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Technical note

Amine reactive dyes: An effective tool to discriminate live and dead cells in polychromatic flow cytometry

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Abstract

Membrane-damaged cells caused by either mechanical trauma or through normal biological processes can produce artifacts in immunophenotyping analysis by flow cytometry. Dead cells can nonspecifically bind monoclonal antibody conjugates, potentially leading to erroneous conclusions, particularly when cell frequencies are low. To date, DNA intercalating dyes (Ethidium monoazaide (EMA), Propidium Iodide, 7AAD, etc.) or Annexin V have been commonly used to exclude dead cells; however, each suffer from technical problems. The first issue with such dyes is the dependence on a consistent dead cell source for fluorescence compensation. Another major issue is the stability of the staining; except for EMA, fixation and permeablization used for intracellular staining procedures can cause loss of fluorescence. EMA requires a UV exposure to covalently bond to DNA; while this dye is effective and is not affected by intracellular treatments it is weakly fluorescent. Here we report on the optimization of a new class of viability dyes, the amine reactive viability dyes (ViD) as a dead cell exclusion marker. The inclusion of ViD into the staining panel was found to be simple, reproducible and can have a significant benefit on the accuracy of identifying appropriate cell populations. We show the fluorescence of cells stained with these dyes correlates with traditional dead cell discriminating markers, even after fixation and permeabilization. Amine reactive viability dyes are a powerful tool for fluorescence immunophenotyping experiments.
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Keywords: Amine reactive dye; Cell viability; Non-specific MAB-conjugate binding

1. Introduction

Immune monitoring and vaccine immunogenicity studies often require the measurement of low frequency cell populations. This inevitably leads to questions of sensitivity and reproducibility, since non-specific binding of monoclonal antibody (mAb)-conjugates to dead cells can lead to significant measurement errors (O'Brien and Bolton, 1995; Schmid et al., 1999; Perfetto et al., 2004; Maecker et al., 2005). Viability dyes may be used to exclude dead cells from analysis; intercalating viability dyes enter damaged cells through open membranes and bind DNA. However, the dye may leak out of cells within a short period of time, leading to significant signal loss (Desrues et al., 1989; Costantino et al., 1995; Clarke and Pinder, 1998). This is particularly problematic when permeablization reagents are used to stain intracellular molecules. This problem is avoided when using ethidium monoazide (EMA), which covalently binds to DNA after exposure to ultraviolet

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(UV) light. While technically challenging, this dye is generally effective and unaffected by intracellular treatments (Riedy et al., 1991).

Amine Reactive Viability Dyes (ViD) offer a new alternative to identifying dead cells. These dyes, in a chemically reactive form, penetrate damaged cell membranes and react with amine groups in the cytoplasm. A fluorescent, and stable, product remains in the cytoplasm. Surface amine groups also will react with this dye, but contribute significantly less fluorescence due to the much lower amount. These dyes come in a variety of excitation and emission wavelengths: green fluorescence (GrViD) at 530±15 nm (excited by the 488 nm laser), orange fluorescence (OrVid) at 630±11 nm (excited by the 638 nm laser), violet fluorescence (ViViD) at 430±11 (excited by the 407 nm laser) or a UV excitable dye (UVid) emitting light at 430 nm. Thus, an important advantage offered by these dyes is their diversity, which allows great flexibility when designing staining panels for multicolor flow cytometry. In this report, we report on the stability and reproducibility of ViD, compare their use to standard viability dyes, and finally, examine the benefit of using them to identify dead cells in the analysis of cell populations under a variety of real-world test conditions.

2. Materials and methods

2.1. Preparation of the amine-reactive viability dye

Each amine reactive dye kit (Molecular Probes, Eugene, OR) comes as 25 μg of lyophilized dye and DMSO. DMSO is placed into a 37 °C waterbath until completely thawed. Into a vial of lyophilized dye (25 ug), 12.5 ul of DMSO is added (2 mg/ml) and mixed thoroughly with a pipet tip. Depending on the dye used the DMSO will turn from clear to colored solution, indicating the dye is dissolved. This stock sample is stored at −20 °C and vials of stock can be thawed and frozen until each aliquot is empty. Each working concentration used must be pre-diluted into distilled water prior to use. Each lot is titered using cultured cells, which were pretested for viability using ethidium-bromide/acridine orange (green fluorescent cells are viable and the red fluorescent cells were dead) and counted on a fluorescent microscope. Cultures with greater than 10% cell death were used for titration experiments.

2.2. Amine labeled beads for fluorescence compensation

Beads coated with active amine groups (Amine Modified Microspheres, Bangs Laboratories, Inc., Fishers, IN) were used as compensation standards and in testing procedures. Stock beads were diluted in staining media (1% heat inactivated fetal calf serum, 0.02% sodium azide in v/v of PBS) to a final concentration of 40×10^6/ml. 40 ul of this bead preparation were mixed with 60 ul of staining media containing a particular concentration of ViD, as determined by preliminary titration experiments (performed on cells). Beads were shielded from light and incubated 20 min at room temperature. After incubation, 100 ul of 1%PFA was added for at least 30 min.

