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A Novel Method for Monitoring Tumor Proliferation In Vivo Using Fluorescent Dye DiD

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Abstract

Monitoring single cell proliferation in vivo is difficult, but optimizing this technique is essential in order to expand our knowledge of the regulation of tumor proliferation. In this study, we used a lipophilic fluorescent dye, DiD, that rapidly and stably integrates into the phospholipid cell membrane. We cultured DiD-stained prostate cancer cell lines for 10 days and isolated cells by flow cytometry based on expression levels of DiD. We found that a decrease in DiD intensity was correlated to the reduction of EdU, where the DiD-high population proliferated more slowly than the DiD-low population and the DiD-low population exhibited a higher mitotic index. We also found that DiD was detected after 3 weeks of implantation in an in vivo setting. Importantly, DiD dye did not have any effect on normal cell growth, whereas a gold standard fluorescent dye for measuring cell proliferation, CFSE, slowed cell proliferation. Although further study is indicated, DiD can be useful for identifying the molecular mechanisms underlying tumor proliferation in vivo. © 2014 International Society for Advancement of Cytometry

Key terms

DiD fluorescent dye; prostate cancer; proliferation; flow cytometry

Introduction

Tumor cells that spread from a primary tumor to distant sites are referred to as "disseminated tumor cells (DTCs)." Recent studies have demonstrated that DTCs of several cancer types are generated at an early stage, and some become and stay dormant for a long period of time (1–3). Once DTCs become dormant, they are effectively resistant to cytotoxic treatments that target actively proliferating cells, and these dormant DTCs, if reactivated, can be the root of cancer recurrence.

To explore the mechanisms of cell dormancy, we must first evaluate cell proliferation. Unfortunately, most assays currently used to determine cell proliferation, such as MTT or XTT assays, BrdU assay, and gene expression analyses by PCR, require the permeabilization of cell membranes and/or the termination of cell culture. Although these assays allow us to analyze cell proliferation, their terminal nature prevents us from further dissecting the biology of tumor dormancy. There is, therefore, a need for a specific tool to analyze and recover live cells. Fluorescent dyes analyzed by flow cytometry are commonly used to track cell proliferation. In addition, incorporated fluorescent dyes enable isolation of live cells without damaging metabolic function, and allow analysis of phenotype in different populations using fluorescent dye–antibody combinations.

Fluorescent dyes are classified into three major groups based on differences in affinity: (i) DNA-binding dye (e.g., Hoechst 33342); (ii) Cytoplasmic dye (e.g., Calcein, CFSE); and (iii) Membrane-bound dye (e.g., PKH26, DiD). DNA-binding dyes have been widely used for cell cycle analysis, since they provide information about DNA content distribution. These DNA-binding dyes are also useful tools for monitor-

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ing cell migration and trafficking, both in vitro and in vivo, because of their high retention (4). However, DNA-binding dyes are not suitable for measuring long-term cell proliferation, since their intensity decreases in a short period of time (5) and they have potential direct effects on cell proliferation (6). Due to their longer retention ability, cytoplasmic dyes have been used for both cell migration and proliferation studies (7–9). However, cytoplasmic dyes exhibit high levels of toxicity to cells (10,11), since they affect the functions of cellular enzymes by binding to cytoplasmic proteins (12). In contrast, membrane-bound dyes are likely to be less toxic (13,14). Additionally, membrane-bound dyes are well recognized for their potential use in cell migration and proliferation assays, made possible by their long retention (15–17).

In the present study, we used a lipophilic fluorescent dye, DiD, that rapidly and stably integrates into the phospholipid membrane, to measure cell proliferation. Although further study is clearly needed, the proposed technique may open new avenues in the investigation of tumor dormancy.

Materials and Methods

Cell Culture

Human prostate cancer cell lines, PC3 and DU145, and murine osteoblastic cell line, MC3T3-E1, were obtained from the American Type Culture Collection. The metastatic subclone of LNCaP C42B, was originally isolated from a lymph node of a prostate cancer patient with disseminated bony and lymph node involvement (18). All prostate cancer cell lines were routinely grown in RPMI 1640 (Life Technologies, Carlsbad, CA, Cat no. 11875-093), and MC3T3-E1 cells were grown in α-minimum essential media (α-MEM, Life Technologies, Cat no. 12561-056). Cultures were supplemented with 10% (vol/vol) fetal bovine serum (FBS, GEMINI Bio-Products, West Sacramento, CA, Cat no. 900-208), 1% (vol/vol) penicillin–streptomycin (Life Technologies, Cat no. 15140-122) and maintained at 37°C, 5% CO_2, and 100% humidity. Doubling time of PC3, DU145, and C4-2B are 36, 30, and 32 h, respectively.

