Tech Tip: Measuring Cell Division in PMBCs by Flow Cytometry

There are many times when it is useful to determine whether cells are actively dividing. For example, immunologists use proliferation assays to monitor lymphocyte activation. In the past, lymphocyte proliferation was evaluated using radioactivity, by measuring incorporation of tritiated thymidine into the newly synthesized DNA of replicating cells. These days, it is safer and easier to use fluorescent cell proliferation dyes (like ViaFluor® SE or the classic CFSE) and measure cell division by flow cytometry.

Summary

In this Tech Tip, we will outline a typical procedure for using fluorescent ViaFluor® SE dyes for measuring lymphocyte proliferation in PBMCs, with helpful tips for getting the best results from your experiment. These protocols and tips can easily be applied to other cell types as well, with some tweaking of the procedures.

Assay Principal

Cell proliferation dyes are used for long-term cell labeling. They are initially non-fluorescent esters that diffuse passively into cells, where they covalently attach to amine groups of cytoplasmic proteins and are converted to fluorescent dyes by esterase enzymes. Immediately after staining, a single bright fluorescent population will be detected by flow cytometry. Each cell division that occurs after labeling is revealed by the appearance of a successively dimmer fluorescent peak on a flow cytometry histogram (Fig. 1). Cell proliferation dyes can be used to track cell divisions *in vivo* or *in vitro*. The staining can withstand fixation and permeabilization for subsequent antibody staining for cell phenotyping.

Assay Considerations:

- **Choosing a proliferation dye**: ViaFluor® SE Cell Proliferation Kits come in two color options, blue or green, so choose the dye that best fits into your preferred flow panel. ViaFluor® 405 and ViaFluor® 488 have low toxicity and show excellent cell division peaks in flow cytometry. We also offer CFSE, the original cell proliferation dye that has been used for decades. However, if you wish to use a green dye, we suggest using ViaFluor® 488 instead of CFSE; ViaFluor® 488 has less toxicity and better dye retention than CFSE, which allows for more division peaks to be detected.
- **Including a viability stain**: For best results, we recommend including a viability stain (such as one of our Live-or-Dye™ Fixable Viability Stains) in your flow panel to exclude dead cells from your analysis. While ViaFluor® dyes are often considered to be live cell specific because their fluorescence is dependent on cleavage by cytoplasmic esterases, they actually do label dead cells, either due to residual esterase activity near dead cells, or passive hydrolysis of the esters by water. In addition, dead cells tend to have very porous membranes that allow a lot more dye to enter than living cells. Therefore, both living and dead cells will stain with ViaFluor® SE dyes (including CFSE) (Fig. 7).

