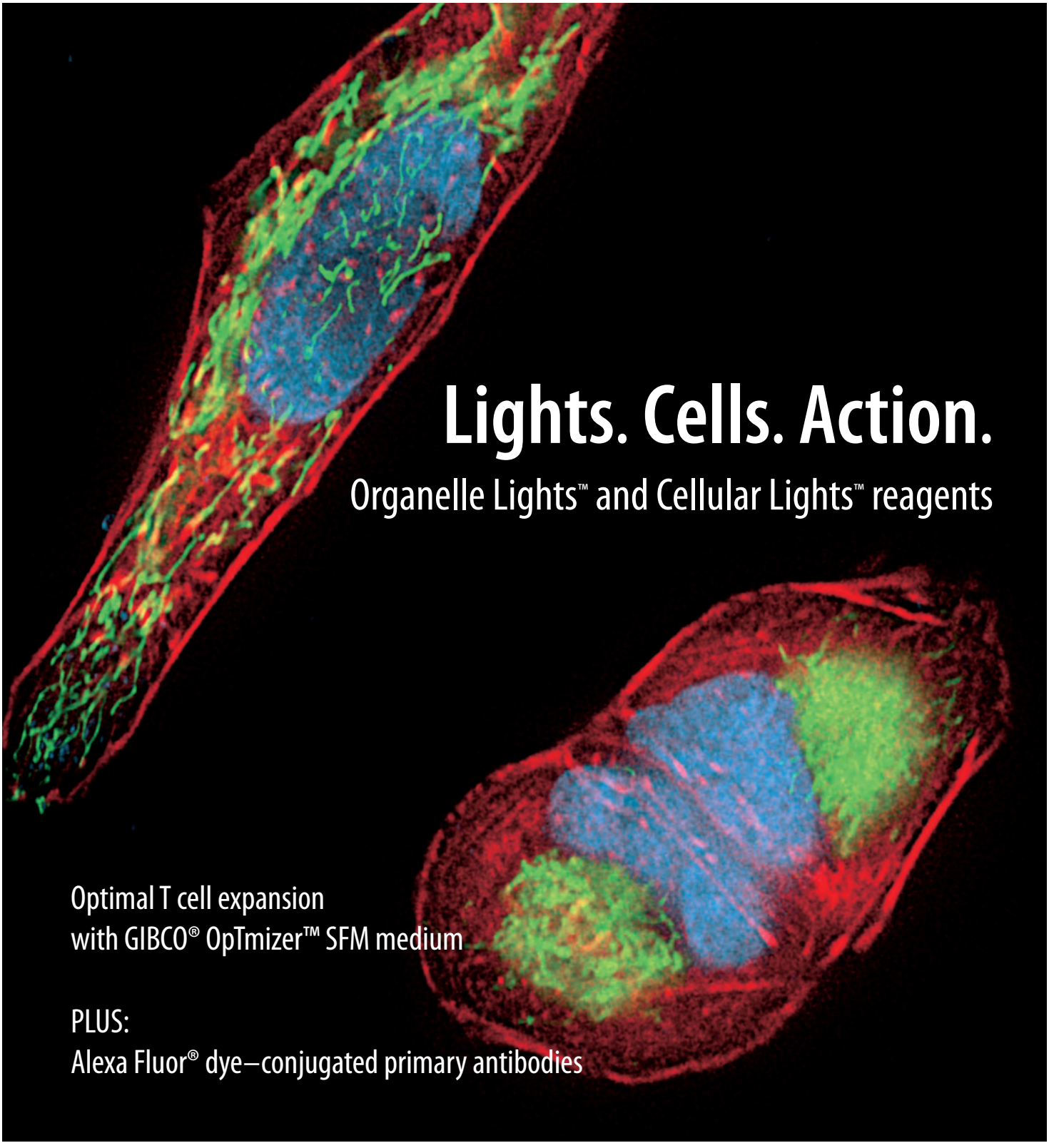
BIOPROBES 59

CELL BIOLOGY BY INVITROGEN

JUNE 2009



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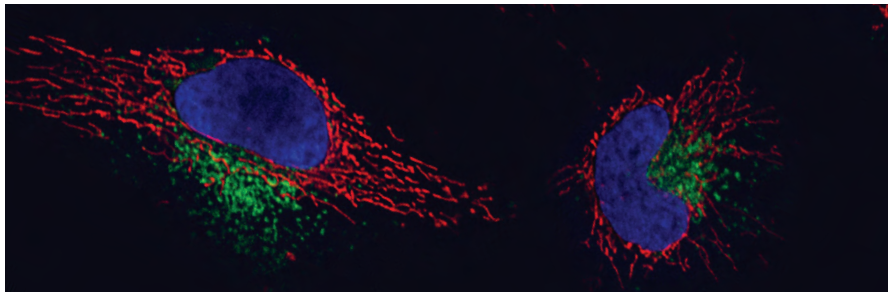

BioProbes 59

Features

Departments

A pHrodo™ dye–based method for monitoring the phagocytic internalization of apoptotic cells

Miksa, M., Komura, H., Wu, R., Shah, K., and Wang, P. (2009) A novel method to determine the engulfment of apoptotic cells by macrophages using pHrodo succinimidyl ester. *J Immunological Methods* 342:71–77.

How can we accurately assay the phagocytosis of apoptotic cells? Apoptosis comprises a multitude of highly regulated pathways through which an organism rids itself of unwanted cells without injuring itself. A key component of apoptosis is the phagocytic removal of cellular debris that results when cells undergo apoptotic self-termination; this clean-up step is carried out by specialized immune cells such as macrophages and neutrophils. Errors (either inborn or acquired) in the complex machinery behind the phagocytosis of apoptotic cells are implicated in a number of medically important conditions; there is therefore a need for researchers to be able to reliably assay phagocytosis in order to better understand these conditions.

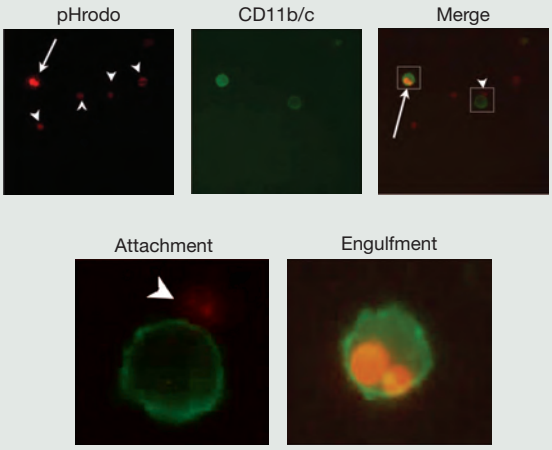

Figure 1. Fluorescent intensity of pHrodo™ SE–labeled apoptotic thymocytes increases after engulfment by macrophages. Splenic macrophages were labeled with FITC anti-CD11b/c (OX42) and thymocytes with pHrodo™ SE (0.02 µg/mL). Cells were coincubated for 60 min, collected, and fixed with 1% PFA prior to fluorescence microscopy. Top three panels show lymphocytes in red (pHrodo-SE+), macrophages in green (CD11b/c), and a merged image of the same area to the right. Boxes in the merged image indicate the 10x magnification shown below. Arrowheads indicate free floating and attached apoptotic thymocytes and arrows indicate engulfed apoptotic cells. Magnification: 400x. Reprinted from *J Immunol Methods*, Vol. 342, Miksa, M., Komura, H., Wu, R., Shah, K., and Wang, P., A novel method to determine the engulfment of apoptotic cells by macrophages using pHrodo succinimidyl ester, pages 71–77, © 2009, with permission from Elsevier.

The phagocytic process can be viewed as occurring in distinct steps: first the binding of the unwanted object by the phagocytic immune cell, followed by the activation of signaling pathways, cytoskeletal rearrangements, and internalization. While many methods have been developed to assess phagocytosis by reporting the initial binding step, these methods can lead to overestimation of the extent of internalization under some circumstances. In this report, Miksa and colleagues describe a simple and elegant method for monitoring the actual internalization step itself, which relies on macrophage-mediated chemical alteration of the internal environment of engulfed particles. In their assay, target cells (apoptotic thymocytes in this study) are loaded with the pH-sensitive fluorescent dye pHrodo™ Succinimidyl Ester (SE). Upon phagocytic engulfment by macrophages, the target cells are lysed and their contents encounter the acidic environment within the phagosome, causing a dramatic increase in pHrodo™ dye fluorescence (Figure 1).

The authors used this assay to monitor the engulfment of apoptotic thymocytes by FACS flow cytometric analysis as well as by direct observation through fluorescence microscopy; their results showed excellent agreement with previously reported results. Additionally, they accurately characterized the limited phagocytic activity of cells deficient in MFGE8 (a key protein required for phagocytosis), and demonstrated the successful recovery of phagocytic activity in these cells upon addition of exogenous MFGE8. Furthermore, this study represents the first application of this approach to eukaryotic apoptotic cells. The authors suggest that this assay could prove useful in future studies of the role of macrophages in apoptosis.

Learn more about pHrodo™ SE at **www.invitrogen.com/bp59**. ■

Product	Quantity	Cat. no.
pHrodo™ Succinimidyl Ester (pHrodo™, SE)	1 mg	P36600

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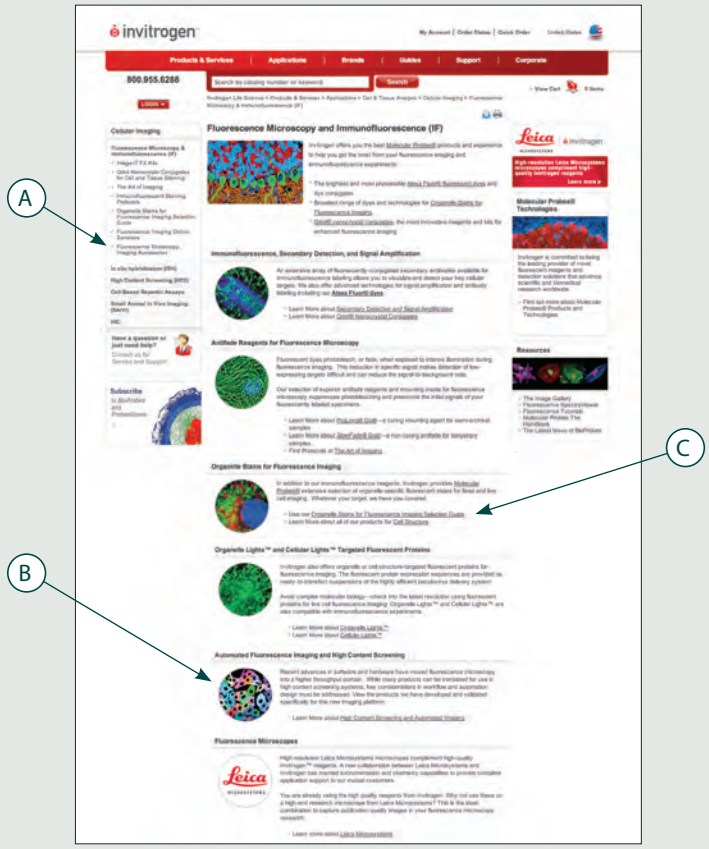
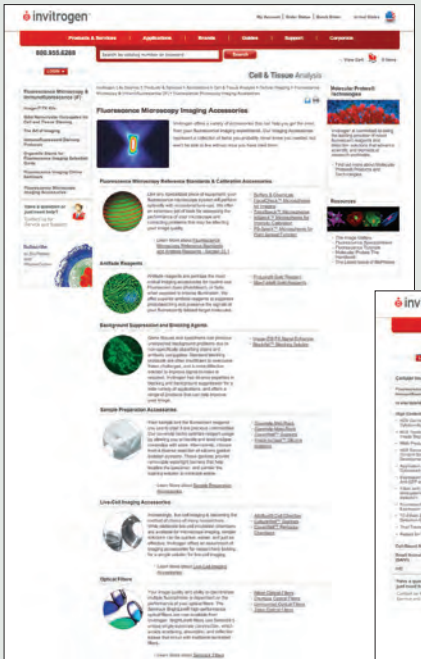


The Fluorescence Microscopy and Immunofluorescence (IF) web page.



A. *The Fluorescence Microscopy Imaging Accessories web page.*

B. *The High-Content Screening (HCS) web page.*

C. *The Organelle Stains Selection Guide web page.*

Faster, easier calcium flux assays

THE FLUO-4 DIRECT™ CALCIUM ASSAY KIT.

Calcium flux assays are widely used for in-cell measurement of agonist-stimulated and antagonist-inhibited calcium signaling through G protein–coupled receptors (GPCRs), a large and active target class in drug discovery. These assays traditionally require the removal of culture media and unquenched calcium dye to limit interfering background fluorescence, and are often time-consuming to perform, particularly under high-throughput screening (HTS) conditions.

The new Fluo-4 Direct™ Calcium Assay Kit offers a unique formulation that eliminates the requirement for culture media removal or a wash step, saving time and making the assays more compatible with automation while reducing variability caused by incomplete liquid removal or physical disruption of the cellular monolayer. The kit also employs a proprietary fluorescence quencher that suppresses background fluorescence due to extracellular fluorescent dye and components of the culture media. The Fluo-4 Direct™ assay can be used to obtain appropriate pharmacological profiles for compounds in both agonist and antagonist modes, and is ideal for HTS experiments.

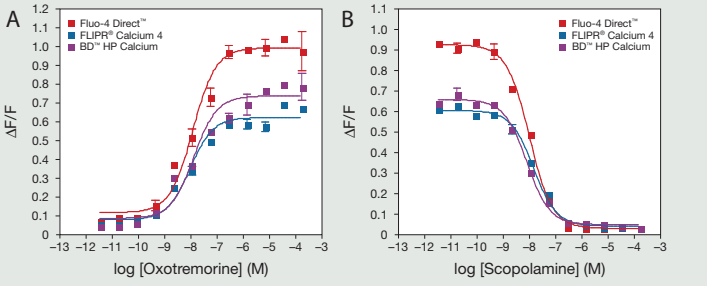


Figure 1. Dose-dependent calcium response to muscarinic 1 (M1) receptor agonist and antagonist. CHO M1 cells were assayed for a calcium response to the M1 agonist oxotremorine (**A**) or to the M1 antagonist scopolamine (**B**), using the Fluo-4 Direct™ Calcium Assay Kit, MDS FLIPR® Calcium 4 Assay Kit, or BD™ High Performance (HP) Calcium Assay Kit. Measurements are expressed as relative fluorescent units [(maximum response – minimum response) / minimum response].

Reduce background fluorescence and increase signal window

We compared the Fluo-4 Direct™ Calcium Assay Kit with competing "addition-only" calcium indicator assays, with agonist and antagonist compound titrations on a CHO cell line expressing the muscarinic 1 (M1) receptor, a well-characterized GPCR. Calcium flux assays were performed using the Fluo-4 Direct™ Calcium Assay Kit, MDS FLIPR® Calcium 4 Assay Kit, or BD™ High Performance Calcium Assay Kit. Compound addition and data acquisition were performed using the Hamamatsu FDSS imaging system.

All three calcium assay kits measured a similar dose-dependent calcium response to the M1 agonist oxotremorine and the M1 antagonist scopolamine. However, the signal window of the Fluo-4 Direct™ assay was larger than those of both the MDS and BD kits (Figure 1), while the EC_{50} and IC_{50} values were comparable between kits.

Get more data from high-throughput screens

To confirm that the Fluo-4 Direct™ assay performs equally well in high-throughput format, we used the kit to conduct a screen for agonists of the muscarinic 3 (M3) receptor using the LOPAC^{1280}™ Navigator compound library (Sigma). Results were compared to those from the MDS FLIPR® Calcium 4 and BD™ High Performance Calcium Assay Kits. CHO cells expressing the M3 receptor were assayed for a calcium response to each of the library compounds, and the resulting data were normalized to percent activation as compared to 100 µM carbachol, a known M3 agonist. Calcium flux agonist compound hits were identified by a response equal to or greater than 30% activation.

The Fluo-4 Direct™ Calcium Assay Kit identified 41 hits, compared to 32 hits identified with each competitor kit. Of the hits, 28 were identified in all three assay formats, 4 were identified in common between the Fluo-4 Direct™ assay and each of the other kits, and 5 were identified by the Fluo-4 Direct™ assay alone (Figure 2). The hits identified with

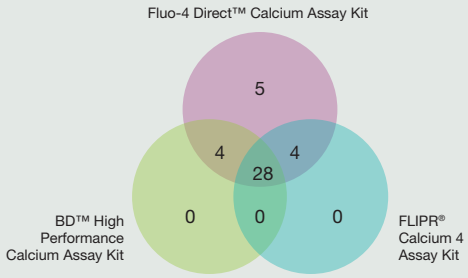


Figure 2. Venn diagram of M3 agonist compound hits identified using three different calcium flux assay kits.

Table 1. Common M3 agonist hits identified using three different calcium flux assay kits.

	% Activation		
	Fluo-4 Direct™	FLIPR® Calcium 4	BD™ HP
Muscarinic receptor agonists			
Arecoline hydrobromide	101	100	71
Acetyl-beta-methylcholine chloride	101	85	86
(±)-Muscarine chloride	100	83	79
(+)-*cis*-Dioxolane iodide	100	90	88
Oxotremorine sesquifumarate salt	96	75	70
Carbachol	94	99	90
OXA-22 iodide	92	93	82
Arecaidine propargyl ester hydrobromide	88	92	66
Oxotremorine methiodide	78	65	74
Bethanechol chloride	72	65	64
Methylcarbamylcholine chloride	52	41	32
(+)-Pilocarpine hydrochloride	48	62	48
Acetylthiocholine chloride	45	43	57
Pilocarpine nitrate	40	54	49
Dopamine receptor agonists (endogenous receptor activation)			
Agroclavine	134	77	34
A-77636 hydrochloride	61	45	41
Ergocristine	76	47	45
P2Y receptor agonists (endogenous receptor activation)			
2-Methylthioadenosine diphosphate trisodium	69	36	52
2-Chloroadenosine triphosphate tetrasodium	65	51	56
2-Methylthioadenosine triphosphate tetrasodium	58	49	42
Ca^{2+} ionophore			
Calcimycin	50	33	32
Autofluorescent compounds			
4-Amino-1,8-naphthalimide	430	101	167
Idarubicin	187	165	107
Dipyridamole	150	110	92
Daunorubicin hydrochloride	134	64	88
P^1,P^4-Di(adenosine-5´)tetraphosphate triammonium	59	45	58
Quinacrine dihydrochloride	123	79	70
Sanguinarine chloride	100	60	57

Table 2. Higher percent activation and more hits identified with the Fluo-4 Direct™ Calcium Assay Kit.

