Tracking Antigen-Driven Responses by Flow Cytometry: Monitoring Proliferation by Dye Dilution

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Abstract
Cell-tracking reagents such as the green-fluorescent protein labeling dye CFSE and the red-fluorescent lipophilic membrane dye PKH26 are commonly used to monitor cell proliferation by flow cytometry in heterogeneous cell populations responding to immune stimuli. Both reagents stain cells with a bright homogeneous fluorescence, which is partitioned between daughter cells during each cell division. Because daughter cell fluorescence intensities are approximately halved after each division, the intensity of a cell relative to its intensity at the time of staining provides information about how many divisions it has undergone. Knowing how many rounds of division have occurred and the relative number of cells in each daughter generation, one can back-calculate the number of cells in the original population (i.e., cells present at the time of stimulus) that went on to respond by proliferating. Using this information, the precursor cell frequencies and extent of expansion to a specific antigen or mitogen of interest can be calculated. Concurrently, the phenotype of the cells can be determined, as well as their ability to bind antigen or synthesize cytokines, providing more detailed characterization of all cells responding to the antigen, not just effector cells. In multiparameter flow cytometric experiments to simultaneously analyze antigen-specific tetramer binding, cytokine production and T-cell proliferation, we found that only approximately half of the cells that exhibited specific binding to influenza tetramer also proliferated, as measured by dye dilution, and synthesized IFNγ in response to antigen. We expect the advent of new cell tracking dyes emitting from the violet to the near infrared combined with the increasing number of lasers and detectors on contemporary flow cytometers to further expand the usefulness of this approach to characterization of complex antigen-driven immunological responses.

Key terms
cell tracking; CellVue dyes; CFSE; dye dilution proliferation assay; flow cytometry; PKH dyes; precursor frequency; proliferation analysis

INTRODUCTION

Bulk vs. Single-Cell Methods

Cell proliferation has historically been monitored by the incorporation of radio-nucleotides such as tritiated thymidine (3H-TdR) into newly synthesized DNA. This bulk assay cannot distinguish small numbers of highly responsive cells from larger numbers of modestly responsive cells (1) and is not compatible with recovery of viable daughter cells for further characterization. In addition to concerns related to handling and disposing of radioisotopes, 3H-TdR uptake reflects proliferation over a relatively narrow window of time, identifying only those cells which have recently synthesized DNA and provides no information on whether labeled cells have gone on to divide or the number of cell divisions that occurred prior to exposure to the 3H-TdR pulse.

Newer methods using flow cytometry address many of the shortcomings of 3H-TdR and enable data rich investigation of immune responses to antigen, which
involve complex interactions among multiple cell types. Cell tracking dyes have proven useful for qualitative and quantitative monitoring of cell division, both in vivo and in vitro (2,3). These dyes generate a fluorescent signal that is relatively stable in nondividing cells but progressively decreases with each round of cell division. Reduction in fluorescence intensity can be quantified by flow cytometry in conjunction with any of several different algorithms to estimate extent of proliferation based on dye dilution. A major advantage of using flow cytometry and cell-tracking dyes to monitor extent of cell division is that the cells can also be stained for expression of other cell surface or intracellular markers to define lineage, functionality, activation state, cytokine expression, etc. (4–6).

**General Classes of Proliferation Tracking Dyes**

Fluorescent cell-tracking dyes that have been used to monitor cell proliferation by flow cytometry are summarized in Table 1 and generally fall into one of the two following classes:

A. **Reactive compounds that form random covalent bonds with amino groups on cellular proteins (general protein labels; Supplemental Fig. S1).** Carboxyfluorescein diacetate succinimidyl ester (CFDA-SE) has been the most widely used dye in this class. Initially a colorless nonpolar analog of fluorescein, it diffuses into the cytoplasm where its acetal substituents are cleaved by nonspecific esterases, forming the fluorescent product carboxyfluorescein succinimidyl ester (CFSE). CFSE reacts with amine groups on peptides and proteins under physiologic conditions, forming a stable amide bond. Weston and Parish (7) were the first to report using CFSE for long-term tracking of lymphocyte migration, but did not appreciate at that time that it could also be used to monitor the division of labeled lymphocytes. Their colleague, Bruce Lyons, subsequently noticed the dilutional effect during long-term tracking studies of B cells in vivo (1). Since then CFSE has been widely used to track differential proliferative responses in immune cell subsets both in vitro (e.g., 1,8–14) and in vivo (e.g., 2,15–18). It has also been reported on in a number of special focus publications (e.g., Immunology and Cell Biology, volume 77 (6), 1999; and Immunological Investigations, volume 36 (5,6), 2007), and increasingly sophisticated methods have been developed to allow estimation of cell division kinetics and precursor cell frequencies based on CFSE intensity distributions following stimulation (1,8,9,11,19–23).

Extremely bright labeling is readily achieved due to trapping of the polar CFSE within the cytoplasm, making it possible to detect six to eight rounds of cell division so long as labeling levels used do not alter protein regions critical for cell function. CFSE is available from the Molecular Probes division of Invitrogen (Carlsbad, CA), Sigma-Aldrich (St. Louis, MO) and a variety of other suppliers. Molecular Probes also makes a succinimidyl ester derivative of their 488-nm excitable Oregon Green carboxylic acid diacetate (carboxy-DFFDA SE), which is reportedly less sensitive to pH and light than CFSE and was reported in one meeting abstract to be less toxic and to give brighter staining than CFSE when used to stain porcine lymphocytes (24). Intracellular trapping of SNARF-1 has been reported to have utility for proliferation tracking, although labeling is not covalent and starting intensities are substantially lower than with CFSE due to the requirement for detection in the far red (well beyond the emission maximum) in order to avoid the pH sensitivity for which this dye is normally used (25). Two reports have also described the use of BRSE (Invitrogen), an orange-emitting amine-reactive analog of BODIPY 558/568, for qualitative proliferation monitoring (26,27). However, this dye also gives lower starting intensities, presumably because it is a neutral, relatively nonpolar fluorophore that is not trapped in the cytoplasm. In addition, although one would expect it to behave similarly to CFSE, it has not, to the best of the authors’ knowledge, ever been formally validated for quantitative dye dilution analysis.

B. **lipophilic compounds that partition stably but noncovalently into the plasma membrane (general membrane labels; Supplemental Fig. S1).** The PKH dyes have been the most widely used dyes in this class. Because of their extreme lipophilicity, homogeneous staining with membrane dyes is best achieved under salt-free, iso-osmotic conditions, where their long aliphatic hydrocarbon “tails” and polar fluorescent “headgroup” facilitate rapid intercalation into lipid bilayers and stable retention through strong noncovalent interactions. As with CFSE, extremely bright labeling is readily achieved and overlabeling must be avoided. However, cellular growth, viability, and proliferation are typically unaffected by membrane dye concentrations that allow detection of six to eight daughter generations (28–34) and membrane dyes have been extensively used for in vitro and in vivo proliferation analysis in normal and abnormal lymphocytes and hematopoietic cells (3,14,30,35–45). Originally developed by Paul Horan and colleagues at Zynaxis Molecular Targeting Technologies, Inc. (West Chester, PA), the PKH dyes became commercially available in 1989 and have been distributed by Sigma-Aldrich (St. Louis, MO) since 1993. The spectral range spanned by lipophilic dyes useful for proliferation analysis has recently been further expanded by the advent of the CellVue® dyes developed by PTI Research (33,34), many of which are commercially available from Sigma-Aldrich, Polysciences, Inc. (Warrington, PA) or Molecular Targeting Technologies, Inc. (West Chester, PA).

