Introduction to Flow Cytometry

UNIVERSITY of ROCHESTER MEDICAL CENTER

Center for Advanced Research Technologies

URMC Flow Cytometry Core

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Purpose of this Lecture

Introduction to URMC Flow Cytometry Core

- Instrumentation
- Training overview
- Policies

Introduction to Flow Cytometry

- What is cytometry
- Instrumentation basics
- Important concepts
- Types of experiments possible

Our Team

Leadership Group

- Tim Bushnell, Ph.D. Scientific Director
- Matt Cochran, Technical Director
- Wojciech Wojciechowski, Development Director
- James Java, Data Analytics

Full time instrumentation specialists

- Lucas Larkin
- Meghann O'Brien
- Zach Nowak
- Steven Polter
- Daria Stekolnikova
- Calvin Tian
- Tom Tam
- Terry Wightman

Aministration etc.

- Sharleen Slaunwhite
- Beth Laffey

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Instruments Available

Traditional Analytical
- 1 Accuri C6+
- FACSDiva Cytometers
  - 2 Fortessa, 5 laser, 18 color
  - 3 LSRII, 4 lasers, 18 colors
  - Symphony A1 is 4 lasers, 16 colors

Full Spectrum Cytometers
- 2 Cytek Auroras (4 and 5 laser)

Mass Cytometer
- Helios

Cell Sorting
- FACSAria-II (17-color)
- FACSAria-II (18-colors)
- BioRad S3e (4 colors)
  - All in secondary safety containment

Metabolomics
- Seahorse XF96

Small Particles
- Nanosight NS300

Imaging Cytometry
- ImagestreamX
- Celigo

Bead based Array
- BioRad BioPlex 200

Additional Services Available

Consultation (office hrs: Zoom as requested)

- Experimental design
- Panel design
- Data interpretation
- FCC_Library – training and educational material

Analysis Computers

- PC workstation
- Multiple analysis programs

Data archiving and transfer

- FCC archives experimental data
  - Code42 automated archiving
- FCC_Transfer provides a space for moving data from cytometers to lab
  - Not for long term storage. Space is cleared once a month.

Data analysis

- Allow approximately a week for analysis
- Ask for a quote for costs

On-going lecture series and webinars

Training Schedule

http://www.urmc.rochester.edu/flow-core/training/

Introduction Lecture must be completed prior to scheduling for any training visits.

Standard Diva Instrument Training**

- Visit 1: 1.5 hour free training on the LSRII using bead, 1 person at a time.
- Visit 2: ~1 hour free training on the LSRII using beads for 1 trainee at a time.

All Other Instruments

- Variable training programs discussed when request is made

Afterhours training

- A separate quiz and practical must be passed for a user to receive after hours access.

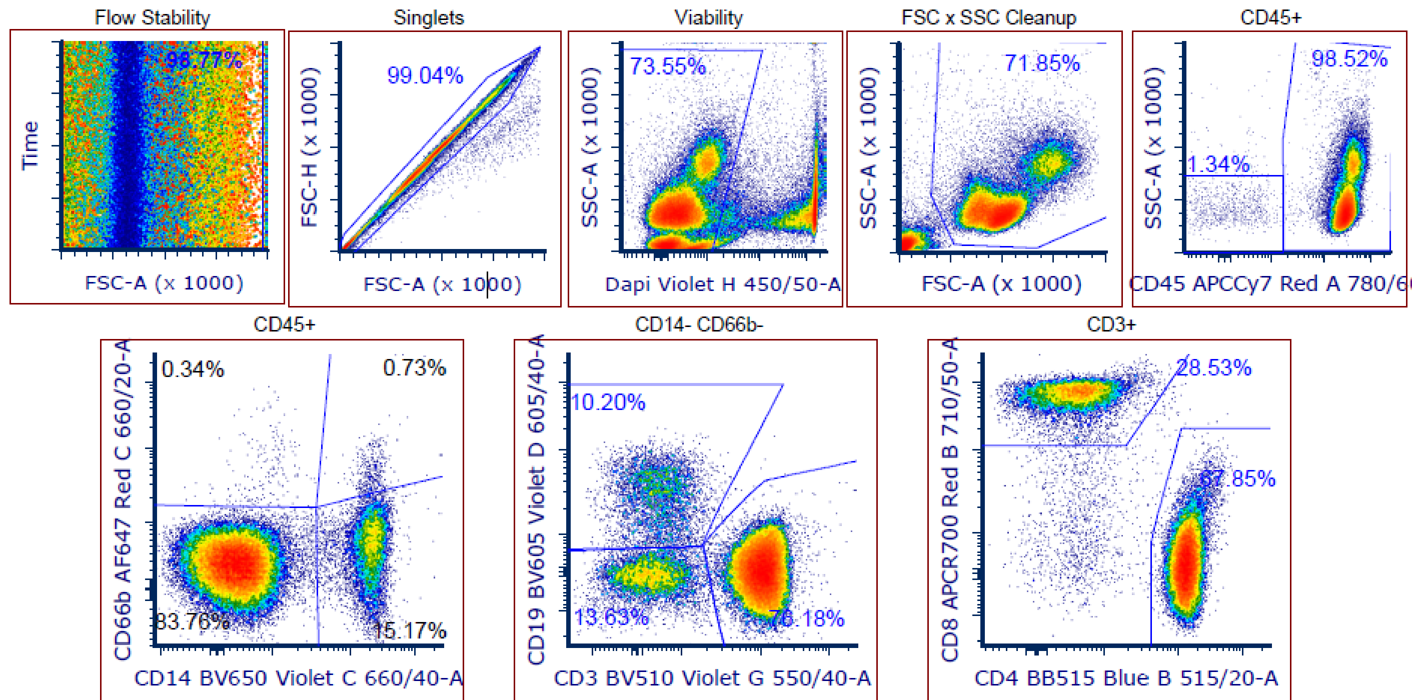
We do not offer training on the Helios or the FACSAria Cell Sorters

Training on the Luminex is not yet open

**** It is critical that we start on time for visits 1 and 2. Anyone more than 10 minutes late will have to reschedule and will be charged for 1 hour of LSRII time.**

What is Cytometry

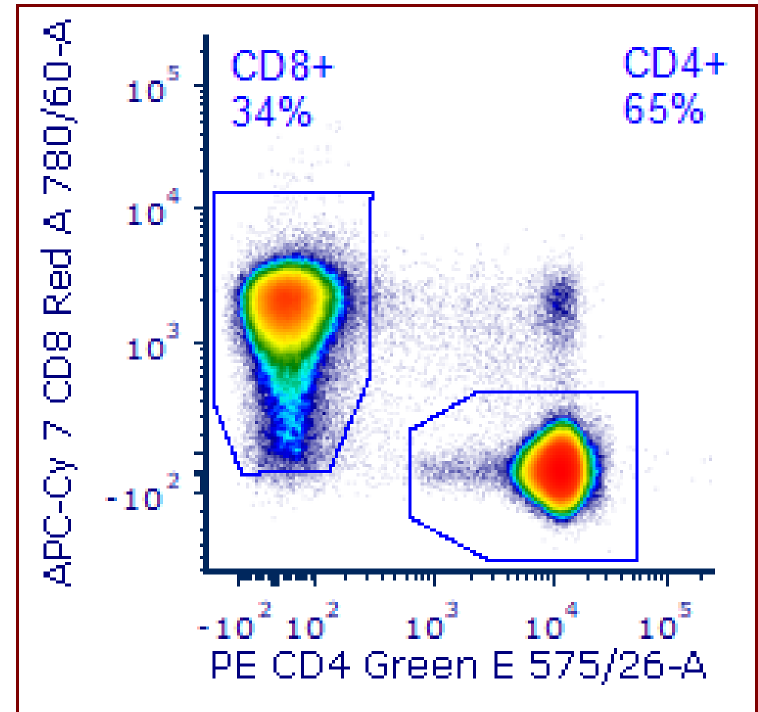
- Cytometry is the measurement of biological processes at the whole cell level
- In Flow Cytometry these measurements are made as the cells, in a fluid suspension, pass one at a time through a measurement apparatus – the Flow Cytometer



Why Flow Cytometry

- We can measure many thousands of cells in a short period of time
- Gather statistical information
  - Sample level, experiment level, project level
- Acquired data can be easily reanalyzed
  - Data analysis does not alter data files
- Basic Tenant – Flow Cytometry provides discrete measurements for every detector, from each cell in the sample, providing a distribution of values, rather than an average.
  - Single cell measurements

