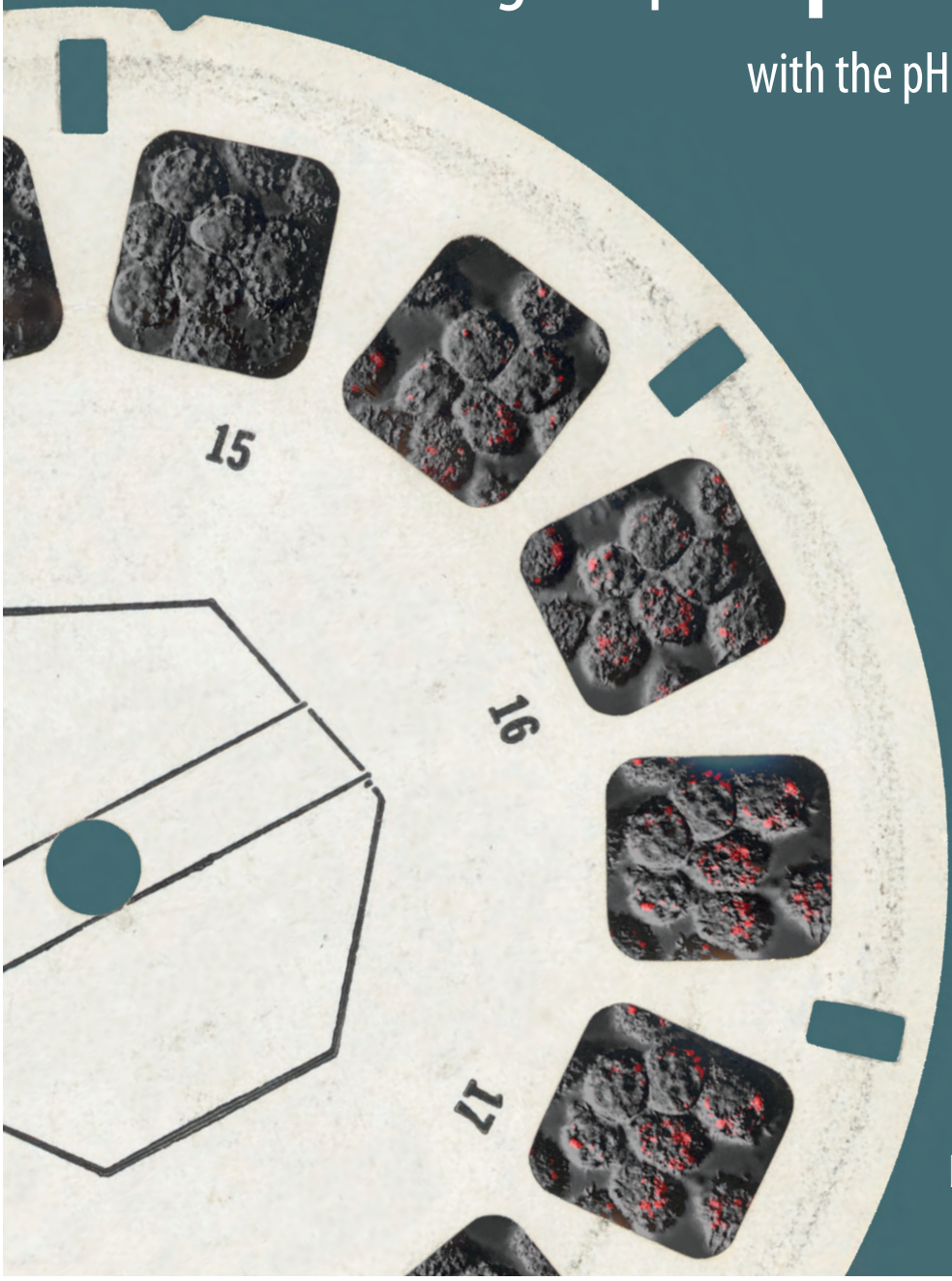
BIOPROBES 53

JULY 2007

Putting the pH in **phagocytosis**

with the pH-sensitive pHrodo™ dye



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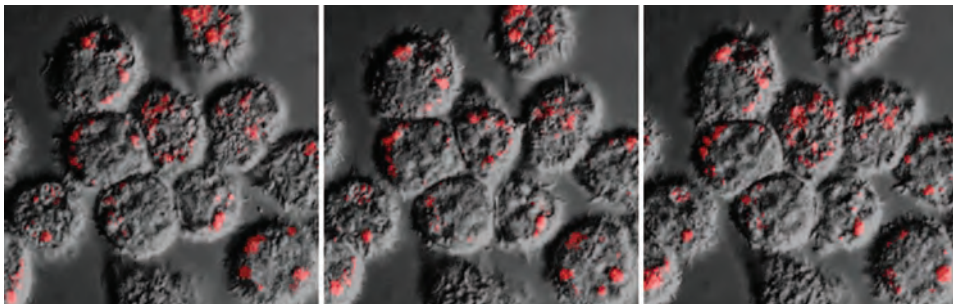

BioProbes 53

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Staining bacterial biofilms: new uses for classic fluorescent dyes

FAMILIAR PROBES TAKE ON A NEW ROLE IN BIOFILM STAINING.

Biofilms are formed when millions of microorganisms accumulate on a solid surface in an aqueous environment, creating a complex structure that functions as a community. These film-forming microbes excrete a glue-like polymeric matrix that anchors them to materials such as metals, plastics, tissues, and soil particles. At the Center for Biofilm Engineering, we have recently identified several fluorescent stains that appear to selectively stain individual biofilm components, providing new tools for the study of biofilm formation.

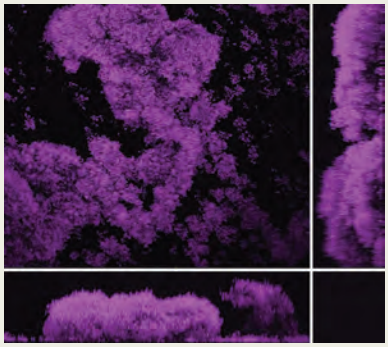

Figure 2—CellTrace™ calcein violet AM applied to a *Staphylococcus epidermidis* biofilm. The esterase substrate CellTrace™ calcein violet AM appears to stain all of the bacteria in the biofilm, suggesting that the bacteria are all actively producing esterase. The image was obtained using a Leica TCS-SP2 AOBS confocal microscope with a 63×/0.9 NA water immersion objective, and a Spectra-Physics Mai Tai® two-photon confocal microscope. Image contributed by Betsey Pitts, Center for Biofilm Engineering, Montana State University, Bozeman, Montana.

The inherent difficulties of biofilm staining

Bacterial biofilms present a unique set of challenges for fluorescent staining and subsequent confocal imaging. A typical biofilm not only exhibits heterogeneous thickness throughout the surface, placing stringent restrictions on stain penetration, but also contains regions of widely varying environmental conditions. Evidence suggests that bacterial cells exist in various physiological states within these biofilm microenvironments. Furthermore, biofilms contain many undefined components (e.g., the extracellular polymeric matrix) that differ with species and conditions.

Given their nonuniform physical, chemical, and biological characteristics, bacterial biofilms would greatly benefit from a multiparametric fluorescent staining strategy. Ideally, fluorescent staining of biofilms would include a cell-specific stain such as an activity stain, an extracellular matrix stain, and a total-cell biomass stain, and these stains could be used together or in series on the same biofilm. Very few, if any, such combinations currently exist. Many fluorescent reagents either interfere chemically with each other or alter the activity they are intended to measure. Even when two or more fluorescent stains are compatible, multicolor labeling presents an array of fluorescence imaging challenges such as bleedthrough, unwanted fluorescence resonance energy transfer (FRET), and photobleaching.

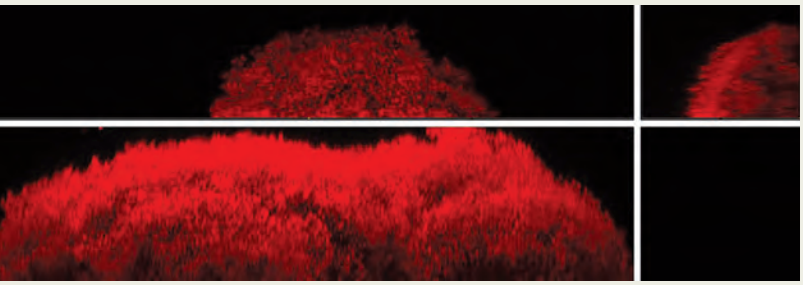

Figure 1—CellTrace™ calcein red-orange AM applied to a *Staphylococcus epidermidis* biofilm. The esterase substrate CellTrace™ calcein red-orange AM appears to stain all of the bacteria in the biofilm, suggesting that the bacteria are all actively producing esterase. The image was obtained using a Leica TCS-SP2 AOBS confocal microscope with a 63×/0.9 NA water immersion objective. Image contributed by Betsey Pitts, Center for Biofilm Engineering, Montana State University, Bozeman, Montana.

Staining individual biofilm components with classic dyes

In collaboration with Invitrogen, the Center for Biofilm Engineering is working to develop a group of fluorescent stains and probes that can overcome the inherent difficulties of staining biofilms and be used either simultaneously or sequentially on bacterial biofilms. Toward that end, we investigated some familiar stains and, in the process, identified several fluorescent reagents that take us one step closer to selectively staining individual biofilm components.

CellTrace™ calcein red-orange AM and CellTrace™ calcein violet AM are proving to be excellent, reliable indicators of esterase activity or cell viability in *Staphylococcus* (Figures 1 and 2). With excitation/emission maxima of ~400/452 nm, CellTrace™ calcein violet AM may be especially useful in combination with green, red, and far-red fluorophores. BODIPY® 630/650-X SE binds to and illuminates matrix components of several strains of *Pseudomonas aeruginosa* (Figure 3) as well as some *Staphylococcus* biofilms. And FM® 1-43 shows tremendous potential as a total-cell biomass stain; this membrane-binding dye has been shown to work equally well on *Pseudomonas aeruginosa*, *Staphylococcus epidermidis,* and *Escherichia coli*, in all cases appearing to stain the entire biofilm uniformly and with exceptional specificity (Figure 4).

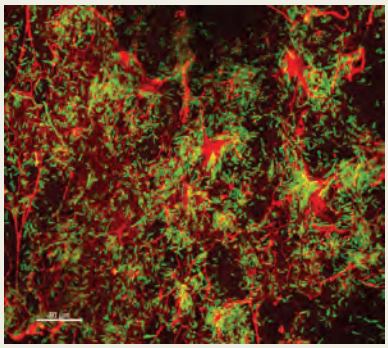

Figure 3—BODIPY® 630/650-X SE applied to a *Pseudomonas aeruginosa* biofilm. BODIPY® 630/650-X SE binds to a *Pseudomonas aeruginosa* PAO1 pMF230 matrix (red). The bacteria are engineered to produce Green Fluorescent Protein (GFP, green). The image was obtained using a Leica TCS-SP2 AOBS confocal microscope with a 63×/0.9 NA water immersion objective. Image contributed by Betsey Pitts, Center for Biofilm Engineering, Montana State University, Bozeman, Montana.

We are continuing to investigate the staining specificity of these fluorescent reagents in biofilms. Next steps include further single-color stain exploration and experimentation with multicolor combinations. ■

Article contributed by Betsey Pitts, Research Associate and Microscopy Facilities Manager at the Center for Biofilm Engineering, Montana State University, Bozeman, Montana.

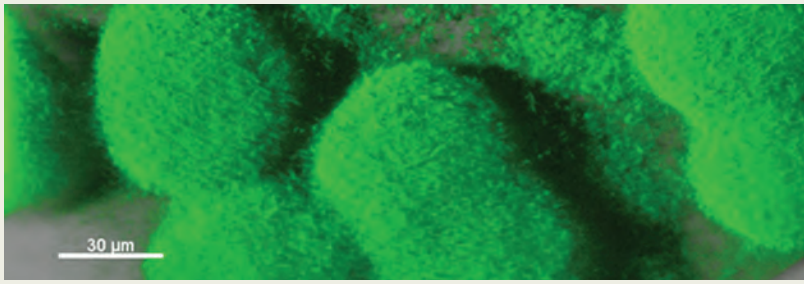

Figure 4—FM® 1-43 applied to a *Pseudomonas aeruginosa* biofilm. FM® 1-43 appears to bind to the cell membrane, and has been shown to work equally well on *Pseudomonas aeruginosa*, *Staphylococcus epidermidis*, and *Escherichia coli*, exhibiting exceptional cell specificity in each case. The image was obtained using a Leica TCS-SP2 AOBS confocal microscope with a 63×/0.9 NA water immersion objective. Image contributed by Betsey Pitts and Ellen Swogger, Center for Biofilm Engineering, Montana State University, Bozeman, Montana.

Product	Quantity	Cat. no.
CellTrace™ calcein red-orange, AM, special packaging	20 × 50 µg	C34851
CellTrace™ calcein violet, AM, for 405 nm excitation, special packaging	20 × 25 µg	C34858
N-(3-triethylammoniumpropyl)-4-(4-(dibutylamino)styryl) pyridinium dibromide (FM® 1-43)	1 mg	T3163
N-(3-triethylammoniumpropyl)-4-(4-(dibutylamino)styryl) pyridinium dibromide (FM® 1-43), special packaging	10 × 100 µg	T35356
6-(((4,4-difluoro-5-(2-thienyl)-4-bora-3a,4a-diaza-*s*-indacene-3-yl)styryloxy)acetyl)aminohexanoic acid, succinimidyl ester (BODIPY® 630/650-X, SE)	5 mg	D10000

Investigating apoptosis-induced loss of SYTO® dye fluorescence

Wlodkowic, D., Skommer, J., and Pelkonen, J. (2007) Towards an understanding of apoptosis detection by SYTO® dyes. *Cytometry A* 71:61–72.

What does loss of SYTO® dye fluorescence tell us about apoptosis? Apoptosis is the major pathway among a variety of processes by which cells initiate and effect their own deliberate destruction. It involves a complex series of orchestrated biochemical events, many of which have proven highly amenable to investigation through the use of fluorescently labeled probes. SYTO® dyes—a family of cell-permeant nucleic acid stains—have found particular success as reliable indicators of the apoptotic state of a cell, especially in conjunction with flow cytometry. It has been suggested that differential SYTO® dye fluorescence observed in viable vs. apoptotic cells is due to self-quenching effects or loss of binding upon chromatin condensation. However, lack of precise knowledge about the mechanism of SYTO® dye fluorescence loss has impeded a detailed understanding of apoptotic events.

