Application Note

Cell Analysis

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Generational Analysis of T Cell Proliferation using Cell Tracking Dyes

Authors

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Abstract

Cell proliferation analysis is essential when monitoring cell growth and differentiation, and tracking cellular responses to various stimuli. Generational proliferation was monitored in peripheral blood mononuclear cells (PBMCs) using cell tracking dyes, with automated flow cytometric analysis with Agilent NovoExpress software. The results demonstrate the utility of using flow cytometry for simultaneous assessment of proliferation with respect to T cell subtype, as well as the value of automated analysis for ensuring the consistency of results.

Introduction

Cell proliferation is one of the most fundamental processes in biology. Quantitative analysis of cell proliferation is crucial for studies of cell growth and differentiation and is used to evaluate compound toxicity and inhibition of tumor cell growth during drug development. Many assays are used to measure cell proliferation, including measurements of mitochondrial enzymes (MTT), adenosine triphosphate (ATP), cell counting, expression of cell cycle associated proteins (Ki-67), evaluation of DNA synthesis though radioactive tracers (^{3}H-thymidine incorporation), nucleotide analogues (BrdU), as well as using cell-tracking dyes. While many of these assays offer bulk population analysis, they do not allow measurements of individual or mixed populations of cells. In contrast, flow cytometry assays are ideal for measuring the proliferation of individual cells, with the ability to identify different cell types in a single sample.

Generational analysis determines how many times a given cell has divided and can measure heterogeneous growth within the same population of cells. This assay is performed by measuring the dilution of a cell-tracking dye as a cell proliferates. There are several fluorescent dyes available for this purpose that typically labels either the cytoplasmic components or the cell membrane. Carboxyfluorescein diacetate succinimidyl ester (CFSE) is a commonly used cytoplasmic tracking dye; it passively diffuses into cells and attaches to cytoplasmic components resulting in a uniform bright fluorescence. As the cell divides, the dye is equally partitioned between the two daughter cells, resulting in cells with half the fluorescence intensity of the parent population. Through this method, 6 to 8 distinct successive generations (in some cases up to 10) of cell division can be labeled. Tracking cell proliferation in this manner is most applicable to studying lymphocytes, stem or progenitor cells, cancer cell lines, as well as yeast and bacteria.

T cells are lymphocytes important for the adaptive immune response to pathogens or cancer. Upon antigen binding to the T cell receptor complex (TCR), T cells become activated and proliferate. Binding of CD3 antibody (part of the TCR complex) to T cells can replicate the T cell activation process *in vitro*, resulting in rapid cellular changes and proliferation. Flow cytometry allows the simultaneous measurement of cell proliferation and immunophenotyping, thus determining the rate of cell proliferation in both the CD4 and CD8 T cell populations simultaneously.

This application note applies flow cytometry generational analysis of proliferation in T cells derived from human peripheral blood, examining differences in cell-tracking dyes as well as titration of activating antibody.

Methods

Generational analysis of T cell proliferation using cell-tracking dyes

To examine the abilities of different cell-tracking dyes to distinguish cell divisions in primary T cells, PBMCs were labeled with either ViaFluor488, CFSE, or CellTrace Violet followed by activation with anti-CD3 antibody.

The T cell proliferation assay in PBMCs was performed as follows:

1. PBMCs were isolated using Ficoll-Paque PLUS following the manufacturer's instructions.
2. Plate was coated with anti-CD3 antibody diluted to final concentration of 0.5 µg/mL in PBS. 400 µL/well was added to a 24-well plate and incubated for 2 hours at 37 °C.
3. Prepared CFSE working solution: 1 to 10 µM CFSE in PBS (5 µM commonly used for PBMCs).
4. Cells were centrifuged and resuspended at 10 to 100 × 10^{6} cells/mL in CFSE working solution for 10 minutes at room temperature.
5. Excess CFSE was removed by adding 5 to 10x the original volume of cell culture medium or PBS containing at least 5% FBS.
6. Cells were centrifuged and resuspended in final cell culture medium with the addition of 100 U/mL of human IL-2. 0.5 × 10^{6} CFSE-labeled PBMCs were plated per well in the anti-CD3 antibody-coated 24-well plate.
7. Cells were allowed to proliferate for 2 to 6 days at 37 °C, 5% CO_2 in an incubator, then CFSE dilution was analyzed on an Agilent Flow Cytometer.