What can we measure with Flow Cytometry



Intrinsic Characteristics

- Size – Forward scatter
- Granularity – Side Scatter
  - Shape
- Natural Fluorescence (chlorophyll, carotenoids)
  - Autofluorescence

Extrinsic Probes

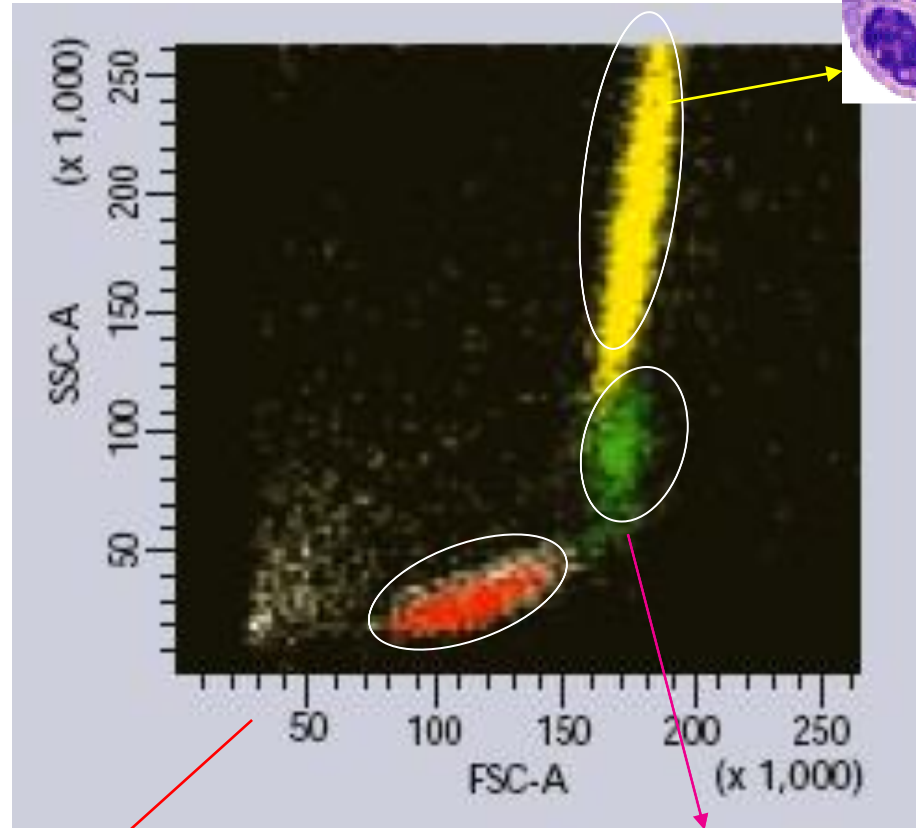
- DNA – Content, Cell cycle
- Apoptosis, Necrosis
- Membrane structure
- Receptors – internal and external (Immunophenotyping CD markers, etc)
- Transfection/infection efficiency (Fluorecent proteins like GFP, mCherry)
- Physiological Parameters – Enzyme activity, Calcium flux

Time

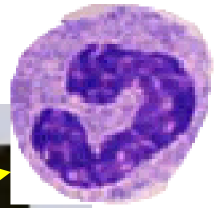
Cell or particle concentration

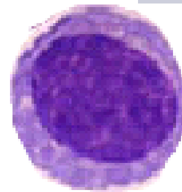
Light Scatter

- Light scatter provides qualitative information related to cell size or internal complexity.
- All objects which pass through a laser beam in a cytometer will scatter light.
- Forward Angle Light Scatter (FSC) is measured in line with the laser and is proportional to size.
- Scatter near 90° (Side Scatter, SSC), is proportional to granularity.



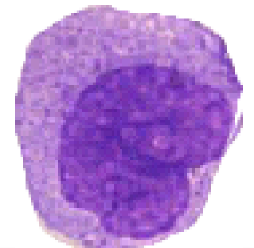
Granulocytes



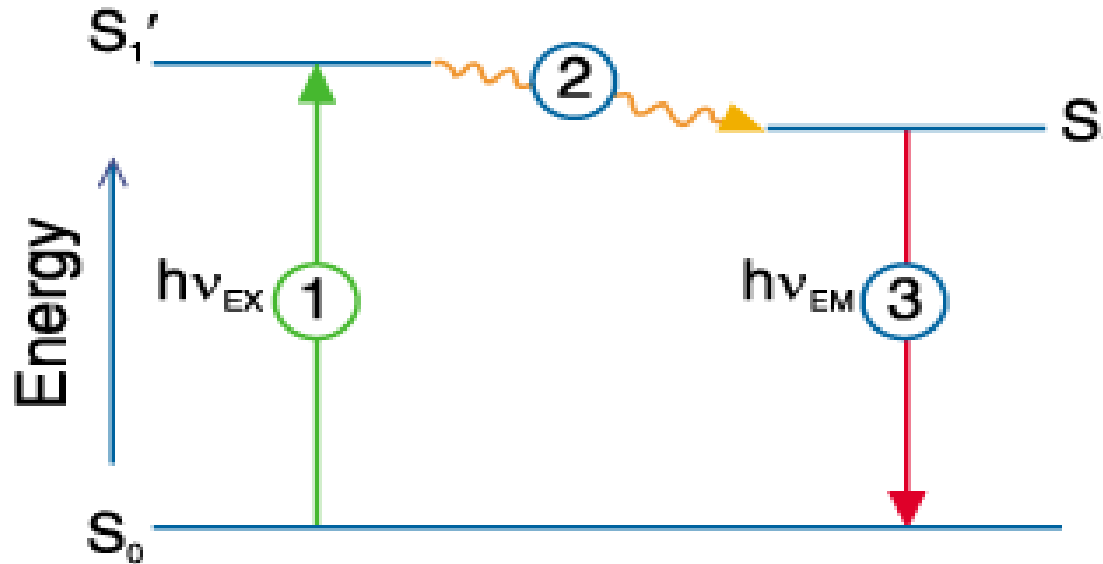


Lymphocytes

Monocytes



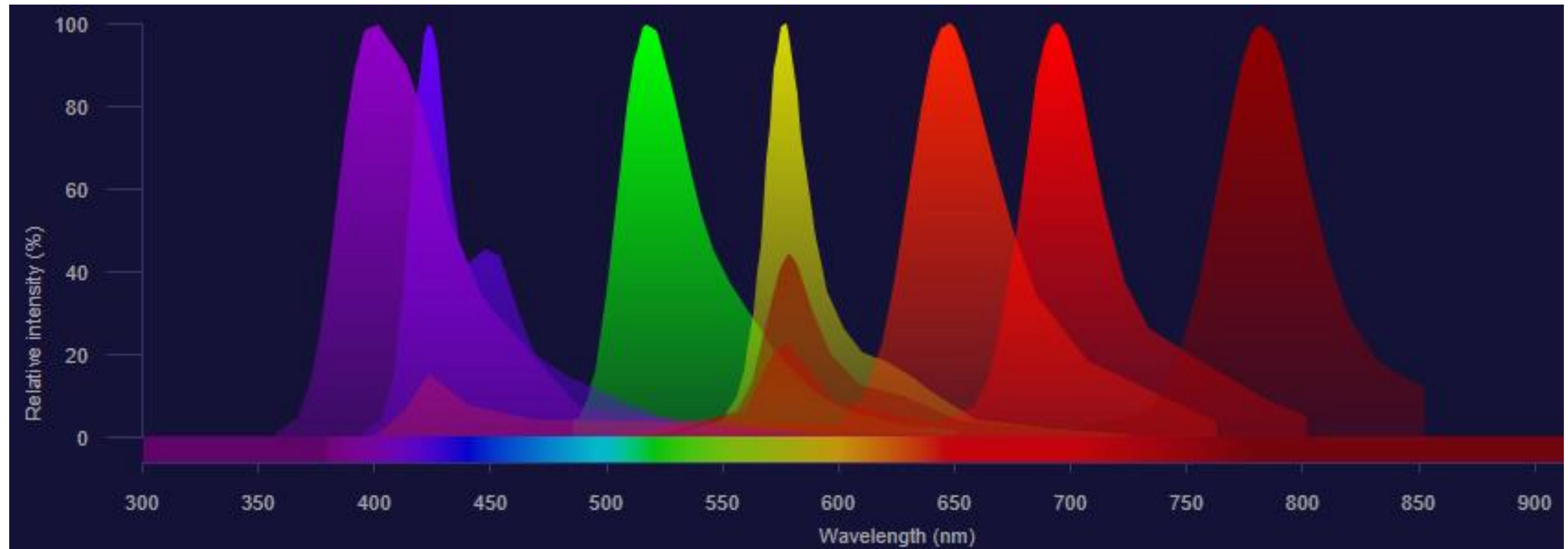
Fluorescence – 1 – Generating fluorescence



1. Fluorescent molecule absorbs energy (excitation) from the photons of light in the lasers
2. Variable amount of energy lost
3. Energy is released from the molecule (emission) in the form of photons of light.