2.3. Labeling cells with ViD and monoclonal antibodies

For experiments requiring only cell surface monoclonal antibody staining, peripheral blood mononuclear cells (PBMCs) at 1×10^6 cells per 100 μl were stained with the following panel of monoclonal antibodies (mAbs, conjugated in-house unless otherwise noted): CD7-Fluorescein isothiocyanate (PharMingen), CD3-Cyanine 7-Allophycocyanin (PharMingen), CD11a-Alexa700-Phycoerythrin, CD28-Allophycocyanin (PharMingen), CD8-Quantum Dot 705, CD27-Cyanine 5-Phycoerythrin, CD57-Quantum Dot 545, CD4-Cyanine 5.5-Phycoerythrin, CCR7-Cyanine 7-Phycoerythrin, CD45RO-Texas Red-Phycoerythrin (Coulter) and CD127-Phycoerythrin (Coulter). In addition, the appropriate amount of ViViD (Invitrogen) was added to the antibody cocktail, which was subsequently brought to a final volume of 100 ul in PBS. After incubation in the dark, for 20 min (room temperature), cells were washed twice in staining media and fixed in a final concentration of 0.5% paraformaldehyde.

2.4. Intracellular staining protocol

PBMCs (1×10^6 cells/ml) were stimulated with streptococcus enterotoxin B (SEB at 1 ug/ml) for 6 h in the presence of brefeldin A (1 ug/ml) at 37 °C in a CO_2 incubator. Cells were washed, resuspended in PBS and surfaced stained with the following monoclonal antibodies (mAbs, conjugated in-house unless otherwise) and ViViD (Invitrogen): CD45RO-Texas Red-Phycoerythrin (Coulter), CD107-Alexa680, CD8-Quantum Dot 705, CD27-Cyanine 5-Phycoerythrin, CD57-Quantum Dot 545, CD4-Cyanine 5.5-Phycoerythrin, ViViD (Invitrogen) and CD14/CD20-Cascade Blue. Cells were fixed and permeabilized (Cytofix/Cytoperm, PharMingen, San Diego, CA), then stained with CD3-Cyanine 7-Allophycocyanin, MIP1β-Phycoerythrin, INFγ-Fluorescein isothiocyanate (PharMingen), IL2-Allophycocyanin (PharMingen) and TNFα-Cyanine 7-Phycoerythrin

for 20 min at room temperature, while protected from light. Finally, cells were washed and fixed with a final concentration of 0.5% PFA.

2.5. Flow cytometry

Samples were collected using an LSR-II (BD, San Jose, CA; configured as described previously; Perfetto et al., 2004). Various ViDs were collected using the following laser and filter combinations: ViViD, 407 nm violet DPSS laser and a 450/50 band-pass filter; GrVid, 488 nm blue laser, with a 515/20 band-pass filter, and 505 LP dichoric mirror; and OrVid, 532 nm green laser, with a 610/20 band-pass filter, and a 600 LP dichoric mirror. Data was analyzed using FlowJo version 6.4 (Tree Star, Inc., Ashland, OR).

