Tracking cell division with flow cytometry

A guide to studying cell proliferation by generational tracing.

Cell proliferation assays provide a critical piece of the puzzle when evaluating cell health, cytotoxicity, and the efficacy of anti-cancer drugs. Moreover, much of our current knowledge of the immune system derives from the ability to initiate an *ex vivo* immune response in isolated T lymphocytes, triggering their proliferation. By labeling cells in a population with a fluorescent dye that is divided evenly between two daughter cells following cell division, researchers can quantify cell proliferation through multiple generations using a high-throughput fluorescence-based platform such as flow cytometry (Figure 1).

CFSE: The original cell tracer

Since its first reference as a cell tracing reagent in 1994 [1], carboxyfluorescein diacetate succinimidyl ester (CFDA SE, or CFSE) has been cited thousands of times in the literature for both *in vivo* and *in vitro* cell proliferation studies. CFSE is a cell-permeant, nonfluorescent dye that enters the cell by diffusion through the plasma membrane. Once inside the cell, this nonfluorescent molecule is converted to a fluorescent derivative by intracellular esterases. At the same time, the active succinimidyl ester group covalently binds to accessible protein amines, resulting in long-term retention of the fluorescent conjugate within the cell. Through subsequent cell divisions, daughter cells receive approximately half of the fluorescent label of their parent cells. Flow cytometric analysis of the fluorescence intensities of individual cells in a population permits the determination of the number of generations through which a cell has progressed since the label was applied. Despite its increased popularity over time, CFSE has a number of technical limitations that should be considered when choosing a fluorescent tracer for cell proliferation measurements.

CellTrace dyes: Improved cell tracers

Like CFSE, the dyes in the Invitrogen™ CellTrace™ Cell Proliferation Kits—including CellTrace Violet, CellTrace Yellow, and CellTrace Far Red—facilitate both *in vivo* and *in vitro* cell proliferation analyses by flow cytometry. CellTrace dyes easily cross the plasma membrane and covalently bind inside cells, where the stable, well-retained fluorescent conjugate offers a reliable fluorescent signal without significantly affecting morphology or physiology. Importantly, these dyes produce fluorescent staining with very little variation between cells within a generation, allowing each generation to be reliably distinguished. The intense fluorescent staining provided by CellTrace dyes permits the visualization of proliferating cells through a minimum of six generations, even after several days in a cell culture environment or following fixation. There are several criteria to consider when choosing the right cell tracer for an experiment, and the following sections focus on the most important technical factors. Also see page 30 for a summary of a recent journal article that discusses methods to assess cell tracing reagents.

Cell tracers must not be toxic to live cells

Cell tracers are used on living, dividing cells and therefore must not have a significant impact on cell health and viability. For example, the concentration of the traditional cell tracer CFSE must be carefully optimized for each cell line and culture condition because high concentrations of CFSE have been reported to impact cell viability and division, whereas low concentrations may limit the number of cell generations detected [1]. At the recommended working concentration of 5 µM, CFSE-labeled Jurkat cells had less than 15% viability after 6 days of staining (Figure 2). Titration studies of CFSE labeling of Jurkat cells demonstrated that concentrations between 0.5 µM and 1 µM were optimal for maintaining cell viability, and concentrations above 2 µM induced →

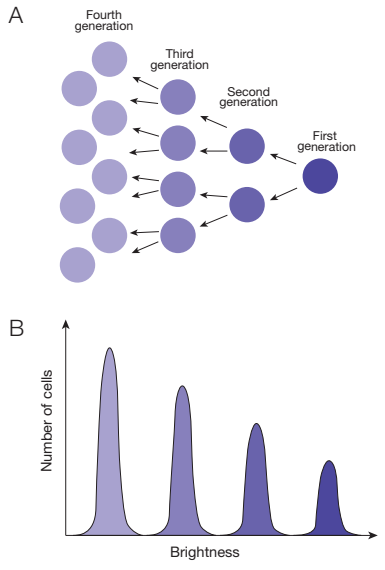

Figure 1. Proliferation analysis using a fluorescent cell tracer that partitions evenly between daughter cells. (A) Illustration of dye dilution over progressive cell divisions. **(B)** Flow cytometric analysis reveals a bright, homogeneous fluorescent signal from the initial population of cells. Subsequent cell divisions result in larger numbers of cells possessing half the fluorescence intensity of their parent cells.

