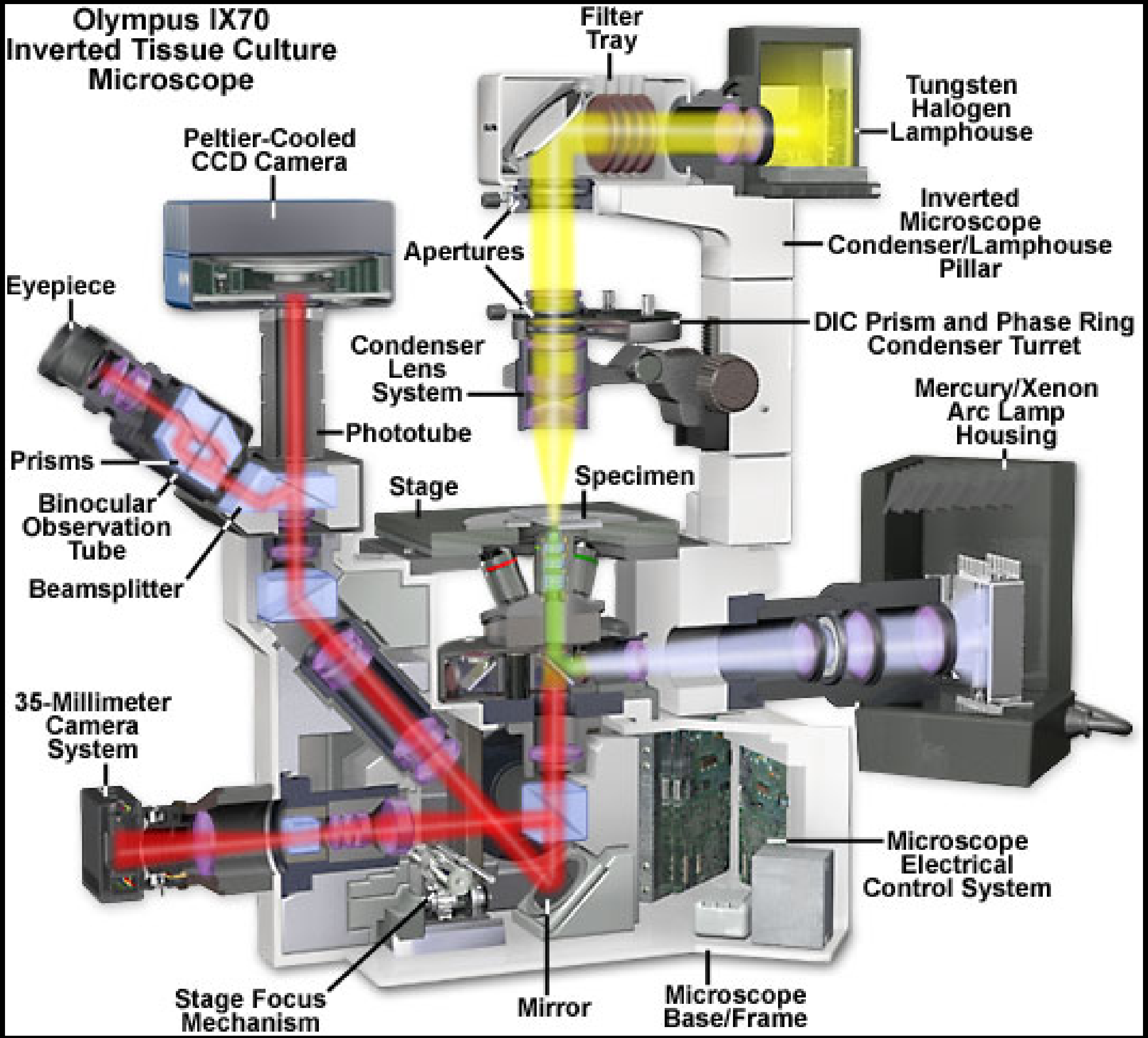
Modes of light microscopy
Choosing the appropriate system

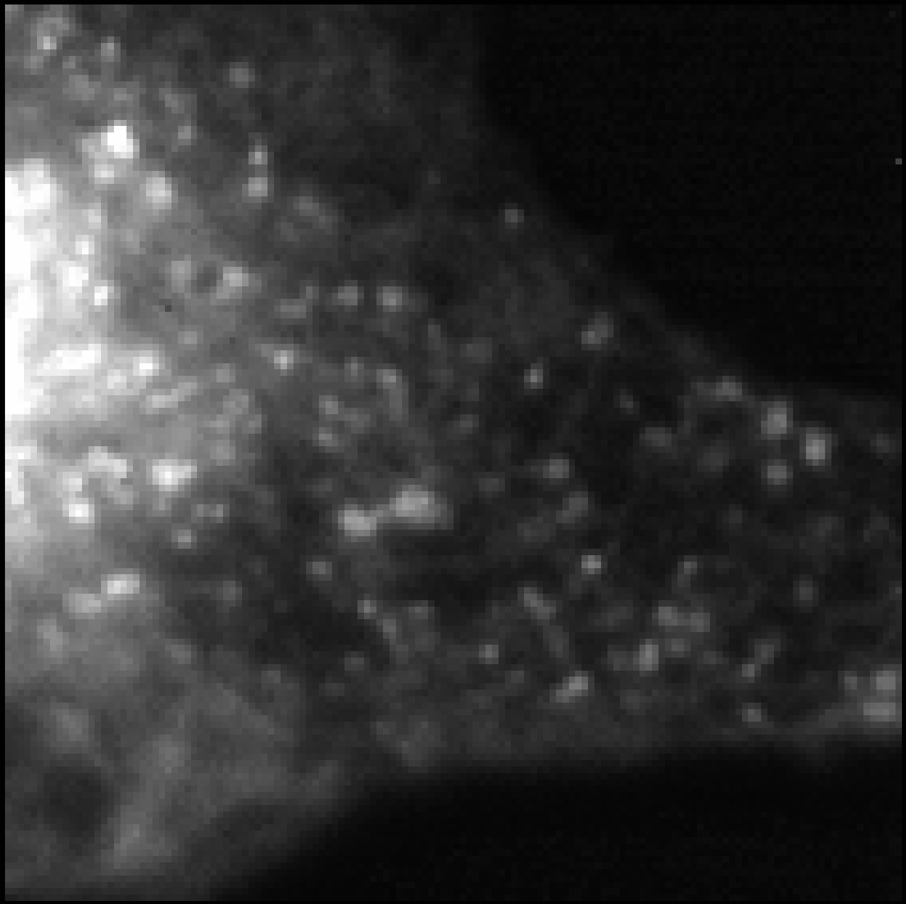
- Wide-field microscopy
- Confocal microscopy
- Multi-photon microscopy

Wide-field, inverted fluorescence microscope

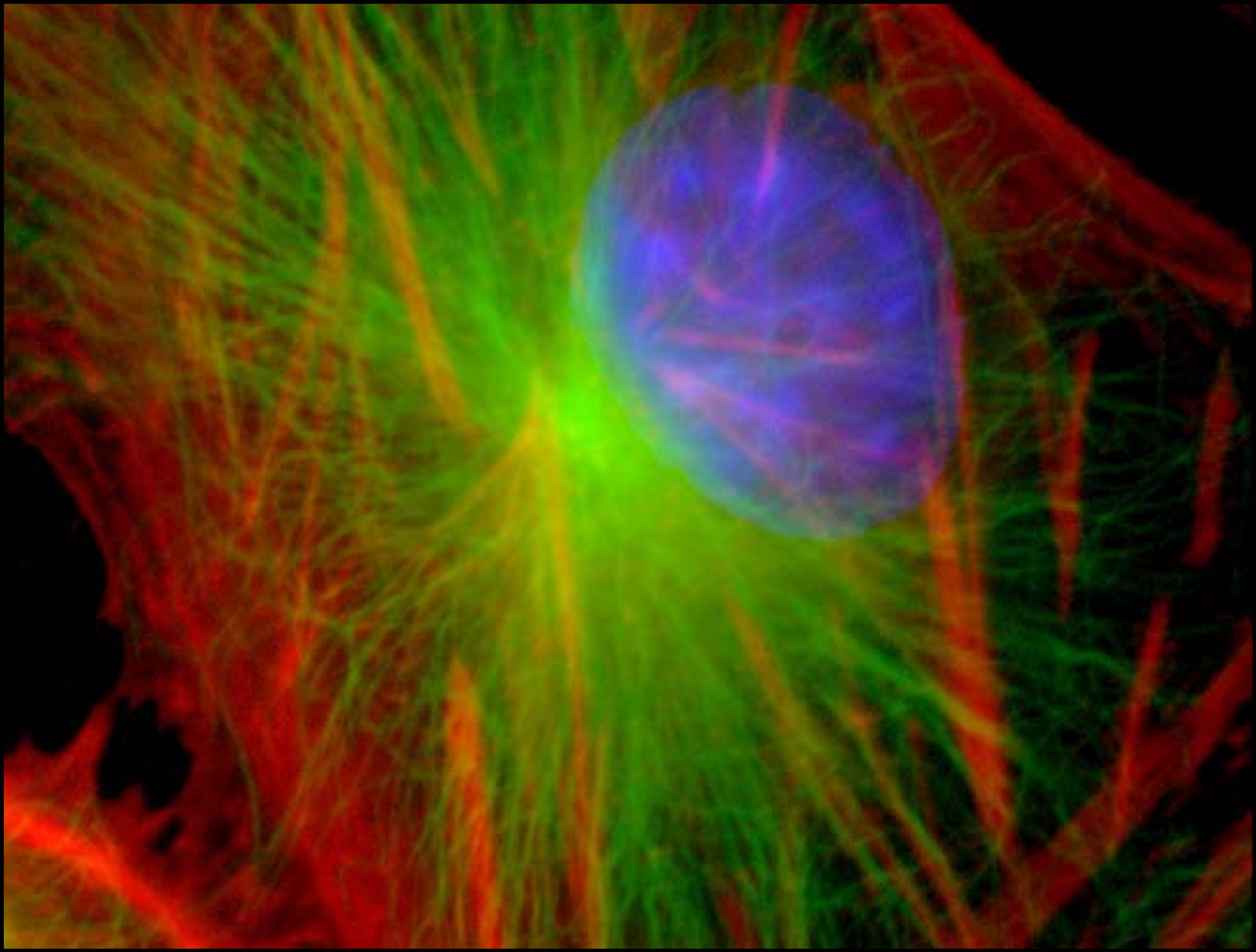


Nikon MicroscopyU

Endosome migration in living cells – imaging a flat cell via wide field microscopy and a CCD

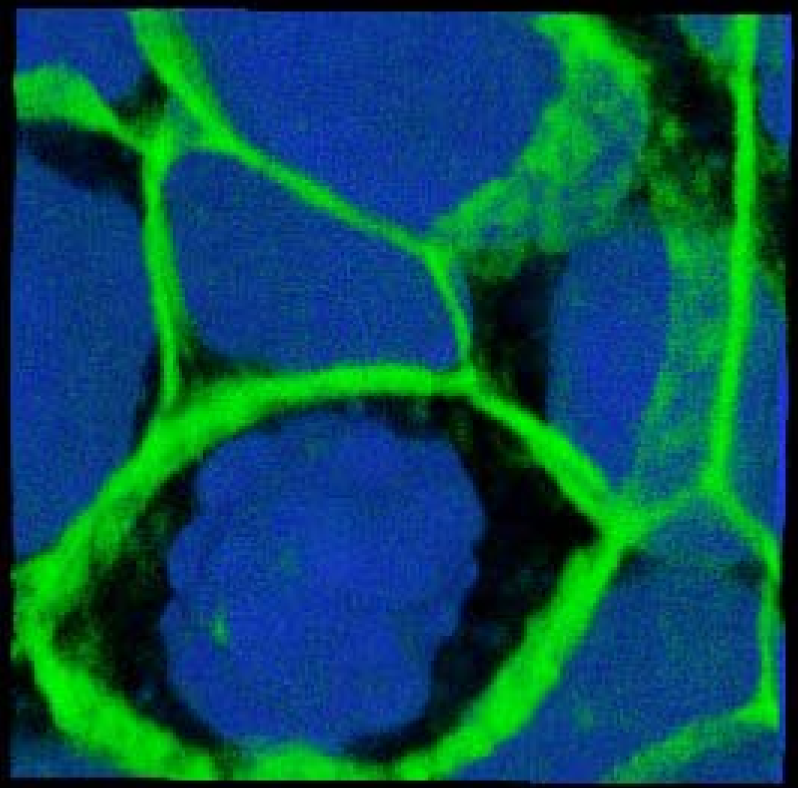


Some cells can be induced to be flat – the cytoskeleton of a fibroblast grown on a solid substrate



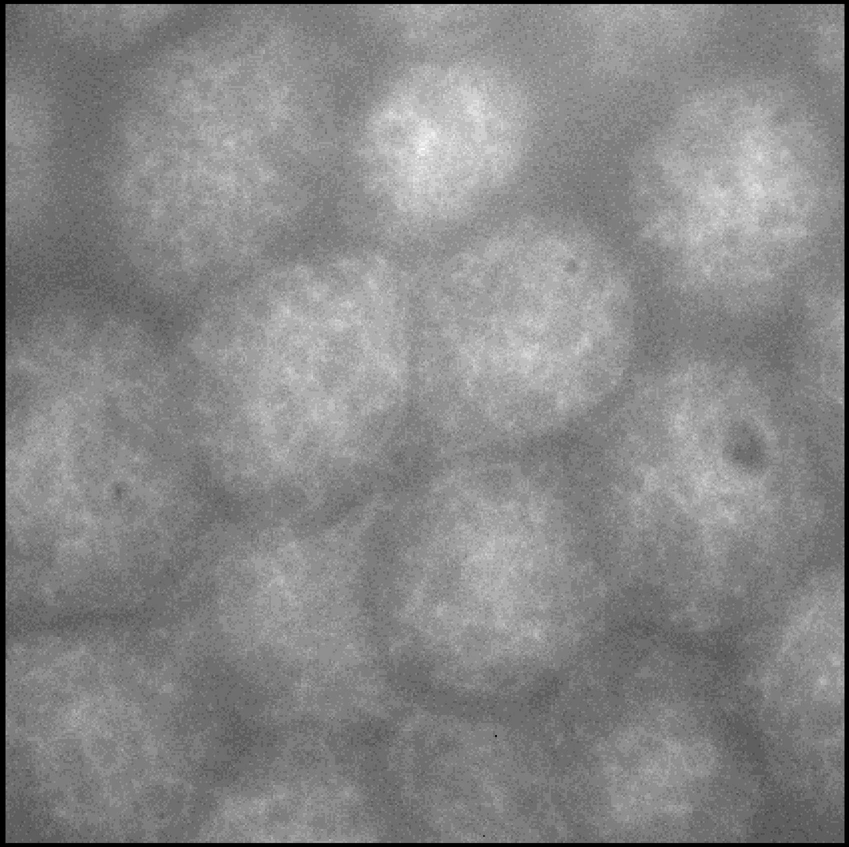
But most cells are 3-dimensional

3D rendering of E-cadherin and nuclei in polarized epithelial cells

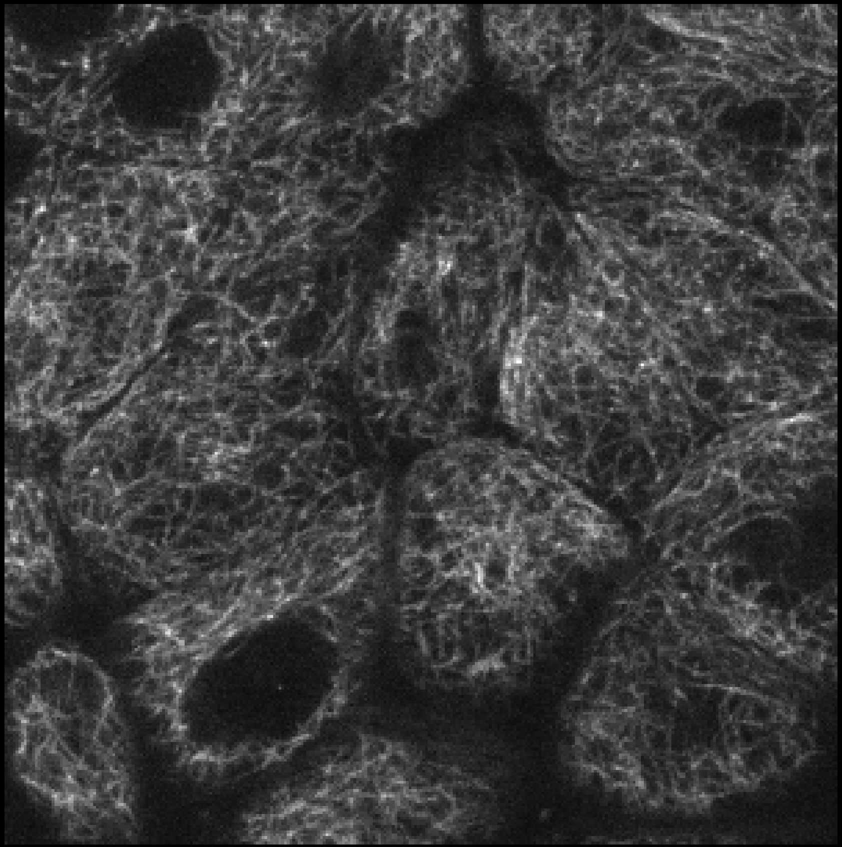


Cells are 3-dimensional – the need for "optical sections"

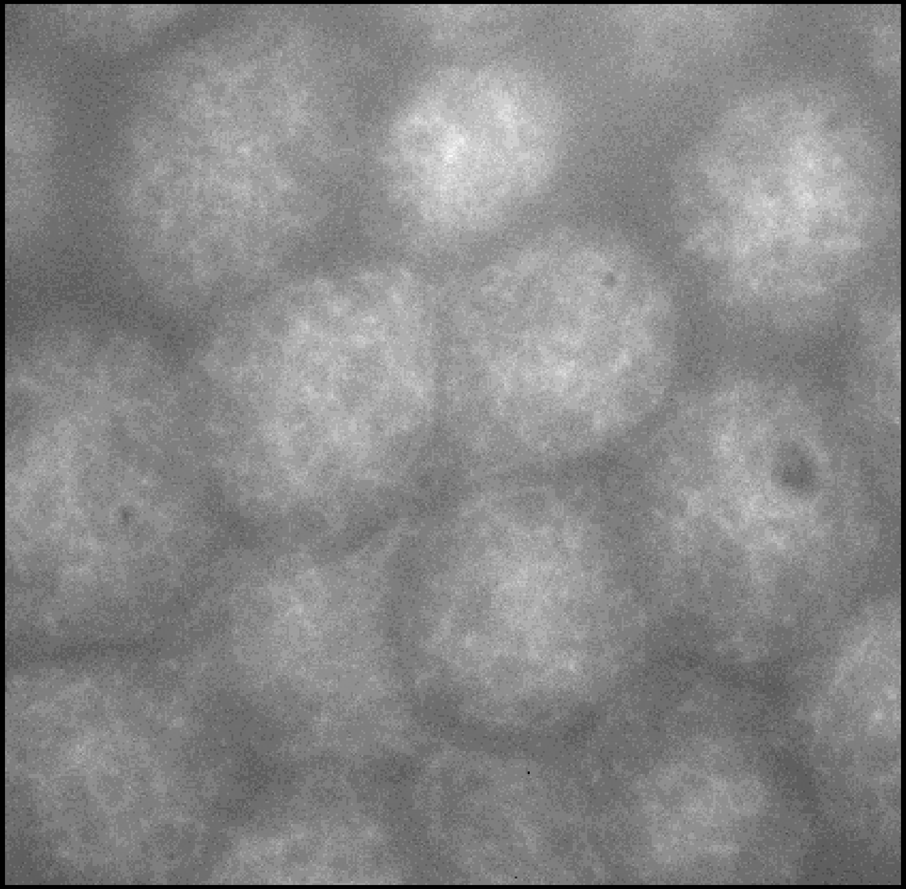
Conventional microscope

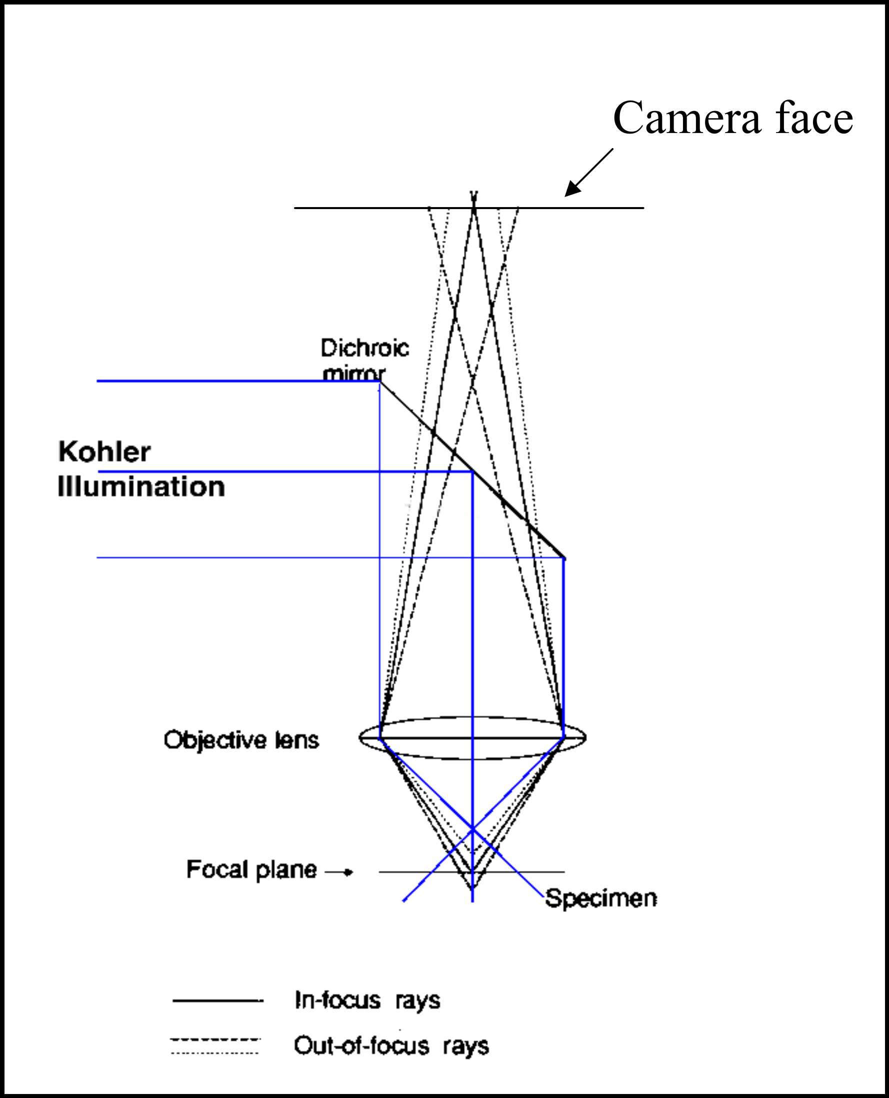


Confocal microscope

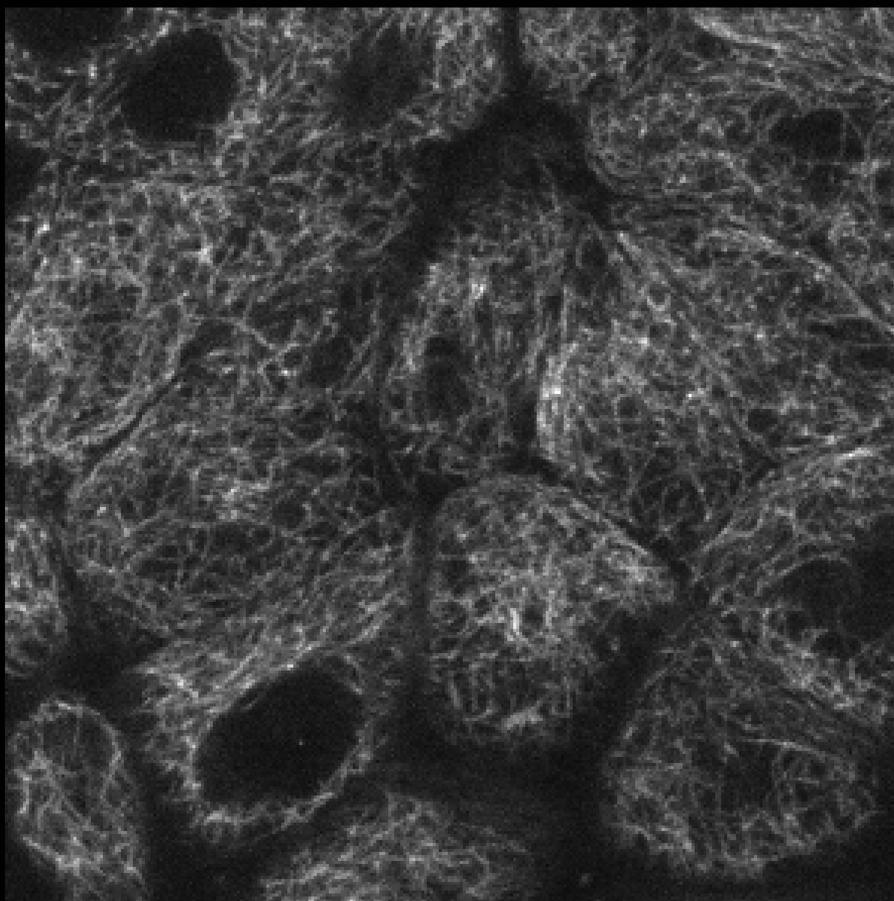


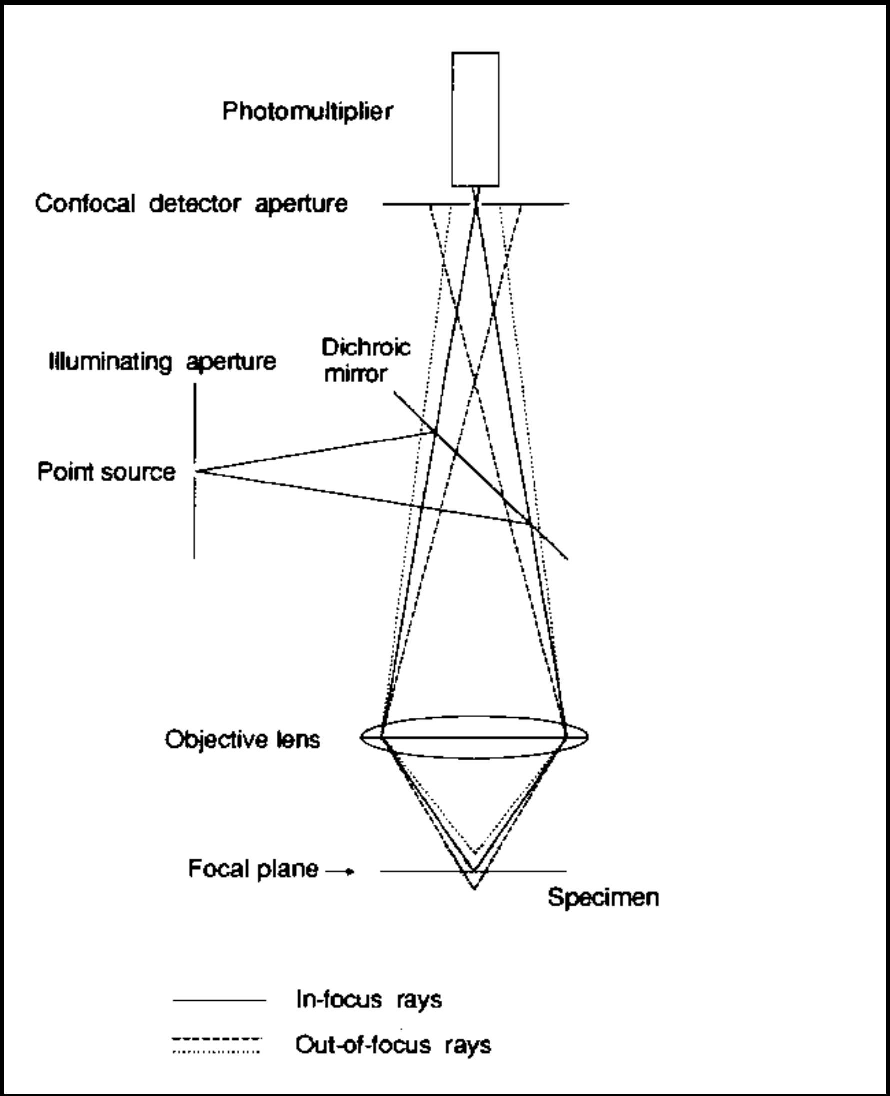
Imaging 3-dimensional structures – conventional epifluorescence



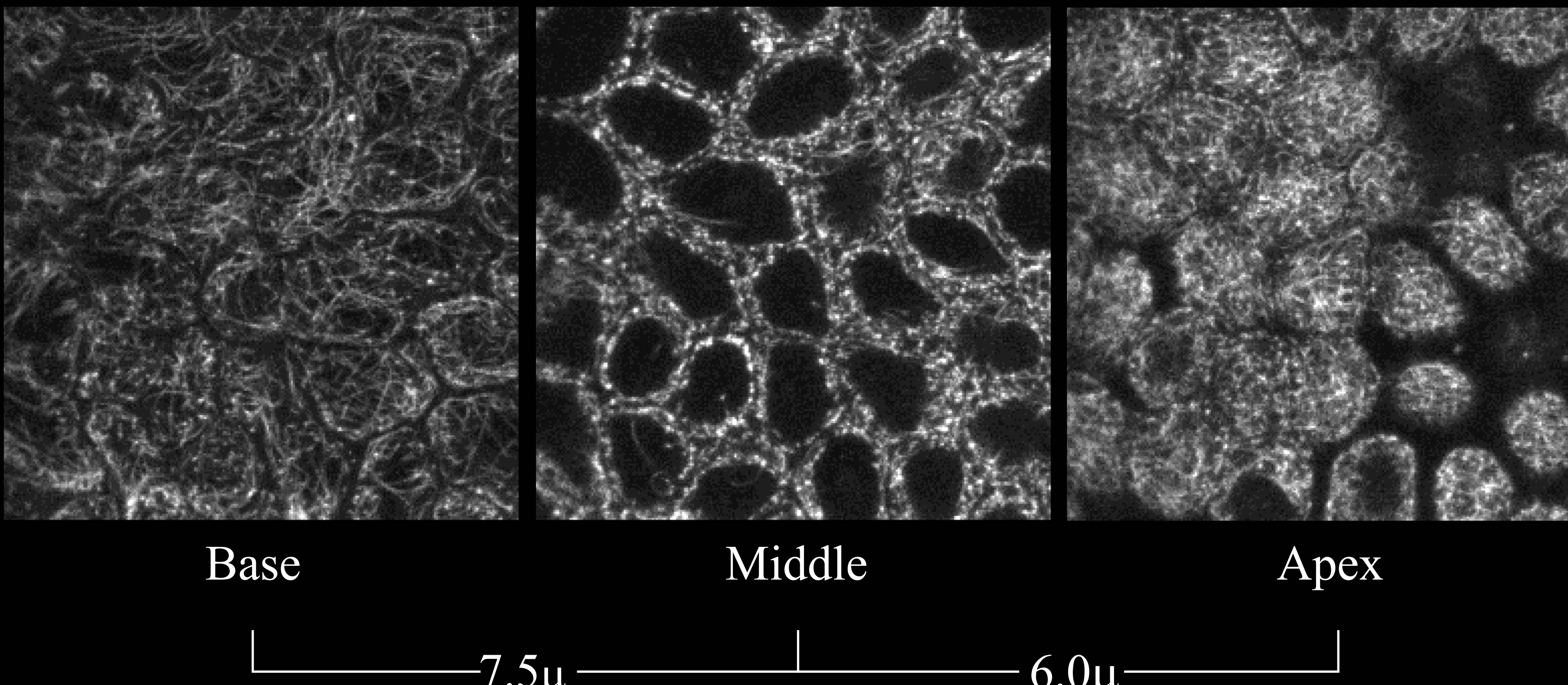


Imaging 3-dimensional structures – Confocal microscopy

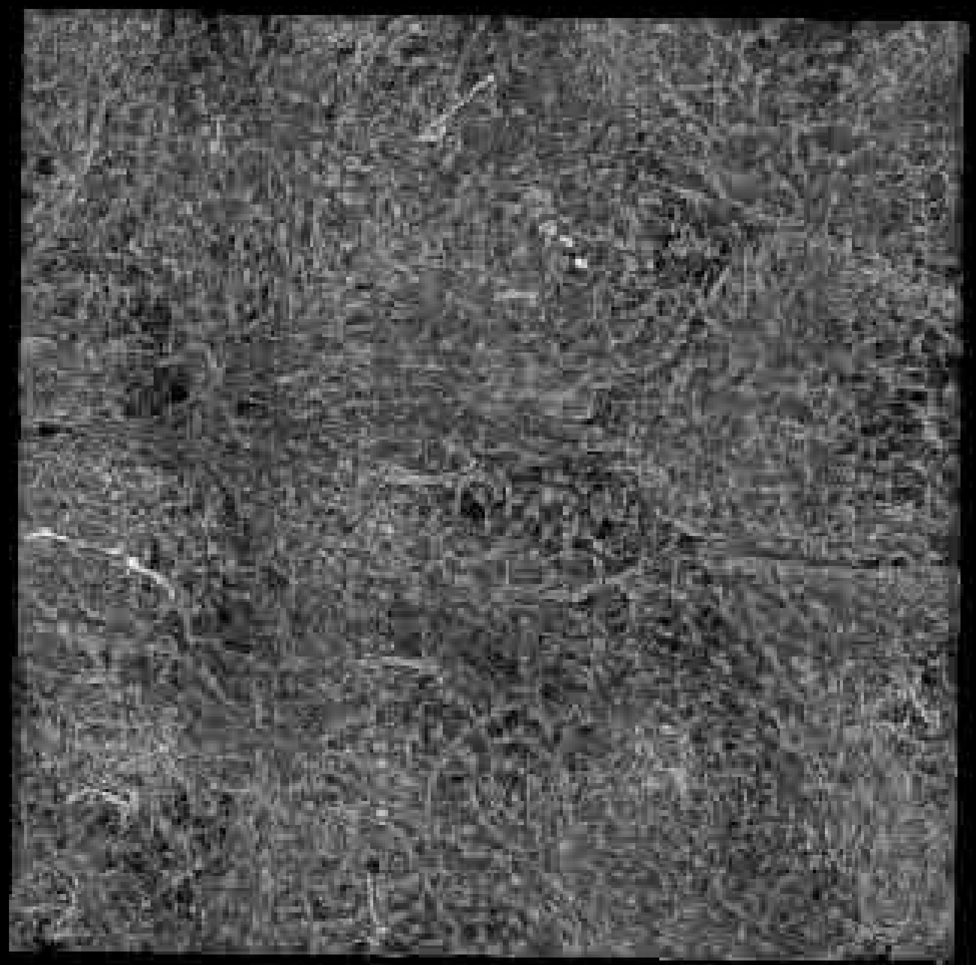




Serial confocal optical sections of the microtubule cytoskeleton of polarized epithelia

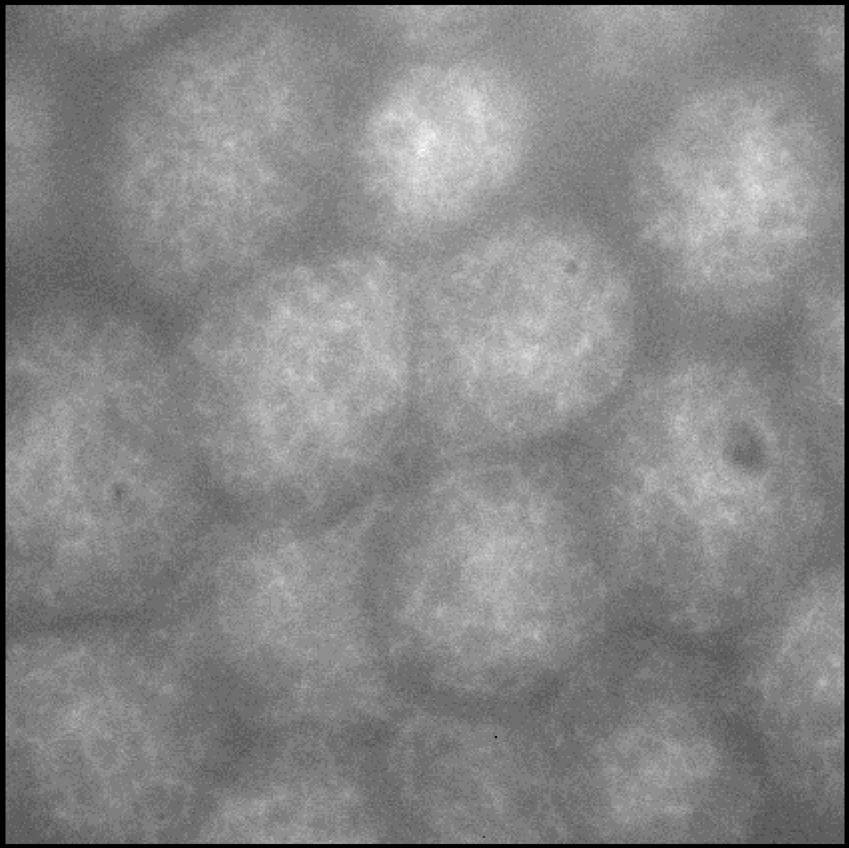


3-D rendering of the organization of microtubules in polarized epithelia – Bob Bacallao

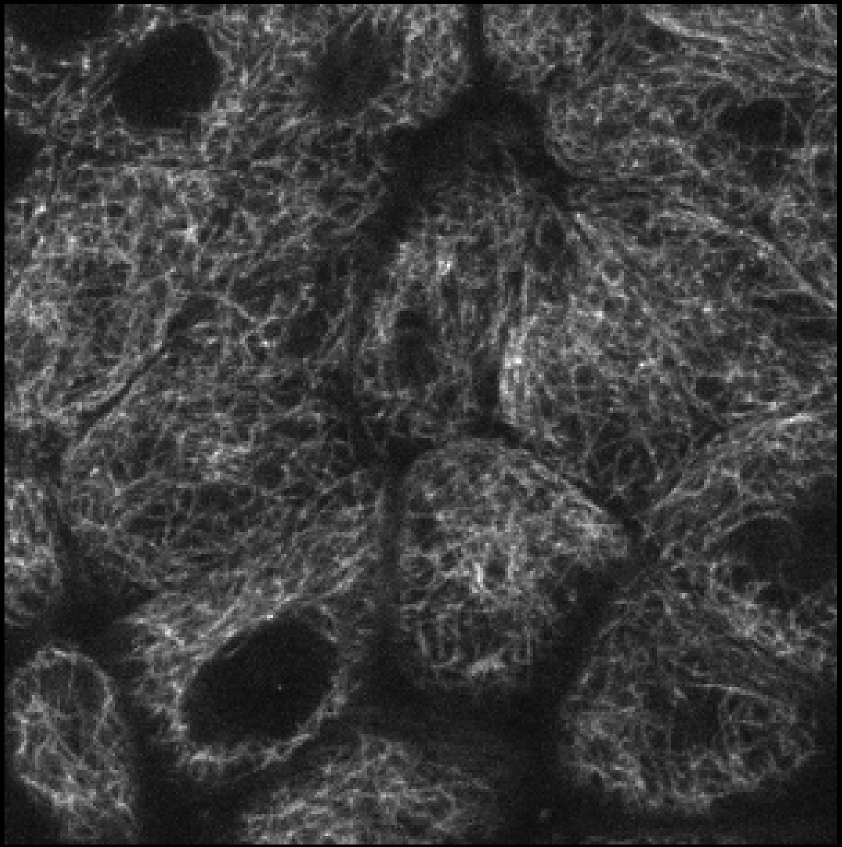


Resolution of confocal microscopy is only ~ 1.4 times better – the big improvement lies in background rejection

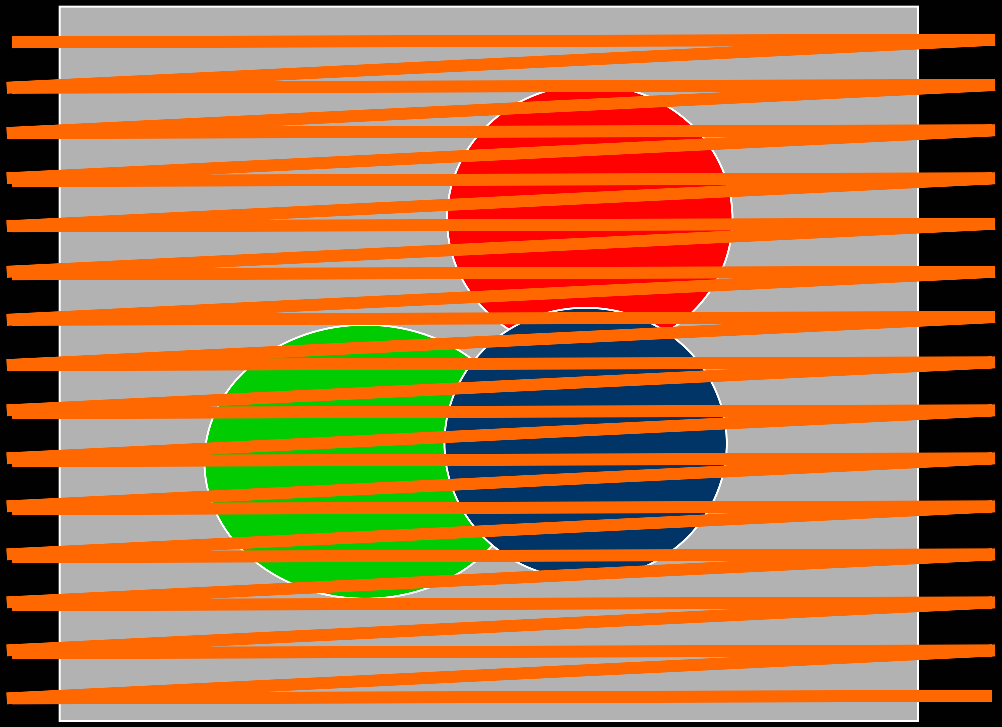
Conventional microscope



Confocal microscope



Confocal microscopy builds an image by point scanning



Need to acquire one point at a time. This limits acquisition to ~1 frame/sec. Limited by number of photons per pixel.

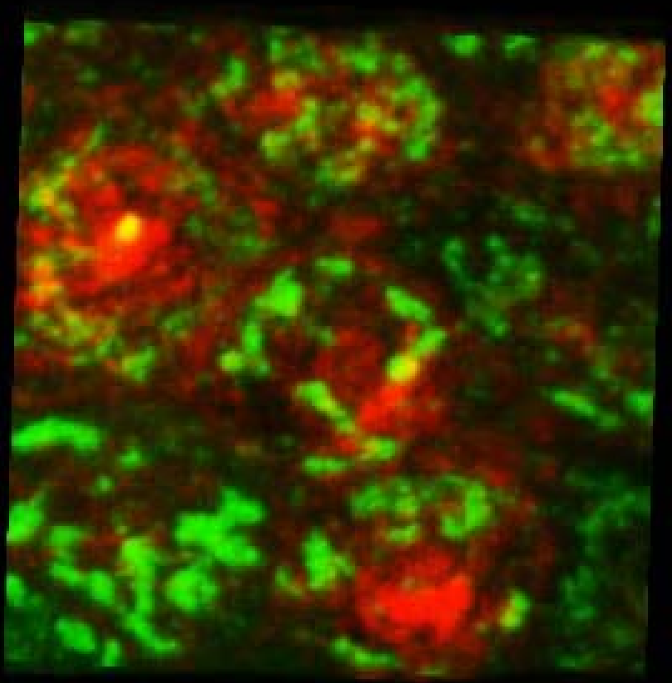
Dave Piston, Vanderbilt

In a 1 second wide-field exposure all pixels are exposed (in parallel) for 1 second.

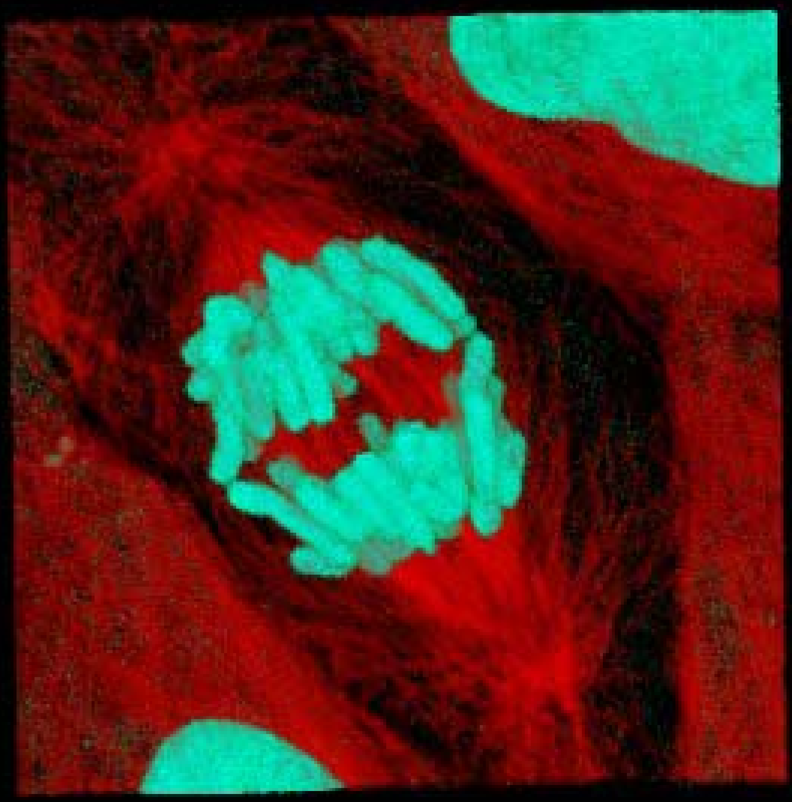
For a 1 second acquisition using a confocal microscope, each pixel is collected for less than 4 μs, requiring 256,000 x higher incident intensity and 256,000 separate measurements.

Point imaging is fundamentally different from wide-field imaging and generally requires lasers, sensitive detectors, and fast computers. These key components became available for common use in the 1980's.

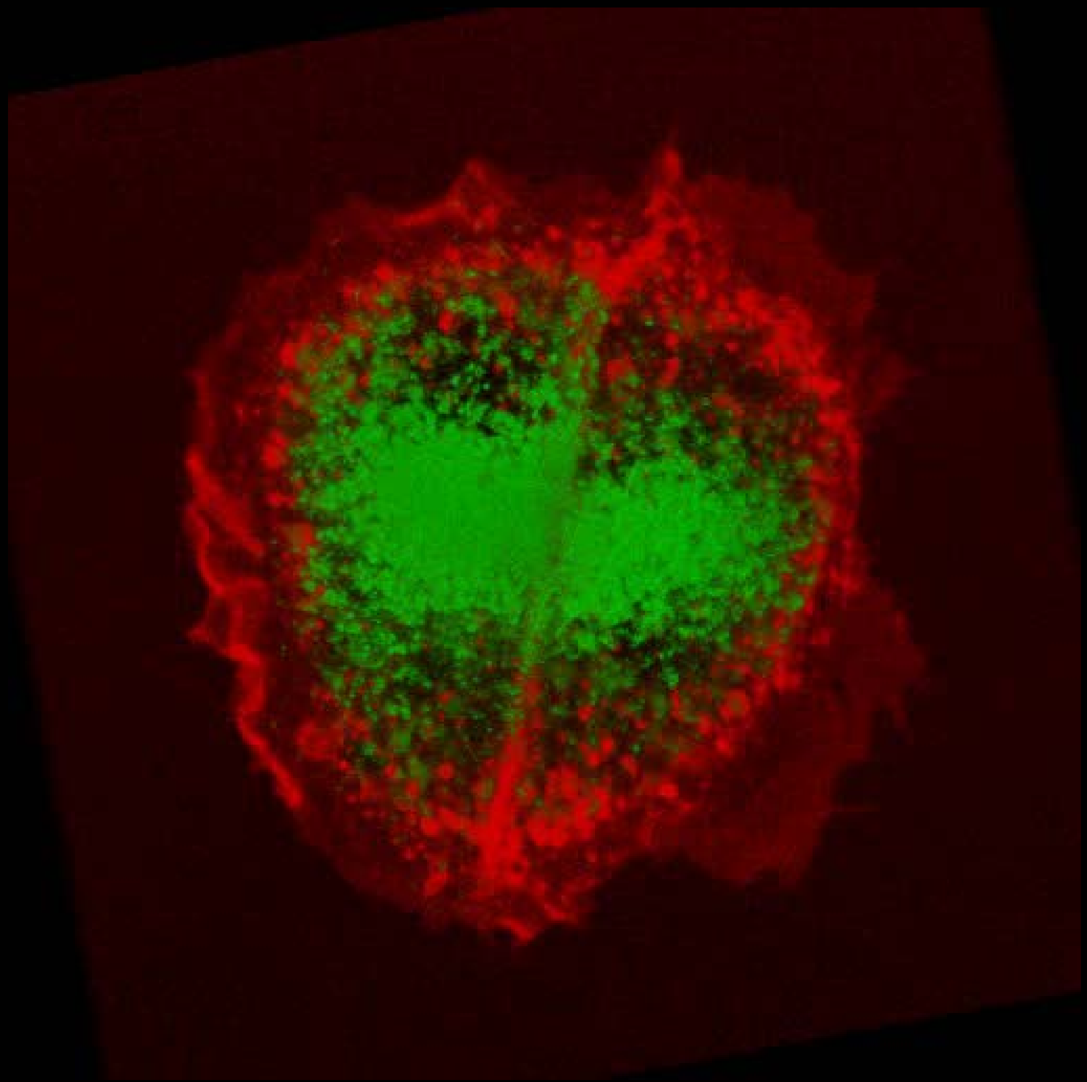
Multi-color confocal microscopy - the apical recycling endosome is distinct from the trans-Golgi network



Simultaneous analysis of multiple parameters - microtubules and chromosomes of a dividing epithelial cell – Ruben Sandoval



Subcellular distributions of internalized transferrin and Rab25 in living MDCK cells



Multicolor confocal microscopy

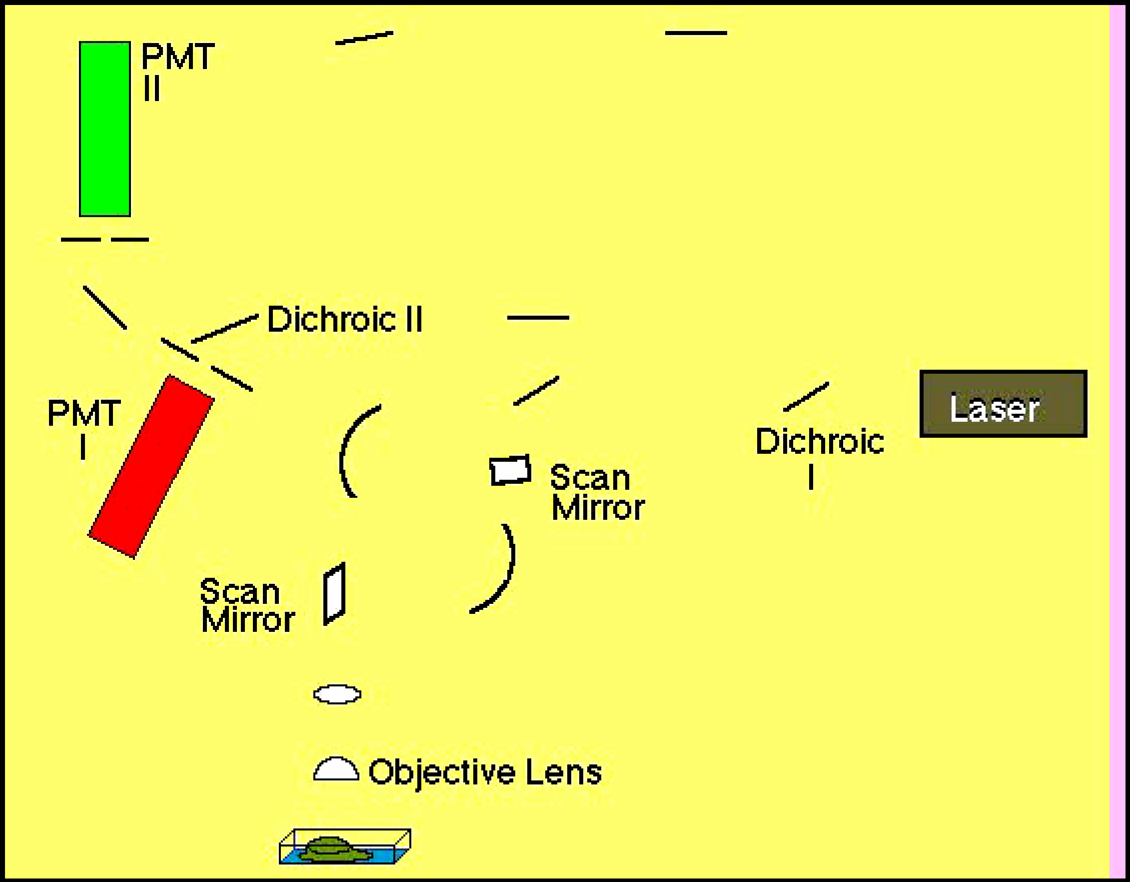
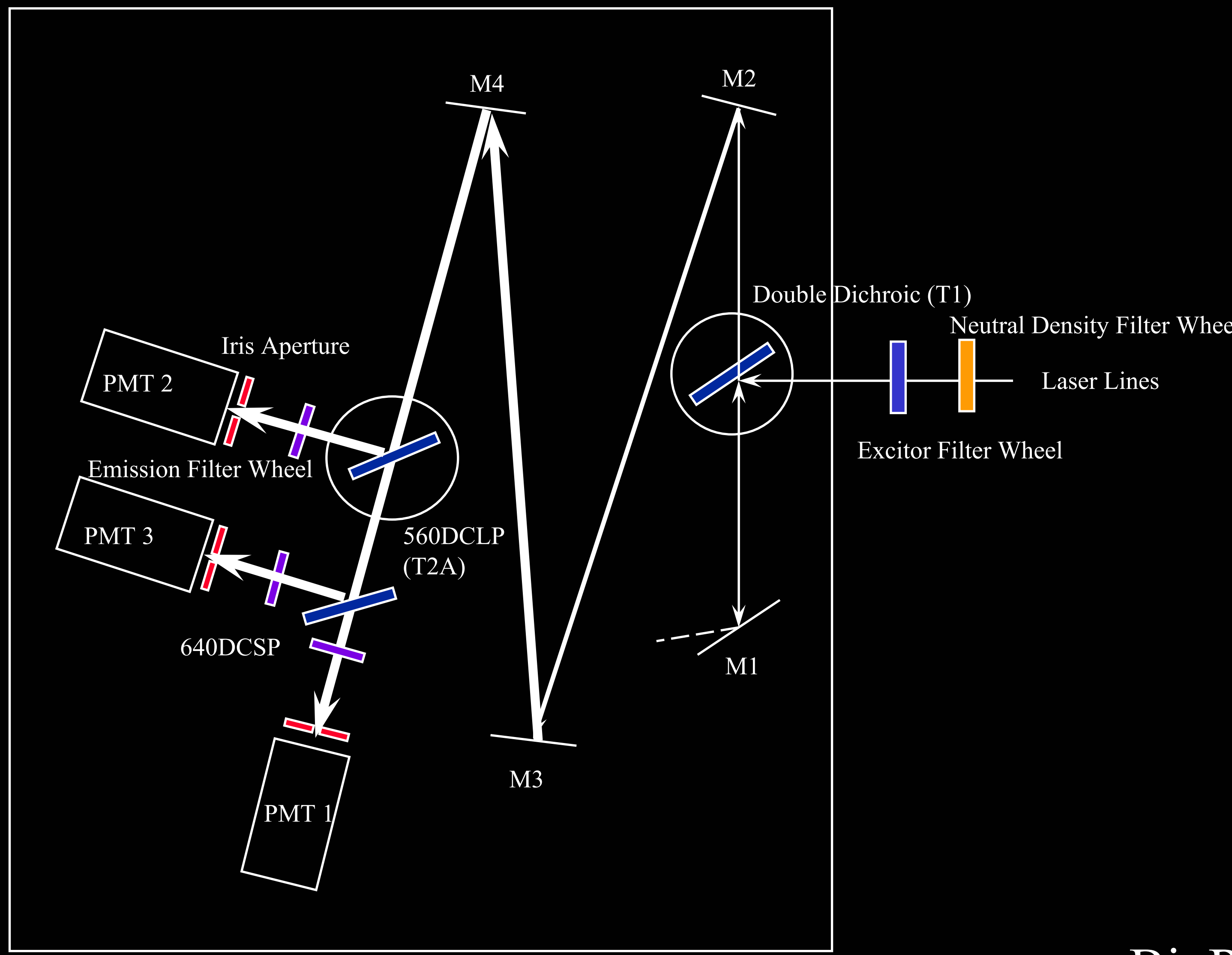


