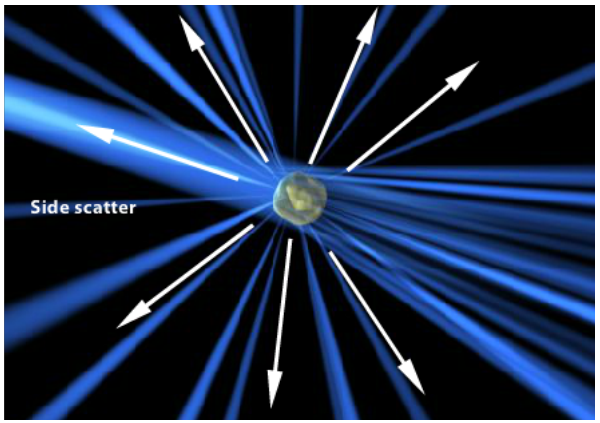
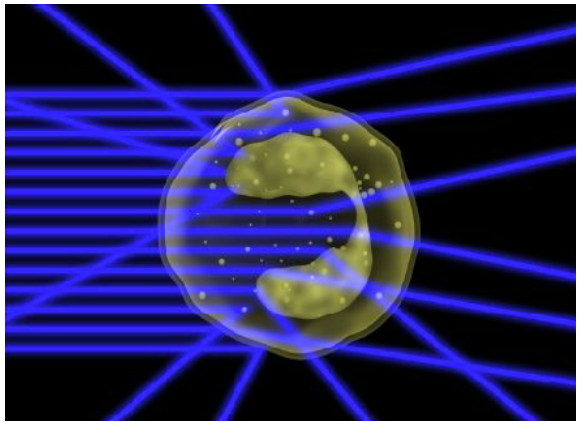
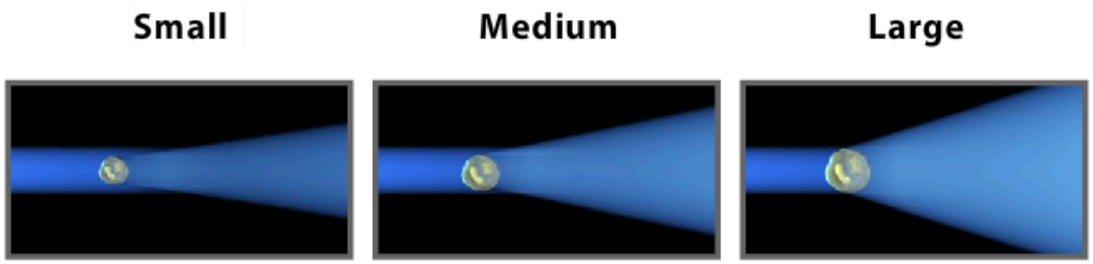
Following photons

- What happens to the photons of the lasers when cells flow through them?...They Scatter!

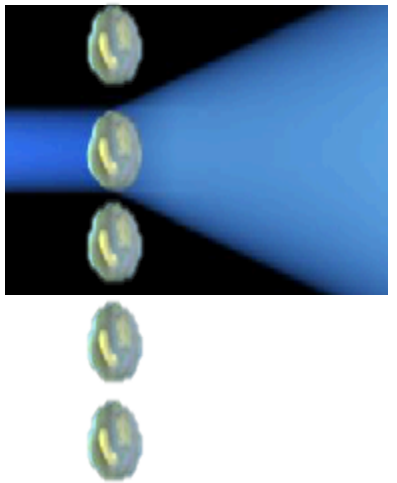




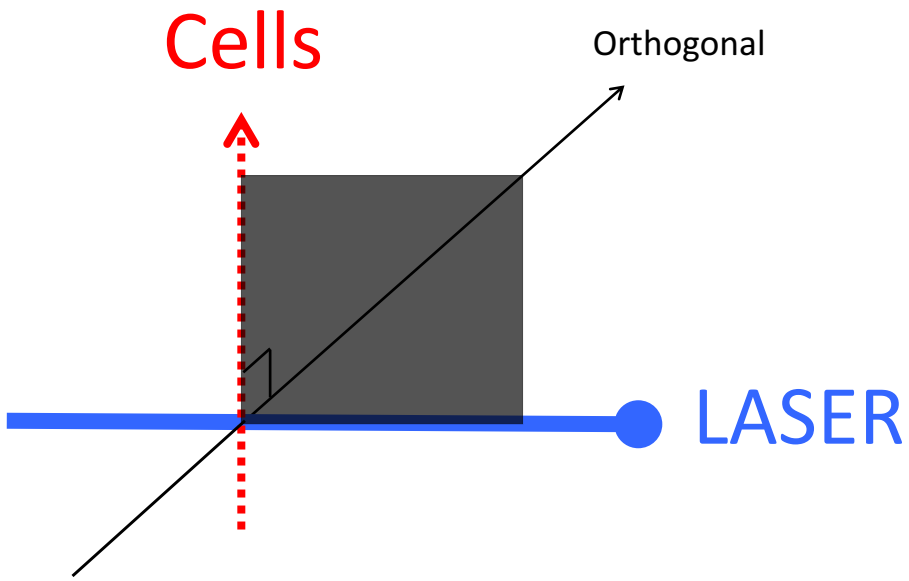


Forward scatter

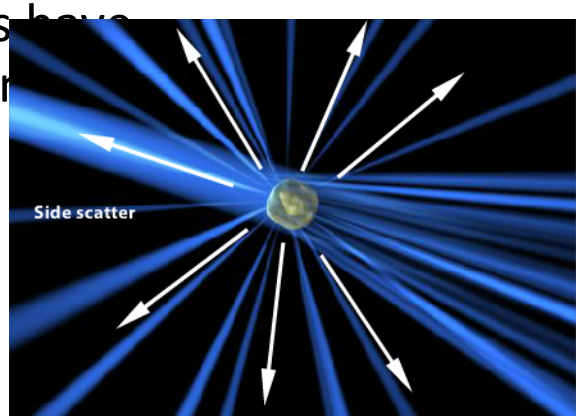
- Forward Scatter (FSC): Laser light scattered in the direction of the laser
  - Has a direct relationship with the size of the cells
  - Larger cells produce larger FSC
  - Is not a quantitative, it's relative
  - Is not effected by fluorescence

