Bioinformatics B: Image Analysis and Flow Cytometry

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FEBRUARY 26, 2019

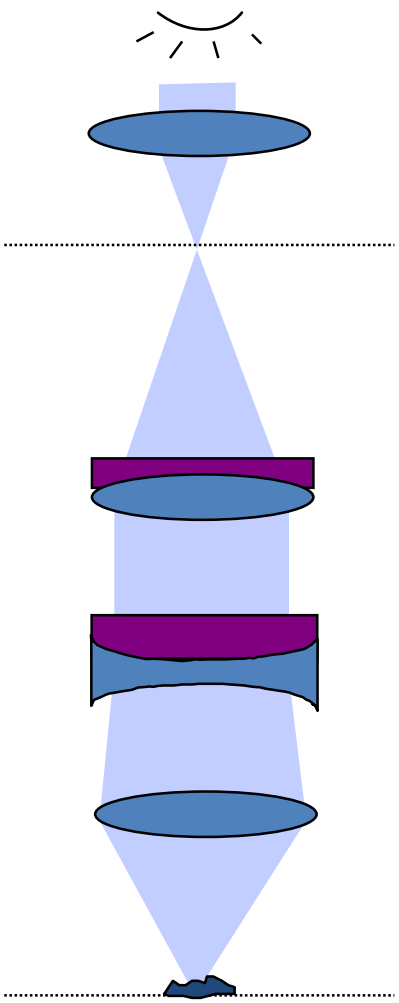
With many valuable slide contributions from
Michael J. McCaughey, Vanderbilt University

and many slides from Purdue University Cytometry Laboratories

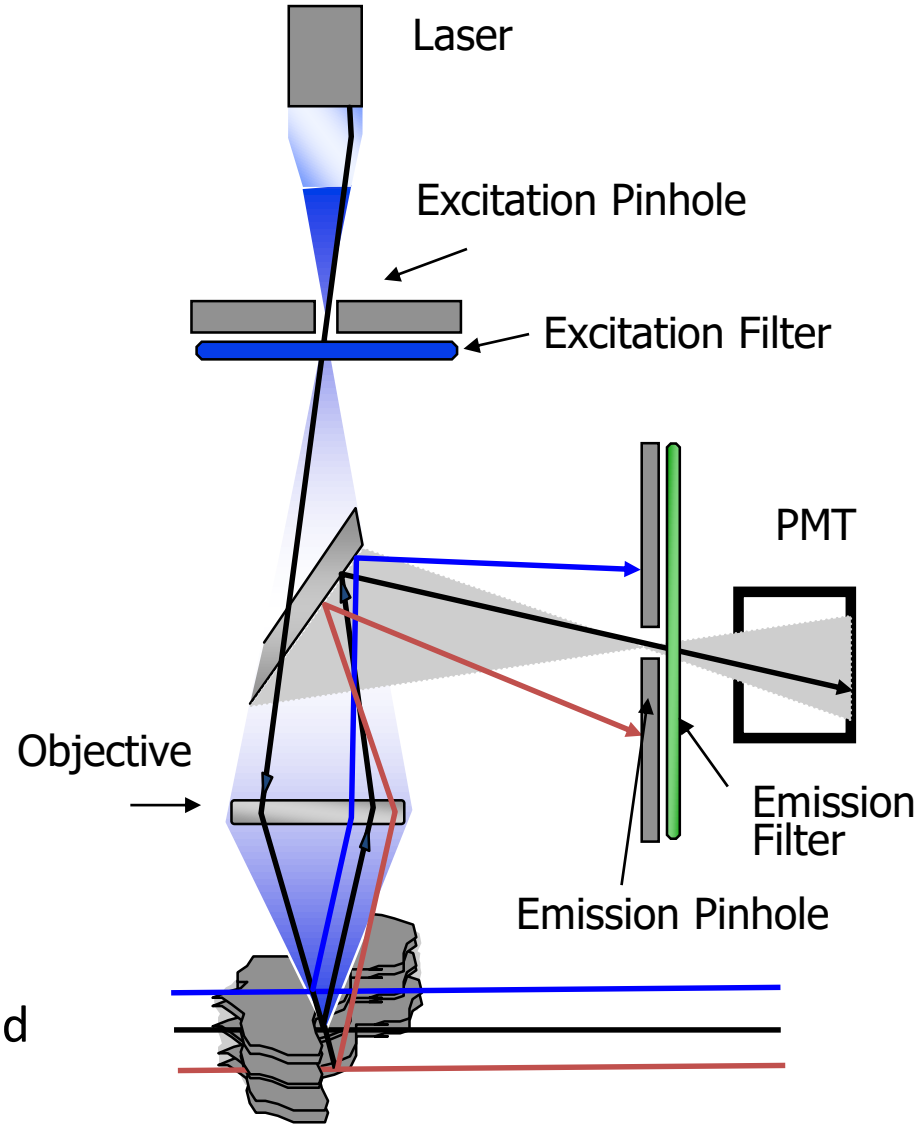
Overview

- Measurements of light intensity play a role in both microscopy and flow cytometry.
- We examine the problems of processing imaging data, with an emphasis on microscopy.
- We will discuss the essentials of flow cytometry since these data are growing so complicated that computer assistance has become required.

Conventional finite optics with telan system



Confocal fluorescence microscope (1957)



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Slide from Purdue University Cytometry Laboratories

Photomultipliers

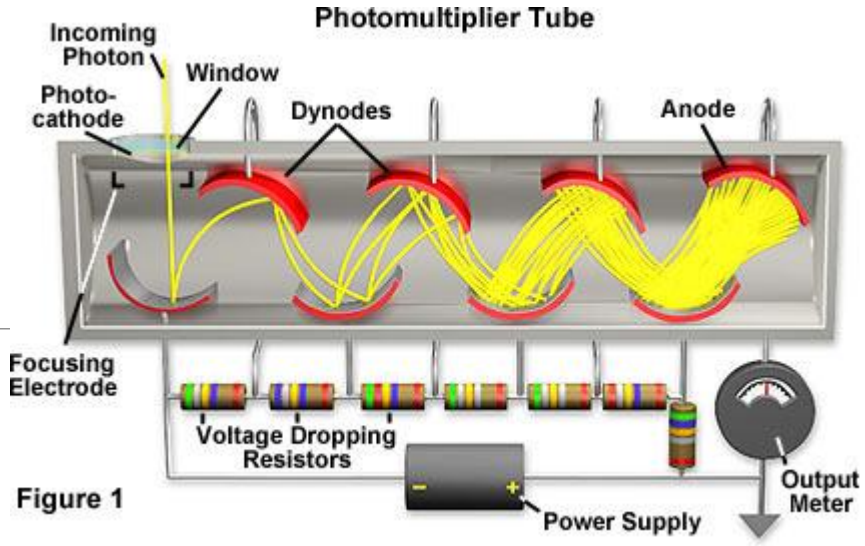

Figure 1

- A PMT is blind to color, but produces a cascade of electrons in response to photons.
- Color is determined by the nature of the light filter used before the PMT.
- Each Charge-Coupled Device (CCD) incorporates millions of PMTs, with density corresponding to resolution of features.

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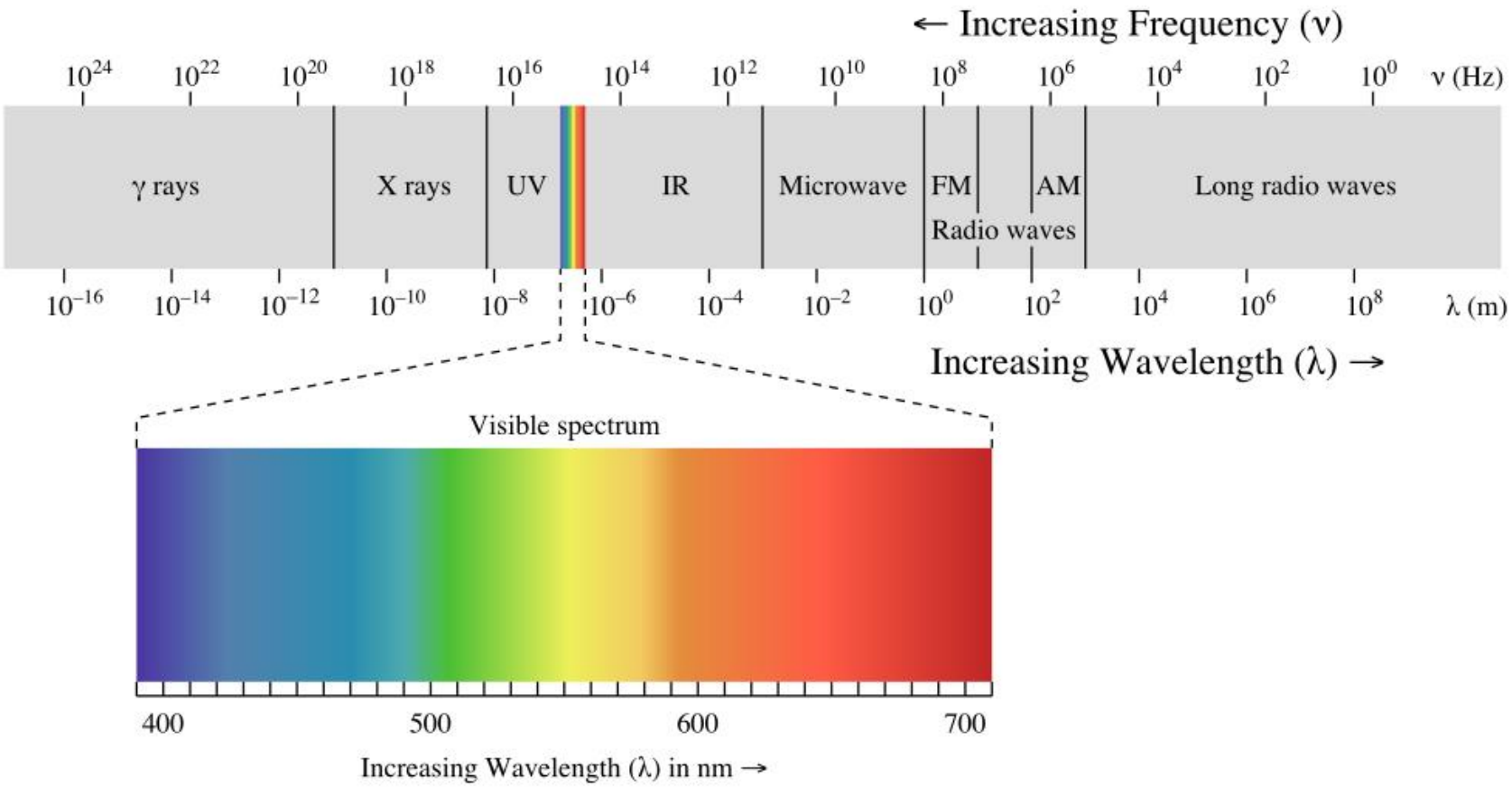
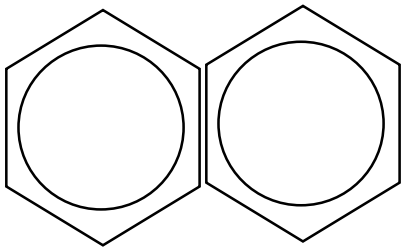
The electromagnetic spectrum: photons are most energetic at low wavelengths



WikiMedia Philip Ronan

Fluorescence

- Chromophores are components of molecules which absorb light.
- Auto-fluorescence from proteins is mostly from the indole ring of tryptophan residue.
- They are generally aromatic rings.

