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APPLICATION NOTE

CellTrace™ Far Red & CellTracker™ Deep Red—long term live cell tracking for flow cytometry and fluorescence microscopy

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Abstract Long term monitoring of live cells *in vitro* or *in vivo* presents considerable challenges. Fluorescent tracers are used as one of the most common methods. However, fluorescent molecules usually are limited by cytotoxicity and/or lack of persistence within live cells over sufficient time periods. In this study, we evaluated our newly developed cell tracing/tracking reagents, CellTrac Far Red and CellTracker Deep Red, as novel red laser-excitable fluorescent dyes for flow cytometry and live cell imaging applications. These dyes cross the plasma membrane and covalently bind proteins inside cells, where the stable, well-retained fluorescent label offers a consistent, reliable fluorescent signal without affecting morphology or physiology and also exhibiting low cellular toxicity. CellTrace Far Red was demonstrated to be an excellent probe for cellular proliferation studies using flow cytometry, allowing measurement of up to eight generations of proliferating cells. CellTracker Deep Red was shown to be an excellent reagent for the study of cell movement, location and morphology by labeling and imaging live-cells with fluorescence microscopy. In addition, CellTracker Deep Red has excellent cell retention characteristics with bright fluorescence intensity even after 72 hours of cell staining, with signal retained after fixation, and can be multiplexed with other fluorescent dyes.

Keywords: proliferation; cell tracking; flow cytometry; Celltrace; CellTracker; multiplex; red fluorescence; microscopy.

INTRODUCTION

There has been a high demand for the development of long-term cellular tracers/trackers due to their pivotal roles in monitoring a wide range of biological processes, pathological pathways, therapeutic effects, etc. over an extended period of time [1-3].

Fluorescent cell tracking dyes have been widely used as live cell imaging reagents, in particular for studies of cell proliferation, migration, chemotaxis, and invasion [4]. Essential requirements for using them as probes for cellular tracking include an efficient labeling of the cells with dyes, their retention within the cells of interest, nontoxicity with live cells and the stability of the label in the cellular micro-environment. Several types of fluorescent dyes fall into this category: lipophilic dyes that hydrophobically insert into the plasma membrane, thiol reactive chloromethyl analog dyes which react with intracellular thiols, and amine reactive dyes which covalently bind to amine group of proteins inside cells. Of all these compounds, carboxyfluorescein diacetate succinimidyl ester (CFDA SE) has become the gold standard for studying immune cell proliferation, differentiation, and function using flow cytometric analysis [5].

CFDA SE enters cells by passive diffusion and its acetate moieties are cleaved by intracellular esterase enzymes to form a fluorescent, amine reactive product, *i.e.* carboxyfluorescein succinimidyl ester (CFSE). After internalization, a bright homogeneous fluorescence is observed and the probe rapidly and irreversibly couples to intracellular lysine side chains and other amine containing components; thus it can tag living cells for long periods. When cells divide, CFDA SE labeling is distributed equally between daughter cells, which are therefore half as fluorescent as the parents. As a result, each successive generation in a population of proliferating cells is marked by a halving of cellular fluorescent intensity with excitation/ emission maxima of 492/517 nm, that is readily detected by a flow cytometer. CFDA SE produces a more homogeneous cellular labeling and, consequently, much better intergenerational resolution than other cell tracking dyes, such as the membrane marker PKH26 [6]. Using flow cytometric analysis of CFDA SE labeling, 8 to 10 successive generations of lymphocytes can be resolved [7]. The signal of CFDA SE can be traced *in vivo* for weeks in transplanted cells [8].

Despite the salutary characteristics of CFDA SE, 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). In order to free up the green channel for multi-color applications, CellTrace Violet, which is functionally analogous to CFDA SE and optimally excited by a 405 nm violet diode laser with blue emission, has recently become commercially available [4]. CellTrace Violet has been successfully used in multicolor flow cytometric analysis of cells in combination with fluorescent antibody conjugates [9]. Still there have been increasing demands for cell tracking dyes which can be excited by the red laser (633 nm) with far-red emission due to reduced harm to live cells and multiplexing capability with other popular reagents,

in particular with GFP.

