

# Amine-Reactive Dyes for Dead Cell Discrimination in Fixed Samples

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## ABSTRACT

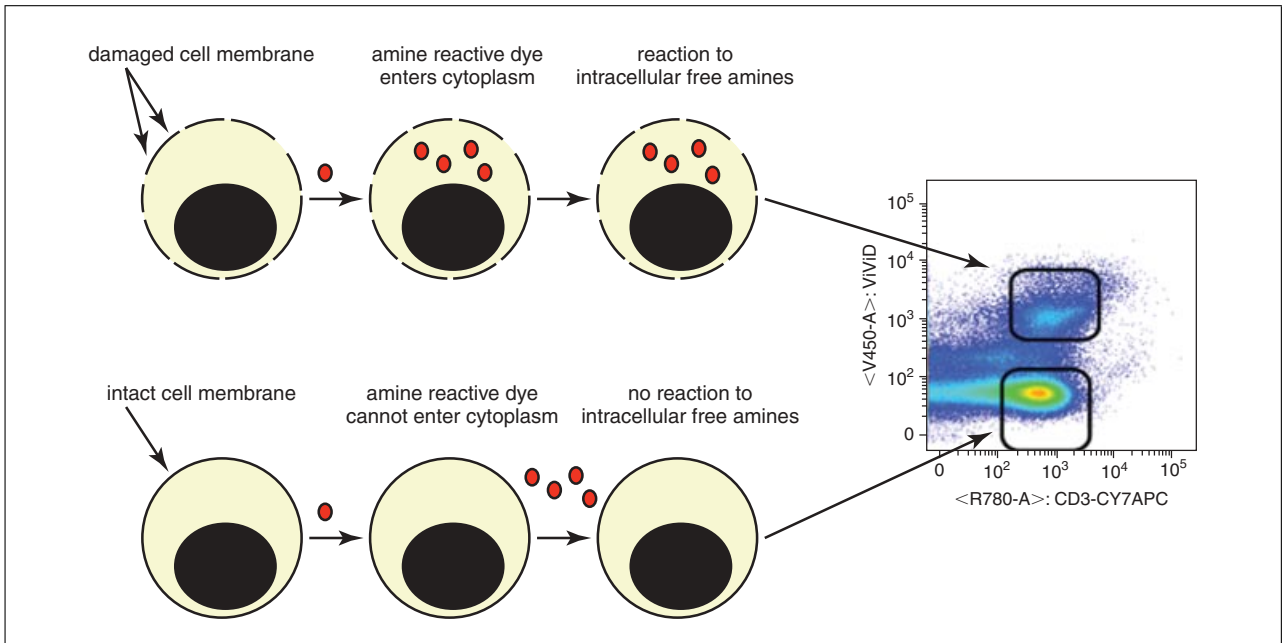
Amine-reactive dyes, also known as LIVE/DEAD fixable dead cell stains, are a class of viability dyes suitable for identifying dead cells in samples that will be fixed. These dyes cross the cell membranes of dead cells, and react with free amines in the cytoplasm. Live cells exclude these dyes because their cell membranes are intact, and free dye is washed away after staining. Notably, the reaction is irreversible; therefore, when cells are fixed and permeabilized (as with intracellular staining procedures), the bound dye remains associated with the dead cells (unlike other viability dyes). Since amine-reactive dyes are fluorescent when excited by lasers, dead cells can be identified by flow cytometry. This unit describes procedures, troubleshooting, and outcomes for using the two most commonly used amine-reactive dyes, ViViD and Aqua Blue. *Curr. Protoc. Cytom.* 53:9.34.1-9.34.14. © 2010 by John Wiley & Sons, Inc.

Keywords: amine reactive dye • LIVE/DEAD fixable cell stain • cell viability • nonspecific binding

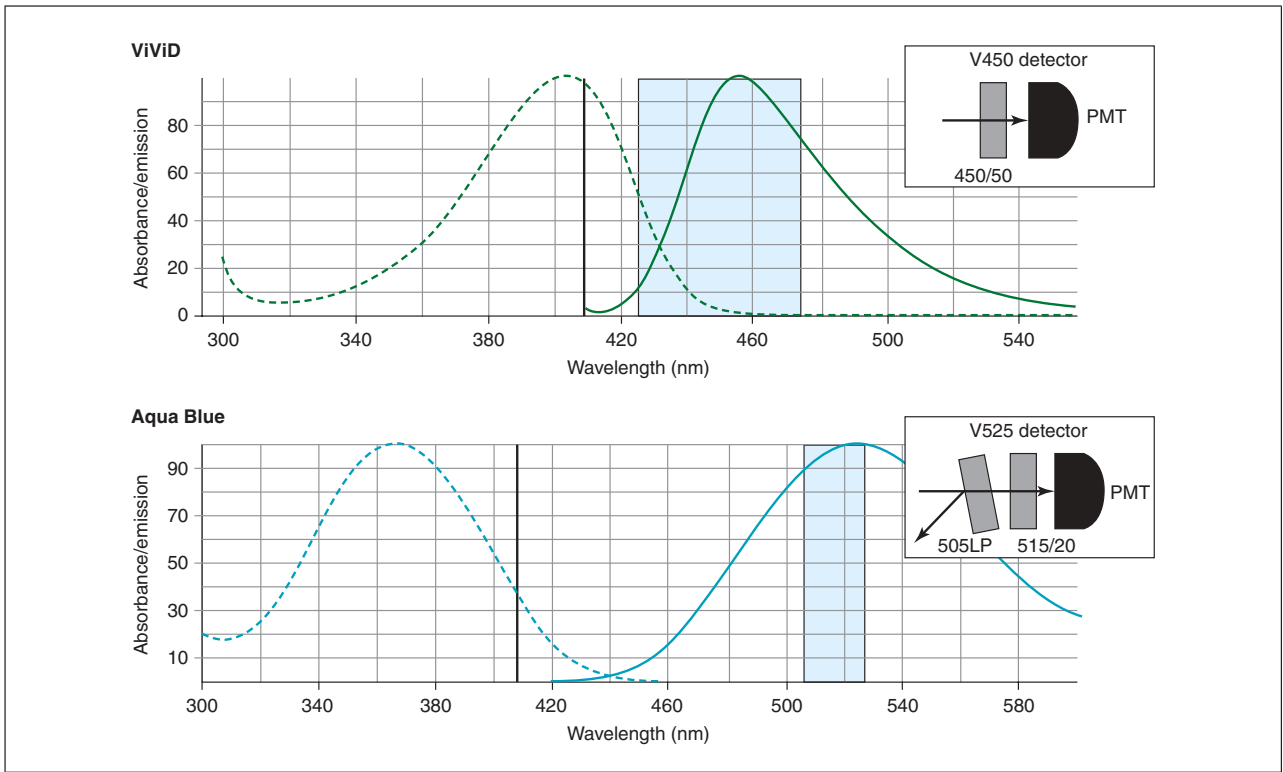
## INTRODUCTION

Amine reactive viability dyes offer an innovative alternative to traditional viability markers. There are eight LIVE/DEAD fixable dead cell stains available in the market today, covering most of the visible and UV spectrum. Two of the most commonly used amine-reactive dyes, ViViD and Aqua Blue, are described in this protocol. These dyes differ from other viability markers because they react with free amines in the cytoplasm. Live cells exclude these dyes because their cell membranes are intact, and free dye is washed away after staining (Fig. 9.34.1). Notably, the reaction is irreversible; therefore, when cells are fixed and permeabilized (as with intracellular staining procedures), the bound dye remains associated with the dead cells (unlike other viability dyes). To measure these two dyes, it is critical to determine the correct filter configuration for detecting signal from each dye on the flow cytometer. For example, Figure 9.34.2 shows the excitation curves (dotted lines) and the emission curves (solid lines) of ViViD and Aqua Blue, which can be excited by a 408-nm violet laser (vertical black line). The shaded area over the emission curve shows the range of detection. Hence, ViViD is measured between 425 to 475 nm (450/50 band pass filter), while Aqua Blue is detected between 525 to 575 nm (505 nm long pass filter + 515/20 nm band pass filter). Additionally, it is important to note that the flow cytometer used to measure amine-reactive dyes should be calibrated and standardized as described (see UNIT 1.3; Perfetto et al., 2006a).

