DEAD CELL STAINS IN FLOW CYTOMETRY: A COMPREHENSIVE ANALYSIS

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Introduction

Loss of membrane integrity is an indicator of cell death in flow cytometric analysis. Cells that exclude a dead cell dye are considered viable, while cells with compromised membranes allow the dye inside the cell to stain an internal component, thus identifying the dead cells. Many dyes for dead cell identification are available for this across a wide range of excitation and emission wavelengths. A comprehensive study of 25 stains (see table 1) that have been used for identification of dead cells based on membrane integrity is presented, which include the classic membrane-impermeant nucleic acid dyes, monomeric cyanine dyes, Annexin V dyes, and amine-reactive dyes. All dyes are tested with a mixture of live and heat-killed Jurkat T cell leukemia cells before formaldehyde fixation, immediately after 2% formaldehyde fixation, and 18 hours after 2% formaldehyde fixation; as well as with aged culture cells; and with camptothecin-induced apoptotic cells. (see figure 1) Dead cells can nonspecifically bind antibody conjugates which can potentially lead to erroneous results, especially in rare event analysis, thus making the identification of dead cells critical to accurate results. (see figure 2) Identification of dead cells following fixation and permeabilization procedures is also important, and requires a dead cell dye that can maintain discrimination of live and dead cells after fixation and permeabilization. (see figure 3)

Table 1 – Dye excitation / emission

Table 1: Excitation laser and bandpass filter used with each dye in figure 1, and excitation/emission max of each dye

Dead Cell Dye	laser & bandpass	excitation & emission max
LIVE/DEAD® Fixable Blue	UV (355 nm) 450/50	350/450
LIVE/DEAD® Fixable Violet	Violet (405 nm) 450/50	416/451
LIVE/DEAD® Fixable Aqua	Violet (405 nm) 525/50	367/526
LIVE/DEAD® Fixable Yellow	Violet (405 nm) 585/42	400/575
LIVE/DEAD® Fixable Green	Blue (488 nm) 530/30	495/520
LIVE/DEAD® Fixable Red	Blue (488 nm) 610/20	595/615
LIVE/DEAD® Fixable Far Red	Red (633 nm) 660/20	650/665
LIVE/DEAD® Fixable Near-IR	Red (633 nm) 780/60	750/775
ethidium monoazide (EMA)	Blue (488 nm) 610/20	462/625
DAPI	UV (355 nm) 450/50	358/461
SYTOX® Blue	Violet (405 nm) 450/50	445/470
SYTOX® Green	Blue (488 nm) 530/30	504/523
SYTOX® AADvanced	Blue (488 nm) 695/40	546/647
SYTOX® Red	Red (633 nm) 660/20	640/658
Ethidium homodimer-1	Blue (488 nm) 610/20	528/617
Propidium Iodide	Blue (488 nm) 610/20	535/617
7AAD	Blue (488 nm) 695/40	546/647
PO-PRO®-1	Violet (405 nm) 450/50	435/455
YO-PRO®-1	Blue (488 nm) 530/30	491/509
TO-PRO®-3	Red (633 nm) 660/20	642/661
AnnexinV Alexa Fluor® 488	Blue (488 nm) 530/30	495/519
AnnexinV Pacific Blue™	Violet (405 nm) 450/50	410/455
AnnexinV r-PE	Blue (488 nm) 575/26	496/578
AnnexinV APC	Red (633 nm) 660/20	650/660
AnnexinV Alexa Fluor® 647	Red (633 nm) 660/20	650/668

Figure 2- Immunophenotyping

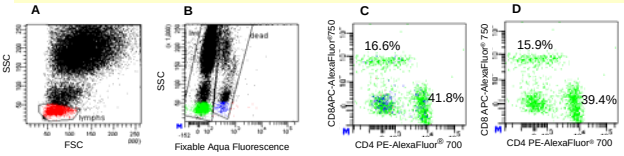
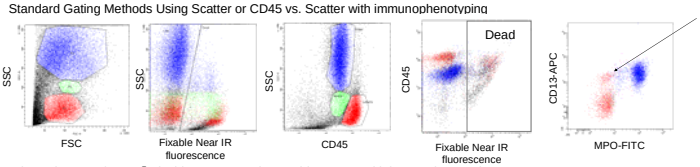


Figure 2: Ammonium chloride lysed Whole Blood was either untreated, or heat treated to generate dead cells (20 min, 60 degrees celsius), and then mixed together. One million cells were stained with a 7-color antibody cocktail containing: CD7 FITC, TCR gamma/delta PE, CD2 PE-AlexaFluor® 610, CD3 Pacific™Blue, CD4 Pe-AlexaFluor® 700, CD5 APC, and CD8 APC-AlexaFluor®750. Cells were washed and then stained with Live/Dead® Fixable Aqua stain. Cells were washed again before data acquisition on a BD LSRII. Standard gating using Scatter parameters (A) include dead cells (blue dots) and live cells (green dots) shown in (C). Gating first using LIVE/DEAD Fixable Aqua dead cell stain (B) allows the elimination of dead cells contaminating the data, as shown in (D).

