BIOPROBES 56

CELL BIOLOGY BY INVITROGEN

JUNE 2008

Seeing green

Alexa Fluor® 488 conjugates outshine the rest

Dynabeads® technology:
Putting regulatory T cells to work

PLUS:
Interrogating the Akt signaling pathway

invitrogen™

Molecular Probes®
invitrogen detection technologies

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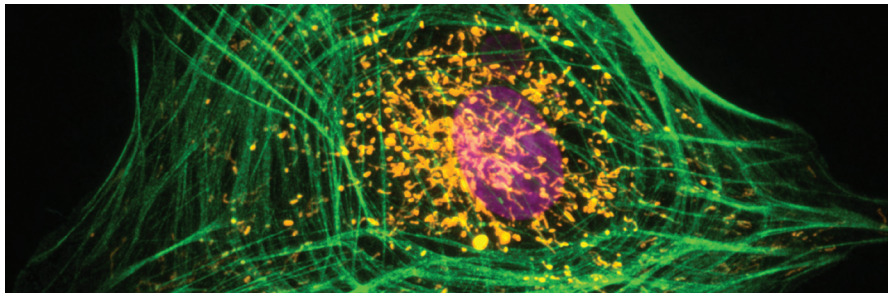

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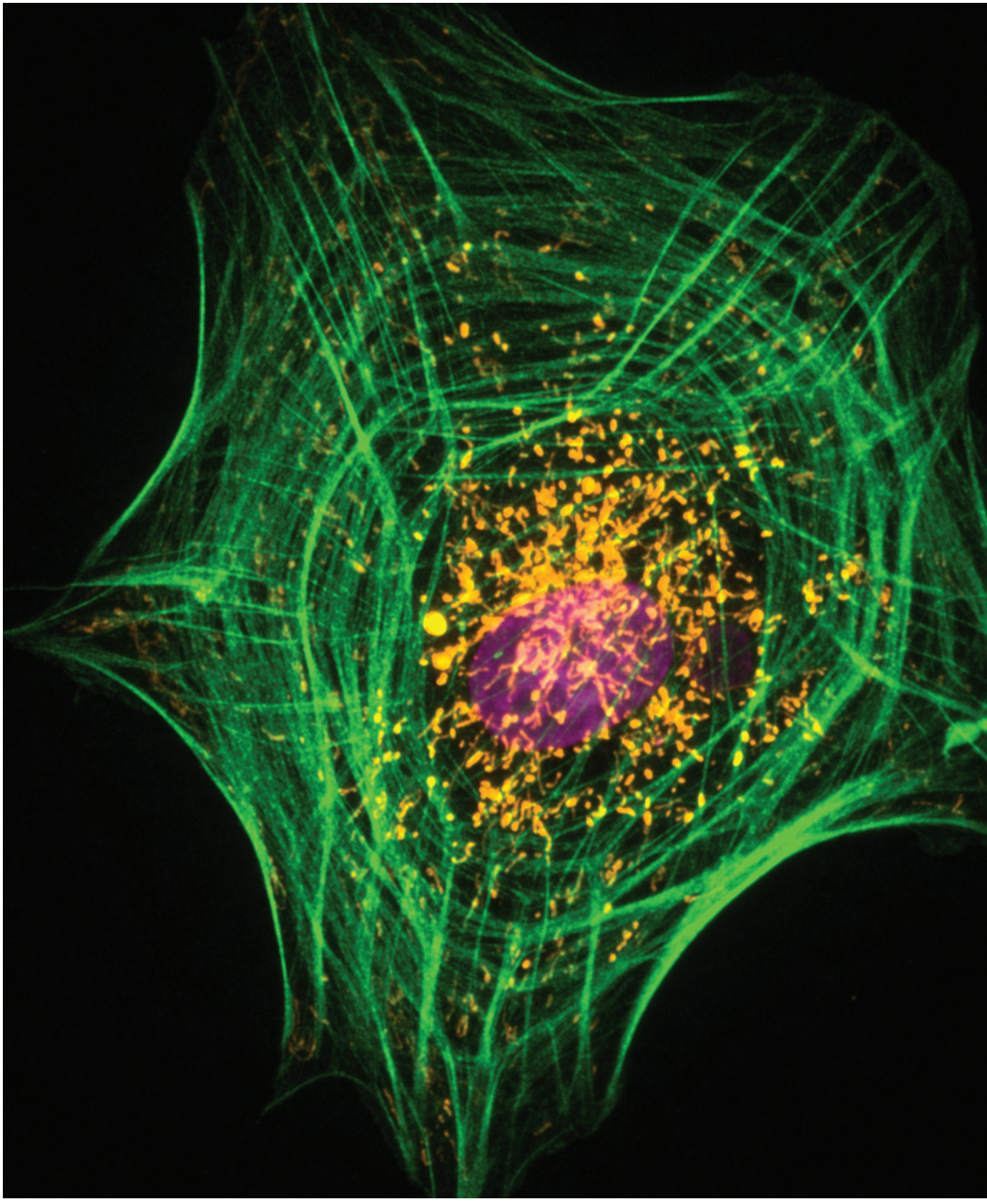
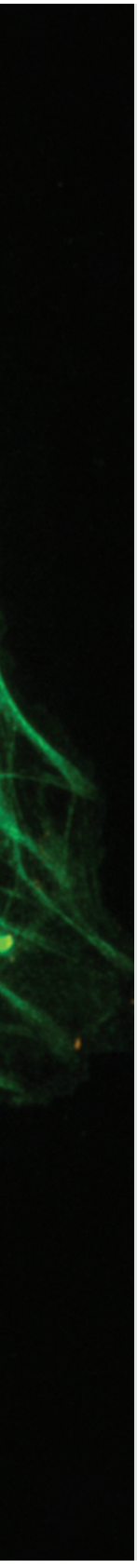




Seeing green

ALEXA FLUOR® 488 CONJUGATES OUTSHINE THE REST.

For more than 100 years, fluorescein was the premier green-fluorescent dye. The fluorophore has much to recommend it: visible light excitation and emission, a high quantum yield, and pH sensitivity in the physiological range (e.g., for studying phagocytosis). However, as fluorescein became more widely used and detection technology improved, some limitations became apparent. For example, although protein conjugates can be made easily using fluorescein, the fluorophore quenches after only a few dyes are attached. Moreover, fluorescein conjugates photobleach quickly, presenting difficulties in fluorescence microscopy, particularly for samples requiring longer and/or iterative exposures to light. Enter the Alexa Fluor® 488 dye, which overcame these obstacles, providing superior performance for fluorescence imaging (Figure 1).

Figure 1—FluoCells® prepared slide #6 (Cat. no. F36925) showing a fixed, permeabilized, and labeled muntjac skin fibroblast. Mitochondria were labeled with anti–OxPhos Complex V inhibitor protein mouse IgG1 and visualized using orange-fluorescent Alexa Fluor® 555 goat anti–mouse IgG (Cat. no. A21422). F-actin was labeled with green-fluorescent Alexa Fluor® 488 phalloidin (Cat. no. A12379), and the nucleus was stained with TO-PRO®-3 iodide (Cat. no. T3605, pseudocolored magenta).

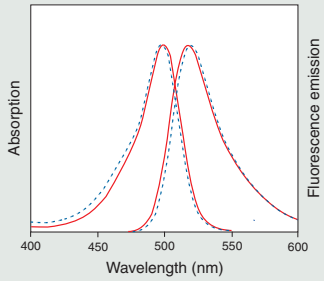


Figure 2—Absorption and fluorescence emission spectra of fluorescein goat anti–mouse IgG antibody (–) and Alexa Fluor® 488 goat anti–mouse IgG antibody (---). The fluorescence intensity of the Alexa Fluor® 488 conjugate was significantly higher than that of the fluorescein conjugate. The data are normalized to show the spectral similarity.

Alexa Fluor® 488 dye—the modern benchmark

Alexa Fluor® 488 dye, which first appeared on the market in 1997, overcame the limitations of fluorescein in a fluorophore that was a near-perfect spectral match (Figure 2). It was, in fact, the first dye to demonstrate a marked improvement over fluorescein, particularly for protein and other conjugates. As an added advantage, researchers weren't required to change equipment or other settings in their experimental setup to use Alexa Fluor® 488 dye. While this made switching to the new fluorophore easy, the real reason to switch was the superior performance of the conjugates. Fluorescein conjugates rapidly quench as more fluorophores are added. The Alexa Fluor® 488 dye allows more fluorophores to attach to the conjugate before self-quenching becomes apparent, leading to significantly brighter conjugates (Figure 3). Photobleaching characteristics of the Alexa Fluor® 488 dye were also significantly improved over fluorescein.

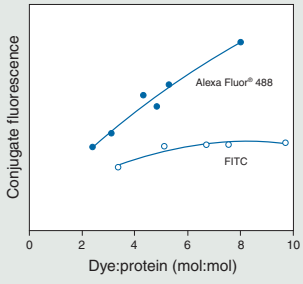


Figure 3—Comparison of the relative fluorescence of goat anti–mouse IgG antibody conjugates prepared from the Alexa Fluor® 488 dye and from fluorescein isothiocyanate (FITC). Conjugate fluorescence is determined by measuring the fluorescence quantum yield of the conjugated dye relative to that of a reference dye and multiplying by the dye:protein labeling ratio.

The excellent photostability of the Alexa Fluor® 488 dye allows more time for image observation and capture, permitting greater sensitivity and simplifying detection of low-abundance targets (Figure 4). Thus, the Alexa Fluor® 488 dye became the new benchmark against which all other green-fluorescent dye conjugates are compared.

Other contenders

More recently, several new contenders have become available (Table 1), most making claims of superior performance. To investigate these claims, we compared all green-fluorescent secondary antibody conjugates available at the time of testing.

Results of the comparison

The fluorophores tested were only those for which commercially available secondary antibody conjugates existed. In all experiments, mitochondria of bovine pulmonary artery endothelial cells were labeled with an antibody against the complex V inhibitor protein of the oxidative phosphorylation system followed by detection with a secondary antibody conjugate. All samples were processed equally and mounted in phosphate-buffered saline.

Initial experiments were performed in triplicate to determine optimal concentration and incubation times for the secondary antibody conjugates. These optimal conditions, based on signal-to-noise ratios for the mitochondrial labeling described above, were employed for subsequent evaluation of photostability and intensity. With the exception of the DyLight™ 488 (Pierce Biotechnology) conjugate, the results showed that all of new secondary antibody conjugates required a much higher concentration to achieve performance comparable to the Alexa Fluor® 488 dye conjugate (Table 1). These results suggest that at comparable concentrations, the Alexa Fluor® 488 dye conjugates are extremely bright compared to the majority of the contenders.

Table 1—Conjugates tested and their optimal working concentrations.

Fluorophore*	Vendor	Working concentration
Alexa Fluor® 488	Invitrogen	1 µg/ml
Chromeo™ 488	Active Motif	15 µg/ml
DyLight™ 488	Pierce	1 µg/ml
HiLyte Fluor™ 488	AnaSpec	25 µg/ml
MFP488	Boca Scientific	15 µg/ml
Northern Lights™ 493	R&D Systems	20 µg/ml
PromoFluor 488	PromoKine	15 µg/ml

* Tested as goat anti-mouse secondary antibody conjugates, except for the Northern Lights™ 493 dye, which was tested as a donkey anti-mouse conjugate.

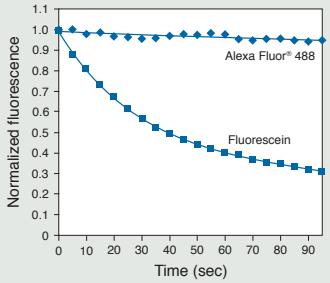


Figure 4—Photobleaching profiles of cells stained with Alexa Fluor® 488 or fluorescein conjugates. Alexa Fluor® 488 or fluorescein conjugates of goat anti–mouse IgG antibody F(ab')$_2$ fragment were used to detect HEp-2 cells probed with human anti-nuclear antibodies. Samples were continuously illuminated and images were collected every 5 seconds with a cooled CCD camera. Normalized intensity data demonstrate the difference in photobleaching rates.

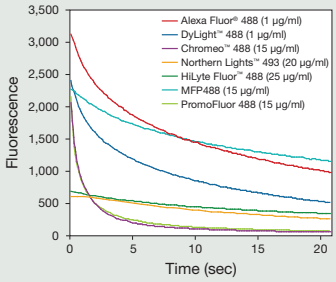


Figure 5—Photobleaching analysis results. Mitochondria in bovine pulmonary artery endothelial cells were detected using an anti-OxPhos Complex V inhibitor protein antibody and secondary antibody conjugate of the dye indicated in the graph. Samples were continuously illuminated and data collected every ~250 milliseconds. The working concentrations are noted in the legend.

Photostability a key factor

The other key characteristic of Alexa Fluor® 488 conjugates is their photostability. Under the optimal conditions determined in the previous set of experiments, dye conjugate–labeled cells mounted on slides were subjected to continuous illumination with a fluorescence microscope, and the intensity was recorded as a function of time. This series of images or "photobleach stacks" was performed in triplicate for each sample (Figure 5). With the exception of the Chromeo™ 488 (ActiveMotif) and PromoFluor 488 (PromoKine) products, the conjugates showed good resistance to photobleaching. A significantly lower signal intensity for the Hilyte Fluor™ 488 (AnaSpec) and Northern Lights™ 493 dye (R&D Systems) conjugates is also evident from the data shown in Figure 5, even under optimal conditions for those products.

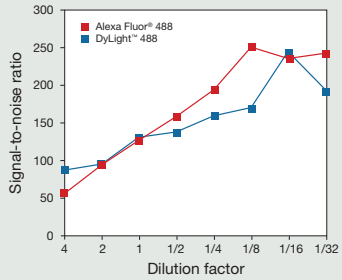


Figure 6—Signal-to-noise comparison of Alexa Fluor® 488 and DyLight™ 488 streptavidin conjugates. Human peripheral blood mononuclear cells were stained with a biotinylated anti-CD3 antibody and detected using the streptavidin conjugates of the indicated dyes. Analysis was done by flow cytometry, and the signal-to-noise ratio was plotted for the indicated dilution series.

Superior signal-to-noise ratios

To determine consistency of the intensity results using a different type of conjugate and detection platform, flow cytometry experiments were performed comparing an Alexa Fluor® 488 dye–labeled streptavidin to a similar streptavidin DyLight™ 488 conjugate. Human peripheral blood mononuclear cells were stained with a biotinylated anti-CD3 antibody and detected using the streptavidin conjugates. The signal-to-noise ratio was determined and is shown in Figure 6; the Alexa Fluor® 488 dye conjugate showed a 15–30% better signal-to-noise ratio than the DyLight™ 488 conjugate for much of the tested range.

The gold standard for dye conjugates

While several of the conjugates tested demonstrate photostability characteristics comparable to the Alexa Fluor® 488 dye conjugate, most products tested were significantly less bright, as reflected in the much higher concentrations needed to achieve fluorescence intensity similar to that of the benchmark Alexa Fluor® 488 dye conjugate. No contender has demonstrated superior performance—and the Alexa Fluor® 488 dye conjugate remains the gold standard for fluorescent dye conjugates. Learn more at **www.invitrogen.com/bioprobes56**. ■

Tools for studying dynamic cellular processes

FLUORESCENT PROTEIN–BASED ORGANELLE LIGHTS™ AND NEW CELLULAR LIGHTS™ BACMAM REAGENTS.

Targeted autofluorescent proteins can provide a window into a living cell, revealing individual cellular organelles and structures without significantly modifying cell function. Organelle Lights™ and new Cellular Lights™ fluorescent reagents are specifically designed for visualizing, tracking, and quantifying dynamic events in live cells with high spatial and temporal resolution. These reagents are provided as ready-to-use fusion constructs prepackaged in baculovirus particles for highly efficient cellular delivery via BacMam technology—that means no plasmids to purify, and no transfection complexes to prepare. The efficient transduction and robust expression of these fusion proteins provide you with highly specific labeling tools for a variety of cell landmarks.

Fluorescent protein–based BacMam reagents for efficient delivery

Designed for simple transduction of mammalian cells, Organelle Lights™ and new Cellular Lights™ reagents are based on recombinant baculoviruses developed for highly efficient, reproducible, and noncytopathic delivery to a variety of cell types, including primary and stem cells (see "The BacMam advantage"). Upon baculovirus entry into mammalian cells, a mammalian promoter directs the expression of fluorescent protein fusions that localize to specific subcellular structures, compartments, and organelles.

Organelle Lights™ and Cellular Lights™ reagents lack overt toxicity, ensuring reproducible and consistent expression. The efficient transduction makes it easy to deliver multiple constructs and to modulate gene expression by adjusting the dose. These versatile tools are easily adaptable to a number of assay formats, and their compatibility with automated liquid handling makes them ideal for high-content imaging applications.