In response, we recently developed CellTrace Far Red, which is a red laser excitable dye with narrow far-red emission (excitation/emission max of 630/660 nm) as an alternative probe to CFDA SE and CellTrace™ Violet. Here we describe the application of this new reagent, as an excellent probe for cellular proliferation studies using flow cytometry. CellTrace Far Red produces a stable and well retained fluorescent signal with very little variance between cells within generations, allowing visualization of proliferating cells up to 8 generations.

In addition, we have developed a novel CellTracker Deep Red dye, which is red laser excitable, nontoxic to living cells at working concentrations, well retained in live cells and brightly fluorescent in physiological environments. Here, we also describe its utility for the study of cell movement, location and morphology by labeling and imaging live-cells with fluorescence microscopy including multi-color applications. CellTracker Deep Red has excellent cell retention characteristics with bright fluorescence intensity even after 72 h of cell staining.

MATERIALS AND METHODS

Peripheral blood mononuclear cell isolation

Whole blood (20 ml) was added to phosphate buffer saline (PBS, 20 ml) and mixed well. Ficoll-Paque™ Plus (15 ml, GE Healthcare) was added to each of two 50 ml centrifuge tubes. The diluted whole blood (20 ml) was carefully layered on top and centrifuged for 30 min at 400 × *g* with the brake off. Peripheral blood mononuclear cell (PBMC) layer was carefully removed. Cells were resuspended in PBS (25 ml) in a 50 ml centrifuge tube and were centrifuged for 5 min at 400 × *g*. The supernatant was decanted and the cells were resupended in PBS (10 ml). Then cells were counted on the Countess Automated Cell Counter (Thermo Fisher Scientific). The final concentration of cells was adjusted to1 × 10^6 cells/ml in PBS.

Labeling procedure with CellTrace Far Red

CellTrace Far Red was made at concentration of 5 µM (20 µl, anhydrous DMSO). The prepared cells (10 ml) in a 50 ml of centrifuge tube were centrifuged at 400 × *g* for 5 min. The supernatant was carefully removed. CellTrace Far Red staining solution (10 µl) was added to the cells suspended in PBS (10 ml) and incubated for 20 minutes in a 37°C water bath, protected from light. Occasional swirling the cells to prevent settling helped to produce more uniform labeling. OpTmizer™ T Cell Expansion SFM medium (40 ml, Thermo Fisher Scientific) was added to the cells to adsorb any unbound dye. After incubation for 5 min at room temperature, protected from light, the cells were centrifuged for 5 min at 400 × *g*, then pouring off the supernatant. The cell pellet was resuspended in a pre-warmed OpTmizer™ T Cell Expansion SFM medium (10 ml).

Stimulating and Culturing T lymphocytes for proliferation analysis

The stained cells were distributed into a 24-well culture plate (at least 1 ml per well). Each 1 ml aliquot of cells was stimulated by adding 100 ng IL-2 (1 µl of 0.1 mg/ml solution), adding resuspended CD3/CD28 Dynabeads Human T-Activator (Thermo Fisher Scientific) to a 12 × 75 mm flow cytometry tube (50 µl of beads for each milliliter of cells to be stimulated), adding the same volume of PBS, or at least 1 ml, and mixing. The tube was placed in the Dynamag-15 magnet for 1 min. The supernatant was decanted from the tube, leaving the beads bound to the magnet. The tube was removed from the Dynamag-15 magnet and the Dynabeads were resuspended in the same volume of PBS as the initial volume of Dynabeads. CD3/CD28 Dynabeads Human T-Activator CD3/CD28 T cell expander beads (2 beads per cell) were added and the cells were incubated at 37°C and 5% CO_2. Every other day in culture, 1 ml of Optimizer CTS T-Cell Expansion SFM medium added. After 5 days of incubation, 2 ml of each sample were removed and transferred to separate tubes. The remaining samples were left in the plate to continue to proliferate. The tube was placed in the Dynamag-15 magnet for 1 min and the supernatant was removed. The cells were pelleted by centrifuging 5 min at 400 × g and decanting the supernatant. The cell pellet was resuspended in 5 ml PBS with 10 % FBS and spun for 5 min at 400 × *g*. The supernatant was decanted. The cells were counted on a Countess Automated Cell Counter (Thermo Fisher Scientific) and the concentration was adjusted to 1 × 10^6 cells/ml in PBS.

