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Monitoring cell proliferation *in vitro* with different cellular fluorescent dyes

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Abstract: There are few methods for quantifying cell proliferation. Those tests describe the proliferation kinetics of a cell population, but they do not report the history of single cells, the number and frequency of cell divisions, or the precursor cell frequency. Cell-tracking assays based on dilution of the green-fluorescent protein labelling dye, CFSE, has become the standard for monitoring cell proliferation. Other labelling dyes, *e.g.* CellTrace Violet and CellVue Claret, are also used for the same purpose. This study aimed to compare these three cell labelling methods for analysing the kinetics of cell viability, proliferation, and precursor cell frequency. Human peripheral blood mononuclear cells stimulated with Concanavalin A (ConA) were used as a model system. After labelling with a cell-tracking dye cells were divided into groups with and without ConA stimulation. From the 5th to 8th day, cells were collected and analysed with flow cytometry. Cell viability was not significantly different between labelled and unlabelled cells that received ConA stimulation. The proliferative fraction, proliferation index, and nonproliferative fraction were not significantly different among lymphocytes labelled with different dyes. Precursor cell frequency was also similar among cells labelled with the three cell-tracing dyes. The practical conclusion from our observations is that the results from cells labelled with different tracers may be compared directly and discussed jointly. *(Folia Histochemica et Cytobiologica 2013, Vol. 51, No. 3, 193–200)*

Key words: cell proliferation, CFSE, CellTrace Violet, CellVue Claret, FACS analysis

Introduction

An important assessment of cellular function is the proliferative capacity of cells under different conditions. There are few methods for quantifying proliferation: cell counting, detection of Ki-67 — a cellular marker of proliferation, BrdU or ^{3}H-thymidine incorporation into DNA during the S-phase of the cell cycle and fluorescence activated cell sorting (FACS) analysis of fixed cells stained with propidium iodide (PI) (cell cycle analysis). Those tests describe the proliferation kinetics of a cell population, but they do not report the history of single cells or the number and frequency of cell divisions. Moreover, the ^{3}H-thymidine incorporation test is potentially biohazardous. Furthermore, none of these methods can determine precursor cell frequency (defined as the proportion of cells in the starting population that were able to proliferate in response to the stimulus) or the fraction of cells in an experiment that underwent division. Those methods are point assays that allow quantification of the overall mitotic behaviour of a population, but give no information on the mitotic history of individual cells.

Cell-tracking, based on the dilution of the green-fluorescent protein labelling dye, carboxyfluorescein diacetate succinimidyl ester (CFSE, 492/517 nm), has become the standard procedure for monitoring cell proliferation [1–3]. The CFSE cytometric cell prolife-

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ration test is based on the observation that, after every division, the cellular fluorescence decreases by half. Other labelling dyes: CellTrace Violet (405/450 nm) [4] and CellVue Claret (655/675 nm) are also commonly used to monitor proliferation [5]. CFSE and CellTrace Violet easily diffuse into cells, where they are cleaved by intracellular esterases to yield highly reactive, fluorescent compounds. The compounds covalently bind to intracellular amines, which results in stable (up to 6 months), well retained fluorescent label. In contrast, the lipophilic compound, CellVue Claret, can stably incorporate into the lipid regions of the cell membrane [5]. Each cell division results in sequential halving of fluorescence, and cell proliferation can be monitored with flow cytometry for up to eight divisions, before the fluorescence decreases (fades) to the background fluorescence level of unlabelled cells. Moreover, the different emission spectra of different fluorescent tracers allow use of optimal supplementary labelling of cells with fluorescently-labelled antibodies to identify the phenotypes of cells.

No previous studies have compared the efficacy of CFSE, CellTrace Violet and CellVue Claret under the same experimental conditions. Therefore, this study aimed to compare three cell labelling methods used to analyse cell proliferation rates. As a model system, we used human peripheral blood mononuclear cells (PBMCs) stimulated with Concanavalin A (ConA).