In this study, Wlodkowic and colleagues have undertaken a detailed comparison of the staining patterns of the blue-fluorescent nuclear stain Hoechst 33342, the red-fluorescent potentiometric dye TMRM (tetramethylrhodamine, methyl ester), and green-fluorescent SYTO® 16 dye, in an effort to further elucidate the exact mechanism behind apoptosis-induced loss of SYTO® dye fluorescence. Analysis of tricolor-stained human follicular lymphoma cells via confocal microscopy revealed that SYTO® 16 dye fluorescence colocalizes only partially with TMRM fluorescence (which is known to be associated with energized mitochondria). Treating the cells for 15 minutes with the mitochondrial uncoupler FCCP caused a loss of TMRM fluorescence, with little change in SYTO® 16 dye fluorescence; longer FCCP treatment led to a progressive loss of SYTO® 16 dye fluorescence followed by cell membrane permeabilization. When the authors pretreated the cells with a pan-caspase inhibitor (z-VAD-fmk) 1 hour prior to FCCP treatment, they observed that the FCCP-induced loss of SYTO® 16 dye fluorescence was blocked, while the loss of TMRM fluorescence persisted.

These results indicate that blocking caspase activation prevents loss of SYTO® dye fluorescence even in cells where mitochondrial rupture is present. The authors conclude that SYTO® dye fluorescence appears to result from caspase-dependent events rather than mitochondrial rupture, though they acknowledge that loss of SYTO® 16 fluorescence may result from caspase-dependent changes in mitochondria. ■

Product	Quantity	Cat. no.
7-aminoactinomycin D (7-AAD)	1 mg	A1310
Hoechst 33342, trihydrochloride, trihydrate	100 mg	H1399
Hoechst 33342, trihydrochloride, trihydrate, 10 mg/ml solution in water	10 ml	H3570
propidium iodide	100 mg	P1304MP
propidium iodide, 1.0 mg/ml solution in water	10 ml	P3566
SYTO® 11 green fluorescent nucleic acid stain, 5 mM solution in DMSO	250 µl	S7573
SYTO® 12 green fluorescent nucleic acid stain, 5 mM solution in DMSO	250 µl	S7574
SYTO® 13 green fluorescent nucleic acid stain, 5 mM solution in DMSO	250 µl	S7575
SYTO® 14 green fluorescent nucleic acid stain, 5 mM solution in DMSO	250 µl	S7576
SYTO® 16 green fluorescent nucleic acid stain, 1 mM solution in DMSO	250 µl	S7578
SYTOX® Green nucleic acid stain, 5 mM solution in DMSO	250 µl	S7020
SYTOX® Red dead cell stain, for 633 or 635 nm excitation, 5 µM solution in DMSO	1 ml	S34859

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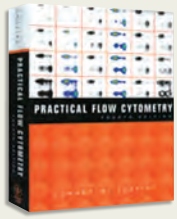
Practical Flow Cytometry by Howard M. Shapiro



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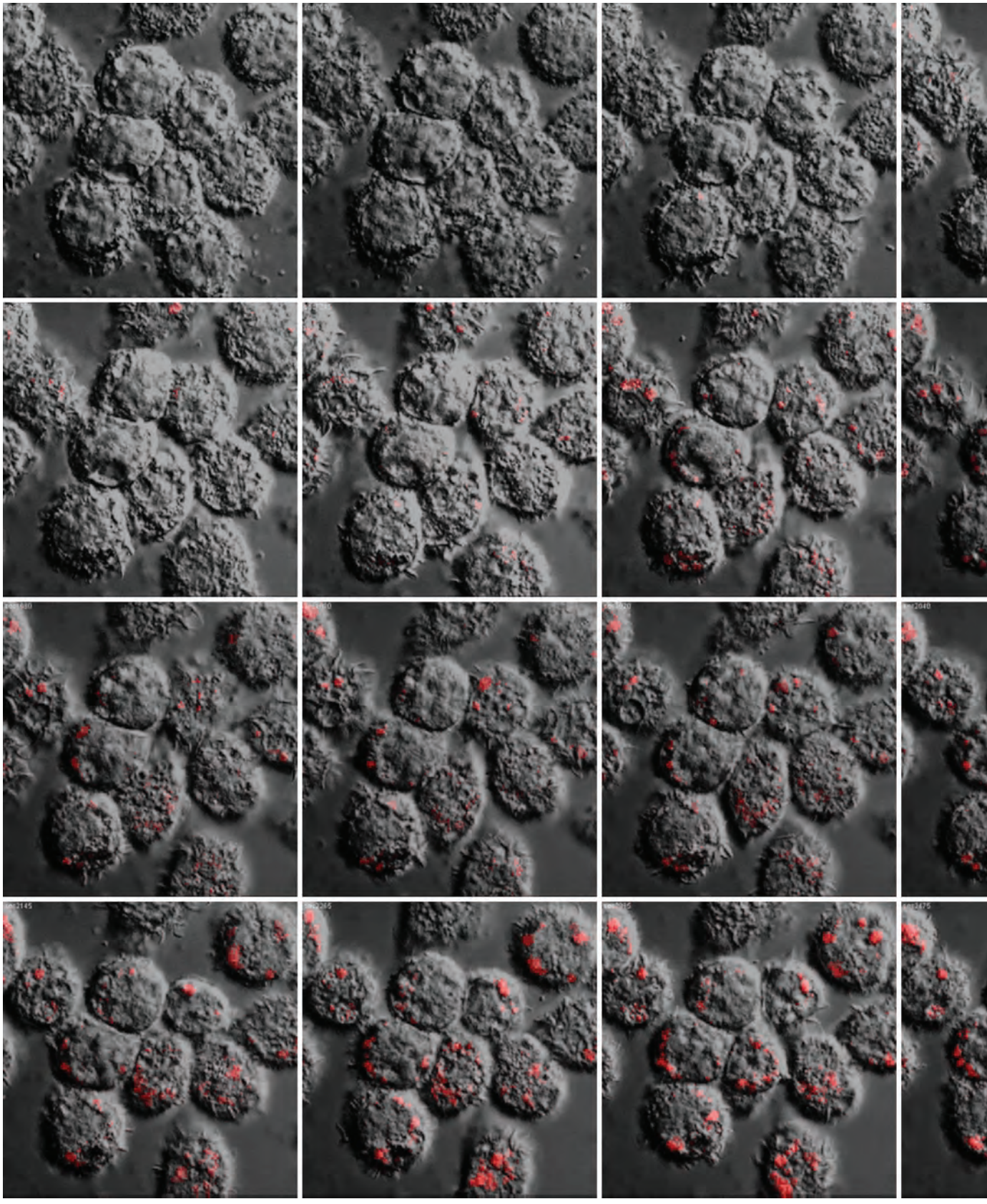

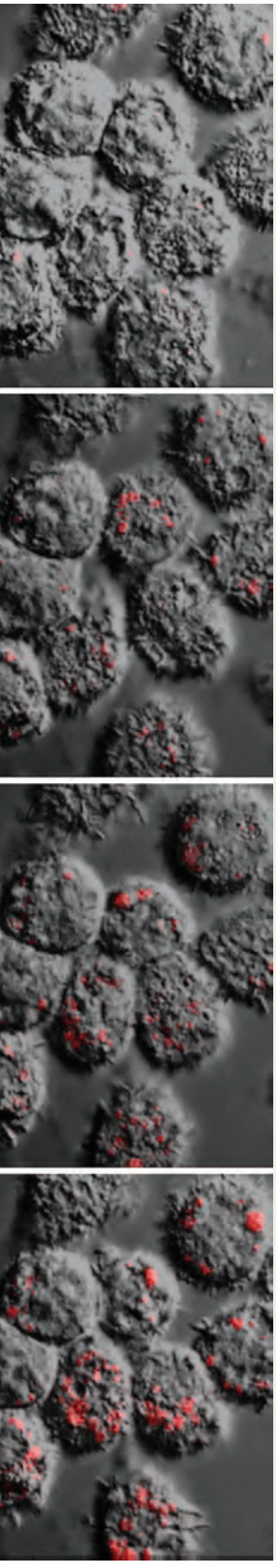

Putting the pH in phagocytosis

A NO-WASH, NO-QUENCH ASSAY BASED ON THE pH-SENSITIVE pHRODO™ DYE.

When invading microbes get under your skin—the body's first line of defense—macrophages, neutrophils, and dendritic cells take up the charge. These phagocytic cells attack bacteria and foreign particles, swarming them with filopodial tentacles and drawing them in for the kill. As the foreign invaders are engulfed, they are subjected to intense acidification,[1] reducing them to harmless bits of protein that can be processed by the cell. Immunologists study the process of phagocytosis to find out how to boost the immune system, to understand what goes wrong in certain disease states, and even to learn how to suppress immune system activity following organ transplants. Toxicologists use phagocytic activity as a measure of the health of an organism's immune system when testing for the effects of harmful pollutants. With the new pH-sensitive pHrodo™ dye, researchers can now take the study of phagocytosis to the next level in sensitivity and convenience. →

Figure 1—Time-lapse images showing internalization and acidification of the pHrodo™ *E. coli* BioParticles® conjugate during phagocytosis. Murine J774A.1 cells were maintained at 37°C using a stage heater. Using excitation with a 561 nm laser at 20% intensity, fluorescence images were recorded in the 570–699 nm emission range and white light DIC images were recorded on a separate PMT. Images were collected every 30 seconds for 83.5 minutes (not all images are shown) using a Leica TCS SP5 laser confocal microscope employing a 63×/1.4 NA oil objective and the resonant galvanometer scanner mode (8000 Hz). Image contributed by Lucy Deriy and Deborah Nelson, University of Chicago.

Figure 2—Imaging of pHrodo™ dye–labeled *E. coli* and fluorescein-labeled *E. coli* with and without trypan blue. Wells containing J774 macrophage cells were treated with (A) 1 mg/ml pHrodo™ *E. coli* BioParticles® conjugate, with no washing or quenching steps; (B) fluorescein-labeled *E. coli,* with no washing or quenching steps; and (C) fluorescein-labeled *E. coli*, followed by a wash step, then treatment with trypan blue. Panels A and C show comparable results, but panel C required the use of a quencher dye with subsequent washes, and the resulting signal was not as bright. Panel B shows the high background fluorescence that results when cells treated with fluorescein-labeled BioParticles® conjugates are not washed and no quencher dye is used. All images were obtained using an Axiovert 200M inverted microscope (Zeiss) at 40× magnification. Exposure times were set at 200 ms, and images were digitized using a CoolSNAP™ camera (Photometrics) and MetaMorph® software (Universal Imaging).

Existing protocols introduce variability

The traditional method of measuring phagocytosis employs fluorescein–labeled bacteria that are ingested by the cells under study. The extracellular fluorescence resulting from unengulfed bacteria must be reduced by washing and then applying a photon-absorbing dye such as trypan blue.[2] Unfortunately, this approach also reduces the signal inside the cells and may not completely quench the fluorescence of the extracellular bacteria, especially those that are attached to the outside of the cell but not internalized. Furthermore, these wash and quench steps frequently lead to assay variability; this is especially challenging in microplate assays, where small differences in technique can vary the number of cells and amount of trypan blue left in the wells, greatly affecting the fluorescence signal.

Eliminate variability with the no-wash, no-quench pHrodo™ dye

To address these issues, we have developed a no-wash, no-quench assay for phagocytosis using *E. coli* conjugated to the novel pHrodo™ dye. Most pH sensors rely on the quenching of fluorescein by acidic pH; in contrast, the fluorogenic pHrodo™ dye dramatically increases in fluorescence as the pH becomes more acidic, providing bright and specific signals (Figure 1). Because the bacteria outside the cell exhibit minimal signal in the relatively alkaline conditions of the saline buffer, there is no need to wash, quench, or even remove the extracellular bacteria. Simply add the pHrodo™ dye–labeled bacteria to your cells, wait for the bacteria to be ingested, and measure fluorescence. Samples can also be fixed with paraformaldehyde and analyzed up to 24 hours later; results may vary based on experimental conditions.

Fewer steps to clearer, brighter images

Figure 2 shows imaging data comparing the traditional method of measuring phagocytosis, fluorescein-labeled *E. coli* with trypan blue, with the new fluorogenic method based on the pH-sensitive pHrodo™ dye. The pHrodo™ *E. coli* BioParticles® conjugate produces brighter images and requires fewer steps to achieve the desired results. A comparison of these techniques in microplate-reader format demonstrates that the pHrodo™ dye signal is significantly greater (285,000 ± 6,360 RFU vs. 194,000 ± 7,370 RFU, $p < 0.0001$, unpaired t-test, $n = 4$) and less prone to variability from experiment to experiment. If desired, you can significantly increase the uptake by macrophage cells with opsonization of the pHrodo™ dye–labeled *E. coli* (644,000 ± 12,600 RFU vs. 353,000 ± 4,500 RFU, $p < 0.0001$, unpaired t-test, $n = 4$). As shown in Figure 3, the microplate-based measurements are sensitive enough to quantitate the effects of both inhibitors and enhancers of phagocytosis. Uptake of the pHrodo™ BioParticles® conjugate in J774.2 cells was dose-dependently inhibited by cytochalasin D, a known inhibitor of phagocytosis, and dose-dependently stimulated by pretreating cells with interferon gamma.

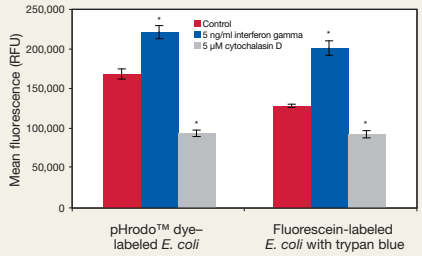

Figure 3—Modulation of uptake by inhibitors and stimulators of phagocytosis. The addition of 5 µM of cytochalasin D, a fungal metabolite that inhibits the function of actin filaments and contractile microfilaments, produced a significant reduction in signal. Overnight treatment with 5 ng/ml of interferon gamma, a known stimulator of phagocytosis, produced a significant increase in signal ($p < 0.001$, one-way ANOVA with Tukey-Kramer multiple comparisons; * indicates statistical significance).

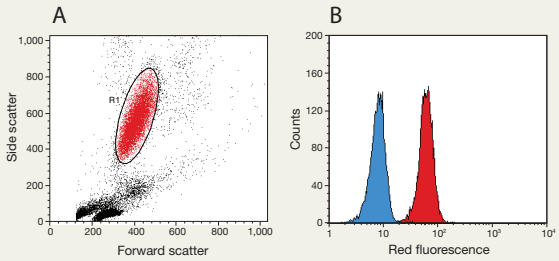

Figure 5—Flow cytometric analysis showing increased fluorescence of granulocytes treated with pHrodo™ dye–labeled *E. coli*. A whole blood sample was collected and treated with heparin, and two 100 µl aliquots were prepared. Both aliquots were treated with pHrodo™ dye–labeled *E. coli* and vortexed. One sample was placed in a 37°C water bath, and the other sample (negative control) was placed in an ice bath. After a 15 minute incubation, red blood cells were lysed with an ammonium chloride–based lysis buffer. The samples were centrifuged for 5 minutes at 500 rcf, washed once, and resuspended with HBSS. The samples were then analyzed on a BD FACSCalibur™ cytometer (BD Biosciences) using a 488 nm argon laser and 564–606 nm emission filter. (A) Granulocytes were gated using forward and side scatter. (B) The sample incubated at 37°C shows the increased fluorescence of the phagocytosed pHrodo™ dye–labeled *E. coli* (red), in contrast to the negative control sample, which was kept on ice to inhibit phagocytosis (blue).

