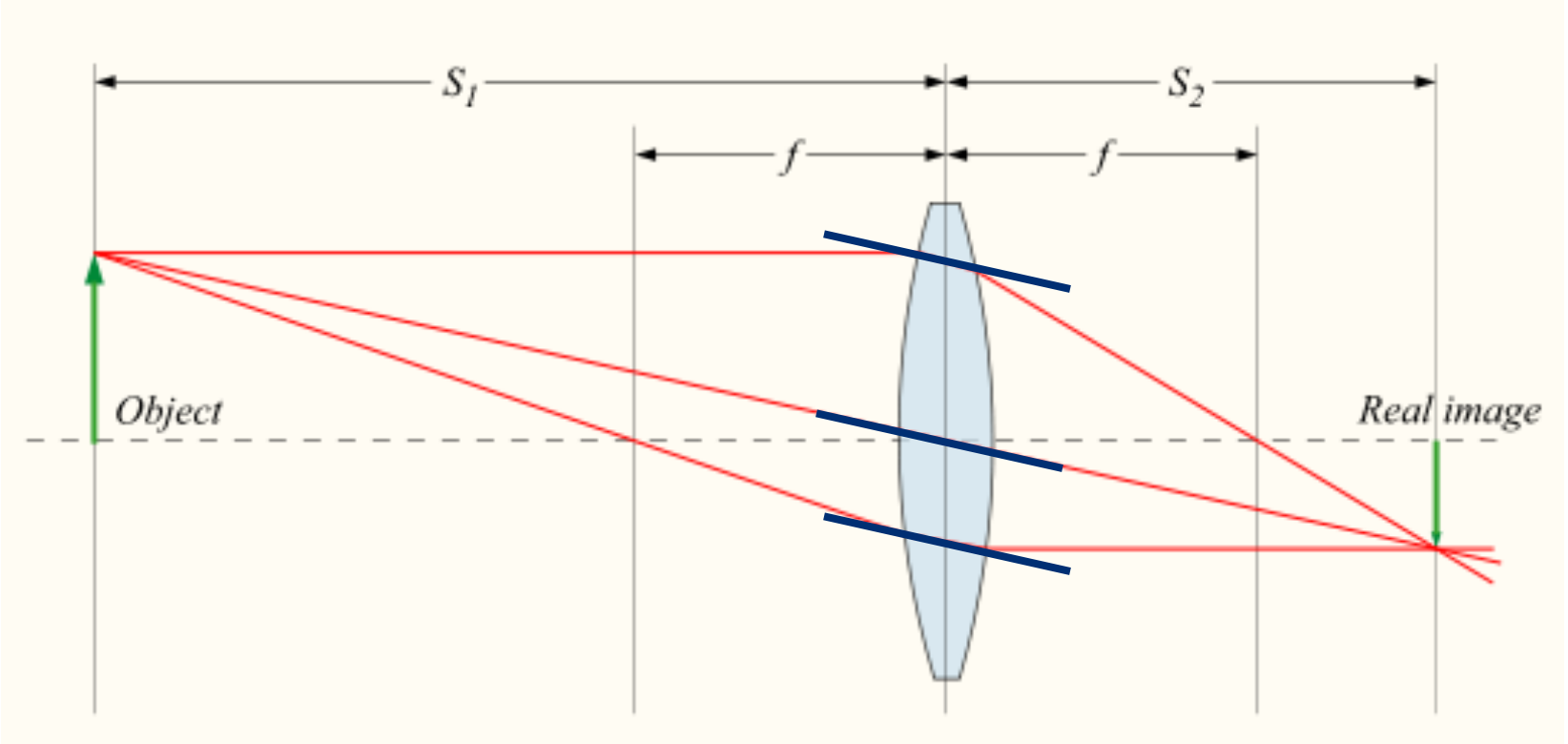
Quantitative Fluorescence Microscopy 2014

Microscope Camp

"Every Photon is Sacred"