Flow Cytometry

The flow cytometric analyses and fluorescence-activated cell sorting (FACS) were performed on a FACS Aria dual-laser flow cytometer (Becton Dickinson, Franklin Lakes, NJ) and data were analyzed with DIVA software (Becton Dickinson). BD cytometer setup and tracking beads kit (BD Biosciences, Franklin Lakes, NJ, Cat no. 642412) are used for the daily instrument standardization and validation procedure. Sorting calibration was performed before each sort by drop-delay using Accudrop beads (BD Biosciences, Cat no. 345249), populations for sorting were gated by forward and side scatter to eliminate the presence of doublets. Sorting of these gated cells was done using a 100-μm nozzle at 20 psi in purity mode.

Cell Labeling with DiD Dye

PCa cell lines were stained with DiD dye (Molecular Probes, Eugene, OR, Cat no. v22887), according to manufacturer directions. Briefly, cells (1×10^6 cells/ml) were incubated with DiD dye (0.5 μM) in serum-free conditions at 37°C for 20 min, and then were washed three times with serum-free medium. The intensity of DiD was analyzed on a FACS Aria dual-laser flow cytometer and data were analyzed with DIVA software. In some cases (Figs. 1B and 1C), cells were co-stained with EdU (10 μM for 48 h; Life Technologies, Cat no. c10425) and DiD to perform further examinations.

Cell Labeling with CFSE Dye

PCa cell lines were stained with carboxyfluorescein succinimidyl ester (CFSE) dye (Molecular Probes, Cat no. C34554) according to manufacturer directions. Briefly, cells (1×10^6 cells/ml) were incubated with CFSE dye (5 μM) in Dulbecco's phosphate buffered saline (DPBS, Life Technologies, Cat no. 14190-144) supplemented with 0.1% (wt/vol) bovine serum albumin (BSA, Sigma, St. Louis, MO, Cat no. A2153) at 37°C for 10 min. Then, cells were transferred into ice-cold culture medium, were incubated on ice for another 5 min, and then were washed three times with culture medium.

Proliferation Assay

PCa cells were plated into 96-well plates at a concentration of 4,000 cells/100 μl/well in growth medium. In some cases (Figs. 3C and 3D), cells were cultured in different serum conditions (1% FBS and 10% FBS). Proliferation was quantified using One Solution Cell Proliferation Assay kit (Promega, Madison, WI , Cat no. G3580), which contains a tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)−5-(3-carboxymethoxyphenyl)−2-(4-sulfophenyl)−2*H*-tetrazolium, inner salt; MTS]. At different time points (24 h and 48 h), optical intensities were read on a multiwell scanning spectrophotometer at optical density (OD) 492 nm (Thermo Labsystems). An automatic blanking measurement was performed immediately before a measurement, and Performance Verification kit (Thermo Labsystems, Milford, MA, Cat no. 24071960) was used to check the photometric accuracy. Cells cultured in medium with 10% FBS served as positive control. Cells cultured in serum-free medium served as negative control.

In Vivo Subcutaneous Implantation of DiD-Stained PC3 Cells

All experimental procedures were approved by the University of Michigan Committee for the Use and Care of Animals (UCUCA). Male 4- to 6-week-old severe combined immunodeficient (SCID) mice (Charles River, Wilmington, MA, CB.17. SCID) were implanted subcutaneously with DiD-stained PC3 cells (2×10^5 cells) within sterile collagen scaffolds ($3 \times 3 \times 3$ mm^3; Gelfoam; Pfizer, New York, NY, Cat no. 09–0315-08) in the mid-dorsal region of each mouse ($n = 3$). Animals implanted with scaffolds alone during surgery were kept as negative controls (surgical control). After 3 weeks, primary tumors were collected and cells were filtered through a 40-μm cell strainer (Fisher, Hampton, NH, Cat no. 22363547) to obtain single-cell suspensions. Thereafter, cells were incubated with a fluorescein isothiocyanate (FITC)-human leukocyte antigens-A, B, and C loci (HLA-ABC) antibody (Biolegend, San Diego, CA, Cat no. 311404) and the intensity of DiD dye in HLA-positive PC3 cells was analyzed by flow cytometry.

