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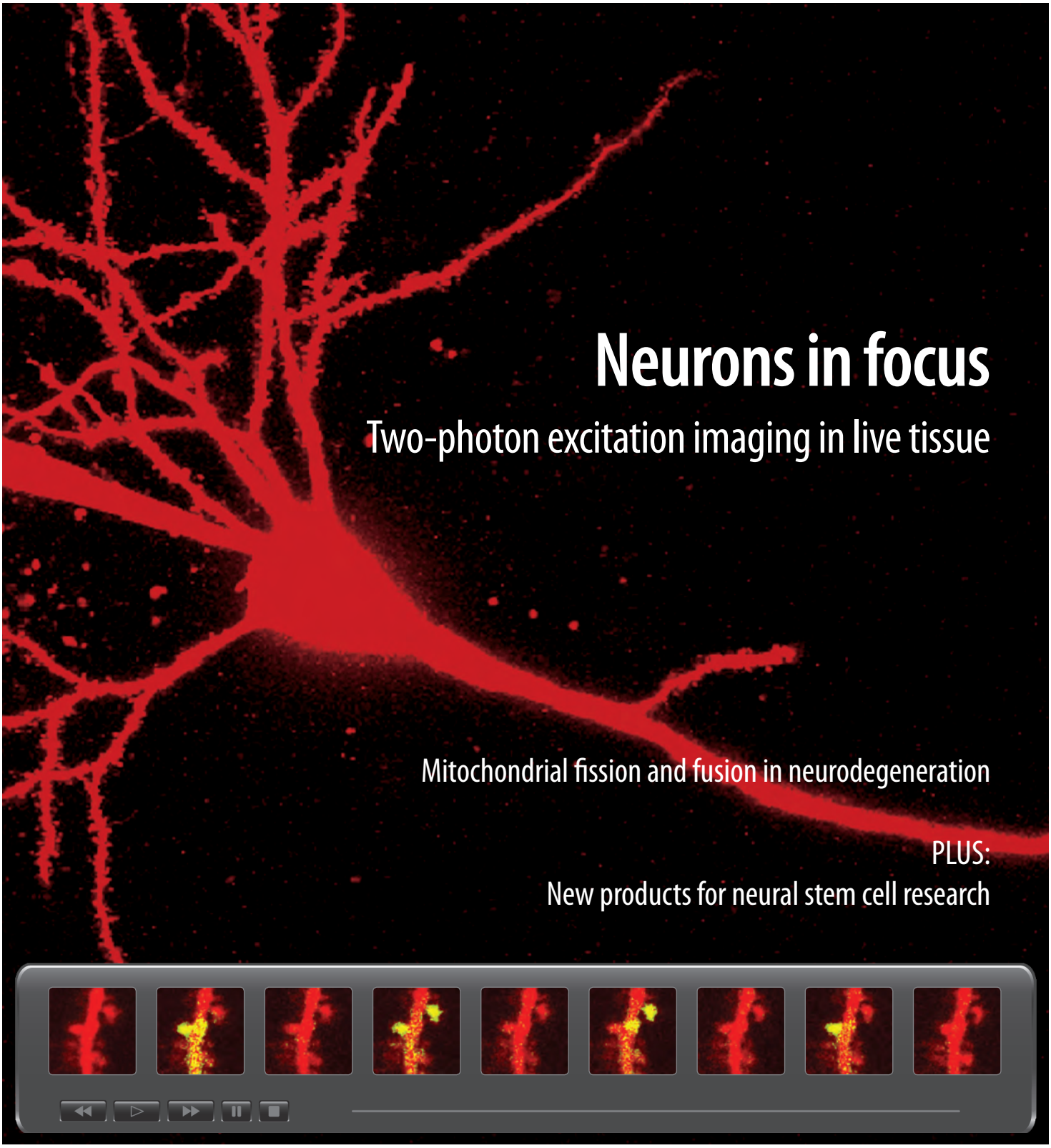

Neurons in focus

Two-photon excitation imaging in live tissue

Mitochondrial fission and fusion in neurodegeneration

PLUS:
New products for neural stem cell research

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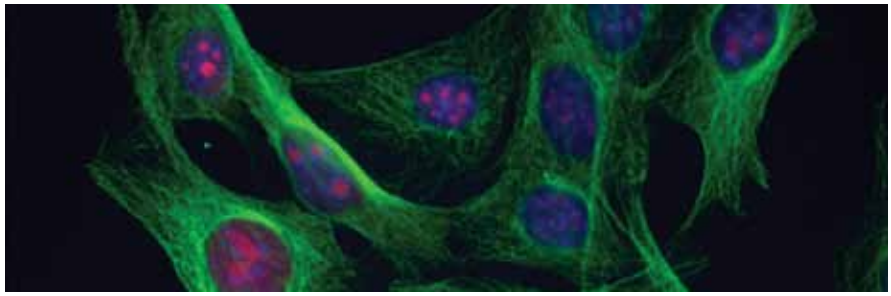

BioProbes 60

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A flow cytometry–based method for isolating neural stem cells

Pastrana E, Cheng LC, and Doetsch F (2009) Simultaneous prospective purification of adult subventricular zone neural stem cells and their progeny. *Proc Natl Acad Sci U S A* 106:6387–6392.

What biochemical characteristics distinguish neural stem cells from their nonproliferative neighbors? Despite their relative rarity, neural stem cells serve the vital function of generating new neurons to provide building blocks for neural circuitry—behavior that suggests vast therapeutic potential. In the adult mammalian brain, neural stem cells represent a subset of astrocytes that exhibit few cellular markers to distinguish them from nonproliferative astrocytes, making their isolation and study difficult. However, a subpopulation of stem cell astrocytes found in the subventricular zone (SVZ) has been shown to express the epidermal growth factor receptor (EGFR), providing a strategy for selective purification of activated stem cell astrocytes away from other SVZ neurons.

In this study, Pastrana and colleagues used Alexa Fluor® 647 dye–labeled EGF, combined with FACS analysis, to identify the activated stem cell subpopulation of SVZ astrocytes in mouse brain and successfully purify cells at different stages of the stem cell lineage (Figure 1). This $EGFR^+$ subpopulation was effectively eliminated by pretreatment of mouse brains with the antimitotic drug Ara-C, demonstrating that this subpopulation represents activated stem cell astrocytes. The researchers were also able to combine their analysis with Vybrant® DyeCycle™ dye staining, which allowed the sorting of cells according to their stage in the cell cycle. Further immunostaining and flow cytometric analysis revealed that the isolated subpopulation was as much as 20-fold enriched in cells exhibiting self-renewal and multipotency potential. The authors suggest that this simple purification strategy will greatly facilitate future molecular and functional studies of stem cell astrocytes, in pursuit of a greater understanding of *in vivo* stem cell behavior and the development of more effective therapeutic strategies.

Learn more about Alexa Fluor® EGF complex and Vybrant® DyeCycle™ Violet stain at **www.invitrogen.com/bp60**. ■

Product	Quantity	Cat. No.
Alexa Fluor® 647 EGF complex	100 µg	E35351
Vybrant® DyeCycle™ Violet stain	200 µL	V35003

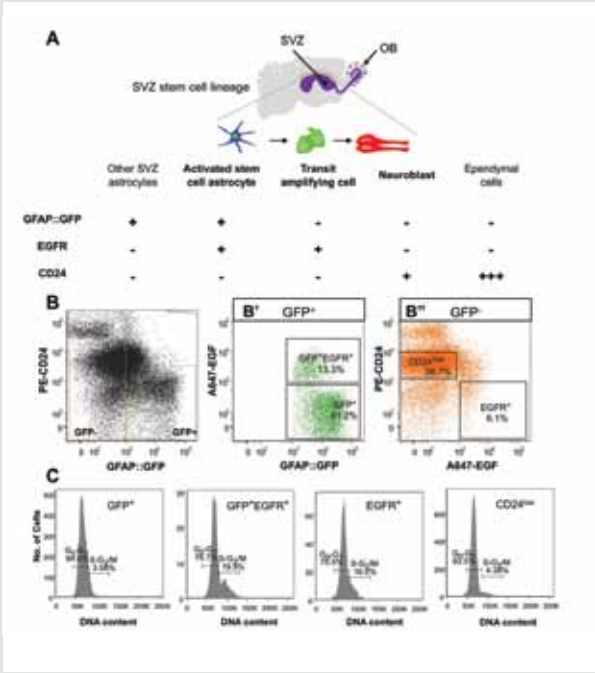

Figure 1. Prospective isolation of stem cells and their progeny from the adult SVZ. (**A**) Schema of a sagittal section of the adult mouse brain showing the lateral ventricle/SVZ (purple) and the SVZ stem cell lineage. Other SVZ astrocytes and ependymal cells lining the ventricles are also present in this region. Table shows the expression of markers used for the prospective isolation of each SVZ cell type. (**B**) FACS plots of the purification of each SVZ cell type from adult GFAP::GFP transgenic mice. GFAP-positive SVZ astrocytes were separated based on GFP expression from other GFP SVZ cells. (**B′**) From the GFP pool, we isolated activated stem cell astrocytes (GFPEGFRCD24) from other SVZ astrocytes (GFPEGFRCD24) based on EGFR expression. The GFPEGFR cell fraction largely corresponds to dim-GFP-expressing cells. (**B″**) From the GFP-negative cell fraction, we isolated transit amplifying cells (GFPEGFRCD24) and neuroblasts (GFPEGFRCD24low). Percentages represented by each population are shown next to the gates and are averages of 10 independent experiments. (**C**) A DNA dye was used to separate the cells according to their cell cycle status (G_0/G_1 phases from S/G_2–M phases). Percentages represented by each population are shown next to the gates. All FACS data are presented using "logical" or biexponential display for 100,000 cells. Reprinted with permission from Pastrana E, Cheng LC, and Doetsch F (2009) Simultaneous prospective purification of adult subventricular zone neural stem cells and their progeny. *Proc Natl Acad Sci U S A* 106:6387–6392.

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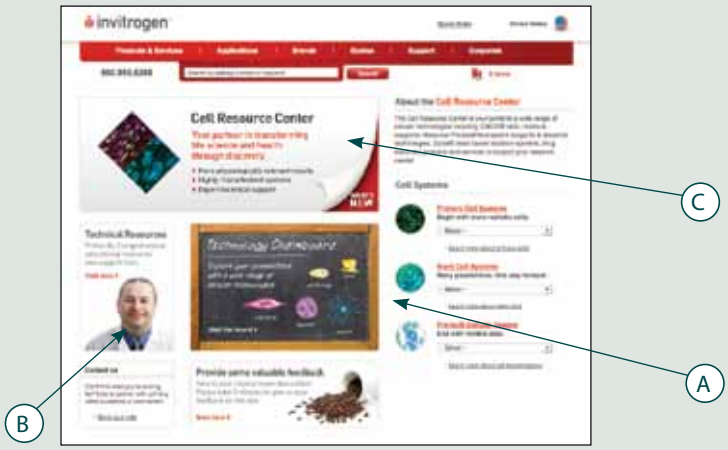

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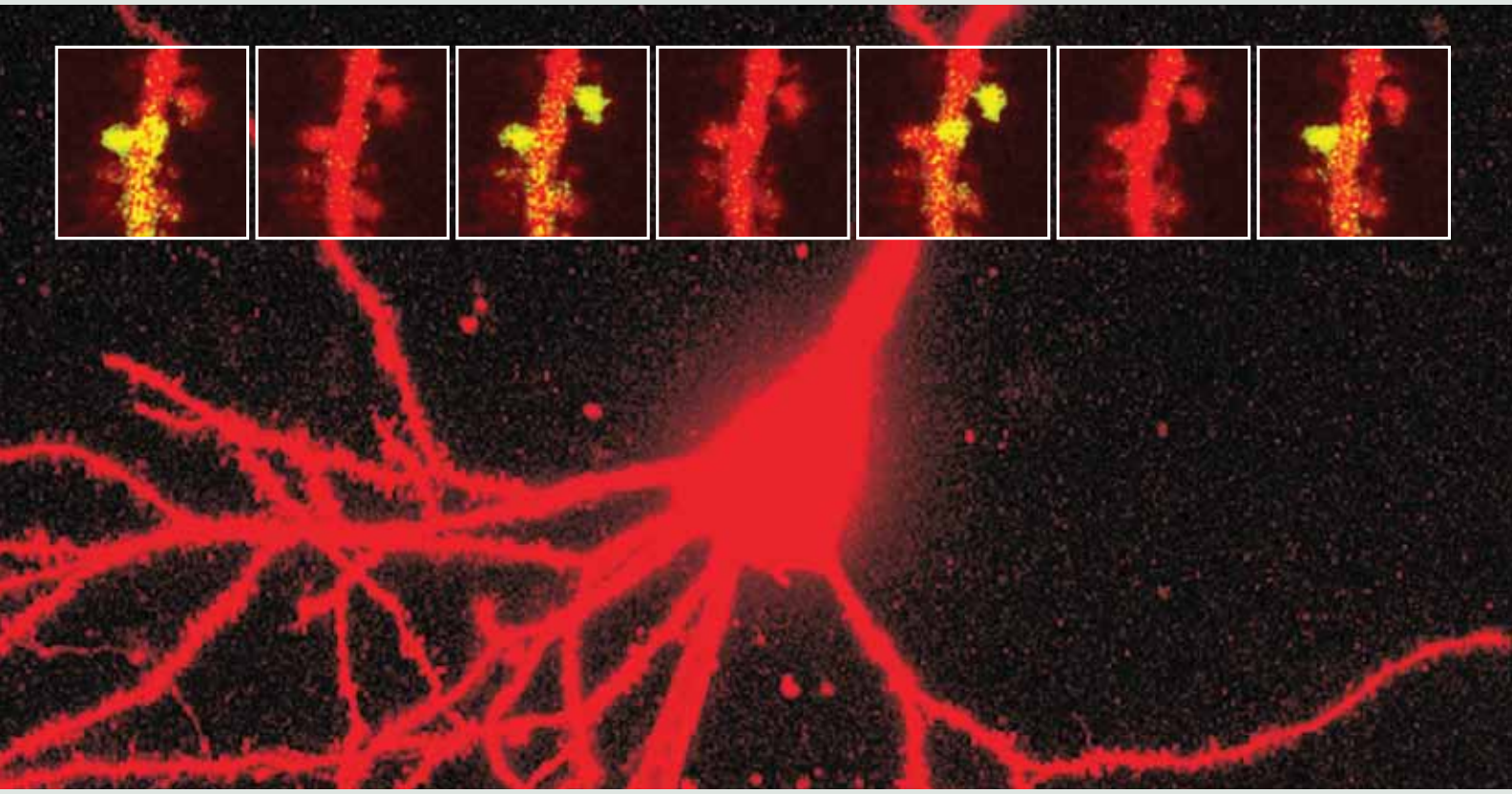

Neurons in focus

TWO-PHOTON EXCITATION IMAGING IN LIVE NEURONAL TISSUE.

Nowhere is preserving native cellular context more important than in studies of the nervous system. This intricate 3D structure is at the core of all of the complex functions we observe—disrupt it, and function is altered. Despite the easy access provided by dissociated neurons in culture, especially in optically based investigations such as fluorescence-based calcium imaging [1], the need for more contextual and system-level analysis has driven the development of techniques capable of exploring tissue slices and even the whole brain. Two-photon excitation (TPE) of fluorescent sensors is one approach giving researchers access to deeper layers of functioning cortex (Figure 1). This article reviews data and observations from the literature about the dye choices compatible with TPE, describes what to consider in making those choices, and lists protocols to optimize their use.

Figure 1 (above). Monitoring synaptically evoked calcium transients by two-photon laser-scanning microscopy in intact brain tissue using fluorescent dyes. A CA1 rat pyramidal neuron was microinjected with 300 µM fluo-5F (Cat. No. F14222), a calcium indicator (K_d 800 nM), and 20 µM Alexa Fluor® 594 hydrazide (Cat. No. A10442). Both dyes were excited at 800 nm using two-photon excitation. In the film insets, several frames of calcium transients are shown following individual evoked action potentials in presynaptic axons (evident by the appearance of a yellow-fluorescent signal against the Alexa Fluor® 594 dye–labeled red-fluorescent background). Using a ratio of the two signals, absolute calcium concentration in individual dendritic spines can be calculated. Such high-resolution optical imaging combined with electrophysiology allows hundreds of transmission events to be recorded at identified synapses, providing temporal and spatial precision that would have been unimaginable just a decade ago. Image courtesy of Thomas Oertner, FMI Institute for Biomedical Research, Basel, Switzerland, and Karel Svoboda, HHMI, Janelia Farms Research Institute, Virginia, US.

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Advantages of TPE for live-tissue analysis

The practical application of TPE microscopy has advanced considerably through the pioneering work of Watt Webb and his colleagues during the last two decades [2,3]. The principle of TPE was first predicted by Maria Göppert-Mayer in 1931 [4], and it is after her that the TPE unit of absorption cross-section or excitation efficiency is named. It took another three decades and the invention of the pulsed ruby laser, followed by the invention of TPE laser scanning microscopy in 1990 [2], to see TPE emerge as a practical application. The principal advantages are increased depth of resolution (due to the use of near infrared light—700 to 1,100 nm), narrow focal plane of the excitation volume (less than 100 µm), and reduced photobleaching of dye above and below the imaged region (~0.1 μm^3) [5]. Thus high-resolution, 3D images of depths up to 1 mm (up to 2 mm in some tissues) can be achieved, compared to less than 200 µm for visible light–based approaches [6] (Figure 2).

The science behind TPE is well reviewed elsewhere [7], but can be conceptualized as a summing of the excitation energies through two long-wave photons (e.g., 1,000 nm) that are focused and concentrated at discrete, small focal volumes (Figure 3). Concentrated in time and space, the two weak photons can sum their energies and excite fluorophores that normally have excitation maxima half that value. Since the excitation beam is concentrated in one focal plane, often as small as 0.1 μm^3, there is reduced photobleaching above and below the plane of interest, which eliminates phototoxicity. Thus TPE is rapidly becoming a preferred excitation source for physiological analysis of live tissue.

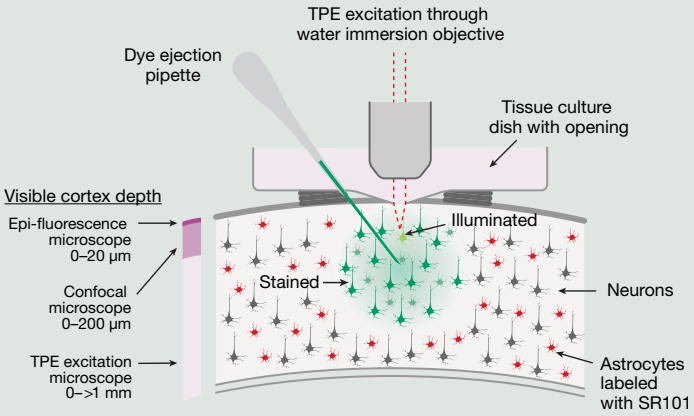

Figure 2. Schematic diagram showing a two-photon excitation (TPE) imaging setup and the depths of resolution of commonly used imaging techniques.

Choosing a fluorophore

In general, any bright one-photon dye can be used in TPE imaging if a somewhat blue-shifted excitation maximum is used (for a complete survey, see the excellent reviews in references 7 and 8). Table 1 lists specific dyes that have been shown to be useful in TPE experiments.

Qdot® nanocrystals have remarkable TPE cross-sectional areas estimated to be three orders of magnitude greater than the best fluorescent dyes or fluorescent proteins. Depending on the size of the Qdot® nanocrystal used, various fluorescent colors can be →

Figure 3. An experiment illustrating ordinary (single-photon) excitation of fluorescence and two-photon excitation. The cuvette contains a solution of the dye safranin O, which normally emits yellow light when excited by green light. The upper lens focuses green (543 nm) light from a CW helium–neon laser into the cuvette, producing the expected conical pattern of excitation (fading to the left). The lower lens focuses pulsed infrared (1,046 nm) light from a neodymium–YLF laser. In two-photon absorption, the excitation is proportional to the square of the intensity; thus, the emission is confined to a small point focus (see arrow), which can be positioned anywhere in the cuvette by moving the illuminating beam. Image contributed by Brad Amos, Science Photo Library, London.

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generated from a single wavelength of excitation, allowing multiplexing experiments to be performed relatively easily and without concern for phototoxicity or photobleaching. The many variations of fluorescent proteins, which also possess excellent cross-sectional absorption areas, are also readily adaptable to TPE.

