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, Dev. Director

Lab/Operations Manager

- Meghann O'Brien

Full time instrumentation/contract specialists

- Theresa Fitzgerald
- Zach Nowak
- Steven Polter
- Calvin Tian
- Terry Wightman





Aministration etc.

- Sharleen Slaunwhite
- Beth Laffey

The Instrumentation – a brief overview

Analytical Flow Cytometry – both traditional and non

- 1 BD Accuri C6+: up to 4 fluorescent parameters, ease of use, limited flexibility
- 4 FACSDiva based cytometers: up to18 fluorescent parameters, excellent flexibility, moderately complex operation.
- 2 Auroras: up to ~40 fluorescent parameters using full spectrum analysis
- Helios: 40+ parameters using mass cytometry – metal labelled tags

Cell Sorters – recover populations of interest with excellent precision/purity

- Aurora CS : Full spectrum benefits
- BD S6: Up to 24 targets

❖Both: 6 way sorting – tube/plate/slide collection - indexing

- FACSAria are being retired

Non Traditional Instruments

- EV and microparticles, Imaging Cytometry, Metabolomics, Bead based array

Configuration/Details Pages

Janice and Zoot - BD Symphony A1 - FACSDiva based

Note that Janice and Zoot have matching configurations and are therefore share a website page.

MIFLOWCYT information specific for this instrument can be found in the FCC_Library.

Detailed information about the QC can be found in the baseline report (available upon request).

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.

Configuration: Low starting voltages as of Jan 2023 posted at the machine and available via FCC_Library.
BLUE 488 LASER (100mW):

Detector	LP	Band Pass	Fluor's (check Fluorofinder for more info)
BLUE B	690	710/50	PerCP-Cy5.5
BLUE C	505	530/30	FITC, GFP, Alexa 488
BLUE E		488/10	SSC
BLUE F		488/10	Small Particle SSC
FSC			

RED 637 LASER (100mW):

Full Spectrum - Constantine, Sweetums and Link

The Aurora Full Spectrum Flow Cytometry instruments are new high end machines from Cytek Biosciences. The URMC FCR is fortunate to have three of these instruments in our facility. The three options are Constantine (4 laser analyzer), Sweetums (5 laser analyzer) and Link Hogthrob (5 laser cell sorter). The full spectrum cytometers use a new type of optical system capturing nearly the entire visible light spectrum and a process called spectral unmixing to resolve individual fluorochromes. The result is a system capable of performing 20+ color analysis (Constantine - 4 lasers) or 30+ colors on the 5 laser systems (Sweetums and Link). In addition to being able to resolve a large number of parameters this technology is also often able to distinguish highly overlapping options such as GFP and YFP or similar but not identical fluorochromes like Brilliant blue 515 and Alexa 488. This opens a new world of panel design options using combinations of fluors that we previously couldn't reliably resolve. Using this technology we can also perform autofluorescence extraction allowing us to resolve negative or dim positive populations that previously were swallowed up by high autofluorescence in our cells of interest.

The two analytical systems also include integrated 96 well plate loaders allowing automated acquisition of a large number of samples. The plate loader includes automated washing to reduce carryover and highly customizable sample acquisition to optimize for each experiment.

The cell sorter (Link) has the same 5 laser configuration as Sweetums which allows direct transfer of whole experiments from one machine to the other. An experiment that is tested and developed on the analyzer can then be transfered to the sorter and expected to work very much the same on both, saving time and reducing troubleshooting needs.

General Information before proceeding

1. Panel design considerations for the full spectrum analyzer are different than for traditional fluorescence flow cytometry. Panels designed for any of the other analyzers will generally work on the Aurora, but to expand panels a discussion of options is recommended. Please see the documents listed below and contact Matt Cochran if you'd like to schedule a time.
2. Please request a training visit in PPMS if you'd like to schedule a time. Training consists of two mandatory visits in front of the instrument for most users.
3. Additional full spectrum cytometry and instrument specific information can be found at the links below.
   - SpectroFlo Software Guide
   - Aurora Operation
   - Video Introduction to Spectroflo
   - Cytek Resources
   - Full Spectrum Viewer - note the Similarity Matrix calculator and the Cytek Cloud link at the top of the page.
   - Aurora User Guide
   - Fluorochrome Selection Guide - 4 laser system with UV (Constantine)
   - Fluorochrome Selection Guide - 5 laser systems (Sweetums and Link)

Support and Services – Communication/Scheduling

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

- Policies and overview – not very dynamic
- Instrument pages for all equipment
- Library contains links and useful information
- Recent updates
  - Cell sorting page overhaul
  - FAQ added to the Library page

PPMS

- Shared between all SRLs
  - Toggle between accounts easily
- Often updated
  - Better control/flexibility for accounting
  - New "Edit" button for existing reservations!
  - Instrument sign in page with account adjustments

Listserve

Support and Services - Data

Analysis Computers

- PC workstations in 3-4151(2), 3-4157
  - Multiple analysis programs: Celigo, Nanosight, Flowjo
  - Dedicated workstation for full spectrum (Aurora) analysis
  - Dedicated workstation for Image Stream analysis

Data archiving and transfer

- FCC archives experimental data
  - Code42 automated archiving
    - Backs up automatically throughout the day
    - Saved indefinitely
    - Files can be retrieved upon request (Instrument used, Exp Title, Date run)
- FCC_Transfer provides a space for moving data from cytometers to lab
  - Not for long term storage. Space is cleared once a month.
- Box is accessible as an alternative

Support and Services - General

Consultation (Zoom or in person as requested)

- Experiment/Panel design
- Data interpretation
- Sorting strategy/setup

Data analysis

- Both Flowjo and FCS Express licenses are available
  - Information, practice data on website and FCC_Library
- High dimensional analysis help is also available

Continuing Education

- FCC_Library share
- Regular seminars, lectures, and demos

Support and Services - General

Instrument/Software assistance

- Slack on all computers – monitored during normal business hours for quick hassle-free assistance
- Full remote software access/control when needed

Full Service Support – Newly Expanded

- Staining and running of sample for flow cytometry
  - Traditional, Full Spectrum, Helios or Cell Sorting
- Sample prep and running of BioPlex assays
- Sample prep and running of sample for Seahorse analysis
  - Probable reagent cost savings as a bonus

Email Matt or Meghann for details or to schedule a consultation

Training Schedule

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

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

Standard FACSDiva and Aurora Training

- Visit 1: Combined training for both instrument types.
- Visit 2: 1 hour training on the machine using beads for 1 trainee at a time.

All Other Instruments

- Variable training programs discussed when request is made

Afterhours training

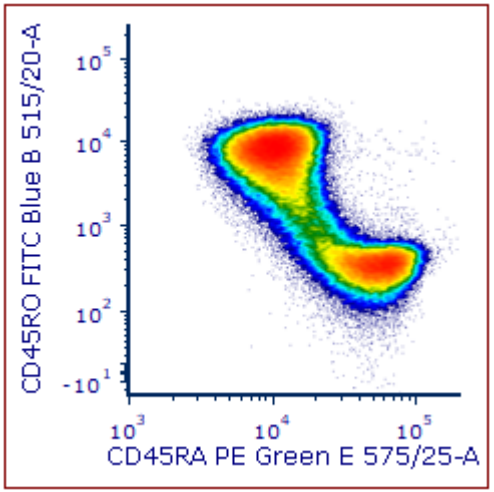
- A separate quiz and practical must be passed for a user to receive after hours access.

