

# Monitoring lymphocyte proliferation *in vitro* and *in vivo* with the intracellular fluorescent dye carboxyfluorescein diacetate succinimidyl ester

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**This protocol outlines the carboxyfluorescein diacetate succinimidyl ester (CFSE) method for following the proliferation of human lymphocytes *in vitro* and mouse lymphocytes both *in vitro* and *in vivo*. The method relies on the ability of CFSE to covalently label long-lived intracellular molecules with the highly fluorescent dye, carboxyfluorescein. Following each cell division, the equal distribution of these fluorescent molecules to progeny cells results in a halving of the fluorescence of daughter cells. The CFSE labeling protocol described, which typically takes <1 h to perform, allows the detection of up to eight cell divisions before CFSE fluorescence is decreased to the background fluorescence of unlabeled cells. Protocols are outlined for labeling large and small numbers of human and mouse lymphocytes, labeling conditions being identified that minimize CFSE toxicity but maximize the number of cell divisions detected. An important feature of the technique is that division-dependent changes in the expression of cell-surface markers and intracellular proteins are easily quantified by flow cytometry.**

## INTRODUCTION

A key feature of the adaptive immune response is the ability of clones of antigen-specific lymphocytes to rapidly proliferate and differentiate into effector cells. Thus, to monitor an immune response, it is important to have procedures that can follow lymphocyte proliferation, both *in vitro* and *in vivo*, with minimal disruption to cell viability and function. The intracellular fluorescent dye, CFSE, has been found to be particularly effective at monitoring lymphocyte division in many experimental situations<sup>1–5</sup>. Indeed, the tracking of lymphocyte division using CFSE has become a routine procedure in many laboratories since its first description by Lyons and Parish in 1994 (see ref. 6). In particular, the procedure has been used for the analysis of cell division *in vivo* in mice and *in vitro* using mouse and human lymphocytes. CFSE has the additional advantage that it can be used to track lymphocyte migration and positioning within tissues and lymphoid organs<sup>1</sup>. In fact, the dye was originally developed for this purpose, being stably incorporated into lymphocytes and allowing the tracking of lymphocytes *in vivo* for many months<sup>7</sup>. CFSE-labeled cells have also been used for *in vivo* and *in vitro* cytotoxicity assays<sup>8–11</sup>.

CFSE has the ability to stably label molecules within cells, with each cell division resulting in a sequential halving of fluorescence. Usually, lymphocyte proliferation can be monitored by flow cytometry for up to eight divisions before CFSE fluorescence is decreased to the background fluorescence of unlabeled cells. An important feature of the technique is that division-dependent changes in the expression of cell surface markers, such as immunoglobulin isotypes<sup>12</sup>, and intracellular proteins, such as cytokines<sup>13,14</sup>, can be easily quantified by flow cytometry by using appropriate antibodies labeled with different fluorochromes. Furthermore, the

proliferation of lymphocyte subsets within complex mixtures of cells can be easily quantified by using appropriate cell surface markers to identify the lymphocyte subpopulation<sup>15</sup>. A mass of highly quantitative data can be generated using the technique, which can be subjected to sophisticated data analysis<sup>16–19</sup>. This aspect of the CFSE procedure will be discussed in detail in an accompanying paper<sup>20</sup>. It should also be noted that although this protocol will describe the use of CFSE to monitor lymphocyte proliferation, the procedure has also been used to measure the proliferation of many other cell types, such as bacteria<sup>21</sup>, fibroblasts<sup>22</sup>, hematopoietic stem cells<sup>23</sup> and airway smooth muscle cells<sup>24</sup>.

To fully understand the use of CFSE to monitor lymphocyte proliferation, the molecular basis of CFSE labeling of cells needs to be outlined (Fig. 1). Initially, carboxyfluorescein diacetate succinimidyl ester is non-fluorescent, owing to the presence of two acetate groups, but these groupings result in the compound being highly membrane permeant and thereby enable the dye to rapidly flux across the plasma membrane of cells (Fig. 1). Once inside a cell, however, the acetate groups are rapidly removed by intracellular esterases to yield the highly fluorescent CFSE that is trapped inside cells owing to its reduced membrane permeability (Fig. 1). The succinimidyl group of CFSE allows it to react at neutral pH with amino groups to form a highly stable covalent bond. A proportion of the resultant carboxyfluorescein (CF) conjugates represent molecules that are able to exit via the plasma membrane or are rapidly degraded (CFR1 in Fig. 1). Conversely, some of the CF conjugates (CFR2 in Fig. 1) are highly stable and remain inside cells. It is these fluorescent conjugates that persist within cells for months but are diluted between daughter cells following cell division, which allows lymphocyte proliferation to be monitored by flow cytometry.

## MATERIALS

### REAGENTS

- Freshly isolated lymphocytes derived from humans or mice. See REAGENT SETUP **! CAUTION** Experiments involving mice and

humans must conform to appropriate national and institutional regulations.

