Applications of Flow Cytometry

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wp.unil.ch/fcf/

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CHUV

What is flow cytometry ?

- **Thousands of events analyzed in a short period of time**
- **Statistical information obtained rapidly**
- **Flexibility of data acquisition**

Main uses include :

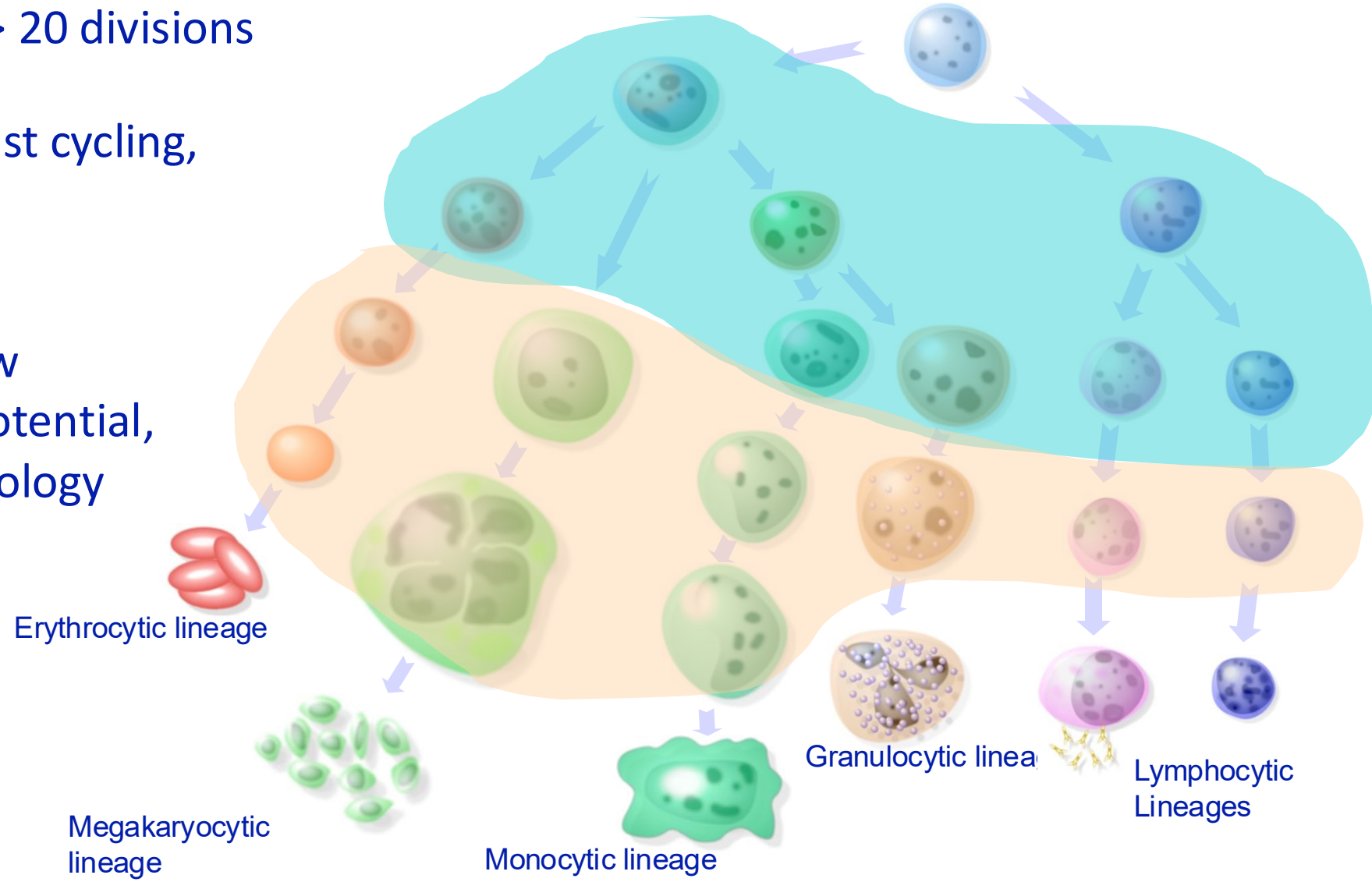
Cell Sorting
Phenotyping
Pharmacokinetic and Pharmacodynamic assays
DNA Analysis
Functional Studies – Proliferation, Activation, Cell Death
Fluorescent Proteins

Application to Stem Cell biology: the hematopoiesis

Stem cells : rare, quiescent,
Potential > 20 divisions

Progenitors : fast cycling,
uni or bipotent

Precursors : low
proliferation potential,
specific morphology



Characterization of Stem Cells

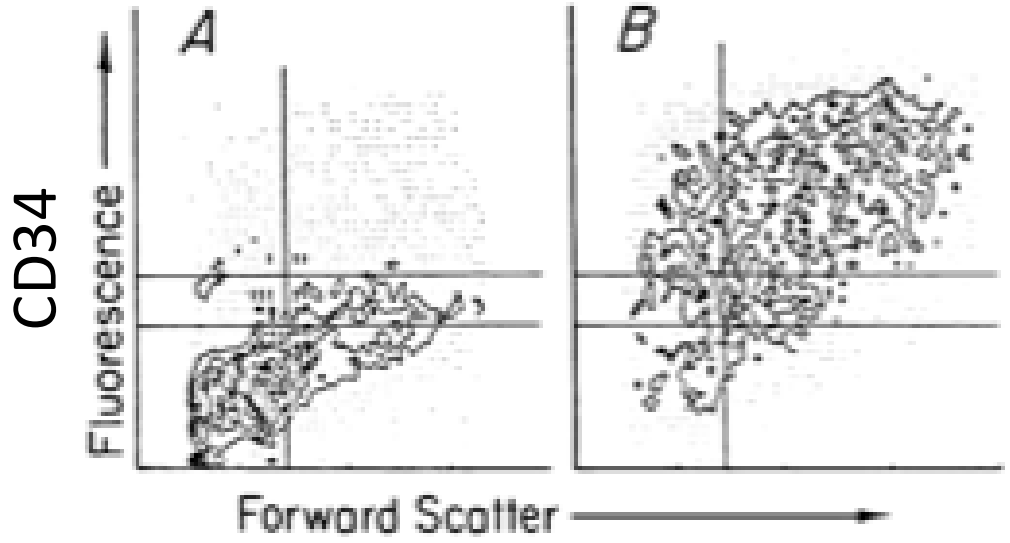
- **Different strategies :**
  - Surface Antigen Expression
  - Quiescence and position in Cell Cycle
  - Metabolic or enzymatic biomarkers

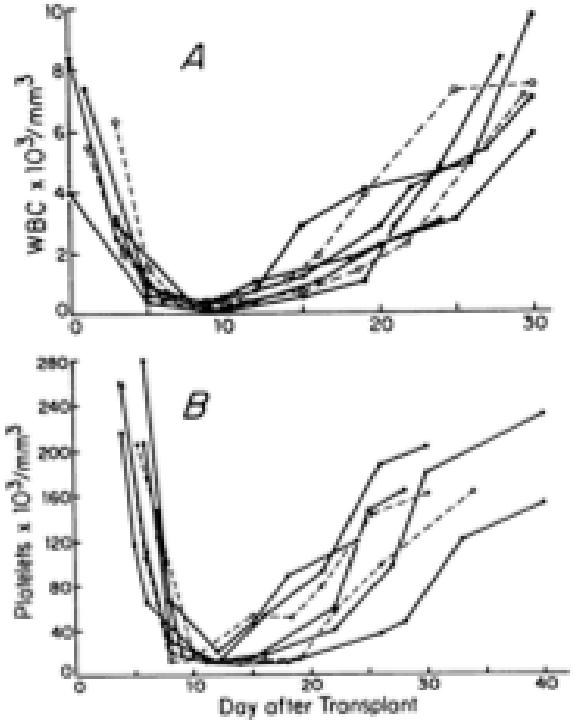
Surface Antigen Expression

Antigen CD34$^+$ Marrow Cells Engraft Lethally Irradiated Baboons

Ronald J. Berenson, Robert G. Andrews, William I. Bensinger, Dale Kalamasz, Glenn Knitter, C. D. Buckner, and Irwin D. Bernstein
Fred Hutchinson Cancer Research Center and the University of Washington Regional Primate Center, Seattle, Washington 98104

J Clin Invest 81:951-955. (1988)



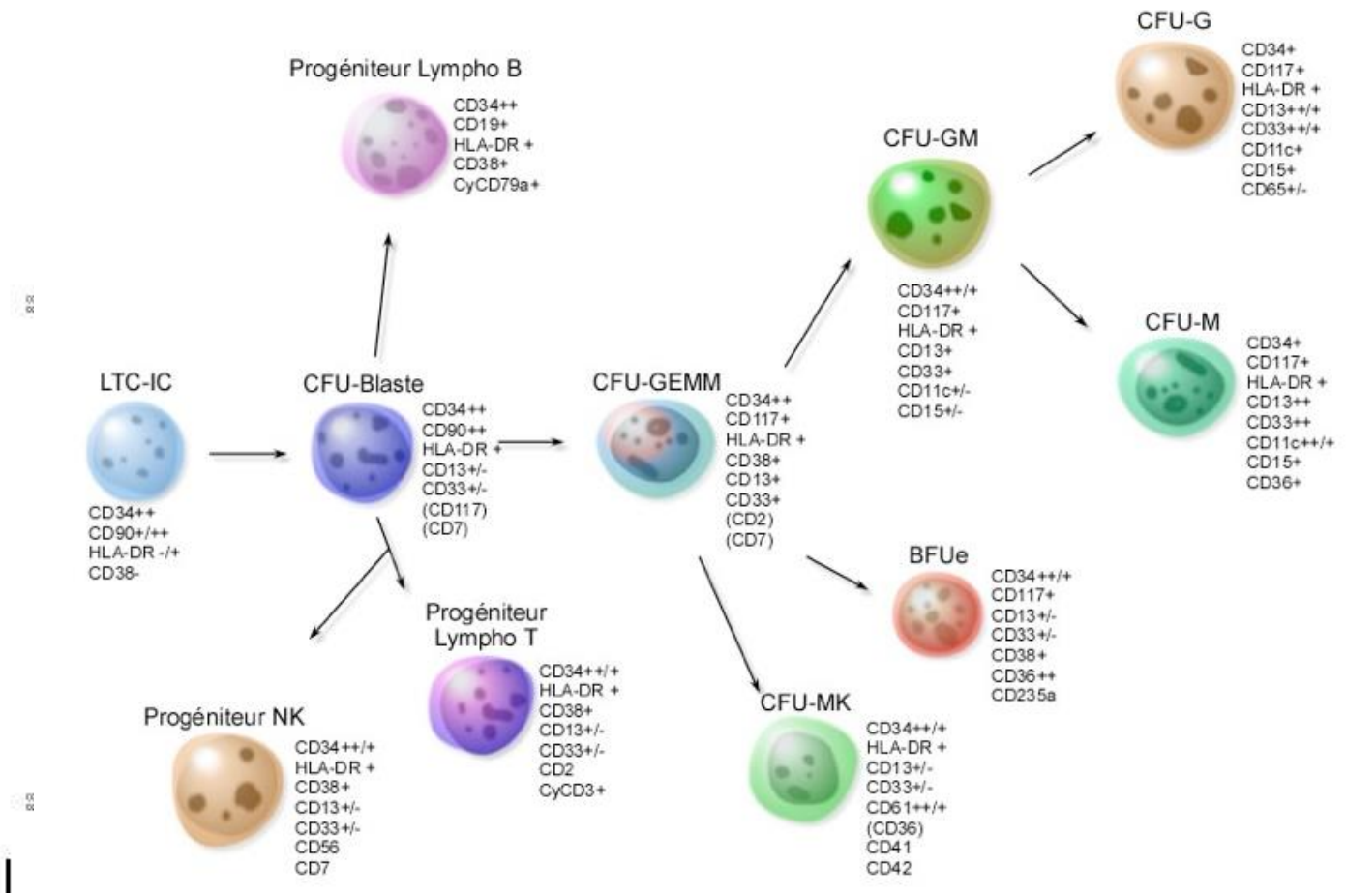


Surface Antigen Expression

CD34+ cells

Clinical Application: about 19’798 allogeneic grafts in 2019 (Europe)

Fundamental Research: too heterogeneous cell population



Surface Antigen Expression

Real Stem Cells are CD34 negative

Bathia M, Bonnet D, Murdoch B *et al*. A newly discovered class of human hematopoietic cells with SCID-repopulating activity.(1998) Nat. Med.

Fujisaki T, Berger MG, Rose John *et al*. Rapid differentiation of a rare subset of adult human Lin^- $CD34^-CD38^-$ cells stimulated by multiple growth factor in vitro.(1999) Blood

In search of new markers :

CD90 (Thy-1)

CD93 (C1qRp)

CD105 (endoglin)

CD110 (TPO receptor)

CD117 (SCF receptor)

CD123 (Il-3 receptor)

CD133

CD135 (Flt-3)

CD143 (Angiotensin Converting enzyme)

CD162 (PSGL-1)

CD202 (Tie-2)

CD243 (MDR-1)

CD309 (KDR VEGF-R2 Flk-1)

CD318 (CDCP1)

CD338 (ABCG2 BCRP1)

Notch

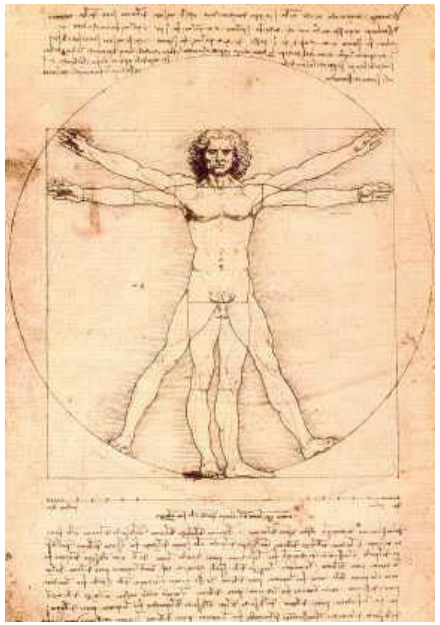
SLAM (CD150)

Robo4

Surface Antigen Expression

Consensus on HSC identification?

For Human



- CD133+ CD34(+/-) CD38- Lin-

Lin = glycophorin-A, CD7, CD33, CD56, CD16, CD3 and CD2

«Eliminate all other factors, and the one which remains must be the truth»

Surface Antigen Expression

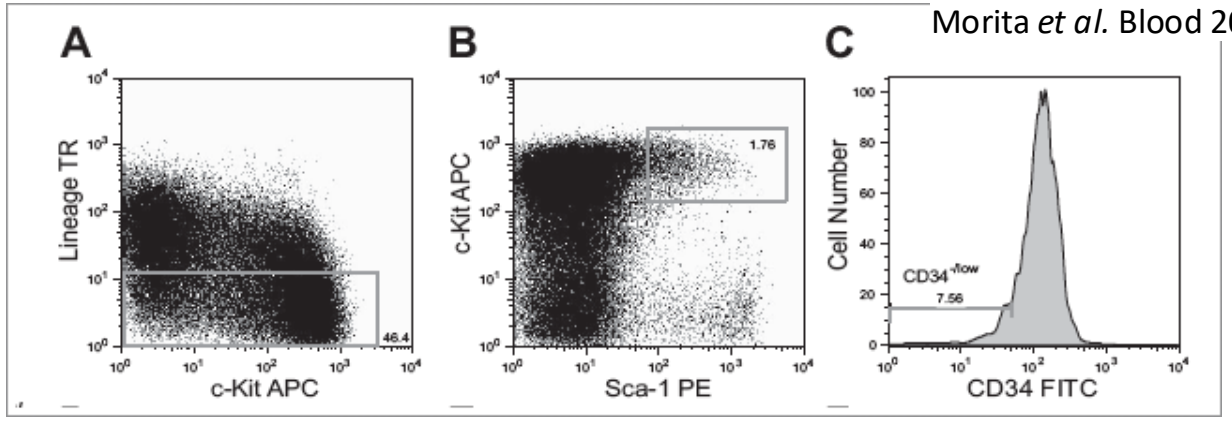
Consensus on HSC identification?

For Mice

KSL Cells: Lin- c-Kit+ Sca-1+

Graft *Single Cell* : 11/44

Morita *et al.* Blood 2006



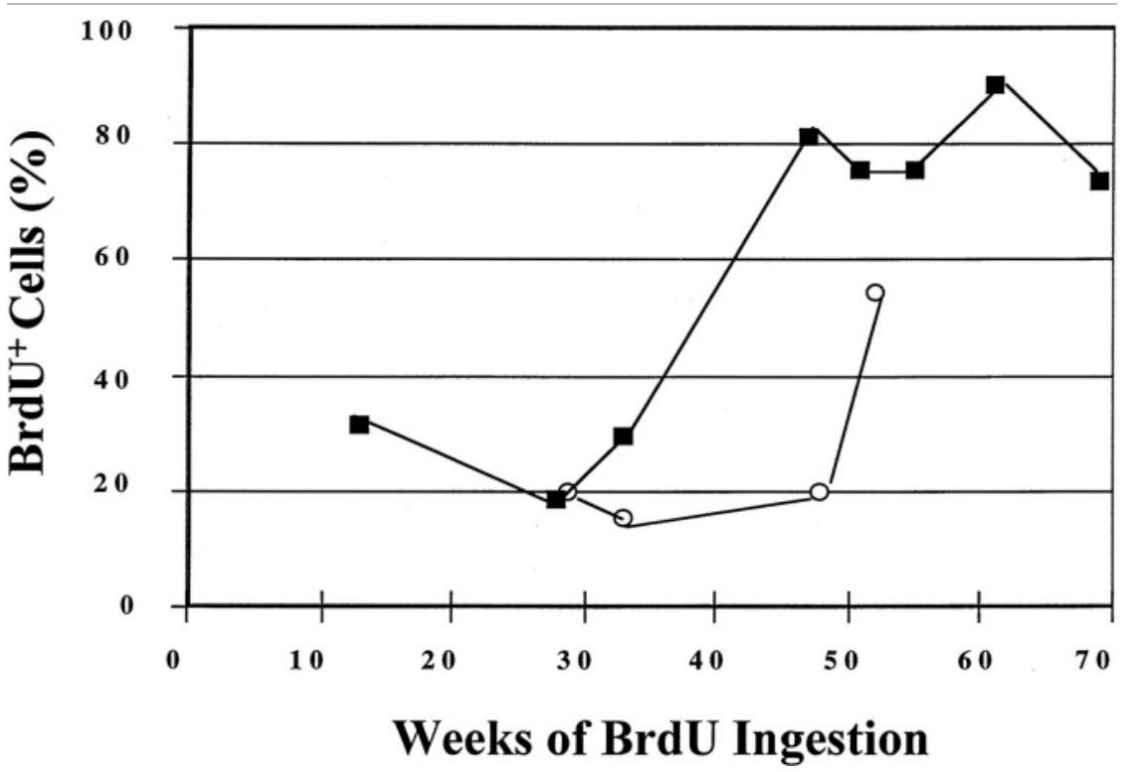
Stem cells are quiescent cells

Stem Cells are quiescent and have a very low proliferation rate

Mahmud, N., S. M. Devine, et al. (2001). “The relative quiescence of hematopoietic stem cells in nonhuman primates.” Blood

BrdU administration to Baboons during more than 85 weeks

Less than 70 % of hematopoietic stem cells incorporated BrdU



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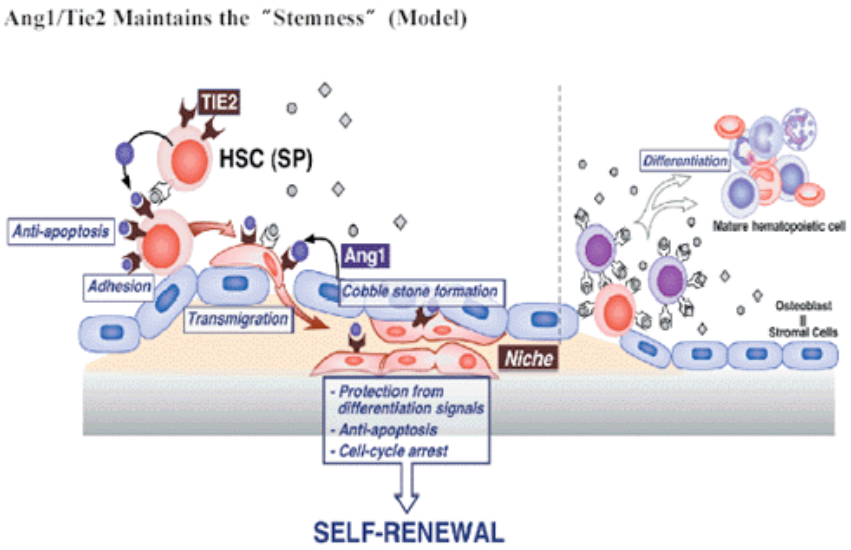
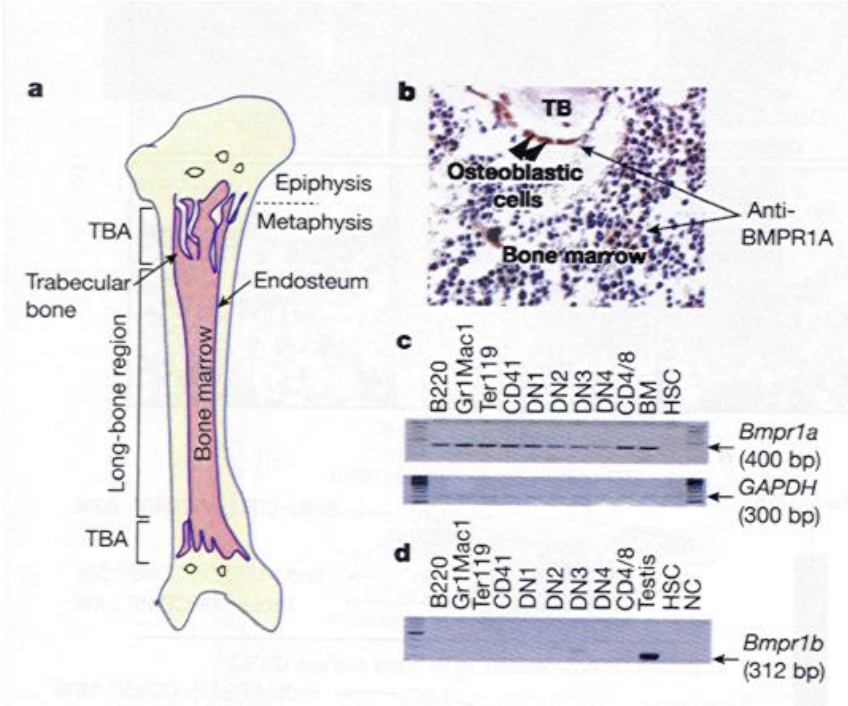