The Microscope is a Combination of Lenses



Not All Objective Lenses Are Created Equal

Figure 2

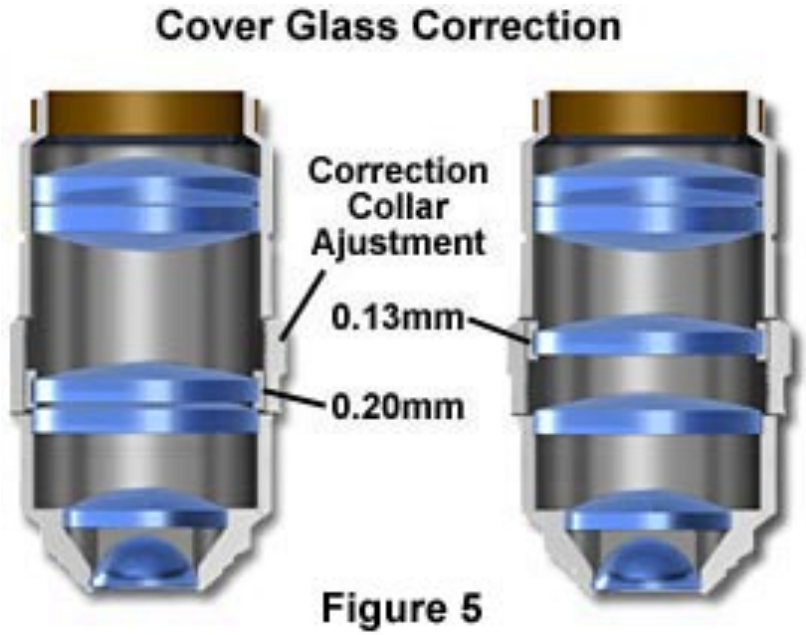


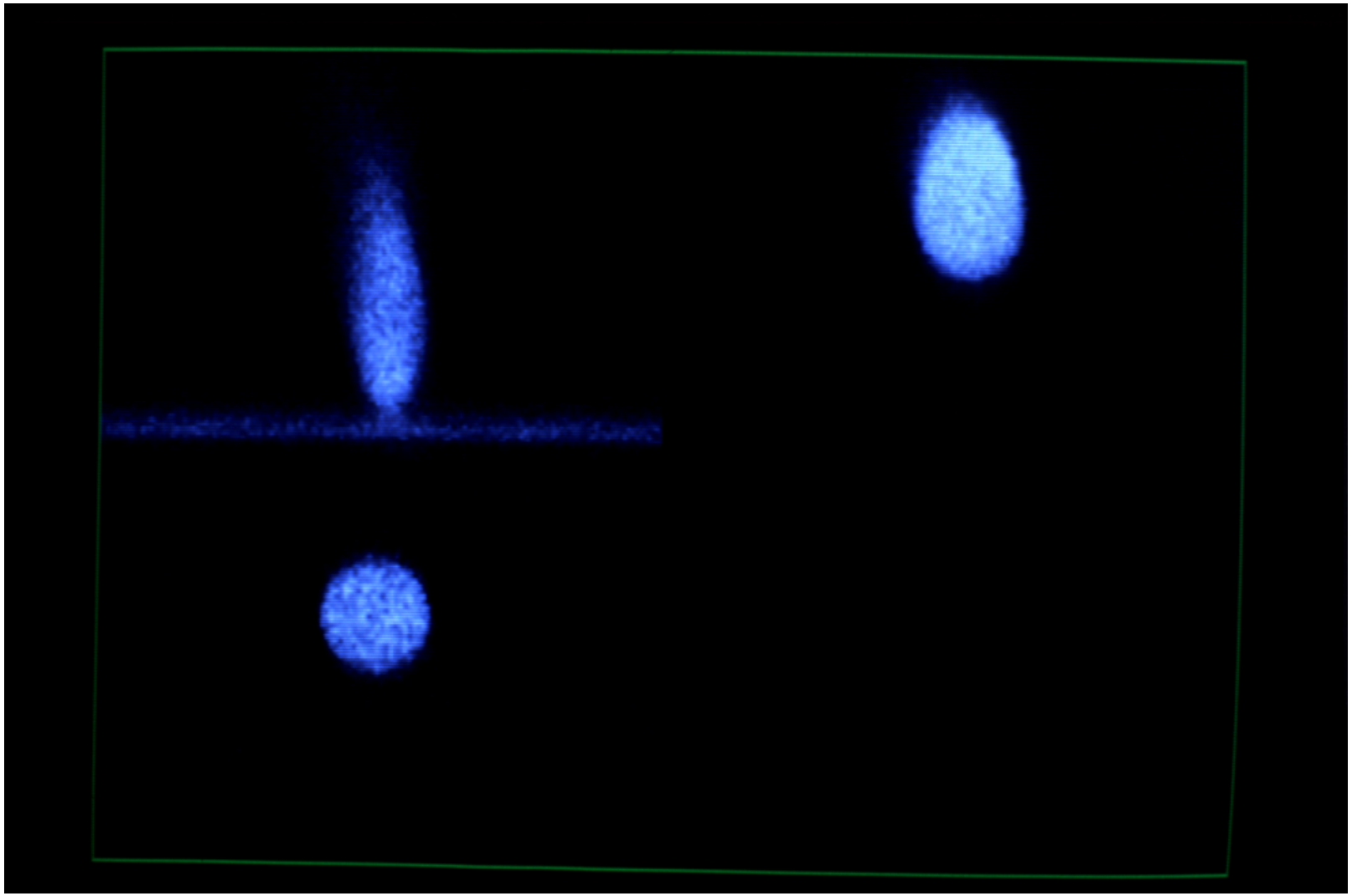
Figure 5

Corrections:

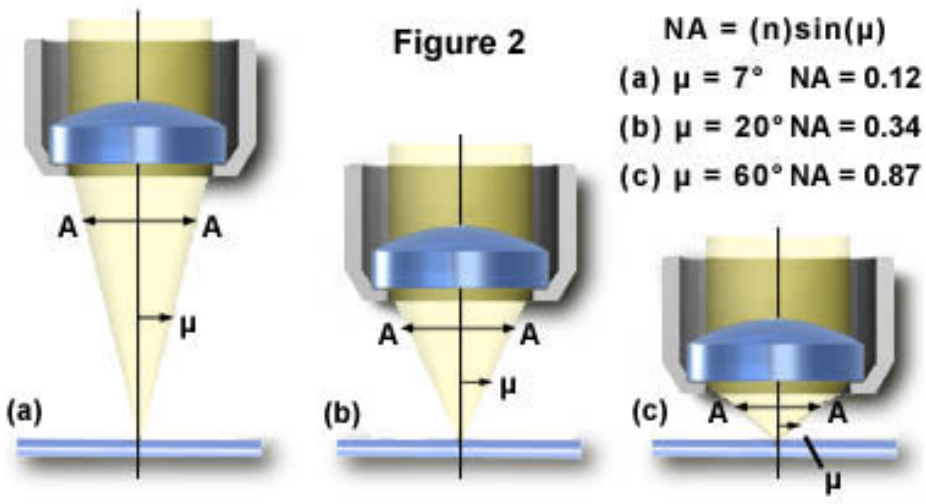
- Color - Achromat, Apochromat
- Flat Field - Plan
- Immersion Media
- Cover Glass
- Polarization
- UV, IR transmission

The more correction that a lens uses, the less transmission

Index mismatch



Resolution is Dictated by Numerical Aperture



Objective Numerical Apertures

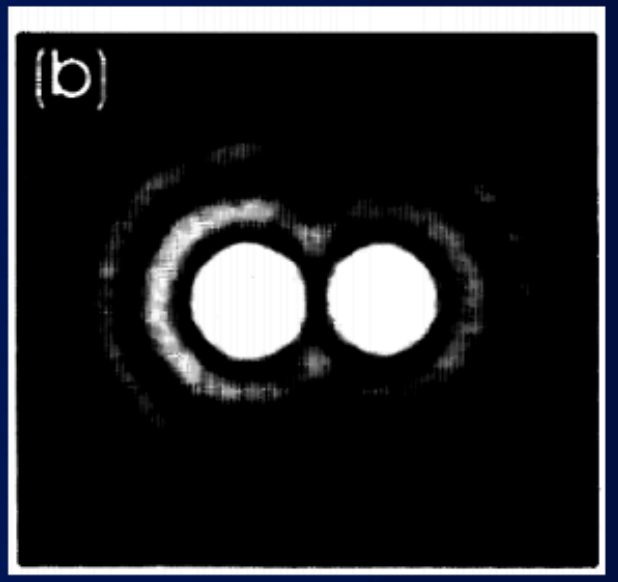
Magnification	Plan Achromat (NA)	Plan Fluorite (NA)	Plan Apochromat (NA)
0.5x	0.025	n/a	n/a
1x	0.04	n/a	n/a
2x	0.06	n/a	0.10
4x	0.10	0.13	0.20
10x	0.25	0.30	0.45
20x	0.40	0.50	0.75
40x	0.65	0.75	0.95
40x (oil)	n/a	1.30	1.00
60x	0.75	0.85	0.95
60x (oil)	n/a	n/a	1.40
100x (oil)	1.25	1.30	1.40
150x	n/a	n/a	0.90