**Advantages of Proliferation Tracking for Monitoring Antigen-Driven Immune Responses**

Dyes from both of the above classes have the advantage of being able to stably, brightly, and homogeneously label a wide range of cell types and to enable proliferation tracking for six to eight divisions before fluorescence dilution and overlap with unlabeled cells begins to make assignment of daughter cell generation number inaccurate. The exact number of generations that can be followed depends upon the initial intensity that can be achieved without compromising cell viability or function, which varies somewhat with the particular dye and instrument being used (34). An important feature of the dye dilution technique is that division-dependent changes in the expression of cell surface markers (e.g., CD25, CD69, and references 14,46,47) intracellular proteins (e.g., cytokines and references 4,9,11), transgene expression (5), antigen binding (4) or other properties of interest can be readily quantified by flow cytometry.
<table>
<thead>
<tr>
<th>STAINING MECHANISM</th>
<th>DYE</th>
<th>EMISSION MAX (NM)</th>
<th>USEFUL LASER LINES (NM)</th>
<th>SPECTRALLY COMPATIBLE WITH</th>
<th>NOTES AND CAVEATS</th>
<th>SELECTED REFERENCES</th>
</tr>
</thead>
<tbody>
<tr>
<td>General protein labeling (random covalent binding to free amino groups)</td>
<td>CFSE</td>
<td>525</td>
<td>488</td>
<td>Hoechst 33342, PE, Cy3, PE-Cy5, PE-Cy7, TR, PI, some red FPs, PerCP, 7-AAD, APC, TO-PRO-3, DRAQ5, APC-Cy7</td>
<td>Most commonly used protein labeling dye</td>
<td>(18,21,65)</td>
</tr>
<tr>
<td></td>
<td>Oregon Green 488, SE</td>
<td>518</td>
<td>488</td>
<td>Hoechst 33342, FITC, CFSE, green FPs, TR, PI, some red FPs, PE-Cy5, 7-AAD, APC, TO-PRO-3, DRAQ5, PE-Cy7, APC-Cy7</td>
<td>SE form of BODIPY 558/568; dimmer staining than CFSE (uncharged dye is not trapped). Correlation of dye dilution with cell growth not shown</td>
<td>(26,27)</td>
</tr>
<tr>
<td></td>
<td>BRSE</td>
<td>568</td>
<td>488, 514, 543</td>
<td>Hoche</td>
<td>Most commonly used protein labeling dye</td>
<td>(18,21,65)</td>
</tr>
<tr>
<td></td>
<td>DDAA-SE</td>
<td>659</td>
<td>633–635, 647</td>
<td>Hoechst 33342, FITC, CFSE; green/yellow FPs, PE, Cy 3, PE-Cy5, PI, TR; some far-red FPs, PerCP, 7-AAD, PE-Cy7, APC-Cy7</td>
<td>Reported to dilute out more rapidly in vivo in mice vaccinated with cognate than control antigen. Correlation of dye dilution with cell growth not shown</td>
<td>(66)</td>
</tr>
<tr>
<td>Intracellular trapping</td>
<td>SNARF-1</td>
<td>&gt;650</td>
<td>488</td>
<td>Hoechst 33342, FITC, PE, APC, TO-PRO-3, DRAQ5, APC-Cy7</td>
<td>10X dimmer than CFSE (far red detection required to avoid pH sensitivity)</td>
<td>(25)</td>
</tr>
<tr>
<td>General membrane labeling (non-covalent intercalation in lipid bilayer)</td>
<td>CellVue Lavender</td>
<td>461</td>
<td>405</td>
<td>FITC, CFSE, some green FPs, TR, PI; yellow-red FPs, PE-Cy5, 7-AAD, APC, TO-PRO-3, DRAQ5, PE-Cy7, APC-Cy7</td>
<td>1-2 fewer generations detectable than with visible and far red proliferation dyes due to higher violet-excited autofluorescence</td>
<td>(34)</td>
</tr>
<tr>
<td></td>
<td>PKH67</td>
<td>502</td>
<td>488</td>
<td>Hoechst 33342, PE, Cy3, PE-Cy5, PE-Cy7, TR, PI, some red FPs, PerCP, 7-AAD, APC, TO-PRO-3, DRAQ5, PE-Cy7, APC-Cy7</td>
<td>Most commonly used green membrane dye (better membrane stability than PKH2)</td>
<td>(4,51,61)</td>
</tr>
<tr>
<td></td>
<td>PKH2</td>
<td>504</td>
<td>488</td>
<td>Hoechst 33342, PE, Cy3, PE-Cy5, PE-Cy7, TR, PI, some red FPs, PerCP, 7-AAD, APC, TO-PRO-3, DRAQ5, PE-Cy7, APC-Cy7</td>
<td>Largely replaced by PKH67</td>
<td>(45,67)</td>
</tr>
<tr>
<td></td>
<td>PKH26</td>
<td>567</td>
<td>488, 514, 543</td>
<td>Hoechst 33342, FITC, CFSE, green FPs, TR, PI, some red FPs, PE-Cy5, 7-AAD, APC, TO-PRO-3, DRAQ5, PE-Cy7, APC-Cy7</td>
<td>Most commonly used orange/red membrane dye</td>
<td>(4,51,61)</td>
</tr>
<tr>
<td></td>
<td>CellVue Plum</td>
<td>671</td>
<td>633–635, 647</td>
<td>Hoechst 33342, FITC, CFSE, green/yellow FPs, PE, Cy 3, PE-Cy5, PI, TR; some far-red FPs, PerCP, 7-AAD, PE-Cy7, APC-Cy7</td>
<td>~ 0.5 log dimmer than CellVue Claret</td>
<td>(34)</td>
</tr>
<tr>
<td></td>
<td>CellVue Claret</td>
<td>675</td>
<td>633–635, 647</td>
<td>Hoechst 33342, FITC, CFSE, green/yellow FPs, PE, Cy 3, PE-Cy5, PI, TR; some far-red FPs, PerCP, 7-AAD, PE-Cy7, APC-Cy7</td>
<td>Comparable to CFSE and PKH26</td>
<td>(33)</td>
</tr>
<tr>
<td></td>
<td>CellVue NIR780</td>
<td>776</td>
<td>780</td>
<td>Hoechst 33342, FITC, CFSE, green/yellow FPs, PE, Cy 3, PE-Cy5, PI, TR; far-red FPs, APC, TO-PRO-3, DRAQ5, PE-Cy7, APC-Cy7</td>
<td>Can be excited at 633-635 nm but fewer generations detectable (~ 10 fold dimmer)</td>
<td>(34)</td>
</tr>
<tr>
<td></td>
<td>CellVue NIR815</td>
<td>814</td>
<td>780</td>
<td>Hoechst 33342, FITC, CFSE, green/yellow FPs, PE, Cy 3, PE-Cy5, PI, TR; far-red FPs, APC, TO-PRO-3, DRAQ5, PE-Cy7, APC-Cy7</td>
<td>780 nm laser required; APD preferable</td>
<td>(68)</td>
</tr>
</tbody>
</table>

* APC, allophycocyanin; 7-AAD, 7-aminoactinomycin D; CFSE, carboxyfluorescein succinimidyl ester; Cy3, cyanine 3; Cy5, cyanine 5; Cy7, cyanine 7; FPs, fluorescent proteins; PE, phycoerythrin; PI, propidium iodide; PerCP, peridinin chlorophyll protein; SE, succinimidyl ester; TR, Texas Red.