Photon emission wavelength is longer (lower energy) than the excitation

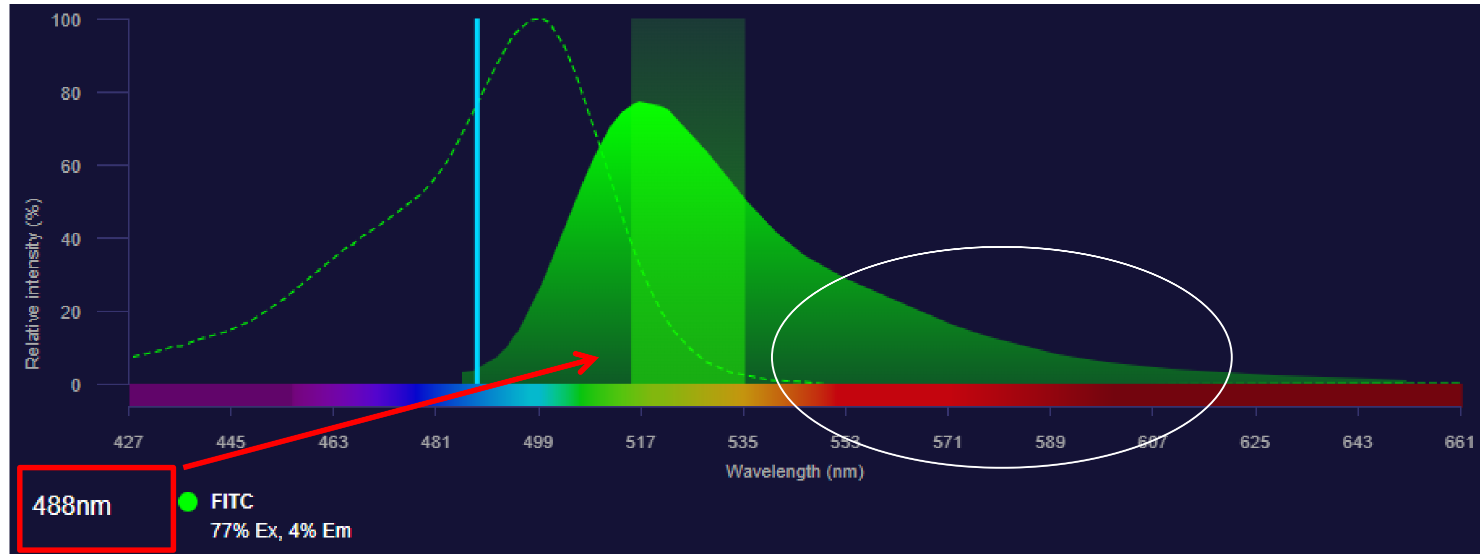
Fluorescence – 2 – The complexity observed

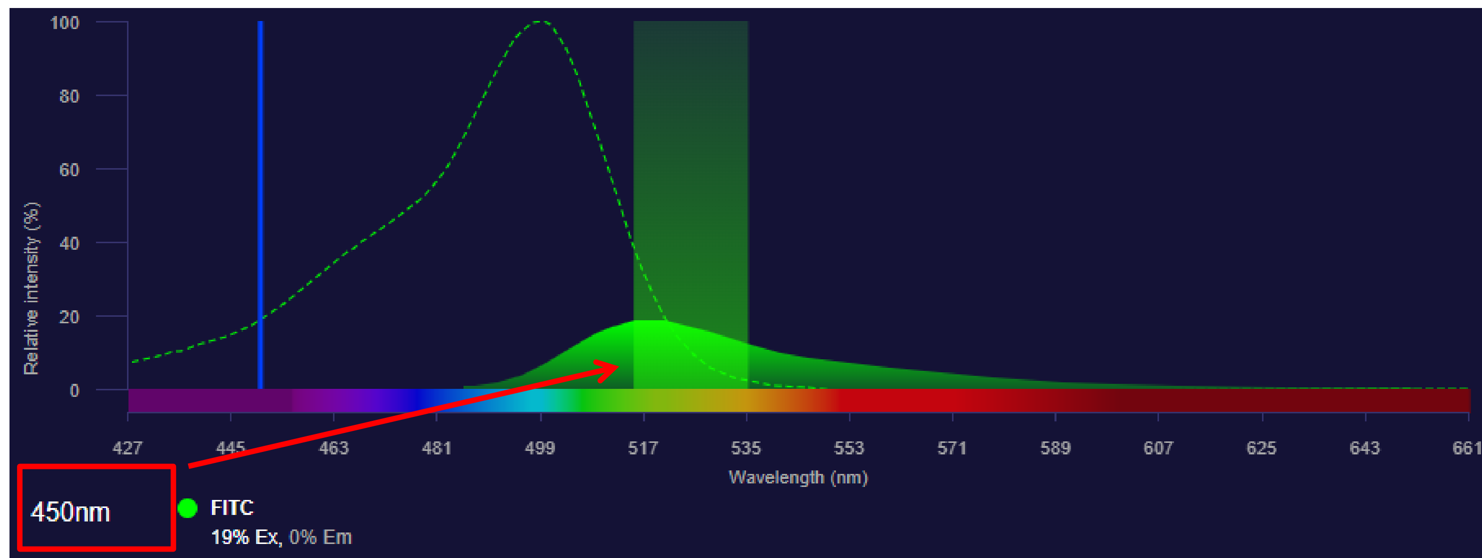


1. Working mostly in the visible light spectrum – high 300s to mid 800s nm
2. Crowding of the space occurs quickly leading to spectral overlap/spillover
   1. Panel design is critical and difficult
   2. Compensation helps with visualization, but not loss of sensitivity
3. Both excitation and emission factor in to output and must be considered

Photon emission wavelength is longer (lower energy) than the excitation

Fluorescence – 3 – Intensity and "color"





Applications

Antibody staining
- Surface Staining
- Intracellular Staining

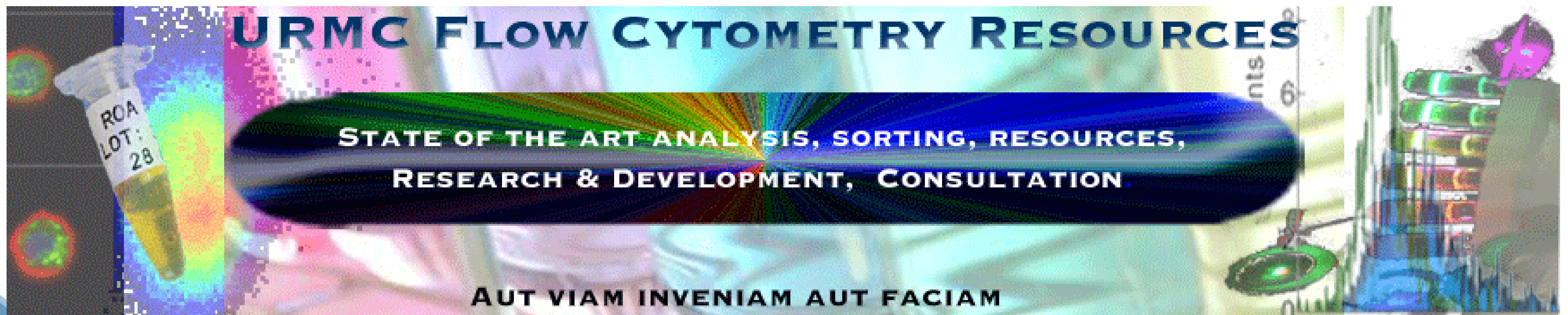
Biological processes
- Cell Cycle/Cell Proliferation
- Apoptosis/Cell Death
- Intracellular processes

Gene Expression
- Fluorescent Proteins (GFP/YFP/etc)

Molecular Cytometry
- Intracellular ions
- Membranes

Plant and Marine Biology
- Pico plankton and bacteria

Cell Isolation
- Sorting



How do we make these measurements?