The utility of amine-reactive dyes is best demonstrated in the context of staining panels, which employ various combinations of fluorochrome monoclonal antibody (mAb)-conjugates, using samples that have been cryopreserved and/or stimulated with antigen. Such samples often include large numbers of dead cells, which can bind mAb-conjugates nonspecifically. In intracytoplasmic cytokine staining (ICS) and peptide-MHC Class I (“tetramer”) assays, (Perfetto et al., 2006b; Chattopadhyay et al., 2008), where detection of rare events is critical, nonspecific binding of mAb-conjugated to dead cells may result



**Figure 9.34.1** The staining theory of amine-reactive dyes. The upper pathway shows cells with damaged membranes; in this example the amine reactive dye ViViD enters through these membranes and reacts with the free amines in the cytosol. After binding with free amines this dye complex is positive for fluorescence in the V450 detect (arrow pointing to the upper cell population or the dead cells). In the lower pathway, the dye is excluded by the intact cell membrane and the cell remains negative for fluorescence (arrow pointing to the lower cell population or the live cell gate). For the color version of this figure go to <http://www.currentprotocols.com/protocol/cy0934>.



**Figure 9.34.2** The instrument configuration for proper detection of two common amine-reactive dyes. The excitation (dashed curve) and emission (solid curve) curves of the ViViD dye (upper panel) and the Aqua Blue dye (lower panel) are shown. These dyes are excited by the violet 408-nm laser indicated by the black line over the excitation curve. Each panel shows the band-pass filter range of detection for the PMT as shown in each insert. In order to detect fluorescence from each emission curve, the V450 detector uses only the 450/50 nm band-pass filter (upper insert) and the V525 detector uses a 505LP dichroic filter combined with a 515/20 nm band-pass filter (lower insert).

**9.34.2**

in significant overestimation of the proportion of antigen-specific cells. In summary, amine reactive viability dyes can be a powerful substitute for more traditional viability dyes and can be used in a variety of panel designs.

## TITRATION OF AMINE-REACTIVE DYES

The purpose of this protocol is to ensure that an optimal amount of amine-reactive dye is used in staining experiments. The optimal concentration is defined as the concentration, which produces the highest signal (MFI) and the lowest background. To this end, amine-reactive dyes should be tested at concentrations above and below the manufacturer's recommended dilution, using samples that contain substantial numbers of dead cells. For example, peripheral blood mononuclear cell (PBMC) samples contain substantial numbers of dead cells when incubated at 37°C for three to five days in RPMI cell culture medium (containing 10% serum). Similarly, the frequency of dead cells is high when frozen cells are thawed under sub-optimal conditions (e.g., extended exposure of frozen cells to ambient temperatures). Once titration is completed, new vials from the same lot of amine reactive dye can be used at the same concentration; titration should be performed with each new lot.

### Materials

Amine reactive dye kit (ViViD, LIVE/DEAD fixable violet dead cell stain or Aqua Blue, LIVE/DEAD fixable aqua dead cell stain; both from Invitrogen) containing:

Dimethyl sulfoxide (DMSO)  
Lyophilized dye  
Phosphate-buffered saline (PBS; Becton Dickinson)  
Cells of interest  
Standard staining medium (see Table 9.34.1)  
Fluorochrome-conjugated mAb mix (e.g., anti-CD3 Cy7APC)

37°C water bath

Flow cytometer (an analyzer or sorter equipped with a violet laser and optics such as shown in Fig. 9.34.2; e.g., LSR-II, Becton Dickinson; alternatively, note in the same figure that the ViViD and Aqua Blue dyes are also well excited by a UV laser)

Flow cytometry data analysis software (e.g., FlowJo software, Treestar)

Statistical analysis software (e.g., JMP software, SAS Institute)

1. Thaw the dimethyl sulfoxide (DMSO) supplied with the kit using a 37°C water bath until completely thawed (~30 sec).

**IMPORTANT NOTE:** *Lyophilized dye is stored desiccated and expires at 60 months under these conditions. DMSO must be stored under desiccated conditions.*

*Each amine reactive dye kit comes with 25 µg of lyophilized dye and DMSO.*

**Table 9.34.1** Standard Staining Medium Preparation

| Reagent  | Volume | Stock concentration | Final concentration |
|--|--------|---------------------|---------------------|
| Neonatal calf sera (HI NCS; Invitrogen) <sup>a</sup> | 20 ml  | 100%                | 4%                  |
| Sodium azide (NaN <sub>3</sub> )                     | 0.5ml  | 20%                 | 0.02%               |
| RPMI 1640 medium (Invitrogen) <sup>b</sup>           | 174 ml | n/a                 | n/a                 |
| Total  | 500 ml |                     |                     |

<sup>a</sup>Thaw NCS and place at 56°C for 1 hr to heat inactivate (HINCS).

<sup>b</sup>RPMI 1640 formula #00-0327DK.

**Table 9.34.2** Titration Guide

| Dilution | Added amount        | DMSO (μl) | Stock concentration (μg/ml) | Working concentration (μg/ml) | Final concentration (μg/ml) |
|----------|---------------------|-----------|-----------------------------|-------------------------------|-----------------------------|
| 1        | 25 μg of dye        | 25        | 500                         | 12.50                         | 0.625                       |
| 2        | 25 μl of dilution 1 | 25        | 250                         | 6.25                          | 0.313                       |
| 3        | 25 μl of dilution 2 | 25        | 125                         | 3.12                          | 0.156                       |
| 4        | 25 μl of dilution 3 | 25        | 62.5                        | 1.56                          | 0.078                       |
| 5        | 25 μl of dilution 4 | 25        | 31.25                       | 0.78                          | 0.039                       |
| 6        | 25 μl of dilution 5 | 25        | 15.62                       | 0.39                          | 0.020                       |

2. Add 50 μl of DMSO into a vial of lyophilized dye as indicated in Table 9.34.2 in order to prepare the first dilution (500 μg/ml).
3. Make six serial dilutions using 25 μl from the first dilution (500 μg/ml) into 25 μl volumes of DMSO resulting in six stock concentrations as listed in Table 9.34.2.
4. Remove 1 μl of each stock concentration and add 39 μl of dH<sub>2</sub>O, resulting in six working concentrations as listed in Table 9.34.2.