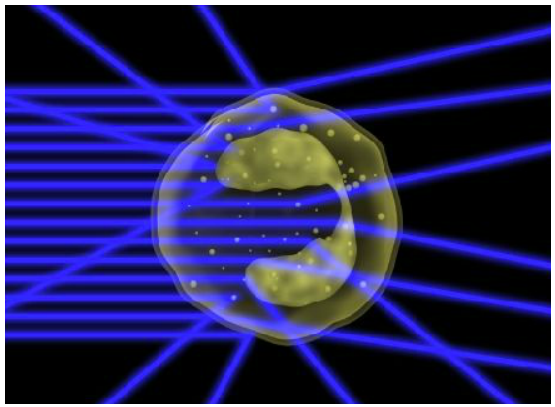


Side Scatter

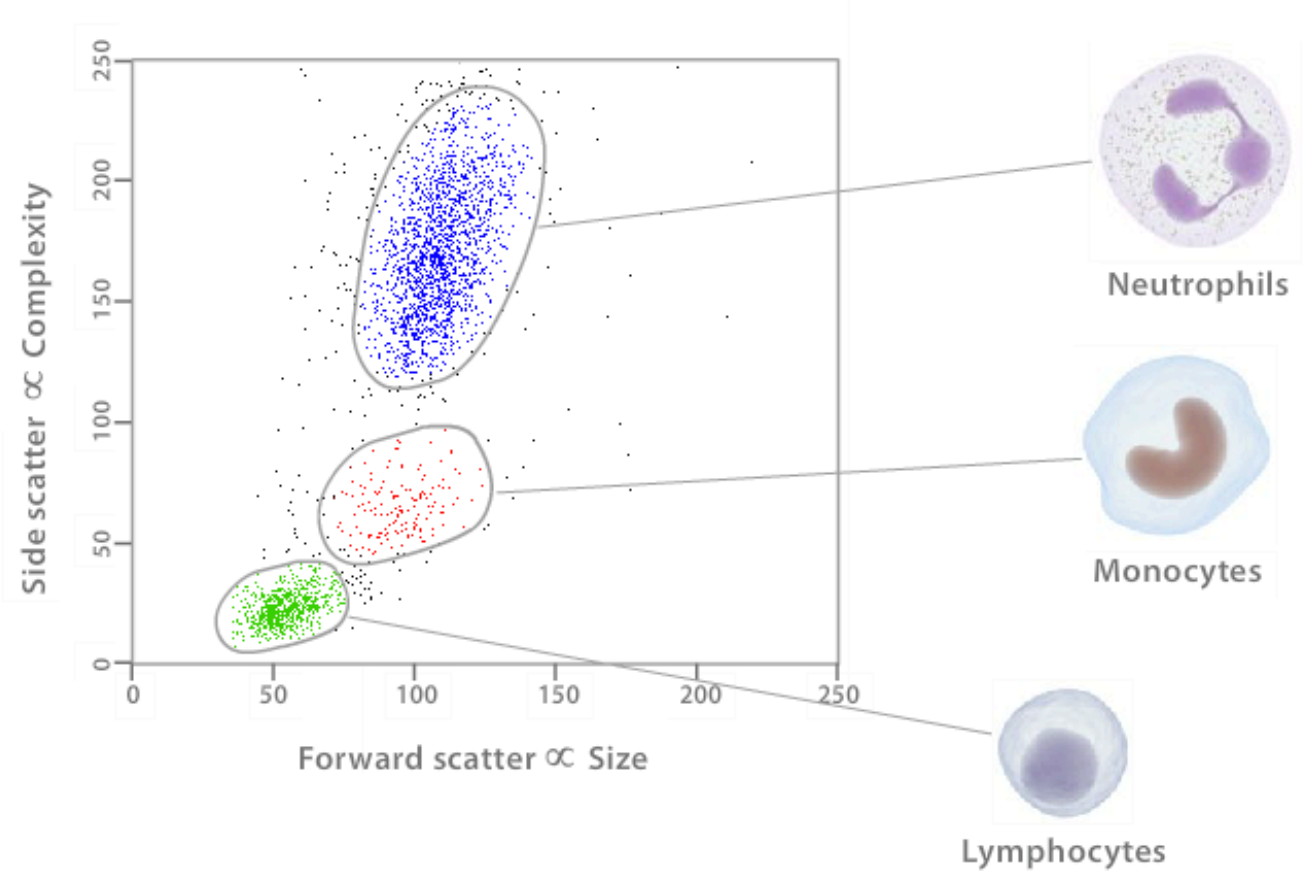


- Side Scatter (SSC): a measurement of laser light scattering in the orthogonal angle
- Measure of relative cellular complexity
- More granular cells have higher cellular inter



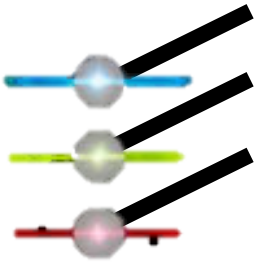


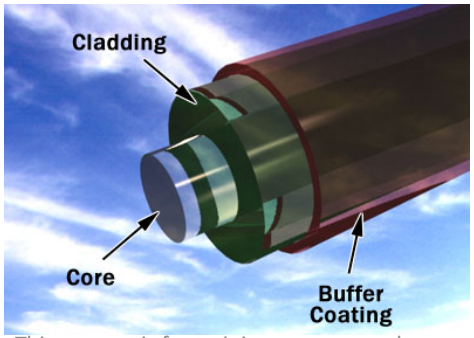
SSC-A vs FSC-a dot plot

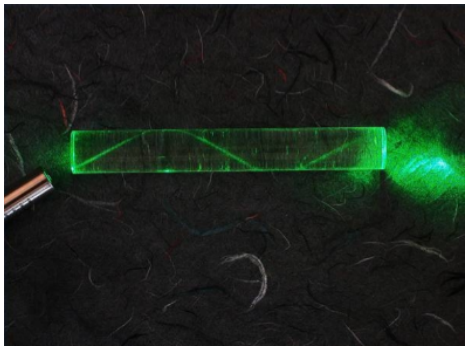


Fluorescent Photons and fibers

- All light particles (laser scatter and fluorescence) are scattered radially
- Fluorescent photons are also collected in the orthogonal angle
- Fluorescent photons will have wavelengths longer than the laser light that excited them
- The orthogonal photons are focused to travel down fiber optic cables
- One fiber per laser allows laser us to determine which laser caused the excitation of the fluorochrome

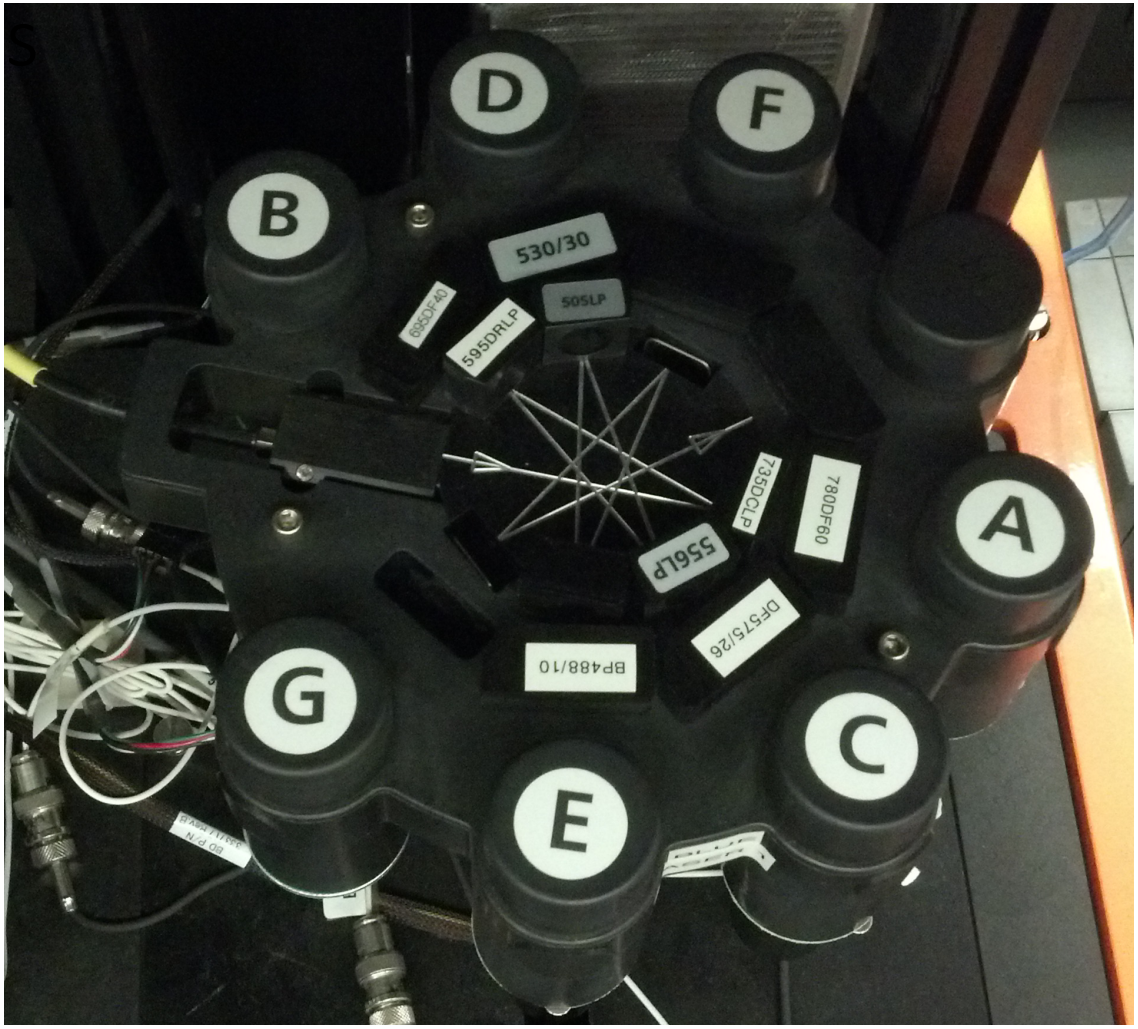






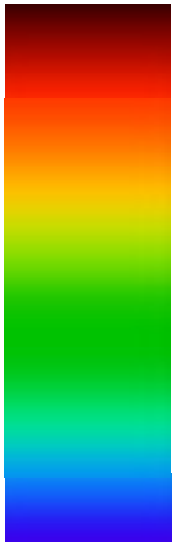
Fibers to carousels

- Fiber optics direct the emitted photons to carousels called “octagons” and “trigons”
- Octagons and trigons can hold 8 and 3 PMTs respectfully
- Equipped with LP mirrors and BP Filters
- Designed to separate photons into groups based upon wavelengths



