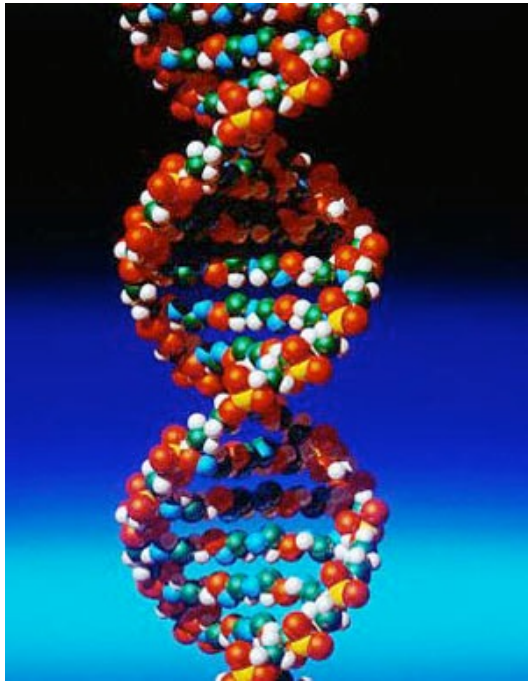
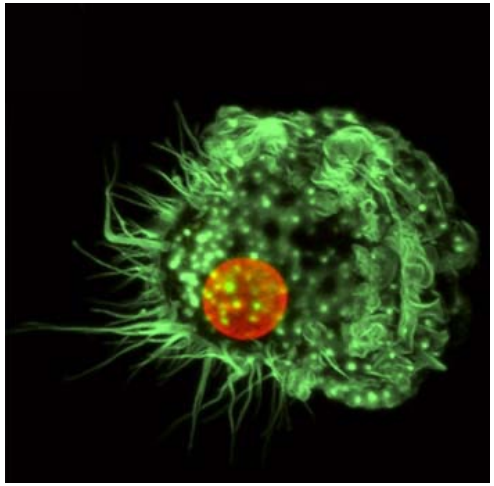
DNA analysis by flow cytometry



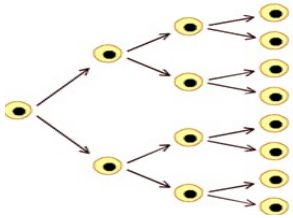
Derek Davies,
The Francis Crick Institute, London, UK

DNA analysis by flow cytometry

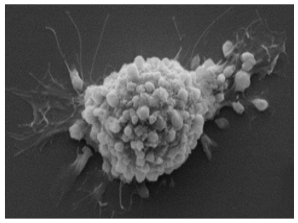
A cell



Proliferation signals

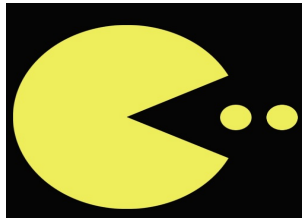


Proliferation



Apoptosis

Cell death



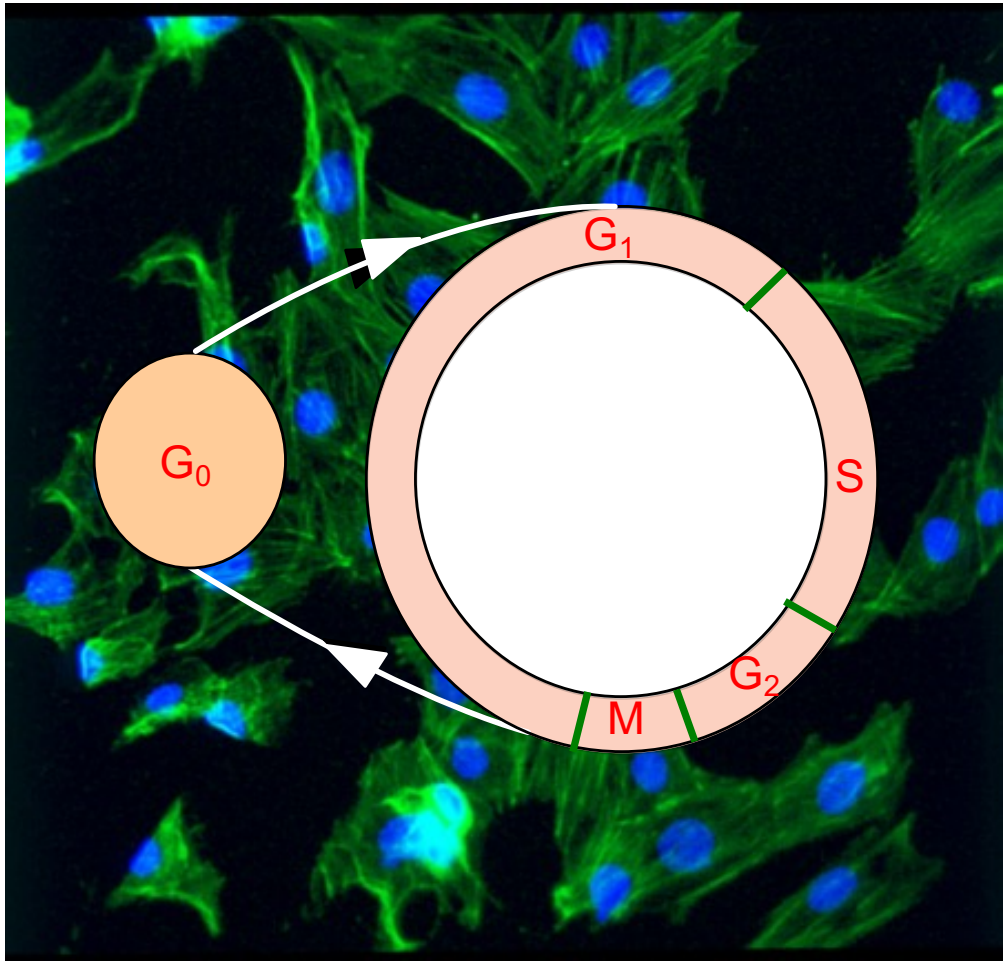
Autophagy

Senescence

Why perform DNA analysis?

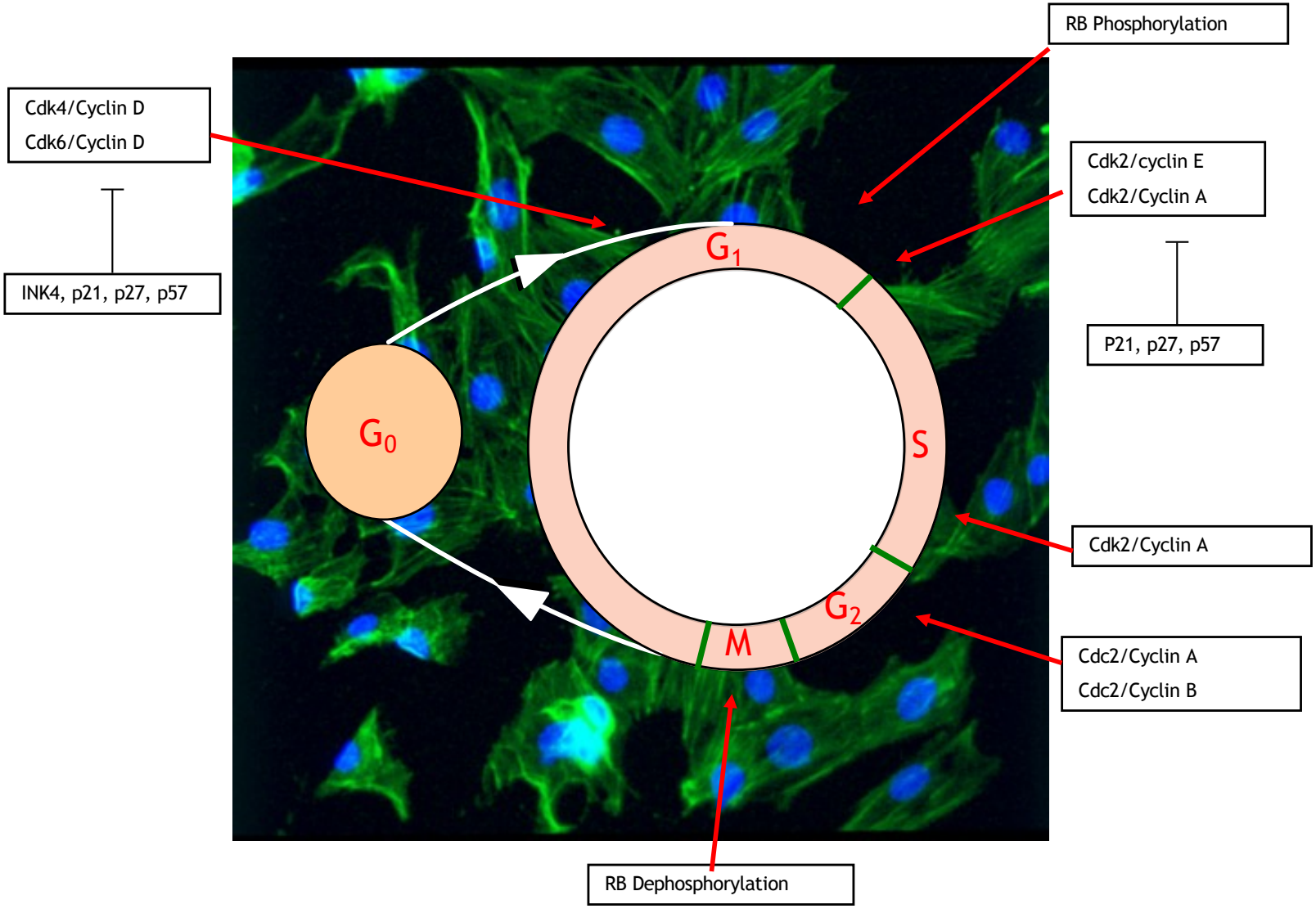
- Disease states e.g. cancer.
- Response to stimulus e.g. infection
- Are my cells growing?
- Important in cell biology and clinical diagnosis.

