

Intelligent Analysis of Biomedical Images

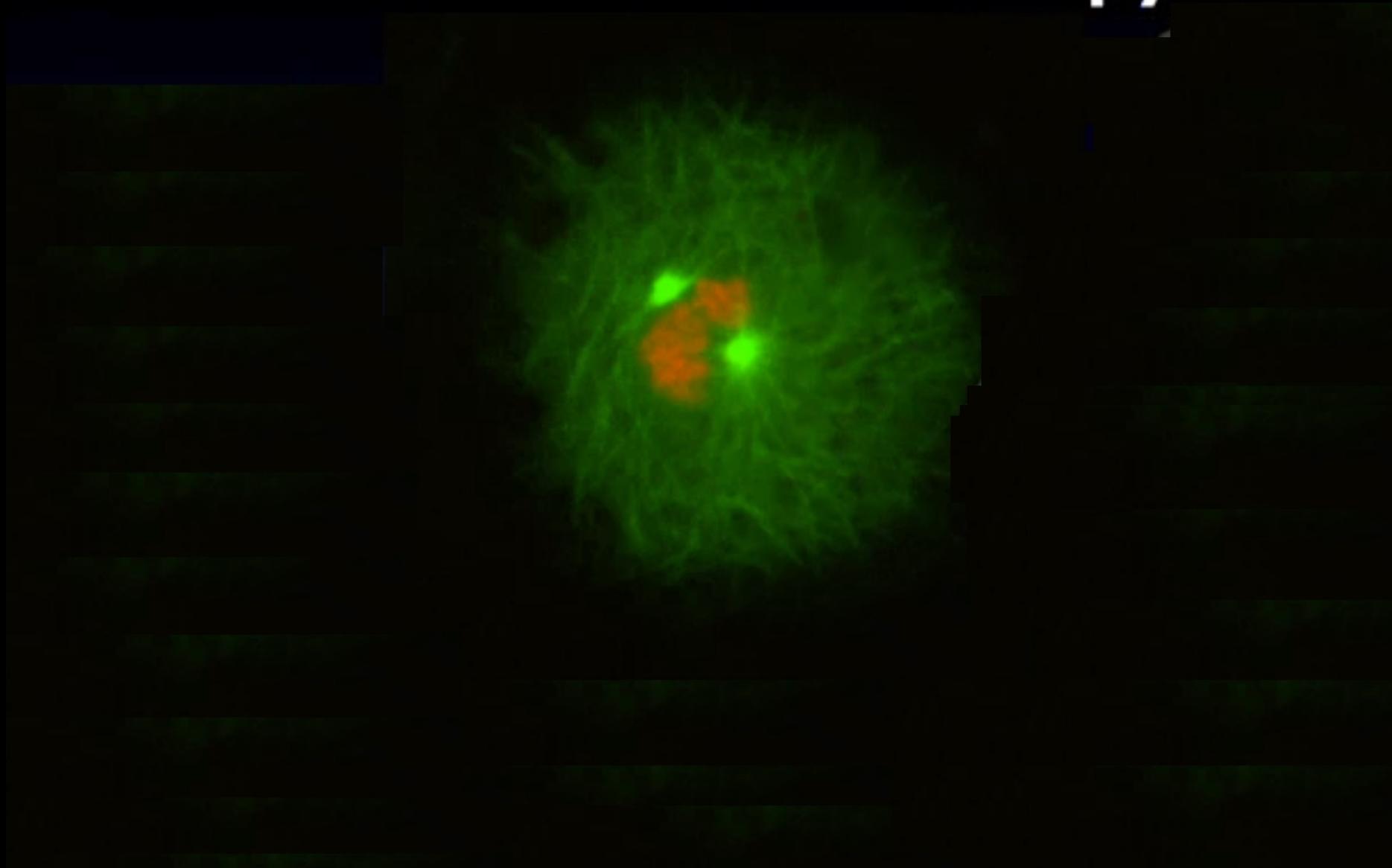
Presenter: Mohammad H. Rohban, Ph.D.

Fall 2023

Courtesy: Some slides are adopted from the videos in iBiology

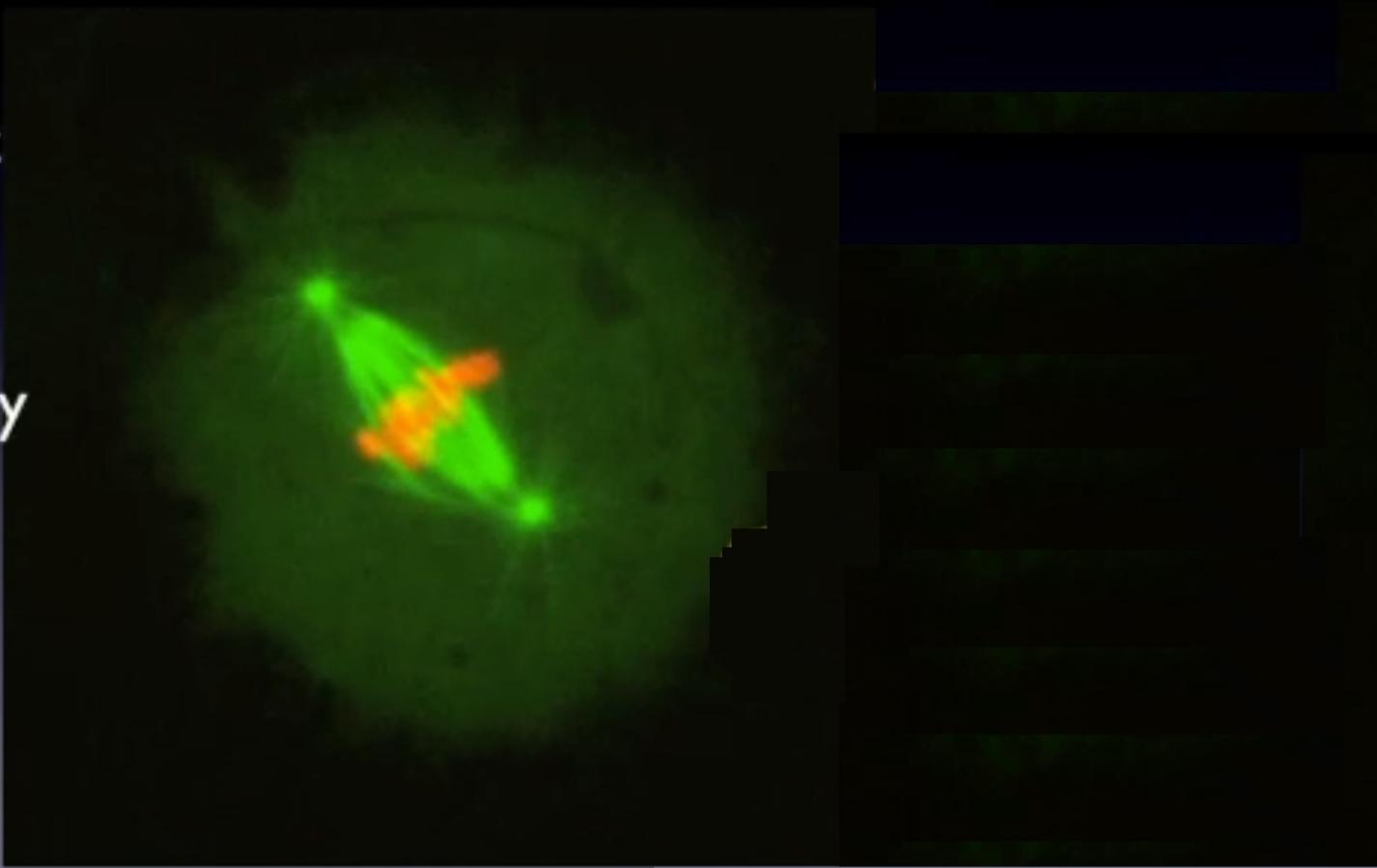
Introduction to Fluorescence Microscopy

Introduction to Fluorescence Microscopy



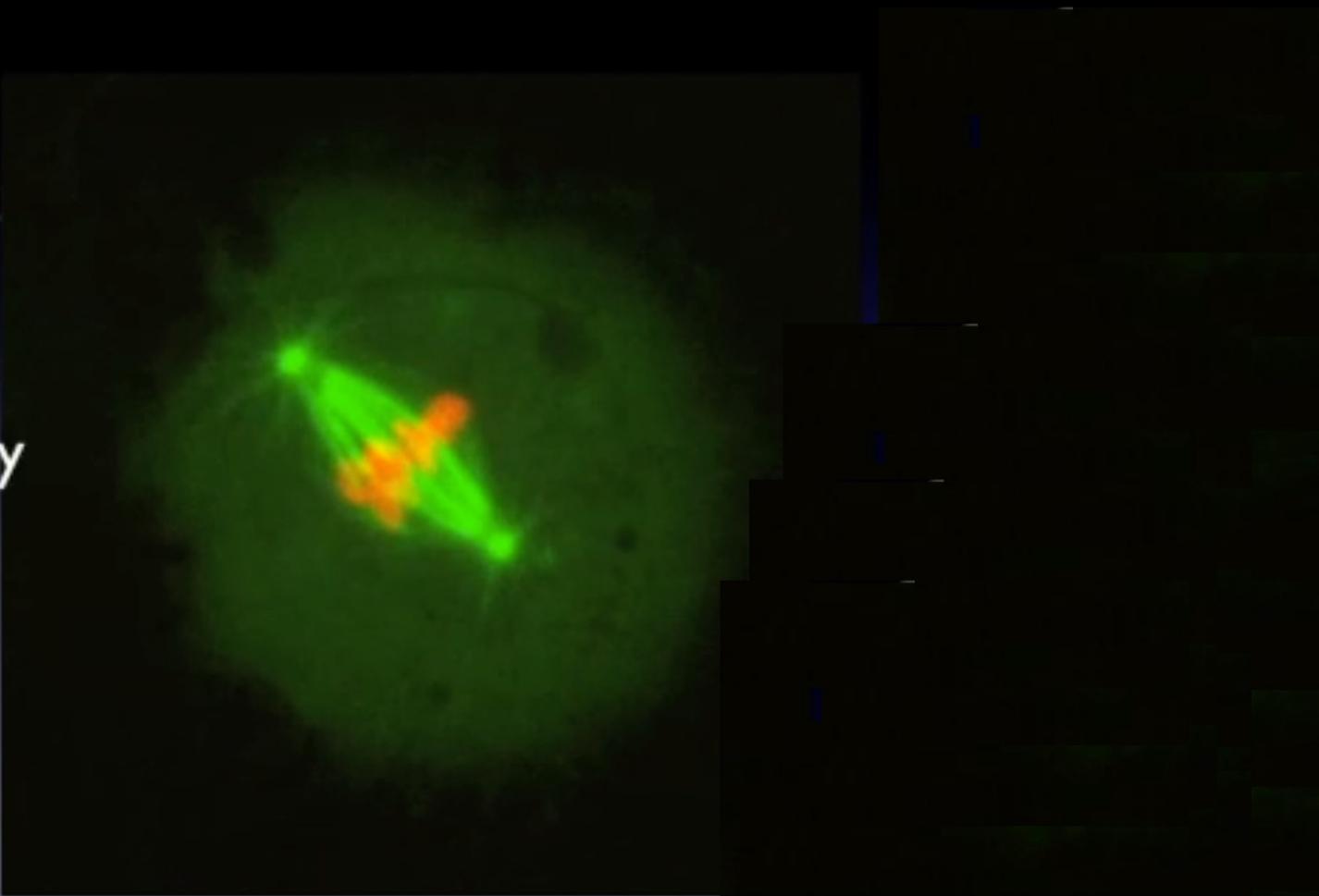
Why Fluorescence?

- High Contrast
- High Specificity
- Quantitative
- Live Cell Imaging



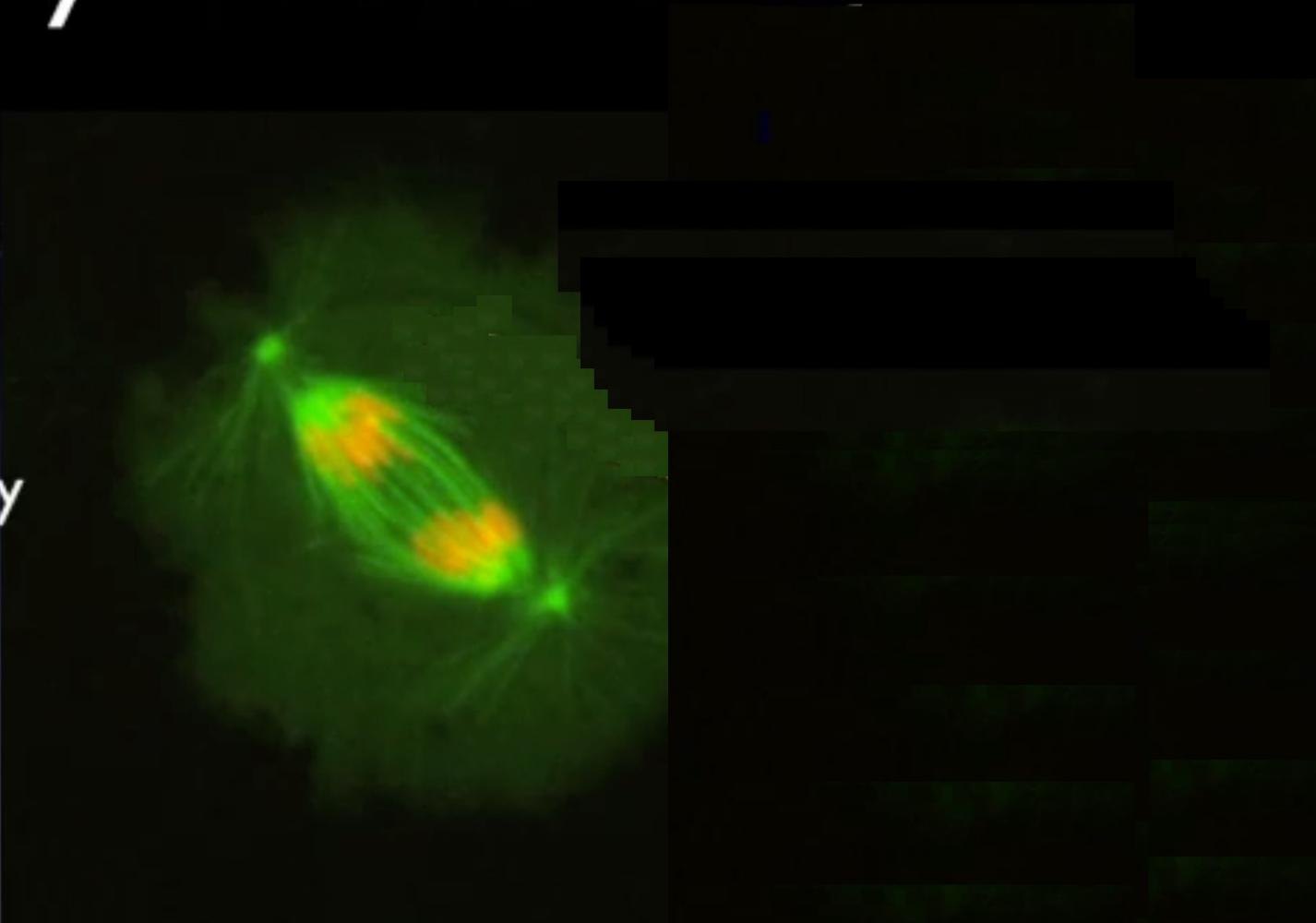
Why Fluorescence?

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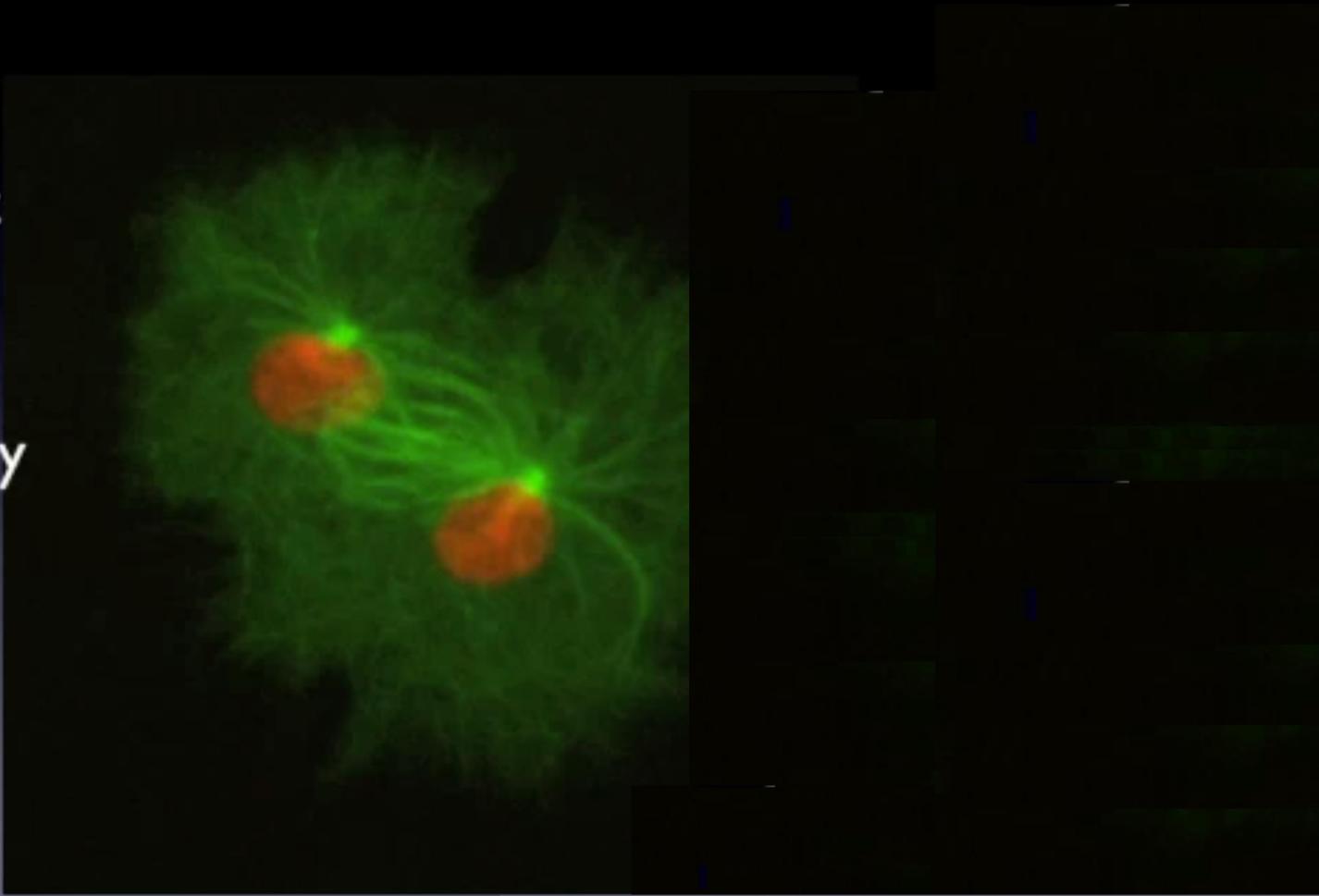
Why Fluorescence?