Identification of quiescent cells

Stem Cells are quiescent and have a very low proliferation rate

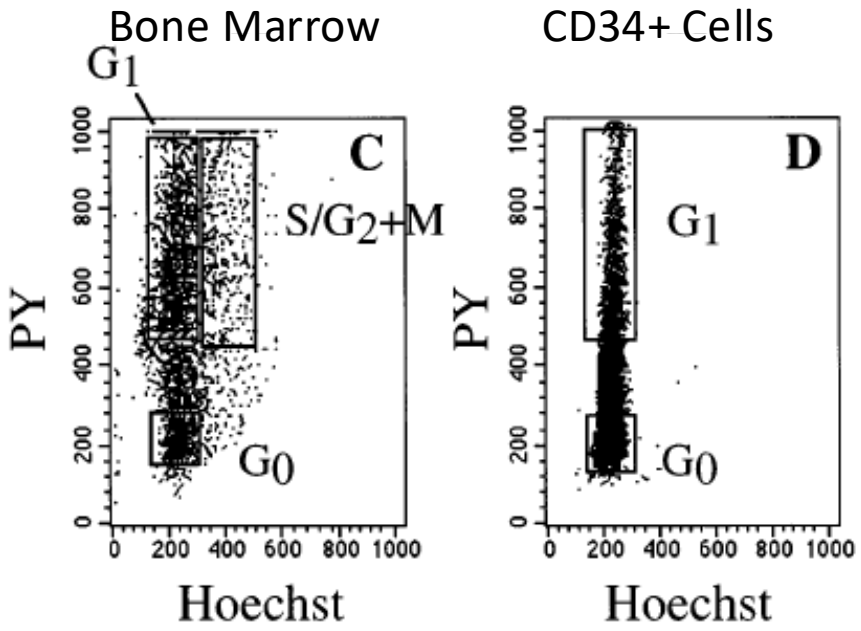
Characterization of quiescent cells by flow cytometry :

Low ATP production
Low mRNA synthesis

Identification of quiescent cells

Analysis of cell cycle : staining with Pyronine Y

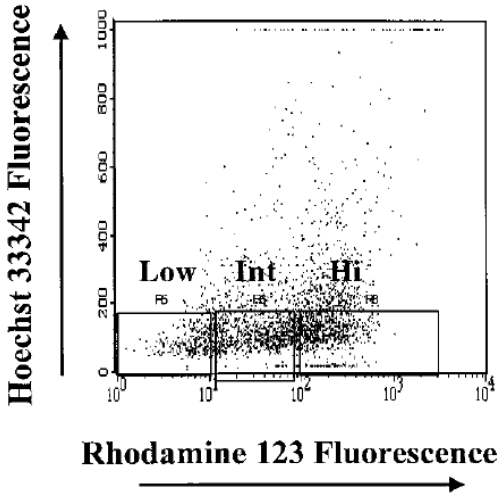
Gothot, A., R. Pyatt, et al. (1997). “Functional heterogeneity of human CD34(+) cells isolated in subcompartments of the G0 /G1 phase of the cell cycle.” Blood



PY Fluorescence intensity is proportional to mRNA content

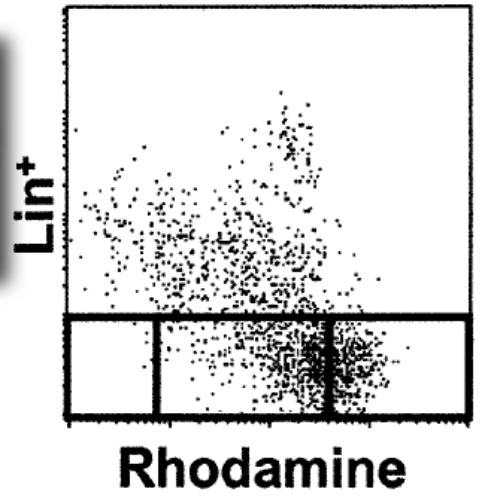
Identification of quiescent cells

Analysis of mitochondrial activity : Rhodamine 123 staining



Mahmud et al Blood 2001

Apoptotic cells may have the same phenotype !!



Ushida *et al* Exp Hematol 2003

Functional characterization: enzymatic activity

Expression of enzymes implicated in chimioresistance processes

ABCG2/BCRP : The «Side Population» phenotype

The SP Cells

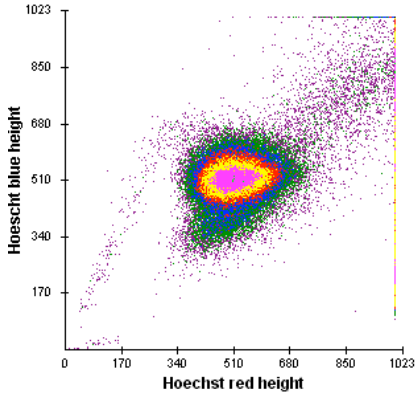
Goodell MA, Brose K, Paradis G, Conner AS, Mulligan RC. Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo. J Exp Med 1996

Stem Cells are able to efflux the vital dye Hoechst 33 342

Functional characterization: The side population

Expression of enzymes implicated in chimioresistance processes

Cells are incubated 2 hours at 37°C with Hoechst 33 342



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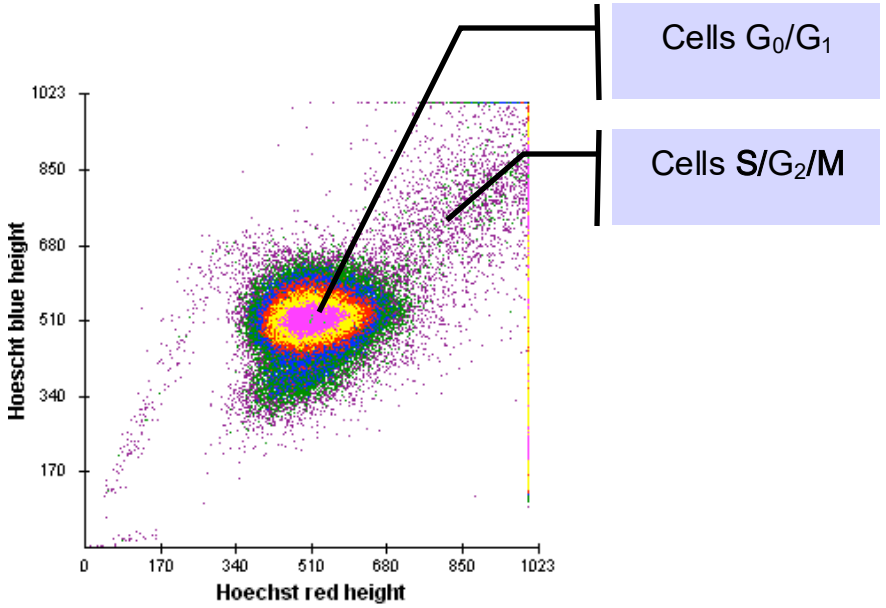

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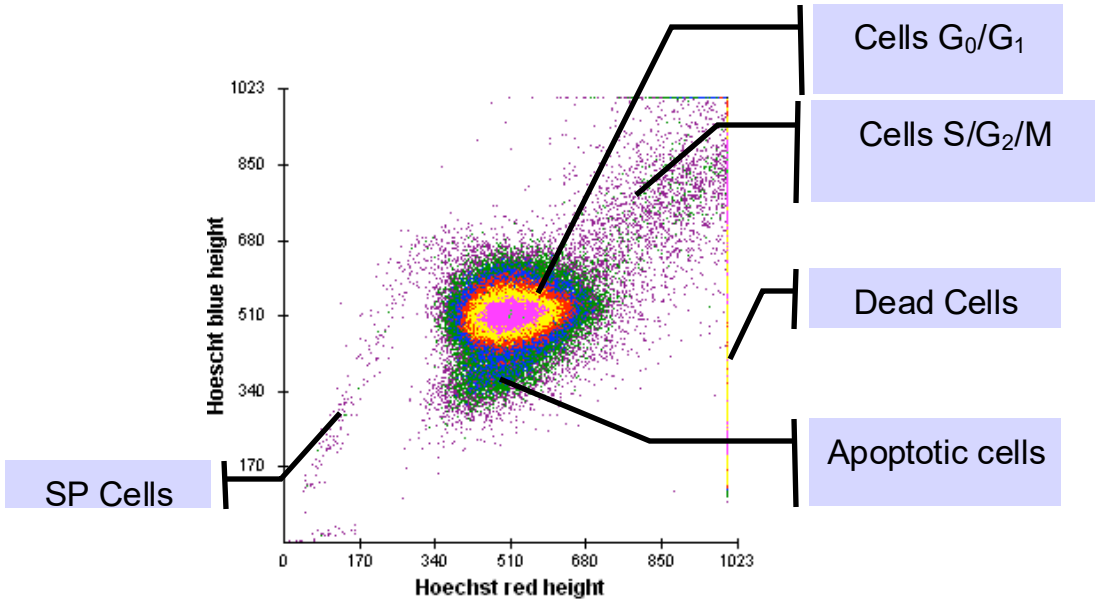
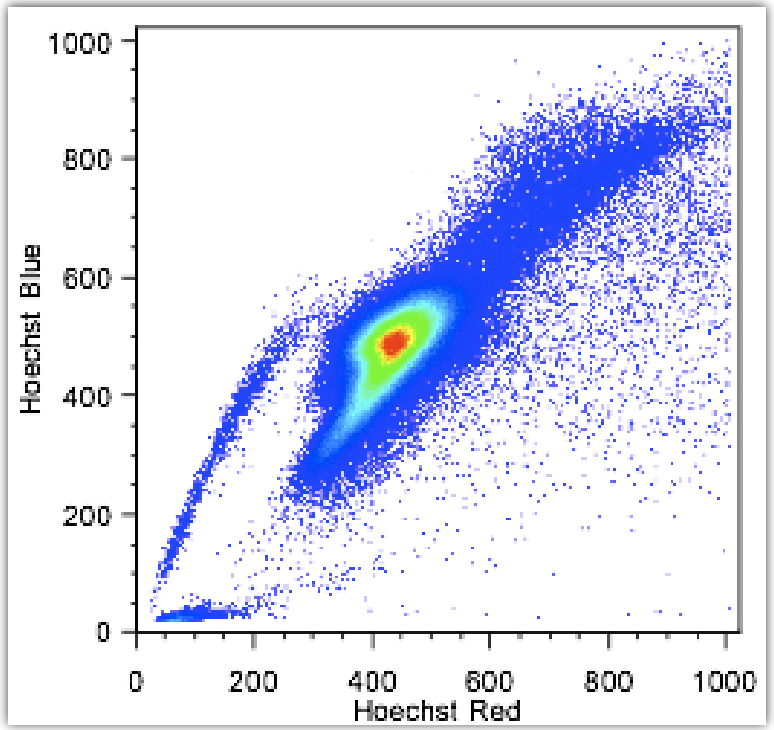


Functional characterization: The side population

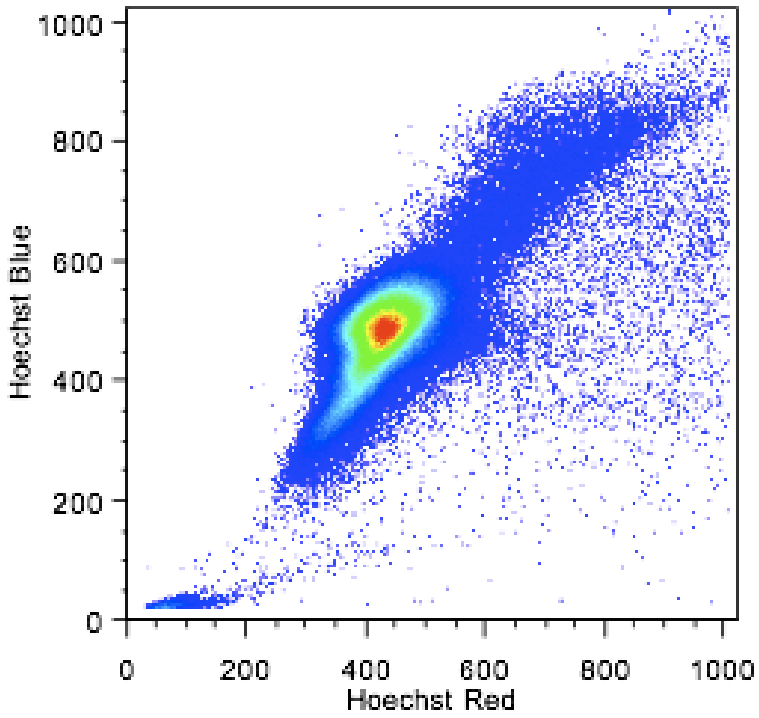
Expression of enzymes implicated in chimioresistance processes

Inhibition of the enzyme activity with Verapamil

Bone Marrow



+ Verapamil



Functional characterization: The side population

Allows the identification of several stem cells:

- in the liver,
- in the skeletal muscle,
- in the nervous central system,
- in the lung,
- in the oesophagus,
- in the mammary gland,
- in the skin,
- in the heart
- in the dental pulp...

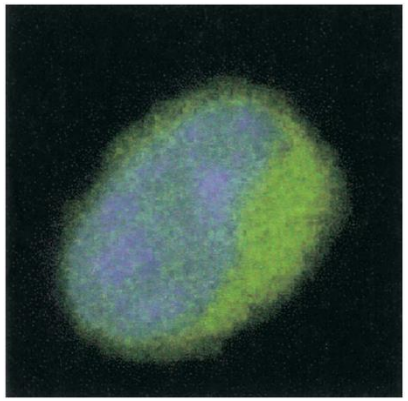
Identification of cancer stem cells:

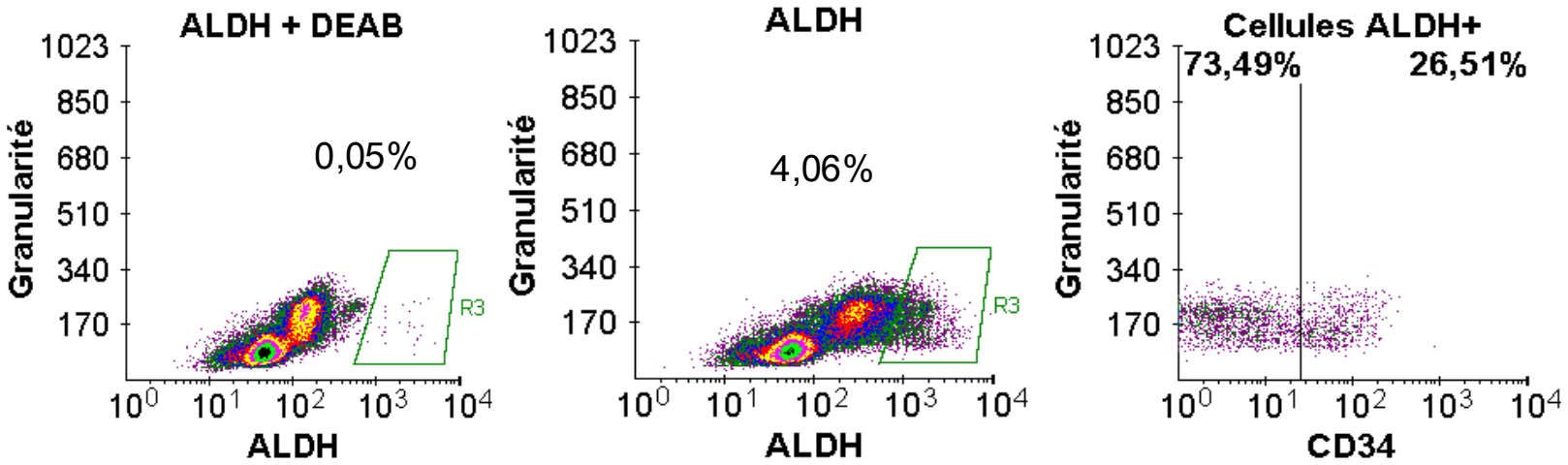
- glioblastoma
- ovarian cancer
- melanoma
- lung cancer,
- hepato-cellular carcinoma
- breast cancer
- leukemia
- gastrointestinal cancer
- ...

Functional characterization: ALDH activity

Detection of aldehyde dehydrogenase (ALDH) using BODIPY

Also known as «Aldefluor»





Bone marrow cells

Potential pitfals

Staining with antibodies ➠ Decrease of the engraftment ability

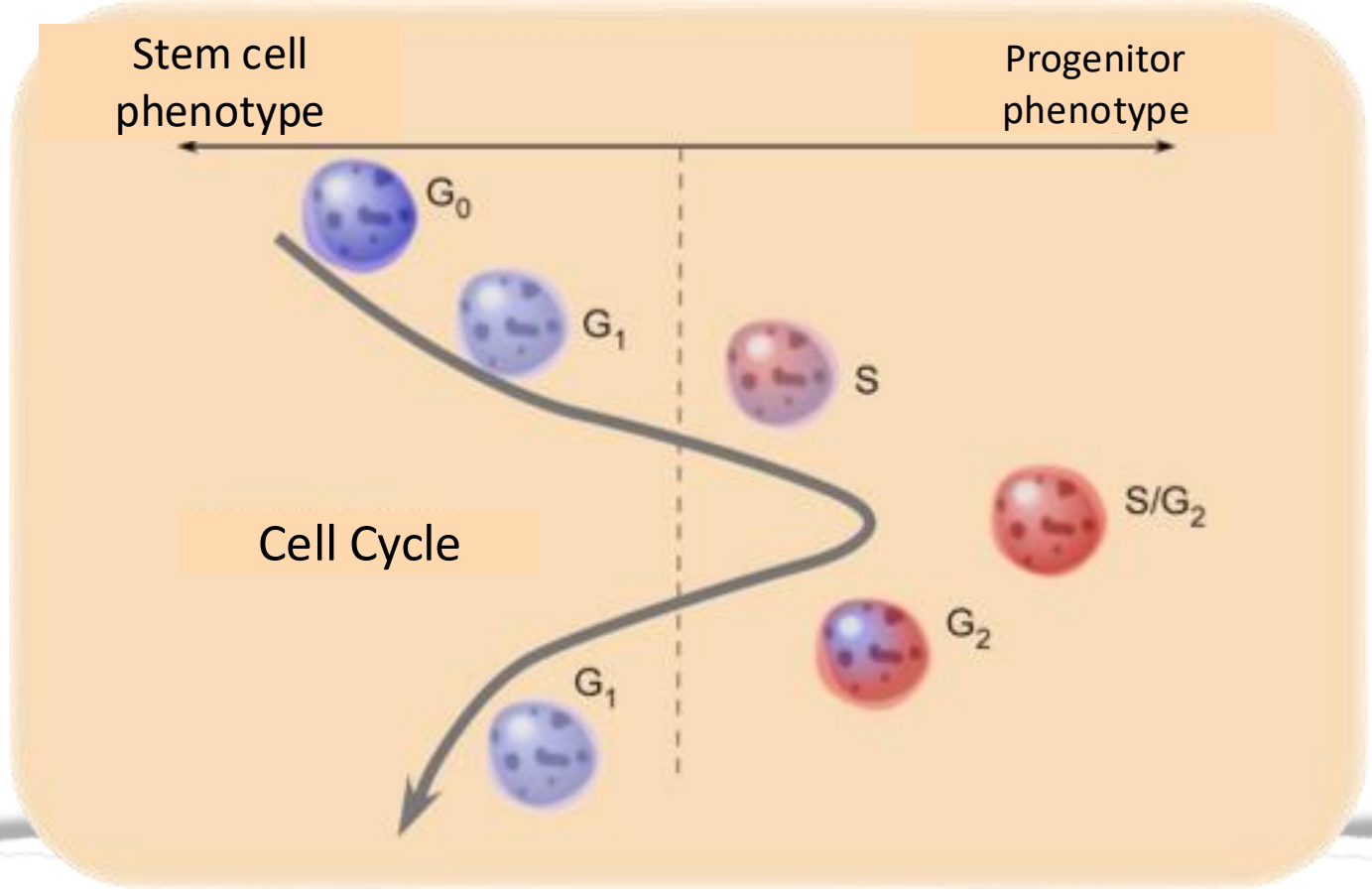
Gilner JB, Walton WG, Gush K, Kirby SL. **Antibodies to Stem Cell Marker Antigens Reduce Engraftment of Hematopoietic Stem Cells.** Stem Cells (2006)

Phenotypic reversal

S. Knaän-Shanzer, I. van der Velde-van Dijke, M.J.M. van de Watering, P.J. de Leeuw, D.Valerio, D.W. van Bekkum, A.A.F. de Vries **Phenotypic and Functional Reversal within the Early Human Hematopoietic Compartment** Stem Cells (2008)

Potential pitfals

Stem Cell Continuum



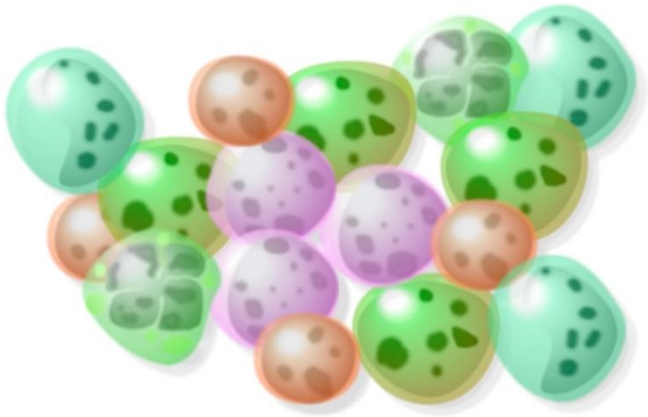
Quesenberry PJ, Colvin G, Dooner G, Dooner M, Aliotta JM, Johnson K. 2007. **The Stem Cell Continuum: Cell Cycle, Injury, and Phenotype Lability**. Ann N Y Acad Sci.

