THE UNIVERSITY OF CHICAGO BIOLOGICAL SCIENCES

Flow Basics 2.1: The Basic Staining Protocol and How to Improve Reproducibility

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The Flow Basics 2.0 Series

Understanding Flow Cytometry Experiments to Get Better Results

- For all scientific experiments the best data is achieved by optimization and consistency!

- This course will go over:
  - A basic staining protocol for surface markers
  - How to improve consistency in sample staining

Staining Protocol

Staining Tubes and Buffers

Staining tube:

- Eppendorf, 5 mL FACS tube, or 96-well round bottom plate
- Choice depends on preference. Main difference is wash volume:

1 mL

1-3 mL

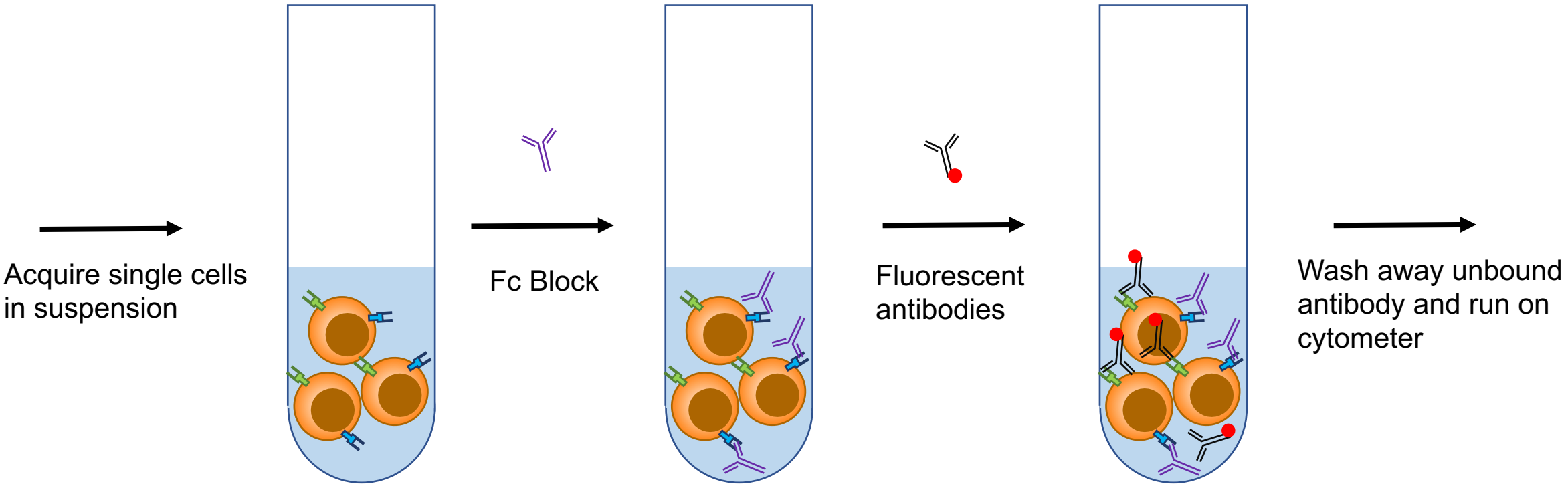
Falcon #352008

0.2 mL

Buffer

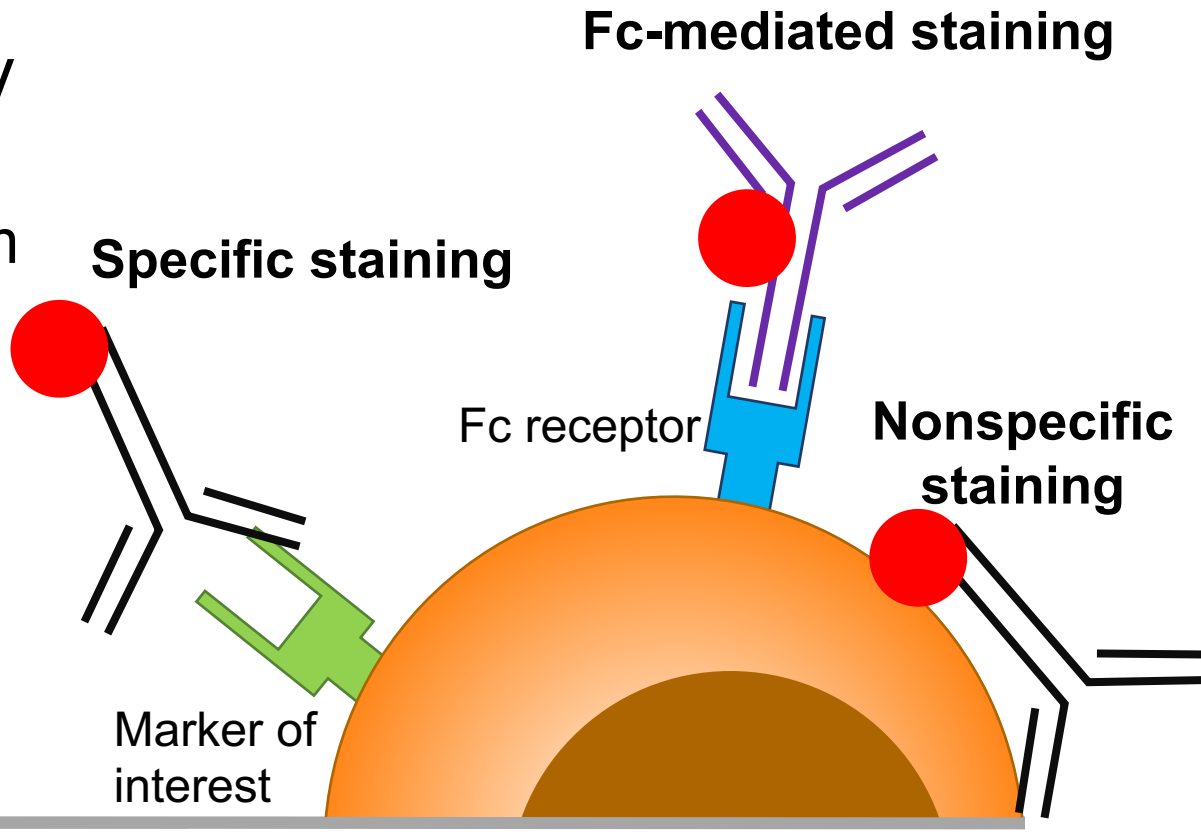
- Choice depends on your cell type
- FACS buffer options:
  - 1X PBS (Ca/Mg2+ free, no phenol red!)
  - Either FBS (1-10%) or BSA (0.1-1%)
  - Commonly used: 1% FBS in 1X PBS (consider filtering FBS to remove debris, especially for cell sorting)
  - Other additives: EDTA (0.5-5mM) if you have very sticky cells, sodium azide