Image is built up through a raster scan requiring approximately 1 second

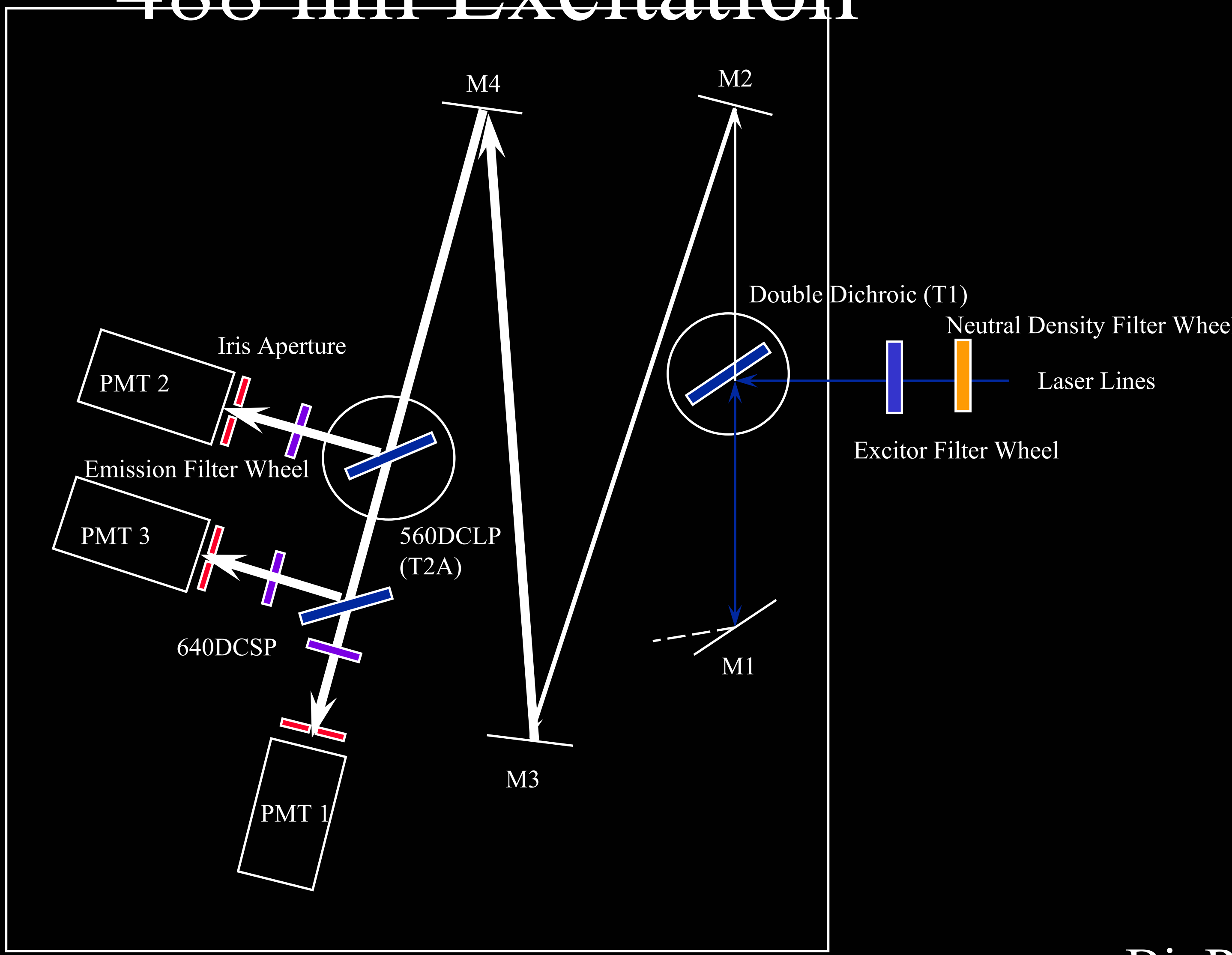
figure through the unknowing courtesy of the Integrated Microscopy Resource, Madison, Wisconsin

