Cell Proliferation by flow cytometry

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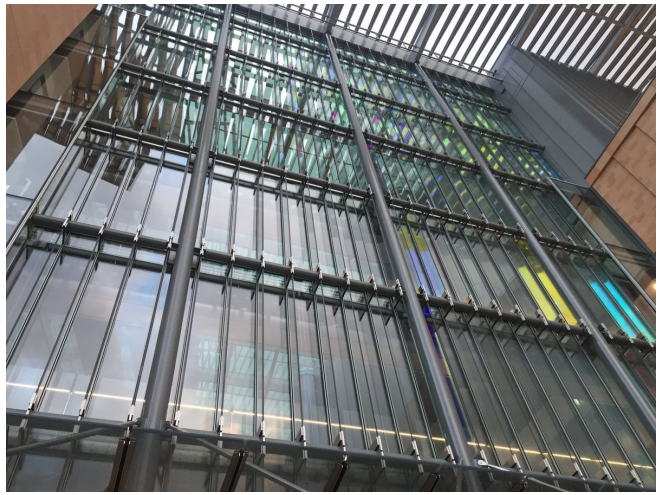
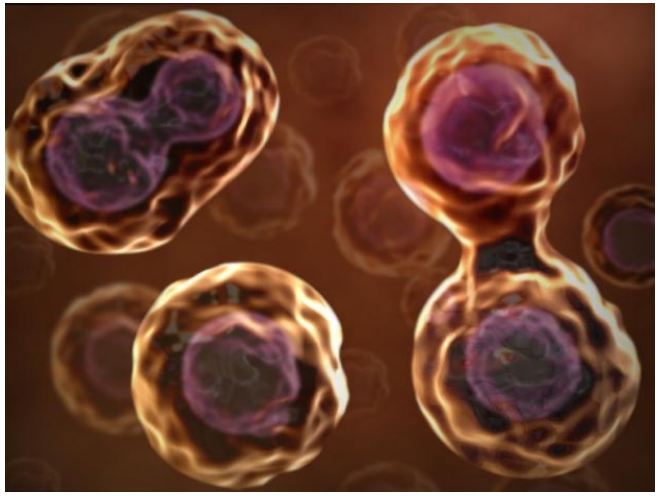
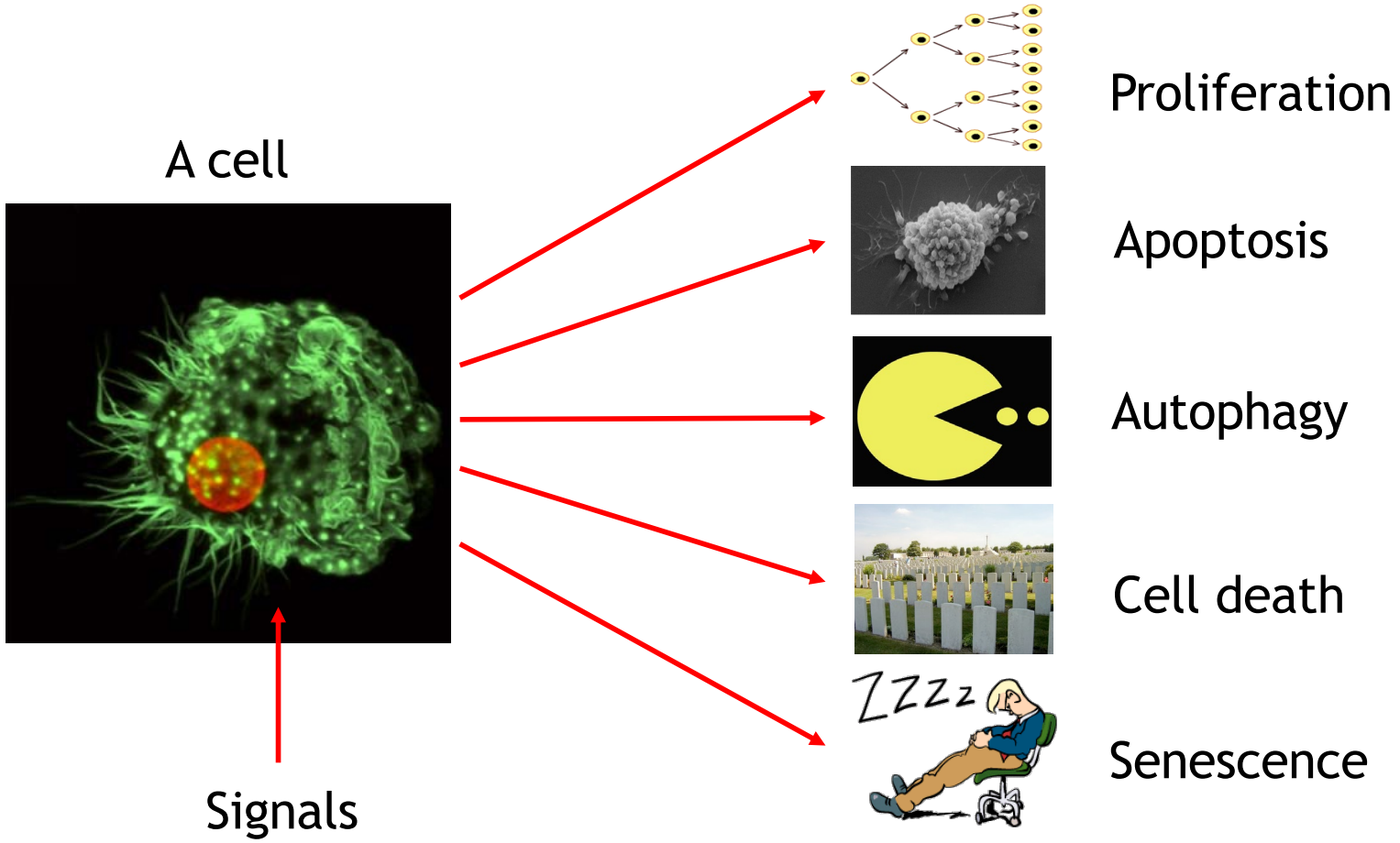
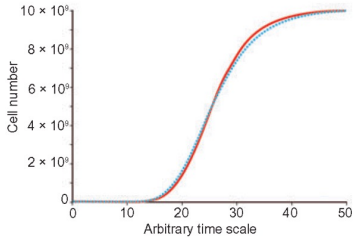
The Francis Crick Institute






A cell
Signals
Proliferation
Apoptosis
Autophagy
Cell death
Senescence
ZZZz

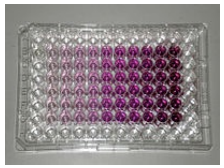
Methods for Measuring Proliferation



Count cells: Know what you put in, count what you get out.