Material and methods

Peripheral blood mononuclear cell isolation. PBMCs were obtained from healthy human donors, aged from 30 to 60 years. PBMCs were isolated by density gradient centrifugation with Histopaque-1077 (Sigma-Aldrich) and resuspended in RPMI-1640 (Gibco), supplemented with 10% heat-inactivated foetal bovine serum (FBS, Gibco) and penicillin/streptomycin (100 UM penicillin + 0.1 mg streptomycin/mL) culture medium. Prior to culture and stimulation, the PBMCs were labelled with one of the three studied proliferation cell-tracking markers. All blood donors provided informed consent to participate in this study. The study protocol was approved by the Bioethical Commission at The Centre of Postgraduate Medical Education in Warsaw.

Labelling procedure with CFSE. CFSE (Fluka, BioChemika) was used as described by the manufacturers. It was dissolved in dry dimethyl sulfoxide (DMSO) to make a 5 mM stock solution stored at –20°C until use. The PBMCs were adjusted to 3×10^6 cells in 1.5 mL phosphate buffered saline (PBS), supplemented with 5% heat-inactivated FBS. An equal volume of CFSE (10 μM in PBS with 5% FBS) was added to the cell suspension to obtain a final CFSE working concentration of 5 μM. Cells were incubated at room temperature and protected from light for 5 min. Uptake of CFSE was stopped by diluting with 5 volumes of PBS with 5% FBS, followed by two washes with the same solution.

Labelling procedure with CellTrace Violet. Cell labelling with CellTrace Violet was performed according to the protocols provided by the manufacturer (CellTrace Violet Cell Proliferation Kit, Invitrogen, Molecular Probes). The compound was dissolved in dry DMSO to make a 5 mM stock solution stored at –20°C until use. PBMCs (3×10^6) were suspended in 1 mL PBS and 1 μL of CellTrace Violet stock solution was added to a final concentration of 5 μM. Cells were incubated at 37°C and protected from light for 20 min. Unbound dye was quenched by diluting with 5 volumes of complete culture medium and incubating for 5 min, followed by two washes with that medium.

Labelling procedure with CellVue Claret. CellVue Claret (CellVue Claret Fluorescent Cell Linker Kit, Sigma-Aldrich) was supplied in a 1 mM stock solution in ethanol. It was used as recommended by the manufacturers. Immediately before staining, 3×10^6 PBMCs were suspended in 1 mL Diluent C (provided with the kit, an iso-osmotic, salt-free, staining solution). In parallel, 4 μL of dye stock solution was added to 1 mL Diluent C; then, the PBMC and dye solutions were rapidly mixed. After 3 min at room temperature, staining was stopped by adding 2 mL of FBS; cells were then washed twice in complete culture medium.

Cell culture and data collection. After labelling with cell -tracking dye, PBMCs were placed in 12-well tissue culture plates with complete culture medium (3 mL/well). The day after labelling, the cells were suspended in fresh culture medium, counted, and divided into two groups: one with and one without 5 μg/mL ConA (Sigma-Aldrich) to stimulate proliferation. Then, cells were placed into 24-well plates (10^5 cells in 1 mL/well). The cultures were incubated at 37°C in 5% CO_2 for 8 days. From the 5th to 8th day, cells were collected, and propidium iodide (PI) was added at a final concentration of 5 μg/mL to distinguish living cells. Cells were analysed with flow cytometry on an apparatus (FACSCanto-II, Becton Dickinson) that had undergone periodic quality control measures. The single cell fluorescence was analysed with flow cytometry collecting 5 000 events from each sample at the fluorochrome characteristic wavelengths: 450 nm for CellTrace Violet, 530 nm for CFSE, and 675 nm for CellVue Claret. Cell viability and the proliferative fraction were analysed with the FacsDiva software. The cell proliferation index, nonproliferative fraction, and precursor frequency were analysed with the ModFitLT application.

Analysis of data collection. Different cellular fluorescent markers for analysis of human PBMC proliferation were compared after ConA stimulation. The experiments assessed

the kinetics of cell viability, proliferation, and precursor cell frequency.

Cell viability was evaluated as the percent of cells that did not bind PI in a tested cell population. PI is a nucleic acid intercalating agent and a fluorescent molecule, which penetrates cells with damaged plasma membranes.

The cell proliferative fraction was calculated as the percent of ConA-stimulated cells below a fluorescence intensity threshold compared to all the cells in the sample. This threshold was set at 98% of cells in an unstimulated PBMC sample.