Flow cytometry data collection

The stained cells were distributed into culture plates, stimulated with 50 µl Dynabeads® Human T-Activator CD3/CD28 per 1 ml of cells, and incubated for desired length of time (4 days) under growth conditions. The cells were harvested and analyzed using the Attune flow cytometer with 633/635 nm excitation and a 660/20 bandpass emission filter.

Cell culture for fluorescence microscopy experiments

The human A549 cell line was obtained, maintained and cultivated twice weekly in MEM medium (GIBCO, Carlsbad, CA) with fetal bovine serum at a final concentration of 10%. Cells were cultured in 5% CO_2 at 37ºC, and subcultures were made with divalent-free phosphate-buffered saline (PBS) rinse followed by dissociation with TrypLE™ Express (Thermofisher Scientific). A Countess ™ automated cell counter (ThermoFisher Scientific) was used to quantitate the cell numbers before plating into 96-well microplates**.**

Fluorescence microscopy

For fluorescent imaging, the EVOS Auto FL microscope (ThermoFisher Scientific) was used. The Cy5 LED cube was used with an excitation wavelength of 655 nm and a 46 nm bandwidth and emission wavelength of 692 nm with a 40 nm bandwidth.**.**

RESULTS AND DISCUSSION

CellTrace Far Red labels lymphocytes, tracking eight generations of cell division

Methods for monitoring cell proliferation, differentiation and function using flow cytometry have enabled investigation of complex biological phenomena, in particular characterization of complex antigen driven immunological responses [10]. Using a fluorescent label that is divided evenly between daughter cells following cell division, researchers can detect and quantify this cell proliferation through multiple generations [11]. In order to assess the capacity of CellTrace Far Red as a reagent to monitor cell proliferation by flow cytometry, fresh peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Paque density gradient centrifugation and stained with 5 µM of the dye, then cells were stimulated with anti hCD3 (clone UCHT1) and supplemented with recombinant hIL2. After proliferation for 3 to 5 days, samples were stained with a viability dye (SYTOX Green) and analyzed by Attune® flow cytometry. When cells were dividing, CellTrace Far Red distributed equally into daughter cells. When cell staining was performed quickly,

the rapid reactivity of CellTrace Far Red made it possible to label a population of cells with a low variation of fluorescence, which resulted in clear visualization of eight discrete fluorescent peaks tracking each cell division as shown in **Figure 1**.