Other applications of Flow Cytometry

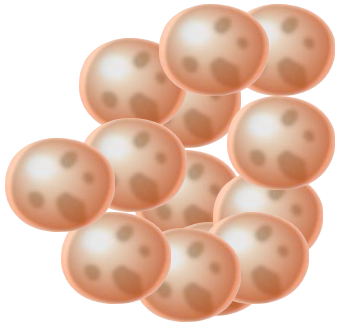
- **Cell sorting**
- **Statistical information obtained rapidly**
- **Flexibility of data acquisition**

Cell Sorting

Heterogeneous cell population



Homogeneous cell population

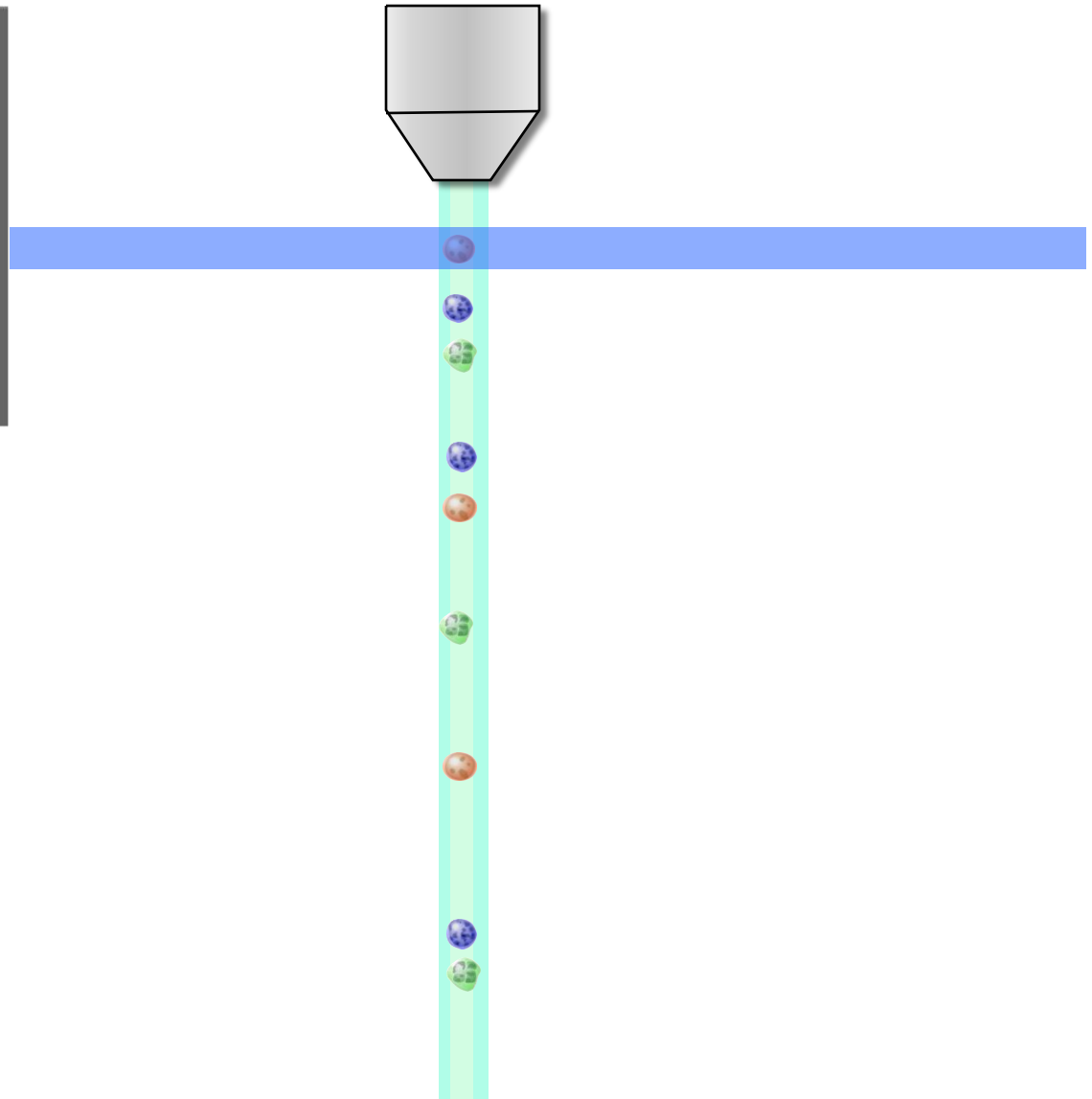


- Cells in suspension
- What is the number of cells required ?
- What will be the yield and the purity ?
- Effect on cell viability

Principle of Cell Sorting

Leonard Herzenberg (1972)

"Jet in air" System

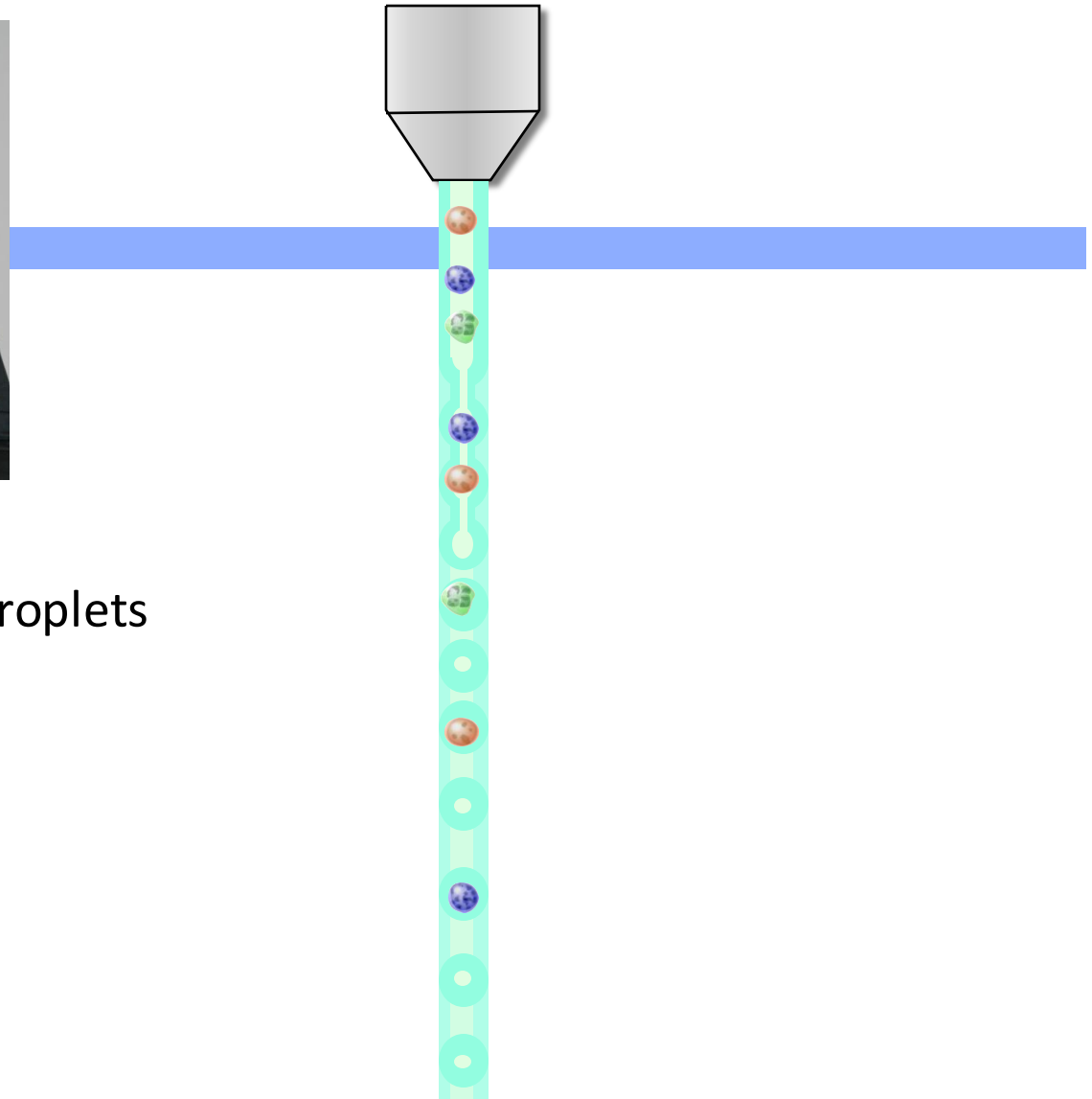


Principle of Cell Sorting

Leonard Herzenberg (1972)

"Jet in air" System

Nozzle vibration: trains of droplets



Principle of Cell Sorting

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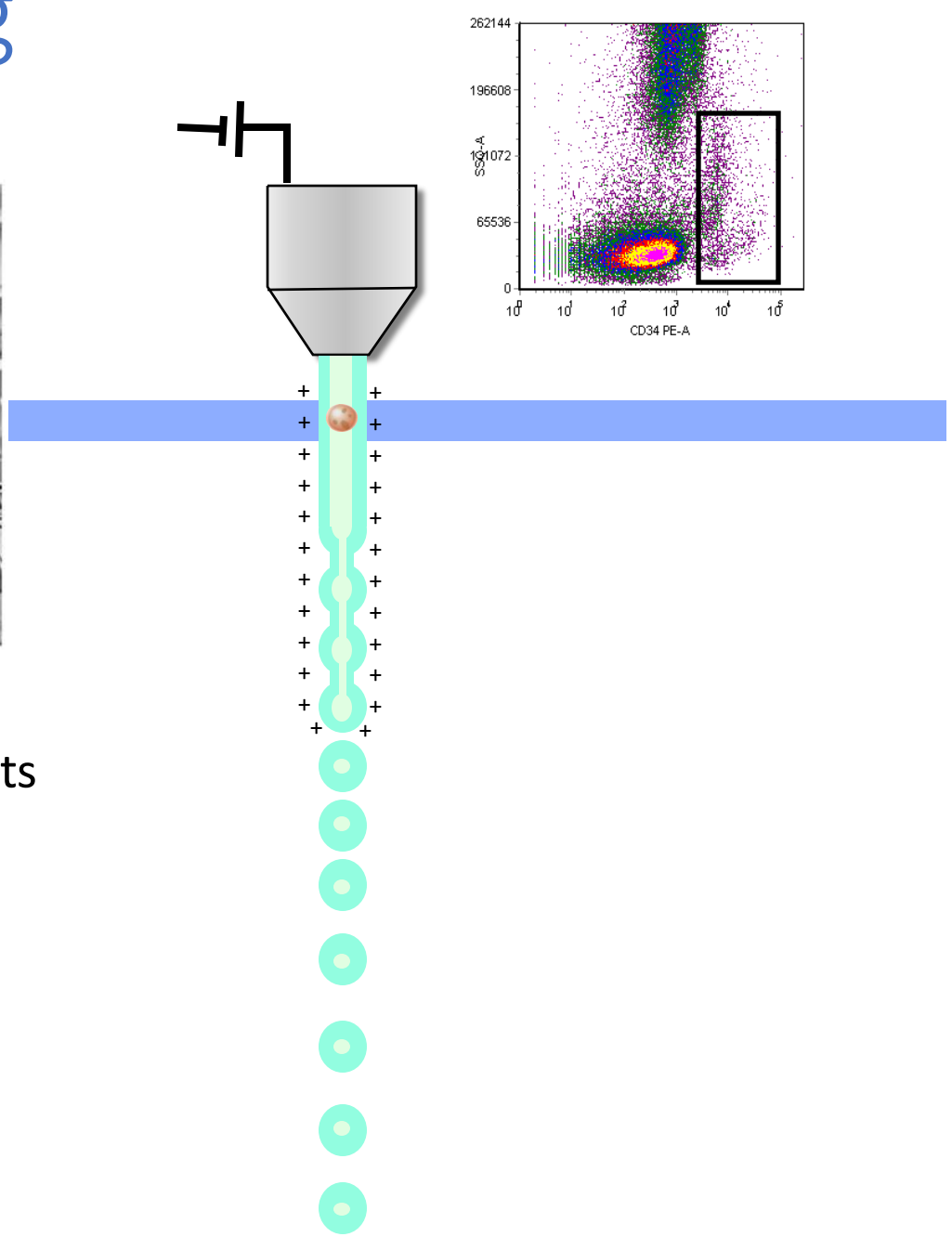
Droplet charged

Drop delay calculation



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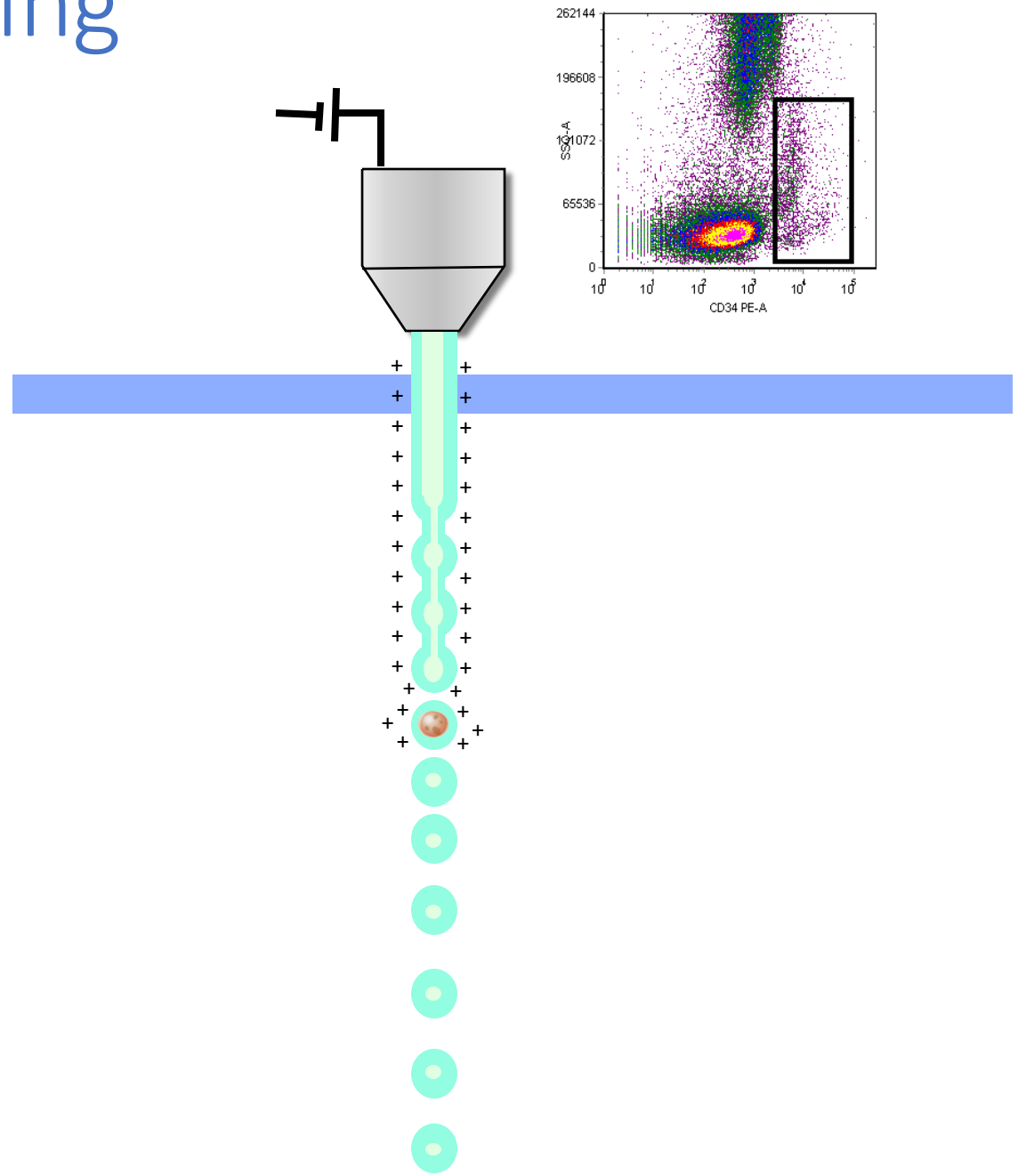
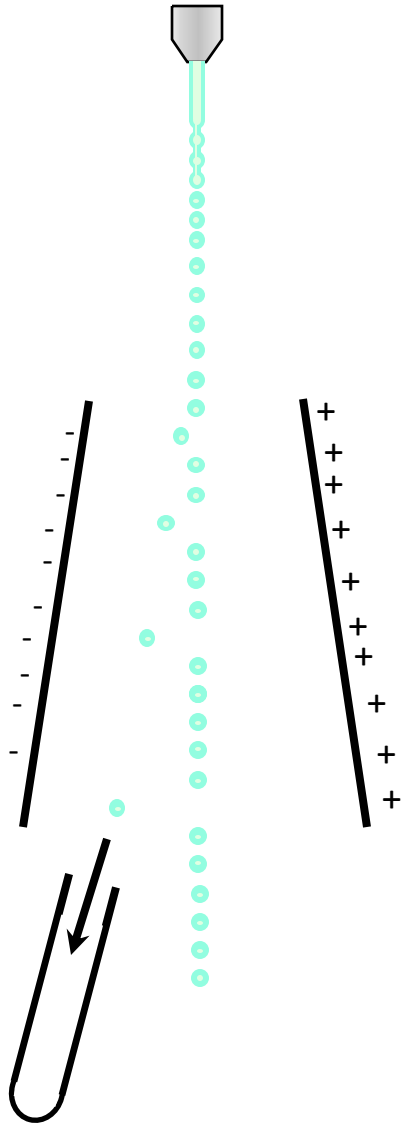


Principle of Cell Sorting

Droplets deflection



Cell Sorting

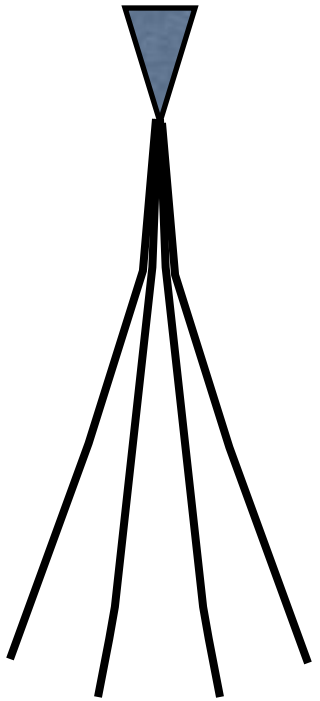
32 Parameters

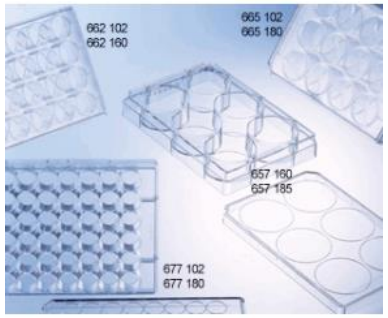
Purity : up to 99,9%

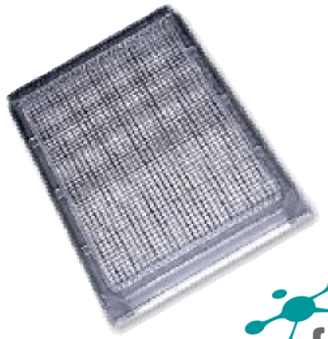
Speed : 70 000 ev/ sec

Sorting up to 8 ways

Yield from 50 to 70%







1536-wells

fc-3 (Bio)

Cell Sorting modes

Mode	Sort ?
Enrichment	Yes
Purity	Yes
Cloning	Yes

Cell Sorting modes

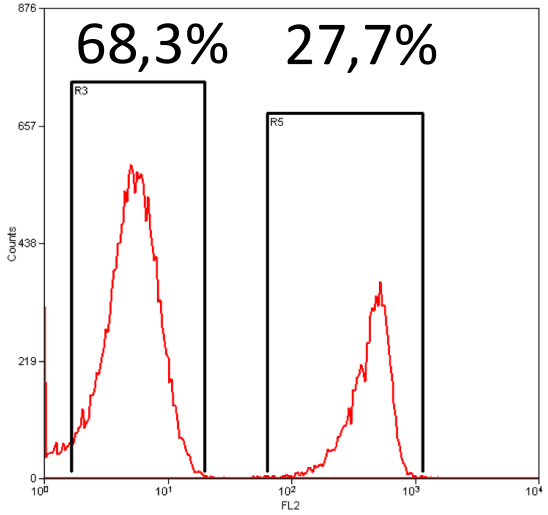
Mode	Sort ?
Enrichment	Yes
Purity	No
Cloning	No