The mammalian cell cycle

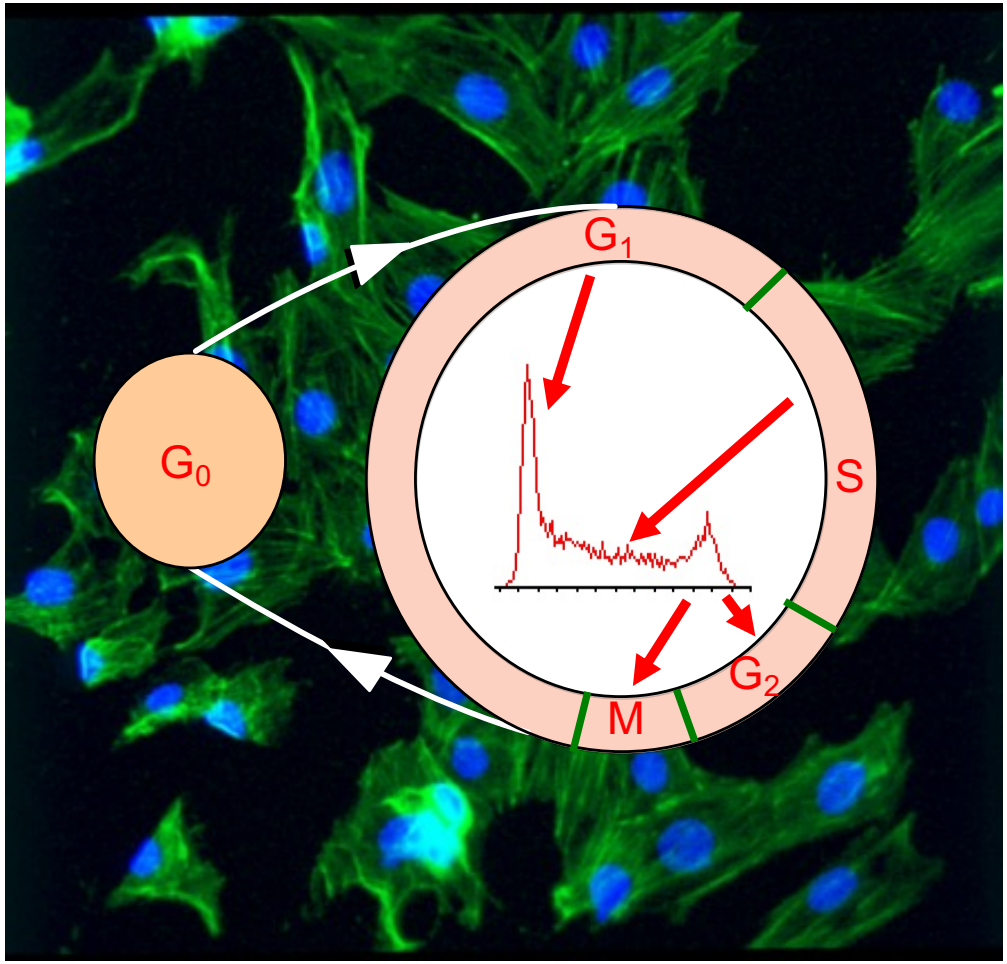


G1: Gap 1
S: Synthetic
G2: Gap 2
M: Mitosis
G0: cells that cease division

The mammalian cell cycle



The mammalian cell cycle

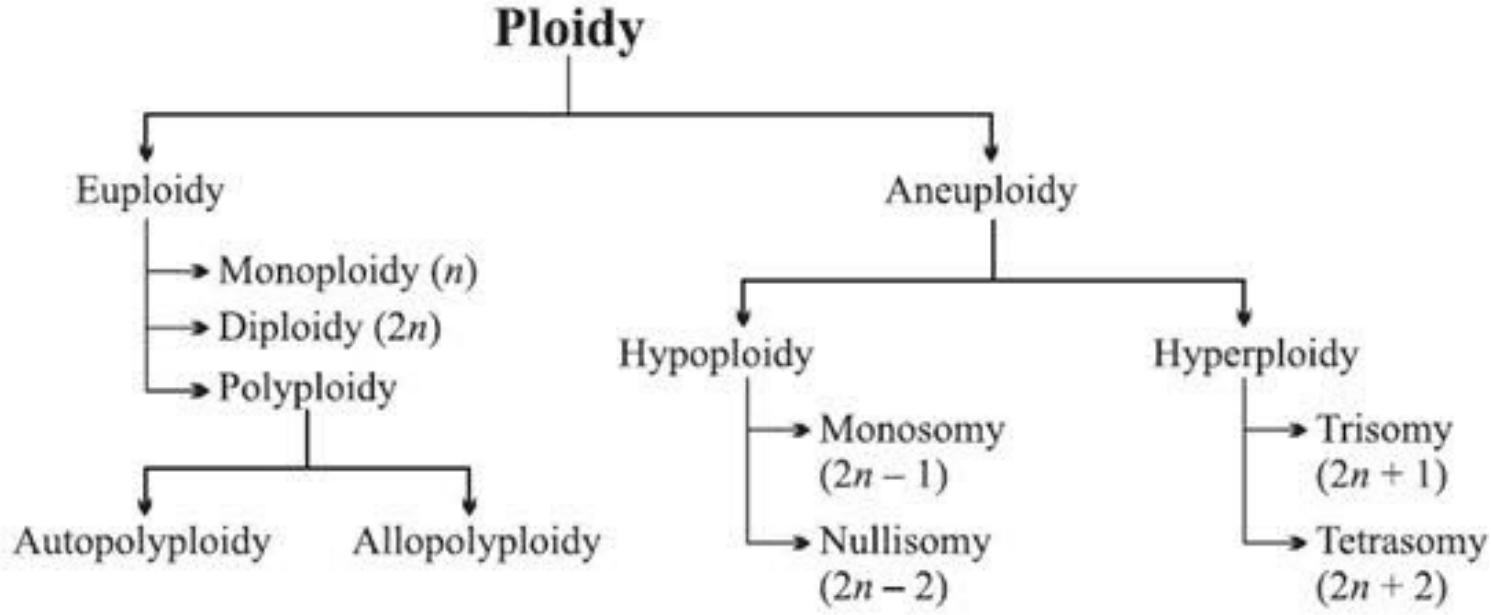


Ploidy

The DNA content of cells is talked about as 1c, 2c, 4c or 2n, 4n, 8n.

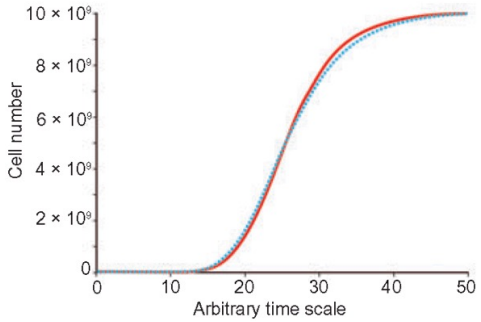
n = number of chromosomes in the nucleus

1n = haploid, 2n = diploid, 4n= tetraploid, >n< = aneuploid

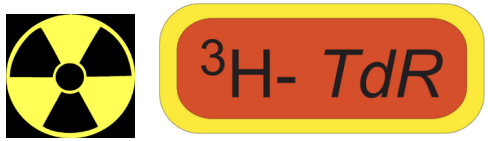


BULK Methods for Measuring Proliferation

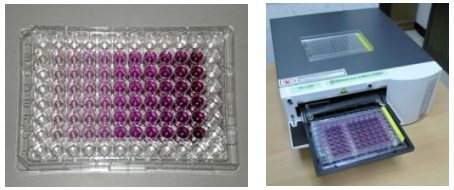
1. Count cells: Know what you put in, count what you get out.



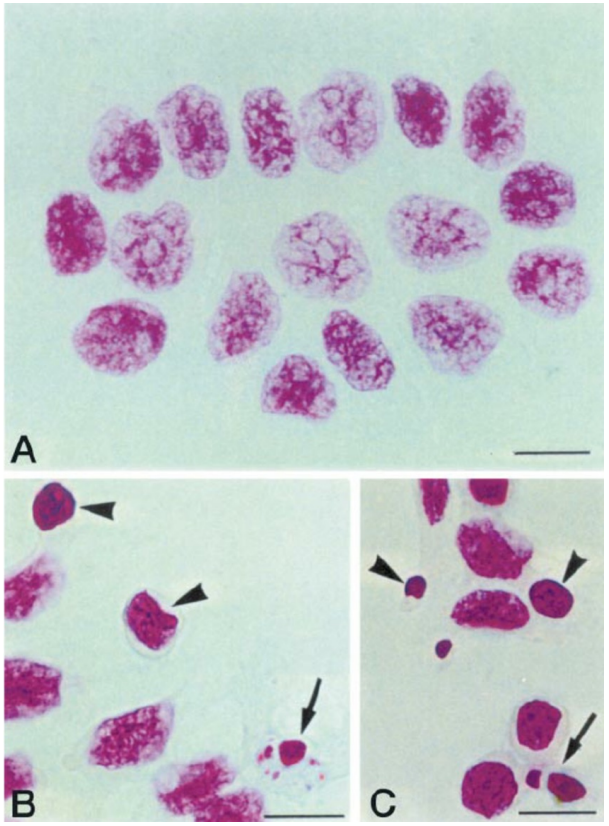
2. Use radioactive Thymidine incorporation



3. Use colorimetric assays such as MTT



Cell Cycle Analysis by Cytometry



Cell Cycle Analysis by Flow Cytometry

It’s nothing new in the flow cytometry world

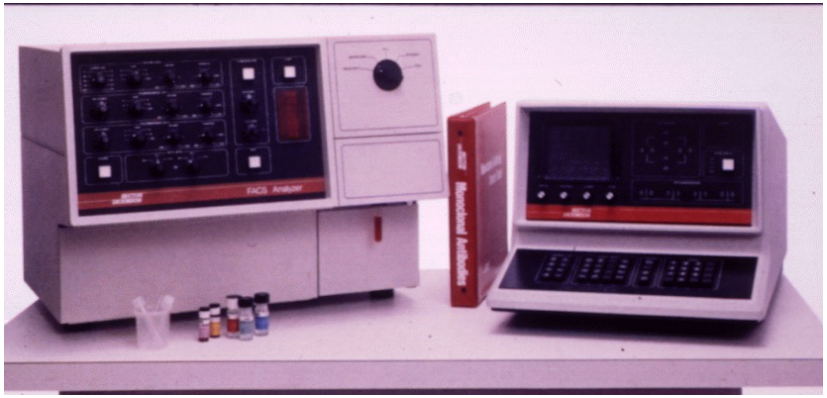
Science. 1974 Jun 21;184(4143):1297-8.