Table 1

The smaller the NA, the bigger the focal spot, and the less resolution obtained – $R \sim \lambda/NA$

Nyquist Theorum

- How many times(*frequency)* must a sample be measured to be sure of the measurement?
  - Temporal and spatial frequency sampling may be considered the same
  - In confocal we deal with spatial frequency
- This is IMPORTANT.
  - More than 2.3 samples resel is a waste and is oversampling
  - less than 2.3 misses useful information



1 sample/resel no separation

2.3 samples/resel

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Resolution

$$d = \frac{0.61\lambda}{NA}$$

Therefore with 488 nm light, and a 100X 1.4 NA oil immersion optic

D = 0.61X488/1.4
= 212 nm

Mag		resolution
10X	0.3NA	1.1
20X	0.5NA	0.7
60X	1.4NA oil	0.2
100X	1.4NA oil	0.2

Even with short wavelength light you cannot exceed 0.2 microns resolution using the best optics available today

IMPORTANT

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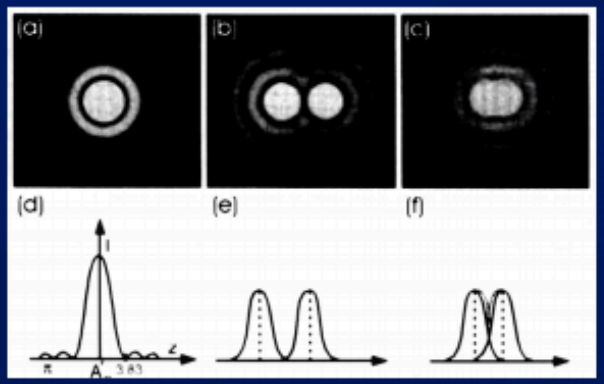
Limits of confocal: Axial resolution



Lateral resolution = 0.61λ/NA

Diffraction limited spot also occurs in the axial or Z dimension.

$Z_{min} = 2\lambda n/(NA)^2$

n = refractive index
NA = numeric aperture
Z_{min} = minimum Z resolution

60X, 1.43NA, λ = 488nm

Lateral resolution = 208 nm
Axial resolution = 477 nm

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Köhler Illumination is Absolutely Required for Good Transmitted Light Contrast.

There are two sets of conjugate Optical planes in the microscope:

1. Aperture or Illumination Plane

2. Focus (Object), Image Plane

These two are Fourier transforms of each other -- This means that they are related in specific ways.

