ABRF Online Workshop
The Future Forward in Flow Cytometry: Remembering the Past, Celebrating the Present, and Shaping the Future

Tuesday, December 6, 2022

The Association of Biomolecular Resource Facilities

Research • Technology • Communication • Education

Webinar Etiquette

- Please mute your microphones.
- You may remain on video if you would like.
- Please place questions in the chat.
- We will hold all questions until all speakers have presented.
- Each speaker will be presenting for 20 minutes.
- A panel will be held at the end of this presentation.
- Please raise your hand during the panel session, and the host will call on your name to ask your question or share your comment.

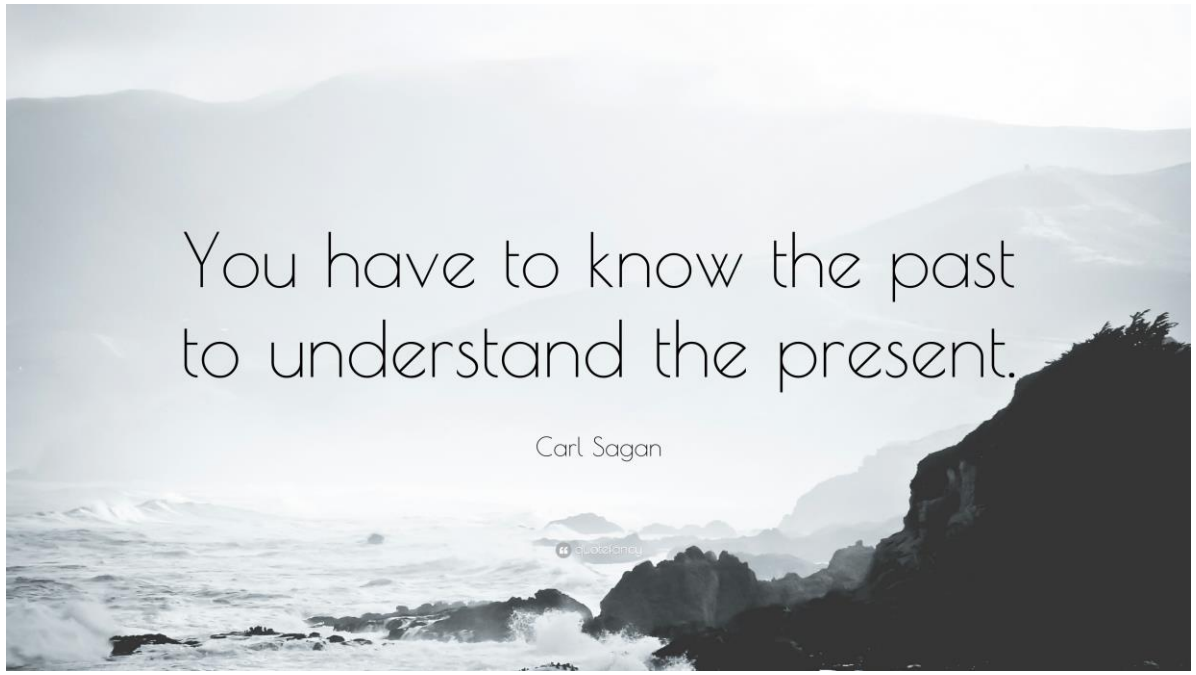
Todays Speakers

- Rachael Walker
  A Brief History of Flow Cytometry
- Rachael Sheridan
  Journey Through A Flow Cytometer
- David Leclerc
  Spectral Flow As A Transformative Technology
- Matthew Cochran
  Cell Sorting: The 10k Ft View
- Tim Bushnell
  The Future is Now!

Rachael Walker, PhD

- Ph.D. in Clinical Engineering from the University of Liverpool, where she was introduced to Flow Cytometry
- Director Babraham Institute Flow Cytometry Core Facility (2012)
- Chair of Mid-Anglia Cytometry Club
- Organizer of Flow Cytometry UK meetings
- ISAC Member, Member of Several Task Forces and Committees
- Elected to ISAC Council (2022-2026).
- Flow and Member of the Royal Microscopical Society (RMS)
- Awarded BBSRC Modular Training Program Grant in 2014 to write flow cytometry courses.
    - Trained over 1500 Scientists in the last 8 years.

https://www.babraham.ac.uk/flow



A brief history of Flow Cytometry*

Dr Rachael Walker

Babraham Institute, Cambridge, UK

* Apologises if anyone is missed

What is Flow Cytometry?

Flow cytometry is a technology that makes measurements on individual particles as they pass through a light source one by one.

There are thousands of Cytometers worldwide, including research, clinical and biopharma labs. They are used for clinical diagnosis; basic research; drug-screening; GMP sorting for patient stem cell transplants and many more

Fluorescence

Activated

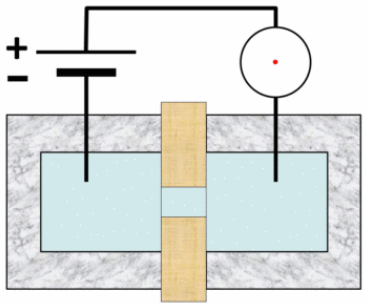
Cell

Sorter

More properly known as Flow Cytometry

Where it all began

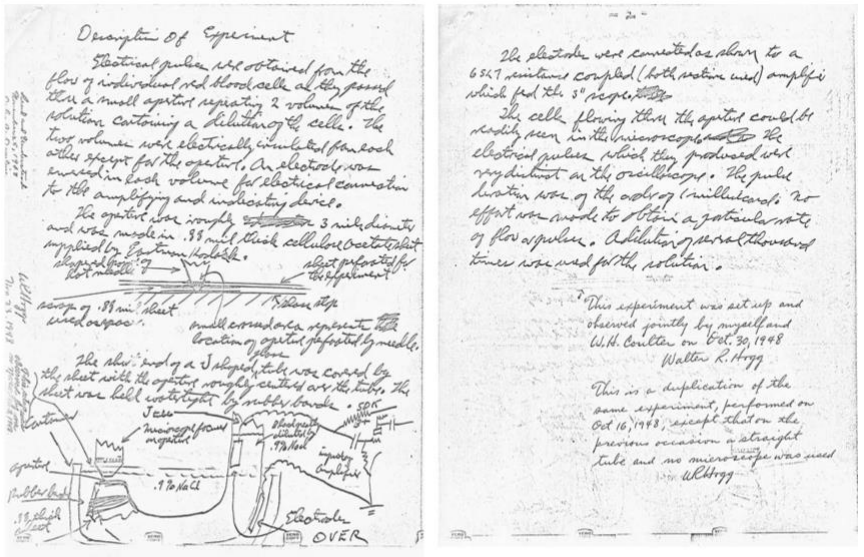
The Coulter Counter



Wallace Coulter - Patented the Coulter counter in 1953

The Coulter Principle and Counter counter detects the size and number of cells based on changes in electrical impedance when cells flow through an orifice

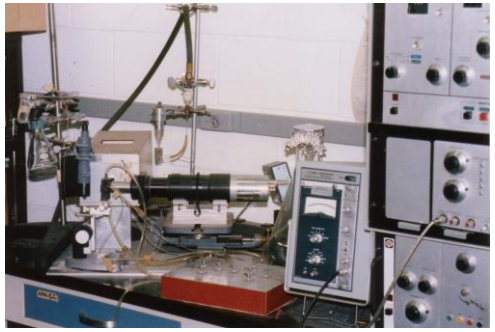
Coulter Counters are still found in all hospitals in the world and are used quick and accurate complete blood counts.



Coulter's lab book from 1948 showing experimental setup yielding electrical detection of red blood cell in diluted blood

https://patents.google.com/patent/US2656508A/en
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4237176/#
https://journals.sagepub.com/doi/10.1016/S1535-5535-03-00023-6

First Cell Sorter



Mack Fulwyler- Invented the first electrostatic cell sorter in 1965

Based on Dick Sweet's inkjet printer, 1965 and Coulter Counter

Electronic Separation of Biological Cells by Volume

Abstract. *A device capable of separating biological cells (suspended in a conducting medium) according to volume has been developed. Cell volume is measured in a Coulter aperture, and the cells are subsequently isolated in droplets of the medium which are charged according to the sensed volume. The charged droplets then enter an electrostatic field and are deflected into a collection vessel. Mixtures of mouse and human erythrocytes and a large volume component of mouse lymphoma cells were separated successfully. In tests with Chinese hamster ovary cells essentially all cells survived separation and grew at their normal rate.*

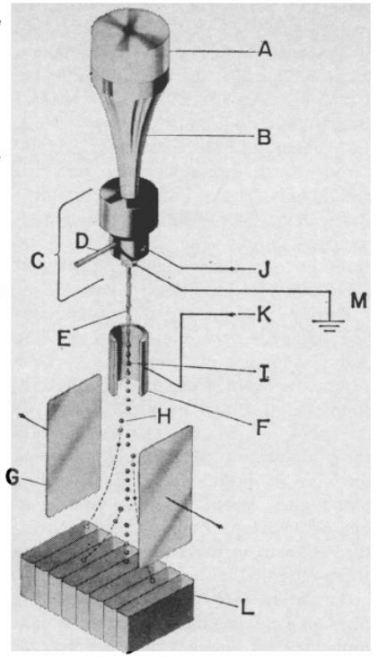


Fig. 1. Cell separator.

https://patents.google.com/patent/US3380584A/en
https://www.science.org/doi/epdf/10.1126/science.150.3698.910
http://www.cyto.purdue.edu/cdroms/cyto10a/media/video/Flowtheinvention.html

Figure taken from Fulwyler's Science paper in 1965 showing the first cell sorter

Len Herzenberg

Len Herzenberg – Added fluorescence to the sorter and commercialised, first paper in Science 1969

Used the white paper from the Fulwyler sorter and added fluorescence detectors

Reprinted from:

THE REVIEW OF SCIENTIFIC INSTRUMENTS VOLUME 43, NUMBER 3 MARCH 1972

Fluorescence Activated Cell Sorting*

W. A. BONNER, H. R. HULETT, R. G. SWEET, AND L. A. HERZENBERG
Department of Genetics, Stanford University School of Medicine, Stanford, California 94305

(Received 6 October 1971; and in final form, 22 November 1971)

An instrument has been developed for sorting biological cells. The cells are rendered differentially fluorescent and incorporated into a small liquid stream illuminated by a laser beam. The cells pass sequentially through the beam, and fluorescent light from the cells gives rise to electrical signals. The stream is broken into a series of uniform size drops downstream of the laser. The cell signals are used to give appropriate electrostatic charges to drops containing the cells. The drops then pass between two charged plates and are deflected to appropriate containers. The system has proved capable of providing fractions containing large numbers of viable cells highly enriched in a particular functional type.

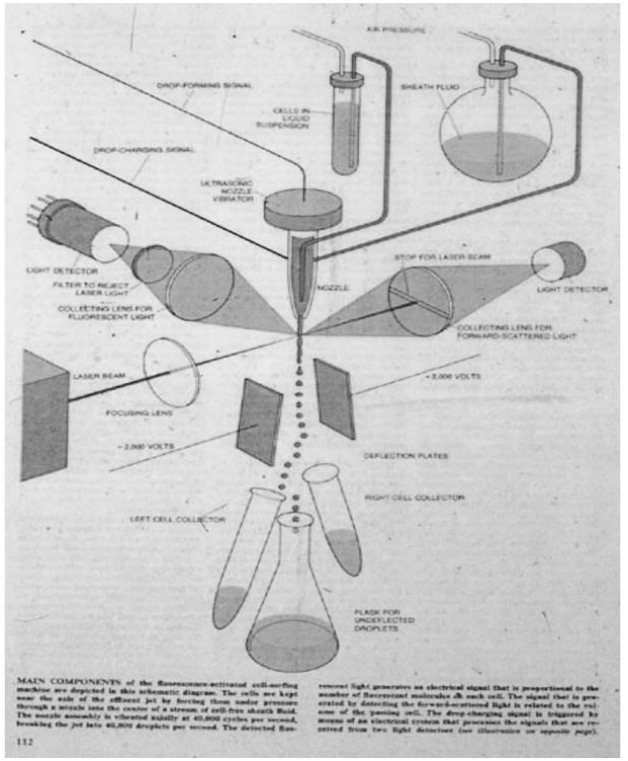


Figure taken from 1974 Scientific America paper showing 1 laser and 2 detectors, one for forward scatter and 2nd for florescence

The fundamentals behind these machines have not changed much since.

https://www.nature.com/articles/504034a
https://herzenberglab.stanford.edu/sites/g/files/sbiybj10046/f/publications/lah048.pdf
http://www.cyto.purdue.edu/cdroms/cyto10a/cytometryhistory/individualhistories/herzenberg.html

Fluorescence Activated Cell sorters: FACS machines

BD commercialised the Fluorescence Activated Cell sorter in 1976

FACS II measured FSC and 530nm fluorescence

The FACS IV was released in 1979 and was the first dual laser system with an additional 568nm krypton laser

These systems have evolved over the next 40 years to the systems we have today

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3279584/
https://www.bd.com/resource.aspx?idx=7301

Applications

Over the years machines were modified and created to help scientists with certain applications…

Chromosome sorting

The Lawrence Livermore National Laboratory in the mid-1980's created the first high speed cell sorter prototypes, for human chromosome separation.

Dual Beam, droplet sorters creating droplets at rate of 220,000 per second &capable of sorting 20,000 events per second.

High-Speed Chromosome Sorting

J. W. Gray, P. N. Dean, J. C. Fuscoe, D. C. Peters, B. J. Trask, G. J. van den Engh, M. A. Van Dilla

Use combination of Hoescht 33258 and Chromomycin A3 to identify the individual chromosomes which can then be sorted

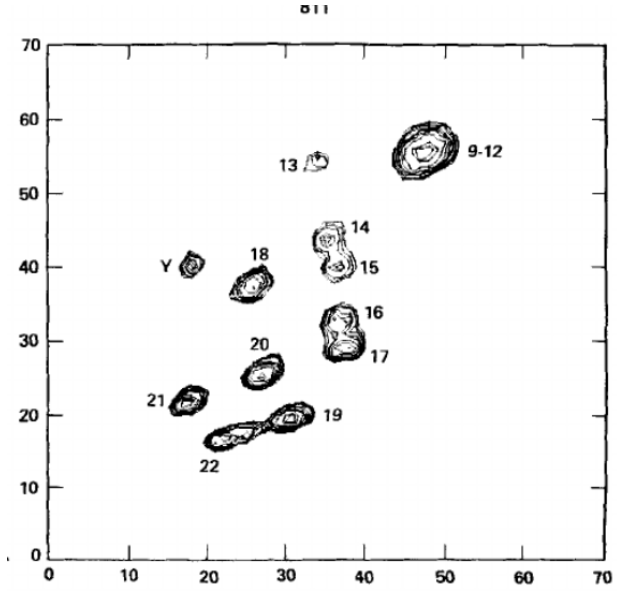
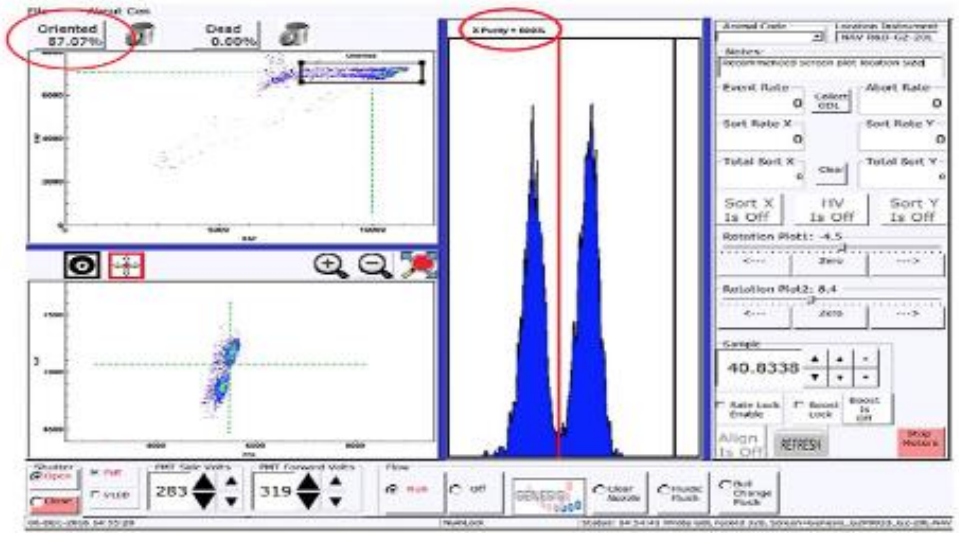


FIG. 7. Contour plot of human chromosomes isolated from the 811 cell line, stained with Hoechst 33258 (2 μg/ml) and Chromomycin A3 (40 μg/ml) and measured with the sorter operating in the dual-beam mode. The analysis rate was 15,000 s^{-1}. The chromosomes producing each peak are indicated on the plot.

https://onlinelibrary.wiley.com/doi/epdf/10.1002/cyto.990060404
https://www.science.org/lookup/doi/10.1126/science.2443974

Figure showing chromosome staining from Peters, 1985

Sex Selection



Cytomation (created MoFlo high-speed cell sorter) , in collaboration with Colorado State University Research Foundation created XY.Inc in 1996.

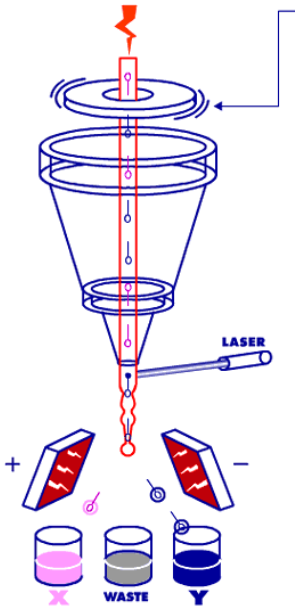


Image from Sexing Technologies & Brito, 2019

Uses FSC and Hoechst 33342

https://www.sexingtechnologies.eu/sorting_process
https://irp-cdn.multiscreensite.com/d25b3de0/files/uploaded/Clinical%20Theriogenology%2011%2C%202019%20%28sexed%20semen%20production%29.pdf

Cell Cycle

The basis of the simple cell cycle studies were do today came from a series of experiments done on early cytometers in late 60's and early 70's.

A lot of work done by Darzynkiewicz looking at DNA, RNA with a range of probes including Acridine Orange. He developed TUNEL and FLICA assays.

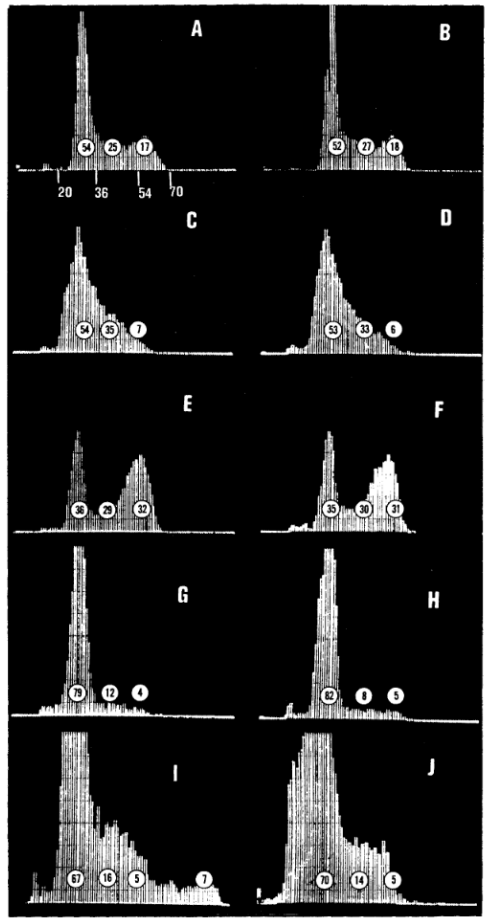


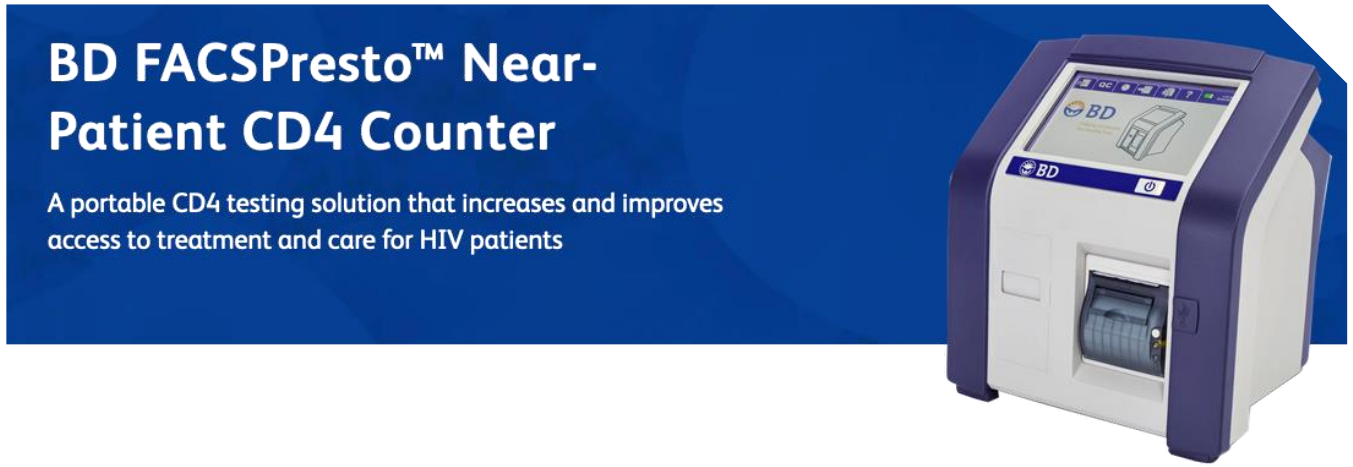
FIGURE 1 Shows DNA per cell frequency distribution histograms of various synchronized populations of CCRF-CEM lymphoblasts in culture (Fig. 1 A–F), and human bone marrow samples (Fig. 1 G–J). Histograms on the left are of samples stained with propidium iodide after fixation in methanol and digestion in RNase, while those on the right side are of identical samples after 5 min of incubation with propidium iodide. Numbers at the bottom of histogram 1 A record the position of the channels, while those recorded in circles on the histograms represent percentage of cells between channels 20–35 (G_1-early S DNA content), 36–53 (S), and 54–70 (late S-G_2-M).

Figure from Krishan, 1975. Showing DNA histograms

https://onlinelibrary.wiley.com/doi/10.1002/cyto.a.20003
https://rupress.org/jcb/article-pdf/66/1/188/1071572/188.pdf
https://onlinelibrary.wiley.com/doi/10.1002/cyto.a.24043

HIV research &diagnosis

The beginning of the AIDS pandemic in mid-1980s coincided with a spike in scientific publications using flow cytometry



Flow cytometry has been vital in detecting a decrease in CD4 T-Cell population. Still used a clinical diagnosis tool especially in Sub-Saharan Africa.