- DMSO anhydrous >99%

- 5- (and 6-) CFSE (Molecular Probes, cat. no. C1157)
- PBS, pH 7.4
- PBS, pH 7.4, containing 5% (v/v) heat-inactivated fetal calf serum (HI-FCS)
- For *in vitro* stimulation: supplemented culture medium. See REAGENT SETUP
- For *in vivo* stimulation: host mice as recipients for adoptively transferred CFSE-labeled cells
- Appropriate stimuli to induce proliferation of lymphocyte subsets (as determined by the investigator; see REAGENT SETUP)
- Cell purification reagents; see REAGENT SETUP
- Fluorochrome-labeled antibodies for the detection of lymphocyte subsets during flow cytometry
- ▲ **CRITICAL** The fluorochromes need to be compatible with CFSE fluorescence (which has a peak emission wavelength of 517 nm). We have routinely used phycoerythrin (PE), PE-Cy7, peridinin chlorophyll protein (PerCP)-Cy5.5, allophycocyanin (APC), Alexa Fluor 594 and APC-Alexa Fluor 750 (or APC-Cy7) conjugated monoclonal antibodies.
- Fluorescent dye for discriminating dead cells from viable cells. See REAGENT SETUP
- Counting beads—if an estimation of absolute cell numbers in *in vitro* proliferation assays is required. We routinely use FLOW-COUNT fluorospheres (Beckman Coulter, cat. no. 7547053)

#### EQUIPMENT

- 10 or 50 ml (depending on the number of cells to be labeled) plastic sterile conical-bottomed tubes
- Centrifuge with swing-out buckets suitable for cell centrifugation
- Biohazard cabinet for handling human blood
- Flow cytometer

#### REAGENT SETUP

**CFSE stock solution** Dissolve CFSE powder in DMSO to a final concentration of 5 mM (typically CFSE, which has a molar mass of 557.47 g mol<sup>-1</sup>, is purchased as a vial containing 25 mg of desiccated powder, which equates to 44.8 μmol; thus, 8.96 ml of DMSO will be required to give a 5 mM solution). This solution can be stored in suitably sized aliquots (typically 50–200 μl) at –20 °C for several months and can be thawed and refrozen several times provided each aliquot is kept above –20 °C for a minimum period of time.

▲ **CRITICAL** CFSE will react rapidly upon exposure to aqueous solutions. It is, therefore, essential that such exposure be avoided until immediately prior to cell labeling.

**Freshly isolated lymphocytes derived from humans or mice** Cultured human lymphocytes following *in vitro* activation can be used, provided that they have reached a quiescent phase. Cryopreserved human peripheral blood mononuclear cells (PBMCs), whether isolated lymphocyte subsets or cultured previously, can also be used for CFSE labeling.

**Cell purification reagents** We have routinely employed negative selection procedures using Magnetic bead technologies (Stem Cell Technologies), positive

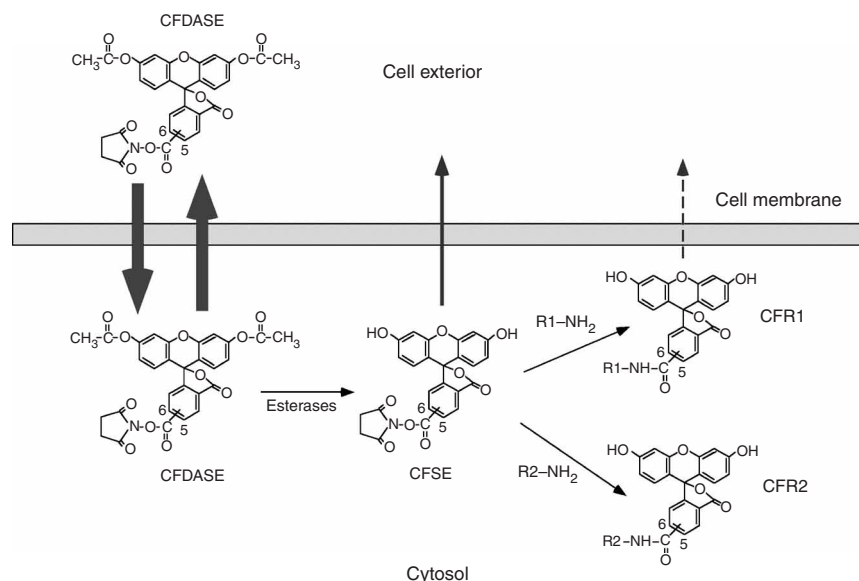


**Figure 1** | A schematic representation of the various molecular events that occur during the labeling of cells with CFSE. For details of the process, see text. CFSE1, carboxyfluorescein conjugated molecule that is lost from the cell; CFSE2, carboxyfluorescein conjugated molecule that is retained by the cell. *Note:* The generic acronym ‘CFSE’, not CFDASE, is used to describe the labeling reagent and the labeling procedure in general. Figure reproduced from Parish<sup>1</sup> with permission of the Australasian Society for Immunology.

or negative selection procedures using magnetic microbeads (Miltenyi Biotec) or cell sorting using immunofluorescence flow cytometry.

**Supplemented culture medium** Culture medium supplemented with antibiotics (100 μg ml<sup>-1</sup> penicillin and 100 μg ml<sup>-1</sup> streptomycin), 2 mM L-glutamine (GIBCO-BRL), 0.1 mM 2-mercaptoethanol (GIBCO-BRL) and 10% (v/v) HI-FCS. Supplement with sodium bicarbonate as recommended for the culture medium of choice. We have routinely used Eagle’s minimal essential medium for human PBMC cultures and DMEM for mouse lymphocyte cultures.

**Appropriate stimuli to induce proliferation of lymphocyte subsets** For the *in vitro* human PBMC studies shown as an example, we used phytohemagglutinin (PHA-P; Sigma, cat. no. L9132) as a polyclonal T-cell mitogen. For the mouse *in vitro* and *in vivo* studies shown, we used ovalbumin (OVA; Sigma, cat. no. A5503), hen egg lysozyme (HEL; Sigma, cat. no. L-7651) and a HEL-OVA conjugate (prepared as previously described<sup>25</sup>) as specific antigens (see ANTICIPATED RESULTS).

**Fluorescent dye for discriminating dead cells from viable cells** Methods based on 4′-6-diamidino-2-phenylindole and Hoechst 33258 are recommended for flow cytometers equipped with a 355 nm ultraviolet (and even a 405 nm violet) excitation source; otherwise, propidium iodide or 7-aminoactinomycin D can be used.