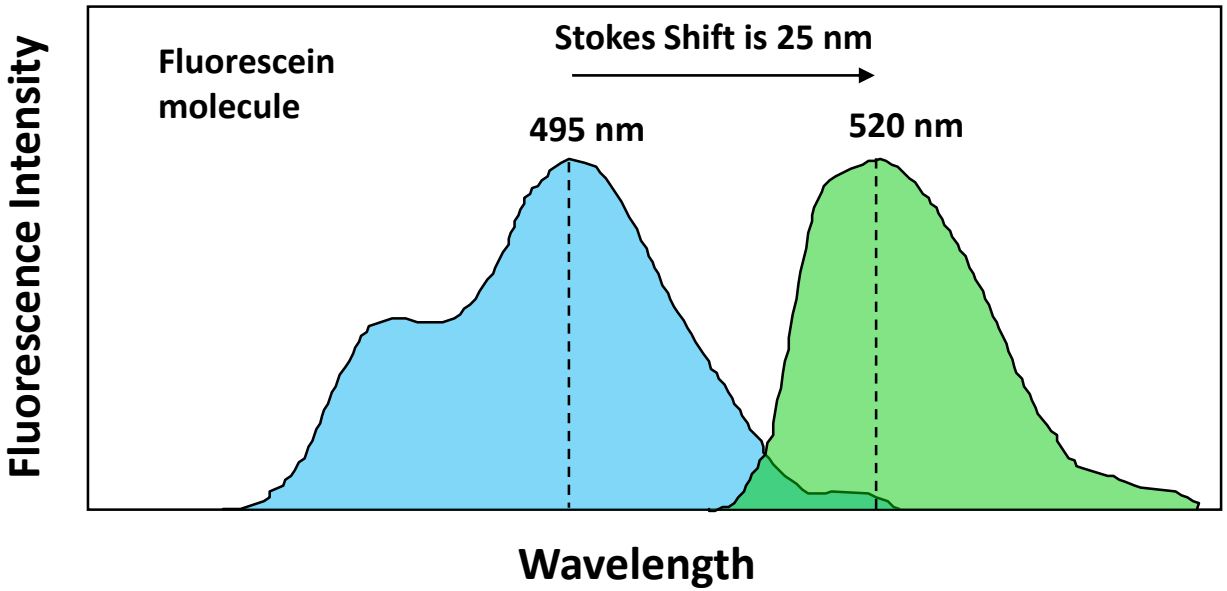


Excitation Sources

- **Lamps**
  - Xenon
  - Xenon/Mercury
- **Lasers**
  - Argon Ion (Ar)
  - Krypton (Kr)
  - Violet 405nm, 380 nm
  - Helium-Neon (He-Ne)
  - Helium-Cadmium (He-Cd)
  - Krypton-Argon (Kr-Ar)
- **Laser Diodes**
  - 400nm – NIR

Stokes Shift

Shift represents the energy difference between the peak of absorbance and the peak of emission. Photon emission results from an electron returning to ground state from excited state.



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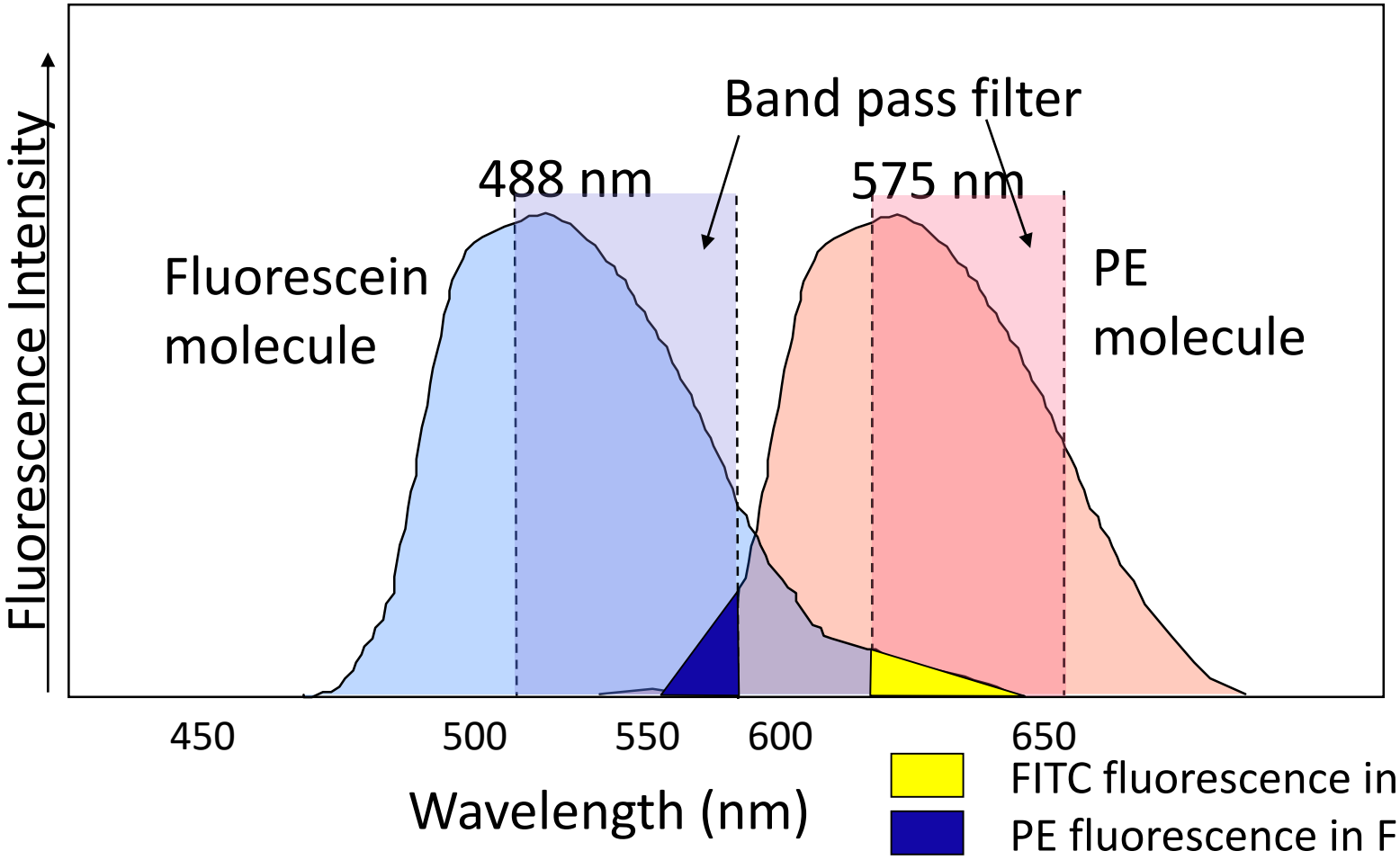
Employing many dyes may overlap fluorescence



Slide from Purdue University Cytometry Laboratories

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Contrast in intensity is where information is stored.

- High signal-to-noise suggests biological information exceeds technical variation.
- *Dynamic range* compares brightest to darkest. If biological variation spans narrow range, it will be harder to detect.
- Optimizing contrast facilitates information inference.