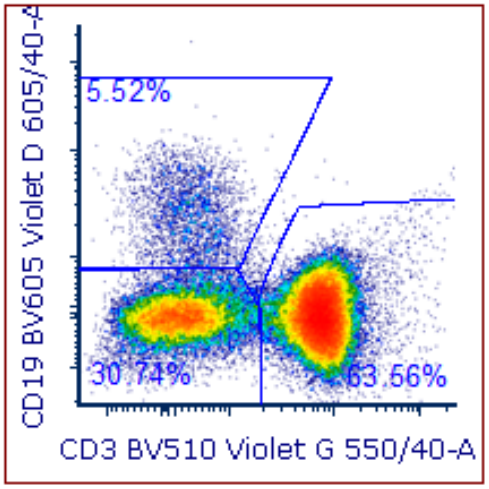
We do not offer training on the Helios or the FACSAria and Aurora Cell Sorters

**** 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 1.5 hours 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

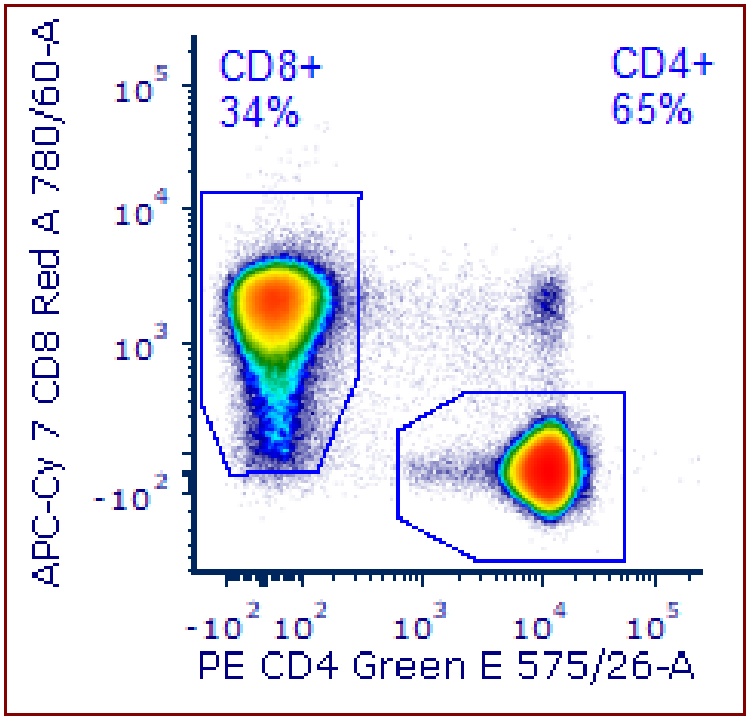




Why Flow Cytometry

- 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't we measure with Flow Cytometry



Intrinsic Characteristics

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

Extrinsic Probes

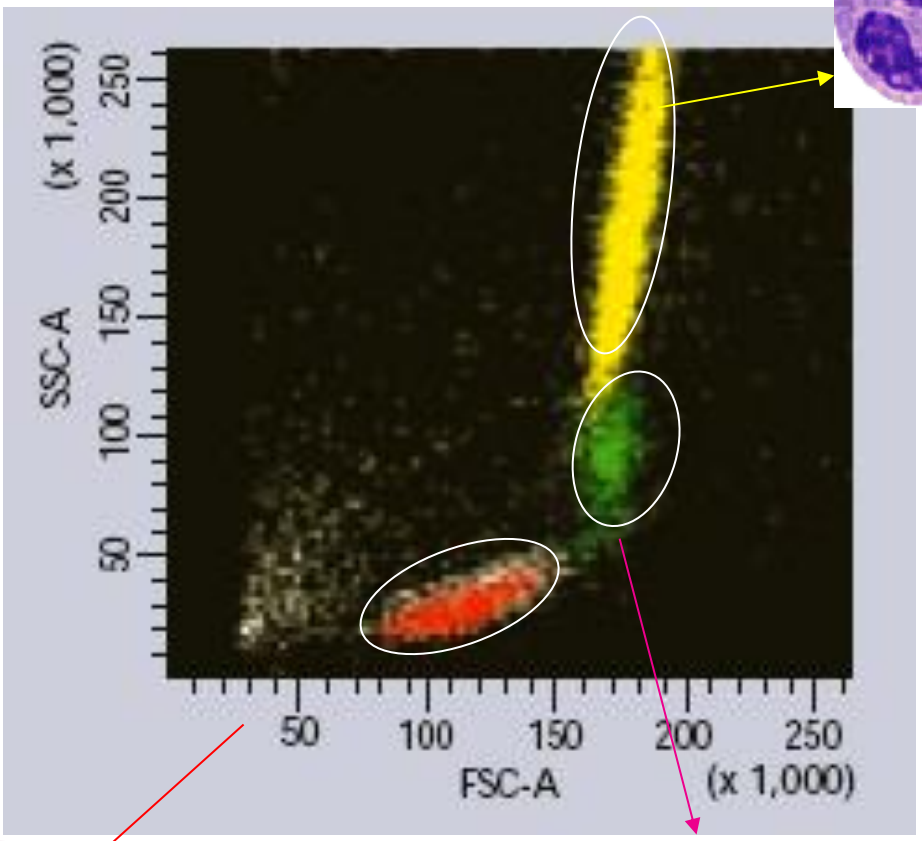
- DNA – Content, Cell cycle
- Apotosis, Necrosis
- Membrane structure
- Receptors – internal and external (Immunophenotyping CD markers, etc)
- Transfection/infection efficiency (Fluorecent proteins like GFP, mCherry)
- Physiological Parameters – Enzyme activity, Calcium flux

Time

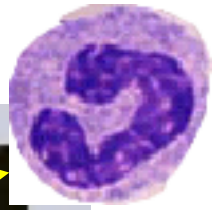
Cell or particle concentration

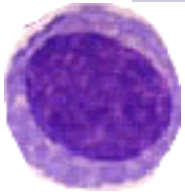
Light Scatter

- 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.