^{3}H- *TdR*

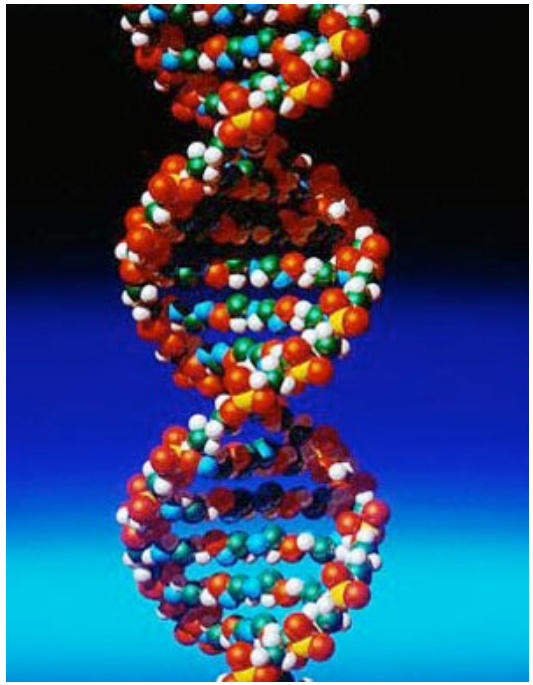
Use radioactive Thymidine incorporation

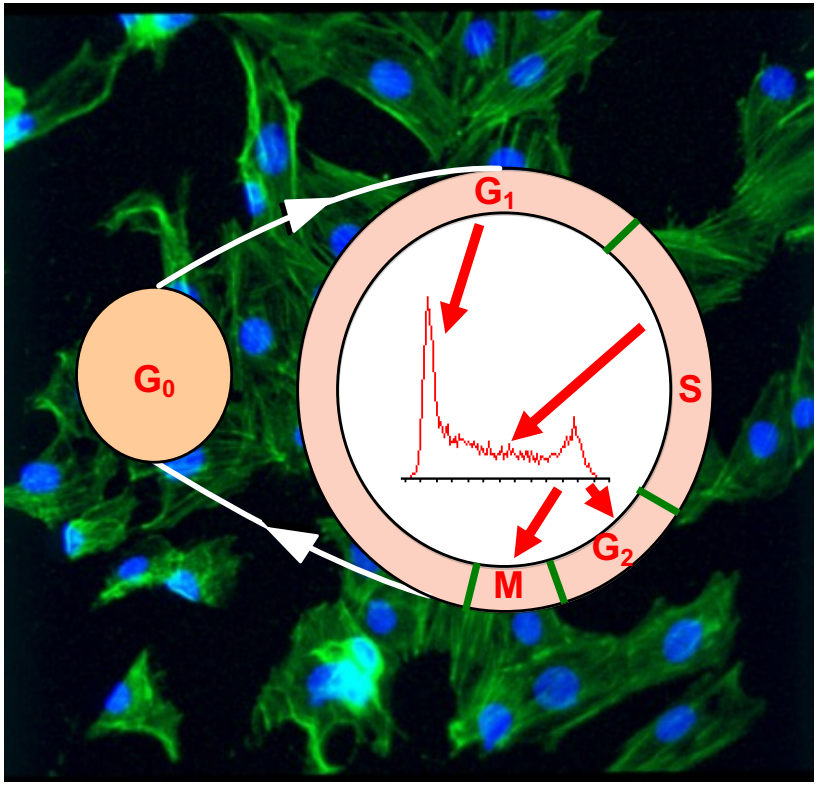


Use colorimetric assays such as MTT

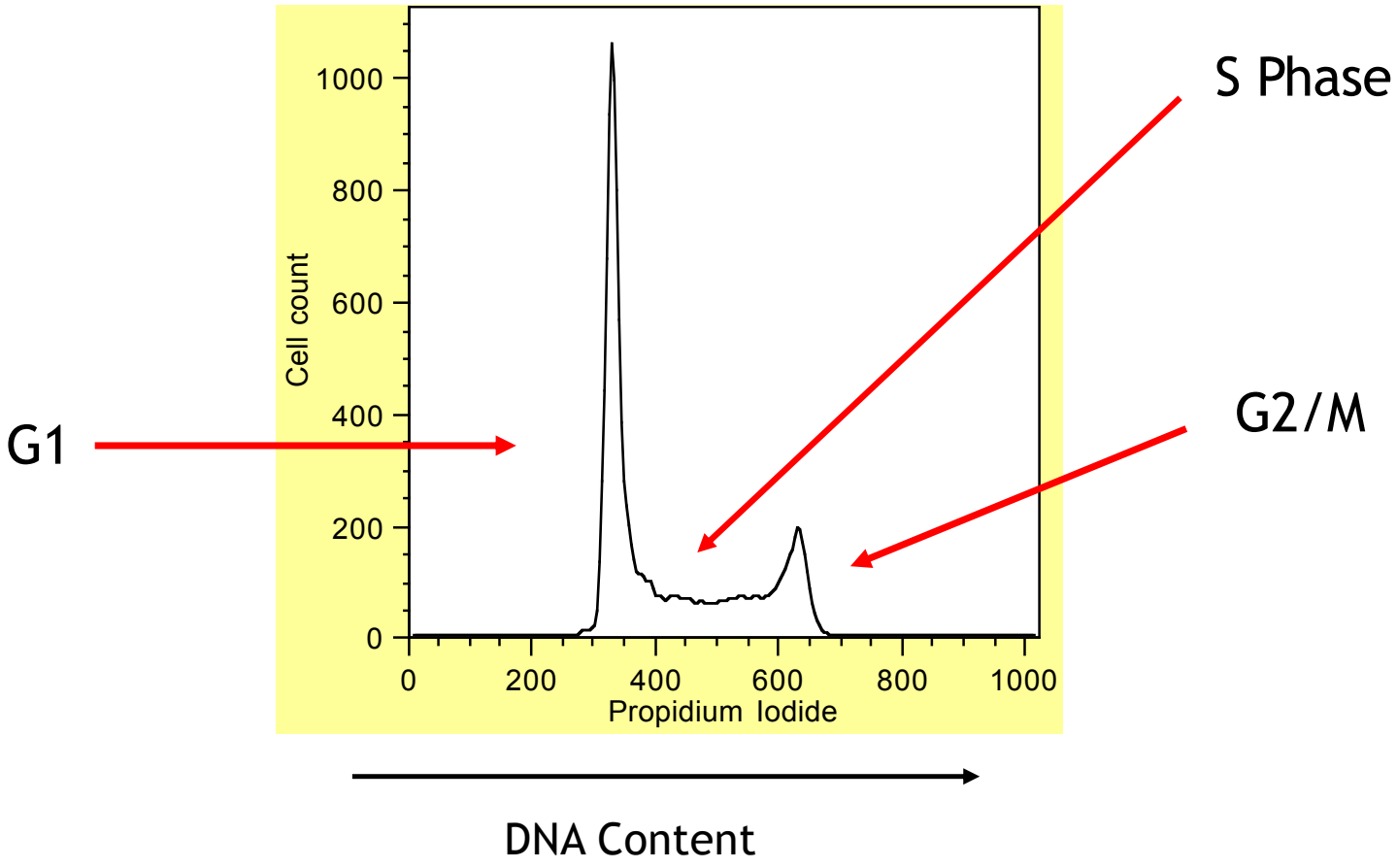
Multi-parameter, single cell analysis
Rapid sample acquisition
Provides fluorescence information on relative scale
Large, statistically robust data sets
Well established protocols

DNA analysis





DNA stained with propidium iodide

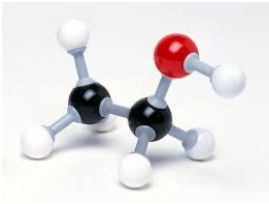


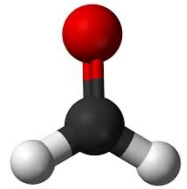
Cell cycle analysis - Bromodeoxyuridine (BrdU) method

- Thymidine analog
- Taken up by cycling cells
- Use for comparative growth rates, pulse labelling, continuous labelling
- Staining procedure involves unwinding DNA
- Combine with Propidium iodide

Cell cycle analysis - Bromodeoxyuridine (BrdU) method

Fixation





Alcohol (70% ethanol or 100% methanol)

Aldehyde (1-4% formaldehyde)

DNA unwinding

Acid (2N HCl)

Alkali (Sodium Borate)

Heat denaturation

Enzyme (DNase)

Cell cycle analysis - Bromodeoxyuridine (BrdU) method

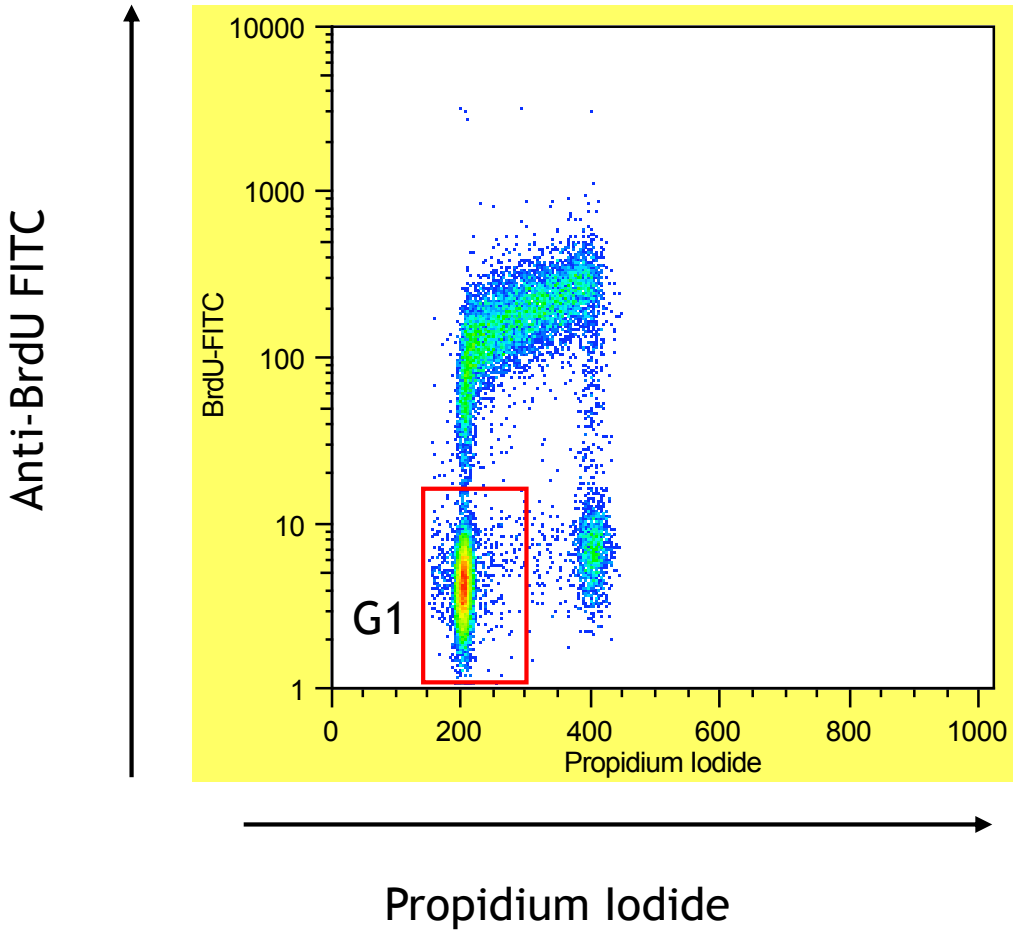
Acid technique

Fix in ethanol
2N HCl 20’ RT
Wash x3 (CRITICAL STEP)
Primary Antibody
Wash
Secondary Antibody
RNAse/PI

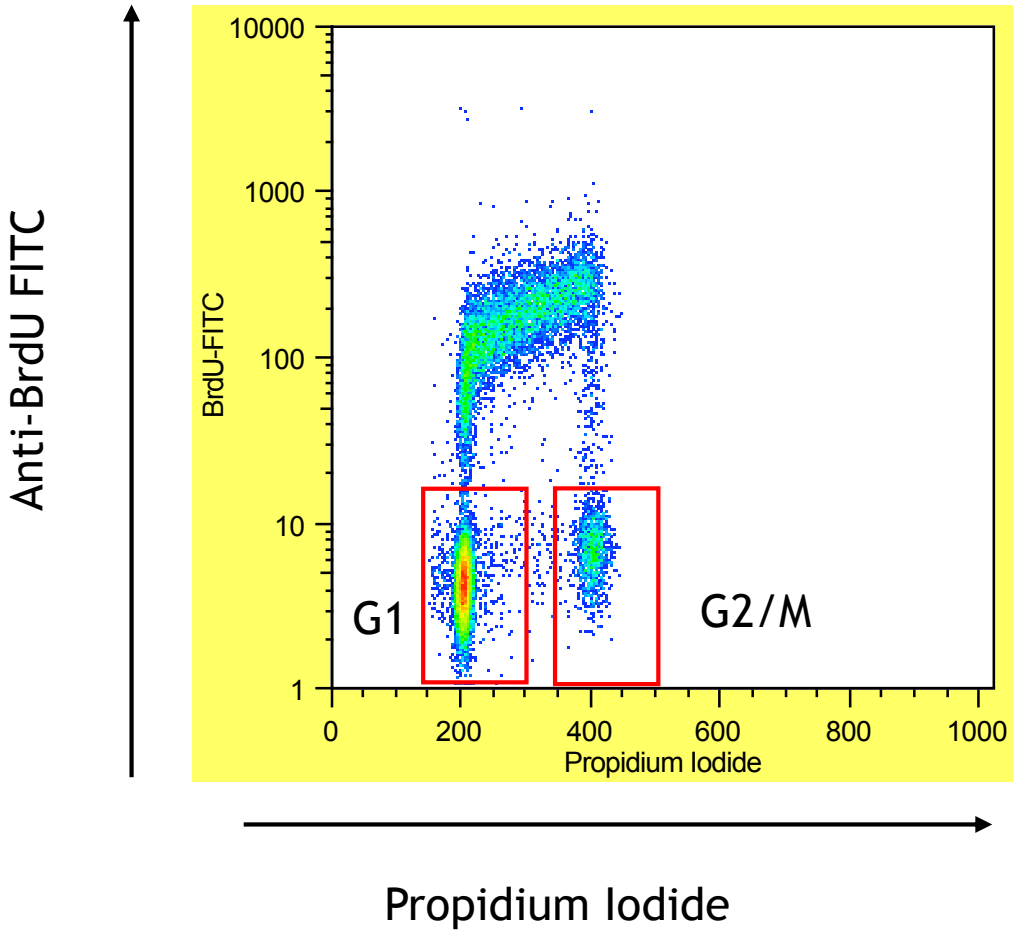
Typical DNAse technique

Fix in formaldehyde
DNAse 60’ 37ºC
Wash
Primary Antibody
Wash
Secondary Antibody
RNAse/PI

