex/Em), in nm. **†** Ex/Em of reaction product, not of substrate. **‡** Ex/Em of membrane-labeling probe. AcLDL = acetylated LDL. BSA = bovine serum albumin. CT-B = cholera toxin subunit B. DHPE = 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine. DMPE = 1,2-ditetradecanoyl-*sn*-glycero-3-phosphoethanolamine. DNP = dinitrophenyl. FFA = free fatty acid. FISH = fluorescence *in situ* hybridization. HDL = high-density lipoprotein. HPA = 1-hexadecanoyl-*sn*-glycero-3-phosphate. HPC = 1-hexadecanoyl-*sn*-glycero-3-phosphocholine. HPM = 1-hexadecanoyl-*sn*-glycero-3-phosphomethanol. HRP = horseradish peroxidase. LDL = low-density lipoprotein. LPS = lipopolysaccharide. NA = not applicable. NBD = 7-nitrobenz-2-oxa-1,3-diazole. PC = *sn*-glycero-3-phosphocholine. PG = 1-hexadecanoyl-*sn*-glycero-3-phosphoglycerol. PtdIns = phosphatidylinositol. py = pyrene. X = 7-atom (aminohexanoyl) spacer. XX = 14-atom (aminohexanoyl)aminohexanoyl spacer.

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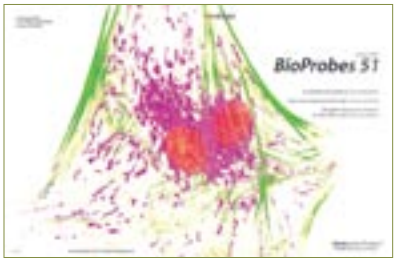

Cover image: detailed protocol

Summary

Filamentous actin in muntjac skin fibroblasts was visualized using green-fluorescent Alexa Fluor® 488 phalloidin. Mitochondria were labeled with an anti–OxPhos Complex V inhibitor protein antibody and visualized using orange-fluorescent Alexa Fluor® 555 goat anti–mouse IgG (pseudocolored magenta). Nuclei were stained with red-fluorescent TO-PRO®-3.

Materials and methods

Cells

Muntjac skin fibroblasts

Materials needed

Phosphate-buffered saline (PBS) (GIBCO)
10X Phosphate-buffered saline (PBS)(GIBCO)
Triton® X-100 (Sigma)
Methanol-free 16% formaldehyde solution (Polysciences)
BlockAid™ Blocking Solution (Molecular Probes)
Bovine serum albumin (BSA; Sigma)
Alexa Fluor® 488 phalloidin (Molecular Probes)
Anti–OxPhos Complex V inhibitor protein, monoclonal 5E2 (Molecular Probes)
Alexa Fluor® 555 goat anti–mouse IgG (Molecular Probes)
TO-PRO®-3 (Molecular Probes)
ProLong® Gold Antifade Reagent (Molecular Probes)

Protocol

All steps are carried out at room temperature unless otherwise noted.

Sample preparation

1. Remove media from live cells, wash in PBS, and fix cells for 15 minutes in 3.7% formaldehyde solution at 37˚C.
2. Wash cells 3 × 5 minutes in PBS (1X).
3. Permeabilize cells by treating for 10 minutes with PBT (0.1% Triton® X-100 in PBS (1X)).
4. Wash cells 3 × 5 minutes in PBS.

Staining

1. Block cells for 1 hour in antibody blocking buffer (BlockAid™ Blocking Solution).
2. Incubate cells with 5 µg/ml of anti–OxPhos Complex V inhibitor protein primary antibody in BlockAid™ Solution for 1 hour.
3. Wash cells 3 × 10 minutes in PBT.
4. Incubate cells with 5 µg/ml Alexa Fluor® 555 goat anti-mouse IgG in 1% BSA/PBT for 1 hour.
5. Wash 3 × 10 minutes in PBS.
6. Incubate cells for 20 minutes with Alexa Fluor® 488 phalloidin, diluted 1:200 from stock solution.
7. Wash 3 × 5 minutes in PBS.
8. Label cells with 1 µM TO-PRO®-3 dye in PBS for 15 minutes.
9. Wash 3 × 10 minutes in PBS.
10. Mount the specimen in ProLong® Gold Antifade Reagent.

Mounting reagent preparation and sample processing

1. Remove the ProLong® Gold Antifade Reagent from the freezer and allow the vial to equilibrate to room temperature. Using an external heat source to warm the vial is not recommended, as this may decrease the long-term stability of the product.
2. Remove any excess liquid from the specimen and apply 1 or 2 drops (depending on the surface area of your sample) of the antifade reagent to the specimen. Cover slide-mounted specimens with a coverslip; for specimens mounted on coverslips, place a drop of antifade reagent onto a clean slide and carefully lower the coverslip onto the antifade reagent to avoid trapping any air bubbles.
3. Allow the mounted sample to cure on a flat surface in the dark. Curing time may vary from a couple of hours to overnight, depending on the thickness of the sample and the relative humidity of the surrounding air. For long-term storage, seal the coverslip to the slide after curing to prevent excessive shrinkage of the mounting medium, which can result in sample distortion. After sealing, store the slide upright in a covered slide box at ≤–20°C. Desiccant may be added to the box to ensure that the slide remains dry.

To view the samples immediately, secure the coverslip at the corners using nail polish or hot wax to prevent the coverslip from moving. Leave the edges clear to allow the preparation to cure.

Note: The antifade properties of ProLong® Gold Antifade Reagent improve slightly the longer it remains in contact with the specimen. To further reduce photobleaching, minimize the exposure of fluorescently labeled specimens to light by using neutral density filters, and expose samples only when observing or recording a signal. Optimize image capture by using a minimum of optics, high-numerical aperture objectives, relatively low magnification, high-quality optical filters, and high-speed film or high-efficiency detectors.

Instrumentation

Microscope

Nikon Eclipse® 800 with MicroMax 1300 YHS 12-bit digital camera

Objective

60x/1.40 NA oil

Filters

Alexa Fluor® 488 dye

Excitation: 475AF40
Dichroic: 505DRLP
Emission: 535AF45

Alexa Fluor® 555 dye

Excitation: 535AF18
Dichroic: 570DRLP
Emission: 590AF18

TO-PRO®-3 dye

Excitation: 630AF25
Dichroic: 650DRLP
Emission: 695AF28

Imaging processing software

MetaMorph® Imaging System

Molecular Probes, Inc.
Registered to ISO 9001
File No. A5974

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