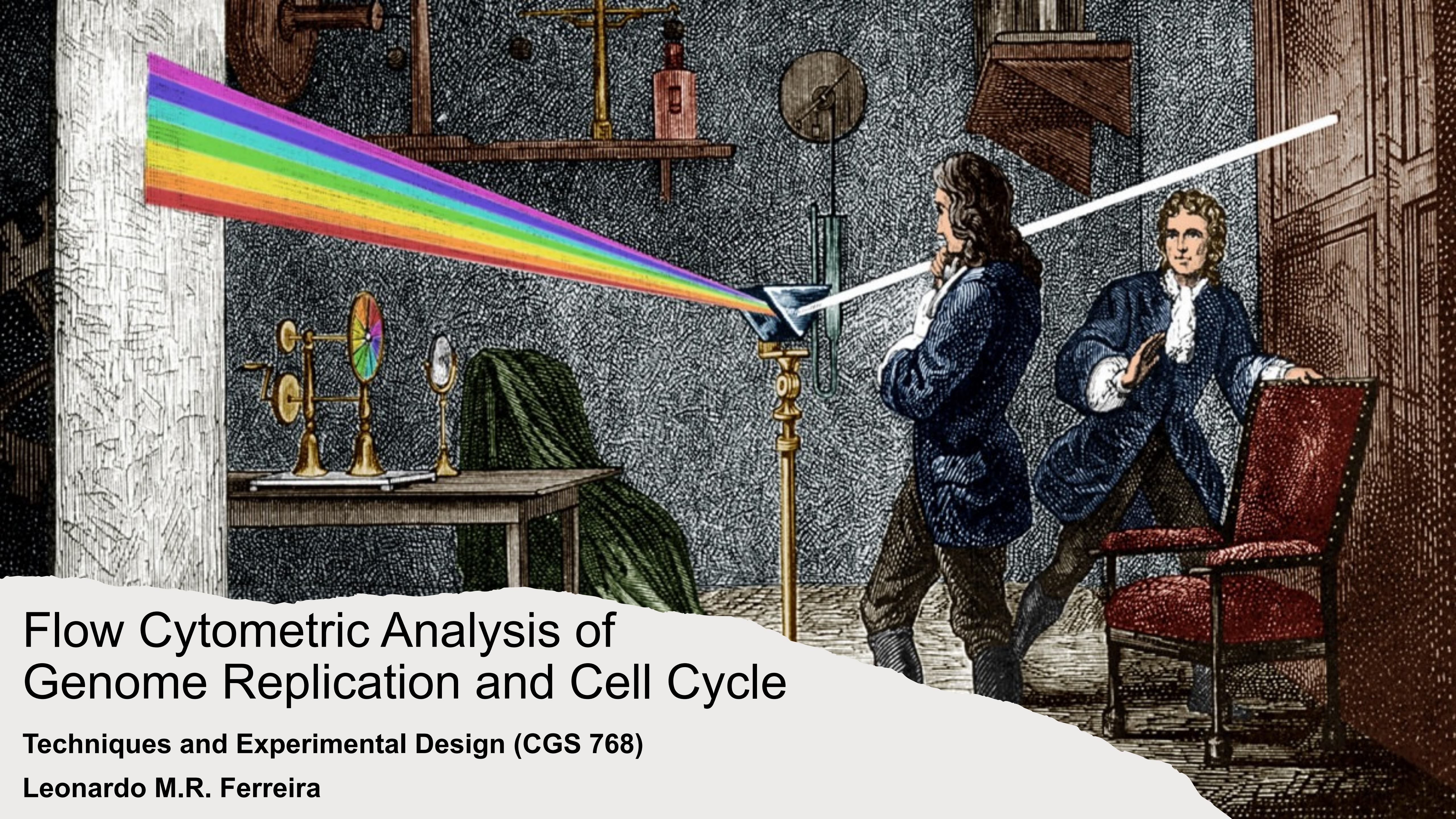


Flow Cytometric Analysis of
Genome Replication and Cell Cycle
Techniques and Experimental Design (CGS 768)
Leonardo M.R. Ferreira

Overview

1. Motivation
2. Cell viability
3. Cell proliferation
4. Cell cycle and DNA replication
5. Cell death
6. Flow cytometry
7. Take-home messages

Why analyze cell viability, division, or death?

- We all come from a single cell – development
- Many diseases consist of the loss of one or more cell types – degenerative diseases
- Some diseases are caused by unwanted proliferation of cells – cancer
- Most genes affect cell size, shape, and/or number

How can we analyze these properties?

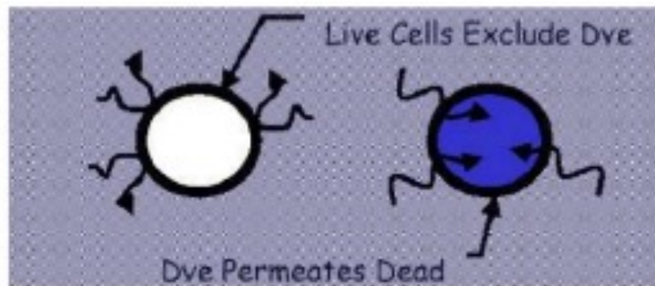
- Microscopy
- Cell counting
- Colorimetric enzymatic assays
- Flow cytometry

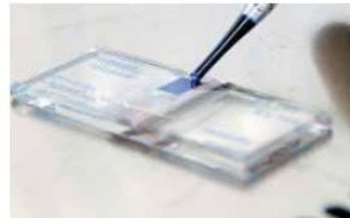
Cell viability

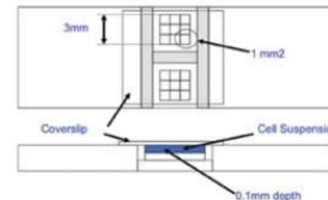
- Dye exclusion
- Cell division
- Metabolic activity

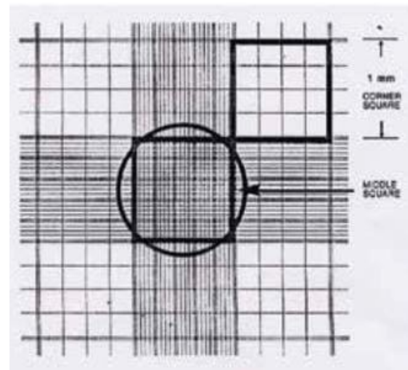
Dye exclusion and cell counting

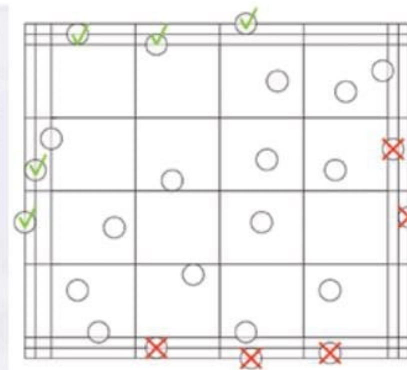
- Some dyes are too large to enter cells with an intact membrane, e.g. Trypan Blue. Hence, dye exclusion indicates a cell is viable.

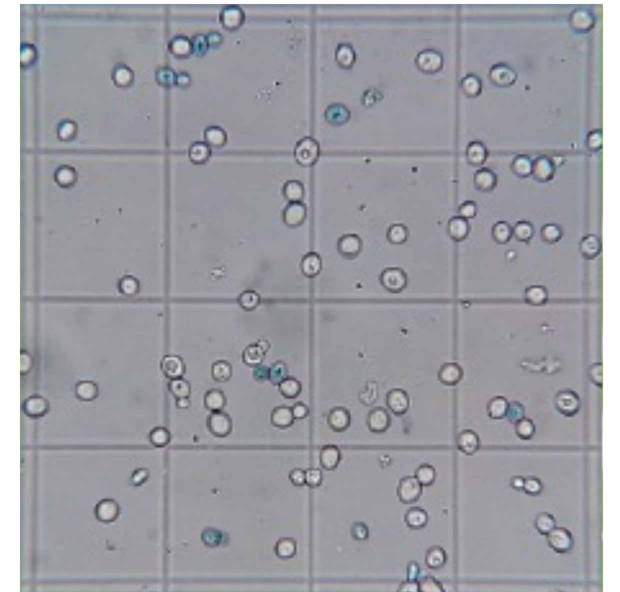






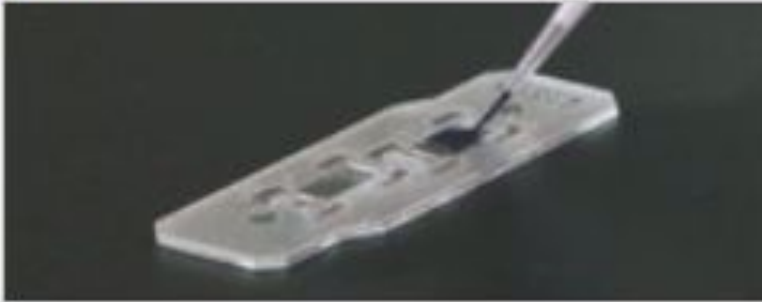






Automated cell counting

Load



Load the sample onto a slide.

Insert

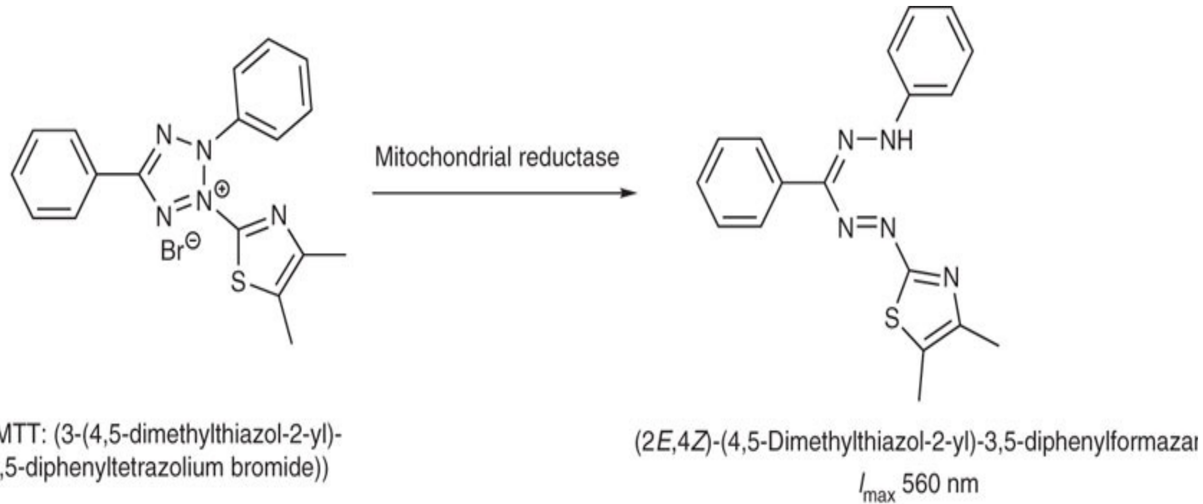
Insert the slide into the TC20 cell counter; counting automatically begins.

View results

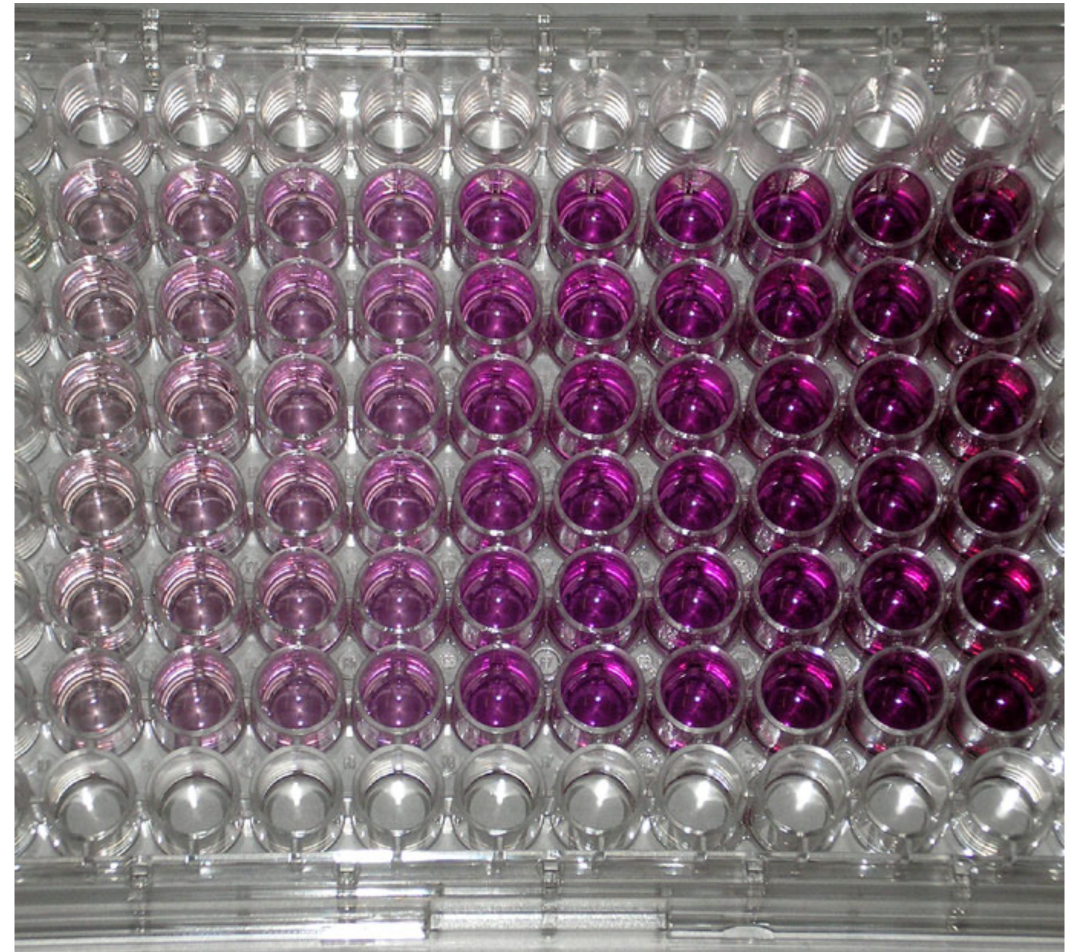
Obtain a total cell count (without trypan blue) or total and live cell counts (with trypan blue) in 30 seconds.

Automated cell counting

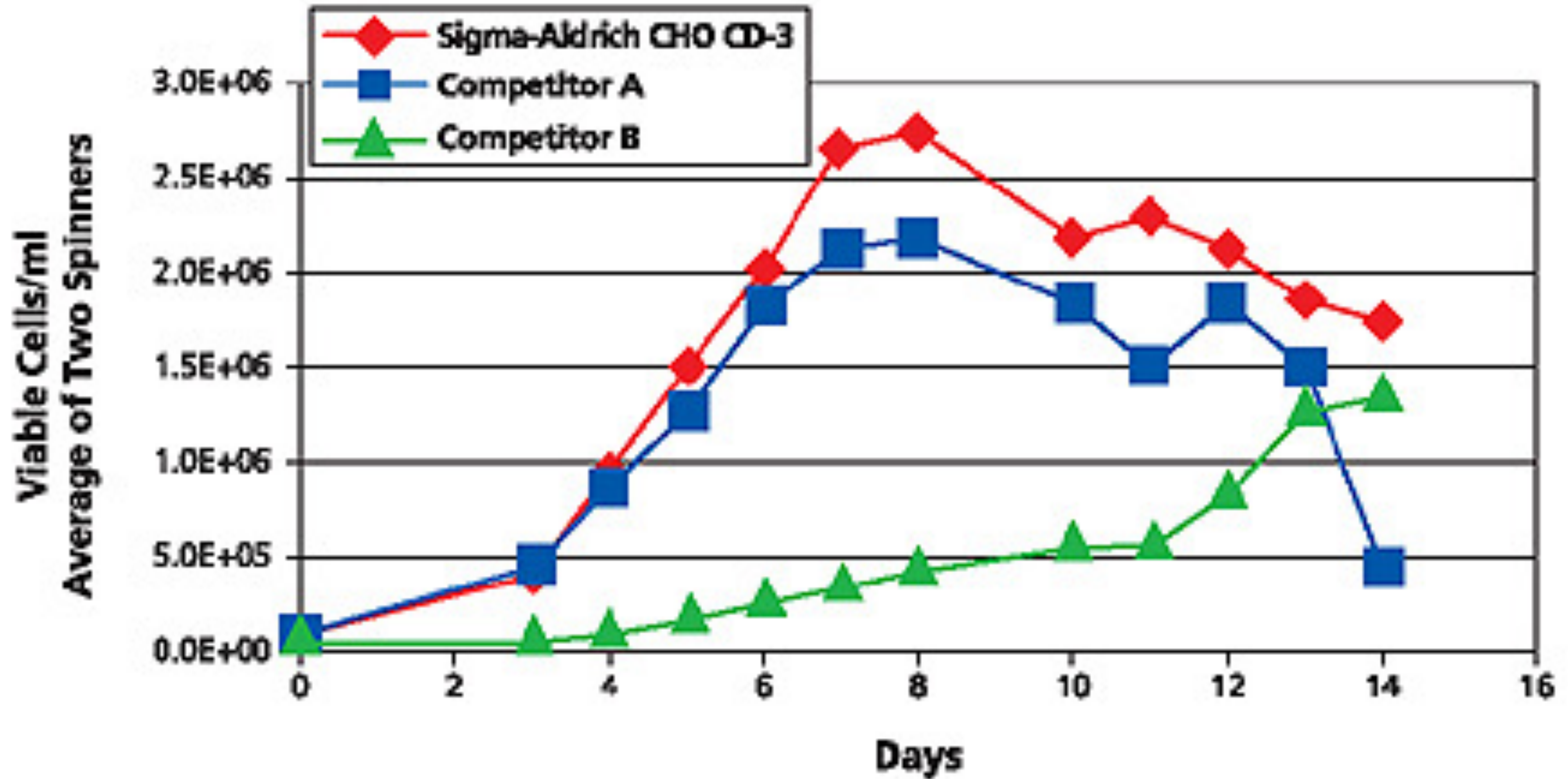
MTT assay for cell viability and proliferation