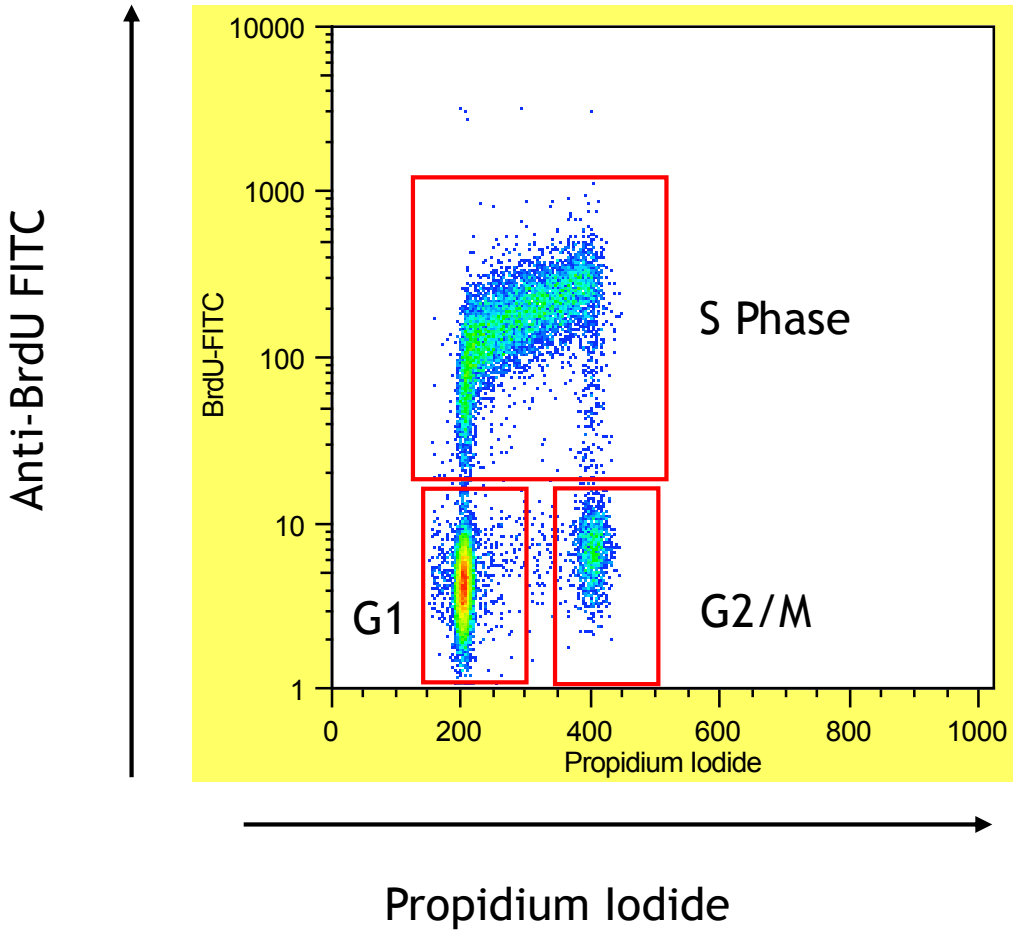
Typical dual parameter plot



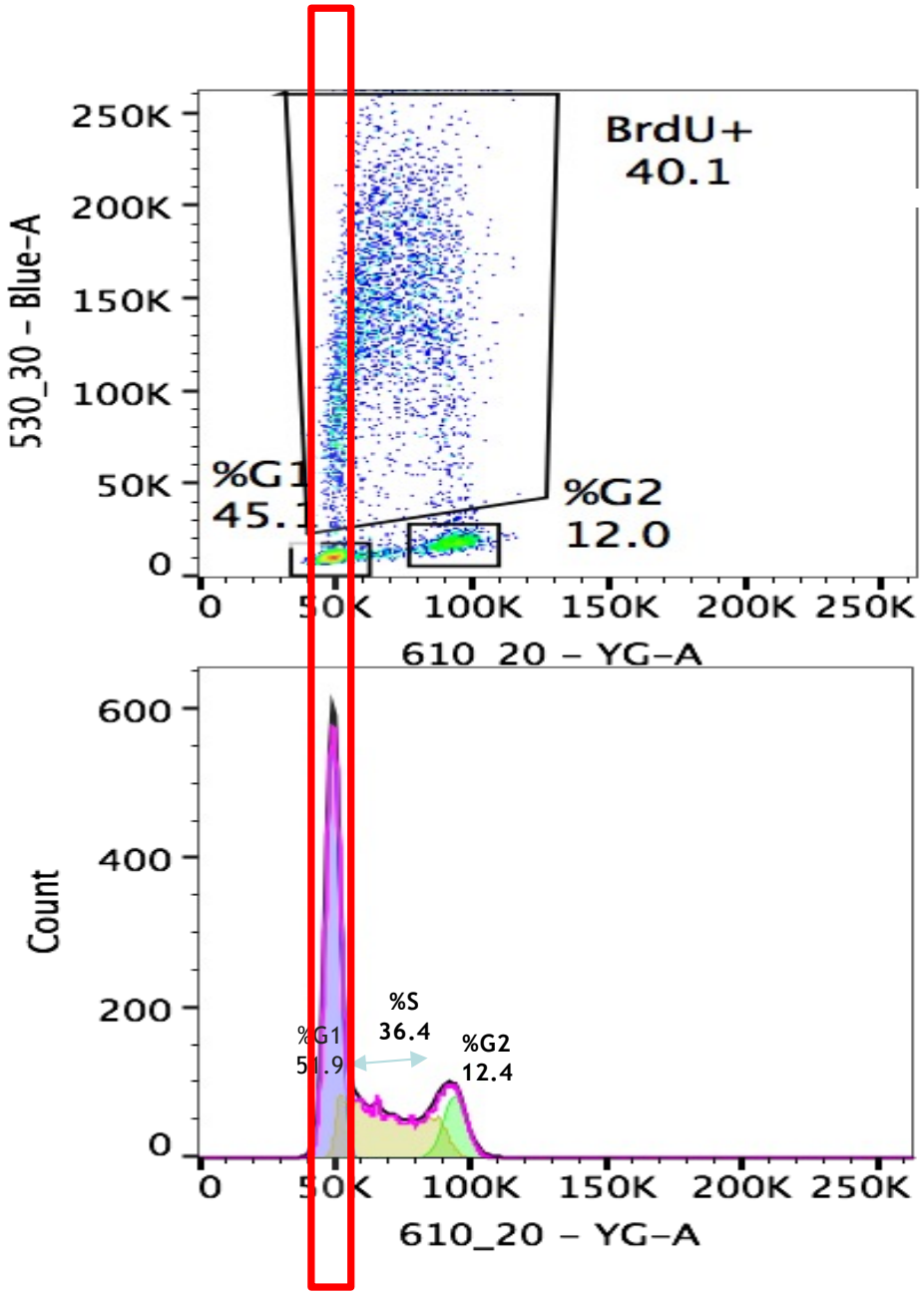
Typical dual parameter plot



Typical dual parameter plot



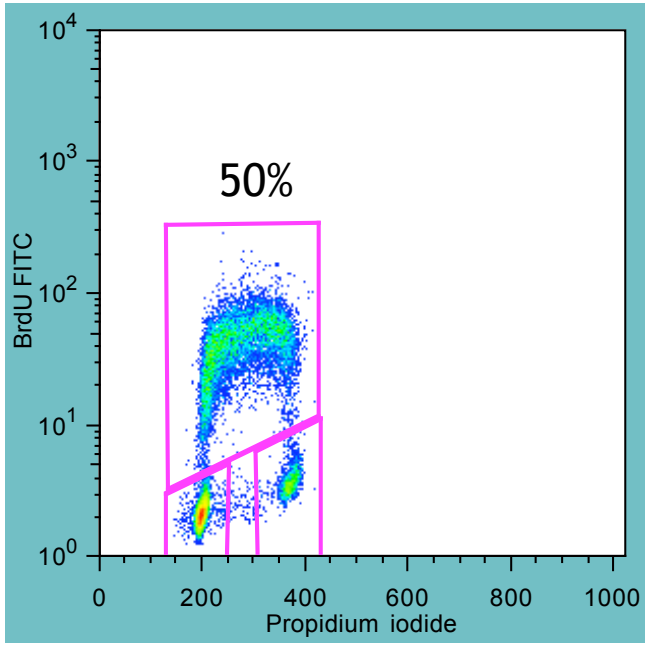
Typical dual parameter plot



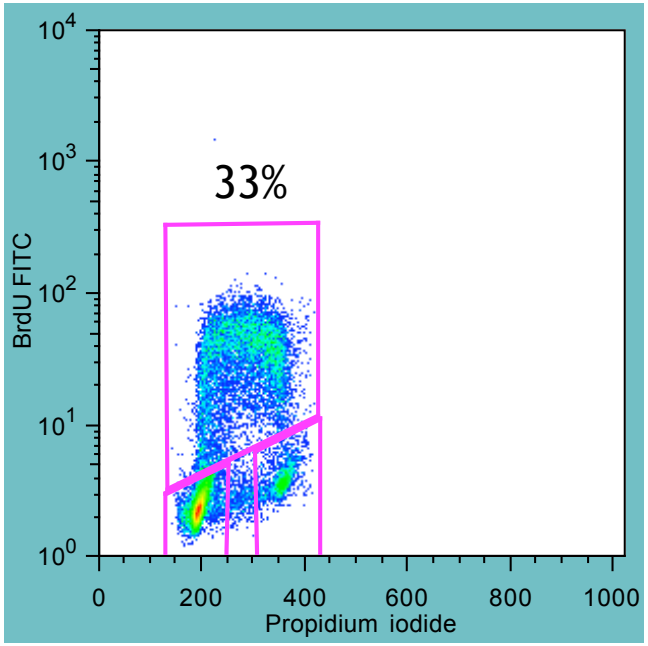
Comparative growth rates

MCF10A Breast epithelial cell line

Control



Drug-treated



Pulse labelling with BrdU - assessing cell cycle phase duration

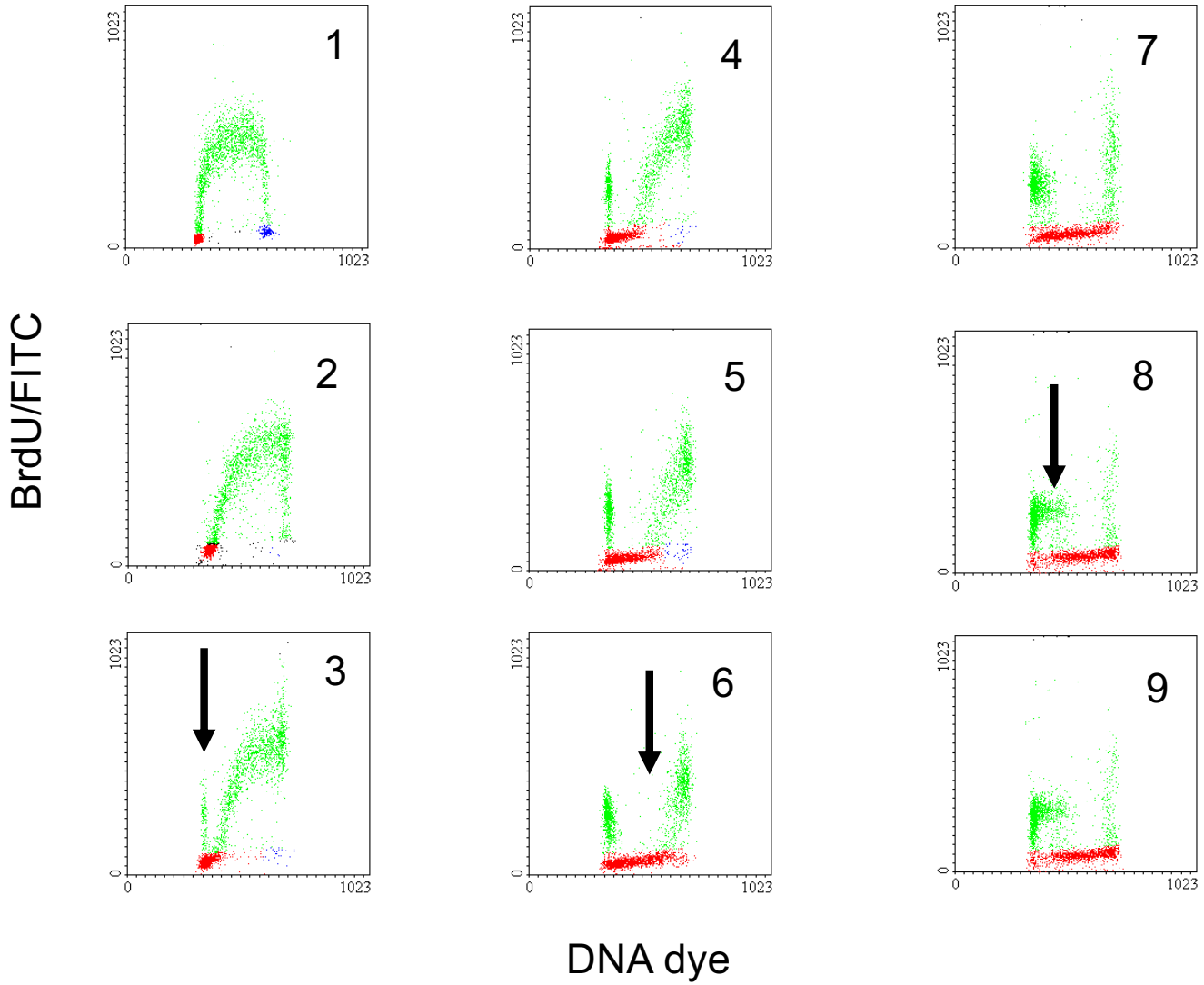
Rationale: Very short pulse (2-30 mins) to label S phase cells