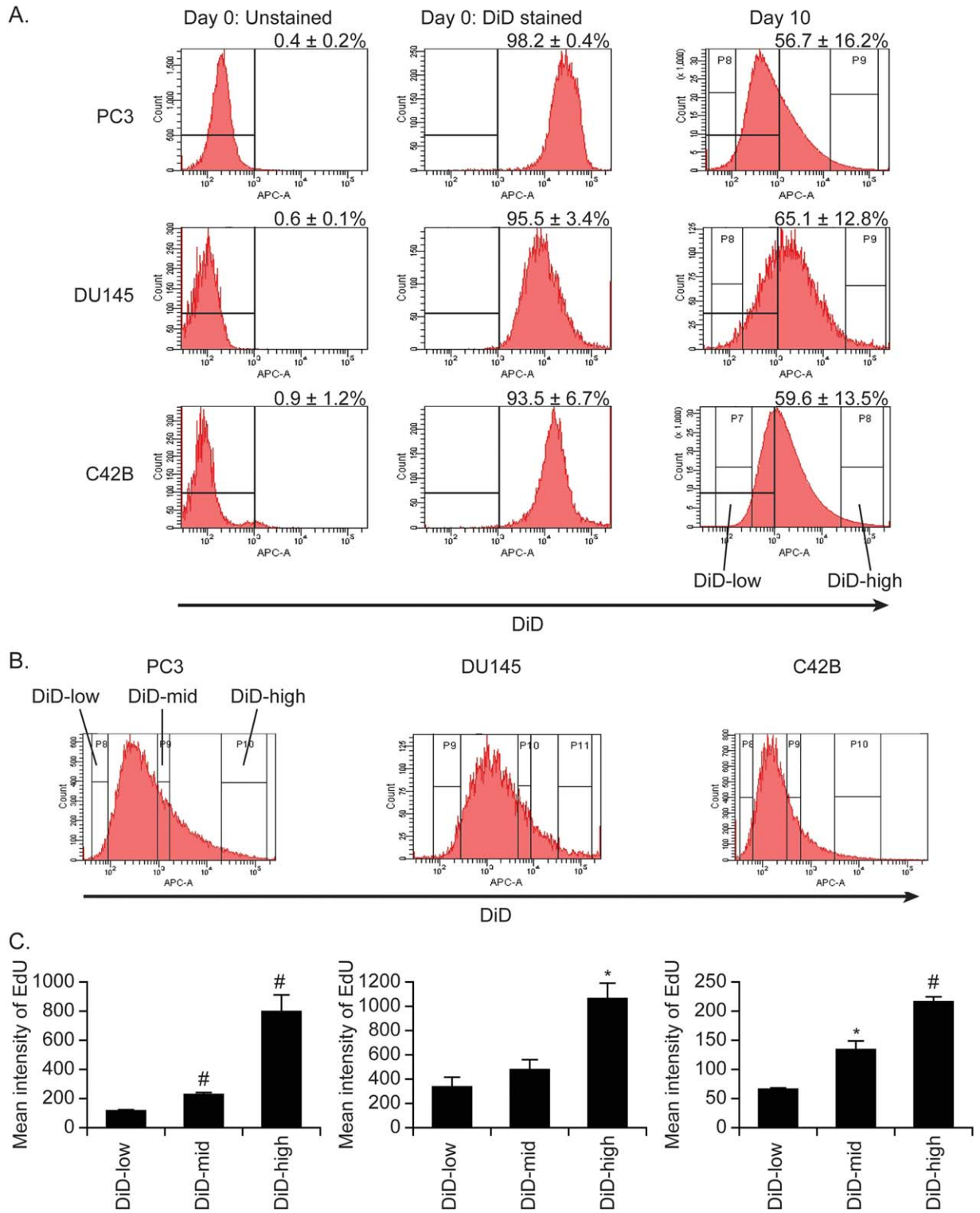

Figure 1. Tracking proliferation of PCa cells with DiD in vitro. **A:** The DiD flow cytometric dilution profiles of PCa cells. PCa cells (PC3, DU145, C42B) were stained with DiD (0.5 μM) and cultured for 10 days. Representative histograms of DiD fluorescence on days 0 and 10 of culture. Data are representative of three replicate experiments. **B:** PCa cells (PC3, DU145, C42B) were co-stained with EdU and DiD. At 7 days of culture, three distinct cell groups were isolated based on DiD fluorescence intensity (low intensity, mid intensity, and high intensity), and (**C**) the retention of EdU was compared between the groups. Data are presented as mean ± standard deviation from triplicate determinations. $^{*}P < 0.05$ and $^{\#}P < 0.01$ vs. low intensity group. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

