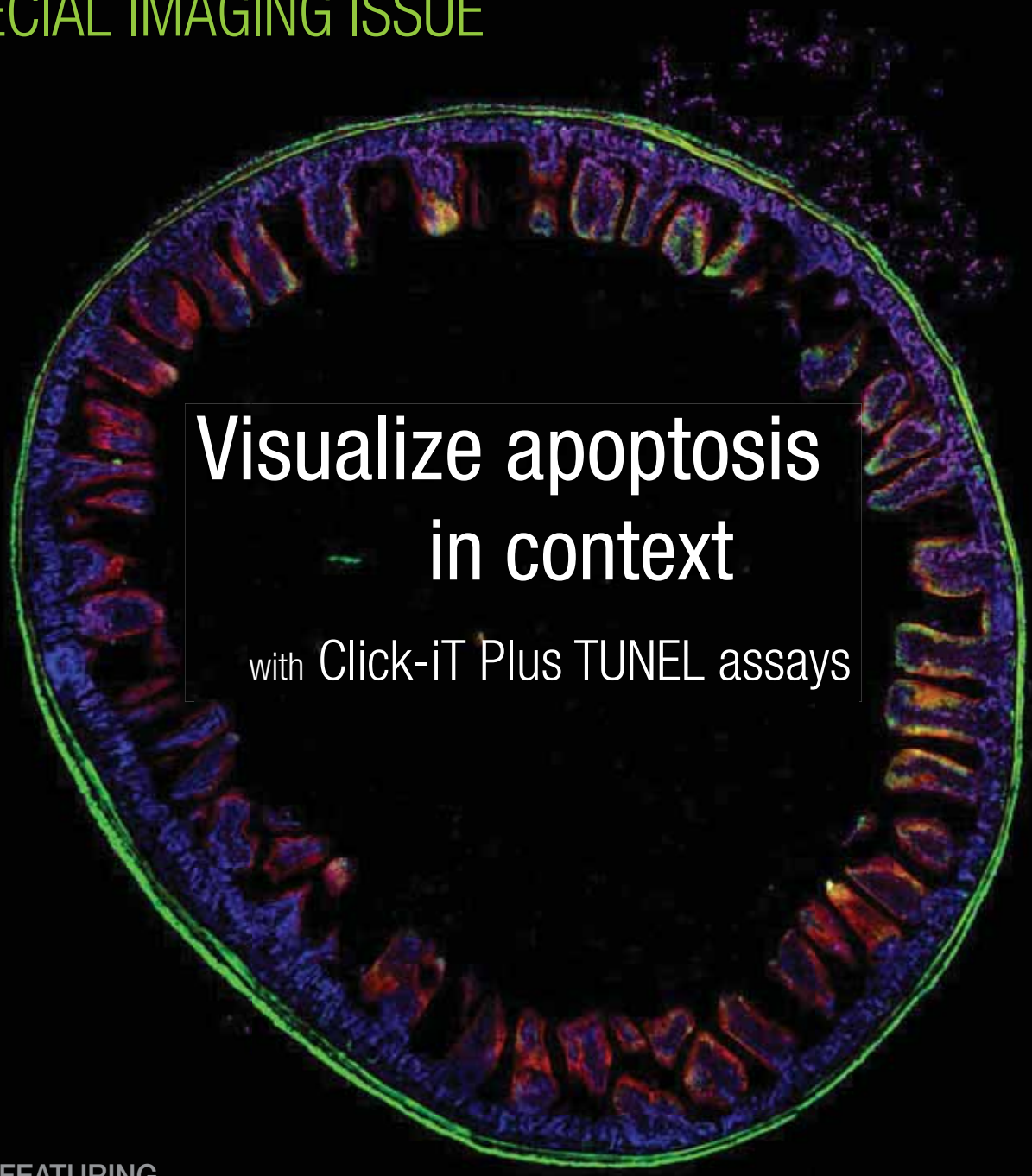
NOVEMBER 2015

BIOPROBES 72

MOLECULAR PROBES JOURNAL OF CELL BIOLOGY APPLICATIONS

SPECIAL IMAGING ISSUE



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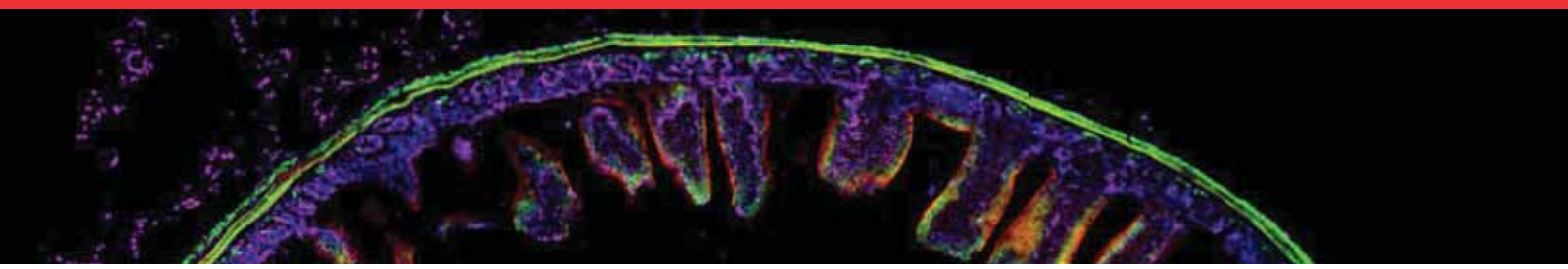

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From the hood to the microscope: Revolutionizing cell-based imaging

Visit our Cell Culture Café archives for the interactive webinar "From the hood to the microscope: Revolutionizing cell-based imaging", which showcases some of the latest advances in cell-based research and imaging. We explore how to obtain superior results through the careful selection of reagents and optimization of your imaging workflow—from growing cells on suitable surfaces through to image capture. Particular attention is given to growing and monitoring cells for imaging, fluorescent labeling of live cells, and critical considerations for live-cell and time-lapse imaging.

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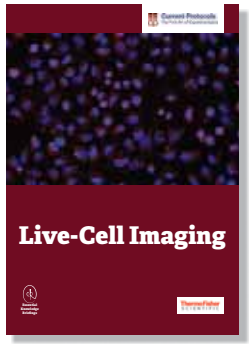
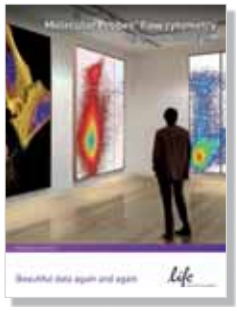
Essential Knowledge Briefing: Live-Cell Imaging



Although prepared slides reveal many details about biological specimens, they always leave you with questions: How does this look when it's alive? How does the organism or molecular component behave in a dynamic natural context? How does it interact with its environment? These questions are at the heart of live-cell imaging. In its most basic form, live-cell imaging allows biologists to examine and analyze live samples. This capability completely changes what can be learned about cell and organismal functions and ultimately determines how that information can be applied in fields as diverse as basic research, biotechnology, and medicine.

Published by Wiley, this *Essential Knowledge Briefing* (*EKB*) introduces readers to the general field of live-cell imaging. It explains the fundamental challenges and solutions, describes applications of live-cell imaging through case studies, and forecasts some of the next advances. Download the *Live Cell Imaging EKB* at **essentialknowledgebriefings.com/live-cell-imaging-ekb-available-now**.

Molecular Probes Flow Cytometry Products and Resource Guide



The Molecular Probes™ Flow Cytometry Products and Resource Guide provides an overview of the products and techniques used in flow cytometry workflows, including:

- Sample preparation—from cell preservation to cell isolation
- Instrument setup and calibration—from alignment to absolute cell counting
- Antigen detection—from primary antibodies to custom services
- Cell analysis—from cell health and viability to phagocytosis

Request your copy today—in print or digital format—at **thermofisher.com/flowguide**.

Molecular Probes cell imaging app: Protocols at your fingertips

The Molecular Probes™ Cell Imaging Reagent Guide and Protocols mobile app is designed to help you find fluorescent reagents, kits, and protocols for cell biology–related fluorescence microscopy applications. Also included are product guides for instrument calibration and setup, protein labeling, and secondary detection. Key features of this app include:

- Straightforward experimental design, with excitation and emission data for every reagent
- Quick and easy protocols, with a built-in protocol timer that runs even if you leave the app
- Examples of data output for every product

Download the Molecular Probes Cell Imaging Reagent Guide and Protocols mobile app today at the App Store™ for iPhone™ and iPad™ devices or at Google Play™ for Android™ phones and tablets, and see our complete selection of mobile and desktop apps at **thermofisher.com/apps**.

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Stay on the leading edge of stem cell research at the third annual 24 Hours of Stem Cells. Join us at this global virtual conference on December 3, 2015, to:

- Access leading scientific content both live and on demand
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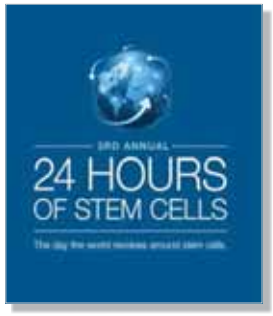
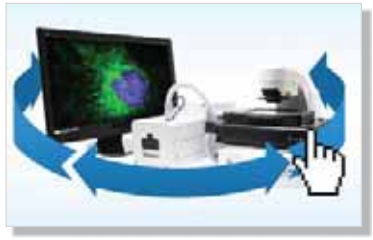


Virtual demonstration of the EVOS FL Auto Imaging System

Explore the innovative features of the EVOS™ FL Auto Imaging System and Onstage Incubator with this interactive virtual demonstration. Spin the unit around and interact with the key features of the system. Experience the simple, intuitive software that allows researchers to perform advanced imaging applications regardless of their skill level. Watch videos of time-lapse images collected by the EVOS FL Auto Imaging System. Choose options from the main menu to learn more about how the EVOS FL Auto Imaging System is optimally designed for:

- Live-cell imaging
- Area scanning
- Time-lapse imaging
- Automated cell counting
- Image tiling/stitching

See for yourself how the EVOS FL Auto Imaging System reduces the complexities of microscopy, allowing you to focus on generating great imaging data; visit **thermofisher.com/evosflauto**.



Molecular Probes School of Fluorescence

Learn the fundamentals of fluorescence imaging from the scientists who develop Molecular Probes™ reagents. We've spent hundreds of hours in the lab and want to share our best tips, tricks, and protocols for capturing beautiful images, including:

- Fundamentals of fluorescence microscopy
- Sample and fluorescent label considerations
- Analysis of your labeled samples
- Live- and fixed-cell staining protocols

It's our aim to include everything that we wish we had known when we started working with fluorescence imaging reagents, and we asked our Technical Support team to review each section to make sure we haven't overlooked anything. Even though we can't be right there in the lab with you when you do your experiments, we hope you'll benefit from the information we've collected. See for yourself at **thermofisher.com/mpsf**.

HCS Studio 3.0 delivers additional tools to high-content analysis users

Newly released Thermo Scientific™ HCS Studio™ 3.0 Cell Analysis Software has even broader utility for assay developers and screeners using Thermo Scientific high-content tools. This newest version provides the ability to rapidly assess the quality of your assay through the software's assay performance tool. Save time by quickly discovering which features will provide the most robust assay, and prevent oversampling in your screen by adjusting your scan's stopping criteria based on Z-prime results.

Integrated within the HCS Studio 3.0 software and the Store Data and Image Database is the ability to create plate maps that show the assignment of compounds or controls and their concentrations in individual wells. Take advantage of the convenience of storing and editing plate maps and assigning them to one or more plate scans. Whether you are printing plate maps for your lab notebook or simply saving them to the database, all the information you need to identify your experimental plate conditions is included, such as well type, compound, concentration, cell type, and antibody. Learn more about HCS Studio 3.0 and the portfolio of high-content screening products at **thermofisher.com/hcs**.

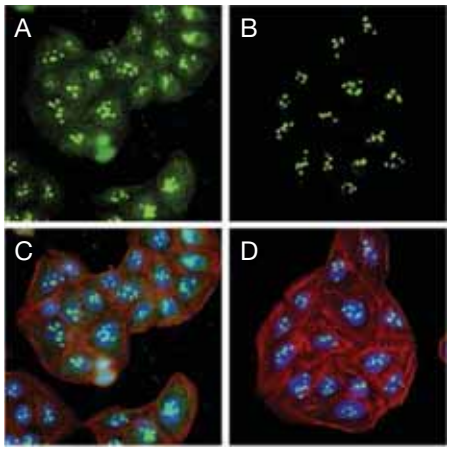
Rapid Z-prime tool to measure assay performance.

Superclonal secondary antibodies provide optimal signal-to-noise ratios for immunodetection

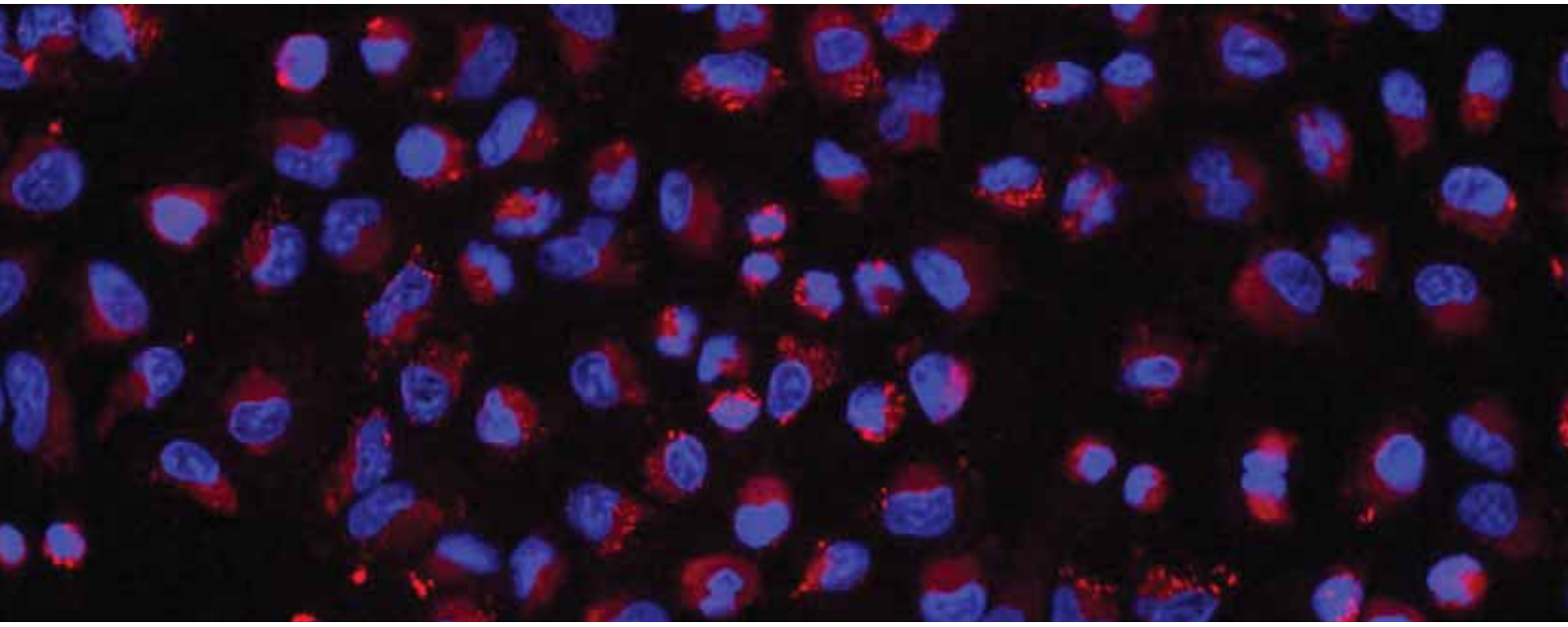
Sensitive immunodetection of antigens in cells and tissues requires brightly fluorescent and specific antibodies that produce minimal background staining. Thermo Scientific™ Superclonal™ secondary antibodies represent a breakthrough in recombinant antibody technology, providing exquisitely sensitive binding to their targets while also showing very low levels of nonspecific staining due to cross-reactivity.

To produce Superclonal secondary antibodies, we employ a proprietary screening and production process that yields mixtures of recombinant goat or rabbit secondary antibodies that are selected for their sensitivity and specificity for the target IgG species. These antibodies bind with the epitope-specific precision of monoclonal antibodies, while also achieving the multi-epitope coverage (e.g., both heavy and light chains of target IgGs) and sensitivity of polyclonal antibodies. By comparison, typical polyclonal secondary antibodies are affinity purified from the serum of immunized animals, resulting in a large, undefined pool of antibodies with an unknown set of epitope-binding characteristics. Although broad epitope coverage is a benefit of traditionally produced polyclonal secondary antibodies, poor lot-to-lot consistency due to animal variability and purification processes can lead to cross-reactivity and high background signals.

Superclonal secondary antibodies enable precise and accurate detection of mouse, rabbit, and goat IgG antibodies in cell imaging, ELISA, and western blot applications, with very little cross-reactivity to other species. Their recombinant origin helps ensure lot-to-lot consistency, minimizing the need to optimize each lot before using them in your assays. Superclonal secondary antibodies are available unconjugated, as well as conjugated with biotin, HRP, and selected Alexa Fluor™ dyes; see them all at **thermofisher.com/superclonal**.



Reduction in nonspecific staining with Superclonal secondary antibodies. HeLa cell nucleoli were labeled with anti-nucleostemin primary antibody, which was then detected with the Alexa Fluor™ 488 conjugate (green) of **(A, C)** highly cross-adsorbed goat anti–mouse IgG (H+L) antibody, or **(B, D)** goat anti–mouse IgG (H+L) Superclonal™ antibody (Cat. No. A28175). Multicolor images also show cell nuclei stained with DAPI (blue, Cat. No. D3571) and actin filaments with Alexa Fluor 594 phalloidin (red, Cat. No. A12381). Superclonal secondary antibodies exhibit significantly less cytoplasmic staining, indicating enhanced specificity.



Breathe new life into hypoxia research

Intracellular detection of low oxygen tension in live cells.

Oxygen homeostasis is an important physiological process that is required to maintain cellular health and function. Hypoxia is a condition of low oxygen tension in tissues and contributes significantly to the pathophysiology of major categories of human disease, including myocardial and cerebral ischemia, cancer, pulmonary hypertension, congenital heart disease, and chronic obstructive pulmonary disease. While generally associated with pathological conditions, hypoxia response pathways are also critical in the normal development of some cell types, such as hematopoietic stem cells. Although the significance of hypoxia in biological processes is well known, creating model systems to accurately control hypoxic conditions is extremely difficult for most researchers without access to elaborate instruments that allow precise control and maintenance of temperature, humidity, and gases (CO_2 and O_2) during an experiment. Fortunately, the EVOS™ FL Auto Imaging System with Onstage Incubator provides an easy-to-use platform that allows for the precise control of oxygen levels, thereby delivering an effective system for researchers to evaluate cellular responses to hypoxia by live-cell fluorescence imaging using the Image-iT™ Hypoxia Reagent (Figure 1).

Figure 1 (above). Imaging hypoxia with the Image-iT Hypoxia Reagent. A549 cells were labeled with Image-iT™ Hypoxia Reagent (red, Cat. No. H10498) and NucBlue™ Live ReadyProbes™ Reagent (blue, Cat. No. R37605) and imaged under 1% O_2 conditions using the EVOS™ FL Auto Imaging System with Onstage Incubator.

Cellular responses to hypoxia

The growth patterns of solid tumors impose low oxygen concentrations on their core cells, and therefore adaptation to hypoxia is advantageous for tumor development and survival [1]. Conversely, disruption of these hypoxic responses can lead to leukemic transformation [2]. Hypoxic responses are also critical for the normal development of hematopoietic stem cells, which reside within a hypoxic bone marrow microenvironment. Hypoxia signaling pathways are used for cell fate decisions leading to normal hematopoiesis [3].

Adaptation to hypoxia is mediated largely by transcriptional activation of genes that facilitate short-term (e.g., glucose transport) and long-term (e.g., angiogenesis) adaptive mechanisms. A key regulator of cellular responses to hypoxic conditions is the transcription factor HIF-1 (hypoxia inducible factor-1), which functions as a master regulator of cellular and systemic homeostatic response to hypoxia by activating transcription of a wide array of genes, including those involved in energy metabolism, angiogenesis, and erythropoiesis, as well as genes whose protein products increase oxygen delivery or facilitate metabolic adaptation to hypoxia.

Methods for studying hypoxia

While the importance of studying the cellular signaling pathways involved in the hypoxic response is clearly understood in a wide range of biological applications, developing model systems to precisely study the effects of low oxygen levels on cells and tissues remains technically challenging for most laboratories. One common method for studying the downstream effects of HIF-1 depletion involves the addition of cobalt chloride ($CoCl_2$) to cells in culture. $CoCl_2$ can mimic the effects of hypoxia by stabilizing the HIF-1 complex and thereby activating HIF-1–inducible genes. However, $CoCl_2$ only impacts the HIF-1 pathway and may not affect other hypoxia-related pathways. In addition, other unknown cellular processes and functions may be disrupted by $CoCl_2$ treatment, inducing phenotypes that are unrelated to the hypoxic response. Techniques for imaging hypoxia include the use of invasive oxygen electrodes to measure tissue oxygen levels, HIF-1 or Glut1 tissue stains to look for indirect evidence of hypoxia in cells, and nitroimidazoles that bind to protein thiols in hypoxic tissue at acute reductions in oxygen levels.

Spheroid culture methods have enabled substantial contributions to both basic cell biology and cancer biology (see "Mimic life in three dimensions" on page 20). The multicellular tumor spheroid (MCTS)—a 3D cell structure with a diameter of 200–500 µm—is a valuable model for cancer biology. Closely mimicking the physiology of small avascular tumors [4], spheroids in this model develop chemical gradients of oxygen, nutrients, and catabolites just like a tumor *in vivo*; they also possess histomorphological and functional features similar to those of tumors.

