AKADEMIE FÜR IMMUNOLOGIE

SPRING SCHOOL ON IMMUNOLOGY

Ettal, Bavaria, March 5 - 10, 2023

Flow and Image Cytometry Essentials with a View of the Future.

Diether Recktenwald, BD Biosciences, retired
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http://www.desatoya.com

Key Reference Abbreviation

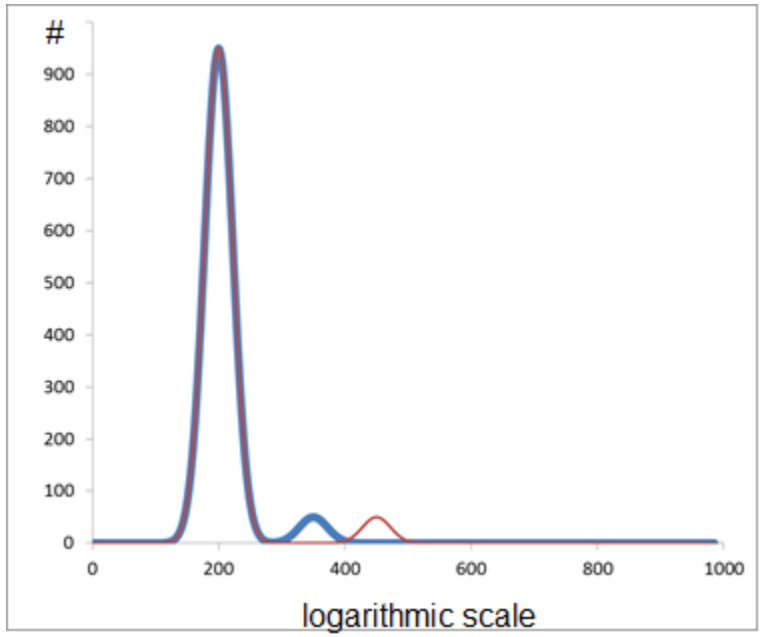
In this document “GUIDELINES” is used for
Cossarizza, Andrea, et al. "Guidelines for the use of flow cytometry and cell sorting in immunological studies." European journal of immunology 49.10 (2019): 1457-1973

The GUIDELINES contain contributions from 337 experienced scientists from more than a hundred institutes worldwide, describing their recommendations for the optimal use of flow cytometry.

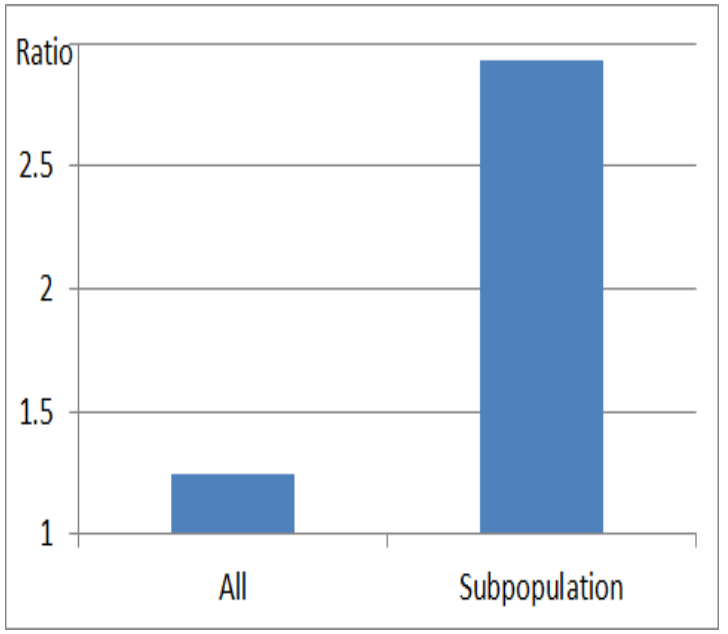
References with a DOI number can be located with an Internet search.

Why Cell Subset Analysis at the Single Cell Level

Intensity Histogram

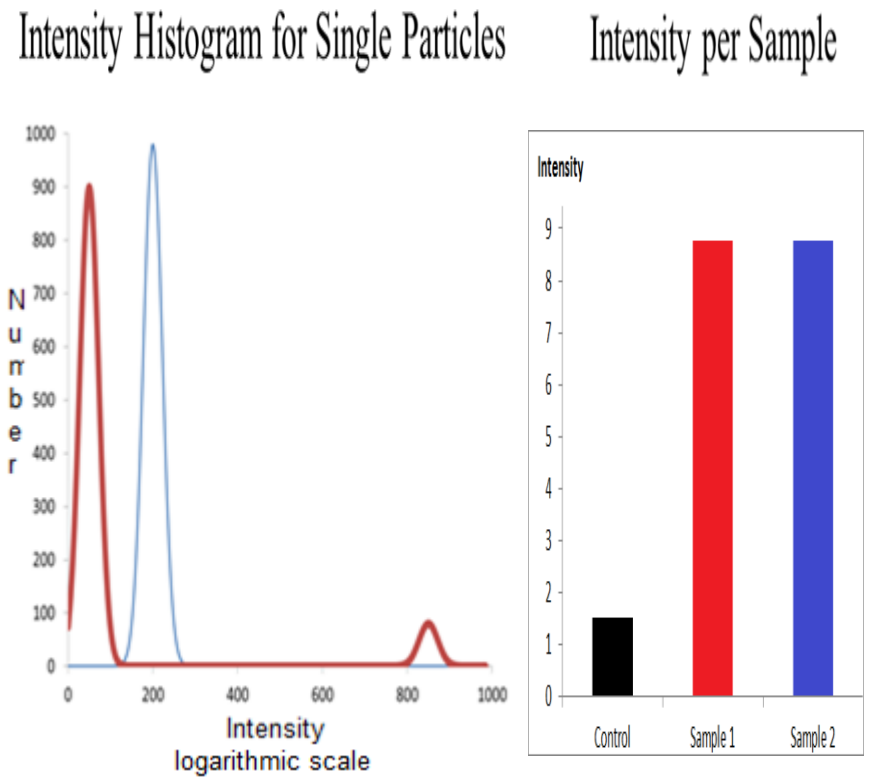


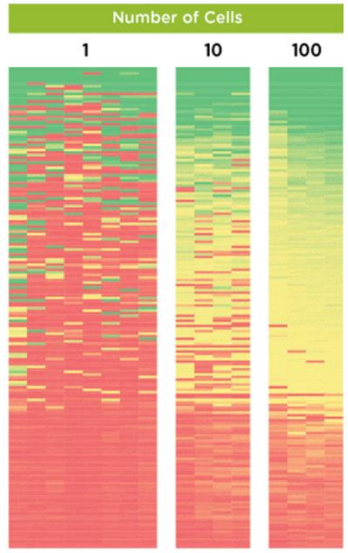
Intensity Ratios



Subpopulation analysis detects changes better, especially for rare subpopulations.

Why Single Cell Analysis

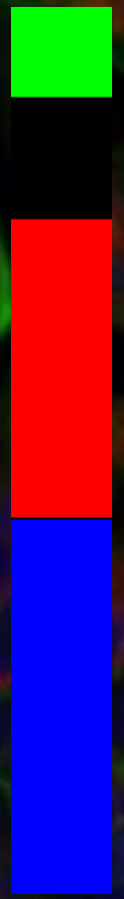




Source: http://www.nanostring.com

Single cell analysis reveals heterogeneity, which is masked by averaging, when analyzing groups of cells.

Single Cell Cytometry vs. Bulk Analysis



Coutesy Dr. Ji

Technologies for single cell analysis

- Microscopy and Digital Imaging
  - Super-resolution
  - High parameter cyclical fluorescence
  - In-vivo
- Single cells in separate defined locations
  - Wells of multi-well plates
  - Aqueous droplets in oil
- Flow cytometry
  - Optical property detection incl. in-vivo
  - Mass label detection (CyTOF)
  - Other parameters

Information from single cell analysis

* Cell-concentration
* Subset fractions
* Cell size
* Cell shape
* Cell arrangement in clusters
* Mass of multiple cellular components per cell
* Distribution of component mass in subsets

- Temporal change of the above parameters
- Gene expression (NGS)

Non direct cell applications

* Highly multiplexed bead-based immunoassays
* Single molecule counting

Flow Cytometer

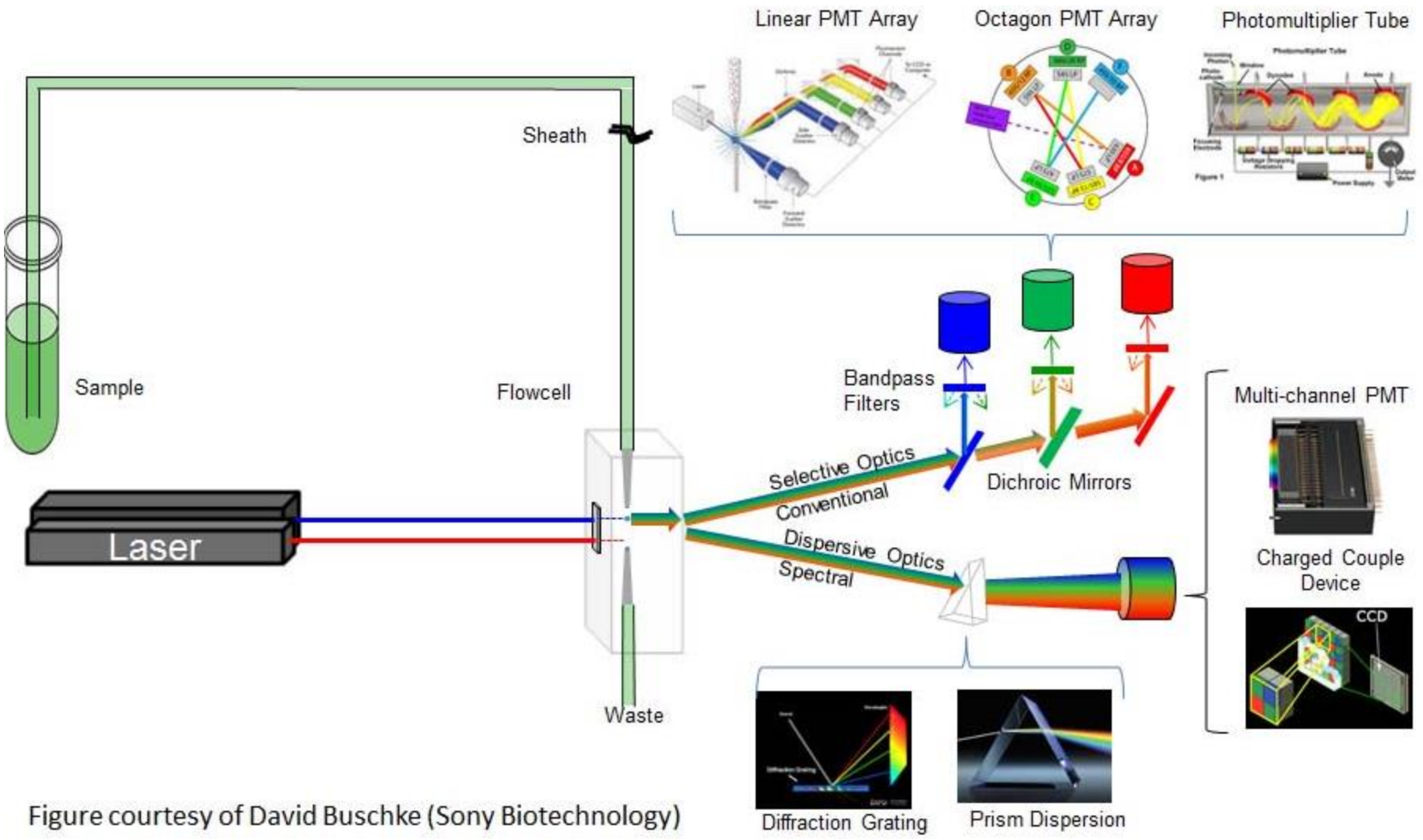


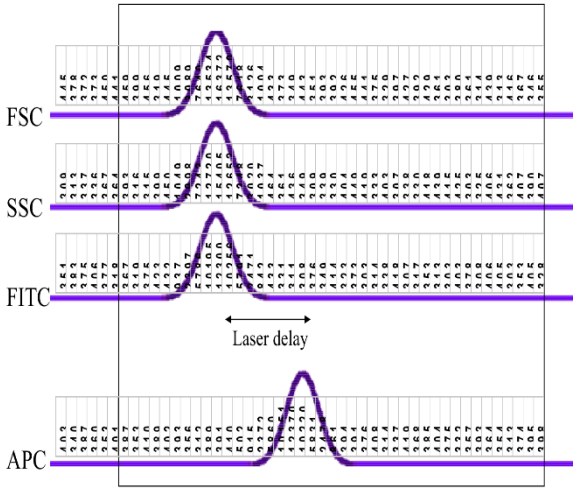
Figure courtesy of David Buschke (Sony Biotechnology)

GUIDELINES Flow cytometers, pages 1478ff

Dichroic filters vs. Multispectral cytometry: Feher K et al.(2016) Cytometry 89A: 681-9