		% Activation		
		Fluo-4 Direct™	FLIPR® Calcium 4	BD™ HP
McN-A-343	M1 receptor agonist	30	7	15
ML-9	Myosin light chain kinase inhibitor	43	19	26
S-(–)-Lisuride	D2 dopamine receptor agonist	36	17	23
MJ33	Inhibitor of phospholipase A2 (PLA2)	44	22	25
I-OMe-Tyrphostin AG 538	IGF-1 receptor tyrosine kinase inhibitor	46	–52	26
(±)-*p*-Chlorophenylalanine	Inhibition of tryptophan hydroxylase	46	33	25
Cephradine	Semisynthetic cephalosporin antibiotic	45	30	25
1-Aminobenzotriazole	Cytochrome P450 inhibitor	45	32	26
5-Azacytidine	DNA methyltransferase inhibitor	43	31	23
Alsterpaullone	Inhibitor of GSK-3beta and CDK5/p25	45	25	34
R-(+)-Lisuride hydrogen maleate	D2 dopamine receptor agonist	61	27	38
Spiperone hydrochloride	D2 dopamine receptor antagonist	60	10	46
Thapsigargin	IP3-independent intracellular calcium releaser	70	26	55

all three kits include known muscarinic receptor full agonists, and the dopamine and P2Y receptor agonists (Table 1).

Only the Fluo-4 Direct™ Calcium Assay Kit identified the muscarinic receptor partial agonist McN-A-343 as an M3 receptor hit. The Fluo-4 Direct™ assay consistently demonstrated greater test compound activation, resulting in a higher number of active compounds qualifying as hits when compared to the competitor kits (Table 2).

A simple calcium flux assay ideal for high-throughput screening

The Fluo-4 Direct™ Calcium Assay Kit eliminates the need to perform laborious plate washing steps, while reducing variability and eliminating background fluorescence. The kit also offers a larger signal window than its competitors, with more hits identified in HTS experiments. For more information about the Fluo-4 Direct™ Calcium Assay Kit and its applications, visit **www.invitrogen.com/bp59**. ■

Product	Quantity	Cat. no.
Fluo-4 Direct™ Calcium Assay Kit, Starter Pack	1 kit	F10471
Fluo-4 Direct™ Calcium Assay Kit, Surveyor Pack	1 kit	F10472
Fluo-4 Direct™ Calcium Assay Kit, High-Throughput Pack	1 kit	F10473

Cytotoxicity and cell proliferation studies

THE CYQUANT® DIRECT CELL PROLIFERATION ASSAY.

Assessing compound cytotoxicity is a critical step in pharmaceutical development. Assays for cell proliferation or metabolism as indicators of cell health are typically performed individually or together in cell-based systems. In oncology research, these assays can be used to test for selective cytotoxicity, or anti-proliferative effects, against a target cell type. Proliferation is also a convenient measure of population dynamics in studies of cytokines or growth factors, or in bioprocess optimization work. To expand the CyQUANT® family of cell proliferation assay kits, we recently introduced the CyQUANT® Direct Cell Proliferation Assay. This fluorescence-based assay is fast, sensitive, and ideal for high-throughput screening (HTS) applications.

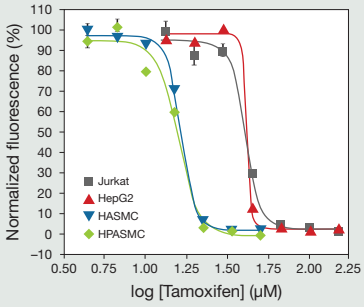


Figure 1. Cytotoxicity measurements using the CyQUANT® Direct Cell Proliferation Assay. Cytotoxicity assays were performed using the CyQUANT® Direct assay across different cell types. Hep G2, Jurkat, human aortic smooth muscle cells (HASMC), and human pulmonary artery smooth muscle cells (HPASMC) were seeded in 384-well plates at a density of 5,000 cells per well in 30 µL medium containing 10% FBS. Increasing concentrations of tamoxifen were added, and following an incubation at 37°C for 48 hr, CyQUANT® Direct reagent (30 µL per well) was added. Fluorescence measurements were made after 60 min. Each data point represents the mean ± SD of 8 measurements. As shown in the figure, the two primary cell types (HASMC and HPASMC) were significantly more sensitive to tamoxifen treatment than the transformed cell lines (adherent Hep G2 and suspension Jurkat cells).

An alternative to metabolism-based assays

Although metabolism-based cytotoxicity assays are widely used, metabolic activity is influenced by a variety of factors that can affect the reliability and reproducibility of results. By comparison, cellular DNA content is tightly regulated, and nucleic acid–based assays provide accurate and sensitive measures of cell proliferation, cell numbers, and cytotoxicity. DNA binding of the CyQUANT® dye is not reliant on metabolic state, resulting in relatively consistent signal windows and intensities across a wide range of assay conditions and cell types.

Accurately measure cell proliferation and cytotoxicity

The CyQUANT® Direct assay is based on a cell-permeant DNA-binding dye that exhibits strong fluorescence enhancement when bound to cellular nucleic acids (excitation/emission maxima ~480/520 nm). In combination with a background suppressor component that masks staining of dead cells and cells with compromised cell membranes, only viable cells are measured, resulting in an accurate and sensitive assay for cell number and for tracking proliferation.

Recent studies using diverse sets of cytotoxic compounds and assay types have shown that cell number quantitation by a nucleic acid readout is among the most sensitive indicators of cytotoxicity.[1–3] The CyQUANT® Direct assay can be used to obtain reliable cytotoxicity data. This was demonstrated by excellent concordance of IC_{50} values

Table 1. Consistent IC_{50} (µM) values for tamoxifen across four cell lines.

	Cell type			
Assay	Hep G2	Jurkat	HASMC	HPASMC
CyQUANT® Direct	40.7	41.5	16.4	15.8
alamarBlue®	44.6	35.5	16.8	16.1
CellTiter-Glo®	39.6	35.8	13.8	12.3
CellTiter 96® AQ_{ueous}	41.9	43.7	15.5	15.8

HASMC = human aortic smooth muscle cells; HPASMC = human pulmonary artery smooth muscle cells.

measured using the CyQUANT® Direct assay with assays based on metabolic activity, including alamarBlue®, CellTiter-Glo®, and CellTiter 96® AQ_{ueous} (Promega) assays (Figure 1 and Table 1). The Z´ values, which are broadly accepted as a standard measure of HTS assay robustness, were >0.5 across all cell types tested using the CyQUANT® Direct assay.

An ideal assay for high-throughput screening

With a homogeneous format and fast "add-mix-read" protocol, the CyQUANT® Direct assay is ideal for HTS applications (Figure 2). The assay can be completed in 1 hour, with no washes, cell lysis, temperature equilibrations, or radioactivity required. The signal is stable for more than 7 hours, offering workflow convenience and robustness in projects with large sample numbers (Figure 3).

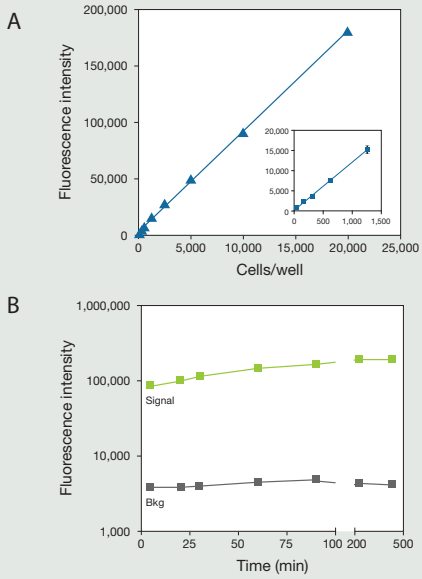


Figure 3. Linearity and stability of CyQUANT® Direct assay signal. (**A**) CHO cells were plated at densities of 0–20,000 per well in a 384-well microplate. Cells were labeled with CyQUANT® Direct reagent according to the microtiter plate assay format protocol. Fluorescence intensities, measured with a fluorescence microplate reader using a FITC filter set, varied linearly with respect to cell number over the range of 40–20,000 cells. The inset shows the measurement range from 0 to 1,250 cells per well. Each data point represents the mean of 8 measurements. (**B**) CyQUANT® Direct 2X detection reagent was added to adherent CHO cells in serum-containing medium, and fluorescence was read from 5 min to 7.5 hr after reagent addition. Fluorescence signal intensity reached a plateau within 30–60 min after reagent addition, and remained stable for more than 7 hr.

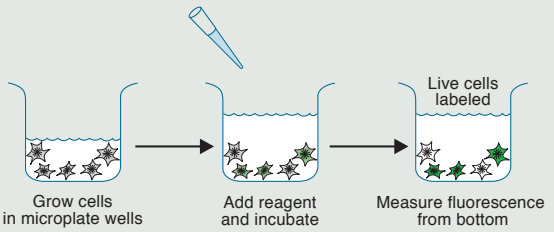


Figure 2. CyQUANT® Direct assay protocol. The CyQUANT® Direct assay is a homogeneous, lysis-free cell proliferation and cytotoxicity assay designed for use with multiwell plates. The reagent is added directly to cells in complete medium and incubated for 30–60 min. Fluorescence is measured using standard plate readers.

The sensitivity of the CyQUANT® Direct Cell Proliferation Assay, as low as 40 to more than 20,000 cells per well for most cell types, means that it can be used in 96-, 384-, or 1,536-well microplate formats (Figure 3). Because cells are not lysed during the procedure, the CyQUANT® Direct assay can be measured using both HTS and HCS fluorescence readers. The assay is available in two configurations: a 10-plate assay kit for smaller sample sizes, and a 100-plate assay kit for higher-throughput applications.

Measure cell viability and proliferation with confidence

The CyQUANT® Direct Cell Proliferation Assay is designed for use with mammalian cells of most adherent and suspension types. Treatments such as lysis and permeabilization are not necessary, nor do the cells have to be metabolically active to be stained. This results in a readout that provides a direct measure of both cell proliferation and viability. The CyQUANT® Direct assay can therefore be used to assess cell growth, cell viability, or compound toxicity in a range of applications, from high-throughput screening to bioproduction. To learn more about the CyQUANT® Direct Cell Proliferation Assay, visit **www.invitrogen.com/bp59**. ■

alamarBlue® is a registered trademark of Trek Diagnostics.

References

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2. O'Brien, P. et al. (2006) *Arch Toxicol* 80:580–604.
3. Abraham, V. et al. (2008) *J Biomol Screening* 13:527–537.

Product	Quantity	Cat. no.
CyQUANT® Direct Cell Proliferation Assay, 10-plate size	1 kit	C35011
CyQUANT® Direct Cell Proliferation Assay, 100-plate size	1 kit	C35012

Optimal T cell expansion

NEW GIBCO® OpTmizer™ SFM AND PROVEN AIM V® MEDIUM.

We survive in a hostile environment of viruses, bacteria, and parasites. The immune system provides a line of defense against these assaults and against neoplastic events, and develops memory to respond quickly to subsequent exposures. Cellular interactions and their exquisite control lie at the heart of the immune response; dissecting these interactions is crucial to understanding how the system functions.

Although the immune system functions in the complex architecture of the body, much of our understanding of this system grew out of triggering a portion of it in partial isolation to produce a response that mimics *in vivo* function. This approach requires tools to identify key cell types, physically separate immune response cells, and support T cell function *in vitro* either for study or to reintroduce *in vivo*. Two effective tools for optimized T cell culture are AIM V® Medium and new OpTmizer™ SFM.

For large T cell population growth, serum-free, at a lower cost

Unlike T cell media that require the addition of 5–10% human serum, the new OpTmizer™ medium provides optimal growth, viability, and cell yield either in the absence of human serum (Figure 1) or at low serum concentrations. Furthermore, serum-free OpTmizer™ medium can effectively compete with media containing up to 5% human serum, and with the addition of only 2% human serum can further boost its performance. OpTmizer™ performs exceptionally well in the Wave Bioreactor® system (Figure 2) and, equally important, cells maintain desired function (Figure 3).

Because OpTmizer™ medium performs well either in the absence of serum or with reduced amounts of serum, this medium can benefit researchers throughout R&D. The absence of all animal-origin components, with the exception of human serum albumin, can ease the transition from research to clinical development and remove or simplify regulatory hurdles. As a result, this medium helps to improve operational efficiency, reduce cycle time, increase productivity, meet regulatory requirements, and reduce the cost of manufacturing a cell product.

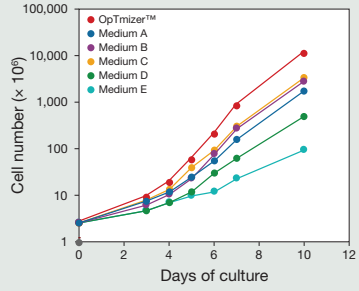

Figure 1. Comparison of T cell growth and viability in various media in the absence of serum. T cells were cultured using Dynabeads® *ClinExVivo*™ CD3/CD28 beads for activation and expansion. Cells were assessed using a Vi-CELL™ cell viability system (Beckman Coulter).

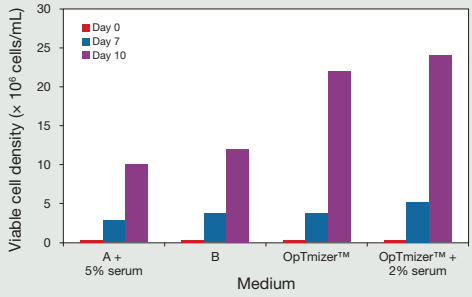

Figure 2. T cell growth in various media (with or without serum) in the Wave Bioreactor® system (Wave Biotech). PBMCs were thawed and mixed with *ClinExVivo*™ CD3/CD28 T cell expansion beads. Bead-bound T cells were concentrated and resuspended in culture medium containing IL-2 and either 2% human serum or no serum. Cells were transferred to Wave Bioreactor® culture bags, and medium was added until day 9. Cells were analyzed for phenotype (CD3, CD4, CD8) and activation marker expression (CD25, CD154), and supernatants were collected for cytokine analysis (IFN-γ).

A proven, FDA 510(K)–cleared choice: AIM V® Medium

AIM V® Medium—the first commercially available chemically defined, serum-free formulation for the proliferation of T cells—is manufactured in compliance with Food and Drug Administration's Current Medical Device Good Manufacturing Practices (cGMP) for *in vitro* diagnostic products, and is the only FDA 510(K)-cleared, therapeutic-grade commercial T cell medium. AIM V® Medium has become the medium of choice in clinical diagnostics and adoptive immunotherapy.

AIM V® Medium replaces human serum–supplemented media in adoptive immunotherapy and has proven to be effective in the *ex vivo* activation of lymphokine-activated killer cells (LAK cells) after supplementation with interleukin-2 (IL-2). As shown in Figure 4, T cells cultured in AIM V® Medium, without addition of human serum, achieved LAK cell lytic activity of lymphocytes comparable with cells maintained in RMPI-1640 supplemented with serum.

AIM V® is the serum-free medium most widely used by research and clinical T cell scientists and clinicians. This medium's utility has expanded beyond LAK cells to include almost all human primary lymphoid cells and cell types such as transformed lymphoid lines (both human [1] and murine), adherent cells (e.g., human fibroblasts), human mononuclear cells,[2] dendritic cells,[3] macrophages,[4,5] and mast cells.[6]

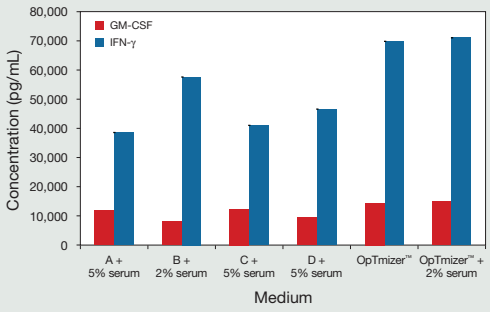

Figure 3. Expression of cytokines by T cells grown in various media (with or without serum). T cells were cultured using Dynabeads® *ClinExVivo*™ CD3/CD28 beads for activation and expansion. Cytokine functional profiles are assessed using day 0, 4, 7, and 10 cell culture supernatants; data shown are from day 4.

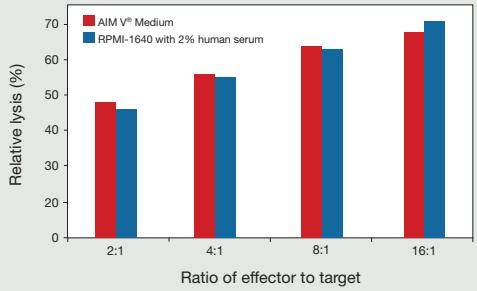

Figure 4. Comparison of LAK cell lytic activity: AIM V® Medium vs. human serum-supplemented medium. Human peripheral blood lymphocytes (PBLs) were cultured in RMPI-1640 with IL-2 plus 2% human serum type AB. Human PBLs were also cultured in serum-free AIM V® Medium.

New and proven paths to optimal cell culture

Learn more about OpTmizer™ SFM, AIM V® Medium, and related T cell products at **www.invitrogen.com/bp59**. ■

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Product	Quantity	Cat. no.
OpTmizer™ T Cell Expansion SFM (bottle)	1 L	A1048501
OpTmizer™ T Cell Expansion SFM (bottle)	500 mL	0080022SA
OpTmizer™ T Cell Expansion SFM	1 L	0080022SC
OpTmizer™ T Cell Expansion SFM	5 L	0080022SD
Therapeutic grade		
AIM V® Medium, liquid	1 L	0870112DK
AIM V® Medium, liquid	10 L	0870112BK
Research grade		
AIM V® Medium, liquid	500 mL	12055091
AIM V® Medium, liquid, contains AlbuMAX® (BSA) substituted for HSA	500 mL	31035025
AIM V® Medium, liquid	1 L	12055083

Near-infrared dye for live cell cycle analysis

VYBRANT® DYECYCLE™ RUBY STAIN.

Live-cell studies of cellular DNA content and cell cycle distribution are useful to detect variations in growth patterns due to a variety of physical, chemical, or biological factors, to monitor apoptosis, and to study tumor behavior and suppressor gene mechanisms. In a given population, cells will be distributed among three major phases of the cell cycle: the G_0/G_1 phase, the S phase, and the G_2/M phase.[1] Applications measuring cellular DNA content require dyes that bind to DNA stoichiometrically. Before the introduction of the Vybrant® DyeCycle™ series of stains, only UV-excited Hoechst dyes[2] were available for studies of DNA content in live cells.

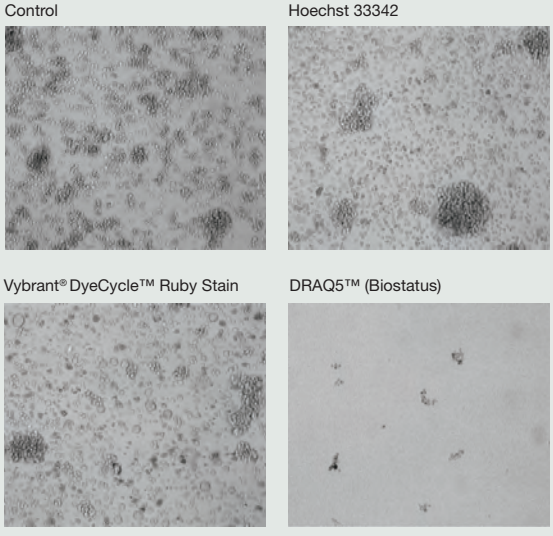

Figure 1. Bright-field images of cells grown after sorting. Live Jurkat cells were stained with either 5 µM UV-excited Hoechst 33342 (Cat. no. H3570), 5 µM Vybrant® DyeCycle™ Ruby Stain (Cat. no. V10309), or 5 µM DRAQ5™ dye (Biostatus Limited); control cells were untreated. The cells were then sorted and cultured for 8 days to examine the ability of the cells to grow after staining and sorting. Characteristic grape clusters of growing Jurkat cells were seen for control cells and cells stained with Hoechst 33342 and Vybrant® DyeCycle™ Ruby stains. The cells stained with DRAQ5™ did not grow, demonstrating that DRAQ5™ is cytotoxic.