*IMPORTANT NOTE: Dilution in dH<sub>2</sub>O is critical. Loss of fluorescence intensity will occur if this dye is prepared in other media sources containing amino acids.*

5. Add 5 μl of each working dilution into 95 μl of cells suspended in PBS. The cell source should contain substantial numbers of dead cells.

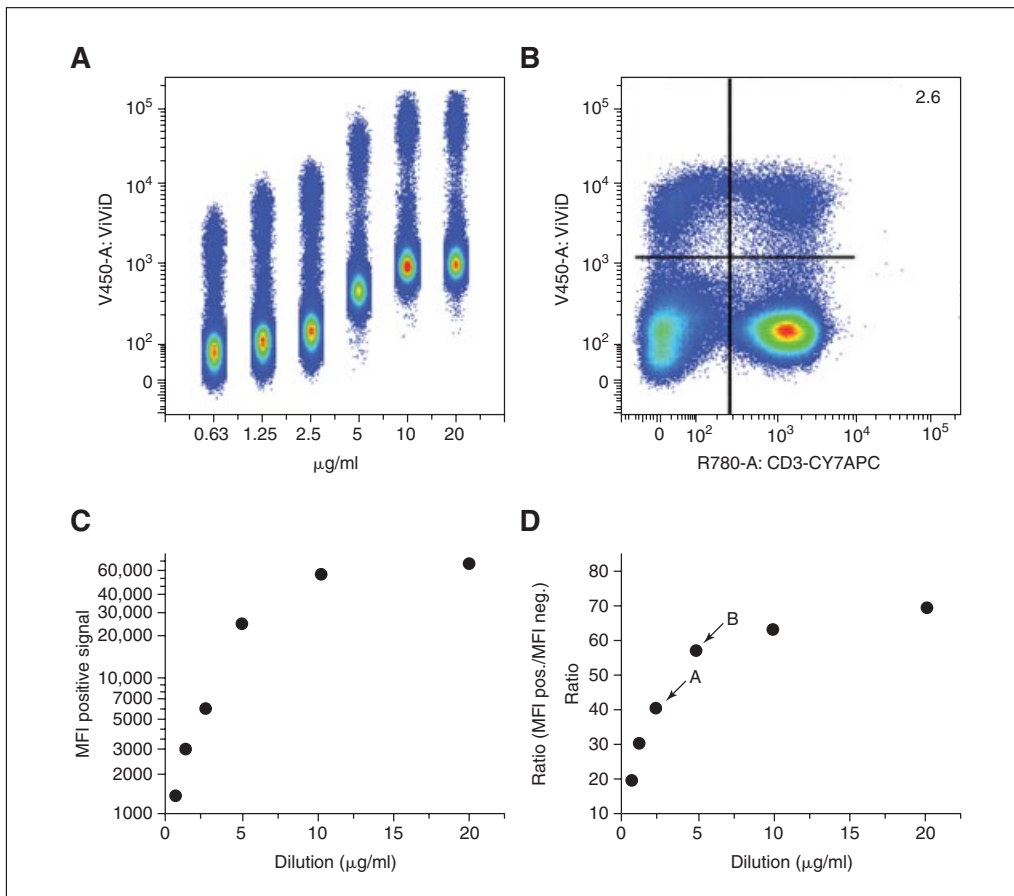
*IMPORTANT NOTE: Suspension in PBS is critical. Loss of fluorescence intensity will occur if this dye is prepared in other media sources containing amino acids. However, standard staining medium may be used in subsequent staining steps.*

6. Mix and incubate 20 min at room temperature (RT), shielded from light.

*IMPORTANT NOTE: This reaction time is optimal. Increased time or temperature does not improve staining intensity of the amine-reactive dyes.*

7. Wash cells twice, each time in 1 ml standard staining medium and stain with fluorochrome-conjugated mAb mix (e.g., anti-CD3 Cy7APC) according to a standard staining procedure as described in UNIT 6.2 and Lamoreaux et al., 2006.
8. Acquire fluorescence measurements for each dilution using a standardized flow cytometer and a detector configuration.

*Figure 9.34.3 shows data from a titration experiment for ViViD. Figure 9.34.3A shows all ViViD dilutions within CD3+ T cells; note the different MFIs for negative- and positive-stained cell populations in the 2.5 and 5 μg/ml dilutions. Figure 9.34.3B is a representative histogram of a single dilution (2.5 μg/ml) showing the ViViD-positive (2.6%) and the ViViD-negative cell populations. Plotting the medians of positive cell populations versus each dye dilution shows the brightest signal or saturation point at 10 μg/ml (Figure 9.34.3C). However, when considering amine-reactive dyes, lowest background signal is more important than maximum separation. Hence, the best separation with the lowest background is dilution 2.5 μg/ml (arrow A); even though dilution 5 μg/ml (arrow B) yields a more positive signal, the background is higher and is judged unacceptable (Figure 9.34.3D).*



**Figure 9.34.3** Representative titration of the ViViD dye used in staining panels. Cells were incubated for 5 days under standard culture conditions and then stained with dilutions of ViViD dye (20, 10, 5, 2.5, 1.25, and 0.625  $\mu\text{g/ml}$ ). After staining was complete, cells were stained with anti-CD3 using standard staining procedures. Gating on only CD3+ cells (live and dead), all dilutions can be concatenated on one histogram (**A**). This display can be used to measure the MFI of the negative and positive cell populations using the appropriate software. Panel **B** is a representative histogram of the working dilution of 2.5  $\mu\text{g/ml}$  of ViViD and anti-CD3. When considering the amine-reactive dyes, the lowest background signal is more important than the absolute positive signal. To illustrate the differences in the positive signal relative to the background staining, panel **C** (positive MFI) can be compared to panel **D** (ratio = positive MFI/negative MFI). The separation is acceptable at either of the lower dilutions (2.5 or 5  $\mu\text{g/ml}$ ); however, since the background is lowest at 2.5  $\mu\text{g/ml}$  (arrow A) as compared to 5  $\mu\text{g/ml}$  (arrow B) the lower dilution is best. For the color version of this figure go to <http://www.currentprotocols.com/protocol/cy0934>.