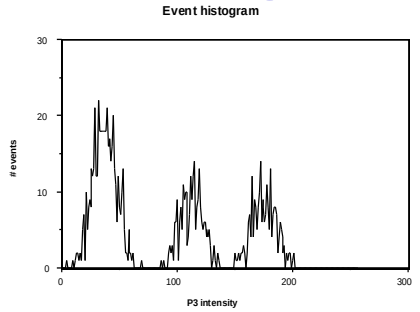
Basic Data Processing

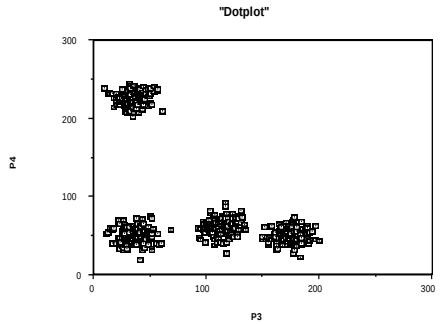
Voltage pulses from detectors

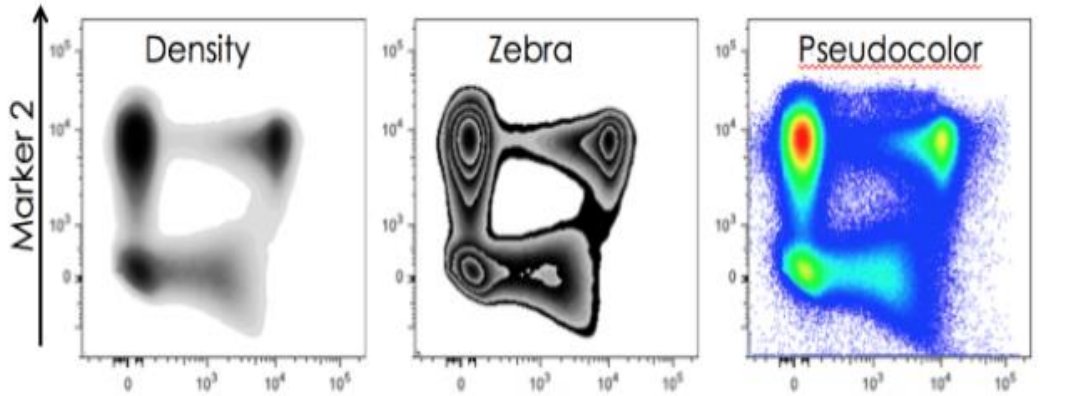


Listmode data after
pulse processing and A/D

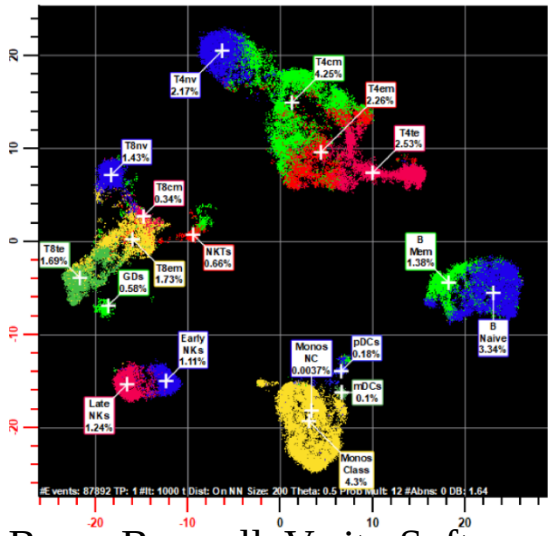
Cell	P1	P2	P3	P4	P5	Pop#
1	242	135	704	175	612	1
2	146	132	690	178	566	1
3	269	147	89	206	580	3
4	442	143	399	250	255	4
5	212	167	155	926	526	2
6	269	2	659	207	575	1
7	204	232	112	171	679	3
8	152	74	160	828	532	2
			…			
9997	215	119	138	936	662	2
9998	244	50	72	261	543	3
9999	214	137	174	1014	597	2
10000	312	87	110	904	560	2







expert.cheekyscientist.com/analyze-facs-data-prepare-cytometry-figures-scientific-papers/

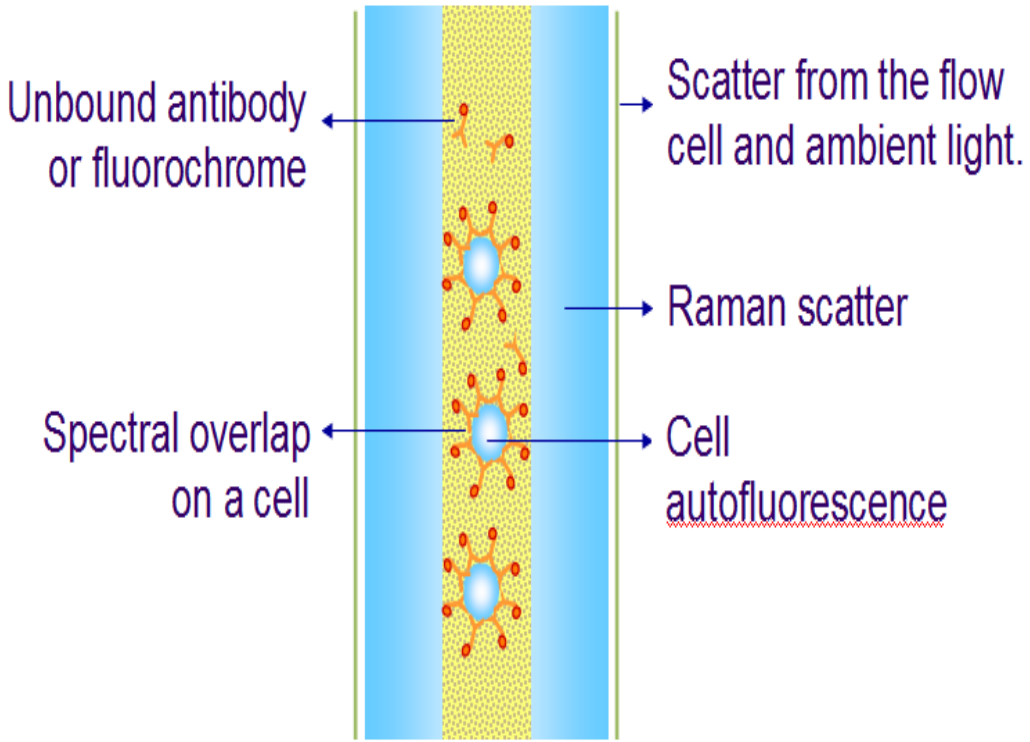


Bruce Bagwell, Verity Software

tSNE
Cen-se’
…
…

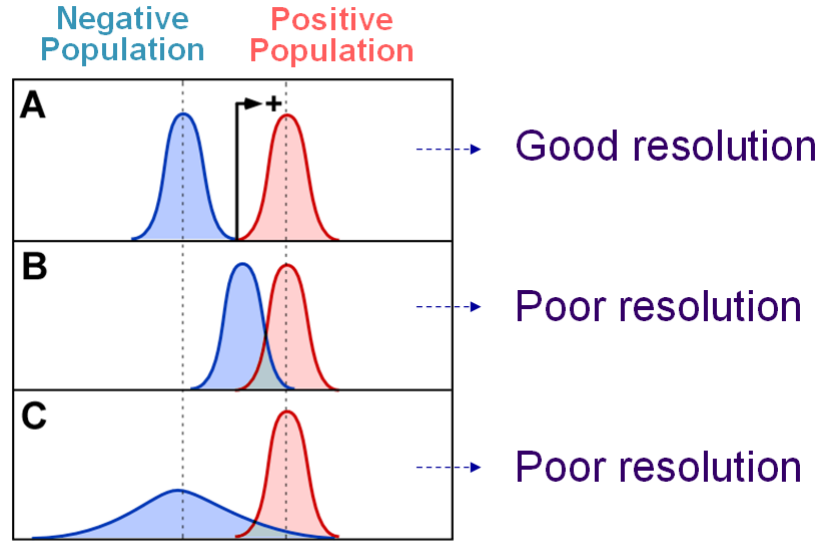
Instrument Evaluation Br, Qr

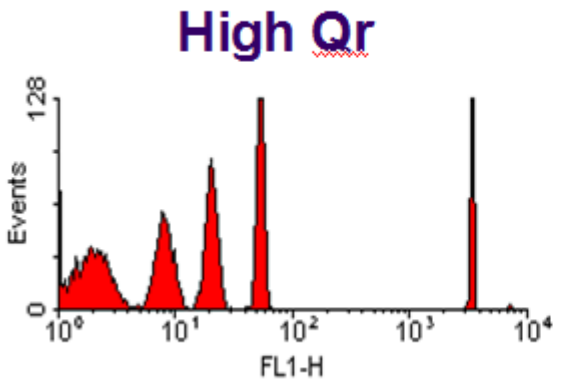
Br is a measure of the photon background in the detector

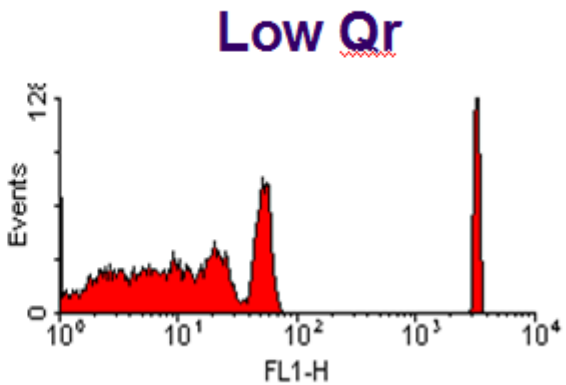


Qr is a systems efficiency for photon detection

$$Qr = \frac{\#\ \text{photoelectrons}}{\#\ \text{fluorescence molecules}}$$



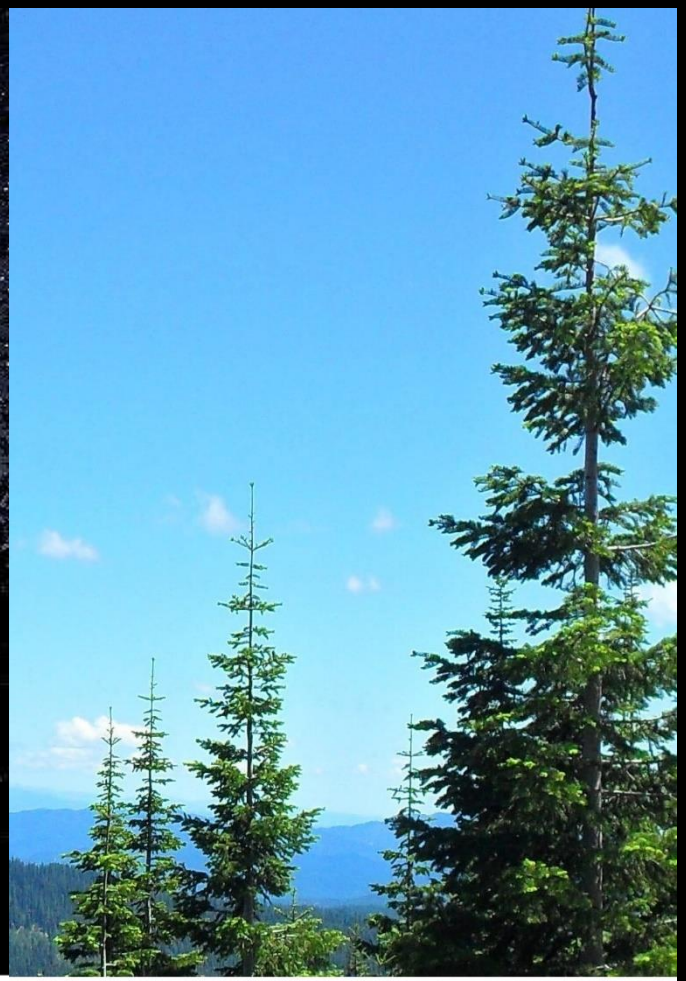




BD Biosciences

Br photon background

https://pbs.twimg.com/media/EWHoc2gXkAAUGlh.jpg



https://www.pikrepo.com/fsizb/green-pine-trees-under-blue-sky-during-daytime

Light background

Spectral overlap

Electronic noise

Photon shot noise

…

Unbound dye

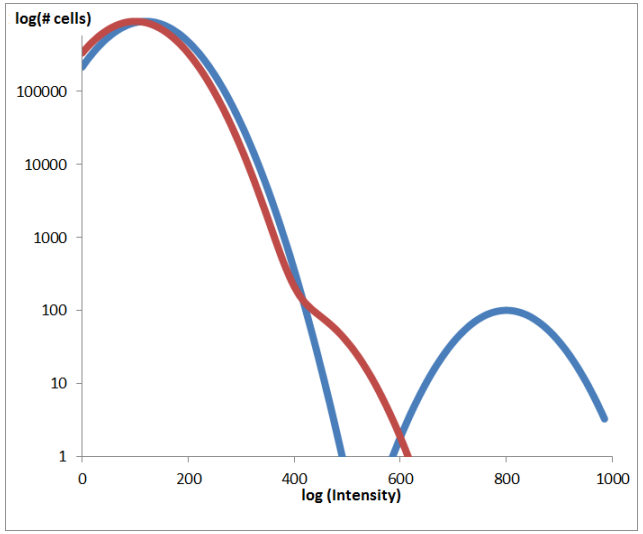
Non-specific binding

Label Selection

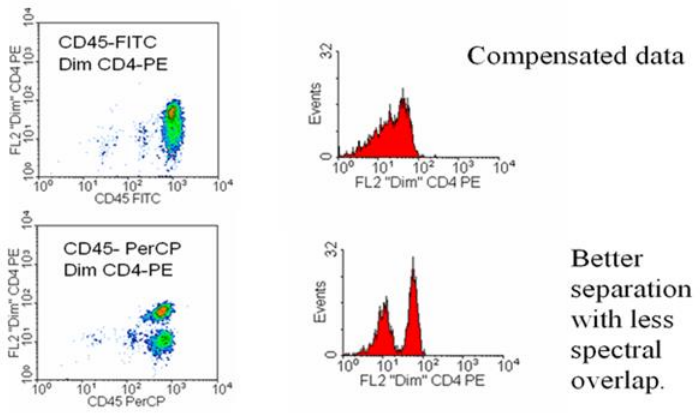
- Detection System
- Brightness
- Spectral Overlap
- Application (surface vs. internal)

Reagent performance

$$\text{Stain index} = \frac{Medium_{pos} - Medium_{neg}}{2 * SD_{neg}}$$



Brightness and Separation



Spectral Overlap and Separation

More info: Maecker HT et al. (2004) Cytometry 62A:169-173

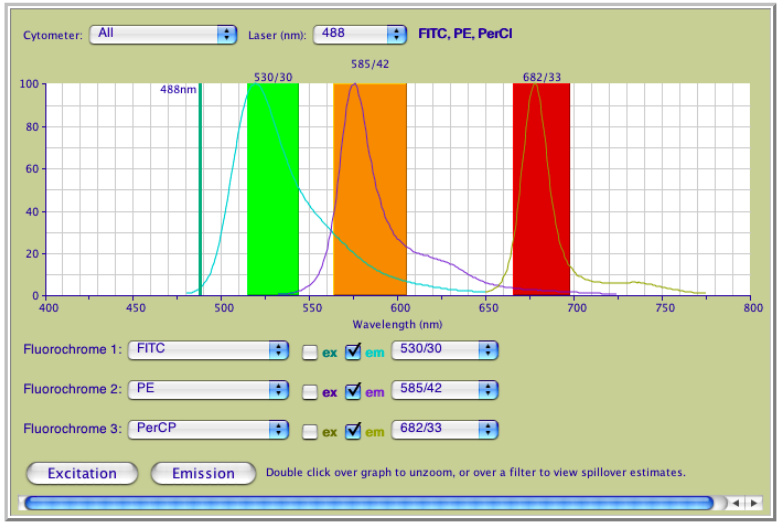
Spectral Overlap and “Compensation”

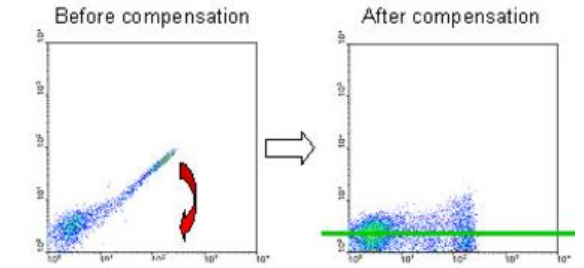
Calculation of concentrations
from optical/mass intensities

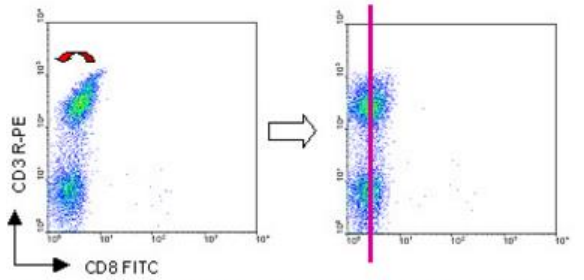
$$I_1 = a_{11} * c_1 + a_{12} * c_2 + a_{13} * c_3$$
$$I_2 = a_{21} * c_1 + a_{22} * c_2 + a_{23} * c_3$$
$$I_3 = a_{31} * c_1 + a_{32} * c_2 + a_{33} * c_3$$

a_{ik} : “compensation” matrix numbers
I_i : measured intensities
c_k : label concentrations

Solve n equations with n unknowns
(in spectral cytometry more complex
calculations are performed.)





