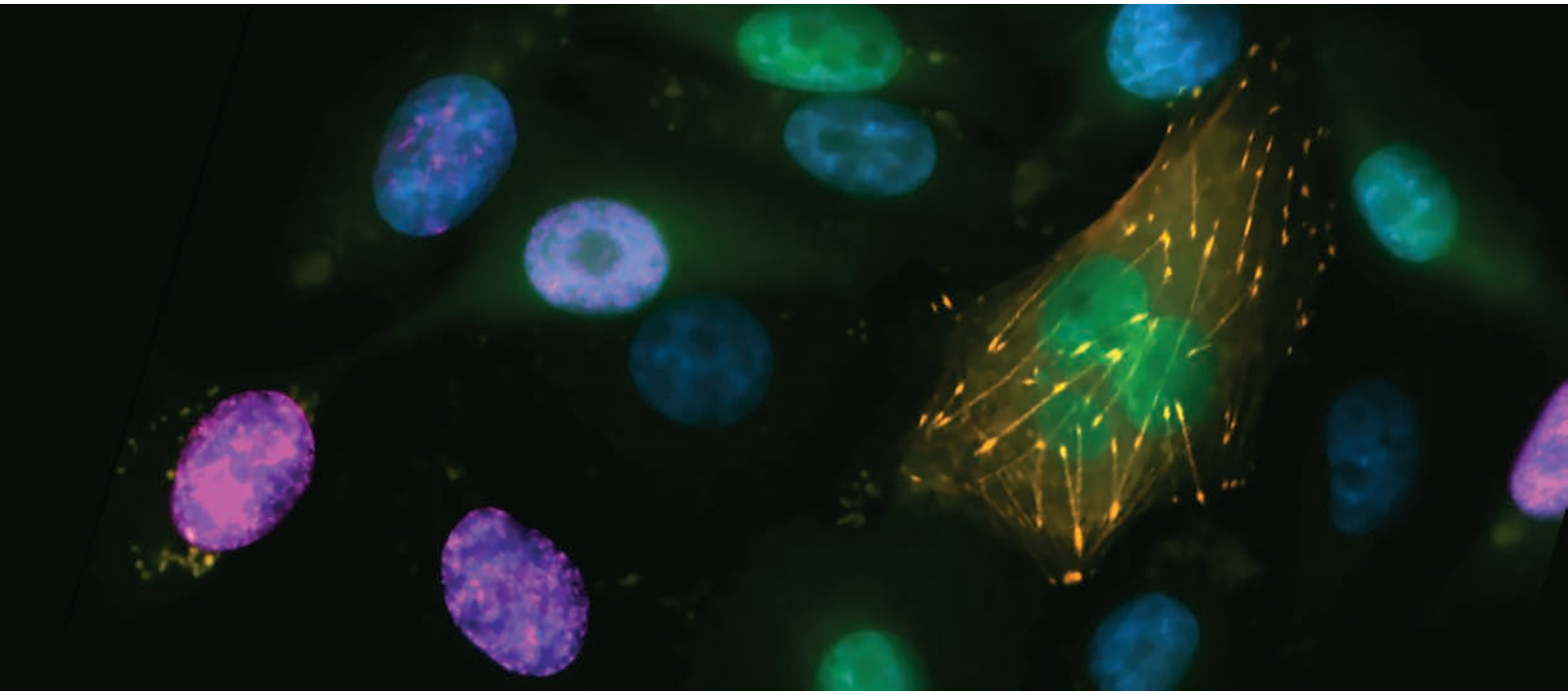

Multiplex your cell proliferation assay with GFP, RFP, and R-PE probes

Click-iT® Plus EdU Proliferation Kits for imaging and flow cytometry.

Cell proliferation assays provide a critical piece of the puzzle when evaluating cell health, genotoxicity, and the efficacy of anti-cancer drugs. Proliferation, however, is rarely assayed in isolation; other cell function probes are often used in concert with proliferation assays to provide a more informative picture of the state of the cell. When compared with traditional antibody-based BrdU methods, the Click-iT® Plus EdU cell proliferation assays not only offer better performance and an easier workflow but are now compatible with an even broader range of commonly used fluorescent probes, including GFP, RFP, and other fluorescent proteins as well as phycobiliproteins.