## CREATING STABLE AMINE REACTIVE DYE-LABELED COMPENSATION BEADS

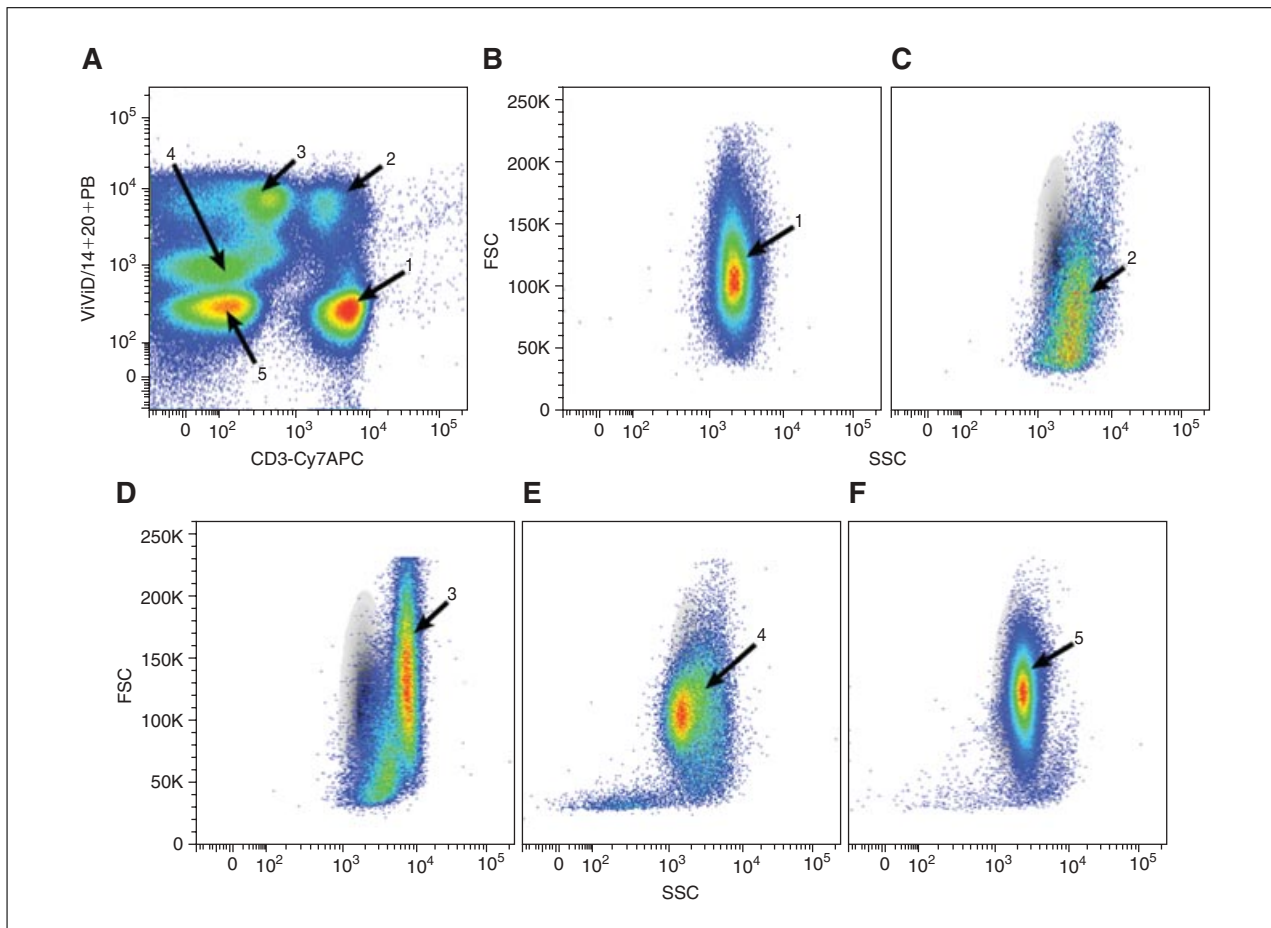
The purpose of this protocol is to create a LIVE/DEAD fixable dye compensation control for correction of spectral overlap in multicolor flow cytometry. The compensation reagents created are reproducible and stable under long-term storage conditions as described in this protocol.

There is one important consideration for using these controls. When dead cells are excluded in the same channel as other cell types (e.g., CD14+ monocytes or CD20+ B cells, also known as a “DUMP Channel”), only one compensation control is necessary. Typically, the LIVE/DEAD fixable dye is the brightest reagent in this detector because only the CD3+ T cells are included in the final gating (negative gating); hence, only the ViViD-stained beads need to be considered as a compensation control. Another advantage of negative gating can be demonstrated in Figure 9.34.4, where ViViD is combined with anti-CD20 Pacific Blue (PB) and anti-CD14 Pacific Blue to exclude dead

## BASIC PROTOCOL 2

### Studies of Cell Function

#### 9.34.5



**Figure 9.34.4** Dump channel analysis. This figure shows the effective use of a “dump” channel, which uses negative gating strategy. In this strategy, ViViD is combined with anti-CD20 Pacific Blue (PB) and anti-CD14 PB to exclude dead cells, B cells, and monocytes (respectively) using a single detector. **(A)** After compensation, five populations can be discriminated based on the ViViD and MAb-conjugates as follows: live CD3+ T cells (1), dead CD3+ T cells (2), monocytes (3), B cells (4), and live CD3-, CD14-, CD20- cells (5). **(B-F)** Overlay of the different populations (1-5) over the lymphocyte gate containing the CD3+ T cells (1). Each population shows the degree of contamination within the traditional lymphocyte gate, dead cells (C), monocytes (D), B cells (E) and live CD3-, CD14-, and CD20- cells (F). For the color version of this figure go to <http://www.currentprotocols.com/protocol/cy0934>.

cells, B cells, and monocytes (respectively) using a single detector. After compensation, five populations can be discriminated based on the ViViD and mAb-conjugate stains (Fig. 9.34.4A); these are as follows: live CD3+ T cells (1), dead CD3+ T cells (2), monocytes (3), B cells (4), and live CD3-, CD14-, CD20- cells (5). Figure 9.34.4B-F shows the overlay of the different populations (1-5) over the lymphocyte gate containing the CD3+ T cells (1). Each population shows the degree of contamination within the traditional lymphocyte gate, dead cells (C), monocytes (D), B cells (E), and live CD3-, CD14-, and CD20- cells (F). Hence, negative cell gating removes many unwanted cells, which might otherwise fall within the gates of interest.

### Materials

- R-NH<sub>2</sub> Beads (SMPLX Amine active beads; Bangs Laboratories)
- Phosphate-buffered saline (PBS)
- Prediluted (reconstituted vial) amine dye at the highest concentration
- Bead storage medium (see Table 9.34.3)
- Flow cytometer (an analyzer or sorter equipped with a violet laser and optics such as shown in Fig. 9.34.2; e.g., LSR-II, Becton Dickinson; alternatively, note in the same figure that the ViViD and Aqua Blue dyes are also well excited by a UV laser)

### Dead Cell Discrimination Using Amine-Reactive Dyes

#### 9.34.6

**Table 9.34.3** Bead Storage Medium Preparation

| Reagent   | Volume  | Stock concentration | Final concentration |
|---|---------|---------------------|---------------------|
| Fetal bovine serum <sup>a</sup> (HIFBS; e.g., Invitrogen) | 1 ml    | N/A                 | 1%                  |
| Sodium azide (NaN <sub>3</sub> ; Sigma)                   | 400 µl  | 5%                  | 0.02%               |
| Phosphate-buffered saline (PBS; Becton Dickinson)         | 98.6 ml | N/A                 | N/A                 |
| Total   | 100 ml  |                     |                     |

<sup>a</sup>Thaw FBS and place at 56°C for 1 hr to heat inactivate (HIFBS).

Flow cytometry data analysis software (e.g., FlowJo software, Treestar)  
Statistical analysis software (e.g., JMP software, SAS Institute)

**NOTE:** For proprietary reasons, the R-NH<sub>2</sub> beads can only be ordered by phone, and is not available in the Bangs Laboratories catalog.