#### PROCEDURE

##### CFSE labeling of lymphocytes ● TIMING 0.5 h

**1** | Resuspend lymphocytes, typically at a concentration of  $0.5 \times 10^6$ – $50 \times 10^6$  ml<sup>-1</sup>, for CFSE labeling.

▲ **CRITICAL STEP** For cells at a low concentration ( $\leq 10 \times 10^6$  ml<sup>-1</sup>), it is essential that cells be labeled in the presence of added protein to buffer the toxic effects of CFSE, which may not induce cell death but can inhibit normal cell division (see ANTICIPATED RESULTS). Therefore, we recommend that cells be prepared in PBS (although culture medium can also be used) that contains 5% (v/v) FCS. Note that if culture medium is used, free amino acids in the medium may lower the labeling efficiency by competing for CFSE.

▲ **CRITICAL STEP** For cells at high concentrations ( $50 \times 10^6$  cells ml<sup>-1</sup>), the presence of added protein or culture medium components can reduce the extent of CFSE labeling, which may reduce the number of detectable division peaks. Therefore, we recommend that the cells be prepared in PBS without the inclusion of protein.

▲ **CRITICAL STEP** Ensure that you have sufficient cells for the experiment, allowing sufficient quantities to perform an appropriate number of replicates and controls.

**2** | Dilute CFSE either directly into the cell suspension or prepare a dilution in aqueous solution and add this in an appropriate volume to give a final concentration of 5 μM in the cell suspension. For cells totaling  $\leq 50 \times 10^6$ , option A can be followed; an

alternative method, which is also appropriate for higher cell numbers, is given in option B. The latter method can also easily be scaled up for larger numbers of cells by increasing, in proportion, the volumes used.

**▲ CRITICAL STEP** As CFSE will react quickly in aqueous solution, it is critical that it is dispersed rapidly in the cell suspension, and that the cells are as close to a homogeneous suspension of single cells as possible, to ensure even labeling of lymphocytes.

**▲ CRITICAL STEP** Although the optimum labeling concentration of CFSE for measurement of cell proliferation is 5  $\mu\text{M}$ , the optimum concentration may have to be determined for each batch prepared and then checked every 6 months to ensure it is effective.

### ? TROUBLESHOOTING

#### (A) For cells totaling $\leq 50 \times 10^6$

- (i) Thoroughly resuspend the cells in a 1 ml volume of PBS containing 5% (v/v) FCS in a fresh tube.
- (ii) Lay the tube horizontally (a non-wetted tube is important to prevent cell suspension movement and premature mixing of solutions).
- (iii) Add 110  $\mu\text{l}$  of PBS to the non-wetted portion of the plastic at the top of the tube (ensure this does not mix with the cells), and in this resuspend 1.1  $\mu\text{l}$  of the 5 mM stock of CFSE.
- (iv) Cap the tube and quickly invert several times and vortex.

#### (B) Alternative method, which is also appropriate for higher cell numbers ( $50\text{--}100 \times 10^6$ )

- (i) Prepare a 10  $\mu\text{M}$  ( $2\times$ ) CFSE solution by adding 2  $\mu\text{l}$  of the 5 mM stock to 1 ml of PBS and quickly add to 1 ml of thoroughly resuspended cells.
- (ii) Mix immediately on a vortex.

3| After thorough mixing, leave cells to label with CFSE by incubating for 5 min at room temperature (20  $^{\circ}\text{C}$ ).

**▲ CRITICAL STEP** Optimum labeling of the cells is complete within only a few minutes at room temperature, and incubation for longer periods can result in toxicity to cells that can affect cell proliferation. This is particularly notable at low cell concentrations in the absence of buffering proteins.

**▲ CRITICAL STEP** It should be noted that upon labeling, CFSE becomes fluorescent and is therefore prone to bleaching by exposure to excessive light, so this should be avoided. For example, cover the tube containing the CFSE-labeled lymphocytes with an aluminum foil.

### ? TROUBLESHOOTING

4| Wash the cells by diluting with ten volumes of 20  $^{\circ}\text{C}$  PBS containing 5% (v/v) HI-FCS, sediment by centrifugation at 300g for 5 min at 20  $^{\circ}\text{C}$  and discard the supernatant. Wash the cell pellet in the same manner two more times.

**▲ CRITICAL STEP** Much of the CFSE at this point is not stably incorporated within the cells and is lost from the cells during the next 24–48 h. As a result, the initial fluorescence intensity is extremely high and can bleed significantly into other flow cytometry detectors (particularly those set for detection of fluorescence at wavelengths close to CFSE, i.e., the PE detector). This should be taken into consideration if cells are to be analyzed or sorted by a flow cytometer within the first few hours after staining, which is not recommended. This problem can be reduced by decreasing the concentration of CFSE during labeling, but this will reduce the number of detectable cell divisions.

## Stimulation of CFSE-labeled lymphocytes

5| The labeled lymphocytes can be stimulated *in vitro* (see option A) or *in vivo* (see option B).