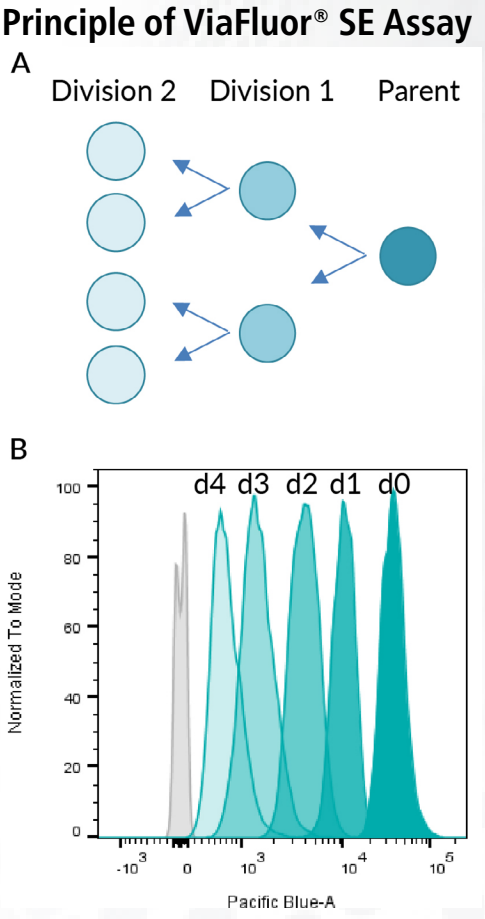

Figure 1. Principle of ViaFluor® SE cell proliferation assay. A. Cells are covalently labeled with ViaFluor® SE dye. With each cell division, the daughter cells inherit half of the fluorescent label. B. Flow cytometry histogram of Jurkat cells labeled with ViaFluor® 405 SE. At day 0 (the day of labeling) a single bright population is detected. Each division of the cells on successive days is revealed by a successively dimmer peak.

Cell Proliferation Assay in PBMCs

This procedure was optimized for measuring T cell divisions in peripheral blood mononuclear cells (PBMCs) (Fig. 3). It can be adapted for measuring cell division of other cell types and cell sources (see Fig. 1 for Jurkat cell proliferation data).

Materials required:

- ViaFluor® SE Cell Proliferation Kit
- Activation reagent(s) (cytokine, peptide, antibody, etc.)
  - **Note:** For T cell activation we use Recombinant Human IL-2 (BioLegend 589102) at 66U/0.4×10^6 cells, and Dynabeads Human T-Activator CD3/CD28 (Thermo Fisher 11161D) at a 1:1 bead: cell ratio.
- Live-or-Dye™ Fixable Viability Stain
- Primary antibody conjugates to detect cell population of interest. Used here:
  - CD3 (RIV9)-CF®488A
  - CD19 (CVID3/429)-CF®647
- Flow buffer (PBS + 2% bovine serum or BSA + 0.02% sodium azide)

Workflow overview:

1. Thaw and count cells.
2. Stain cells with ViaFluor®.
3. Quench unbound dye and wash cells.
4. Induce cells of interest to divide.
5. Incubate for desired time.
6. Stain cells with Live-or-Dye™ dead cell stain.
7. Stain with antibodies to identify the surface markers for cell type(s) of interest.
8. (Optional) Fix and permeabilize cells.
9. (Optional) Stain intracellular markers of interest.
10. Analyze by flow cytometry.

Procedure:

1. Thaw frozen PBMCs in pre-warmed cell culture media. The cells can be used immediately or cultured overnight.
2. Count the cells. Determine how many total samples you will have, including controls, in your experiment (i.e., single stains, isotype controls, etc.). Start with 0.4 million (0.4×10^6) cells per sample, and spin down the total number of cells for the samples that will be stained with the cell proliferation dye (i.e., for 4 samples, spin 1.6 million cells). If you want to have samples without proliferation dye, aliquot those into a separate tube and set them aside for now.
3. Dissolve a vial of ViaFluor® SE dye in anhydrous DMSO. These dyes are prone to hydrolysis, so for best results, use a freshly dissolved aliquot of dye. Prepare a staining solution of 1 uM ViaFluor® cell proliferation dye in an amine-free buffer such as HBSS or PBS.
    - **Tip:** The dye concentration may need to be optimized for your cell type to maximize signal while minimizing toxicity. We recommend testing a range of 1-5 uM.
4. Resuspend the pelleted cells in the ViaFluor® staining solution at 0.8 million cells/mL. Incubate the cells for 10-15 minutes at 37°C to allow uptake of the dye.
5. Add an equal volume of cell culture medium and incubate 5 more minutes, to hydrolyze any free dye.
6. Pellet the cells and resuspend in cell culture medium.
7. Induce the cells of interest to divide using the activation method of your choice.
    - **Tip:** For activation of bulk T cells in human PBMCs, we use a combination of 66U recombinant IL-2 and 0.4 million CD3/CD28 beads for 0.4 million cells. Antigen-specific T cells can be activated by incubating with the peptide antigen.
    - **Tip:** For activation of B cells in human PBMCs, there are multiple treatments that can be used, including anti-CD40 antibody, LPS, IL-7, and PMA+ionomycin. IL-2 can also weakly stimulate B cell proliferation (Fig. 4).
8. Place the cells in the incubator and culture for the desired time. For T cells activated as in step 7, 4 days of induction is enough time for approximately five cell division cycles (Fig. 3).
9. When you are ready to assay, aliquot the cells into FACS tubes, pellet, and resuspend in 1 mL PBS.
10. Add 1 uL of Live-or-Dye™ Fixable Viability Dye to each tube to stain the dead cells. Incubate for 30 minutes on ice. Pellet the cells and wash once with PBS.
    - **Tip:** Using a viability dye to exclude dead cells from the analysis will result in cleaner proliferation peaks, because proliferation dyes stain dead cells strongly (Fig. 7).
11. Stain with fluorescently labeled primary antibodies against cell type markers, to allow selective analysis of the cell type of interest. For example, if you are studying T cells, stain with an anti-CD3 antibody (here we used CD3 (RIV9)-CF®488A). For B cells, use an anti-CD19 antibody (we used CD19 (CVID3/429)-CF®647). Incubate for 30 minutes on ice. Pellet the cells.
12. (Optional) If intracellular targets are to be stained, perform fixation and permeabilization using your method of choice, followed by antibody staining.
13. Resuspend the cells in flow buffer and run on a flow cytometer, detecting fluorescence in the appropriate channels.
14. During the data analysis set up the following gates:
    - Use FSC/SSC to gate on the lymphocyte population (Fig. 2A)
    - Gate on Live-or-Dye™ negative cells to eliminate dead cells (Fig. 2B)
    - Gate on CD3+ cells to specifically analyze T cells (Fig. 2C)
    - For the live T cell population, plot the ViaFluor® SE signal as a histogram, comparing uninduced to induced cells, and count the number of peaks to determine the number of cell divisions that have taken place (Fig. 2D)