Systems within a Flow Cytometer

Fluidics

- Getting cells from a your sample tube to the intercept point(s)

Optics

- Move and separate the light
  - Move the lasers to the intercept point(s)
  - Separate the emitted fluorescence after cells pass the intercept(s)

Electronics

- How photons of light become electrons
- How electrons become a digital data file

Data Analysis

- Making sense of the digital output

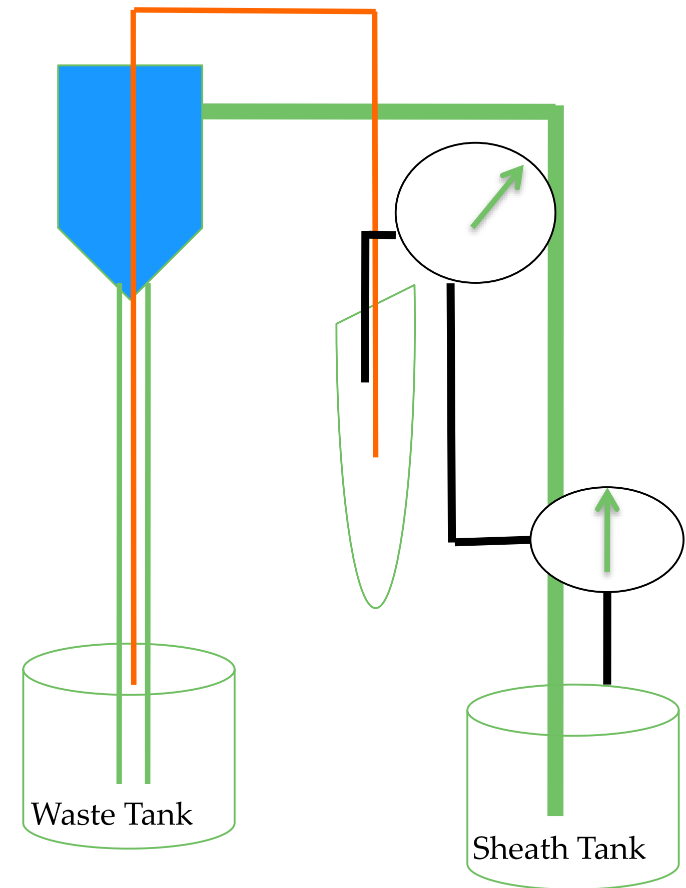
The fluidics system moves the cells

Pressure is applied to both sheath and sample

- Pressure can be applied via compressor (air) or pumps/syringes (physical)

Sheath pressure typically sets the fluid flow rate

Difference between sheath and sample sets sample acquisition rate

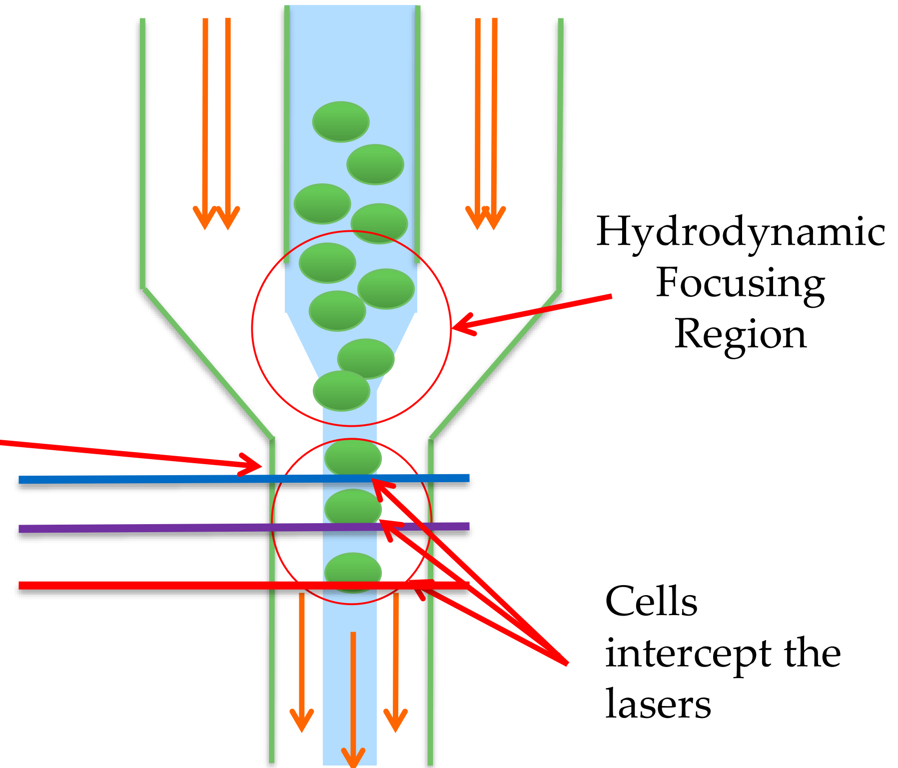


Fluidics

Cells are hydrodynamically focused by injection into a stream of ‘sheath’ fluid

Cells pass single file through the interrogation region

Sample flows with the sheath fluid in laminar flow



IMPORTANT: sample and sheath do not mix

Flow Rate

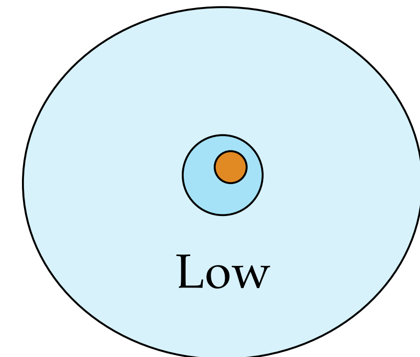
Sheath fluid runs at a constant velocity

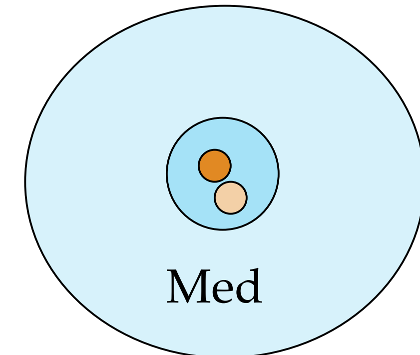
Therefore, to increase sample throughput (i.e. speed), we need to increase the pressure differential

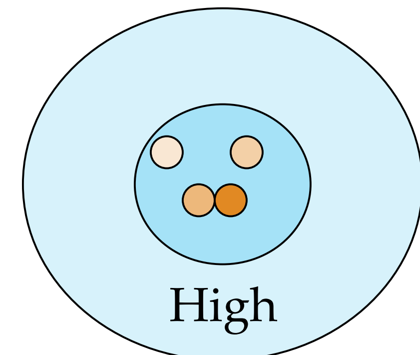
This increases the core stream size, so more cells pass the intercept per unit time

This can be bad

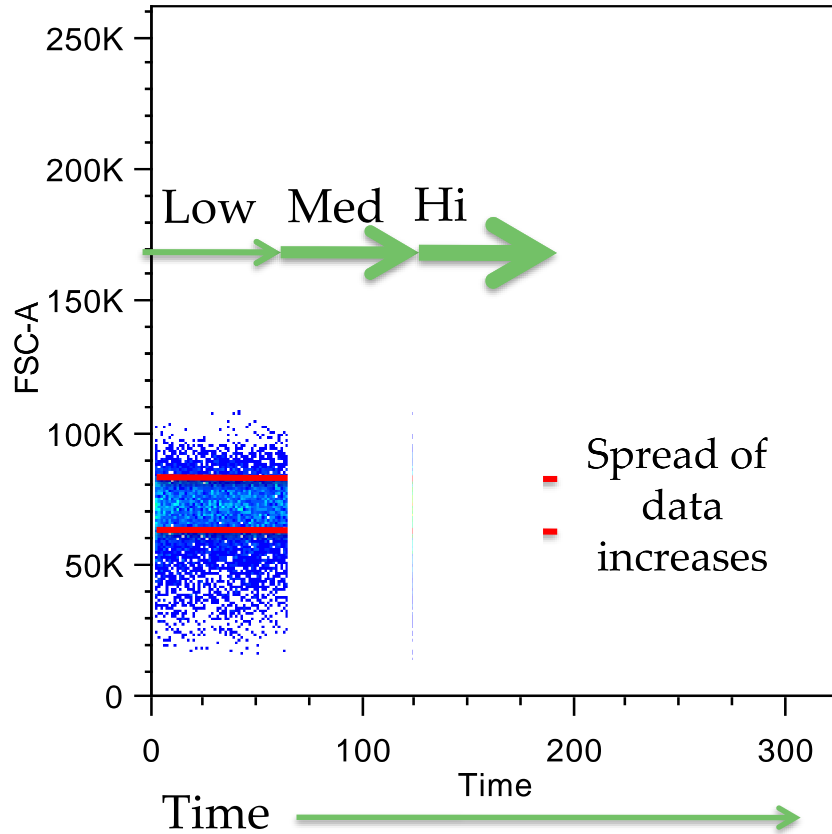
- Increases coincident events
- Increases spread of fluorescent signals

