ABRF Meeting, Palm Springs CA March 2022

Spectral flow cytometry: the evolution of high parameter particle analysis.

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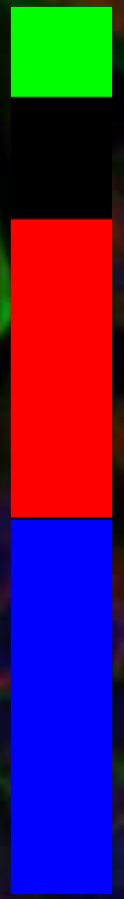
Outline

- Why single cell resolution flow cytometry (FCM)
- History of single cell analysis
- Present approaches for high parameter cell analysis
- Essentials of fluorescence-based FCM
- Historical development of full spectrum FCM
- System performance evaluation (full spectrum vs. dye specific)
- Selected applications
- A look into the future of high parameter FCM

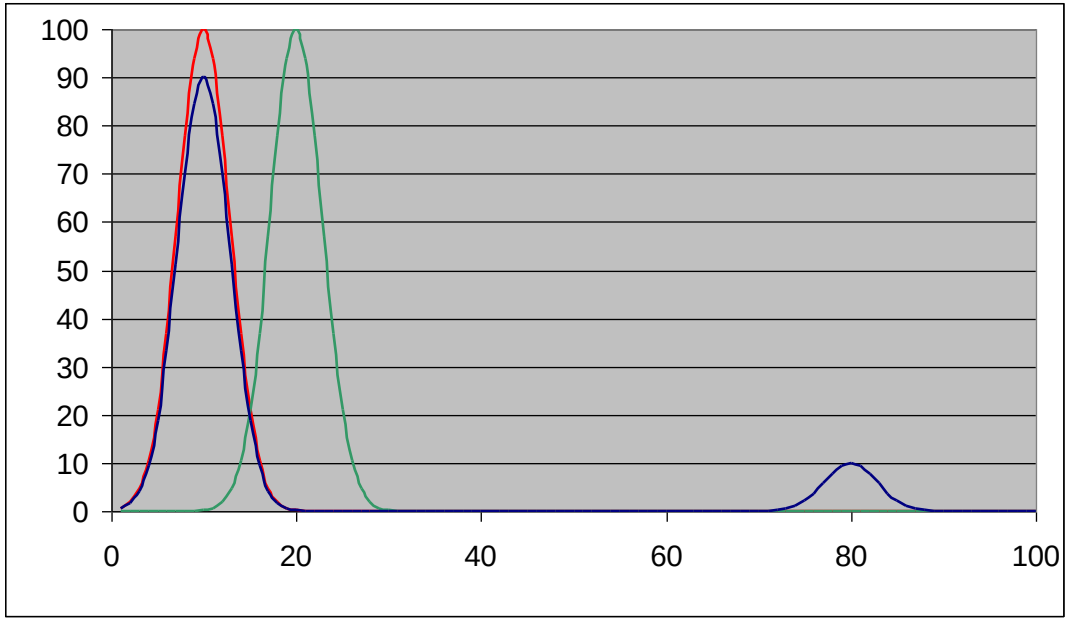
Single Cell Cytometry vs. Bulk Analysis



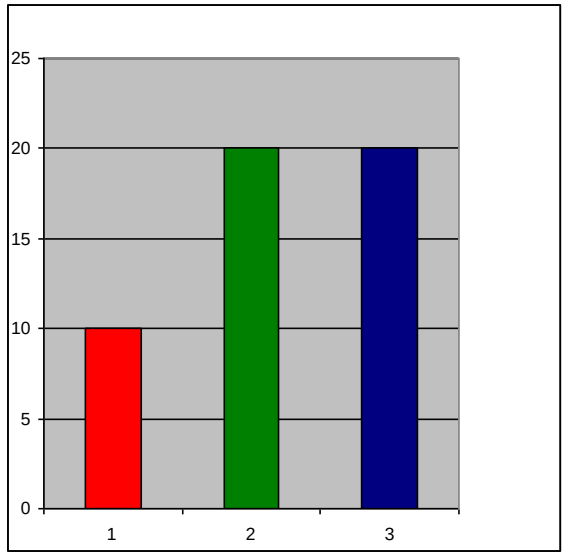
Coutesy Dr. Ji

Single Cell Cytometry vs. Bulk Analysis

Intensity Histogram for Single Particles



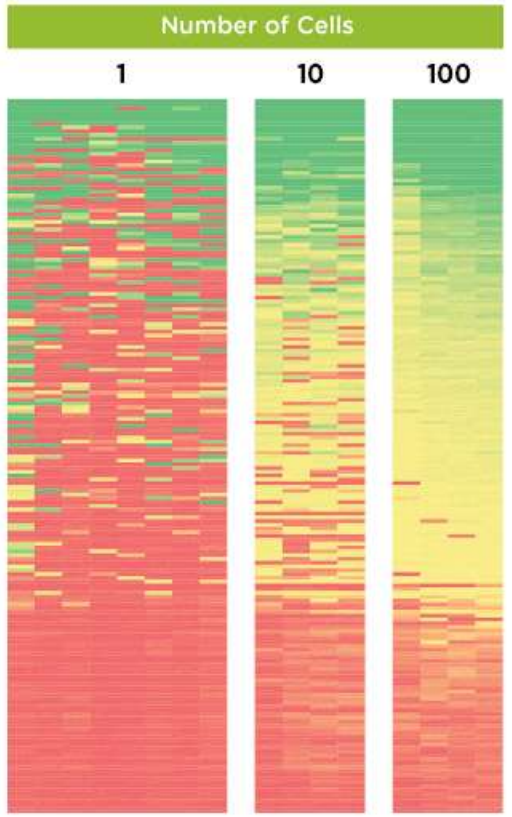
Intensity per Sample



Cell by cell intensity analysis detects population heterogeneity.

Single Cell Genomics

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



Source:
http://www.nanostring.com

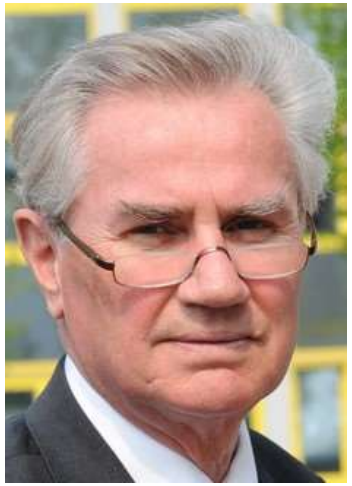
Technical advancements in polychromatic flow cytometry combined with an increased understanding of T cell biology have precipitated a number of flow-cytometry-based approaches, that interrogate T cell function.
… the integrated complexity of the immune system mandates that part of the evaluation of Tcell therapy focus on the impact that treatment has on the broader immune system. Although this relatively new concept has not yet been broadly implemented in clinical trials, one approach that has been implemented with some success has been to evaluate systemic cytokine levels in patients during treatment. … this strategy has revealed that engineered T cell activation and antitumor activity result in broad and potent cytokine-driven effects… the hypothesis-agnostic interrogation of cytokines in these trials unexpectedly identified IL-6 as a major cytokine induced by CAR therapy: As a result of this observation, the anti-IL-6 receptor antagonist antibody tocilizumab was successfully deployed to mitigate the observed cytokine-induced toxicity, a treatment now being applied more systematically to counteract cytokine-release syndrome.
(Maus MV et al. in Ann.Rev.Immunol.,2014, 32:189, Adoptive Immunotherapy)

Carl June, developer of CAR_T cancer therapy

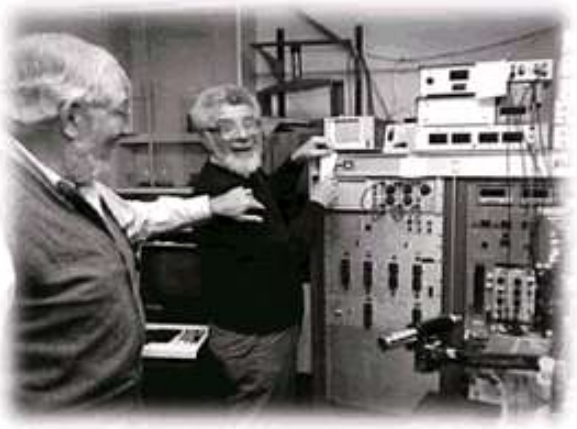
The Beginnings of Single Cell Analysis

- 1665 – English physicist, Robert Hooke used a microscope lens to observe “pores” in cork
- 1674 – Anton van Leeuwenhoek built a simple microscope with only one lens to examine blood cells
- 1872 – Ernst Abbe calculated the maximum resolution in microscopes
- ...
- …
- 1971 – Intel launches 4-bit 4004

First Fluorescence Based Instruments



1968 1st fluorescence-based flow cytometry device (ICP 11) by Prof. Göhde from the University of Münster, Germany, and first commercialized in 1968/69 by German developer and manufacturer Partec through Phywe AG in Göttingen.



1971 Cytofluorograph, Ortho

1973 PAS 8000, Partec

1974 1st FACS instrument, BD

1977 Epics Instrument,Coulter

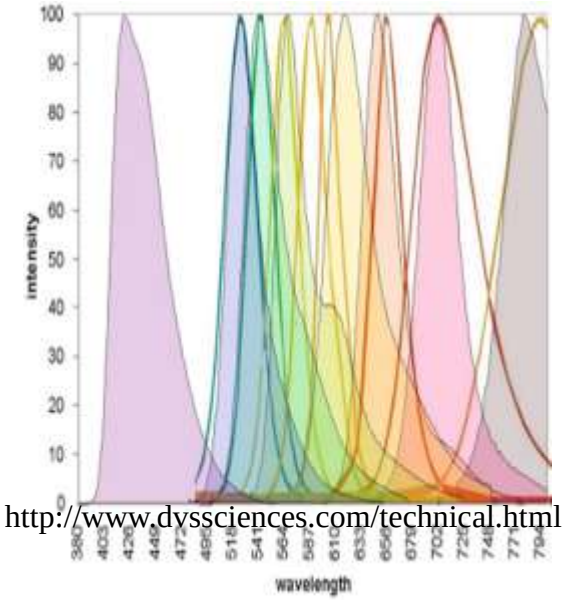
Multi-beam Flow Cytometers

Howard M. Shapiro - 1973-76

Shapiro and the Block instruments designed a series of multibeam flow cytometers that did differentials and multiple fluorescence excitation and emission

From Beckman-Coulter website 2013

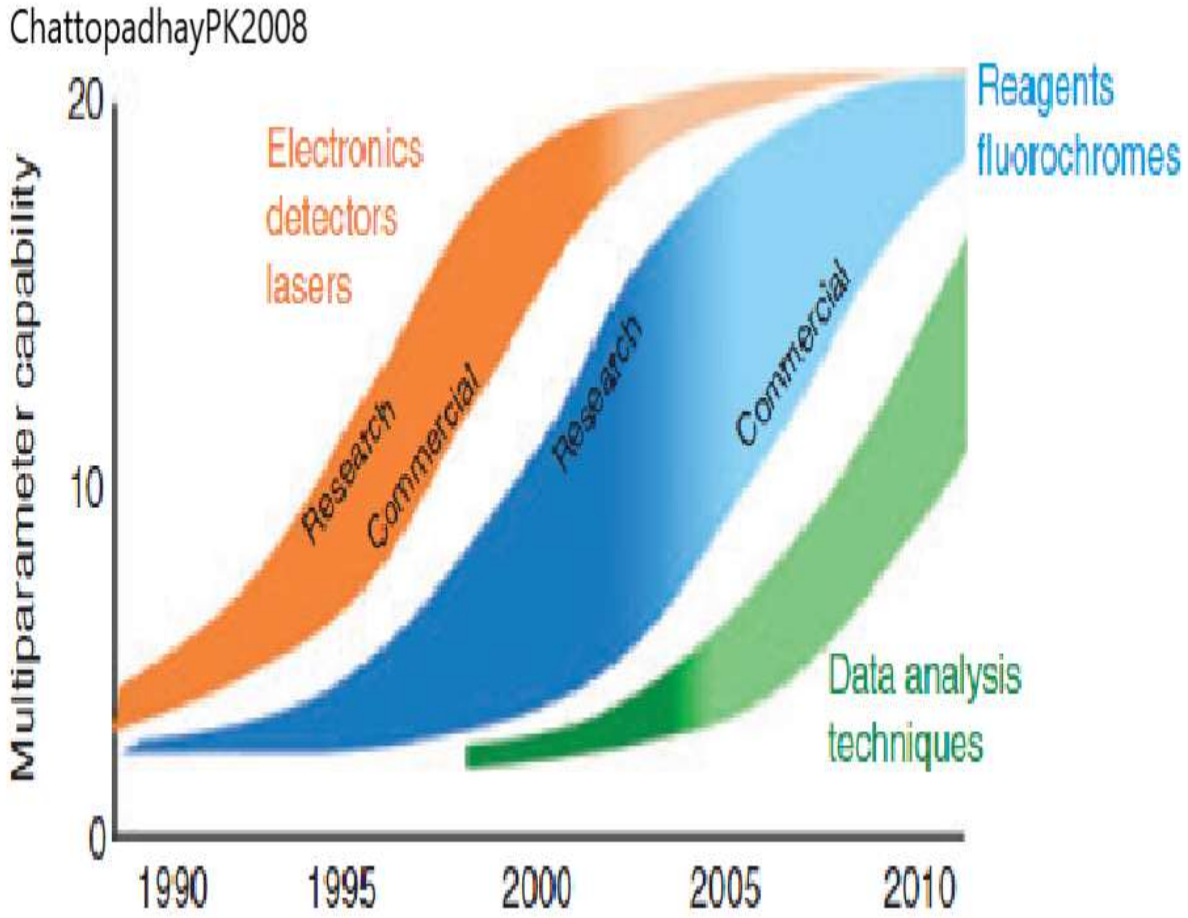
Technology Developments For Changes in Cytometry



http://www.dvssciences.com/technical.html

- Labels
  - Fluorescent dyes
  - Raman labels
  - Energy transfer dyes
- Light sources
  - Arc lamps
  - Gas lasers
  - Solid state lasers
  - LEDs
- Detectors
  - Photomultipliers and Arrays
  - CCD and CMOS detectors
  - APDs
- Computing
  - Fast multi-parallel processing