The BacMam advantage

The BacMam delivery system uses an insect virus (baculovirus) to efficiently deliver and express genes in mammalian cells. Baculoviruses have been used for protein production in insect cells for more than two decades, but their use in mammalian cells is relatively recent. Advantages of BacMam technology include:

- Biosafety level 1 and noncytopathic delivery, because baculoviruses do not replicate in mammalian cells
- Highly efficient delivery in many cell types, including primary and stem cells
- Prepackaged, very easy-to-use delivery and transient expression system
- Ability to reproducibly transduce one or more BacMam reagents
- Easy adjustment of expression levels by simply varying the dose

Ready-to-use fluorescent protein constructs

Organelle Lights™ and Cellular Lights™ reagents are provided ready to use—there's no need to purify plasmids or worry about vector integrity and quality. No lipids, dye-loading chemicals, or other potentially harmful treatments are required. In addition, because each batch of these reagents is packaged at the same concentration, transduction conditions only need to be optimized once per cell type.

These fluorescent protein–based reagents are available in a variety of colors and a wide range of destination peptides and functional fusions, including mitochondria, lysosomes, ER, Golgi apparatus, actin, histone 2B, and MAP4. This allows convenient multiplexing, colocalization studies, and studies of dynamic cellular processes. Organelle Lights™ and Cellular Lights™ reagents allow visualization of specific live-cell structures using fluorescence microscopy (Figure 1), flow cytometry, or high-content imaging, and are compatible with subsequent fixation and immunocytochemical processing. Each reagent is supplied with an enhancer solution for increased expression.

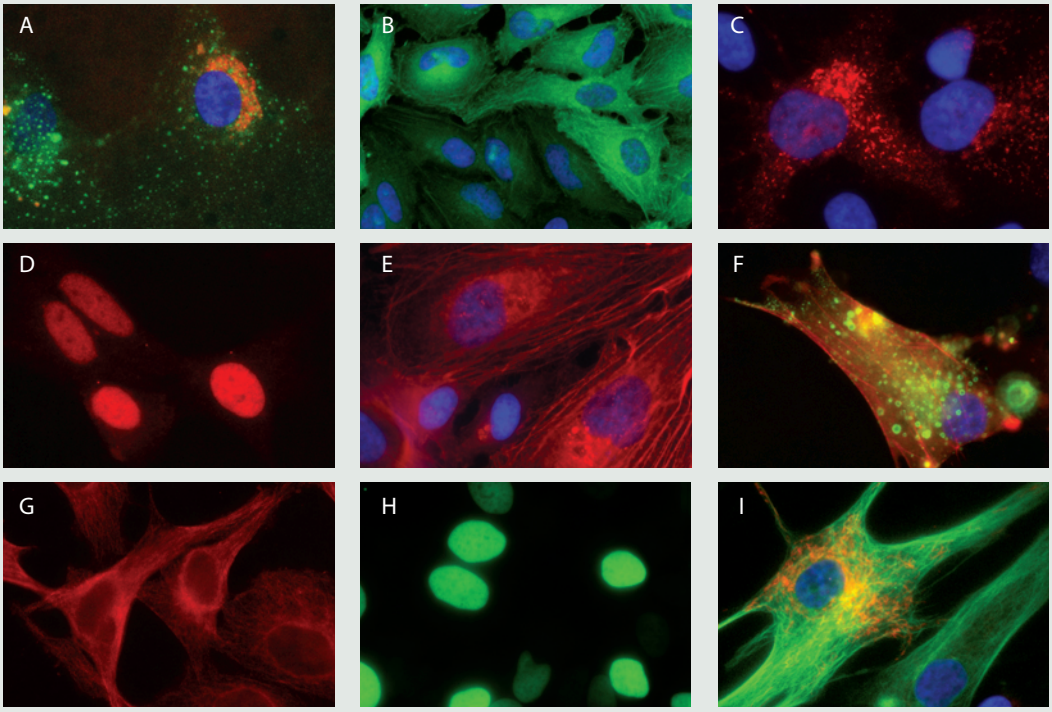


Figure 1—Live cells transduced with Organelle Lights™ or Cellular Lights™ reagents. (A) Cascade Biologics® human aortic smooth muscle cells (HASMC, Cat. no. C-007-5C) transduced with Organelle Lights™ Endosomes-GFP (Cat. no. O10104), Organelle Lights™ Golgi-OFP (Cat. no. O36224), and Hoechst 33342 (Cat. no. H24192); **(B)** HeLa cells transduced with Cellular Lights™ Actin-GFP (Cat no. C10126) and Hoechst 33342, viewed at 40×; **(C)** HeLa cells transduced with Organelle Lights™ Lysosomes-RFP (Cat. no. O10100) and Hoechst 33342, viewed at 40×; **(D)** HeLa cells transduced with Cellular Lights™ Histone 2B-RFP (Cat. no. C10129), viewed at 40×; **(E)** HeLa cells transduced with Cellular Lights™ Actin-RFP (Cat. no. C10127) and Hoechst 33342, viewed at 63×; **(F)** Cascade Biologics® HASMC transduced with Cellular Lights™ Actin-RFP (Cat. no. C10126), Organelle Lights™ Endosomes-GFP (Cat. no. O10104), and Hoechst 33342; **(G)** HeLa cells transduced with Cellular Lights™ Tubulin-RFP (Cat. no. C10112); **(H)** HeLa cells transduced with Cellular Lights™ Histone 2B-GFP (Cat. no. C10128); **(I)** Cascade Biologics® HASMC transduced with Cellular Lights™ Map4-GFP (Cat. no. C10105), Organelle Lights™ Mito-OFP (Cat. no. O36222), and Hoechst 33342.

Red fluorescent protein now available

Invitrogen now offers red fluorescent protein (RFP) versions of Organelle Lights™ and Cellular Lights™ reagents. TagRFP from Evrogen is a novel monomeric red fluorescent protein based on the wild-type RFP from the sea anemone *Entacmaea quadricolor*.[1] Approximately three times brighter than mCherry protein,[2] TagRFP is the brightest monomeric red fluorescent protein available.

Free yourself from making your own constructs

Organelle Lights™ and Cellular Lights™ reagents offer efficient, noncytopathic delivery and reproducible expression in multiple cell types with full rights to use. Invitrogen's newest Organelle Lights™ and Cellular Lights™ reagents are listed at right. To learn more and view the full list of these reagents, visit **www.invitrogen.com/bioprobes56**. ■

References

1. Merzlyak, E.M. et al. (2007) *Nat Methods* 4:555–557.
2. Shaner, N.C. et al. (2004) *Nat Biotechnol* 12:1567–1572.

Product	Quantity	Cat. no.
Organelle Lights™ Golgi-RFP	1 kit	O10098
Organelle Lights™ Nuc-RFP	1 kit	O10099
Organelle Lights™ Lysosomes-RFP	1 kit	O10100
Organelle Lights™ Endosomes-GFP	1 kit	O10104
Cellular Lights™ Actin-GFP	1 kit	C10126
Cellular Lights™ Actin-RFP	1 kit	C10127
Cellular Lights™ Tubulin-GFP	1 kit	C10106
Cellular Lights™ Tubulin-RFP	1 kit	C10112
Cellular Lights™ MAP4-GFP	1 kit	C10105
Cellular Lights™ MAP4-RFP	1 kit	C10140
Cellular Lights™ Histone 2B-GFP	1 kit	C10128
Cellular Lights™ Histone 2B-RFP	1 kit	C10129
Cellular Lights™ Null (Control)	1 kit	C10130

Measuring mitochondrial protein levels and enzyme activity

GET BOTH READOUTS WITH MITOPROFILE™ DIPSTICK AND MICROPLATE ASSAYS.

Mitochondria play an important role in a variety of biological functions, the most significant of which is energy production through oxidative phosphorylation using glucose, fatty acids, or amino acids as substrates. The mitochondrial oxidative phosphorylation (OxPhos) chain consists of five protein complexes that release energy by a series of redox reactions. Inefficiencies in this system can lead to the production of reactive oxygen species and oxidative stress. Dysfunction of the mitochondrial OxPhos system has been linked to diseases such as Alzheimer's, Parkinson's, diabetes, and cancer.

Until now, measuring activity and expression has necessitated isolating mitochondria from the sample, a time-consuming process that requires large initial amounts of material. Invitrogen's MitoProfile™ assays employ a new approach that uses high-specificity, high-affinity immunocapture antibodies and gentle solubilization buffers to assay functionally active OxPhos complexes without the need to isolate mitochondria. MitoProfile™ assays are provided in both rapid and high-throughput options. They require only a small amount of sample (nanograms to micrograms) and are compatible with various sample types including whole cells, tissue, or blood.

MitoProfile™ dipstick assays—a rapid and simple approach

The dipstick assays offer a quick and simple method to measure mitochondrial protein quantity or enzyme activity. These assays are ideal for performing measurements on microgram amounts of tissue in animal studies or on a single drop of blood or a cheek swab for human subjects. Semiquantitative results can be collected within two hours. Dipstick assays have an immobilized monoclonal antibody, specific to the target protein, bound to the nitrocellulose membrane of the dipstick (Figure 1). To measure protein quantity, the target protein is first immunocaptured onto the dipstick, then immersed in a second monoclonal antibody conjugated to gold particles. The signal intensity that develops on the dipstick correlates directly to the protein quantity. Similarly, dipstick assays for enzyme activity measurement first immunocapture the target enzyme onto the dipstick. Once the enzyme is bound, the dipstick is immersed in a buffer containing the enzyme substrate and a precipitating reporter dye. The signal intensity of the precipitate, which can be measured quantitatively using an imaging system, corresponds to the enzyme activity levels (Figure 2).

MitoProfile™ microplate assays—a high-throughput approach

The microplate assays allow up to 96 samples to be run simultaneously with high reproducibility. The kits are ready to use and are supplied with all necessary reagents, including a 96-well plate precoated with a monoclonal antibody that binds the target protein. The amount of protein captured is determined by the reaction of a second monoclonal antibody against a different epitope in the protein complex, with the signal reported by an

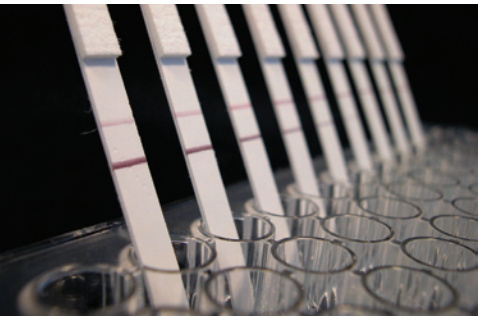


Figure 1—MitoProfile™ dipstick assays. The capture antibody and a goat anti-mouse (GAM) positive control antibody are bound to the nitrocellulose membrane of the dipstick (GAM is the upper line), and the detector antibody (conjugated to colloidal gold) is in the bottom of the microplate well. After the sample and buffers are added to the well, the dipstick is inserted and the sample solution wicks up through the lines of embedded antibody, with the gold-conjugated mAb binding to the target protein creating a visible and quantitative signal.

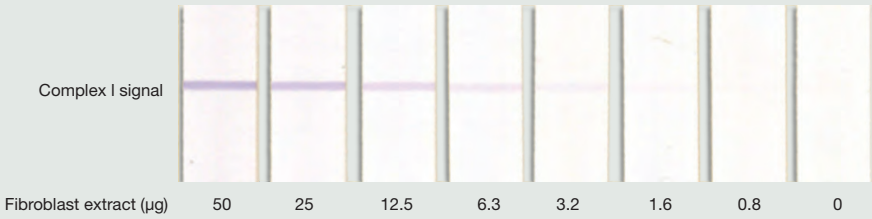


Figure 2—Activity measurements for Complex I from a dilution series of cultured fibroblasts demonstrating assay sensitivity. A clearly visible signal is evident when only 3.2 µg of material is used.

appropriate alkaline phosphatase–conjugated secondary antibody. In the case of activity microplates, the enzyme is immunocaptured by the plate-bound monoclonal antibody. The enzyme's activity is then measured by following the oxidation reaction at either 340 nm or 550 nm (Figure 3).

Get the data you need

Whether you use the rapid dipstick format or the high-throughput microplate format, MitoProfile™ assays can yield more data with less sample. Learn more about MitoProfile™ dipstick and microplate assays at **www.invitrogen.com/bioprobes56**. ■

MitoProfile™ is a trademark of Mitoscience LLC.

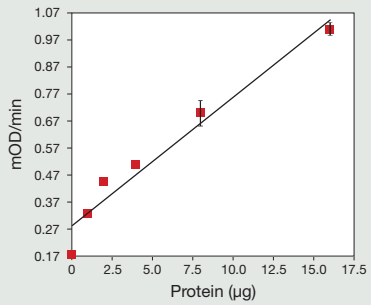


Figure 3—Activity measurements for Complex V (ATP synthase) using the MitoProfile™ Complex V Activity Kit. Complex V activity was measured from a HepG2 cell lysate dilution series to show linearity of results.

Product	Quantity	Cat. no.
MitoProfile™ Dipstick Kit for Complex I Activity	1 kit	KHM1011
MitoProfile™ Dipstick Kit for Human Complex I Quantity	1 kit	KHM1111
MitoProfile™ Dipstick Kit for Rodent Complex I Quantity	1 kit	KRM1111
MitoProfile™ Dipstick Kit for Human Complex IV Activity	1 kit	KHM1041
MitoProfile™ Dipstick Kit for Human Complex IV Quantity	1 kit	KHM1141
MitoProfile™ Dipstick Kit for Rodent Complex IV Activity	1 kit	KRM1041
MitoProfile™ Human Complex IV Activity	1 kit	KHM2041
MitoProfile™ Human Complex IV Quantity	1 kit	KHM2141
MitoProfile™ Human Complex IV Activity and Quantity	1 kit	KHM2241
MitoProfile™ Mouse Complex IV Activity	1 kit	KMM2041
MitoProfile™ Mouse Complex IV Quantity	1 kit	KMM2141
MitoProfile™ Mouse Complex IV Activity and Quantity	1 kit	KMM2241
MitoProfile™ Rat Complex IV Activity	1 kit	KRM2041
MitoProfile™ Complex V Activity	1 kit	KHM2051
MitoProfile™ Complex V Quantity	1 kit	KHM2151
MitoProfile™ Complex V Activity and Quantity	1 kit	KHM2251
MitoProfile™ Mitochondria Isolation Kit for Rodent Tissue	1 kit	KRM3011
MitoProfile™ Mitochondria Isolation Kit for Rodent Tissue with Homogenizer	1 kit	KRM3021
MitoProfile™ Mitochondria Isolation Kit for Cultured Cells	1 kit	KHM3031
MitoProfile™ Mitochondria Isolation Kit for Cultured Cells with Homogenizer	1 kit	KHM3041
MitoProfile™ Dipstick Kit for Frataxin Quantity	1 kit	KHM1171
MitoProfile™ Dipstick Kit for PDH Activity	1 kit	KHM1061
MitoProfile™ Dipstick Kit for PDH Quantity	1 kit	KHM1161
MitoProfile™ Apoptosis Detection Kit	1 kit	KHM4011

The next generation in protein profiling

CLICK-IT™ AHA FOR NASCENT PROTEIN SYNTHESIS.

Detecting newly synthesized protein is key for researchers studying protein biosynthesis, trafficking, and degradation. Click-iT™ AHA (L-azidohomoalanine) provides a fast, sensitive, nontoxic, and—most importantly—nonradioactive alternative to the traditional radioactive technique, ^{35}S-methionine, for the detection of nascent protein.

Make comprehensive protein profiling a snap

L-AHA is an amino acid analog of methionine containing a very small modification: an azido moiety (Figure 1). Click-iT™ AHA is fed to cultured cells and incorporated into proteins during active protein synthesis. Detection of the amino acid is carried out through a chemoselective ligation, or "click" reaction, between an azide and an alkyne; the azido-modified protein is detected with TAMRA, Dapoxyl®, or biotin alkyne (Figure 2) included in the Click-iT™ Protein Analysis Detection Kits. These reagents provide detection sensitivity comparable to that obtained using the radioactive method (Figure 3), and are compatible with downstream LC-MS/MS and MALDI MS analysis or with Multiplexed Proteomics® reagents for differential analyses of newly synthesized protein together with total glycoprotein, total phosphoprotein, or total protein (Table 1).

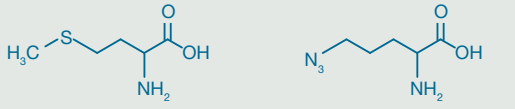


Figure 1—Structures of methionine and Click-iT™ AHA.

