Flow cytometry: checking vital signs

A SURVEY OF MOLECULAR PROBES® VIABILITY AND VITALITY TECHNOLOGIES.

Viability and cytotoxicity assays are principally used to enumerate the proportion of live and dead cells in a population, and most commonly rely on a simple check of membrane integrity. However, cell vitality comprises a range of cell interactions with the environment, and can be judged based on virtually any cell process from membrane function to proliferative capacity (Figure 1). The diversity of live cells and their environments makes it impossible to devise a single assay applicable to all cell types, and assays deemed useful must also meet the needs of individual researchers. Invitrogen offers a range of flow cytometry assays that can be used with available lasers and emission channels.

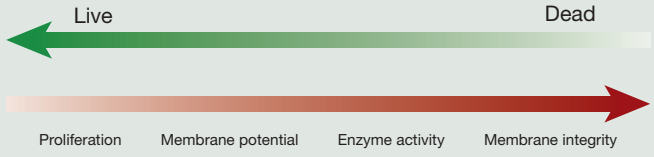

Figure 1—Cell viability comprises a spectrum of cell responses to the environment.

Membrane-impermeant DNA dyes

Loss of membrane integrity is the ultimate indicator of cell death, and is easily detected with impermeant DNA dyes. These dyes are excluded from cells with intact membranes, but enter cells with compromised

Table 1—Spectral characteristics of reagents used for viability and vitality measurements.

Excitation source	UV	405 nm			488 nm	
Emission color	Blue	Blue	Green	Yellow	Green	Yellow
Impermeant dyes	• DAPI (D3571)	• SYTOX® Blue dye (S34857)	• SYTOX® Blue dye (S34857)		• SYTOX® Green dye (S7020)	• Dead cell discriminator (DCD00) • Propidium iodide (P3566)
Fixable dead-cell dyes	• Fixable blue dye (L23105)	• Fixable violet dye (L34955)	• Fixable aqua dye (L34957)		• Fixable green dye (L23101)	
Mitochondrial membrane potential					• JC-1 (M34152) • $DiOC_2(3)$ (M34150) • Rhodamine 123 (R302)	• JC-1 (M34152) • $DiOC_2(3)$ (M34150) • MitoTracker® Red CMXRos (M7512)
Metabolic and enzymatic activity	• CellTrace™ calcein blue AM (C34853)	• CellTrace™ calcein violet AM (C34858)			• Calcein AM (C3100MP)	• C_{12}-resazurin (V23110) • CellTrace™ calcein red-orange AM (C34851)
Other cell responses (calcium flux, pH)	• Indo-1 AM (I1223)			• Fura Red™ AM (F3021)	• Fluo-3 AM (F1242) • Fluo-4 AM (F14201)	• Fura Red™ AM (F3021) • SNARF®-1 AM dye (C1272)
Cell division: nucleoside incorporation		• Click-iT™ EdU Pacific Blue™ (A10034)			• Click-iT™ EdU Alexa Fluor® 488 (C35002) • Alexa Fluor® 488 anti-BrdU (A21303) • FITC anti-BrdU (MD5401) • ABSOLUTE-S™ SBIP (A23150)	
Cell division: cell cycle for intact cells	• Hoechst 33342 (H3569)	• DyeCycle™ Violet dye (V35003)			• DyeCycle™ Green dye (V35004)	• DyeCycle™ Orange dye (V35005)
Cell division: cell cycle for permeabilized cells	• DAPI (D3571) • Hoechst 33342 (H3569)	• DyeCycle™ Violet dye (V35003) • SYTOX® Blue dye (S34857)	• SYTOX® Blue dye (S34857)		• DyeCycle™ Green dye (V35004) • SYTOX® Green dye (S7020)	• DyeCycle™ Orange dye (V35005) • Propidium iodide (P3566)
Cell division: tracer dye analysis					• CFSE (C34554) • Oregon Green® SE (C34555) • DiO (V22886)	
Cell count	• CountBright™ beads (C36950)	• CountBright™ beads (C36950)	• CountBright™ beads (C36950)	• CountBright™ beads (C36950)	• Caltag™ Counting Beads (PCB-100) • CountBright™ beads (C36950)	• Caltag™ Counting Beads (PCB-100) • CountBright™ beads (C36950)

Red = compatible with fixation. Example product numbers are provided; additional configurations may be found at www.invitrogen.com.

membranes and undergo significant fluorescence enhancement when they bind DNA. Propidium iodide (PI) is the most common dye in this group, but there are other dyes for the verification of membrane integrity that fit with every excitation source (Table 1). These dyes are often used in a "dump channel," with gating on the viable cells for further analysis (Figure 2). Because these dyes bind in equilibrium with DNA, external dye concentration must be maintained during analysis, and the dye should not be washed out. Impermeant DNA dyes are not compatible with fixation or intracellular staining protocols when used as viability probes.