Technology Development History



Today, March 2022:

Instrumentation >100

Fluorochromes 50
NA barcoding >100

Data analysis >100

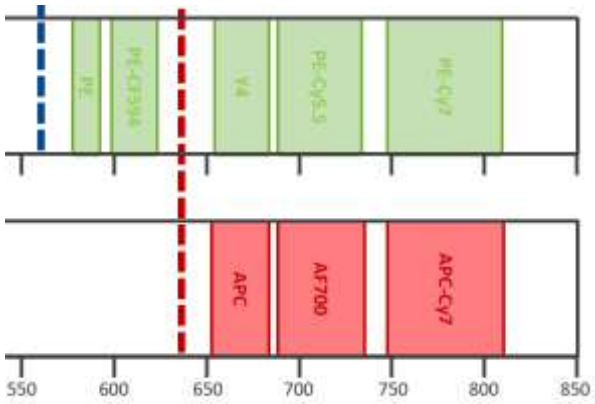
Flow Cytometry Features

Single cell resolution analysis with

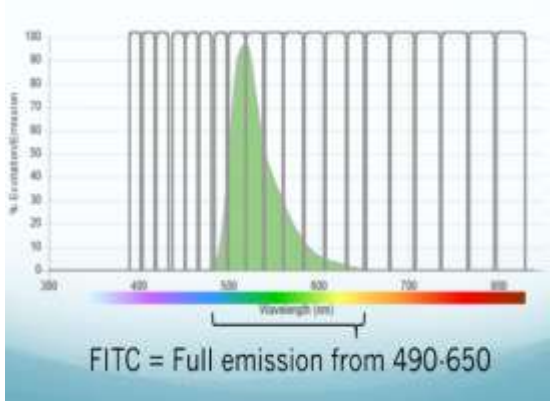
- High analysis rates to ~10^5 particles sec^{-1}
- High sensitivity (single molecule sensitivity by fluorescence)
- Wide dynamic range (10^3 to 10^7 cells mL^{-1})
- High precision fluorescence measurement (1%CV)
- Many simultaneous measurements
- Viable cells can be by sorted (e.g. for culture)
- Good ease-of-use

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

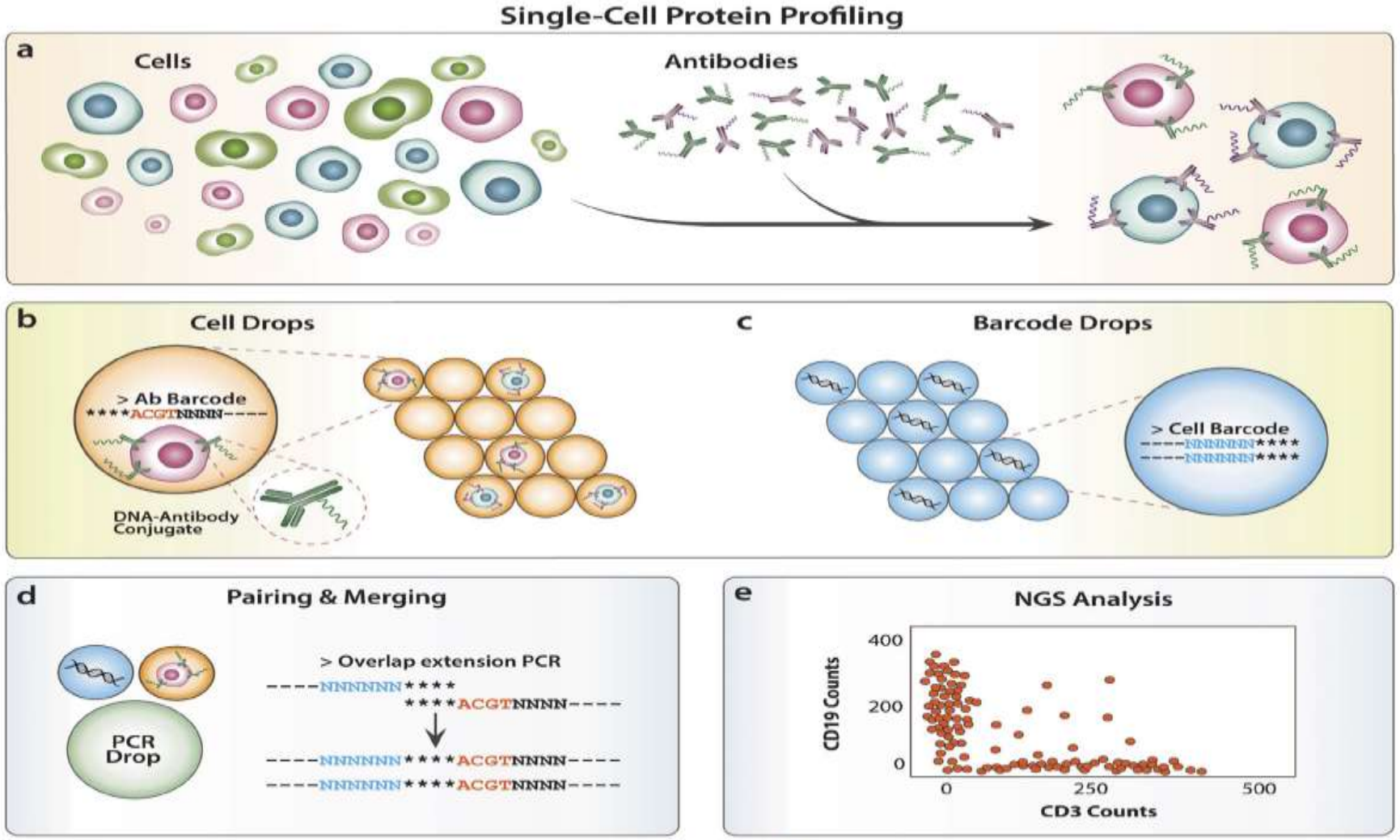


dye specific



full spectrum

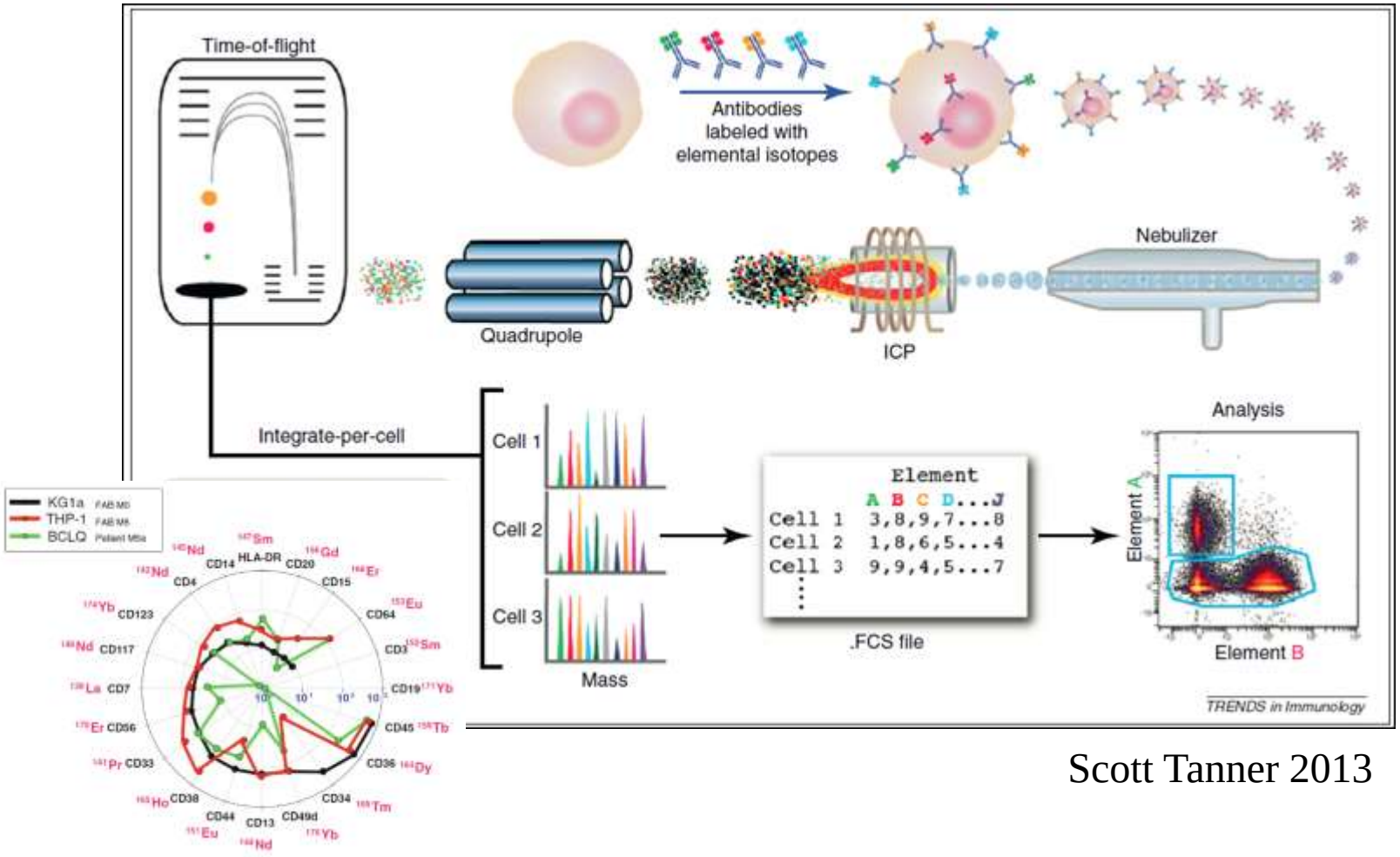
Sequence Barcodes



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.

MS for Flow Cytometry



Scott Tanner 2013

Flow Cytometry with Fluorescence Detection

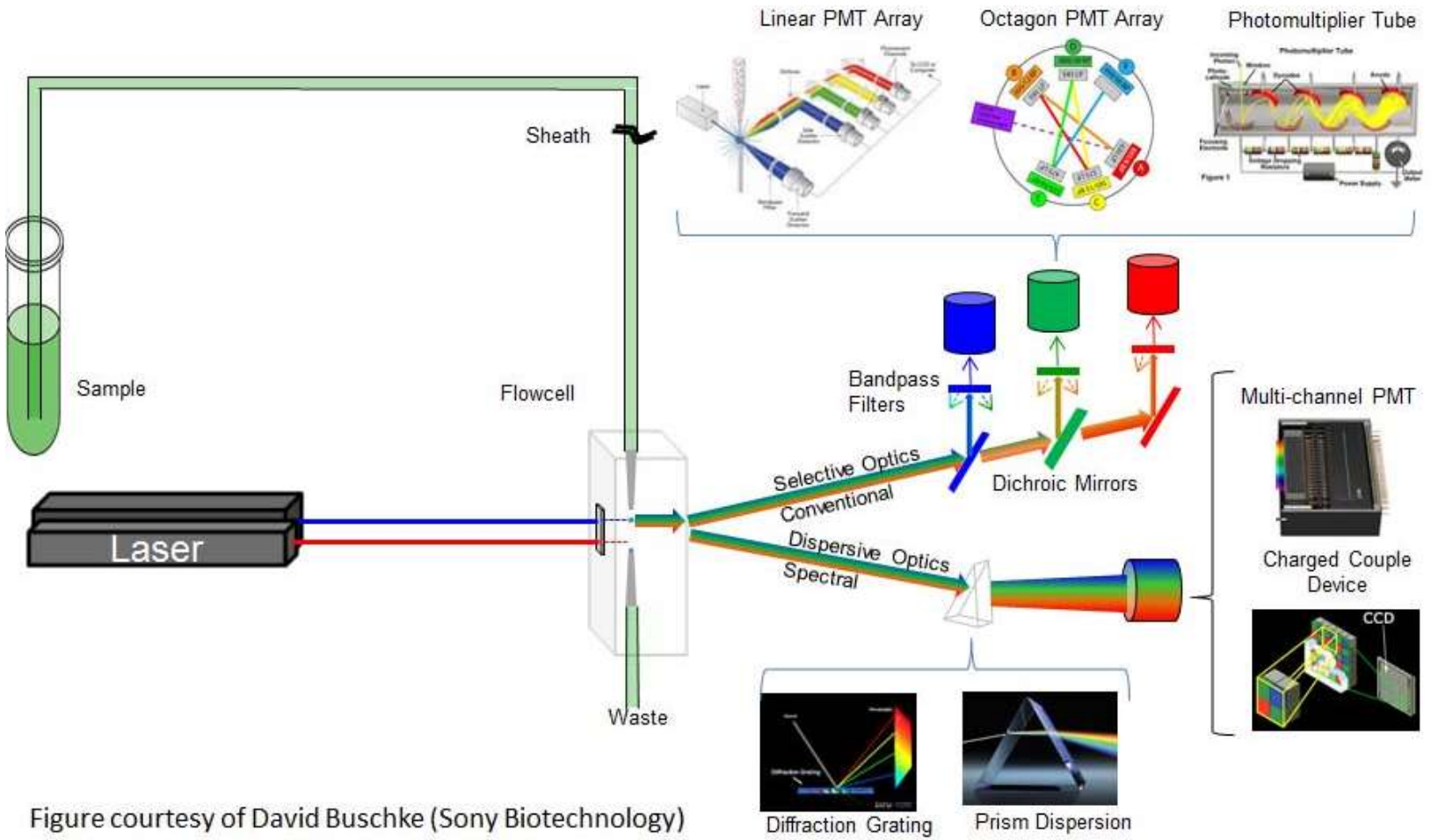
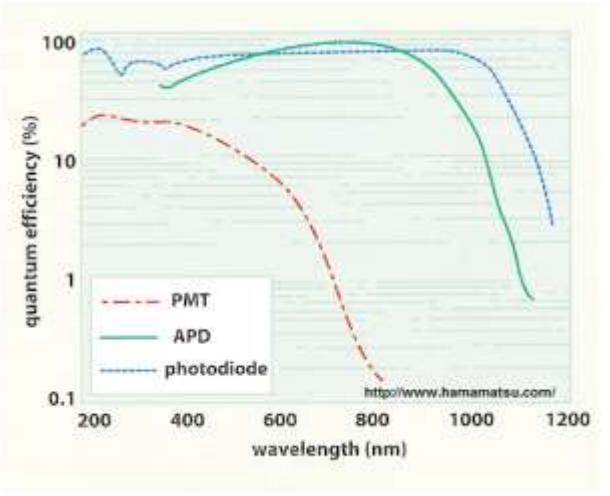
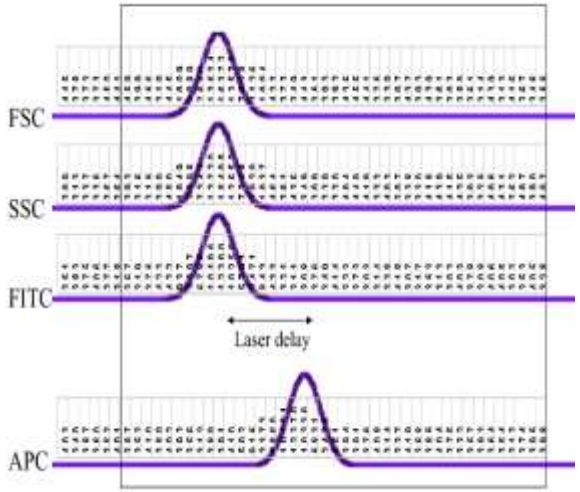