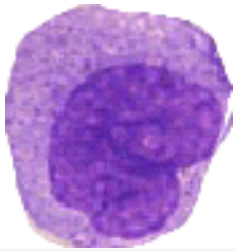
Granulocytes



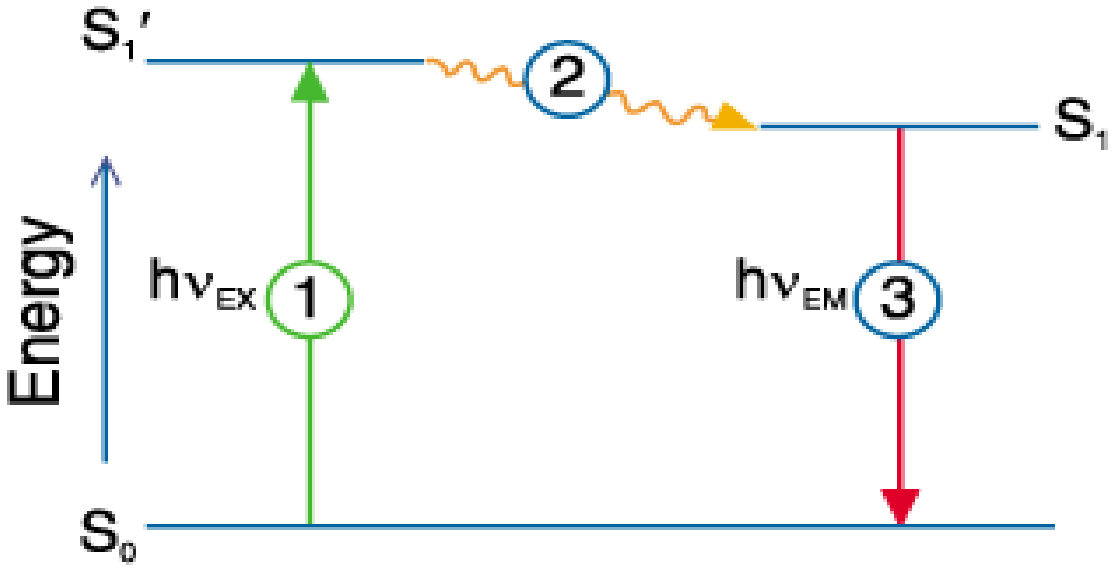


Lymphocytes

Monocytes



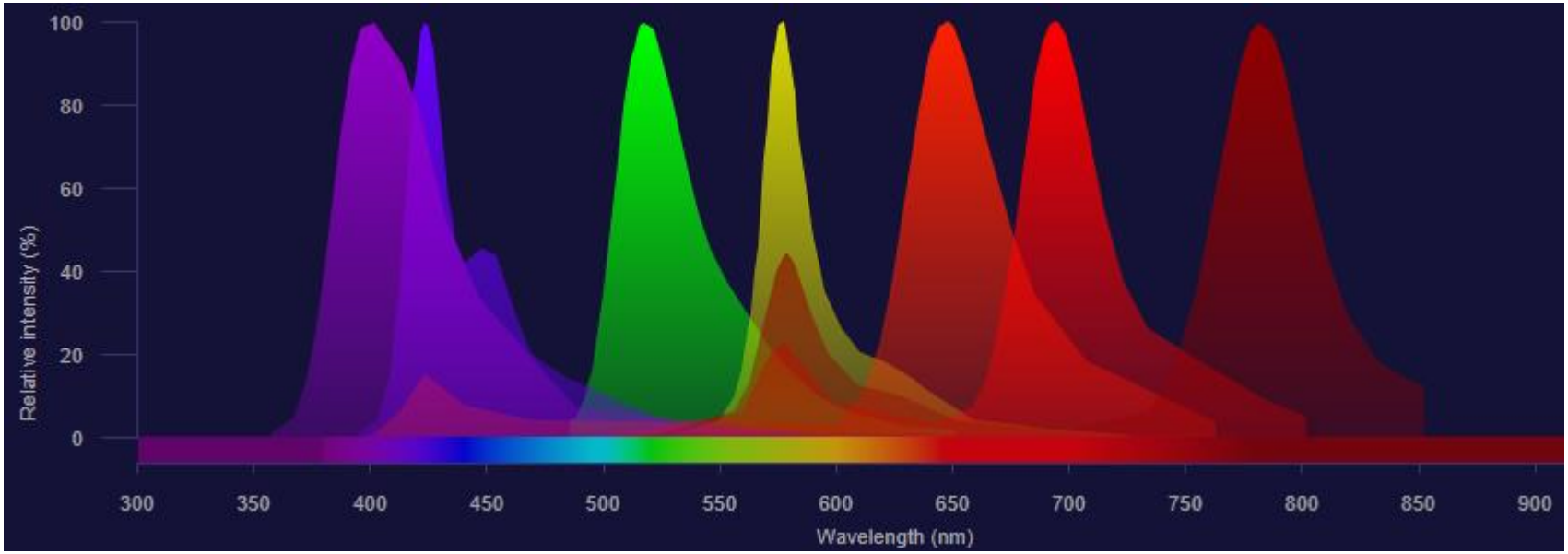
Fluorescence – 1 – Generating fluorescence



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.

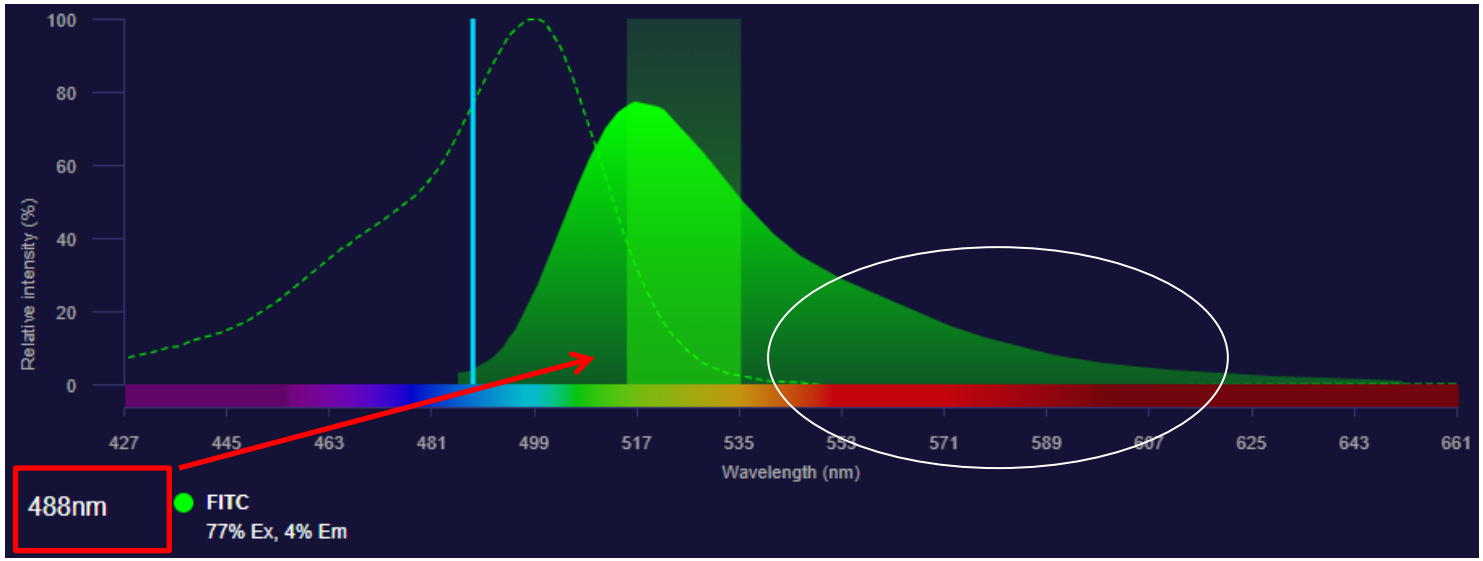
Photon emission wavelength is longer (lower energy) than the excitation

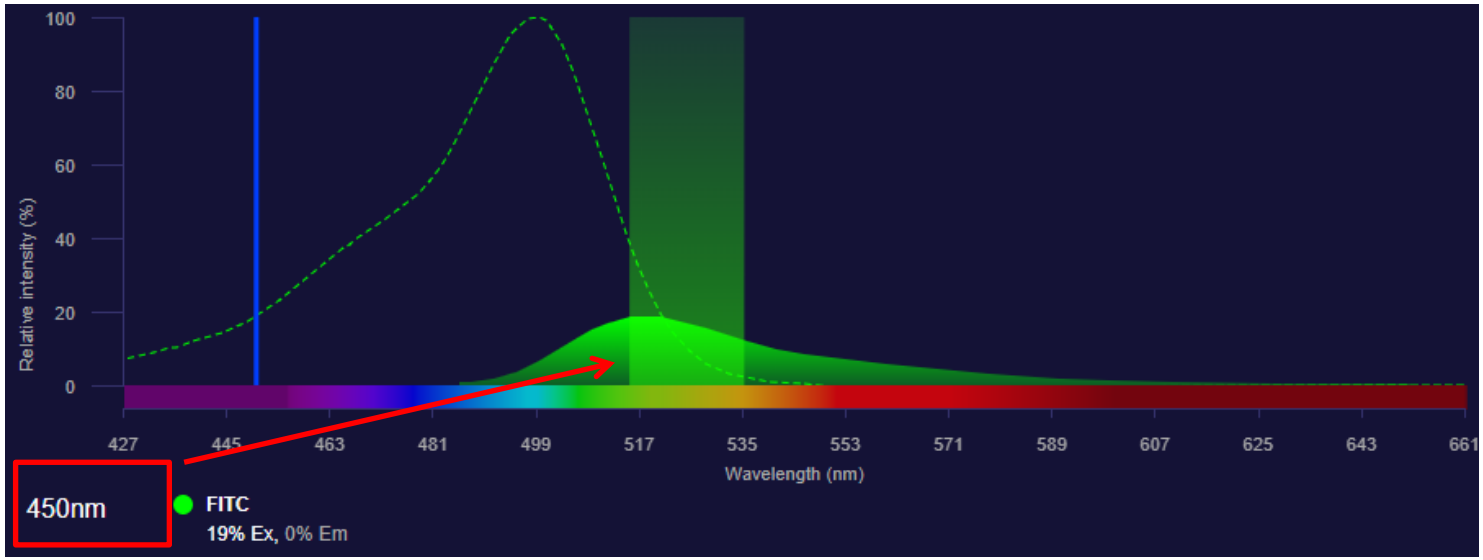
Fluorescence – 2 – The complexity observed



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

Fluorescence – 3 – Intensity and "color"