#### **Create a stable compensation control for the amine-reactive dyes**

1. Dilute the R-NH<sub>2</sub> beads to a concentration of  $8.0 \times 10^7$  beads/ml in PBS (e.g., use 10 µl of R-NH<sub>2</sub> bead stock at  $2.8 \times 10^9$  beads/ml in a total volume of 350 µl in PBS).

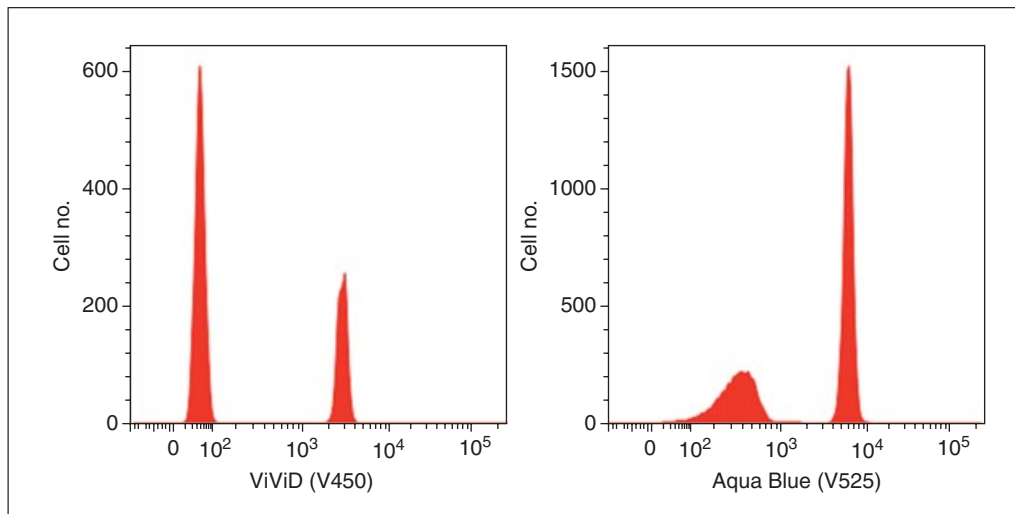
**IMPORTANT NOTE:** *Since the concentration of beads is in large excess relative to the dye concentration, the beads will require dilution against the highest dye concentration to determine the correct number of beads per vial of dye. The following steps are used to create this control.*

*The R-NH<sub>2</sub> beads are pre-labeled with amine dye and contain the matching negative control. The expiration time is one year.*

2. Perform a series of bead dilutions with PBS down to  $\sim 2.5 \times 10^6$  beads using total volumes of 175 µl per dilution.
3. Wash all bead dilutions twice, each time in 1 ml PBS. Pellet beads by centrifuging 3 min at  $400 \times g$ , 4°C and dry the pellet at room temperature to completely remove PBS.
4. Add 5 µl of prediluted (reconstituted vial) amine dye at the highest concentration (i.e., 500 µg/ml) to each bead dilution (see step 2 of Basic Protocol 1). For example, add 50 µl of 500 µg/ml dye into 50 µl of PBS to a final concentration of 250 µg/ml to all the bead dilution tubes, keeping the dye concentration constant but varying the concentration of beads.
5. Incubate 60 min at room temperature and shield from light.
6. Wash twice, each time in 1 ml PBS. Centrifuge 3 min at  $400 \times g$ , 4°C. Resuspend in 100 µl of bead storage medium.
7. Add 100 µl of equal concentration of unstained beads in bead storage medium.
8. Run on a standardized flow cytometer and determine the bead dilution with maximum MFI (e.g., in our tests,  $10 \times 10^6$  beads produced the highest MFI per one vial of dye stock concentration).

*Using the bead:dye ratio determined in this step, a larger batch of beads can be created following the procedure above. As an example, if the ideal bead concentration were determined to be  $10 \times 10^6$  beads per one vial of dye stock concentration (250 µg/ml), then  $20 \times 10^6$  beads would require 2 vials, etc. This means that it requires 1.25 µg of dye to stain  $10 \times 10^6$  beads to produce the maximum MFI.*

*Figure 9.34.5 shows the results of negative and positive beads labeled with the ViViD dye (left panel) and the Aqua Blue dye (right panel) as prepared by this protocol.*



**Figure 9.34.5** Prelabeled compensation controls used in cell staining panels. This figure illustrates two prelabeled compensation controls used in staining panels, one labeled with ViViD and the other labeled with Aqua Blue. These controls contain both the negative control beads (lower intensity) and the positive control beads (high intensity).

### BASIC PROTOCOL 3

## STAINING, GATING, AND ANALYSIS OF AMINE-REACTIVE DYES

The purpose of this protocol is to demonstrate how to use amine-reactive dyes in multi-color cell panels to identify and remove dead cells from the gating analysis. As previously discussed, dead cells can nonspecifically bind mAb-conjugates, which can result in artifacts and erroneous labeling of cell populations (Perfetto et al., 2006b). Hence, only viable cells should be used in most gating analysis strategies. In this protocol, an example of the standard gating strategy using a ViViD dump channel (negative gating) and anti-CD3 will illustrate the utility of the ViViD amine reactive dye to describe gating and analysis strategies.

### Materials

Amine reactive dye kit (ViViD, LIVE/DEAD fixable violet dead cell stain or Aqua Blue, LIVE/DEAD fixable aqua dead cell stain; both from Invitrogen) containing:

Dimethyl sulfoxide (DMSO)

Lyophilized dye

Phosphate-buffered saline (PBS)

37°C water bath

Standard staining medium (see Table 9.34.1)

Flow cytometer (an analyzer or sorter equipped with a violet laser and optics such as shown in Fig. 9.34.2; e.g., LSR-II, Becton Dickinson; alternatively, note in the same figure that the ViViD Aqua Blue dye is also well excited by a UV laser)

Flow cytometry data analysis software (e.g., FlowJo software, Treestar)

Statistical analysis software (e.g., JMP software, SAS Institute)

1. Thaw the dimethyl sulfoxide (DMSO) supplied with the kit using a 37°C water bath until completely thawed (approximately 30 sec). Add the amount of DMSO into a vial of lyophilized dye as determined in the titration protocol (see Basic Protocol 1 and Fig. 9.34.3).

**IMPORTANT NOTE:** *Lyophilized dye is stored desiccated and expires at 60 months under these conditions. DMSO must also be stored under desiccated conditions.*



- Mix thoroughly with a pipet tip.

*Depending on the dye used, DMSO will turn from clear to colored, indicating the dye is dissolved.*

- Store the vials up to 3 months at  $-20^{\circ}\text{C}$ . These vials can be thawed and frozen until the vial is empty.

**IMPORTANT NOTE:** *Do not store as aliquots in buffered medium or media containing amino acids.*

- Add  $1\ \mu\text{l}$  of stock concentration into  $39\ \mu\text{l}$  of  $\text{dH}_2\text{O}$  (see Basic Protocol 1).

**IMPORTANT NOTE:** *Dilution in  $\text{dH}_2\text{O}$  is critical. Loss of fluorescent intensity will occur if this dye is prepared in other media sources containing amino acids.*

- Add  $5\ \mu\text{l}$  of the working concentration (from step 4) into cells suspended in  $95\ \mu\text{l}$  of PBS.