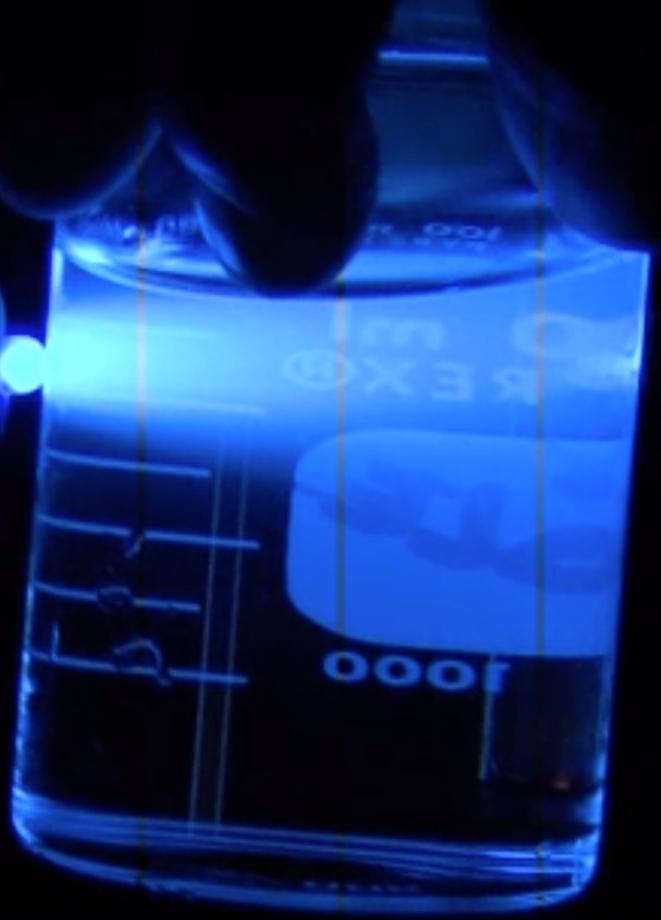
- High Contrast
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Why Fluorescence?

- High Contrast
- High Specificity
- Quantitative
- Live Cell Imaging





Quinine

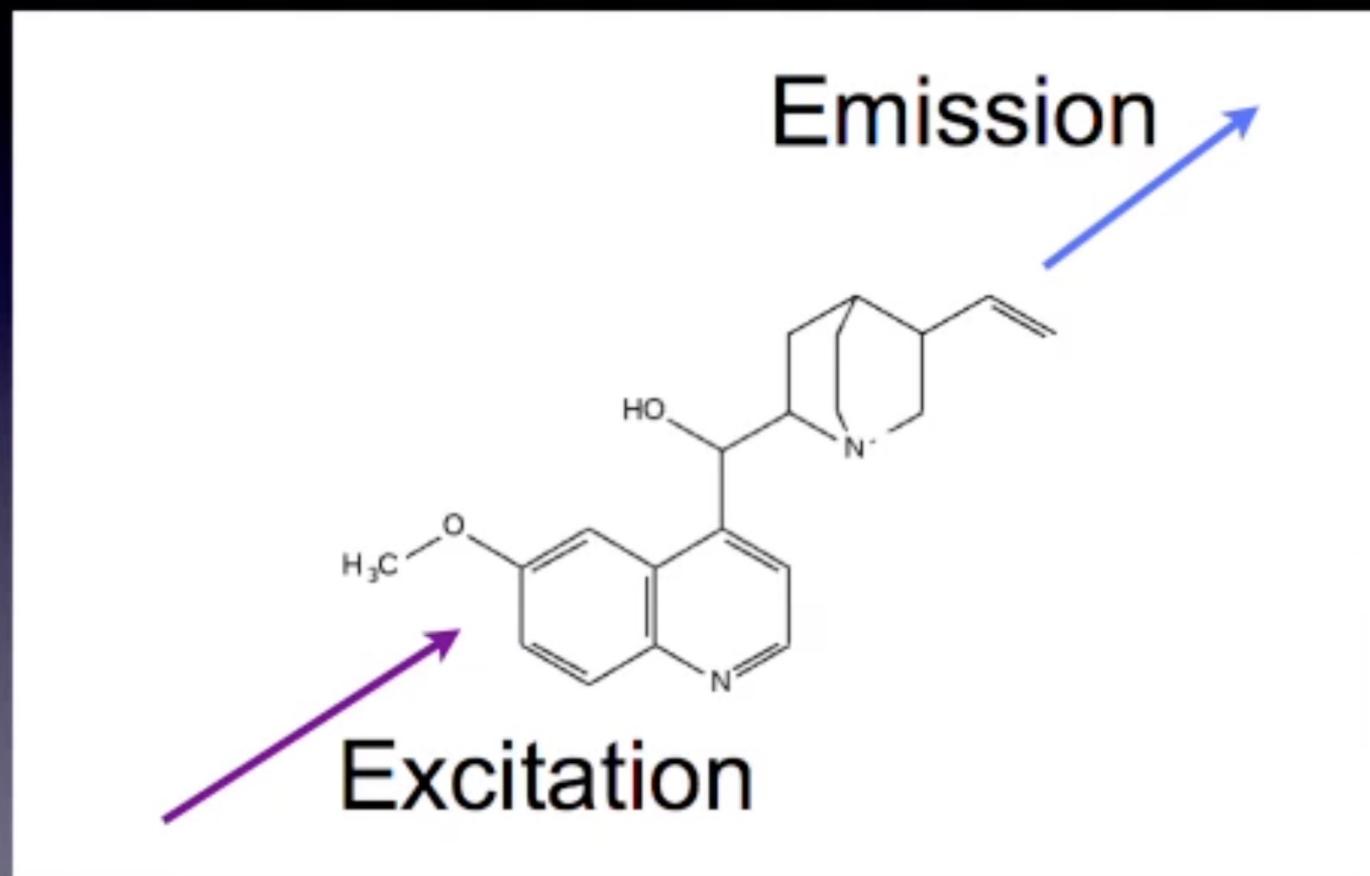


Fluorescein



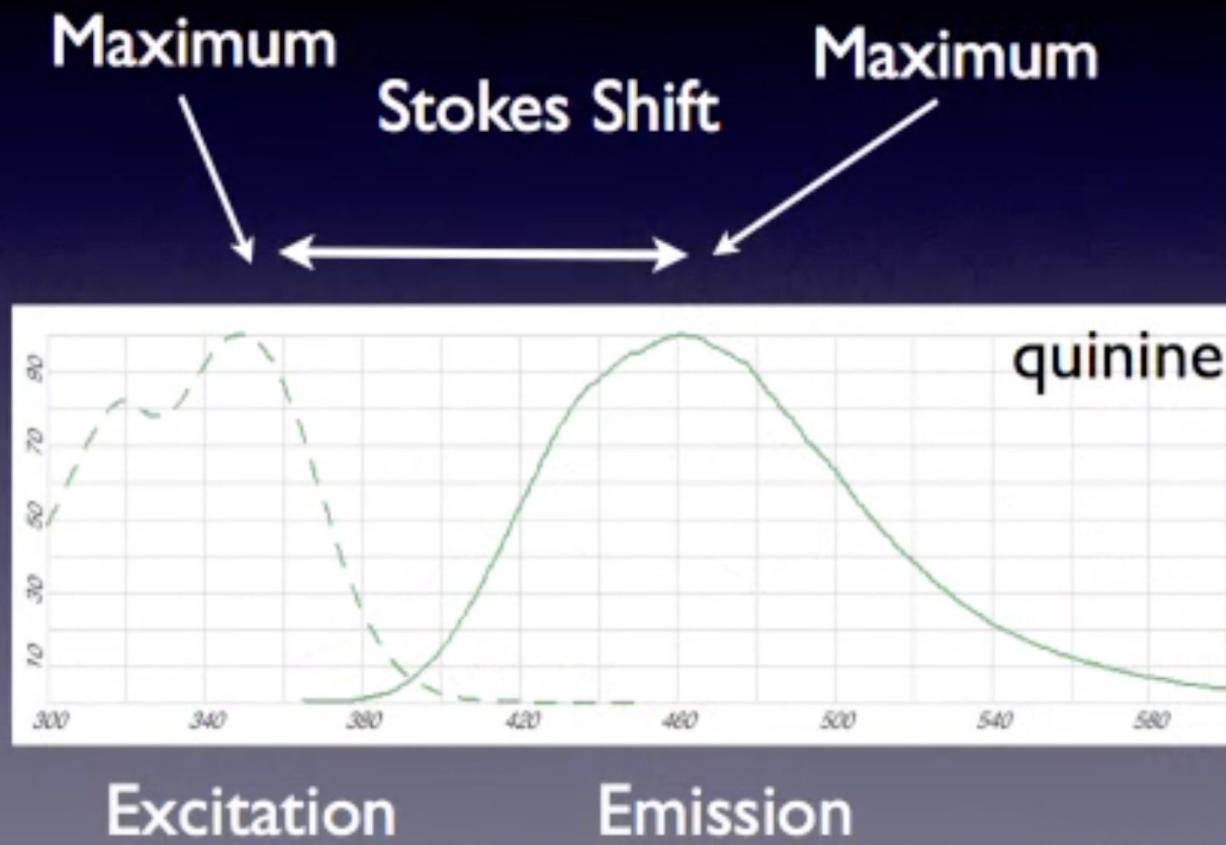
Rhodamine

Excitation/Emission

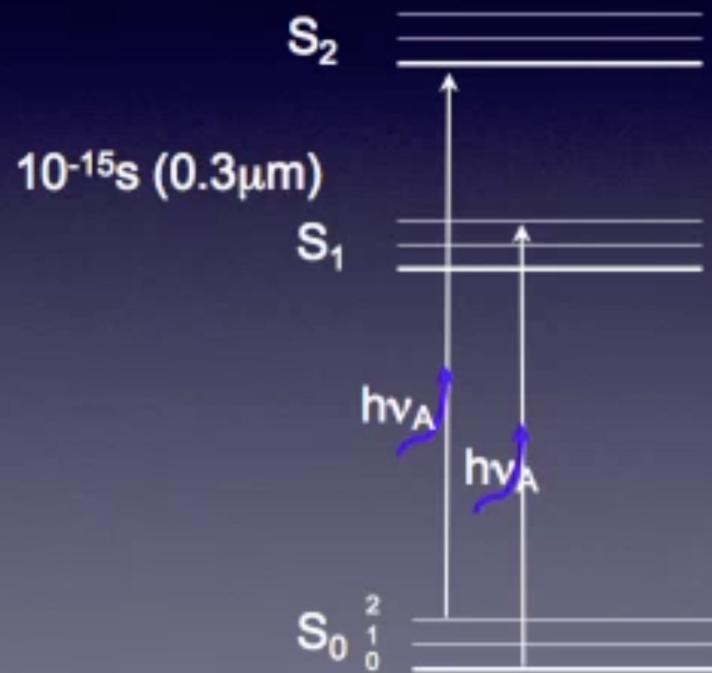


Emission light is longer wavelength
(lower energy) than excitation light

Fluorescence Spectrum

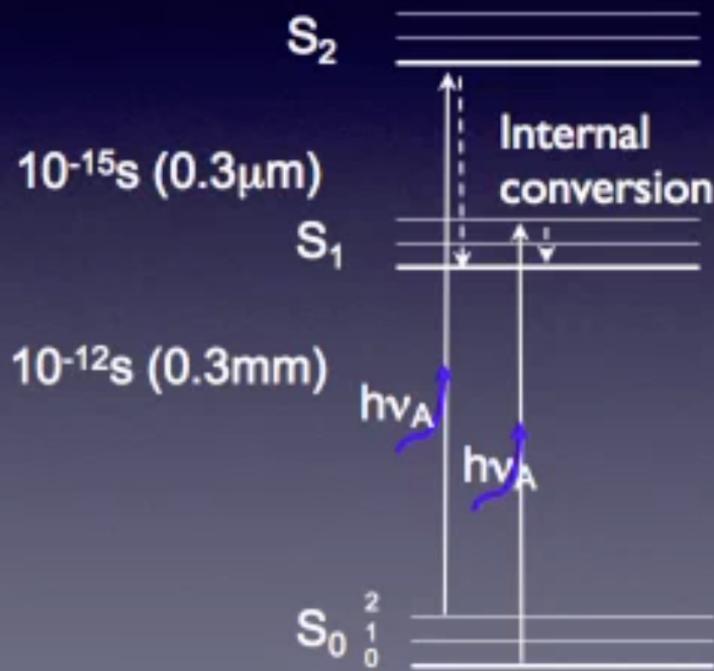


Jablonski diagram

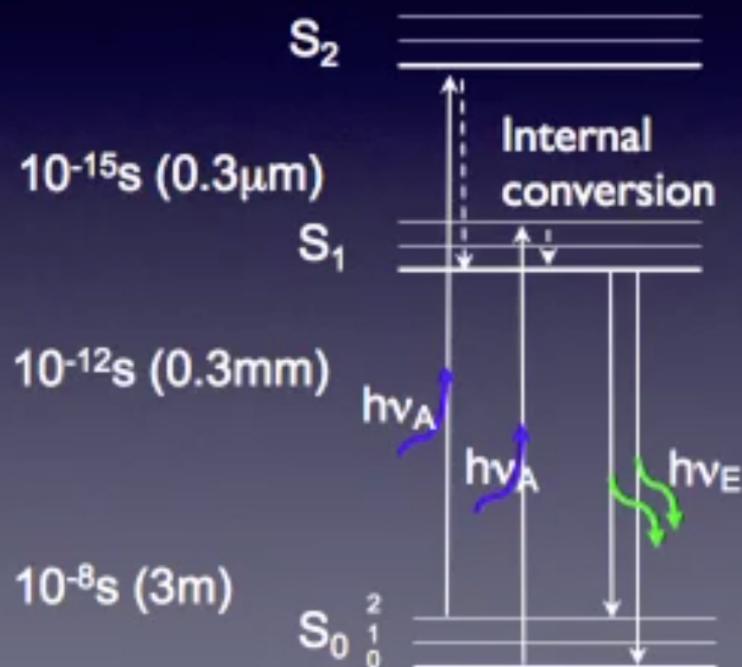


10^{-15}s ($0.3\mu\text{m}$)