Fixable dead-cell dyes

The fixable dead-cell dyes, which covalently interact with available amino groups, are a new class of viability dyes. Like impermeant dyes, the fixable dyes are excluded from the cytosol of healthy cells. The dyes react with surface proteins of healthy cells, but also label proteins throughout the cytoplasm of cells with damaged membranes, causing dead cells to have at least 50-fold greater fluorescence than live cells. Because the labeling is covalent, labeled cells can be aldehyde fixed and permeabilized without losing viability →

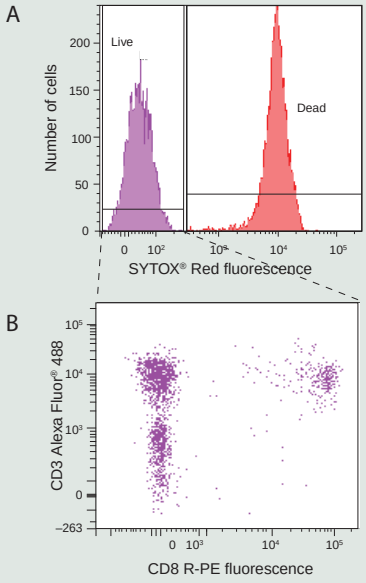

Figure 2—Viable cell gating with an impermeant DNA dye. A mixture of heat-treated and untreated human peripheral blood leucocytes (PBL) was stained with antibody conjugates, then stained with 5 nM SYTOX® Red stain before being analyzed by flow cytometry with 488 nm and 635 nm excitation. The dot plot showing cells stained for CD3 and CD8 antigens **(B)** was gated on live cells (left peak from the SYTOX® Red stain, panel **A**).

		532 nm			633 nm	
Orange	Red	Yellow	Orange	Red	Red	Infrared
• 7-AAD (A1310) • Dead cell discriminator (DCD00) • Propidium iodide (P3566)	• 7-AAD (A1310) • Dead cell discriminator (DCD00) • Propidium iodide (P3566)	• Dead cell discriminator (DCD00) • Propidium iodide (P3566)	• 7-AAD (A1310) • Dead cell discriminator (DCD00) • Propidium iodide (P3566) • SYTOX® Orange dye (S11368)	• 7-AAD (A1310) • Dead cell discriminator (DCD00) • Propidium iodide (P3566)	• SYTOX® Red dye (S34859)	
• Fixable red dye (L23102)			• Fixable red dye (L23102)		• Fixable far red dye (L10120)	• Fixable near IR dye (L10119)
• JC-1 (M34152) • $DiOC_2(3)$ (M34150)		• MitoTracker® Red CMXRos (M7512)			• $DiIC_1(5)$ (M34151)	
		• C_{12}-resazurin (V23110)				
• SNARF®-1 AM dye (C1272)		• Fura Red™ AM (F3021) • SNARF®-1 AM dye (C1272)	• SNARF®-1 AM dye (C1272)			
					• Click-iT™ EdU Alexa Fluor® 647 (A10202) • Alexa Fluor® 647 anti-BrdU (A21305)	• Alexa Fluor® 680 anti-BrdU (A31859)
		• DyeCycle™ Orange dye (V35005)				
• 7-AAD (A1310) • Propidium iodide (P3566)	• 7-AAD (A1310) • Propidium iodide (P3566)	• Propidium iodide (P3566)	• 7-AAD (A1310) • Propidium iodide (P3566)	• 7-AAD (A1310) • Propidium iodide (P3566)	• SYTOX® Red dye (S34859)	
		• DiI (V22885)			• DDAO-SE (C34553) • DiD (V22887)	
• Caltag™ Counting Beads (PCB-100) • CountBright™ beads (C36950)	• CountBright™ beads (C36950)	• CountBright™ beads (C36950)	• CountBright™ beads (C36950)	• CountBright™ beads (C36950)	• CountBright™ beads (C36950)	• CountBright™ beads (C36950)

discrimination (Figure 3). Cells must be stained for viability in the absence of extraneous protein. These reagents are ideal for researchers who want to fix samples before analysis and also maintain dead-cell discrimination during intracellular staining.

