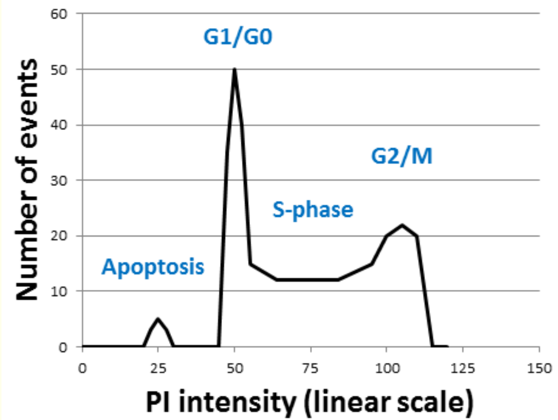
Detmold Family Cytometry Facility

Cell cycle analysis

How it works?

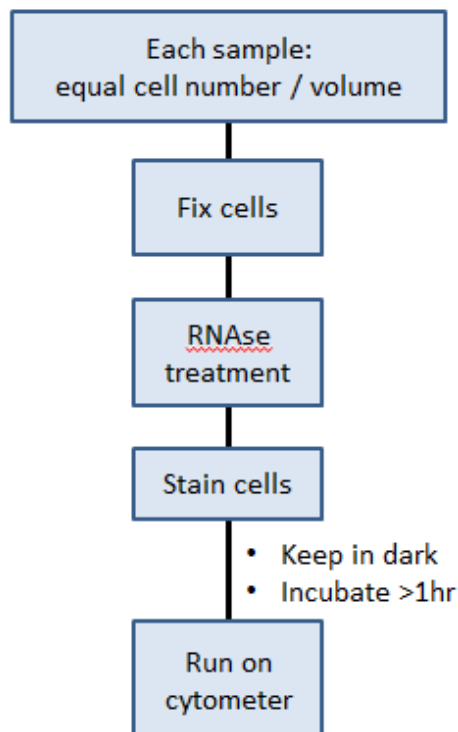
Propidium iodide (PI), DAPI and 7-AAD are dyes that can be used to quantify cellular DNA content. By measuring a cell's DNA content, you can determine its cell cycle status and viability. These dyes bind directly to DNA, but are impermeable to the cell (fixation required).

- A cell in G2/M will have double the DNA content and twice the PI signal of a cell in G1/G0.
- A cell in S-phase has more DNA than G1/G0 cells, but less than G2/M cells.
- An apoptotic cell will have less DNA content less than a cell in G1, as a result of apoptotic DNA fragmentation.



Flow cytometry is used to measure these dyes, allowing for high accuracy and throughput.

Flow chart



Next page, detailed protocol, then gotchas

Preferred cell cycle protocol (adapted from Joey Puccini)

<table>
<tr><td>Harvest</td></tr>
<tr><td>• Harvest cells in PBS

/!\ Must have cells in single suspension before fixation. Cells will not separate once fixed. Cell aggregates will critically affect flow acquisition and analysis.</td></tr>
<tr><td>Fix – can replace with a fixation method optimised for your cell line.</td></tr>
<tr><td>• Resuspend cells in 1.2 mL ice cold PBS. Add ethanol (100%, 2.8 mL, -20°C), dropwise to the side of the tube whilst gently vortexing

• Store cells at -20°C overnight (can be stored for up to 2 weeks).

• Centrifuge cells at 1200 rpm for 5 minutes and discard supernatant (cells will form a loose, white pellet).

• Rehydrate pellet by adding 2 mL of PBS and disperse by vortexing at a high speed. Centrifuge cells at 1200 rpm for 5 minutes, decant supernatant. Repeat.

/!\ Do not resuspend cell pellet by pipetting. Cells are very sticky following ethanol fixation and will stick to inside of pipette tip.

• Resuspend cell pellet in 0.25% Triton X-100/PBS with gentle pipetting to avoiding making bubbles. Centrifuge cells at 1200 rpm for 5 minutes, decant supernatant.</td></tr>
<tr><td>Stain / RNase treatment – can swap out PI for alternate dyes</td></tr>
<tr><td>• Resuspend cells in 300-600 μL of staining solution (made in PBS) in FACS tubes:
- propidium iodide (25 μg/mL)
- RNase A (40 μg/mL)

/!\ RNase A must be DNase-free to prevent DNA degradation. Denature DNases by heating RNase A stock solution (10mg/mL in dH_2O) at 95°C for 15 minutes. Aliquot and store at -20°C (DO NOT STORE at 4°C).