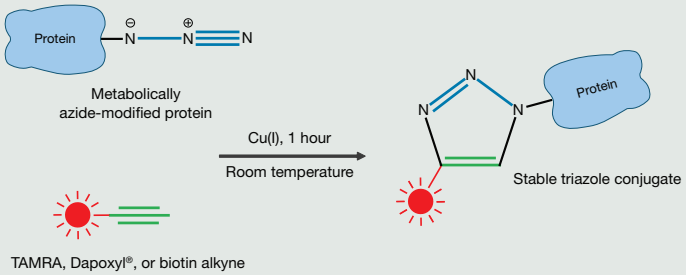


Figure 2—Click-iT™ azide/alkyne reaction.

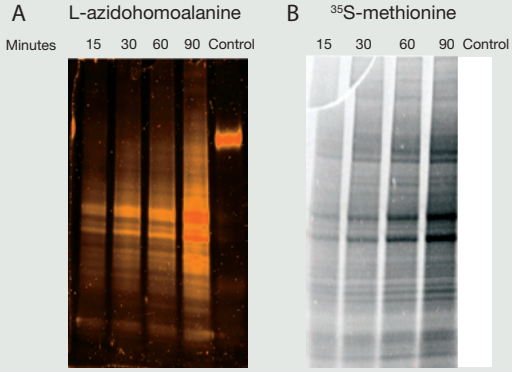


Figure 3—Comparison of ^{35}S-methionine and Click-iT™ AHA sensitivity. Cortical neurons were metabolically labeled with either Click-iT™ AHA (L-azidohomoalanine) or ^{35}S-methionine in a time series for 15–90 minutes. (**A**) Newly synthesized proteins labeled with Click-iT™ AHA were detected with the Click-iT™ TAMRA Protein Analysis Kit (C33370) and visualized using 532 nm excitation. (**B**) Proteins labeled with ^{35}S-methionine were blotted onto Immobilon®-P membrane (Millipore) and detected by storage phosphorimaging. The time course for incorporation of Click-iT™ AHA vs. ^{35}S-met shows similar increases over a time frame of 15–90 minutes. The sensitivity of Click-iT™ AHA with TAMRA alkyne detection is comparable to that of ^{35}S-Met; the fluorescence of the TAMRA image was quantified in the two most prominent bands, and the incorporation was linear across the entire time series (data not shown). Data contributed by Pate Skene, Duke University.

Table 1—Compatibility of the Click-iT™ protein analysis detection kits.

		Compatibility with detection methods			Spectral compatibility with Multiplexed Proteomics® stains		
Click-iT™ protein analysis detection kit	Cat. no.	1D or 2D gel	Western blot	Mass spectrometry	SYPRO® Ruby protein gel stain	Pro-Q® Emerald glycoprotein gel stain	Pro-Q® Diamond phosphoprotein gel stain
Click-iT™ Tetramethylrhodamine (TAMRA) Protein Analysis Detection Kit	C33370	●	●	●	●	●	
Click-iT™ Dapoxyl® Protein Analysis Detection Kit	C33371	●		●	●		●
Click-iT™ Biotin Protein Analysis Detection Kit	C33372		●	●			

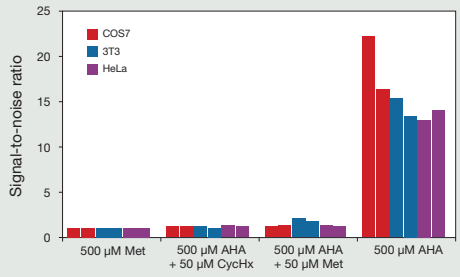


Figure 4—Inhibition of Click-iT™ AHA incorporation. COS-7, 3T3-L1, and HeLa cells were treated for 1 hour with 500 µM methionine (Met); 500 µM AHA + 50 µM cycloheximide (CycHx); 500 µM AHA + 50 µM methionine; or 500 µM AHA and detected using the Click-iT™ TAMRA Protein Analysis Detection Kit. AHA incorporation is suppressed by the native amino acid methionine and the protein synthesis inhibitor cycloheximide.

Experience a radioactivity-free lab

"We are out of the radioactivity business."
—Pate Skene, Professor of Neurobiology, Duke University

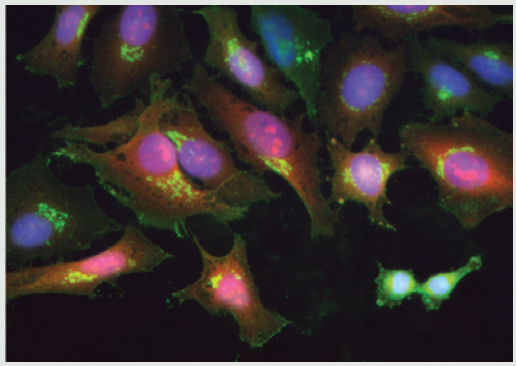
Click-iT™ AHA replaces one of the last applications requiring the use of radioactive reagents. Click-iT™ AHA has proved to be a successful substitute for methionine in many cell types (Figure 4), including COS-7, 3T3-L1, HeLa, HEK 293, and Jurkat cells. Note that cells should be labeled in methionine-free media, as methionine is the preferred substrate for methionyl tRNA transferase, and supplemented media (i.e., methionine-free DMEM) should be used in place of HBSS to achieve greater Click-iT™ AHA incorporation at lower concentrations (data not shown).

Leave the handling and disposal hassles behind

Click-iT™ AHA not only enables you to rid your lab of the costly handling and disposal of radioactive materials, but provides multiplexing capabilities not possible with standard ^{35}S-methionine labeling techniques. Learn more about Click-iT™ AHA at **www.invitrogen.com/bioprobes56**. ■

The future of click chemistry is available today

Click chemistry is designed not to replace existing detection methodologies, but to enable or enhance detection—something that isn't always possible, or optimal, when using antibodies, streptavidin, or directly labeled compounds. Invitrogen is committed to developing new and innovative tools based on click detection technology and offers Click-iT™ reagents, such as an alkyne-modified mannosamine and Alexa Fluor® 594 alkyne, by custom synthesis. Find out how to make your experiments a snap with click chemistry—email our Custom Services department at **probescustom@invitrogen.com** with your research goals.



HeLa cells stained using click technology. HeLa cells were cultured for 20 hours with 50 µM of an alkyne-containing mannosamine (available by custom synthesis). The medium was replaced with DMEM without methionine for 30 minutes, then the medium was replaced again with DMEM containing 50 µM Click-iT™ AHA and cells were incubated for 30 minutes. Following fixation and permeabilization, cells were stained with green-fluorescent Alexa Fluor® 488 azide (from the Click-iT™ EdU Alexa Fluor® 488 Imaging Kit, Cat. no. C10083) to detect the alkyne-containing mannosamine, washed, then stained with red-fluorescent Alexa Fluor® 594 alkyne (available by custom synthesis) to detect the AHA. Finally, nuclei were stained with blue-fluorescent Hoechst 33342 before imaging.

Product	Quantity	Cat. no.
Click-iT™ AHA (L-azidohomoalanine), for nascent protein synthesis	5 mg	C10102

Seeing clearly: Accurate cell identification

CELLMASK™ STAINS FOR FLUORESCENCE-BASED IMAGING.

Fluorescence-based imaging techniques, including both conventional fluorescence microscopy and high-content screening (HCS) assays, are powered by increasingly sophisticated application software and automation hardware. The algorithms introduced by these new technologies require accurate, dependable, and distinguishable signals from cell compartments to make assignments formerly made by a microscopist. New Molecular Probes® CellMask™ plasma membrane stains and HCS CellMask™ cytoplasmic/nuclear stains offer reliable cell delineation tools in a choice of fluorescent colors from blue to deep red for multiparametric cell analysis in a broad array of image-based applications.

Outlining cells with CellMask™ plasma membrane stains

The newest CellMask™ products—CellMask™ Orange and CellMask™ Deep Red (Figures 1 and 2) plasma membrane stains—deliver uniform staining of the plasma membrane across a wide range of mammalian cell types. These two CellMask™ stains provide very fast labeling of the entire plasma membrane (often staining is complete in a few seconds or minutes), and the resulting membrane staining is relatively uniform compared with that achieved using labeled lectins. Moreover, unlike traditional lipophilic membrane stains such as DiI and DiO, CellMask™ plasma membrane stains are very slow to internalize, providing a much broader window for observation in living cells. And finally, the labeling pattern is well retained after formaldehyde fixation. LIke other small molecular weight, organic dye–based membrane stains, CellMask™ plasma membrane stains cannot withstand detergent extraction and therefore cannot be used with probes that require permeabilization.

CellMask™ Orange and CellMask™ Deep Red plasma membrane stains can serve as general tools for cell identification in both live and fixed cells using either traditional or automated imaging and analysis. They are especially useful for labeling the plasma membrane for short-term tracing in live-cell studies, colocalization with cell-surface expression of GFP chimeras, and automated measurement of translocation events.

Filling in cells with HCS CellMask™ cytoplasmic and nuclear stains

HCS CellMask™ cytoplasmic/nuclear stains fluorescently label fixed mammalian cells, producing a stronger stain of the nuclear area with a weaker overall stain of the cytoplasmic area. These stains have been designed for and validated on a variety of HCS platforms and have been shown to provide accurate cell identification by common HCS algorithms that identify whole cells. Available in a choice of fluorescent colors, HCS CellMask™ cytoplasmic/nuclear stains can be applied to cells immediately after fixation or in the last step of multiplexing protocols, and they are compatible with detergent extraction steps in cell permeabilization protocols.

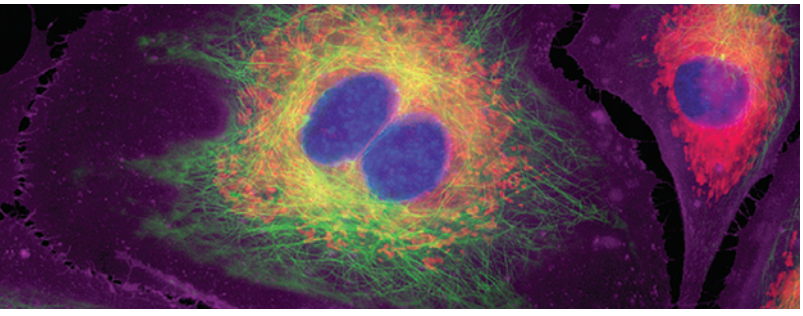


Figure 1—Live-cell labeling using CellMask™ Deep Red plasma membrane stain. Live bovine pulmonary arterial endothelial cells were labeled with CellMask™ Deep Red plasma membrane stain (Cat. no. C10046), the tubulin-selective TubulinTracker™ Green dye (Oregon Green® 488 Taxol, bis-acetate, Cat. no. T34075), mitochondrion-selective MitoTracker® Red CMXRos dye (Cat. no. M7512), and blue-fluorescent Hoechst 33342 nuclear stain (Cat. no. H24192). Labeled cells were then imaged using standard fluorescence microscopy, and the image was deconvolved using Huygens software (Scientific Volume Imaging).

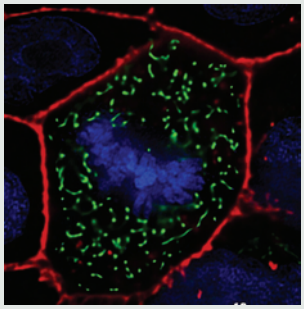


Figure 2—Live-cell labeling using CellMask™ Deep Red plasma membrane stain. HaCaT cells (an immortalized human keratinocyte cell line) transfected with a mitochondrion-targeted eGFP construct were incubated for 15 minutes in a solution containing 1 µM Hoechst 33342 (Cat. no. H21492) and then for 5 minutes in a fresh solution containing 8 µg/ml CellMask™ Deep Red plasma membrane stain (Cat. no. C10046); staining took place at 37°C in the presence of 5% CO_2. After washing twice with DMEM (including 10% FBS) at 37°C, the cells were imaged with a Zeiss Cell Observer HS microscope fitted with a 100× oil alpha Plan-Apochromat objective and a BFP/GFP/HcRed filter set from Zeiss (62HE). The image stack was deconvolved with the Zeiss AxioVision 3D Deconvolution module with the point spread function acquired using a 200 nm TetraSpeck™ fluorescent microsphere (Cat. no. T7280). Metaphase chromosomes (pseudocolored blue, Hoechst 33342), plasma membrane (pseudocolored red, CellMask™ Deep Red plasma membrane stain), and mitochondria (pseudocolored green, eGFP) are all clearly visible. Image submitted by Christian Junker, University of Saarland, Department of Biophysics, Homburg, Germany.

Cell identification strategies in high-content screening

With the resolution inherent in an imaged-based methodology and the productivity of high-throughput assays, high-content screening (HCS) provides a powerful tool for studying cell biology in a spatial and temporal context. Cell identification (or object identification) is the first step of the automated image acquisition and analysis process. For many software algorithms, the cell identification process begins with the detection of fluorescently stained nuclei. Then, using the position of the stained nucleus as a guide, the software extrapolates to build a mask that marks the probable position of the cytoplasmic region.

For some applications, however, cell identification based on nuclear staining alone is not adequate because the cytoplasmic region assigned by the algorithm does not match that defined by the actual cell boundaries. The inadequacies of these types of algorithms become apparent when the analysis includes detection of cytoplasmic features revealed in other fluorescent channels. The suite of CellMask™ stains described here facilitates a thorough description of a cell's anatomy, providing an accurate backdrop against which the features of interest can be assessed.

Furthermore, the staining pattern of HCS CellMask™ Red cytoplasmic/nuclear stain can be modulated to produce both nuclear and cytoplasmic staining or more prominent nuclear staining, a property that can be exploited to achieve a labeling pattern that better fits the design of particular image analysis algorithms. At low concentrations (0.5 µM), this stain labels the entire cell, and the staining pattern is easily processed by algorithms that make use of both nuclear labeling and faint cytoplasmic labeling. As the probe concentration is increased (up to 5 µM), nuclei become more prominently labeled for processing by software that relies on a nuclear signal. The ratio of nuclear/cytoplasmic staining can be further adjusted by combining an HCS CellMask™ cytoplasmic/nuclear stain with a nuclear stain of similar spectral properties. For example, HCS CellMask™ Blue cytoplasmic/nuclear stain has been successfully combined with a blue-fluorescent DNA-binding dye to enhance the nuclear signal.

HCS CellMask™ cytoplasmic/nuclear stains have proven useful for autofocusing, automated cell identification, and characterizing morphological changes to cells.

More tools for fluorescence-based imaging techniques

Invitrogen continues to expand its collection of HCS tools, offering products that have been validated on several HCS platforms and are provided in convenient, automation-compatible formulations. Learn more about tools for HCS at **www.invitrogen.com/bioprobes56.** ■

Product	Ex/Em (nm)	Quantity	Cat. no.
CellMask™ Orange plasma membrane stain, 5 mg/ml solution in DMSO	554/567	100 µl	C10045
CellMask™ Deep Red plasma membrane stain, 5 mg/ml solution in DMSO	649/666	100 µl	C10046
HCS CellMask™ Red cytoplasmic/nuclear stain, 5 mM solution in DMSO, for high-content screening and cellular imaging	622/645	125 µl	H32711
HCS CellMask™ Blue cytoplasmic/nuclear stain, for high-content screening and cellular imaging	346/442	1 set	H34558
HCS CellMask™ Deep Red cytoplasmic/nuclear stain, for high-content screening and cellular imaging	650/665	1 set	H34560

Putting regulatory T cells to work

ISOLATE AND EXPAND CD4$^+$ CD25$^+$ TREG CELLS USING DYNABEADS® MAGNETIC SEPARATION TECHNOLOGY.

Defined by their functional ability to regulate or suppress immune responses, regulatory CD4$^+$ CD25$^+$ T cells (Treg cells) are a specialized subpopulation of T cells that act to maintain homeostasis within the immune system. Naturally occurring Treg cells (CD4$^+$ CD25$^+$ FOXP3$^+$ T cells) have been identified in nonmanipulated rodents and humans and comprise cells of the adaptive immune system.[1,2] In addition, it has been reported that an uncommitted T cell can be skewed toward a regulatory function (Tr1 and Th3 cells) in the presence of suppressive cytokines such as IL-10 and TGFb.[3,4] Treg cells are hyporesponsive to T cell receptor (TCR) stimulation *in vitro*, but exogenous IL-2 and strong costimulation with anti-CD28 can overcome their anergic state. On polyclonal or antigen-specific TCR stimulation, Treg cells potently suppress the proliferation and cytokine production of effector CD4$^+$ and CD8$^+$ cells by inhibiting IL-2 gene transcription. The interest in Treg cells has been accelerated by evidence from experimental mouse and human models demonstrating that the immunosuppressive potential of these cells can be utilized in the treatment of various diseases such as autoimmunity, infectious diseases, and cancer.[5–11] Invitrogen's Dynabeads® Treg products provide a path from isolation to stimulation to analysis of this therapeutically relevant class of immune cells.