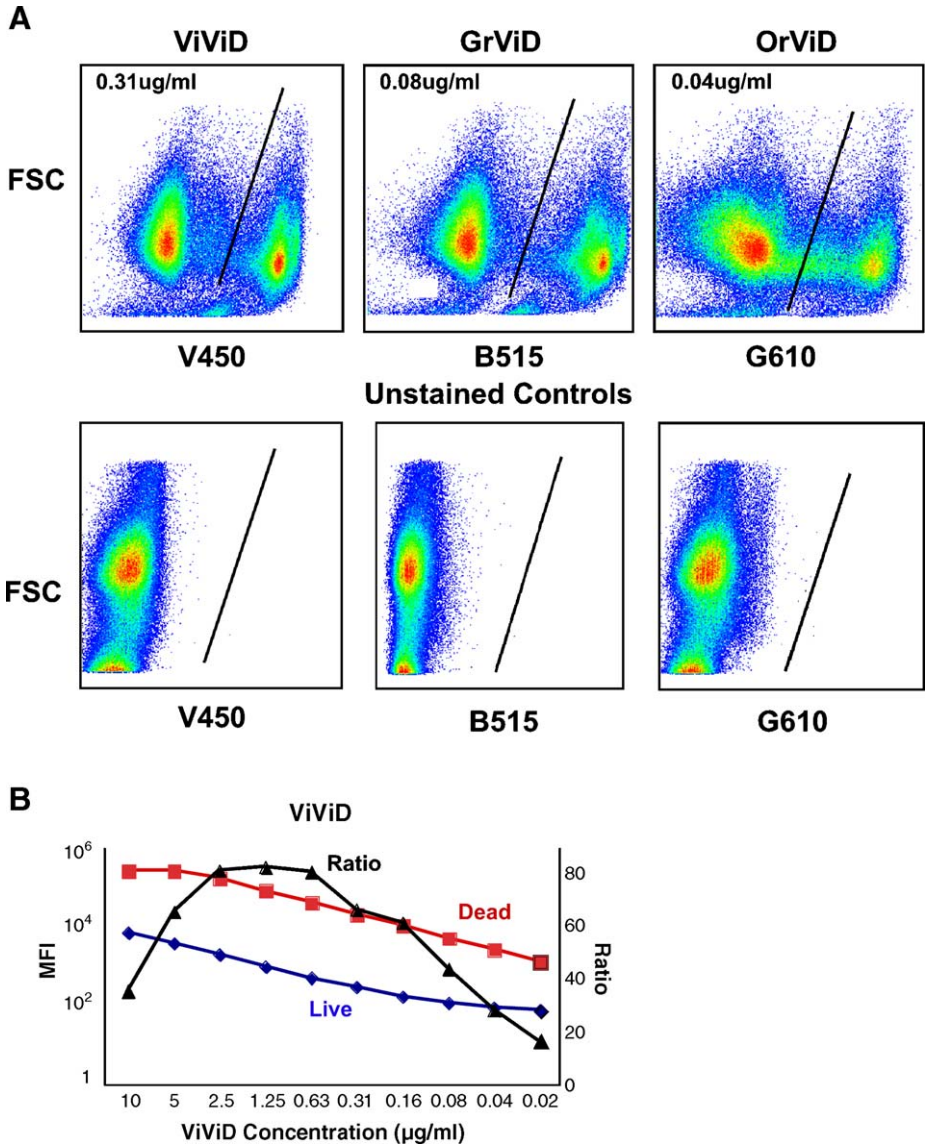

Fig. 1. Staining with amine-reactive viability dyes. A) The top three histograms illustrate the staining profile of a culture of live and dead cells using three different types of amine-reactive viability dyes. Cells located to the right of the black line on each histogram are dead cells (ViD+) while cells to the left of this line are live cells (ViD−). The first histogram is of ViViD and forward scatter, demonstrates a typical staining pattern and the second and third histograms illustrate staining of two other ViDs, the green amine-reactive viability dye (GrViD) and the orange amine-reactive viability dye (OrViD). The concentrations of the dyes used in the staining, shown in the graphics, was determined by titration experiments such as that shown in (B): The first curve is the median fluorescent intensity of the dead cells (red line), the second curve represents the median fluorescent intensity of the live cells (blue line) and the last curve represents the ratio of the median fluorescent intensity of the dead cells to the median fluorescent intensity of the live cells (black line).

3. Results

3.1. Amine-reactive viability dye require titration

To ensure minimal background staining and maximum separation, amine reactive viability dyes were titrated using fresh PBMCs prior to use. Fig. 1A (first histogram) shows the typical staining pattern of PBMCs labeled with ViViD, which includes small cells (lymphocytes and apoptotic cells) and larger cells (likely monocytes). ViViD+ cells are clearly separated from negative cells. In general, we find that viable monocytes stain to a larger extent with this dye than lymphocytes, but could still be well separated from dead cells. Additionally, in Fig. 1A we demonstrate the fluorescent intensity of two of the other amine reactive viability dyes, e.g. GrViD and OrViD. As would be expected from the fluorescence spectra, the relative brightness increases from ViViD to GrViD to OrViD. The optimal concentration for use was determined as shown in Fig. 1B. For ViViD, we found that a 0.31 ug/ml concentration yielded the lowest background and the highest separation ratio. In titrating these reagents, it may be advantageous to select a concentration which gives low background (live cell) staining even if the signal to background is somewhat lower than maximum; the trade-off is between increasing separation of the dead cells from the live cells and increasing staining of live cells.