Features of Vybrant® DyeCycle™ Ruby Stain

Vybrant® DyeCycle™ Ruby Stain is a membrane-permeant, DNA-selective dye that undergoes moderate fluorescence enhancement upon binding DNA. This stain binds stoichiometrically to DNA in live or fixed cells and requires no RNase treatment. Vybrant® DyeCycle™ Ruby Stain can be used with any excitation source from 488 nm to 690 nm, with fluorescent emission in the near-infrared region.

DNA content measurement in live cells offers sorting options

Vybrant® DyeCycle™ Ruby Stain facilitates DNA content analysis in live cells. This dye takes advantage of the commonly available 488 nm and 633 or 635 nm excitation sources, with emission at >670 nm. Because Vybrant® DyeCycle™ Ruby Stain is significantly less cytotoxic than DRAQ5™ dye, this stain can be used to determine the cell proliferation rate and offers the possibility of cell sorting based on DNA content (Figure 1). To learn more about flow cytometry assays for live cells, visit **www.invitrogen.com/bp59**. ■

References

1. Pozarowski, P. and Darzynkiewicz, Z. (2004) *Methods Mol Biol* 281:301–311.
2. Darzynkiewicz, Z. et al. (2004) *Cytometry A* 58:21–32.

Product	Quantity	Cat. no.
Vybrant® DyeCycle™ Ruby Stain, 2.5 mM solution in DMSO, 400 assays	400 µL	V10273
Vybrant® DyeCycle™ Ruby Stain, 2.5 mM solution in DMSO, 100 assays	100 µL	V10309
Vybrant® DyeCycle™ Violet Stain, 5 mM in water, 200 assays	200 µL	V35003
Vybrant® DyeCycle™ Green Stain, 5 mM solution in DMSO, 200 assays	400 µL	V35004
Vybrant® DyeCycle™ Orange Stain, 5 mM solution in DMSO, 200 assays	400 µL	V35005

Specific detection of gap junction proteins

ALEXA FLUOR® DYE–CONJUGATED PRIMARY ANTIBODIES.

Invitrogen offers primary antibodies conjugated to Alexa Fluor® 488 and Alexa Fluor® 594 dyes for immunocytochemistry (ICC), immunofluorescence (IF), and immunohistochemistry (IHC). Invitrogen's fluorescent primary antibodies directed against gap junction and cytoskeletal proteins provide highly specific detection of their target proteins; a selection of these fluorescent primary antibodies is shown in Table 1. These high-quality, high-affinity primary antibodies are directly conjugated to Alexa Fluor® dyes, eliminating extra secondary antibody detection steps and providing bright, crisp images. Furthermore, when secondary antibodies aren't used for detection, you are free to use primary antibodies from the same host for colocalization studies.

Bright fluorochromes on high-quality gap junction antibodies

Alexa Fluor® 488 and Alexa Fluor® 594 dyes are brighter substitutes for FITC and Texas Red®-X dyes, respectively, but exhibit similar fluorescence spectra, so you won't need to change optics when using these dyes. Because Alexa Fluor® fluorophores resist fluorescence quenching, more dyes can be conjugated to each antibody for a brighter signal. Our optimized protocols allow conjugations of most fluorochromes without quenching or loss of specificity. By using high-quality gap junction or tubulin primary antibodies conjugated to Alexa Fluor® dyes, you may be able to detect targets that otherwise would need to be detected using traditional secondary detection (Figures 1 and 2).

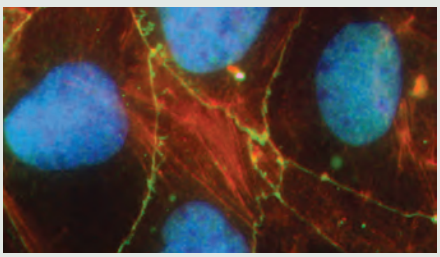

Figure 1. Bright, crisp imaging with highly specific dye-conjugated primary antibodies. Human Caco-2 cells labeled with Alexa Fluor® 488 mouse anti–claudin-4 antibody (Cat. no. 329488). Actin is stained with Alexa Fluor® 568 phalloidin (Cat. no. A12380); DNA is stained with blue-fluorescent Hoechst 33258 (Cat. no. H3569).

Taking the next step

These primary labeled antibodies against gap junction and cytoskeletal proteins will give you bright, crisp images and are validated for multiple applications. Learn more at **www.invitrogen.com/bp59**. ■

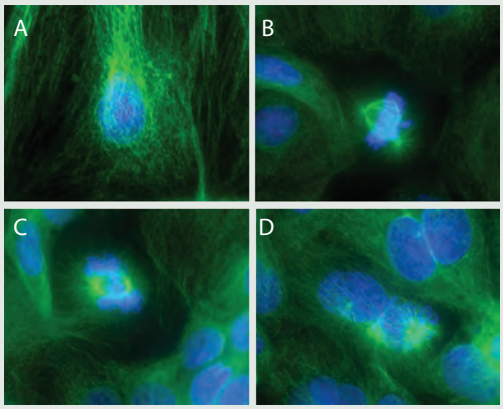

Figure 2. Different phases of mitosis in human Caco-2 cells stained with Alexa Fluor® 488 mouse anti-tubulin antibody (Cat. no. 322588). (**A**) Interphase, (**B**) metaphase, (**C**) anaphase, and (**D**) telophase. DNA is stained with blue-fluorescent Hoechst 33258 (Cat. no. H3569).

Table 1. A selection of Invitrogen's fluorescent primary antibodies.

Target	Fluorophore	Ex/Em (nm)	Quantity	Cat. no.
ZO-1	Alexa Fluor® 488	495/519	100 µg	339188
ZO-1	Alexa Fluor® 594	590/617	100 µg	339194
Occludin	Alexa Fluor® 488	495/519	100 µg	331588
Occludin	Alexa Fluor® 594	590/617	100 µg	331594
Claudin-1	Alexa Fluor® 488	495/519	100 µg	374988
Claudin-4	Alexa Fluor® 488	495/519	100 µg	329488
Claudin-4	Alexa Fluor® 594	590/617	100 µg	329494
Claudin-5	Alexa Fluor® 488	495/519	100 µg	352588
α-Tubulin	Alexa Fluor® 488	495/519	100 µg	322588
Connexin 43	Alexa Fluor® 488	495/519	100 µg	138388

Dead cell stains in flow cytometry: A comprehensive analysis

VIABILITY DETECTION IN EVERY COLOR.

Loss of membrane integrity is the ultimate indicator of cell death in flow cytometric analysis. Cells that exclude a dead cell dye are considered viable, while cells with a compromised membrane allow dye inside the cell to stain an internal component, thus identifying the cell as dead. Many dyes across a wide range of excitation and emission wavelengths are available for dead cell identification. This article summarizes a survey of 27 such dyes. All dyes were tested using a mixture of live and heat-killed Jurkat T cells before and after formaldehyde fixation to test whether the dye can withstand fixation, and using Jurkat cells treated with camptothecin, an inducer of apoptosis, to test whether the dye can identify apoptotic cells. The study included five classes of dyes: traditional (or impermeant) nucleic acid dyes, dimeric cyanine dyes, LIVE/DEAD® fixable dead cell stains (amine-reactive dyes), annexin V conjugates, and monomeric cyanine dyes.

Traditional impermeant nucleic acid dyes

Impermeant nucleic acid dyes enter cells with compromised membranes and bind DNA, where they undergo significant fluorescence enhancement.[1] Propidium iodide is the most commonly used dye in this group, but many other traditional DNA dyes that are compatible with every excitation source can be used for the verification of membrane integrity, including the versatile SYTOX® stains. Because these dyes bind in equilibrium with DNA, external dye concentration must be maintained during analysis, and the dye should not be washed out. Impermeant DNA dyes are not compatible with fixation or intracellular staining protocols, and generally do not identify apoptotic cells (Figure 1A).

Dimeric cyanine dyes

Invitrogen's dimeric cyanine nucleic acid dyes are among the highest-sensitivity fluorescent probes available for nucleic acid staining, and are excluded from cells with intact cell membranes. In addition to their high affinity for nucleic acids, cyanine dimers are essentially nonfluorescent in the absence of nucleic acids and exhibit 100- to 1,000-fold fluorescence enhancement upon binding to DNA.[2] The extinction coefficients and fluorescence quantum yields of cyanine dimers bound to DNA are high, resulting in very bright fluorescent signals.[3] Impermeant cyanine dimer dyes are not compatible with fixation or intracellular staining protocols, and do not identify apoptotic cells (Figure 1B).

LIVE/DEAD® fixable dead cell stains

Based on the reaction of a fluorescent reactive dye with cellular proteins, the LIVE/DEAD® Fixable Dead Cell Stain Kits are often referred to as amine-reactive dyes. Because these dyes cannot penetrate an intact cell membrane, only cell surface proteins are available to react with the dye, resulting in relatively dim staining. However, the reactive dye can enter compromised membranes and stain the interior of the cell as well as the cell surface. This results in more intense dead cell staining, typically greater than 50-fold, allowing complete, simultaneous discrimination of live and dead cell populations. Because the dyes react covalently with proteins, the discrimination is completely preserved following fixation of the sample with formaldehyde under conditions that inactivate pathogens. LIVE/DEAD® fixable dead cell stains are completely compatible with aldehyde-based fixation and intracellular staining protocols,[4] and do not identify apoptotic cells (Figure 1C).

Annexin V conjugates

Highly fluorescent annexin V conjugates provide a quick and reliable detection method for studying the externalization of phosphatidylserine (PS), an indicator of intermediate stages of apoptosis.[5,6] Apoptosis is distinguishable from necrotic death by characteristic morphological and biochemical changes, including compaction and fragmentation of nuclear chromatin, shrinkage of the cytoplasm, and

loss of membrane asymmetry.[5] In normal viable cells, PS is located on the cytoplasmic surface of the cell membrane. In apoptotic cells, however, PS is translocated from the inner leaflet to the outer leaflet of the plasma membrane, a process known as membrane flipping, exposing PS to the external cellular environment. Annexin V conjugates bind to PS on apoptotic cell surfaces in the presence of Ca^{2+}, but can also pass through the compromised membranes of necrotic cells and bind to PS in the interior of the cell. Therefore, it is recommended that a cell-impermeant dead cell stain be used in combination with annexin V conjugate staining to distinguish necrotic from apoptotic cells. Annexin V conjugates are compatible with fixation, and identify apoptotic cells (Figure 1D). →

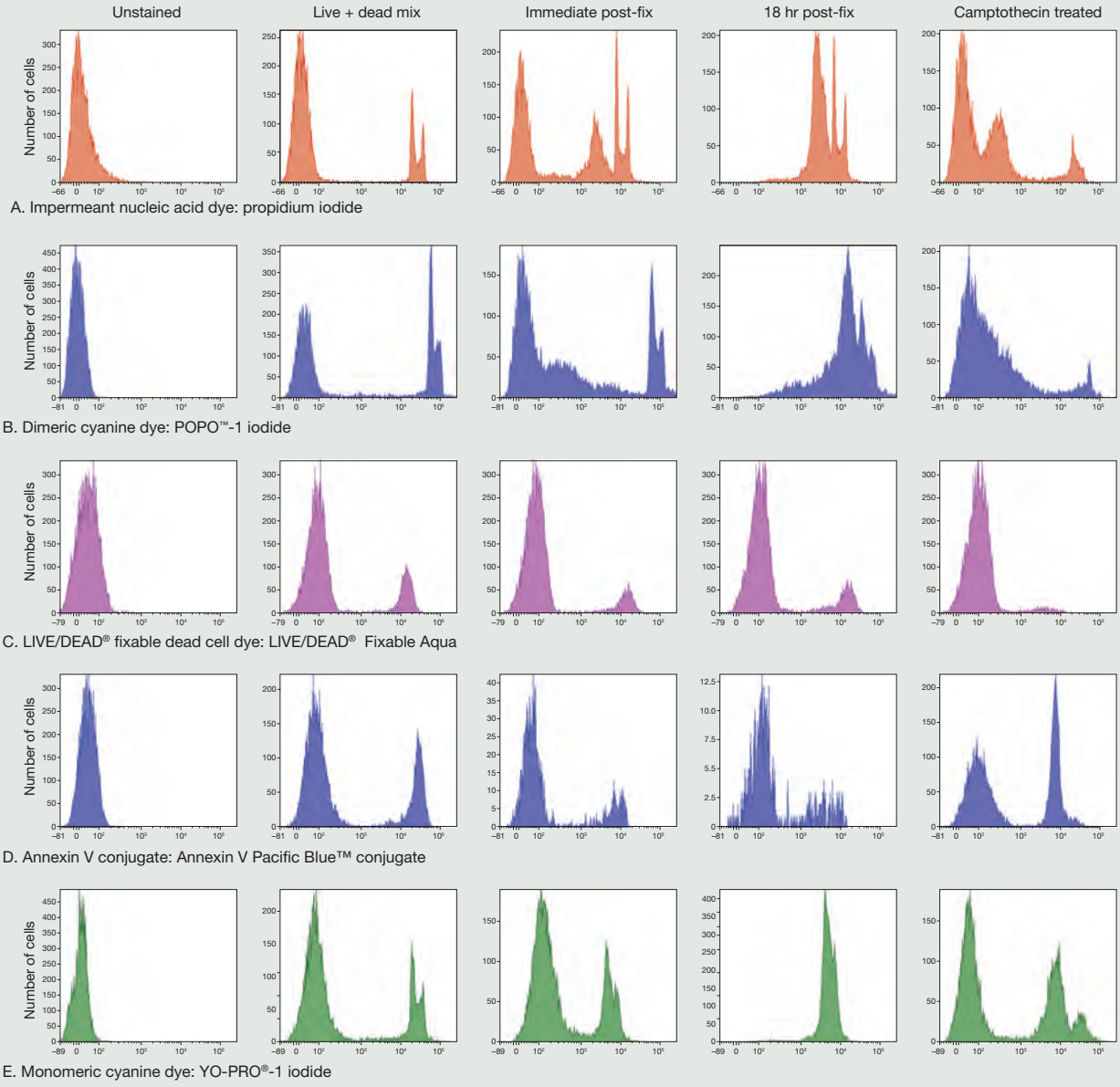

Figure 1. Jurkat T cell leukemia cells stained with examples of each class of dead cell dye. The first four columns use a mixture of live- and heat-killed cells. The last column shows Jurkat cells treated with camptothecin to induce apoptosis.

Monomeric cyanine dyes

During loss of membrane asymmetry in apoptosis, plasma membrane permeability changes to the point that some normally cell-impermeant dyes can enter these cells. Monomeric cyanine dyes enter these apoptotic cells, while propidium iodide and other classic impermeant nucleic acid dyes cannot.[7] The relatively narrow emission bandwidths of monomeric cyanine dyes facilitate multicolor applications. These dyes do not require special staining buffers like annexin V does, and can be used with trypsinized adherent cells. Monomeric cyanine dyes identify apoptotic cells but are not compatible with fixation (Figure 1E).

More about dead cell stains

To learn more about flow cytometry assays for assessing viability and vitality, visit **www.invitrogen.com/bp59**. You'll also find a comprehensive survey of dead cell stains, available as a downloadable PDF. ■

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1. Arndt-Jovin, D.J. and Jovin, T.M. (1989) *Methods Cell Biol* 30:417–448.
2. Rye, H.S. et al. (1992) *Nucleic Acids Res* 20:2803–2812.
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4. Perfetto, S.P. et al. (2006) *J Immunol Methods* 313:199–208.
5. Darzynkiewicz, Z. et al. (1997) *Cytometry* 27:1–20.
6. Koopman, G. et al. (1994) *Blood* 84:1415–1420.
7. Idziorek, T. et al. (1995) *J Immunol Methods* 184: 249–258.

Product	Excitation laser	Emission wavelength/ bandpass (nm)	Quantity	Cat. no.
Traditional nucleic acid dyes				
4′,6-Diamidino-2-phenylindole, dihydrochloride (DAPI)	UV	450/50	10 mg	D1306
SYTOX® Blue Dead Cell Stain, 1 mM solution in DMSO	405 nm	450/50	1 mL	S34857
SYTOX® Green Nucleic Acid Stain, 5 mM solution in DMSO	488 nm	530/30	250 µL	S7020
Propidium iodide, 1 mg/mL solution in water	488 nm	585/42, 610/30	10 mL	P3566
Ethidium homodimer-1 (EthD-1)	488 nm	610/30	1 mg	E1169
Ethidium homodimer-2 (EthD-2), 1 mM solution in DMSO	488 nm	610/30	200 µL	E3599
Ethidium monoazide bromide (EMA)	488 nm	610/30	5 mg	E1374
7-Aminoactinomycin D (7-AAD)	488 nm	695/40	1 mg	A1310
SYTOX® AADvanced™ Dead Cell Stain Kit (100 assays)	488 nm	695/40	1 kit	S10349
SYTOX® AADvanced™ Dead Cell Stain Kit (500 assays)	488 nm	695/40	1 kit	S10274
SYTOX® Red Dead Cell Stain, 5 µM solution in DMSO	633–635 nm	660/20	1 mL	S34859
Monomeric cyanine dyes				
PO-PRO™-1 iodide (435/455), 1 mM solution in DMSO	405 nm	450/50	1 mL	P3581
YO-PRO®-1 iodide (491/509), 1 mM solution in DMSO	488 nm	530/30	1 mL	Y3603
TO-PRO®-3 iodide (642/661), 1 mM solution in DMSO	633–635 nm	660/20	1 mL	T3605
Dimeric cyanine dyes				
POPO™-1 iodide (434/456), 1 mM solution in DMSO	405 nm	450/50	200 µL	P3580
YOYO®-1 iodide (491/509), 1 mM solution in DMSO	488 nm	530/30	200 µL	Y3601
TOTO®-1 iodide (514/533), 1 mM solution in DMSO	633–635 nm	660/20	200 µL	T3600
LIVE/DEAD® fixable dead cell stains				
LIVE/DEAD® Fixable Blue Dead Cell Stain Kit	UV	450/50	1 kit	L23105
LIVE/DEAD® Fixable Violet Dead Cell Stain Kit	405 nm	450/50	1 kit	L34955
LIVE/DEAD® Fixable Aqua Dead Cell Stain Kit	405 nm	530/30	1 kit	L34957
LIVE/DEAD® Fixable Yellow Dead Cell Stain Kit	405 nm	575/25	1 kit	L35959
LIVE/DEAD® Fixable Green Dead Cell Stain Kit	488 nm	530/30	1 kit	L23101
LIVE/DEAD® Fixable Red Dead Cell Stain Kit	488 nm	610/30	1 kit	L23102
LIVE/DEAD® Fixable Far Red Dead Cell Stain Kit	633–635 nm	660/20	1 kit	L10120
LIVE/DEAD® Fixable Near-IR Dead Cell Stain Kit	633–635 nm	680/60	1 kit	L10119
Annexin V conjugates				
Annexin V, Pacific Blue™ Conjugate	405 nm	450/50	400 µL	A35122
Annexin V, R-phycoerythrin Conjugate (R-PE annexin V)	488 nm	585/42, 610/30	250 µL	A35111
Annexin V, Alexa Fluor® 647 Conjugate	633–635 nm	660/20	500 µL	A23204

Is it time to go direct?