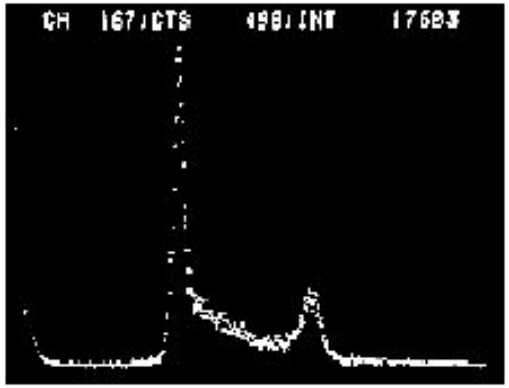
Cell-cycle analysis in 20 minutes.

Crissman HA, Tobey RA.

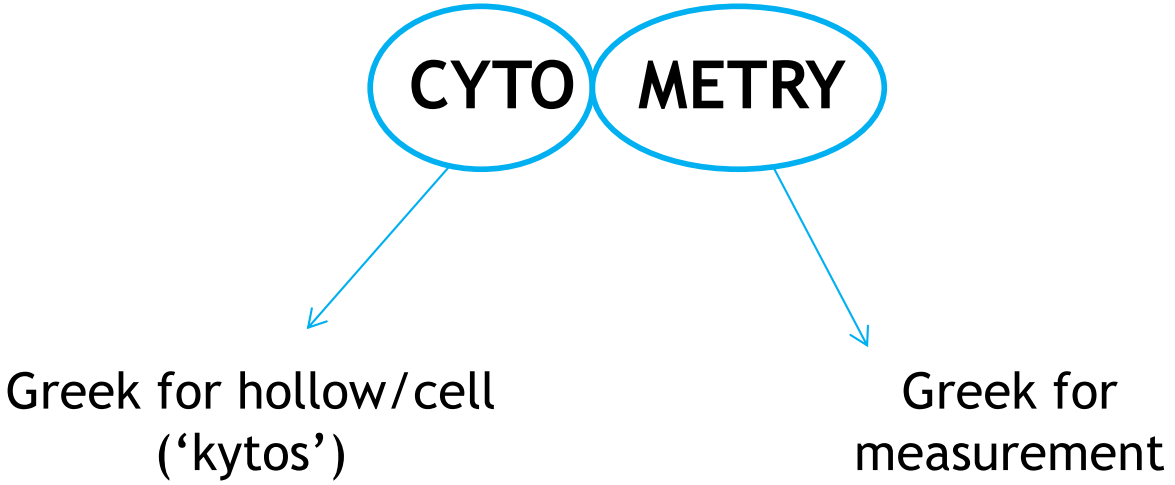
Abstract

Mithramycin added to mammalian cells fixed in aqueous ethanol is bound to DNA and fluoresces in direct proportion to the cellular DNA content. Quantitative fluorescence measurement by means of a high-speed flow system instrument provides a rapid method for cell-cycle analysis and for the first time permits continuous monitoring of cell-cycle kinetics during ongoing experiments.





But what is Flow Cytometry?



"Flow Cytometry is a technology where physical and chemical measurements are made on particles as they flow one by one through a flow chamber at rates of several thousand per second."

But what is Flow Cytometry?

Fluidics : Separation and alignment of particles.

Optics : Light source(s), detectors, spectral separation (filters, dichroic mirrors).

Detectors : Collection and analysis of optical signals; data display.

Data Analysis : Dots on the screen

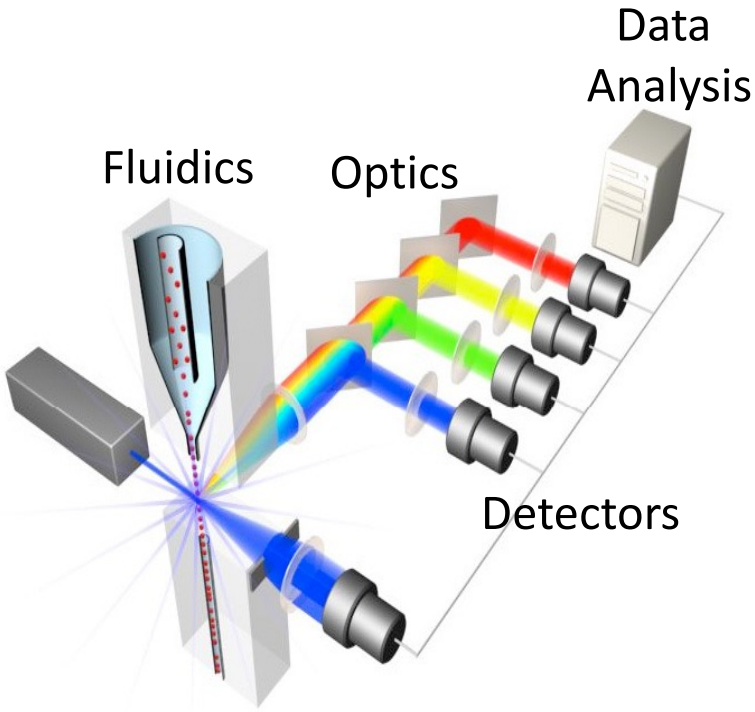


Diagram taken from www.invitrogen.com

But what is Flow Cytometry?

Need knowledge of:

- Fluorochromes
- Lasers
- Optical filters
- Signal capture and measurement
- Data analysis
- Biology!

DNA/RNA Probes

- Propidium Iodide
- Ethidium Bromide
- Hoechst dyes
- Cyanine dyes eg TO-PRO-3, SYTO/SYTOX dyes
- Acridine Orange
- Pyronin Y
- Styryl Dyes eg LDS-751
- Mithramycin, Chromomycin
- 7 Amino-actinomycin D (7-AAD)
- Diamino-2-phenylindole (DAPI)
- DRAQ5, DRAQ7

Which probe to use?

Excitation wavelength available	UV: Hoechst, DAPI 488: PI, 7AAD 633: TO-PRO-3
Specificity	None: PI A-T: Hoechst G-C: 7AAD, Chromomycin
Viability	Hoechst 33342 DRAQ5 DyeCycle dyes (Molecular Probes)

Cell cycle analysis by flow cytometry

Cells must be permeable - can use detergent or fixation (ethanol is best)

DNA in cells can be stained with a fluorescent dye

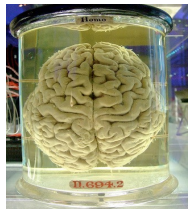
DNA probes like PI are stoichiometric and increase fluorescence on binding

Basic protocol - fix, wash twice, remove RNA and stain with DNA-binding dye

Fix or permeabilise?

Dehydrating, coagulating fixative.

Ethanol, methanol



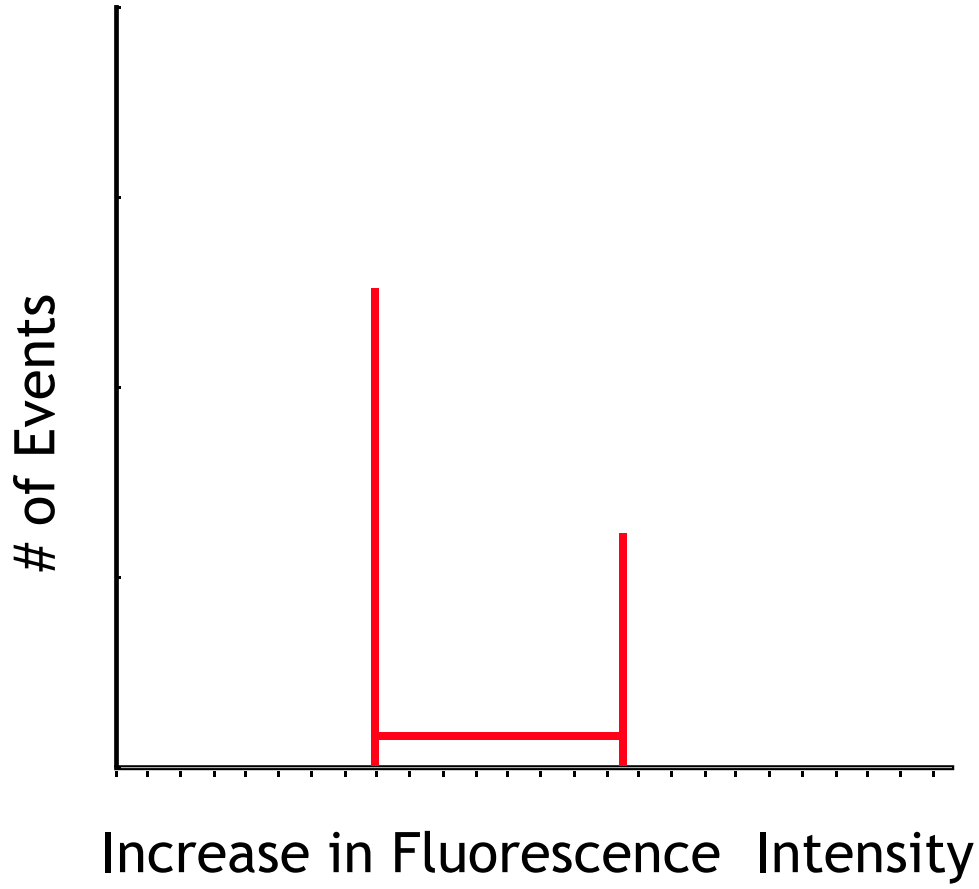
Cross-linking fixative.