Automated analysis of cellular proliferation

Integration of flow cytometry software, such as NovoExpress, allows quick, thorough, and automatic analysis of cell proliferation, providing more information from this dye dilution assay. NovoExpress uses data modeling to determine the number of peaks/divisions represented in the population, and fits the model to the sample.

Proliferation assays typically focus on three calculations for cell analysis:

- **Frequency Divided:** Percentage of original cells that have undergone cell division
- **Division Index:** Average number of divisions of total cells
- **Proliferation Index:** Average number of divisions of responding cells (cells that have undergone at least one division)

The automatic data analysis provides this additional information about the proliferation of the cells. Replicating the automatic analysis of cell proliferation by manual means may seem easy, however, setting gates to avoid peak overlap is difficult, and cells typically become over-represented after the second round of division. Data modeling can determine a more accurate percentage of dividing cells by correcting the frequency of each generation.

Results and discussion

Five days after activation, cell division was examined in both the CD4 and CD8 T cell populations (Figure 1). The addition of anti-CD3 antibody induced proliferation of both CD4 and CD8 T cell populations compared to controls as seen by a decrease in fluorescence compared to the unstimulated control (red histogram). To analyze the proliferation of CD4 and CD8 T cells in each sample, the cell proliferation module in NovoExpress was used to automatically determine each cell division (Figure 1B). The cell proliferation module could resolve seven division peaks in ViaFluor488-labeled cells, instead of eight divisions in cells that were labeled with CFSE or CellTrace Violet (Figure 1B). The resolution of cell division was comparable when using either CFSE or CellTrace violet and resulted in similar numbers for the frequency of dividing cells, division index, and proliferation index (data not shown).