Multiplexing capabilities of the pHrodo™ dye

With emission in the red wavelengths, peaking at ~590 nm, the pHrodo™ dye is ideal for use in cells expressing Green Fluorescent Protein (GFP) or for multiplexing with other green-fluorescent indicators such as fluo-4 or calcein AM (Figure 4). Although its peak excitation is around 560 nm, the pHrodo™ dye can be excited by the 488 nm laser line in a flow cytometer. Figure 5 shows a clear (roughly 10-fold) increase in the fluorescence of freshly isolated granulocytes incubated with pHrodo™ dye–labeled *E. coli* at 37°C, compared with a negative control sample stored on ice.

The future of pHrodo™ dye–based assays

This pH-sensitive dye is available as a pHrodo™ *E. coli* BioParticles® conjugate or as an amine-reactive pHrodo™ succinimidyl ester. The pHrodo™ *E. coli* BioParticles® conjugate can be used in both microplate-based and imaging assays of macrophage activity. pHrodo™ dye–labeled *E. coli* have also proven useful in flow cytometry measurements of primary granulocyte phagocytic activity. The amine-reactive pHrodo™ SE dye, available separately, can potentially be used for labeling bacteria and yeast to study how these microbes interact with immune cells. Alternatively, pHrodo™ dye–labeled antibodies or peptides may be useful for following internalization and other pH-dependent processes. To learn more about the novel pHrodo™ dye, visit **probes.invitrogen.com**. ■

References

1. Di, A. et al. (2006) *Nat Cell Biol* 8:933–944.
2. Wan, C.P. et al. (1993) *J Immunol Methods* 162:1–7.

Product	Quantity	Cat. no.
pHrodo™ *E. coli* BioParticles® conjugate for phagocytosis	5 x 2 mg	P35361
pHrodo™, succinimidyl ester (pHrodo™, SE)	1 mg	P36600
Escherichia coli BioParticles® opsonizing reagent	1 U	E2870

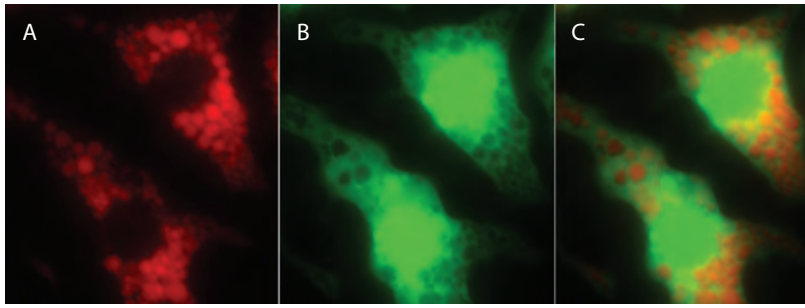

Figure 4—Multiplex imaging with the pHrodo™ dye. Macrophage cells were incubated in HBSS with 1 mg/ml pHrodo™ *E. coli* BioParticles® conjugate (A), then with 5 µM calcein AM (B). Cells were imaged on a Axiovert 200M inverted microscope (Zeiss) at 40× magnification. The green-fluorescent calcein AM was used as a cytosolic stain to show the outlines of the macrophage cells, demonstrating that the pHrodo™ dye–labeled *E. coli* are relatively nonfluorescent outside the cell. Only after internalization and acidification of the phagosome is the red fluorescence clearly visible. As shown in the overlay (C), the red emission of the pHrodo™ dye allows easy multiplexing with green-fluorescent probes.

Cells from the inside out

ORGANELLE LIGHTS™ TARGETED FLUORESCENT PROTEINS.

Context is everything

When cellular events are studied in isolation from the rest of the cell, part of the story is inevitably lost. Likewise, fixed-endpoint measurements can capture an accurate snapshot, but their utility is limited when examining dynamic cellular processes. The loss in complexity inherent in these types of static measurements can be a useful first step in unraveling a tangled series of interactions. Ultimately, however, examining a particular cellular pathway in both its spatial and temporal context is critical for understanding cell development and functioning.

In the context of a functioning cell, fluorescent organelle probes can serve as important landmarks, allowing you to map a subcellular location, track morphological changes, and follow cell development. Molecular Probes® Organelle Lights™ reagents combine the selectivity of a targeted autofluorescent protein with the transduction efficiency of BacMam technology to enable unambiguous visualization of organelles and other subcellular structures in live mammalian cells by fluorescence microscopy (Figure 1). These reagents open up a new avenue for studying dynamic cellular events in real space and time.

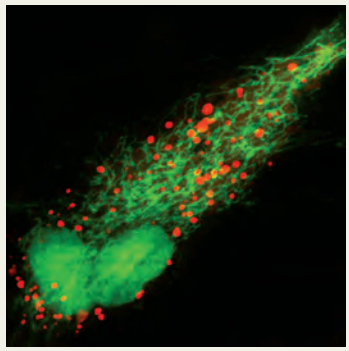

Figure 1—Multiplex analysis with Organelle Lights™ reagents and Qtracker® nanocrystals. Porcine primary skeletal muscle cells were transduced with Organelle Lights™ ER-GFP and Organelle Lights™ Nuc-GFP. Simultaneously, cells were incubated with far-red–fluorescent Qtracker® 655 nanocrystals, which contain a targeting peptide that directs their uptake into cytoplasmic vesicles. After 30 minutes at room temperature, cells were treated with the Organelle Lights™ enhancer and then incubated overnight at 37°C before imaging by fluorescence microscopy using a 63× objective. Image contributed by Ann Bazar, Michael Rutten, and Kenton Gregory, Oregon Medical Laser Center Bioimaging Suite, Providence St. Vincent Hospital, Portland, Oregon.

Table 1—Organelle Lights™ reagents and their targeting sequences.

Organelle or other subcellular structure	Targeting sequence	Ref	CFP (435/485)*	GFP (485/520)*	YFP (500/535)*	OFP (550/580)*
Cytoplasm	Nuclear export sequence (C-terminus)	1		O36227		
Endoplasmic reticulum (ER)	ER signal sequence of calreticulin and KDEL (ER retention signal)	2		O36212		O36223
Golgi apparatus	Human Golgi-resident enzyme N-acetylgalactosaminyltransferase-2	3		O36215		O36224
Mitochondria	Leader sequence of E1alpha pyruvate dehydrogenase	4		O36210		O36222
Nuclear envelope (NE)	Nesprin 1alpha C-terminal transmembrane domain (aa 923–982)	5		O36213		
Nucleus	SV40 nuclear localization sequence (C-terminus)	6	O36218	O36209	O36219	O36220
Peroxisomes	Peroxisomal C-terminal targeting sequence	7		O36211		O36225
Plasma membrane (PM)	Myristoylation/palmitoylation sequence from LCK tyrosine kinase	8	O36216	O36214	O36217	O36226

*Excitation and emission maxima, in nm; CFP (Cyan Fluorescent Protein), GFP (Green Fluorescent Protein), and YFP (Yellow Fluorescent Protein) can all be seen through a fluorescein (FITC) optical filter, whereas OFP (Orange Fluorescent Protein) can be seen through a tetramethylrhodamine (TRITC) optical filter.

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Visualize subcellular structures *in vivo* with Organelle Lights™ reagents

Organelle Lights™ reagents provide a convenient and highly efficient tool for delivering organelle- and membrane-targeted autofluorescent proteins (e.g., GFP and its color mutants) to mammalian cells. Each Organelle Lights™ reagent is based on a baculovirus that, upon entry into a mammalian cell, directs the expression of autofluorescent proteins targeted to specific subcellular compartments by means of a well-documented signal sequence or targeting domain (Table 1). Available autofluorescent proteins include the blue-fluorescent CFP, green-fluorescent GFP, yellow-fluorescent YFP,[1] and orange-fluorescent OFP,[2] with emission maxima of 485, 520, 535, and 580 nm, respectively. Genes for these proteins have been fused in frame with targeting sequences to produce autofluorescent fusion proteins that selectively localize in the endoplasmic reticulum (ER), Golgi apparatus, mitochondria, peroxisomes, nucleus, nuclear envelope, plasma membrane, or cytoplasm[3] (Figures 1 and 2).

BacMam technology delivers the goods

Delivery of these genetically encoded autofluorescent proteins is achieved using BacMam technology, a highly efficient method for using a modified baculovirus to introduce genes into mammalian cells.[4–6] Baculoviruses are a family of insect viruses that have been

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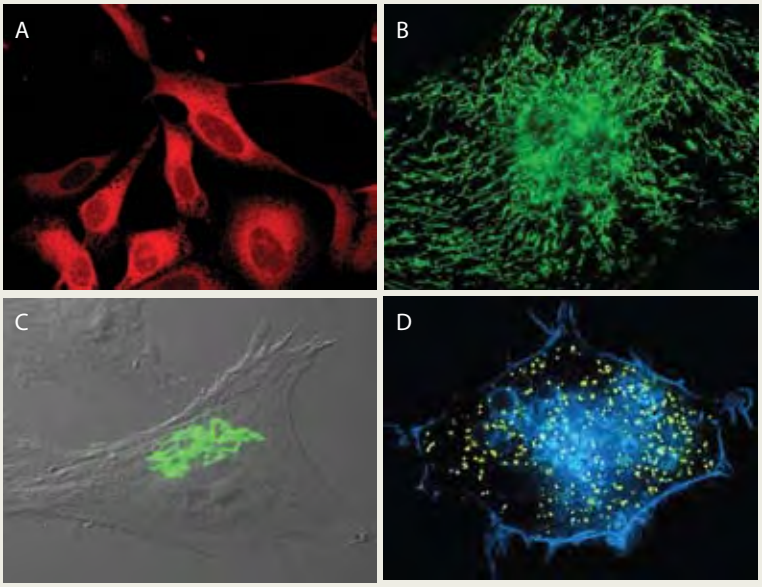

Figure 2—Organelle Lights™ reagents targeted to a variety of cellular organelles and membranes. (A) U2OS cells were transduced with Organelle Lights™ ER-OFP and imaged in live-cell format using a 40× objective. (B) Indian Muntjac deer epidermis fibroblast cells were transduced with Organelle Lights™ Mito-GFP and then fixed prior to imaging using a 100× objective. (C) FoLu (Gray Fox Lung) cells were transduced with Organelle Lights™ Golgi-GFP and then fixed prior to imaging by fluorescence and DIC microscopy using a 100× objective. (D) HEK 293 cells were transduced with Organelle Lights™ PM-CFP and Organelle Lights™ Peroxi-YFP and then imaged in live-cell format using a 100× objective. Images in (B) and (C) were contributed by Michael Davidson, Molecular Expressions, Tallahassee, FL.

Synchronicity: Genetically encoded fluorescent probes paired with the BacMam delivery system

Working in concert with collaborators in both academia and industry, we are developing a suite of genetically encoded fluorescent probes—including the Organelle Lights™ reagents and Premo™ cameleon calcium sensor—that use the BacMam viral transduction system. These qualitative and quantitative measurement tools provide real-time measurements in functioning cells, even difficult-to-transfect progenitor and primary cell types.

Premo™ cameleon calcium sensor is a genetically encoded, FRET-based calcium indicator capable of ratiometric calcium measurements in live cells. Active for several days, Premo™ cameleon sensor allows iterative calcium assays with a ratiometric readout that normalizes artifactual variations in fluorescence signals. For more information on the Premo™ sensor, see the opening article in *BioProbes* 52 (which can be found at **probes.invitrogen.com/bioprobes**) or visit **www.invitrogen.com/premo**. And watch for additional genetically encoded fluorescent probes from Invitrogen in the coming year.

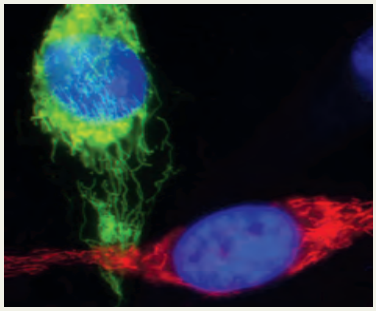

Figure 3—Differentiating two Organelle Lights™ reagents in the same experiment. HeLa cells were transduced with either Organelle Lights™ Mito-GFP or Organelle Lights™ Mito-OFP (pseudocolored red) and counterstained with Hoechst 33342. These cell populations were then mixed together and imaged in live-cell format using a 40× objective.

employed to produce engineered protein in insect cells for over two decades; however, use of baculoviruses to deliver genes to mammalian cells is relatively new. As an enabling technology, BacMam delivery system has several important advantages, including highly reproducible and titratable expression, reliance on a biosafety level 1 reagent (baculoviruses do not replicate in mammalian cells and no viral genes are expressed), and potential for simultaneous delivery of multiple genes. These features, coupled with its lack of microscopically observable cytopathic effects in commonly studied cell types, make the BacMam delivery system a straightforward and reliable method of gene delivery for mammalian cells.

Organelle Lights™ reagents are prepackaged as baculoviral particles for convenient transduction of a broad range of mammalian cell types, including primary and stem cells, without the need for lipids or dye-loading protocols that can perturb cell growth and viability. Because the Organelle Lights™ reagents are ready to use and provided at a constant concentration, there is no need to purify plasmids or prepare transfection complexes. Just add Organelle Lights™ reagent to your cells for two hours, treat with the provided enhancer solution to increase chimera expression, wash, and incubate overnight to visualize results. Each Organelle Lights™ reagent comes with a detailed protocol, as well as full rights to use the CFP, GFP, YFP, and OFP vectors in this application.

Organelle Lights™ reagents are compatible with a variety of other fluorescent labels

Organelle Lights™ reagents can be multiplexed with other Organelle Lights™ reagents of different colors (Figures 2D and 3), as well as with fluorescent dyes and Qdot® nanocrystals, allowing you to colocalize multiple labels to a single organelle or membrane or to monitor multiple cellular structures in a single cell (Figure 4). As proteins, the Organelle Lights™ organelle- and membrane-targeted probes are also compatible with fixation and subsequent immunocytochemical analysis. Moreover, they complement our wide selection of organelle-selective, organic dye–based fluorescent probes for live cells (Table 2), which include MitoTracker®, LysoTracker®, and ER-Tracker™ reagents. To find out more about Organelle Lights™ reagents, visit **www.invitrogen.com/olights**. ■

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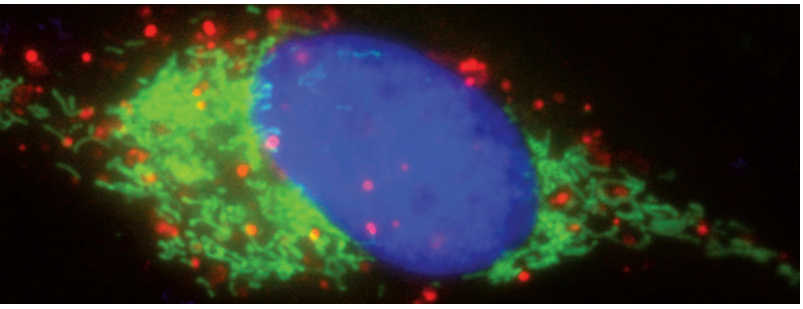

Figure 4—Multiplex analysis with Organelle Lights™ Mito-GFP and LysoTracker® Red DND-99. U2OS cells were transduced with green-fluorescent Organelle Lights™ Mito-GFP and then stained with red-fluorescent LysoTracker® Red DND-99, which selectively labels acidic organelles. Following nuclear counterstaining with blue-fluorescent Hoechst 33342, cells were imaged in live-cell format using a 63× objective.

