Functional Characterization of a Novel Fluorescent Dye for Proliferation Analysis

Chris L. Langsdorf, Jolene Bradford, Gayle Buller, Nick Dolman, Kyle Gee, Hee Chol Kang, and Jixiang Liu
Life Technologies, 29851 Willow Creek Road, Eugene, OR, 97402 USA

ABSTRACT

The current study was undertaken to evaluate the performance of a new violet-excitable tracing dye in several multicolor applications.

INTRODUCTION

A class of fluorescent compounds known as cell tracing dyes has been used for several years to evaluate the proliferation and growth of cells *in vivo* and *in vitro*. Dyes of this category are available in many different varieties, but they all share two common characteristics: stability and extremely bright staining. The popular tracing dye carboxyfluorescein diacetate succinimidyl ester (CellTrace™ CFSE) quickly enters live cells where it covalently binds and is cleaved to create very bright, long-lasting fluorescence. Though CellTrace™ CFSE can be effectively used to track cell populations, its spectral properties prevent it from being combined with many popular fluorophores, such as Alexa Fluor® 488, R-Phycoerythrin (RPE), and Green Fluorescent Protein (GFP). This study will attempt to demonstrate that the spectral properties of CellTrace™ Violet permit multiplexing with many common flow reagents, such as GFP, RPE, and Alexa Fluor® 488.

MATERIALS AND METHODS

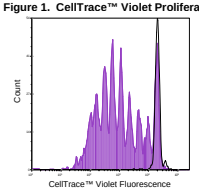
Human peripheral blood mononuclear cells were isolated from whole blood using a Ficoll-Paque® Plus density gradient (GE Healthcare). For the first part of the experiment, cells were stained with 10 µM CellTrace™ Violet and resuspended in OpTmizer™ T-Cell Expansion Buffer (GIBCO) containing L-glutamine, penicillin and streptomycin. Stained cells were stimulated to proliferate with 200 ng mouse anti-human CD3 antibody (clone S4.1) and 100 ng Interleukin-2 (IL-2) per milliliter cells and incubated at 37°C and 5% CO_2 for 7 days. Mouse anti-human CD8 R-phycoerythrin and SYTOX® AADvanced™ Dead Cell Stain were used to gate on live CD8+ lymphocytes. Cells were analyzed on a Becton Dickinson (BD™) LSRII flow cytometer and proliferation analysis was performed with ModFit LT™ (Verity Software House).

For the second part of the experiment, cultured human osteosarcoma cells were transduced with GFP and stained with 5 µM CellTrace™ Violet. Cells were analyzed on a BD™ LSRII flow cytometer and a DeltaVision® Pro fluorescence microscope using standard FITC and DAPI channels.

For the third part of the experiment, proliferating human lymphocytes were stained with 10 µM CellTrace™ Violet, stimulated with mouse anti-human CD3 and IL-2, and grown in culture as previously described. After seven days, cells were fed 10 µM EdU (5-ethynyl-2´-deoxyuridine), a modified nucleoside, for two hours. Cells were then stained with LIVE/DEAD® Fixable Yellow Dead Cell Stain, washed with media, fixed 30 minutes in 4% formaldehyde, and treated with Click-iT® saponin-based perm for 20 minutes. Click-iT® Reaction Cocktail containing Alexa Fluor® 488 azide was added for 30 minutes to complete the Click reaction. Cells were run on a BD™ LSRII flow cytometer with 405 nm, 488 nm, 532 nm, and 633 nm lasers and analyzed with BD FACSDiva™ 6.0 and Modfit LT™ software.