The proliferation index, the precursor cell frequency, and the nonproliferative fraction were calculated from a model for cell division (Proliferation Wizard Model) that predicted a doubling of cell number as a cell proliferated through each daughter generation. The proliferation index was the sum of the cells in all generations divided by the number of original parent cells present at the start of the experiment. It measured the increase in cell number in a culture over the course of the experiment.

The precursor frequency was defined as the proportion of cells in the starting population that were able to proliferate in response to the stimulus. According to the model, it was calculated as the proportion of total cells that exhibited "true proliferation" during the course of cell culture, where "true proliferation" excluded cells that only divided once.

The nonproliferative fraction was the number of cells in the parent generation at the time of data collection divided by the number of cells present in the original culture. It represented the fraction in the original culture of cells that did not proliferate during the course of the experiment.

The fading of tracer signal was also analysed. The fluorescence signal in labelled cells faded over time. The median fluorescence intensity (MFI) of unstimulated cells was compared each day to the signal measured on the 5th day.

Statistics. Values represent medians and 25th to 75th percentile (P_{25}–P_{75}) values from 5 independent experiments. Statistically significant differences were calculated with the Mann-Whitney *U* test, assuming significance at $p < 0.05$.

Results

Experiments were performed on freshly isolated PBMCs from healthy individuals. The proliferation analyses were performed with live lymphocyte fractions, gated on Forward Scatter / Side Scatter (FSC/SSC), CD14CD45 graph and Forward Scatter/ /Propidium Iodide (FSC/PI) graphs after PI labelling (Figure 1A and B, respectively). ConA caused stimulation of T-lymphocyte proliferation (Figure 1C). In contrast, B-lymphocytes, visible before ConA stimulation (Figure 1C, left graph), were nearly absent in the lymphocyte population after ConA stimulation (Figure 1C, right graph). This change the proportion of B cells to T cells about 20 times. Stimulation with ConA-induced proliferation was observed and analysed from 5th day of the culture.

Survival and proliferation of labelled cells

Survival in cultured samples was stable throughout the 8-day observation. Among the cells labelled with different dyes, survival was dependent on ConA addition: 97–99% of unstimulated cells and 90–93% of ConA-stimulated cells were viable between days 5 and 8 (Figure 2A). Both labelled and unlabelled cells showed significant differences in survival with ConA stimulation. However, no significant differences in viability were observed between ConA-stimulated labelled cells and ConA-stimulated unlabelled cells, as well as between ConA-unstimulated labelled and ConA-unstimulated unlabelled cells.

The proliferation of stimulated cells was asynchronous. The three tested proliferation tracer dyes were stable in cells, and sequential cell divisions caused the expected reductions by half in fluorescence content. An example of cell fluorescence analysis on the 5th day after ConA stimulation is presented in Figure 3. Lymphocytes labelled with the three tested tracer fluorochromes were compared to unlabelled and unstimulated control cells.

The proliferative fraction was the percent of proliferating cells, including cells that had undergone only one cell division. The proliferative fraction of cells was similar for different lymphocyte samples labelled with different cell tracers, and the fraction rose from day 5 to day 8, by about 2.3- to 2.7-fold, depending on the fluorescent marker (Figure 2B). The proliferative fraction of cells labelled with CellTrace Violet was slightly lower on days 6 and 7; however, the difference was not significant compared with the fractions observed with CFSE or CellVue Claret (Figure 2B).

The proliferation index was the increase in the number of living cells over the observation time, and it increased between the 5th and 8th day of the experiment (Figure 2C). The proliferation index was below 2 (1.4–1.6) until day 5; then, it increased to 3.8–4.7 by the 8th day of observation. However, the dispersion of the results was elevated in the final phase of the observation. Similar cell behaviour was observed independantly from the cell-tracer used.

Precursor cell frequency and nonproliferative cell fractions

Within the lymphocyte population, some cells were not stimulated to proliferate after the addition of ConA.