Formaldehyde

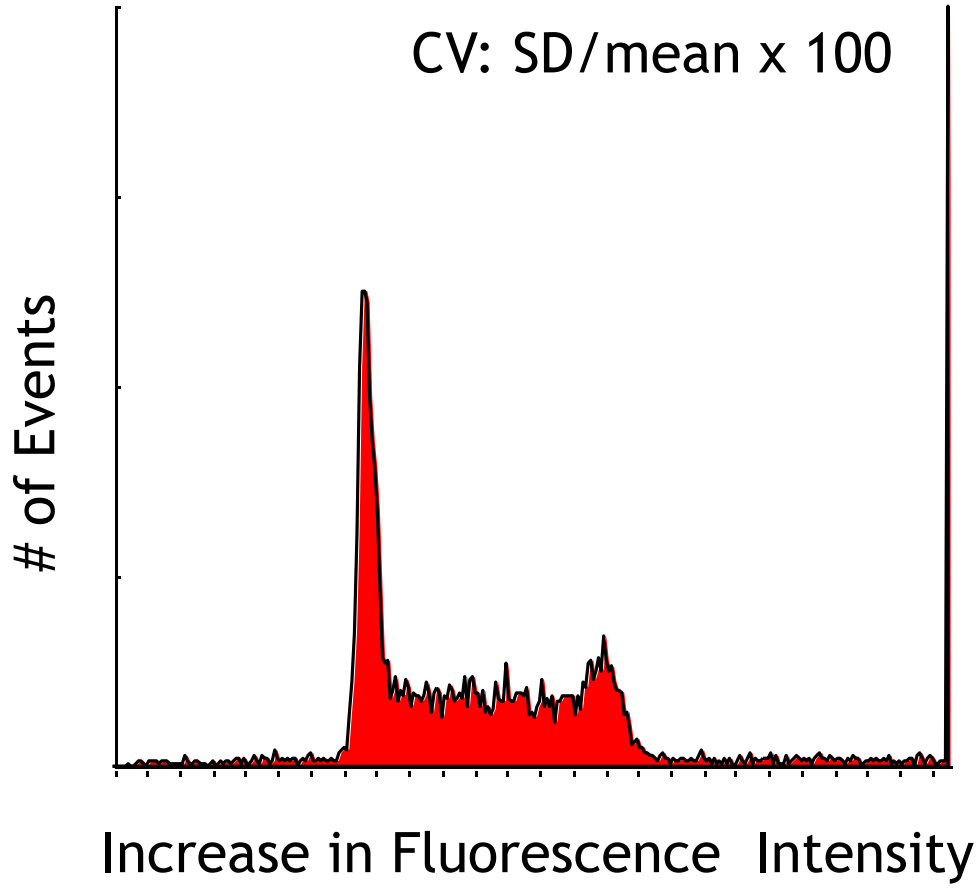
Detergent disruption of plasma and nuclear membranes

Triton, NP-40, Saponin

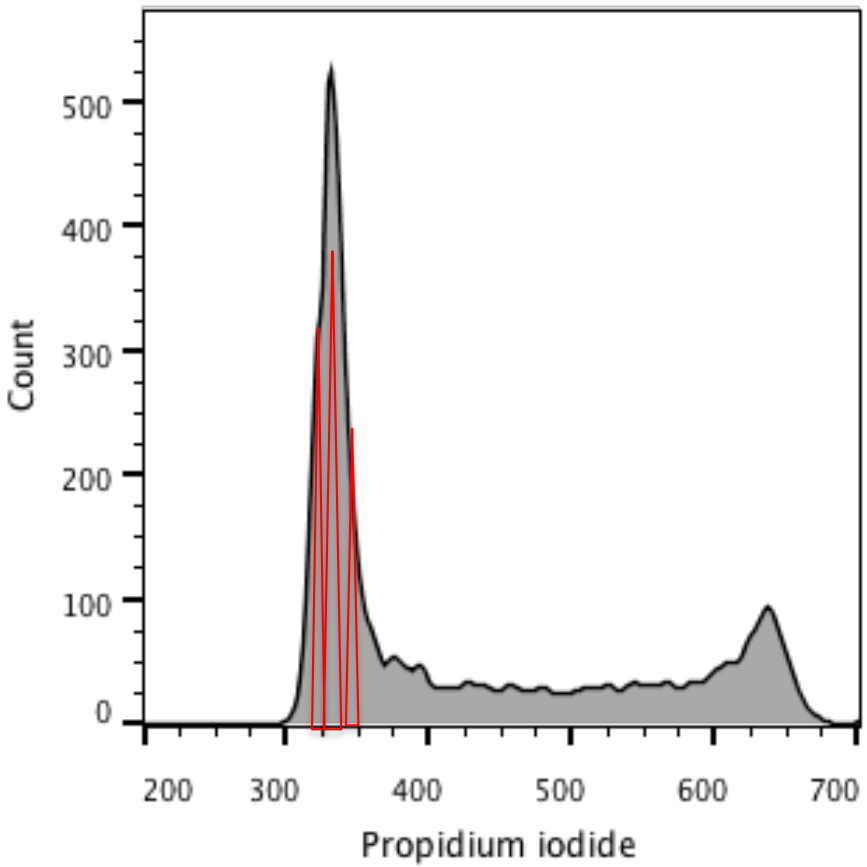
In an ideal world…….



In the real world…….

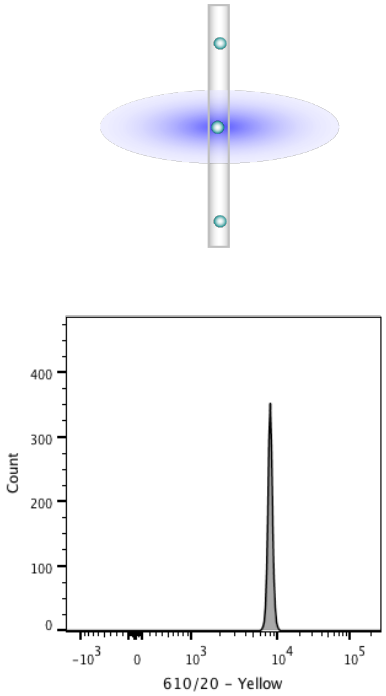


Causes of heterogeneity: Biological variation (subclones)