MRC-1024 Lightpath



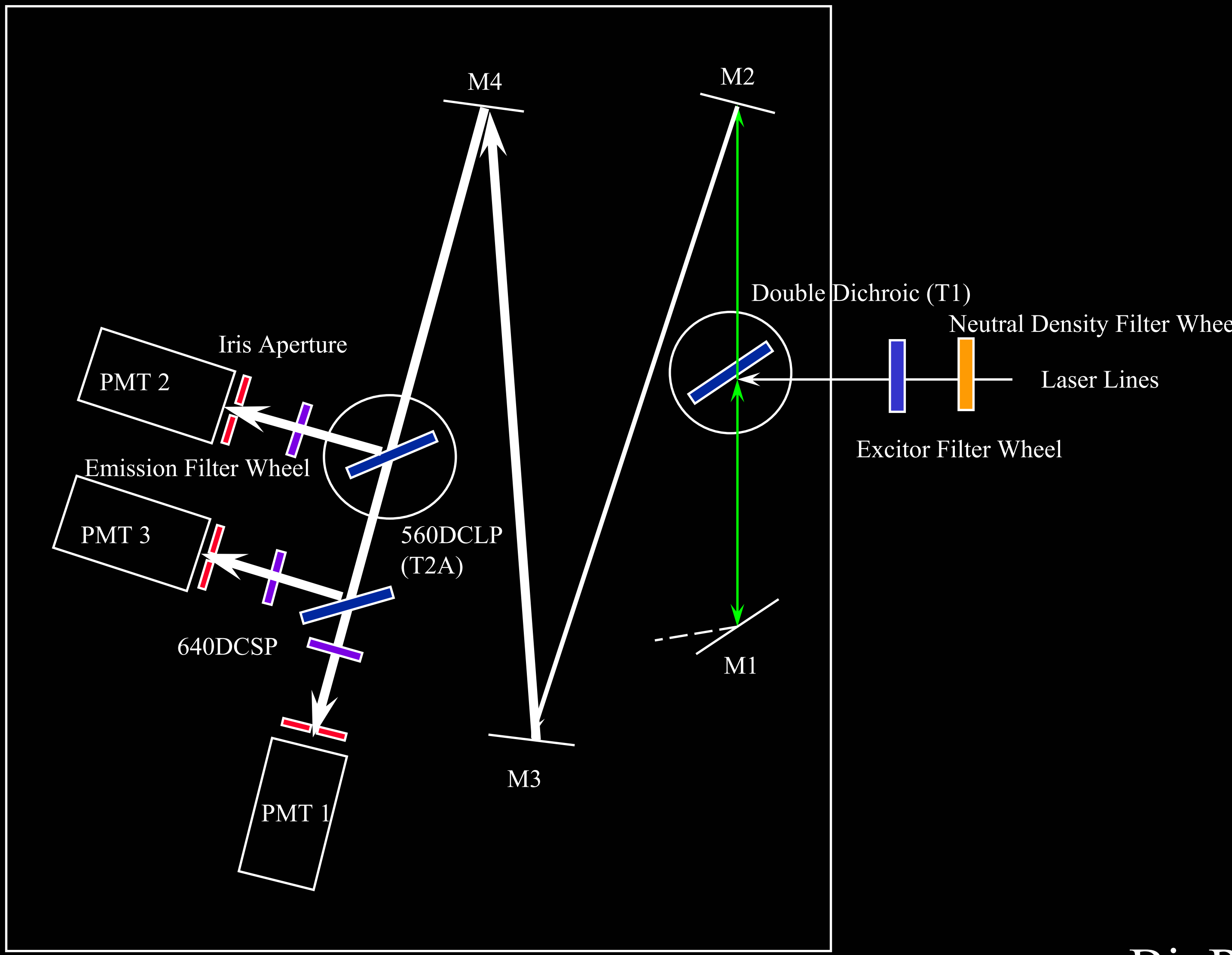
BioRad

488 nm Excitation



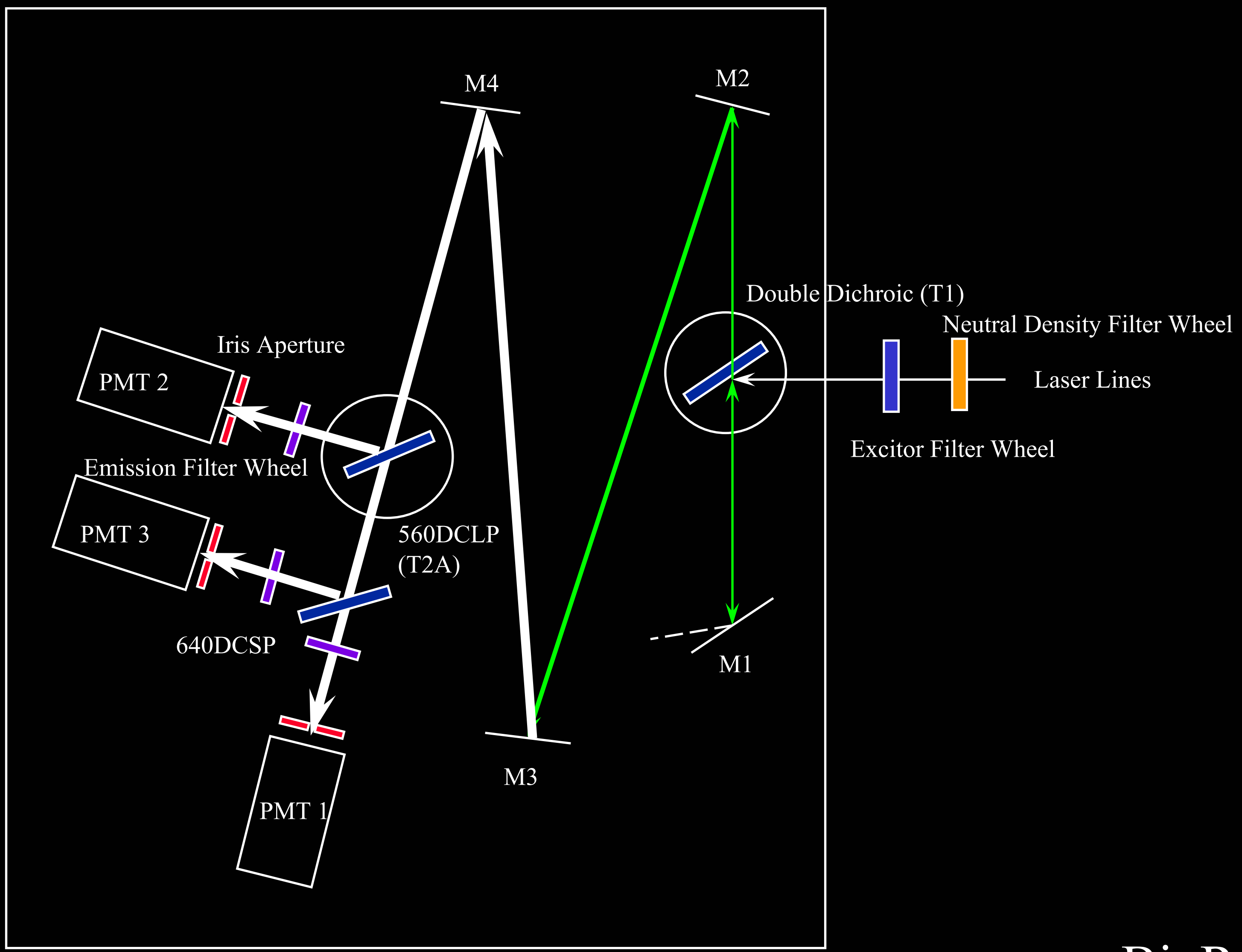
BioRad

Fluorescein Emission



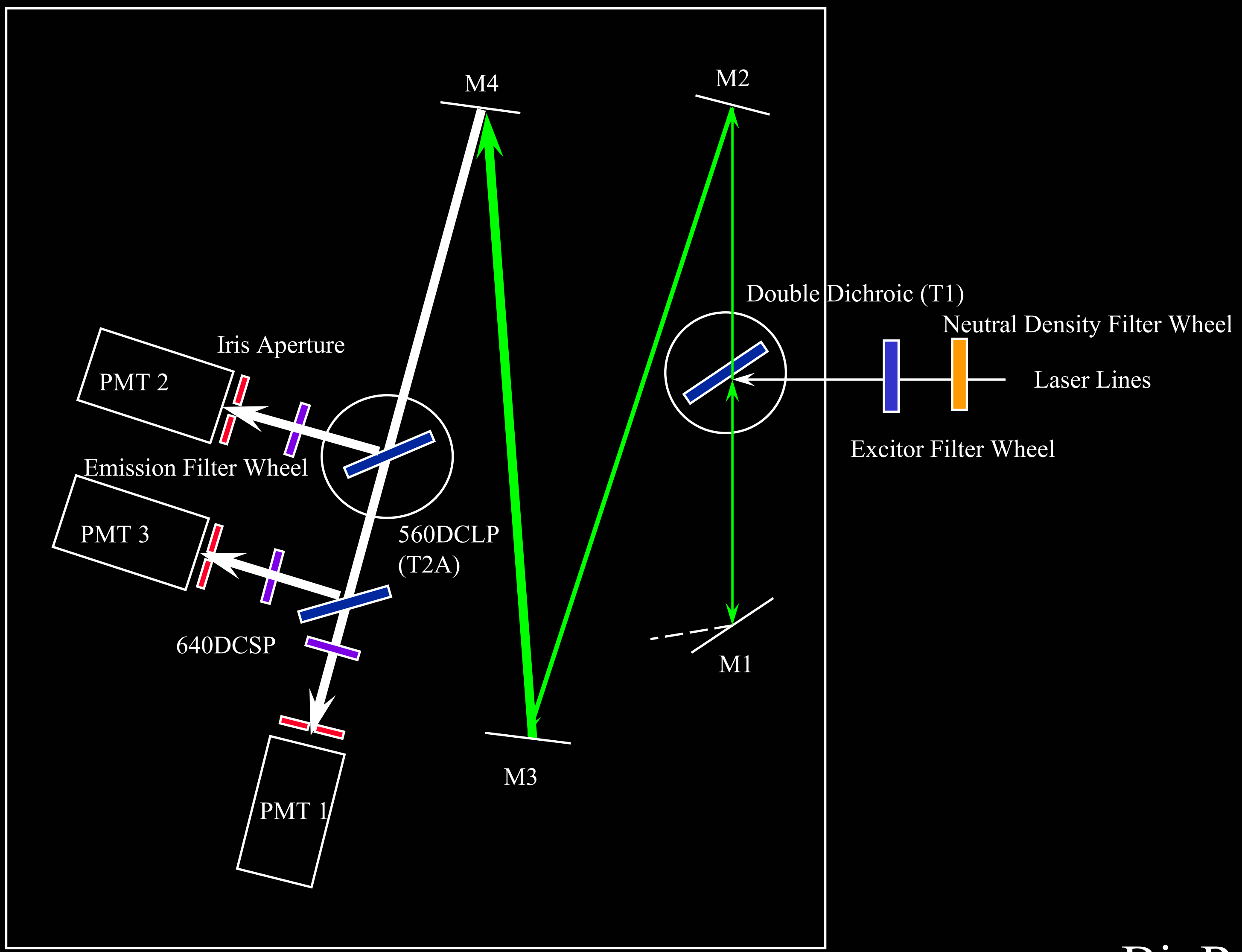
BioRad

Fluorescein Emission



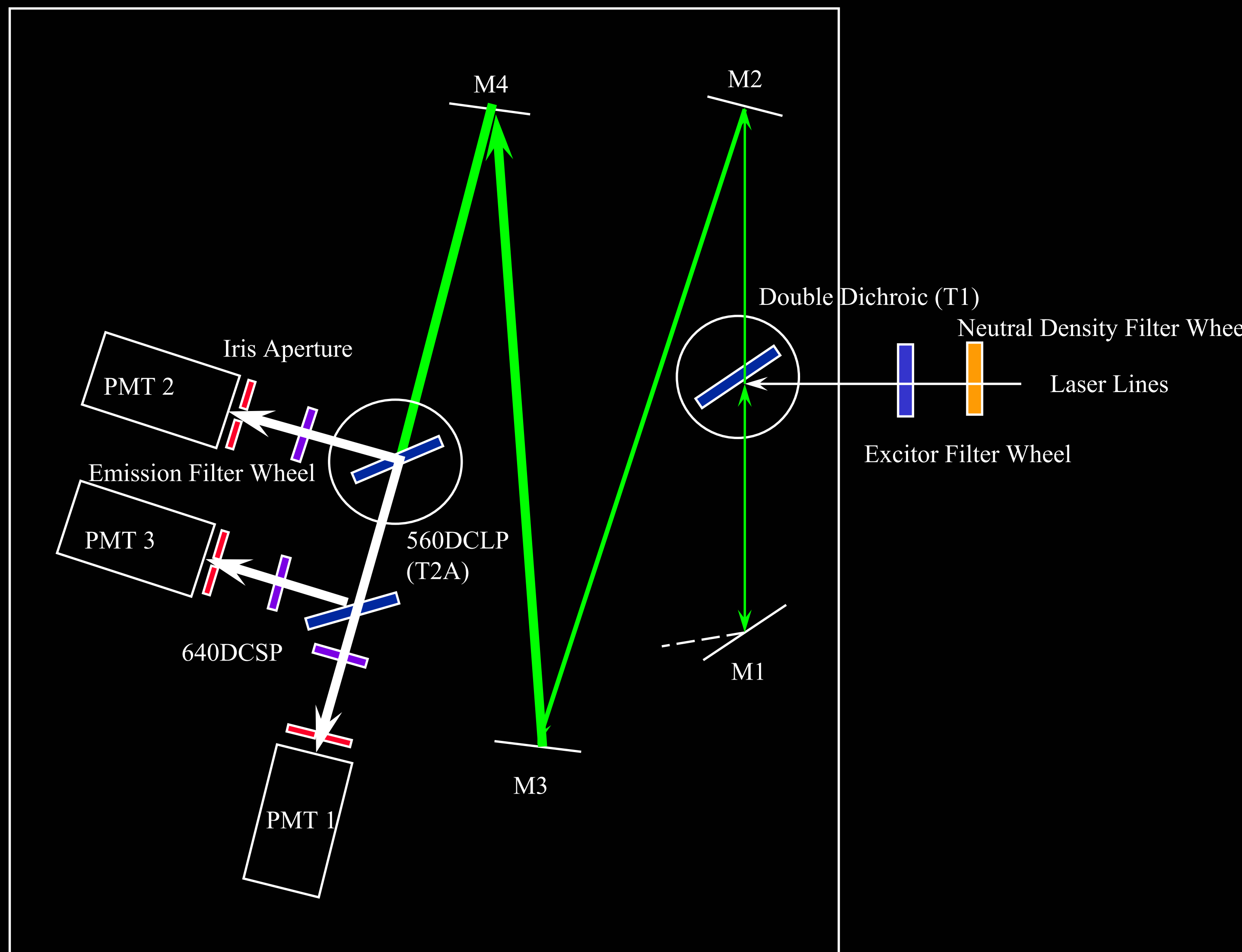
BioRad

Fluorescein Emission



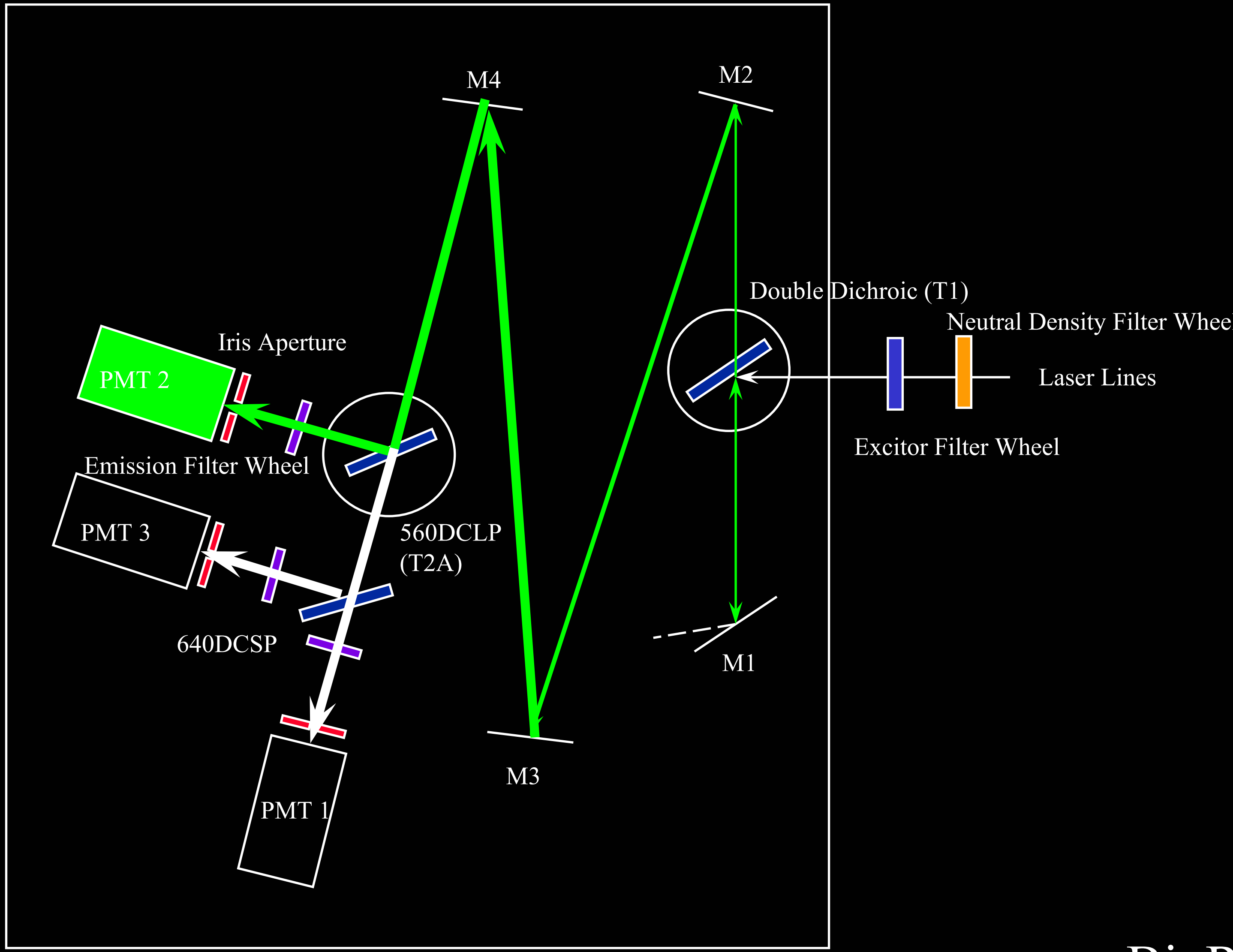
BioRad

Fluorescein Emission



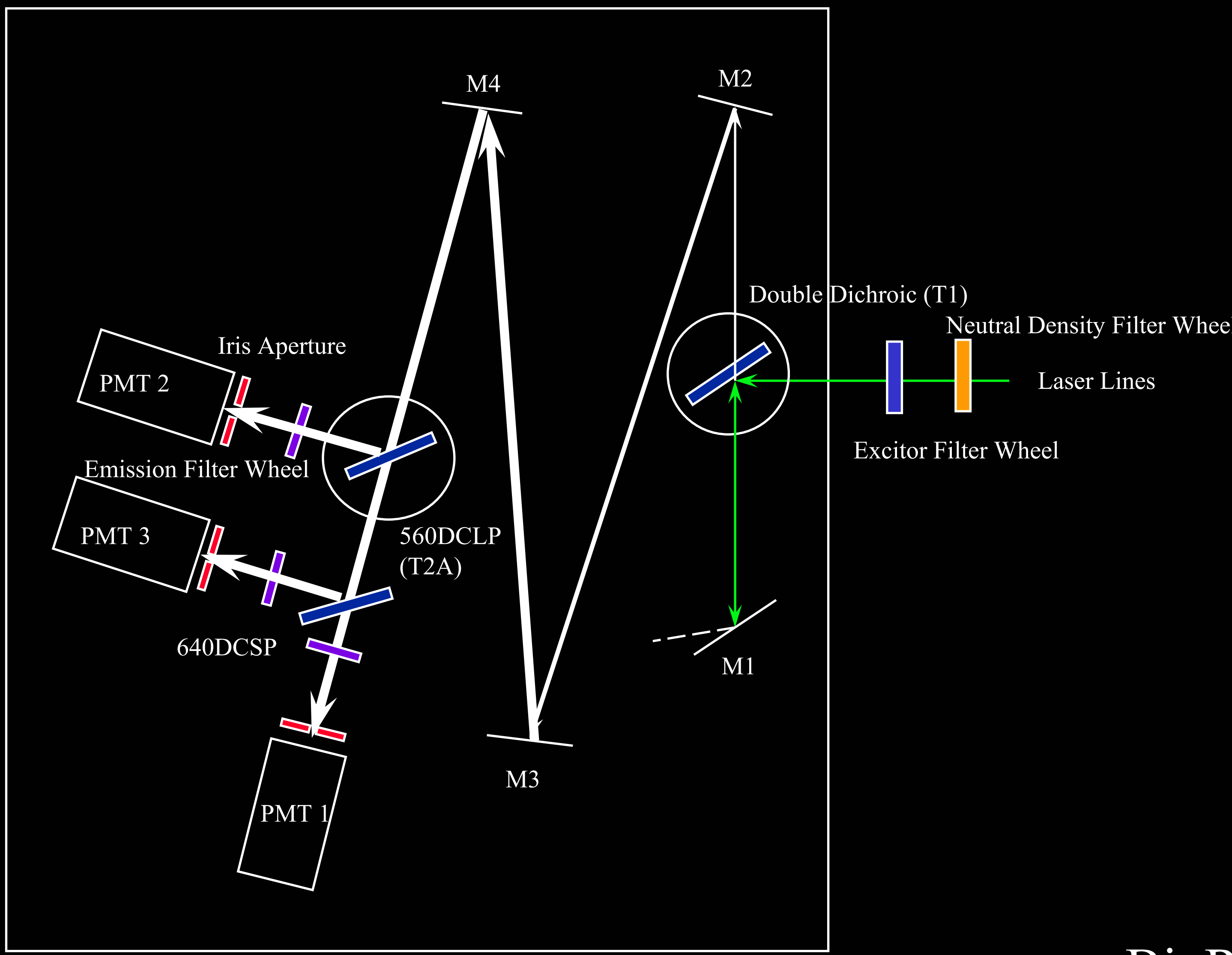
BioRad

Fluorescein Emission



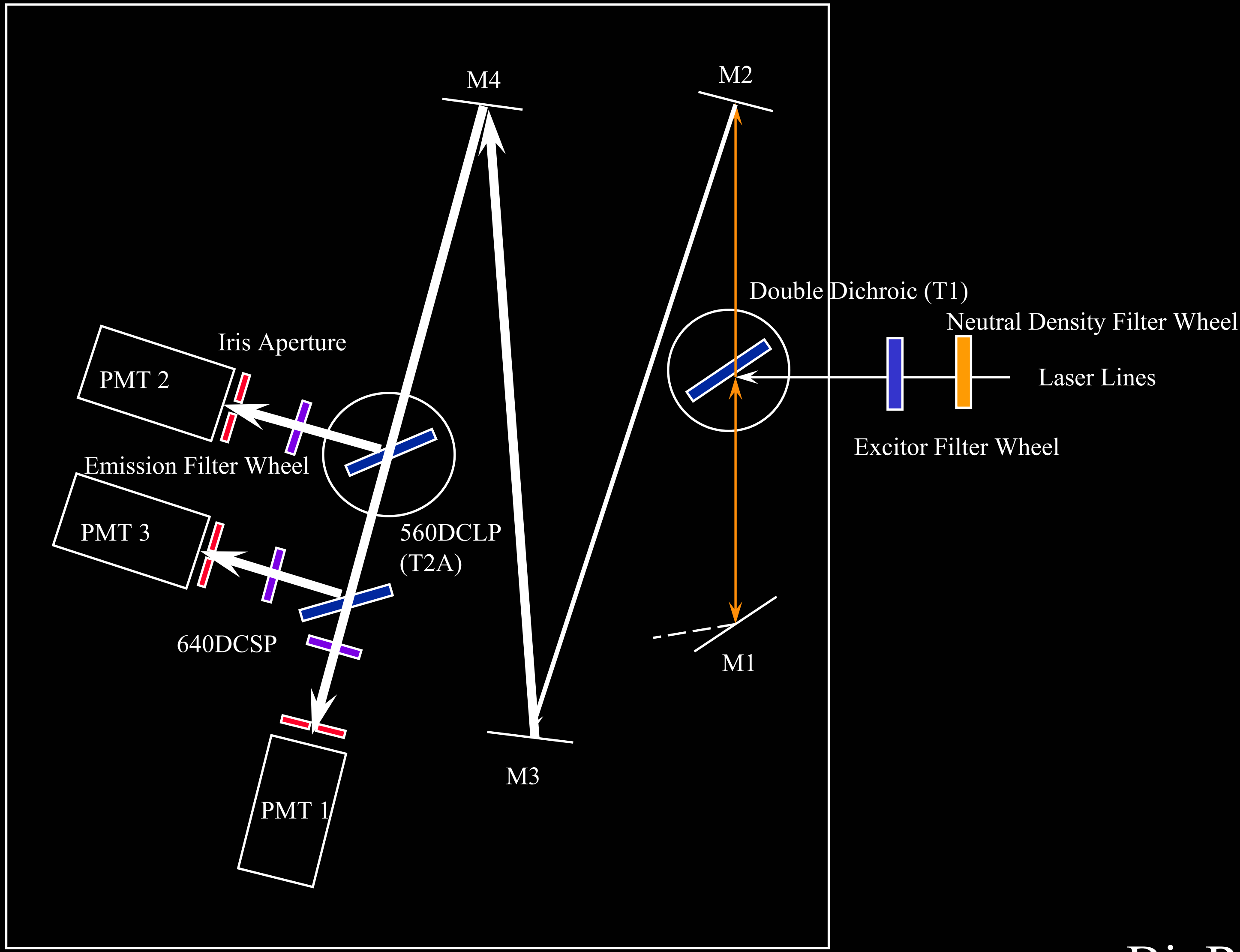
BioRad

568 nm Excitation



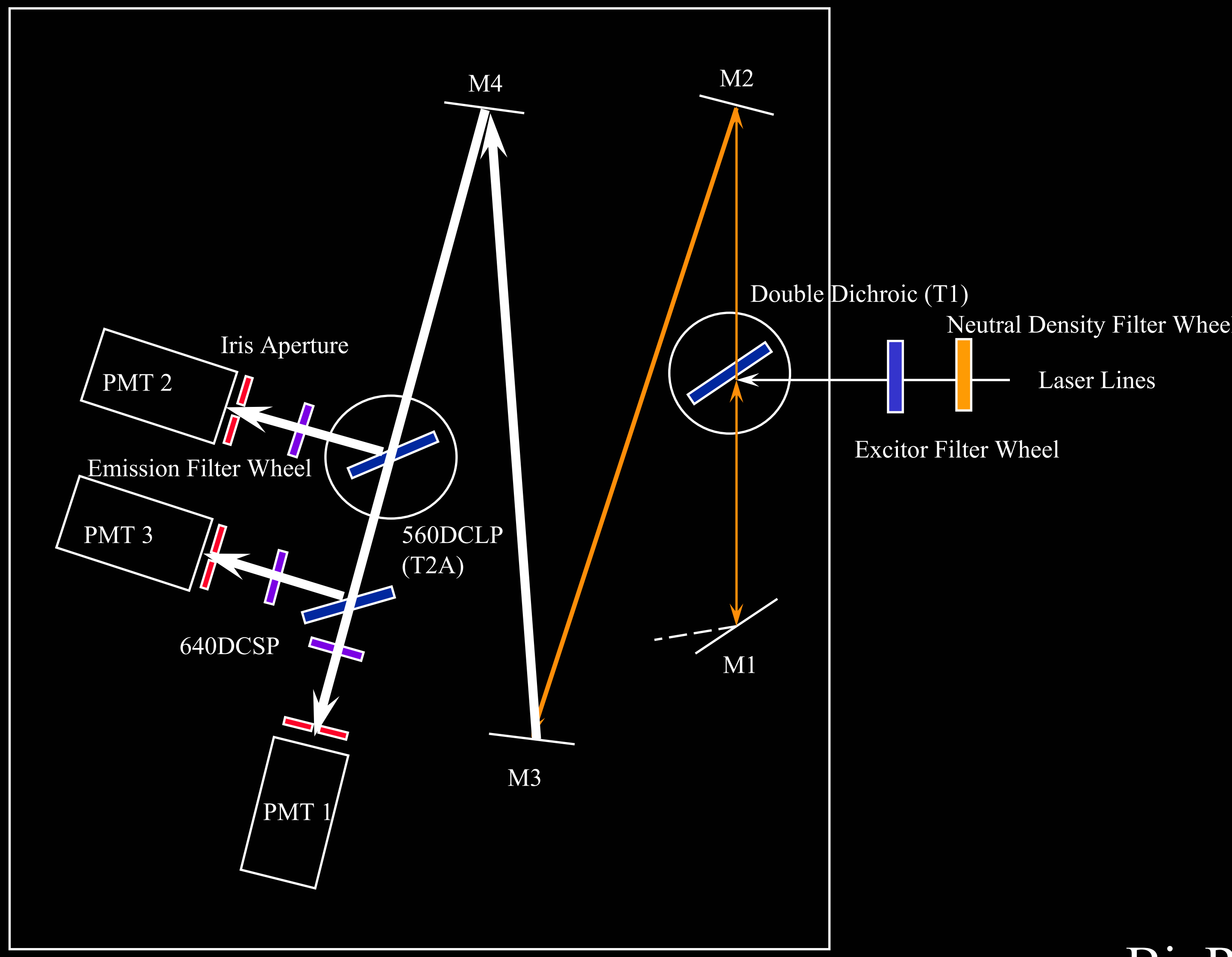
BioRad

Rhodamine Emission



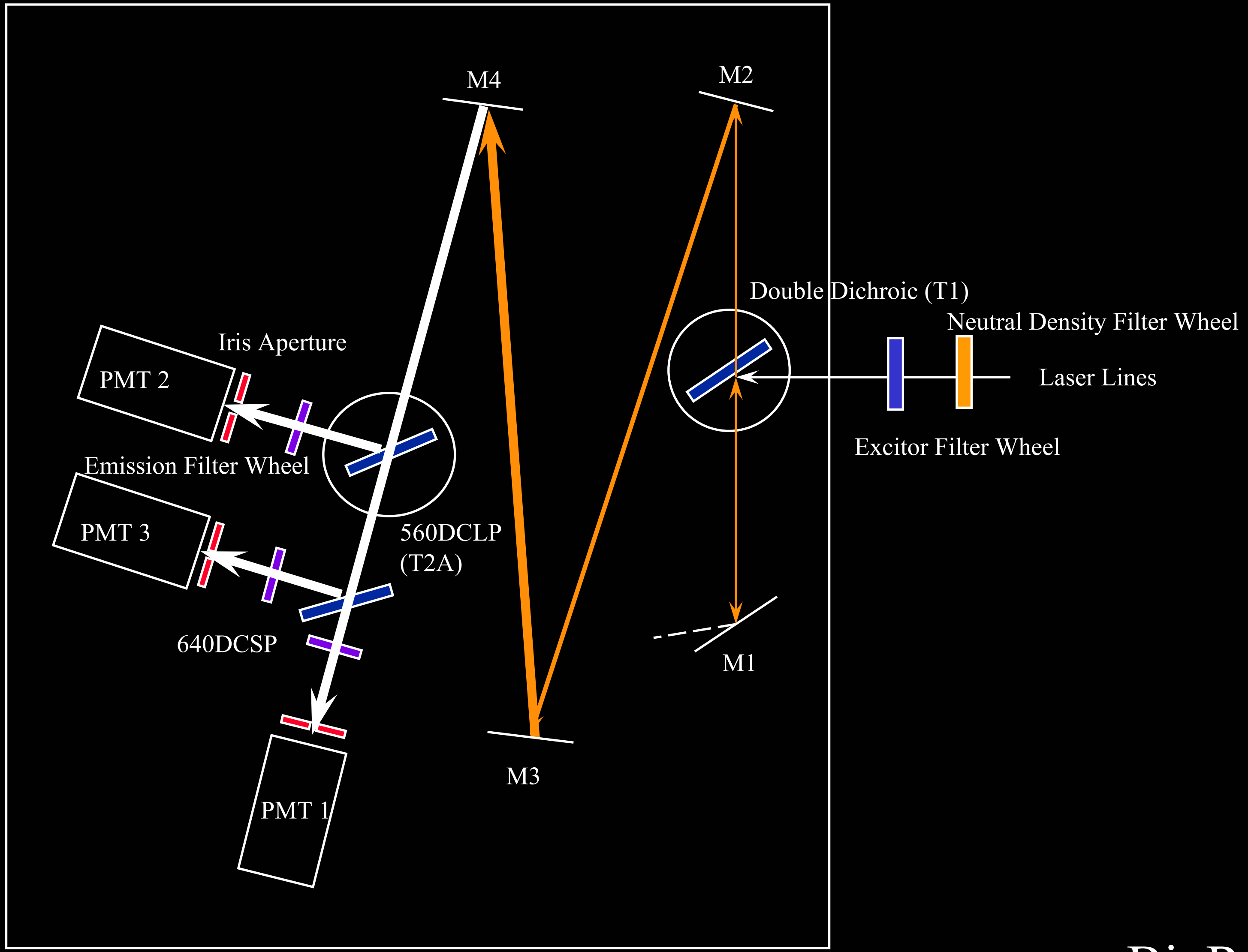
BioRad

Rhodamine Emission



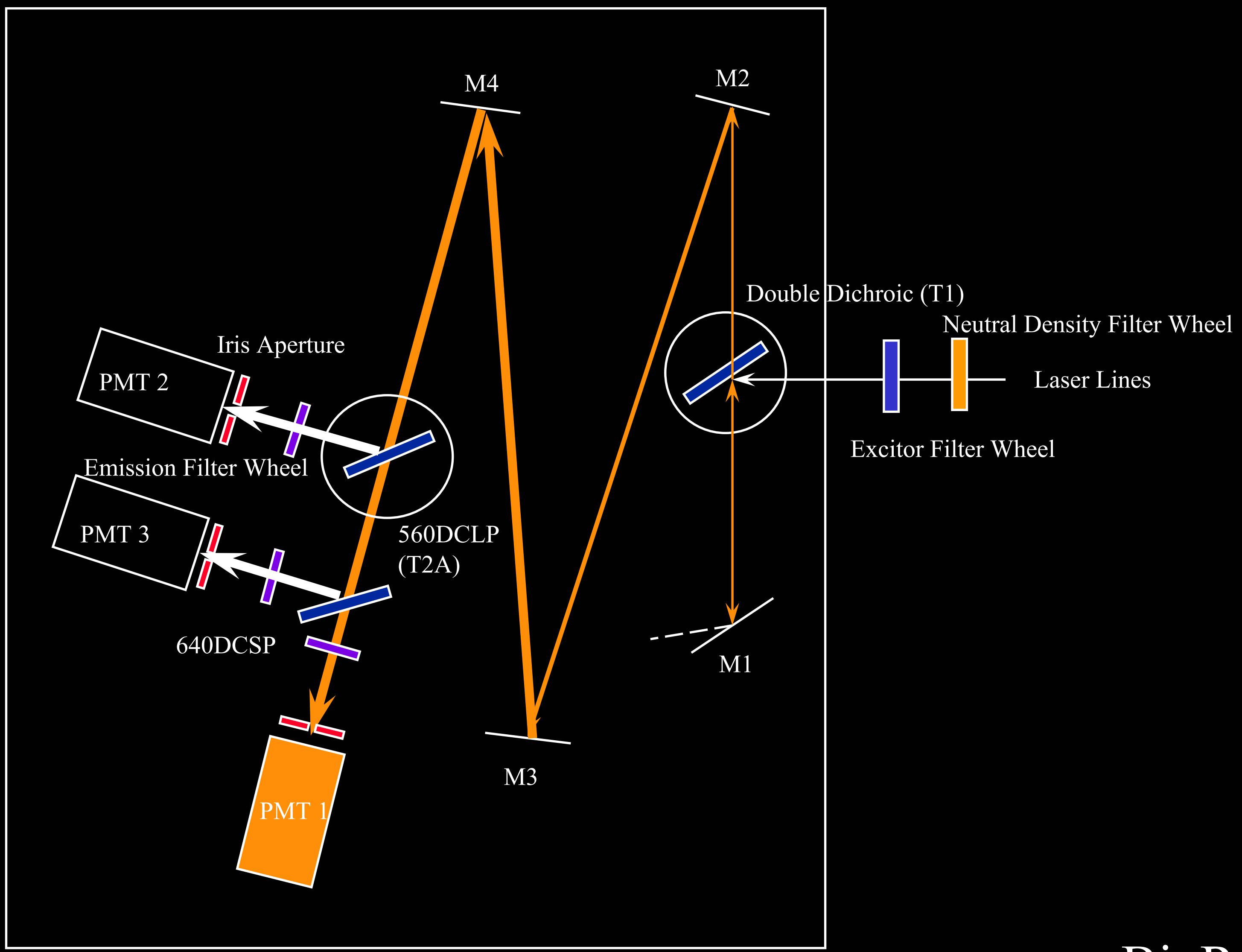
BioRad

Rhodamine Emission



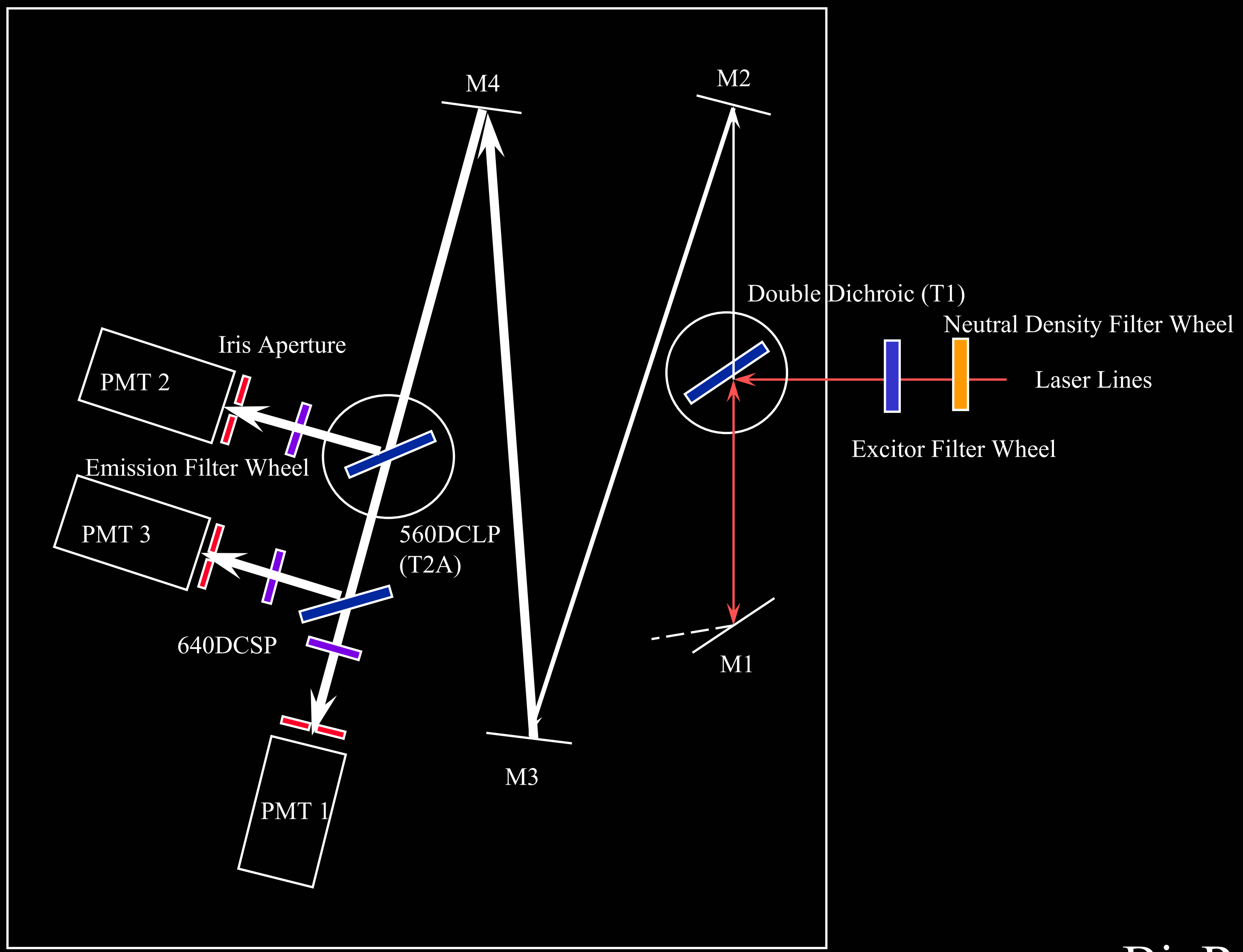
BioRad

Rhodamine Emission



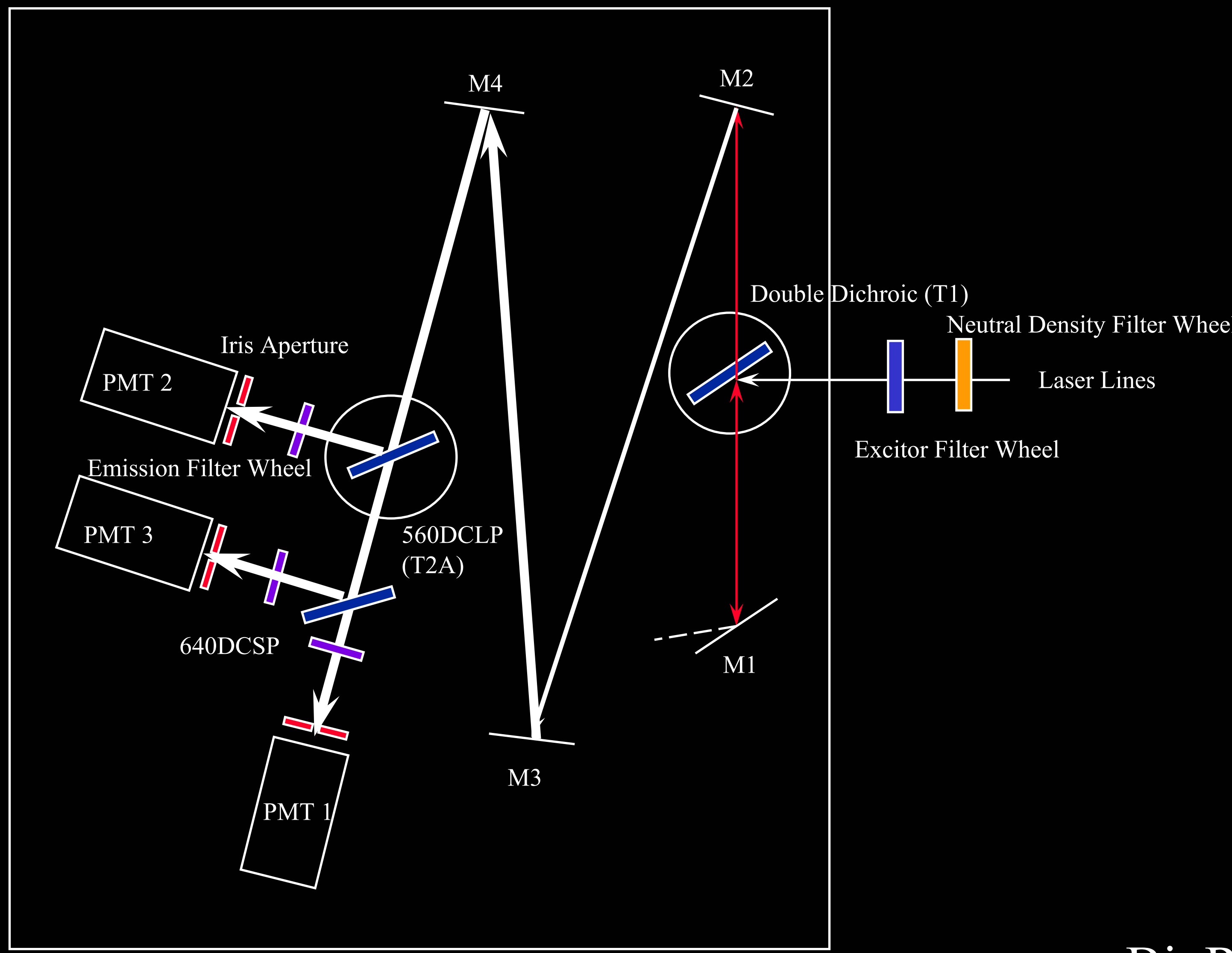
BioRad

647 nm Excitation



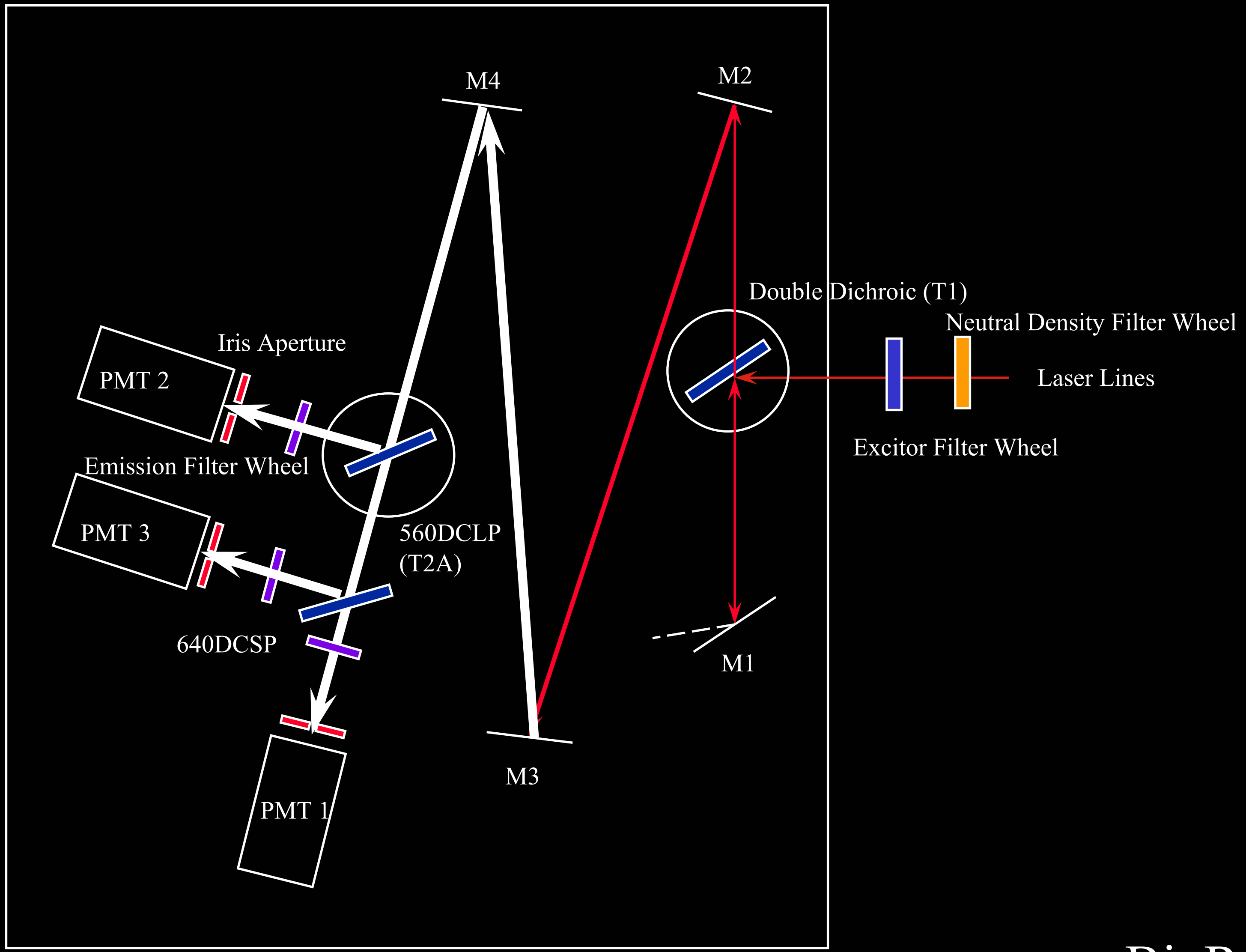
BioRad

Cy-5 Emission



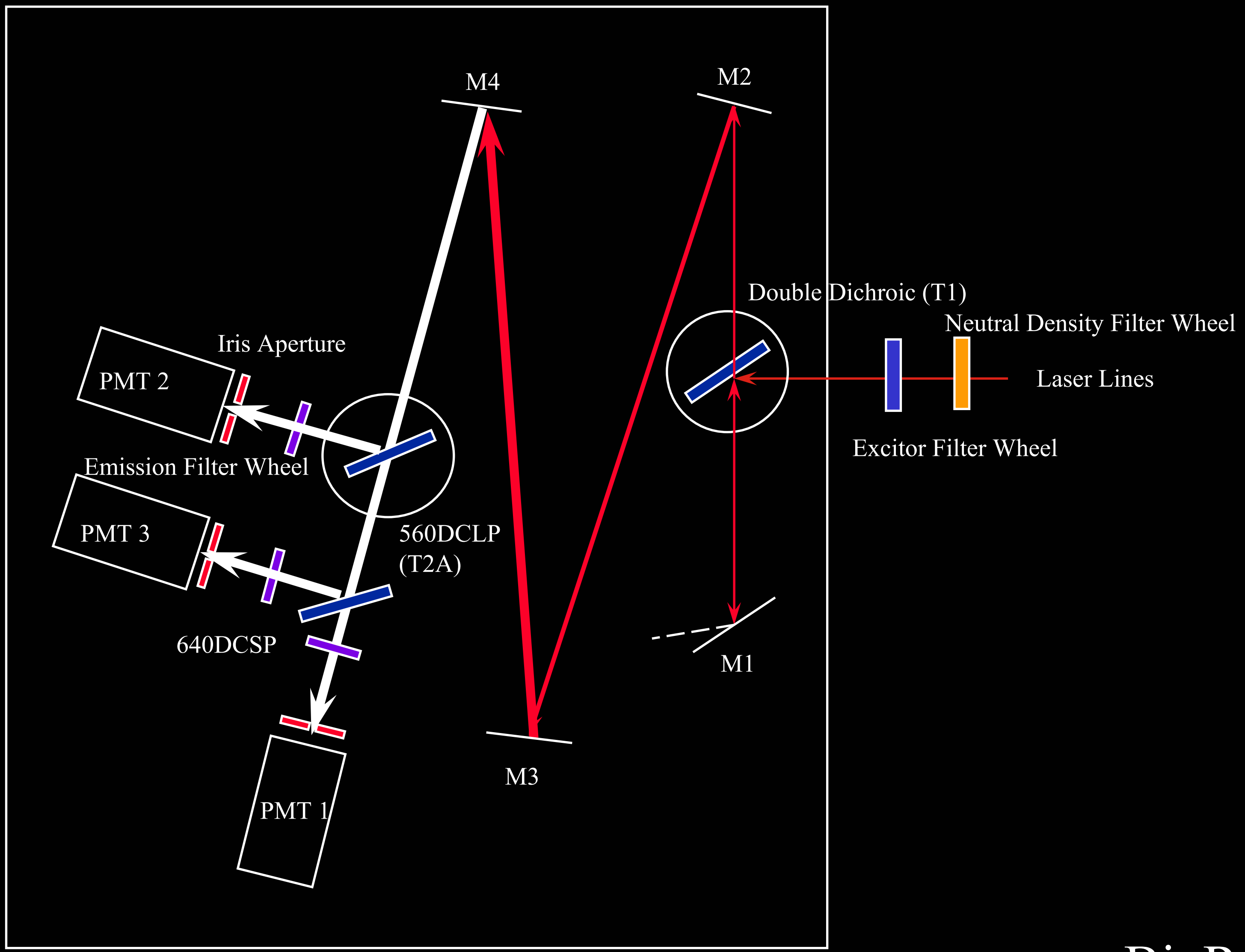
BioRad

Cy-5 Emission



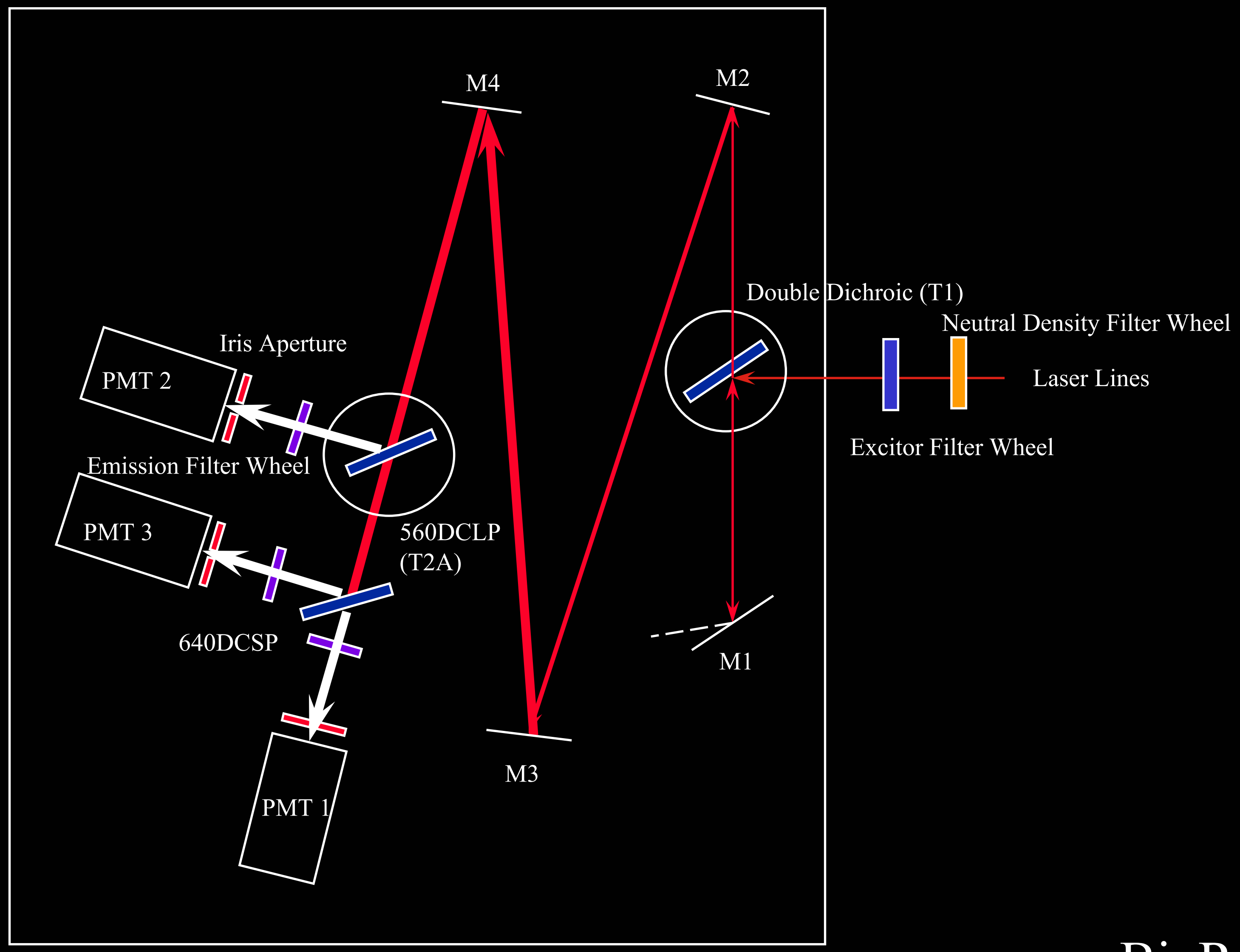
BioRad

Cy-5 Emission



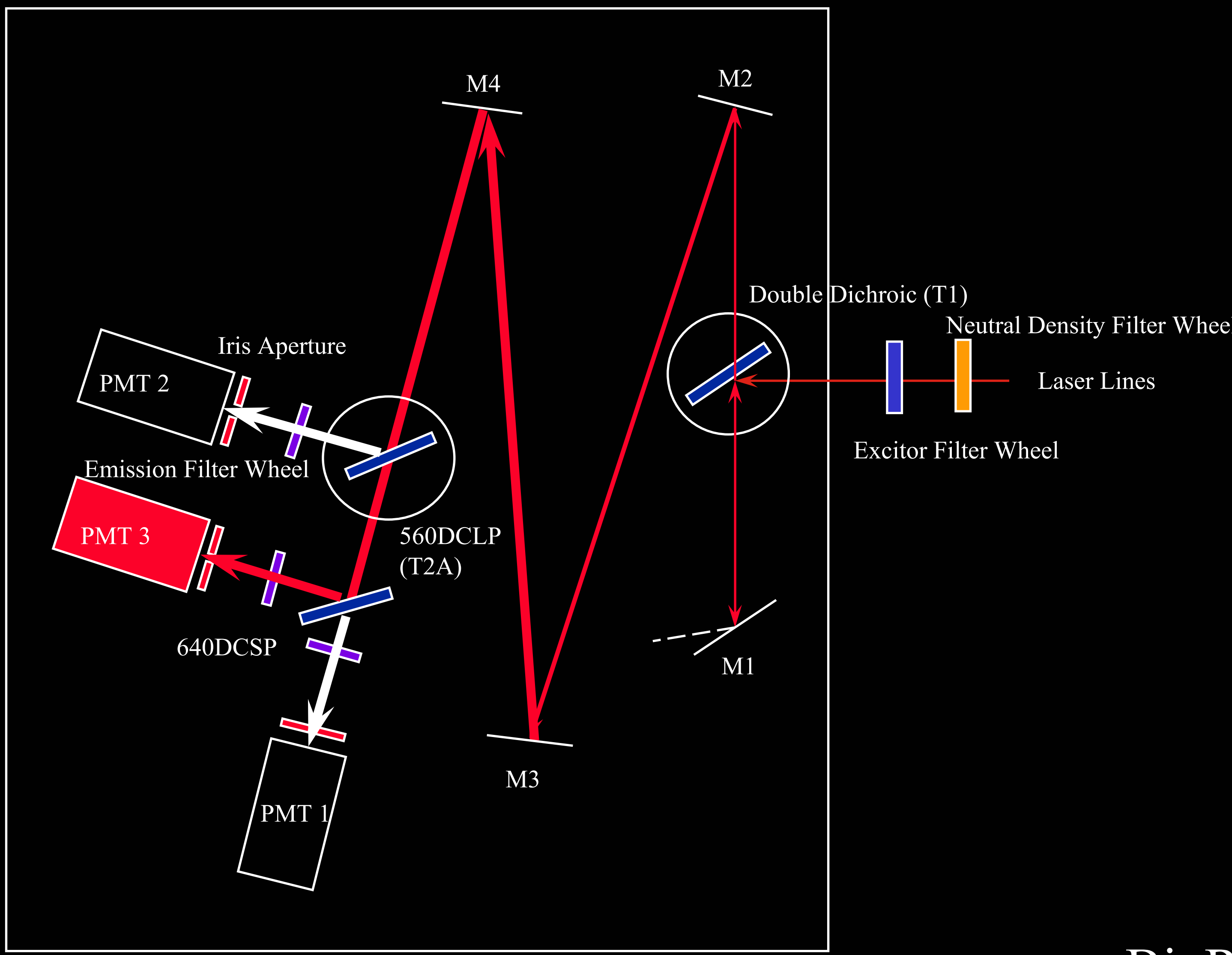
BioRad

Cy-5 Emission



BioRad

Cy-5 Emission



BioRad

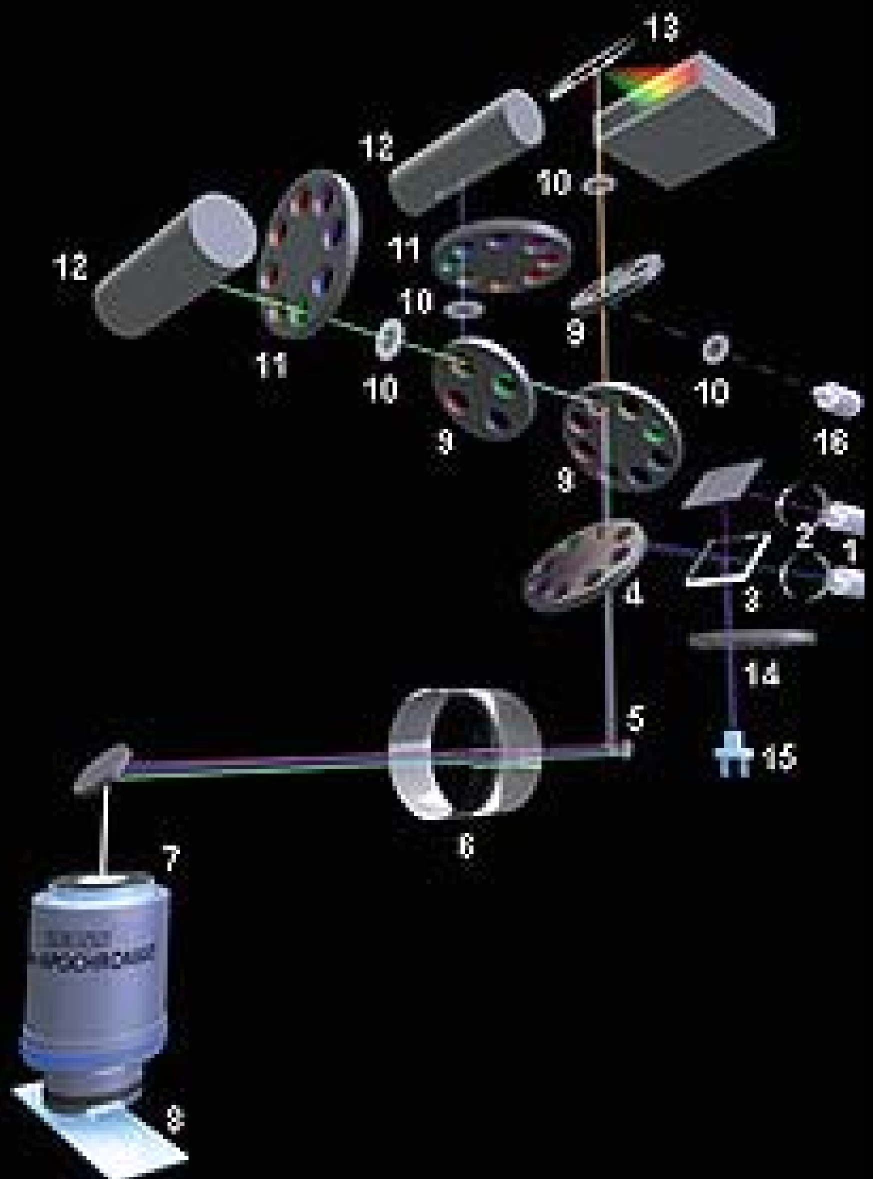
Advantages of scanning by Acousto-optical tuneable filters (AOTFs)

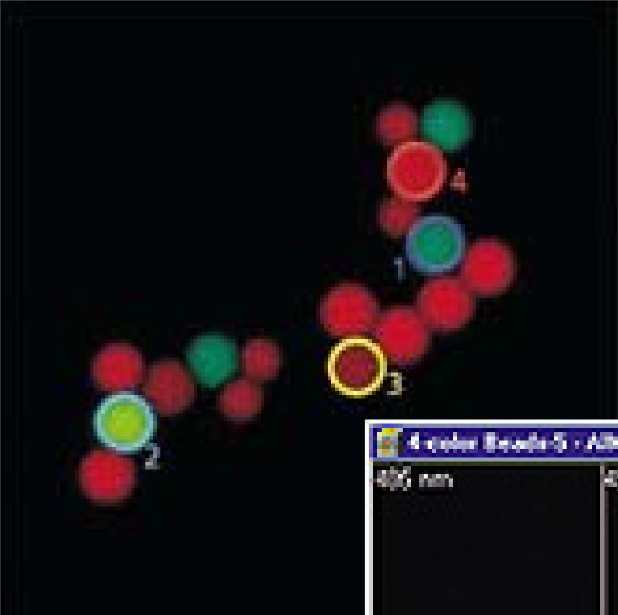
- Modulate individual lines
- Sequential multicolor
- Blank retrace
- Continuous modulation

Spectral confocal microscopy –
Zeiss LSM510-Meta detector system

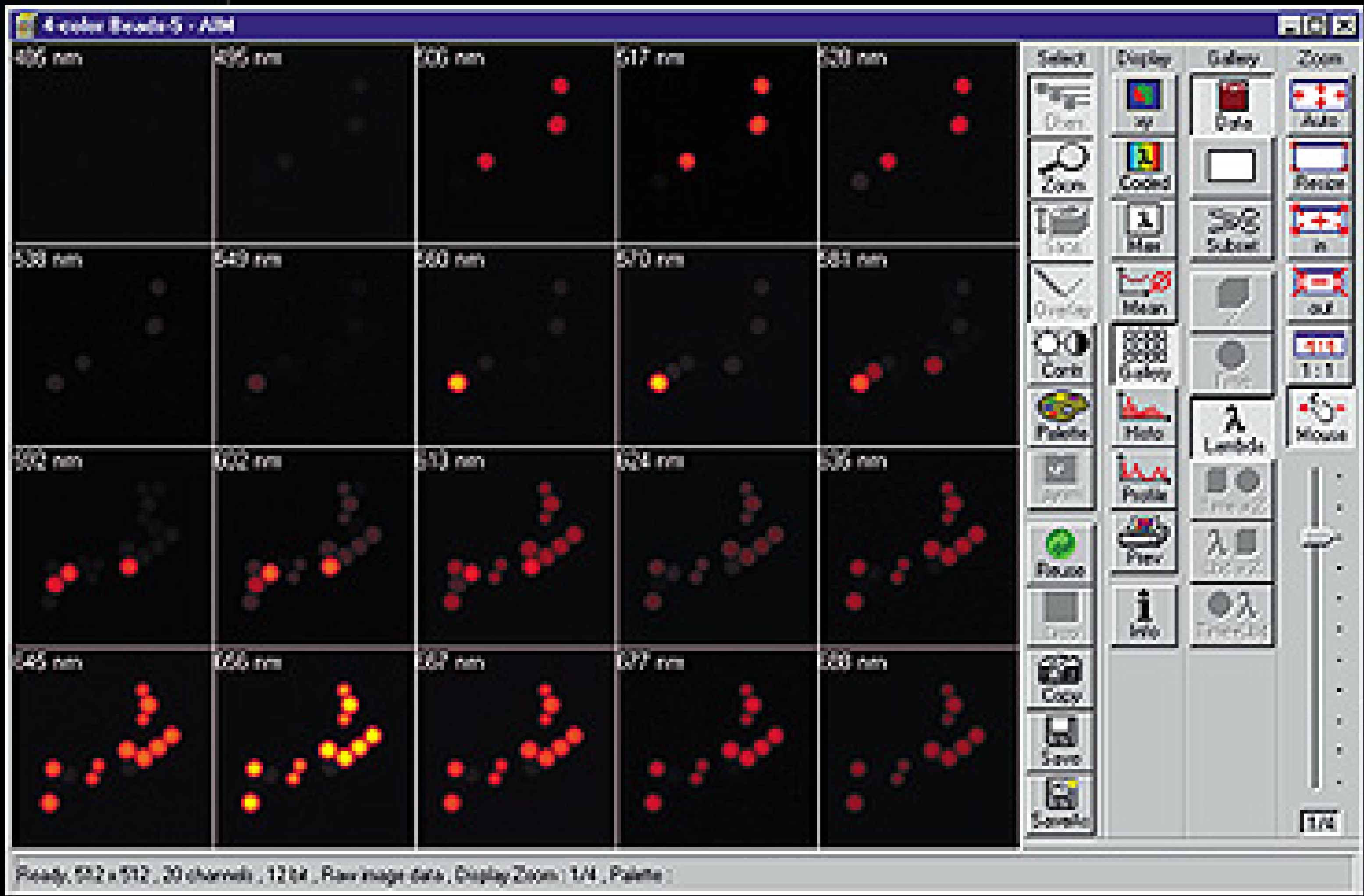
- 32 channel Metadetector collects spectrum of each pixel – deconvolution permits distinction of multiple, closely spaced fluorophores – linear unmixing

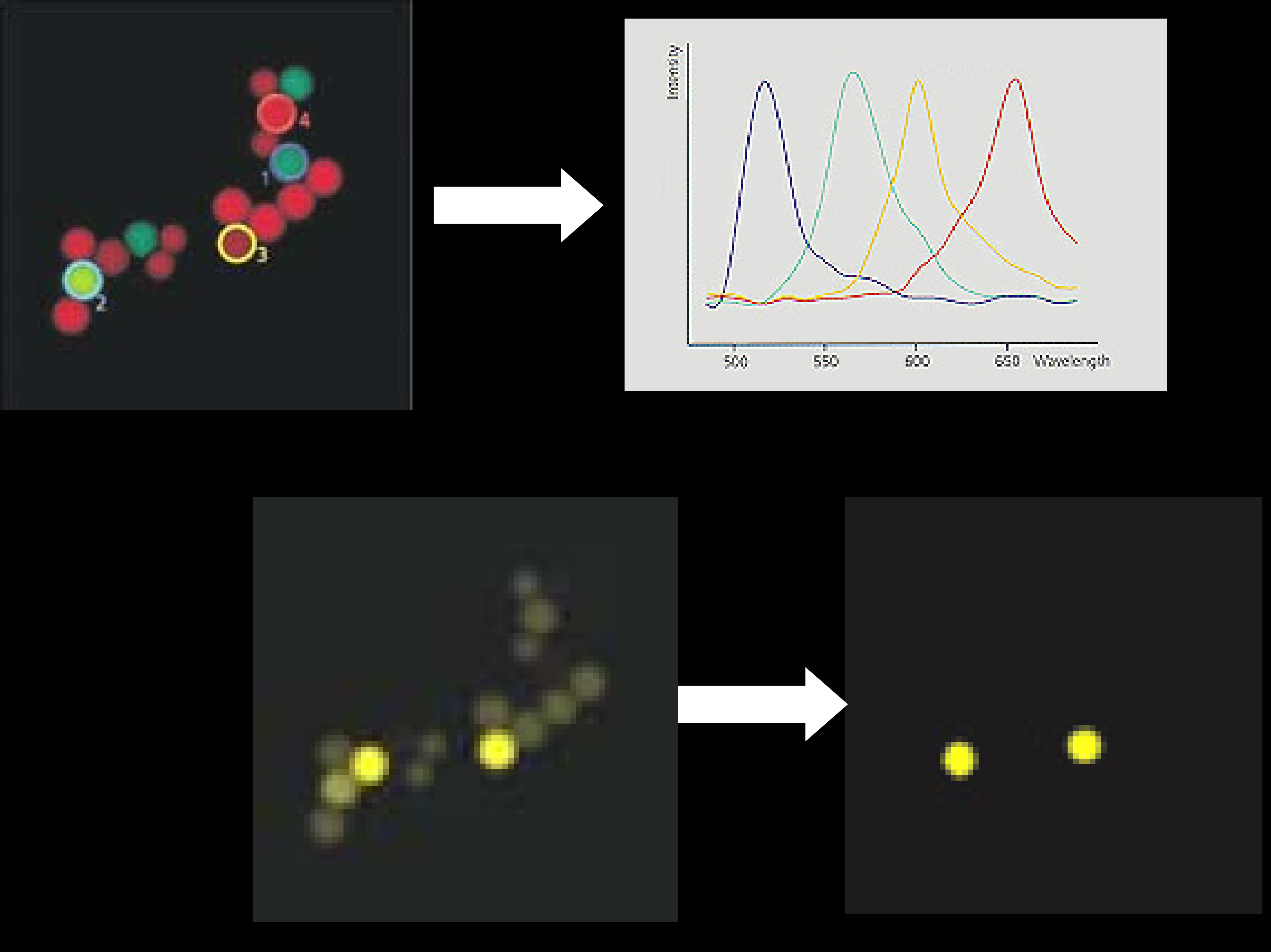
Zeiss LSM510-Meta detector system



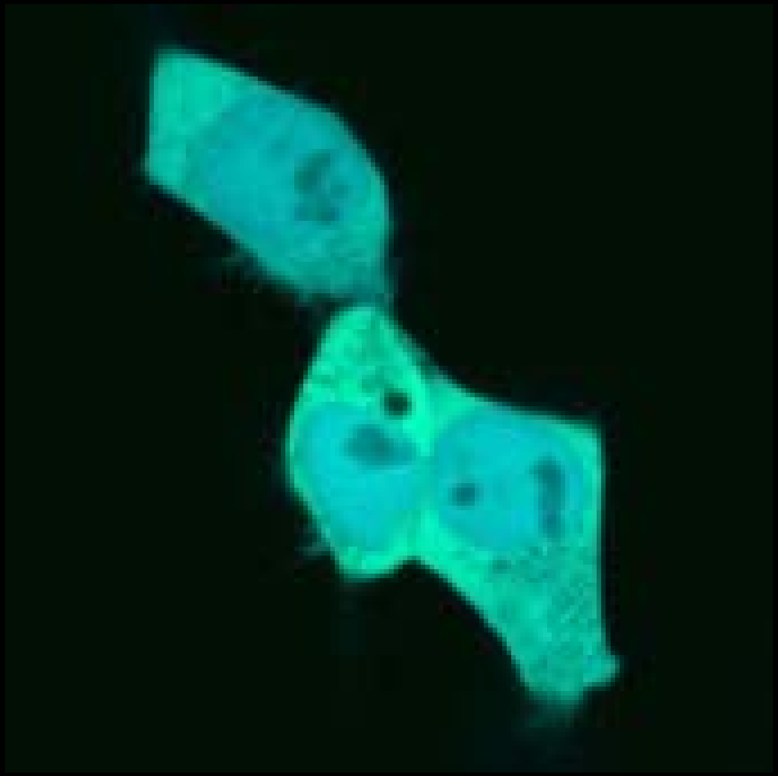


Zeiss LSM510-Meta system – 20 channels of fluorescence




4
1
3
2
Intensity
500
550
600
650
Wavelength

Zeiss 510 META
linear unmixing of GFP and YFP



Original image

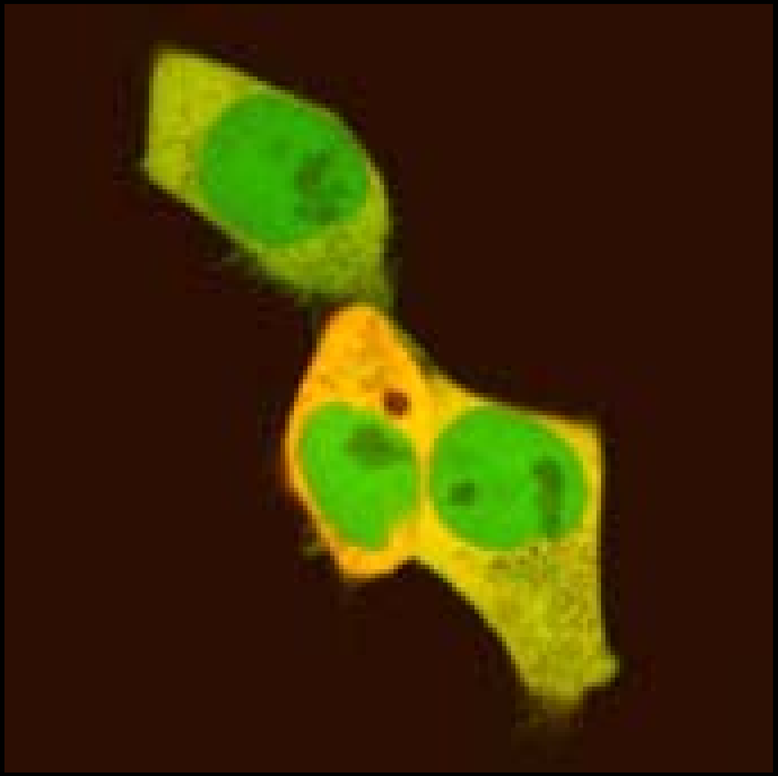
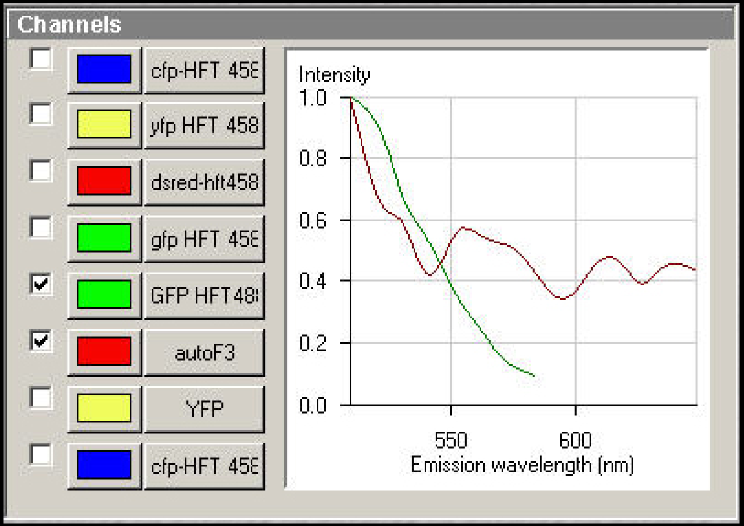
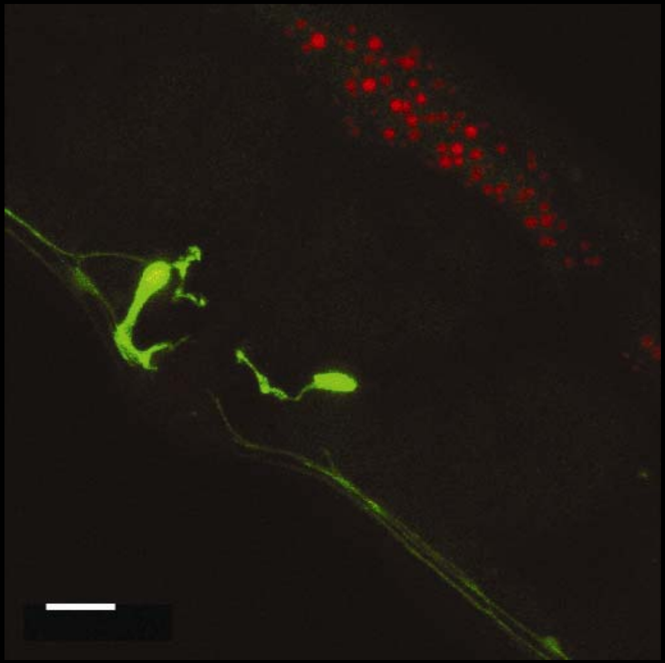


Image after linear unmixing

GFP versus autofluorescence

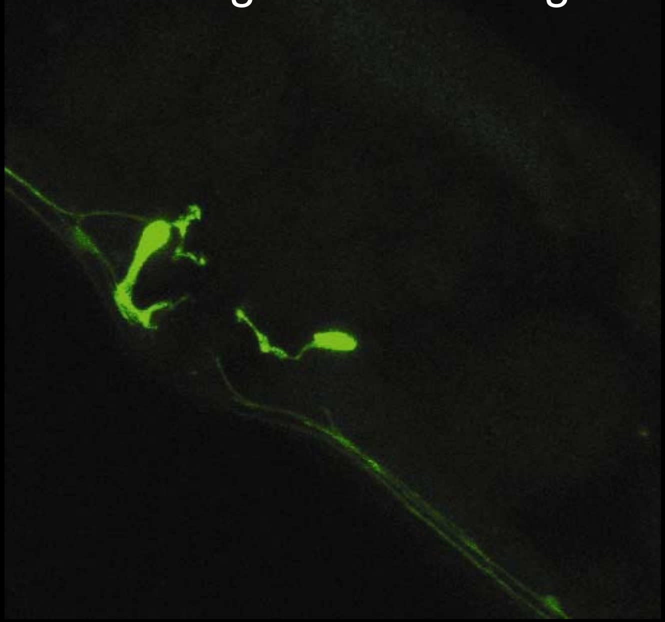


VC4 and VC5 motor neurons (green) + gut autofluorescence (red)



Imaged by David Miller

Removal of autofluorescence using spectral fitting and “unmixing”



Sam Wells, Vanderbilt

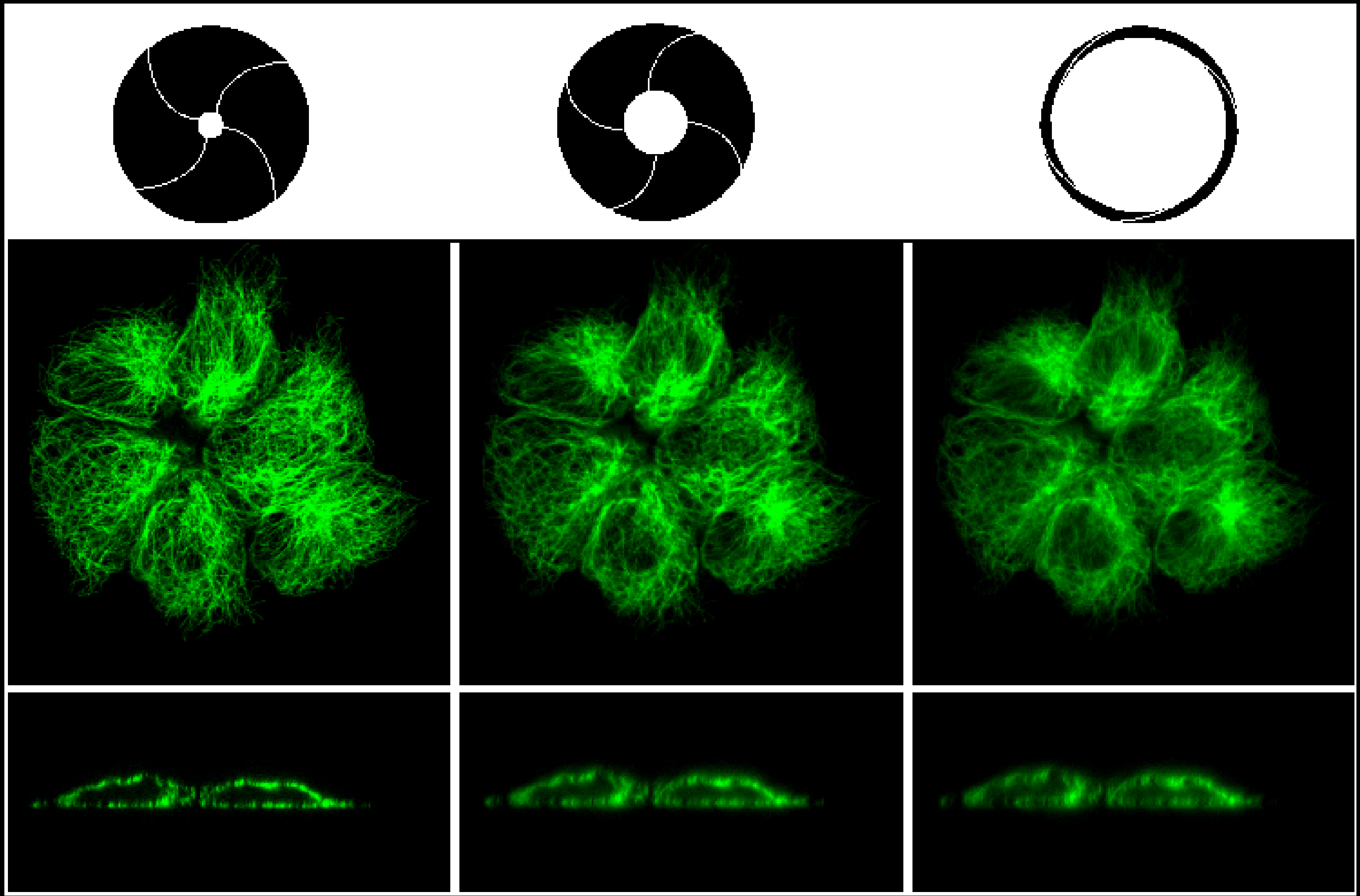
Practical confocal microscopy

- Image collection settings
- Objective choice

Practical confocal microscopy

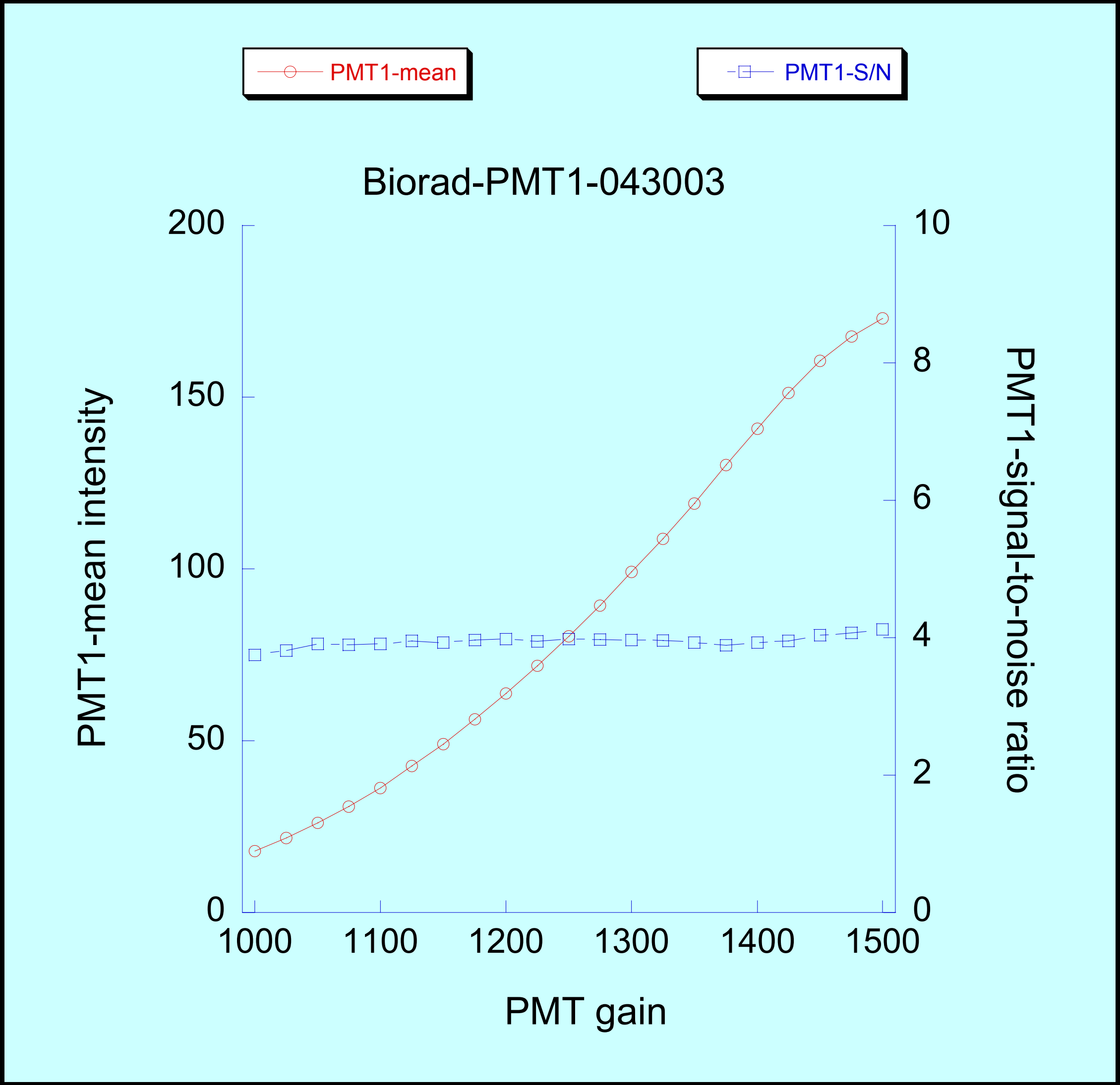
- Image collection settings
  - prioritizing the pinhole, PMT and laser power

Effect of pinhole diameter on image quality

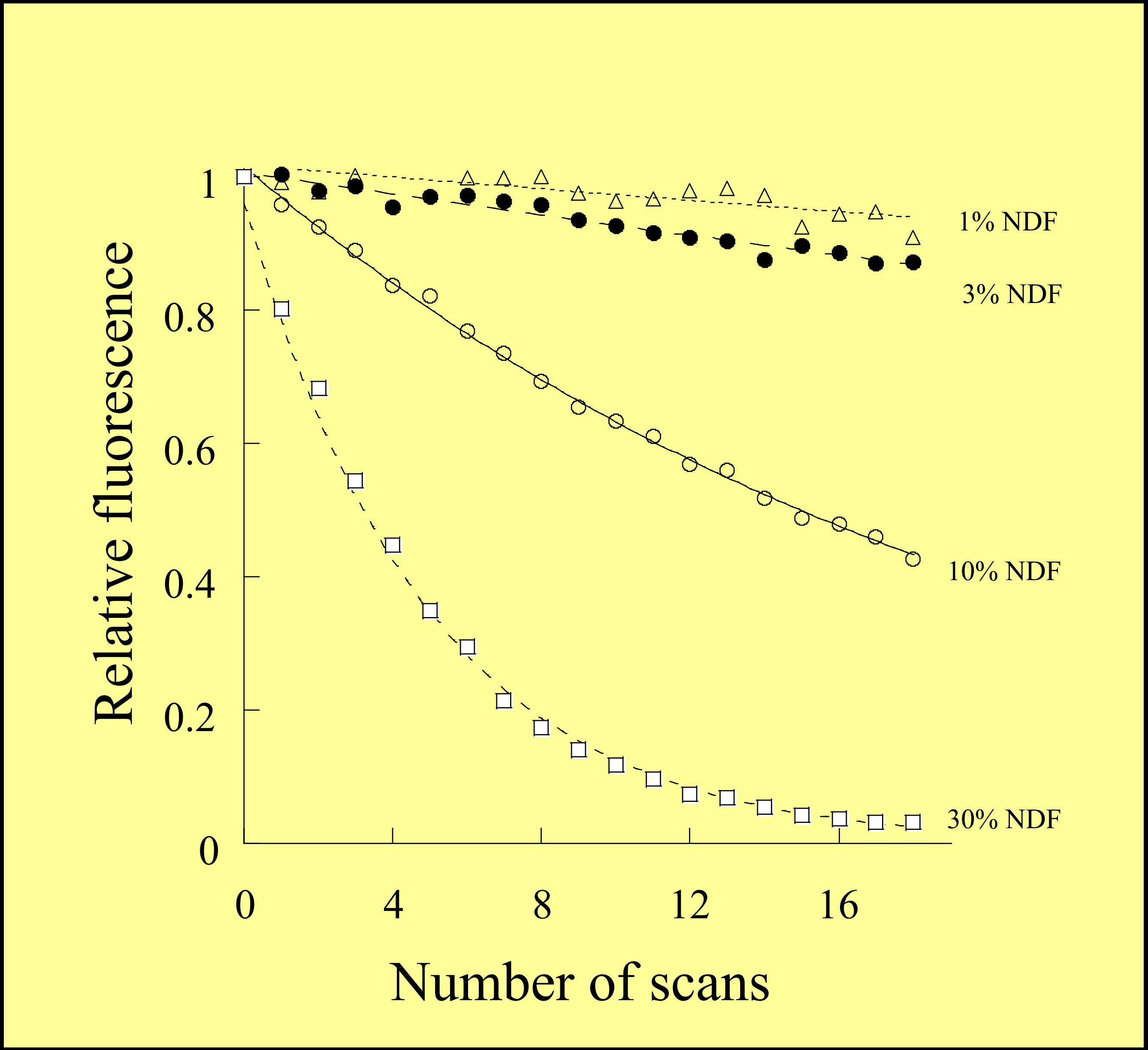


Institut Jacques Monod

Effect of PMT Voltage on Signal, and Signal-to-Noise Ratio



Effect of Laser Power on Signal



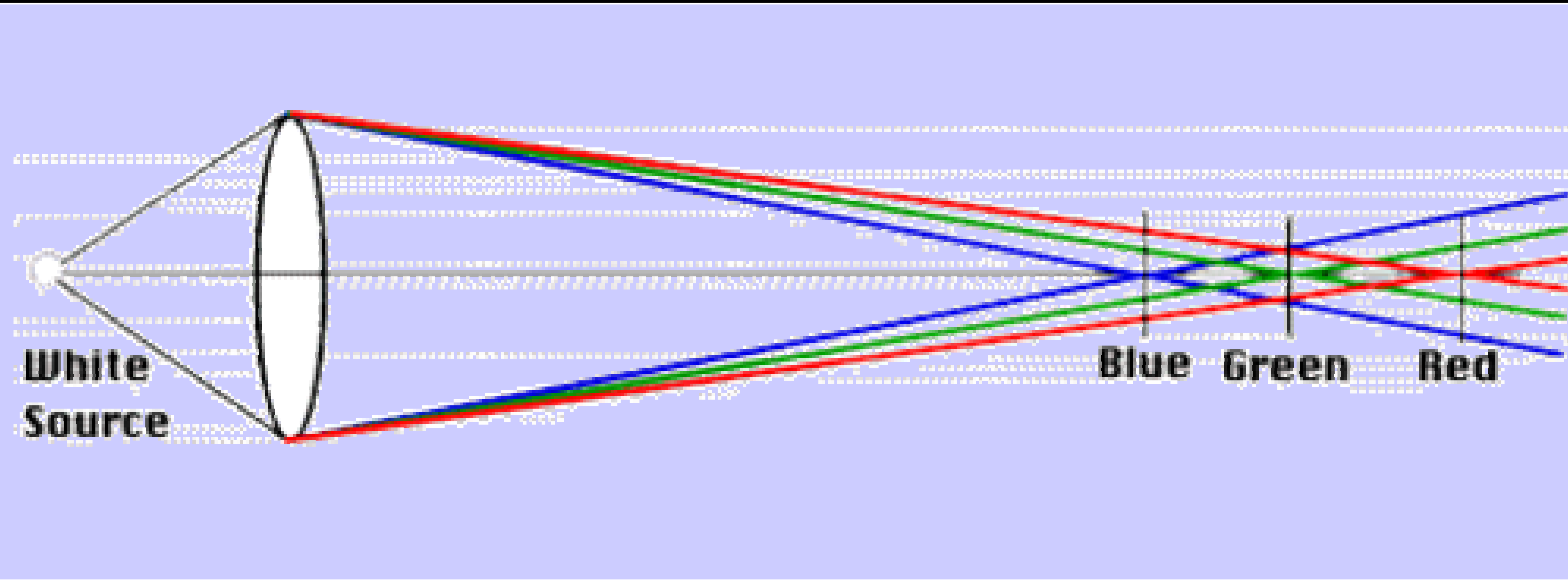
Practical confocal microscopy

- Image collection settings
- Objective choice

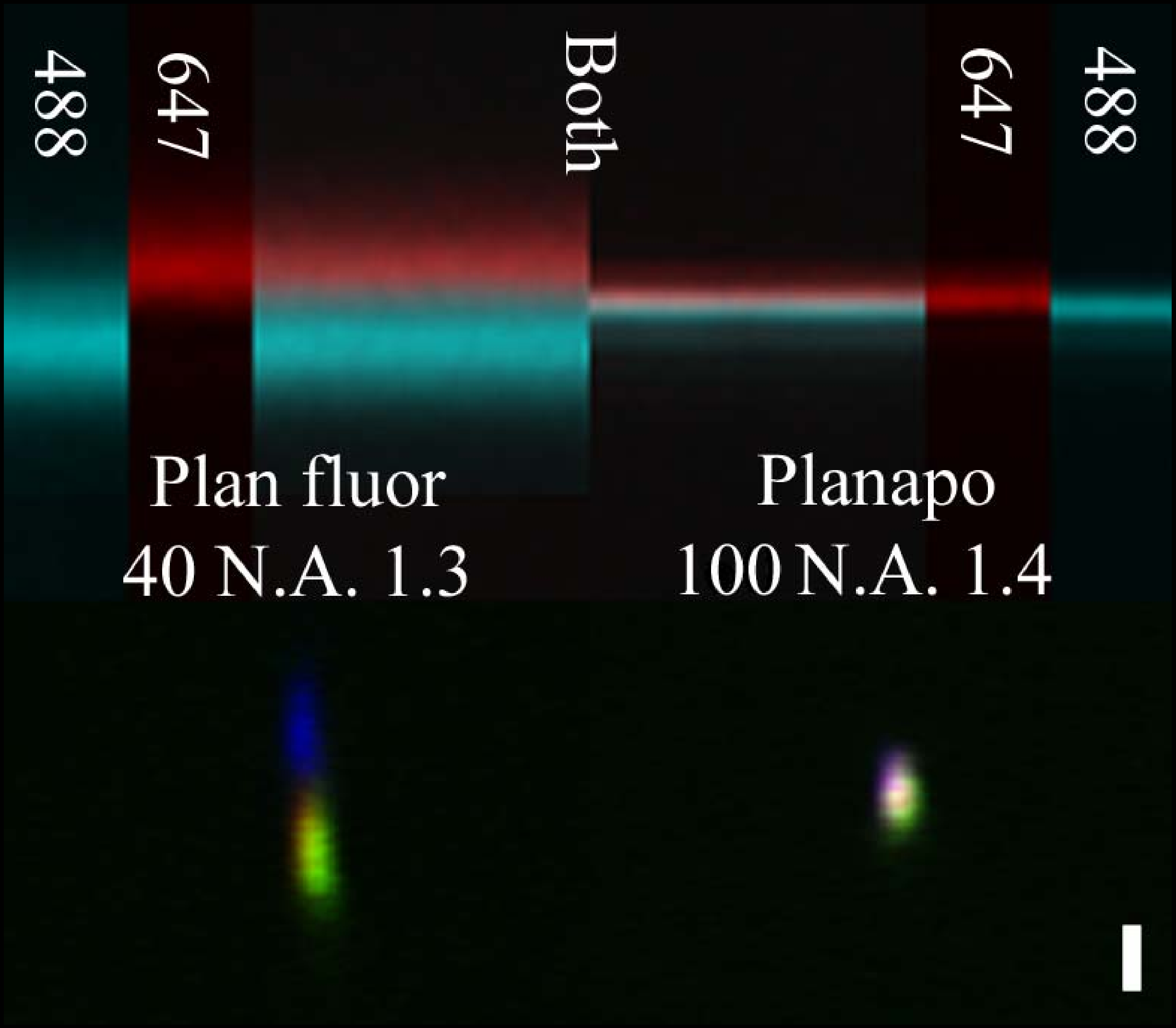
Practical confocal microscopy

- Image collection settings
- Objective choice
  - Chromatic aberration

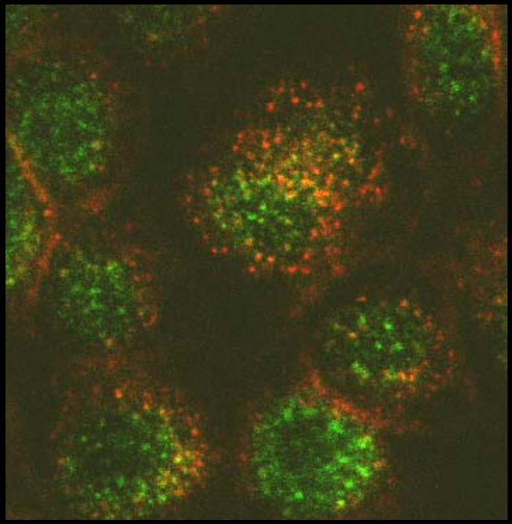
Axial chromatic aberration



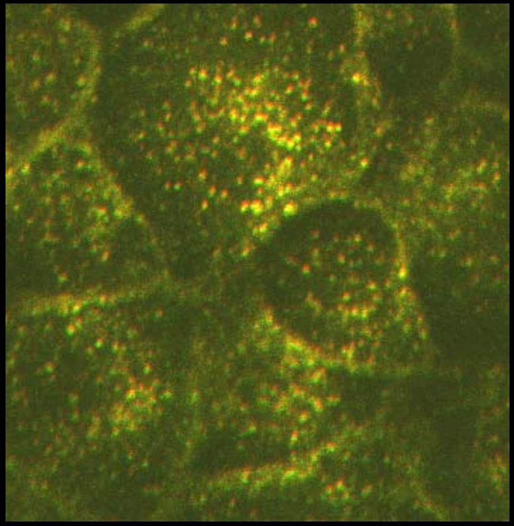
Axial chromatic aberration



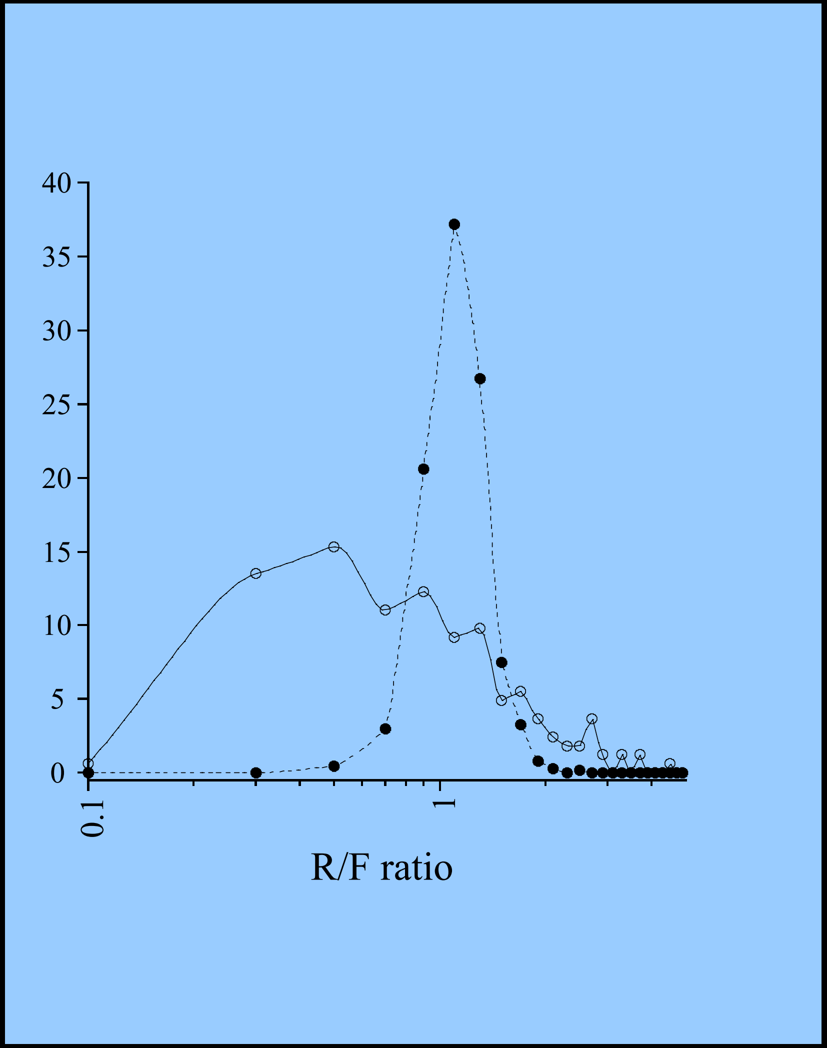
Chromatic aberration - F-Cy5 ratios and the Plan fluor 40