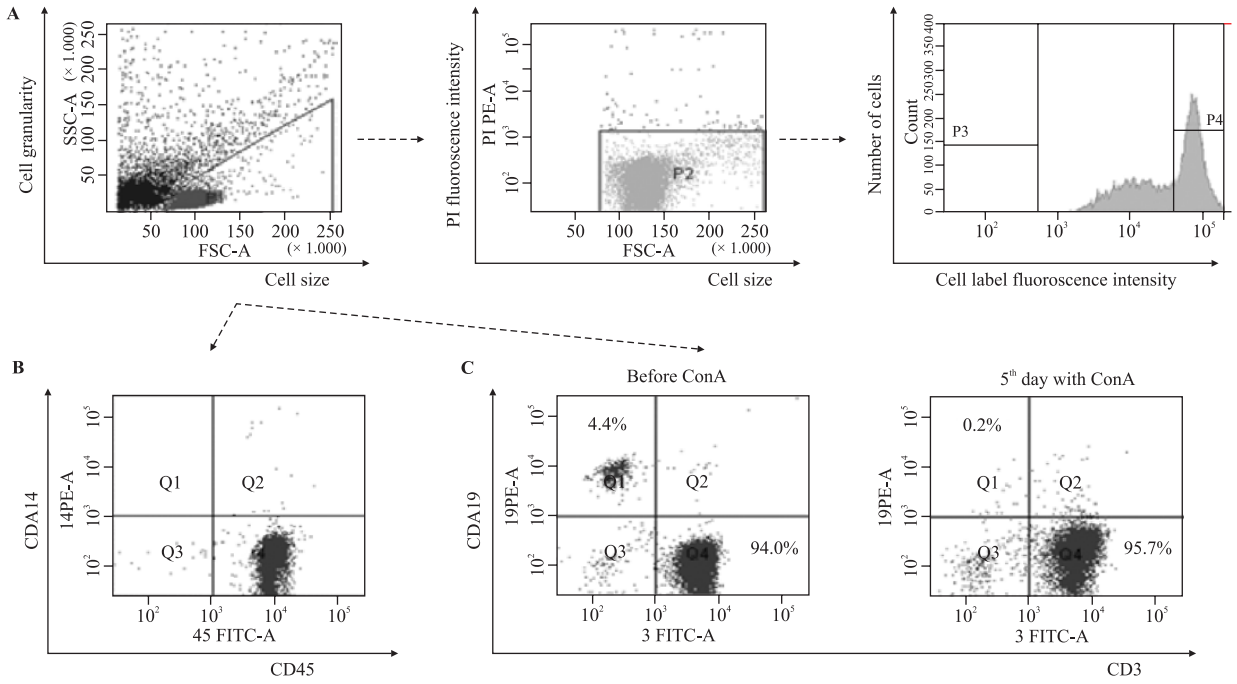

Figure 1. Gating strategy for PBMC proliferation analysis and immunophenotyping. Cells were analysed on the 5th day of culture. **A.** Cells were defined by first gating on FSC/SSC graphs (left graph) and then on FSC/PE graphs (middle graph). The P2 gating included viable cells, which did not bind propidium iodide (PI).The proliferation analyses (histogram) were performed on the cell fraction from the P2 gate. **B.** The gating on FSC/SSC included lymphocytes ($CD45^+CD14^-$), evidenced by the CD14/CD45 graph. **C.** ConA primarily activated T-lymphocyte ($CD3^+CD19^-$) proliferation; the B-lymphocyte population ($CD3^-CD19^+$) was very small, and is only slightly detectable on the graph

We applied the concentration of ConA (5 μg/mL) most frequently used in this type of test. Performed analysis for "true proliferating" cells, which included cells that were stimulated to divide at least twice, however some cells divided several times. In the ConA-stimulated population of lymphocytes, the precursor frequency was about 0.05 until day 5; then, on days 6 to 8, the precursor frequency increased (to 0.2–0.4), and was accompanied by high dispersion in the results (Figure 4A). The observed cell behaviour did not depend on the cell-tracer. On the 7th day after ConA stimulation, the precursor cell frequency in CFSE-labelled populations was significantly higher than in CellTrace Violet-labelled cell populations, but not significantly different from that observed in CellVue Claret-labelled cell populations (Figure 4A). However, at either earlier or later times during the experiment, precursor cell frequency was not significantly different between cells labelled with CFSE and those labelled with CellTrace Violet.

The nonproliferative fraction on day 5 was about 0.9, and then it decreased with all tested cell-tracers to about 0.78 on day 8 (Figure 4B). The cells labelled with CFSE had a slightly lower nonproliferative fraction than cells labelled with CellTrace Violet or CellVue Claret; however, the difference was not significant (Figure 4B). Thus, the nonproliferative fraction was similar for cell populations with different cell-tracer labels.