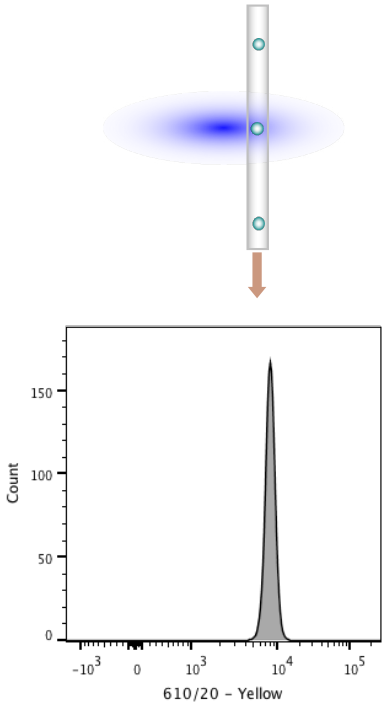


Causes of heterogeneity: Laser alignment

Laser Properly Aligned

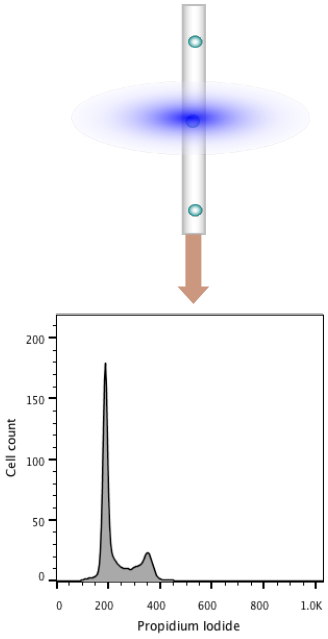


Misaligned Laser

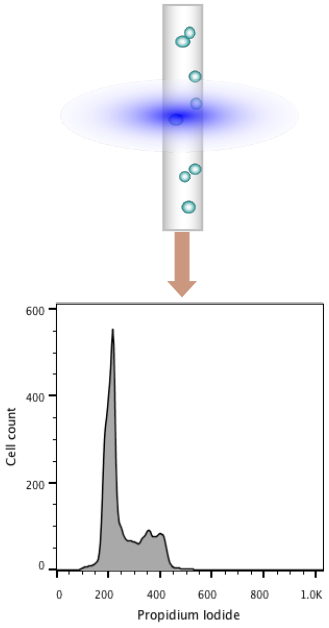


Causes of heterogeneity: Flow rate

Narrow Sample Stream: Low Flow Rate

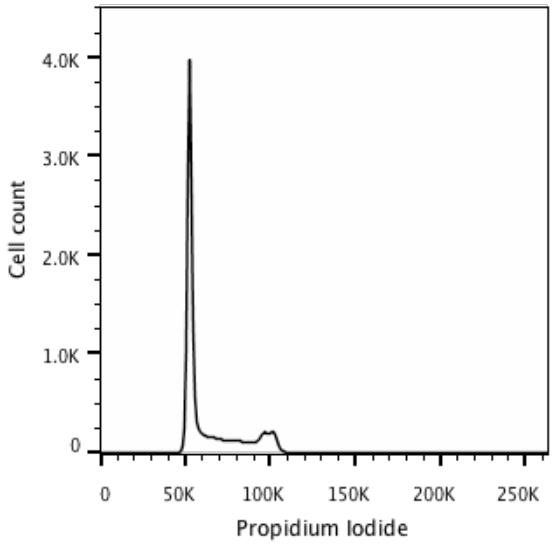


Wide Sample Stream: High Flow Rate

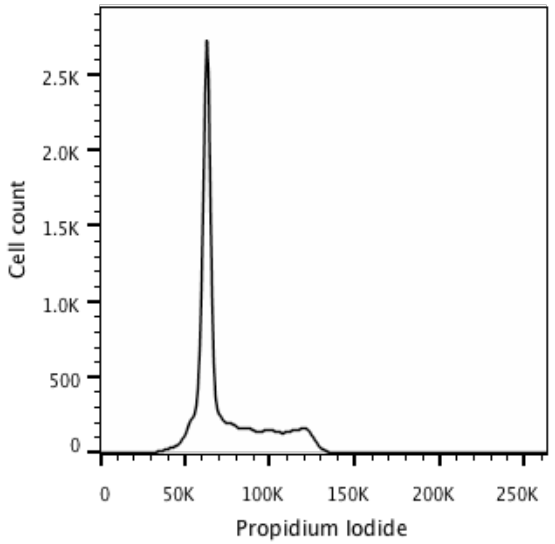


Causes of heterogeneity: Fixation

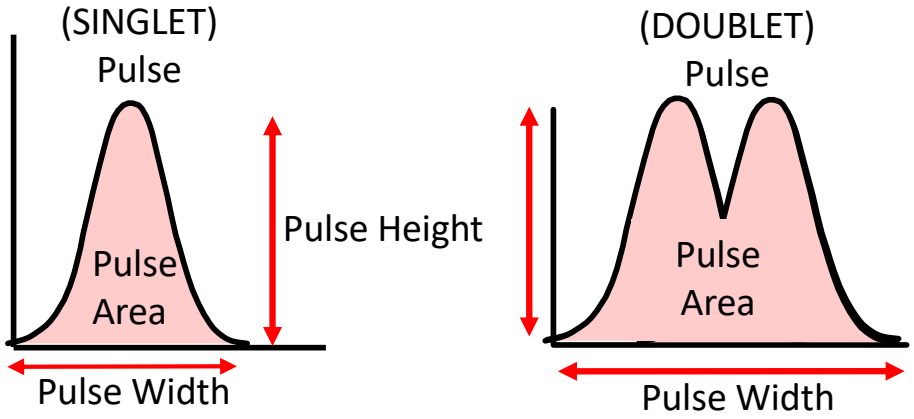
70% ethanol

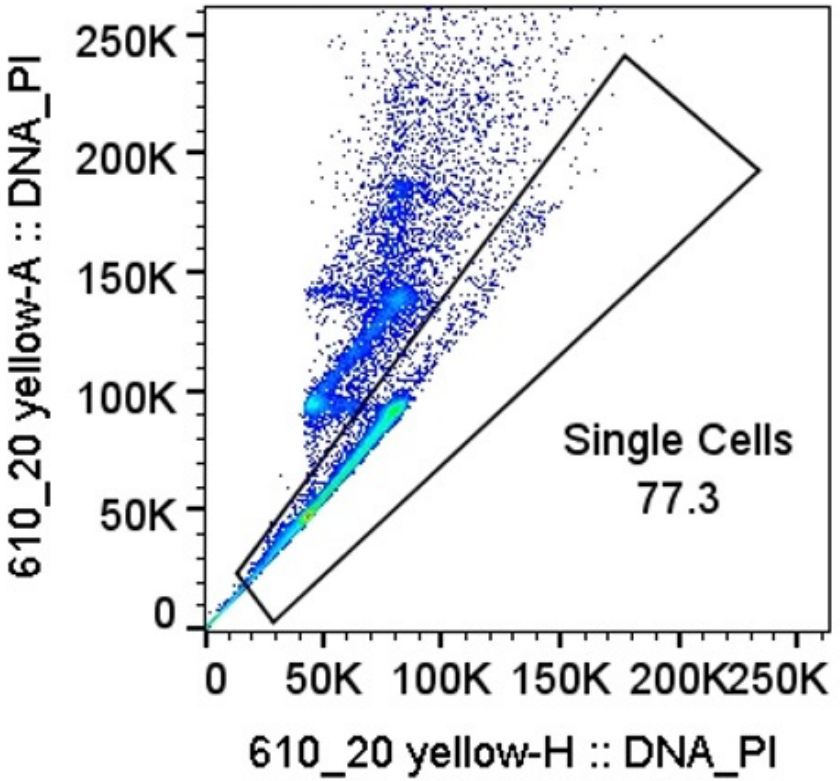


2% formaldehyde



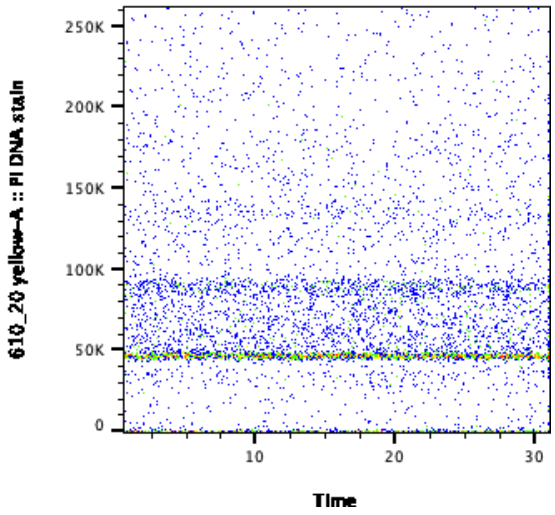
Causes of heterogeneity: Doublets



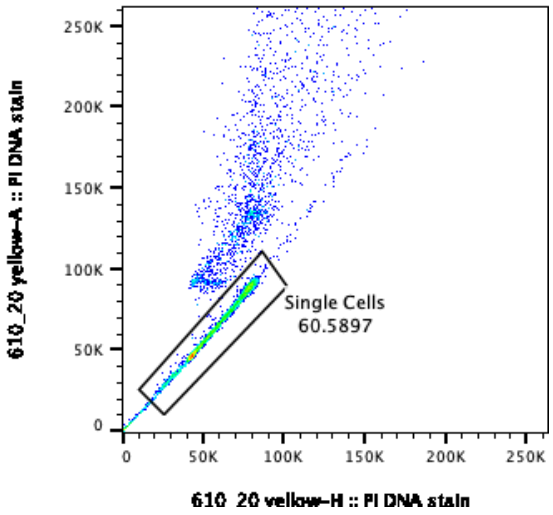


Cell cycle analysis – Data clean-up/QC