Flow is also used to confirm that subsets of CD4+ T cells were targeted by HIV and other immunological studies. Detection and progression of HIV to AIDS is done by flow.

https://academic.oup.com/labmed/article/46/3/e59/2657953
https://www.nature.com/articles/nrmicro1998
https://www.mybeckman.uk/resources/research-areas/hiv/cd4-cell-counting

President Obama, NIH, Maryland, 2014

Flow is everywhere

- Marine, Space
- Food safety – levels of bacteria and yeast in food
- Blood Doping

Astronaut Chris Hadfield on ISS with Microflow flow cytometer

https://www.youtube.com/watch?v=X6K3kQJQ-K4
https://academic.oup.com/jimb/article/36/8/999/5993588
https://onlinelibrary.wiley.com/doi/epdf/10.1002/ajh.21196

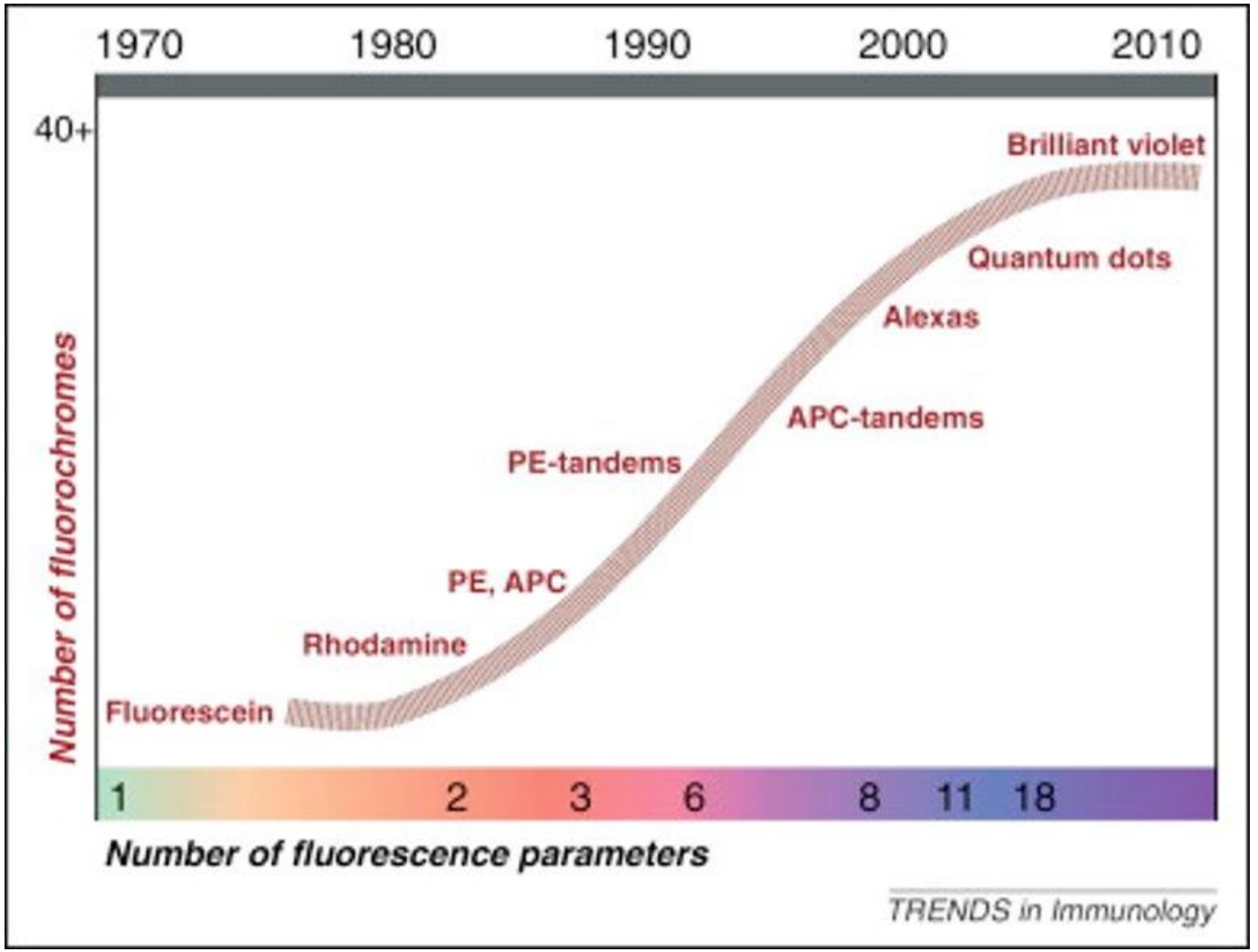
Give me more more more…

Fluorochrome Technology

Increase in recent years in number of fluorochromes available.

Mario Roederer's lab produced the first 17 colour panel in 2005

Paper from Bendall *et al* claimed 18 colours was maximum



https://pubmed.ncbi.nlm.nih.gov/15286731/

History of Fluorochrome development, Bendall *et al* 2012

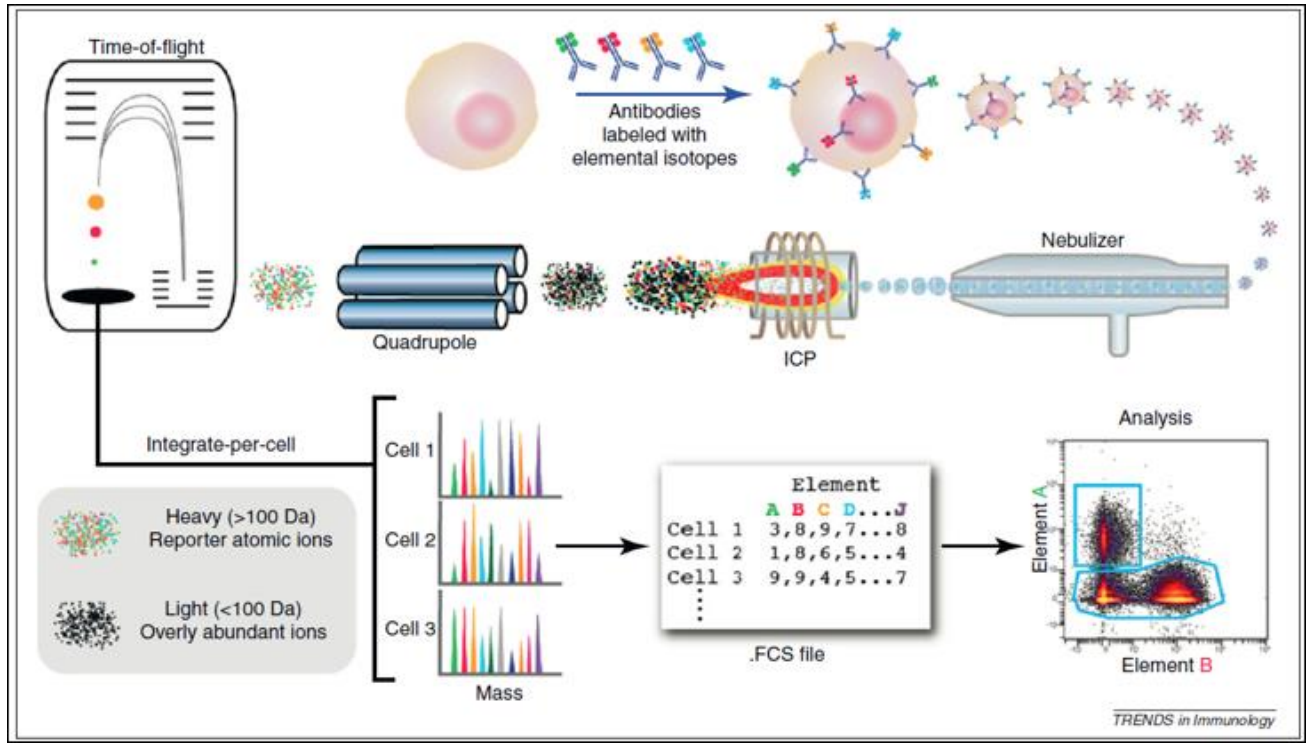
CyTOF

Cytometry by Time of Flight – marries cytometry and Mass Spectroscopy.

Uses stable metal isotopes conjugated to antibodies.
The Helios can use metals 79 to 209, so allows ~100 parameters to be measured.

In reality ~40 parameters are used.

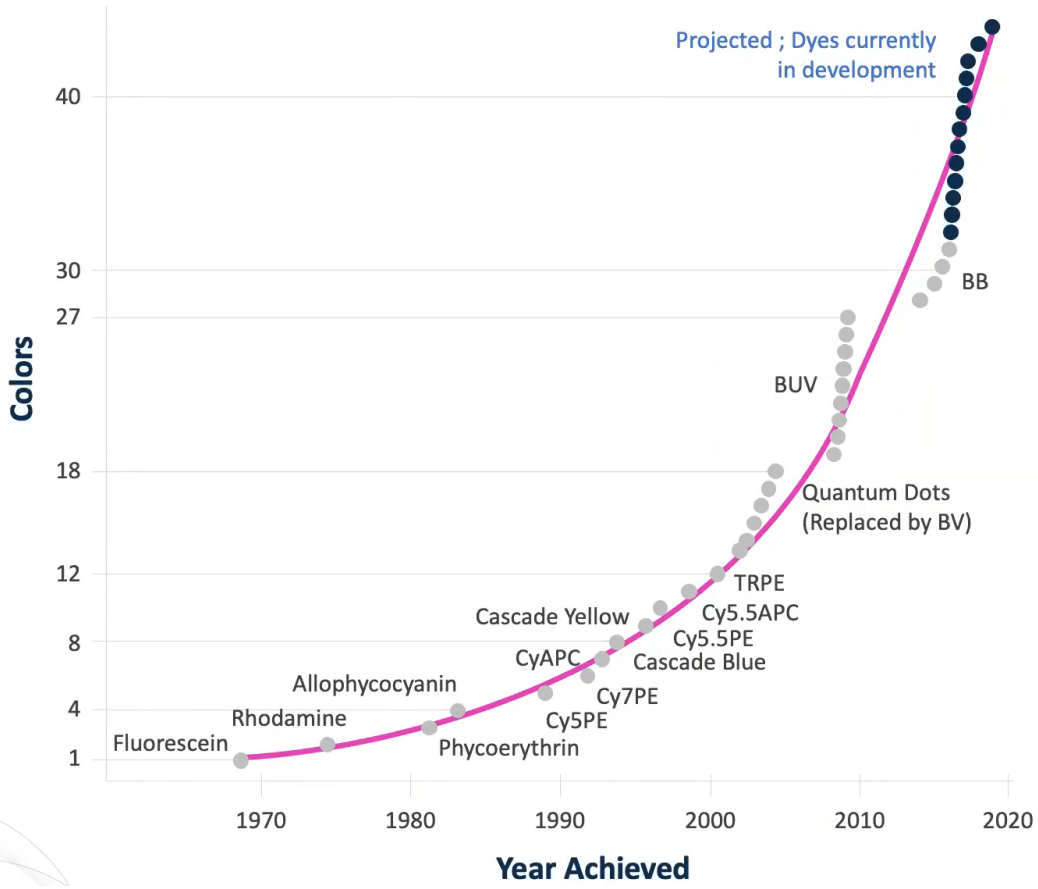
CyTOF is an expensive instrument to set up and run and a lot of sample is lost in the process



https://pubmed.ncbi.nlm.nih.gov/22476049/

Fluorochrome Technology

Increase in recent years in number of fluorochromes available, which has led to 40+ colour cytometry being carried out.



Roederer's Law for Flow Cytometry :

The number of colors doubles every 10 years

Figure from Phitonex

BD launched the Symphony instruments to be able to see the Brilliant dyes.

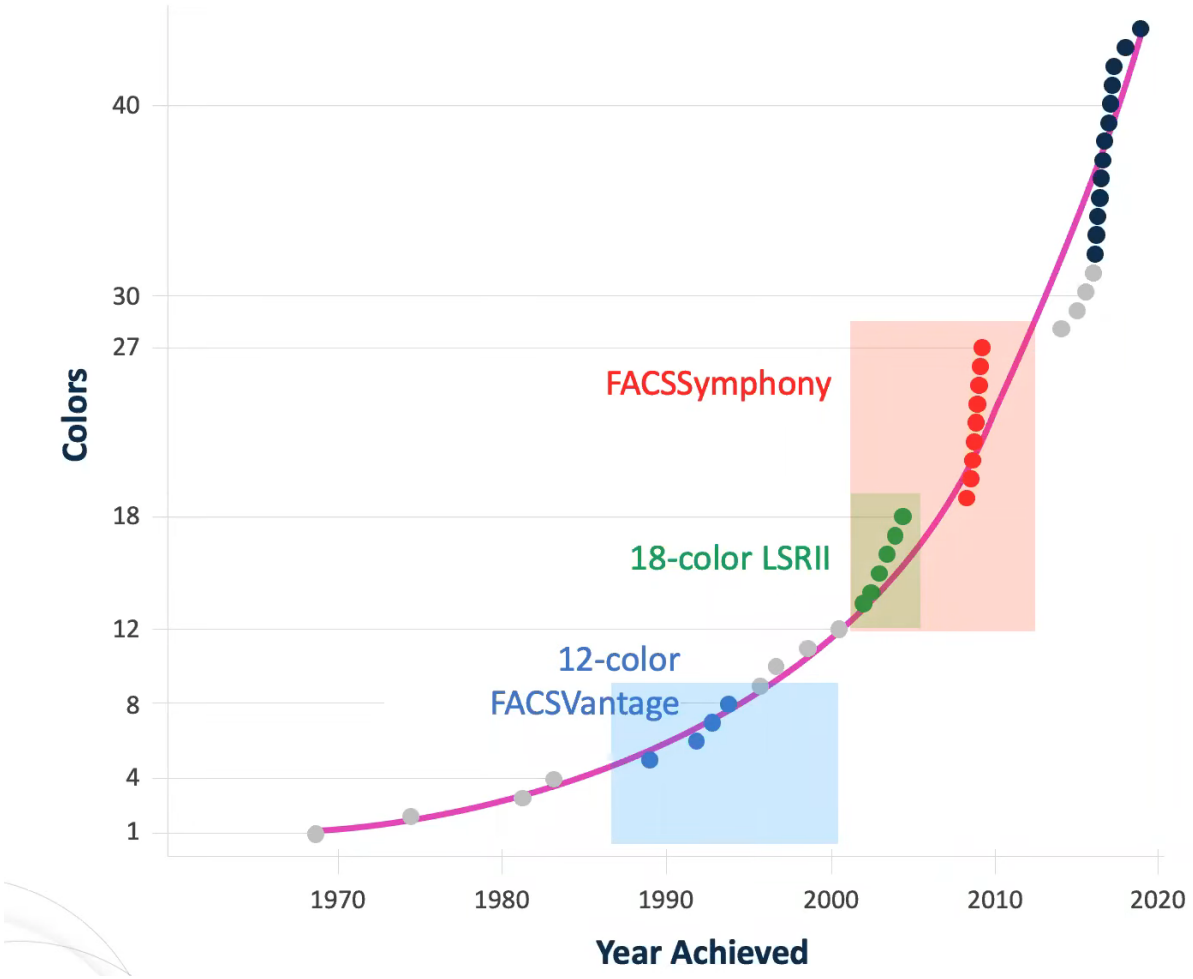
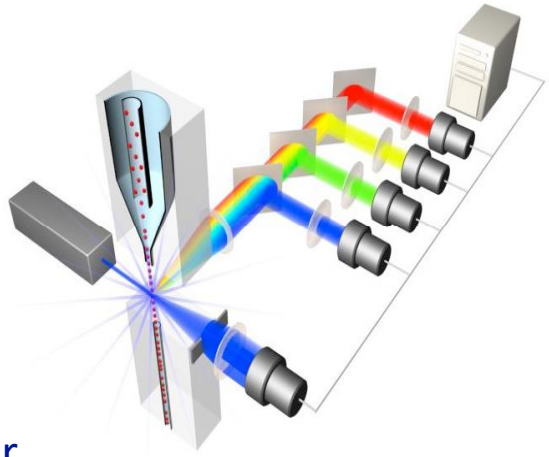


Figure from Phitonex

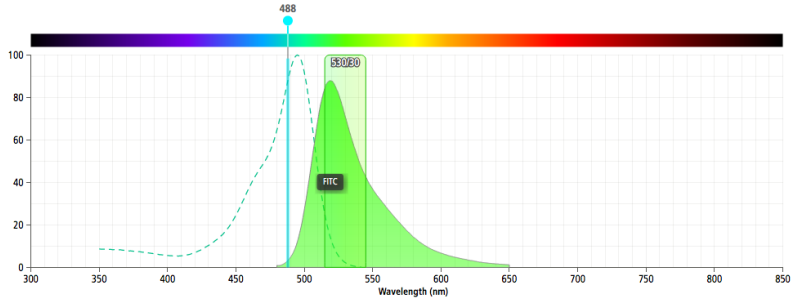
https://www.youtube.com/watch?v=evFfvH_3QuQ

Spectral flow

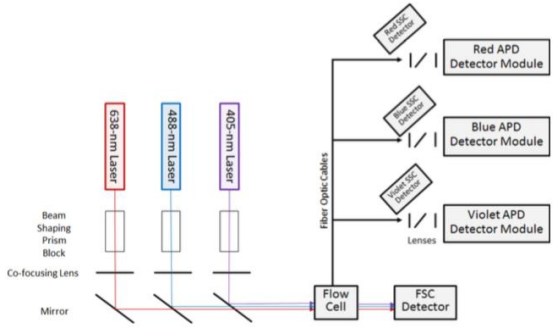
Conventional Flow Cytometers

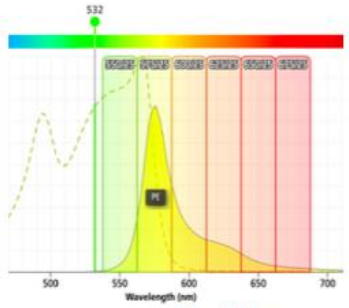


Each detector collects photons within a certain wavelength window (using bandpass filters e.g. 530/30)

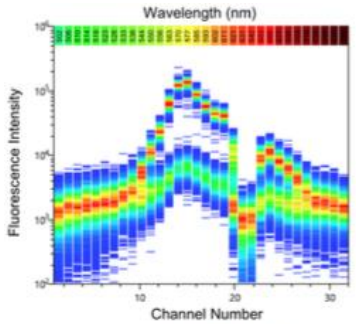


• Spectral Analysers





Adapted from BD Biosciences



Adapted from Sony Biotechnology

Spectral Analysers collect photons across an array of detectors across the whole spectrum (400nm- 800nm)

http://www.cyto.purdue.edu/Spectral%20Flow%20Cytometry
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3556726/pdf/nihms-433336.pdf
https://currentprotocols.onlinelibrary.wiley.com/doi/epdf/10.1002/cpz1.222

Getting more from each cell

Increase in recent years in number of fluorochromes available, which has led to 40+ colour cytometry being carried out.

OMIP

ISAC CYTOMETRY Journal of Quantitative Cell Science PART A

MIFlowCyt

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

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

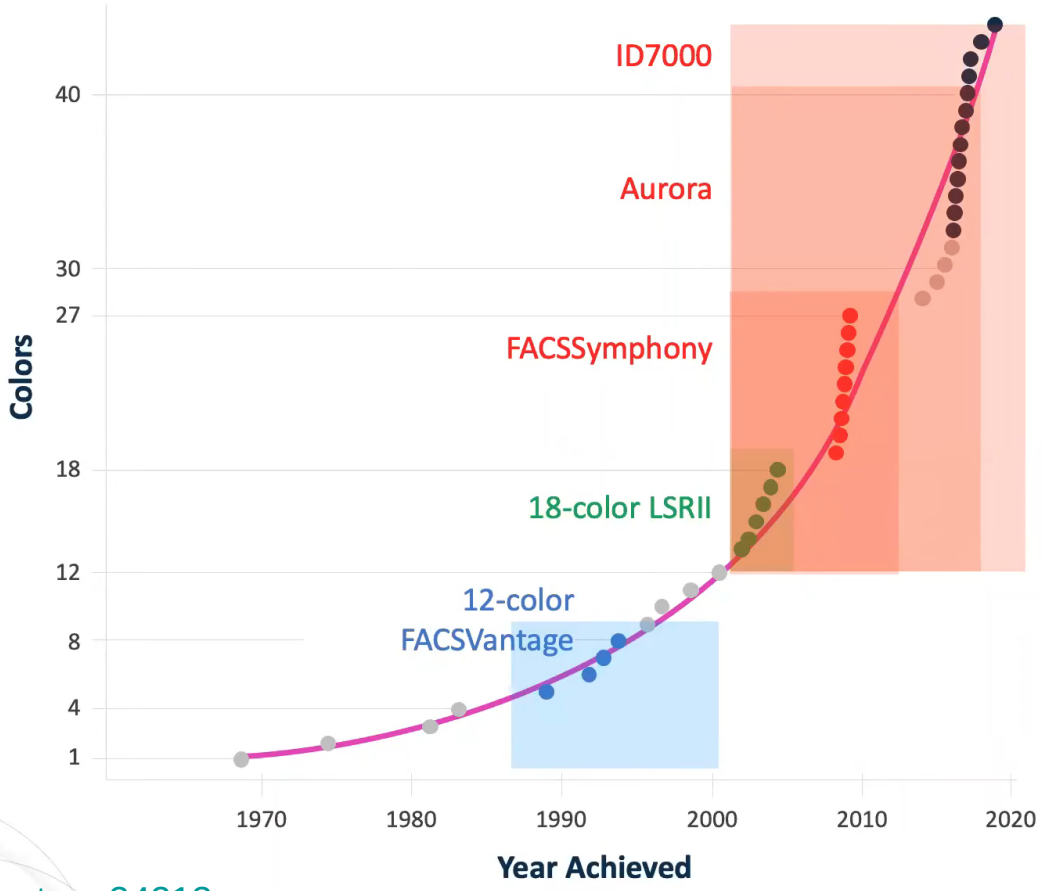
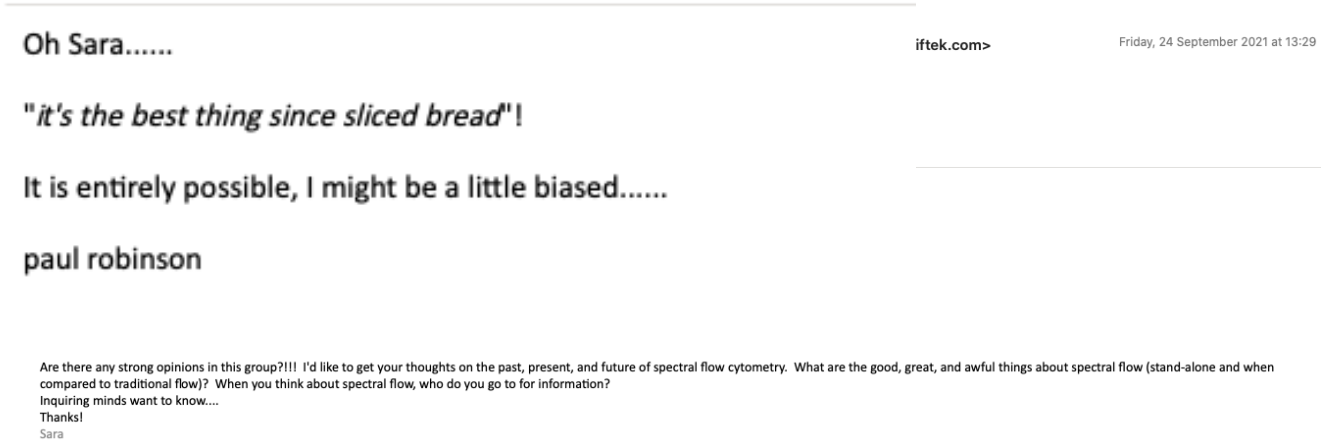


Figure from Phitonex

https://onlinelibrary.wiley.com/doi/epdf/10.1002/cyto.a.24213

The future…

Garry Nolan Tweet, May 2020



Paul Robinson, email, September 2021

https://www.nature.com/articles/s42003-020-0896-2.pdf

Babraham Institute FLOW CYTOMETRY

Rachael Walker – rachael.walker@babraham.ac.uk

Flow Cytometry Facility, Michael Wakelam Building, Babraham Institute, Cambridge, UK

Rachael Sheridan, PhD, SCYM(ASCPP) ^{CM}

- Director of Flow Cytometry Core Facility at Van Andel Institute in Grand Rapids, MI
- Worked in a Flow Core since 2013
- ISAC emerging leader (2020-2024)
- Member ABRF FCRG
- GLIIFCA Board of Directors
- Interest in Flow Cytometry Education, Process Improvement, Intersectionality of flow, and other technologies (ex. Genomics and metabolomics)

VAN ANDEL INSTITUTE®

Journey Through a Flow Cytometer

Rachael Sheridan, PhD SCYM(ASCP)^{CM}

Director, Flow Cytometry Core

Van Andel Institute, Grand Rapids, MI

ISAC SRL Emerging Leader 2020-2024

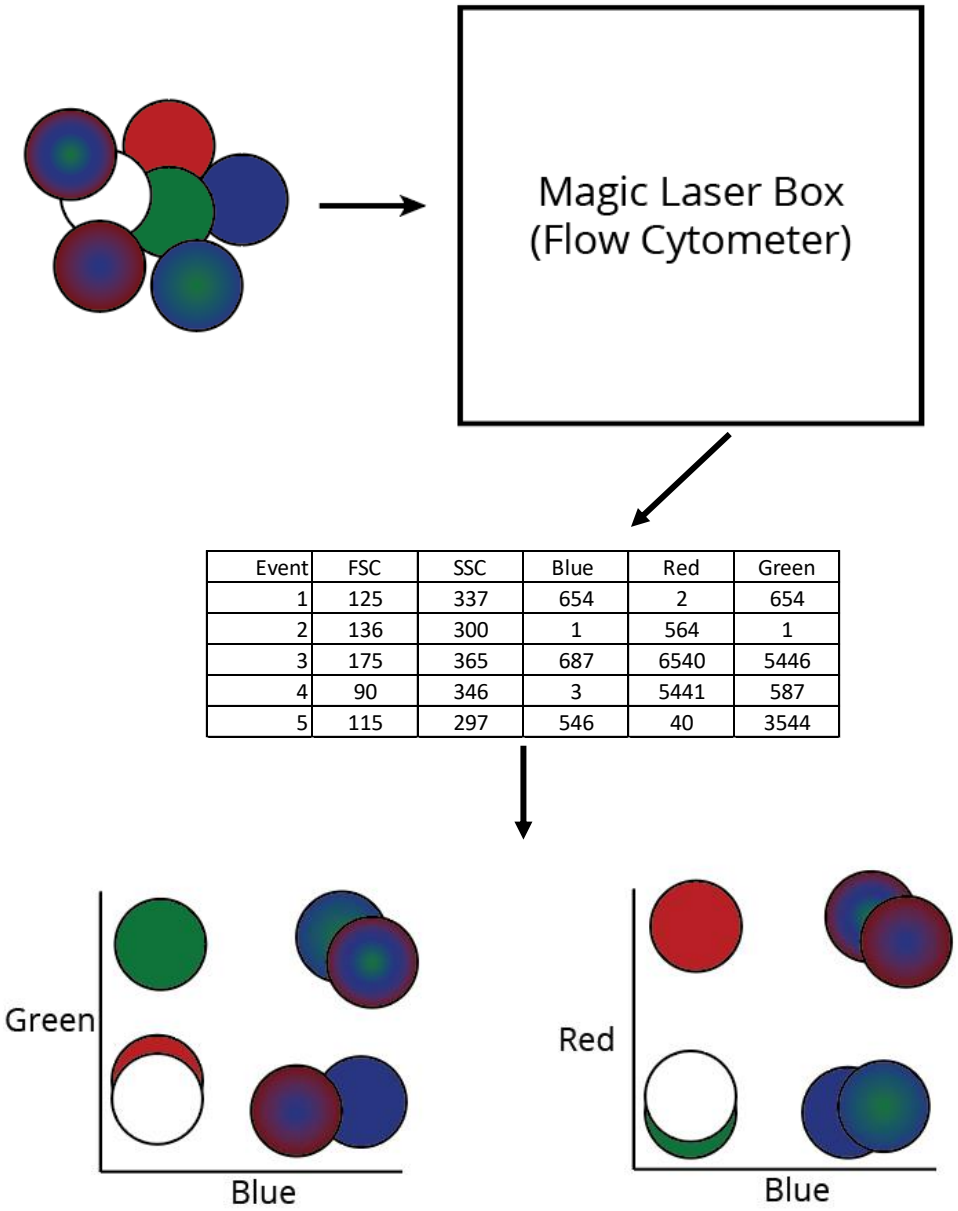
Flow Cytometry: The Original (high throughput) Single Cell Technique

The central goal of Cytometry is to unravel the complexity of biology and to identify and analyze the extremely diverse phenotype and function of individual cells in complex cell systems.

