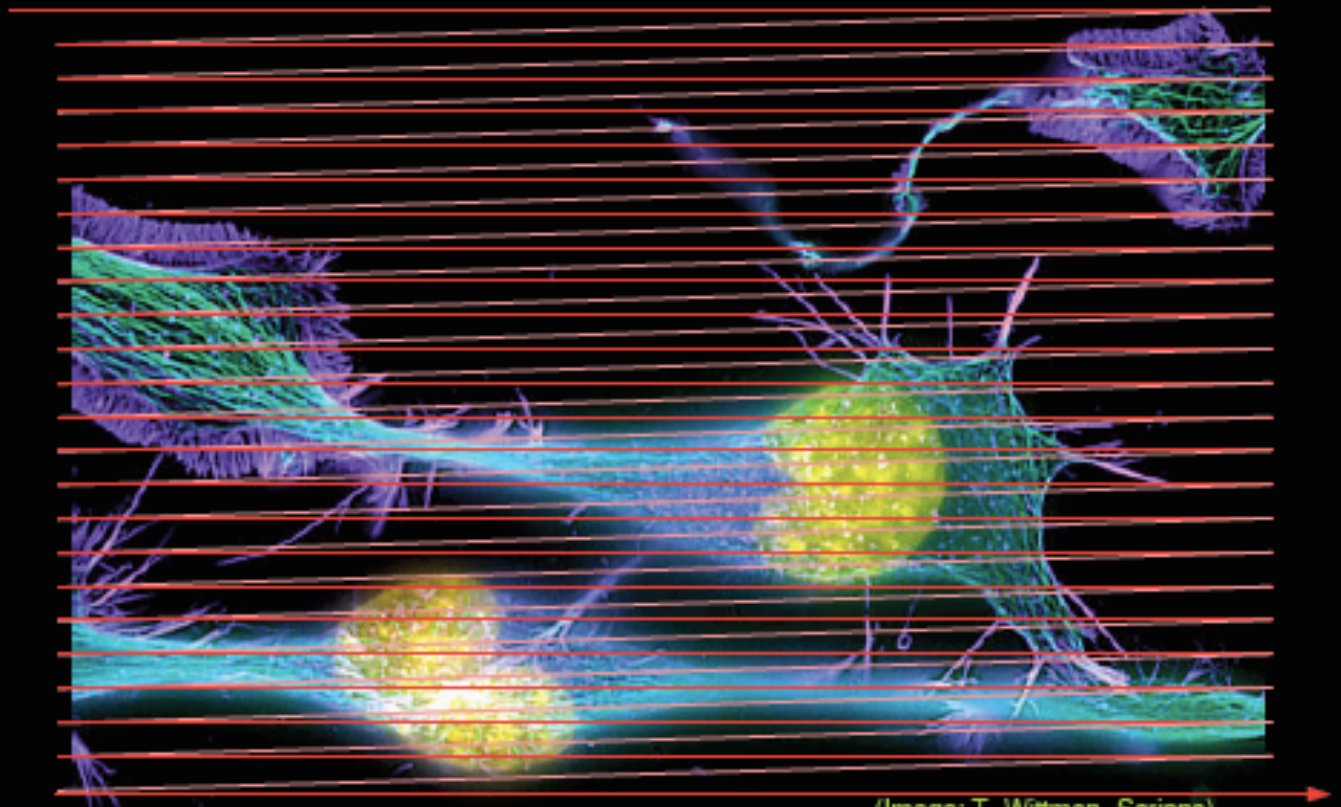
Scanning Microscopy

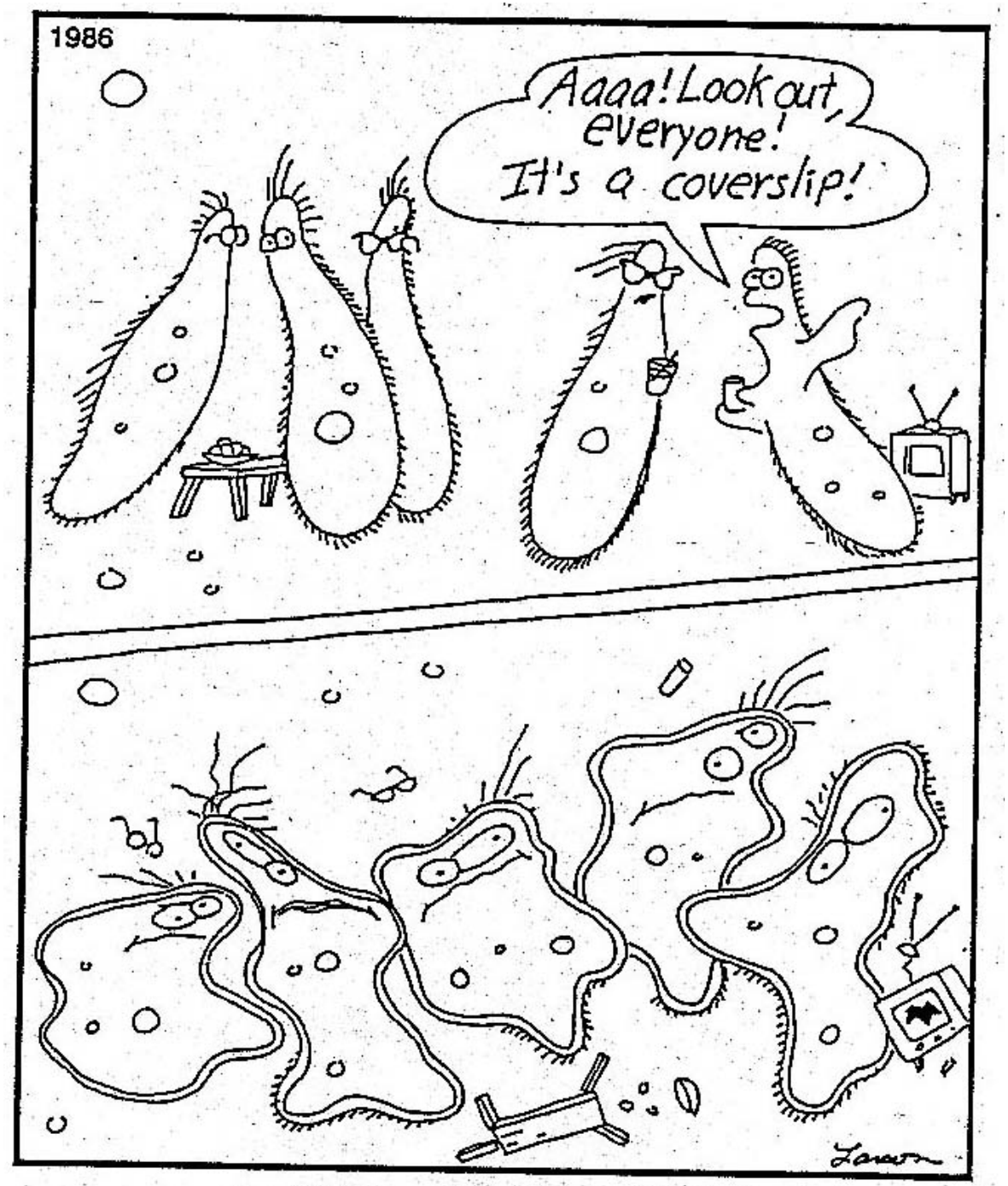


Edited by: Zvi Kam, Weizmann
For Advance Light Microscopy course

From Larson diary

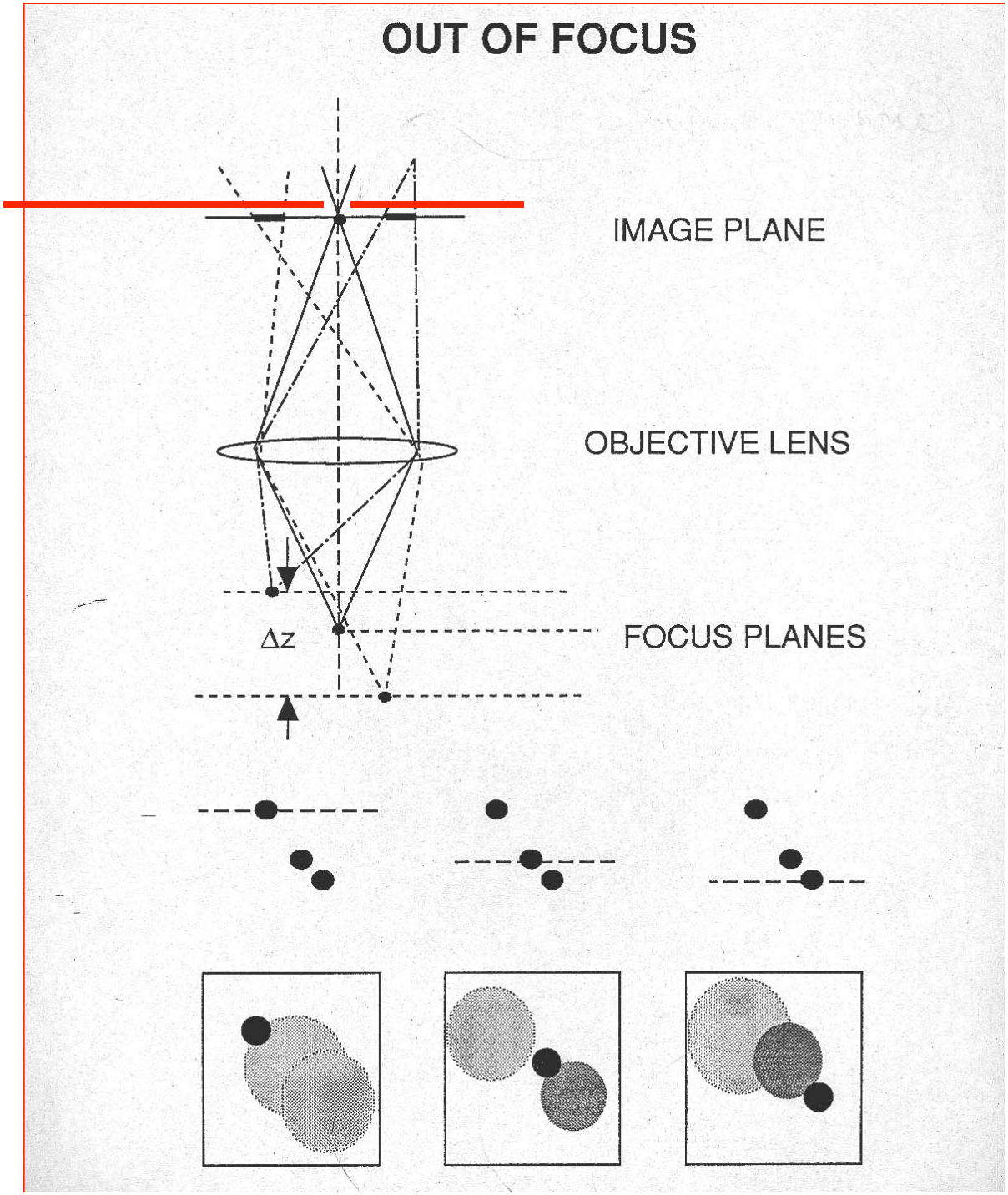
Three-Dimensional Imaging.