toxicity (Figure 2A). In contrast, the other CellTrace dyes did not exhibit toxic side effects after 6 days of staining at the concentrations tested (Figure 2B). In particular, CellTrace Violet, CellTrace Yellow, and CellTrace Far Red showed minimal effects on cell viability at concentrations of 5 µM, 10 µM, and 1 µM, respectively, substantially increasing the window of effective reagent concentration for cell proliferation analysis.

Cell tracers must show minimal cell-to-cell dye transfer

CFSE is known to undergo nonspecific cell-to-cell dye transfer. This dye transfer decreases the observed resolution of cell generations, thereby reducing accuracy when identifying cell division populations. Although most succinimidyl esters also undergo membrane-exchange mechanisms that negatively affect peak resolution, CellTrace Violet dye is unique in that it shows minimal transfer between adjacent cells [2]. Consequently, CellTrace Violet dye provides better resolution between division peaks and therefore more accurate quantitation in generational analysis.

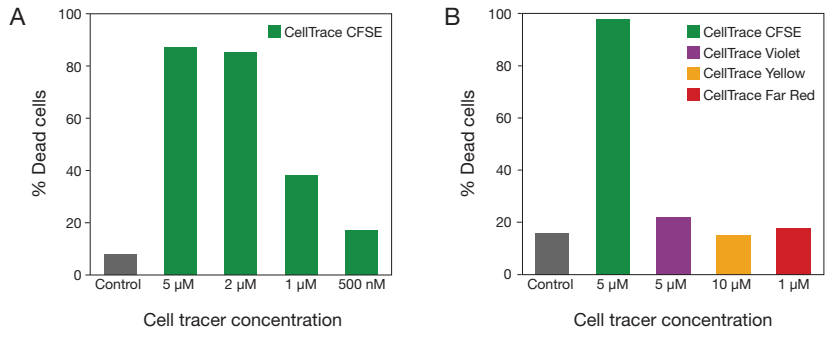

Figure 2. Viability analysis of Jurkat cells labeled with fluorescent cell tracers. (A) Jurkat cells were labeled with CellTrace™ CFSE (Cat. No. C34554) at concentrations between 5 µM and 0.5 µM for 20 min, diluted 5-fold with fresh culture medium (containing protein to bind any free dye), and incubated for 6 days before viability analysis using SYTOX™ Red Dead Cell Stain (Cat. No. S34859); control cells were incubated with 5 µM DMSO. **(B)** Jurkat cells were labeled with the recommended concentrations of CellTrace CFSE, CellTrace™ Violet (Cat. No. C34557), CellTrace™ Yellow (Cat. No. C34567), or CellTrace™ Far Red (Cat. No. C34564) dye, and then incubated for 6 days before viability analysis.