Cell Sorting modes

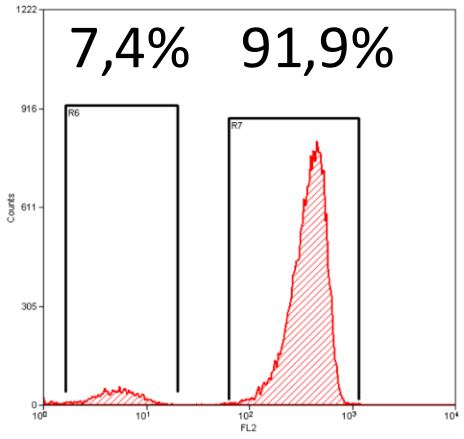
Mode	**Sort ?**
Enrichment	Yes
Purity	Yes
Cloning	No

Cell Sorting modes

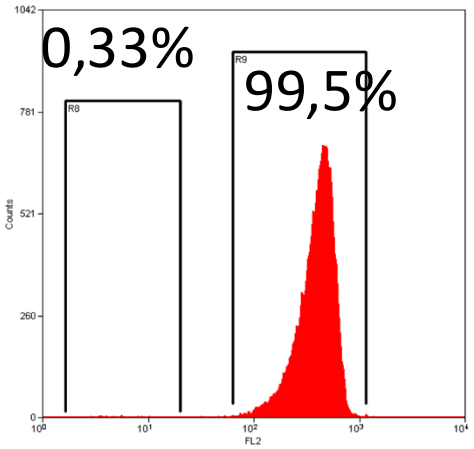
Before cell sorting



Enrichment



Purity

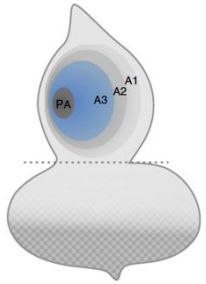


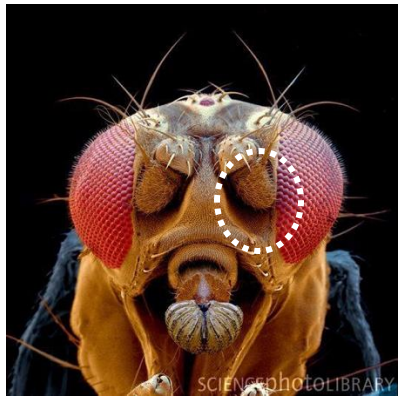
Cell Sorting Applications

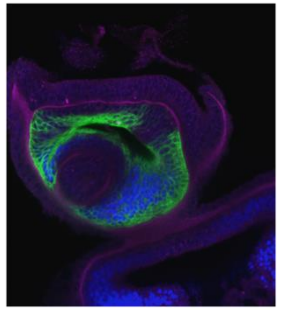
Isolate and purify different subsets

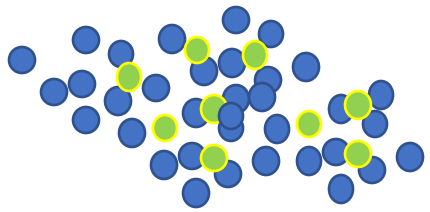
- Bulk or Single Cell sorting
- Culture or transfer in vivo
- Selection based on gene/tg expression
- RNA expression
- Gene expression screens
- DNA analysis
- Transfection
- Protein expression
- Cloning

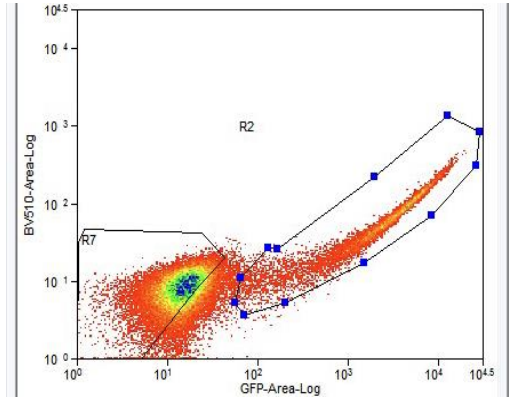
Tracing Olfactory Sensory neurone Identity at the Single Cell Level

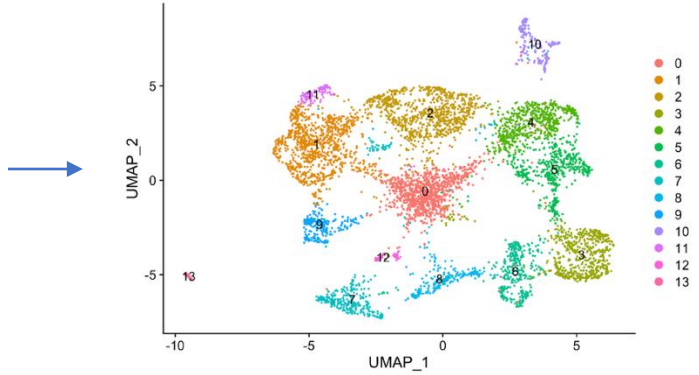








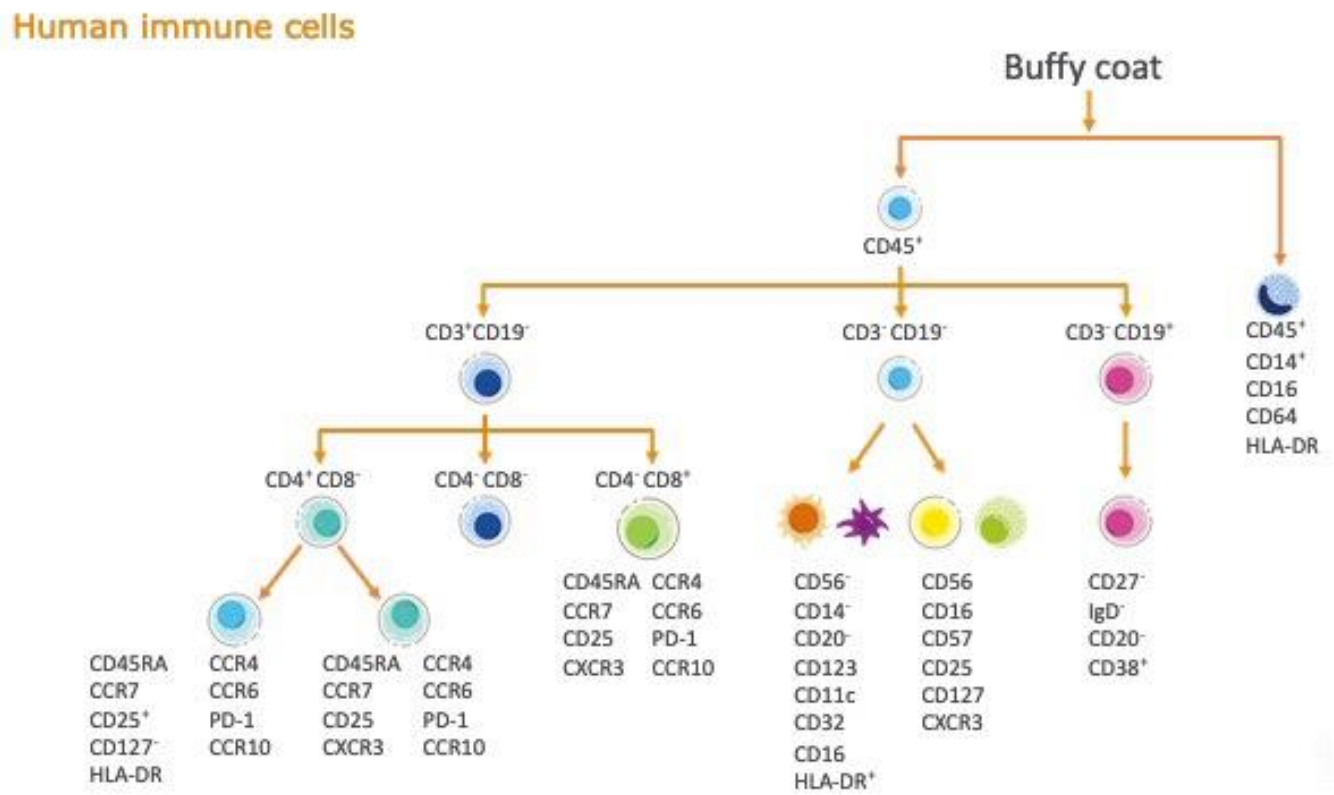




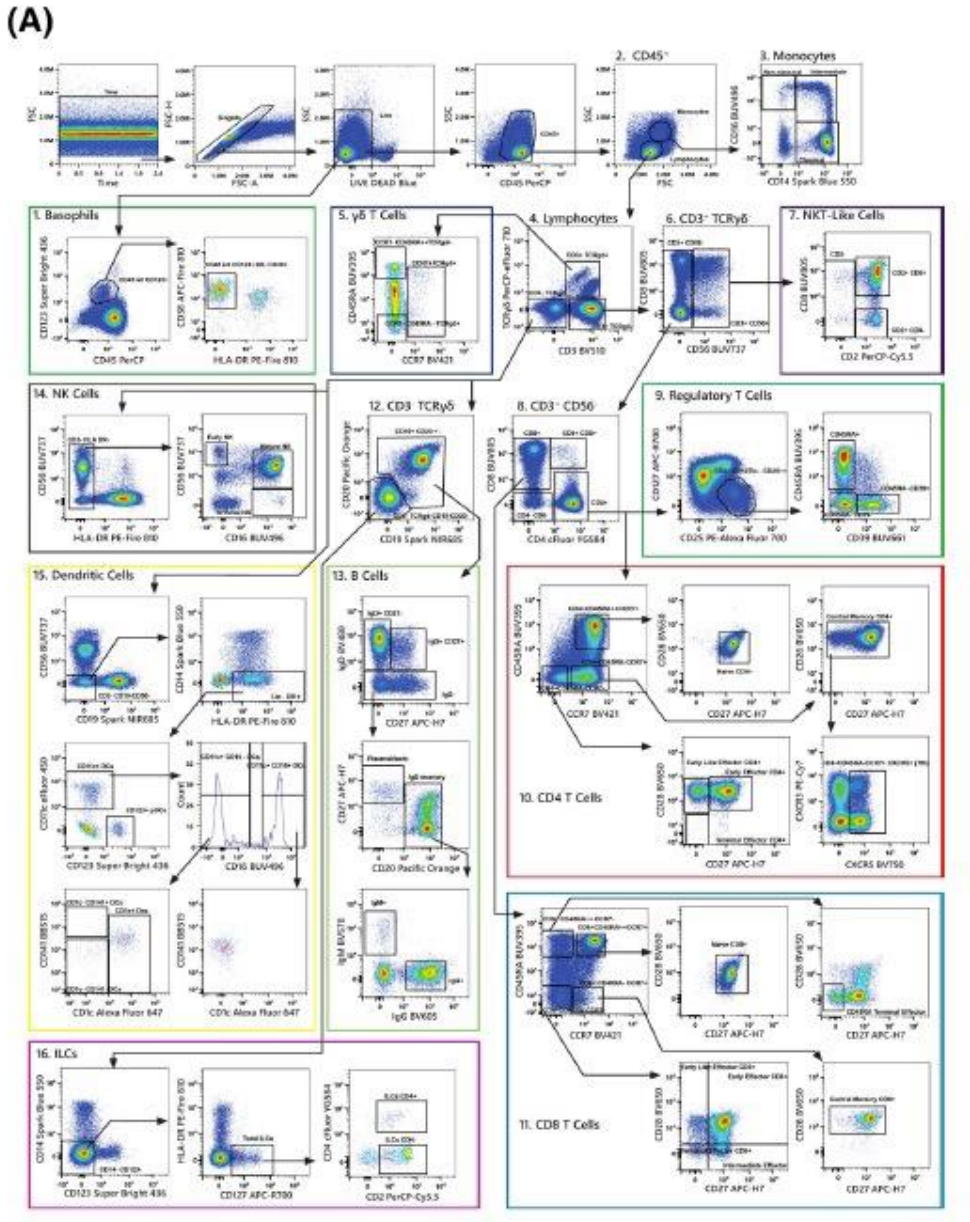
Jeremy Mermot, CIG, UNIL

Immunophenotyping

27-Colour Immunophenotyping



Immunophenotyping



40-color Immunophenotyping

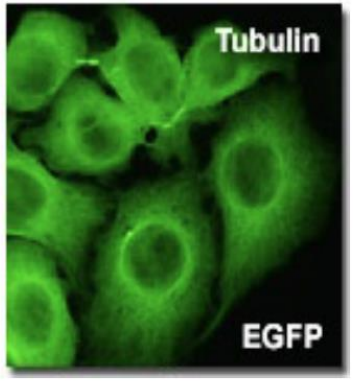
ISAC CYTOMETRY Journal of Quantitative Cell Science PART A

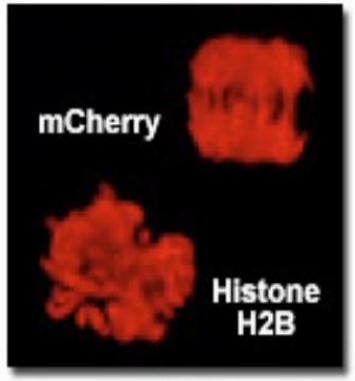
MIFlowCyt

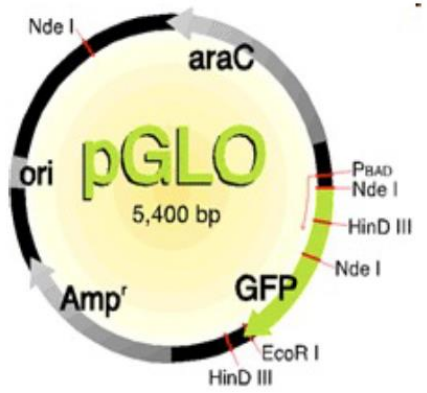
OMIP-069: Forty-Color Full Spectrum Flow Cytometry Panel for Deep Immunophenotyping of Major Cell Subsets in Human Peripheral Blood

Lily M. Park,[1] Joanne Lannigan,[2] Maria C. Jaimes[3*]

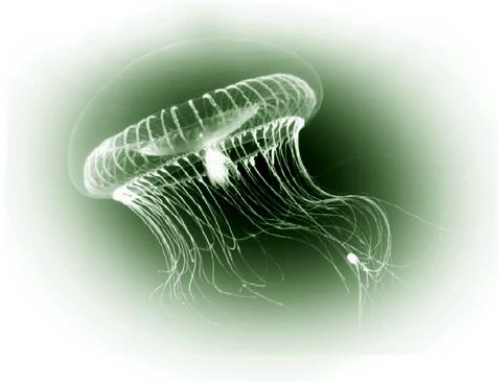
Fluorescent protein expression analysis







Protein (Acronym)	Excitation Maximum (nm)	Emission Maximum (nm)	Relative Brightness (% of eGFP)
eGFP	484	507	100
eCFP	439	476	39
eYFP	514	527	151
mRFP1	584	607	37
mCherry	587	610	47
DsRed	558	583	176
eBFP	383	445	27

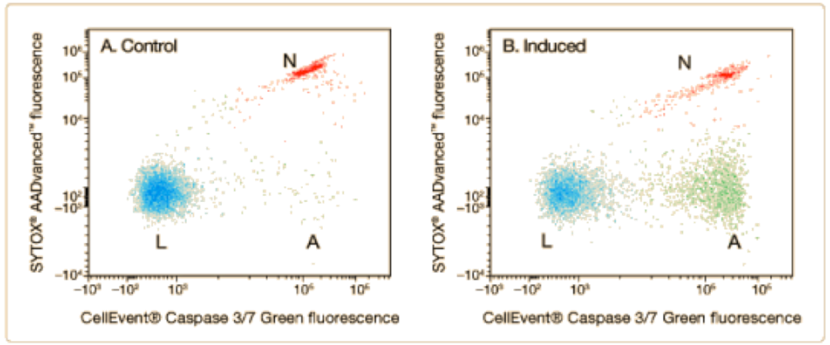


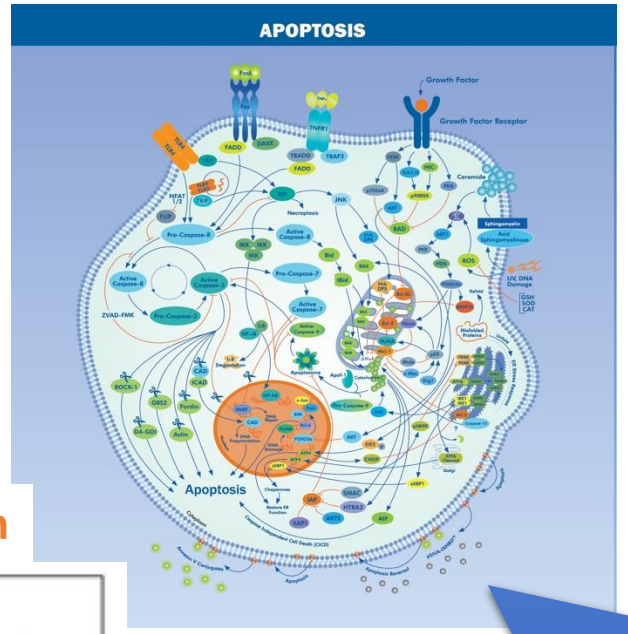
Aequorea victoria

http://www.microscopyu.com/articles/livecellimaging/fpintro.html

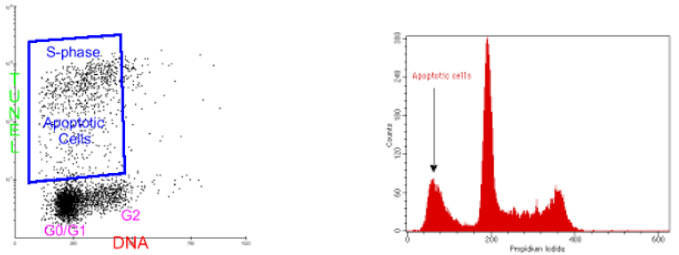
Cell functionality monitoring – programmed cell death

Caspases activation

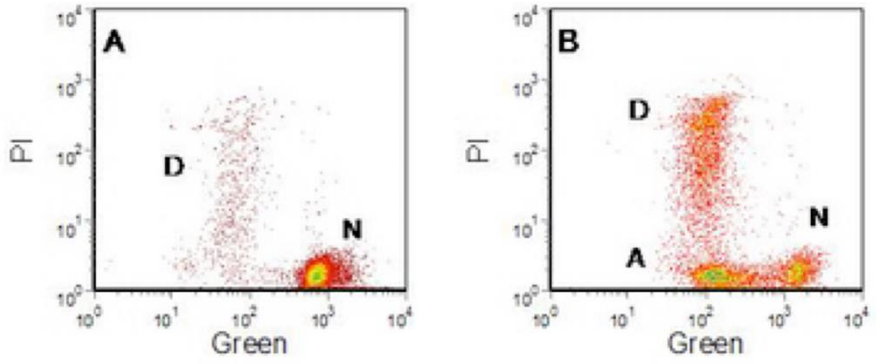




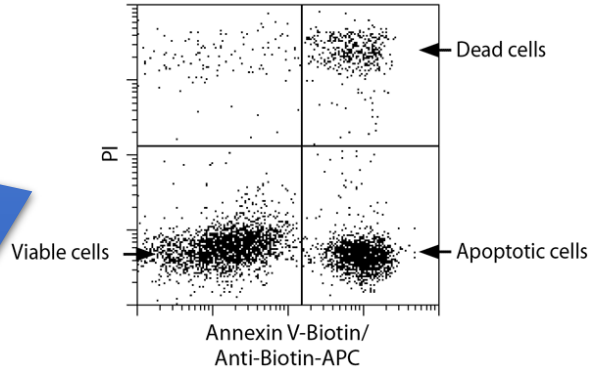
DNA Fragmentation



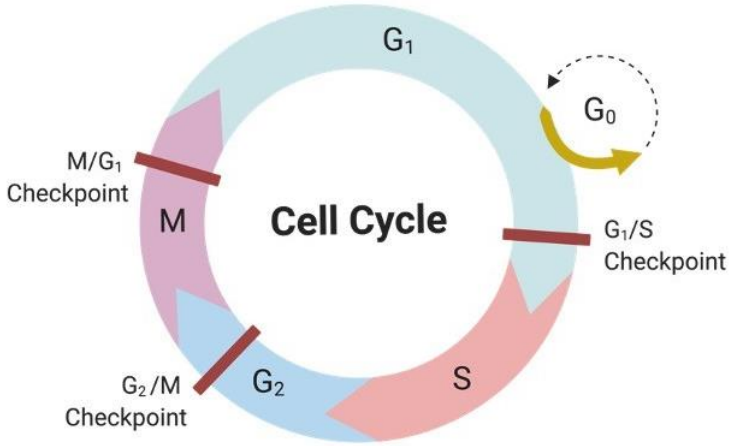
Mitochondrial potential dissipation

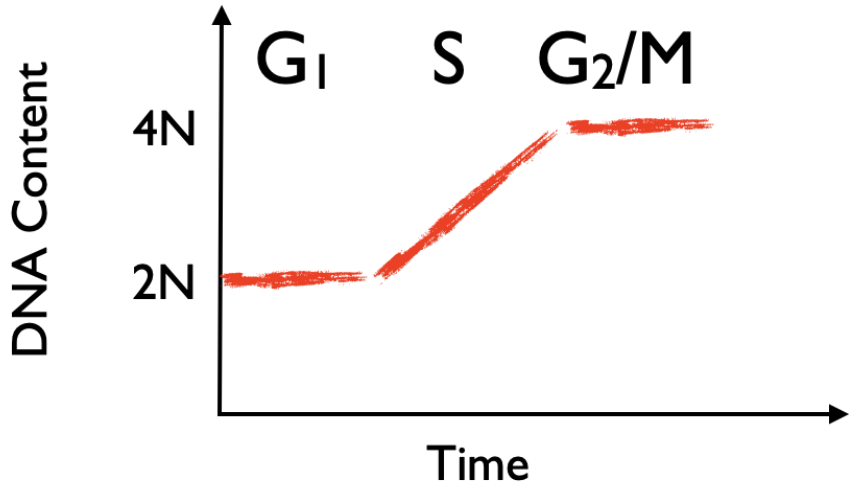


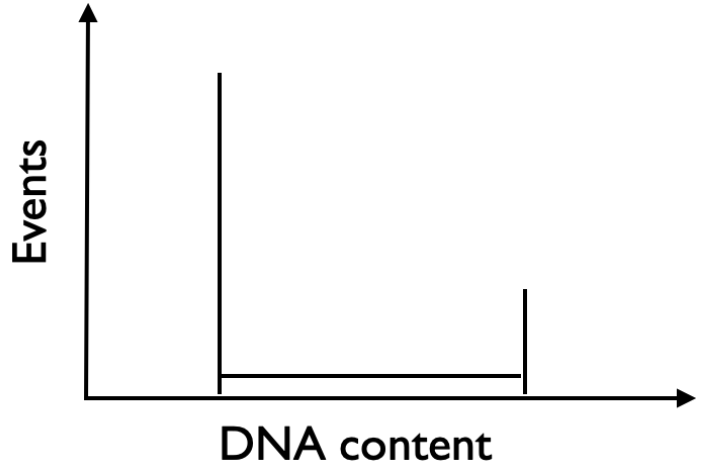
Phosphatidylserine Externalization
Annexine V staining



