Flow Cytometry Platform CB UTechS

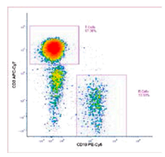
Flow Cytometry Technology

What is Flow Cytometry

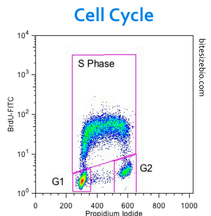
Flow Cytometry was probably the first high-throughput **single-cell analysis** technique to be developed, over 50 years ago!

Flow Cytometry measures the properties of cells and particles in a stream of fluid, allowing multiparametric analysis at a single-cell level. Fluorescently-labeled cells in suspension are run on flow cytometers where they pass in file, one by one, through one or more lasers of different wavelengths. Scattered laser light or emitted fluorescence are collected and transmitted through optical pathways and amplified/digitized for downstream analysis.

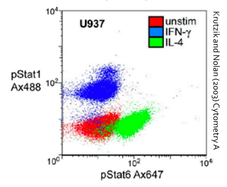
Immunophenotyping



Cell Cycle



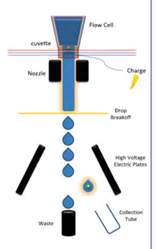
Phosphorylation



In **Cancer Research,**

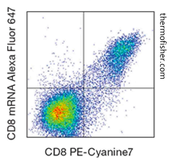
Flow Cytometry can be applied to answer an assortment of different biological questions through a variety of applications that include the measurement of **DNA content**, **immunological phenotyping**, **cell death**, **proliferation**, **phosphorylation** and **RNA expression**, among many other parameters.

Cell Sorting

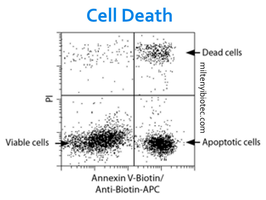


Cell sorters are an extension of this technology which allow for isolation of specific populations from heterogeneous samples for a variety of downstream applications, such as single-cell sequencing or in vivo studies.

RNA Expression

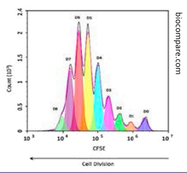


Cell Death



Multidimensional Analysis

Cell Proliferation



Sample Preparation Tips for Cell Sorting

Cell Viability, autofluorescence and cell aggregation may all affect the quality of cell sorting experiments. Good sample preparation is crucial and will result in better sort purity, yield and post-sort cell function and viability.

1. Buffer Suggestions

- Use Ca^{++}/Mg^{++} free buffers → reduces cell aggregation
- Use BSA (0.1 – 1%) or dialyzed FBS (1 – 5%)
  - Minimal amount of BSA decreases autofluorescence, increases population resolution, and improves flow rate in instrument
  - Avoid non-dialyzed FBS as it facilitates cell-cell adhesion by replacing Ca and Mg
- Add EDTA (2 – 5 mM) → helps prevent cell adhesion
- Add 10 – 25 mM of HEPES to improve pH stability
- Add DNAse I (25 – 50 ug/mL) and 5 mM of MgCl2 → digests free DNA released by dead cells.

2. Single Cell Suspension

- Filter samples immediately before sorting
- Avoid keeping cells at unnecessarily high concentration. Keep cell suspension at 1 – 10 million/mL during processing.
- Always check cells under the microscope as you are preparing them to ensure they are in a single cell suspension.

3. Dead Cell Discrimination

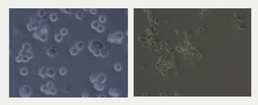
- Strongly recommended to use a Dead Cell Exclusion Dye with any cell sorting experiment. It will greatly reduce autofluorescence and lower non-specific baselines, which will result in increased population resolution.

To keep in mind:

- ✦ Use minimal speed to centrifuge the cells. *A good starting point is 300 g for 10 minutes.*
- ✦ Avoid vigorous vortexing.
- ✦ Do not generate a dry pellet at any time during processing.
- ✦ Avoid introducing air bubbles, since surface tension forces can kill cells.
- ✦ Keep cells on ice, unless otherwise required by a specific protocol.