We noted an increase in precursor cell frequency and a decrease in the nonproliferative fraction between days 5 and 8 after ConA stimulation. Both changes were significant within the observed period of time, and they showed increased dispersion (P_{25}–P_{75}) over time. These results suggested that the changes might reflect a dynamic transformation in the population pool from nonproliferating cells to precursor cells.

Fading of tracers

Some fading of tracers was noted during the experiments. The labelled cells lost the tracer signal over time noted as a decrease in the MFI of unstimulated lymphocytes. For the days following day 5, the median ratios of MFIs were 0.99 for day 6, about 0.98 for day 7, and about 0.97 for day 8, independent of the tracer (Figure 5).

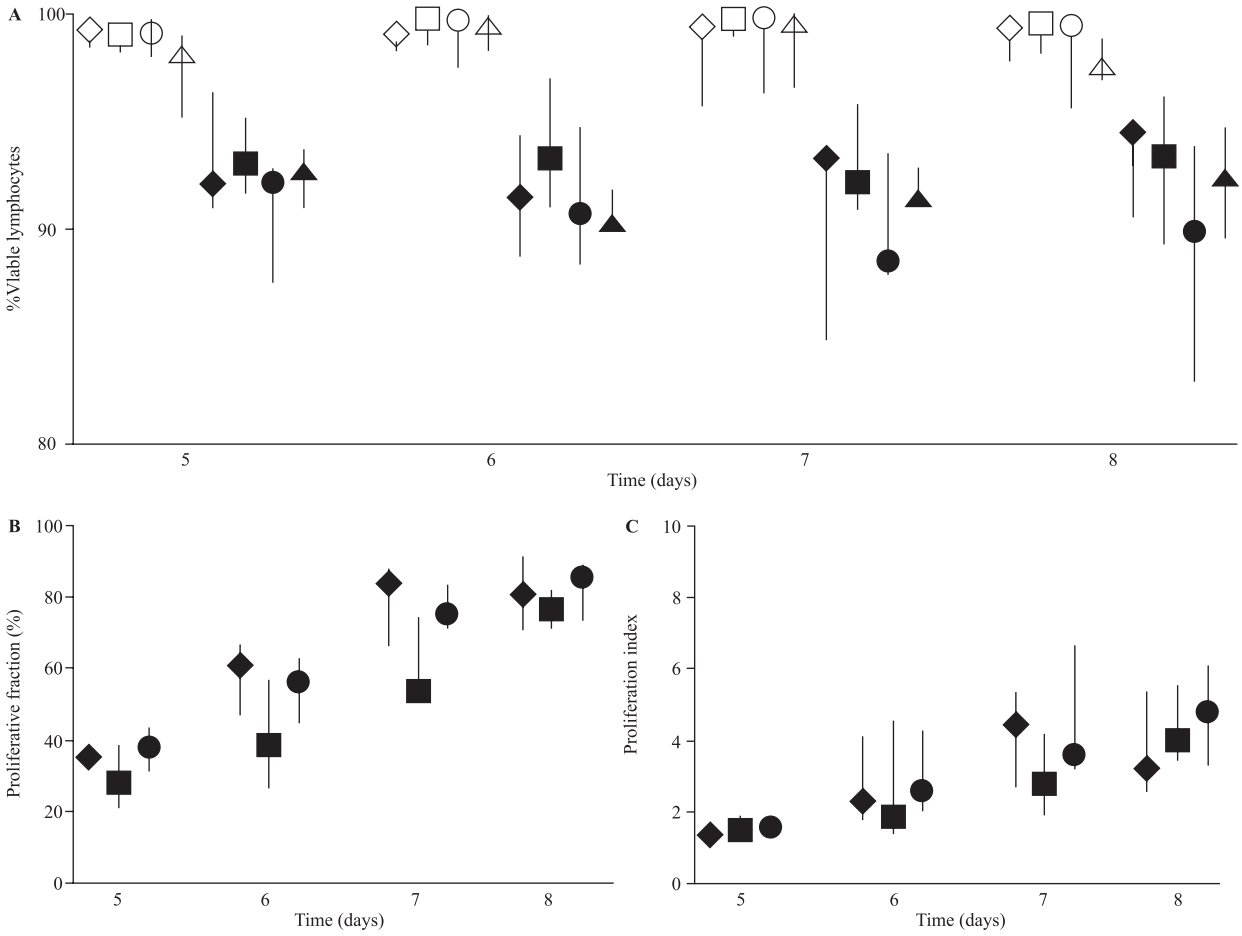

Figure 2. Viability in unstimulated or ConA-stimulated lymphocytes and the fraction of proliferating lymphocytes and the proliferation index for differently labelled populations.