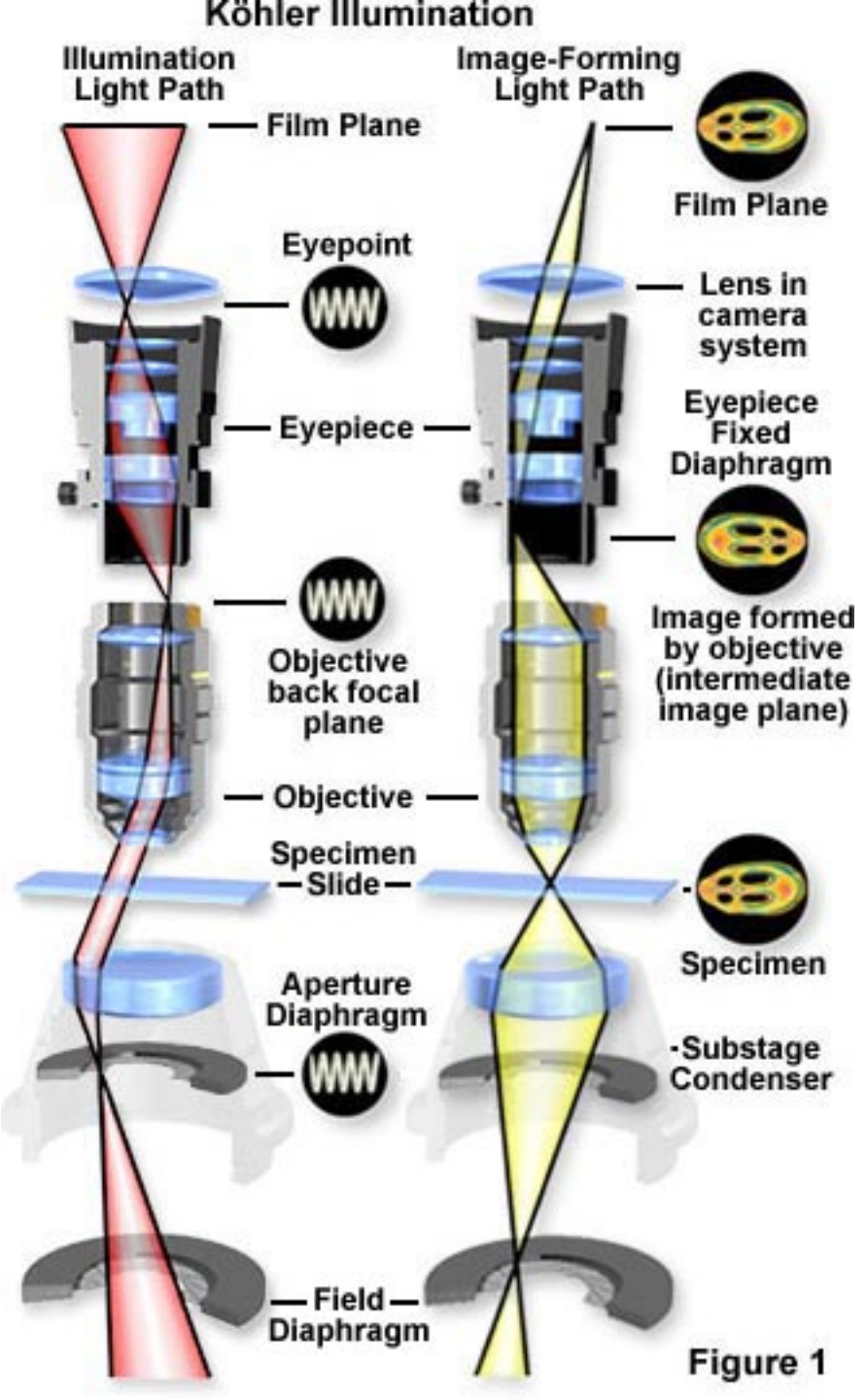
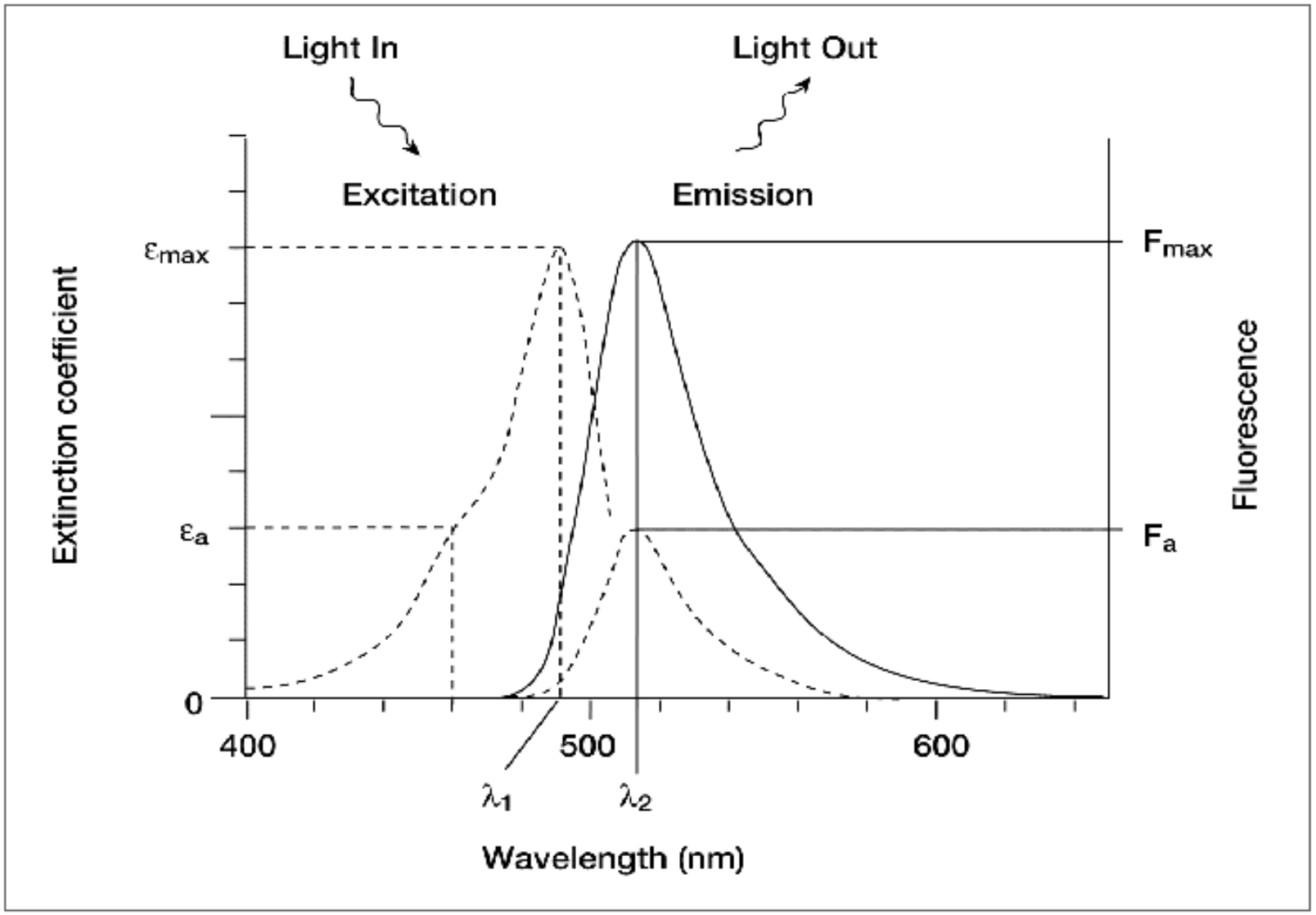
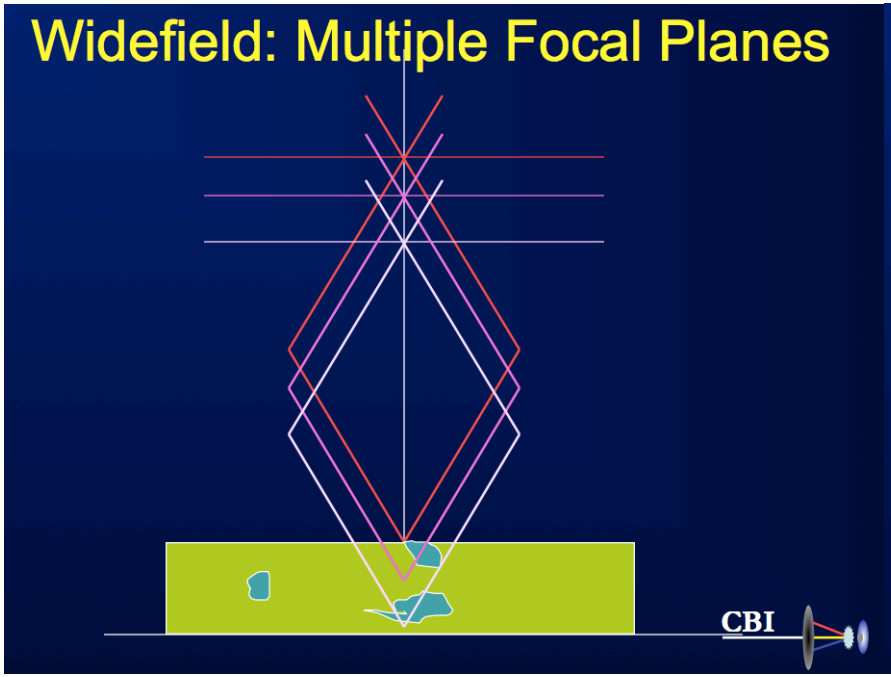