Harvest cells at time points post-labelling

Stain for BrdU incorporation

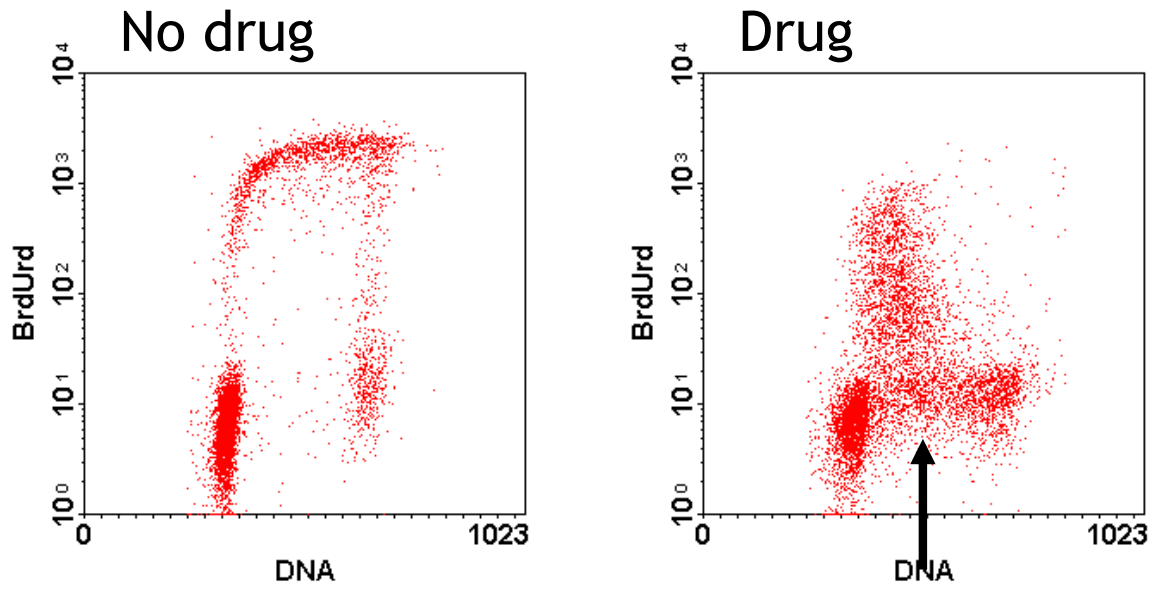
Follow labelled cohort through cell cycle

Pulse labelling with BrdU - assessing cell cycle phase duration

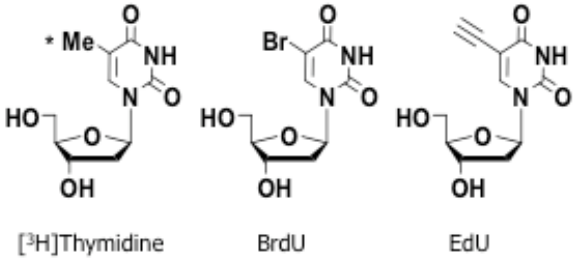


Drug effects on cell cycle: pulse label after treatment

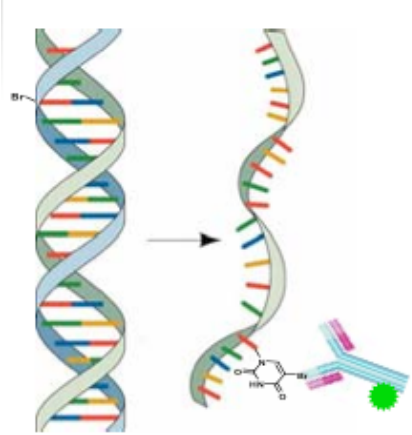
Incubated for 2h with cisplatin 24h earlier



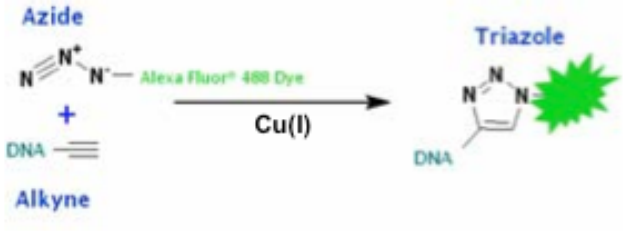
Detection of proliferation - EdU



EdU = 5-ethynyl-2'-deoxyuridine

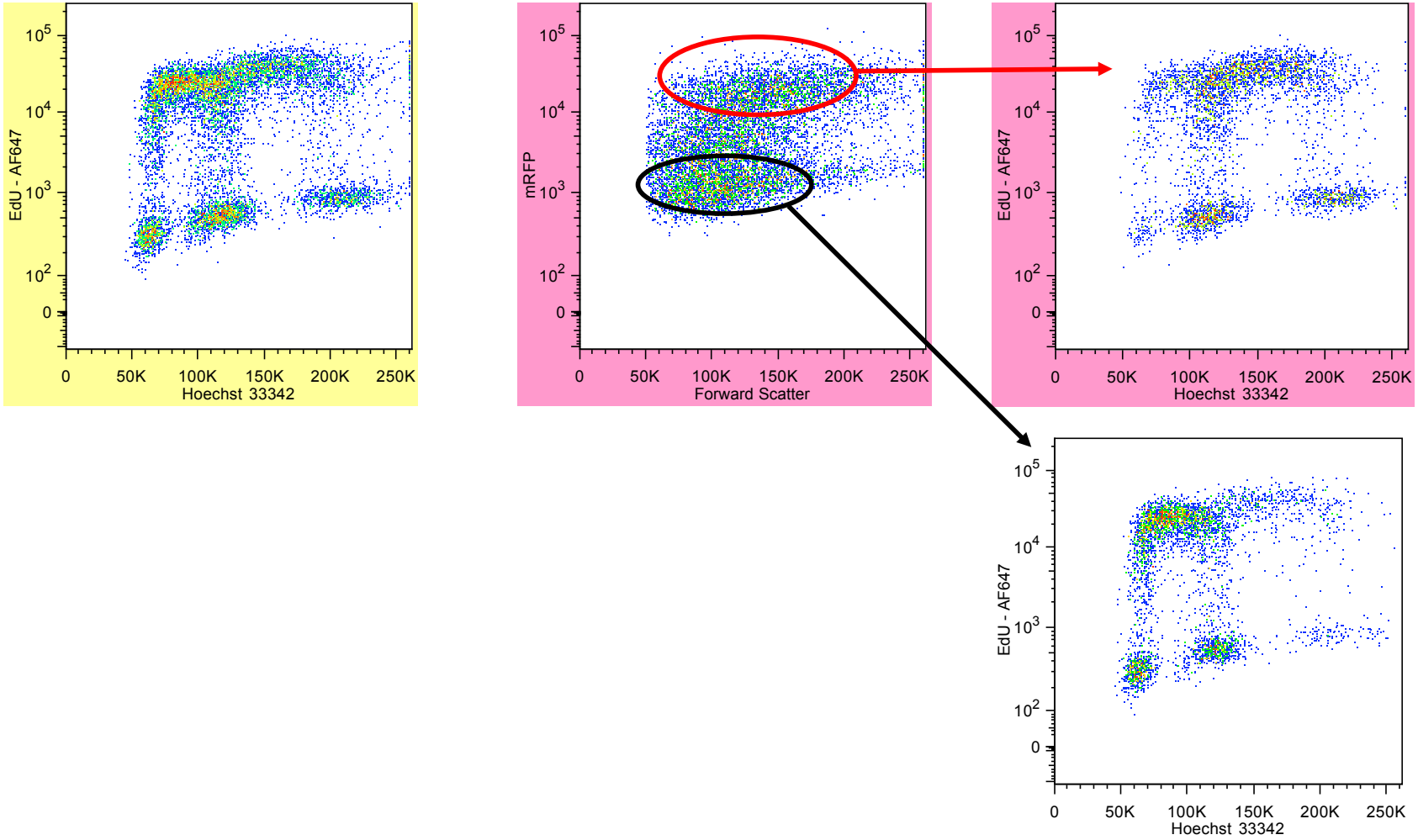


BrdU detection needs unwinding of DNA. Can be incompatible with surface staining or Fluorescent Protein detection.

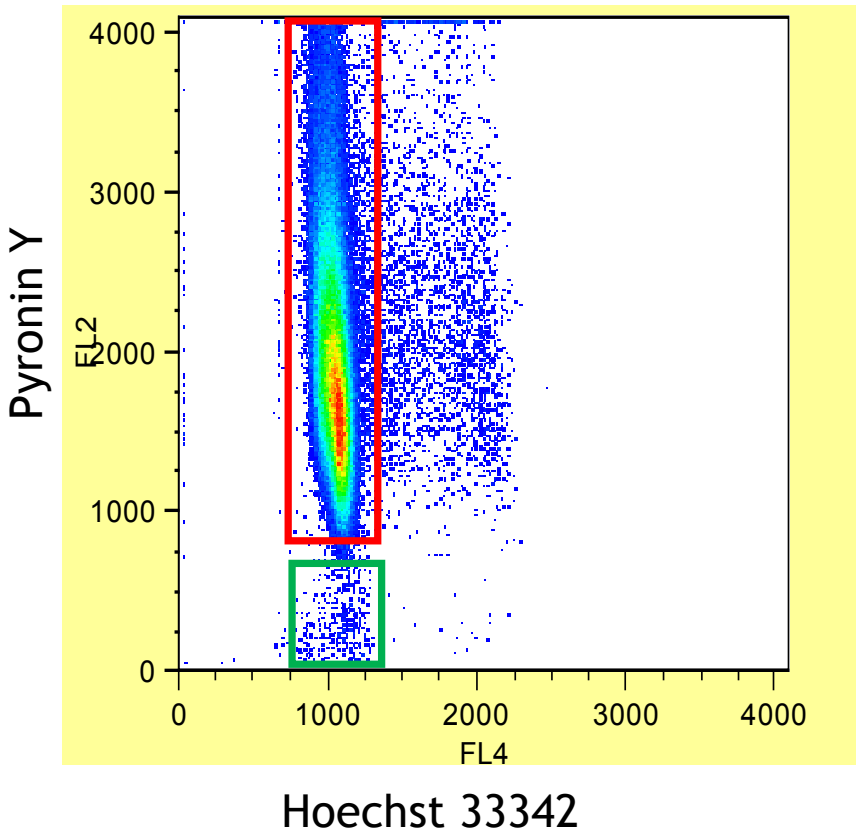


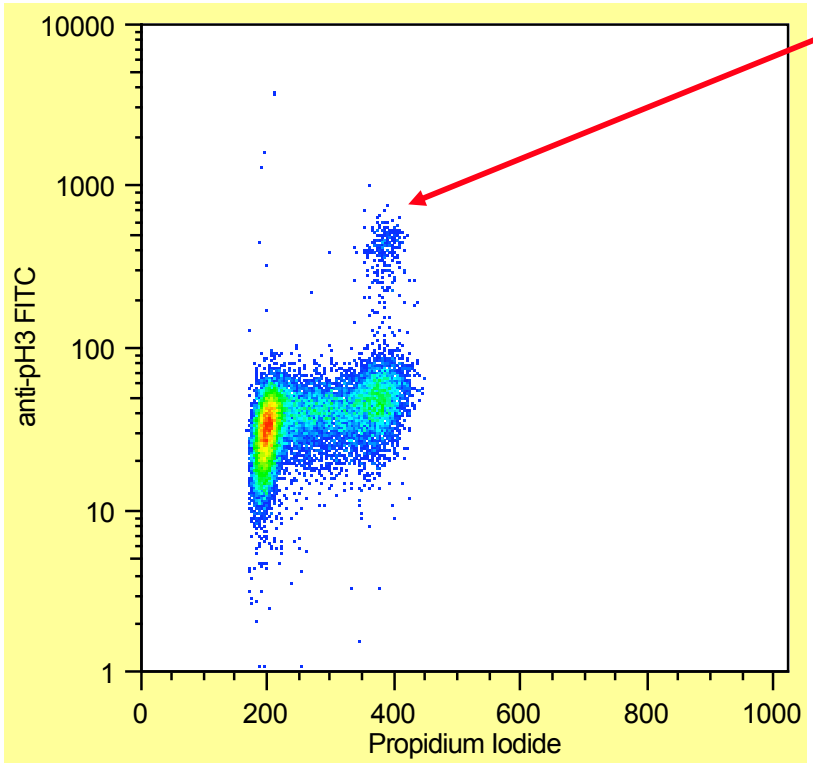
Cu-catalysed click reaction

Detection of proliferation - EdU

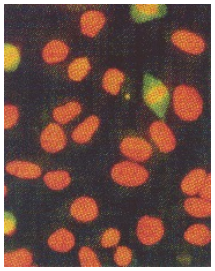


How about G0/G1 and G2/M?