Table 2—Selected probes for staining organelles and other subcellular structures in live cells.

Probe	Fluorescence emission*				Key attributes
	Blue	Green and yellow	Orange and red	Far-red	
Cytoplasm					
CellTracker™ probes	C2110	C7025	C2927 C34552		Cell-permeant, thiol-reactive CellTracker™ probes provide uniform cytoplasmic staining and are well retained in cells through generations.
Cytoskeleton					
TubulinTracker™ Green probe		T34075			TubulinTracker™ Green is a cell-permeant, fluorescent paclitaxel derivative that stains polymerized tubulin in live cells.
Endoplasmic reticulum (ER)					
ER-Tracker™ Green and ER-Tracker™ Red probes		E34251	E34250		These cell-permeant BODIPY® glibenclamide derivatives provide ER-selective staining with minimal mitochondrial staining.
Golgi apparatus					
BODIPY® ceramides and BODIPY® ceramides complexed with BSA		D3521 B22650	D7540 B34400		BODIPY® ceramides selectively stain the Golgi apparatus; accumulation of the green-fluorescent BODIPY® FL C_5-ceramide in the trans-Golgi is sufficient to form red-fluorescent excimers.
Lysosomes					
LysoTracker® probes	L7525	L7526	L7528		LysoTracker® probes are cell-permeant, fluorescent acidotropic reagents for labeling acidic organelles at nanomolar concentrations.
Image-iT™ LIVE Lysosomal and Nuclear Labeling Kit			I34202		This kit contains LysoTracker® Red DND-99 and Hoechst 33342 for selectively staining lysosomes and nuclei, respectively.
LysoSensor™ probes	L7533	L7534 L7545			LysoSensor™ probes are pH indicators that partition into acidic organelles and exhibit pH-dependent fluorescence increase upon acidification.
Mitochondria					
MitoTracker® probes		M7514†	M7510 M22425† M7512	M22426	MitoTracker® probes are cell-permeant, mitochondrion-selective dyes that contain a mildly thiol-reactive chloromethyl moiety and are concentrated by active mitochondria and generally well retained during fixation.†
Image-iT™ LIVE Mitochondrial and Nuclear Labeling Kit			I34154		This kit contains MitoTracker® Red CMXRos and Hoechst 33342 for selectively staining mitochondria and nuclei, respectively.
Reduced MitoTracker® probes			M7511 M7513		Reduced MitoTracker® probes are not fluorescent until oxidized to the mitochondrion-selective probe and sequestered in the mitochondria.
JC-1		T3168†			At high concentrations or high membrane potentials, the green-fluorescent JC-1 monomer forms red-fluorescent J-aggregates, making it a useful dual-emission indicator of mitochondrial membrane potential.
Nucleoli					
SYTO® RNASelect™ Green stain		S32703			This cell-permeant RNA-selective stain exhibits prominent nucleoli staining, with weaker nuclear, cytoplasmic, and mitochondrial staining.
Nucleus					
Hoechst 33342	H1399 H3570				The cell-permeant Hoechst 33342 dye has been extensively used for staining the nuclei of live cells.
SYTO® nucleic acid stains		S7576	S11363 S11341	S11343	SYTO® stains are cell-permeant cyanine dyes that exhibit strong fluorescence enhancement when bound to nucleic acids.
Plasma membrane					
FM® 1-43FX and FM® 4-64FX			F35355	F34653	Amphiphilic FM® dyes are easily applied to cells, where they rapidly bind to the plasma membrane with strong fluorescence enhancement.
Alexa Fluor® wheat germ agglutinin (WGA)	W11263	W11261	W32464 W11262	W21404 W32466 W32465	Fluorescent WGA conjugates bind to sialic acid and *N*-acetylglucosaminyl residues on the outer surface of plasma membranes.
Image-iT™ LIVE Plasma Membrane and Nuclear Labeling Kit		I34406			This kit contains Alexa Fluor® 488 WGA and Hoechst 33342 for selectively staining plasma membranes and nuclei, respectively.
Intracellular membranes					
CellTrace™ BODIPY® TR methyl ester			C34556		This CellTrace™ probe selectively stains mitochondria and endomembranous organelles but does not localize in the plasma membrane.
Image-iT™ LIVE Intracellular Membrane and Nuclear Labeling Kit			I34407		This kit contains CellTrace™ BODIPY® TR methyl ester and Hoechst 33342 for selectively staining intracellular membranes and nuclei, respectively.

*Fluorescence colors are based on the emission maximum of each probe, regardless of how broad the spectrum is; selective filters can be used to limit the emission wavelengths detected. Visit **probes.invitrogen.com** for complete spectral information. Except for those marked with an †, these probes are fixable by aldehyde fixatives for subsequent immunochemical analysis.

Breakthrough cell proliferation assay for flow cytometry

THE CLICK-IT™ EDU KIT.

Detection of cell proliferation is a fundamental method for the assessment of cell health, determination of genotoxicity, and evaluation of anticancer drugs. The most accurate method uses the direct measurement of new DNA synthesis. Traditionally, this is performed by the incorporation of the nucleoside analog bromodeoxyuridine (BrdU) into DNA followed by detection with an anti-BrdU antibody. Although effective, this method requires DNA denaturation (using HCl or DNase) to expose the BrdU to the antibody—a step that is often lengthy and difficult to perform consistently. As a result, many BrdU-based assays require significant fine-tuning for use in flow cytometry applications. An alternative assay to the BrdU method monitors the incorporation of radioactive ^{3}H-thymidine, but involves all of the safety and waste disposal difficulties inherent in radioisotope-based methods. Additionally, assays that measure total DNA (e.g., CyQUANT® or DAPI staining) can be useful, but really only give information about cell populations rather than individual cells.

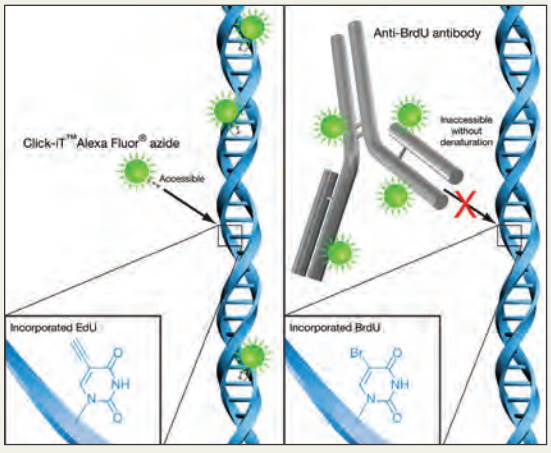

Figure 1—Overview of the Click-iT™ EdU assay compared with BrdU-based detection.

Click-iT™ chemistry—Small functional groups, huge potential

Click-iT™ chemistry is a powerful method for joining biomolecules quickly and specifically, and offers significant advantages over BrdU assays. Click-iT™ chemistry involves a copper-catalyzed covalent reaction between an alkyne and an azide, an extremely efficient reaction that utilizes functional groups that do not occur naturally in biological systems. This uniqueness virtually eliminates the nonspecific binding or high background that can compromise other widely used detection methods, including antibody/antigen and biotin/streptavidin interactions.

The Click-iT™ EdU assay

In the Click-iT™ EdU Alexa Fluor® 488 Cell Proliferation Assay Kit, the alkyne is a modified nucleoside (5-ethynyl-2´-deoxyuridine, or EdU) similar to BrdU, and the azide is a modified fluorescent dye (Alexa Fluor® 488 azide). The small size of the Alexa Fluor® 488 azide allows for efficient access to the DNA without the need for harsh cell treatment, and simplifies the assay considerably (Figures 1 and 2). By using the advantages of the Click-iT™ reaction, the Click-iT™ EdU Alexa Fluor® 488 Cell Proliferation Assay Kit eliminates the need to denature DNA, providing a superior alternative to the standard BrdU antibody–based method for measuring cell proliferation by flow cytometry (Figure 3). The reaction is complete in 15–30 minutes, can be used with adherent cells or cell suspensions, and forms a highly stable covalent bond for bright and specific fluorescence detection.

Quantitate S-phase cells

Recent experiments have illustrated the utility of the Click-iT™ EdU assay for the high-throughput quantitation of S-phase adherent

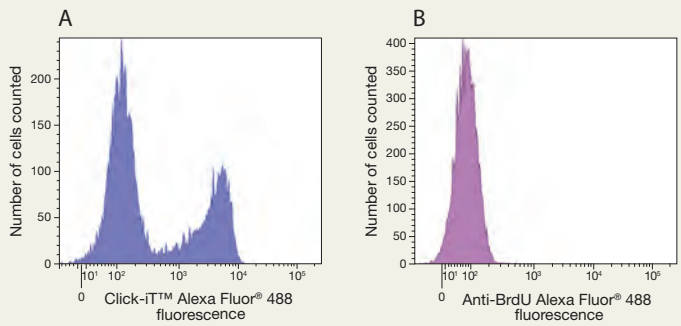

Figure 2—Click-iT™ chemistry works without a harsh denaturation step. Jurkat cells were analyzed using a standard aldehyde-based protocol. (A) Results obtained using the Click-iT™ detection method. (B) The antibody-based assay is not able to reach the incorporated BrdU without DNA denaturation.

cells in a population, an important characteristic for evaluating the cellular response to certain drugs. Treatment of A549 (human alveolar basal epithelial) cells with etoposide (a potent S-phase inducer) followed by Click-iT™ EdU labeling produced an S-phase population that was clearly visible by flow cytometry and fluorescence microscopy. The method is amenable to multiplexing with other chemical- and antibody-based fluorescence labeling techniques for content-rich assays.

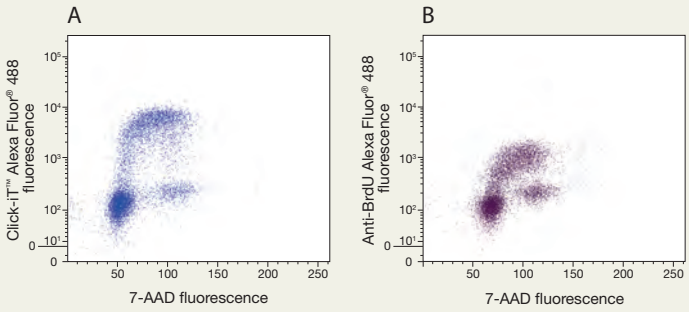

Figure 3—Results obtained using the Click-iT™ EdU reagents typically surpass those from the BrdU assay. (A) Results obtained using the new Click-iT™ EdU detection method, showing a dual-parameter plot of Click-iT™ Alexa Fluor® 488 azide vs. 7-AAD cell cycle staining. (B) Results using the standard acid denaturation method for the antibody-based detection of incorporated BrdU, showing a dual-parameter plot of anti-BrdU Alexa Fluor® 488 vs. 7-AAD cell cycle staining.

Everything you need, easy to perform

The Click-iT™ EdU protocol is based on the aldehyde fixation and detergent permeabilization steps typically used for immunohistochemical antibody labeling. In just two hours and three steps, you'll be ready to analyze your cell proliferation data:

1. Fix cells for 15 minutes; wash once.
2. Permeabilize cells for 30 minutes; wash once.
3. Incubate cells with Click-iT™ labeling cocktail for 30 minutes; wash once. (Optional: incubate with cell cycle stains for 15–30 minutes.)

The Click-iT™ EdU Cell Proliferation Assay Kit provides everything you need to perform 50 assays, including the nucleoside analog EdU and all components for fixation, permeabilization, and labeling. Two red-fluorescent cell cycle stains are included for use with 488 nm or 633 nm excitation. The EdU assay can also be multiplexed with surface and intracellular marker detection using antibodies labeled with small organic dyes such as the Alexa Fluor® 647 dye. If fluorescent proteins including phycobiliproteins such as R-PE and its tandems are used, the staining step must occur after the click labeling step.

Learn more about the Click-iT™ EdU assay at **probes.invitrogen.com/flowcytometry**. And while you're there, explore CytoGenie™ reagent selection software (see page 5) and see how easy multicolor flow cytometry can be. ■

Product	Quantity	Cat. no.
Click-iT™ EdU Alexa Fluor® 488 Cell Proliferation Assay Kit, for flow cytometry	1 kit, 50 assays	C35002

Looking for apoptosis?

NEW CLEAVED PARP–SPECIFIC ANTIBODIES FOR DETECTING AND QUANTIFYING ULTRALOW LEVELS OF APOPTOSIS.

PARP cleavage is an important apoptosis marker

Apoptosis, or programmed cell death, is essential to the development, immunological competence, and homeostasis of life. In a heterogeneous cell population, detecting those cells undergoing apoptosis requires the ability to see and measure key molecular events along this pathway. PARP cleavage is an established and reliable apoptosis indicator downstream of caspase activation.

During apoptosis, poly(ADP-ribose) polymerase (PARP)—a 116 kDa nuclear protein that normally functions in DNA damage detection and repair—is cleaved by caspase-3 [1–4] and caspase-7 [2] between Asp214 and Glyn215 to yield the p85 and p25 fragments. This cleavage effectively neutralizes the ability of PARP to participate in DNA repair, and contributes to a cell's commitment to undergo apoptosis.[3] Sensitive detection of PARP cleavage therefore allows you to distinguish different types of cell death and quantify apoptotic cells in a population.

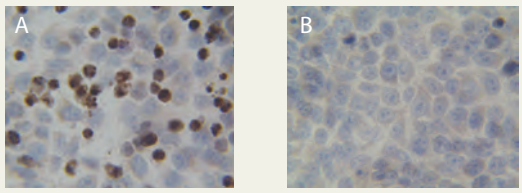

Figure 1—Detection of PARP cleavage in apoptotic HeLa cells by immunocytochemistry. HeLa cells were treated with 0.5 µM staurosporine for 5 hours to induce apoptosis (A) or left untreated as a control (B). Cells were then fixed in cold acetone for 5 minutes and incubated with anti–PARP p85 antibody (20 µg/ml) and biotinylated goat F(ab')$_2$ anti–rabbit IgG antibody, followed by ABC and DAB.

Detect apoptosis with antibodies specific for PARP cleavage

Invitrogen has developed an antibody highly specific to the p85 fragment of cleaved PARP (Table 1). Significantly, this cleavage site–specific

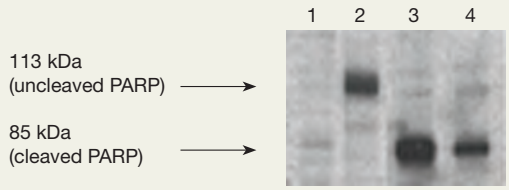

Figure 2—Detection of PARP cleavage in cell extracts by western blotting. Cell extracts were prepared from HeLa cells induced with staurosporine as described in Figure 1. Proteins from these cell extracts were resolved by SDS-PAGE on a 4–20% Tris-glycine gel and transferred to PVDF membranes, which were then incubated with either 1 µg/ml anti-PARP antibody or 1 µg/ml anti–PARP p85 antibody, followed by alkaline phosphatase–labeled goat F(ab')$_2$ anti–rabbit IgG antibody and BCIP/NBT. Lane 1, extracts from uninduced cells incubated with anti-PARP antibody; lane 2, uninduced cells, anti–PARP p85 antibody; lane 3, induced cells, anti-PARP antibody; lane 4, induced cells, anti–PARP p85 antibody.