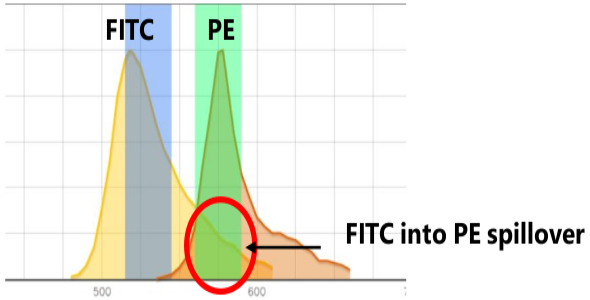


regmed.musc.edu/flowcytometry/Compensation.html

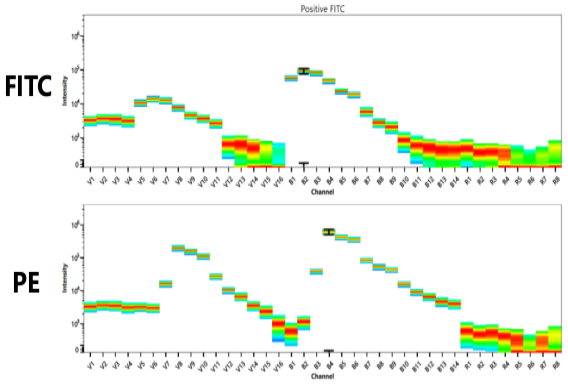
GUIDELINES Compensation, pages 1484-88

Measurement data transformation (dye-specific vs. full spectrum)

To obtain meaningful data for the researcher the multiple light intensities are converted to fluorophore masses per particle.

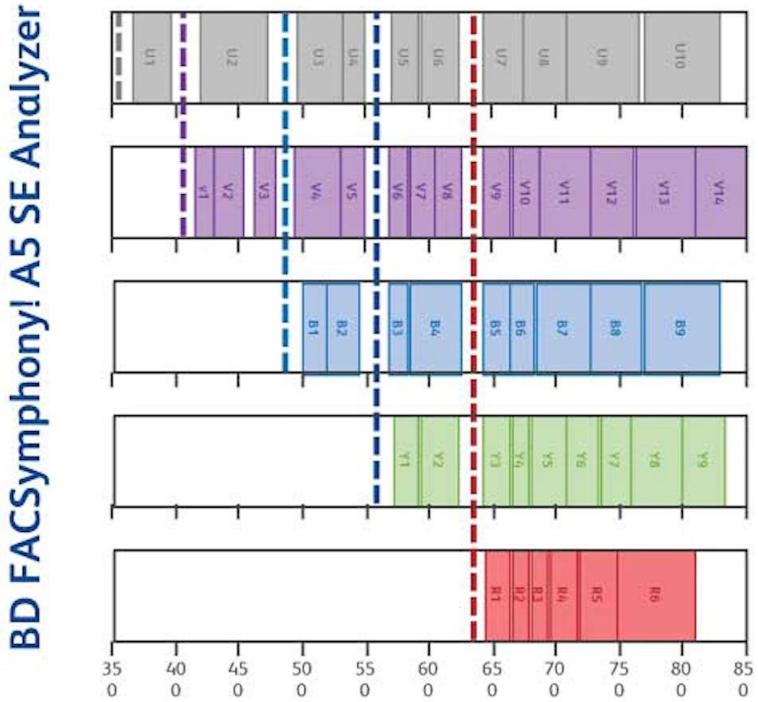


- Each fluorochrome is detected in ONE channel
- Detector # = Fluor #
- Single stained controls establish spillover
- Compensation mathematically subtracts the amount of light contribution from non-primary colors into the primary detector
- A compensation matrix is calculated: n x n (square matrix)



- Each fluorochrome is detected in ALL channels
- Detector # ≥ Fluor #
- Single stained controls establish reference signature
- Unmixing determines which combination of reference controls **best fits** the signature of the multicolor sample
- An unmixing matrix is calculated: n x channel number

“Spectral” Flow Cytometry



BD Biosciences website

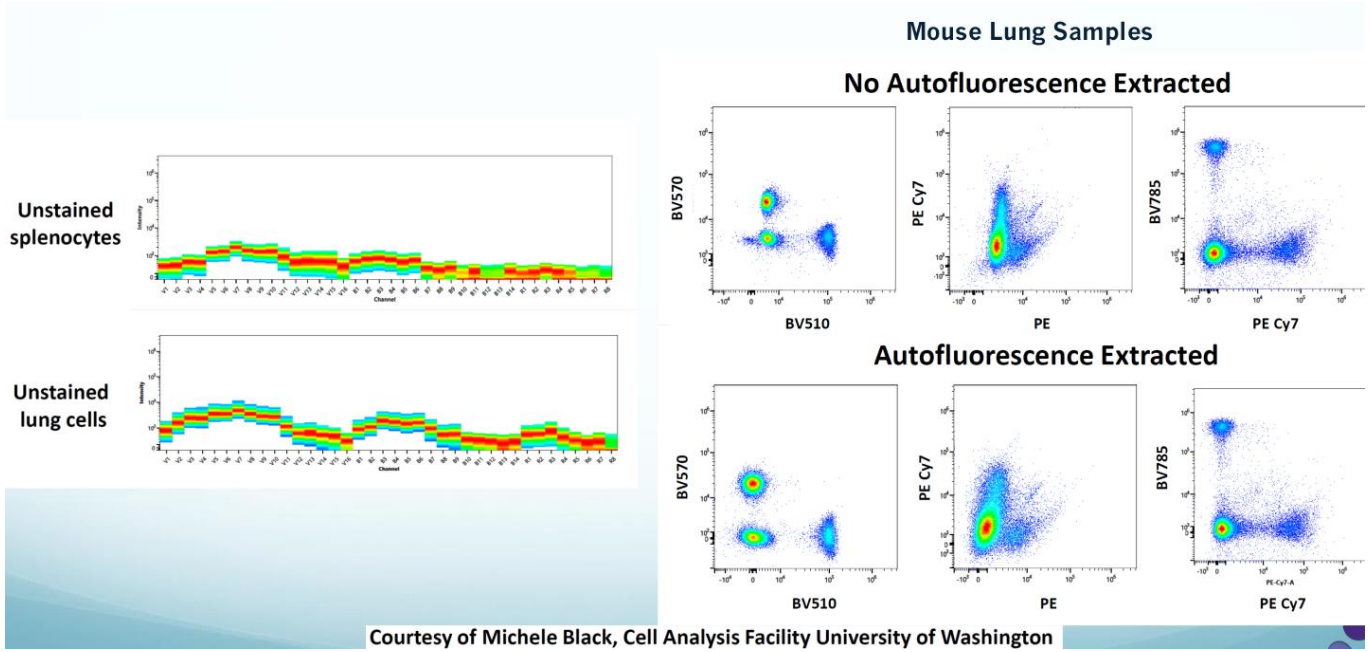
Table 3. Comparison of background (B), Q value, and detection limit (DL) of standard filter setting and multispectral filter setting for QSC microspheres stained with CD4 FITC or CD4 PE

PARAMETER	530/30	585/40	MULTISPECTRAL
Detection wavelength (nm)	515–545	565–605	505–810
Q_{FITC} (phe$^-$/ABC)	0.004	–	0.04
Q_{PE} (phe$^-$/ABC)	–	0.02	0.14
B (phe$^-$)	9	32	63
DL_{FITC} (ABC)	320	–	59
DL_{PE} (ABC)	–	875	231

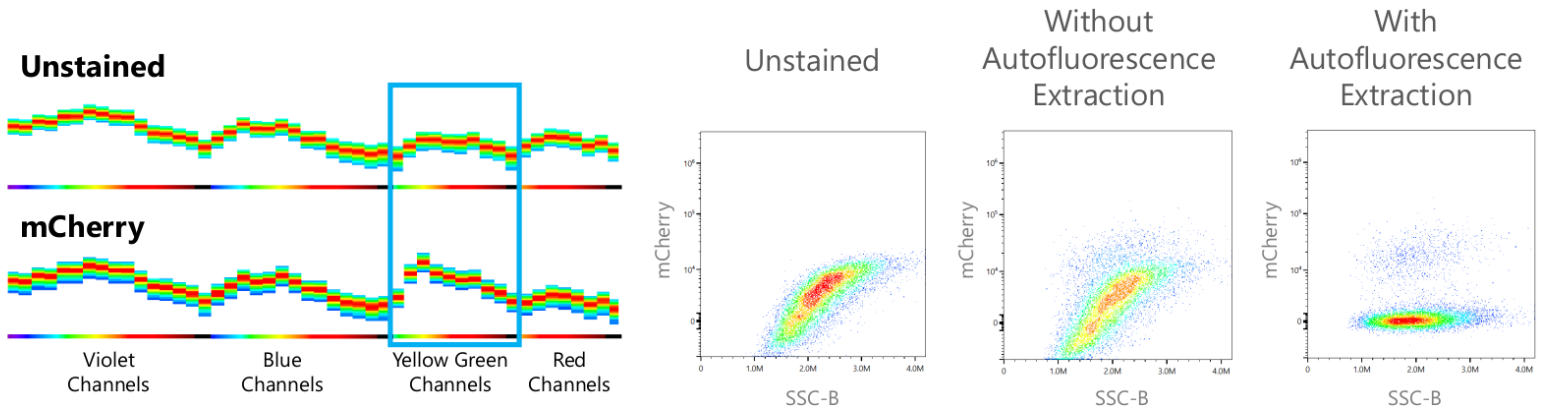
FeherK2016 DOI: 10.1002/cyto.a.22888

Full spectrum analysis generally collects more photons and as a result a lower limit of detection for fluorescence is achieved.

Autofluorescence

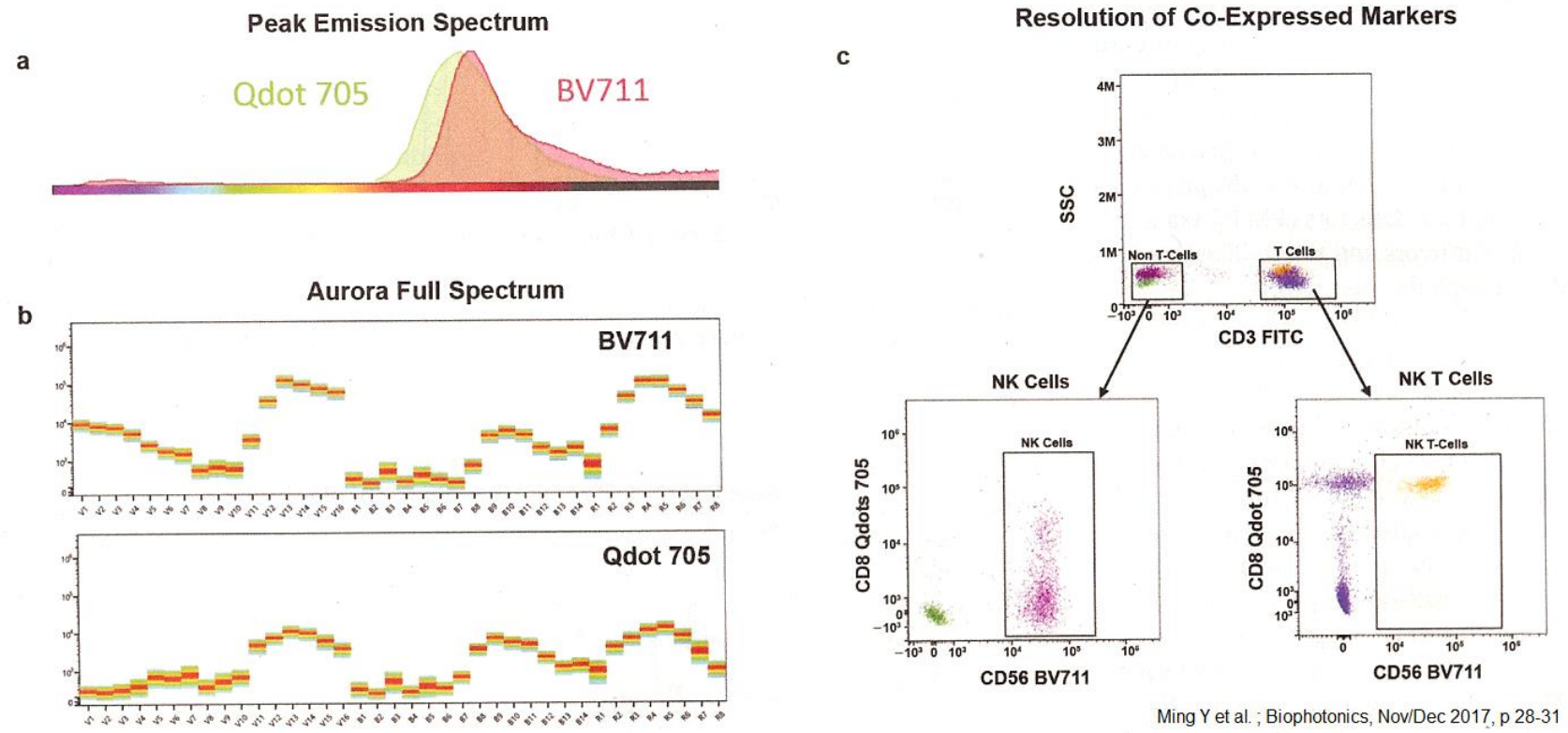


HeLa human cells were transformed with a CRISPR-Cas9 target vector carrying an mCherry reporter



Malte Paulsen, Flow Cytometry and Cell Sorting Facility, EMBL

Resolving Spectrally Similar Dyes



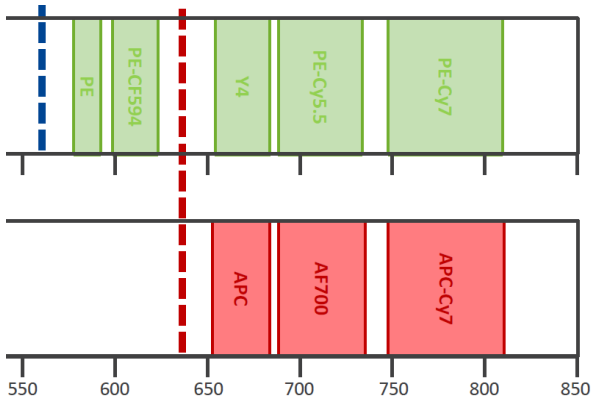
Ming Y et al. ; Biophotonics, Nov/Dec 2017, p 28-31

Figure 4. The peak emission spectra of the dyes Qdot 705 and BV711 highly overlap and cannot be used together on a conventional flow cytometer **(a)**. However, these two dyes have distinct signatures, and because of this, they can be used in combination with full-spectrum cytometry **(b)**. This means these dyes can be used in combination to identify cell populations of interest such as T cells and non-T cells that co-express CD8 and CD56 **(c)**. The new technology can fully resolve cells that express one or both markers at different levels.