Lymphocytes were unlabelled (▲, Δ) or labelled with different proliferation dyes, including CFSE (♦, ◊) , CellTrace Violet (■, □), and CellVue Claret (●, ○). All cells were cultured for 8 days with (filled symbols) or without (open symbols) ConA stimulation. Symbols indicate the median and P_{25}–P_{75} percentile values from 5 independent experiments. **A.** Viabilities are significantly lower in ConA-stimulated cultures (▲, ♦, ■, ●) compared to unstimulated cultures (Δ, ◊, □, ○); $p < 0.05$. Viabilities of lymphocytes labelled with proliferation dyes were comparable to those of unlabelled cells. **B.** The proliferative fraction and **C.** The proliferation index of labelled lymphocytes did not differ significantly among cells labelled with the different dyes

Discussion

This study aimed to compare three methods of labelling PBMCs, and then analyse their response to polyclonal stimulation with ConA. The experiments assessed the kinetics of cell viability, proliferation, and precursor cell frequency. We compared the changes in these read-out parameters due to different methods of lymphocyte cell labelling on days 5 to 8 from ConA stimulation. Our observations suggested that the three different cell labelling methods produced similar results for the proliferative fraction of cells, the precursor frequency, and the proliferation index.

Our comparison of CellTrace Violet and CSFE, which are complementary probes to CellVue Claret, cell membrane lipophilic tracer, may be instructive for polychromatic studies of immune cell proliferation and function. Within heterogeneic lymphoid cells, there are several T-cell subpopulations that respond to ConA stimulation with different numbers of sequential divisions. After cell labelling, both CD8+ and CD4+ enriched T-cell populations respond to ConA [6]. Some T cells after activation induce physiological apoptotic process [7]. Although commonly used in diagnoses, the cell tracer CSFE [8–11] emits fluorescence in the range frequently used for antibody labelling; therefore, it may be inconvenient in some studies. In those cases CellVue Claret [5] or CellTrace Violet labelling may be preferred. In particular, the latter may be useful in combination with new applications,