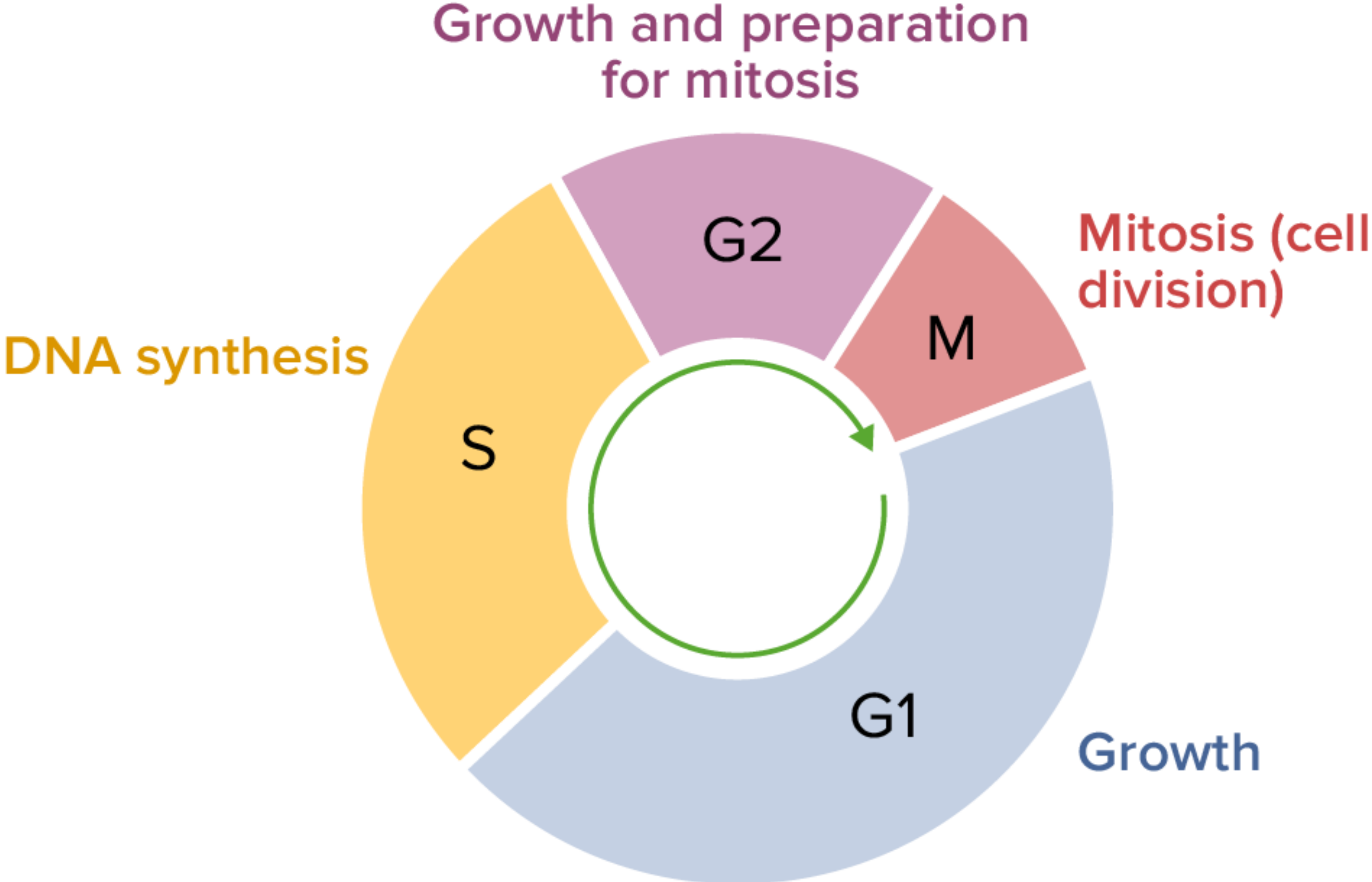
Mitochondrial reductase in viable cells converts MTT to formazan. Formazan is purple, emits light in the near red wavelength, and can be read by a plate reader



Cell growth curves



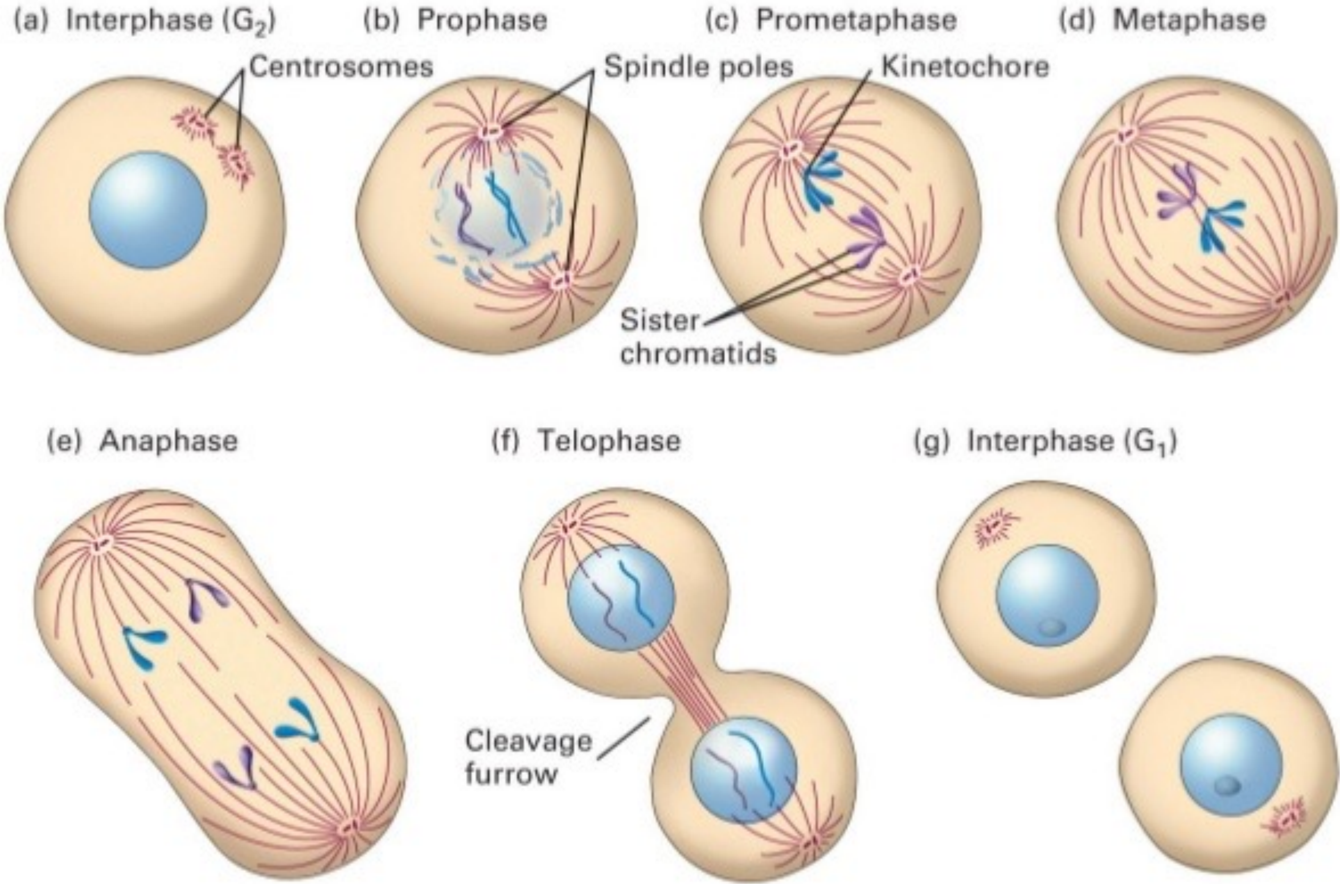
Cell cycle



Cell cycle

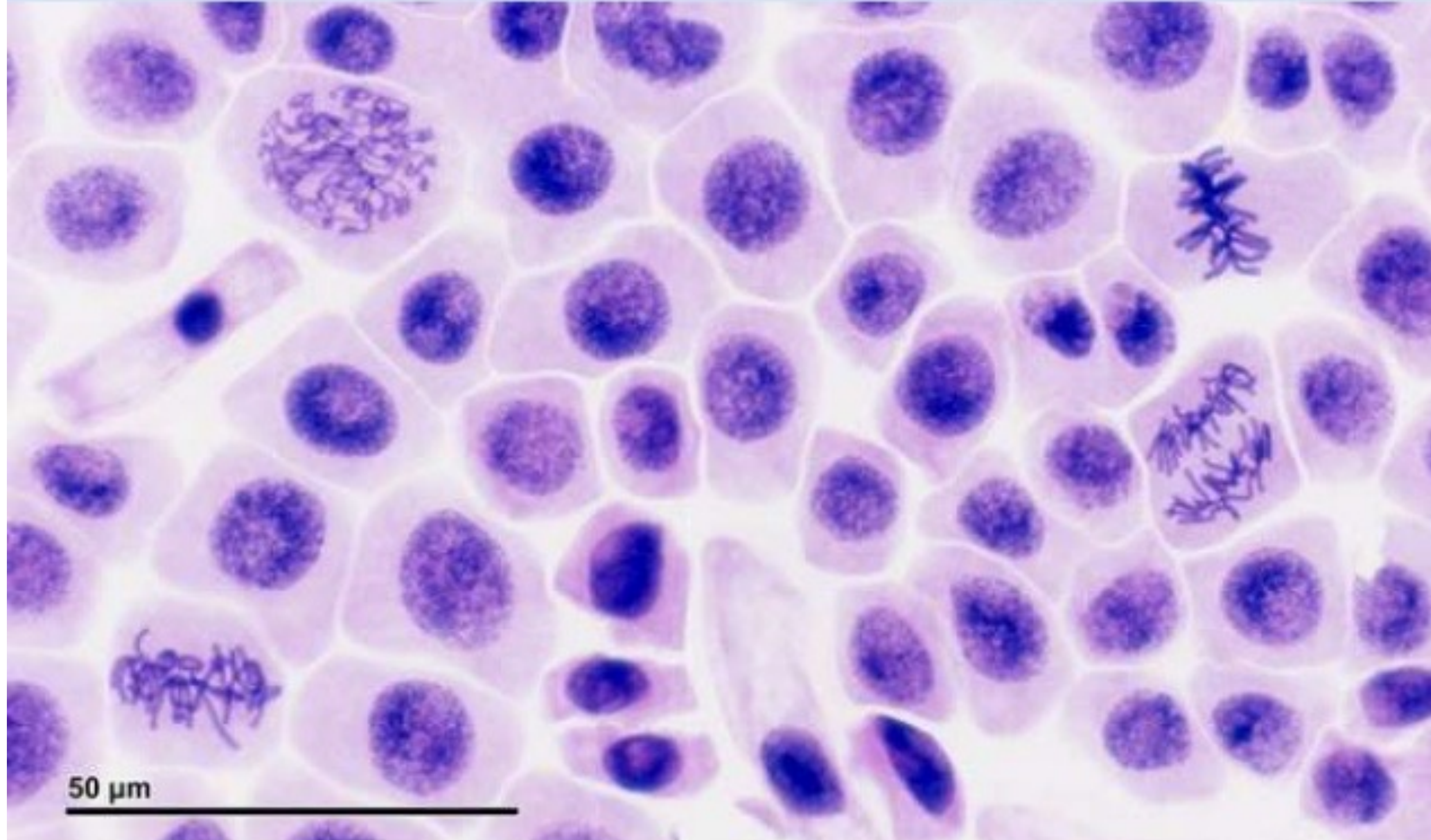
- Interphase G1 – preparation for next round of cell division (some cells exit the cell cycle at this point – G0)
- S-phase – active replication of genomic DNA
- Interphase G2 – organelle replication but not yet actively dividing
- M-phase (mitosis) – active cell division

Cell cycle



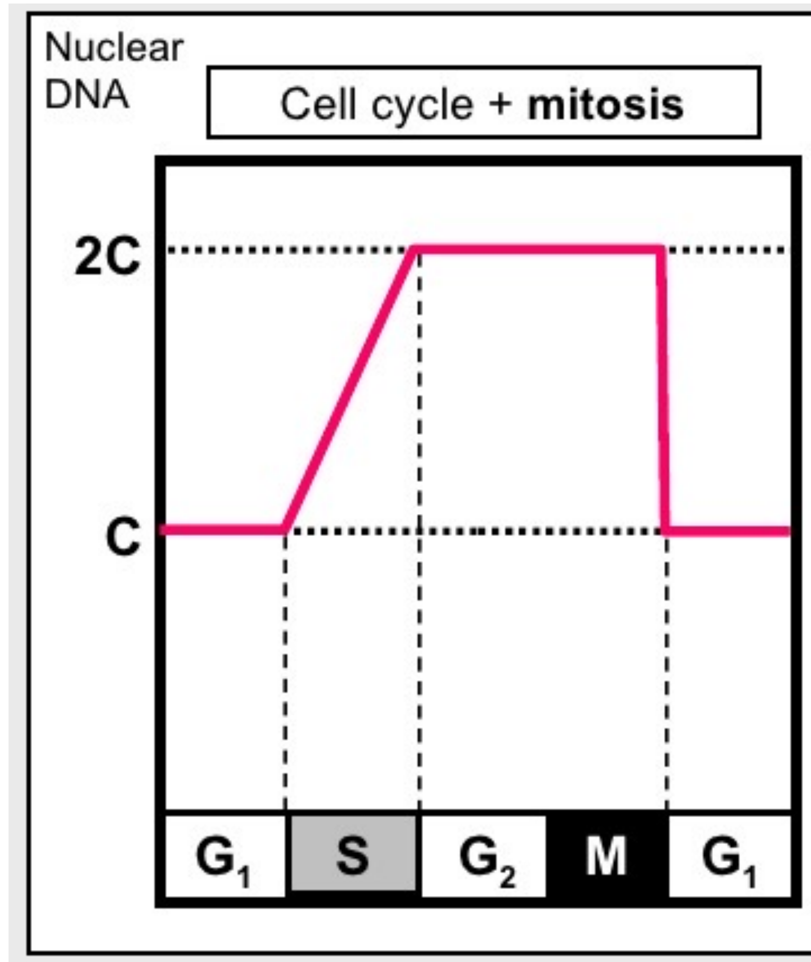
Mitotic index

1.6.9 Determination of a mitotic index from a micrograph.



$$\text{Mitotic index} = \frac{\text{number of cells in mitosis}}{\text{total number of cells}}$$