Both spheroids and tumors exhibit a heterogeneous distribution of cell types, expression patterns, and physiology. Cells located at the surface of a spheroid secrete specific compounds as a tumor would *in vivo*. Internally, spheroids possess the same hypoxic core seen in tumors; this hypoxic core is one of the most distinct characteristics of spheroid cultures that cannot be successfully reproduced with classic 2D culture methods. The MCTS model thus mimics *in vivo* solid tumors in which cells rapidly outgrow the blood supply, leaving the center of the tumor with an extremely low oxygen concentration (Figure 2). →

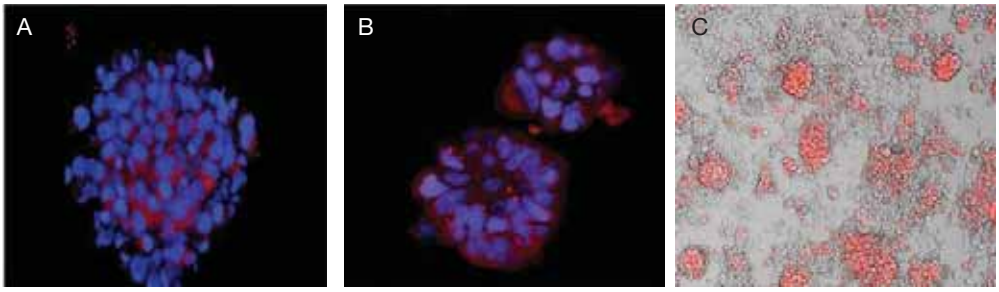


Figure 2. Tumor spheroids visualized with the Image-iT Hypoxia Reagent. (A, B) HeLa cells were grown on Thermo Scientific™ Nunclon™ Sphera™ 96-well U-bottom plates for 2 days in complete medium to allow for spheroid formation. Spheroids were stained with 5 µM Image-iT™ Hypoxia Reagent (red, Cat. No. H10498) for 3 hr. NucBlue™ Live ReadyProbes™ Reagent (blue, Cat. No. R37605) was used as nuclear counterstain. Images were acquired on an EVOS™ FL Auto Imaging System. **(C)** HeLa cells were grown in complete medium on NanoCulture Plates (Scivax Life Sciences) for 6 days to allow for spheroid formation, and then spheroids were stained with 10 µM Image-iT Hypoxia Reagent for 1 hr. Images were acquired on a Zeiss™ LSM 710 confocal microscope. Hypoxic conditions at the core of the spheroids are represented by red staining.

The EVOS FL Auto Imaging System with Onstage Incubator: Live-cell imaging under precisely controlled oxygen levels

Incubation chambers have been used to lower oxygen concentrations to allow for long-term cell growth in hypoxic conditions. However, real-time visualization of cellular processes in response to hypoxic conditions becomes problematic when transferring cells from the incubator to a microscope for imaging. Not only is it difficult to achieve precise control of oxygen levels in an incubator, but reoxygenation may create misleading results during the time required to image hypoxic cells.

The EVOS FL Auto Imaging System with Onstage Incubator includes an environmental chamber allowing for the precise control of oxygen levels, temperature, and humidity, thereby delivering an effective system for researchers to evaluate cellular responses to hypoxia over long time periods by live-cell fluorescence imaging. The onstage incubator contains port connections for air, O_2, and N_2. Gas concentrations are controlled by the software on the EVOS FL Auto system, allowing cells to be cultured using precise O_2 concentrations over an extended period of time. The EVOS FL Auto Imaging System with Onstage Incubator is easy to use: Simply input the desired O_2 level and the onstage incubator will equilibrate to the selected conditions. The incubator is designed specifically for the EVOS automated imaging system, which combines live-cell imaging, area scanning, image stitching, and time-lapse imaging in a single user-friendly platform. EVOS imaging systems make multichannel fluorescence microscopy accessible to both novice users and high-throughput core imaging facilities (see "The EVOS FL Cell Imaging System: A key component of an imaging core facility" on page 9).

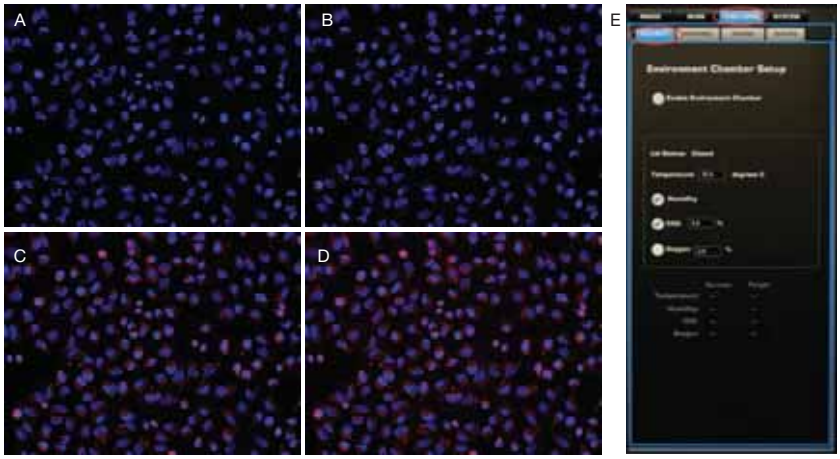


Figure 3. Imaging hypoxia with the Image-iT Hypoxia Reagent using the EVOS FL Auto Imaging System with Onstage Incubator. A549 cells were labeled with Image-iT™ Hypoxia Reagent (red, Cat. No. H10498) to visualize the cellular response to changing oxygen levels, and NucBlue™ Live ReadyProbes™ Reagent (blue, Cat. No. R37605) was used to label nuclei in all cells. **(A)** Under normal conditions (20% O_2), the Image-iT Hypoxia Reagent is nonfluorescent. Fluorescence increases as oxygen levels are decreased to **(B)** 5% O_2, **(C)** 2.5% O_2, and **(D)** 1% O_2. The NucBlue Live ReadyProbes Reagent, a formulation of Hoechst™ 33342, is used as a counterstain for autofocusing throughout the experiment; its fluorescence remains relatively unchanged under hypoxic conditions. **(E)** The user interface of the EVOS™ FL Auto Imaging System with Onstage Incubator shows the environmental chamber setup.

Image-iT Hypoxia Reagent: A real-time oxygen detector

With the EVOS FL Auto Imaging System, live-cell imaging can be performed in real time under hypoxic conditions using the Image-iT Hypoxia Reagent. The Image-iT Hypoxia Reagent is a fluorogenic, cell-permeant compound for measuring hypoxia in live cells. This reagent is nonfluorescent in an environment with normal oxygen concentrations (approximately 20%) and becomes increasingly fluorescent as oxygen levels are decreased (Figure 3). Unlike nitroimidazoles (such as pimonidazole) that respond only to very low oxygen levels (<1%) [5,6], the Image-iT Hypoxia Reagent begins to fluoresce when oxygen levels drop below 5%.

Because it responds quickly to a changing environment, Image-iT Hypoxia Reagent can serve as a real-time oxygen detector, with a fluorescent signal that increases as atmospheric oxygen levels drop below 5% and decreases if oxygen concentrations increase. In addition, Image-iT Hypoxia Reagent is very easy to use; just add it to cell culture medium and image. These properties make this reagent an ideal tool for detecting hypoxic conditions in tumor cells, 3D cultures, spheroids, neurons, and other tissues used in hypoxia research. Reagents with similar applications have been reported to detect tumors in small animals, and their fluorescence properties have been shown to correspond with increased HIF-1α expression and translocation in hypoxic environments [7]. →

Together again!

In the coming months, you will once again see the Molecular Probes brand closely affiliated with the Invitrogen brand. You can expect the same superior Molecular Probes products, technical support, educational tools, and customer service that we have provided these past 40 years, with the same commitment to your success.

The EVOS FL Cell Imaging System: A key component of an imaging core facility

Bret L. Judson, Imaging Facility Manager, Boston College

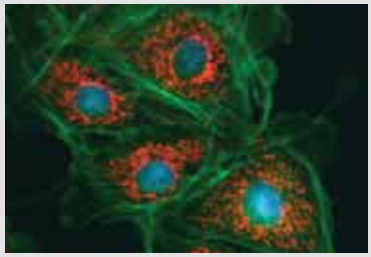
As a core facility manager, one often struggles with new users wanting to use the "latest and greatest" equipment that is available. Often, the "latest and greatest" will intimidate and deter new users from making the gains in their research that they are capable of. For example, training on a confocal microscope may take three or more sessions to reach independence from the core facility manager. When a new user is trying to learn the specifics of the lab protocols in addition to the complexities of the equipment, frustration can set in. A microscope that is easy to use, while remaining flexible for a variety of techniques, is a welcome addition.

At the Boston College Imaging Facility, we needed a microscope that streamlined training while also remaining flexible. I met with my local representative of the EVOS™ FL Imaging System and also spoke with several more traditional microscope representatives to test their offerings in this capacity. I was quickly impressed with the EVOS FL microscope: it took less than 5 minutes to unbox the instrument and to have it operational. Other key features of the EVOS FL system were the objective choices, on-screen viewing (no eyepieces), stage flexibility, ease of use, camera integration, and environmental consciousness.

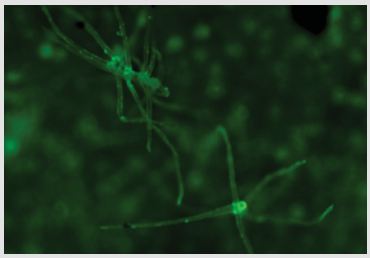
When choosing an objective, we are not limited to low-power observation; all objectives are supported, including oil immersion. In a core facility that uses all specimen size scales, having a range of objectives is very important. In addition, the camera integrated into the EVOS FL microscope body allows for easy image capture and simple movie creation. Files are typically saved as TIFF images for ease of integration into image processing programs like ImageJ. All observation is done via an LCD screen, making imaging and training very easy—you can easily point out features of interest and eliminate guesswork.

The fact that the EVOS FL system is easy to use is in no way indicative of how powerful it is. Even for absolute beginners, training is very intuitive and requires almost no time; a typical training session might be 10–15 minutes before users can operate the system unassisted. In the end, the choice to fill this particular need within my core facility was an easy one. Our EVOS FL system has been in near-daily use for the last two years, and I highly recommend it to anyone considering a microscope purchase as part of a sound decision-making strategy. ■

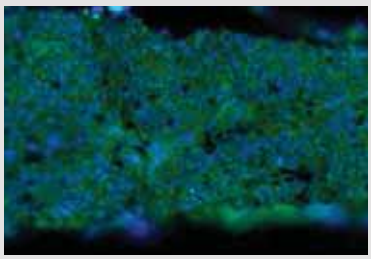
Bret L. Judson is the Imaging Facility Manager at Boston College within the Department of Biology, and teaches a cell imaging course in addition to managing the core facility. His research interests lie in the field of cell biology, with an emphasis on using microscopy-based approaches to solve biological questions. All images shown were taken by students in the Advanced Lab in Cell Imaging course at Boston College.



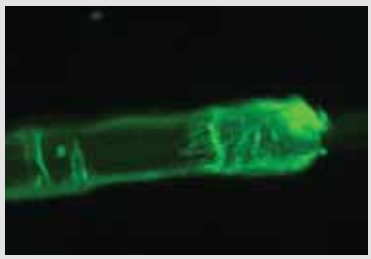
FluoCells™ Prepared Slide #1 (Cat. No. F36924) imaged at 40x on the EVOS™ FL Imaging System with the EVOS DAPI, GFP, and Texas Red™ Light Cubes.



A low-magnification view of a sycamore leaf with attached filamentous fungi imaged on the EVOS™ FL Imaging System with the EVOS GFP Light Cube.



A section of a red mulberry leaf imaged on the EVOS™ FL Imaging System with the EVOS DAPI and GFP Light Cubes.



A human hair showing the follicle and shaft imaged on the EVOS™ FL Imaging System with the EVOS GFP Light Cube.

Immunodetection of HIF-1 in fixed cells

In fixed-cell imaging, HIF-1 expression is commonly used as a marker for monitoring cells following exposure to hypoxic conditions. The HIF-1α mouse monoclonal antibody provides a highly specific probe for evaluating HIF-1α expression in fixed-cell samples. We offer a number of Thermo Scientific™ Pierce™ antibodies that recognize HIF-1α and have been verified to perform in various applications, including immunocytochemistry (ICC), immunohistochemistry (IHC), western blot analysis, and immunoprecipitation; Figure 4 shows an example of ICC using the anti–HIF-1α mouse monoclonal antibody, clone mgc3, which has citations describing its use in all of the above applications. Also available is the well-validated anti–HIF-1α mouse monoclonal antibody, clone H1alpha67, which has proven useful in at least 10 different types of immunoassays, including western blotting (Figure 5).

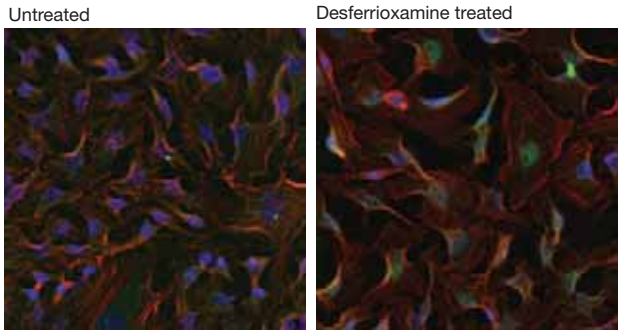


Figure 4. Immunofluorescence analysis of HIF-1α localization in HeLa cells after treatment with desferrioxamine. HeLa cells were either left untreated (left panel) or treated with 100 µM desferrioxamine mesylate (a hypoxia-mimetic agent, right panel) for 16 hr. After fixation with formalin, cells were permeabilized with 0.1% Triton™ X-100 in TBS for 15 min, blocked with 0.3% BSA for 15 min, and labeled with anti–HIF-1α mouse monoclonal antibody (clone mgc3, Cat. No. MA1-516) at a dilution of 1:100 for 1 hr at room temperature. Cells were then washed with PBS, and incubated with a DyLight™ 488 goat anti–mouse IgG (H+L) secondary antibody (green, Cat. No. 35502) at a dilution of 1:500 for 30 min at room temperature. F-actin was stained with DyLight 594 Phalloidin (red, Cat. No. 21836), and nuclei were stained with Hoechst™ 33342 (blue, Cat. No. 62249). Images were taken on a Thermo Scientific™ ArrayScan™ instrument at 20x magnification.

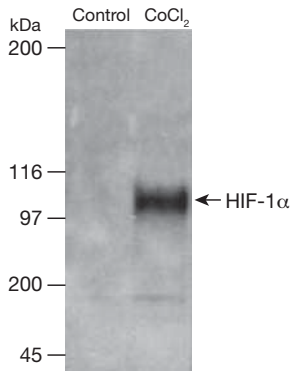


Figure 5. Western blot analysis of HIF-1α localization in COS-7 nuclear extracts after treatment with cobalt chloride. COS-7 cells were treated with cobalt chloride ($CoCl_2$), which stabilizes the HIF-1 complex, mimicking the effects of hypoxia. Nuclear extracts were prepared and subjected to western blot analysis using anti–HIF-1α mouse monoclonal antibody (clone H1alpha67, Cat. No. MA1-16504).

Accelerating the pace of hypoxia research

The EVOS FL Auto Imaging System with Onstage Incubator allows for the precise control of oxygen concentrations, overcoming technical hurdles that have plagued hypoxia research in the past. This easy-to-use system, in combination with the Image-iT Hypoxia Reagent, is ideal for studying the role of cellular responses to hypoxia in both basic biological processes and disease mechanisms. Visit **thermofisher.com/evosflauto** to download a step-by-step guide for setting up hypoxic conditions using the EVOS FL Auto Imaging System with Onstage Incubator, and find out how easy it is to request a demonstration of the EVOS imaging systems in your own lab. ■

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Product	Quantity	Cat. No.
EVOS™ FL Auto Imaging System	1 each	AMAFD1000
EVOS™ Onstage Incubator	1 each	AMC1000
HIF-1α Mouse Monoclonal Antibody (clone mgc3)	100 µL	MA1-516
HIF-1α Mouse Monoclonal Antibody (clone H1alpha67)	100 µL	MA1-16504
Image-iT™ Hypoxia Reagent	1 mg	H10498
NucBlue™ Live ReadyProbes™ Reagent	1 kit	R37605
Nunclon™ Sphera™ 96 U-Well Plates	1 per pack, 8 per case 5 per pack, 50 per case	174925 * 174929 *

* These Thermo Scientific™ cell culture products are available at **thermoscientific.com**.

Optimize nanoscale imaging at every step

Practical considerations for super-resolution microscopy.

Fluorescence microscopy offers an unparalleled view into the spatial and temporal functioning of cells in an intact system. Until recently, however, the fundamental diffraction properties of light limited the resolution of standard fluorescence microscopy to a few hundred nanometers. In the last few years, concurrent innovations in optics, instrumentation, and software have circumvented this limit, providing nanometer-scale views into the arrangement and functions of intracellular components (Figure 1). These methods include structured illumination microscopy (SIM) and stimulated emission depletion (STED) microscopy, as well as single-molecule localization techniques such as photoactivated localization microscopy (PALM), stochastic optical reconstruction microscopy (STORM), direct STORM (dSTORM), and ground-state depletion followed by individual molecule return (GSDIM).

For an excellent primer on super-resolution microscopy (SRM) techniques, see the article by Galbraith and Galbraith [1]. There are a number of critical considerations when preparing samples and labeling for SRM. Several recent reviews have provided detailed protocols and discussions of these considerations [2–5]; here we will summarize the main points from these protocol guides.

Sample preparation

Optimal sample preparation is a critical step in any fluorescence microscopy workflow. With SRM techniques, any nonspecific binding or perturbation of cellular structure will be even more noticeable. It is imperative to select the optimal fixative for the structure under investigation. Ice-cold methanol, glutaraldehyde, and formaldehyde have all been successfully used as fixatives, but the best fixative choice is dependent on each primary antibody and must be empirically determined [2,5]. Allen and coworkers [2] and Whelan and Bell [5] provide detailed protocols and recipes for each fixation protocol, as well as examples of images resulting from suboptimal fixation [5]. In addition to the fixative choice, the temperature and subsequent wash protocols will also affect image quality and should be optimized for a given label [5].

Nonspecific labeling is especially problematic in SRM, and optimizing the blocking protocols for each antigen is critically important [2,3,5]. A possible protocol addition is a prefixation extraction step [4,5]. During extraction, the plasma membrane is permeabilized, thereby removing cytoplasmic components that may bind nonspecifically to the primary antibody [4]. Note that an extraction step does not improve the fidelity of labeling for every antibody; for example, extraction has been shown to be beneficial for labeling of microtubules but not mitochondria [4].

The final step in sample preparation is the labeling of the structure of choice with a fluorescent dye. When using a secondary antibody conjugated to a fluorophore, it is important to consider that the large size of an IgG can limit your effective resolution [3] and that an additional antibody step introduces another point of nonspecific binding. Wherever possible, use highly cross-adsorbed secondary antibodies to reduce nonspecific binding [4]. The use of antibody fragments, such as Fab and $F(ab')_2$ [2] or nanobodies [6], should be considered because these labels are smaller in size than an intact IgG. Furthermore, fluorophores can be coupled directly to primary antibodies using a number of approaches, such as NHS ester conjugation [4,7], eliminating the need for a secondary antibody.

Fluorophore performance

Optimal fluorophore choice will vary with the application and the SRM strategy used. Here are practical considerations for some of the different SRM techniques.

Structured illumination microscopy (SIM)—In SIM, the sample is first illuminated with patterned light, and then the information in the Moiré fringes that lie outside of the normal range of observation is analyzed. Reconstruction software deciphers the images to give a resolution limit of about 100 nm (two-fold higher resolution than the diffraction limit of 250 nm). Unlike techniques such as STED and STORM, SIM does not require fluorophores with specialized photochemical properties. →

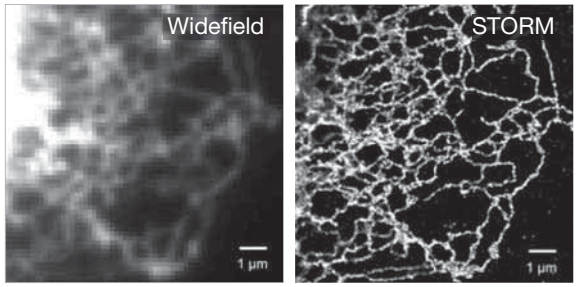

Figure 1. Improvement in resolution with STORM imaging. Widefield (diffraction-limited) and STORM images of a BSC-1 cell expressing a GFP-SEC61B fusion, which labels the endoplasmic reticulum, and stained with an Alexa Fluor™ 647 conjugate of an anti-GFP antibody. Images courtesy of Joshua Vaughan, University of Washington, and acquired at an SRM workshop on a custom-built STORM microscope.

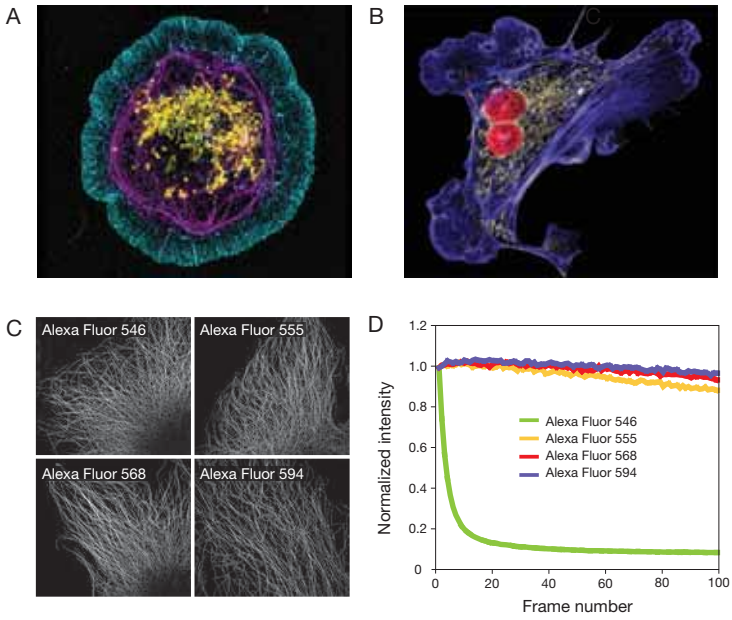

Figure 2. Multicolor SIM imaging. (A) *Drosophila* S2 cells labeled with Alexa Fluor™ 488 phalloidin (blue, Cat. No. A12379), anti-tubulin antibody followed by a secondary antibody conjugated to Alexa Fluor 568 (magenta), and MitoTracker™ Deep Red FM (gold, Cat. No. M22426). **(B)** BPAE cell labeled with DAPI (red, Cat. No. D3571), Alexa Fluor 488 phalloidin (purple, Cat. No. A12379), and MitoTracker Red FM (orange, Cat. No. M22425). **(C)** SIM imaging of HeLa cells labeled with anti–alpha-tubulin antibody (clone DM1A) conjugated to orange-red Alexa Fluor dyes (Alexa Fluor 546, Alexa Fluor 555, Alexa Fluor 568, or Alexa Fluor 594), with **(D)** bleach curves over 100 frames of SIM imaging (15 images per frame). Images courtesy of **(A)** William Voss and Catherine Galbraith, Oregon Health & Science University, and acquired on a Zeiss™ Elyra SR SIM system, and **(B–D)** Talley Lambert, Harvard Medical School, and acquired on a DeltaVision OMX™ 3D-SIM system.