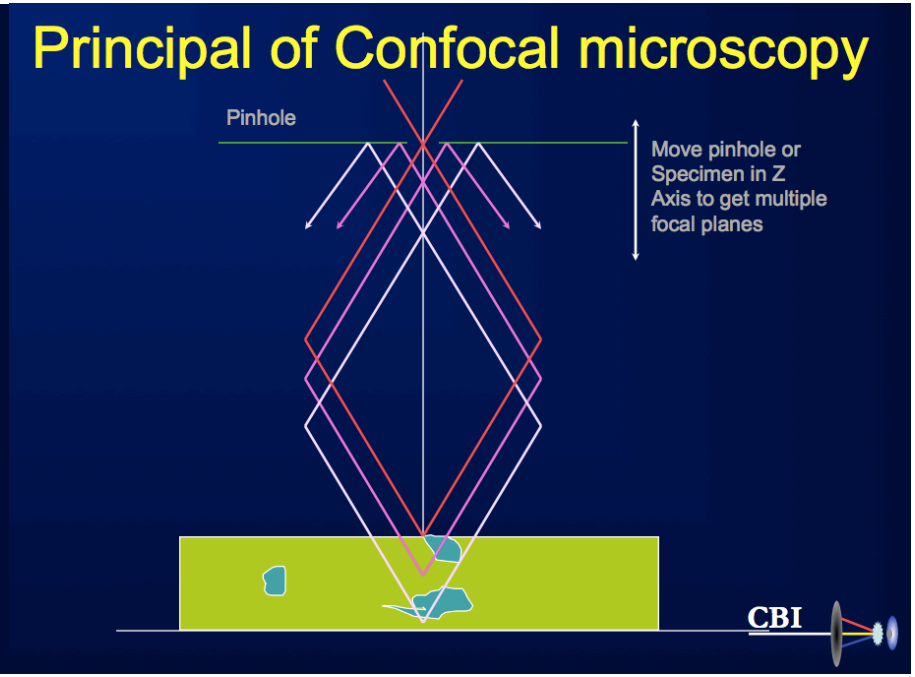
Figure 1

Spectral Features of Fluorescent Molecules

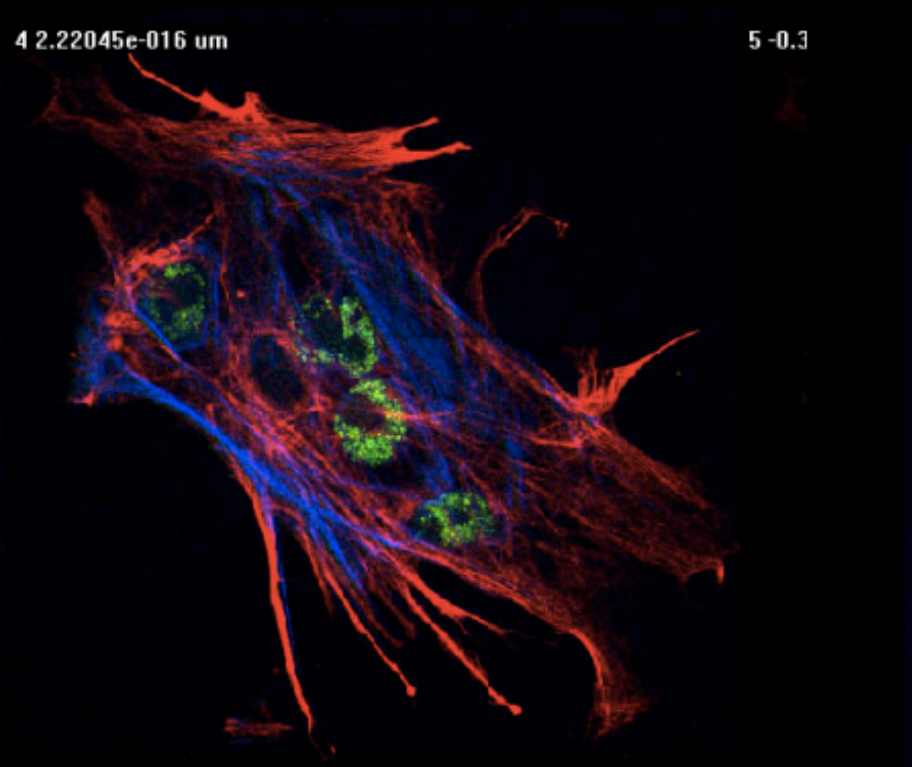


Confocal vs Widefield

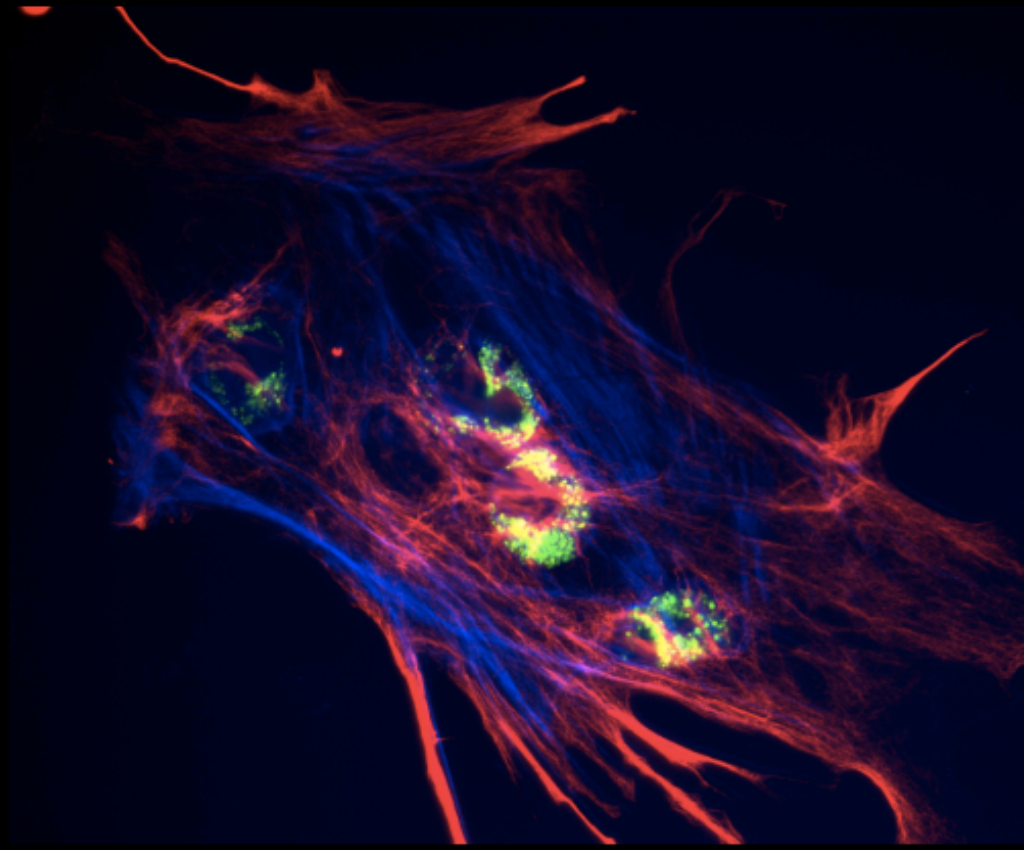




Confocal *vs* widefield



T=10 sec min!
Will bleach rapidly



T=0.3 secs
Bleaches much more slowly

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Confocal Microscopy: Primary Uses