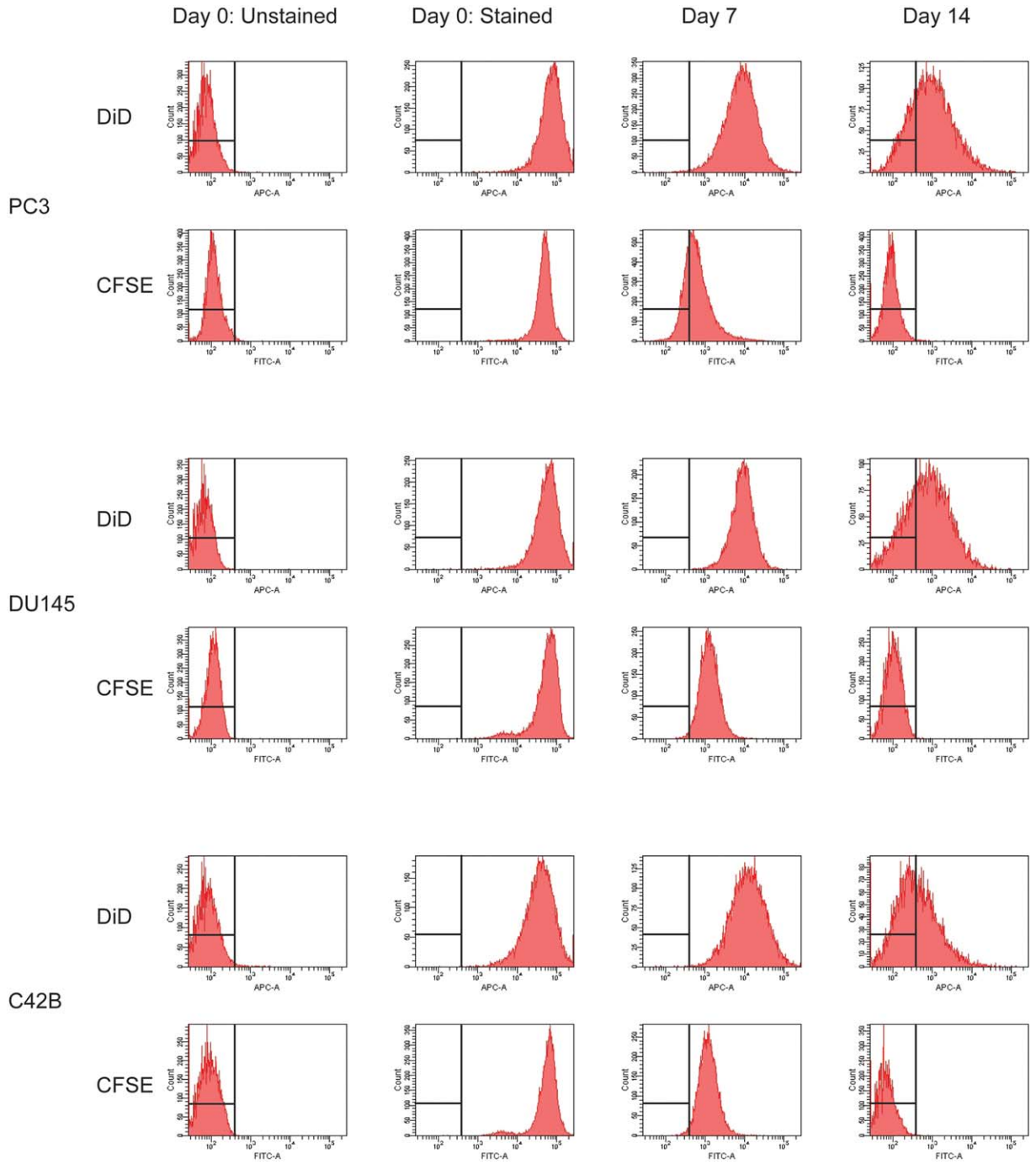

Figure 2. Tracking proliferation of PCa cells with DiD and CFSE in vitro. The DiD and CFSE flow cytometric dilution profiles of PCa cells (PC3, DU145, C42B). PCa cells were stained with DiD (2 μM) or CFSE (5 μM), and cultured for three weeks. Representative histograms of DiD and CFSE fluorescence on days 0, 7, and 14 of culture. Data are representative of three replicate experiments. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Co-culture

DiD-labeled (2 μM) PC3 cells (3×10^5 cells) were co-cultured with murine osteoblast cell line MC3T3-E1 cells (3×10^5 cells) in 10 cm dishes for 8 days. The initial cell mixture was incubated with a FITC-conjugated human specific HLA-ABC antibody, and greater than 99% of the PC3 cells were positively stained, but not MC3T3-E1 cells. For the following culture period, any transfer of DiD dye from PC3 cells to MC3T3-E1 cells was evaluated using flow cytometry.

Statistical Methods

All numerical data are expressed as mean $\pm$ standard deviation unless specified otherwise. Two-tailed, unpaired Student's *t*-test was used for data analysis, with $P < 0.05$ considered to be statistically significant.

