Modern Methods in Cell Biology

Flow cytometry

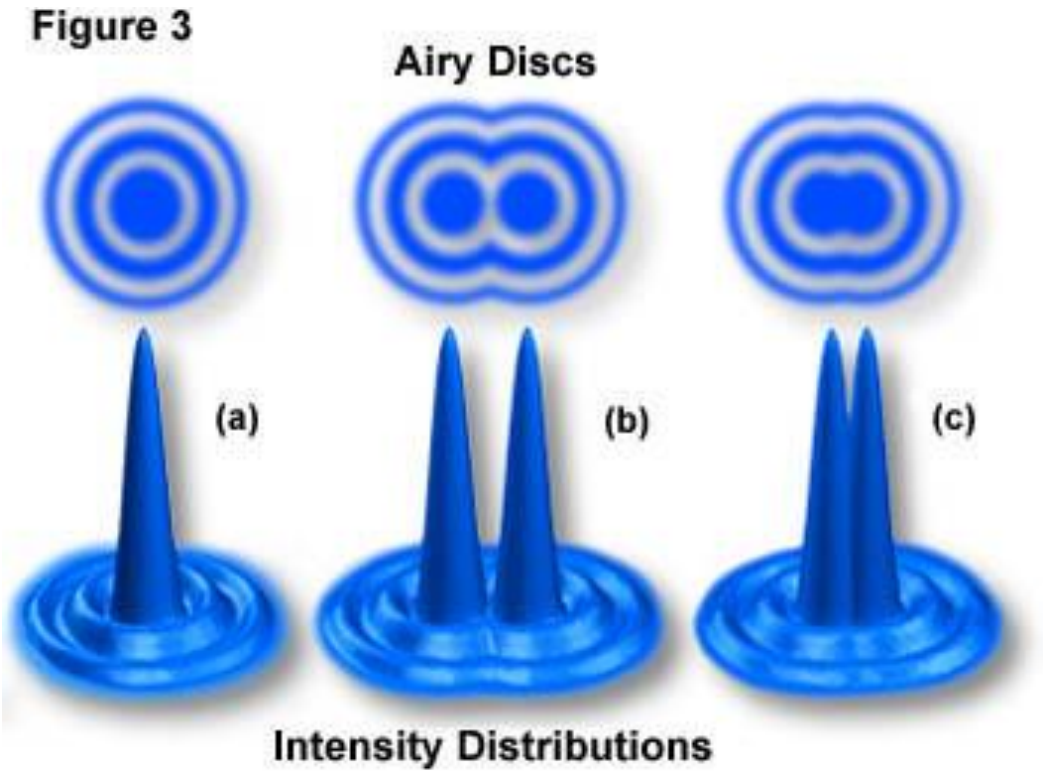
Imaging cytometry

Approaches to problems in cell biology

- Biochemistry-You can define a enzyme reaction (protein) and then try to figure what does it, when, where and under what control
- Genetics- You can make a mutation and then try to figure out what you mutated (knock-out; conditional knock-out, siRNA etc)
- Cell Biology- You can **visualize** a process and try to understand it- for instance cell division was one of the earliest
- Today- there are no distinctions. You cannot be just one thing, or be knowledgable about one thing. You need to take integrated appoaches to problems using the appropriate tools when needed. If you limit your approach, you limit your science

Resolution of instruments in cellular biology

Resolution describes the minimal distance of two points that can be distinguished.



Picture taken from http://microscopy.fsu.edu/primer/anatomy/numaperture.html

Resolution of instruments in cell biology (2 objects)

- Visible light is 400-700nm
- Dry lens(0.5NA), green(530nm light)=0.65μm=650nm
- for oil lens (1.4NA) UV light (300nm) = 0.13 μm
- for electron microscope
- l=0.005nm but NA 0.01 so =30-50nm
- Conventional flow cytometer > 300 nm
- Imaging flow cytometer – 300nm scatter

Sizes of objects

- Eukaryotic cell- 20μm
- Procaryotic cell-1-2μm
- nucleus of cell-3-5μm
- mitochondria/chloroplast- 1-2μm
- ribosome- 20-30nm
- protein- 2-100nm
- Exosome – 40-100 nm
- Microparticle – 100-1000 nm

Basic info expected from flow cytometry experiment (*2 cellular populations*):

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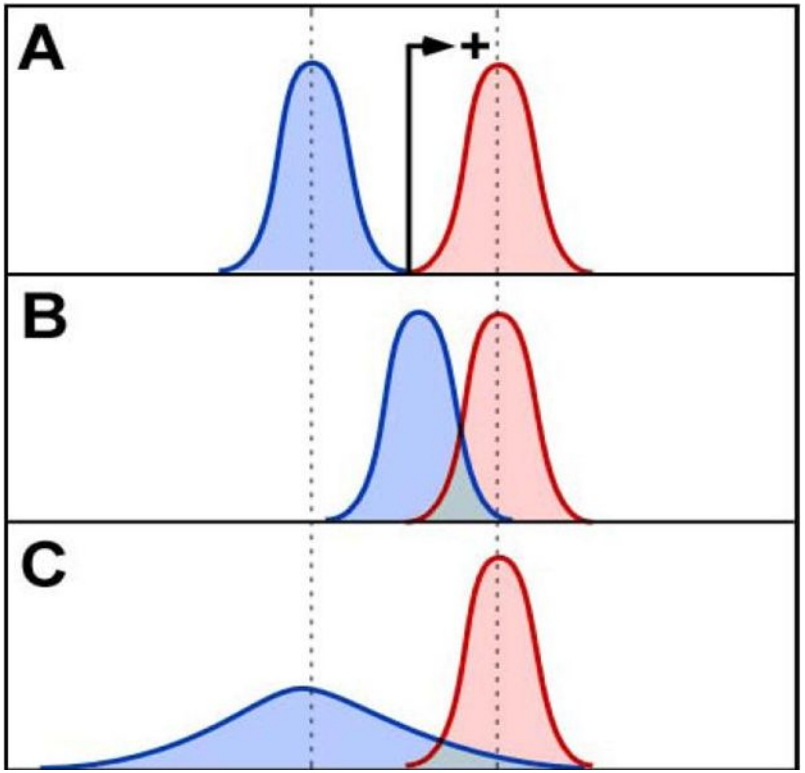
- **Whether a cell of interest is positive or negative for a given marker?**

Separation of positive and negative cellular populations



Analysis of Cellular subpopulations by different methods (*How many parameters to measure?)*

- **Conventional flow cytometry -4---12---18 fluorescent** parameters+ 2-3 light scattering parameters (FSC-A, FSC-H, FSC-W, SSC-A, SSC-H, SSC-W); fluorescence: mean fluorescent intensity (MFI)

- **CYTOF** (mass-cytometry) **50 fluorescent** parameters

- **Imaging Flow Cytometry (Imagestream X Mark II)**

  Bright Field+SSC+**10** fluorescent channels **x**

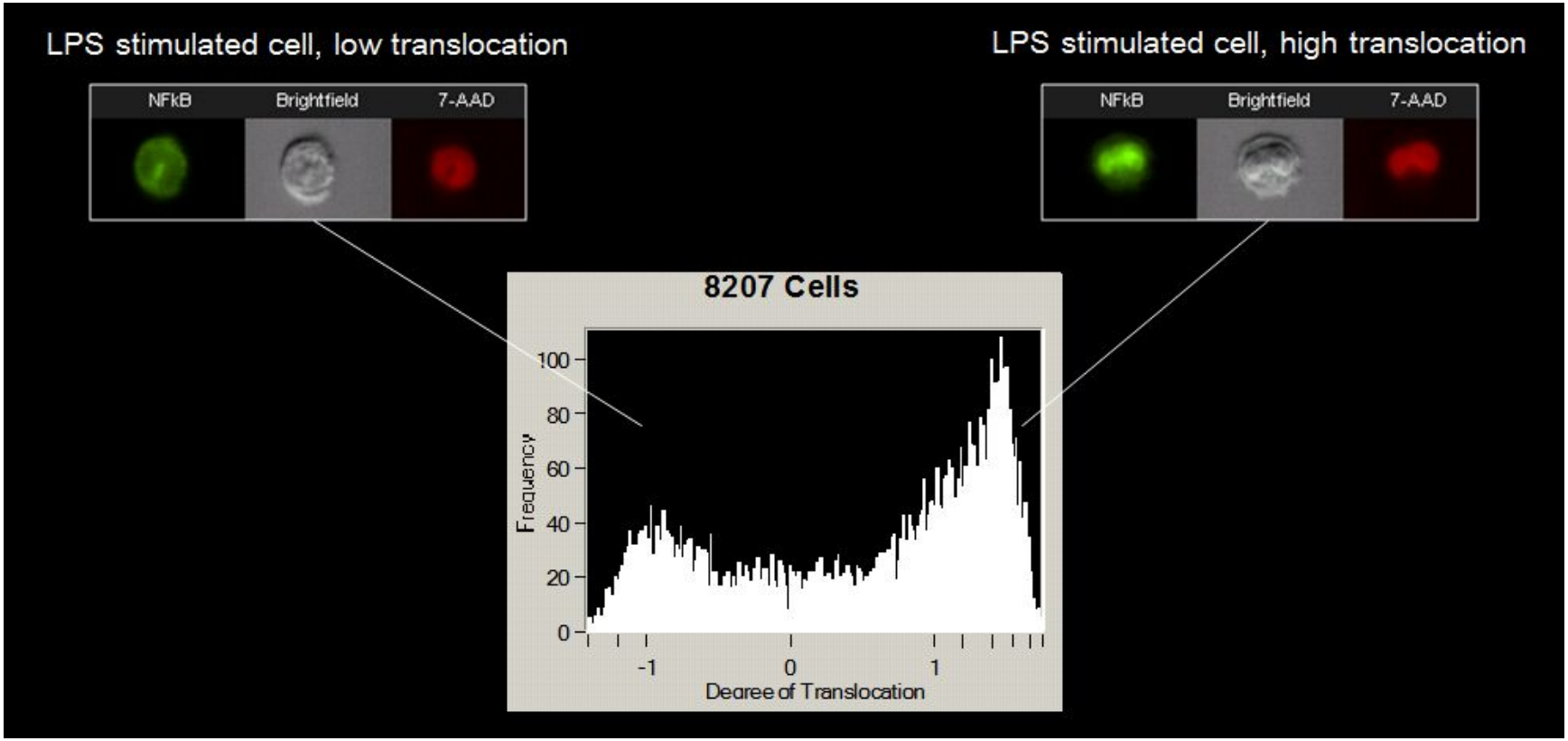
~ **200** morphological parameters > 2,000 parameters

Speed and Statistics (*How fast? How precise?)*

- Microscopy (20x-100x objective) – 20-100 cells/per slide or well – subjective factor;
- **High-throughput microscopy** (**20x** objective)
- **Conventional** flow cytometry **3-25,000** events/sec
- **Imagestream –high-throughput microscopy In Flow or Imaging flow cytometry:** up to **5,000** events/sec with 20x-**60**x objectives

Zeno’s paradox

STATISTICS: How many cells we really need to count?



It depends from heterogeneity of cell population, % of antigen expression etc etc

- SD = $\sqrt{r}$
- CV = $100/\sqrt{r}$
- Where r is the number of positive events.

- From CV = $100/\sqrt{r}$ follows r = [100/CV]2
- For CV=5%: r = [100/5]2 = 400

- If the rare cell subset is present at 5% of total events: acquire 8,000 total events.
- If 0.5%, then 80,000 total events needs to be acquired, and so on.

File size for Imagestream imaging flow cytometer –up to 100,000 events (cell images) allows to work with RARE events (<0.05%)

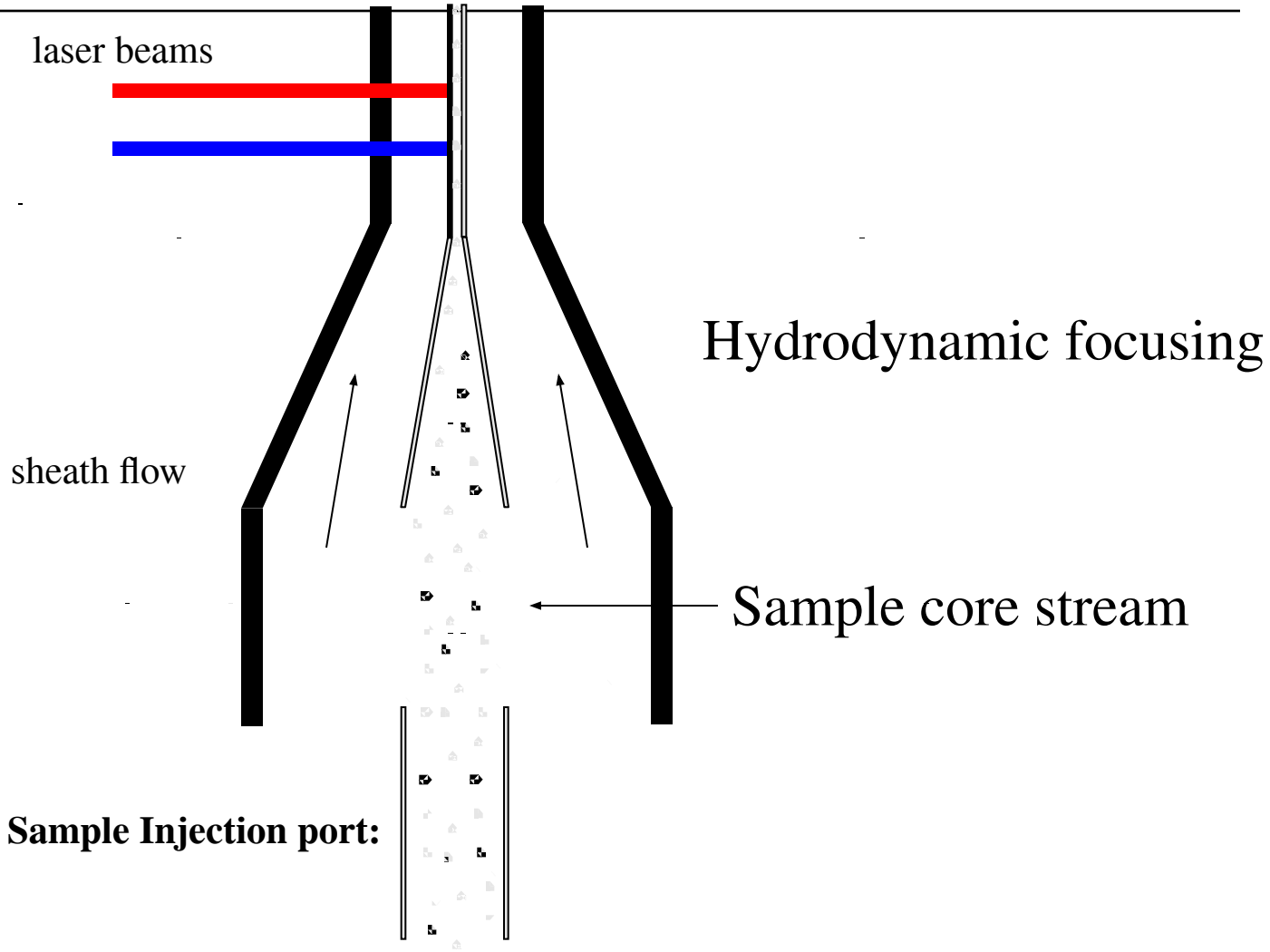
Conventional flow cytometer > 10,000,000 cell events per file

Basic Flow Cytometer

- How does it work?
  - Fluidics (stream)
  - Optics/excitation sources
  - Electronics

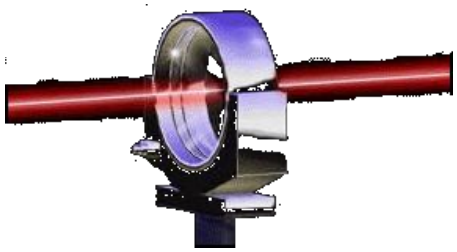
- Fluidics
  - Hydrodynamic focusing of sample stream within a sheath fluid
  - Sheath fluid needs similar refractive index as sample fluid
  - For sorting: electolyte solution

Sample cells at interrogation point



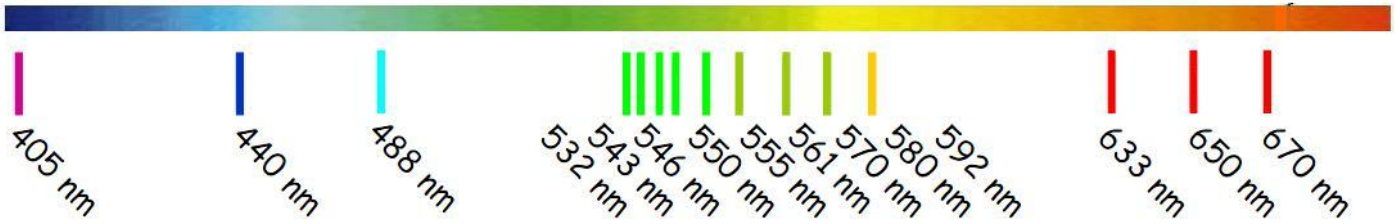
Optics:Light Sources

- **L**ight **A**mplification by the **S**timulated **E**mission of **R**adiation s



  - Can provide a single wavelength of light
  - Can provide from milliwatts to watts of light
  - Can be unexpensive, air-cooled units or expensive, water-cooled units
  - Provide coherent light at uniform wavelength, phase,polarity
  - Can be tightly focused

- Arc Lamps:
  - Provide mixture of wavelengths that must be filtered to receive desirable wavelength;
  - Provides miliwatts of light
  - Unexpensive air-cooled units
  - Provide uncoherent light



- Solid state lasers-small, reliable, easy to integrate in existing technology and are rapidly decreasing in the cost, available practically in any color