Jablonski diagram

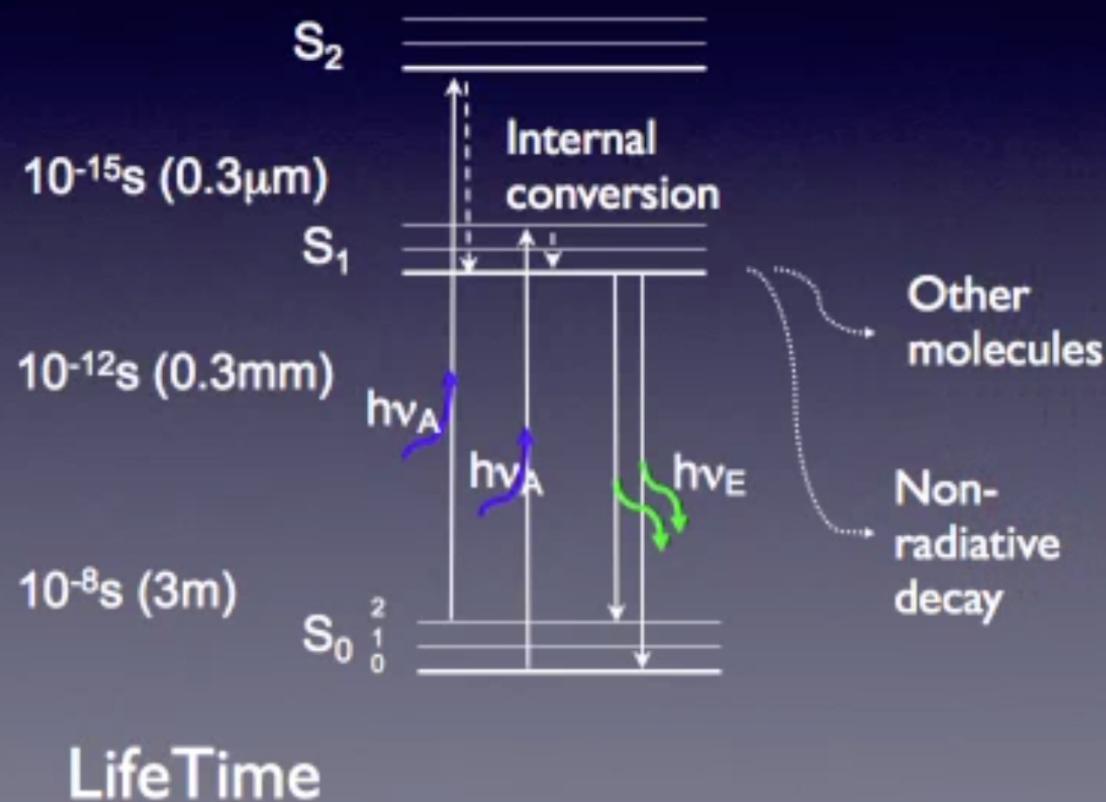


Jablonski diagram

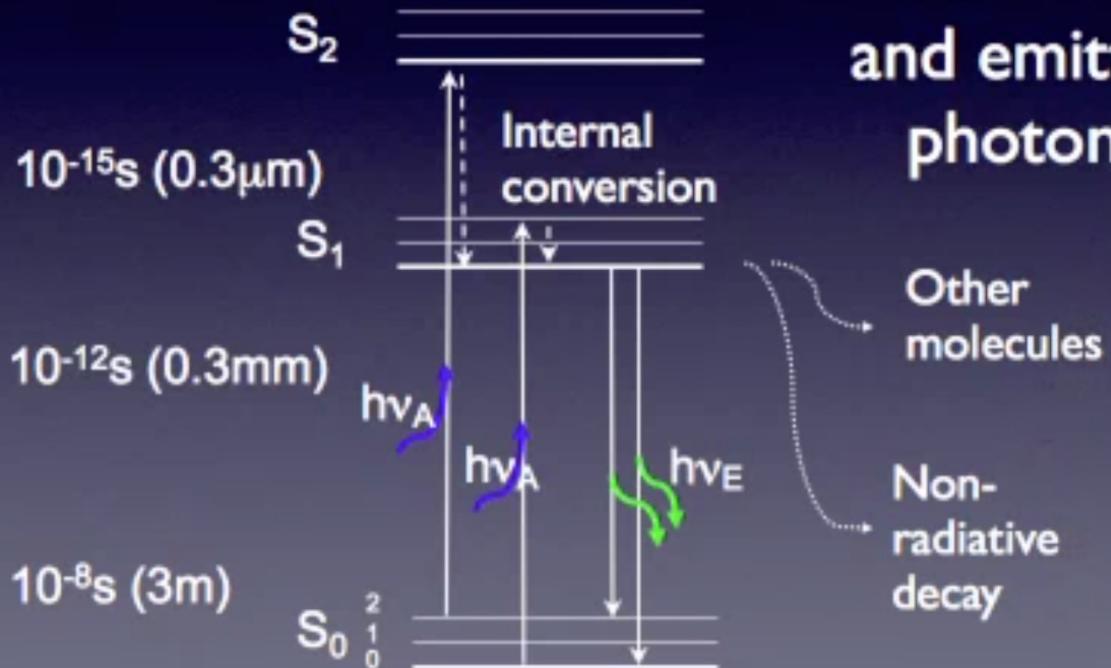


LifeTime

Jablonski diagram



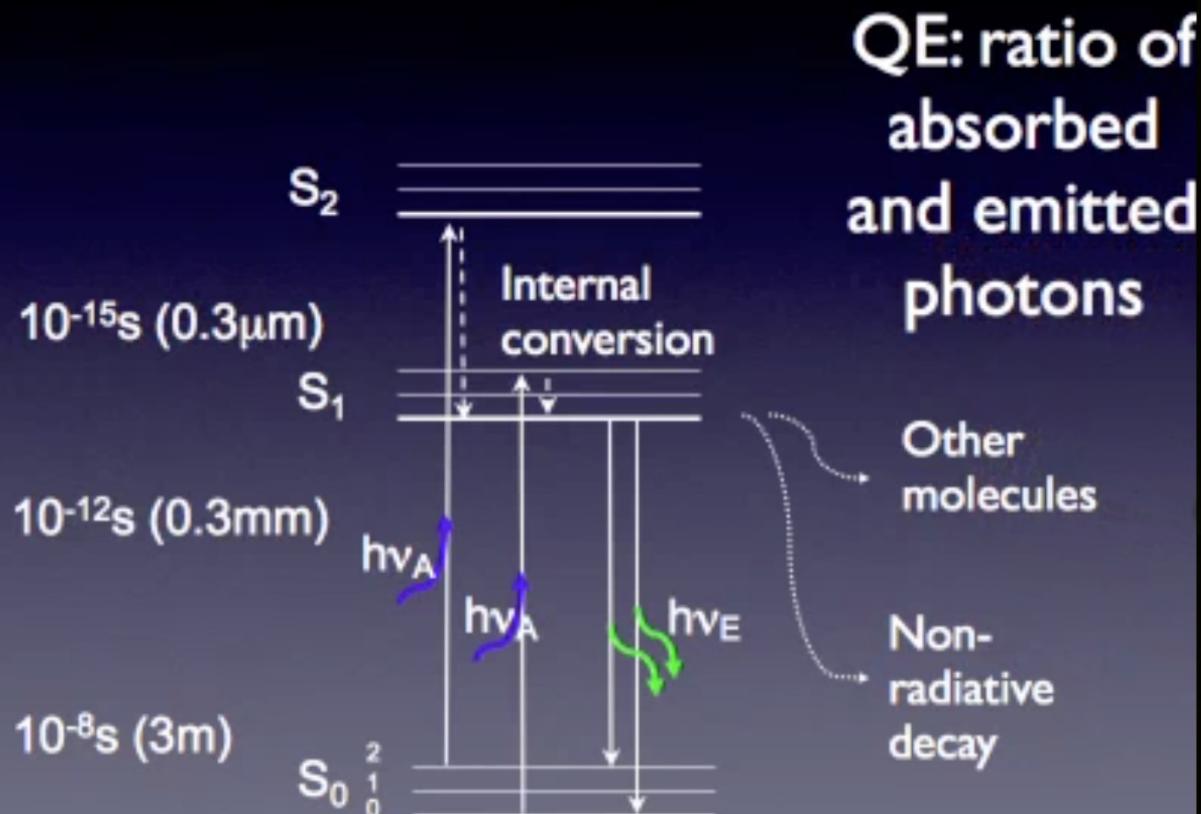
Jablonski diagram



QE: ratio of
absorbed
and emitted
photons

LifeTime

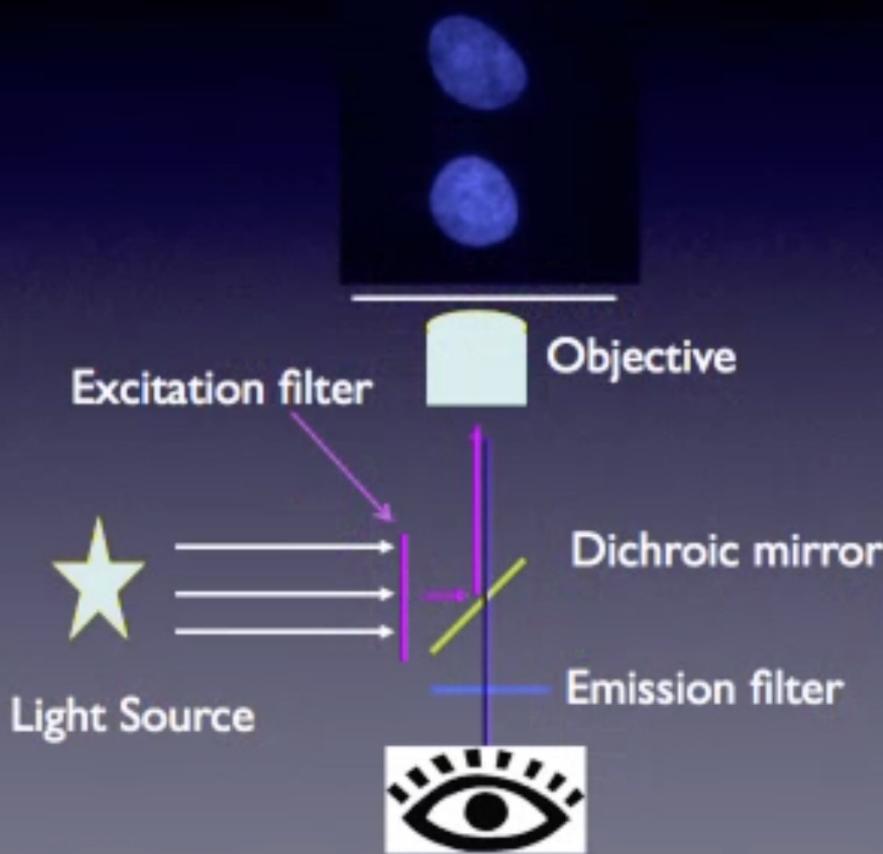
Jablonski diagram



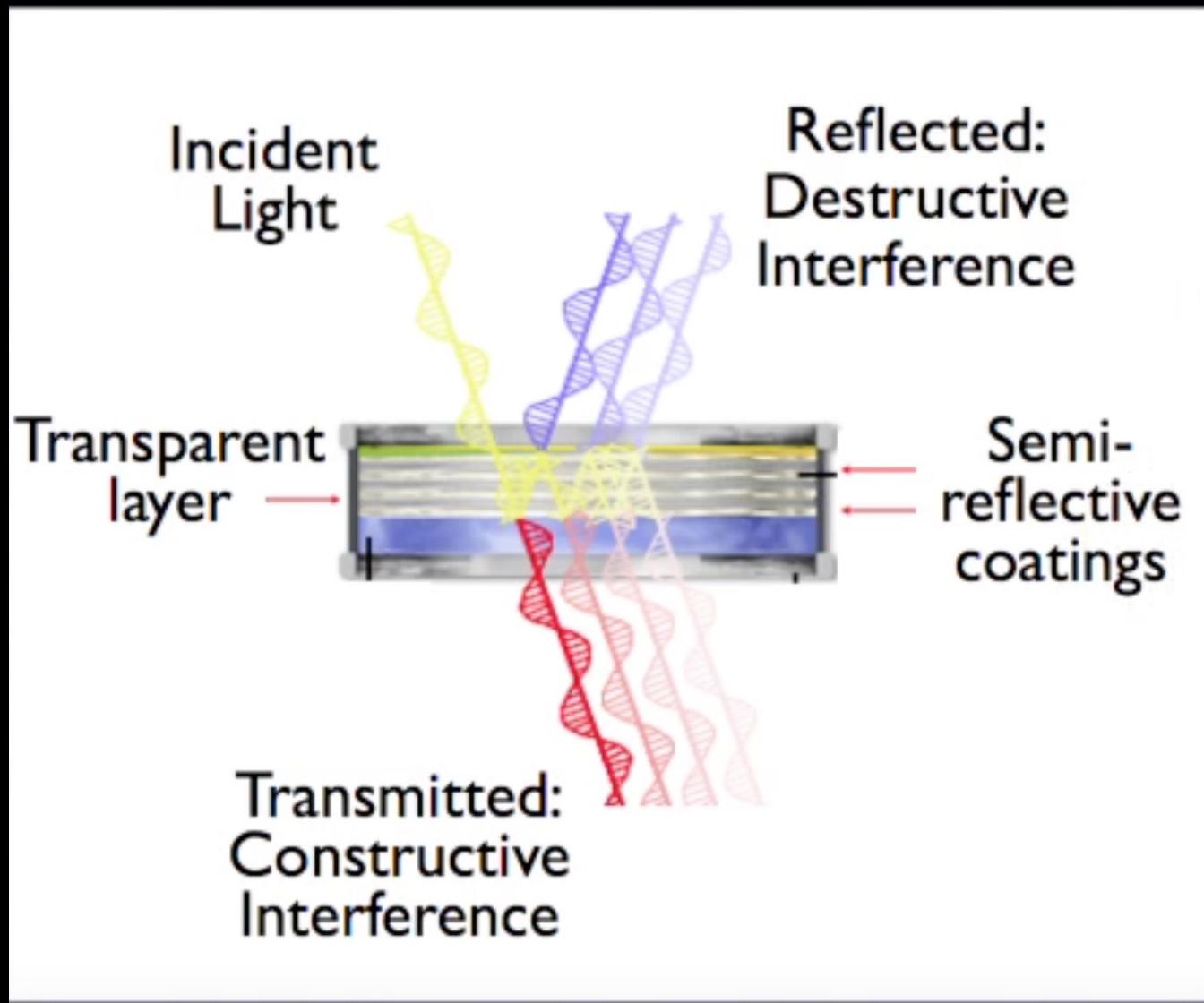
LifeTime

Brightness: determined by absorption coefficient and QE

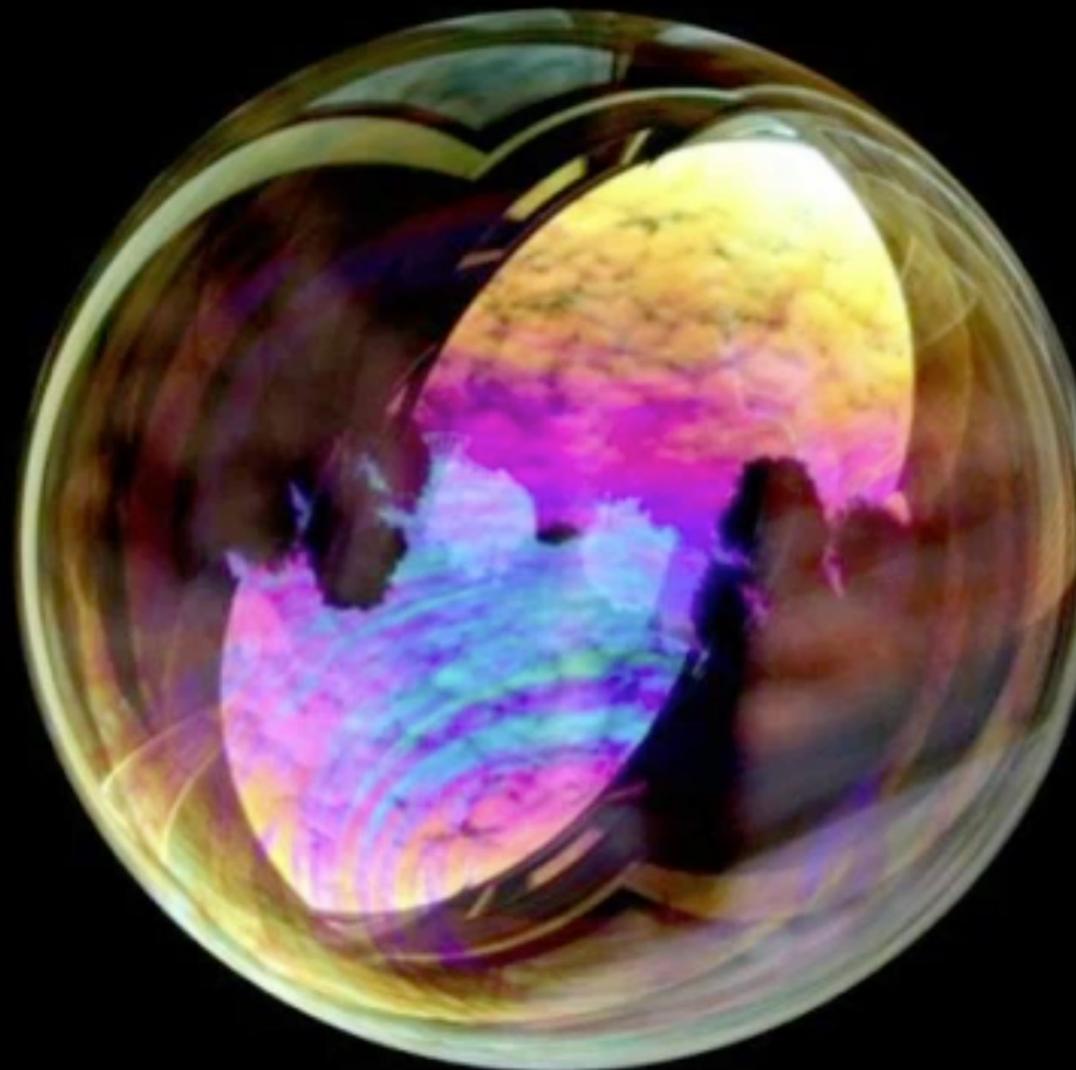
Fluorescence Microscope



Interference Filters



Interference Filters

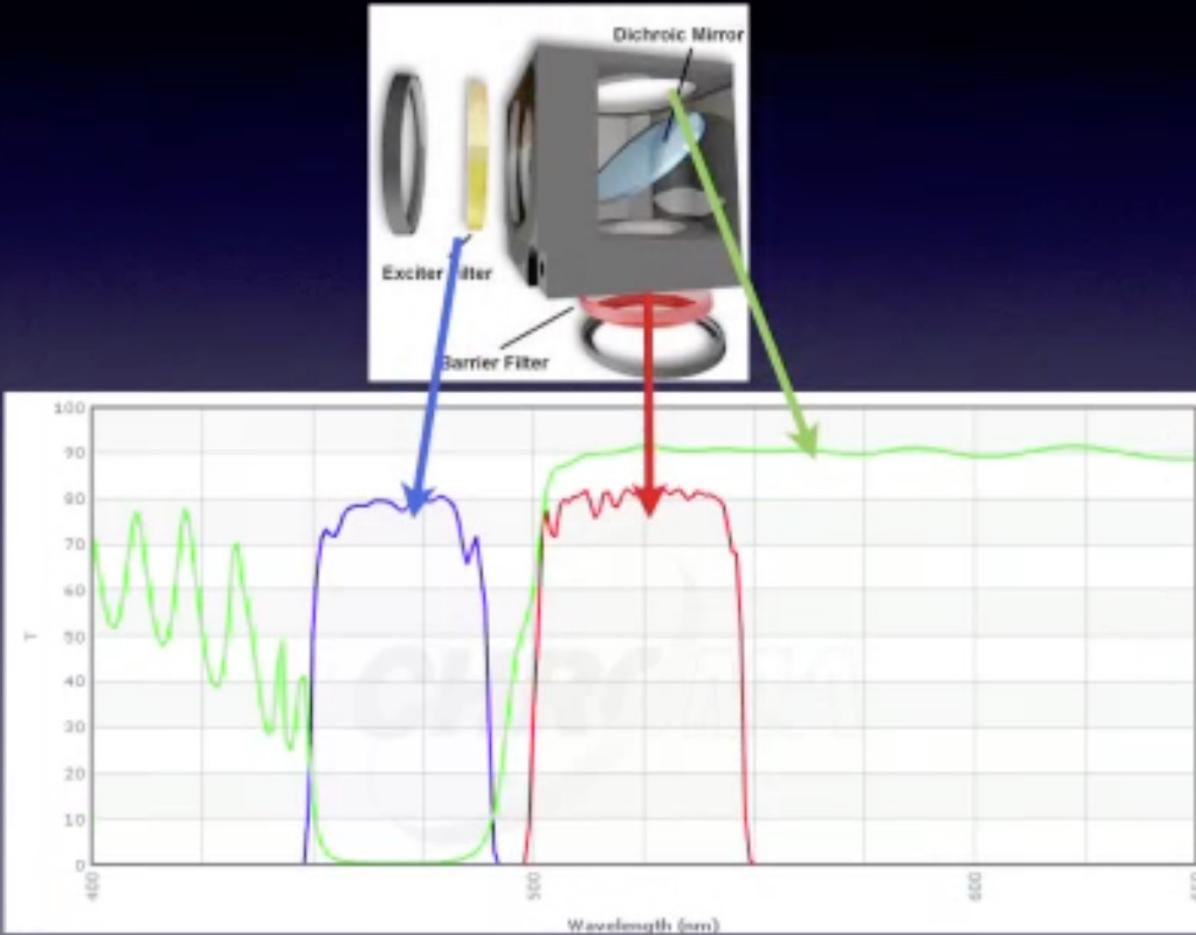


The Open Source Micro

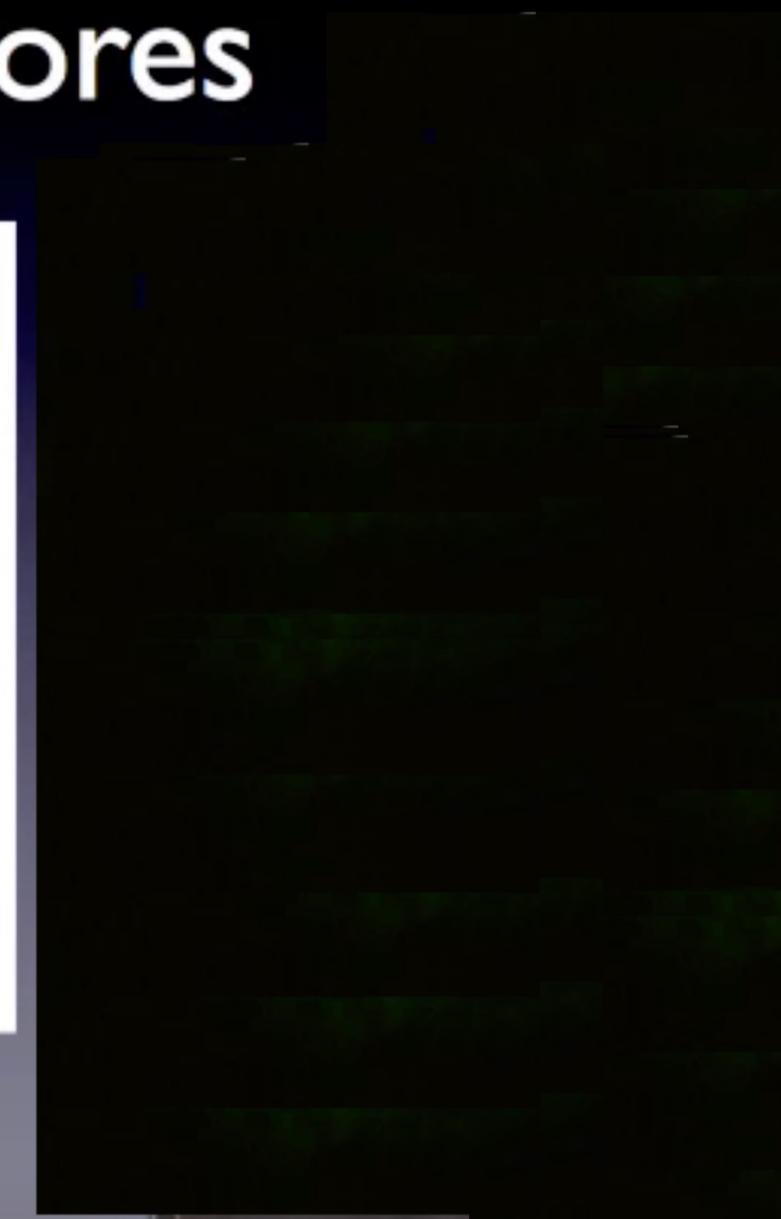
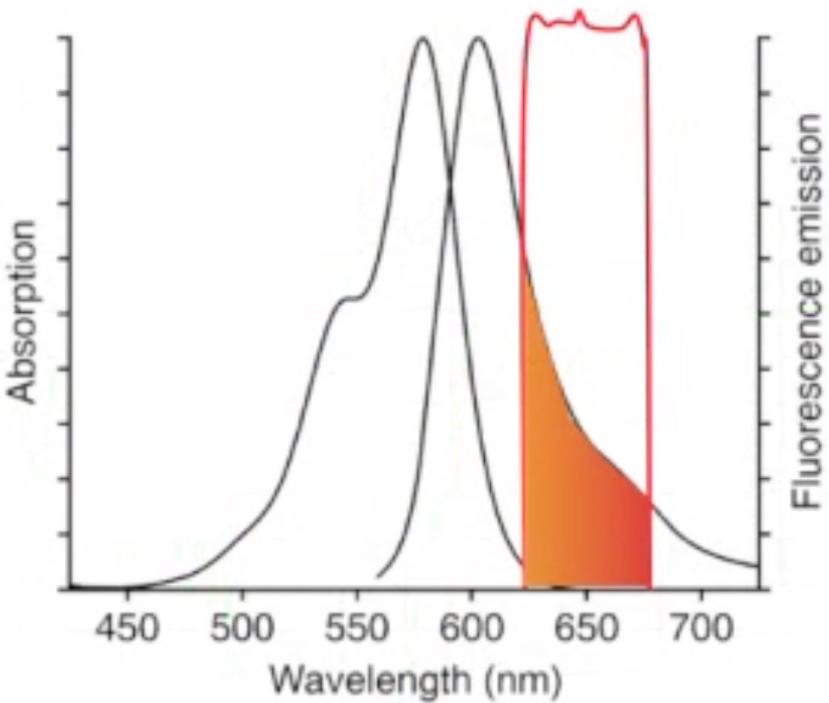
iBiology.org