Example of photon grouping

- Diagram of trigon on the fortessa



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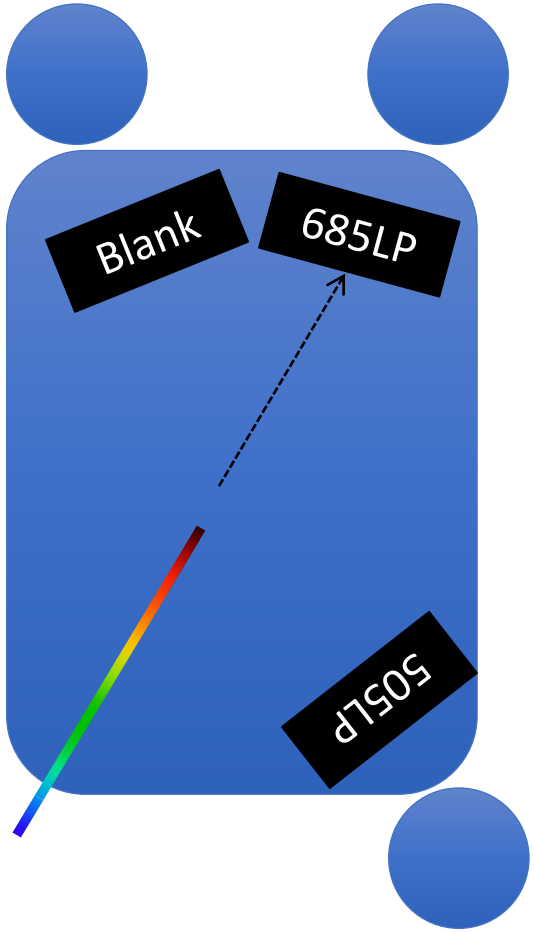
Potential Wavelengths



Example of photon grouping

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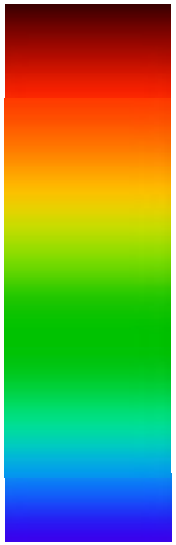
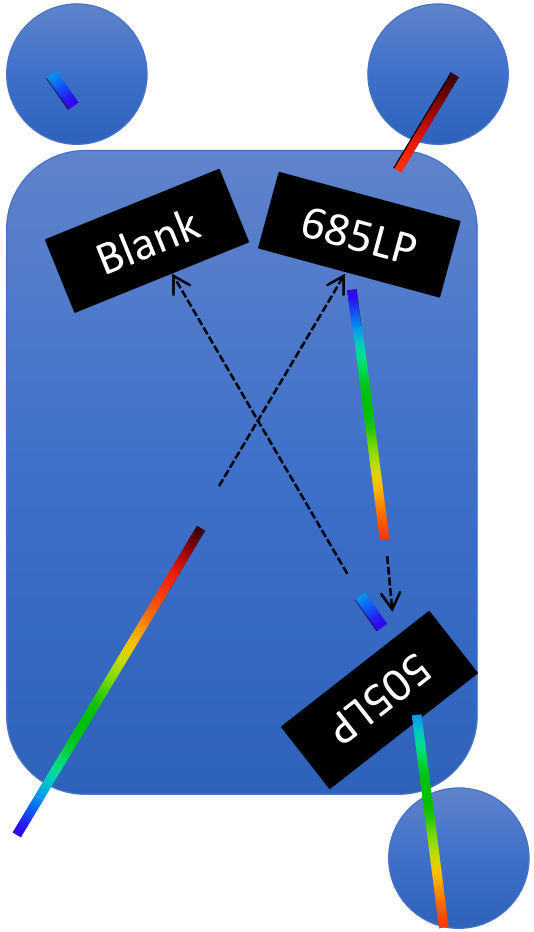
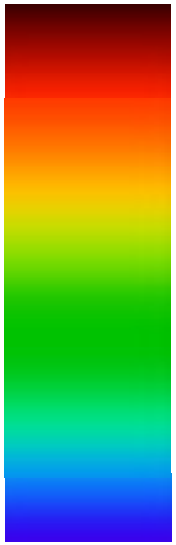


Potential Wavelengths



Signal refinement

- BP Filters are used to refine the samples before they enter the PMT



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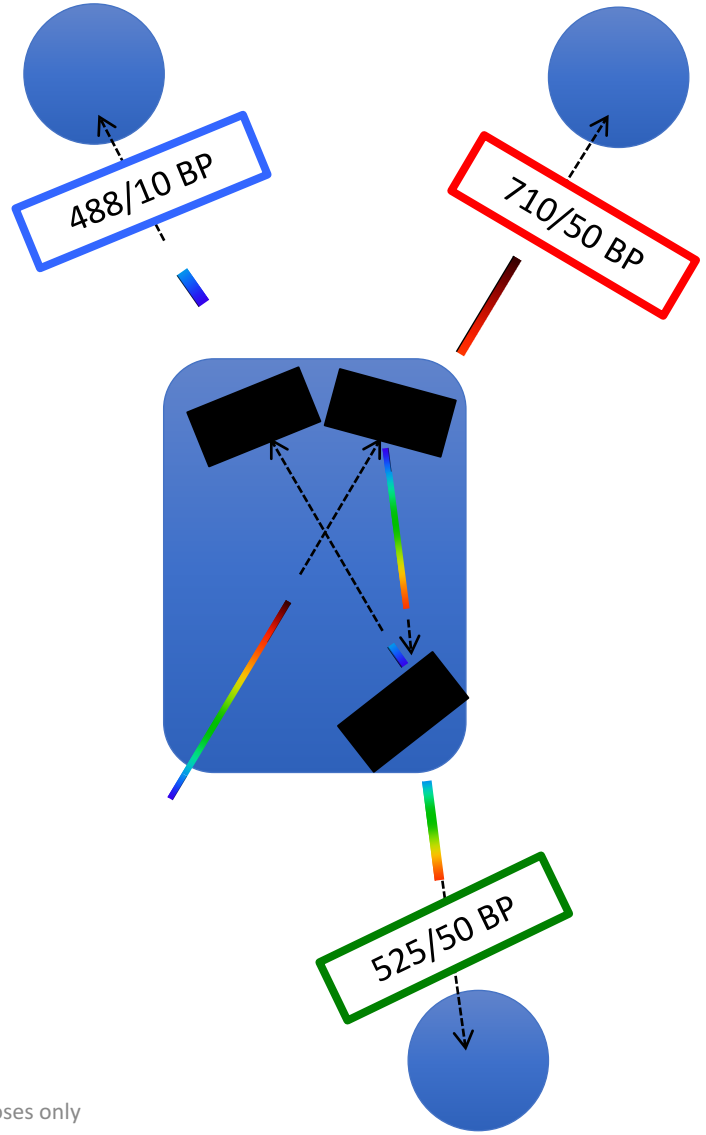

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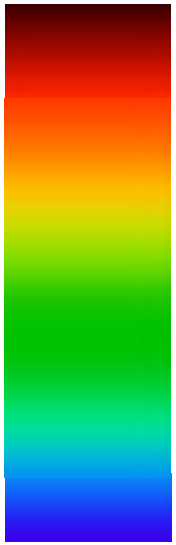

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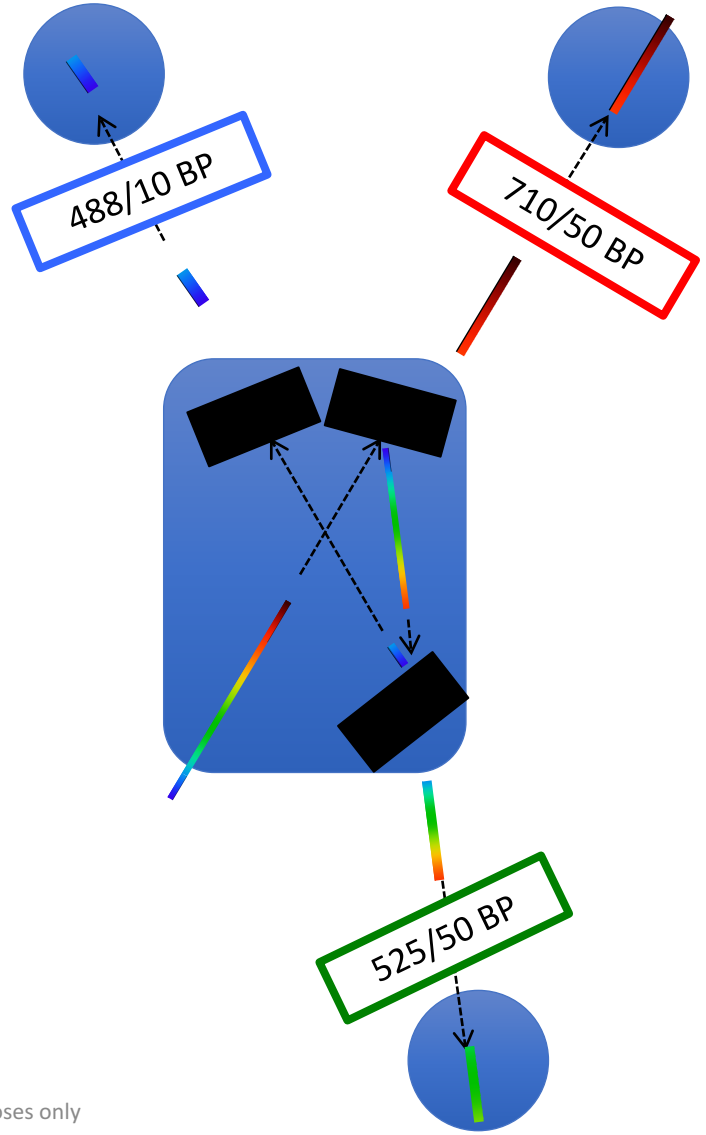
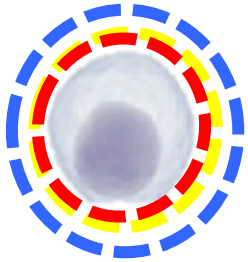
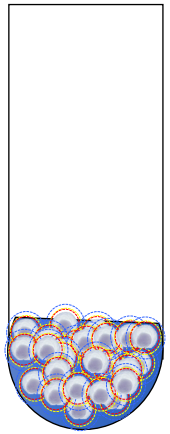
Potential Wavelengths