Table 1—PARP-specific products for detecting and quantifying apoptosis.

Product	Conjugate	Reactivity*	Applications†	Quantity	Cat. no.
anti-PARP, mouse monoclonal IgG1κ, clone C-2-10 (Zymed®)	unconjugated	Hu, Ms, Rt, B, Mk, Rb	WB, ELISA, IF, IHC	100 µl	33-3100
anti–PARP [214/215] cleavage product p85, rabbit polyclonal IgG (BioSource™)	unconjugated	Hu, Ms, Rt, B	WB, IHC	100 µl	44-698G
anti–PARP [214/215] cleavage product p85, rabbit polyclonal IgG (BioSource™)	FITC conjugate	Hu, B	FC	100 tests	44-699
PARP Cleavage [214/215] ELISA Kit (BioSource™)	NA	Hu	ELISA	96 tests	KHO0741

*Hu = human; Ms = mouse; Rt = rat; B = bovine; Mk = monkey; Rb = rabbit. †WB = western blot; ELISA = enzyme-linked immunosorbent assay; FC = flow cytometry; IF = immunofluorescence; IHC = immunohistochemistry.

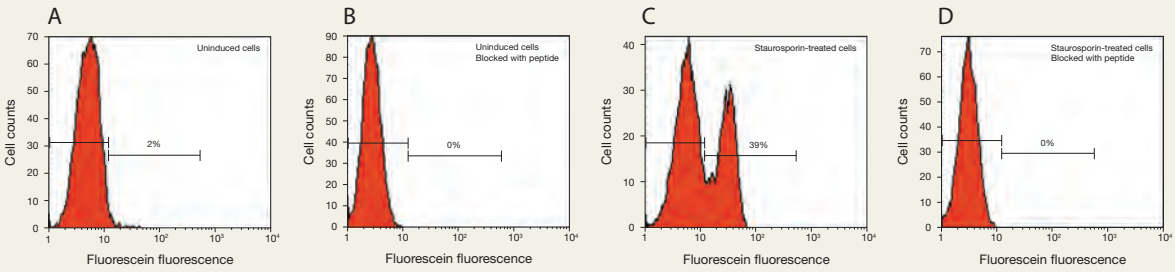

Figure 3—Detection of apoptotic HeLa cells by flow cytometry using FITC-conjugated anti–PARP p85 antibody. HeLa cells were treated with 0.5 µM staurosporine for 5 hours at 37°C to induce apoptosis (C, D) or left untreated as a control (A, B). Following fixation, cells were stained with 10 µl FITC-conjugated anti–PARP p85 antibody for 30 minutes and analyzed by flow cytometry. The data show that FITC-conjugated anti–PARP p85 antibody stains a subset of the induced HeLa cells (C, 39%) but not the uninduced HeLa cells (A). Furthermore, the positive staining in apoptotic cells was effectively blocked with a synthetic peptide corresponding to the C-terminus of cleavage site 214/215 on human PARP (D), demonstrating the specificity of the FITC-conjugated anti–PARP p85 antibody.

antibody to PARP can be used in several different experimental formats for detecting and quantifying apoptosis.

In conjunction with labeled secondary antibodies, the anti–PARP p85 antibody can efficiently detect cleaved PARP in fixed and permeabilized apoptotic cells using immunocytochemical techniques (Figure 1). In western blots, the anti–PARP p85 antibody easily identifies the cleaved PARP fragment in extracts prepared from apoptotic cells, yet does not react with native PARP in uninduced-cell extracts (Figure 2). In contrast, the conventional anti-PARP antibody reacts with both native PARP and the p85 fragment and therefore cannot be used to detect apoptotic cells by immunocytochemistry or flow cytometry.

This cleavage site–specific antibody to PARP is also compatible with flow cytometry, which offers a rapid and convenient method for quantifying apoptotic events in a cell population. Populations of apoptotic and nonapoptotic cells could be readily resolved by flow cytometry using a FITC-conjugated anti–PARP p85 antibody to detect cleaved PARP (Figure 3). Furthermore, the anti–PARP p85 antibody labeling of apoptotic cells was completely blocked when a synthetic peptide corresponding to the C-terminus of the PARP cleavage site was included during the antibody incubation, further demonstrating the specificity of the anti–PARP p85 antibody.

ELISA is 100× more sensitive than colorimetric caspase-3 assays

The new PARP Cleavage [214/215] ELISA Kit employs the anti–PARP p85 antibody in a solid-phase sandwich assay to detect and quantify ultralow levels of the human PARP p85 fragment. With the convenience of a ready-to-use format and a detection level 100× lower than that achieved with caspase-3 protease assays (Figure 4), this kit allows you to detect apoptosis that might otherwise be missed with traditional enzyme assays. And like the anti–PARP p85 antibody products, this new ELISA kit offers high specificity for cleaved PARP, superior signal-to-noise ratios, and excellent lot-to-lot consistency. ■

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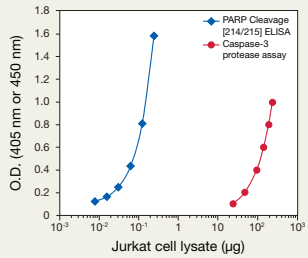

Figure 4—Comparison of the sensitivity of the PARP Cleavage [214/215] ELISA Kit and the Caspase-3 Colorimetric Kit for detecting apoptosis. Jurkat cells were treated with 1 µM staurosporine for 3 hours to induce apoptosis. Cell extracts were prepared, serially diluted, and analyzed with the PARP Cleavage [214/215] ELISA Kit in parallel with the Caspase-3 Colorimetric Kit. The amount of cell lysate assayed (in logarithmic scale) was plotted against the corresponding optical density (O.D.). The PARP Cleavage [214/215] ELISA Kit was 100× more sensitive than the Caspase-3 Colorimetric Kit for detecting apoptotic events in cell extracts.

Important additions to the Alexa Fluor® dye series

A NEW AMINE-REACTIVE FORM OF THE ALEXA FLUOR® 488 DYE, AND THE NEW NEAR-IR ALEXA FLUOR® 790 DYE.

Alexa Fluor® dyes are a series of revolutionary fluorophores that span the spectrum from the near-UV to near-IR wavelengths. These dyes, without exception, produce the brightest and most photostable conjugates we have ever tested, providing many options for multicolor detection using a wide range of fluorescence instrumentation.

Efficient protein conjugation with Alexa Fluor® active esters

Active esters such as the *N*-hydroxysuccinimidyl (NHS) ester (succinimidyl ester, SE) and tetrafluorophenyl (TFP) ester are preferred for protein conjugations. The strong carboxyamide bond formed between the ester and a primary amine (Figure 1) makes it easy to attach Alexa Fluor® active esters to proteins and amine-modified oligonucleotides for imaging and flow cytometry applications. Furthermore, the resulting conjugates are stable, even during prolonged storage.

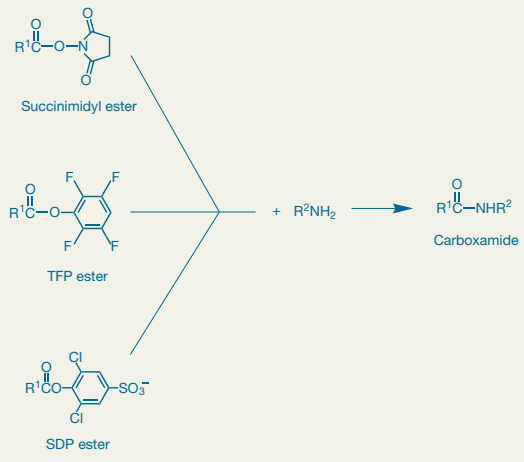

Figure 1— Reaction of a primary amine with a succinimidyl, tetrafluorophenyl (TFP), or sulfodichlorophenyl (SDP) ester.

Improving the labeling efficiency of amine-reactive Alexa Fluor® 488

Although they provide an easy and efficient means for attaching fluorophores, active esters are subject to hydrolysis. Alexa Fluor® 488 NHS ester is the least hydrolytically stable of the reactive Alexa Fluor® dyes. To achieve efficient labeling, researchers must compensate for hydrolyzed reagent by using an excess of Alexa Fluor® 488 NHS ester. Alexa Fluor® 488 TFP ester, developed as an alternative to the NHS ester, offers improved hydrolytic stability and provides a higher degree of labeling (DOL) under similar reaction conditions (Figure 2). However, this TFP ester is still subject to hydrolysis, particularly at elevated pH, where most reactions take place.

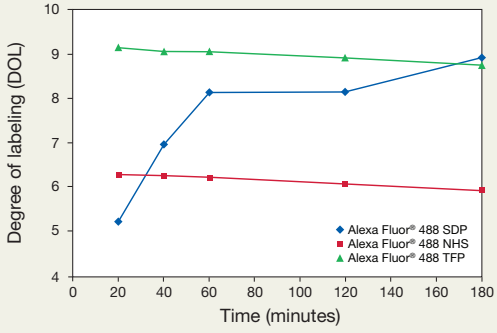

Figure 2—Comparison of reaction kinetics. The reaction kinetics for Alexa Fluor® 488 sulfodichlorophenyl (SDP) ester, Alexa Fluor® 488 tetrafluorophenyl (TFP) ester, and Alexa Fluor® 488 *N*-hydroxysuccinimidyl (NHS) ester with goat anti–mouse IgG antibody were compared under standard amine-conjugation reaction conditions. After normalizing the SDP, TFP, and NHS ester to 100% reactivity, each compound was reacted with the antibody at a molar ratio of 12, and the resulting degree of labeling (DOL) was measured at various time points.

Alexa Fluor® 488 SDP ester: superior reaction control

The novel sulfodichlorophenyl (SDP) ester offers a new level of control over your labeling reactions. The SDP ester is significantly less susceptible to hydrolysis (Figure 3), making this form of the Alexa Fluor® 488 dye the preferred one for amine conjugations. In addition to adjusting the molar ratio, you can now increase the reaction time to control the DOL. As a result, you need less dye to achieve optimized labeling.

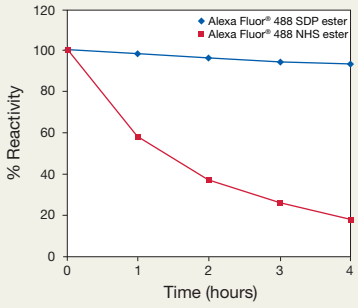

Figure 3—The sulfodichlorophenyl (SDP) ester is significantly less susceptible to hydrolysis than the *N*-hydroxysuccinimidyl (NHS) ester under typical amine-conjugation reaction conditions. Equivalent amounts of the SDP and NHS ester were normalized to 100% reactivity and incubated in phosphate buffer at pH 8.6. At identified time points, each compound was reacted with an excess of *n*-butylamine and analyzed by HPLC to determine the remaining % reactivity.

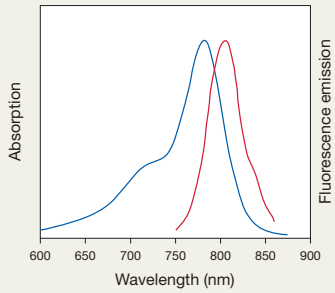

Figure 4—Fluorescence excitation and emission spectra of the Alexa Fluor® 790 succinimidyl ester.

Alexa Fluor® 790 NHS ester: the longest-wavelength Alexa Fluor® dye

On the other end of the spectrum, the new Alexa Fluor® 790 NHS ester is the longest-wavelength Alexa Fluor® dye available. With excitation/emission maxima of ~784/814 nm (Figure 4), Alexa Fluor® 790 dye has spectral properties similar to those of indocyanine green (ICG) and IRDye® 800 dye (LI-COR Biosciences). This fluorophore will be useful for researchers who require an amine-reactive, near-IR label for small animal *in vivo* imaging (SAIVI) applications and researchers preparing conjugates for multicolor analysis with Alexa Fluor® 680 dye and the LI-COR® Odyssey® infrared imaging system. The Alexa Fluor® 790 NHS ester is supplied in a 100 µg unit size, enough to label ~1 mg of IgG antibody. To learn more about these and other Alexa Fluor® dyes, please visit **probes.invitrogen.com**. ■

Product	Quantity	Cat. no.
Alexa Fluor® 488 5-SDP ester (Alexa Fluor® 488 sulfodichlorophenyl ester)	1 mg	A30052
Alexa Fluor® 488 carboxylic acid, 2,3,5,6-tetrafluorophenyl ester (Alexa Fluor® 488 5-TFP), 5-isomer	1 mg	A30005
Alexa Fluor® 488 carboxylic acid, succinimidyl ester, mixed isomers	1 mg	A20000
Alexa Fluor® 488 carboxylic acid, succinimidyl ester, mixed isomers	5 mg	A20100
Alexa Fluor® 790 carboxylic acid, succinimidyl ester, penta(triethylammonium) salt	100 µg	A30051

Effortless protein conjugations for small animal *in vivo* imaging

SAIVI™ RAPID ANTIBODY LABELING KITS.

The high pharmacokinetic impact of heavily labeled antibodies

Retaining native targeting properties while minimizing the pharmacokinetic impact of the label is the goal of functional labeling. Invitrogen has developed two new antibody labeling kits, featuring near-IR Alexa Fluor® dyes, for achieving a degree of labeling (DOL) that is optimized for *in vivo* imaging applications. The DOL of fluorescent targeting antibodies influences the signal associated with the targeted site as well as clearance of the antibody from circulation. Many labeling protocols are developed with the intent of producing maximal fluorescence by conjugating multiple fluorophores (>3) to each antibody; however, antibodies with higher DOL have decreased target signal *in vivo*, likely due to hepatic clearance of the high-DOL antibodies from circulation. The optimal fluorescent antibody conjugate for *in vivo* imaging produces an intense fluorescent signal at the targeted site that persists throughout the study, without significant clearance or redistribution of the probe.

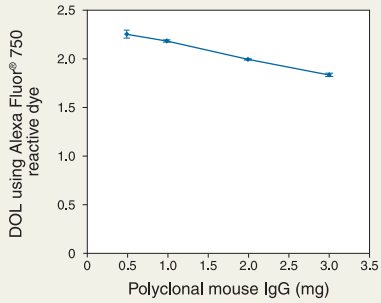

Figure 1—Four different amounts of mouse polyclonal IgG were labeled using the SAIVI™ Alexa Fluor® 750 Rapid Antibody Labeling Kit. Over this 6-fold protein concentration range, the degree of labeling (DOL) obtained using this kit was ~2 fluorophores per antibody, which is ideal for *in vivo* imaging applications.