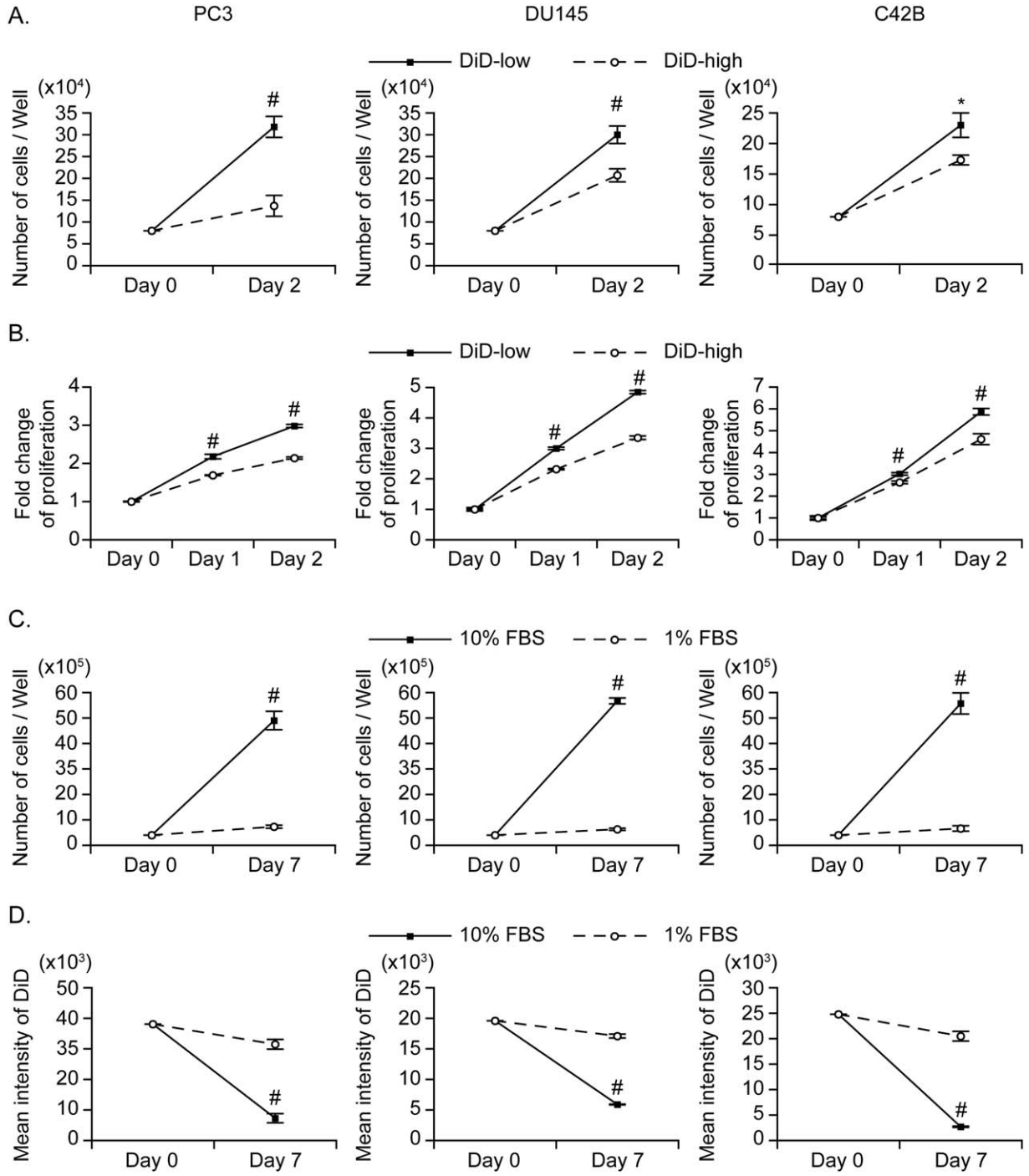

Figure 3. Retention of DiD is correlated to PCa cell proliferation. **A, B:** PCa cells (PC3, DU145, C42B) were stained with DiD and cultured for 10 days. Thereafter, DiD-high and DiD-low cell subpopulations were isolated by FACS and cultured for 2 days, after which: (**A**) The total cell number was counted using trypan blue exclusion, and (**B**) Cell proliferation was measured by MTS assay. Data are presented as mean ± standard deviation from triplicate determinations. $^{*}P < 0.05$ and $^{\#}P < 0.01$ vs. DiD-high subpopulation. **C, D:** PCa cells (PC3, DU145, C42B) were stained with DiD and cultured in media with either 10% FBS or 1% FBS for 7 days. C: The total cell number was counted using trypan blue exclusion and (D) the retention of DiD was measured by flow cytometry. Data are presented as mean ± standard deviation from triplicate determinations. $^{\#}P < 0.01$ vs. 1% FBS condition.

Results and Discussion

DiD can be Used to Indicate Cell Proliferation

To first determine whether DiD is segregated into daughter cells after cell division or mitosis, PCa cell lines (PC3, DU145, C42B) were stained with DiD, and the intensity of fluorescent dye was analyzed by flow cytometry. All or nearly all PCa cells expressed DiD on their surfaces (Fig. 1A). After 10 days of culture, the mean level of DiD fluorescence was significantly