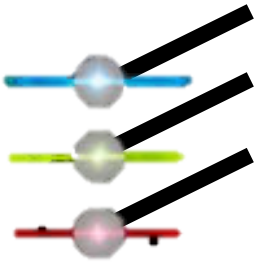


Example experimental panel

- PE
  - Excitation (max) = 561nm
  - Emission (max) = 575nm
- PE-Cy7
  - Excitation (max) = 561nm
  - Emission (max) = 760nm
- APC-Cy7
  - Excitation (max) = 640nm
  - Emission (max) = 760nm
- Lasers available in the cytometer
  - 488nm (blue)
  - 532nm (green)
  - 640nm (red)







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  - Excitation (max) = 561nm
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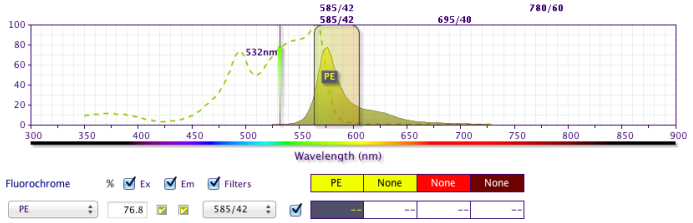
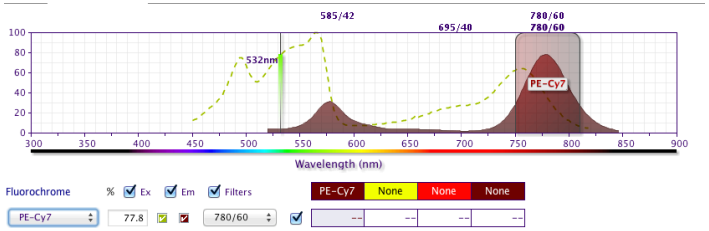
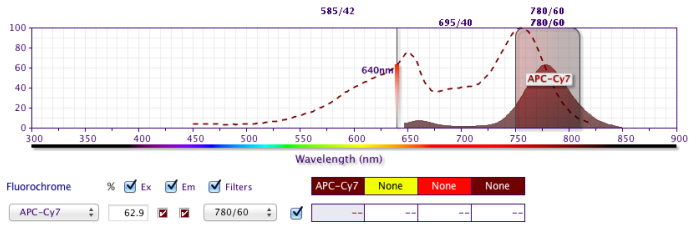
- APC-Cy7
  - Excitation (max) = 750nm
  - Emission (max) = 760nm
- Lasers available in the cytometer
  - 488nm (blue)
  - 532nm (green)
  - 640nm (red)







Laser specific excitation

- PE
  - Excitation (max) = 561nm
  - Emission (max) = 575nm
- PE-Cy7
  - Excitation (max) = 561nm
  - Emission (max) = 760nm
- APC-Cy7
  - Excitation (max) = 640nm
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- Lasers available in the cytometer
  - 488nm (blue)
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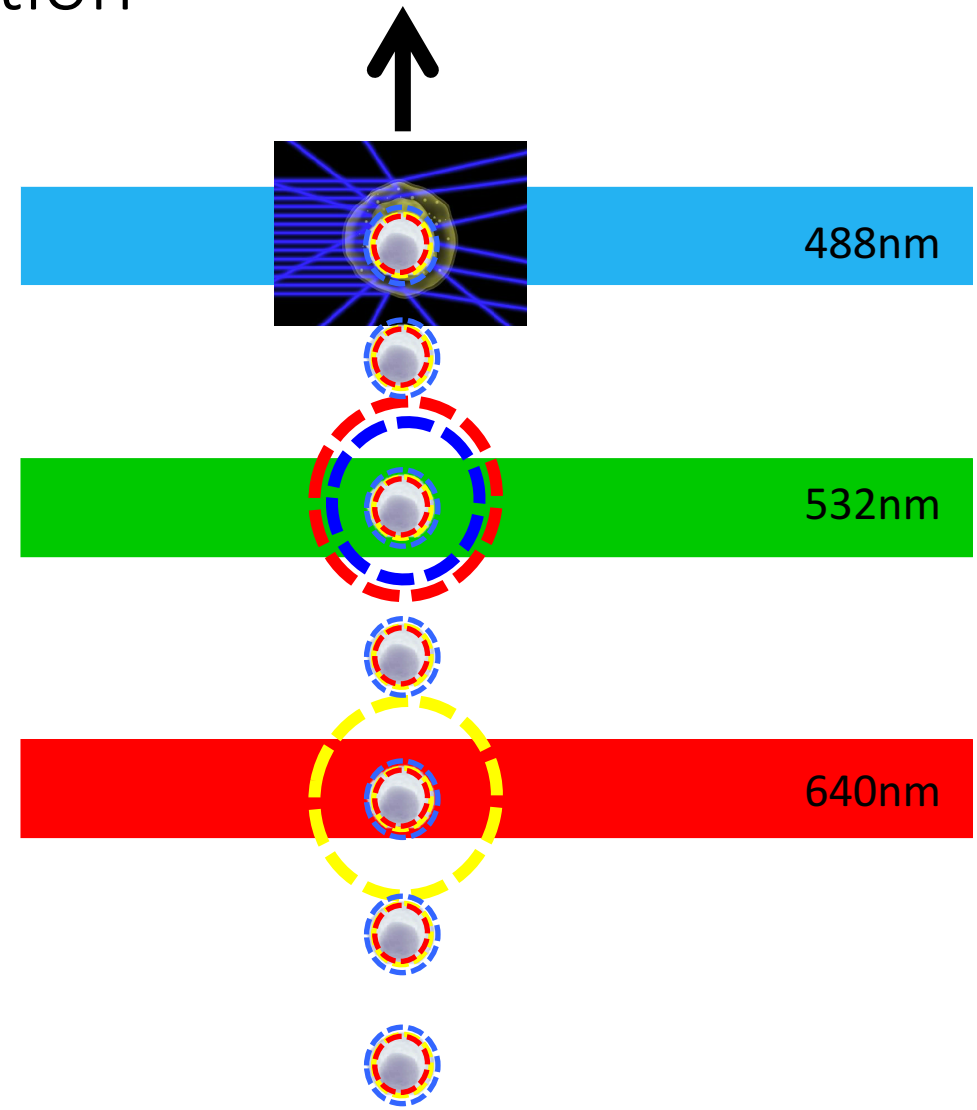
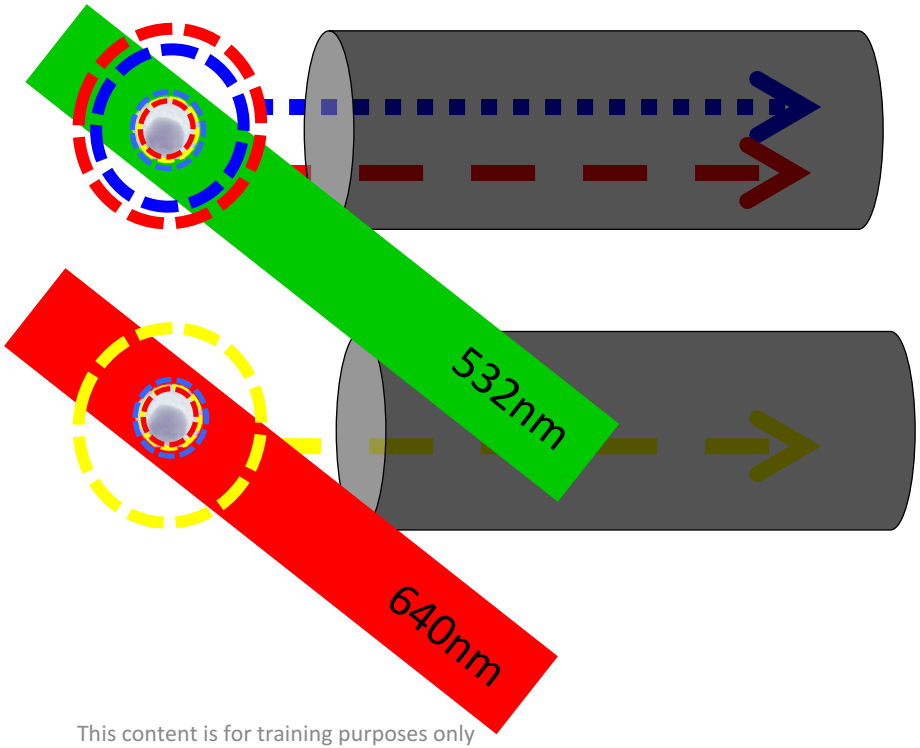