Multi-cell bolus labeling (MCBL)

The simplest way to label cells is to use a micropipet to place dye in the region of interest in a technique called multi-cell bolus labeling (MCBL, Figure 2). Easier than achieving either whole-cell patch clamp or intracellular contact, MCBL can be used to deliver calcium-sensing dyes and dyes that selectively label astrocytes (sulforhodamine 101 (SR101) or its fixable version, Texas Red® hydrazide) in one 5-second injection pulse [9,10]. Many cells stained in this way are observed to have taken up the dye within 20 minutes [11].

This approach offers two main benefits: (1) individual cells (micro) as well as groups of cells (macro) can be studied in the same field; and (2) signal can be precisely attributed to astrocytes or neurons. This approach is effective in lower vertebrates and mammals, including whole alert mice and rats, at dye concentrations as low as 100 µM [8]. For this application, the dyes chosen must be cell permeant. Oregon Green® 488 BAPTA-1 AM has been used, but fluo-4 and others can be considered, and, when used with Pluronic® F-127 (Invitrogen Cat. No. P6867) (BASF Corporation), their uptake is increased. It is necessary to consider various outcomes when optimizing the experimental setup: for example, signal gain vs. signal-to-noise ratio, speed of response, the binding affinity of the dye, and color choice. These attributes are summarized in Tables 1 and 2, and a range of options exists. For example, Oregon Green® 488 BAPTA, when equimolar to fluo-4, is three times brighter, so three times less excitation could be used to achieve the same response. Reduced light excitation is a key factor in maintaining cell health. Oregon Green® 488 BAPTA also produces faint staining at low calcium or pre-stimulation levels, which can be helpful in finding the cells. But fluo-4 and its variants are used when a much bigger signal change is needed. For example, 100 µM of fluo-4 or fluo-5F can provide as much as a 50% signal increase in pyramidal cells [7]. For on/off sensing at individual neurons, Oregon Green® 488 BAPTA is a good choice, but for submicron regions like dendritic spines, the greater signal change with fluo-4 is preferred.

Intracellular injection

While more difficult to perform, whole-cell patch clamping and delivery of dyes into cells via microelectrodes allow more precise calcium ion concentration determinations, control of electrical behavior of the

Table 1. Excitable fluorophores for calcium detection for use in two-photon excitation (TPE) microscopy.

	Fluorophore	K_d in solution at 22–24°C*	Φ TPE cross-section†	TPE optimal excitation	Emission
Blue and green indicators	Oregon Green® 488 BAPTA-1 and -2	170 nM	ND	810–880 nm	530 nm
	Fluo-3	325 nM	10 GM/5 GM	810 nm	526 nm
	Fluo-4	345 nM	ND	800 nm	516 nm
	Fluo-5F	2.3 µM	ND	810 nm	520–530 nm
	Fluo-4FF	9.7 µM	ND	810 nm	516 nm
	Calcium Green™-1 +/– Ca	190 nM	30 GM/2 GM	820 nm	530 nm
	Fura-2 +/– Ca	140 nM	6 GM/0.2 GM	800 nm	505/362 nm
	Indo-1 +/– Ca	230 nM	3.5 GM/1.5 GM	590/700 nm	490/400 nm
Red indicators	X-rhod-1	0.7 µM	ND	900 nm	602 nm
	X-rhod-5F	1.6 µM	ND	770–800 nm	603 nm
	X-rhod-FF	17 µM	ND	ND	603 nm
	Rhod-FF	19 µM	ND	ND	603 nm

* K_d can change inside cells (see reference 11). Data shown are according to Molecular Probes data taken at 22°C. † Expressed in Göppert-Mayer Units (GM); 1 GM = 10^{-50} cm^4 s. For a more complete listing of TPE experiment–compatible dyes, see **www.invitrogen.com/bp60**. **ND** = Not determined.

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Table 2. Other useful stains for use in two-photon excitation (TPE) microscopy.

Stain	Target	Φ TPE cross-section*	TPE optimal excitation	Emission
CoroNa™ Green	NA	ND	770 nm	516 nm
CoroNa™ Red	NA	ND	~1,000 nm	570 nm
Sulforhodamine 101 (SR101) or Texas Red® hydrazide (fixable version of SR101)	Astrocytes	ND	840–890 nm	605 nm
Alexa Fluor® 594 hydrazide	Ca^{2+}-insensitive intracellular marker	ND	810 nm	594 nm
Fluorescent proteins	General expression tag	100–200 GM	800–1,030 nm	505–610 nm
Rhodamine B	General cell stain	100–200 GM	840 nm	600 nm
Lucifer yellow CH L453, L682, L1177	Gap junctions	ND	850 nm	533 nm
DAPI	DNA/nuclei	0.16 GM	700 nm	450 nm
FM® 1-43	Vesicle recycling	ND	840 nm	575–600 nm†
Qdot® nanocrystals	NA	Up to 47,000 GM	Broad	8 colors from 545–800 nm

* Expressed in Göppert-Mayer Units (GM); 1 GM = 10^{-50} cm^4 s. † Strongly environment dependent. **NA** = Not applicable. **ND** = Not determined.
For a more complete listing of TPE experiment–compatible dyes, see **www.invitrogen.com/bp60**.

cell, and delivery of intracellular agonists to second messengers. High affinity dyes such as fluo-4, fluo-5F, X-rhod-1, or X-rhod-5F are used to detect single action potentials or small synaptically evoked signals whereas lower affinity dyes such as fluo-4FF, X-rhod-FF, or rhod-FF are used to sense trains of action potentials or larger synaptically evoked signals [12]. It has been noted that the apparent affinities of nearly all dyes will be affected by other ions already free in the sample; thus the affinities offered in the table generally need to be halved to estimate their behavior in cells. Moreover, the interaction of calcium with the dye will also impact the free calcium levels, competing for ions that might otherwise be signaling.

While ratiometric dyes (e.g., the excitation ratio fura dyes or the emission ratio indo-1 dyes) are optimal for deriving accurate calcium concentrations, TPE does not readily lend itself to this approach. A far easier approach is to co-inject a red dye, such as Alexa Fluor® 594 dye, and monitor ratio changes in levels of indicator dye against the constant value of the Alexa Fluor® 594 dye for that cell [7]. One group has shown that multiple sensing dyes can be co-injected, which opens a diverse range of indicator options. In their experiments, they filled neurons via intracellular injection with a sodium-sensing reagent, CoroNa™ dye, and a red-shifted calcium indicator, X-rhod-5F. Both can be excited by 770 nm light, conveniently far from the wavelengths used to excite Green Fluorescent Protein (GFP)-labeled neurons (commonly employed to observe cell morphology and to identify candidate cells), yet their emissions are monitored separately. In doing so, they found that currents for sodium and calcium were co-distributed at action potential initiation sites in cochlear interneurons [13].

The future of TPE imaging

Using the TPE technique, experiments considered impossible several years ago are now moving ahead. Possibly the most remarkable and complex of these approaches involves analysis of the contribution of NMDA-induced calcium transients to secondary downstream signaling such as RAS [14] at the level of individual dendritic spines of hippocampal neurons in brain slices. NMDA pulses are delivered with regional accuracy by using the same TPE to uncage MNI-caged glutamate [15]. Alternatively, fluorescent protein–tagged protein pairs can be co-injected and FRET interactions studied through lifetime changes, as regulatory elements combine in response to ion fluxes [13]. Tools like these, available from Invitrogen (Tables 1 and 2), are allowing researchers to explore the molecular substrates of learning and memory at the most basic structural level—the minute spines that decorate dendrites throughout the brain. Learn more at **www.invitrogen.com/bp60**. ■

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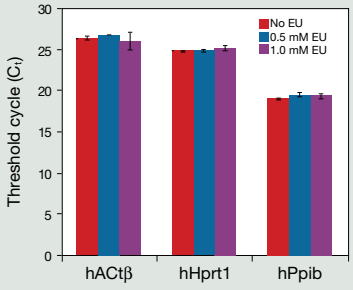

Figure 1. Housekeeping genes are unaffected in cells incubated with EU. Real-time RT-PCR was conducted to determine if EU incorporation affected housekeeping gene expression levels. RNA was isolated using TRIzol® LS reagent (Cat. No. 102906-010) from NIH3T3 cells fed with 0.5 mM EU and 1.0 mM EU. RNA concentrations were determined for each sample, and then 250 ng of RNA and specific primer sets for hACtβ, hHprt1, and hPpib were used in a reaction volume of 50 µL to perform one-step qPCR using the SuperScript® III Platinum® SYBR® Green One-Step qRT-PCR Kit (Cat. No.11736-051) and the Mx3000P system (Stratagene). The threshold cycle (C_t) values were obtained using MxPro software. The data clearly show that mRNA levels of housekeeping genes do not change in the presence of the EU concentrations tested.

Take a snapshot of RNA synthesis with Click-iT® RNA assays

Click-iT® RNA assays are ideal for imaging global RNA synthesis in multiplex analyses using traditional fluorescence microscopy or high-content screening (HCS). The assays employ an alkyne-modified nucleoside, EU (5-ethynyl uridine), that is fed to cells and incorporated into nascent RNA [3]. The small size of the alkyne tag (MW ~25) enables efficient incorporation by RNA polymerases without any apparent changes to the RNA levels of several housekeeping genes (Figure 1). Detection is accomplished using an Alexa Fluor® azide, which has a small "footprint" (MW <1,000) compared with an IgG antibody (MW ~150,000), and results are available in 30 minutes, eliminating long antibody incubations. Following EU incubation, simply fix and permeabilize, then perform the copper-catalyzed click reaction. Click-iT® RNA assays are compatible with other cell-labeling methods such as immunocytochemistry and small-molecule labeling of cellular structures (Figure 2). The multiplexing

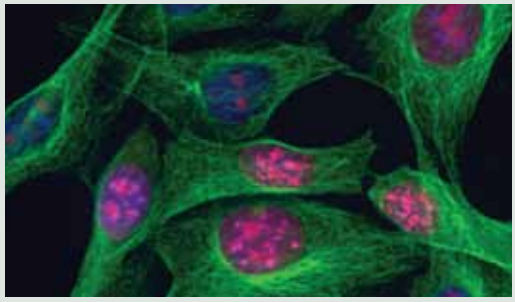

Figure 2. Multiplex imaging with Click-iT® RNA assays. NIH3T3 cells were incubated with 1 mM EU for 1 hr, then formaldehyde fixed and permeabilized with Triton® X-100. EU incorporated into newly synthesized RNA (red) in some cells was detected using the Click-iT® RNA Alexa Fluor® 594 Imaging Kit (Cat. No. C10330). Tubulin (green) was detected with mouse anti-tubulin IgG (Cat. No. A11126) and visualized with Alexa Fluor® 488 goat anti–mouse IgG (Cat. No. A11001). Nuclei (blue) were stained with Hoechst 33342 (Cat. No. H3570).

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Table 1. Quantitative analysis of RNA inhibition.

Cell	Alexa Fluor® azide	CV (%)	Signal change
NIH3T3*	Alexa Fluor® 488	6.4	8.9
	Alexa Fluor® 594	10.5	21.4
HeLa†	Alexa Fluor® 488	8.6	7.7
	Alexa Fluor® 594	19.0	11.1

Cells were treated with 250 nM actinomycin D for 18 hr, and inhibition of RNA synthesis was assayed with the indicated Click-iT® RNA HCS Assay. The data shown represent the average %CV and fold change in signal intensity of treated samples (Max) from either two† or three* Min/Max plates.

capability of the assays makes them ideal for toxicological profiling or interrogation of disease models using high-content imaging platforms (Table 1, Figure 3).

Go viral with Click-iT® RNA assays

Little is understood about the sites of viral RNA synthesis, including the transport and fate of nascent RNA inside and outside the viral replication complex [4]. The Click-iT® RNA Imaging and Click-iT® RNA HCS kits provide exciting new alternatives to antibody-based BrU or BrUTP assays for studying viral RNA synthesis in infected cells (Figure 4). Unlike BrUTP, EU does not require a transfection reagent, and the small size of the Alexa Fluor® azide relative to the anti-BrdU antibody enables detection of both ssRNA and dsRNA. The Click-iT® RNA imaging assays provide the tools necessary to determine levels of viral RNA synthesis as well as the subcellular localization of RNA in host cells, which will aid in understanding viral gene expression and genome replication.

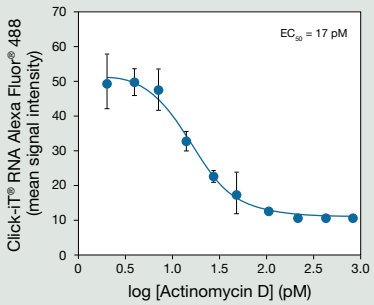

Figure 3. Dose response for actinomycin D in HeLa cells using the Click-iT® RNA Assay. HeLa cells were treated with the indicated amounts of actinomycin D for 18 hr, followed by a 1 hr incubation with 5-ethynyl uridine (EU). Cells were then fixed and permeabilized, and EU incorporated into newly synthesized RNA was detected using green-fluorescent Alexa Fluor® 488 azide. Quantitative analysis was performed using the Cellomics® ArrayScan® VTI and Compartmental Analysis Bioapplication (Thermo Scientific).

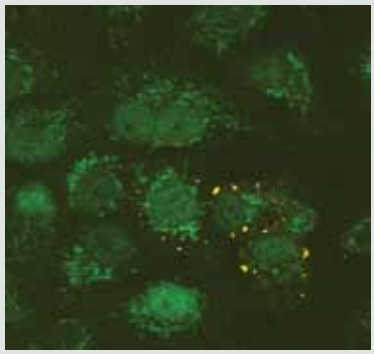

Figure 4. Subcellular localization of RNA in Vero cells. Vero cells were pretreated with 2 µM actinomycin D to inhibit host cell transcription, then infected with Tacaribe virus. Infected cells were incubated with 2 mM EU for 1 hr, followed by cold methanol fixation and permeabilization with Triton® X-100. Nascent RNA (green) was detected with the Click-iT® RNA Alexa Fluor® 488 Imaging Kit (Cat. No. C10329). Viral nucleoprotein was detected with a directly labeled Alexa Fluor® 594 monoclonal antibody (red). Colocalization of EU and virus nucleoprotein indicates transcription sites in the host cells (yellow).

Taking a new look at RNA

Click-iT® RNA Imaging Kits and Click-iT® RNA HCS Assays allow you to apply the advantages of click chemistry to the study of RNA synthesis. Learn more at **www.invitrogen.com/bp60**. ■

References

1. Saxon E and Bertozzi CR (2000) *Science* 287:2007–2010.
2. Kolb HC et al. (2001) *Angew Chem Int Ed Engl* 40:2004–2021.
3. Jao C and Salic A (2008) *Proc Natl Acad Sci U S A* 105:15779–15784.
4. Kopek BG et al. (2007) *PLOS Biology* 5:2022–2034.

Product	Quantity	Cat. No.
Click-iT® RNA Alexa Fluor® 488 HCS Assay, 2-plate size	1 kit	C10327
Click-iT® RNA Alexa Fluor® 594 HCS Assay, 2-plate size	1 kit	C10328
Click-iT® RNA Alexa Fluor® 488 Imaging Kit, for 25 coverslips	1 kit	C10329
Click-iT® RNA Alexa Fluor® 594 Imaging Kit, for 25 coverslips	1 kit	C10330
5-ethynyl uridine (EU)	5 mg	E10345

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Isolate T cells directly from whole blood

SAVE TIME WITH DYNABEADS® FLOWCOMP™ ISOLATION TECHNOLOGY.

Fast and easy isolation of pure cells directly from whole blood samples is beneficial for basic research and patient monitoring (e.g., analysis of circulating tumor cells, prenatal diagnosis, T cell numeration in HIV, chimerism analysis in transplantation) as well as for drug discovery and development. Dynabeads® FlowComp™ technology offers rapid, pure, and gentle isolation of human $CD3^+$, $CD4^+$, and $CD8^+$ T cells directly from whole blood. The beads are released from the cells after isolation to avoid any possible artifacts in your downstream applications.

Dynabeads® FlowComp™ technology

Dynabeads® FlowComp™ technology uses a highly specific biotin-streptavidin technology to positively isolate T cells. Included in the kit is a release buffer that removes the cells from the beads (Figure 1). The process is fast and gentle, ensures very high cell viability, and eliminates the use of columns.

Pure and viable cells isolated directly from whole blood

You can isolate $CD3^+$, $CD4^+$, or $CD8^+$ T cells directly from whole blood, obtaining >95% pure T cells with viability exceeding 98%. There is no need for gradient centrifugation or lysis of red blood cells before isolation, saving several hours compared to isolation from peripheral blood mononuclear cells (PBMCs). Compared with a column-based protocol, the purity and viability of T cells isolated using Dynabeads® FlowComp™ is superior (Figure 2). Plots of forward scatter to side scatter (FSC/SSC) and histograms of the selected population illustrate the difference in purity and the presence of apoptotic and aggregated cells using the column-based method.

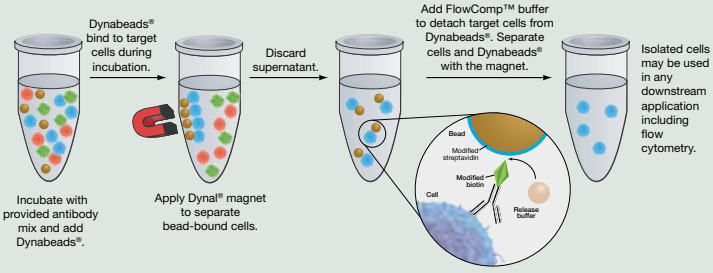

Figure 1. Positive isolation of T cells in less than 50 min directly from whole blood using Dynabeads® FlowComp™ technology.

Isolated T cells proliferate when activated

To study the proliferative capacity of $CD3^+$ T cells isolated with Dynabeads® FlowComp™ Human CD3, isolated cells were stimulated *in vitro* using Dynabeads® Human T-Activator CD3/CD28 in a 1:1 bead-to-cell ratio. Proliferation was measured using a Click-iT® EdU assay; from days 2 to 3, more than 40% of the cells had incorporated EdU into newly synthesized DNA (Figure 3), indicating T cell proliferation.

Tetanus toxoid stimulates antigen-specific $CD3^+$ T cell proliferation

$CD3^+$ T cells isolated with Dynabeads® FlowComp™ Human CD3 were stained with carboxyfluorescein diacetate succinimidyl ester (CFSE) after isolation and added to PBMCs pulsed with tetanus toxoid. In a proliferation assay, the T cells proliferated in an antigen-specific manner (Figure 4).

"For the positive isolation of T cells, the kit works beautifully well. I end with a very pure $CD3^+$ population. We demonstrate that the CD3 FlowComp™ kit allows for the isolation of functional T cells and that these cells are capable of being activated in an antigen-specific manner."

—Amir Horowitz, London School of Hygiene and Tropical Medicine, UK

Dynabeads® FlowComp™ technology—the flexible solution

For more information on mouse and human FlowComp™ products and technology, visit **www.invitrogen.com/bp60**. ■

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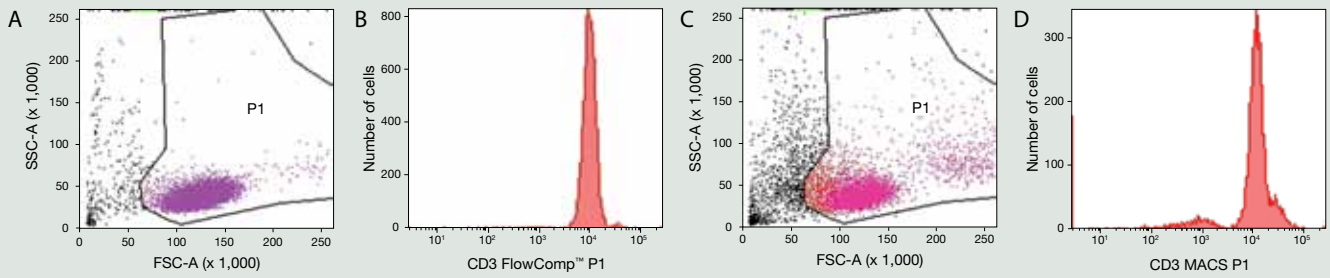

Figure 2. CD3$^+$ T cells isolated with FlowComp™ technology are highly pure and viable. T cells were isolated from whole blood with either Dynabeads® FlowComp™ Human CD3 or a column-based system, then analyzed by flow cytometry. The cells isolated using FlowComp™ technology (P1 area) are a highly uniform population with >99% pure CD3$^+$ T cells (**A, B**). In contrast, the cells isolated using the column-based method contained more red blood cells (red signal) and smaller cells typical of apoptotic cells or erythrocytes (**C**). In addition, a CD3$^+$ population with higher FSC is visible, indicating aggregation of isolated T cells (**D**).