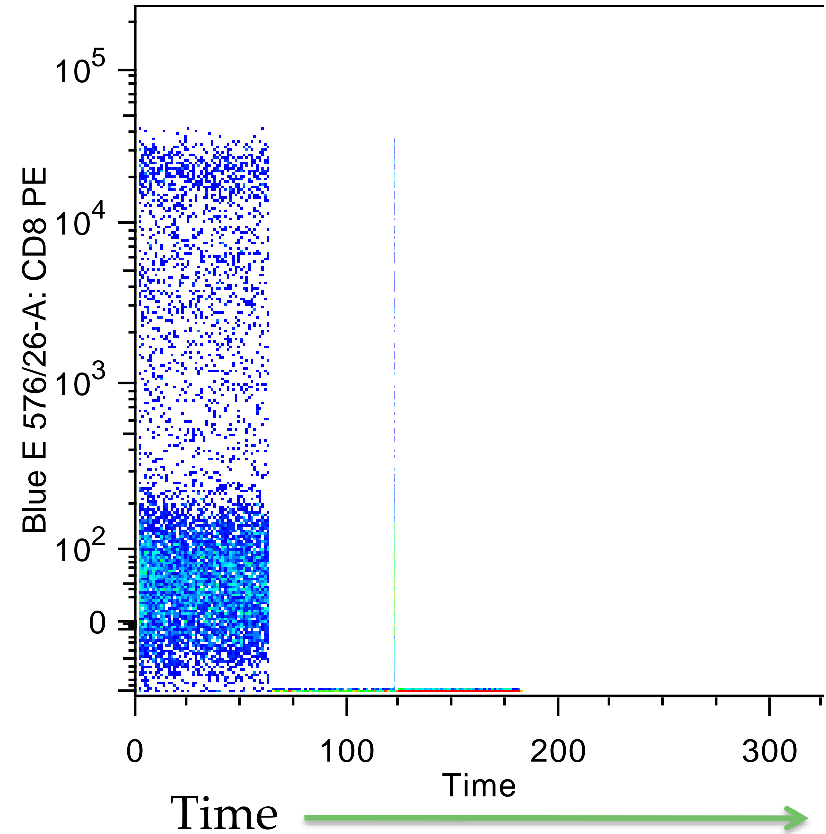






Changing Flow Rate





Time

Time

Take home message

To increase acquisition rate
concentrate your sample

** **smaller volumes with higher concentration can be diluted but samples that are too dilute are stuck**

Optics

Three optical systems

- Excitation source
- Filter System
- Photon capture system

Excitation source

- Lasers or Hg Arc lamps
- Dictate excitation lines

Filters

- Long pass, short pass, band pass
- Set the sensitive range of the detectors

Photon capture system

- PD
- PMT
- CCD

Filters – separate light based on wavelength

Dichroic mirrors (traditional)

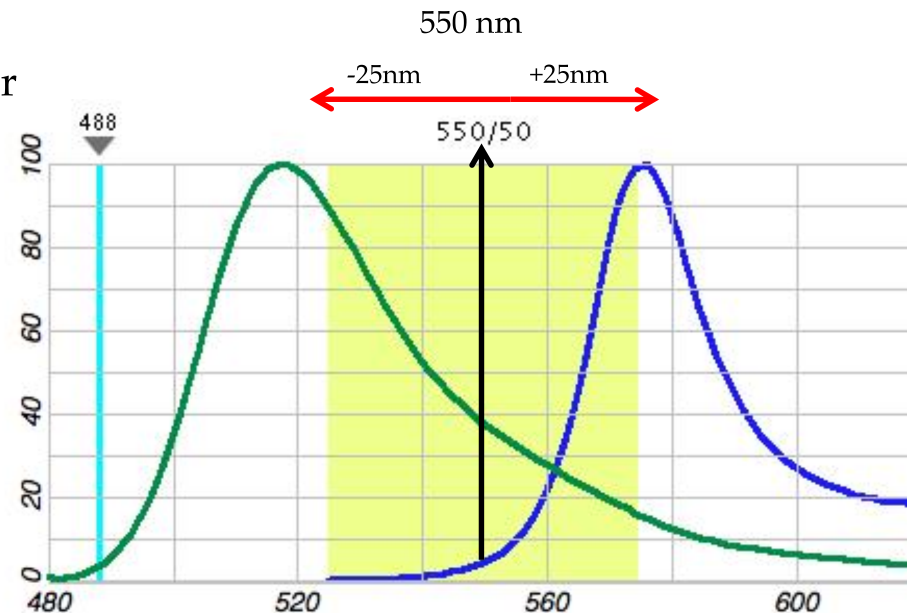
- Pass light of one signal, deflect the remainder

Long or Short pass (e.g LP560 or SP560)

- Transmits light above or below the stated wavelength

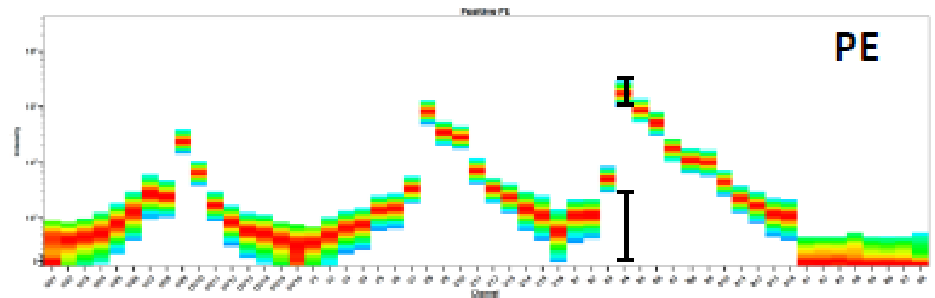
Bandpass (550/50)

- Transmits light between a given range
- Centered at 550 nm and +/- 25 nm



CWDM – coarse wavelength division multiplexing (spectral)

- Split light into bands of wavelengths



Configuration/Details Pages

Oscar the Grouch Details

MIFLOWCYT information specific for this instrument can be found in the FCC_Libra

Detailed information about the QC can be found in the baseline report (available u

Reservations can be made online by following this link.

You will need an approved USER account. Apply for one here.

To design a panel using Fluorofinder please click here.

Training videos can be accessed here.

LSRII Configuration: Low starting voltages as of Sept 2019
BLUE 488 LASER (20mW):

Detector	LP	Band Pass	Fluor's	LowVoltage
BLUE A	685	710/50	PerCP-Cy5.5	650
BLUE B	505	515/20	FITC, CFSE, GFP, Alexa 488	600
BLUE C		488/10	SSC	
FSC				

RED 633 LASER (100mW):

Detector	LP	Band Pass	Fluor's	Low Voltage
RED A	740	780/60	APC-Cy7, APC-Alexa 750	450
RED B	685	710/50	APC-Cy5.5, Alexa 700	350
RED C		660/20	APC, Alexa 647	350

Detecting Photons

- Photodiodes/Avalanche Photodiodes (PD/APD)
  - FSC often with PD
  - Aurora uses APD
- Photomultiplier Tube (PMT)
  - All BD instruments (LSR, Aria)
- CCD Camera
  - Image Stream, Celigo