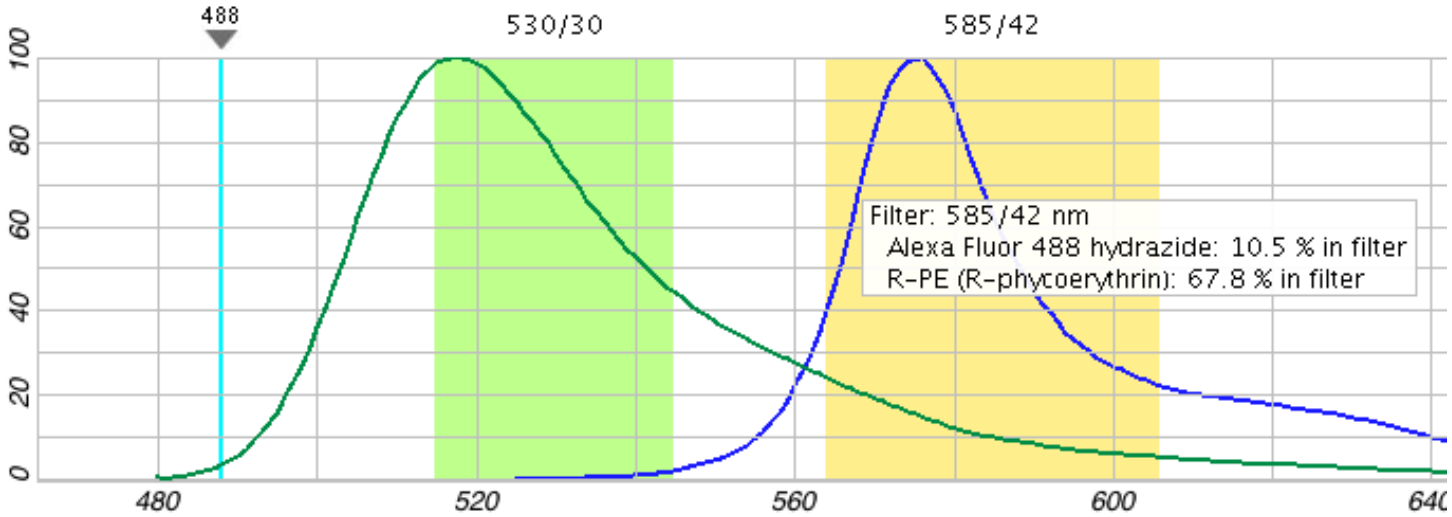
Spectral Overlap and Compensation/Unmixing

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

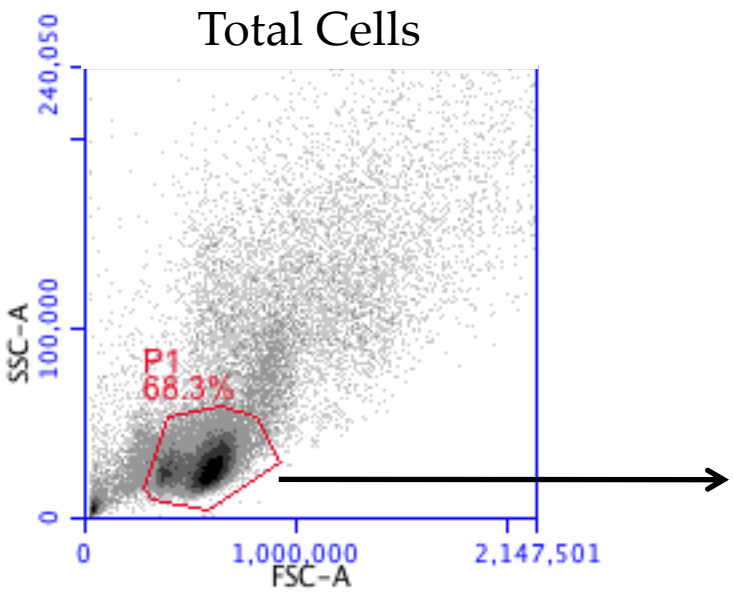
- Data visualization tool, does not alter original data, or correct error

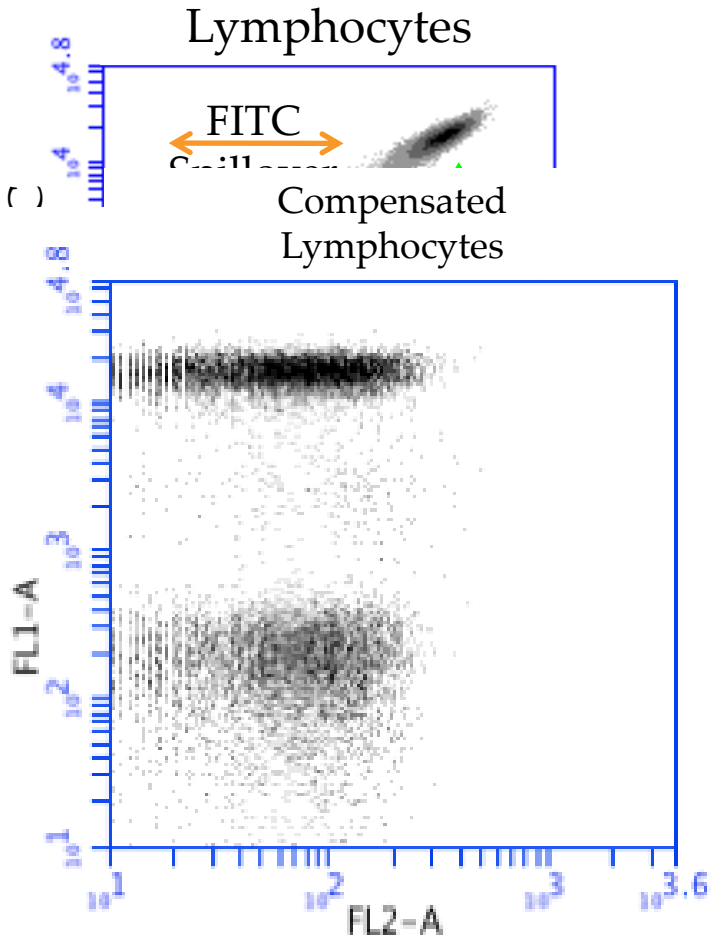
Necessary due to the overlap of emission spectra

- Typical overlap from shorter into longer wavelength



What does spectral overlap look like?





ignal

CD4 FITC labeled PBMC
Single stained control

Uncompensated
Compensated

Need single stained controls for every fluorescent 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