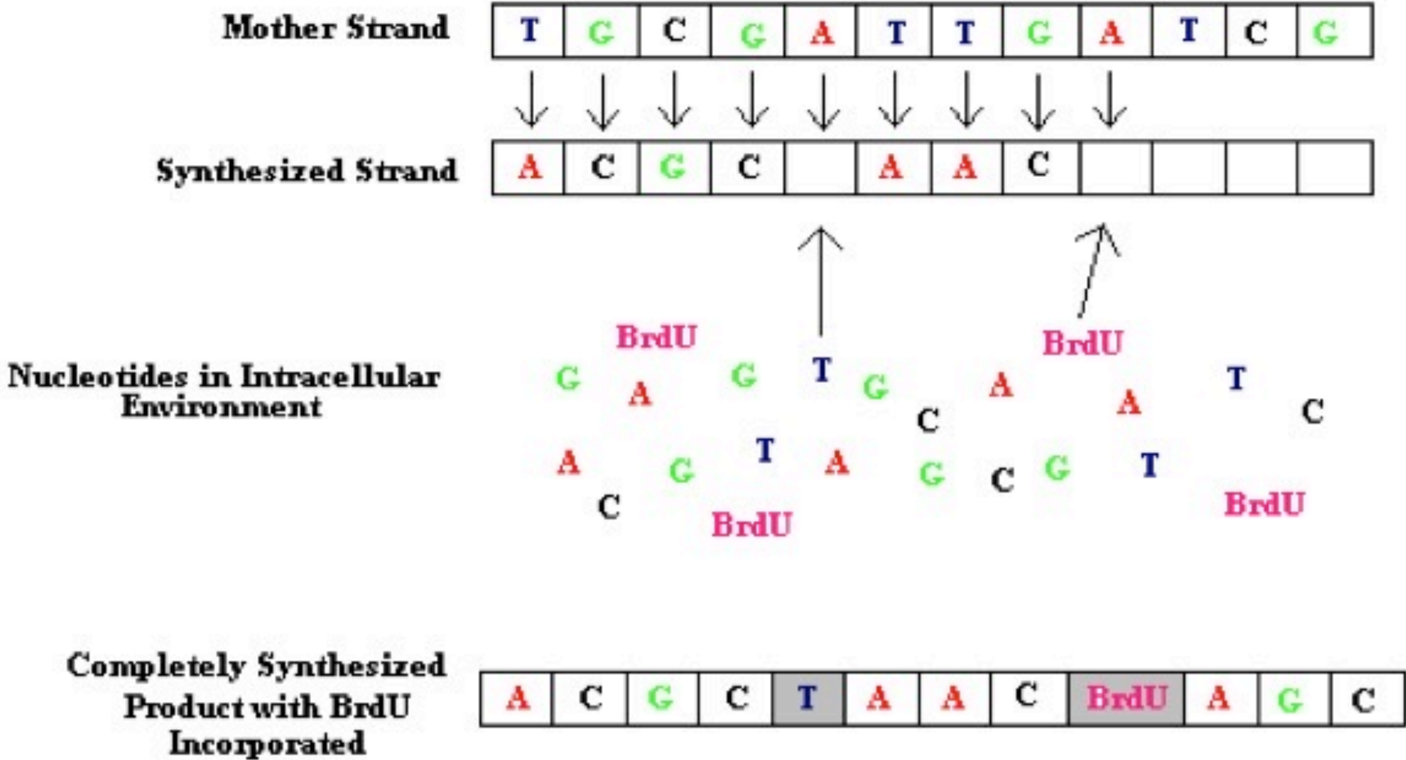
DNA content and cell cycle



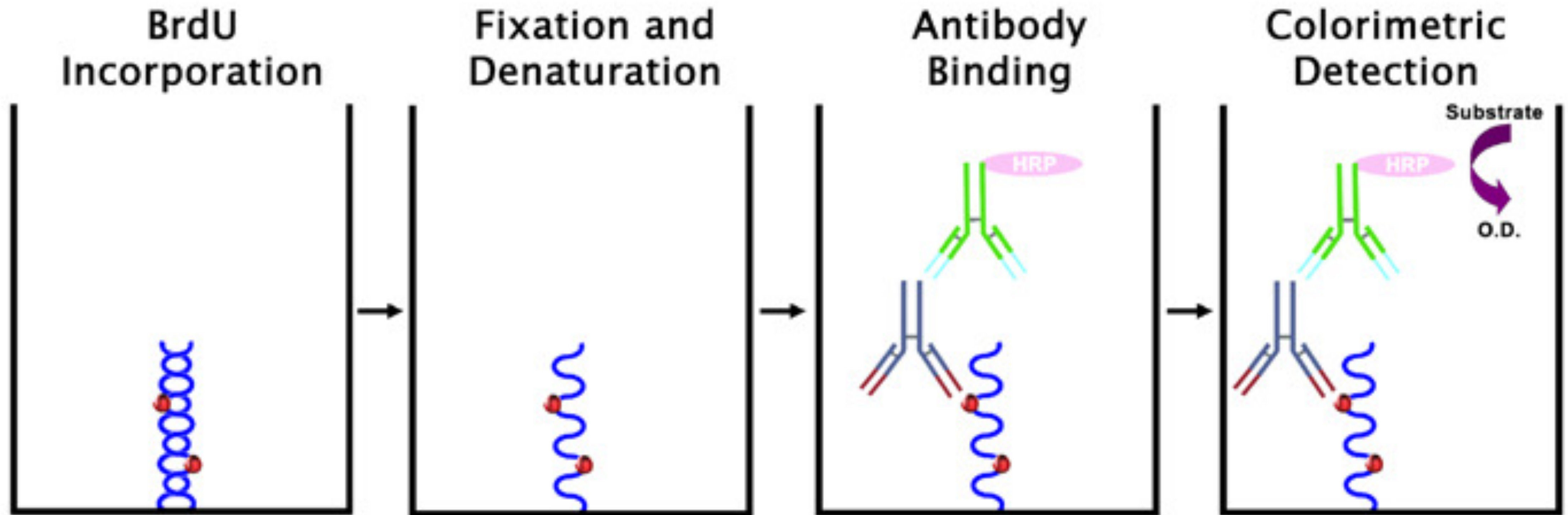
Total nuclear DNA content doubles during S-Phase and then returns to normal levels after mitosis.

BrdU incorporation

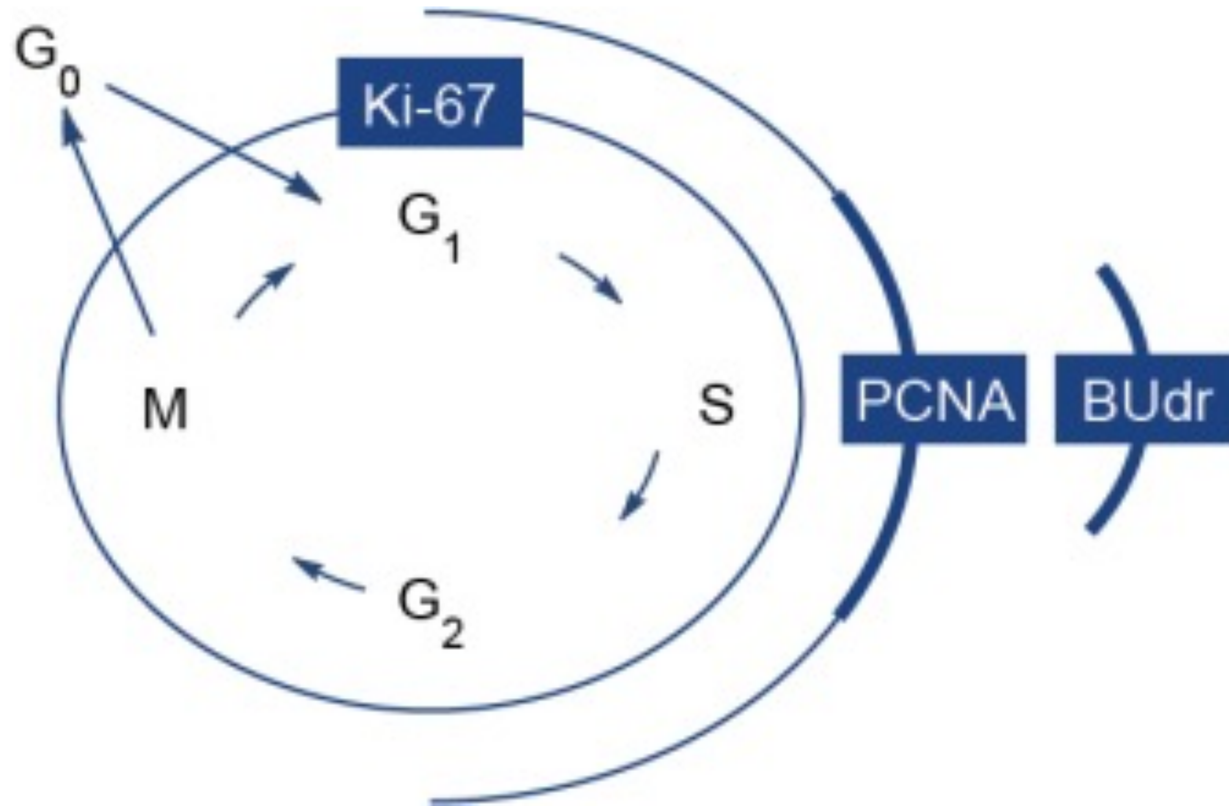
- Bromodeoxyuridine (5-bromo-2'-deoxyuridine, BrdU) and 5-ethynyl-2 deoxyuridine (EdU) are synthetic analogs of thymidine



BrdU detection



Labeling cells in different phases of the cell cycle



Ki -67 is absent from cells in rest phase, marking cells in any phase of proliferation

BrdU marks cells in S-Phase

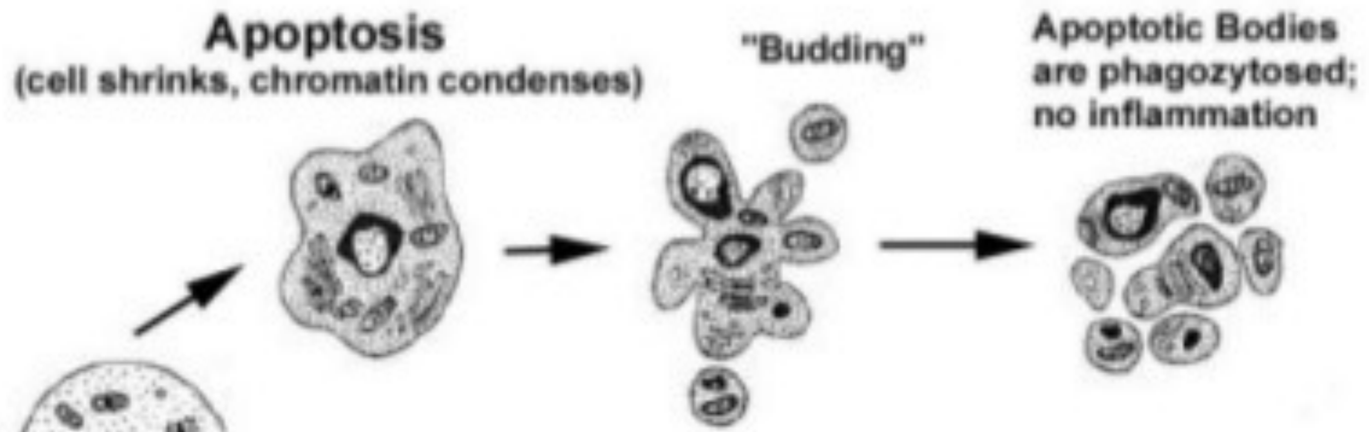
PCNA marks all proliferating cells except those actively dividing (M-phase)

Cell death

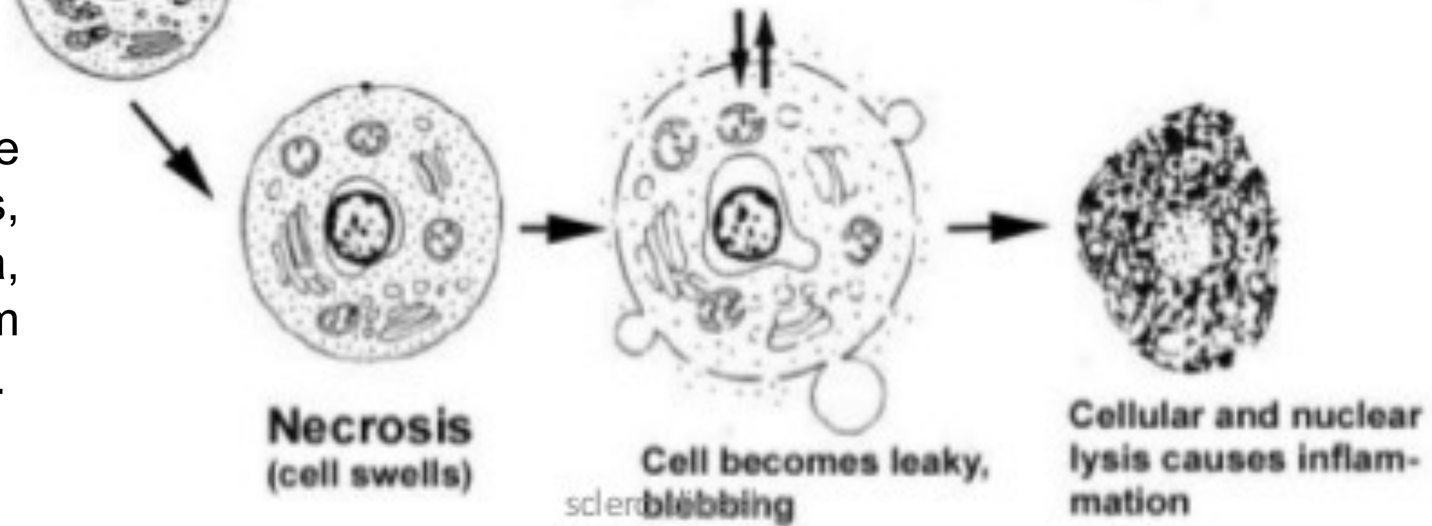
- Loss of cell membrane integrity – uptake of Trypan Blue
- Failure of electron transport chain – low mitochondrial transmembrane potential
- Cleaved caspase-3 – apoptosis
- Condensed chromatin
- DNA fragmentation – TUNEL assay

Main cell death mechanisms

Programmed cell death



Unexpected cell damage caused by toxins, radiation, heat, trauma, hypoxia resulting from blood flow blockade, etc.



Programmed cell death

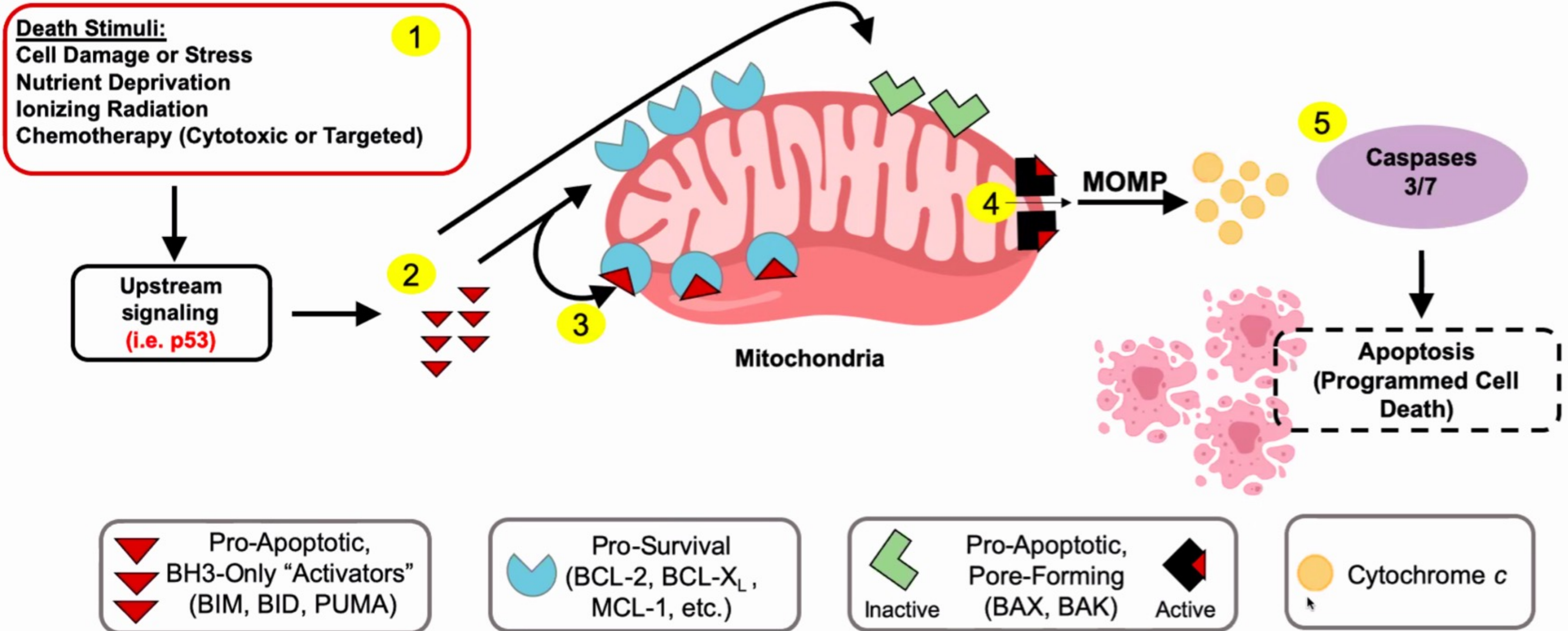
- Any type of cell death that is mediated by an intracellular, genetic "program".