Basic staining protocol

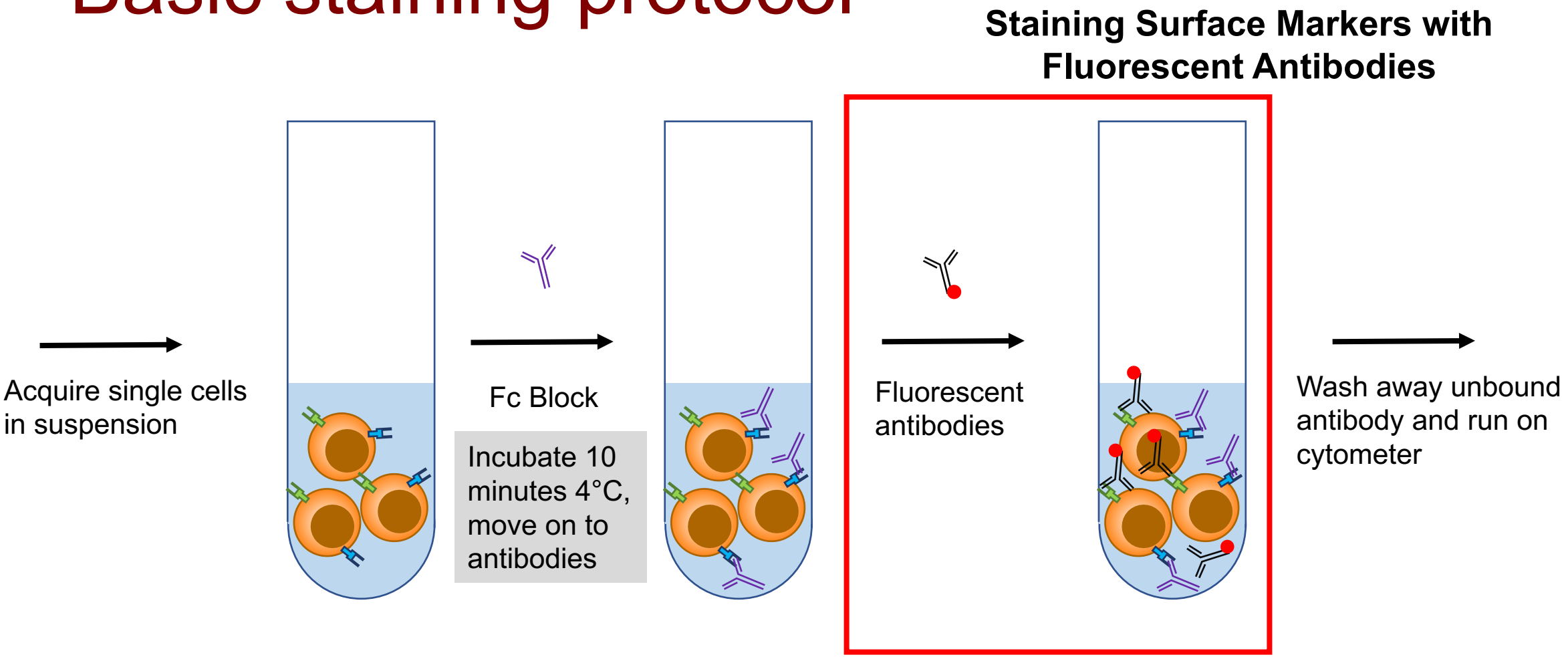


Blocking to reduce nonspecific and Fc-mediated staining

- Immune cells have Fc receptors that bind IgG antibodies. Most antibodies used for flow cytometry are IgG
  - Fc Block inhibits IgG antibodies from binding to Fc receptors
    - In mice, Fc block is anti-CD16/CD32
  - Serum could also be used
    - E.g. Rabbit serum for rabbit antibodies, goat serum for goat antibodies
    - Also BSA/FBS in staining buffer helps



Basic staining protocol



Antibody Staining is Affected by Five Factors

1. Time
2. Temperature
3. Volume
4. Cell number
5. Antibody amount

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Time and temperature

- Typically 30 minutes at 4°C
- Time is often between 15-60 minutes, sometimes overnight
- Temperature is often 4°C or room temp
- Some antibodies/dyes require something else – read manufacturer protocols!

Pick a time and temperature and don't vary the protocol between experiments!

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Staining Volume

- Typically 100 µL
- Some antibodies/dyes require a different volume
- Flow Basics 2.2 will discuss when changing the staining volume should be considered

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Cell Number and Antibody Amount

- Typical starting point is $1x10^6$ cells and 0.1 µg of antibody (usually 0.5-1 µL)
- Will discuss further in Flow Basics 2.2

Pipetting – another source of variability

- Avoid pipetting small volumes of antibody
  - If you must pipette less than 1-2 µL, make a 1:10 dilution (pipette 5 µL instead of 0.5 µL)
  - Make a master mix of your antibody cocktail to ensure all of your samples are stained with the same amount of antibody

- Simplify your pipetting
  - You can add Fc block to your cells in 50 µL
  - Add FACS buffer to master mix to bring up to 50 µL
  - To stain, add 50 µL cells and 50 µL master mix for a total staining volume of 100 µL