Good Sample Preparation

Poor Sample Preparation



Controls in Flow Cytometry

Controls are essential in any experiment in order to distiguish your results from background, set appropriate gates, and more. Here we will discuss some crucial controls for Flow Cytometry you must consider to ensure publication quality data.

Parental Cells Unstained
The same cell type as your experimental samples, without any added fluorochromes, fluorescents proteins or viability.
This control is necessary to define the autofluorescence of your cells of interest.

Viability Alone
A single color control with only viability dye added to determine live cell and fluorescent gates, excluding dead cells.
Cells of interest should be used. In the case of amine-reactive viability dyes for fixed cells,
Arc-Amine beads may be used instead for compensation.

Single Color
Cells or beads single stained with just one fluorochrome or expressing only one fluorescent protein.
Necessary in multicolor experiments to calculate compensation.
Single color controls need to be run for each experiment and compensation should not be re-used from day to day.
Ab/fluor should be matched to your experiment.

Secondary Ab alone
Cells stained whith only the secondary Ab, if you are not using a directly conjugated Ab. This helps you determine if there is non-specific binding of the secondary antibody.

Negative Control
When possible, negative control cells are advisable to verify your Ab is not binding to other epitope not of interest.

Positive Control
When possible, positive control cells are advisable to verify your Ab is binding as expected.

Unstimulated Control
If carrying out stimulation experiment, a sample that has not been stimulated should be stained/run to determine baseline levels of expression for your markers of interest.

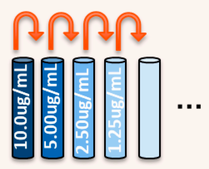
FMO Controls (Fluorescence Minus One)
Sample with all the fluorochromes in the panel with the exception of one. This is a gating control that allows you to set a gate which takes into account the spreading of the data.
Strongly advised in all multicolor experiments when a panel is first being developed or when positivity is difficult to determine.

Antibody Titration for Flow Cytometry

Antibody Titration is one of the most important steps in Panel optimization to ensure **best Resolution**. It allows you to find the **optimal concentration** of antibody that results in the **brightest signal of the positive population** while **avoiding background** staining, thus maximizing **signal-to-noise ratio**. Additionally, it helps **save money and reagents.**

Sample Staining Tips

1. Titrate your antibodies under the **same conditions as your experiment** (same cell type, temperature, incubation time, total volume, same number of cells)
2. You must include **Viability Dye** for reliable data due to non-specific binding of antibody to dead cells
3. Adding **FC block** is critical when doing titration to prevent FC mediated binding
4. If your marker of interest is expressed at low levels, adding another marker may help you resolve the populations (e.g., CD45 can be added in an heterogeneous tissue to pull out leukocytes)
5. Start your Ab concentration at 10 µg/mL and do 8 serial 2-fold dilutions

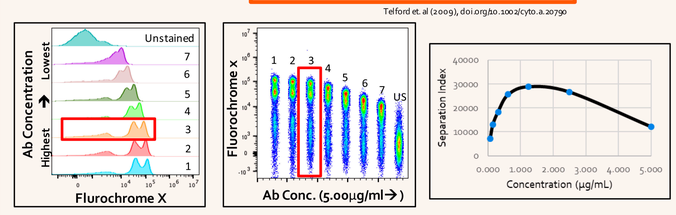


Analysis Tips

1. Record at least 1,000 live cells expressing your marker of interest
2. Gating strategy: Live cells ➔ FSC/SSC ➔ Singlets ➔ Positive and Negative populations
3. Positive and Negative **gates need to be adjusted** for each sample since the populations will shift
   Note: population percentages should be approximately the same.
4. Visual analysis of your data alone should never be used to determine the optimal concentration
5. Calculate the **Separation Index (SI)** for each condition using the MFI of the negative and positive populations
   Choose optimal antibody concentration where the SI is maximized. This can be adjusted to a lower concentration to allow for reduced spread in your multicolor experiments).

$$Separation\ Index = \frac{MedianPositive - MedianNegative}{(84\%Negative - MedianNegative)/0.995}$$