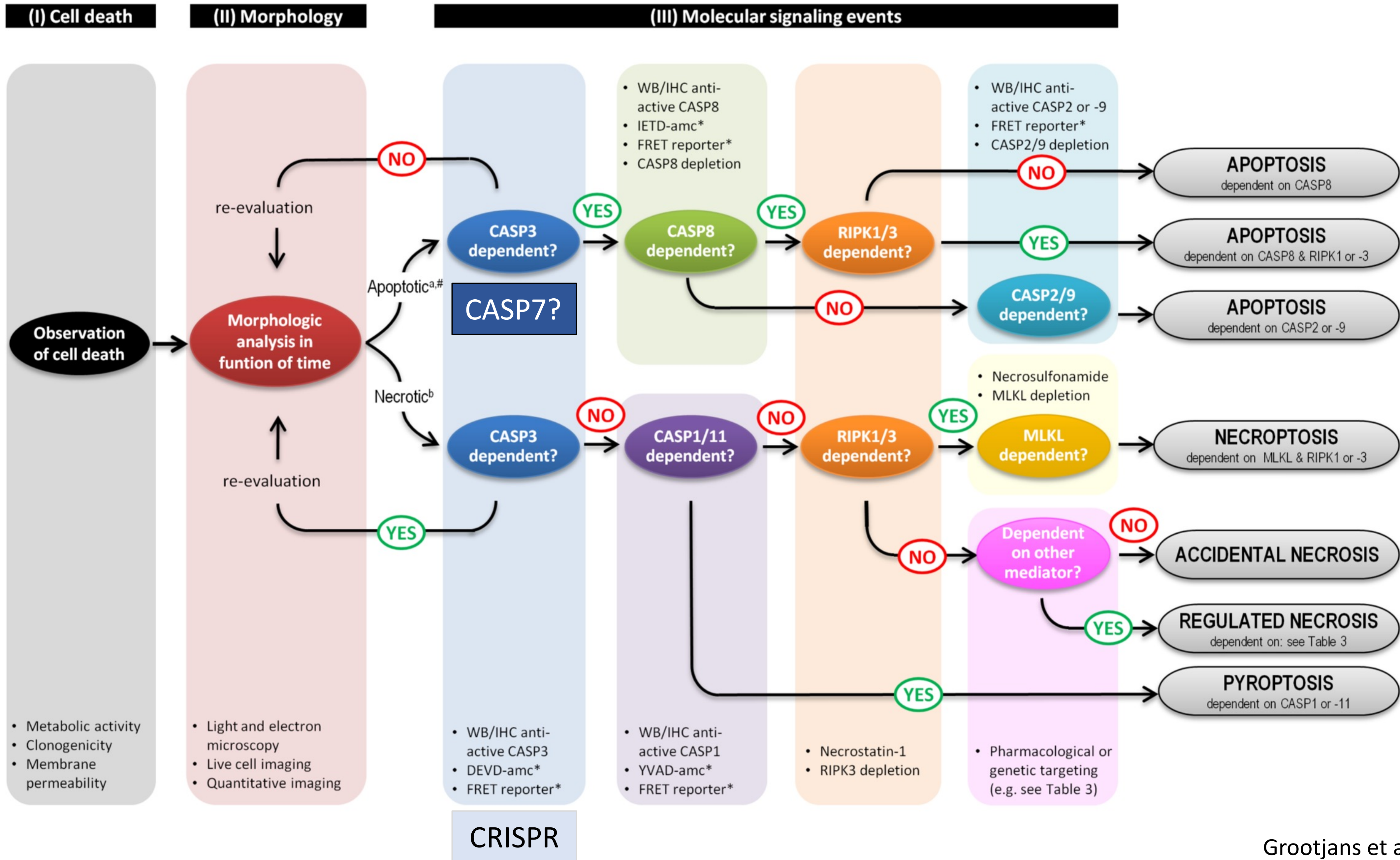
- Apoptosis is the most widely studied type of programmed cell death

  - Like necrosis, apoptosis is marked by specific morphological changes

  - Apoptosis is mediated by a cascade of genetic changes that mark it for death

Apoptosis





Grootjans et al, 2014, in *Cell Death in Biology and Diseases*

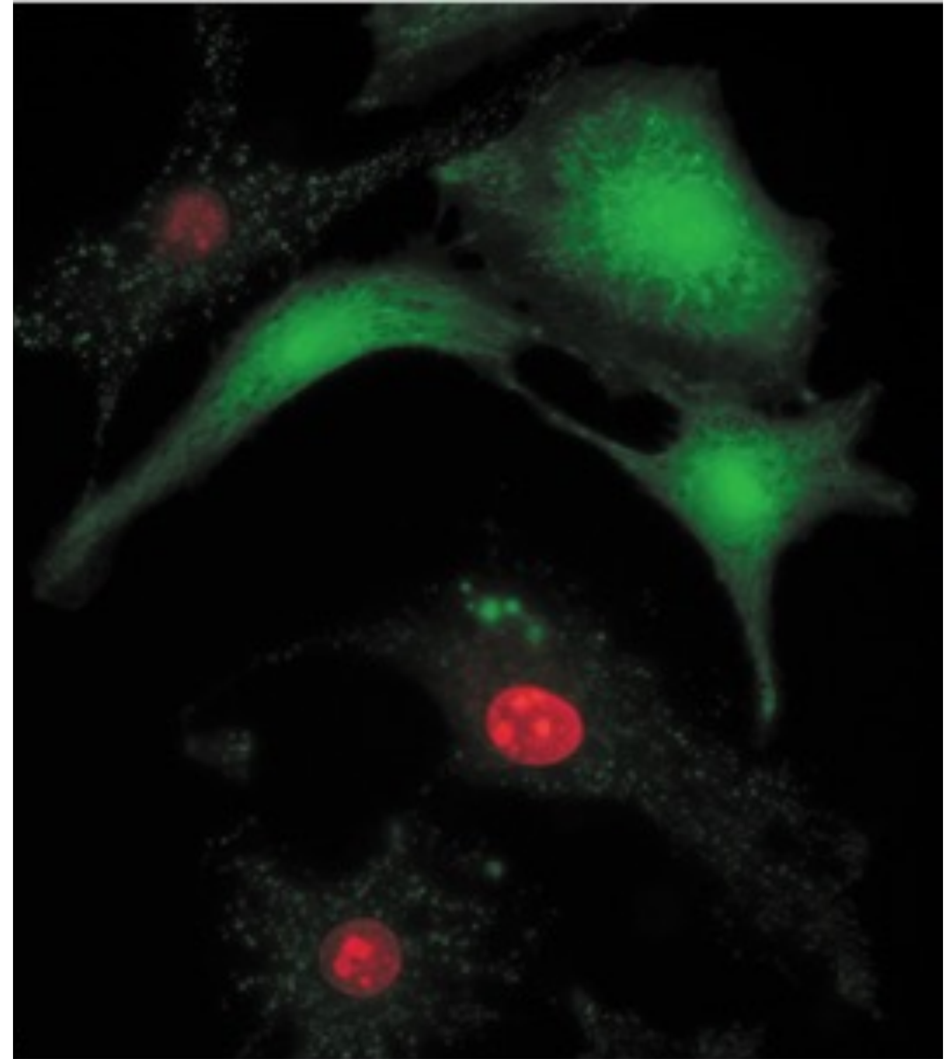
Cell death assays

- Dye exclusion
- Pathway-specific antibody staining
- Microscopy
- Flow cytometry

Fluorescent dye assay

Calcein AM (Green) marks live cells. The non-fluorescent form is easily taken up by all cells and cleaved to the fluorescent form by esterases present in live cells.

Ethidium Homodimer (Red) marks the nuclei of dead cells (live cells exclude this dye).

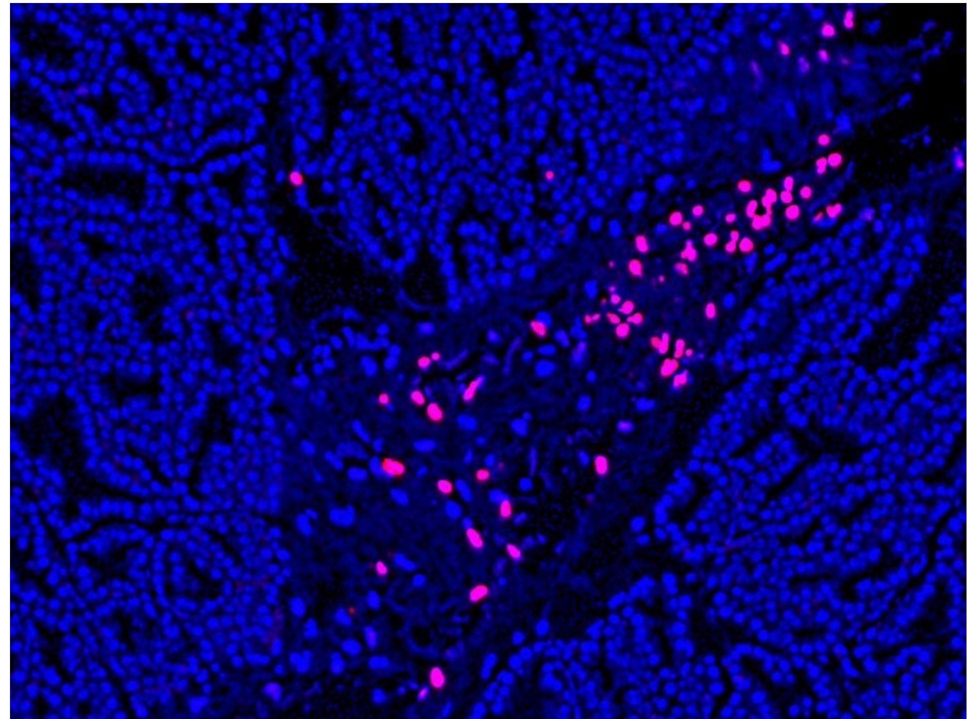


TUNEL assay

Terminal deoxynucleotidyl transferase dUTP nick end labeling

TdT is an enzyme that recognizes nicked ends of DNA.

A series of amplification steps allows the bound TdT to be visualized in a fluorescence microscope.



Needed

A technique that can be used to assess cell viability, proliferation, and death

A technique that can be used to simultaneously measure thousands if cells

A technique that is highly quantitative and reproducible

Is there one such technique?

Flow cytometry

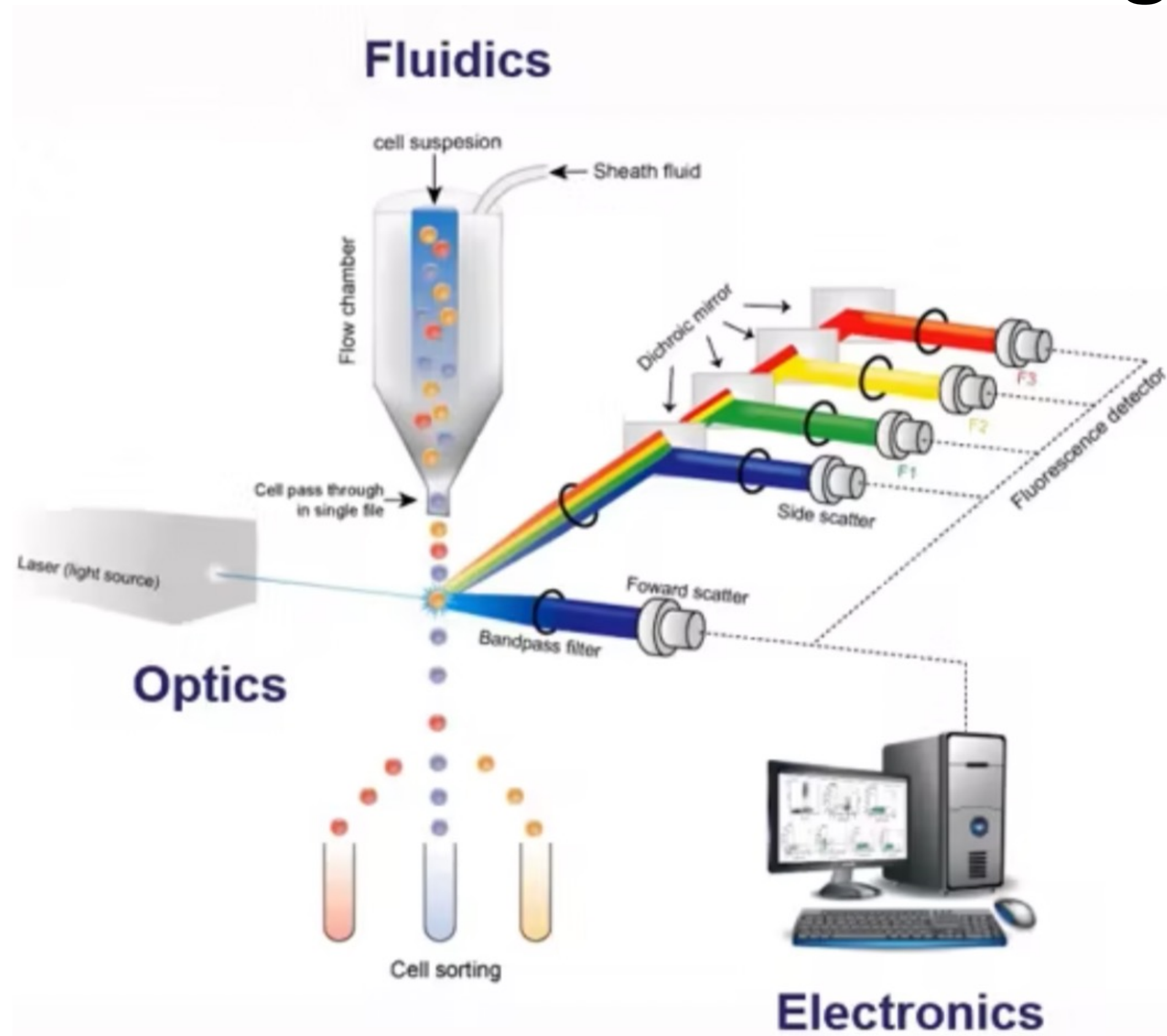
Flow cytometry/Fluorescence-assisted cell sorting (FACS) is a versatile technique

- Cell isolation
- Cell viability
- Ploidy
- Apoptosis
- DNA damage
- Autophagy
- Oncosis
- Calcium flux
- FRET
- NADH quantitation
- RNA analyses
- DNA modifications
- Dye exclusion
- Target enrichment/ multiple pass sorting
- Target multiple populations

Cell cycle
Cell senescence
Flow FISH
Chromosome Isolation
Apoptosis
Necroptosis
Necrosis
Rare event sorting
Multiplex analyses
Single cell deposition

Yeast
Bacteria /Algae
Exosomes

Fluorescence-assisted cell sorting (FACS)



Fluorescence-assisted cell sorting (FACS)

Flow cytometry

Fluorescence-assisted cell sorting (FACS)

- Cells are diluted in PBS and passed in a steady stream of single cells through capillary fluidics.

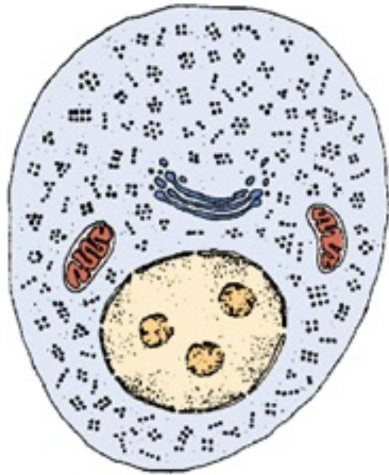
- As each cell passes through the analysis zone they are illuminated by a laser.