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

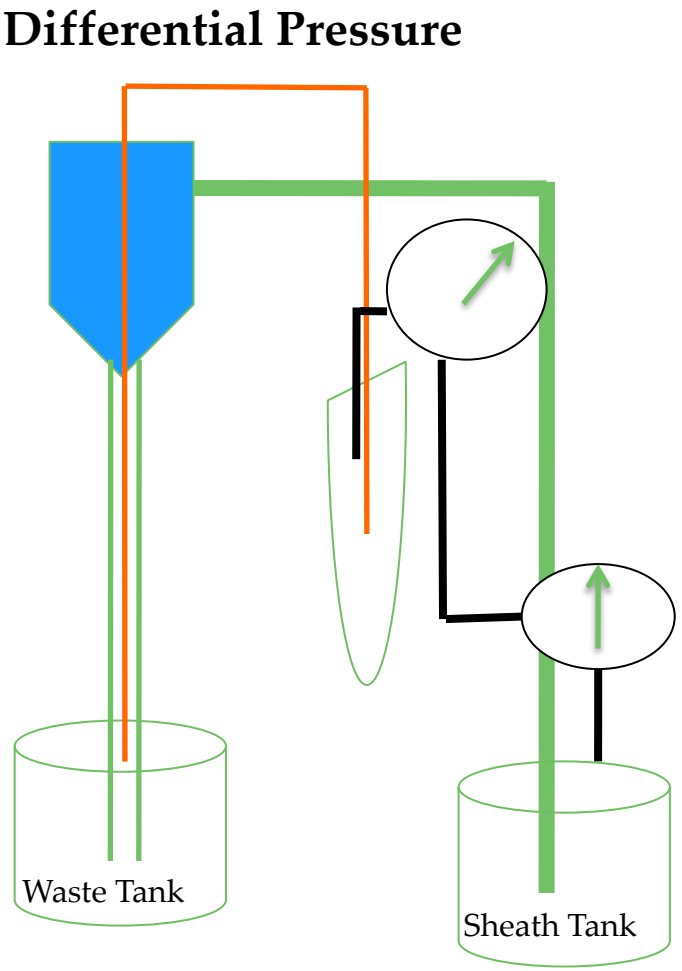
The fluidics system moves the cells

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

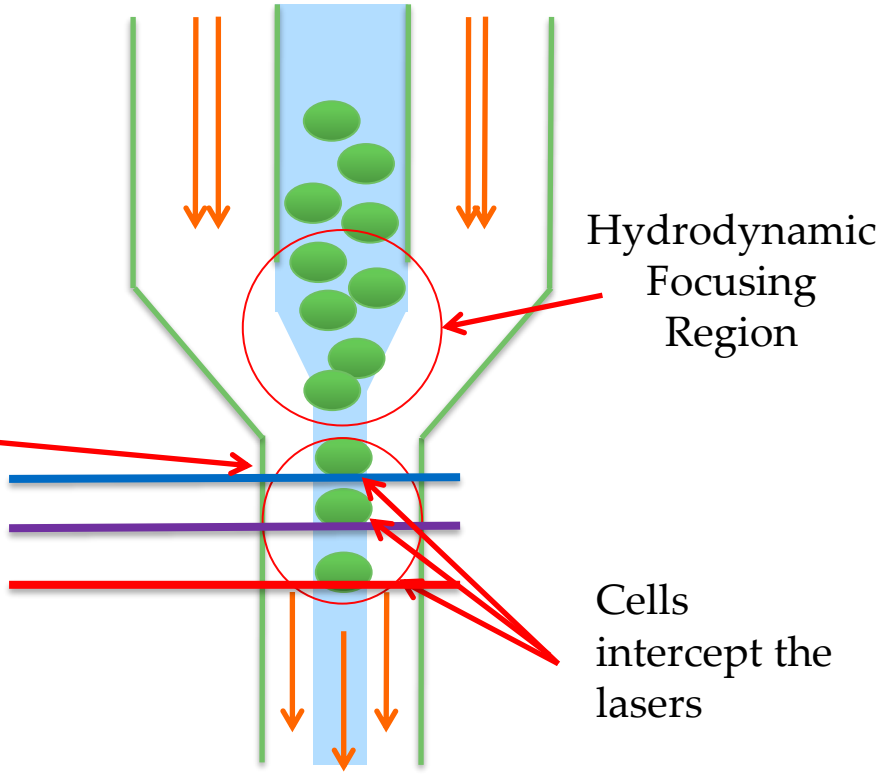


Fluidics

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

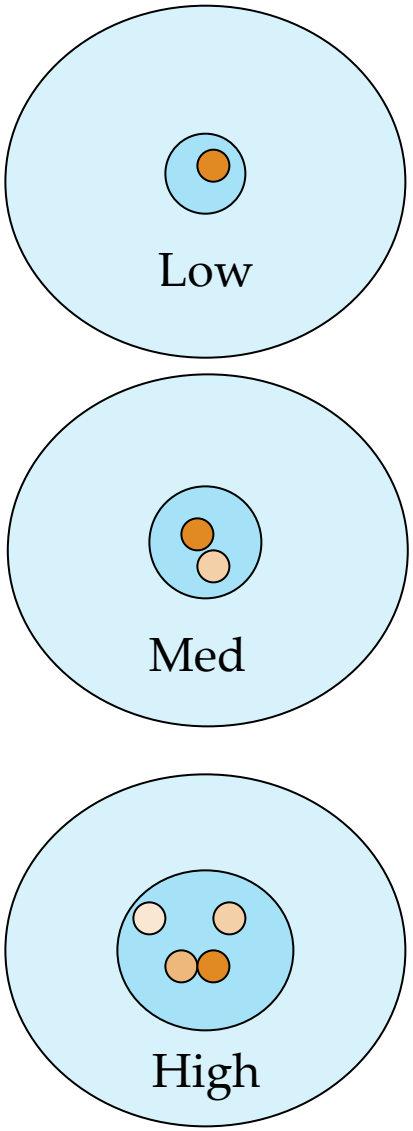
Sheath fluid runs at a constant velocity

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

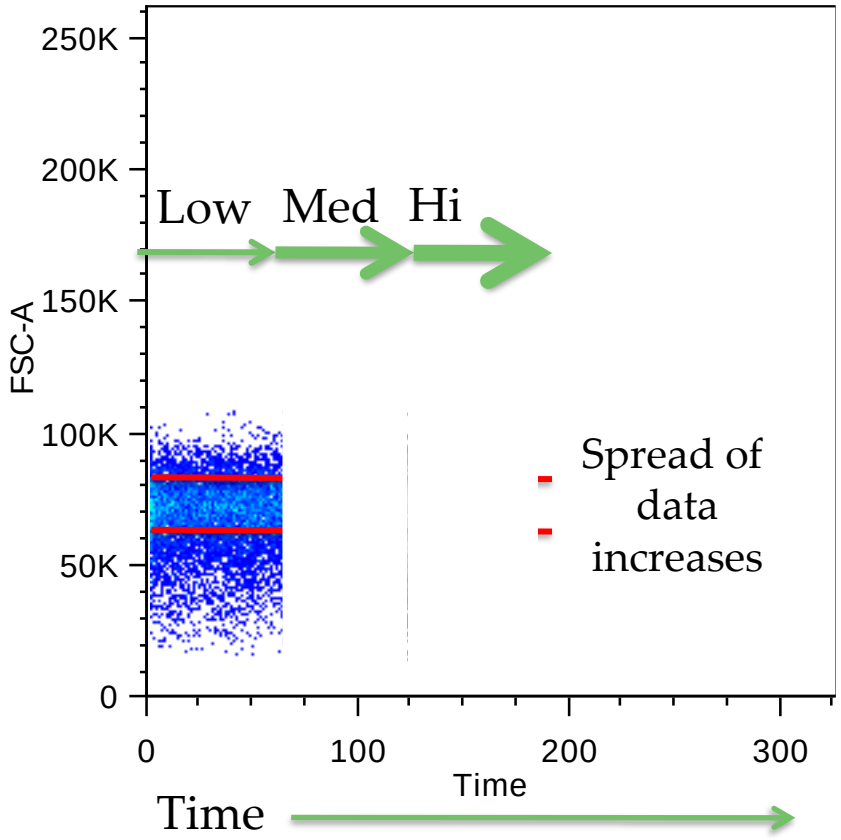
This increases the core stream size, so more cells pass the intercept per unit time

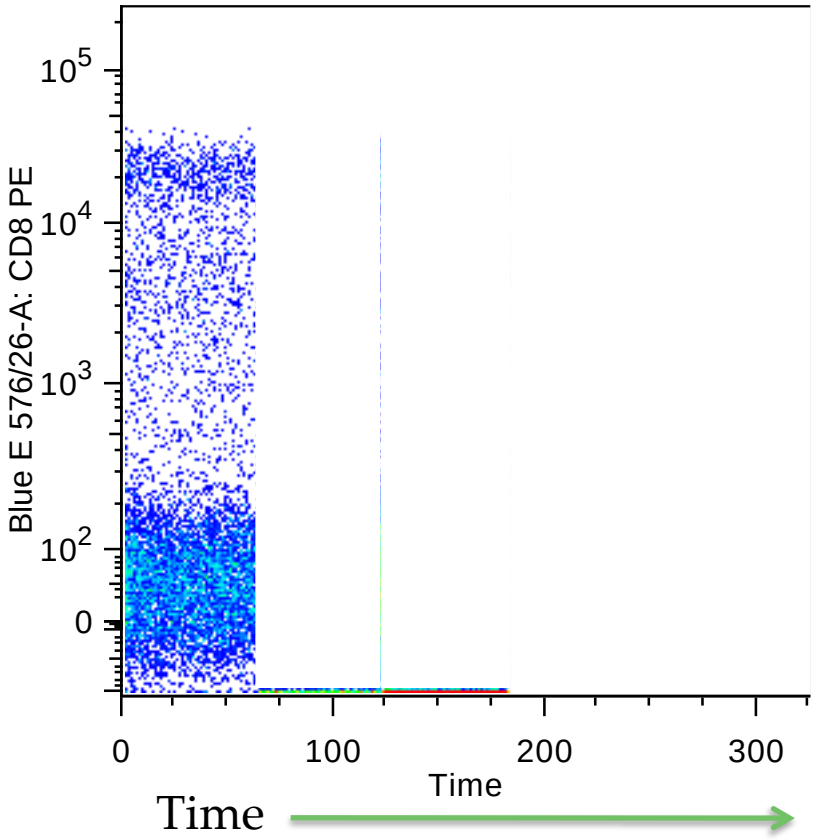
This can be bad

- Increases coincident events
- Increases spread of fluorescent signals



Changing Flow Rate





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)