Figure courtesy of David Buschke (Sony Biotechnology)



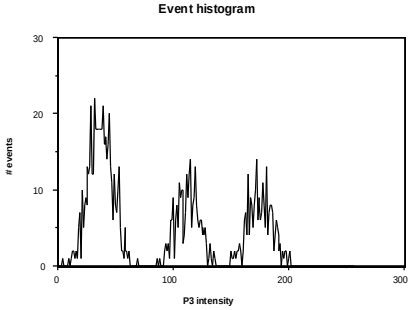
Basic Data Processing

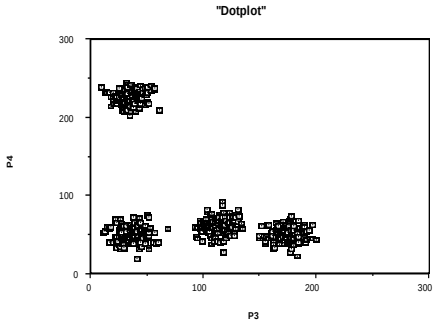
Voltage pulses from detectors

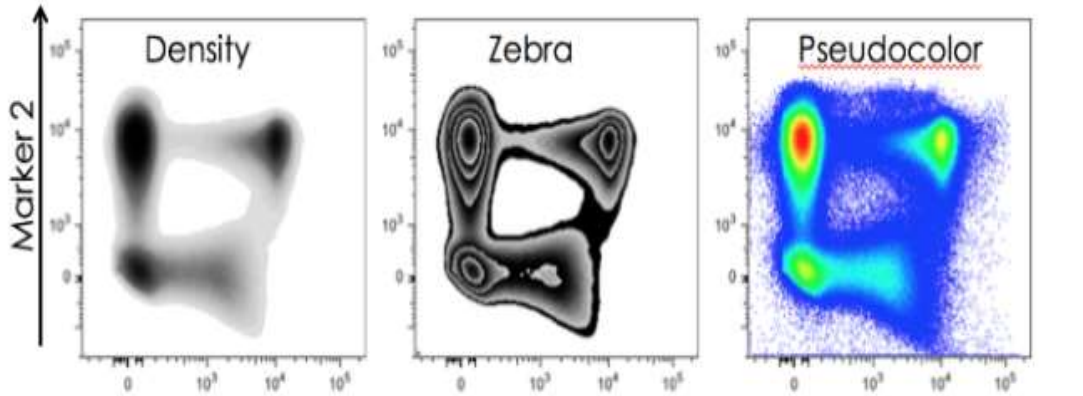


Listmode data after
pulse processing and A/D

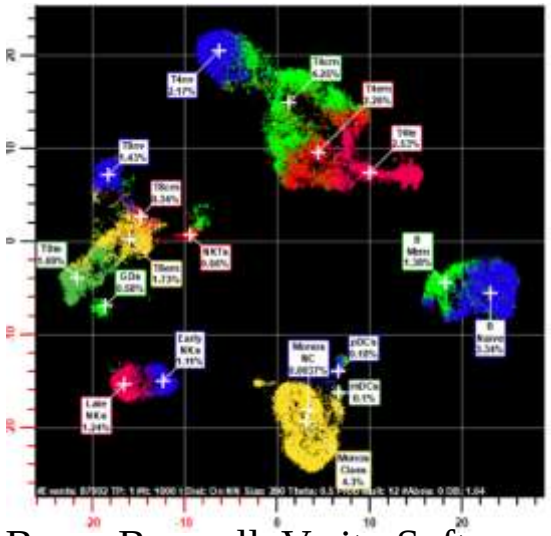
Cell	P1	P2	P3	P4	P5	Pop#
1	242	135	704	175	612	1
2	146	132	690	178	566	1
3	269	147	89	206	580	3
4	442	143	399	250	255	4
5	212	167	155	926	526	2
6	269	2	659	207	575	1
7	204	232	112	171	679	3
8	152	74	160	828	532	2
			…			
9997	215	119	138	936	662	2
9998	244	50	72	261	543	3
9999	214	137	174	1014	597	2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







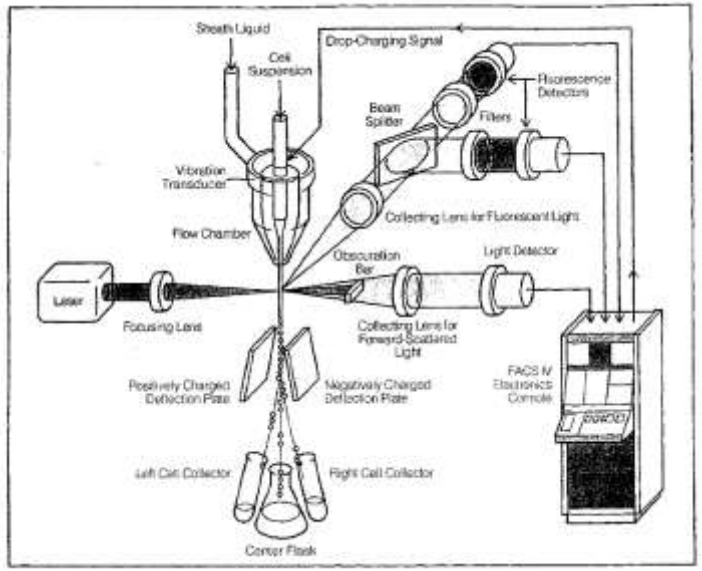
expert.cheekyscientist.com/analyze-facs-data-prepare-cytometry-figures-scientific-papers/

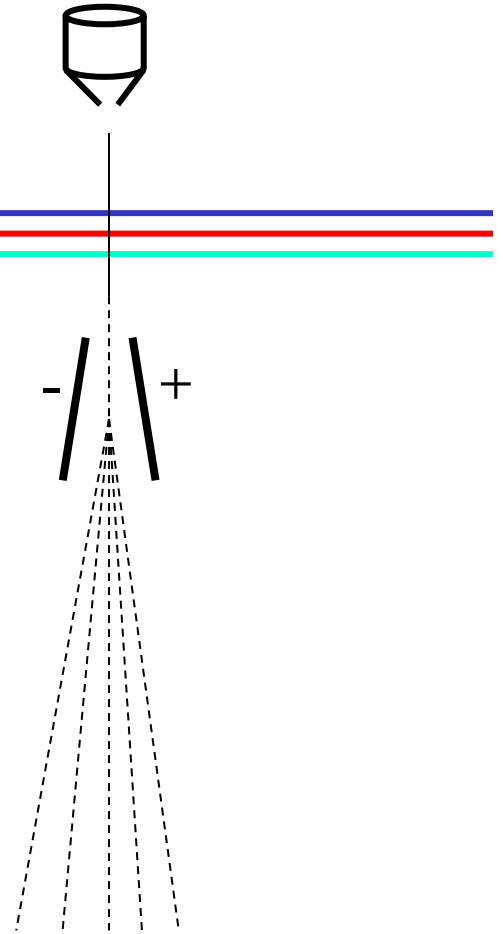


tSNE
Cen-se’
…
…

Bruce Bagwell, Verity Software

“Droplet-based” Sorting





BD Biosciences

Historical Development of Full Spectrum Flow Cytometry

Reference	Approach	Comments
Wade et al, 1979(Wade et al., 1979)	Grating spectrograph and silicon intensified detector	Measured average spectra of many cells
Steen and Stokke, 1986(Steen and Stokke, 1986)	Grating monochromometer and PMT	Measured average intensity at 10 nm intervals
Buican, 1990(Buican, 1990)		Fourier transform FC
Gauci et al, 1996(Gauci et al., 1996)	Prism and intensified photodiode array	Measured spectra of single cells and microspheres
Fuller and Sweedler, 1996(Fuller and Sweedler, 1996)	Grating spectrograph and CCD	Measured spectra of single microspheres an liposomes
Asbury et al, 1996(Asbury et al., 1996)	Scanning monochromometer and PMT	Constructed population average spectra from many single cells, single wavelength measurements
Dubelaar et al, 1999(Dubelaar et al., 1999)	Grating spectrograph and a 7 pixel hybrid PMT	Used 3 of 7 detector elements to measure light scatter and two colors of fluorescence
Isailovic et al, 2005(Isailovic et al., 2005)	Grating and ICCD camera	Measured spectra of bacterial cells in a capillary flow system
Robinson et al, 2005(Robinson et al., 2005);Gregori et al 2012(Grégori et al., 2011)	Prism or grating and multianode PMT	Measured spectra of fluorescence-stained cells, PCA analysis
Goddard et al, 2006(Goddard et al., 2006)	Grating and CCD	Measured spectra from single beads and cells
Watson et al, 2008(Watson et al., 2008); Nolan et al, 2012(Nolan and Sebba, 2011)	Imaging spectrograph and CCD	Measured fluorescence and SERS spectra from individual beads and cells, PCA and linear unmixing analysis

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Parallel advances in optics, electronics, computing, and reagents created the present superior tool for life science work.

Full Spectrum Analysis 1979

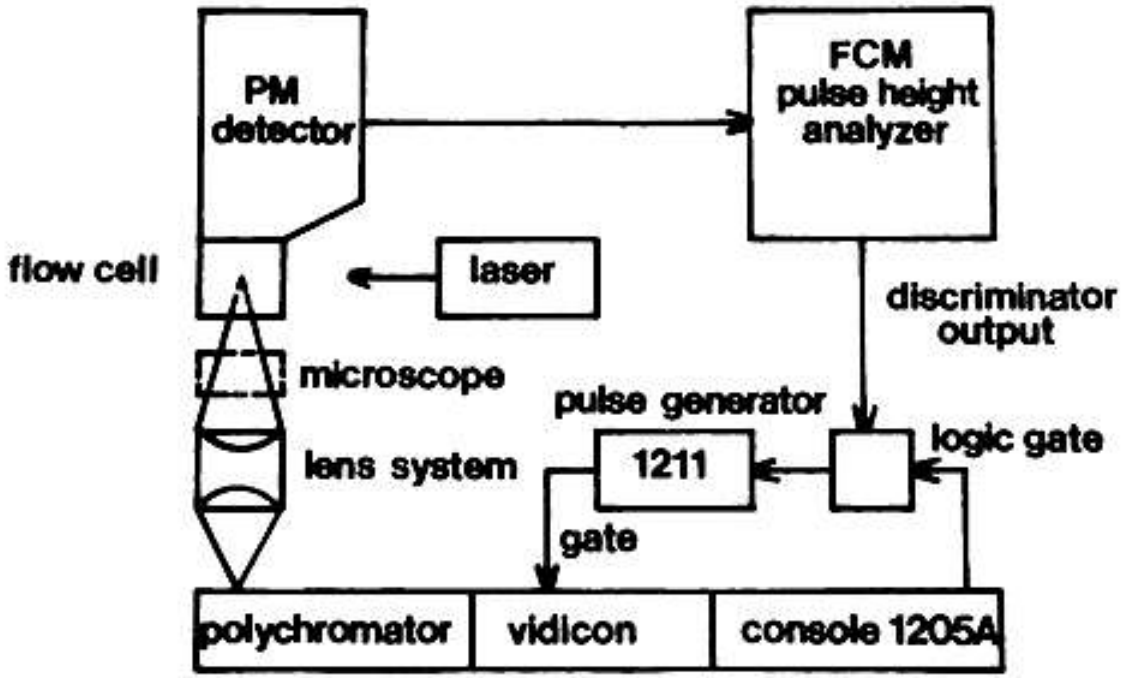


FIG. 1. Arrangement of FCM and vidicon systems (top view). The discriminator, logic gate, and gate generator were used only on the Lawrence Berkeley Lab system.