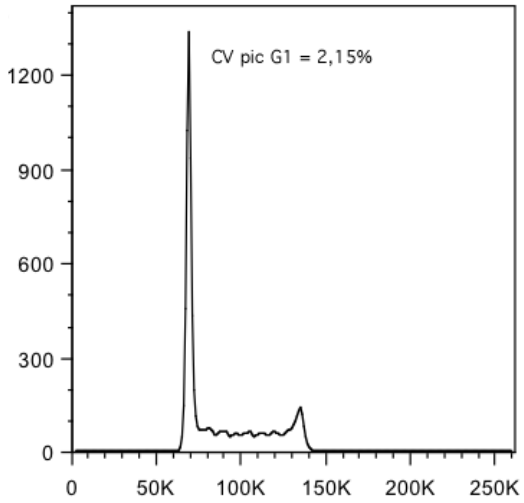
Cell functionality monitoring – cell cycle

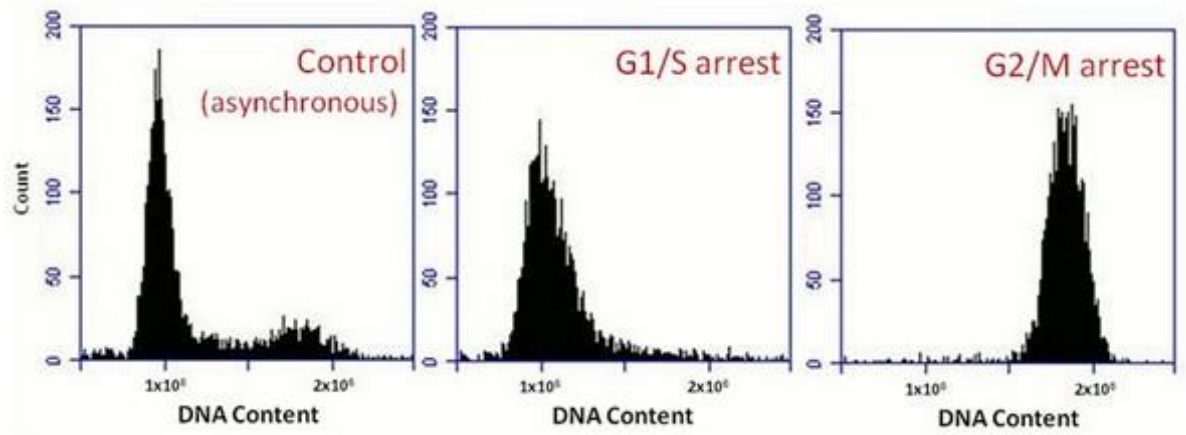






Monoparametric cell cycle analysis

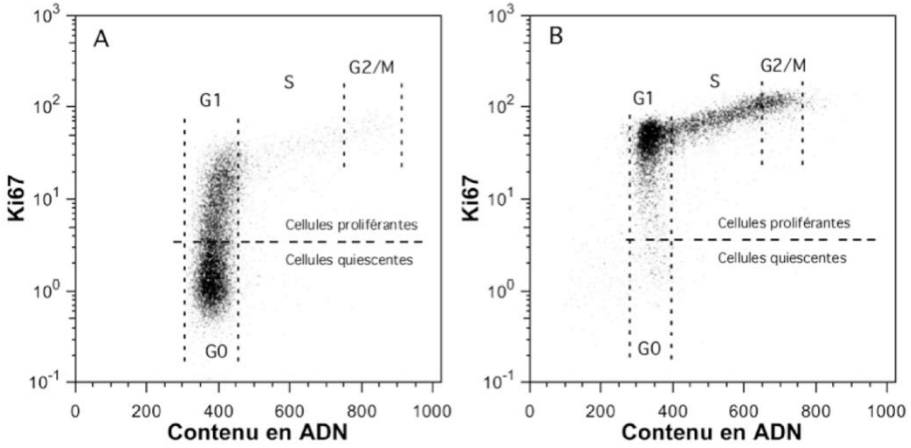




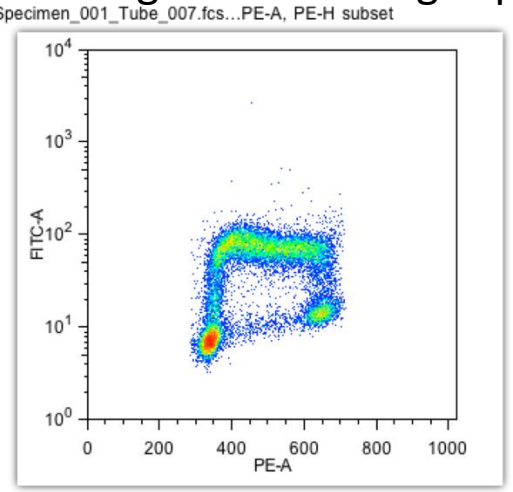
Cell functionality monitoring – cell cycle

Multi-parametric cell cycle analysis

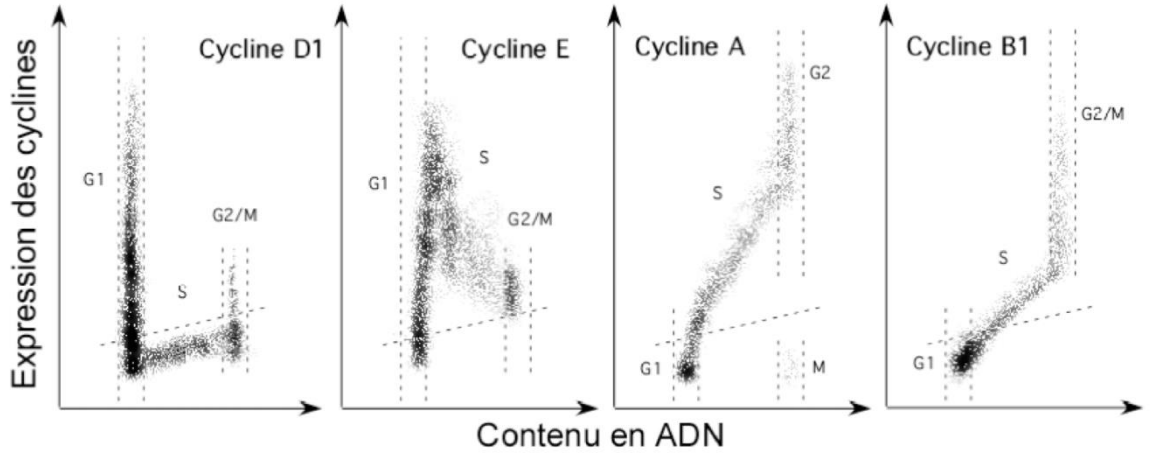
Ki67 staining: proliferating cells



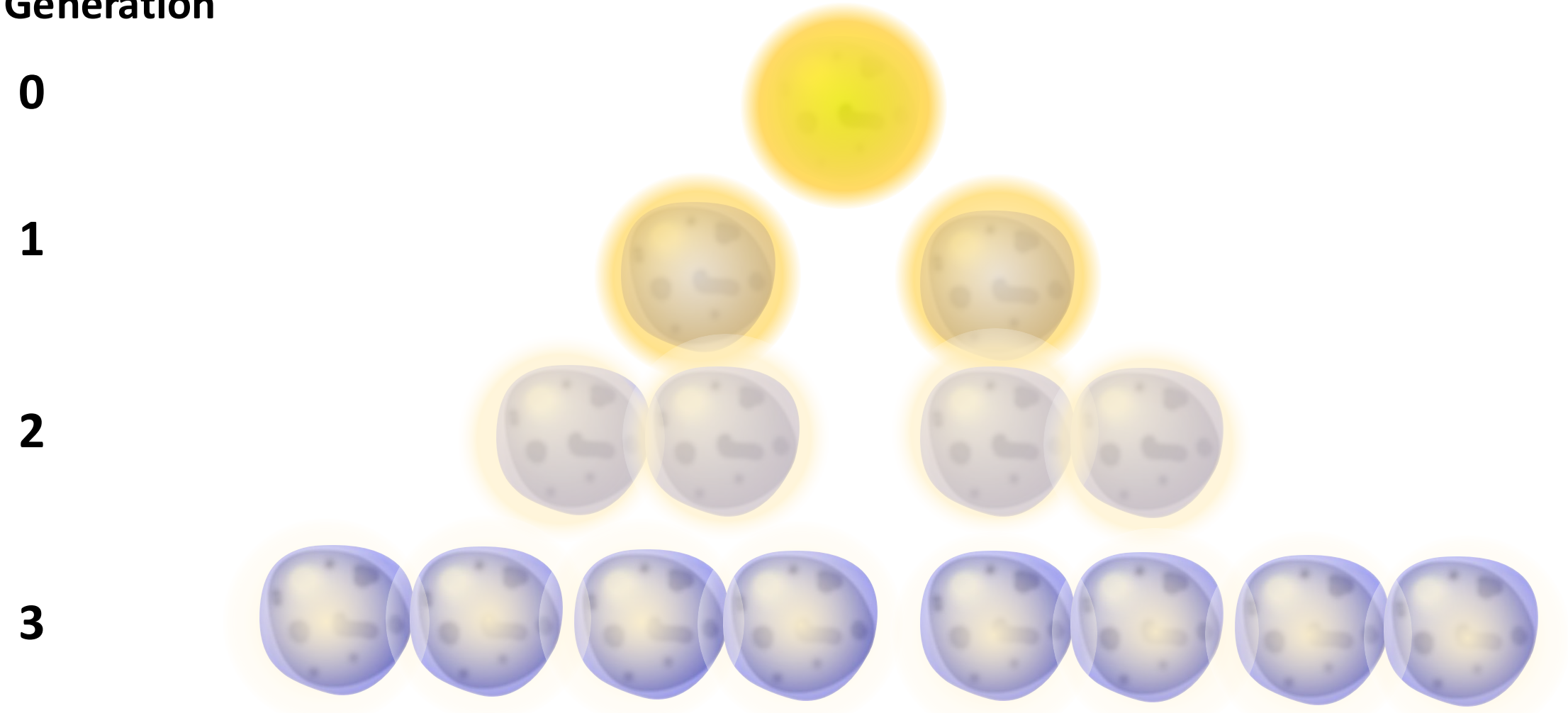
BrdU integration staining: S phase



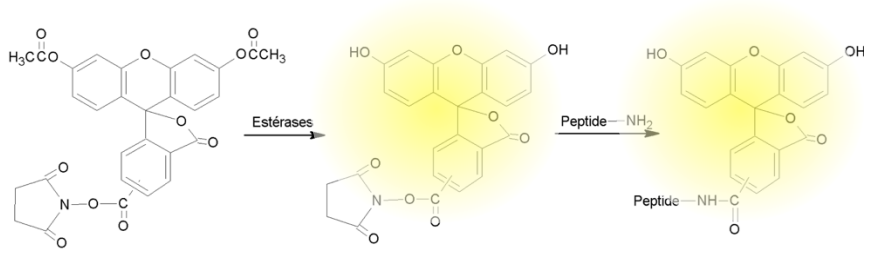
Cyclin stainings



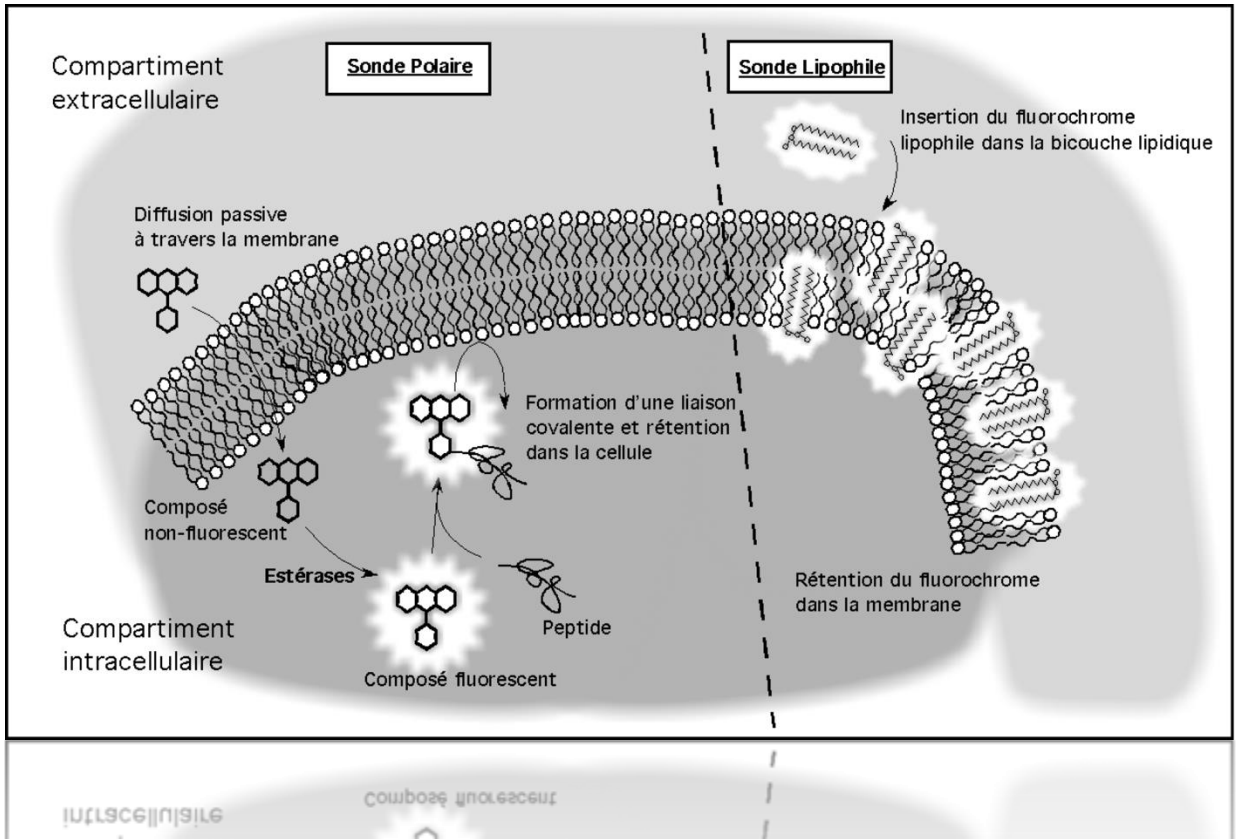
Cell functionality monitoring – cell proliferation