- Laser light is scattered by the cells and is picked up by a sensor

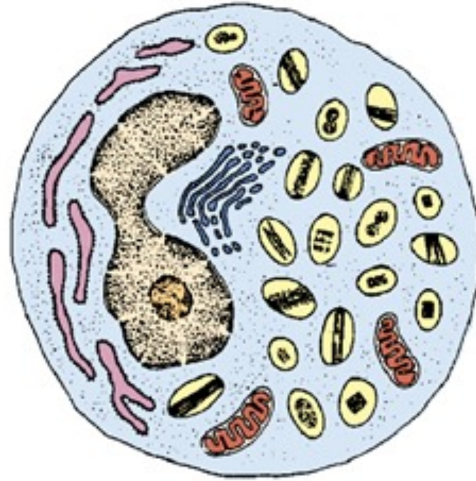
Counting cells using flow cytometry

- The number of times that the laser beam is interrupted indicates the number of cells
- The amount and direction of light scatter tell us about size and internal complexity
- One can simultaneously quantify cell viability by adding exclusion dyes
  - Propidium Iodide (PI), 7-amino-actinomycin (7AAD), DAPI, etc.
- Many other cell parameters can be queried using antibodies

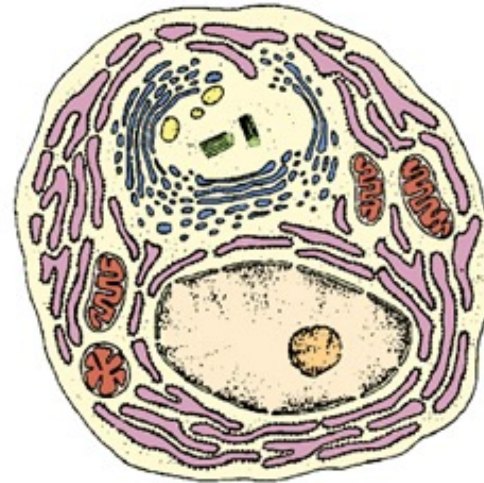
Counting specific cells using flow cytometry



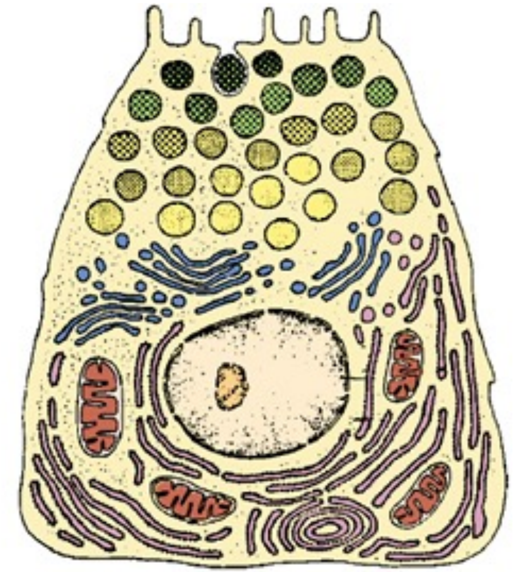
a Erythroblast



b Eosinophilic leukocyte



c Plasma cell



d Pancreatic acinar cell

Different cells can be identified by their size and shape

- Since FSC ~ size and SSC ~ internal structure, a correlated measurement between them can allow for differentiation of cell types in a heterogenous cell population

Lymphocytes

Granulocytes

Monocytes

RBCs, Debris, Dead Cells

SSC

FSC

0 50 100 150 200 250

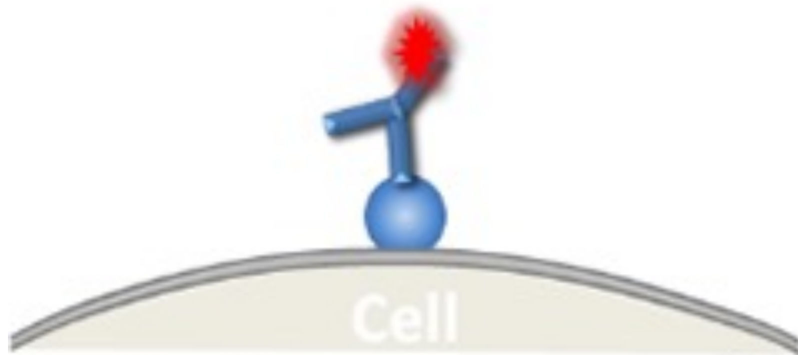
Different cells can be identified by their size and shape

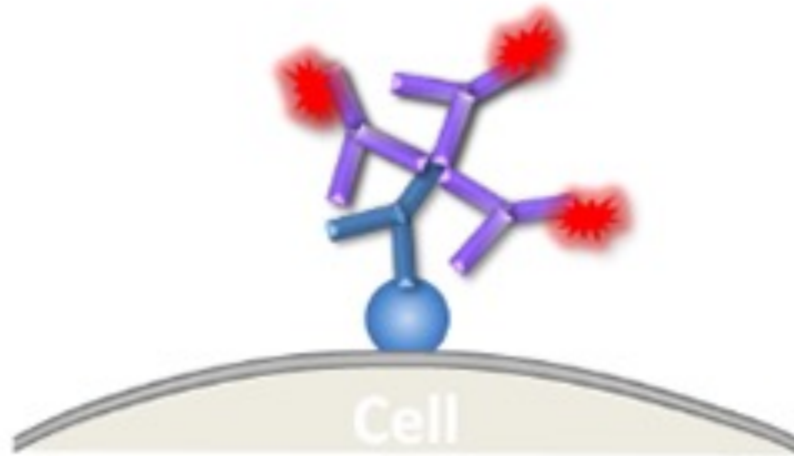
- FSC – forward-scatter – cell size
- SSC – side-scatter – cell granularity

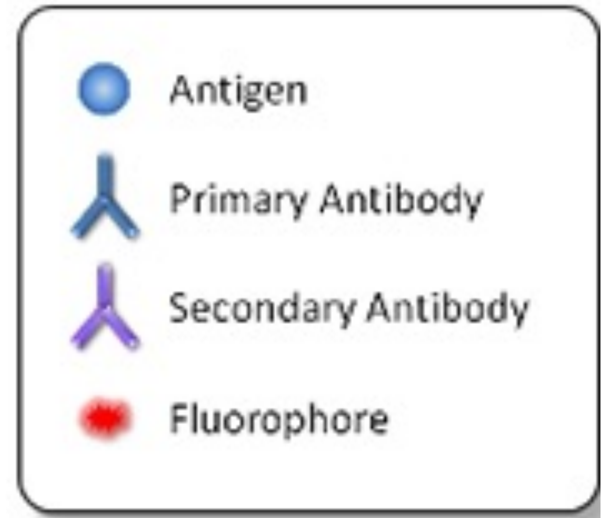
Sample preparation in flow cytometry is very important

- Prepare a single-cell suspension for labeling and subsequent analysis
- Titrate antibodies
- Use directly conjugated antibody whenever possible
- Use monoclonal antibody whenever possible
- Stain same number of cells for every sample
- Each time a new lot of antibody is acquired, repeat titration
- Fluorophore choice is crucial – compensation

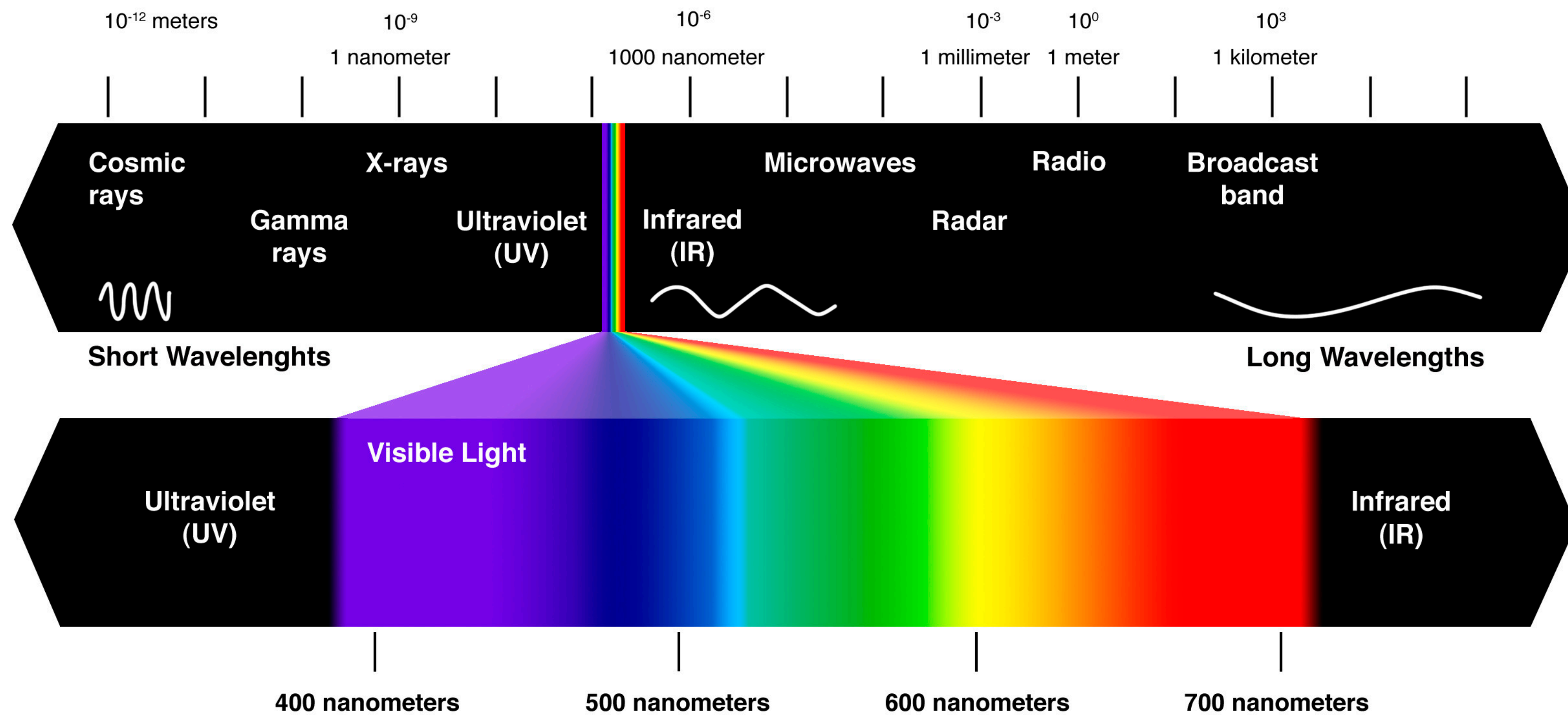
Fluorophore-conjugated antibodies for flow cytometry



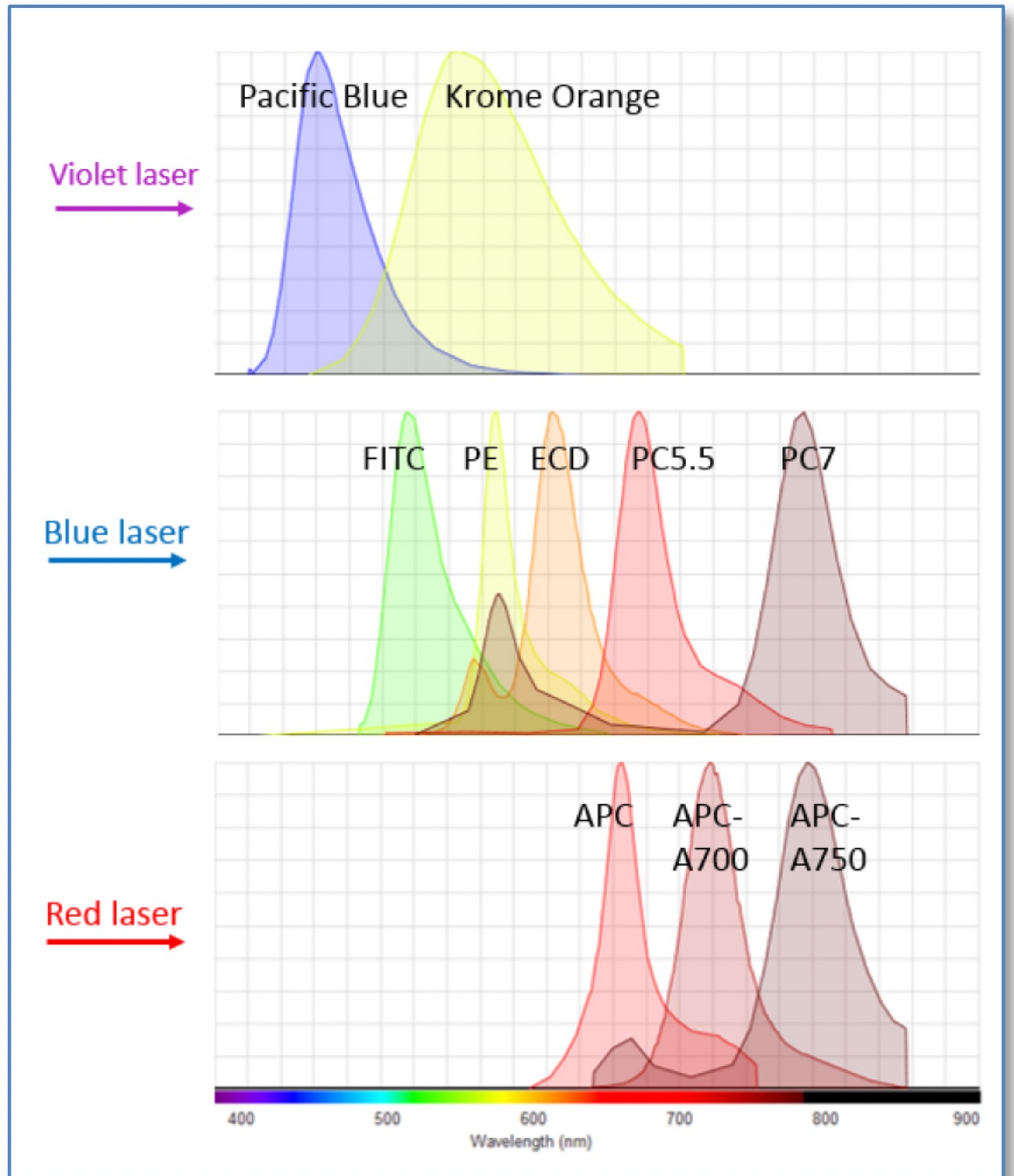




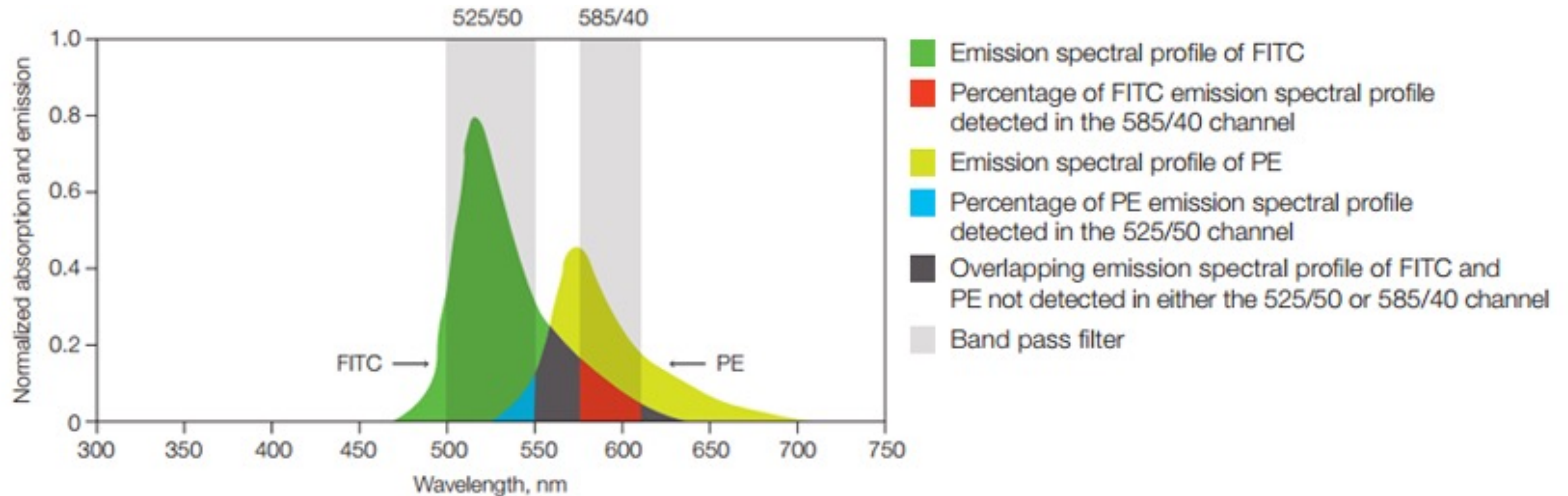
Electromagnetic spectrum



Different fluorophores are excited by different wavelengths and emit in different wavelengths

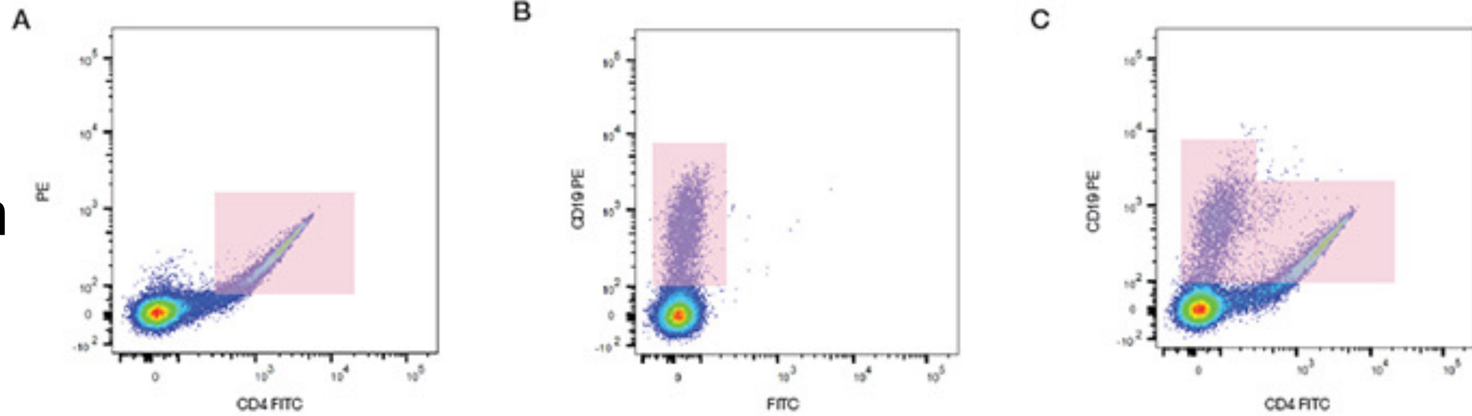


What if antibodies are conjugated to fluorophores with partial overlap?