Capturing Treg cells from clinical samples

Treg cells can be isolated from human blood, lymphoid organs, umbilical cord blood, and the thymus, and comprise 1–10% of the total CD4$^+$ T cell population in peripheral blood. Based on Dynabeads® technology, we have developed a new isolation and expansion protocol for human CD4$^+$ CD25$^+$ regulatory T cells with characteristic phenotype and suppressive capacity by using Dynabeads® Regulatory CD4$^+$ CD25$^+$

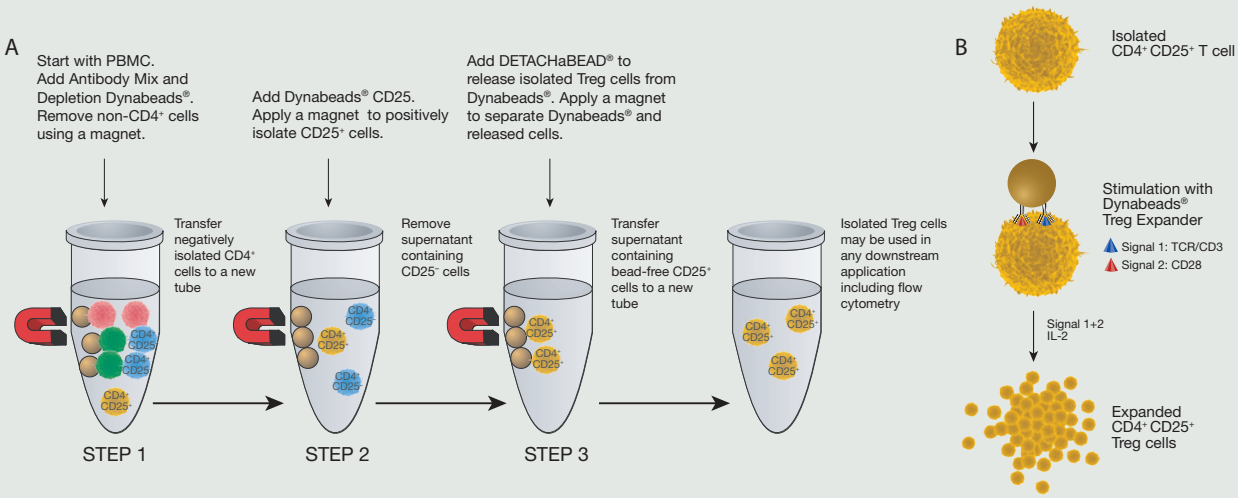


Figure 1—An overview of the isolation and expansion protocol for human Treg cells. (A) CD4+ CD25+ regulatory T cells are isolated from peripheral blood mononuclear cells (PBMC) with the Dynabeads® Regulatory CD4+ CD25+ T Cell Kit by negative isolation of CD4+ T cells followed by positive isolation with Dynabeads® CD25 and bead detachment with DETACHaBEAD® reagent. Isolated CD4+ CD25+ regulatory T cells are bead- and antibody-free. **(B)** Expansion of CD4+ CD25+ Treg cells using Dynabeads® Treg Expander.

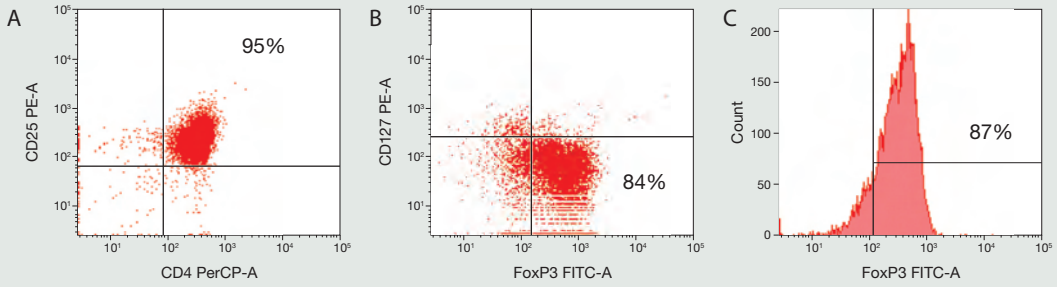


Figure 2—Treg phenotype after isolation. Isolation of Treg cells with the Dynabeads® Regulatory CD4+ CD25+ T Cell Kit. **(A)** The isolated Treg cells are 95% pure. **(B)** Within the CD4+ CD25+ Treg cell population, 84% express FoxP3 and are negative for CD127. **(C)** In total, 87% of the CD4+ CD25+ Treg cells express FoxP3.

T cell Kit and Dynabeads® Treg Expander (Figure 1). The unique positive isolation method allows the release of both the bead and the anti-CD25 antibody, which eliminates undesirable effects that occur when the IL-2α chain (CD25) becomes bound by antibody.[12] It is also possible to isolate both T effector ($CD4^+$ $CD25^-$) and T regulatory ($CD4^+$ $CD25^+$) populations using this kit.

Isolated Treg cells are phenotypically correct and retain their suppressive capacity

Highly pure (≥95%) regulatory $CD4^+$ $CD25^+$ T cells were isolated from the peripheral blood mononuclear cells (PBMC) of healthy blood donors using the Dynabeads® Regulatory $CD4^+$ $CD25^+$ T Cell Kit. A large majority of these cells (≥80%) expressed the FoxP3 transcription factor (Figure 2). Comparison of Dynabeads® technology with a column-based isolation technology revealed that the Dynabeads® tube-based isolation strategy resulted in a significantly higher number of $CD25^+$ as well as $FoxP3^+$ T cells (Figure 3).

To assay suppressive capacity, $CD4^+$ $CD25^-$ effector T cells were stained with CFSE (Cat. no. C34554) and mixed with $CD4^+$ $CD25^+$ Treg cells. Dynabeads® CD3 (1 bead/cell) were added to activate the effector $CD25^-$ T cells, and suppression was measured 4 days later using CFSE staining. Treg cells suppressed the proliferation of $CD4^+$ $CD25^-$ effector T cells in the presence of CD3 activation by up to 96% (Figure 4), showing that the isolated Treg cells retain their normal function.

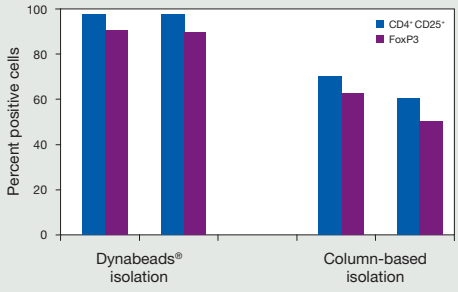


Figure 3—Comparison of the Dynabeads® tube-based isolation strategy and a column-based method. Treg cells were isolated from two different donors using the Dynabeads® Regulatory CD4+ CD25+ T Cell Kit or a column-based method. Purity was assessed by flow cytometric analysis with anti-CD25 and anti-FoxP3 antibodies.

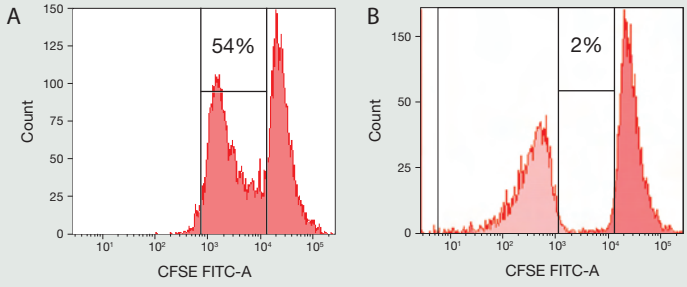


Figure 4—Suppressive capacity of isolated Treg cells. (A) CD4+ $CD25^-$ cells were stained with CFSE and stimulated with Dynabeads® CD3 (1 bead:cell) for four days. On day 4, 54% of the cells were dividing as identified by flow cytometry. **(B)** CD4+ $CD25^-$ cells stained with CFSE were stimulated with Dynabeads® CD3 in the presence of CD4+ CD25+ Treg cells in a 1:1 ratio. After four days, only 2% of the CD4+ $CD25^-$ cells were dividing and 96% suppression of cell division was achieved in the presence of CD4+ CD25+ Treg cells (unstained CD4+ CD25+ cells shown in light red).

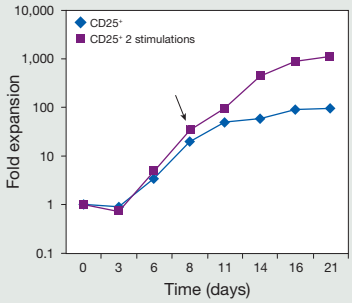


Figure 5—Expansion of isolated regulatory T cells. Highly pure Treg cells (>95%) isolated with the Dynabeads® Regulatory T Cell Kit can be expanded 100-fold while retaining their Treg phenotype with one round of stimulation using Dynabeads® Treg Expander. High initial purity of the isolated Treg cells (>97%) allows restimulation at day 8 (arrow) to obtain even higher expansion levels (1,000-fold) without overgrowth of non-Treg cells.

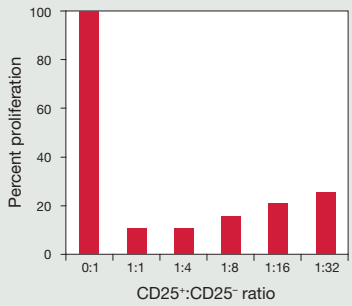


Figure 6—Suppressive capacity of rapamycin-generated Treg cells. $CD4^+$ T cells expanded with Dynabeads® CD3/CD28 and rapamycin were cocultured with Dynabeads® CD3/CD28 only to evaluate the suppressive capacity of the rapamycin-expanded cells. At day 4, proliferation was analyzed in a thymidine incorporation assay. Suppression of ~80% could be maintained with a 1:32 $CD25^+$:$CD25^-$ ratio.

Expand the possibilities for functional studies

Low numbers of regulatory T cells can be a roadblock for scientists wishing to perform functional and/or adoptive cell transfer experiments.[13] To expand the $CD25^+$ Treg population, Dynabeads® Treg Expander reagent was added to 1×10^6 cells/ml (3 beads/cell) for 14 days. The cultures were supplemented with 500 U/ml of IL-2. As shown in Figure 5, there is robust expansion (up to 100-fold) of Treg cells using the Dynabeads® CD3/CD28 approach.

For clinical research, Treg cells can be generated by expanding $CD4^+$ T cells (Dynal® CD4 Negative Isolation Kit) with Dynabeads® CD3/CD28 in the presence of rapamycin for 2 weeks (Figure 6).

Treg cells in clinical applications

The Dynabeads® Regulatory $CD4^+$ $CD25^+$ T Cell Kit can be used to isolate ≥95% pure $CD4^+$ $CD25^+$ Treg cells, and more than 80% of the isolated $CD25^+$ cells express the FoxP3 transcription factor. Dynabeads® Treg Expander reagent can expand human $CD4^+$ $CD25^+$ Treg cells up to 100-fold during 2–3 weeks of culture while retaining their suppressive phenotype and expression of CD25 and FoxP3. Such expansion will facilitate further characterization of Treg cells as well as the evaluation of their potential in clinical applications. For more information or to place an order, visit **www.invitrogen.com/bioprobes56**. ■

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Product	Application	Quantity	Cat. no.
Dynabeads® Regulatory $CD4^+$ $CD25^+$ T Cell Kit	Treg cell isolation	2 × 5 ml	113.63D
Dynabeads® Human Treg Expander	Treg expansion	2 ml	111.29D
Dynabeads® CD3	Short-term stimulation/suppression assays	5 ml	111.51D
Dynabeads® CD3/CD28	T cell expansion in preclinical research	10 ml	111.41D
Dynabeads® *ClinExVivo*™ CD3/CD28	T cell expansion in clinical research	10 ml	402.03D
Dynal® CD4 Negative Isolation Kit	$CD4^+$ T cell isolation	5 ml	113.17D
CellTrace™ CFSE Cell Proliferation Kit	Cell proliferation kit for flow cytometry	1 kit	C34554

Cell-based tools for interrogating the PI3K/Akt/mTor pathway

CELLSENSOR® AND LANTHASCREEN™ GFP CELLULAR ASSAYS.

The PI3K/Akt signaling pathway is central to cell growth and survival, entry into the cell cycle, and regulated cell death (Figure 1). Aberrant activation of this signaling cascade is linked to diseases including cancer, diabetes, cardiovascular conditions, and neurological disorders.[1] Moreover, compounds that target this network (e.g., rapamycin and its analogs) have proven efficacious as approved drugs targeting such diverse indications as renal cell carcinoma, transplant rejection, and the prevention of restenosis following balloon angioplasty. These observations make numerous components of the pathway attractive as therapeutic targets, and have fueled drug discovery and high-throughput screening (HTS) efforts in this area. The considerable degree of complexity, crosstalk, and feedback regulation that exists within the PI3K/Akt pathway—especially as applied to the regulation of the mammalian target of rapamycin (mTOR) and its complexes (mTORC1 and mTORC2)—underscores the need for cell-based methods to properly identify and characterize small-molecule modulators of the pathway. Because cellular intricacies are lost when using purified components in a biochemical experiment, Invitrogen is introducing a more holistic approach to pathway analysis. Two cell-based tools, both HTS-compatible and fluorescence-based, have been developed to interrogate the PI3K/Akt/mTor pathway: (1) a traditional CellSensor® reporter gene–based system that provides an endpoint measurement of global compound effects on the PI3K/Akt/Foxo3 arm of the pathway, and (2) a LanthaScreen™ GFP cellular assay format that enables detection of changes in the phosphorylation status of specific kinase targets within the pathway, including readouts for both mTORC1 and mTORC2 activity.

CellSensor® products

Invitrogen has extensive experience developing CellSensor® cell lines that use GeneBLAzer® technology to provide a reliable, rapid, and sensitive method for analyzing the response of disease-relevant signal →

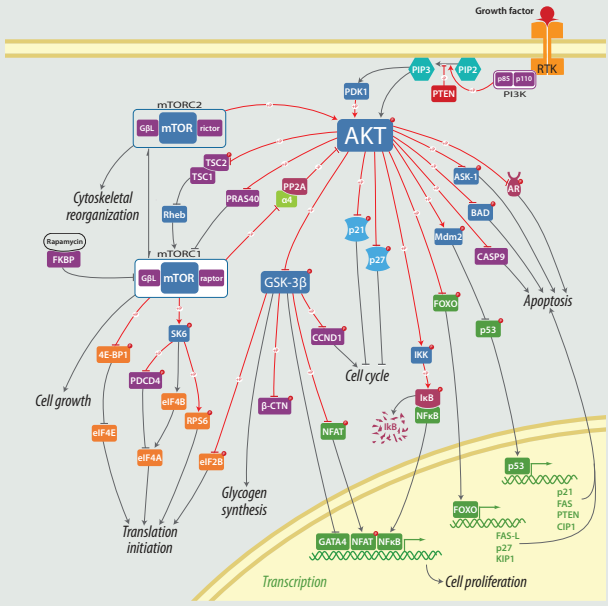

Figure 1—The Akt signaling pathway. This cell signaling cascade is involved in numerous biological processes, including the regulation of metabolism, cell growth and survival, cell-cycle progression, and transcription and translation. AKT resides downstream of phosphoinositide 3-kinase (PI3K) signaling, which is activated upon binding of growth factors (e.g., insulin) to receptor tyrosine kinases (RTKs) on the cell surface. Activated AKT phosphorylates a range of substrates, including PRAS40 and FOXO3. The mammalian target of rapamycin (mTOR) is a serine/threonine kinase that assembles into two distinct complexes inside the cell (mTORC1 and mTORC2) and has been placed on both sides of the Akt signaling hub. mTORC1 (the rapamycin-sensitive complex with raptor) resides downstream of AKT, while mTORC2 (the rapamycin-insensitive complex with rictor) is able to fully activate AKT by direct phosphorylation at Ser473.