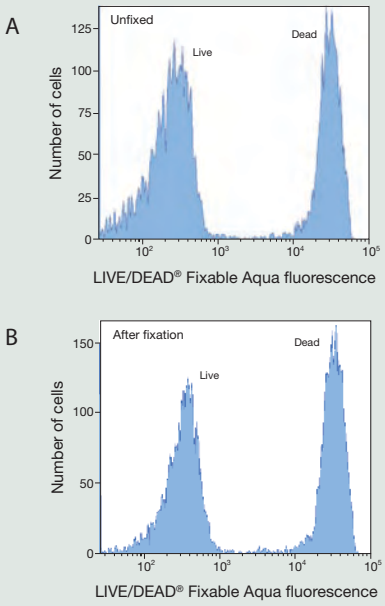

Figure 3—Use of fixable dead cell dyes. The reagents in the LIVE/DEAD® Fixable Aqua Stain Kit were used to differentially stain a mixture of live (left peak) and heat-treated Jurkat cells (right peak). The cells in **(A)** were not fixed; the cells in **(B)** were fixed in 3.7% formaldehyde following the staining reaction. Samples were analyzed by flow cytometry using 405 nm excitation and ~525 nm emission.

Indicators for metabolic, enzymatic, and other processes

Cells require metabolic and enzymatic functions to survive, and these activities are readily detected with a variety of fluorogenic reagents. General reductase activity can be measured with C_{12}-resazurin; esterase activity can be detected with calcein reagents (Figure 4), available for several excitation sources. These reagents are often paired with dead-cell dyes for better live/dead resolution. Although fluorescence is well retained, samples should be analyzed soon after staining. These reagents are not compatible with fixation protocols.

Monitoring mitochondrial membrane potential

Mitochondrial activity depends on an active membrane potential, which can change in response to a wide variety of environmental effects. Mitochondrial membrane potential dyes are sensitive indicators of cell stress.[1] These probes are generally positively charged molecules that sequester in the matrix of mitochondria that have active membrane potentials, causing a fluorescence increase or spectral shift. JC-1 is commonly used with apoptosis models. $DiIC_1(5)$ also allows membrane potential to be observed with red excitation, leaving 488 nm excitation available for other reagents (Figure 5). Due to the chemical properties of MitoTracker® Red dye, it becomes covalently linked to sulfhydryl moieties in active mitochondria, which in turn allows the stained sample to be fixed. JC-1, $DiOC_2(3)$, and $DiIC_1(5)$ are not compatible with fixation.

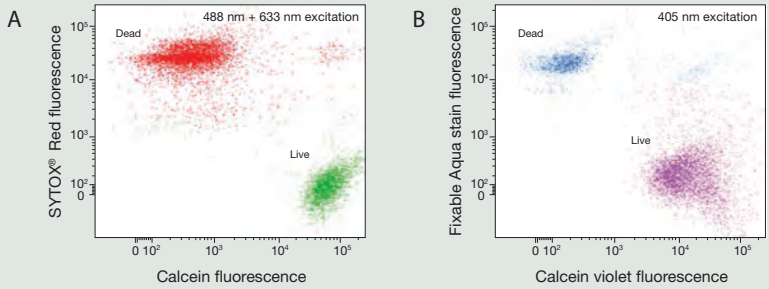

Figure 4—Combination of esterase substrates with dead-cell dyes using mixtures of heat-treated and untreated cells. (A) Jurkat cells were reacted with calcein, then stained with the impermeant DNA stain, SYTOX® Red dye, and analyzed by flow cytometry using 488 nm and 635 nm excitation. **(B)** Chinese hamster ovary (CHO) cells were stained according to the protocol in the LIVE/DEAD® Violet Viability/Vitality Kit (calcein violet and fixable violet dead-cell stain). Cells were analyzed by flow cytometry using 405 nm excitation.