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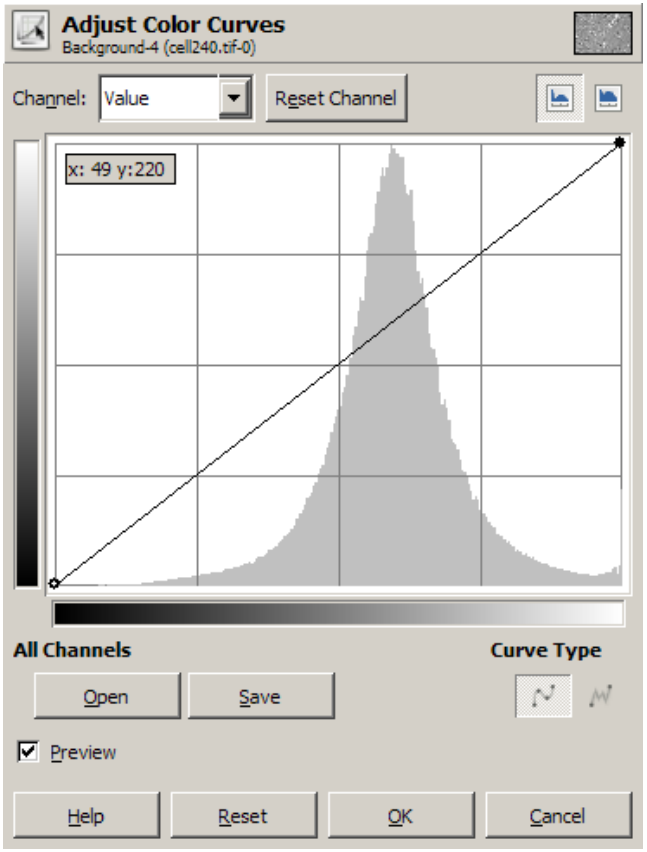
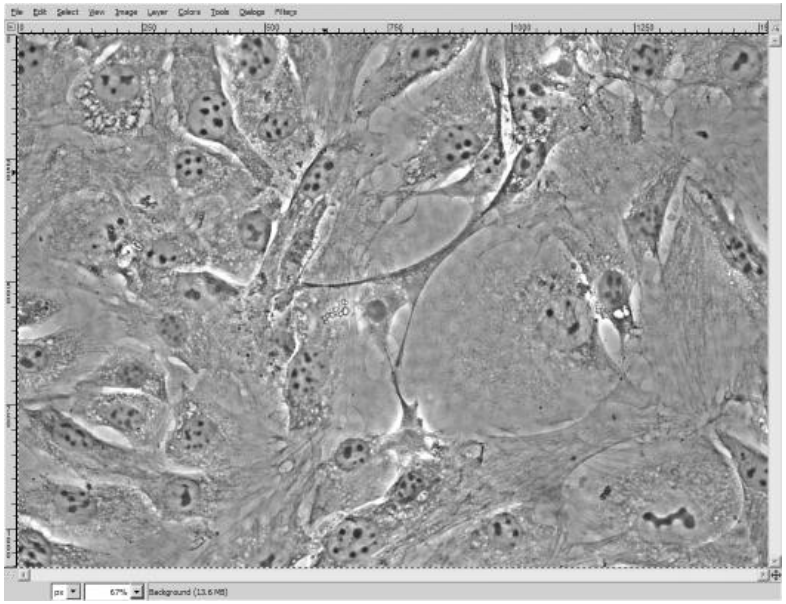
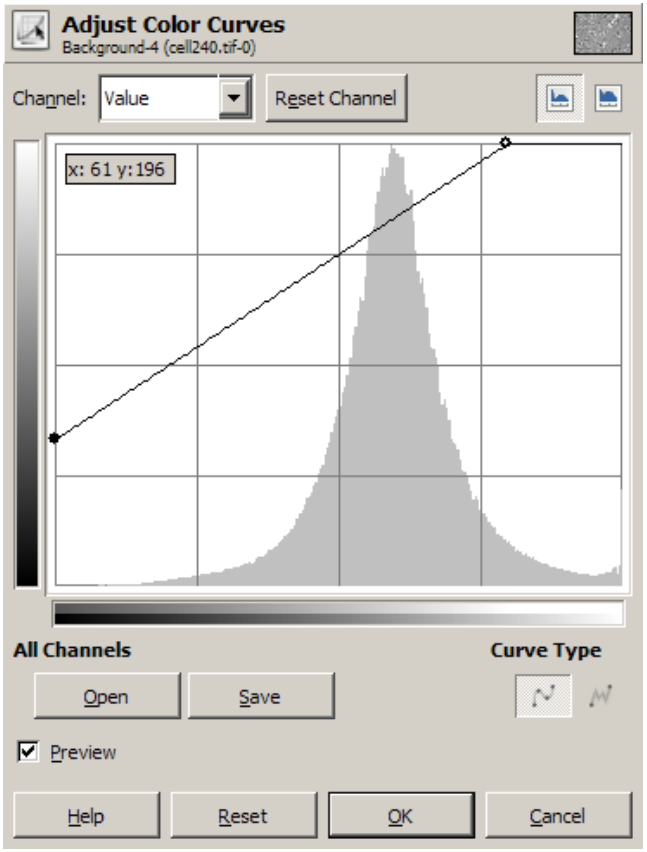
Input-Output Curve

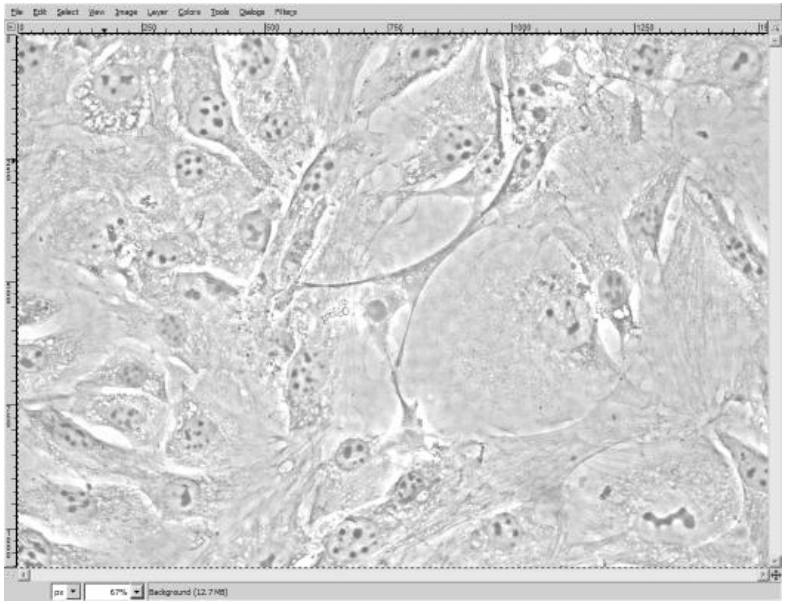




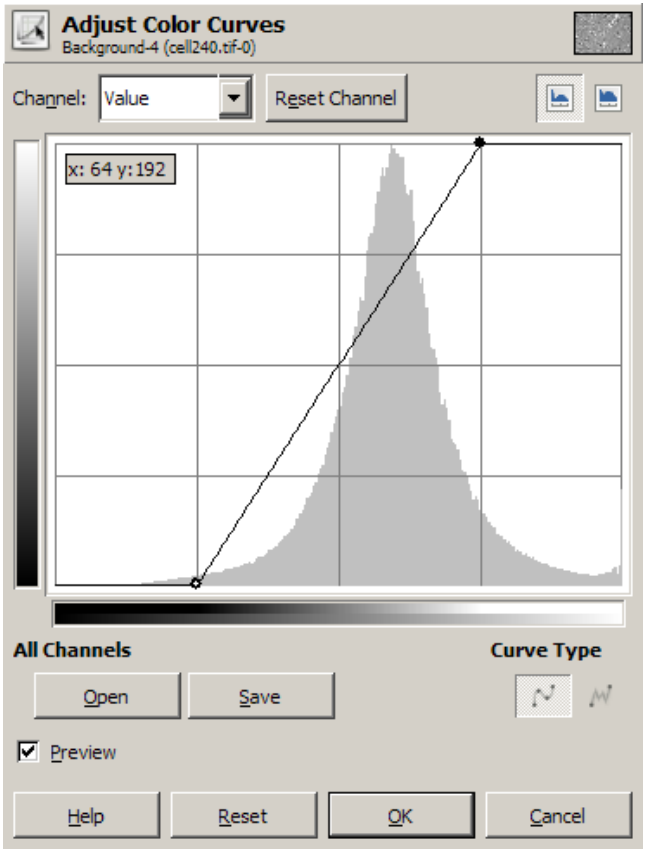
Slide from Purdue University Cytometry Laboratories

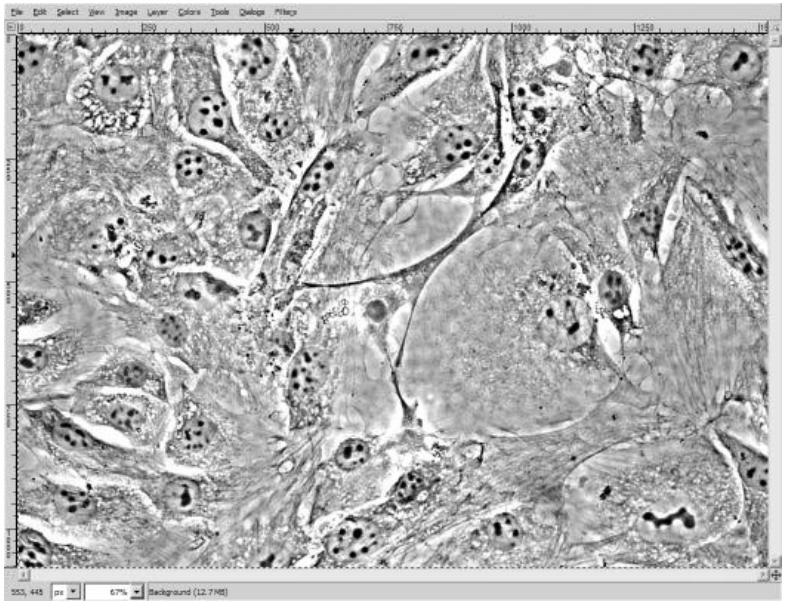
Input-Output Curve





Input-Output Curve





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Mechanics of kernel convolution