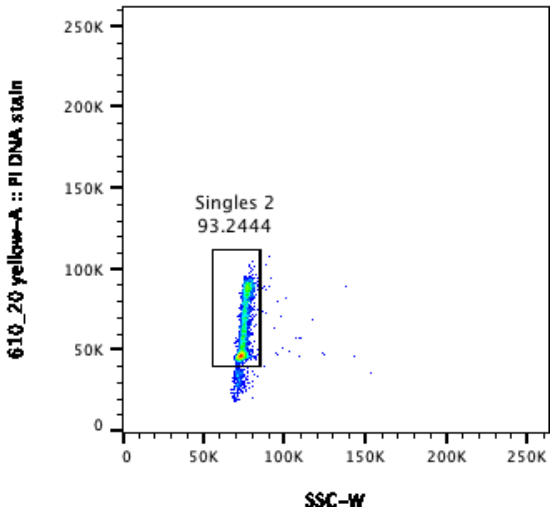
Time QC



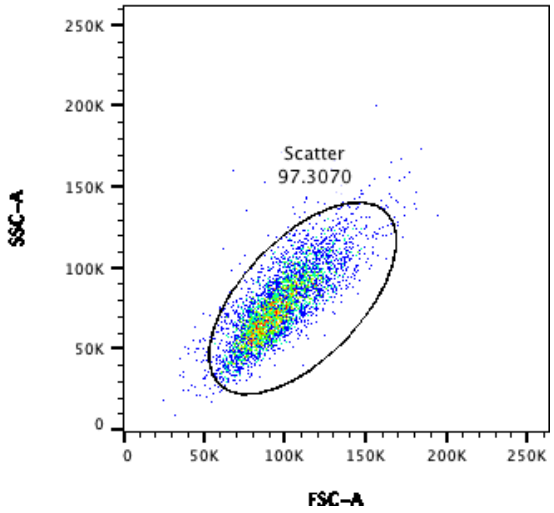
Singlet gate 1



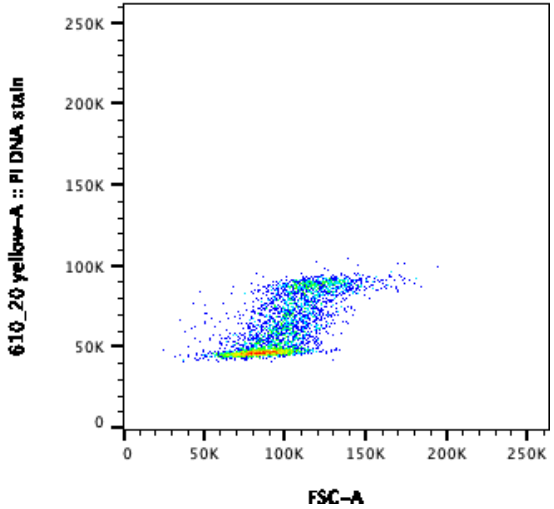
Singlet gate 2



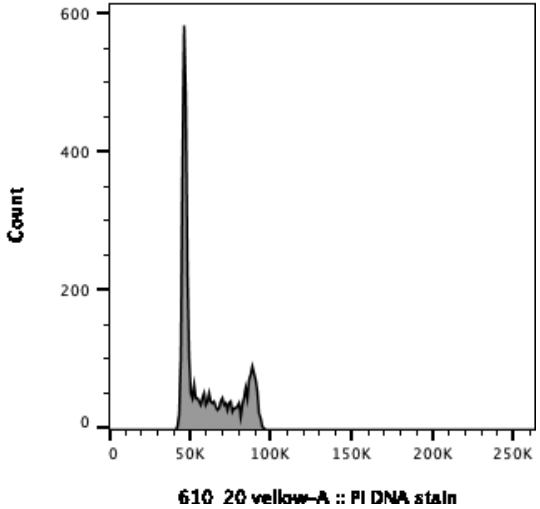
Scatter gate



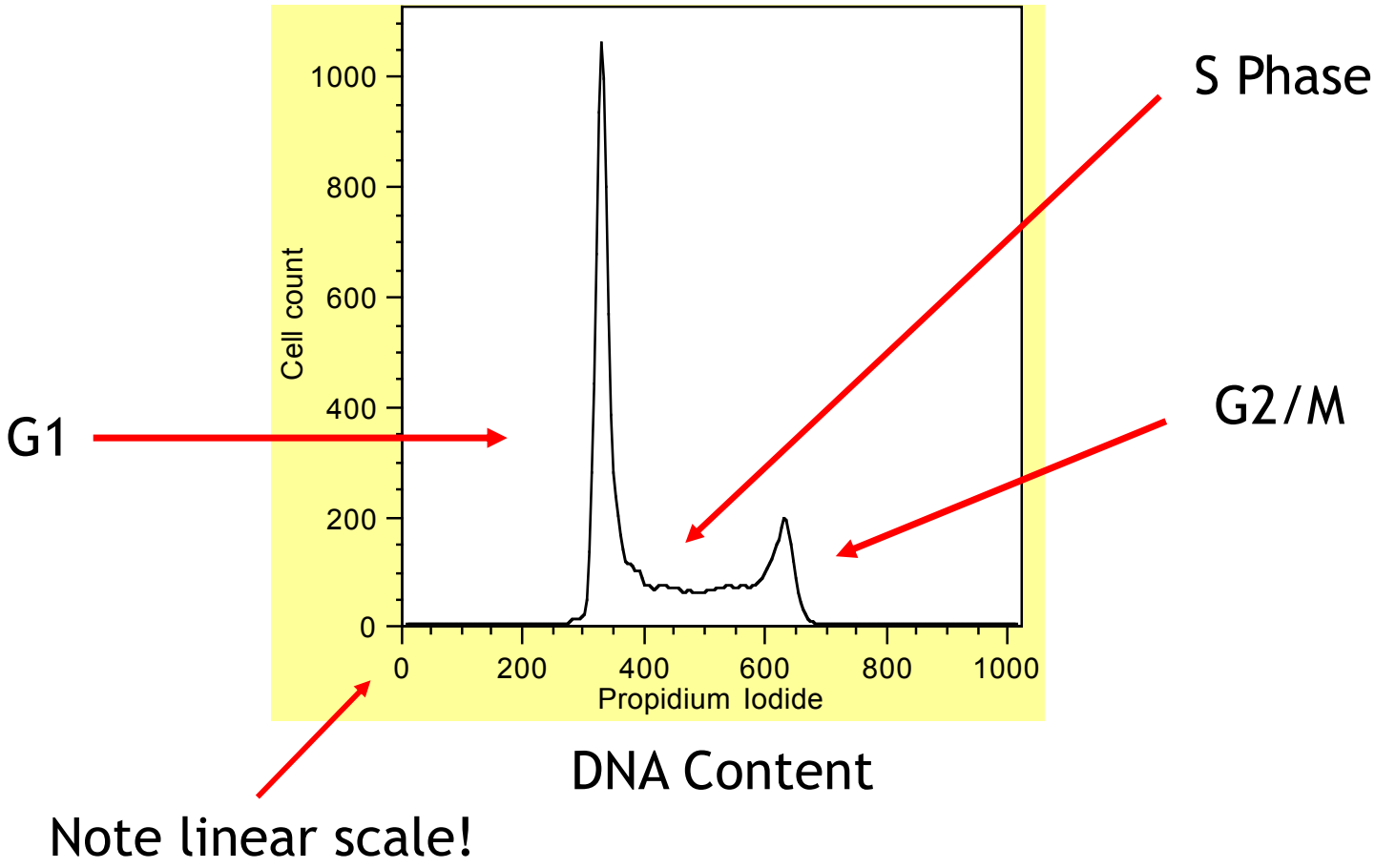
Machine QC



DNA profile



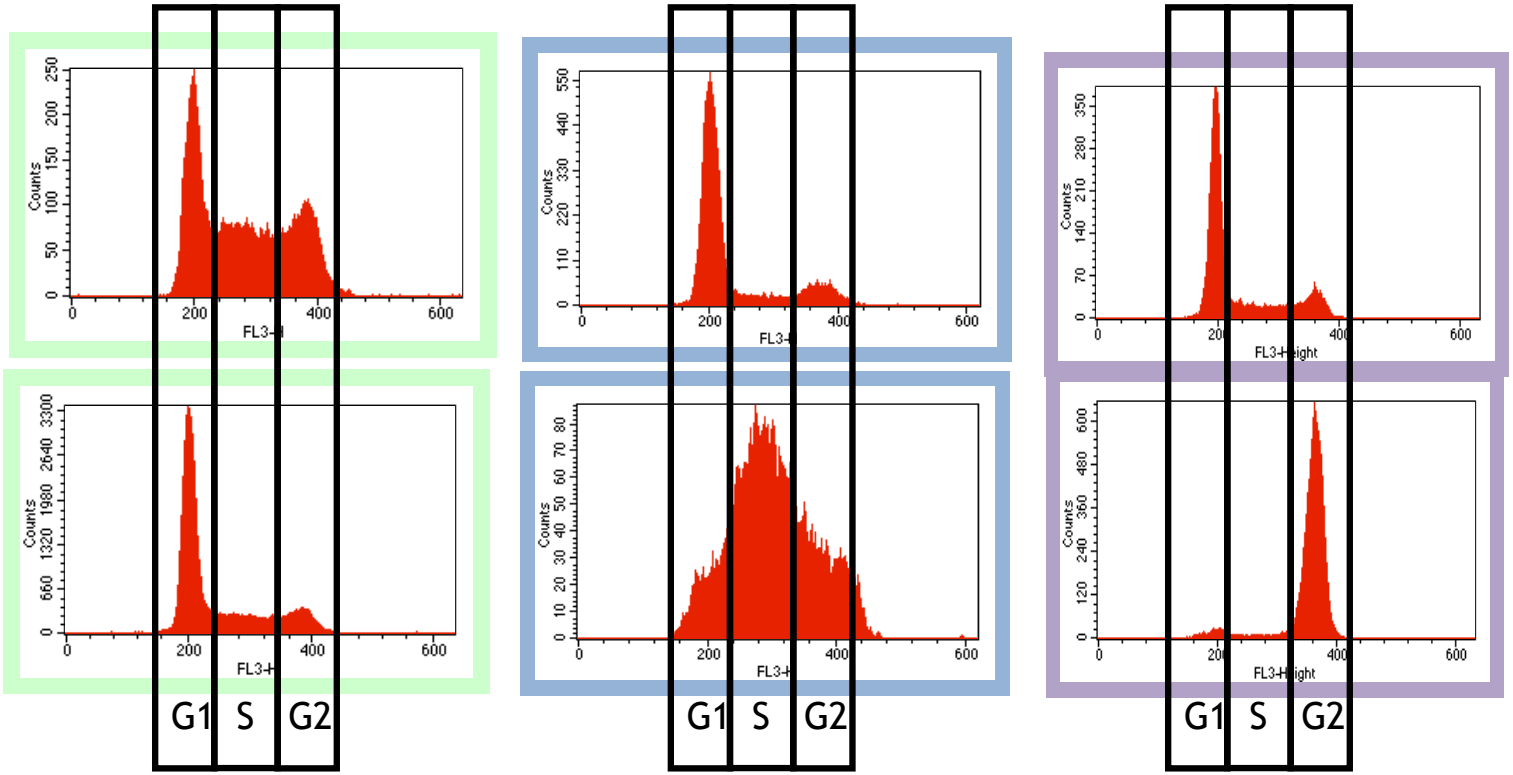
DNA stained with propidium iodide



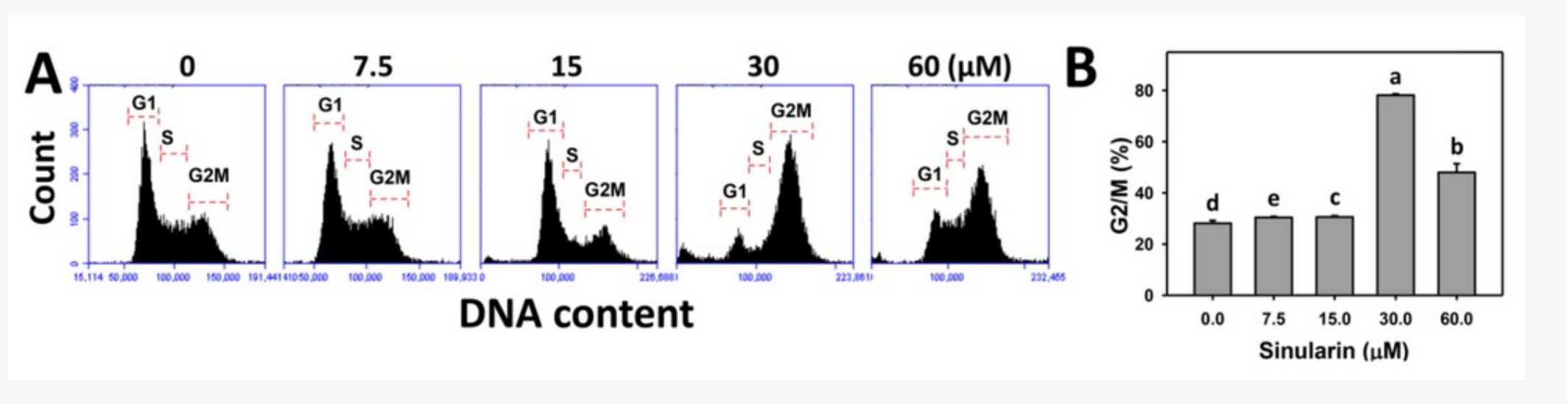
Example 1: Compare cycles

Example 2: S phase block

Example 3: M block



Cell Cycle Perturbations

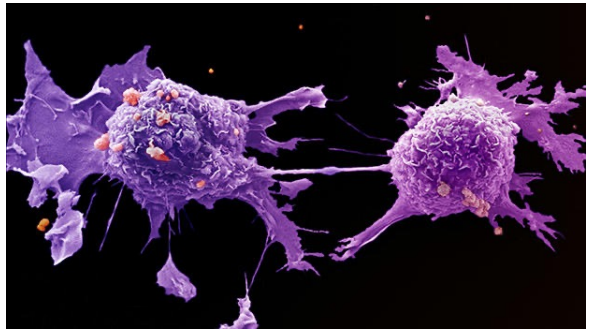


Huang et al 2018, Molecules, 23, 1670

DNA analysis in the clinic

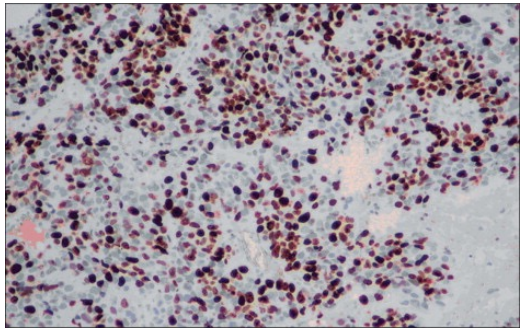
Many tumours show altered DNA content