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Figures

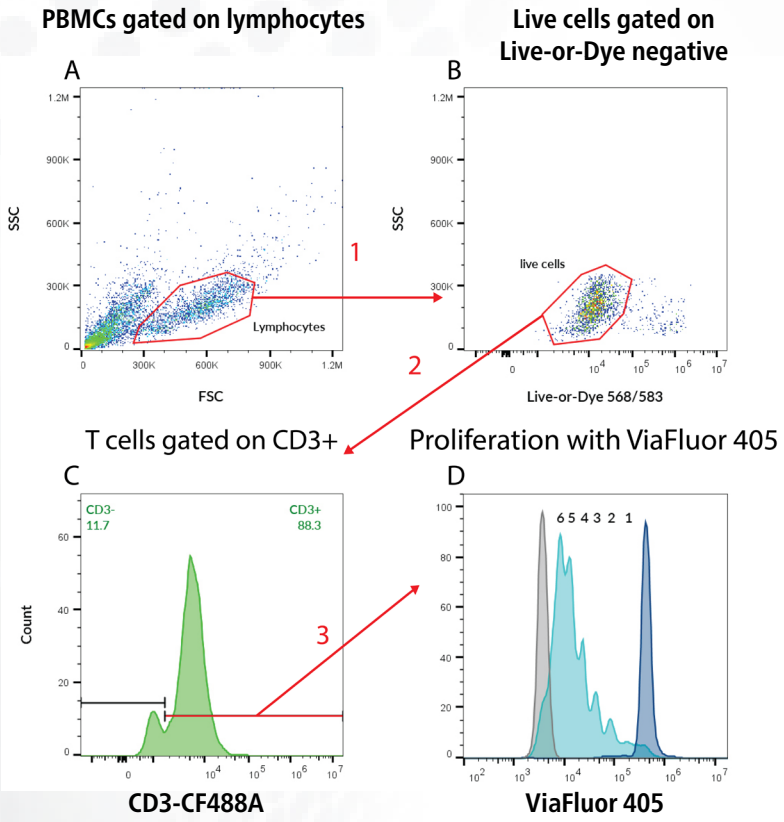

Figure 2. Gating scheme for monitoring T cell proliferation in PBMCs. PBMCs (in this case, depleted of granulocytes by density gradient) were labeled with ViaFluor® 405 SE, cultured for 5 days, and then stained with Live-or-Dye™ 568/583 dead cell stain and CD3 (RIV9)-CF®488A. A) Total PBMCs were viewed with FSC/SSC, and the lymphocytes were gated based on known FSC/SSC profile (see Fig. 5). B) The lymphocytes were viewed in the PE channel to measure Live-or-Dye™ 568/583 dead cell stain levels, and the negative population (i.e., live cells) were gated. C) The live cells were viewed in the FITC channel to detect CD3+ T cells using CD3-CF®488A. Note that CD3 levels on T cells decrease after activation (see Fig. 6), so this gate can be set using unstimulated PBMCs. The CD3+ cells were gated. D) The CD3+ live T cells were viewed in the Pacific Blue channel to monitor proliferation using ViaFluor® 405 SE. Activated T cells (cyan) were compared to unstimulated T cells (dark blue), as well as cells that had not been stained with ViaFluor® 405 (gray).

CD3+ T Cell Proliferation

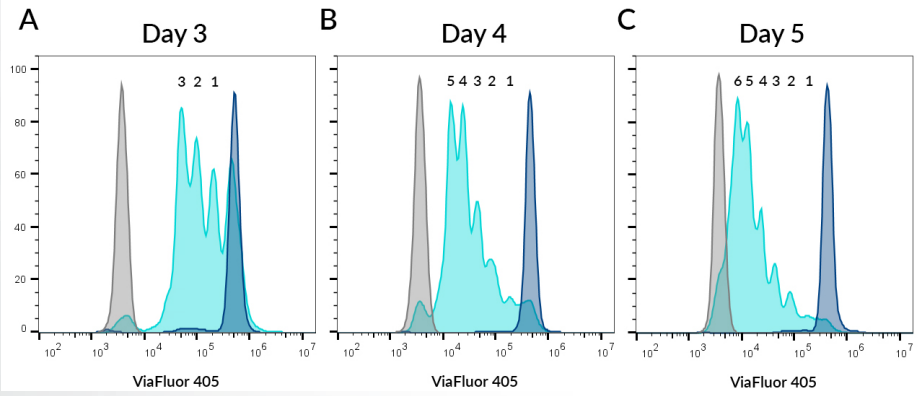

Figure 3. CD3+ T cell proliferation. PBMCs were labeled with 1 uM ViaFluor® 405 SE, incubated with IL-2 and CD3/CD28 beads to induce T cell activation, and then live CD3+ T cells were analyzed after the indicated times. By viewing the ViaFluor® 405 peak patterns, we can determine how many cell divisions the T cells underwent. A) At day 3, 3 distinct cell division peaks were seen (cyan); B) 5 peaks were visible at day 4; C) 6 peaks were visible at day 5. Unstimulated T cells (dark blue peak) did not undergo any cell division. T cells that were not stained with ViaFluor® 405 are shown in gray.