Single section (solid line)



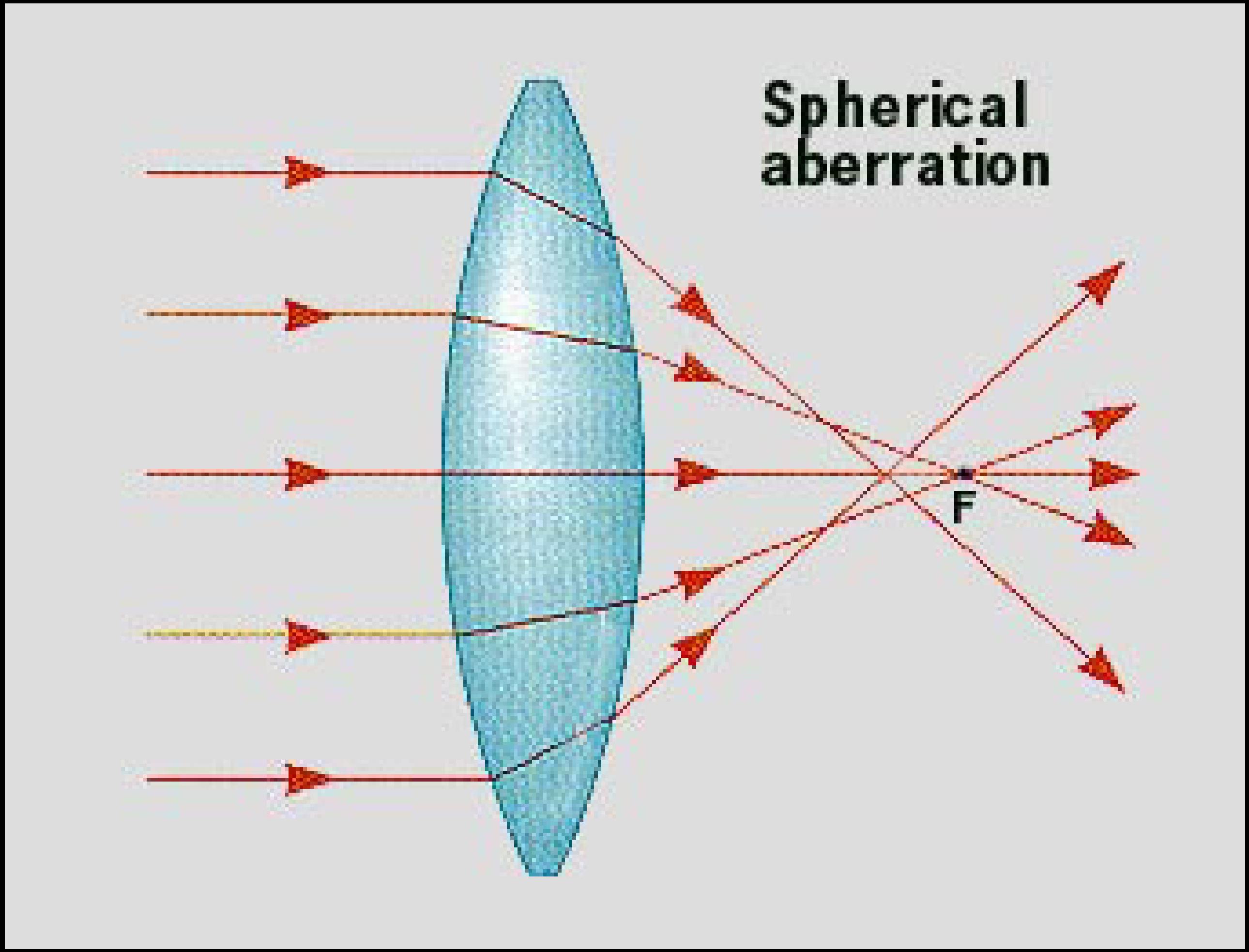
Projection (dashed line)



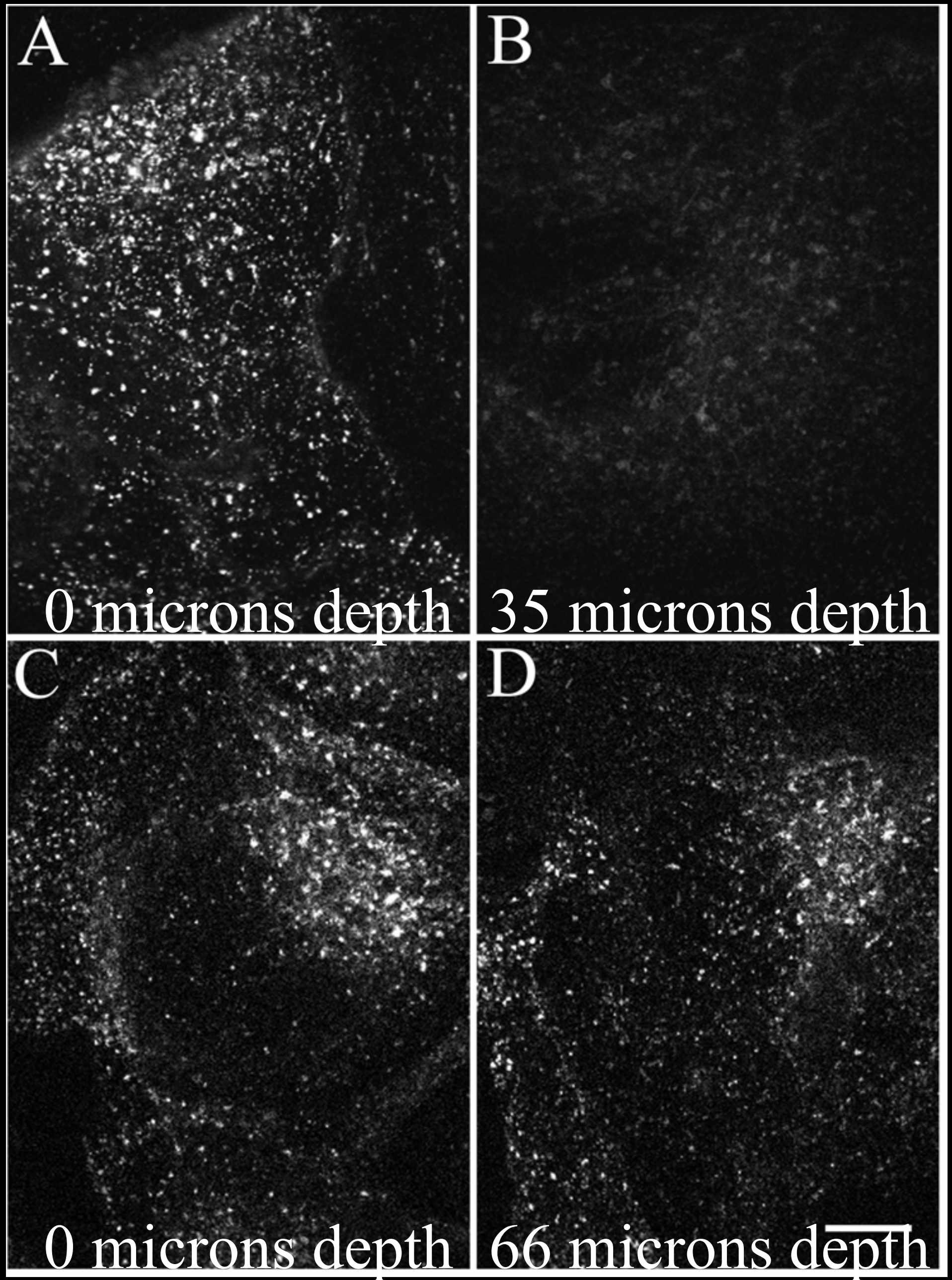
Practical confocal microscopy

- Image collection settings
- Objective choice
  - Chromatic aberration
  - Spherical aberration

Spherical aberration



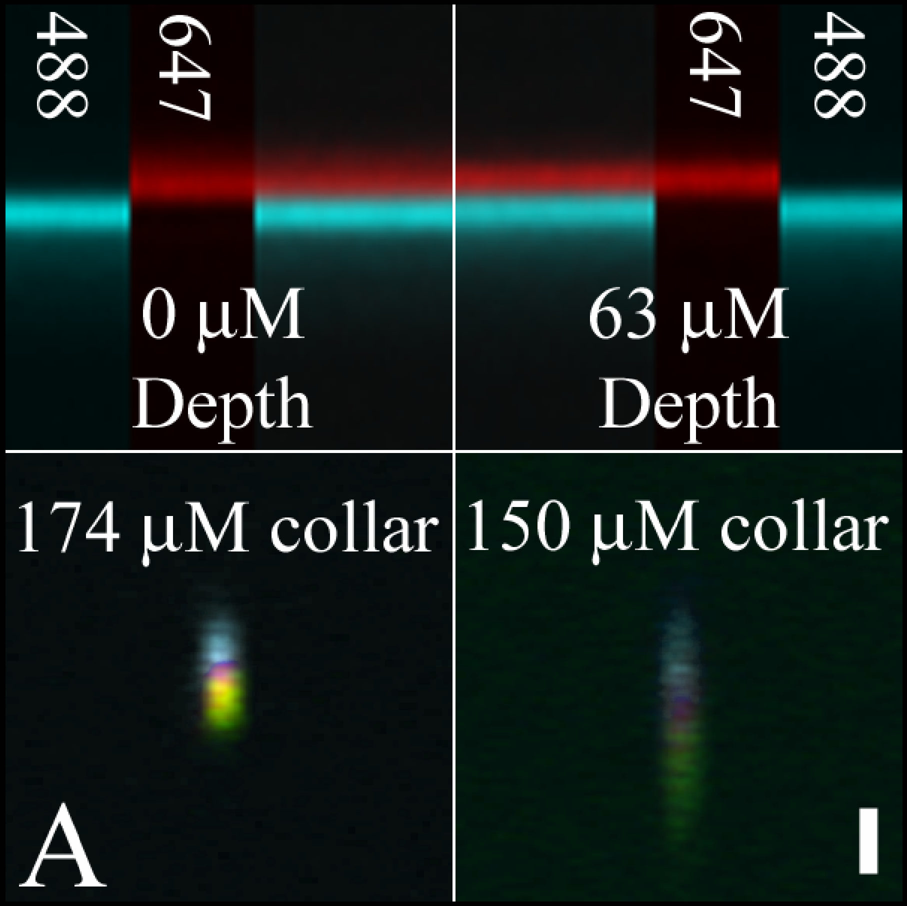
100x, Planapo, Oil immersion objective



Spherical aberration

60x, Planapo, Water immersion objective

Collar adjustment of the 60x water immersion objective



Collar adjustment of the 60x water immersion objective

Ruben Sandoval

Objective corrections require multiple lens elements

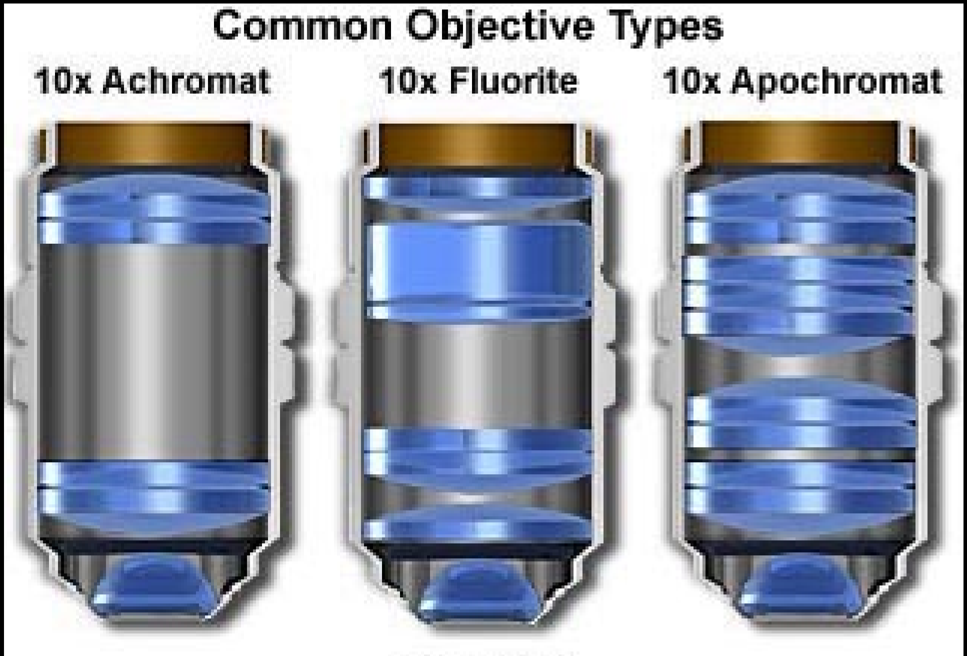


Figure 2

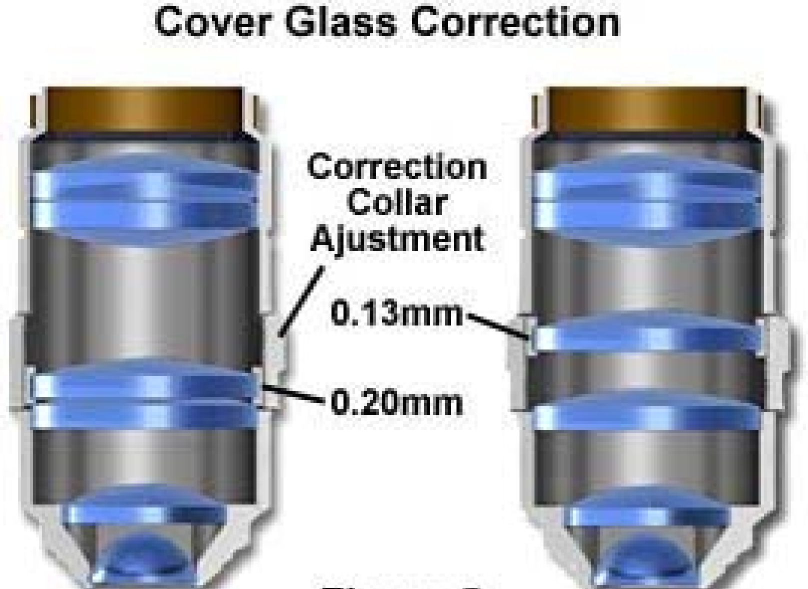


Figure 5

<u>Corrections:</u>

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

The more correction that a lens uses, the less transmission

	Light collection efficiency $10^4 \times (NA^2/mag)^2$	Corrections (# elements - transmission)
100X, Oil immersion, NA 1.4	3.84	Planapochromat 10 – 66%
40X, Oil immersion, NA 1.4	24.01	Plan-fluor 6 – 79%
60X, Oil immersion, NA 1.4	10.67	Planapochromat 10 – 66%
60X, Water immersion, NA 1.2	5.76	Planapochromat 10 – 66%
20X, Water immersion, NA .75	7.91	Plan-fluor 6 – 79%

Ask yourself–

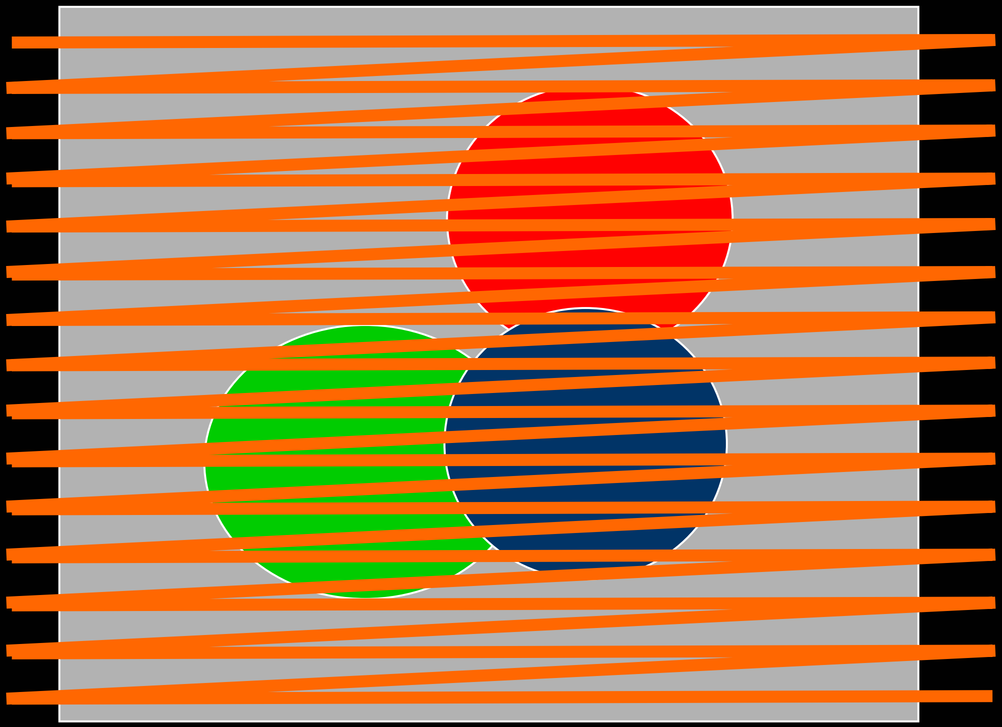
Is correction for chromatic aberration important for you?

	Light collection efficiency $10^4 \times (NA^2/mag)^2$	Corrections (# elements - transmission)
100X, Oil immersion, NA 1.4	3.84	Planapochromat 10 – 66%
40X, Oil immersion, NA 1.4	24.01	Plan-fluor 6 – 79%
60X, Oil immersion, NA 1.4	10.67	Planapochromat 10 – 66%
60X, Water immersion, NA 1.2	5.76	Planapochromat 10 – 66%
20X, Water immersion, NA .75	7.91	Plan-fluor 6 – 79%

Ask yourself –
Is correction for chromatic aberration important to you?

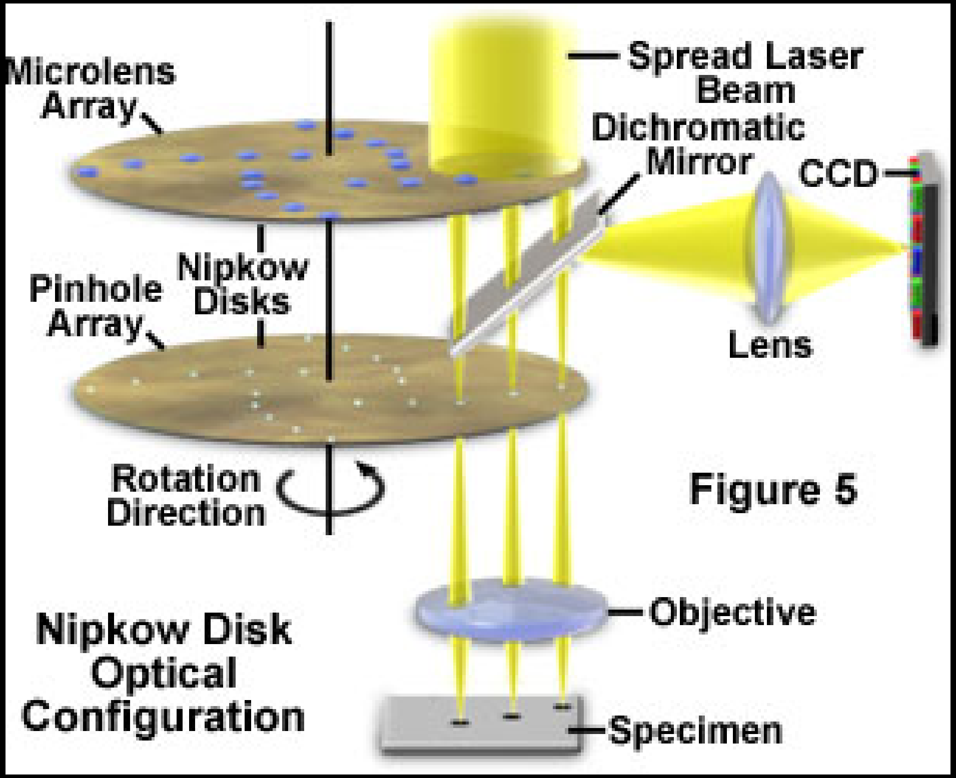
Confocal microscopy
- 2-dimensional imaging detectors

Confocal microscopy builds an image by point scanning – and so is slow



Need to acquire one point at a time. This limits acquisition to ~1 frame/sec. Limited by number of photons per pixel.

Dave Piston, Vanderbilt

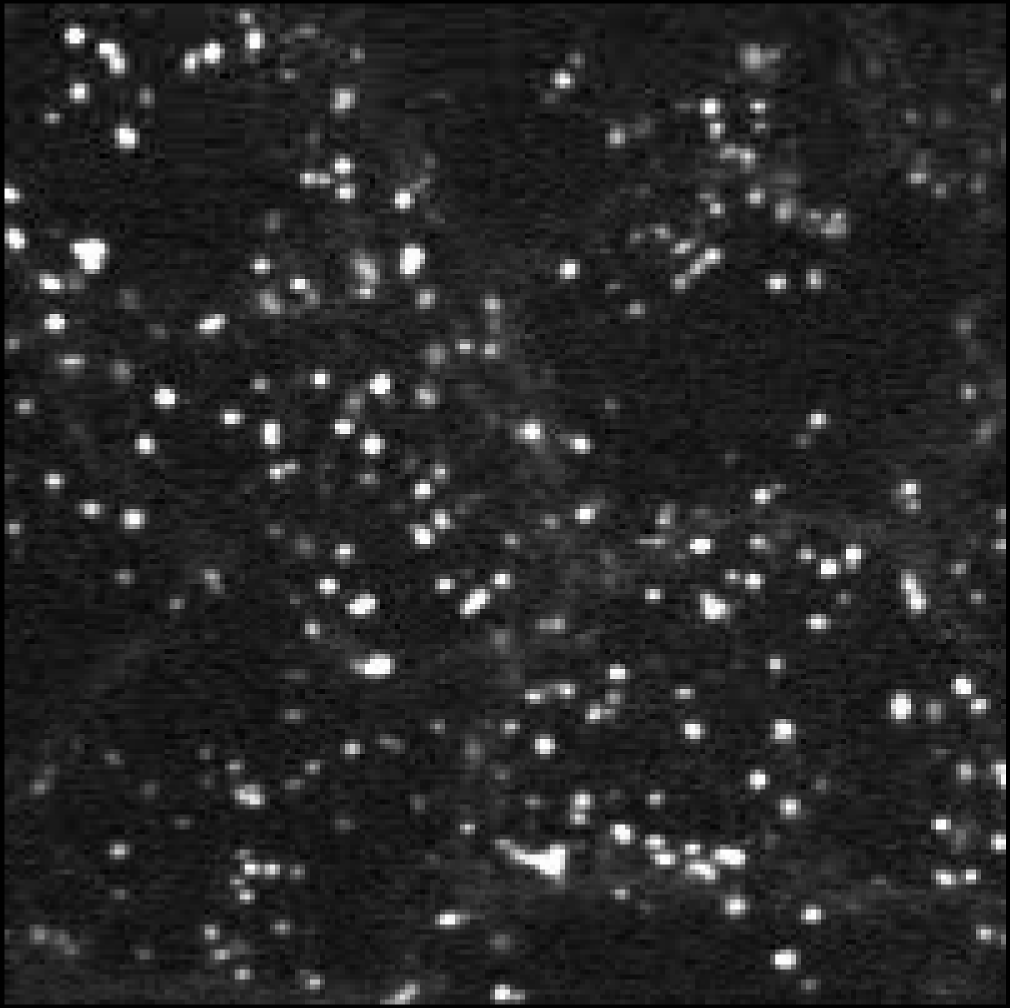


Spinning disk confocal microscope

- Spinning disk scans 1200 spots across a field at 30 Hz
- 360 Hz frame rate, we've collected at up to 40 Hz
  - Characterize rapid dynamics
- Signal to noise better than conventional confocal systems
  - Reduce illumination for reduced phototoxicity and photobleaching

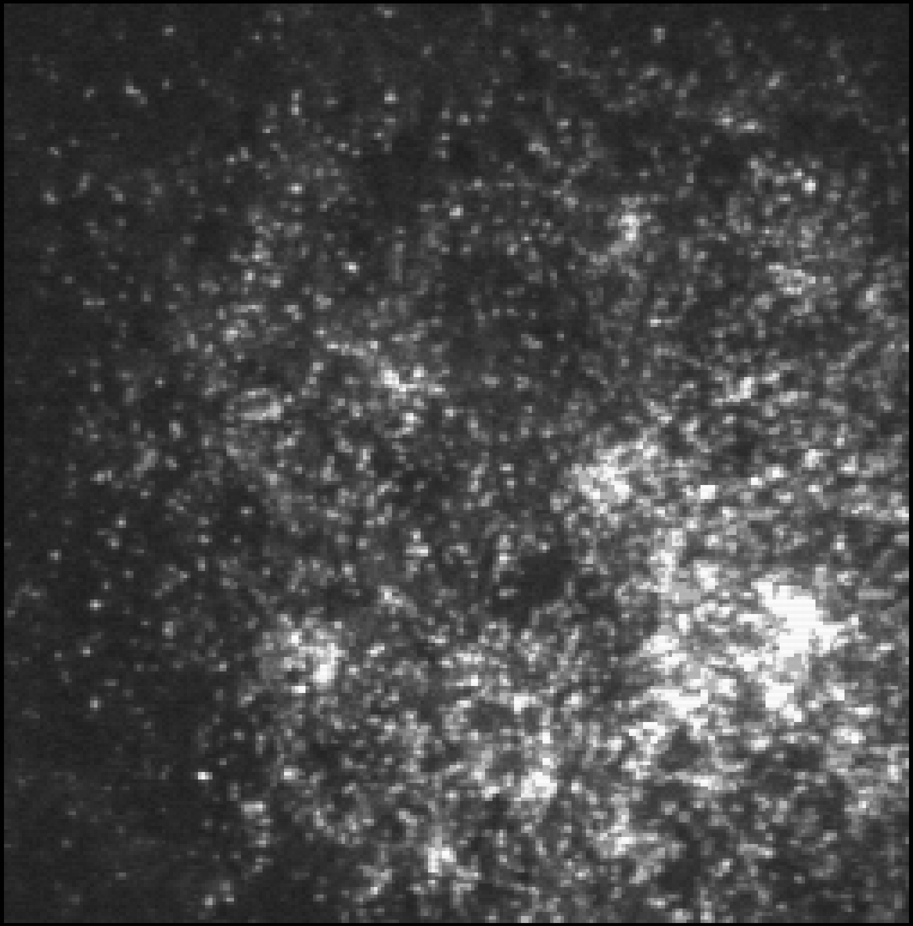
Figure courtesy of Florida State Univ.

Transferrin labeled endosomes migrating around an MDCK cell – Perkin-Elmer/Yokagawa spinning disk video-rate confocal microscope



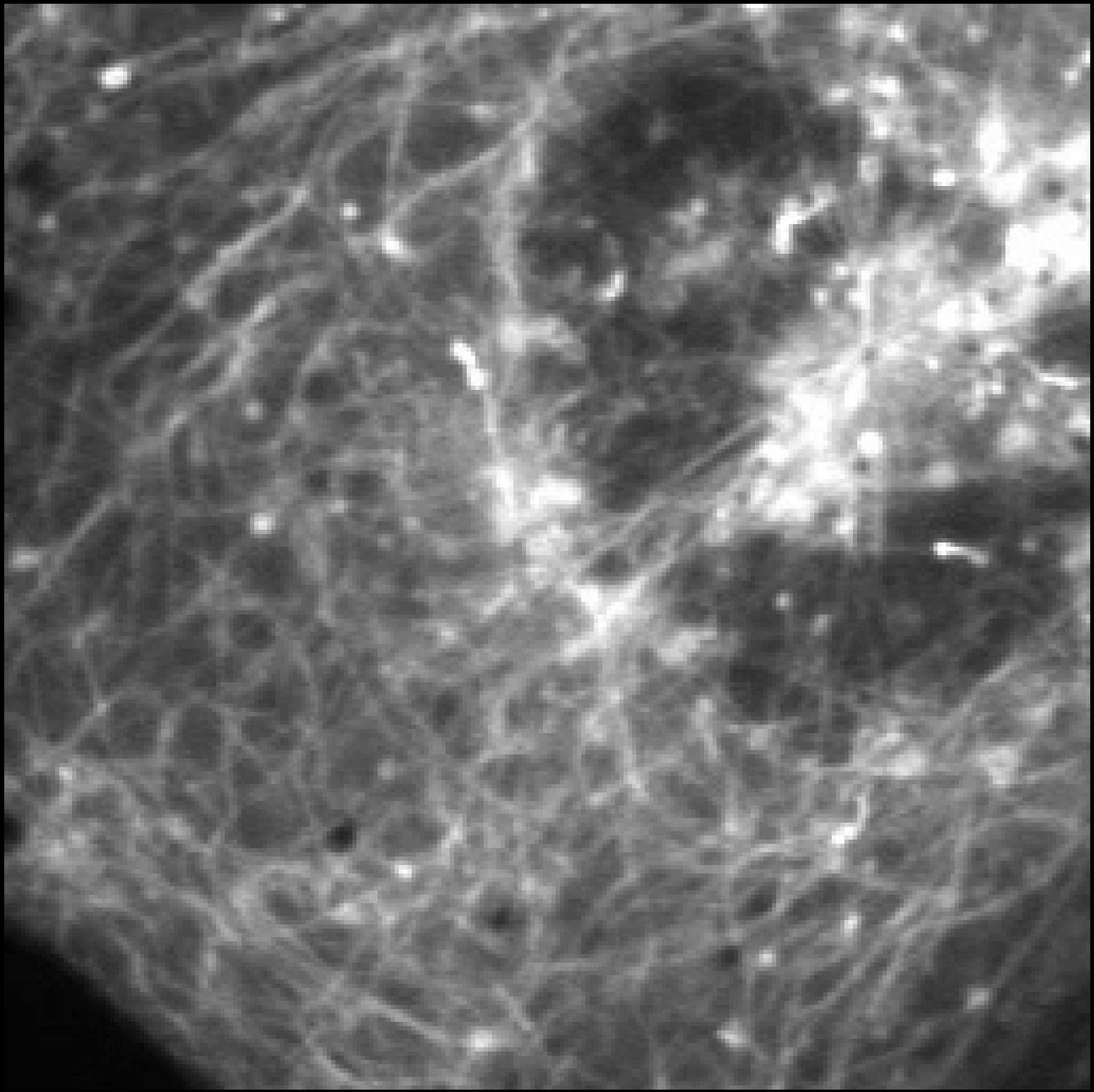
1100 time points collected at 11 fps over 100 seconds

Real-time movement of Rab25 around an MDCK cell – Perkin-Elmer/Yokagawa spinning disk confocal microscope

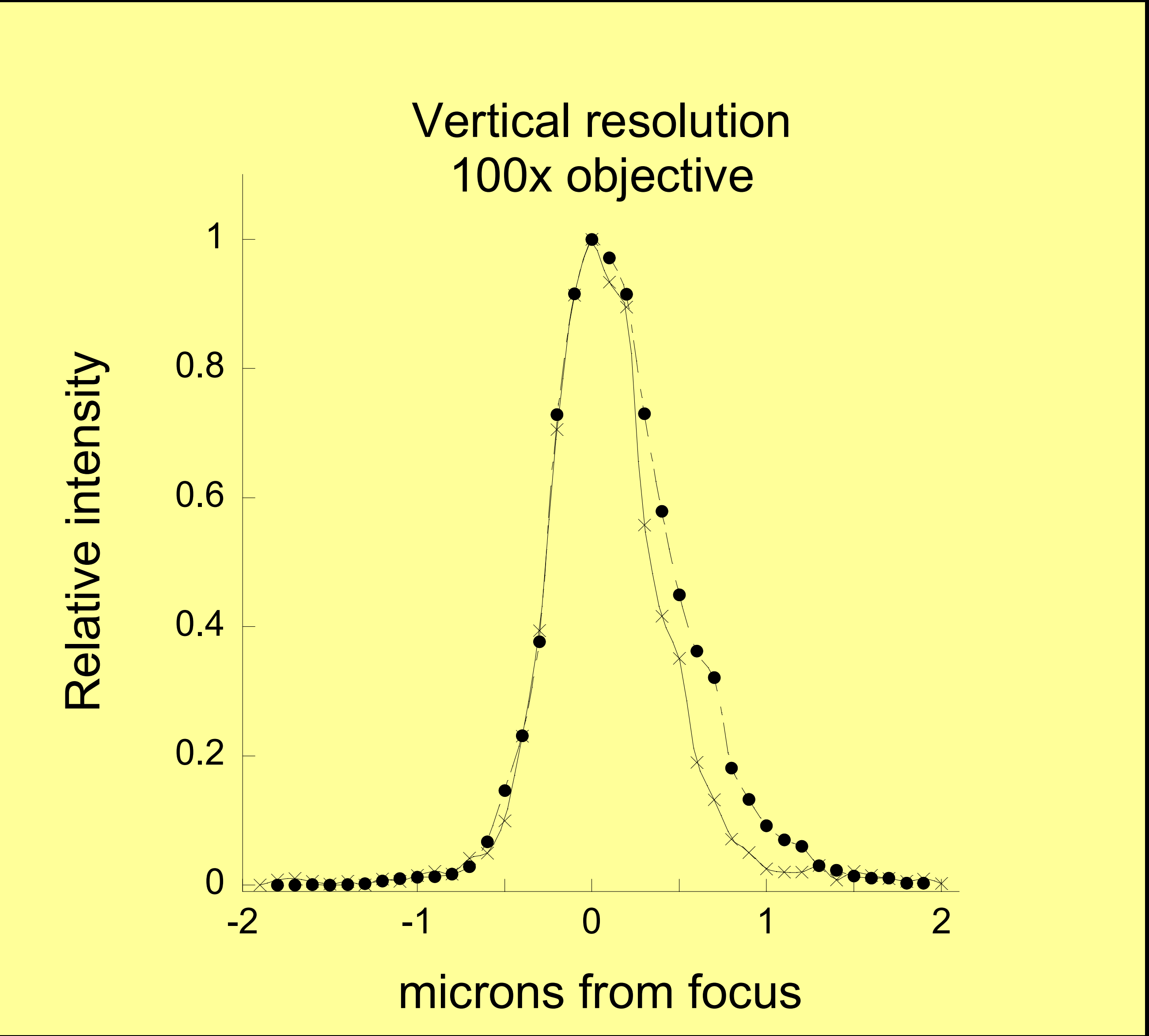


600 time points collected and displayed at 20 fps over 30 seconds

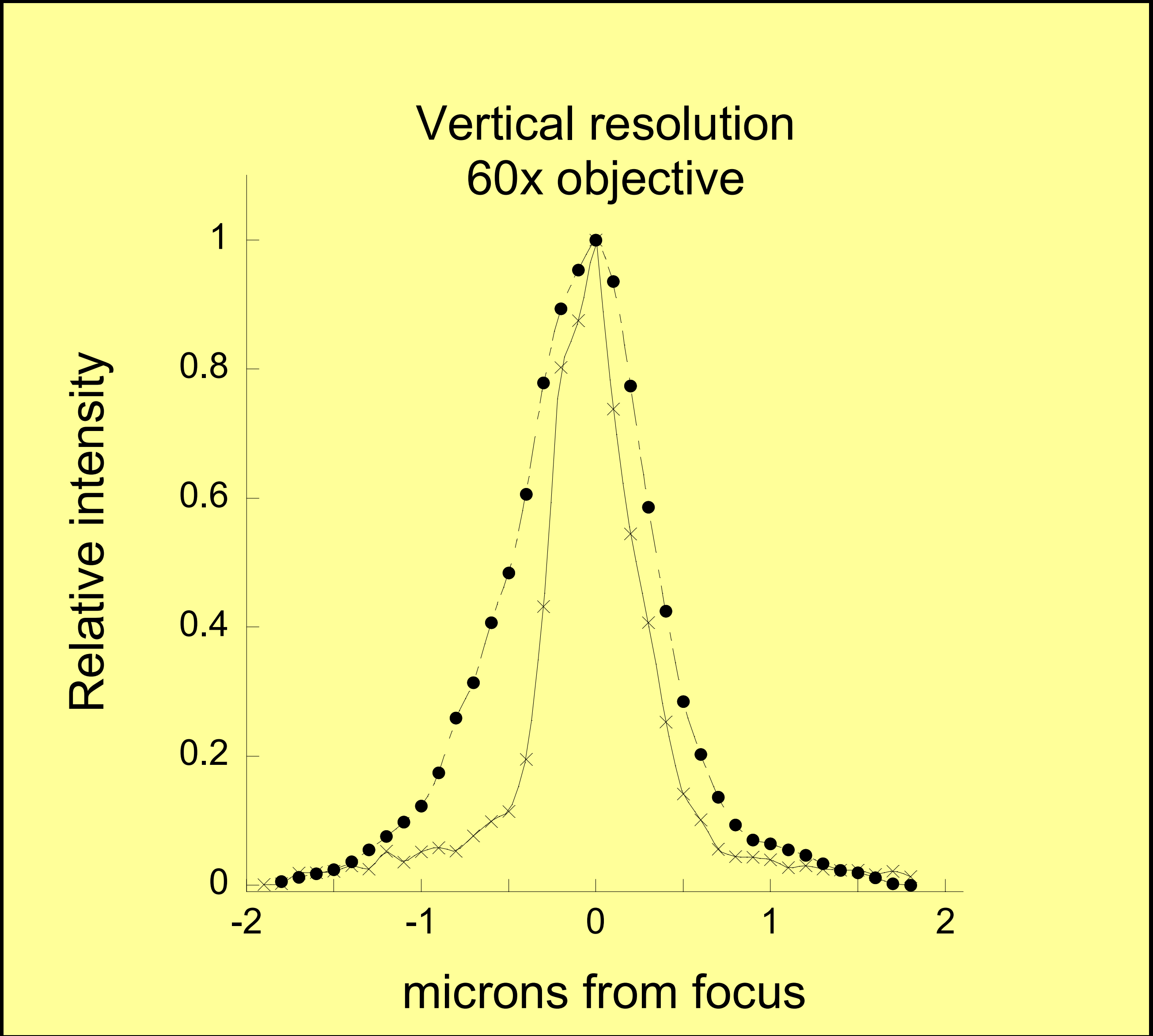
Living MDCK cells expressing GFP-tubulin and GFP-Rab7 – Perkin-Elmer/Yokagawa spinning disk video-rate confocal microscope



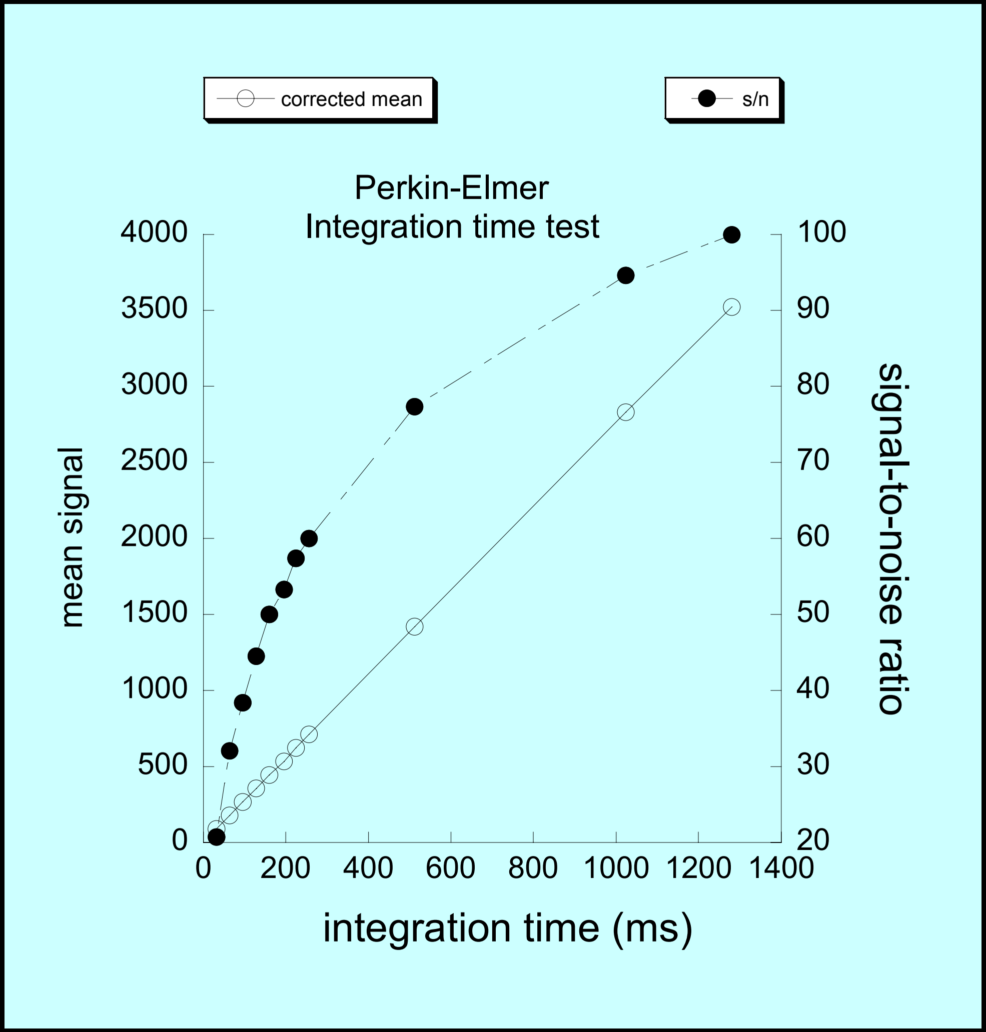
Axial resolution of the Ultraview and the Zeiss 510 confocal microscopes – 100x objectives



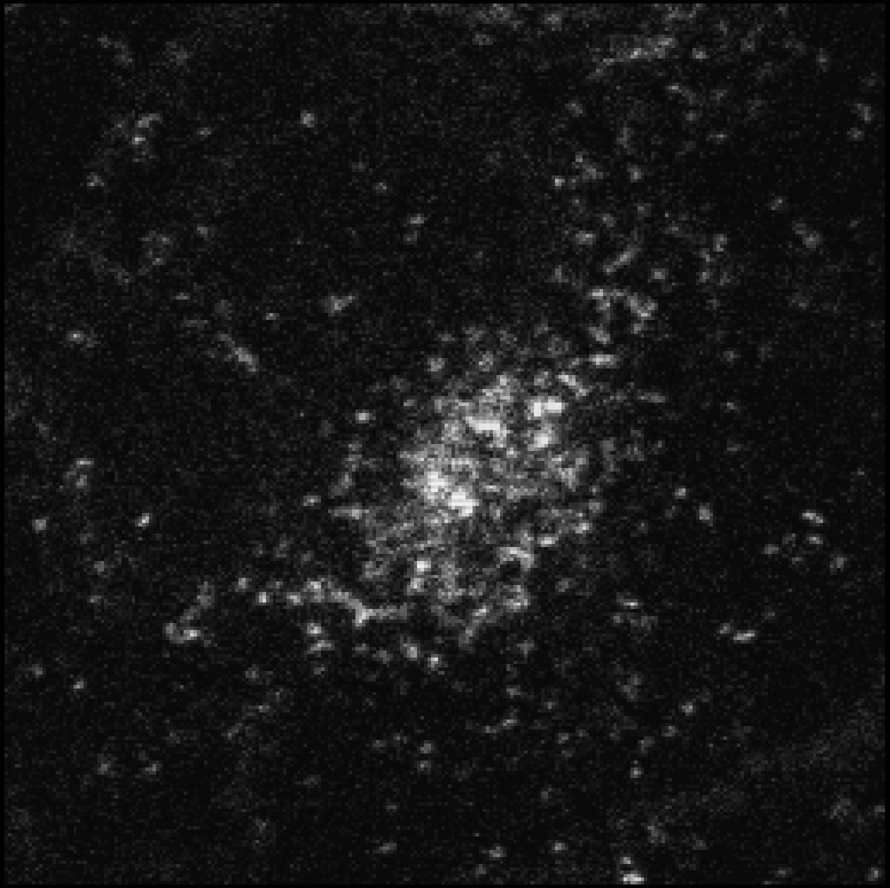
Axial resolution of the Ultraview and the Zeiss 510 confocal microscopes – 60x objectives



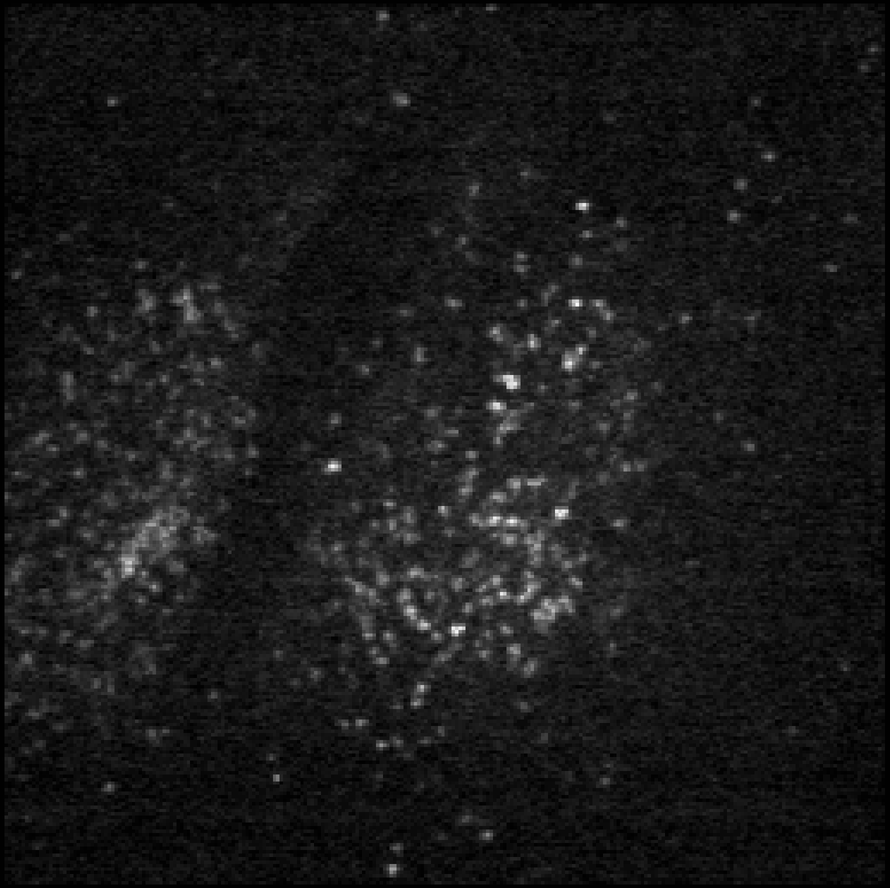
Ultraview system shows large linear range characteristic of CCD detectors



The Ultraview system for imaging living cells
Rab25 in MDCK cells

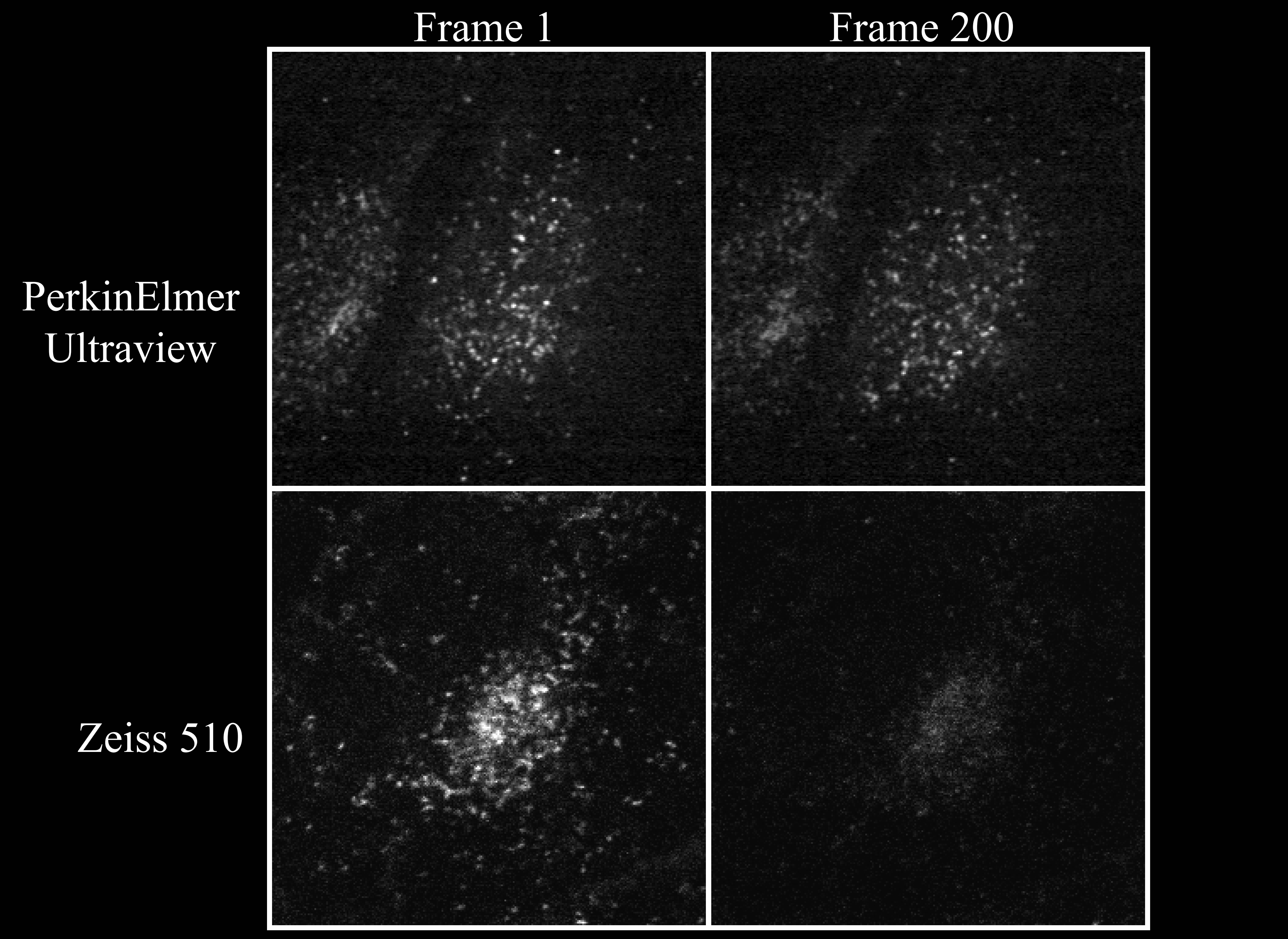


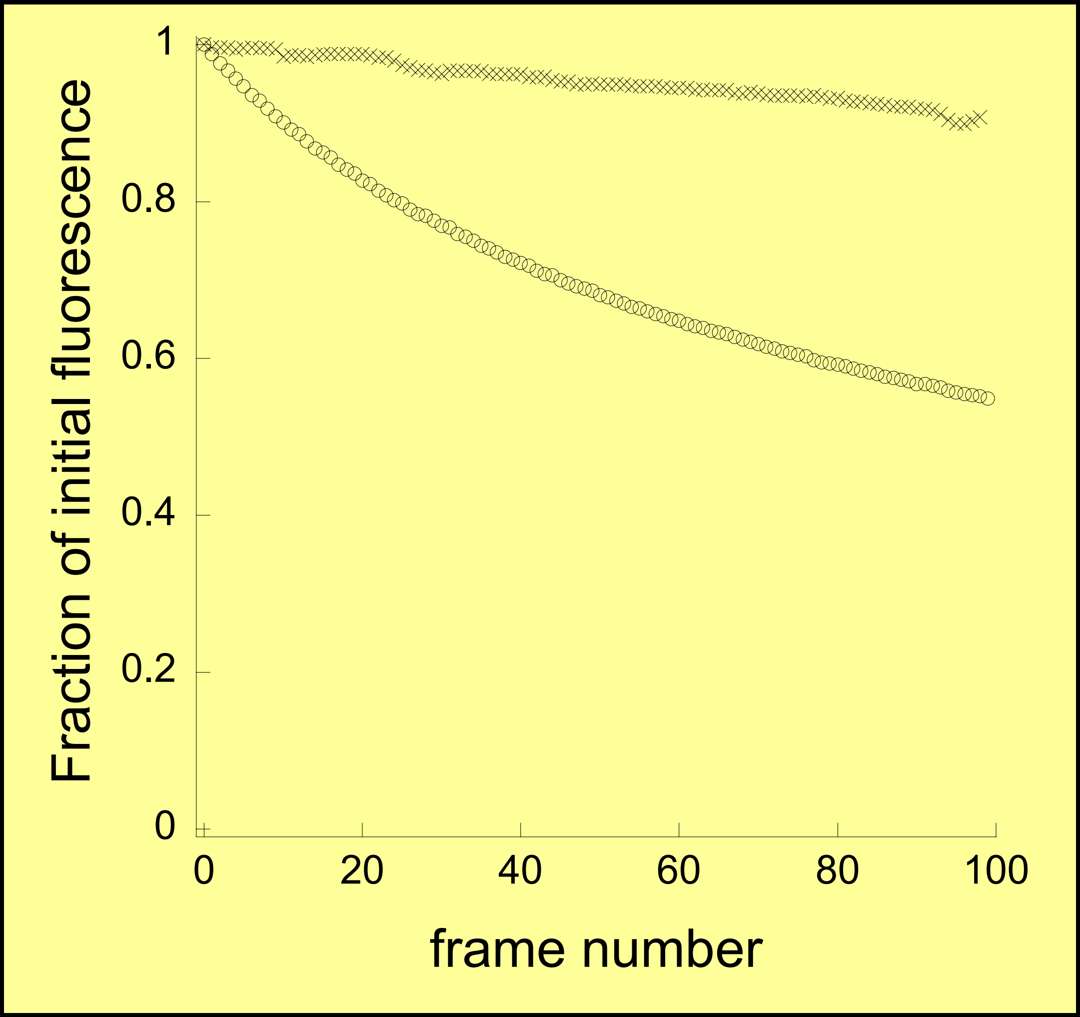
Zeiss 510



PerkinElmer Ultraview

200 images collected at 2 frames per second, 0.13 micron pixels, 12 bit pixel depth


Frame 1
Frame 200
PerkinElmer Ultraview
Zeiss 510



Comparison of imaging performance –

Zeiss 510 versus PerkinElmer Ultraview

Images collected at 1.7 frames per second, 0.067 micron pixels, 12 bit pixel depth

	S/N - initial	S/N - final
Ultraview	56.5	54.2
Zeiss	22.0	14.2

Why is the Ultraview so good?