Product	Quantity	Cat. No.
Dynabeads® FlowComp™ Human CD4	Processes 2 x 10^9 cells	113-61D
	Processes 4 x 10^8 cells	113-71D
Dynabeads® FlowComp™ Human CD8	Processes 2 x 10^9 cells	113-62D
Dynabeads® FlowComp™ Human CD3	Processes 2 x 10^9 cells	113-65D
Dynabeads® FlowComp™ Flexi	Processes 2 x 10^9 cells	110-61D
Dynabeads® Human T-Activator CD3/CD28 for cell expansion and activation	0.4 mL	111-61D
	2 mL	111-31D
	5 x 2 mL	111-32D
DynaMag™-15 magnet	Holds 4 x 5 mL FACS tubes or 4 x 15 mL tubes	123-01D
DynaMag™-50 magnet	Holds 2 x 50 mL tubes	123-02D
DynaMag™-2 magnet	Holds 16 x 1.5–2 mL microcentrifuge tubes	123-21D
OpTmizer™ T-Cell Expansion SFM	500 mL	0080022SA
Click-iT® EdU Alexa Fluor® 647 Flow Cytometry Assay Kit	1 kit	A10202

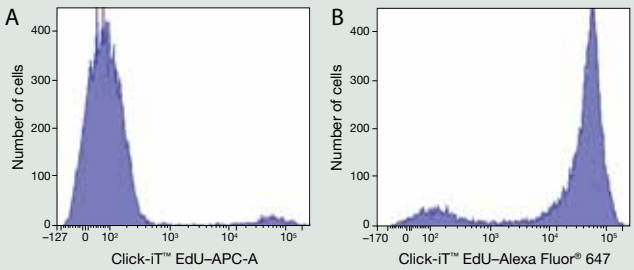

Figure 3. Isolated CD3$^+$ T cells proliferate when activated. Freshly isolated CD3$^+$ T cells were stimulated with Dynabeads® Human T-Activator CD3/CD28 for 3 days, and proliferation was measured by the Click-iT® EdU Alexa Fluor® 647 Flow Cytometry Assay (**A**); 44% of the cells proliferated, as detected by flow cytometry (**B**).

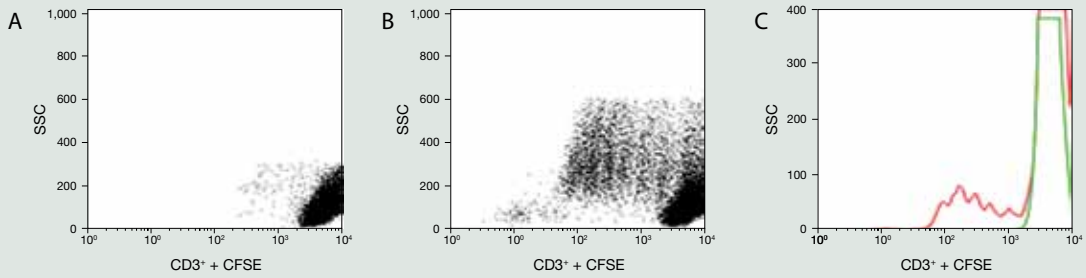

Figure 4. Proliferation assay demonstrating tetanus toxoid–specific T cell response. CD3$^+$ T cells isolated by Dynabeads® FlowComp™ Human CD3 were prestained with CFSE, then incubated with antigen-presenting cells (**A**) with no stimulus or (**B**) with tetanus toxoid (10 µg/mL). After 120 hr, the cells were analyzed by flow cytometry. (**C**) shows the nondividing cells from A (green) and the dividing cells from B (red) in a histogram.

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Chemiluminescent immunodetection and reporter gene assays

HIGH-QUALITY, HIGH-PURITY SUBSTRATES FOR ULTRASENSITIVE DETECTION.

Chemiluminescence is the conversion of chemical energy to light energy. 1,2-dioxetanes are one class of synthetic chemical structures that produce light energy upon chemical or enzymatic cleavage. These chemical-based light-producing reactions are employed in a wide variety of laboratory applications, including biological assays, clinical diagnostic assays, biosensors, and hygiene monitoring. These applications are enabled by the high-intensity signal, low background, wide dynamic range, rapid signal production, and assay format compatibility provided by chemiluminescence.

Invitrogen's high-quality, high-purity 1,2-dioxetane chemiluminescent substrates enable ultrasensitive detection of biomolecules. These substrates are available as stand-alone reagents or in convenient kit format for immunoassays, enzyme assays, reporter gene assays, and cAMP measurement.

Principles of enzyme-activated chemiluminescence

Figure 1 diagrams the production of light by the enzyme-catalyzed decomposition of a 1,2-dioxetane containing a cleavable phosphate residue. In this example, Invitrogen's CSPD® substrate is dephosphorylated to form a metastable intermediate that produces a phenolate anion that then decomposes, emitting light in the process. The enzyme-catalyzed reaction ultimately produces a steady light glow that can be detected by simple instrumentation or with light-sensitive film. The enhancer partitions water molecules away from the site of signal production, reducing the quenching of the light signal by water molecules and improving overall signal intensity.

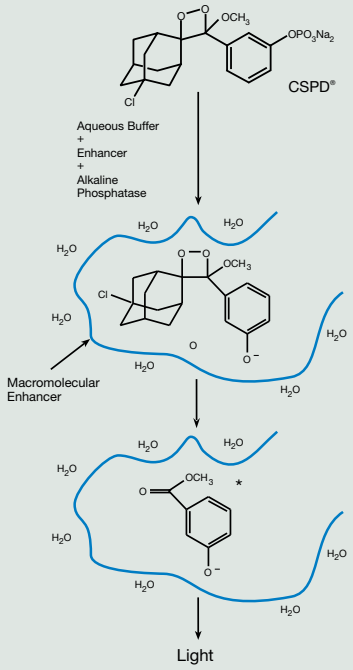

Figure 1. The light emission mechanism of CSPD® substrate in the presence of enhancer. The diagram depicts the reaction process for the enzyme-catalyzed decomposition of a 1,2-dioxetane using CSPD® as an example. Following enzymatic cleavage, the resulting product produces a phenolate anion that decomposes, producing light. The enhancer partitions water away from the reactive species to improve light output.

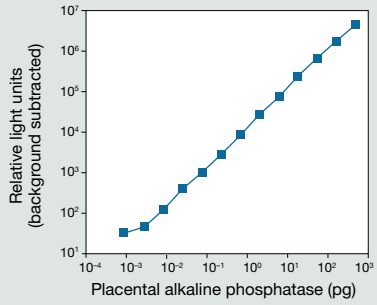

Figure 2. Detection of placental alkaline phosphatase with the NovaBright™ Secreted Placental Alkaline Phosphatase Enzyme Reporter Gene Chemiluminescent Detection Kit. The data demonstrate the linear response of the kit over 5 orders of magnitude.

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Advantages of 1,2-dioxetane substrates

Enzyme-triggered 1,2-dioxetane chemiluminescent substrates offer several important advantages. The inherent low background and high signal intensity results in exceptionally high assay sensitivity, allowing femtogram detection (Figure 2). These favorable signal characteristics also result in the exceptional dynamic range of up to 7 orders of magnitude (Figures 2 and 3). Rapid light emission kinetics permits rapid assay completion, often in less than one hour.

Invitrogen's 1,2-dioxetane enzyme substrate–based kits meet the challenging demands of a broad variety of applications. The kits employ CDP-Star® and CSPD® substrates for alkaline phosphatase, and Galacton-Plus® and Galacton-Star® substrates for β-galactosidase. The high quality, purity, and lot-to-lot consistency of these ready-to-use substrates enable excellent reproducibility and low background.

Alkaline phosphatase substrates

CDP-Star® and CSPD® substrates offer key advantages relative to colorimetric and fluorometric methods for detecting alkaline phosphatase or alkaline phosphatase conjugates. For immunoassays, 1,2-dioxetane substrates improve sensitivity and generate results faster than colorimetric methods [1]. These substrates are ideally suited for the detection of placental alkaline phosphatase (PLAP) and secreted placental alkaline phosphatase (SEAP) in reporter gene assays [2,3].

CDP-Star® or CSPD® is incorporated into the Invitrogen™ Chemiluminescent Alkaline Phosphatase ELISA Kits for the construction of direct-capture or competitive immunoassays using an alkaline phosphatase conjugate. The NovaBright™ Secreted Placental Alkaline Phosphatase (SEAP) Enzyme Reporter Gene Chemiluminescent Detection Kit uses the CSPD® substrate to provide highly sensitive detection of the reporter gene product SEAP in a convenient microplate format. The Invitrogen™ cAMP Chemiluminescent Immunoassay Kit also uses the CSPD® substrate in a competitive ELISA assay for the detection of cAMP using either a manual assay or an automated high-throughput screening protocol (Figure 4).

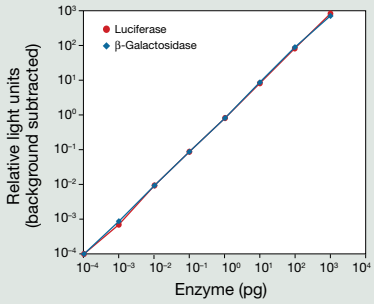

Figure 3. Detection of luciferase and β-galactosidase from a single sample. The assay demonstrates linear measurement of firefly luciferase and β–galactosidase concentrations over 7 orders of magnitude, from the femtogram to nanogram range.

β-Galactosidase substrates

Galacton-Plus® and Galacton-Star® substrates are widely used and have become the gold standards for sensitive quantitation of β-galactosidase in reporter gene assays in both mammalian and yeast cells. →

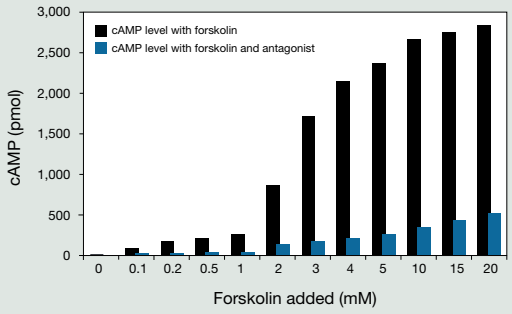

Figure 4. The effect of forskolin on cAMP levels using the Invitrogen™ cAMP Chemiluminescent Immunoassay Kit. Microplate luminometer data showing the effect of forskolin, a known inducer of cAMP, and the effect of a forskolin antagonist on the levels of cAMP.

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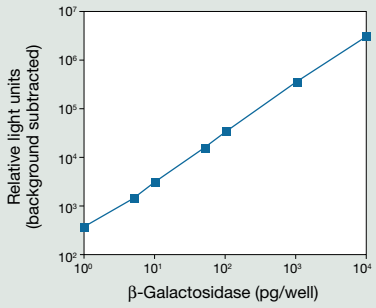

Figure 5. Sensitivity of the NovaBright™ β-Galactosidase Enzyme Reporter Gene Chemiluminescent Detection Kit. β-galactosidase assays using purified β-galactosidase diluted in PBS showing linearity over 4 orders of magnitude of enzyme. Assays were measured on a microplate luminometer.

The chemiluminescent substrates for β-galactosidase exhibit over 3 orders of magnitude greater sensitivity than colorimetric assays.

NovaBright™ β-Galactosidase Enzyme Reporter Gene Chemiluminescent Detection Kits for Mammalian or Yeast Cells use Galacton-Star® substrate to quantitate the β-galactosidase reporter gene enzyme from lysates of mammalian or yeast cells (Figure 5). The assay protocol is very efficient, requiring the addition of a single reagent.

NovaBright™ β-Galactosidase and Firefly Luciferase Dual Enzyme Reporter Gene Chemiluminescent Detection Kits use Galacton-Plus® in combination with the luciferase substrate, luciferin, in a dual reporter enzyme detection assay. A single cell lysate sample is used, and the light emission for each enzyme is measured sequentially (Figure 3). The kit allows the detection of reporter gene activity from the same sample with a large dynamic range (6–7 orders of magnitude).

Take advantage of ultrasensitive chemiluminescent detection

Invitrogen's 1,2-dioxetane enzyme substrate–based kits provide ultrasensitive detection for measuring reporter gene activity, cAMP activity, and alkaline phosphatase ELISAs. Learn more at **www.invitrogen.com/bp60**. ■

References

1. Albrecht S, Ehle H, Schollberg K et al. (1991) Chemiluminescent enzyme immunoassay of human growth hormone based on adamantyl dioxetane phenyl phosphate substrate, *Bioluminescence and Chemiluminescence: Current Status.* pp 115–118.
2. Bronstein I et al. (1989) *Clin Chem* 35:1441–1446.
3. Bronstein I et al. (1994) *Biotechniques* 17:172–178.

Product	Quantity	Cat. No.
Chemiluminescent Alkaline Phosphatase ELISA Kit #1 with CSPD® Substrate and Sapphire™-II Enhancer	1,000 assays	C10552
Chemiluminescent Alkaline Phosphatase ELISA Kit #2 with CSPD® Substrate and Emerald™-II Enhancer	1,000 assays	C10553
Chemiluminescent Alkaline Phosphatase ELISA Kit #3 with CDP-Star® Substrate and Sapphire™-II Enhancer	1,000 assays	C10554
Chemiluminescent Alkaline Phosphatase ELISA Kit #4 with CDP-Star® Substrate and Emerald™-II Enhancer	1,000 assays	C10555
Chemiluminescent Alkaline Phosphatase ELISA Substrate/Enhancer Sampler Kit	1,000 assays	C10556
cAMP Chemiluminescent Immunoassay Kit	2 plates	C10557
cAMP Chemiluminescent Immunoassay Kit	10 plates	C10558
NovaBright™ Secreted Placental Alkaline Phosphatase (SEAP) Enzyme Reporter Gene Chemiluminescent Detection Kit	400 assays	N10559
NovaBright™ Secreted Placental Alkaline Phosphatase (SEAP) Enzyme Reporter Gene Chemiluminescent Detection Kit	1,200 assays	N10560
NovaBright™ β-Galactosidase and Firefly Luciferase Dual Enzyme Reporter Gene Chemiluminescent Detection Kit	200 assays	N10561
NovaBright™ β-Galactosidase and Firefly Luciferase Dual Enzyme Reporter Gene Chemiluminescent Detection Kit	600 assays	N10562
NovaBright™ β-Galactosidase Enzyme Reporter Gene Chemiluminescent Detection Kit for Mammalian Cells	200 assays	N10563
NovaBright™ β-Galactosidase Enzyme Reporter Gene Chemiluminescent Detection Kit for Mammalian Cells	1,000 assays	N10564
NovaBright™ β-Galactosidase Enzyme Reporter Gene Chemiluminescent Detection Kit for Yeast Cells	200 assays	N10565
NovaBright™ β-Galactosidase Enzyme Reporter Gene Chemiluminescent Detection Kit for Yeast Cells	1,000 assays	N10566

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Live-cell imaging of cell cycle and cell division

INTRODUCING THE PREMO™ FUCCI CELL CYCLE SENSOR.

The Premo™ FUCCI Cell Cycle Sensor enables live-cell imaging of cell cycle and division—as cells progress through the cell cycle, nuclear fluorescence changes from red to green. Based on the BacMam gene delivery system, the genetically encoded and prepackaged fluorescent protein (FP)–based reagents are ready for immediate use and optimization for your live-cell applications.

Color-coded G_1/S and G_2/M

Miyawaki and colleagues developed the fluorescence ubiquitination cell cycle indicator (FUCCI), a sensor that uses two different FPs fused to regulators of the cell cycle: Cdt1 and geminin [1]. Ubiquitin E3 ligases add ubiquitin to Cdt1 and geminin, thereby targeting these proteins to the proteasome for degradation. Temporal regulation of E3 ligase activity results in the biphasic cycling of geminin and Cdt1 through the cell cycle. In G_1, geminin is degraded, leaving only Cdt1 tagged with Red Fluorescent Protein (RFP), resulting in red-fluorescent nuclei. During the G_1/S transition, Cdt1 levels fall as geminin levels rise; because both proteins are present, both Green Fluorescent Protein (GFP) and RFP fluorescence is visible, and the cell appears yellow when green and red images are overlaid. In S and G_2 into M phase, Cdt1 is degraded, leaving only geminin tagged with GFP, so that cells in G_2 and M phase appear green. This color change from red to yellow to green serves as an important marker of progression through cell cycle and division (Figure 1A).

Ready-to-use Premo™ FUCCI Cell Cycle Sensor

Invitrogen has combined FUCCI with the powerful BacMam gene delivery system to form the Premo™ FUCCI Cell Cycle Sensor. BacMam uses a modified baculovirus that contains the FP chimeras. 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. Transduction is efficient and reproducible in most cell types, including primary and stem cells, without apparent cytopathic effects. No lipids, dye-loading chemicals, or other potentially harmful treatments are required. In addition, BacMam technology allows you to precisely titrate expression levels to optimize the reagent for your experiment. Simply add the reagent to your cells for 1–2 hours, treat with the enhancer for 1–2 hours, wash, incubate overnight, then visualize cell cycle progression in populations of cells using fluorescence microscopy or high-throughput imaging platforms (Figure 1B). Learn more about the Premo™ FUCCI Cell Cycle Sensor at **www.invitrogen.com/bp60**. ■

Reference

1. Sakaue-Sawano et al. (2008) *Cell* 132:487–498.

Product	Quantity	Cat. No.
Premo™ FUCCI Cell Cycle Sensor	1 kit	P36232

Figure 1. Imaging cell cycle progression in live cells with Premo™ FUCCI Cell Cycle Sensor. (**A**) Schematic of cell cycle progression with nuclear fluorescence changes. (**B**) U2OS cells were transduced with Premo™ FUCCI Cell Cycle Sensor. Images were collected over 15 hours.

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Advances in neural stem cell culture

SUPERIOR NSC EXPANSION WITH STEMPRO® NSC SERUM-FREE MEDIUM.

Neural stem cells (NSCs) are self-renewing, multipotent cells of the nervous system that can differentiate into neurons or glial cells (oligodendrocytes and astrocytes). NSCs can be isolated from the fetal or adult central nervous system (CNS) [1,2] or derived from embryonic stem cells (ESCs) [3,4]. Isolated adult NSCs are often limited in their differentiation potential, with the propensity to differentiate toward the predominant neural lineage surrounding the NSCs prior to isolation. In contrast, ESC-derived NSCs have the capacity to differentiate into all three lineages. Because of their versatility, NSCs are a valuable tool for basic research and for clinical applications to treat neurodegenerative diseases and neurological disorders.

When NSCs were first isolated from adult tissue, scientists initially relied on serum-containing media for NSC culture and expansion [5]. NSCs have the propensity to differentiate into neurons and glial cells in serum-containing media, and the development of serum-free alternatives such as the GIBCO® Neurobasal® Medium/N-2 combination alleviated this problem. However, the stringent culture conditions required with this system often resulted in suboptimal expansion of NSCs.

To address the need for a serum-free solution optimized for both fetal- and ESC-derived NSCs, we developed StemPro® NSC SFM, enabling superior NSC expansion efficiency of adherent and neurosphere suspension cultures while maintaining normal NSC multipotency, phenotype, and karyotype.

Proliferation of human neural stem cells in StemPro® NSC SFM

Human NSCs (hNSCs) represent a very small fraction of the total CNS cell population; thus, expansion is critical for generating a sufficient number of cells to study differentiation pathways and explore the downstream clinical applications hNSCs. In addition, hNSCs can be passaged only a limited number of times before exhibiting reduced proliferation and differentiation potential; therefore, maximizing the total hNSC yield per passage is essential.