IgG ANTIBODY LABELING METHODS AS VERSATILE AS YOUR IMAGINATION.

Invitrogen offers a number of labeling strategies for attaching fluorescent organic dyes, phycobiliproteins, or nanocrystals to an IgG antibody for use in fluorescence imaging or flow cytometry applications. Although directly labeled antibodies may not yield signals as bright as those observed with secondary antibodies, their use eliminates the background commonly associated with nonspecific secondary antibody binding. In addition, directly labeled antibodies allow you to use more than one same-species antibody in a single experiment. Here we provide guidance on selecting the best method—whether direct or indirect (Figure 1)—for conjugating a fluorophore or other label to your target antibody.

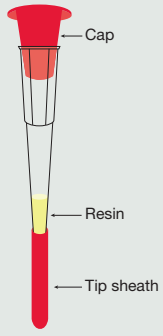


Figure 2. APEX antibody labeling technology allows convenient elution of contaminants through the filter tip.

Labeling techniques from A to Z

This overview focuses primarily on the unique APEX Antibody Labeling Kits and Zenon® IgG Antibody Labeling Kits, which are specifically designed to label IgG antibodies regardless of quantity or purity issues, as well as tools to covalently attach phycobiliproteins and nanocrystals. A comprehensive list of Invitrogen's innovative and easy-to-use antibody labeling kits (including the APEX and Zenon® labeling kits) can be found in Table 1.

APEX Antibody Labeling Kits use a solid-phase labeling technique that captures the IgG on the resin inside the APEX labeling tip (Figure 2). Contaminants (e.g., stabilizing proteins and amine-containing buffers) are eluted through the tip, and an amine-reactive label is then applied. Subsequent wash steps remove unreacted dye, and the fluorescent IgG conjugate is eluted and ready for use in as little as 2 hours with very little hands-on time (Figure 3). →

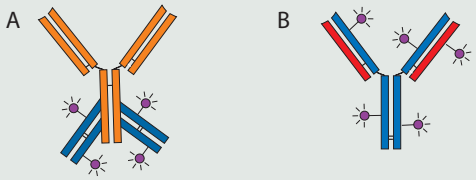


Figure 1. Noncovalent vs. covalent labeling. (**A**) Noncovalent Zenon® antibody labeling. (**B**) Covalent APEX, microscale, monoclonal, cross-linking, and protein labeling. Covalent attachment generates a stable conjugate that can last for years, whereas noncovalently attached labels can dissociate over time (Zenon® conjugates last from several hours to several days).

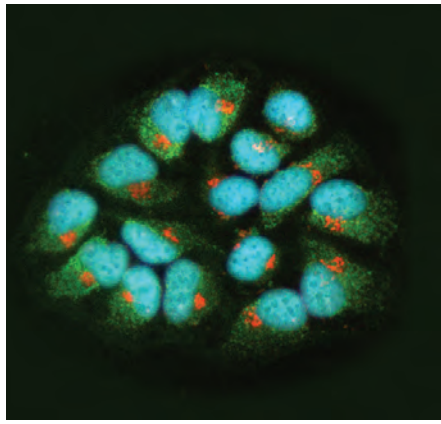


Figure 3. Mitochondrial and Golgi complex labeling in HeLa fixed and permeabilized cells. The Golgi complex was detected with an anti–golgin-97 mouse monoclonal antibody labeled using the APEX Alexa Fluor® 555 Antibody Labeling Kit (Cat. no. A10470, orange fluorescence). Mitochondria were detected with an anti–OxPhos Complex V inhibitor protein mouse monoclonal antibody labeled using the APEX Alexa Fluor® 488 Antibody Labeling Kit (Cat. no. A10468, green fluorescence). Nuclei were stained with blue-fluorescent DAPI (Cat. no. D1306).

Table 1. IgG antibody labeling kits.

Amount of IgG	Product	Notes	Method of attachment	Available fluorophores and labels *	Applications
<1–20 µg	Zenon® IgG Labeling Kits	• Antibodies ready to use in 10 min • Isotype-specific labeling • Compatible with stabilizing proteins	Noncovalent	Organic dyes, phycobiliproteins	FC, ICC
10–20 µg	APEX Antibody Labeling Kits	• Antibodies ready to use in 2 hr (~15 min hands-on time) • Compatible with stabilizing proteins	Covalent	Organic dyes	FC, ICC, IHC
20–100 µg	Microscale Protein Labeling Kits	• Antibodies ready to use in 2 hr (~30 min hands-on time) • Optimized for 10–150 kDa proteins, including IgG antibodies (~150 kDa) • Stabilizing proteins must be removed from sample before labeling	Covalent	Organic dyes	FC, ICC, IHC
100 µg	Monoclonal Antibody Labeling Kits	• Antibodies ready to use in 90 min (~15 min hands-on time) • Optimized for both monoclonal and polyclonal IgG antibodies • Stabilizing proteins must be removed from sample before labeling	Covalent	Organic dyes	FC, ICC, IHC
300 µg	Qdot® Antibody Conjugation Kits	• Antibodies ready to use in 4–5 hr • Includes amine-derivative PEG-coated nanocrystals and an amine–thiol crosslinker, SMCC • Requires mild reduction of IgG antibody to expose thiols	Covalent	Nanocrystals	FC, ICC, IHC
0.2–3 mg	Protein–Protein Crosslinking Kit	• Antibodies ready to use in 3 hr	Covalent	Phycobiliproteins	FC
0.5–3 mg	SAIVI™ Rapid Antibody Labeling Kits	• Antibodies ready to use in 75 min (~10 min hands-on time) • Includes degree-of-labeling regulator • Uses no organic solvents and produces azide-free conjugates	Covalent	Organic dyes	ICC, IHC, *in vivo* imaging, FC
1 mg	Protein Labeling Kit	• Antibodies ready to use in 2 hr (~30 min hands-on time) • Designed to label monoclonal and polyclonal IgG antibodies • Stabilizing proteins must be removed from sample before labeling	Covalent	Organic dyes	FC, ICC, IHC

* See "Choosing the right fluorescent label for your antibody" for additional information on fluorophores and labels. FC = flow cytometry; ICC = immunocytochemistry; IHC = immunohistochemistry. Learn more about IgG antibody labeling kits at **www.invitrogen.com/bp59**.

As an alternative to direct chemical labeling, Zenon® technology provides one of the most versatile and easy-to-use methods for the fluorescent labeling of even submicrogram amounts of mouse, rabbit, goat, and human IgG. Zenon® antibody labeling technology takes advantage of the immunoselectivity of the antibody binding reaction (Figure 4); simply mixing the labeled Zenon® reagent (supplied in the kit) with the corresponding primary antibody quantitatively produces the Fab–antibody complex in under 10 minutes, with no pre- or post-labeling purification required. The labeled antibody can be used immediately to stain cells in the same manner as a covalently labeled primary antibody (Figure 5).

Labeling an IgG antibody with a molecule other than an organic dye requires a slightly different method. Forming a stable complex between an IgG antibody and a phycobiliprotein or Qdot® nanocrystal requires chemical crosslinking. The most common scheme involves the multistep coupling of an amine group on one biomolecule to a thiol

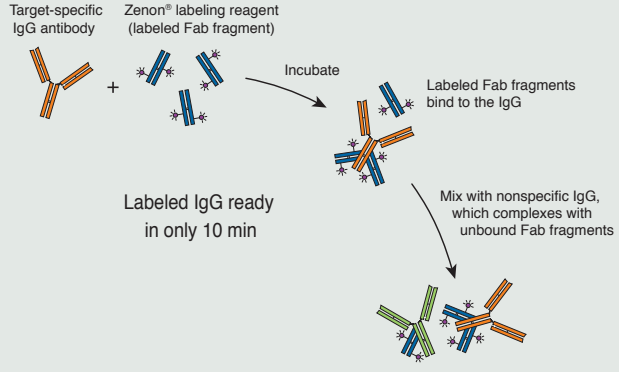


Figure 4. The Zenon labeling scheme. An unlabeled IgG is incubated with the Zenon® labeling reagent, which contains a fluorophore-labeled Fab fragment. The labeled Fab fragment binds to the Fc portion of the IgG antibody, and excess Fab fragment is bound by the addition of a nonspecific IgG. The addition of nonspecific IgG prevents cross-labeling of the Fab fragment in experiments where multiple primary antibodies of the same type are present. Note that the Fab fragment used for labeling can be coupled to a fluorophore, an enzyme, or biotin.

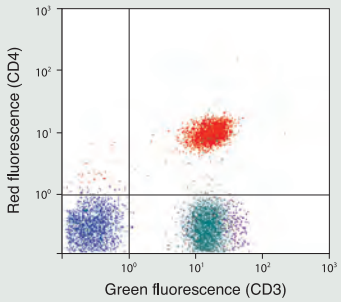


Figure 5. Human peripheral blood mononuclear cells stained with markers for CD3 and CD4 and detected using a lymphocyte gate. The cell sample was stained with an anti-CD3 mouse IgG1 antibody prelabeled using the Zenon® Alexa Fluor® 488 Mouse IgG1 Labeling Kit (Cat. no. Z25002) and an anti-CD4 mouse IgG1 antibody prelabeled using the Zenon® Alexa Fluor® 647–R-Phycoerythrin Mouse IgG1 Labeling Kit (Cat. no. Z25021). Plots of CD3 vs. CD4 demonstrate good signal separation. The samples were analyzed on a Coulter Elite™ flow cytometer using excitation at 488 nm and appropriate bandpass emission filters.

group on a second biomolecule. The Protein–Protein Crosslinking Kit (Cat. no. P6305) provides all of the reagents needed to crosslink and purify small amounts of two proteins, such as an IgG antibody and a phycobiliprotein. Qdot® Antibody Conjugation Kits follow a similar strategy for attaching nanocrystals to target antibodies.

Start labeling today

To explore the labeling technologies highlighted here, as well as our broad selection of labels for fluorescence imaging and flow cytometry applications, visit **www.invitrogen.com/bp59**. ■

Choosing the right fluorescent label for your antibody

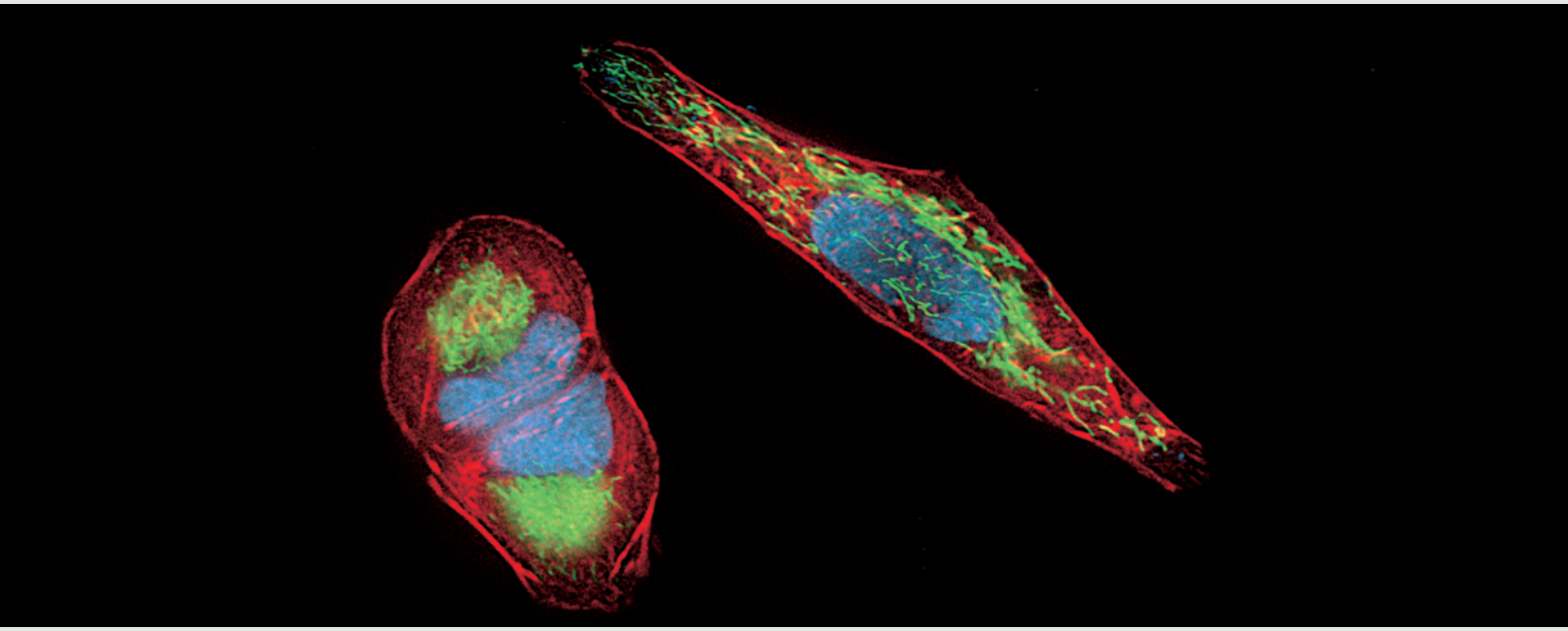
There are several considerations to keep in mind when selecting a fluorescent label:

- Match the dye or fluorescent label to the optical characteristics of your detection system. Superior dye brightness or photostability is irrelevant if the dye cannot be efficiently excited or detected.
- Minimize the spectral overlap between multiple dyes in an application.
- Consider target abundance—rare targets require brighter fluorophores, whereas more abundant targets are visible with less bright fluorophores.

Key differentiating characteristics of the fluorophores and fluorescent labels used to label primary antibodies are provided here. For further assistance in choosing the ideal fluorophore for your application or instrumentation, use our online SpectraViewer at **www.invitrogen.com/spectraviewer**. Using the SpectraViewer, you can not only plot the excitation and emission spectra of up to five fluorophores, but also include excitation or emission filters or laser excitation lines to customize the program for your instrument.

Fluorescent labels

	Organic dye	Phycobiliprotein	Nanocrystal
Product examples	Alexa Fluor® dye series (e.g., Alexa Fluor® 488 dye)	R-phycoerythrin (R-PE), allophycocyanin (APC), Alexa Fluor® 680–R-phycoerythrin	Qdot® nanocrystals (e.g., Qdot® 625 nanocrystal)
Instrument platform compatibility	Fluorescence imaging, flow cytometry	Flow cytometry	Flow cytometry, fluorescence imaging
MW range	~250–1,800 daltons	~100,000–240,000 daltons	NA
Extinction coefficient range	20,000–250,000 $cm^{-1}M^{-1}$	700,000–2,410,000 $cm^{-1}M^{-1}$	140,000–8,000,000 $cm^{-1}M^{-1}$
Emission range	~400–850 nm	~575–800 nm	~500–850 nm
Key features	• Photostable • Widest selection of fluorophores from near-UV to near-IR • High extinction coefficient and quantum efficiency	• Large Stokes shifts • Extremely high extinction coefficients	• Narrow emission • Extremely large Stokes shifts • Extremely long-term photostability • Multiplex capable with single excitation source (405 nm)
Antibody labeling products	• Amine-reactive fluorophores available as stand-alone products or as part of antibody labeling kits (see Table 1) • Thiol-reactive fluorophores available as stand-alones • Zenon® antibody labeling technology for noncovalent attachment of the label	• Protein–Protein Crosslinking Kit for covalent attachment of antibody to the phycobiliprotein • Zenon® antibody labeling technology for noncovalent attachment	• Qdot® antibody labeling kits



Lights. Cells. Action.

LIVE-CELL IMAGING WITH ORGANELLE LIGHTS™ AND CELLULAR LIGHTS™ REAGENTS.

When cellular events are studied out of context—isolated from the rest of the cell—part of the story is inevitably lost. Fixed-endpoint measurements can capture an accurate snapshot, but don't provide any insight into dynamic cellular processes. Ultimately, examining a particular cellular pathway in both its spatial and temporal context is critical for understanding cell development and functioning. Molecular Probes® Organelle Lights™ and Cellular Lights™ reagents combine the selectivity of a targeted fluorescent protein with the transduction efficiency of BacMam technology, enabling unambiguous visualization of organelles and other cellular structures in live mammalian cells by fluorescence microscopy (Figure 1). Provided in ready-to-use format—simply add, incubate, and visualize—these reagents open up new avenues for multiparametric study of dynamic cellular events in context.

Figure 1 (above). Visualize cytoskeletal and mitochondrial dynamics and organization with Cellular Lights™ Talin-RFP and Organelle Lights™ Mito-GFP. Cellular Lights™ reagents use fluorescent protein–signal peptide fusions for accurate and specific targeting to subcellular structures in living cells. Their high spatial and temporal resolution enable unique and powerful insights into biological systems without modifying cell function. To create this image, HeLa cells were incubated with Cellular Lights™ Talin-RFP (Cat. no. C10324) and Organelle Lights™ Mito-GFP (Cat. no. O36210) for ~2 hr. Cellular Lights™ Talin-RFP facilitates the visualization of focal adhesions in live cells via talin, a protein that links actin to the extracellular matrix; Organelle Lights™ Mito-GFP enables the visualization of mitochondria independent of mitochondrial membrane potential. Nuclei were stained with Hoechst 33342 (Cat. no. H3570). Imaging was performed on live cells using a Delta Vision Core microscope and standard DAPI/FITC/TRITC filter sets.

Lights: Powerful reagents for labeling cellular structures

Organelle™ Lights and Cellular Lights™ reagents express fluorescent protein–signal peptide fusions that provide accurate and specific targeting to subcellular compartments and structures (Figure 2) for live-cell imaging. Available in a range of colors, these reagents label a variety of subcellular structures, including nuclear and plasma membranes, mitochondria, and the cytoskeleton (Figures 1 and 3), for convenient multiplexing and colocalization studies. Unlike many conventional stains, Cellular Lights™ and Organelle Lights™ reagents stain independently of function (e.g., membrane potential). As a result, these reagents can be used as a baseline for ratiometric measurements when used with classic stains such as tetramethylrhodamine methyl ester (TMRM, Figure 3). The signal is well retained following aldehyde-based fixation, and the fluorescence can be further amplified using Alexa Fluor® dye–labeled anti–green fluorescent protein (GFP) antibodies.

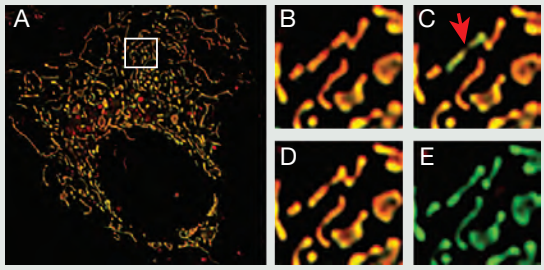


Figure 3. Dynamic imaging of mitochondrial membrane potential using Organelle Lights™ reagents. (**A**) HeLa cells were transduced with Organelle Lights™ Mito-GFP (Cat. no. O36210) and loaded with 50 nM TMRM (Cat. no. T668) for 10 min at 37°C. Colocalization of TMRM and GFP can be clearly seen, confirming the specific accumulation of TMRM in mitochondria. Images were acquired at 5 sec intervals for 90 sec, revealing heterogeneity in mitochondrial membrane potential regulation (**B–E**), transient depolarization in individual mitochondria (**C**, red arrow), and, following application of CCCP, a global, irreversible loss of mitochondrial membrane potential (**E**).