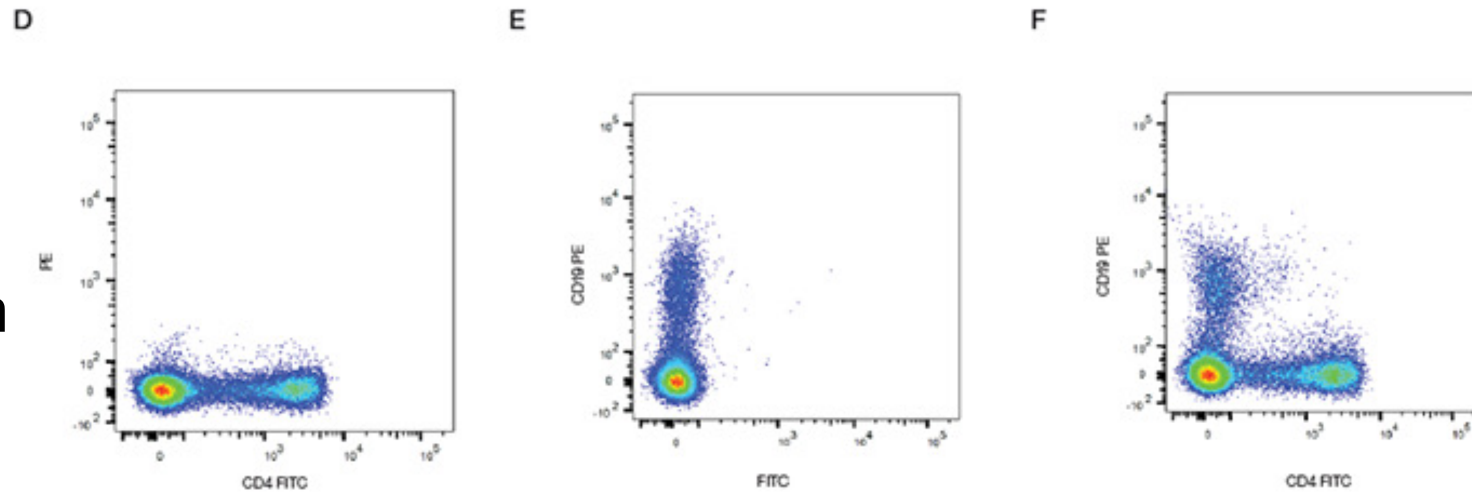


Compensation

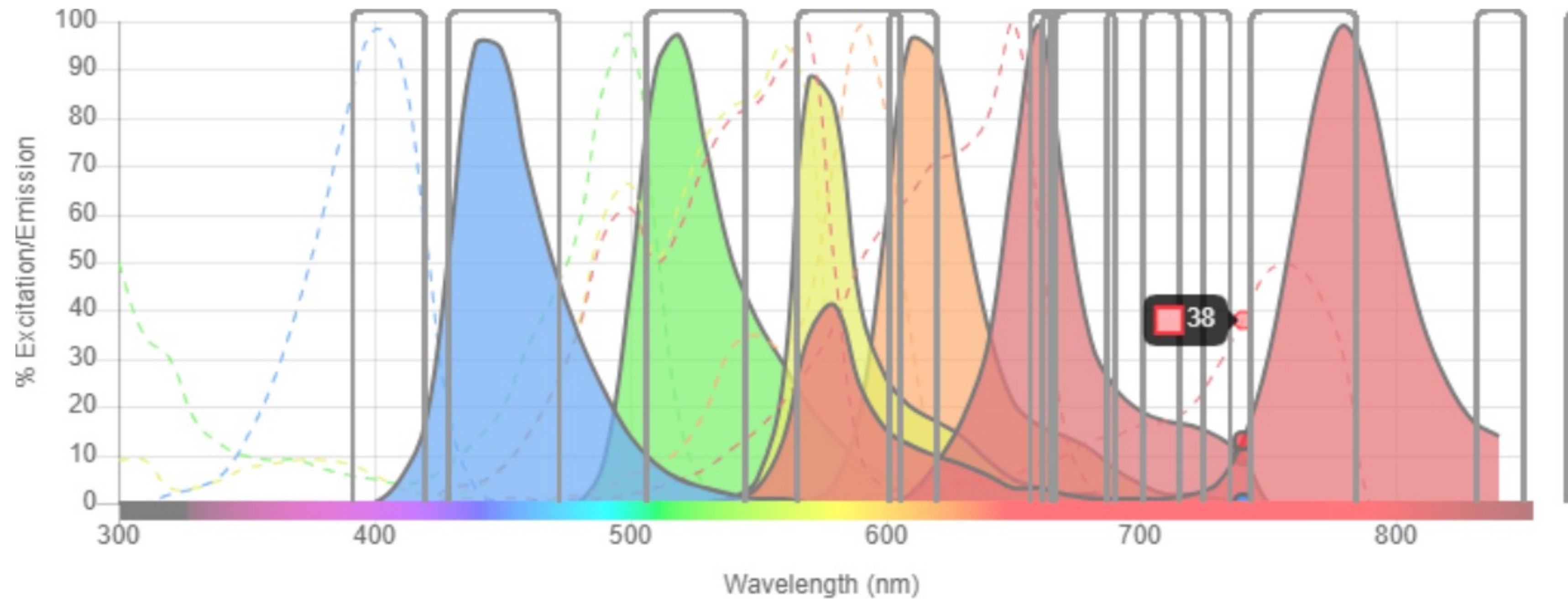
Before compensation



After compensation



Multicolor flow cytometry



Multicolor flow cytometry

Filter Coverage

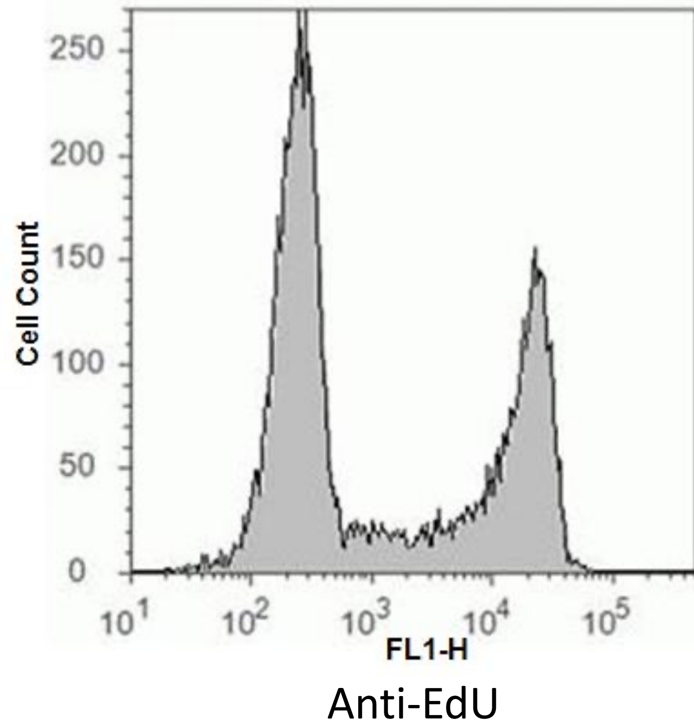
	Fluorophores / Filters	405/30	525/40	675/30	450/45	525/40	610/20	660/10	763/43	525/40	610/20	690/50	585/42	610/20	675/30	710/50	763/43	660/10	712/25	763/43	840/20	885/40
FOXP3	eFluor 450	7%	4%	0%	**72%**	4%	0%	0%	0%	4%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
CD3	PE-Cy7	0%	0%	1%	0%	0%	11%	3%	**69%**	0%	11%	1%	28%	11%	1%	1%	**69%**	3%	1%	**69%**	14%	0%
EGFR	APC	0%	0%	**40%**	0%	0%	5%	**99%**	0%	0%	5%	**31%**	0%	5%	**40%**	18%	0%	**99%**	17%	0%	0%	0%
HELIOS	PE	0%	0%	1%	0%	0%	18%	3%	0%	0%	18%	1%	**59%**	18%	1%	0%	0%	3%	0%	0%	0%	0%
Viability	Live-or-Dye 594/614	0%	0%	10%	0%	0%	**94%**	16%	0%	0%	**94%**	7%	20%	**94%**	10%	3%	0%	16%	2%	0%	0%	0%
CD4	FITC	0%	**78%**	0%	0%	**78%**	3%	0%	0%	**78%**	3%	0%	12%	3%	0%	0%	0%	0%	0%	0%	0%	0%

Flow cytometry panel design tools

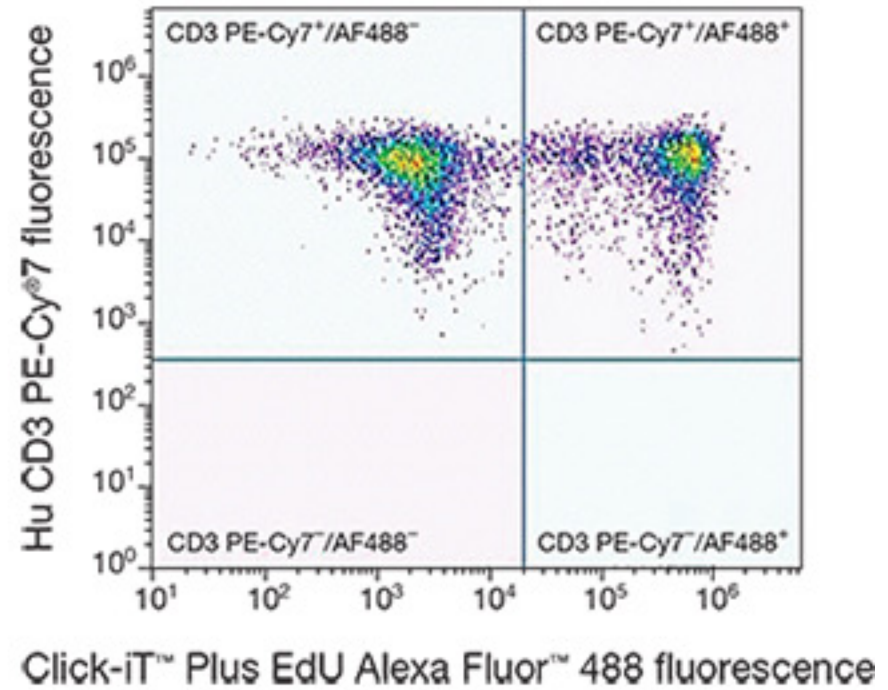
- https://www.bdbiosciences.com/en-us/applications/research-applications/multicolor-flow-cytometry/product-selection-tools

- https://www.bdbiosciences.com/en-us/applications/research-applications/multicolor-flow-cytometry/product-selection-tools/spectrum-viewer

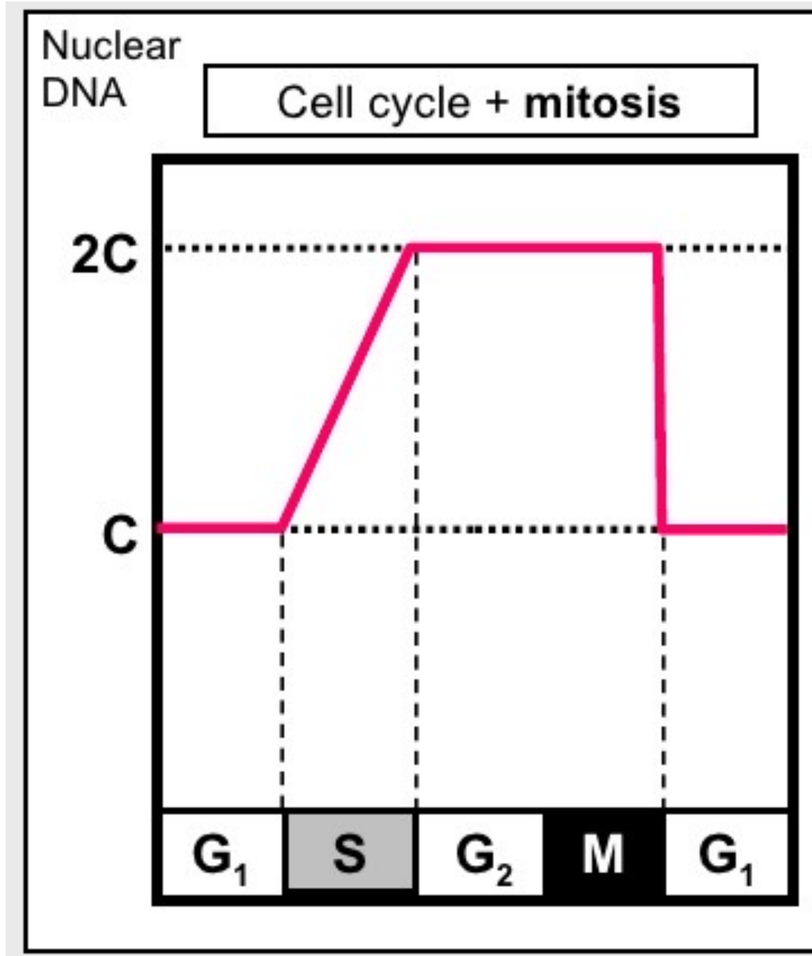
Analysis of cell proliferation by flow cytometry



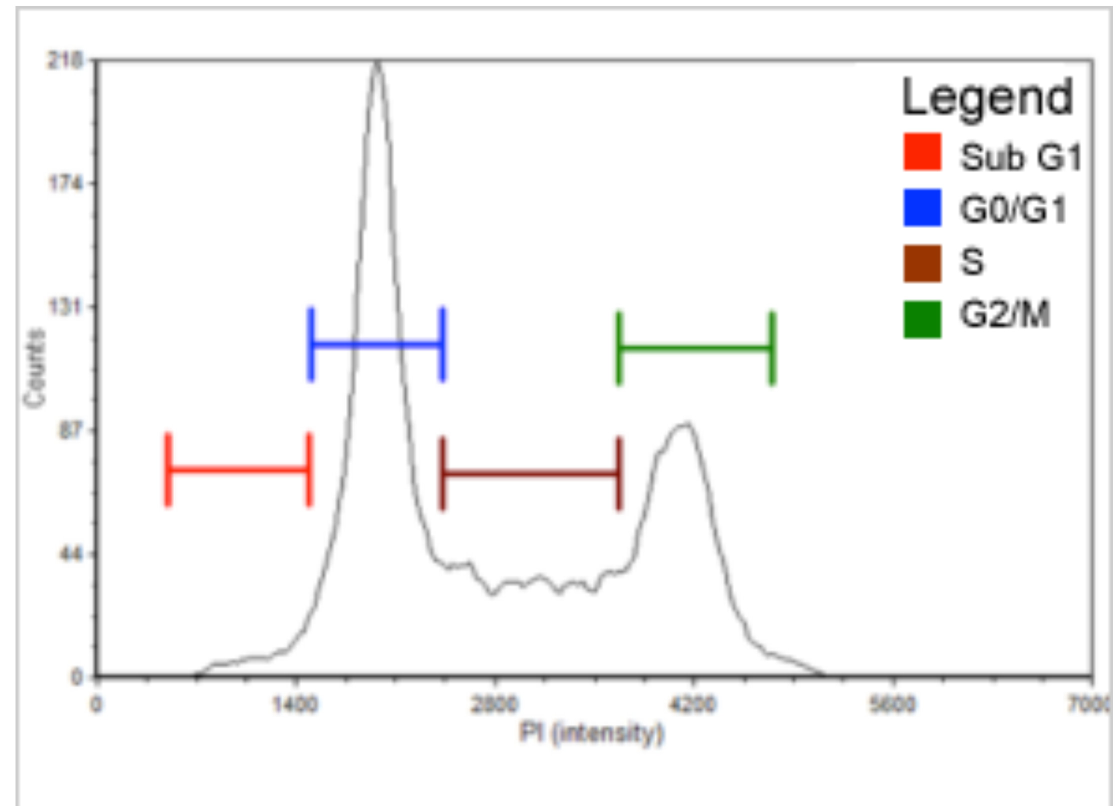
Anti-EdU



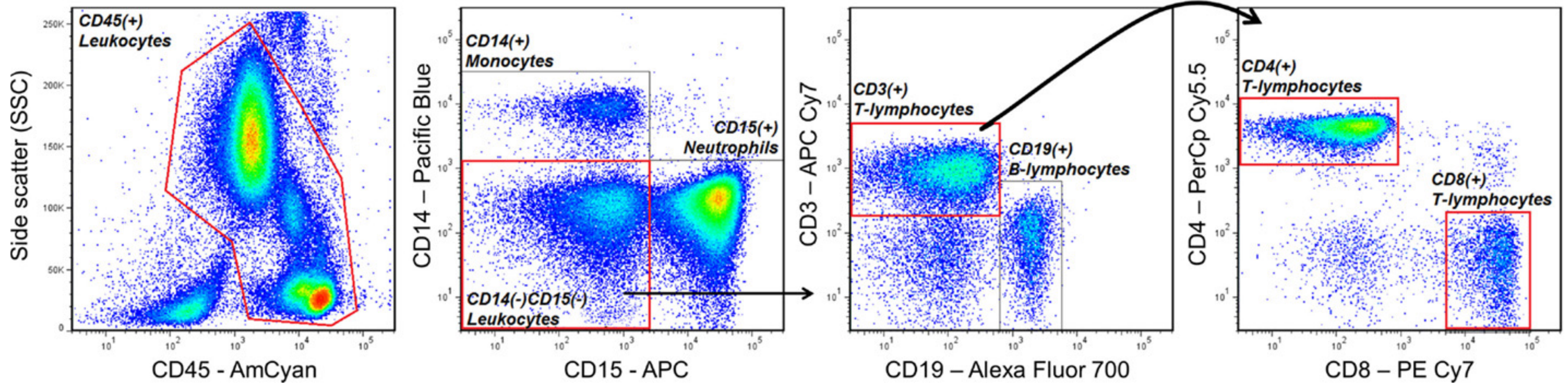
Analysis of cell cycle by flow cytometry