Diploid index may have prognostic significance

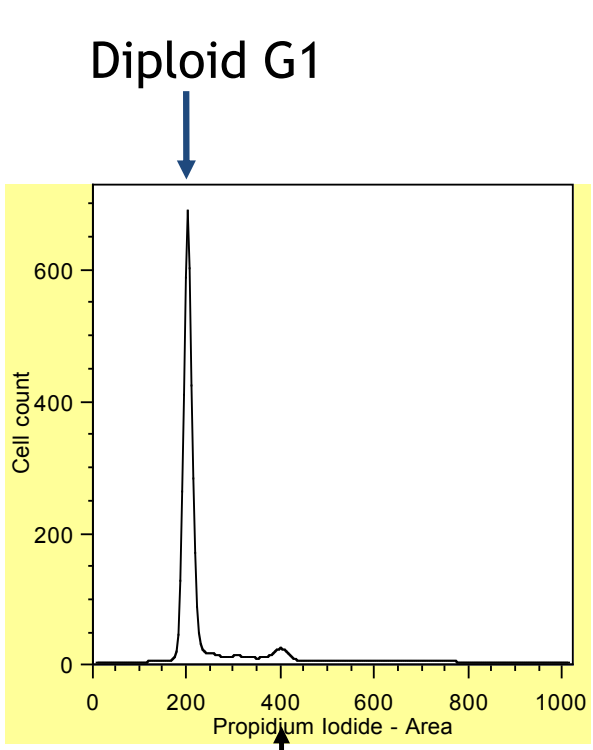


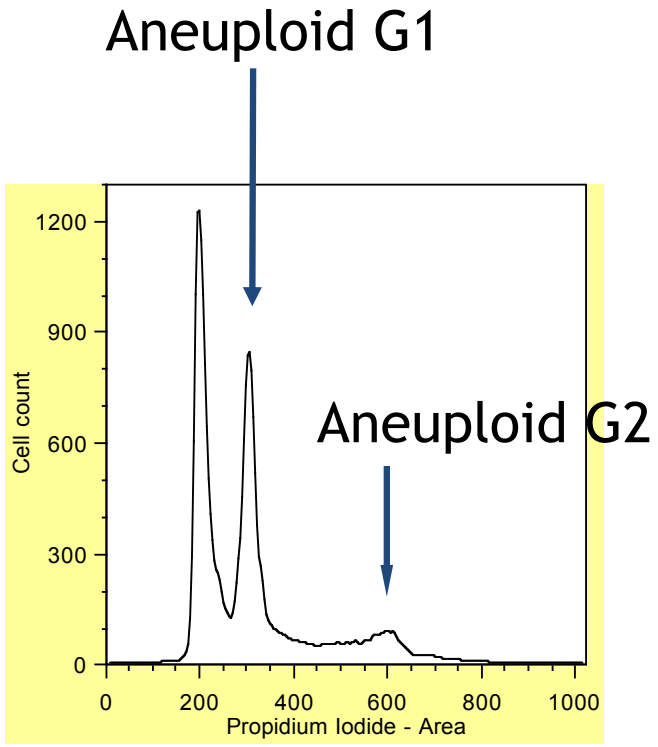
Many tumours show increased proliferation

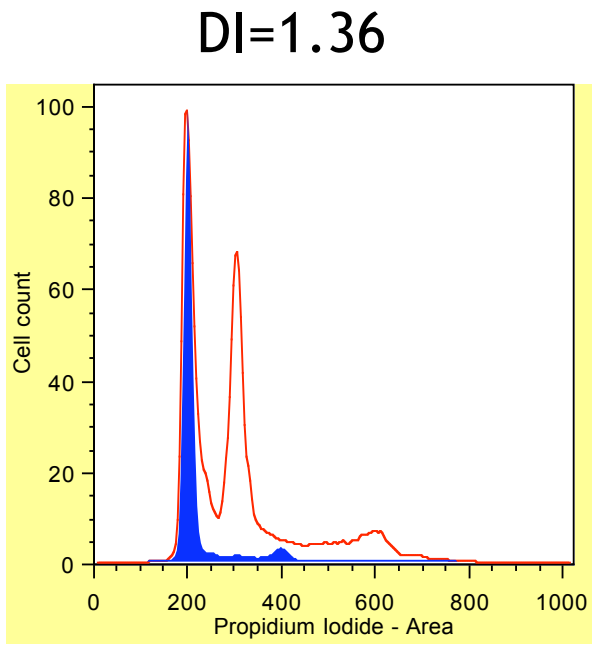
S phase fraction may have prognostic significance



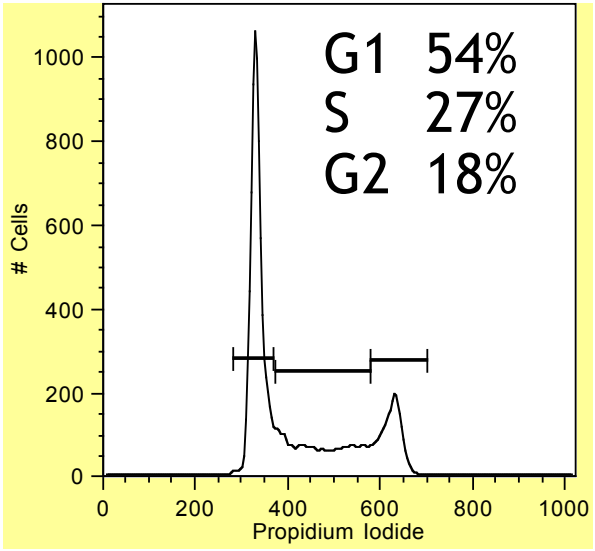
DNA analysis in tumours: Aneuploid colorectal carcinoma



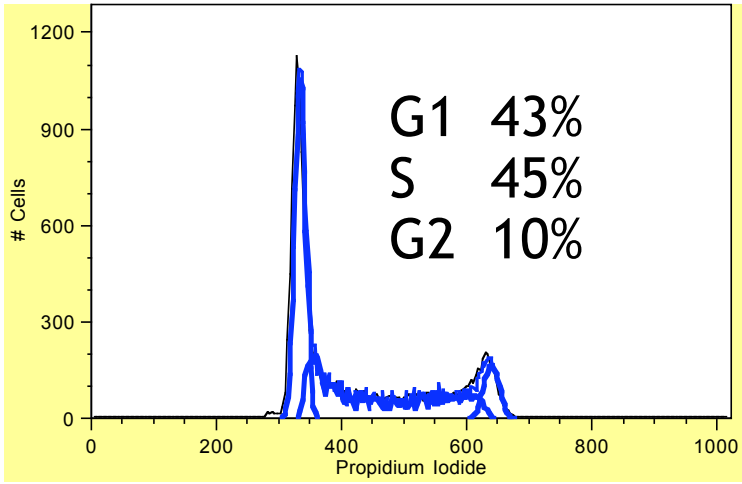




Analysis of DNA histograms - pitfalls and a better approach…



The use of markers gives a good indication but is only an estimate!



Mathematical modeling is a better approach but still not ideal!

Computer estimation of cell cycle phases

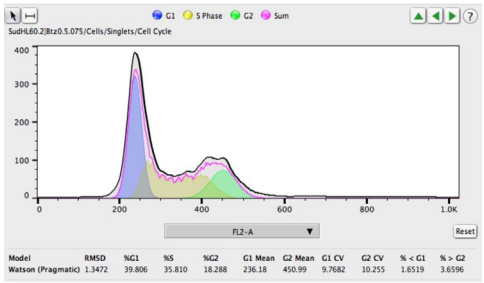
Several algorithms and programs available

