A Novel Non-Cytotoxic Fluorescent Dye for Cell Proliferation Analysis in Flow Cytometry

Penny Melquist and Mike O'Grady
Thermo Fisher Scientific, 29851 Willow Creek Road, Eugene, OR, USA, 97402

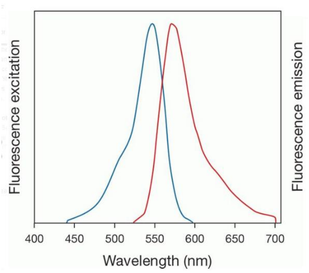
INTRODUCTION

Cell proliferation analyses are crucial for cell growth and differentiation studies, and are often used to evaluate compound toxicity and/or inhibition of tumor cell growth during drug development. Tools for measuring cell proliferation include probes for analyzing the average DNA content and cellular metabolism in a population, as well as single-cell indicators of DNA synthesis and cell cycle–specific proteins, and tracking population doubling by dye dilution. Successful proliferation analysis by dye dilution requires an extremely bright dye to distinguish fluorescently labeled cells after several cell divisions. The intense fluorescent staining provided by CellTrace™ Yellow dye enables the visualization of six or more generations of proliferating cells. CellTrace Yellow reagent covalently binds intracellular proteins where the stable, well-retained fluorescent dye offers a consistent signal, even after several days in cell culture. Division of a cell labeled with a CellTrace Yellow reagent results in equal partitioning of dye between daughter cells and approximately half of the fluorescence intensity in the progeny. When analyzed by flow cytometry using either a 532 nm or 561 nm laser, this partitioning of the CellTrace Yellow dye provides a direct indication of cell proliferation without compromising cell health. In this study, we demonstrate that the spectral properties of CellTrace Yellow reagent permits multiplexing with many common flow reagents (cell cycle, viability, proliferation, traceability, and resolution) excited by violet, green, yellow, and red laser lines without introducing cytotoxicity.

MATERIALS AND METHODS

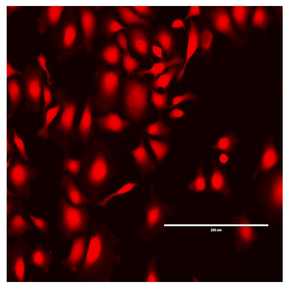
Human peripheral blood mononuclear cells were isolated from whole blood using a Ficoll-Paque Plus density gradient. Cells were stained with 10 µM CellTrace Yellow reagent and resuspended in OpTmizer™ T-Cell Expansion media. Stained cells were stimulated to proliferate with 50 µL CD3/CD28 Dynabeads per milliliter cells and incubated at 37° C and 5% CO_2 for 6 days. After six days, cells were fed 10 µM EdU for two hours. Cells were then washed and stained with LIVE/DEAD™ Fixable Near-IR Dead Cell Stain for 30 min, washed, and fixed for 15 minutes in 4% formaldehyde. Cells were treated with Click-iT™ saponin-based perm for 20 minutes. The Click-iT Reaction Cocktail containing Alexa Fluor™ 488 azide was added for 30 minutes to complete the Click reaction. Cells were labeled with 5 µL mouse anti-human CD4 APC for 20 minutes and washed. Finally, cells were labeled with FxCycle™ Violet for 30 min and analyzed on a Attune™ NxT Flow Cytometer with 405 nm, 488 nm, 561 nm, and 638 nm lasers.