- Optical sectioning with high resolution Z sections of known thickness
- 3 Dimensional reconstruction and measurement
- Computer aided densitometry
- Fluorescence Recovery After Photo-bleaching (FRAP)
- Photo-activation

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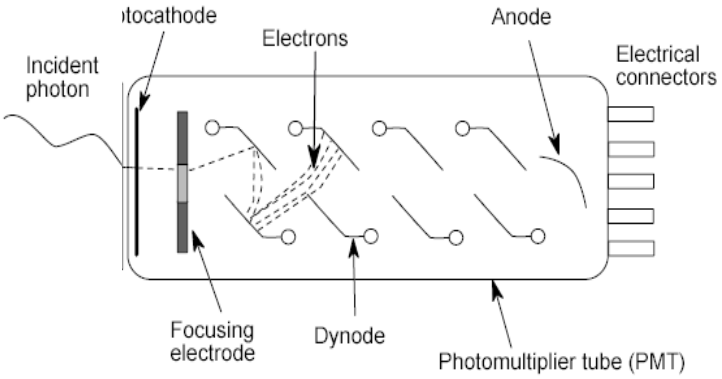
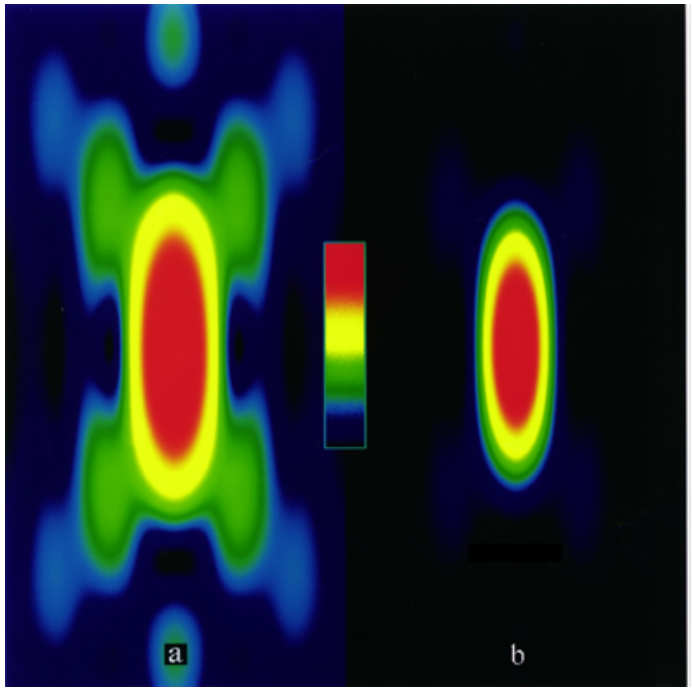
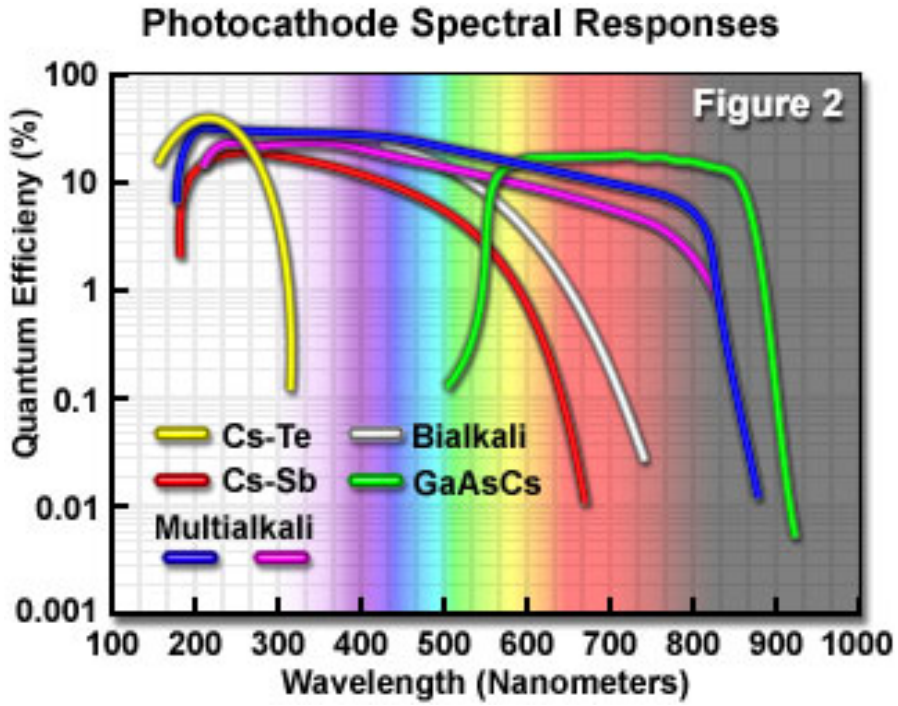
Photomultiplier Tube (PMT)