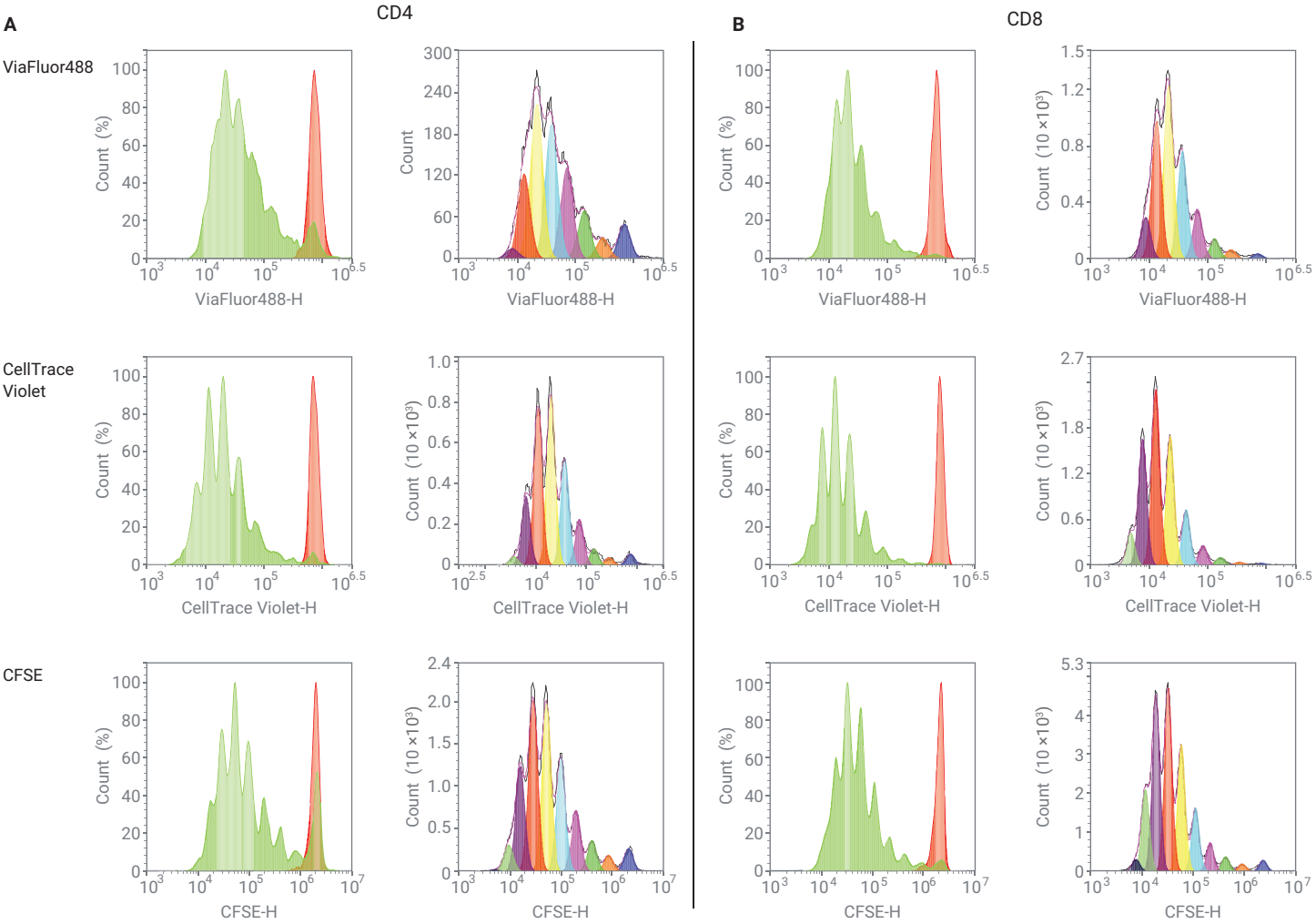

Figure 1. Difference in the resolution capabilities of commercially available cell tracing dyes. PBMCs were isolated from a healthy donor using Ficoll–Paque. A 48-well plate was pre-coated with anti-CD3 antibody (5 µg/mL) for 1 hour and washed with PBS prior to addition of cells. PBMCs were stained with 1 µM of indicated dye (CFSE, ViaFluor488, or CellTrace Violet) and seeded at 2×10^5 cells/well in complete RMPI media and 100 ng/mL IL-2. After 5 days stimulation, cells were stained with anti-CD3 ECD, anti-CD4 PE-Cy7, and CD8 APC antibodies. Cells were then analyzed on the NovoCyte Quanteon for proliferation of both the CD4 (A) and CD8 (B) T cell populations. The left plot in each section shows cell proliferation with activating anti-CD3 antibody (green) compared to wells in which no anti-CD3 antibody was added (red). The right plot demonstrates the data fitting done by the cell proliferation analysis module in the Agilent NovoExpress software.

Notably, in all samples, proliferation of the CD8 T cell population was higher than in the CD4 population. For example, 35% of the CFSE-labeled CD4+ T cells were proliferating, while in the same sample 70.5% of the CD8+ T cells were proliferating.

Concentration of anti-CD3 antibody dramatically affects T cell proliferation

Cell proliferation analysis allows you to differentiate between individual cell divisions and identify key differences between populations. The correct titration of activating compounds is important in these types of experiments since too much proliferation decreases the resolution of cell division, while too little proliferation will result in no cell division.