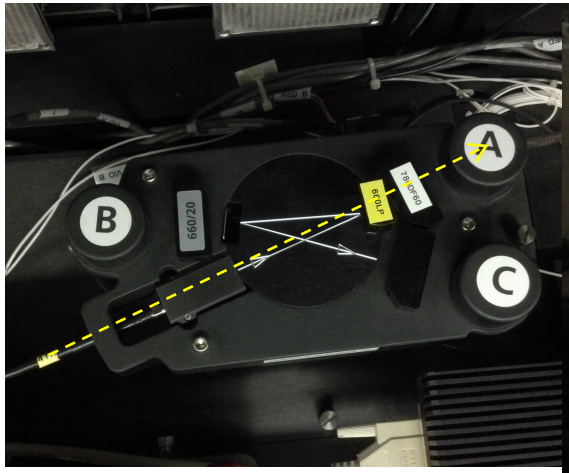
Excitation specific photon trafficking

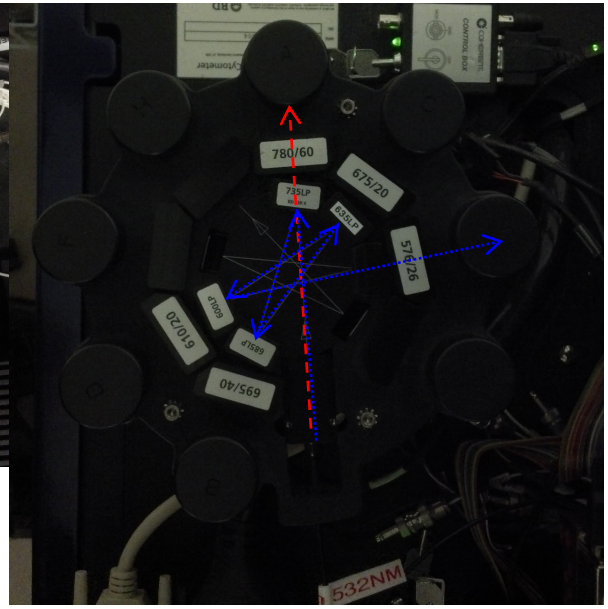
- PE and PE-Cy7 travel down the same fiber optic
  - PE-Cy7 = 760nm
  - PE = 575nm
- APC-Cy7 travels alone
  - APC-Cy7 = 760nm



Emission based sorting

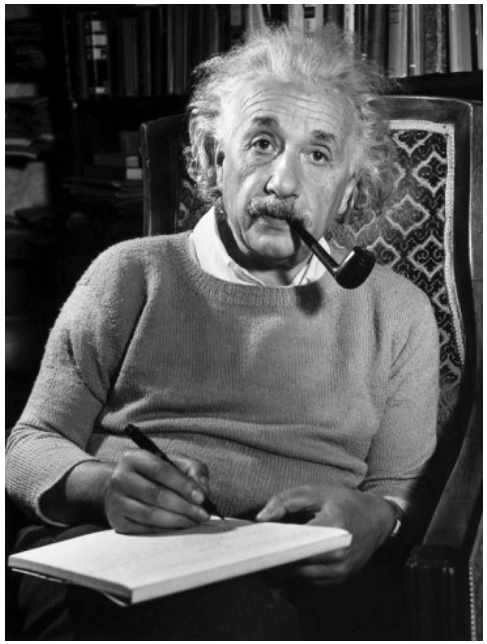
- PE and PE-Cy7 travel down the same fiber optic
  - PE-Cy7 = 760nm
  - PE = 575nm
- APC-Cy7 travels alone
  - APC-Cy7 = 760nm

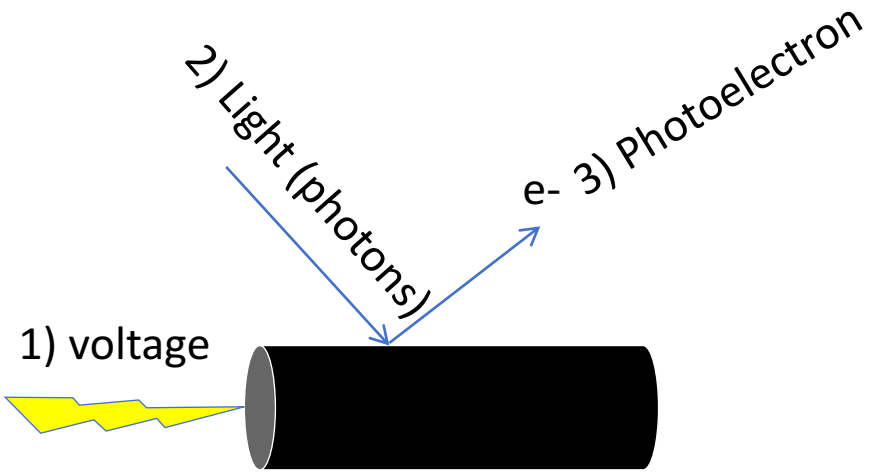




Photon detection

- Photomultiplier Tube (PMT)
- Converts electromagnetic energy (light) into electrical pulses (voltage pulse)
- Works by way of the photo electric effect





Photomultiplier tubes

- Photomultiplier tubes convert light energy into a current
- The light comes from fluorescent molecules while a cell crosses the laser
- This results in a voltage pulse
- These PMTs exploit the photo electric effect

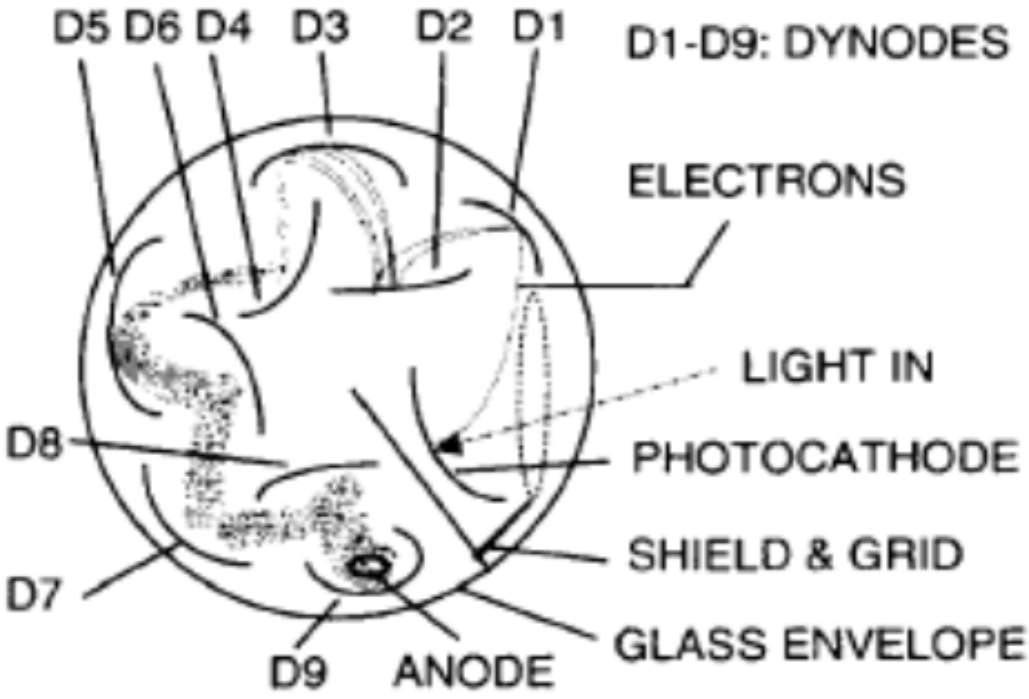
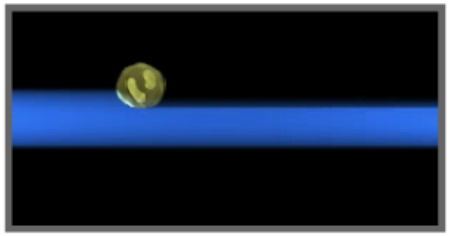
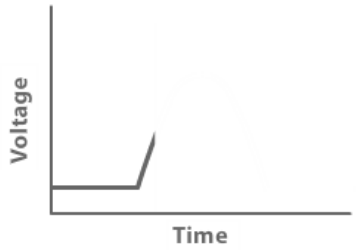


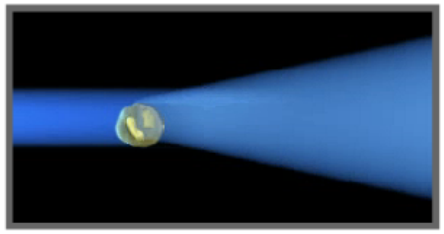
Figure 4-34. Elements of a photomultiplier tube (PMT).