Cytometry A 2008:73A(4):333-340

Overview

- Fluorescently labeled SINGLE CELL sample
  - Labeled antibodies
  - Functional dyes
  - DNA dyes
  - Other
- Flow cytometer reads barcode and generates single cell data file
- Data analysis can tell you which barcodes are present and how many of each



Event	FSC	SSC	Blue	Red	Green
1	125	337	654	2	654
2	136	300	1	564	1
3	175	365	687	6540	5446
4	90	346	3	5441	587
5	115	297	546	40	3544

So, what's in the box?

- Fluidics
  - Move sample through instrument
  - Focus cells for analysis
- Optics
  - Excite fluorochromes
  - Separate and capture photons
- Electronics
  - Convert electronic signals into usable data
  - Control critical instrument timing/sensitivities

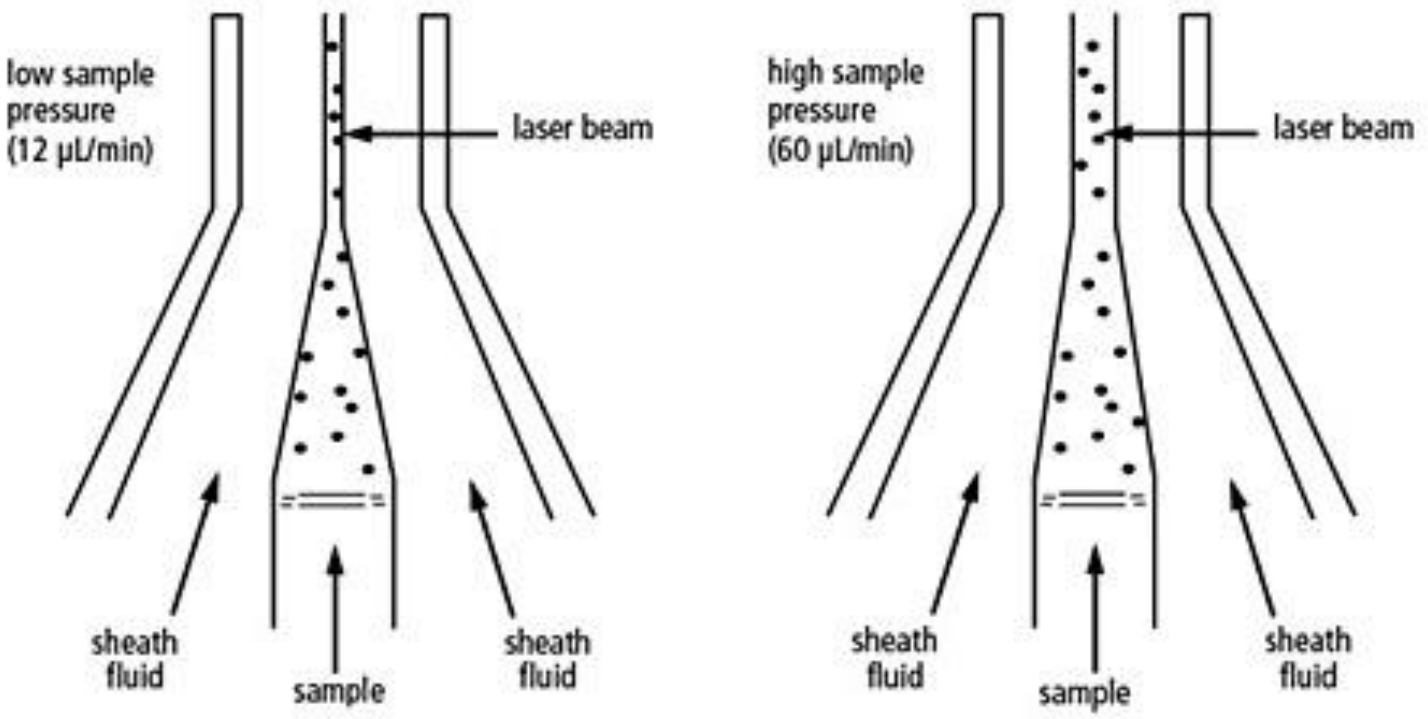
https://www.123rf.com/photo_119519466_mystery-box-isolated.html?vti=nwhehqxo55ql9focrj-1-38

Get in line! Hydrodynamic Focusing

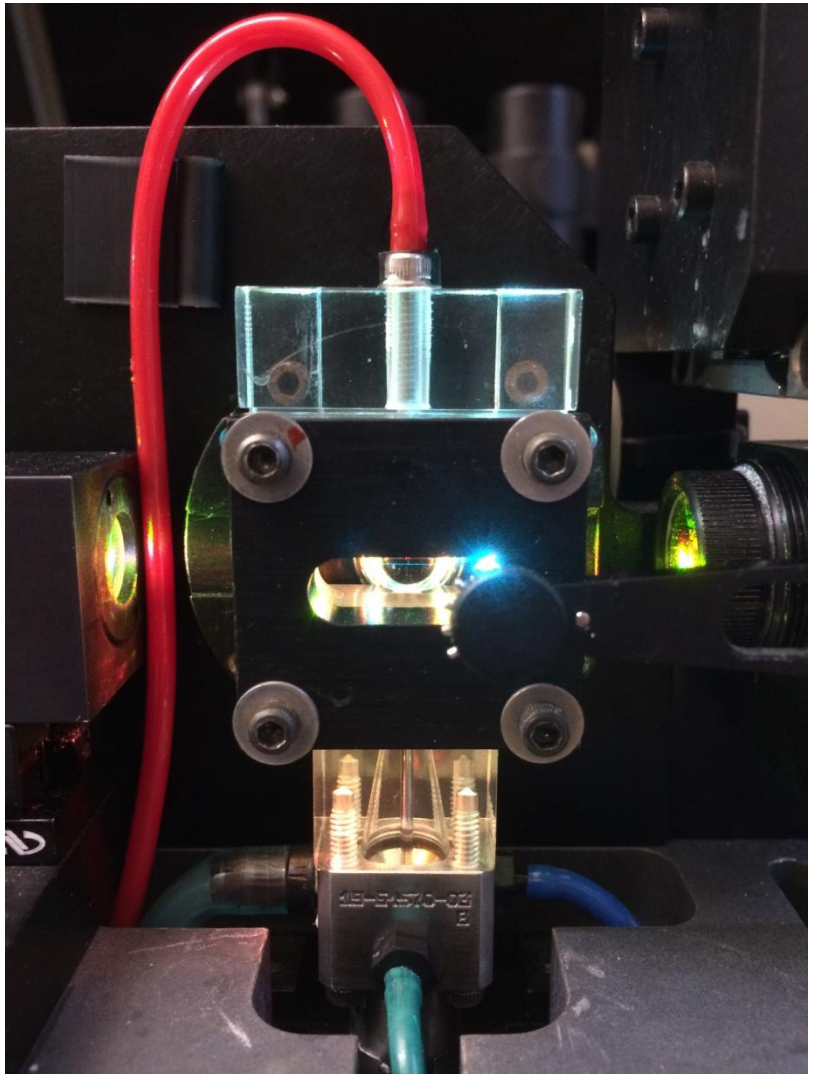
- Constant pressure on sheath fluid
- Slightly higher sample pressure allows it to push through
- Laminar flow!
- Delivers sample to interrogation point in a (mostly) orderly fashion

The Hobbit: The Desolation of Smaug

Anatomy of a flow cell

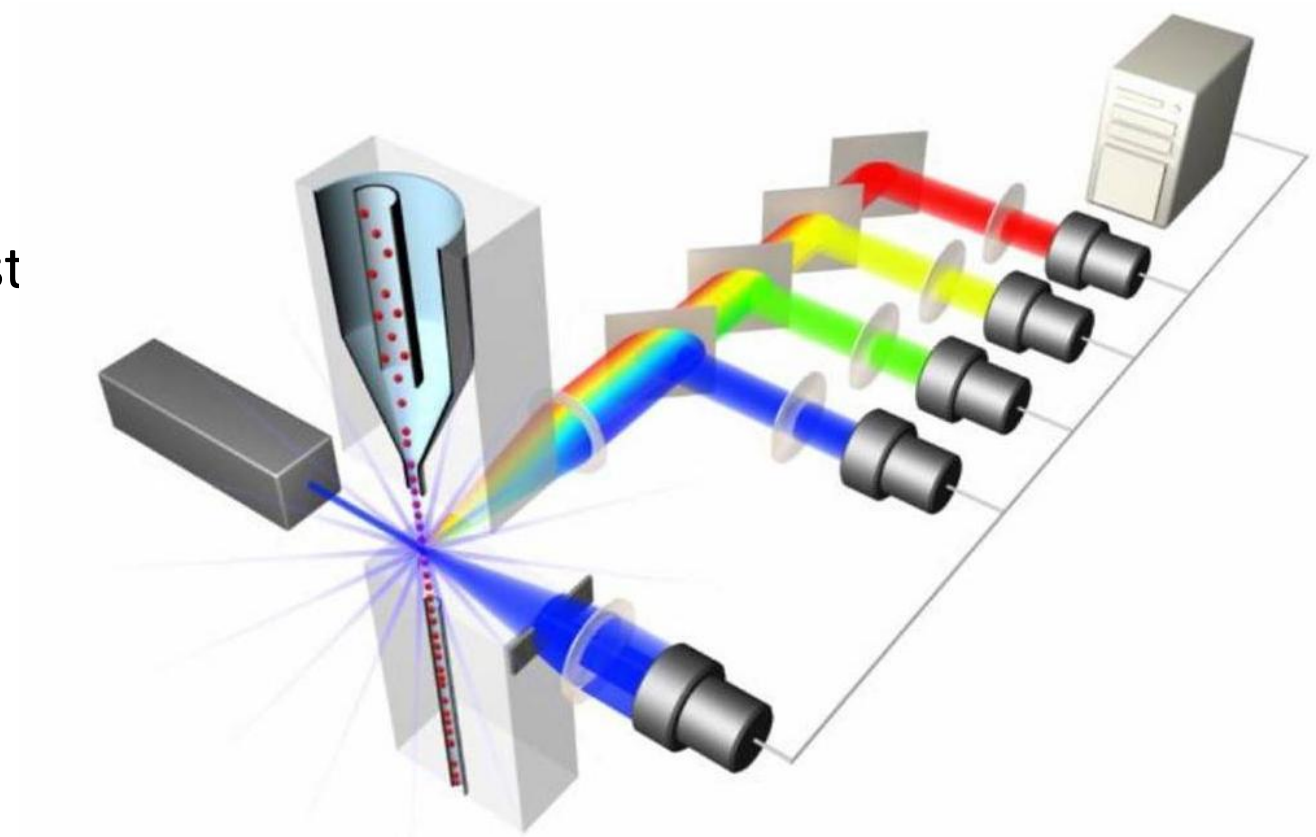


HTTP://WWW.BDBIOSCIENCES.COM/ANZ/WCMIMAGES/FACSCANTO_FLUIDICS_LRG.JPG



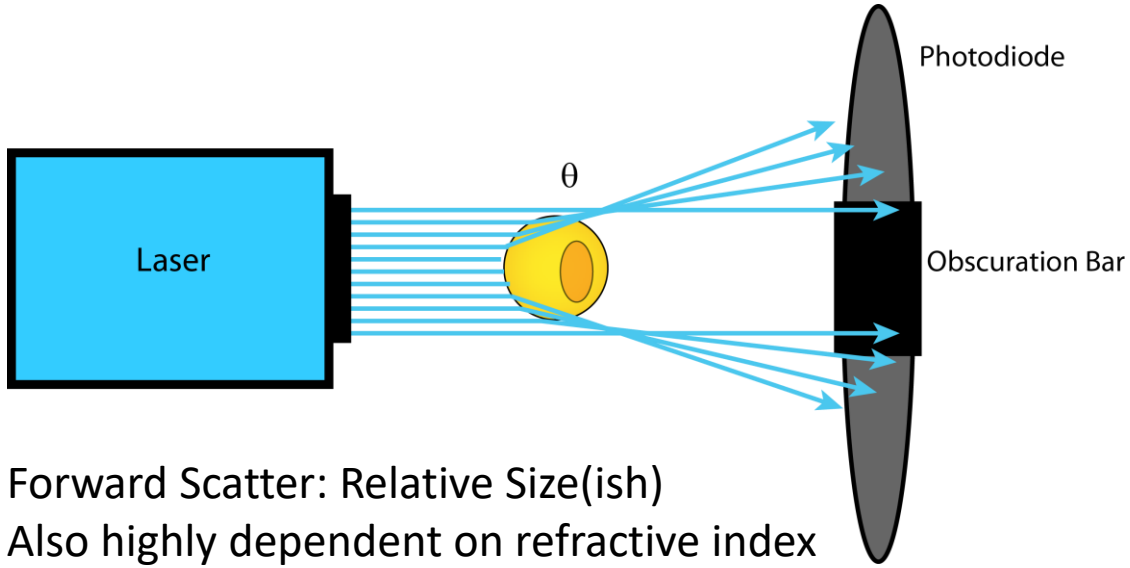
Follow those photons! The optical system

- Excitation source(s)
  - Lasers! Typically, 1-5 of them.
- Optical glass
  - Isolate wavelengths of interest
- Photon detectors
  - Photodiode
  - Avalanche Photodiode
  - Photomultiplier tubes
  - CCD (ImageStream)

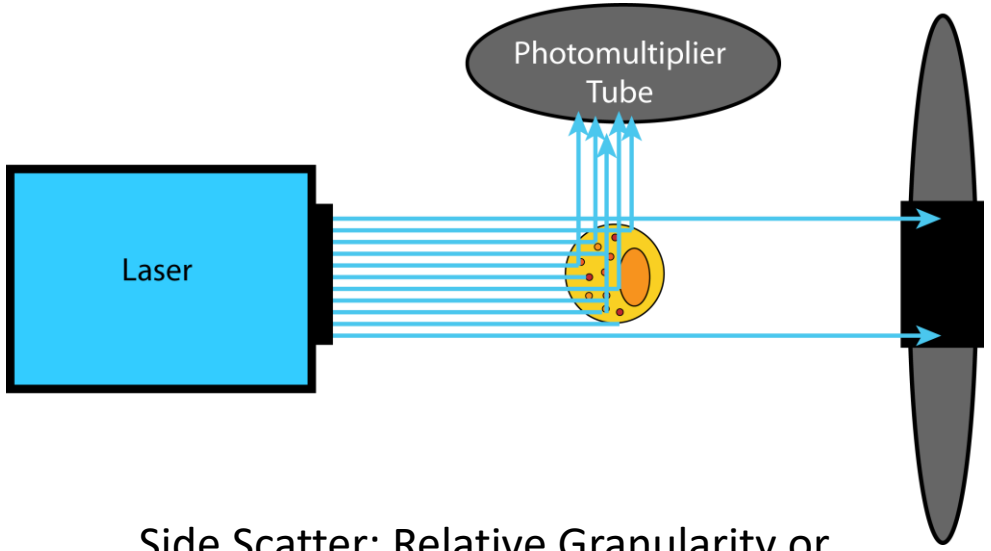


LifeTech

Label-free measurement: Scatter

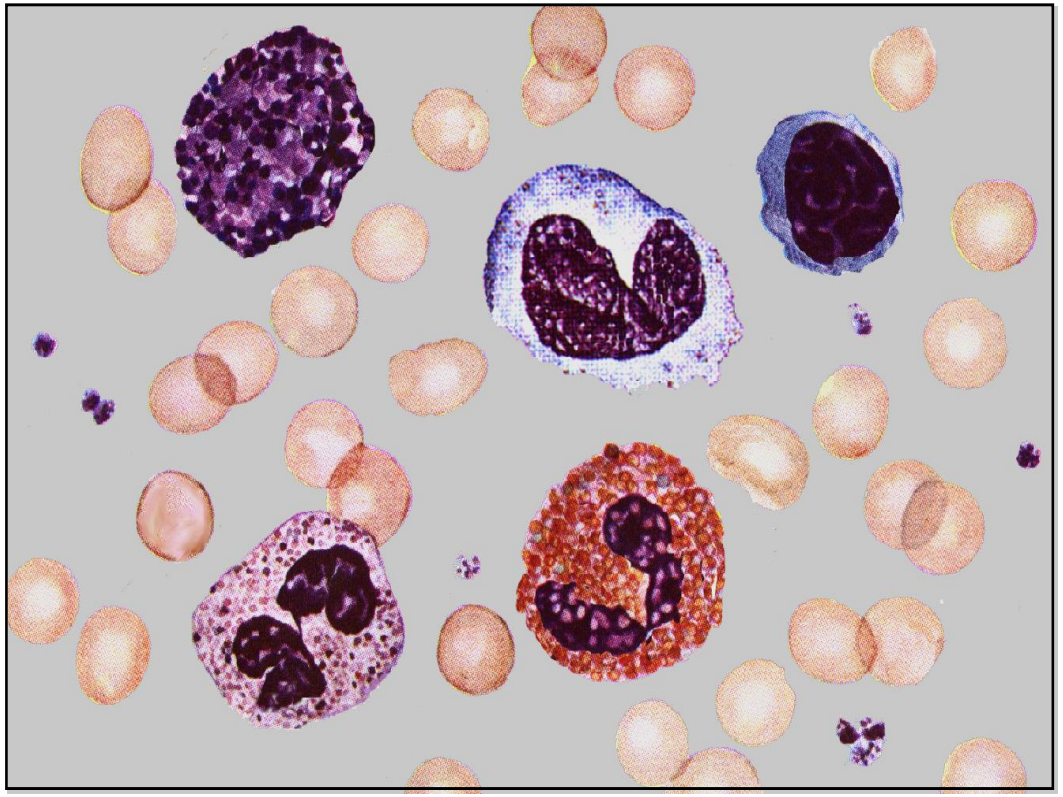


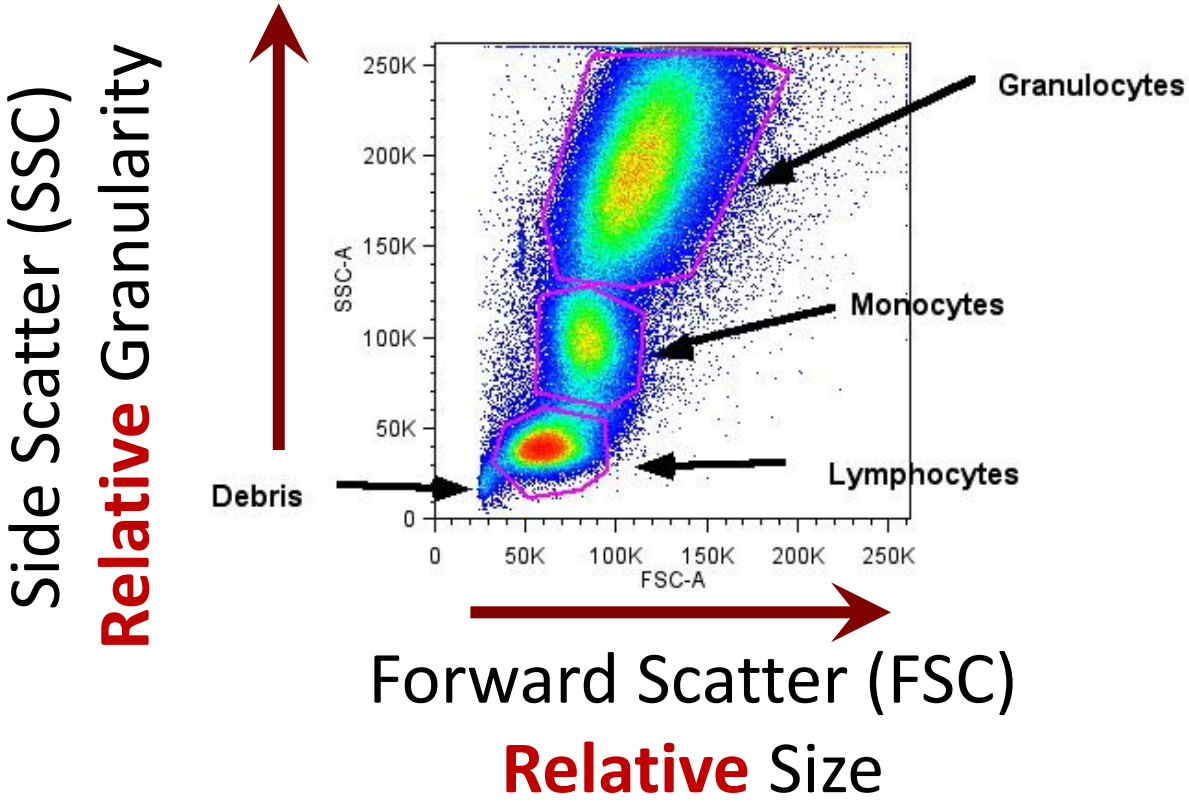
Forward Scatter: Relative Size(ish)
Also highly dependent on refractive index
If you need accurate sizing, find a scope
With a scale bar



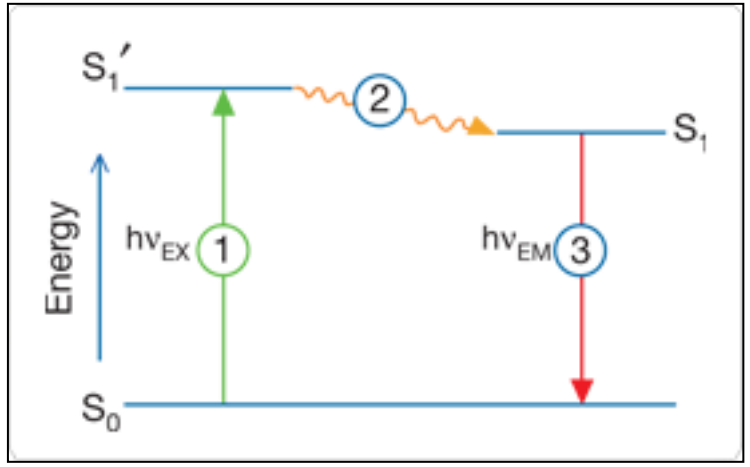
Side Scatter: Relative Granularity or
“Complexity”