In the examples shown here, the coating anti-CD3 antibody was titrated from 0 to 1 µg/mL to determine the ideal concentration to induce cell proliferation (Figure 2). As expected, increasing the amount of anti-CD3 antibody resulted in more proliferation of T cells, however, the resolution of division peaks was improved using 1 µg/mL anti-CD3 antibody compared to 0.25 µg/mL (Figure 2A).

Also, there are apparent differences between the proliferation of CD4 and CD8 T cells. The frequency of dividing cells and division indices were the same in both populations at all concentrations of CD3, but the proliferation index was higher in the CD8 T cell population (Figure 2B). Although the percentage of cells that are dividing are equal in both CD4 and CD8 populations, the cells that have divided in the CD8 T cell population have undergone more cell divisions.

These data demonstrate the importance of using automated cell proliferation modeling for data analysis, since often more information can be obtained by assessing more than just the percentage of dividing cells.

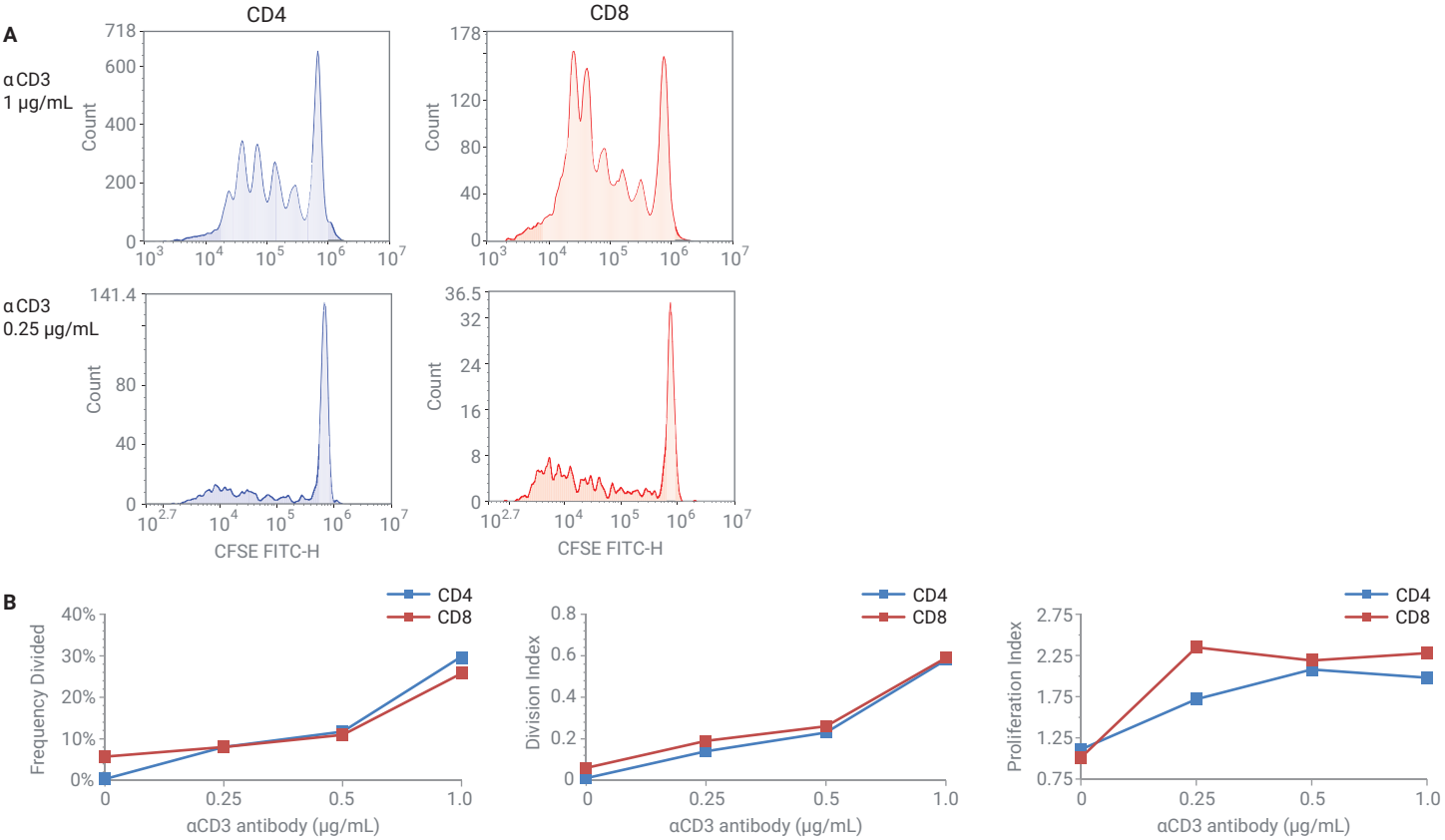

Figure 2. Increasing the concentration of anti-CD3 antibody increases T cell proliferation. PBMCs were isolated from a healthy donor using Ficoll–Paque. A 24-well plate was pre-coated with indicated anti-CD3 antibody (0, 0.25, 0.5, or 1 µg/mL) for 2 hours and washed with PBS prior to addition of cells. PBMCs were stained with 5 µM CFSE and seeded at 0.5×10^6 cells/well in complete RMPI media and 100 ng/mL IL-2. After 4 days stimulation, cells were stained with anti-CD3 PE, anti-CD4 BV421, and CD8 APC antibodies. Cells were then analyzed on the Agilent NovoCyte Quanteon for cell proliferation of both the CD4 and CD8 T cell populations. Representative plots of CFSE dilution for CD4 (blue) and CD8 (red) T cells shown in the left panel. The cell proliferation analysis module in NovoExpress was used to calculate the frequency of divided cell, division index, and proliferation index.

Optimizing the CFSE cell proliferation assay of PBMCs

To optimize the resolution of cell division in your dye dilution assay, several aspects of your protocol might require optimization, including:

- Titration of activating compound/antibody
- Titration of tracking dye concentration
- Collection timepoint