Figure 1. CellTrace Yellow Excitation and Emission Spectra



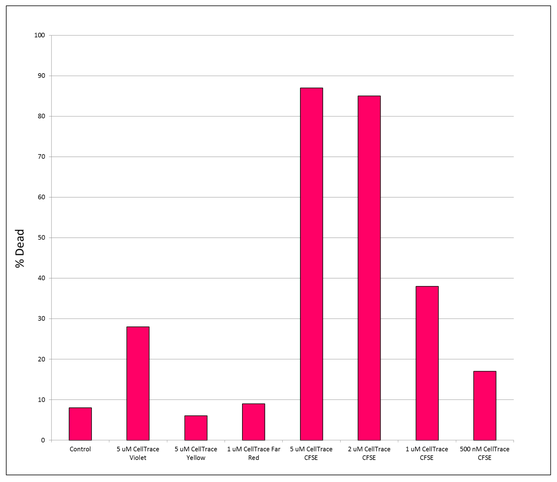
CellTrace Yellow reagent is excited by 532 nm and 561 nm lasers

Figure 2. CellTrace Yellow Uniform Staining



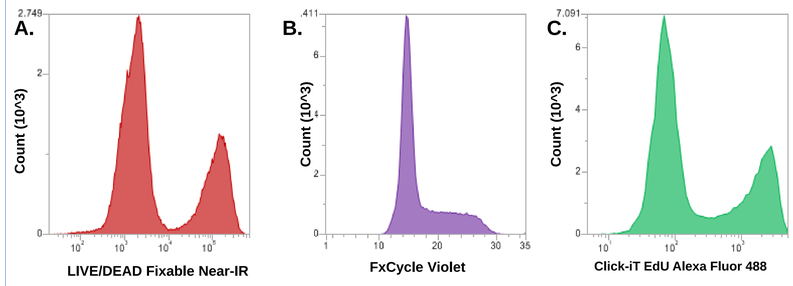
HeLa cells were labeled with 10 µM CellTrace Yellow reagent and incubated for 1 hr at 37° C / 5% CO_2. After 1 hr. cells were washed and imaged in Live Cell Imaging Solution using the EVOS-FL imaging system and a 20X objective.

Figure 3. CellTrace Yellow Reagent Cytotoxicity



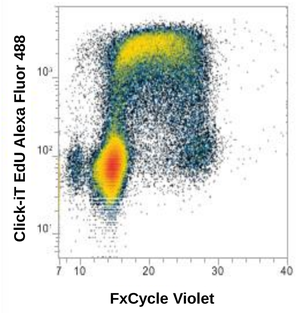
Jurkat cells were labeled with 5 µM CellTrace Violet reagent, 10 µM CellTrace Yellow reagent, 1 µM CellTrace Far Red reagent, & 5 µM, 2 µM, 1 µM, and 500 nM CellTrace CFSE reagent. Samples were incubated in RPMI + 10% FBS Medium at 37° C / 5% CO_2 for 6 days. After 6 Days, samples were harvested and analyzed in complete media without washing. Samples were analyzed using SYTOX™ Green or SYTOX Red dead cell stains on the Attune NxT Flow Cytometer at 200 µL/ min. % dead in CellTrace Yellow, Far Red, and Violet reagents show minimal toxicity. CellTrace CFSE reagent demonstrates significant toxicity until you reach 500 nM.

Figure 4. Multiplexing Cell Cycle and Proliferation with CellTrace Yellow



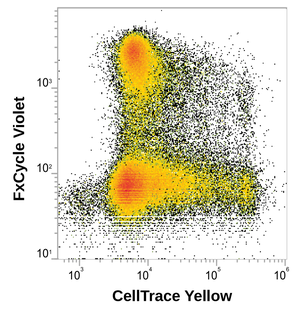
Proliferating human lymphocytes were stained with 10 µM CellTrace Yellow reagent, grown in culture for six days, harvested, and multiplexed to look at cell cycle, proliferation, and viability. Fluorescent histograms show (A) LIVE/DEAD Fixable Near-IR Dead Cell Stain was used to identify live cells. (B) DNA content analysis using FxCycle Violet depicts G_0/ G_1 and G_2/M phase histogram peaks separated by the s-phase distribution, (C) Click-iT Plus EdU Alexa Fluor 488 was used to analyze DNA replication in proliferating cells. Samples were acquired and analyzed on the Attune NxT Acoustic Flow Cytometer at 200 µL/min.