Figure 1 (above). GFP and RFP compatibility with the Click-iT® Plus EdU assay. Erk2-GFP–expressing A375 melanoma cells were transduced with CellLight® Talin-RFP (Cat. No. C10612, orange) overnight, and then pulsed with 10 µM EdU for 2 hr and labeled using the Click-iT® Plus EdU Alexa Fluor® 647 Imaging Kit (Cat. No. C10640, pink) and Hoechst® 33342 nucleic acid stain (blue). Coverslips were mounted with ProLong® Gold Antifade Mountant (Cat. No. P10144) and imaged using a Nikon® ECLIPSE® E800 microscope with Semrock® DAPI, FITC, TRITC, and Cy®5 optical filter sets. Proliferating cells have pink nuclei; the nuclei in nonproliferating cells appear blue due to Hoechst® 33342 staining and green due to Erk2-GFP expression.

Click-iT® Plus EdU: A breakthrough cell proliferation assay

The Click-iT® Plus EdU assay represents a significant breakthrough in the evolution of cell proliferation measurements (Figure 1). The most accurate cell proliferation assays directly quantitate newly synthesized DNA by following the incorporation of a deoxyribonucleoside analog that contains a detectable tag. In the 1950s, the original cell proliferation measurements were based on the incorporation of radioactive nucleosides (i.e., ^{3}H-thymidine) into DNA. Thirty years later, a nonradioactive proliferation assay was introduced based on the detection of the thymidine analog 5-bromo-2´-deoxyuridine (BrdU) by anti-BrdU antibodies.

Although it eliminates the complications of working with radioactivity, the BrdU proliferation assay is both difficult to perform consistently and time consuming, typically requiring 6–24 hr to complete. In the standard BrdU assay, cells are incubated with BrdU and then treated with acid, heat, or enzymes to denature the DNA and facilitate detection of the incorporated BrdU molecules by anti-BrdU antibodies (Figure 2A). These harsh treatments can adversely affect cell morphology and antigen recognition sites, as well as image quality. The denaturants also limit the ability of the BrdU assay to be multiplexed with fluorescent proteins (e.g., GFP, RFP, mCherry) or phycobiliproteins (e.g., R-PE, R-PE tandems), which are regularly used in imaging or flow cytometry applications.

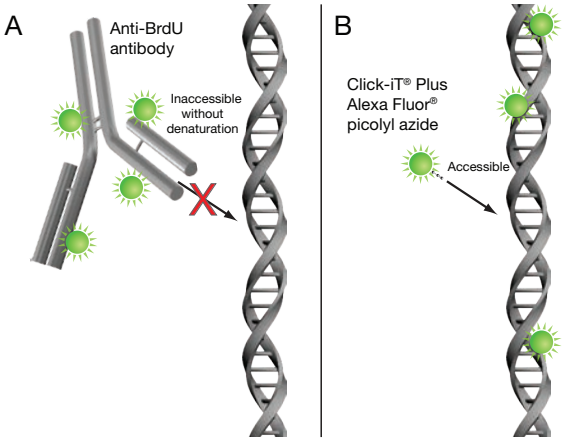

Figure 2. Detection of incorporated BrdU with an anti-BrdU antibody, compared with detection of incorporated EdU with the Alexa Fluor® picolyl azide. (A) Without DNA denaturation, BrdU is inaccessible to antibodies used for detection. **(B)** The small size of the EdU detection reagent, Alexa Fluor® picolyl azide, eliminates the need to denature the DNA for the detection reagent to access the nucleotide.

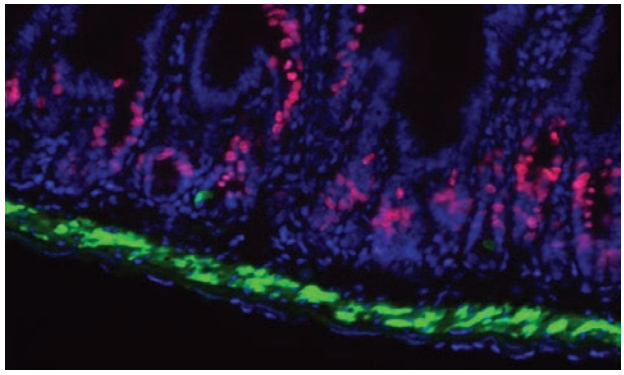

Figure 3. Detection of cell proliferation and GFP fluorescence in mouse tissue. A transgenic mouse was injected intraperitoneally with 50 µg EdU per gram body weight 4 hr before sacrifice. The Click-iT® Plus EdU Alexa Fluor® 555 Imaging Kit (Cat. No. C10638) was used to detect newly synthesized DNA in mouse duodenum tissue. Constitutively expressed β-actin–GFP fusion (green) is seen in the smooth muscle band below the bright Click-iT® Plus EdU–labeled proliferating cells (red) of the intestinal villi; the tissue was counterstained with DAPI nucleic acid stain (blue). Image provided by Jessica-Sordet Dessimoz, École Polytechnique Fédérale de Lausanne, Lausanne, Switzerland.