When an activating compound/antibody is used to induce cell proliferation, titrating the concentration is important to achieve an optimal rate of proliferation and resolve as many distinct cell divisions as possible. Most cell tracking dyes can resolve approximately 6 to 8 cell divisions—and in some cases up to 10—but if the cells are dividing too rapidly, the resolution will be lost between distinct divisions. However, if no activating compound (or too little) is added when it is essential to induce proliferation, the cells will not grow, and no divisions will be detected.

Additionally, titration of the cell-tracking dye concentration and staining time is also important for your cell proliferation assay. Successful proliferation analysis requires an extremely bright dye that stains cells uniformly so that it is easy to distinguish fluorescently labeled cells after several cell divisions. The ideal concentration may differ with the specific cell types, but for CFSE it is usually in the range of 1 to 10 µM for 10 million cells/mL. Some cell-tracking dyes can affect the viability of the cell, either immediately or after long-term culture; therefore, we recommended to use the lowest concentration of dye that allows you to see at least 6 to 8 peaks of cell division.

Finally, determining the best time point is crucial for data analysis. When using PBMCs for T cell proliferation analysis, most assays allow 4 to 6 days for cell growth. However, when working with different cell types, the optimal time point can vary. In addition, including these two crucial controls in your experiment is important: 1) unstained control, and 2) unstimulated control. These controls determine the background fluorescence and the location of unstimulated parent cells that have not undergone cell division. Using these tips for cell proliferation studies will help you optimize your assay and extract the most information from your experiments.

Conclusion

Cell proliferation analysis is useful for characterizing cellular response, especially T cell activation. Evaluating single-cell generational analysis by flow cytometry is particularly well suited for the study of heterogeneous mixtures of cells. New advances in flow cytometry software, such as NovoExpress, provide increased information and an automated method to analyze proliferation.

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