*b Significant compensation typically required in spectral windows adjacent to the window used to collect proliferation dye signal [3].
Another advantage of proliferation tracking using dye dilution is that at the end of the experiment it is possible to assign a generation number for each cell and assess the relative frequency of cells in each daughter generation. With this information it is possible to calculate the minimum number of cells in the original inoculum that had to divide and the number of divisions each had to undergo in order to obtain the observed histogram. As discussed in more detail in Data Analysis and Results, this permits calculation of: (1) proliferation index, a measure of the total extent of expansion in a given cell population in response to a stimulus, analogous to the stimulation index calculated in a \(^3\)H-TdR assay; and (2) precursor frequency, a measure of the frequency of cells in the starting population able to proliferate in response to the stimulus, analogous to the frequency of antigen-specific cells ascertained from tetramer binding or ELISPOT assays.

Comparison with Other Methods for Assessing Antigen-Driven Immune Responses

Clearly, proliferation tracking has many advantages for monitoring antigen-driven immune responses. However, several differences from other methods should be kept in mind when planning a dye dilution study. First, as discussed in Technical Considerations and Variables, it is critical to verify that intensities bright enough for the desired application can be achieved without altering the viability or function of the cells to be tracked. This is true no matter which tracking dye is chosen. Second, dye dilution studies typically take longer to complete (at least 4 days and as long as 8–10 days) than measuring precursor frequencies based on early consequences of antigen exposure such as tetramer binding (48), ELISPOT or cytokine expression (1–2 days; 49, 50) or even \(^3\)H-TdR uptake (3–4 days). This is in part because \(^3\)H-TdR is a measure of DNA synthesis in preparation for division, whereas additional time must be allowed for the cells to actually undergo division in the dye dilution technique. However, the added time also makes it easier to visualize and accurately quantify small populations of proliferating cells because they have undergone multiple divisions, expanding their frequency relative to nonresponding cells (51) and thus increasing the difference in fluorescence intensity between responders and nonresponders due to dye dilution. Finally, dye dilution assays typically require more cells than other measures of antigen-specific responses. In return, they enable simultaneous identification and characterization of which immune cell subsets respond to antigen by proliferating, which subsets do not, and how number of cell divisions affects other markers of activation and/or effector function.

Methodology

Overview

Peripheral blood mononuclear cells (PBMCs) isolated by Ficoll-Hypaque density gradient centrifugation from heparinized peripheral blood under sterile conditions, cryopreserved PBMCs, or cell lines are all suitable for staining. Prior to culture and stimulation, PBMCs are stained with the appropriate proliferation-tracking dye, which is usually selected based on spectral compatibility with other fluorochromes to be used in the study and the ability to achieve acceptable starting intensity without adverse effect on cell viability or function (18,51–53). Culture time required will depend on the stimulus used. Typically, 4 or 5 days are sufficient for a strong polyclonal stimulus such as a mitogen or anti-CD3 combined with CD28 or IL-2. However, 6 to 8 days are recommended when using either a protein that must be processed and presented or a peptide antigen. At the end of the culture period, the cells are stained for surface phenotype, tetramer binding, intracellular cytokines, etc. and then analyzed by flow cytometry. If cells are to be counterstained for cytokine expression, they should be restimulated with antigen for the last 16 h of culture in the presence of Brefeldin A (4,53). Blood used in all studies reported here was collected from consented healthy donors at Roswell Park Cancer Institute, Buffalo, NY, or Dartmouth Hitchcock Medical Center, Lebanon, NH according to the guidelines and recommendations of each respective institution’s IRB.

Staining procedure for CFSE (adapted from 18). If CFSE is purchased as a powder, it should be made up in DMSO as a 5 mM stock solution (MW 557.47 g/mol). Aliquots can be stored at \(-20^\circ\)C for several months but must be kept moisture-free to prevent degradation due to ester hydrolysis. Alternatively, dye stock may be prepared by dissolving preweighed CFSE in single-use vials in DMSO at the beginning of the labeling protocol. Cells are washed twice in a protein-free medium such as Hanks Buffered Salt Solution (HBSS) to remove extraneous proteins, counted and resuspended at a final concentration of \(1 \times 10^7\) cells per mL in HBSS warmed to 37 C. CFSE is typically used at a final concentration of 0.5–5.0 \(\mu\)M, which is prepared by diluting the CFSE stock from 5 mM to 0.5 mM in PBS and then rapidly dispensing the appropriate volume directly into the cell suspension to ensure homogeneous labeling. Cells are incubated with the dye, with occasional mixing, for varying time periods at ambient temperature or 37 C, with or without added protein as needed to prevent cell toxicity. Staining is halted by the addition of an equal volume of fetal bovine serum (FBS) to react with excess dye or five volumes of medium containing at least 10% FBS. Cells are then washed twice with medium containing serum, counted and resuspended in culture medium at an appropriate concentration (typically 1.0–1.5 \(\times\) \(10^6\)/mL). Although cells are considered stained at this point, their initial intensity does not represent the starting point for a proliferation assay since a substantial fraction of total dye is lost during the first 24 h (see Fig. 1), as the cells clear damaged or short-lived proteins and those destined for export. As also discussed in Technical Considerations and Variables, one cannot simply assume that dye concentration(s) reported in the literature will work for every cell type or experimental setting. Rather, they must be tested and modified as necessary to assure that, after 24 h in culture, labeled cells exhibit high viability, unaltered function, bright homogeneous staining, and acceptable...
ability to compensate for spectral overlap with other fluorochromes, particularly phycoerythrin (PE).

Staining procedure for lipophilic dyes (adapted from 53). Lipophilic dyes used for proliferation tracking are typically supplied by the manufacturer as 1 mM stock solutions in ethanol, since they are poorly soluble in aqueous solutions. An iso-osmotic, salt free staining vehicle (Diluent C) that maximizes dye solubility is also supplied with the PKH or CellVue dyes. Because of their lipophilicity and the fact that staining occurs by noncovalent partitioning into the cell membrane, minimizing the amount of salts present and insuring rapid homogeneous dispersal of cells in dye are the key elements for successful labeling with these reagents. Briefly, cells are washed twice in HBSS to remove exogenous proteins, taking care after the second wash to ensure that the supernatant is aspirated as completely as possible (ideally no more than 25–50 µL should remain) without removing any cells. Immediately before staining, cells are dispersed in Diluent C at a concentration twice the desired final staining concentration (e.g., 1 × 10⁸ cells/mL if the final staining concentration is to be 5 × 10⁷ cells/mL). In parallel, a 2X solution of dye is prepared by diluting the ethanolic dye stock into an appropriate volume of Diluent C at a concentration twice the desired final staining concentration (e.g., 1 × 10⁶ cells/mL if the final staining concentration is to be 5 × 10⁵ cells/mL). Staining is initiated by rapidly dispensing and admixing an equal volume of 2X cell solution into the 2X dye solution. After 2–3 min at room temperature, staining is stopped by adding a volume of FBS equal to that in the final staining step, or five volumes of culture medium containing at least 10% FBS. Stained cells are then washed twice in medium, counted, and resuspended at an appropriate concentration in culture medium. For maximum yields, cells should be left in Diluent C no longer than is needed to complete the brief staining procedure, since the lack of physiologic salts enhances dye solubility and staining efficiency but can impair cell viability over longer time periods. Similarly, the 2X dye solution should be prepared just prior to the labeling reaction, as dye molecules will slowly aggregate in this aqueous vehicle. For best results, it is also recommended that staining be done in polypropylene tubes and that after the first wash the resuspended cells are transferred to a fresh tube to eliminate carryover of dye nonspecifically bound to the plastic. Again the indicated concentrations are starting points that have been successfully used for PBMC. However, appropriateness of concentrations should always be verified by titering, since the amount of dye required will increase as total number and/or size of cells to be stained increases (i.e., as total amount of membrane increases). As with CFSE, the goal is to achieve bright homogeneous staining without altering cell viability or function or compromising ability to detect other dyes due to spectral overlap. However, unlike CFSE, parental intensity can be assessed immediately after staining since membrane binding is very stable and dye losses are typically minimal (33) and slow (30) unless washing has been incomplete. Skewed or broadened intensity distributions at time 0 can usually be resolved by more careful attention to removal of salts, insuring complete dispersion of the cell pellet to single cells when resuspending in diluent, and/or improved mixing technique when cells are added to dye.