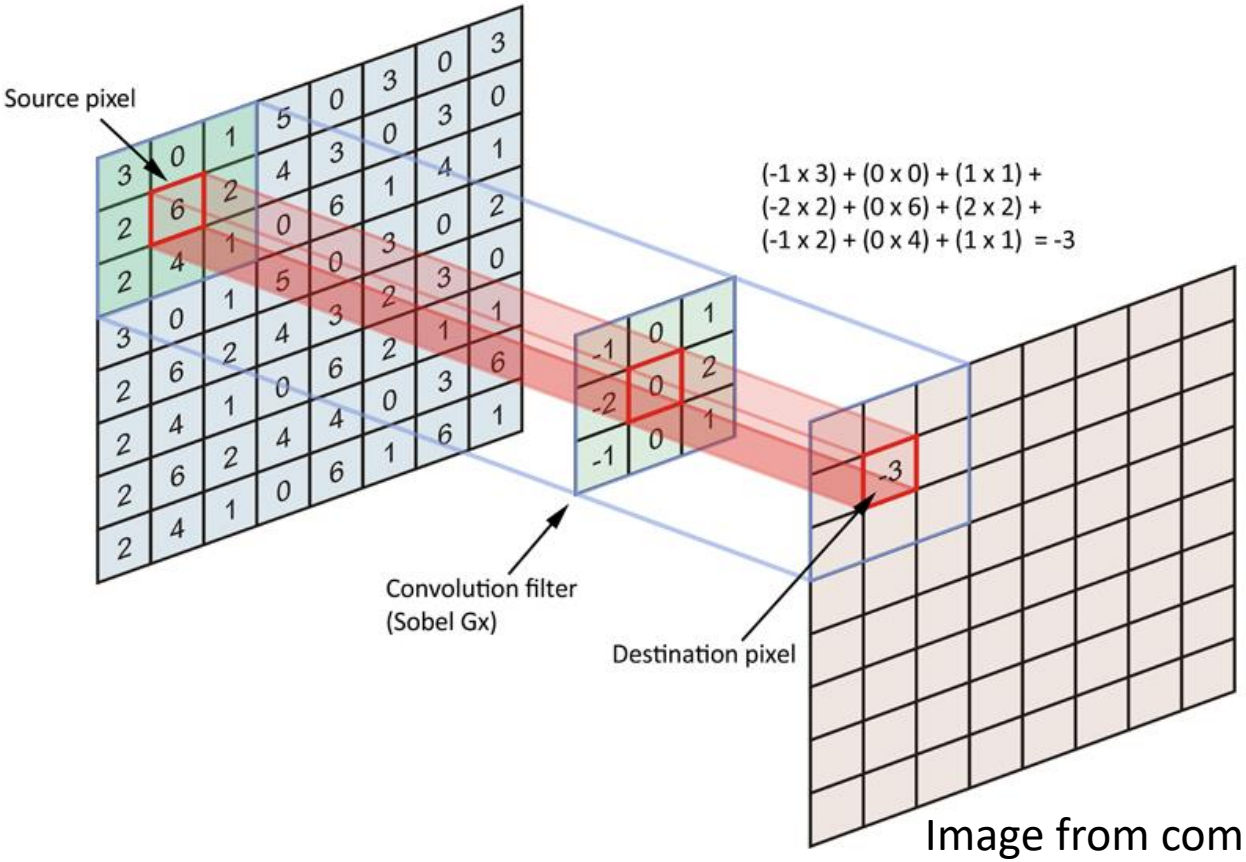


Image from community.arm.com

http://www.aishack.in/tutorials/image-convolution-examples/

Gaussian kernel filter

1	2	1
2	4	2
1	2	1

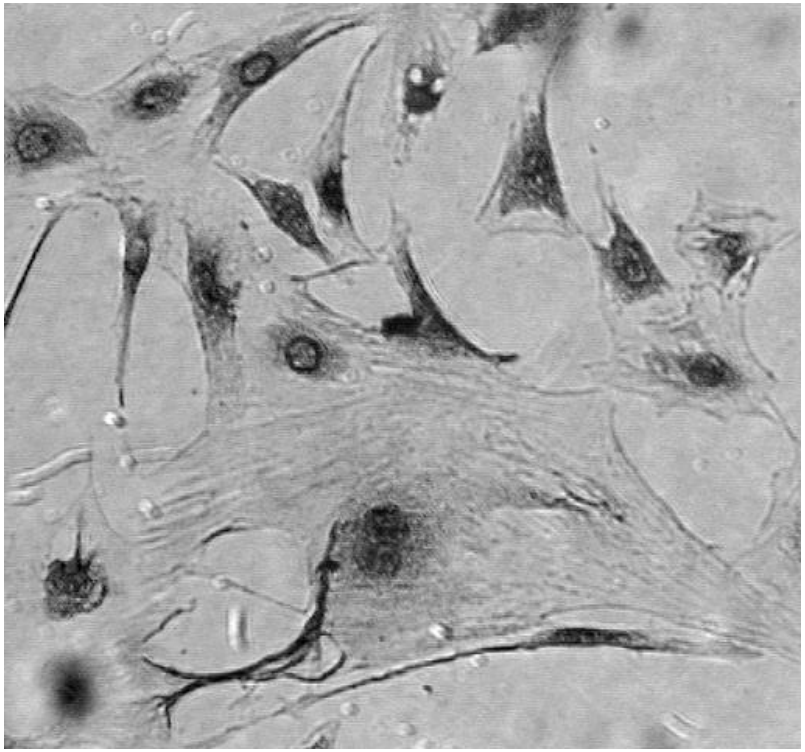
Divisor=1+2+1+2
+4+2+1+2+1=16

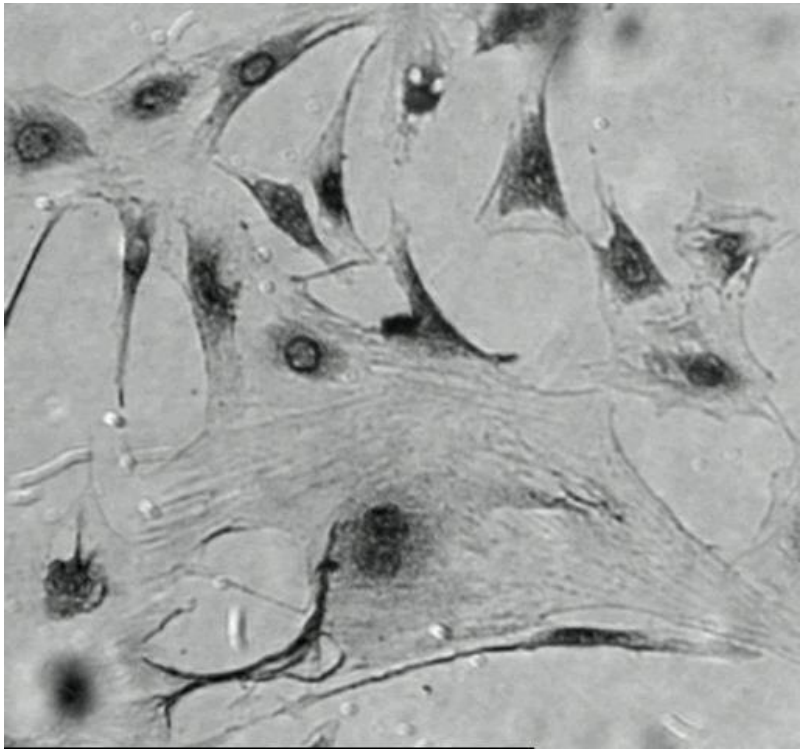
Section of image file

127	129	123	121	124	130
139	134	133	134	137	137
127	130	133	134	132	131
123	122	131	129	129	125
118	122	122	124	123	122
124	121	117	116	114	118
127	121	114	110	109	114
136	124	122	117	111	109

Gaussian filters reduce noise at the cost of low-intensity detail.

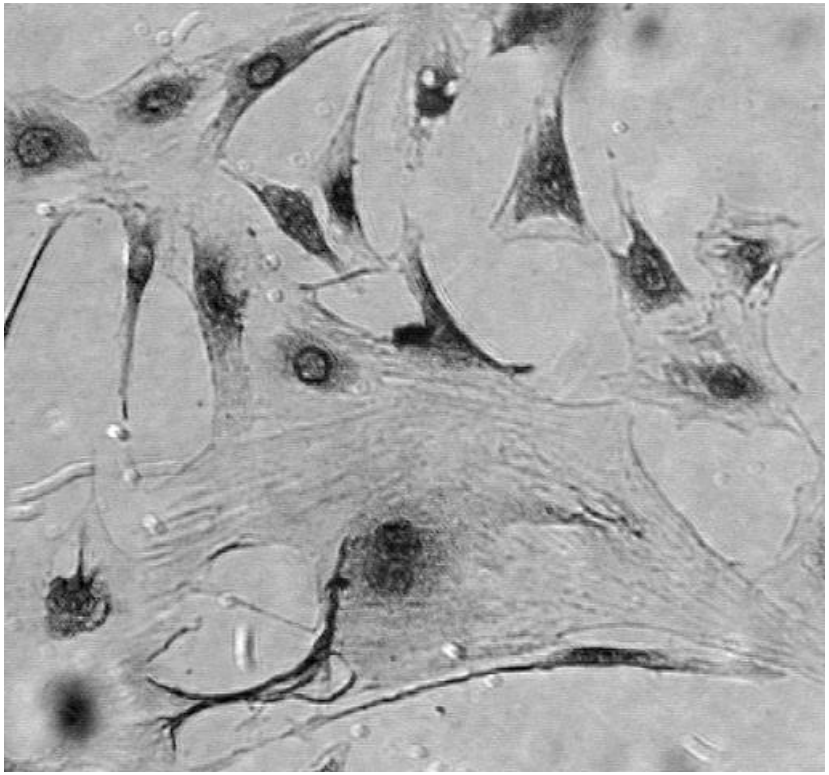
Gaussian Filter		
1	2	1
2	4	2
1	2	1

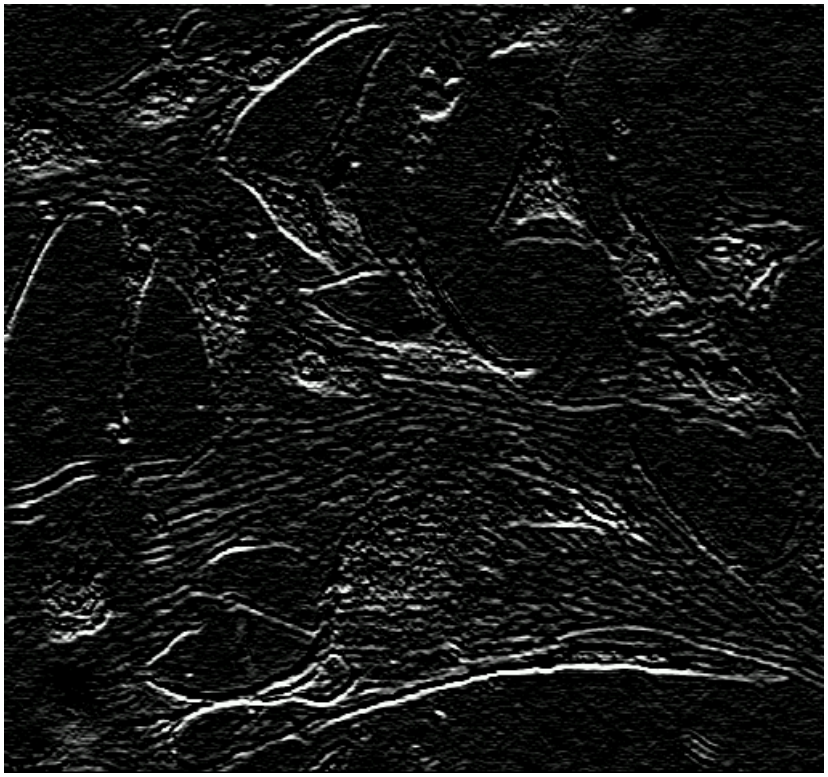




Sobel filters intensify edges.