Detectors convert photons of light into electric current

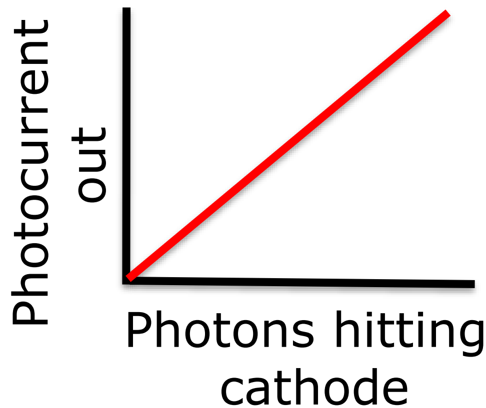
Inside the PMT

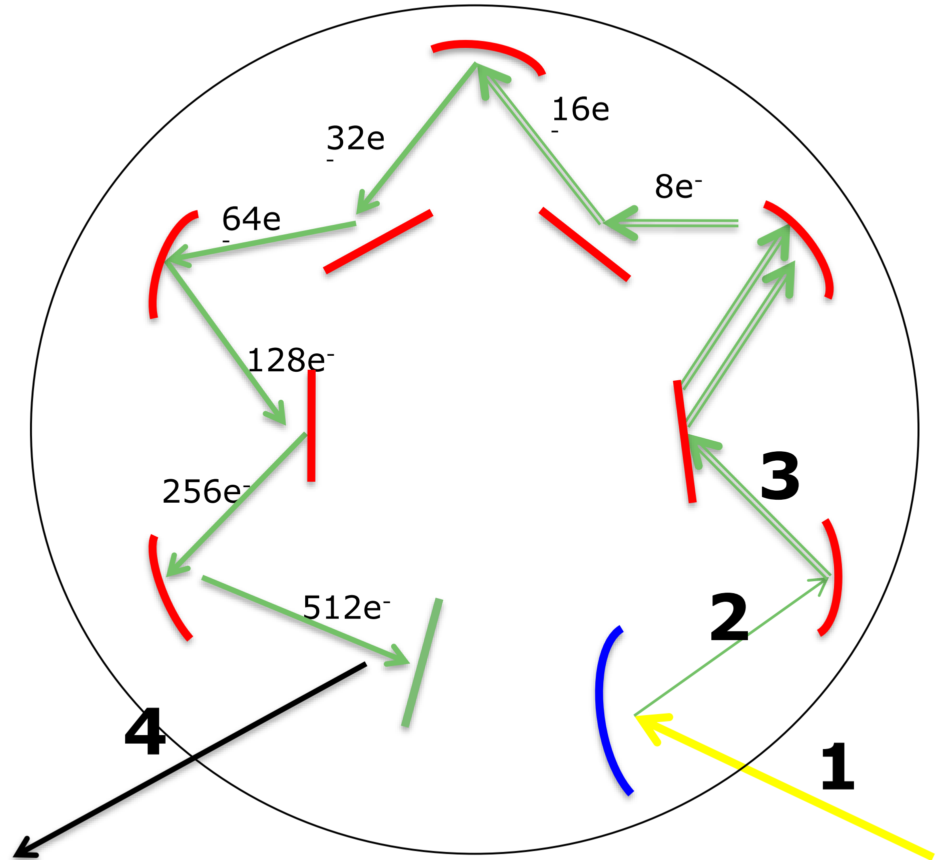
① Light strikes the photocathode

② An electron is discharged towards a dynode

③ 2 electrons are ejected with each electron hitting a dynode

④ Electrons reaching the photoanode produce the photocurrent





Anatomy of an electronic pulse

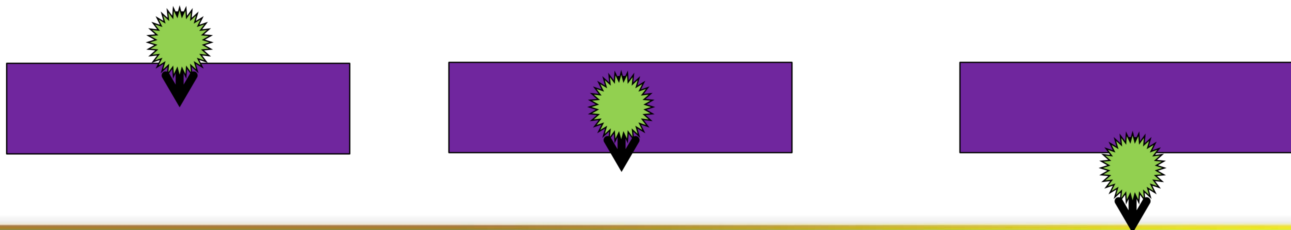
Pulse Width (time of flight)

Pulse Area

$$\int_a^b f(x)\,dx$$

Pulse Height

THRESHOLD



Data Files

Photocurrent is a continuous signal, it must be converted to a discrete digital number to be stored in the data file

Binning of data – plot scales differ based on number of bins

The samples are digitized using an analog to digital converter (ADC)

- LSR and Aria are 18 bit cytometers (~262k bins)
- Accuri C6+ (~17 million bins)
- Aurora (~6 million bins)
- FACSCalibur (~1k bins)

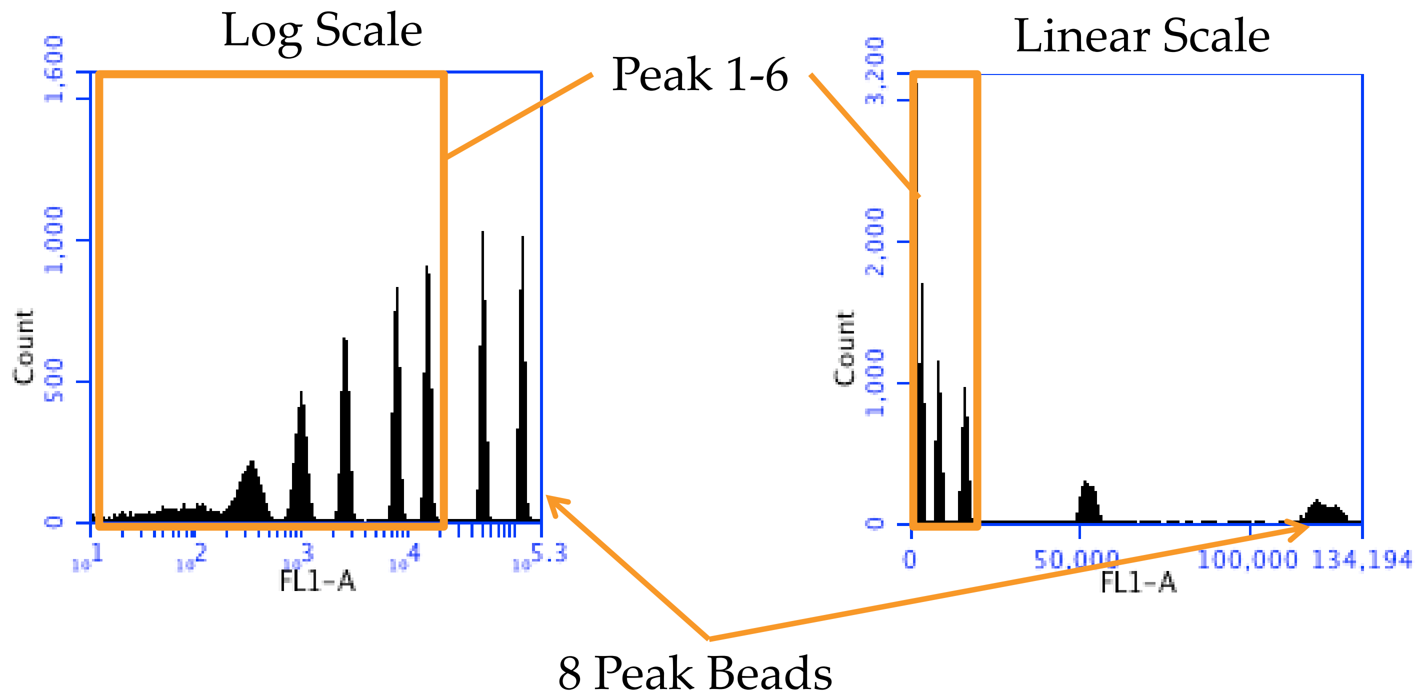
Data is stored in 'fcs' format – Flow Cytometry Standard

- Includes data in a 'spreadsheet'
- Additional header information
    - Keywords for reviewing and organizing files

Data Scales

- Fluorescence phenotyping
- Small sized particles (bacteria, nuclei) are often shown with size in log scale

- Size, complexity (Scatter)
- DNA cell cycle analysis (fluorescence)
- Calcium flux (fluorescence)