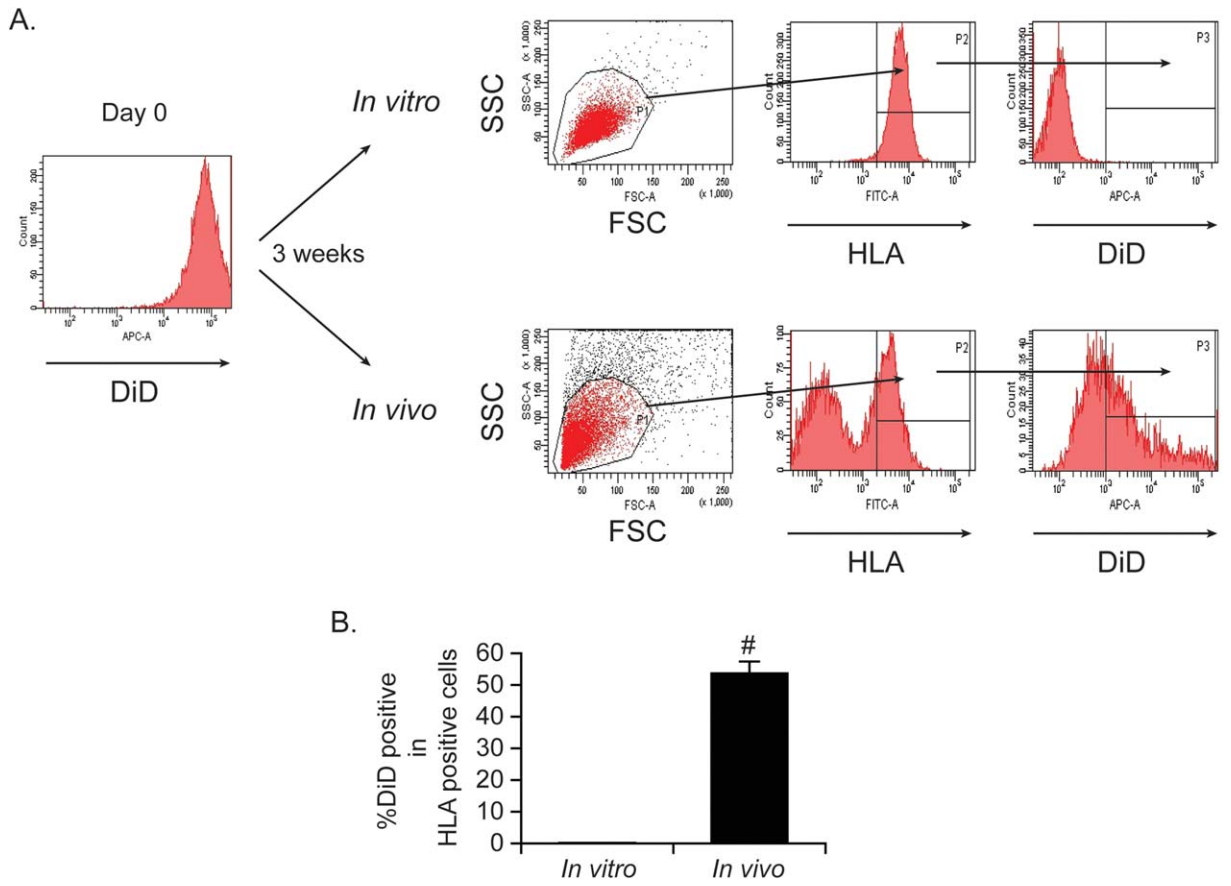

Figure 4. DiD detection in vivo. To determine whether DiD is retained in cells in an in vivo situation, SCID mice were implanted with DiD-stained PC3 cells subcutaneously ($n = 3$ per group). After 3 weeks, the primary tumors were collected and cells were filtered through a 40-μm cell strainer to obtain single-cell suspensions without enzymatic digestion. The resulting cells were incubated first with a FITC-conjugated HLA-ABC antibody, where greater than 99% of the PC3 cells were positively stained. Thereafter, the intensity of DiD was analyzed with flow cytometry by gating on HLA-ABC. In vitro cultured PC3 cells served as a time control for tracking fluorescent intensity. **A:** Representative flow cytometric analyses. **B:** Quantification of (A). Data are presented as mean ± standard deviation from triplicate determinations. $^{\#}P < 0.01$ vs. in vitro cultured cells. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

decreased, and a DiD-negative population was revealed (Fig. 1A and Supporting Information Fig. 1). To determine whether the DiD dye could be retained over a longer period, PCa cell lines were stained with either DiD dye or CFSE dye (for comparison) and fluorescent intensities were compared over time. Surprisingly, cells containing DiD retained fluorescence significantly longer than those stained with CFSE dye (Fig. 2). To verify whether a decrease in DiD intensity represented cell division, a separate experiment was performed in which the coincident retention of EdU, an analog of BrdU, and DiD in dual labeled PCa cells was tracked over the course of culture. As expected, the intensity of DiD dye was well correlated to the retention of EdU (Figs. 1B and 1C and Supporting Information Fig. 1).

To determine whether DiD intensity correlates with cell proliferation, a DiD high intensity population and a DiD low intensity population were isolated by FACS, and proliferation in both populations was compared. Greater cell proliferation was detected in the DiD-low population compared to the DiD-high population by manually counting cell number (Fig. 3A) and MTS proliferation assay (Fig. 3B and Supporting Information Figs. 2 and 3A and 3C). In addition, a higher mitotic index was observed in the DiD-low population by measuring the level of a mitosis marker, phosphorylated-Histone H3 (Ser28) (Data not shown). To determine whether DiD intensity decreases as cells proliferate, DiD-stained cells were cultured in different serum conditions (1% FBS vs. 10% FBS) to manipulate cell proliferation. As expected, cells cultured in 1% FBS proliferated more slowly than those cultured with 10% FBS (Fig. 3C). Critically, the DiD intensity of cells cultured in 1% FBS remained high, while the DiD intensity of cells in 10% FBS dramatically decreased (Fig. 3D).