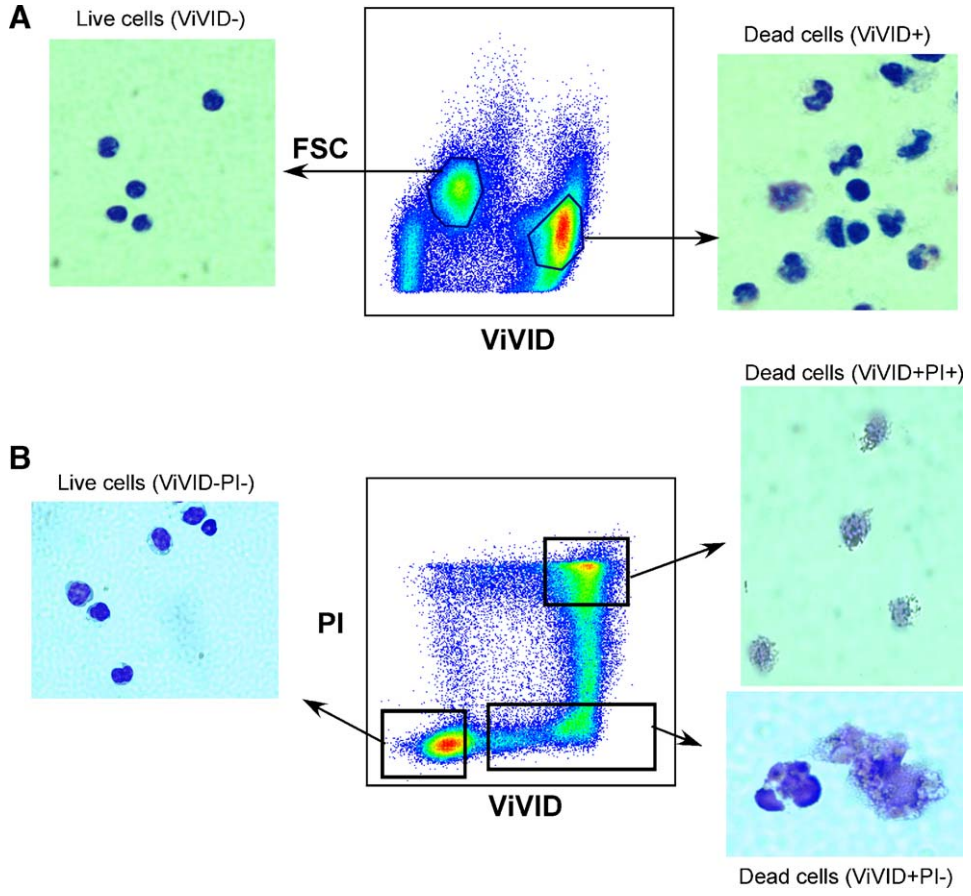

Fig. 2. The cell morphology of ViViD+ cells is consistent with post apoptotic and/or necrotic cells. A) ViViD+ and ViViD negative cells were sorted and stained with Wright's stain. The ViViD negative cells (live cells) show cells with the typical morphology of normal lymphocytes. These cells show intact and compact nuclei with a small cytoplasmic compartment. The ViViD+ cells (dead cells) show cells with deformed and bledding nuclei. The cytoplasmic compartment was clear and the nuclei were non-compact, suggesting membrane and DNA damage. These morphological changes are characteristic of post apoptotic or necrotic cells. B) Cells co-stained with PI and ViViD were sorted and stained with Wright's stain. The histogram shows three or perhaps four phenotypes; ViViD−PI−, ViViD+PI−, ViViD+PI+ and ViViD−PI+. The morphology of ViViD−PI− and ViViD+PI+ is consistent with cells stained only with ViViD and described in part A of this figure. ViViD+PI− cells show extreme cellular damage, which have likely lost their nuclei or degraded the DNA to an extent that PI fluorescence is absent.

3.2. Cells stained with ViViD show characteristic morphology of dead cells

ViViD+ cells were compared to ViViD negative (live cells) after cell sorting and staining with Wright's stain. Fig. 2A shows ViViD negative cells with compact nuclei and little cytoplasm, consistent with normal lymphocyte morphology. ViViD+ cells show multi-lobular nuclei, increased cytoplasmic volume and nuclear blebbing, suggesting that these cells show signs of morphological membrane damage and are either post apoptotic or necrotic. When cells were stained with a combination of propidium iodide (PI) and ViViD, three or perhaps four cell populations are evident (Fig. 2B). The ViViD−PI− cells were clearly live cells and as described above demonstrated

