Flow Cytometry Platform CB UTechS

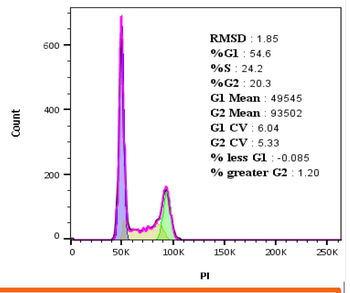
Flow Cytometry Technology

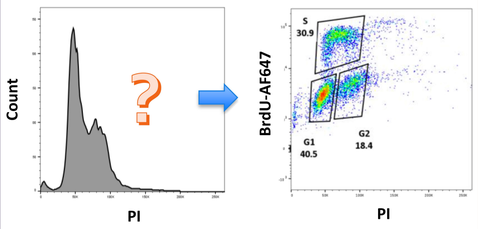
Cell Cycle

Studying **Cell cycle** by Flow Cytometry can be performed by staining cells or nuclei with **fluorescent DNA markers** or **nucleoside analogs** and measuring the signal output. By using DNA dyes that bind stoichiometrically we can assess DNA ploidy level, cell cycle stage, the presence of apoptotic cells and performance of drugs for treatment of disease states .

Cell cycle analysis can be carried out with **live or fixed cells**. When fixing cells for cell cycle analysis, alcohols (e.g. 70% EtOH) are suggested over crosslinking fixatives (e.g. aldehydes) for cell cycle analysis. Propidium Iodide (**PI**) and 4',6-diamidino-2-phenylindole (**DAPI**) are commonly used dyes. If using fluorochromes that bind both DNA and RNA (such as PI), then inclusion of RNAse A is required.

Alternatively, live cell cycle analysis can be done with dyes that cross membranes. When this is done, an efflux pump blocker may be required. If using **Hoechst 33342** consider adding dye DiOC5(3) or Verapamil. **DRAQ5** is another supravital dye available, but care should be taken as it can be cytotoxic for cultured cells. There are also a variety of **Vybrant DyeCycle** fluorochromes available which can be used across a variety of instrument configurations.





Addition of a nucleoside analogs, such as a fluorescently tagged **BrdU** or **EdU**, allow a more accurate evaluation of the different cell cycle phases.

Experimental Tips and Tricks

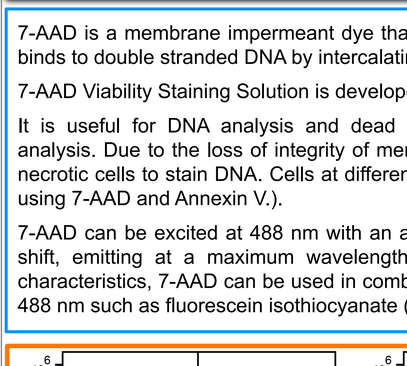
- Use the same number of cells per sample and concentrate appropriately to run at a low flow rate to ensure best resolution. $1x10^6$ cells/mL is a good start.
- Optimize dye concentration for best resolution of each cell cycle stage.
- Before adding fixative, ensure cells are in a good single cell suspension.
- For univariate cell cycle data analysis, mathematical modeling approaches available in various analysis programs are suggested. Manual gating is not advised.
- **Data acquisition** and **analysis** of all cell cycle data should be done with the DNA fluorescent parameter on a linear scale.

Apoptosis Detection

Apoptosis, or programmed death cell, is a highly controlled and complex process that occurs naturally in cells, differing from necrosis or accidental cell death. Apoptosis is a fundamental mechanism in the normal development of a living being. Apoptosis dysregulation can cause several diseases, including cancer. **The study of the cell changes during apoptosis can be done with flow cytometry.**

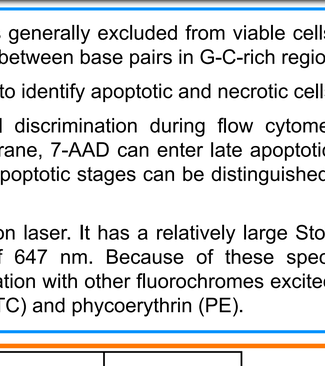
Plasma membrane modifications

A unique feature of apoptosis is the externalization of phosphatidylserine (PS) in the phospholipid bilayer. **Annexin V can bind externalized PS**. The conjugation of a fluorochrome to Annexin V coupled with a DNA-binding dye results in the evaluation of apoptosis.



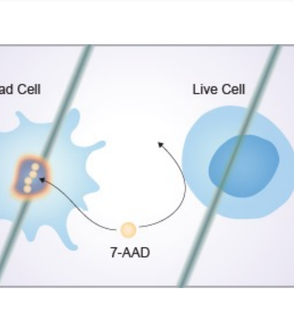
Activation of caspases

Another characteristic of apoptosis is the **activation of caspases** through caspase dimerization or cleavage. By staining caspase with specific antibodies it is possible to quantify the number of cells dying through apoptosis.