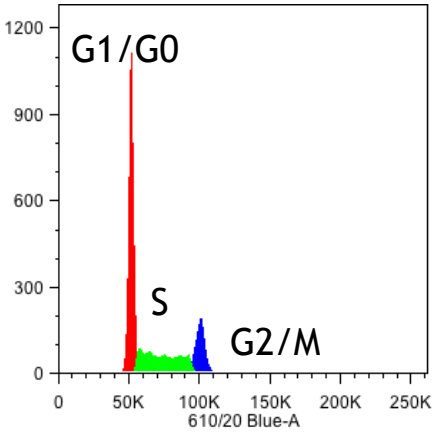


Mitotic cells

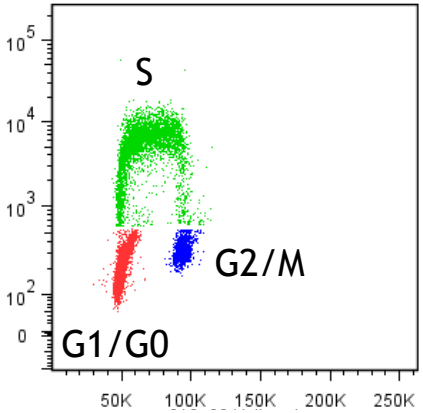


Cell Proliferation - the story so far

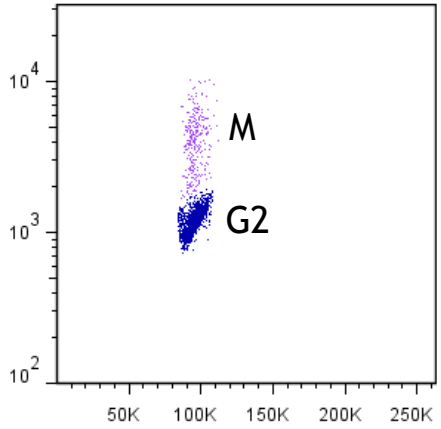
1D analysis

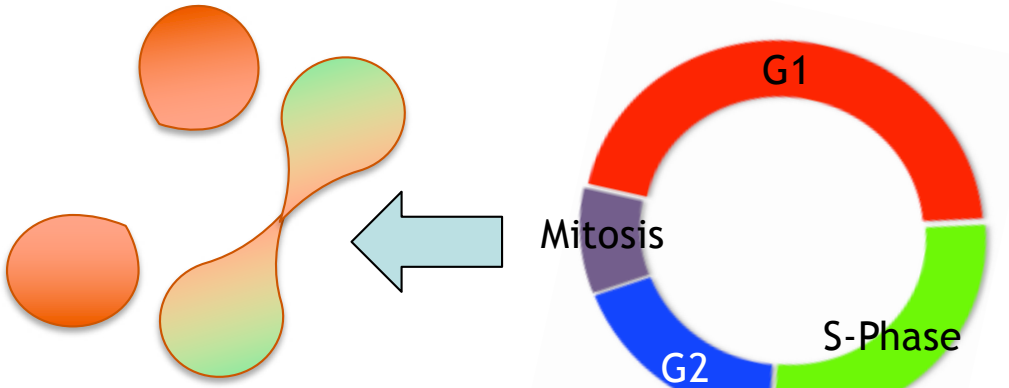


2D analysis



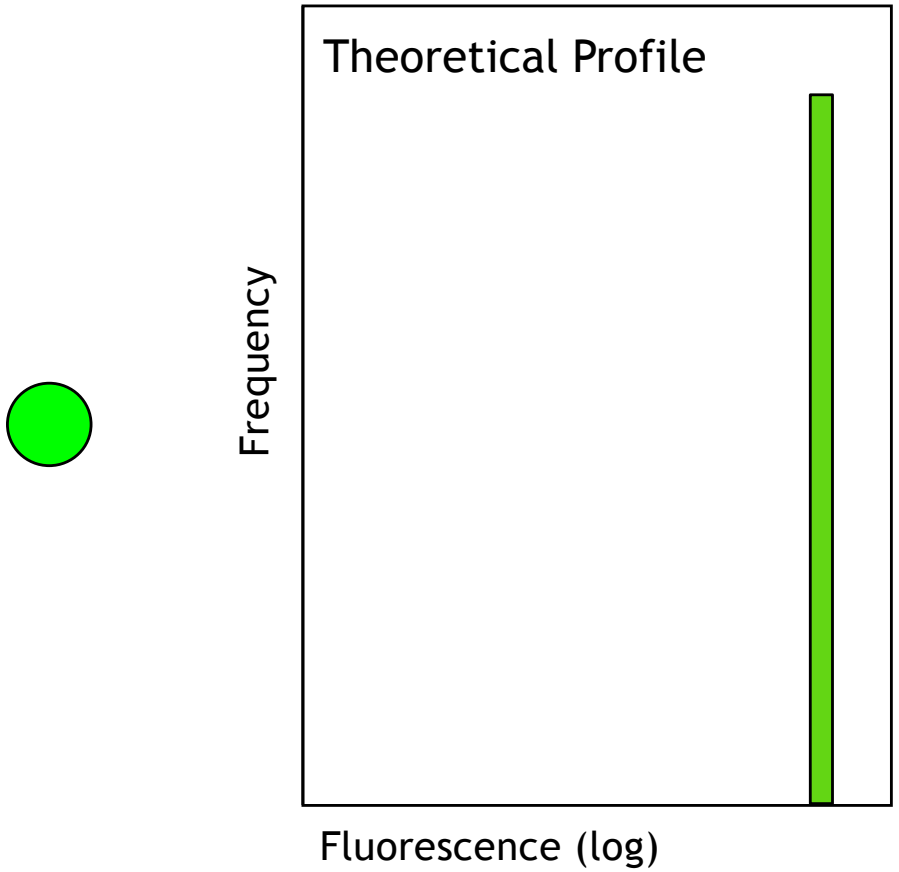
3D analysis



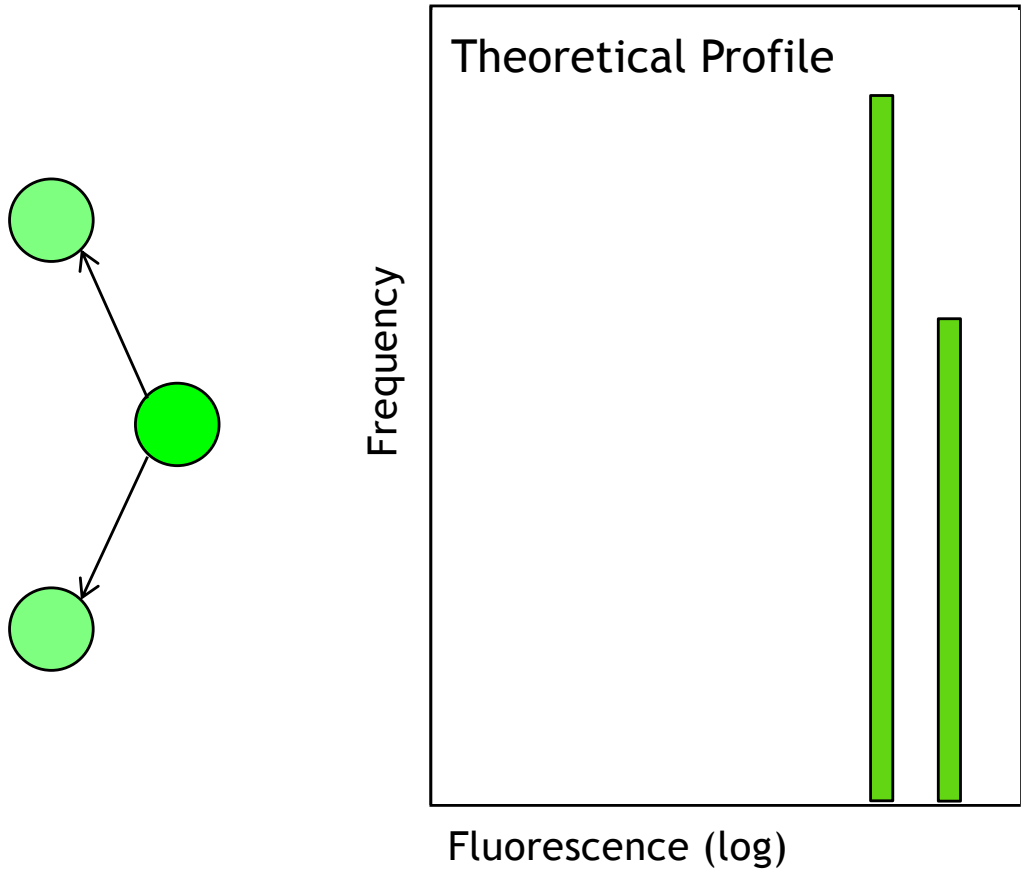


4D analysis

Measuring Proliferation by Fluorescent dye dilution



Measuring Proliferation by Fluorescent dye dilution



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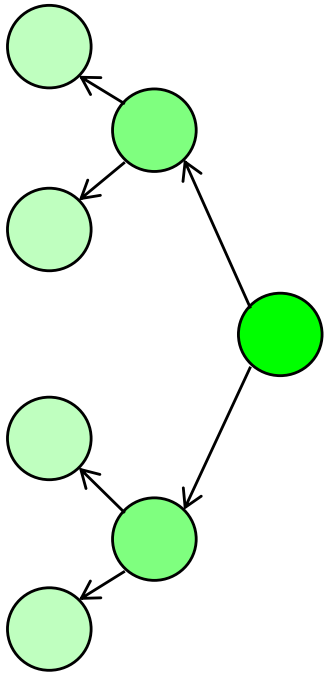
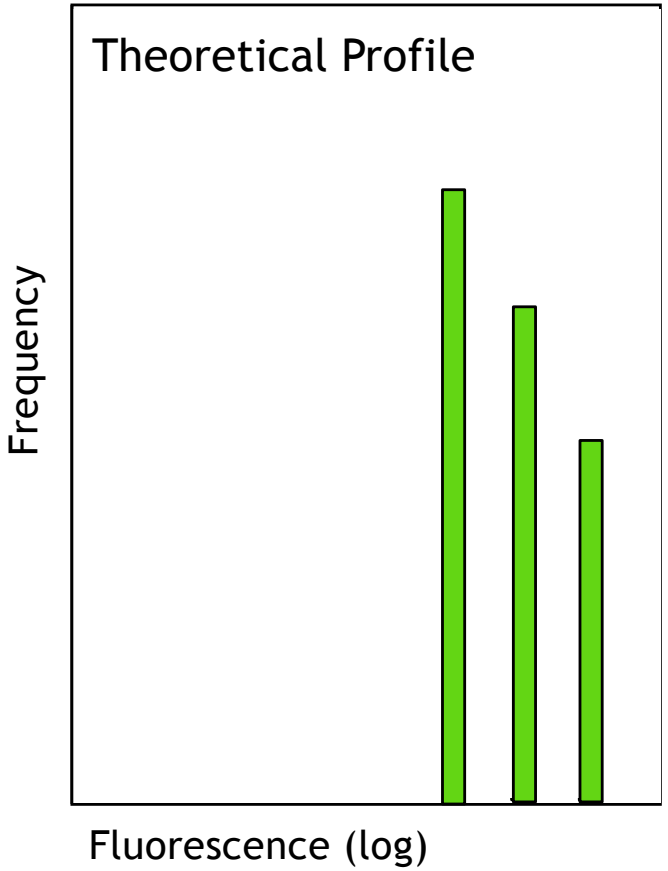
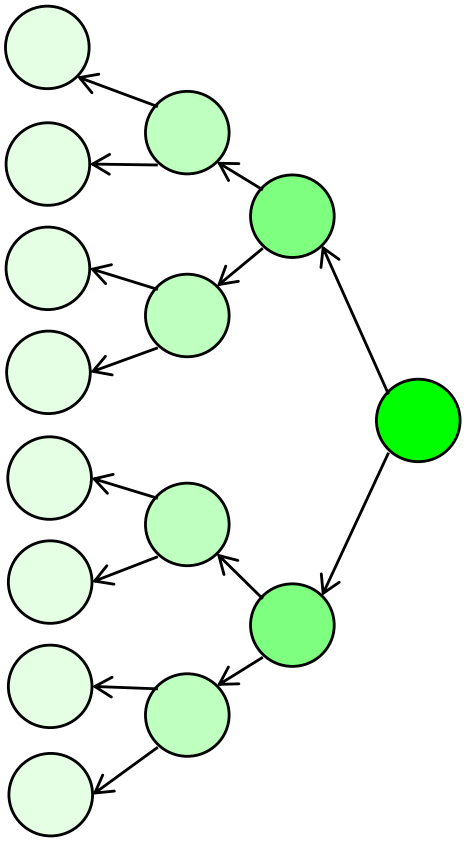
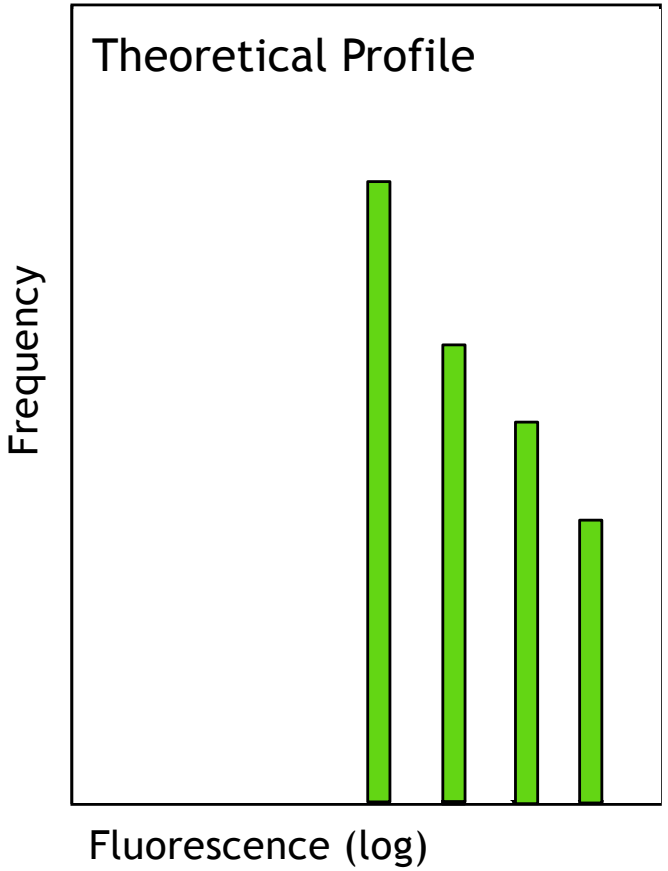