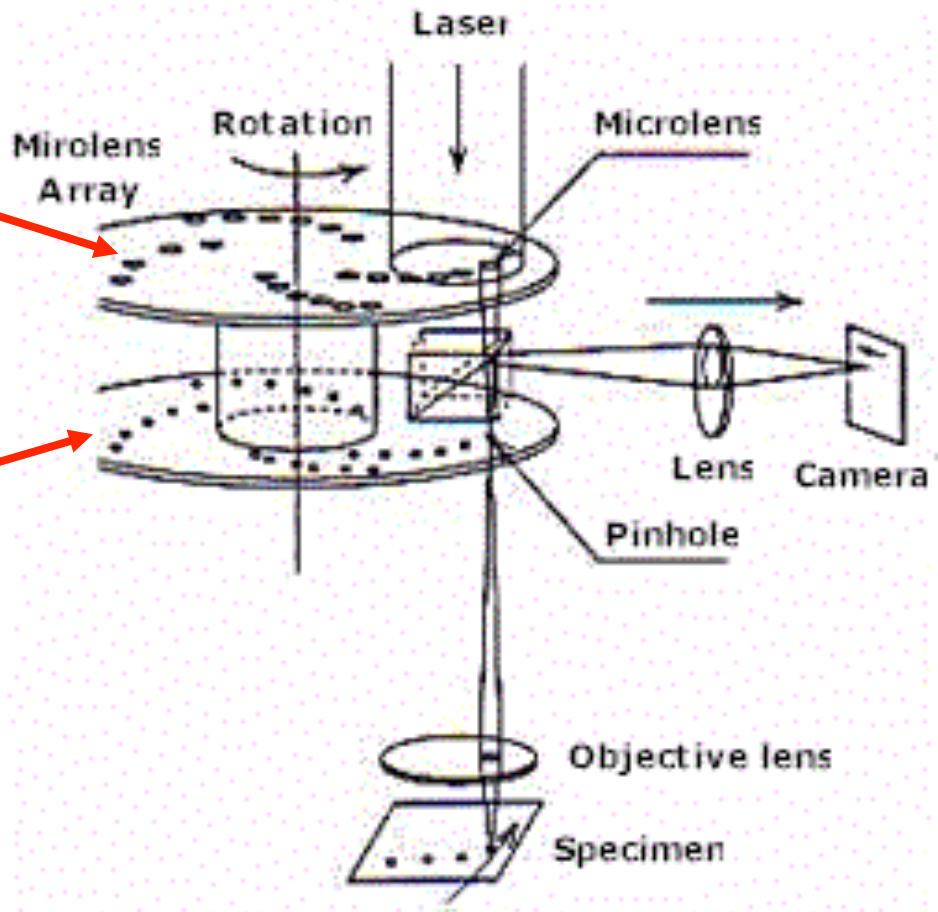




Spinning Disk with Microlens Array

Lens array directs >50% of the excitation light through the Nipkow Disk pinholes

Only 1% of Nipkow disk is holes, this leads to low excitation rates



But, faster acquisition means faster photobleaching!