To evaluate StemPro® NSC SFM performance, we compared the proliferation rates of hESC-derived NSCs grown in StemPro® NSC SFM vs. Neurobasal® Medium and competitor media, and analyzed the expression of key NSC markers. NSCs cultured in StemPro® NSC SFM proliferated at higher rates than NSCs grown in Neurobasal® Medium or competitor media (Figure 1). NSCs grown in StemPro® NSC SFM expressed the NSC markers Nestin and Sox2 (Figure 2), demonstrating

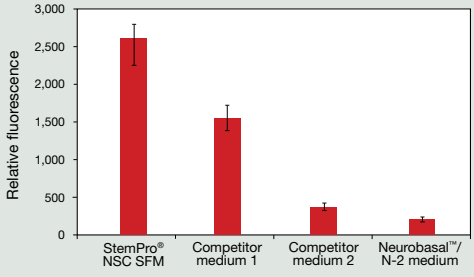

Figure 1. Proliferation of hNSCs cultured in StemPro® NSC SFM, competitor medium 1, competitor medium 2, or Neurobasal™ N2-supplemented medium. ESC-derived hNSCs were seeded at 1 x 10^4 cells per well in CELLstart™ substrate–coated 96-well plates for 3 days in each respective medium. Indirect cell counts were obtained with the CyQUANT® Cell Proliferation Assay Kit (Cat. No. C35006). Data represent the mean relative florescence units from stained cells (n = 6).

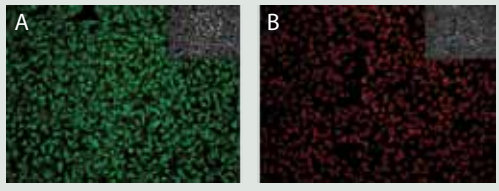

Figure 2. Phenotype marker expression in neural stem cells cultured in StemPro® NSC SFM through passage 17. NSCs expressed the normal phenotypic markers Nestin (**A**) and Sox2 (**B**), in the absence of ESC marker Oct4. The inset image in each panel shows DAPI nuclear staining.

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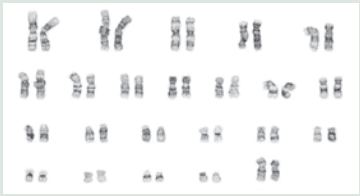

Figure 3. Maintenance of normal NSC karyotype after 5 passages in StemPro® NSC SFM.

retention of the NSC phenotype. Maintenance of NSC karyotype was also shown (Figure 3).

Differentiation potential of NSCs cultured in StemPro® NSC SFM

hNSCs are defined by the ability to differentiate to three distinct lineages—neurons, oligodendrocytes, and astrocytes. To demonstrate that hNSCs cultured in StemPro® NSC SFM retain their multipotent differentiation potential, we induced differentiation of hNSCs cultured in StemPro® NSC SFM to these three lineages (Figure 4). We also examined whether the neurons were physiologically active using an indirect measurement of action potential. Neurons emitted fluorescence upon treatment with the neurotransmitters acetylcholine, glutamate, norepinephrine, and ATP, demonstrating activity (Figure 5).

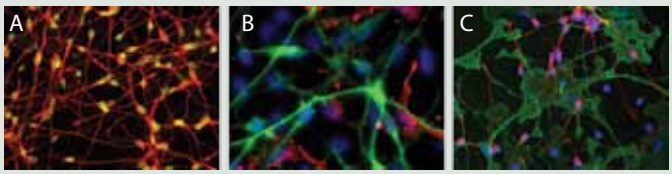

Figure 4. Differentiation potential of hNSCs cultured in StemPro® NSC SFM. hNSCs were cultured in StemPro® NSC SFM and were differentiated into neurons and glial cells. (**A**) Neurons were labeled with an anti-HuC/D antibody (green) and an anti-Dcx antibody (red). (**B**) Cells with an oligodendrocyte lineage were labeled with an anti-GalC antibody (red). Cell nuclei were labeled with DAPI (blue), and neurons were labeled with an anti-Dcx antibody (green). (**C**) Cells with an astrocyte lineage were labeled using an anti-CD44 antibody (green). Cell nuclei were labeled with DAPI (blue), and neurons were labeled with an anti-Dcx antibody (green).

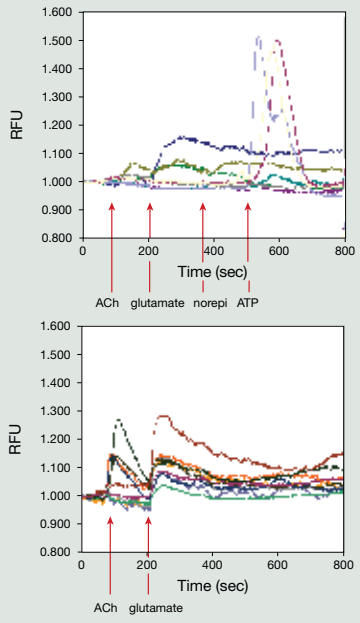

Figure 5. Differentiated neurons maintain expected physiological properties in StemPro® NSC SFM. Cells were exposed to various neurotransmitters, and responses were evaluated by indirect measurement of calcium concentration (fluo-4 emission intensity). Differentiated neurons cultured in StemPro® NSC SFM retain physiologically reactive responses to the neurotransmitters acetylcholine, glutamate, norepinephrine, and ATP. Each colored line represents measurement of an individual cell body. The arrows designate timepoints when the indicated neurotransmitter was added and a reading was taken.

Learn more about Invitrogen's neural stem cell products

StemPro® NSC SFM enables superior expansion of human and rodent NSCs derived from either embryonic stem cells or fetal tissue, and supports long-term growth and expansion of both adherent and neurosphere suspension cultures. StemPro® NSC SFM is produced under cGMP regulations and is qualified using an hNSC performance assay, providing excellent batch-to-batch consistency. For more information about StemPro® NSC SFM and other products for neurobiology, visit **www.invitrogen.com/bp60**. ■

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Product	Quantity	Cat. No.
StemPro® NSC SFM	1 kit	A1050901
CELLstart™ substrate	2 mL	A1014201
N-2 Supplement (100X)	5 mL	17502048
FGFb Rec Hu	10 µg	13256029
Neurobasal™ Medium (1X)	500 mL	21103049
B-27 Serum-Free Supplement (50X)	10 mL	17504044

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Optimal growth of neurons and neural stem cells

GIBCO® NEUROBASAL® MEDIA AND SUPPLEMENTS.

During the last two decades, the field of neurobiology has seen great advances, leading to effective treatments for diseases such as Alzheimer's, Parkinson's, and multiple sclerosis. Research in these areas has been enabled by the availability of GIBCO® serum-free neural cell culture products such as Neurobasal® Medium, B-27 Supplement, N-2 Supplement, G-5 Supplement, and the new StemPro® Neural Supplement. These cGMP-manufactured products support optimal growth of neural stem cells and all the major neural cell types (Table 1).

Neurobasal® media

Neurobasal® media are serum-free formulations that meet the special requirements of neuronal cells [1–4]. When supplemented, they support the long-term growth and normal phenotypes of neuronal cells, and enable highly enriched populations of neuronal cells to be maintained in the absence of an astrocyte feeder layer. Neurobasal® media are available in two formulations: Neurobasal® Medium is optimized for long-term growth of fetal hippocampal neurons and many other neurons of the CNS and PNS; Neurobasal®-A Medium is optimized for the long-term growth and viability of postnatal and adult neurons of the CNS and PNS. For added stability, Neurobasal® media are formulated without L-glutamine. Addition of a serum-free supplement, discussed in the following sections, is required before use.

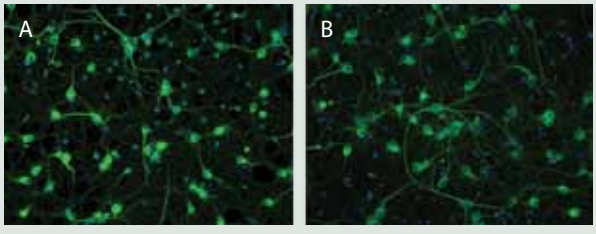

Figure 1. Immunofluorescence detection of primary rat cortex neurons (A) and primary rat hippocampus neurons (B). Primary neurons were cultured in Neurobasal® Medium supplemented with B-27 and GlutaMAX™-I for 1 week. Neurons were labeled with mouse anti-MAP2 antibody and detected using Alexa Fluor® 488 goat anti–mouse IgG antibody (green). Nuclei were stained with DAPI (blue).

B-27 Supplement

B-27 Supplement [5–12] is an optimized serum substitute that supports the long-term growth and viability of hippocampal neurons in Neurobasal® Medium without an astrocyte feeder layer (Figure 1). The lack of the excitatory amino acids glutamate and aspartate makes this supplement ideal for neurotransmitter response studies. Because of these

Table 1. Choosing the right medium for your neural cell type.

	Neural cell types	Basal media	Supplements
CNS			
Neurons	Hippocampus, striatum, substantia nigra, septum, cortex, retina, brainstem, cerebellum (granule cells, Purkinje cells), GABAergic, glutaminergic, motor, dopaminergic	Neurobasal®, Neurobasal®-A, DMEM, DMEM/F-12	B-27, N-2, StemPro®
Glial cells	Astrocytes*		
	Oligodendrocytes, radial and other specialized glia		
PNS			
Neural crest	Dorsal root ganglion, spinal ganglion, sympathetic ganglia	Neurobasal®, DMEM, DMEM/F-12	B-27, N-2
Placodes	Cranial ganglion (ears and eyes)	Neurobasal®, DMEM/F-12	B-27
Other			
Stem cells	Neural stem cells, neural precursor cells, neuroepithelial cells	Neurobasal®, KnockOut™ DMEM/F-12	B-27, N-2, StemPro®
Cell lines	Neuronal tumor stem cell lines	Neurobasal®, DMEM/F-12	B-27, N-2
Pancreatic cells	Islet cells	Neurobasal®, DMEM/F-12	B-27
Microglial cells		Neurobasal®, DMEM/F-12	B-27, G-5

* G-5 Supplement can also be used to support the growth of astrocytes.

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properties, researchers have extended applications of B-27 to the culture of neurons from various brain parts including striatum, substantia nigra, septum, cerebral cortex, cerebellum, and dentate gyrus, as well as neural stem cells, neuronal tumor cell lines, and some nonneuronal cell types.

B-27 without antioxidants, or without retinoic acid

B-27 Supplement Minus AO excludes antioxidants that could interfere with the analysis of free radical damage, apoptosis, or age-related neurodegenerative diseases. B-27 Supplement without retinoic acid is available for the growth of CNS progenitors or for stem cells that require controlled levels of vitamin A in the culture.

StemPro® Neural Supplement

Due to their scarce availability, the *in vitro* expansion of neural stem cells (NSCs) and neural progenitor cells has been a challenge to academic and clinical researchers. To address this challenge, we developed StemPro® Neural Supplement—a convenient and cost-effective serum-free supplement for optimal proliferation of human and rat NSCs and neural progenitor cells such as glial precursor cells (GPCs) (Figure 2).

N-2 Supplement

N-2 Supplement is a chemically defined additive for Neurobasal® media. N-2 can be used for the growth of rat primary embryonic neurons, embryonic neurospheres, and CNS progenitor cells when combined with the growth factors bFGF and EGF in Neurobasal® Medium or DMEM. N-2 can also be used for the growth and expansion of post-mitotic neurons and tumor cells with neuronal phenotypes [13–15].

G-5 Supplement

G-5 Supplement is a chemically defined additive for the growth of glial cells in Neurobasal® media or other basal formulations such as DMEM, DMEM/F-12, and EMEM. G-5 also supports growth of the primary and serial tumor lines of the astrocytic phenotype [16,17].

Products for optimal neural cell culture

Learn more about Neurobasal® media, supplements, and other related products for neural cell culture at **www.invitrogen.com/bp60**. ■

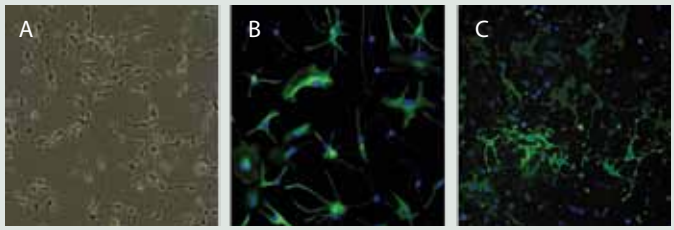

Figure 2. Proliferation and differentiation of human glial progenitor cells (hGPCs) in serum-free medium. (**A**) Proliferation of hGPCs in serum-free medium supplemented with StemPro® Neural Supplement for 3 passages. hGPCs differentiated into (**B**) astrocytes expressing the phenotype marker GFAP (green) and (**C**) oligodendrocytes expressing the phenotype marker GalC (green). Cell nuclei were labeled with DAPI (blue).

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Product	Quantity	Cat. No.
Neurobasal® Medium (1X)	500 mL	21103049
Neurobasal® Medium without Phenol Red (1X)	500 mL	12348017
Neurobasal®-A Medium (1X)	500 mL	10888022
Neurobasal®-A Medium without Phenol Red (1X)	500 mL	12349015
B-27 Supplement (50X)	10 mL	17504044
B-27 Supplement Minus AO (50X)	10 mL	10889038
B-27 Supplement Minus Vitamin A (50X)	10 mL	12587010
StemPro® Neural Supplement (50X)	10 mL	A1050801
N-2 Supplement (100X)	5 mL	17502048
G-5 Supplement (100X)	1 mL	17503012
GlutaMAX™-I Supplement	100 mL	35050061
KnockOut™ DMEM/F-12	500 mL	12660012

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Ready-to-use, high-quality neural cells

GIBCO® RAT NEURAL PRIMARY AND STEM CELLS.

As an alternative to freshly isolated neural primary cells and neural stem cells (NSCs), Invitrogen now offers GIBCO® rat fetal neural stem cells, rat glial precursor cells, rat primary cortical astrocytes, and rat primary cortex and hippocampal neurons. These ready-to-use cells have been developed for optimal performance with high purity, minimal lot-to-lot variability, and superior post-thaw viability.

Rat fetal neural stem cells

GIBCO® rat fetal neural stem cells are isolated from the cortex of Sprague-Dawley rats at day 14 of gestation, and can be used for both adherent cell culture and neurosphere suspension culture. These cells can be expanded in StemPro® NSC SFM (see page 16) for up to 3 passages without differentiation. During expansion, more than 75% of the NSCs retain their undifferentiated phenotype (Figure 1). GIBCO® Rat Fetal NSCs can also spontaneously differentiate into neurons, oligodendrocytes, and astrocytes as shown by immunocytochemistry.

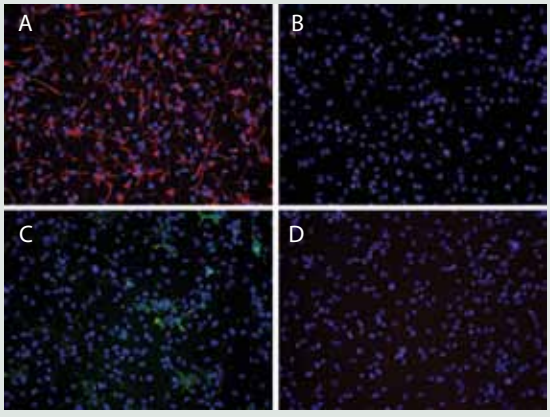

Figure 1. Marker expression in undifferentiated neural stem cells (NSCs). Rat fetal NSCs were cultured in StemPro® NSC SFM for 10 days to passage 3. While approximately 90% of the cells stained positive (red) for the undifferentiated NSC marker nestin (**A**), less than 10% of the cells stained positive for differentiated cell type markers doublecortin (DCX) (<5%) (**B**), GalC (<5%) (**C**), and GFAP (<8%) (**D**). Nuclei were stained with DAPI (blue) in all images. Magnification: 20x.

Rat glial precursor cells

Glial precursor cells (GPCs) are restricted progenitors; the majority of their downstream progeny are oligodendrocytes and astrocytes. GIBCO® rat glial precursor cells are isolated from the cortex of newborn Sprague-Dawley rats. More than 80% of the GPCs retain the undifferentiated phenotype marker A2B5 upon proliferation (Figure 2), and retain the ability to differentiate into oligodendrocytes and astrocytes.

Rat primary cortical astrocytes

Astrocytes constitute the most numerous cell type in the central nervous system (CNS), and have critical roles in adult CNS homeostasis. GIBCO® rat primary cortical astrocytes are isolated from the cortex of

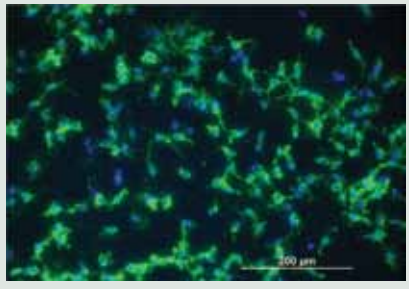

Figure 2. Rat glial precursor cells (GPCs) stained by indirect immunofluorescence for the cell surface marker A2B5 (green). Nuclei were stained with DAPI (blue). Cells were maintained in the undifferentiated state in GPC recovery media for 3 days prior to 4% paraformaldehyde fixation and staining.

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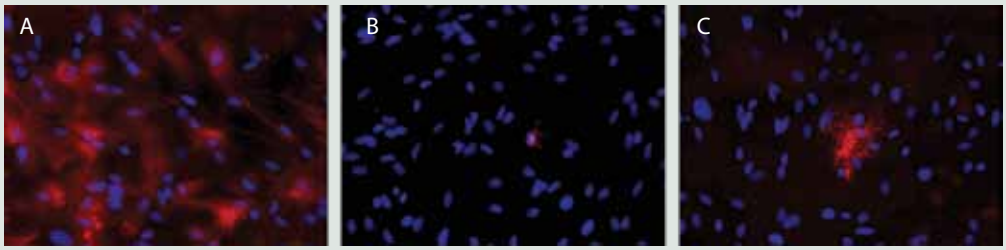

Figure 3. GFAP expression in rat cortical astrocytes. Cells were cultured for 3 days to passage 2. While approximately 80% of the cells stained positive (red) for the astrocyte marker GFAP (**A**), less than 10% of the cells stained positive for the immature neuronal marker doublecortin (DCX) (**B**) and the oligodendrocyte marker galactosylceramide (GalC) (**C**). Nuclei were stained with DAPI (blue) in all images. Magnification: 20x.

Sprague-Dawley rats at day 19 of gestation and are cryopreserved at the end of first passage. These astrocytes demonstrate superior post-thaw viability and can be further expanded for at least two additional passages. More than 80% of GIBCO® rat primary cortical astrocytes stain positive for the astrocyte-specific marker glial fibrillary acidic protein (GFAP) upon expansion (Figure 3).

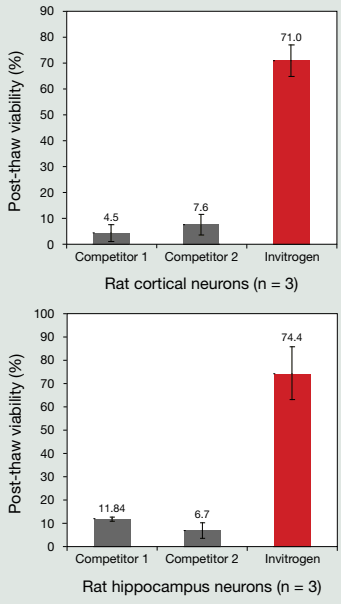

Figure 4. Superior post-thaw viability of Invitrogen's GIBCO® rat primary cortex and hippocampus neurons compared to competitor neurons.

Rat primary cortex and hippocampus neurons

GIBCO® rat primary cortex and hippocampus neurons are isolated from Fischer 344 rats at gestation day 18, then cryopreserved in a medium containing 10% DMSO. These cells exhibit high purity with a low glial cell background (<10%). Neurons are extremely sensitive to the freezing and thawing process, which can result in cell death. However, GIBCO® rat primary cortex and hippocampus neurons consistently exhibit post-thaw viability in the range of 50–80%, unlike competitor neurons, which exhibit post-thaw viability in the range of 4–20% (Figure 4).

Find the right neural stem cells for your research

Learn more about primary and stem cell systems available for your research at **www.invitrogen.com/bp60**. ■

Product	Quantity	Cat. No.
GIBCO® Rat Fetal Neural Stem Cells (~2 x 10^6 cells)	1 mL	N7744100
GIBCO® Rat Fetal Neural Stem Cell Kit	1 kit	N7744200
GIBCO® Rat Glial Precursor Cells (~1 x 10^6 cells)	1 mL	N7746100
GIBCO® Rat Primary Cortical Astrocytes (~1 x 10^6 cells)	1 mL	N7745100
GIBCO® Rat Primary Cortex Neurons (~1 x 10^6 cells)	1 mL	A1084001
GIBCO® Rat Primary Cortex Neurons (~4 x 10^6 cells)	1 mL	A1084002
GIBCO® Rat Primary Hippocampus Neurons (~1 x 10^6 cells)	1 mL	A1084101

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Tools for the study of Alzheimer's disease

AMYLOID BETA ANTIBODIES, PEPTIDES, ELISAS, AND LUMINEX® KITS.