Because of this property, SIM lends itself to multicolor imaging (Figure 2). Given the illumination protocols required for SIM, however, the more photostable the dye, the better the data. Photostability profiles for dyes under standard microscopy techniques are a good guide when choosing dyes for SIM (Figure 2D). Bright, photostable fluorophores that match the excitation and emission filters on the given SIM system should be selected. This recommendation is especially relevant for time-lapse or 3D SIM, where numerous images are acquired in either time or space. The addition of a high–refractive index mounting medium that contains an effective antifade reagent improves photostability and therefore SIM image quality. Also note that SIM is a widefield technique and therefore image quality will decrease in thicker samples; consequently, samples should be thin and mounted as close to the coverslip as possible.

Stimulated emission depletion (STED) microscopy—STED microscopy uses two laser pulses to localize fluorescence at each focal spot; the first pulse is used to excite a fluorophore to its fluorescent state, and the second pulse is a modified beam used to de-excite (through stimulated emission depletion) any fluorophores surrounding the excitation focal spot. STED is a universal property of all fluorescent dyes and proteins—the challenge is to find those dyes photostable enough to withstand the high-intensity illumination required while also matching the excitation and depletion laser lines available on commercial STED systems. Despite these criteria, many fluorophores work well for STED, including several Molecular Probe™ dyes—Oregon Green™ 488, Alexa Fluor™ 488, Alexa Fluor 532 (Figure 3), Alexa Fluor 568, and Alexa Fluor 594 dyes—as well as GFP. For dual-color STED, it is often beneficial to pair a dye exhibiting a standard Stokes shift with a dye exhibiting a long Stokes shift (e.g., Alexa Fluor 488 and Pacific Orange™ dyes [8]).

Single-molecule localization microscopy (STORM, dSTORM, and GSDIM)—Single-molecule localization techniques place the most stringent demands on fluorophores. Dyes need to be induced to "blink" from a dark state to an on state, thereby enabling the precise localization of the actual position of each fluorophore from its point-spread function. The SRM method STORM utilizes stochastic activation and time-resolved localization of photoswitchable fluorophores to generate high-resolution images. Photoswitching dyes must have high photon outputs per switch, coupled with a low duty cycle (i.e., they are in a non-emitting state longer than in an emitting state). With appropriate

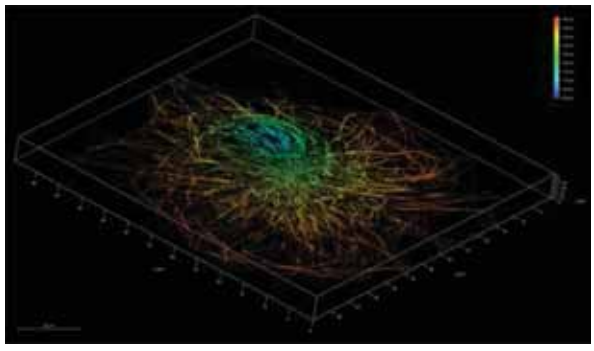

Figure 3. 3D STED projection. COS-7 cells were fixed and labeled with anti-tubulin antibody followed by F(ab´)$_2$ fragments conjugated to Alexa Fluor™ 532 dye (depletion wavelength, 660 nm). Image courtesy of Jana Doehner, University of Zurich, and acquired on a Leica™ TCS SP8 STED 3X system.

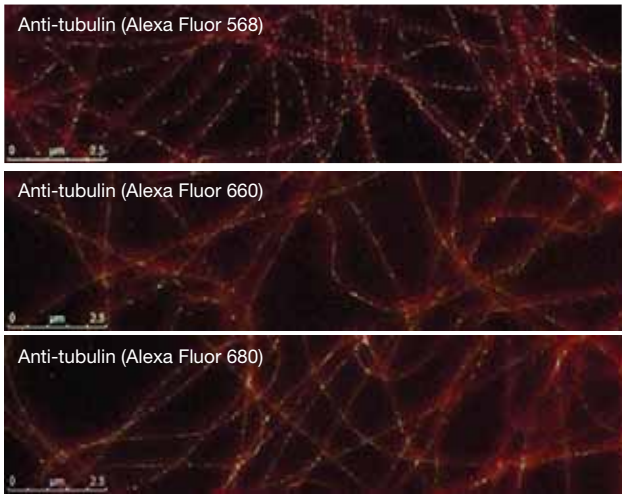

Figure 4. Use of Alexa Fluor dyes in single-molecule localization microscopy. COS-7 cells were fixed and labeled with anti-tubulin antibody followed by a secondary antibody conjugated to either Alexa Fluor™ 568, Alexa Fluor 660, or Alexa Fluor 680 dye, and imaged in the presence of GLOX and MEA (see Allen et al. [3] and Halpern et al. [4] for protocols). Widefield images (red) are overlaid with ground-state depletion followed by individual molecule return (GSDIM) images (green). Images courtesy of Jana Doehner, University of Zurich, and acquired on a Leica™ SR GSD microscope.

dye–buffer combinations, an optimized STORM system can generate images with 17 nm resolution [9].

Two forms of STORM exist. In the first method, two fluorescent dyes are coupled to the antibody of choice—an "activator dye" to induce switching and a "reporter dye" from which emission is detected. A good starting point, according to Bates and coworkers [7], is a degree of labeling (DOL) between 2 and 4 for the activator and 0.1 and 1 for the reporter (see "Tools for studying antibody internalization and trafficking" on page 24 for a summary of antibody labeling methods). For the activator–reporter STORM technique, many different Alexa Fluor dyes have been used as effective activators and both Alexa Fluor 647 dye and Alexa Fluor 750 dye have been shown to be reliable reporters. The availability of multiple activator–reporter pairs facilitates multicolor STORM applications.

The second method, commonly known as dSTORM or GSDIM, relies upon direct switching of a single fluorophore through specific excitation parameters. When using dSTORM or GSDIM, it is possible to use commercially available secondary antibodies with a DOL between 2 and 4 as a starting point [3]. Lower DOLs prove beneficial for densely labeled specimens; however, if the DOL (or indeed the labeling density with the primary antibody) is too low, then the continuity of the structure may be lost [3]. The accuracy of localizing individual fluorophores is critically dependent on the blinking properties. Many recent studies have characterized optimal dye properties and buffer conditions for single-molecule detection, and these are succinctly summarized by Allen and coworkers [3]. For single-molecule localization techniques, STORM, dSTORM, and GSDIM imaging of most dyes occurs in an oxygen-buffering system, such as a glucose oxidase and catalase with a primary thiol (e.g., β-mercaptoethylamine (MEA)) [3,4,9]. Figure 4 shows single-molecule GSDIM imaging using direct switching of either Alexa Fluor 568, Alexa Fluor 660, or Alexa Fluor 680 dye.

Alexa Fluor 647 dye is used extensively in dSTORM (Figure 1) because it exhibits extremely good photoswitching properties—namely, high photon output, cycle number, and survival fraction, along with a low duty cycle; it is the most forgiving dye for the novice user [4] and has been used in test samples to optimize STORM systems [10]. Moreover, even when the fluorescent label used is not ideal for single-molecule detection, such as occurs with a GFP chimera, the use of an anti-GFP antibody conjugated to Alexa Fluor 647 dye enables imaging of the GFP chimera by dSTORM or GSDIM (Figure 1). For two-color dSTORM, the best resolution is reportedly achieved with Alexa Fluor 647 and Alexa Fluor 750 dyes under phosphine quenching conditions [11]. It is also possible to perform two-color dSTORM with other Alexa Fluor dyes: for example, Alexa Fluor 568 and Alexa Fluor 647 dyes [12] or Alexa Fluor 532 and Alexa Fluor 647 dyes [13].

Optimize SRM imaging in your lab

Imaging below the diffraction limit of light is revolutionizing fluorescence microscopy. The optimization of sample preparation and fluorophore choices is central to super-resolution imaging. The references provided here are an excellent starting point for those considering applying SRM techniques to their studies. For a complete list of compatible probes and supporting references, go to **thermofisher.com/srmbp72**. ■

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At long last, protection from photobleaching for live cells

ProLong Live Antifade Reagent protects fluorescent proteins and dyes in live-cell imaging.

One of the key pain points when labeling cells and tissues with fluorescence is photobleaching, the degradation of fluorescent signals as samples are exposed to light. Photobleaching is a complex photodynamic process whereby a photoexcited fluorophore interacts with molecular oxygen, resulting in the destruction of the fluorophore and the production of highly reactive singlet oxygen ($^1O_2$) that can further degrade neighboring dye molecules. This loss of signal is particularly problematic when attempting to collect time-course data, image rare targets with low signal-to-noise ratios, or quantitatively compare fluorescently labeled samples. In this article we describe the recently introduced ProLong™ Live Antifade Reagent, which helps protect fluorescent proteins and dyes from photobleaching in live cells.

How to protect live cells from photobleaching

Researchers using fixed-cell systems have long had the luxury of a number of commercial antifade mounting media to choose from, including the recently released ProLong™ Diamond and SlowFade™ Diamond Antifade Mountants (see page 16). These fixed-cell mountants contain antioxidants and free radical scavengers that greatly reduce the rate of photobleaching; these formulations, however, are not intended for use with live-cell systems. Factors such as pH, osmolarity, and nutrient content in fixed-cell mountants are not balanced for live cells, and the antifade components themselves may be cytotoxic.

Consequently, when imaging live cells, researchers have relied on their own in-lab antifade formulations, such as Trolox™ antioxidant [1,2] or ascorbic acid added to cell environments. Unfortunately, the protection from photobleaching provided by these additions (at concentrations that do not perturb cell function) is not significant for most fluorescent dyes and virtually nonexistent for fluorescent proteins. Without an effective live-cell antifade option, the best course of action for live-cell imaging has been to minimize light exposure as much as possible [3,4] by limiting exposure time, light intensity, and frequency of sampling [3], and to use only the most photostable dyes, which severely reduces fluorophore choices.

ProLong Live Antifade Reagent protects live cells...

After a comprehensive review of many different formulations, we offer ProLong Live Antifade Reagent. Based on Oxyrase™ technology [5], ProLong Live reagent contains enzymes from the plasma membrane of naturally occurring *E. coli*. These enzymes metabolize environmental components that exacerbate photobleaching, and they are not cell permeant so intracellular functions are minimally affected.

ProLong Live Antifade Reagent is diluted into cell medium or a suitable imaging buffer, such as FluoroBrite™ DMEM, and then added directly to cells for a 15- to 120-minute incubation. After incubation, imaging can be performed for up to 24 hours with continuous protection from photobleaching. ProLong Live Antifade Reagent has been validated to provide protection for a range of live-cell–compatible organic dyes, including Hoechst™ 33342, MitoTracker™, LysoTracker™, and CellTracker™ dyes; Figure 1 shows representative photobleaching curves. Furthermore, ProLong Live Antifade Reagent can be used for dyes across the spectrum without any initial quenching or increased background fluorescence.

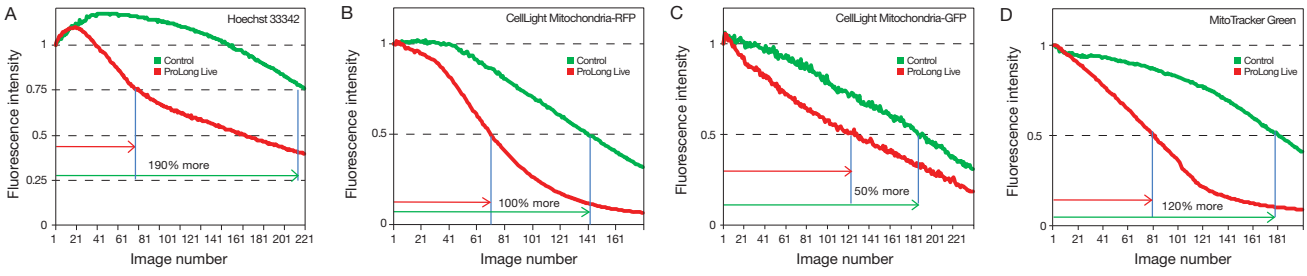

Figure 1. Quantitative analysis of protection from photobleaching by ProLong Live Antifade Reagent for key dyes and fluorescent proteins. HeLa or U2OS cells were stained with **(A)** Hoechst™ 33342 (Cat. No. H21492), **(B)** CellLight™ Mitochondria-RFP (Cat. No. C10505), **(C)** CellLight™ Mitochondria-GFP (Cat. No. C10508), or **(D)** MitoTracker™ Green FM (Cat. No. M7514) reagents. Cells were incubated for 2 hr in the dark either in complete medium (control) or in complete medium containing ProLong™ Live Antifade Reagent (Cat. No. P36974). After incubation, cells were imaged every 15 sec using optimal but consistent excitation/emission imaging conditions with the Thermo Scientific™ ArrayScan™ VTI HCS Reader.

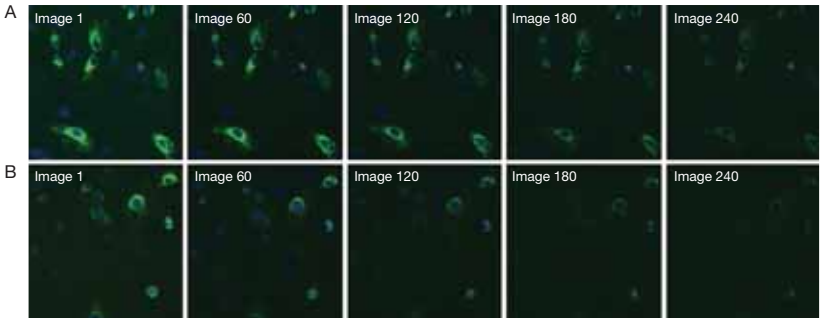

Figure 2. Qualitative assessment of protection from photobleaching by ProLong Live Antifade Reagent for Hoechst 33342 and GFP. HeLa cells were transduced with CellLight™ Mitochondria-GFP (Cat. No. C10508) for 24 hr, then stained with Hoechst™ 33342 (Cat. No. H21492) for 15 min. ProLong™ Live Antifade Reagent (Cat. No. P36974) was added to one sample, while the second sample remained in complete medium. After a 2 hr incubation, samples were imaged every 15 sec for a total of 240 total images, using the same exposure conditions for each image and sample. The sample incubated with **(A)** ProLong Live Antifade Reagent retained more signal at all time points, when compared to the sample in **(B)** medium alone.

Importantly, ProLong Live Antifade Reagent also protects fluorescent proteins from photobleaching. Compared with cells in medium only, use of ProLong Live Antifade Reagent with emGFP or TagRFP allows you to acquire many more images before the sample fades to half of the initial fluorescence intensity (Figures 1B and 1C). The images in Figure 2 show this protection from photobleaching over time for HeLa cells labeled with both emGFP and Hoechst 33342 dye.

...Without affecting cell viability or proliferation

ProLong Live Antifade Reagent has been rigorously tested and shows little to no measurable effect on cell vitality, proliferation, or incidence of apoptosis over 48 hours (Figure 3). Using Dead Red Stain (a component of the LIVE/DEAD™ Cell Imaging Kit, Figure 3A), which only enters and fluoresces in dead cells, we showed that after 48 hours there was no significant decrease in the percentage of live HeLa cells in the presence of ProLong Live reagent. Likewise, cells stained with PrestoBlue™ Reagent (Figure 3B), which is converted to fluorescent resorufin by cellular metabolism, showed no significant decrease in fluorescence after 48 hours in the presence of ProLong Live reagent, indicating no change in cell vitality. And for cell proliferation, the cells' ability to divide normally in the presence of ProLong Live reagent for up to 48 hours was demonstrated with the Click-iT™ Plus EdU Assay (Figure 3C), which measures DNA synthesis, and the CyQUANT™ Direct Assay (data not shown), which measures total DNA. Finally, using CellEvent™ Caspase-3/7 Green Detection Reagent (Figure 3D), we have shown that ProLong Live Antifade Reagent does not induce apoptosis over a 48-hour time period.

Get live-cell protection from photobleaching

ProLong Live Antifade Reagent can help you maintain longer imaging times for scans or time-lapse experiments, and enable you to detect low-abundance targets without sacrificing cell health. To learn more, go to **thermofisher.com/prolonglivebp72**. ■

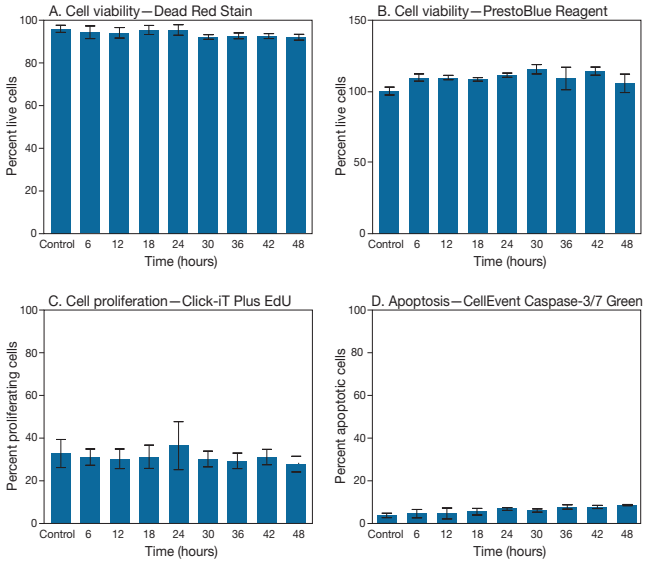

Figure 3. Assessment of cell viability and proliferation in the presence of ProLong Live Antifade Reagent. HeLa cells were plated at a concentration of 1,000 cells/well in a 96-well plate using Gibco™ MEM (Cat. No. 11095-080) with 10% FBS (Cat. No. 16000-036). A working concentration of ProLong™ Live Antifade Reagent (Cat. No. P36974) was added to 8 replicate wells, every 6 hr, for up to 48 hr, while 8 wells without antifade reagent served as a control. Cell viability was detected using **(A)** Dead Red Stain (a component of the LIVE/DEAD™ Cell Imaging Kit (488/570), Cat. No. R37601) and **(B)** PrestoBlue™ Cell Viability Reagent (Cat. No. A13261), **(C)** cell proliferation was detected with Click-iT™ Plus EdU Reagent (Cat. No. C10639), and **(D)** apoptosis was detected with CellEvent™ Caspase-3/7 Green Detection Reagent (Cat. No. C10423) using the ArrayScan™ VTI HCS Reader. These data demonstrate that the presence of ProLong Live Antifade Reagent has no statistically significant effect on cell vitality, proliferation, or incidence of apoptosis over a 48 hr time period. Error bars = standard deviation.

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Product	Quantity	Cat. No.
ProLong™ Live Antifade Reagent, for live-cell imaging	1 mL	P36975
	5 x 1 mL	P36974

Superior protection from photobleaching for fixed cells

ProLong Diamond and SlowFade Diamond Antifade Mountants for fixed-cell imaging.

The choice of mounting medium is an integral part of the fluorescence imaging workflow and is critically important when trying to obtain the highest-quality images from fixed cell and tissue samples. In immunocytochemical (ICC) and immunohistochemical (IHC) analyses, aqueous mounting media allow rapid mounting of labeled samples from water or buffer. Aqueous mounting media are generally categorized as soft- or hard-setting, depending on whether the mountant contains a gelling agent. In this article, we describe our newest antifade mounting media: hard-setting ProLong™ Diamond Mountant and soft-setting SlowFade™ Diamond Mountant. These two aqueous mounting formulations enable outstanding image quality as well as unparalleled fluorescent signal retention across the spectrum of traditional dyes, premium dyes, and even fluorescent proteins (Figure 1).

ProLong Diamond Antifade: A hard-setting mountant

ProLong Diamond Antifade Mountant is a premium hard-setting mounting medium offered in convenient 2 mL dropper bottles or in a 10 mL bottle. The mountant contains a proprietary polymeric constituent that semipermanently affixes the coverslip to the slide, allowing extended storage of the sample without the need for sealing. In addition to archivability, the polymer increases the refractive index of the mounting medium to 1.47 upon curing, close to that of glass and common immersion oils, providing greater resolution when imaging at higher magnification. The higher viscosity of the hard-setting ProLong Diamond mountant may also slow the off-rate and diffusion rate of low-affinity labels.

SlowFade Diamond Antifade: A soft-setting mountant

SlowFade Diamond Antifade Mountant is a premium soft-setting mounting medium. Unlike ProLong mountants, the SlowFade mountant contains no polymer and is instead a buffered glycerol solution. SlowFade Diamond Antifade Mountant is recommended when images must be obtained quickly, as samples can typically be imaged immediately after mounting. Also, SlowFade mountants can be washed easily from the coverslip, allowing additional staining of samples (Figure 2). Because the mountant is not permanent, longer-term storage requires sealing the coverslip with paraffin, VALAP (a mixture of Vaseline™ petroleum jelly, lanolin, and paraffin), or a solvent-based polymer mixture (such as nail polish). SlowFade Diamond mountant has a lower refractive index (1.42) than ProLong Diamond mountant; therefore, water- or glycerol-matched objectives are recommended for higher-magnification imaging.