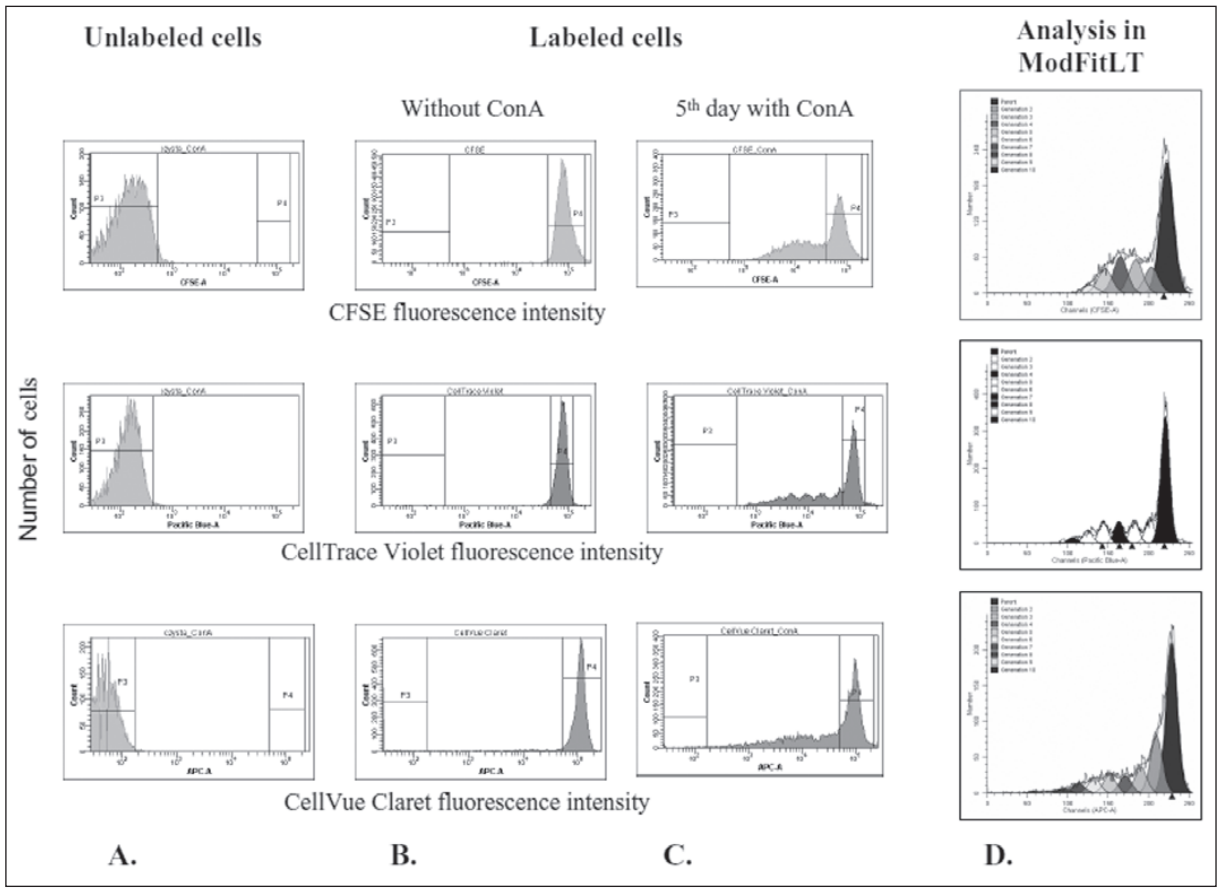

Figure 3. The characteristic subpopulations of dividing lymphocytes analysed with FacsDiva and ModFitLT application. Cell fluorescence was analysed on the 5th day after the ConA stimulation. Lymphocytes were (**A**) unlabelled or (**B, C, D**) labelled with individual proliferation tracking dyes (upper row for CFSE, middle for CellTrace Violet, and lower for CellVue Claret). Cells were cultured (B) without or (C, D) with ConA stimulation (data not shown for unlabelled cells). Cells in panels A, B, and C were analysed with FACSDiva; cells in panel D were analysed with the ModFitLT application