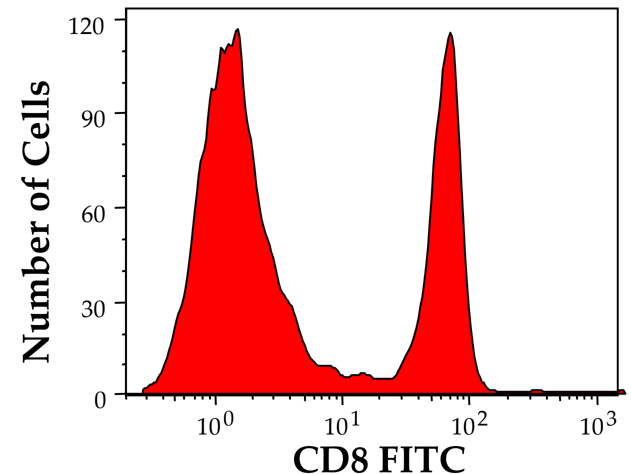
Histograms – Univariate Plot

The **Y axis** on a histogram

- The number of events per channel

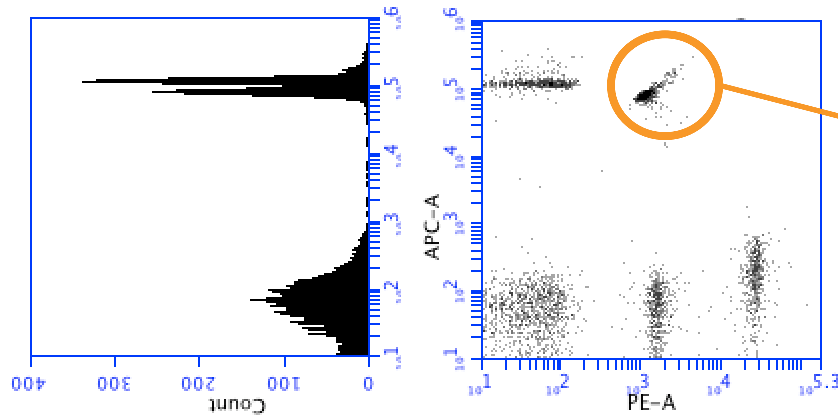
The **X axis** on a histogram

- Intensity of the fluorescence
- Increases as it moves to the right

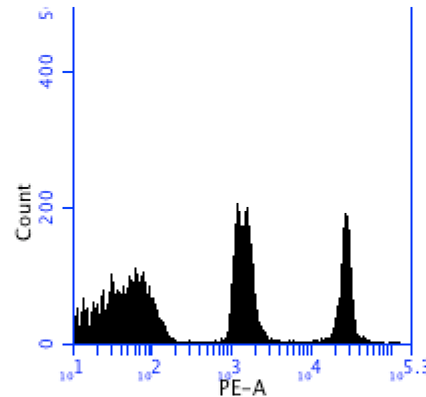


The Bivariate plot

The **Y axis** on a histogram
- Intensity of the fluorescence
- Increases as it moves to the top

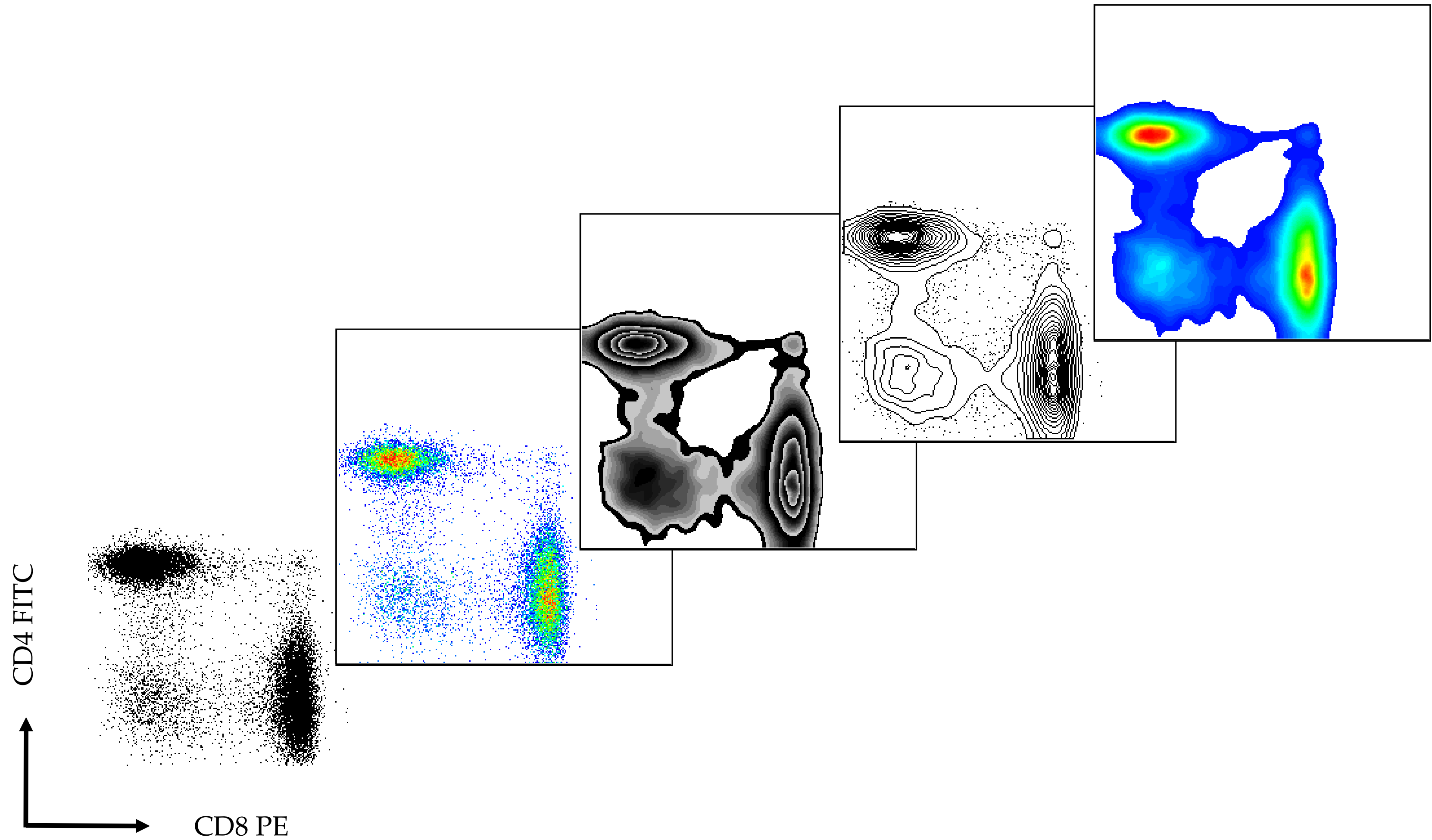


Double Positive cells not readily detectible on univariate plot



The **X axis** on a histogram
- Intensity of the fluorescence
- Increases as it moves to the right

Lots of display options – Use them



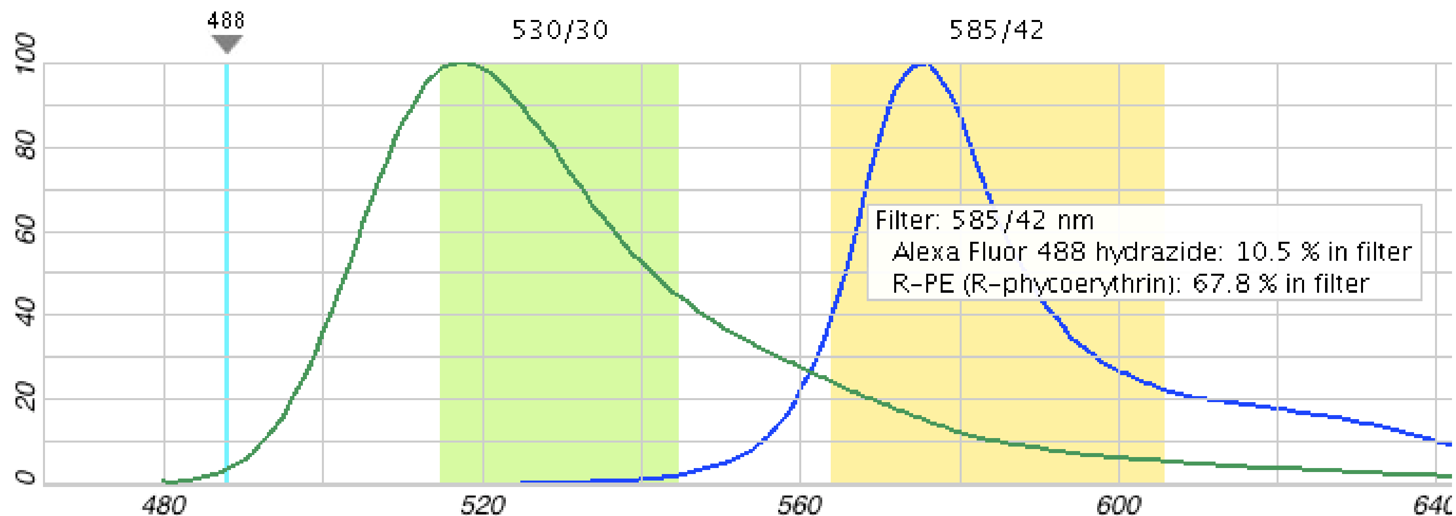
Spectral Overlap and Compensation

Compensation is the process for obtaining the measurement of a single reagent in the presence of two or more reagents