Live cell Imaging require special sample preparation and mounting.

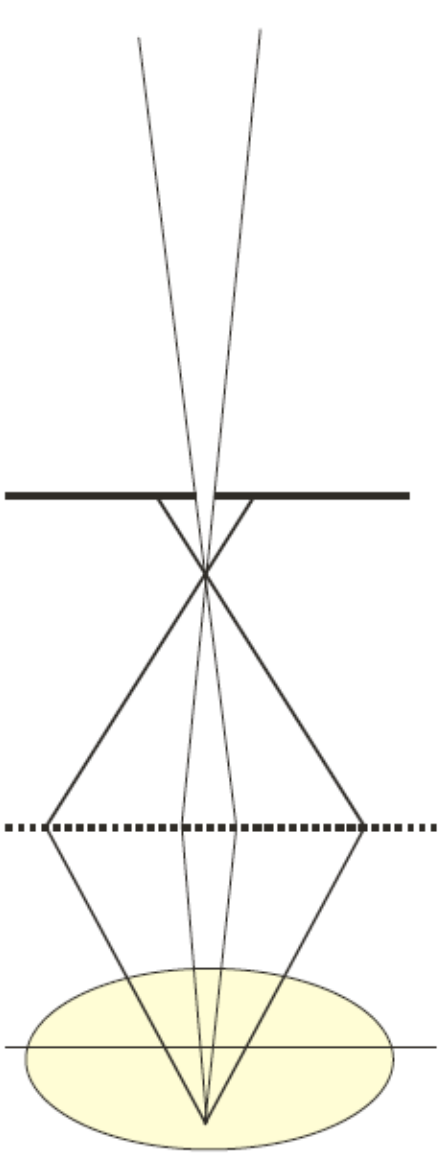


Life on a microscope slide

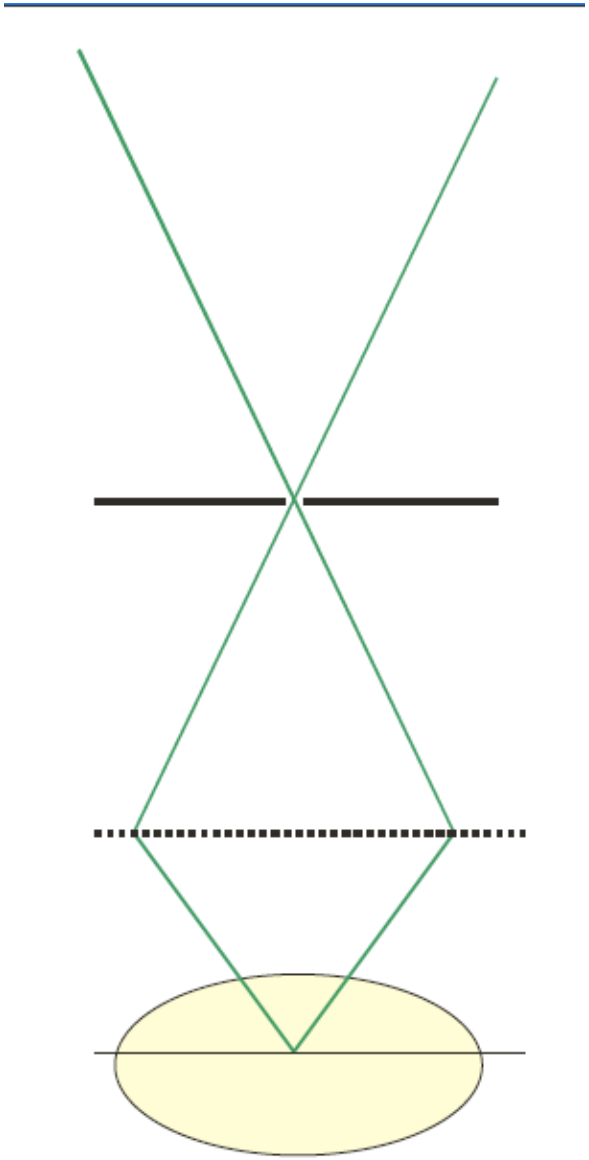
Confocal Pinhole



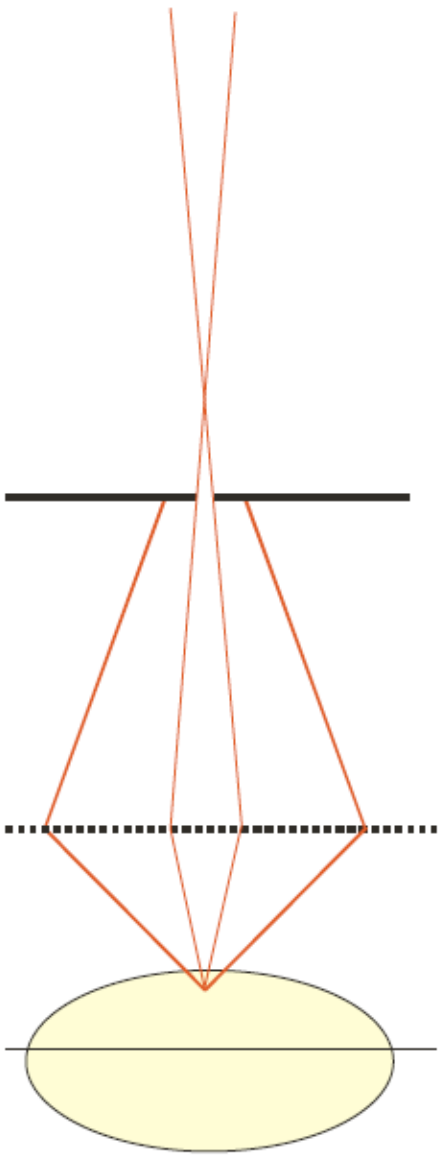
Debluring
By deconvolution



Below the focus



In focus



Above the focus

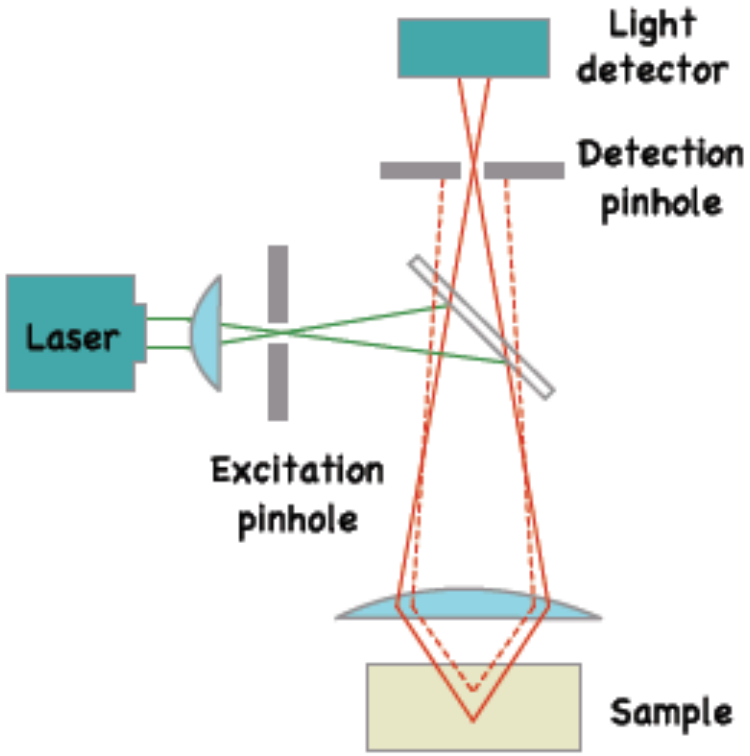
3D fluorescence microscopy