The Open Source Micro

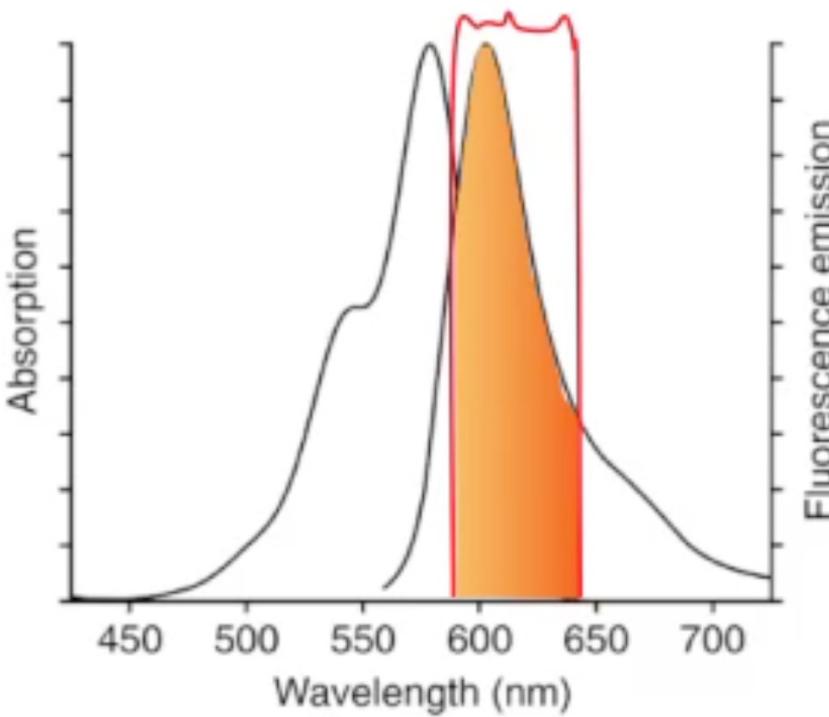
Filter Cube (after Ploem)

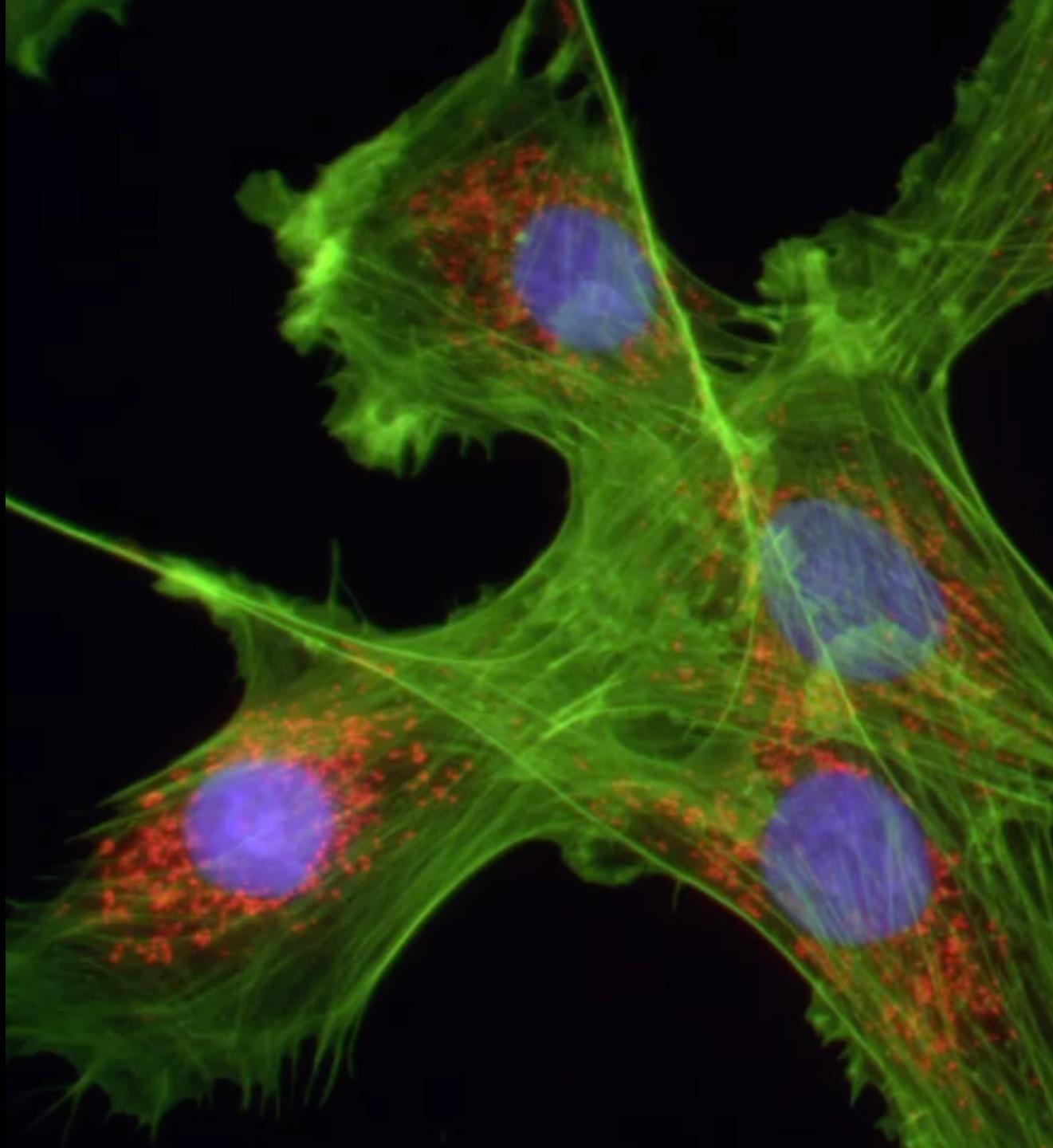


Matching Filters and Fluorophores



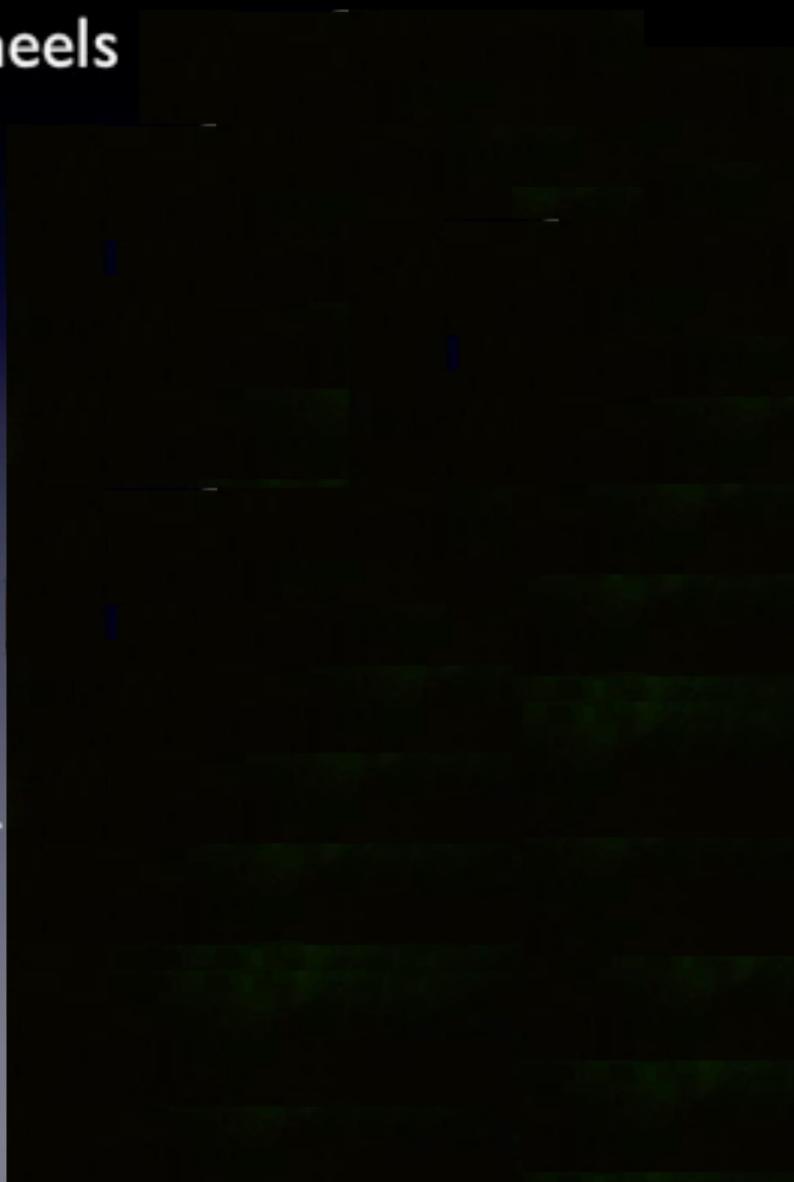
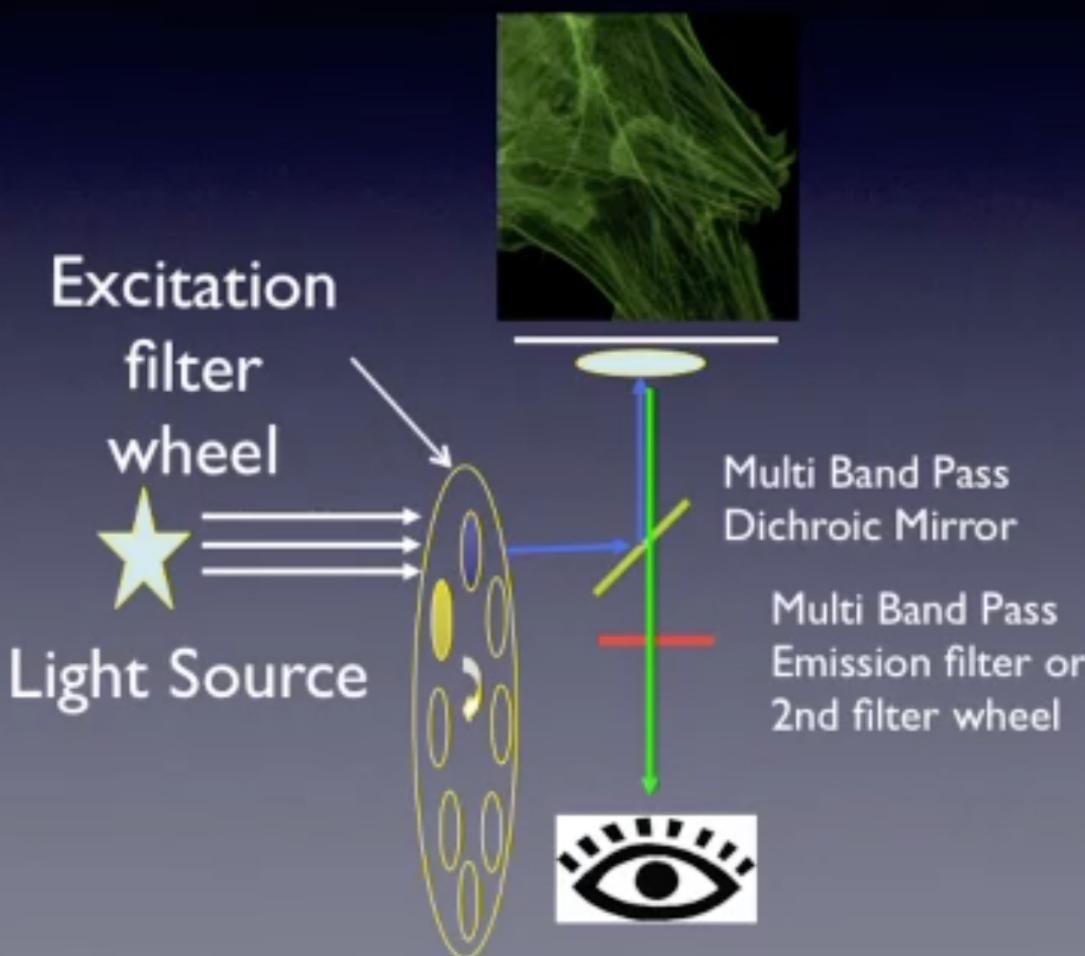
Matching Filters and Fluorophores





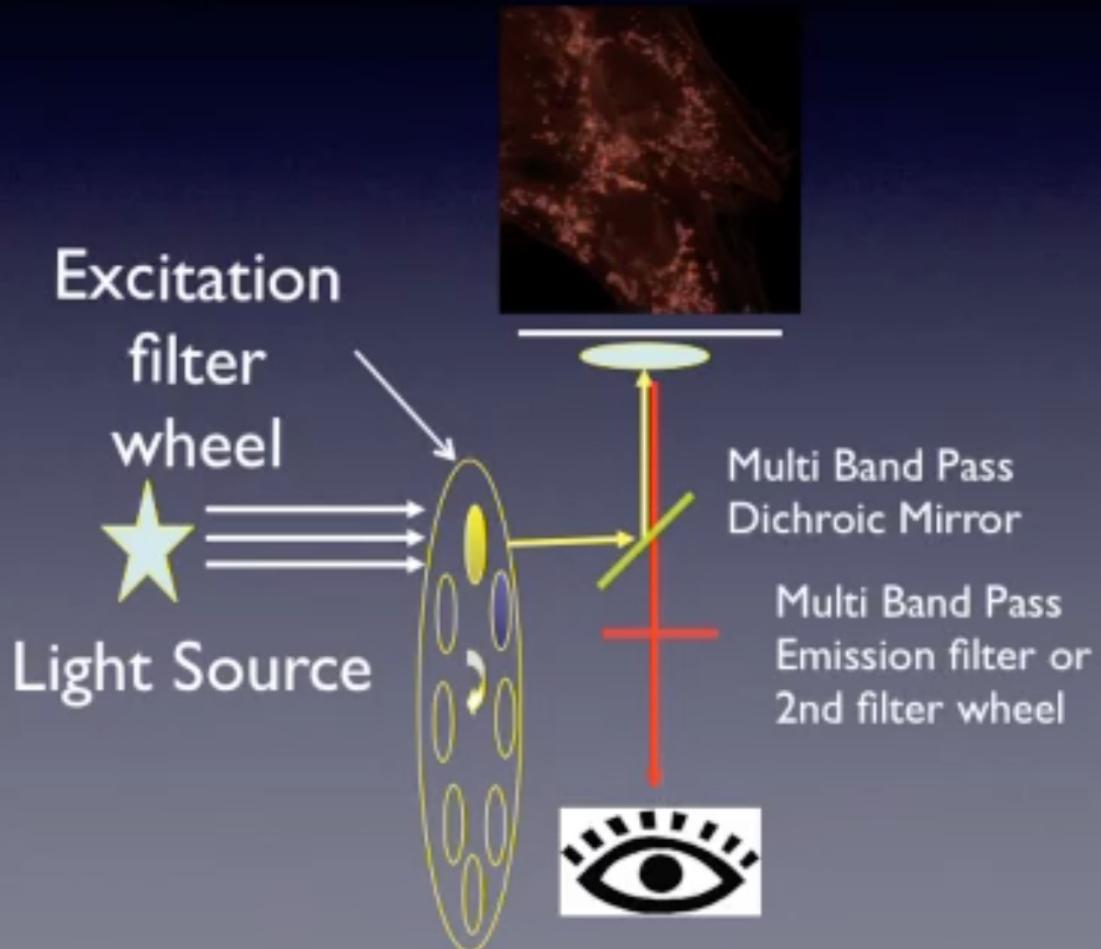
Faster Wavelength Selection

Multi Band Pass Filters & Filter Wheels



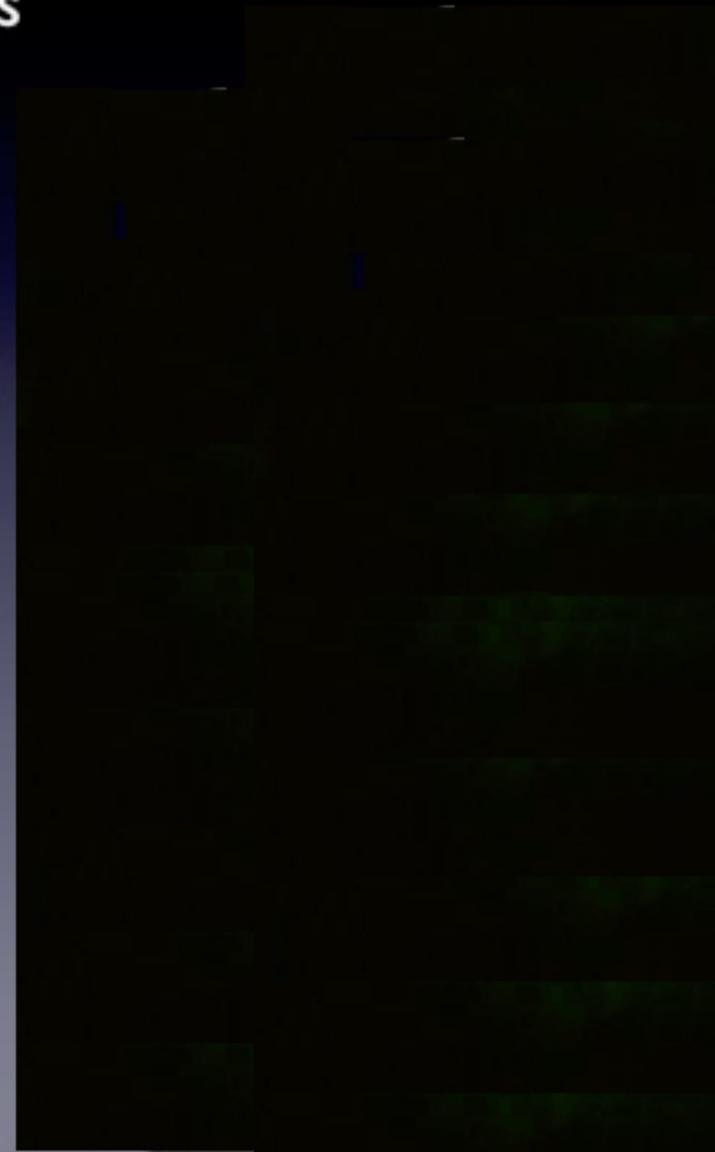
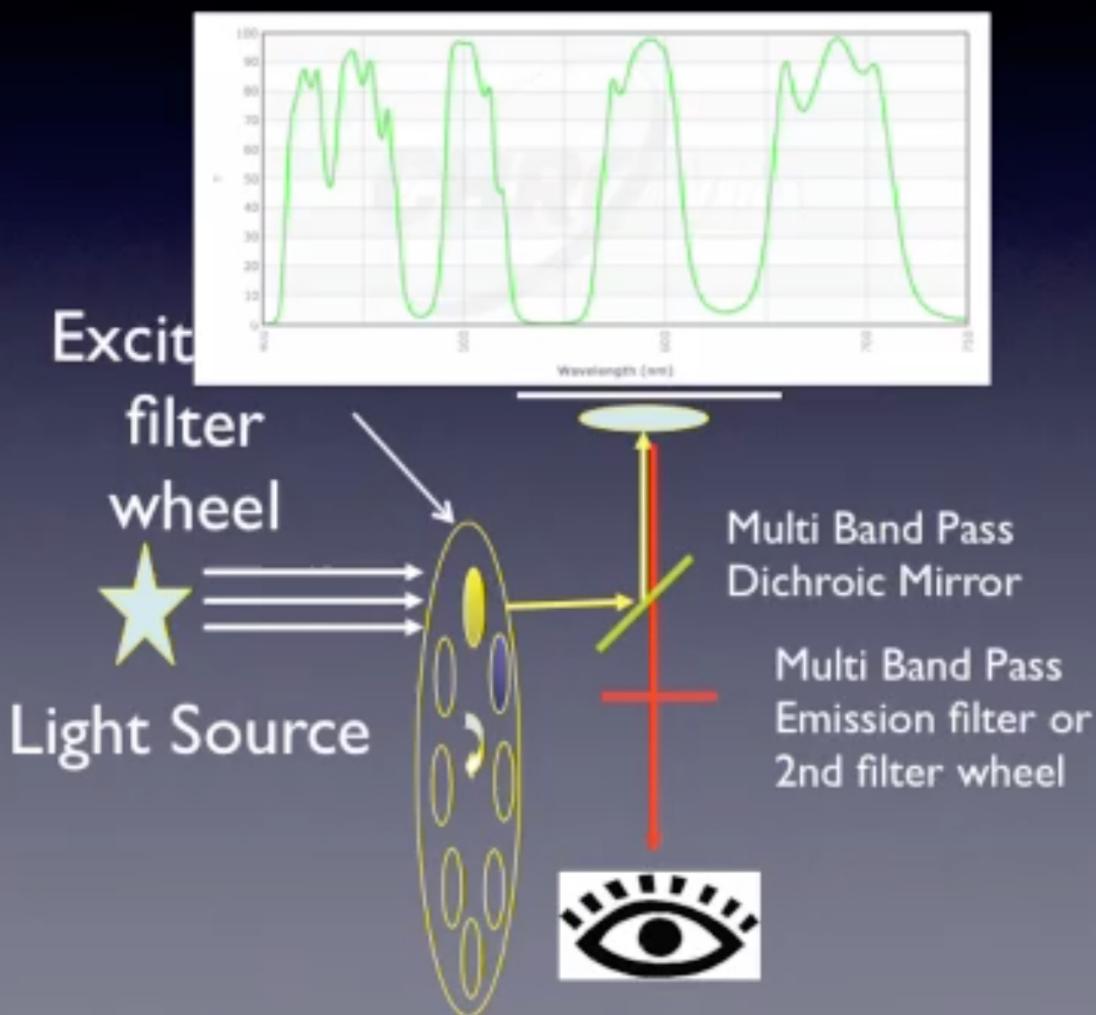
Faster Wavelength Selection