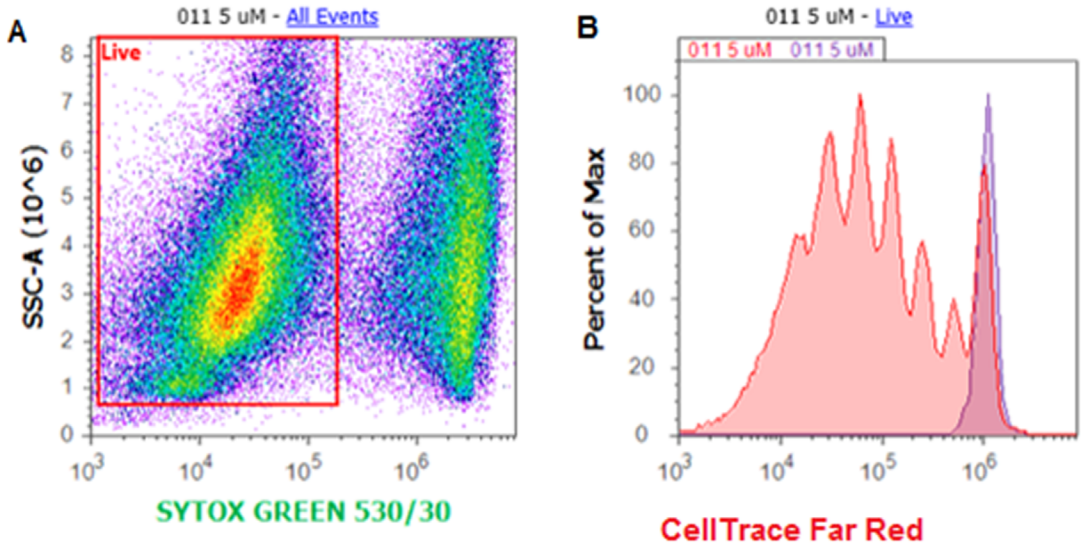

Figure 1. Generational tracing using CellTrace Far Red. A. Proliferation of human T lymphocytes stimulated by anti-hCD3 antibody and cultured for 5 days. The gating strategy employs a SYTOX® Green dye to focus on the live population. Peripheral blood mononuclear cells were harvested and stained with CellTrace Far Red prior to stimulation with anti-human CD3. **B.** Histogram of generational peaks of live T lymphocyte cells analysed using Atune® Acoustic Focusing Cytometer with 638 nm excitation and 660/20 nm bandpass emission filter.