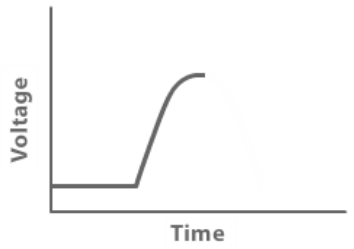
Voltage pulses

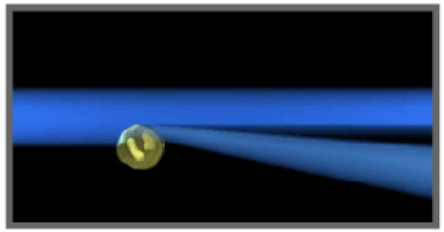
- The voltage pulse is specifically what the cytometer measures
- Measurements of pulse height and width can be made
- The pulse area can be calculated
- The size of the voltage pulse is directly related to the intensity of the signal
  - Is a function of intensity over time
  - The geometries of these pulses can be helpful in analysis and utilized in single cell sorting

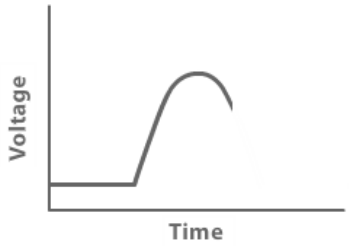




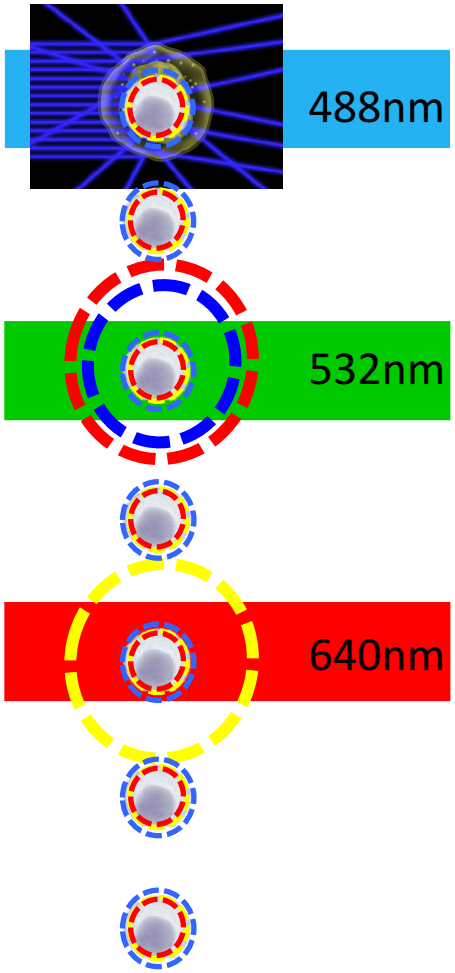


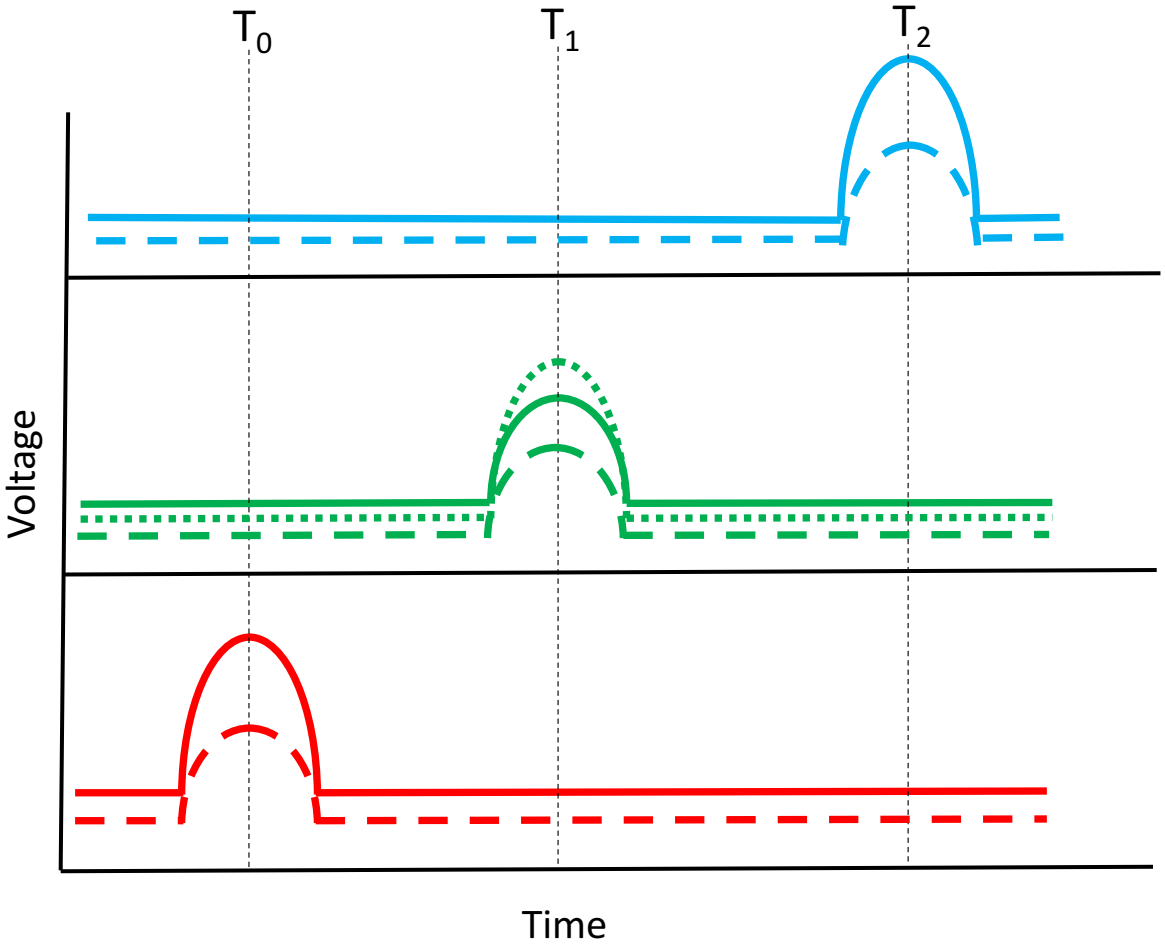






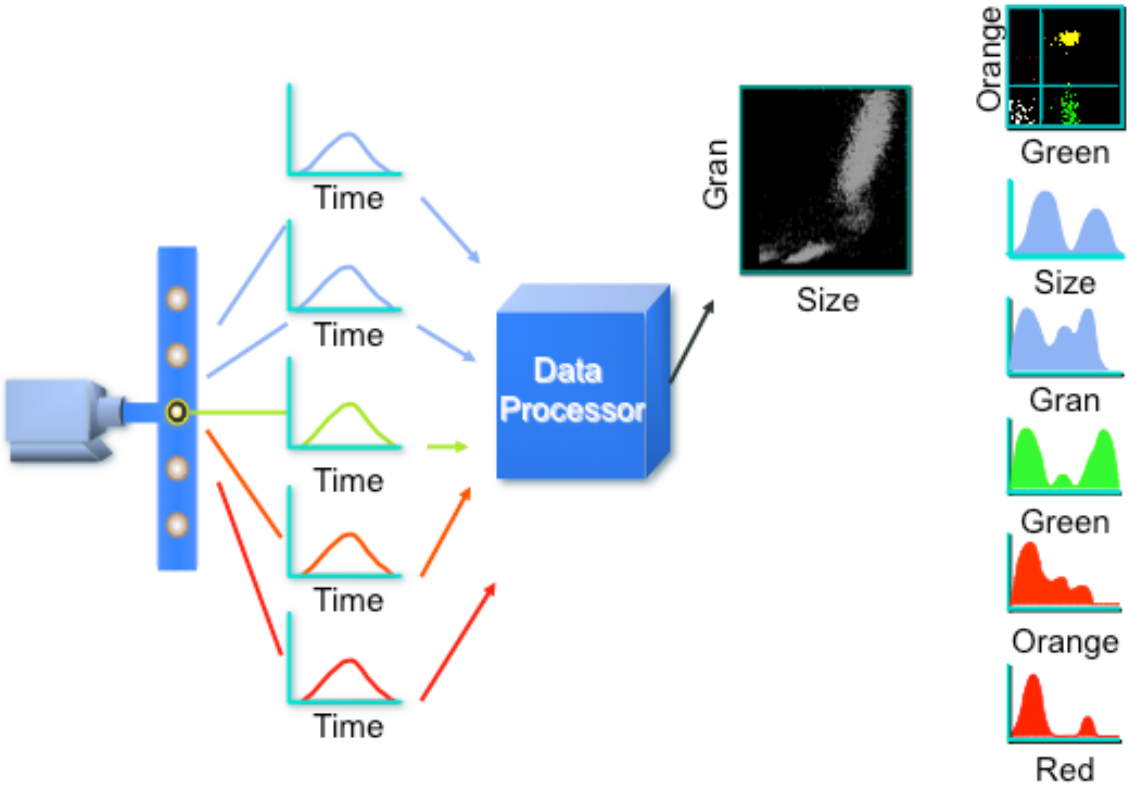
Laser Delays





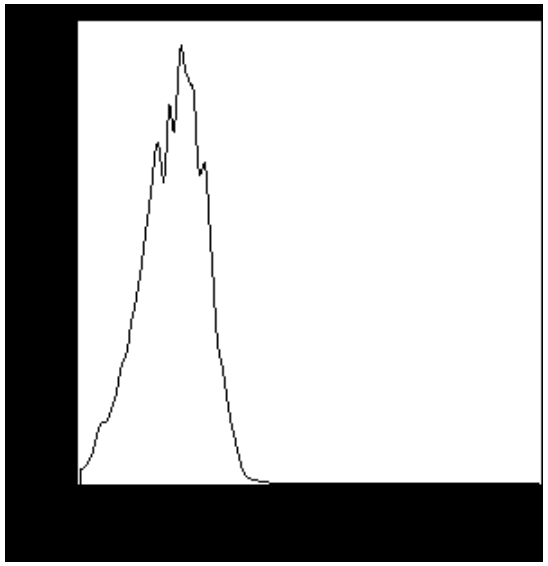
Fluorescent signals

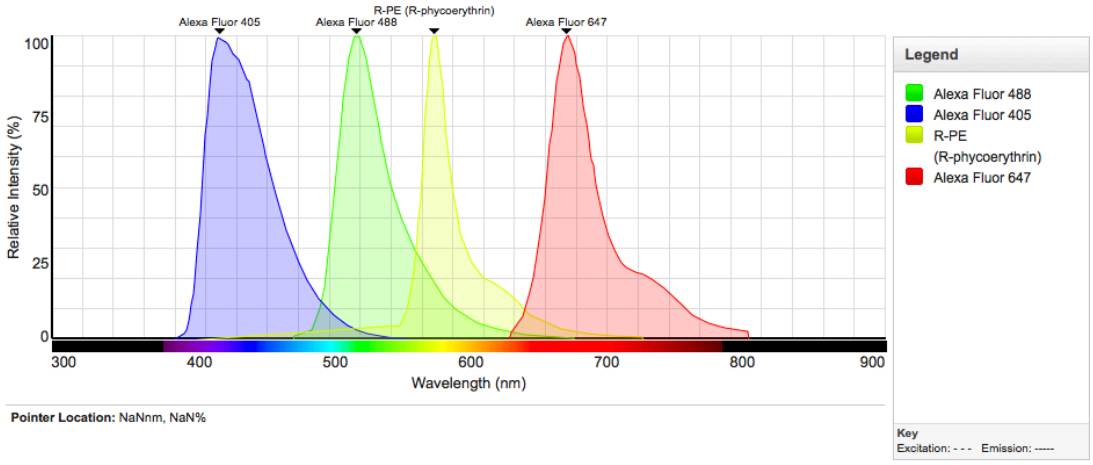
- PMTs that detect fluorescence produce voltage pulses as well
- The signals are separated by laser position first, then emission spectrum
- Typically measured as percent positive or MFI
- What could be the benefits of measuring MFI? or % or Both?



Setup Controls

- We need these to provide a basis for our instrument settings and compensation