CD19+ B Cell Proliferation

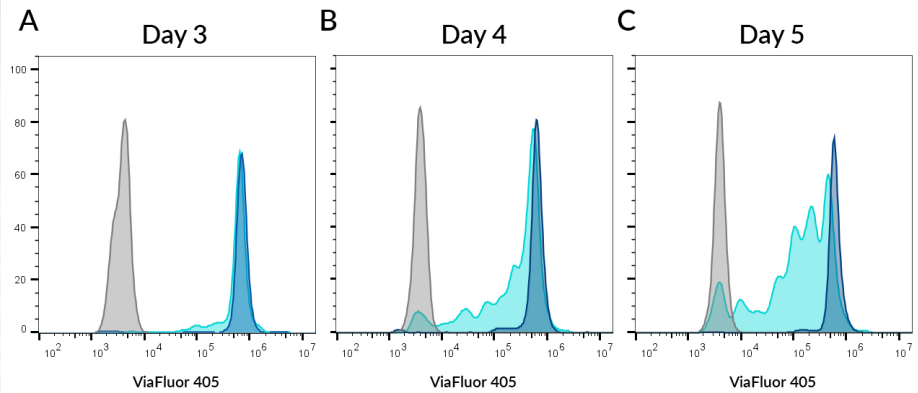

Figure 4. CD19+ B cell proliferation. PBMCs were labeled with 1 uM ViaFluor® 405 SE, incubated with IL-2 and CD3/CD28 beads, and then live CD19+ B cells were analyzed after the indicated times. We see that IL-2 treatment did induce some B cell proliferation, but it was less complete and took longer than T cells (see Fig. 3). A) At day 3, no cell division peaks were seen (cyan); B & C) at days 4 and 5 some small cell division peaks began to appear. Unstimulated B cells (dark blue peak) do not undergo any cell division. B cells that were not stained with ViaFluor® 405 are shown in gray.