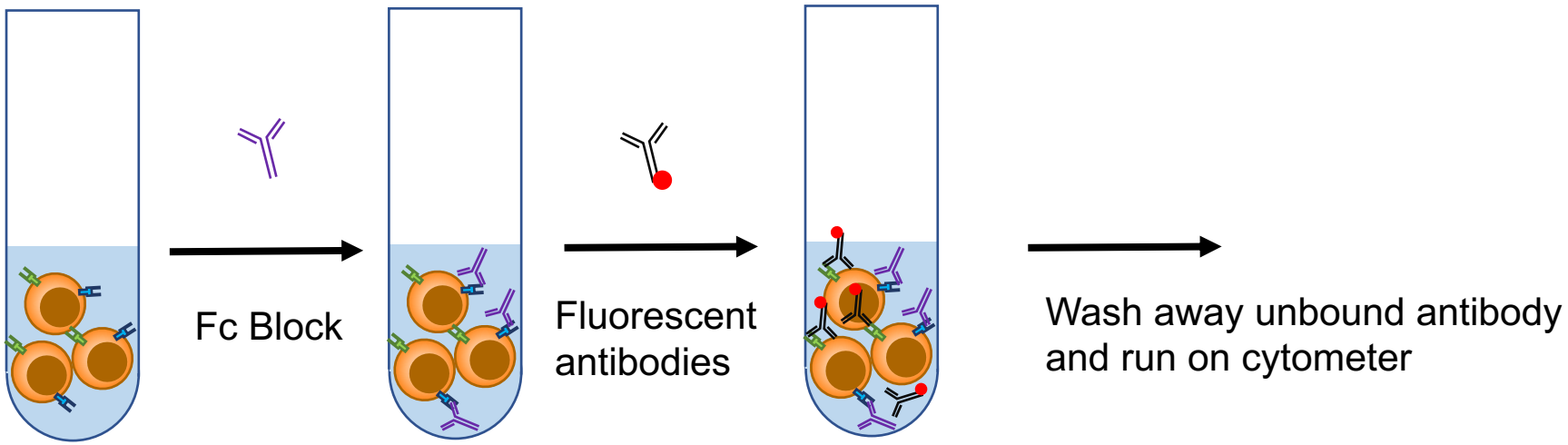
	1X	10X
FITC	1	10
APC	5	50
PE	0.5	5
FACS Buffer	43.5	435
TOTAL	50	500

Always protect fluorophores from light!

- Once fluorescent antibodies have been added to the cells, be sure to keep the cells in the dark as much as possible
- Most common: Cover tubes with foil and incubate in a dark place (fridge for 4°C or a drawer or cabinet for room temperature)

- Note: cells with fluorescent protein(s) should always be protected from light

Basic staining protocol



Wash and centrifuge

- Volume depends on tube – 1 mL, 1-3 mL, or 0.2 mL
- Wash buffer with protein (BSA/FBS) helps prevent cell loss compared to PBS without protein
- Aspirate with dumping or pipetting, careful with vacuum

Resuspending Cells to Run on a Cytometer

- If you are new to flow cytometry, it is safer to resuspend your cells (especially controls) in a larger volume. This will allow you to properly set the instrument settings without running out of sample
  - As you become more comfortable with the cytometer, you can use more concentrated controls so that they run faster on the instrument
- The volume to resuspend cells in depends on the instrument
  - Fortessas
    - 150 µL **absolute minimum**, 300-500 µL minimum for new users, 150-300 µL for advanced users
    - In general, run samples at less than 35,000 events/sec
  - Attune: see chart here
  - Sorters
    - 500 µL **absolute minimum**
    - 70um tip (lymphocytes, splenocytes, small cells): 20-25 million cells per mL
    - 100um tip (most cell culture, lung and liver cells, etc): 10-15million cells per mL