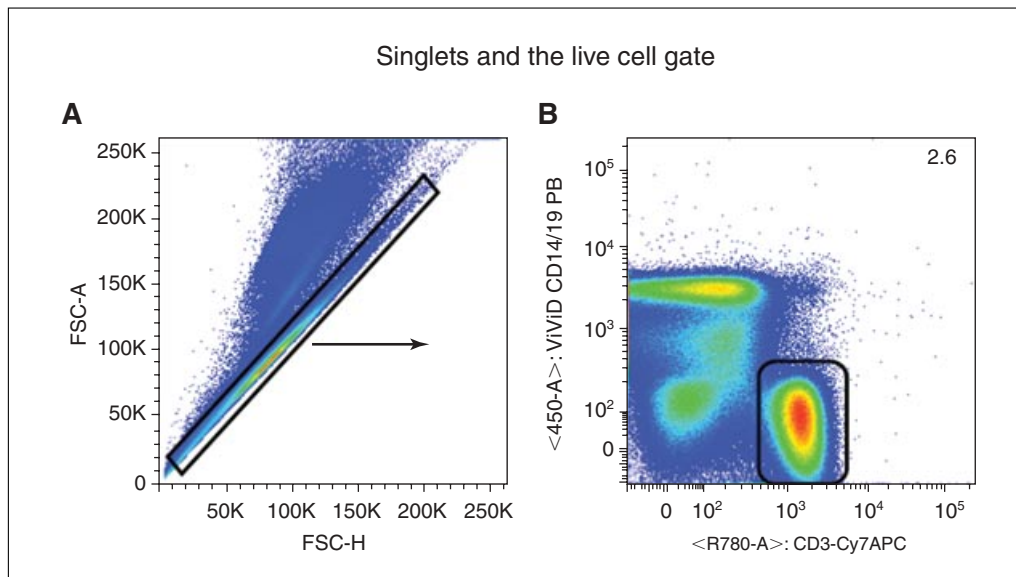
**IMPORTANT NOTE:** *Because some mAb-fluorochromes and free amino acids in media can compete with amine-reactive dyes, all other cell surface stains should be added in a separate step to avoid the loss of dye effectiveness. In addition, PBS is used in this staining step but all other wash steps can use media containing heat-inactivated fetal bovine serum (HIFBS) or other enriched media containing amino acids.*

- Mix and incubate 20 min at room temperature shielded from light.

**IMPORTANT NOTE:** *This reaction time is optimal. Increased time or temperature does not improve staining intensity of the amine-reactive dyes.*

- Wash once in 1 ml standard staining medium or medium containing HIFBS.

- Prepare fluorochrome mAb conjugates, typically in a master mix brought to a volume of  $100\ \mu\text{l}/\text{test}$ ; mix thoroughly and incubate according to each panel standard



**Figure 9.34.6** Common gating strategy to remove doublets and dead cells in the cell analysis. This figure shows the gating strategy used to gate on the live  $\text{CD}3^+$  T cells. The first histogram (A) uses the measurements of forward scatter area and forward scatter height to determine the single cells (inside the diagonal gate) from the doublets (above the diagonal gate). From the single cell gate the viability gate (DUMP channel using ViViD, CD14, and CD19) is set up with CD3 (B). This negative gate contains only the live  $\text{CD}3^+$  T cells while excluding dead cells, monocytes, B cells and live cells not stained with CD14, CD19, or CD3. For the color version of this figure go to <http://www.currentprotocols.com/protocol/cy0934>.

operating procedure (see UNIT 6.2; Lamoreaux et al., 2006; Chattopadhyay et al., 2008).

**IMPORTANT NOTE:** *PERM/FIX reagents such as Cytofix/Cytoperm or 0.5% PFA will not change the stability of this dye nor will the dye leak out of the cells.*

9. Run compensation controls and all stained cells using a standardized flow cytometer.

*Figure 9.34.6 shows a typical gating strategy when collecting cell samples to verify correct gate position and efficiency of the viability stain. The first histogram uses the measurements of forward scatter area and forward scatter height to determine the single cells (inside the diagonal gate in panel A) from the doublets (above the diagonal gate in panel A). From the single cell gate, the viability gate (DUMP channel using ViViD, anti-CD14 and anti-CD19) is set up with anti-CD3 to identify the live CD3+ T cells (panel B), which are gated for further analysis depending on the staining panel characteristics.*

## COMMENTARY

### Background Information

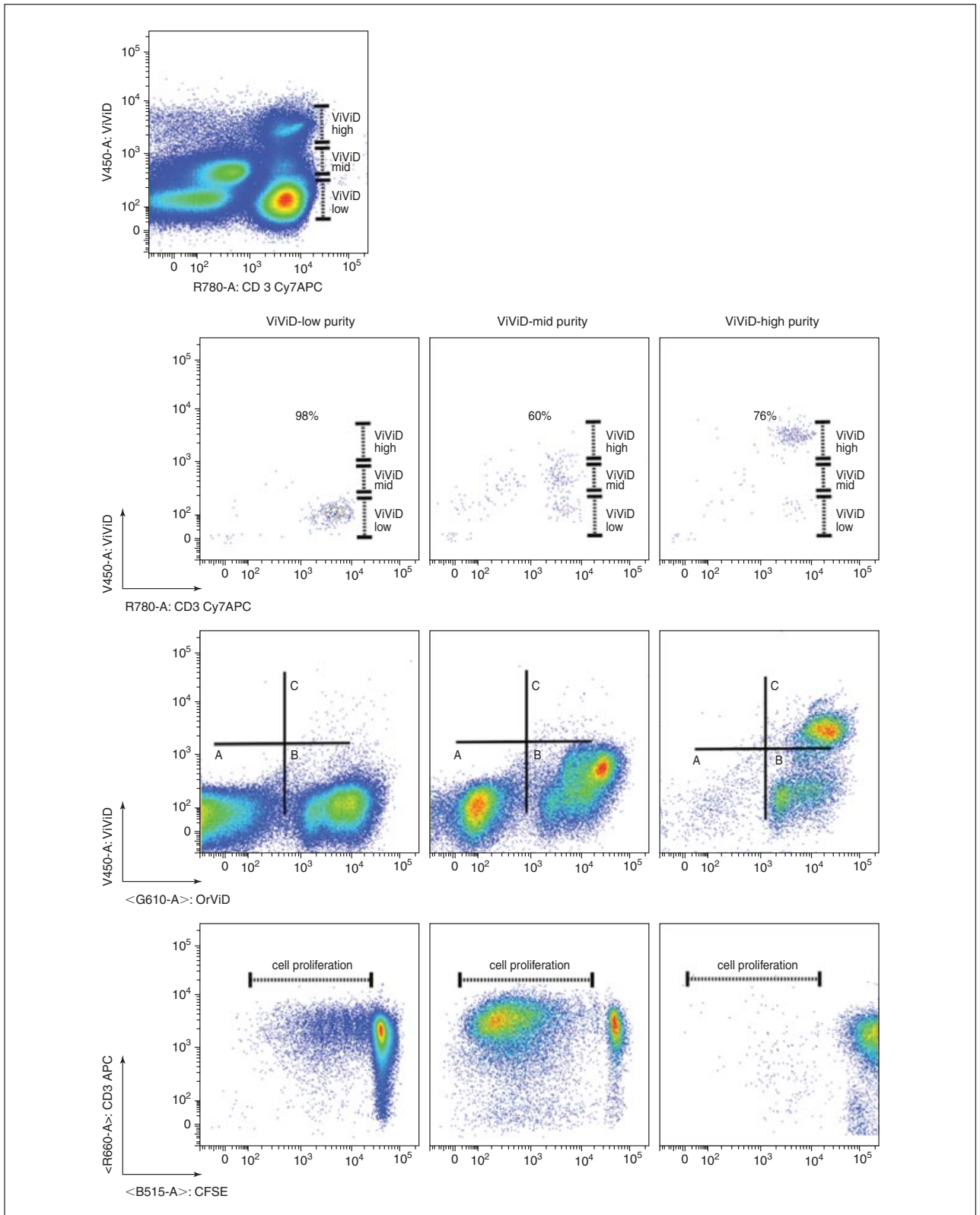
Immune monitoring and vaccine immunogenicity studies often require the measurement of low-frequency cell populations in samples that have been cryopreserved. This inevitably leads to questions of sensitivity and reproducibility, since dead cells in the sample may nonspecifically bind mAb-conjugates and cause significant artifacts (O'Brien and Bolton, 1995; Schmid et al., 1999; Perfetto et al., 2004a; Maecker et al., 2005). Fortunately, viability dyes may be used to exclude dead cells from analysis. Historically, viability dyes that enter damaged cells via compromised cell membranes and intercalate into the DNA were employed; propidium iodide (PI) is an example of such a dye. However, PI may leak out of cells within a short period of time, leading to significant loss of signal (Desrués et al., 1989; Costantino et al., 1995; Clarke and Pinder, 1998). This is particularly problematic when permeabilization reagents are used to stain intracellular molecules (such as cytokines), as is often the case in immune monitoring and immunogenicity studies. To avoid this problem, ethidium monoazide (EMA) may be used. This dye covalently binds to DNA after exposure to ultraviolet (UV) light. Although this dye