Cell culture. After staining with tracking dye, cells are counted to check viability and recovery, adjusted to 1.5 × 10⁶
cells per mL and placed into either a 24-well tissue culture plate (2 mL/well; 3 \times 10^6 total cells) or a 12-well tissue culture plate (3.3 mL/well; 5 \times 10^6 total cells). Antigenic proteins or peptides are added to the appropriate wells at the beginning of the culture period at a concentration previously established to induce proliferation. As a positive proliferation control, ConA at 5 \mu g per mL final concentration, PHA at 2 \mu g per mL final concentration or anti-CD3 (100 ng/mL) plus IL-2 (200 IU/mL) are added for the last 4–5 days of culture. As discussed in Technical Considerations and Variables, a stained but unstimulated control should always be included to allow estimation of parental peak position in the tracking dye histogram, since undivided parental cells may not be present at longer time points in samples treated with polyclonal stimuli. The cultures are incubated at 37°C in 5% CO₂ for 4 to 8 (or even 10) days depending on the strength of the proliferative response. Cultures should be checked regularly and replenished with fresh culture medium as required. If the cells are not to be stained for phenotypic or activation markers, intracellular proteins, or tetramer-binding, they can be spun down at the end of the culture period, resuspended in 0.2–0.4 mL of washing buffer and analyzed directly by flow cytometry. Alternatively, after washing, they can be stained using standard surface or intracellular labeling protocols. In general, it is recommended that a viability probe be included wherever possible (see Supplemental Technical Issues: Critical Controls).

Instrumentation and data collection. Essentially any multi-parameter flow cytometer can be used for proliferation tracking studies so long as it has excitation sources and emission filters compatible with the tracking dyes selected and with any other reagents needed to 1) correlate extent of proliferation with antigen binding, activation and effector function, or 2) correlate precursor frequency with stimulus, co-stimulus, etc. Selection of appropriate compensation conditions, discussed in more detail in Supplemental Technical Issues: Critical Controls, is arguably both more difficult and more critical for proliferation monitoring than for standard immunophenotyping. The most obvious concern is that maximizing the intensity of undivided cells to enable tracking as many daughter generations as possible can lead to significant compensation problems in adjacent spectral windows. Another concern, although often overlooked, is that (a) the phenotypic markers used may also overlap in the spectral window used to detect the proliferation dye, and (b) under-(or over-) compensation for this overlap will selectively elevate (or decrease) apparent signal for marker positive cells, leading to selective under-(or over-)estimation of proliferation for those cells (31). Careful characterization of instrument linearity is also essential in cytometers that use hardwired log amplifiers (51). This is because the use of metrics such as proliferation index or precursor frequency to quantify extent of proliferation requires assumptions to be made about how daughter cells are partitioned among adjacent peaks, even with CFSE. Such analyses are typically done using modeling software, which requires information about linearity and slope of the logarithmic amplifiers being used.

Technical Considerations and Variables

Considerations for Selecting a Proliferation Tracking Dye

Listing the properties desired for an ideal proliferation tracking agent dye is much easier than creating a probe that meets all of them. An ideal dye would: (1) give extremely bright initial staining intensities without altering expression or function of receptors involved in antigen processing and presentation or otherwise affecting functional or proliferative responses; (2) exhibit minimal cell-to-cell transfer; (3) exhibit stable intensity in non-dividing cells over the entire period of study; and (4) accomplish all of the above using simple, rapid labeling protocols that are readily reproduced from laboratory to laboratory, and label 100% of cells with 100% recovery and 100% viability. As summarized below, each of the major classes of proliferation tracking dyes has not only significant advantages, but also distinct limitations, and both classes have some advantages and limitations in common. Selecting the “best” tracking method (or combination of methods) therefore typically requires determining which of the available dyes represents the best compromise between the limitations of each specific probe and the needs of a particular study or experimental system.

Advantages and Limitations of General Protein-Reactive Dyes for Proliferation Tracking

Major advantages for CFSE and related dyes include:

1. A rapid, simple labeling procedure that gives bright initial staining with essentially all cell types;
2. A rate of dye dilution that, after an early period of rapid efflux (see below and Fig. 1), correlates well with rate of cell division as measured by cell count (20,21);
3. Ability in some, but not all, systems to visualize subpopulations corresponding to different daughter generations (for representative examples see 1,33,54,55).

The most important limitations for general protein labeling dyes arise because stained cells exhibit not only proliferation-related dye dilution but also significant biphasic dye loss that is proliferation independent:

1. During the first 24 h postlabeling, mean intensity typically decreases 2–10 fold in nonproliferating cells due to clearance of labeled proteins destined for secretion and turnover of short-lived proteins (Fig. 1A) (1,20). Some investigators use an additional 30–60 min incubation at 37°C in fresh media after removal of unbound dye, but this appears to be only partially effective (34). Consequences of the early phase of proliferation-independent dye loss include difficulty achieving staining bright enough to track proliferation for six to eight daughter generations without altering cell viability or function. To some extent, it may be possible to offset this by using a higher concentration in the labeling step. However, this also increases the probability of random covalent labeling of critical residues, leading to altered expression or malfunction of critical proteins involved in
Advantages and Limitations of General Membrane-Labeling Dyes for Proliferation Tracking

Advantages of general membrane labeling with lipophilic proliferation tracking dyes include:

1. A rapid, simple labeling procedure that gives bright initial staining with essentially all cell types.
2. No waiting period required for intensity to stabilize (Fig. 1B). This simplifies analysis of proliferative responses in continuously proliferating cell lines or populations where cell division begins soon after labeling and stimulation (35,42). Strong hydrophobic interactions between the hydrocarbon tails of the dyes and those of membrane phospholipids provide fluorescence intensities that remain stable for weeks to months in nondividing cells (28,31,37) but are halved with each successive daughter generation (30–34). Rate of loss is somewhat greater for nucleated cells stained with lipophilic dyes (P. Wallace, personal observations) but still relatively slow (30).
3. Less difficulty achieving bright staining without altering protein (and cell) function since labeling occurs by intercalation into lipid regions of cell membrane and dye efflux is minimal (53).

The most significant limitations of general membrane dyes for proliferation tracking arise from the fact that labeling occurs by noncovalent partitioning rather than by equilibrium binding or covalent reaction:

1. Obtaining optimal intensity distributions requires careful attention to mixing technique and to cell concentration as well as dye concentration (3,53). Although CFSE also requires rapid mixing for homogeneous staining (18), lipophilic dye staining is essentially instantaneous upon admixing with cells in non-ionic media. Slow or inhomogeneous mixing results in skewed or broadened initial intensity distributions that are inappropriate as a starting point for proliferation monitoring. Because staining occurs by partitioning, variations in both cell and dye concentrations affect final staining intensity and must be controlled (and reported!) for reproducible results. Increasing dye concentration and/or decreasing cell concentration gives brighter staining, with the upper limit being compromised membrane integrity and loss of viability. However, less extreme over-labeling can cause a shift in fluorescence spectrum (58) and a less than two-fold decrease (or, in extreme cases, a slight increase) in intensity due to dye quenching before the first cell division (A. Bantly, personal communication; P. Wallace, unpublished observations).