Acquire a "focal series" (stack) of images

Problem:
Each image contains out-of-focus blur from other focal planes

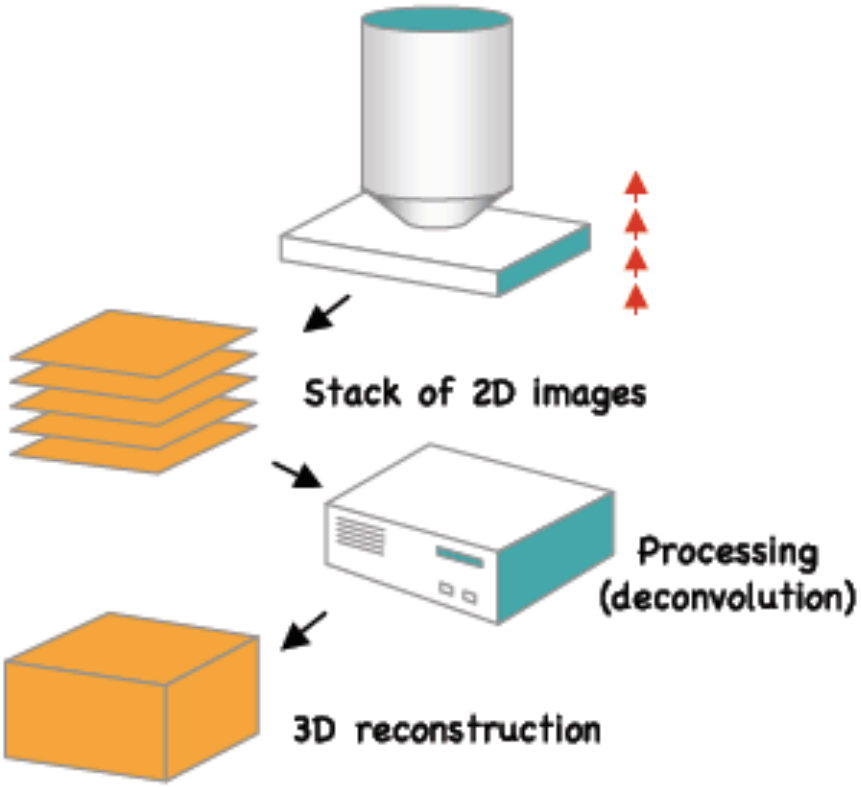
Approach 1

Physically exclude the blur
Example: confocal microscopy



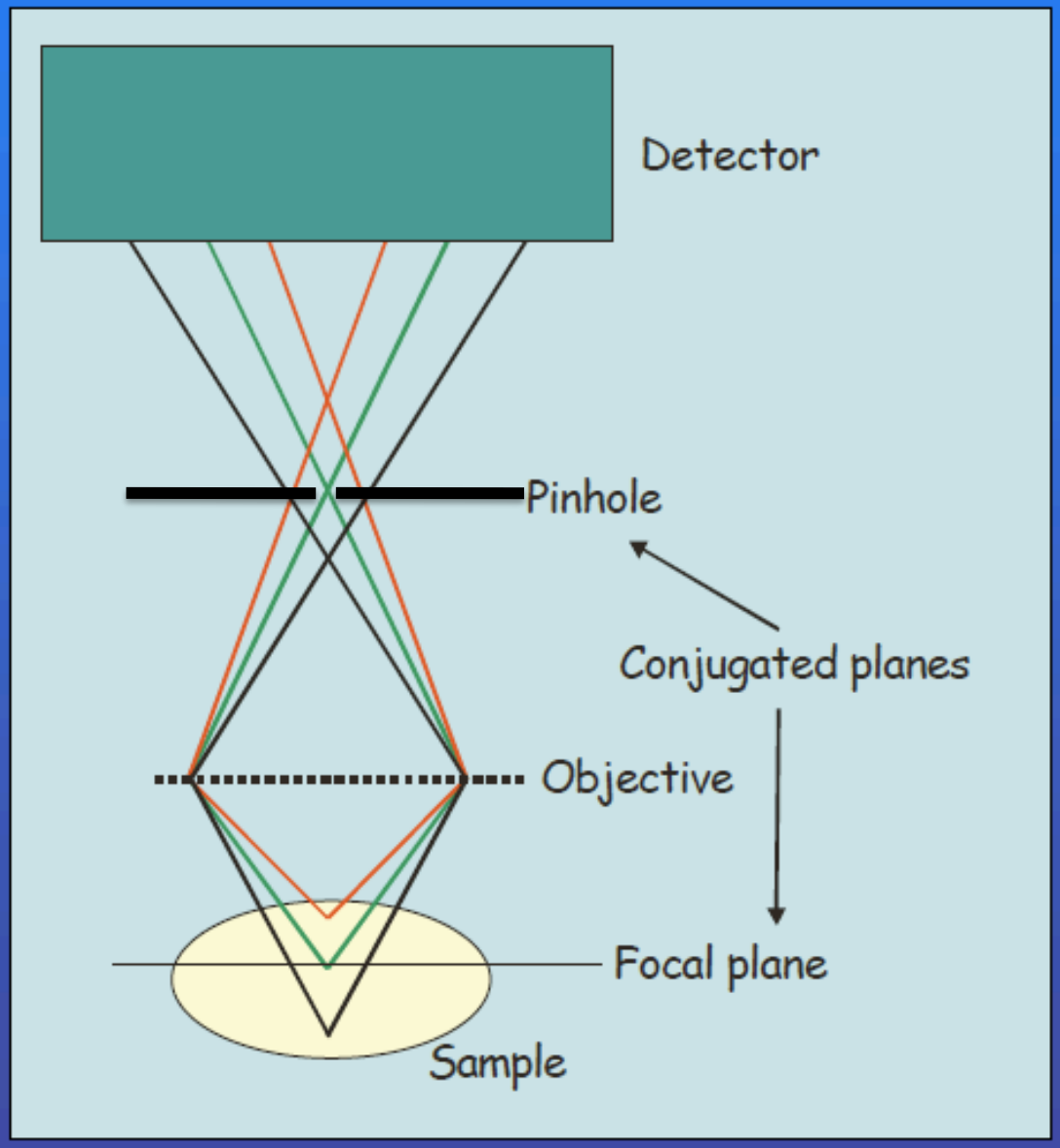
Approach 2

Remove the blur computationally

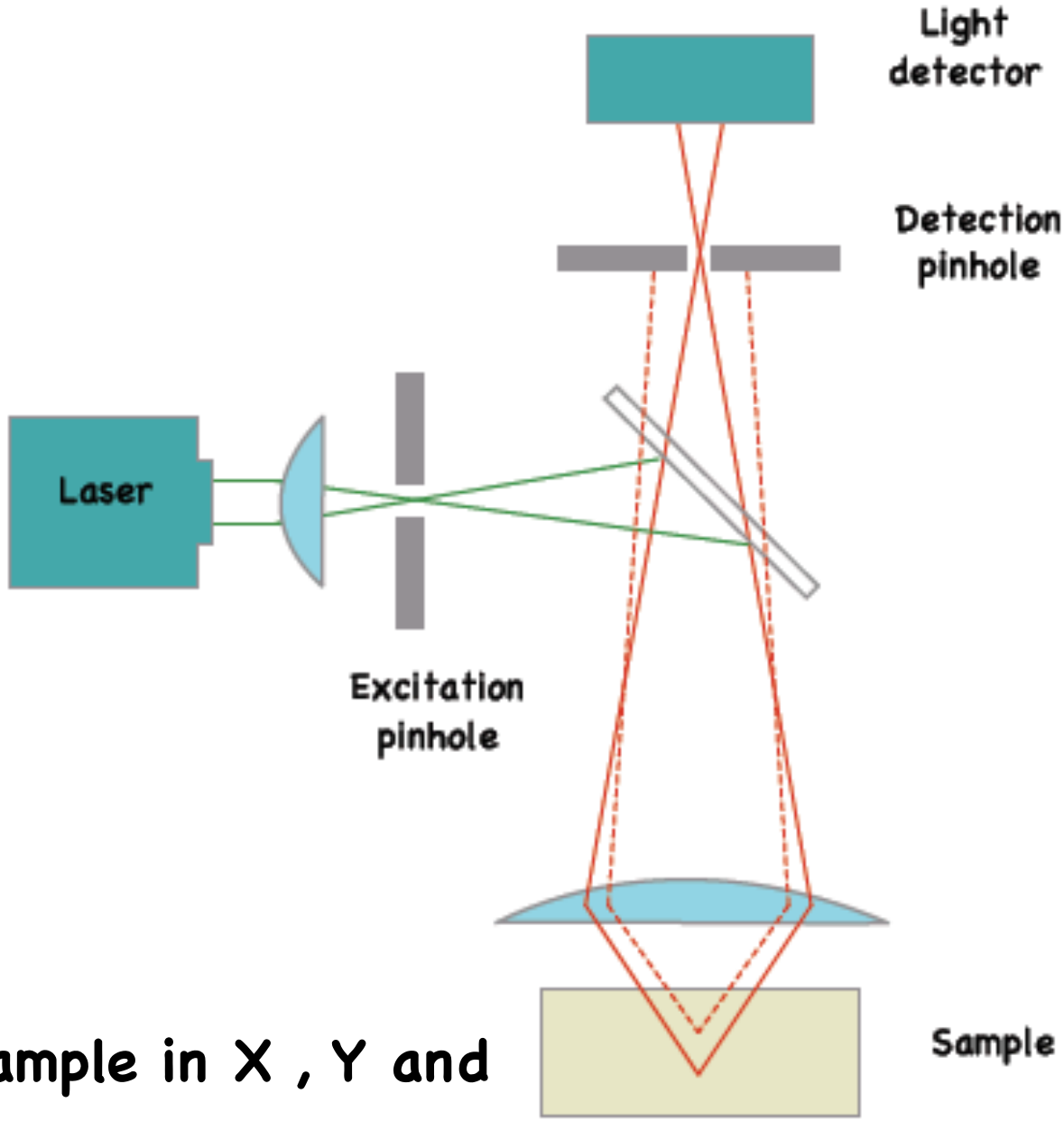


How well can this be done?