Multiple lasers in modern flow cytometer

LSR2 7 lasers

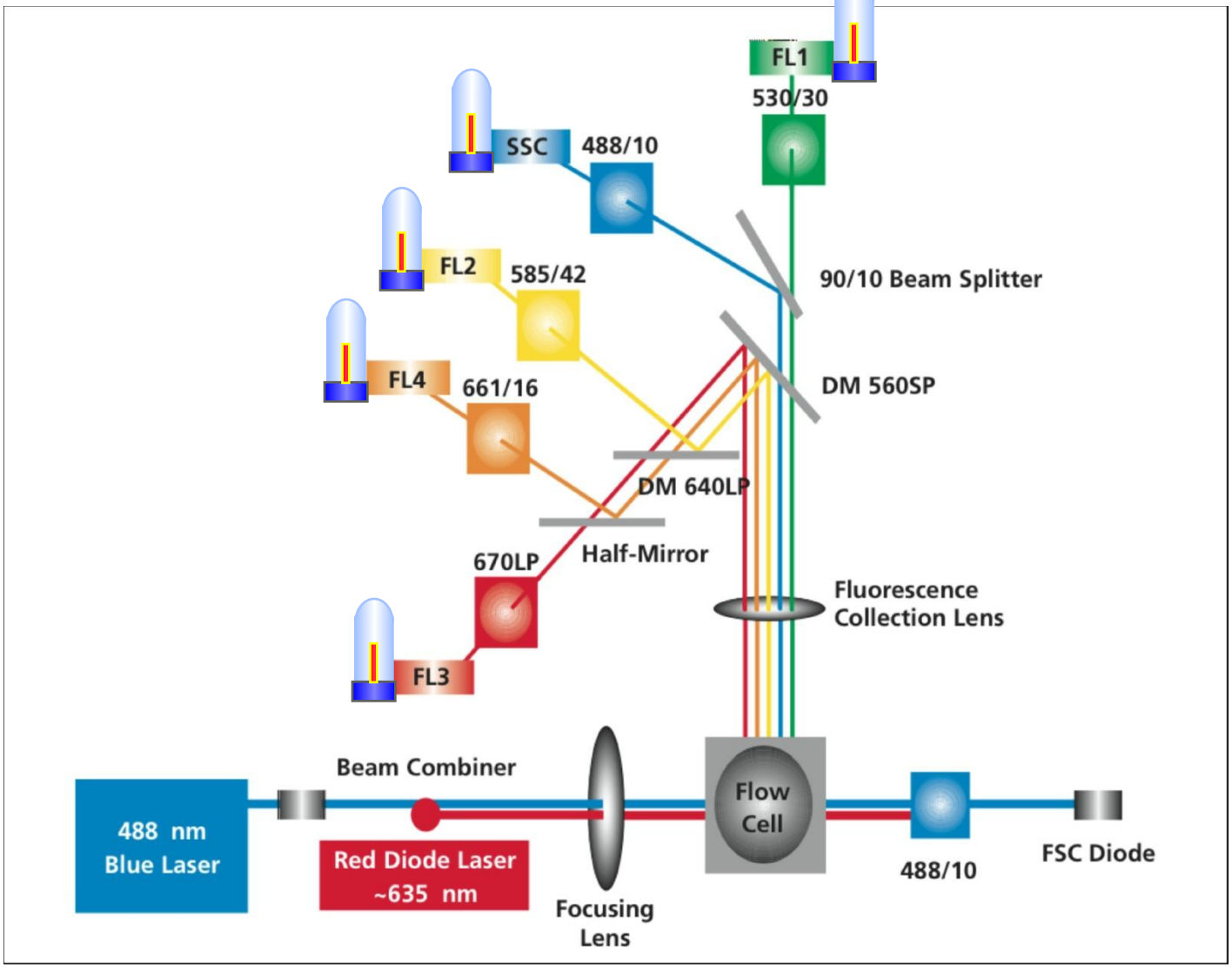
LSRFortessa
5 lasers

Stratedigm
4 lasers

Influx…
6 lasers

FACS Aria sorter

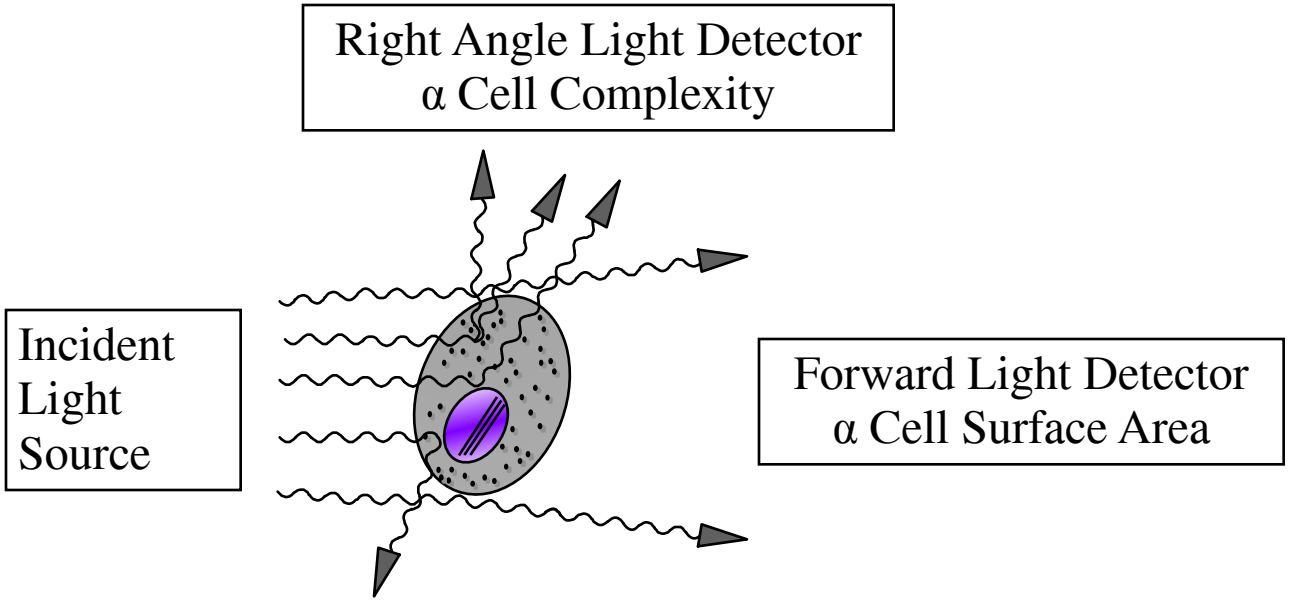
FACSCalibur flow cytometer



Optics: Forward Scatter Channel/Side Scatter Channel

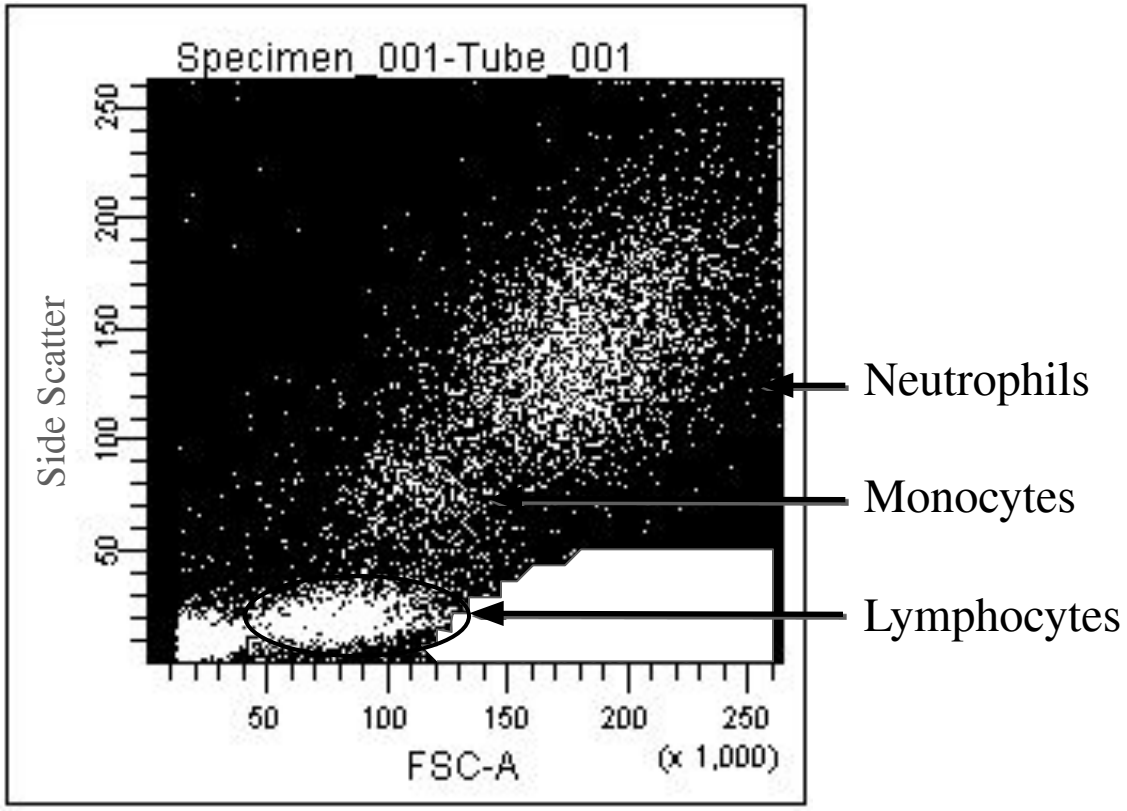
- FSC influenced by particle size and shape;
  - Allows the computer discriminate between particulate matter of minimal size and elctronical or optical noise; used as threshold;
- SSC(90^o –side scatter)-is also influenced by size, but also by surface structure,”granularity”;
- Combination of FSC and SSC allows live/dead cell gating and gives some information on size and structure

Light Scattering properties of cells

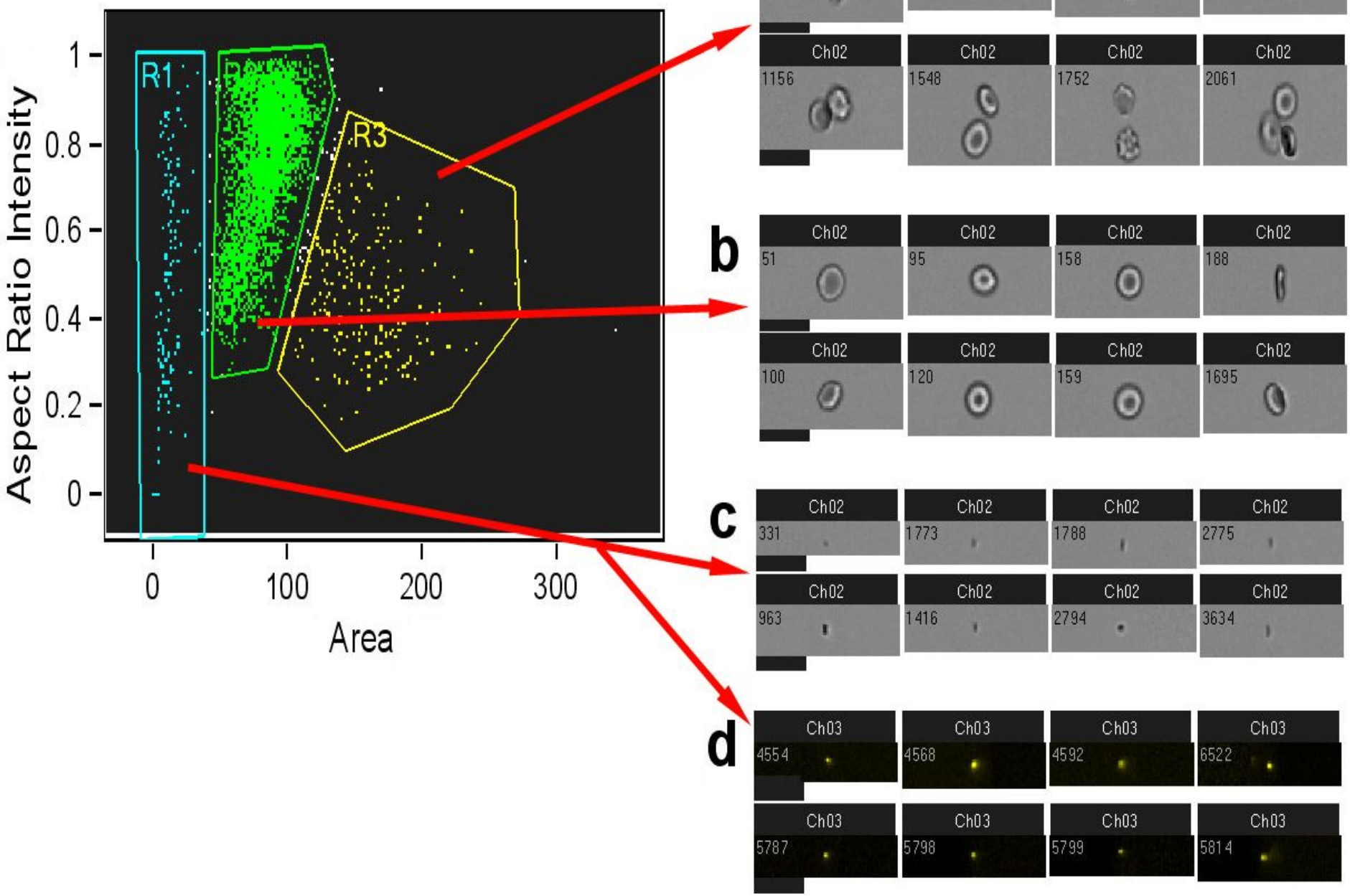


Analyze (gate on) cells of interest

Lysed Whole Blood



Forward Light Scatter


Aspect Ratio Intensity
Area
R1
R2
R3
0
0.2
0.4
0.6
0.8
1
0
100
200
300
a
b
c
d
Ch02
Ch03
2338
2578
2984
3139
1156
1548
1752
2061
51
95
158
188
100
120
159
1695
331
1773
1788
2775
963
1416
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3634
4554
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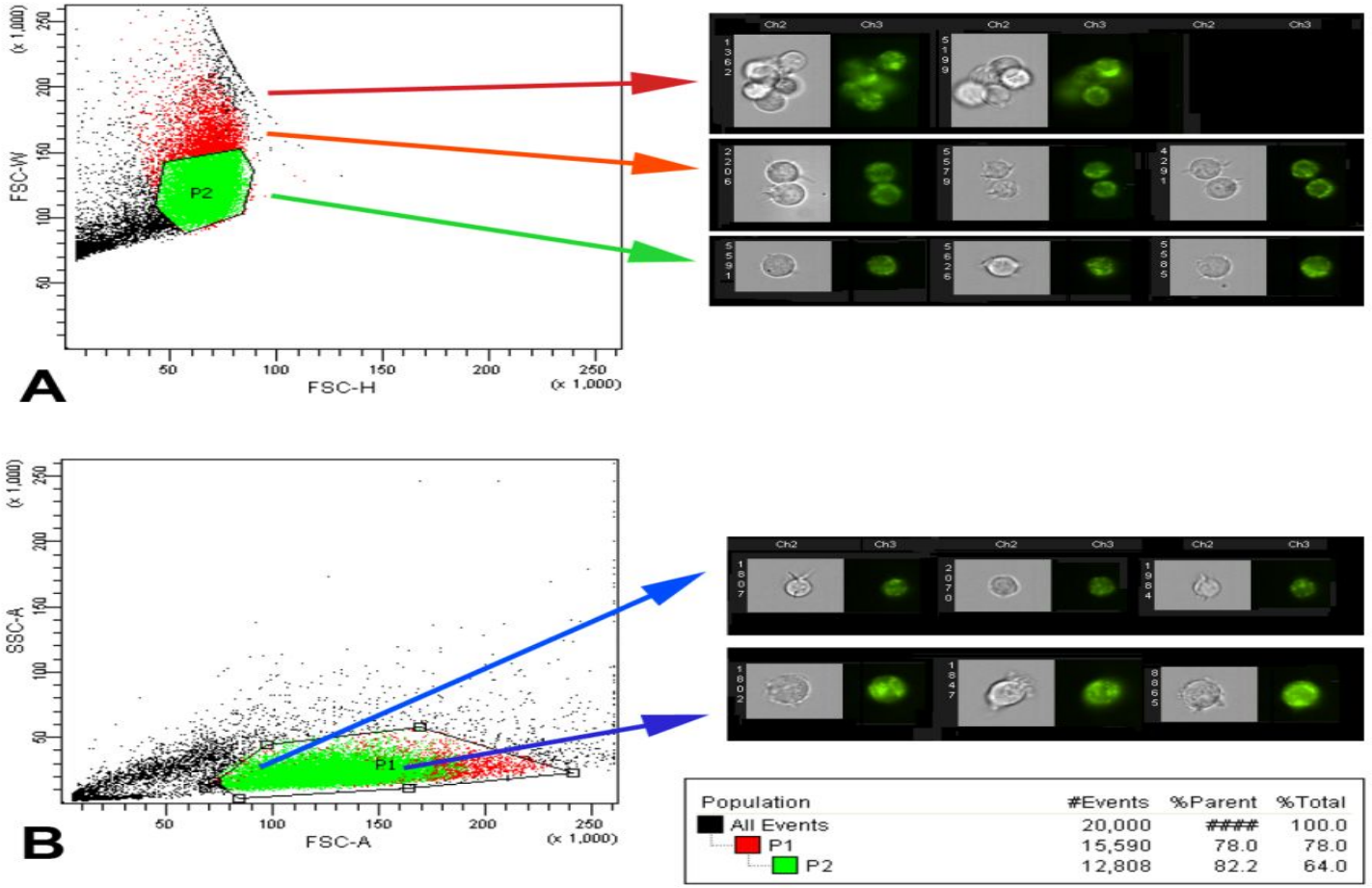
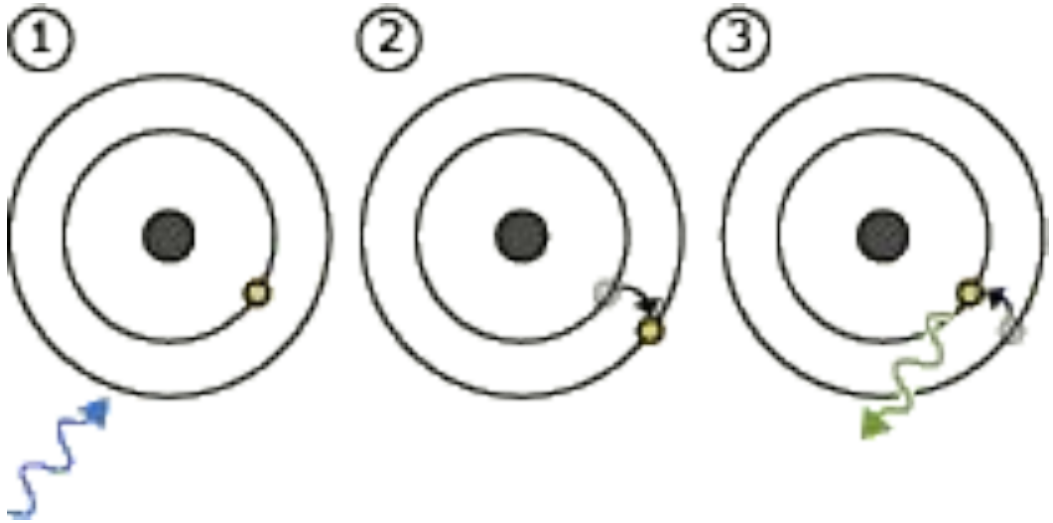
4592
6522
5787
5798
5799
5814

Scatter (Size parameter)-by conventional flow cytometry and IFC



Barteneva et al, BBA Reviews on Cancer 2013, 1836: 105-122

Principle of fluorescence



Principle of Fluorescence

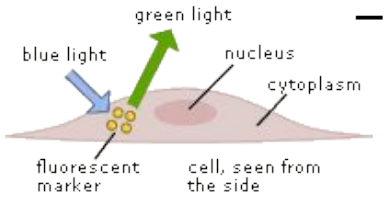
1. Energy is absorbed by the atom which becomes excited.
2. The electron jumps to a higher energy level.
3. Soon, the electron drops back to the ground state, emitting a photon (or a packet of light) - the atom is fluorescing.

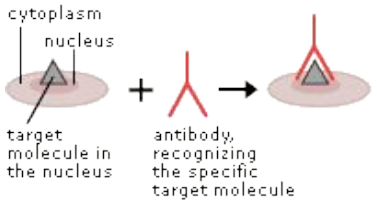
FLUORESCENT methods in the research laboratory

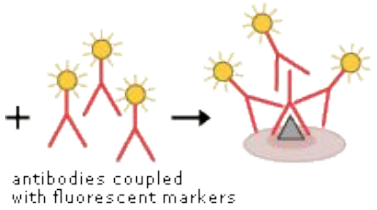
- State-of-the art Fluorescent Microscopy and Confocal Microscopy
- High dimensional Flow Cytometry (FACSAria, CYFLEX etc)
- High speed FACS-based cell sorting
- ...
- High-throughput single-cell analysis
- Super-Resolution microscopy
- **Imaging Flow Cytometry-high-dimensional analysis of correlations between cellular fluorescence and cellular morphology**

Advantages of fluorescent methods

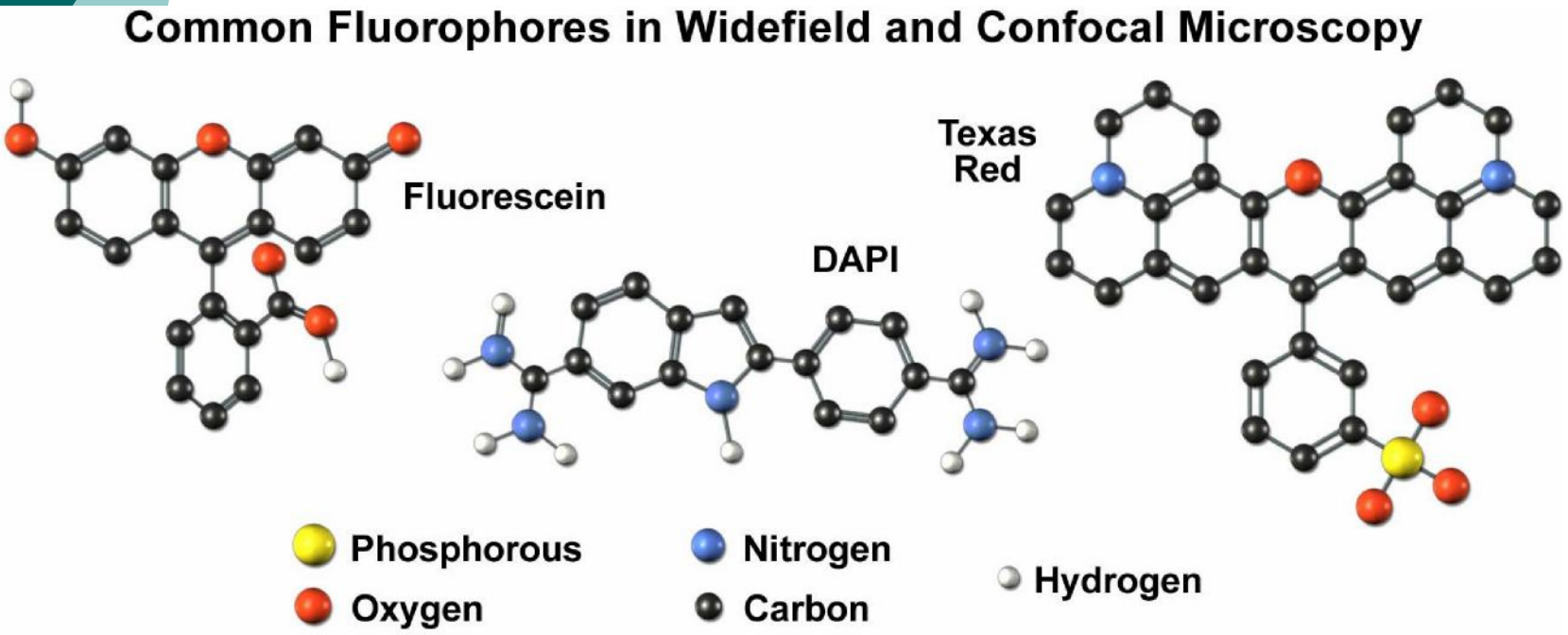
- Highly sensitive method (high resolution)
- Highly sophisticated fluorescent probes (multi-)
  - Fluorescent dyes that accumulate in different cellular compartments or are sensitive to pH, ion gradients
  - Fluorescently tagged antibodies to specific cell features
  - Endogenously expressed fluorescent proteins
    - » Really endogenous
      - NADH/FAD: enzymes involved in ATP production
      - structural proteins: collagen/elastin
      - amino-acids: tryptophan/tyrosine
    - » After gene modification
      - Green fluorescent protein and variants