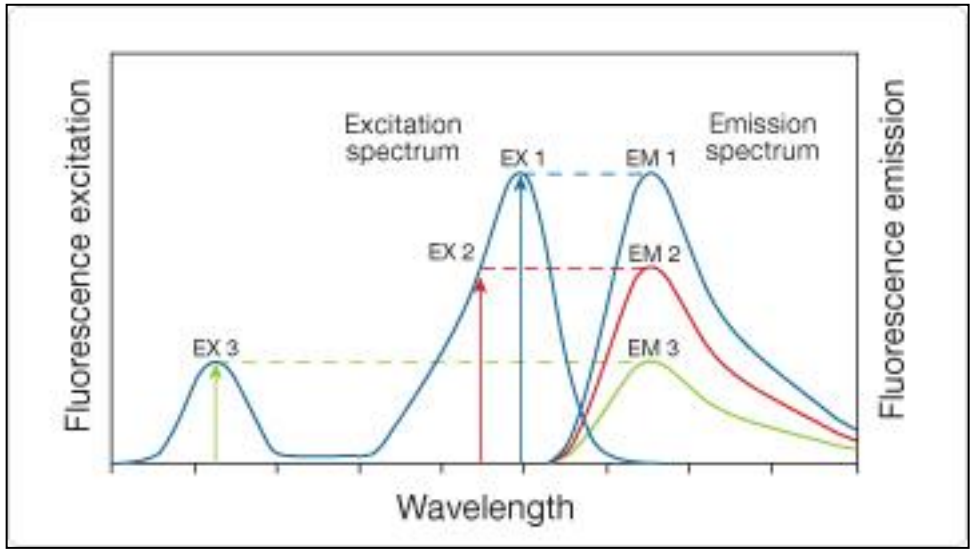
Scatters of blood cells



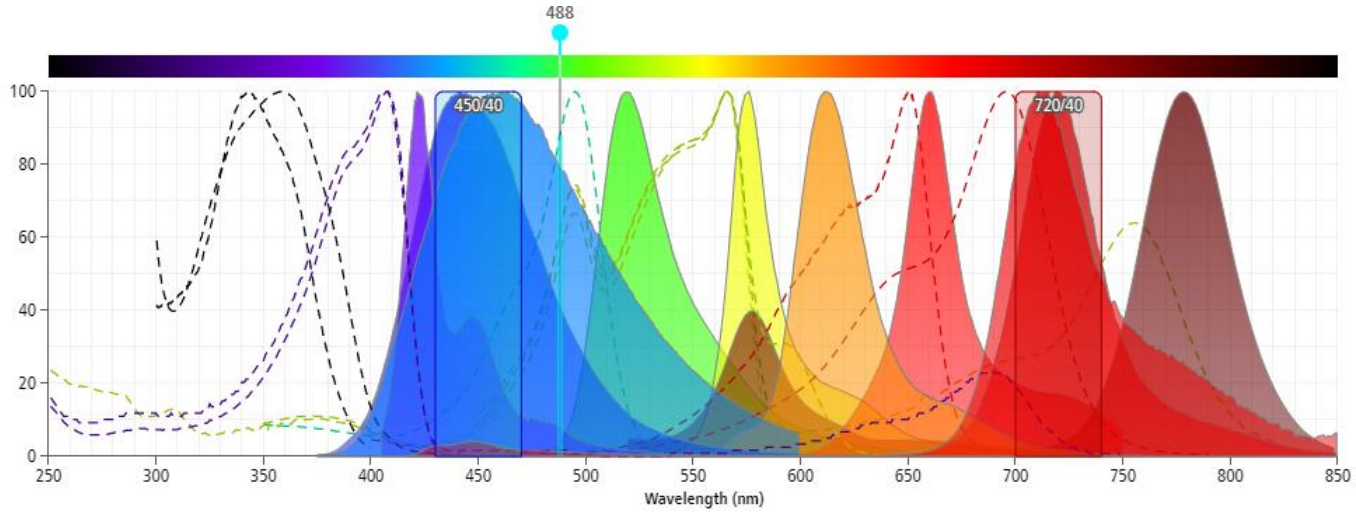


The Rainbow Connection: Fluorescence Measurements



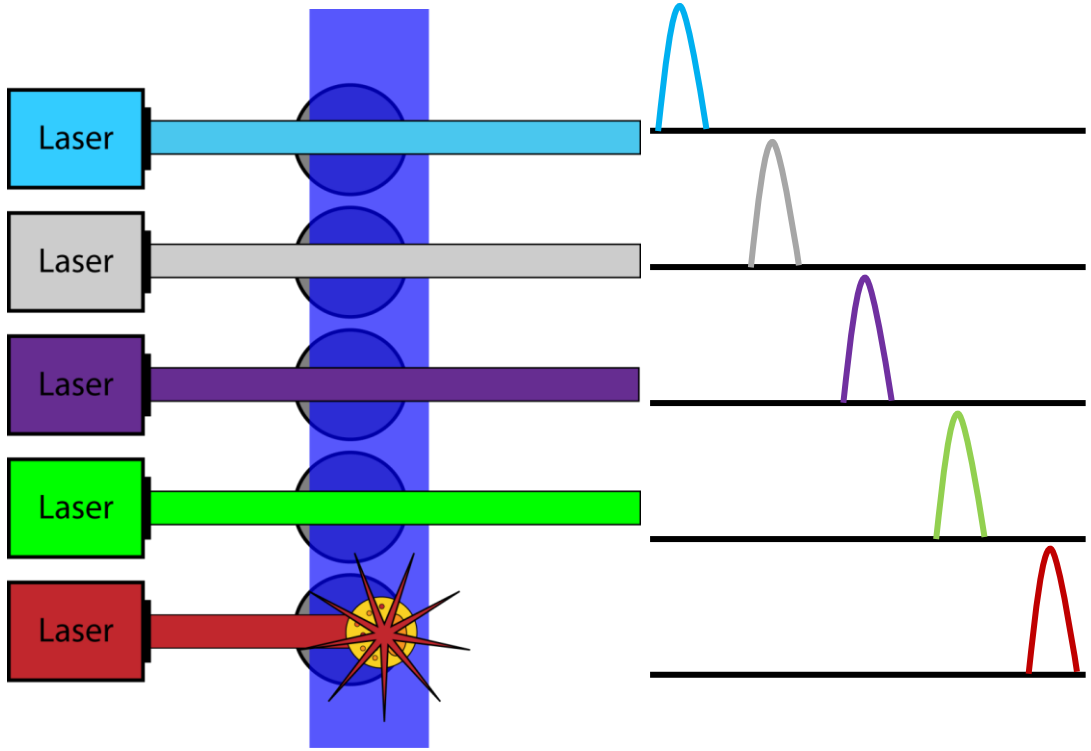


Molecular Probes Handbook



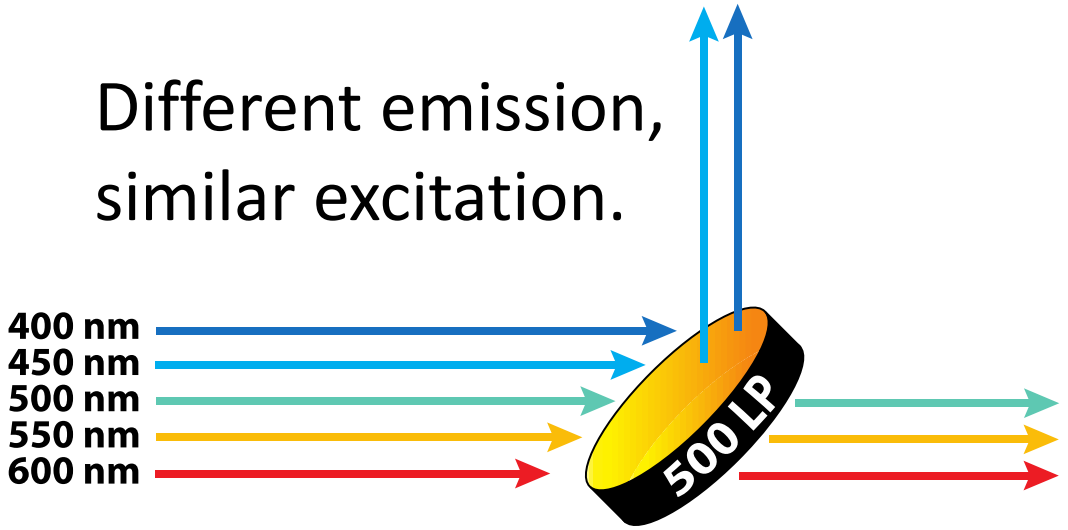
Narrow excitation bands (because lasers) but full emission spectra.
More colors, more problems…

Hardware design approaches to tackling overlapping emission



Similar emission, different excitation.

Different emission, similar excitation.



Dichroic Mirrors: Take all incoming photons, split them into two chunks based on wavelength.

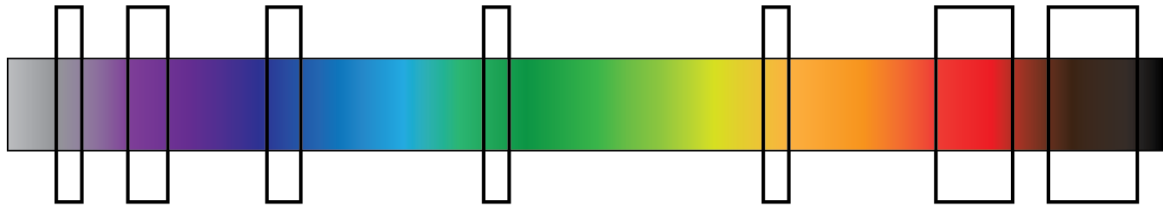
Bandpass Filters: Transmit specific range of wavelengths, destroy the rest. 525/50 = 525 nm+/- 25 nm.

Maps and Maths

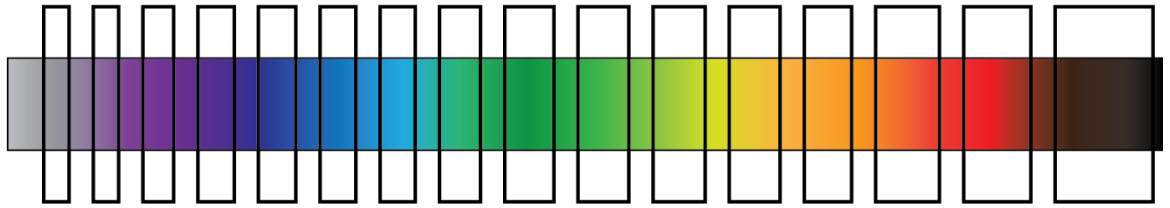
Violet (405)				Blue (488)		Yellow-Green (561)				Red (640)		
450/45	525/40	610/20	660/20	525/40	690/50	585/42	610/20	690/50	780/60	660/20	712/25	780/60
BV421	BV510	BV605	BV650	FITC	PerCP	PE	RFP	PE-Cy5	PE-Cy7	APC	Alexa700	APC-H7
DAPI	L/D Aqua	L/D Yellow		Alexa488	PerCP-Cy5	L/D Red	mCherry	PE-Cy5.5		Alexa647	APC-Alexa700	APC-Cy7
V450	Ghost510			BB515	PerCP-Cy5.5	Alexa555	mKate	7-AAD		Cy5	eFluor710	APC-eF780
PacBlue	V500			GFP	7-AAD	Dylight555	mStrawberry	mPlum		L/D Far Red	APC-R700	

Conventional cytometry places filters at key peak emission wavelengths and typically assigns one color to one detector. Spectral cytometry uses a more distributed approach to capture critical off-peak signatures and uses data from all the detectors. Compensation vs unmixing.

Conventional Cytometry



Spectral Cytometry

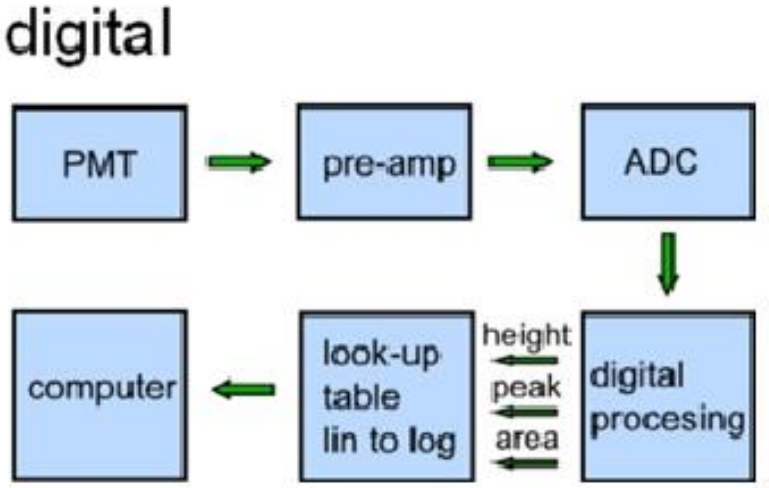


It’s not perfect, we still need controls

- Single stain controls
  - Provide fluorochrome spectral overlap ‘truth’
  - Must be of high quality- we have rules
- Fluorescence Minus One (FMO)
  - Sample stained with all colors EXCEPT one
  - Show spillover spreading in context of your samples
  - Allow for gating of positive vs negative populations

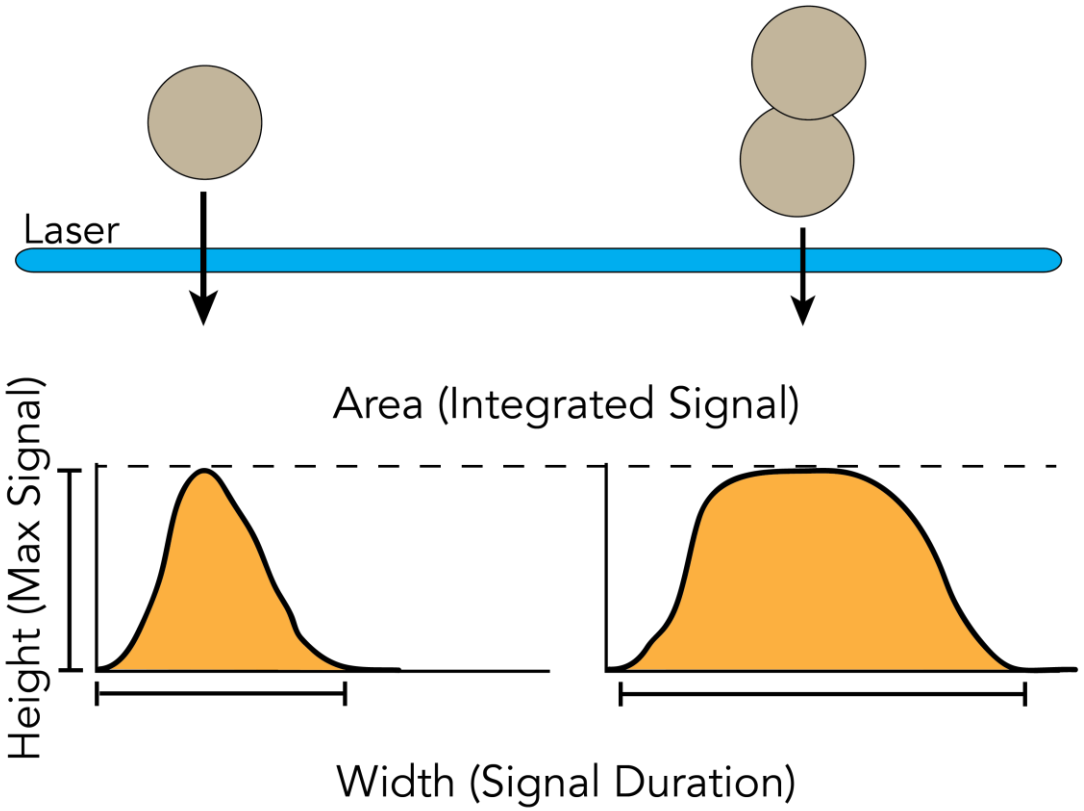
PMT, PMT, what do YOU see? A brief mention of electronics

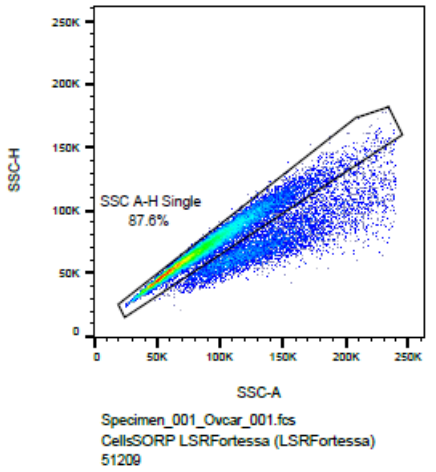
- We need an electronics system to process photodetector output and make listmode files for dots-on-plots
- Control what we do or do not see (threshold)
- Signal characteristics can be informative too

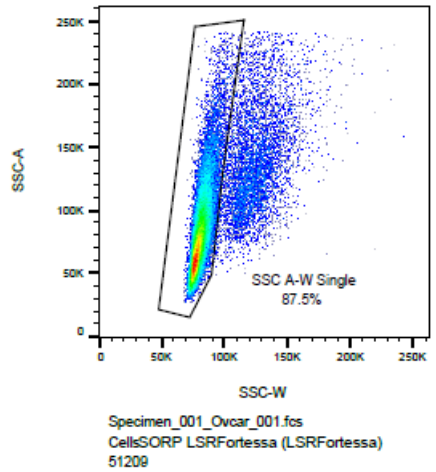


http://flowbook.denovosoftware.com/Flow_Book/

How do you know it’s just one cell?





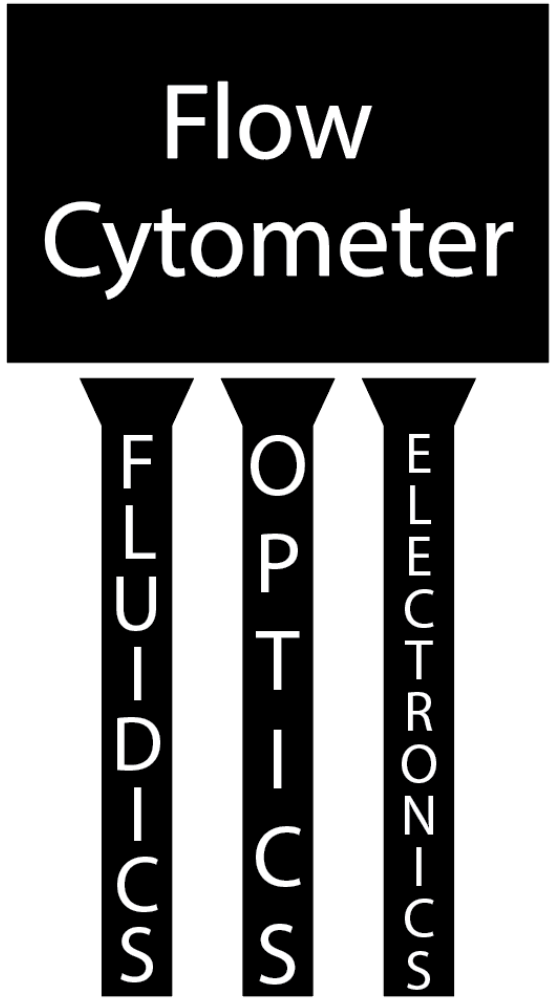


Critical to remove doublets/aggregates from analysis or sort!

Where did my cells go??

- Analysis?
  - They went to the great culture flask in the sky…
  - (Usually a waste tank for decontamination)
- Sorting?
  - Hopefully in your tube or plate!
  - Some still end up in the waste tank

Thank you!



Rachael Sheridan

rachael.Sheridan@vai.org

VAN ANDEL INSTITUTE®

David Leclerc

- Director, Cytometry, and Antibody Technology Facility at the University of Chicago (2016)
- Member GLIIFCA Board of Directors
- Leads Chicago Users Group in Flow Cytometry (ChUG).
- Primary role is in acquisition of technology for flow cytometry and strategy development for adoption of new technologies at UoC

THE UNIVERSITY OF CHICAGO BIOLOGICAL SCIENCES

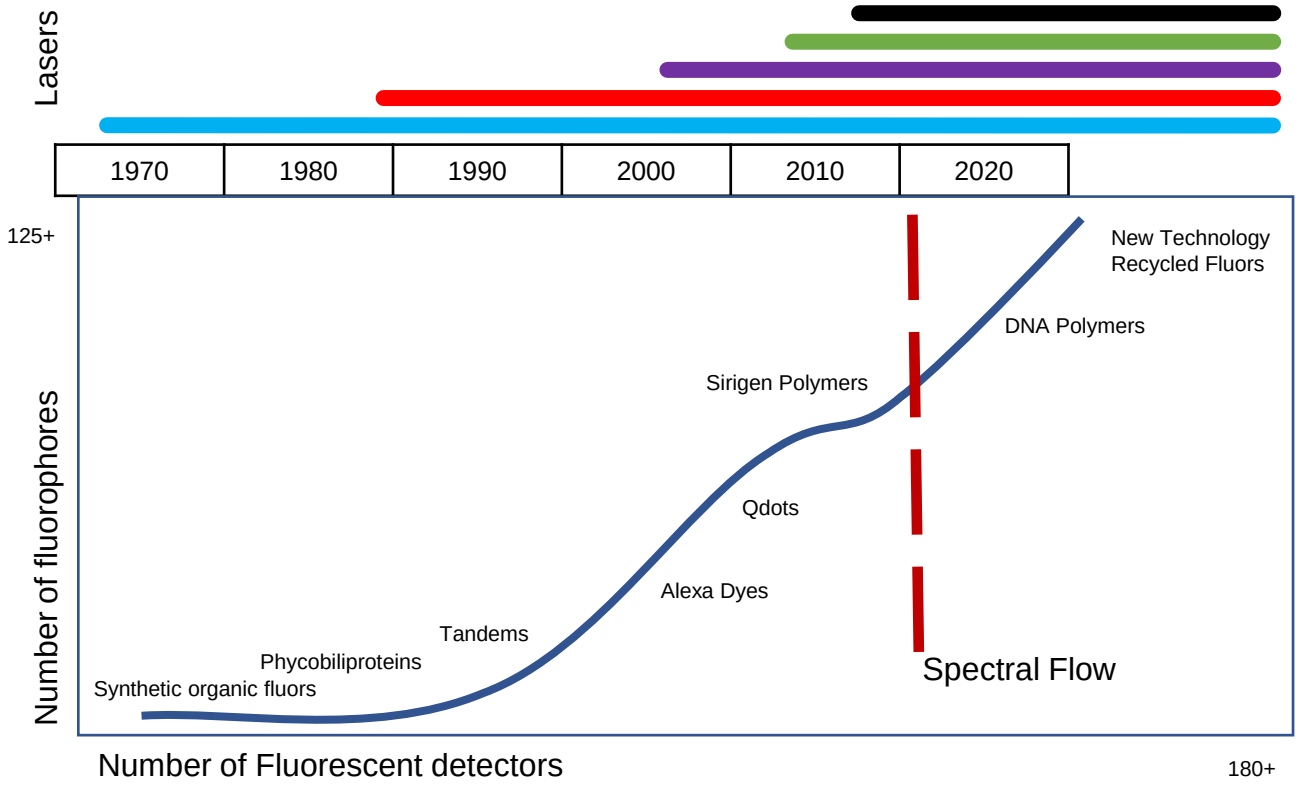
CAT FACILITY

Spectral Flow as a Transformative Technology

Recent advances in the field of Flow Cytometry

David Leclerc, Director
Cytometry and Antibody Technology Facility
University of Chicago
December 6th, 2022

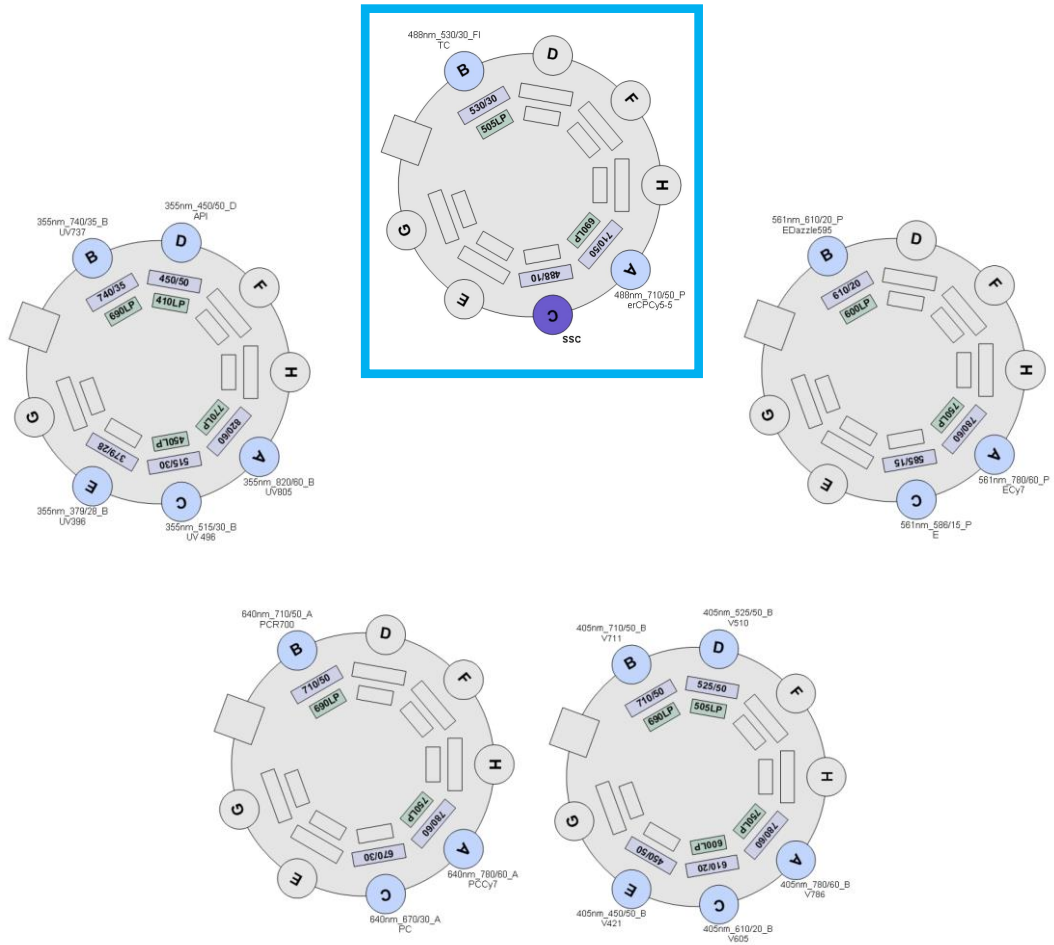
Evolution of instrumentation and fluorophores