Confocal Principle

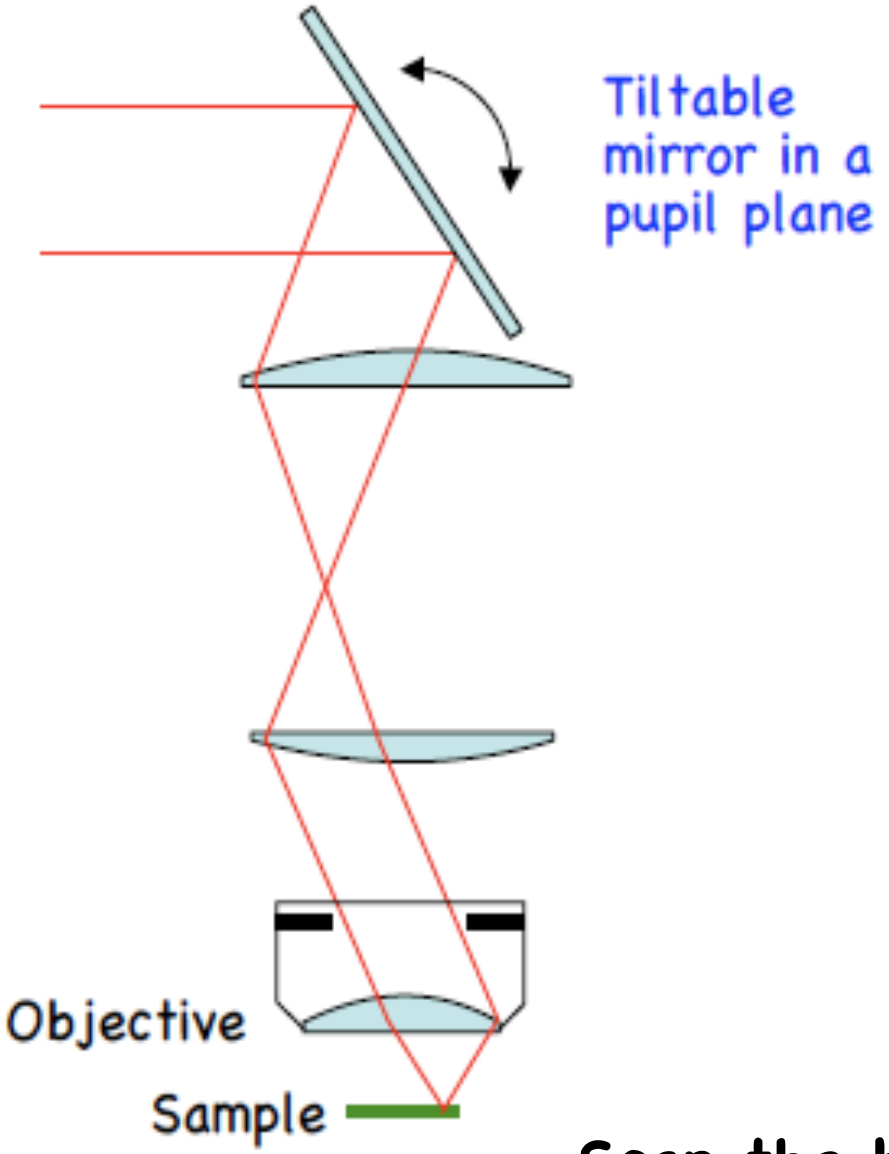


Confocal Microscopy



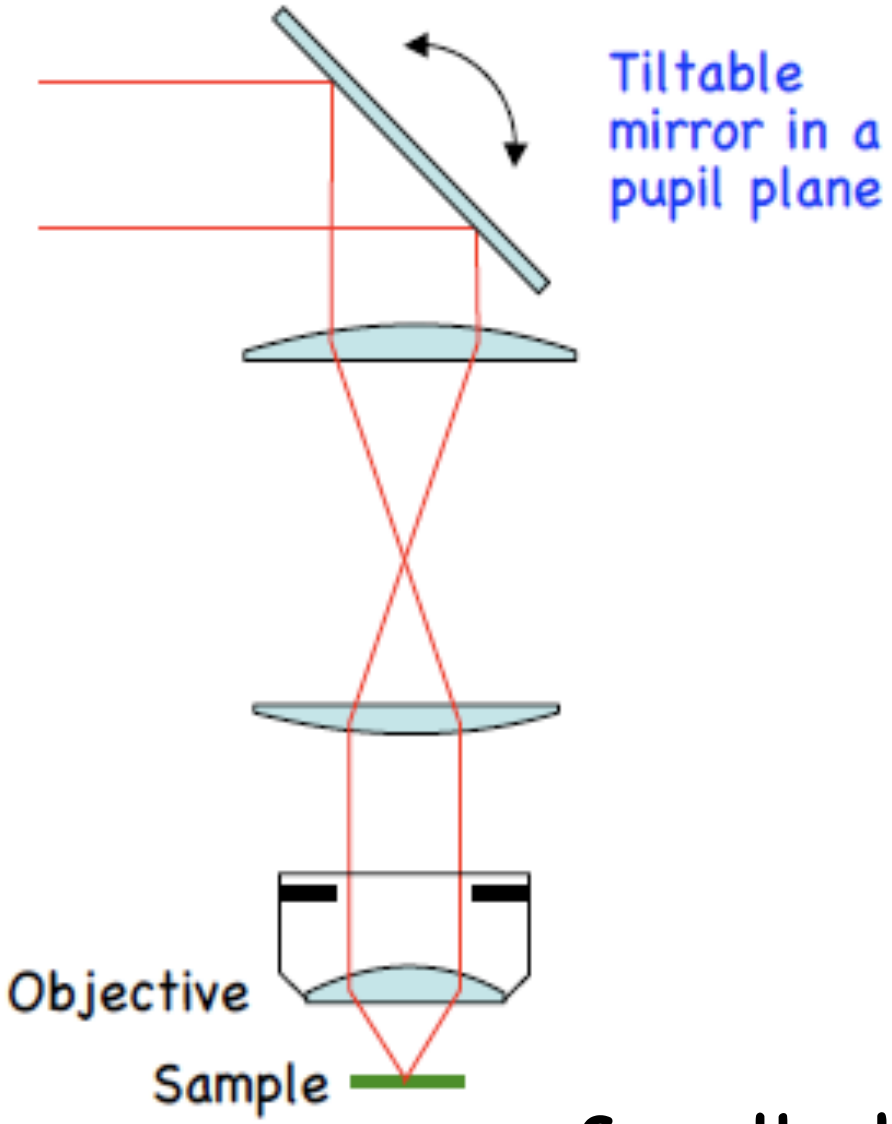
Scan the sample in X , Y and Z

Laser scanning



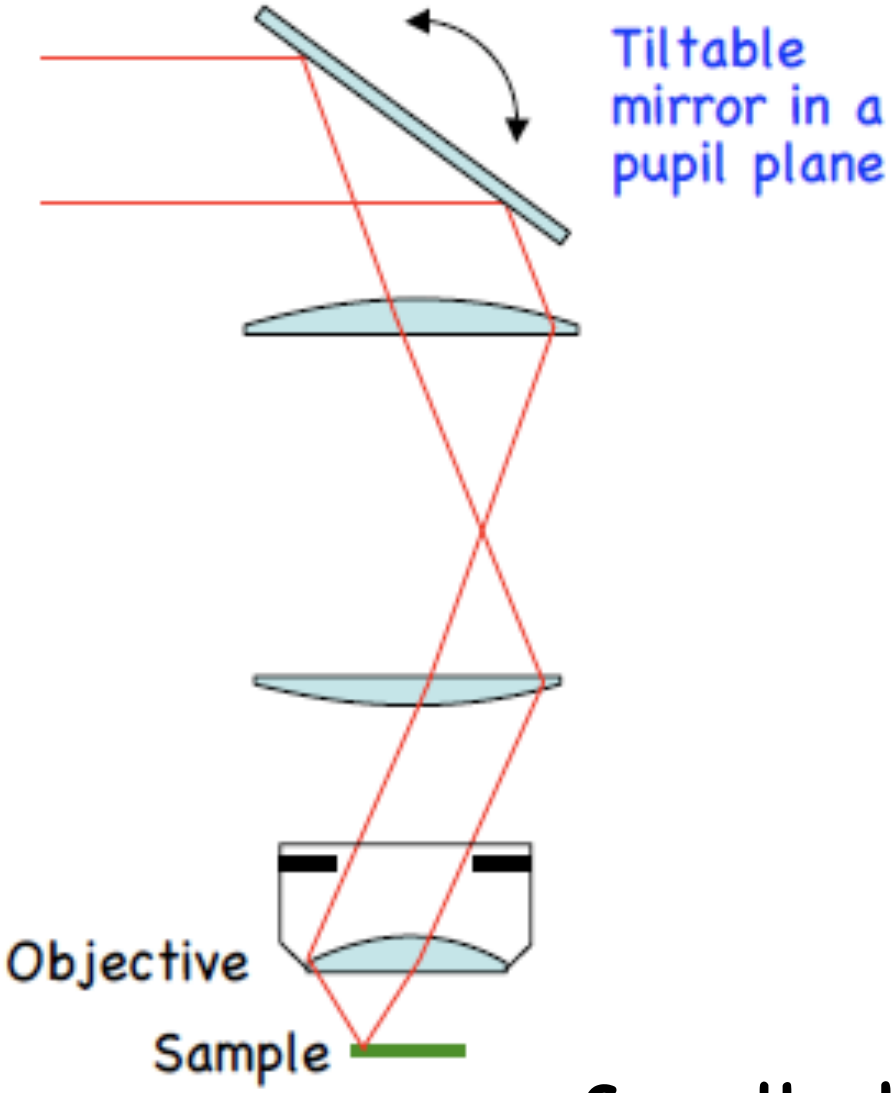
Scan the beam X , Y &

Laser scanning



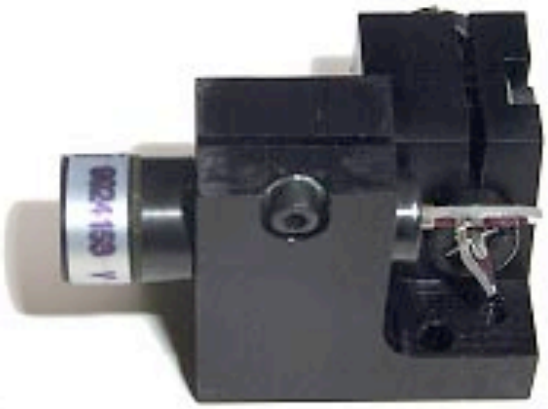
Scan the beam X , Y &

Laser scanning



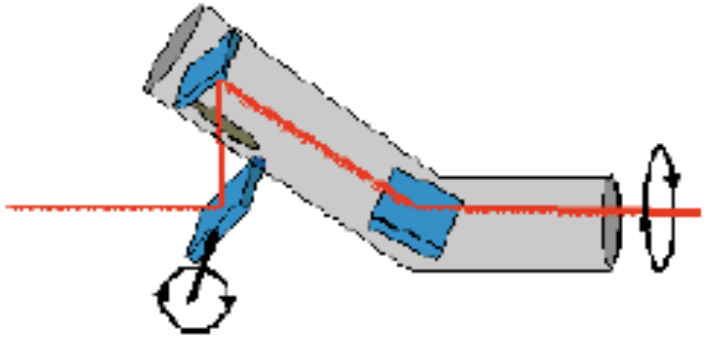
Scan the beam X , Y &

