EMBL European Molecular Biology Laboratory

Cell Viability Assessment

Cell viability can be easily determined in Flow Cytometry by adding dead cell dyes at relatively low concentration to a suspension of cells. Live cells with intact membranes are distinguished by their ability to exclude these dyes, while cells with a compromised membrane will allow the entrance of the dyes to stain internal components, thus identifying these cells as dead.

Identifying and removing dead cells from **analysis and sorting** is a critical step in ensuring accurate results, dead cells tend to bind nonspecifically to many reagents, giving rise to false positive signals. The use of exclusion dyes help to distinguish live and dead cell populations more accurately than by relying on scatter characteristics alone.

A common approach is to use DNA binding dyes like propidium iodide (PI), but there is a wide selection of dyes that can be used, which include 7-AAD, DAPI, SYTOX® and To-Pro-3 among others. The procedure is very simple and the stained (dead) cells are thousand of times brighter than viable cells, making them easy to identify.

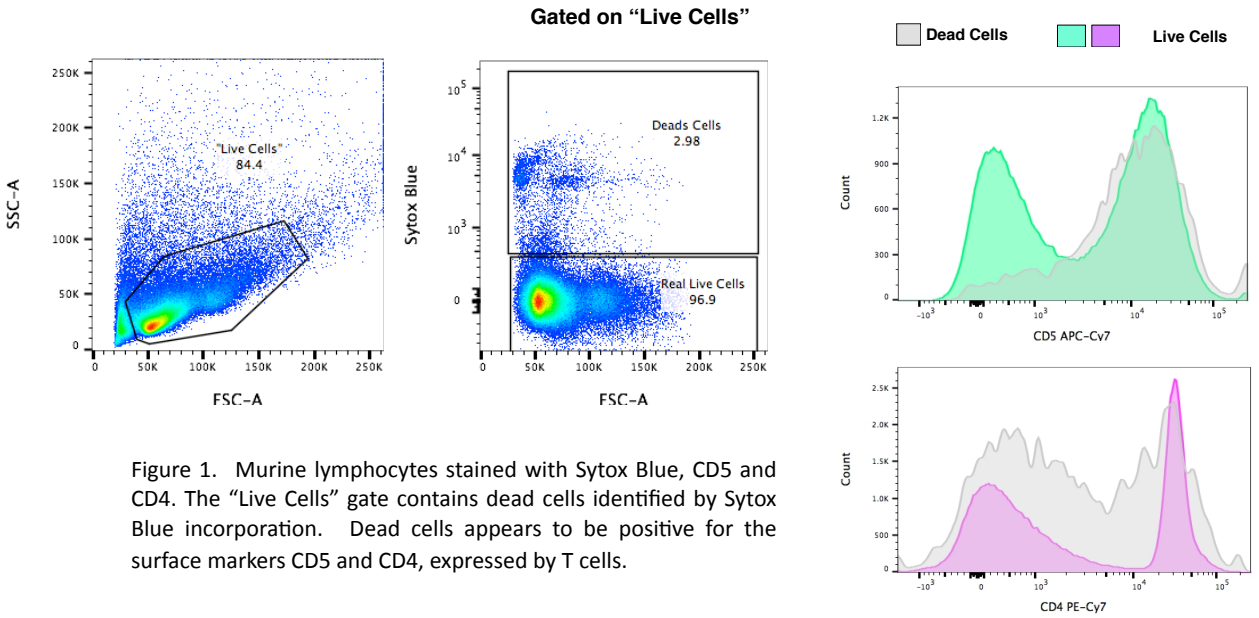
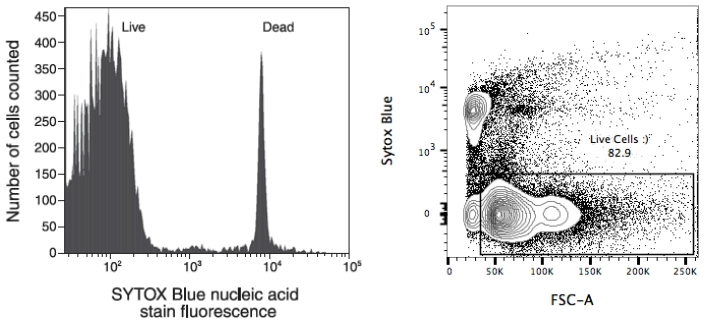


Figure 1. Murine lymphocytes stained with Sytox Blue, CD5 and CD4. The "Live Cells" gate contains dead cells identified by Sytox Blue incorporation. Dead cells appears to be positive for the surface markers CD5 and CD4, expressed by T cells.