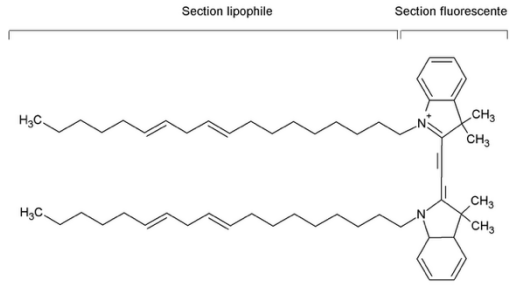


Cell functionality monitoring – cell proliferation



Ex: CFSE, Cell Trace, …



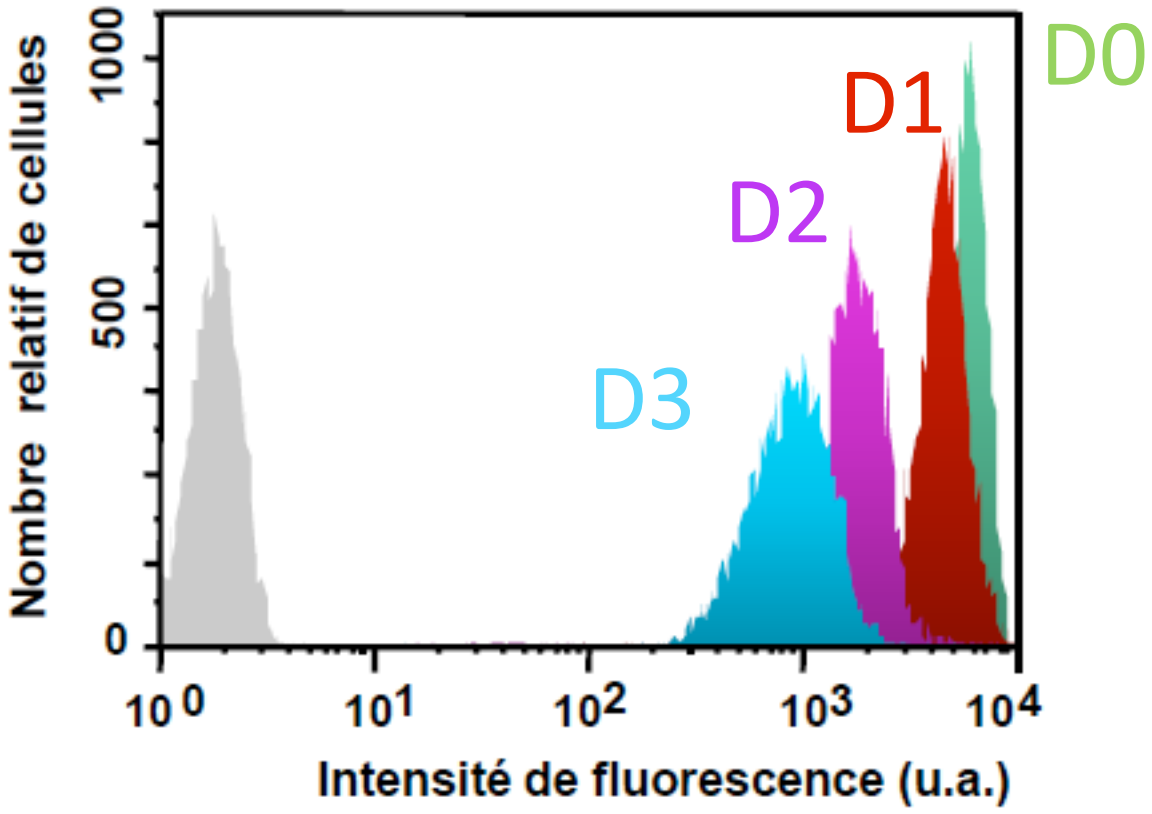


Ex: PKH

Cell functionality monitoring – cell proliferation

Data interpretation

Clonal or Oligo-clonal cell lines



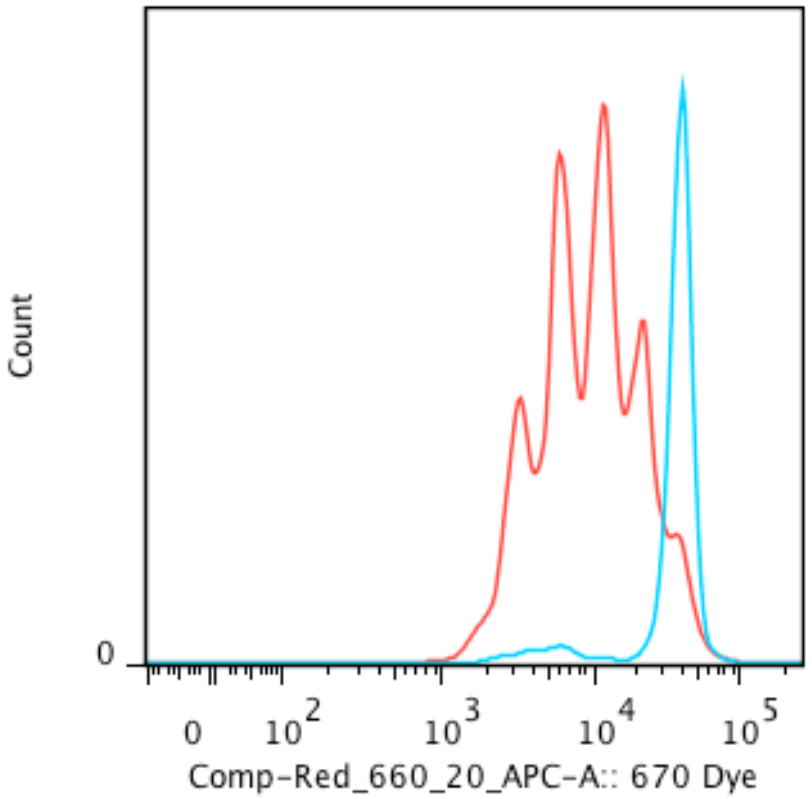
Proliferation index

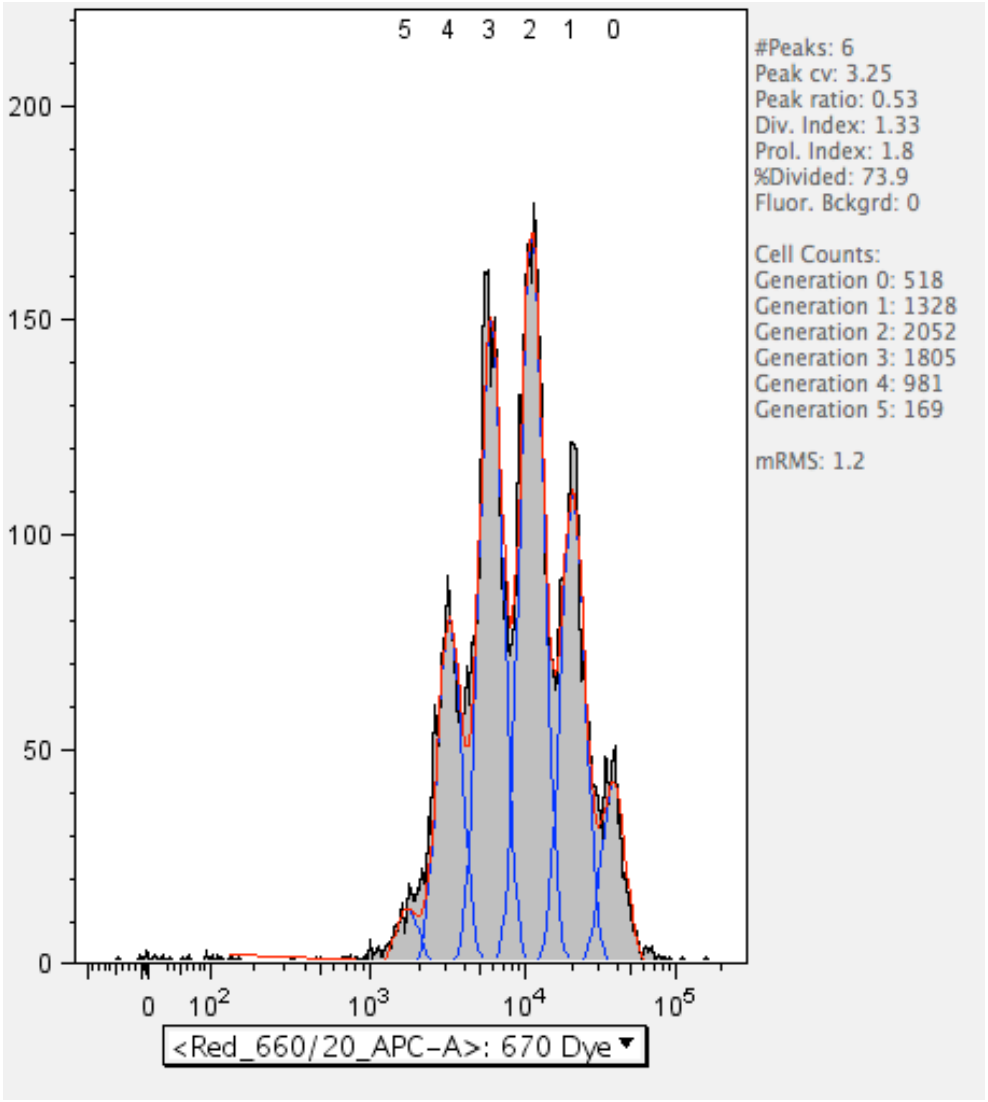
$$\frac{\text{Mean Fluo D0}}{\text{Mean Fluo Dn}}$$

Cell functionality monitoring – cell proliferation

Data interpretation

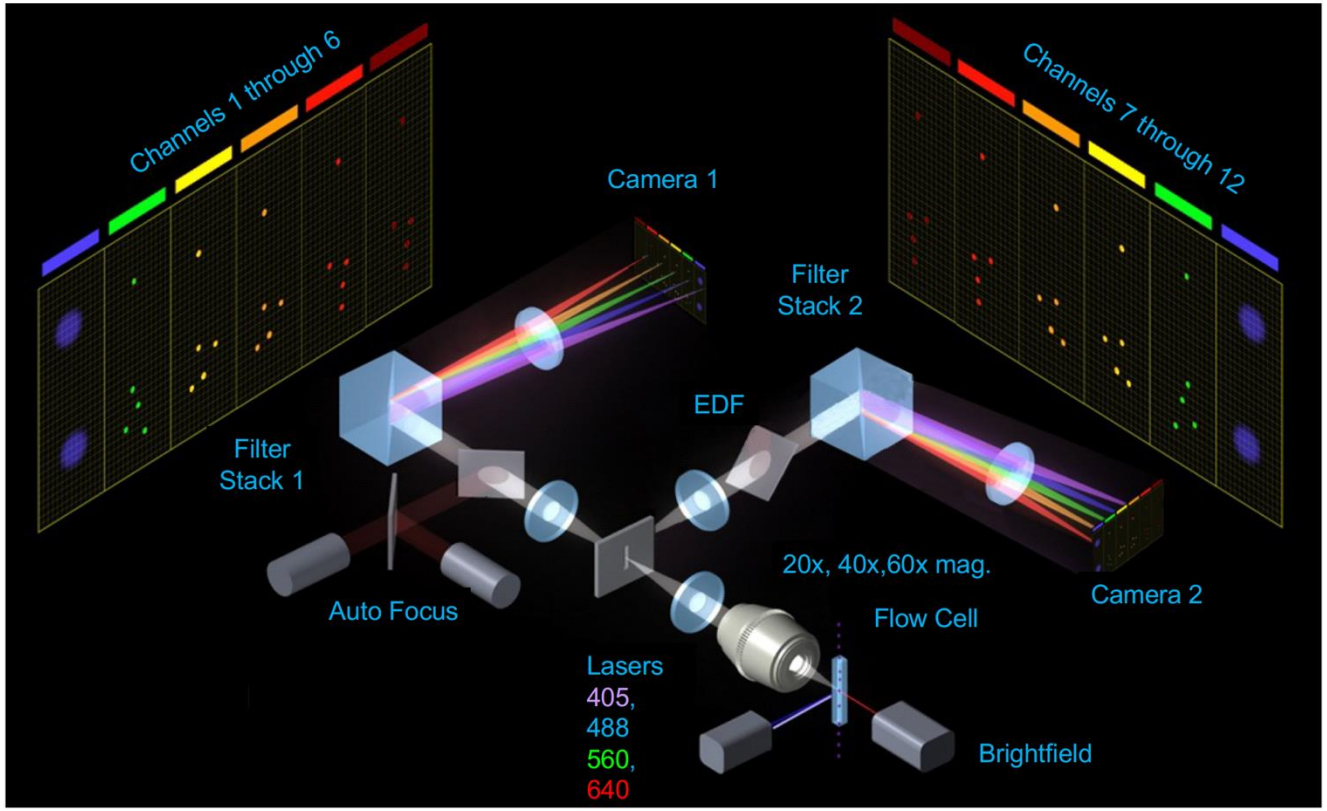
Polyclonalcell lines





Non-conventional Flow cytometry: Imaging Flow Cytometry

4 lasers, 12 channels: BrightField x 2, 10 colours or 9 colours plus SSC
CCD Cameras instead of PMTs or APDs to measure fluorescence intensity

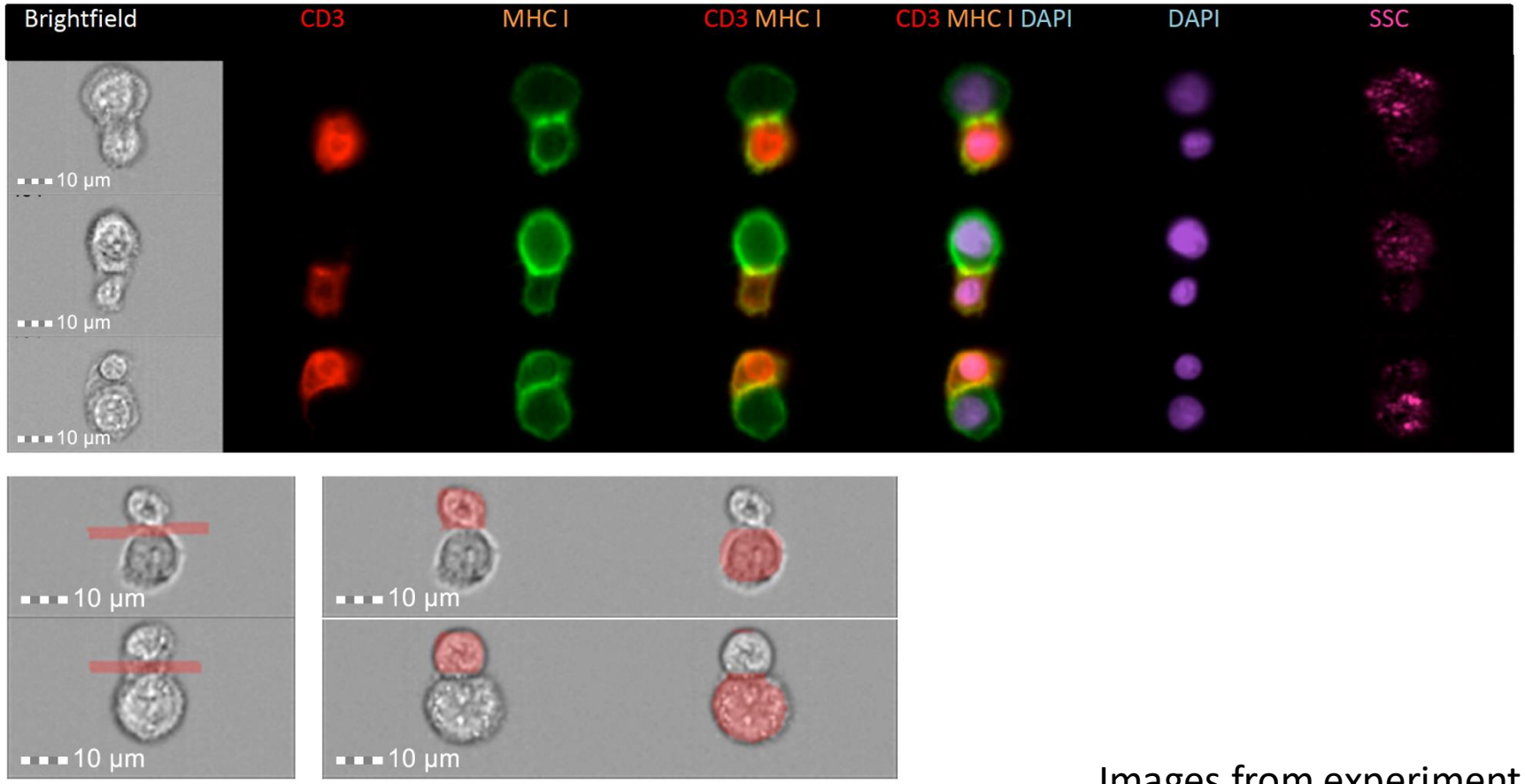


Applications:

- Cell cycle analysis (mitotic phases)
- Cell-cell interactions
- Immune synapse visualization
- Apoptosis vs Necrosis
- Transcription factor translocation
- Colocalization with organelles
- Autophagy
- Internalization of particles
- Phagocytosis

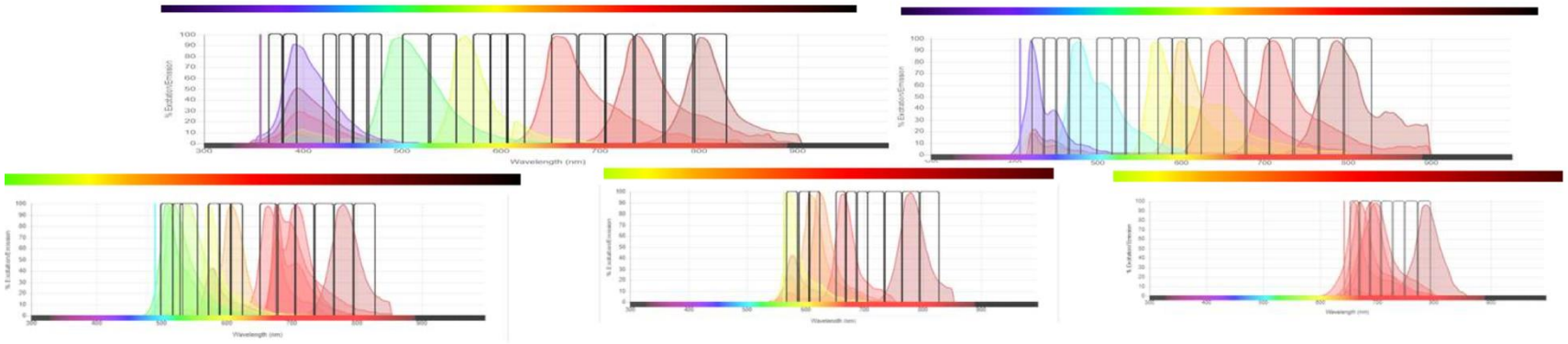
Non-conventional Flow cytometry: Imaging Flow Cytometry

Analysis of the immune synapse



Images from experiment of N. Dilek (DO)

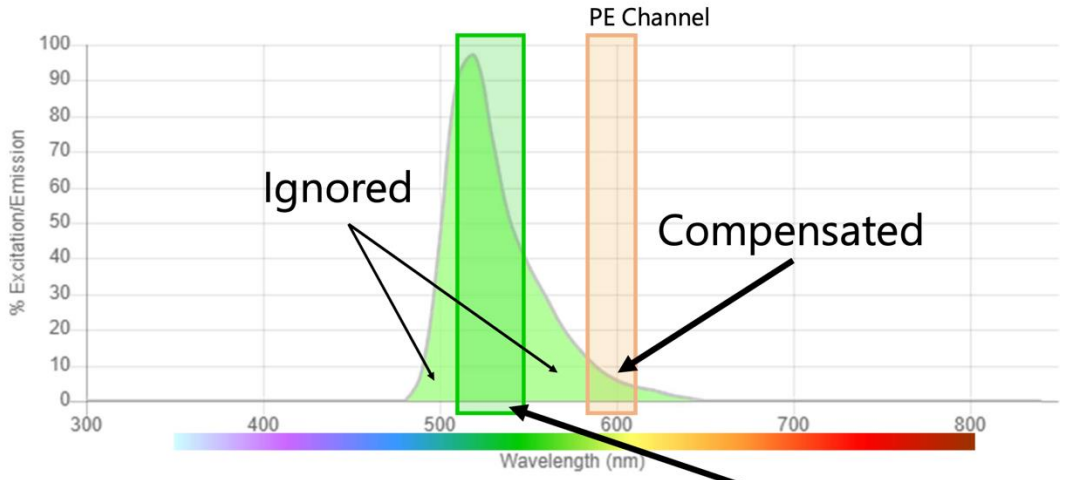
Non-conventional Flow cytometry: Spectral Flow Cytometry



Non-conventional Flow cytometry: Spectral Flow Cytometry

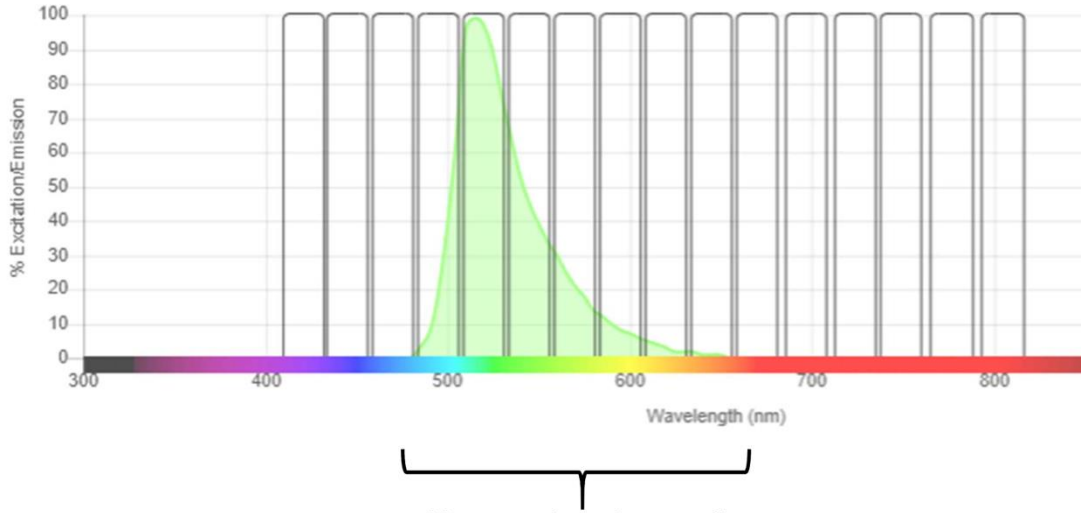
Full Spectrum analysis: higher sensitivity

Classical Flow Cytometer

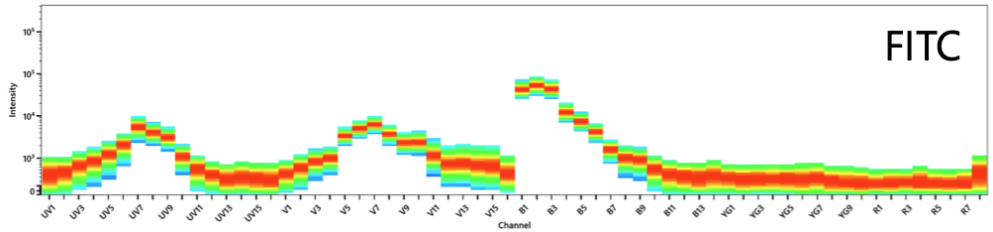


FITC = Green! … *or emission from 515-545 (530/30 BP)*

Spectral Flow Cytometer

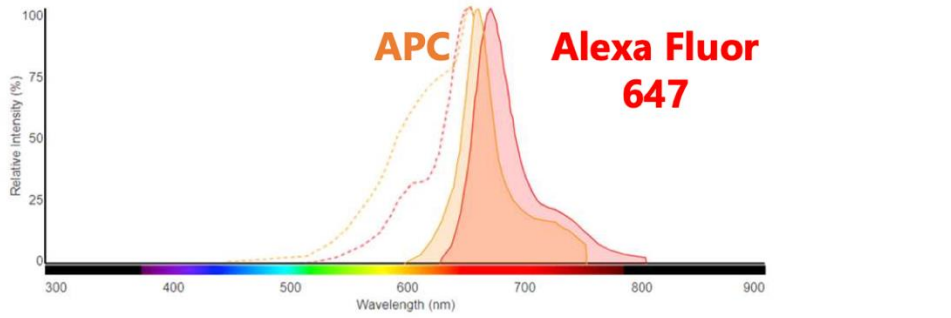


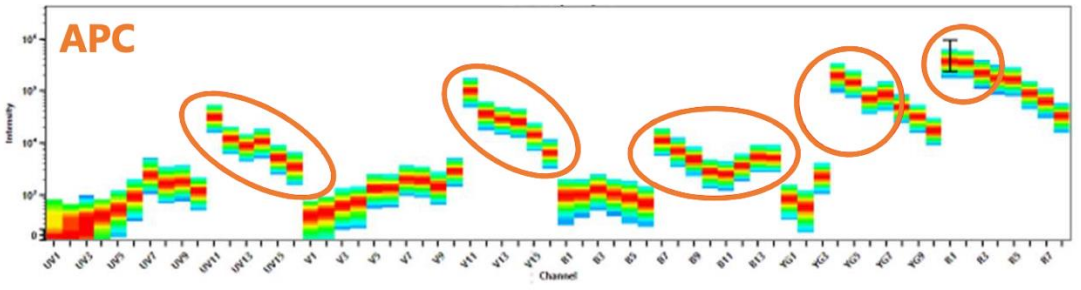
FITC = Full emission from 490-650

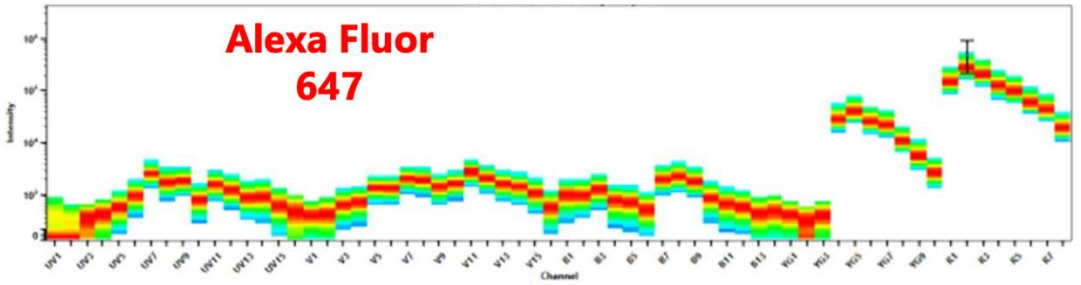


Non-conventional Flow cytometry: Spectral Flow Cytometry

Simultaneous use of highly overlapping dyes: increased number of colors







Approximate Emission Wavelength (nm)	UV	Violet	Blue	Yellow-Green	Red
395	BUV395				
420		BV421			
440		Super Bright 436			
450	LIVE/DEAD™ Blue	eFluor 450			
480		BV480			
500	BUV496		BB515		
520		BV510	FITC		
550		Pacific Orange	Spark Blue 550		
570	BUV563	BV570		PE	
580				cFluor YG584	
600	BUV615	BV605		PE/Dazzle 594	
660	BUV661	BV650		PE-Alexa Fluor 610	APC
680			PerCP	PE-Cy5	Alexa Fluor 647
690			PerCP-Cy5.5		Spark NIR 685
700		BV711	PerCP-eFluor 710	PE-Alexa Fluor 700	APC-R700
730	BUV737				
750		BV750			
780		BV785		PE-Cy7	APC-H7
800	BUV805			PE/Fire 810	APC/Fire 810