- 1/1200 the intensity – less saturation
- Integrate 8 μs x 360 per second vs. 4 μs
- 2-fold higher quantum efficiency
- 360 breaks between illuminations
- Less digitizer and amplifier noise

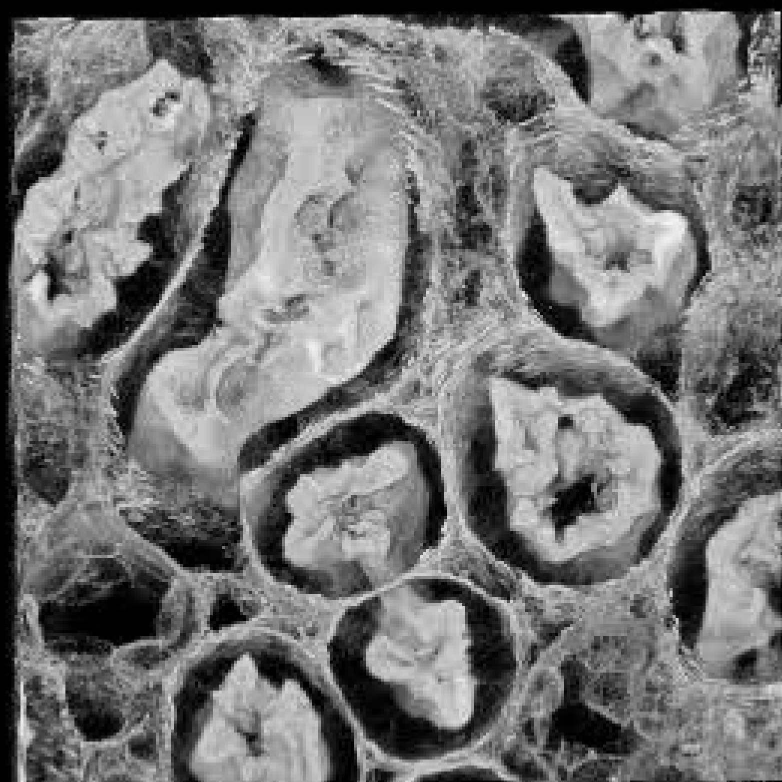
Field imaging (Ultraview) versus point scanning confocal systems

- Field imaging detectors
  - CCDs are very clean, with wide dynamic range
  - Images can be collected rapidly
  - Different colors must be collected sequentially, or on different detector arrays

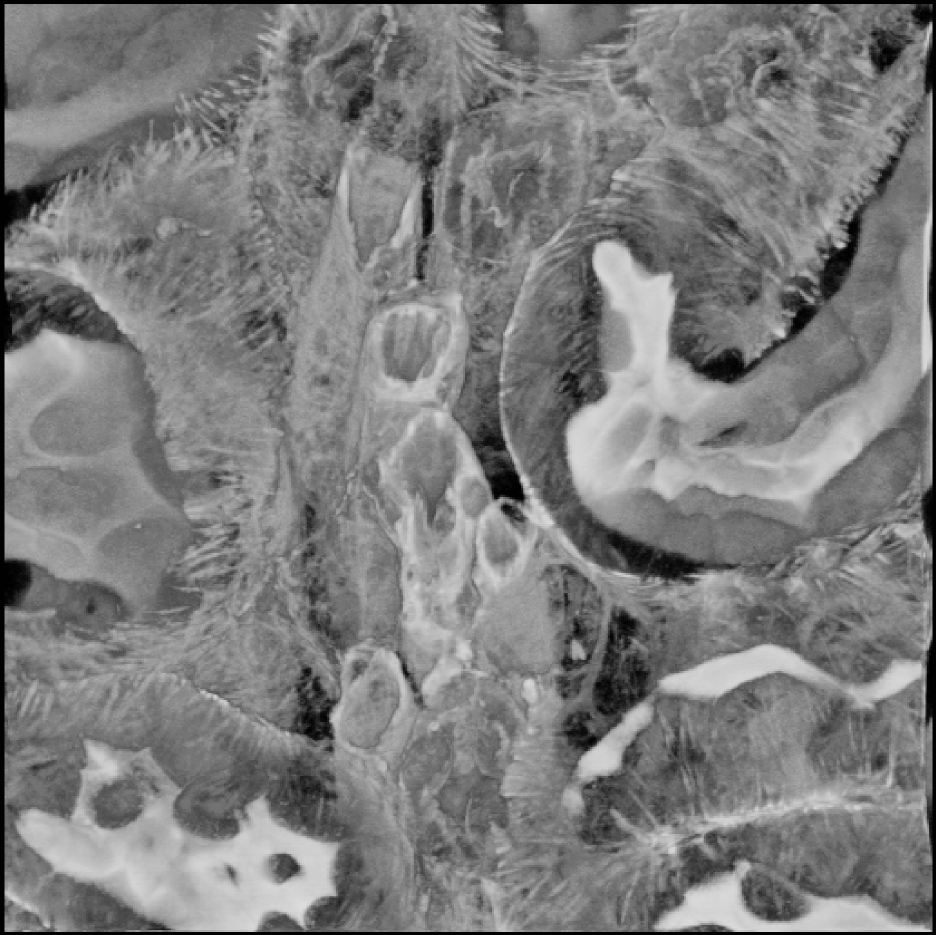
- Point scanning detectors
  - Multiple colors can be collected simultaneously
  - PMTs are noisier than CCD systems
  - Slow image acquisition and reasonable frame rates require very brief collection per pixel – high illumination and few photons

Multi-photon microscopy

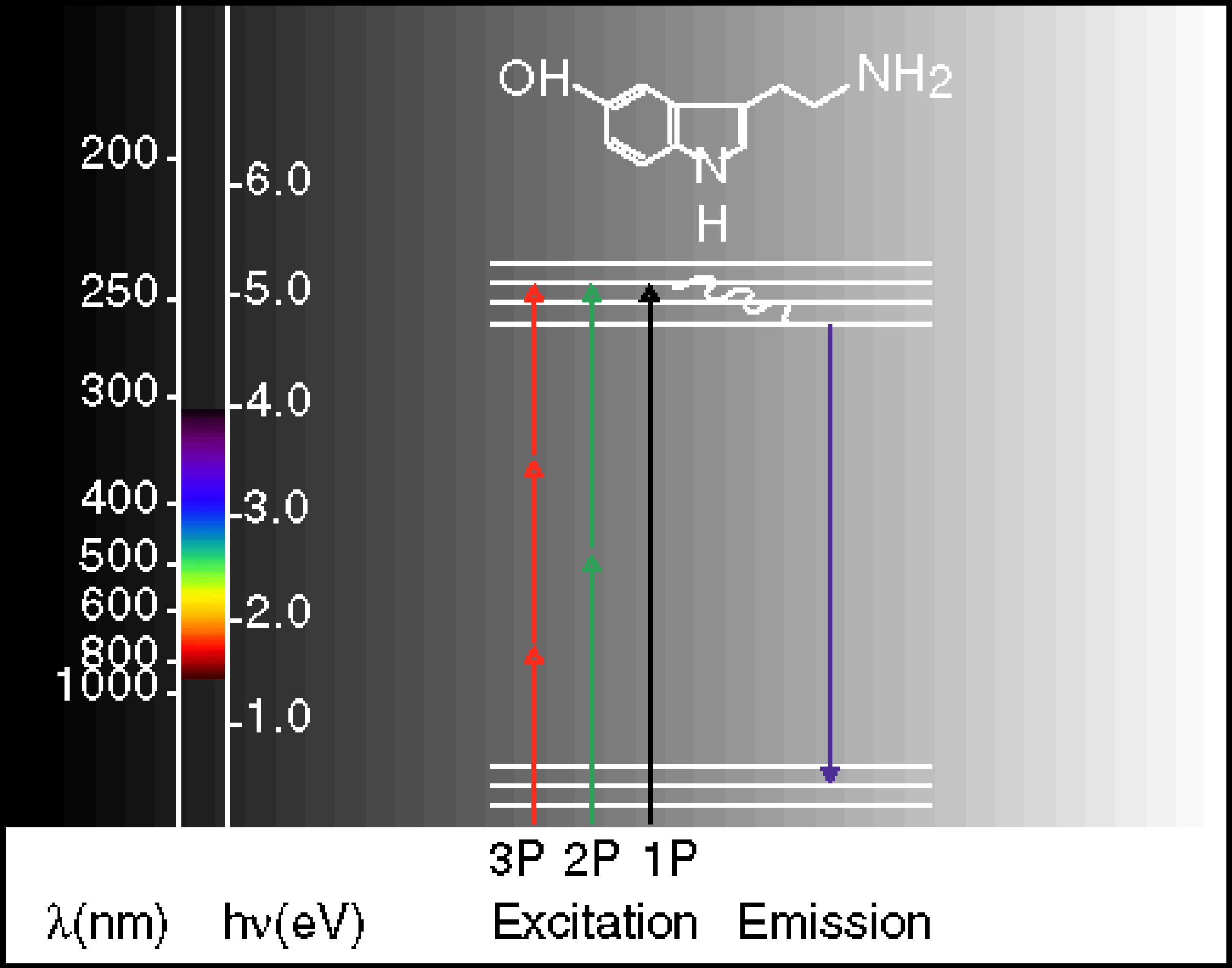
3D imaging of kidney tissue by confocal microscopy



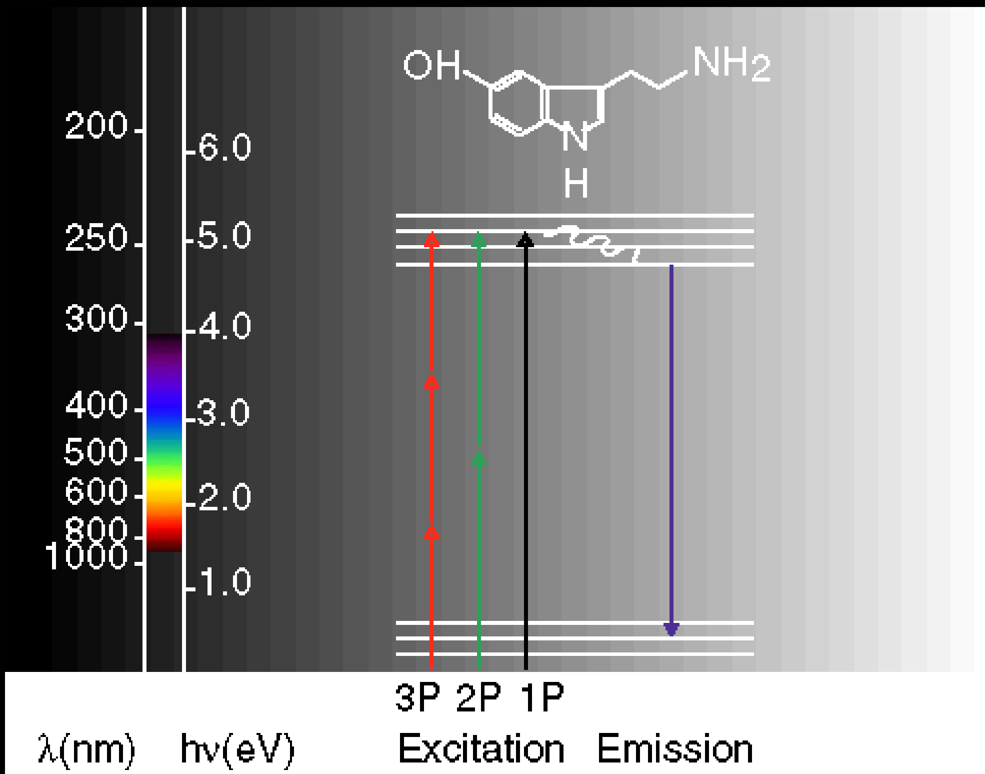
3D imaging of kidney tissue by 2-photon microscopy



Multi-photon fluorescence excitation depends upon the simultaneous absorption of multiple photons

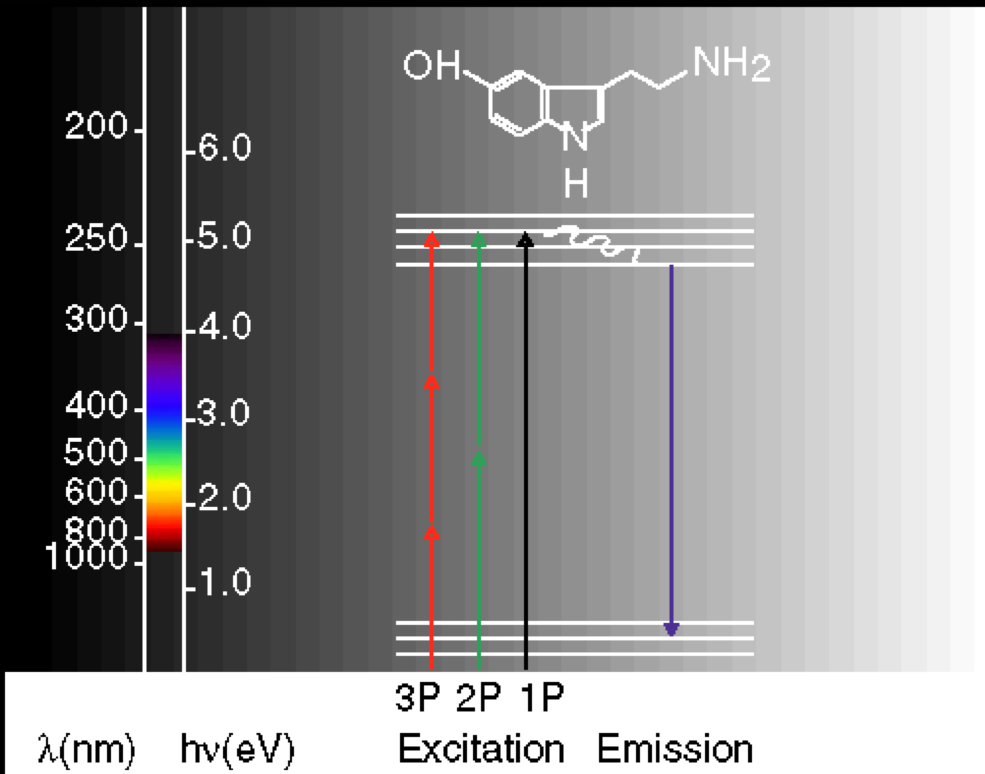


Multi-photon fluorescence excitation depends upon the simultaneous absorption of multiple photons



What is “simultaneous”?

Multi-photon fluorescence excitation depends upon the simultaneous absorption of multiple photons

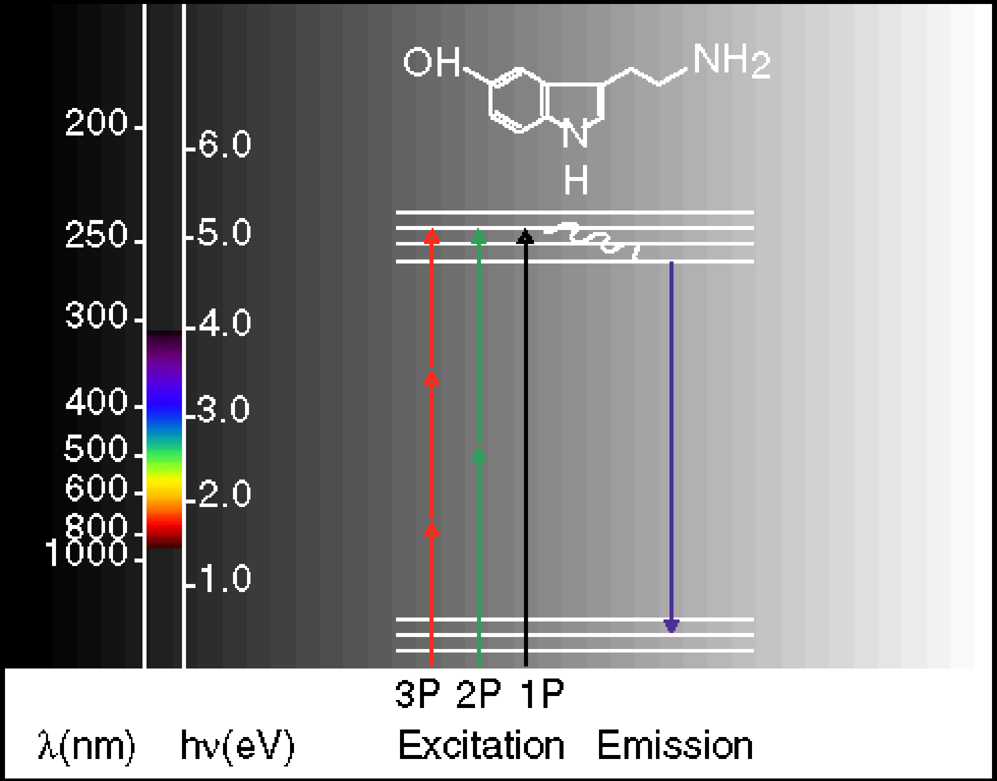


What is "simultaneous"?

Multiple photons must arrive within the duration of the intermediate virtual state of the electron

~ 1 attosecond (10^{-18} seconds)

Multi-photon fluorescence excitation depends upon the simultaneous absorption of multiple photons



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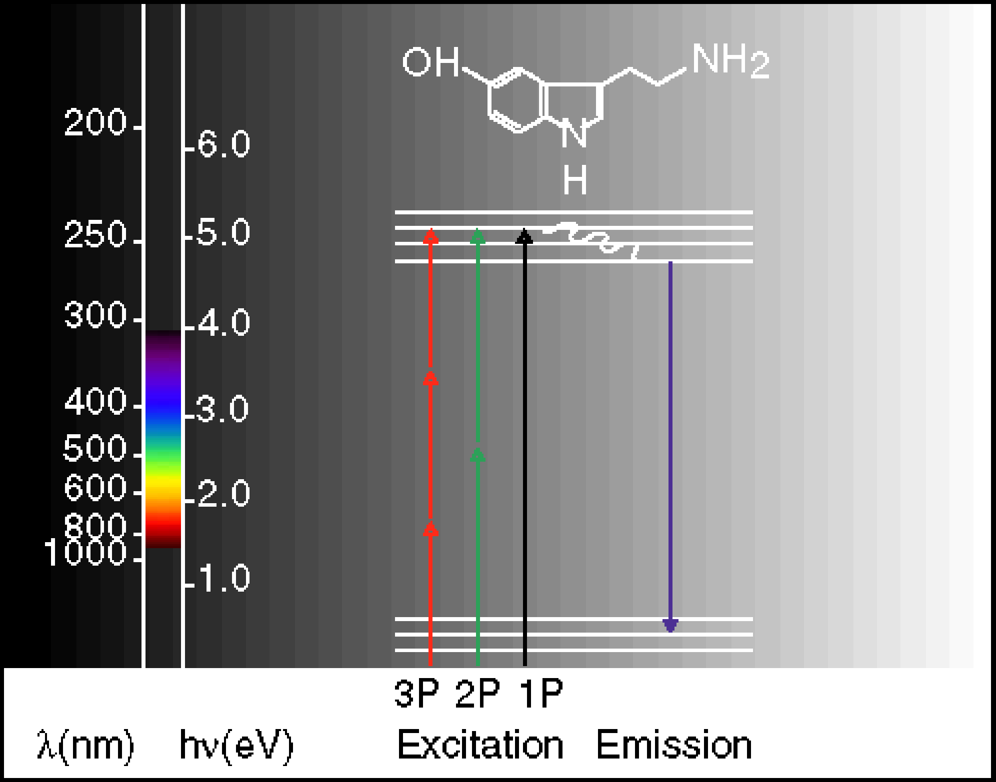
What is the relative frequency of such absorptions?

Multi-photon fluorescence excitation depends upon the simultaneous absorption of multiple photons



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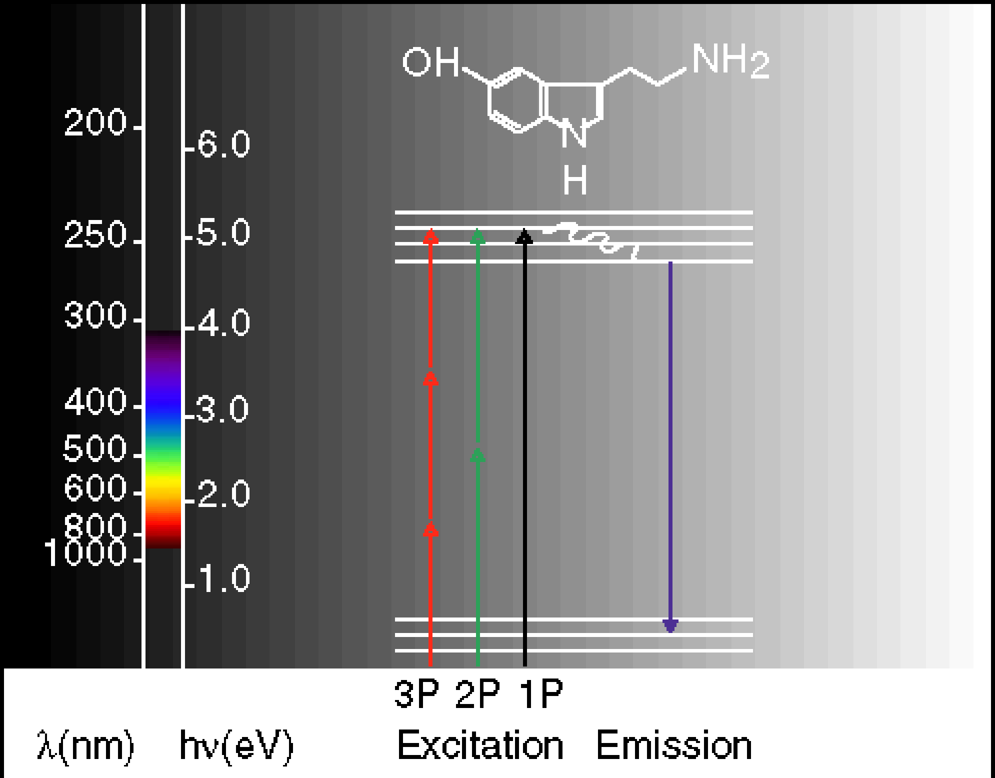
Winfried Denk calculated that a molecule of rhodamine B exposed to direct sunlight will experience:

- A one-photon absorption around once per second.

Multi-photon fluorescence excitation depends upon the simultaneous absorption of multiple photons



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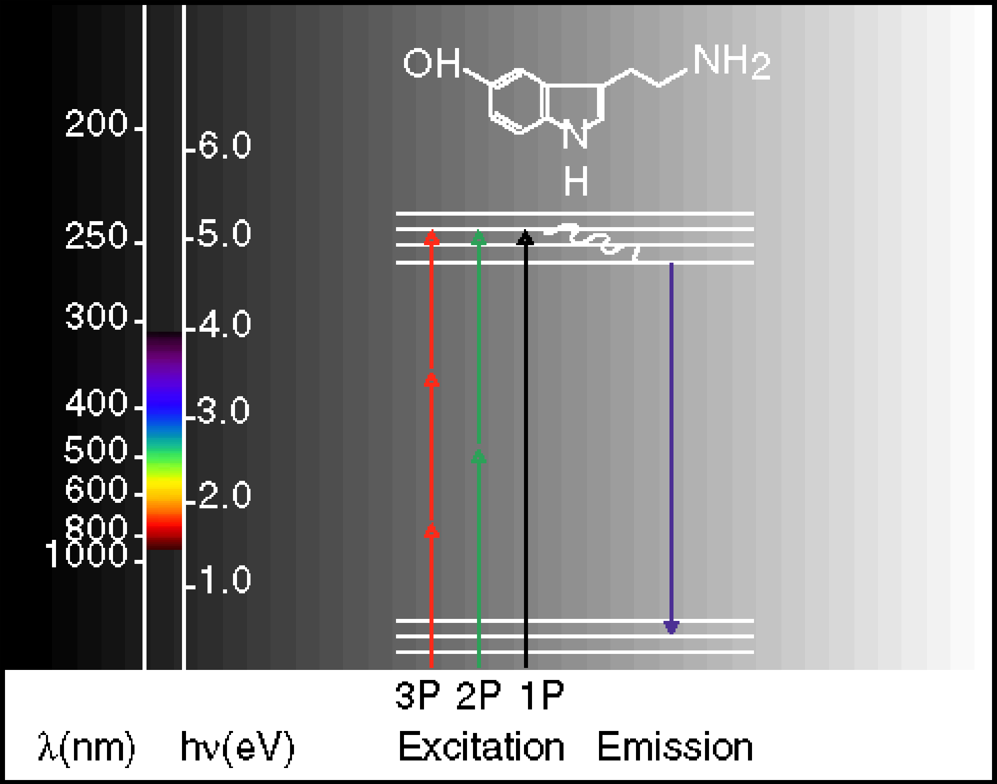

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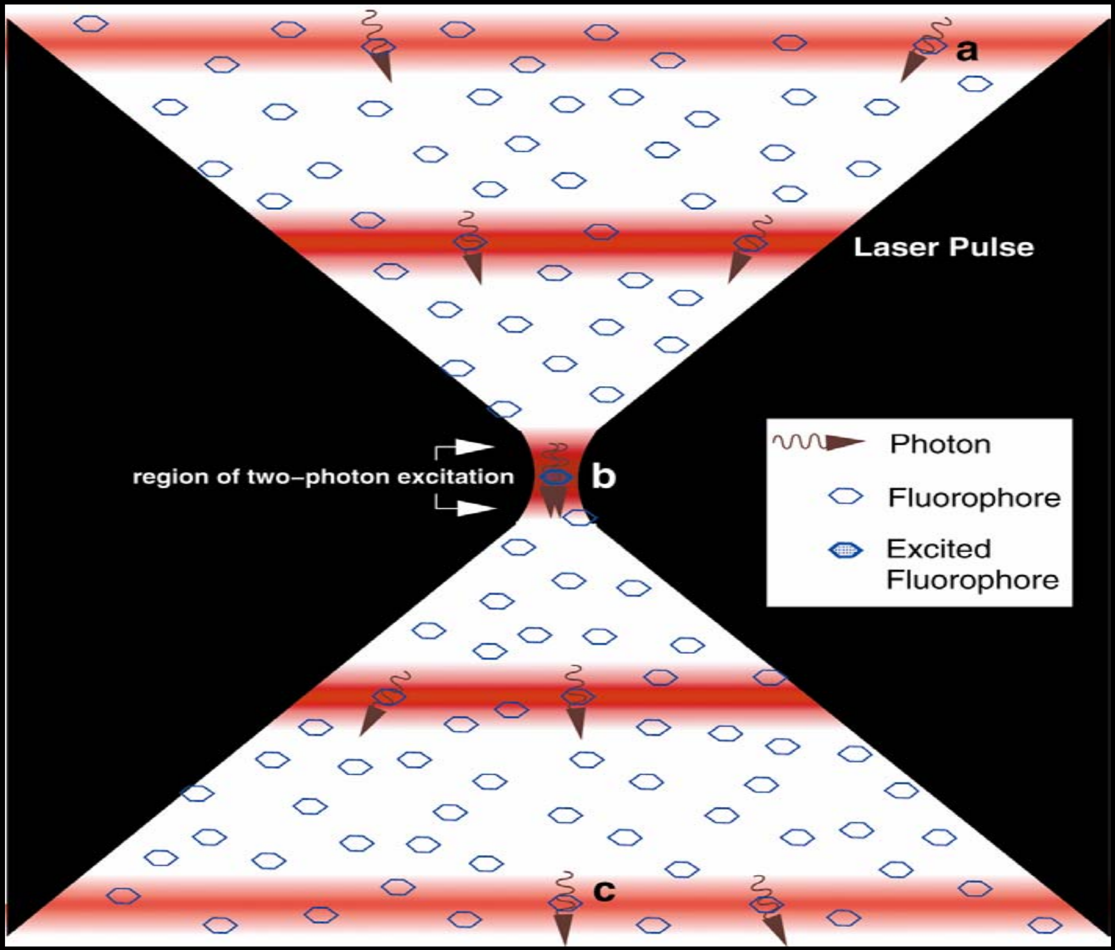
- A one-photon absorption around once per second.
- A two photon absorption once every 10,000 years.
- A three-photon absorption . . . well actually never in the history of the universe.

How to increase the probability of multi-photon absorption for multiphoton microscopy?

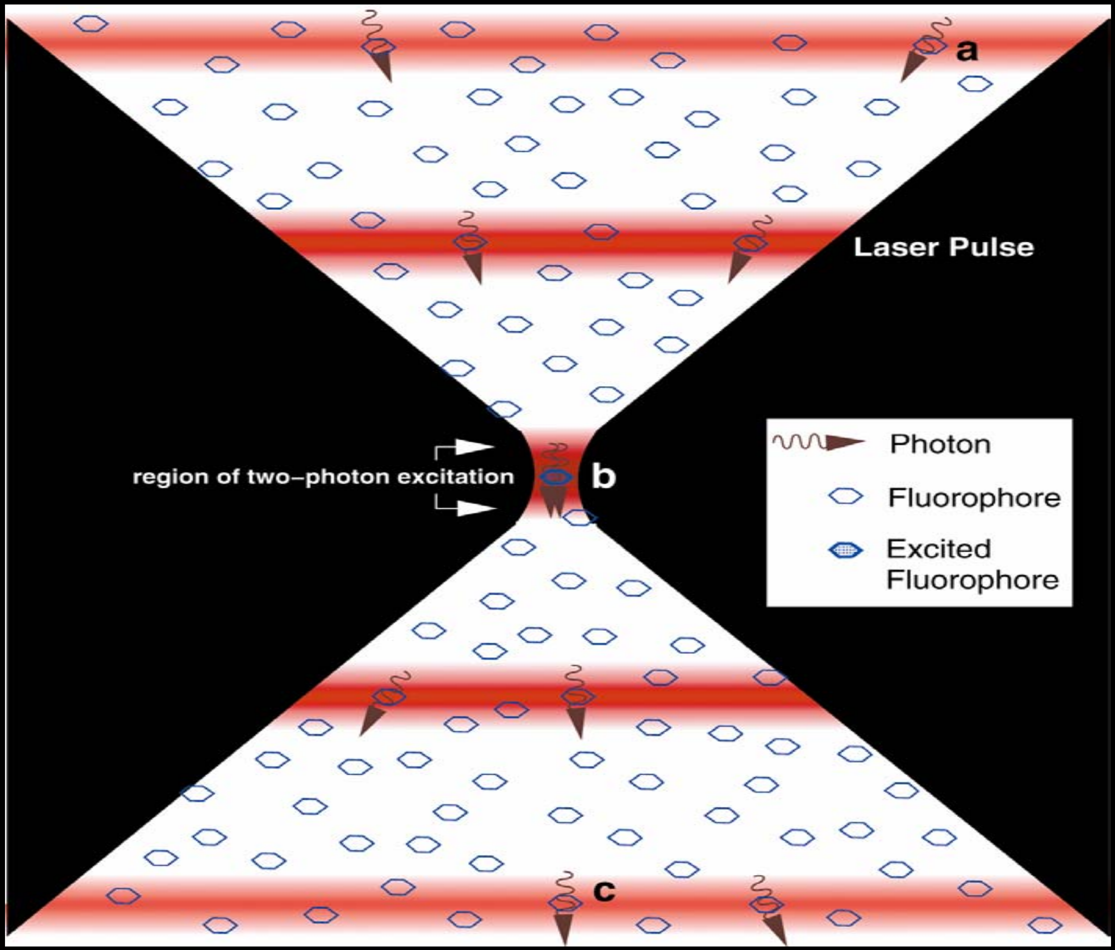
You could increase illumination 600,000 fold.

How is the probability of multi-photon absorption increased in multiphoton microscopy? – Photon crowding



Dave Piston, Vanderbilt

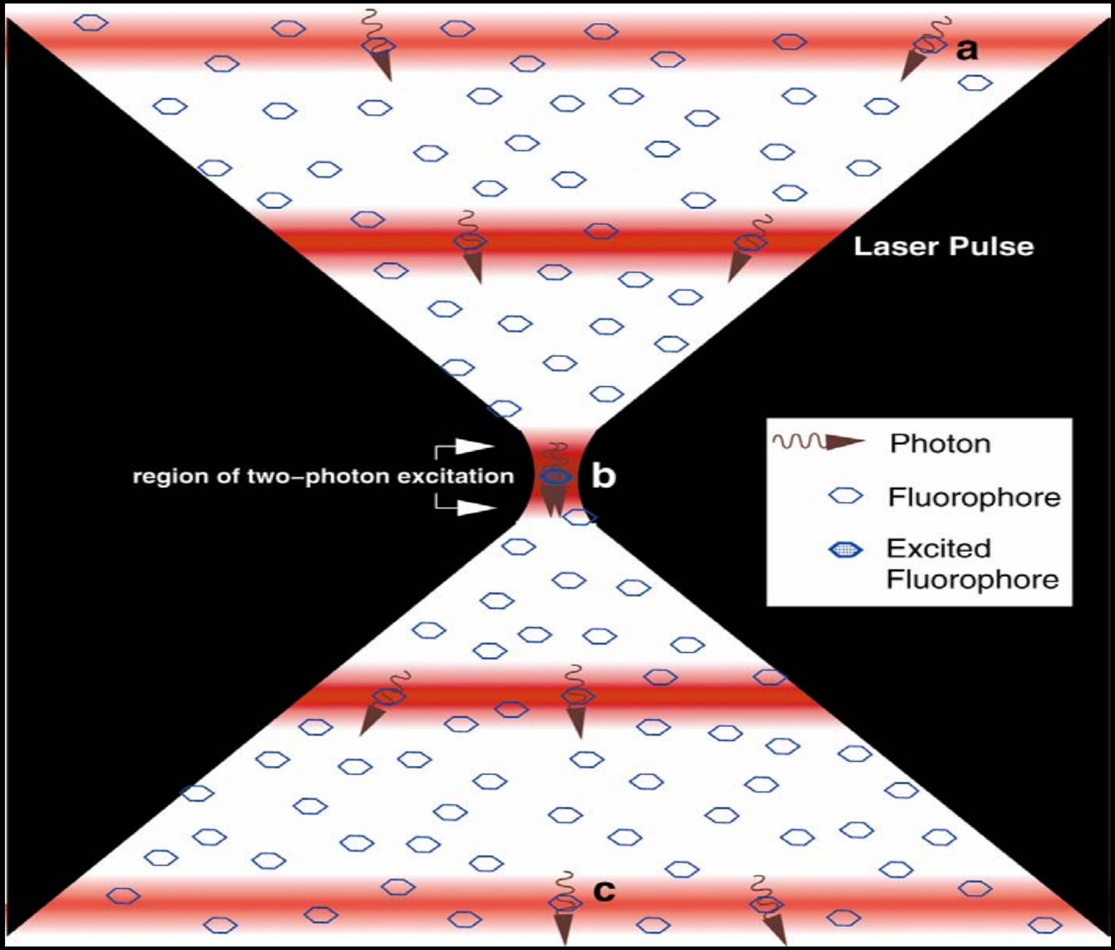
How is the probability of multi-photon absorption increased in multiphoton microscopy? – Photon crowding



Photon crowding in space

- the cross-sectional density of photons is highest at the focal point.

How is the probability of multi-photon absorption increased in multiphoton microscopy? – Photon crowding



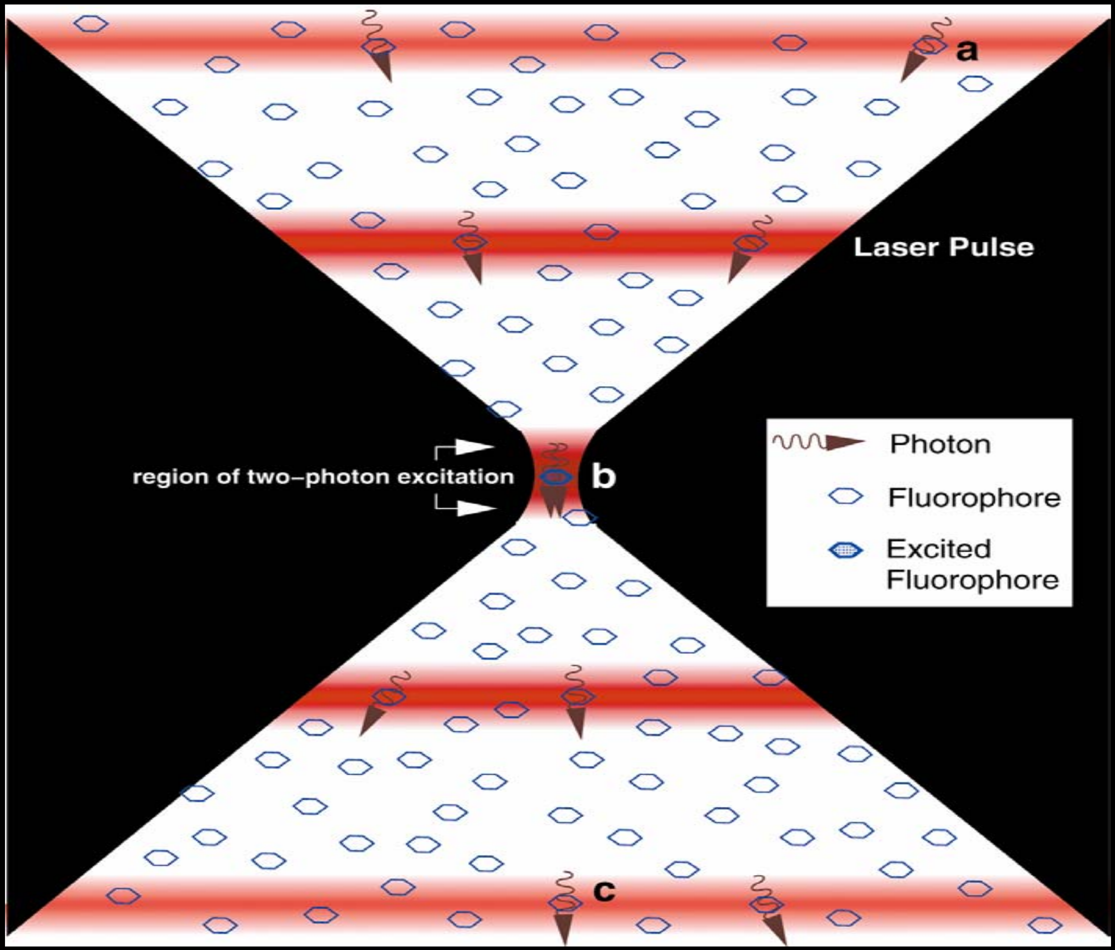
Photon crowding in space

- the cross-sectional density of photons is highest at the focal point.

Photon crowding in time

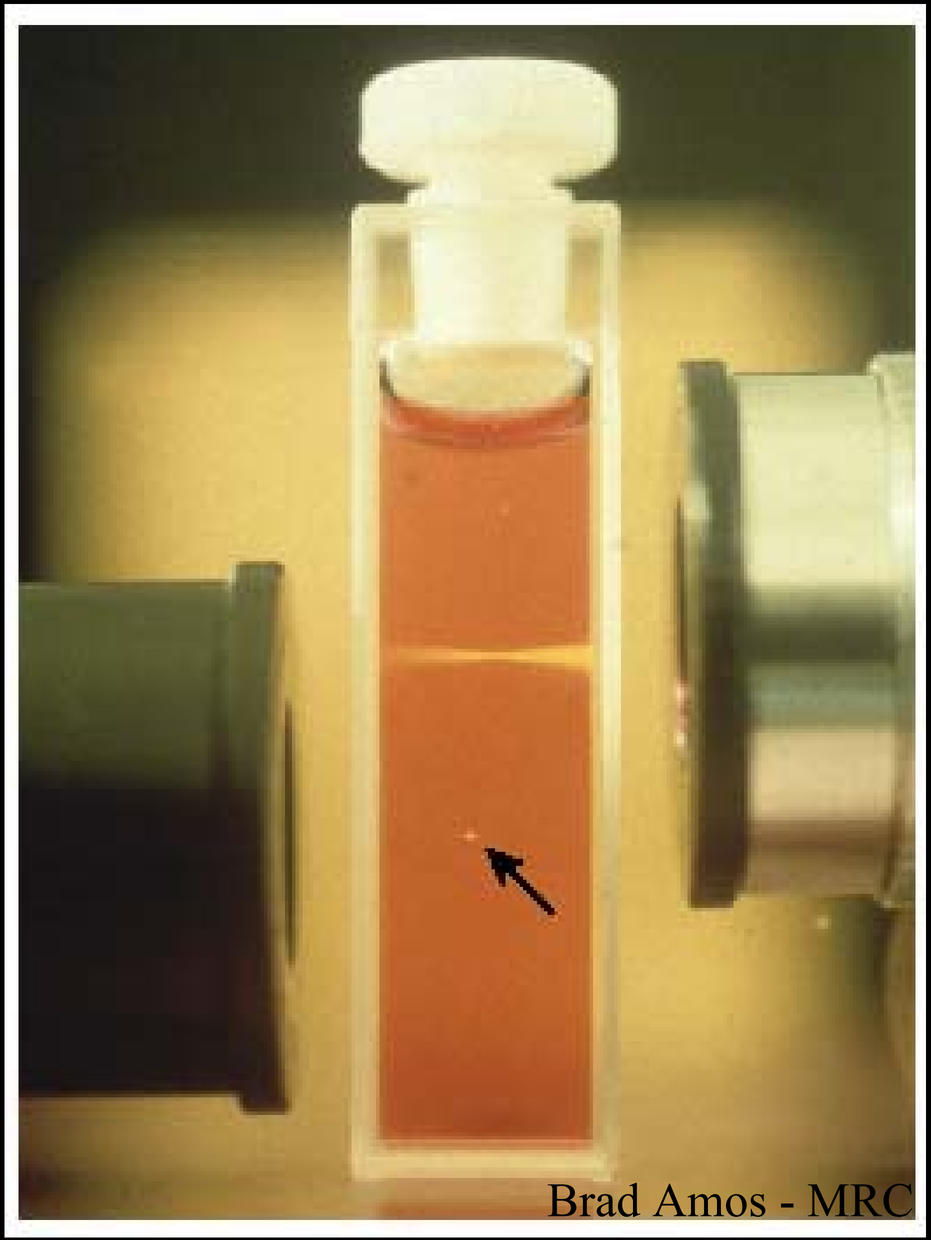
- laser emissions are pulsed into brief (~100 femtosecond) packets

Pulsed laser emissions provide for power sufficient for multiphoton absorption without photo-damage



- Pulsed laser provides low average power but peak power high enough for 2-photon absorption
- Sample illuminated for only 8 one millionth of the pixel dwell time

One and two photon fluorescence excitation



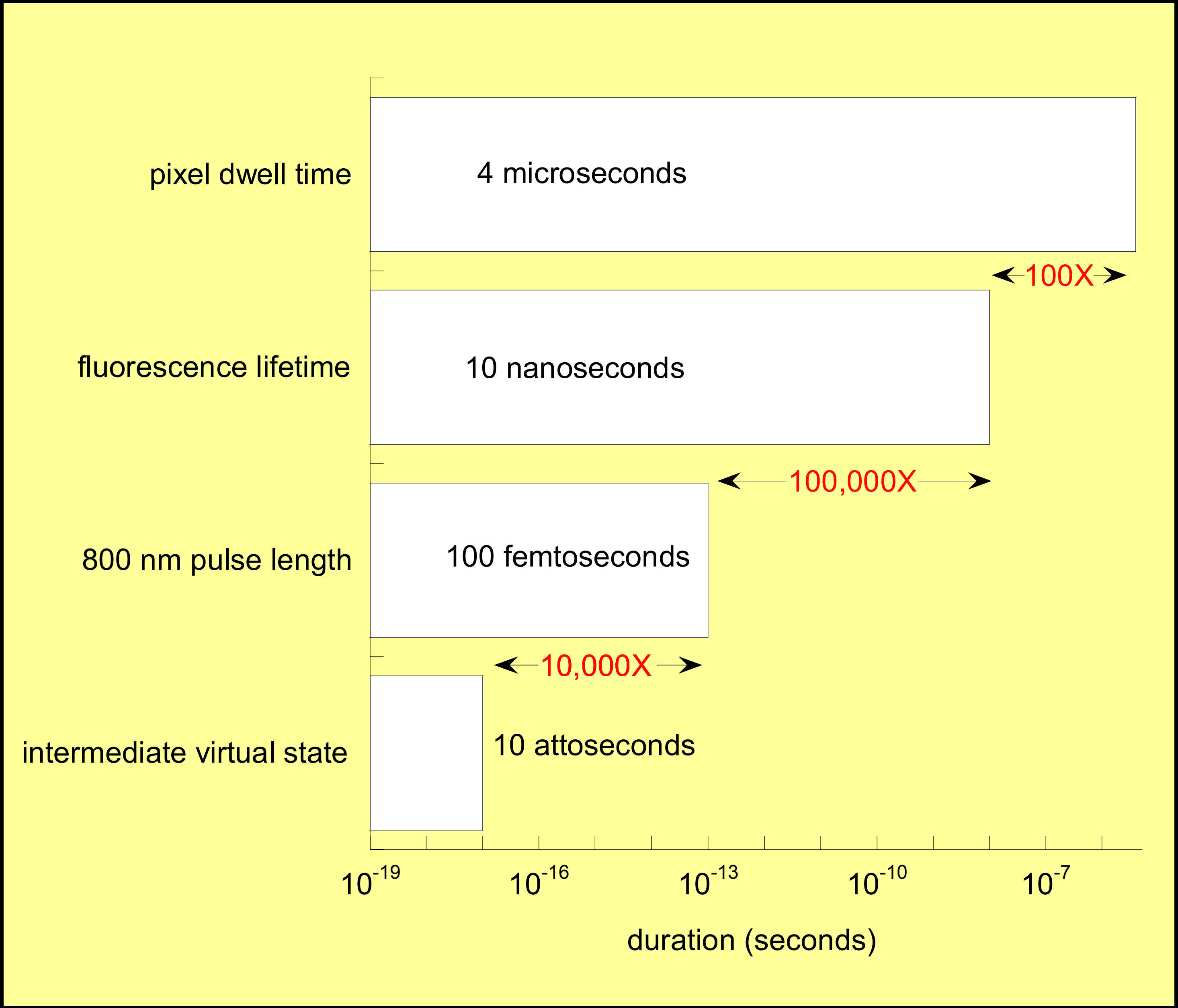
One photon absorption is proportional to illumination

- Fluorescence is stimulated throughout the lightpath

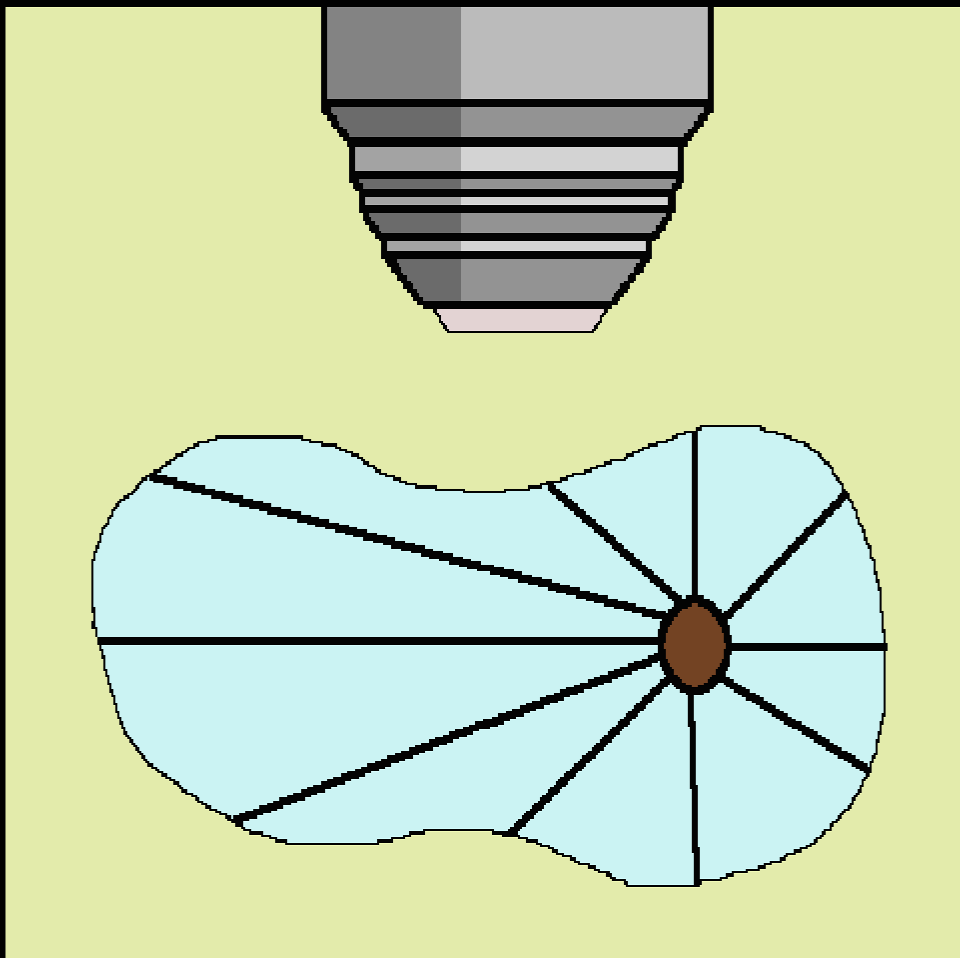
Two-photon absorption is proportional to the squared power of illumination

- Photon density sufficient to excite fluorescence occurs only at the focal point

Fluorescence microscopy time



Benefits of Multi-Photon Microscopy



- no photobleaching in out-of-focus planes

figure through the unknowing courtesy of the Integrated Microscopy Resource, Madison, Wisconsin

Multiphoton photobleaching is limited to the focal point

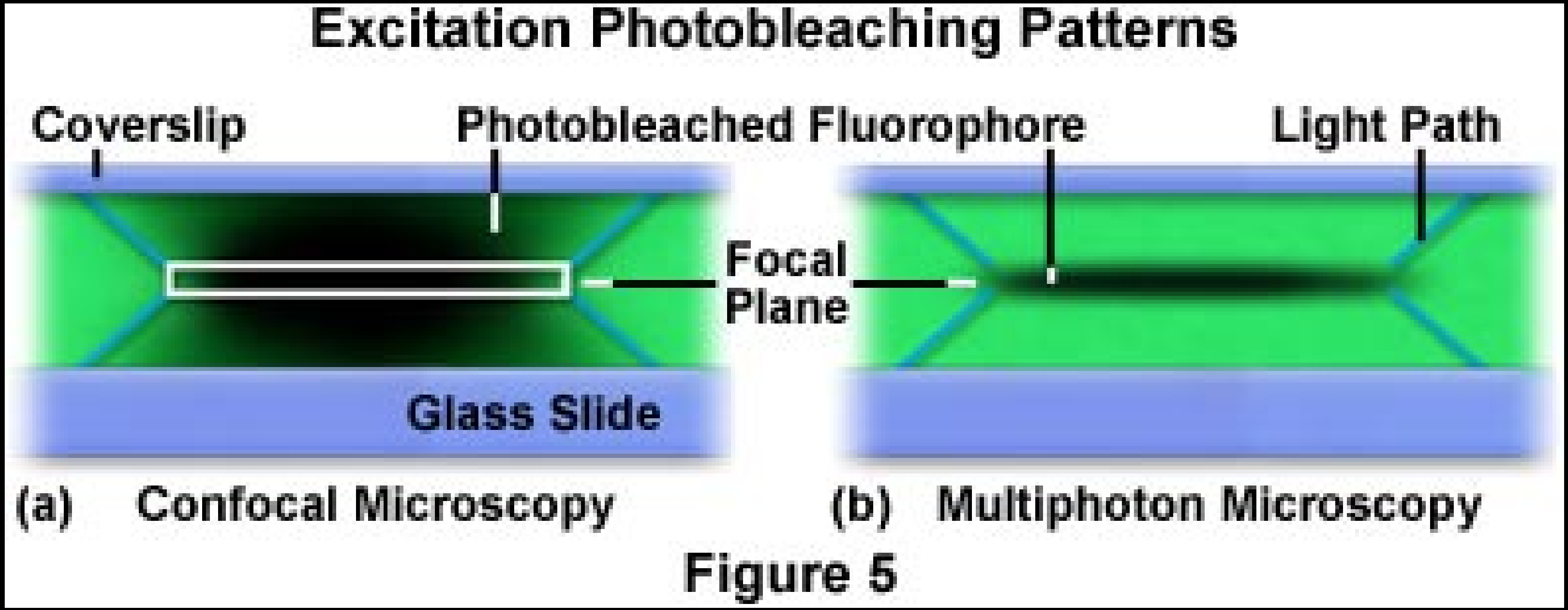
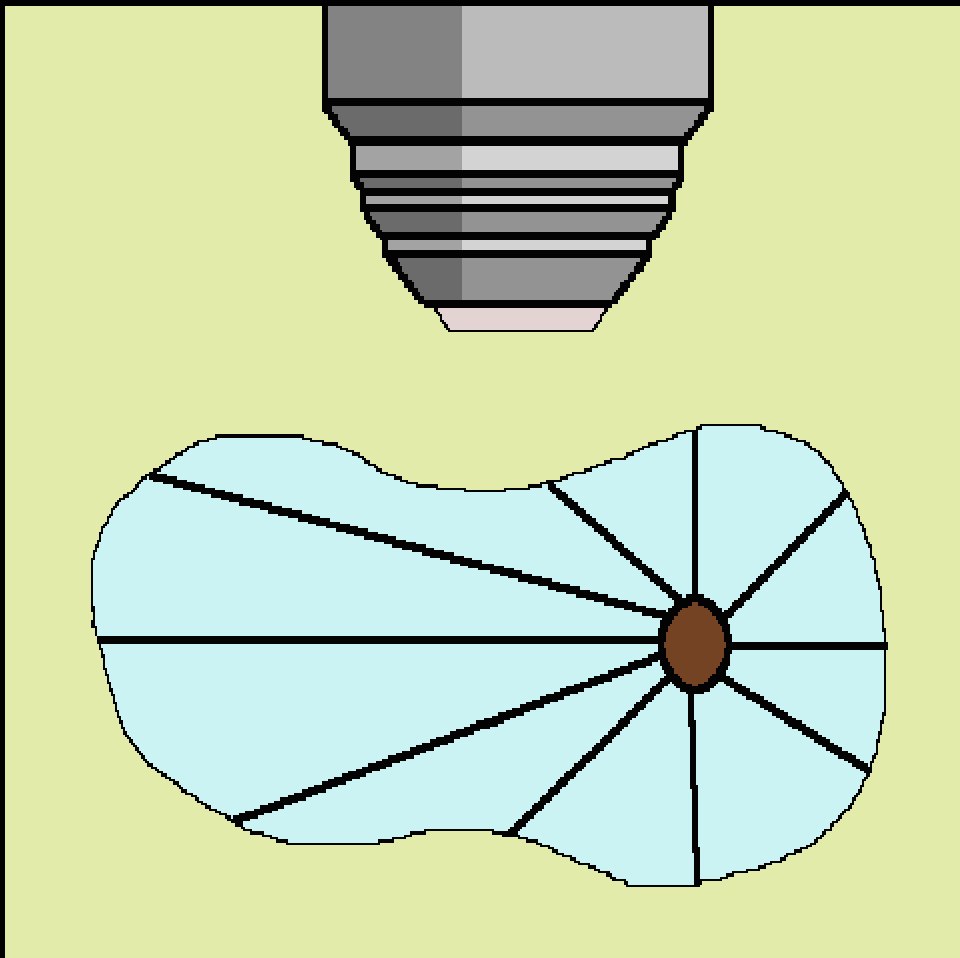


Figure courtesy of the National High Magnetic Field Laboratory – Florida State University And Dave Piston, Vanderbilt Univ.