Assessing cell proliferation

The proliferative state of a population of cells is an important parameter when studying live-cell function, particularly in cancer and drug-discovery research. Invitrogen offers several fluorescence-based kits and reagents for assessing cell proliferation (Table 1), including detection via nucleic acid stains, nucleoside incorporation, cell division, and overall cell counts.

Cell cycle profiles as a measure of proliferation

Detection of DNA content provides a snapshot of cells in a population that are in different stages of the cell cycle. Flow cytometry, in conjunction with modeling algorithms, provides a powerful tool to assess cells in G_0/G_1 phase versus S-phase, G_2, or polyploid.[2–4] DNA content is readily assessed in fixed cells with any of the impermeant DNA dyes, often requiring RNAse treatment. The DyeCycle™ dyes offer the ability to stain for DNA profile in live cells (Figure 6) with 405, 488 or 532 nm excitation. These dyes are generally used with a viability dye to exclude dead cells from the analysis. The dyes are not cytotoxic, allowing stained cells to be sorted and otherwise cultured or assessed with functional assays after staining.

Proliferation measured by nucleoside incorporation

Nucleoside incorporation provides direct measurement of new DNA synthesis. Traditionally, this has been performed by incorporating the nucleoside analog bromodeoxyuridine (BrdU) into DNA, followed by detection with an anti-BrdU antibody. Although effective, this method requires DNA denaturation (using HCl, heat, or DNase) to expose the BrdU to the antibody—a step that can be lengthy and difficult to perform consistently, and can adversely affect sample quality. The Click-iT™ EdU Cell Proliferation Assay eliminates the need to denature DNA, providing a superior alternative to the standard BrdU antibody-based method for measuring cell proliferation by flow cytometry. The Click-iT™ advantage is in the chemistry—small, unique, and bioorthogonal labeling and detection moieties that react very efficiently and specifically with one another. EdU (5-ethynyl-2′-deoxyuridine) is a nucleoside analog containing an alkyne. →

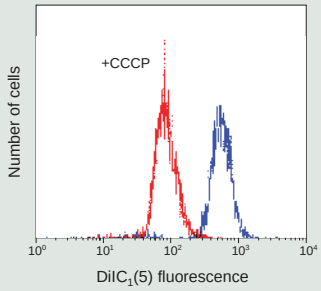

Figure 5—Decrease in $DiIC_1(5)$ fluorescence with the loss of mitochondrial membrane potential. Jurkat cells were stained with 50 nM $DiIC_1(5)$ alone (blue line) or in the presence of 50 µM CCCP (carbonyl cyanide 3-chlorophenylhydrazone, red line), used to disrupt mitochondrial membrane potential. Cells were analyzed by flow cytometry using 635 nm excitation and ~660 nm emission.

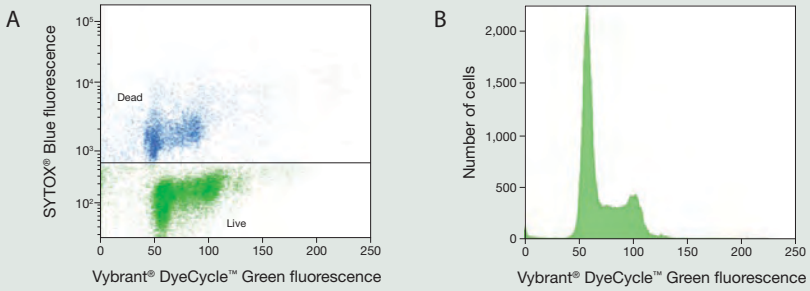

Figure 6—Viable cell gating with DyeCycle™ dyes. Jurkat cells from an overgrown culture were stained with Vybrant® DyeCycle™ Green stain, then stained with SYTOX® Blue dye and analyzed by flow cytometry using 488 nm and 405 nm excitation. The histogram **(B)** was gated on live cells **(A)** and shows DNA content distribution in live cells: G_0/G_1 and G_2/M phase histogram peaks are separated by the S-phase distribution. Inclusion of the dead cells would have given aberrant live-cell results.