To address how DiD-stained cells behave in vivo, PC3 cells were implanted into SCID mice subcutaneously. Surprisingly, more than 50% of PC3 cells still expressed DiD on their surface after 3 weeks of implantation, as compared to PC3 cells in in vitro culture, which expressed little or no DiD after 3 weeks (Figs. 4A and 4B).

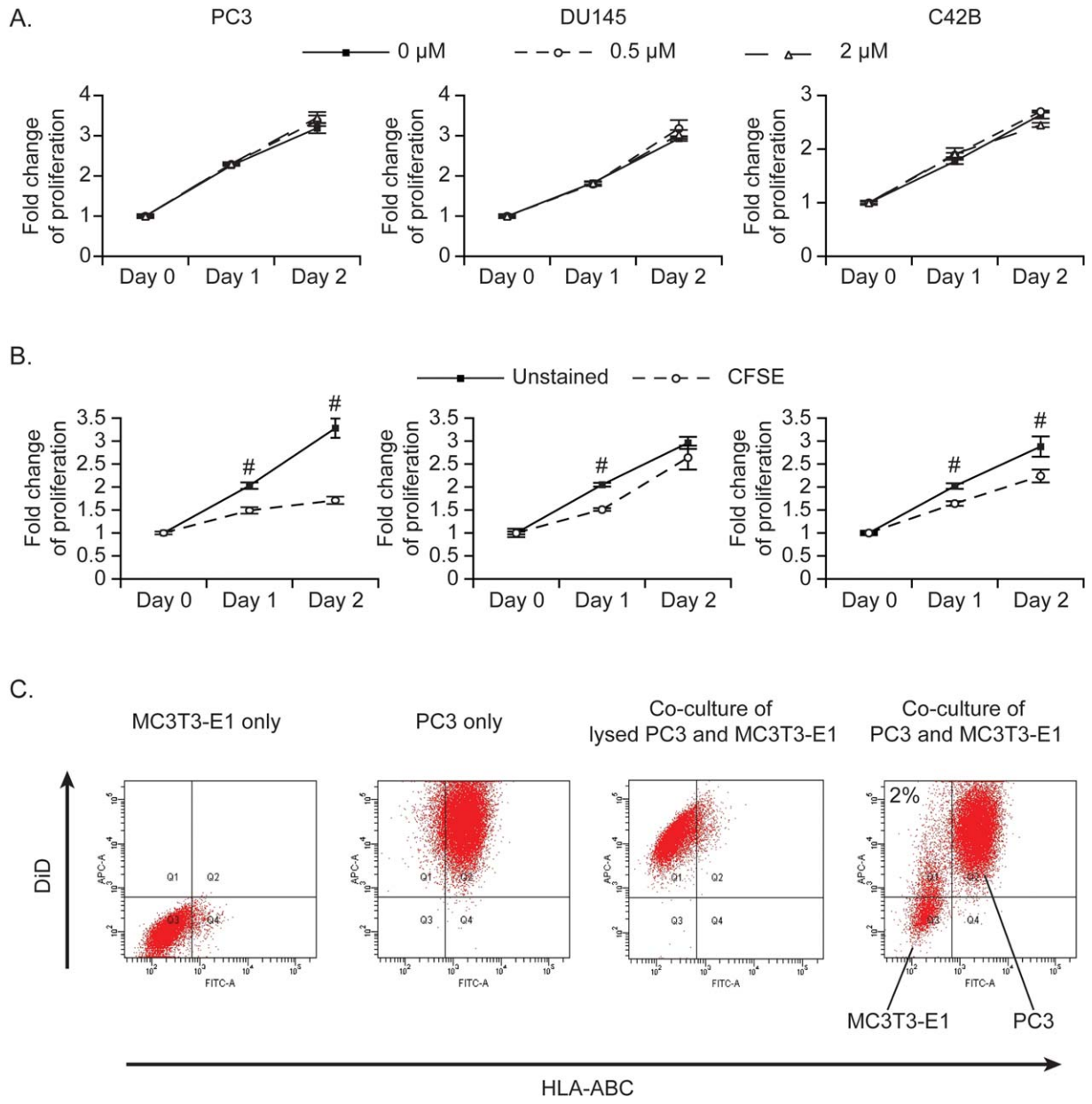

Figure 5. Effects of DiD on cell proliferation, and evaluation of DiD retention in labeled cells in co-culture. To determine possible adverse effects of fluorescent dyes on cell function, PCa cells (PC3, DU145, C42B) were stained with (**A**) DiD (0.5 μM and 2 μM) and (**B**) CFSE (5 μM). Cell proliferation was measured by MTS assay. Data are presented as mean $\pm$ standard deviation from triplicate determinations. $^{\#}P < 0.01$ vs. unstained cells. **C**: To determine whether DiD fluorescent dye transfers to neighboring cells, co-culture of DiD-labeled PC3 cells with non-labeled MC3T3-E1 cells was performed. At 8 days of co-culture, the resulting cells were incubated first with a FITC-conjugated HLA-ABC antibody to distinguish murine MC3T3-E1 cells (HLA-ABC negative) from human PCa cells (HLA-ABC positive). Greater than 99% of PC3 cells were positively stained. Thereafter, the DiD intensity of both PC3 cells and MC3T3-E1 cells was analyzed by flow cytometry, gating on HLA-ABC. The number indicated in the right dot plot represents the percentage of DiD positive MC3T3-E1 cells. As a positive control, DiD-stained PC3 cells were lysed and added onto the MC3T3-E1 cells. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

DiD did not Affect Cell Proliferation or Leak from the Cells

As a prerequisite for long-term studies, labeling of cells must not adversely affect cell viability or function. To test whether DiD has adverse effects on normal cellular metabolism, a dose fourfold higher than that routinely used was included in these functional experiments. First, cell proliferation was compared between unstained cells, DiD-stained cells, and CFSE-stained cells. There were no differences between unstained and DiD-stained cells in cell proliferation as measured by MTS assay, even at higher levels of DiD (Fig. 5A and Supporting Information Figs. 2, and 3B and 3C), while cell

proliferation was inhibited in CFSE-stained cells (Fig. 5B and Supporting Information Figs. 2 and 3C and 3D).

Finally, to examine whether DiD leaks from the cells during cell culture and is taken up by unlabeled cells, direct cell-to-cell co-culture with a murine osteoblastic cell line, MC3T3-E1, was performed. As expected, when PC3 cells were lysed prior to co-culture, MC3T3 cells picked up DiD dye, however little or no DiD was detected in MC3T3-E1 cells even after 8 days of co-culture when PC3 cells were viable (Fig. 5C).