Non-conventional Flow cytometry: Spectral Flow Cytometry

Autofluorescence extraction

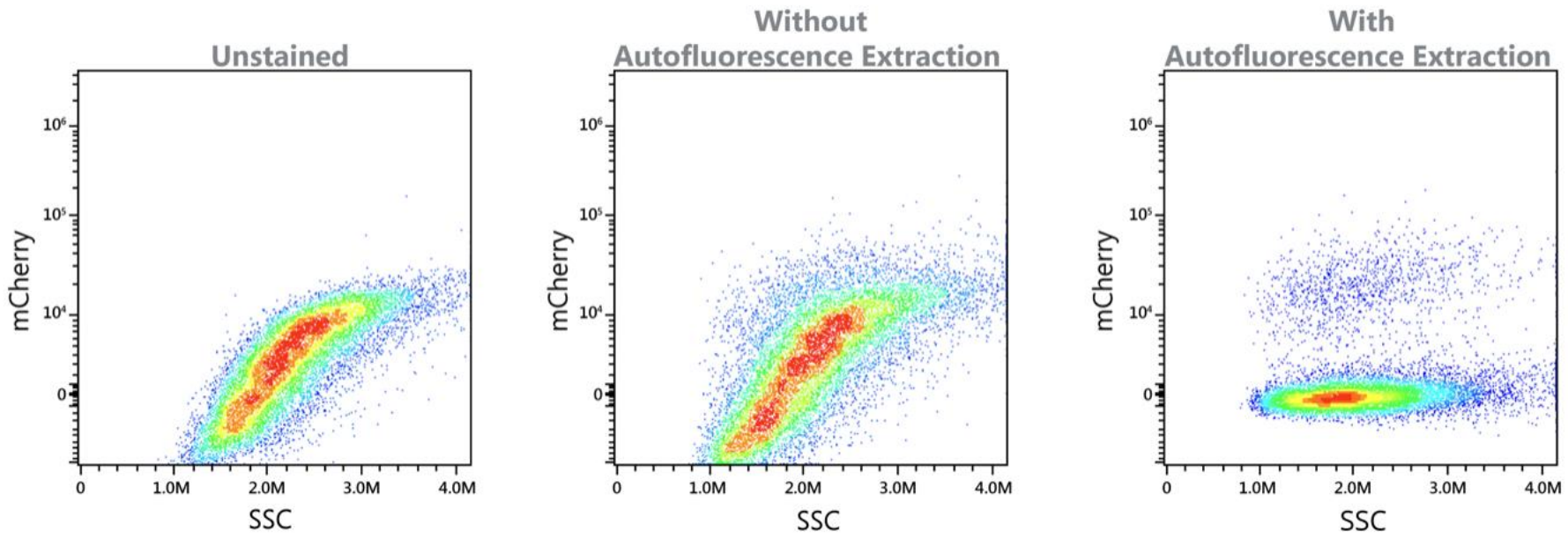
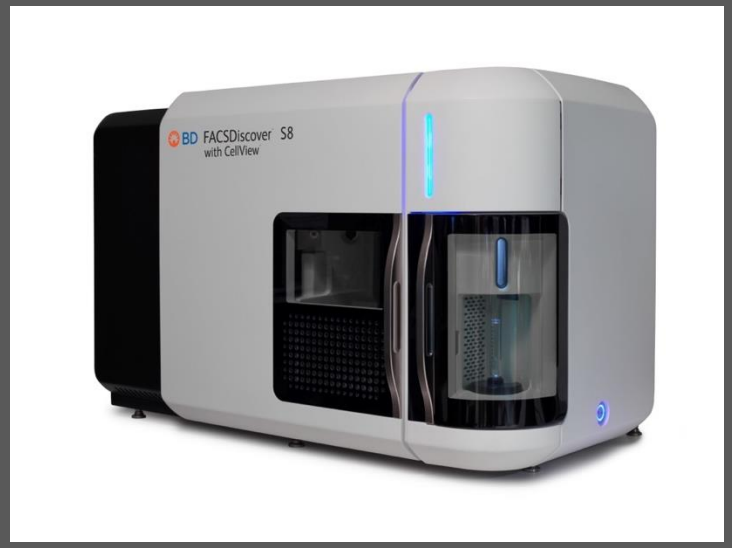
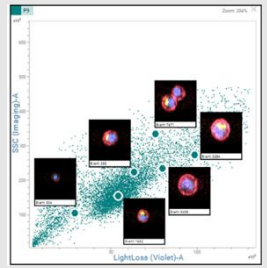
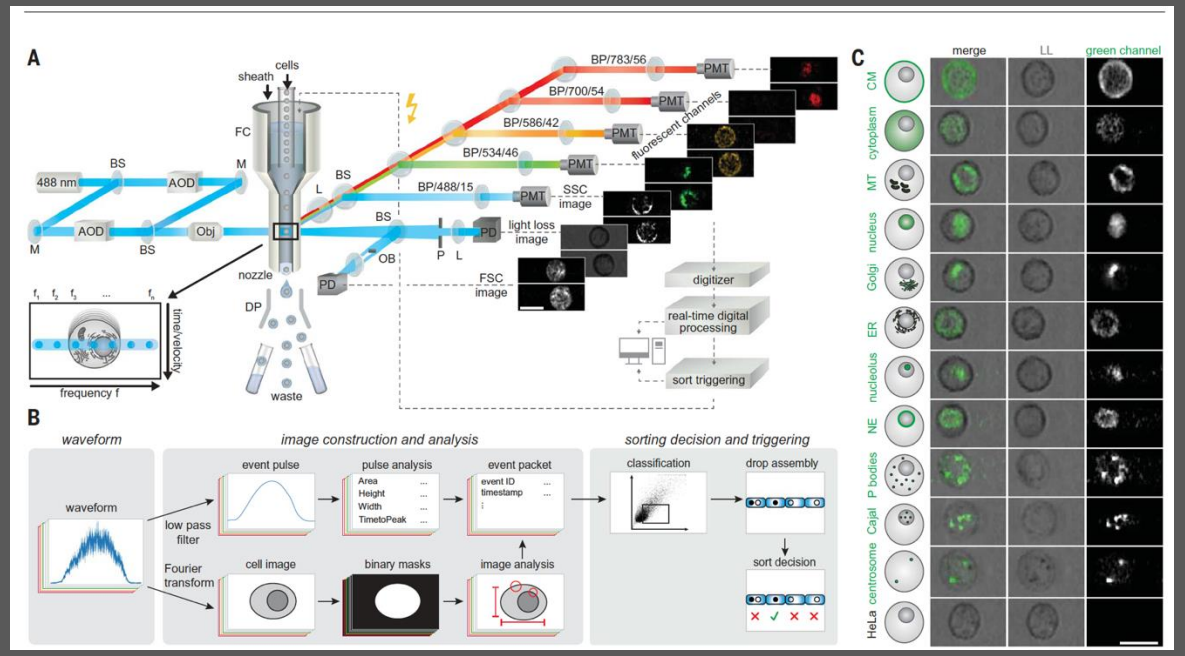


Image-enabled and spectral cell sorting

FACS Discover S8 with Cell View



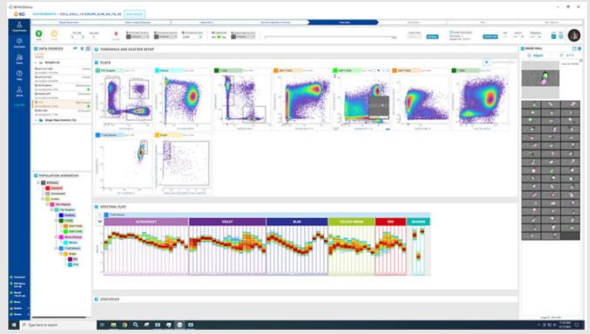




Imaging

- 1 laser (488nm)
- 6 imaging detectors (3 for fluorescence)
- Includes image features such as eccentricity, max intensity, size, radial moment, correlation and delta center of mass
- Sorting up to 10’000 cells/sec with imaging

Spectral



- 5 lasers
- 78 detectors
- Autofluorescence substraction
- Deep Immunoprofiling

Applications:

- Cell-cell interaction
- Cell cycle
- Fluorescent localization (ex: NFκB translocation)
- 6-ways sorting
- …

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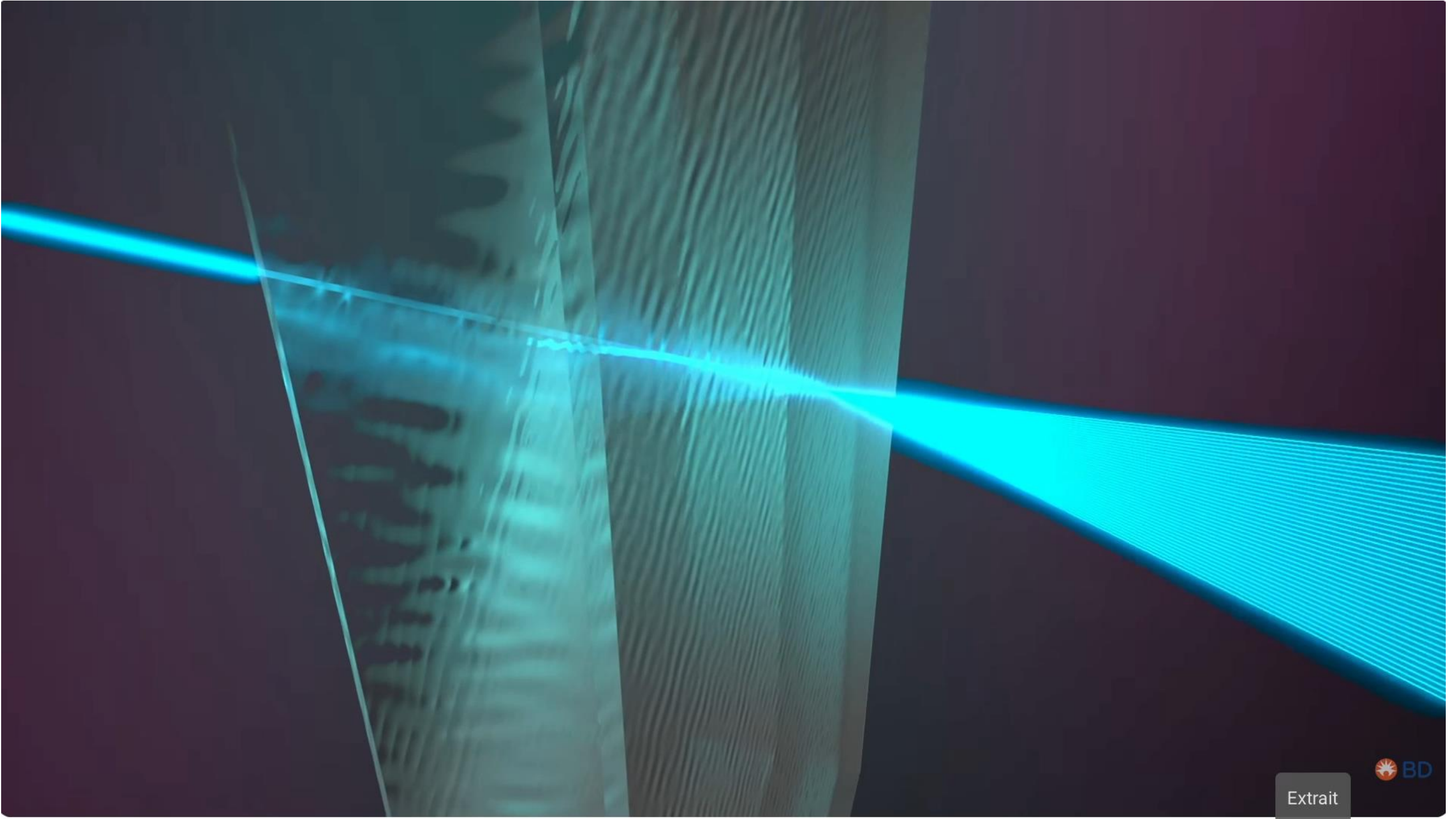
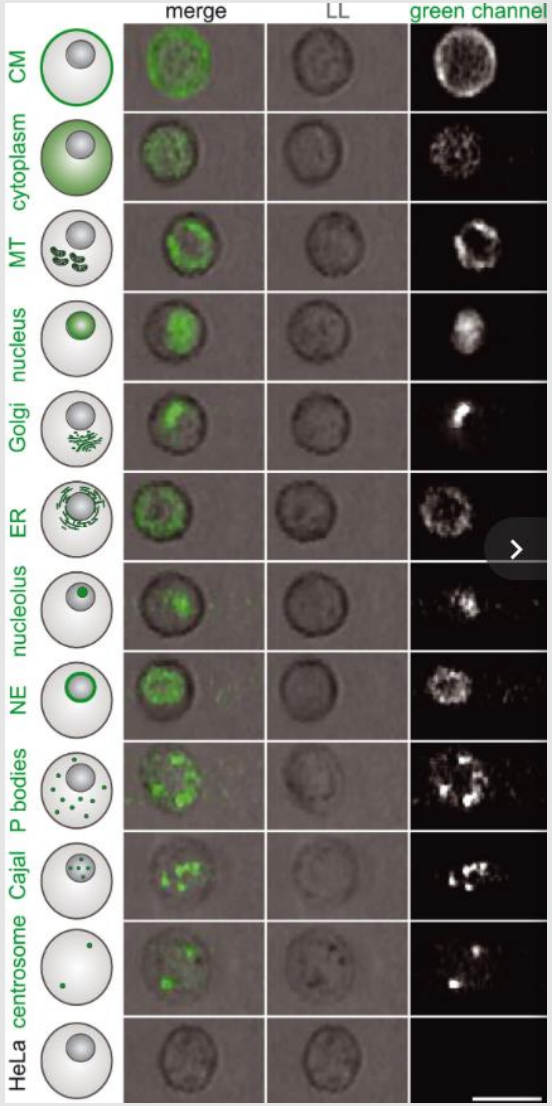
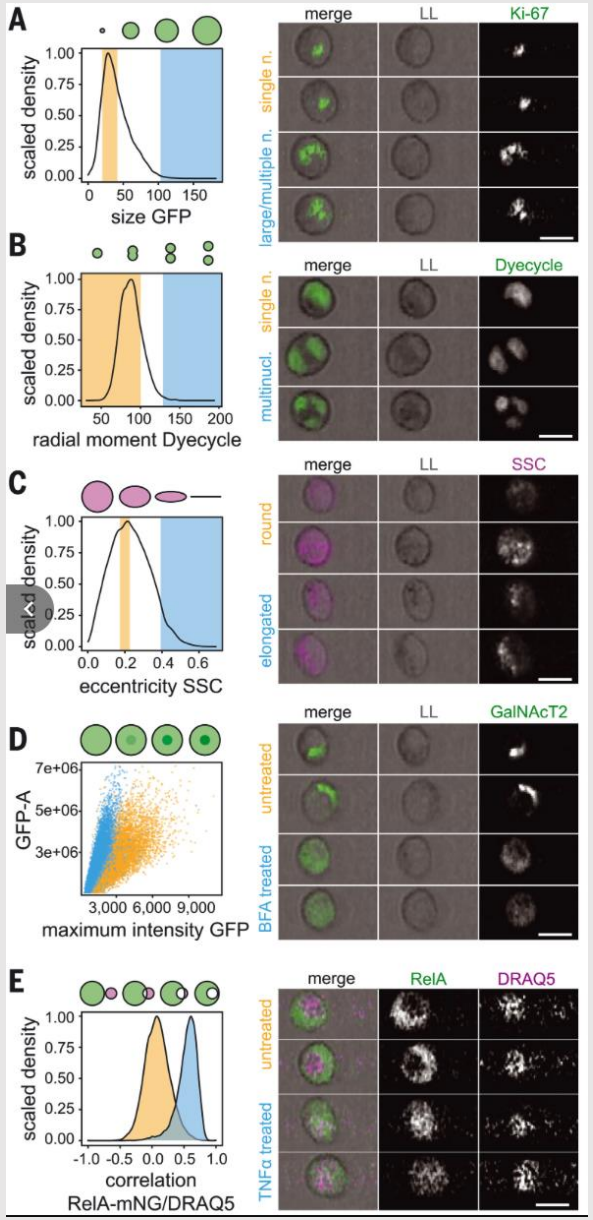
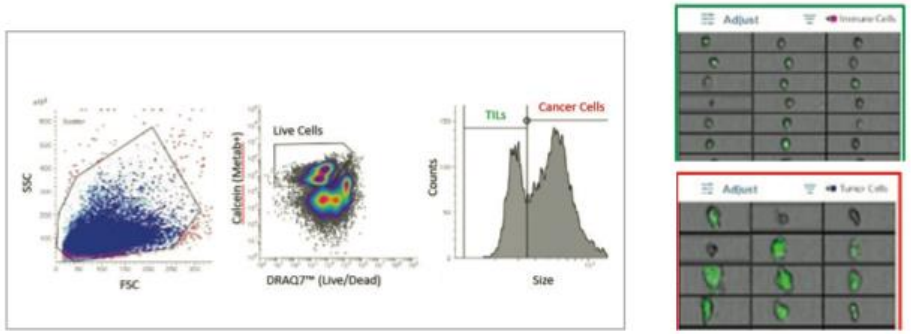

BD
Extrait

FACS Discover S8: some applications





FACS Discover S8: some applications



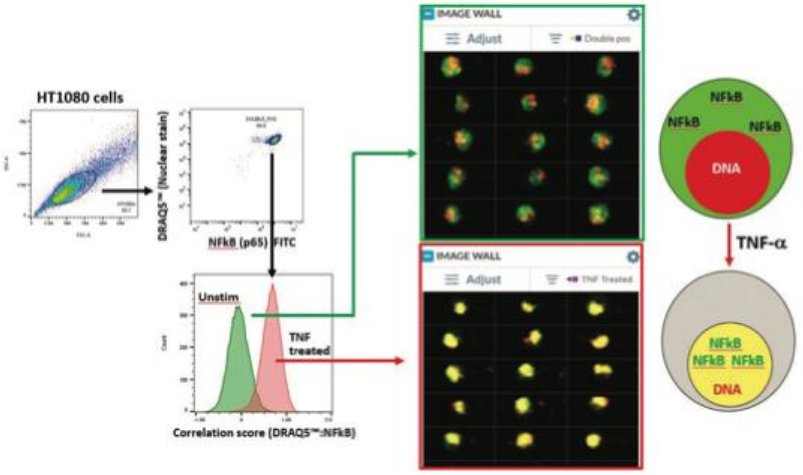
Label-free identification of TILs and cancer cells based on size

Label-free sorting

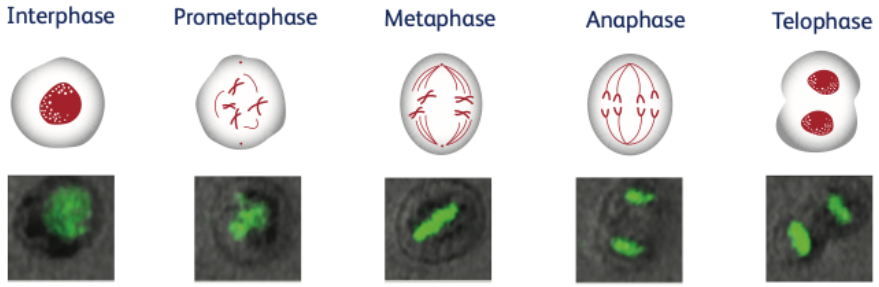
Minimize sample preparation and sort precious, sensitive and transiently expressing cells using image-enabled FSC, SSC and light loss detectors to enable accurate cell characterization without fluorescent antibody labeling.

Fluorescent localization

Reveal the spatial context of fluorescent signals hidden in flow cytometry. Track the subcellular movement of a protein across organelle boundaries within the cell, such as the NFκB translocation from the cytoplasm to the nucleus.



FACS Discover S8: some applications

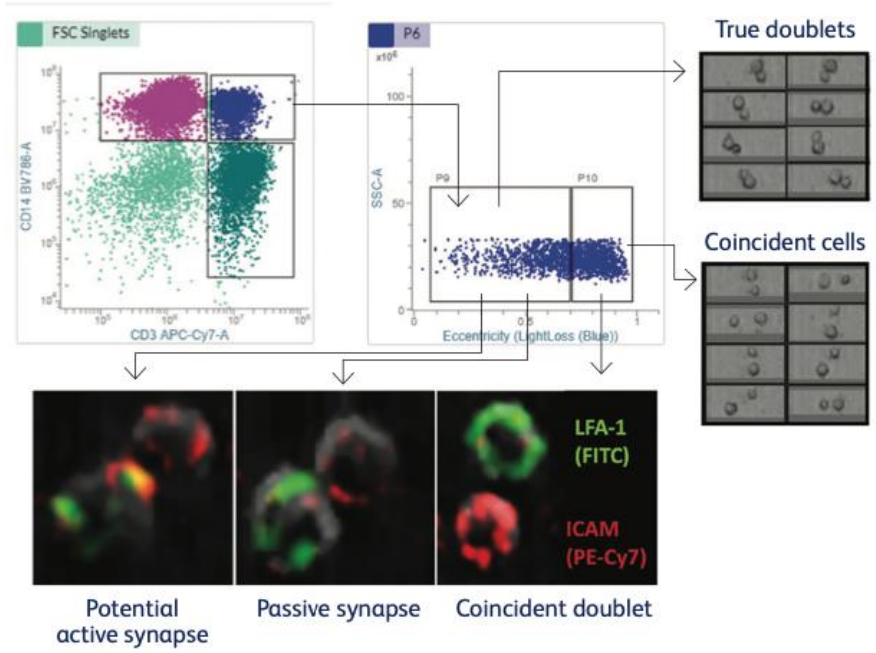


Cell cycle analysis

Flow cytometry methods only rely on a single indicator of DNA content for cell cycle classification, which is incomplete. Image feature analysis can provide insight into DNA distribution information to differentiate the phases of the cell cycle.