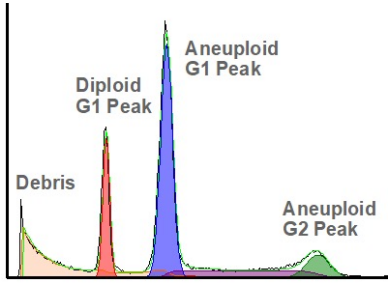
FlowJo

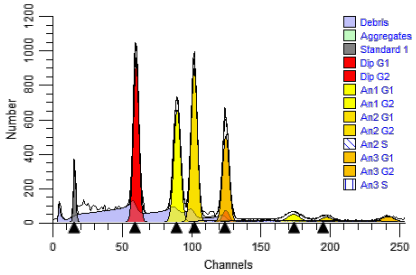
FCS Express

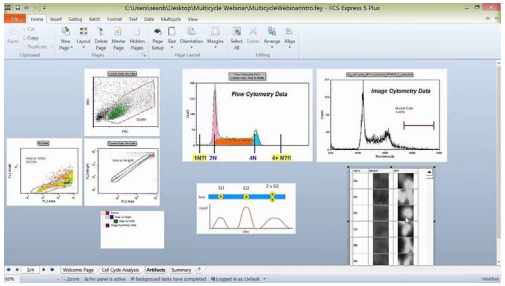
ModFit

MultiCycle

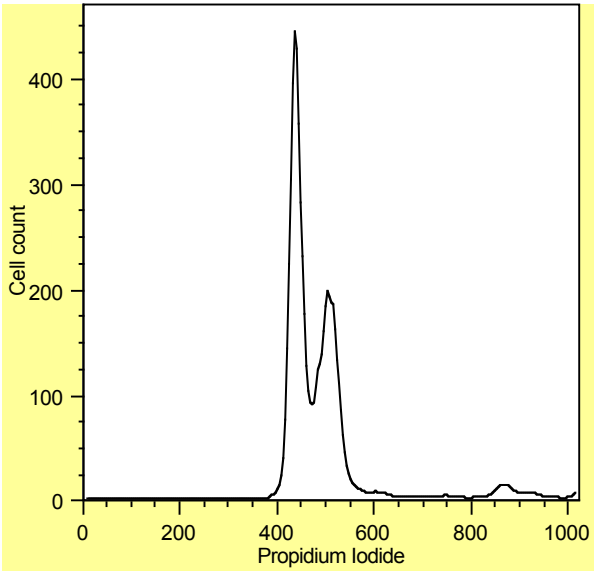


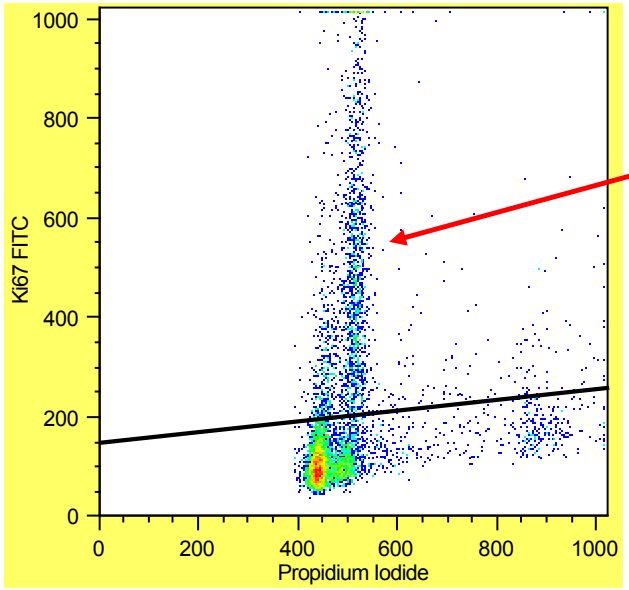






DNA analysis in tumours: DNA isn't always enough

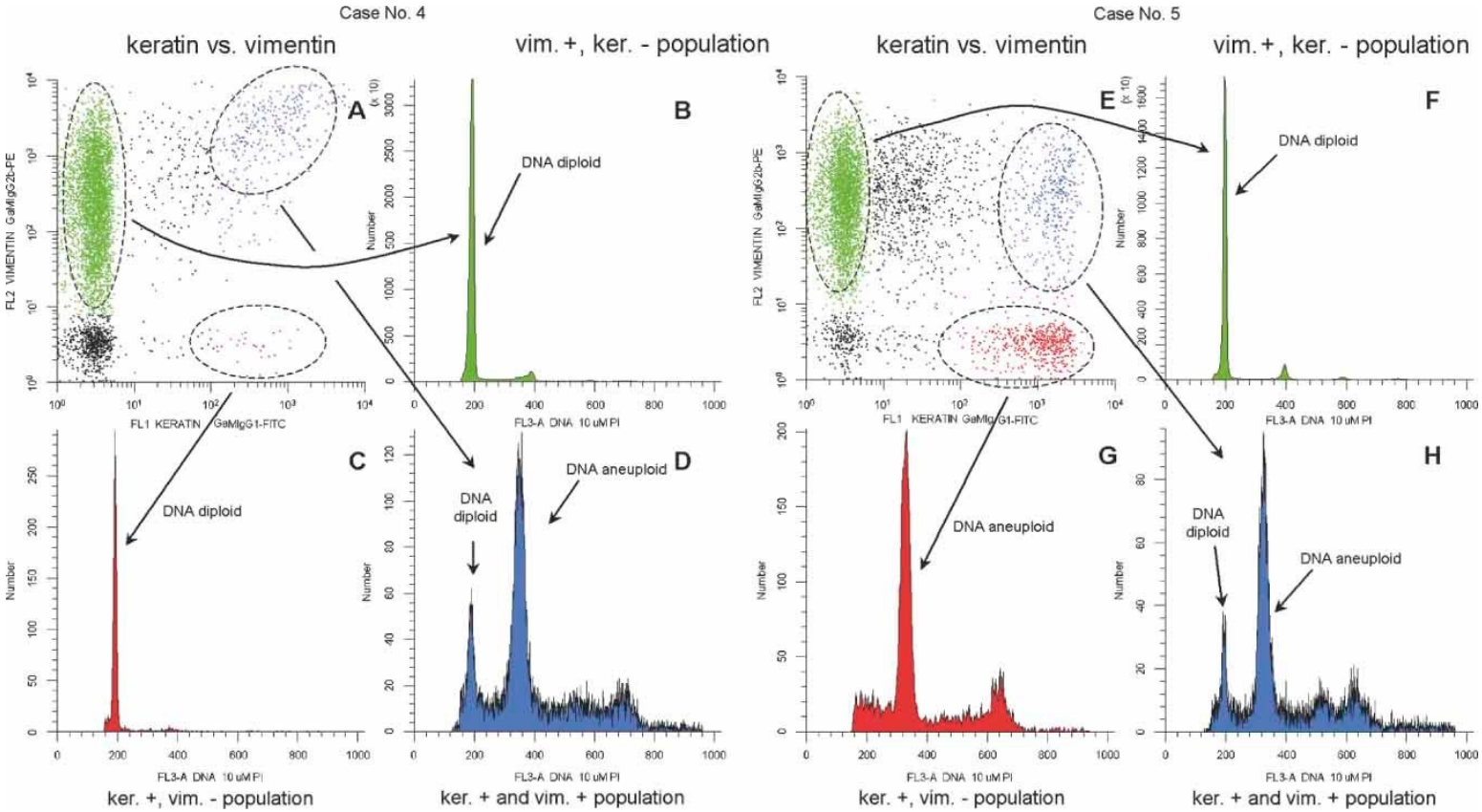




Ki-67 +ve

DNA plus antigen staining

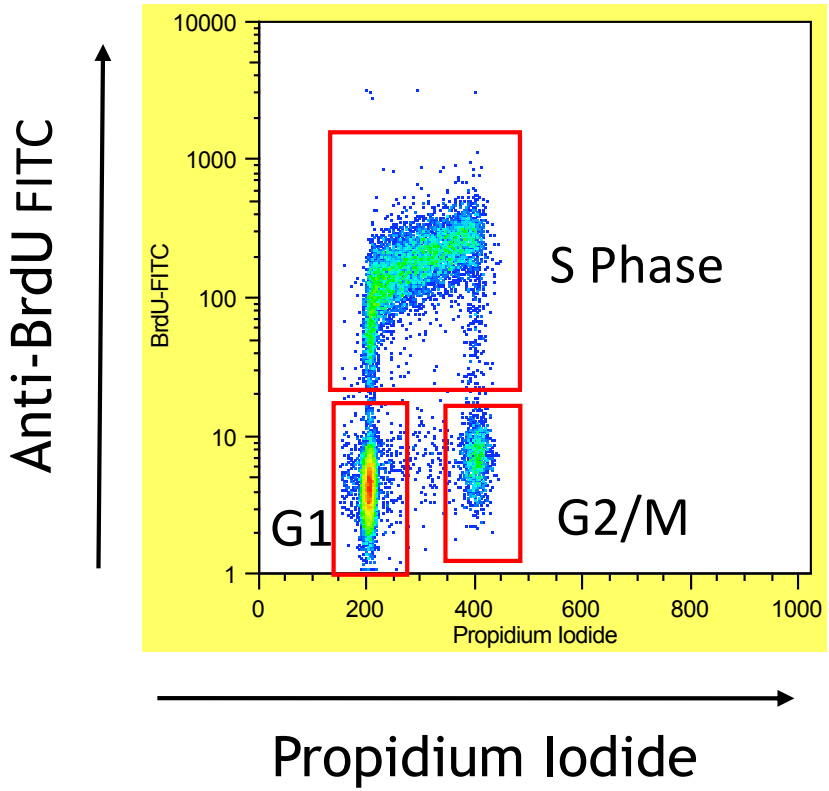
The more colours, the more information….



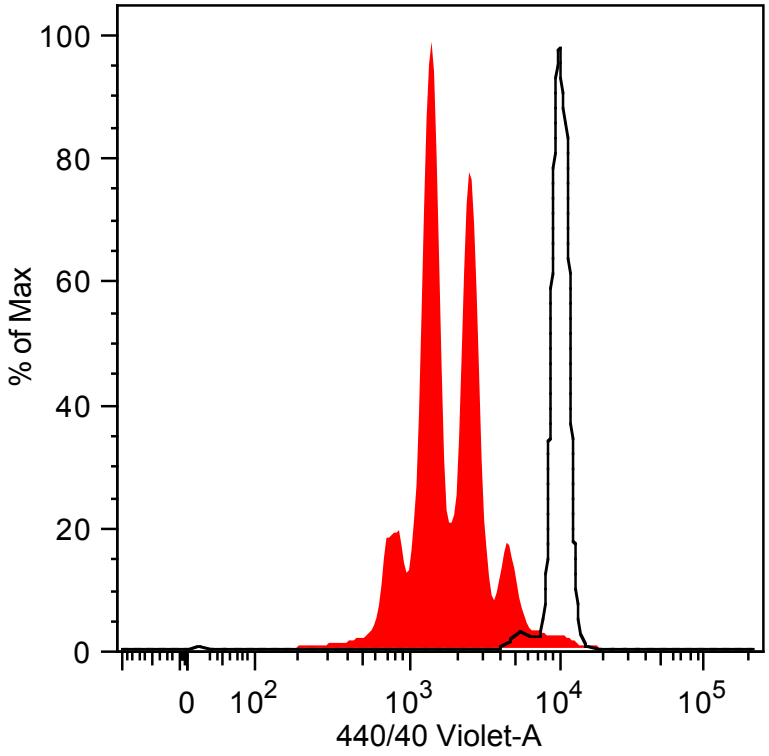
Corver et al J Pathol 2005; 206: 233–241

Advanced proliferation studies

Thymidine analog incorporation



Dye dilution

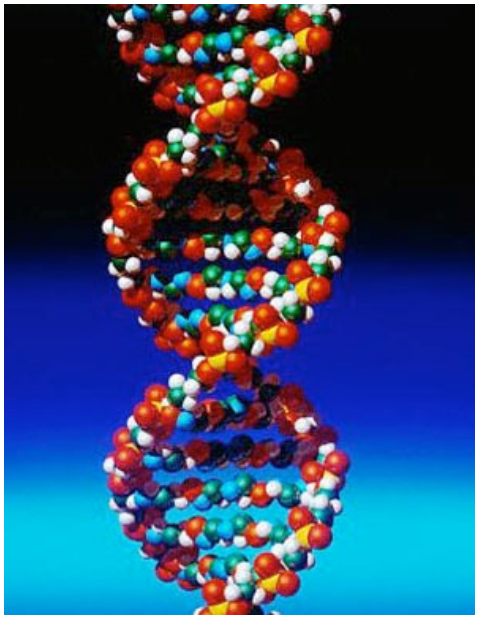


Summary and contacts

DNA content analysis by flow cytometry is widespread and relatively straightforward and can be run on ANY flow cytometer BUT…

Always think about:

- Biology
- Sample preparation
- The cytometer
- Data analysis/interpretation



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