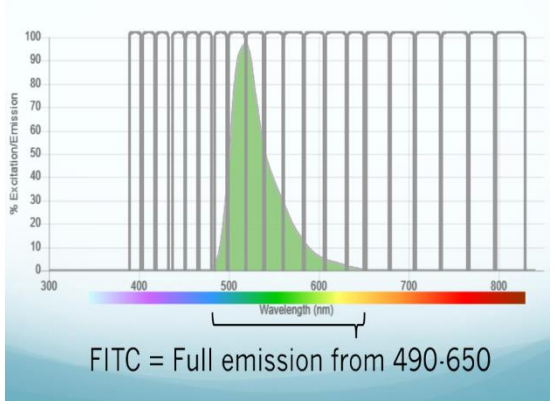
Ming Yan 2017

Approaches for Multi-parameter Single Cell Analysis

- NA barcodes as labels for sequencing
  - aqueous droplets in oil
  - multiwell plates
- High speed flow stream
  - multiparameter MS
  - conventional dye specific fluorescence
  - full spectrum fluorescence

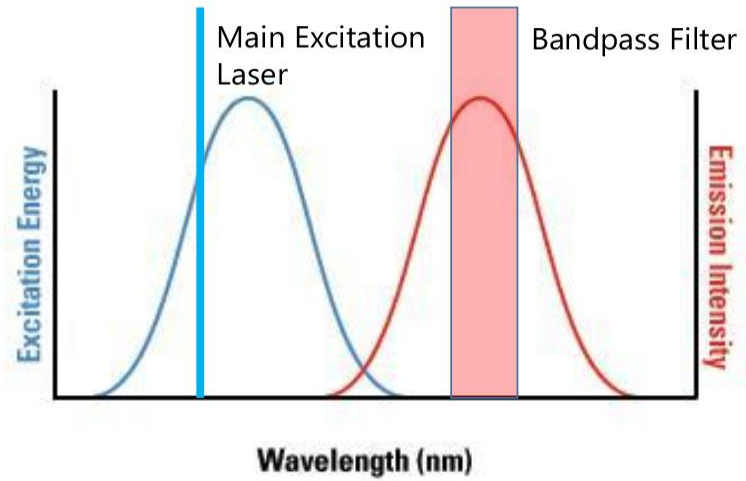


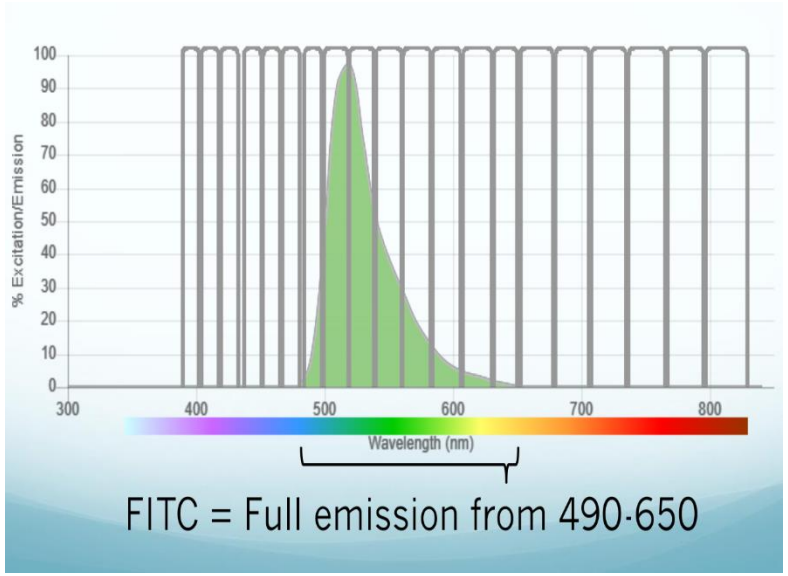
dye specific



full spectrum

Full Spectrum Optics





Unique Optical Design

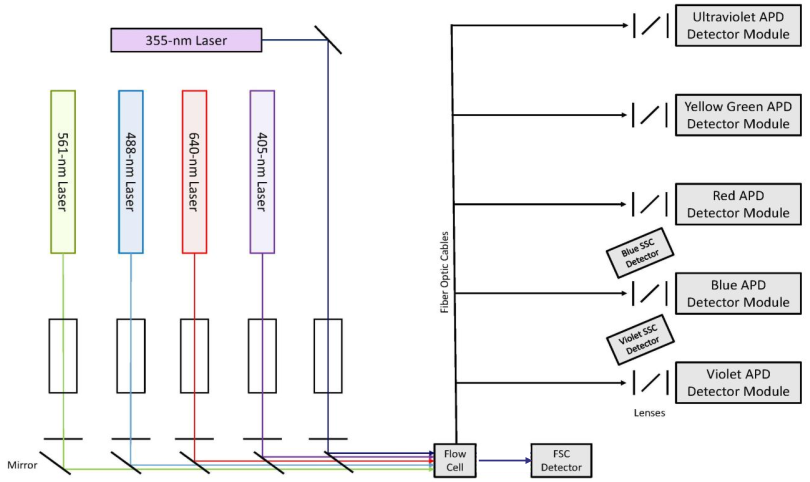
- High Sensitivity Collection Optics
- Lasers are spatially separated
- Dedicated detector array

Full Spectrum Analysis

- Spectral signature created via capture of the entire emission spectrum

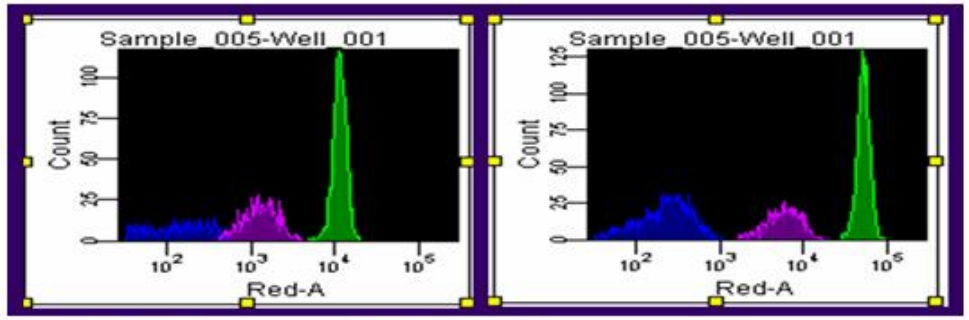
Spectral Unmixing

- Calculates the contribution of each known fluorophore's spectra to the total

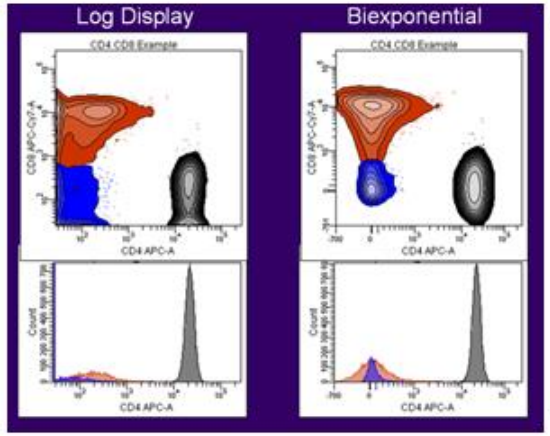


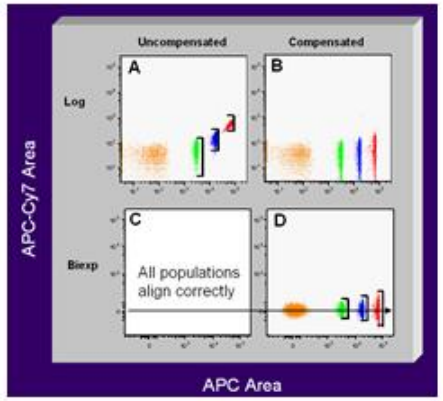
Optimizing cytometry measurements

- Gain (PMT, CMOS, CCD) settings

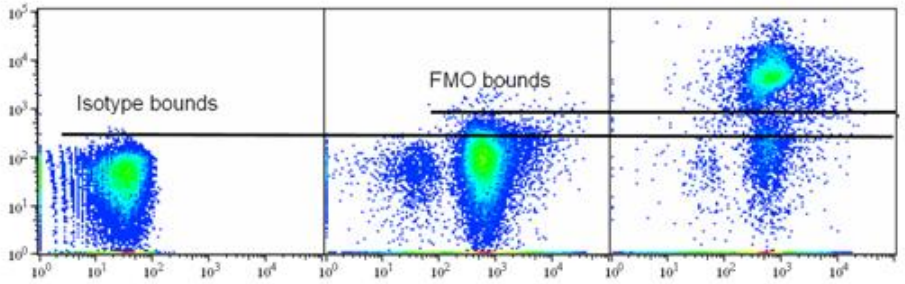


- Data Display





- Controls



J. Trotter, BD Biosciences

Multi-marker Cell Analysis
Points To Consider

- Know your instrument status e.g. Qr & Br for different channels
- Use optimal detector settings e.g. high gain to maximize sensitivity (check to avoid off-scale events)
- An poor separation conditions for a single marker analysis will be even worse for a multi-marker measurement
- Use high sensitivity labels for low expression markers
- High sensitivity does not help against non-specific binding
- For energy transfer fluorophors beware of spectral drifts by photo-degradation
- Internal controls are essential
- Be aware of counting statistics limitations for low count populations

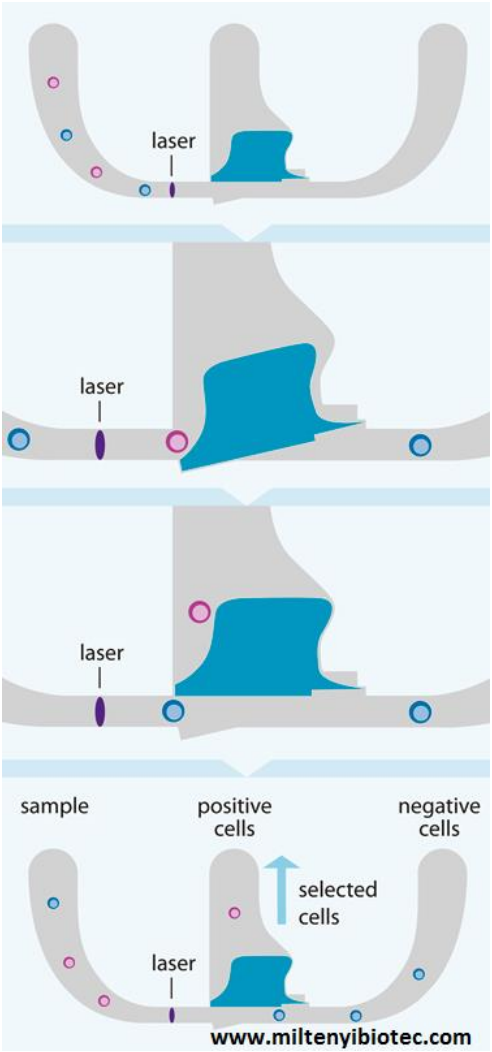
Cell Sorting Technologies

- Classical droplet sorters (FACS™)
- Single Cell dispensers
- Tyto/OWL
- DEP sorter
- …

- BulkSorting

(Magnetic, Gravity, Acoustic, …)

DEPArray™ System

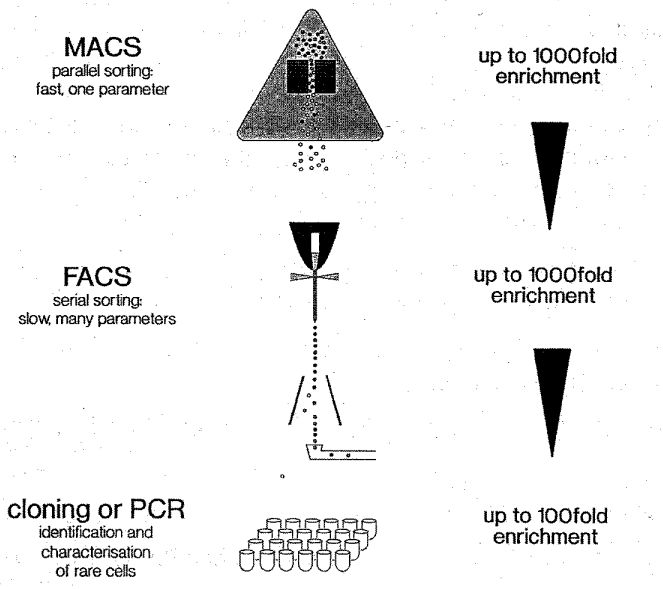


MACSQuant®Tyto™

Rare Cell Analysis

Examples CD34, AC133, antigen specific cells, CTCs

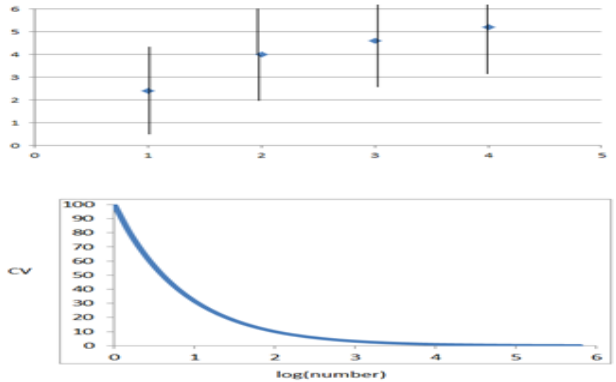
- Poisson count statistics
- Population Separation
- Subset pre-enrichment