Modified from Trends in Immunology, Volume 33, Issue 7, July 2012, Pages 323-332

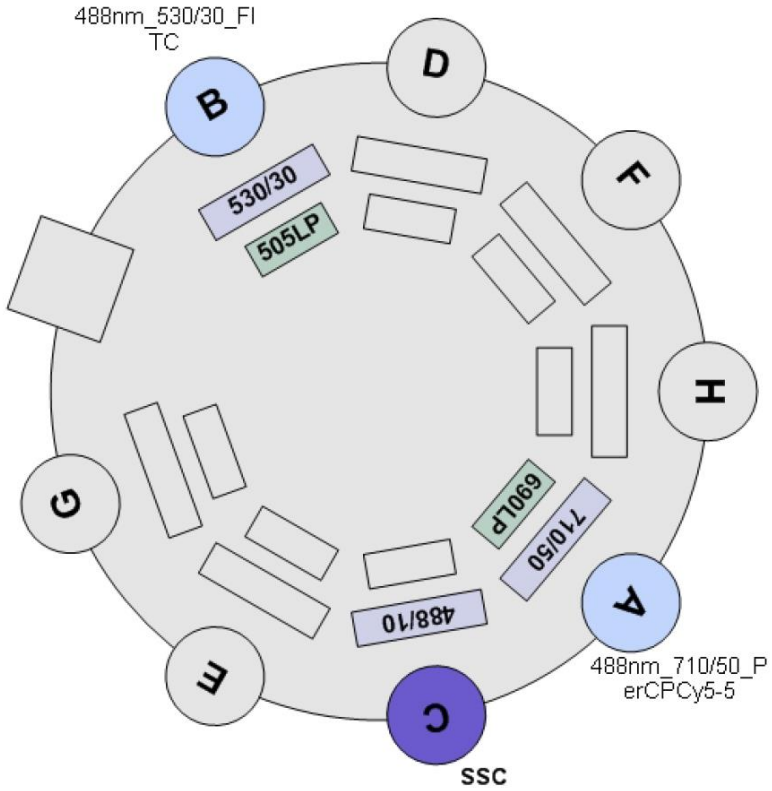
The Traditional Flow Paradigm

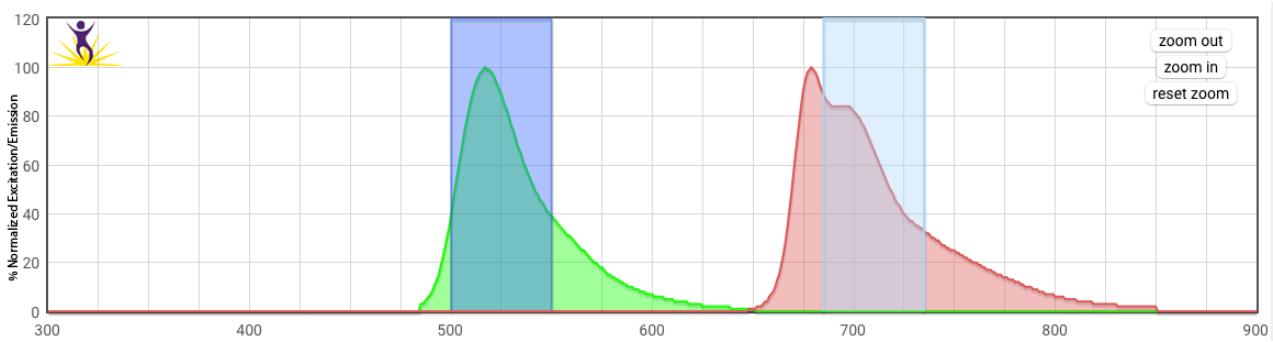
- One detector per fluorophore in the assay
- Filter selection based on fluorophores commercially available
- Panel design is baked in



CAT facility Fortessa X20 (RIP 2016-2021)
5 lasers optical layout

488nm laser restrained potential





Why did Spectral Flow had such an impact?

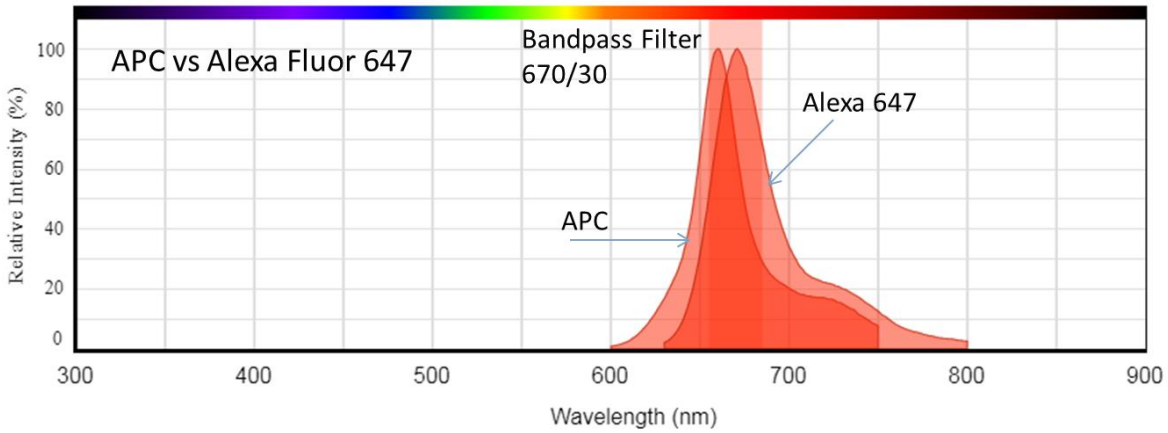
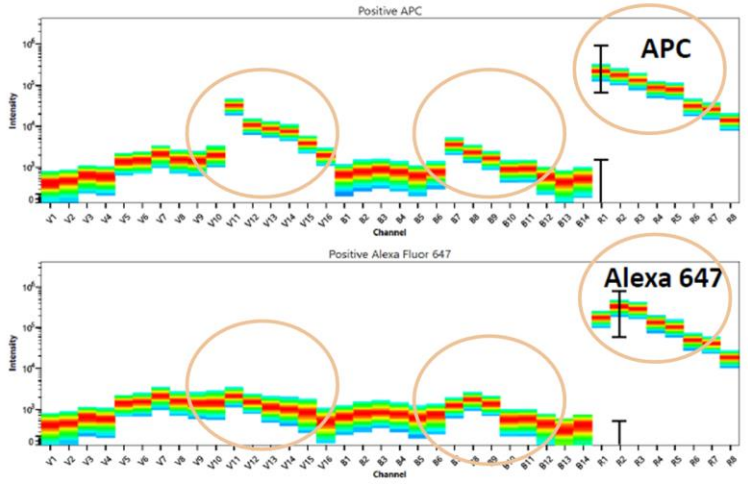
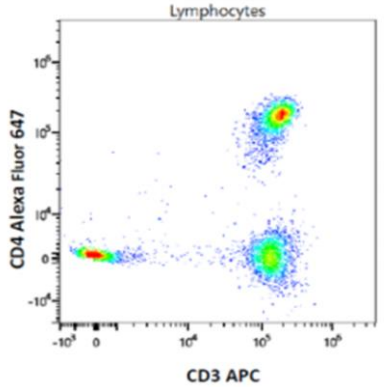


Image courtesy AAT Bioquest, Inc. (https://www.aatbio.com)



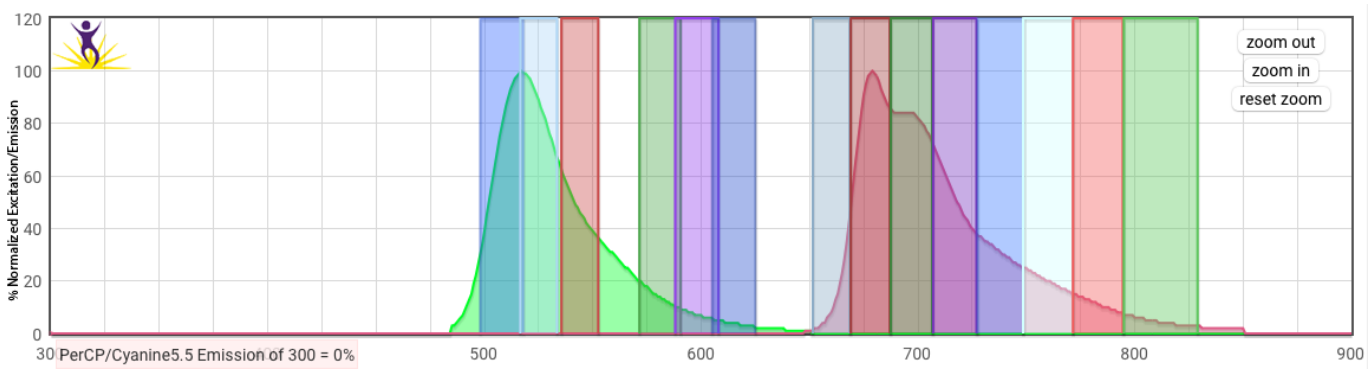


Gated on lymphocytes

- Never have to worry about filter sets again
- Separate fluorophores with similar emission
- Manage larger number of markers more efficiently

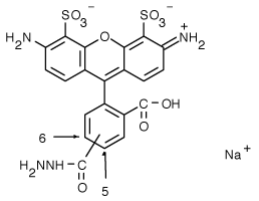
Get more from your 488nm laser!

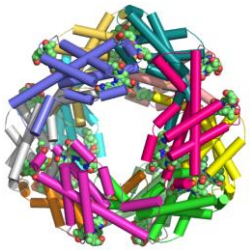
- More room to interrogate
- Fluorophores now required!

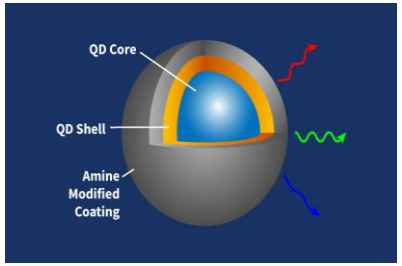


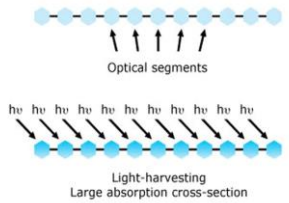
Fluorophores then and now

Family	What we had	What was released
Organic dyes	Alexa Fluors, Dylight	CF Dyes, spark/fire dyes
Proteins	PE, APC, GFP, …	
Tandems	Bunch!	PE/Fire810 / APC/Fire 810
Nanocrystals	Qdots	StarBright dyes
Organic Polymers	Sirigen brilliant dyes, SuperBright	Kiravia, BB755, BB790
DNA Polymers		NovaFluors
What else?	Some dyes were available but could not be used	New Tech: BD RealBlue, RealYellow, …





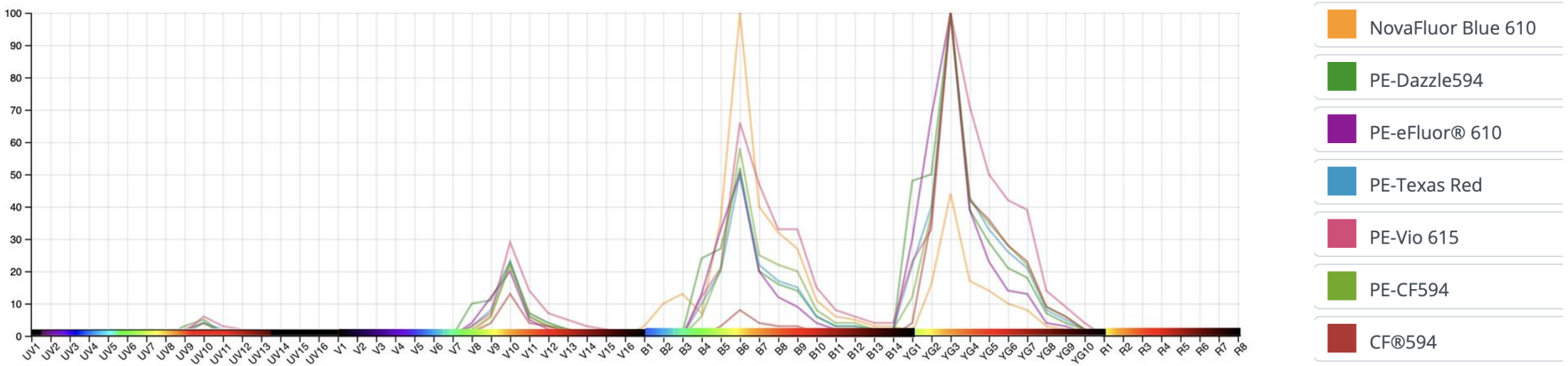




- Exploit unused part of the spectrum
- Reduce spillover by limiting cross-excitation / narrowing emission
- Increased solubility, brightness, …

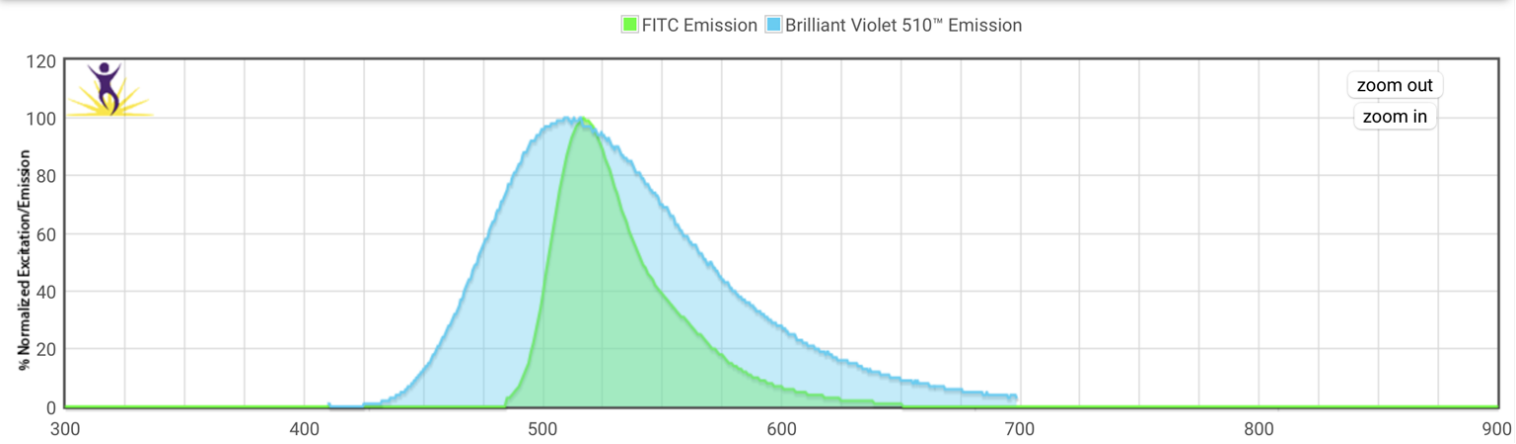
- Many of these new dyes require special buffers
- New dyes have limited catalog options
- Some rebranding, or substitutes with limited value

Some fluorophores are redundant and repeat themselves



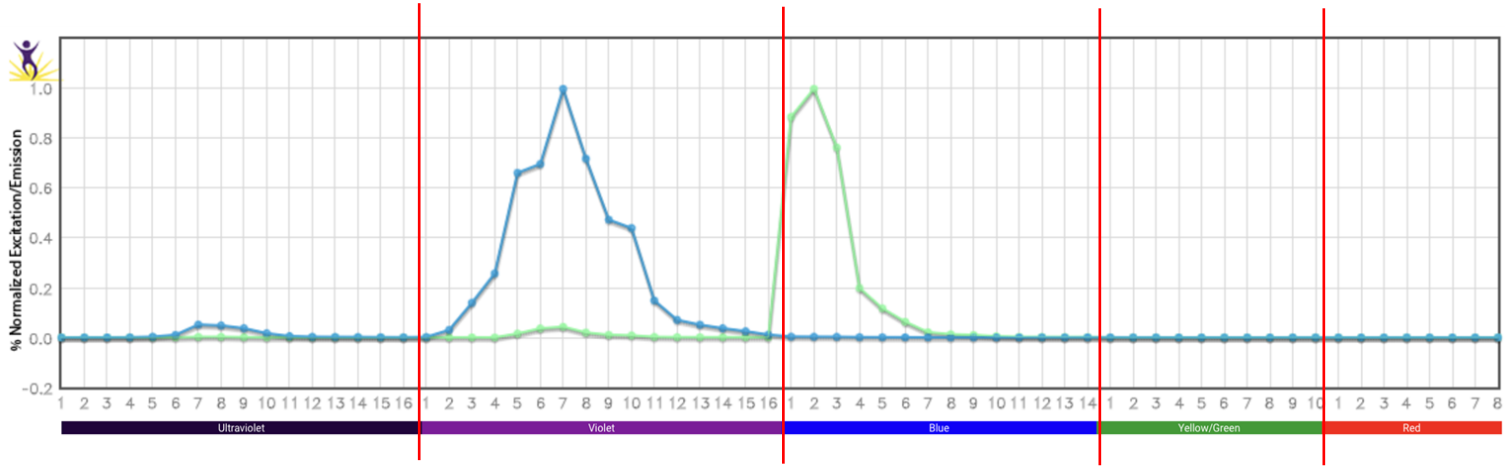
Yet they may still have advantages…

Hello Spectra analyzers!

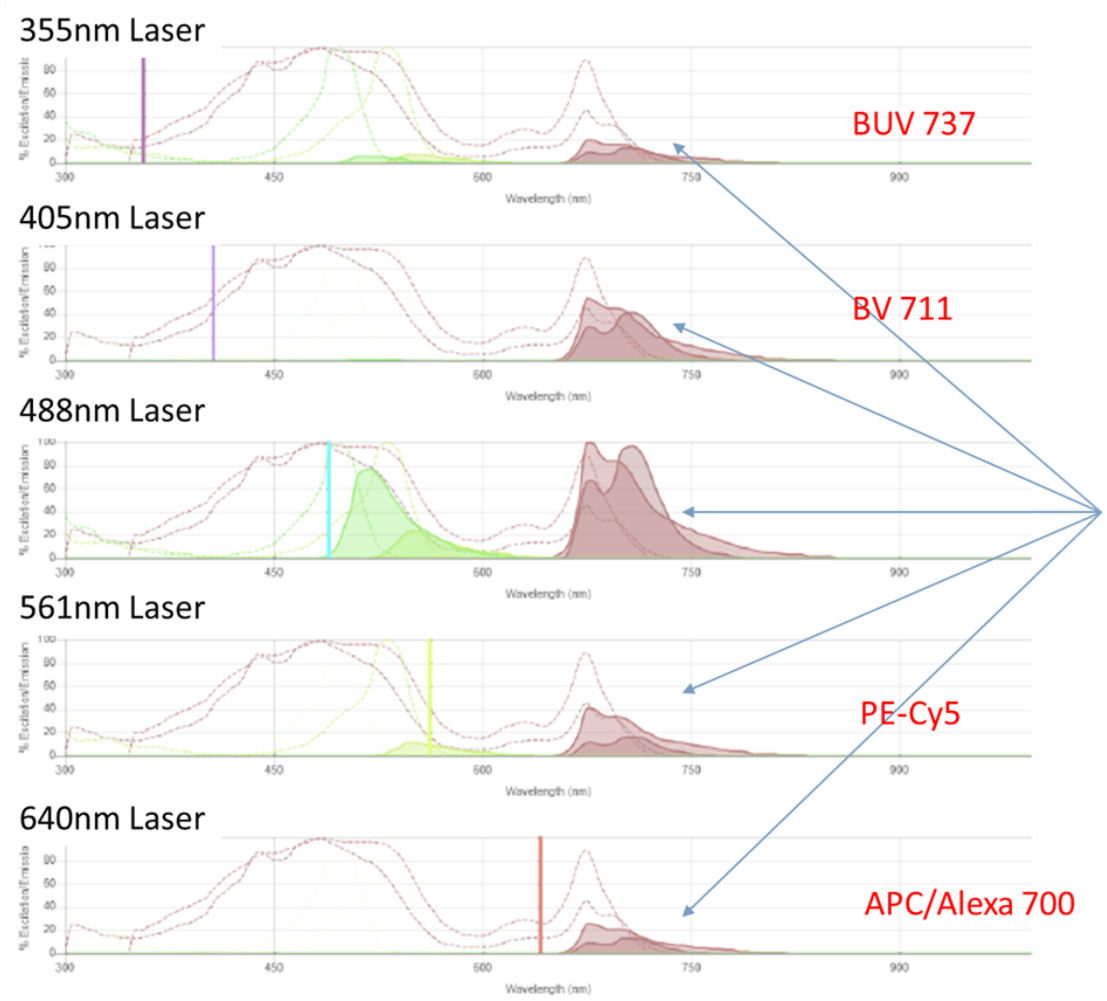


FITC → Green

BV510 → Blue



Get rid of bad habits

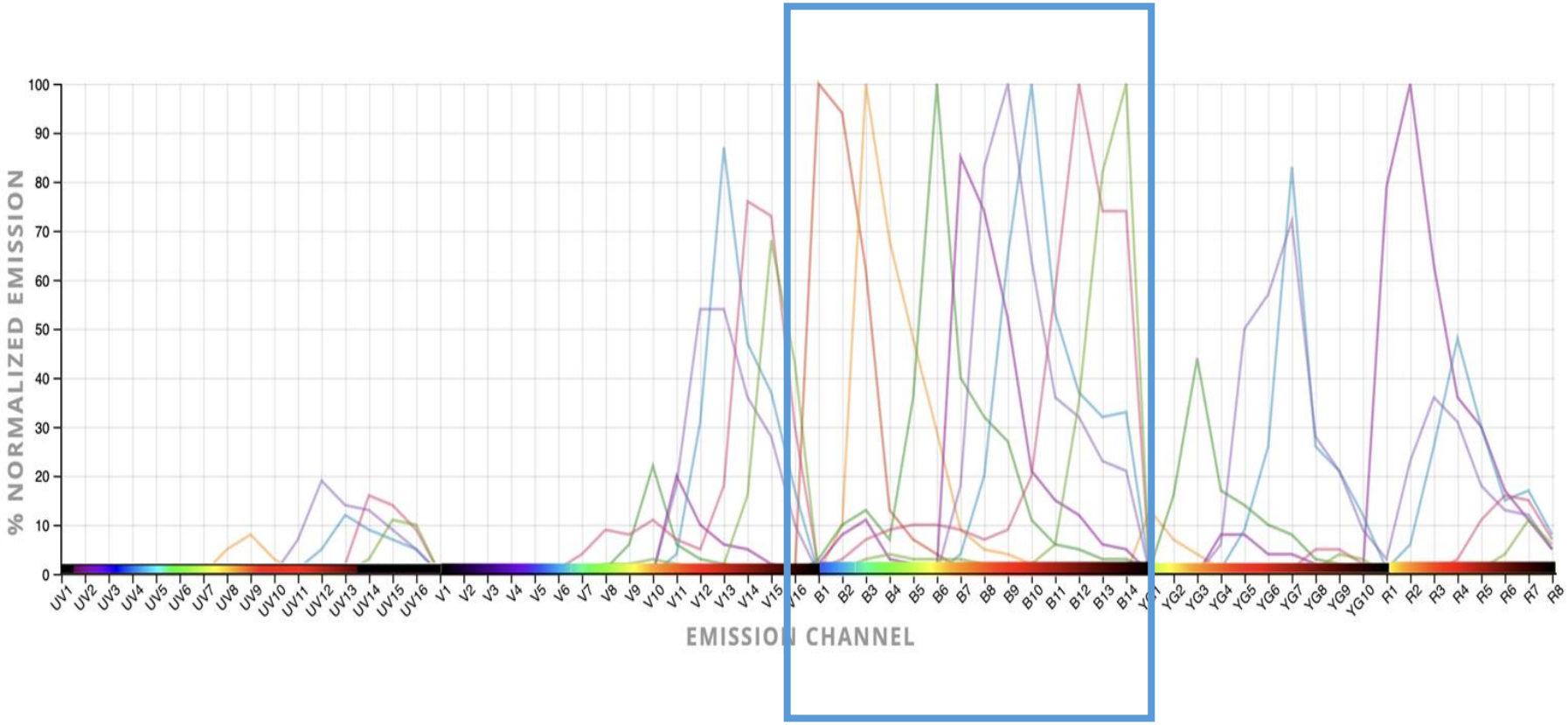


Cross-laser spillover

Cross-laser excitation of PerCP-tandems leads to spread in other key channels

https://fluorofinder.com

Eight markers on the 488nm laser



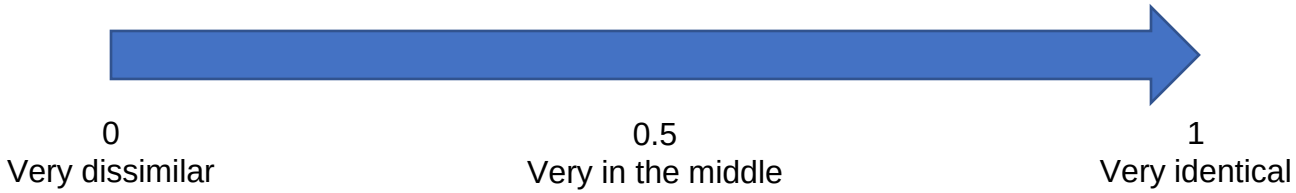
- BB515
- AF532
- NF610
- NF660
- PerCP-Cy5.5
- PerCP-eFluor 710
- Brilliant Blue 755
- Brilliant Blue 790