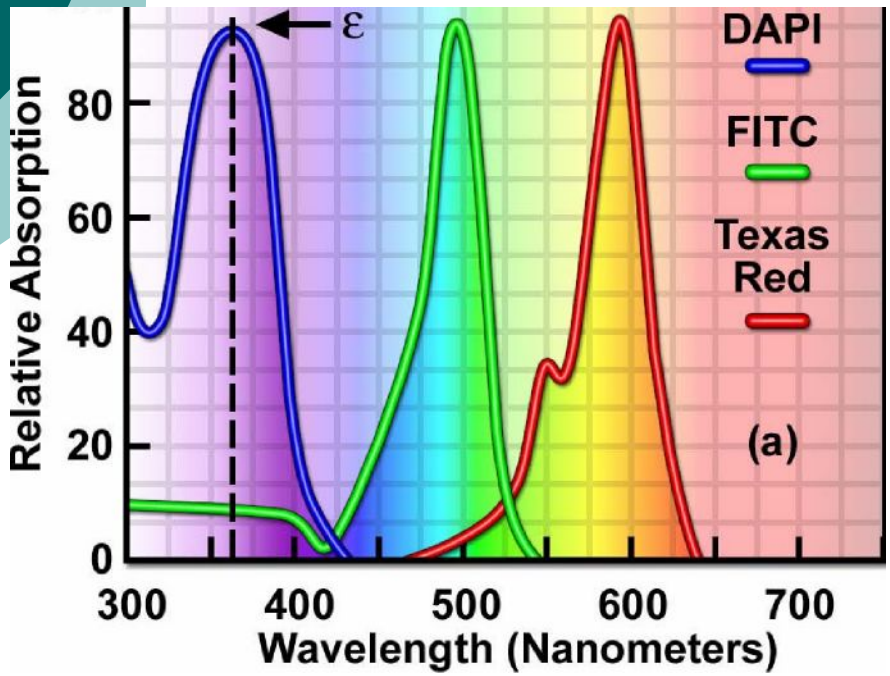


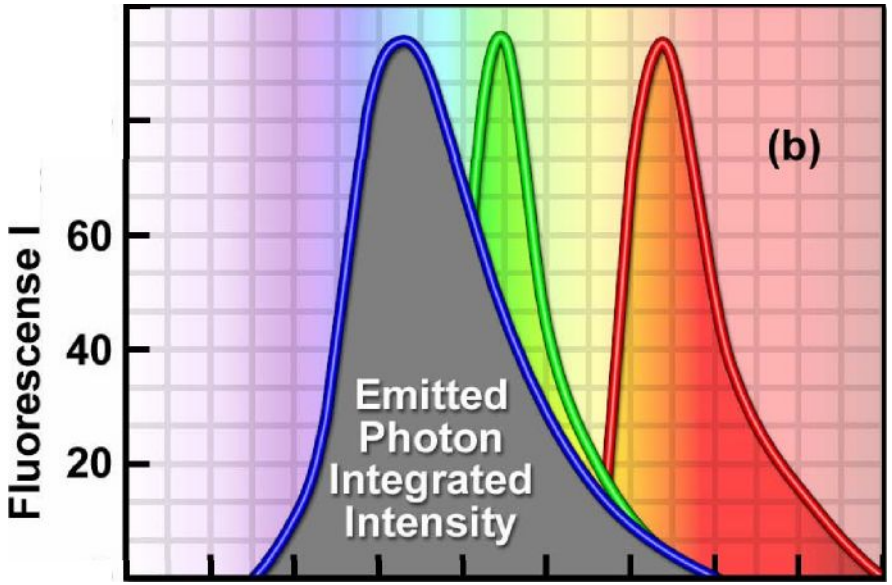


FLUORESCENT dyes are typically composed of ring structures

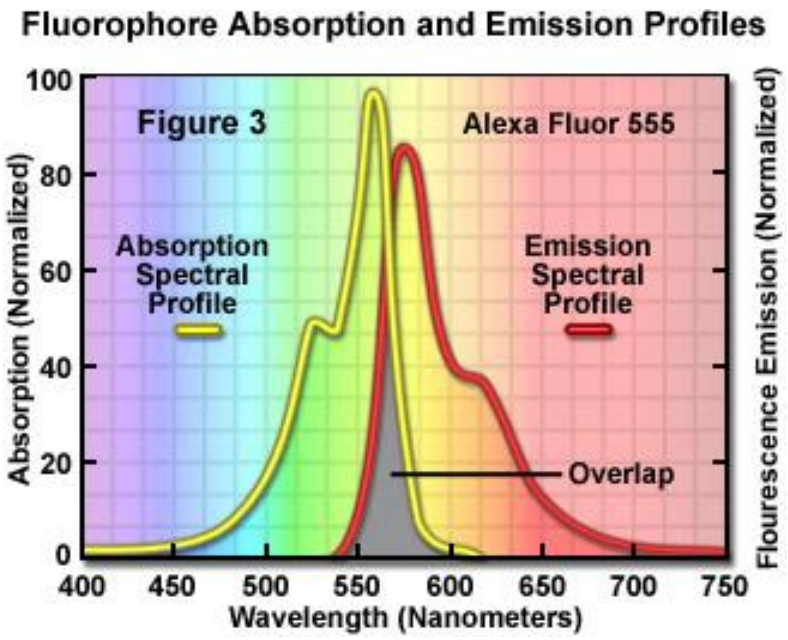


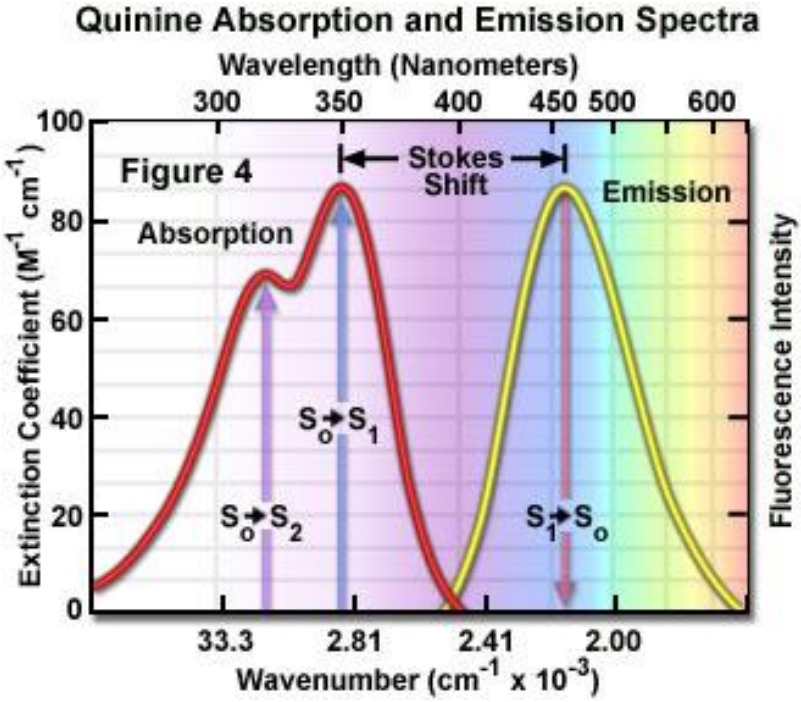
Absorption and Emission Spectra of some traditional fluorophores





Fluorescence Stoke's shift



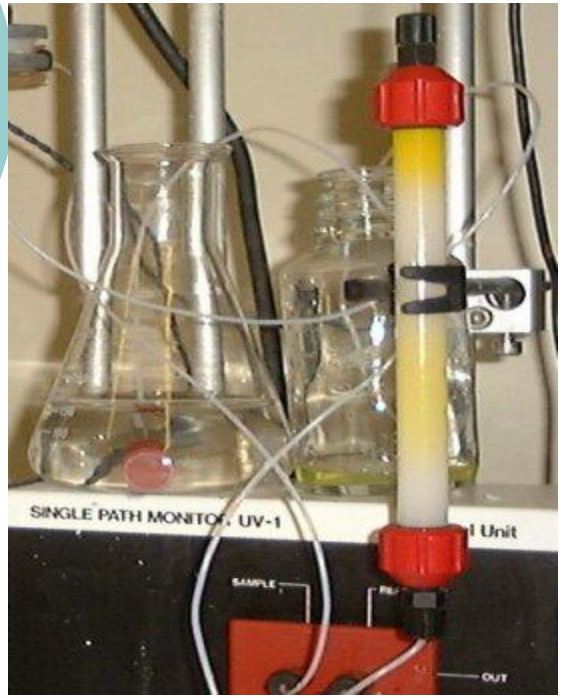


- Fluorescence emission peak wavelength is red-shifted with respect to absorption peak wavelength
- This shift may vary typically from 5 to more than 100 nm, depending on the electronic structure of the molecule

USE OF FLUORESCENT DYES

- Labeling of proteins - antibodies, streptavidin
- Labeling of nucleic acids – DNA, RNA
- Labeling cell membranes and organells, mitotracker,
- lysotracker, rhodamine ceramide (Golgi complex)
- Sensors: pH, membrane potential, redox potential
- Quenching and dequenching reactions

FITC (Fluorescein isothiocyanate)



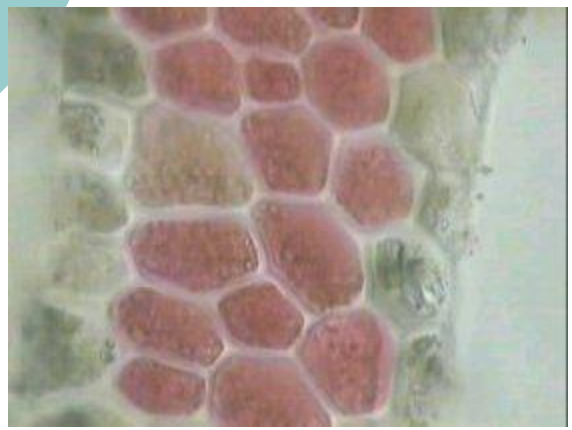
-Because of the large difference in molecular weight between FITC (389 Da) and immunoglobulin proteins (150,000 Da), simple gel filtration procedures are sufficient to separate free (unreacted) dye from FITC-labelled antibody

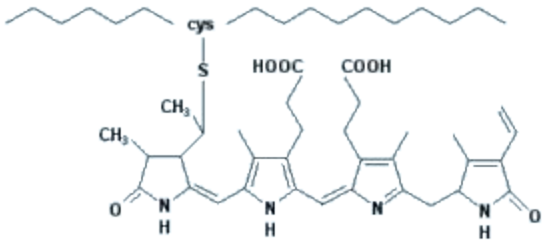
Fluorescein isothiocyanate is a yellow-green colored low molecular weight dye which couples to proteins via reaction with primary amine groups at high pH. FITC is excitable at 488nm, close to its absorption maximum at 494nm, and produces maximum fluorescence emission around 520nm

Phycobiliproteins-

The phycobiliproteins are 'antenna' pigments used by some classes of plants to increase the efficiency of photosynthesis by collecting light energy at wavelengths over which chlorophyll absorbs poorly.

PE=phycoerythrin- extracted from Corralina officinalis





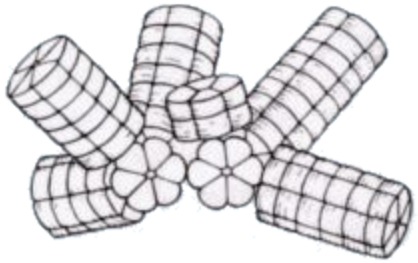
R-PE - R symbolises its red-algal origin – it is a bright orange-red colored protein, with a molecular weight of 250 kDa and containing 34 chromophore prosthetic groups.

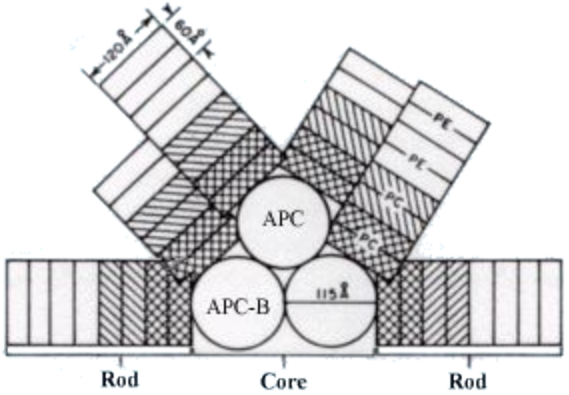
-With absorption maxima at 492 and 565nm it is excitable by the 488nm argon-ion laser,

and has emission maxima around 578nm

APC(allophycocyanin)

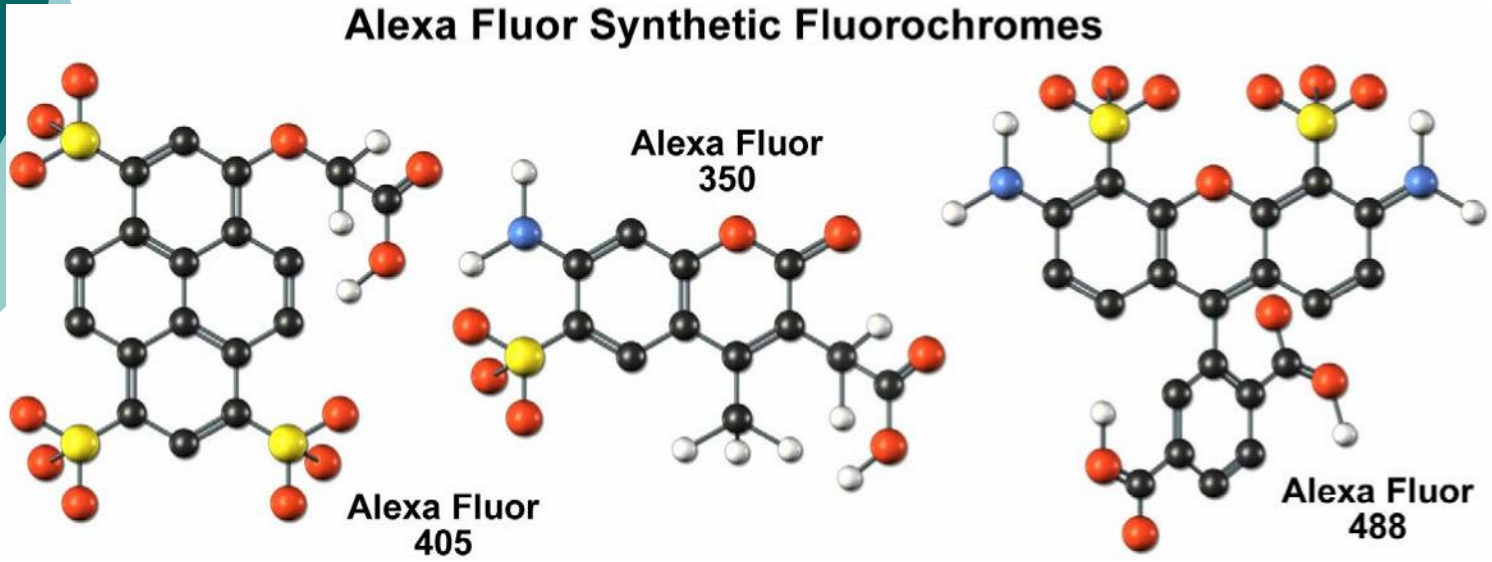
Rod-and-core structure of cyanobacterial phycobilisome. Left-hand diagram shows stacks of hexameric phycocyanin complexes comprising the rods. The right-hand diagram shows phycoerythrin- and phycocyanin-containing rods, with a three-cylinder core consisting of APC and APC-B. *[Adapted from AN Glazer. Phycobilisome: a macromolecular complex optimized for light energy transfer. Biochemica et Biophysica Acta, 1984, p29-51]*



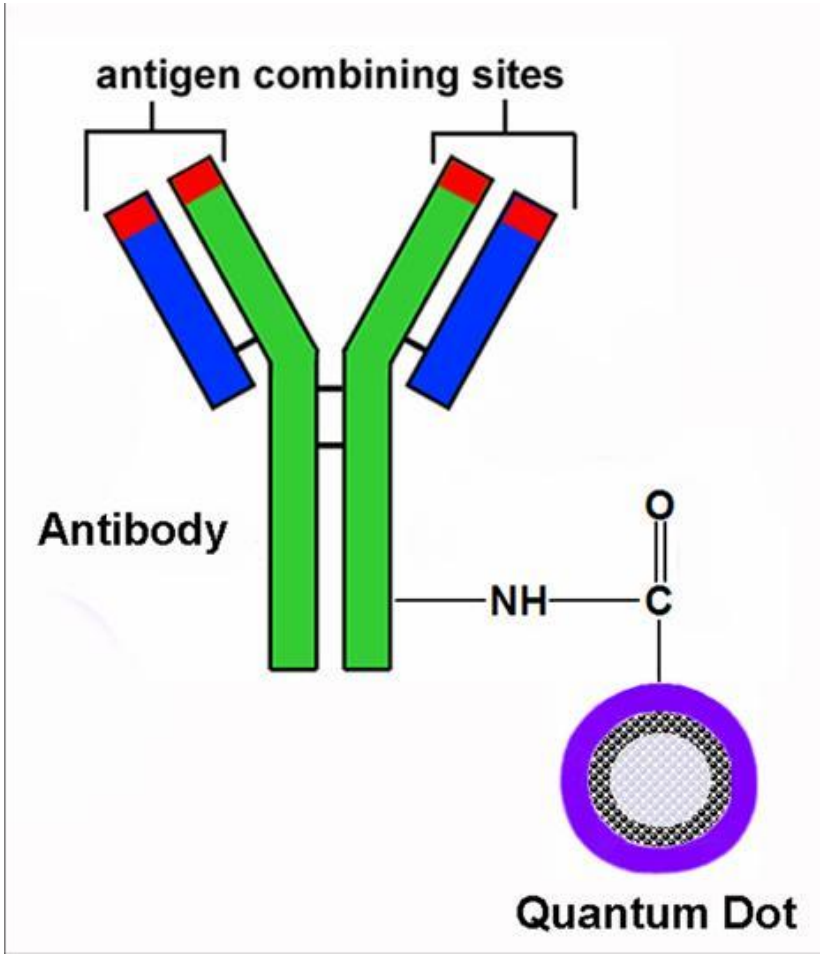


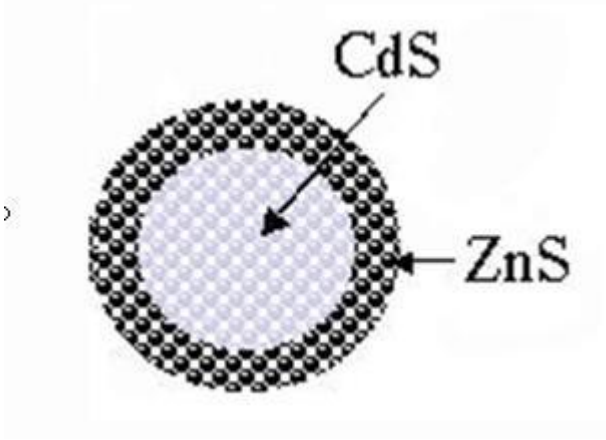
APC and allophycocyanin-B constitute the core of the phycobilisome, with other biliproteins constituting the rods. Light energy is transmitted down the rods to the core, then to chlorophyll which is embedded in the 'thylakoid' membranes of the photosynthetic chloroplasts. The normal sequence of energy transfer is:
phycoerythrin - phycocyanin - allophycocyanin - allophycocyanin B - chlorophyll a

ALEXA family:brighter, more photostable, less environmental sensitive



Quantum Dot-conjugated antibody





Quantum Dots advantages

- Extremly photostable
- Narrow emission spectrum, hence small spectral overlap
- Broad absorption spectrum (disadvantage at some situations-excited by all standard lasers)
- Capacity for multiplexing

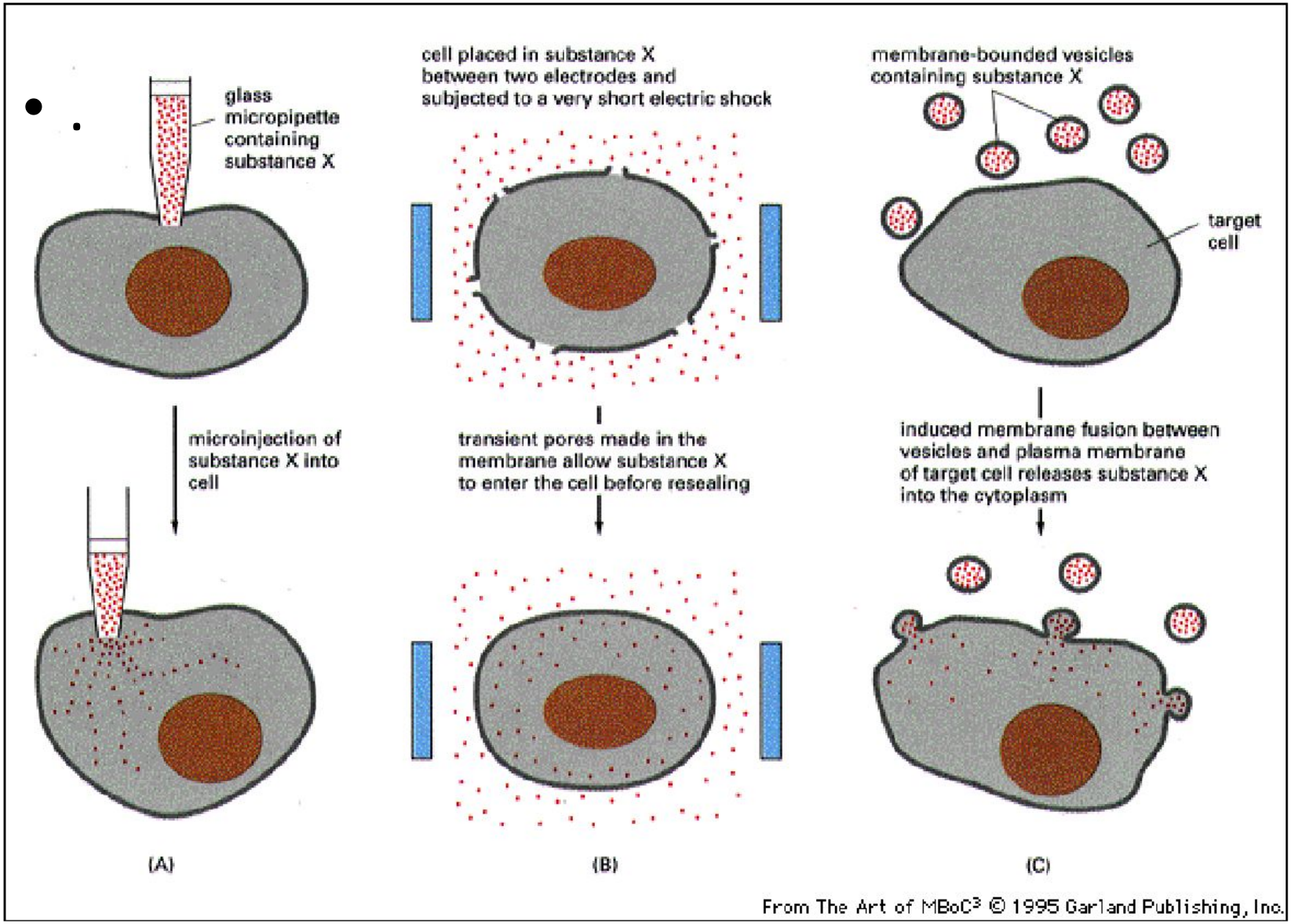
QDots Brightness

Brightness Index=Extinction Coefficient x Quantum Yield/1000

How do we get fluorescent probes into cells

- Kill the cell and make the membrane permeable
- Live cells
  - Diffusion: some can cross membrane
  - Microinjection- stick and tiny needle through membrane
  - Trauma: rip transient holes in membrane by mechanical shear (scrape loading) or electrical pulse (electroporation)
  - Lipid vesicles that can fuse with membrane
  - Transfect with fluorescent protein vector

How to load cells (microscopy)

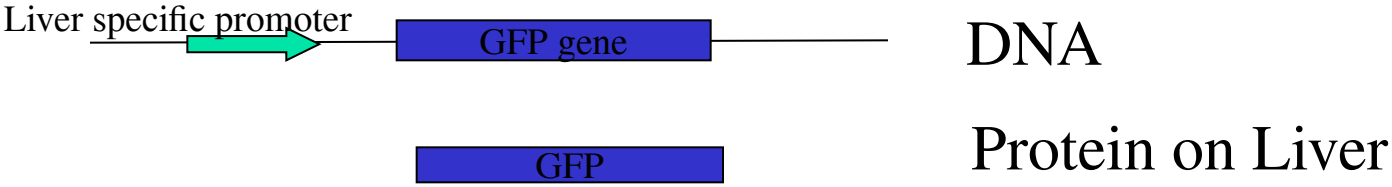


Immunofluorescent staining of proteins in fixed/dead cells

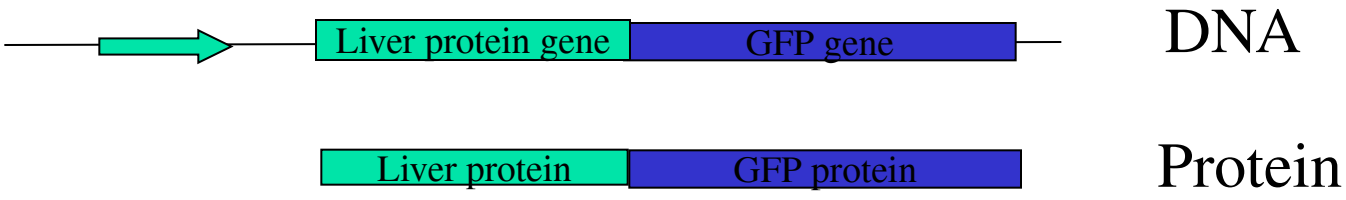
- You can purify almost any protein from the cell (Biochemistry)
- Make an antibody to it by injecting it into a rabbit or mouse (primary antibody)
- Use the antibody to bind to the protein in the fixed cell
- Fixed cells can be made permeable so antibodies can get into interior
- Use a fluorescent “secondary antibody” (anti-rabbit or mouse) to localize the primary antibody
- Amplify secondary label (tyramide etc)

Green Fluorescent Protein (GFP)- An Ongoing Revolution in Cell Biology

- Protein from fluorescent jellyfish
- The protein is fluorescent
- Now cloned, sequenced and X-ray structure known
- If you express it in a cell, the cell is now fluorescent!
- Use a liver promoter to drive gene expression, and you get a fluorescent liver! All cells in the liver make GFP which fills the cytoplasm with fluorescence.