Get optimal DOL, regardless of protein concentration

SAIVI™ Rapid Antibody Labeling Kits provide a convenient method for achieving an optimal DOL (~2 fluorophores per antibody, ideal for *in vivo* imaging applications) over a 6-fold antibody concentration range, with no need to adjust the reaction volume or dye or antibody concentration. These kits supply either Alexa Fluor® 680 (excitation/emission maxima ~679/702 nm) or Alexa Fluor® 750 (excitation/emission maxima ~749/775 nm) near-IR dye as a water-soluble activated ester, lyophilized and ready to use, as well as a regulator solution for controlling the DOL (Figure 1). Purification of the dye-labeled conjugate takes less than 10 minutes, with excellent reproducibility. Using this procedure, you can quickly and efficiently produce Alexa Fluor® dye–labeled antibodies for applications that require azide-free reagents, such as live-cell imaging or direct injection into animals.

Take control of your near-IR labeling

To learn more about the advantages of SAIVI™ labeling kits, and to view a full list of Molecular Probes® reagents and kits for *in vivo* imaging applications, visit **probes.invitrogen.com/saivi**. ■

Product	Quantity	Cat no.
SAIVI™ Rapid Antibody Labeling Kit, Alexa Fluor® 680	1 kit, 3 labelings	S30045
SAIVI™ Rapid Antibody Labeling Kit, Alexa Fluor® 750	1 kit, 3 labelings	S30046

The latest in stem cell research

NEW ANTIBODIES AND GROWTH FACTORS FOR KEY EMBRYONIC AND MESENCHYMAL STEM CELL APPLICATIONS.

Stem cells (SC) possess a unique capacity to renew themselves and to give rise to specialized cell types. Unlike other cells in the body, which are committed to execute specific functions, a stem cell remains uncommitted until it receives a signal, usually a particular growth factor binding to a cell surface receptor, to activate and specialize its development. Because SC have these unique properties, they offer great hope for treating and potentially curing many life-threatening diseases. Significant work involving SC has been performed in several areas, including transplantation research (restoring bodily functions), therapeutic delivery systems (delivering genes to specific tissues), and basic research applications (understanding embryonic development). Much of SC research involves identifying types of SC—for example, embryonic, mesenchymal, hematopoietic, and neuronal SC—and their properties, including cell surface marker identification and response to growth factors that promote growth, differentiation, and survival. Invitrogen offers a growing selection of SC reagents for these and other applications, including antibodies for characterizing cell surface molecules, and recombinant growth factors for expansion and differentiation experiments (Tables 1 and 2, pages 22–23).

Discovery of key growth receptors on embryonic SC

Embryonic SC are pluripotent cells derived from the inner mass of fertilized human blastocysts. Researchers have recently shown that key growth factors, including fibroblast growth factor (FGFb), transforming growth factor β1 (TGFβ1), activin-A, bone morphogenic proteins (BMPs), hepatocyte growth factor (HGF), and others, bind to receptors on the ES cell surface.[1] Each growth factor has a unique differentiation or cell selection effect, but none directs differentiation exclusively to one cell type. Significant research is also being performed to determine which cell surface markers are expressed in the presence of different growth factors. Tables 1 and 2 (pages 22–23) list a selection of Invitrogen's embryonic SC reagents.

Mesenchymal SC retain CD34$^+$ phenotype in bone marrow cultures

Mesenchymal SC are pluripotent cells in the bone marrow (Figure 1) that can differentiate into cells of osteogenic, chondrogenic, tendonogenic, adipogenic, and myogenic lineages. Using flow cytometry analysis, researchers recently found that mesenchymal SC are a homogeneous population devoid of hematopoietic cells, and that mesenchymal SC retained their CD34$^+$ hematopoietic progenitor lineage in long-term bone marrow cultures.[2] Researchers also found steady-state levels of IL-1α–induced G-CSF and GM-CSF. Significant work is also being performed to determine the impact that different growth factors have on mesenchymal growth, survival, and differentiation. Tables 1 and 2 (pages 22–23) list a selection of Invitrogen's mesenchymal SC reagents.

Stem cell reagents from Invitrogen

Invitrogen offers a growing collection of embryonic and mesenchymal SC reagents. For a complete list of antibodies and growth factors, visit **www.invitrogen.com/antibodies** and **www.invitrogen.com/proteins**.

References

1. Schuldiner, M. et al. (2000) *Proc Natl Acad Sci U S A* 97:11307–11312.
2. Majumdar, M.K. et al. (1998) *J Cell Physiol* 176:57–66.

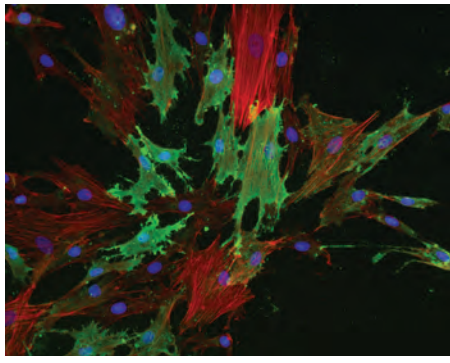

Figure 1—Immunofluorescence analysis of bone marrow–derived mesenchymal stem cells. Stromal elements were detected with Zymed® mouse anti-Stro-1 and labeled using Alexa Fluor® 488 goat anti-mouse IgM (green). Tubulin was stained with Alexa Fluor® 594 phalloidin (red), and nuclei were stained with DAPI (blue). The sample was mounted in ProLong® Gold antifade reagent.

Table 1—The latest stem cell antibodies from Invitrogen.

Target protein	Stem cell lineage	Reactive species	Applications	Quantity	Cat. no.
CD10	Hematopoietic, mesenchymal	Hu	FC (ASR)	0.5 ml	CD1000
CD105 (Endoglin)	Mesenchymal	Hu	FC (ASR)	0.5 ml/100 µg	MHCD10500
CD152	Hematopoietic	Ms	FC, IP, IHC, functional inhibitor	100 µg	41-0500
CD29	Mesenchymal	Hu	FC (ASR)	0.5 ml/100 µg	CD2900
CD30 (Ki-1, TNFRSF8)	All	Hu	FC (ASR)	0.5 ml	MHCD3000
CD34	Mesenchymal	Hu	IHC, FC, WB, IF	100 µg	07-3403
CD44	Hematopoietic, mesenchymal	Hu	FC (ASR)	0.5 ml	MHCD4400
CD44v3	Hematopoietic, mesenchymal	Hu	IHC, WB	1 mg	AHS4441
CD44v6	Hematopoietic, mesenchymal	Hu	IHC, WB	100 µg	33-6700
CD45/LCA	Mesenchymal	Hu	FC, IHC	0.1 mg	AHS4552
CD45RO	Mesenchymal	Hu	FC (ASR)	0.5 ml	MHCD45RO00
CD56 (N-CAM)	Neuronal	Hu	FC (ASR)	100 µg	MHCD5600
CD73	Hematopoietic	Hu	FC, IP, IHC	100 µg	41-0200
CD9	Embryonic	Hu, Rb, Ms	FC, IHC	1 ml	AHS0907
CD90.2 (THY-1.2)	Mesenchymal	Ms	FC	1 ml/200 µg	MM2000
E-cadherin	Embryonic	Ms	WB, IP, IF, IHC, FC, ICC	100 µg	13-1900
Galectin-1	Mesodermal	Hu, Ms, (B, Ch, Ov, Sw, Rt)	WB	100 µg	42-5800
GATA-4	Endodermal	Hu (Ms, Rt)	WB	100 µg	48-1300
GATA-4 [pS105]	Endodermal	Ms, (Hu, Rt, Ch)	WB	100 µl (10 blot)	44-948
GFAP	Neuronal	Hu, B, Ms, Rt	IHC, FC, WB, IF	100 µg	13-0300
Glast-1	Neuronal	Hu, Ms	WB, IP	100 µg	42-8100
Isl-1	Neuronal	Hu (Ms, Rt)	WB	100 µg	48-1500
Ki-67 (FITC)	All	Hu	IF	100 µg	33-4711
MYF5	Mesenchymal, skeletal muscle	Hu, Ms	WB	100 µg	42-7400
N-cadherin	Neuronal	Hu, Ms, Rt, Ch, Sw	WB, IF, IHC, IP	100 µg	33-3900
NG2	Oligoprecursor/oligodendrocyte	Rt, Hu	IHC, IP, E	100 µg	37-2300
Nkx2.8	Mesodermal	Hu	WB, IP	100 µg	42-7600
Nkx3.1	Mesodermal	Hu, Ms	WB, IHC, E	100 µg	35-9700
Nkx5.2	Mesodermal	Ms, (Hu)	WB	100 µg	42-3900
Notch1	Neuronal, hematopoietic	Ms, Hu	WB, IP, IF, FC	100 µg	41-3500
Nucleostemin	Embryonic, mesenchymal, neuronal	Hu	WB, IP	100 µg	48-1800
Oct-2	Hematopoietic	Hu, (Ms, Rt)	WB, E, IHC	100 µg	39-5400
Olig1	Oligodendrocyte	Hu, Rt	WB	100 µg	42-8200
Olig3	Neuronal	Hu, Ms, Rt	WB	100 µg	42-3600
Osteocalcin	Mesenchymal	Hu, B	IHC, WB, E	100 µg	33-5400
Osteopontin	Mesodermal, osteoblasts	Ms, Rt, Hu	WB, E, IHC	100 µl	42-7701
Runx2	Mesenchymal	Hu, (Ms, Rt)	WB, E	100 µg	41-1400
Sigma-1 receptor	Neuronal	Rt, Ms, (Hu, B, Ch)	WB	100 µg	42-3300
SOX-9 [pS181]	Oligodendrocyte, endodermal, mesenchymal	Hu, Ms, B	E, WB, IP	200 µl (10 blot)	44-440A
SSEA-1	Embryonic	Hu, Ms	FC, IF	100 µg	41-1200
SSEA-3	Embryonic	Hu, Ms	FC, IF	100 µg	41-4400
SSEA-4	Embryonic	Hu, Ms	FC, IF	100 µg	41-4000
STRO-1	Mesodermal	Hu	ICC, FC	100 µl	39-8401
Tenascin	Mesenchymal, neuronal	Hu	WB, E, IHC	100 µg	37-8500

Table 1—The latest stem cell antibodies, continued.

Target protein	Stem cell lineage	Reactive species	Applications	Quantity	Cat. no.
Tenascin-R	Mesenchymal, neuronal	Ms, Rt, Ch	WB, IF, IHC	100 µg	41-3700
Tie-2	Hematopoietic	Hu	WB	100 µg	42-5100
TRA 1-60	Embryonic	Hu	WB, IP, IF	100 µg	41-1000
TRA 1-81	Embryonic	Hu	WB, IP, IF	100 µg	41-1100
Tyrosine hydroxylase	Neuronal	All mammals	WB, IP, IHC	100 µg	32-2100
Tyrosine hydroxylase (Ser19)	Neuronal	Rt	WB, IF, IHC	100 µl	36-9800
Tyrosine hydroxylase (Ser31)	Neuronal	Rt	WB, IF	100 µl	36-9900
Tyrosine hydroxylase (Ser40)	Neuronal	Rt, Hu, Ms	WB, E, IHC, IP	100 µl	36-8600
VE-cadherin	Hematopoietic	Hu	WB, E	50 µg	36-1900
VEGFR2/Flk-1	Mesenchymal	Hu, Ms	WB, IP	10 blot	44-1053G
VEGFR2/Flk-1 [pY1054/pY1059]	Mesenchymal	Ms, (Hu, Rt)	WB	100 µl (10 blot)	44-1047G
VEGFR2/Flk-1 [pY1054]	Mesenchymal	Ms, (Hu, Rt)	WB	100 µl (10 blot)	44-1046
VEGFR2/Flk-1 [pY1214]	Mesenchymal	Hu, Ms (Rt)	WB	100 µl (10 blot)	44-1052
VEGFR2/Flk-1 [pY949]	Mesenchymal	Ms	WB	10 blot	44-1041G
VEGFR2/Flk-1 [pY951]	Mesenchymal	Hu	WB	100 µl (10 blot)	44-1040
β-Tubulin III (TuJ-1, Clone:2-28-33)	Neuronal	Hu, Ms, Rt, *C. elegans*	IF, WB	100 µg	32-2600

Ms = mouse; Rt = rat; Hu = human; Ov = ovine; B = bovine; Ch = chicken; Sw = swine; Rb = rabbit; IHC = immunohistochemistry; ICC = immunocytochemistry; FC = flow cytometry; WB = western blot; E = ELISA; IF = immunofluorescence; ASR = analyte specific reagent; IP = immunoprecipitation. To view a complete list of stem-cell related antibodies and fluorescent conjugates, visit www.invitrogen.com/antibodies.

Table 2—The latest stem cell growth factors from Invitrogen.

Target protein	Stem cell lineage	Reactive species	Quantity	Cat. no.
Recombinant human Ang-1	Embryonic, mesenchymal	Hu	10 µg	PHC1514
Recombinant human Ang-2	Embryonic, mesenchymal	Hu	10 µg	PHC1524
Recombinant human BDNF	Neuronal	Hu	10 µg	PHC7074
Recombinant human BMP-2	Embryonic, mesenchymal	Hu	10 µg	PHC7094
Recombinant human EGF	Embryonic, mesenchymal	Hu	200 µg	PHG0064
Recombinant human Erythropoietin (EPO)	Embryonic, mesenchymal	Hu	500 IU	PHC2054
Recombinant human FGFb full length (FGF-2)	Embryonic, mesenchymal	Hu	10 µg	PHG0264
Recombinant human FGF-8b	Embryonic, mesenchymal	Hu	10 µg	PHG0274
Recombinant human G-CSF	Embryonic, mesenchymal	Hu	5 µg	PHC2034
Recombinant human GDNF	Neuronal	Hu	10 µg	PHC7045
Recombinant human GM-CSF	Embryonic, mesenchymal	Hu	10 µg	PHC2015
Recombinant human HGF	Embryonic, mesenchymal	Hu	10 µg	PHG0254
Recombinant human IL-3	Embryonic, mesenchymal	Hu	10 µg	PHC0034
Recombinant human IL-4	Embryonic, mesenchymal	Hu	10 µg	PHC0044
Recombinant human IL-6	Embryonic, mesenchymal	Hu	10 µg	PHC0065
Recombinant human neurotrophic factor 3	Embryonic, mesenchymal	Hu	5 µg	PHC7034
Recombinant human neurotrophic factor 4	Embryonic, mesenchymal	Hu	5 µg	PHC7024
Recombinant human noggin	Embryonic, mesenchymal	Hu	20 µg	PHC1506
Recombinant human SCF/c-kit	Embryonic, mesenchymal	Hu	10 µg	PHC2115
Recombinant human TGF-beta1	Embryonic, mesenchymal	Hu	10 µg	PHG9204
Recombinant human TPO	Embryonic, mesenchymal	Hu	10 µg	PHC1144

Hu = human. To view a complete list of recombinant proteins, visit **www.invitrogen.com/proteins**.

Mercator™ multiplex phosphoantibody-based array

A NOVEL PLATFORM FOR PHOSPHOPROTEOMIC APPLICATIONS.