Figure 3-Dead cell identification with Fix & Perm

3A Standard Gating Methods Using Scatter or CD45 vs. Scatter with immunophenotyping



3B Gating using LIVE/DEAD® Fixable Near IR stain vs. Side Scatter with immunohenotyping

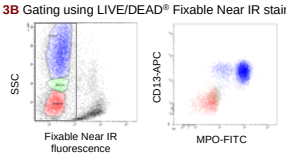


Figure 3A & 3B: Two day old Whole Blood was ammonium chloride lysed. Cells were stained with CD13 APC and CD45 PE-Cy7. Cells were washed, stained with LIVE/DEAD® Fixable Near IR dead cell stain, washed, fixed with Caltag Fix and Perm reagent A, washed, permeabilized with Caltag Fix and Perm reagent B, incubated with MPO FITC, and washed and analyzed. 3A Results show gating using the standard gating parameters of scatter or CD45 vs. Side Scatter are not adequate to eliminate dead cells from data, thus producing aberrent cell populations as indicated by the arrow. 3B Suggested method of using LIVE/DEAD cell gating to eliminate dead cells, and then further subgating is recommended to get the most accurate results.

3C Functional assay for cell proliferation using LIVE/DEAD® Fixable Violet stain to identify dead cells

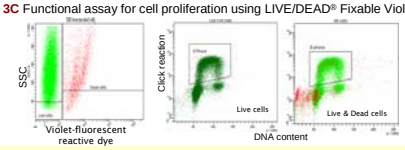


Figure 3C: Jurkat cells were treated with 10uM EdU for one hour, harvested, and washed. Cells were then stained with LIVE/DEAD Fixable Violet stain & washed again. Cells were then paraformaldehyde fixed & permeabilized with a saponin buffer. Labeling with the Click-iT™ reaction mixture using AlexaFluor 488 azide dye was followed by a wash before DNA content labeling with Click-iT™ Cell Cycle Red 633 stain. Dead cells were identified in a SSC vs. LIVE/DEAD stain plot. Click reaction vs DNA content show gating on either live cells only, or on all cells; Red dots represent dead cells.

Results and Conclusions

All dyes studied identify dead cells in live + heat killed samples. The amine-reactive dyes maintain dead cell distinction after formaldehyde fixation while this discrimination was lost with other dyes. The annexin V conjugates maintained cell population separation although there was cell loss over time. The monomeric cyanine & annexin V conjugates identified early & late apoptotic cells, while the DNA intercalating and amine-reactive dyes generally identified late apoptotic/necrotic cells.

Just as it is important to know the spectral qualities of a dead cell dye in experimental design, it is important to understand how a particular dye performs in it's intended application. Excluding dead cells in immunophenotyping and functional assays improves the accuracy of the results. The identification and exclusion of dead cells is an important aspect in flow cytometric assays.

Figure 1-Jurkat cells stained with dead cell dyes

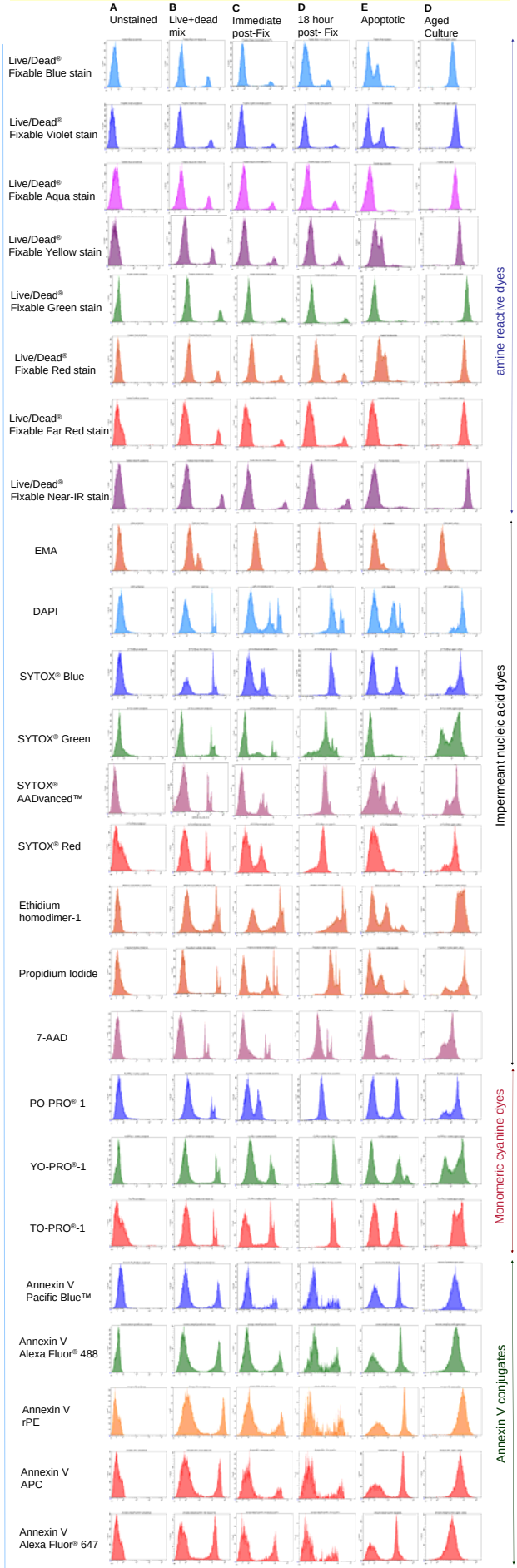


Figure 1: Jurkat T-cell leukemia cells stained with 25 different dyes which enter cells via compromised membranes to label internal cellular components to identify the cells as dead. Column **A** unstained cells, **B** live+dead mix, **C** live+dead mix immediately after formaldehyde fixation, **D** live+dead mix 18 hours after formaldehyde fixation, **E** camptothecin-induced apoptotic cells, **F** aged culture cells