Galvanometer scanners



Problem: want *both* x and y mirror in the same pupil plane

Just put them close...

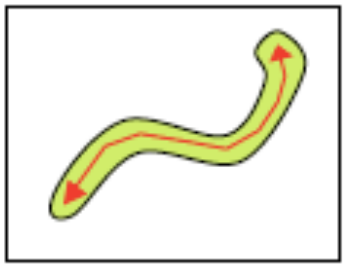


...or engineer something

(Leica "K" scanner)

Resonant scanners: very fast (video rate) but inflexible

Closed-loop scanners: slower but fully controllable →



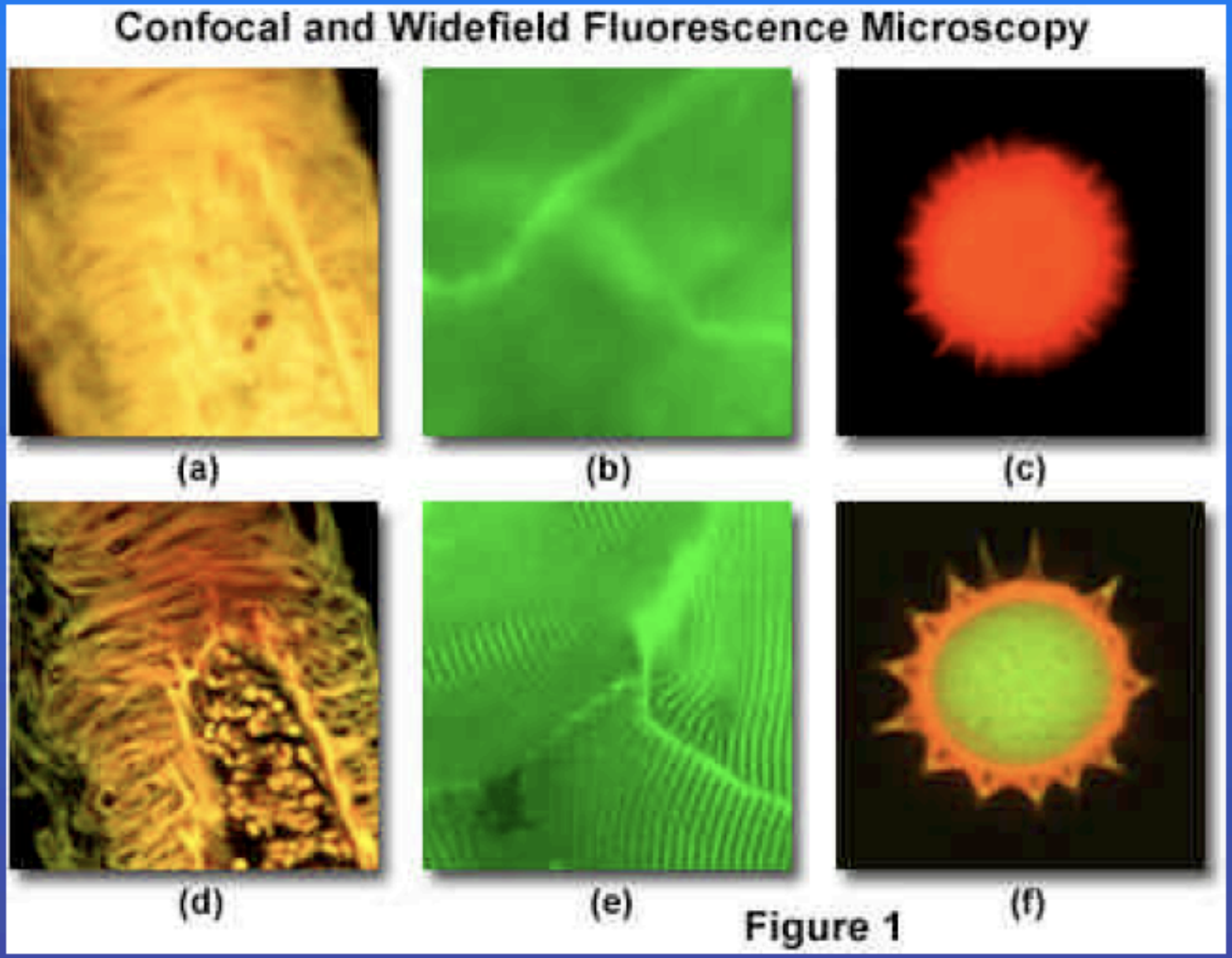
Arbitrary path

Some microscopes have both types (Leica SP5)