Alzheimer's disease is a neurodegenerative disorder characterized by the aggregation of the amyloid beta (also known as Aβ or β-amyloid) peptide in the brain. This peptide, a 39 to 43 amino acid cleavage product of the amyloid precursor protein (APP), is the major constituent of the protein plaques observed in the brains of Alzheimer's patients. Because the disease is currently incurable, many efforts are focused on understanding the role of amyloid beta in the onset and progression of the disease. Aβ antibodies, synthetic peptides, ELISAs, and Luminex® kits are valuable tools for the detection and characterization of APP and Aβ cleavage isoforms. Here we provide an overview of these tools and describe their applications for Alzheimer's disease research.

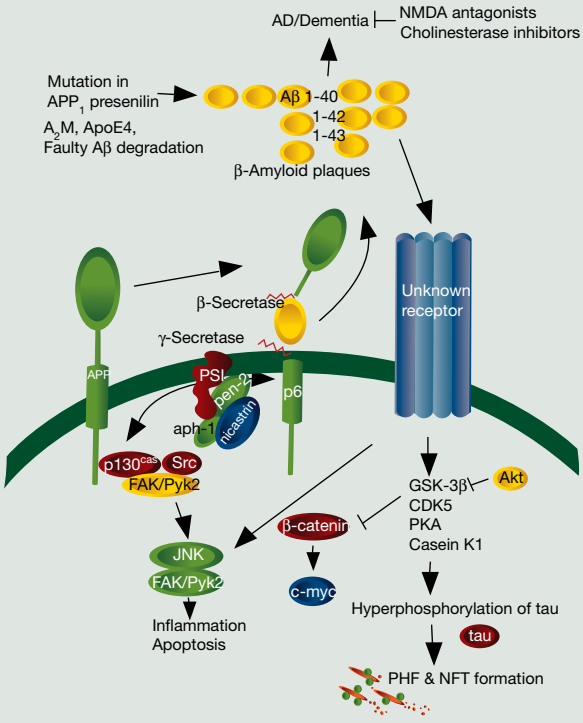

Figure 1. β-Amyloid pathway. Illustration of various β-amyloid signal transduction pathways and their interactions with tau phosphorylation and cytokine production.

Amyloid beta antibodies

Cleavage of APP by β-secretase generates a variety of Aβ isoforms, ranging from 39 to 43 amino acids in length (Figure 1). The most common isoforms are $A\beta_{40}$ (Aβ [1–40]) and $A\beta_{42}$ (Aβ [1–42]), with $A\beta_{42}$ having a greater tendency to aggregate. A wide selection of antibodies targeting both APP and its cleavage isoforms is available (Table 1). These antibodies are available both unconjugated and biotin-labeled, from a variety of

Table 1. Anti-APP and anti-At antibodies.

Antibody	Reactive species	Applications	Quantity	Cat. No.
Anti-APP	Hu	E, ICC, IF, IHC, WB	100 µL	130200
Anti-APP	Hu, Ms	IHC, IP, WB	100 µg	366900
Anti-Aβ (N-term)	Hu	E, IF, IHC, IP, WB	100 µg	374200
Anti-Aβ (N-term)	Hu	DB, E, IHC, RIA	50 µg	4433850
Anti-Aβ (N-term)	Hu	E, IHC	100 µL	AHB0121
Anti-Aβ (N-term)	Hu	DB, E, IHC, RIA	100 µg	44338100
Anti-Aβ [1–20]	Hu	E, WB	50 µg	AHB0272
Anti-Aβ [1–40]	Hu	DB, E, IHC, RIA	100 µg	44136
Anti-Aβ [1–40]	Hu, Ms,* Rt*	DB, E, RIA	25 µg	44348A
Anti-Aβ [1–40], biotin conjugate	Hu, Ms,* Rt*	DB, E, RIA	25 µg	443489
Anti-Aβ [1–42]	Hu, Ms	E, IHC	25 µg	437900
Anti-Aβ [1–42]	Hu, Ms, Rt	DB, E, IHC, RIA	25 µg	44344
Anti-Aβ [1–42], biotin conjugate	Hu, Ms, Rt	DB, E, RIA	25 µg	443449
Anti-Aβ [1–43]	Hu	DB, E, RIA	25 µg	44340

*Reactivity predicted but not tested.

Reactive species: **Hu** = human, **Ms** = mouse, **Rt** = rat
Applications: **DB** = dot blot, **E** = ELISA, **IC** = immunocytochemistry, **IF** = immunofluorescence, **IHC** = immunohistochemistry, **IP** = immunoprecipitation, **RIA** = radioimmunoassay, **WB** = western blot.

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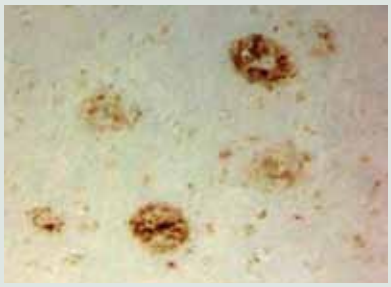

Figure 2. Immunohistochemical staining of amyloid plaques. Plaques in the parietal cortex of the brain of a patient with Alzheimer's disease were detected using a rabbit monoclonal antibody, anti-Aβ 42 (Cat. No. 437900), and visualized with a secondary antibody–HRP conjugate (Cat. No. 878963).

host species, and are validated for diverse applications including western blotting, immunoassays, immunoprecipitation, immunocytochemistry, and immunohistochemistry. Immunostaining of Aβ enables the detection of Aβ deposits in the brains of human Alzheimer's disease patients or in animal model systems (Figure 2). Anti-Aβ monoclonal antibodies have also been shown to reduce amyloid plaque formation *in vitro* and in animal studies [1,2].

Amyloid beta peptides

Because amyloid plaques are usually too fragile to extract from the brains of diseased patients, researchers have developed techniques to study synthetic plaque assembly and growth *in vitro* [3]. Invitrogen offers $A\beta_{40}$ and $A\beta_{42}$ synthetic peptides in addition to an inactive control peptide for *in vitro* studies of Aβ aggregation and deposition (Table 2).

Table 2. Aβ synthetic peptides and peptide fragments.

Peptide	Quantity	Cat. No.
Aβ [1–40] peptide	1 mg	03136
Aβ [1–40] peptide, biotin	0.5 mg	03243
Aβ [1–40], ultra pure	1 mg	03138
Aβ [40–1] (inactive control) peptide	1 mg	03245
Aβ [40–1] (inactive control) peptide, biotin	0.5 mg	03246
Aβ [1–40, Gly5, Phe10, Arg13] peptide, rat homolog	1.0 mg	03189
Aβ [1–42] peptide	1 mg	03112
Aβ [1–42] peptide	0.5 mg	03111
Aβ [42–1] (inactive control) peptide	1.0 mg	03247
Aβ [1–12] peptide fragment	0.5 mg	03260
Aβ [1–28] peptide fragment	0.5 mg	03142

Table 3. ELISA and Luminex® kits for Alzheimer's disease research.

Marker	ELISA kits	Luminex® kits
α-Synuclein	KHB0061	NA
APP	KHB0051	NA
Aggregated Aβ	NA	LHB3491
$A\beta_{40}$	KHB3481, KHB3482, KMB3481	LHB3481
$A\beta_{42}$	KHB3544, KHB3441, KHB3442, KMB3481	LHB3441
Tau (total)	KHB0042, KHB0041	LHB0041

NA = not applicable.

Amyloid beta ELISAs and Luminex® kits

Enzyme-linked immunosorbent assays (ELISAs) [4] or Luminex® bead-based assays [5] can be used for sensitive, quantitative detection of APP, Aβ, and α-synuclein, another component of Alzheimer's plaques, from cerebrospinal fluid (CSF), brain homogenate, or tissue culture supernatant samples (Table 3). In a recent study, the levels of $A\beta_{42}$ and the tau protein in CSF were found to correlate with the transition from latent Alzheimer's disease to the onset of minor cognitive impairment [5].

Advancing neuroscience research

Start exploring Invitrogen's comprehensive selection of tools for neuroscience research, including antibodies, fluorescent probes, neural primary and stem cells, and neural media. Learn more at **www.invitrogen.com/bp60**. ■

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A growing list of tools for the violet laser

A GUIDE TO VIOLET-EXCITED REAGENTS FOR FLOW CYTOMETRY.

Violet lasers are critical to achieving the full analytical capability of modern flow cytometry. Violet-excited reagents allow researchers to take significant steps forward in the analysis of cellular function—far beyond what has been achieved with immunophenotyping alone. Here we describe a comprehensive collection of Molecular Probes® violet-excitable reagents and assays for immunophenotyping, viability, cell cycle, and apoptosis. Because these reagents are excited by your flow cytometer's violet laser, you have more options when using the crowded blue and red excitation lines (Table 1).

Multicolor immunophenotyping

Immunophenotyping applications represent the majority of flow cytometer use. Violet-excitable reagents allow three to four colors to be easily added to standard antibody panels. Pacific Blue™ dye (emission maximum ~455 nm) is commonly used and can easily be paired with Pacific Orange™ dye (emission maximum ~551 nm) (Figure 1A). Both dyes are available as direct, Zenon®, and second-step conjugates.

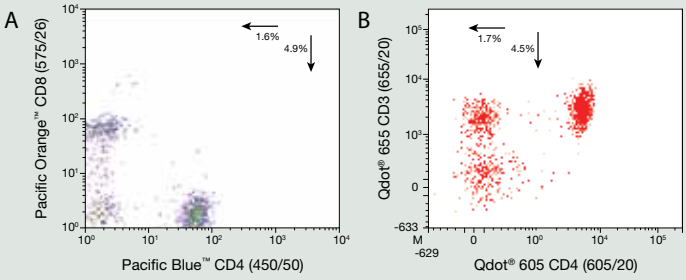

Figure 1. Violet-excited fluorophores for antibody staining. Human peripheral blood leukocytes (PBLs) were stained with either (**A**) Pacific Blue™ anti-CD4 and Pacific Orange™ anti-CD8 or (**B**) Qdot® 605 anti-CD4 and Qdot® 655 anti-CD3 antibodies. Samples were analyzed on a BD LSR™ II flow cytometer using violet diode laser excitation. Plots are gated on lymphocytes by scatter. Axes are labeled with the bandpass filters used; plots are labeled with compensation values (arrows).

Qdot® nanocrystal–conjugated primary antibodies deliver the power of more than seven colors in flow cytometry. The bright fluorescence and narrow emission spectra of Qdot® nanocrystals make them ideal labels for this platform. They work well even with relatively low compensation (Figure 1B), and can be combined with conventional fluorophores in the same experiment. Qdot® nanocrystals are also available attached to a range of human and mouse markers and as second-step reagents.

Viability and vitality dyes

Nonspecific staining from damaged cells can interfere with data collected on critical cell populations; the use of viability dyes allows you to exclude dead cells from analysis. SYTOX® Blue Dead Cell Stain (Cat. No. S34857), a traditional viability reagent, is a high-affinity dye that exhibits >500-fold fluorescence enhancement upon binding to nucleic acid. The dye easily penetrates cells with compromised plasma membranes but will not cross intact cell membranes.

Fixable dead-cell dyes comprise a new class of viability reagents that covalently react with available amines. In healthy cells, these dyes are excluded from the interior and therefore only label surface proteins. In dead cells they enter and label proteins throughout the cytoplasmic volume. Dead cells have at least 50-fold greater fluorescence than live cells, and the covalently labeled cells can be aldehyde-fixed and permeabilized for intracellular staining protocols without losing viability discrimination (Figure 2). There are three options for use with violet excitation: LIVE/DEAD® Fixable Violet (~460 nm emission, Cat. No. L34955), Aqua (~530 nm emission, Cat. No. L34957), and Yellow (~560 nm emission, Cat. No. L34959) Dead Cell Stains. In addition, ArC™ amine-reactive beads (Cat. No. A10346) are now available to help researchers set compensation with fixable dead-cell reagents.

Cell vitality can be measured by the esterase activity that is ubiquitous in the cytosol of live cells. Cell-permeant calcein violet AM (Cat. No. C34858) is cleaved by esterases to form highly fluorescent calcein violet (emission ~452 nm), which is retained in the cell.

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Cell cycle analysis

There are several dyes available for analyzing the DNA distribution in both fixed and viable cells. When loaded, these dyes allow the clear distinction of the different phases of the cell cycle: G_0/G_1 (2N), S (DNA replication), and G_2/M (4N). FxCycle™ Violet Stain (Cat. No. F10347) is a violet laser–excited dye used for cell cycle analysis in fixed cells (Figure 3) that exhibits little spectral overlap into channels of other lasers.

Vybrant® DyeCycle™ Violet (emission maximum ~440 nm, Cat. No. V35003) is used for DNA content analysis in live cells. DyeCycle™ Violet Stain is DNA selective, cell membrane–permeant, and essentially non-fluorescent until bound to double-stranded DNA. Vybrant® DyeCycle™ Violet Stain is excited by 405 nm and near-UV laser lines. This live-cell dye is essentially nontoxic and offers the possibility of cell sorting based on DNA content. Vybrant® DyeCycle™ Violet Stain has also been shown to identify stem cell side populations in hematopoietic tissues using the violet laser [1] and can be used to detect condensed chromatin in studies of apoptosis (see Table 1).

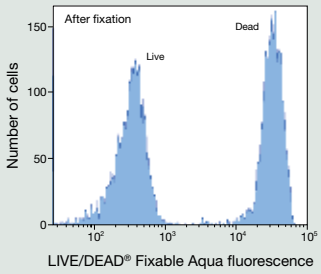

Figure 2. Use of fixable dead-cell dyes. The reagents in the LIVE/DEAD® Fixable Aqua Dead Cell Stain Kit were used to differentially stain a mixture of live (left peak) and heat-treated (right peak) Jurkat cells, and were fixed in 3.7% formaldehyde following the staining reaction. Unfixed cells show equivalent separation. Samples were analyzed by flow cytometry using 405 nm excitation and ~525 nm emission.

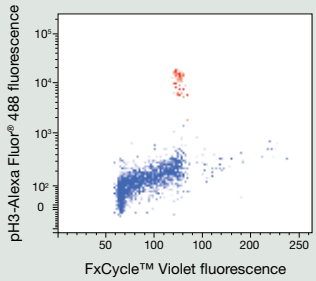

Figure 3. Multiparametric cell cycle and immunophenotypic analysis using FxCycle™ Violet Stain. TF-1 erythroblast cells were alcohol-fixed overnight, washed, and then suspended in 0.1% Triton® X-100/PBS/1% BSA before staining with anti–histone H3 [pS10] purified antibody (Cat. No. 441190G) complexed with Zenon® Alexa Fluor® 488 Rabbit IgG labeling reagent and FxCycle™ Violet Stain. For cell cycle analysis, 405 nm excitation was used with a 440/40 nm bandpass filter. The pH3 signal (red) identifies cells that are in mitosis.

Cell proliferation assays

Nucleoside incorporation provides direct measurement of new DNA synthesis in cells and has traditionally been detected by →

Table 1. Violet-excited reagents listed by function and approximate emission range.

Function	Blue: ~440–460 nm	Green: ~530 nm	Orange: ~565–585 nm	Red: >600 nm
Antibody fluorochromes	• Pacific Blue™ • Alexa Fluor® 405	• Qdot® 525	• Pacific Orange™ • Qdot® 565 • Qdot® 585	• Qdot® 605 • Qdot® 800
Traditional viability dyes	• SYTOX® Blue • FxCycle™ Violet	• SYTOX® Blue		
Fixable dead-cell dyes	• Fixable Violet	• Fixable Aqua	• Fixable Yellow	
Metabolic activity	• Calcein violet			
Cell division: nucleotide incorporation	• Pacific Blue™ EdU			
Cell division: cell cycle for living cells	• DyeCycle™ Violet			
Cell division: cell cycle for fixed cells	• FxCycle™ Violet • DyeCycle™ Violet • SYTOX® Blue	• SYTOX® Blue		
Apoptosis: membrane asymmetry	• Pacific Blue™ Annexin V • PO-PRO™-1			
Apoptosis: nuclear changes	• DyeCycle™ Violet			
Apoptosis: membrane integrity	• SYTOX® Blue • Fixable Violet	• SYTOX® Blue • Fixable Aqua	• Fixable Yellow	
Calcium flux				• Fura Red™
Cell count	• CountBright™ Beads	• CountBright™ Beads	• CountBright™ Beads	• CountBright™ Beads

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incorporation of the nucleoside analog bromodeoxyuridine (BrdU) into DNA, followed by the addition of an anti-BrdU antibody. This method requires DNA denaturation (using HCl, heat, or DNase) to expose the BrdU to the antibody—a step that can be lengthy and difficult to perform consistently. The Click-iT® EdU Pacific Blue™ Flow Cytometry Assay Kit (Cat. No. A10034) eliminates the need to denature DNA. The Click-iT® advantage is in the chemistry—small, unique, and low-background labeling and detection moieties that react specifically and covalently with one another. 5-ethynyl-2′-deoxyuridine (EdU) is a nucleoside analog containing an alkyne. In a copper-catalyzed reaction, the alkyne reacts with a Pacific Blue™ dye–labeled azide, forming a stable covalent bond. The small size of the azide reagent allows efficient access to the DNA without the need for harsh cell treatment, which simplifies the assay considerably but delivers the same results (Figure 4). Click-iT® EdU labeling is compatible with most fixation protocols.

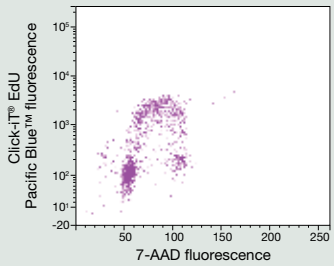

Figure 4. Flow cytometric analysis of cell proliferation using Click-iT® technology. Jurkat cells were treated with 10 µM EdU for 2 hr, then fixed and permeabilized, stained via the click reaction, washed, and counterstained for cell cycle analysis using 7-aminoactinomycin D (7-AAD). Cells were analyzed using a flow cytometer with 405 and 488 nm excitation.

Detection of apoptosis

Apoptosis is the carefully regulated process of cell death that occurs as a part of normal development. Three of our apoptosis kits incorporate violet-excitable stains. The Pacific Blue™–annexin V conjugate (Cat. No. A35136) detects changes in membrane asymmetry characteristic of apoptotic cells (Figure 5A). Slight changes in permeability to dyes such as PO-PRO™-1 dye (Cat. No. A35123) can also be used to identify apoptotic cells (Figure 5B). Unlike annexin conjugates, PO-PRO™-1 dye can provide efficient staining of both trypsinized adherent and suspension cells [2]. In addition, DyeCycle™ Violet stain (Cat. No. A35135) can detect chromatin condensation, a late event in apoptosis (Figure 5C).

A growing list of violet-excited reagents

The list of Molecular Probes® violet laser–compatible applications continues to grow as we develop new fluorophores specifically for this laser. In addition to the applications above, violet excitation can be used with Fura Red™ dye for measurement of calcium flux and with CountBright™ Absolute Counting Beads to quantify cell concentrations in samples. As a result, researchers will be able to easily expand their multispectral capabilities on flow cytometers. Find out more about our entire line of violet-excitable reagents at **www.invitrogen.com/bp60**. ■

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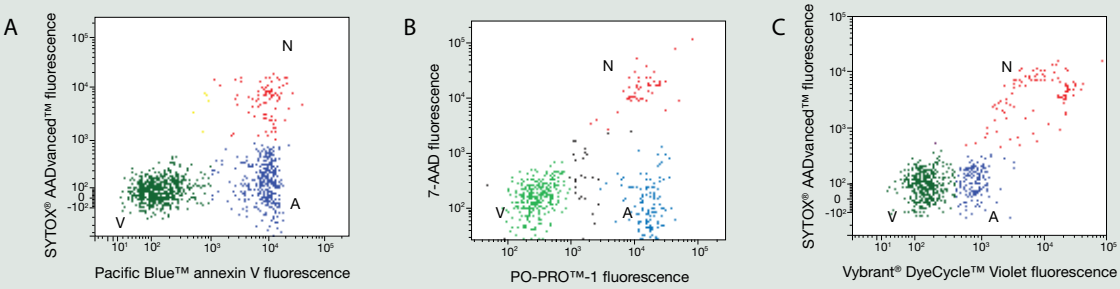

Figure 5. Violet-excited detection of apoptosis. Jurkat cells were treated with 10 µM camptothecin for 4 hr and then stained with (**A**) the reagents in the Apoptosis Kit–Pacific Blue™ Annexin V/SYTOX® AADvanced™, (**B**) PO-PRO™-1 and 7-AAD stains, which can be used for adherent or suspension cells, or (**C**) Vybrant® DyeCycle™ Violet and SYTOX® AADvanced™ stains. Cells were analyzed by flow cytometry using 405 nm and 488 nm excitation. Viable (**V**), apoptotic (**A**), and necrotic (**N**) populations are labeled in each plot.