WadeCG1979JHistochemCytoChem27_1049

Spectra from Single Particles using Diffraction Grating

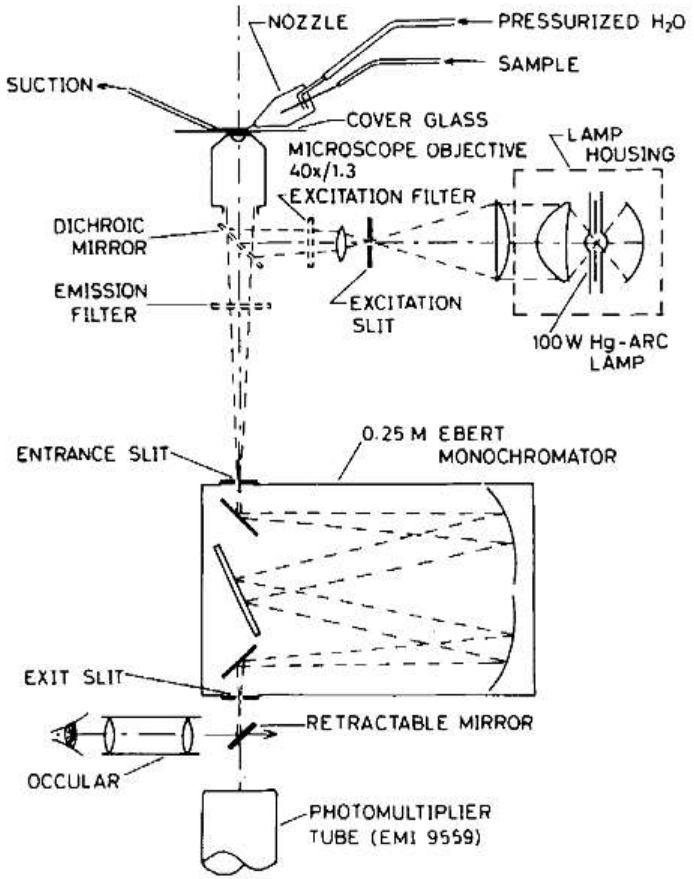


FIG. 1. Optical outline of the flow cytometer including a grating monochromator for monochromatic fluorescence detection.

SteenHB1986Cytometry7_104

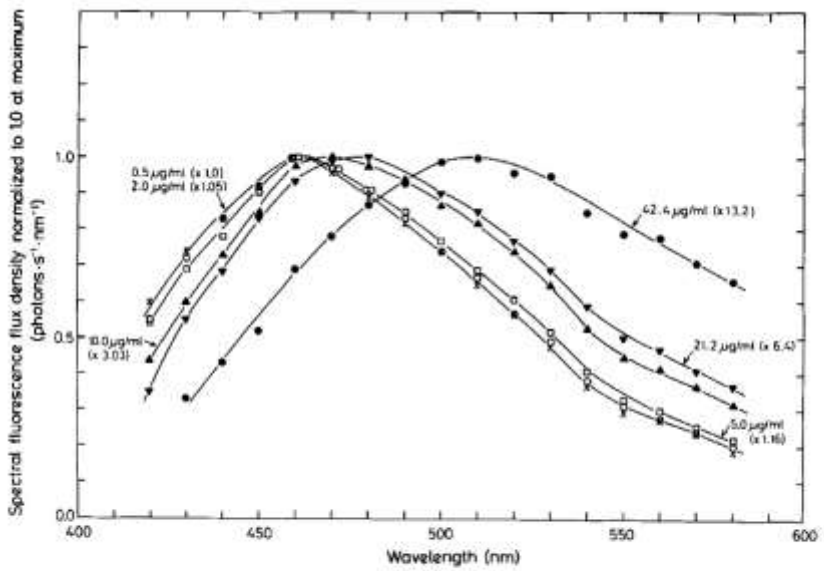


FIG. 2. Corrected fluorescence spectra of rat thymocytes fixed in ethanol and stained with Hoechst 33258 in the concentrations indicated on the curves. Each spectrum has been multiplied by the normalization factor given in parentheses. The total fluorescence intensity is thus approximately inversely proportional to this factor. The similarity between the spectra obtained with the two lowest dye concentrations is an indication of the reproducibility of the measurements.

SteenHB1986Cytometry7 104

Sensitivity Reducing Factors

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

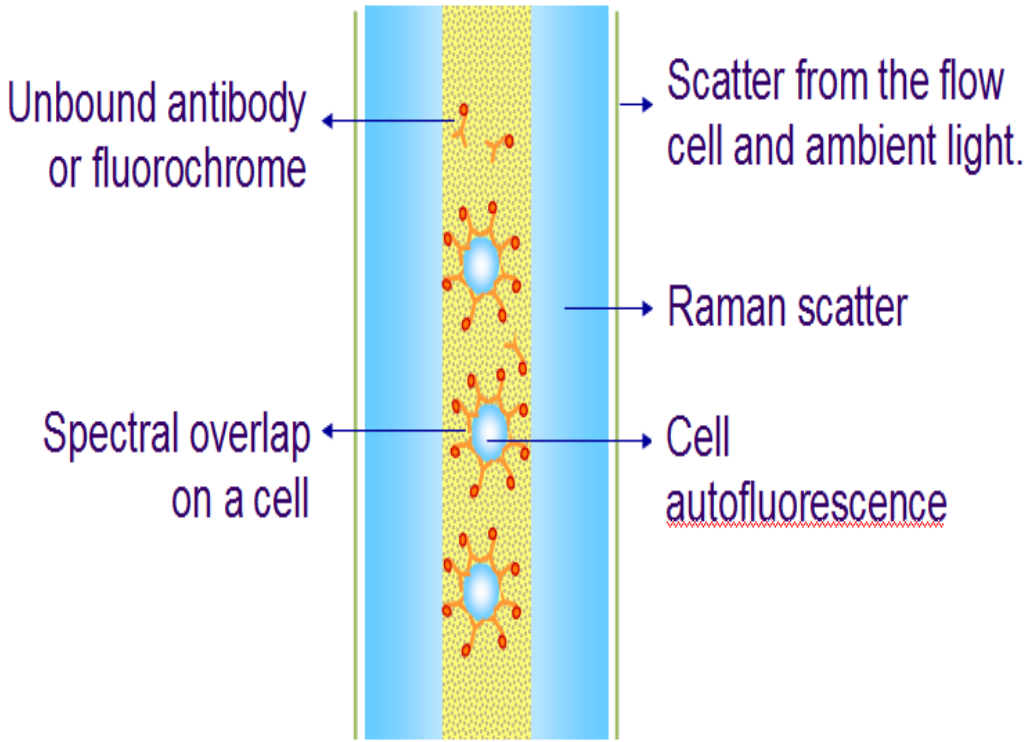
…

Unbound dye

Non-specific binding

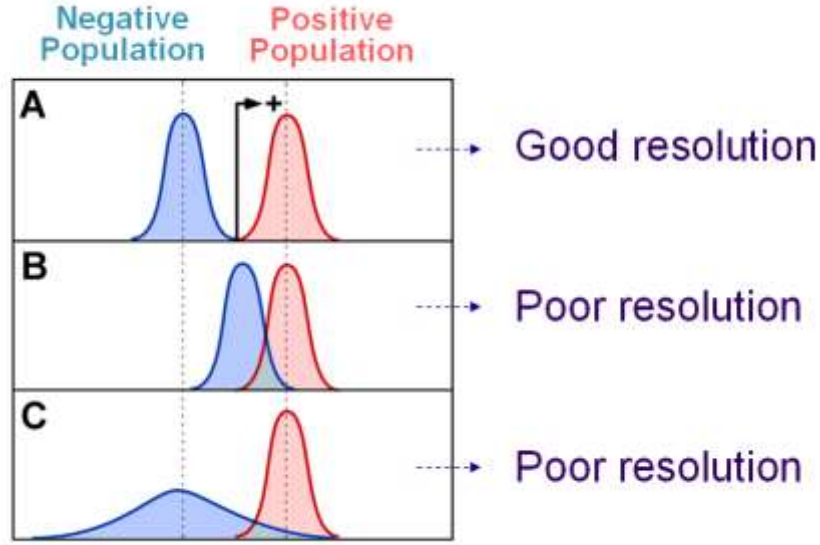
Instrument Evaluation Br, Qr

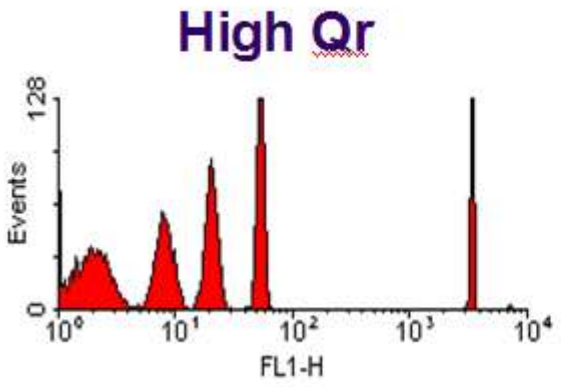
Br is a measure of the photon background in the detector

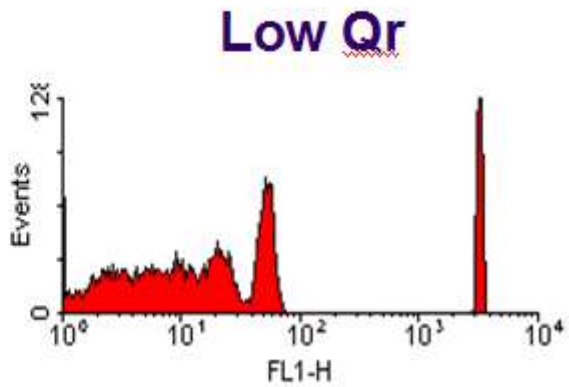


Qr is a systems efficiency for photon detection

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







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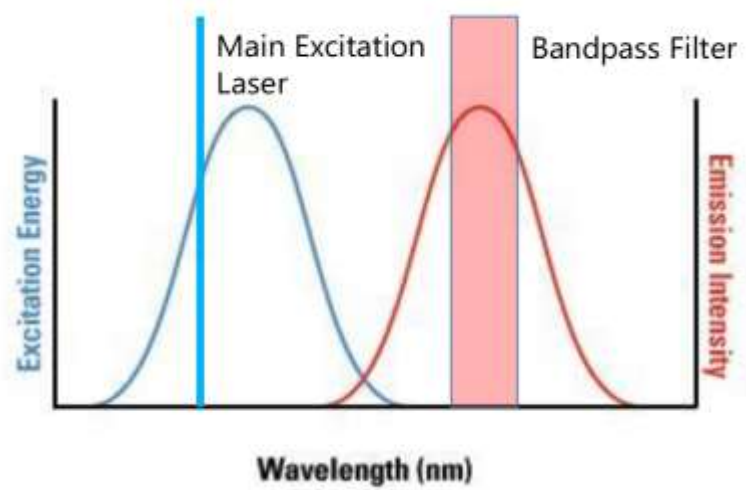
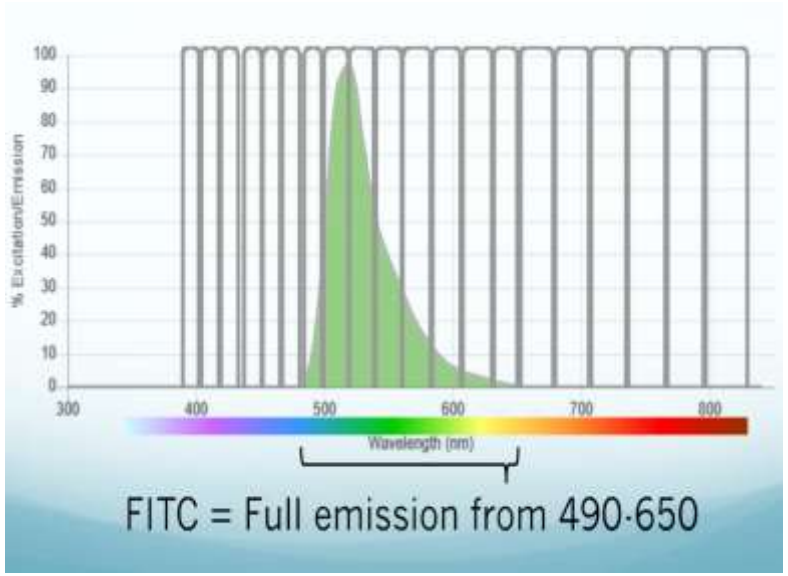
Full Spectrum Optics





Unique Optical Design

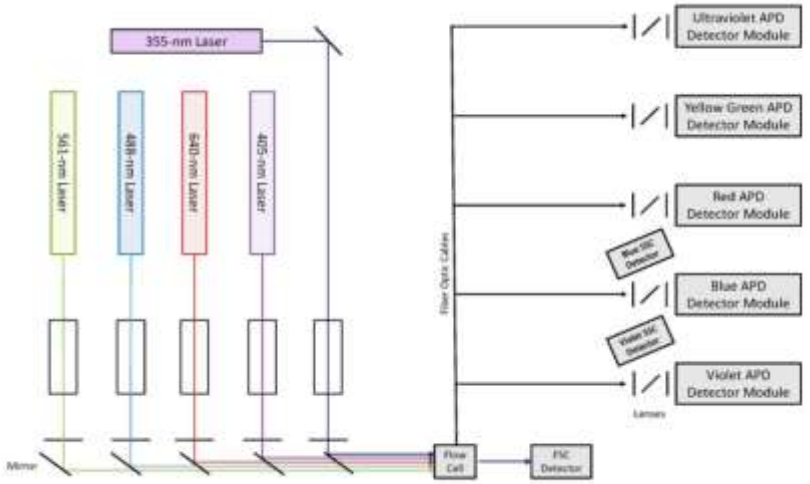
- High Sensitivity Collection Optics
- Lasers are spatially separated
- Dedicated detector array