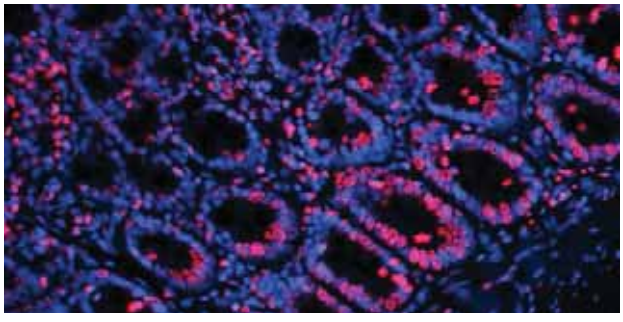

Figure 1. ProLong Diamond Antifade Mountant enables the acquisition of high-quality images from fixed tissues. Formalin-fixed, paraffin-embedded (FFPE) rat intestinal tissue was labeled with an antibody against histone 2B. This antibody was then detected with tyramide signal amplification using the TSA Kit #15 with Alexa Fluor™ 594 tyramide (pink, Cat. No. T20925). Nuclei were counterstained with NucBlue™ Fixed Cell ReadyProbes™ Reagent (blue, Cat. No. R37606) and then mounted in ProLong™ Diamond Antifade Mountant (Cat. No. P36965). This image was taken on a fluorescence microscope using a 20x objective.

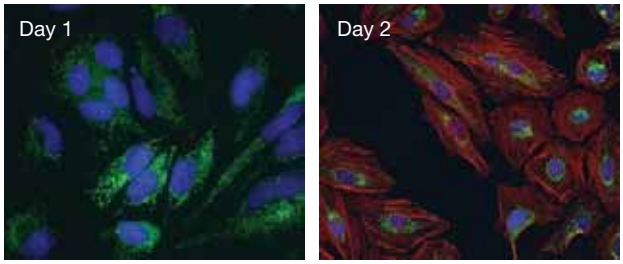

Figure 2. SlowFade Diamond Antifade Mountant can be washed off easily to accommodate restaining. HeLa cells were fixed, permeabilized, blocked, probed with antibody to complex V inhibitor protein followed by Alexa Fluor™ 488 goat anti–mouse IgG (H+L) antibody (Cat. No. A11005), and stained with NucBlue™ Live ReadyProbes™ Reagent (Cat. No. R37605). Labeled cells were mounted in SlowFade™ Diamond Antifade Mountant (Cat. No. S36967) and imaged (day 1). Slides were stored overnight at 4°C. On day 2, coverslips were removed, and cells were washed, further stained with Texas Red™ Phalloidin (Cat. No. T7471), remounted using SlowFade Diamond Antifade Mountant, and imaged (day 2), with no loss of signal.

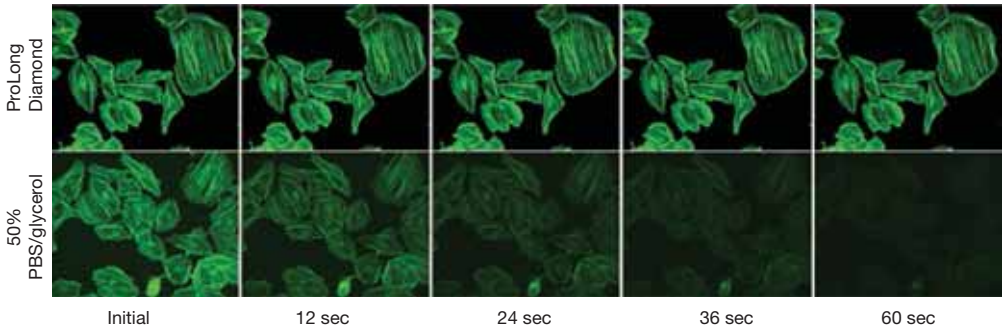

Figure 3. A 60-second time course shows the resistance to photobleaching afforded by ProLong Diamond Antifade Mountant. Fixed HeLa cells were labeled with FITC Phalloidin (Cat. No. F432) and mounted in ProLong™ Diamond Antifade Mountant (Cat. No. P36965) or 50% PBS/glycerol. Images were acquired at 12 sec intervals using a 20x objective with continuous illumination from a standard 100-watt Hg-arc lamp.

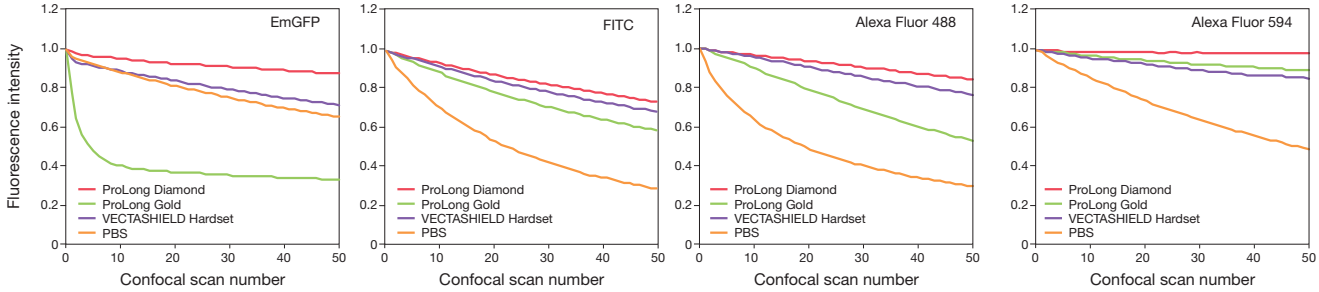

Figure 4. ProLong Diamond Antifade Mountant provides unparalleled protection against photobleaching for fluorescent proteins and dyes. HeLa or U2OS cells were stained and mounted using standard ICC protocols, and photobleaching resistance was quantified on an Zeiss™ LSM 710 confocal microscope. Five regions within three fields of view were scanned 50 times with a 1.58 µsec dwell time per pixel. Excitation wavelength and intensity were optimized for the fluorophore being measured.

Antifades that protect fluorescent dyes and proteins

ProLong Diamond and SlowFade Diamond Antifade Mountants offer superior protection from photobleaching for Alexa Fluor™ and DyLight™ dyes and traditional dyes (such as FITC, Texas Red™, and CyDye® fluorophores), as well as fluorescent proteins including emGFP, TagRFP, and mCherry (Figure 3, Figure 4). Unlike other antifade solutions, ProLong and SlowFade mountants do not significantly quench CyDye fluorophores or significantly increase background (Figure 5), helping to ensure that the best-quality high-resolution images can be obtained with the highest signal-to-noise ratios. It is important to note that fixed-cell mounting media are not intended for live-cell imaging, as the components of most fixed-cell mounting media will compromise the health of live cells or even lead to cell death. To protect live cells from photobleaching, we recommend using ProLong Live Antifade Reagent, which is described on page 14.

Choose the antifade mountant that works best for you

ProLong Diamond and SlowFade Diamond Antifade Mountants offer superior protection against photobleaching and provide excellent signal-to-noise ratios, allowing for exceptional fixed-cell imaging across the spectrum of dyes and fluorescent proteins. Find out more about the complete line of Molecular Probes™ antifade reagents at **thermofisher.com/antifadesbp72**. ■

Figure 5. ProLong Diamond and SlowFade Diamond Antifade Mountants provide images with high signal-to-noise ratios (S:N). HeLa cells were fixed, permeabilized, blocked, probed with mouse anti–beta-tubulin antibody (Cat. No. 322600), and then labeled with Alexa Fluor™ 647 goat anti–mouse IgG (H+L) antibody (Cat. No. A11005). Labeled cells were mounted in the four antifade mountants indicated, stored at room temperature overnight, and then imaged using a 20x objective.

Product	Quantity	Cat. No.
ProLong™ Diamond Antifade Mountant	2 mL	P36965
	10 mL	P36970
SlowFade™ Diamond Antifade Mountant	2 mL	S36967
	10 mL	S36972

Accessible technology for high-content analysis

Cellinsight CX7 High Content Analysis Platform.

High-content analysis (HCA) integrates fluorescence microscopy, image processing, automated measurements, and informatics into a complex tool that has not only facilitated fundamental discoveries in biology but also enabled the progression of many compounds through the drug discovery process. Since their introduction in 1999, the Thermo Scientific™ ArrayScan™ HCA readers have been cited in over 1,000 publications for application areas such as oncology, neuroscience, and toxicology. HCA (also referred to as high-content screening or HCS) has become a staple of systems biology research, particularly when multiple cellular events are tracked in response to a stimulus or treatment.

Wanted: An advanced yet accessible HCA instrument

As researchers address a broader range of phenotypes with HCA, they need more capabilities from their hardware and software tools. Sample types were once limited to microscope slides and 96-well plates, but they now include 3D matrices and all SBS (Society of Biomolecular Screening) microwell formats. The biological content of the samples also demands a wider range of techniques, from confocal imaging to brightfield microscopy, each with multiple wavelength options and software tools for analysis.

In this changing application landscape, selecting an instrument platform is challenging for scientists who don't want to limit the scope of their experiments. And because budgets are tight, many laboratories are reluctant to commit the resources necessary for top-of-the-line HCA platforms. The new Thermo Scientific™ Cellinsight™ CX7 High Content Analysis Platform is an integrated benchtop instrument that interrogates multiple sample types with a wide range of techniques, all within a physical and fiscal footprint to meet the needs of most laboratories (Figure 1).

Meet the Cellinsight CX7 HCA Platform

With the Cellinsight CX7 platform, your HCA assays can combine any of the imaging modes—brightfield, widefield, and confocal (Figure 2)—to extract the information you need from your samples. Each modality takes advantage of a proprietary laser-based autofocus technique that enables fast and reproducible read times, even when sample wells are sparsely populated.

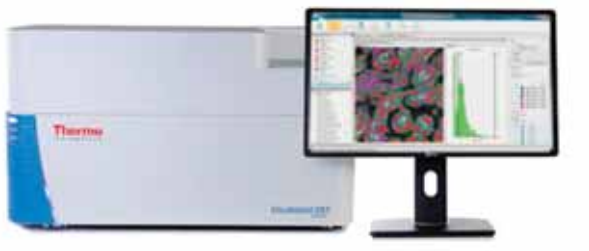

Figure 1. Cellinsight™ CX7 High Content Analysis Platform.

For brightfield imaging, colorimetric absorbance measurements from histology samples are achieved using the LED array for RGB and amber illumination. Moreover, you can multiplex classic stains like hematoxylin and eosin (H&E) with fluorescent probes, offering new possibilities for data correlation in tissue sections. For confocal imaging, high-speed CrEST™ spinning-disk confocal technology with 40 µm or 70 µm pinholes is built into the optical path to provide sharp imaging in thick tissue samples and 3D matrices. The widefield imaging mode occupies the same light path as the confocal mode, sharing a 7-color solid-state LED light engine that provides a wide spectrum of excitation and maximizes your capabilities for performing multiplex assays. You can exploit the range of Molecular Probes™ fluorescent reagents—from HCS CellMask™ Blue stain to Alexa Fluor™ 750 dye—to minimize spectral overlap and improve assay quantitation.

A Photometrics™ X1 CCD camera with a 2,200 x 2,200 pixel array provides high sensitivity and resolution in all imaging modes. Together, the X1 camera and the 7-color light engine provide fast channel-switching times and minimize intensity fluctuations to help reduce scan times and boost quantitative performance. And getting started is easy: the Cellinsight CX7 platform is ready to accept screening plates from a robotic handler, or you can individually load your samples.

Efficiently analyze HCS data

For HCS to be truly accessible, it must meet the needs of novice users without sacrificing the power that experienced users have come to depend on. Thermo Scientific™ HCS Studio™ 3.0 Cell Analysis Software provides icon-driven guidance, starting with plate maps and annotation tools to set up assays and efficiently manage your experimental design. New users have a choice of more than 30 established assays that

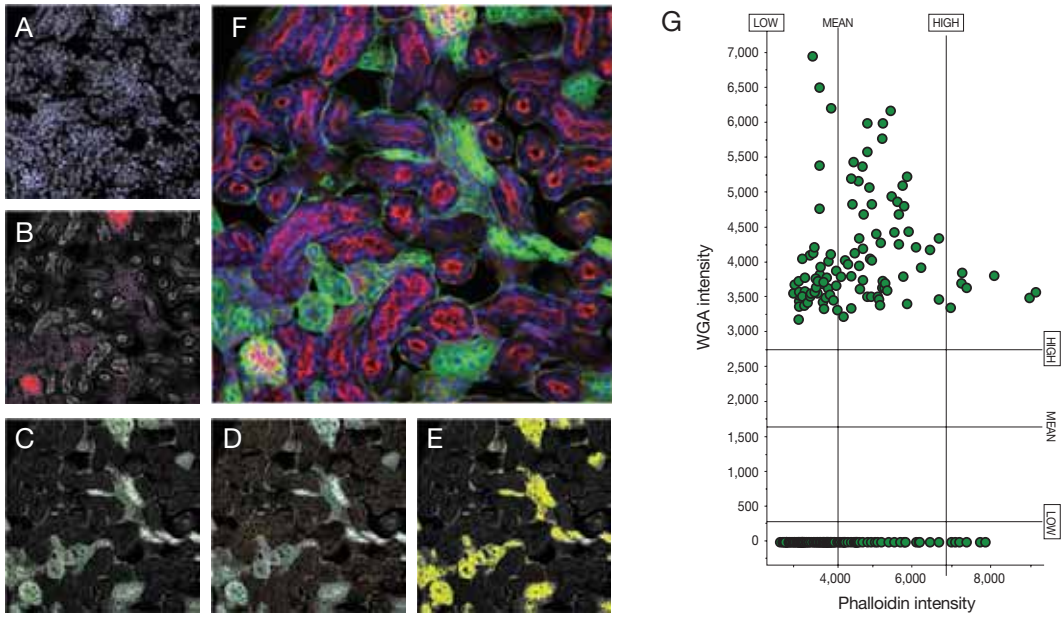

Figure 2. Confocal image analysis of a fluorescently stained mouse kidney. A 16 µm cryostat section of mouse kidney was stained with Alexa Fluor™ 488 Wheat Germ Agglutinin (green, Cat. No. W11261), Alexa Fluor 568 Phalloidin (red, Cat. No. A12380), and DAPI (blue, Cat. No. D1306) and imaged on the Cellinsight CX7 HCA Platform using laser autofocus and confocal acquisition at 20x magnification: **(A)** nuclear identification and segmentation (blue); **(B)** phalloidin detection in WGA+ cells (red); **(C)** WGA+ cell selection (green); **(D)** WGA mask modification (green); **(E)** spot detection for quantifying WGA signal; **(F)** composite confocal image. **(G)** Scatter plot shows relative labeling intensity of the WGA vs. phalloidin conjugates in order to characterize phenotypes using intensity cutoffs.

can be optimized to suit a particular cell line or phenotype. Using the intuitive icons, you can simply choose the assay and magnification of the optimized protocol, confirm settings, and begin to gather data on processes such as:

- Apoptosis
- Autophagy
- Cell cycle
- DNA damage
- Invasion
- Motility
- Myotube formation
- Neurite outgrowth
- Synaptogenesis

Experienced users can build their own assays from scratch using the flexible software tools. With instant feedback they can control hundreds of options, including:

- Background correction
- Object segmentation
- Spot/granule detection
- Regions of interest
- Detection sensitivity
- Projected image format
- Image contrast
- Phenotypic gating

For a researcher running assays on any scale, time-to-results is a critical measure. The sooner you have a result, the sooner you can make a decision, adjust a parameter, qualify an answer, or repeat an experiment. HCS Studio software users quickly recognize the benefits of intelligent software that processes data in real time, limiting acquisition to only the data required to generate a statistically significant result. For an assay like neurite outgrowth measurement in 96 wells, the Cellinsight CX7 platform can read the plate and report results in less than 4 minutes. Furthermore, the Assay Performance Tool of the HCS Studio software allows you to measure assay performance over multiple measurement criteria and select the parameters that give the best Z-prime result. At the end of the assay, you have results you can work with directly, without the delays of data spooling and off-line processing.

Explore your HCA options

The Cellinsight CX7 platform makes HCS accessible to more researchers by providing a range of high-performance tools in a convenient benchtop format. This technology platform builds on a 16-year legacy of HCS instrument and software development with ArrayScan readers and HCS Studio software, and almost 40 years of fluorescence expertise represented by Molecular Probes reagents. Contact us for more information or request an in-lab demonstration at thermofisher.com/cellinsightcx7bp72. ■

Product	Quantity	Cat. No.
Cellinsight™ CX7 High Content Analysis Platform	1 each	CX7A1110

Mimic life in three dimensions

Formation of uniform and reproducible 3D cancer spheroids in high-throughput plates.

The physiological characteristics of a traditional monolayer culture of cells growing on a flat 2D tissue culture substrate can differ considerably from those of cells in a 3D environment. Increasingly, spheroids grown in 3D cell culture are being used in cancer research to more closely mimic the environment associated with tumors. These 3D cancer spheroids have several physiological traits in common with tumors, including overall morphology, formation of cell–cell contacts, decreased proliferation rates, increased survival rates, and a hypoxic core. However, variability in cancer spheroid formation has been a persistent problem for researchers in this field. The reproducibility of spheroid formation appears to be linked to medium composition and volume, cell density, duration in cell culture, and most importantly, the interactions of cells with the culture dish itself. More consistent results can reportedly be achieved using high-quality cultureware with low cell-binding characteristics.

Here we describe the formation and characterization of uniform and reproducible 3D cancer spheroids *in vitro* using Thermo Scientific™ Nunclon™ Sphera™ plates. These 96-well U-bottom plates have a polymer-coated culture surface that inhibits the binding of extracellular matrix (ECM) proteins, which typically mediate cell adhesion. Furthermore, cancer spheroids form quickly in the Nunclon Sphera wells without the formation of "satellite colonies", demonstrating superior quality when compared with spheroids formed in methylcellulose-containing media on nontreated (also called non–tissue culture treated or non–TC treated) polystyrene plates. Cell viability and cell function can be conveniently evaluated *in situ* using fluorescence-based and colorimetric assays; drug treatment can also be administered directly to spheroids growing in Nunclon Sphera plates. The consistent formation of cancer spheroids in the Nunclon Sphera plate makes it an ideal platform for modeling 3D tumor growth for cell-based drug discovery procedures, co-culture studies, and high-throughput screening.

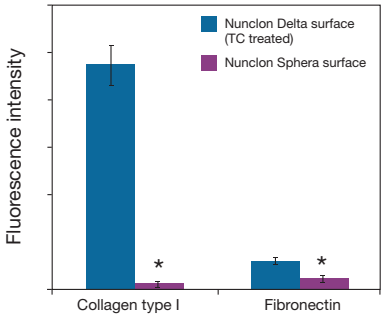

Figure 1. Adsorption of ECM proteins collagen and fibronectin to the Nunclon Sphera surface is extremely low compared with adsorption to the standard cell or tissue culture treated surface. Thermo Scientific™ Nunclon™ Sphera™ 96-well U-bottom plates (Cat. No. 174925) and Thermo Scientific™ Nunclon™ Delta 96-well plates (also called TC treated plates, Cat. No. 143761) were each coated with 100 µL/well of a FITC bovine collagen type I conjugate (24 µg/mL in D-PBS) and incubated for 24 hr at 2–8°C. Another set of plates were coated with a TAMRA fibronectin conjugate (20 µg/mL in D-PBS) and incubated for 16 hr at room temperature. After the plates were washed three times with 200 µL/well PBS-T (PBS with 0.05% Tween™ 20), the fluorescence intensity was detected using excitation/emission of 495/525 nm for FITC collagen or 543/570 nm for TAMRA fibronectin. * $p < 0.01$ for Student's *t*-test.

The Nunclon Sphera plate surface exhibits extremely low adsorption of ECM proteins

For anchorage-dependent or adherent cells to form spheroids in suspension, the culture vessel must promote the aggregation of cells through cell–cell binding while preventing the ECM from binding to the culture vessel surface. Figure 1 demonstrates that both collagen I and fibronectin adsorption are minimal on the Nunclon Sphera plate surface when compared with adsorption on the Thermo Scientific™ Nunclon™ Delta plate surface (the standard cell or tissue culture treated, also called TC treated, polystyrene surface). These ECM protein–binding assays suggest that, unlike the TC treated surface of cell culture vessels, the Nunclon Sphera surface has minimal binding interactions with the ECM, which discourages cells from attaching to the cultureware.