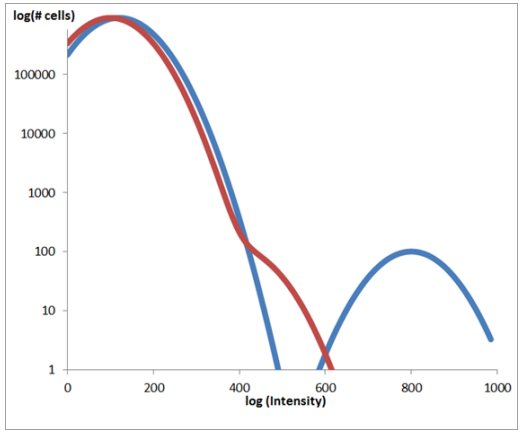
Pre-enrichment

Weichel W et al (1999)
Springer Lab Manual

	Sample 1	Sample 2	Sample 3	Sample 4
	6	2	6	8
	3	7	1	6
	1	3	5	3
	1	4	5	6
	1	4	6	3
Mean	2.4	4	4.6	5.2
St.Dev	2.2	1.9	2.1	2.2
		Overall	Mean	4.1
			St.Dev	2.2



Ignoring Counting Statistics Can Lead to Erroneous Conclusions



Population Separation

Limit of Detection

Routine >0.2%

Optimized instrument >0.01%

Optimized system $>10^{-7}$

GUIDELINES Rare Cells: General Rules, pages 1523 – 26, 1846-7

Conclusions / Caveats

- For optimal results use an adequate technology

  (flow cytometry has enormous capabilities, but is not always the adequate technology to use e.g. single cell kinetics)

- Understand the limitations of the system

  (complexity, limits of detection, non-specific binding of reagents, …)

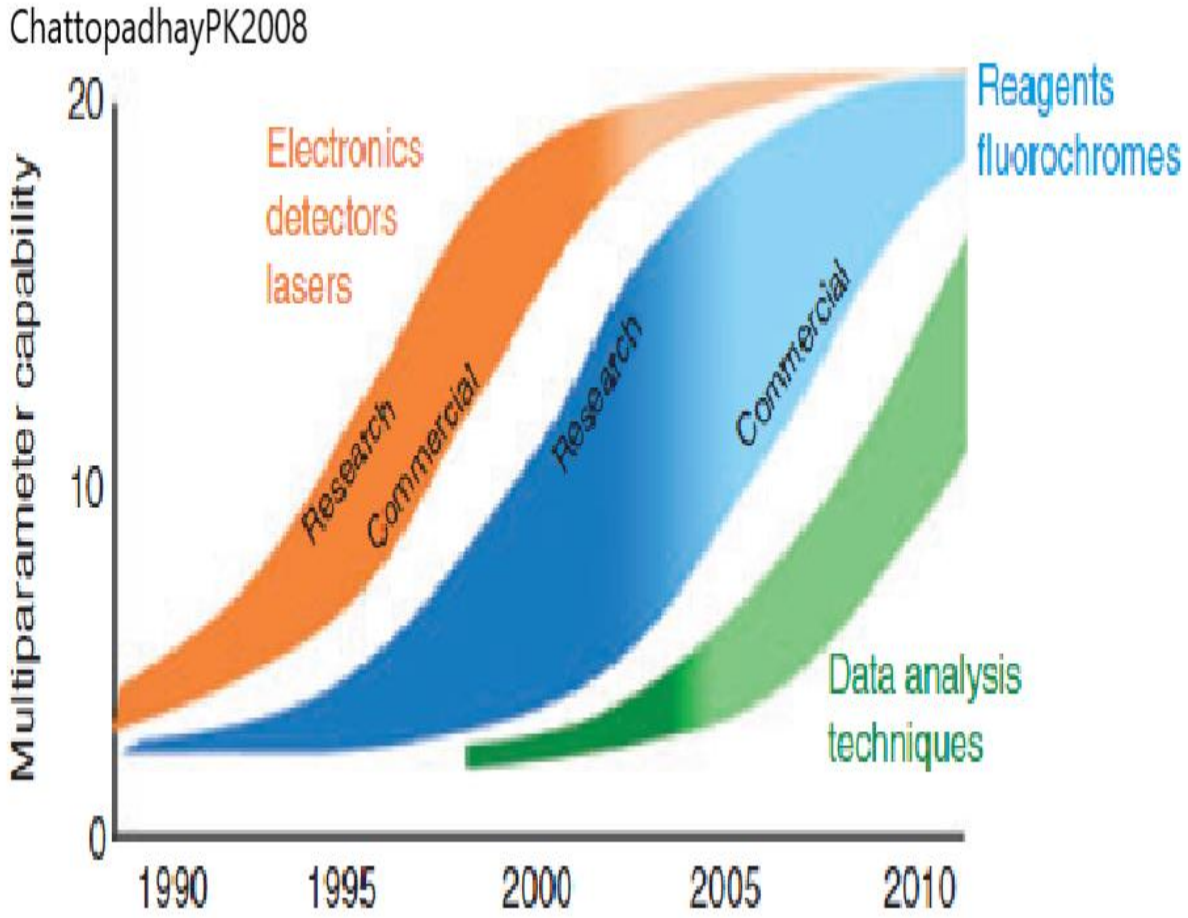
- Use appropriate statistical methods

  (understand variance of very low counts during rare cell analysis)

- Validate results with appropriate controls

Emerging Technologies

Technology Development History



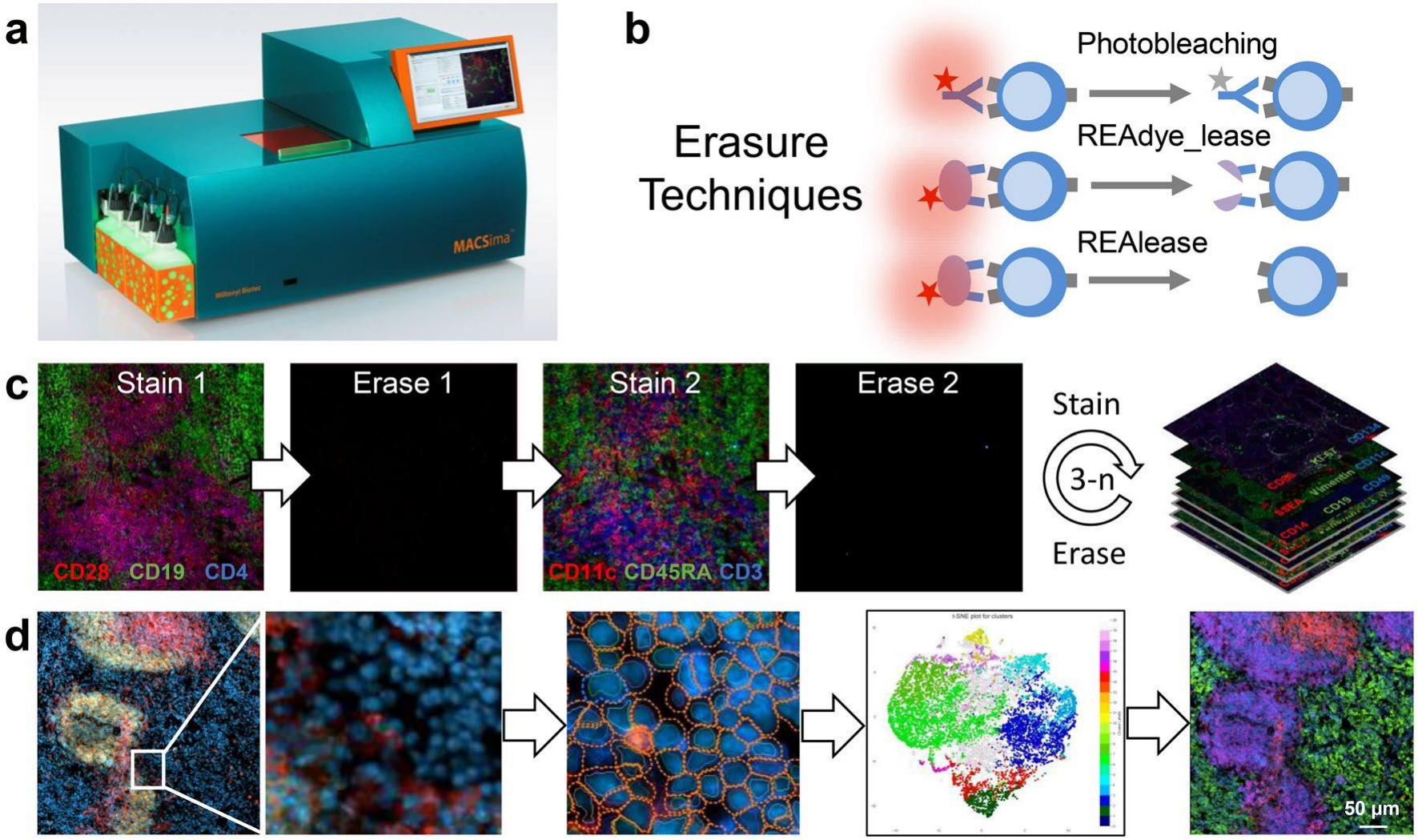
Today, March 2023:

Instrumentation >100

Fluorochrome >40
NA barcoding >100

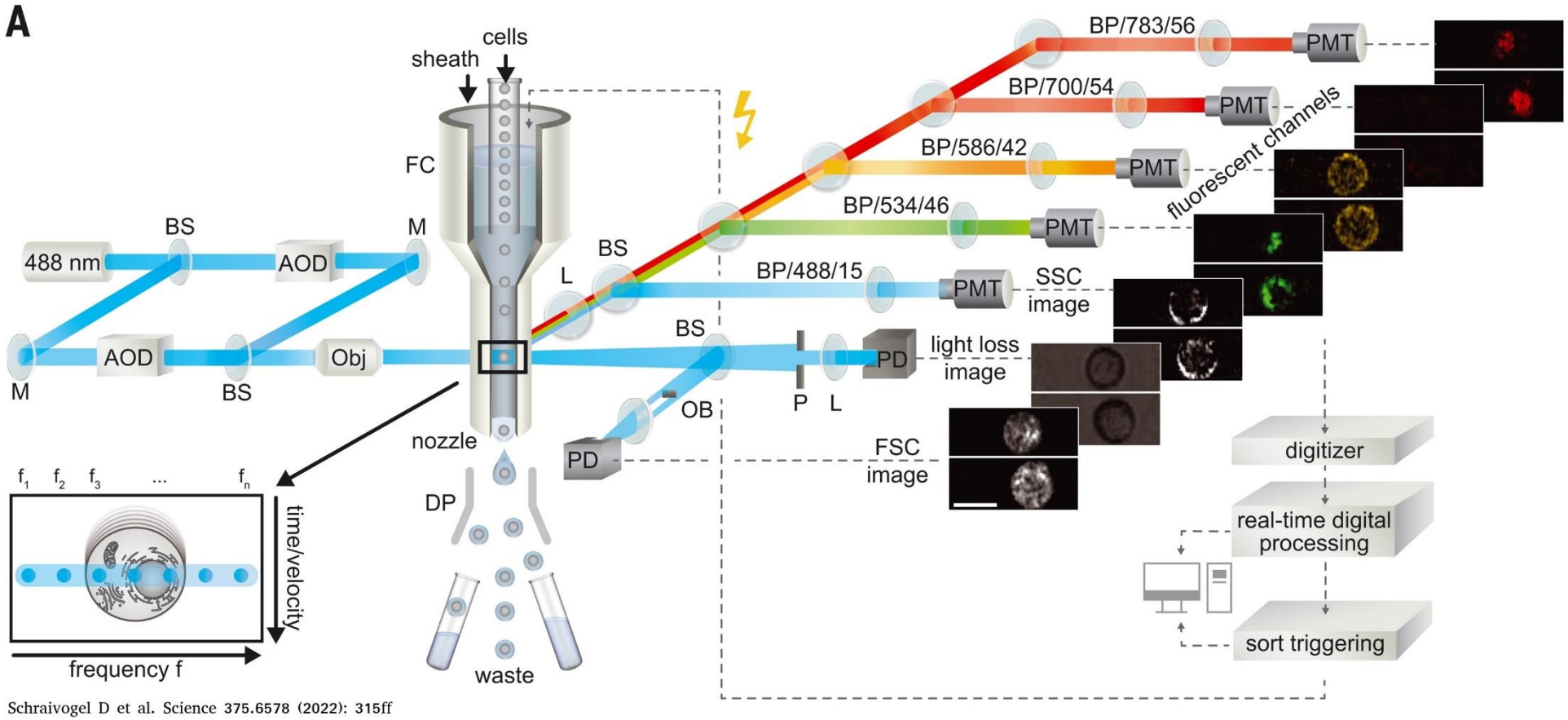
Data analysis >100

Cyclic Staining Fluorescence Microscopy



Scientific Reports | (2022) 12:1911 | https://doi.org/10.1038/s41598-022-05841-4

High speed imaging flow cytometry with droplet sorting capability



Schraivogel D et al. Science 375.6578 (2022): 315ff

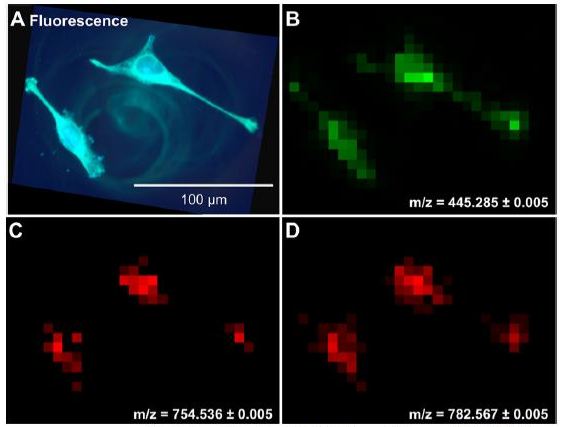
Technology originated at UCLA in Prof. Bahram Jalali's group.
An example of successful physical and life sciences collaboration.