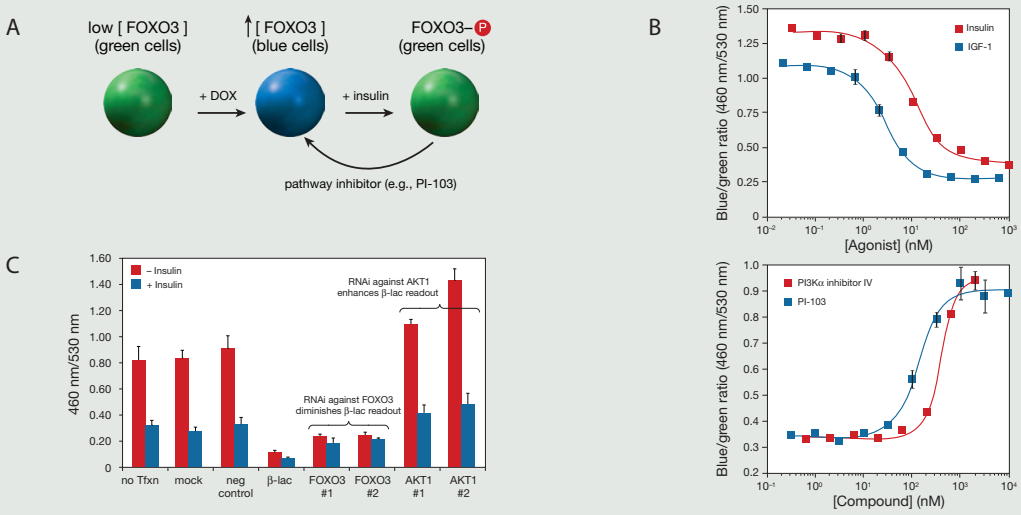

Figure 2—CellSensor® reporter gene readout for PI3K/Akt pathway analysis. (A) The T-REx™ FOXO3 DBE-*bla* HeLa cell line (Cat. no. K1468) has a FOXO3 response element (driving beta-lactamase (BLA) expression (DBE-*bla*)), tetracycline repressor, and tetracycline-inducible Foxo3 constructs. Addition of doxycycline (DOX, a tetracycline analog) upregulates FOXO3-driven BLA expression. Activation of the endogenous PI3K/Akt signaling cascade with insulin (Cat. no. 12585-014) leads to phosphorylation/inactivation of FOXO3 and concomitant suppression of BLA. Interruption of the pathway with inhibitors restores FOXO3 transcriptional activity and thus BLA expression (green to blue cells). (**B**, top) The cellular response to growth factor stimulation was tested with IGF-1 and insulin, and the EC_{50} values obtained were 1.6 nM and 5 nM, respectively. (**B**, bottom) The small-molecule inhibitors PI-103 and PI3Kα inhibitor IV were evaluated, and the IC_{50} values obtained were 110 nM and 330 nM, respectively. **(C)** RNAi knockdown experiments of FOXO3 and AKT for target validation were performed. Cells were reverse-transfected with Lipofectamine™ RNAiMAX (Cat. no. 13778-075) and 20 nM of a panel of RNAi duplexes, including the FOXO3 Validated Stealth™ RNAi DuoPak (Cat. no. 12937-07), the AKT1 Validated Stealth™ RNAi DuoPak (Cat. no. 12935-001), Stealth™ RNAi oligonucleotides against beta-lactamase, and the medium GC control from the Stealth™ RNAi Negative Control Kit (Cat. no. 12935-100).

transduction pathways upon exposure to drug candidates or other stimuli.[2] The growing portfolio of cell-based beta-lactamase reporter assays addresses (as part of the SelectScreen™ cell-based profiling service) more than 20 different signaling pathways, as well as specific protein targets (e.g., kinases) that are involved in the endogenous pathways.

To build a PI3K/Akt pathway–specific CellSensor® cell line, we stably engineered the beta-lactamase reporter under the control of a FOXO3 response element (DBE-*bla*) into HeLa cervical cancer cells. Additionally, we further designed this cell line to feature tetracycline-inducible FOXO3 expression (via the T-REx™ mechanism), because overexpression of FOXO3 is capable of triggering apoptosis through transcription of cell death genes (e.g., FAS-L), and precise regulation of FOXO3 levels was therefore necessary. When cells are left untreated and then loaded with LiveBLAzer™ substrate, the FRET-based beta-lactamase substrate remains green (no BLA present) (Figure 2A). Expression of BLA (induced by tetracycline and driven by FOXO3), however, results in cleavage of the fluorescent substrate molecule, disrupting the energy transfer, and cells turn blue. Activation of PI3K signaling upon growth factor binding (e.g., insulin or IGF-1; Figure 2B) to a cell-surface receptor tyrosine kinase leads to increased downstream activity of AKT and subsequent phosphorylation of the FOXO3 transcription factor (among other targets) to promote cell survival and oppose apoptosis. This modification at Thr32 inactivates FOXO3, leading to its translocation out of the nucleus and concomitant suppression of BLA expression to yield green cells. Application of pathway inhibitors (e.g., PI-103 or PI3Kα Inhibitor IV; Figure 2B) or Stealth™ RNAi (e.g., against AKT1; Figure 2C) restores FOXO3 activity and BLA expression, turning the cells back to blue.

Advantages of the CellSensor® assay system include the use of a sensitive fluorescent reporter readout that is amenable to low-volume HTS formats, ratiometric FRET-based data analysis that improves data quality, and a live-cell signal (blue/green cells) that is highly suitable for microscopic imaging and cell sorting. The T-REx™ FOXO3 DBE-*bla* HeLa cell line provides an effective endpoint readout of PI3K/Akt/Foxo3 pathway alteration upon exposure to a variety of ligands.

LanthaScreen™ GFP cellular assays

LanthaScreen™ assays use a technology based on time-resolved FRET (TR-FRET) to monitor phosphorylation of a specific kinase substrate in

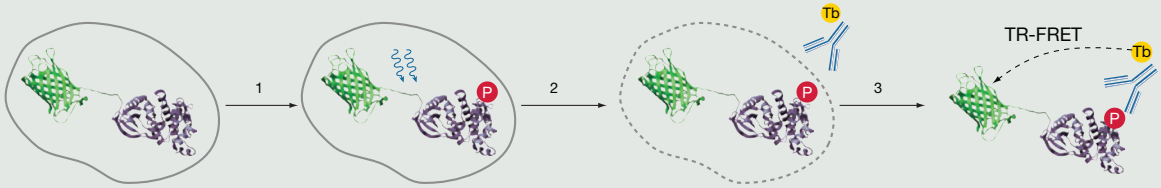

Figure 3—LanthaScreen™ GFP cellular assay schematic. Cells stably expressing a GFP-fusion of the kinase target are stimulated to activate the pathway and the substrate is phosphorylated (step 1; "*in vivo* kinase assay"). Next, the cells are lysed and a Tb-labeled PSSA is added (step 2). The FRET signal is measured (step 3) on a fluorescence plate reader in a time-resolved manner.

an endogenous signaling pathway.[3] By stably expressing the protein of interest as a fusion with green fluorescent protein (GFP), cells can be treated with an agonist (e.g., insulin or IGF-1) to stimulate the pathway and phosphorylate the kinase target. Following cell lysis, the modification is quantitatively detected by a phosphorylation site–specific antibody (PSSA) that is labeled with terbium (Tb) chelate, which serves as the FRET donor partner (Figure 3). Due to the long emission lifetime of the Tb donor, the FRET signal can be measured after interference from autofluorescent molecules or from scattered light has decayed. By translating the LanthaScreen™ technology into a cellular format with GFP-fusion substrates, we have preserved the physiological complexity of live cells to assay the endogenous protein kinase within the context of the native signaling pathway. The robust, homogeneous (no-wash) format of the LanthaScreen™ GFP cellular assay offers an attractive alternative to complex image-based assays or traditional methods such as western blots or ELISAs. There is little hands-on time with this assay, and it is amenable to automation. Moreover, the use of a single antibody simplifies the assay and improves performance relative to other two-antibody "sandwich" approaches.

To interrogate the PI3K/Akt pathway using the LanthaScreen™ GFP cellular assay technology, specifically as it applies to the regulation of mTOR, we have generated three different cell lines, each with a GFP fusion of a key pathway marker: PRAS40, PDCD4, and AKT (Figure 4A). Importantly, each of these assays are built in a HEK 293E cell background, where (unlike HEK 293T cells), the mTOR pathway is strongly regulated by serum and insulin.[4]

A cell line to monitor immediately downstream of Akt was generated that contains a GFP fusion of the 40 kDa, proline-rich AKT substrate of PRAS40. This protein is phosphorylated at Thr246 by AKT in response to insulin. This modification of PRAS40 is known to promote interaction with 14-3-3 adaptor proteins, leading to its dissociation from mTORC1 and subsequent inactivation. Consequently, mTORC1 activity and downstream signaling is restored.[3] Because AKT phosphorylation of PRAS40 is upstream of mTOR, it is affected by PI3K inhibitors (e.g., PI-103, →

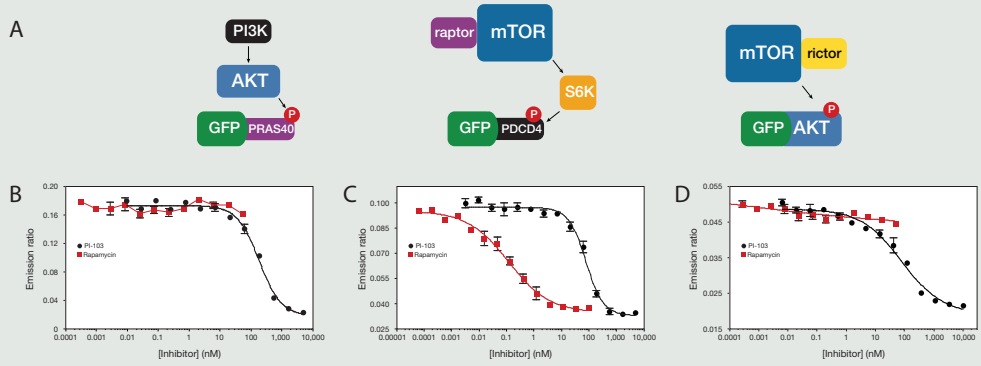

Figure 4—Assessing compound action on the PI3K/Akt/mTor signaling pathway. (A) GFP fusions of key pathway markers enable dissection of signaling complexity. Comparison of small-molecule inhibitor titrations against **(B)** LanthaScreen™ PRAS40 (Cat. no. K1528), **(C)** LanthaScreen™ PDCD4 (Cat. no. K1593), and **(D)** LanthaScreen™ AKT (Cat. no. K1615), all in HEK 293E cells. PI-103 (a dual PI3K and mTOR inhibitor) has global effects on the pathway and shows activity in each assay readout (IC_{50} values ~100 nM). Rapamycin (Cat. no. PHZ1233), however, only inhibits the phosphorylation of PDCD4 (IC_{50} <1 nM) in the mTORC1-dependent (complexed with raptor) assay.

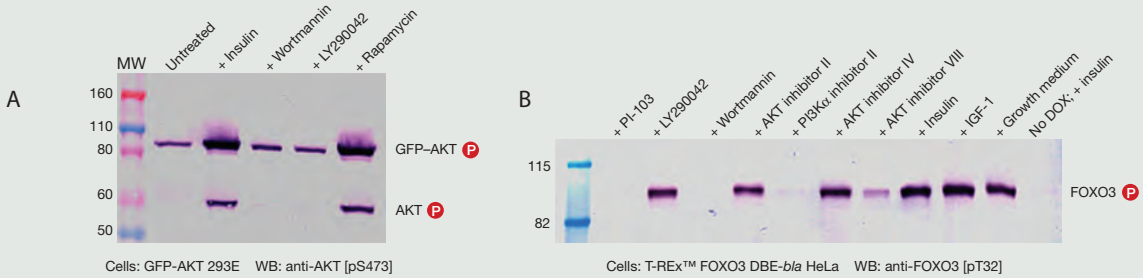

Figure 5—Validation of LanthaScreen™ and CellSensor® cell lines via western blot. (A) LanthaScreen™ GFP-AKT HEK 293E cells were treated under the indicated conditions, including IGF-1 (Cat. no. PHG9071), LY294002 (Cat. no. PHZ1144), and wortmannin (Cat. no. PHZ1301). Cell extracts were resolved by SDS-PAGE and transferred to nitrocellulose with the iBlot® Gel Transfer Device (Cat. no. IB1001). The membrane was then probed with the anti-AKT [PS473] PSSA (Cat. no. 44-621G), and the signal was detected using a WesternBreeze® Chromogenic Kit (Cat. no. WB7105). **(B)** CellSensor® T-REx™ FOXO3 DBE-*bla* HeLa cell lysates were generated by treatment with doxycycline to induce FOXO3 expression, followed by incubation with the indicated stimulants or inhibitors and lysis. The nitrocellulose membrane was prepared and developed as in **(A)**, except that the PSSA used in this case was anti-FOXO3 [PT32] (Cat. no. 44-1240G).

LY294002, and wortmannin) but is insensitive to rapamycin (Figure 4B). Interestingly, the mTOR kinase is able to phosphorylate PRAS40 directly at Ser183 in a rapamycin-sensitive manner, which further complicates the role of this protein within the mTor pathway.

To monitor pathway activation downstream of the mTORC1 complex, a cell line was developed that contains a GFP fusion of programmed cell death protein 4 (PDCD4). This protein is a tumor suppressor that inhibits translation initiation by binding to eIF4A. In response to insulin stimulation, PDCD4 is phosphorylated by p70 S6 kinase (at Ser457), which is immediately downstream of (and activated by) mTORC1. This phosphorylation event marks PDCD4 for $SCF^{\beta TRCP}$-mediated ubiquitination and subsequent degradation.[6] Inactivation of PDCD4 is necessary for efficient protein synthesis, and ultimately for cell growth and proliferation. Because PDCD4 is part of the PI3K/Akt pathway and is linked to mTORC1 activity, its phosphorylation is sensitive to both PI-103 and rapamycin (Figure 4C).

To examine pathway activation downstream of the multiprotein mTOR complex with rictor (mTORC2), a cell line containing a GFP fusion of AKT was constructed. Also known as protein kinase B (PKB), AKT has emerged as one of the most important and most actively studied kinases due to its versatility in regulating protein function and influencing human disease. Although AKT is often placed at the "beginning" of the pathway, it has also been shown to be a substrate of mTORC2, which phosphorylates AKT at Ser473.[7] This modification is required for full activation of AKT, and it leads to the repression of pro-apoptotic events. The precise mechanism and consequences of Ser473 phosphorylation remain controversial, yet this site has been shown to be insensitive to acute treatment with rapamycin (Figure 4D).

The future of PI3K/Akt/mTor pathway analysis

Each of the cell lines described here is a clonal population isolated by flow cytometry. The corresponding HTS assays have been optimized in 384-well format by testing a variety of parameters (e.g., DMSO tolerance, cell number, and stimulation time) and provide excellent statistical data (Z´-factor ≥0.5). Additionally, we have validation data for several known agonists and commercially available ligands, and the observed pharmacology (EC_{50} or IC_{50} values) is in agreement with reported literature data. Most importantly, we have compared these data to those obtained using alternative technologies from Invitrogen (e.g., western blotting with PSSA (Figure 5); phosphoELISA™ and Mercator™ Phospho-AKT Pathway Array; data not shown), with excellent correlation seen. Taken together, these cell lines constitute a validated set of tools for studying a complex pathway in a simplified format that complement our existing biochemical assays. These cell-based assays will facilitate the identification of novel modulators of PI3K/Akt/mTor signaling. Learn more about Akt pathway–related products at **www.invitrogen.com/bioprobes56**. ■

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What your colleagues are doing with the Qubit® quantitation platform

RESEARCHERS WEIGH IN ON THE ADVANTAGES OF THE QUBIT® PLATFORM.

The Qubit® quantitation platform, a fluorescence-based method for measuring DNA, RNA, and protein, provides higher accuracy and higher sensitivity than UV absorbance–based quantitation does. Why is this important for your research? Researchers who have converted to the Qubit® quantitation platform have shared their thoughts below.[1] You'll see that accurate quantitation leads not just to better quantitation, but also to better experimental results downstream.

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Improve your workflow with the Qubit® quantitation platform. Learn more about the Qubit® platform and Quant-iT™ assays for DNA, RNA, and protein at **www.invitrogen.com/bioprobes56**. ■

Reference

1. Results from Qubit® Owner Survey 2008, conducted February 25–29, 2008, 52 respondents.

How the Qubit® platform improved the workflow in your colleagues' labs

"Troubleshooting qPCR is easier when starting material is properly characterized, and results from different experimental conditions can be confidently compared knowing that the starting amount of template was exactly the same. Also helps first-strand cDNA reactions to perform optimally. The same general idea goes for DNA and protein as well."

"I'm able to measure things more quickly, so I measure more things, or things at more steps."

"I have been able to really quantify my RNA and proteins. RNA was especially important because we are collecting from a very low cell number."

"I am more sure about the amount of sample I have."

"It's made it easier to measure protein concentration."

"Better accuracy in quantitation of DNA or RNA yields more reproducible results."

"Using our UV spectrophotometer was a lot of extra work, so I dreaded measuring my samples. The Qubit is easy and accurate."

"Cloning speed and efficiency increased dramatically, and qRT-PCR is more precise."

"Yes, I can analyze my samples quickly and trust the results more completely."

"Faster quantification of DNA, faster sequencing."

"I no longer worry about losing sample to measuring how much there is."

"We are more confident in the accuracy of our quantitations. People perform quantitations more often rather than estimating."

"It has changed my workflow because it is more accurate and sensitive than UV spec. I am so happy with Qubit."

"I am more confident about my downstream design and results."

"We use it for routine PCR. It offers time savings and accuracy."