2. Daughter generations are often less easy to detect visually than with general protein labels (particularly CFSE). The ability to visually distinguish daughter generations depends on the CV of the parental staining distribution and the extent to which that CV broadens in subsequent generations. CFSE has the advantage that its distributions tend to narrow somewhat in the first 24 h post-labeling, presumably because cell-to-cell variation in the complement of stable proteins is less than that of cell size, whereas membrane dye distributions show minimal change. In addition, membrane dye distributions tend to broaden slightly at later daughter generations (30,34). For some membrane dyes, the difference from CFSE is modest; for others it is greater. However, despite differences in starting CV, results such as those shown in Figure 2 and reported by Bantly et al. (33) indicate that CFSE, PKH26, and CellVue Claret give comparable proliferation indices and precursor frequencies, whether or not successive daughter cell generations give visually distinguishable peaks.

Limitations and Caveats Common to Both Classes of Proliferation Tracking Dyes

Several other issues must also be considered, no matter which type of dye is chosen. These include:

1. Brighter staining enables tracking of more daughter generations but complicates filter selection and color compensation for multiparameter studies. Accurate identification of weakly positive populations in the spectral window(s) immediately adjacent to the proliferation dye is often problematic, although this is somewhat less of an issue for membrane dyes than protein dyes because of the wider range of colors available (Table 1). In either case, reducing the concentration used for labeling can address this problem but also limits the number of daughter generations that can be tracked before generation number becomes uncertain due to overlap with autofluorescence from unstained cells.

2. Effects of fixation on dye retention must be considered if samples are to be held for batch analysis at the end of a
study. Although the general protein labels form covalent bonds that are stable to most fixatives, procedures that permeabilize without crosslinking can lead to dye loss as labeled proteins leak out of the cells (20). Fixatives that extract lipids (e.g., ethanol, methanol, acetone) can also extract membrane dyes to varying extents (29,59). How-

Figure 2. Proliferation index and precursor frequencies are comparable for stimulated lymphocytes labeled with CFSE or PKH26. Peripheral blood mononuclear cells were labeled with optimized concentrations of either CFSE (0.5 μM, 5 x 10⁶ cells/mL) or PKH26 (5 μM, 1 x 10⁷ cells/mL) and cultured with anti-CD3 (0.25 μg/mL) and anti-CD28 (0.5 μg/mL) for 4 days. Cells from duplicate cultures were harvested daily and dye dilution was assayed as described for Figure 1, except that the lymphocyte light scatter gate was expanded to include lymphoblasts while excluding dead cells and debris. Panels A (CFSE) and B (PKH26): Unstained controls (open histograms) and stained, unstimulated controls (filled histograms) from 96 hour cultures. Panels C (CFSE) and D (PKH26): Proliferation profiles from 96-h cultures stimulated with anti-CD3 and anti-CD28. Histograms were analyzed by ModFit LT (Verity Software House, Topsham, ME) using the floating (CFSE) or standard (PKH26) proliferation models to fit the raw data. The brightly stained parental population and dimmer daughter populations were modeled by ModFit LT and the resultant best fits as determined by the reduced chi square (RCS) for each histogram are shown, along with the corresponding proliferation index and precursor frequency results calculated from each proliferation profile. Panels E and F: Data shown compares the average proliferation index (Panel E) and precursor frequency (Panel F) of cells stained with CFSE (-_-) or PKH26 (-_) over the 4-day culture period. Error bars indicate ±1 SD, n = 2 replicate cultures. Precursor frequencies and proliferation index did not differ significantly between the two dyes (P > 0.1 by paired t test using all time points). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com]
ever, membrane dye intensities are typically stable to fixation with formaldehyde and/or permeabilization with 0.1% saponin (53). In either case it is important to include unstimulated controls from each time point to allow accurate assessment of fixation effects on intensity of undivided cells (see Supplemental Technical Issues: Critical Controls).

3. Apoptotic and/or necrotic cell discrimination is essential. Dead and dying cells lose variable amounts of protein- or membrane-associated dye due to shedding of apoptotic vesicles or loss of membrane integrity, which may cause overestimation of extent of proliferation if the intensity reduction due to apoptosis is not recognized (8,12,33,53). Use of viability probes such as 4′,6-diamidino-2-phenylindole, propidium iodide, 7-aminoactinomycin D, TO-PRO-3 or Annexin V is therefore strongly recommended so that such cells can be either excluded from or separately accounted for in the proliferation analysis. For studies where cells must be fixed (e.g., to enable counterstaining for intracellular cytokines), labeling with one of the recently introduced LIVE/DEAD fixable dyes (Invitrogen), after proliferation but prior to fixation, may represent a better alternative than attempting to gate out dead/apoptotic cells using light scatter. If dead cells are not to be further analyzed, the fixable dye can be potentially multiplexed with other probes in a “dump” channel.

### Selection of Appropriate Instrument Setup and Method Controls

Specifics will vary as a function of the experimental question being addressed, and it is impossible to be comprehensive. As discussed in greater detail in Supplemental Technical Issues: Critical Controls, appropriate compensation and biological/method controls are vital in any dye dilution proliferation study to assure consistent and valid results (18,33,51). Depending on the number of fluorochromes used and their degree of spectral overlap, single color and/or fluorescence-minus-one (FMO) controls will be required to properly establish compensation and analysis regions. An unstained control is essential, both for setting up compensation and for verifying, using an alternate method such as 3H-TdR incorporation, that cells labeled with proliferation dye still respond appropriately to stimulation. A positive method control is also needed to verify that the particular cell preparation used is able to respond to an appropriate polyclonal stimulus, and a negative method control to verify that there is minimal response in the absence of stimulation or to an irrelevant stimulus.

### Sensitivity and Background

The sensitivity of the dye dilution method depends on (a) the intensity of stained but undivided parental cells relative to the autofluorescence of unstained cells present in the assay system; (b) the homogeneity (CV) and symmetry of the stained cell population; (c) the dynamic range of the cytometer’s detection system vs. the staining intensity achievable without toxicity; (d) the total number of events that can be collected; and (e) how well rare populations of interest can be resolved by using gating strategies to discriminate against irrelevant cells and other sources of background.

### Minimum Detectable Response

To maximize sensitivity, the stained population should be as bright, homogeneous, and symmetric as possible, consistent with good recovery (at least 70% and preferably 85–90%), high viability and the ability to compensate for spectral overlap in spectral windows used for detection of other probes (29,33,34). Where possible without loss of function, it is desirable to start with intensities that fall in the uppermost decade on a four decade log scale when using instrument settings adjusted to place unstained cells in the lowest decade, but no stained cells offscale in the highest channel. Starting with an intensity ~1,000-fold brighter than unstained cells (e.g., mean intensity of 5,000 if autofluorescence is 5) translates to the ability to detect up to seven to eight divisions before fluorescence completely overlaps unstained cells, and one or two less (depending on CV) before generational assignments become unreliable. Increasing the number of generations detectable requires increasing starting intensity, decreasing starting CV, or both. Assuming seven daughter generations distinguishable from autofluorescence and ability to reliably detect a population which represents 1% of total cells, sensitivity is conservatively estimated at one responding cell in 10^4 or 0.01% (51). A gating strategy allowing reliable detection of 0.1% of total cells would theoretically extend sensitivity to 0.001%.