Multi Band Pass Filters & Filter Wheels



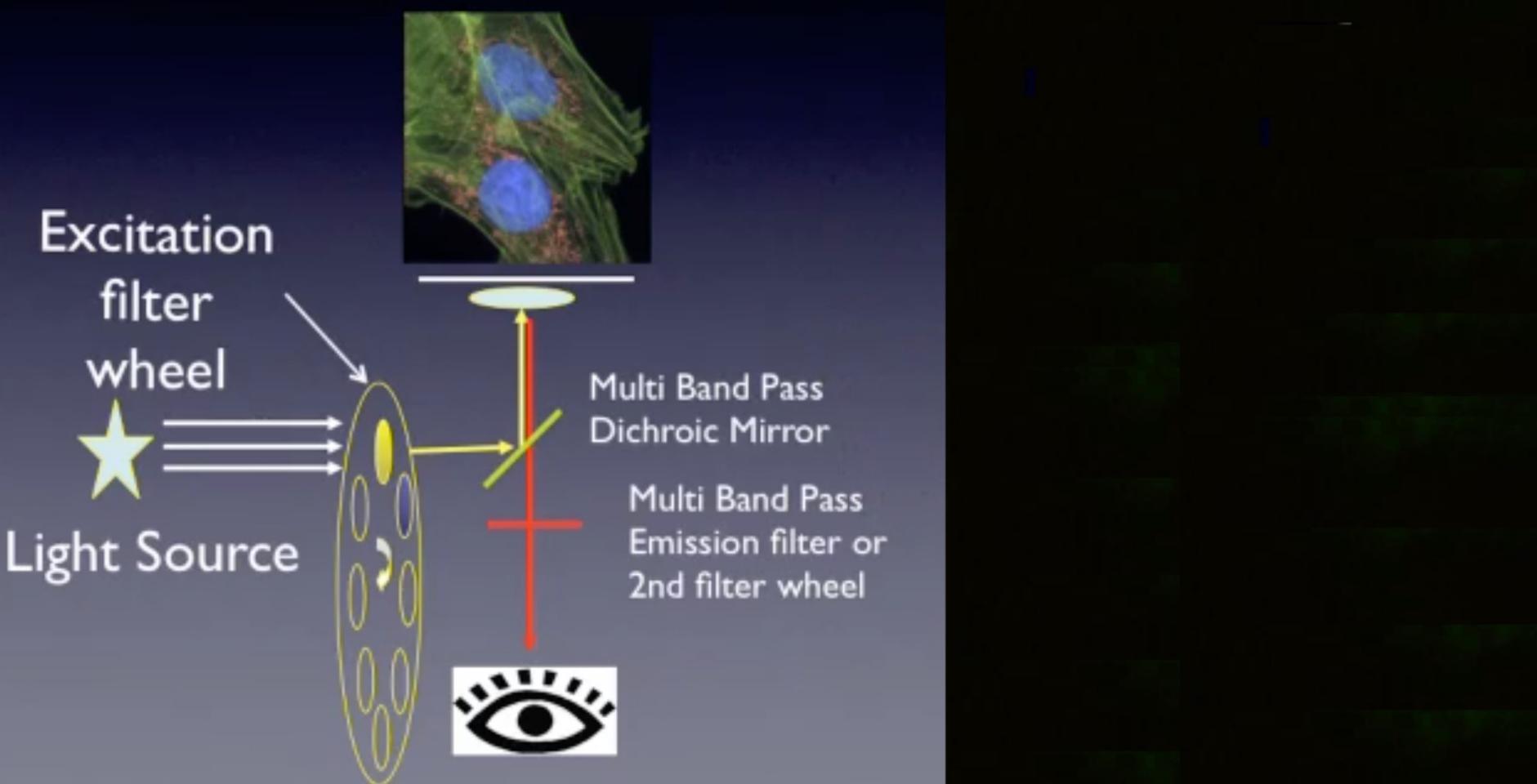
Faster Wavelength Selection

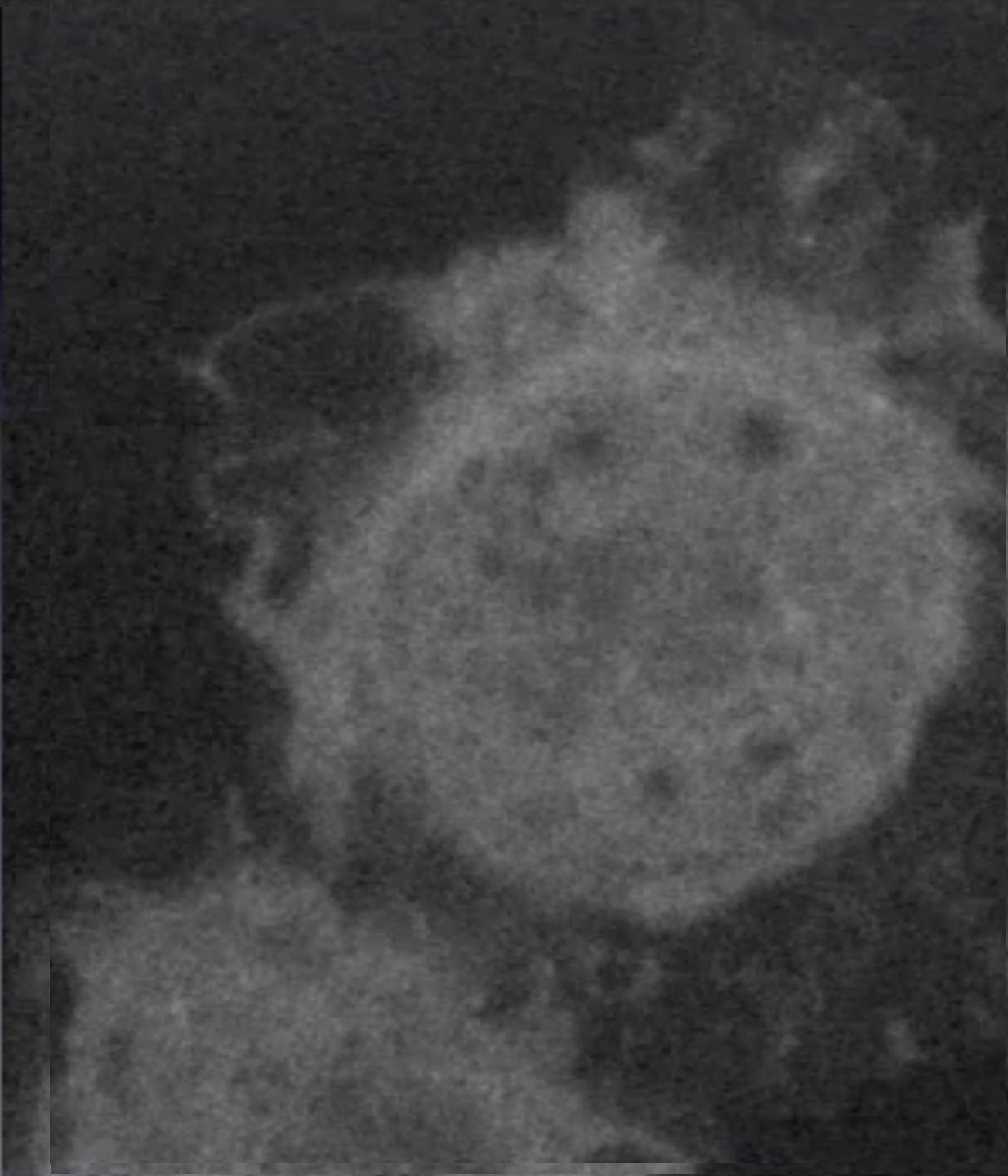
Multi Band Pass Filters & Filter Wheels



Faster Wavelength Selection

Multi Band Pass Filters & Filter Wheels

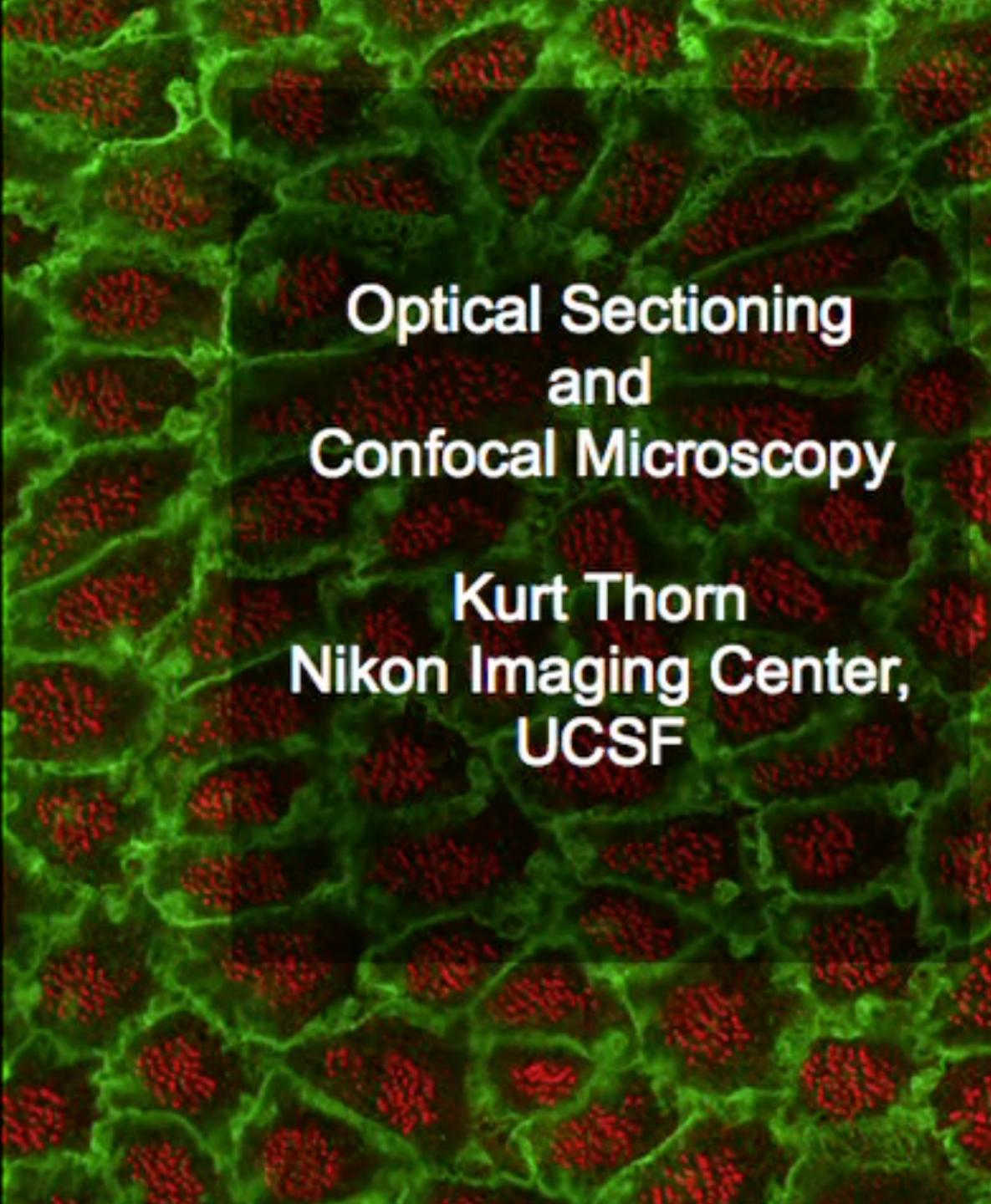




What to do about PhotoBleaching?

- Select fade-resistant dyes
- Label densely
- Decrease bleaching by anti-fade compounds
- Budget the photons you have
 - Only expose when observing
 - Minimize exposure time & excitation power