- Data visualization tool, does not alter original data

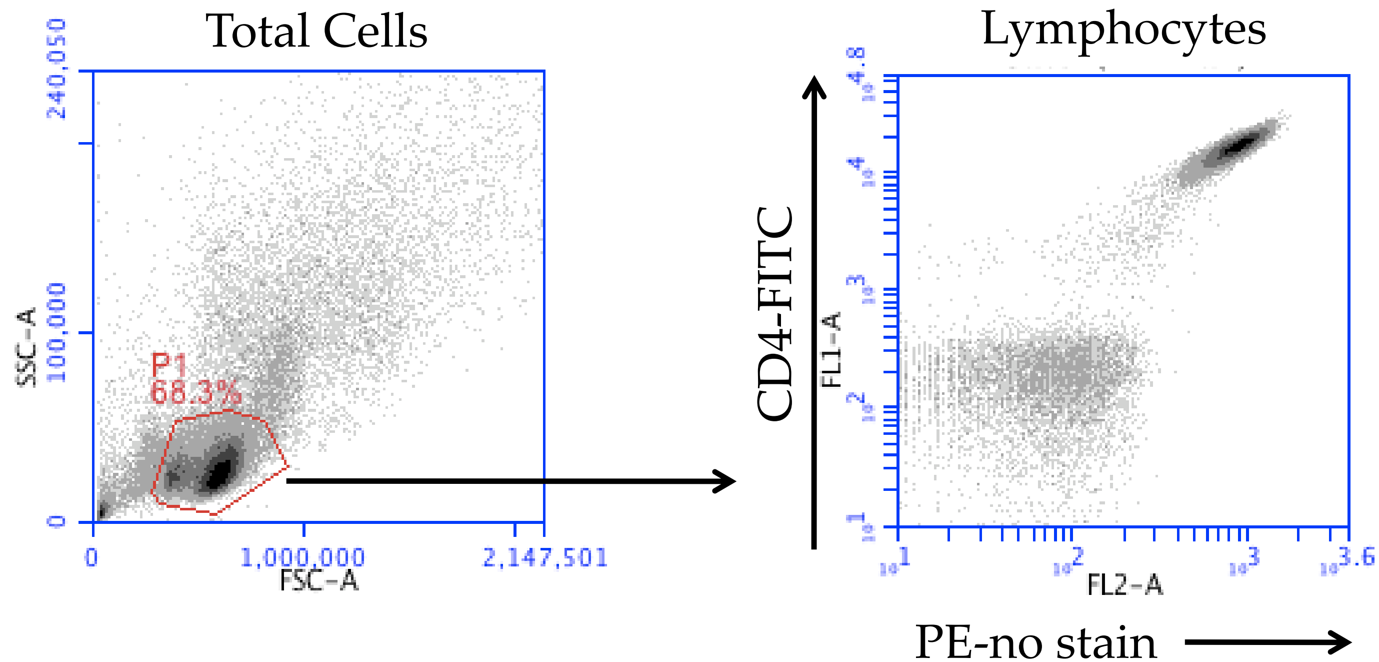
Necessary due to the overlap of emission spectra

- Typical overlap from shorter into longer wavelength



What does spectral overlap look like?

CD4 FITC (only) labeled PBMC – Uncompensated Data

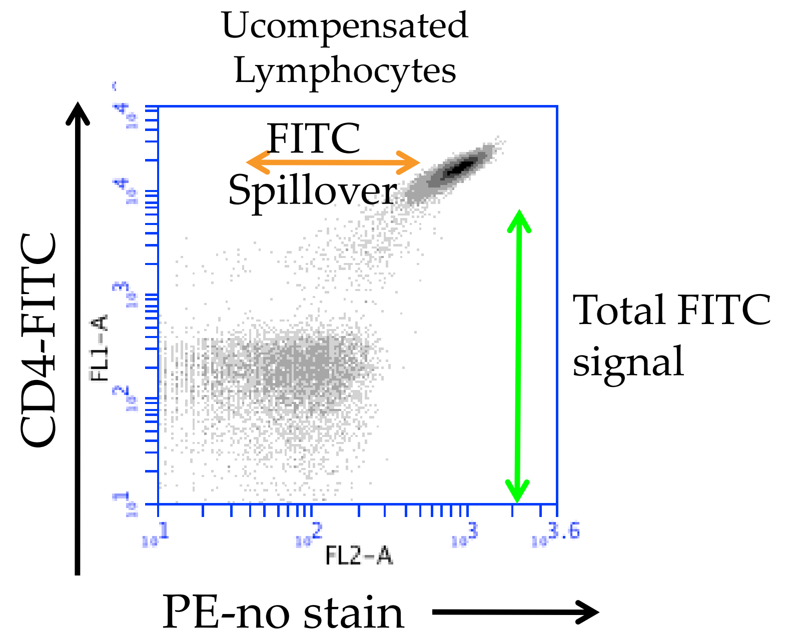


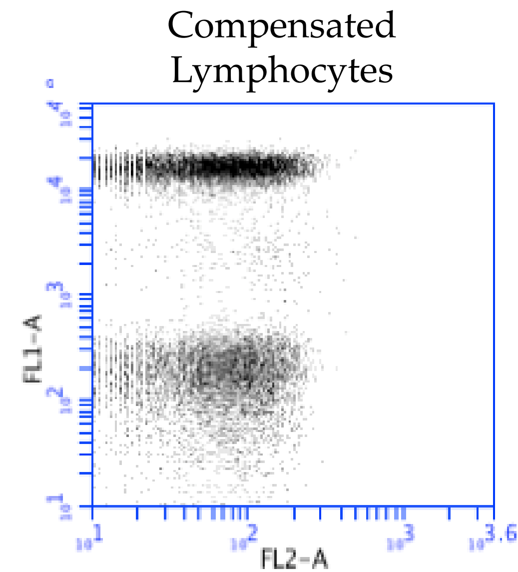
What does this mean?

This sample has NO PE stain

Therefore the observed signal in the PE channel comes from the FITC molecule only.

The signal we see is the observed signal, we need to determine the true signal





How do I generate a compensation matrix?

Matched Median Compensation

Spillover-Matrix

Adjusted by hand-eye

Susceptible to overcompensation

Time consuming

NOT RECOMMENDED

Software Compensation

Inverted matrix

Calculated mathematically

Fast, reproducible and accurate if done with proper controls

Need to have proper controls

Cytometry Controls

Compensation Controls

- Unstained Control
- Single Stained Cells or Beads

Gating Controls

- Fluorescence minus one (FMO)

Blocking controls

- Isotype controls (to determine NSB)

Experimental Controls

- Untreated vs treated
- Healthy vs disease

Welcome to the URMC Flow Cytometry Core

Website: http://www.urmc.rochester.edu/flow-core

- Latest information on the core
- Policies and procedures
- Upcoming lectures, demos, etc

Listserv: UR_Cytometry

- Announcement list
- Quick way to connect with others at URMC

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PPMS for the Flow Cytometry Core - FCC

Home Book Order Request Documents Schedules Reports Publications Profile Logout

User rights Browse incidents Trainings

Home

Current user: Adams Ashley

Book a system:

Systems available: book

Order a service or a consumable:

Services/consumables available: order

Make a new request: request a training request/start a new project

Restore Default Sections

+ Add a New Section to the Home Page

Report a Publication

Please report any publication that used FCC resources.

This is very important for us when we submit grant applications or grant renewals, so thanks in advance for your help!

Report a publication

Orders - New

No new orders on this core.

Orders - Accepted

- Tracking system for all cores
- Linked from the Flow Core website
- Controls scheduling/billing/tracking
- Log in to schedule a training, book time, track usage, see incidents, report publications

Now what?

Training can be scheduled through the PPMS system under the “Request” heading.

All training sessions are scheduled as soon as possible.

- Unfortunately due to current circumstances scheduling is somewhat difficult and can take longer than we’d like.

Review training documents at:

- http://www.urmc.rochester.edu/flow-core/training/

WELCOME TO THE WONDERFUL WORLD OF FLOW CYTOMETRY