The Nunclon Sphera plate surface is superior for culturing cancer spheroids

To demonstrate the formation of 3D cancer spheroids in Nunclon Sphera plates, HCT 116 (human colon carcinoma) cells were seeded into Nunclon Sphera 96-well U-bottom plates in complete DMEM. Cells were similarly seeded into nontreated 96-well U-bottom plates in complete DMEM containing 3% methylcellulose. Figure 2A →

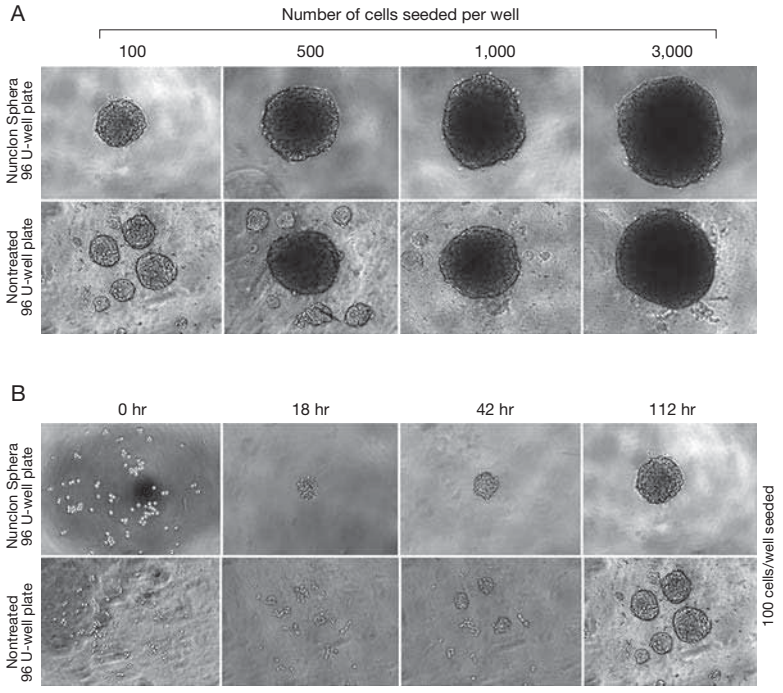

Figure 2. Comparison of cancer spheroid formation using Nunclon Sphera plates vs. nontreated plates containing methylcellulose. HCT 116 cells, maintained in Nunclon™ Delta cell culture flasks (Cat. No. 136196), were seeded in Nunclon™ Sphera™ 96-well U-bottom plates (Cat. No. 174925) at densities of 100–3,000 cells/well in 200 µL/well Gibco™ DMEM (with high glucose, GlutaMAX™ supplement, and pyruvate; Cat. No. 10569-010) containing 10% FBS (Cat. No. 26400-036), 1X MEM Non-Essential Amino Acids (Cat. No. 11140-050), 100 U/mL penicillin–streptomycin, and 25 mM HEPES. Traditional plates not treated for cell or tissue culture (non–TC treated plates) were similarly seeded in complete DMEM medium that also contained 3% methylcellulose. Plates were briefly centrifuged at 250 x *g* for 5 min and then incubated at 37°C and 5% CO_2; cells were re-fed every 72 hr by carefully removing 100 µL of medium from each well and replenishing with 100 µL of fresh growth medium. Formation and growth of spheroids were imaged. **(A)** After 112 hr incubation, cancer spheroids grown in Nunclon Sphera plates show more uniform shape, better-defined edges, and cleaner backgrounds than those grown in nontreated plates at all seeding densities. **(B)** At a seeding density of 100 cells/well, the early time-course of spheroid formation in the Nunclon Sphera plate reveals spheroids after only 18 hr and many fewer satellite colonies than in the nontreated plate. Images used with permission from Helmut Dolznig, Institute of Medical Genetics, Medical University of Vienna.

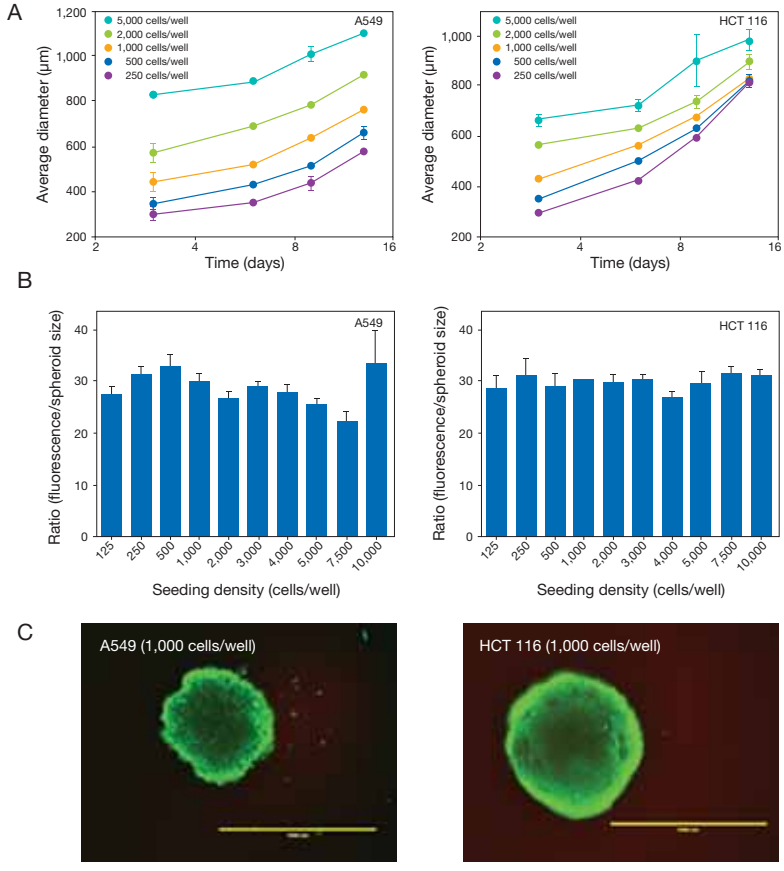

Figure 3. Assessments of spheroid growth, cell health, and cell viability on Nunclon Sphera plates. (A) Growth kinetics of A549 and HCT 116 cancer spheroids grown in Nunclon™ Sphera™ plates (Cat. No. 174925) at increasing seeding densities were evaluated over a period of 13 days. Data represent the mean ± SD of 3 replicates for each cell number. **(B)** Spheroid cell health was assessed *in situ* using PrestoBlue™ Cell Viability Reagent (Cat. No. A13261). After 12–13 days of spheroid culture, 20 µL of 10X PrestoBlue Cell Viability Reagent was added to each well of the Nunclon Sphera plates, which were then incubated at 37°C and 5% CO_2 for an additional 2–5 hr before reading on a fluorescence-based microplate reader (Ex/Em ~560/590 nm). The fluorescence signals were normalized by spheroid size; a higher ratio indicates healthier spheroids. **(C)** Spheroid cell viability was evaluated using the LIVE/DEAD™ Viability/Cytotoxicity Kit (Cat. No. L3224). After 12–13 days of spheroid culture, the LIVE/DEAD kit reagents were added to the Nunclon Sphera plates, which were then incubated for 30–45 min, rinsed at least 3 times with a half-volume change of D-PBS, and imaged using a fluorescence microscope; live cells fluoresce green, dead cells fluoresce orange. Data were analyzed using ImageJ image analysis software; scale bar = 1,000 µm.

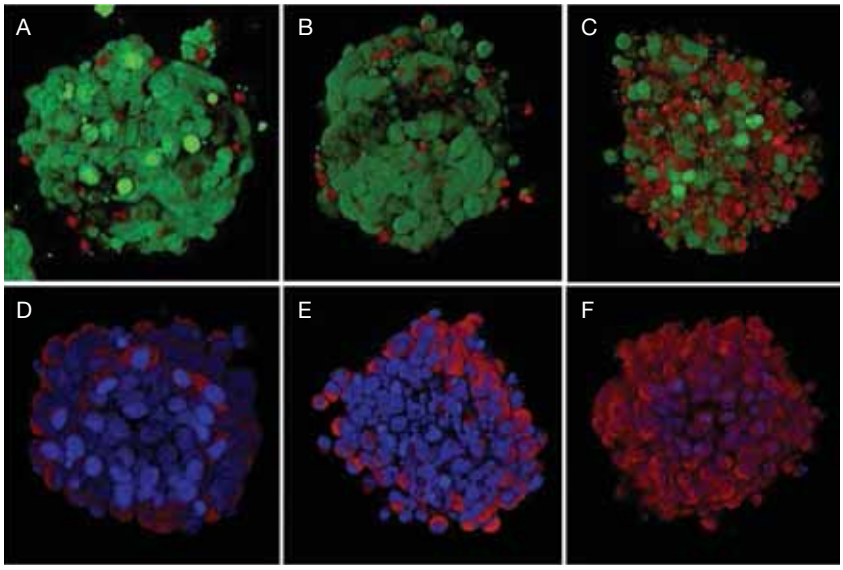

Figure 4. Assaying cell viability and oxidative stress in drug-treated HeLa spheroids. HeLa cells were grown in Gibco™ MEM (Cat. No. 11095-080), seeded at 600 cells/well in a Nunclon™ Sphera™ 96-well U-bottom plate, centrifuged at 200 x *g* for 5 min, and cultured for 3 days to allow spheroid formation. One set of HeLa spheroids was **(A)** left untreated, or treated with **(B)** 100 nM niclosamide or **(C)** 10 µM niclosamide for 24 hr, and then stained using the LIVE/DEAD™ Cell Imaging Kit (Cat. No. R37601). A second set of HeLa spheroids was **(D)** left untreated, or treated with **(E)** 100 nM menadione or **(F)** 10 µM menadione for 1 hr at 37°C to induce oxidative stress, and then stained with NucBlue™ Live ReadyProbes™ Reagent (Cat. No. R37605) and CellROX™ Deep Red Reagent (Cat. No. C10422). After staining, spheroids were transferred to Nunc™ glass-bottom dishes with 200 µL pipette tips (with tip ends cut off) and imaged on a Zeiss™ LSM 710 confocal microscope with EC Plan-Neofluar™ 10x/0.3 objective and 488 nm and 561 nm lasers, in addition to the 488/561 nm main beam splitter; stacks were projected using 3D shadow rendering. **(A–C)** In the spheroids assayed with the LIVE/DEAD Cell Imaging Kit, live cells fluoresce green, and dead cells with permeable membranes fluoresce red. **(D–F)** In the spheroids stained with CellROX Deep Red Reagent and NucBlue Live ReadyProbes Reagent, cells showing oxidative stress fluoresce red, and live-cell nuclei fluoresce blue.

shows that, after 112 hr incubation, HCT 116 cancer spheroid formation in the Nunclon Sphera plates exhibited more uniform shape, better-defined edges, cleaner backgrounds, and fewer "satellite colonies" at all seeding densities when compared with spheroid formation in the nontreated plate, leading to higher-quality spheroids. Moreover, at the lowest seeding density of 100 cells/well, the HCT 116 cancer spheroids formed after only 18 hours of incubation in the Nunclon Sphera plate (Figure 2B). These findings show that uniform and reproducible spheroids are formed after less than a day of incubation when using Nunclon Sphera 96-well U-bottom plates and complete DMEM.

Assaying the cell health of cancer spheroids

To monitor spheroid growth over time, the A549 (human lung carcinoma) and HCT 116 cancer cell lines were seeded at different densities in Nunclon Sphera plates and then cultured for 2 weeks. Both cell types showed adequate spheroid growth, as demonstrated by size measurements (Figure 3A) throughout the incubation period. Additionally, the cell health of A549 and HCT 116 spheroids was assessed using the PrestoBlue™ cell viability assay, which detects the reducing power of live cells (Figure 3B), and the LIVE/DEAD™ viability/cytotoxicity assay, which detects plasma membrane integrity and intracellular esterase activity (Figure 3C). Each of these assays showed that spheroids from both the A549 and HCT 116 cell lines were viable and healthy, indicating that Nunclon Sphera 96-well U-bottom plates are reliable and convenient tools for both routine and high-throughput cancer spheroid applications.

To further demonstrate the health and vitality of the cancer spheroids grown in the Nunclon Sphera plates, we used a third fluorescence-based viability assay, as well as a probe for oxidative stress. First we subjected the spheroids to treatment either with niclosamide, a drug that inhibits oxidative phosphorylation, or with menadione, which induces oxidative stress. The niclosamide-treated spheroids were assayed using the LIVE/DEAD Cell Imaging Kit, which provides a sensitive two-color fluorescence cell viability assay that is optimized for FITC (green) and Texas Red™ (red) optical filters. Increasing the niclosamide concentration resulted in a higher proportion of dead cells in the spheroids, as expected (Figure 4A–C).

Next, the menadione-treated spheroids were assayed with CellROX™ Deep Red Reagent, a cell-permeant dye that exhibits bright red fluorescence upon oxidation by reactive oxygen species. With increasing menadione concentration, we observed an increasing number of cells in the spheroid undergoing oxidative stress (Figure 4D–F). These results indicate that both the LIVE/DEAD Cell Imaging Kit and CellROX Deep Red Reagent provide effective and convenient fluorescence assays of cell function in multicellular spheroid structures.

Detecting hypoxic cores in cancer spheroids

One feature of 3D cancer spheroids that distinguishes them from cells grown in monolayer culture is a low-oxygen (hypoxic) core. Importantly, this hypoxic core is also present in solid tumors *in vivo*, where cells rapidly outgrow the blood supply, leaving cells at the center of the tumor in an environment with an extremely low oxygen concentration.

To detect low-oxygen conditions in the cancer spheroids, we used Image-iT™ Hypoxia Reagent, a cell-permeant compound that is nonfluorescent in an environment with normal oxygen concentrations and becomes increasingly fluorescent as oxygen levels are decreased. Because it responds quickly to a changing environment, Image-iT Hypoxia Reagent can serve as a real-time oxygen detector, with a fluorescent signal that increases as atmospheric oxygen levels drop below 5% and decreases if oxygen concentrations increase up to 5%. Figure 5 shows HeLa cells cultured in a Nunclon Sphera 96-well U-bottom plate for 2 days in complete medium, stained *in situ* with Image-iT Hypoxia Reagent, and counterstained with NucBlue™ Live ReadyProbes™ Reagent. The red-fluorescent staining beneath the spheroid surface provides evidence of a hypoxic core, mimicking the physiological conditions inside a tumor.

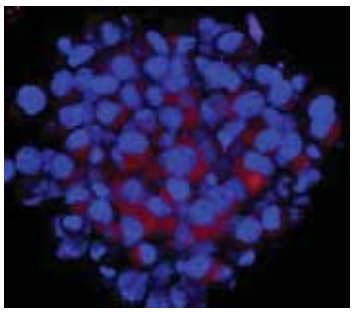

Figure 5. Assessment of the hypoxic core in a single HeLa spheroid. HeLa cells (250 cells/well) were cultured on Nunclon™ Sphera™ 96-well U-bottom plates (Cat. No. 174925) for 2 days in complete medium. The spheroids were then incubated *in situ* with 5 µM Image-iT™ Hypoxia Reagent (red, Cat. No. H10498) for 3 hr, and nuclei were counterstained with NucBlue™ Live ReadyProbes™ Reagent (blue, Cat. No. R37605). The stained spheroids were transferred by pipetting using wide-bore pipette tips to a Nunc™ Glass Bottom Dish (12 mm, Cat. No. 150680), and images were taken on a confocal microscope.

Bring 3D spheroid culture to your lab

We have demonstrated that the surface of the Nunclon Sphera 96-well U-bottom plates exhibits extremely low ECM-binding properties, effectively discouraging cell attachment and promoting spheroid formation. These plates have been shown to support uniform and reproducible formation and growth of cancer spheroids across commonly used cancer cell lines, and are compatible with *in situ* fluorescence assays of cell health. The presence of hypoxic cores in cancer spheroids indicates that 3D cancer spheroid culture in Nunclon Sphera plates presents an effective *in vitro* system for modeling tumor growth. For more information on Nunclon Sphera flasks, dishes, and microplates, visit **thermofisher.com/nunclonspherabp72**. ■

Product	Quantity	Cat. No.
CellROX™ Deep Red Reagent, for oxidative stress detection	5 x 50 µL	C10422
DMEM, high glucose, GlutaMAX™ Supplement, pyruvate	500 mL	10569-010
Fetal Bovine Serum (FBS), dialyzed, US origin	100 mL	26400-036
Image-iT™ Hypoxia Reagent	1 mg	H10498
LIVE/DEAD™ Viability/Cytotoxicity Kit	1 kit	L3224
LIVE/DEAD™ Cell Imaging Kit	1 kit	R37601
MEM	500 mL	11095-080
MEM Non-Essential Amino Acids Solution (100X)	100 mL	11140-050
NucBlue™ Live ReadyProbes™ Reagent	1 kit	R37605
Nunc™ Cell Culture Treated Flasks, with filter caps (25 cm^2 with angled neck)	20 per pack, 160 per case	136196 *
Nunc™ Glass Bottom Dish (12 mm inside bottom diameter)	1 per pack, 20 per case	150680 *
Nunclon™ Sphera™ 96 U-Well Plates	1 per pack, 8 per case 5 per pack, 50 per case	174925 * 174929 *
Nunc™ MicroWell™ Plates with Nunclon™ Delta Surface, without closure	1 per pack, 50 per case	143761 *
Nunc™ MicroWell™ Plates with Nunclon™ Delta Surface, with closure	1 per pack, 50 per case	163320 *
PrestoBlue™ Cell Viability Reagent	25 mL	A13261

* These Thermo Scientific™ cell culture products are available at **thermoscientific.com**.

Tools for studying antibody internalization and trafficking

Applications for therapeutic monoclonal antibodies.

Therapeutic monoclonal antibodies (mAbs) and their derivatives represent an exciting and rapidly developing class of medicines that are used to treat serious diseases such as cancer, autoimmunity, and metabolic disorders [1]. These agents include antibody–drug conjugates (ADCs) and bi-specific variants, which are engineered to recognize two different antigens. Produced using recombinant DNA technology, therapeutic mAbs bind specific soluble or membrane-bound target molecules to alter cell signaling events. Because they closely resemble the human body's own immunoglobulin (Ig) molecules, therapeutic mAbs are generally well tolerated and have favorable safety profiles. Several different mechanisms of action for mAb-based molecules are shown in Figure 1.

With the intensifying interest in mAb technologies from the biopharmaceutical sector as well as academic investigators, there is a growing need for improving and expanding the range of tools for studying therapeutic mAbs as they engage with their cellular and subcellular targets. In the case of an ADC, for example, the internalization and trafficking of the conjugate to specific cellular compartments are fundamental to the drug's mechanism of action [2]. Investigators may need to assess the propensity of a therapeutic mAb candidate for degradation versus recycling pathways as a component of pharmacokinetic analysis [3]. In addition, preclinical safety and efficacy studies may require whole-animal noninvasive imaging studies to track the biodistribution of a drug or surrogate in tissues [4]. Scientists at Thermo Fisher Scientific have developed a wide range of solutions to address these and other important questions for investigators engaged in the discovery and advancement of therapeutic mAbs.

Selected technologies for fluorescent antibody labeling

During target validation and selection of lead candidates, it is often advantageous to rapidly and reliably label mAbs with fluorescent dyes to facilitate their detection in imaging or flow cytometry studies. We offer a variety of conjugation technologies designed to help researchers generate fluorescent antibodies custom-labeled with Molecular Probes™ dyes (Table 1).

APEX™ Antibody Labeling Kits are the preferred method when the goal is to quickly label a small quantity (10–20 µg) of antibody that is suspended in a buffer containing other proteins, such as albumin or gelatin, which cannot easily be dialyzed away. These kits utilize a solid-phase labeling technique that captures the IgG antibody on a resin inside an APEX antibody labeling tip. By immobilizing the IgG within a micro-column, stabilizing proteins are easily eluted prior to the solid-phase conjugation step, which uses an amine-reactive fluorophore that reacts with the antibody's lysines. The fluorescent IgG conjugate is ready to use in an imaging or flow cytometry assay in as little as 2.5 hours, with minimal hands-on time.

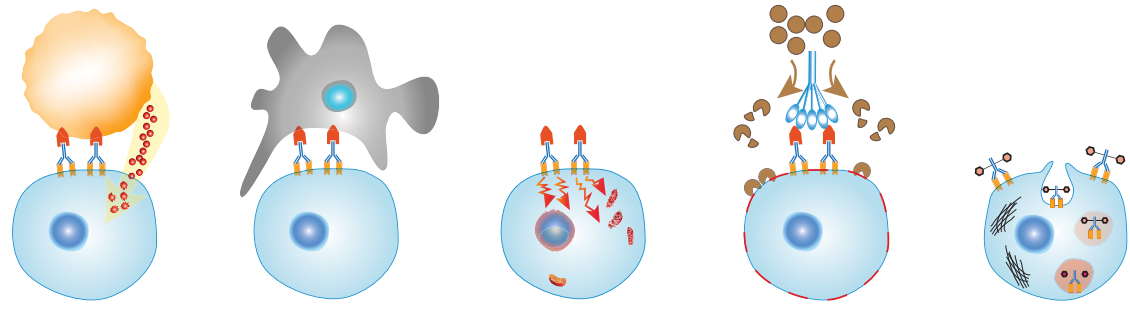

Figure 1. Therapeutic mAbs and their derivatives can affect target cell function and viability via several different mechanisms. For oncology indications where directed tumor cell lysis is the desired endpoint, mAbs can be used to engage cytotoxic or phagocytic effector cells (ADCC or ADCP, respectively), to drive pro-apoptotic signaling via ligation of death receptors (PCD), or to activate complement cascades (CDC). An antibody–drug conjugate (ADC) consists of a mAb directed against a tumor cell antigen coupled to a small cytotoxic molecule, resulting in a highly specific and targeted chemotherapeutic agent.

Table 1. Molecular Probes™ antibody labeling kits.

Amount	Antibody labeling kit*	Notes
10–20 µg	APEX™ Alexa Fluor™ Antibody Labeling Kits (also available with Pacific Blue™ and Oregon Green™ 488 dyes)	• Labeled IgG antibodies ready to use in 2.5 hr (~15 min hands-on time) • Solid-phase labeling using a pipette tip preloaded with resin • Efficient labeling in serum, ascites fluid, or hybridoma supernatants
1–20 µg	Zenon™ Antibody Labeling Kits	• Labeled IgG antibodies ready to use in 10 min • Isotype-specific, noncovalent labeling with dyes, haptens, or enzymes • Efficient labeling in serum, ascites fluid, or hybridoma supernatants
20–100 µg	Alexa Fluor™ Microscale Protein Labeling Kits (also available for biotin labeling)	• Labeled proteins ready to use in 2 hr (~30 min hands-on time) • Optimized for proteins between 10 and 150 kDa • Stabilizing proteins must be removed from sample before labeling
100 µg	Alexa Fluor™ Monoclonal Antibody Labeling Kits (also available with Pacific Blue™ and Pacific Orange™ dyes)	• Labeled proteins ready to use in 90 min (~15 min hands-on time) • Optimized for small-scale labeling of any protein >40 kDa • Stabilizing proteins must be removed from sample before labeling
100–125 µg	SiteClick™ Antibody Labeling Kits (available with R-PE and Qdot™ labels)	• Labeling occurs over 3 days, with ~3 hr hands-on time • Modular, click chemistry–mediated method for enzymatically labeling an antibody on its heavy chain *N*-linked glycans, far from the antigen-binding site
1 mg	Alexa Fluor™ Protein Labeling Kits (also available with Pacific Blue™, Pacific Orange™, fluorescein, Oregon Green™ 488, and Texas Red™ dyes and the biotin hapten)	• Labeled proteins ready to use in 2 hr (~30 min hands-on time) • Optimized for large-scale labeling of IgG antibodies (~150 kDa) • Stabilizing proteins must be removed from sample before labeling
0.5–3.0 mg	SAIVI™ Alexa Fluor™ Antibody Labeling Kits	• Labeled proteins ready to use in 90 min (~10 min hands-on time) • Control for optimal degree of labeling for Alexa Fluor near-IR dyes • Produces azide-free conjugates for *in vivo* imaging applications

* In addition to these antibody labeling kits, we offer custom antibody conjugation. Please email us at custom.services@thermofisher.com about your project requirements.