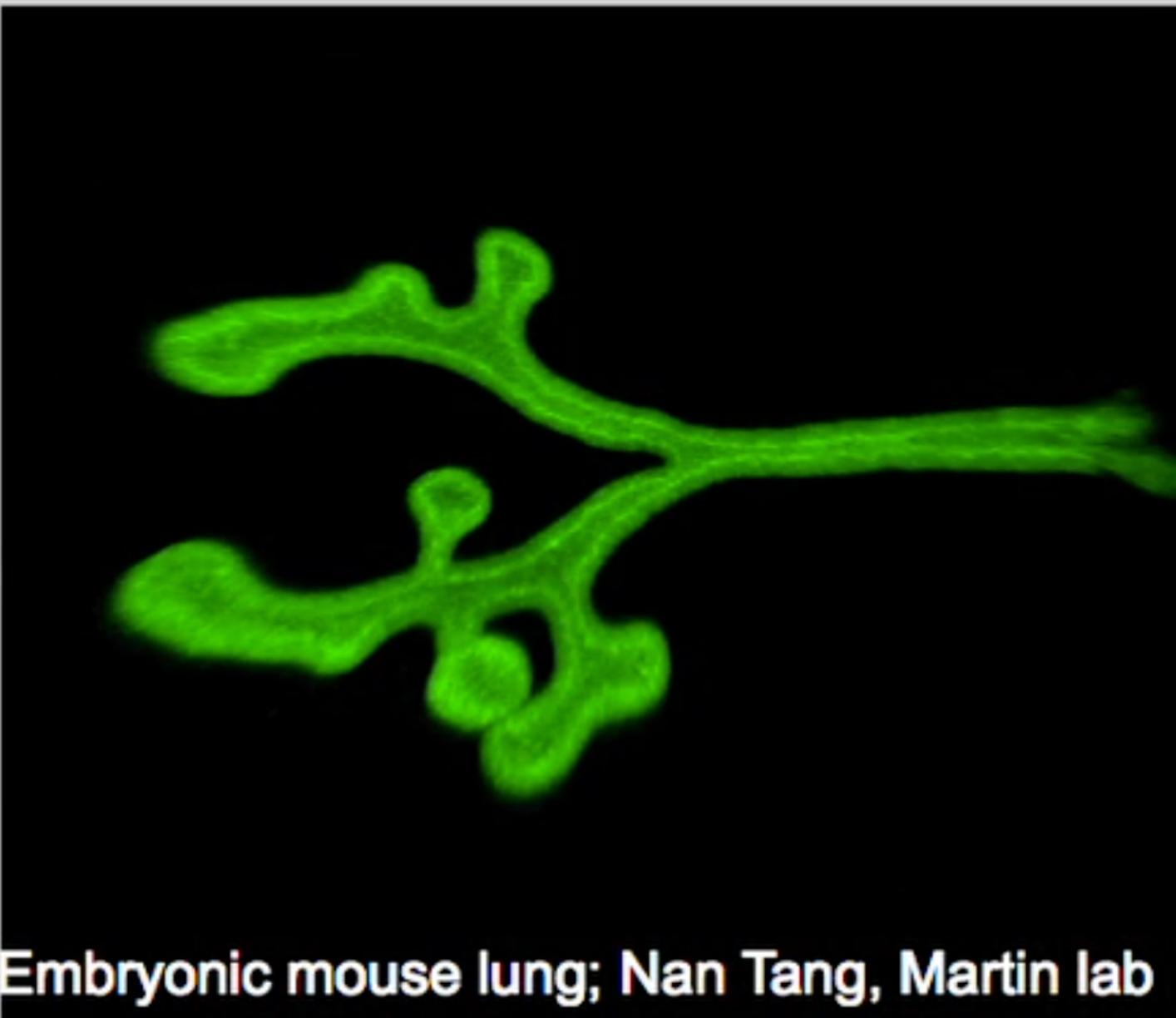
Introduction to Confocal Microscopy



Optical Sectioning
and
Confocal Microscopy

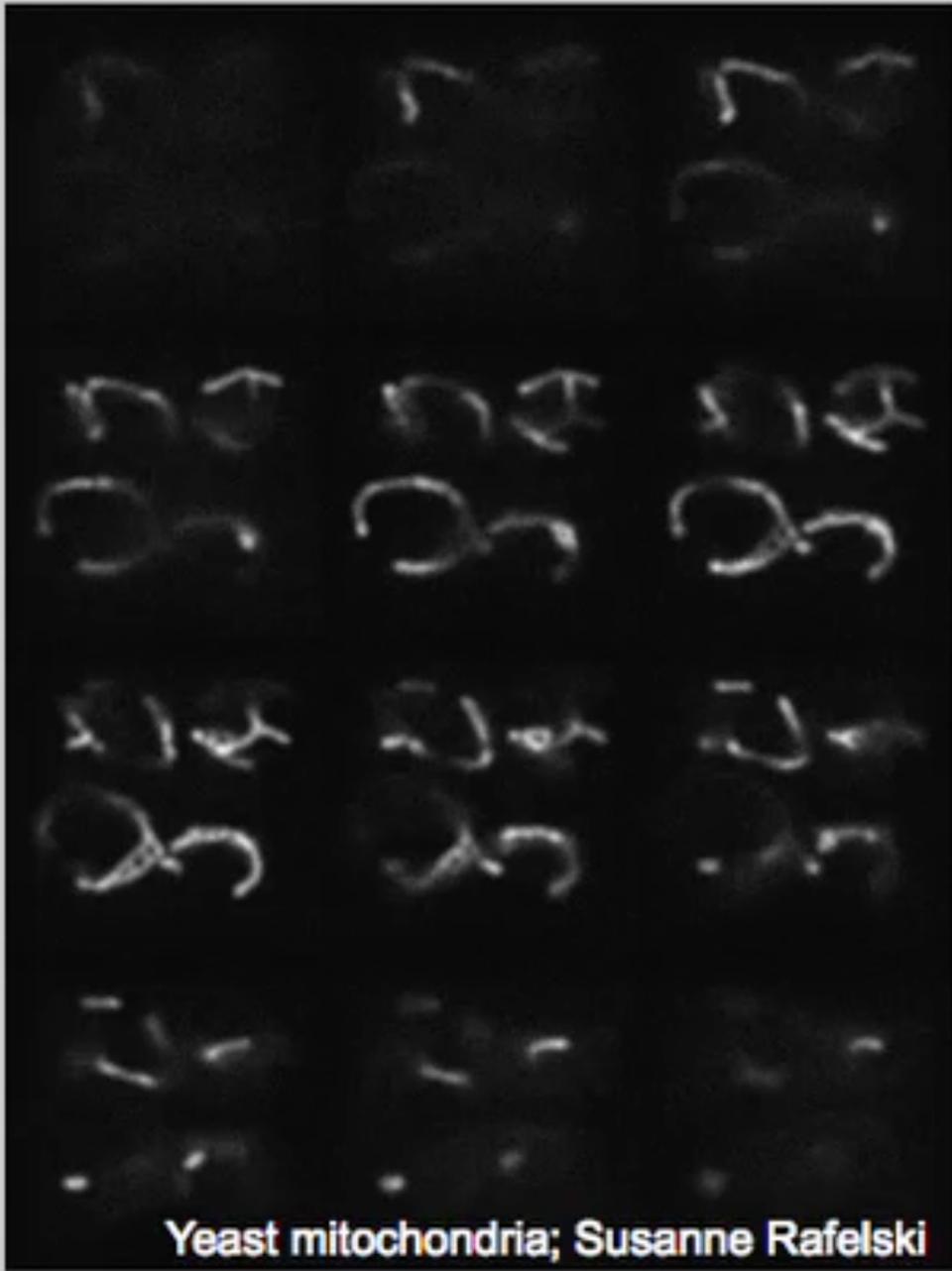
Kurt Thom
Nikon Imaging Center,
UCSF

The goal: build 3D images of biological samples



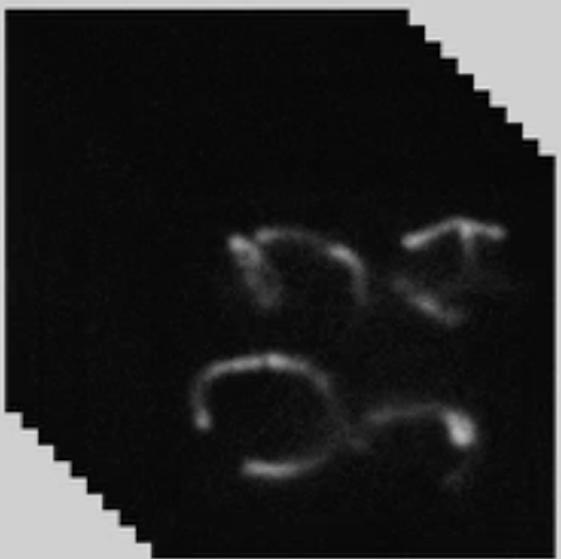
Embryonic mouse lung; Nan Tang, Martin lab

A series of optical sections

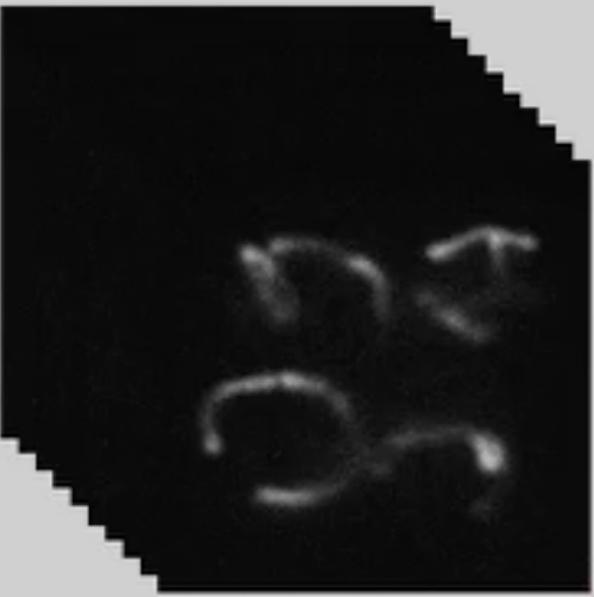


Yeast mitochondria; Susanne Rafelski

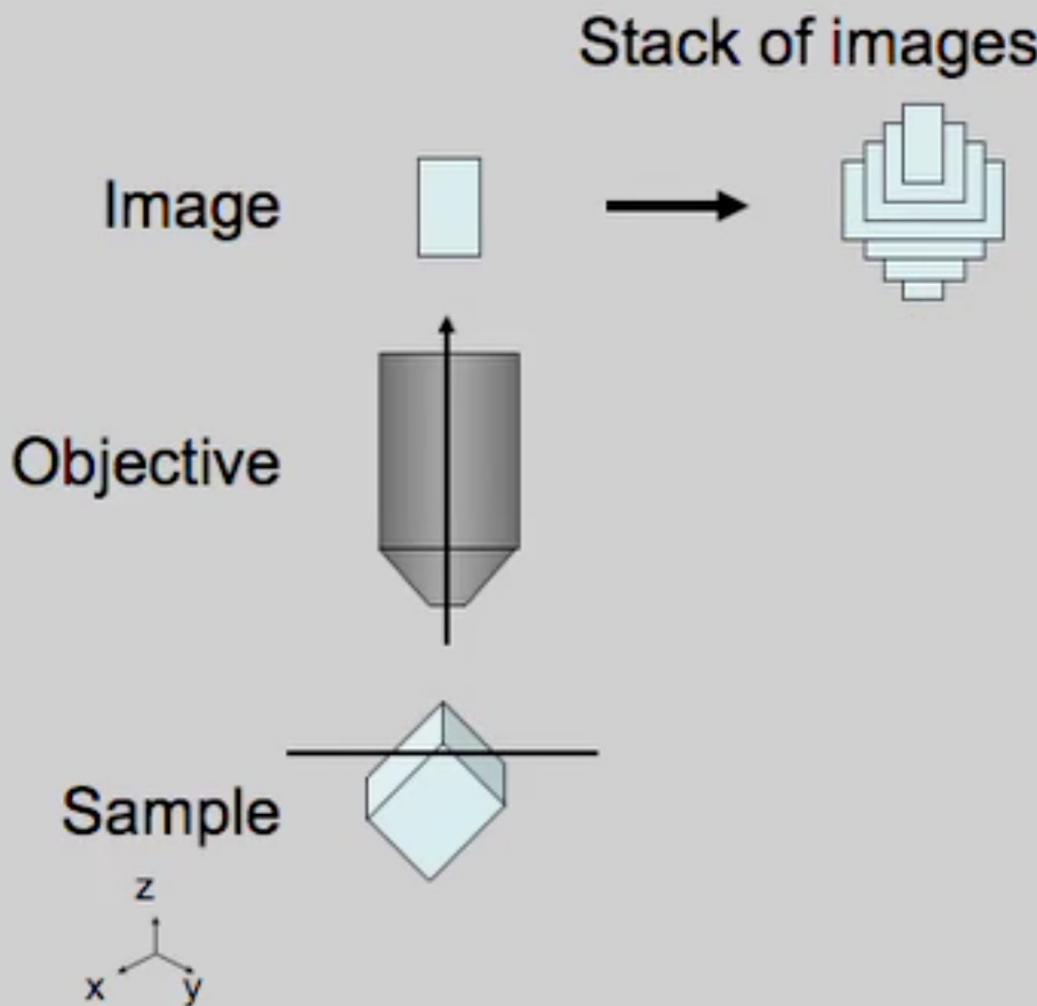
Optical sectioning and 3D reconstruction