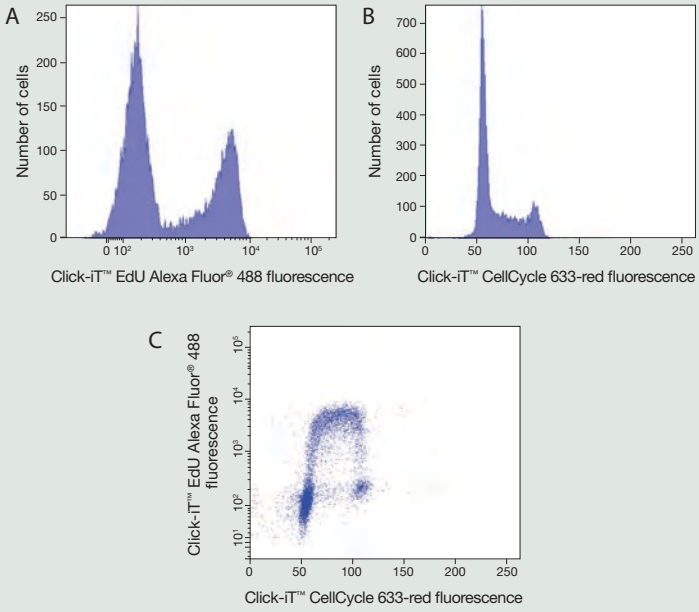

Figure 7—Cell proliferation using the Click-iT™ EdU Alexa Fluor® 488 Flow Cytometry Assay Kit. Jurkat cells were treated with 10 µM EdU for one hour and tested according to staining protocol. **(A)** Cell staining with Alexa Fluor® 488 azide using 488 nm excitation; clear separation of proliferating cells (which have incorporated EdU) and nonproliferating cells is demonstrated. **(B)** Cell staining with Click-iT™ CellCycle 633-red using a 633 nm excitation, showing DNA content distribution where G_0/G_1 and G_2/M phase histogram peaks are separated by the S-phase distribution. **(C)** Combination of the DNA content with the labeling of proliferating cells incorporating EdU; co-positive staining of cells provides the percentage of cells in S-phase (DNA synthesis).

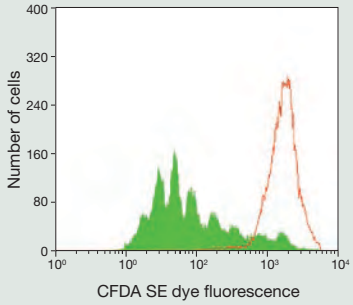

Figure 8—Human peripheral blood lymphocytes were harvested and stained with CFDA SE dye on Day 0. A portion of the population was arrested at the parent generation using mitomycin C (red peak). The rest of the sample was stimulated with phytohemagglutinin and allowed to proliferate for 5 days. Solid green peaks represent successive generations.

In a copper-catalyzed reaction, the alkyne reacts with a dye-labeled azide, forming a stable covalent bond. The small size of the azide reagents allows for efficient access to the DNA without the need for harsh cell treatment, thus simplifying the assay considerably, yet generating the same results (Figure 7). Click-iT™ EdU labeling is compatible with fixation protocols. For more details about Click-iT™ EdU, see pages 3–6.

New fixable dead-cell stains for your red laser

The LIVE/DEAD® Fixable Far Red and LIVE/DEAD® Fixable Near IR Dead Cell Stain Kits are some of the many Molecular Probes® products that Invitrogen has developed for red laser–equipped flow cytometers. The most up-to-date list can be found at **probes.invitrogen.com/products/flowcytometry**. Check back often to see what's new in flow cytometry tools.

Monitoring cell proliferation via generation analysis

Carboxyfluorescein diacetate, succinimidyl ester (5(6))-CFDA SE, also commonly called CFSE, spontaneously and irreversibly couples to cellular proteins by reaction with lysine side chains and other available amines. When cells divide, CFDA SE labeling is distributed equally between the daughter cells, and each successive generation in a population of proliferating cells is marked by a halving of cellular fluorescence intensity (Figure 8). Eight to ten successive generations have been identified this way.[5,6] It is also possible to perform multiplex analysis of CFDA SE and other markers to correlate cell division status with other cellular markers.[5,7,8]

To learn more about flow cytometry assays for assessing viability and vitality, visit **www.invitrogen.com/flowcytometry**. ■

References

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