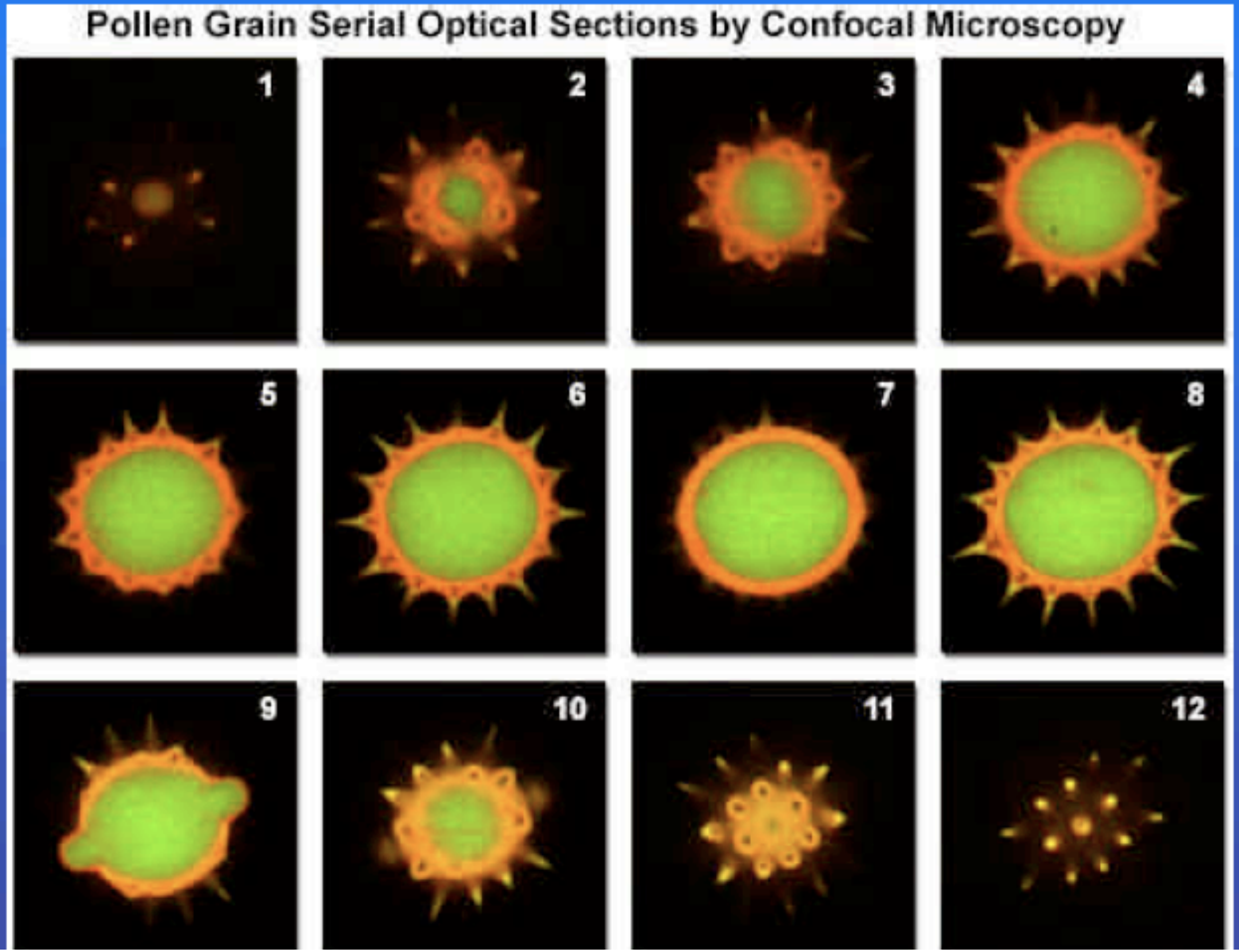
Removal of out-of-focus light

Widefield

Confocal



Optical sectioning



Scanning basics

- *Optical layout of wide field epifluorecence microscope*
- *Unlike wide field microscope, illumination is from a laser, and focused to a diffraction limited spot*
- *Fluorescence from specimen is directed to a photomultiplier*
- *Out of focus light is rejected by the confocal pinhole*
- *Spot is scanned over specimen (raster scanning)*
- *Image is composed in computer (image is not visible by eye!)*

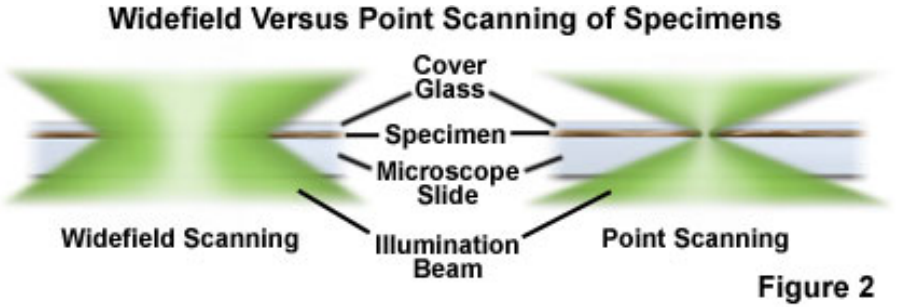
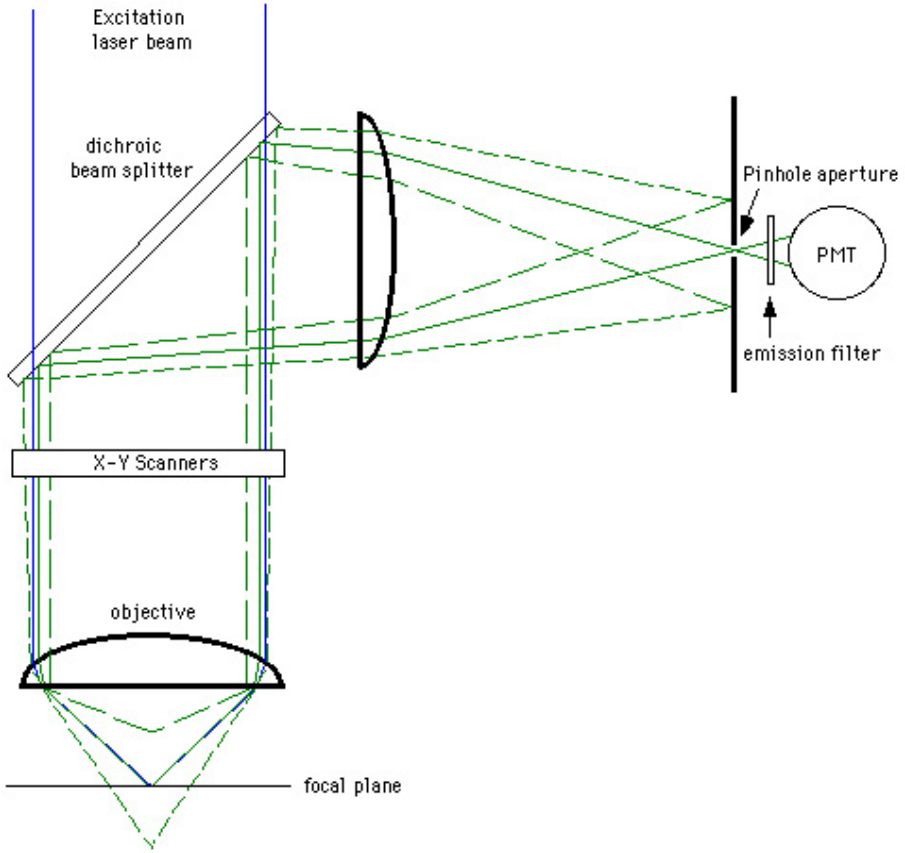


Figure 2



Scanning basics

- *In laser scanning confocal microscopes spot is scanned over specimen*
- *Scanning is typically realized by a pair of mirrors that rotate back and forth at high rates (up to 1000 cycles/sec)*
- *One mirror (the fast mirror) scans the beam in one direction (usually in X)*
- *The other (the slow mirror) scans the beam in the other direction (usually in Y)*
- *Mirrors are connected to scanner galvanometers – precision positioners driven by special electronics*
- *As good as these are, there are limits to scanning rates, resulting in relatively slow frame rates (at most a few frames per second)*
- *By positioning the mirror in a conjugate plate of the objectives back aperture, angle translates precisely to position in the image plane*

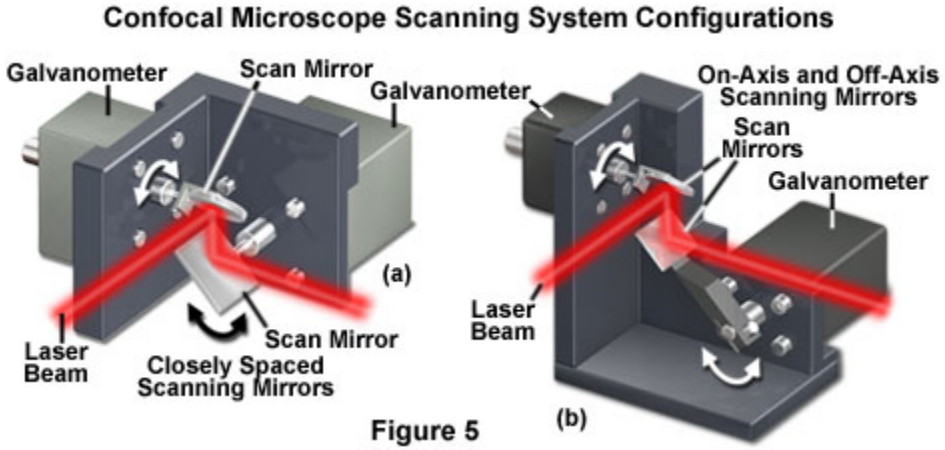
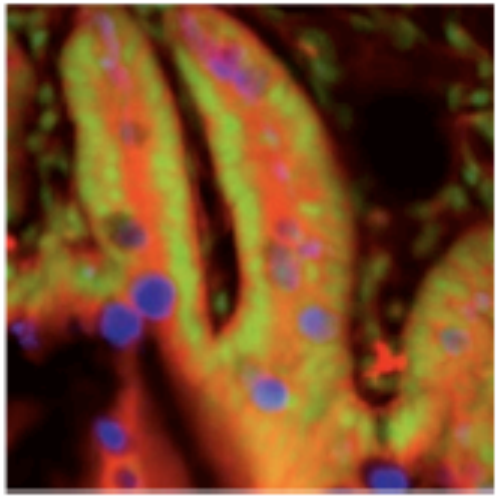