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



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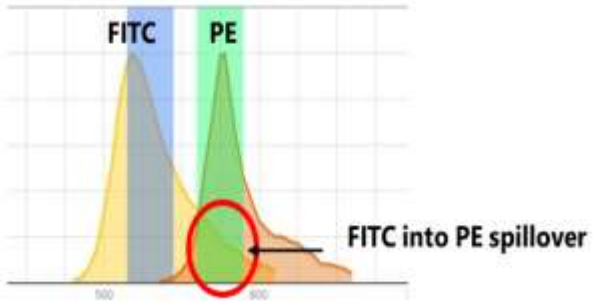
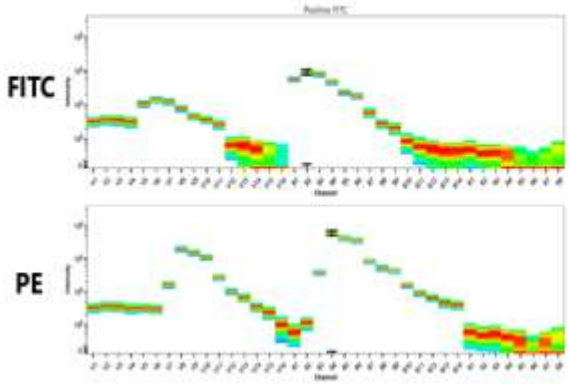
Measurement data transformation
(dye-specific vs. full spectrum)

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

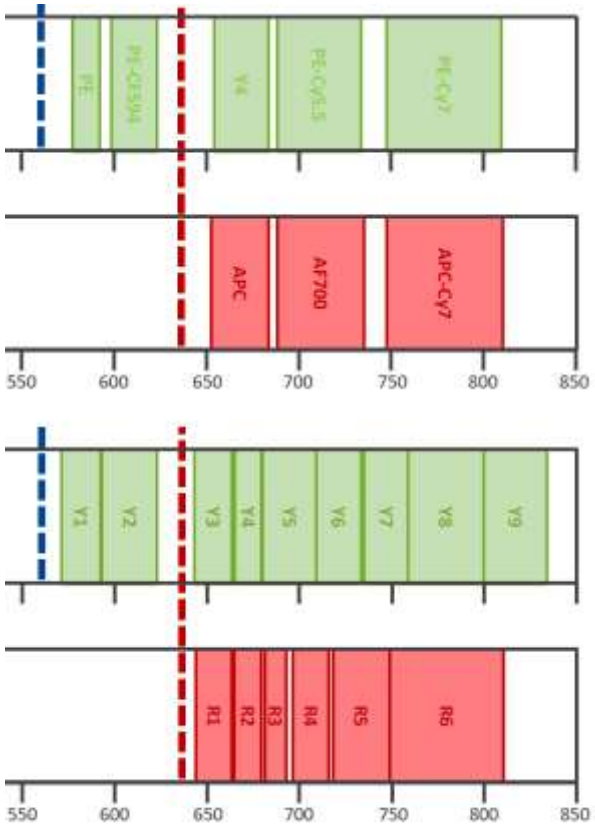


- Each fluorochrome is detected in ONE channel
- Detector # = Fluor #
- Single stained controls establish spillover
- Compensation mathematically subtracts the amount of light contribution from non-primary colors into the primary detector
- A compensation matrix is calculated: n x n (square matrix)



- 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

Limit of Detection



BD Biosciences website

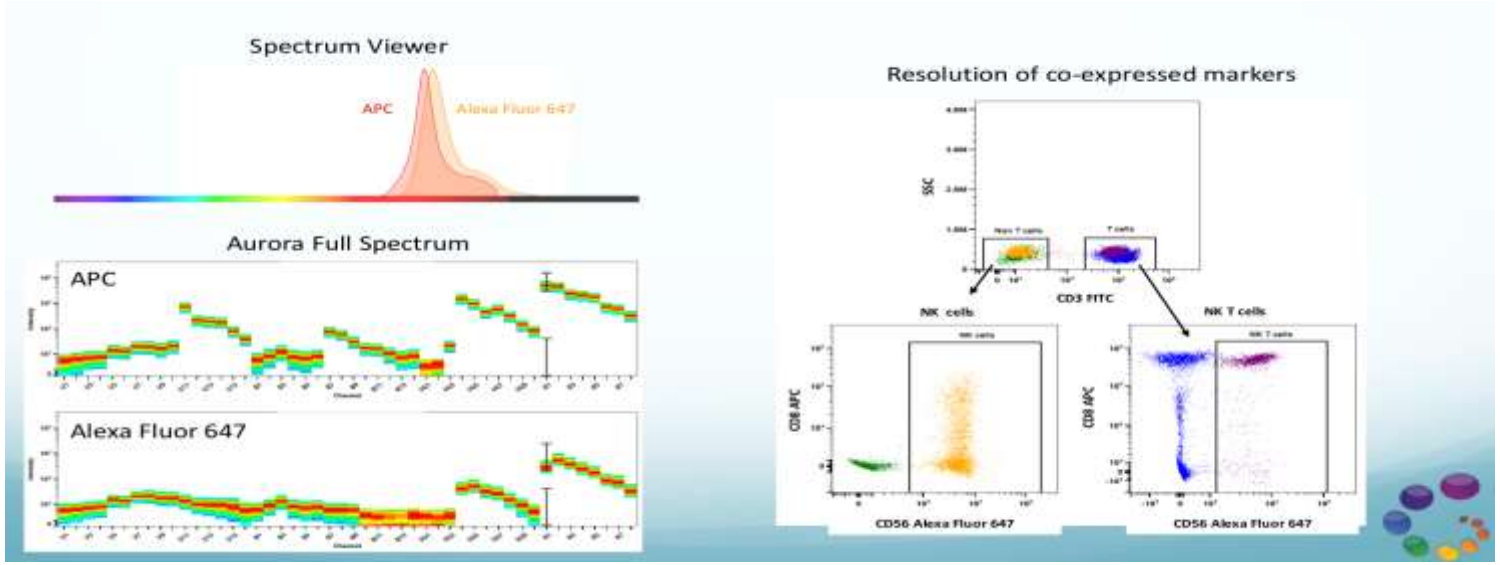
Table 3. Comparison of background (B), Q value, and detection limit (DL) of standard filter setting and multispectral filter setting for QSC microspheres stained with CD4 FITC or CD4 PE

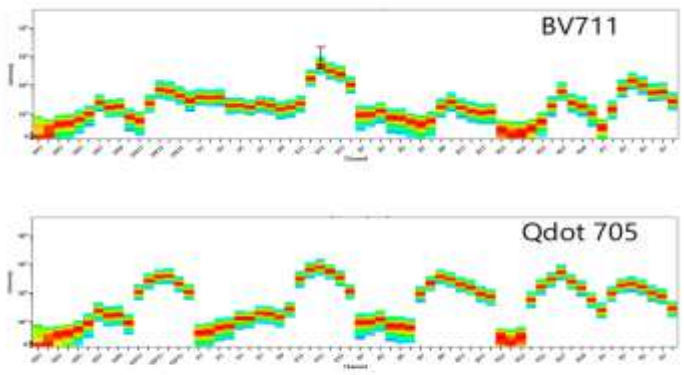
PARAMETER	530/30	585/40	MULTISPECTRAL
Detection wavelength (nm)	515–545	565–605	505–810
Q_{FITC} (phe^-/ABC)	0.004	–	0.04
Q_{PE} (phe^-/ABC)	–	0.02	0.14
B (phe^-)	9	32	63
DL_{FITC} (ABC)	320	–	59
DL_{PE} (ABC)	–	875	231

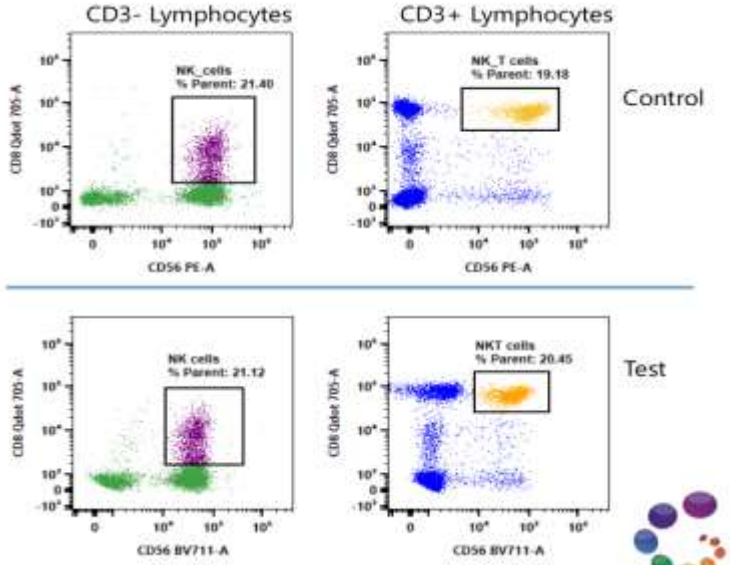
FeherK2016 DOI: 10.1002/cyto.a.22888

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

Resolving Spectrally Similar Dyes







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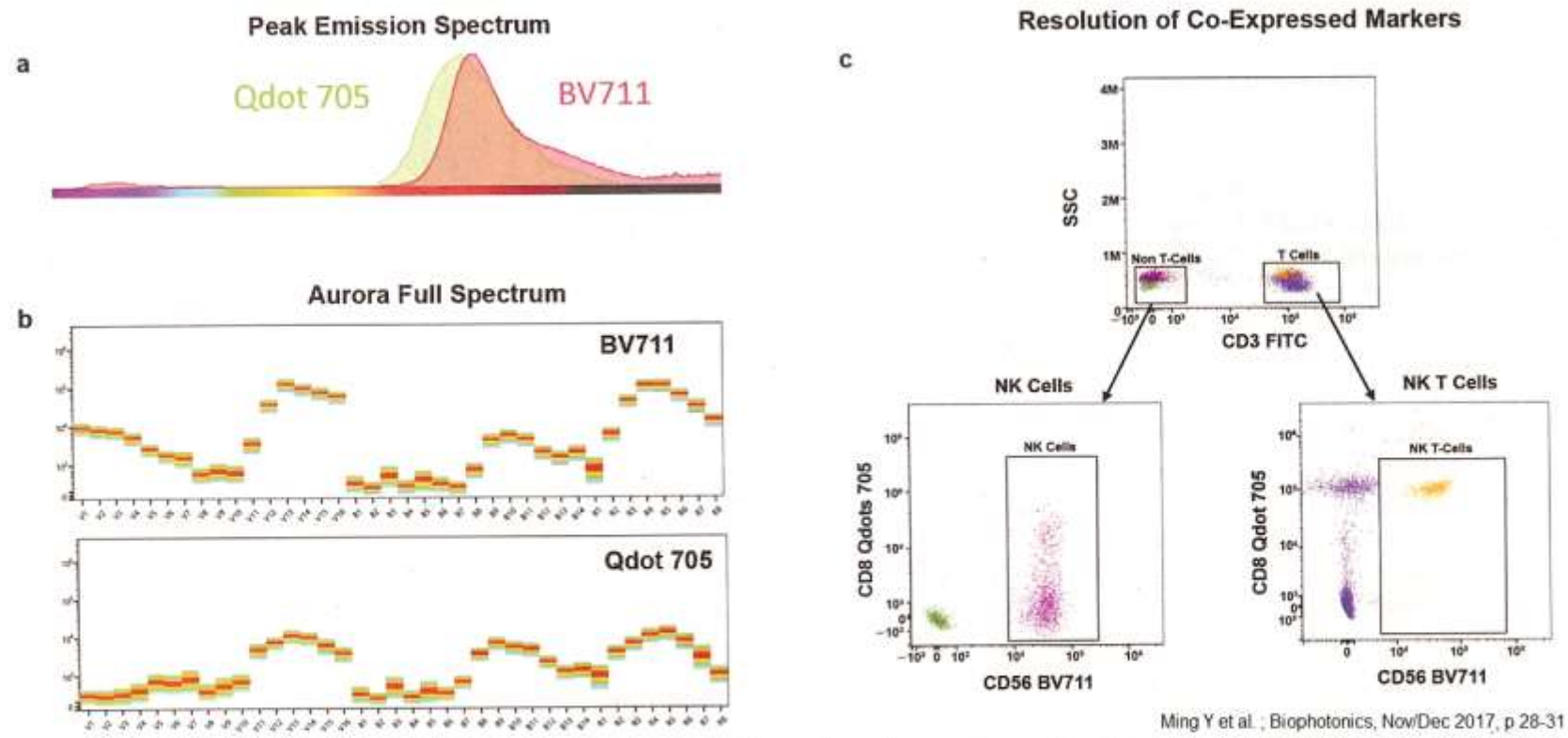
Resolving Spectrally Similar Dyes