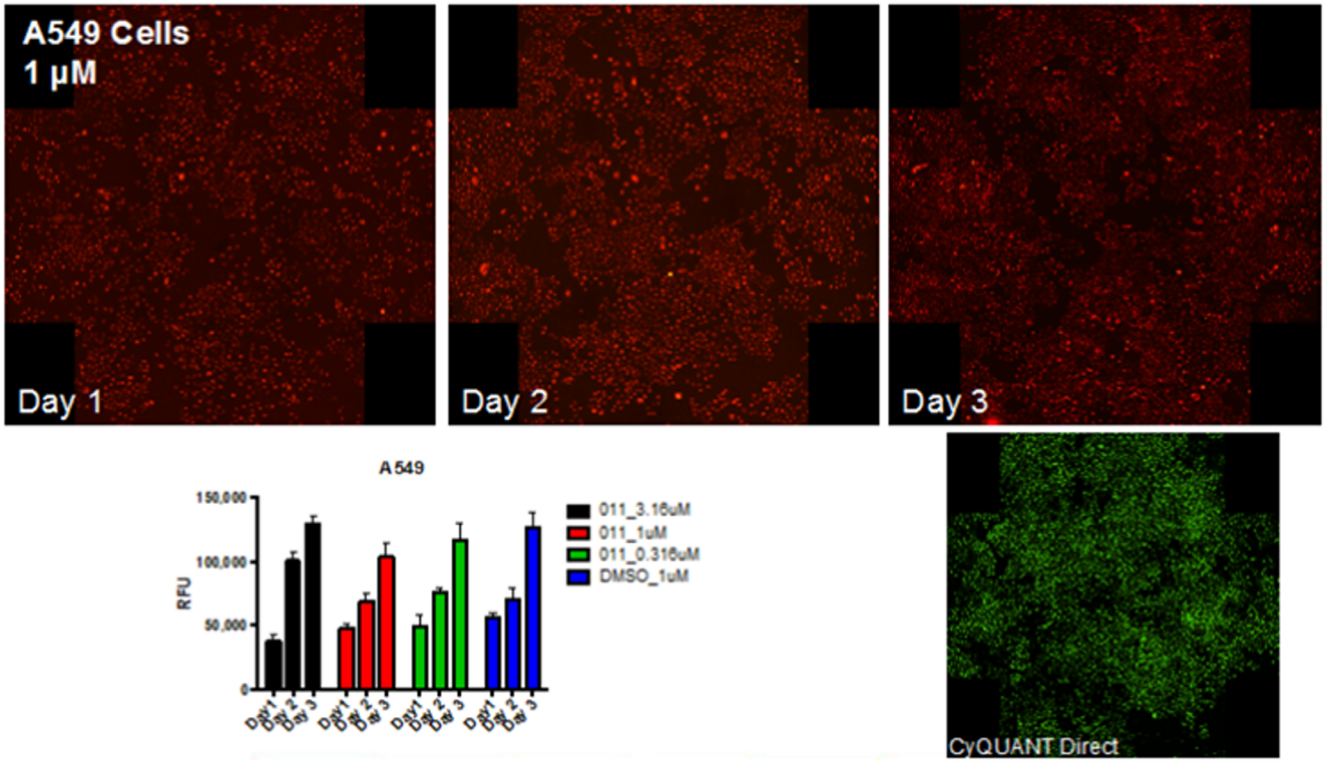

Figure 2. CellTracker Deep Red has long term cell retention. Proliferation of A549 cells labeled with CellTracker Deep Red at concentration of 3.16, 1, 0.316 µM with 1µM DMSO as a control. Cells were incubated for 60 mins at 37°C and then loading media was aspirated, cells washed 1X in LCIS, and complete media added back to wells for culturing, cells were washed and LCIS added for imaging and CyQUANT® Direct assay on days 1, 2, and 3 post reagent addition. A 10% area of the well was imaged using the scan function of the EVOS® FL Auto Imaging System, and then CyQUANT® Direct Cell Proliferation Assay reagent was used to measure cell number. This experimental protocol was repeated for days 2 and 3 and represented as scanned and stitched area images and graphed to show cell proliferation assay results.

CellTrace Far Red stain easily crosses the plasma membrane and covalently binds inside cells where the stable, well-retained fluorescent dye shows a consistent signal, even after several days in a cultured environment. It is optimally excited at 633 nm and has a narrow emission band with a 660 nm maximum. The spectral properties make it ideal for multiplexing applications due to the limited spectral overlap with other common dyes (Alexa Fluor™ 488, FITC, RPE) and fluorescent proteins (Green Fluorescent Protein (GFP) and mCherry). There are a few commercially available red-emitting cell tracing products from various sources including DDAO-SE, eFluor 670, PKH26, CellVue Claret Far Red [9,12,13]. However, these reagents either barely trace generations of cell proliferation or do not have discrete generational peaks. During our work on this study, it has recently been reported that CellTrace Far Red was particularly bright compared to CellVue Claret Far Red and eFluor 670 [14]. Also observed was that eFluor 670 was transferred to bystander cells and inherited in a significantly more variable manner than CellTrace Far Red. In contrast, CellTrace Far Red was not liable to transfer to bystander cells while it was inherited and distributed in a highly symmetrical fashion across the plane of cytokinesis [14].

CellTracker Deep Red labels A549 cells with high longevity and low toxicity

It has been known that fluorescent dyes are gradually metabolized by enzymes in cells even though they are covalently attached to cellular proteins and other cellular components [15]. Additionally, the instability of incorporated fluorescent dyes in cells and the release of peripheral fragments of cell cytoplasm contribute to the decrease of cell retention of fluorescent dyes. The longer the flurorescent tags can stay inside cells while not compromising cellular functions, the more useful they are for researchers. To determine whether CellTracker Deep Red can be retained in cells with high longevity and low toxicity, A549 cells were labeled with the dye at concentration of 3.16 µM, 1 µM, and 0.316 µM. Live cell imaging was performed on an EVOS microscope on day 1, day 2, and day 3. DMSO at 1 µM was used as control and cell proliferation was quantified with CyQuant Direct® which contains a cell-permeable DNA-binding dye and an extracellular masking dye reagent.

The live cell imaging results at 1 µM from day 1 to day 3 are shown in **Figure 2**. A significant red fluorescent signal was observed after day 3. This indicated that CellTracker Deep Red was retained in cells after 72 h. The covalent attachment of the dye to cells appears to contribute to this longevity. From the zoomed images, cells were labeled uniformally. The primary reason for the low variance is most likely similar to the case of CFSE, the rapid reactivity renders cells to react covalently so that the dyes are distributed throughout cells uniformly. Cell growth quantified with CyQuant Direct® demonstrated that cells were proliferating well compared to DMSO control.