Measuring Proliferation by Fluorescent dye dilution





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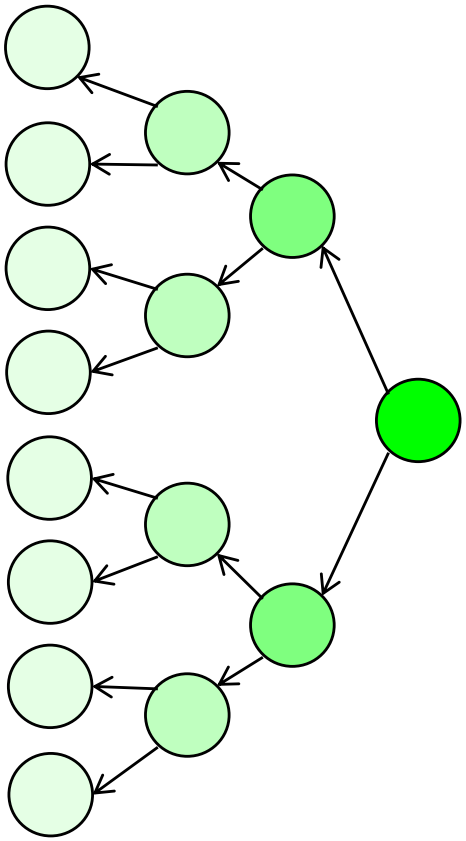
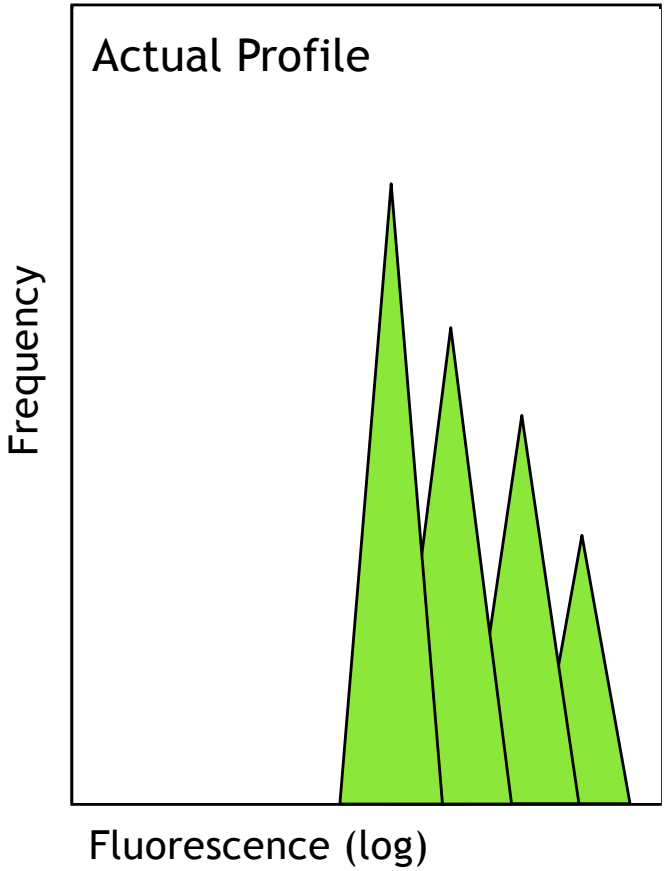
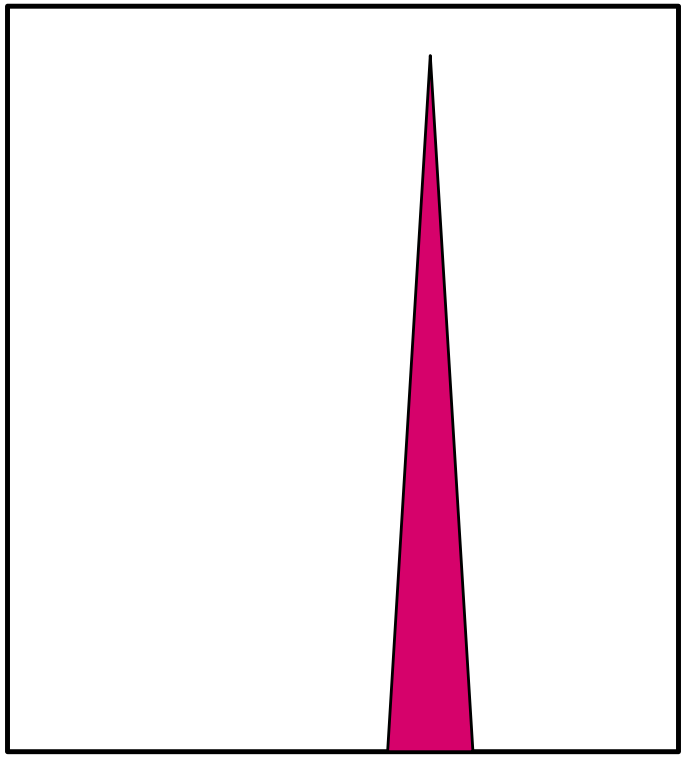


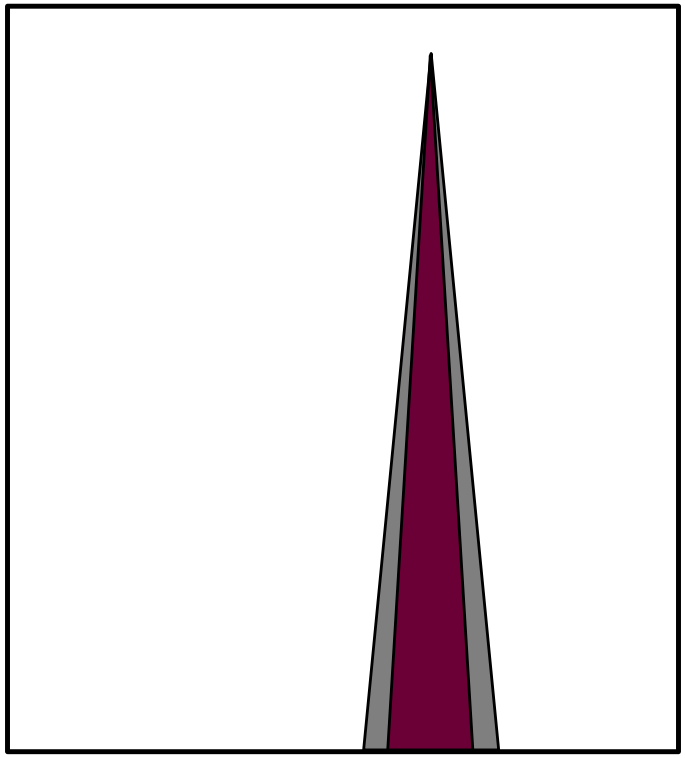


Measured “width” comes in 3 parts



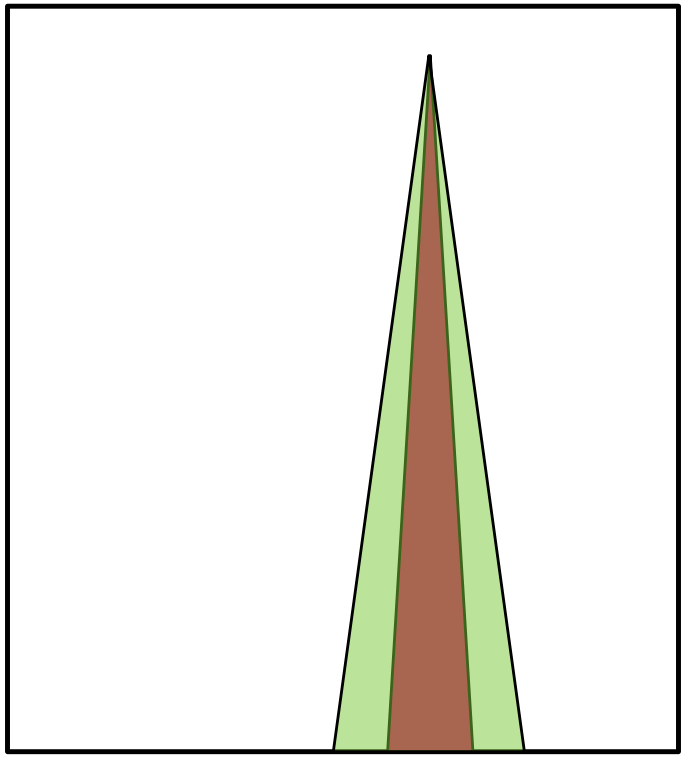
1. Cell heterogeneity (protein content)

Measured “width” comes in 3 parts



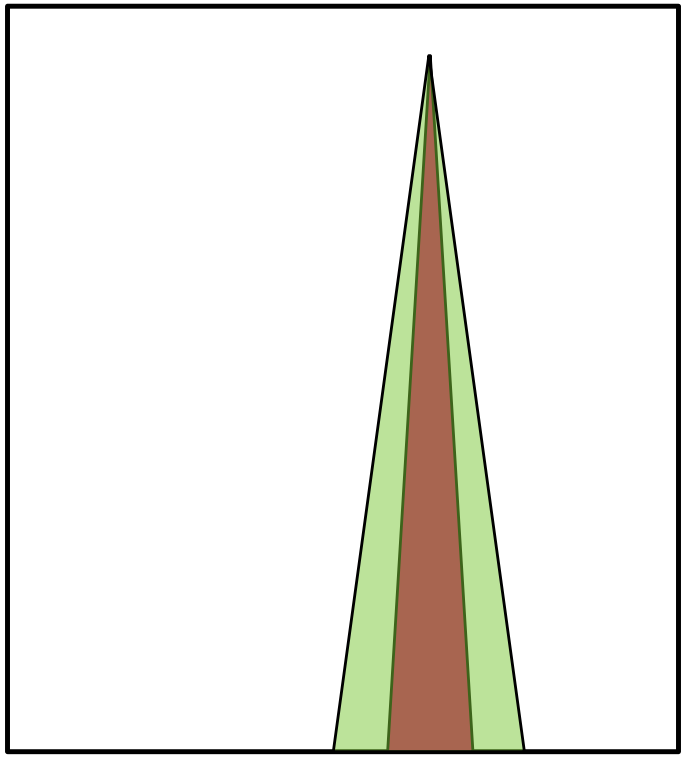
1. Cell heterogeneity (protein content)
2. How well the dye labels cells

Measured “width” comes in 3 parts



1. Cell heterogeneity (protein content)
2. How well the dye labels cells
3. Cytometer performance

Measured “width” comes in 3 parts



1. Cell heterogeneity (protein content)
2. How well the dye labels cells
3. Cytometer performance

We can control and influence ALL of these parameters to improve peak resolution.