Eric D. Diebold, BrandonW. Buckley, Daniel R. Gossett and Bahram Jalali, Nature Photonics, (2013)

Examples of New Detection Technologies

MALDI imaging

(high parameter in-vitro imaging using mass spectrometry)



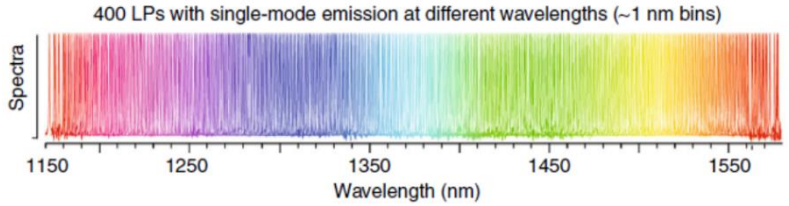
Schober Y et al. (2012) Anal.Chem. 84, 6293ff

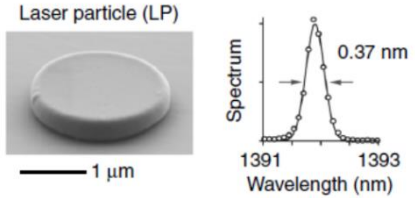
Label-free imaging with Raman

(measuring cellular components by their Raman spectra)

Microlasers for high parameter cytometry

(ultra-narrow bands of light emission)





Laser Particles as Labels for Cell Analysis
Kwok S.J.J. et al (2019)

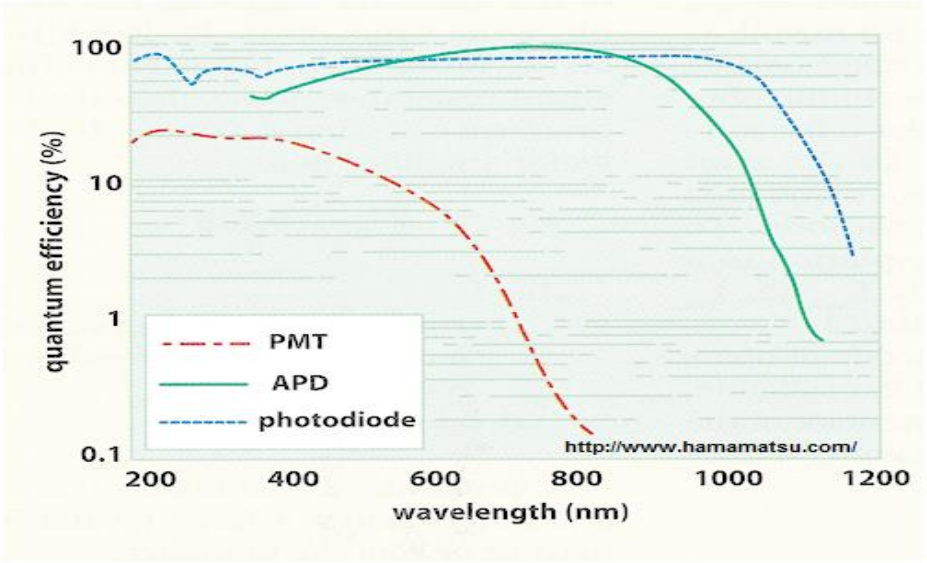
https://doi.org/10.1038/s41377-019-0183-5

Label-free medium resolution NMR imaging

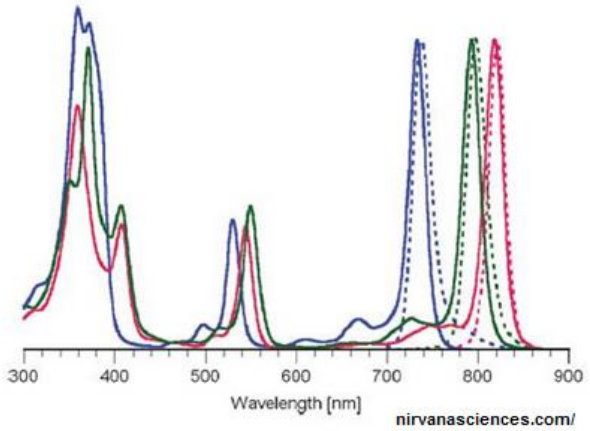
(chemical environment sensing)

New Detector-Label Combinations

- New photodetectors extend the available spectrum
  (Si avalanche photodiodes extend detection into the far infrared)

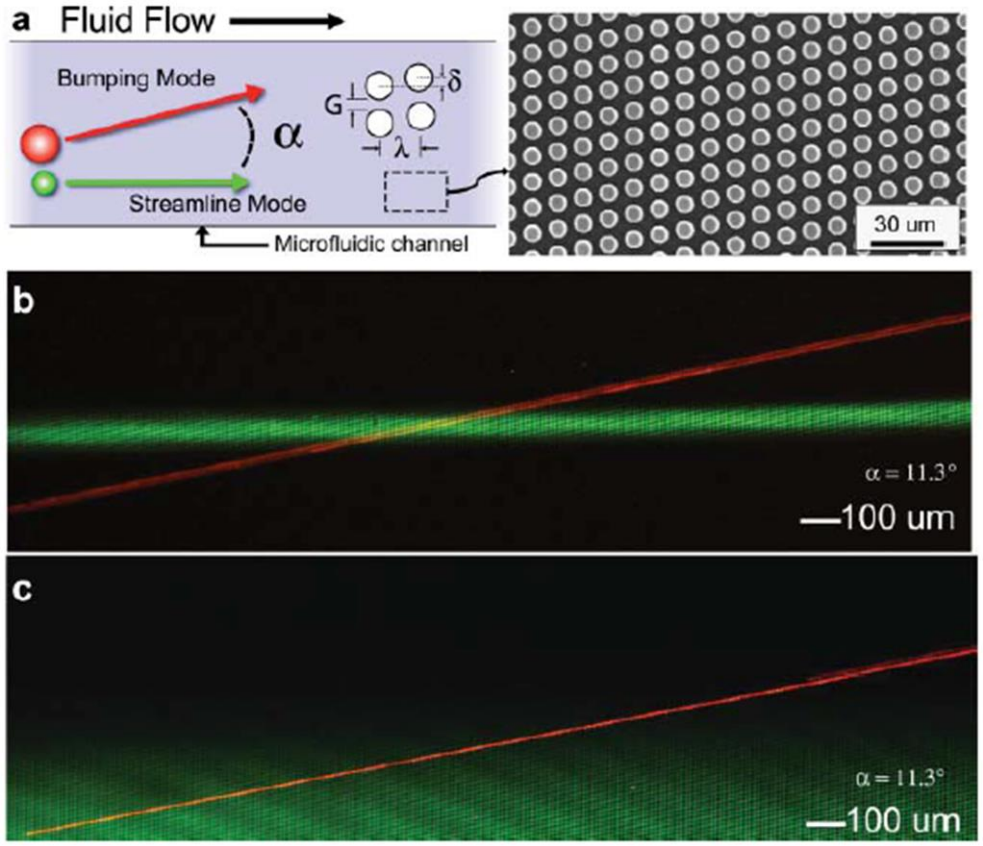


- New dyes add excitation in the UV, some detection in the IR
  (Fluorescent polymers, bacteriochlorins, …)



Automatable Sample Preparation

Microfluidic system for leukocyte isolation and automated staining and cell washing (deterministic lateral displacement)

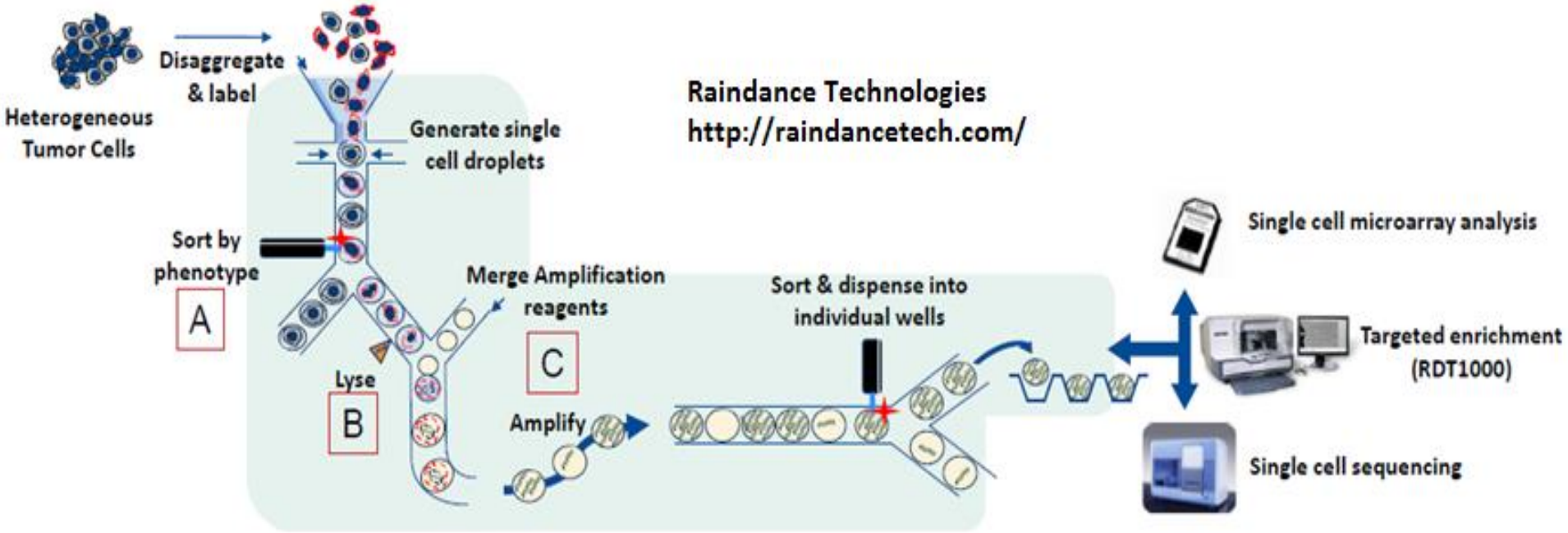


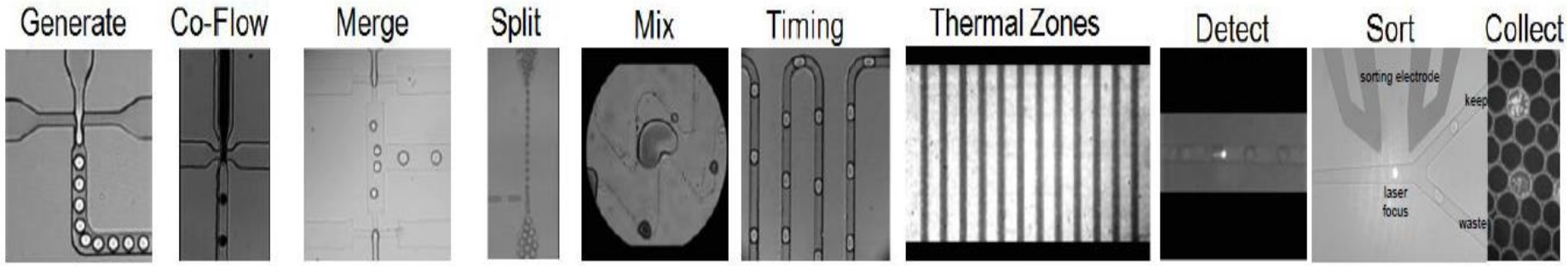
also:

- acoustic focusing
- microfluidic filters
- inertial flow
- magnetic nanoparticles
- high density particles
- dielectropheresis
- optical traps
- …

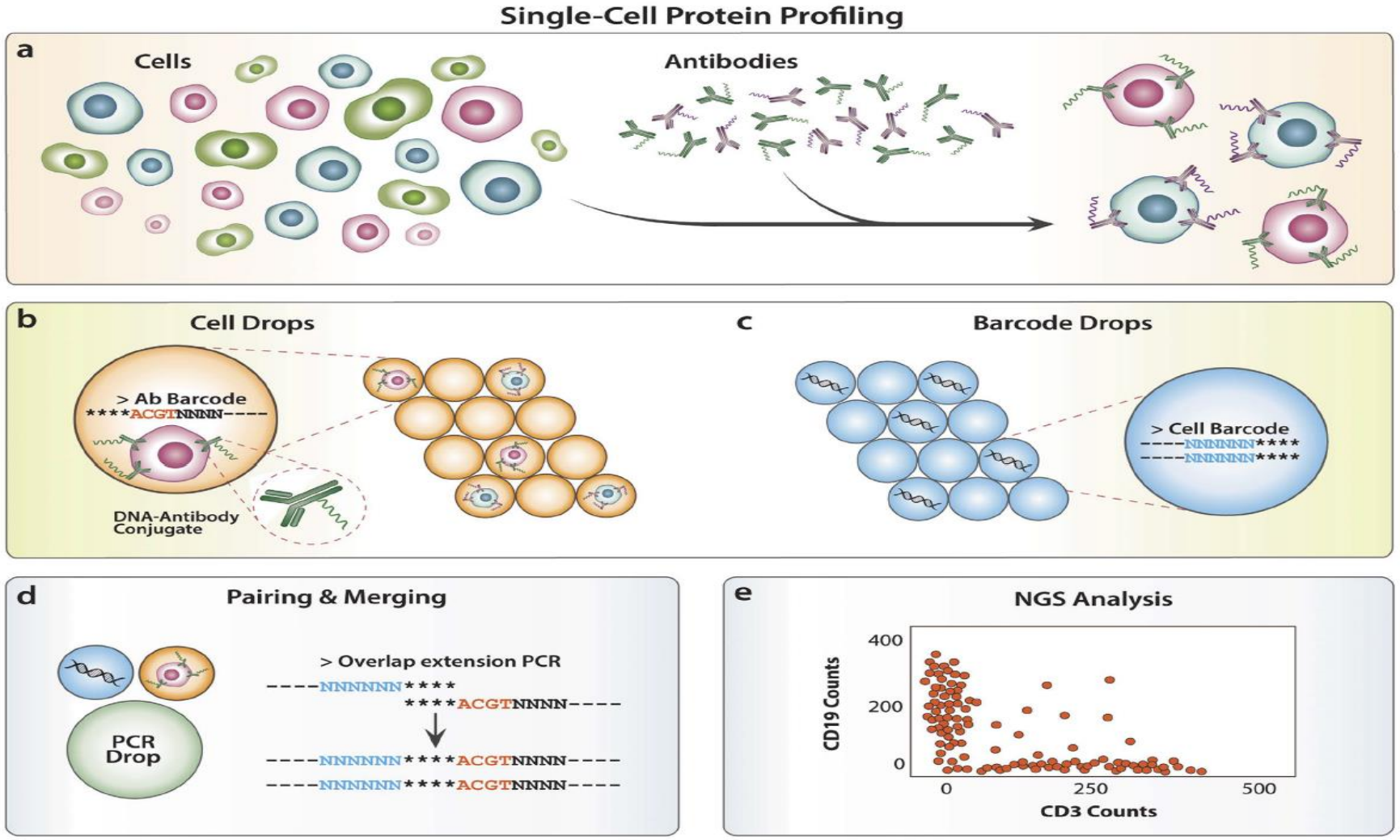
1. Davis JA et al (2006) PNAS 103: 14779ff
2. Morton KJ et al (2008) Lab on a Chip 8: 1448ff
3. Cyto 2012 poster, Liping Yu et al,
4. Sturm JC et al. (2014) Interface Focus 4: 1-9

Droplet-based Integrated Bio-Assay System Technology





Sequence Barcodes



Shahi P et al. (2017) Abseq; DOI: 10.1038/srep44447

Conceived to measure unlimited number of markers with single molecule sensitivity (PCR) and sequencing readout.