Additionally low cellular toxicity when using CellTracker Deep Red was confirmed in Hela cells as well by comparison with other existing cell tracking products. Hela cells were stained with CellTracker Deep Red at 1 µM along with CellTracker Blue, CellTracker Green, and CellTracker Red with DMSO as control. Cell proliferation for all stained cells was quantified with CyQuant Direct® analysis**.** As shown in **Figure 3**, cell toxicity of CellTracker Deep Red is no greater than the other tracking reagents after day 3.

CellTracker Deep Red can be multiplexed with GFP and other fluorophores

The spectral characteristics (excitation/emission maxima ~630/650 nm) of CellTracker Deep Red make it suitable for multiplexing with commonly used 488 nm excitable fluorophores such as GFP, Alexa Fluor 488, and fluorescein-based dyes (*e.g.*, FITC) as well as commonly used violet-excitable fluorescent dyes. The multiplexing capabilities are desirable for researchers to have flexibility choosing functional dyes that are compatible with commonly used dyes and fluorescent proteins, without compromising their results. To determine multiplexing capability of CellTracker Deep Red, Hela cells transfected with Cell Lights Mito GFP and Golgi RFP were stained with CellTracker Deep Red and NucBlue Live. The live cell image is shown in **Figure 4** with distinct fluorescence for Mito GFP in green, Golgi RFP in red, NucBlue in blue, CellTracker Deep Red in white.

In another experiment, cells transfected with Cell Lights MitoGFP were labeled with CellTracker Deep Red and then fixed. Vimentin was detected with a Vimentin primary Antibody and an Alexa Fluor 750 labeled secondary antibody. The imaging with distinct fluorescence for MitoGFP in green, Vimentin in white, NucBlue in blue, CellTracker Deep Red in pink shows that CellTracker Deep Red still had retention after fixation with formaldehyde and permeabilization with Triton. Both experiments indicate that CellTracker Deep Red can be multiplexed with commonly used fluorescent labeling reagents in blue, green, orange, and near IR.

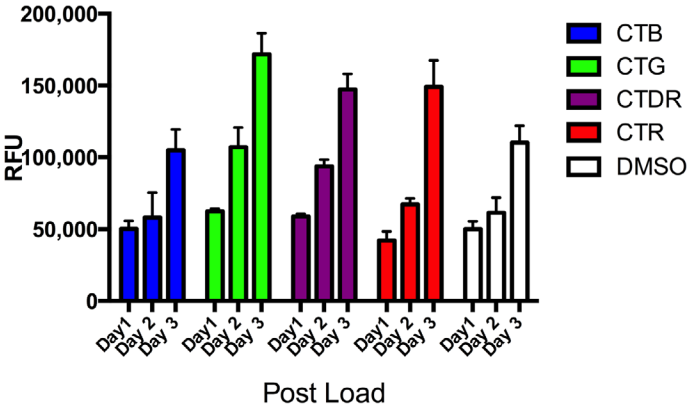

Figure 3. Low toxicity of CellTracker Deep Red. Relative fluorescence on the Y-axis is proportional to cell population viability. HeLa cells were plated in 96-well plates at a density of 2500 cells per well. The next day the cells (across 3 separate 96 well plates) were loaded with the indicated CellTracker at 1 µM concentration in Live Cell Imaging Solution(LCIS). DMSO in LCIS was applied to cells for an internal control. Cells were incubated for 60 mins at 37°C and then loading media was aspirated, cells washed 1× in LCIS, and complete media added back to wells for culturing. On days 1, 2,and 3 post reagent addition, CyQUANT® Direct Cell Proliferation Assay reagent was used to measure cell proliferation.

CellTracker Deep Red can be used to monitor wound healing activity

Neonatal human dermal fibroblasts were grown to confluency and then labeled with 1µM CellTracker Deep Red for 30 minutes in LCIS. LCIS was removed and media was placed back on cells. A scratch wound was made in the cells. The entire wound was scanned on EVOS Auto FL (Thermo Fisher Scientific) using the scan function. The same area was re-scanned 24 and 48 h later to monitor how the wound was healing. Results are shown in **Figure 5**.