Fix and permeabilize cells
Add PI



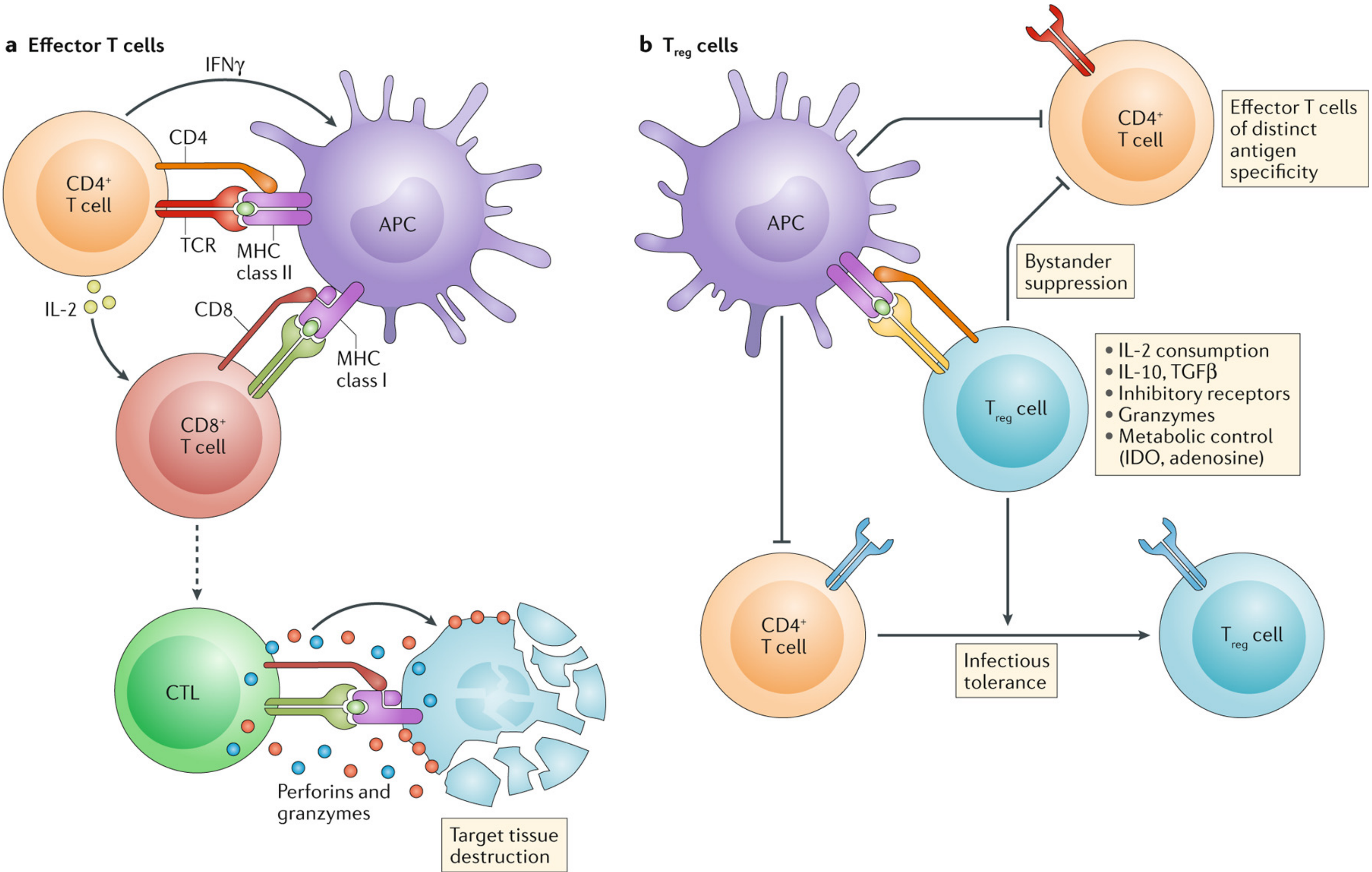
Subsetting cells using flow cytometry



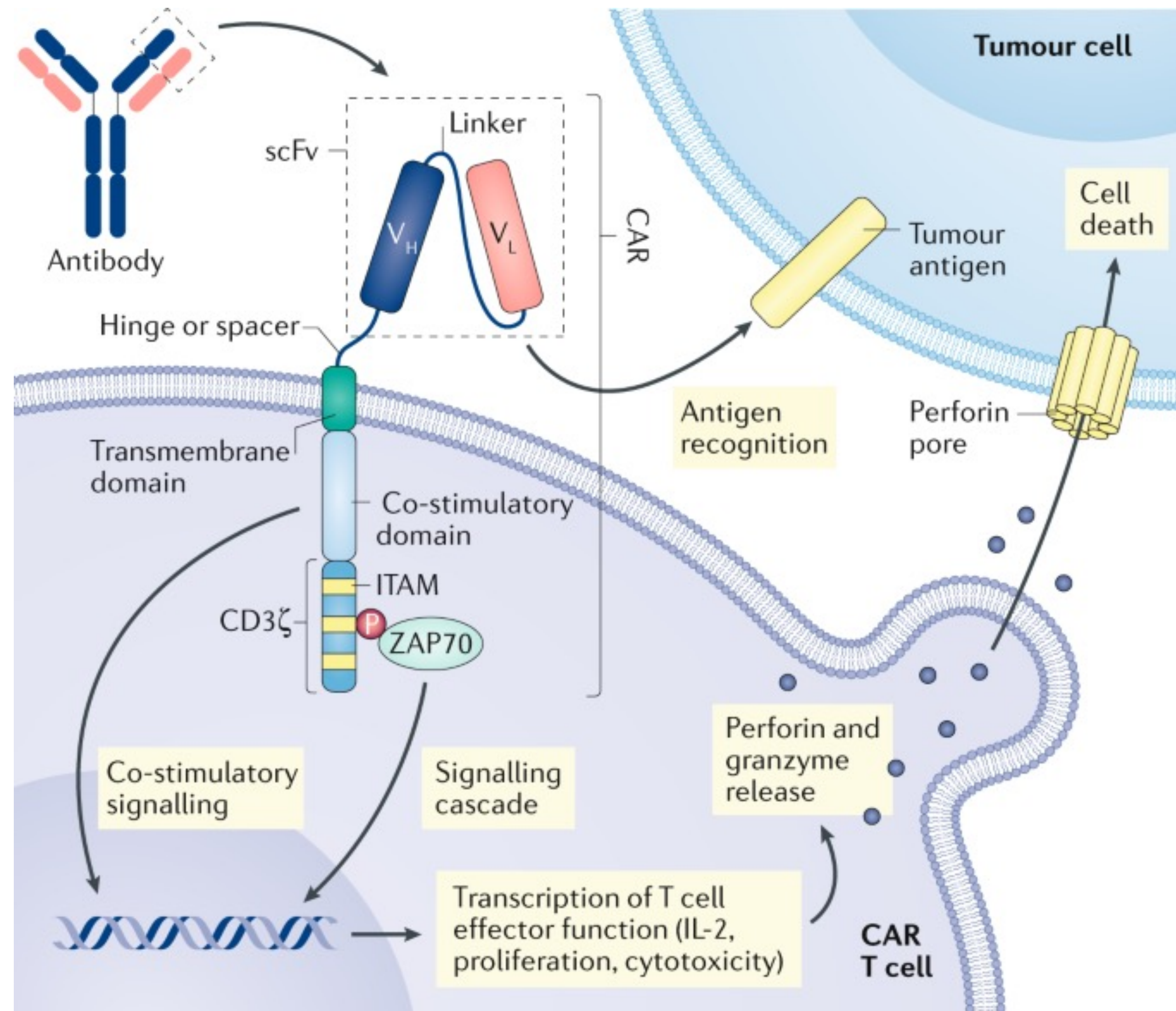
CD45 – blood cells
CD14 – monocytes
CD15 – neutrophils
CD19 – B cells
CD3 – T cells

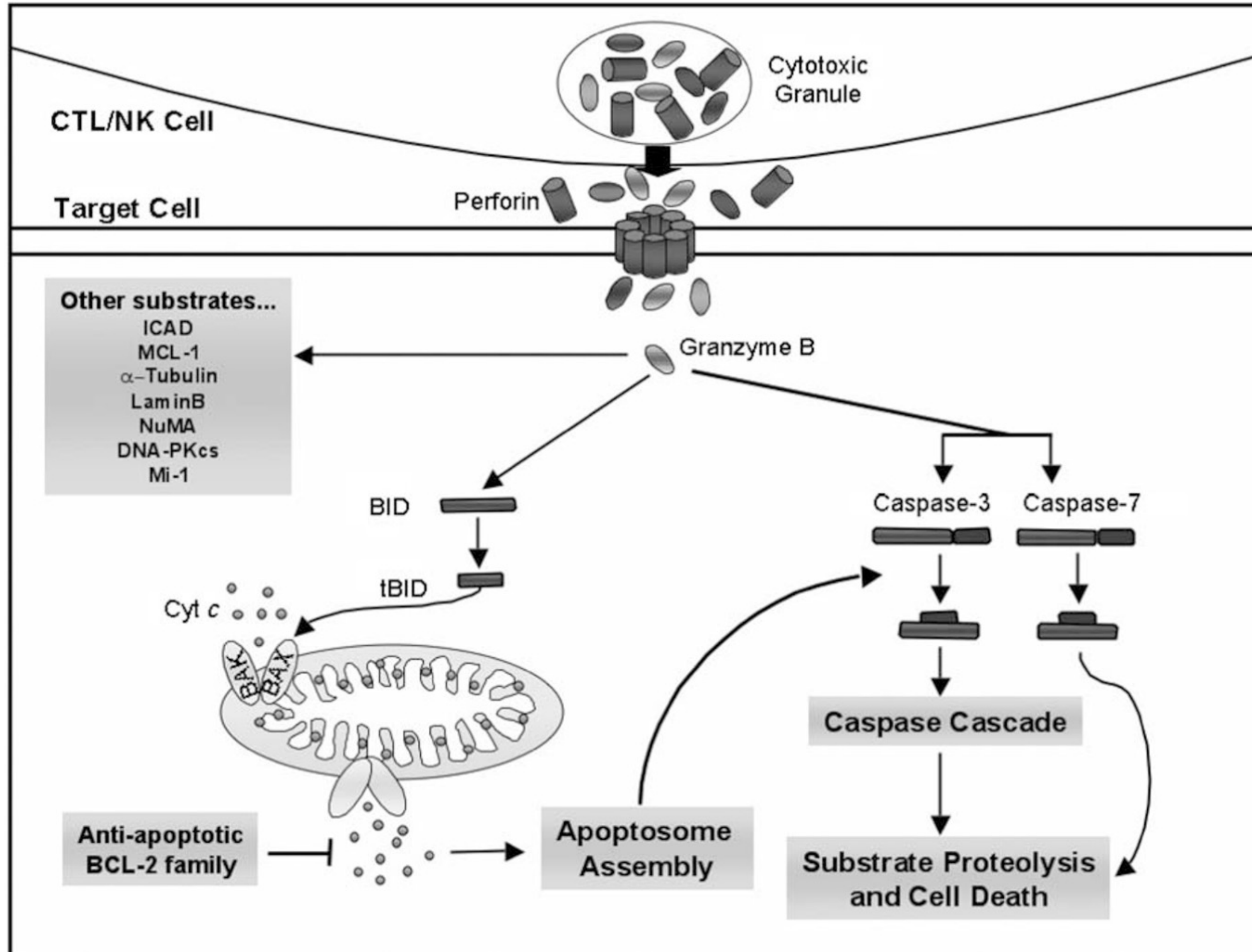
CD4 – helper T cells
CD8 – cytotoxic T cells

T cells and their regulation

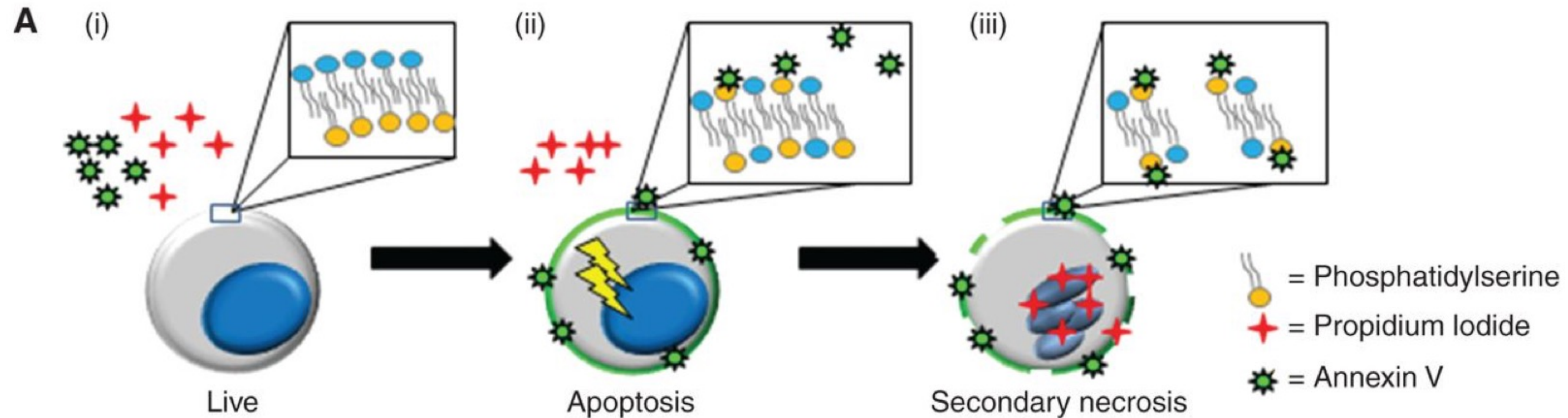


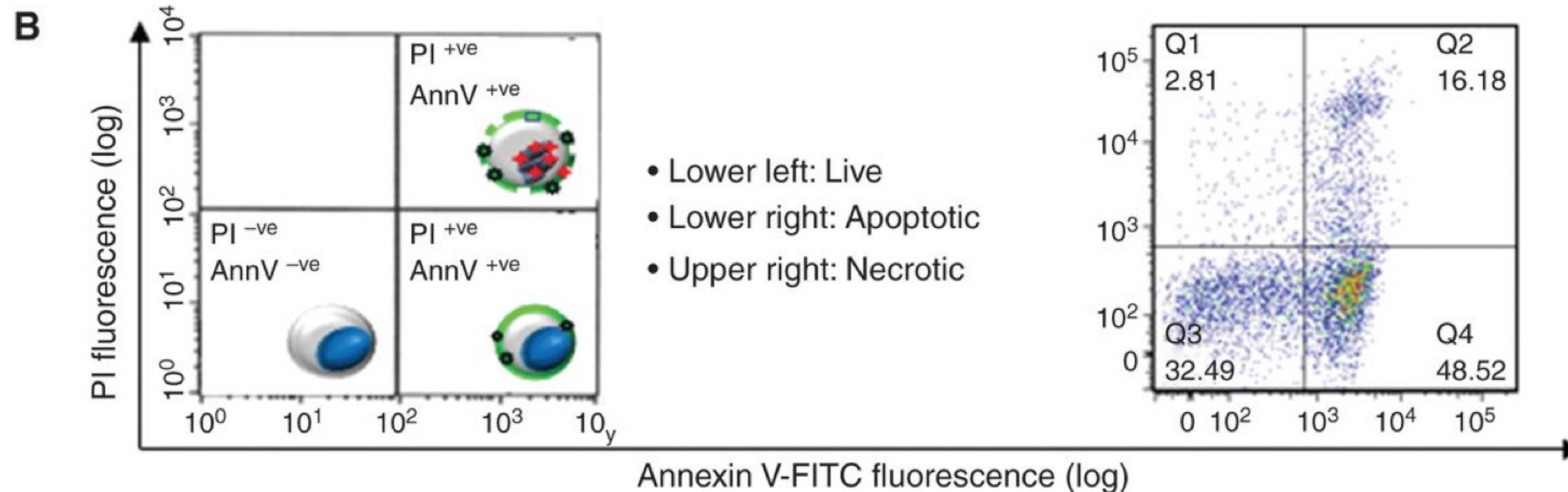
Chimeric antigen receptor (CAR) T cells




Cytotoxic Granule
CTL/NK Cell
Target Cell
Perforin
Other substrates...
ICAD
MCL-1
α–Tubulin
LaminB
NuMA
DNA-PKcs
Mi-1
Granzyme B
BID
tBID
Cyt c
BAK
BAX
Caspase-3
Caspase-7
Caspase Cascade
Anti-apoptotic BCL-2 family
Apoptosome Assembly
Substrate Proteolysis and Cell Death