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A fluorogenic, target-specific reagent for small animal *in vivo* imaging

ALEXA FLUOR® 680 DYE–LABELED PROBE DRAMATICALLY IMPROVES TUMOR DETECTION, STAGING, AND MONITORING.

Fluorescent near-infrared (NIR) dye–based *in vivo* imaging holds significant promise for understanding tumor growth and treatment strategies. However, creating fluorescent bioconjugates suitable for *in vivo* studies is difficult due to intramolecular quenching and rapid clearance through the liver. To avoid these issues, we offer SAIVI™ antibody/protein kits that control the degree of labeling (DOL) to 2–3 dye molecules per protein. But in a recent paper, researchers approach the problem in reverse—rather than avoiding intramolecular quenching, the team intentionally causes quenching by overlabeling an antibody with Invitrogen's Alexa Fluor® 680 dye. The result is a highly specific, fluorogenic reagent that targets only tumor cells and is not cleared to the liver.

Creating a targeted, fluorogenic NIR reagent for tumor imaging

Ogawa and coworkers detail a protocol using Alexa Fluor® 680 dye and a therapeutic, humanized monoclonal HER-2 antibody, trastuzumab (Genentech) [1]. By overlabeling with 7–9 fluorophores per protein molecule, the authors create a quenched probe that is not cleared to the liver. This probe, when injected into animals or presented *in vitro*, targets only the tumor cells; after binding to the cells, the probe is internalized. When the complex degrades, only the affected cells fluoresce as the quenching interactions are uncoupled. Traditional nonquenched probes contribute generalized fluorescence at the tumor site due to leaky vasculature, a hallmark of tumor neovessels. By creating a quenched probe, the authors overcame this barrier; the probe was only detectable when bound and internalized by targeted cells (Figure 1).

When the authors compared Alexa Fluor® 680 dye–labeled conjugates to those prepared using an equivalent NIR dye, Cy®5.5 (GE Healthcare), they found that only the Alexa Fluor® dye–based probe showed high levels of specific targeting and remained in circulation, avoiding rapid clearance through the liver (data not shown).

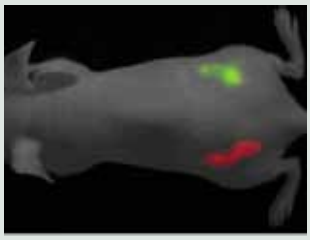

Figure 1. *In vivo* fluorescence images of tumor-bearing mice two days after injection. Self-quenched trastuzumab (Herceptin) Alexa Fluor® 680 conjugates were injected intravenously into mice bearing 3T3/HER2$^+$ and Balb/3T3/ZsGreen Fluorescent Protein–tagged tumor cells. The image shows targeting of the probe to the HER2$^+$ tumor (red), but not the HER2$^-$/ZsGreen$^+$ tumor (green), with minimal background signal from the self-quenched conjugate. Image submitted by Hisataka Kobayashi, National Cancer Institute, NIH.

Two conjugation strategies for *in vivo* imaging with NIR dyes

Our scientists have previously demonstrated that a high DOL causes rapid clearance to the liver when mouse and rabbit antibodies are used for *in vivo* imaging. However, humanized therapeutic antibodies have been designed to stay in circulation for days, and this paper shows that overlabeling these antibodies with Alexa Fluor® 680 dye—although not with other NIR dyes—does not increase clearance to the liver.

Thus, two conjugation strategies are available for NIR dye–based *in vivo* imaging. The SAIVI™ Rapid Antibody Labeling Kits may be best suited for research applications using mouse and rabbit antibodies, where internalization and degradation are not expected, to ensure low DOL, minimal clearance to the liver, and minimal quenching. Using a high DOL with the Alexa Fluor® 680 dye, as described by Ogawa and coworkers, should be considered for humanized monoclonal antibodies that are intended to be internalized, and thus unquenched. Learn more about Alexa Fluor® dyes for *in vivo* imaging at **www.invitrogen.com/bp60**. ■

Reference

1. Ogawa M, Regino CA, Choyke PL et al. (2009) *Mol Cancer Ther* 8:232–239.

Product	Quantity	Cat. No.
For high degree of labeling for fluorogenic, quenched probes		
Alexa Fluor® 680 Protein Labeling Kit	3 labelings	A20172
Alexa Fluor® 680 carboxylic acid, succinimidyl ester	1 mg	A20008
Alexa Fluor® 680 carboxylic acid, succinimidyl ester	5 mg	A20108
Alexa Fluor® 680 C_2-maleimide	1 mg	A20344
For low degree of labeling		
SAIVI™ Alexa Fluor® 647 Antibody/Protein 1 mg-Labeling Kit	3 labelings	S30044
SAIVI™ Rapid Antibody Labeling Kit, Alexa Fluor® 680	3 labelings	S30045
SAIVI™ Rapid Antibody Labeling Kit, Alexa Fluor® 750	3 labelings	S30046

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Interrogating potential drug targets for Parkinson's disease

BIOCHEMICAL AND CELL-BASED ASSAYS FOR DOPAMINE D2 AND LRRK2.

Parkinson's disease (PD), a degenerative disorder of the central nervous system caused by a loss of dopamine-secreting neurons, results in altered neuronal activity within regions of the brain that regulate movement. Classical symptoms of the disease include resting tremors, bradykinesia, rigidity, and postural instability. Current therapies have focused on treating the symptoms of PD; however, recently identified genetic mutations may spur the development of new therapies to address the cause of the disease. This complex disease involves many cellular components and pathways, offering a variety of potential targets—including the G-protein–coupled receptor (GPCR) dopamine D2 and the serine/threonine protein kinase LRRK2 (leucine-rich repeat kinase 2)—for drug therapeutics (Figure 1). Invitrogen offers biochemical and cell-based assays for studying these key targets within the pathways regulating PD. Alternatively, you can outsource your screening and profiling projects to Invitrogen's SelectScreen® Service.

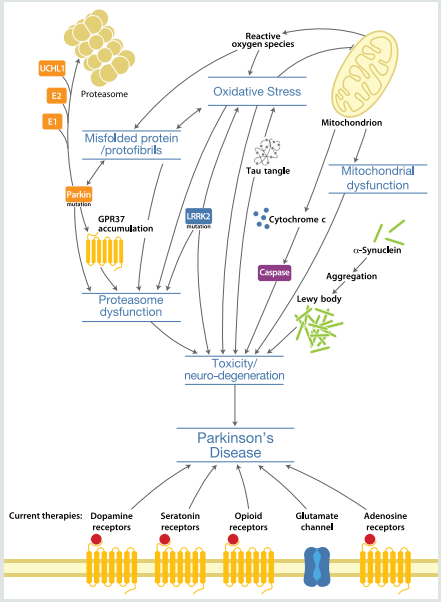

Figure 1. Schematic of cellular and pathway components involved in Parkinson's disease, along with current therapy approaches.

Measuring dopamine D2 activation using GeneBLAzer® technology

The first-line treatment for PD is the use of dopamine agonists, many of which act through the dopamine D2 GPCR. Invitrogen has developed a GeneBLAzer® cell-based assay to monitor activation of this GPCR via a beta-lactamase reporter technology. GeneBLAzer® technology employs a membrane-permeant fluorescence resonance energy transfer (FRET)-based substrate that allows detection of a functional response in live cells. The dual-emission wavelength readout significantly reduces experimental variables. To aid in the identification of possible

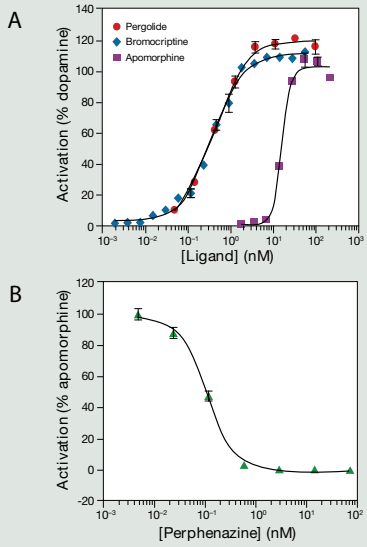

Figure 2. Measuring dopamine D2 activation or inhibition using GeneBLAzer® technology. (**A**) GeneBLAzer® D2-Gqo5-NFAT-*bla* CHO-K1 (Cat. No. K1708) cells were activated by incubation with serial dilutions of pergolide, bromocriptine, or apomorphine and then loaded with LiveBLAzer™-FRET B/G Substrate (Cat. No. K1095). (**B**) Inhibition of apomorphine-induced activation by perphenazine. GeneBLAzer® D2-Gqo5-NFAT-*bla* CHO-K1 cells were incubated with serial dilutions of perphenazine prior to stimulation with 18 nM apomorphine. Cells were then loaded with LiveBLAzer™-FRET B/G Substrate. Fluorescence emission ratios were normalized such that 0% is equivalent to the absence of stimulant while 100% equals that observed with (**A**) 1 µM dopamine or (**B**) 18 nM apomorphine.

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agonists of the dopamine receptor, the GeneBLAzer® D2-Gqo5-NFAT-*bla* CHO-K1 cell line was developed to monitor activation of the D2 GPCR via a beta-lactamase reporter gene. The cell line contains the stably integrated human dopamine receptor 2 (D2), a beta-lactamase (*bla*) reporter gene under control of the nuclear factor of activated T cells response element (NFAT), as well as the chimeric G-protein Gqo5 in the CHO-K1 cell line. The results show that D2 receptor agonism can be quantified for the Parkinson's disease drugs bromocriptine, pergolide, and apomorphine; furthermore, D2 receptor antagonism was readily quantified using apomorphine as the agonist (Figure 2).

Alternatively, this cell line may be used to determine the specificity of receptor agonists and/or antagonists. The SelectScreen® Cell-Based GPCR Profiling Service allows you to not only identify new agonists and/or antagonists for the D2 receptor, but also determine the selectivity of your compounds for the receptor by profiling against a panel of any of our 132 available GPCR cell lines.

LanthaScreen® TR-FRET assay for LRRK2

Mutations in the kinase-encoding gene LRRK2 have been identified in association with PD [1]. At least 20 such mutations have been discovered, scattered across the large, multidomain protein. The most prevalent mutation, G2019S, has been demonstrated to increase the kinase activity of LRRK2. Discovering an LRRK2 inhibitor could lead to new therapeutic agents for treating the disease. Invitrogen has developed a collection of high-throughput screening tools for wild-type LRRK2 and the LRRK2 G2019S mutant. We have generated highly purified and active LRRK2 enzymes and the kinase-dead mutant LRRK2 D1994A (Figure 3), which can be used to screen potential inhibitors in a fully validated LanthaScreen® TR-FRET kinase assay. This assay provides sensitive and specific detection of LRRK2 kinase activity using a peptide substrate derived from ezrin/radixin/moesin (ERM) labeled with fluorescein. Phosphorylation of the peptide recruits a terbium-labeled phosphospecific antibody, resulting in FRET. Due to the TR-FRET readout, the assay is highly resistant to compound interference and is also suitable for evaluating inhibitor potencies (Figure 4).

Let us enable your search for potential mediators of LRRK2 activation by screening your compound libraries using our SelectScreen® Library Screening Service. We can also profile your lead candidates using the SelectScreen® Biochemical Kinase Profiling Service.

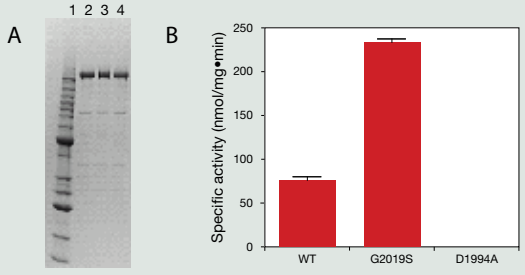

Figure 3. Analysis of wild-type LRRK2 and various LRRK2 mutants. **(A)** Purity was determined by SDS-PAGE. Lanes contain BenchMark™ Ladder (lane 1), and wild-type (Cat. No. PV4873), G2019S (Cat. No. PV4881), and D1994A (inactive) LRRK2 variants (lanes 2–4, respectively). **(B)** Activity of each LRRK2 variant was determined using a radiometric assay. LRRK2 D1994A shows no activity using this method, confirming that the kinase is inactive.

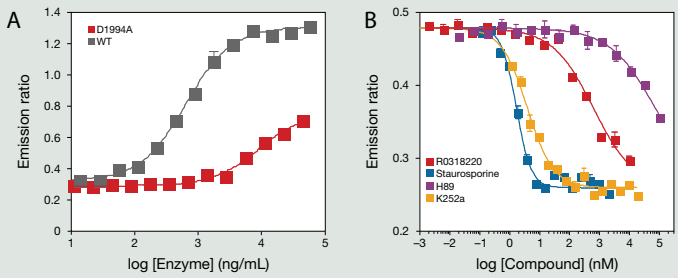

Figure 4. Validation of the LanthaScreen® assay for LRRK2. **(A)** Differentiation of response between wild-type and kinase-dead LRRK2, which was not observed with other substrates. Assay components used were the LanthaScreen® Tb-anti-ERM (pLRRKtide) Antibody (Cat. No. PV4899) and the Fluorescein-ERM (LRRKtide) Substrate (Cat. No. PV4901). **(B)** Inhibition of LRRK2 with small-molecule kinase inhibitors using the LanthaScreen® format.

Tools for studying potential PD drug targets

Take advantage of Invitrogen's biochemical and cell-based assays for dopamine D2 and LRRK2, and rely on Invitrogen's SelectScreen® Service for outsourcing your screening and profiling projects. Learn more about these assays and services at **www.invitrogen.com/bp60**. ■

Reference

1. Cookson M et al. (2007) *J Neurosci* 27:11865–11868.

Product	Quantity	Cat. No.
GeneBLAzer® D2-Gqo5-NFAT-*bla* CHO-K1	2 x 10^6 cells	K1708
LiveBLAzer™-FRET B/G Substrate	200 µg	K1095
LanthaScreen® Tb-anti-ERM (pLRRKtide) Antibody	25 µg	PV4899
Fluorescein-ERM (LRRKtide)	1 mg	PV4901
LRRK2 recombinant protein	10 µg	PV4873
LRRK2 G2019S recombinant protein	10 µg	PV4881

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Mitochondrial fission and fusion in neurodegeneration

APPROACHES FOR VISUALIZING MITOCHONDRIAL MORPHOLOGY.

Defects in neuronal mitochondrial function are key indicators in many neurodegenerative diseases, including Alzheimer's disease, amyotrophic lateral sclerosis, Parkinson's disease, and ischemic stroke, along with many other disease states outside the nervous system. For example, cytochrome *c* leakage, imbalance in reactive oxygen and nitrogen species, and activation of apoptotic pathway genes and proteins have all been described in one or more of these diseases.

Shifting the balance to mitochondrial fragmentation

Recent evidence suggests a parallel morphological correlation in which the balance between fission and fusion of the mitochondrial syncytia is disrupted. Nitric oxide, amyloid-β, rotenone, ApoE4 protein, and oxidative and nitrosative stress can all shift the balance from fusion to fission (fragmentation) [1–5] (Figure 1). These same stresses can alter mitochondrial distribution and morphology in cells—possibly via a key motor protein that induces fission [6]. A number of GTPases are critical for fusion events and have also been implicated, with upregulation of their activity protecting against neurodegeneration [7,8]. Simple morphological visualization of mitochondria in live or fixed cells can thus be used as a key phenotypic marker and indicator of stress and as an effective model in understanding inducers of neurological pathologies.

Options for visualizing mitochondria

Invitrogen offers a variety of reagents available for staining mitochondria (Table 1), including MitoTracker® probes (Figure 2), other potential-sensitive mitochondrial probes (e.g., JC-1, JC-9, and tetramethylrhodamine methyl and ethyl esters), mitochondrial-targeted Organelle Lights™ reagents (Figure 3), and primary antibodies for detecting specific proteins involved in oxidative phosphorylation or other mitochondrial pathways. Learn more about mitochondrial staining at **www.invitrogen.com/bp60**. ■

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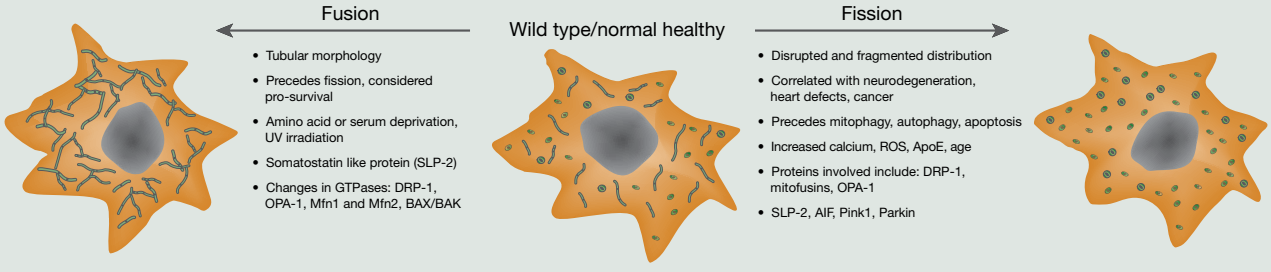

Figure 1. Changes in mitochondrial morphology. Many cellular events and proteins have been described that can regulate and shift the balance of mitochondrial distribution between hyper-fused (tubular) to hyper-fissioned (fragmented). This figure summarizes some of the recent data from the literature [1–8].

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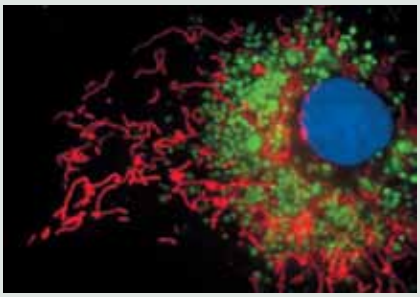

Figure 2. Dye-based mitochondrial labeling. Live bovine pulmonary artery endothelial cells were incubated simultaneously with MitoTracker® Deep Red FM (Cat. No. M22426), LysoTracker® Green DND-26 (Cat. No. L7526), and Hoechst 33342 (Cat. No. H3570).

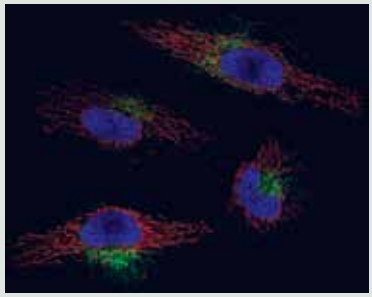

Figure 3. Fluorescent protein–based mitochondrial labeling. Live HeLa cells were transduced with Organelle Lights™ Lysosomes-GFP (Cat. No. O36228) and Organelle Lights™ Mito-RFP (Cat. No. O36229), then stained with Hoechst 33342 (Cat. No. H3570).

Table 1. Products for visualizing mitochondria.