Sobel Filter		
1	2	1
0	0	0
-1	-2	-1



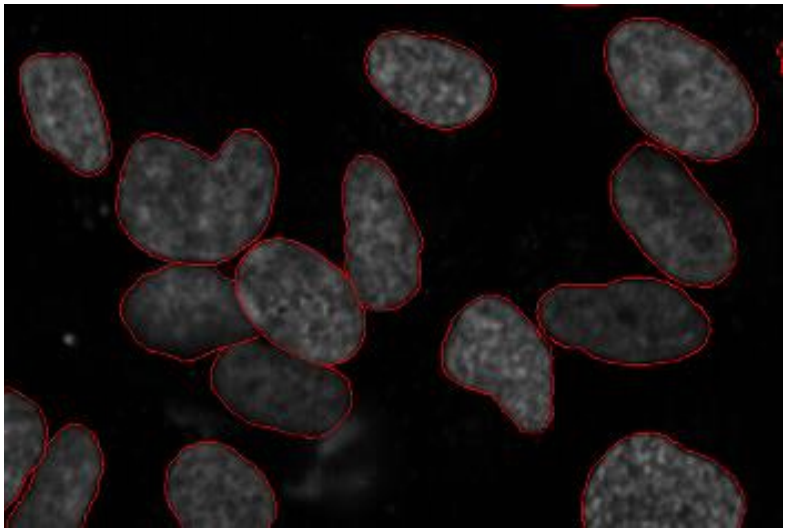


Slide from Purdue University Cytometry Laboratories

Key activities in image analysis

- *Segmentation*: separating objects and background in the visual field
- *Registration*: aligning two images of the same scene by mapping objects seen in both
- *Motion analysis*: tracing the path of an object among multiple time slices
- *Quantitation*: estimating volumes, speeds, or other metrics in image data

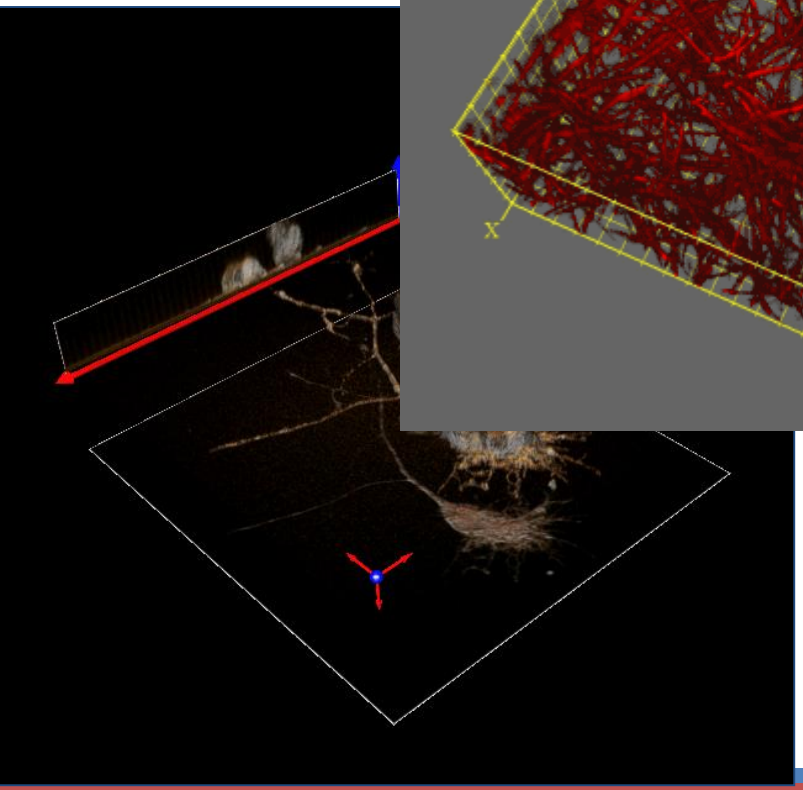
Segmentation

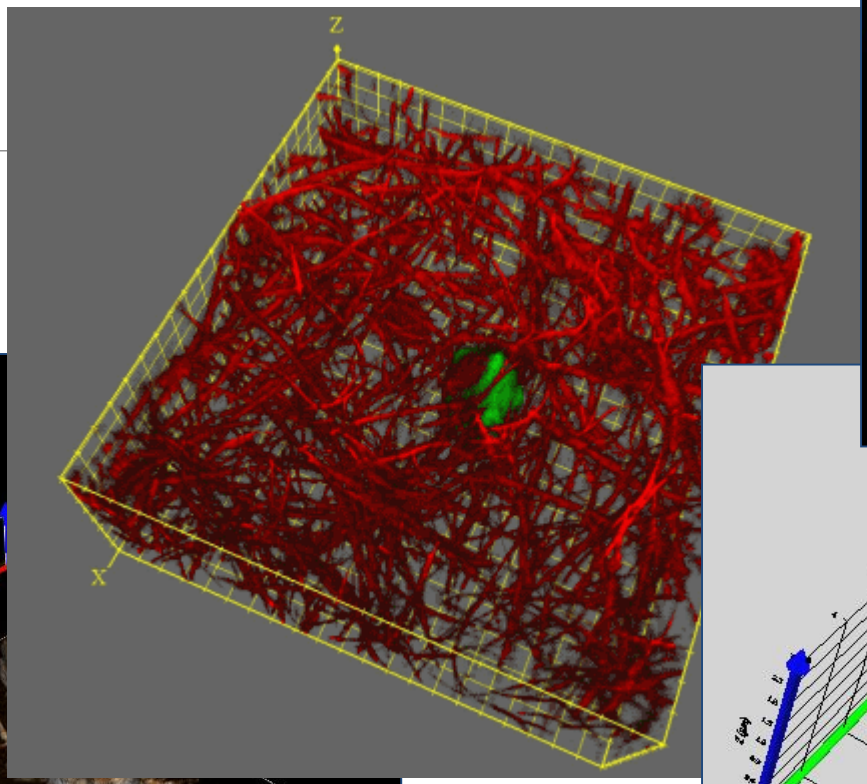


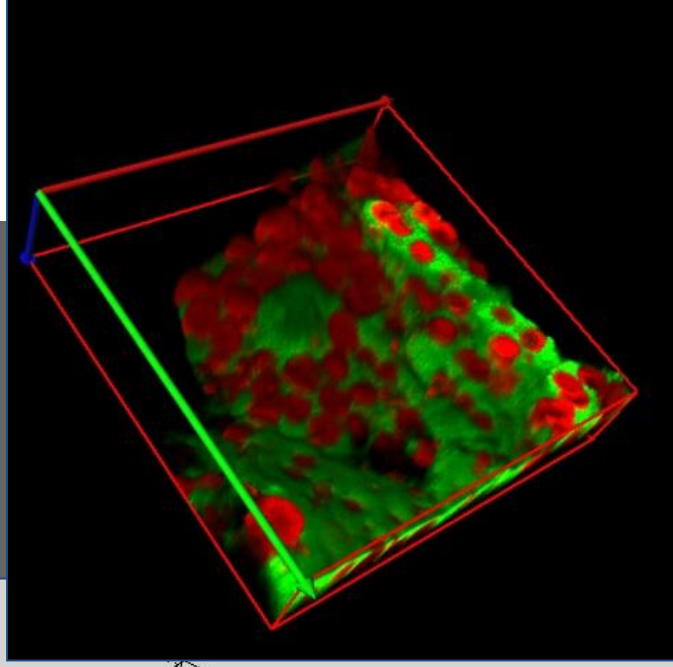
nuclei of mouse NIH 3T3, stained with Hoechst

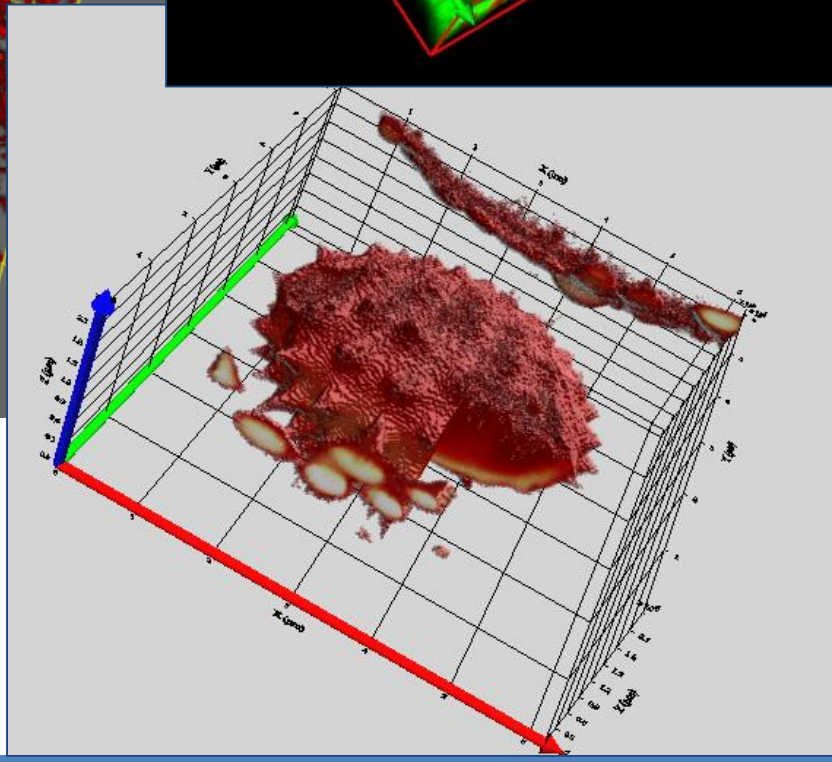
Separating cells from the background benefits from contrast; untangling overlaps adds to challenge.

3D reconstruction *registers* objects across “Z-stack”