### Number of Cells to Collect

To reliably detect 1 in 10,000 responder cells, enough events must be collected to accurately estimate the intensity distribution of proliferating cells, particularly if modeling is to be used to calculate the precursor frequencies or the proliferation index (see DATA ANALYSIS AND RESULTS). When dealing with very low frequencies of responding cells, it is critical to determine beforehand the number of events that should be collected based on the estimated frequency of the smallest population of interest. For small numbers of events, the standard deviation (SD) of the number counted (N) is the square root of N and the coefficient of variation (CV) is \( \frac{\text{SD}}{N} \). Counting 100 positive events therefore gives an estimate of the true mean with CV = 10%. Assuming a normal distribution for repeat determinations gives a 68% confidence that the true value lies in the interval 100 ± 10 (mean ± 1SD), a 90% confidence interval of 100 ± 17 (mean ± 1.75SD) and a 95% confidence interval 100 ± 20 (mean ± 1.96SD). Generally speaking, a CV of 10% for number counted is considered to be minimally acceptable, while the acceptable confidence interval around the experimentally determined mean will depend on the certainty desired that the true value is within the confidence interval. To be 95% certain that the true frequency lies within ±10% of the experimentally determined value requires counting 1,537 positive events \( N = \text{SQRT}(2 \times 1.96/10\%) \). This means that if the proliferating population represents 1% of the total lymphocytes, a minimum of 153,700 lymphocyte-gated events should be collected. Correspondingly more events will be needed if the population of interest is a subset of prolif-
erating cells (e.g., CD3+CD4+ cytokine producing cells). Fortunately, the relative frequency of small subsets of antigen specific cells will increase as they proliferate (51).

VALIDATION ISSUES

Comparison with Traditional Proliferation Assays

When properly performed, the dye dilution assay correlates well with the 3H-TdR assay. Fulcher and Wong (60) assayed T cell function using both CFSE and 3H-TdR incorporation assays and concluded that they compared favorably. However there are several caveats: (a) the cells can not be overlabeled with tracking dye; (b) the dye dilution assay should be read out at least 24 h later than 3H-TdR incorporation; and (c) although the relative magnitudes of responses are comparable, the actual values are not. A second, more direct way to validate the dye dilution assay is to compare rate of increase in cell count with rate of decrease in fluorescence intensity in a cell population where cell death is minimal, as illustrated in Supplemental Fig. S2 for logarithmically growing U937 cell cultures stained with optimal concentrations of PKH26. Under these circumstances, the decrease in mean fluorescence intensity (MFI $T_o/T_x$) for proliferating cells should be inversely proportional to the increase in cell number (Count $(T_o/T_x)$), at least until the intensity of highly divided cells begins to overlap significantly with autofluorescence. When triplicate cultures, split every three days to maintain logarithmic growth, were sampled over seven days, the slope of the resulting line was 1.1, correlating well with the predicted slope of 1.0. In similar experiments we have verified that rate of dye dilution correlates with cell growth rate using CFSE (beginning 24 h post-labeling; J. Tario, unpublished observations) and the new CellVue dyes Plum, NIR780 (34) and Claret (B. Gray, E. Breslin and K. Muirhead, unpublished data).

Reproducibility of Dye Dilution Assays

In any proliferation study it is always preferable to set up duplicate or triplicate cultures and report the average. In the U937 cultures used to verify that rate of dye dilution directly paralleled cell growth rate (Supplemental Fig. S2), reproducibility was excellent, with an average CV of 2.3% over the six-day study (mean MFI ± SD: 2102 ± 22 on Day 1, 634 ± 13 on Day 3, and 178 ± 2.6 on Day 4; autofluorescence 2.8 ± 0.5 over all days). We have obtained similarly reproducible results with both CFSE and the CellVue dyes in this model system (unpublished observations and 34). In a more complex system, Givan et al. (30) examined the intra-run reproducibility of precursor frequency determinations at multiple time points in a PKH26 dye dilution study using mononuclear cells from three different donors cultured with tetanus toxoid. As expected, total number of daughter cells increased over time, but calculated precursor frequency remained relatively constant over the period of 6–14 days, indicating that neither dividing nor non-dividing cells were being selectively lost (or excluded by light scatter gating) over time. In the same study, Givan et al. evaluated the reproducibility of repeated precursor frequency measurements over time by collecting blood from the same 10 donors at each of three separate clinic visits. Overall, the data for each donor varied little over the three visits, demonstrating the inter-run reproducibility of this method.

One important application of the dye dilution assay is to determine whether vaccination results in an increase in the proportion of immune cells able to mount an antigen specific response. We therefore tested whether precursor frequency was increased after administration of the annual influenza vaccine in nine healthy volunteers. Blood was drawn pre-exposure, each volunteer was vaccinated, and approximately 13 days later a second blood sample was obtained. The frequency of flu-specific CD4 and CD8 T cell precursors was determined in duplicate for each volunteer using PKH26 dye dilution and the average change from Day 0 to Day 13 was compared. Variability between duplicates was low, although somewhat greater for CD8 T cells than CD4 T cells (Figs. 3A and 3B, respectively). In 9/9 donors there was an absolute increase in either CD4 or CD8 precursor frequencies, and in 7/9 donors, both CD4 and CD8 precursor frequencies increased (the exceptions being donors 4 and 6 for CD4 and CD8, respectively). The increase was statistically significant for CD4 T cells ($P = 0.005$) and nearly significant for CD8 T cells ($P = 0.08$) (Fig. 3C), demonstrating that the dye dilution assay was able to detect altered precursor frequencies following administration of a standard vaccine.

DATA ANALYSIS AND RESULTS

Numerous ways have been devised to compare the extent of proliferation based on dye dilution profiles and these can generally be divided into three groups: descriptive, semi-quantitative, and quantitative. Those most commonly found in the literature are discussed below. Any flow cytometric analysis program may be used for either descriptive or semi-quantitative approaches. Quantitative analysis methods will yield more information but require specially designed peak modeling software to estimate the number of cells in each generation.

Descriptive Analysis

Describing trends in antigen expression across generations is an easy and perfectly satisfactory approach to analyzing how expression of surface antigens or cytokines changes across daughter generations (9). Figure 4 (Panels 4A and 4C) illustrates this approach in a study where CD4+ cells were separated into CD25+ and CD25− fractions, stained with CFSE, and then stimulated with anti-CD3 and CD28 for 7 days. Qualitative examination of this figure reveals that in the CD4+CD25− fraction (Panel A) nonproliferating cells are CD25 negative but CD25 expression is seen as early as the first generation of daughter cells and is retained through subsequent daughter generations. In contrast, in the CD4+CD25+ fraction (Panel C) nearly half of the nonproliferating cells lack CD25 expression and overall extent of proliferation is less than in the CD4+CD25− fraction.

Semiquantitative Methods

Proliferative fraction. In this model-independent approach, no generational information is incorporated. The results are
simply expressed as percent of cells that have undergone one or more divisions (Figs. 4B and 4D). The stained but unstimulated control (not shown) is used to set the upper boundary for enumeration of daughter cells, selecting an intensity that gives an acceptably low value for dividing cells in the absence of stimulus (e.g., 1–5%). If the test sample contains unstained cells (e.g., accessory cells, allogeneic stimulators, etc.), an unstained control containing these cells, but treated similarly to the stained sample, is used to define the lower boundary for enumeration of proliferating cells, selecting an intensity that gives an acceptably low value for dividing cells in the absence of proliferation dye. The Proliferative Fraction is then defined as the percentage of proliferating cells with fluorescence intensity less than the stained but unstimulated control and more than the unstained control. The Proliferative Fraction is defined as the percentage of proliferating cells with fluorescence intensity less than the stained but unstimulated control and more than the unstained control. Using this approach gives a Proliferative Fraction of 83% for the CD4CD25 sample after 7 days in culture (Panel B) vs. 62% for the CD4CD25 sample (Panel D), confirming reduced proliferation in the latter population. This approach was also used by Bantly et al. to characterize a new far-red tracking dye, CellVue Claret, which gave results comparable to CFSE and PKH26 for lymphocytes stimulated with anti-CD3 and IL-2 (33).