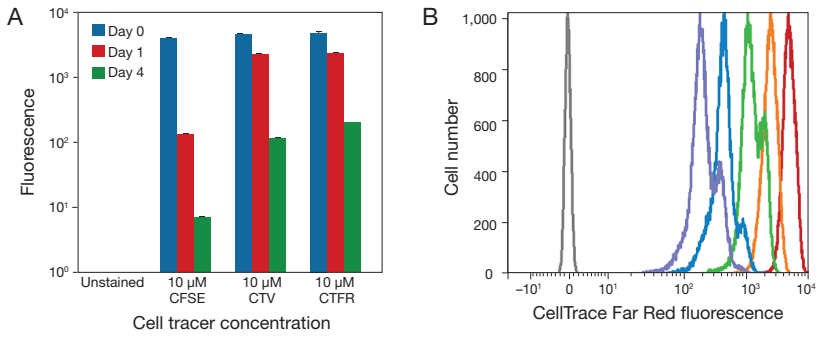

Figure 3. Monitoring the initial decrease in fluorescence for three cell tracers. (A) U937 cells were left unlabeled or labeled with CellTrace™ CFSE (Cat. No. C34554), CellTrace™ Violet (CTV, Cat. No. C34557), or CellTrace™ Far Red (CTFR, Cat. No. C34564) dye at a concentration of 10 µM and then analyzed on a BD™ LSR II flow cytometer on the indicated day after treatment. **(B)** Histogram showing representative fluorescence measurements for U937 cells labeled with CTFR. In this histogram, data from each successive day in the experiment are shown as follows: red = day 0, orange = day 1, green = day 2, blue = day 3, violet = day 4; for reference, the fluorescence distribution from unlabeled U937 is also presented on the overlay histogram (grey). Data used with permission from Joseph D. Tario Jr. and Paul K. Wallace, Roswell Park Cancer Institute, Buffalo, New York.

Cell tracers must exhibit bright and stable fluorescence in labeled cells

To enable detection of cell proliferation through many generations, the ideal cell tracer should produce a bright fluorescent signal that is still detectable after several rounds of division, despite the dilution of the signal with each successive generation. CFSE-labeled cells, however, show a significant decrease in fluorescence after the first 24 hours. It has been reported that this is likely due to the cells' clearing of short-lived proteins that were initially labeled with the dye [3]. Although this loss of fluorescence may not affect every experiment in which CFSE is used, it suggests that there will be less tracer available for generational analysis over time. In contrast, independent testing by Joseph Tario Jr. at the Roswell Park Cancer Institute demonstrates that, unlike CFSE, neither CellTrace Violet dye nor CellTrace Far Red dye shows the unwanted initial decrease in fluorescence (Figure 3).

Multiplexable cell tracers increase options in panel design

Cell proliferation assays are often used in concert with other cell function assays to provide a more informative picture of the state of the cell. Although widely used for proliferation analysis, CFSE (excitation/emission = 492/517 nm) requires one of the most popular flow cytometry channels—the same channel used by FITC, Alexa Fluor™ 488 dye, and GFP. In contrast, the CellTrace