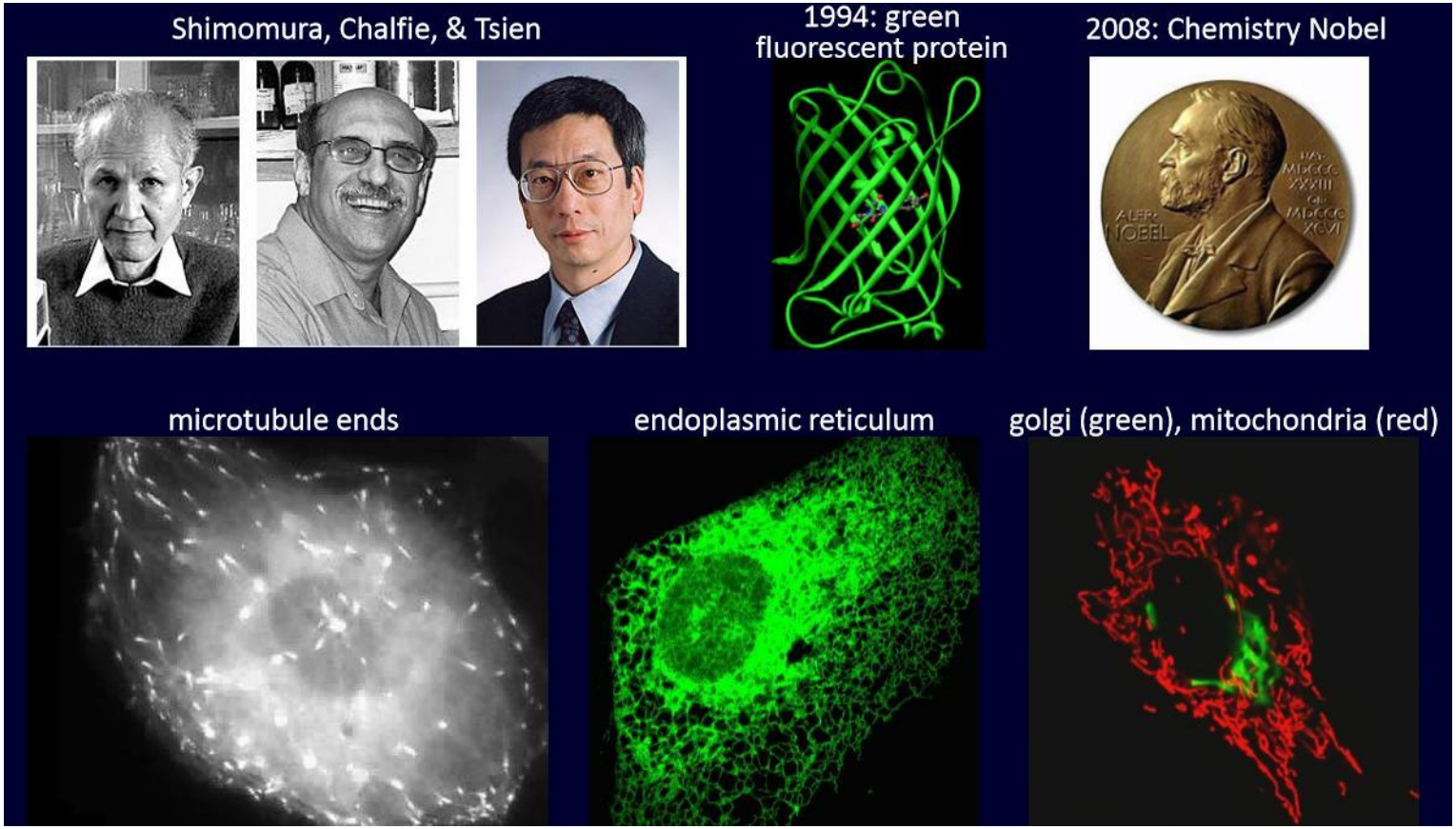


- Fuse the DNA sequence of a protein to the DNA sequence of GFP and the cell will express it and make a fusion protein which has two domains. Wherever that protein is in the cell, you will see fluorescence!



- Allows you to do live cell dynamic localization of specific proteins

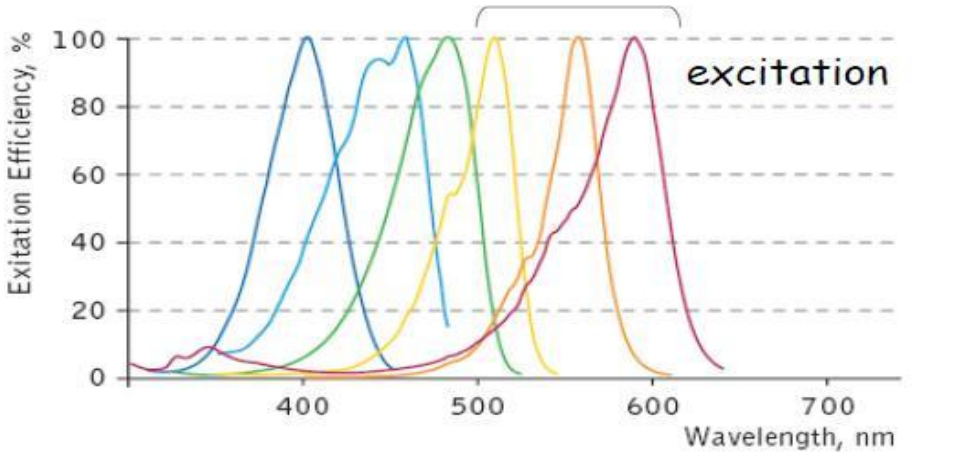
Discovery of fluorescent proteins

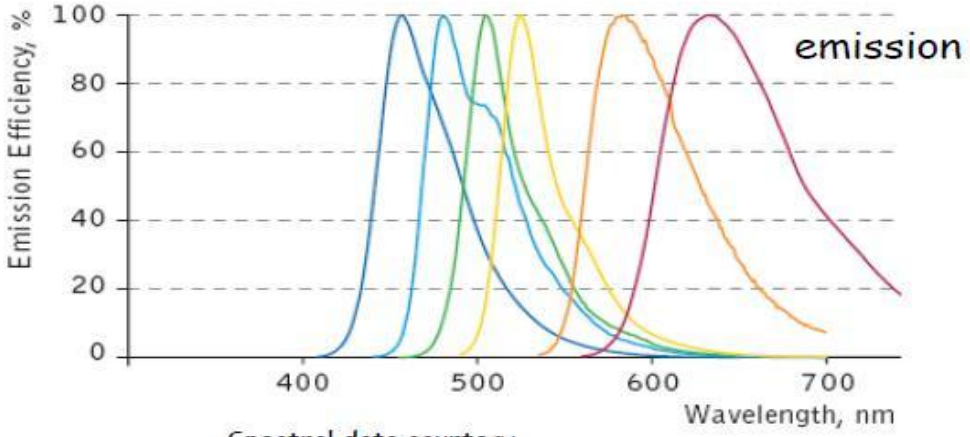


Evrogen proteins (Lukianov Lab)

The Evrogen jellyfish and sea anonome derived fluorescent proteins are used for fusion proteins and organelle targeting.

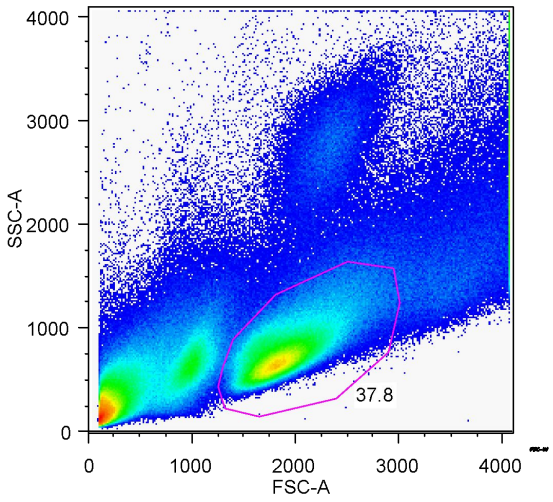
- TagBFP
- TagCFP
- TagGFP2
- TagYFP
- TagRFP
- mKate2

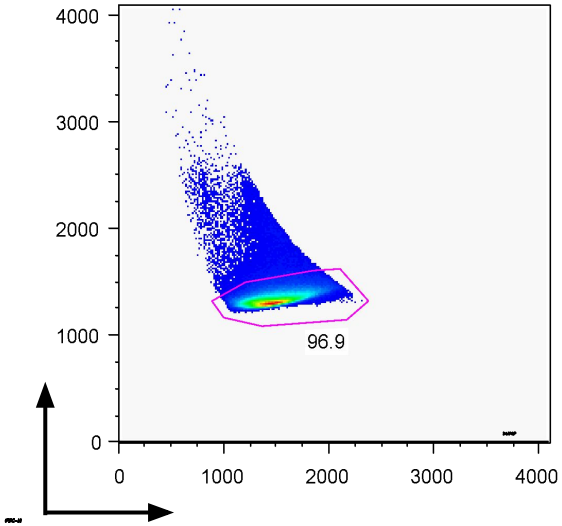


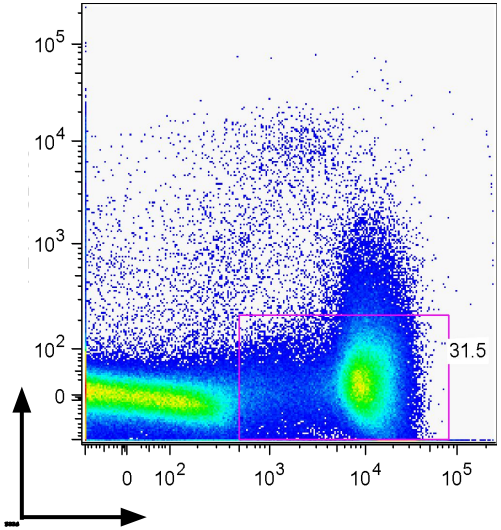


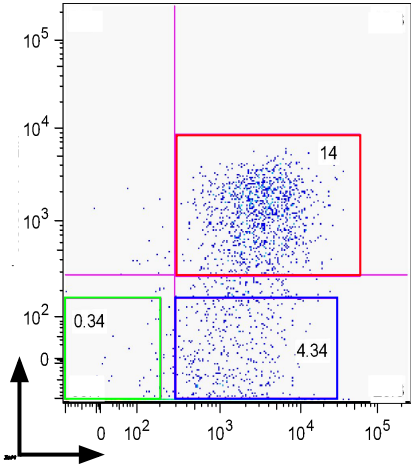
Spectral data courtesy of Evrogen

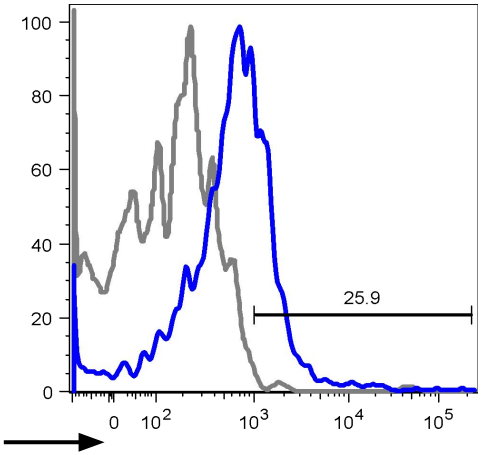
Conventional flow cytometry (Example: scattering+5 colors)

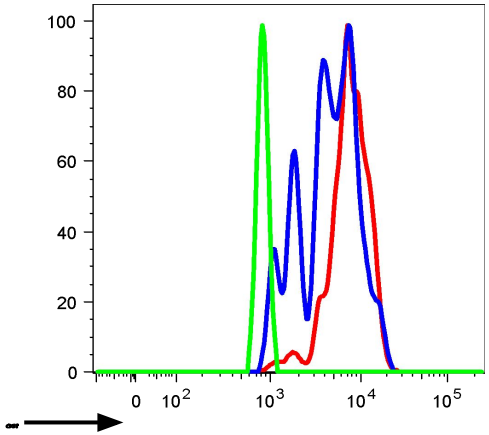






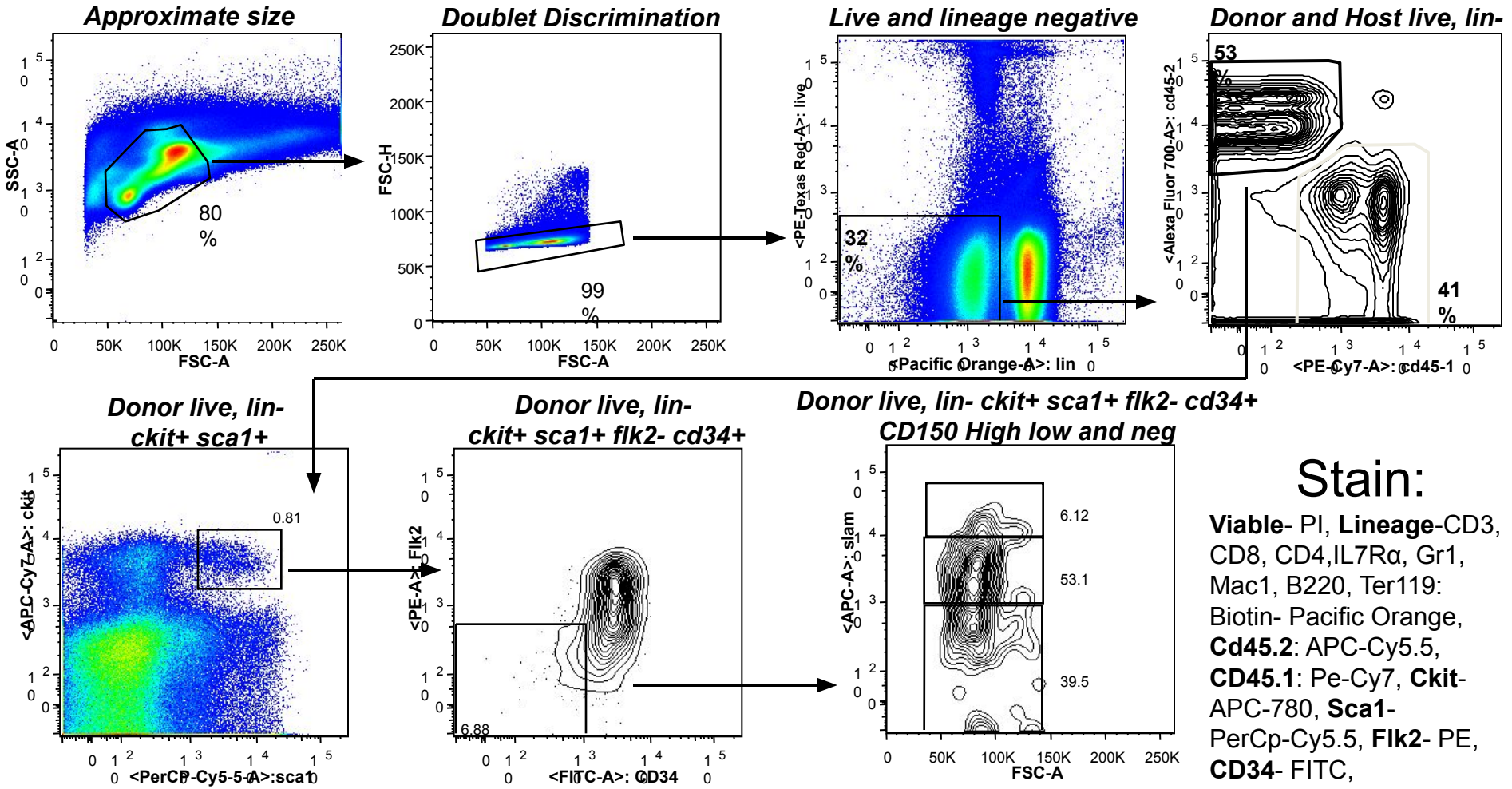






9 colors: Murine Hematopoietic Stem Cells Sort from Transplant

Objective: **To serially transplant subpopulations of hematopoietic stem cells (HSC's)** Cell surface phenotype of HSC: Ckit^{+} Sca1^{+} CD34^{+} Flk2^{+} Lin^{-}. Donor Mouse was CD45.2: Recipient Mouse: CD45.1 CD150 gates for the HSC compartment defined as follows: Slam Neg: ckit^{+}sca1^{+} CD34+ Flk2^{+} Slam Low: ckit^{+} Sca1^{-} CD34^{-} Flk2^{-}, Slam High: Ckit^{+} Sca1^{+} CD34^{-} Flk2^{-} above Slam Low gate.

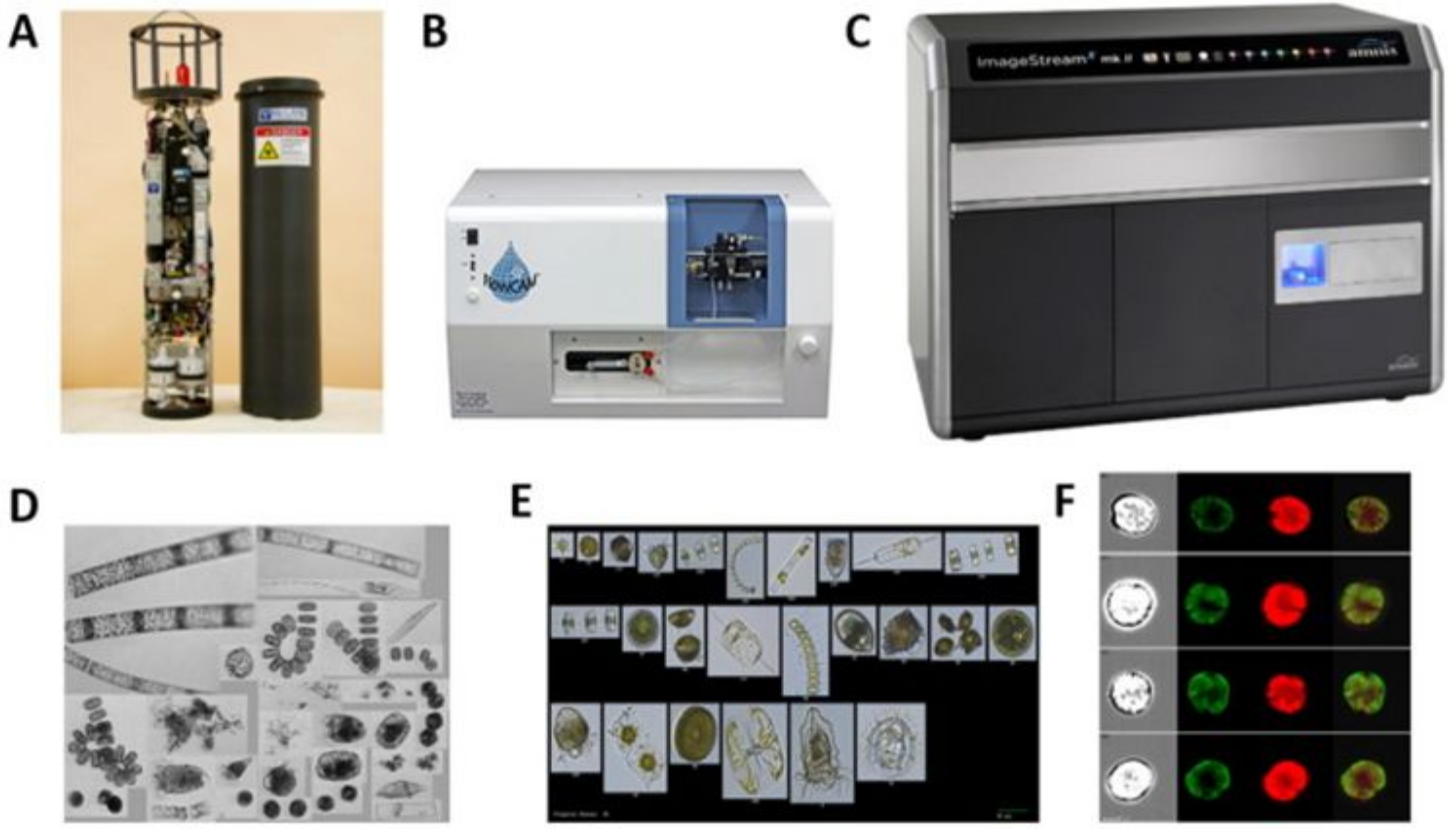


Stain:

Viable- PI, **Lineage**-CD3, CD8, CD4,IL7Rα, Gr1, Mac1, B220, Ter119: Biotin- Pacific Orange, **Cd45.2**: APC-Cy5.5, **CD45.1**: Pe-Cy7, **Ckit**- APC-780, **Sca1**- PerCp-Cy5.5, **Flk2**- PE, **CD34**- FITC, **CD150/Slam**: APC

Isabel Beerman/PCMM

Imaging flow cytometers provide alternative for cellular analysis and characterization