Slide from Purdue University Cytometry Laboratories

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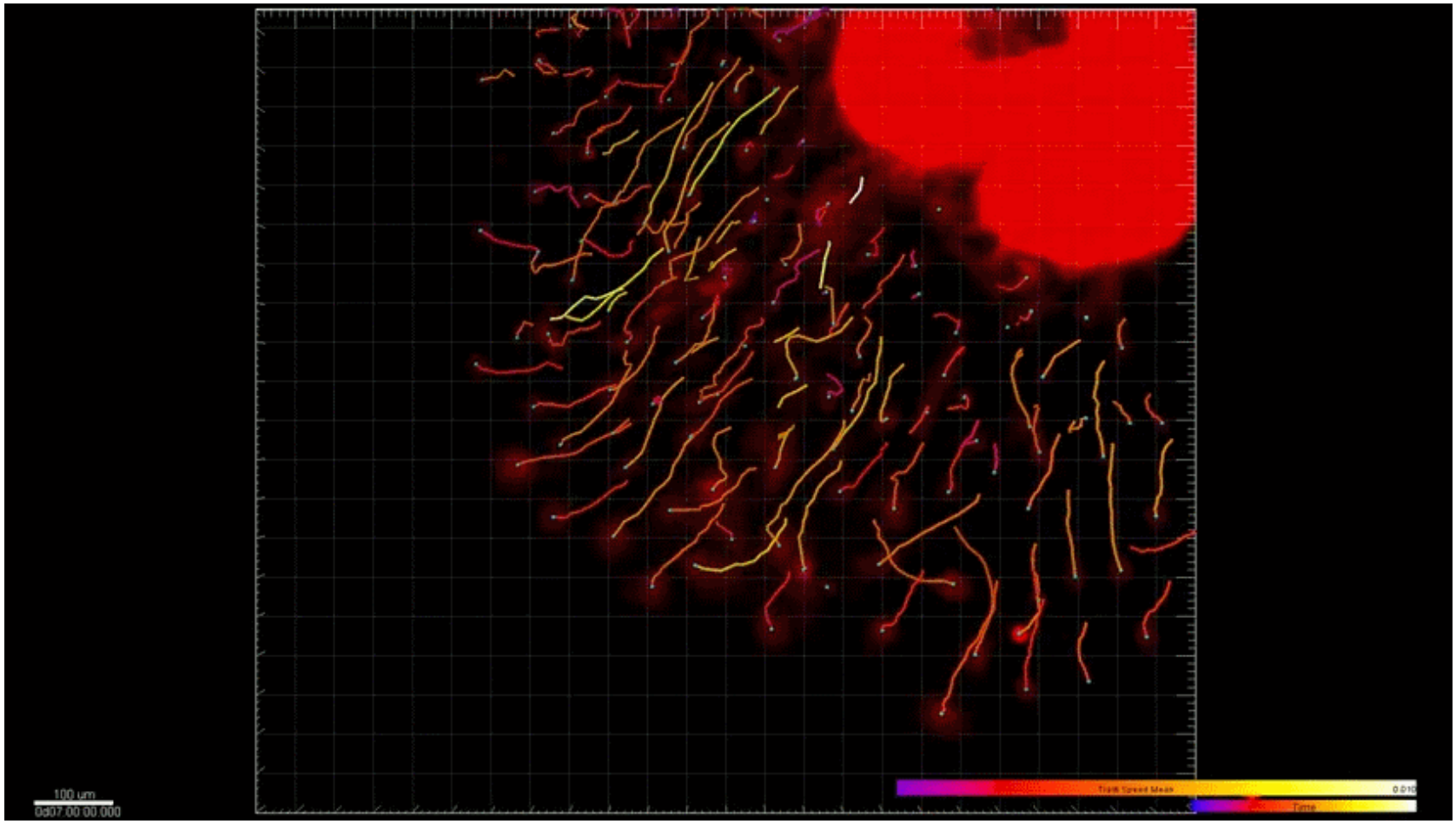
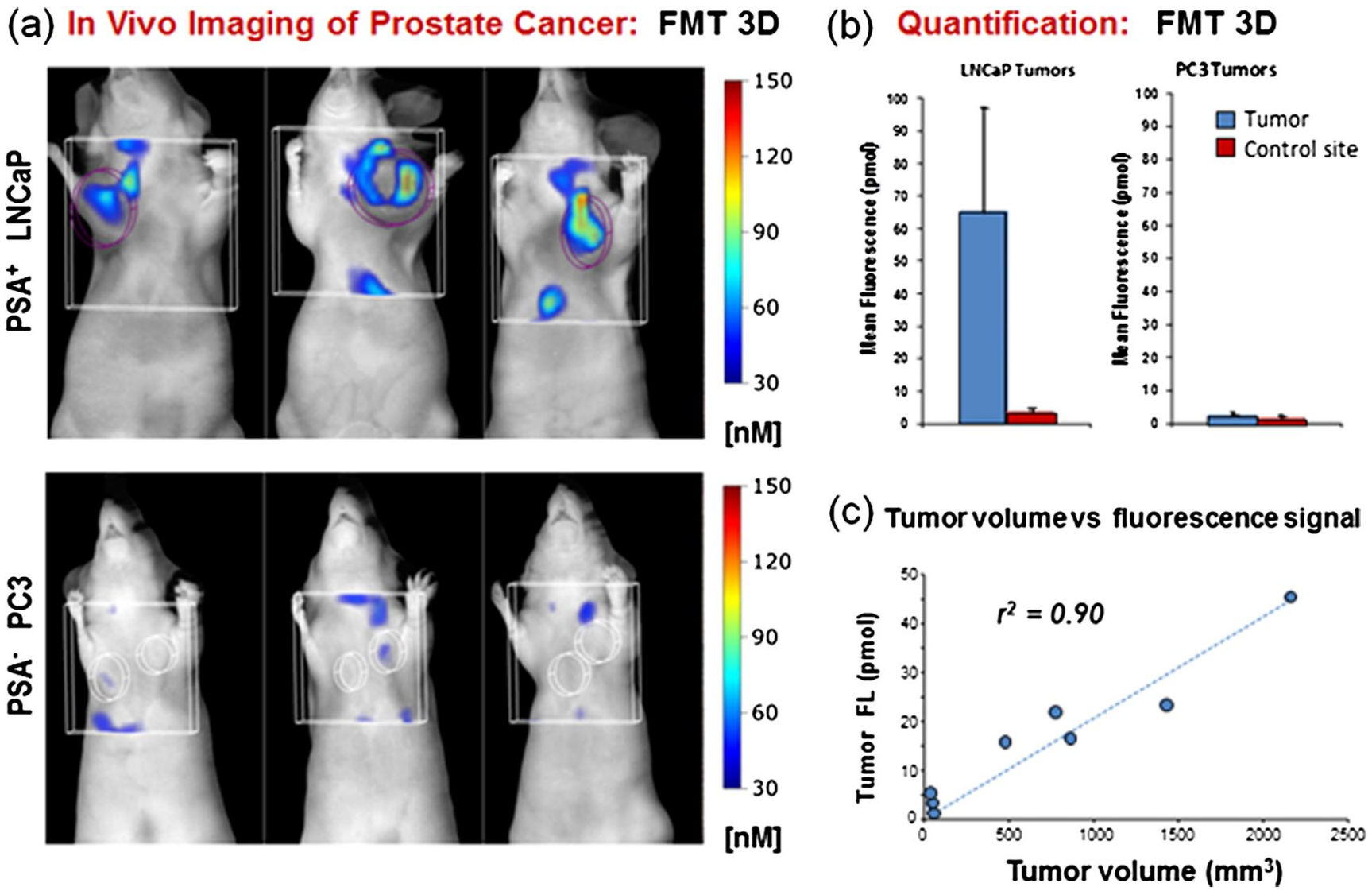
Motion Analysis

Live cell imaging of cells emerging from the embryonic quail heart



YouTube MSL: https://youtu.be/-U12539tHig

Quantitation



Ho et al. *J. Biomed. Optics*. (2013) 18: 101319

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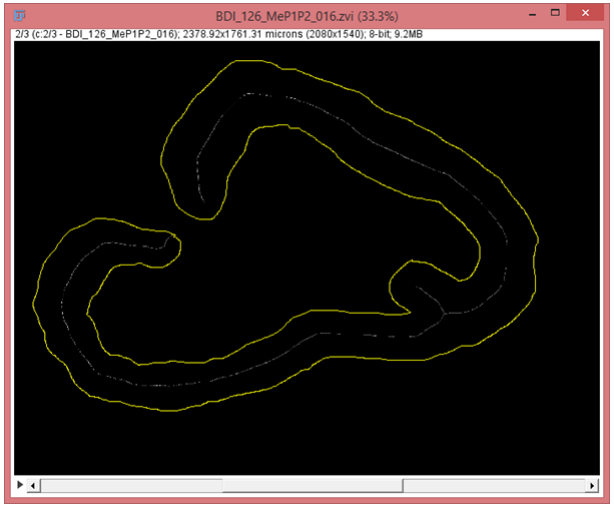
ImageJ

- ✓Extremely popular, free image analysis software
- ✓Written in Java for cross-platform compatibility
- ✓Wide variety of analysis plug-ins available



https://imagej.nih.gov/ij/docs/examples/ http://forum.imagej.net

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What can Flow Cytometry do?

- Enumerate particles in suspension
- Determine “biologicals” from “non-biologicals”
- Separate “live” from “dead” particles
- Evaluate 10^5 to $5x10^6$ particles in a min
- Measure particle-scatter as well as innate fluorescence or 2° fluorescence
- Sort single particles for subsequent analysis

Slide from Purdue University Cytometry Laboratories

The Coulter Principle

- Cells are relatively poor conductors.
- Blood is a suspension of cells in plasma, which is a relatively good conductor.
- Previously it was known that the cellular fraction of blood could be estimated from the conductance of blood.
- As the ratio of cells to plasma increases, the conductance of blood decreases.