Antibody-based protein microarrays are high-content/high-throughput (HCS/HTS) platforms used to screen for changes in protein expression and regulation.[1–4] The Mercator™ Cell Adhesion Array is a robust, phosphoantibody-based multiplex platform for quantitative screening of protein expression and phosphorylation and provides a high-throughput alternative to standard methods such as western blotting and ELISA.

The Mercator™ Cell Adhesion Array uses eight different phosphorylation site–specific antibodies to concurrently screen and delineate signaling events as determined by the phosphorylation status of eight markers (EGFR, FAK, Src, paxillin, Akt, p38, HSP27, and ATF2). Because of its multiplexed design, the Mercator™ platform allows quantitative and simultaneous detection of site-specific phosphorylation on each of these markers using minimal amounts of crude lysates derived from cells or tissues. This approach enables researchers to study multiple pathways simultaneously and gain a more comprehensive assessment of the complexity of protein/pathway activation or inactivation. This method may also be used as part of a high-throughput screen for activators or inhibitors of specific protein kinases and phosphatases, and has the potential to identify therapeutic markers and profile drug responses.

Mercator™ chip configuration: optimized for high throughput

The Mercator™ Cell Adhesion assay system uses coated glass slides, each fitted with 16 high–binding capacity nitrocellulose pads that serve as the solid support in the assay (Figure 1A). The very high binding capacity of this nitrocellulose format allows the Mercator™ array to achieve much higher sensitivity and a wider linear range (typically 3–4 logs) as compared to other immunoassay formats. Each nitrocellulose pad is prespotted in triplicate with eight monoclonal antibodies that capture the protein of interest independently of the phosphorylation state. Positive and negative controls are also prespotted onto the pad in triplicate (at two locations) (Figure 1B). The capture monoclonal antibodies on the nitrocellulose pads bind to and immobilize the target analytes in the Array Standard and the experimental samples. Detection of the captured protein is achieved by the addition of a mixture of eight highly selective phosphorylation site–specific antibodies (PSSAs).[5] In the case of the Mercator™ Phospho 8-plex, these detector antibodies bind to their target analytes only when the corresponding proteins are phosphorylated at the appropriate residues. In the Mercator™ Total 8-plex, the detector antibodies bind phosphorylated and nonphosphorylated proteins alike. Each slide allows 384 individual determinations to be made (16 pads per slide × 8 antibody signals per pad × 3 spots per antibody).

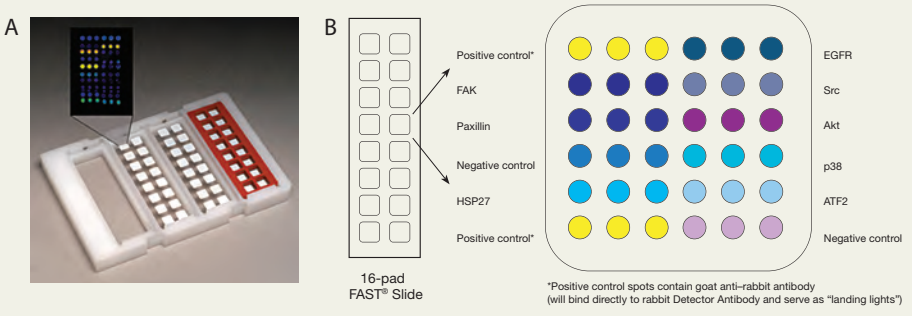

Figure 1—Expanded view of the Mercator™ Cell Adhesion Array platform and 8-plex layout. (A) Slide layout with frame. (B) Spotting configuration for each pad.

A panel of rigorous quality control tests have been conducted on each lot of Mercator™ Array slides to ensure high sensitivity, specificity, reproducibility, consistency to single-plex results, and comparability to other methods.

Specific capture and detection

Figure 2 shows the Mercator™ Cell Adhesion Array assay workflow. The nitrocellulose pads are wetted and blocked before adding the experimental sample or the standard (which contains known concentrations of eight recombinant phosphoproteins that will bind to the spotted capture antibodies). The slide is then covered and incubated with shaking for 3 hours at room temperature (or overnight at 2–8°C) to allow the capture antibodies arrayed onto the nitrocellulose pads to bind to and immobilize target analytes. Unbound materials are subsequently removed from the pads by washing. Next, the Detector Antibody solution (containing a mixture of highly selective affinity-purified rabbit (polyclonal) antibodies) is added to the pads and incubated for 1 hour at room temperature. Following a wash step, the Secondary Antibody solution (Alexa Fluor® 647 goat (polyclonal) anti–rabbit IgG fluorescent conjugate) is added for 45 minutes at room temperature. At the completion of this incubation step, unbound conjugate is removed by washing, the array is allowed to dry, an image is acquired, and the data are analyzed. Once dried, the Mercator™ array is stable for up to three months at 2–8°C when protected from light.

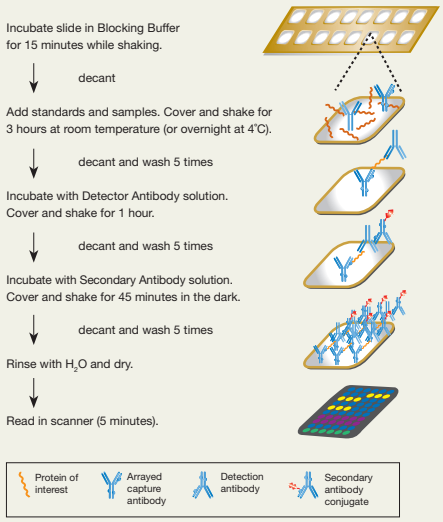

Figure 2—Mercator™ Cell Adhesion Array assay workflow.

Comparison of Mercator™ Array and phosphoELISA methods

For this experiment, we compared the signal sensitivity and linear range for both phosphoprotein levels and total-protein levels using the Mercator™ Array assay and standard single-plex ELISA. The same protein standards and detection antibody pairs were used for both platforms. The results indicated good correlation between the assays and also revealed the dramatically enhanced assay sensitivities obtained with Mercator™ Arrays (Table 1). Linearity for both phosphorylation- and

→

Table 1—Comparing protein expression level data obtained using Mercator™ Cell Adhesion Array and phosphoELISA.

Marker	Mercator™ Phospho 8-plex		PhosphoELISA		Mercator™ Total 8-plex		Total ELISA	
	Detection range, U/ml	Fold	Detection range, U/ml	Fold	Detection range, U/ml	Fold	Detection range, U/ml	Fold
EGFR	0.07–1,080	15,429	0.3–100	333	3–46,000	15,333	160–10,000	62
FAK	0.14–2,184	15,600	0.9–100	111	64–250,000	3,906	1,600–100,000	62
Src	0.11–1,670	15,182	1.6–100	62	17–283,000	16,647	1,600–50,000	31
Paxillin	0.06–950	15,833	NA		5–86,000	17,200	NA	
Akt	0.17–2,694	15,847	0.8–100	125	4–270,000	67,500	100–20,000	200
p38	0.14–2,128	15,200	0.8–100	125	6–85,000	14,167	31–2,000	64
HSP27	0.16–2,518	15,738	1–100	100	40–630,000	15,750	800–50,000	62
ATF2	0.07–1,062	15,171	1–100	100	20–106,200	5,310	1,100–70,000	63
Average		15,500		137		19,477		78

NA = not applicable.

protein-expression–level signals across a wider range of recombinant standards and cell lysate samples (3–4 logs) was observed for the Mercator™ Array platform. In fact, the results from the Mercator™ assay were superior to any other immunoassay format we evaluated, including standard ELISA. This feature is particularly significant, given the quantitative capabilities of the Mercator™ Cell Adhesion Array.

Simultaneous profiling of protein level and phosphorylation of eight multiplexed markers

In this study, human embryonic kidney (HEK 293) cells were either untreated or stimulated with anisomycin (100 ng/ml, 1 hour) (Figure 3). A 70 µg sample (10×10^6 cells) from each treatment was lysed, and the lysates were analyzed using the Mercator™ Cell Adhesion Array. Samples were either immunoprobed with the 8-Plex Phospho-Detector Antibody (for phosphorylation signal) or with the 8-Plex Total Detector Antibody (for total-protein expression signal). The slides were then scanned and analyzed. The levels of protein expression and of phosphorylation for each of the eight markers were simultaneously evaluated and cross-validated with western blotting analysis using the same total and phosphoantibodies. This multiplexed protein expression experiment illustrates the power of the Mercator™ Array platform.

Future directions for the Mercator™ Array screening platform

The Mercator™ Cell Adhesion Array is a robust multiplex platform for quantitative screening of protein phosphorylation and expression. This platform provides a comprehensive and selective tool to help delineate the signaling network, and promises to advance our understanding of molecular mechanisms of disease pathology with demonstrated reproducibility and accuracy. Future work will focus on the development of potential applications of this platform; we are currently at work on a larger multiplex assay for the Akt pathway targeting high-profile markers in metabolic, diabetic, and cancer signaling pathways. To learn more about the Mercator™ Array platform, visit **www.invitrogen.com**. ■

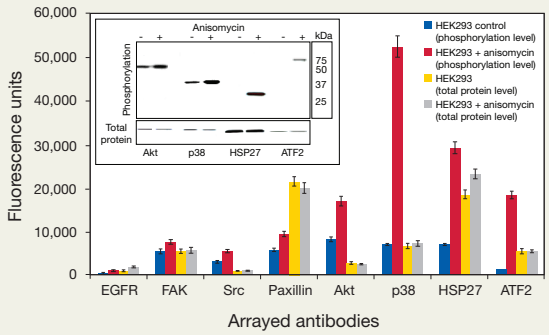

Figure 3—Multiplex phosphoregulation of eight markers following anisomycin treatment. All measurements were performed in triplicate, and the results revealed high signal uniformity and reproducibility. Low detection limits were obtained, making the Mercator™ Array platform ideal for very low-abundance antigens.

References

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2. Haab, B.B. (2005) *Mol Cell Proteomics* 4:377–383.
3. Kingsmore, S.F. (2006) *Nat Rev Drug Discov* 5:310–320.
4. Raggiatchi, R. et al. (2005) *Biosci Rep* 25:33–44.
5. Mandell, J.W. (2003) *Am J Pathol* 163:1687–1698.

Product	Quantity	Cat. no.
Mercator™ Cell Adhesion Array Phospho 8-plex, 1 slide	128 tests	BHM9021
Mercator™ Cell Adhesion Array Phospho 8-plex, 4 slides	512 tests	BHM9024
Mercator™ Total Cell Adhesion Array 8-plex, 1 slide	128 tests	BHM9031
Mercator™ Total Cell Adhesion Array 8-plex, 4 slides	512 tests	BHM9034
Mercator™ Array Slide Holder	4 slides	BHM0004
Mercator™ Array Slide Holder	1 slide	BHM0001

How much DNA, RNA, or protein is really in your sample?

THE QUBIT™ QUANTITATION PLATFORM.

The revolutionary Qubit™ quantitation platform is changing the way DNA, RNA, and protein are quantitated. Fundamentally different from nonspecific absorbance readings that measure everything in the sample, the Qubit™ platform uses special Quant-iT™ dyes that specifically detect only DNA, RNA, or protein. For the first time, you'll know exactly what's in your sample.

Why absorbance readings don't work

An absorbance measurement at 260 nm does not distinguish between DNA, RNA, or even protein contamination. Taking the ratio of sample absorbances at 260 nm and 280 nm will provide you with more information; however, since the 260 nm measurement is unreliable, the ratio still only provides a best guess. To demonstrate, a 10 ng/µl DNA sample with varying amounts of RNA contamination was measured using the Qubit™ platform and absorbance. As shown in Figure 1, the absorbance reading is only accurate for the pure DNA sample. In fact, the absorbance reading showed as much as an 8-fold variation in the RNA-contaminated samples.

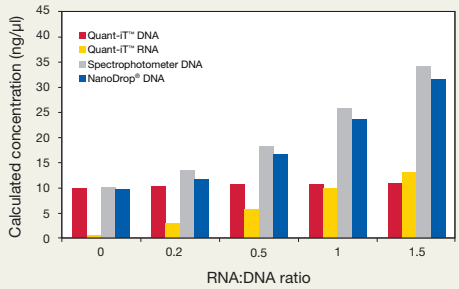

Figure 1—Accurate DNA quantitation using the Qubit™ platform. Five samples, each containing 10 ng/µl DNA and 2–50 ng RNA, were analyzed using four methods: the Quant-iT™ DNA assay and Qubit™ fluorometer; the Quant-iT™ RNA assay and Qubit™ fluorometer; A_{260} absorbance; and absorbance measured using the NanoDrop® spectrophotometer (NanoDrop Technologies). The Qubit™ platform provides accurate results for each sample, while the absorbance readings show as much as 8-fold variation.

Get an accurate quantity assessment

"Based on the Qubit™ measurements, Nanodrop [spectrophotometer] overestimated the amount of RNA in the blood spot samples by about 10 times.... We used Qubit™ for some of our other troublemaker RNA samples and it worked like a charm!

—Julia Busik, Assistant Professor, Michigan State University

How much DNA, RNA, or protein is in your sample? The only way to truly know is by using the molecule-specific Qubit™ quantitation platform. Quant-iT™ dyes become fluorescent only upon binding to the molecule of interest (DNA, RNA, or protein), so only the concentration of your specific molecule is reported, not contaminants, for accurate results (Figure 1). You get better specificity and accuracy, and that means greater confidence when using the sample in your experiment. To learn more, visit **probes.invitrogen.com/qubit**. ■

Product	Quantity	Cat. no.
Quant-iT™ dsDNA BR Assay Kit, 100 assays, 2–1,000 ng	1 kit	Q32850
Quant-iT™ dsDNA BR Assay Kit, 500 assays, 2–1,000 ng	1 kit	Q32853
Quant-iT™ dsDNA HS Assay Kit, 100 assays, 0.2–100 ng	1 kit	Q32851
Quant-iT™ dsDNA HS Assay Kit, 500 assays, 0.2–100 ng	1 kit	Q32854
Quant-iT™ RNA Assay Kit, 100 assays, 5–100 ng	1 kit	Q32852
Quant-iT™ RNA Assay Kit, 500 assays, 5–100 ng	1 kit	Q32855
Quant-iT™ Protein Assay Kit, 100 assays, 0.25–5 µg	1 kit	Q33211
Quant-iT™ Protein Assay Kit, 500 assays, 0.25–5 µg	1 kit	Q33212
Qubit™ Quantitation Starter Kit	1 kit	Q32860
Qubit™ Quantitation Lab Starter Kit	1 kit	Q32861
Qubit™ assay tubes, set of 500	1 set	Q32856
Qubit™ fluorometer	each	Q32857
Qubit™ fluorometer USB cable	each	Q32858

Simultaneously visualize surface and intracellular markers

FIX & PERM® CELL PERMEABILIZATION KIT AND ZENON® LABELING COMPLEXES.

Some staining methods enable you to see what's on the outside of a cell. Others allow you to visualize the inside of the cell. Very rarely are these technologies compatible, forcing you to decide which set of markers you want to study. With the FIX & PERM® Cell Permeabilization Kit and Zenon® labeling technology, there's no need to choose. Using these two methods, you can stain the cell surface without affecting inside morphology, label the same population with Zenon® primary antibodies specific to your target(s), and then analyze the cell population in your flow cytometer. You'll get the whole picture in one experiment.