What makes a good proliferation tracking dye?

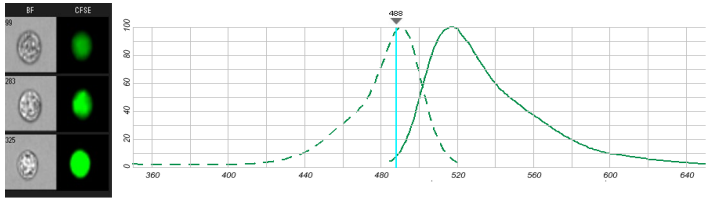
- Dye must be taken up by live cells
- Dye must have low toxicity yet bright staining
- Dye must be compatible with machine and panel
- Dye must be well retained (not leaky)
- Dye must be equally apportioned between daughter cells

1. Lipophilic dyes that label cell membrane (PKH dyes)
2. Succinimidyl Ester dyes that label intracellular proteins

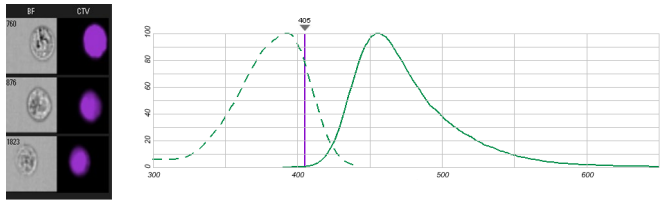
Succinimidyl Ester Dyes

- Are highly cell permeable
- Non-fluorescent until cleaved by Intracellular esterases
- Covalently couples via amine group to intracellular proteins
- Succinimidyl dyes are NOT highly transferred to adjacent cells
- Are retained for extremely long time periods

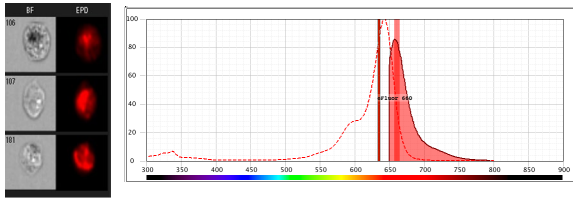
- Suitable for *in vitro* and *in vivo* proliferation tracking



CFSE



CellTrace Violet



eFluor 670

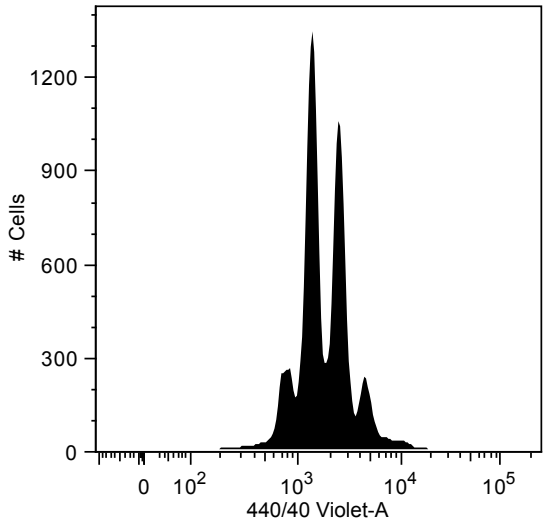
Generic Protocol for succinimidyl dye labelling

1. Pre-warm labelling solution (0.1-10 uM)
2. Harvest, count and wash cells, take care to remove protein
3. Re-suspend at 1- 20 million/ml in labelling solution
4. 30 min at 37 degrees
5. Add protein to 5% volume, leave for 5-10 min
6. Spin down and count
7. Check labelling intensity, uniformity and viability

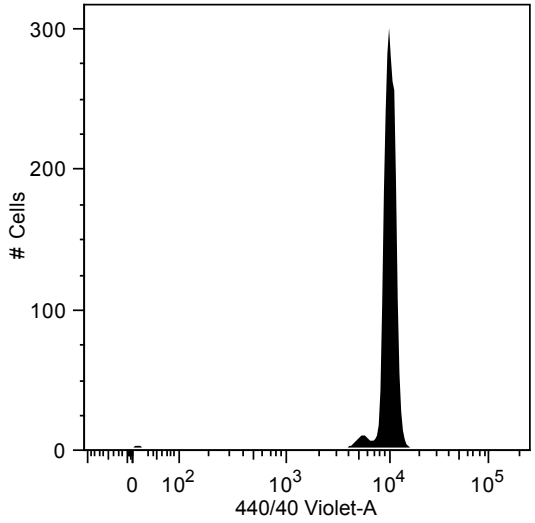
    1. The brighter the signal the better
    2. The lower CV the better
    3. The higher the viability the better

The identity of the undivided peak must be established

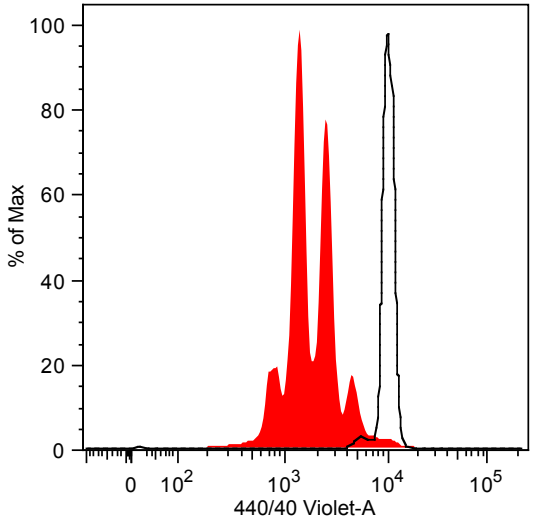
Stimulated



Unstimulated*



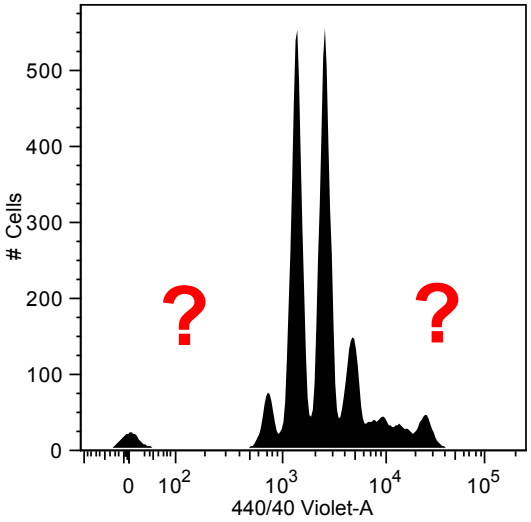
Overlay



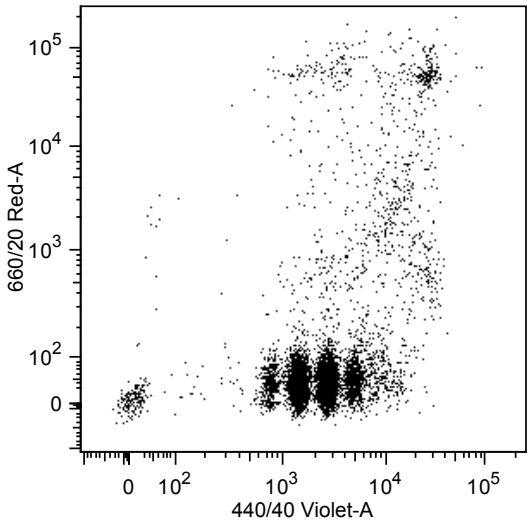
- *For a cell line, serum free, for primary T cells no stimulus + 1ng/ml IL-7 to keep cells from dying.
- Unstimulated sample MUST be harvested and analysed at SAME time because of non-proliferation dependent signal decay.