#### (A) *In vitro* stimulation of CFSE-labeled lymphocytes ● TIMING 1 h

- (i) Prepare unlabeled and CFSE-labeled cells at a suitable concentration and stimulate with and without antigens and mitogens of interest. For the human PBMC examples given in this protocol (**Fig. 2**), PBMCs (untreated or CFSE-labeled) were prepared at  $5 \times 10^5 \text{ ml}^{-1}$  in supplemented Eagle's minimal essential medium (see REAGENT SETUP), 0.1 ml was added per well of a 96-well flat-bottomed plate and then 0.1 ml of culture medium (control) or PHA ( $10 \mu\text{g ml}^{-1}$ ) prepared in culture medium was added. For the mouse lymphocyte examples shown (**Fig. 3**),  $\text{CD4}^+$  T cells purified from the spleen and lymph nodes of T-cell receptor (TCR)-transgenic (OVA-specific) OT-II mice ( $1 \times 10^5 \text{ cells well}^{-1}$ , untreated or CFSE-labeled) were cultured with different numbers ( $1\text{--}30 \times 10^4 \text{ well}^{-1}$ ) of splenic B cells from B-cell receptor (BCR)-transgenic (HEL-specific) MD4 mice in a total volume of  $200 \mu\text{l well}^{-1}$  of supplemented DMEM (see REAGENT SETUP) in round-bottomed wells of a 96-well plate and in the presence of  $100 \text{ ng ml}^{-1}$  of a HEL-OVA conjugate as antigen.

**▲ CRITICAL STEP** It is important to have CFSE-labeled, unstimulated cells as a control, as well as stimulated cells (both unlabeled and CFSE-labeled). The autofluorescence of proliferating lymphocytes is higher than that of resting lymphocytes and thus, as an unlabeled population, provides a measurement of the autofluorescence of an activated lymphocyte population. CFSE-labeled unstimulated cells provide a fluorescence measurement of non-dividing lymphocytes. Using these controls, one can therefore calculate the cell division number of the proliferating, CFSE-labeled lymphocytes.

**▲ CRITICAL STEP** Avoid using the outer wells on a plate for lymphocyte cultures, as these are most subject to evaporation and temperature fluctuations over time. The outer wells can be filled with PBS.

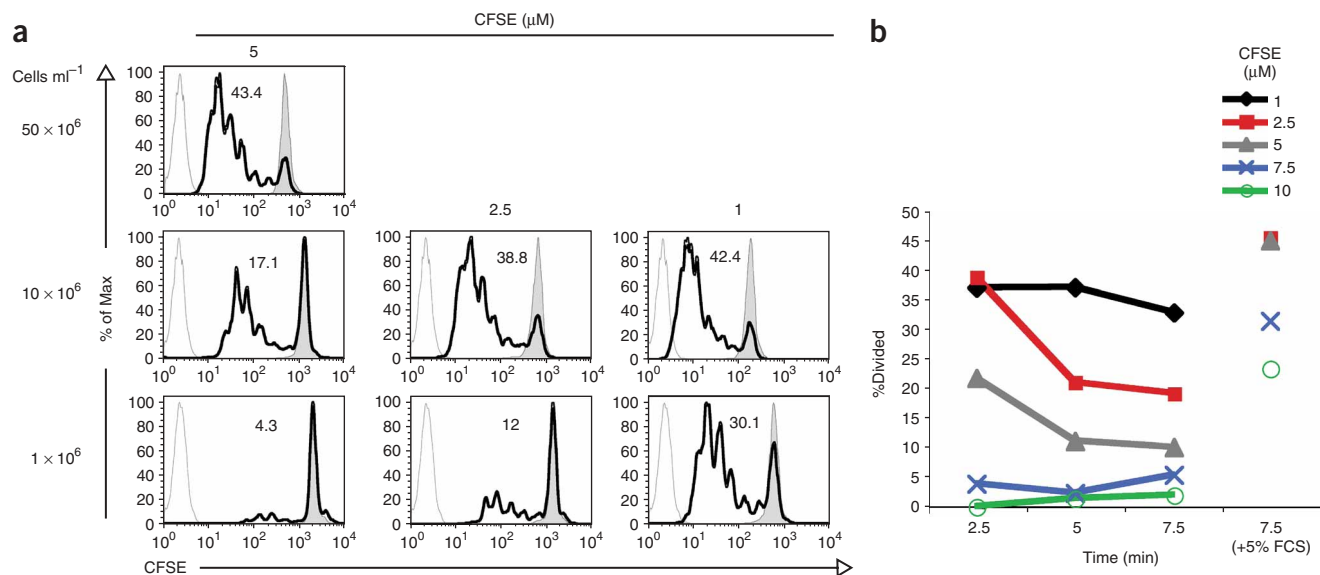


**Figure 2 | Demonstration of the effect of different CFSE labeling conditions on the ability of human PBMCs to respond to the polyclonal T-cell mitogen PHA.** (a) Human PBMCs were labeled with different CFSE concentrations at different cell concentrations and then assessed for their ability to proliferate when stimulated with PHA for 4 d. Cells were labeled for 5 min at 20 °C in protein-free PBS and then PBS containing 5% (v/v) FCS was added to halt the reaction. The bold numbers within each histogram represent the %Divided cells in response to PHA as measured by CFSE dilution. The results show that when 5 μM CFSE was used, the capacity of the lymphocytes to fully respond to PHA was preserved only when labeling was performed at the highest cell concentration (50 × 10<sup>6</sup> ml<sup>-1</sup>). At lower cell concentrations, 5 μM CFSE impaired PHA-induced proliferation. In contrast, at lower CFSE concentrations (1 μM), lower concentrations of lymphocytes (10 × 10<sup>6</sup> ml<sup>-1</sup>) were effectively labeled without evidence of CFSE toxicity. Note that the intensity of CFSE labeling of the unstimulated cells (shaded histogram) is an indication of the extent of CFSE uptake under the various labeling conditions. (b) Data showing the PHA responsiveness of human PBMCs labeled with different CFSE concentrations and for different labeling times. PBMCs (1 × 10<sup>6</sup> ml<sup>-1</sup>) were labeled in protein-free PBS, with the data showing the percentage of cells that divided in response to PHA. It is evident that CFSE labeling is rapid (2.5 min) and becomes toxic for cells following more prolonged incubation. In contrast, the presence of protein (5% (v/v) FCS) during the labeling reaction can protect cells from CFSE toxicity. These data were derived in part from a previous publication<sup>5</sup>.