In this study, we demonstrated that DiD fluorescent dye can be a useful tool to trace the proliferation of cells by flow cytometry without requiring permeabilization or perturbing cellular function. As expected, a marked decrease of fluorescent intensity was detected in DiD-stained PCa cells after 10 days of in vitro culture, compared to freshly stained cells. The EdU retention assay revealed that the decrease seen in DiD intensity correlates with cell division. Following this observation, determination of whether the distribution of fluorescence in cells is related to proliferative activity was performed. DiD-high and DiD-low cell populations were isolated using flow cytometry, then plated to evaluate any differences in proliferation. MTS assays revealed that the DiD-low population grew faster than the DiD-high population. Interestingly, DiD was retained for more than 3 weeks in vivo, much longer than in vitro retention. Importantly, MTS assay indicated that DiD did not affect proliferation of the cells, even when using a relatively high concentration of dye (2 μM).

Fluorescent dye staining of cells is widely used to measure cell proliferation, since most of the dye is typically distributed equally between the daughter cells with each cell division, resulting in a reduction in intensity of fluorescence by half. In order to continue studies of these cells over time, however, it is important that the stained cells are analyzed and recovered using flow cytometry without fixation and/or permeabilization. This is beneficial, since it allows us to isolate living cells, as well as to perform further analyses to address new research questions about tumor dormancy. In this study, DiD showed similar cell proliferation index to that assessed by EdU, which is widely used to measure cell proliferation. However, labels like BrdU and EdU require cell fixation and permeabilization for the detection of cell proliferation, which limits the ability to perform other genetic or cell-based analyses. In our study, the retention of DiD was inversely correlated to cell proliferation. Slowly dividing cells retained the high fluorescent intensity of DiD, while rapidly dividing cells lost DiD fluorescence over time. These findings suggest that DiD fluorescent dye enables the detection of cell proliferation ability over time, a marked advantage over the single time point methods. We also found that CFSE inhibits cell proliferation, while DiD did not affect cellular function. Although further study would be needed, this suggests that cytoplasmic dyes are not appropriate tools for studying cell proliferation. Additionally, the intensity of DiD in cells implanted in animals was retained longer than that in in vitro cell culture. This finding follows the hypothesis that tumor cells become dormant in vivo. However, in our co-culture study, DiD leaked out to adjacent cells when labeled cells died. As a result, DiD dye may not always be the best fluorescent dye to recover the cells from animals. However, our findings suggest that DiD is a useful tool to monitor cell division if viable cells are identified with other cell surface markers (e.g., HLA, green fluorescent protein (GFP)).

In conclusion, our study demonstrated that DiD provides stable fluorescent labeling of live cells, which is useful to monitor cell proliferation over time both in vitro and in vivo without damaging cell proliferation. Moreover, this system does not require any genetic manipulation. Although further analysis is necessary, our study indicates that DiD can be a safe and powerful tool for identifying cell proliferation.

Acknowledgment

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