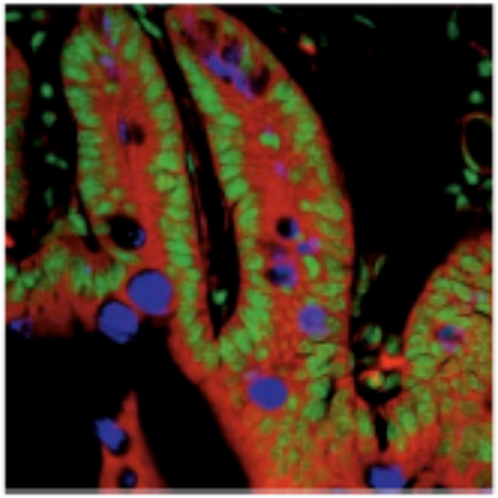
Figure 5

Point-scanning Confocal Microscopy

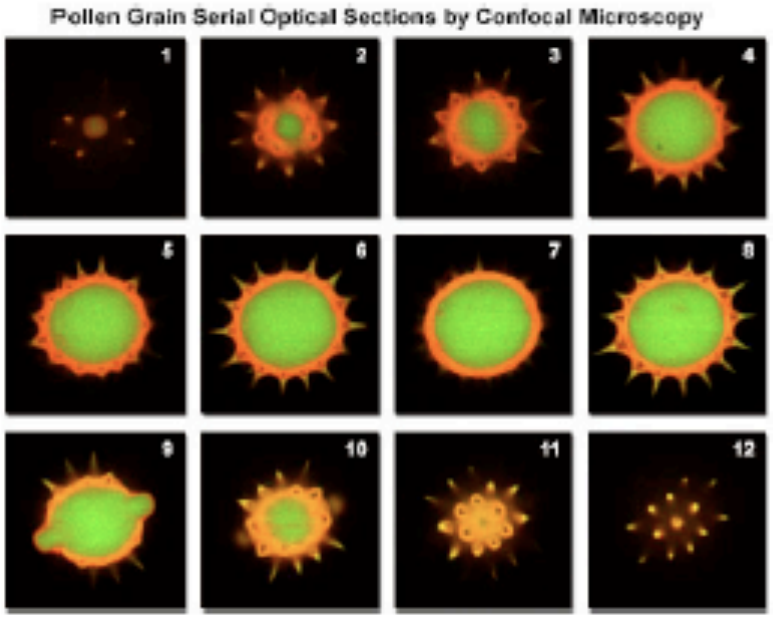
Widefield

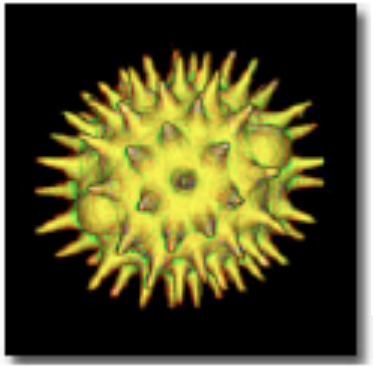


Confocal



mouse intestine section





Confocal strengths:

Optical sectioning no out-of-focus
Theory: gain sqrt(2)in resolution
Practice: higher contrast ->
better resolved features

Weaknesses:

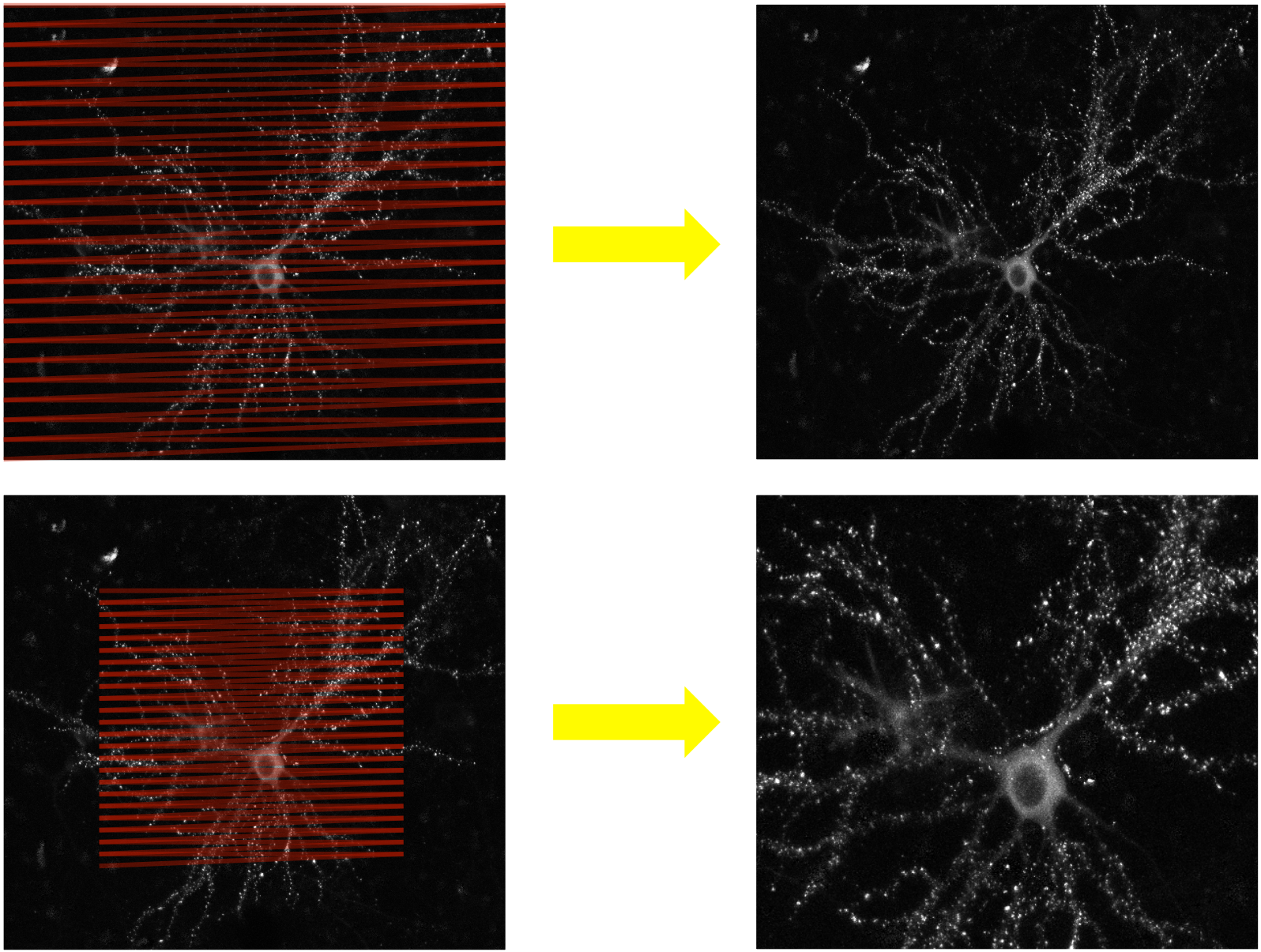
Slower
Lower sensitivity (PMT <5% QE)
->Higher photodamage
->Lower signal
->Saturation of dyes
->Saturation of Detector
Hard to quantify intensities
Heavy and expensive instrument
Multiple Lasers for colors

Additional advantages

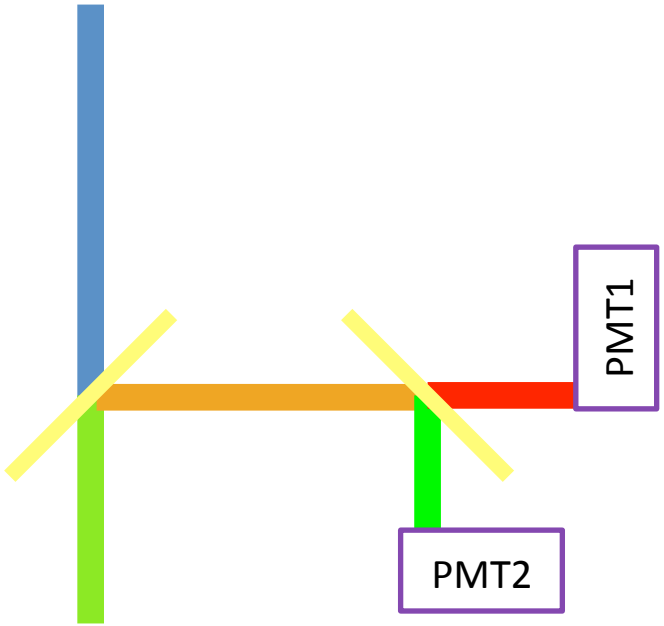
Although the major advantage of laser scanning confocal microscopy relates to image quality, other advantages are significant

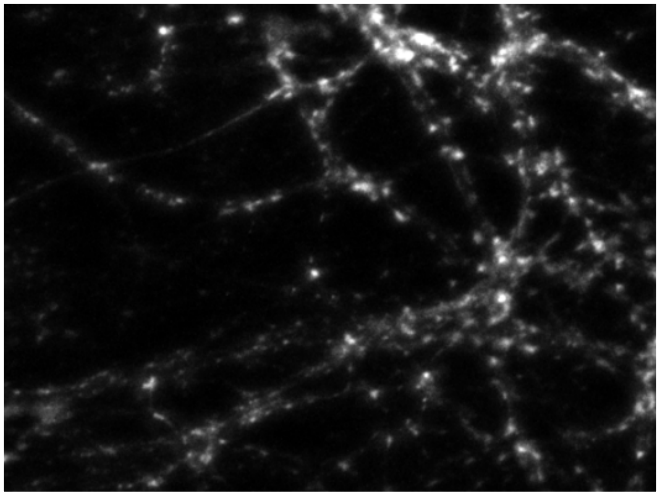
- *Electronic zoom, pan and rotation – changing the scanning parameters can be used to rapidly change magnification, region of interest and image orientation*
- *Multiple channels – by splitting the emission between several PMTs, images of multiple fluorophores can be acquired simultaneously with all images in perfect registration*
- *Spectral unmixing -multiple channel imaging can be used to separate fluorphores with overlapping emission spectra*
- *Transmitted light imaging – by adding a detector in the transmitted light path, brightfield, DIC/Nomarski images can be collected simultaneously*
- *Region of Interest scanning –by using AOTFs, scanning can be limited to specific arbitrary regions (FRAP, photoactivation)*
- *Line scanning – by scanning the same line, data from small regions can be collected at very high rates (500-1000 samples /sec)*
- *3D reconstruction is simple and allows real-time display*