Product	Notes	Quantity	Cat. No.
MitoTracker® probes			
MitoTracker® Red CMXRos	Most popular product; add to live cells, then fix	20 x 50 µg	M7512
Image-iT® LIVE Mitochondrial and Nuclear Labeling Kit	M7512 in kit form with nuclear stain; compatible with GFP staining	1 kit	I34154
MitoTracker® Green FM	Color option to M7512, accumulates in polarized mitochondria where it binds to thiols, and thus is retained if mitochondria are depolarized [9]. Has been successfully used to calculate total mitochondrial mass and has been shown to be retained in fixed cells.	20 x 50 µg	M7514
MitoTracker® Orange CMTMRos	Color option to M7512, with similar properties	20 x 50 µg	M7510
MitoTracker® Red FM	Color option to M7512, with similar properties	20 x 50 µg	M22425
MitoTracker® Deep Red FM	Color option to M7512, with similar properties	20 x 50 µg	M22426
MitoTracker® Red CM-H_2XRos	Reduced form of M7512, fluorescence requires both oxidation and mitochondrial membrane potential	20 x 50 µg	M7513
MitoTracker® Orange CM-H_2TMRos	Reduced form of M7510, fluorescence requires both oxidation and mitochondrial membrane potential	20 x 50 µg	M7511
Organelle Lights™ reagents			
Organelle Lights™ Mito-GFP	BacMam-based fluorescent proteins	1 kit	O36210
Organelle Lights™ Mito-OFP	BacMam-based fluorescent proteins	1 kit	O36222
Organelle Lights™ Mito-RFP	BacMam-based fluorescent proteins	1 kit	O36229
Other live-cell dyes to assess mitochondrial physiology			
MitoSox™ Red mitochondrial superoxide indicator	Accumulates in polarized mitochondria; functional status indicator	10 x 50 µg	M36008
Dihydroethidium (hydroethidine)	Cytosolic superoxide indicator; intercalates within DNA on oxidation	10 x 1 mg	D11347
Image-iT® LIVE Mitochondrial Transition Pore Assay Kit	A live-cell apoptosis assay for visualizing the mitochondrial permeability transition	1 kit	I35103
HCS Mitochondrial Health Kit	For high-content imaging of mitotoxicity and cytotoxicity	1 kit	H10295
JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolyl-carbocyanine iodide)	Green at low concentrations; forms red aggregates in healthy, polarized mitochondria	5 mg	T3168
JC-9 (3,3′-dimethyl-α-naphthoxacarbocyanine iodide)	Functions like JC-1, but baseline green signal more stable over range of depolarization	5 mg	D22421
Classic mitochondrial stains (accumulate in live, polarized mitochondria, but unlike most MitoTracker® probes, are not retained upon fixation)			
Rhodamine 123	Green-fluorescent sensor of mitochondrial membrane potential	25 mg	R302
Tetramethylrhodamine, methyl ester, perchlorate (TMRM)	Red-orange–fluorescent sensor of mitochondrial membrane potential	25 mg	T668
Tetramethylrhodamine, ethyl ester, perchlorate (TMRE)	Red-orange–fluorescent dye	25 mg	T669
Primary antibodies			
For antibodies and assays to study mitochondrial proteins and determine their role in disease states or pathways, visit **www.invitrogen.com/bp60**.			

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Visualizing tumor metastasis

CELLTRACKER™ DYES, DQ™ COLLAGEN, AND GELTREX™ MATRIX.

Cancer is projected to become the leading cause of death worldwide by 2010 [1], while even more lives are severely disrupted by traumatic surgeries and follow-on drug treatments. While some tumors are toxic *in situ*, most morbidity is associated with cancer cell–associated degradation of the basement membrane and subsequent extravasation to spread throughout the body. Here we provide an overview of Invitrogen's Molecular Probes® reagents that can be used for the study of cancer metastasis.

Breaching boundaries

A variety of normal cellular processes involve the crossing of cell and tissue borders or boundaries, collectively termed basement membranes or extracellular matrices. For example, macrophages undergo a cascade of adhesive events and selective extracellular matrix (ECM) degradation to depart from the blood system and invade infected tissues. A similar mechanism has been characterized for endothelial cells during the generation of neovessels.

Many of the molecular strategies used in normal cell migration are also employed by tumor cells during the process of metastasis, and therapeutic approaches have focused on blocking these pathways. The process of metastasis and accompanying neovessel formation can be studied using fluorescent dyes and natural protein substrates that facilitate visualization of cell movement and the selective degradation of ECM components constituting tissue boundaries.

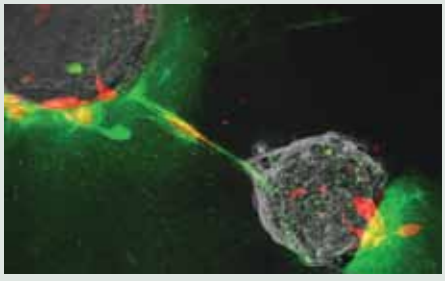

Figure 1. Interaction of tumor spheroids with stromal fibroblasts results in extensive degradation of type IV collagen. HCT116 human colon carcinoma cells were cocultured with human colon fibroblasts prestained with CellTracker™ Orange in reconstituted basement membrane containing DQ™ collagen IV. Extensive proteolysis, resulting in fluorescence of DQ™ collagen IV (green), occurs at the site of interaction between the tumor spheroids and the fibroblasts (orange), with yellow regions denoting areas of overlap. Magnification: 40x. Image supplied by Drs Mansoureh Sameni and Bonnie Sloane, Wayne State University.

CellTracker™ dyes—tracking tumor cell migration

Molecular Probes® CellTracker™ (thiol-reactive) and most CellTrace™ (amine-reactive) dyes freely diffuse through the membranes of live cells and once inside, the action of intracellular esterase converts them to fluorescent forms that survive fixation. Cells labeled in this manner remain viable and fluorescent for days to weeks, even after 5–6 divisions. For cell migration studies, these dyes can be used to follow cells as they migrate through tissue, either artificial or real, and can be multiplexed with functional probes (such as DQ™ collagen, discussed below) for the simultaneous detection of cell migration and ECM degradation. The Sloane lab and colleagues have used CellTracker™ and CellTrace™ dyes extensively [2–5] to visualize the migration of tumor cells through three-dimensional (3D) extracellular matrices, their interaction with stromal fibroblasts, and the simultaneous digestion of the ECM protein collagen IV (Figure 1).

DQ™ collagen—visualizing tumor-associated proteolysis

A variety of proteases from tumor cells and tumor-associated cells are critical to the process of metastasis; these proteases facilitate the breakdown of various ECM components during cell migration. The fluorescein conjugate DQ™ collagen can be used to directly monitor

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collagenase activity of tumor cells. This heavily conjugated collagen substrate readily self-quenches, but quenching is relieved upon hydrolysis to single dye–labeled peptides.

DQ™ collagen can be used in live-cell studies [2] (Figure 1) or for *in situ* zymography on unfixed cryosections [6]. When multiplexed with CellTracker™ dyes, the simultaneous visualization of cell migration and proteolysis can be accomplished, under conditions that mimic the *in vivo* tumor architecture and its environment (using Geltrex™ matrix, discussed below). Figure 2 demonstrates the experimental setup used for imaging CellTracker™-labeled tumor cell migration through a DQ™ collagen substrate suspended in a 3D basement membrane matrix.

DQ™ collagen assays are ideal for the identification of inhibitors of tumor proteolysis. Sameni and colleagues [4] recently demonstrated the use of DQ™ collagen IV to visualize inhibition of matrix metalloproteinases (MMPs), cysteine cathepsins, and serine proteases in cocultures of breast carcinoma cells and fibroblasts (Figure 3).

Geltrex™ matrix—mimicking *in vivo* conditions

Geltrex™ matrix is a soluble form of basement membrane extract purified from continuous sheets of specialized ECM that form an interface between Engelbreth-Holm-Swarm (EHS) tumor cells. Like naturally occurring basement membrane, Geltrex™ matrix not only supports cells and cell layers but also plays an essential role in tissue organization that affects cell adhesion, migration, proliferation, and differentiation. Because it mimics *in vivo* conditions, Geltrex™ matrix provides an ideal setting for *in vitro* metastasis assays when combined with DQ™ substrates (Figure 2).

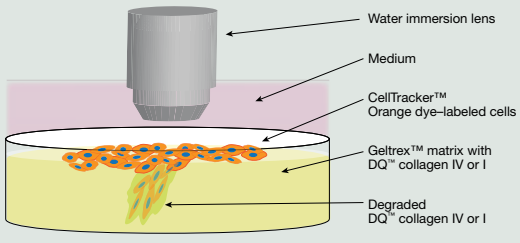

Figure 2. Imaging tumor cell migration *in vitro*. Tumor cells are labeled with CellTracker™ dye and migrate through DQ™ collagen substrate suspended in three-dimensional matrix gel. Green fluorescence indicates the region of collagen degradation by secreted proteases. In this schematic, cells are labeled orange with CellTracker™ Orange dye, and nuclei are stained blue with Hoechst 33342.

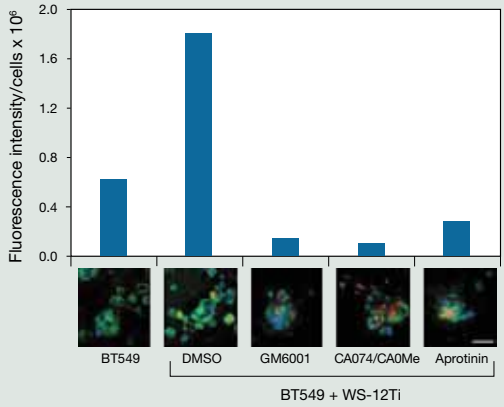

Figure 3. Inhibition of proteolysis of DQ™ collagen IV. Cocultures of BT549 breast carcinoma cells and WS-12Ti fibroblasts (prelabeled with CellTrace™ Far Red, Cat. No. C34553) were treated with inhibitors of MMPs (GM6001), cysteine cathepsins (CA074/CA0Me), and serine proteases (aprotinin). The bar above each image represents fluorescence intensity per cell. DQ™ collagen degradation appears green, and nuclei were stained blue with Hoechst. With kind permission from Springer Science+Business Media: Sameni M et al. (2009) Imaging and quantifying the dynamics of tumor-associated proteolysis. *Clin Exp Metastasis* 26:299–309, Figure 3.

More reagents for tumor metastasis studies

To find all available assays for visualizing tumor metastasis including CellTracker™ dyes, Qdot® nanocrystals, and fluorescent protein–based Cellular Lights™ reagents, visit **www.invitrogen.com/bp60**. ■

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Product	Quantity	Cat. No.
DQ™ collagen, type IV from human placenta, fluorescein conjugate	1 mg	D12052
DQ™ collagen, type I from bovine skin, fluorescein conjugate	1 mg	D12060
Geltrex™ Reduced Growth Factor Basement Membrane Matrix	1 mL	12760013
CellTracker™ Blue CMAC	5 mg	C2110
CellTracker™ Green CMFDA	20 x 50 µg	C7025
CellTracker™ Orange CMTMR	1 mg	C2927
CellTracker™ Orange CMRA	20 x 50 µg	C34551
CellTracker™ Red CMTPX	20 x 50 µg	C34552
Vybrant® CFDA SE Cell Tracer Kit	1 kit	V12883
CellTrace™ Far Red DDAO	20 x 50 µg	C34553

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Innovative solutions for ion channel research

CELL-BASED AND BIOCHEMICAL ASSAYS FOR ION CHANNEL ACTIVITY.

Ion channels are transmembrane proteins that facilitate ion flow across cells, thereby controlling the membrane potential and membrane excitability. Sodium ions are concentrated outside cells, and potassium ions are concentrated inside cells; there is also a charge gradient from net positive in the extracellular fluid to negative in the cytosol. Ion-selective channels open in response to stimuli and conduct electrical currents across cells in accordance with these gradients to regulate many important processes in biology.

An estimated 400 ion channel genes exist [1]. Functional diversity is increased by the formation of heteromeric channels from multisubunit complexes and the interaction of specific channels with accessory proteins [2], generating a very large number of functional ion channel signatures. Study of these ionic currents in voltage clamp experiments is a major goal of heterologous expression, and a major challenge where subunit stoichiometry is critical in cellular function of the complex.

Ion channels are critical to a number of cellular functions and processes. They mediate information transfer and control cellular homeostasis. Ion channels underlie nerve impulses and signal transmission across synapses and make up the quantal units of communication in the central nervous system. In addition, they are involved in a wide range of biological processes that require rapid cellular changes, such as muscle contraction, epithelial transport of nutrients and ions, T cell activation, and hormonal release. As a consequence, ion channel dysfunctions have been associated with many disease states [1,3].

Despite the large number of potential targets and their physiological importance, ion channels remain relatively unexploited as pharmaceutical targets. This has been due to challenges in the development of assays and cellular models suitable for drug discovery efforts [4,5]. Methods for the detection of ion channel activity appropriate for modern drug discovery requirements have lagged behind those of other target classes. Recently, however, tools for measuring ligand displacement, membrane potential, and ion flux and automated patch instruments have enabled high-throughput screening of ion channel targets.

A second challenge is generating the cellular models required for experimentation and screening. The traditional approach of using stable cell lines has been difficult due to toxicity, clonal drift with passage, and other factors. Investigators are therefore increasingly turning to inducible or transient expression systems.

Molecular Probes has been a leader in the development of reagents for cell-based assays, including those for ion channels. Fluo-4 may be the most widely used reagent for studies of calcium flux, either via calcium ion channels or as a second messenger in G-protein–coupled receptor activation. Recently, we introduced a novel suite of reagents to enable holistic studies of ion channel activity in a variety of contexts with cellular and biochemical assays. Broadly, these can be classified into two categories: sensors to measure ion channel activity in high throughput and transient expression of ion channel genes.

Potassium channels

Potassium ion channels are represented by more than 80 genes in human cells [6]. Most potassium channels have a tetrameric structure. Both homotetrameric and heterotetrameric complexes exist in nature, generating a wealth of pharmacological, structural, and functional diversity. Potassium channels are the most widely distributed type of ion channel and are virtually ubiquitous in mammalian cell types, controlling a wide variety of functions. In excitable cells such as neurons, muscle, and vascular cells, they shape action potential duration and set the resting membrane potential, affecting every other voltage-gated channel type in the membrane. Because potassium ion channels regulate the action potential duration in cardiac muscle, pharmacological blockage or malfunction of their activity may cause life-threatening arrhythmias and other cardiovascular diseases. Potassium channel activity also regulates cellular processes such as the secretion of hormones, and their malfunction in this context has been linked to several disease states such as diabetes, cardiac disease, immune disorders, and hypertension [1,7].

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Sodium channels

Sodium channels are classified according to whether the trigger that opens the channel is voltage- or ligand-gated [8]. Voltage-gated channels are responsible for action potential initiation and propagation in most excitable cells, including nerve, cardiac, muscle, and neuroendocrine cell types. This family of sodium channels has nine known members, with amino acid identity >90% in the pore, voltage sensor, transmembrane, and extracellular loop regions [8]. These proteins are formed from a large pore-forming α subunit (~220–260 kDa) that associates with regulatory β subunits and other factors to regulate fast electrical events in excitable tissues of the central and peripheral nervous system. Their size and extensive homology have made recombinant α subunits particularly challenging to express stably. All pharmacological agents that act on sodium channels have well-described receptor sites on the α subunits, but with only subtle differences within the family, drugs that can discriminate subtypes have been extremely difficult to identify.

Chloride channels

Chloride channels are also involved in a very broad range of cellular processes, so defects in their activity are typically severe, causing cystic fibrosis, Bartter syndrome, myotonia congenita, and neuronal degeneration [9]. A number of drugs for the treatment of insomnia, anxiety, convulsions, and mood disorders also target neuronal chloride channels [10].

Types of assays for ion channel activity

Traditional patch-clamp measurements of ion channel activity are too expensive and labor-intensive for large-scale screening. Membrane potential dyes can report transmembrane voltage changes when channels are opened or closed, and have supported some high-throughput screening (HTS), though these dyes do not discriminate ion-selective activity. Fluorescent calcium indicators such as fluo-4 allow the detection of intracellular calcium and therefore assay ion-specific activity, which has already been successful for HTS of a handful of calcium ion channels.

FluxOR™ potassium channel assays

The FluxOR™ assay is a universal potassium ion channel assay that produces relevant pharmacological data using standard fluorescence microplate readers. While ion channel activity is most accurately measured with patch-clamp techniques, even automated systems are too labor-intensive for most screening efforts. The FluxOR™ assay is an easy-to-use, robust HTS assay that can measure the activity of any potassium-selective ion channel.

The basis for the FluxOR™ assay is the bright fluorescence of the FluxOR™ dye upon binding thallium ions, which serve as surrogate ions for potassium. Drugs that inhibit potassium channels diminish the response, while drugs that activate the channels increase it. The fluorescent signal is proportional to potassium channel activity (Figure 1). FluxOR™ dye does not have significant affinity for calcium, which may interfere with measurements using other thallium-sensitive dyes, and off-target effects from other ion channels in the cells do not interfere with the signal. This specificity reduces the number of false positives in drug screening campaigns, where millions of compounds have to be tested for activity at a specific ion channel. Because the FluxOR™ assay is cell based, the channels are observed in an intact biological system, which means the pharmacology will be more closely related to that of assays downstream of the primary screening. The FluxOR™ platform also reports the activity of allosteric or modulatory compounds that control potassium ion channel activity, not just those that close the pore.

Assays based on voltage sensor probes (VSPs)

Another method for measuring ion channel activity in microplate format uses membrane potential dyes. All active ion channels affect cellular membrane potential, and fluorescent dye kits have been developed that report when channels of any type are opened or closed. VSPs report the potential across the cell membrane via a fluorescence resonance energy transfer (FRET) principle. VSP kits combine an →

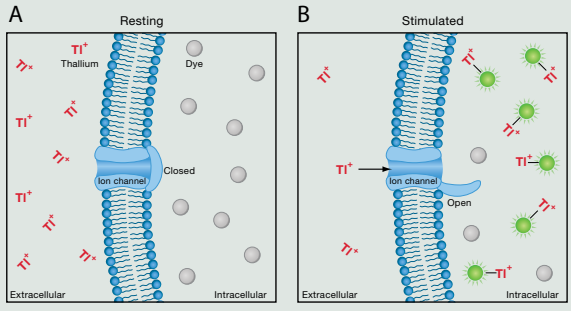

Figure 1. Principle of the FluxOR™ assay. Basal fluorescence from cells loaded with the FluxOR™ dye is low (**A**) until potassium channels are stimulated. Thallium (I) ions added to the assay with the stimulus flow into the cells through the open channels, activating the dye (**B**).

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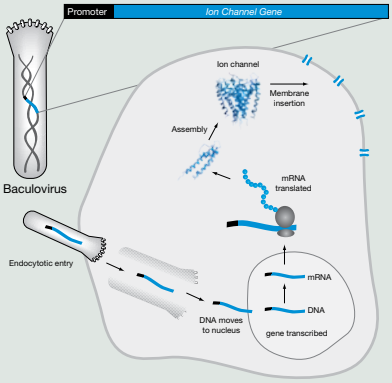

Figure 2. BacMam-mediated gene delivery and expression. An ion channel gene is cloned into a modified baculovirus vector driving mammalian expression. BacMam particles are endocytosed, and translated protein is inserted into the membrane, forming functional ion channels.

outer membrane–bound donor and a mobile acceptor that traverses the membrane in response to changes in membrane potential. The FRET-based measurement is restricted to the plasma membrane and is therefore more relevant than non-FRET approaches that measure signals throughout the cell.

While sodium-specific sensors suitable for HTS assays are lacking, VSPs faithfully respond to voltage-gated sodium channel activity in a variety of cellular models [11,12]. Sodium ion movement through open channels causes membrane depolarization that can be measured with a large and reproducible signal. Benefits include rapid, high-throughput, high-sensitivity detection, minimal well-to-well variation, and applicability to any target that changes the membrane potential.

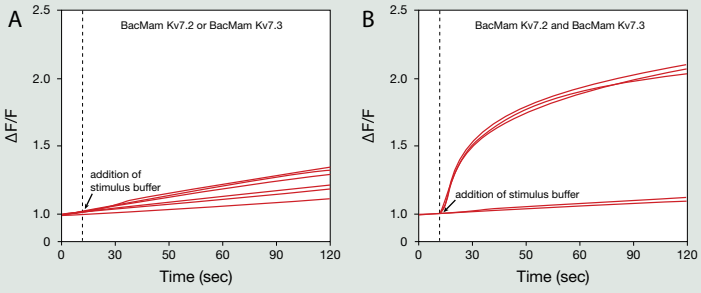

Figure 3. Functional testing of M channel components Kv7.2 and Kv7.3 with the FluxOr™ assay. (**A**) With BacMam constructs of either component expressed independently, the channel is not functional. (**B**) When the two subunits are expressed stoichiometrically, a functional heterotetramer channel is formed, evidenced by the dramatic increase in fluorescence.