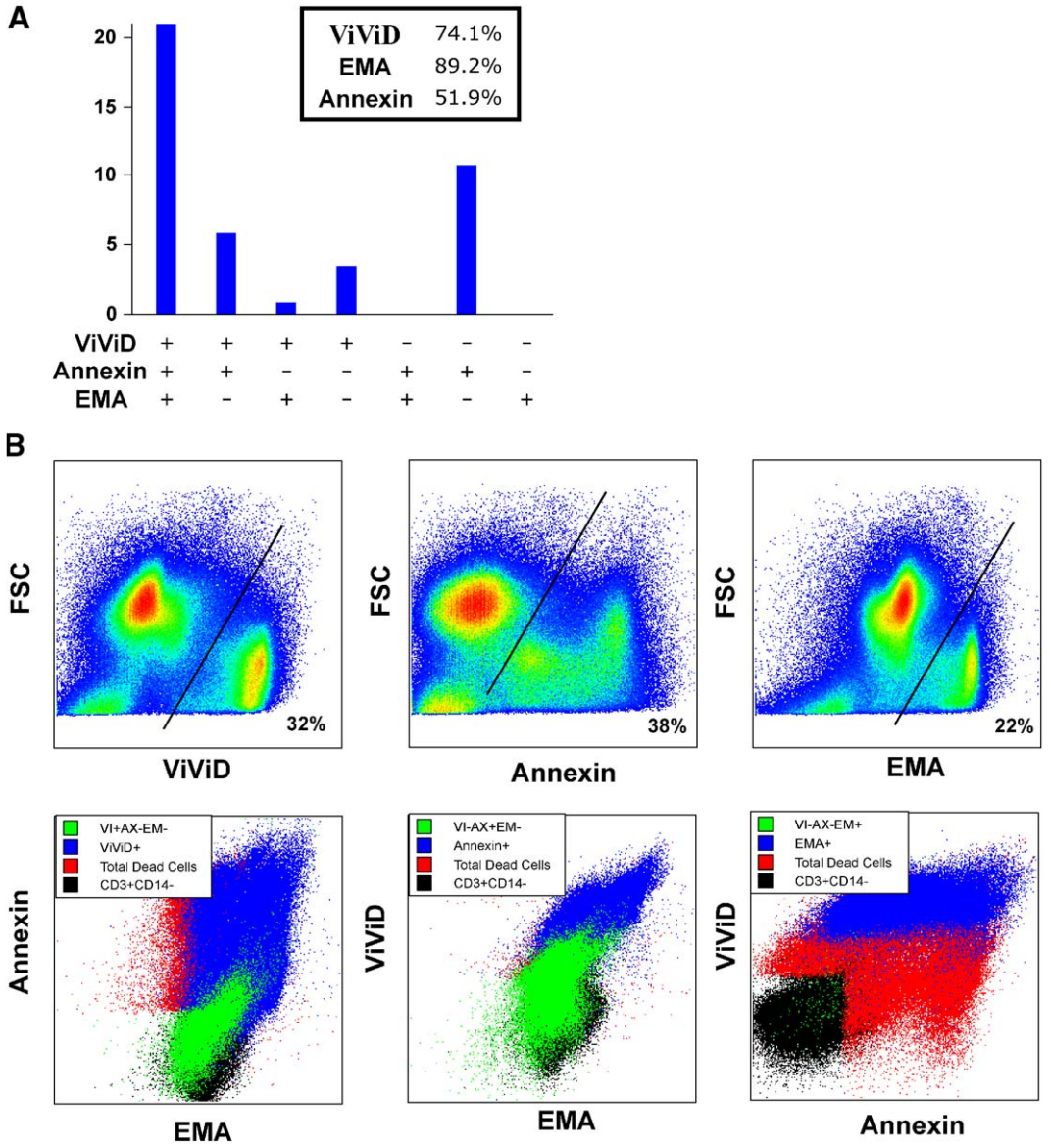

Fig. 3. Correlation of ViViD staining with other viability dyes. A) This chart illustrates the percentage of all of the possible combinations of a cell sample stained with ViViD, annexin and EMA. The fraction of cells that fall into each combination is shown for a cell sample that had nearly 40% dead cells. The total fraction of the dead and apoptotic cells identified by each of the individual stains is shown in the legend. B) Cells stained with three viability markers were gated independently to calculate the individual cell combinations as illustrated in Fig. 3A. Overlay plots indicate the distribution of the total dead and apoptotic population (e.g. ViViD+ *or* EMA+ *or* Annexin+; red dots), together with the location of the cells identified by each of the stains (blue dots), and those that are identified *only* by each of the stains (green dots).

normal lymphocyte morphology. ViViD+PI+ were dead cells displaying morphology consistent with cells with damaged membrane. ViViD+PI− cells show extensive membrane damage, and represent cells that have lost nuclei or have degraded DNA to an extent that no PI fluorescence is detectable. Notably, these fragments may still stain with antibodies and yet would not be excluded by DNA-based viability dyes.

3.3. Viability determined by ViD staining is comparable to EMA and annexin staining

Typically dead cells which have a low FSC and high SSC generally can be excluded by the lymphocyte gate (Ohen and Loken, 1982; McGann et al., 1988). However, some dead cells are found within the lymphocyte gate, indicating that gating by light scatter alone cannot exclude all dead cells. To demonstrate this, cells were stained with

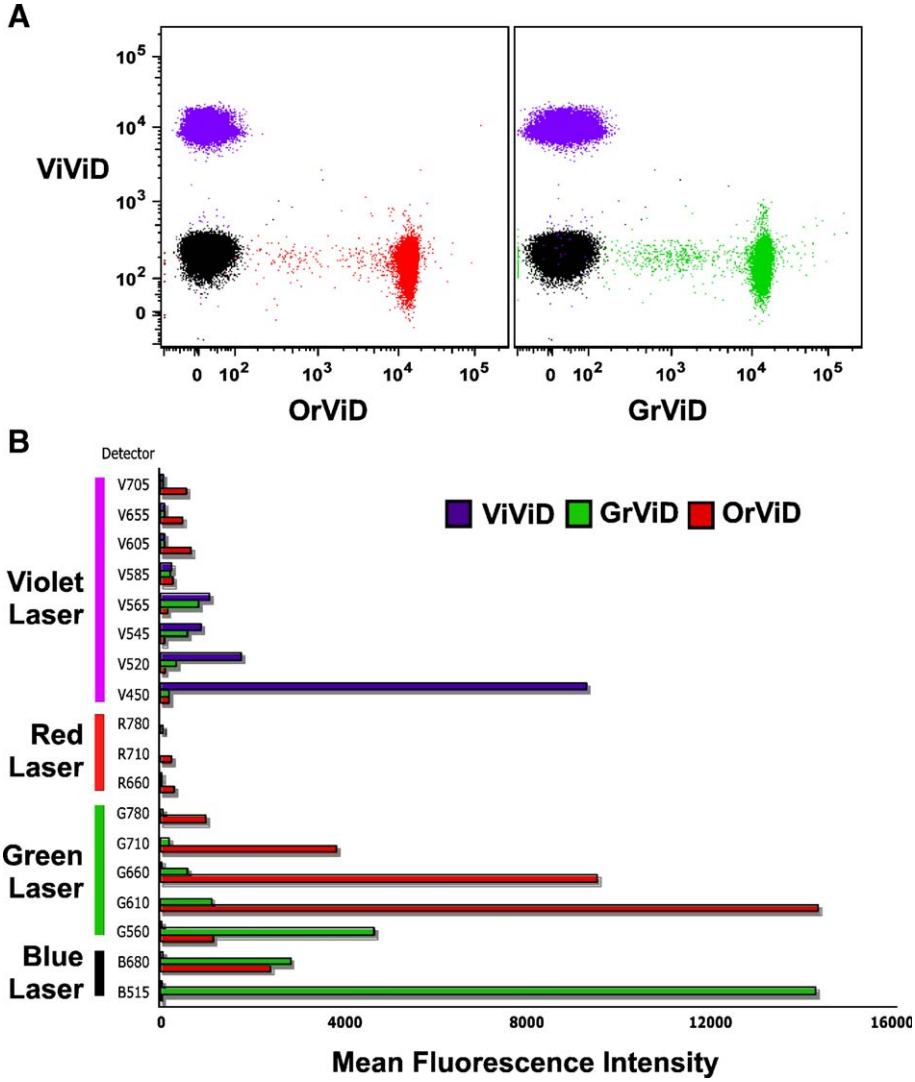

Fig. 4. Amine beads (R-NH_2) can be used as a control for compensation. A) R-NH_2 Beads were reacted with one of each of the different amine reactive viability dyes (ViViD, GrViD or OrViD). The resulting dot plot shows the separation between the unstained bead sample (black) and the positively stained samples. This separation is greater than that seen for stained cell populations, indicating that the beads can be good controls for setting fluorescence compensation. B) This graphic illustrates the spectral overlap measured in various fluorescence detectors for all three of the ViDs. As an example, the violet laser excites ViViD and the primary emission can be detected in the V450 channel with only a fraction of light detected in the V520, V545 and V565 detectors. None of the other lasers used excited ViViD. The channels are named by a letter identifying the excitation laser (V, 405 nm violet; B, 488 nm blue; G, 532 nm green, and R, 638 nm red) and the wavelength of the center of the bandpass filter in nm to detect the emission. The spectral profile of other detectors on a standard blue laser instrument (e.g. PE, Cy5PE, etc.) should be similar to that of the green-laser detectors shown here.