Benefits of Multi-Photon Microscopy



- no photobleaching in out-of-focus planes
- no emission aperture - less loss to scattering

figure through the unknowing courtesy of the Integrated Microscopy Resource, Madison, Wisconsin

Multi-photon microscopy is less sensitive to light scatter by tissues – attenuation of signal

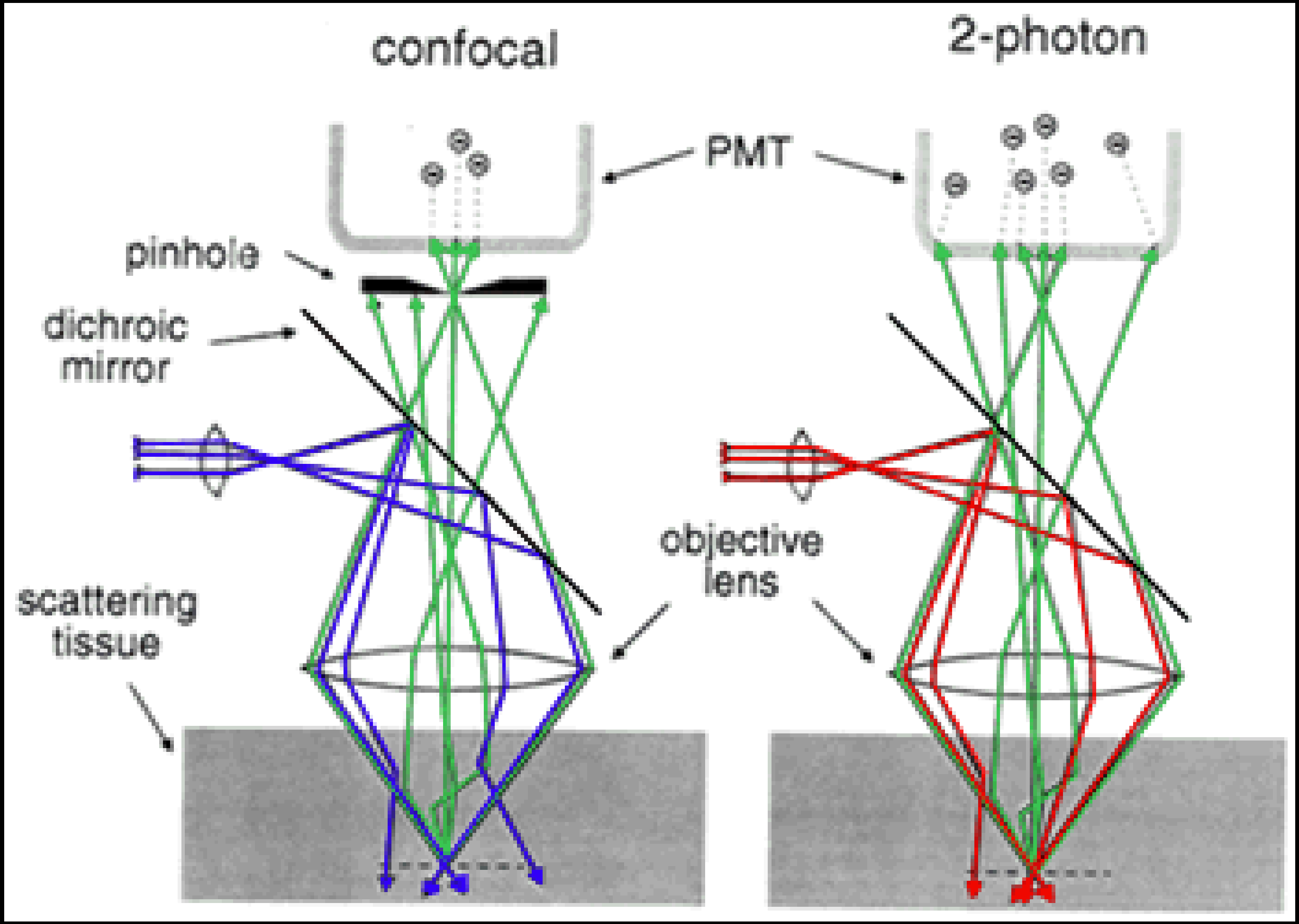
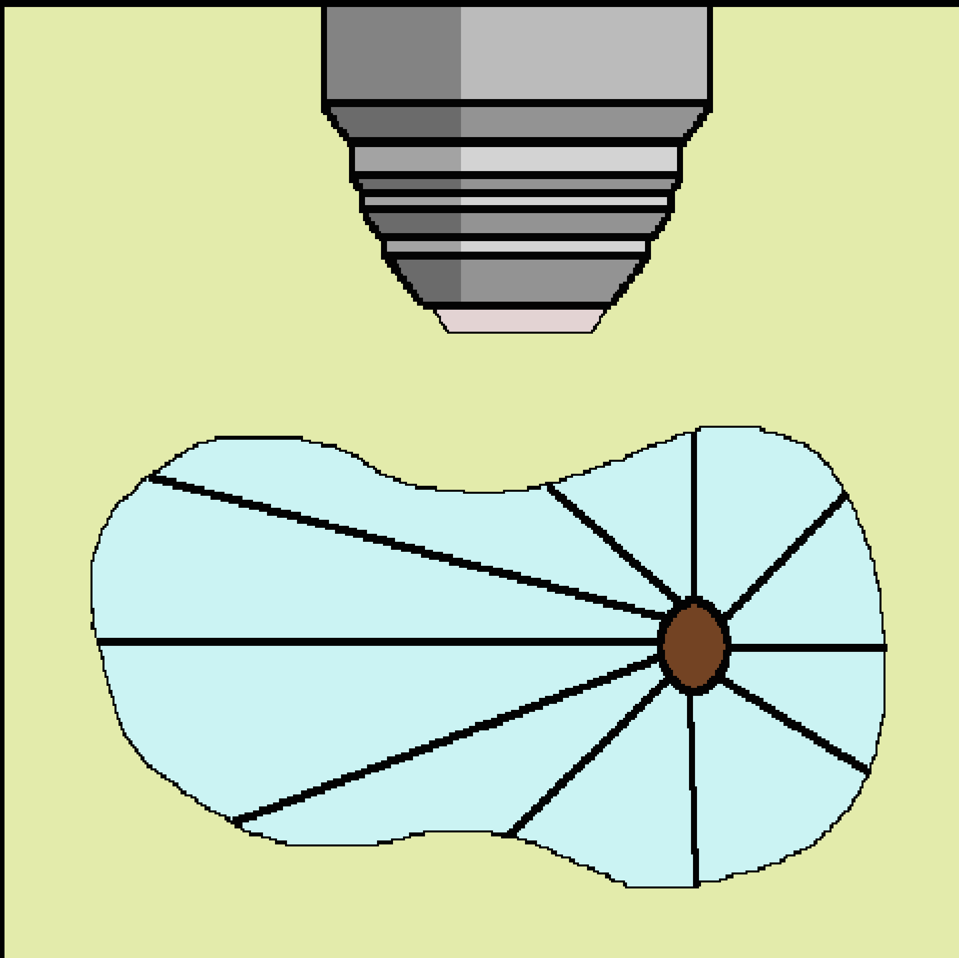


Image courtesy of Istituto Nazionale per la Fisica della Materia - Genoa

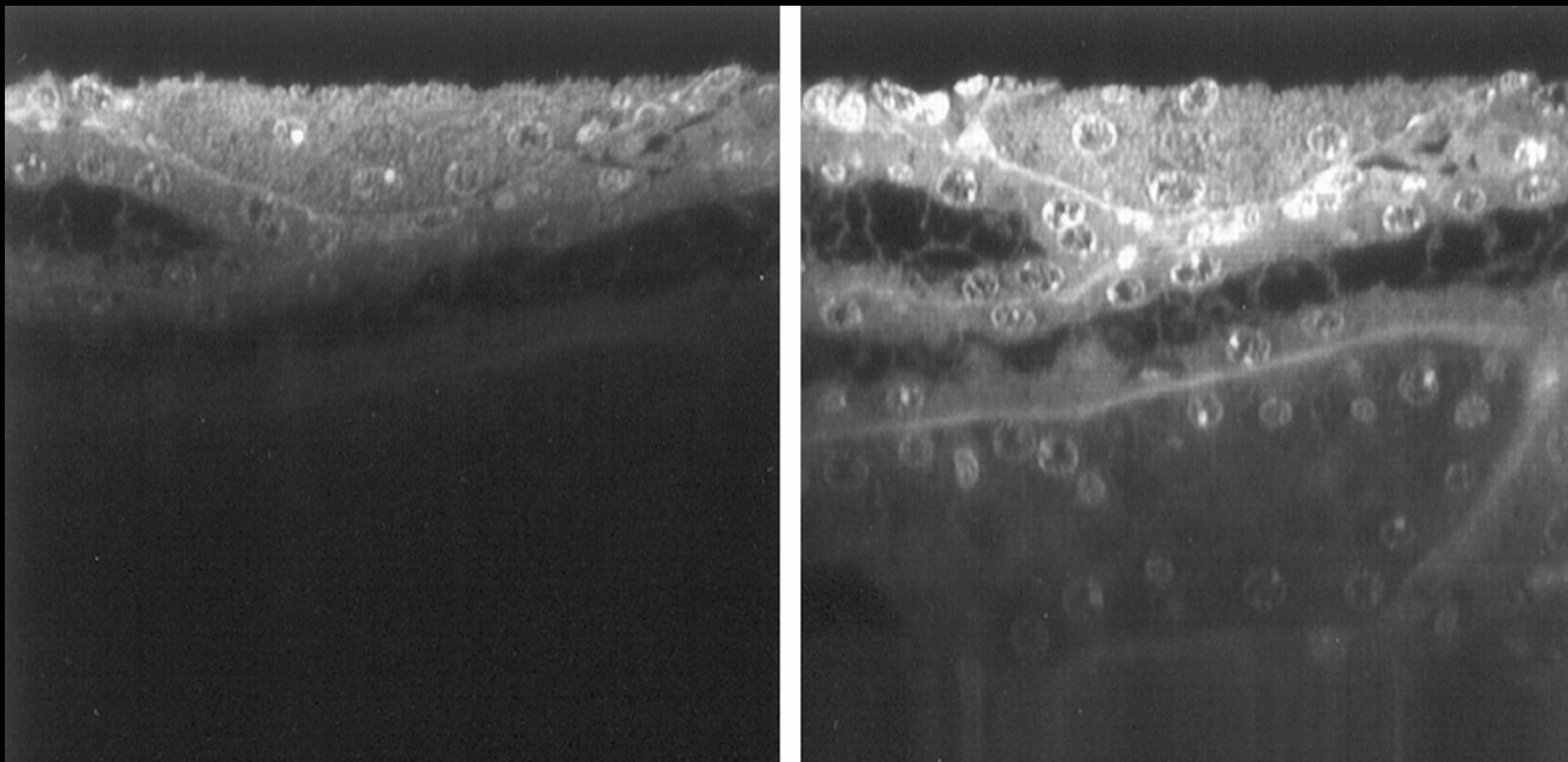
Benefits of Multi-Photon Microscopy



- no photobleaching in out-of-focus planes
- no emission aperture - less loss to scattering
- IR light penetrates deeper, with less damage

figure through the unknowing courtesy of the Integrated Microscopy Resource, Madison, Wisconsin

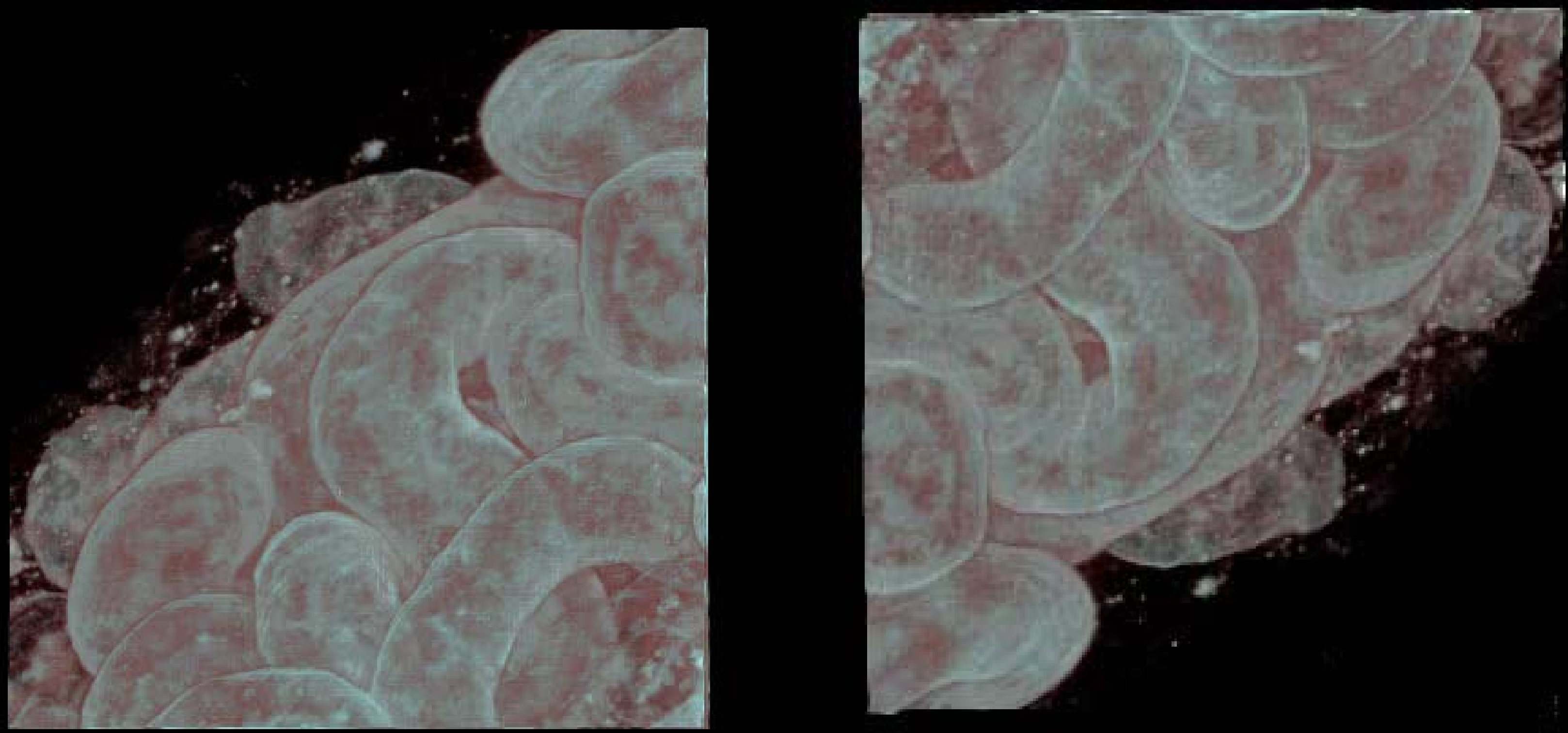
Multi-photon Excitation Allows Deeper Imaging in Intact Tissue



Confocal **Two-Photon Excitation**

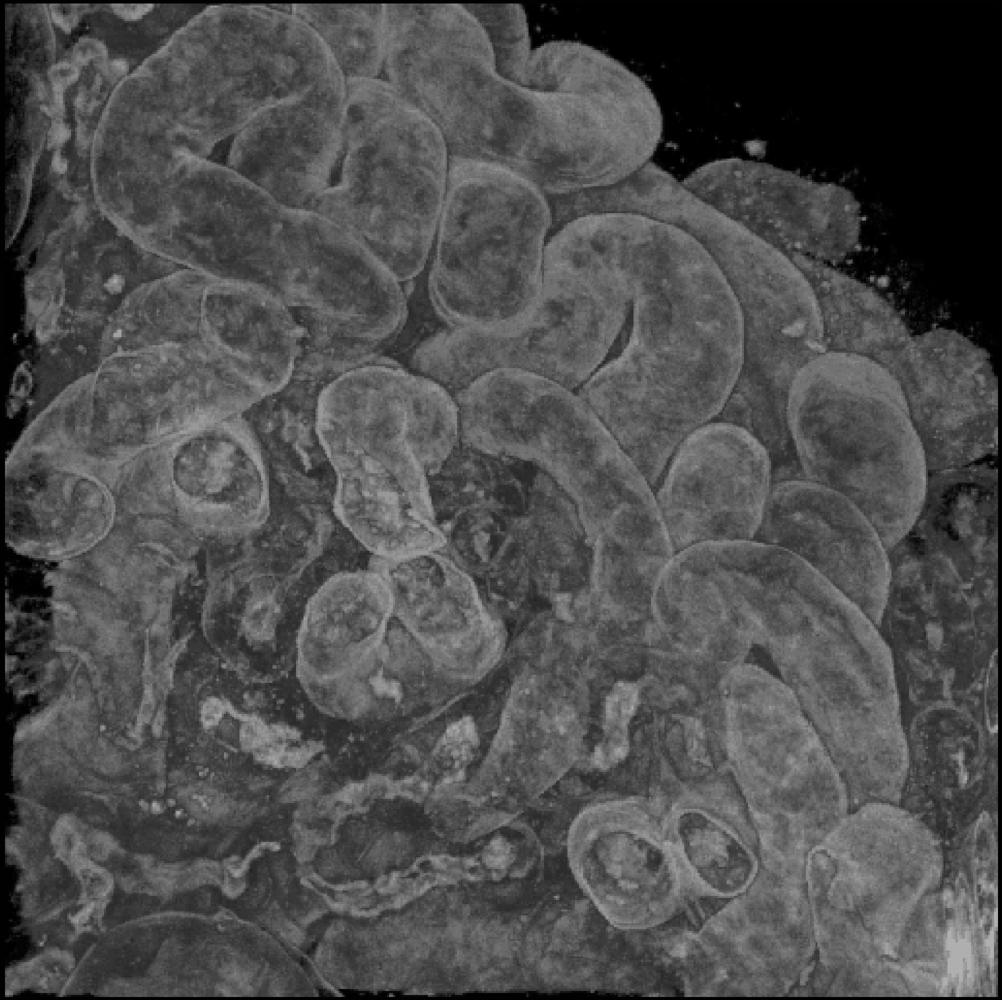
Dave Piston, Vanderbilt

Imaging complex structures – Kidney tubules of a newborn mouse kidney



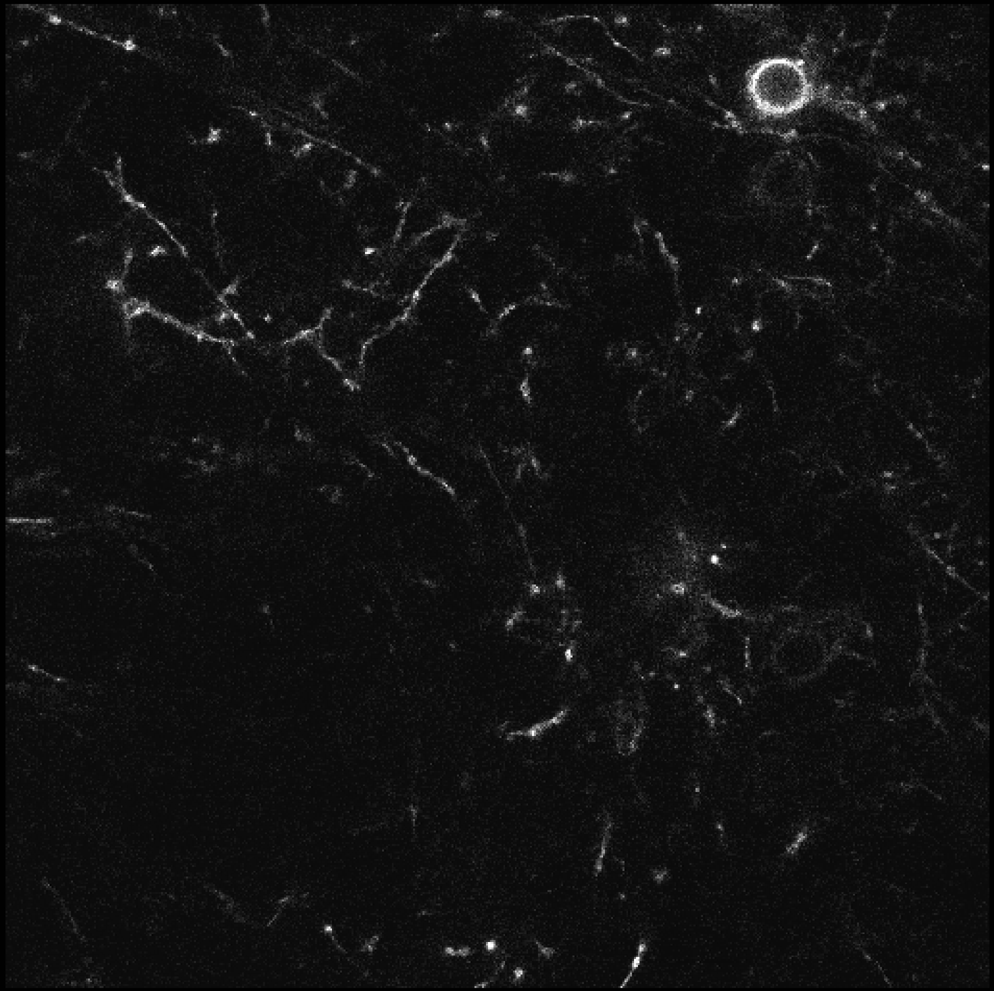
Carrie Phillips, Nephrology and Pathology

Imaging complex structures – Kidney tubules of a newborn mouse kidney

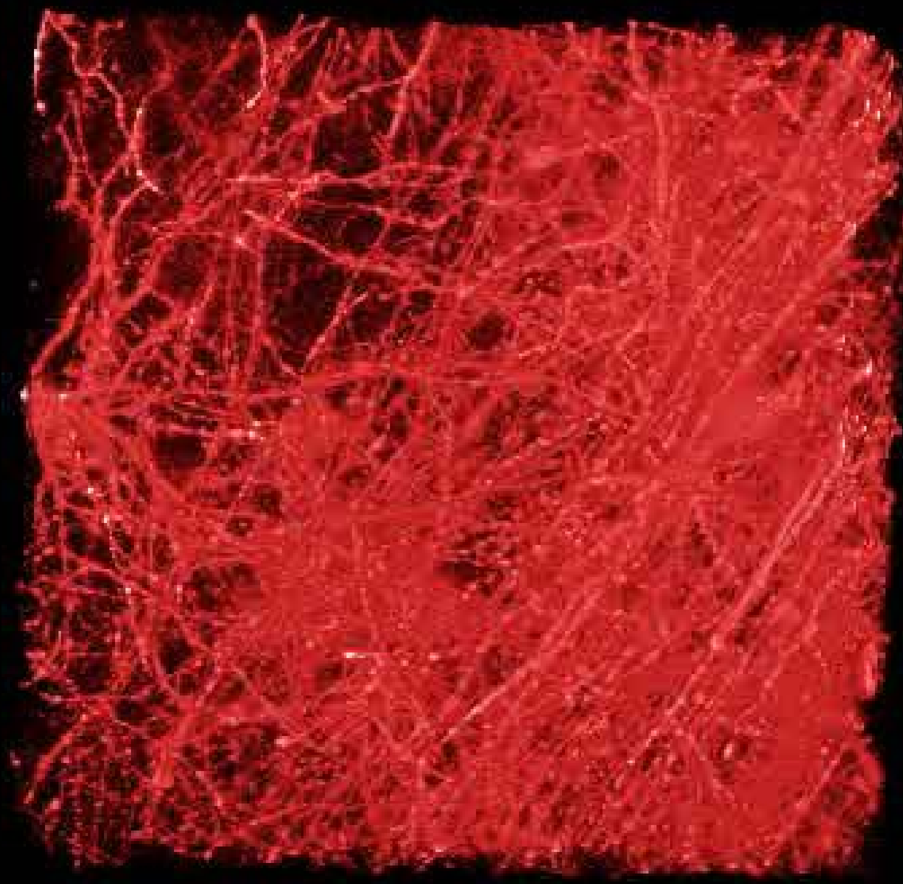


Carrie Phillips, Nephrology and Pathology

Single optical section of neural network



Imaging complex structures – Neural network in mouse brain adapted to chronic alcohol

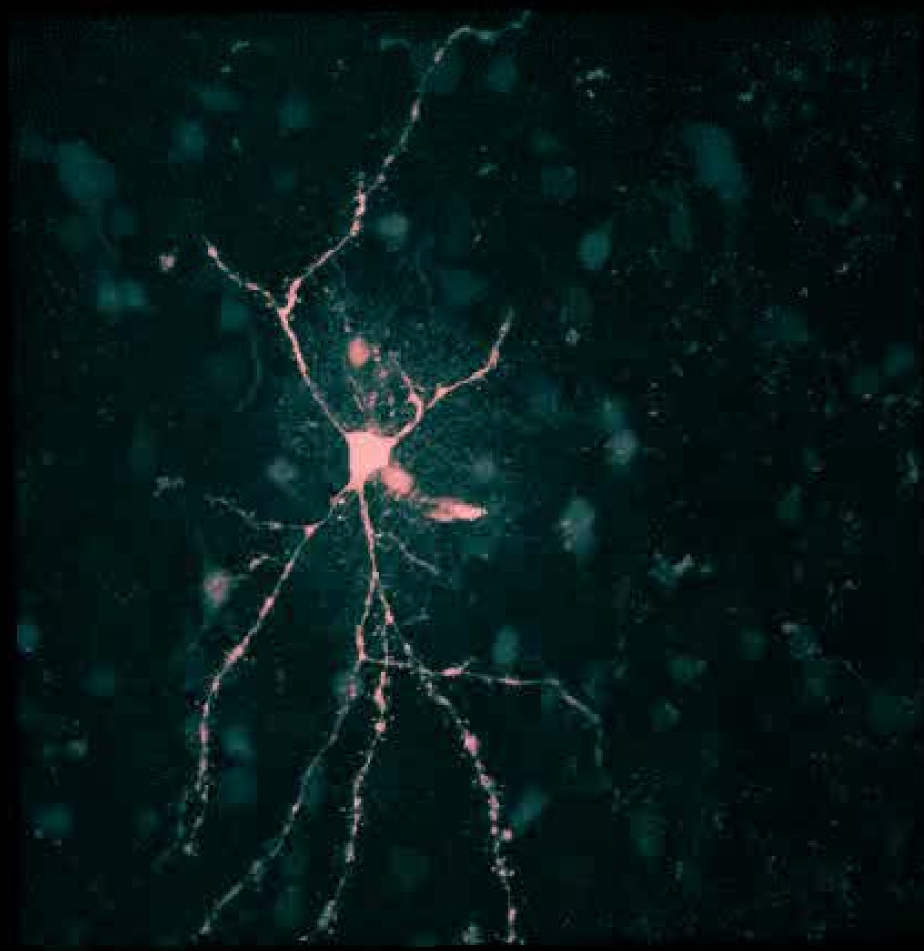


Feng Zhou, Anatomy and Cell Biology

Imaging complex structures – Segmentation of a single neuron

Feng Zhou, Anatomy and Cell Biology

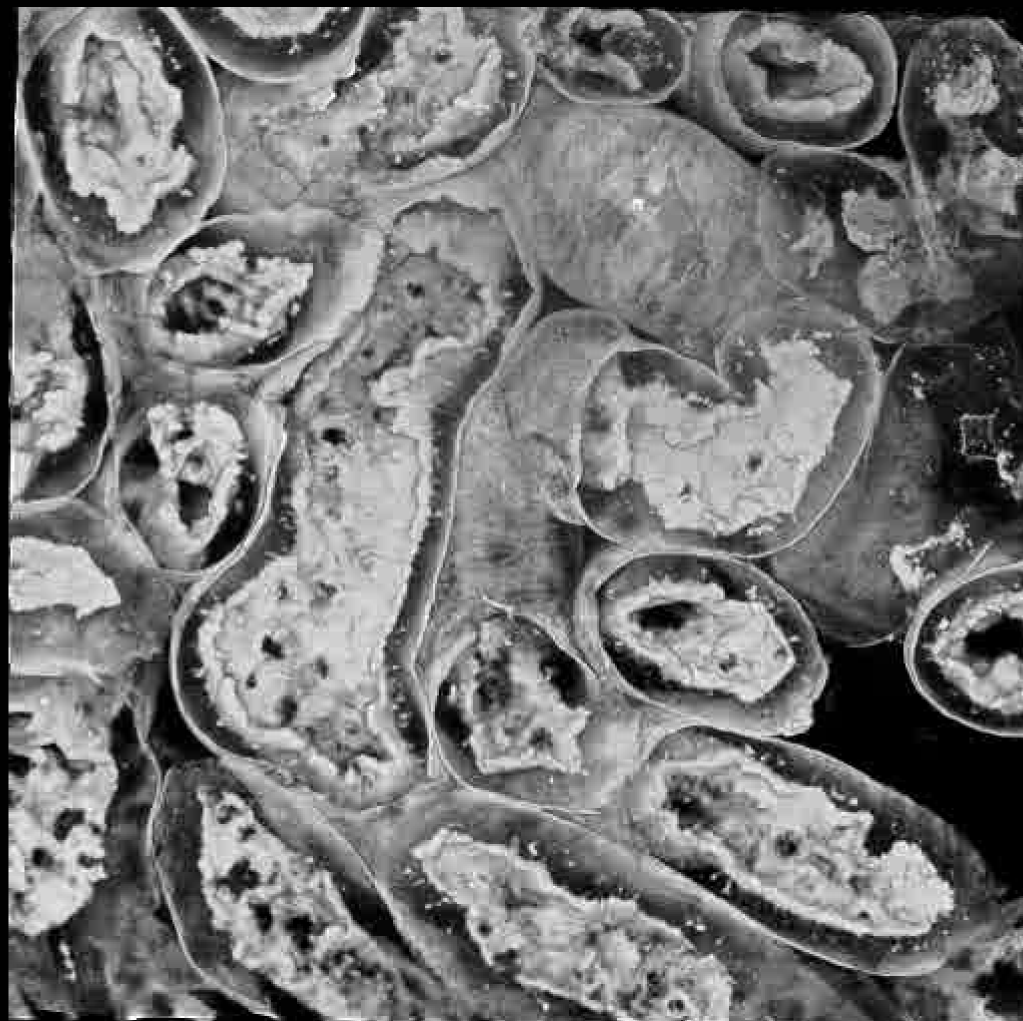
Imaging complex structures –
Neural network in mouse brain adapted to chronic alcohol



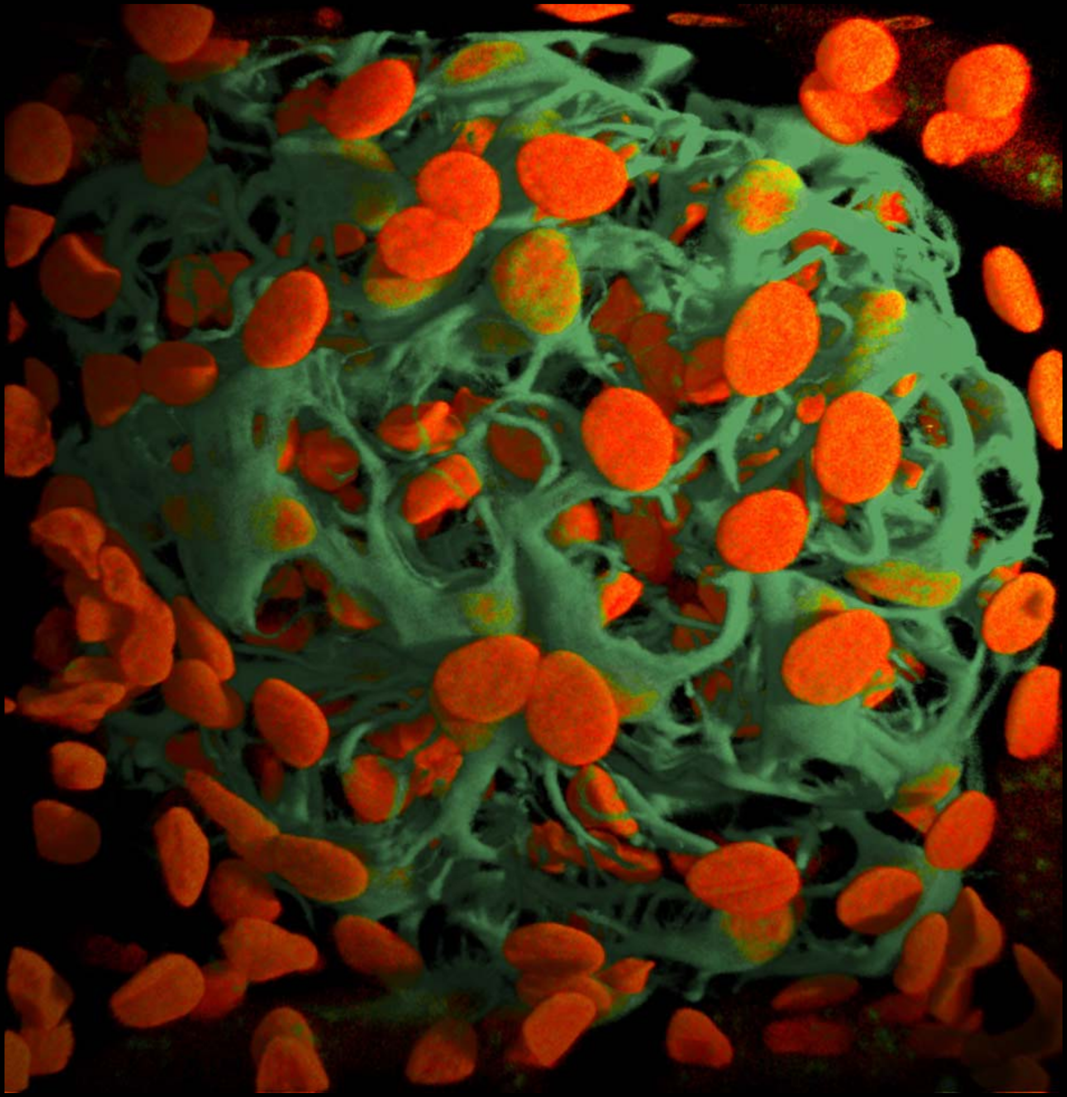
Feng Zhou, Anatomy and Cell Biology

Multiphoton imaging of kidney

3D imaging of newborn mouse kidney by 2-photon microscopy – Carrie Phillips

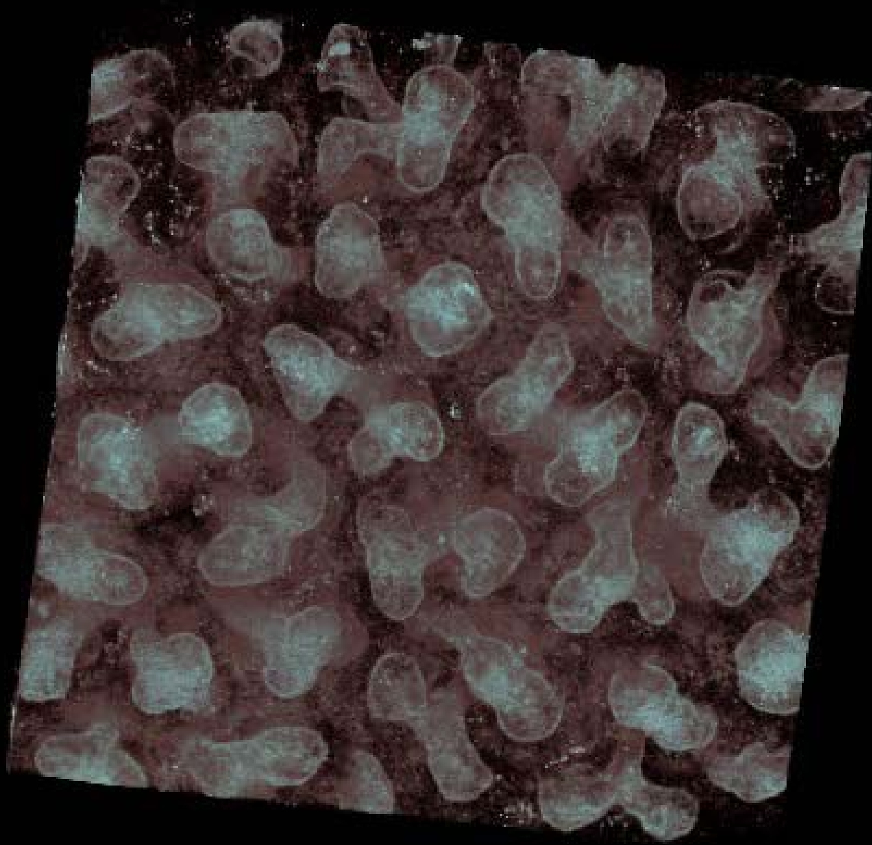


Imaging complex structures – Glomerulus of a newborn mouse kidney



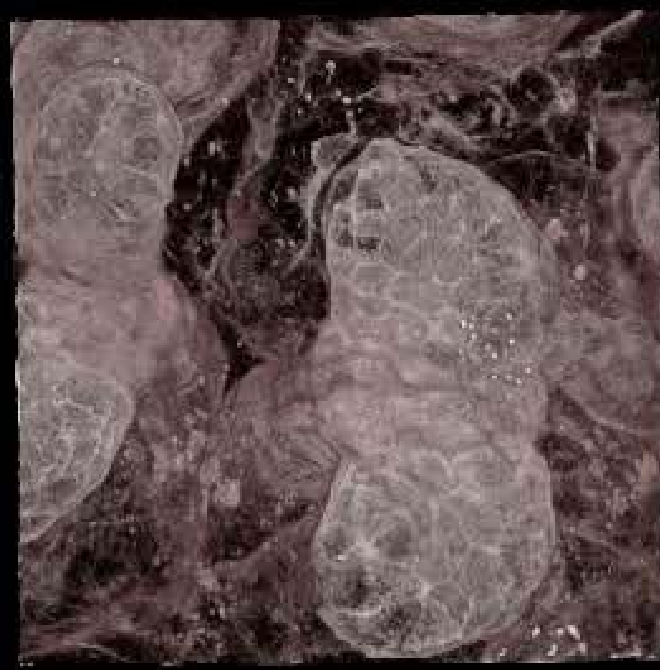
Carrie Phillips, rendered by Anatomical Travel, Inc.
www.anatomicaltravel.com

Imaging complex structures – branching tubulogenesis in an embryonic mouse kidney



Carrie Phillips, Nephrology and Pathology

140 micron thick volume of embryonic mouse kidney - branching tubulogenesis – Carrie Phillips



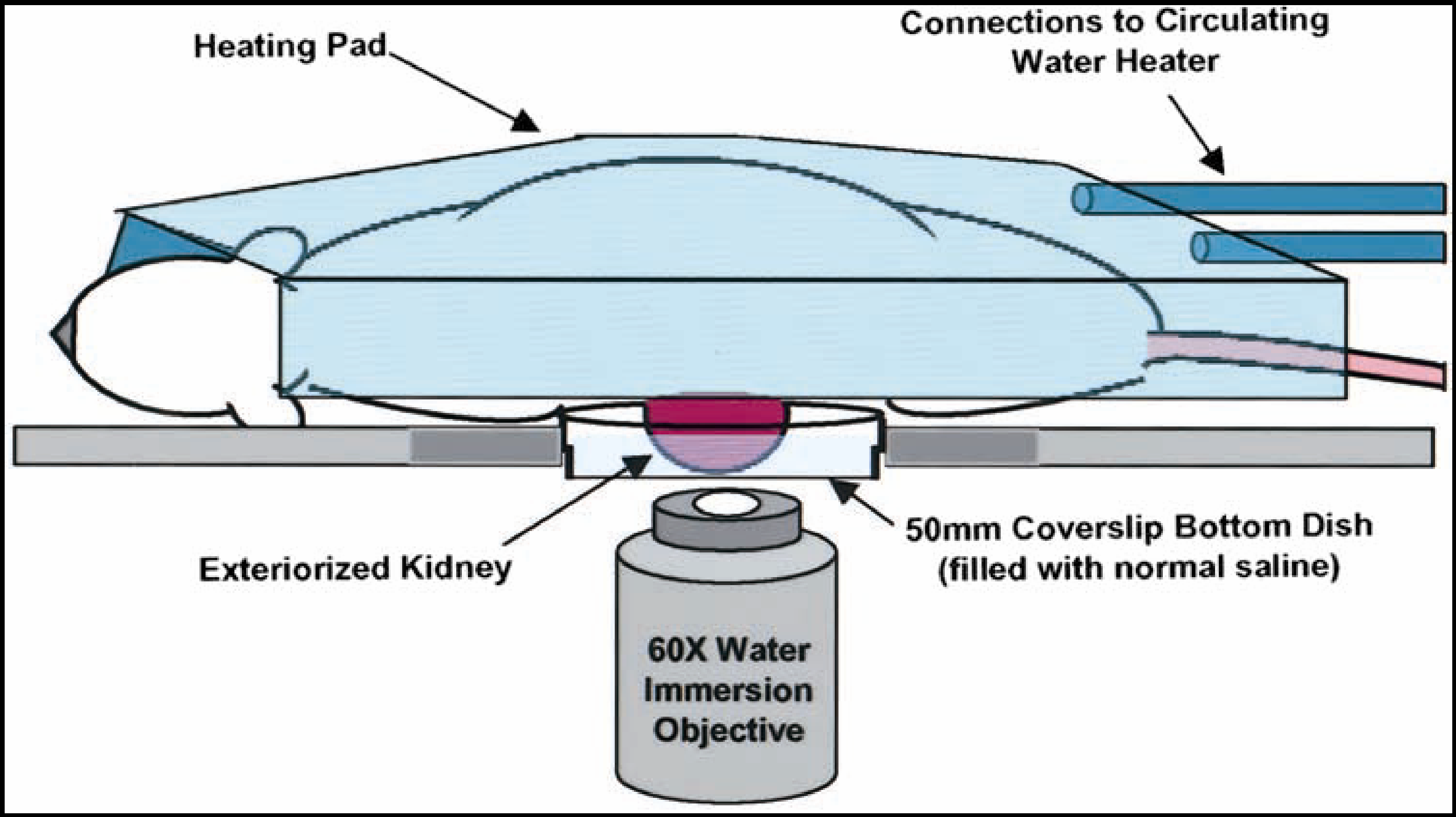
. . . and real time rendering on a PC using Voxx software – Jeff Clendenon

Vital imaging by multiphoton microscopy

- The extended depth provided by multi-photon microscopy permits high-resolution imaging of the cells of living animals.

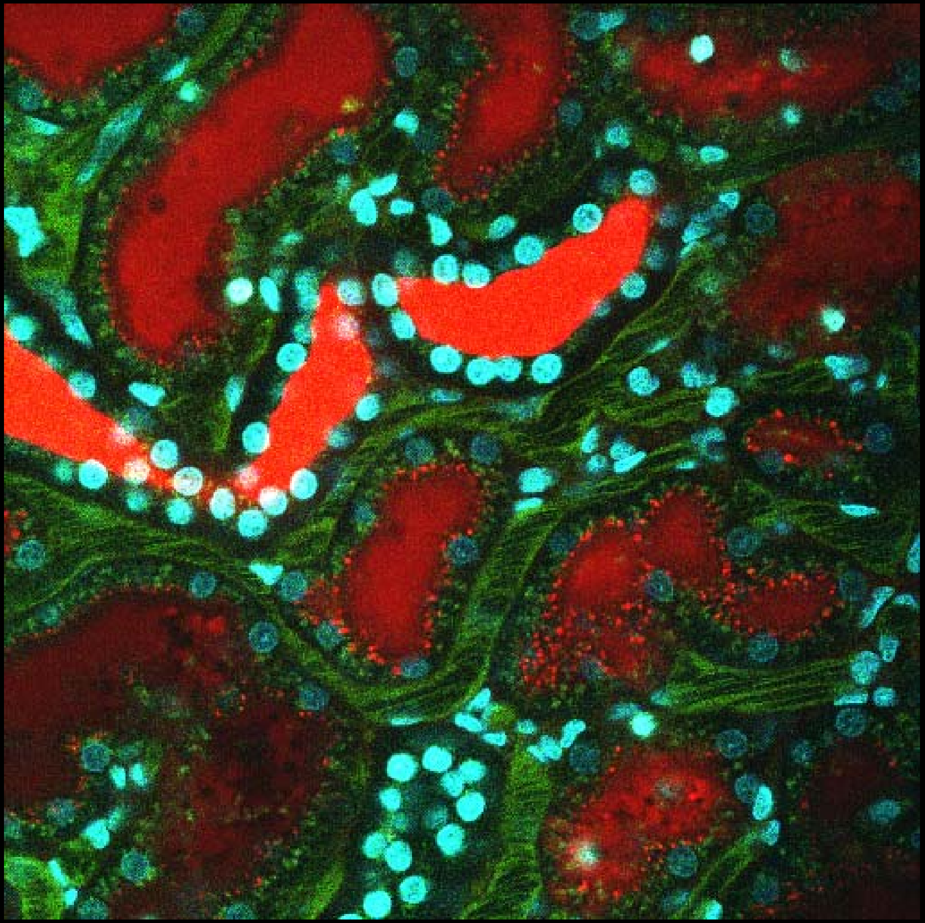
- The ability to image multiple fluorophores supports correlations of multiple proteins and physiological processes.

Experimental arrangement for intravital imaging of rat kidney



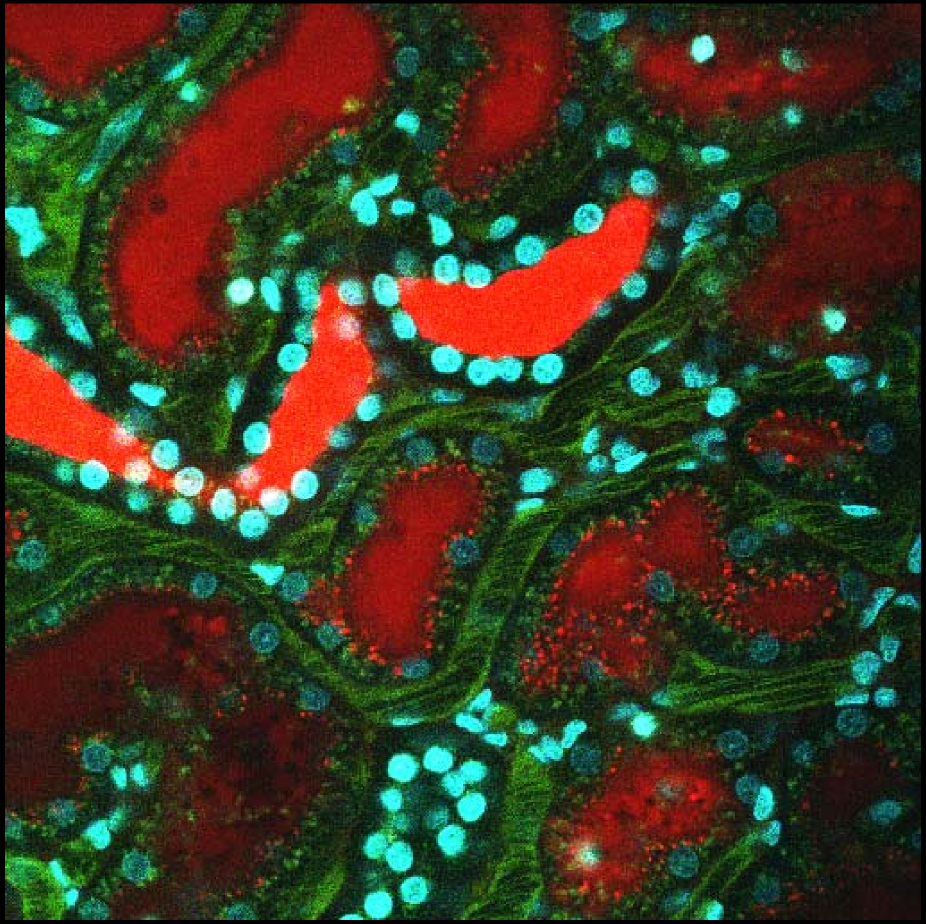
Anesthesia is provided via 1% halothane and low-flow oxygen. Fluorescent probes are administered via tail-vein injection. Blood gases are monitored via femoral artery.