Flow cytometer innards

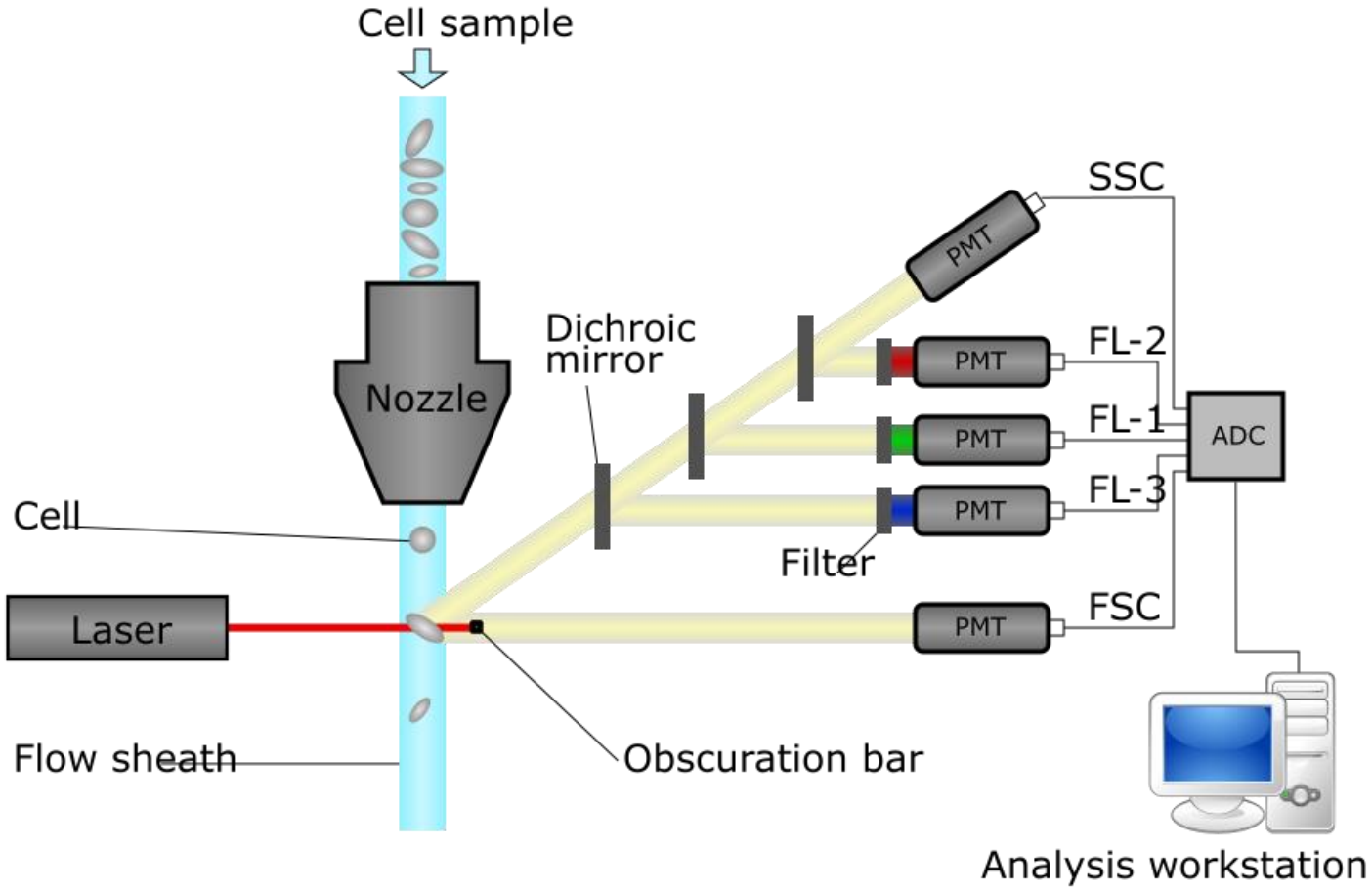

Diagram from WikiMedia by user Kierano

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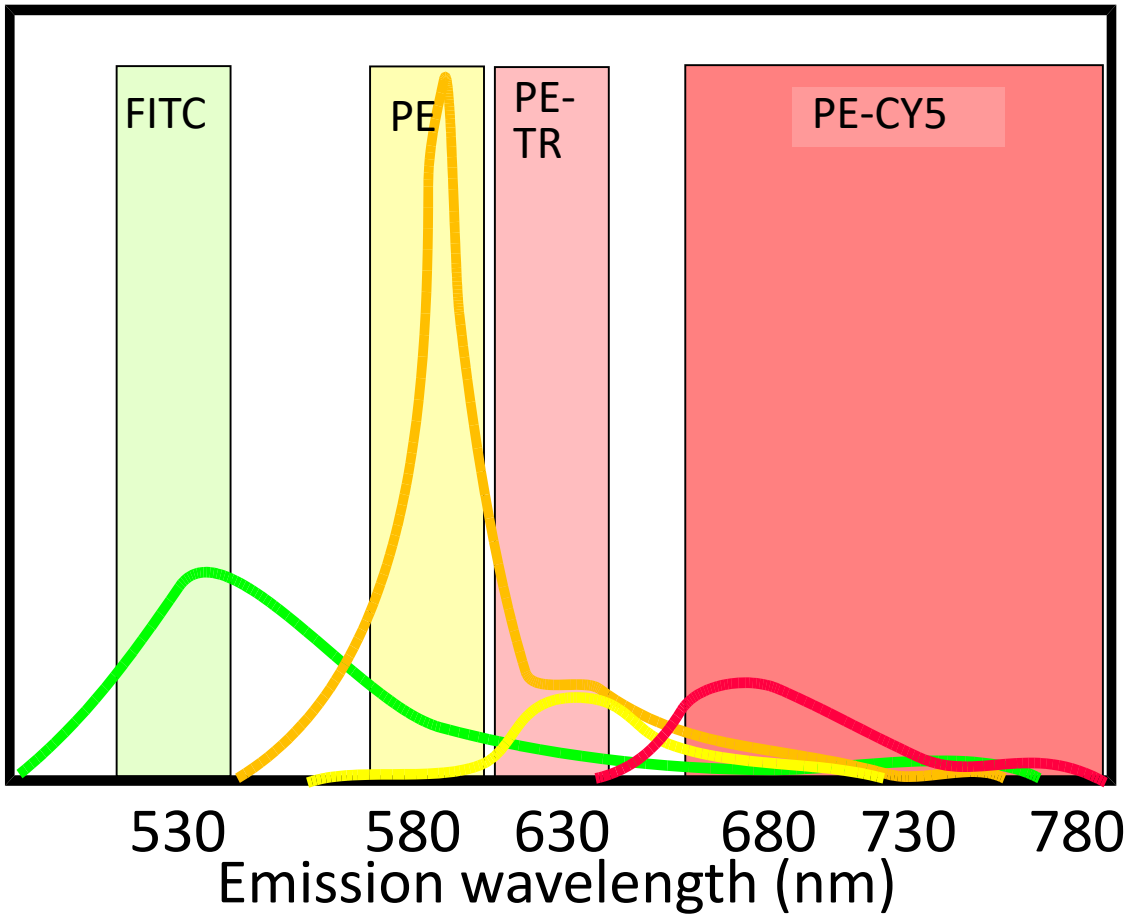
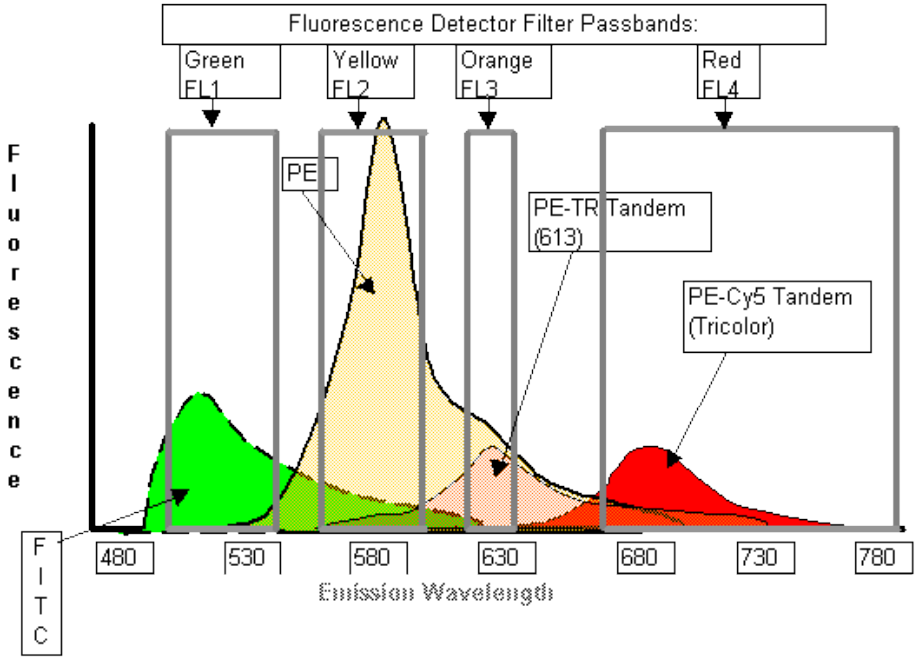
4 colors - simultaneous collection



Slide from Purdue University Cytometry Laboratories

Compensation

- The shoulder of one dye can add intensity to another channel.
- Compensation reduces the intensity in each channel by the amount overlapped from neighboring channels.



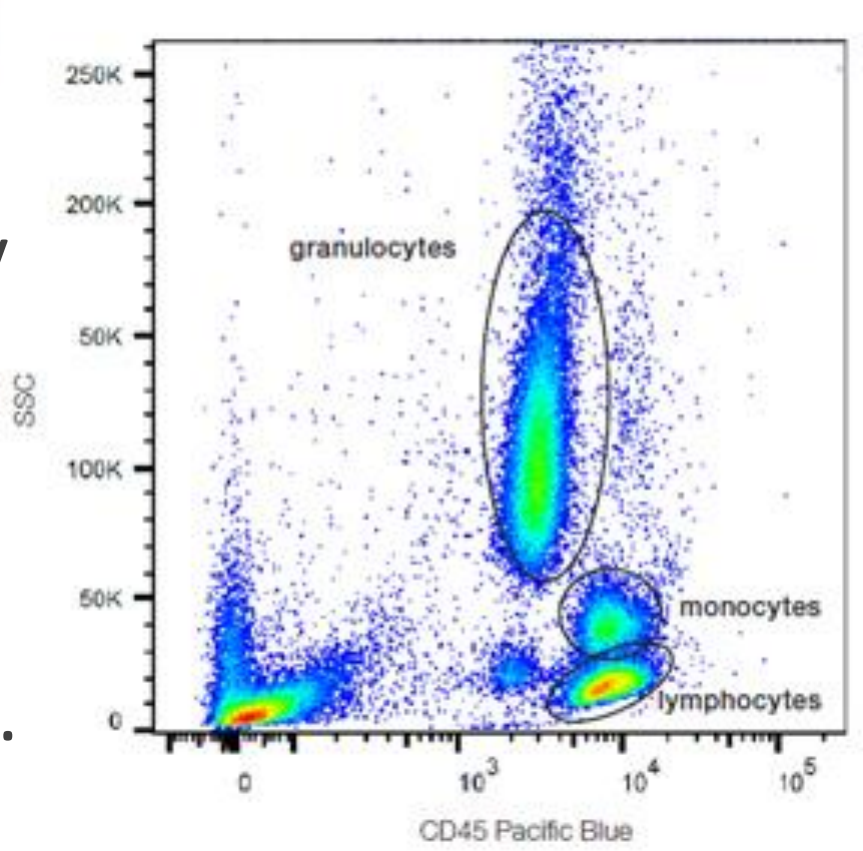
http://cybertronic.com/flow-cym.html

Real-Time vs. Software Gating

- Real-time or live gating: Only record to disk the "events" that meet a particular criterion.
- Software or analysis gating: In evaluating data from a completed flow experiment, consider only the "events" that meet a particular criterion.

Why do we gate?