- Compensation controls are required for every experiment

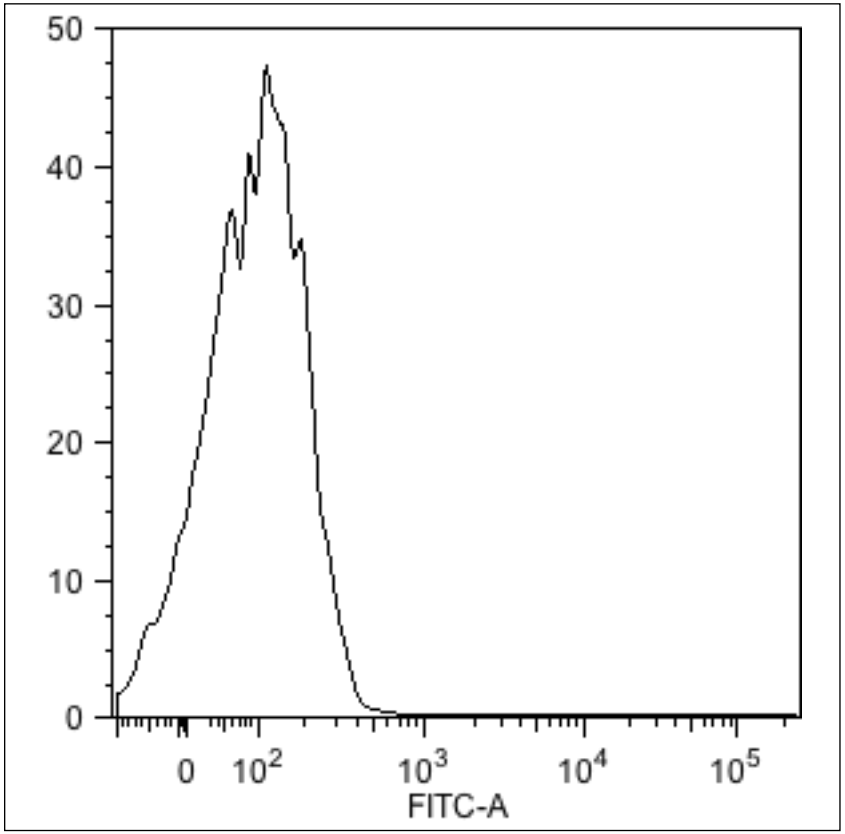




A Universal Negative or Unstained Control

- We can use this type of control to set acceptable baseline PMT voltages
  - This method is not perfect
  - Mixtures of different cell types (i.e. whole blood) might result in optimal settings for one, poor settings for another
  - It's a good idea, especially the more fluorochromes you have, to look at your positive controls before saving your unstained control

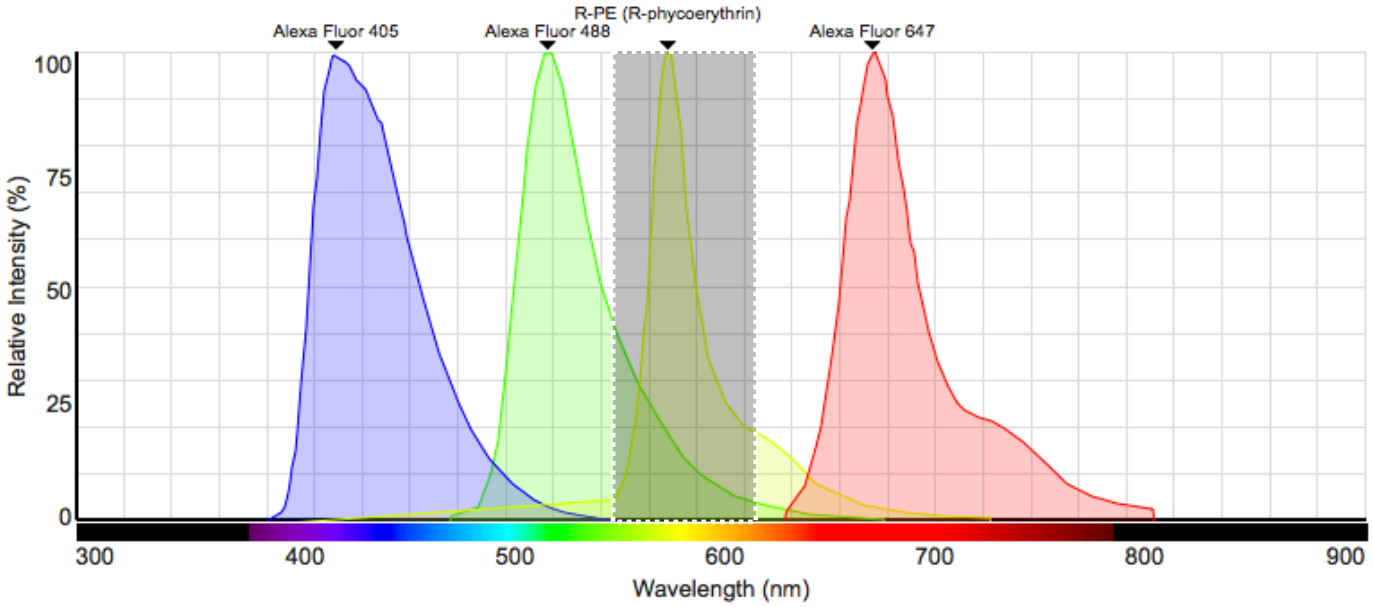
Universal Negative (Unstained)



Adjust voltage gains until the peaks on each histogram are centered over the second log decade

Compensation Controls

- We need these to determine the amount of spectral overlap between fluorochromes and correct for this
- The ideal control would be 50% positive, 50% negative and positive staining at least as bright as the sample
- The actual experimental reagents should be used, fluorchrome to protein ratios are going to be different for each reagent and each lot of the same reagent
- Sometimes you do have to get creative!