Cells: Efficient transduction via BacMam delivery technology

Cellular transduction is mediated by an insect virus (baculovirus) that is nonreplicating in mammalian cells, and thus safe to handle (Biosafety level 1). The genetically encoded and prepackaged reagents are ready for immediate use—there's no need to purify plasmid or worry about vector integrity and quality. Furthermore, no lipids, dye-loading chemicals, or other potentially harmful treatments are required. Transduction is efficient and reproducible in most cell types, including primary and stem cells, without apparent cytopathic effects. Simply add the reagent to your cells for 1–2 hours, treat with the enhancer for 1–2 hours, wash, incubate overnight, then visualize your results. For the most up-to-date list of cell types successfully tranduced with proven BacMam technology,[1,2] visit **www.invitrogen.com/bp59**.

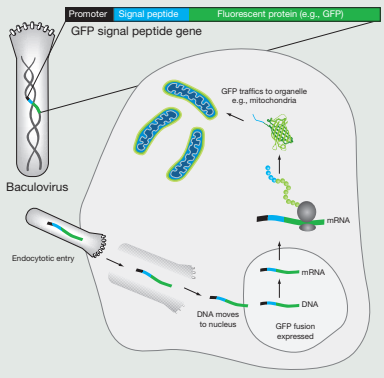


Figure 2. How Organelle Lights™ and Cellular Lights™ fluorescent protein–based reagents work.

The efficient transduction afforded by BacMam technology makes it easy to deliver multiple constructs and to modulate gene expression simply by adjusting the dose. The effective delivery and the genetic content of these reagents also permit transduction of large quantities of cells in batch mode that can be separated into aliquots and stored for use as needed, approximating the consistency of stable cell lines without the risk of genetic drift. Expression of the fluorescent protein is unaffected by freezing, and upon plating, cells can remain brightly fluorescent for more than 120 hours, depending on the cell line.

Action: Putting it all together

Organelle Lights™ and Cellular Lights™ reagents are ideal for multiplexing with other fluorescent proteins, organic fluorescent dyes, or Qdot® nanocrystals. 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.

With the simplicity, ease of use, and reproducibility of Organelle Lights™ and Cellular Lights™ reagents, you can focus less on your imaging reagents and more on experimental design, imaging, and data analysis. To learn more and view a full list of these reagents, visit **www.invitrogen.com/bp59**. ■

References

1. Kost, T.A. et al. (2005) *Nat Biotechnol* 23:567–575.
2. Kost, T.A. and Condreay, J.P. (2002) *Trends Biotechnol* 20:173–180.

The envelope, please: Best picture for biofilms

ADVANCING BIOLOGY THROUGH DIRECT FLUORESCENCE VISUALIZATION.

Fluorescence-based contrast reagents have advanced discoveries not only in cell biology, but also in microbiology. Recently, identifying, highlighting, and differentiating the components of microbial communities known as "biofilms" have become important in areas as diverse as food safety, chronic wound healing, personal health products, antimicrobial product efficacy testing, and bioremediation.

What are biofilms?

Biofilms are complex, adherent microecosystems that potentially contain many types of organisms, architectural features, and structural building blocks. Direct visualization of biofilms via fluorescence microscopy has demonstrated the presence of two dominant constituents: cell bodies, consisting of organisms in different physiological and nutrient states, and the extracellular polymeric substance (EPS), the glue that holds the microbial community together and acts as a barrier to chemicals and anti-microbial agents [1] and inhibits immune responses.[2] Fluorescence visualization tools such as LIVE/DEAD® *Bac*Light™ bacterial viability stains, acridine orange, DAPI, FUN® 1, and calcofluor white have allowed researchers to observe biofilms in association with antibiotic treatment evaluations,[1] oral hygiene product efficacy,[3] and contact lens care solution examination,[4] but until recently, the choice of effective staining reagents has been limited.

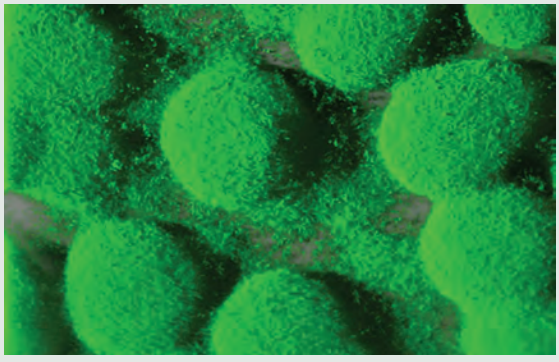


Figure 1. FilmTracer™ FM® 1-43 green biofilm cell stain applied to a *Pseudomonas aeruginosa* biofilm. FilmTracer™ FM® 1-43 appears to bind to the cell membrane, and works equally well on *P. aeruginosa*, *S. epidermidis*, and *E. coli*, exhibiting exceptional cell specificity in each case. Image was obtained using a Leica TCS-SP2 AOBS confocal microscope with a 63×/0.9 NA water immersion objective. Image contributed by Betsey Pitts and Ellen Swogger, Center for Biofilm Engineering, Montana State University.

A new line of biofilm staining reagents

Molecular Probes, the leading brand in fluorescence microbial visualization reagents, has introduced a line of fluorescent reagents specifically identified for biofilm staining. These new tools enable the observation of cells in the context of their EPS, giving researchers the potential to uncover new details about biofilms. A better understanding of biofilms will lead to a better understanding of how to monitor and control microbes in their communities.

FilmTracer™ FM® 1-43 green biofilm cell stain

The FilmTracer™ FM® 1-43 green biofilm cell stain (Cat. no. F10317) labels *Pseudomonas aeruginosa*, *Staphylococcus epidermidis*, and *Escherichia coli* cells within a single-species biofilm. The FM® 1-43 dye is a lipophilic compound that labels cell membranes with exceptional cell specificity (Figure 1).

FilmTracer™ calcein products

FilmTracer™ calcein products stain *P. aeruginosa* and *S. epidermidis* cells within a single-species biofilm. FilmTracer™ calcein biofilm stains may work better in gram-positive than gram-negative bacteria; these stains did not work reliably with *E. coli*, perhaps due to differences in the cell

membranes. These dyes are acetoxymethyl ester derivatives of calcein-based molecules that are, with the exception of calcein red-orange AM, nonfluorescent until cleaved by nonspecific esterases, thereby producing a fluorescence signal. The staining pattern of the biofilm appears as a fluffy cloud with some cell specificity (Figure 2A and 2B).

FilmTracer™ SYPRO® Ruby stain

The FilmTracer™ SYPRO® Ruby biofilm matrix stain (Cat. no. F10318) stains the matrix of *P. aeruginosa*. The SYPRO® Ruby Stain is classically used as a protein gel stain and may stain protein components of certain biofilm matrices.

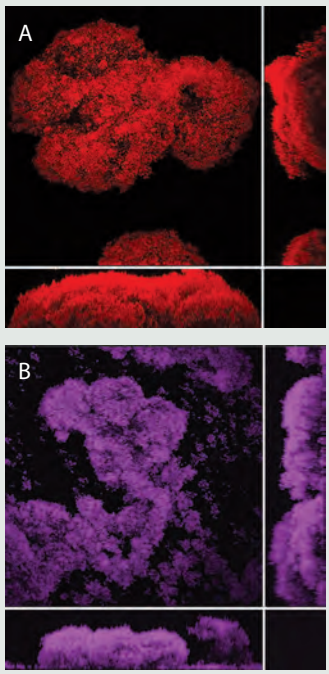


Figure 2. FilmTracer™ calcein biofilm stains applied to *Staphylococcus epidermidis* biofilms. The esterase substrates calcein red-orange AM (**A**) and calcein violet AM (**B**) appear to stain all of the bacteria in the biofilms, suggesting that the bacteria are all actively producing esterase. The images were obtained using a Leica TCS-SP2 AOBS confocal microscope with a 63×/0.9 NA water immersion objective. Images contributed by Betsey Pitts, Center for Biofilm Engineering, Montana State University.

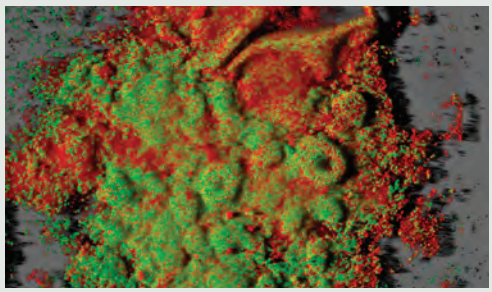


Figure 3. Stains from the FilmTracer™ LIVE/DEAD® Biofilm Viability Kit applied to a *Staphylococcus epidermidis* biofilm. The green cells and clusters indicate bacterial cells with intact membranes (live). The red cells indicate bacterial cells with damaged membranes (dead). Image contributed by Betsey Pitts, Center for Biofilm Engineering, Montana State University.

FilmTracer™ LIVE/DEAD® Biofilm Viability Kit

The FilmTracer™ LIVE/DEAD® Biofilm Viability Kit (Cat. no. L10316) differentially stains live and dead cells in biofilms, based on membrane integrity. Live bacteria exhibit green fluorescence and dead bacteria exhibit red fluorescence, even when the population contains a mixture of bacterial species (Figure 3). This kit is the same as the classic LIVE/DEAD® *Bac*light™ Kit (Cat. no. L7012), but with a protocol specific for biofilm staining.

Get the best picture with our new, wider range of biofilm staining reagents. Learn more at **www.invitrogen.com/bp59**. ■

References

1. O'Connell, H.A. et al. *Appl Environ Microbiol* (2006) 72:5013–5019.
2. Hentzer, M. and Givskov, M. *J Clin Invest* (2003) 112:1300–1307.
3. Guggenheim, B. et al. *J Dent Res* (2001) 80:363–370.
4. Imamura, Y. et al. *Antimicrob Agents Chemother* (2008) 52:171–182.

Product	Quantity	Cat. no.
FilmTracer™ FM® 1-43 green biofilm cell stain	1 mg	F10317
FilmTracer™ calcein green biofilm stain	20 x 50 µg	F10322
FilmTracer™ calcein violet biofilm stain	20 x 25 µg	F10320
FilmTracer™ calcein red-orange biofilm stain	20 x 50 µg	F10319
FilmTracer™ SYPRO® Ruby biofilm matrix stain	200 mL	F10318
FilmTracer™ LIVE/DEAD® Biofilm Viability Kit	1 kit	L10316

Click. Discover. Publish.

MORE THAN 600 PUBLICATIONS DEMONSTRATE THE POWER OF CLICK CHEMISTRY LABELING.

Click chemistry describes an extremely powerful class of chemical reactions that occur between biologically unique moieties (e.g., an azide and an alkyne). Researchers are able to take advantage of the specificity of the reaction between the azide–alkyne tag pair to label molecules of interest in complex biological samples, then detect those molecules with unprecedented sensitivity and extremely low background. The azide and alkyne moieties can be used interchangeably, and either one can be used to tag the molecule of interest while the other is used for subsequent detection (Tables 1 and 2). This article highlights just a few of the more than 600 publications to date that report the utility of click chemistry for biomolecule labeling and detection.

Nascent DNA exposed

For fast, direct, and accurate detection of new DNA synthesis, Click-iT® EdU assays are first class. Click-iT® EdU assays avoid the harsh treatments required by antibody-based BrdU assays (including HCl, DNase, and heat), providing a method that is not only more reliable and easier to perform, but also faster. In addition, antigen recognition sites and dsDNA integrity are preserved, enabling truly in-depth, multiplexed analyses. New applications include dual-pulse labeling and detection of newly synthesized mitochondrial DNA, the latter requiring the aid of signal amplification strategies. Signal amplification can potentially be achieved using Oregon Green® 488 azide as a bio-orthogonal hapten followed by an anti-fluorescein/Oregon Green® antibody conjugated to HRP (Cat. no. A21253), which is capable of generating fluorescence with a variety of fluorogenic peroxidase substrates. Successful implementation of click chemistry labeling in studies of DNA repair synthesis, proliferation, and cell signaling can be found in these recent publications:

- Limsirichaikul, S. et al. (2009) A rapid non-radioactive technique for measurement of repair synthesis in primary human fibroblasts by incorporation of ethynyl deoxyuridine (EdU). *Nucleic Acids Res* 37:e31.
- Chehrehasa, F. et al. (2009) EdU, a new thymidine analogue for labeling proliferating cells in the nervous system. *J Neurosci Methods* 177:122–130.
- Kharas, M.G. et al. (2008) Ablation of PI3K blocks BCR-ABL leukemogenesis in mice, and a dual PI3K/mTOR inhibitor prevents expansion of human BCR-ABL+ leukemia cells. *J Clin Invest* 118:3038–3050.

Table 1. Azide- or alkyne-modified biomolecules.

Biomolecule	Azide or alkyne	Cat. no.	Use
L-Azidohomoalanine	Azide	C10102	Monitor nascent protein synthesis
L-Homopropargylglycine	Alkyne	C10186	
EdU (5-ethynyl-2´-deoxyuridine)	Alkyne	A10044, E10187	Explore nascent DNA synthesis
5-Ethynyl uridine (EU)	Alkyne	E10345	Detect nascent RNA synthesis
Fucose alkyne	Alkyne	C10264	Identification of fucosylated proteins
GalNAz (tetraacetylated *N*-azidoacetylgalactosamine)	Azide	C33365	Identification of *O*-linked glycoproteins including *O*-GlcNAc
GlcNAz (tetraacetylated *N*-azidoacetylglucosamine)	Azide	C33367	Identification of *O*-GlcNAc-modified glycoproteins
ManNAz (tetraacetylated *N*-azidoacetyl-D-mannosamine)	Azide	C33366	Identification of sialic acid–modified glycoproteins
Geranylgeranyl alcohol, azide	Azide	C10249	Identification of geranylgeranylated proteins
Palmitic acid, azide (15-azidopentadecanoic acid)	Azide	C10265	Identification of protein fatty acylation
Myristic acid, azide (12-azidododecanoic acid)	Azide	C10268	

Table 2. Azide- or alkyne-modified fluorophores and haptens for detection.

Label	Abs/Em *	Azide or alkyne	Cat. no.	Use	Detection technique
Alexa Fluor® 488	495/519	Azide	A10266	Fluorescent dye or hapten	Flow cytometry, HCS, fluorescence microscopy
		Alkyne	A10267		
Alexa Fluor® 594	590/617	Azide	A10270	Fluorescent dye	HCS, fluorescence microscopy
		Alkyne	A10275		
Alexa Fluor® 647	650/668	Azide	A10277	Fluorescent dye	Flow cytometry, HCS, fluorescence microscopy
		Alkyne	A10278		
Biotin †	NA	Azide	B10184	Hapten	Flow cytometry, HCS, fluorescence microscopy, western blot, mass spectrometry
		Alkyne	B10185		
Oregon Green® 488	496/524	Azide	O10180	Fluorescent dye or hapten	Flow cytometry, HCS, fluorescence microscopy
		Alkyne	O10181		
Tetramethylrhodamine (TAMRA)	555/580	Azide	T10182	Fluorescent dye or hapten	HCS, western blot,‡ fluorescence microscopy, 1D or 2D gel, mass spectrometry
		Alkyne	T10183		

* Absorption and fluorescence emission maxima, in nm. † Requires streptavidin. ‡ Use with anti-tetramethylrhodamine (TAMRA) antibody.

Image global RNA transcription

A recent publication has established for the first time the use of an alkyne-modified nucleotide, 5-ethynyl uridine (EU, Cat. no. E10345, Table 1), to image and quantitate global RNA transcription both *in vitro* and *in vivo*. Jao and Salic demonstrate that EU can be efficiently incorporated into RNA by several polymerases, but they also provide evidence that DNA does not incorporate EU (*Proc Natl Acad Sci USA* (2008) 105:15779–15784). This exciting new development will enable detection of not only temporal but spatial expression of RNA together with RNA-interactive proteins using antibodies on imaging-based platforms.

Cutting-edge tools for protein exploration and discovery

Researchers have a number of tools in their arsenals for the detection and in-depth analysis of proteins, including traditional techniques such as western blots and mass spectrometry. Detecting critical posttranslational modifications (PTMs) is not always straightforward; suitable antibodies do not always exist for western protocols, and very labile PTMs can be lost during mass spectrometry. As a result, the role of many PTMs in normal and disease states remains a mystery. Several recent publications reveal that click chemistry–based tools are able to reliably and sensitively detect the presence of these PTMs, delivering detection data in hours or days as opposed to the weeks or months required when radiolabeling techniques are used.

- Martin, D.D. et al. (2008) Rapid detection, discovery, and identification of post-translationally myristoylated proteins during apoptosis using a bio-orthogonal azidomyristate analog. *FASEB J* 22:797–806.
- Kostiuk, M.A. et al. (2008) Identification of palmitoylated mitochondrial proteins using a bio-orthogonal azido-palmitate analogue. *FASEB J* 22:721–732.
- Wang, Z. et al. (2009) Site-specific GlcNAcylation of human erythrocyte proteins: potential biomarker(s) for diabetes. *Diabetes* 58:309–317.

Invitrogen offers several click-modified reagents for in-depth protein analysis (Table 1). The azide- or alkyne-containing biomolecule is fed to cells or animals and becomes actively incorporated into proteins, giving researchers radioisotope-free detection of key posttranslational modifications and nascent protein synthesis. In addition to our growing list of labeled biomolecules, there is also a Click-iT® O-GlcNAc Enzymatic Labeling System (Cat. no. C33368) for *in vitro* labeling of *O*-GlcNAc–modified glycoproteins. Once labeled, the modified protein is detected with the corresponding alkyne-containing dye or hapten using either the Click-iT® Cell Reaction Buffer Kit (Cat. no. C10269) or the Click-iT® Protein Buffer Kit (Cat. no. C10276). With the Click-iT® Cell Reaction Buffer Kit, cells can be analyzed by fluorescence microscopy, flow cytometry, or high-content imaging and analysis. With the Click-iT® Protein Reaction Buffer Kit, proteins are compatible with common analyses including downstream LC-MS/MS and MALDI MS analysis, and detection sensitivity in 1D gels and western blots is in the low femtomole range.

Visit **www.invitrogen.com/bp59** for the latest information on our Click-iT® assays for flow cytometry, microscopy, high-throughput imaging and analysis, and microplate readers. ■

Traffic lights: Illuminating internalization pathways

PHRODO™ CONJUGATES.

The internalization of plasma membrane, via either endocytosis or phagocytosis, underlies a diverse array of physiological events, ranging from nutrient acquisition to receptor desensitization. In response to the need for visual indicators of membrane internalization, Invitrogen has developed a pH-sensitive, fluorogenic rhodamine derivative: the pHrodo™ dye—a superior alternative to other fluorophores for imaging endocytosis or phagocytosis. The pHrodo™ dye increases its emission intensity upon internalization into acidic compartments (Figure 1).

pHrodo™ dye conjugation leverages the amine-reactive, succinimidyl form of the dye for reaction with available primary amines on ligands or "cargo" molecules (e.g., EGF or transferrin), enabling the development of endocytosis and trafficking assays for a variety of detection platforms. In this article, the use of two pHrodo™ conjugates in the context of high-content imaging is presented, providing validated pharmacological data to support their use in high-throughput drug discovery.