Mitochondrial function

The increase in mitochondrial membrane permeability can be reflected in **decrease of membrane potential** and a **dysregulation in the redox activity**. Dyes like TMRM, $DiOC_6$, $DiIC_1$ or JC-1 that can measure potential difference, mitochondrial membrane disruption or redox state, can be used to study

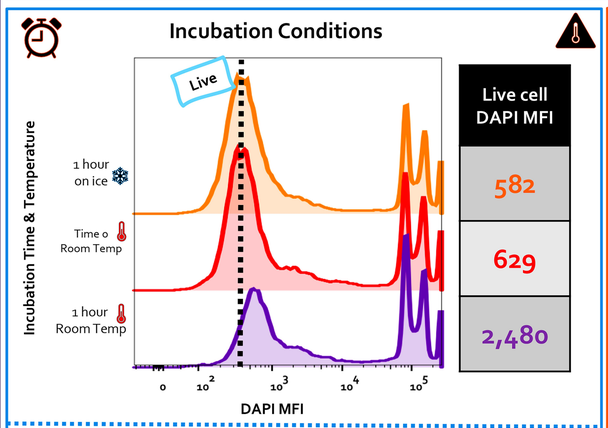


*Suggested reading: Wlodkowic, D. (2009) doi:10.1007/978-1-60327-017-5_2

DAPI Permeability

DAPI (4',6-diamidino-2-phenylindole) is a fluorescent stain often used to differentiate between live and dead cells for **viability measurements** in flow cytometry. This reagent is a popular choice due to its short incubation time and high relative brightness. Similar to other reagents, DAPI staining conditions must be optimized for best experimental results.

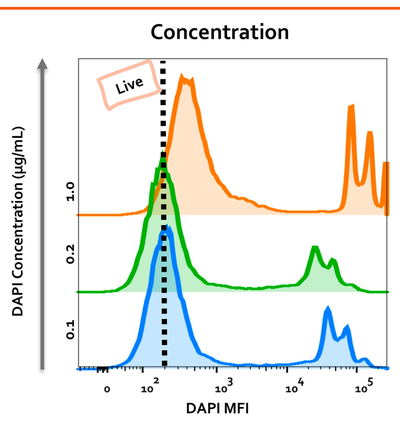
Incubation Conditions



Live cell DAPI MFI
582
629
2,480

Samples in figure above were all stained at a final concentration of 1.0 µg/mL. When left at room temperature for 1 h the live population experienced a shift in DAPI signal, whereas the sample kept on ice did not, indicating colder temperatures can be beneficial in preventing DAPI uptake in viable cells.

Concentration



Live cell DAPI MFI
582
241
259

Figure above shows the the impact of staining with increasing concentrations of DAPI. All samples were kept on ice and acquired immediately after DAPI was added. 1.0 µg/mL shows a shift of the live cell population, indicating the concentration is too high.

DAPI is a fast acting, bright fluorochrome which binds to the Adenine-Thymine regions in dsDNA. It is important to remember that DAPI can be permeable to live cells. **DAPI uptake by live cells can be prevented by optimizing concentration, adding it immediately prior to acquisition and controlling sample temperature**, understanding that ideal conditions can vary across cell types.

7-AAD Viability Staining Solution

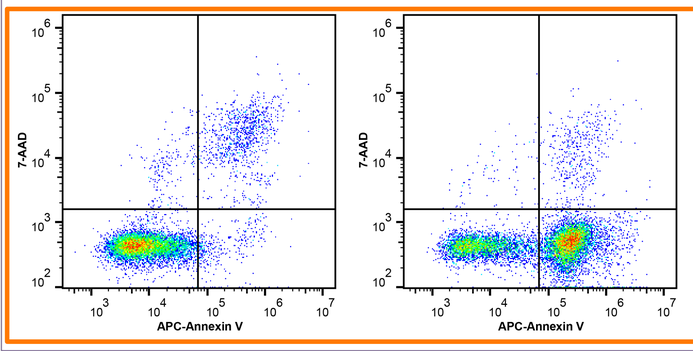
7-AAD Viability Staining Solution can be used as a viability probe for methods of non viable cell exclusion.

7-AAD is a membrane impermeant dye that is generally excluded from viable cells. It binds to double stranded DNA by intercalating between base pairs in G-C-rich regions.

7-AAD Viability Staining Solution is developed to identify apoptotic and necrotic cells.

It is useful for DNA analysis and dead cell discrimination during flow cytometric analysis. Due to the loss of integrity of membrane, 7-AAD can enter late apoptotic or necrotic cells to stain DNA. Cells at different apoptotic stages can be distinguished by using 7-AAD and Annexin V.).

7-AAD can be excited at 488 nm with an argon laser. It has a relatively large Stokes shift, emitting at a maximum wavelength of 647 nm. Because of these spectral characteristics, 7-AAD can be used in combination with other fluorochromes excited at 488 nm such as fluorescein isothiocyanate (FITC) and phycoerythrin (PE).



Jurkat cells were treated with 5 µM Camptothecin (**Right**) or not (**Left**) for 4 h. Annexin V-APC single-positive cells were early apoptotic cells, Annexin V-APC and 7-AAD double-positive cells were necrotic or late apoptotic cells, and PI single-positive cells were nude nuclear cells.