Fix and permeabilize the cells

The FIX & PERM® Cell Permeabilization Kit allows mild fixation and permeabilization of cells while leaving the morphological scatter characteristics of the cells intact. This enables you to accurately identify previously undetectable intracellular markers, such as cytoplasmic or nuclear enzymes, oncoproteins, cytokines, and immunoglobulins, in addition to extracellular markers. The FIX & PERM® Kit is suitable for flow cytometric analysis of normal and malignant leukocyte

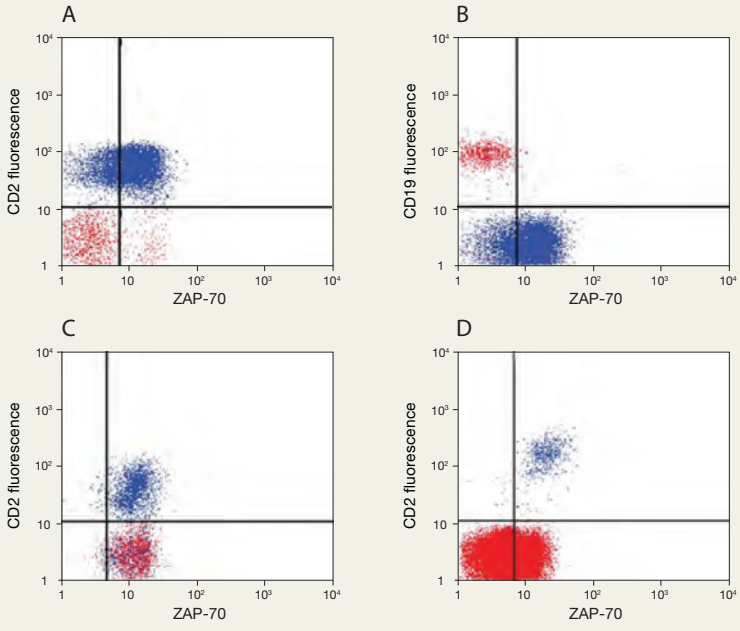

Figure 1—Intracellular ZAP-70 staining combined with surface CD2 and CD19 staining of human lymphocytes using Zenon® labeling reagents. Samples were treated with anti-CD19 and Zenon® R-PE mouse IgG1 labeling reagent (molar ratio 3), anti-CD2 and Zenon® Alexa Fluor® 680 R-PE mouse IgG1 labeling reagent (molar ratio 3), and anti–ZAP-70 and Zenon® Alexa Fluor® 488 mouse IgG1 labeling reagent (molar ratio 3). A lymphocyte gate was used. (A) Normal patient sample showing a normal ZAP-70 staining pattern copositive with CD2 lymphocytes. (B) Normal patient sample showing a normal ZAP-70 staining pattern mutually exclusive with CD19 lymphocytes. (C, D) Two different patients with B cell chronic lymphocytic leukemia showing different ZAP-70 expression patterns in dual parameter testing with CD2, gated on lymphocytes. Data collected in collaboration with M. Suter, Oregon Medical Laboratories, Eugene, OR.

populations derived from various human biological samples (blood, bone marrow, and others). The dependable FIX & PERM® Kit offers:

- compatibility with analysis of most cellular antigens
- reduced background staining
- proven protocols

The FIX & PERM® Cell Permeabilization Kit provides two reagents: Fixation Medium for fixing cells in suspension, and Permeabilization Medium for permeabilizing the cell membranes.

Label the primary antibodies

Zenon® technology provides an easy, versatile, and truly unique method of labeling your primary antibodies with Molecular Probes® premier dyes. The fast labeling reaction is complete in 10 minutes and does not require any pre- or post-labeling purification steps. The Zenon® reagent labels the Fc portion of the antibody only, leaving the binding sites fully functional. This eliminates all guesswork involved in labeling primary antibodies with reactive dyes. Zenon® technology is:

- efficient—label nearly 100% of the primary antibody, even when the antibody is not pure
- economical—label submicrogram amounts of antibody and store for later use
- reliable—clean labeling of cells, with minimal nonspecific background (Figure 1)

Zenon® Labeling Kits are currently available for use with mouse IgG1, mouse IgG2a, mouse IgG2b, rabbit IgG, goat IgG, and human IgG antibodies.

Analyze your cell population

Once you've labeled the desired surface and intracellular markers, analyze cells via flow cytometry. Just set the appropriate parameters on your instrument and go.

One complete picture

Combining the FIX & PERM® Kit with Zenon® labeling can provide you with a complete look at specific extracellular and intracellular markers in one experiment. The protocol in the sidebar shows you how. To learn more about these technologies, visit **www.invitrogen.com**. ■

FIX & PERM® is a registered trademark of An Der Grub Bioresearch GmbH.

Product	Quantity	Cat. no.
FIX & PERM® Cell Permeabilization Kit	50 tests	GAS-003
FIX & PERM® Cell Permeabilization Kit	200 tests	GAS-004

Protocol for combining surface staining with intracellular staining using Zenon® labeling complexes and the FIX & PERM® Cell Permeabilization Kit

Antibody labeling

1. Collect whole blood or prepare a cell suspension in a suitable staining buffer such as PBS/BSA.
2. Add 100 µl cell suspension or 100 µl whole blood to a 12 × 75 mm flow cytometry tube.
3. Prepare Zenon® complex with primary antibody for surface staining, according to the Zenon® Kit instructions.

Surface marker staining

4. Add the entire Zenon® labeling mixture for surface staining (or directly conjugated antibody) to the cell suspension.
5. Incubate the sample for 30 minutes at room temperature, protected from light.
6. Fix the cell sample by adding 100 µl FIX & PERM® Reagent A and incubating for 15 minutes at room temperature.
7. Wash the cells once: fill the flow tube with staining buffer, centrifuge the sample to pellet the cells, and then decant the supernatant.

Intracellular staining

8. Permeabilize the cell sample by adding 100 µl FIX & PERM® Reagent B.
9. Prepare Zenon® complex with primary antibody for intracellular staining, according to the Zenon® Kit instructions.
10. Add the entire Zenon® labeling mixture for intracellular staining to the cell suspension containing permeabilization reagents.
11. Incubate the sample for 30 minutes at room temperature, protected from light.
12. Fill the flow tube with staining buffer and let rest for 5 minutes at room temperature, protected from light.
13. Centrifuge the sample to pellet the cells, and decant the supernatant.
14. Resuspend the cells in staining buffer.
15. Analyze the sample using appropriate instrument parameters.

Seeing superoxide in a radical new light

AN IMPROVED METHOD FOR SELECTIVELY DETECTING MITOCHONDRIAL SUPEROXIDE WITH MITOSOX™ RED PROBE.

Superoxide formation can lead to oxidative stress in mitochondria

In aerobic organisms, the generation of reactive oxygen species (ROS) is an inevitable side reaction of cellular respiration. Mitochondria consume 85–95% of a cell's oxygen to power oxidative phosphorylation,[1] and almost all of this mitochondrial oxygen is completely reduced to water by a tightly coupled electron transport chain. However, a small portion (estimates range between 0.1 and 4%) of electrons leak from the respiratory chain, reducing oxygen to superoxide ($O_2^{\bullet-}$).[2,3] Although $O_2^{\bullet-}$ is the predominant ROS in mitochondria, its presence initiates a cascade of reactions that produce other ROS, including peroxynitrite, hydrogen peroxide, and hydroxyl radical.[4,5] Because mitochondrial DNA lacks protective histones and has only rudimentary repair mechanisms, mitochondria are particularly vulnerable to damage by ROS, subsequent respiratory chain breakdown, and eventual activation of apoptosis.[5] Accordingly, mitochondrial dysfunction caused by oxidative stress has been implicated in aging as well as in a variety of pathological states, including neurodegeneration, cancer, and diabetes.[1–5]

MitoSOX™ Red superoxide indicator localizes in mitochondria

Detecting $O_2^{\bullet-}$ within the dynamic context of live cells is critical to understanding antioxidant defenses in healthy cells and their breakdown in disease states. Traditionally, hydroethidine (HE)—a cell-permeant, two-electron reduced form of the nucleic acid intercalator ethidium—has been used to monitor $O_2^{\bullet-}$ in live cells. HE exhibits weak cytosolic blue fluorescence; once oxidized, however, this probe binds nucleic acids, staining the nucleus, and especially nucleoli, with bright red fluorescence (Figure 1A).

To help further investigations into the primary intracellular source of $O_2^{\bullet-}$ production, we developed the MitoSOX™ Red mitochondrial superoxide indicator, comprising HE covalently linked to a triphosphonium cation through a hexyl carbon chain. The positive charge on the phosphonium group selectively targets this cell-permeant HE analog to mitochondria, where it accumulates as a function of mitochondrial membrane potential and exhibits bright red fluorescence upon oxidation and subsequent binding to mitochondrial nucleic acids. Because ethidium was assumed to be the oxidation product of HE, the red fluorescence of oxidized HE and MitoSOX™ Red probes was routinely measured using 510 nm excitation (Figure 1B).

Distinguishing superoxide oxidation of MitoSOX™ Red probe using shorter excitation wavelengths

In controlled solutions of oxidants, both MitoSOX™ Red probe and HE have been shown to be oxidized by $O_2^{\bullet-}$ in a highly selective fashion.[6] In live cells, however, the selectivity of these probes for superoxide is complicated by autooxidation as well as by other nonsuperoxide-dependent oxidation processes catalyzed by intracellular peroxidases, oxidoreductases, and cytochromes, all of which generate ethidium or,

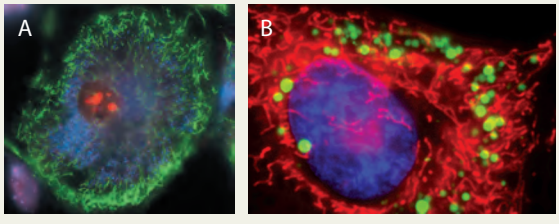

Figure 1—Live-cell labeling with two superoxide indicators: hydroethidine and MitoSOX™ Red probe. (A) Live bovine pulmonary artery endothelial cells (BPAECs) were incubated with hydroethidine (HE) and the green-fluorescent MitoTracker® Green FM probe. (B) Live BPAECs were treated with antimycin A, which stimulates mitochondrial superoxide production, and then incubated with MitoSOX™ Red mitochondrial superoxide indicator. Lysosomes were labeled with green-fluorescent LysoTracker® Green DND-26 probe, and the nucleus was counterstained with blue-fluorescent Hoechst 33342.

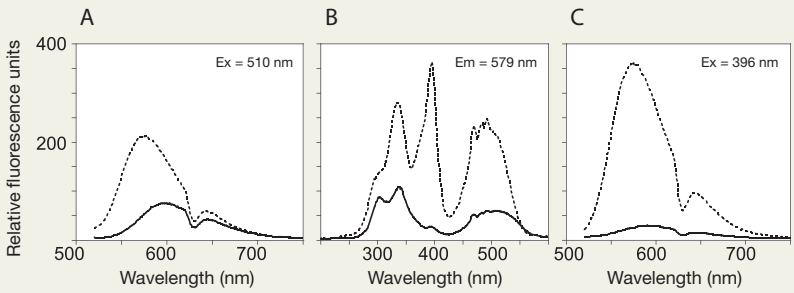

Figure 2—Selective excitation of the superoxide-dependent MitoSOX™ Red oxidation product at 396 nm. (A) With 510 nm excitation, the fluorescence emission of the two-electron MitoSOX™ Red oxidation product (an ethidium analog, —) overlapped 40% with that of an equimolar concentration of the superoxide-dependent MitoSOX™ Red oxidation product (a 2-hydroxyethidium analog, ---). (B) Excitation spectra (emission at 579 nm) reveal that the 2-hydroxyethidium analog (---) has a distinct excitation at 396 nm that is not present for the ethidium analog (—). (C) As compared with 510 nm excitation, 396 nm excitation enhances the fluorescence emission of the 2-hydroxyethidium analog (---) by 70% and reduces spectral overlap with the ethidium analog (—) to 10%. Image reproduced with permission from *Proc Natl Acad Sci U S A* 103:15038–15043 (2006), © 2006 National Academy of Sciences, USA.

in the case of MitoSOX™ Red probe, an ethidium analog. Furthermore, in 2003 Zhao and coworkers reported that the fluorescent oxidation product of HE and $O_2^{\bullet-}$ is not ethidium but 2-hydroxyethidium, a hydroxy adduct that is difficult to distinguish from ethidium using the conventional excitation wavelength of 510 nm.[7]

Building on these findings, Robinson and colleagues have recently determined that the hydroxy adduct resulting from $O_2^{\bullet-}$ oxidation of both HE and MitoSOX™ Red probes can be selectively excited at 396 nm, with minimal interference from ethidium[6] (Figure 2). Significantly, in isolated mitochondria, the oxidation of MitoSOX™ Red probe measured with 396 nm excitation was reported to be 30% slower than that measured with 510 nm excitation after antimycin A stimulation.[6] These observations indicate that 396 nm excitation provides a more selective detection of superoxide-oxidized MitoSOX™ Red probe, and traditional measurements using 510 nm excitation alone can potentially overestimate mitochondrial $O_2^{\bullet-}$ production in live cells.[6]

Ensuring mitochondrial health during live-cell measurements

Given a mitochondrial membrane potential of –180 mV, probes that accumulate in mitochondria by a membrane potential–dependent mechanism can concentrate in this organelle up to 1,000-fold over extracellular levels. Such high concentrations can easily stress mitochondria, priming the mitochondrial permeability transition pore or even completely rupturing these organelles. To avoid cytotoxicity when using the MitoSOX™ Red mitochondrial superoxide indicator, probe concentration must be determined empirically for each cell type. For example, loading endothelial cells with 2 µM but not 0.5 µM MitoSOX™ Red probe caused mitochondrial stress and relocalization of oxidation products to the cytosol and nuclei,[6] underscoring the importance of optimizing staining protocols for each experimental model. In our studies, the optimal concentration of MitoSOX™ Red probe generally ranged between 0.1 and 2.5 µM, depending on the cell type. A mitochondrial potential sensor, such as MitoTracker® Deep Red 633 probe, can be used in conjunction with MitoSOX™ Red probe to monitor mitochondrial health during experimental treatments.

Refining your protocols

By determining the optimal staining concentration for your cells and then monitoring the oxidation of MitoSOX™ Red probe with 396 nm excitation, you can more selectively detect mitochondrial $O_2^{\bullet-}$ production and distinguish it from other nonspecific intracellular oxidative processes in live cells. Visit **probes.invitrogen.com** to find out more about these and other ROS indicators. ■

Kristine M. Robinson, Ph.D., and Joseph S. Beckman, Ph.D., Oregon State University (Corvallis, Oregon) contributed to this article.

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Product	Quantity	Cat. no.
MitoSOX™ Red mitochondrial superoxide indicator, for live-cell imaging	10 × 50 µg	M36008
dihydroethidium (hydroethidine), special packaging	10 × 1 mg	D11347

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