Visualize endocytosis in a high-throughput format

To study endocytosis in a high-content imaging format, we used pHrodo™ conjugates to create dose response curves for the inhibition of endocytosis by dynasore, a dynamin-specific inhibitor. pHrodo™ succinimidyl ester was conjugated to an amine-derivatized 10 kDa dextran, and cells were treated with serial dilutions of dynasore to create dose response curves for endocytosis inhibition (Figure 2). The loss of pHrodo™ dextran accumulation in punctuate structures within the cell was observed as a function of dynasore concentration.

The excitation and emission profiles of pHrodo™ dextran (542/581 nm) facilitate multiplexing with other fluorophores in high-content imaging studies. Figure 3 demonstrates the multiplexing capability of pHrodo™ conjugates, where early endosomes (green), acidic endocytotic vesicles (purple), and internalized transferrin (red), along with the nucleus (blue), were clearly distinguished. It was also possible to discriminate acidic and nonacidic early endosomes that either contain or lack transferrin molecules.

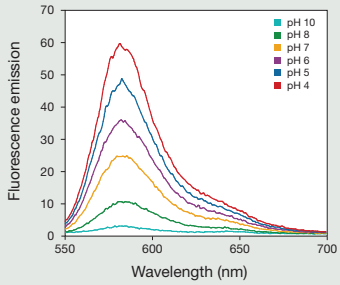


Figure 1. The pH sensitivity of pHrodo™ conjugates. pHrodo™ dextran was reconstituted in HEPES (20 mM)-buffered PBS, and adjusted to pH values ranging from 4 to 10. The intensity of fluorescence emission increases with increasing acidity, particularly in the pH 5–8 range.

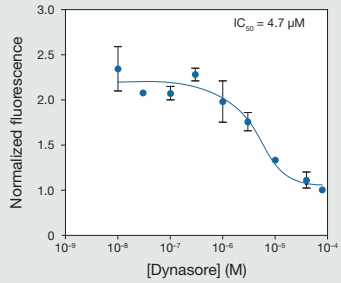


Figure 2. Tracking endocytosis inhibition with pHrodo™ dextran conjugates. HeLa cells were plated in 96-well format and treated with dynasore for 3 hr at 37°C prior to the pHrodo™ assay. Then, 40 µg/mL of pHrodo™ dextran synthesized from pHrodo™ succinimidyl ester (Cat. no. P36600) and amine-reactive 10 kDa dextran (Cat. no. D1860) was incubated for 30 min at 37°C. Cells were stained with HCS NuclearMask™ Blue Stain (Cat. no. H10325) for 10 min to reveal total cell number and demarcation for image analysis. Images were acquired on the BD Pathway™ 855 High-Content Bioimager (BD Biosciences).

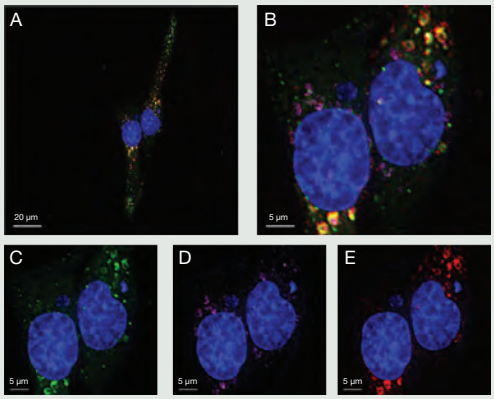


Figure 3. Multiplexed analysis with pHrodo™ dextran conjugate. (**A**) HeLa cells were transduced with Organelle Lights™ Endosome-GFP (Cat. no. O10104). The following day, the medium was replaced with serum-free medium plus 50 µM deferoxamine. After 24 hr, the cells were washed and incubated with 1 µg/mL Hoechst 33342 (Cat. no. H3570), 10 µg/mL pHrodo™ dextran, and Alexa Fluor® 647 transferrin (Cat. no. T23366) for 5 min at 37° C before imaging. (**B**) Magnified perinuclear regions of the cells in (**A**). (**C, D, E**) Organelle Lights™ Endosome-GFP, pHrodo™ 10kDa dextran, and Alexa Fluor® 647 transferrin, respectively.

Visualize spatial and temporal aspects of phagocytosis

The ability to track the extent of drug-induced changes in phagocytosis over time in high-throughput format is highly valuable to the drug discovery process (Figures 4 and 5). We used a known inhibitor of phagocytosis, cytochalasin D, to construct dose response curves using pHrodo™ BioParticles®: pHrodo™ dye conjugated to heat-inactivated *E. coli* or *S. aureus* bacteria. Analysis was performed in fixed cells, allowing archiving of plates and subsequent re-scanning. This assay can be used with compound libraries in 96- or 384-well format for screening of drug-induced alteration of phagocytosis.

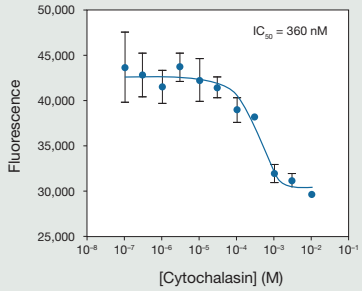


Figure 5. Application of pHrodo™ BioParticles® to high-throughput imaging of phagocytosis. Cells were treated with various concentrations of cytochalasin D for 2 hr prior to addition of the dye conjugates. Cells were incubated in the presence of 500 µg/mL pHrodo™ *E. coli* BioParticles® conjugate (Cat. no. P35361) for 2 hr at 37°C. Cells were washed in PBS before fixation in 4% formaldehyde. Data were acquired using the Acumen eX3 high-content imaging platform and provided courtesy of Paul Wylie, TTP LabTech, Royston, UK.

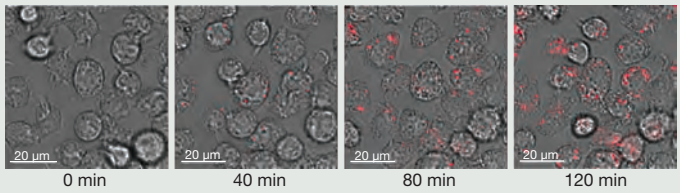


Figure 4. Time course of pHrodo™ BioParticles® uptake by MMM cells. Cells plated in glass-bottom 96-well plates were imaged at 37°C in the continued presence of 100 µg/mL pHrodo™ BioParticles®. Uptake of pHrodo™ BioParticles® can be seen as early as 20 min and reaches a plateau within 2–3 hr.

pHrodo™ indicators—enlightening internalization pathways

pHrodo™ dextran and pHrodo™ BioParticles® conjugates enable critical insights into the biology of membrane internalization, and can be used in automated imaging applications to probe compounds that alter endocytosis or phagocytosis in mammalian cells. Furthermore, pHrodo™ conjugates can be multiplexed with other fluorophores to enable multiparametric analysis of events related to membrane internalization and trafficking. Learn more about pHrodo™ indicators at **www.invitrogen.com/bp59**. ■

Product	Quantity	Cat. no.
pHrodo™ *S. aureus* BioParticles® conjugate for phagocytosis	5 x 2 mg	A10010
pHrodo™ *E. coli* BioParticles® Phagocytosis Kit, for flow cytometry	100 tests	A10025
pHrodo™ Phagocytosis Particle Labeling Kit, for flow cytometry	100 tests	A10026
pHrodo™ *E. coli* BioParticles® conjugate for phagocytosis	5 x 2 mg	P35361
pHrodo™, succinimidyl ester (pHrodo™, SE)	1 mg	P36600
Organelle Lights™ Endosomes-GFP	1 kit	O10104
Transferrin from human serum, Alexa Fluor® 647 conjugate	5 mg	T23366
Hoechst 33342, trihydrochloride, trihydrate, 10 mg/mL solution in water	10 mL	H3570
HCS NuclearMask™ Blue stain, for 10 x 96-well plates, 1,000X concentrate	65 µL	H10325
Dextran, amino, 10,000 MW	1 g	D1860

Take advantage of brighter fluorogenic readouts for HRP-based ELISAs

AMPLEX® ULTRARED REAGENT.

The fundamental technology behind enzyme-linked immunosorbent assays (ELISAs) has remained unchanged for decades, but detection options continue to expand and improve. ELISAs differ in the enzyme used to report, or detect, bound antibody and in the substrate used. Many substrate options are available for horseradish peroxidase (HRP), the most popular and reliable enzyme. Suitable for absorbance-based assays, the chromogen 3,3´,5,5´-tetramethylbenzidine (TMB) yields a blue color when oxidized by hydrogen peroxide (catalyzed by HRP), with major absorbance peaks at 370 nm and 652 nm. The color then changes to yellow on the addition of sulfuric or phosphoric acid, with maximum absorbance at 450 nm. TMB-based absorbance assays provide a reliable, inexpensive readout that is a vast improvement over older colorimetric substrates like 2,2-azino-di(3-ethylbenzothiazoline) sulfonic acid (ABTS) or diaminobenzidine (DAB). However, more sensitive fluorescent subtrates offer improved signal strength and quantitation over a larger analyte range.

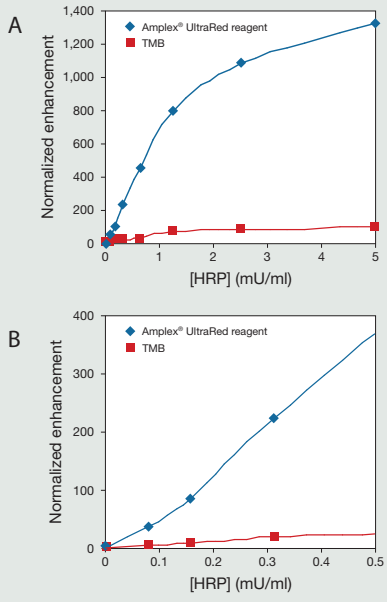


Figure 1. Enhanced signal in ELISA using Amplex® UltraRed reagent. Dilution series of (**A**) 0–5 mU HRP and (**B**) 0–0.5 mU HRP demonstrates higher fluorescence signal with Amplex® UltraRed reagent than the absorbance signal attainable with the colorimetric HRP substrate TMB.

Brighter assays with Amplex® UltraRed reagent

Amplex® UltraRed reagent has many features similar to Amplex® Red reagent, but with a brighter signal. In the presence of HRP, both Amplex® Red and Amplex® UltraRed reagents react in a 1:1 stoichiometry with hydrogen peroxide to produce a highly fluorescent product with excitation at 571 nm and emission at 585 nm. These reagents are only fractionally more expensive than TMB, and although assays using them require a fluorescent plate reader, they offer a broader, more sensitive dynamic range—adding accuracy in sample-to-sample comparisons. A study comparing colorimetric readouts (e.g., absorbance with TMB) to fluorescence readouts (with Amplex® reagents) found a 5–6-fold increase in signal-to-noise ratio.[1] These gains from switching to fluorescent mode were considered vital to increased precision of the ELISAs, as well as allowing kinetic reads and a larger dynamic range.

The improved signal obtained by use of fluorescent Amplex® UltraRed reagent compared to TMB is shown in Figure 1, where the signal enhancement for Amplex® UltraRed reagent is clearly superior to that attainable with TMB.

Improved signal strength by changing readout pH

At all pH values, Amplex® UltraRed reagent is brighter than Amplex® Red reagent, with the greatest gain obtained by shifting the readout conditions to pH 6 (FIgure 2). As can be seen from an HRP dilution series (Figure 2A), Amplex® UltraRed reagent, when used in pH 6 buffer, had greatly improved signal gain and sensitivity relative

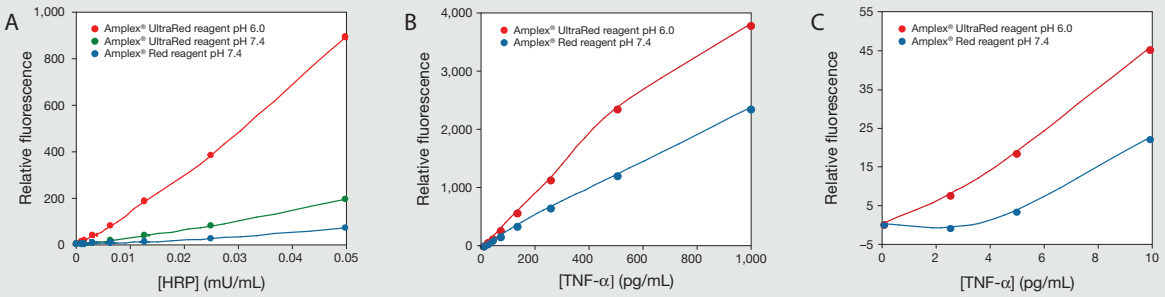


Figure 2. Comparison of signals obtained with Amplex® Red and Amplex® UltraRed reagents. (**A**) In a dilution series with HRP, both high- and low-end signal gain were superior using Amplex® UltraRed reagent at pH 6 compared to either Amplex® UltraRed or Amplex® Red reagent at pH 7.4. Detection of high (**B**) and low (**C**) concentrations of TNF-α in ELISA format (KRC3011) shows the increased signal and sensitivity obtained by switching the assay to use of Amplex® UltraRed reagent at pH 6. All assays were measured at 15 min.

to Amplex® Red reagent at pH 7.4 (with a more modest improvement in signal observed with Amplex® UltraRed reagent at pH 7.4). Though less dramatic, signal gain was also seen in ELISAs designed to detect TNF-α, at both high and low concentrations of TNF-α. In these assays, the overall dynamic range, signal enhancement, and sensitivity achieved with Amplex® UltraRed reagent were superior (Figure 2A and 2B).

Amplex® UltraRed reagent not only is brighter than Amplex® Red reagent, but also produces a more stable reaction product (Amplex® UltroxRed product) than Amplex® Red reagent (resorufin). In addition, when Amplex® Red/UltraRed stop reagent is used, ELISA plates can be stored for up to 24 hours and read later with little signal loss.

Adapting assays to Amplex® UltraRed reagent

We offer stand-alone reagents or kits to optimize your existing HRP-based ELISA assays for Amplex® UltraRed reagent or to create your own ELISAs. For existing TMB-based ELISAs, the conversion to Amplex® UltraRed reagent requires merely swapping the reagents and adjusting the pH, followed by detection on a fluorescent plate reader. Amplex® ELISA development kits provide a comprehensive set of components for creating an ELISA based on either absorbance or fluorescence using a mouse or rabbit primary antibody. For more information on tailoring assays to take advantage of the signal strength of Amplex® UltraRed reagent, visit **www.invitrogen.com/bp59**. ■

Reference

1. Meng, Y. et al. (2005) *Anal Biochem* 345:227–236.

Product	Quantity	Cat. no.
Amplex® UltraRed reagent	5 x 1 mg	A36006
Amplex® Red reagent	5 mg	A12222
Amplex® Red/UltraRed stop reagent	1 set of 5 vials, 500 tests	A33855
Amplex® ELISA Development Kit for Rabbit IgG with Amplex® UltraRed reagent	500 assays	A33851
Amplex® ELISA Development Kit for Mouse IgG with Amplex® UltraRed reagent	500 assays	A33852

Isolation and expansion of mouse Treg cells using Dynabeads® technology

DYNABEADS® FLOWCOMP™ MOUSE CD4$^+$CD25$^+$ TREG CELLS.

Regulatory T (Treg) cells are defined by their functional ability to suppress immune responses, which act to regulate and maintain homeostasis within the immune system. Interest in Treg cells continues to grow, based on evidence from experimental mouse and human models demonstrating that the immunosuppressive potential of these cells can be used in the treatment of diseases such as autoimmunity, infectious diseases, and cancer.[1–7] One of the hurdles in mouse Treg cell research is to obtain sufficiently high recovery of pure, functional cells after isolation. Treg cells are phenotypically CD4$^+$CD25$^+$Foxp3$^+$, a subset of the CD4 population that is only 3–10% of the total.[8,9] Thus, a good isolation method is critical to obtain cells with high recovery, high purity, and true Treg functionality. Isolation kits that incorporate Dynabeads® technology have led to improved recovery and purity of various cell types, and also facilitate related studies due to their common platform. For mouse Treg applications, the Dynabeads® FlowComp™ Mouse CD4$^+$CD25$^+$ Treg Cells kit isolates pure and functional Treg cells that can be easily expanded up to 10-fold using Dynabeads® Mouse T-Activator CD3/CD28.

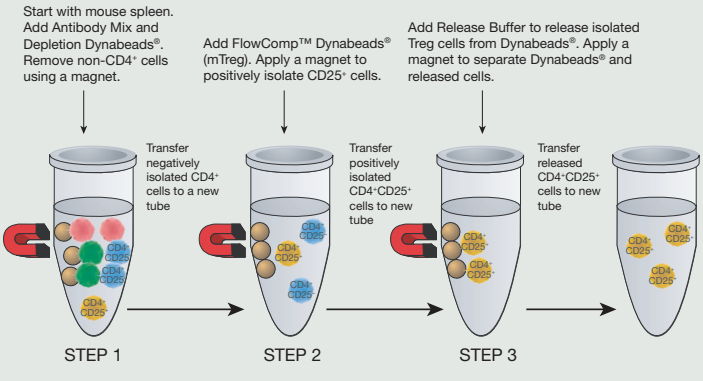


Figure 1. Isolation of CD4$^+$CD25$^+$ Treg cells from secondary lymphoid organs with Dynabeads® FlowComp™ Mouse CD4$^+$CD25$^+$ Treg Cells. Mouse CD4$^+$CD25$^+$ Treg cells are obtained by negative isolation of CD4$^+$ T cells followed by positive isolation of CD25$^+$ cells and bead release using FlowComp™ Release Buffer. The isolated cells are pure and bead-free.

Isolated Treg cells are phenotypically correct

Highly pure (≥90%) regulatory CD4$^+$CD25$^+$ T cells were isolated from mouse secondary lymphoid organs (i.e., spleen and lymph nodes) using Dynabeads® FlowComp™ Mouse CD4$^+$CD25$^+$ Treg Cells (Figure 1). The isolated Treg cells were phenotypically correct. A large majority of these cells express the Foxp3 transcription factor (avg ≥88%).[10] Cells isolated in this manner contain a significantly higher number of Foxp3$^+$ cells than those obtained by column-based isolation technology (Figure 2).

Treg population expansion for functional and *in vivo* transfer studies

To expand the CD25$^+$ Treg population, Dynabeads® Mouse T-Activator CD3/CD28 was added to 1 x 10^6 cells/mL (2 beads/cell) for 10 days and the cultures were supplemented with 1,000 U/mL of IL-2. More than 50% of the expanded cells showed Foxp3 expression after expansion in this manner (Figure 2). They are thus suitable for functional and adoptive transfer experiments.