Figure 3. Changes in precursor frequency of CD4 and CD8 cell subsets are detectable following vaccination. Peripheral blood samples were drawn from healthy, consented volunteers pre- and 13 days post-vaccination with licensed, inactivated, trivalent influenza vaccine (Aventis Pasteur, Inc., Swiftwater, PA; Donor 7 was 20 days post vaccination), and used to prepare mononuclear cells, which were stored frozen until assay. To determine the precursor frequency of influenza specific T cells, paired pre- and post-vaccination samples from the same donor were thawed and stained with PKH26 (1 μM, 2.5 × 10⁶ cells/mL) then plated with (or without) influenza trivalent vaccine (0.1 μg/mL) for 8 days. At culture termination, cells were harvested, stained with anti-CD4 FITC or anti-CD8 FITC, and analyzed by flow cytometry, gating on viable lymphocytes and lymphoblasts as defined by forward and side scatter and on the relevant phenotypic antibody (for detailed instrument setup and gating strategies, see Ref. 53). Precursor frequencies were calculated for stimulated samples from each donor using ModFit LT (as described in Fig. 2) and data were normalized for inter-donor differences in background response by subtracting the corresponding calculated precursor frequency from unstimulated cultures for the same donor. Increases in precursor frequency post-vaccination were seen in both CD4 and CD8 subsets from most volunteers, with the most robust response observed in the CD4 population. Mean precursor frequencies (percent ± SD from replicate wells) for samples taken pre- (hashed bars) and post-vaccination (filled bars) are shown for the CD4 (Panel A) and CD8 (Panel B) subsets. Using paired analysis of pre- and post-vaccination samples, change in the precursor frequency was found to be highly significant for the CD4 subset (P = 0.005) and nearly significant (P = 0.080) in the CD8 subset (Panel C).
Stimulation index. This ratiometric descriptor measures effect of stimulation on relative number of cells that have divided at least once, calculated as

$$SI = \frac{\text{Proliferative fraction (stimulated)}}{\text{Proliferative fraction (unstimulated)}}$$

It has been used, for example, to compare CD4+ T cell responses to allergen stimulation in healthy, allergic, and atopic subjects (43).

Mitotic index. First described by Wells et al. (8), this index estimates total number of divisional events during the culture period. With very homogeneous initial staining and a strong polyclonal stimulus, individual peaks can frequently be distinguished in a CFSE or lipophilic dye dilution profile, making it possible to visually estimate where specific daughter generations fall (Fig. 2) (33). Generational boundaries are defined as being the lowest point between adjacent daughter peaks and used to calculate percentage of cells falling in each daughter peak. When combined with total number of cells in the culture at the time of harvest, this allows calculation of total number of cell divisions at the time of harvest. Since baseline resolution is almost never seen, even in ideal cases, drawing a generational boundary at the lowest point assumes the cells in the boundary region are equally divided between adjacent generations. This is unlikely to be true when adjacent peaks are of unequal height, as is typically the case. Consequently, mitotic index has been largely replaced by more quantitative methods that use modeling to determine how to partition daughter cells falling in inter-generational channels.

Quantitative Methods

Quantitative methods employ peak modeling software to more accurately extract details from the dye dilution histogram and estimate the number of cells in parental and daughter generations. There are currently two commercially available flow cytometric analysis programs, ModFit LT (Verity Software House, Topsham, ME) and FlowJo (TreeStar, Ashland, OR), that can be used for this purpose. These programs use a nonlinear least squares analysis to iteratively find the best fit to the raw data by changing the position, height, and CV (or width) of each Gaussian (Figs. 2 and 5). The area under each generational Gaussian is taken as a measure of the relative number of cells in that generation and the sum of all Gaussians corresponds to the relative number of cells in the total population. These values can then be used to calculate various descriptors such as proliferation index, precursor frequency, etc.
Figure 5. Frequency of flu-peptide specific CD8+ T cells in peripheral blood of a healthy influenza-vaccinated volunteer as determined by tetramer staining, IFNγ production, ELISPOT, and dye dilution assays. CD8+ T cells specific for the M158-66 influenza epitope (GILGFVFTL) were used as a model system to compare assays for antigen specificity (for details of cell isolation, staining, and precursor frequency analysis, see 4). In brief, a healthy, consented HLA-A2 donor was vaccinated against influenza virus (trivalent vaccine, Aventis Pasteur, Inc.), circulating peripheral blood mononuclear cells were isolated two weeks later, and CD8+ T cells were purified magnetically using positive selection with anti-CD8 beads. For Panels A and B, CD8+ T cells were stained with influenza tetramers or assayed by ELISPOT to determine the ex vivo frequency of cells able to specifically bind M158-66. For Panels C-F, CD8+ T cells were additionally stained with PKH26 (2μM PKH26, 5 x 10^6 cells/mL) or PKH67 (2μM PKH67, 1 x 10^7 cells/mL), and incubated with autologous dendritic cells loaded with M158-66. On Day 5, the cells were restimulated with peptide-loaded dendritic cells in the presence of Brefeldin A to boost IFNγ production. On Day 6, the cells were harvested, stained with tetramers (HLA-A*0201/M158-66), anti-CD8 antibody, and antibody against intracellular IFNγ. Data were collected by gating on forward scatter vs. side scatter to eliminate very large or very small events (respectively, aggregates or debris) and on viable (TO-PRO-3 negative) CD8+ T cells. Panel A: For tetramer staining, results are expressed as the percentage of viable CD8+ cells that were tetramer positive (HLA-A*0201/M158-66) minus the percentage of viable CD8+ cells that were positive for staining with a negative control tetramer (data not shown). Panel B: In the ELISPOT method, results are expressed as the mean number of spots for triplicate wells stimulated with M158-66 at each of three different cell concentrations after subtracting the values for non-specific stimulation with unloaded dendritic cells. Panel C: For the dye dilution assay, results are expressed as precursor frequency of cells proliferating in response to 6 days of stimulation with dendritic cells loaded with M158-66 as calculated using ModFit LT software, and subtracting the value in control wells incubated with unloaded dendritic cells (not shown). For Panels D-F, quadrant statistics are expressed as either the percentage of viable CD8+ cells in Day 6 cultures (Percent by Quadrant on Day 6) or precursor frequency in the viable CD8+ population (i.e., percentage of cells present in day 0 cultures that went on to proliferate during the 6-culture period; Precursor Frequency by Quadrant). Panel D: PKH67 versus tetramer staining. Panel E: PKH67 versus IFNγ production. Panel F: Tetramer staining versus IFNγ production.
Proliferation index. This descriptor is analogous to a \(^3\)H-TdR stimulation index in that it is a measure of the increase in cell number over the course of the assay. Given the generation number and relative number of cells in that generation, it is possible to back calculate how many parent cells must have been present in the original culture that went on to divide in order to obtain the observed histogram as fit by the model. Proliferation index is then calculated as the sum of cells in all generations at the time of harvest divided by the number of parent cells computed to have been present at the start of the experiment (for details see Ref. 3). Proliferation index is useful for comparing the effects of different stimuli on a population of cells or, as shown in Figure 2, responses from differentially treated cells to a common stimulus. As shown, comparable values were obtained for proliferation index using either CFSE or PKH26 (2.47 and 2.18, respectively on Day 4; \(P = 0.26\); Figs. 2C–2E).

Precursor frequency. This descriptor is a measure of the fraction of cells in a population that is able to respond to a particular antigen. Modeling the dye dilution histogram allows back calculation of the relative number of cells in each generation and therefore the number of cells in the original population that went on to proliferate in response to a specific stimulus. Precursor frequency is then calculated as the percentage of cells in the original population that proliferated in response to the stimulus: as shown in Figures 2C, 2D, and 2F, CFSE and PKH26 gave comparable precursor frequencies (30.2% and 32.1%, respectively), confirming that visually evident daughter peaks are not required for accurate analysis using quantitative approaches. Precursor frequency is particularly useful in vaccine trials to determine if treatment increased the number of antigen specific immune cells (Fig. 3) (61). Using influenza vaccination as a model, we have extensively validated the application of the dye dilution technique to determining precursor frequencies, and Givan et al. have detailed both the applications and pitfalls of this method (30,51,53).