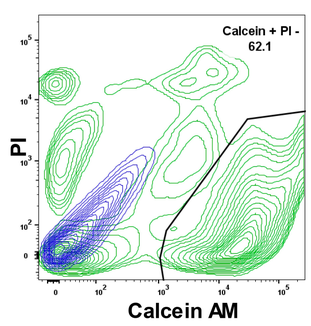
Vital Dyes

Why should we use vital dyes?

- Distinguishing debris from small cells in tissue preps for Flow Cytometry can often be difficult. Dead cell removal (through nuclear or amine reactive viability dyes) and scatter gating alone ***cannot*** be used in scenarios such as these to pull out live cells for analysis or sorting. **Remember that debris will not always be stained by your viability dye**.
- The use of a vital dye in conjunction with a dead cell exclusion dye allows researchers to eliminate dead cells and debris through clear visualization of metabolically active live cells.
- Vital dyes are available in a variety of wavelengths for excitation and emission, making them compatible for multicolor panel design.

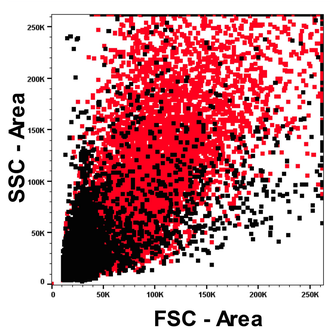
Helpful Tips

- Calcein can enter through the membrane of live cells. Esterases present in these metabolically active cells cleave the acetoxymethyl ester group of calcein, resulting in fluorescence and subsequent live cell identification by flow cytometry/microscopy.
- Titration of vital dyes is highly suggested. Excess dye can result in cytotoxicity.
- Adding vital dyes is recommended when removal of debris is essential for accurate analysis or successful downstream applications after sorting (96 or 384 well plate sorting, sequencing, etc.)
- DNA binding Draq5 and Hoechst dyes can also be used to identify live cells.



Sample (+) PI/Calcein AM

Sample (-) PI/Calcein AM



Calcein AM **Positive**

Calcein AM **Negative**

Overlay above shows the same sample. The addition of Calcein AM and PI showed that only 62% of these events were live cells excluding debris. **Note:** Calcein AM and PI double positive events indicate cells that are in the process of dying.

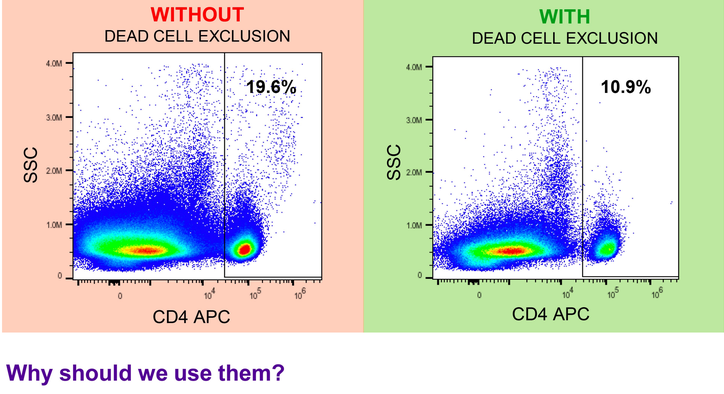
Sample above pre gated to exclude dead cells and small debris as seen by scatter. Red events are Calcein-AM positive, whereas black events were Calcein-AM negative. Overlapping events show that the vital dye is necessary for live cell identification

Fixable Viability Dyes

Fixable Viability dyes should be included in **every experiment** with surface and/or intracellular staining where fixation is required.

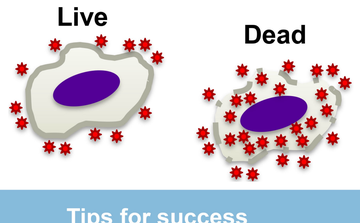
What are fixable viability dyes?

All mammalian cells have amines that are present both on the surface and inside of the cell. Fixable viability dyes are amine reactive and will covalently bond to these amines, resulting in an irreversible staining. Dead cells can be distinguished due to a higher level of fluorescence intensity due to compromised cell membranes.



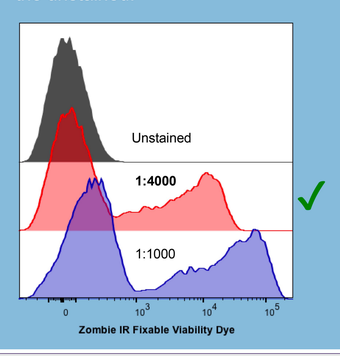
Why should we use them?

Antibodies can **non-specifically bind dead cells**, resulting in false positive results and poor statistical data. This makes the inclusion of a viability dye vital for a successful Flow Cytometry experiment. Fixable viability dyes are available from a variety of vendors and offer the flexibility of assessing cell viability while staining for both surface and intracellular markers of interest. It is amenable to fixation and permeabilization due to the nature of the binding.



Tips for success

- No protein/serum can be in the buffer when staining for these dyes
- Fixable viability dyes should be titrated so the live cells do not shift up in fluorescence from the unstained.



Reference:

Flow Post-its Memorial Sloan Kettering Cancer Center

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