Isolated Treg cells retain their suppressive capacity

To assay suppressive capacity, CD4$^+$CD25$^-$ effector T cells were stained with carboxyfluorescein diacetate succinimidyl ester (CFSE) and mixed with CD4$^+$CD25$^+$ Treg cells. Dynabeads® coated with anti–mouse CD3 (3 beads/cell) were added to activate the CD4$^+$CD25$^-$ effector T cells, and suppression of their proliferation was measured 4 days later. Treg cells suppressed the proliferation of CD4$^+$CD25$^-$ effector T cells in the presence of CD3 activation up to 60% (Figure 3), showing that the isolated Treg cells retained their normal function.

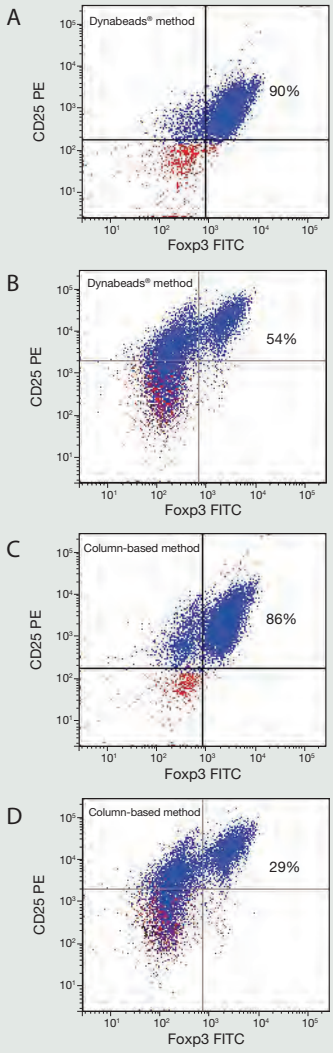


Figure 2. Expansion of isolated regulatory T cells. Treg cells isolated with Dynabeads® FlowComp™ Mouse $CD4^+CD25^+$ Treg Cells can be activated with Dynabeads® Mouse T-Activator CD3/CD28 and expanded up to 10-fold while retaining their Treg phenotype. Foxp3 and CD25 expression by Treg cells isolated with Dynabeads® technology before expansion (**A**) and after 12 days of expansion (**B**) shows that these cells retained the majority of Foxp3-expressing cells after expansion. In contrast, comparison of Treg cells isolated with column-based technology before (**C**) and after expansion (**D**) shows loss of the majority of Foxp3-expressing cells.

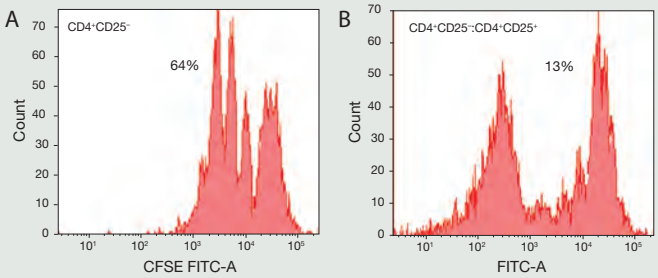


Figure 3. Suppressive capacity of isolated Treg cells. $CD4^+CD25^-$ cells were stained with CSFE and stimulated with Dynabeads® coated with anti-mouse CD3 (3 beads/cell) for 4 days. On day 4, in the absence of $CD4^+CD25^+$ Treg cells, 64% of the cells were dividing as identified by flow cytometry (**A**), but in the presence of $CD4^+CD25^+$ Treg cells at a 1:1 ratio, only 13% of all $CD4^+CD25^-$ cells were dividing and 60% suppression of cell division was achieved (**B**). Unstained $CD4^+CD25^+$ cells are shown in light red.

Facilitating studies in the mouse model system and beyond

Dynabeads® FlowComp™ Mouse $CD4^+CD25^+$ Treg Cells can be used to isolate ≥95% pure $CD4^+CD25^+$ Treg cells, and >80% of the isolated $CD25^+$ cells routinely express the Foxp3 transcription factor. Dynabeads® Mouse T-Activator CD3/CD28 can expand human $CD4^+CD25^+$ Treg cells up to 10-fold during 2 weeks of culture while retaining their expression of CD25 and Foxp3. Such expansion will facilitate further characterization of Treg cells in functional and *in vivo* transfer studies, as well as evaluation of their potential in clinical applications. For more information on products and technologies for T cell research, visit www.invitrogen.com/bp59. ■

References

1. Curiel, T.J. et al. (2004) *Nat Med* 10:942–949.
2. Cohen, J.L. et al. (2002) *Exp Med* 196:401–406.
3. Edinger, M. et al. (2003) *Nat Med* 9:1144–1150.
4. Taylor, P.A. et al. (2002) *Blood* 99:3493–3499.
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7. Kukreia, A. et al. (2002) *J Clin Invest* 109:131–140.
8. Sakaguchi, S. (2004) *Annu Rev Immunol* 22:531–562.
9. Shevac, E.M. (2002) *Nat Rev Immunol* 2:389–400.
10. Ökern, G. et al. (2007) https://tools.invitrogen.com/content/sfs/posters/Mouse-Treg-poster-040608.pdf.

Product	Application	Quantity	Cat. no.
Dynabeads® FlowComp™ Mouse $CD4^+CD25^+$ Treg Cells	Positive isolation of mouse Treg cells	Processes ~1 x 10^9 cells	114-63D
Dynabeads® Mouse T-Activator CD3/CD28 for physiological activation of mouse T cells	Expansion of mouse T cells and Treg cells	0.4 mL 2 mL 10 mL	114-56D 114-52D 114-53D
CD25, Rat Anti-Mouse (Alexa Fluor® 488)	Flow cytometry staining antibody for CD25	1 mL	RM6020
CD4, Rat Anti-Mouse (R-PE)	Flow cytometry staining antibody for CD4	0.5 mL	MCD0404
CellTrace™ CFSE Cell Proliferation Kit	Cell proliferation kit for flow cytometry	1 kit	C34554

High-content analysis of cytotoxicity

MOLECULAR PROBES® TOOLS MEET THE ACUMEN® eX3 PLATFORM.

Toxicological profiling is a key component of both drug discovery and drug safety. Cell-based assessment of toxicity is best served by a multiparametric approach in order to establish both phenotypic and mechanistic information and, ultimately, to make decisions about drug development. Using ready-to-use kits and Molecular Probes® reagents developed by Invitrogen for high-content screening (HCS), researchers can perform simultaneous interrogation of multiple aspects of cytotoxicity and cell health, including apoptosis, cell proliferation, cell viability, DNA damage, and mitochondrial health. Coupling these tools with instruments such as the next-generation laser-scanning Acumen® eX3 microplate cytometer from TTP LabTech enables cytotoxicity data to be captured from every cell in every well, while generating throughputs of >300,000 wells per day. In this article, we demonstrate that a number of key cytotoxic phenomena can be analyzed using Invitrogen HCS kits on the Acumen® eX3 platform for fast, robust, and high-throughput analysis of cytotoxicity.

Robust, quantitative analysis of cell viability

To demonstrate the use of the Acumen® eX3 for high-throughput cell viability analysis, HeLa cells were treated with staurosporine, and the HCS LIVE/DEAD® Green Kit was used for the automated detection of cell viability on the Acumen® eX3 platform (Figure 1). The Image-iT® DEAD Green™ viability stain, a component of the kit, labels only cells with compromised plasma membranes and is amenable to fixation and permeabilization, enabling multiplexing with other markers of cytotoxicity or antibody-based target detection. HCS NuclearMask™ Deep Red stain is included in this kit as a total cell stain and while optimally excited at 633 nm, may also be adequately excited at 488 nm.

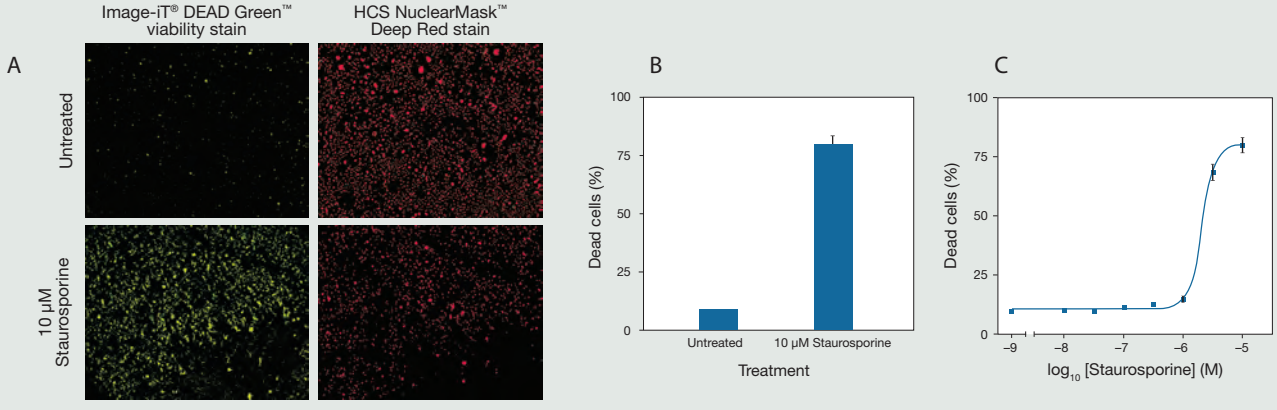


Figure 1. Cell viability analysis using Image-iT® DEAD Green™ viability and HCS NuclearMask™ Deep Red stains on the Acumen® eX3 microplate cytometer. HeLa cells were treated with 10 µM staurosporine for 4 hr, then labeled with Image-iT® DEAD Green™ viability stain and HCS NuclearMask™ Deep Red stain and scanned on the Acumen® eX3 microplate cytometer. (**A**) Treated cells showed bright green fluorescence labeling compared to untreated control cells. (**B**) This labeling, along with staining of the total cell population with HCS NuclearMask™ Deep Red stain, enabled quantitation of the percentage of dead cells in the staurosporine-treated population. (**C**) The dose response curve was used to calculate an EC_{50} value of 2 µM (mean ± SEM; n=3) for staurosporine. Data contributed by Dr. Paul Wylie, TTP LabTech.

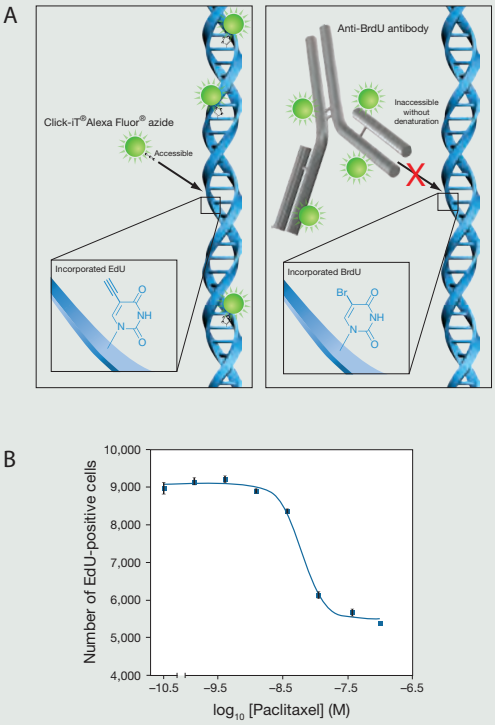


Figure 2. The Click-iT® EdU Proliferation Assay on the Acumen® eX3 microplate cytometer. (**A**) Comparison of assay methodology between the Click-iT® EdU method and the BrdU method. (**B**) Dose response curve for paclitaxel with EdU-positive cells, which was used to calculate an IC_{50} of 6 nM (mean ± SEM; n=5). Data contributed by Dr. Paul Wylie, TTP LabTech.

A superior alternative to BrdU for high-content cytotoxicity screening

Traditionally, analysis of cell proliferation has been performed by incorporating the nucleoside analog bromodeoxyuridine (BrdU) into DNA, followed by detection with an anti-BrdU antibody. Although effective, this method requires DNA denaturation (using HCl, heat, or DNase) to expose the BrdU for antibody access—a step that can be lengthy and difficult to perform consistently, and may adversely affect the sample.[1] The Click-iT® EdU cell proliferation assay eliminates the need to denature DNA, providing a superior alternative to the standard BrdU antibody-based method for measuring nascent DNA synthesis and cell proliferation. Furthermore, Click-iT® EdU cell proliferation assays can be easily performed on the Acumen® eX3 platform (Figure 2), demonstrating its use as a valuable tool for cell health and cytotoxicity studies.

Get a comprehensive view of cytotoxicity

The strength of the Acumen® eX3 platform, combined with robust Invitrogen HCS kits and reagents, enables the high-throughput analysis of a wide variety of parameters for cytotoxicity. The Acumen® eX3's multi-laser excitation and ability to acquire up to 4 channels of data per laser complements these innovations in fluorescence labeling and detection. For more information on Molecular Probes® kits and reagents for HCS, including a reagent selection guide and posters featuring more assays validated on the Acumen® eX3 platform, visit www.invitrogen.com/bp59. ■

References

1. Young, D. et al. (2008) *Nat Chem Biol* 4:59–68.

Product	Quantity	Cat. no.
HCS LIVE/DEAD® Green Kit, 2-plate size	1 kit	H10290
Image-iT® DEAD Green™ viability stain, 1 mM solution in DMSO	25 µL	I10291
HCS NuclearMask™ Blue stain, for 10 x 96-well plates	65 µL	H10325
HCS NuclearMask™ Red stain, for 10 x 96-well plates	65 µL	H10326
HCS NuclearMask™ Deep Red stain, 250X concentrate in DMSO	400 µL	H10294
HCS CellMask™ Blue stain, for 10 x 96-well plates	1 set	H32720
HCS CellMask™ Green stain, for 10 x 96-well plates	1 set	H32714
HCS CellMask™ Orange stain, for 10 x 96-well plates	1 set	H32713
HCS CellMask™ Red stain, for 10 x 96-well plates	1 set	H32712
HCS CellMask™ Deep Red stain, for 10 x 96-well plates	1 set	H32721
Click-iT® EdU Alexa Fluor® 488 High-Throughput Imaging (HCS) Assay	1 kit, 2 plates 1 kit, 10 plates	A10027 A10028
Click-iT® EdU Alexa Fluor® 594 High-Throughput Imaging (HCS) Assay	1 kit, 2 plates 1 kit, 10 plates	A10209 C10081
Click-iT® EdU Alexa Fluor® 647 High-Throughput Imaging (HCS) Assay	1 kit, 2 plates 1 kit, 10 plates	A10208 C10082
Click-iT® AHA Alexa Fluor® 488 Protein Synthesis HCS Assay	1 kit	C10289
HCS DNA Damage Kit, 2-plate size	1 kit	H10292
Mitochondrial Health Kit, 2-plate size	1 kit	H10295
HCS Mitotic Index Kit, 2-plate size	1 kit	H10293
HCS LipidTOX™ Phospholipidosis and Steatosis Detection Kit	1 kit, 2 plates 1 kit, 10 plates	H34157 H34158
HCS LipidTOX™ Green phospholipidosis detection reagent, 1,000X aqueous solution, 10-plate size	1 each	H34350
HCS LipidTOX™ Red phospholipidosis detection reagent, 1,000X aqueous solution, 10-plate size	1 each	H34351
HCS LipidTOX™ Green neutral lipid stain, solution in DMSO	1 each	H34475
HCS LipidTOX™ Red neutral lipid stain, solution in DMSO	1 each	H34476
HCS LipidTOX™ Deep Red neutral lipid stain, solution in DMSO	1 each	H34477

Improve your image

TWO EASY STEPS TO REDUCE BACKGROUND NOISE AND PRESERVE SIGNAL STRENGTH.

For high signal-to-noise ratios in your imaging experiments, we offer two quick and easy solutions. First, to reduce background signals due to nonspecific binding, consider our proprietary Image-iT® FX Signal Enhancer. Second, to preserve signal strength, use our superior ProLong® Gold antifade reagents.

Reducing nonspecific binding of dye-labeled reagents:

The unique Image-iT® FX Signal Enhancer is highly effective at blocking background staining that results from nonspecific interactions of a wide variety of fluorescent dyes with cell and tissue constituents. Background staining seen with fluorescent conjugates of streptavidin, goat anti–mouse IgG antibodies, or goat anti–rabbit IgG antibodies is largely eliminated when Image-iT® FX Signal Enhancer is applied to fixed and permeabilized cells prior to staining. Background reduction can be dramatic when confronting highly problematic tissues like brain samples rich in myelin (Figures 1A and 1B). It is important to note that Image-iT® Signal Enhancer is not a substitute for standard blocking reagents, which should still be used to minimize protein–protein interactions.

Signal preservation with superior antifade reagents

Although great effort is made in preparing fluorescently labeled samples for imaging, the last step—mounting the coverslip to the

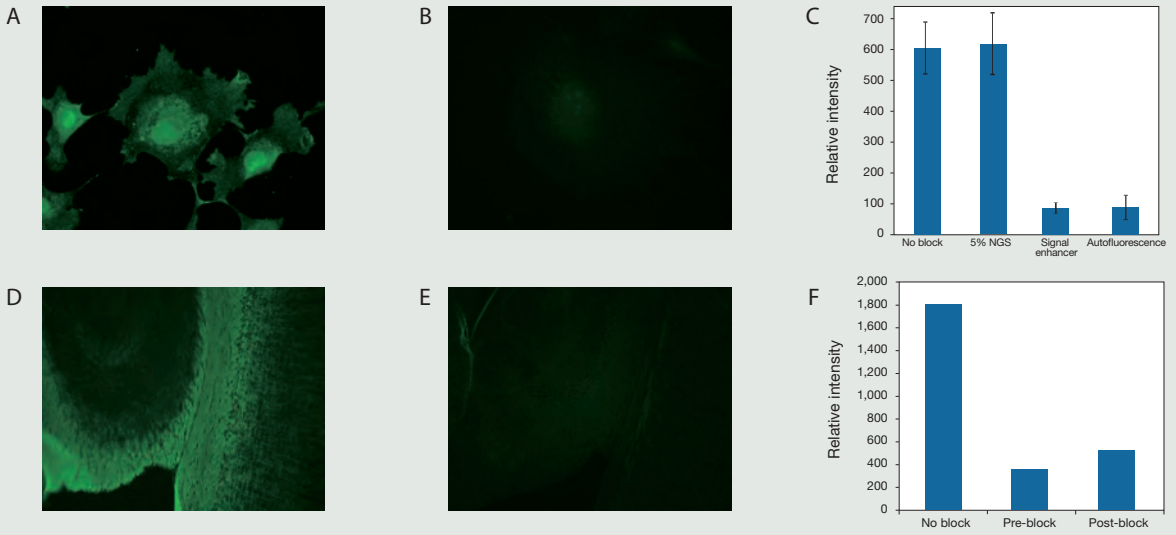


Figure 1. Performance of Image-iT® FX Signal Enhancer. Fixed and permeabilized BPAE cells were labeled with 5 µg/mL Alexa Fluor® 488 goat anti-rabbit secondary antibody and no primary antibody. Cells that were not blocked (**A**) exhibited substantial on-cell background. Cells blocked with Image-iT® FX Signal Enhancer (**B**), however, showed no significant difference from autofluorescence (**C**). Adjacent mouse brain cryosections were rehydrated, permeabilized, and treated for antigen retrieval, then labeled with 5 µg/mL Alexa Fluor® 488 goat anti-mouse secondary antibody and no primary antibody. Sections that received no Image-iT® FX Signal Enhancer treatment (**D**) showed considerable nonspecific labeling of white matter, while treated sections showed near-complete blocking of nonspecific labeling (**E**), and appeared more than four times dimmer (**F**).