Staining Protocol: Unfixed Samples
(Using PI, DAPI or SYTOX® Blue).
Materials:
-FACS Buffer: PBS 1X + 2% BSA + 2.5 mM EDTA.
-PI working solution: From a stock solution (1000 µg/ml), prepare a dilution 1/10 in PBS 1X and use it as your 100X PI solution.
-DAPI working solution: From a stock solution (1000 µg/ml), prepare a dilution 1/10 in PBS 1X and use it as your 100X DAPI solution.
-SYTOX® Blue working solution: Prepare a dilution 1/20 in PBS 1X and use it as your 100X SYTOX® solution.

1. Collect and wash the cells
2. Re-suspend the cells in 300µl of Facs Buffer.
3. Add 3µl of the diluted dye (100X).
4. Vortex, incubate for 3 minutes protected from light and acquire.
5. In the LSR Fortessa Analyser:
   For PI detection use channel: **561-610/20**
   For DAPI detection use channel: **355-450/50 or 405-450/50**
   For SYTOX® Blue detection use: **405-525/50**

   Open a histogram and adjust the PMTs of the involved channel, placing lived cells in the left side of the histogram, making sure bright cells are in scale. After adjusting the PMTs values, identify nonviable cells on a bivariate plot of Viability Dye vs. forward light scatter (FSC).



DAPI and SYTOX® dyes bind in equilibrium with DNA, therefore, external dye concentration must be maintained during analysis. Dyes should be added last in any staining protocol and should not be washed out before data acquisition. Impermeant DNA dyes are not compatible with fixation or intracellular staining protocols.

Assessment of Cell Viability in Fixed Cells

For reasons of safety or convenience, it is frequently necessary to fix cells prior to analysis. In this case, the protocol described above will not work since fixation will render all cells permeable. There are, however, reactive dyes that stain dead cells and withstand fixation, such as the LIVE/DEAD® Fixable Dead Cell Stain Dyes from Invitrogen.

Fixable Dead Cell Stain LIVE/DEAD® utilise the loss of membrane integrity for dead cell discrimination. Often referred to as amine-reactive dyes, these stains are based on the reaction of a fluorescent reactive dye with cellular proteins. In live cells, only surface proteins bind to the reactive dye, resulting in dim fluorescence. The reactive dye can enter

dead cells and label proteins in the interior of the cell, producing at least a 50-fold increase in fluorescence.

Because the LIVE/DEAD® dyes react covalently with proteins, dead cell discrimination is completely preserved following fixation and/or permeabilisation of the sample with formaldehyde under conditions that are commonly used for intracellular staining and to inactivate human samples or pathogens. The staining protocol is also very simple; involve the staining of the sample before the fixation/permeabilisation step and **should be perform in the absence of FCS or BSA**.

Staining Protocol- Fixed Samples

1. Centrifuge a sample of cells in suspension containing $1X10^6$ cells. Discard the supernatant.
2. Wash the cells once with 1 mL of PBS 1X.
3. Re-suspend the cell pellet in 100 µl of LIVE/DEAD® dye (previously diluted 1/1000 PBS 1X).
4. Incubate at RT for 20 minutes protected from light.
5. Wash the cells with 1mL of PBS
6. Continue with your regular surface staining or fixation protocol.
7. For acquisition in the LSR Fortessa Analyser follow the steps previously described. The detection channel will depend on the fluorescent dye used; eight colours of amine-reactive dyes are available, offering flexibility in experimental design.

Reagent Test

The Flow Cytometry Core Facility has a collection of small supplies of reagents, which can be tested by the users without any cost before buying these relatively expensive reagents.

Name	Excitation Source	Emission Peak	Channel
Propidium Iodide	561 nm	617	561-610/20
DAPI	355-405 nm	452	355-450/50 405-450/50
Sytox Blue	405 nm	480	405-525/50
Live Dead Fixable Blue	UV	450	355-450/50
Live Dead Fixable Violet	405 nm	451	405-450/50
Live Dead Fixable Aqua	405 nm	526	405-525/50
Live Dead Fixable Yellow	405 nm	575	405-540/30
Live Dead Fixable Green	488 nm	520	488-530/30
Live Dead Fixable Red	635 nm	615	640-670/14
Live Dead Fixable F Red	635 nm	665	640-670/14
Live Dead Fixable Near IR	635 nm	775	640-780/60