## (B) *In vivo* stimulation of CFSE-labeled lymphocytes ● TIMING 2.5 h

- For *in vivo* stimulation, adoptively transfer CFSE-labeled lymphocytes into host mice, typically up to 5 × 10<sup>7</sup> cells animal<sup>-1</sup> in a 200 μl PBS bolus i.v.
- Challenge these mice, typically 2–24 h later, with specific antigen in PBS or PBS alone (200 μl i.v.), the control (PBS-injected) mice providing a fluorescence measurement of non-dividing CFSE-labeled cells as well as the autofluorescence of recipient (host) lymphocytes. The fluorescence measurements in the control mice allow discrimination of the number of cell divisions occurring in the antigen-stimulated, CFSE-labeled lymphocyte populations (see ANTICIPATED RESULTS).

## Harvesting and detection of proliferating lymphocytes

**6 |** Harvest lymphocytes for CFSE analysis after ≥ 18 h *in vitro* (option A) or *in vivo* (option B), which gives sufficient time for loss of much of the non-stably incorporated CFSE and consequently reduces the detection problems associated with extremely fluorescent cells. Cell proliferation, however, is not usually seen until > 48 h after culture initiation or adoptive transfer (see ANTICIPATED RESULTS). Using the above method it is anticipated that up to nine fluorescent peaks of CFSE can be detected. Thus, if cell division has proceeded beyond eight divisions, cells will be at or close to the autofluorescence level of unlabeled cells.

▲ **CRITICAL STEP** It should be noted that CFSE has a peak excitation wavelength of 492 nm and a peak emission wavelength of 517 nm. The excitation wavelength, however, is compatible with most standard flow cytometers with a 488 nm excitation laser.

## (A) *In vitro* cultures ● TIMING 2 h

- Harvest cells from culture wells and analyze directly by flow cytometry for cell proliferation. Cells can be analyzed in their culture medium without washing.
- If fluorescent antibody staining is required, perform this as normal, taking into consideration the fact that CFSE has a strong peak fluorescence intensity at 517 nm, which precludes the use of similar fluorescent conjugates and may also require substantial compensation to reduce the bleeding of fluorescence into some flow cytometer channels.

▲ **CRITICAL STEP** Successful fluorescent compensation requires the use of single stained control samples for each fluorescent label used as well as a non-stained sample. Owing to its high fluorescence, it is critical to use CFSE-labeled cells without fluorescent antibody staining to enable correct compensation to be determined on the flow cytometer.