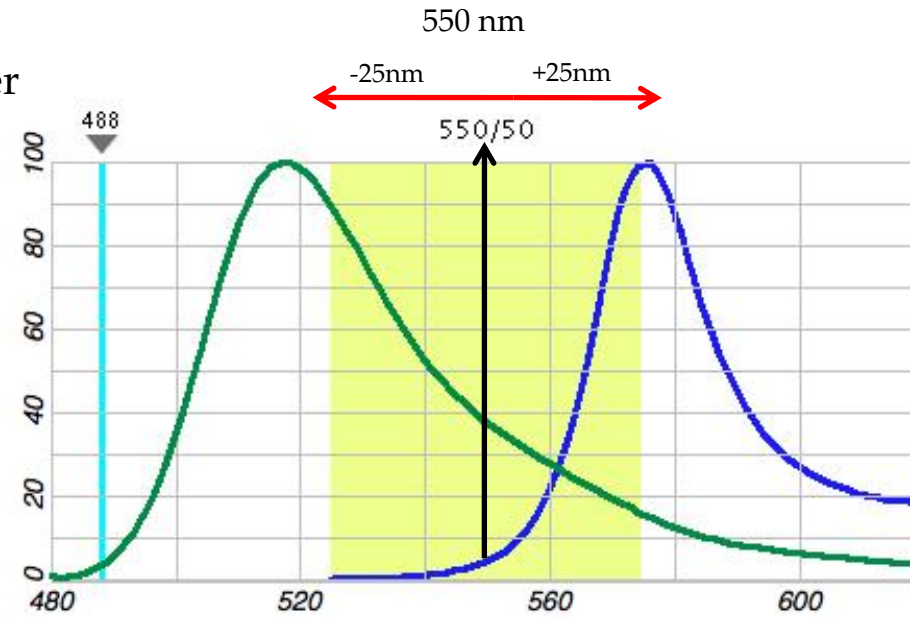
- Pass light of one signal, deflect the remainder

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

- Transmits light above or below the stated wavelength

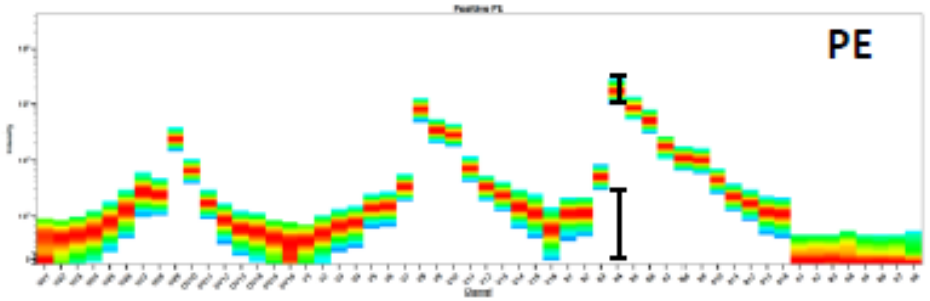
Bandpass (550/50)

- 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



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

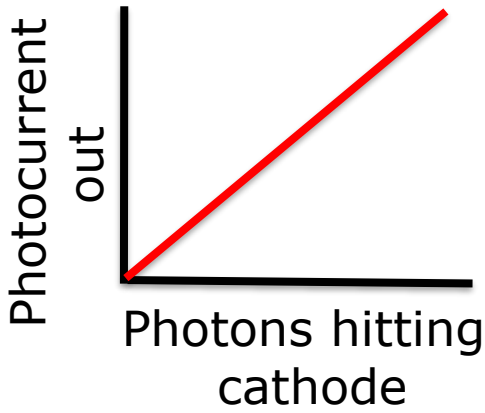
Inside the PMT

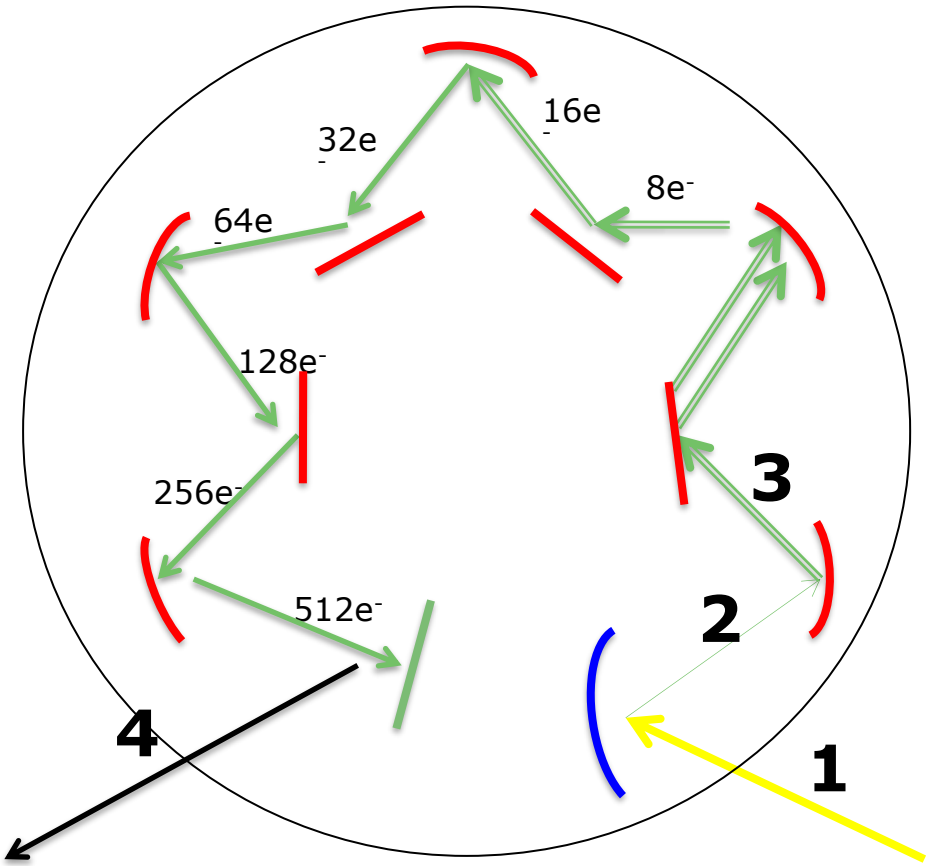
① Light strikes the photocathode

② An electron is discharged towards a dynode

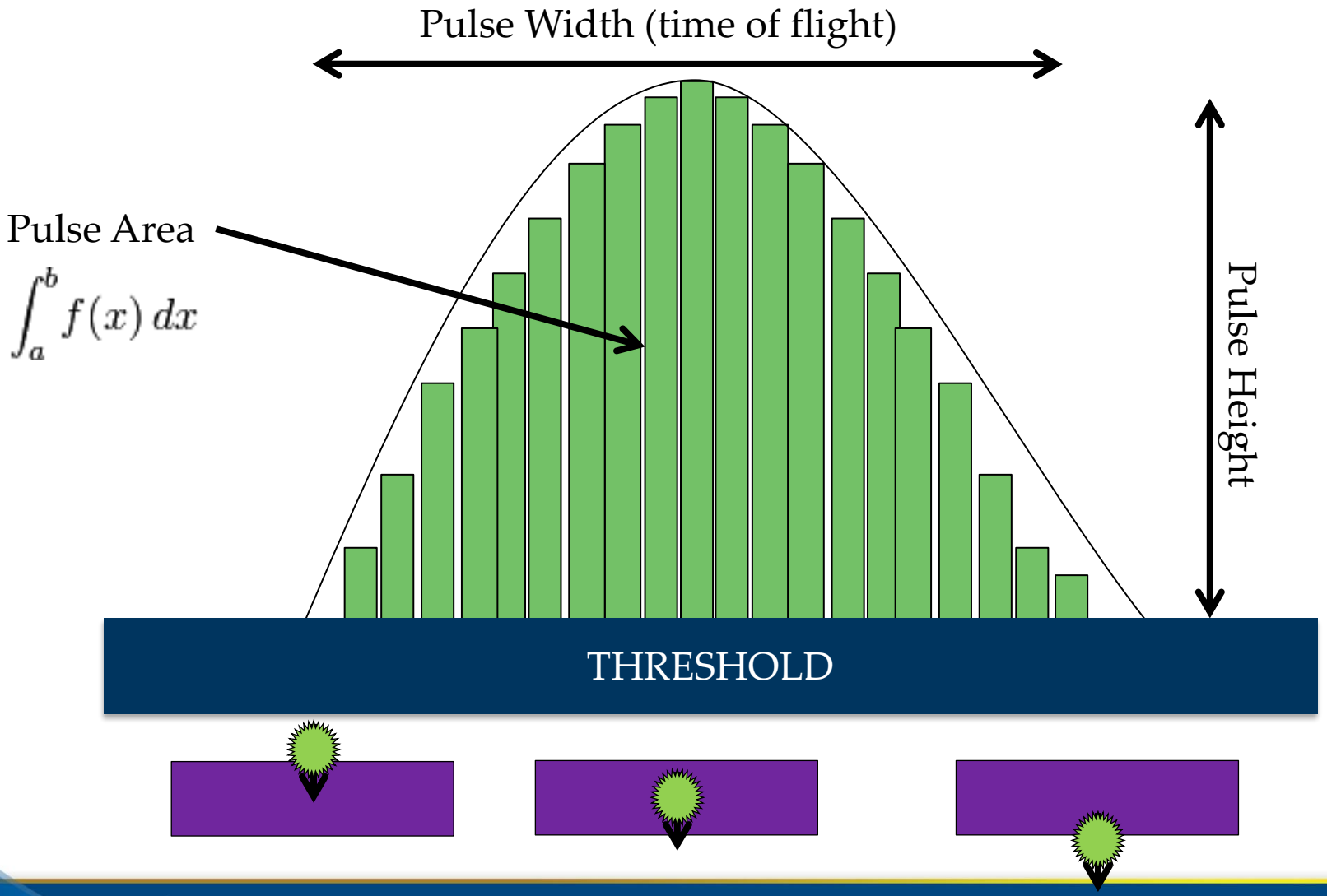
③ 2 electrons are ejected with each electron hitting a dynode