Imagestream 100 imaging flow cytometer

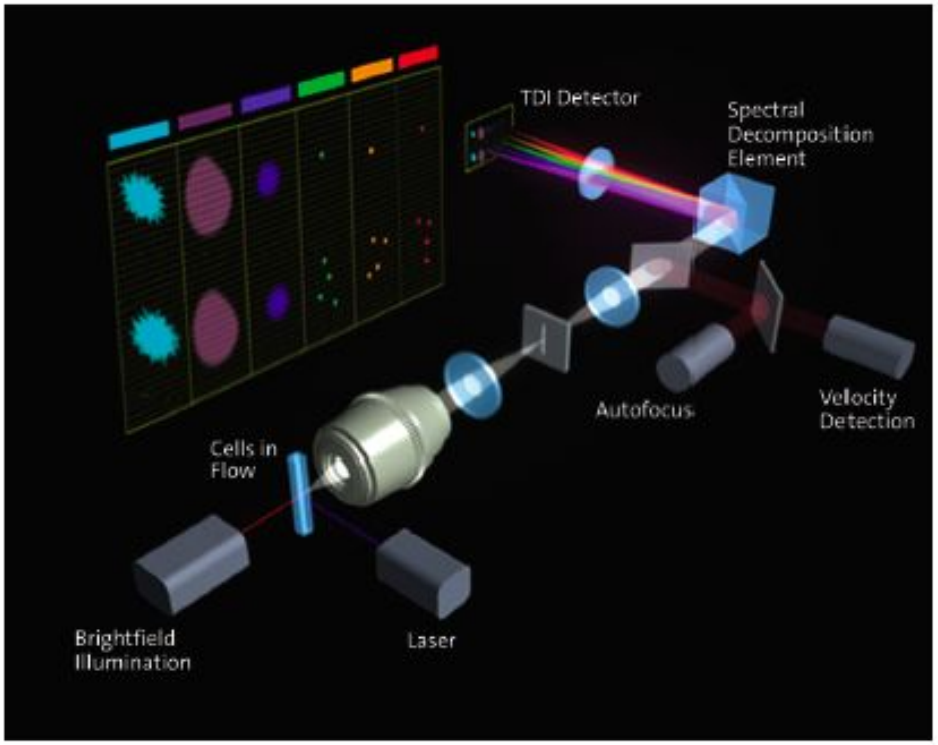
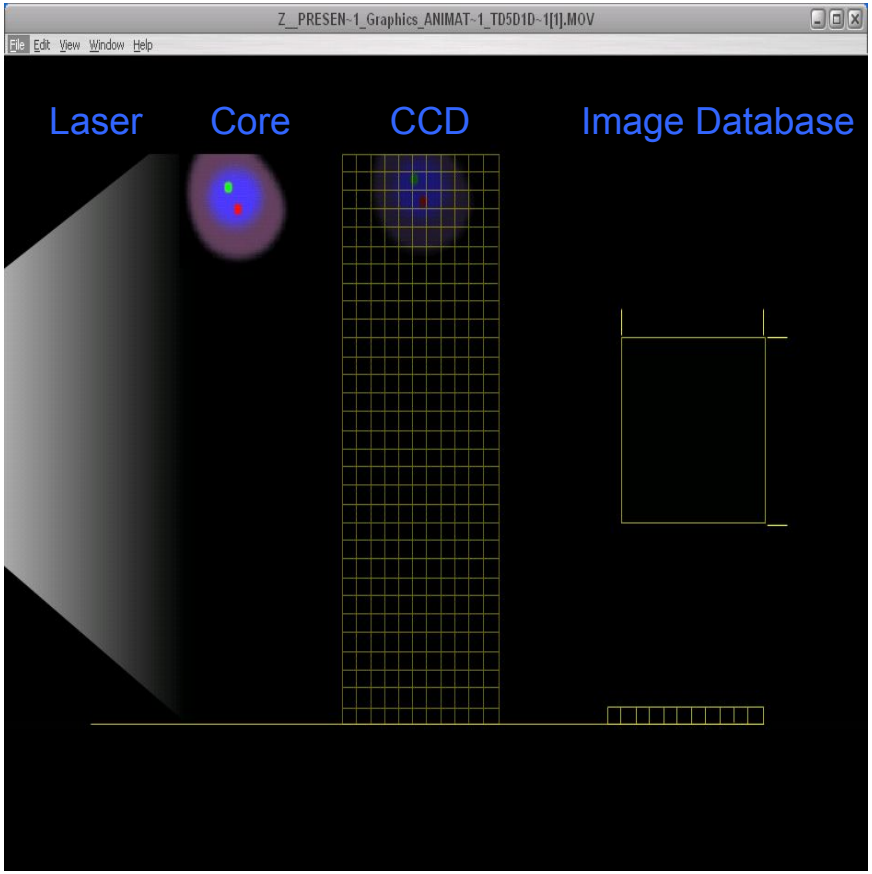


Figure 1. Data collection on the ImageStream 100. Cells passing through the flow cuvette are illuminated both by a brightfield source and a 488nm laser. Composite cell images are decomposed into six separate spectral imaging bands and projected on a proprietary CCD camera that has six independent data collection channels. Using Time Delay Integration (TDI) technology, the system amplifies the signal intensities as cells move through the flow cuvette, allowing imaging of even relatively faint fluorescent probes. Data collected on the system are processed and analyzed with the IDEAS image analysis software package.

ImageStream 100 Specifications

Sensitivity	1000 MESF
Dynamic Range	10 bits per pixel
Collection Numeric Aperture	0.75 NA
Fluorescence Excitation Wavelengths	488nm standard 200 milliwatt laser
Spectral Imaging Bands	
Deep blue	400-470nm
Darkfield and visible	470-500nm
Fluorescein (FITC) fluorescence	500-560nm
Phycoerythrin (PE) fluorescence	560-595nm
Texas Red, PE-conjugates	595-660nm
Brightfield default; user selectable	660-730nm
Sample Characteristics	10^7 - 10^8 cells/ml 50-500 microliters
Data Analysis	Over 200 standard image parameters per cell unlimited user-defined image parameters software crosstalk compensation
Instrument Operation	automatic sample load,empty, flush, purge automtic focus and core position tracking multiple self-diagnostic systems
Requirements	90-240 VAC, 50-60 Hz, 1000W 10/100 ethernet minimum No external air / water needed 36" x 24" x 24"

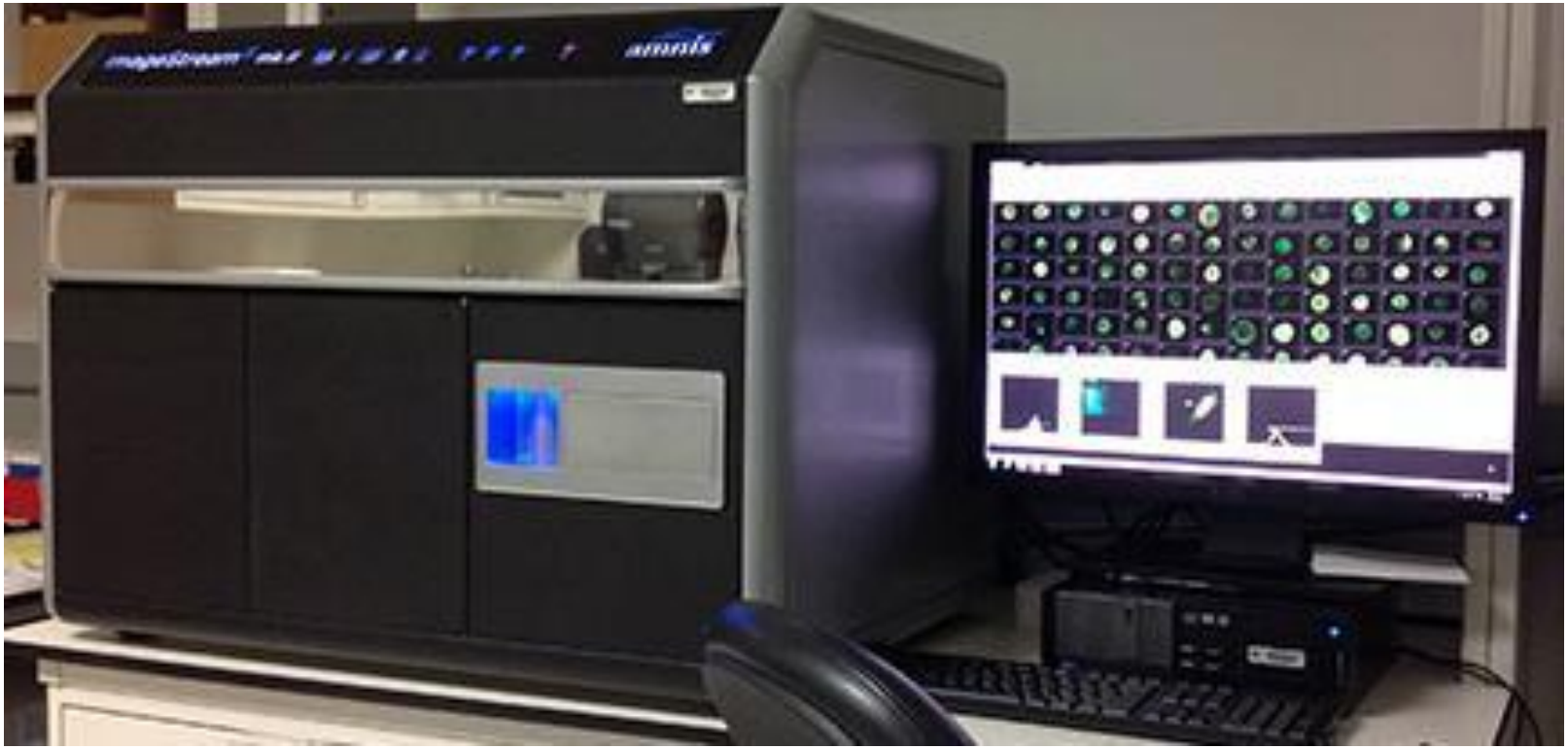
Time Delay Integration



TDI CCD

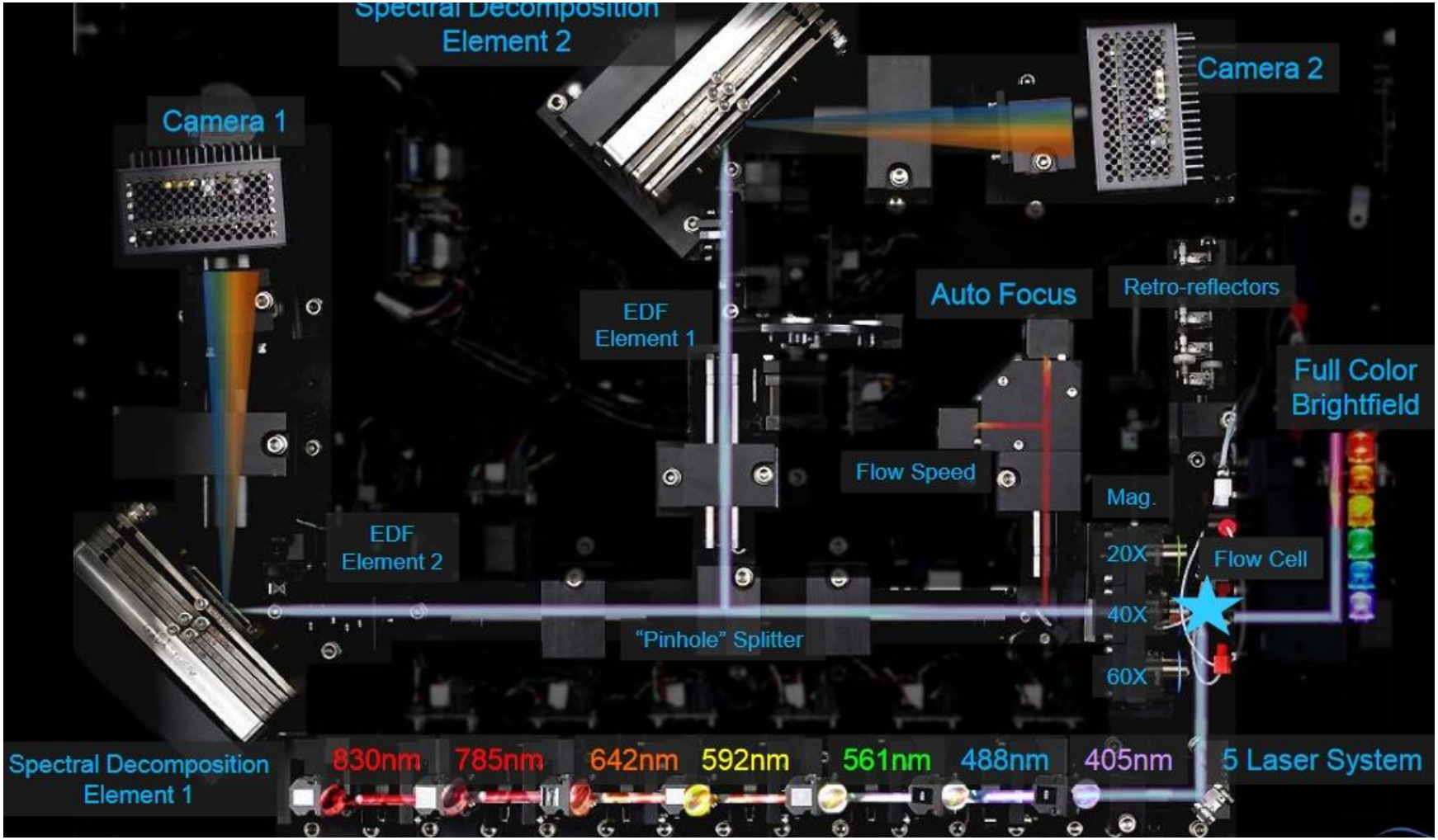
- Excite fluorescence over the entire height of the detector
- Light is detected in the first pixel row and transferred to the pixel below in exact synchrony with the velocity of the cell as it goes streaming by.
- Light is integrated over the entire height of the detector to achieve high photonic sensitivity
- Images don't streak or blur and maintain 0.3 um per pixel resolution.

Imagestream X Mark II



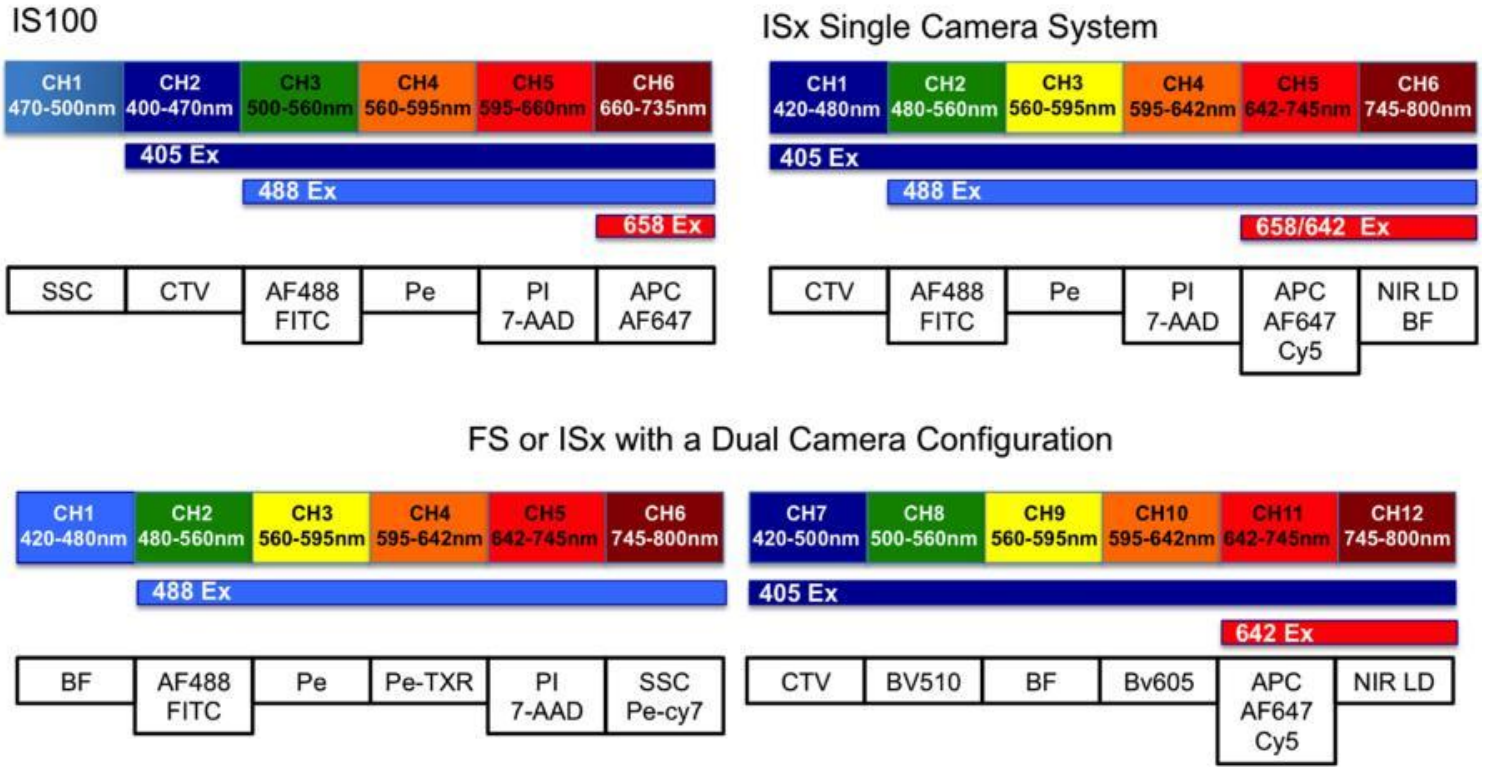
x60 objective; higher acquisition speed; 10 fluorescent channels; +561 nm laser

Imagestream X Mark II



Amnis Inc

Imagestream (s) optical configurations and fluorescent channels



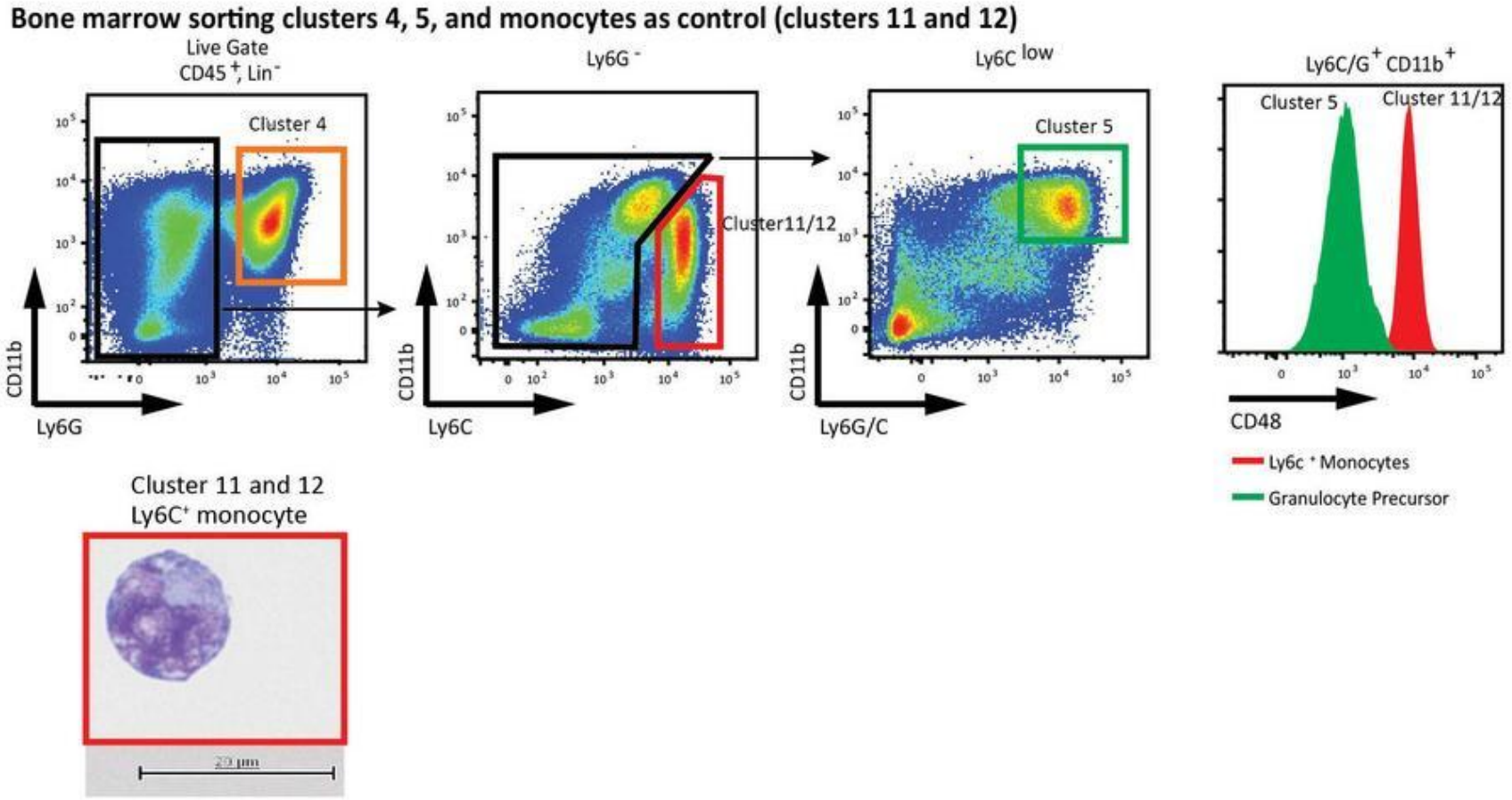
Channel 1	Channel 2	Channel 3	Channel 4	Channel 5	Channel 6	Channel 7	Channel 8	Channel 9	Channel 10	Channel 11	Channel 12
468/76	532/56	578/36	628/64	690/60	760/80	468/76	532/56	578/36	628/64	690/60	760/80
Brightfield	FITC	PE	ECD	PerCP	SSC	DAPI	PacOrng	Brightfield	TxR	AF647	APC Cy7
	AF488	Cy-3	PE-TxR	Draq5	PE-Cy7	PacBlu	AF430		AF594	AF660	APC AF750
	GFP	AF555	PI	PerCp5	PE-750	MarBlu			AF568	Cy5	SSC
	YFP	DS-Red	7AAD	PE-647	SSC	Hoechest			AF610	APC	
	Syto		PE-610	PE-Cy5						APC-Cy5.5	

Adapted from A.Filby, 2015

Cellular analysis by conventional Flow Cytometry

- Traditional markers to define cell populations (human, rat, mouse)
- Relies on fluorescence-based analysis; no morphological parameters (only size-parameter)