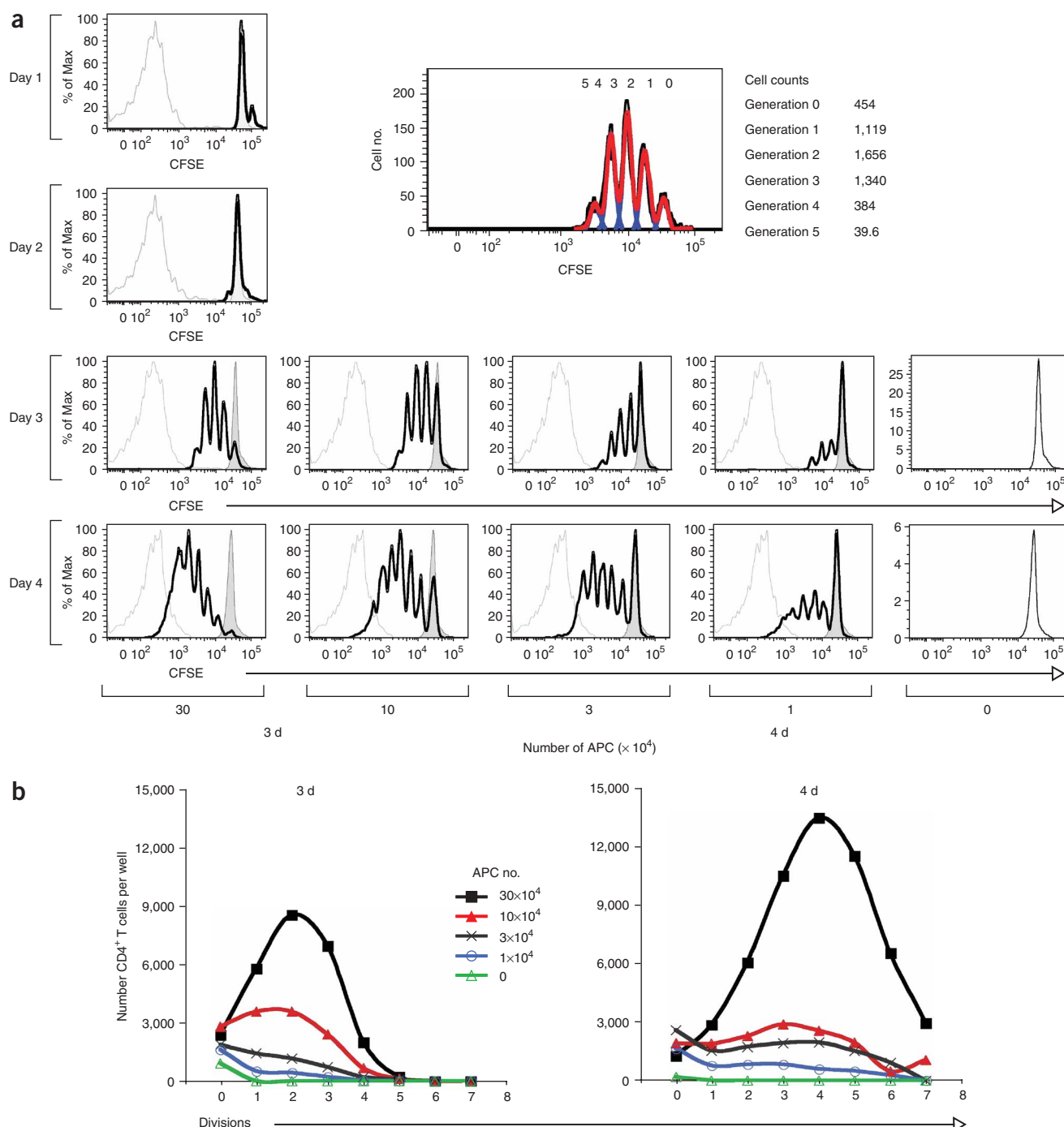


**Figure 3** | Measurement of CD4<sup>+</sup> T-cell proliferation *in vitro* by CFSE dilution. **(a)** Histogram plots of CFSE fluorescence of OVA-specific TCR-transgenic CD4<sup>+</sup> OT-II T cells after 1–4 d culture with decreasing numbers of HEL-specific BCR-transgenic MD4 B cells in the presence of a HEL-OVA conjugate as antigen. CD4<sup>+</sup> T cells purified from spleen and lymph nodes of OT-II mice were labeled with CFSE and cultured with the indicated numbers of B cells (antigen-presenting cells (APCs)) purified from the spleen of MD4 mice and in the presence of 100 ng ml<sup>-1</sup> of a HEL-OVA conjugate. Cells in each well at each time point were doped with 2,000 FLOW-COUNT beads, stained with anti-B220-PerCP, anti-CD4-APC and anti-CD90.2-APC-Cy7 antibodies to delineate lymphocyte subpopulations, stained with Hoechst 33258 to identify dead cells and then analyzed by flow cytometry. The plots for each day show the CFSE profiles of viable CFSE-labeled OT-II CD4<sup>+</sup> T cells stimulated with either different numbers of APC (B cells—black open histogram) or no APCs (gray filled histogram) as well as the autofluorescence of CD4<sup>+</sup> T cells not labeled with CFSE (open gray histogram). The enlarged CFSE plot shows a FlowJo-generated CFSE profile, the red line depicting the overall proliferation profile and the blue lines forming individual histograms for each cell division, the latter plots being used to calculate the number of cells in each generation (0–5) (data from day-3 CFSE plot obtained with 30  $\times 10^4$  APCs). **(b)** Total number of CD4<sup>+</sup> T cells in each culture well from **a** that have undergone 0–8 cell divisions, as calculated by the FlowJo curve-fitting software and using FLOW-COUNT counting beads to calculate absolute cell number.

(iii) For an estimate of cell number, we recommend that a known number of FLOW-COUNT fluorospheres are added to each well. The number added should be such that  $>100$  beads sample<sup>-1</sup> are collected or in the range of 5–10% minimum of the total events measured. The beads should be added to the culture in 10–50  $\mu$ l in PBS.

▲ **CRITICAL STEP** Beads should be added before any antibody staining procedures, so that any loss in cells during sample preparation will be accompanied by a proportional loss of beads.

▲ **CRITICAL STEP** Mix the bead stock thoroughly and transfer the required amount to be used for the experiment to a smaller tube (we often mix this with the antibody cocktail to be used to stain the cells). Again mix well and transfer the required volume of beads (or antibodies) to each well using the same pipette tip to reduce error.

(iv) To simultaneously assess bead and lymphocyte numbers, set the side scatter parameter on the flow cytometer on a logarithmic scale.

▲ **CRITICAL STEP** It is not essential to harvest the whole sample, as cell number can be determined using the ratio of cells to beads. It is essential, however, to thoroughly mix the samples so that the beads are evenly dispersed with the cells in solution.

(v) To assess cell death, add propidium iodide or an alternative fluorescent DNA binding protein (see REAGENT SETUP) compatible with fluorescent antibody staining, just before collection on the flow cytometer.

## (B) *In vivo* stimulation ● TIMING 3 h

(i) Extract lymphoid tissues and make single-cell suspensions.

▲ **CRITICAL STEP** It should be pointed out that when CFSE-labeled lymphocytes are adoptively transferred into host mice, the content of transferred cells in host lymphoid organs may be quite low (in the order of 1% or less). It is therefore important that enough cells are prepared for a sufficient number of CFSE-labeled cells to be collected for analysis. We routinely prepare  $4 \times 10^6$  cells sample<sup>-1</sup> for antibody staining, such that at least  $1 \times 10^6$  cells can be processed by the flow cytometer to yield at least  $1 \times 10^4$  CFSE-labeled cells. It is also important to remember that if lymphocytes have undergone extensive division, they may not contain detectable CFSE. In these cases, it is important to detect the presence and number of transferred cells with another marker, for example, by the use of recipient animals that express a different allotype of the leukocyte common antigen (CD45) than the adoptively transferred cells<sup>15</sup>.

(ii) Stain cells with compatible fluorochrome-labeled antibodies and/or fluorescent dyes and analyze by flow cytometry as normal (as described in Steps 6A(ii) and (v)).