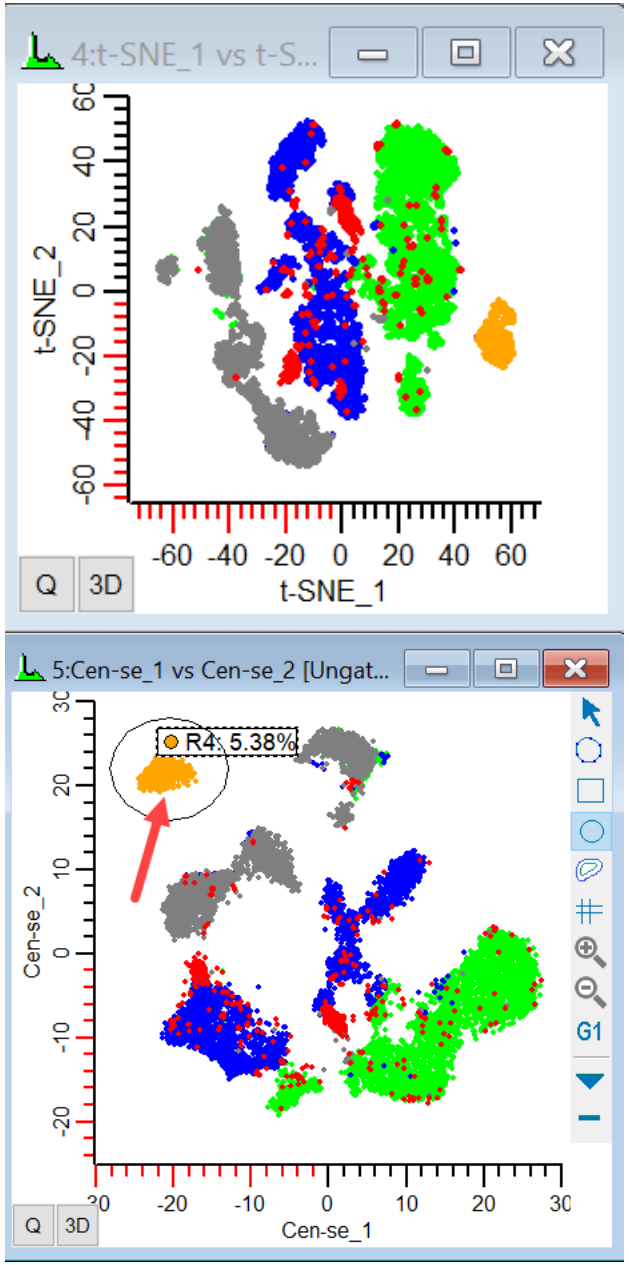
Automated Data Analysis

- Algorithms for fully automated analysis
- Artificial intelligence (AI)/ machine learning
- Even more advanced displays

Literature:

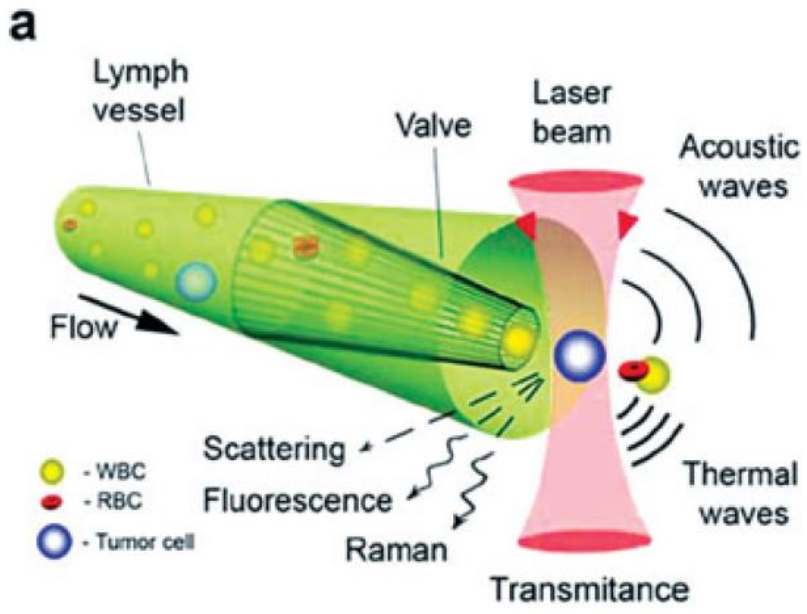
https://repository.lboro.ac.uk/articles/thesis/Defining_confidence_in_flow_cytometry_automated_data_analysis_software_platforms/20254551 (thesis with a lot of detail about automated data analysis)

doi: 10.3389/fimmu.2021.787574 (machine learning review 2022)