Labeling antibodies for use as imaging reagents in whole-animal biodistribution studies presents unique challenges. Importantly, the degree of labeling (DOL), which refers to the number of dye molecules per antibody, must be tightly controlled to maximally preserve the antibody's binding characteristics. Therefore, the dyes themselves must have high quantum yield for accurate and sensitive detection in the near-infrared spectrum. Also, these experiments typically require larger amounts of antibody than many labeling kits can accommodate. The SAIVI Rapid Antibody Labeling Kits were specifically designed to address these needs, allowing researchers to conjugate their own primary antibodies of choice at milligram scale to the extremely bright and photostable near-infrared–fluorescent Alexa Fluor™ 680 and Alexa Fluor 750 dyes with a high degree of precision and confidence. Fluorescent antibody conjugates are eluted in sterile azide-free buffer and do not require additional dialysis steps prior to *in vivo* administration.

A different approach to fluorescently labeling therapeutic mAb candidates during screening and lead selection phases utilizes Zenon™ antibody labeling technology. The Zenon labeling reagent is a fluorophore-, biotin-, or enzyme-labeled Fab fragment directed against the Fc portion of a primary IgG antibody (Figure 2). As such, their binding does not interfere with the complementarity-determining region (CDR) of the antibody, and therefore affinity and specificity are unaffected. Zenon technology can be used to rapidly generate Alexa Fluor, allophycocyanin (APC), or R-phycoerythrin (R-PE) antibody conjugates, which can then serve as flow →

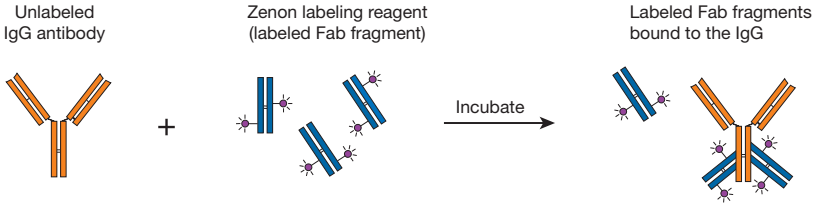

Figure 2. Humanized or fully human IgG antibodies can be noncovalently coupled with fluorescent dyes using Zenon antibody labeling technology. Unconjugated antibodies are incubated with fluorescently labeled Fab fragments directed against the Fc portion of a human IgG antibody. This labeling reaction leaves the complementarity-determining region (CDR) of the target antibody intact and free from obstruction, while providing a consistent degree of labeling (DOL) of 3 to 5 Fab molecules per primary IgG antibody. Zenon™ antibody labeling technology is also available for mouse and rabbit IgG antibodies.

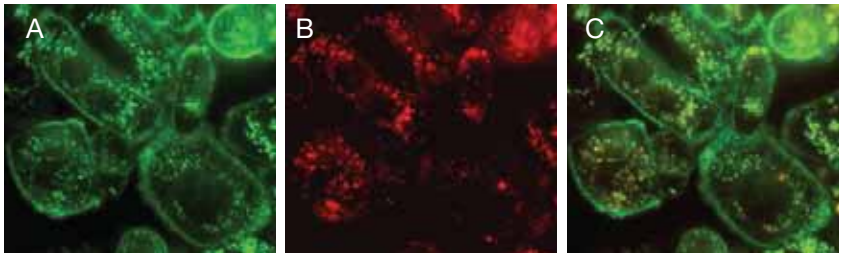

Figure 3. Immunodetection using Herceptin labeled with Zenon Alexa Fluor 594 Human IgG Labeling Reagent. Herceptin™ was labeled with Zenon™ Alexa Fluor™ 594 Human IgG Labeling Reagent, producing a DOL of approximately 3 Fab fragments per mAb. SK-BR-3 breast carcinoma cells, which highly express HER2/ErbB2, were treated with 1 µg/mL Herceptin/Zenon Alexa Fluor 594 complex and 50 nM LysoTracker™ Deep Red Reagent in complete medium for 30 min at 37°C. Live cells were imaged to detect **(A)** surface-bound as well as internalized Herceptin/Zenon Alexa Fluor 594 complex (pseudocolored green) and **(B)** Lysotracker Deep Red labeling (pseudocolored red). **(C)** Within the 30 min incubation period, it is apparent that some of the Herceptin/Zenon complex has reached lysosomes, shown by colocalization with the LysoTracker Deep Red fluorescence.

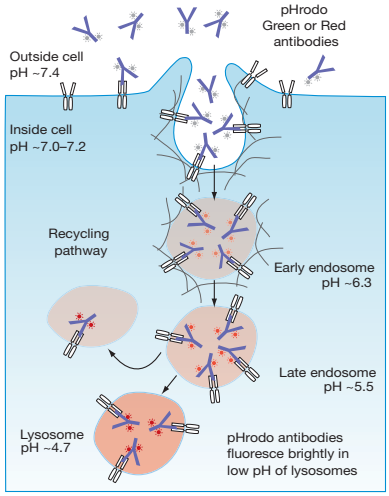

Figure 4. Gradient of acidification in vesicles of the endosome–lysosome pathway. As antibodies are taken up by cells from the extracellular space (pH 7.4), they are sequestered within early endosomes (pH ~6.3), then trafficked to late endosomes (pH ~5.5) and finally to lysosomes (pH ~4.7), where they are degraded. In some cases, antibodies may escape lysosomal degradation by being targeted to recycling endosomes, which direct them back to the cell surface for exocytosis.

cytometry detection reagents for target validation across cell line panels or for characterization of transient and stable cell lines. Although the interaction between the Zenon Fab fragment and the primary antibody is noncovalent, the binding is sufficient to allow detection of internalization and trafficking of the conjugates within cells. Figure 3 shows an example of SK-BR-3 breast cancer cells treated for 30 minutes with Herceptin™ (trastuzumab, Roche) labeled using the Zenon Alexa Fluor 594 Human IgG Labeling Reagent. Both cell surface–bound and internalized pools of labeled Herceptin bound to its target HER2/ErbB2 are clearly visualized. Trafficking of the Herceptin/Zenon Alexa Fluor 594 complex to lysosomes is confirmed by colocalization with LysoTracker™ Deep Red Reagent.

The SiteClick™ Antibody Labeling Kits provide a modular, click chemistry–mediated method for enzymatically labeling essentially any antibody on its heavy chain *N*-linked glycans—far from the CDR—providing excellent reproducibility from labeling to labeling and from antibody to antibody. This site-selective strategy is especially important when labeling monoclonal antibodies that contain lysine residues in or around the CDR, as labeling of these sites with amine-reactive dyes can disrupt antigen binding. While SiteClick Antibody Labeling Kits are currently available

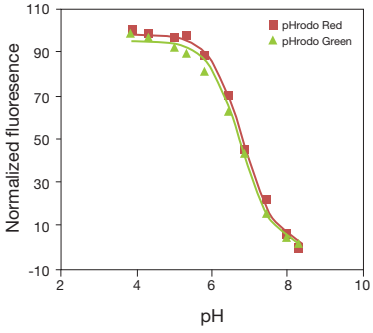

Figure 5. pH-dependent fluorescence emission profiles of pHrodo™ Red and pHrodo™ Green dextran conjugates.

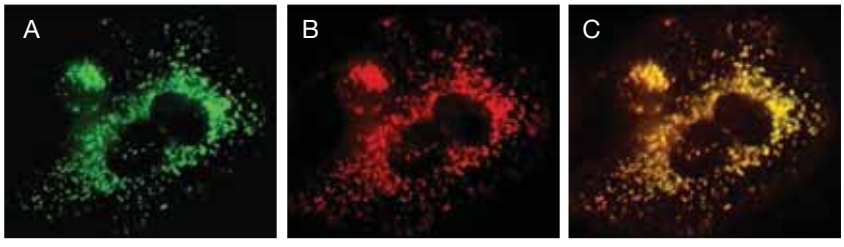

Figure 6. Immundetection using Herceptin labeled with a custom Zenon pHrodo Red Human IgG Labeling Reagent. pHrodo™ Red was conjugated to Fab fragments directed against the Fc portion of a human IgG antibody in order to generate a custom Zenon™ reagent for directly detecting Herceptin™ trafficking to lysosomes. SK-BR-3 cells were incubated with 1 µg/mL Herceptin/Zenon pHrodo Red complex and 50 nM LysoTracker™ Deep Red Reagent in complete medium for 30 min at 37°C. **(A)** Herceptin/Zenon pHrodo Red complex labeling (pseudocolored green). **(B)** LysoTracker Deep Red labeling (pseudocolored red). **(C)** Merged image. Images acquired from live cells clearly demonstrate the localization of internalized Herceptin/Zenon pHrodo Red complex within lysosomes. Because pHrodo Red is essentially nonfluorescent at neutral pH values, the Herceptin/Zenon pHrodo Red complex bound to the cell surface is not detected in this experiment.

for creating your own fluorescent R-PE and Qdot™ conjugates, the SiteClick technology can be applied to your monoclonal antibody using a variety of Molecular Probes dyes through our Custom Services. For more information, please send your inquiry to custom.services@thermofisher.com.

Tools for following antibody internalization and trafficking

As therapeutic mAbs interact with membrane-bound targets at the cell surface, they are often internalized via clathrin-mediated endocytosis and trafficked to lysosomes, where they are catabolized by proteolytic enzymes. This internalization and lysosomal degradation can have a profound influence on the pharmacokinetic properties of therapeutic mAbs, including accelerated clearance from the circulation that could significantly affect the dosing regimen. In other cases, notably for ADCs, lysosomal degradation of the peptide linker between the antibody and the cytotoxic payload is required for efficacy. Thus, there is an emerging appreciation for understanding the disposition of therapeutic mAbs after they interact with their cellular targets.

A powerful method to visualize trafficking of therapeutic mAbs in live cells takes advantage of the pH gradient that exists between the extracellular space and vesicular compartments of the endolysosomal pathway. Upon internalization, mAbs are exposed to an increasingly acidic environment as they are trafficked from early endosomes to late endosomes and eventually to lysosomes (Figure 4). pHrodo™ Green and pHrodo™ Red dyes are minimally fluorescent at neutral and basic pH values; these dyes, however, show increasing fluorescence emission with decreasing pH conditions (Figure 5). Thus, conjugates made using pHrodo dyes can be used to directly determine when mAbs are trafficked to endosomes and lysosomes in live cells using microscopy or flow cytometry [5]. Alternately, pHrodo and Zenon technologies can be combined to produce a reagent that enables rapid labeling of mAbs with a pH-sensitive dye for internalization and trafficking studies. In Figure 6, SK-BR-3 cells were treated for 30 minutes at 37°C with Herceptin labeled using a custom Zenon pHrodo Red Human IgG Labeling Reagent. The internalized Herceptin/Zenon pHrodo complex is readily observed in live cells, and trafficking to lysosomes is confirmed by colocalization with LysoTracker Deep Red Reagent. Note that there is no fluorescence emitted by membrane-bound Herceptin/Zenon complex, consistent with the pH-dependent emission properties of pHrodo dyes and their conjugates. For more information on the Zenon pHrodo Labeling Reagents, please send your inquiry to **custom.services@thermofisher.com**.

An alternate method for discriminating internalized versus cell surface–bound pools of therapeutic mAbs is to utilize the fluorescence-quenching capabilities of rabbit anti–Alexa Fluor 488 dye antibody. When primary antibodies conjugated to Alexa Fluor 488 dye are added to live cells under conditions that promote surface binding and internalization, the cells can then be incubated with saturating concentrations of anti–Alexa Fluor 488 dye antibody. This treatment effectively quenches the fluorescence from the antibody bound to the surface without attenuating the signal from the internalized fluorescent label. Herter and colleagues combined this approach with flow cytometric analysis to demonstrate that different anti-CD20 variants display different internalization dynamics that correlate with drug efficacy [6].

Find the best solution for your antibody labeling experiments

We offer a number of ready-to-use antibody labeling kits for the covalent or noncovalent attachment of a broad range of intensely fluorescent dyes to your antibody, at scales from as little as 1 µg up to 3 mg IgG. To learn more, visit **thermofisher.com/antibodylabelingbp72**. For this emerging area, however, we also have scientists available to help determine the best solution for your research; contact our Technical Support staff at **techsupport@thermofisher.com** with questions about our protein labeling options or **custom.services@thermofisher.com** to inquire about custom reagents. ■

References

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6. Herter S, Herting F, Mundigl O et al. (2013) *Mol Cancer Ther* 12:2031–2042.

Product	Quantity	Cat. No.
Anti–Alexa Fluor™ 488 dye, rabbit IgG fraction	500 µL	A11094
APEX™ Alexa Fluor™ 488 Antibody Labeling Kit	1 kit	A10468
LysoTracker™ Deep Red Reagent	5 x 50 µL	L12492
pHrodo™ Green 10,000 MW Dextran, for endocytosis	0.5 mg	P35368
pHrodo™ Red 10,000 MW Dextran, for endocytosis	0.5 mg	P10361
pHrodo™ Green STP Ester	500 µg	P35369
pHrodo™ Red, succinimidyl ester (pHrodo Red SE)	1 mg	P36600
SAIM™ Rapid Antibody Labeling Kit, Alexa Fluor™ 680	1 kit	S30045
SAIM™ Rapid Antibody Labeling Kit, Alexa Fluor™ 750	1 kit	S30046
Zenon™ Alexa Fluor™ 594 Human IgG Labeling Kit	1 kit	Z25407

Visualize apoptosis in context

Multiplexable Click-iT Plus TUNEL Assays for *in situ* apoptosis detection.

The late stages of apoptosis are characterized by changes in nuclear morphology, chromatin condensation, nuclear envelope degradation, and ultimately fragmentation of cellular DNA. While DNA-binding dyes (e.g., Hoechst™ 33342 and DAPI) are typically used to monitor nuclear morphology and chromatin condensation, DNA fragmentation is routinely detected *in situ* with the terminal deoxynucleotidyl transferase–dUTP nick end labeling (TUNEL) assay. Here we describe the limitations of conventional TUNEL assays and introduce the Click-iT™ Plus TUNEL Assays for *In Situ* Apoptosis Detection. Click-iT Plus technology enables you to perform specific and sensitive TUNEL assays that can be multiplexed with other fluorescence-based cell function assays, including those that incorporate fluorescent proteins and a variety of dyes.

The TUNEL assay…then

Since its introduction in 1992, the TUNEL assay has been widely used for the *in situ* detection of apoptosis. The TUNEL assay is based on the incorporation of modified thymidine analogs by the enzyme terminal deoxynucleotidyl transferase (TdT) at the 3´-OH ends of fragmented DNA. The modification on the nucleotide can be as simple as a bromine atom (5-bromo-2´-deoxyuridine triphosphate or BrdUTP) or a more complex molecule such as a fluorophore or hapten (e.g., biotin-dUTP). Incorporated BrdU is typically detected with an anti-BrdU antibody, followed by a secondary detection reagent. A fluorescently modified nucleotide (e.g., fluorescein-dUTP) can be detected directly, whereas biotin is detected indirectly by the addition of a fluorescent streptavidin conjugate.

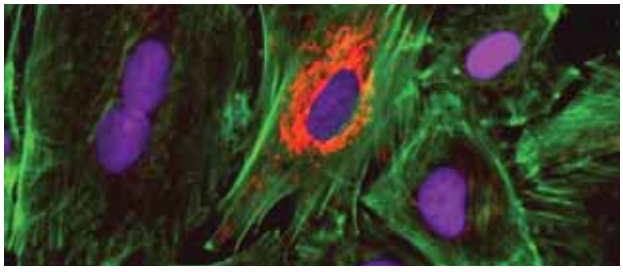

Figure 1. The Click-iT Plus TUNEL Assay detects DNA strand breaks. HeLa cells transduced with CellLight™ Mitochondria-RFP, BacMam 2.0 (Cat. No. C10505) were treated with DNase I to induce TUNEL-positive DNA strand breaks. After performing the Click-iT™ Plus TUNEL Assay with Alexa Fluor™ 647 dye (Cat. No. C10619), filamentous actin was stained with ActinGreen™ 488 ReadyProbes™ Reagent (Cat. No. R37110). Clearly identified are the mitochondria (red), the filamentous actin (green), and the TUNEL signal (purple), which is localized to the nuclei.

The extensive fixation and permeabilization required to give antibodies access to the incorporated BrdU can erase important physical and antigenic characteristics of the tissue being examined. Nonspecific background issues associated with the use of biotin–streptavidin detection systems make fluorescence-based TUNEL assays preferable. However, the size of the fluorophores used to modify the nucleotides can cause a reduction in incorporation rates, decreasing the sensitivity of the TUNEL assay. Additionally, the fluorophores used in most currently available TUNEL assays suffer from high rates of photobleaching, further reducing the assay sensitivity, and they often exhibit significant spectral overlap with other fluorescent dyes and proteins, limiting the ability to multiplex with other fluorescence-based probes.

And now…Click-iT Plus TUNEL Assays

The Click-iT Plus TUNEL Assays for *In Situ* Apoptosis Detection were developed to address the issues affecting sensitivity, photobleaching, and multiplexability. These assays use an EdUTP (a dUTP nucleotide modified with a small alkyne moiety), which is incorporated at the 3´-OH ends of fragmented DNA by the TdT enzyme. Detection is based on a highly specific click reaction, a copper-catalyzed covalent reaction between a fluorescent Alexa Fluor™ picolyl azide dye and the alkyne moiety on the EdUTP. Because of the small size of the alkyne moiety, the EdUTP nucleotide is more readily incorporated by TdT than other modified nucleotides. In addition, the small size of the Alexa Fluor picolyl azide allows easier access to the incorporated nucleotides, negating the need for harsh DNA denaturing techniques required for antibody-based detection of the nucleotide. Moreover, the Alexa Fluor picolyl azide dye serves as a brightly fluorescent and photostable detection reagent and is available in three emission colors for flexibility in multiplex assays.

The Click-iT Plus technology improves upon the first-generation Click-iT TUNEL Assays by the use of the picolyl azide combined with a copper protectant to reduce the exposure of fluorescent proteins, such as Green Fluorescent Protein (GFP) or Red Fluorescent Protein (RFP), to the damaging effects of free copper. In addition, standard aldehyde-based fixation and detergent permeabilization are sufficient for the Click-iT Plus EdU detection reagent to gain access to the DNA; no harsh denaturants are required. The gentle reaction and detection conditions of the Click-iT Plus TUNEL Assay enable you to multiplex this assay

with fluorescent proteins, labeled phalloidins, and other copper-sensitive fluorophores (Figure 1).

Specific, multiplexable TUNEL assays

To demonstrate the specificity of the Click-iT Plus TUNEL Assay in a variety of cell types, four formalin-fixed, paraffin-embedded (FFPE) tissue sections were obtained. The mouse colon, heart, liver, and intestine tissue sections were deparaffinized, fixed, and permeabilized. As a substitute for the DNA nicking that occurs in late-stage apoptosis, the tissue sections were treated with DNase I. Tissue sections without DNase I treatment were used as controls. After treatment, the nicked DNA representing the apoptotic signal was detected using the Click-iT Plus TUNEL Assay with Alexa Fluor 594 dye; no significant fluorescence was detected in the control samples (Figure 2).

To demonstrate the ability to multiplex with the Click-iT Plus TUNEL Assays, we used FFPE intestine tissue sections from a transgenic mouse in which GFP expression was localized to the muscularis externa (muscular layer surrounding the intestine). The tissue sections were subjected to deparaffinization, permeabilization, and DNase I treatment. After treatment with the Click-iT Plus TUNEL Assay (Alexa Fluor 594 dye), the tissue sections were stained with Alexa Fluor 647 dye–conjugated phalloidin and Hoechst 33342 dye. Figure 3 clearly shows that all four fluorescent signals—fragmented DNA labeled with Alexa Fluor 594 dye, muscularis externa expressing GFP, nuclei labeled with Hoechst 33342 dye, and actin staining with Alexa Fluor 647 phalloidin—can be visualized in a single tissue section.

Choose from three fluorescent colors

The Click-iT Plus TUNEL Assays for *In Situ* Apoptosis Detection are available with detection reagents that fluoresce green (Alexa Fluor 488 picolyl azide), red (Alexa Fluor 594 picolyl azide), or deep red (Alexa Fluor 647 picolyl azide). These assays have been optimized and contain all the components necessary to label and detect apoptotic cells from FFPE tissue sections or adherent cells grown on coverslips. The kits include sufficient reagents for labeling 50 coverslips (18 x 18 mm) and can be configured for 50 independent TUNEL apoptosis tests. To learn more, go to **thermofisher.com/apoptosisforimagingbp72.** ■

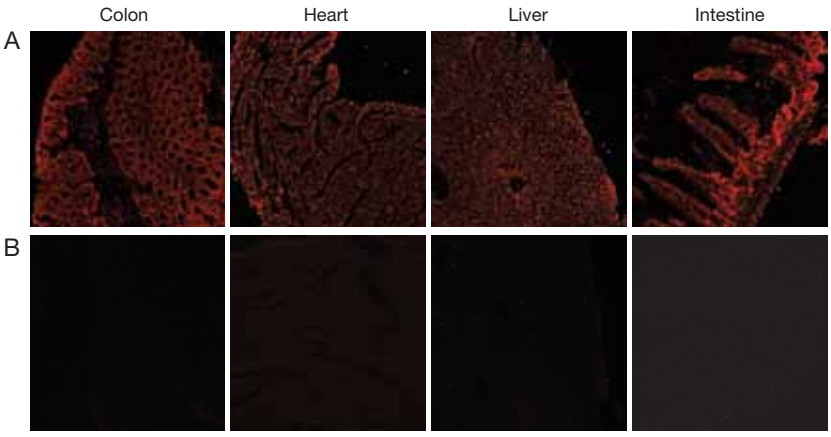

Figure 2. The Click-iT Plus TUNEL Assay detects nicked DNA in mouse tissue sections. Formalin-fixed, paraffin-embedded (FFPE) mouse tissue from colon, heart, liver, and intestine was assayed for nicked DNA, **(A)** with or **(B)** without DNase I treatment, using the Click-iT™ Plus TUNEL Assay with Alexa Fluor™ 594 dye (Cat. No. C10618). Very few positive signals were detected in the sections that were not treated with DNase I.