can resolve dead cell populations and is unaffected by intracellular treatments, the need for a UV light source is inconvenient (Riedy et al., 1991). In addition, the degree of membrane damage in apoptotic cells can be variable, leaving some Annexin V+ cells with intermediate levels of PI or EMA staining (Matteucci et al., 1999; Waters et al., 2002). The amine-reactive dyes as discussed in this protocol avoid many of the disadvantages of these traditional viability markers. Hence, these are a good alternative for measuring and removing dead cells from the cell analysis.

### Critical Parameters and Troubleshooting

The amine-reactive dyes have distinct advantages over traditional viability staining. Firstly, they are simple to use, they are stable, and they can be used with other mAb-conjugates after complete interaction with free amines in the cytosol of the dead cell. Secondly, the amine-reactive dyes are sold with a variety of emission and excitation wavelengths and can therefore be included in many cell-staining panels. This flexibility allows for many traditional mAb-conjugates combinations to remain while “fitting in” a viability dye.

**Figure 9.34.7** (*appears on next page*) CD3+ T cells that are dimly stained with amine-reactive dyes should be included in cell analysis. This figure shows the proliferative capacity of three CD3+ ViViD-stained populations, ViViD-Low, ViViD-Mid, and ViViD-High after staining with CFSE, ViViD, and anti-CD3-Cy7APC. The second row shows the sort purity results for each of the sorted cell populations as illustrated in the histogram in the first row. The third and fourth rows show results of post-incubation for five days in the presence of SEB followed by staining with OrViD (amine reactive dye measured in the G610 detector) and anti-CD3-APC. These additional stains were added to measure dead cells as a result of a 5-day incubation and the total number of CD3+ T cells. In the third row three populations can be described, live cells (A, ViViD-OrViD-), dead cells as a result of incubation (B, ViViD-OrViD+), and dead cells from the original sort (C, ViViD+OrViD+). In both the ViViD-Low and ViViD-Mid cell sorts, live cells were observed and these cells were also proliferating (fourth row). The dead cells from the ViViD-High cell sort show no cell proliferation (fourth row). For the color version of this figure go to <http://www.currentprotocols.com/protocol/cy0934>.



**Figure 9.34.7** (legend appears on previous page)

**Table 9.34.4** List of Common Problems and Solutions

| Problem   | Possible cause  | Solution   |
|---|---|--|
| Dead cells are dimly fluorescent and appear to separate poorly from the live cell population                          | ViViD dye was used at the incorrect concentration                     | See Basic Protocol 2 for details   |
|   | Dye not resuspended properly  | When initially resuspending dye in DMSO, mix well using a pipet  |
|   | DMSO is contaminated  | Oxidized DMSO can cause a loss in dye solubility. Use only DMSO that is part of the kit (stored at $-30^{\circ}\text{C}$ ) or minimize exposed to oxygen.  |
| Poor amine-reactive dye compensation control. Dim staining as compared to dead cells stained with amine-reactive dye. | Free amines competing for dye   | Stain cells only in PBS or medium containing a low concentration of free amines  |
|   | Amine-reactive dye not stained according to protocol                  | Compensation beads must be stained according to Basic Protocol 2. Incubation time and concentration are critical.  |
|   | Unmatched negative beads  | Beads used for the stained compensation control (positive) MUST be the same bead source as the negative  |
|   | Did not use amine-reactive dye stained beads                          | Amine-coated beads as described in Basic Protocol 2 are unique for this control.   |
|   | Used an incorrect amine-reactive dye                                  | Use correct amine-reactive dye to match the detector configuration.  |
| Poor DUMP compensation control. amine-reactive dye appears not to be subtracted.                                      | Did not use amine-reactive dye stained beads for compensation control | When mixing other mAb-conjugates with the amine-reactive dye, only the amine-reactive compensation control is needed to establish the compensation matrix. |