Unlike these traditional cell proliferation assays, the Click-iT® Plus EdU proliferation assay does not rely on radioactivity or antibodies for detection of the newly incorporated deoxyribonucleoside. In the Click-iT® Plus EdU assay, the alkyne-containing thymidine analog EdU (5-ethynyl-2´-deoxyuridine) is incorporated into DNA during active DNA synthesis [1]. The incorporated EdU is then detected by a click reaction—a copper-catalyzed azide–alkyne cycloaddition—using a fluorescent Alexa Fluor® or Pacific Blue™ dye containing a picolyl azide moiety (Figure 2B). The use of the picolyl azide combined with a copper protectant is the basis of the upgraded Click-iT® Plus EdU technology, which achieves the same sensitive, reliable detection of cell proliferation as the original Click-iT® EdU assay while also preserving the fluorescence of GFP, RFP (Figures 1, 3, and 4), and R-PE. 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 click reaction and subsequent wash steps are typically completed in 60 minutes, and newly synthesized DNA can be detected and quantified using image-based techniques or flow cytometry. →

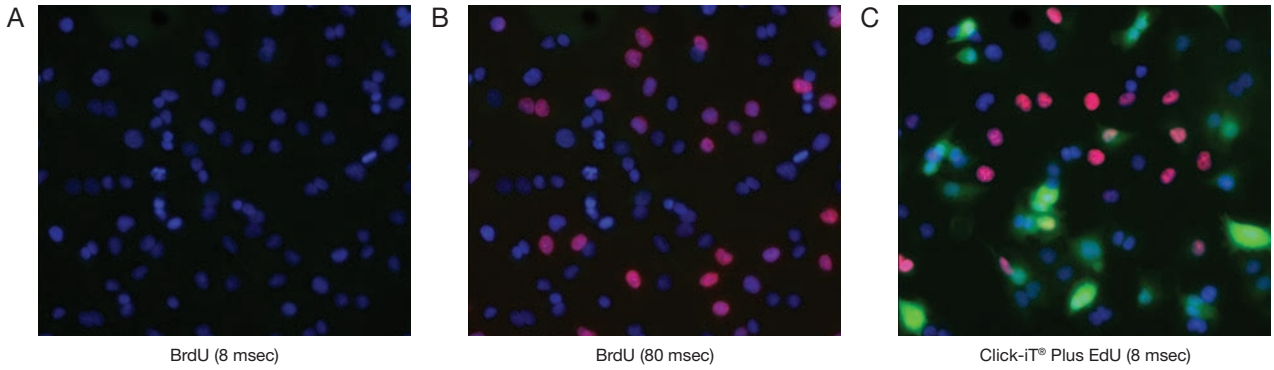

Figure 4. Cell proliferation detected using the BrdU assay or the Click-iT® Plus EdU assay. (A, B) After incubation with BrdU, Erk2-GFP–expressing A375 melanoma cells were treated with HCl, resulting in a loss of GFP signal, and incubated with Alexa Fluor® 594 anti-BrdU antibody (clone MoBU-1, Cat. No. B35132), producing moderately bright detection of proliferation (red), shown here with **(A)** an 8 msec exposure and **(B)** an 80 msec exposure. **(C)** In contrast, Erk2-GFP A375 cells processed using the reagents and fixation/detection protocol provided in the Click-iT® Plus EdU Alexa Fluor® 594 Imaging Kit (Cat. No. C10639) retained their GFP signal (green), and the EdU-based detection of proliferation was very bright (red, 8 msec exposure). Both cell samples were treated with Hoechst® 33342 nucleic acid stain (blue); high-content analysis was performed using the Thermo Scientific® Cellomics® ArrayScan® VTI HCS Reader.

Choose Click-iT® Plus EdU assays over BrdU assays

To demonstrate its superior performance, the Click-iT® Plus EdU assay was directly compared with the traditional BrdU assay. The proliferation signal from A375 melanoma cells expressing an Erk2-GFP fusion was detected using either BrdU or Click-iT® Plus EdU (Figure 4). The harsh treatment required for the antibody-based BrdU assay resulted in the loss of the GFP signal, as seen by the absence of green fluorescence in Figures 4A and 4B. Furthermore, the BrdU proliferation signal required a 10-fold longer exposure time (Figure 4B) to generate results comparable to those obtained with the Click-iT® Plus EdU assay (Figure 4C).