④ Electrons reaching the photoanode produce the photocurrent





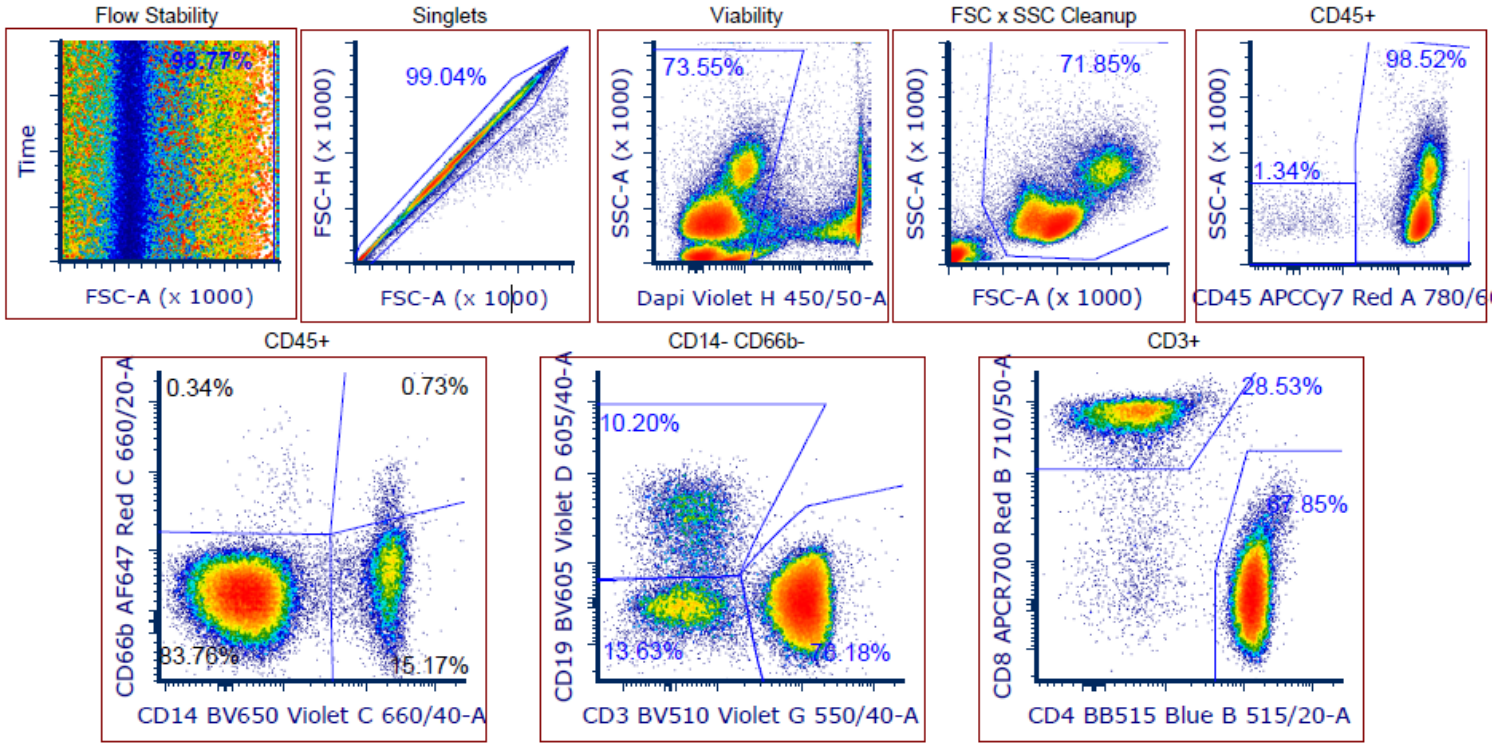
Photoelectric current from the detector, must be converted to a discrete digital number to be stored in the data file



Data Files

Data is stored in ‘fcs’ format – Flow Cytometry Standard

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



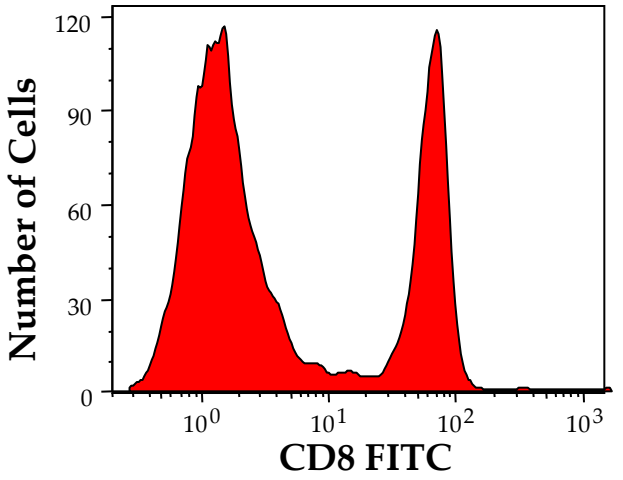
Histograms – Univariate Plot

The **Y axis** on a histogram

- The number of events per channel

The **X axis** on a histogram

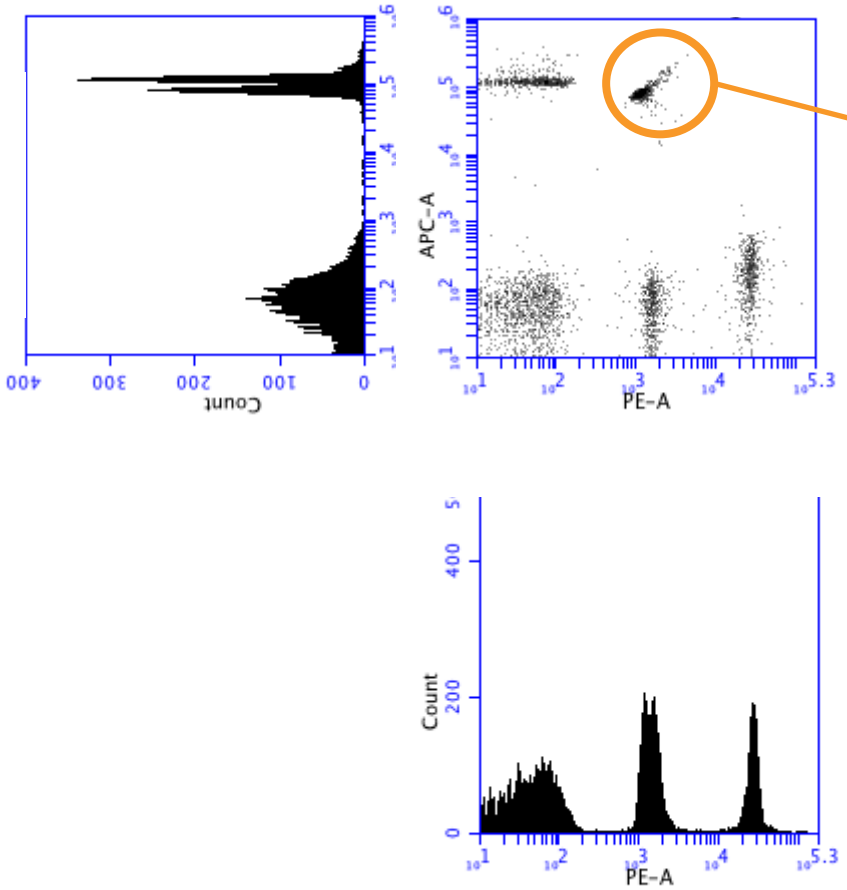
- Intensity of the fluorescence
- Increases as it moves to the right