Time course analysis. Substantial additional information about division rates and death rates can be extracted from time course studies of the type illustrated in Figure 2. Quantitative methods developed by Hodgkin and coworkers (2,11) reveal in detail how lymphocyte proliferation and survival are regulated and altered by signals such as those received from co-stimulatory molecules, drugs, and genetic polymorphisms. Sampling at different time points allows calculations to be made for time to first division, rate of proliferative fraction, and exodus due to cell death using generally available procedures and software (Excel, Microsoft, Inc., Redmond, WA; Matlab, Mathworks, Inc., Natick, MA) (2), and applications to stem cell differentiation have also been described (21).

Comparison to Other Technologies

Using CD8\(^+\) T cells specific for the M138-66 influenza epitope as a model system, determination of precursor frequency by the dye dilution technique was compared with two different and completely independent methods: (a) staining with MHC/peptide tetrameric complexes (48), and (b) the ELISPOT assay for IFN\(\gamma\) production (50). Figure 5 shows representative results from one donor, in whom HLA-A*0201/ M138-66 tetramer positive cells represented 0.50% of CD8\(^+\) T cells (Fig. 5A). For the same donor, the frequency of flu-specific IFN\(\gamma\)-secreting T cells by ELISPOT was 0.22% of CD8\(^+\) T cells (Fig. 5B), or about half the number of tetramer specific T cells. For this same donor, using PKH26 dye dilution gave a precursor frequency for flu-specific CD8\(^+\) T cells of 0.18% (Fig. 5C), a value very similar to that determined by IFN\(\gamma\) ELISPOT assay. Given that these three independent methods assay different properties of T cells, the agreement among assays was quite good for this donor and similar results were obtained for other donors (4).

Despite the similarities, results from the three different assays were not identical, so we further dissected the relationships among them using multiparameter flow cytometry to simultaneously measure antigen-specific tetramer binding, T-cell proliferation by dye dilution, and cytokine production, another independent measure of antigen-driven response (4,48,49). Results from a representative donor are shown in Figure 5 (panels 5D–5F). Stimulation with flu-loaded dendritic cells (DC) led to an increase in tetramer positive cells (13.7% of total events by Day 6; Fig. 5D), almost all of which displayed decreased PKH fluorescence intensity, indicating that they had proliferated (13.3% of total events; Fig. 5D) and the majority of which secreted IFN\(\gamma\) (9.7% of total events; Fig. 5F). We also detected a small populations of tetramer positive cells that had not proliferated (0.4% of total events; Fig. 5D) or synthesized detectable amounts of IFN\(\gamma\) (3.6% of total events; Fig. 5F) in response to flu-peptide stimulation. In fact, all possible combinations of tetramer binding, cytokine producing, and proliferating cells were found.

As antigen-responsive T cells proliferated in response to flu-peptide-pulsed DC during the 6-day culture period, their relative frequency increased. Precursor frequency was therefore calculated to estimate the proportion of CD8\(^+\) T cell subsets in the ex vivo (Day 0) population. The importance of assessing precursor frequencies can be seen by comparing the observed Day 6 percentage of cells in each quadrant with the calculated precursor frequencies (Day 0 frequency) for the same quadrant (see quadrant statistics for Figs. 5D–5F). For example, 0.59/(0.59 + 0.25) = 70.2% of parental (Day 0) tetramer positive cells produced IFN\(\gamma\), whereas 29.8% did not. Importantly, while almost all of the tetramer-positive cells present on Day 6 had proliferated (13.3/13.7 = 97.1%; Fig. 5D), examination of precursor frequencies indicated that only 55.4% of parental tetramer positive cells had responded to stimulation by proliferating whereas an almost equal number (44.6%) had not. From these and similar data, we concluded that only approximately half of the cells that bound specific tetramers actually had the capacity to proliferate and synthesize IFN\(\gamma\) in response to antigen. This further confirmed the small but consistent elevation in values for antigen specific CD8\(^+\) T cells noted for the tetramer binding assay as compared with these other methods of estimating antigen specific T cells (4).
CHALLENGES AND FUTURE DIRECTIONS

Dye dilution-based proliferation assays have proven extremely useful for basic and clinical research because of their ability to correlate a critical functional outcome, expansion of antigen responsive cells, with earlier events that do (or do not) lead to that endpoint. In particular, they have aided in the identification and validation of simpler surrogate measures such as phenotype or cytokine expression profile. Full realization of their potential, however, will require surmounting several challenges that have so far limited their use in routine clinical studies. The most important are lack of uniform methods (including those for data analysis), relatively longer assay times, and requirement for sample collection and preservation procedures able to provide the necessary numbers of functional cells. Future efforts at miniaturization, as has been done for ELISPOT (62), and methodological standardization, as has been done with Class I tetramers (48), will be important. Equally important will be recognition that obtaining reliable results requires collection of data on adequate numbers of cells per sample and use of replicate samples, just as is true for other assays that measure antigen specific responses.

Tracking cell proliferation using the dye dilution technique exploits the power of multicolor, multiparameter flow cytometry to track multiple cell divisions over time and define specific populations of proliferating (and nonproliferating) cells. We and others have used this approach to determine which T cell subset(s) proliferate in response to a specific antigen and to simultaneously characterize patterns of cytokine production in responding cells. More recently, different tracking dyes have been combined to simultaneously monitor the proliferation of multiple lymphocyte populations in the same culture (25,33). This approach is particularly useful for regulatory T cells (14), which do not exhibit a single, discrete phenotype but are functionally defined as anergic and suppressive. Clinical settings where dye dilution proliferation assays would complement other measures of antigen-specific responses include vaccine trials and infectious disease studies, where knowing the type of responding cells as well as simply being able to quantify them might better predict outcomes (61,63,64). As the spectral range covered by commercially available proliferation dyes (Table 1) continues to expand in parallel with the spectral capabilities of modern flow cytometers, we expect that this methodology will continue to facilitate investigation of even more complex cellular interactions of interest to investigators in immunological and biological sciences.

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LITERATURE CITED

TECHNICAL NOTE

30. Givan AL, Fisher JL, Waugh M, Ernstoff MS, Wallace PK. A flow cytometric method to estimate the precursor frequencies of cells proliferating in response to specific anti-
32. Poon RY, Ohlsson-Wilhelm BM, Bagnell CB, Muirhead KA. Use of PKH membrane
33. intercalating dyes to monitor cell trafficking and function. In: Diamond RA, DeMa-
37. 655.
42. proliferation assay: Application of the DDPA to identify tumor-specific T cell precor-
44. Li Pira G, Ivaldi F, Dentece E, Righi P, Del Bono V, Viscoli C, Manca F. Minimizations
45. and automation for measuring antigen specific T-cell responses. 2008, Presented at the third MASIR conference, La Plagne, France.
47. Yu J, Chen H, Horton H, Bansaal A, McElrath JM, Reichman R, Geoppert P, Jin X. Interleukin-2 reconstitutes defective human immunodeficiency virus (HIV), and cy-
49. Lyons AB. Divided we stand: Tracking cell proliferation with carboxyfluorescein di-
52. Boyd FT. Identification of growth inhibited cells by retention of a lipophilic fluores-
57. Roederer M. Spectral compensation for flow cytometry: Visualization artifacts, lim-