Cell-cell interaction

Reveal the spatial context of cells using image feature analysis to identify combinations of engaged cells. Distinguish between two cells that are coincident (passed through the interrogation point in close proximity) and true doublets (cells that are actually touching each other). Further image analysis can reveal receptor accumulation at the site of the cell-cell synapse (active synapse).



FACS Discover S8: some applications

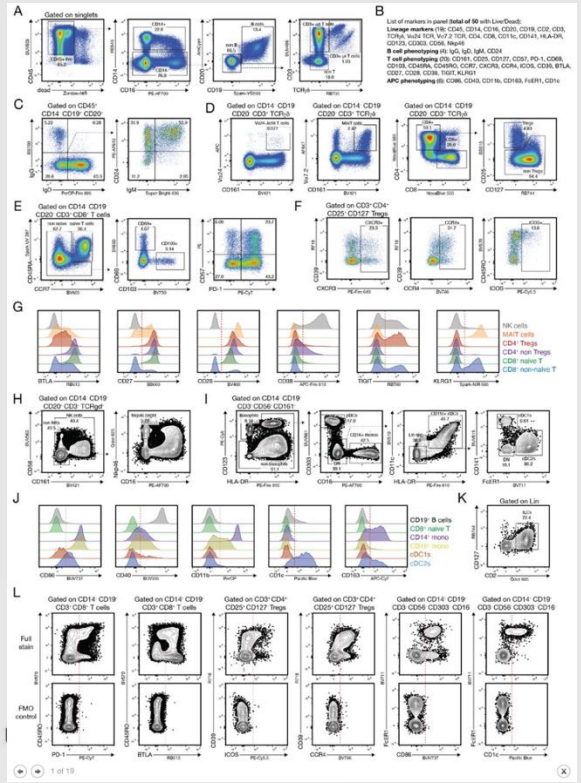
› bioRxiv. 2023 Dec 15:2023.12.14.571745. doi: 10.1101/2023.12.14.571745. Preprint

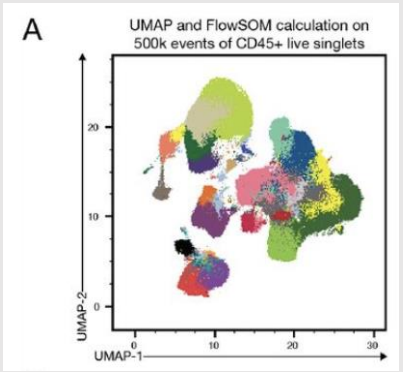
50-color phenotyping of the human immune system with in-depth assessment of T cells and dendritic cells

Andrew J Konecny [1 2], Peter Mage [3], Aaron J Tyznik [4], Martin Prlic [1 2], Florian Mair [1 5]

Affiliations + expand

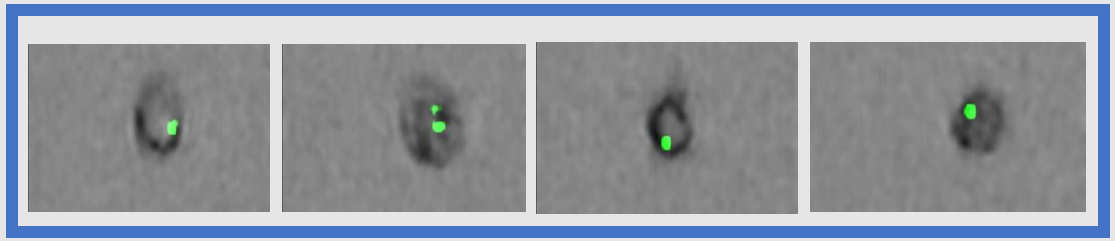
PMID: 38168221 PMCID: PMC10760076 DOI: 10.1101/2023.12.14.571745



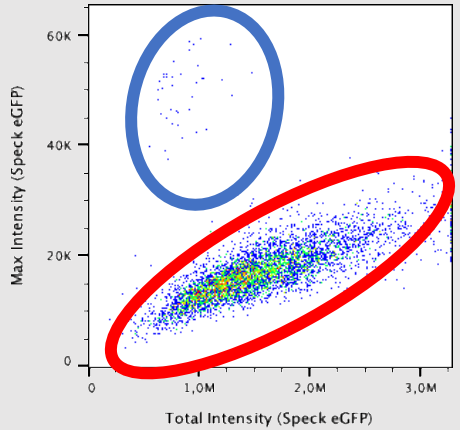


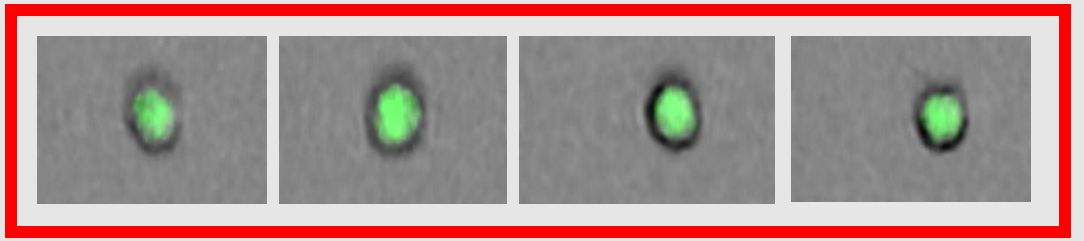
29 - TCRlow γδ T cells
28 - HLA-DRhigh CD14+ monocytes
27 - CD14+ CD16+ monocytes
26 - CD161+ MAIT T cells
25 - CD14+ CD86+ monocytes
24 - CD1c+ CD14int cDC3
23 - Mono-T Doublets
22 - CD16+ monocytes
21 - CD1c+ cDC2s
20 - CD161+ CD4+ T cells
19 - PD1+ TIGIT+ CD8+ T cells
18 - CD141+ cDC1s
17 - CD303+ pDCs
16 - FcER1+ Basophils
15 - TCRhigh γδ T cells
14 - CD45 low events
13 - CD56bright CD16- NK cells
12 - CD56dim CD16+ NK cells
11 - Mono-B Doublets
10 - CD4+ CD25+ Tregs
9 - CXCR3+ CD8+ T cells
8 - IgG+ B cells
7 - CD45RO+ CD4+ T cells
6 - CD24- B cells
5 - CD24+ B cells
4 - CD45RO+ Tregs
3 - naive CD4+ T cells
2 - naive CD8+ T cells
1- B cells
0 - IgD+ B cells

Speck eGFP expressing cells: Inflammasome



Cells with activated inflammasome

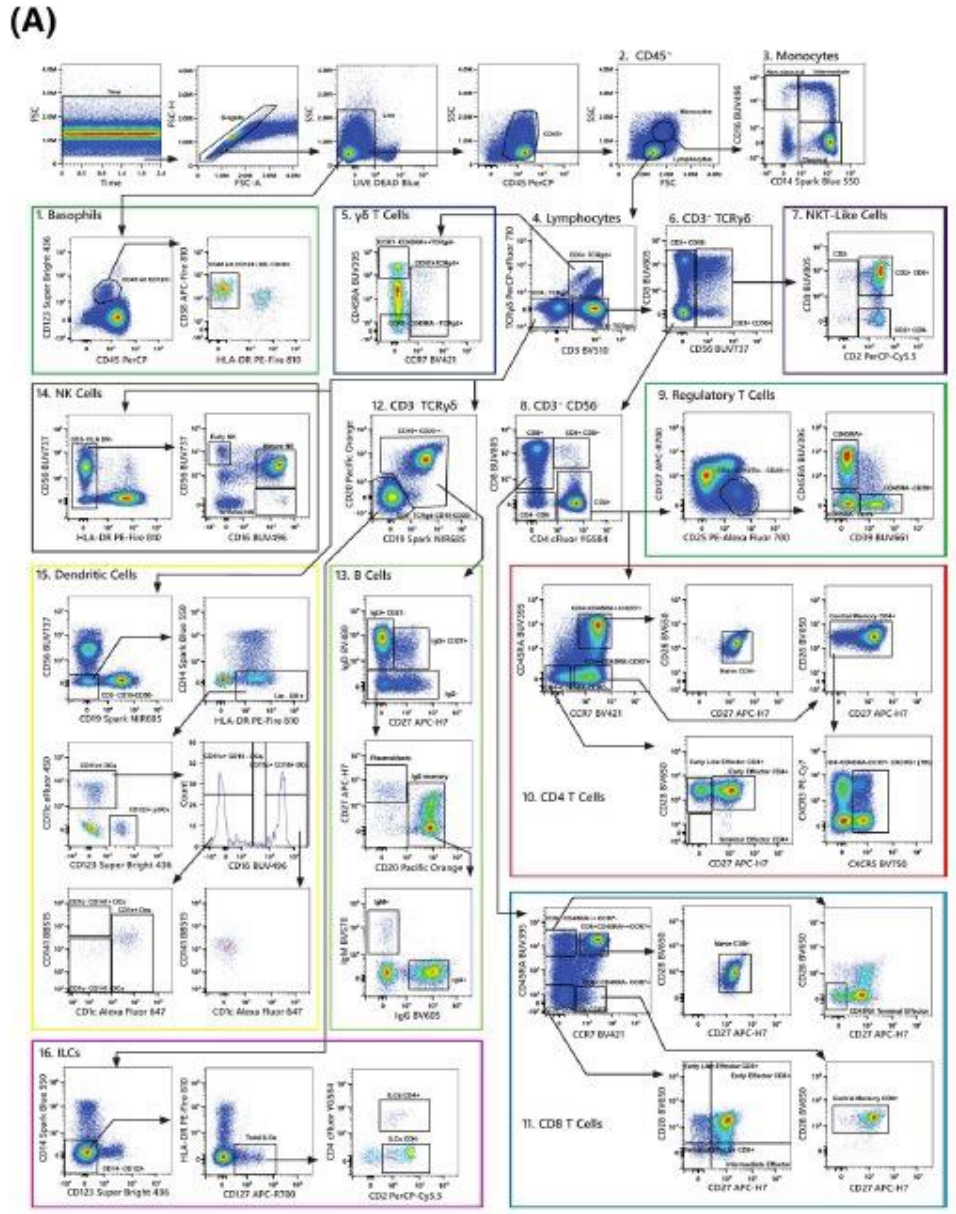




Cells without activated inflammasome

(By courtesy of Zhuo Wang – Fabio Martinon – UNIL)

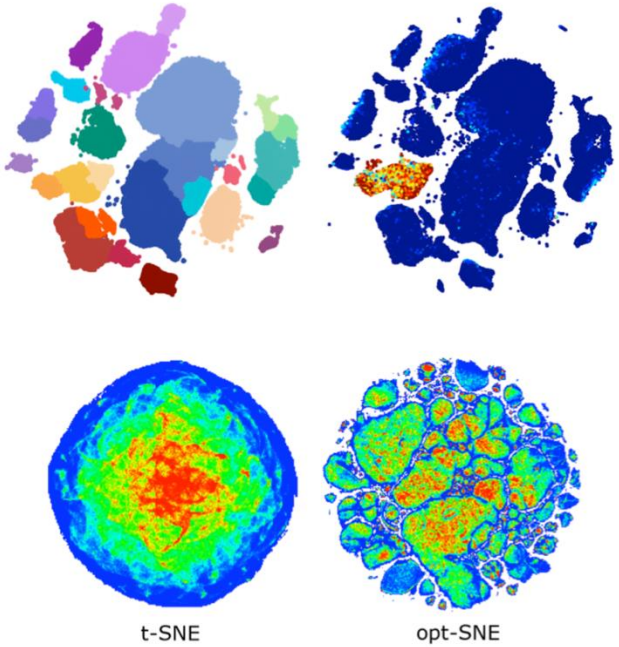
Data Interpretation



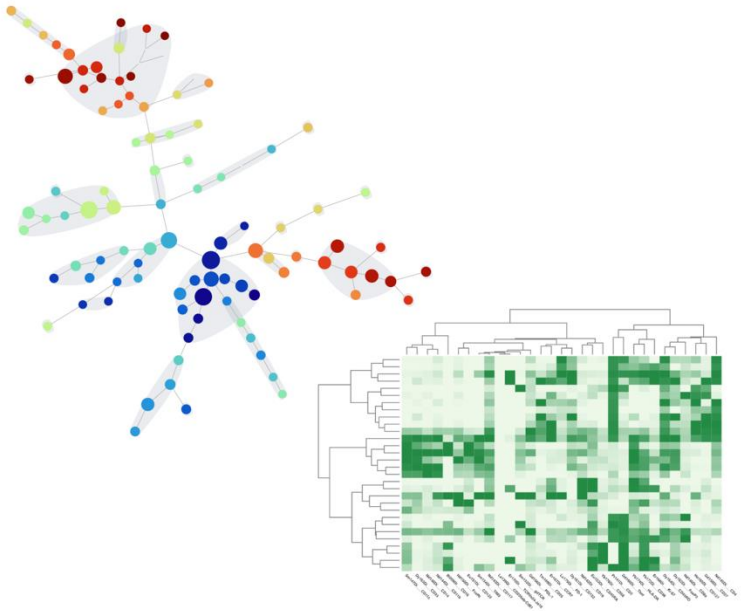
Complex gating strategies

Data Interpretation: computational flow cytometry

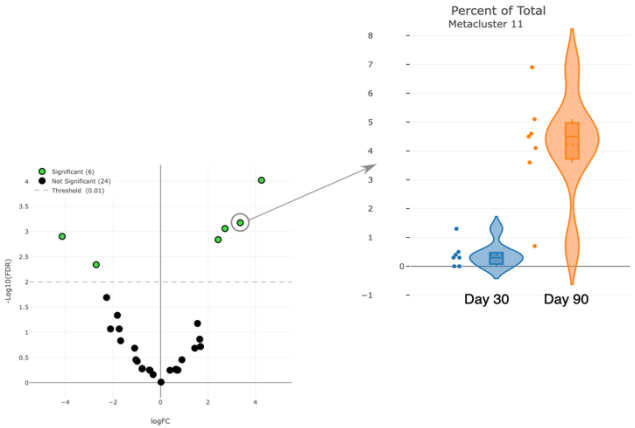
Dimentionality reduction



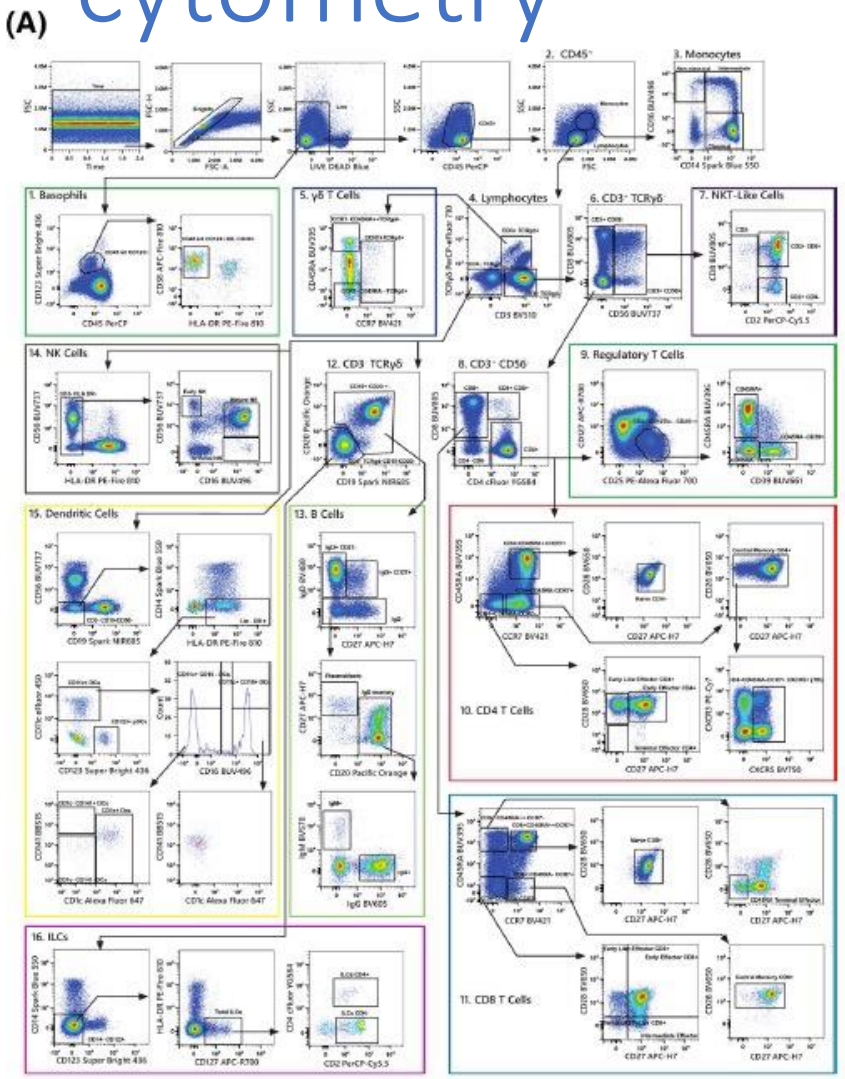
Clustering

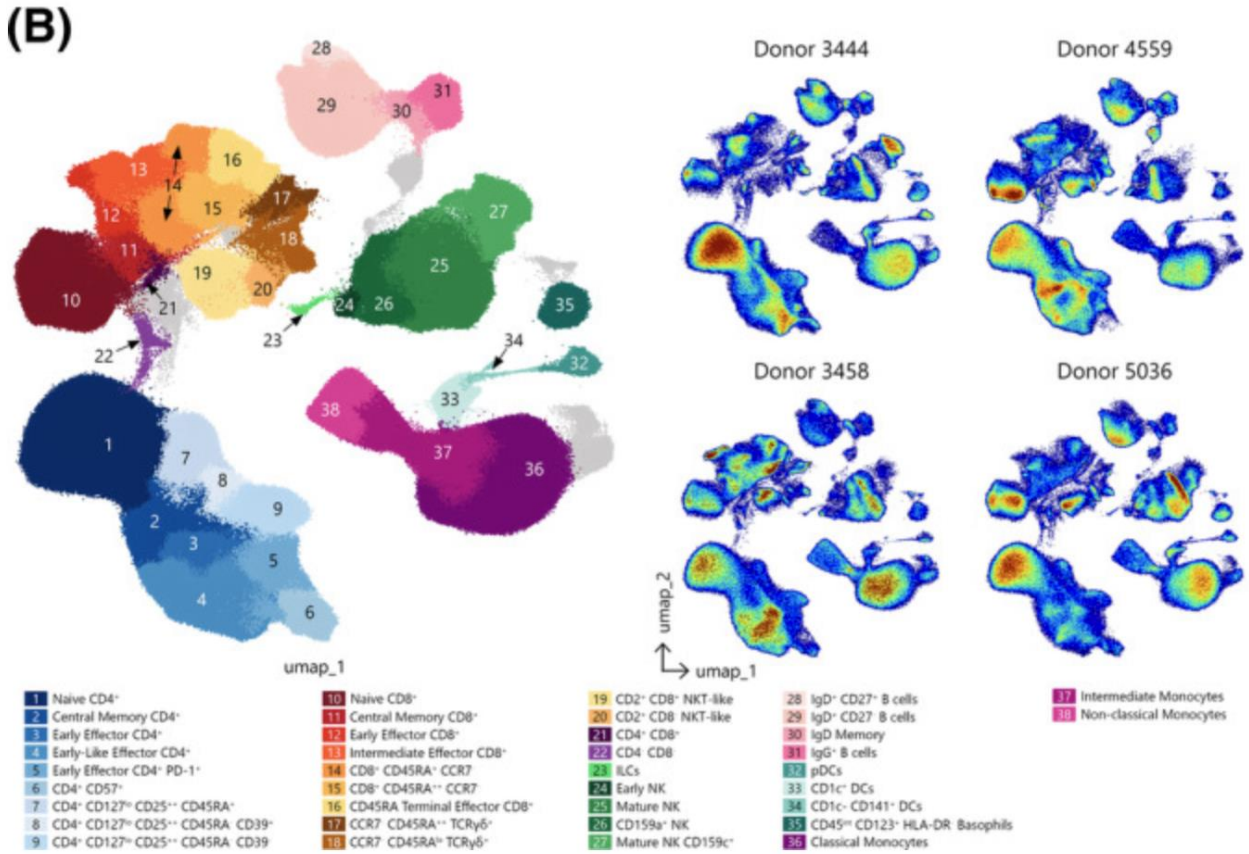


Statisical analysis



Data Interpretation: computational flow cytometry





Benefits of flow cytometry

- Measurement of single cells, identification of sub-populations
- Wide area of application in fundamental and clinical resaerch as well as in diagnosis
- Efficient and fast

Limitations of flow cytometry

- Cannot tell the intracellular location and distribution of proteins
- Aggregates and/or debris can give false results
- Pre-tretment of the cell for fluorescent staining is time-consuming
- Samples such as solid tissues have to be treated for generating a cell suspension