Assessing apoptosis using flow cytometry

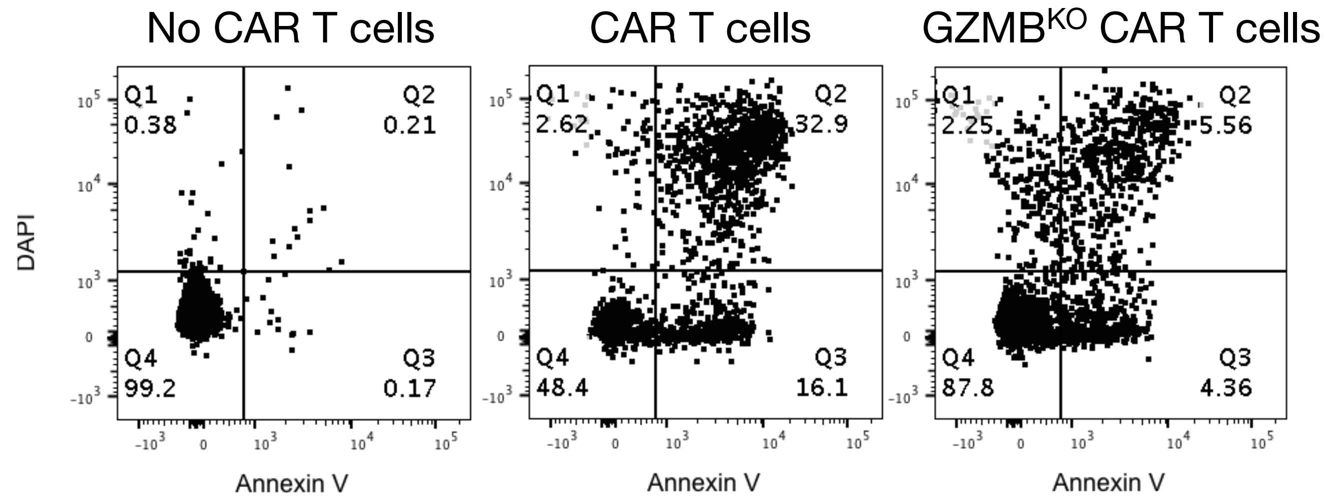


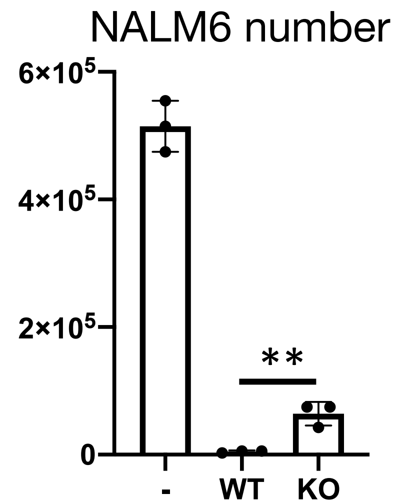


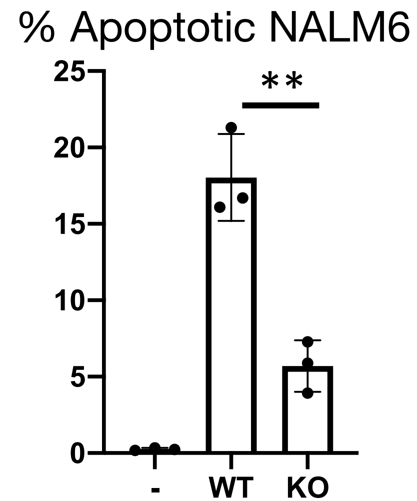
- Lower left: Live
- Lower right: Apoptotic
- Upper right: Necrotic

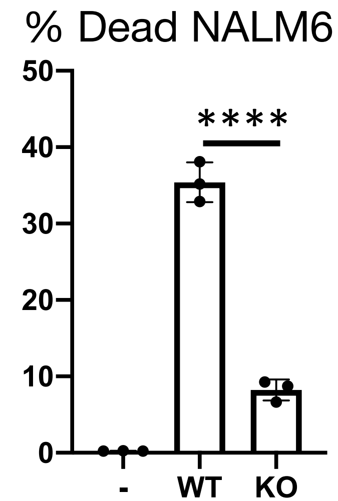
Crowley et al ,2016, CSH Protocols

Assessing apoptosis using flow cytometry



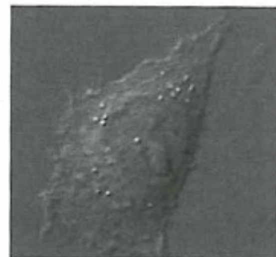


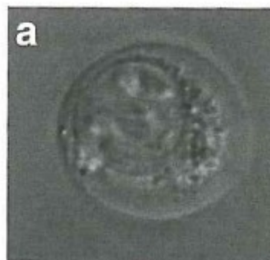


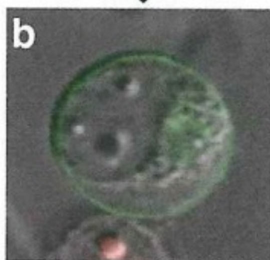


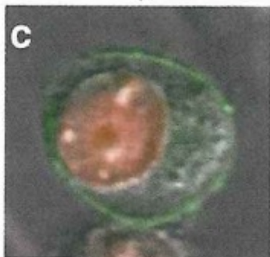
Necrosis

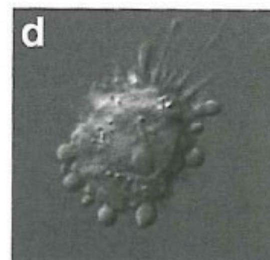
Expected:
Higher SSC
(granularity)

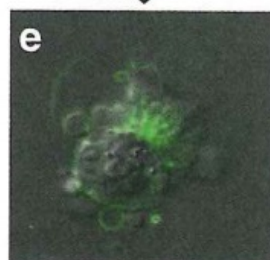


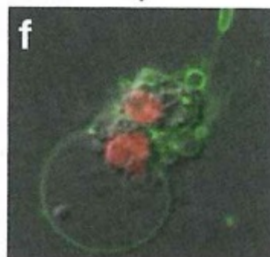










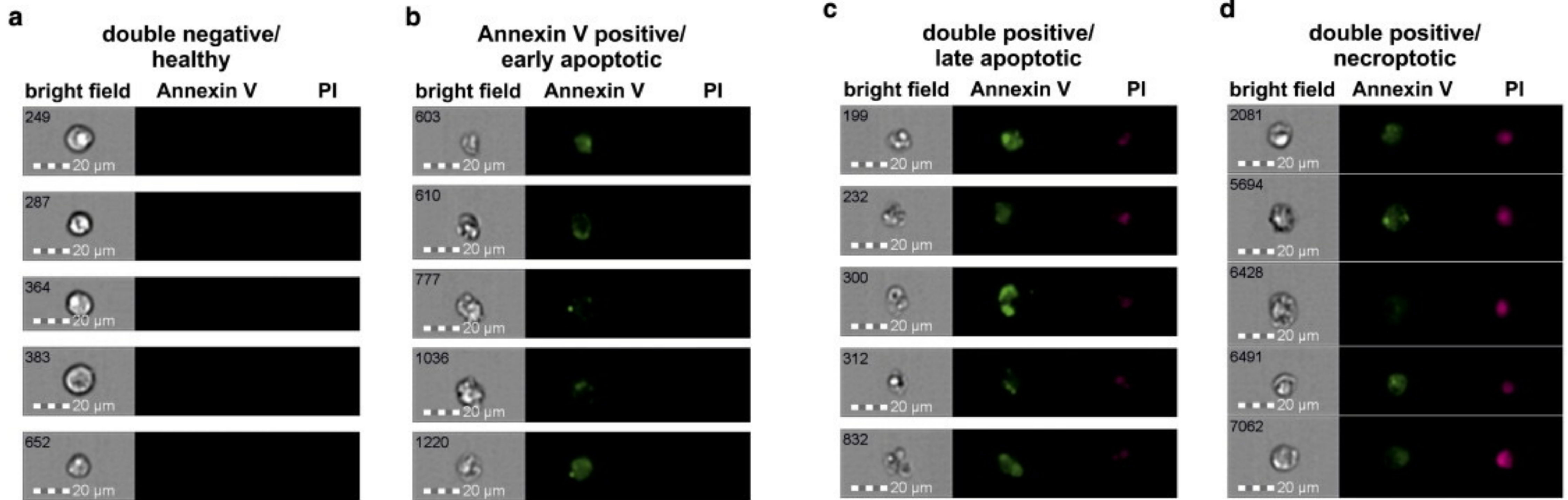


Apoptosis

Expected:
Lower FSC
(cell size)

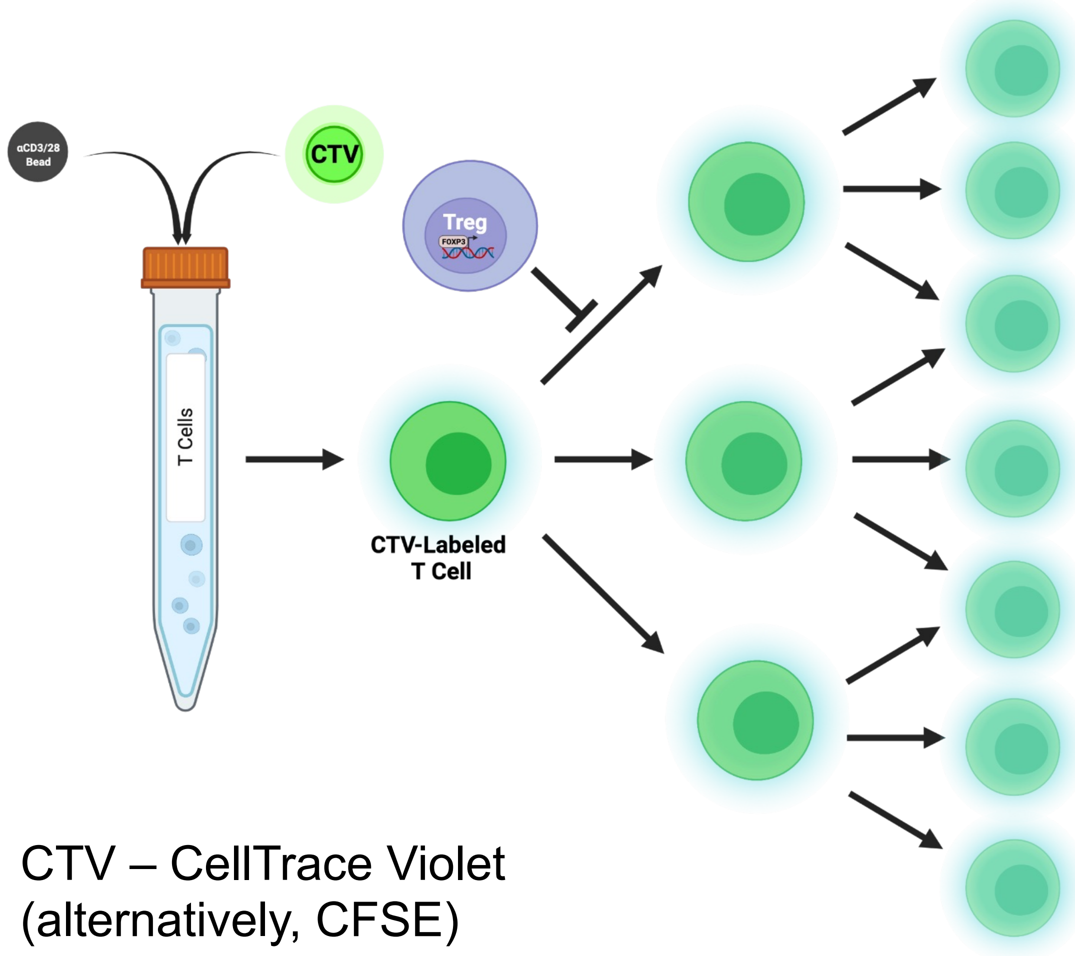
Grootjans et al, 2014, in *Cell Death in Biology and Diseases*

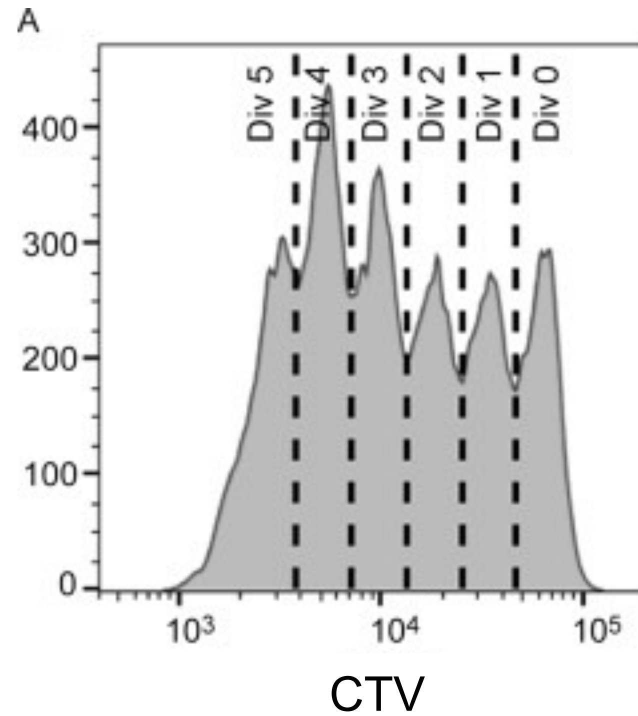
PI brightness in late apoptosis vs. necroptosis

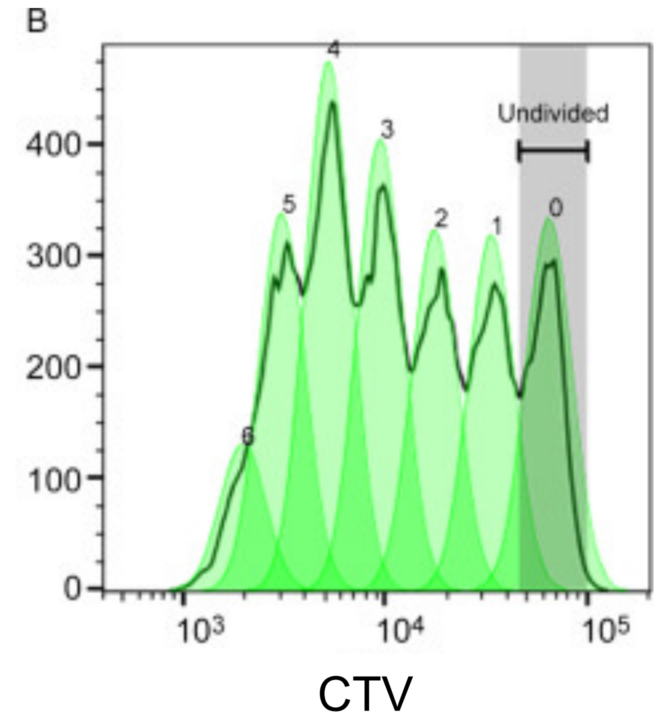


Pietkiewicz et al, 2015, JIM

Measuring cell division using flow cytometry







CTV – CellTrace Violet
(alternatively, CFSE)

Take-home messages

- Analyses of the cellular processes that drive development, homeostasis and disease, as well as the impact of gene targeting, often include quantitation of cell number, proliferation, and death.
- A broad array of reagents and technologies is available for these analyses.
- Thoughtful selection of reagents and technology platform permits simultaneous quantitative analyses of one or several cell processes.
- Flow cytometry stands out as a technique that allows for simultaneous multiparameter analysis of many single cells, either live or fixed, in a mixed population.

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