Two-photon image of kidney of living rat injected with Hoechst, 500 Kd fluorescein dextran and 3 Kd Texas-Red dextran



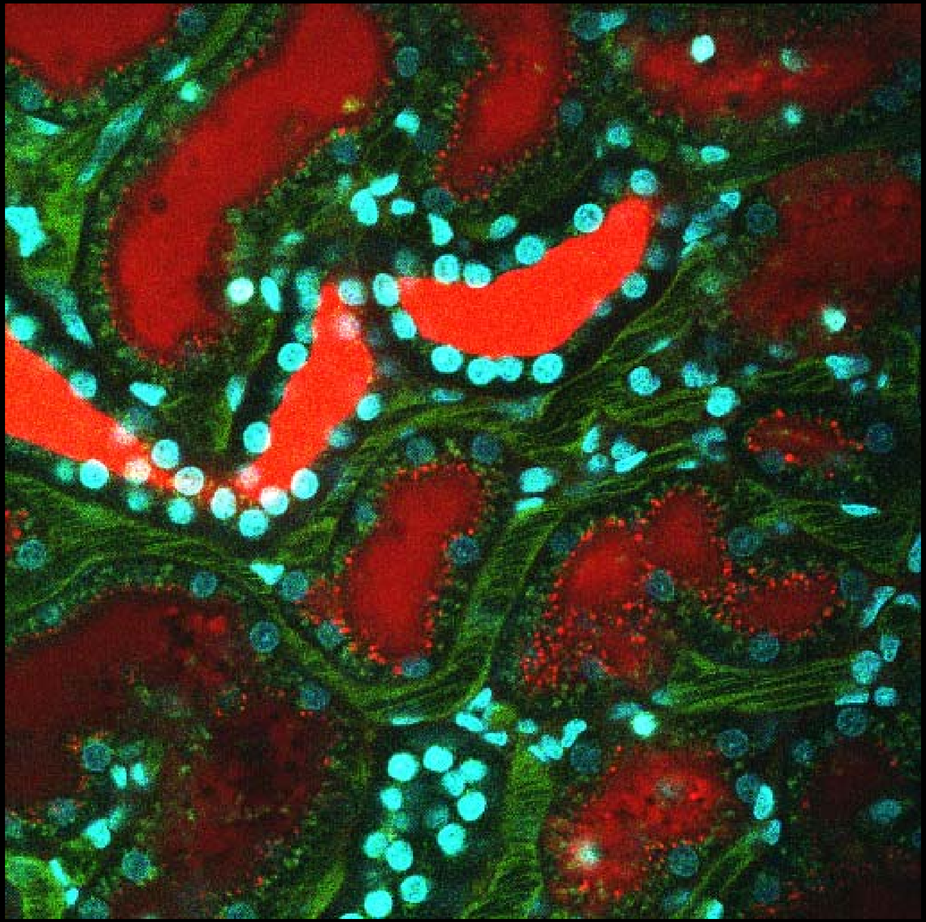
– Ruben Sandoval and Katrina Kelly

Multiple functions apparent in images of kidneys of animals injected with large and small dextrans



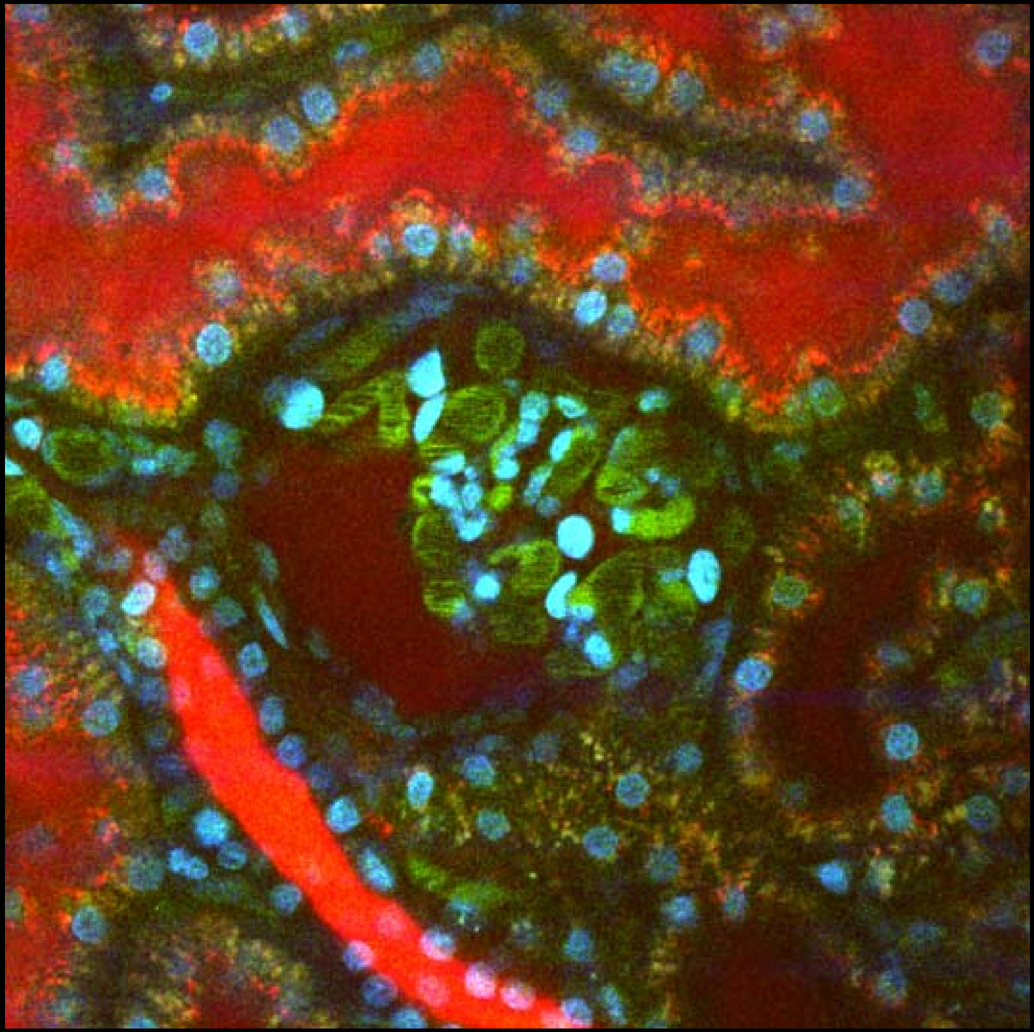
- proximal tubule endocytosis
- glomerular filtration
- tubular solute concentration
- capillary blood flow

Multiple functions apparent in images of kidneys of animals injected with large and small dextrans



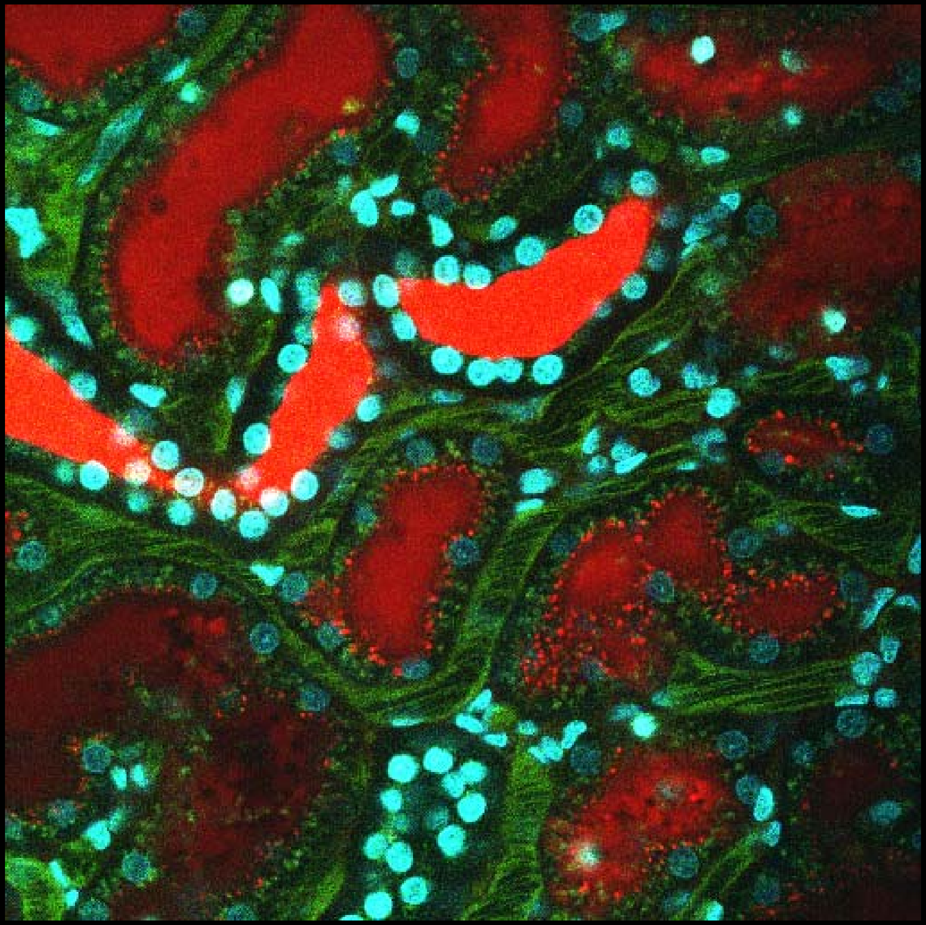
- proximal tubule endocytosis
- glomerular filtration
- tubular solute concentration
- capillary blood flow

2-photon microscopy of glomerular filtration in a living rat



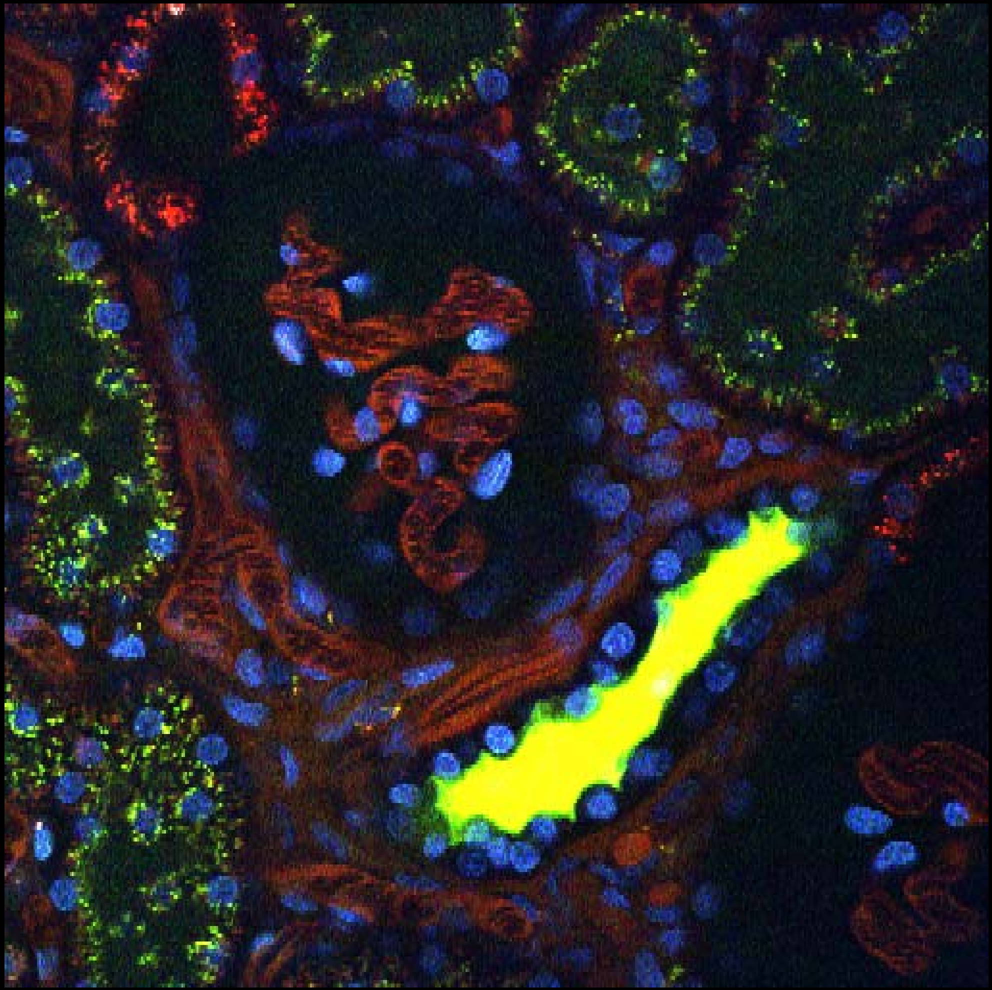
Hoechst-labeled nuclei, 500 Kd fluorescein dextran in blood, 3 Kd Texas-Red dextran in lysosomes and tubule lumens – Ruben Sandoval and Katrina Kelly

Multiple functions apparent in images of kidneys of animals injected with large and small dextrans



- proximal tubule endocytosis
- glomerular filtration
- tubular solute concentration

2-photon microscopy of tubular solute concentration in a living rat



Hoechst-labeled nuclei, rhodamine-albumin in blood, 3 Kd fluorescein dextran in lysosomes and tubule lumens – Ruben Sandoval and Katrina Kelly

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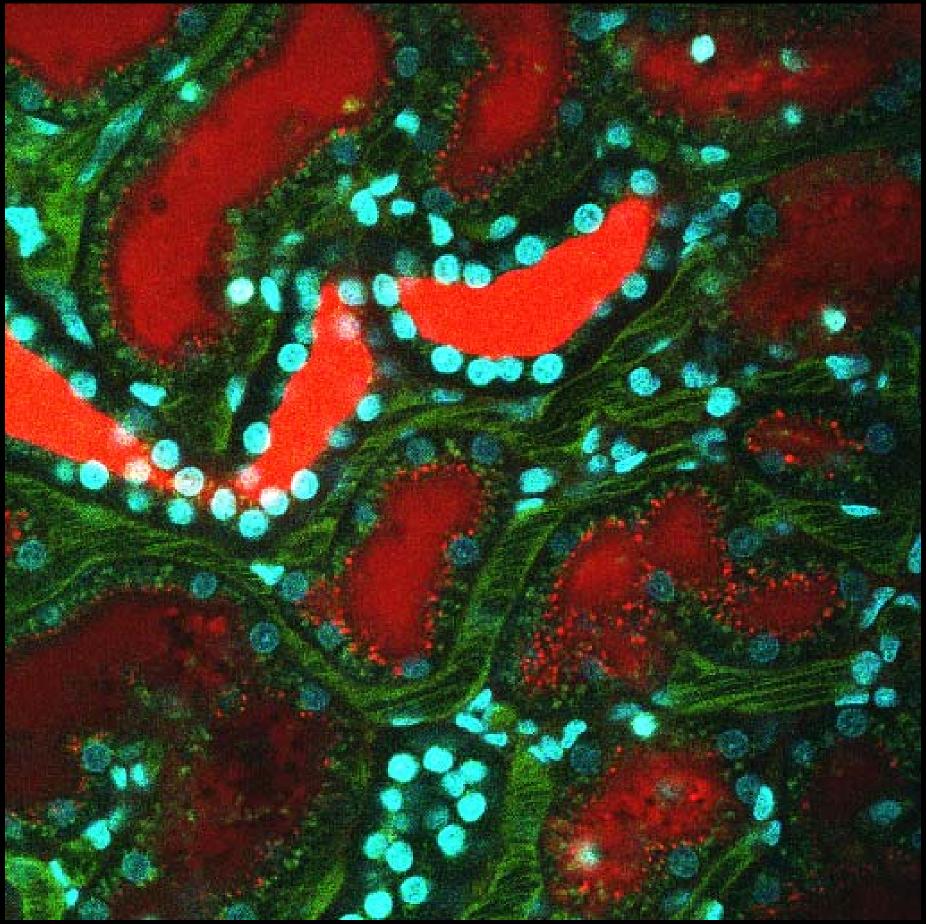
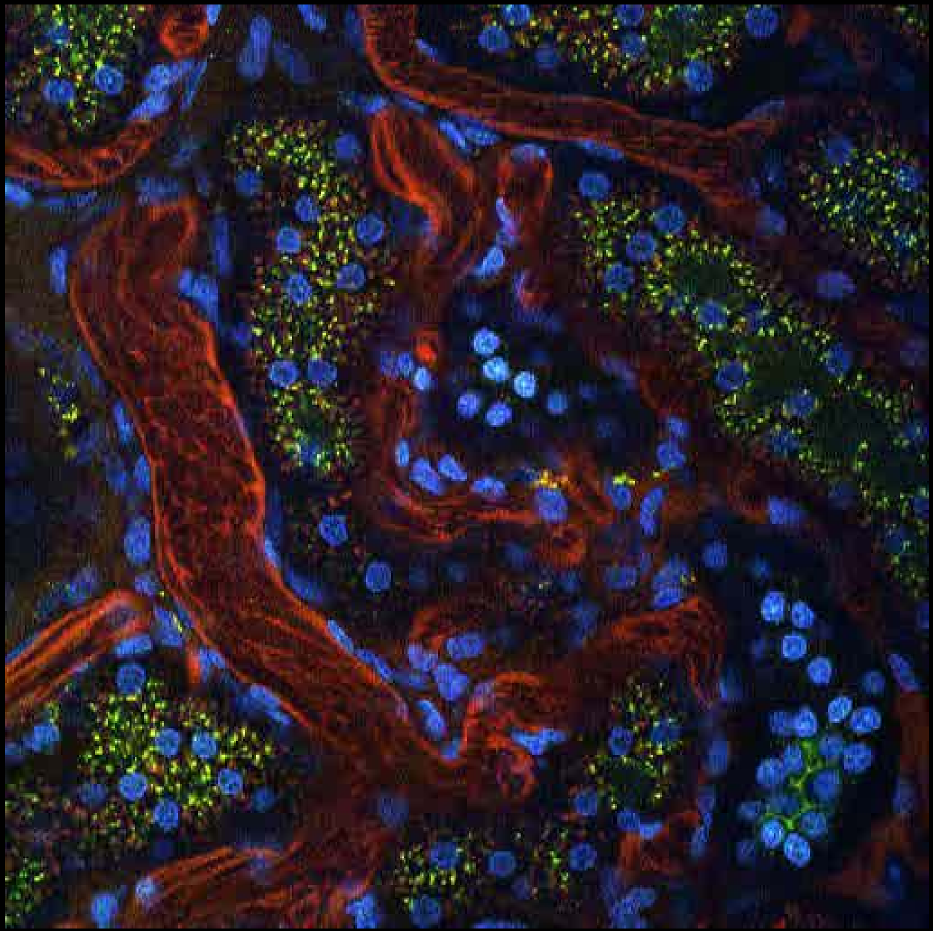


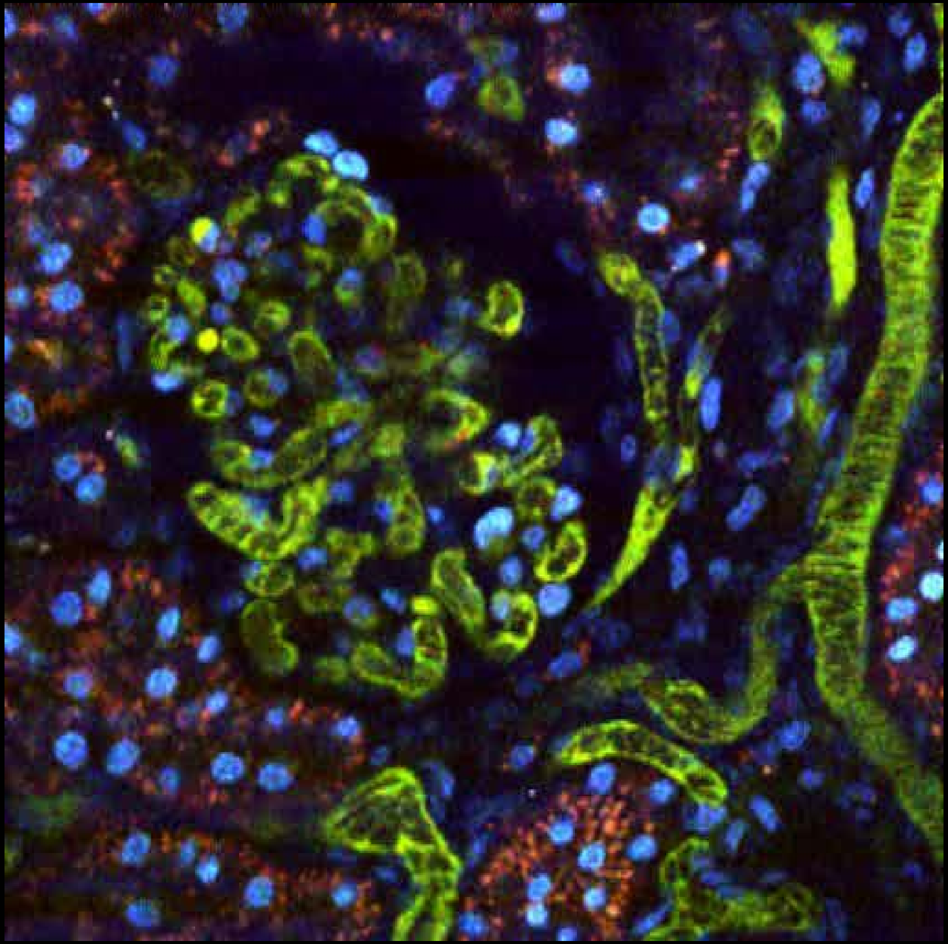
- proximal tubule endocytosis
- glomerular filtration
- tubular solute concentration
- capillary blood flow

Imaging capillary blood flow by 2-photon microscopy



Rhodamine-albumin, 3K fluorescein dextran and Hoechst

Imaging capillary blood flow (and proximal tubule autofluorescence) by 2-photon microscopy



500K fluorescein dextran
and Hoechst

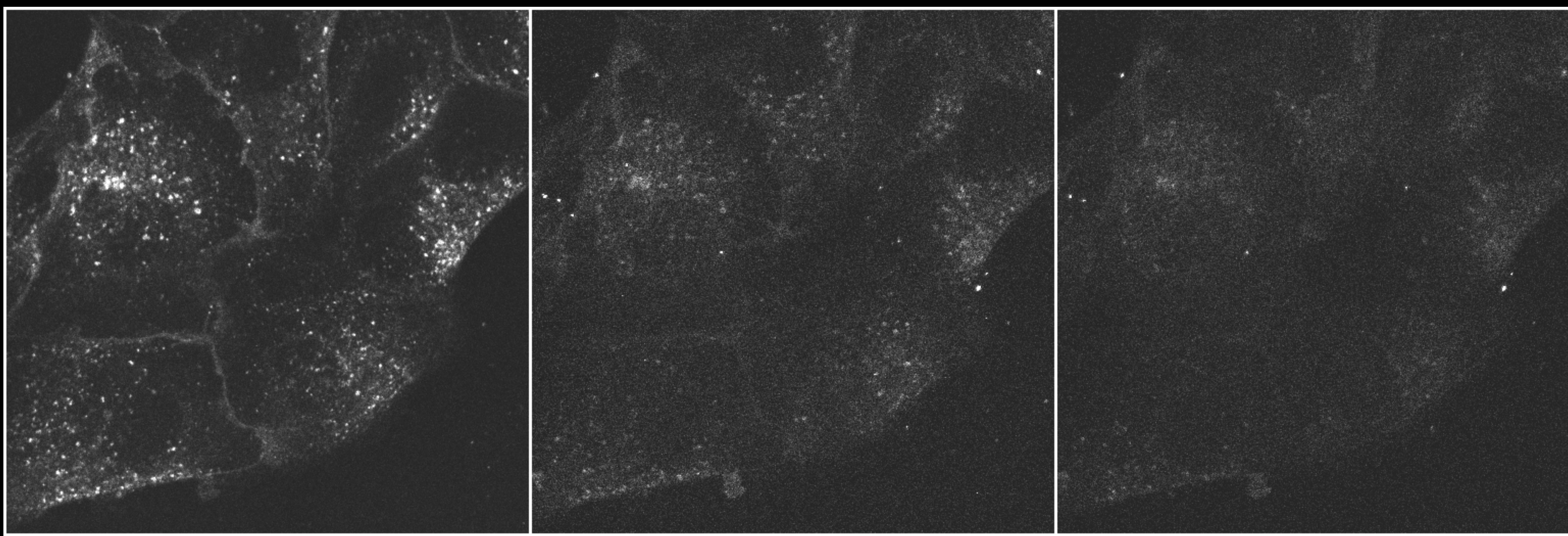
Practical multiphoton microscopy

Practical multiphoton microscopy

- Photobleaching
- Resolution
- Multiphoton fluorescence dyes
- Imaging multiple colors
- Laser choices
- Detector options

2 photon versus confocal microscopy

POP go the endosomes



One photon image at first, tenth, whatever scan

Two photon image

Two photon image at fourth scan

Practical multiphoton microscopy

- Photobleaching
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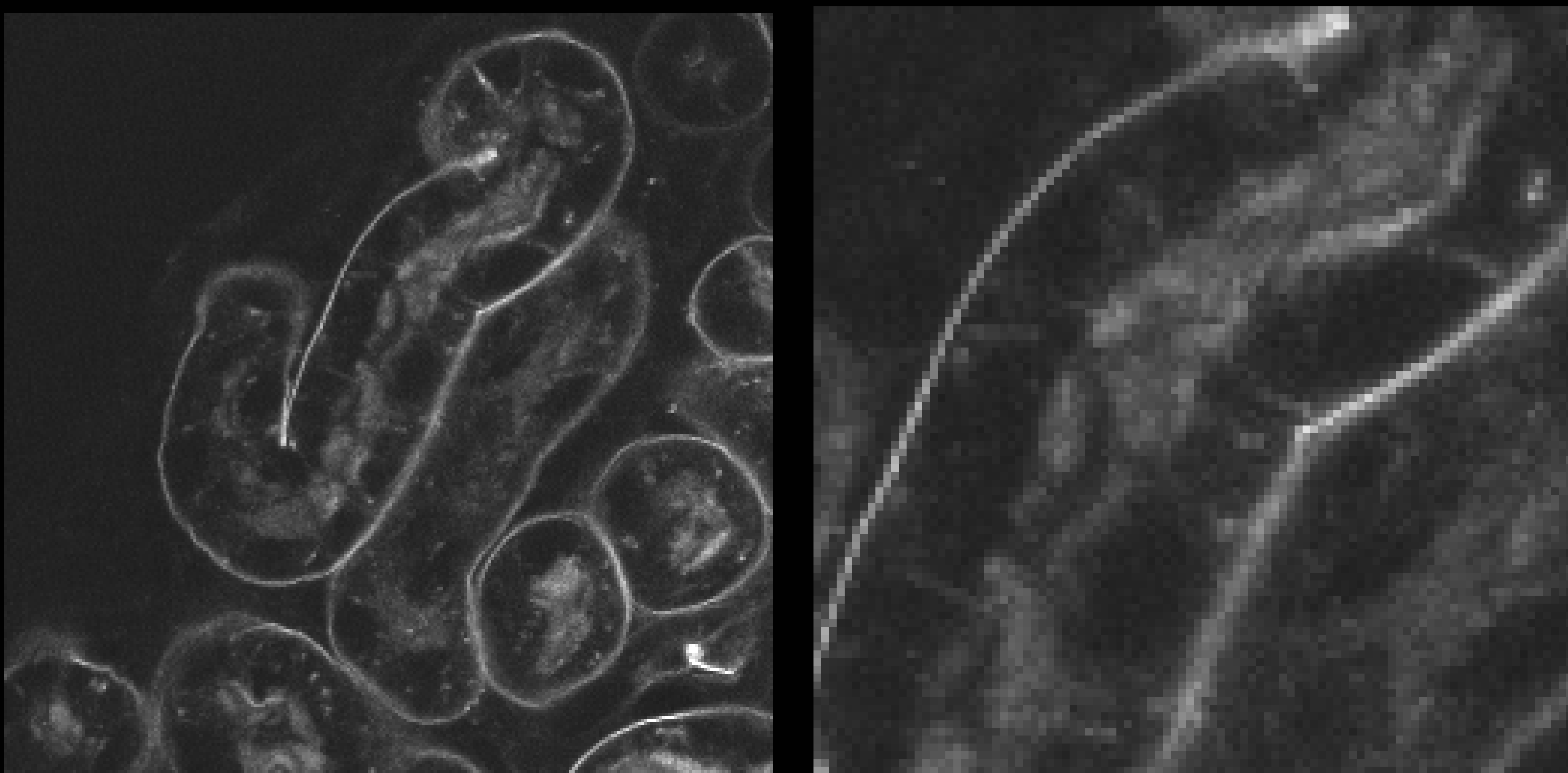
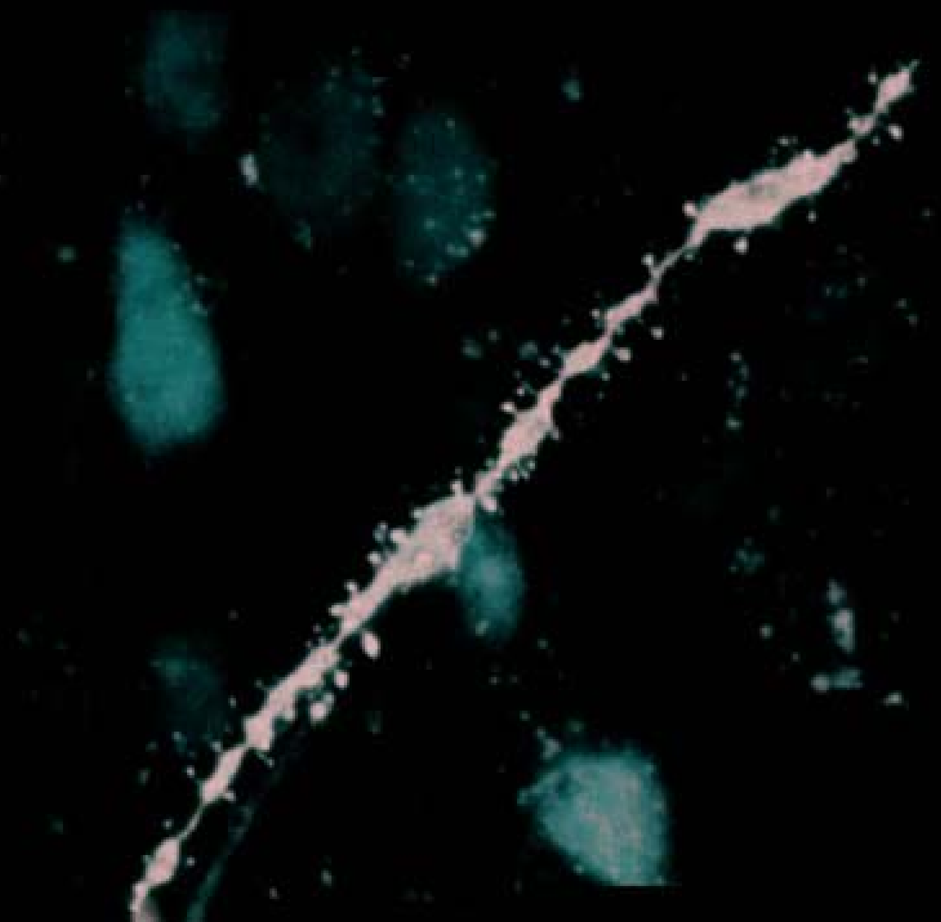
Deep tissue imaging by multi-photon microscopy



2 photon image collected 120 microns into kidney section

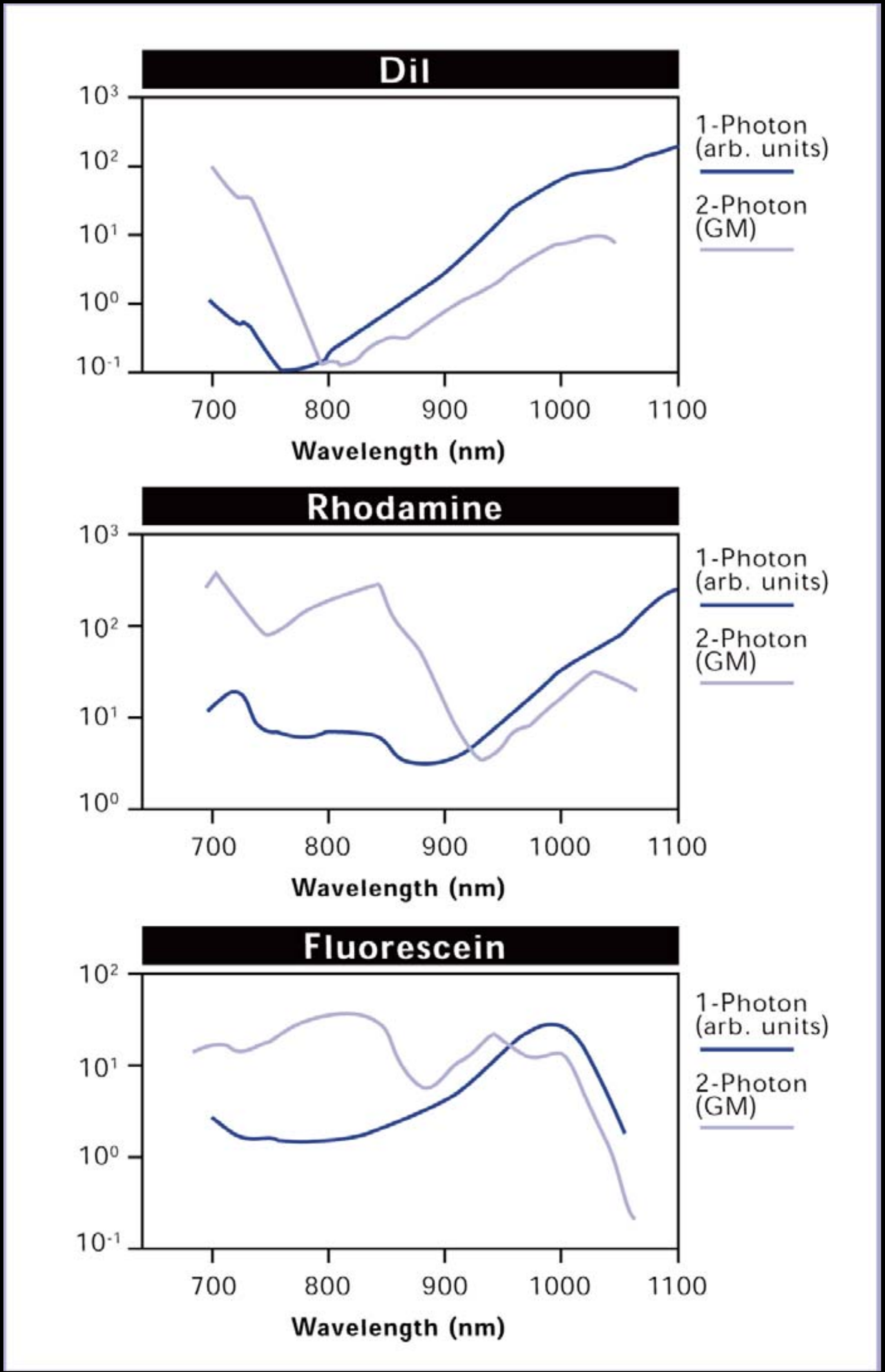
Imaging complex structures –
Dendritic spines in mouse hippocampal neuron



Feng Zhou, Anatomy and Cell Biology

Practical multiphoton microscopy

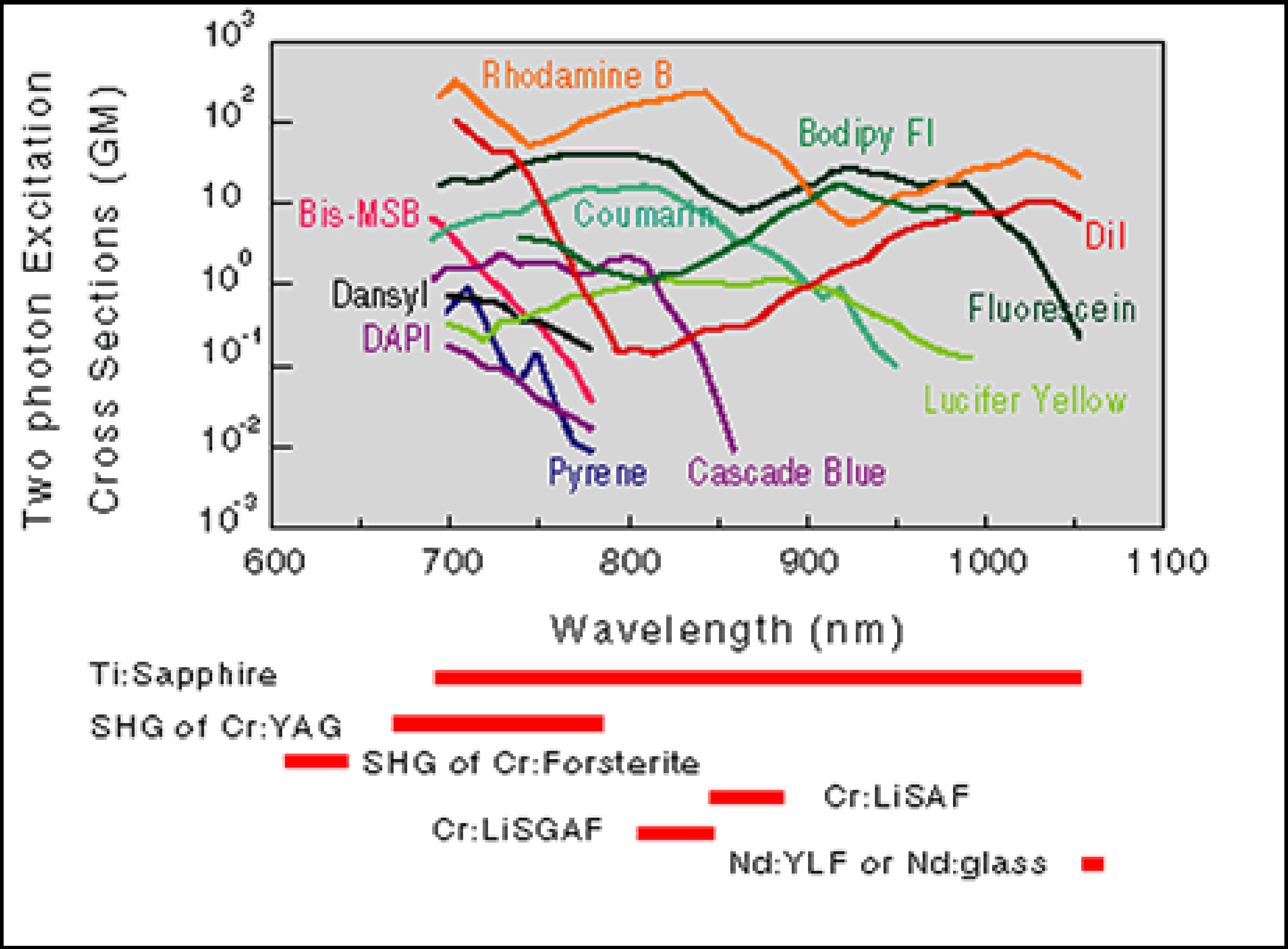
- Photobleaching
- Resolution
- Multiphoton fluorescence dyes



2-photon cross sections are not necessarily predicted by single photon excitation spectra

BioRad

2-photon fluorescence excitation – 2-photon cross sections

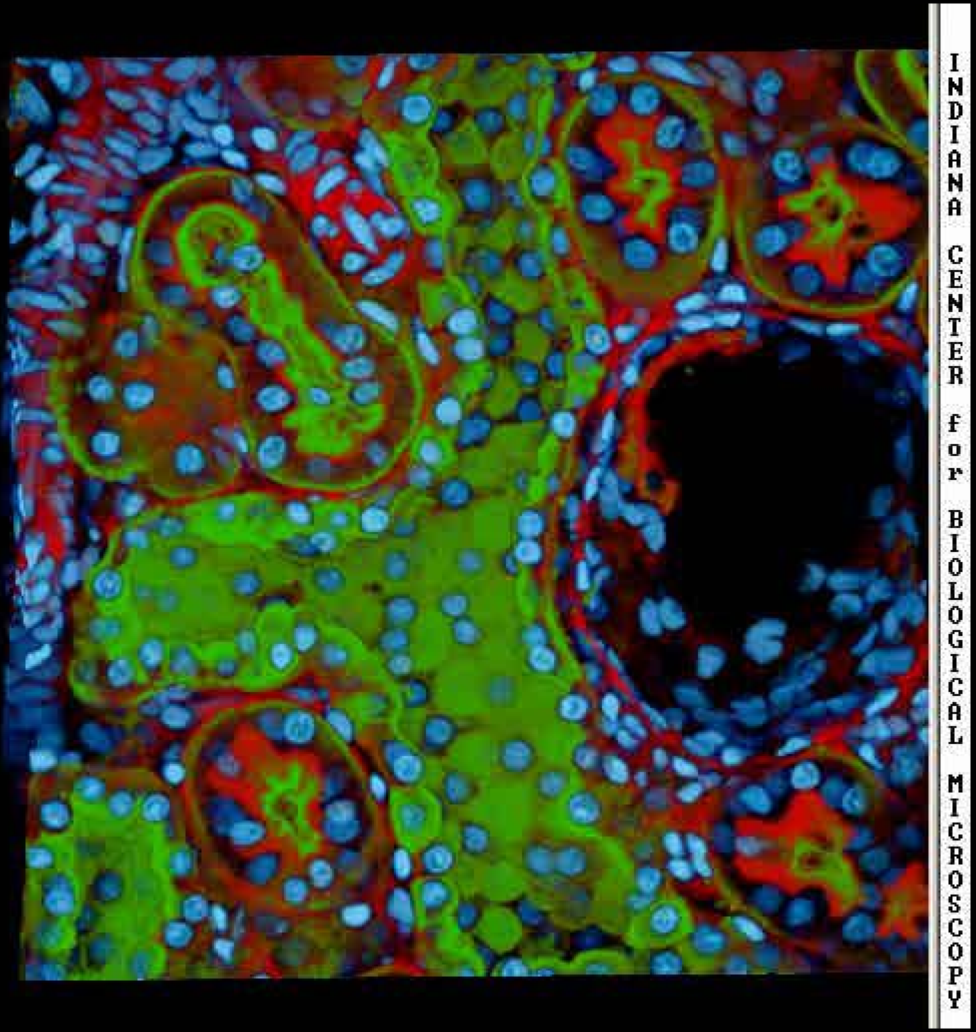


Xu et al., BioImaging, 1996

Practical multiphoton microscopy

- Photobleaching
- Resolution
- Multiphoton fluorescence dyes
- Imaging multiple colors
- Laser choices
- Detector options

Imaging complex structures – Comparing multiple probes in kidney tissue



Ruben Sandoval, Nephrology

Practical multiphoton microscopy

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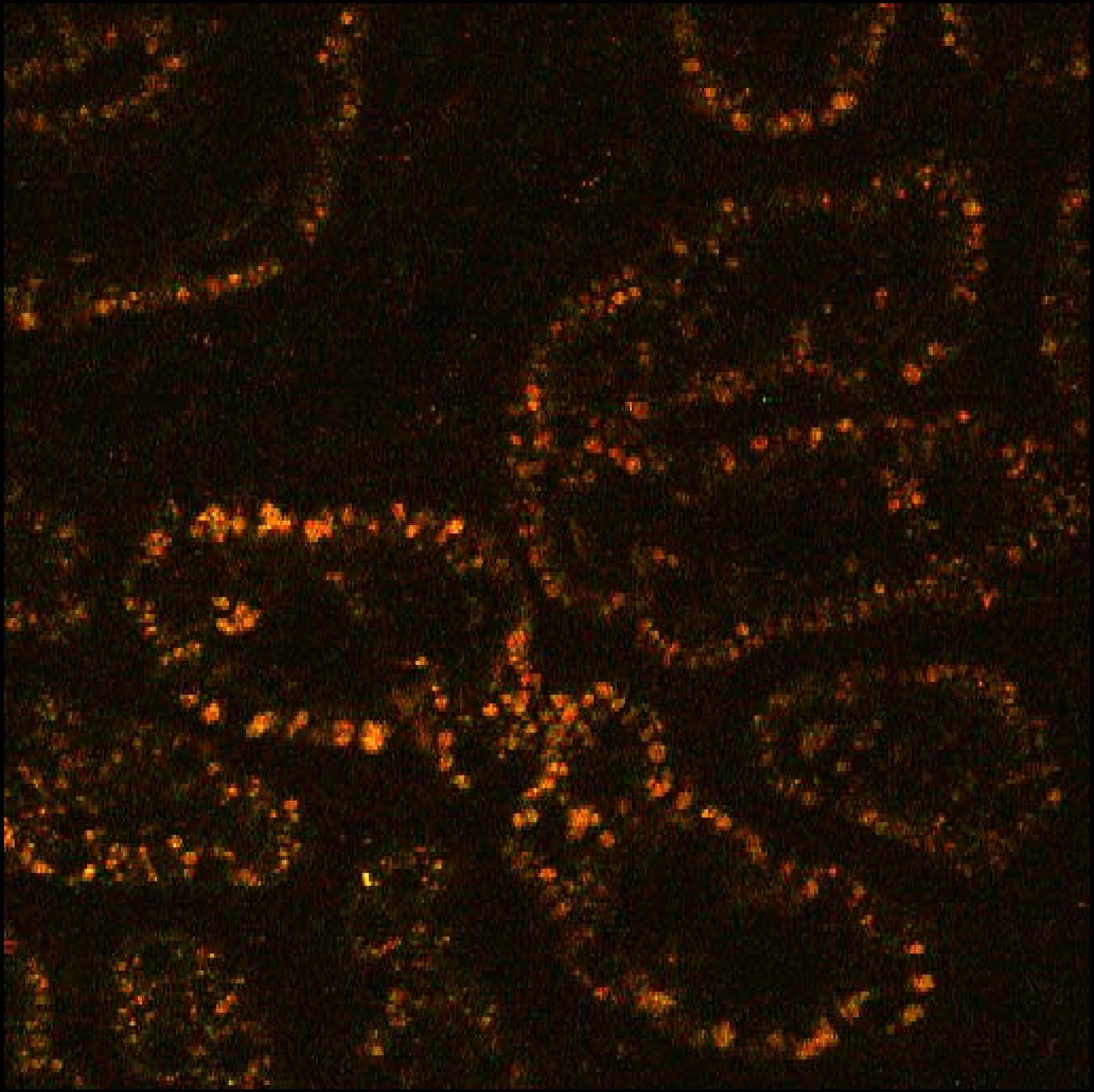
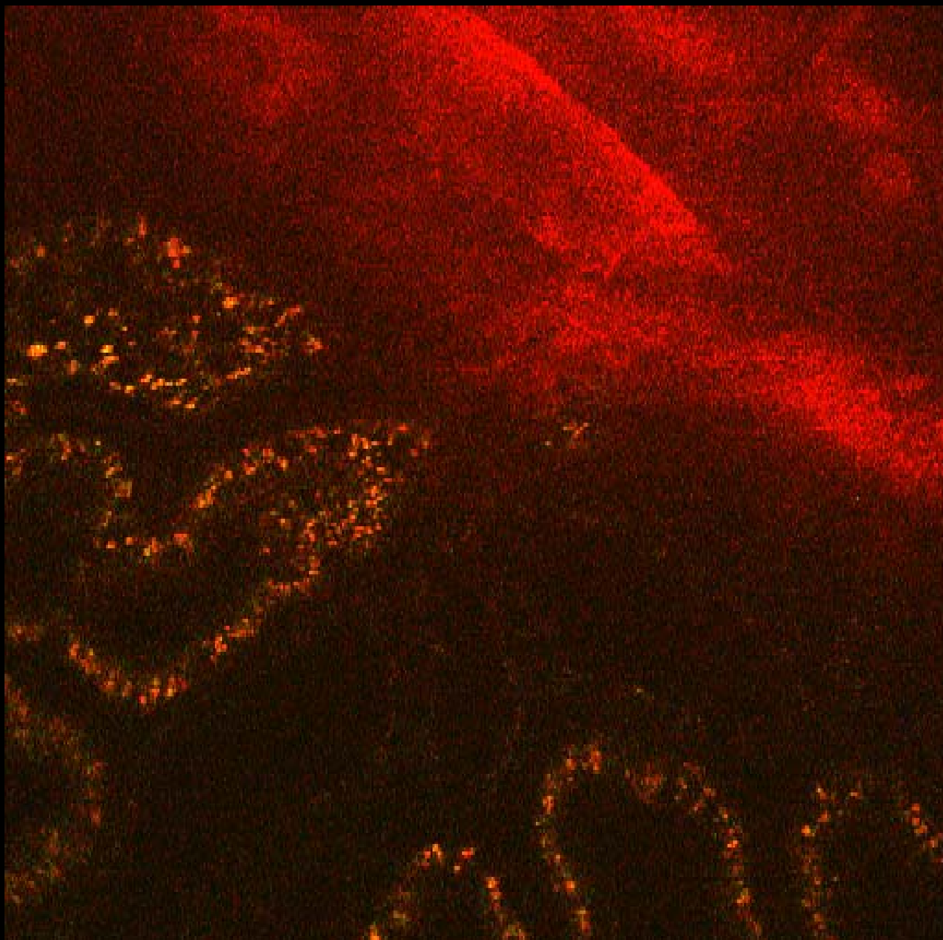
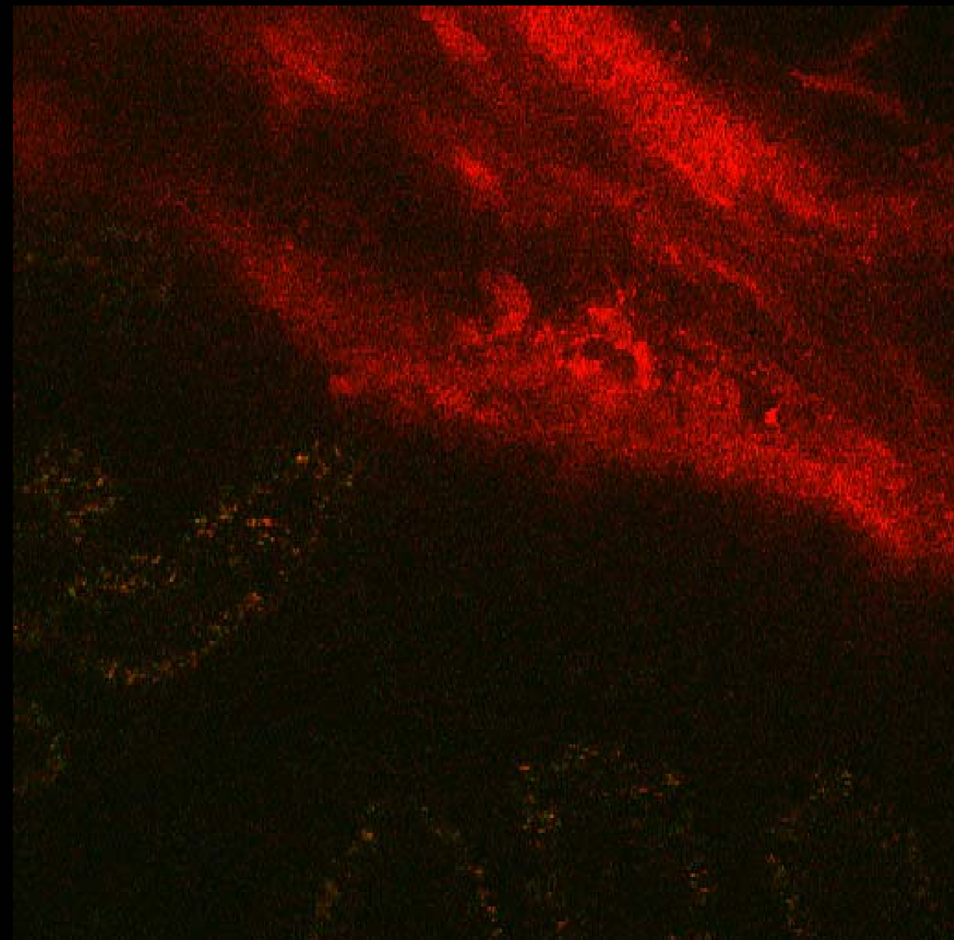
2-photon image volume of kidney of a living rat
(before any fluorescence labeling)



2-photon image autofluorescence and Texas-Red gentamicin
A tuneable laser is a good idea



820 nm excitation



860 nm excitation

Practical multiphoton microscopy

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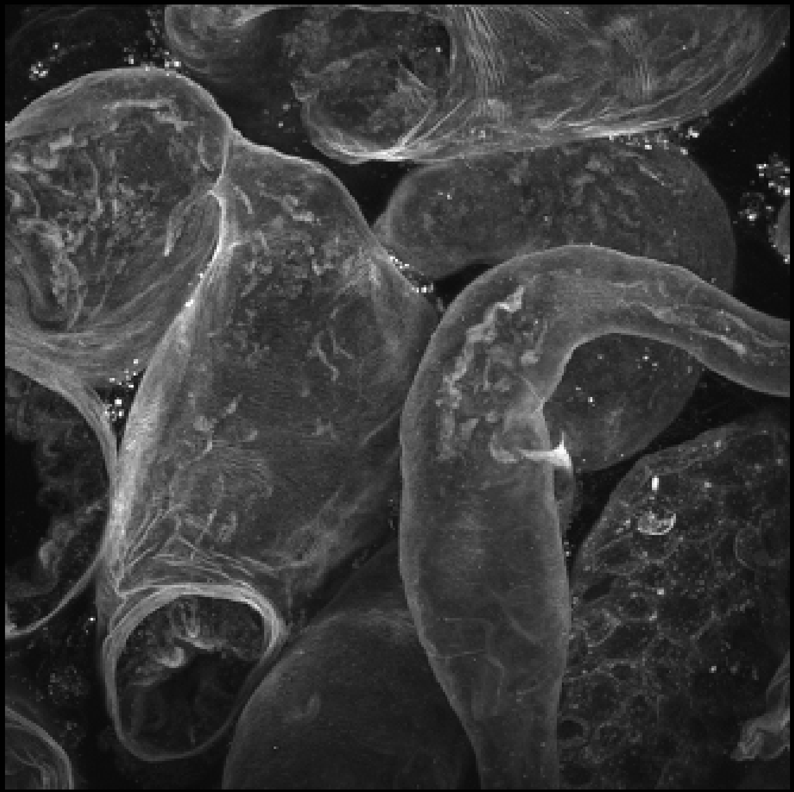
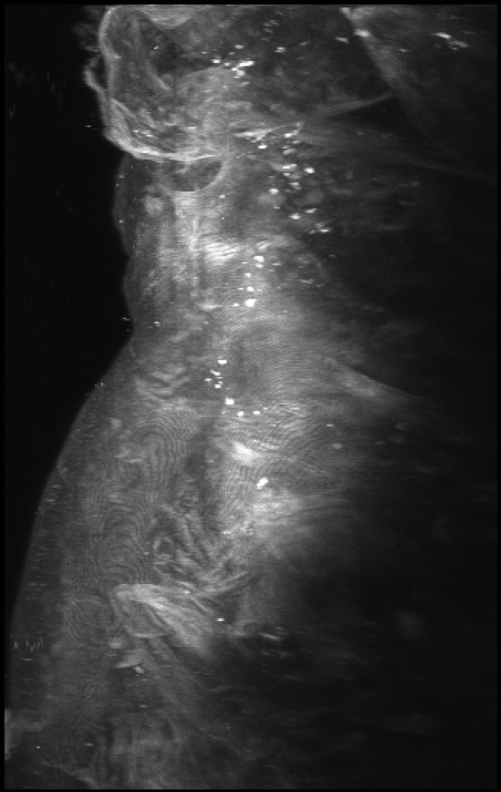
Signal attenuation with depth into fixed tissues - Multiphoton microscopy