Standard approach to verify FACS-defined cellular subset:cell sorting+microscopy

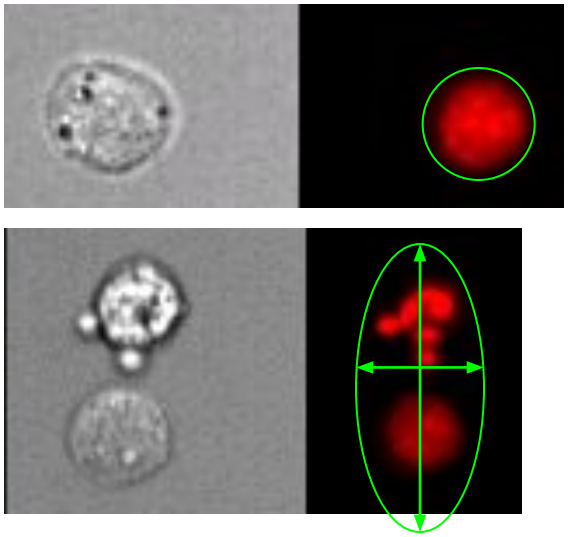


From Becher et al, Nature Immunology, 2014

Limitations of FACS sorting/microscopy approach

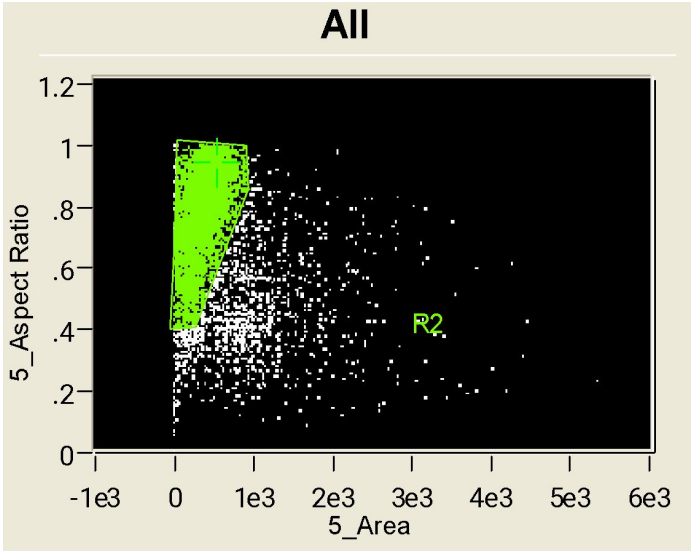
- Purity of sorted subpopulation (never 100%)-can be 85% or less for some sorted subsets
- Difficult or not possible to sort/perform microscopy on low expressing (<1%) and rare cell (<0.1%) populations
- Manipulations related to cell sorting may induce maturation and activation of cell subsets (e.g. DC), leading to negative impact on outcome of experiment
- Viability and/or fluorescence of sorted cells can be affected
- Cells can be not identifiable by morphology
- Advanced spectral compensation not available in microscopy

Identifying Singlets by IFC (Aspect Ratio Intensity/Area)



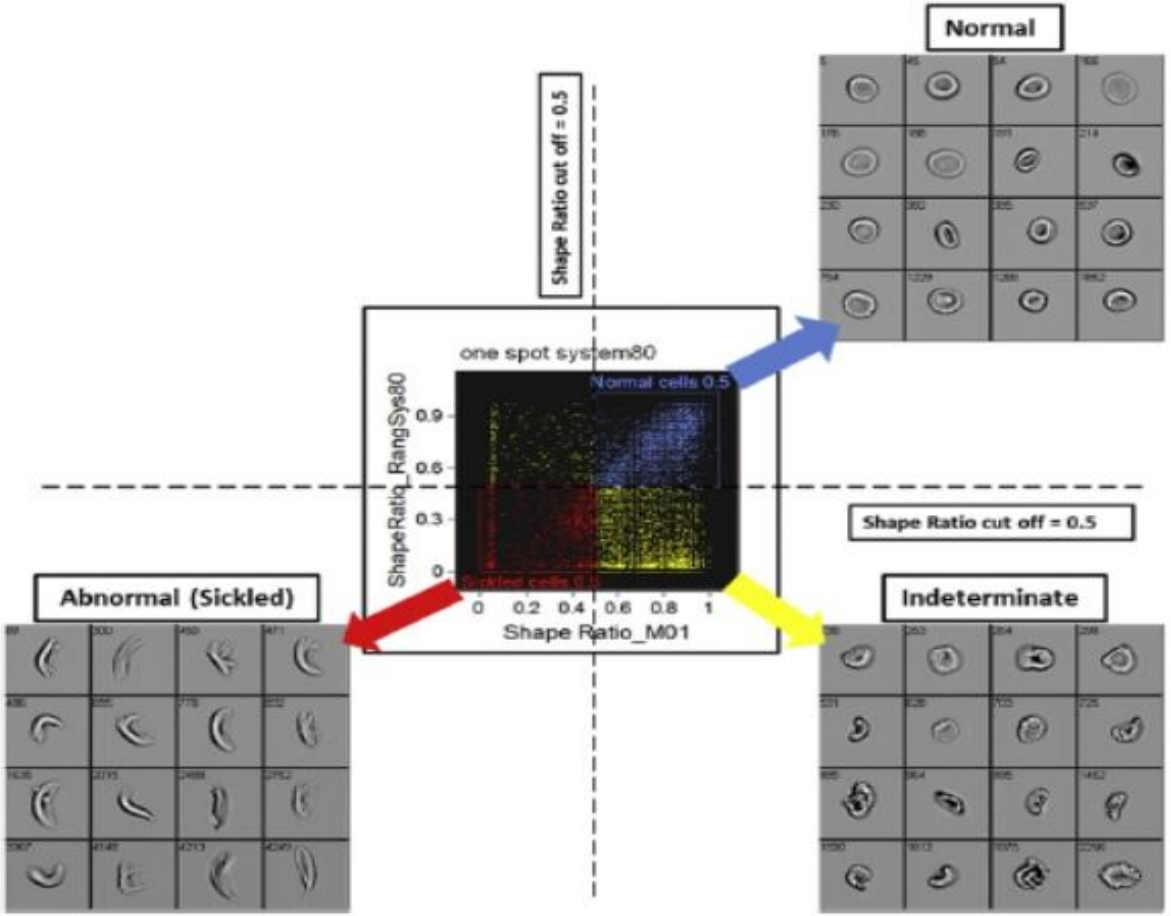
Aspect Ratio Intensity is the minor axis intensity divided by the major axis intensity.

•Identifying single cells vs. doublets and multiple events

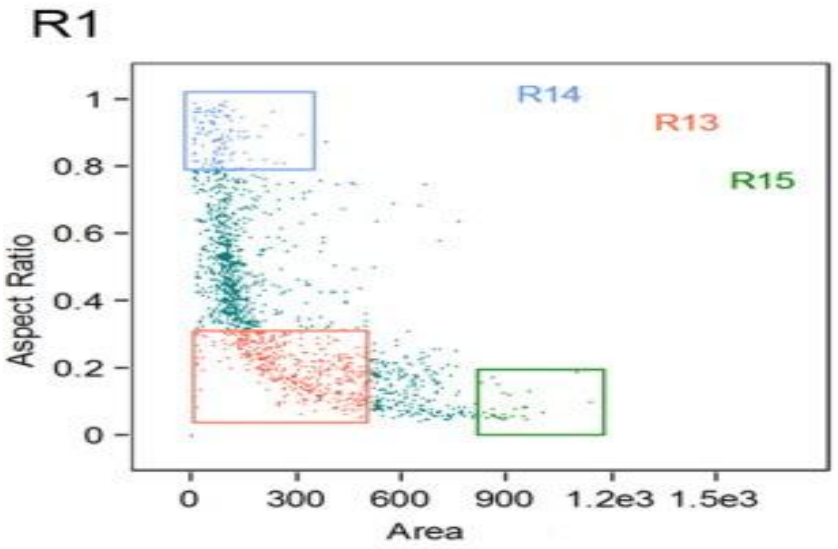


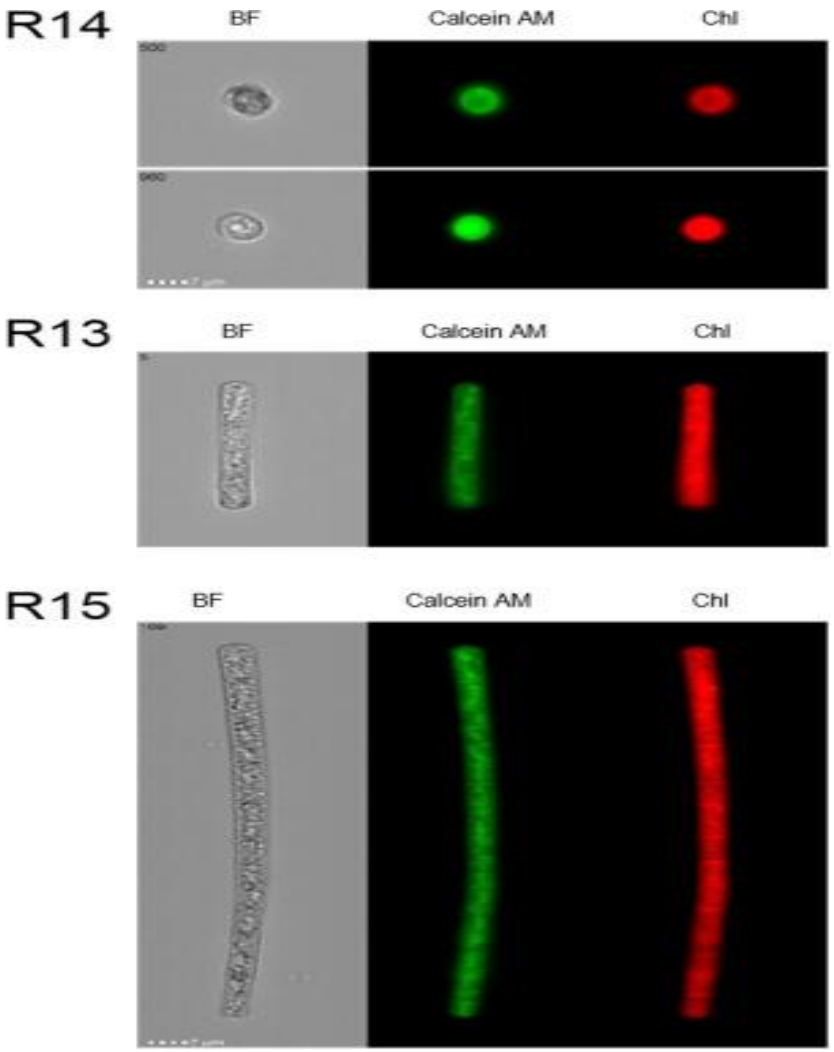
Shape parameters in defining erythroid sickle anemia cells

(Samsel, McCoy Jr, 2016)



Size/Shape distribution analysis
(Aphanizomenon sp. Cells, our data)

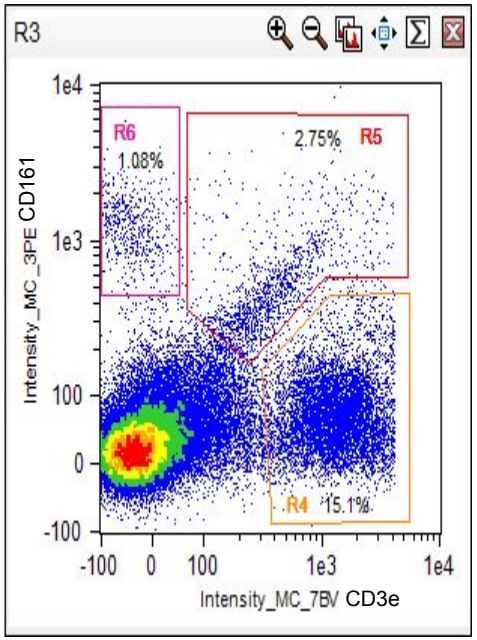


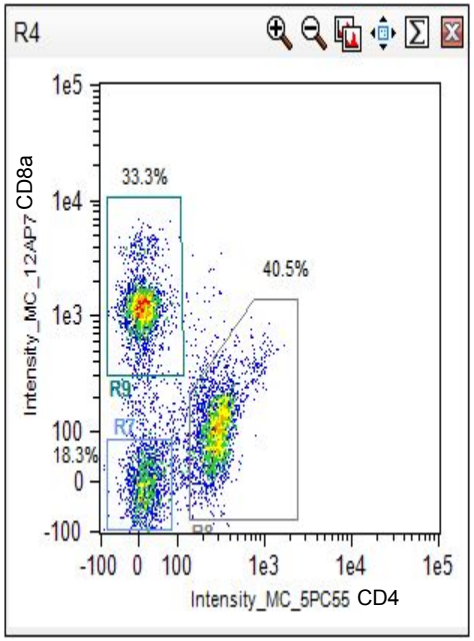


Fluorescence-based analysis by Imagestream

- DNA/RNA dyes (PI, Sytox Blue, SYTOX Green etc)
- Lipid dyes (DiO, DiA, BODIPY family_
- Fluorochrome-tagged Annexin V
- Fluorescently-tagged probes-fluorescent probes (GFP and others) and/or or lectins
- AUTOFLUORESCENCE as a parameter

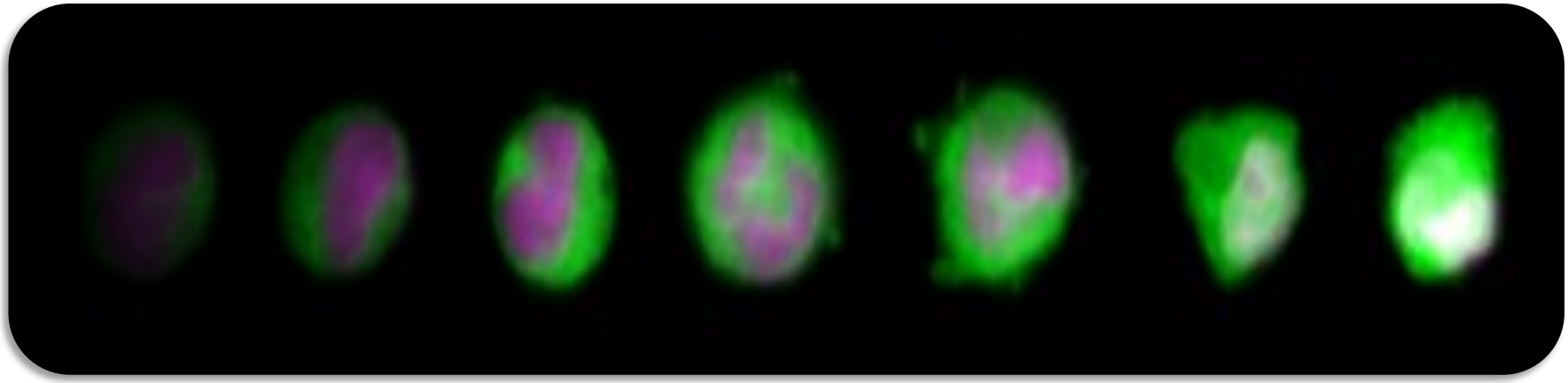
Intensity: Total Fluorescence



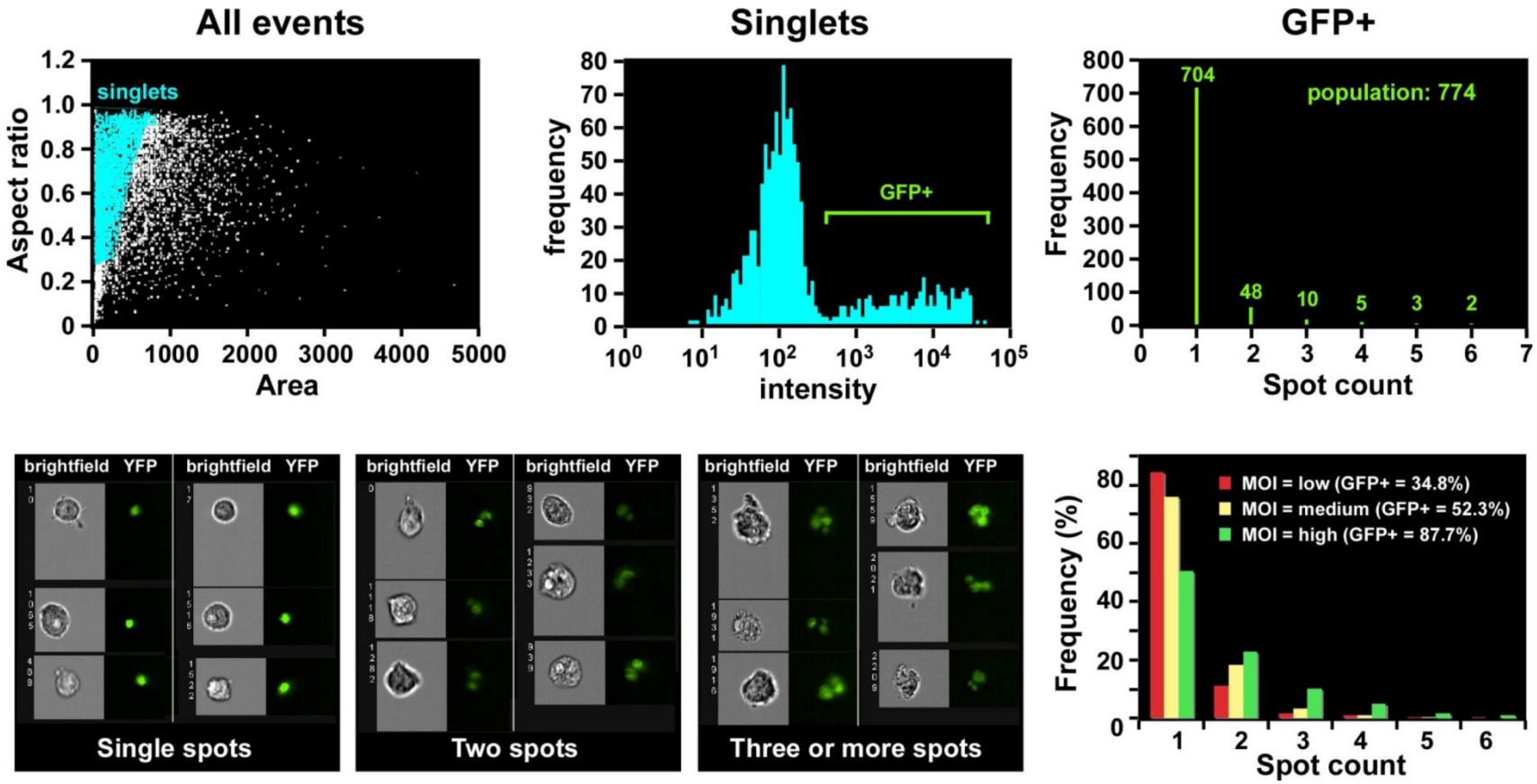


Description:

Intensity is the sum of all the pixel values in the mask, background subtracted.

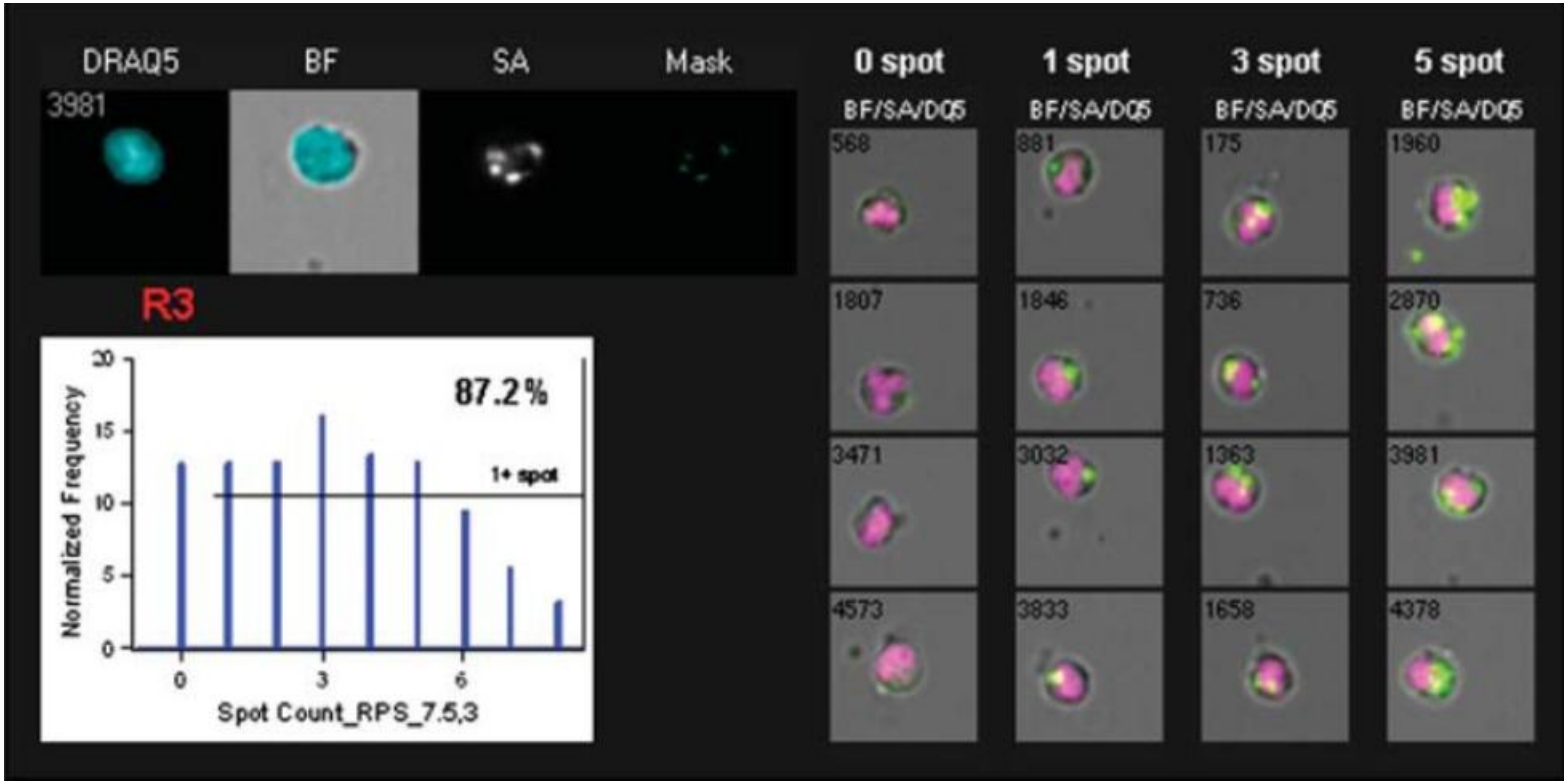


Quantification of *Toxoplasma gondii*



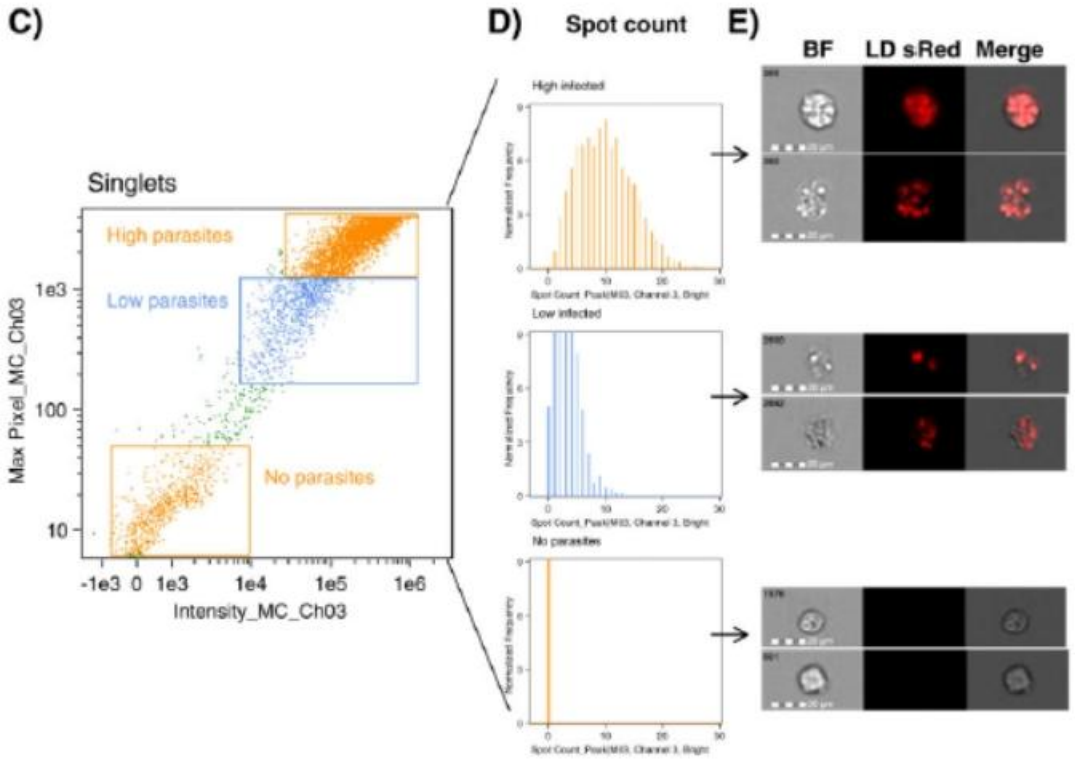
Muskavitch et al, 2008

Number of ingested by neutrophils *S. aureus* bacteria (Ploppa et al, 2011)