Point, click, and learn

Join us from the comfort of your desk for a series of free, live, online technical seminars covering topics in cell biology and fluorescence imaging. Our scientists host a new seminar every two weeks, each focusing on imaging-related applications. We also welcome your suggestions for additional topics in cell biology. The presentations last approximately 45 minutes, followed by 15 minutes for live Q&A.

Upcoming seminars will be announced each month by email. If you would like to be notified of our upcoming seminars, sign up at **www.invitrogen.com/imaging-edu**.

To view a complete list of our scheduled online seminars and to access recordings of previous presentations, visit **www.invitrogen.com/imaging-webinars**.

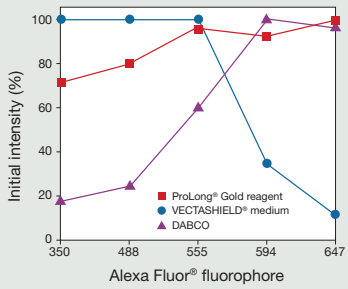


Figure 2. ProLong® Gold Antifade Reagent outperforms competitors' antifades across the visible spectrum. Competitors' reagents preserve signal strength only at specific wavelengths, whereas ProLong® Gold Reagent offers stability across the entire spectrum.

microscope slide—can have the most impact. To perform this step with anything less than the best antifade mounting medium is to compromise the hours and sometimes weeks that go into sample creation. Signal stability is critical not just for image quality, but also for improved quantitation and sample-to-sample comparisons. Molecular Probes® antifade mounting reagents are unsurpassed for signal preservation—both initial and long term—for the widest range of dyes possible. One of these reagents, ProLong® Gold Antifade Reagent, was found to be better than other mountants for quantitative immunohistochemistry [1] and superior for general imaging (Figure 1). For details, see **www.biocompare.com/ProductReview.aspx?r=1044**.

Knowing that you need a mountant is different from knowing which mountant to use. Table 1 lists the characteristics of our current line of antifade reagents. Our ProLong® Gold and *SlowFade*® Gold reagents offer the ultimate in antifade performance without impacting a dye's initial fluorescence (Figure 2). For your convenience, they come with or without DAPI and in different packaging formats. Please note that Qdot® reagents are not compatible with these mounting reagents. For customers still using our original ProLong® and *SlowFade*® antifade products, we strongly recommend that you upgrade to ProLong® Gold or *SlowFade*® Gold Antifade Reagent to take advantage of the ease of use, improved antifade properties, and cost-effectiveness of these reagents.

Two ways to improve your image

For a quick and easy way to higher signal-to-noise ratios, consider using both Image-iT® FX Signal Enhancer and ProLong® Gold Antifade Reagent or *SlowFade*® Gold Antifade Reagent for all of your fixed samples, especially those that require long exposures involving rare event detection. Start improving your image at **www.invitrogen.com/bp59**. ■

Reference

1. Huang, D. et al. (2007) *Cancer Epidemiol Biomarkers Prev* 16:1371–1381.

Table 1—Product selection table.

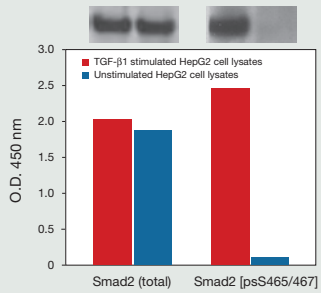
Product	Description	Quantity	Cat. no.		Notes
Image-iT® FX Signal Enhancer		10 mL	I36933		Largely eliminates background staining
			Without DAPI	With DAPI	
ProLong® Gold Antifade Reagent	Curing	10 mL	P36930	P36931	Best antifade performance; cure overnight at room temperature
		5 x 2 mL	P36934	P36935	
SlowFade® Gold Antifade Reagent	Non-curing	10 mL	S36936	S36938	Ideal for thick samples and for short-term or immediate use
		5 x 2 mL	S36937	S36939	

SMAD2 AND β-CATENIN ELISA KITS

The SMAD2 [pSpS465/467] ELISA Kit and SMAD2 (Total) ELISA Kit are designed for quantitative measurements of Smad2 in cell lysates. SMAD2, one of the key components in TGF-β pathways, carries signals from the cell surface directly to the nucleus. In response to TGF-β, Smad2 is phosphorylated on COOH-terminus Ser465/467 by activin type 1 receptor kinases, disassociates from the receptor complex, and accumulates in the nucleus. In the nucleus, Smad2 can target a variety of DNA binding proteins to regulate gene transcription and expression.

The β-Catenin (Total) ELISA Kit provides quantitative measurements of β-catenin in cell lysates. β-catenin is a cytoplasmic protein that is the major player in the canonical Wnt cascade. This pathway plays an essential role in normal development through the activation of many different transcriptional programs responsible for cell–cell interactions as well as cell growth and differentiation. β-catenin also plays a role as a component in the adherens junctions of epithelial cells by binding to the cytoplasmic tails of different cadherins to form a stabilizing interaction.

For a complete list of phosphoELISA™and ELISA kits, visit **www.invitrogen.com/elisa**.

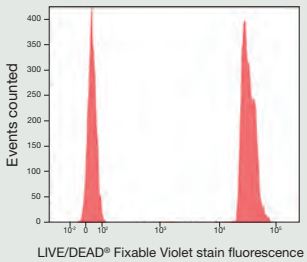


TGF-β1 stimulates phosphorylation of Smad2 at serine site 465/467 in HepG2 cells.

Product	Quantity	Cat. no.
SMAD2 [pSpS465/467] ELISA Kit	96 tests	KHO2011
SMAD2 (Total) ELISA Kit	96 tests	KHO2021
β-Catenin (Total) ELISA Kit	96 tests	KHO1211

ArC™ AMINE REACTIVE COMPENSATION BEAD KIT

The ArC™ Amine Reactive Compensation Bead Kit provides a consistent, accurate, and easy-to-use technique for setting flow cytometry compensation. The kit is specifically optimized for use with the LIVE/DEAD® Fixable Dead Cell Stain Kits, which label dead cells prior to intracellular staining. Two types of specially modified polystyrene microspheres are included in the ArC™ bead kit: ArC™ reactive beads, which bind the LIVE/DEAD® fixable stains, and ArC™ negative beads, which have no reactivity. After incubation with an amine-reactive dye such as a LIVE/DEAD® fixable stain, the two components provide distinct negative and positive bead populations that can be used to accurately set compensation. Learn more at **www.invitrogen.com/bp59**.

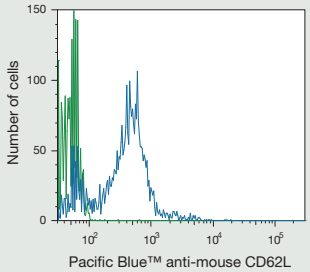


Staining profile of ArC™ Amine Reactive Bead Kit (Cat. no. A10346) components incubated with the LIVE/DEAD® Fixable Violet Dead Cell Stain Kit (Cat. no. V34955). ArC™ beads stained with the LIVE/DEAD® Fixable Violet Dead Cell Stain were analyzed by flow cytometry.

Product	Quantity	Cat. no.
ArC™ Amine Reactive Compensation Bead Kit	1 kit	A10346

ANTIBODY CONJUGATES FOR FLOW CYTOMETRY

Invitrogen's rapidly expanding menu of directly conjugated primary antibodies includes a variety of colors to help maximize multicolor analysis by flow cytometry. These antibodies are useful in studies of complex biological questions relating to cancer, immunology, stem cell research, and activation studies. The antibodies allow simultaneous measurement of different specificities, revealing more information about individual cells with less time and less sample. To view a complete list of new antibodies for flow cytometry, visit **www.invitrogen.com/bp59**.



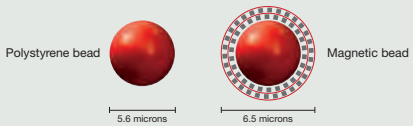
Flow cytometry analysis using anti-mouse CD62L. C57BL/6 mouse spleen cells were stained with Pacific Blue™ Rat Anti-Mouse CD62L (Cat. no. RM4328, blue line) and gated on cells positive for mouse CD3, which were detected with Hamster Anti-Mouse CD3 (Cat. no. HM3401). The green line represents spleen cells with isotype control.

RESEARCH ANTIBODIES AND ASSAYS

New research antibodies and assays are released each month. For a complete list of products validated for western blotting, immunofluorescence, flow cytometry, and more, visit **www.invitrogen.com/bp59**.

INFLAMMATORY CYTOKINE MOUSE MAGNETIC 4-PLEX PANEL

The Inflammatory Cytokine Mouse Magnetic 4-Plex Panel provides simultaneous measurement of four mouse cytokines: GM-CSF, IL-1β, IL-6, and TNF-α. The assay uses magnetic beads that are internally labeled with fluorescent dyes and have magnetite encapsulated in a functional polymer outer coat; the beads offer completely automatable washing and setup without vacuum filtration. The magnetic 4-plex panel was carefully designed and tested for maximum sensitivity, range, and correlation. The assay is performed in a 96-well plate format and analyzed with a Luminex® instrument, which monitors the spectral properties of the capture beads while simultaneously measuring the quantity of associated fluorophore. Standard curves generated with this assay extend over several orders of magnitude of concentrations, and the sensitivity and quantitation capability of the assay is comparable to that of ELISAs. Assay standards are calibrated to NIBSC reference preparations, when available, to assure accurate and reliable results. Learn more about the magnetic bead panel at **www.invitrogen.com/bp59**.



Schematic representation of polystyrene and magnetic beads. The encapsulated magnetite layers around the polystyrene core account for the larger size of the magnetic bead.

Product	Quantity	Cat. no.
Inflammatory Cytokine Mouse Magnetic 4-Plex Panel	100 tests	LMC0003M

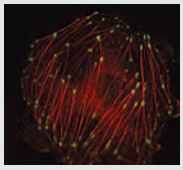
FXCYCLE™ STAINS FOR FLOW CYTOMETRY

FxCycle™ Far Red stain and FxCycle™ Violet stain provide measurements of DNA content in fixed cells by flow cytometry. FxCycle™ Far Red stain uses the common 633/635 nm excitation sources, with emission ~660 nm; FxCycle™ Violet stain uses the popular 405/407 nm laser, with emission ~450 nm. FxCycle™ Far Red stain labels RNA as well as dsDNA, whereas FxCycle™ Violet stain is DNA selective, with no RNase required. The FxCycle™ stains free the common 488 nm laser and detection channels for other markers, making these dyes ideal for DNA content analysis in multicolor cell cycle studies.

Product	Quantity	Cat. no.
FxCycle™ Far Red stain	1 kit	F10348
FxCycle™ Violet stain	1 kit	F10347

CELLULAR LIGHTS™ REAGENTS FOR FOCAL ADHESIONS

Cellular Lights™ reagents use fluorescent protein–signal peptide fusions that provide accurate and specific targeting to subcellular structures in living cells for both live- and fixed-cell imaging applications. Their high spatial and temporal resolution enables unique and powerful insights into biological systems without modifying cell function. Cellular Lights™ Talin-GFP and Cellular Lights™ Talin-RFP, the newest additions to this powerful series of reagents, facilitate the visualization of focal adhesions via talin, a protein that links actin to the extracellular matrix. Learn more about Cellular Lights™ reagents and other tools based on this powerful technology at **www.invitrogen.com/bp59**, or see pages 18–19.

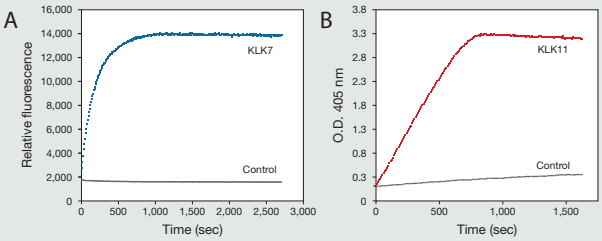


Live-cell imaging with Cellular Lights™ Talin-GFP. HeLa cells were transduced with Cellular Lights™ Talin-GFP (Cat. no. C10323) and Cellular Lights™ Actin-RFP (Cat. no. C10127). Imaging was performed on live cells using a Delta Vision Core microscope and standard FITC/TRITC filter sets.

Product	Quantity	Cat. no.
Cellular Lights™ Talin-GFP	1 kit	C10323
Cellular Lights™ Talin-RFP	1 kit	C10324

SERINE PROTEASES FOR INFLAMMATORY SKIN DISEASE AND CANCER RESEARCH

Kallikreins (KLK) are serine proteases with conserved catalytic regions, regulated in many cases by steroid hormones. At least 15 tissue kallikreins belong to a large multigene family on chromosome region 19q13.4. Kallikreins are of particular interest for their use as potential biomarkers for disease and their biological roles. Human KLK7 is found in the skin, CNS, kidney, breast, and many other tissues. Overexpression of KLK7 has been associated with ovarian carcinomas and skin diseases including pathological keratinization and psoriasis. Human KLK11 is expressed in the prostate gland and other tissues. Elevated serum levels of KLK11 were observed in a large percentage of individuals with ovarian or prostate cancer. Human KLK4 is expressed in human embryonic kidney 293 cells. Learn more about biologically active hKLK7, hKLK11, and hKLK4 at **www.invitrogen.com/bp59**.



Enzymatic activity of kallikrein proteins. (**A**) KLK7 enzymatic activity is measured by its ability to cleave a fluorogenic peptide substrate Mca-Arg-Pro-Lys-Pro-Val-Glu-Nval-Trp-Arg-Lys(Dnp)-NH2. Cleavage of this peptide can be measured using fluorescence spectroscopy. (**B**) KLK11 enzymatic activity is measured using a colorimetric peptide substrate D-Val-Leu-Lys-ThioBenzyl ester in the presence of DTNB and detected at a wavelength of 405 nm. The specific activity for KLK7 is >300 pmol/µg/min and KLK11 is >2200 pmol/µg/min.

Recently published

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

***In vivo* imaging: Characterization of Qdot® 800 nanocrystal–conjugated epidermal growth factor.** The near-infrared emission spectrum, strong fluorescence output, and extreme photostability of Qdot® 800 nanocrystals make them ideal optical markers for *in vivo* imaging of tumor-associated epidermal growth factor (EGF) receptor overexpression. Accordingly, researchers from the University of Texas M. D. Anderson Cancer Center used the Qdot® 800 Antibody Conjugation Kit to couple Qdot® 800 ITK amino (PEG) quantum dots to EGF sulfhydryl groups via the amine–thiol crosslinker SMCC. Intravenous injection of the Qdot® 800 EGF conjugate into immunocompromised mice bearing xenografted tumors resulted in tumor-specific fluorescence that reached maximal levels ~4–6 hours after administration and could be blocked by pretreatment with the anti-EGFR antibody cetuximab. Fluorescence representing nonspecific accumulation of the probe was primarily associated with the liver, spleen, and lymph nodes.

Diagaradjane, P., Orenstein-Cardona, J.M., Colón-Casasnovas, N.E., Deorukhkar, A., Shentu, S., Kuno, N., Schwartz, D.L., Gelovani, J.G., Krishnan, S. (2008) Imaging epidermal growth factor receptor expression *in vivo*: Pharmacokinetic and biodistribution characterization of a bioconjugated quantum dot nanoprobe. *Clin Cancer Res* 14:731–741.

Autoimmune pathology: Correlating CNS tissue damage with anti-apoptotic protein expression in a mouse model of multiple sclerosis. Promoting apoptosis of myelin-reactive immune cells in diseases such as multiple sclerosis (MS) may delay disease progression and decrease the frequency and severity of relapses. A team of researchers from Dalhousie University (Halifax, NS) used FluoroMyelin™ Green fluorescent myelin stain and DAPI in a mouse MS surrogate model to visualize demyelinated lesions and cellular infiltration into the spinal cord, respectively, and examined the relationship of these pathophenotypes to T cell expression of X-linked inhibitor of apoptosis (XIAP), a potent anti-apoptotic protein. Flow cytometric analysis with FITC annexin V was used to detect and quantify T cell apoptosis. Changes in IAP gene expression were analyzed by quantitative RT-PCR of total RNA isolated from whole blood or T cells using TaqMan® EZ RT-PCR Core Reagents and TaqMan® β-actin Detection Reagents.

Moore, C.S., Hebb, A.L., Robertson, G.S. (2008) Inhibitor of apoptosis protein (IAP) profiling in experimental autoimmune encephalomyelitis (EAE) implicates increased XIAP in T lymphocytes. *J Neuroimmunol* 193:94–105.

Virology: Imaging HIV translocation across virological synapses. The spread of HIV between immune cells is greatly enhanced by cell–cell adhesions called virological synapses. Seeking deeper insights into the underlying mechanisms, a research team from Mount Sinai School of Medicine, New York, and University of California, Davis, used real-time imaging of HIV incorporating green fluorescent protein (GFP)-tagged Gag proteins interacting with primary $CD4^+$ T cells labeled with CellTracker™ Orange CMTMR, CellTracker™ Orange CMRA, or CellTracker™ Blue CMF_2HC. Viral transfer events were observed to form virus-laden internal compartments within target cells, indicating that HIV dissemination may be enhanced by virological synapse-mediated cell adhesion coupled to viral endocytosis. Measurements of GFP expression in CellTracker™ Blue CMF_2HC–labeled target cells by flow cytometry were used to show that cell-associated infection is inhibited by cytochalasin D and is therefore actin-dependent.

Hübner, W., McNerney, G.P., Chen, P., Dale, B.M., Gordon, R.E., Chuang, F.Y., Li, X.D., Asmuth, D.M., Huser, T., Chen, B.K. (2009) Quantitative 3D video microscopy of HIV transfer across T cell virological synapses. *Science* 323:1743–1747.

Genetic toxicology: Click-iT® EdU detection of unscheduled DNA synthesis. Seeking improvements in methods based on detection of unscheduled DNA synthesis (UDS) for diagnosis of the autosomal recessive genetic disorder xeroderma pigmentosum (XP), researchers at the Atomic Bomb Disease Institute at Nagasaki University turned to the Click-iT® EdU Alexa Fluor® 488 Imaging Kit. The accuracy and resolution of the UDS assay based on incorporation of 5-ethynyl-2´-deoxyuridine (EdU) in primary human fibroblasts and chemoselective detection with Alexa Fluor® 488 azide was found to be comparable to conventional ^3H thymidine autoradiography and superior to BrdU immunodetection. Furthermore, the total time required to perform the assay was reduced dramatically compared to autoradiography and more modestly compared to the BrdU-based assay. Compatibility of the EdU assay with immunostaining was exploited to demonstrate that UDS could be detected in quiescent fibroblasts as well as proliferating populations using rabbit anti-ki-67 proliferation marker in combination with Alexa Fluor® 594 goat anti–rabbit IgG.

Limsirichaikul, S., Niimi, A., Fawcett, H., Lehmann, A., Yamashita, S., Ogi, T. (2009) A rapid non-radioactive technique for measurement of repair synthesis in primary human fibroblasts by incorporation of ethynyl deoxyuridine (EdU). *Nucleic Acids Res* 37:e31.

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