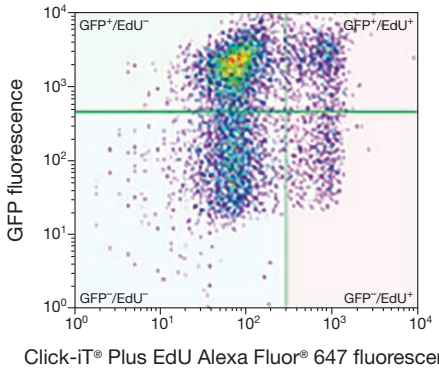

Figure 5. Dual-parameter plot of fluorescence from cells labeled with the Click-iT® Plus EdU Alexa Fluor® 647 Flow Cytometry Assay Kit and GFP. Erk2-GFP–expressing A375 melanoma cells were treated with 10 µM EdU for 2 hr and detected according to the Click-iT® Plus EdU staining protocol (Cat. No. C10634). Data were collected and analyzed using the Attune® Acoustic Focusing Cytometer with 635 nm excitation and a 660/20 nm bandpass emission filter for detection of the Alexa Fluor® 647–labeled EdU, and with 488 nm excitation and a 530/30 nm bandpass emission filter for detection of GFP.

Multiplex Click-iT® Plus EdU assays with fluorescent proteins

The ability to multiplex Click-iT® Plus EdU assays with other fluorescent probes opens the door to a more complete analysis of cell function. For example, Figure 1 shows proliferating A375 melanoma cells that are expressing both GFP and RFP fusion proteins; additionally, the red-fluorescent talin-RFP fusion protein confirms the presence of an intact cytoskeletal structure. New DNA synthesis and GFP expression were also detected by fluorescence microscopy in tissue samples from a transgenic mouse model (Figure 3) and by flow cytometry in Erk2-GFP–expressing A375 melanoma cells (Figure 5). In addition, mCherry fluorescence was preserved when mCherry-expressing A549 cells were assayed for cell proliferation using the Click-iT® Plus EdU Alexa Fluor® 488 Kit (Figure 6).

Likewise, phycobiliproteins (e.g., R-PE and R-PE tandems) can be multiplexed with Click-iT® Plus EdU cell proliferation assays. Figure 7 shows co-labeling of Jurkat cells with Click-iT® Plus Alexa Fluor® 488 picolyl azide to detect incorporated EdU and with PE-Cy®7–conjugated mouse anti–human CD4 antibody. This dual-parameter flow cytometry experiment demonstrates the preservation of the phycobiliprotein fluorescence as well as of the antigen recognition sites on the primary antibody.