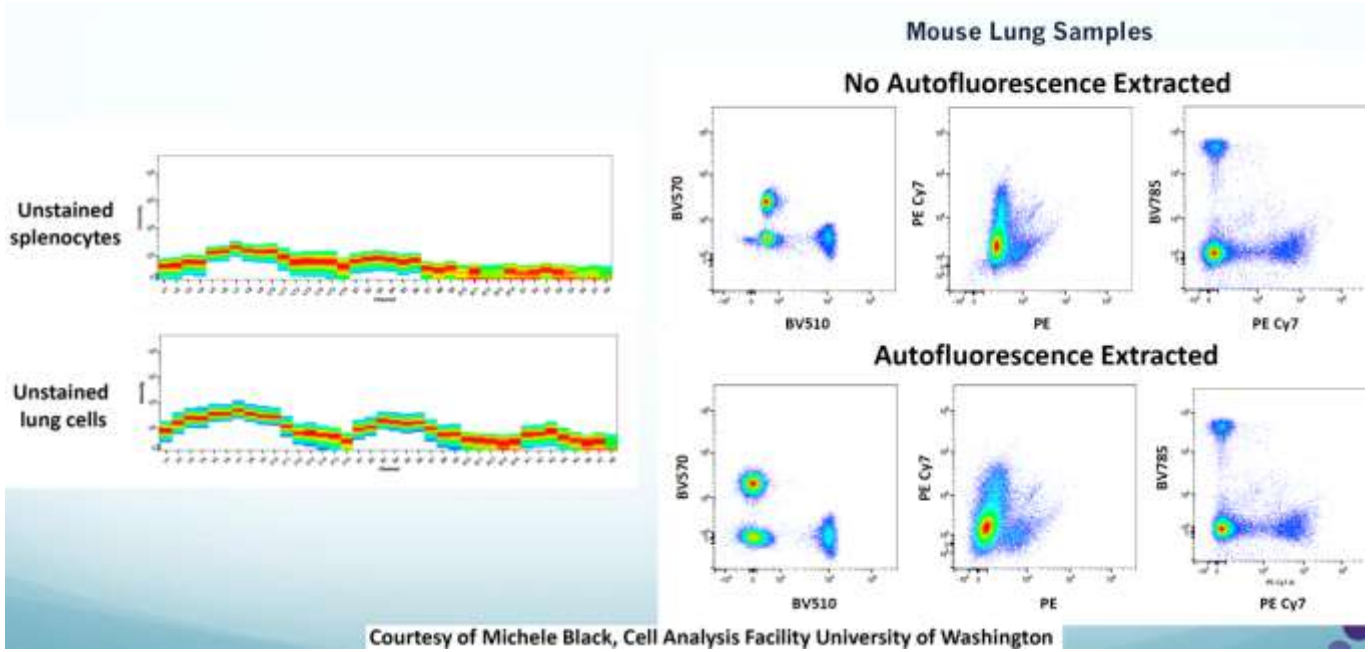


Ming Y et al. ; Biophotonics, Nov/Dec 2017, p 28-31

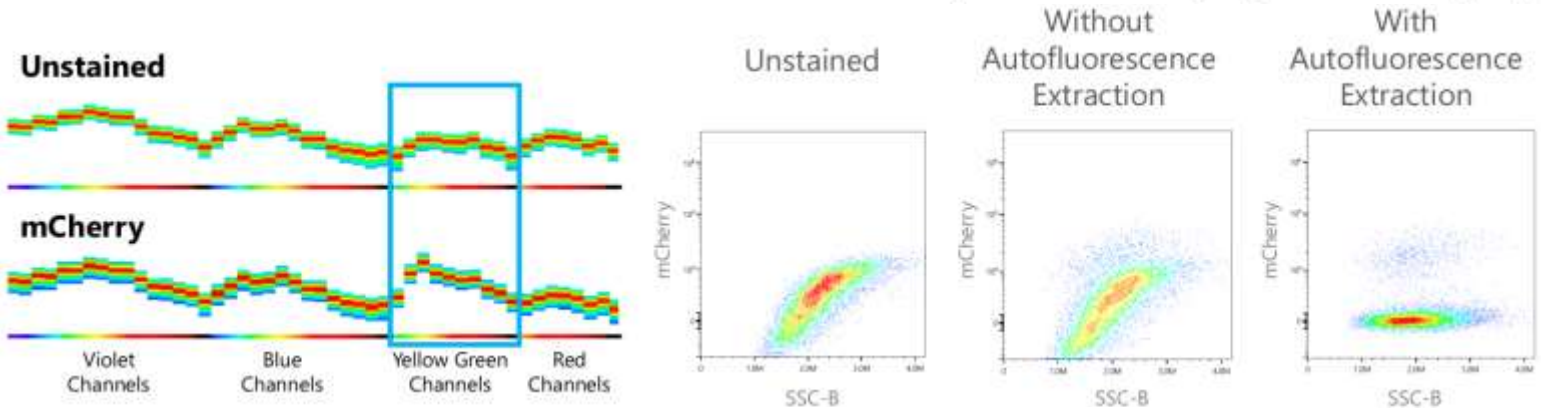
Figure 4. The peak emission spectra of the dyes Qdot 705 and BV711 highly overlap and cannot be used together on a conventional flow cytometer **(a)**. However, these two dyes have distinct signatures, and because of this, they can be used in combination with full-spectrum cytometry **(b)**. This means 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

Autofluorescence

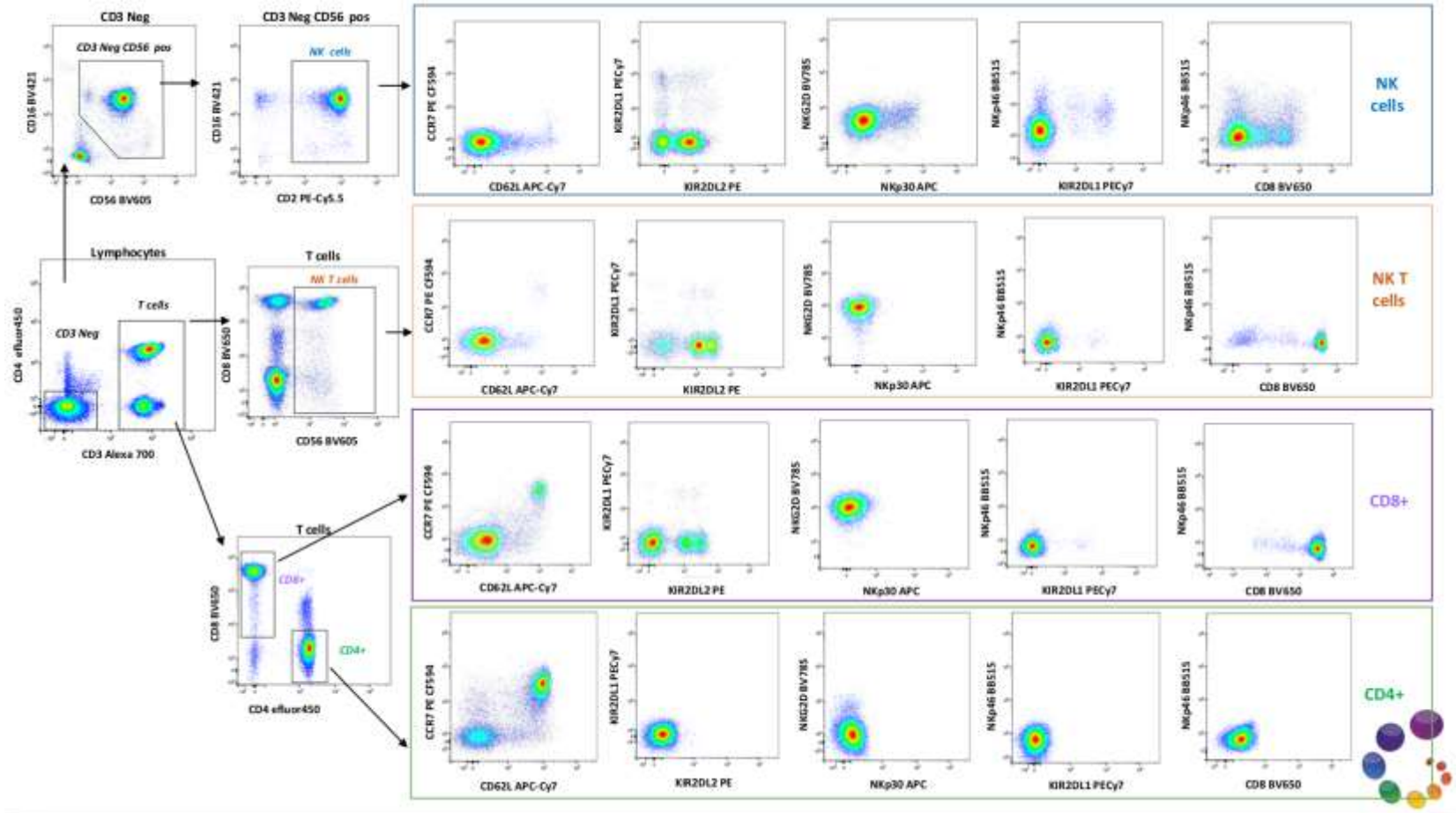


HeLa human cells were transformed with a CRISPR-Cas9 target vector carrying an mCherry reporter



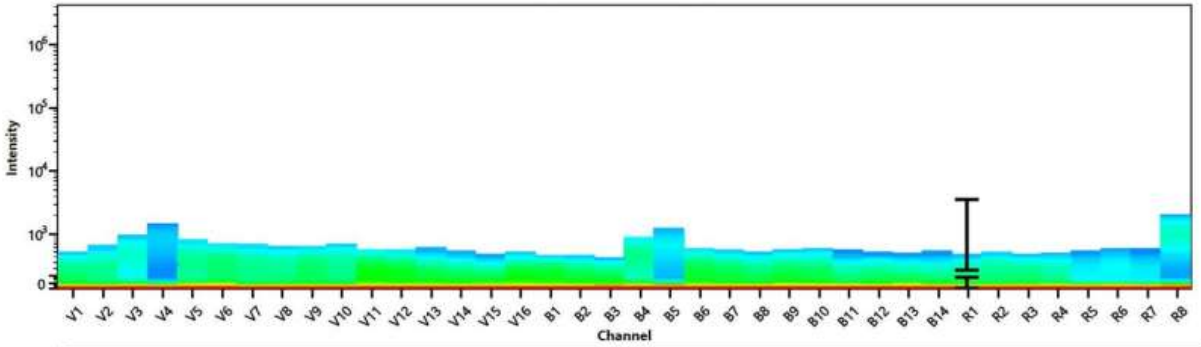
Malte Paulsen, Flow Cytometry and Cell Sorting Facility, EMBL