## Analysis of proliferating lymphocytes

7| Calculate the number of dividing lymphocytes. Further details of various methods available for calculating the number of dividing lymphocytes (both *in vitro* and *in vivo*) are given in an accompanying paper (ref. 20). In the examples presented here

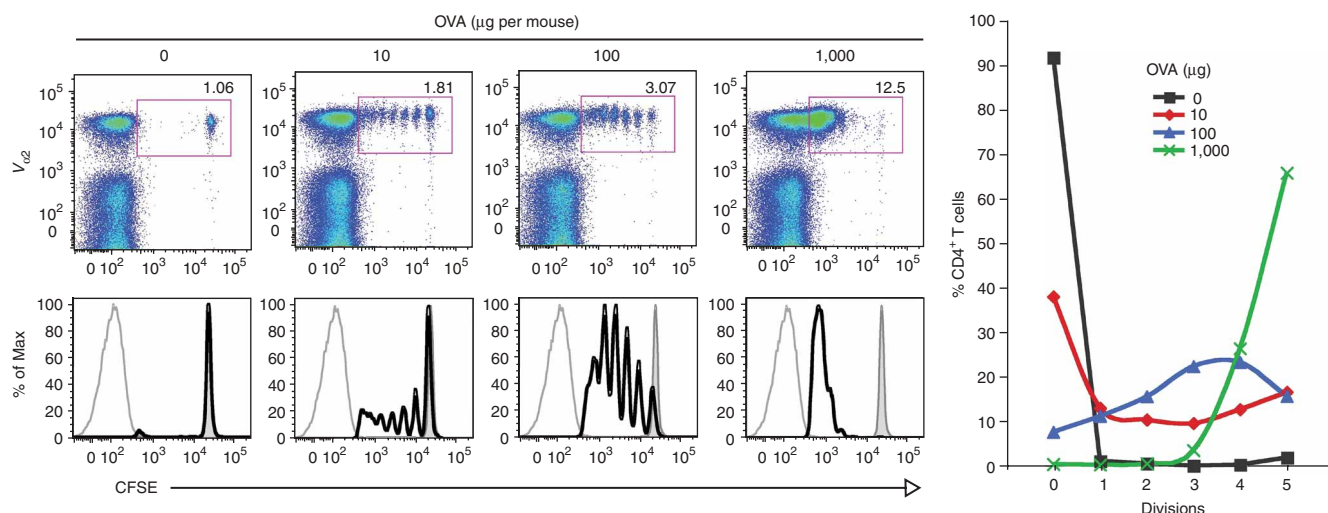


**Figure 4** | Measurement of CD4<sup>+</sup> T-cell proliferation *in vivo* by CFSE dilution. CFSE fluorescence of OVA-specific TCR-transgenic CD4<sup>+</sup> OT-II T cells after 3 d *in vivo* in the presence of increasing concentrations of OVA. CD4<sup>+</sup> T cells purified from the spleen and lymph nodes of male OT-II mice were labeled with CFSE and  $9 \times 10^6$  cells were injected i.v. into the lateral tail vein of male C57BL/6J mice. One day later, the mice were injected i.v. with 10–1,000  $\mu$ g of OVA. After an additional 3 d, spleens from the mice were collected, made into a single-cell suspension, labeled with anti-B220-PerCP, anti-CD4-APC and anti-V $\alpha$ 2-APC-Cy7 (present on all OT-II CD4<sup>+</sup> T cells) antibodies to delineate lymphocyte subsets, stained with Hoechst 33258 to identify dead cells and then analyzed by flow cytometry. Two-color density plots show expression of CFSE and V $\alpha$ 2 by viable CD4<sup>+</sup>, B220<sup>-</sup> cells, with the gated region showing the percentage of the population expressing both CFSE and V $\alpha$ 2, which represents the transferred CD4<sup>+</sup> OT-II T cells. The histogram plots show the CFSE profiles of viable CD4<sup>+</sup> OT-II T cells (as defined above) at each concentration of OVA (black open histogram) relative to the generation 0 CFSE profile of viable transferred CD4<sup>+</sup> OT-II T cells in the absence of OVA (gray filled histogram) and the autofluorescence of host CD4<sup>+</sup>, B220<sup>-</sup> cells (open gray histogram). The line graph shows the percentage of transferred CD4<sup>+</sup> OT-II T cells in each animal that have undergone 0–8 cell divisions as calculated by the FlowJo curve-fitting software (as in Fig. 3a).

(see ANTICIPATED RESULTS), we have used ‘%Divided cells’ as calculated by the FlowJo software, which is defined as the percentage of cells from the original sample that have divided, assuming no cell death.

### ● TIMING

It takes 30 min to label the lymphocytes with CFSE. Preparation and plating for *in vitro* stimulation takes 30 min to 1 h depending on the number of treatment groups. Injection into mice for *in vivo* experiments takes 30 min to 1 h depending on the number of recipient mice. Lymphocytes are usually harvested 18 h to 4 d after *in vitro* or *in vivo* stimulation. Fluorescent staining of lymphocytes after stimulation takes 2 h, with an additional 1 h being required for the preparation of lymphocytes from the recipient mice used in *in vivo* experiments.

### ? TROUBLESHOOTING

The amount of CFSE used to label cells must be sufficiently high to maximize the number of detectable cell divisions above the autofluorescence of unlabeled cells, while not being so high that, owing to CFSE toxicity, it impairs the ability of cells to divide. The above protocol gives an overall method that takes into consideration this important aspect of the procedure. Nevertheless, it is possible for there to be some variation in the optimum CFSE labeling method owing to reagent preparation and the cell type being labeled. **Table 1** lists TROUBLESHOOTING solutions to some of these problems.