3D Reconstruction

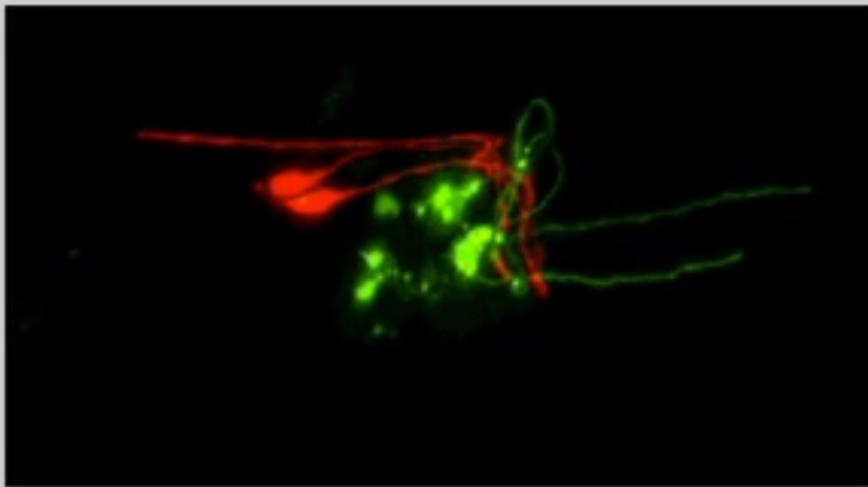


Optical sectioning and 3D reconstruction



Optical Sectioning and 3D reconstruction

3D reconstruction

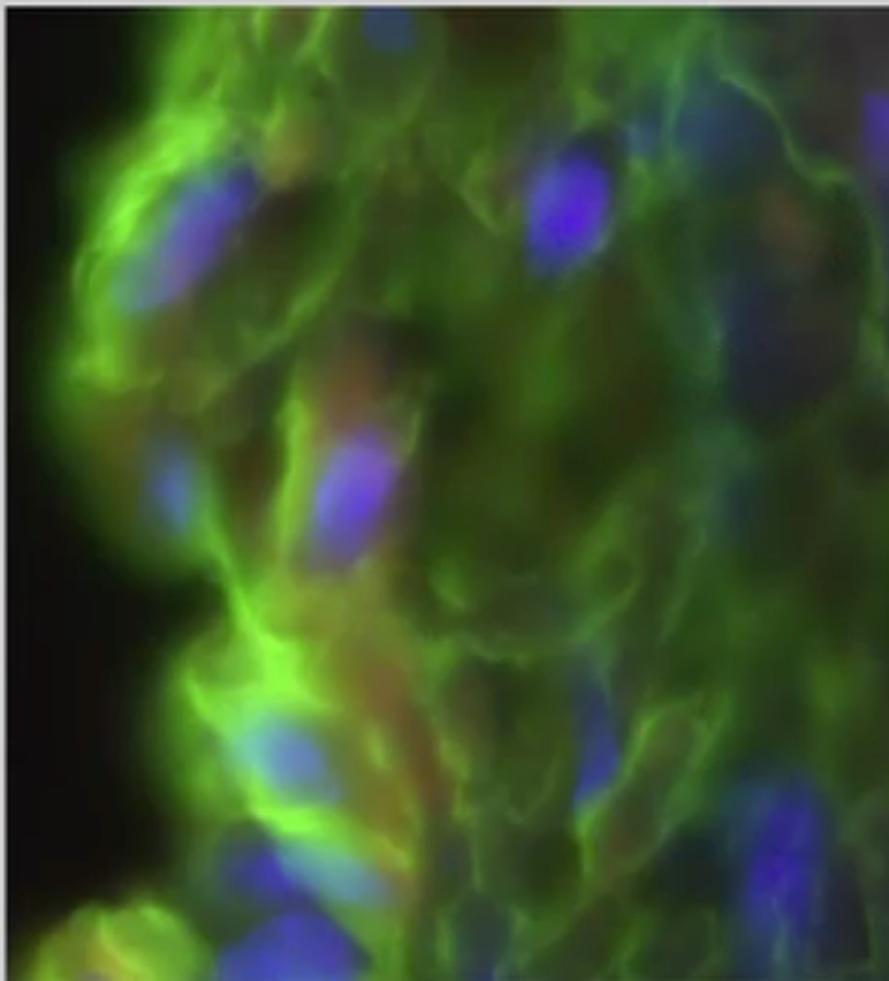


C. elegans with two different sensory neurons
expressing GFP, DsRed; 85 Z slices, 250 nm apart

A problem: out of focus light

Conventional microscopes see both in-focus and out of focus light

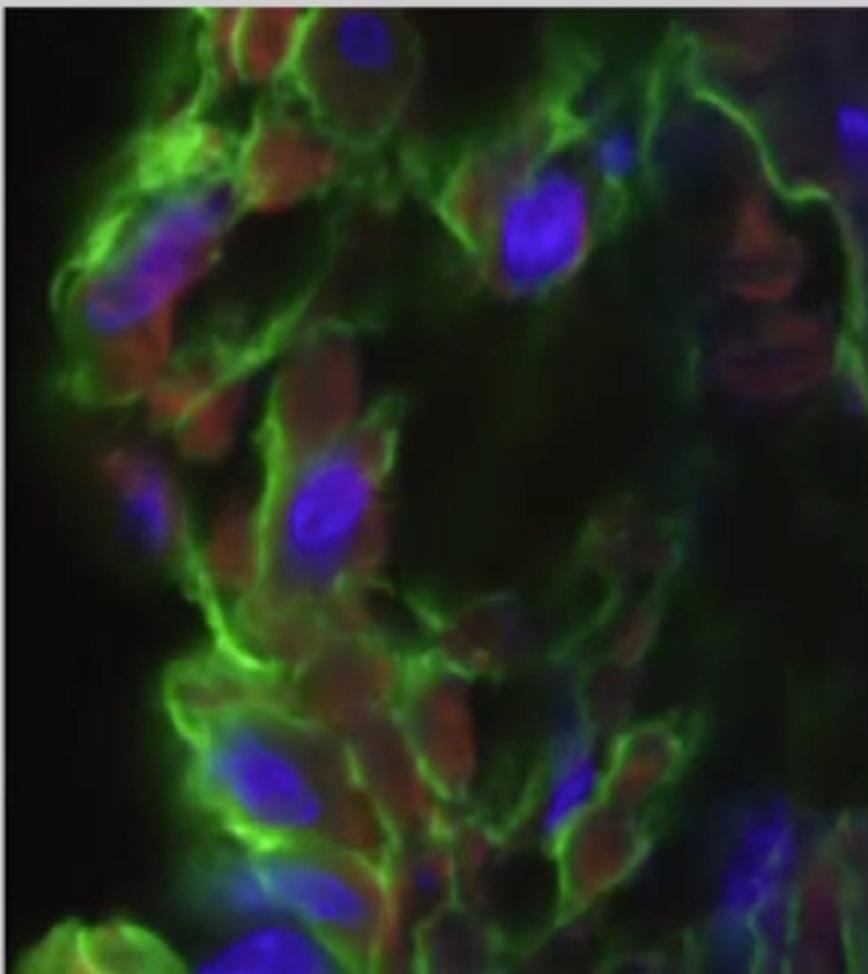
Conventional



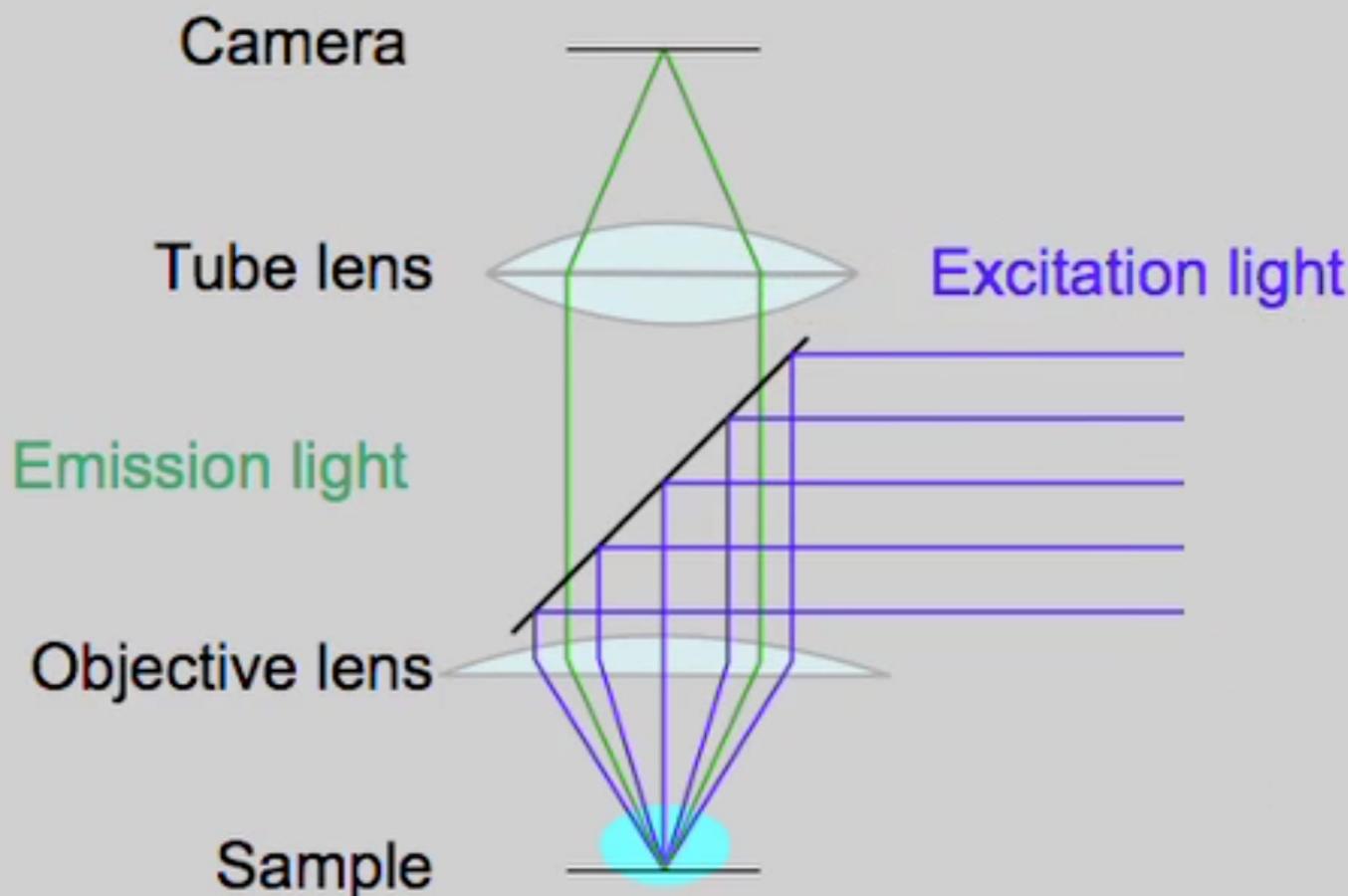
A problem: out of focus light

A confocal microscope blocks the out of focus light

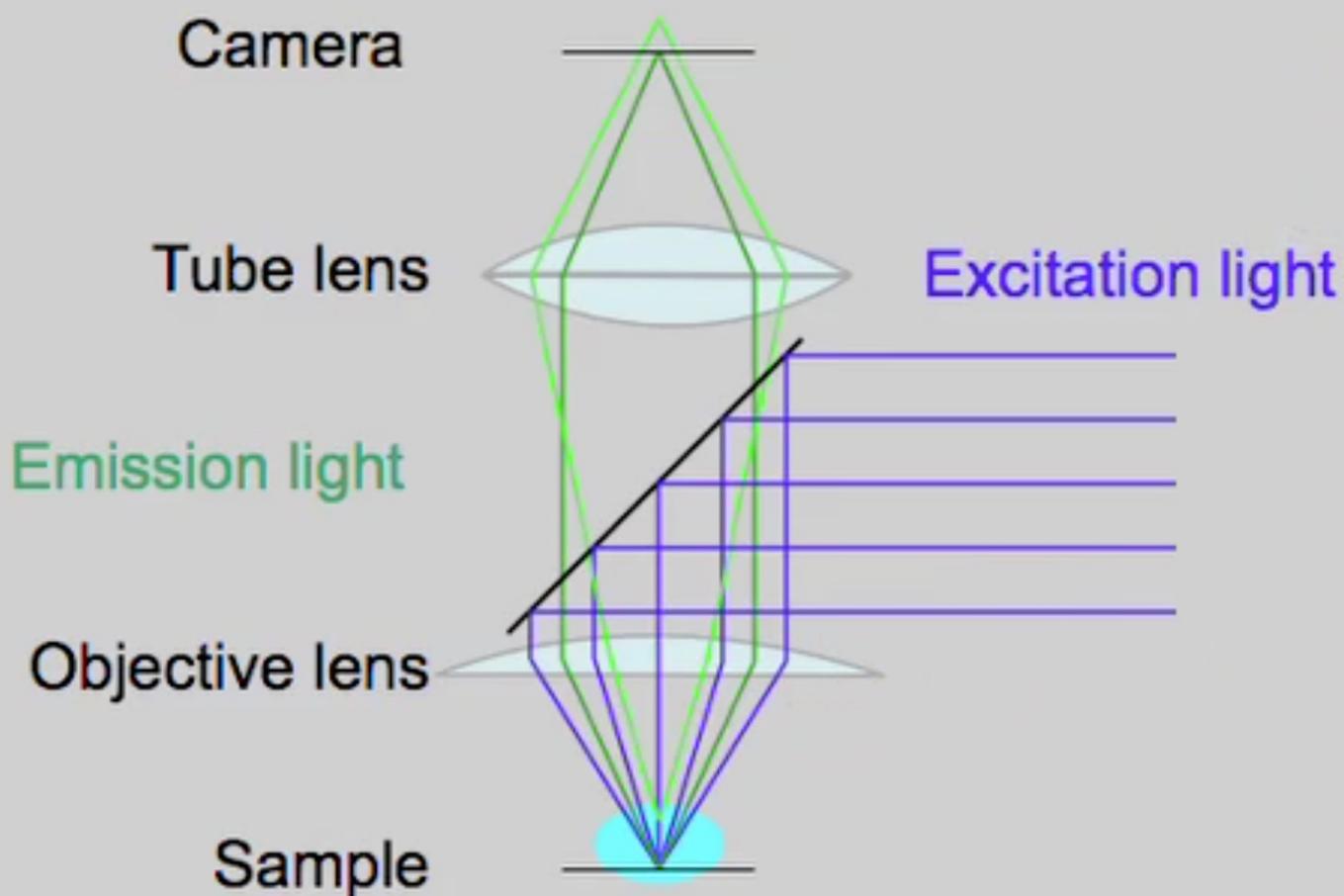
Confocal



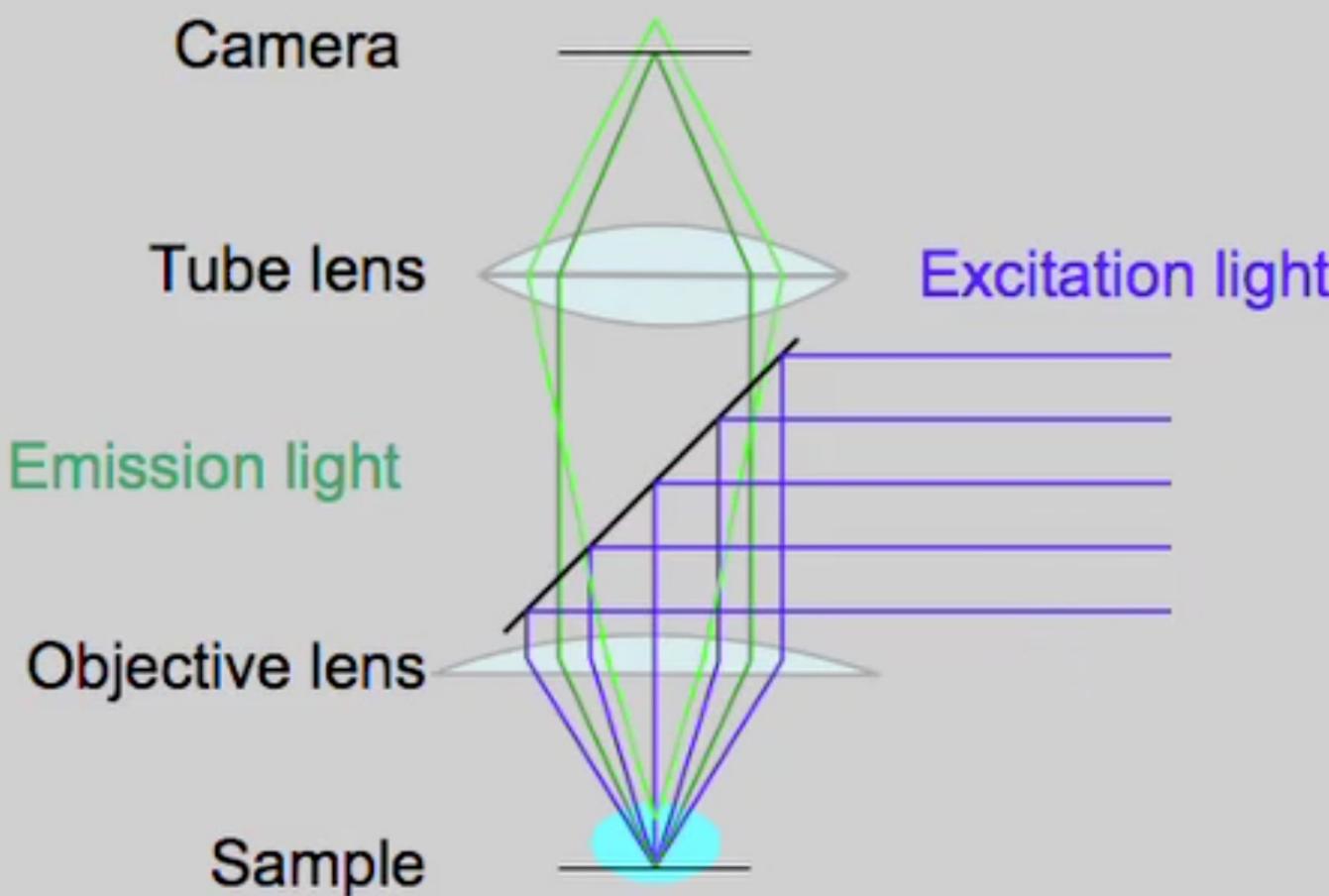
Fluorescence Illumination of a single point



Fluorescence Illumination of a single point



Fluorescence Illumination of a single point



Problem – fluorescence is emitted along entire illuminated cone, not just at focus

The Confocal Microscope

Use a pinhole to block out-of-focus light

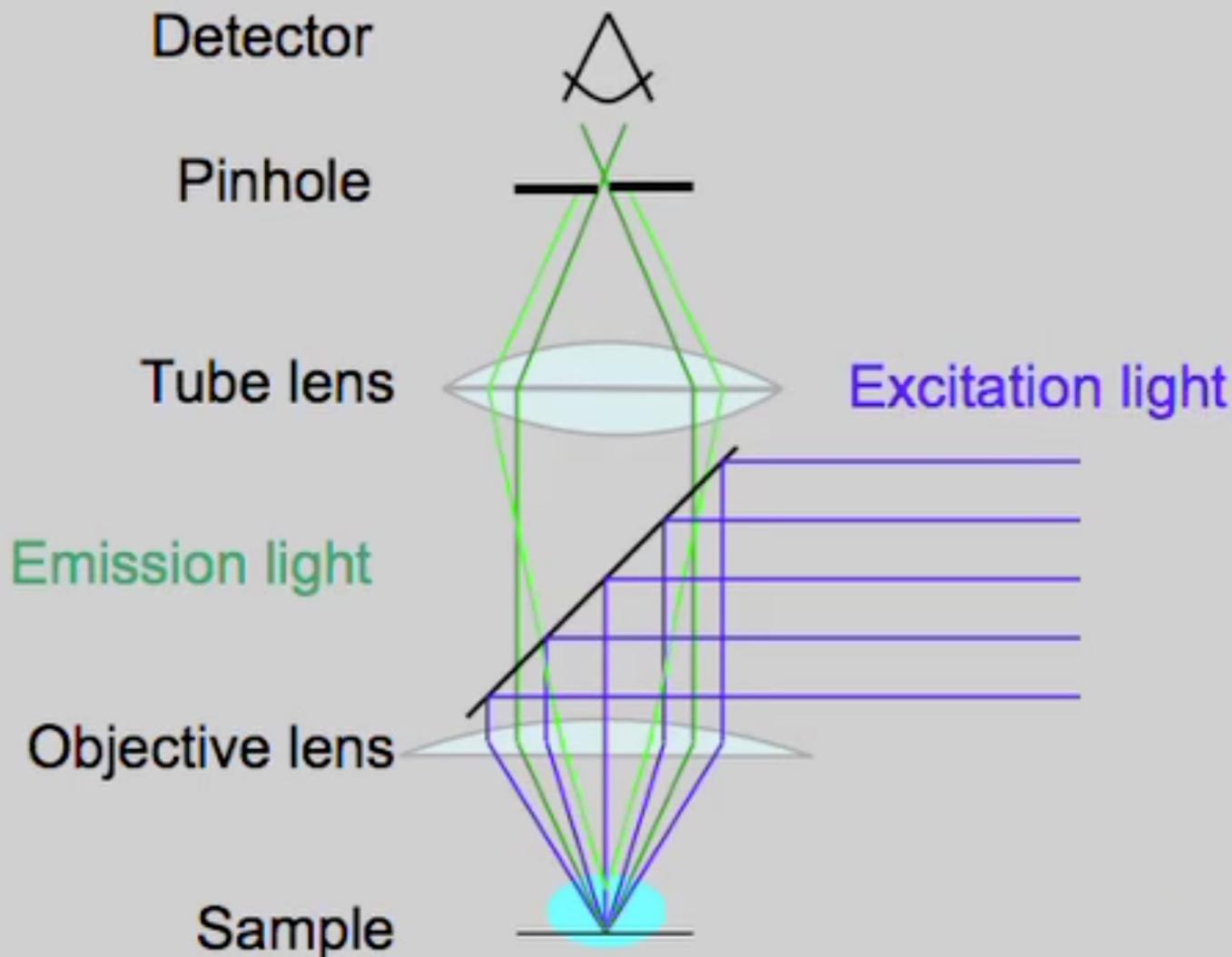


Image by raster scanning

Create image by scanning
laser point-by-point over
sample and recording intensity
at each spot.

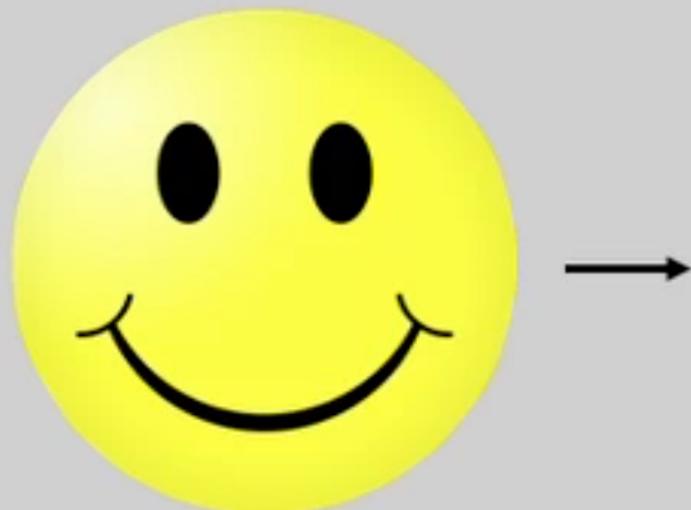


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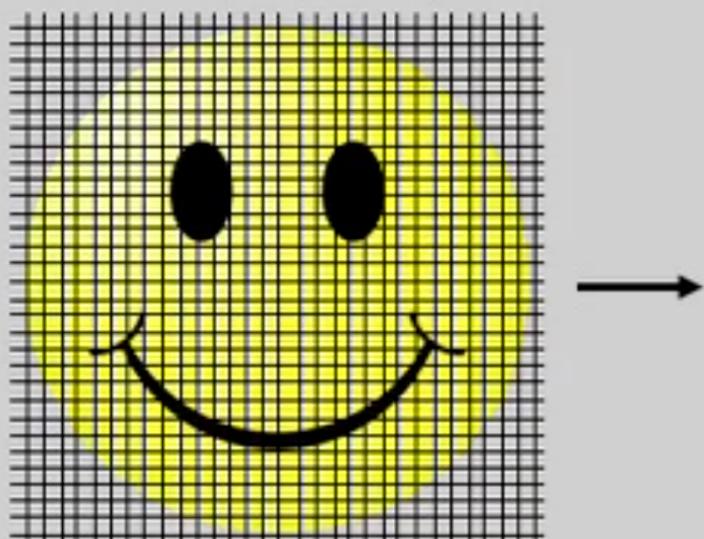
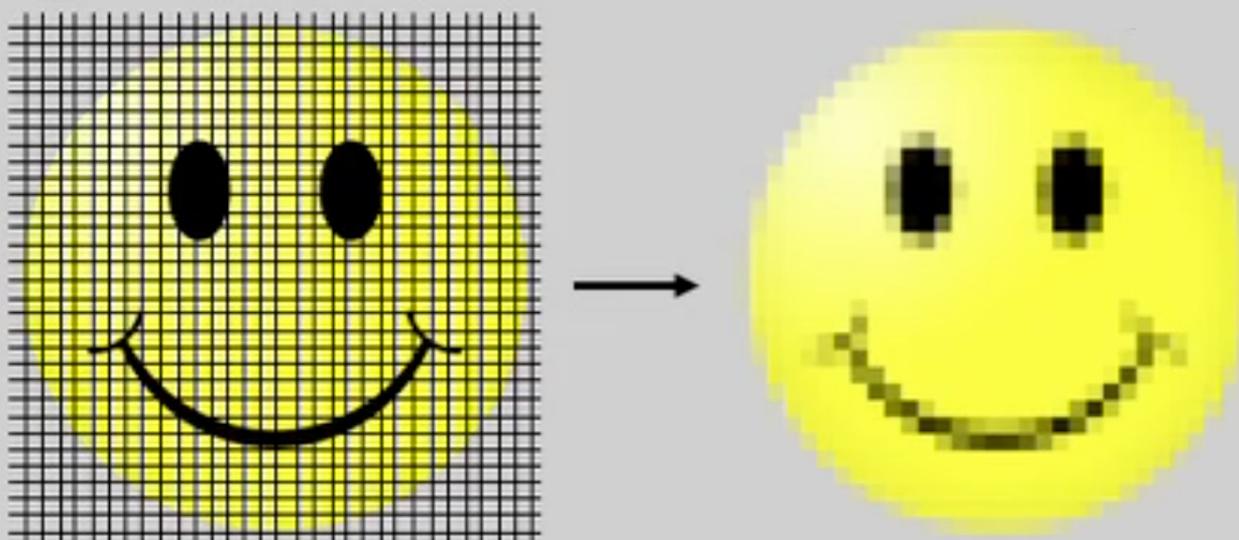


Image by raster scanning

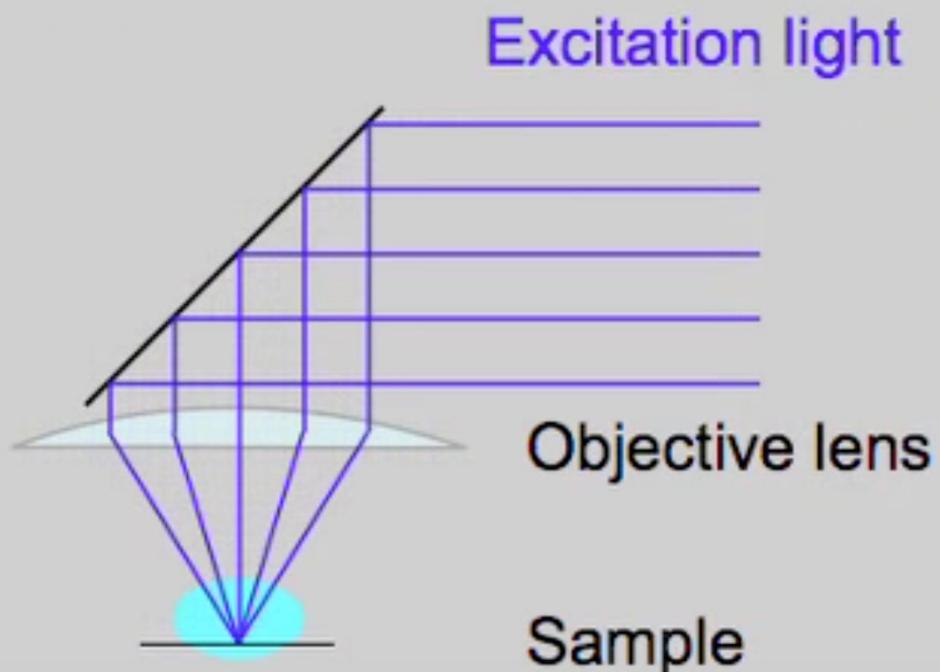
Create image by scanning
laser point-by-point over
sample and recording intensity
at each spot.