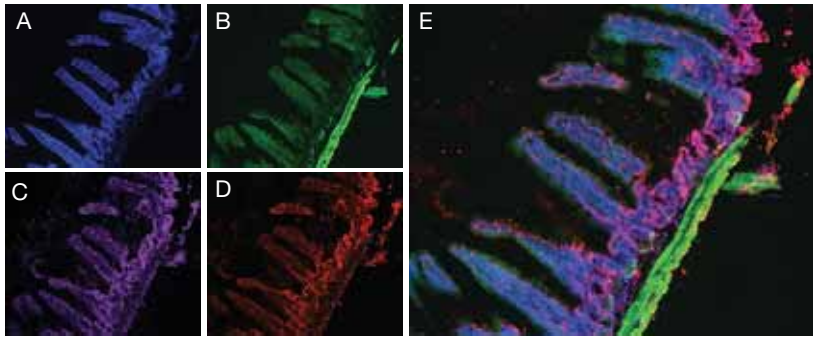

Figure 3. The Click-iT Plus TUNEL Assay can be multiplexed with a variety of other fluorescent probes. Formalin-fixed, paraffin-embedded (FFPE) tissue from a transgenic mouse expressing GFP in intestinal muscle was treated with DNase I, followed by the Click-iT™ Plus TUNEL Assay with Alexa Fluor™ 594 dye (Cat. No. C10618), and then stained with Hoechst™ 33342 dye (Cat. No. H3570) and Alexa Fluor 647 Phalloidin (Cat. No. A22287). **(A)** Cell nuclei are stained by Hoechst 33342 dye (blue), **(B)** the GFP signal (green) is detected in the surrounding muscular layer, **(C)** filamentous actin is stained by Alexa Fluor 647 Phalloidin (purple), and **(D)** the TUNEL-positive signal resulting from DNase I treatment is clearly defined by the Click-iT Plus TUNEL Assay with Alexa Fluor 594 dye (red). The last panel **(E)** is the multiplexed image resulting from an overlay of the four fluorescent signals.

Product	Quantity	Cat. No.
Click-iT™ Plus TUNEL Assay for *In Situ* Apoptosis Detection, Alexa Fluor™ 488 dye	1 kit	C10617
Click-iT™ Plus TUNEL Assay for *In Situ* Apoptosis Detection, Alexa Fluor™ 594 dye	1 kit	C10618
Click-iT™ Plus TUNEL Assay for *In Situ* Apoptosis Detection, Alexa Fluor™ 647 dye	1 kit	C10619

Incorporate automation into your cell analysis workflows

Fluorescence-based viability assays for the Countess II FL Automated Cell Counter.

Many biological research applications require accurate and precise measurement of cell number and viability prior to downstream analysis. The Countess™ II FL Automated Cell Counter is a three-channel (brightfield and two optional fluorescence channels) benchtop assay platform with state-of-the-art optics and image analysis software for automated cell counting. With autofocusing and auto–brightfield lighting controls, as well as multiple gating settings (size, brightness, circularity, fluorescence intensity), this automated counter allows researchers to analyze cells in suspension for a range of characteristics, including cell viability, cell cycle phase, apoptotic stage, and fluorescent protein expression. A number of Molecular Probes™ fluorescence-based assays are compatible with the Countess II FL instrument, which enables quick and easy checks of the health of your cells before committing them to existing workflows for imaging, flow cytometry, and high-content analysis (HCA).

Minimize counting variability

When manually counting cells using a hemocytometer and a microscope, count-to-count variability of a single sample by an experienced cell biologist is typically 10% or more. Counting variability between multiple users commonly exceeds 20%. In addition to helping save time, automated cell counters minimize the subjective nature of manual counting as well as user-to-user differences in total cell count assays. Users of the Countess II FL Automated Cell Counter typically observe count-to-count variabilities of less than 5%, while spending as little as 10 seconds per sample count.

Assessing cell viability is both a key step in daily cell manipulation and often a requirement for subsequent processing and analysis. Moreover, knowing the viability of your cell sample prior to application of costly reagents or booking core facility time can be extremely valuable in today's fast-paced research environment. Trypan blue staining coupled with brightfield imaging is a common method for viability analysis, but many fluorescence options are becoming commonplace in both microscopy and flow cytometry workflows. With fluorescence-based viability assays on the Countess II FL Automated Cell Counter, you can simplify your workflow by obtaining cell count and viability information directly from your samples, immediately prior to advanced analysis.

Detect cell viability with a one-color fluorescence assay

There are many choices for one-color fluorescence viability assays, depending on your experimental needs. For assays where you need to distinguish live cells from dead cells after a fixation step, we recommend the LIVE/DEAD™ Fixable Dead Cell Stains. Only requiring a single fluorescence channel, the LIVE/DEAD Fixable Dead Cell Stains enable discrimination of live and dead cells based on membrane permeability and amine-reactive fluorescent dye chemistry. In viable cells, only surface proteins are available to react with these probes, resulting in relatively dim staining; conversely, in dead cells with compromised plasma membranes, these probes react with both intracellular proteins and those on the cell surface to produce intense fluorescent staining. The roughly 50-fold difference in intensity between live and

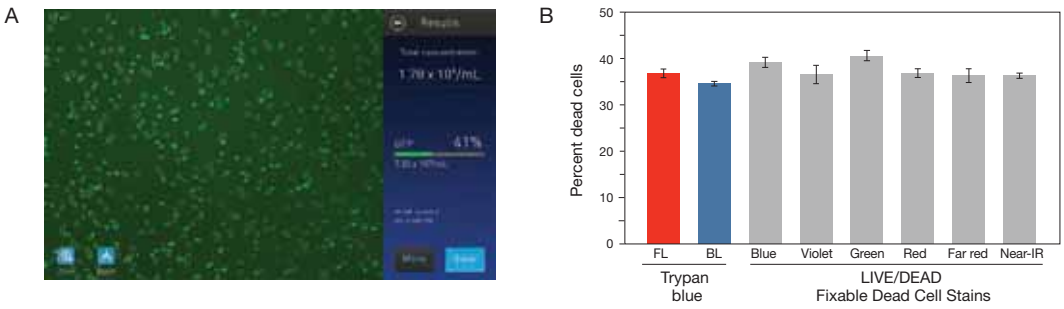

Figure 1. Cell viability detection using single-color viability assays. Live and ethanol-killed Jurkat cells were mixed (~2:1 live:dead), stained with trypan blue or with various colors of the LIVE/DEAD™ Fixable Dead Cell Stains, and analyzed on the Countess™ II FL Automated Cell Counter equipped with the appropriate EVOS™ Light Cubes. **(A)** A cell population treated with LIVE/DEAD Fixable Green Dead Cell Stain (Cat. No. L34969) for 30 min at 37°C is analyzed with the Countess II FL instrument equipped with the GFP EVOS Light Cube. Dead cells represented 41% of the total cell population as seen by positive staining. **(B)** For each stain, a total cell count was acquired using the brightfield image. A dead cell count for trypan blue staining was determined by brightfield (BL) or fluorescence (FL) imaging. For the LIVE/DEAD Fixable stains, the dead cell count was determined using the fluorescence channel. The percentages of dead cells indicated by all the dyes are in good agreement, but differ from the theoretical 2:1 starting ratio (33%) because the "live" population contained some dead cells.

dead cells is easily distinguished on the Countess II FL instrument equipped with standard EVOS™ Light Cubes (Figure 1). Figure 1B shows that the results of dead-cell detection with the LIVE/DEAD Fixable Dead Cell Stains are comparable with those obtained using the conventional trypan blue assay.

When a fixable dead-cell indicator is not required, we offer several other single-color viability markers—including the cell-impermeant propidium iodide, ethidium homodimer, and SYTOX™ nucleic acid stains—which selectively stain dead cells that have compromised plasma membranes. These nonfixable nucleic acid stains are commonly employed for multiplex analysis with functional or structural fluorescent probes, including fluorescent proteins or cell-surface markers. Figure 2 demonstrates the ability of the Countess II FL instrument equipped with a Cy®5 EVOS Light Cube to count dead cells stained with the SYTOX Red Dead Cell Stain. A total cell count can be acquired using the brightfield image, providing a simple method for calculating the ratio of live (unstained) to dead (SYTOX Red stained) cells within the population (Figure 3).

Detect cell viability with a two-color fluorescence assay

A more robust determination of cell viability can be achieved using an assay that simultaneously measures two different parameters of cell health. We recommend using the LIVE/DEAD™ Viability/Cytotoxicity Kit with the Countess II FL Automated Cell Counter. This viability assay relies on the use of two fluorescent probes: ethidium homodimer-1 is a high-affinity, red-fluorescent nucleic acid stain that is only able to pass through the compromised membranes of dead cells, and calcein AM is a cell-permeant fluorogenic esterase substrate that is hydrolyzed by active esterases to a green-fluorescent product (calcein) upon entering a live cell. Cells stained using the LIVE/DEAD Viability/Cytotoxicity Kit can be evaluated on the Countess II FL instrument equipped with Texas Red™ and GFP EVOS Light Cubes (Figure 4). With the viability assessment in hand, the cell sample can then be subjected to further functional analysis on other imaging platforms or by flow cytometry.

Save time and simplify your existing workflows

Fluorescence-based counting and viability assays on the Countess II FL Automated Cell Counter can be integrated seamlessly into your existing imaging, flow cytometry, and HCA workflows, allowing you to assess cell samples prior to committing them to more complex analyses. To learn more about the Countess II FL instrument as well as compatible reagents and assays, visit **thermofisher.com/countessbp72**. ■

Product	Quantity	Cat. No.
Countess™ II FL Automated Cell Counter	1 each	AMQAF1000
Countess™ Cell Counting Chamber Slides	50 slides	C10228
Countess™ II FL Reusable Slide	1 each	A25750
EVOS™ Light Cubes	1 each	Assorted
LIVE/DEAD™ Viability/Cytotoxicity Kit, for mammalian cells	1 kit	L3224
LIVE/DEAD™ Fixable Aqua Dead Cell Stain Kit	1 kit	L34965
LIVE/DEAD™ Fixable Green Dead Cell Stain Kit	1 kit	L34969
SYTOX™ Red Dead Cell Stain	1 mL	S34859

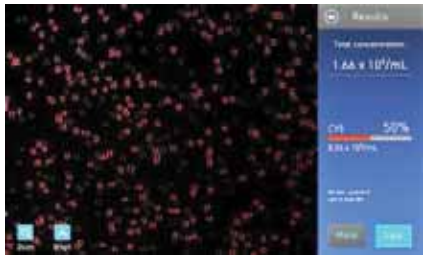

Figure 2. Discrimination of live and dead cells on the Countess II FL Automated Cell Counter. Live and formaldehyde-killed Jurkat cells were mixed (~1:1), stained with SYTOX™ Red stain (Cat. No. S34859), and analyzed on the Countess™ II FL instrument equipped with the Cy®5 EVOS™ Light Cube. A total cell count was acquired using the brightfield image, and a SYTOX Red–positive cell number was generated using the fluorescence channel, allowing the percentage of dead cells to be calculated for the population.

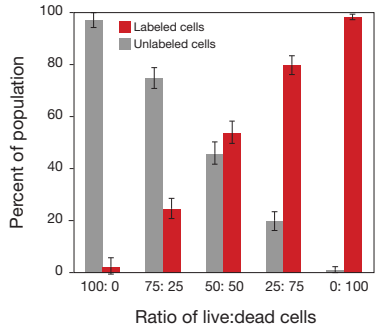

Figure 3. Cell viability assay with SYTOX Red Dead Cell Stain. Live and formaldehyde-killed Jurkat cells were mixed in the indicated ratios, stained with SYTOX™ Red stain (Cat. No. S34859), and analyzed using the Countess™ II FL instrument equipped with the Cy®5 EVOS™ Light Cube (see example in Figure 2).

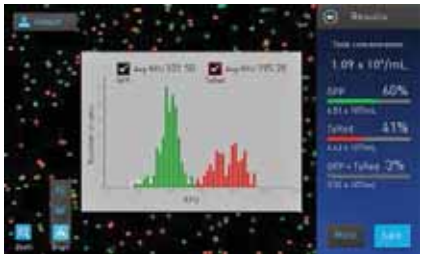

Figure 4. Cell viability assay with the LIVE/DEAD Viability/Cytotoxicity Kit. Live and heat-killed cells were mixed, stained with calcein AM and ethidium homodimer-1 supplied in the LIVE/DEAD™ Viability/Cytotoxicity Kit (Cat. No. L3224), and analyzed on the Countess™ II FL instrument equipped with GFP and Texas Red™ EVOS™ Light Cubes. The histogram shows the counts of live cells in the population, which fluoresce green, and of dead cells, which fluoresce red.

Multiplex your mitochondrial data

Tools to study mitochondrial morphology and function.

Mitochondria can make up as much as 10% of the cell volume of eukaryotic cells. Changes in mitochondrial morphology and function are good indicators of cell health, and multiplexing mitochondrial morphology reagents with probes that assess function can provide more in-depth information about mitochondrial health. We have developed a wide range of Molecular Probes™ reagents to investigate mitochondria in both live- and fixed-cell imaging applications. In this article, we highlight a few of our most referenced mitochondrial probes.

Mitochondrial morphology probes

Mitochondria can have a fragmented morphology, with many spheroid-shaped mitochondria, or a reticulated morphology, in which the mitochondrion is a single, many-branched structure [1,2]. The number of mitochondria is a function of several variables, including cell type, cell cycle or differentiation stage, cellular energy level, and overall cell health. Simply staining mitochondria and observing their morphology through a microscope can provide a significant amount of information about their overall biology and functional state (Table 1). Figure 1 shows mitochondria with normal reticulated morphology, as well as spheroid-shaped mitochondria being cleared by autophagy.

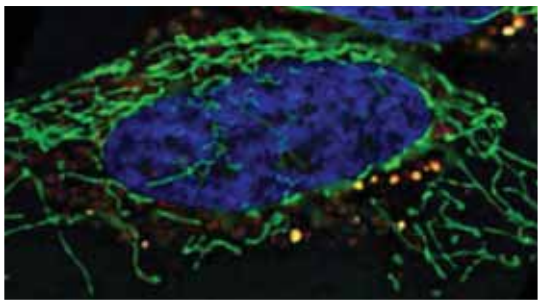

Figure 1. Mitochondrial morphology during mitophagy. HeLa cells labeled with Hoechst™ 33342 dye (blue, Cat. No. H3570) and expressing CellLight™ Mitochondria-GFP (green, Cat. No. C10600) and Premo™ Autophagy Sensor LC3B-RFP (red, Cat. No. P36236) were treated with CCCP to depolarize mitochondria. Loss of mitochondrial membrane potential triggers the targeted clearance of damaged mitochondria via mitophagy, as reflected through the colocalization of the autophagosomal marker LC3B-RFP with mitochondrial spheroids.

Mitochondrial functional tools

Mitochondrial dysfunction is associated with various diseases [4], and is a hallmark of cellular toxicity. We offer a variety of Molecular Probes reagents to study mitochondrial function from many perspectives, including probes for mitochondrial membrane potential, calcium flux, oxidative phosphorylation, autophagy/mitophagy, and cytosolic pH (Table 2).

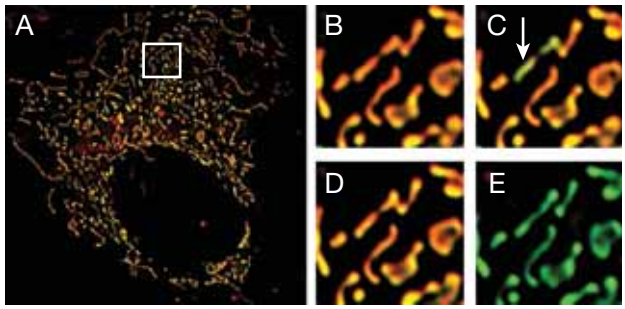

Figure 2. Dynamic imaging of mitochondrial membrane potential and organelle integrity. HeLa cells were transduced with CellLight™ Mitochondria-GFP (Cat. No. C10600) and loaded with 50 nM TMRM (Cat. No. T668) for 10 min at 37°C. **(A–E)** Images were acquired at 5 sec intervals for 90 sec following treatment with the uncoupler CCCP; zoomed sections **B–D** reveal heterogeneity in mitochondrial membrane potential regulation. Transient depolarization was observed in one but not all mitochondria (**C**, arrow), as indicated by loss of orange TMRM signal; GFP fluorescence was maintained during depolarization, indicating an intact mitochondrion. Loss of mitochondrial membrane potential was evident by 90 sec post-CCCP treatment **(E)**; however, mitochondria were still intact, information that would have been lost using TMRM alone.

Table 1. Various Molecular Probes tools to study mitochondrial morphology.

Probe	Mechanism of action
MitoTracker™ Green, Red, and Deep Red probes	MitoTracker Green (Cat. No. M7514), MitoTracker Red FM (Cat. No. M22425), and MitoTracker Deep Red FM (Cat. No. M22426) dyes are sequestered by functioning mitochondria. However, cells stained with these dyes retain their fluorescent staining patterns even if mitochondrial function is disrupted or if cells are subjected to fixation and permeabilization. This property makes them useful morphology markers that, once bound, are independent of mitochondrial function [3].
CellLight™ probes	CellLight Mitochondria-GFP (Cat. No. C10600) and CellLight Mitochondria-RFP (Cat. No. C10601) stain mitochondria independent of their functional state. These probes use a one-step protocol—based on BacMam gene delivery and expression technology—to label all the mitochondria in cells with either GFP or RFP. After application, mitochondria can be imaged in live cells, or cells can be fixed and permeabilized for further study.
Antibodies	Antibodies are a powerful tool for staining mitochondria in fixed cells. Antibodies such as the anti–Complex V monoclonal antibody (Cat. No. 459000) can stain all mitochondria in cells for morphological studies.

Table 2. Various Molecular Probes tools to study mitochondrial function.

Function	Probe recommendations
Mitochondrial membrane potential	TMRM (Cat. No. T668) is a classic dye for studying mitochondrial membrane potential because it accumulates in mitochondria with intact membrane potential and, upon loss of potential, leaks into the cytoplasm.
Mitochondrial calcium flux	The calcium indicator rhod-2 AM (Cat. No. R1244) has long been used to measure mitochondrial calcium flux because of its preferential accumulation in mitochondria (Figure 3).
Oxidative phosphorylation	Mitochondria generate various reactive oxygen species (ROS), particularly superoxides [5]. MitoSOX™ Red dye (Cat. No. M36008) is a mitochondria-targeted superoxide sensor. CellROX™ Orange (Cat. No. C10443) and CellROX™ Deep Red (Cat. No. C10422) Reagents are general oxidative stress indicators, but their signals can be localized to mitochondria when used together with mitochondrial morphology probes (Figure 4).
Autophagy/mitophagy	Cells routinely recycle dysfunctional mitochondria through a specific autophagy process called mitophagy. This mechanism can be detected by multiplexing CellLight Mitochondria-GFP or CellLight Mitochondria-RFP with one of the Premo™ Autophagy Sensors (Figure 1).
Cytosolic pH	Disruption of mitochondrial function can alter cytosolic pH [6]. pHrodo™ Green AM (Cat. No. P35373) or pHrodo™ Red AM (Cat. No. P35372) can be used to detect changes in cytosolic pH.

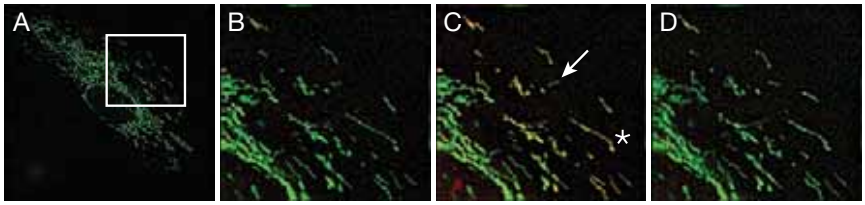

Figure 3. Multiplex imaging of mitochondrial calcium levels and dynamics. (A) HeLa cells were labeled with CellLight™ Mitochondria-GFP (Cat. No. C10600) and 5 µM rhod-2 AM (Cat. No. R1244) for 15 min at 37°C before imaging live over 100 sec. **(B–D)** The region outlined in **(A)** is enlarged to show individual mitochondria within a single cell over time. **(C, D)** Calcium is released from internal stores following application of 10 µM histamine. Mitochondria in close proximity to the calcium release are revealed by the increase in the orange-red fluorescence of rhod-2. The arrow in **(C)** denotes a mitochondrion that may have impaired calcium uptake, a detail that would have been missed using rhod-2 AM alone. The asterisk marks a mitochondrion that shows a transient elevation in calcium levels.

The power of multiplexing

The study of mitochondria can be improved by multiplexing functional probes with morphology probes. As an example, CellLight Mitochondria-GFP or CellLight Mitochondria-RFP can be combined with potential-sensitive dyes such as TMRM to monitor mitochondrial structural integrity while also assessing mitochondrial membrane potential (Figure 2). Figure 3 demonstrates the power of multiplexing the mitochondrial calcium flux indicator rhod-2 AM with potential-independent mitochondrial markers such as CellLight Mitochondria-GFP, which enables the visualization of mitochondrial fission, fusion, and motility before, during, and after calcium uptake. With the help of multiplexing, mitochondrial function can be determined even with tools that are not specifically targeted for mitochondria, when they are used in conjunction with mitochondria-targeted probes. When multiplexed with CellLight Mitochondria-GFP, CellROX Orange, a probe of general oxidative stress, can indicate the oxidative stress status of mitochondria (Figure 4).