ViViD, Annexin and EMA and analyzed for all of the possible staining combinations. Fig. 3A shows the percentages associated with all combinations for the ViViD positive and the ViViD negative cells. Data show that the majority of dead cells identified by any of the three stains were identified by ViViD (Fig. 3A). The two major populations were stained with all markers or co-stained with Annexin and ViViD. Fig. 3B shows the location of all combinations tested as overlay plots gated on either ViViD+ or the ViViD− cell populations. A small percentage of cells (Annexin+ in the first overlay as illustrated in red) are not stained with ViViD, suggesting these cells represent a true apoptotic cell population that has not yet suffered loss of membrane integrity. Further, we identified a small percentage of cells that stain only with ViViD, suggesting that this dye may be more sensitive for identification of dead cells. Back-gating on this population shows that the vast majority of this phenotype displays low FSC and high SSC, which is characteristic of dead cells (data not shown).

The next overlay illustrates the majority of the EMA+ or ViViD+ are annexin+ (e.g. very few cells are colored red). Cells, negative for both and positive for annexin (VI−AX+EM−, represented in green) are apoptotic cells, which have intact membranes. The last overlay plot (far right) illustrates the location of cells, which are not stained by EMA (represented in red) from those that are stained (represented by blue and green). The presence of the VI+AX−EM− and VI+AX+EM− populations reflect an increased level of sensitivity of the ViViD compared to EMA. Such cells would not be excluded in an analysis procedure if EMA were used and therefore could have an impact on background sensitivity.

3.4. ViD stained amine beads (R-NH_2) for use as fluorescence compensation and stability testing

Data from beads coated with amine groups (R-NH_2) and incubated separately with ViViD (purple), GrViD (green) and OrViD (red) and an unstained bead control (black) are overlayed in Fig. 4A. The results showed a significant reaction of these dyes to the beads, as indicated by a significant increase in fluorescent signal as compared to the unstained control. Compared to ViD staining of dead cells, the signal obtained with beads is much higher and therefore is ideal as a compensation control. As shown in Fig. 4B, ViViD, GrViD and OrViD-bound beads were used to examine primary fluorescence spillover into other (secondary) channels. ViViD fluoresces most in the primary detector (V450, violet laser, peak emission=450 nm), with only minimal light contamination in the neighboring violet detectors (V520>V545>V565). No light was detected in any of the red, green or blue laser detectors. OrViD labeled beads showed a strong primary signal into the G610 detector (the 610 nm emission as excited by either a 488 nm blue or 532 nm green laser). Finally, GrViD showed a strong primary signal into the B515 (fluorescein) detector but was emitted into the orange and red regions.

Stained beads incubated at 37 °C produced a slightly lower fluorescence signal than those incubated at room temperature, while incubation at 4 °C improved fluorescence intensity slightly compared to RT. Comparison of incubation periods indicated that 20 min was optimal, with slight reductions in fluorescence thereafter. Lower pH (pH=6.0) reduced fluorescence intensities compared to neutral pH buffers (pH=7.2). ViD prepared and stored in DMSO (2 mg/ml) showed the best stability over the 6 months tested without loss fluorescent intensity (data not shown, stability testing is still under investigation).

3.5. Cells retain ViD after treatment with permeablization reagents or PFA

Clinical and research studies of immune function often require cell stimulation and staining of intracellular

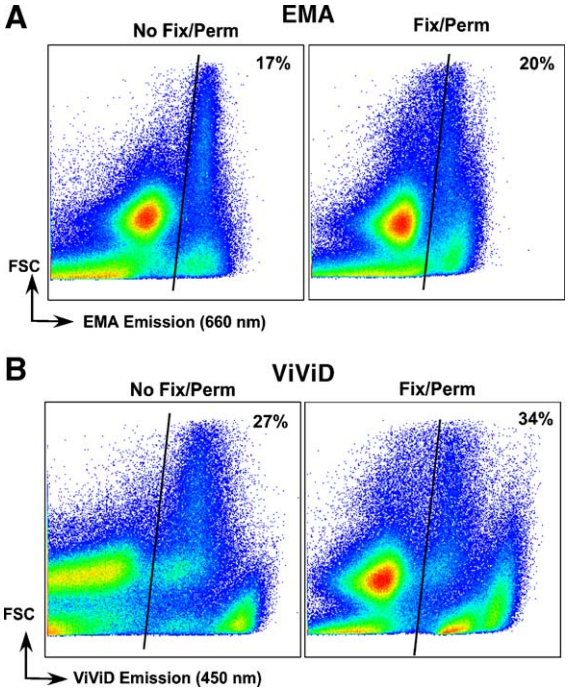

Fig. 5. Amine reactive dyes are stable after fixation and permeablization reagents. EMA labeled cells (A) and ViViD labeled cells (B) show no loss of fluorescence after treatment with fixation and permeablization reagents. As shown in Fig. 3, ViViD identifies a larger fraction of the dead cells present in the culture (prior to fixation and permeabilization).

antigens such as cytokines; therefore, we tested ViDs and EMA after exposure to common fixation and permeablization (fix/perm) reagents (Fig. 5). The proportion of dead cells identified by EMA (17% and 20%, 5A) and ViViD (27% and 34%, 5B) were similar, further validating ViD as a method for detection of dead cells. Furthermore, with both dyes, the proportion of dead cells increased marginally but did not decrease after treatment with the fix/perm reagents. As expected this was coupled with a typical decrease in FSC after treatment. As noted earlier, an increase in ViViD staining as compared to EMA demonstrates the increased sensitivity of this marker.