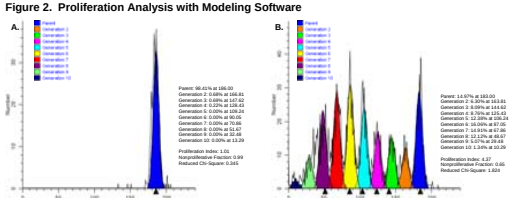
RESULTS

Figure 1. CellTrace™ Violet Proliferation Analysis



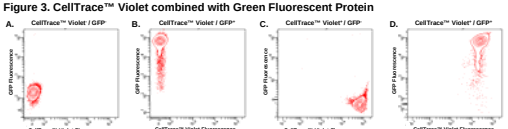
Human peripheral blood mononuclear cells were stained with 10 µM CellTrace™ Violet and incubated in OpTmizer™ T-cell Expansion Medium at 37°C / 5% CO_2 for 7 days. SYTOX® AADvanced™ and mouse anti-human CD8 RPE were used to gate on live $CD8^+$ lymphocytes. The peak outlined in black represents control cells which were not stimulated to divide. The discrete violet peaks represent individual generations of cells which divided several times after being stimulated with mouse anti-human CD3 and IL-2. Unstimulated cells labeled with CellTrace™ Violet had very little change in fluorescence intensity over the course of the experiment. Stained cells had a very low level of cell death which was similar to unstained cells. Stimulated cells were observed to proliferate through several generations during the course of the experiment, with each generation visible as a discrete peak on a fluorescence histogram.

Figure 2. Proliferation Analysis with Modeling Software



Human $CD8^+$ lymphocytes were stained with 10µM CellTrace™ Violet and incubated in OpTmizer™ T-cell Expansion Medium at 37°C for 7 days. (A) Unstimulated cells. (B) Cells stimulated with 200ng mouse anti-human CD3 antibody and 100ng IL-2 per milliliter cells. Proliferation analysis was performed with ModFit LT™ software. The modeling software uses a non-linear least squares analysis to estimate the location of each generation of cells and calculate statistics to quantify the proliferation.

Figure 3. CellTrace™ Violet combined with Green Fluorescent Protein



Demonstration of the spectral compatibility of CellTrace™ Violet with GFP in cultured osteosarcoma cells. (A) Unstained cells without GFP expression. (B) Unstained cells stably expressing GFP. (C) 5 µM CellTrace™ Violet stained cells without GFP expression. (D) GFP expressing cells stained with 5 µM CellTrace™ Violet. The fluorescence excitation and emission properties of GFP and CellTrace™ Violet permit the simultaneous use of these two reagents.

Figure 4. CellTrace™ Violet combined with Cellular Lights® Talin-GFP

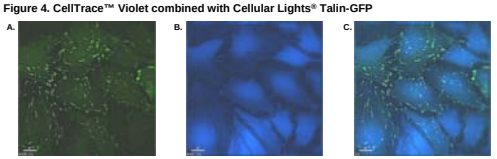
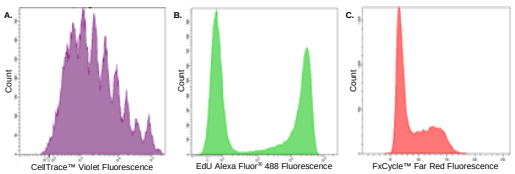


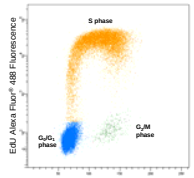
Figure 3 – Fluorescence microscopy demonstration of the spectral compatibility of CellTrace™ Violet with Green Fluorescent Protein. Cultured osteosarcoma cells were transduced with Cellular Lights® Talin-GFP, stained with 5 µM CellTrace™ Violet, and imaged on a DeltaVision® Core fluorescence microscope. (A) When imaged with a standard FITC filter only the GFP signal is detected. (B) When imaged with a standard DAPI filter only the CellTrace™ Violet fluorescence is detected. (C) When imaged with both FITC and DAPI filters the Cellular Lights® Talin-GFP and CellTrace™ Violet signals are both detected.