Telford et. al (2009), doi.org/10.1002/cyto.a.20790



Doublets

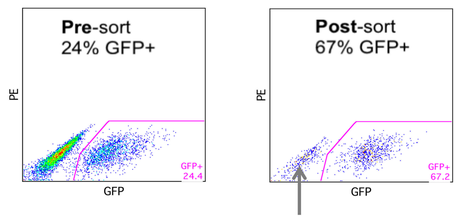
What is a clumpy sample?
Contains aggregates of cells

Why is it a problem?
1 - Clumps clog instrument
2 - Sorting doublets causes poor sort purity
  * Singlet gatting does not exclude all doublets
3 - Analyzing doublets gives false double positives

How-to prevent :
1 - Filter samples to remove clumps
  * filter as the last step of preparation
  * filters do not remove doublets!
2 - Adjust sample preparation
  * Add EDTA (1-2mM) to remove Ca++ and Mg++
  * Add DNaseII (10 U/ml)
  * Add Accumax
  * keep samples on ice to prevent aggregation

Common mistakes :
Make sure cells are happy in their media
  * PBS without protein can damage cells

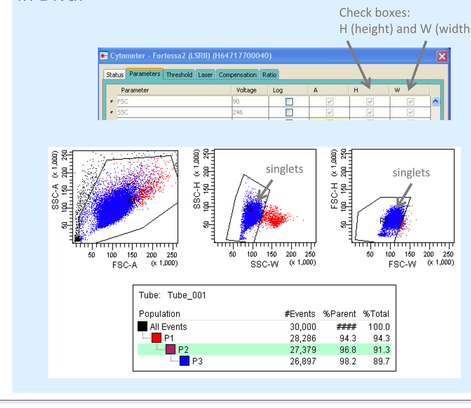
Use a microcope during sample preparation to track stats of cells



GFP-:GFP+ doublets are sorted and fall apart

Problem	Consequence	Prevention
Clumpy sample	Poor sort purity; false double positives	Adjust sample preparation

In Diva:

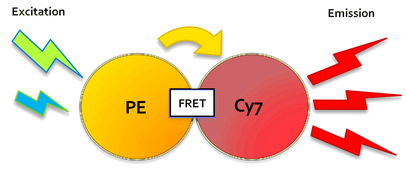


Tandem Dyes

When conjugating two dyes where one fluorochrome's emission spectra (donor) overlaps the excitation spectra of a second fluorochrome (acceptor) a phenomenon called **Förster Resonance Energy Transfer (FRET)** occurs, creating a **new dye** with the excitation maximum of the donor and the emission maxima of the acceptor.
The resulting dye is called a **Tandem dye.**

Naturally occurring stokes shifts are generally small, meaning that the emission maximum wavelength is typically close to the excitation maximum. The use of tandem dyes allows for an increase in the stokes shift, and the simultaneous use of several of these dyes excited by the same laser but with increasingly larger stokes shifts can expand the capabilities of multicolor flow cytometry,.

The donor fluorochrome is excited by laser light and gives off light at a longer wavelength. This emitted light from the donor then excites the acceptor fluorochrome, which then emits the light at an even longer wavelength.



PE-Cy7 (Ex_{max}566 nm/Em_{max}785 nm)

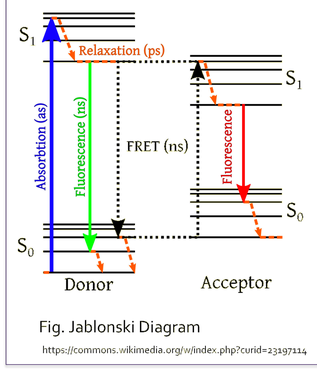


Fig. Jablonski Diagram
https://commons.wikimedia.org/w/index.php?curid=23197114

Helpful Hints

- Antibodies conjugated to tandems and any subsequent samples stained with these antibodies should be **protected from light and kept at 4C** to prevent degradation.
- Due to lot-to-lot variation, the **same antibody/tandem dye conjugate should be used** for your **controls** and **experimental samples**. In fact, considering the sensitivity of tandem dyes to light and temperature, the **same vial** should be used.
- For fix/perm, **confirm stability of the tandem dye** under experimental conditions
- **Note:** many of the brilliant & super bright fluorochromes are tandems and should be treated as such (eg. Brilliant Violet 605, Super Bright 702, etc.).

Reference:

Flow Post-its Memorial Sloan Kettering Cancer Center

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