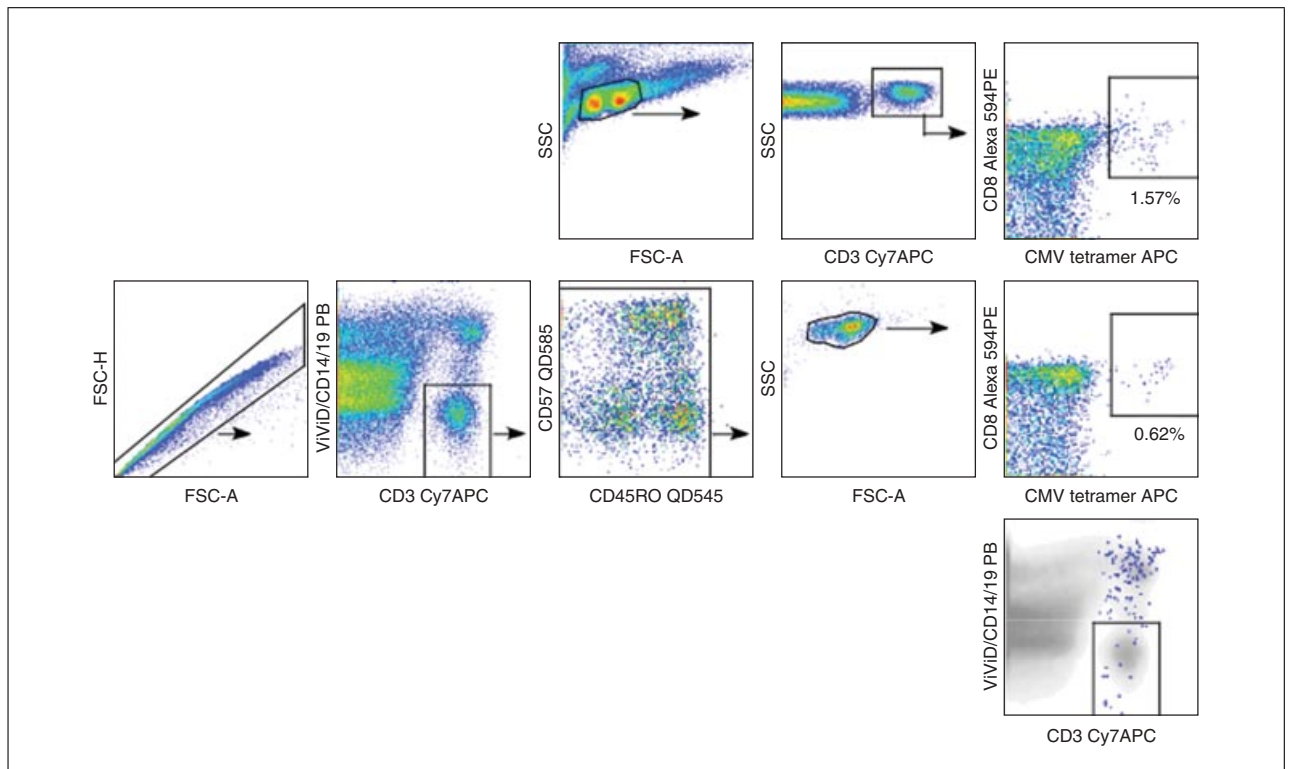
In some cases, greater intensity of the amine-reactive dye can be seen above background but below expected positive intensity. In these situations, it is important to determine if these cells should be excluded because they are dead or to include them because they are viable. To this end, the immunophenotype, cytokine production, or proliferation of the ViViD dim and negative cells may be compared. If the ViViD dim population shows a similar expression profile as the negative cells, then the former should be included in the analysis. For example, we compared three cell populations sorted from the viability histogram in the first row of Figure 9.34.7; these include: live CD3+ T cells (ViViD-Low), dim ViViD+ CD3+ cells (ViViD-Mid), and ViViD+ CD3+ dead cells (ViViD-High). In addition to cell surface stains, the cells were loaded with the proliferation marker CFSE. The sorted cells (Fig. 9.34.7, second row) were then cultured in the presence of streptococcus enterotoxin B (SEB) for five days using standard culture

procedures. After this incubation, cells were restained for CD3 (anti-CD3-APC) and OrViD (a second amine-reactive dye) and measured on the flow cytometer. OrViD positive cells died as a result of the 5-day incubation process and they were excluded from the analysis (Fig. 9.34.7, third row). The sorted population of cells that was ViViD-Mid progressed through as many (or more) rounds of division as the ViViD negative population (ViViD-Low), suggesting that these cells should be included in the final analysis (Fig. 9.34.7, fourth row). Notably, ViViD staining is reduced in proliferating cells, presumably as a result of dilution of bound dye into the daughter cells. These results suggest that protein expression is increased for cell populations that are activated in vivo or in vitro, resulting in a slightly increased ViViD binding (ViViD Mid) and fluorescence.

For a list of common problems and solutions, see Table 9.34.4.

### Dead Cell Discrimination Using Amine-Reactive Dyes

#### 9.34.12



**Figure 9.34.8** An example of gating strategy used to eliminate aberrant binding events due to dead cells that may change tetramer analysis. This figure shows an example of the utility of using amine-reactive dyes to eliminate erroneous frequencies due to nonspecific binding of dead cells. In this example, CMV antigen specific CD8<sup>+</sup> T cells are measured in a staining panel containing a specific CMV tetramer reagent. The first row shows a traditional gating strategy. The gate at the far right indicates that 1.57% of the CD8<sup>+</sup> T cells are tetramer positive. However, when including the viability gate (second row) the CD8<sup>+</sup> tetramer<sup>+</sup> T cells were determined to be at a lower frequency (0.62%) than reported using traditional gating. Overlaying the total CD8<sup>+</sup> tetramer<sup>+</sup> T cells from the first row onto the viability gate (bottom row) shows that the majority of the cells are in fact ViViD<sup>+</sup> and hence should not be included in the final analysis. For the color version of this figure go to <http://www.currentprotocols.com/protocol/cy0934>.

### Anticipated Results

The problem of nonspecific binding is particularly notable when analyzing rare events within cryopreserved samples, since these samples are likely to contain significant numbers of dead cells. When viability markers are not included in these analyses, the frequency of the cells of interest can be dramatically misrepresented because dead cells can nonspecifically bind reagents. For example, in Figure 9.34.8, a rare population (CMV-specific CD8<sup>+</sup> T cells) was examined in bone marrow samples. Data was analyzed with and without excluding cells in the dump channel, which included antibodies against CD14 and CD19 (conjugated to Pacific Blue) and ViViD. The top row shows a “traditional” gating strategy (without dump channels) that might be used on a six-parameter flow cytometer; tetramer<sup>+</sup> cells are identified in a bivariate plot with CD8 after gating to identify T cells on the basis of CD3 expression. The second row shows a polychromatic gating

strategy to eliminate aberrant binding events. In this analysis, live CD3<sup>+</sup> T cells were distinguished from dead cells, monocytes, and B cells, which could bind tetramer and mAbs nonspecifically. Notably, CD8<sup>+</sup> T cells specific for the CMV epitope could be cleanly identified with the polychromatic gating strategy (second row, right panel, 0.62%), whereas tetramer-binding cells are overrepresented in the top panel (1.57%). Backgating analysis shows that many of these cells are binding reagents in the dump channel, suggesting that much of the binding is nonspecific (bottom panel); tetramer<sup>+</sup> events are shown in blue overlaid on the total population in a bivariate CD3 versus CD14/CD19/ViViD plot). Thus, it is possible that sample-to-sample variation in viability, or in the frequency of B cells and monocytes, could have a profound impact on the proportion of antigen-specific cells identified, thereby skewing study results. This demonstrates very clearly the need for amine-reactive dyes in cell-staining panels.

## Time Considerations

The time needed for cell staining with viability dyes and multicolor antibody conjugates is typically no more than 30 min. It would be tempting to shorten this procedure by mixing the mAb-conjugates with the viability dye; however, this is not recommended because the staining medium used for antibody staining often contains proteins. Moreover, some antibody preparations typically contain high levels of proteins as stabilizers. These proteins introduce free amines, which compete with free amines from the cytosol of dead cells to bind to the amine-reactive dyes. This competition significantly decreases the fluorescence intensity of the dye, and increases background staining. Therefore, we recommend prestaining cells (resuspended in PBS) with amine-reactive dyes, washing the cell sample, and then incubating with multicolor antibodies in the staining medium of choice.

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## Disclaimer

The views expressed here are the opinions of the authors and are not to be considered as official or reflecting the views or policies of the Vaccine Research Center/National Institutes of Health/Department of Health and Human Services nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S. Government.

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