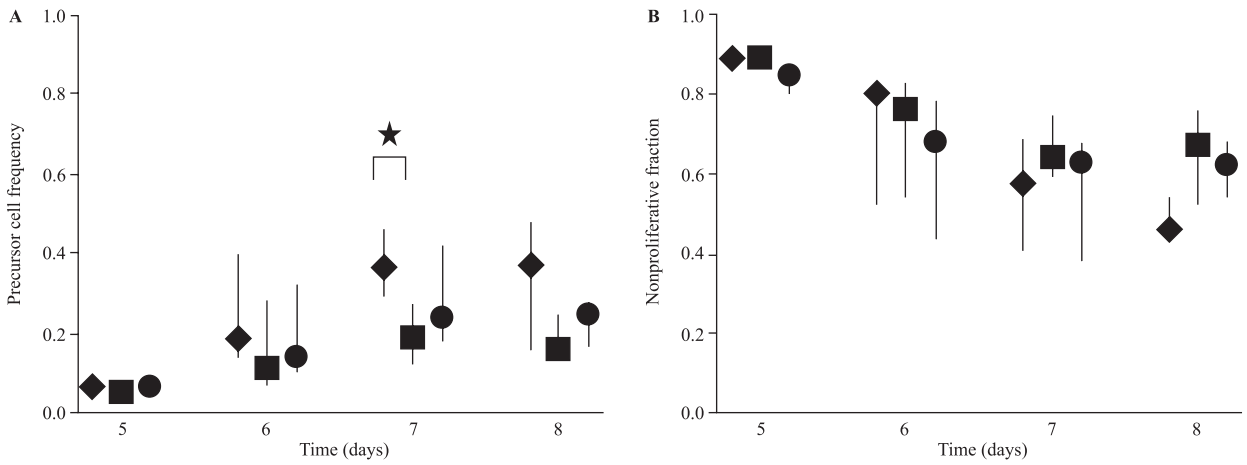

Figure 4. The precursor cell frequency and the nonproliferative fraction for differently labelled populations**.** PBMCs labelled with CFSE (♦), CellTrace Violet (■), or CellVue Claret (●) were cultured for 8 days with ConA. Symbols indicate the median and P_{25}–P_{75} percentile values from 5 independent experiments. **A.** The precursor cell frequency of lymphocytes labelled with CFSE was significantly higher than that of lymphocytes labelled with CellTrace Violet ($p < 0.05$) on the 7th day after stimulation with ConA. The precursor cell frequencies of lymphocytes labelled with the other dyes did not differ significantly. **B.** The nonproliferative fraction of labelled lymphocytes did not differ significantly among cells labelled with different dyes

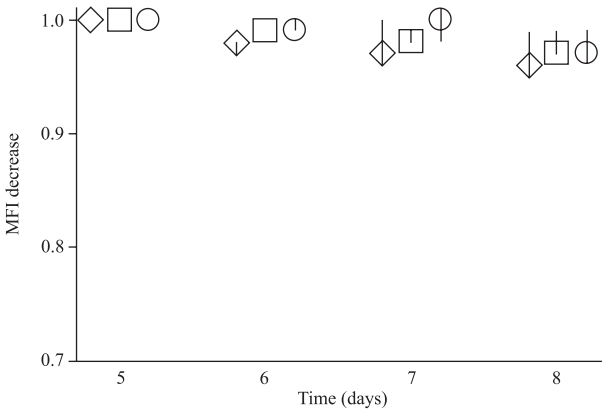

Figure 5. Median Fluorescence Intensity (MFI) fading for differently labelled populations.

Lymphocytes labelled with CFSE (◊), CellTrace Violet (□), or CellVue Claret (○) were cultured for 8 days without ConA. The rate of MFI fading was calculated for each day relative to day 5. Symbols indicate the median and P_{25}–P_{75} percentile values from 5 independent experiments. The MFI fading in labelled lymphocytes did not differ significantly among cells labelled with different dyes

like the Infinicyt, which allows multidimensional study of single flow cytometry files. CellTrace Violet emits fluorescence in a range outside the range most often used for monoclonal fluorescence labelling. In this application, it is indispensable to read in each sample, apart from the FSC and SSC, one joint fluorochrome that can be merged later into shared files, in addition to fluorochrome tracers for other cell markers.

The slow decay observed in CFSE intensity, demonstrated by the decline in the undivided peak of fluorescence, was consistent with previous findings [12]. It was shown that fluorescence fading was not connected to CFSE loss from cells during the first 24–48 h after labelling, which is due to unstably incorporated CFSE within the cells at that time [13]. In contrast, the fluorescence intensity may decrease due to the release of peripheral fragments of cell cytoplasm (bubbling) and cell membrane, independent of the cell cycle. Here, we could not define the factors responsible for the fading of the tracers; thus, we could not exclude a potential contribution from bubbling (release) of the cell peripheral cytoplasm and membrane fragments. It was noted, however, that the tested cellular fluorescent labels faded at similar rates within the observation period, despite the fact that the CellVue Claret label was incorporated into the membrane lipid bilayer [5].

In conclusion, the labelling techniques described above can be used for separate quantifications of the number of cells in the culture that have not divided and those that have divided a specific number of times. Cell tracer labelling is a powerful tool for monitoring cell migration *in vivo* and for quantitatively analysing cell division, both *in vivo* and *in vitro* [14]. Over the 8 days of ConA stimulation of lymphocytes, the survival and proliferation of labelled cells, the precursor cell frequency, and nonproliferating fraction of cells were similar for the three tested cell-tracers. It follows that the results obtained with the tested intracellular and cell membrane lipophilic tracers may be directly compared. This is particularly valuable information, because previous results obtained with CFSE can be readily compared with future results obtained with multiparameter applications.

We also noted another novel observation with all three tracer-labelled cells. We found that the nonproliferative fraction of cells could transform into precursor cells between days 5 and 8 with constant presence of ConA in the culture medium.

The practical conclusion from this study was that results from cells labelled with different tracers may be compared, and they can be discussed jointly.

Acknowledgments

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