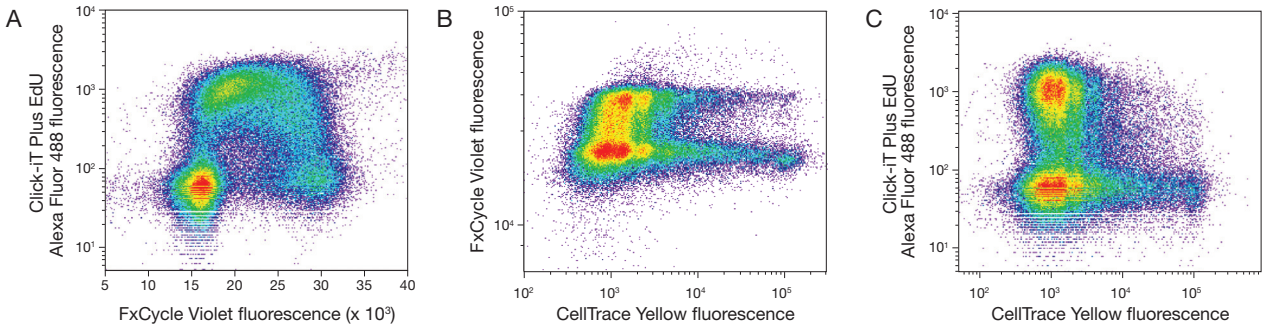

Figure 4. Multiplex flow cytometry analysis of cell proliferation. Human peripheral blood mononuclear cells isolated from whole blood were stained with 10 µM CellTrace™ Yellow dye (provided in the CellTrace Yellow Cell Proliferation Kit, Cat. No. 34567) and then resuspended in fresh medium. Stained cells were stimulated to proliferate using Dynabeads™ Human T-Activator CD3/CD28 for T Cell Expansion and Activation (Cat. No. 11161D) and incubated at 37°C and 5% CO_2 for 6 days. After incubation, cells were fed 10 µM EdU (provided in the Click-iT™ Plus EdU Alexa Fluor™ 488 Flow Cytometry Assay Kit, Cat. No. C10632) for 2 hr, washed, stained with LIVE/DEAD™ Fixable Near-IR Dead Cell Stain (Cat. No. L34975) for 30 min, washed, and fixed for 15 min in 4% formaldehyde. Cells were treated with the Click-iT reaction cocktail containing Alexa Fluor 488 azide for 30 min to complete the click reaction, labeled with APC mouse anti–human CD4 antibody for 20 min, and washed. Finally, cells were labeled with FxCycle™ Violet Stain (Cat. No. F10347) for 30 min and analyzed on a 4-laser Attune™ NxT Flow Cytometer (with 405, 488, 561, and 638 nm lasers, Cat. No. A24858). **(A)** This dual-parameter density plot shows newly synthesized DNA, as determined with the Click-iT Plus EdU assay, vs. DNA content, as determined with FxCycle Violet Stain. It is used to analyze the cell cycle phases of proliferating cells in the population. **(B)** This dual-parameter density plot shows DNA content, as determined with FxCycle Violet Stain, vs. progression of generations, as determined with CellTrace Yellow dye. This plot displays the progression of cell generations on the x-axis and DNA content on the y-axis. Cells that are not currently dividing (G_0/G_1) are shown in blue; cells from each generation that are actively synthesizing DNA or are about to divide are seen at the top of the plot. **(C)** This dual-parameter density plot shows the S-phase analysis provided by Click-iT EdU labeling vs. the generational analysis provided by CellTrace Yellow labeling. The parent-cell generation can be seen on the far right of the plot, with successive generations represented by clusters of dots to the left of the initial generation. Cells from each generation that are actively synthesizing DNA are seen at the top of the figure.

Violet, CellTrace Yellow, and CellTrace Far Red dyes are excited by the 405 nm, 532 (or 561) nm, and 633 (or 635) nm lasers, respectively. Not only do these tracers allow for greater flexibility in flow cytometry panel design, but each dye can be multiplexed with 488 nm–excitable green-fluorescent probes. Figure 4 shows a multiplex experiment that incorporates CellTrace Yellow dye for measuring cell proliferation, Invitrogen™ FxCycle™ Violet Stain for fixed-cell DNA content analysis, the Invitrogen™ Click-iT™ Plus EdU Alexa Fluor™ 488 Flow Cytometry Assay Kit for detecting newly synthesized DNA, Invitrogen™ LIVE/DEAD™ Fixable Near-IR Dead Cell Stain for assessing viability, and a fluorescent antibody conjugate for immunophenotyping. This multicolor analysis demonstrates the depth of information that can be gathered when additional functional reagents are used to assay the cell population of interest.

Explore the CellTrace Cell Proliferation Kits

When compared with traditional cell tracers such as CFSE, the CellTrace dyes provide significant advantages, including their low toxicity and bright and stable fluorescence. In particular, CellTrace Violet dye has been cited in several comparative studies as superior to CFSE for cell proliferation analyses [2,4]. Learn more about the entire line of CellTrace Cell Proliferation Kits at thermofisher.com/celltracebp73. ■

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References

1. Lyons AB, Blake SJ, Doherty KV (2013) *Curr Protoc Cytom* Chapter 9:9.11.1–9.11.12.
2. Filby A, Begum J, Jalal M et al. (2015) *Methods* 82:29–37.
3. Wallace PK, Muirhead KA (2007) *Immunol Invest* 36:527–561.
4. Quah BJ, Parish CR (2012) *J Immunol Methods* 379:1–14.

Product	Laser type	Ex/Em*	Quantity	Cat. No.
CellTrace™ Violet Cell Proliferation Kit, for flow cytometry	405 nm	405/450	180 assays 20 assays	C34557 C34571
CellTrace™ Yellow Cell Proliferation Kit, for flow cytometry	532 or 561 nm	546/579	180 assays	C34567
CellTrace™ CFSE Cell Proliferation Kit, for flow cytometry	488 nm	492/517	180 assays 20 assays	C34554 C34570
CellTrace™ Far Red Cell Proliferation Kit, for flow cytometry	633 or 635 nm	630/661	180 assays 20 assays	C34564 C34572

*Fluorescence excitation (Ex) and emission (Em) maxima, in nm.