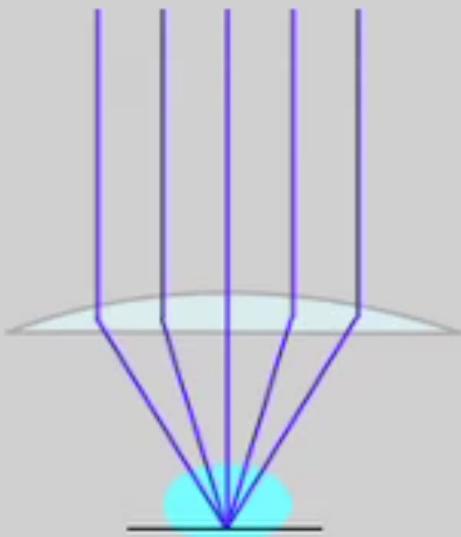
Light sources

Excitation light must be focused to a diffraction limited spot

Enter the laser:
Perfectly collimated and high power

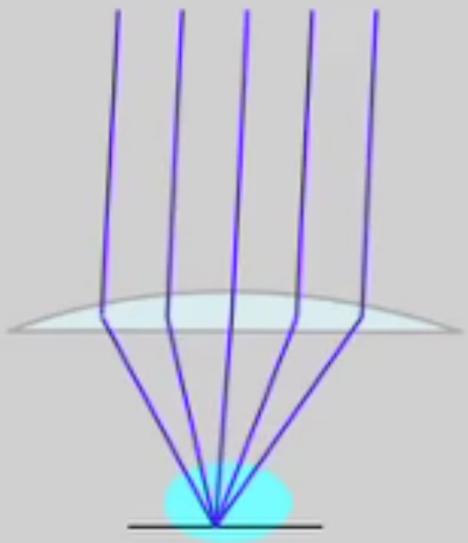


Scanning



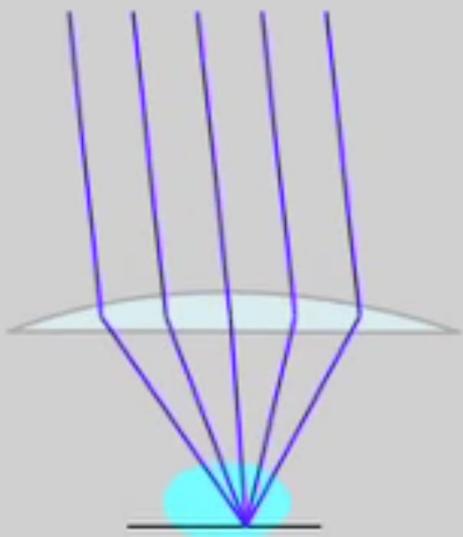
Changing entrance angle
of illumination moves
illumination spot on
sample

Scanning



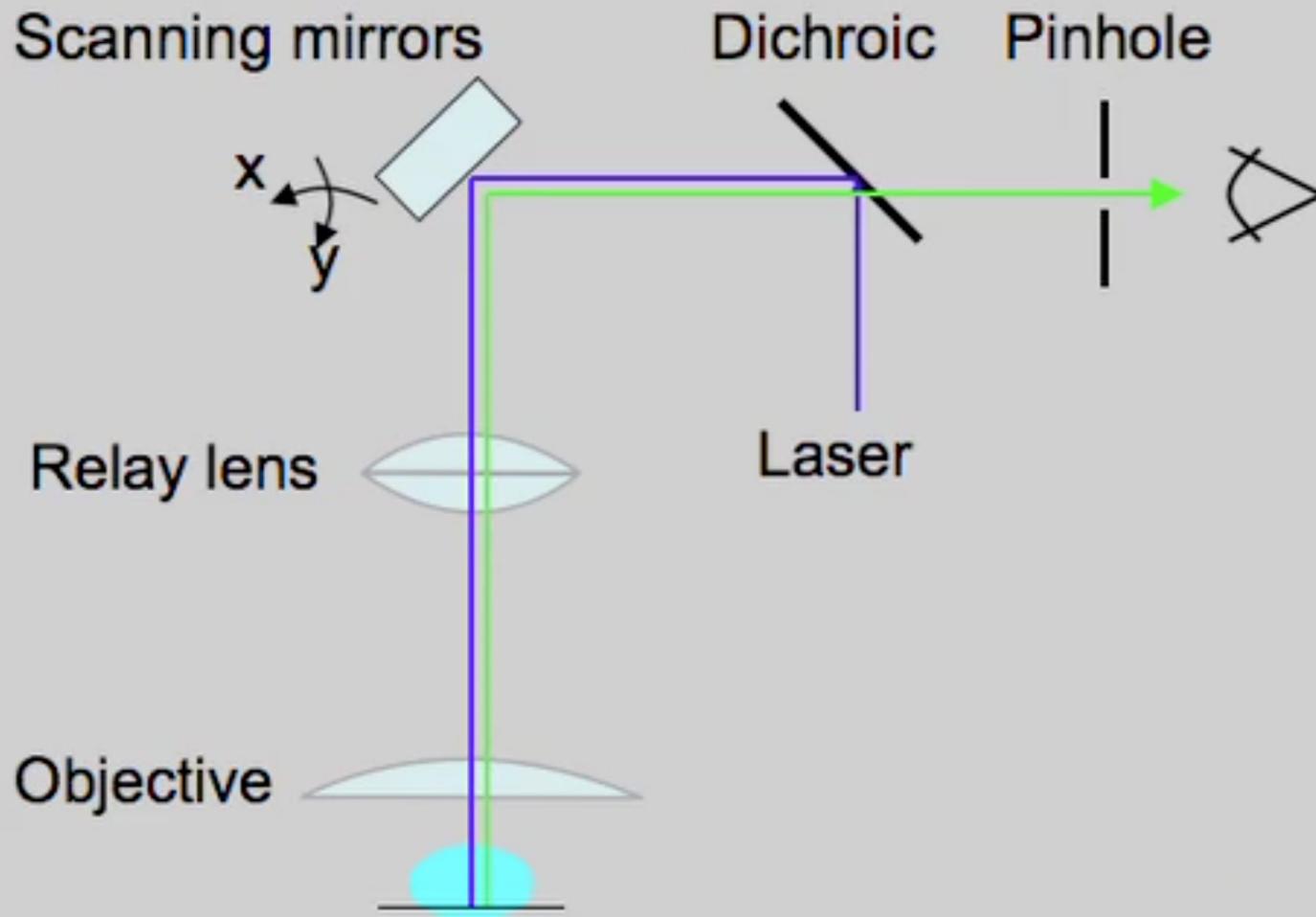
Changing entrance angle
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Scanning



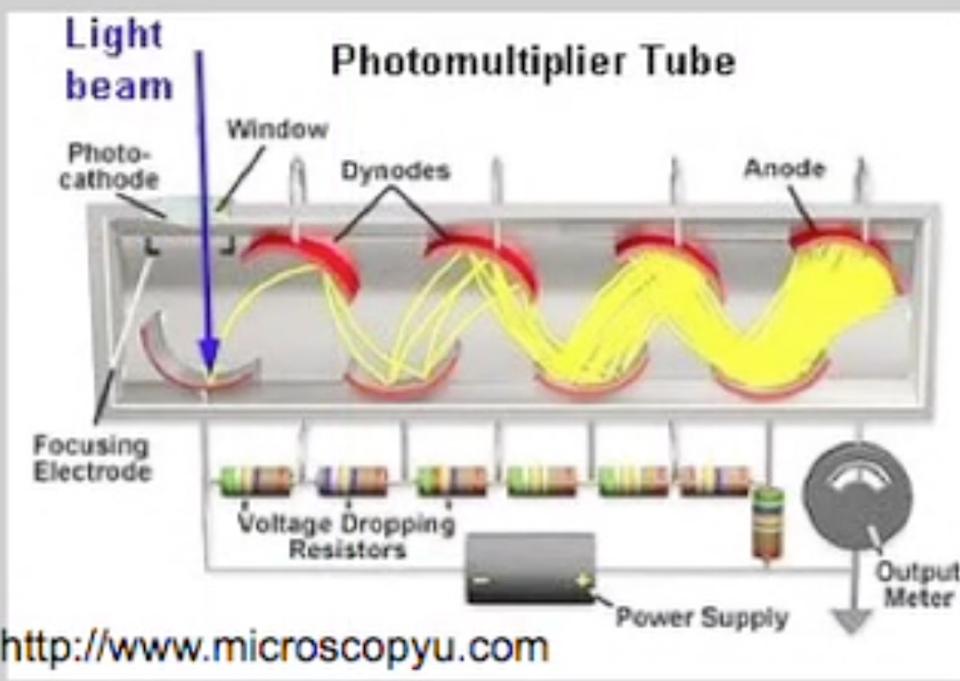
Changing entrance angle
of illumination moves
illumination spot on
sample

Confocal optical path



Detectors - photomultiplier tube (PMT)

- Must be fast – confocal beam spends only a few μ s on each pixel



Confocal microscope drawbacks

Scans excitation spot point-by-point to build up image

Slow (~1 sec to acquire an image)

Low light efficiency (due to use of PMT as detector)

Solution:

Use multiple pinholes and a camera

A Solution: Spinning Disk Confocal

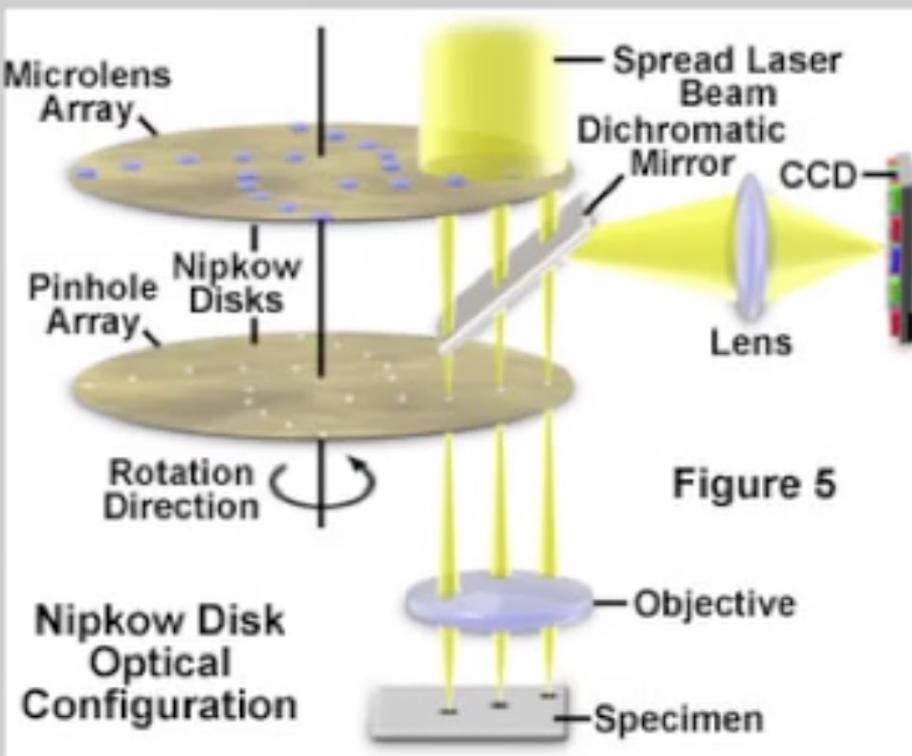


Figure 5

Image with many pinholes at once, so fast

Use CCD as detector, so high light efficiency

A Solution: Spinning Disk Confocal

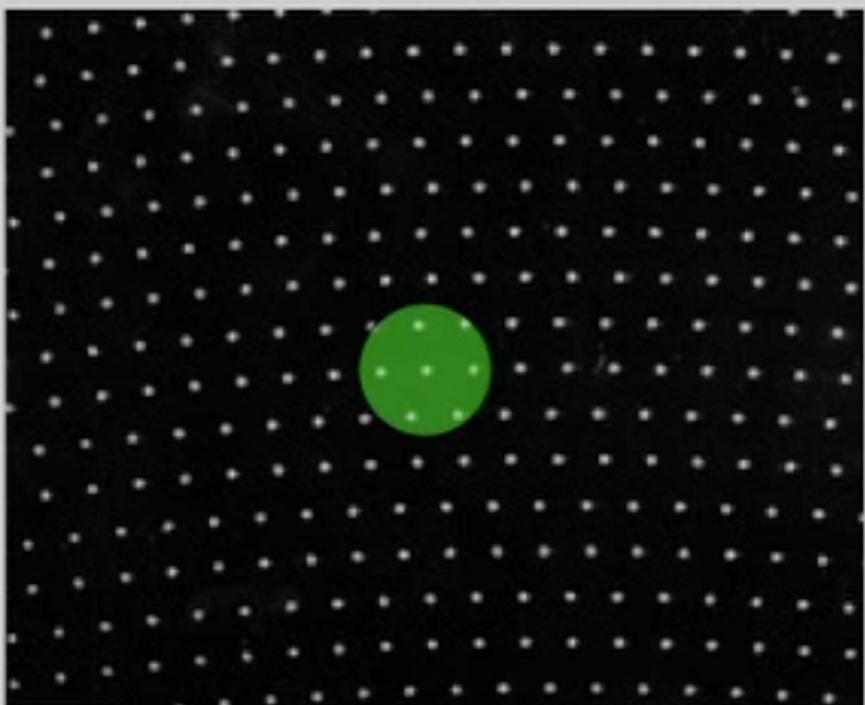


Image with many pinholes at once, so fast

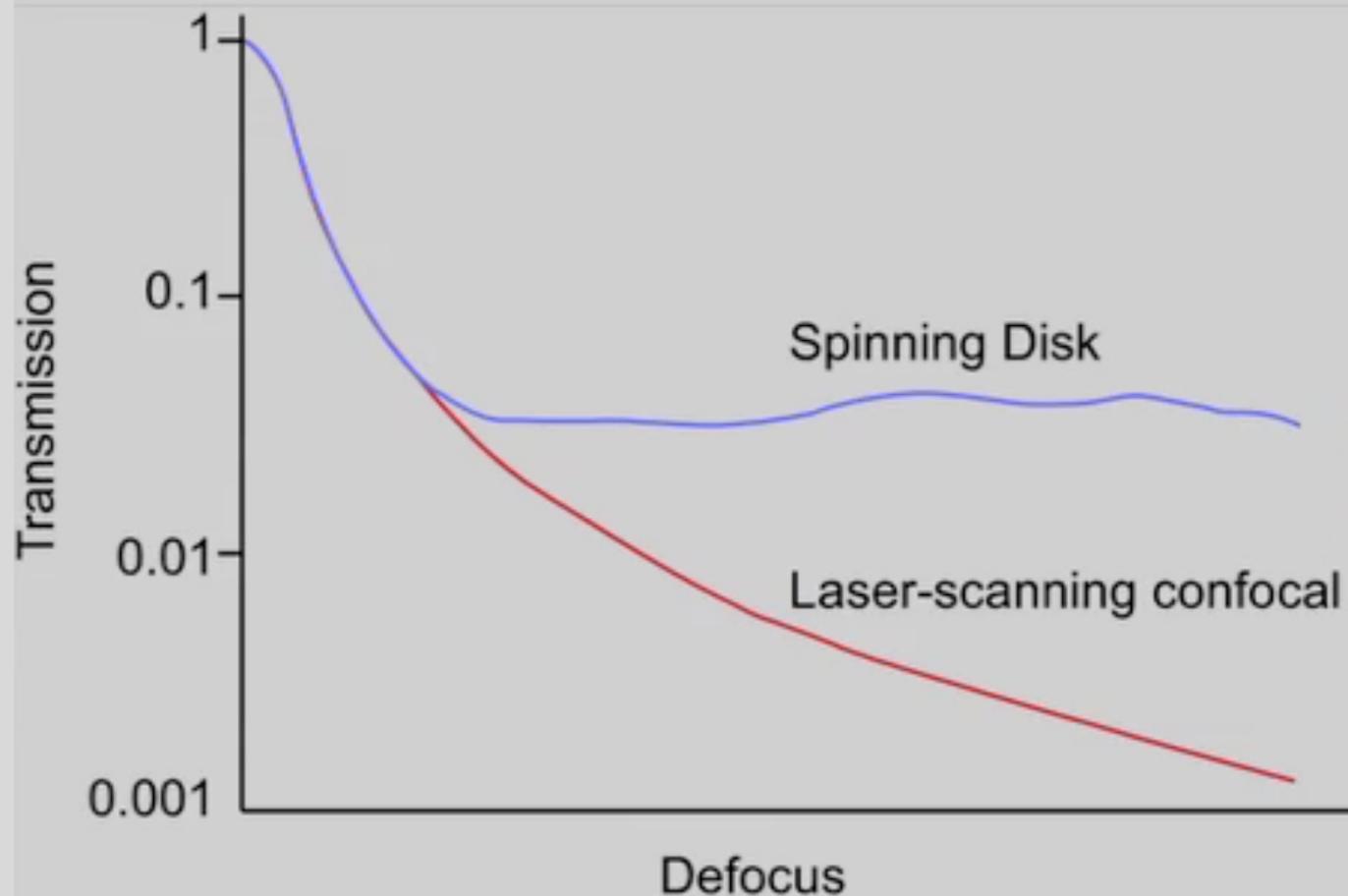
Use CCD as detector, so high light efficiency

The downside to the spinning disk

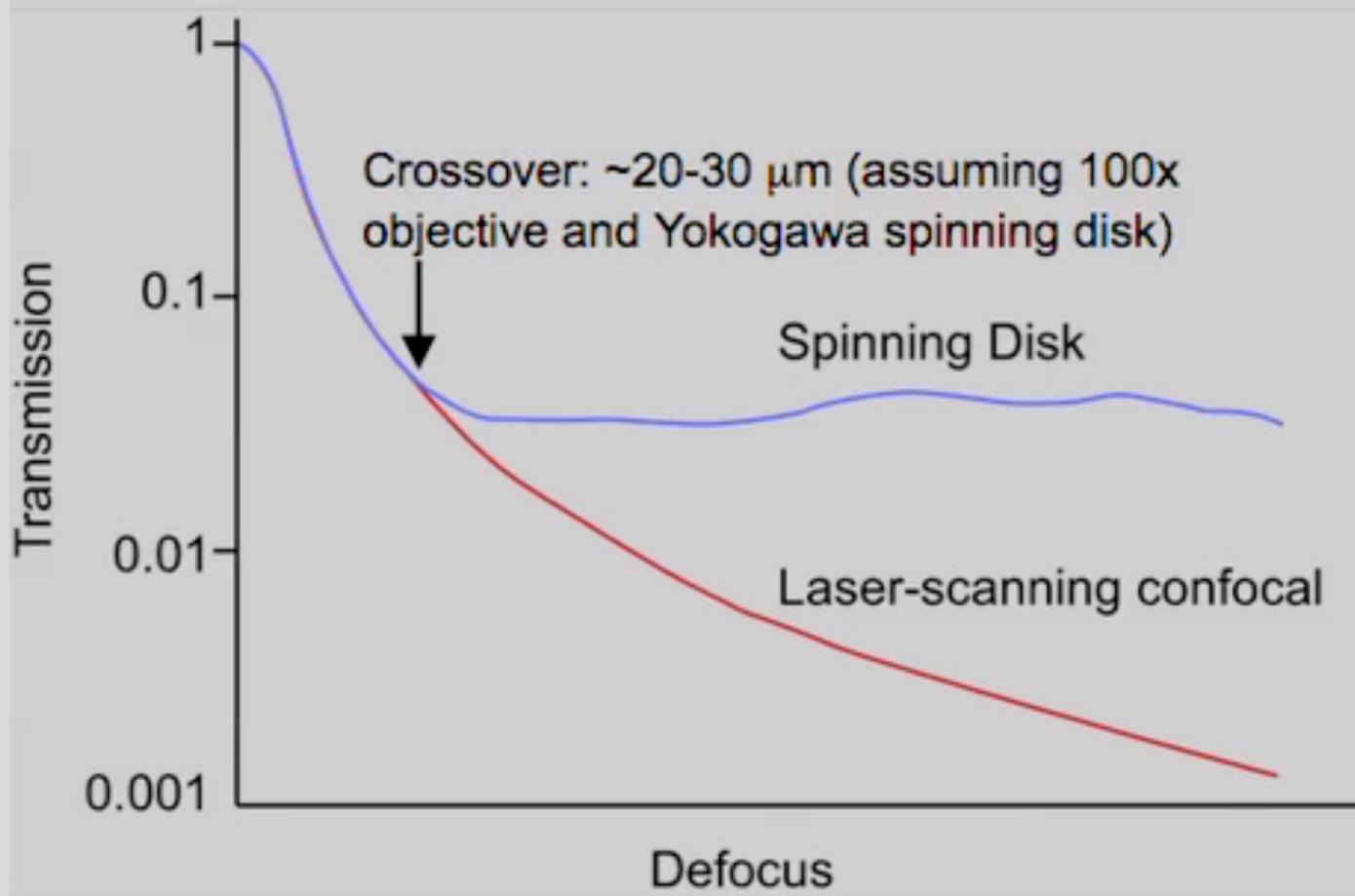
Limited out of focus rejection



Out of focus rejection for spinning disk and laser-scanning confocal



Out of focus rejection for spinning disk and laser-scanning confocal



When to use confocal?

Fixed samples:

Thin and/or low mag:

Widefield

Thick ($>10 \mu\text{m}$), high mag

Laser scanning confocal

Thick ($>30 - 50 \mu\text{m}$):

Laser scanning confocal

Thick ($>100 - 200 \mu\text{m}$):

2-photon, other specialized techniques