Product	Quantity	Cat. no.
Qubit® Fluorometer	each	Q32857
Qubit™ Quantitation Starter Kit	1 fluorometer and 4 kits	Q32860
Qubit™ Quantitation Lab Starter Kit	5 fluorometers and 4 kits	Q32861

IgG antibody labeling from A to Z

ALEXA FLUOR® TO ZENON® IgG ANTIBODY LABELING KITS FOR IMAGING AND FLOW CYTOMETRY.

Directly attach an intensely fluorescent Alexa Fluor® dye, R-phycoerythrin (R-PE), or even biotin to your IgG antibody with one of Invitrogen's superior antibody labeling kits. Using directly labeled antibodies can eliminate the background commonly observed when secondary antibodies bind nonspecifically to the sample. In addition, directly labeled antibodies allow you to use more than one same-species antibody in a single staining experiment. All of the antibody labeling kits described here make it easy to go direct.

How to choose an antibody labeling kit

There are three main considerations involved in choosing among the various protein labeling kits offered by Invitrogen. The primary consideration is the amount of antibody that the kit is optimized to label (Table 1). Next is whether to use the noncovalent attachment of Zenon® technology or an amine-reactive dye to covalently attach the label to the antibody (Figure 1). A covalent attachment of a label to an antibody generates a very stable conjugate lasting several months, whereas conjugates with a noncovalent attachment of the label can dissociate over time, lasting several hours to days. However, because amine-reactive moieties are used, other amine-containing compounds such as BSA must be removed prior to the reaction. The third consideration is the label—Zenon® technology provides the most comprehensive offering, including the premium Alexa Fluor® dyes as well as fluorescent proteins such as R-phycoerythrin (R-PE), and enzymes such as horseradish peroxidase (HRP). The antibody labeling kits using amine-reactive fluorophores are available with Alexa Fluor® dyes, classic fluorophores, and biotin.

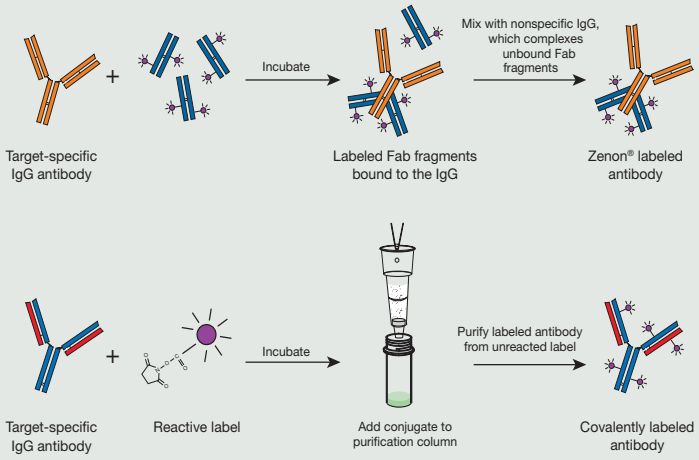

Figure 1—Noncovalent labeling with Zenon® antibody labeling vs. covalent labeling with the Microscale, Monoclonal, and Protein Labeling Kits.

Zenon® antibody labeling technology

Zenon® labeling technology provides a versatile, easy-to-use system for labeling mouse IgG1, IgG2a, and IgG2b antibodies and rabbit IgG antibodies using a labeled Fab fragment directed against the Fc portion of an intact IgG antibody to form a labeling complex (Figure 1A). Zenon® labeling is simple and efficient; the entire labeling procedure takes only 10 minutes. Antibodies labeled using Zenon® technology display fluorescence intensity or enzymatic activity similar to that observed for directly labeled antibody conjugates (Figure 2). The extensive selection of labels (a complete listing is available at **www.invitrogen.com/zenon**) and the versatility of this labeling technology make it easy to experiment with different color combinations for multicolor applications in both fluorescence imaging and flow cytometry.

Covalent antibody labeling kits with fluorescent dyes and biotin

Zenon® technology is great for quickly labeling small amounts of IgG antibody—for instance, if you want to change colors or test whether a particular fluorophore is suitable for your application or antibody. However, because the rapidly formed complex can slowly dissociate

Table 1—Invitrogen's IgG antibody labeling kits.

IgG antibody labeling kit	Amount of IgG labeled per reaction	Number of reactions	Sample requirements	Covalent attachment	Total time	Purification method
Zenon® IgG Antibody Labeling Kits	1–20 µg	10–50*	None, compatible with BSA or other stabilizing proteins and azide	No	10 minutes	None required
Microscale Protein Labeling Kits	20–100 µg	3	No BSA or other stabilizing proteins, no azide	Yes	2–3 hours (~15 minutes hands on)	Size-exclusion chromatography; spin filter
Monoclonal Antibody Labeling Kits	100 µg	5	No BSA or other stabilizing proteins, no azide	Yes	1.5–2 hours (~15 minutes hands on)	Size-exclusion chromatography; spin column
Protein Labeling Kits	1 mg	3	No BSA or other stabilizing proteins, no azide	Yes	2–3 hours (~30 minutes hands on)	Size-exclusion chromatography; gravity column

*The Zenon® Mouse IgG Labeling Kits contain sufficient reagents for either 50, 25, or 10 labelings, depending upon the kit. One labeling is defined as the amount of Zenon® labeling reagent required to label 1 µg of an intact, affinity-purified mouse IgG antibody at a Fab:antibody molar ratio of 3:1.

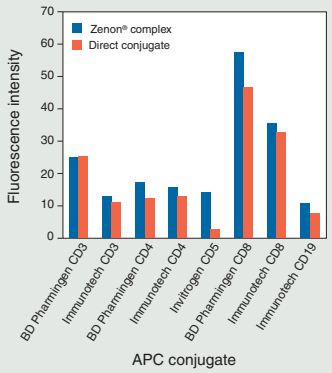

Figure 2—Zenon® labeling produces antibody conjugates with brightness comparable to or better than those obtained from direct conjugates. Antibodies to various lymphocyte markers were labeled either covalently with allophycocyanin (APC) or noncovalently with 1 µg of a Zenon® APC complex. Labeled antibodies were then used to stain samples of human peripheral blood lymphocytes. The brightness of the Zenon® staining complex can be further enhanced by increasing the ratio of Zenon® labeling reagent to the primary antibody used in the preparation of the complex.

Table 2—Antibody labeling kits with Alexa Fluor® dyes, Pacific Blue™ dye, Pacific Orange™ dye, or biotin.

Label	Ex/Em	Microscale Protein Labeling Kit	Protein Labeling Kit	Monoclonal Antibody Labeling Kit
Alexa Fluor® 350	346/442		A10170	A21080
Pacific Blue™	410/455		P30012	P30013
Alexa Fluor® 488	495/519	A30006	A10235	A20181
Pacific Orange™	400/551		P30016	P30014
Alexa Fluor® 532	531/554		A10236	A20182
Alexa Fluor® 546			A10237	A20183
Alexa Fluor® 555	555/565	A30007	A20187	A21087
Alexa Fluor® 568	578/603		A20184	A20184
Alexa Fluor® 594	590/617	A30008	A20185	A20185
Alexa Fluor® 647	650/668	A30009	A20173	A20186
Alexa Fluor® 680	679/702		A20172	
Biotin	NA	B30010; B30756 *		

*The FluoReporter® Biotin Quantitation Assay (F30751) is a fluorescence-based assay that requires only 600 ng of protein; the traditional colorimetric HABA assay requires ~1 mg of sample for accuracy.

over time, Zenon® antibody labeling is not recommended for applications requiring long or overnight incubations. The antibody labeling kits that include amine-reactive fluorophores are ideal for labeling larger amounts of antibody and can be used in applications requiring short (~30 minute) to long, overnight, or multiday incubations (Figure 3). To use, simply add the antibody to the premeasured dye, incubate for ~1 hour at room temperature, and purify the optimally labeled conjugate using size-exclusion chromatography. The difference between the MW of the label (~500–1,500 Da) and the IgG antibody (~150,000 Da) makes the purification quite easy. The kit you choose depends primarily on the total amount of IgG antibody and the label itself (Table 2).

Go direct

Invitrogen offers many choices when it comes to creating your perfect directly labeled antibody. For more information or to place your order, visit www.invitrogen.com/bioprobes56. ■

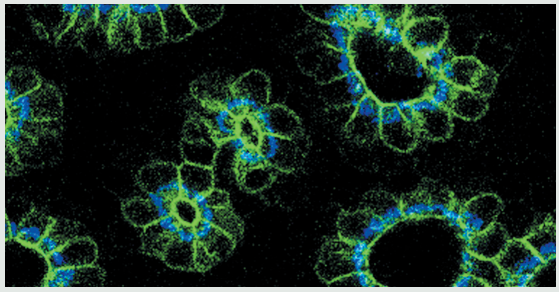

Figure 3—Two-color confocal image of a human epidermal whole mount. A monoclonal antibody to β1 integrin was labeled with Alexa Fluor® 488 dye (green fluorescence, Cat. no. A20181), and α6 integrin was labeled using a primary antibody followed by an Alexa Fluor® 594 secondary antibody (pseudocolored blue, Cat. no. A11005). Image contributed by Uffe Birk Jensen, University of Aarhus, Denmark.

Evaluating cellular viability in human primary cells

ALAMARBLUE® REAGENT FOR CELL VIABILITY AND CELL PROLIFERATION.

Evaluating compound toxicity is a key step in the drug development process. Before new chemical entities can progress into ADME studies—long before being administered to a patient—their toxicity profiles must be well understood. To this end, a system that most closely models the patient is ideal for identifying compounds with undesirable effects. Toxicity assessment early in the drug discovery process often uses cultured cells; the most physiologically relevant model may therefore be primary cells that are isolated directly from human patients, rather than transformed cell lines that are more suitable for evaluating cancer drug candidates.

Compound toxicity in cultured cells can be examined by studying several cellular properties, including DNA content, enzyme activity, presence of ATP, membrane integrity, or metabolic activity. Specially designed cell viability indicators have been developed for sensing the different cellular characteristics and providing some sort of visual readout of cell health. All indicators have positive and negative attributes; however, their ease of use, cost, robustness, sensitivity, reliability, and compatibility with physiologically relevant cell lines are the most important factors in determining their ultimate utility.

Ease of use and cost

alamarBlue® reagent is a cell viability and cell proliferation indicator that uses the inherent reducing power of live cells as an indicator of metabolic activity.[1] alamarBlue® reagent consists of the redox indicator resazurin in a proprietary stabilization formula; the reducing capability of metabolically active cells converts resazurin to the fluorescent reporter molecule resorufin (Figure 1). alamarBlue® reagent is easy to use—just add the reagent to cells in culture, incubate, then read either the fluorescence or absorbance of the sample (Figure 2). This reagent is established as a very reliable and sensitive cytotoxicity indicator that provides reproducible data and is readily scalable from single-well to high-throughput format. alamarBlue® reagent can be used on adherent and nonadherent cells, and is a less expensive option compared to other commonly used resazurin- or ATP-based cell viability and proliferation reagents (Table 1).

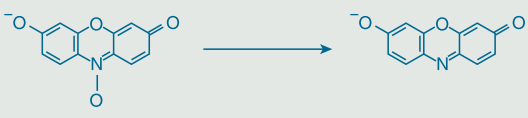

Figure 1—alamarBlue® reagent works as a cell viability and proliferation indicator through the conversion of resazurin to resorufin. Resazurin, a nonfluorescent indicator dye, is converted to highly red-fluorescent resorufin via redox reactions in metabolically active cells. The magnitude of the fluorescence signal is proportional to the number of living cells.

Assay robustness and sensitivity

alamarBlue® reagent can be used to detect as few as 50 cells per well in a 96-well plate (Figure 3). In this experiment, alamarBlue® reagent was added to a serial dilution of human umbilical vein endothelial cells (HUVEC) at 1/10 volume in a black, clear-bottom 96-well plate, and fluorescence was measured after 40 minutes and 18 hours. After 40 minutes, the fluorescence intensity of alamarBlue® reagent was directly proportional to number of cells present, within the range of 500–50,000 cells (Figure 3). Further incubation (18 hours) with alamarBlue® reagent yielded more sensitive detection in the 50–5,000 cell range.

Primary cells

In order to assess its performance in primary cells, alamarBlue® reagent was used to analyze the toxicity of tamoxifen on human primary cells versus immortalized human cell lines. The primary cell types (Cascade Biologics® human aortic smooth muscle cells (HASMC) and HUVEC) were compared to immortalized cell lines available from the ATCC (HepG2, a liver-derived cell line used in toxicity studies, and SH-SY5Y,

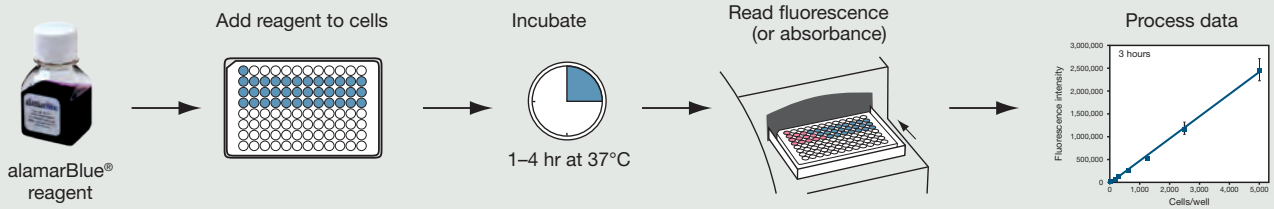

Figure 2—The alamarBlue® cell viability assay protocol. A 96-well plate containing the cells and the compounds to be tested is prepared using standard methods. alamarBlue® reagent is added directly to each well, the plates are incubated at 37°C to allow cells to convert resazurin to resorufin, and the fluorescence (or absorbance) signal is measured. Results are evaluated by plotting the fluorescence (or absorbance) signal vs. compound concentration. This schematic depicts an assay carried out in a 96-well plate, but the procedure is readily adaptable to other formats as well (including 384-well plates and tubes of various volumes). If tubes are used, the sample is transferred to a cuvette prior to spectrophotometric analysis.

a neuroblastoma cell line originally derived from a metastatic bone tumor). Cells were treated with tamoxifen, an anti cancer drug used in the treatment of breast cancer, for 24 hours prior to the addition of alamarBlue® reagent to evaluate cell viability and proliferation. Decreasing fluorescence from alamarBlue® reagent as tamoxifen concentration was increased clearly indicates tamoxifen toxicity at higher concentrations (Figure 4). Interestingly, the two primary cell types were 5–10 times more susceptible to tamoxifen than immortalized cell lines. While these results may not generally apply to all primary cell vs. cell line comparisons, they do bring to light a potentially important difference: immortalized cell lines may have a greater resistance to compound toxicity than most primary cells.[2] When evaluating compound cytotoxicity, it is therefore important to first determine the general sensitivity to reference drugs on the cell type used in the study.

Table 1—Cost per well for popular resazurin- and ATP-based cell viability and proliferation reagents.

Product	Cost/well (¢)*
alamarBlue® reagent	5.4
CellTiter-Blue® reagent (Promega)	9.0
ATPLite™ reagent (PerkinElmer)	34.3
CellTiter-Glo® reagent (Promega)	81.0

*Cost per well was determined for a 96-well plate containing 100 µl of medium plus cells, according to the manufacturers' instructions for how much reagent to add to each well. Prices were based on the smallest available sizes from website data on March 6, 2008.

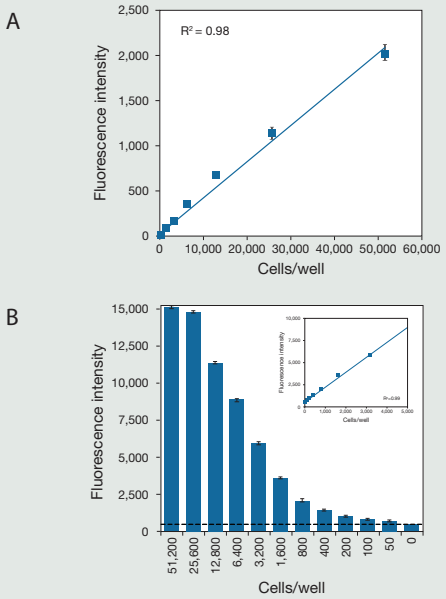

Figure 3—Linearity and sensitivity of alamarBlue® reagent on HUVEC cells. (**A**) Fluorescence of the alamarBlue® reagent is linear over the range 500–50,000 cells after a 40 minute incubation with cells. (**B**) The same 96-well plate read after an 18 hour incubation shows the sensitivity of alamarBlue® reagent. The horizontal line at ~450 RFU represents the background fluorescence in the experiment, which was calculated as three times the standard deviation of the no-cell control. The inset graph shows alamarBlue® reagent is linear over the range from 50 to 5,000 cells/well after an 18 hour incubation with cells. Error bars show ±SEM.