• Incubate at room temperature in dark for at least 2 hours. Can store for several days at 4°C in dark before analysis.</td></tr>
<tr><td>Run on cytometer</td></tr>
<tr><td>- set flow stream to low to make sure only one cells passes through laser at a time (high flow streams will give broader peaks and unreliable statistics)
- to achieve optimal resolution of peaks adjust voltage so that the mean fluorescence of G0/G1 peak is approximately 50,000 (Fortessa)
- fluorescence of G2/M population (4n DNA content) should be approximately double that of G0/G1 population (2n DNA content)
- exclude doublets/debris based on non-linearity with a doublet discriminator plot (FSC-H vs. FSC-A)
- acquire at least 50,000 events (100,000 events is optimal for statistical analysis)</td></tr>
</table>

Gotchas /!\

There are a number of critical steps for acquiring usable cell cycle data by flow cytometry

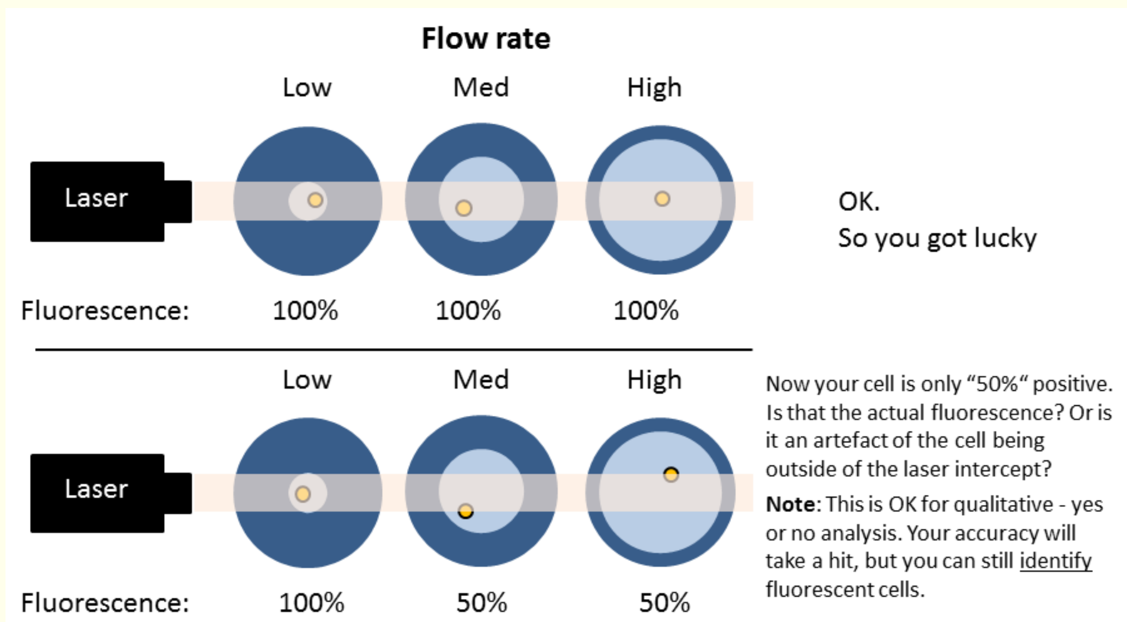
- Cells MUST be in a single cell suspension
    - **Action required**: Fixation method needs to be optimised.
    - **Consequence**: Two G1 cells stuck together will look like a cell in G2 phase
- Cellular RNA interferes with this assay
    - **Action required**: Treat cells with RNAse prior to staining
    - **Consequence**: DNA-dyes can also bind to RNA. Your DNA-staining profile will just be a random smear if you do not treat cells with RNAse.
- Use the slowest flow rate on the cytometer
    - See below
- Cell staining Staining per cell number critical
    - **Action required**: Add the same PI-weight per cell to each sample. Sample A: $2x10^6$ cells, add 25µg PI. Sample B $5x10^6$ cells, add 62.5µg PI.
    - **Consequence**: Samples will have variable staining profiles (left/right histogram shift), which makes data analysis difficult and or less accurate
- You need 100,000 (single cell) events or more for a high-quality analysis
- Make sure you use linear scale for fluorescence acquisition
- Fixed cells have different FSC/SSC profiles – make a layout from scratch on the acquisition software

Using the lowest flow rate will take longer :(

It does take longer, but it is critical when acquiring **quantitative** flow data.

Increasing the flow rate, increases the width of the stream (light blue).

This can cause **suboptimal fluorescence** (below), or **increase doublets** in your data (allows cells to fit side-by-side).



So your data will contain values equal to, or less than a cells actual fluorescence. The histogram will have broad peaks and you will not be able to accurately determine the cell cycle phase-boundaries.