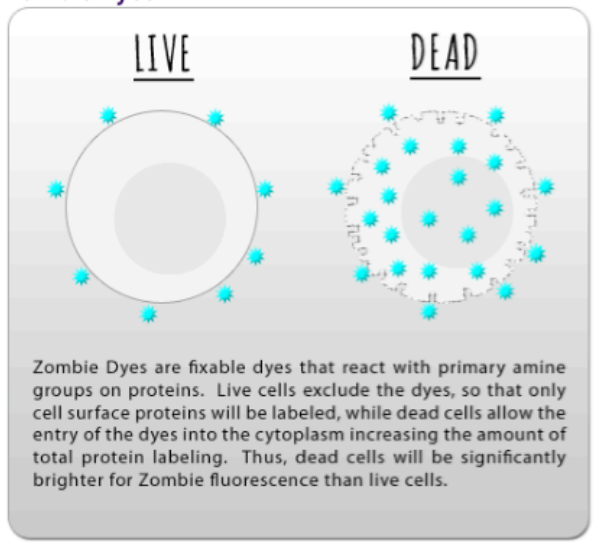
Basic Staining Protocol: Additional Notes

- Use a viability stain to remove dead cells from analysis
- Using primary antibodies vs primary and secondary antibodies?
- Should a fixative be used?

Viability Dyes

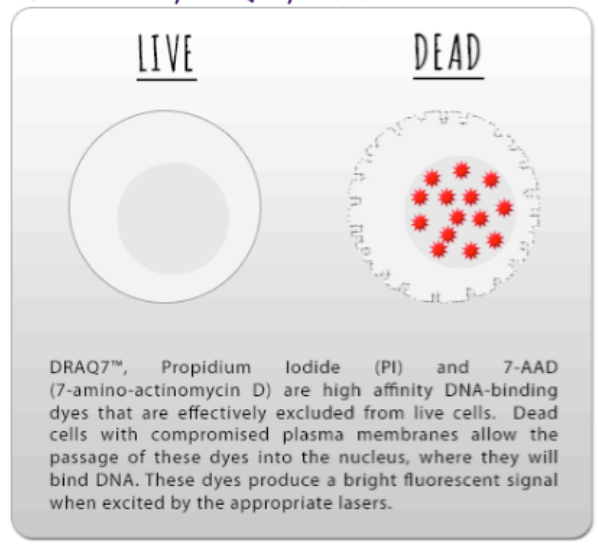
- Dead cells will:
  - Bind some antibodies non-specifically
  - Skew statistics
- DNA-binding (DAPI, PI, etc.)
  - Non-fixable
  - Stain ***after antibodies***, right before acquisition
- Amine-binding
  - Fixable (Biolegend Zombie dyes or ThermoFisher Fixable Live/Dead dyes)
  - Stain ***before antibodies*** and **before** fixation (fixation not required)
  - Staining buffer must be free of protein – read the manufacturers protocol!!

Zombie Dyes



*DRAQ7™ is a trademark of Biostatus Limited.

Helix NP™ NIR, DRAQ7™, PI and 7-AAD



Product Links: Helix NP™ NIR, DRAQ7™, PI, 7-AAD

More info: https://www.biolegend.com/en-us/live-dead

Using Primary and Secondary Antibodies

- Flow cytometrists prefer to use fluorophore-conjugated primary antibodies as opposed to unconjugated primary antibodies and fluorophore-conjugated secondary antibodies
  - Using a primary antibody instead of a primary and secondary:
    - Only requires one incubation time and wash steps instead of two
    - Requires less optimization (one antibody instead of two)
    - Makes the staining protocol easier
    - Avoids chances of nonspecific/background staining from the secondary antibody

- You are welcome to use primary and secondary antibodies if you want – just titrate both antibodies!

Fixatives

- Fresh cells (BSL1) can be run on all of our instruments
- Cells with infectious particles (BSL2 and above) are required to be fixed prior to running on the benchtop analyzers

- There are many options for fixatives: 1-4% paraformaldehyde, methanol, etc.

- Concerns with fixation:
  - Fixing cells before antibody staining may alter the cell's epitope, leading to altered or no staining
  - Fixing cells after antibody staining may decrease the signal intensity of some fluorophores
  - Fixing cells could increase cell autofluorescence, which may decrease resolution of markers

Resources for Fixation

- https://www.biolegend.com/en-us/blog/fix-now-fix-later-considerations-for-the-use-of-paraformaldehyde-fixation-in-flow-cytometry
- https://bitesizebio.com/22141/fixation-and-flow-cytometry/

Stay Tuned for the Rest of the Flow Basics 2.0 Series