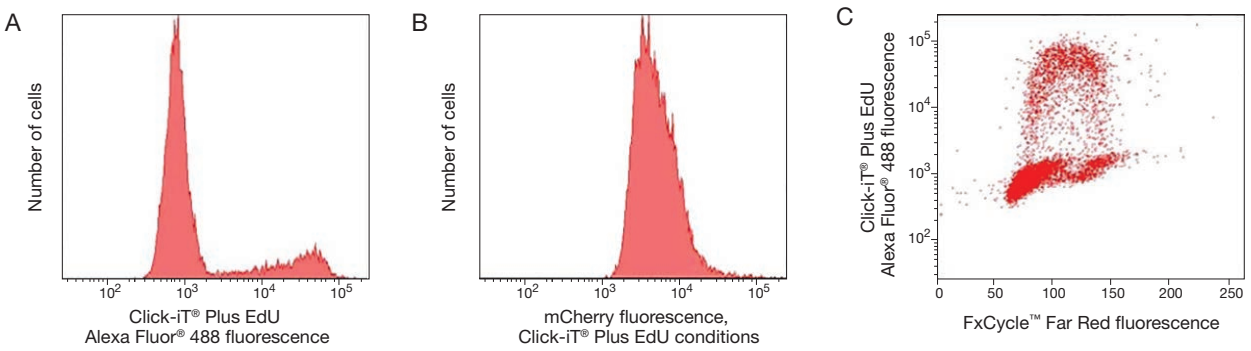

Figure 6. Flow cytometric analysis of mCherry-expressing cells labeled with the Click-iT® Plus EdU Alexa Fluor® 488 Flow Cytometry Assay Kit and FxCycle™ Far Red Stain. mCherry-expressing A549 cells were treated with 10 µM EdU for 2 hr and detected according to the Click-iT® Plus EdU staining protocol (Cat. No. C10632). **(A)** Cells in S phase are identified by the fluorescence of the Alexa Fluor® 488-labeled EdU. **(B)** mCherry fluorescence is readily detected in cells under the conditions used for Click-iT® Plus EdU labeling and is not significantly different from that seen in the no-copper positive control (data not shown). **(C)** Dual-parameter plot of Alexa Fluor® 488–labeled EdU fluorescence (indicating newly synthesized DNA) and FxCycle™ Far Red Stain fluorescence (indicating DNA content analysis, Cat. No. F10348). This plot's typical inverted U-shaped pattern identifies proliferating cells with bright EdU staining and nonproliferating cells with dim EdU staining that are either in G_0/G_1 phase (with 2N DNA content) or in G_2/M phase (with 4N DNA content).

Even more Click-iT® Plus tools

In addition to the imaging and flow cytometry kits, the Click-iT® Plus technology is available in the Click-iT® Plus Alexa Fluor® Picolyl Azide Toolkits, which contain the reagents needed to perform copper-catalyzed click reactions with copper-sensitive compounds. These toolkits provide Alexa Fluor® picolyl azide and Click-iT® reaction buffers, as well as copper sulfate and copper protectant. They allow you to optimize your own Click-iT® Plus detection of alkyne-containing biomolecules *in vitro*, in cells, or in tissue samples.

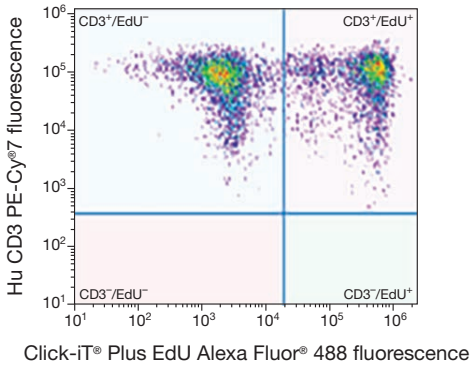

Figure 7. Dual-parameter plot of fluorescence from cells labeled with the Click-iT® Plus EdU Alexa Fluor® 488 Flow Cytometry Assay Kit and the PE-Cy®7 anti–Hu CD3 antibody. Jurkat cells were treated with 10 µM EdU for 2 hr, stained with the PE-Cy®7 conjugate of anti–Hu CD3 antibody (Cat. No. MHCD0312), and detected according to the Click-iT® Plus EdU staining protocol (Cat. No. C10632). Data were collected and analyzed using the Attune® Acoustic Focusing Cytometer with 488 nm excitation and a 530/30 nm bandpass emission filter for detection of the Alexa Fluor® 488–labeled EdU, and with 488 nm excitation and a 780/60 nm bandpass emission filter for detection of the PE-Cy®7 anti–Hu CD3 conjugate.

Learn about the latest advances in Click-iT® Plus technology

The Click-iT® Plus technology advances our original Click-iT® protocols and offers more flexibility for multiplexing with other cell function assays. Find out more about the Click-iT® Plus technology and upcoming Click-iT® Plus products at **lifetechnologies.com/clickitplusbp70**. ■

Reference

1. Salic A, Mitchison TJ (2008) *Proc Natl Acad Sci U S A* 105:2415–2420.

Product	Quantity	Cat. No.
Click-iT® Plus EdU Alexa Fluor® 488 Imaging Kit	1 kit	C10637
Click-iT® Plus EdU Alexa Fluor® 555 Imaging Kit	1 kit	C10638
Click-iT® Plus EdU Alexa Fluor® 594 Imaging Kit	1 kit	C10639
Click-iT® Plus EdU Alexa Fluor® 647 Imaging Kit	1 kit	C10640
Click-iT® Plus EdU Alexa Fluor® 488 Flow Cytometry Assay Kit	50 assays	C10632
	100 assays	C10633
Click-iT® Plus EdU Alexa Fluor® 647 Flow Cytometry Assay Kit	50 assays	C10634
	100 assays	C10635
Click-iT® Plus EdU Pacific Blue™ Flow Cytometry Assay Kit	50 assays	C10636
Click-iT® Plus Alexa Fluor® 488 Picolyl Azide Toolkit	1 kit	C10641
Click-iT® Plus Alexa Fluor® 555 Picolyl Azide Toolkit	1 kit	C10642
Click-iT® Plus Alexa Fluor® 647 Picolyl Azide Toolkit	1 kit	C10643