Premo™ Halide Sensor assays

The Premo™ Halide Sensor combines fluorescence detection with BacMam technology, which uses an insect cell virus (baculovirus) to efficiently deliver and express genes in mammalian cells [13,14] (Figure 2). The sensor uses a halide-sensitive Yellow Fluorescent Protein (YFP) variant to detect changes in local halide concentration.

The assay is based on the permeability of chloride channels to iodide, the strong gradient across the plasma membrane of this ion, and higher quenching of fluorescence by iodide than chloride. YFP can be detected using standard GFP/FITC or YFP filter sets. Iodide-sensitive fluorescent proteins have been successfully used in HTS applications to screen activators of the cystic fibrosis transmembrane regulator, calcium-activated chloride channels, and GABA A receptor chloride channels [15,16]. Use of a YFP-based biosensor for chloride channel activity in a drug discovery setting has recently been reviewed [9].

Premo™ Halide Sensor assays enable real-time kinetic measurements, allowing assessment of the modulation of chloride channel conductance with previously unavailable sensitivity, reliability, and precision.

BacMam-mediated ion channel expression

Cell-based assays have typically been based on the stable expression of a target in a transformed cell line. For some protein classes, including ion channels, constitutive expression is frequently associated with cytotoxicity and clonal drift upon passage, leading to variable experimental results. Researchers have therefore begun to explore transient expression approaches, and one of the most promising is BacMam technology [13,14]. BacMam technology has successfully driven discovery campaigns at diverse targets, including ion channels and transporters [17–20]. As mammalian cells do not support baculovirus replication, transduction is very well tolerated and generally lacks visible cytopathic effects; baculoviruses are considered safe research reagents, with a BSL 1 classification.

For multicomponent systems that require stoichiometric expression, the ability to control transient expression by simply varying the dose enables screening in a wide range of cell types (Figure 3). Gene expression is unaffected by freezing of transduced cells, so it is easy to generate a large batch of assay-ready cells. The BacMam system thus provides flexibility and faster assay development timelines.

These reagents have proven particularly useful in discovery using automated patch-clamp platforms [21]. The ability to simultaneously deliver

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more than one BacMam ion channel to a cell enables interrogation of multiple channels, which increases throughput and data quality (Figure 4).

Holistic approach to ion channel interrogation

The ability to combine target expression and robust assays opens new research avenues. Fluo-4 was a breakthrough for high-sensitivity calcium detection in live cells and propelled G-protein second-messenger detection and calcium channel activity research to a new level. The tools described here help fill a similar need for other ion channel targets. For example, BacMam technology, which provides transient expression of potassium channels, and the FluxOR™ assay [22], which measures potassium channel activity, make a powerful combination [23]. This combination makes potassium ion channel targets available in a mix-and-go format, with cells ready to screen the next day, scalable from target identification to HTS.

The activities of ion channels can now be interrogated with an unprecedented level of throughput, pharmacological fidelity, and predictive capacity. For more information on assays to further ion channel research, visit **www.invitrogen.com/bp60**. ■

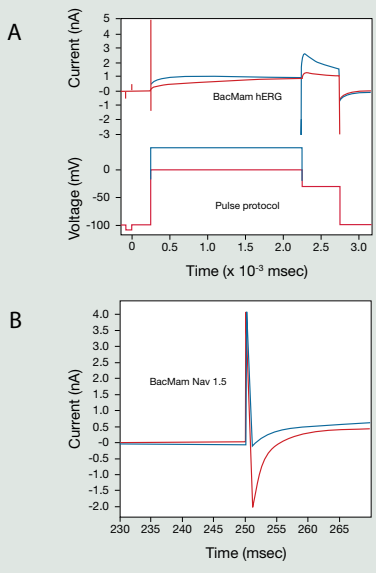

Figure 4. Automated patch-clamp measurement of a U2OS cell coexpressing BacMam hERG (Kv11.1) and Nav1.5 ion channels. (**A**) Lower trace shows the applied voltage. The cells were held at –100 mV and a tail current applied (blue trace) to measure the outward hERG-mediated current (upper trace). A second 0t mV step (red trace) was made to capture the peak inward current carried by Nav1.5, shown on an expanded timescale in (**B**).

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Product	Quantity	Cat. No.
BacMam hERG, for 10 microplates	1 kit	B10019
BacMam Kv1.1, for 10 microplates	1 kit	B10331
BacMam Kv1.3, for 10 microplates	1 kit	B10332
BacMam Kv2.1, for 10 microplates	1 kit	B10333
BacMam Kir1.1, for 10 microplates	1 kit	B10334
BacMam Nav1.5, for 10 microplates	1 kit	B10335
BacMam Nav1.2, for 10 microplates	1 kit	B10341
BacMam Kir2.1, for 10 microplates	1 kit	B10146
BacMam Kv7.2 and Kv7.3, for 10 microplates	1 kit	B10147
Premo™ Halide Sensor, for 10 microplates	1 kit	P10229
FluxOR™ Thallium Detection Kit, for 10 microplates	1 kit	F10016
FluxOR™ Thallium Detection Kit, for 100 microplates	1 kit	F10017
PowerLoad™ concentrate, 100X	5 mL	P10020
Probenecid, water soluble	10 x 77 mg	P36400
Voltage Sensor Probes Set, $DiSBAC_2(3)$ and CC2-DMPE	1 mg	K1016
Voltage Sensor Probes Set, $DiSBAC_4(3)$ and CC2-DMPE	1 mg	K1046
VABSC-1 Dye (Voltage Assay Background Suppression Compound)	1 g	K1019
CC2-DMPE	1 mg	K1017
CC2-DMPE	5 mg	K1020
CC2-DMPE	10 mg	K1070
$DiSBAC_2(3)$	1 mg	K1018
$DiSBAC_2(3)$	5 mg	K1021
$DiSBAC_4(3)$	5 mg	K1022
$DiSBAC_4(3)$	1 mg	K1047
Fluo-4 Direct™ Calcium Assay Kit, Starter Pack	1 kit	F10471
Fluo-4 Direct™ Calcium Assay Kit, High-Throughput Pack	1 kit	F10473
Fluo-4, AM, packaged for high-throughput screening	5 x 1 mg	F14202

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Resources for cytokine research

ANTIBODIES, RECOMBINANT PROTEINS, ELISAS, AND LUMINEX® KITS.

Cytokines are secreted cell signaling proteins or peptides that regulate many aspects of the immune system. Approaches to studying cytokine function are varied and include the use of antibodies, recombinant proteins, and ELISA and Luminex® kits. Invitrogen offers a diverse array of products for investigating cytokines, their cell surface receptors, and downstream signaling pathways for a variety of applications.

Cytokine antibodies

Invitrogen's extensive portfolio of cytokine antibodies, including phosphospecific and fluorescently labeled antibodies, are validated for multiple applications including western blotting, ELISAs, immunocytochemistry, immunohistochemistry, and flow cytometry. Find antibodies for your cytokine or downstream pathway of interest at **www.invitrogen.com/bp60**.

Recombinant cytokine proteins

Invitrogen's recombinant proteins and growth factors exhibit high purity and potent biological activity. Rigourous in-house testing of biological activity includes assays that measure cell proliferation, cytotaxis, chemotaxis, calcium flux, secondary cytokine upregulation, and induction of surface antigen expression (Figure 1). Find recombinant proteins for your cytokine research at **www.invitrogen.com/bp60**.

ELISA kits for cytokines

Invitrogen has a wide menu of ELISA kits with world class sensitivity, proven specificity, and consistent performance. ELISA and phospho-ELISA™ kits can be used for precise quantitation of expressed cytokines, with high sensitivity and specificity (Figure 2). Learn more at **www.invitrogen.com/bp60**.

Luminex® assays for multiplex cytokine analysis

Invitrogen offers extracellular marker assays for accurate quantitation of cytokines, chemokines, and growth factors. Available as singleplex or multiplex kits, the assays can detect picogram levels of protein and can be run in ~4 hours. Find our full range of Luminex® assays at **www.invitrogen.com/bp60**. ■

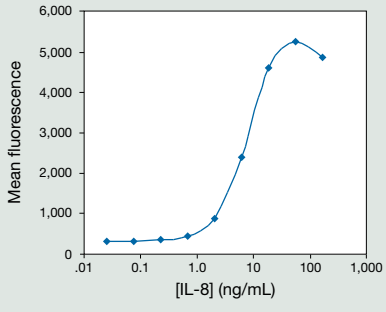

Figure 1. Biological activity of recombinant human IL-8. Chemotaxis assay using human polymorphonuclear leukocytes (PMNs) fluorogenically labeled with BCECF-AM. Serial dilutions of IL-8 (Cat. No. PHC0884) were incubated with PMNs in a chemotaxis chamber. Fluorescence was measured in a fluorometer using excitation/emission of 485/530 nm.

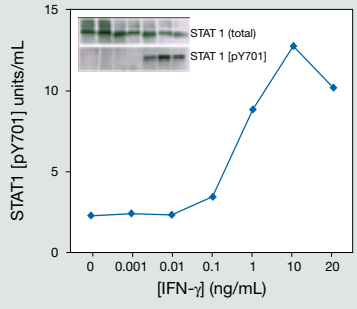

Figure 2. IFN-γ activation of STAT1. HeLa cells were treated with recombinant human IFN-γ (Cat. No. PHC4031) at varying concentrations for 20 min, then lysed. Phosphorylation of tyrosine 701 increases with dosage of IFN-γ as measured by the STAT1 [pY701] phosphoELISA™ kit (Cat. No. KHO0271). The results correlated well with western blot analysis of the same samples.

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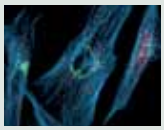
CLICK-IT® EDU IMAGING AND HCS KITS

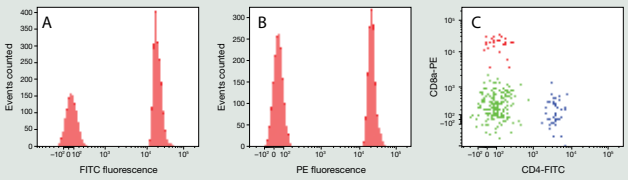


The fastest, easiest, and most accurate method for detecting cell proliferation via nascent DNA synthesis now offers even more advantages. The Click-iT® EdU Imaging and HCS kits have been reformulated to further streamline the assay protocol, and now include a wash buffer to maximize the signal-to-noise ratio. Learn more at **www.invitrogen.com/bp60**.

Product	Quantity	Cat. No.
Click-iT® EdU Alexa Fluor® 488 Imaging Kit	1 kit/50 coverslips	C10337
Click-iT® EdU Alexa Fluor® 555 Imaging Kit	1 kit/50 coverslips	C10338
Click-iT® EdU Alexa Fluor® 594 Imaging Kit	1 kit/50 coverslips	C10339
Click-iT® EdU Alexa Fluor® 647 Imaging Kit	1 kit/50 coverslips	C10340
Click-iT® EdU Alexa Fluor® 488 HCS Assay	1 kit/2 plates	C10350
	1 kit/10 plates	C10351
Click-iT® EdU Alexa Fluor® 555 HCS Assay	1 kit/2 plates	C10352
	1 kit/10 plates	C10353
Click-iT® EdU Alexa Fluor® 594 HCS Assay	1 kit/2 plates	C10354
	1 kit/10 plates	C10355
Click-iT® EdU Alexa Fluor® 647 HCS Assay	1 kit/2 plates	C10356
	1 kit/10 plates	C10357

AbC™ ANTI-RAT/HAMSTER BEAD KIT

Compensation is crucial for accurate multicolor experimentation. Because many antigens are not highly expressed, cells may not always generate the fluorescence signal needed for correcting spectral overlap, and dimly stained cells could lead to improper compensation settings. The AbC™ Anti-Rat/Hamster Bead Kit, for use with conjugates of any rat or hamster IgG, overcomes this problem. Each kit provides polystyrene microspheres that are either coated with capture antibody (positive) or uncoated (negative). After mixing with a fluorophore-conjugated rat or hamster antibody, the two components provide distinct positively and negatively stained bead populations, which can then be used to set compensation. Learn more at **www.invitrogen.com/bp60**.

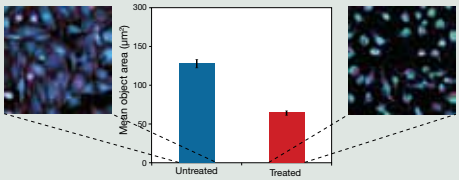


Compensation using the AbC™ Anti-Rat/Hamster Bead Kit. (**A**) FITC-conjugated rat anti-mouse CD4 (Cat. No. MCD0401) labels the AbC™ Capture Beads for a positive signal, and the negative beads provide a negative signal. (**B**) Phycoerythrin-conjugated rat anti-mouse CD8a antibody (Cat. No. RM2204) labels the AbC™ Rat/Hamster Capture Beads for a positive signal, and the negative beads provide a negative signal. (**C**) Dual-parameter plot showing gated mouse splenocytes labeled with both FITC-conjugated rat anti-mouse CD4 and phycoerythrin-conjugated rat anti-mouse CD8a using compensation settings obtained using the AbC™ Anti-Rat/Hamster Bead Kit.

Product	Quantity	Cat. No.
AbC™ Anti-Rat/Hamster Bead Kit	100 tests	A10389

ACCURATE CELL IDENTIFICATION FOR HCS PLATFORMS

Molecular Probes® HCS CellMask™ and HCS NuclearMask™ stains provide reliable cell delineation with a choice of fluorescent colors ranging from blue to deep red. HCS CellMask™ stains label the whole cell, including cytoplasm and nucleus, and are applied to cells immediately after fixation and permeabilization or after antibody labeling. The versatile HCS NuclearMask™ stains measure DNA content in addition to enabling robust cell demarcation of live and formaldehyde-fixed cells. Learn more about tools for high-content imaging and analysis at **www.invitrogen.com/bp60**.

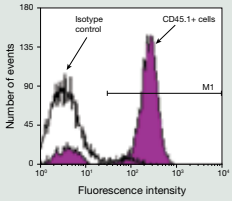


The effects of cytochalasin D treatment on HeLa cell size as measured by HCS CellMask™ Blue Stain. HeLa cells were treated with a DMSO vehicle (left) or 10 µM cytochalasin D (right) for 3 hr before fixation and permeabilization. Samples were then labeled with HCS CellMask™ Blue stain, Alexa Fluor® 488 phalloidin to visualize filamentous actin (red), Alexa Fluor® 555 goat anti–mouse IgG to detect anti–α-tubulin IgG (green), and TO-PRO®-3 iodide to stain nuclei (magenta). The bar graph represents quantitative measurements of cell size as indicated by HCS CellMask™ Blue stain to show the effects of cytochalasin D treatment.

Product	Quantity	Cat. No.
HCS CellMask™ Red stain	10 plates	H32712
HCS CellMask™ Orange stain	10 plates	H32713
HCS CellMask™ Green stain	10 plates	H32714
HCS CellMask™ Blue stain	10 plates	H32720
HCS CellMask™ Deep Red stain	10 plates	H32721
HCS NuclearMask™ Blue stain	10 plates	H10325
HCS NuclearMask™ Red stain	10 plates	H10326
HCS NuclearMask™ Deep Red stain	10 plates	H10294

NEW ANTIBODY CONJUGATES FOR FLOW CYTOMETRY

Invitrogen has a rapidly expanding menu of directly conjugated primary antibodies with human and mouse reactivity that are available in a variety of colors to help maximize multicolor flow cytometric analysis. These antibody conjugates can help answer complex biological questions relating to cancer, immunology, stem cell research, cell signaling, and studies of T, B, and NK cells, and more. In addition, these conjugates allow simultaneous measurement of different specificities to help reveal more information about each individual cell with less time and less sample. View all of our new antibodies at **www.invitrogen.com/bp60**.



SJL spleen cells stained with mouse anti-mouse CD45.1-FITC. Lymphocytes were gated by scatter and analyzed on a FACScan™ flow cytometer (BD Biosciences).

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Recently published

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

Neuroscience: *In situ* optical recording of action potentials from mouse nerve terminals. The quest to understand neural processing at its most fundamental level requires development of optical techniques capable of discrete detection of action potentials at single-cell resolution in three-dimensional tissue preparations to augment invasive electrode array techniques with limited spatial resolution. Researchers from the University of Pennsylvania have advanced this quest using two-photon excitation of the potentiometric styryl dye di-3-ANEPPDHQ in nerve terminals of intact mouse neurohypophysis preparations. They demonstrated single-trial recordings of action potentials with signal-to-noise ratios of 5:1 and fractional fluorescence changes of up to 10%. Because the potentiometric response of fluorescent dyes is highly specimen-dependent, careful *in situ* optimization of the two-photon excitation wavelength was required to obtain this level of performance. The selection of di-3-ANEPPDHQ for these measurements was based on its relatively low phototoxicity and its resistance to internalization from the plasma membrane, which results in diminished background fluorescence and therefore increased signal-to-noise.

Fisher JA, Barchi JR, Welle CG, Kim GH, Kosterin P, Obaid AL, Yodh AG, Contreras D, Salzberg BM (2008) Two-photon excitation of potentiometric probes enables optical recording of action potentials from mammalian nerve terminals *in situ*. *J Neurophysiol* 99:1545–1553.

Stem cells: Using proteins to induce pluripotency in somatic cells. The reprogramming of somatic cells into pluripotent stem cells has previously been achieved by transforming those cells with genetic sequences that code for factors necessary for the reprogramming. However, the introduction of exogenous genetic material carries the risk of unpredictable genetic modifications of the target cell genome. In their recent report, Zhou and colleagues describe the generation of induced pluripotent stem cells from mouse embryonic fibroblast cells following transduction with the recombinant reprogramming proteins *Oct4*, *Sox2*, *Klf4*, and *c-Myc* containing a protein transduction domain fused at the C-terminus. Pluripotency of the resulting cells was demonstrated through immunolabeling experiments using Alexa Fluor® dye-labeled antibodies directed toward known pluripotency markers. These protein-induced pluripotent stem cells (piPSPs) were stably and homogeneously expanded for more than 30 passages; the cells were morphologically indistinguishable from mouse embryonic stem cells (mESCs), and were observed to efficiently incorporate and differentiate *in vivo*.

Zhou H, Wu S, Joo JY, Zhu S, Han DW, Lin T, Trauger S, Bien G, Yao S, Zhu Y, Siuzdak G, Schöler HR, Duan L, Ding S (2009) Generation of induced pluripotent stem cells using recombinant proteins. *Cell Stem Cell* 4:381–384.

Astrobiology: Amino acid detection in Martian analogue samples using Pacific Blue™ dye. The search for evidence of life on other planets has centered on the detection of amino acids, due to their ubiquitous presence in terrestrial organisms. For an upcoming Mars mission, the European Space Agency has developed a portable capillary electrophoresis instrument—the Mars Organic Analyzer (MOA)—for the *in situ* detection and analysis of bioamines. Utilizing the amine-reactive succinimidyl ester form of Pacific Blue™ dye for fluorescence-based detection, the development team has demonstrated successful detection, and subsequent identification and chirality analysis, of amino acids at levels as low as 75 pM (sub–parts-per-trillion) in prepared samples. This represents a 200-fold increase in sensitivity as compared to detection based on fluorescamine; the team used this greater sensitivity for the successful analysis of amino acids at the parts-per-billion level in terrestrial (Atacama Desert) and astrobiological (Murchison meteorite) samples.

Chiesl TN, Chu WK, Stockton AM, Amashukeli X, Grunthaner F, Mathies RA (2009) Enhanced amine and amino acid analysis using Pacific Blue and the Mars Organic Analyzer microchip capillary electrophoresis system. *Anal Chem* 81:2537–2544.

Evolutionary biology: Fluorescence analysis of the evolution of neural structures necessary for vocalization. In an effort to establish the ancestral origin of vocal communication, Bass and colleagues have made extensive use of Alexa Fluor® dye–labeled probes, including Alexa Fluor® 488 dextran amine and Alexa Fluor® 546 dextran amine, to map the development of vocal neural networks in larval ray-finned fish. Their results demonstrate vocal network development in a hindbrain-spinal region; taxonomic comparison showed this development pattern to be consistent with that observed in studies of multiple lineages of vocal vertebrates. Their results suggest the origin of a vocal network in a common ancestor of ray-finned and lobe-finned fish; further study on the highly conserved nature of this vocal network region could significantly push back the common origin of this neural basis for vocalized social signaling.

Bass AH, Gilland EH, Baker R (2008) Evolutionary origins for social vocalization in a vertebrate hindbrain-spinal compartment. *Science* 321:417–421.

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