Figure 5. DNA Content with S-phase Identification



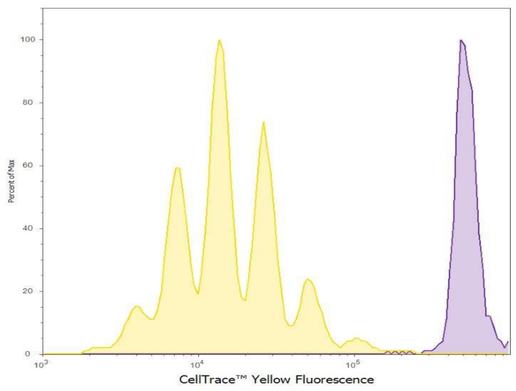
The DNA content cell cycle dye FxCycle Violet easily crosses the plasma membrane of fixed and permeabilized cells, where it binds to DNA and becomes fluorescent. The fluorescent signal is proportional to the quantity of DNA in each cell, so that cells with a double complement of DNA will be twice as bright as those that have not duplicated their DNA. The thymidine analog 5-ethynyl-2´-deoxyuridine (EdU), is incorporated into cells that are actively duplicating DNA. A copper-catalyzed Click reaction is used to covalently link Alexa Fluor 488 azide to EdU, resulting in fluorescently labeled DNA. This figure combines DNA content analysis with Click-iT EdU S-phase labeling to provide a complete cell cycle depiction of the proliferating cells in this experiment. Samples were acquired and analyzed on the Attune NxT Acoustic Flow Cytometer at 200 µL/min.

Figure 6. Cell Cycle with Generational Analysis



The DNA content information acquired from FxCycle Violet can be combined with generational information provided by CellTrace Yellow reagent to give a detailed view of the growth of a population of cells. This figure displays the progression of cell generations on the x-axis and DNA content on the y-axis. Cells which are not currently dividing (G_0/G_1) are shown in closest to the X-axis Cells from each generation which are actively synthesizing DNA or are about to divide are seen at the top.. Samples were acquired and analyzed on the Attune NxT Acoustic Flow Cytometer at 200 µL/min.

Figure 7. Generational Tracing of CellTrace Yellow



Human peripheral blood mononuclear cells were stained with 10 µM CellTrace Yellow reagent. Dynabeads Human T-Activator CD3/CD28 were used for T Cell Expansion and Activation. Samples were incubated in OpTmizer T-cell Expansion Medium at 37° C / 5% CO_2 for 7 days. After 7 days, cells were removed from culture and Dynabeads were removed from samples. SYTOX Green Dead Cell Stain was used to exclude dead cells and mouse anti-human CD4 APC was used to gate on live $CD4^+$ lymphocytes. The peak in purple represents unstimulated control cells. The discrete yellow peaks represent individual generations of cells which proliferated during the course of the experiment.

CONCLUSIONS

Results indicate that CellTrace Yellow reagent can be used to successfully track multiple generations of proliferating cells. The bright, uniform staining produces minimal fluorescence variation between cells in a population, resulting in the ability to visualize resolve distinct generations by flow cytometry. The yellow excitation at 546 nm and emission at 579 nm of CellTrace Yellow dye make it ideal for multiplexing due to the limited spectral overlap with other common dyes. Results of this experiment suggest that CellTrace Yellow reagent is a useful tool to track and characterize cells as they proliferate through several generations and shows little cytotoxicity, with minimal observed effect on the proliferative ability or biology of cells.

REFERENCES

1. Wallace, et al. Cytometry Part A 73A: 1019-1034, 2008.
2. Lyons AB, Parish CR. *Journal of Immunological Methods* 1994; 171:131-37.
3. Givan AL, et al. *Journal of Immunological Methods* 1999; 230:99-112.

TRADEMARKS/LICENSING

© 2016 Thermo Fisher Scientific Inc. All rights reserved. All trademarks are the property of Thermo Fisher Scientific and its subsidiaries unless otherwise specified.
Ficoll-Paque is a trademark of GE Healthcare

For research use only. Not for use in diagnostic procedures.