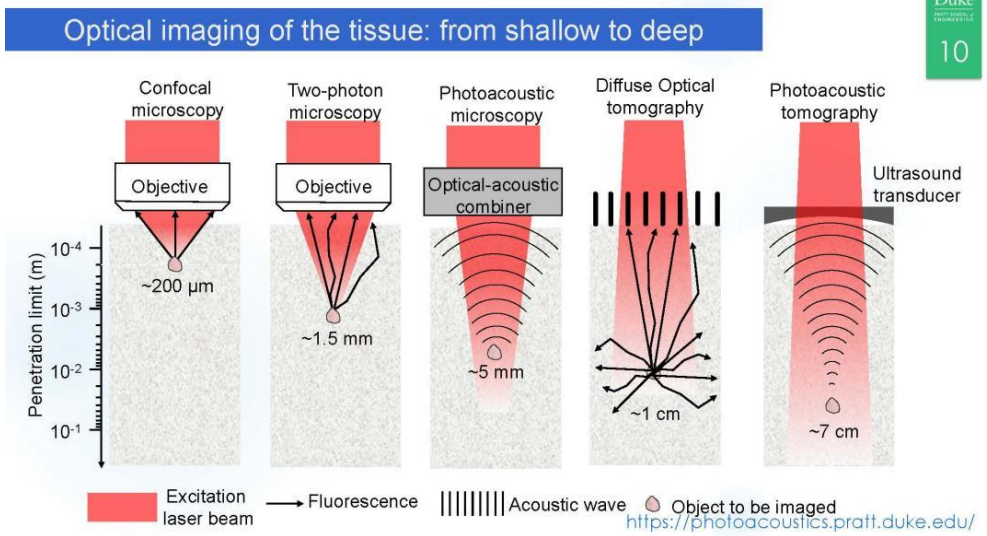


https://www.vsh.com/documentation/winlist/image/tsne_bcells.png

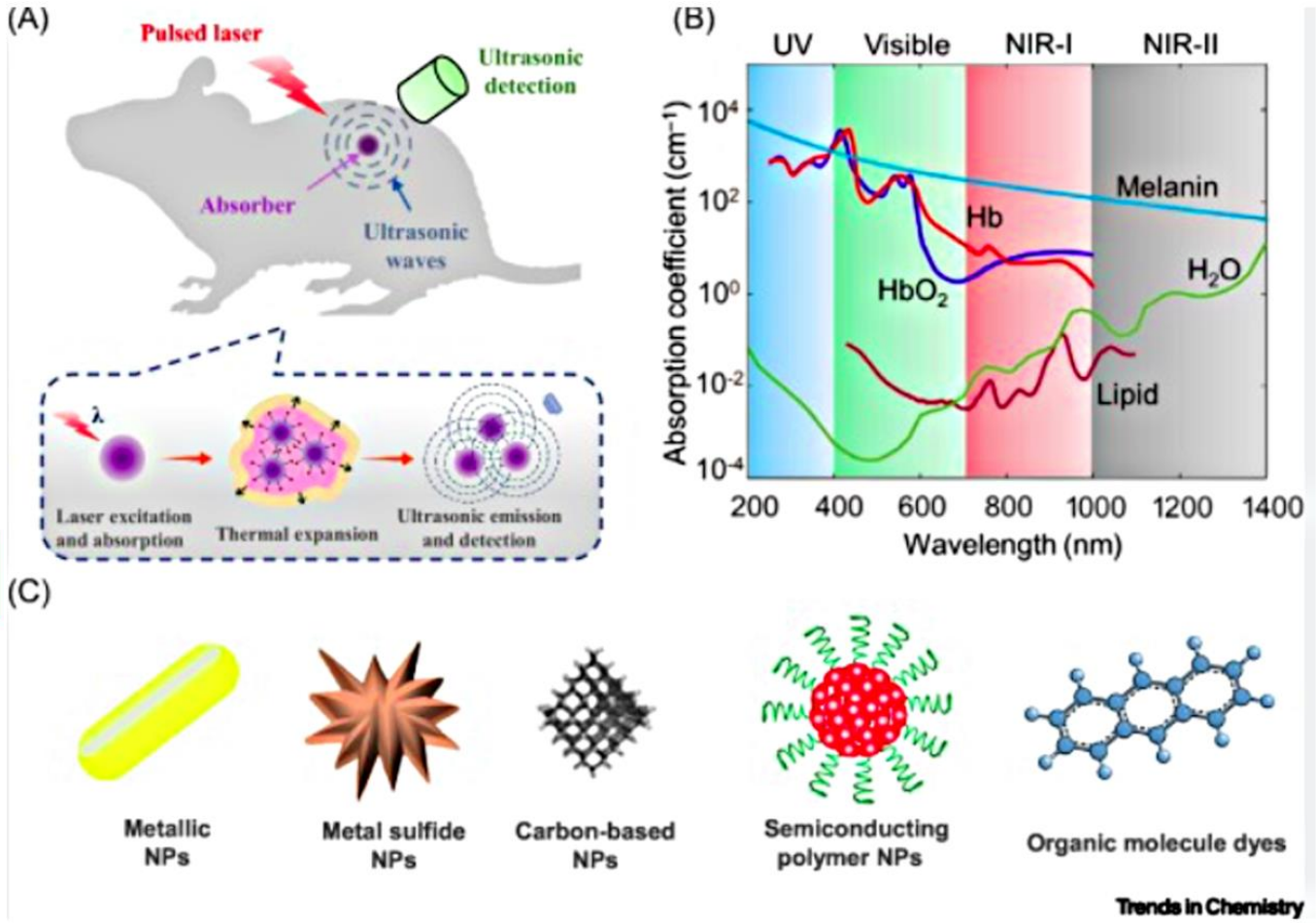
In-vivo Cytometry



VP Zharov Group, DOI: 10.1002/cyto.a.20587

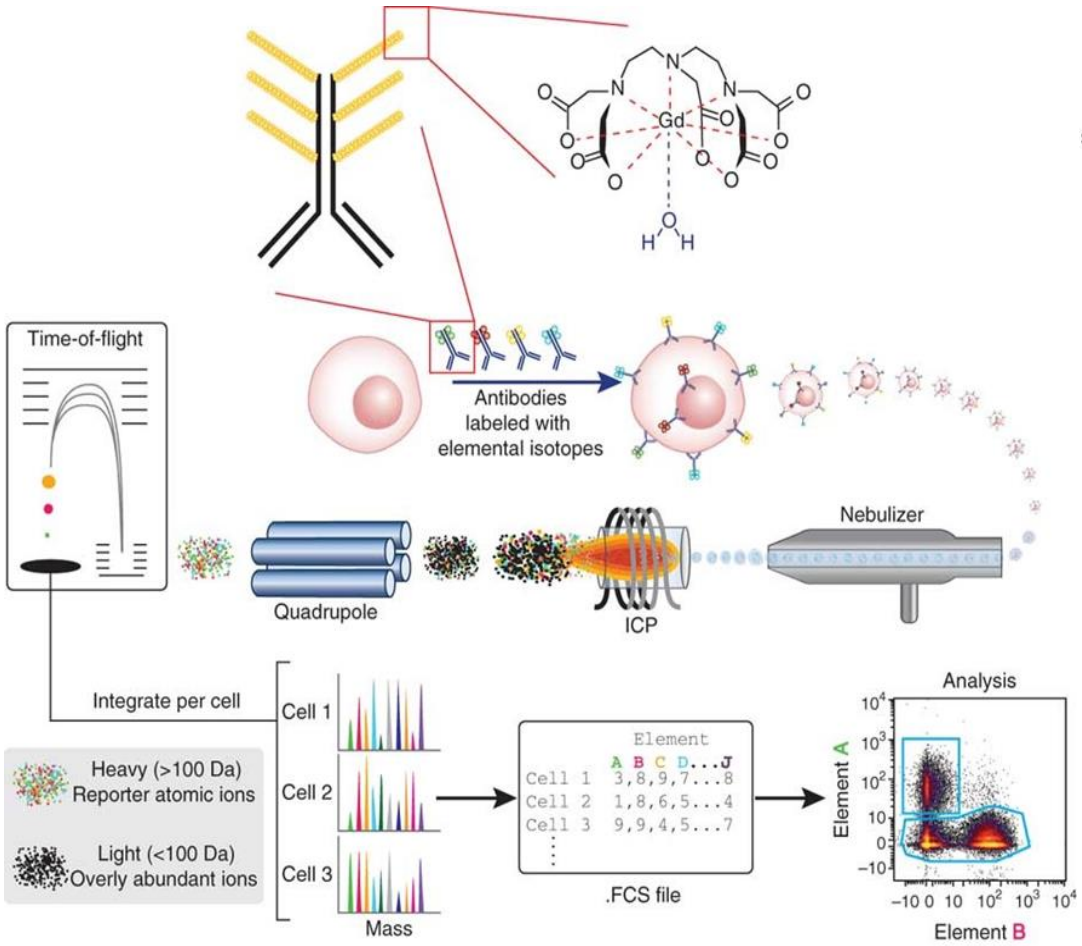


Duke 10

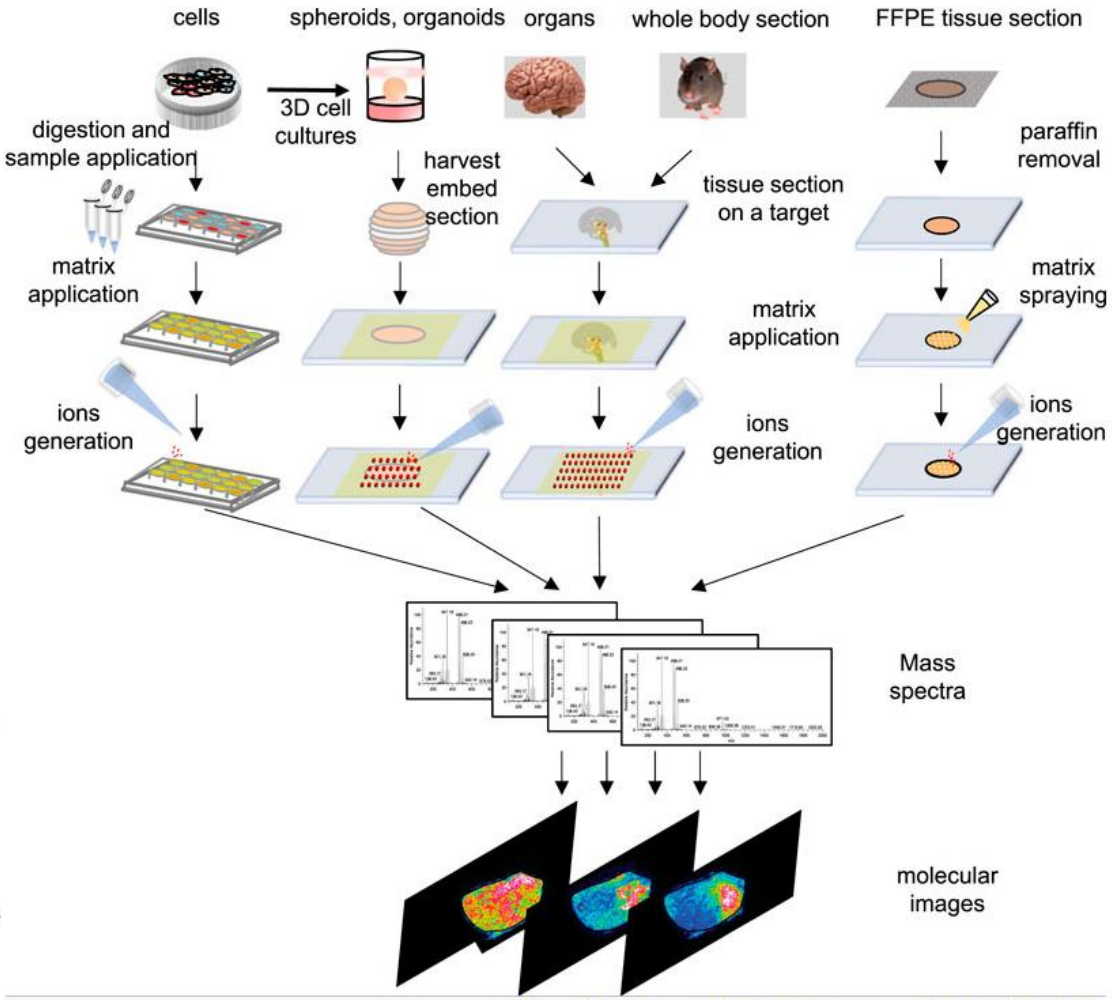


Zhang J et al. (2021) https://doi.org/10.1016/j.trechm.2021.01.002

Mass Cytometry

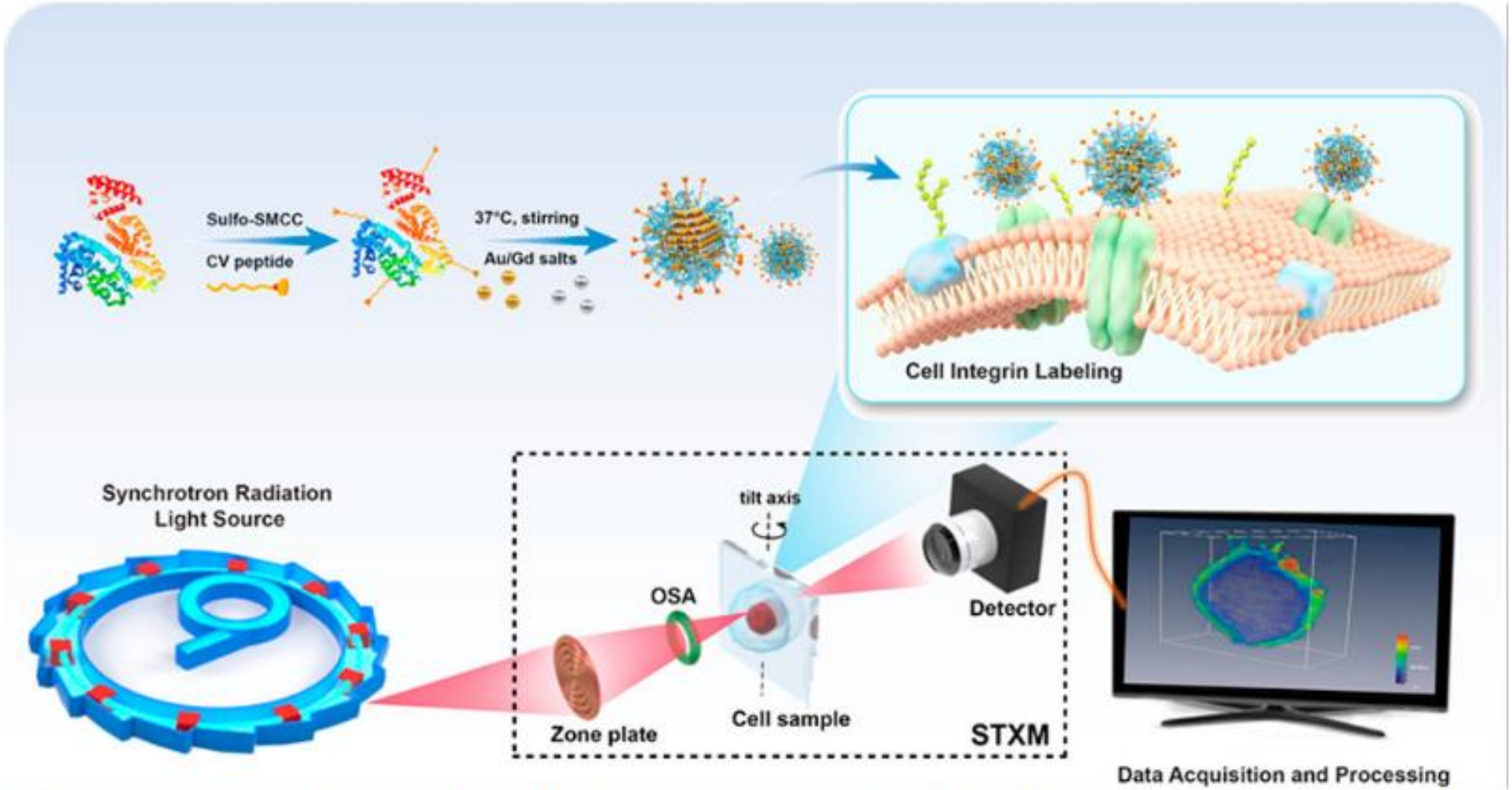


Sean C Bendall & Garry P Nolan (2012) Nature Biotechnology 30, 639–647



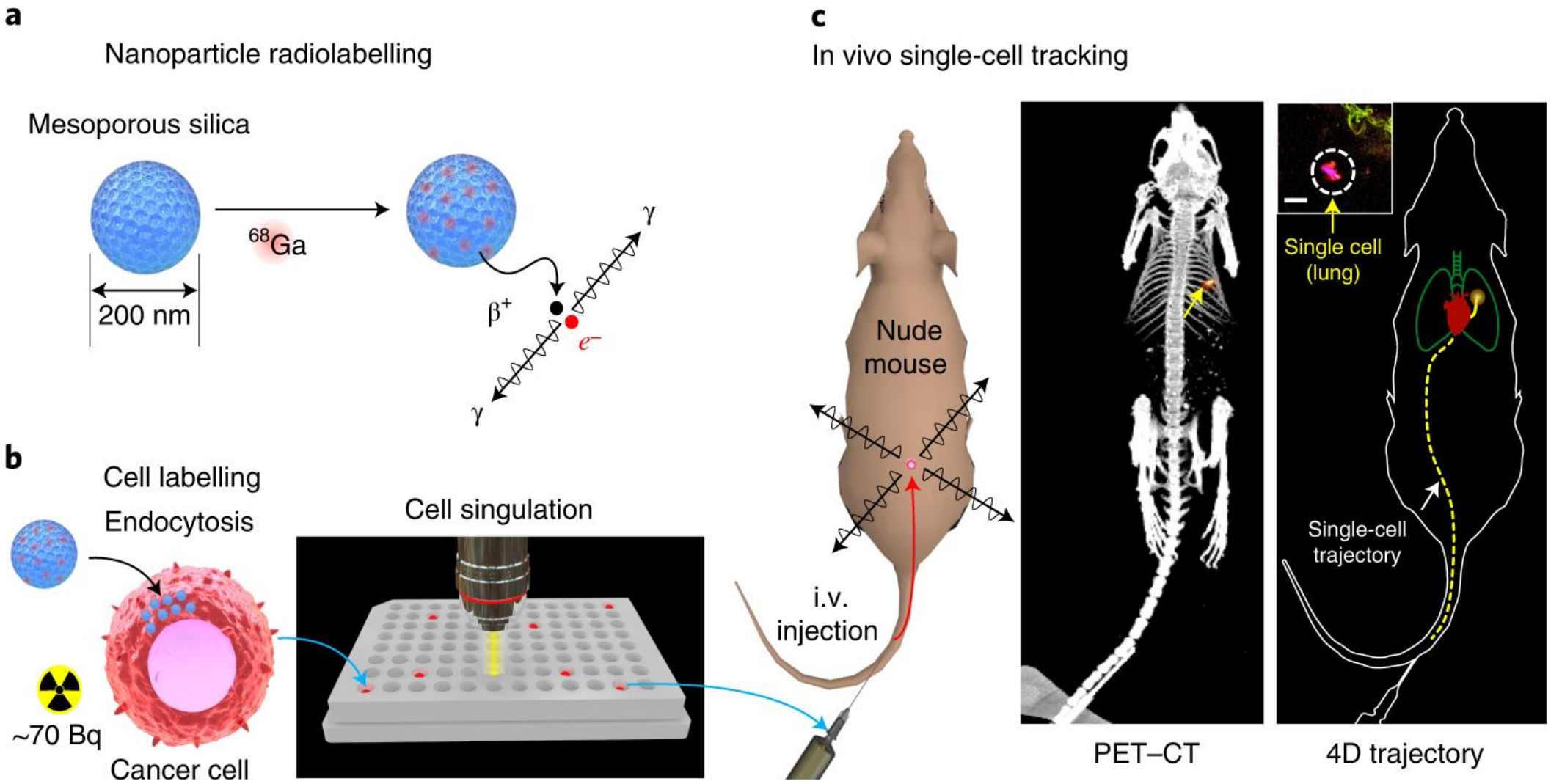
https://doi.org/10.3389/fchem.2021.782432

X-Ray Microscopy Tomography



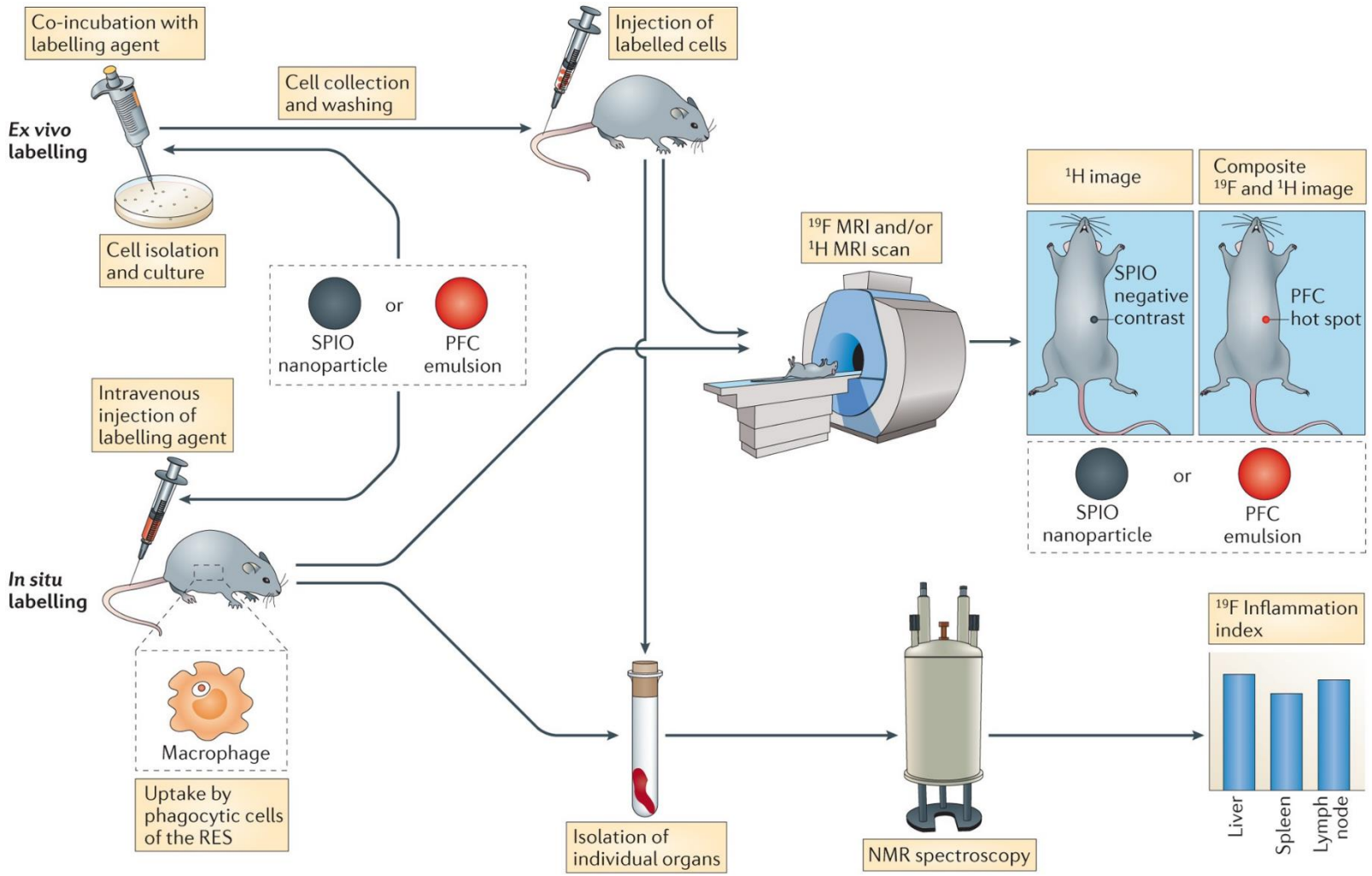
X-Ray microscopy tomography 2021 https://dx.doi.org/10.1021/acs.analchem.0c04662

Positron Emission Tomography



Nature Biomedical Engineering volume 4, pages835–844 (2020)

Single Cell Tracking with Magnetic Resonance Imaging



Nature Reviews Immunology volume 13, pages755–763

Conclusions

Evolving Technologies

Technology developments in algorithms, computing, detectors, electronics, nanotechnology, microfluidics, organic chemistry, and recombinant protein technology create the basis for new reliable analytical approaches for a deeper molecular understanding of living systems.

There is substantial value in working with other scientific disciplines.

Acknowledgements

- Joe Trotter
- Bob Hoffman
- Ed Goldberg
- Hrair Kirakossian
- Brent Gaylord
- Mike Brasch
- Ben Verwer
- Eric Diebold
- Brian Warner
- …

(all while at BD)

- Holden Maecker, Stanford
- Martin Büscher, Miltenyi
- Christian Dose, Miltenyi
- Ming Yan, CYTEK
- Eric Chase, CYTEK
- Maria Jaimes, CYTEK
- Janette Phi, Thinkcyte
- **...**

- BD Biosciences
- Miltenyi Biotec
- CYTEK Biosciences
- …

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More science detail and references: http://www.desatoya.com