The Bivariate plot

The **Y axis** on a histogram

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

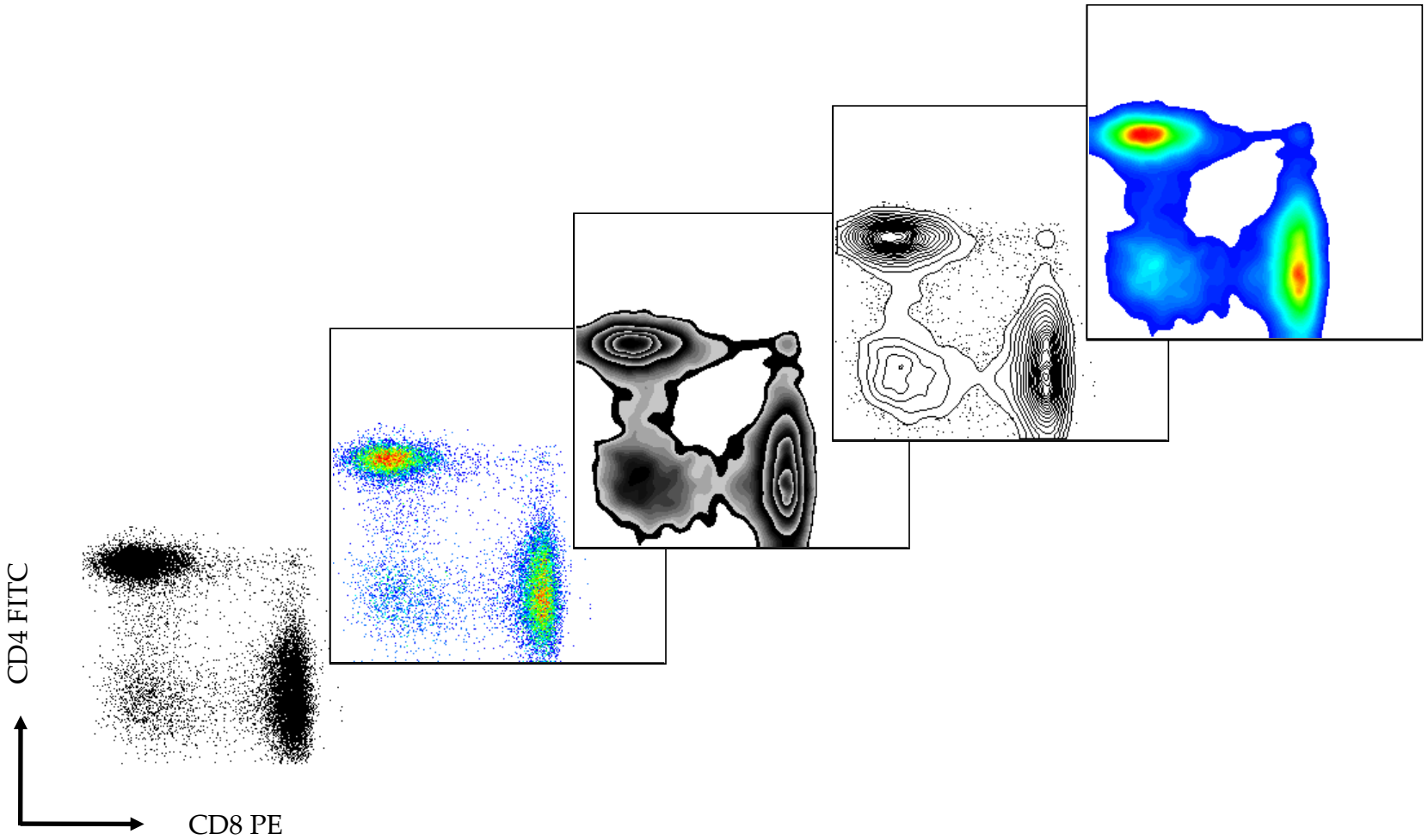


Double Positive cells not readily detectible on univariate plot

The **X axis** on a histogram

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

Lots of display options – Use them



Cytometry Controls

Compensation Controls

- Unstained Control
- Single Stained Cells or Beads

Gating Controls

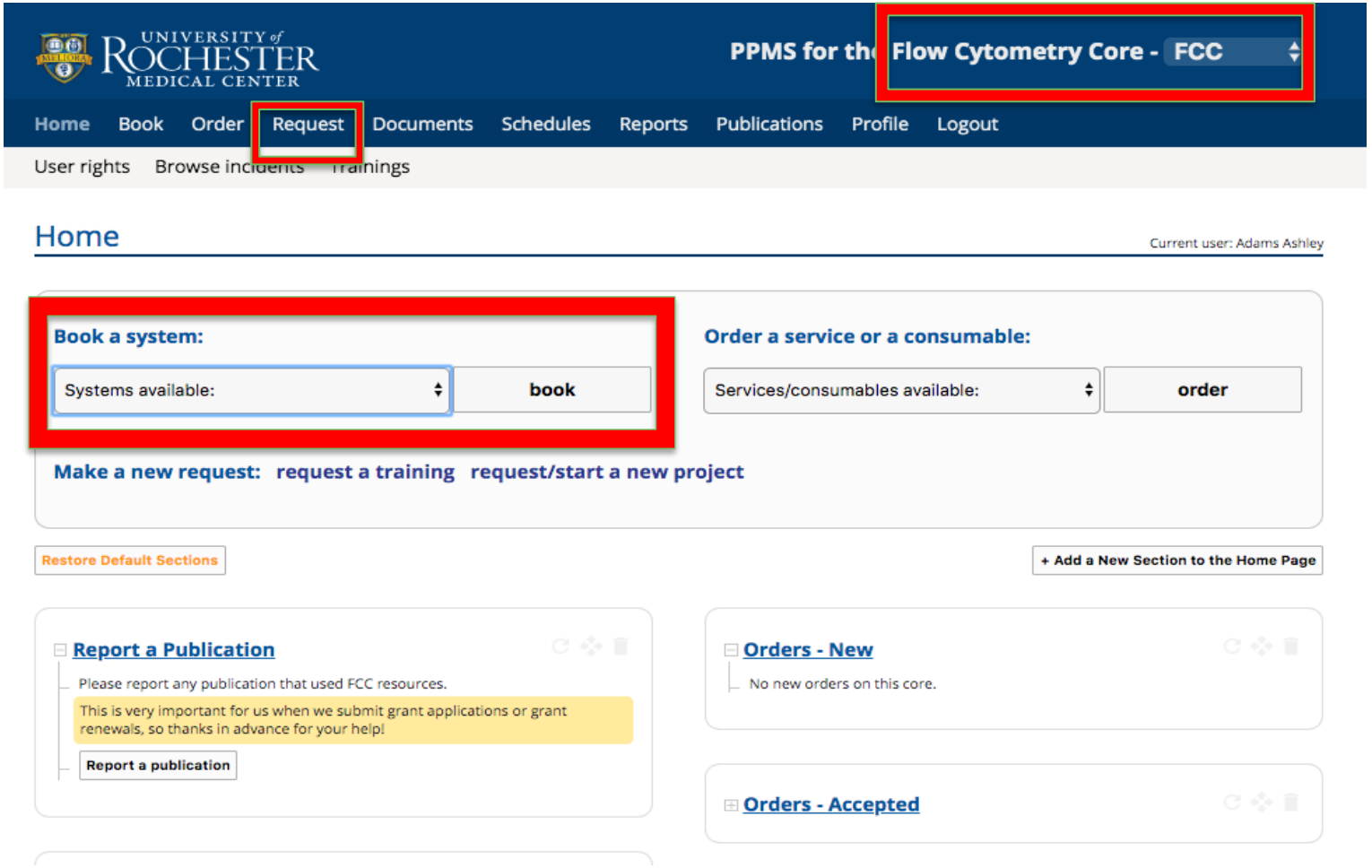
- Fluorescence minus one (FMO)

Blocking controls

- Isotype controls (to determine NSB)

Experimental Controls

- Untreated vs treated
- Healthy vs disease



- 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.

- After a training is requested our team will reach out via email to schedule times. A request does not confirm a day/time but there is an option to input availability.

Review training documents at:

- https://www.urmc.rochester.edu/research/flow-core/services/training.aspx

WELCOME TO THE WONDERFUL WORLD OF FLOW CYTOMETRY

MEDICINE *of* THE HIGHEST ORDER

UNIVERSITY *of* ROCHESTER MEDICAL CENTER