**TABLE 1** | Troubleshooting table.

| Step    | Problem                                                                             | Possible reason                                                                                                                       | Solution                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                        |
|---------|-------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| 2 and 3 | Poor proliferative response                                                         | CFSE labeling is too high                                                                                                             | Decrease the concentration of CFSE used to label cells and include 5% (v/v) FCS in the labeling medium to limit the toxic effects of CFSE                                                                                                                                                                                                                                                                                                                                                                                                                                                                       |
| 2       | Insufficient number of division peaks above the autofluorescence of unlabeled cells | CFSE labeling is too low<br>CFSE stock is too old<br>DMSO used to prepare CFSE stock solution is not anhydrous                        | Reassess the optimum CFSE concentration used to label cells<br>Prepare new CFSE stock solution using anhydrous DMSO<br>Do not label in medium containing amino acids                                                                                                                                                                                                                                                                                                                                                                                                                                            |
| 2       | Division peaks are not distinct                                                     | Cell labeling is not uniform<br><br>Cell suspension is heterogeneous in size, with individual cells consequently labeling differently | Ensure that addition of CFSE to cells occurs rapidly and with immediate mixing<br>Ensure cells are fully dissociated into a single-cell suspension before labeling<br><br>Using the flow cytometer, sort cells, before CFSE labeling, into a homogeneous population based on size (forward scatter) and granularity (side scatter). Conversely, sort cells after CFSE labeling into a homogeneous fluorescent population <sup>16</sup><br>The use of markers to delineate specific lymphocyte subsets will increase the resolution of division peaks, particularly if multiple subpopulations are proliferating |

### ANTICIPATED RESULTS

A critical aspect of the CFSE labeling procedure is to ensure that the labeling of lymphocytes is optimal to enable the tracking of a maximum number of cell divisions, without CFSE concentration reaching a level that is toxic for the cells. The four key factors that determine effective, non-toxic labeling of lymphocytes are the cell concentration, the labeling time, the presence or absence of protein in the medium in which the cells are suspended during labeling and the CFSE concentration. **Figure 2** highlights the effect of these variables on CFSE labeling efficiency and the subsequent proliferative potential of the labeled PBMCs. At high cell concentrations ( $50 \times 10^6$  cells  $\text{ml}^{-1}$ ), 5  $\mu\text{M}$  CFSE resulted in a labeled lymphocyte population that exhibited maximal proliferative potential (43.4%; **Fig. 2a**). In contrast, when lower concentrations of lymphocytes ( $1\text{--}10 \times 10^6$  cells  $\text{ml}^{-1}$ ) were labeled with 5  $\mu\text{M}$  CFSE, although the undivided cell populations displayed heightened levels of fluorescence, the proliferative capacity of the labeled cells was dramatically reduced (**Fig. 2a**). However, the toxic effects of CFSE can be prevented by reducing the CFSE concentration when labeling lower concentrations of cells, with 1  $\mu\text{M}$  CFSE effectively labeling cells at a concentration of  $10 \times 10^6$   $\text{ml}^{-1}$  (**Fig. 2a**). Alternatively, CFSE toxicity can be reduced by including protein (5% (v/v) FCS) in the labeling medium, this approach being particularly effective when low concentrations of cells are available for labeling (**Fig. 2b**). The data presented in **Figure 2b** also highlight the fact that CFSE labeling of cells is extremely rapid, good

labeling occurring in 2.5 min but excessive labeling increasingly becoming a problem with longer labeling times (**Fig. 2b**). Finally, it should be noted that a characteristic feature of CFSE toxicity is that the over-labeled cells remain viable, in terms of exclusion of vital dyes, but are unable to proliferate. Based on the above discussion, it is probably wise, when a laboratory is undertaking CFSE labeling of lymphocytes for the first time, to initially CFSE label a readily obtainable lymphocyte population and test the mitogen responsiveness of the labeled cells.

A typical T-cell proliferation experiment, as measured by CFSE dye dilution, is depicted in **Figure 3**. In this example, the *in vitro* responsiveness of TCR-transgenic CD4<sup>+</sup> T cells to different numbers of antigen-presenting cells (APCs) is shown, in this case the APC being antigen-specific (BCR-transgenic) B cells. **Figure 3a** displays the proliferation profiles, with T-cell proliferation clearly being dependent on the concentration of APC and the time of culture. It should be noted, however, that substantial T-cell proliferation is evident only after 3 d of culture, a common feature of T and B cells responding to mitogens and specific antigens. Also there is a steady decline in the CFSE fluorescence of undivided T cells from days 1 to 4 of culture, with a concomitant increase in the autofluorescence of unlabeled activated T cells. These two effects can result in fewer rounds of cell division being detectable before the CFSE fluorescence of the dividing cells merges with the autofluorescence of unlabeled cells.

**Figure 3a** also provides an example of the use of the FlowJo curve-fitting software to calculate the number of cells in each division (generations 0–5). This approach, in combination with counting beads, can be used to calculate the absolute number of cells in each culture well that have undergone different numbers of cell divisions (**Fig. 3b**).

Data obtained from a typical *in vivo* CFSE tracking experiment are shown in **Figure 4**. In this case, the proliferative response of CFSE-labeled, OVA-specific, TCR-transgenic CD4<sup>+</sup> T cells to different concentrations of OVA was assayed 3 d after adoptive transfer of the T cells into naïve recipient mice. In the absence of antigen (OVA), the transferred, CFSE-labeled T cells failed to proliferate, but increasing proliferation and expansion in the number of transferred CD4<sup>+</sup> T cells was observed when increasing amounts of OVA were injected into the animals (**Fig. 4**). In fact, at the highest OVA dose (1,000 µg), all the CFSE-labeled T cells proliferated and the fluorescence of the dividing cells approached the autofluorescence level of unlabeled CD4<sup>+</sup> T lymphocytes in the recipient animal (**Fig. 4**). To overcome the problem of distinguishing highly proliferating donor cells from non-proliferating recipient cells, CFSE-labeled lymphocytes can be discriminated from recipient lymphocytes using the CD45.1 and CD45.2 allo-typic markers<sup>15</sup>. Finally, the number and percentage of donor cells in each division can be calculated using the FlowJo software (**Fig. 4**, right-hand panel). More sophisticated ways of analyzing data is presented in detail in an accompanying protocol<sup>20</sup>.

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