Cells separate by fluorescent intensity in combos of dyes. By defining a region in a dye-vs-dye plot, we are specifying a sub-population of cells for retention or exclusion.



https://www.bio-rad-antibodies.com/flow-cytometry-gates-regions.html

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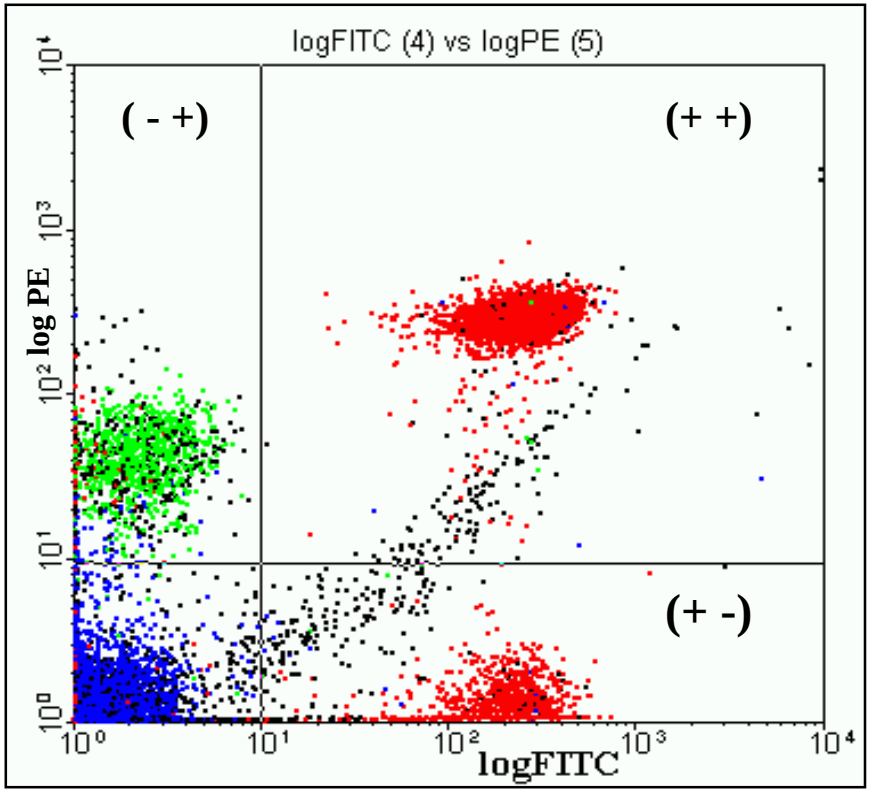
Quadrant Analysis



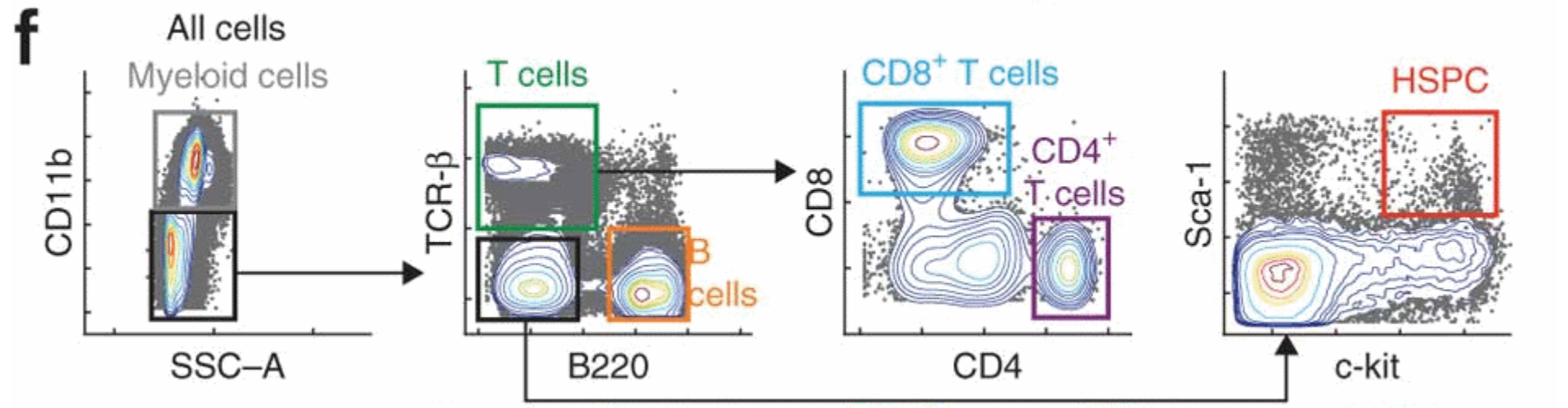

```
Multiple Document Interface for Flow Cytometry
WinMDI Version 2.4 - Windows 3.10/DOS 20.30
Thu May 01 09:22:31 1997
File: 70000048.LMD Sample ID:
Date: 18-Oct-91 Parameters: 7
Total Events 15973 Gated Events 15973  100.00%
System: DOS 4.0
Cytometer: Elite
Log Parameter Means: Geometric
logFITC(Log) vrs logPE(Log) Quad Stats
Location: x 256 y 248
```

Quad	X-mean	Y-mean	Events	% Gated
(1)-UL	2.1	38.2	1376	8.61
(2)-UR	246.4	251.3	2718	17.02
(3)-LL	1.3	1.2	9812	61.43
(4)-LR	170.5	1.3	2067	12.94

Slide from Purdue University Cytometry Laboratories

Sequential gates plus logic

Layering of sequential gates allows for extensive discrimination of cell populations.



Qiu et al. *Nat. Biotechnol*. (2011) 29: 886-891.

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Models at SUN FHMS

- BD LSR II: 12 channels
- BD FACS Canto II: 8 channels
- BD FACS Calibur (2): 4 channels
- BD FACS Count: 2 channels
- BD FACSJazz: 6 channels

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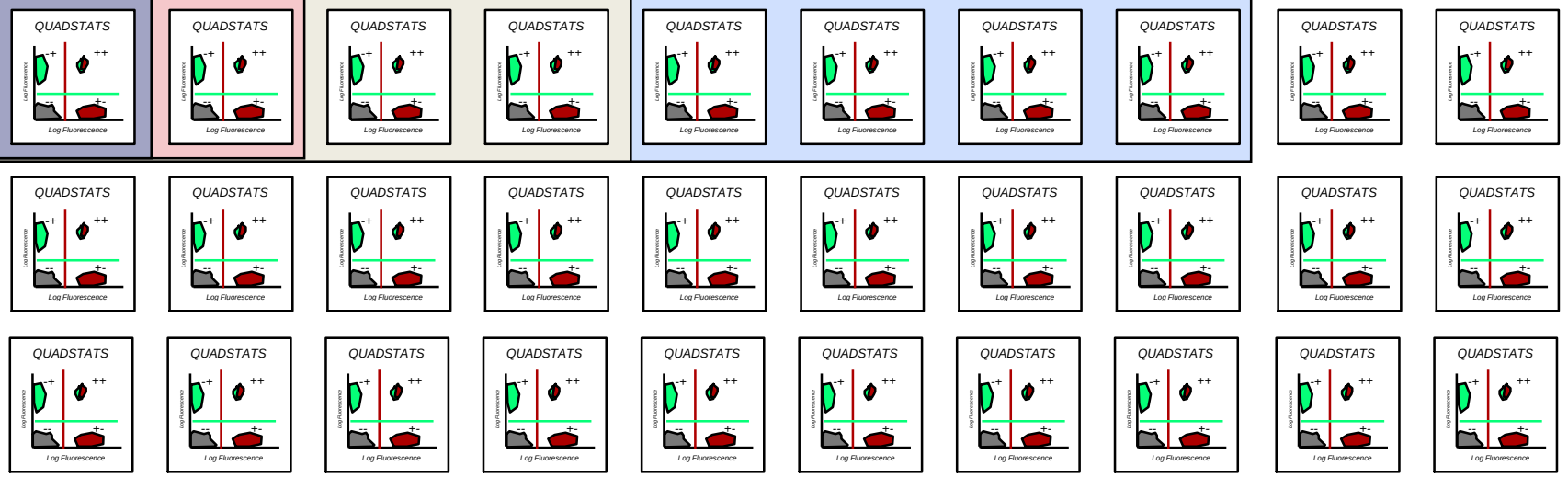
Multi-color studies generate a lot of data

3 color

4 color

5 color

1 2 3 4 5 6 7 8 9 10



Slide from Purdue University Cytometry Laboratories

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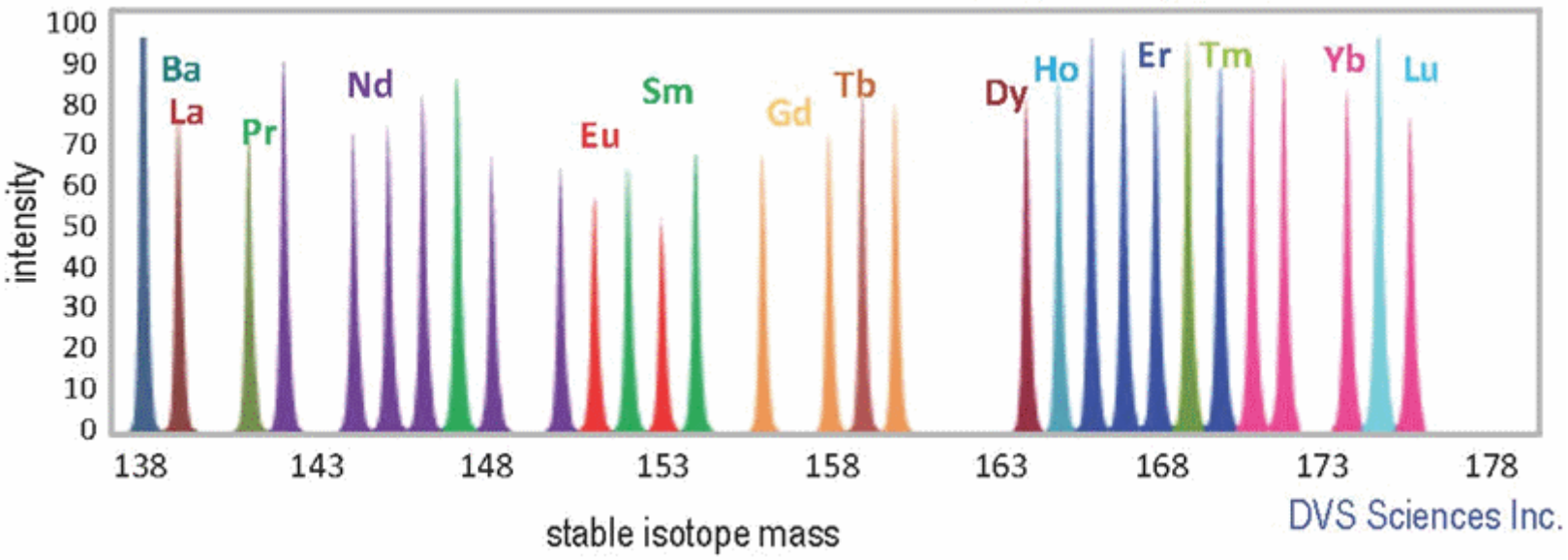
CyTOF measures up to 30 markers via mass spectrometry

Computer-assisted gating is particularly necessary as the number of markers in an experiment rises. Decisions may combine markers through PCA.



DVS Sciences (now part of Fluidigm)

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FCS: file format for cytometry

- Created by Intl. Society for Advancement of Cytometry (ISAC) Data Standards Task Force
- v1.0: 1984 v2.0: 1990 v3.0: 1997.
- v3.1 update added internationalization, compensation features, display defaults, sample metadata, and data provenance.

J Spidlen et al. *Cytometry A*. (2010) 77: 97-100.

Conclusions

- Key imaging concepts:
  - Contrast and Resolution
  - Segmentation
  - Registration
- Key flow cytometry concepts:
  - Compensation
  - Gating
  - FCS files