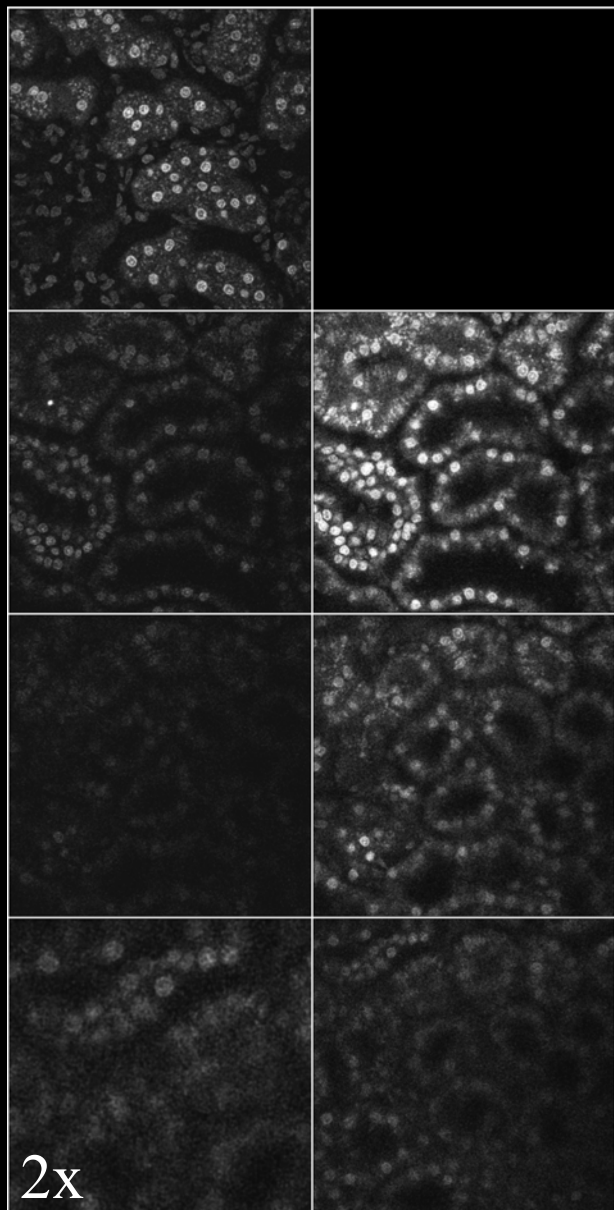


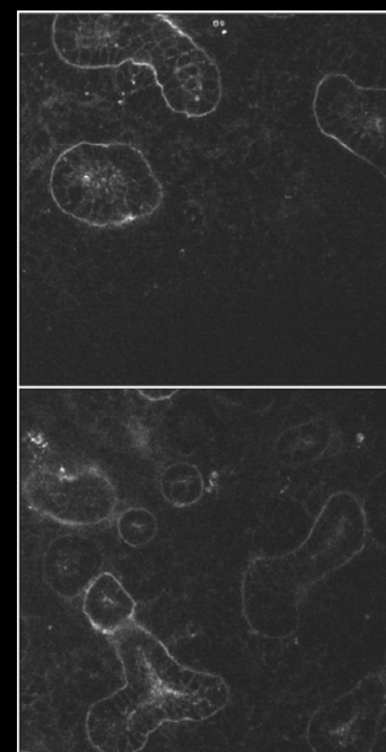
Top view



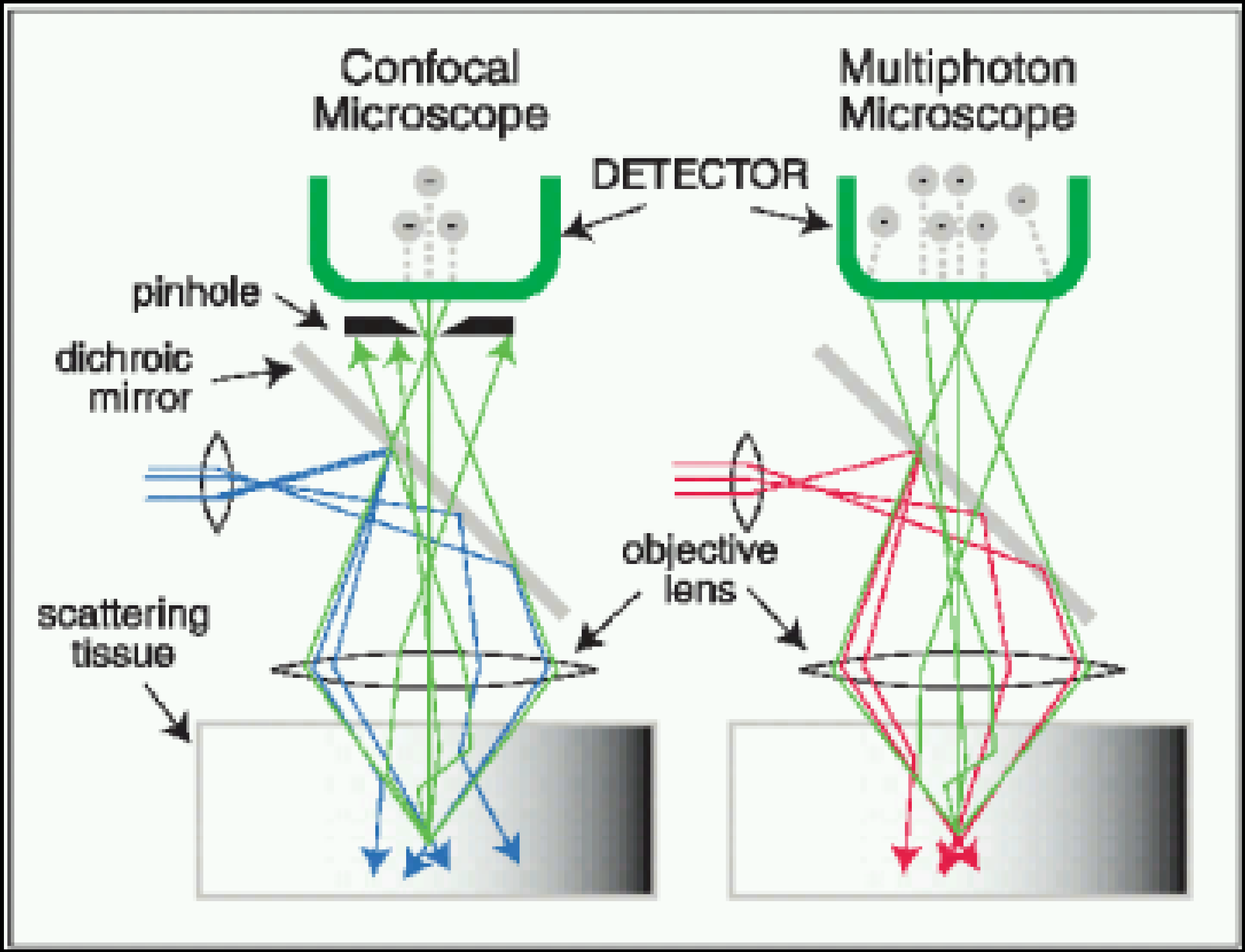
Side view

Signal attenuation with depth in multiphoton microscopy



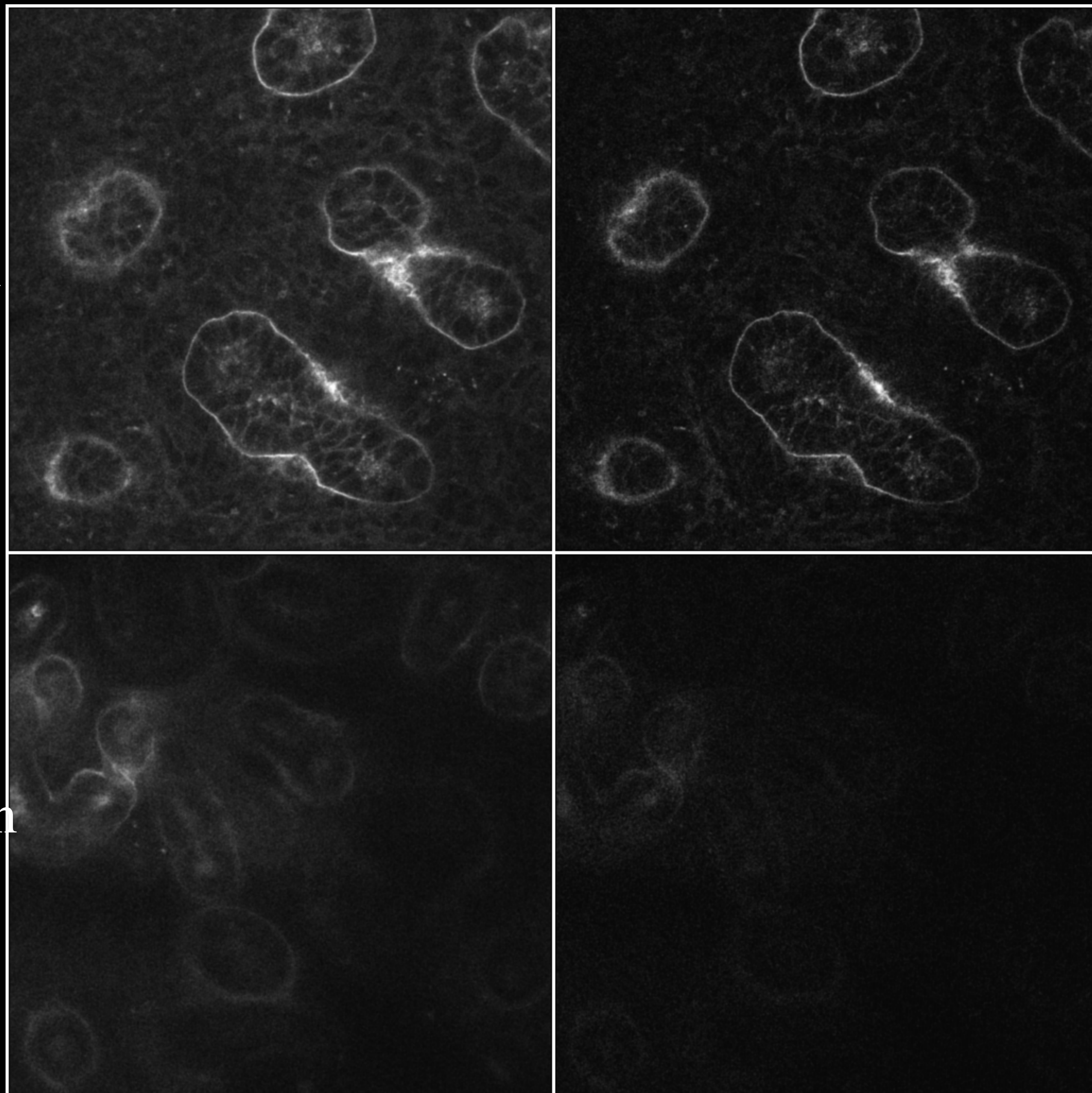


Multi-photon microscopy is less sensitive to light scatter by tissues – attenuation of signal



Sources of signal attenuation at depth

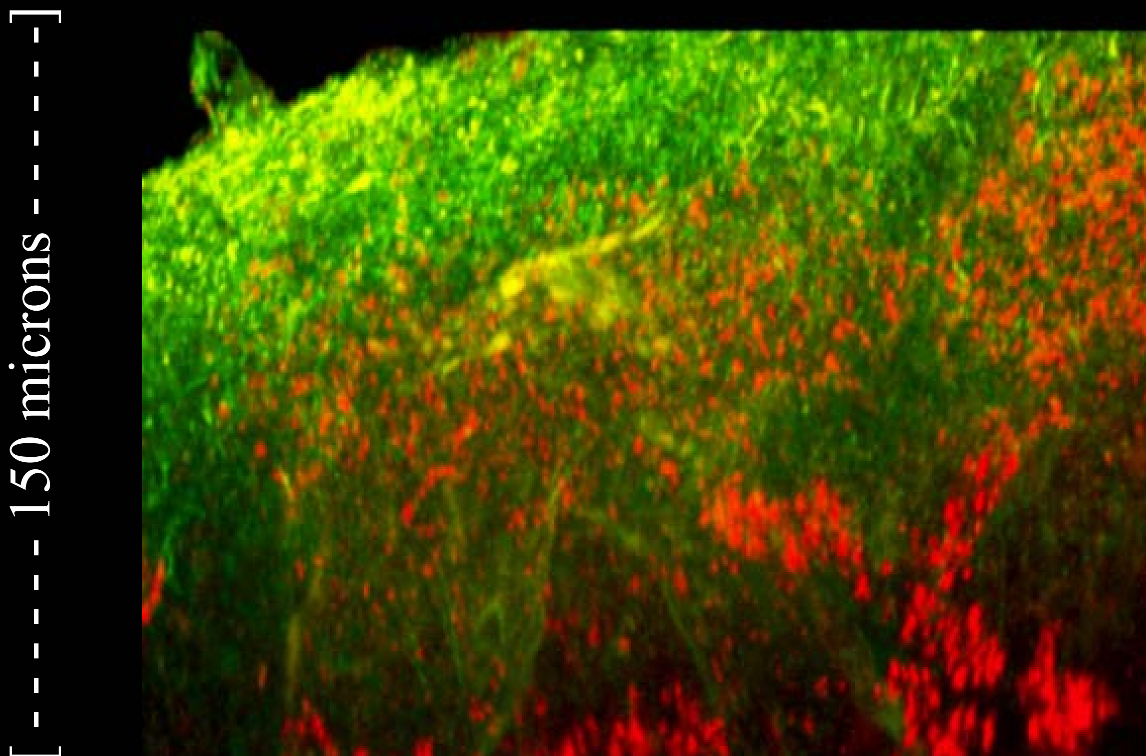
- Fluorescence emissions
  - scattering
  - refraction
    - spherical aberration



Use Red fluors – scattering decreases as the fourth power of wavelength

860 nm excitation

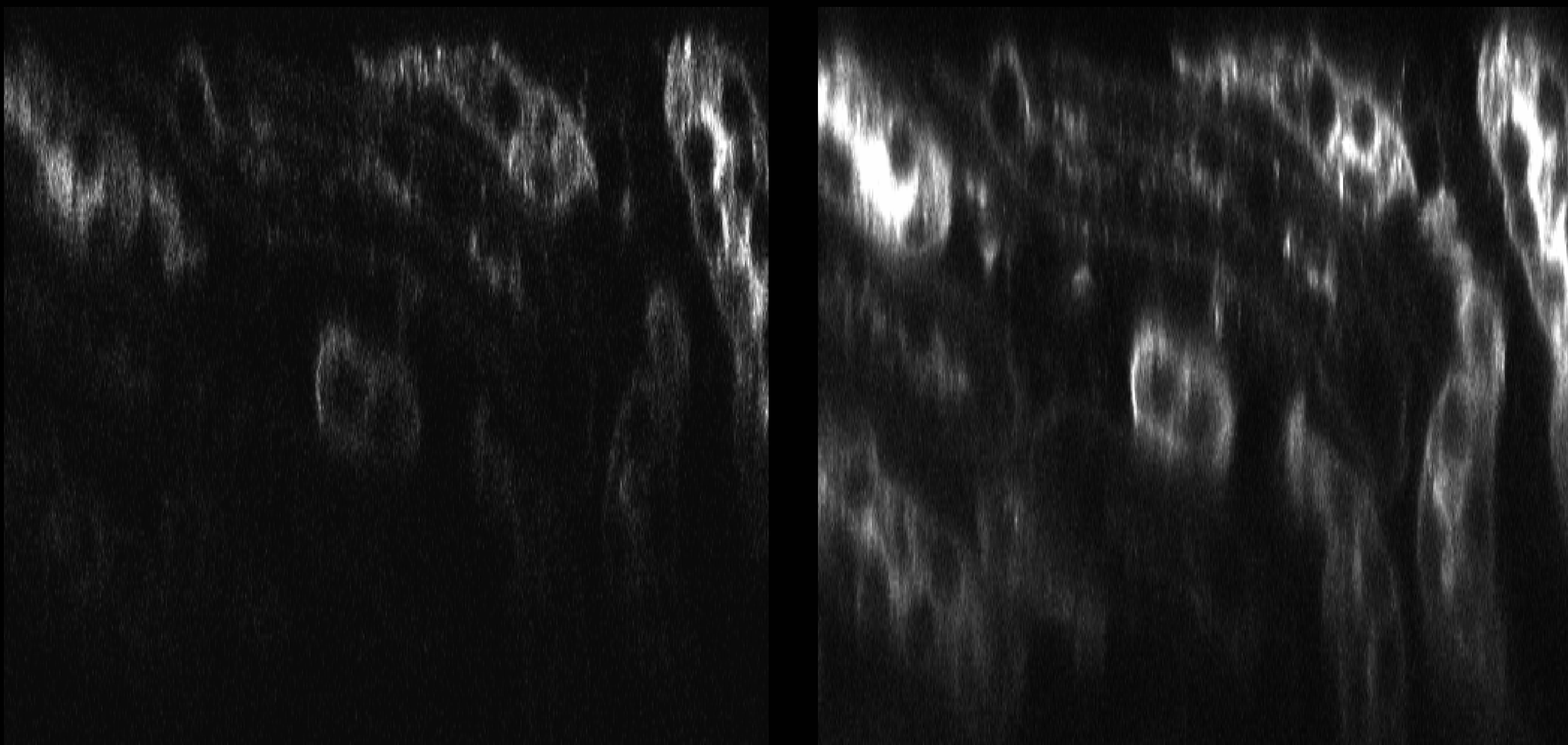
Two color 2-photon imaging of 13 day old embryonic mouse kidney



Red - peanut agglutinin

Green - Len culinaris agglutinin

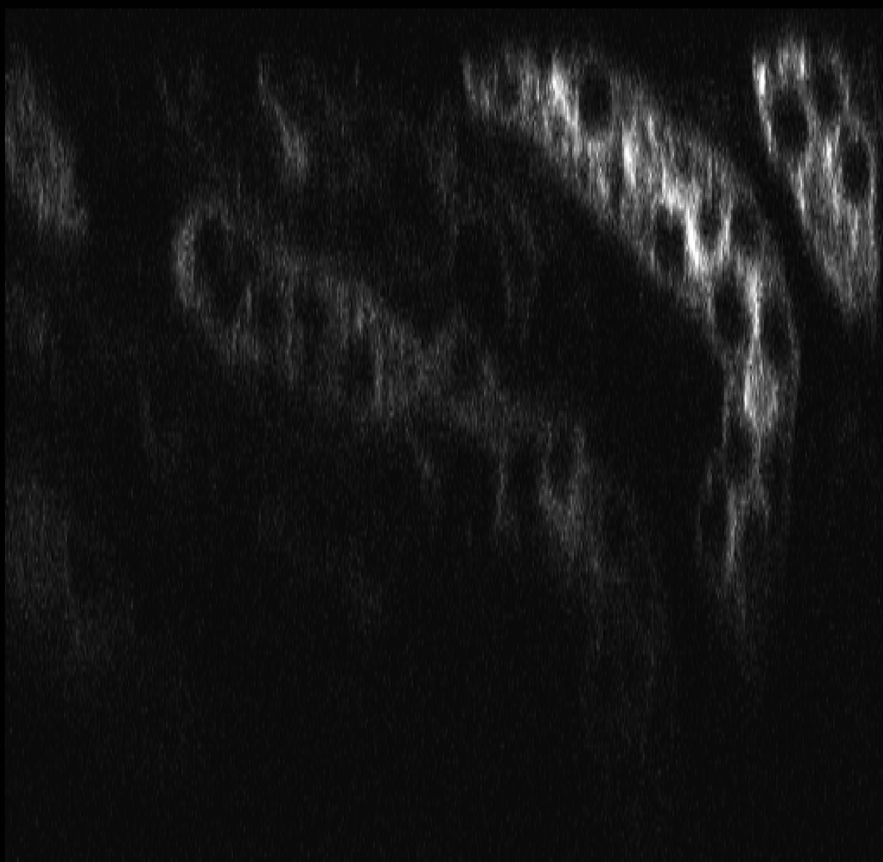
Non-descanned detectors collect scattered light more efficiently



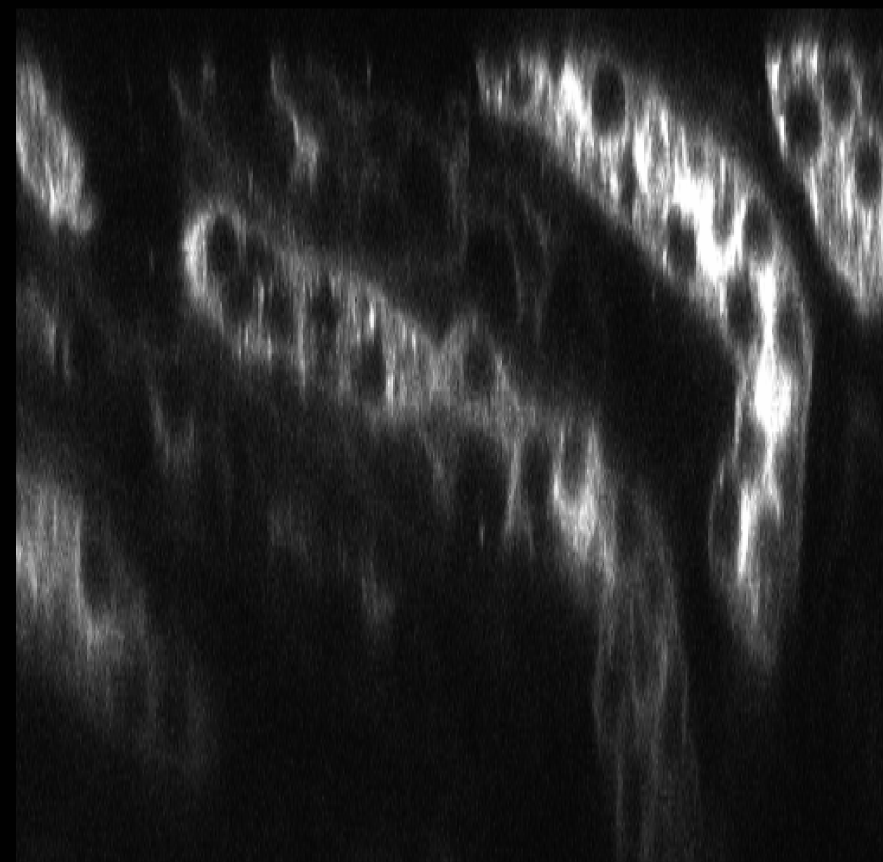
Descanned PMT

Non-descanned PMT

Non-descanned detectors collect scattered light more efficiently



Descanned PMT

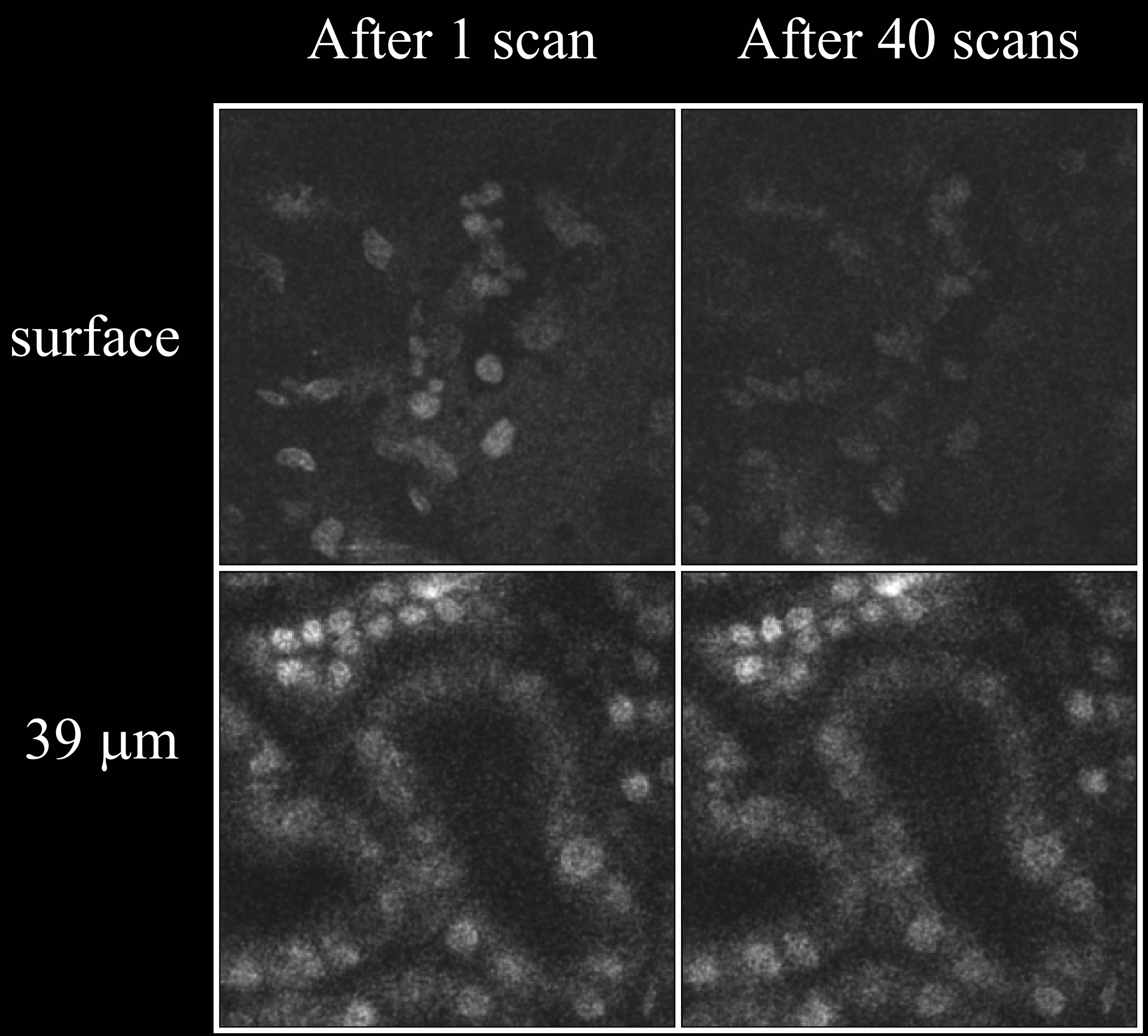


Non-descanned PMT

Sources of signal attenuation at depth

- Fluorescence emissions
  - scattering
  - refraction
    - spherical aberration
- Illumination
  - scattering
  - refraction
    - spherical aberration

Attenuation of illumination is reflected by lower photobleaching at depth

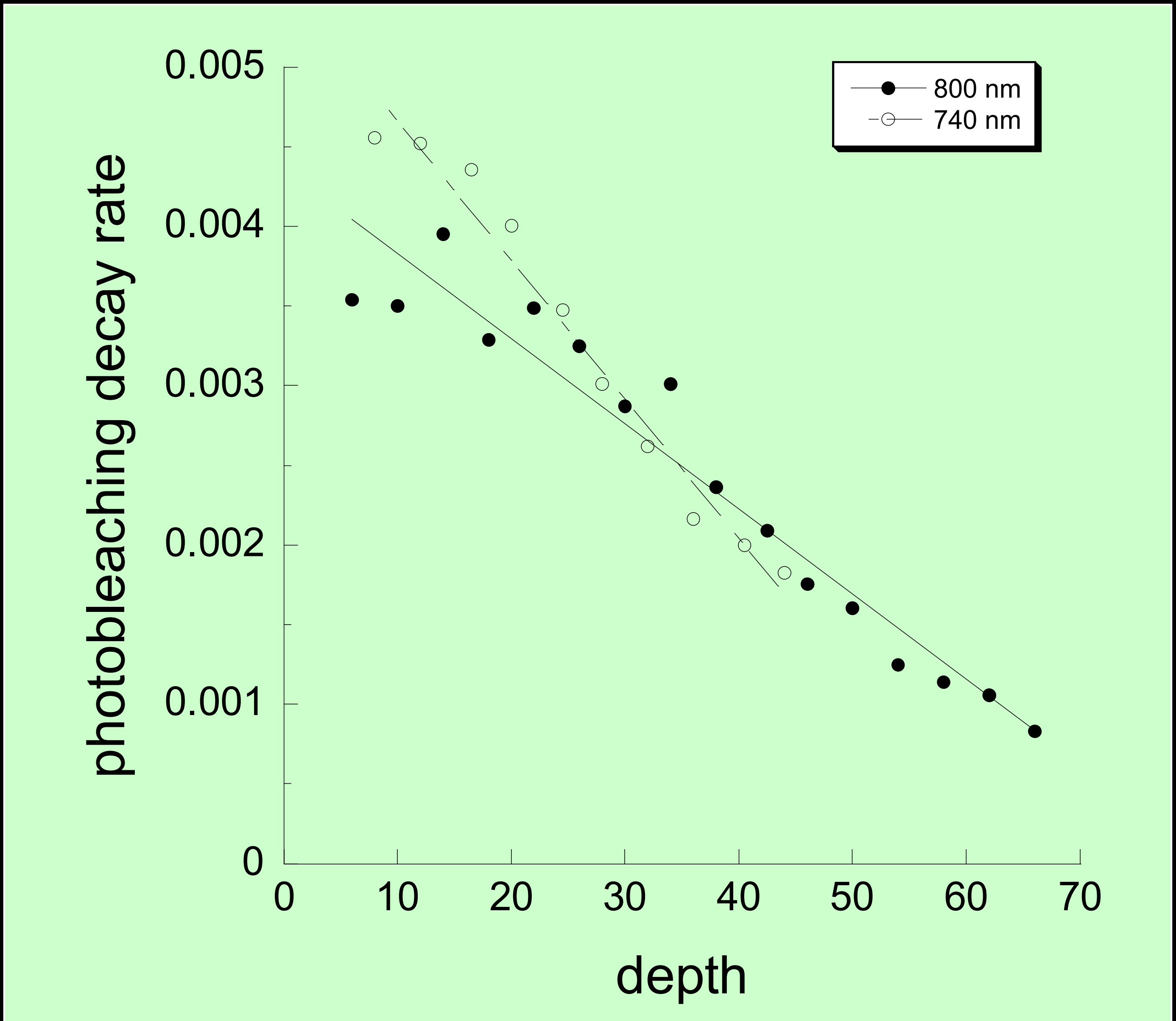


Attenuation of illumination – photobleaching rates

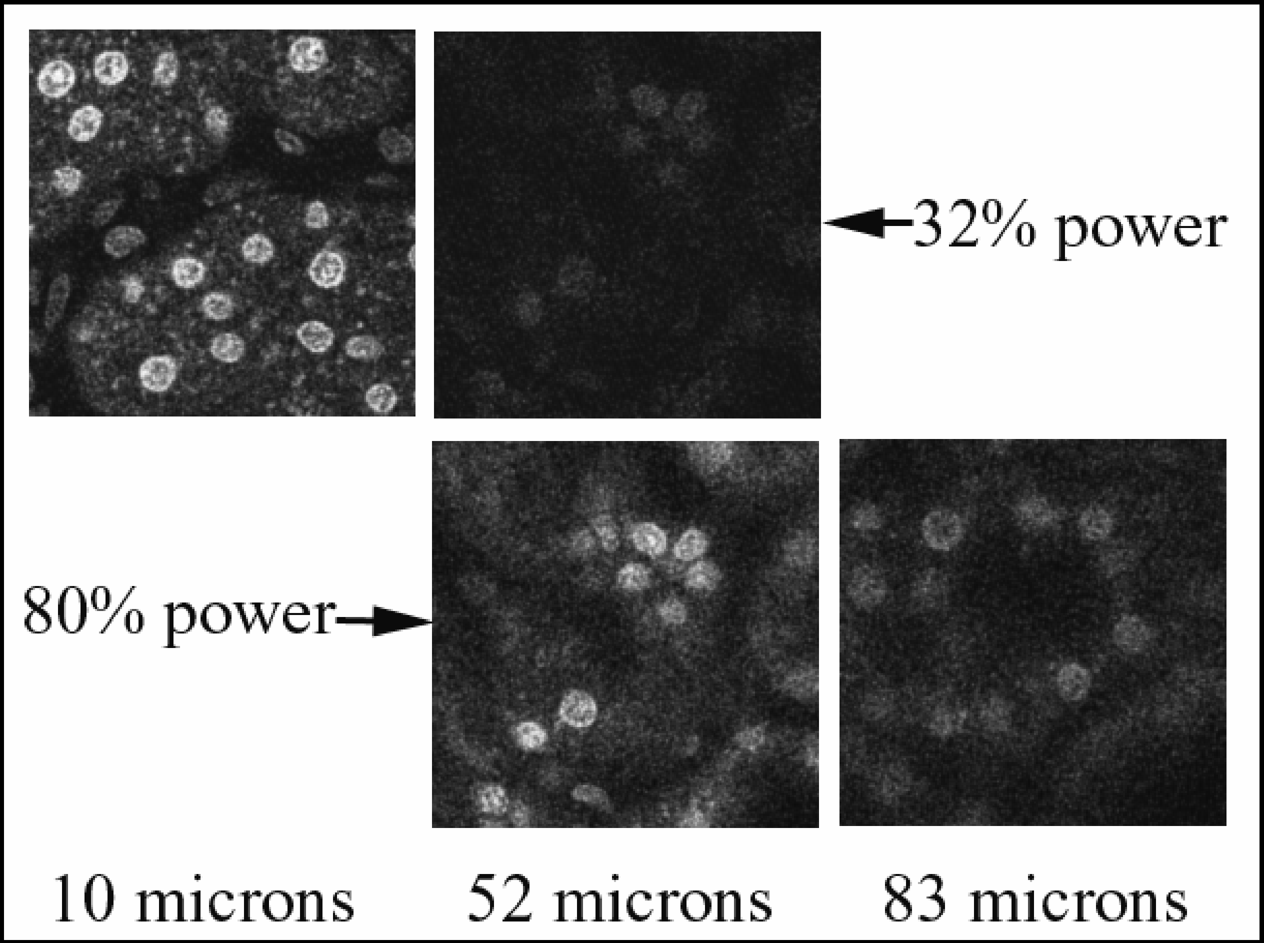
Live rat, 800 nm excitation, Hoechst

17ocbleach

Illumination attenuation decreases at longer wavelengths – light scatter decreases with wavelength



Signal attentuates with depth into kidneys, but can be regained with increased illumination



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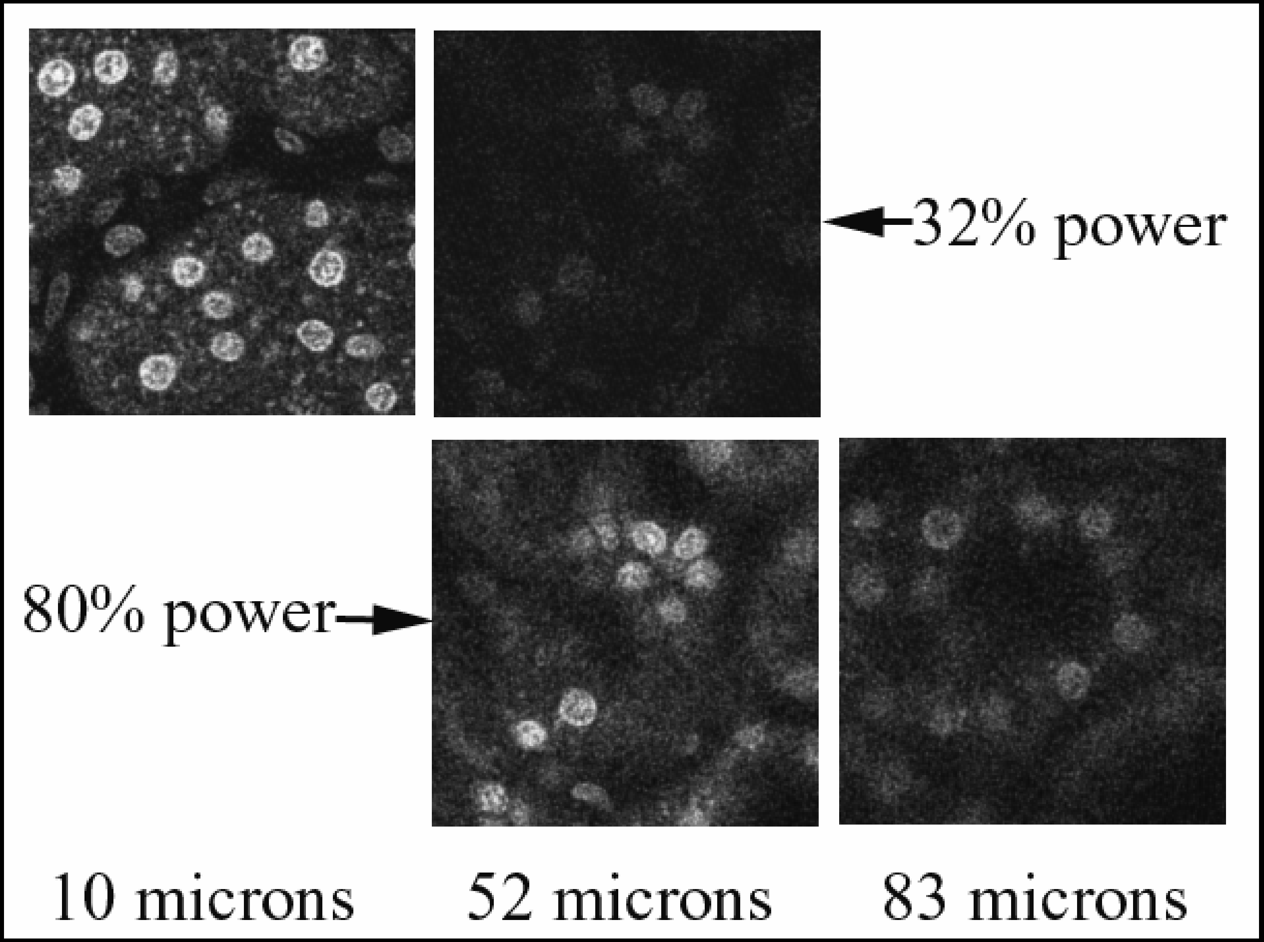

And, unlike confocal microscopy, scatter has minimal effect on resolution

Practical multiphoton microscopy

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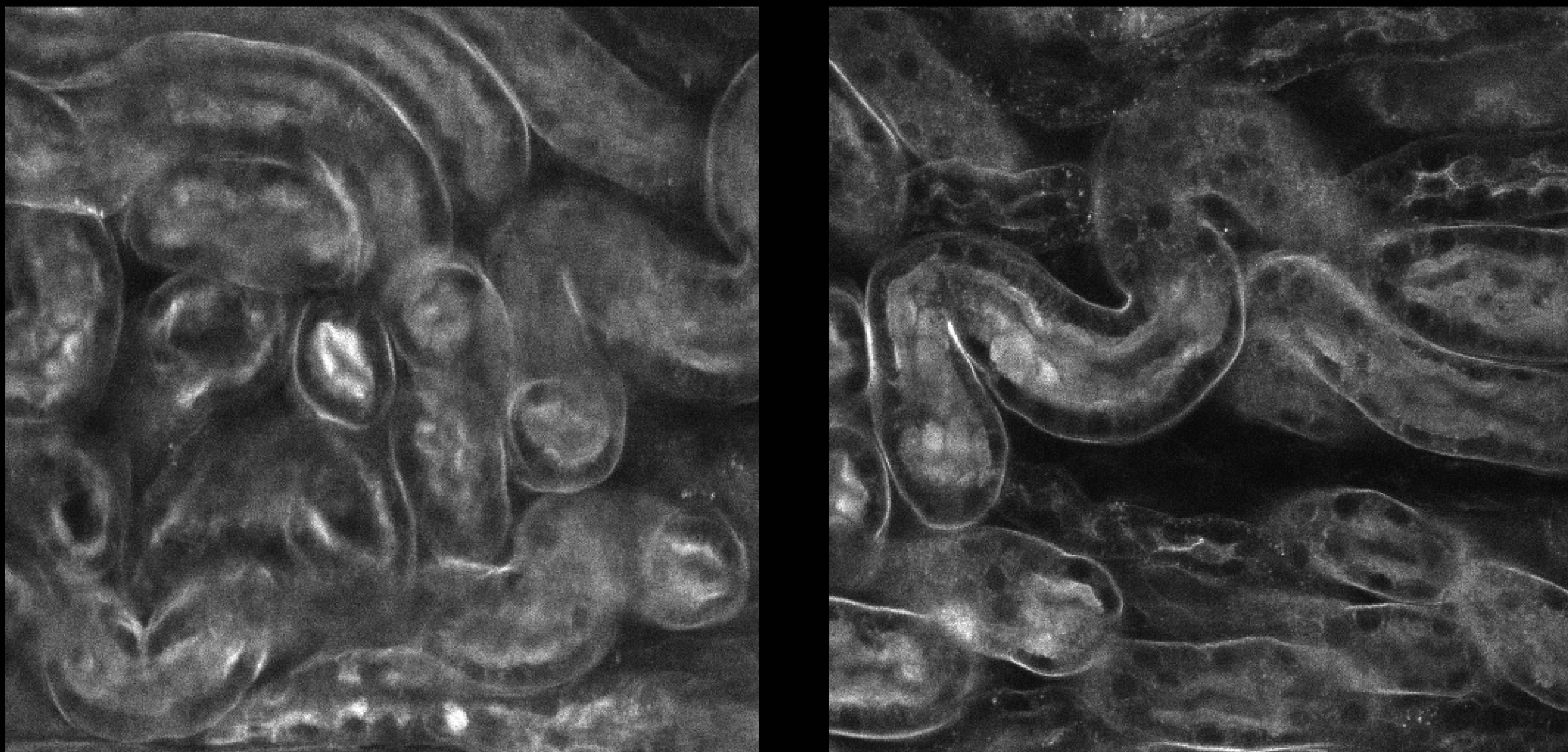
- Objective choice

Objectives for Multiphoton Microscopy

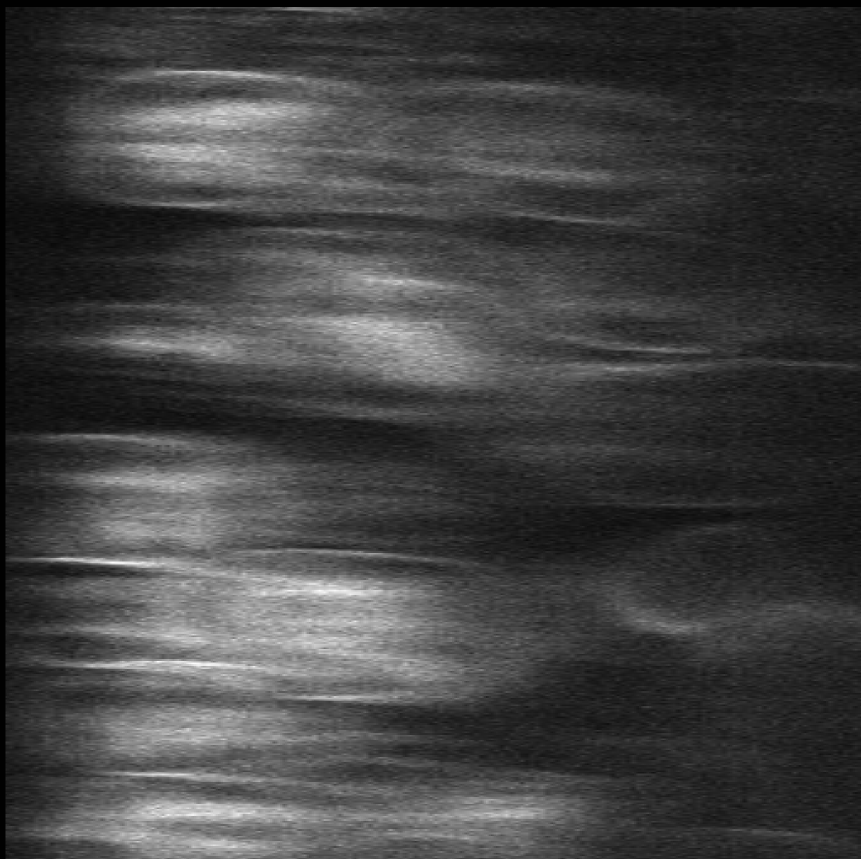
A new generation of microscope objectives is being developed that is optimized for infrared transmission, and designed for light collection rather than image formation.

The importance of high numerical aperture to 3d microscopy - XY planes of a kidney sample

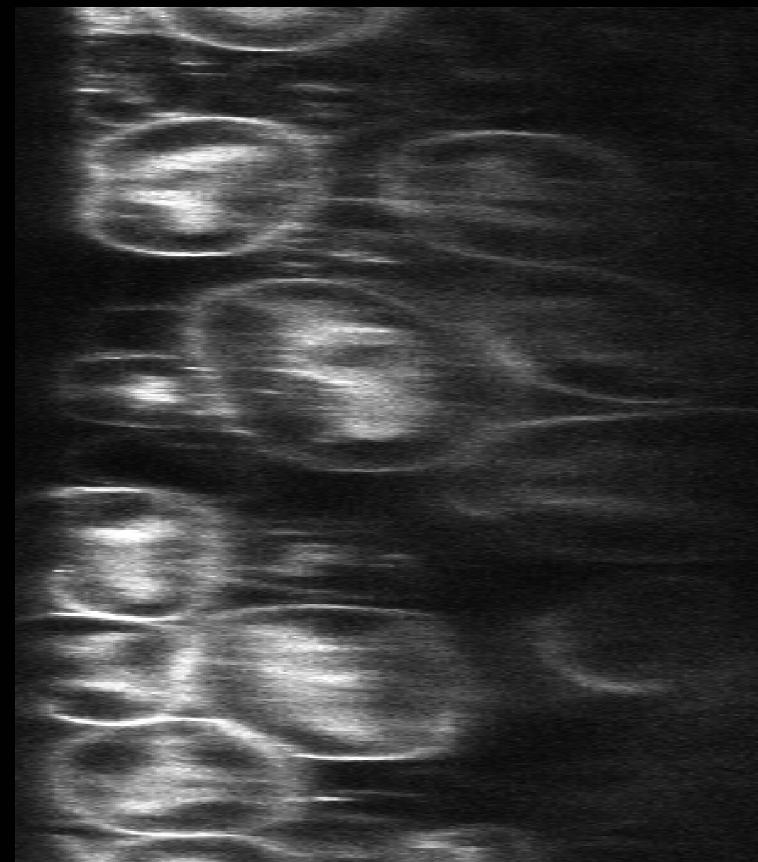


20x, NA .75   60x, NA 1.2

The importance of high numerical aperture to 3d microscopy - XY planes of a kidney sample

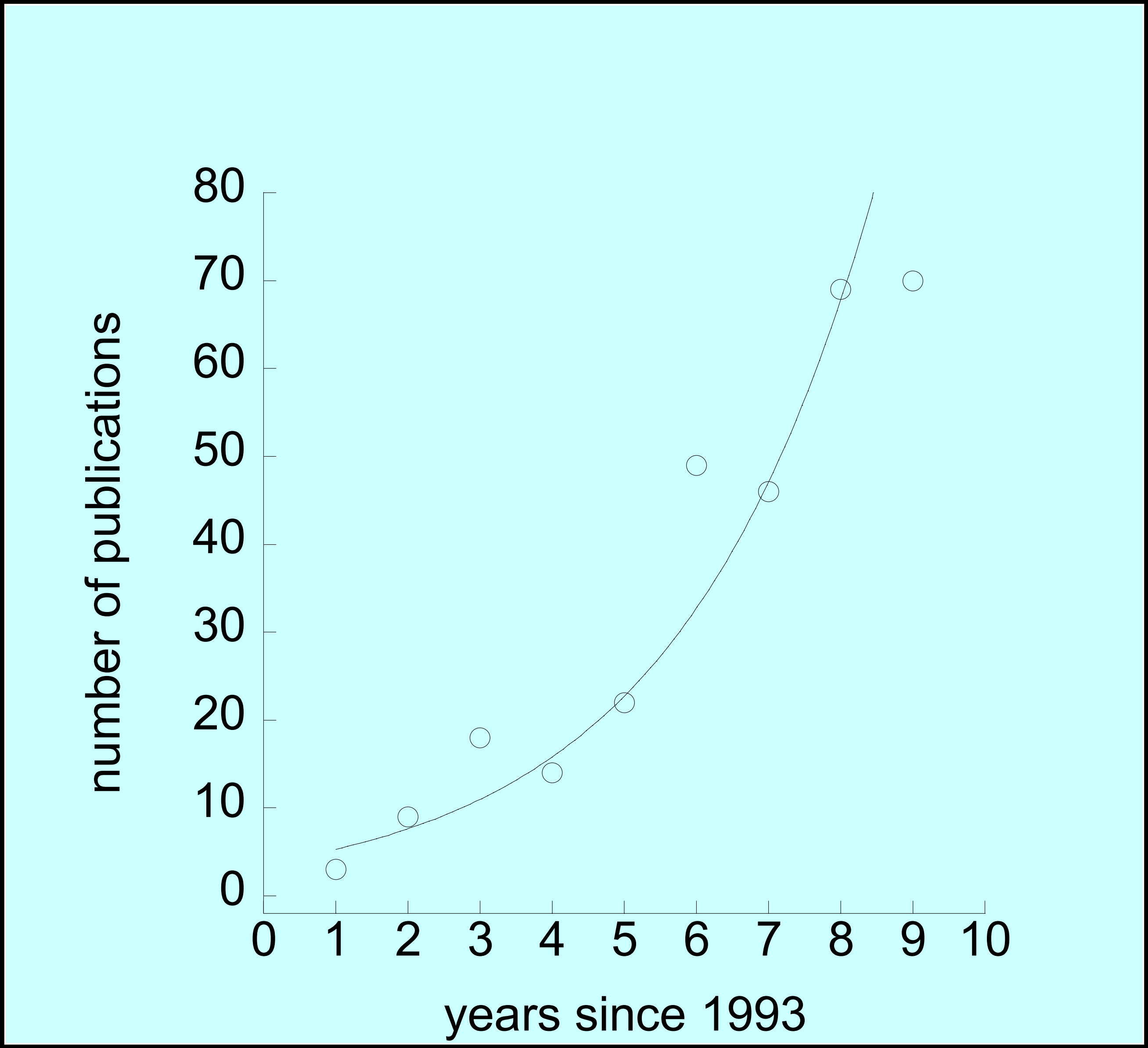


20x, NA .75

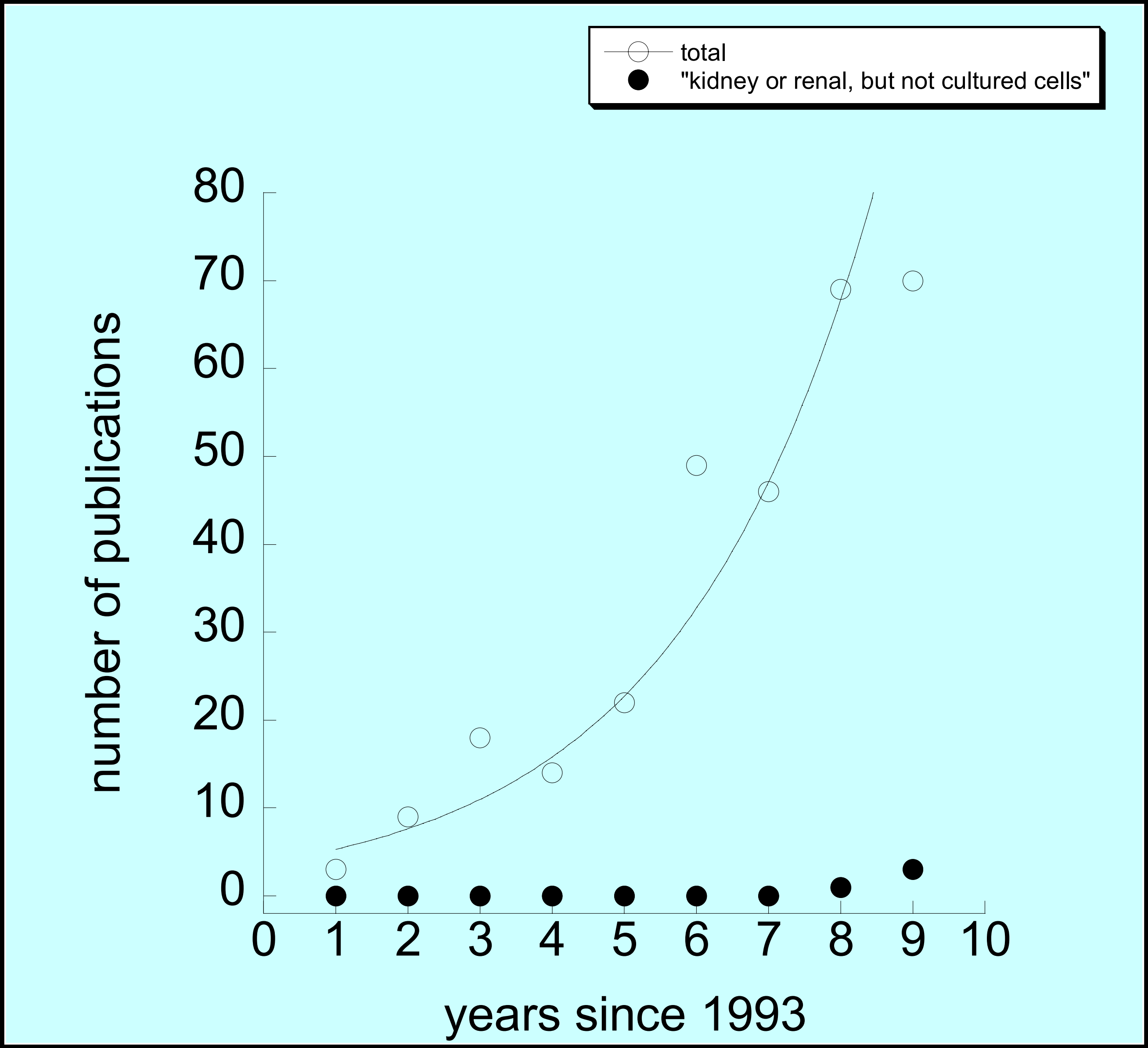


60x, NA 1.2

Increase in the use of multiphoton microscopy since 1993



Increase in the use of multiphoton microscopy since 1993



Summary – Which system to choose?

- Do you need to image?
- Do you need optical sections?
- Confocal or image deconvolution?
- Confocal or multi-photon?

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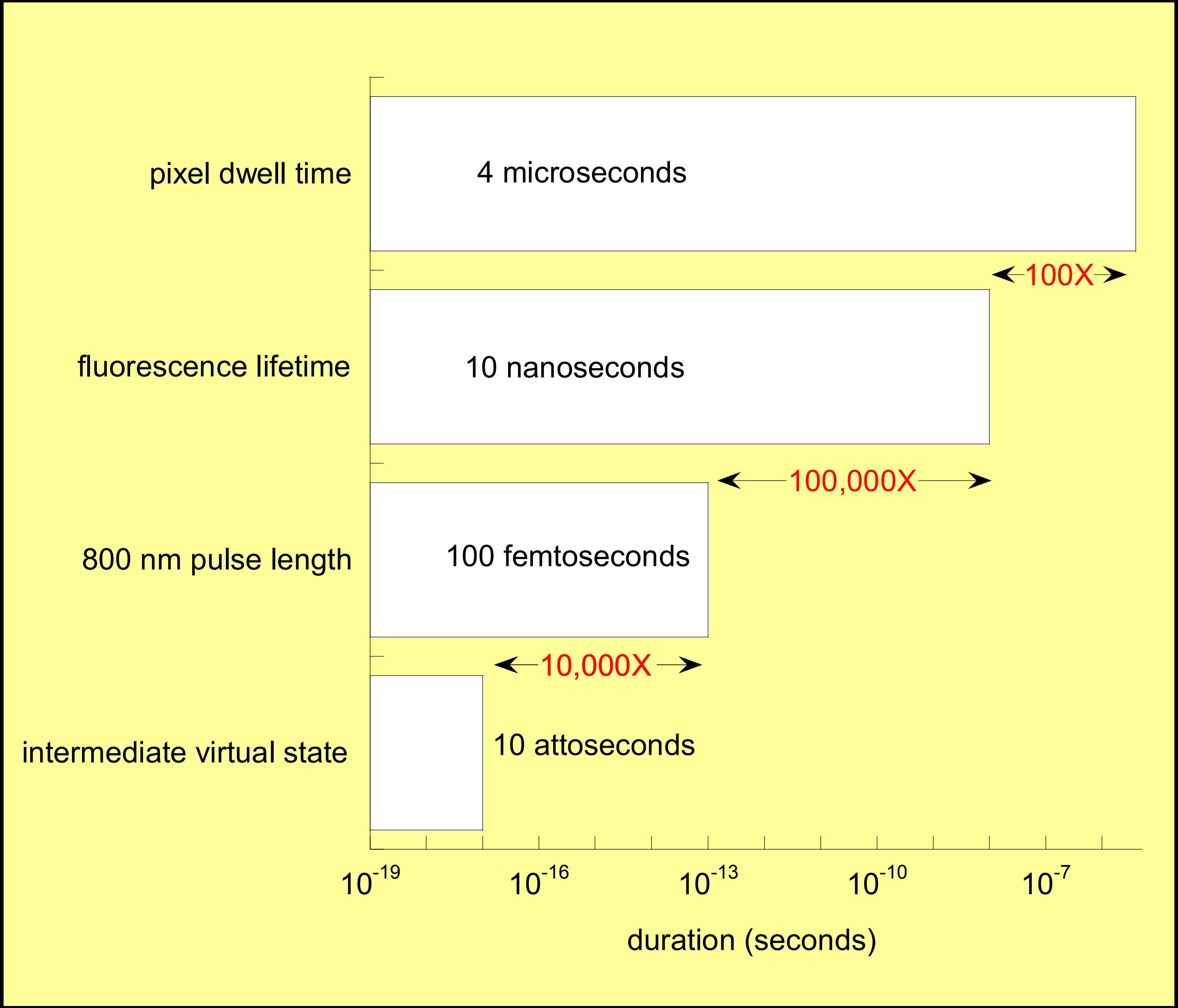

pixel dwell time
4 microseconds
100X
fluorescence lifetime
10 nanoseconds
100,000X
800 nm pulse length
100 femtoseconds
10,000X
intermediate virtual state
10 attoseconds
10^{-19}
10^{-16}
10^{-13}
10^{-10}
10^{-7}
duration (seconds)