Figures

Lymphocyte changes after stimulation

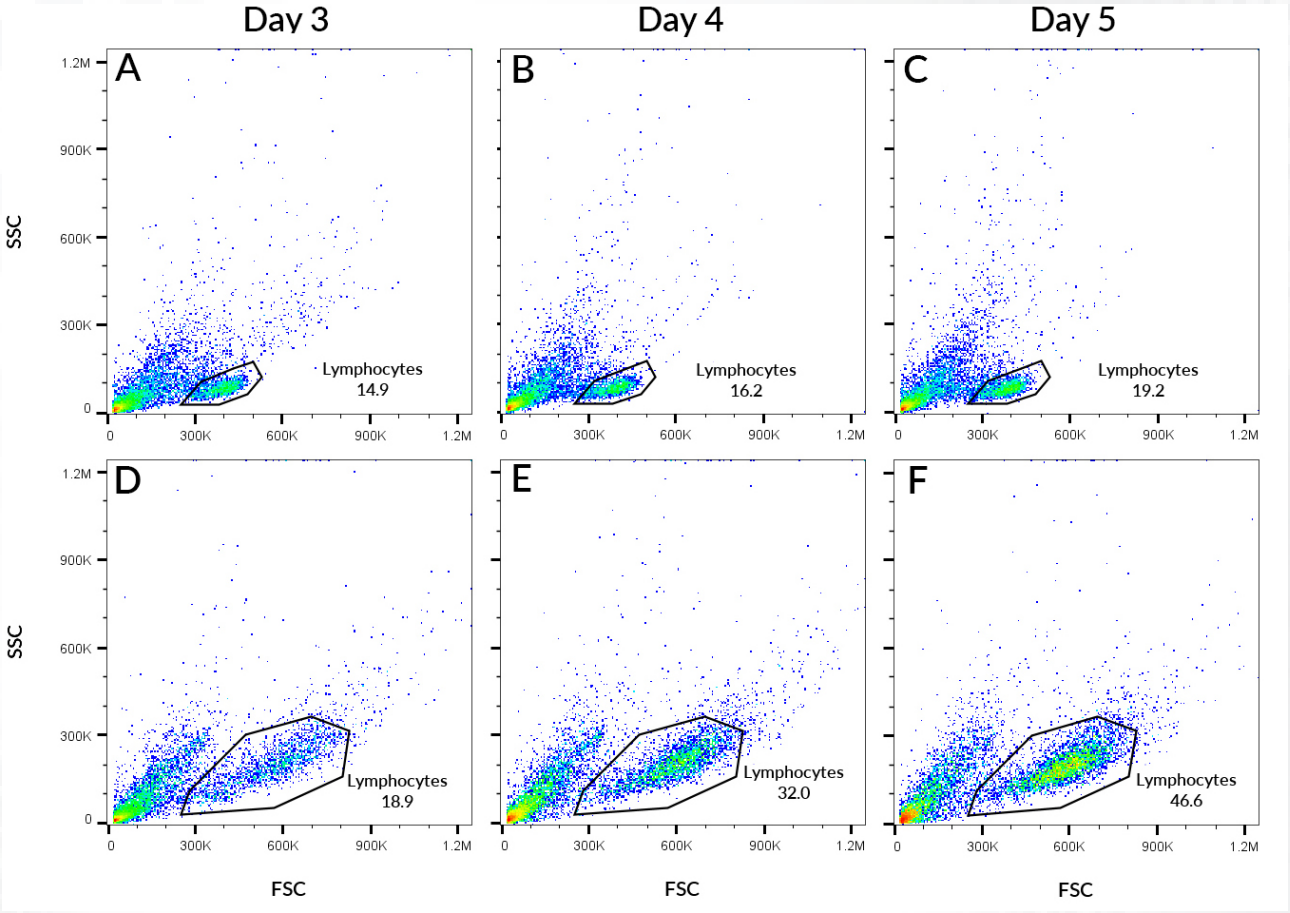

Figure 5. Lymphocyte changes after stimulation. PBMCs were incubated with IL-2 and CD3/CD28 beads to induce T cell activation, and then analyzed by FSC/SSC after the indicated times. In the top row (A-C), we can see that in unstimulated PBMCs, the cells in the lymphocyte population remained the same and at roughly the same frequency in the population after (A) 3, (B) 4, and (C) 5 days in culture. In the bottom row (D-F), we can see that after stimulation with IL-2 and CD3/CD28 beads, the cells in the lymphocyte population became larger; the size change is reflected in an extended FSC/SSC profile, apparent after (D) 3 days in culture and even more obvious after (F) 5 days. In addition, the frequency of cells in the lymphocyte gate increased from ~19% to ~47% between 3 and 5 days, reflecting the fact that the T cells were actively dividing.