Pointer Location: NaNnm, NaN%

Legend

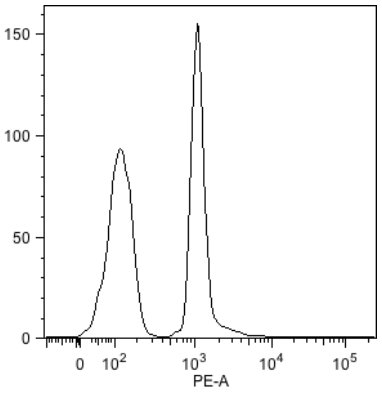
- Alexa Fluor 488
- Alexa Fluor 405
- R-PE (R-phycoerythrin)
- Alexa Fluor 647

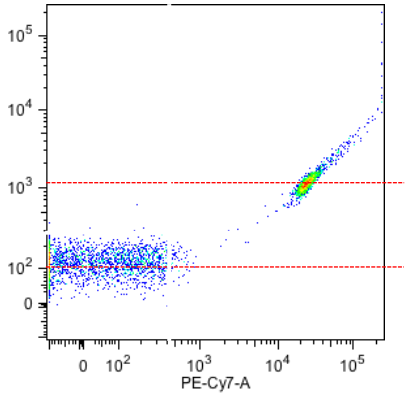
Key
Excitation: - - - Emission: -----

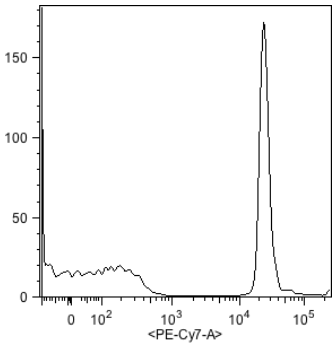
Compensation Beads

- Polystyrene beads that will bind to any mouse Ig k light chain containing antibody
- Used as a replacement for Cells to stain for your compensation controls
- Why use them?
  - If the cells themselves do not express the surface marker of interest at a high percentage
  - If the cells to be tested are very rare (and you have low yields)

Before Compensation



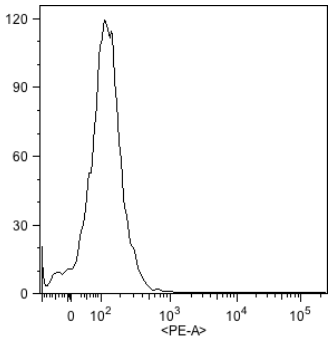


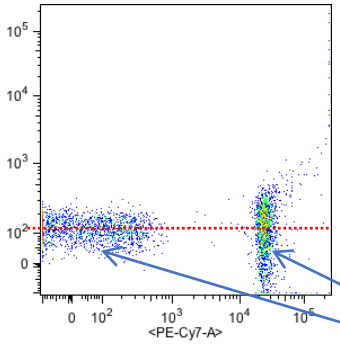


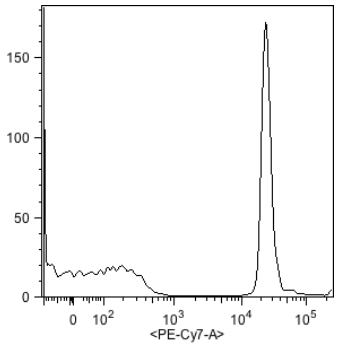
PE-Cy7 Only Control

- Mean Fluorescent Intensity of PE-Cy7 is above background in the PE Channel
- Why is this a problem?
  - It's difficult to accurately gate "double positive" cells if the single positive have double positive signals

After Compensation





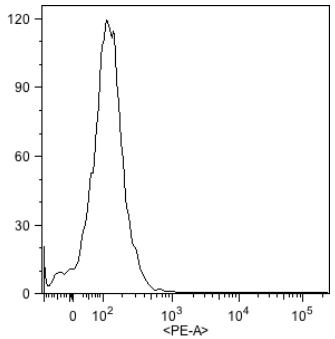


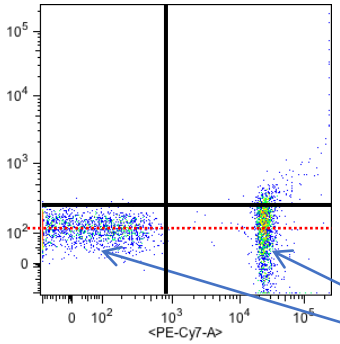
Spectral overlap from PE-Cy7 is subtracted from PE channel

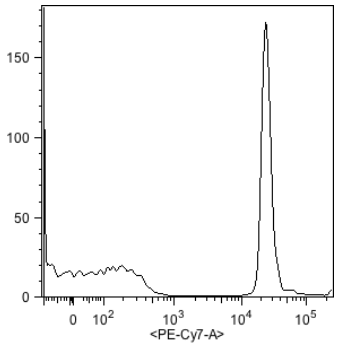
Compensation calculation is made so the median of these two populations match

It's not always perfect!

After Compensation







Spectral overlap from PE-Cy7 is subtracted from PE channel

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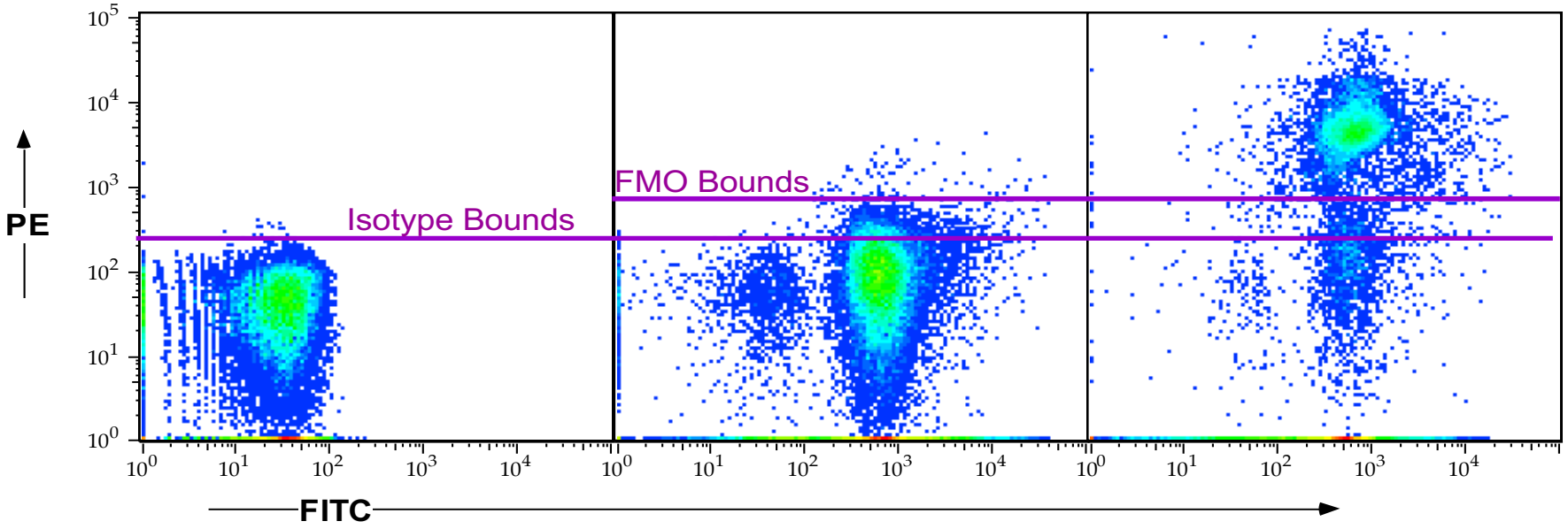
It's not always perfect!

Gating Controls

- Isotypes may not be the most specific controls for gating
  - They are very useful for determining non-specific binding, but better controls can be used in multi-color panels
- The best control to determine the gating boundaries for a particular reagent would be Fluorescence Minus One (FMO) controls
  - A sample that has all the other reagents EXCEPT the reagent you are gating on
- Not needed necessarily if you have an easily distinguished target population or have arbitrary gating criteria (top 5%, etc)

	Unstained Control	FMO Control	Fully Stained
FITC	Ğ	CD3	CD3
PE	Ğ	Ğ	CD4
Cy5PE	Ğ	CD8	CD8
Cy7PE	Ğ	CD45RO	CD45RO



Biological Controls

- These are user/experiment specific controls
- Consider your experiment variables:
  - Stimulation
  - Transfection/Infection
  - Kinetics
  - Temperature (Ca++)