Figure 5A. Multiplexed Cell Cycle and Proliferation Analysis



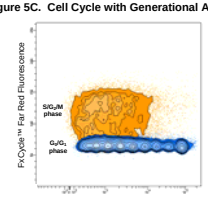
Proliferating human lymphocytes were stained with 10 µM CellTrace™ Violet and grown in culture for seven days. Live/Dead® Fixable Yellow Dead Cell Stain and mouse anti-human CD4 RPE were used to identify live $CD4^+$ cells. A gating strategy was employed to eliminate debris and doublets from analysis. Cells were stained with FxCycle™ Far Red for to evaluate DNA content. Click-iT® EdU Alexa Fluor® 488 labeling was used to identify cells in S phase of the cell cycle. Fluorescence histograms show (A) several generations of proliferating cells stained with CellTrace™ Violet, (B) s-phase analysis as indicated by Click-iT® EdU Alexa Fluor® 488 dye, (C) DNA content analysis with FxCycle™ Far Red showing cells in G_0/G_1, S, and G_2/M phases of the cell cycle.

Figure 5B. DNA Content with S-phase Identification



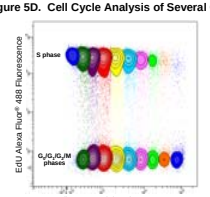
The cell cycle dye FxCycle Violet™ easily crosses the plasma membrane of permeabilized cells, where it binds to DNA and becomes highly fluorescent. The fluorescent signal is proportional to the quantity of DNA in each cell, so that cells with a double complement of DNA will be twice as bright as those 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.

Figure 5C. Cell Cycle with Generational Analysis



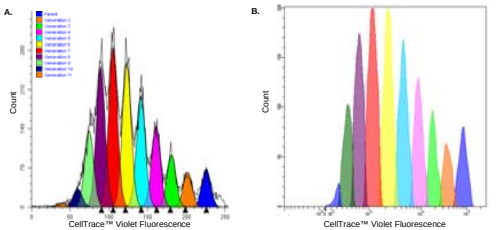
The DNA content information acquired from FxCycle™ Far Red can be combined with generational information provided by CellTrace™ Violet to give a detailed view of the growth of a population of cells. This figure displays the progression of cell generations on the x-axis and DNA content on the y-axis. Cells which are not currently dividing (G_0/G_1) are shown in blue. Cells from each generation which are actively synthesizing DNA or are about to divide are seen at the top of the figure in orange.

Figure 5D. Cell Cycle Analysis of Several Generations



The S-phase analysis provided by Click-iT® EdU can be combined with CellTrace™ Violet to provide more information about the cells in this experiment. This dual-parameter contour plot shows s-phase analysis as indicated by Click-iT® EdU Alexa Fluor® 488 fluorescence on the y-axis. The parent generation of cells 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 which are actively synthesizing DNA are seen at the top of the figure.

Figure 5E. Software Model Compared to Fluorescence Histogram



(A) The proliferation module in ModFit LT™ analysis software was used to model each generation of proliferating cells in this experiment. Generations are indicated by the colored peaks. Unlike many cell tracing dyes, CellTrace™ Violet can often be used to visualize individual generations of cells without the need for modeling software. (B) This fluorescence histogram was created in FACSDiva™ by placing a unique gate on each generation of proliferating cells.

CONCLUSIONS

Experimental results indicate that CellTrace™ Violet can be used to successfully track several generations of proliferating cells. The extremely bright, homogenous staining produces very little fluorescence variation between cells in a population, resulting in the ability to visualize distinct generations in a fluorescence histogram, even without complex modeling software. CellTrace™ Violet was found to be spectrally compatible with several blue, green, and red excitable fluorophores including Alexa Fluor® 488 dye, R-Phycoerythrin, PE-Cy7 tandem, Green Fluorescent Protein, LIVE/DEAD® Fixable Yellow Dead Cell Stain, SYTOX® AADvanced™ Dead Cell Stain, and FxCycle™ Far Red stain. Results of this experiment suggest that CellTrace™ Violet provides an excellent tool to track and characterize cells as they proliferate through several generations.

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.

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TRADEMARKS/LICENSING