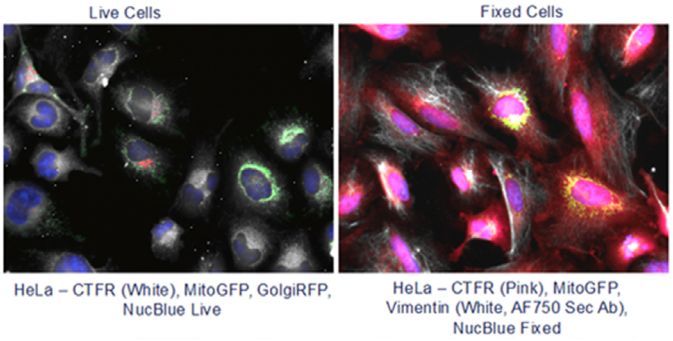

Figure 4. Multiplexing capability of CellTracker Deep Red. A. HeLa cells were treated withCellTracker Deep Red (white), CellLight® Mitochondrial-GFP, BacMam 2.0 (green), CellLight Golgi-RFP, BacMam 2.0 (red), and NucBlue® Live ReadyProbes® Reagent (blue). **B.** HeLa cells treated with CellTracker Deep Red (pink), CellLight® Mitochondrial-GFP, BacMam 2.0 (green), anti-vimentin and goat-anti-rabbit Alexa Fluor 750 (white), and NucBlue® Fixed ReadyProbes® Reagent (blue).

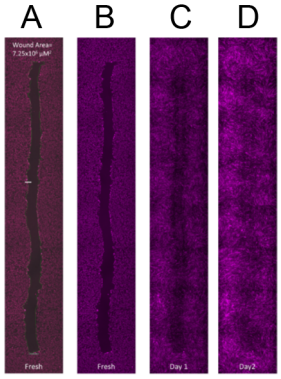

Figure 5. Wound healing activity of fibroblast using CellTracker Deep Red. Proliferation of Neonatal human dermal fibroblasts after staining with CellTracker Deep Red followed by manual formation of a scratch wound. The entire wound was scanned on EVOS Auto FL on Day1 and Day2.

After labeling with CellTracker Deep Red, the wound was clearly visible (dark, nonfluorescent) under microscope. The initial scan of the fresh wound was analyzed using the measure function on the EVOS Auto FL to calculate the area of the wound (7.25×10^6 µm^2). On day 1, cells were growing and confluent into the center of wound with some wound remaining. On day 2, the area of the wound under the microscope was almost completed replaced with new cells emitting red fluorescence with retention of the CellTracker Deep Red dye, indicating benign dye behavior at this concentration within actively motile cells.

In summary, we assessed the capacity of the novel red-emitting fluorescent CellTrace Far Red and CellTracker Deep Red dyes as cell tracing and tracking agents in different cell lines utilizing flow cytometry and fluorescence microscopy, respectively.

CellTrace Far Red can label lymphocytes with stable and bright red fluorescence upon red laser excitation with long lived fluorescence of low variance and low cellular toxicity and trace cell proliferation with clear visualization of up to 8 discrete generational peaks following cell division; it is superior to other available red-emitting tracking agents based on the reported cell tracing results. It can resolve cell proliferation as well as, or better than, the gold standard CFSE, providing an alternative to CFSE for measuring cell division with its distinct spectra.

CellTracker Deep Red has excellent cellular retention characteristics with bright fluorescence after 72 h of cell staining. It doesn't interfere with cell proliferation during this period of time while maintaining low cellular toxicity. CellTracker Deep Red is also retained after fixation and permeabilization methods commonly used in Immuno Fluorescence studies giving researchers the flexibility of multiplexing with commonly used fluorescent labeling reagents in distinct colors including blue, green, orange and red. **Note:** CellTrace Far Red and CellTracker Deep Red are for research use only. Not for use in diagnostic procedures.

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