Dead cells can really affect the data

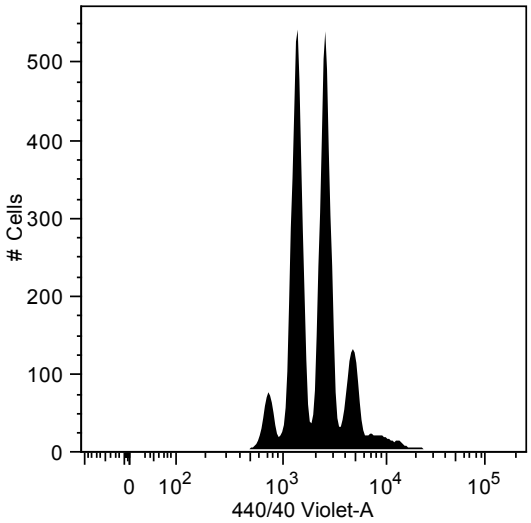
All events



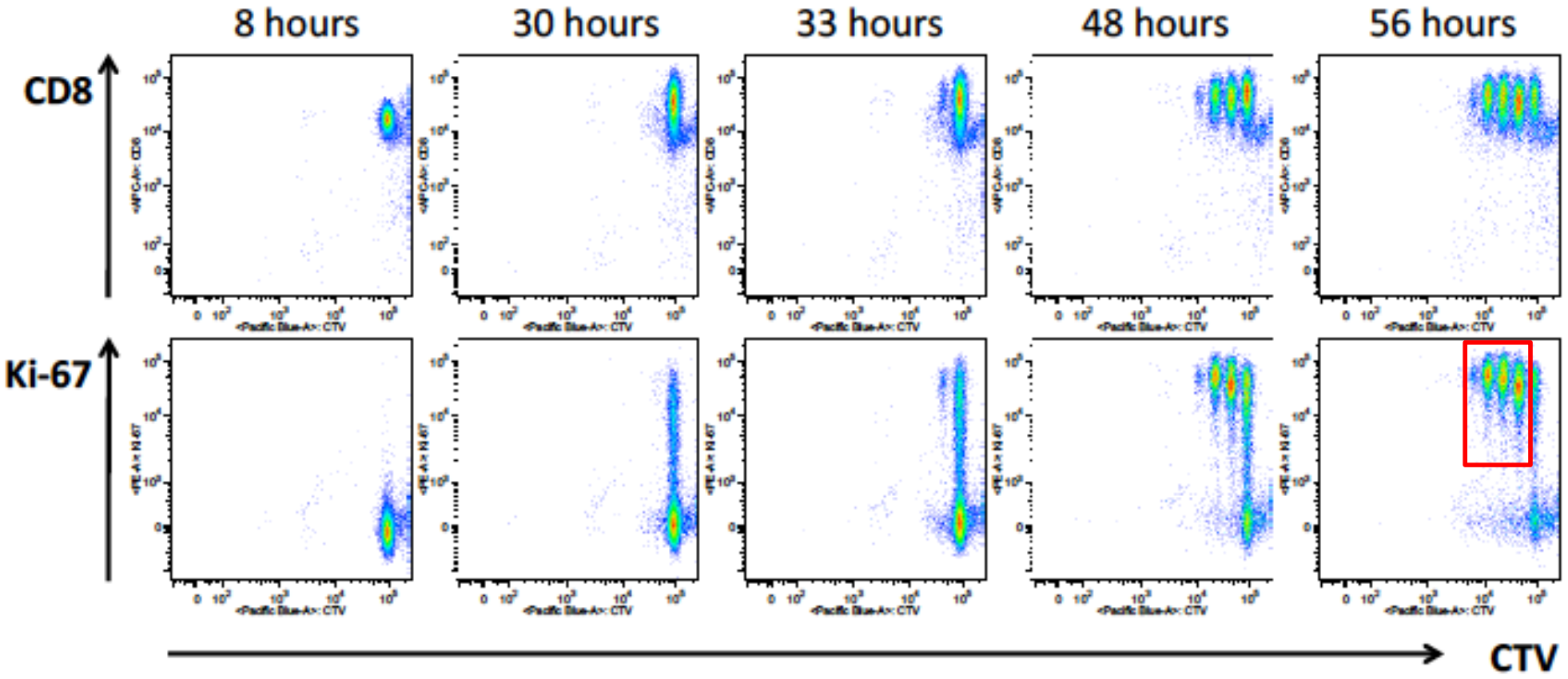
To-Pro-3



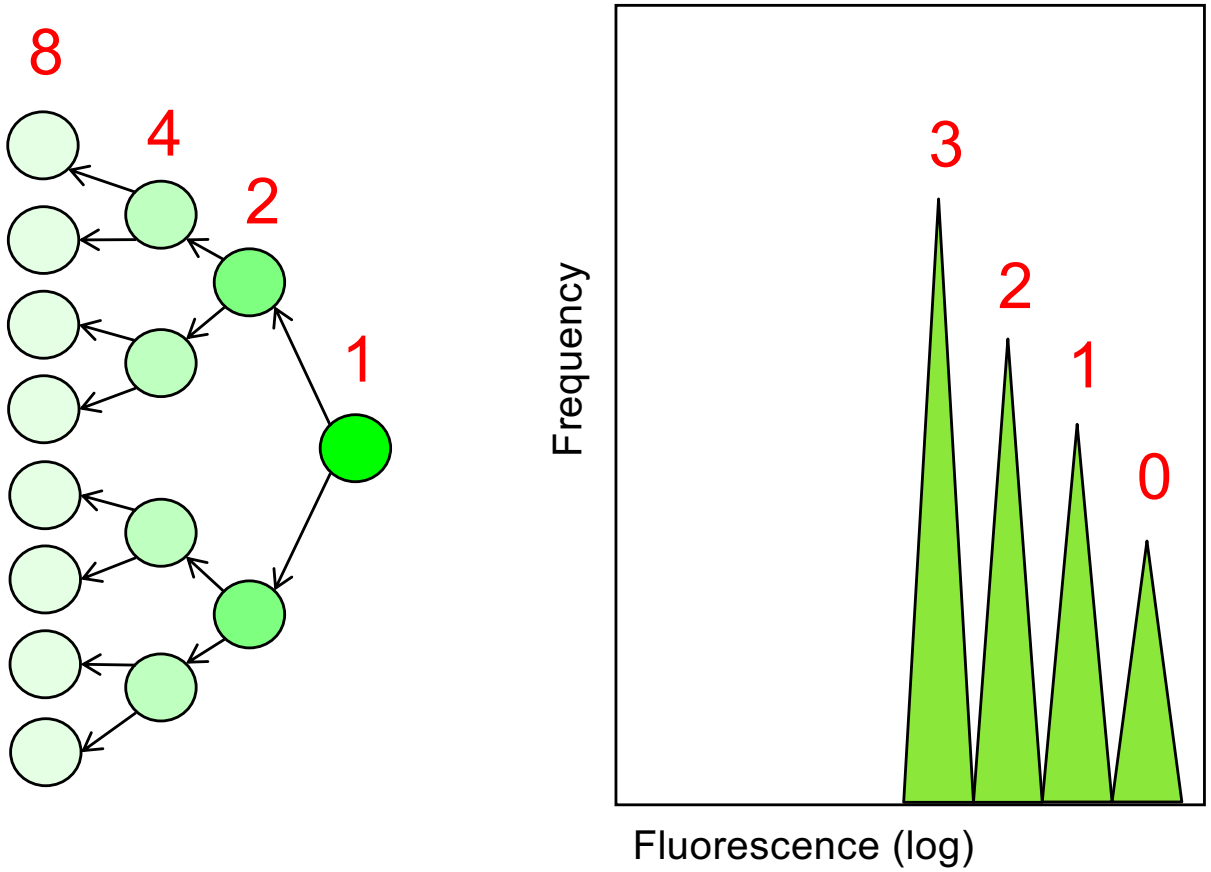
Live gated



Analyse the data correctly

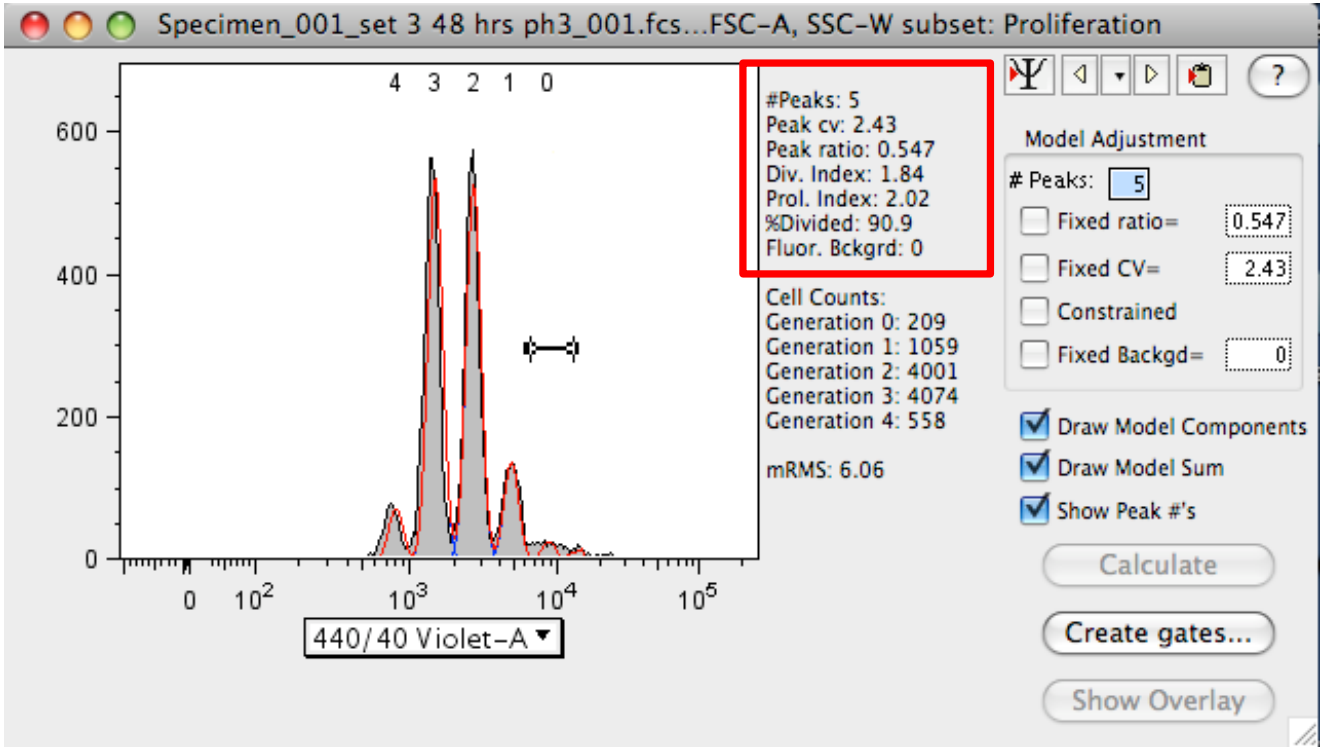


Principles of proliferation analysis by dye dilution



Each time INPUT cell divides it becomes over-represented by 2^n where n = division round

Software packages can help with the maths



1. % Divided
   - The % of the INPUT population that has entered division
   - Gives you an idea of triggering threshold

2. Proliferation index or Burst Size
   - This is the mean division number EXCLUDING undivided
   - Average division of triggered cells ONLY

Resources

ORIGINAL ARTICLE

Cytometry PART A
Journal of the International Society for Advancement of Cytometry

MIFlowCyt

An Imaging Flow Cytometric Method for Measuring Cell Division History and Molecular Symmetry During Mitosis

Andrew Filby,[1*] Esperanza Perucha,[2,3] Huw Summers,[4] Paul Rees,[4] Prabhjoat Chana,[3] Susanne Heck,[3] Graham M. Lord,[2,3] Derek Davies[1]

December 2013

Volume 83, Issue 12
Pages 1047–1125

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ISAC

ORIGINAL ARTICLE

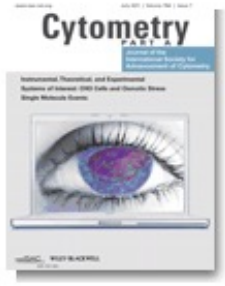
Cytometry PART A

MIFlowCyt

A Method for Evaluating the Use of Fluorescent Dyes to Track Proliferation in Cell Lines by Dye Dilution

Julfa Begum,[1] William Day,[1] Carl Henderson,[1] Sukhveer Purewal,[1] Joana Cerveira,[1] Huw Summers,[2] Paul Rees,[2,3] Derek Davies,[1] Andrew Filby[1*]

Issue



Cytometry Part A
Volume 79A, Issue 7, pages 496–506, July 2011

THE FRANCIS CRICK INSTITUTE

Cell proliferation - summary

Single parameter DNA analysis is simple but limited

BrdU/EdU allows cell kinetic information

More information is obtained by combining DNA analysis with other markers

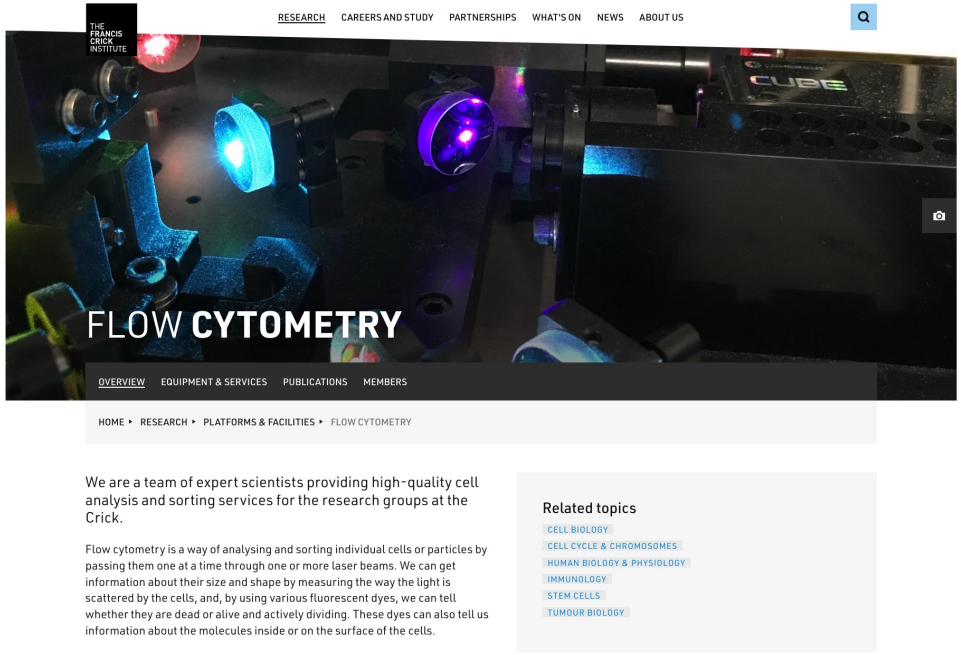
Nucleotide incorporation (BrdU/EdU) – 1-2 divisions

Dye dilution – 5-6 divisions

Know your cytometer
Know your dyes
Know your strengths and weaknesses

Contacts and Resources

derek.davies@crick.ac.uk



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