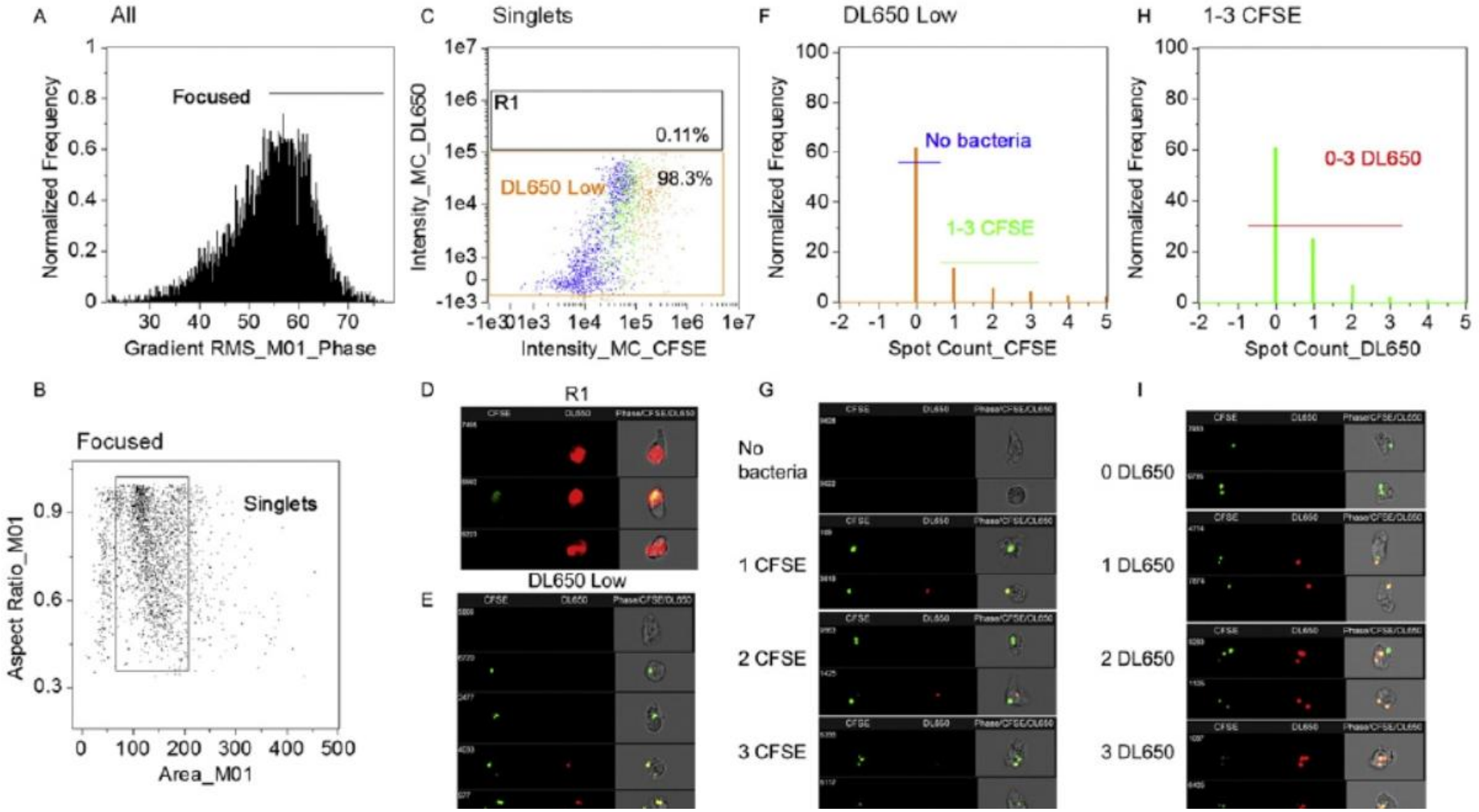


Counting of *Leishmania donovani* (% infected cells and #parasites/cell)

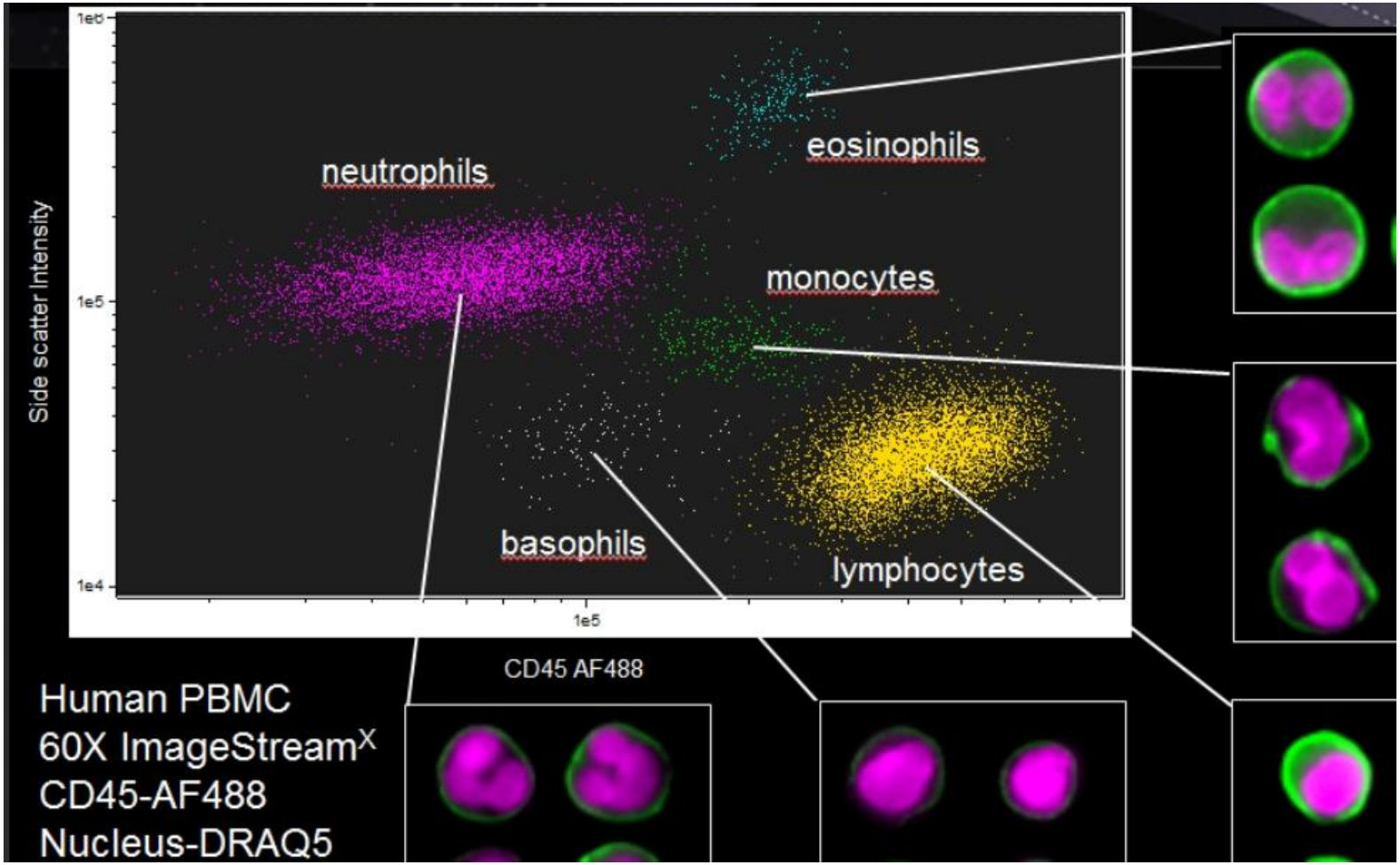
(Torrezas et al, 2015)



Internalization of CSFE-stained N.gonorrhoeae bacteria (Smirnov et al, 2015)



Human PBMC -morphology

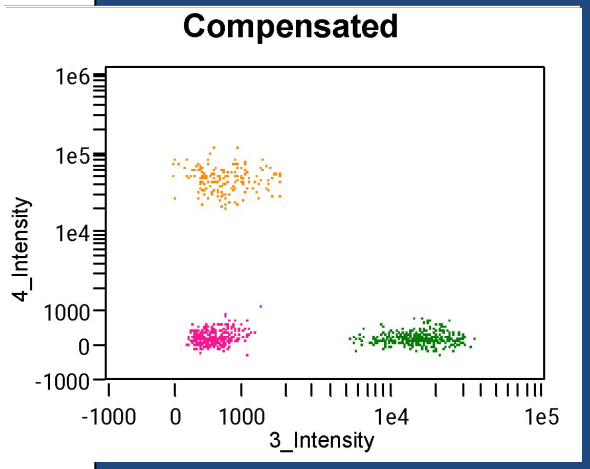


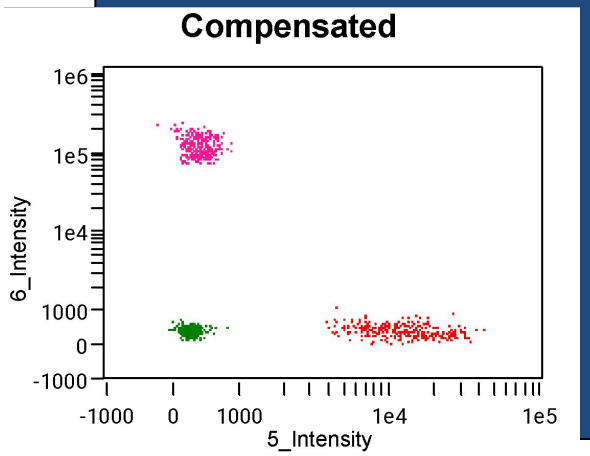
(from B.Hall)

Spectral Compensation

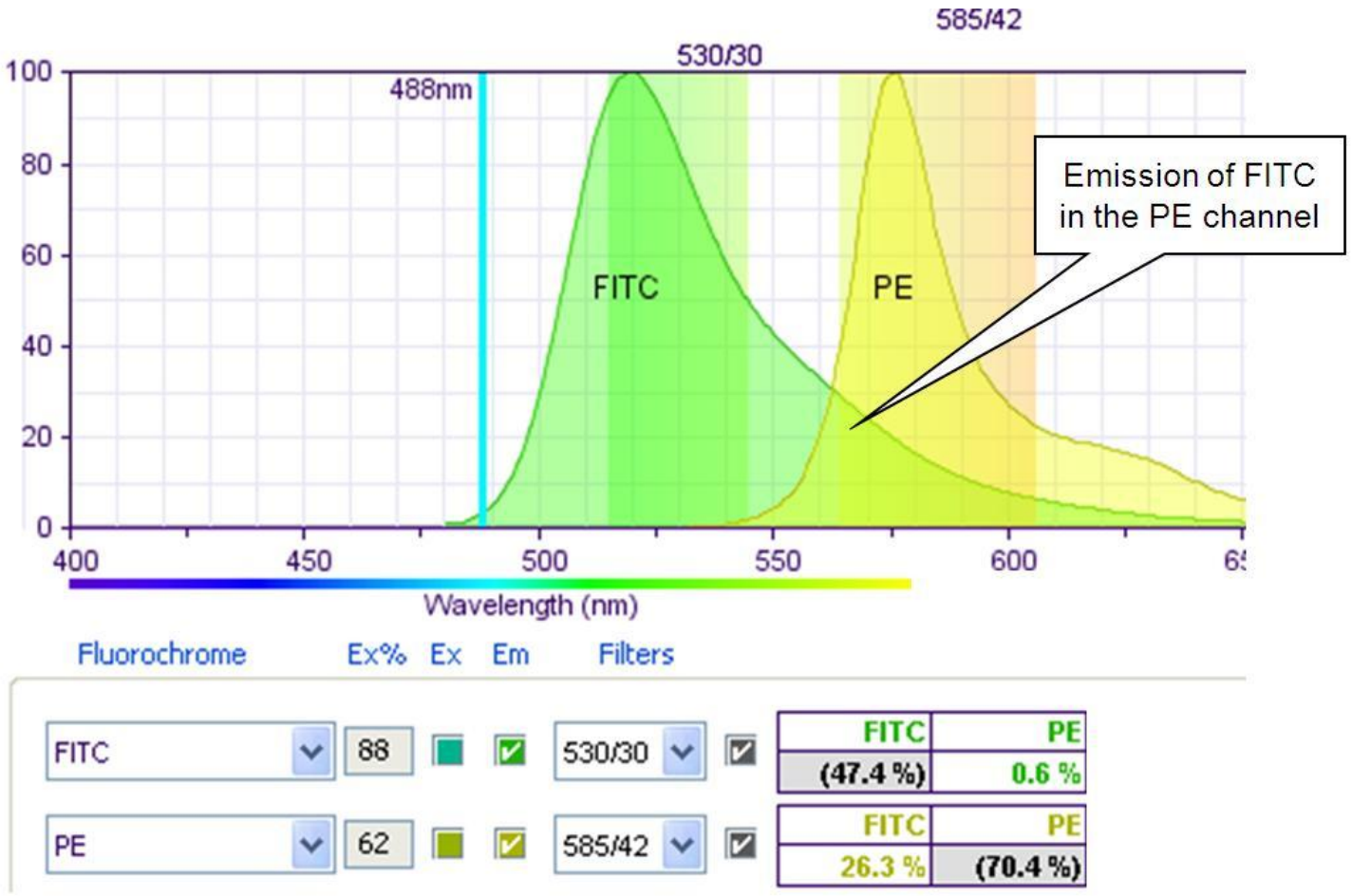
(Imagestream 100, Amnis Corp)

Post-acquisition compensation is applied to images on a pixel by pixel basis in IDEAS.

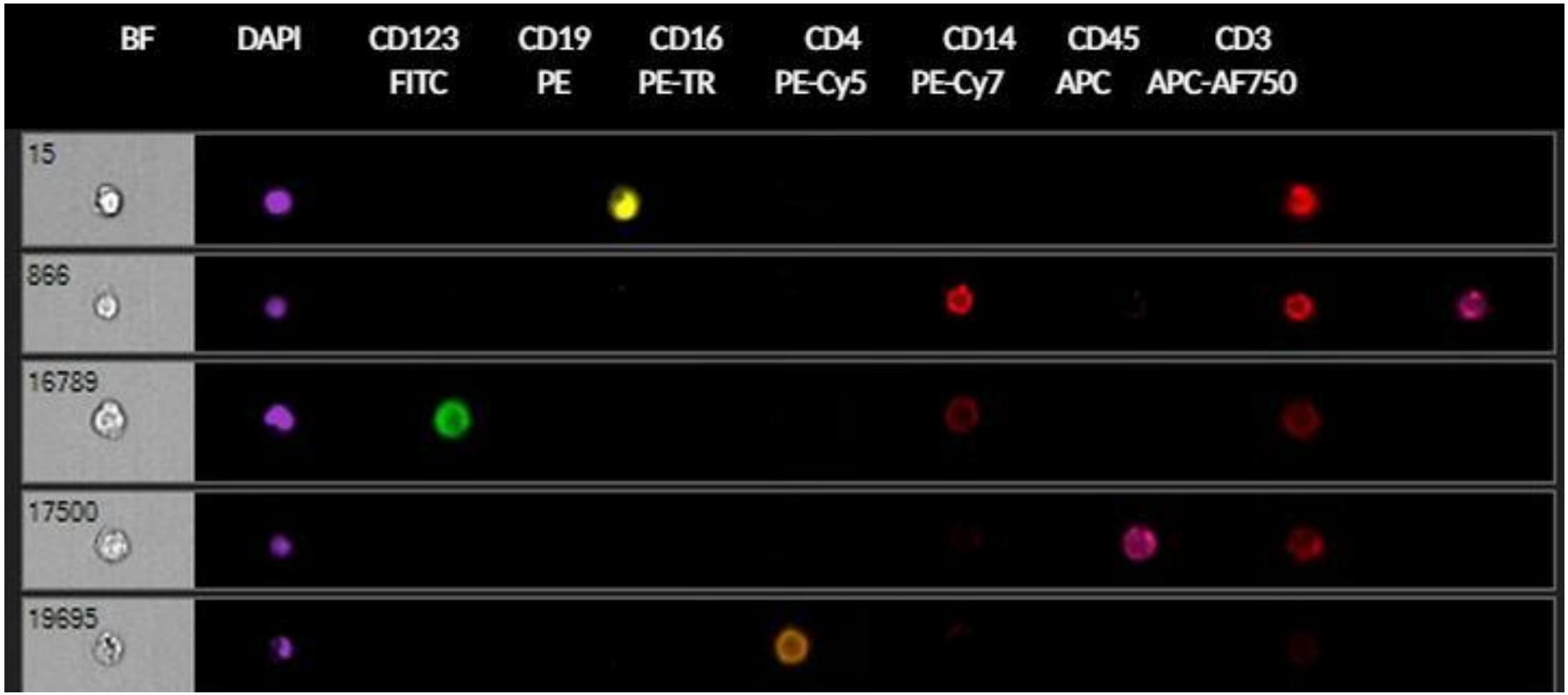




Spectral compensation is assymetric

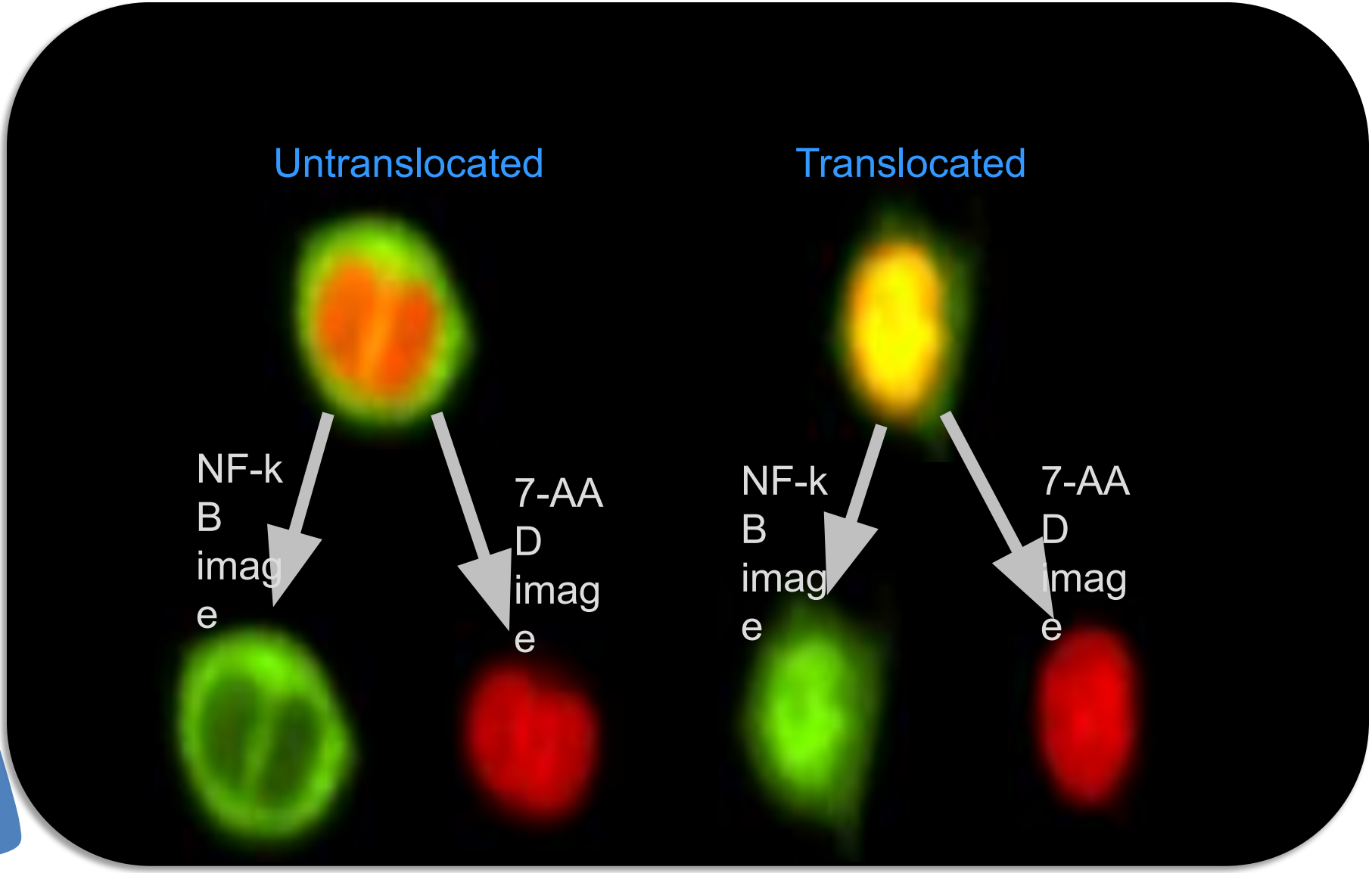


From 3-4 colors for images (microscopy) to 8-colors immunophenotyping (external staining) with Imagestream X Mark II