Modified from R.Duggan, ChUG 2022 Workshop

Similarity / Complexity Index

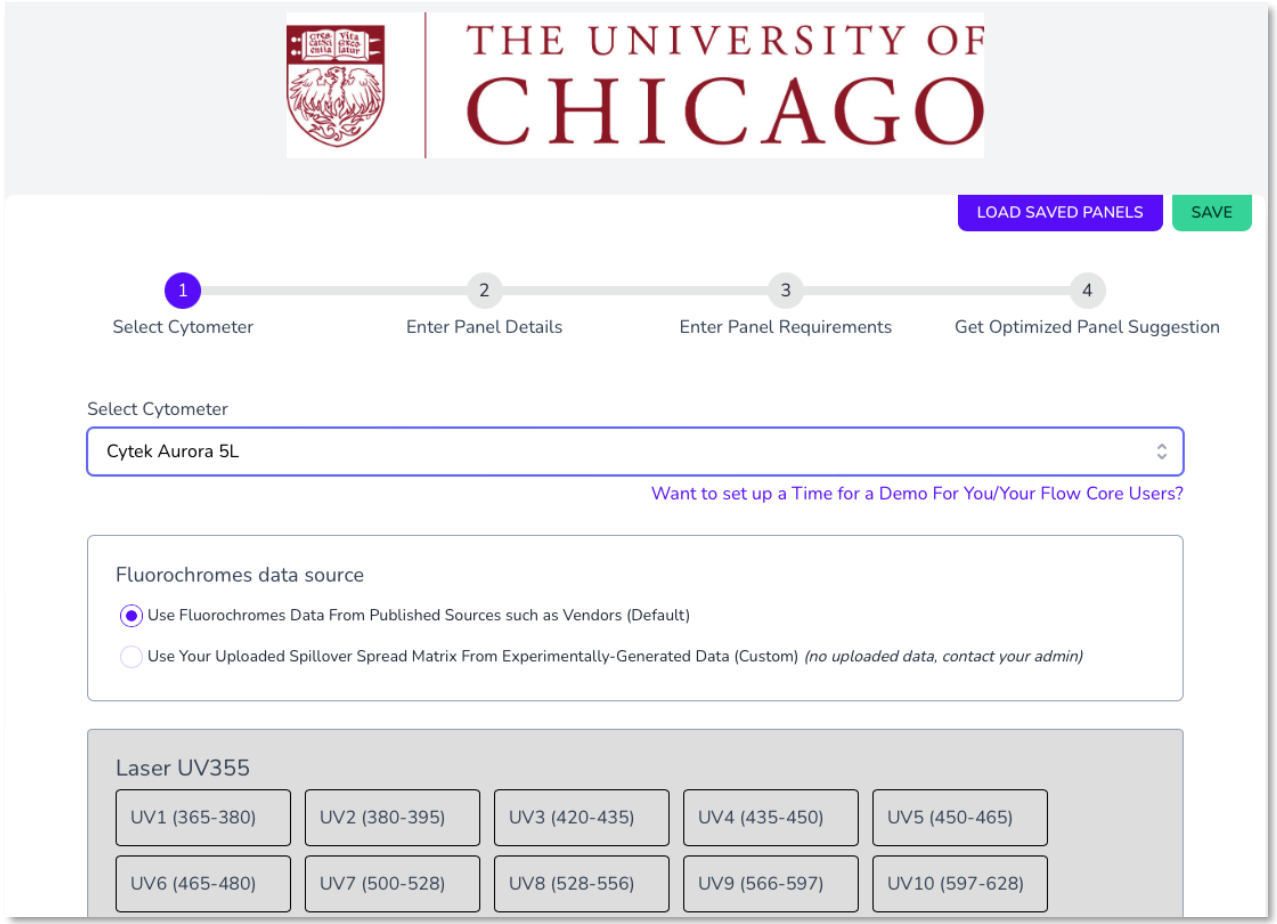
BB515	1							
Alexa Fluor 532	0.41	1						
NovaFluor Blue 610	0.14	0.4	1					
NovaFluor Blue 660	0.06	0.11	0.31	1				
PerCP-eFluor 710	0	0.02	0.2	0.37	1			
PerCP-Cy5.5	0.01	0.03	0.32	0.56	0.86	1		
BB755	0.04	0.09	0.17	0.18	0.57	0.44	1	
BB790	0.02	0.04	0.08	0.08	0.37	0.27	0.82	1
	BB515	Alexa Fluor 532	NovaFluor Blue 610	NovaFluor Blue 660	PerCP-eFluor 710	PerCP-Cy5.5	BB755	BB790
Complexity™ Index: 5.58								

- Similarity Index



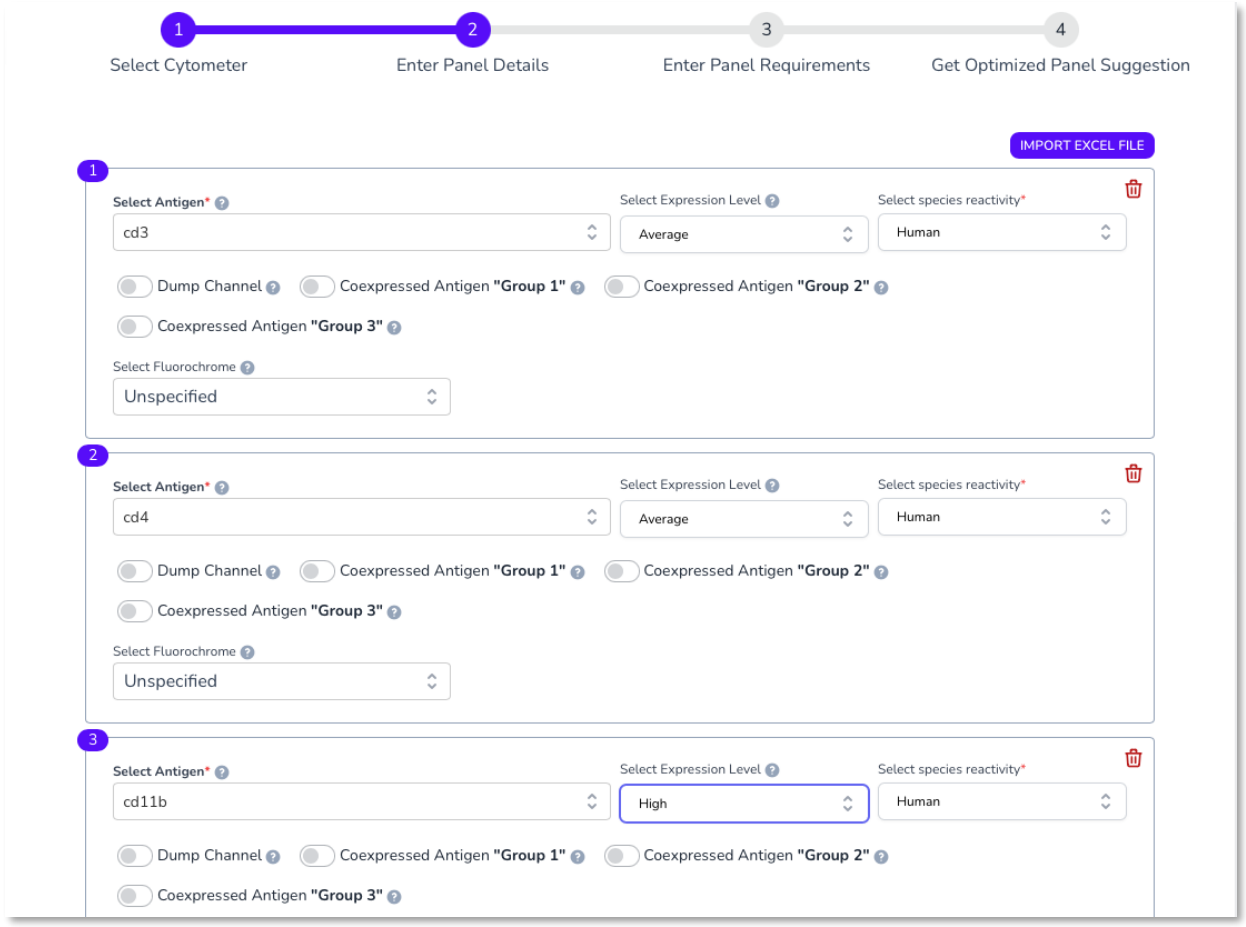
- Complexity Index
  - Lower the better
  - Small Panel → 2 to 3
  - Large Panel → 40 to 50

Online Panel Design Services (EasyPanel)



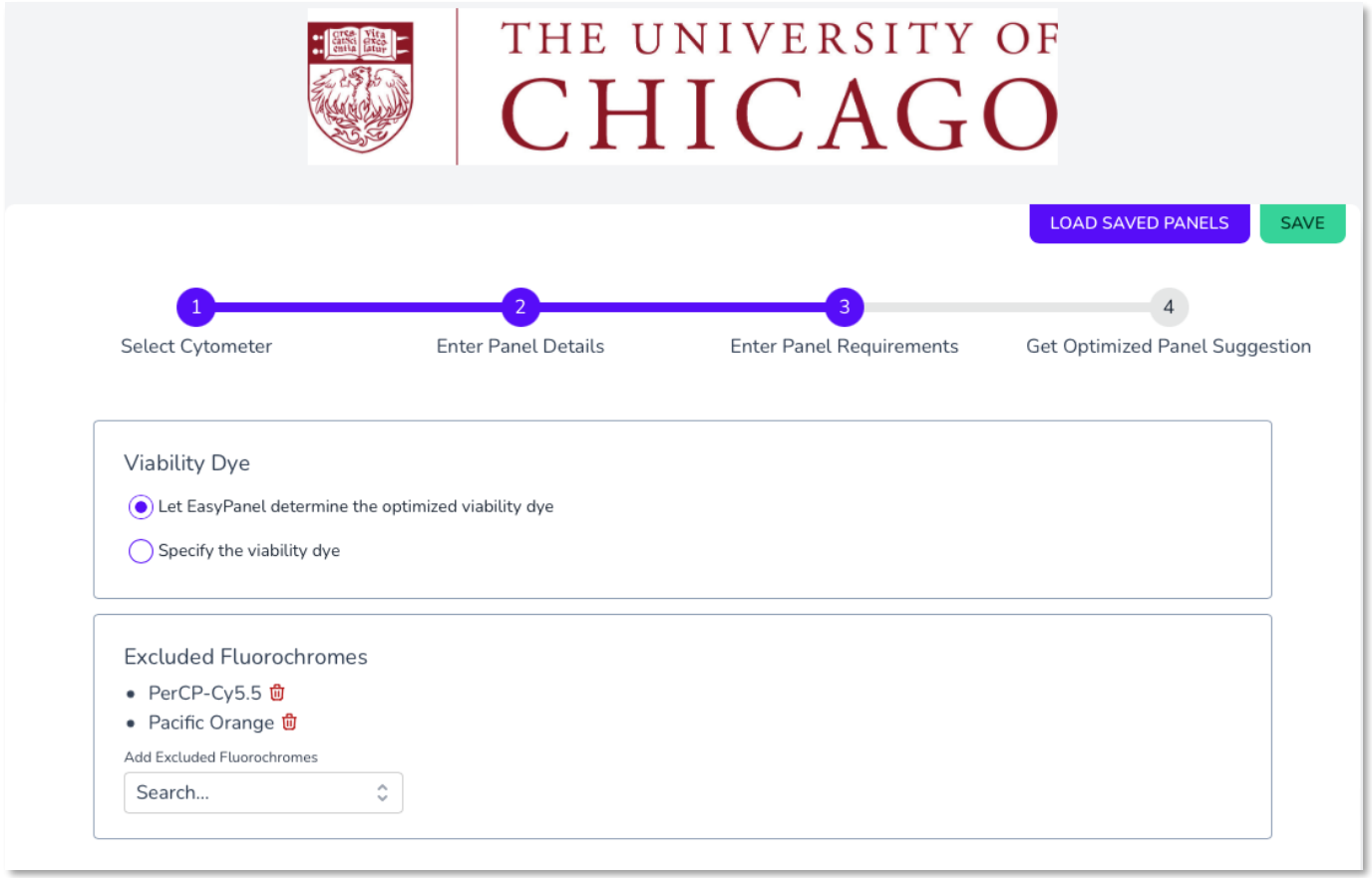
Step 1: Choose your instrument

Online Panel Design Services (EasyPanel)



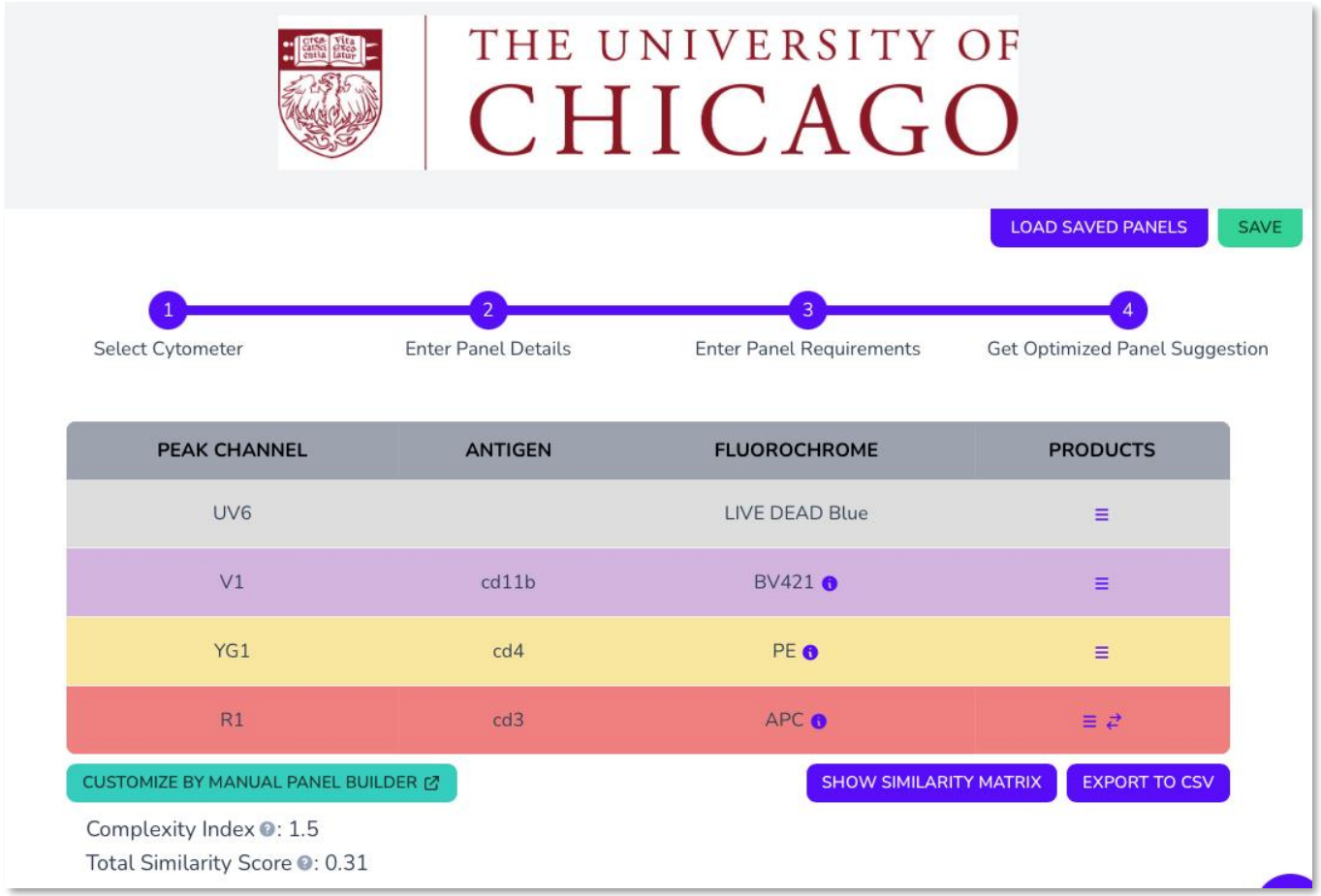
Step 2: Choose your markers

Online Panel Design Services (EasyPanel)



Step 3: Select specific requirements

Online Panel Design Services (EasyPanel)



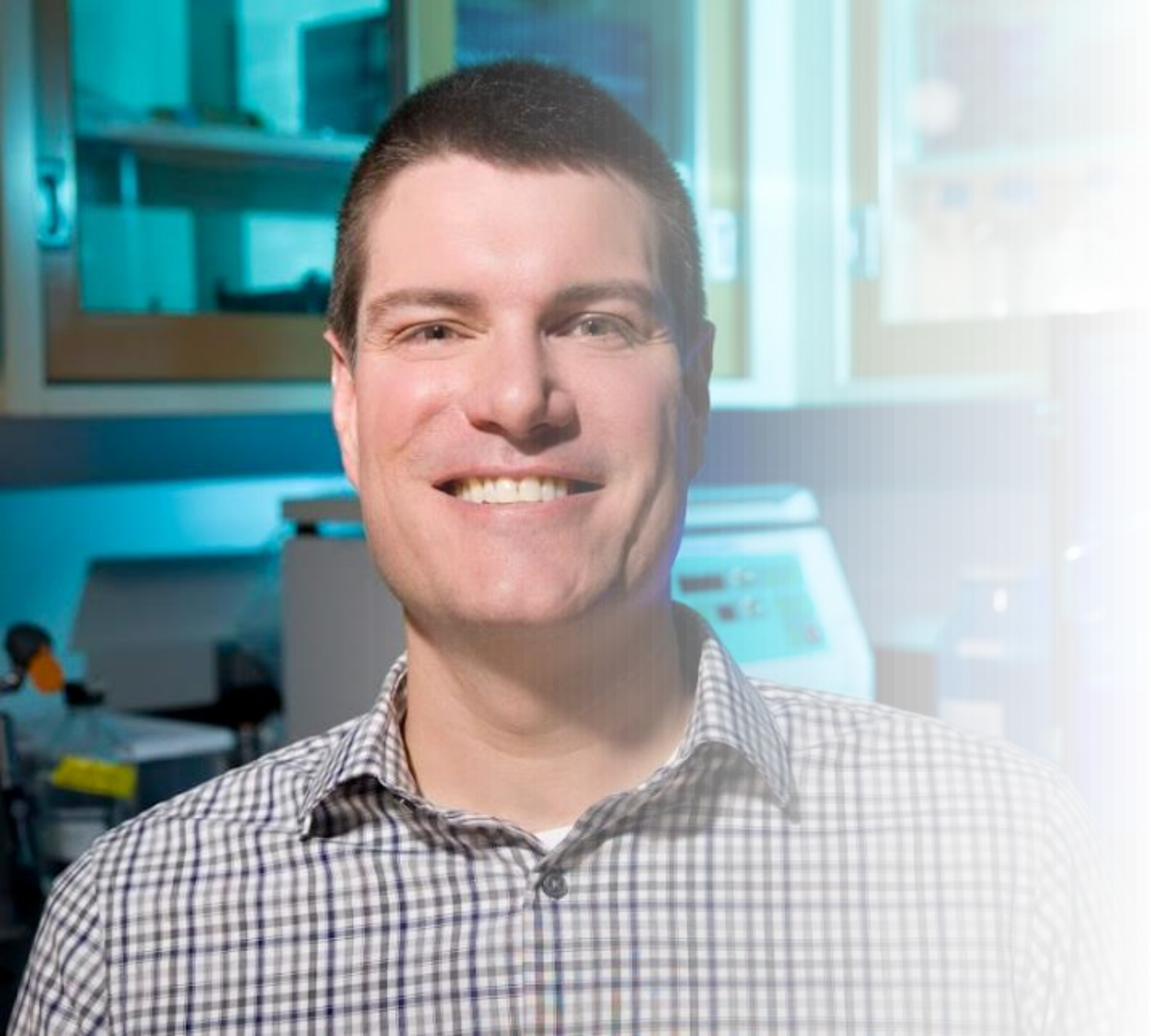
Step 4: Profit!

Thank you!

Visit the CAT Facility Website for more information

- With Spectral Flow came:
  - New fluorophores
  - Improved panel design tools

David Leclerc, Director
dleclerc@bsd.uchicago.edu



Matt Cochran, MS

- Flow Cytometry since 2003
- Technical Director for the URMC Flow Cytometry Resource
- Member GLIIFCA Board of Directors
- ISALC SRL Content Task Force
- Past Member of ABRF FCRG

Cell Sorting:
the 10k ft view

Center for Advanced Research Technologies

URMC Flow Cytometry Resource

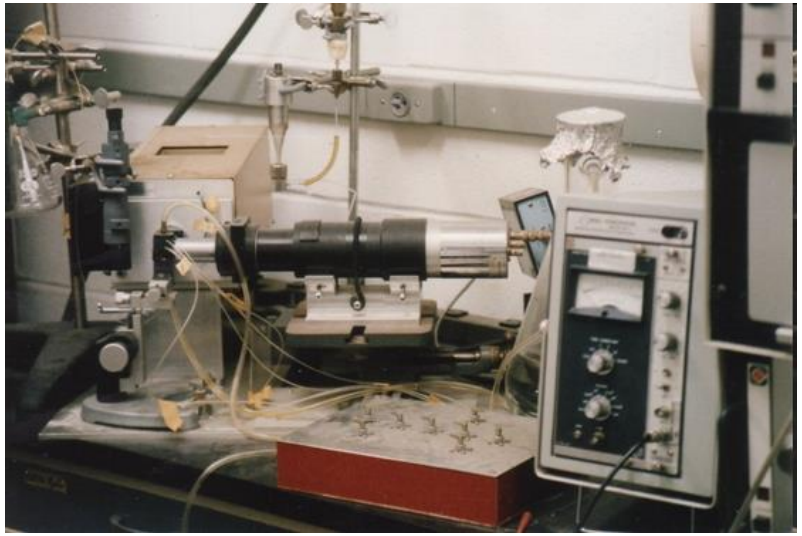
Matt Cochran, Technical Director

Matthew_Cochran@urmc.rochester.edu

Mack Fulwyler and Len Herzenberg

Mack Fulwyler combined

- Coulter principle for identification of cells
- Ink jet printing theory (Richard Sweet)
- 1965!



The first electrostatic sorter

Extended the Fulwyler plan by adding

- Arc lamp (1969)
- Argon laser (1972)

Coined term Fluorescence Activated Cell Sorter (FACS)

Len Herzenberg with FACs I circa 1972

What?

Cell sorting is the physical separation of specific cells from a heterogeneous population.

- Single cell sorting
  - Generating single cells and collecting them one at a time in separate vessels
- Bulk sorting
  - Separating many (>1) single cells with the same characteristics and collecting them in one vessel

Enrichment is bulk sorting though its typically focused on greater yield and lower purity

- Not necessarily done via single cell identification
- Sometimes followed by single cell methods

Why?

Rapidly generate, highly purified populations of cells that are used for any number of downstream purposes.

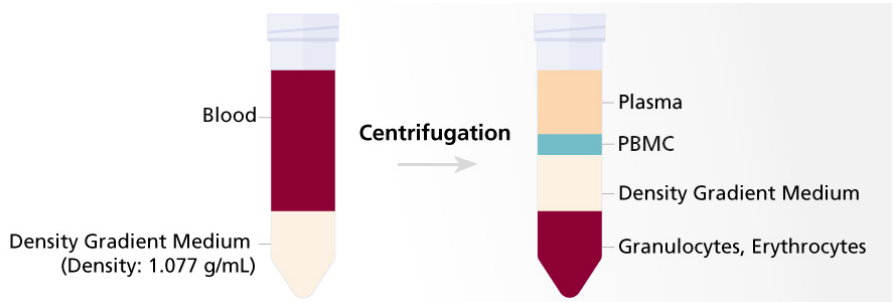
- Functional assays
- Transplantation studies
- Tissue culture
- Complex characterization (Genomics, proteomics)

How?