Figures

T Cell changes after stimulation

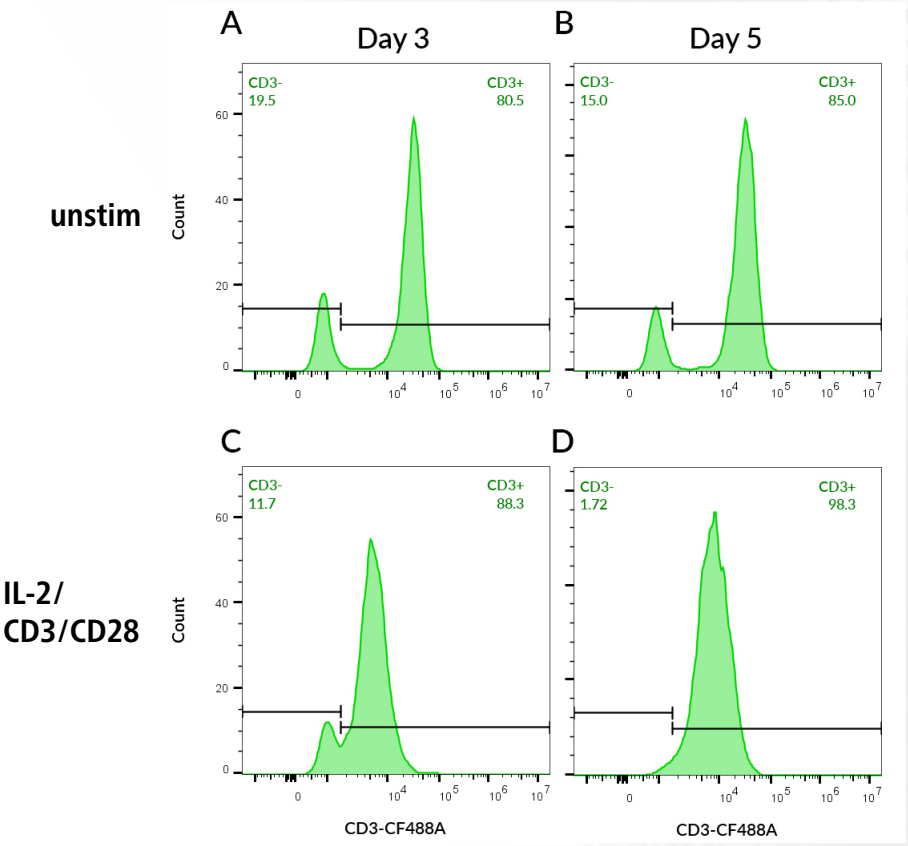

Figure 6. T cell changes after stimulation. PBMCs were incubated with IL-2 and CD3/CD28 beads to induce T cell activation, and then stained with CD3-CF®488A to label T cells after the indicated times. The cells were gated on live lymphocytes, then CD3 expression was analyzed in the FITC channel. In the top row (A-B), we can see that in unstimulated PBMCs, the CD3+ T cells showed relatively high CD3 signal, and good separation between CD3+ and CD3- lymphocytes. Also, the frequency of CD3+ cells remained relatively constant after (A) 3 and (B) 5 days in culture. In the bottom row (C-D), we can see that after stimulation with IL-2 and CD3/CD28 beads, the CD3 signal on the CD3+ T cells went down, as receptor was internalized after activation. In addition, the frequency of CD3+ cells increased from 88% to 98%, reflecting the fact that the T cells were actively dividing.

Live vs. dead T cells

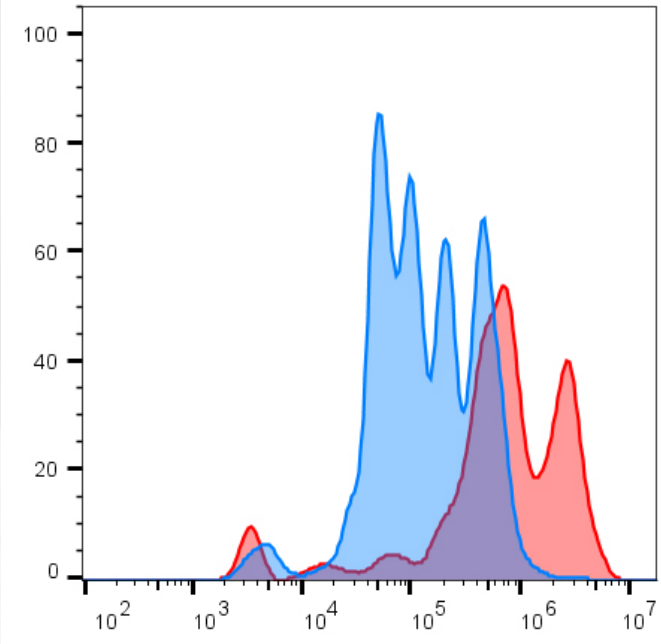

Figure 7. ViaFluor® 405 signal in live and dead T cells. PBMCs were cultured for 3 days with IL-2 and CD3/CD28 beads to induce T cell activation, and then gated on CD3+ T cells. The cells were then gated on live or dead cells using the dead cell stain Live-or-Dye™ 568/583, and then viewed in the Pacific Blue channel to monitor cell proliferation via ViaFluor® 405 staining. While the live T cells (blue) showed 4 distinct cell divisions peaks (representing 0, 1, 2, and 3 cell divisions), the dead cells (red) showed two bright peaks, because dead cells with porous cell membranes are able to absorb a lot of dye. For this reason, dead cells should be excluded when performing cell proliferation analysis.