CD3+ T-cells; CD4+ helper T-cells; CD16+granulocytes; CD19+ B-cells; CD14+ monocytes; CD123+ pDC/basophils; Nuclear morphology

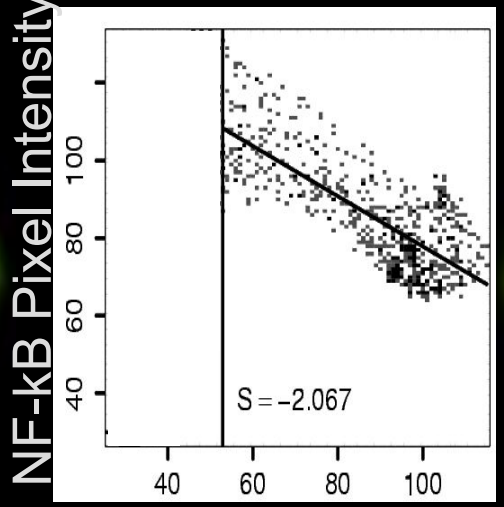
NFkB Translocation Using The Similarity Algorithm (Amnis)



NFkB Translocation Using The Similarity Algorithm (Amnis)

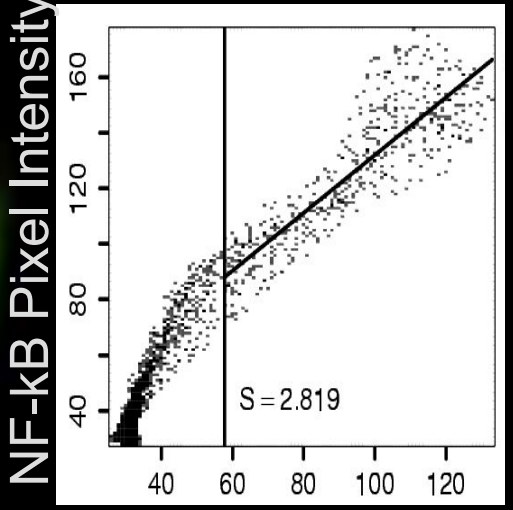
S = -2.07

Untranslocated

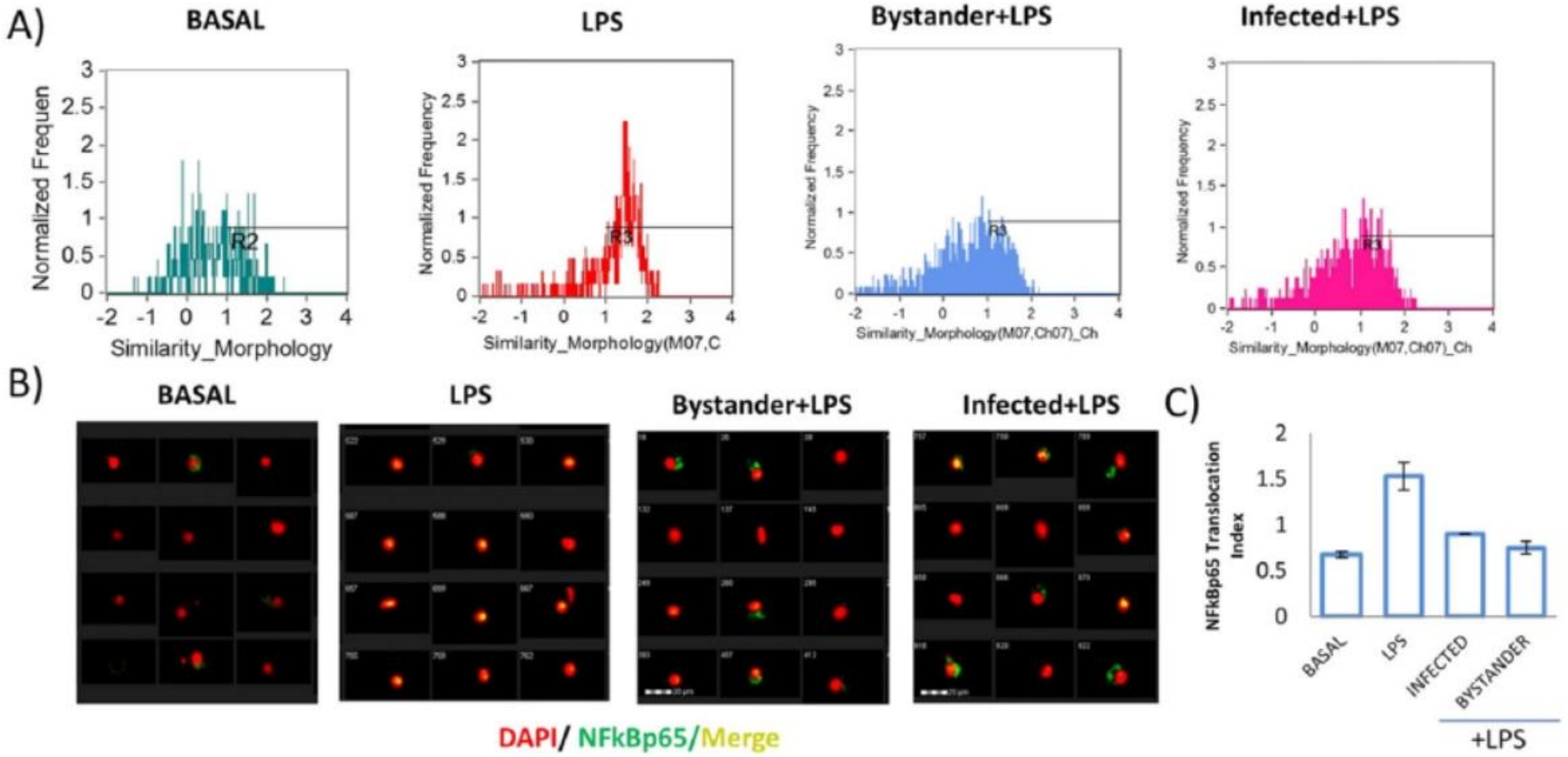


S = +2.82

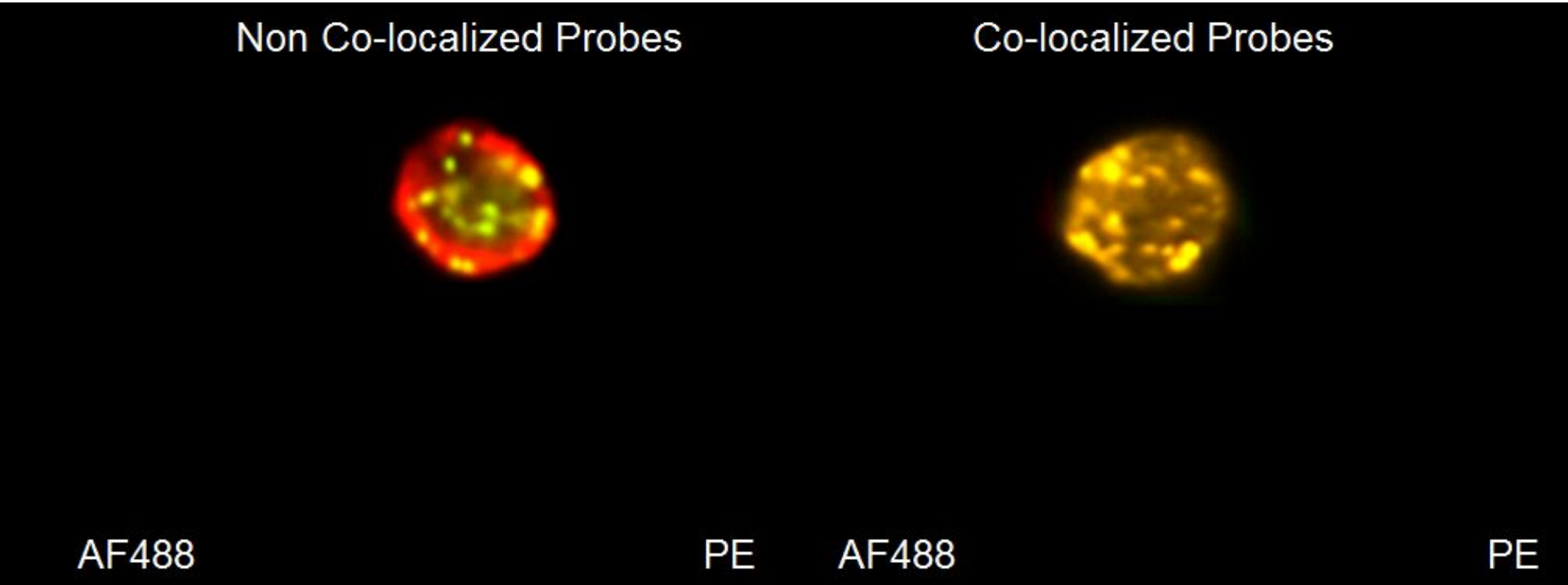
Translocated



Bystander MFs have impaired NFkappaBeta translocation to the nucleus (Torrez et al, 2015)



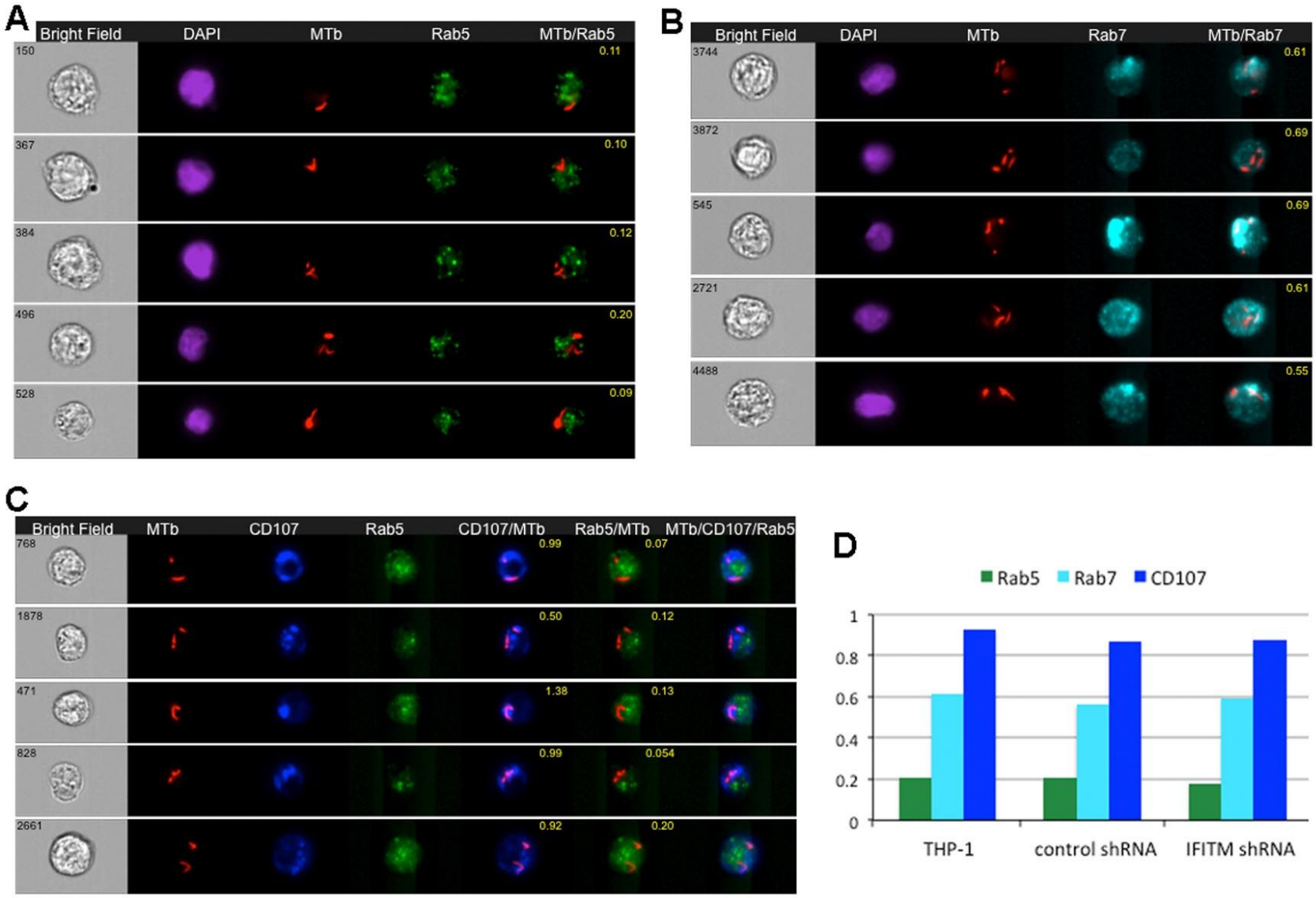
Co-localisation



- The degree of probe co-localization is measured by performing cross-correlation analysis on the small bright regions in image pairs of the same cell
- The feature is called Similarity Bright Detail (SBD).

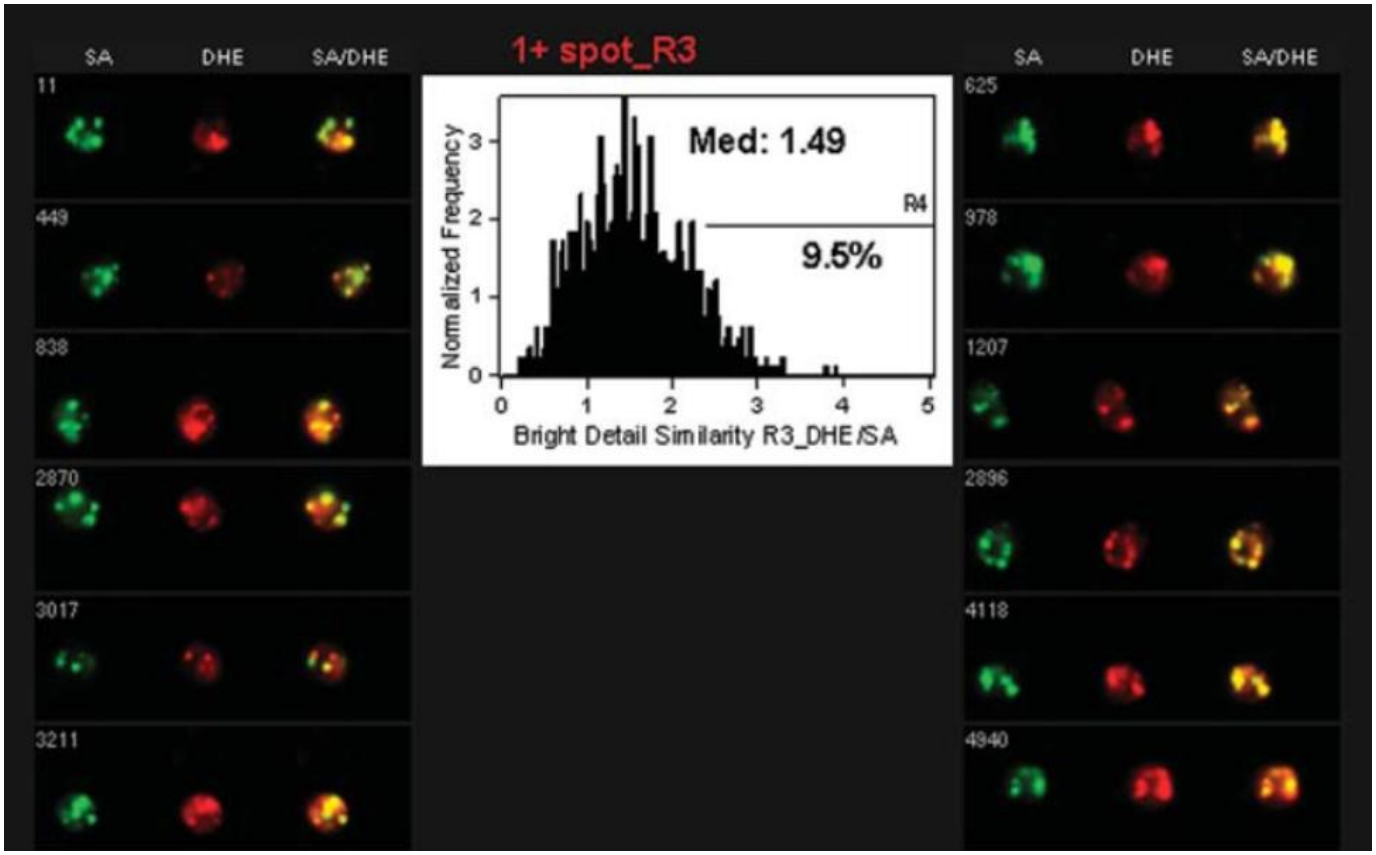
Case 1: Co-localisation M.tuberculosis with Rab5 and Rab7

(From Haridas et al, 2016)

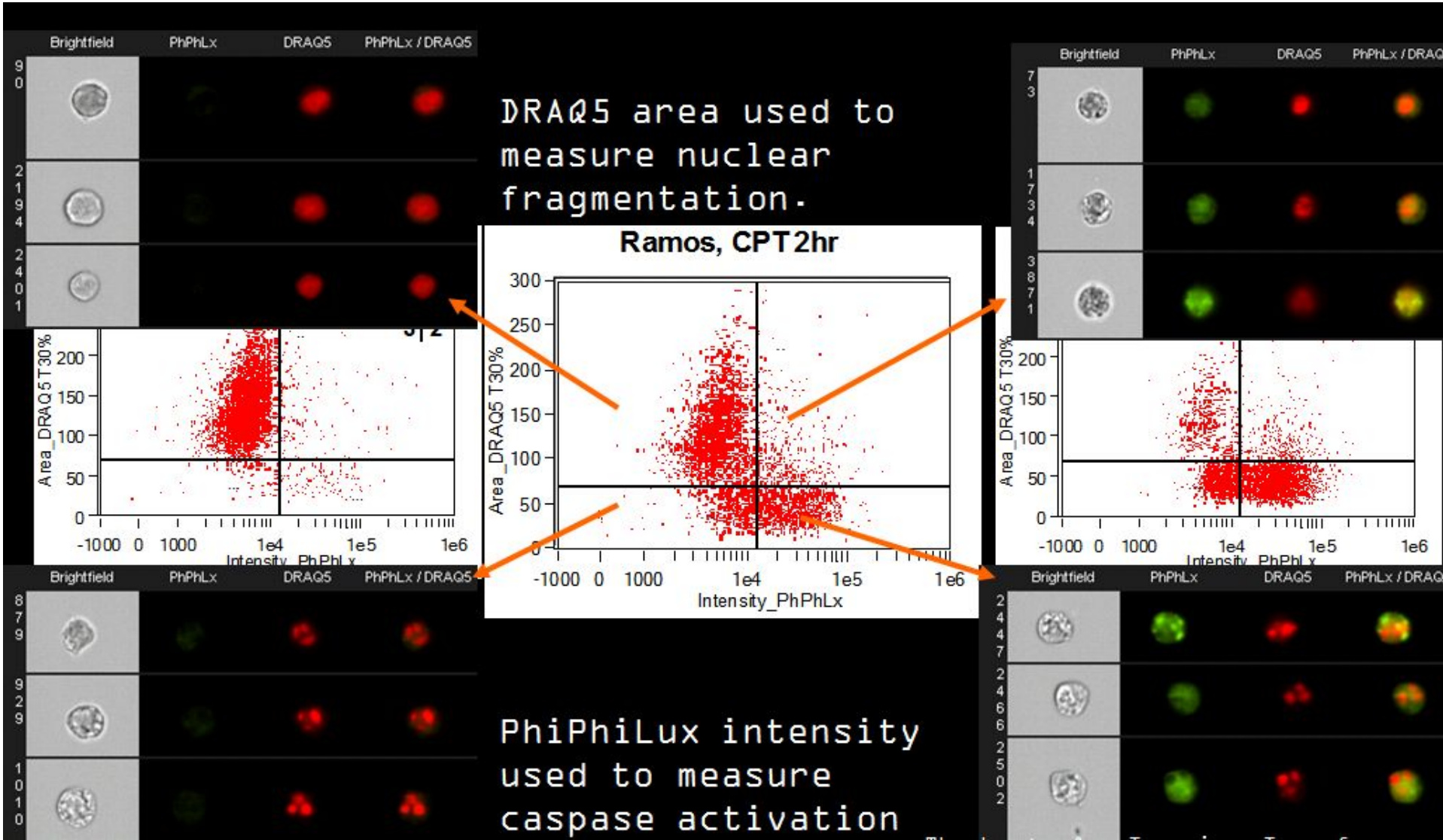


Co-localisation of *S.aureus*/dihydroethidium (oxidative burst in human whole blood)

(Ploppa et al, 2011)

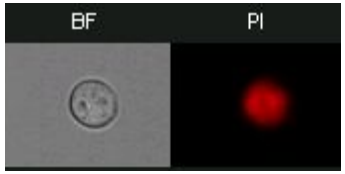


Nuclear fragmentation/caspase activity

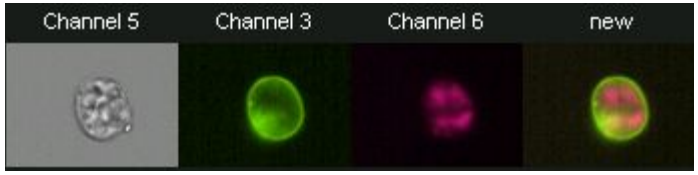


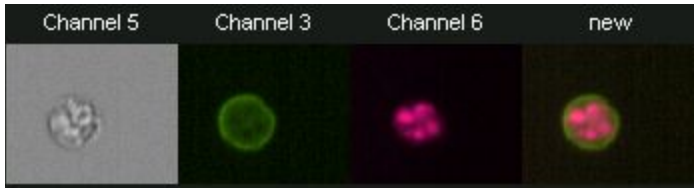
Hallmarks of apoptosis (Morphology)

•DNA condensation & nuclear fragmentation

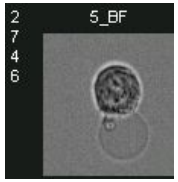


•Phosphatidylserine exposure on cell surface





•Membrane blebbing



•Caspase activation