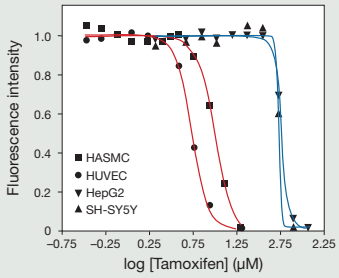

Figure 4—Primary cells differ in susceptibility to tamoxifen compared to immortalized cell lines. Cells were plated in 96-well plates and treated with various concentrations of tamoxifen. After 24 hours, alamarBlue® reagent was added and fluorescence was read on a FlexStation® plate reader. Apparent IC_{50} values for tamoxifen were determined to be 10.3 and 5.6 µM for HASMC and HUVEC primary cells, respectively (red); immortalized cell lines exhibited apparent IC_{50} values of 57.7 and 54.1 µM for HepG2 and SH-SY5Y cells, respectively (blue). Values were normalized for ease of comparison due to varying cell numbers between cell types. Graphs were generated using GraphPad Prism® with n = 4 and ±SEM for each data point.

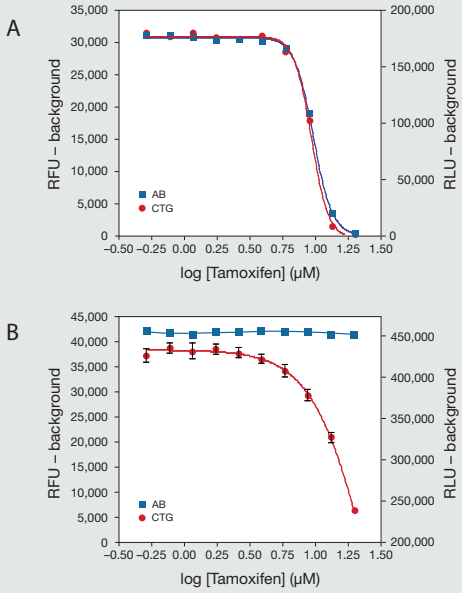

Figure 5—Comparison of alamarBlue® and CellTiter-Glo® reagents. Log phase–growing HUVEC cells **(A)** and confluent HASMC cells **(B)** were treated with tamoxifen for 24 hours prior to analysis of cell health with alamarBlue® (AB) or CellTiter-Glo® (CTG) reagents. Results were collected on a BMG POLARstar Optima with n = 8 and ±SEM for each data point.

Comparison to other assays

In other experiments, alamarBlue® reagent was compared to CellTiter-Glo® reagent, a luminescence-based assay that detects the presence of ATP as an indicator of cell health. Primary cells were again treated with various tamoxifen concentrations; results determined using alamarBlue® reagent correlated well with those obtained using CellTiter-Glo® reagent for HUVEC cells in log-phase growth (Figure 5A). Both assay formats revealed toxicity by a decrease in signal intensity, yielding IC_{50} values between 9 and 10 µM, in good agreement with literature values.[3] When this experiment was repeated on HASMC cells at confluence (Figure 5B), CellTiter-Glo® indicated cell death at higher tamoxifen concentrations, whereas alamarBlue® reagent did not. These results are likely due to the fact that CellTiter-Glo® reagent measures ATP as an indicator of cell health, as opposed to alamarBlue® reagent, which measures reducing capability of cells. Further experiments performed with known cytotoxic vs. cytostatic reagents will help to elucidate the observed differences between these indicators of viability.

Radioactive tracers such as ^{3}H-thymidine have been largely replaced by assays using tetrazolium salts such as MTT and its derivates (XTT, MTS), which produce intense color upon reduction in cells. Since tetrazolium-based indicators work by the same reduction principle as alamarBlue® reagent, independent comparisons have been performed. Hamid and coworkers [4] compared alamarBlue® reagent to MTT for high-throughput screening and found that both assays were in good agreement; however, alamarBlue® reagent gave increased sensitivity, presumably due to the inherent sensitivity of fluorescence measurements compared to absorbance readouts.[4] Since both colorimetric and fluorescence changes are associated with the reduction of resazurin to resorufin, alamarBlue® reagent is perhaps the most versatile, easy-to-use, and reliable cell viability indicator available. Learn more about alamarBlue® reagent at **www.invitrogen.com/bioprobes56**. ■

References

1. Ahmed, S.A. et al. (1994) *J Immunol Methods* 170:211–224.
2. Krebs, F.C. et al. (2002) *Antimicrob Agents Chemother* 46:2292–2298.
3. Riss, T.L. and Moravec, R.A. (2004) *Assay Drug Dev Technol* 2:51–62.
4. Hamid, R. et al. (2004) *Toxicology in Vitro* 18:703–710.

Product	Quantity	Cat. no.
alamarBlue® reagent	25 ml	DAL1025
alamarBlue® reagent	100 ml	DAL1100
Human aortic smooth muscle cells (HASMC)	1 ml	C-007-5C
Human umbilical vein endothelial cells (HUVEC)	1 ml	C-003-5C

Analyzing signaling events with phosphorylation site–specific antibodies

INSIGHTS INTO FUNCTIONAL PROTEOMICS.

The number of protein isoforms for each gene and the degree of post-translational modification together are recognized as a dominant source of functional diversity and a critical avenue for generating drugs that will effectively ameliorate human disease. Importantly, site-specific phosphorylation by protein kinases (acting at individual Ser, Thr, or Tyr residues) and cleavage (as catalyzed by proteases such as caspases, calpain, cathepsins, secretases, etc.) can result in dramatic changes in the activity of a protein or the nature and location of specific complexes within the cell. Invitrogen produces highly selective, high-avidity antibodies to study individual posttranslational modifications, including more than 300 phosphorylation site–specific antibodies (PSSAs) and cleavage site–specific antibodies (CSSAs).

Rigorous manufacturing process yields highest-quality PSSAs

Invitrogen's PSSA and CSSA reagents are of the highest quality, thanks to a rigorous R&D and manufacturing process for generating polyclonal antibodies. Key steps in that process include immunogen design and conjugation, use of different immunization strategies, and affinity purification chromatography (both negative and positive selection). The result is high-specificity rabbit polyclonal preparations that work in multiple applications (e.g., western blotting, ELISA, immunocytochemistry (ICC), immunohistochemistry (IHC), TR-FRET, and flow cytometry).

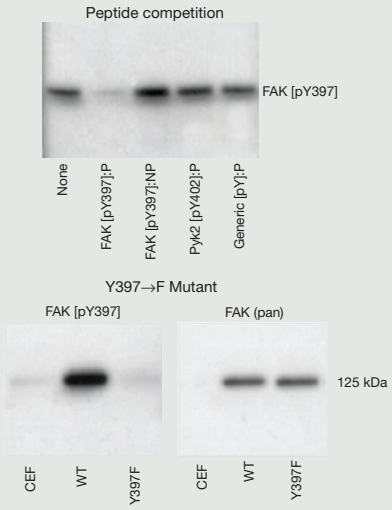

Figure 1—Specificity of FAK [pY397] PSSA. The high specificity of FAK [pY397] PSSA is demonstrated by peptide competition (only the appropriate phosphopeptide competes the signal) and site-directed mutant analysis (the Y397F mutant is not detected by the PSSA, although it is seen by the pan-FAK antibody).

Confidently interpret phosphorylation states

The high specificity of these antibodies for the targeted modification is a critical attribute. PSSAs must recognize the target protein only when it is phosphorylated at the specific Ser, Thr, or Tyr residue and must not recognize the nonphosphorylated version of the protein. In addition, these reagents must not cross-react with other phosphorylated sites in the target protein or other proteins found in the sample. Specificity is rigorously tested using a combination of peptide competition analysis, signal induction by an appropriate stimulus, phosphatase stripping, and site-directed mutant analysis, where the corresponding Ser (S), Thr (T), or Tyr (Y) residue has been converted to a residue that can't be phosphorylated (typically Ala for Ser/Thr residues and Phe for Tyr residues) (Figure 1). These specificity tests provide the confidence to interpret the signal patterns obtained in different applications (e.g., subcellular localization in cells and tissues, Figure 2), allowing critical insights about biomarker function. →

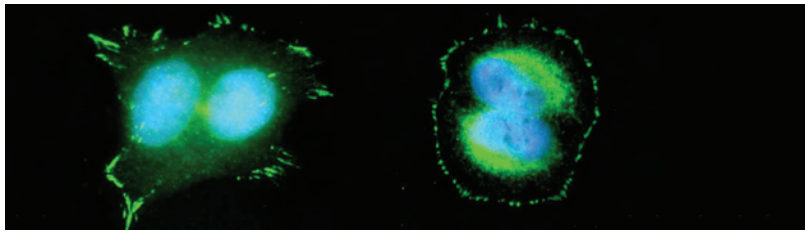

Figure 2—Visualizing focal adhesion kinase with FAK [pY397] PSSA. HeLa cells were fixed and permeabilized using ice-cold 95% methanol. FAK [pY397] PSSA was labeled with Alexa Fluor® 488 dye (green fluorescence, Molecular Probes® Zenon® Alexa Fluor® Rabbit IgG Labeling Kit, Cat. no. Z25302), and nuclei were counterstained with DAPI (blue fluorescence, Cat. no. D21490). The image was captured using a Zeiss Axioplan 2 fluorescence microscope fitted with a 100× oil immersion lens.

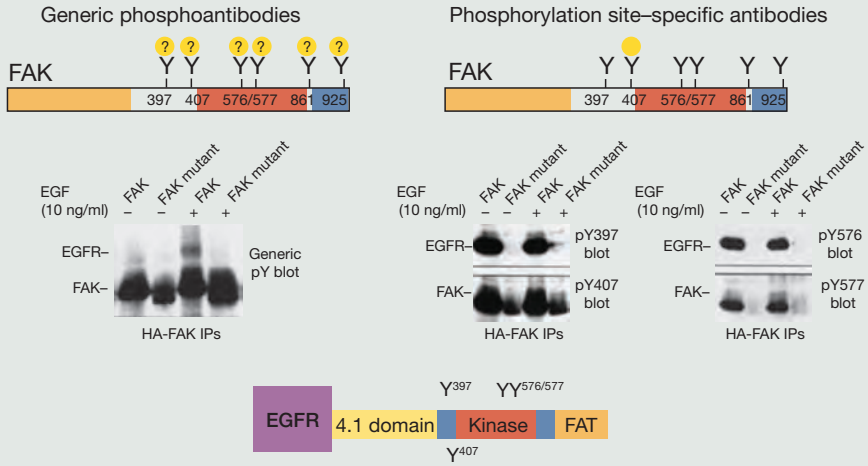

Figure 3—Differential regulation of FAK tyrosine phosphoryation. PSSAs provide the means to understand the roles of individual sites as part of the circuitry that controls functional proteomics. Using generic (anti-pY) antibodies to study protein phosphorylation misses important information, such as differential regulation of individual sites, seen with phosphorylation site–specific reagents. Portions of this figure reprinted with permission from Macmillan Publishers Ltd: Sieg, D.J. et al. (2000) *Nat Cell Bio* 2:249–256.

Gain valuable insight with high-specificity PSSAs

Using highly selective modification site–specific antibodies to study posttranslational changes provides important information on how individual proteins are regulated in different cells or tissues, in response to different treatments, and in normal or disease states. The rapid development of instrumentation with which these high-value antibody reagents can be used is providing new opportunities to better understand basic biology and to accelerate the development of new drugs to ameliorate disease at the level of functional proteomics.

We can see how critical precise dissection of protein phosphorylation events is to the understanding of protein regulation through a study of focal adhesion kinase (FAK). FAK plays an important role in many cell types and signaling events, functioning both as a scaffold protein and as a protein Tyr kinase.[1] Autophosphorylation of FAK at Y397 allows Src and PI3-kinase to bind via their SH2 domains, setting in motion phosphorylation of FAK at other sites and the activation of specific signaling pathways that control focal adhesion assembly and disassembly, and thereby dictating whether cells will be adherent or migratory.[2] FAK [pY397] PSSA provides a valuable readout to detect the activation state and subcellular location of FAK and to study the invasive phenotype in tissues.[3] FAK is a large 125 kDa protein with ~30 different phosphorylation sites (data from the CMC phosphoproteome at www.cellmigration.org/resource/proteomics/data/fak_total.shtml), many of which have been shown to be differentially phosphorylated.[3–7] Invitrogen offers multiple PSSAs to study the role of individual sites on FAK (Tables 1 and 2), providing insight that would be missed using generic anti-pY, -pS, or -pT antibodies. This complex circuitry is an important element of functional proteomics (Figure 3).

Antibodies with the specificities and labels you need

Invitrogen generates PSSAs routinely in large-scale batches with minimal lot-to-lot variability. In addition, Invitrogen combines antibody expertise with in-house fluorescence labeling technologies (e.g., Alexa Fluor® dyes, Qdot® nanocrystals, europium, and terbium) as applied to primary and secondary antibodies for use in a variety of detection formats for your specific application needs. Tables 1 and 2 show a sample of the PSSA antibodies and sampler packs we offer; for a complete listing, visit **www.invitrogen.com/bioprobes56**. ■

References

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7. Jiang, X. et al. (2007) *Cell Signal* 19:1000–1010.

Invitrogen offers more than 300 PSSAs and CSSAs for 13 major signaling pathways/biomarker groups

Receptor tyrosine kinases: 14 RTKs and 52 sites: EGFR, ErbB2, FGFR, FLT3, IGF1R, IR, Kit, Met, PDGFRα/β, Ret, Tie-2, TrkA, VEGFR2/KDR

Cell adhesion signaling: Cadherins, cofilin, FAK (7 sites), integrins α4/β1/β3, LIMK, MAPKs, PAK1-3, paxillin, Pyk2 (5 sites), Src

Cytokine/immune receptor signaling: Hck, JAK1/2, Lck, MAPKs, STAT1/3/5, Syk, ZAP70

Adaptor proteins: AS160, BAD, BCL, BIM, BLNK, cortactin, DAB, DOK2, filamin, GAB1, HSP27, IRS1/2 (10 sites), LAT, PAG3, paxillin, PED/PEA15, PRAS40, Shc, VAV1/2, vinculin

Akt/mTor/AmpK pathways: AKT, AMPK, AS160, GSK3, GS, LKB, mTOR, p70S6K, PED/PEA15, PRAS40, PTEN, RSK, S6, SGK

MapK pathways: Raf, COT, MEK1-4/6/7, ERK1/2, ERK5, p38, JNK, MAPKAPs

PKA and PKC pathways: PKC (10 isoforms, 18 sites), PKA (catalytic and regulatory sites)

NFκB/IKK/IκB signaling: IKK, IκB, MLK3, NFκB, PKR

Neurodegeneration markers: APP, β-amyloid CSSAs, CAMK2, FAK, Pyk2, Ret, Src, Tau (16 sites), Tau CSSA, Tau-kinases (ERK1/2, JNK, p38, PKA, GSK3), α-synuclein, TrkA

Cell-cycle markers: Aurora, CDKs, CK2β, histone-H3, inhibitor-2, p53, Rb, TTK

Transcription factors: ATF2, β-catenin, c-Fos, c-Jun, CREB, ELK-1, ER-α, ETS1/2, FOXO1/3, GATA-4, IκB, NFAT1, NFκB, SMAD2/3, SOX9

Translation factors: 4E-BP1, eIF2α, eIF4ε, eIF4G

Apoptosis signaling: BAD, Bcl-xL, Bid CSSA, BimEL, caspase-9 CSSA, eNOS, HSP27, JNK1/2, p38, PARP CSSA, PKR, Pyk2

Table 1—PSSAs for FAK* (protein Tyr kinase, nonreceptor focal adhesion kinase).

Product	Upstream kinase and functional significance of phosphorylation site	Applications tested	Quantity	Cat. no.
FAK [pY397]	Autophosphorylation (initial activation step) site; allows Src and p85 subunit of PI3-kinase to bind; activates cell migration and invasion	WB, ICC, IHC (does not detect Pyk2)	100 µl, 10 western blots	44-624G
FAK [pY397], rabbit monoclonal	Autophosphorylation (initial activation step) site; allows Src and p85 subunit of PI3-kinase to bind; activates cell migration and invasion	WB, ICC (cross-reacts with Pyk2)	100 µl, 10 western blots	44-625G
FAK [pY407]	Pyk2 site, activated via VEGF and TGF-beta but not EGF; can be phosphorylated under conditions where there is reduced phosphorylation of Y397	WB, ICC	100 µl, 10 western blots	44-650G
FAK [pY576]	Src site, kinase activation loop (YY motif); sites appear to be differentially regulated by stimuli	WB, ICC, IHC	100 µl, 10 western blots	44-652G
FAK [pY577]	Src site, kinase activation loop (YY motif); sites appear to be differentially regulated by stimuli	WB, ICC, IHC	100 µl, 10 western blots	44-614 G
FAK [pS732]	CDK5 site; function unknown, but must play role in cell-cycle regulation, especially in brain	WB	100 µl, 10 western blots	44-590G
FAK [pS843]	Activated via cell adhesion and during mitosis; thought to function in opposition to multiple tyrosine phosphorylation events on FAK	WB	100 µl, 10 western blots	44-594G
FAK [pY861]	Src site (major); promotes binding to the cytoplasmic tail of integrins	WB, ICC, IHC	100 µl, 10 western blots	44-626G
FAK [pS910]	Activated via cell adhesion and during mitosis; thought to function in opposition to multiple tyrosine phosphorylation events on FAK	WB	100 µl, 10 western blots	44-596G

* Corresponding pan antibodies for FAK: AHO0502/AHO0672. All antibodies listed are affinity-purified (both negative and positive) rabbit polyclonal antibodies except FAK [pY397], which is a rabbit monoclonal antibody. WB = western blot; ICC = immunocytochemistry; IHC = immunohistochemistry.