MICROSCOPE CAMP
QFM 2014

Super Resolution

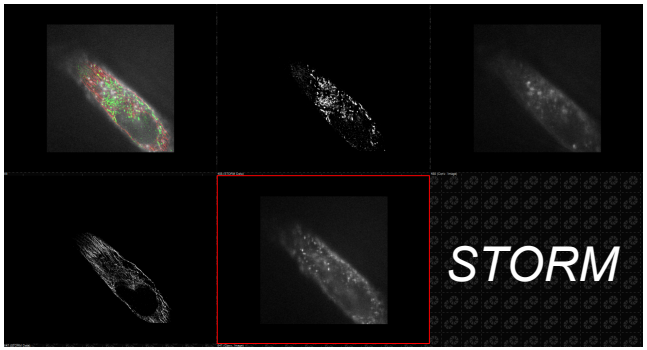


Image Processing

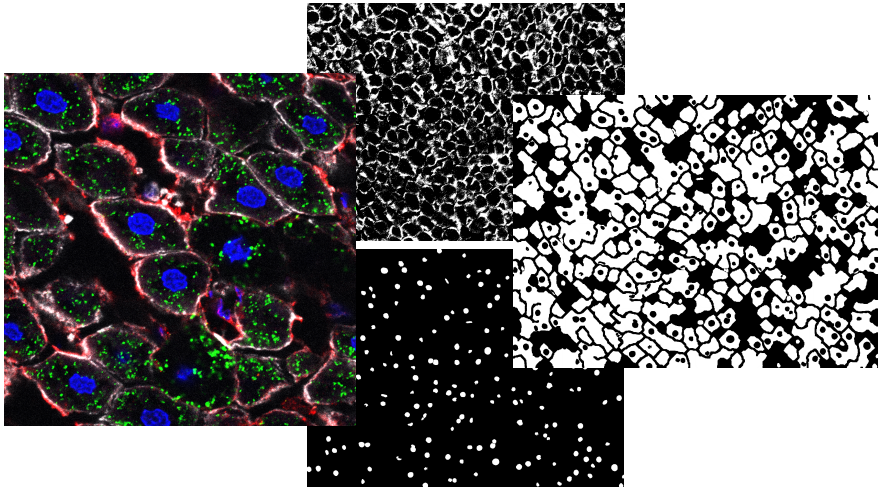
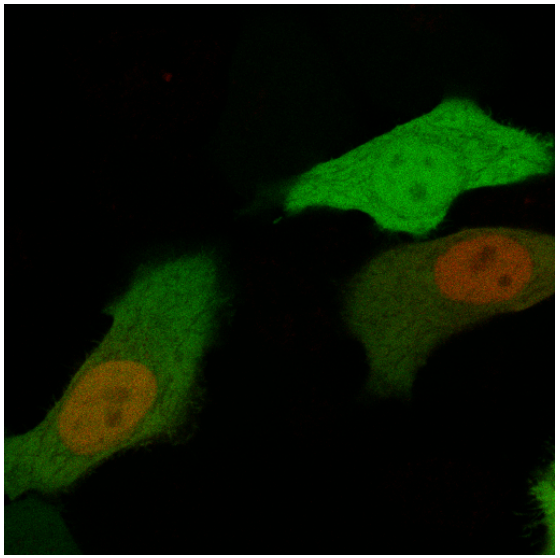
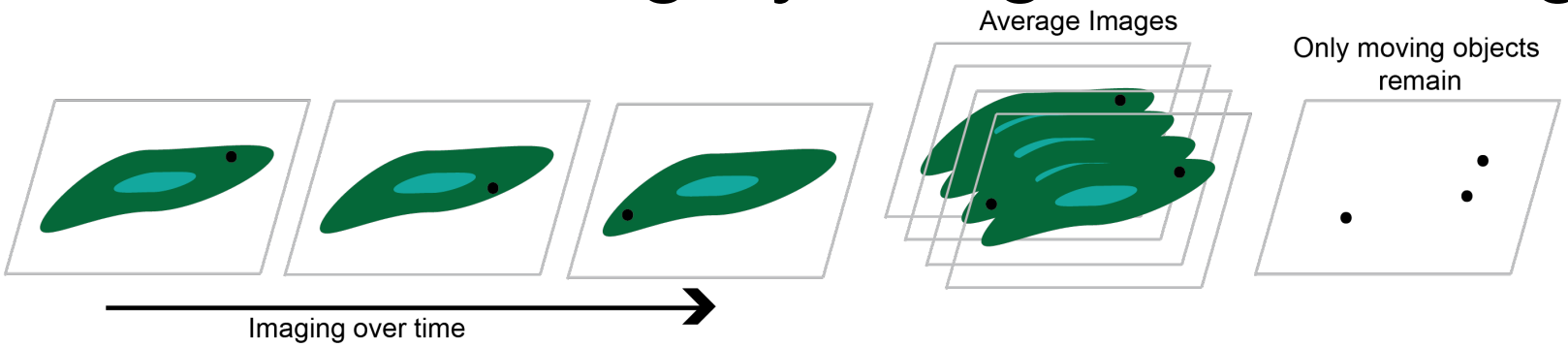
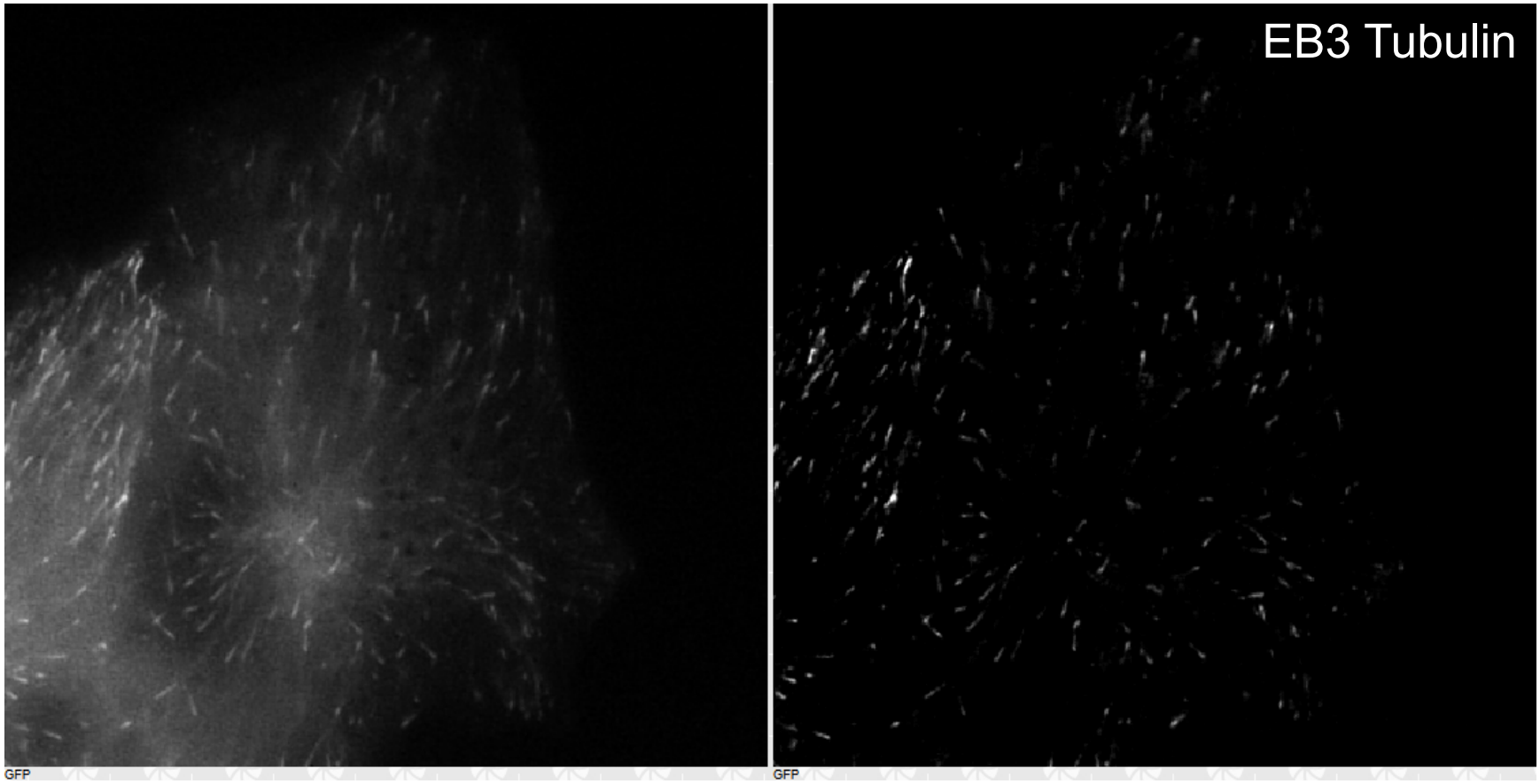


Photo bleaching/activation



Feature Extracting by Image Processing





Selective Photo bleaching by binary selection

QFM Class Information

- http://www.mdibl.org/courses/Quantitative_Fluorescence_Microscopy/159/