- Density gradient centrifugation and elutriation
- Capture bead based
- Acoustic field based
- Microfluidic chip based
- Droplet based - Electrostatic

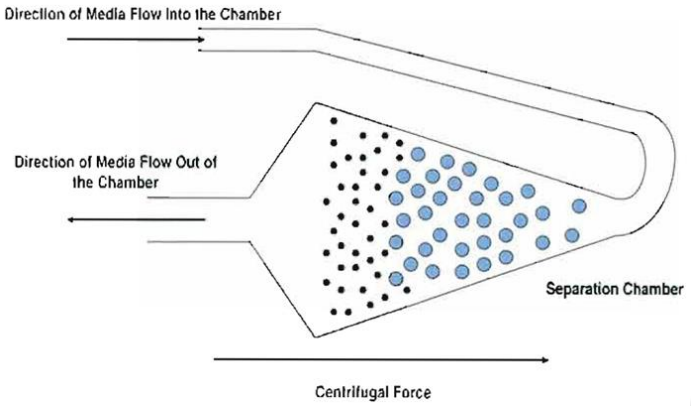
How?

- Density gradient centrifugation and elutriation
  - Classic liquid density gradient separation
    - Large volume blood processing
    - Well understood
    - Limited cellular stresses
    - Finicky
    - Purely enrichment (limited specificity)
  - Serial centrifugation
    - Small particle work
  - Centrifugal elutriation
    - Large volume and number of cells (billion
    - Limited cellular stress
    - Limited current usage or expertise
    - Enrichment (size and density)



Stemcell Technologies

Biocyclopedia



How?

- ❖Capture bead based
  - Magnetic beads
    - Easy to use (minor equipment limitations)
    - Large cell numbers (hundreds of millions)
    - Well known, highly available
    - Good purity, recovery and viability
    - Pos selection – single target specificity
    - Neg selection – enrichment
    - Either way its bulk collection

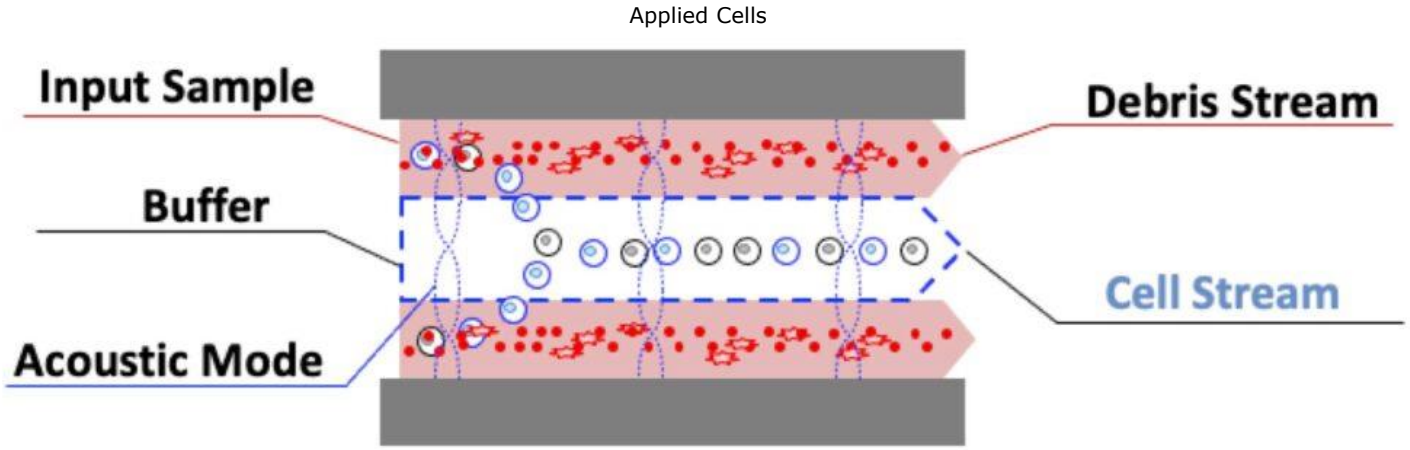
Miltenyi Biotec

  - Buoyancy beads
    - Very easy to use (no equipment at all)
    - Limited cell numbers
    - Pos vs Neg selection
    - Bulk collection

Akadeum Life Sciences

How?

- ❖Acoustic field based
  - Emerging technology for cell separation
  - Gentle (??) should mean high viability
  - Cell numbers, throughput?
  - Bulk enrichment
    - Purity?

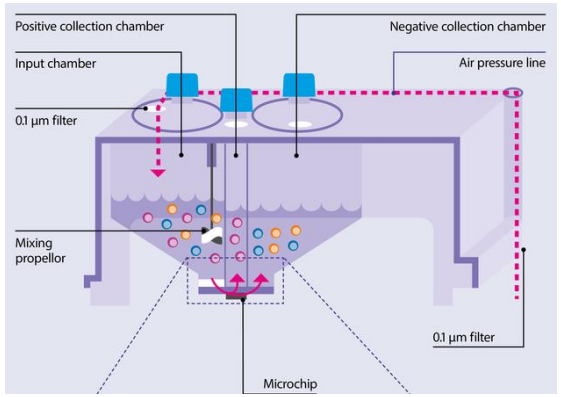


- Pairing with other technologies, primarily microfluidics opens many options for advancement.

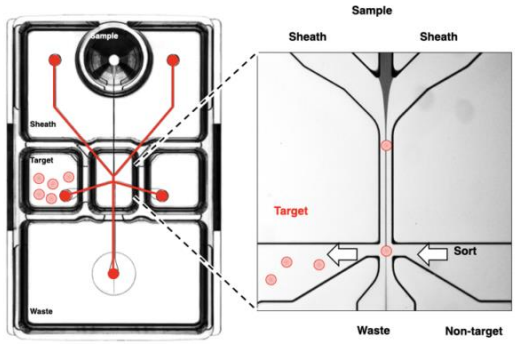
How?

- Microfluidic chip based
  - Full chip sorting
    - Fewer biosafety or contamination/carryover issues
    - Extremely gentle
    - Often single population
      - Repeat sorts for additional populations
    - Limited fluorescence capabilities, but growing
    - Bulk single cell capture

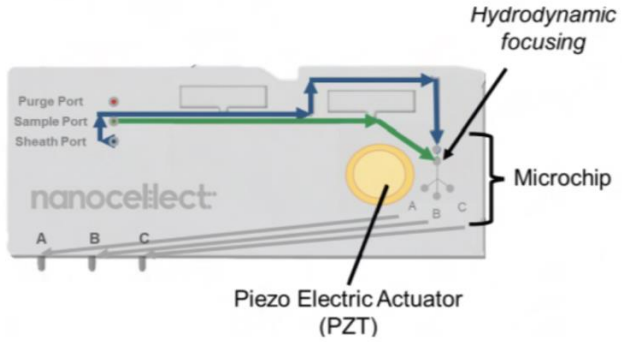
Miltenyi Biotec



On-Chip

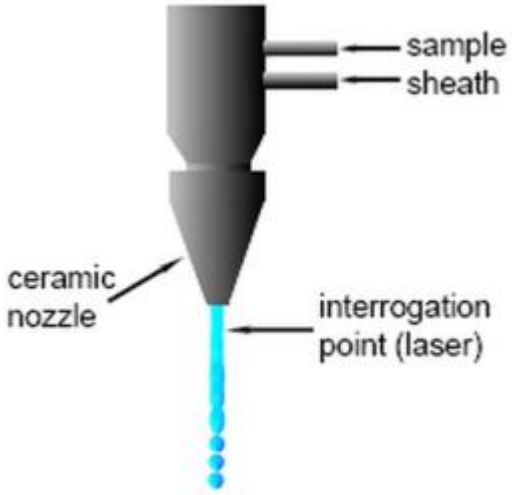


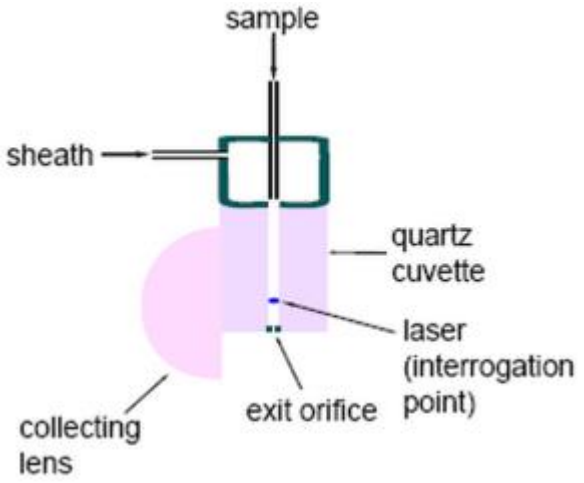
Nanocellect Biomedical



How?

- Droplet based
  - Broadly, two types
    - Jet-in air : laser intercept occurs outside nozzle, “in air”
    - Cuvette based : laser intercept in a cuvette, prior to nozzle orifice.
      - Microfluidic options act as single use flow cells
        - May be electrostatic or other.
  - Droplet’s (containing cells) are generated as stream exits the nozzle.
  - Droplets are charged and target cells are deflected into collection vessel.



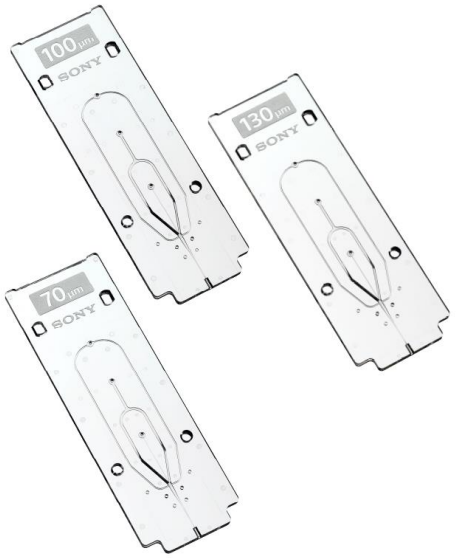


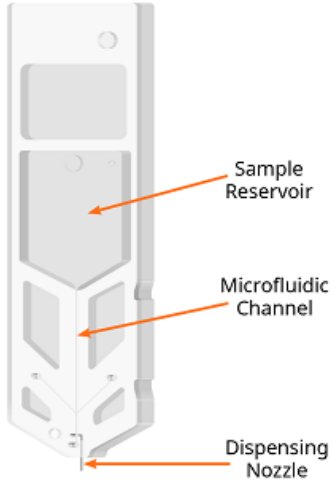
“Flow Cytometry. A Basic Introduction” by M. Ormerod

How?

- Microfluidic chip with droplets
  - Variable biosafety concerns
    - Electrostatic options generate aerosolizable droplets.
  - Limited contamination/carryover issues
  - Gentle, typically (relatively lower pressure)
  - Bulk or single cell options
  - Good fluorescence capabilities

Sony Biotechnology

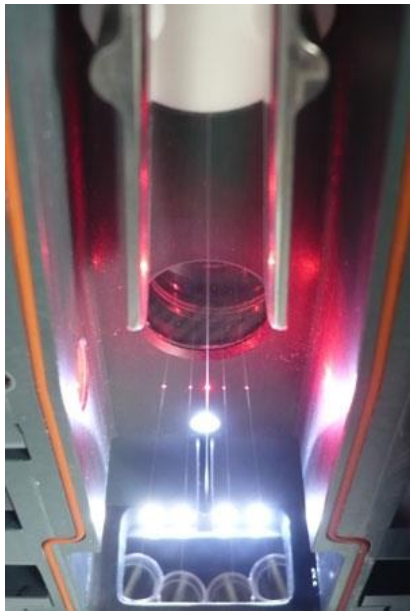




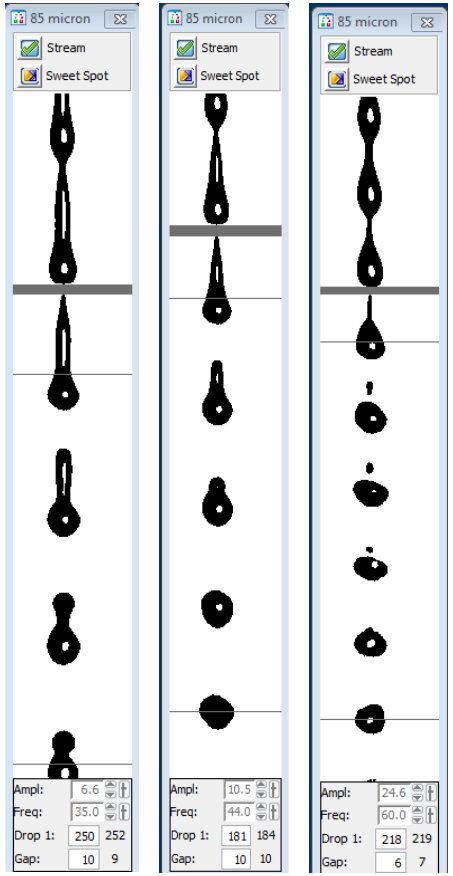
Namocell, Bio-techne

Why?

- Electrostatic droplet based
  - Single cell or bulk capture
  - Fluorescence flexibility
  - Up to 6 populations at once
  - High speed (10s-100s of million per hour)
  - Highly manipulatable
    - Variable collection vessels – tubes, plates, slides, custom
    - Variable droplet size/collection volume
      - Nozzle changes
      - Pressure changes
      - Frequency changes
    - Variable Speed



Same nozzle – varied frequency

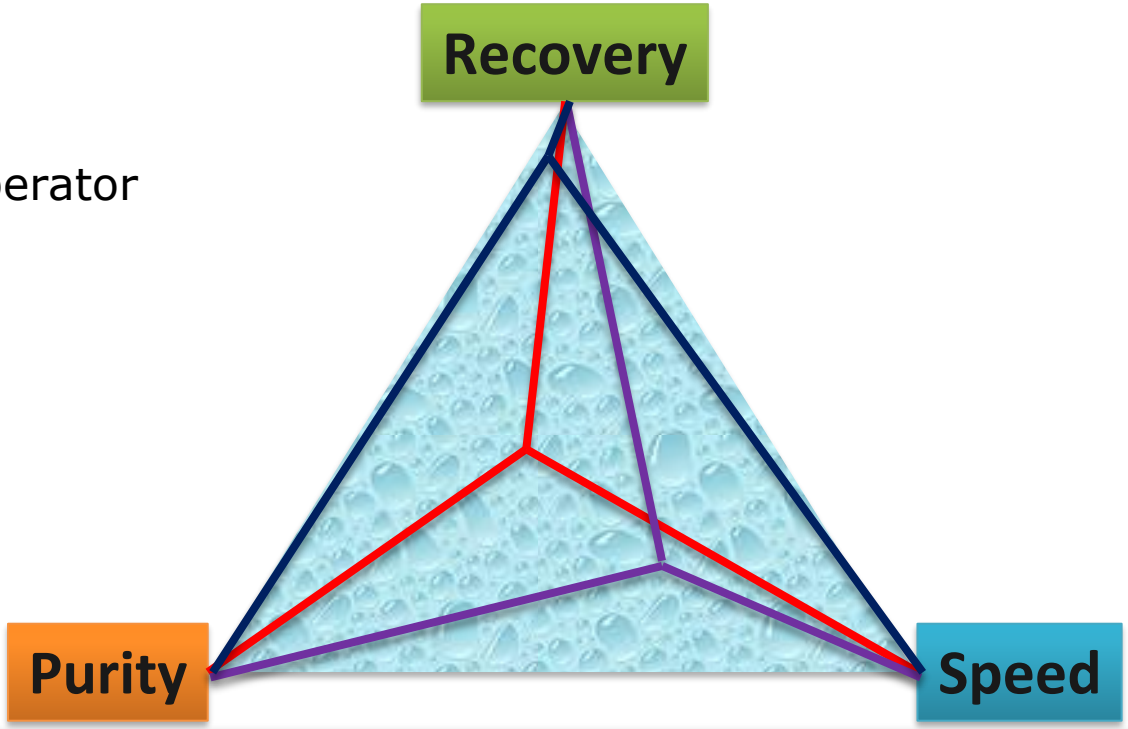


What else?

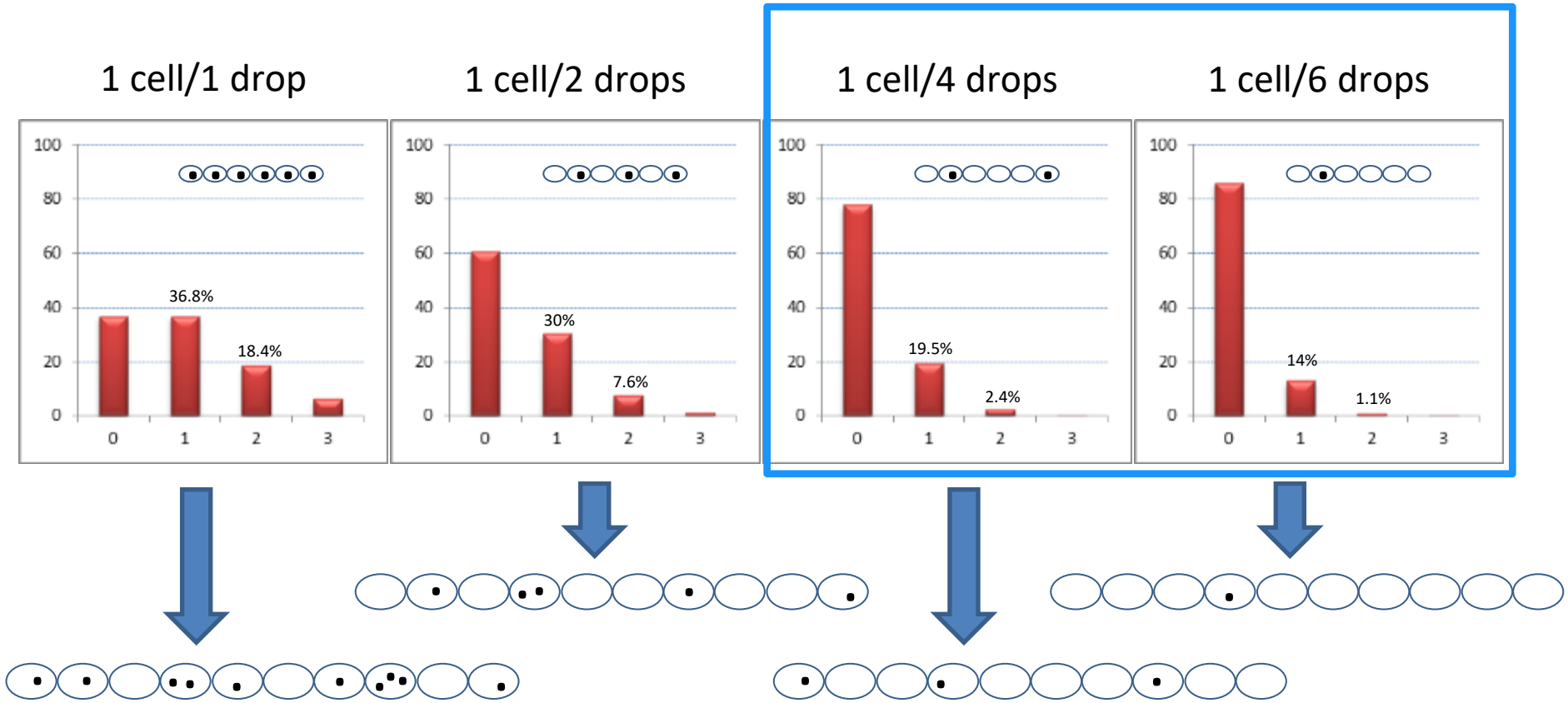
- Variable operation options lead to variable outputs
- Moderately steep learning curve but sky is the limit.

"**With great power comes great responsibility**“ – Stan Lee

- Expectations vs reality
  - Communication between operator and investigator is crucial
  - Pre-sort requirements
  - Post sort requirements
    - Downstream needs



Poisson Distribution



At 40k Hz you better not be over 10k events/sec.

Adapted from a slide by Rui Gardner

Pre-sort Information

Experiment prep

- Controls, controls, controls
- Compensation
- Cell size
- Nozzle size

Choose the right instrument

- Know the configuration and capabilities

Collection

- Tubes, plates, or slides
- Collection buffer (coated vessel)
- Volume

Sample Prep!!!!

Post-sort Information

Still gathered pre-sort

Where are the cells going?

- Viability needs
  - Can you sort in lysis buffer?
- Concentration needs
  - Nozzle changes
- Total count/yield needs
  - Expectations vs reality

- Do you need a post sort test?
  - Can we do a post sort test?
    - Volume/cell number

Shapiro's laws and Sample Prep

Practical Flow Cytometry – Howard Shapiro

Shapiro's Zeroth Law – There is no magic

- We're dealing with physics and chemistry, not magic
  - It just feels like it sometimes

Shapiro's First Law – a 51uM particle will clog a 50uM orifice

- Know your cells!
  - On its surface its about picking the right nozzle, but very much also about sample prep and single cell suspensions

Shapiro's Seventh Law – You can't make good data from bad data

- Closely related to "Garbage in, Garbage out"

Sample prep is the single biggest factor in the quality of the cell sorting process.

- Easier said than done, but….
- Everything suffers if sample quality is poor

That being said: Does Sorting Affect my Cells?

Pressurised to 60 psi

Accelerated to 20m/sec in a fraction of a second

Forced through a vibrating nozzle

Depressurised to atmospheric pressure

Passed through laser beams

Charged to 150 volts

Pass through an electric field of 10,000V/cm

Hit a liquid surface (hopefully) at 20/m sec

And we worry about poor recovery ?????

Adapted from a slide by Terry Hoy

Summary

Cell sorting and/or enrichment can be accomplished in many ways

- There is no single “best” option
- The tools are getting better every year

Single cell sorting technologies are extremely powerful but can’t be taken lightly

- What tool is the right tool
- How well do you understand your tool
- How well does the investigator understand the tool
- Does the investigator know what they want
- Do you know what the investigator wants

Sample prep is the single biggest factor in the quality of the cell sorting process.

- Easier said than done, but….
- Everything suffers if sample quality is poor

THANK YOU

Tim Bushnell, PhD, MBA

- Discovered Flow as a post-doctoral student.
- Flow Core Director in 2003 at URMC
- Director of Shared Resource Technologies in 2012
- Reorganized into the Center for Advanced Research Technologies (CART) in (2021).
- Served on ISAC Council and GLIIFCA Board of Directors
- Continues to serve on ISAC Committees
- NERLSCD Organizing Committee

Center for Advanced Research Technologies

The Future is Now!

Timothy Bushnell, PhD, MBA
Associate Professor, Pediatrics
Director, Center for Advanced Research Technologies
Scientific Director, Flow Cytometry Resource

Tim_bushnell@urmc.Rochester.edu

MEDICINE *of* THE HIGHEST ORDER

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What we have covered so far

- A history of Flow Cytometry
- How a Flow Cytometer Works
- The Spectral Revolution and Fluorescence
- Cell Sorting

What we learned so far

- Cytometers are composed of three key elements
- Flow cytometry works best on single cell suspensions
- A label is needed to identify our targets
- A detector must convert photons to electrons
- It is possible to isolate cells based on their physical and biochemical characteristics

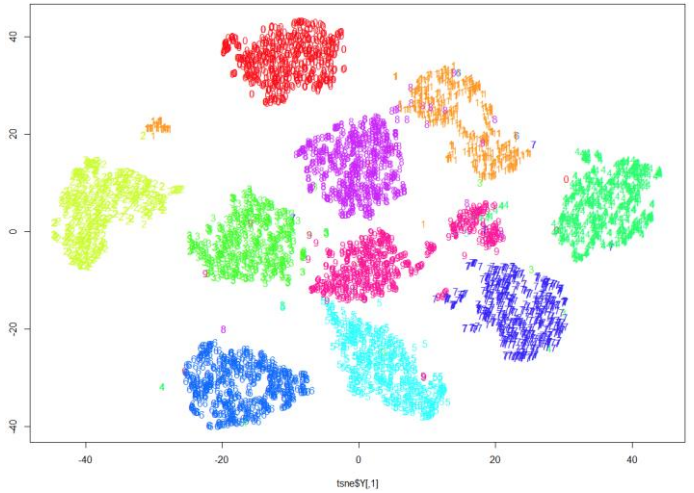
- Why do we want to push beyond current technology?
- How do we combine the detectors and the labels to push beyond current technology?
- Can we overcome the single-cell suspension requirement?
- What's next?