Look closely at mitochondrial health

Healthy mitochondria are responsible for the bulk of a cell's energy production and play roles in a variety of other critical cell functions. To learn about our extensive collection of Molecular Probes tools for detecting mitochondrial morphology, membrane potential, and function, go to thermofisher.com/mitochondriabp72. ■

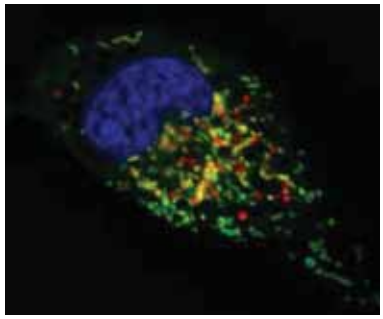

Figure 4. Multiplex imaging of mitochondrial structure and function. Human osteosarcoma (U2OS) cells expressing CellLight™ Mitochondria-GFP (green, Cat. No. C10508) were treated with 200 µM *tert*-butyl hydroperoxide (TBHP, an inducer of oxidative stress) for 2 hr. A stain solution containing 5 µM CellROX™ Orange (orange, Cat. No. C10443) and 2 drops of NucBlue™ Live Cell Stain (blue, Cat. No. R37605) per mL of cell sample was applied for 30 min at 37°C. Cells were washed and imaged with Live Cell Imaging Solution (Cat. No. A14291DJ) using a confocal microscope. While the green-fluorescent mitochondria with normal morphology indicate healthy mitochondria, the orange-fluorescent spheroids suggest that a fraction of the mitochondria are showing signs of oxidative stress after TBHP treatment.

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High-content analysis applied to autophagy

Automating cell imaging with Thermo Scientific HCA systems.

High-content analysis (HCA)—also known as high-content screening (HCS), image cytometry, quantitative cell analysis, or automated cell analysis—is an automated method for identifying substances that alter the phenotype of a cell in a desired manner. Primarily used in biological research and drug discovery, this technology combines fluorescence microscopy and automated cell calculations with phenotype analysis using image processing algorithms and informatics tools that enable the user to make decisions about the data. In this article, we describe the use of Thermo Scientific™ HCA systems (for example, see a description of the new Cellinsight™ CX7 HCA Platform on page 18) and Molecular Probes™ reagents to examine the process of autophagy and the effects of various compounds on that process.

Autophagy defined

Long-lived proteins, damaged cellular organelles, and invading microorganisms are cleared from healthy cells via a bulk-catabolic process known as autophagy. It was originally described by Christian De Duve in the 1960s [1] as a response to nutrient deprivation, but the last decade has provided more insight into the process, including characterization of receptors that mediate specific forms of autophagy, the autophagosome that engulfs cargo destined for clearance, and the mechanisms that allow fusion of the autophagosome with the lysosome [2]. Multiple scenarios have been described whereby aberrant mechanisms lead to a variety of disease states, and autophagy is now recognized as an area for therapeutic modulation [3].

Interrogating autophagy as a therapeutic target requires high-throughput analytical techniques. Current cell-based methods include identification of the characteristic double-membrane autophagosome through electron microscopy (EM) or localization of microtubule-associated proteins 1A/1B light chain 3B (LC3B) to the autophagosomal membrane using either fluorescence microscopy or western blotting. Of these three analysis platforms, fluorescence microscopy has proven to be the most amenable to high-throughput automation. Automated imaging and analysis platforms—integrated as high-content analysis [4]—have been used effectively to elucidate the hallmarks and regulators of autophagy [5,6].

An important feature of the assays presented here is the use of appropriate controls to determine whether or not the phenotypes being measured are a product of autophagy (with siRNA knockdown controls), as well as to ascertain if these phenotypes are a result of stimulating autophagy or inhibiting autophagic progress or flux (with chloroquine controls). Such controls are essential to validate assay protocols and to characterize candidate autophagy-moderating compounds under study.

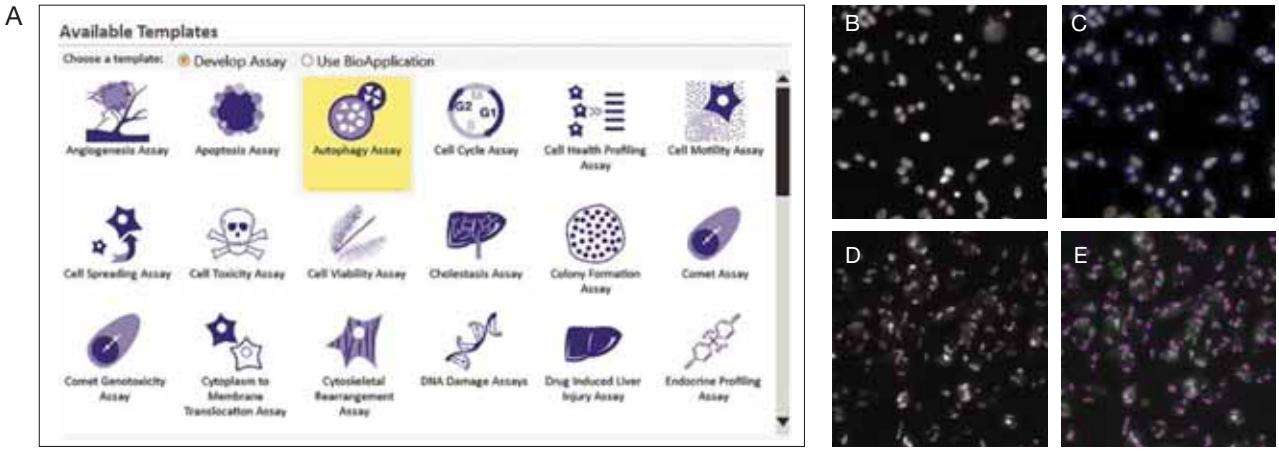

Figure 1. High-content image segmentation and analysis strategies. (A) Autophagy BioApplication analysis software on the Thermo Scientific™ Cellinsight™ CX5 HCS Platform. IBMK cells stably expressing GFP-LC3B were labeled with 1 µg/mL Hoechst™ 33342 **(B)**; subsequent identification of nuclei by the software allowed segmentation **(C)**. Once cells were identified, the autophagosomes expressing GFP-LC3B **(D)** were identified using the spot detector, and then segmented for analysis **(E)**. Images were acquired using a 20x objective on the Cellinsight CX5 HCS Platform.

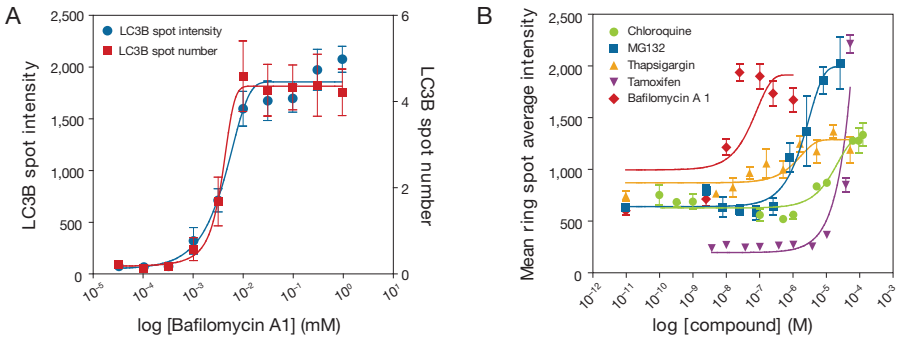

Figure 2. Dose-dependent LC3B labeling. (A) Dose-dependent correlation between the intensity of anti-LC3B staining and the number of autophagosomes detected via LC3B puncta. **(B)** Dose-response curves for five modulators of autophagy using mean LC3B spot intensity as an indicator of autophagy. Images were acquired using a 20x objective on the Thermo Scientific™ Cellinsight™ CX5 HCS Platform, and anti-LC3B staining was quantified using the Autophagy BioApplication analysis software.

Image segmentation and analysis

High-content analysis of autophagy requires identification of cells (via nuclear segmentation) and subsequent identification of LC3B puncta, which is efficiently accomplished using the Autophagy (or similar Compartmental Analysis) BioApplication software available on Thermo Scientific™ Cellinsight™ and ArrayScan™ HCS platforms. Figure 1 shows immortalized baby mouse kidney (IBMK) epithelial cells stably expressing GFP-LC3B and labeled with the blue-fluorescent nuclear stain Hoechst™ 33342. Once nuclei were identified, LC3B puncta were segmented and quantified using the Cellinsight CX5 HCS Platform.

A number of measurements can be taken to quantify autophagosome formation. Figure 2A shows the dose-dependent accumulation of LC3B puncta, as detected with anti-LC3B primary antibody and Alexa Fluor™ 647 secondary antibody, following inhibition of autophagic flux with bafilomycin A1. The relationship between LC3B puncta intensity and the number of LC3B puncta per cell is identical across bafilomycin A1 concentrations. The dose-response curves for five autophagy modulators were rapidly analyzed on the Cellinsight CX5 HCS Platform using mean LC3B spot intensity as a measure of autophagy (Figure 2B).

Mechanism of action and specificity

When examining the nature of autophagosome formation, it is critical to confirm that the signal being measured is specific to autophagy. Knockdown of key autophagy genes, such as *ATG5* or *ATG7*, is the best way to establish a given autophagy reporter is specific. Chloroquine is a known inhibitor of autophagic flux through the pathway, and chloroquine treatment results in an increase of LC3B puncta in otherwise normal cells. In contrast, siRNA-mediated knockdown of *ATG7* expression results in a loss of LC3B puncta after chloroquine treatment (Figure 3). →

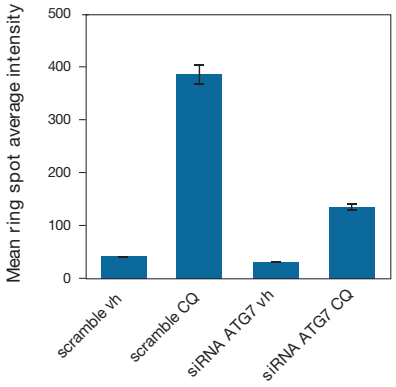

Figure 3. siRNA-mediated knockdown of key autophagy genes to confirm specificity of the label. LC3B puncta significantly increased upon treatment of A549 cells with chloroquine (CQ), as compared with treatment with vehicle alone (vh, in this case water). This increase is much reduced when cells are transfected with siRNA against *ATG7*, but not when cells are transfected with the scrambled version of the siRNA (scramble). Anti-LC3B staining was quantified using the Compartmental Analysis BioApplication software on a Thermo Scientific™ ArrayScan™ VTI HCS Reader.

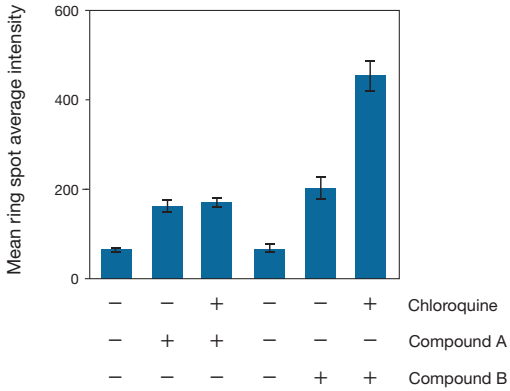

Figure 4. LC3B puncta resulting from induction of autophagy or inhibition of autophagic flux. Treatment of cells with compound A or B significantly increases anti-LC3B staining. Combining chloroquine with compound A or B causes a further increase only with compound B. Anti-LC3B staining was quantified using the Compartmental Analysis BioApplication software on a Thermo Scientific™ ArrayScan™ VTI HCS Reader.

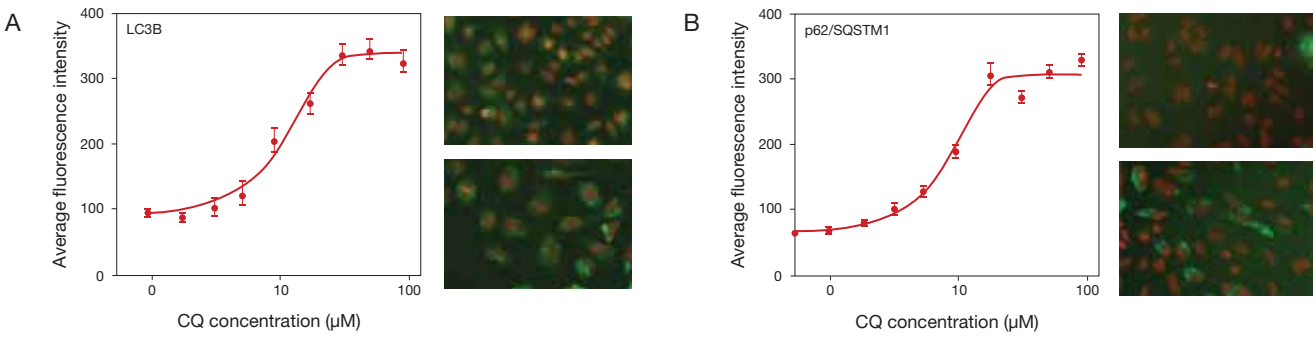

Figure 5. Measuring autophagy with transient expression of GFP-based biosensors. U2OS cells were transduced using either the Premo™ Autophagy Sensor LC3B-GFP (Cat. No. P36235) or the Premo™ Autophagy Sensor GFP-p62 (Cat. No. P36240). High-content imaging of autophagy is shown in cells expressing fluorescent protein–based biosensors for either **(A)** LC3B or **(B)** the autophagy receptor p62/SQSTM1. Images were quantified using the Compartmental Analysis BioApplication software on a Thermo Scientific™ ArrayScan™ VTI HCS Reader.

An increase in LC3B puncta in the cytoplasm can arise either from agents that stimulate autophagy or from those that block autophagic flux. To identify which of these possibilities is occurring, a known autophagic flux inhibitor, such as chloroquine, may be used. Figure 4 shows this approach in practice. Two compounds, A and B, are applied in the presence or absence of chloroquine. Both compound A and B cause an increase in LC3B labeling; however, only compound B shows additional LC3B staining in the presence of chloroquine. Therefore, compound B is stimulating autophagy, whereas compound A is acting on the autophagy pathway at a point similar to that of chloroquine (i.e., inhibiting autophagic flux).

Measuring autophagy with transient expression of GFP-based biosensors

An alternative to antibody-based monitoring of autophagy is the use of cells transformed with a biosensor that is a fusion of a fluorescent protein (FP) and an autophagy marker. Stable cell lines, once generated, are ready to assay; however, the process of generating stably transformed cell lines is expensive and time consuming, and assaying different cell types requires the creation of new cell lines. Fortunately, the delivery of FP-based biosensors can be performed quickly and easily using the BacMam gene delivery and expression system, and the transiently transduced cells can be used in autophagy assays the next day. Figure 5 shows U2OS cells transduced with either Green Fluorescent Protein (GFP)-LC3B or GFP-p62 using BacMam 2.0 technology. High-content imaging demonstrates the dose-dependent accumulation of both markers when autophagic flux is inhibited using chloroquine.

More resources for high-content analysis

With the wealth of Molecular Probes fluorescence assays available and the automated analysis power of Thermo Scientific HCA platforms, researchers have the tools to more efficiently screen compounds affecting complex cellular processes such as autophagy. Learn about our products for high-content analysis at **thermofisher.com/hcsbp72**. ■

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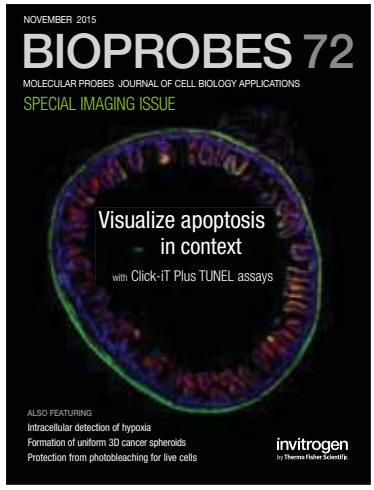
Product	Quantity	Cat. No.
ArrayScan™ XTI High Content Platform	1 each	ASN00002P
Cellinsight™ CX5 High Content Screening (HCS) Platform	1 each	CX51110
Cellinsight™ CX7 High Content Analysis Platform	1 each	CX7A1110
LC3B Antibody Kit for Autophagy	1 kit	L10382
Premo™ Autophagy Sensor LC3B-GFP (BacMam 2.0)	1 kit	P36235
Premo™ Autophagy Sensor GFP-p62 (BacMam 2.0)	1 kit	P36240



Cover image

Click-iT Plus TUNEL assays can be multiplexed with a variety of other fluorescent probes. Formalin-fixed, paraffin-embedded (FFPE) tissue from a transgenic mouse expressing GFP in intestinal muscle was treated with DNase I, followed by the Click-iT™ Plus TUNEL Assay for *In Situ* Apoptosis Detection with Alexa Fluor™ 647 dye (Cat. No. C10619), and then stained with Hoechst™ 33342 dye (Cat. No. H3570) and ActinRed™ 555 ReadyProbes™ Reagent (Cat. No. R37112). Cell nuclei are stained by Hoechst 33342 dye (blue), the GFP signal (green) is detected in the surrounding muscular layer, filamentous actin is stained by ActinRed 555 reagent (red), and the TUNEL-positive signal resulting from DNase I treatment is clearly defined by the Click-iT Plus TUNEL Assay with Alexa Fluor 647 dye (purple).

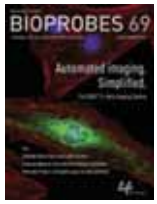
Previous issues

BIOPROBES 71

This issue is devoted to flow cytometry applications and instrumentation. We highlight flow cytometric detection of exosomes, fluorescent proteins, and rare events, as well as assays for hyperploidy and cell death. Also described in detail are the Attune™ NxT Acoustic Focusing Cytometer, flow cytometry panel design and color compensation, and no-lyse, no-wash assays.

BIOPROBES 70

This issue features pHrodo™ pH sensors, multiplexable Click-iT™ Plus EdU proliferation assays, and Qubit™ and Quant-iT™ microRNA assays. Also discussed are two next-generation benchtop instruments—the Attune™ NxT Acoustic Focusing Cytometer and the Countess™ II FL Automated Cell Counter—as well as time-lapse microscopy and super-resolution imaging.



BIOPROBES 69

In this issue, we describe the EVOS™ FL Auto Imaging System for fully automated multichannel fluorescence microscopy and time-lapse imaging. You can also explore the Molecular Probes™ cell health assays for flow cytometry, and the SiteClick™ antibody labeling system, as well as fluorescent probes for oxidative stress, autophagy, and live-cell staining of pluripotent stem cells.

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Thermo Fisher Scientific
5791 Van Allen Way
Carlsbad, California 92008
United States
Tel: +1 760 603 7200
Toll-Free Tel: 800 955 6288
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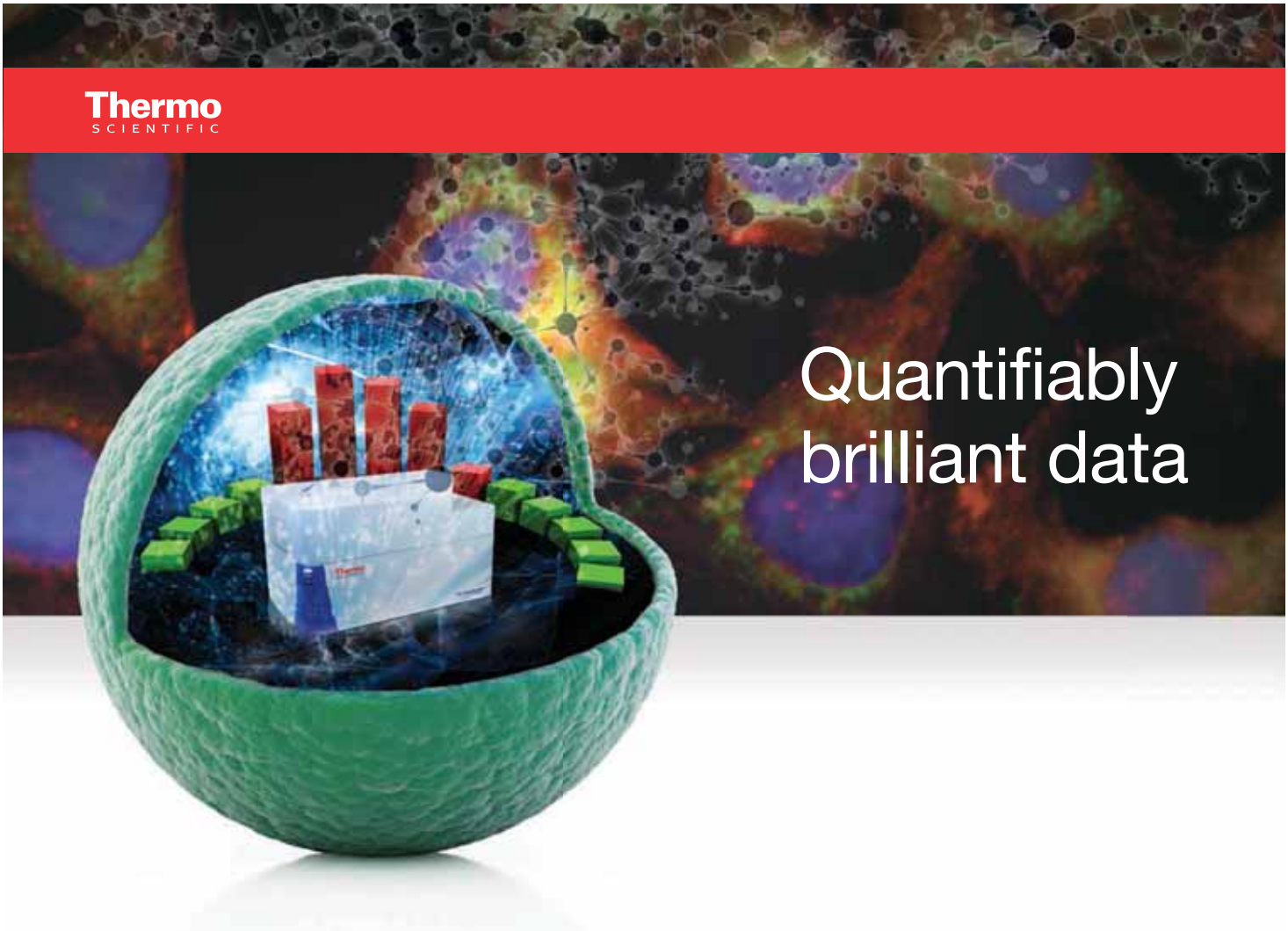

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