3.6. ViD reduces artifacts when used in 12-color cell experiments

To demonstrate the utility of ViViD in polychromatic flow cytometry, we used 12-color staining panels to query surface and intracellular reagents after exclusion of ViViD+ dead cells. Fig. 6 demonstrates the artifacts introduced by dead (ViViD+) cells. As illustrated, cells were gated through a singlet gate, a lymphocyte gate and the viability gate. Phenotypes were based on either the live cell or the dead cell gates. Dead cells showed few negative cells and few CD8+ cells. CD4+ gated cells

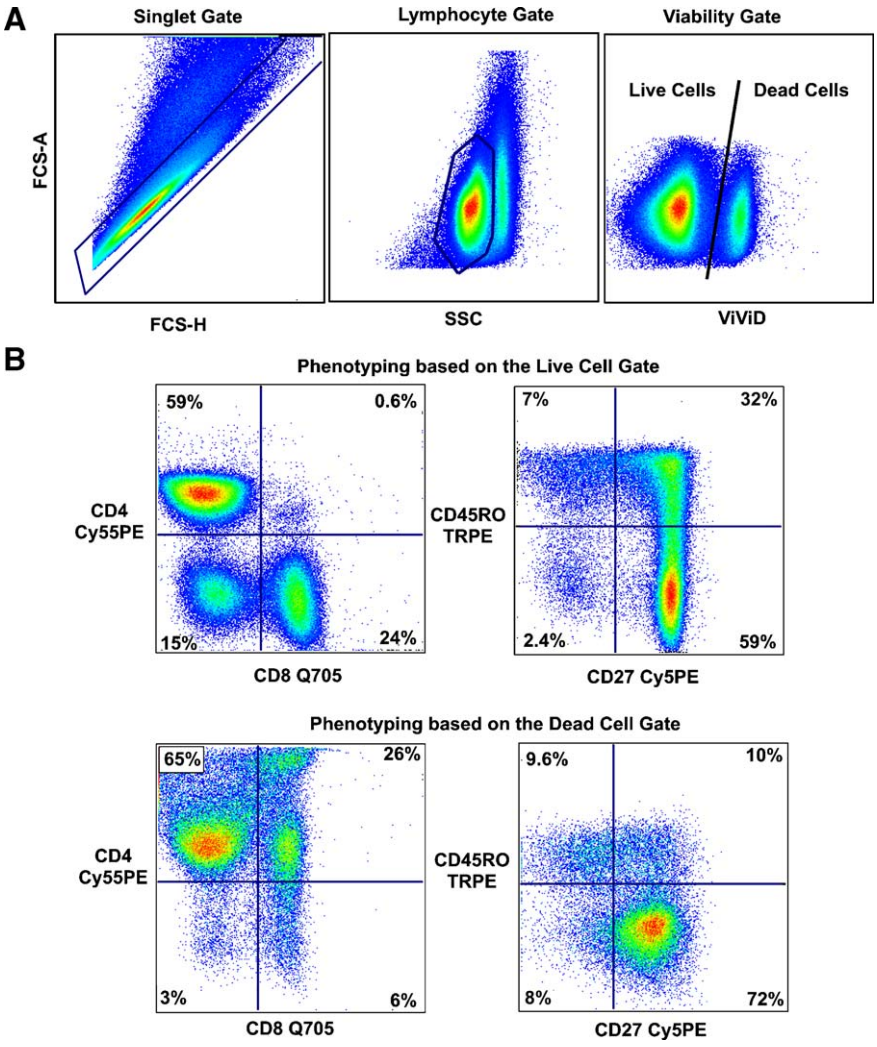

Fig. 6. Inclusion of dead cells adds significant staining artifacts to analysis. A) In a comparison between gated live cells (ViViD−) and dead cells (ViViD+), staining artifacts are illustrated as cells are gated through a singlet gate, a lymphocyte gate and a viability gate. Note that there are a significant number of dead cells despite a scatter gate. B) The phenotype of the dead cells (bottom) is distinct from that of live cells (top), largely due to nonspecific binding of antibodies. The lack of exclusion of the dead cell population would thereby skew the phenotypes assessed by this kind of analysis.