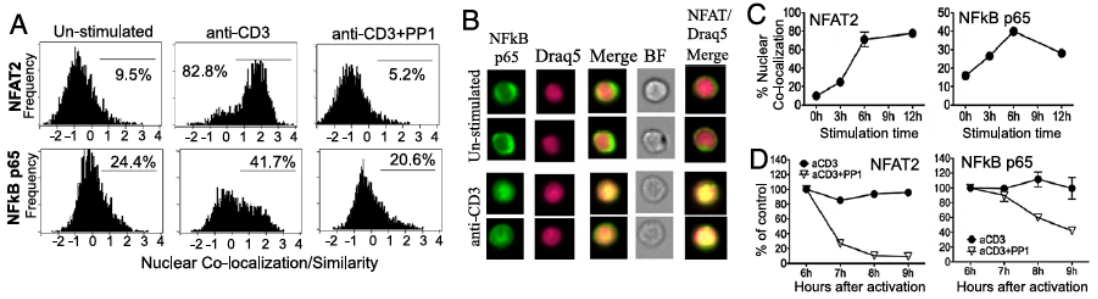
Why the interest in high-dimensional flow cytometry

- More colors allows for us to describe more populations
  - Width – look at lots of populations to get a picture of the different populations in a sample. Good for initial discovery work
  - Depth – look at fewer populations in detail to understand the biology of these cells
  - Discovery – identify previously undiscovered poplations (Cyto Incognito)

Options for High Dimensional Flow Cytometry

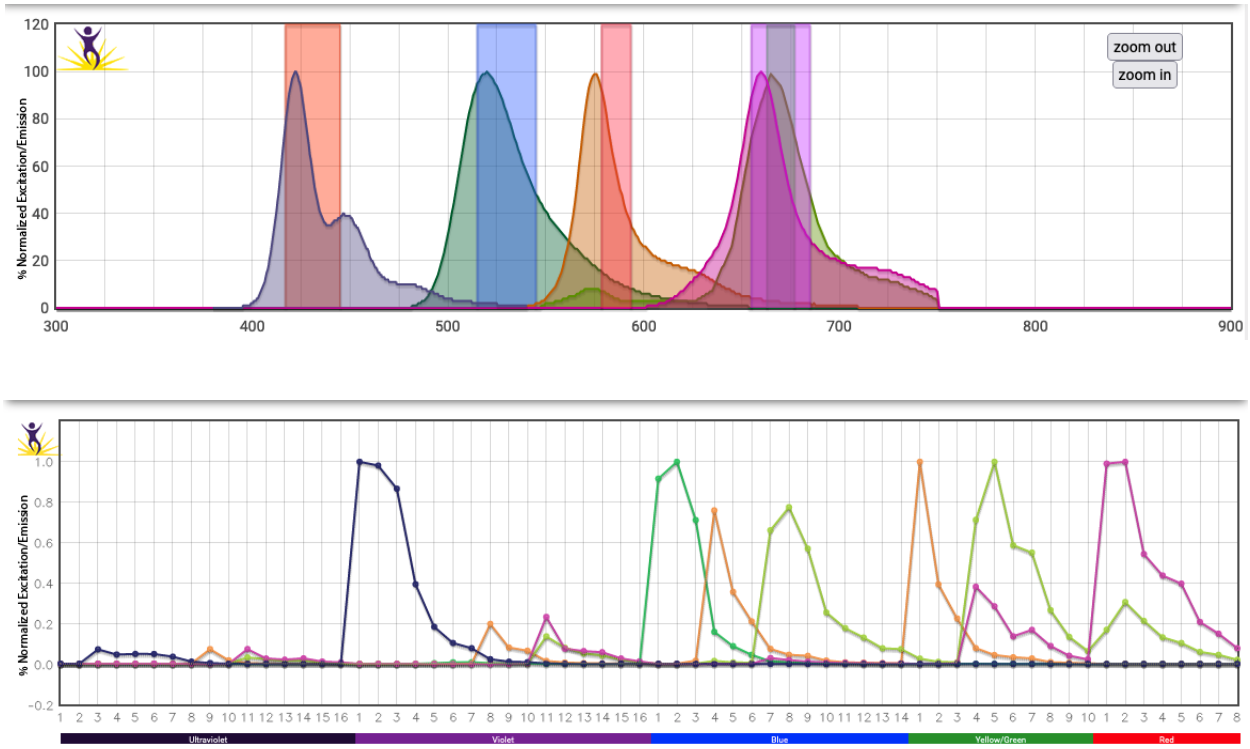
- Traditional Flow Cytometry
- Spectral Cytometry
- Mass Cytometry
- Raman Cytometry
- Imaging Cytometry





Fluorochromes

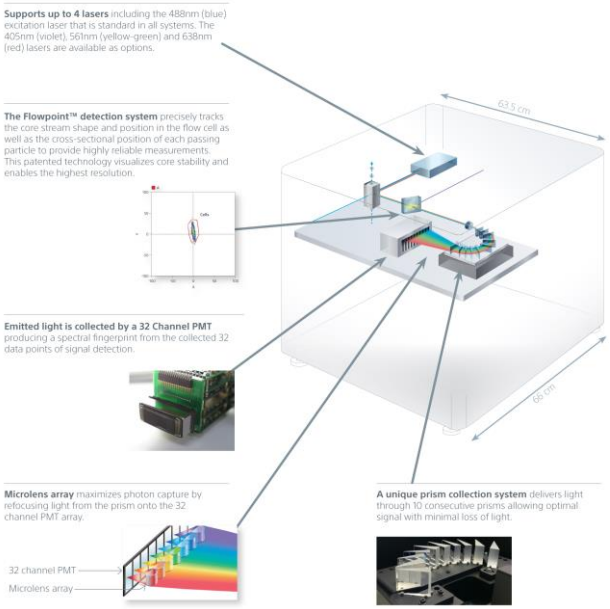
- Spectral viewers are your friend



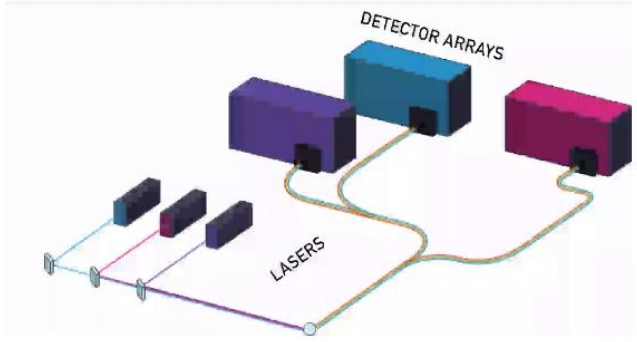
Spectral Cytometry

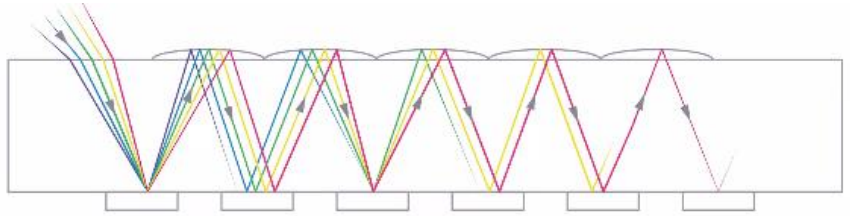
- Caveat -all flow cytometers can generate spectral data IF one or more detectors is left blank

Sony System



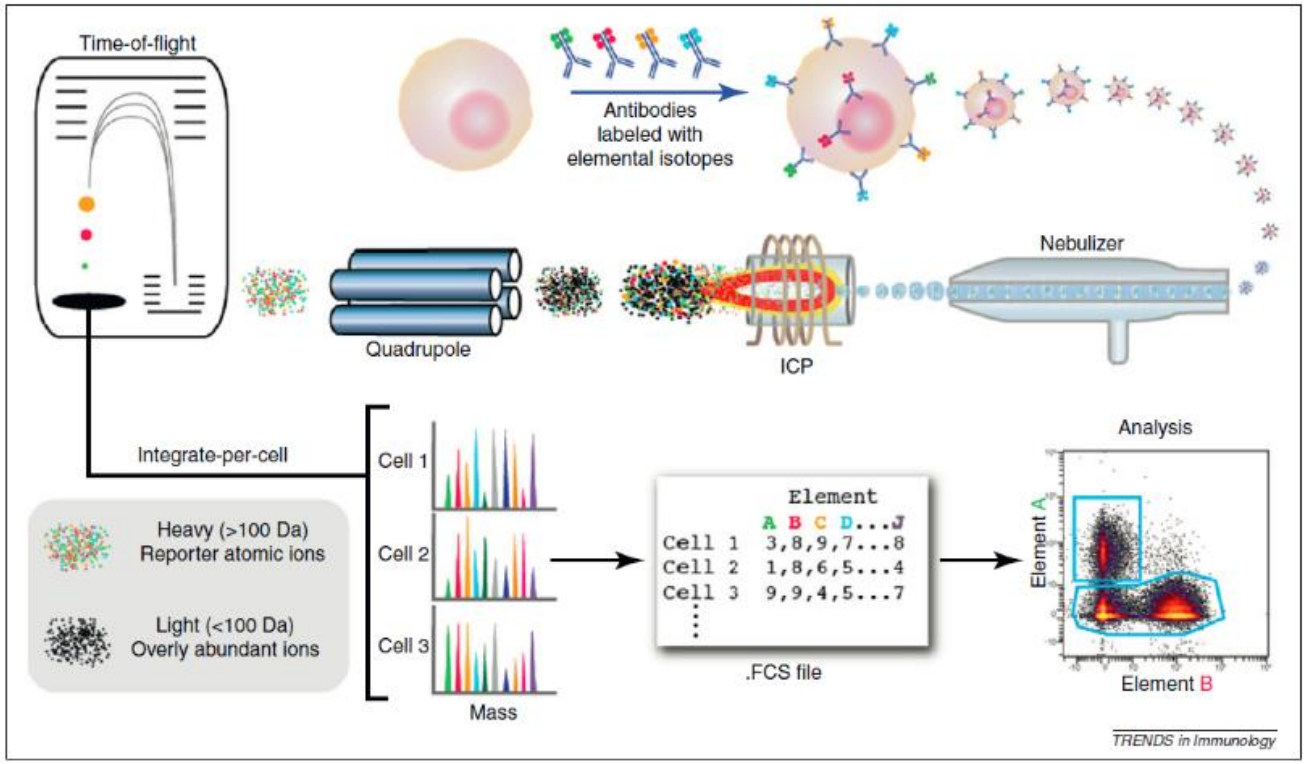
Cytek System





Mass Cytometry

- Imaging replacing the fluorochromes with metal tags and the PMTs for a mass spectrometer?



Bendall S. *et al*. (2012). Trends in Immunology, **33** (7), 323-332

Mass Cytometry

Pro's

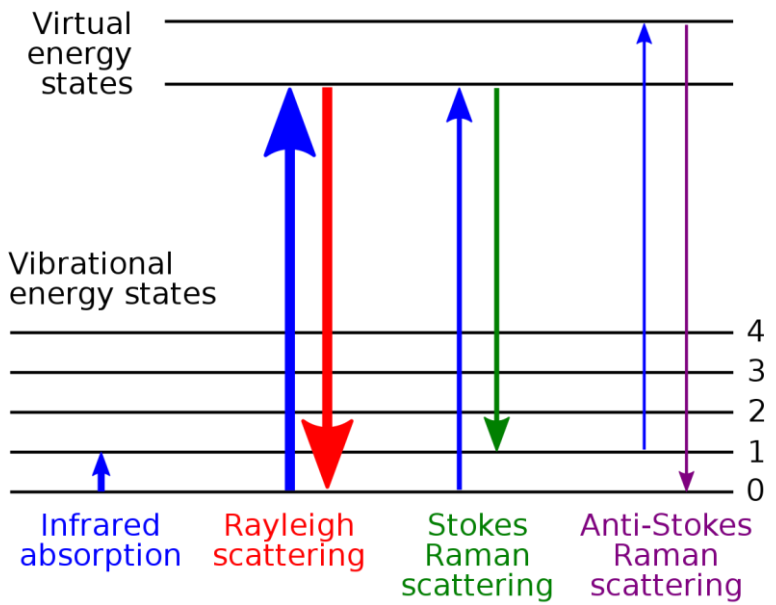
- Easier to design panels
- More predictable 'spillover'
- Stability of samples
- Barcoding
- *Tissue Mass Cytometry*

Con's

- Invest in new reagents (expensive)
- Dedicated instrument and new infrastructure
- Need large userbase
- Sample is destroyed

Raman Cytometry

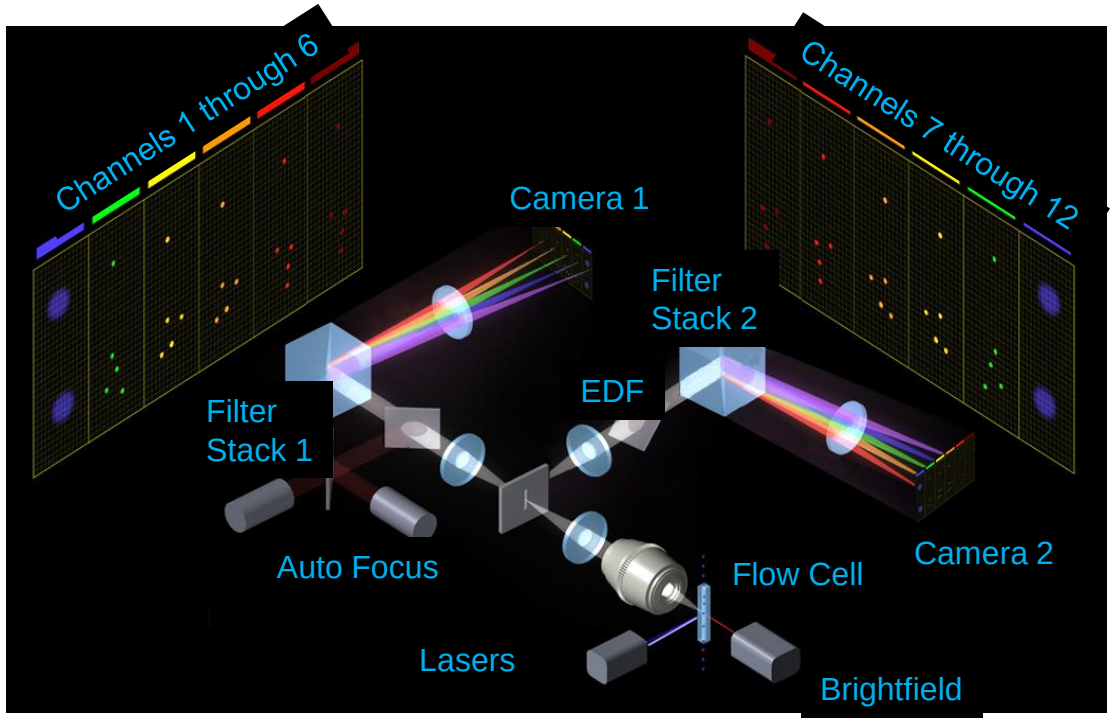
- Raman spectroscopy measures the inelastic scattering of photons
- Label-free cytometry
- Relatively weak, but techniques to enhance signal
- Efforts to improve speed and sensitivity
- Measures metabolic compounds not amenable to fluorescent flow



By Moxfyre, based on work of User:Pavlina2.0 - vectorization of File:Raman energy levels.jpg, CC BY-SA 3.0, https://commons.wikimedia.org/w/index.php?curid=7845122

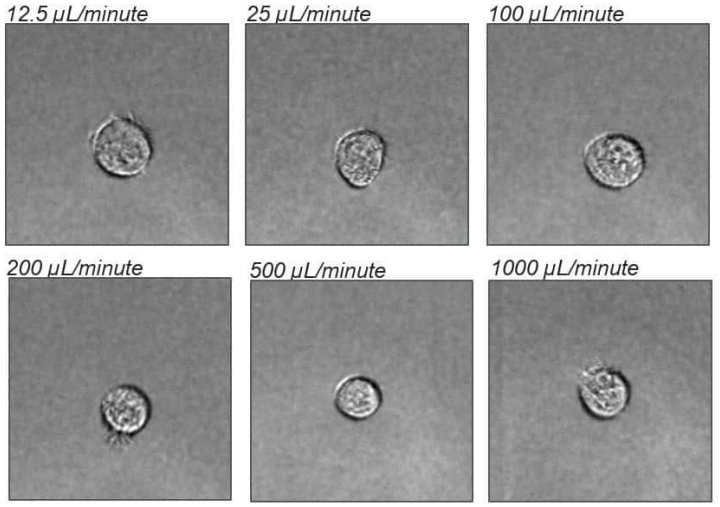
Imaging Cytometry

- A picture is worth a thousand words
- Allows for characterization and location of the signals of interest
- Originally commercialized by Amnis (now part of Luminex)



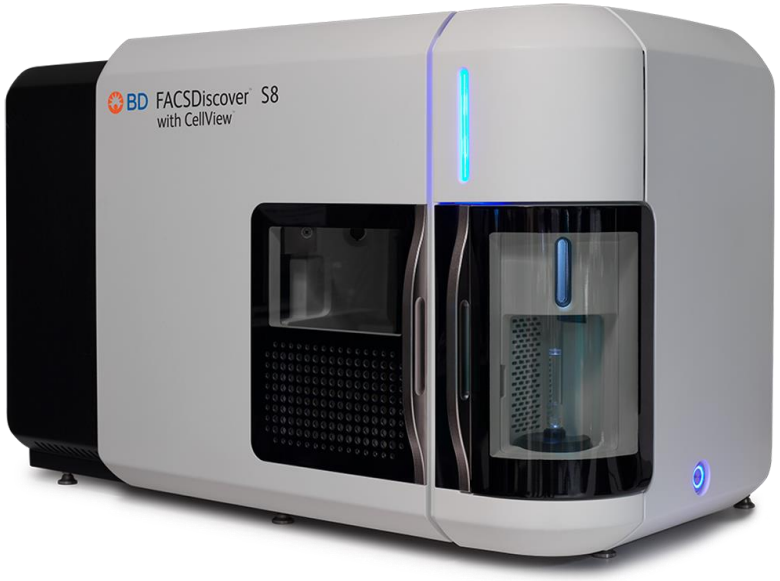
But wait – there is more!

Attune CytPix system has a brightfield camera allowing for brightfield images of cells in motion



https://www.thermofisher.com/us/en/home/life-science/cell-analysis/flow-cytometry/flow-cytometers/attune-nxt-flow-cytometer/models/cytpix.html#image-enhance-flow-cytometry-applications

Spectral and Imaging and Sorting Cytometry in one box



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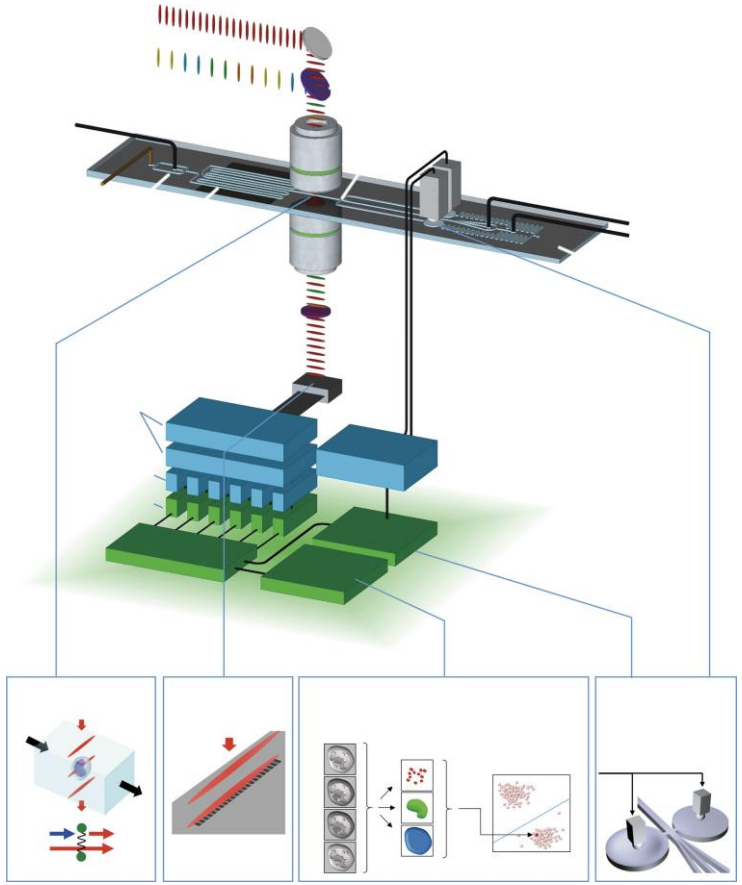
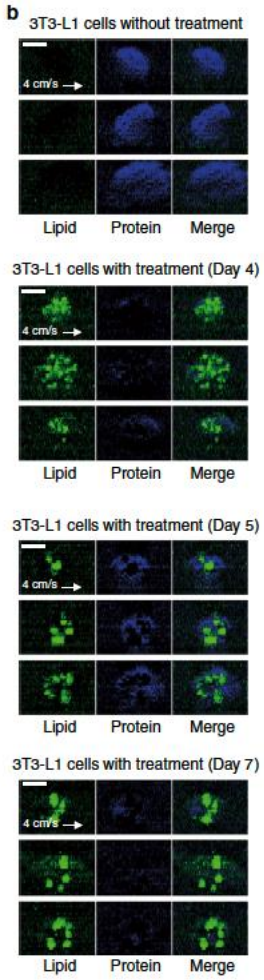
Raman imaging sorter



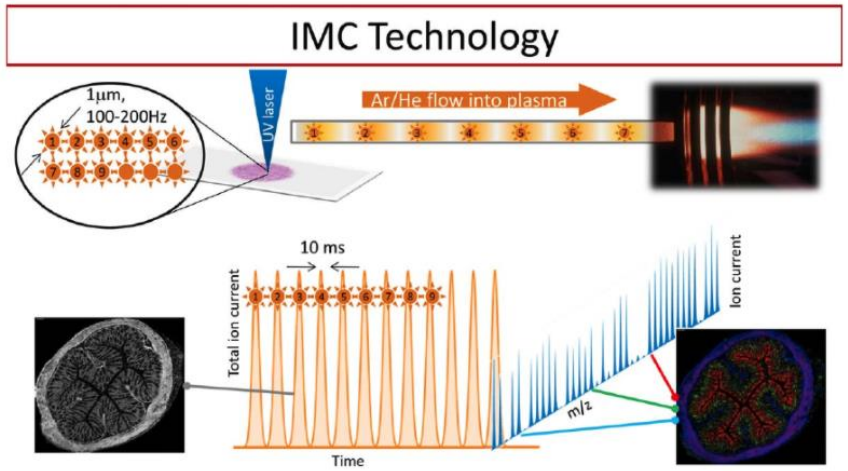


NATURE COMMUNICATIONS | https://doi.org/10.1038/s41467-020-17285-3

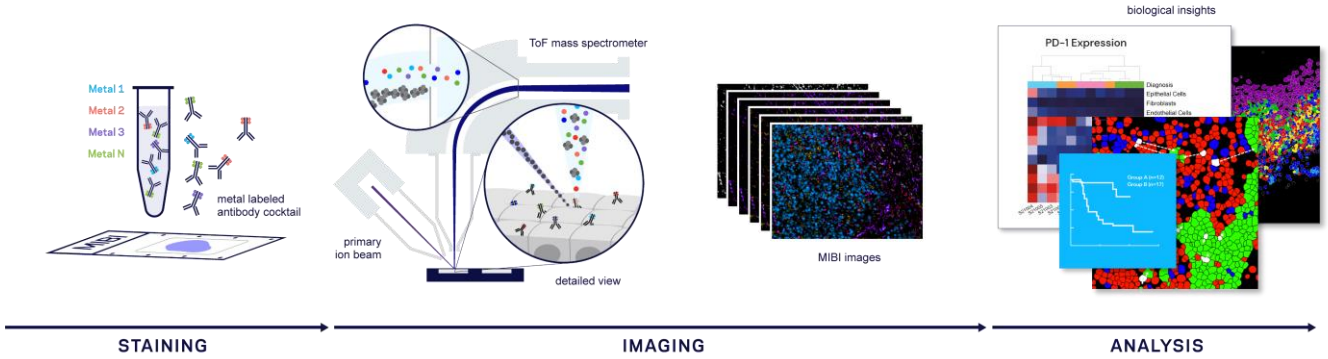
Overcoming single cell suspensions

- Location is critical to understanding how cells can impact the function of neighbors
- This is lost in solution-based flow cytometry
- Traditional microscopy techniques are limited in the number of parameters that can be interrogated
- Spatial proteomics

Imaging Mass Cytometry



Chang et al (2017) - DOI: 10.1002/cyto.a.23053



https://www.ionpath.com/mibi-technology/

The Future?

- For a 50+ year old technology, flow cytometry continues to innovate
- Allows one to confirm data from RNAseq type experiments.
  - Is the protein there?
  - Is it being modified (e.g. Phosphorylated)
- Also allows for functional assays and isolation of cells for downstream applications
- Spatial ‘Omics and meta ‘Omic data analysis is the next big step

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