Immune Cell Profile with 35 fluorophors

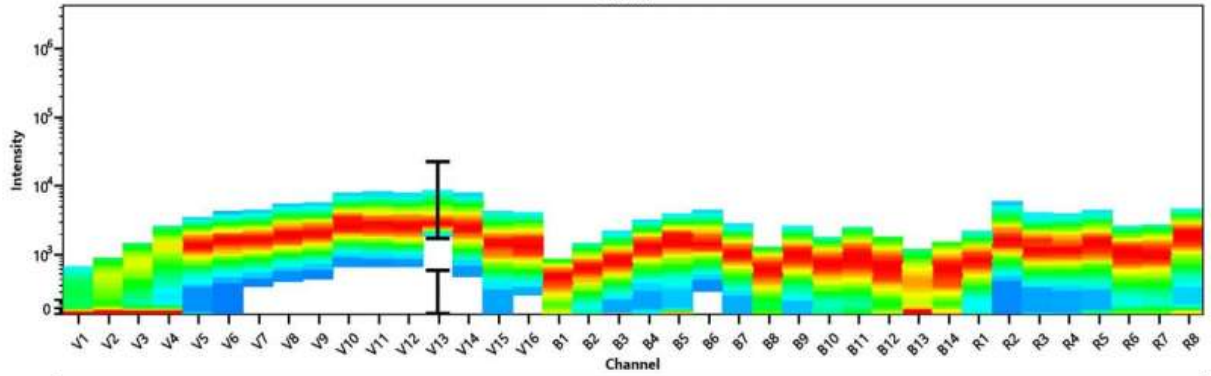


Cytek Biosciences

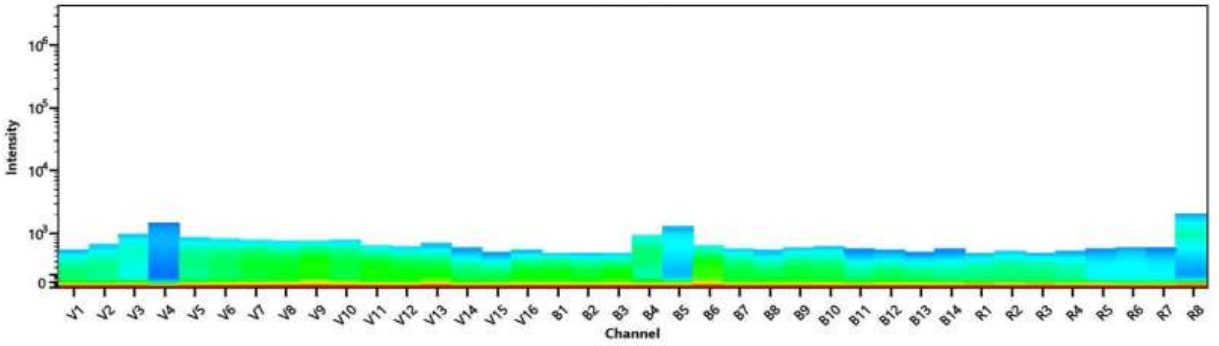
Autofluorescence of EVs



Plasma EVs – 21K Xg

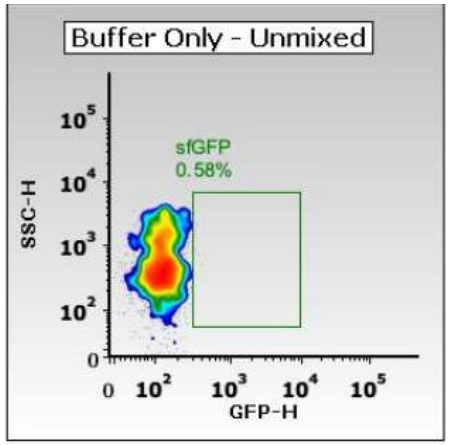


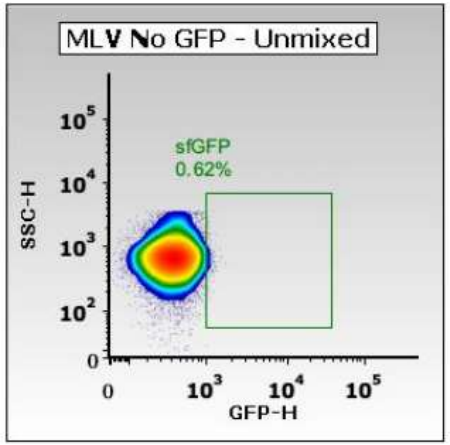
Urine EVs – 21K Xg

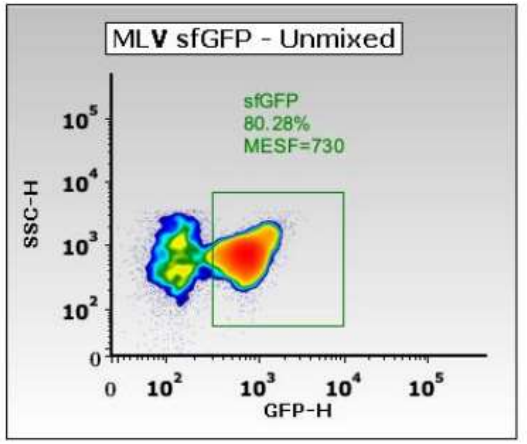


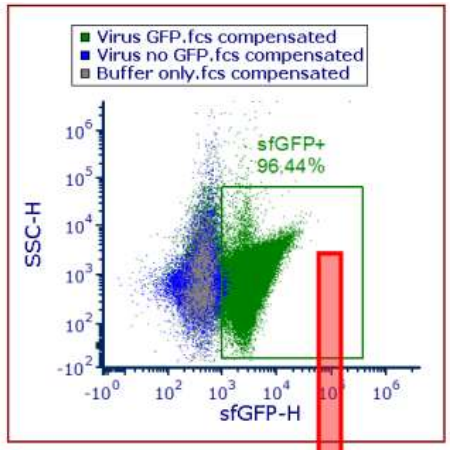
Saliva EVs – 21K Xg

Labelled MLV virus

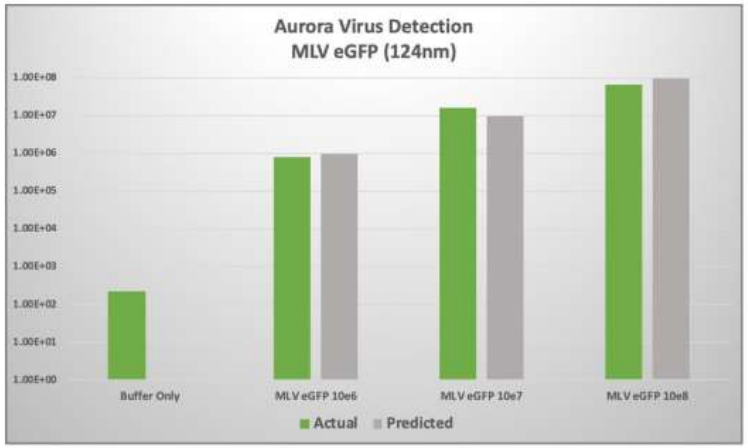




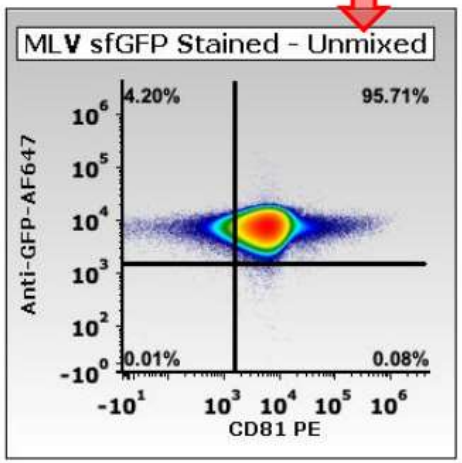




Gated on GFP+



sfGFP virus stained with anti-CD81 PE and Anti-GFP AF647



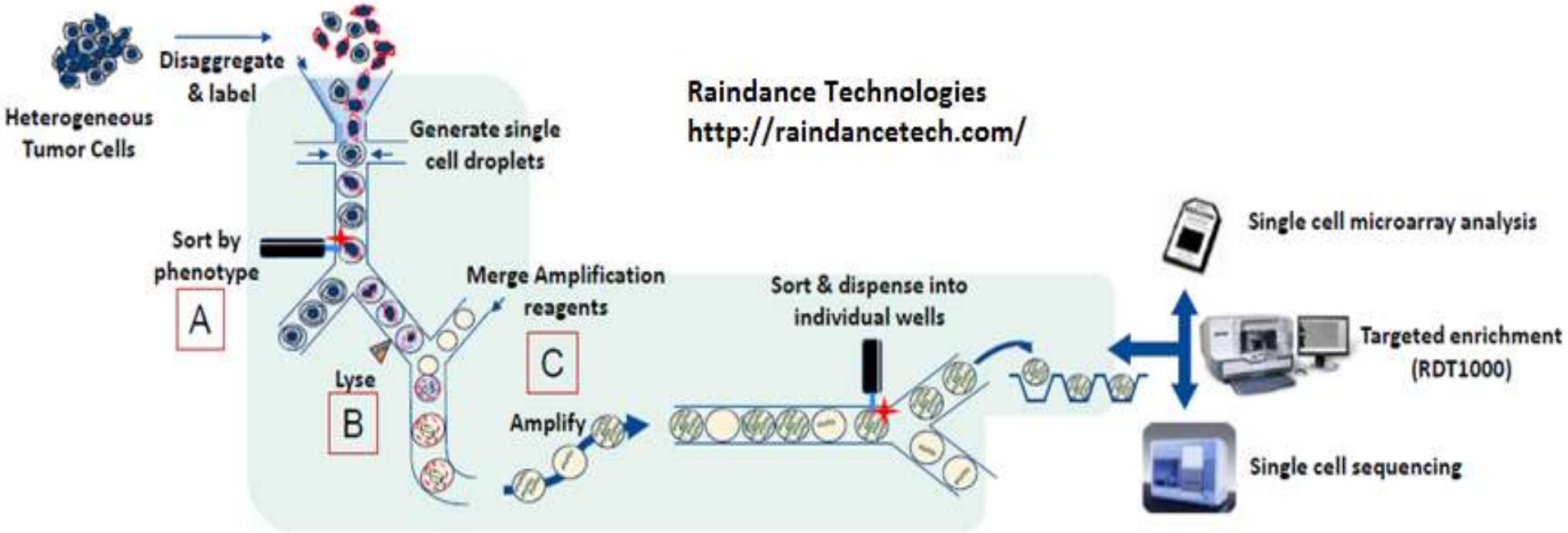
Virus courtesy of ViroFlow Technologies

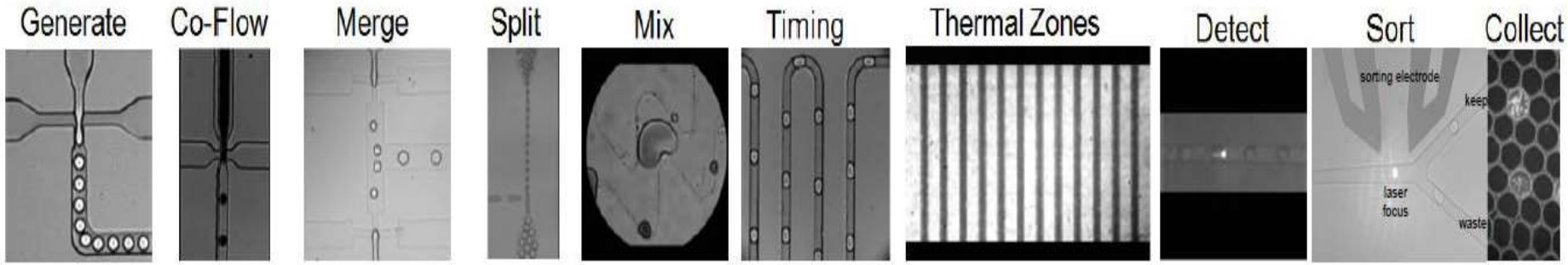
Conclusion

After more than 30 years of flow cytometry and work in academic institutions, several full spectrum systems have become available commercially. This provides new capabilities for discoveries in biology, higher quality in monitoring of biotechnological processes, and better patient care through clinical diagnostics and cellular therapy. Advanced software is making the technology directly accessible to the biological and medical researcher. This value has already been demonstrated by creating new insights rapidly into the virus immune system interaction in the present pandemic.

Thoughts About the Future

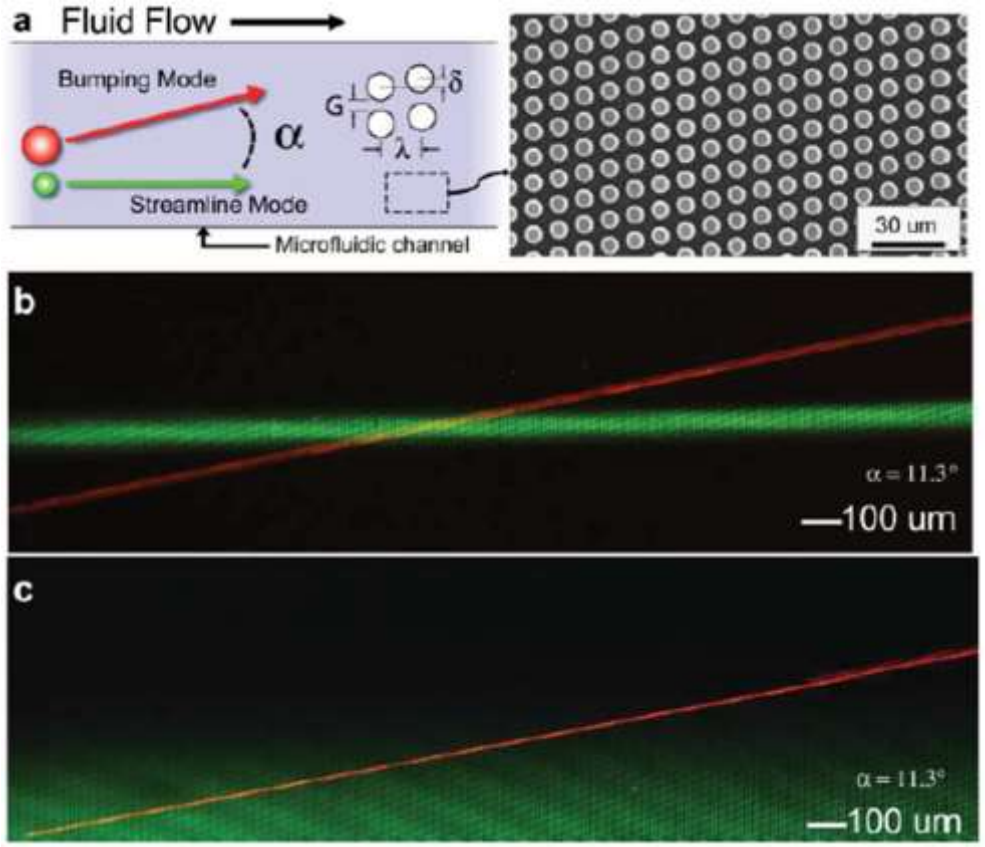
Droplet-based Integrated Bio-Assay System Technology





Automatable Sample Preparation

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

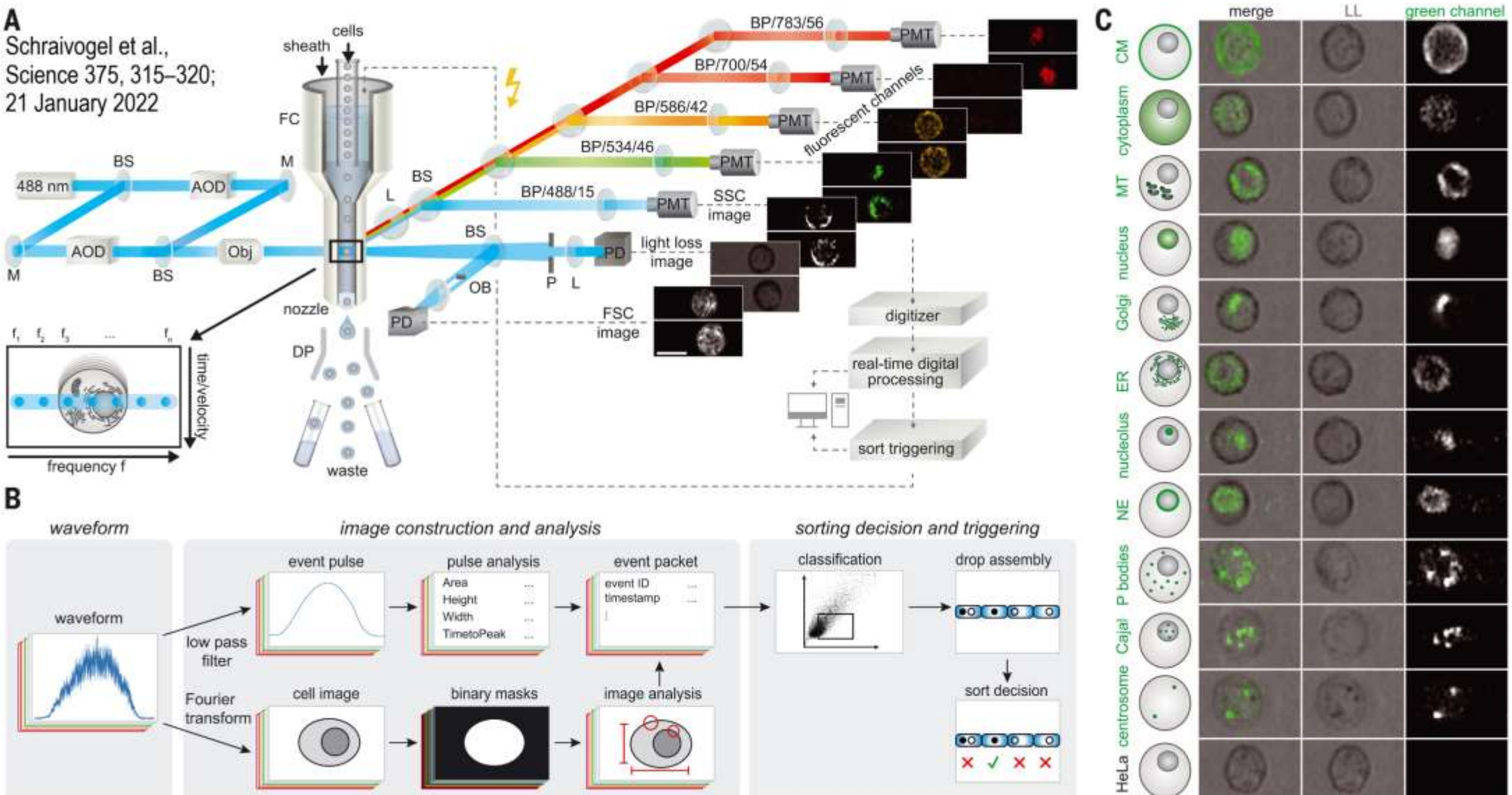


also:

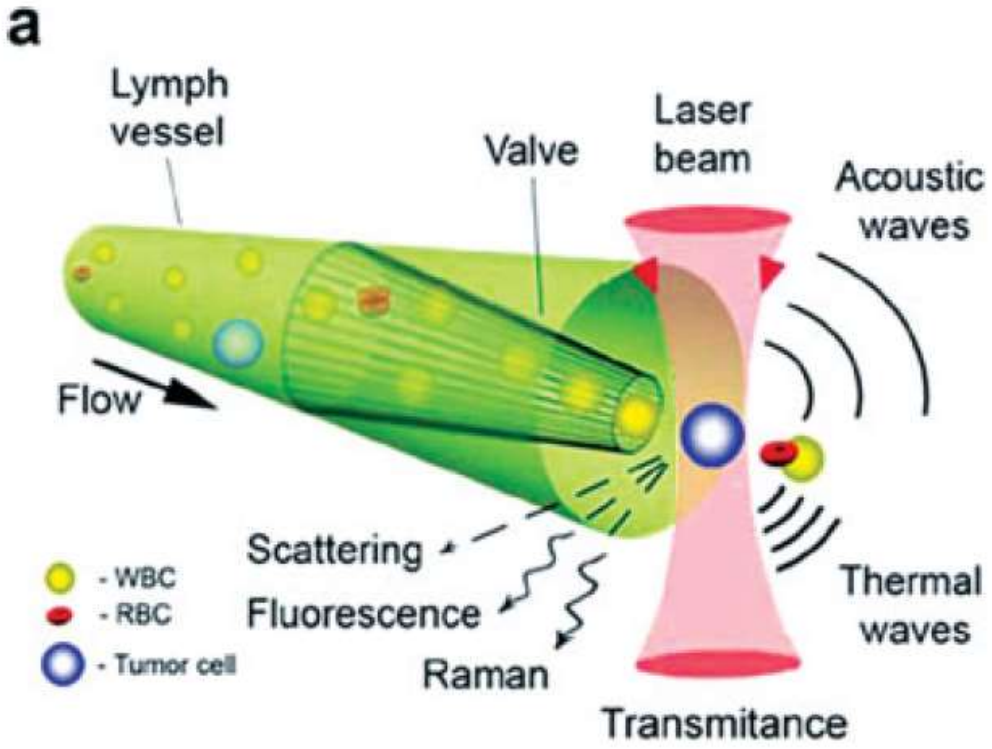
- acoustic focusing
- microfluidic filters
- inertial flow
- magnetic nanoparticles
- high density particles
- dielectropheresis
- optical traps
- …

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

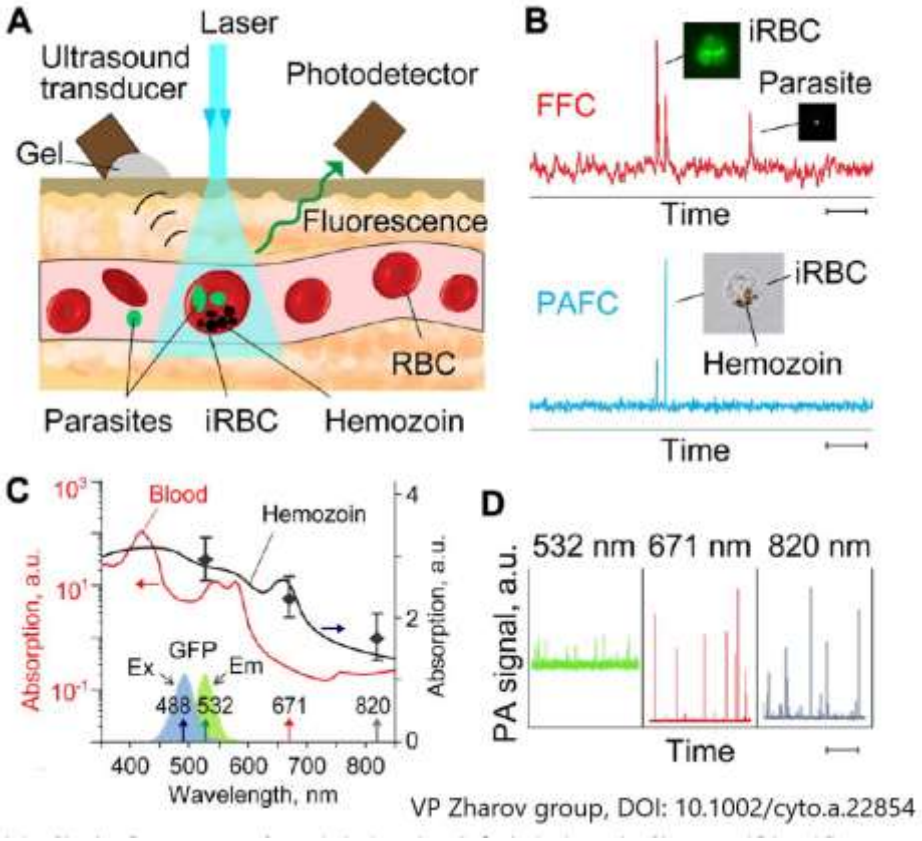
Flow Sorting based on Morphology



In-vivo Flow Cytometry

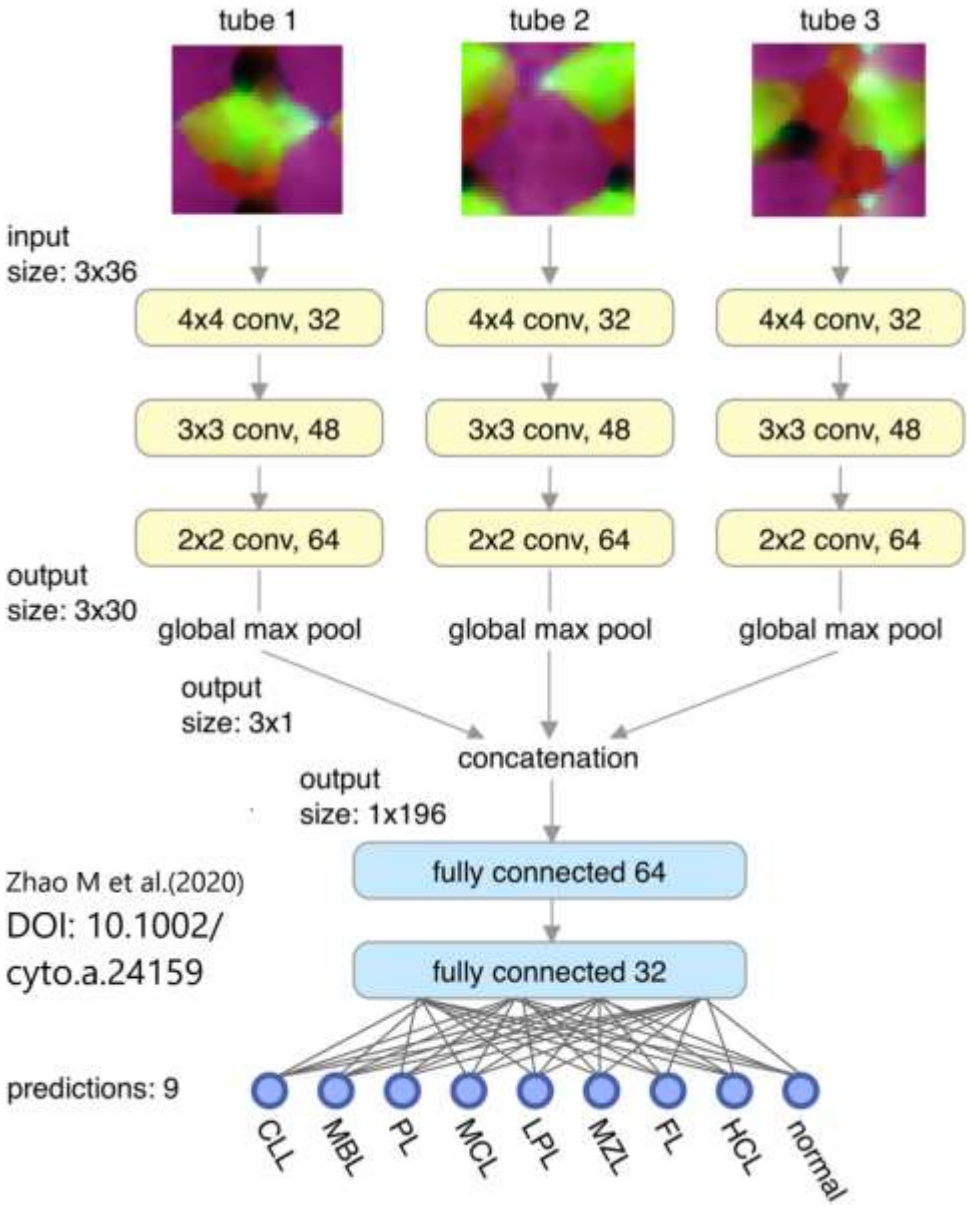


VP Zharov Group, DOI: 10.1002/cyto.a.20587



VP Zharov group, DOI: 10.1002/cyto.a.22854

Automated Data Analysis

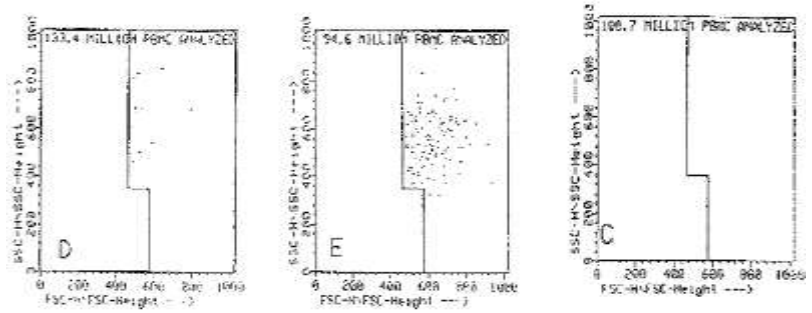


Zhao M et al.(2020)
DOI: 10.1002/
cyto.a.24159

- Algorithms for fully automated analysis
- Applications of artificial intelligence technologies incorporating biological and medical knowledge
- Even more advanced displays

Rare Cell Analysis

- Ag-specific T-cells
- Ag-specific B-cells
- Circulating epithelial cells
- Circulating endothelial cells
- Fetal cells in maternal blood
- …



10^{-6} **10^{-5}** **control**

Gross HJ et al, Cytometry 14 (1993) 519-526

Gross HJ et al, PNAS 92 (1995) 537-541

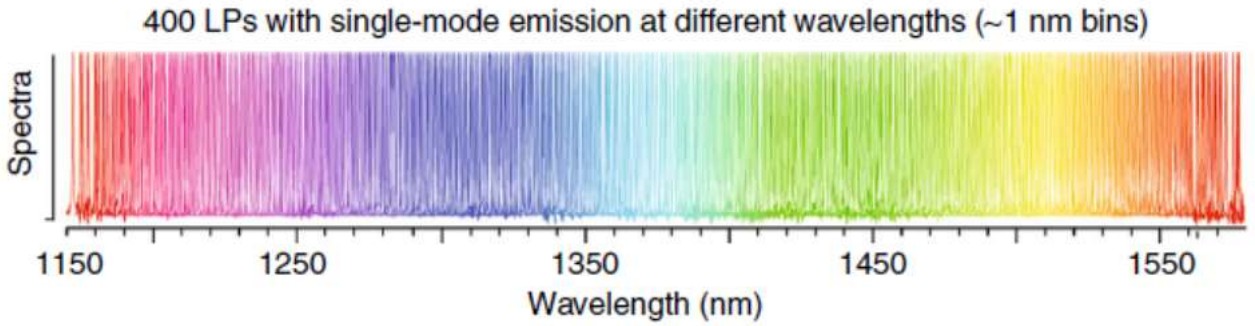
Limit of Detection

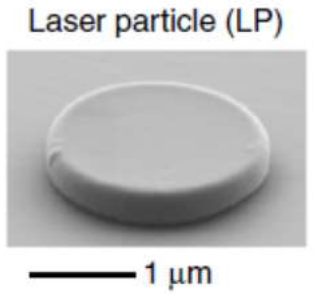
Routine >0.2%

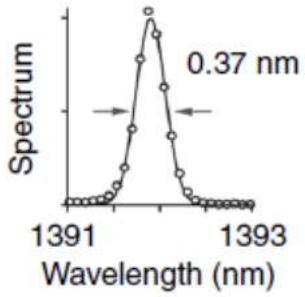
Optimized instrument >0.01%

Optimized system $>10^{-7}$

New Labels







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

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

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- Miltenyi Biotec

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