Table 2—Antibody sampler packs for PSSAs and/or CSSAs.

Product*	Quantity	Cat. no.
Progrowth Signal PSSA Sampler Pack (includes FAK pY397, Src pY418, ERK1&2 pTpY, JAK2 pYpY, Akt/PKB pS473, paxillin pY31, and PLCγ pY783)	7 vials (2 blots each)	44-586
FAK pY PSSA Sampler Pack (includes pY397, pY407, pY576, pY577, pY861, and FAK pan)	6 vials (2 blots each)	44-631

* Antibodies included are affinity-purified rabbit polyclonal antibodies.

Recent news in neurodegeneration

Xie, Y., Dong, Y., Maeda, U., Alfille, P., Culley, D., Crosby, G., and Tanzi, R. (2006) The common inhalation anesthetic isoflurane induces apoptosis and increases amyloid β protein levels. *Anesthesiology* 104:988–994.

Alzheimer's disease (AD) is a slow, progressive, and irreversible disease that results in memory loss, impaired cognitive ability, and changes in behavior and personality. Alzheimer's disease is characterized by the presence of extracellular plaques and intracellular neurofibrillary tangles (NFTs) in the brain (Figure 1). The major component of these plaques is β-amyloid peptide (Aβ), a 40–43 amino acid peptide cleaved from amyloid precursor protein (APP). Increased release of the longer forms of Aβ peptide, Aβ42 or Aβ43, which have a greater tendency to aggregate than Aβ40, occurs in individuals expressing certain genetic mutations or Apo alleles, and may also involve other still undiscovered factors. Surgeries, especially among the elderly, often result in short- and long-term memory loss and even more severe cognitive dysfunction. This has prompted researchers to investigate the role of commonly used inhalants on the development of neurodegenerative disease, especially with respect to Aβ protein levels.

Xie et al. recently performed a study to determine whether the commonly used anesthetic inhalant isoflurane increased levels of the Aβ protein and apoptosis in neuroglioma cells. H4 human neuroglioma cells stably transfected to express human full-length wild-type APP were exposed to 2% isoflurane for 6 hours. The cells and media were harvested at the end of the 6 hour treatment. Caspase-3 activation, processing of APP, cell viability, and Aβ levels were measured by western blot, a cell viability kit, and ELISA. The results indicated that 2% isoflurane caused apoptosis, altered processing of APP, and increased production of Aβ in the H4 human neuroglioma cell lines. The isoflurane-induced apoptosis was independent of changes in Aβ and APP holoprotein levels. However, isoflurane-induced apoptosis was influenced by the increased levels of APP C-terminal fragments. Xie and coworkers concluded that a clinically relevant concentration of isoflurane induces apoptosis, alters APP processing, and increases Aβ production in the human neuroglioma cell line. The researchers noted that because altered processing of APP leading to accumulation of Aβ is a key player in the pathogenesis of Alzheimer's disease, these findings may have implications for use of this anesthetic agent in individuals with excessive levels of cerebral Aβ and in elderly patients at increased risk for postsurgical cognitive dysfunction.

For a complete list of peptides, inhibitors, and growth factors from Invitrogen, visit **www.invitrogen.com/bioprobes56**. ■

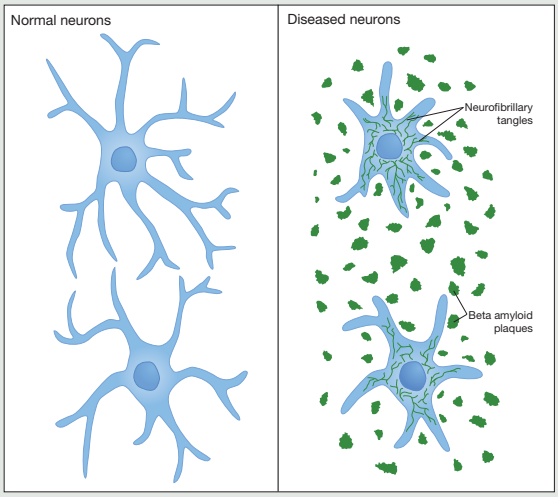


Figure 1—β-amyloid plaques and neurofibrillary tangles result in lost connectivity to the neuronal network.

Table 1—The latest β-amyloid (Aβ) peptides and peptide fragments from Invitrogen.

Product	Quantity	Cat. no.
Aβ [1-40] peptide	1.0 mg	03-136
Aβ [1-40] peptide, biotin	0.5 mg	03-243
Aβ [1-40] peptide, 5-carboxyfluorescein	0.1 mg	03-219
Aβ [1-40] peptide, ultrapure	1.0 mg	03-138
Aβ [1-40] peptide, all D amino acid	0.5 mg	03-134
Aβ [40-1] peptide	1.0 mg	03-245
Aβ [40-1] peptide, biotin	0.5 mg	03-246
Aβ [1-40, Gly21] peptide, (AD) Dutch variant	1.0 mg	03-209
Aβ [1-40, Gly5, Phe10, Arg13] peptide, rat homolog	1.0 mg	03-189
Aβ [1-40, Nle35] peptide, substituted form	1.0 mg	03-229
Aβ [1-42] peptide	0.5 mg	03-111
Aβ [1-42] peptide	1.0 mg	03-112
Aβ [42-1] peptide	1.0 mg	03-247
Aβ [1-12] peptide fragment	0.5 mg	03-260
Aβ [1-20] peptide fragment	1.0 mg	03-264
Aβ [1-28] peptide fragment	0.5 mg	03-142
Aβ [35-25] peptide fragment	1.0 mg	03-244

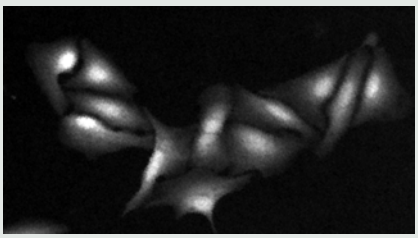
CELLTRACKER™ VIOLET BMQC

CellTracker™ Violet BMQC rounds out the CellTracker™ product line with a violet laser offering (either UV or 405 nm excitation with 520 nm emission). Brighter than the blue CellTracker™ products, CellTracker™ Violet BMQC is a robust reagent that provides reproducible results. As with most CellTracker™ products, CellTracker™ Violet BMQC passes through the cell membrane, binds intracellular thiols, and becomes impermeant. CellTracker™ Violet BMQC not only is bright, but also survives formaldehyde fixation and detergent extraction. Learn more at www.invitrogen.com/bioprobes56.

Product	Quantity	Cat. no.
CellTracker™ Violet BMQC	5 × 0.1 mg	C10094

THIOLTRACKER™ VIOLET GLUTATHIONE DETECTION REAGENT

ThiolTracker™ Violet reagent is an easy-to-use thiol-reactive probe that is ten times brighter than the bimanes traditionally used for glutathione (GSH) detection and oxidation–reduction. This live cell–permeant stain survives formaldehyde fixation and detergent extraction, provides reproducible results, and is useful for multiplex assays, including those for cytotoxicity. ThiolTracker™ Violet reagent can be used with a conventional UV or 405 nm laser and 510 nm filter, and is particularly useful for fluorescence microscopy, flow cytometry, and high-content imaging. Because reduced glutathione is the most abundant thiol in cells, ThiolTracker™ Violet reagent can be used to detect GSH depletion. GSH is important for understanding redox status, the effects of drugs, and detoxification mechanisms. Learn more at www.invitrogen.com/bioprobes56.

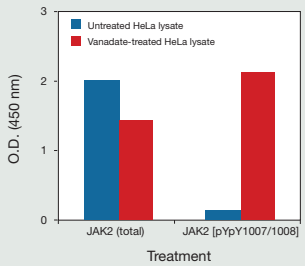


U-2 OS cells stained with ThiolTracker™ Violet dye (20 mM in DPBS).

Product	Quantity	Cat. no.
ThiolTracker™ Violet (Glutathione Detection Reagent)	180 assays	T10095
ThiolTracker™ Violet (Glutathione Detection Reagent)	5 plates	T10096

PHOSPHOELISA™ KITS

phosphoELISA™ kits provide quantitative measurements of specific phosphoproteins in cell lysates. These immunoassays are inherently sensitive and fast (complete in 4 hours). For a complete list of phosphoELISA™ kits, visit www.invitrogen.com/bioprobes56.



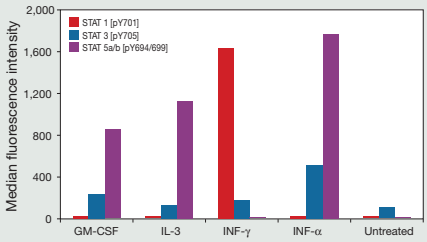
JAK2 (total) and JAK2 [pYpY1007/1008] ELISAs on vanadate-treated HeLa cells. HeLa cells were either untreated or treated with vanadate, then measured using the JAK2 [pYpY1007/1008] ELISA Kit (Cat. no. KHO5621) and the JAK2 (Total) ELISA Kit (Cat. no. KHO5521). The JAK2 (Total) ELISA Kit was used to normalize the data.

Product	Quantity	Cat. no.
JAK2 [pYpY1007/1008] ELISA Kit	96 tests	KHO5621
JAK2 (Total) ELISA Kit	96 tests	KHO5521
Paxillin [pY118] ELISA Kit	96 tests	KHO6551

LUMINEX® BEAD ANTIBODY KITS

STAT 1, 3, 5 Phospho-Plex combines the efficiency of multiplexing three phosphoproteins in the JAK/STAT signaling pathway with reproducibility similar to that of ELISA methods. Multiplex immunoassays provide simultaneous analysis, saving sample, time, and money. Learn more about Luminex® technology at www.invitrogen.com/bioprobes56.

Luminex® is a registered trademark of Luminex Corporation.



STAT 1, 3, and 5 phosphorylation measured in one sample using the Luminex® bead-based platform for multiplexing capabilities. TF-1 cells were serum starved overnight, then stimulated with specific cytokines for 15 minutes. Untreated cells were used as an unstimulated control. Measured levels of STAT proteins were as expected following specific treatments using the STAT 1, 3, 5 Plex Antibody Bead Kit (Cat. no. LHO0005).

Product	Quantity	Cat. no.
STAT 1, 3, 5 Phospho-Plex Bead AB Kit	96 tests	LHO0005

Recently published

A LOOK AT HOW YOUR FELLOW RESEARCHERS ARE USING INVITROGEN™ PRODUCTS.

Signal transduction: Simultaneous imaging of Na^+ and Ca^{2+}. The transient receptor potential V1 (TRPV1) is a nonselective cation channel that functions as a molecular integrator of pain perception. Researchers from Hokkaido University recently reported the discovery of a novel mechanism for activation of TRPV1 triggered by extracellular sodium depletion, using fura-2 AM and CoroNa™ Green AM to simultaneously image intracellular Ca^{2+} and Na^+, respectively. In some experiments, Premo™ Cameleon Calcium Sensor was used for Ca^{2+} detection in place of fura-2. TRPV1 expression vectors were constructed using Gateway® technology and transfected into HEK 293 cells using Lipofectamine™ 2000 reagent. Experiments were also conducted in cultured DRG neurons expressing endogenous TRPV1 verified by immunofluorescent staining with rabbit anti-TRPV1 serum in combination with Alexa Fluor® 488 goat anti–rabbit IgG secondary antibody.

Ohta, T., Imagawa, T., and Ito, S. (2008) Novel gating and sensitizing mechanism of capsaicin receptor (TRPV1). *J Biol Chem* 283:9377–9387.

Cell biology: Fluorescence *in situ* hybridization (FISH) detection of mRNA export. Seeking confirmation for functional coupling between mRNA splicing and export as a promoting mechanism for gene expression, researchers from Harvard Medical School used FISH to assess the nucleocytoplasmic distribution of mRNAs transcribed from plasmids microinjected into cell nuclei. The FISH probes were 70-mer oligonucleotides labeled at the 5´ end with Alexa Fluor® 546 succinimidyl ester. Fluorescein-labeled dextran, 70,000 MW, was mixed with plasmid DNA to identify microinjection locations. Splicing-dependent enhancement of the cytoplasmic-to-nuclear ratio of mRNA was observed for three different genes in two different cell backgrounds, with the enhancement ranging between 6- and 10-fold, depending on the gene in question.

Valencia, P., Dias, A.P., and Reed, R. (2008) Splicing promotes rapid and efficient mRNA export in mammalian cells. *Proc Natl Acad Sci U S A* 105:3386–3391.

High-throughput screening: Oxidative/nitrosative stress–activated Ca^{2+} channels. TRPM2 is a voltage-independent Ca^{2+} channel belonging to the transient receptor potential melastatin (TRPM)–related family. Activation of TRPM2 induced by oxidative/nitrosative stress leads to an increase in intracellular free Ca^{2+}. To facilitate the identification of compounds able to specifically modulate TRPM2 function in primary cells or animal models, Song and coworkers have developed and validated a cell-based functional assay for TRPM2 based on Invitrogen's Fluo-4 NW Calcium Assay Kit in conjunction with stable expression of TRPM2 in nonadherent DT40 B lymphocytes.

Song, Y., Buelow, B., Perraud, A.L., and Scharenberg, A.M. (2008) Development and validation of a cell-based high-throughput screening assay for TRPM2 channel modulators. *J Biomol Screen* 13:54–61.

Histotechnology: *In situ* DNA quantitation in fixed tissue sections. Conventional histotechnological processing methods do not properly conserve the location, amount, and conformation of DNA in paraffin-embedded fixed tissues, with negative consequences for subsequently applied analytical procedures such as *in situ* hybridization, laser-capture microdissection, *in situ* PCR, and TUNEL assays. Accordingly, a team of researchers from New Jersey Medical School and New York Institute of Technology has developed improved methods for quantitative analysis of DNA in fixed tissue sections, combining rapid microwave-vacuum oven fixation, optimized fixative formulations, dsDNA staining with PicoGreen® dsDNA Quantitation Reagent, and densitometric image analysis.

Gagna, C.E., Kuo, H.R., Chan, N.J., Mitacek, E.J., Spivak, A., Pasquariello, T.D., Balgobin, C., Mukhi, R., and Lambert, W.C. (2007) Novel DNA staining method and processing technique for the quantification of undamaged double-stranded DNA in epidermal tissue sections by PicoGreen probe staining and microspectrophotometry. *J Histochem Cytochem* 55:999–1014.

Virology: Inhibition of human papillomavirus infection (HPV) by monoclonal antibodies. Infection with a subset of sexually transmitted human papillomaviruses (HPVs), particularly HPV16, is considered a necessary factor in the development of virtually all cases of cervical cancer. A neutralizing antibody response to L1, the major structural viral protein, is known to effectively prevent infection, as demonstrated by the success of the recently developed HPV vaccine. However, better understanding is needed of the various mechanisms by which these neutralizing antibodies act to prevent infection. Accordingly, a team from the National Cancer Institute recently analyzed mechanistic differences in the inhibitory effects of a panel of three neutralizing monoclonal antibodies. Fluorescence microscopy of HPV16 pseudovirus labeled with Alexa Fluor® 488 5-TFP reactive dye was used to assess binding of antibody-complexed virions to extracellular matrix (ECM). Immunofluorescent staining to assess monoclonal antibody–mediated effects on cell-surface binding and endocytosis of HPV16 was performed using Alexa Fluor® 488 and Alexa Fluor® 594 dye–labeled anti–mouse IgG and anti–rabbit IgG secondary antibodies.

Day, P.M., Thompson, C.D., Buck, C.B., Pang, Y.Y., Lowy, D.R., and Schiller, J.T. (2007) Neutralization of human papillomavirus with monoclonal antibodies reveals different mechanisms of inhibition. *J Virol* 81:8784–8792.

The publications summarized here are just a few of the recent additions to the 59,000+ references describing applications of Invitrogen™ products in our searchable bibliography database. Visit www.invitrogen.com/support to search for citations by product.

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