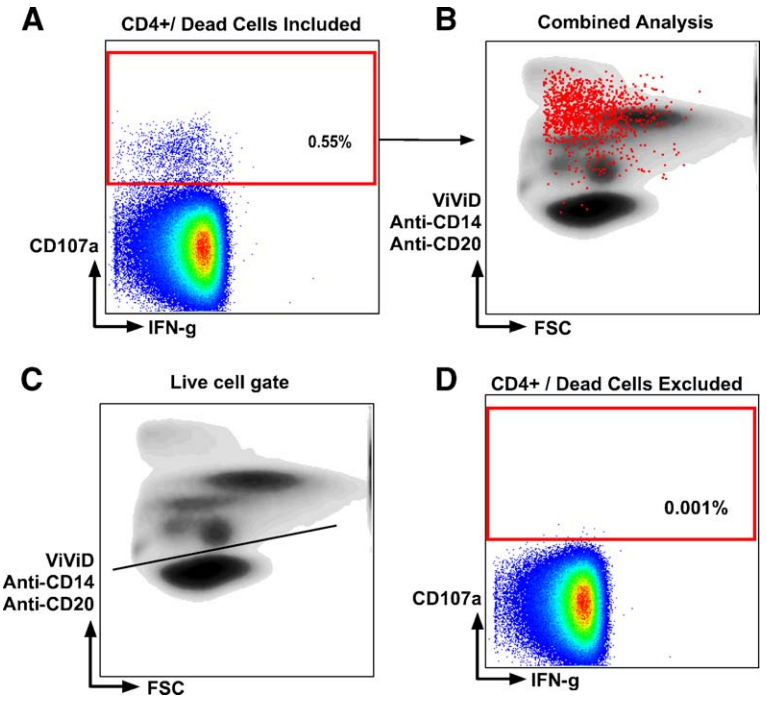

Fig. 7. Back-gated, ViViD+ cells show that the majority of "background" stimulated cells is due to the inclusion of dead cells. PBMC were treated under typical stimulatory conditions to identify antigen-specific T cells, but with no antigen stimulus. Cells producing cytokine under these conditions are considered "background" stimulated cells; the more background stimulated cells, the less sensitive the assay for quantifying antigen-specific cells. A) When dead cells were not excluded, background-stimulated cells are apparent (0.55%) in a control sample. B) Back-gating on the apparently responsive cells illustrates that these cells are all ViViD+; not necessarily low FSC. Using the live cell gate (C) to exclude the dead cells (ViViD+), the background staining was reduced to nearly zero (D).

showed a completely different phenotype when comparing the CD45RO vs. CD27 gated histograms. The inclusion of dead cells introduces a population artifact, which in this example, as illustrated by unusually high naïve CD4+ T cells (CD45RO−CD27+). Thus, the inclusion of dead cells can lead to errors in CD4 and CD8+ T-cell enumeration and the subgated cell populations. The exclusion of ViViD+ dead cells eliminated these problems, as most naïve cells displayed the expected CD45RO−CD27+ phenotype.

In the setting of immunogenicity experiments, it is important to reduce background staining when trying to detect low frequency T-cell populations (such as those responding to peptide stimulation). Fig. 7A shows that a sample stimulated only with CD28 and CD49d (no peptide) has 0.55% "background" staining of CD107a+ CD4+ T-cells. Back-gating and overlaying the ViViD+ cells shows that all of the background cells are ViViD positive (Fig. 7B). When excluding the ViViD+ cells (excluding dead cells, Fig. 7C) the background staining in the CD107a+ gate decreased to 0.001% (Fig. 7D).

4. Discussion

Amine reactive dyes are potentially powerful tools to identify and remove dead cells which potentially bind mAb-conjugates non-specifically, thereby greatly improving the sensitivity and accuracy in immunological analysis. Our results show that the ViDs can be used as an effective substitute for EMA, since all EMA positive cells are also ViD positive. Morphological changes consistent with necrotic or post apoptotic cell characteristics was demonstrated in all sorted cell populations which were ViViD+. This demonstrated the specificity of the amine reactive dyes. Furthermore, cells that are PI−ViViD+ have likely lost so much DNA that PI will no longer bind, suggesting ViViD staining is more sensitive than DNA-intercalating dyes. When comparing ViViD to EMA and annexin, nearly all ViViD+ stained cells accounted for all "dead" cells, with or without annexin or EMA co-staining. Biological changes in the cell membrane such as the increase in phosphotidylserine, which can potentially have compromised membrane integrity and therefore bind antibody-conjugates non-specifically, should be removed from analysis. Hence,

the small population, which is positive only for annexin, most likely represents apoptotic cells with intact membranes.

Staining with the ViD dyes is simple; they can be added together with mAbs during any pre-fixation staining step for 20 min. In addition, the amine-reactive beads provide an effective, non-cellular based consistent fluorescence compensation control; they too require a 20 min incubation at room temperature. Thus, the use of the ViDs introduces no significant change to standard staining protocols.

Finally, these dyes were shown to be stable for extended periods of time under −20C conditions but storage of pre-diluting aliquots in staining media demonstrates a loss of activity. This suggests the dye should only be stored in DMSO at −20 °C until used.

ViD stained cells showed no loss of fluorescent intensity after standard fixation and permeablization conditions. Surface stained cells with the ViD reveals dead cells, which produce artifactual cell populations. It is critical to point out that mAb-conjugates stained dead cells is a non-specific binding event and the populations shown in these results only represent artificial cell staining patterns. Hence, a dead cell stained with an anti-CD4 conjugates may or may not represent cells with the CD4 molecule. Similarly, cells stained with intracellular mAb-conjugates showed improved functional measurement by the reduction in background staining as show in the example of CD107a stained cells.

In summary, the amine reactive viability dyes are simple to use, reproducible and eliminate cells with increased background staining. These dyes were found to be stable with no loss in fluorescent intensity when treated with permeablization or fixation reagents. Since these dyes are excited by a variety of lasers and emit light energy at a variety of detectors, they can be easily incorporated into different panel strategies. In addition, other mAb-conjugates can be added with ViD to be used as a dump channel (e.g. anti-B-cell, anti-monocyte and anti-NK cell markers for the analysis of T cell subsets).

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