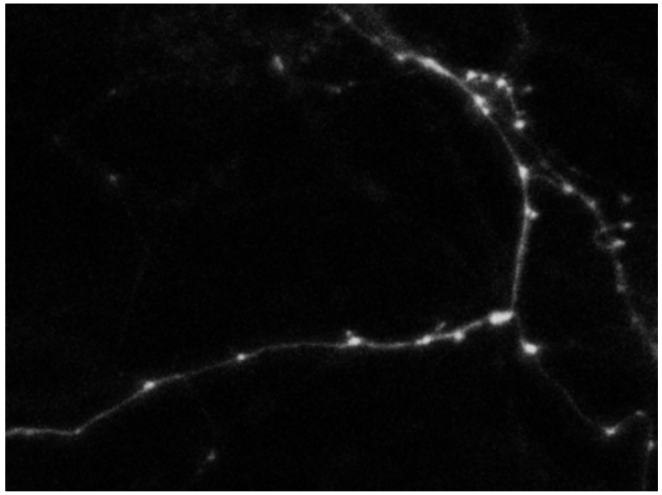
“Electronic” zoom

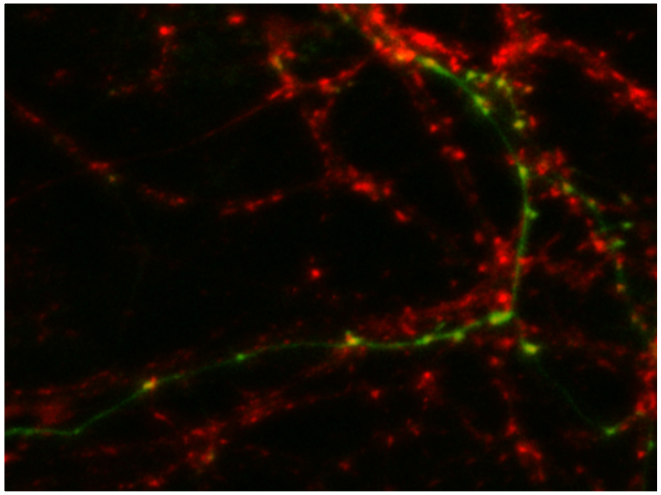


Multiple Channel imaging

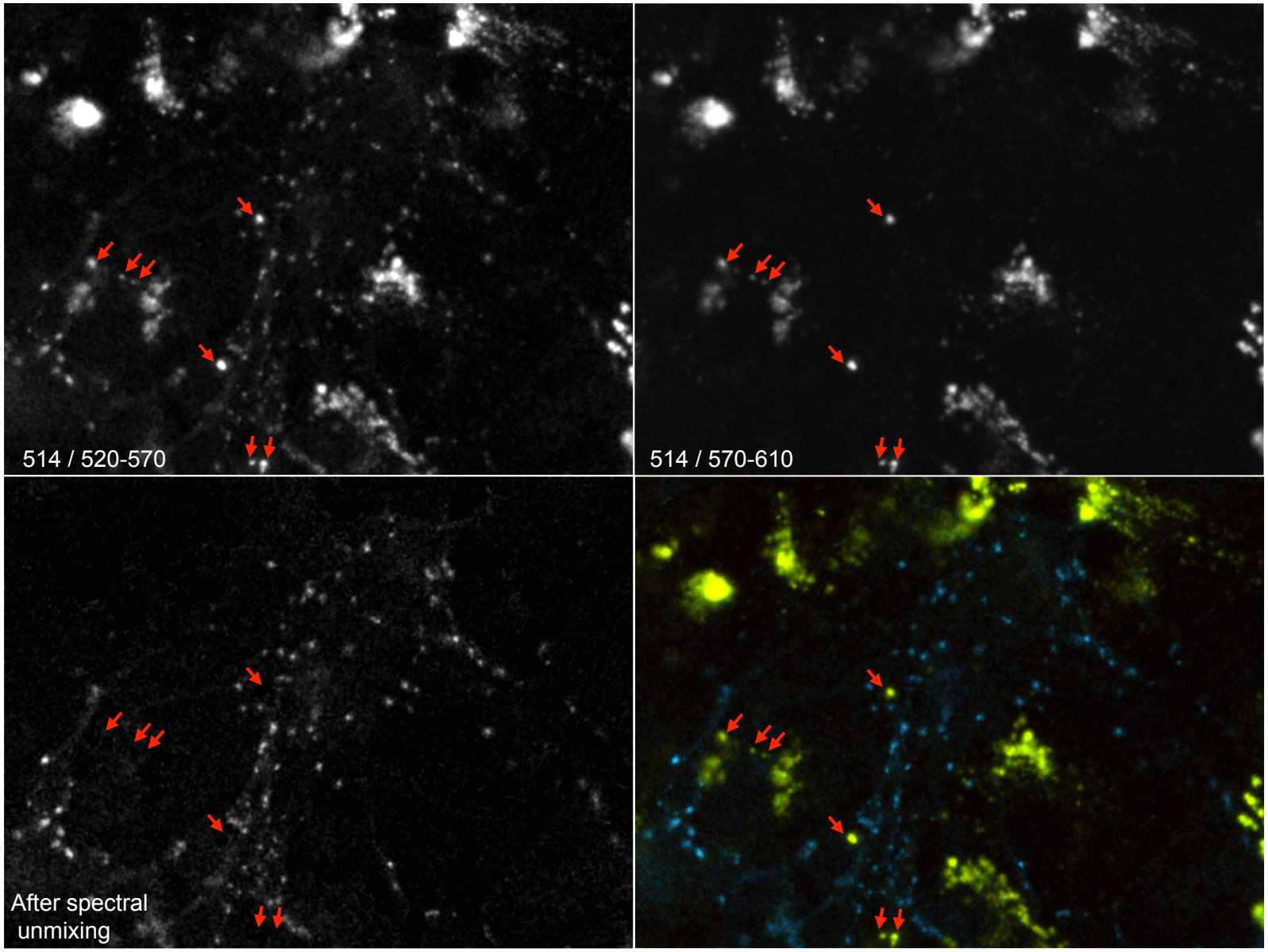




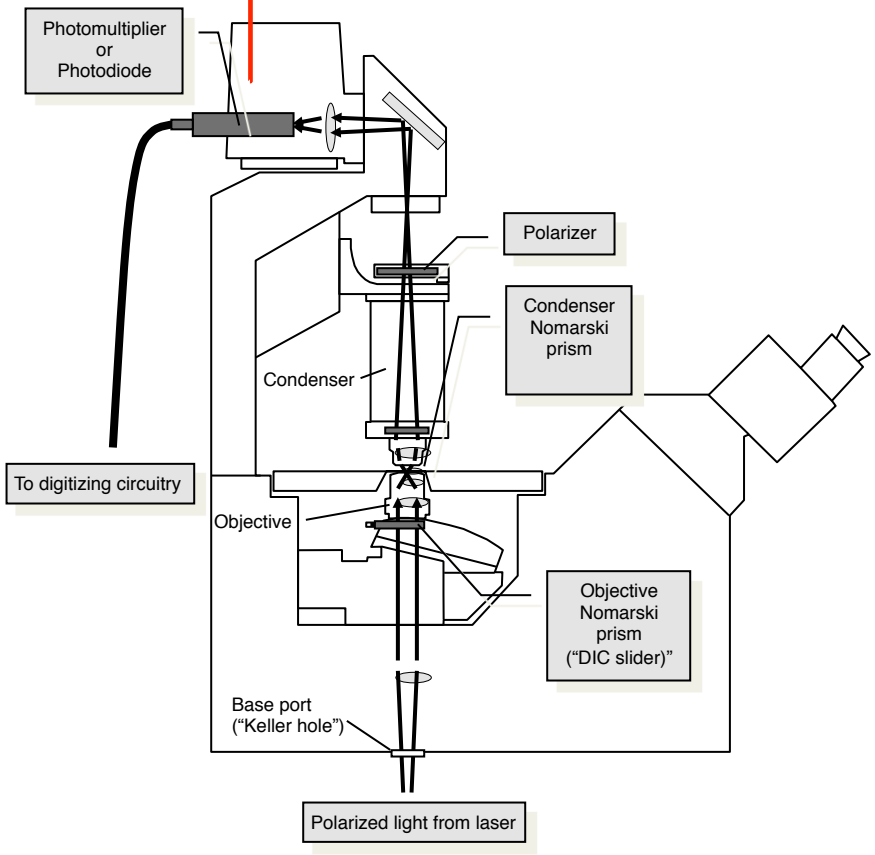


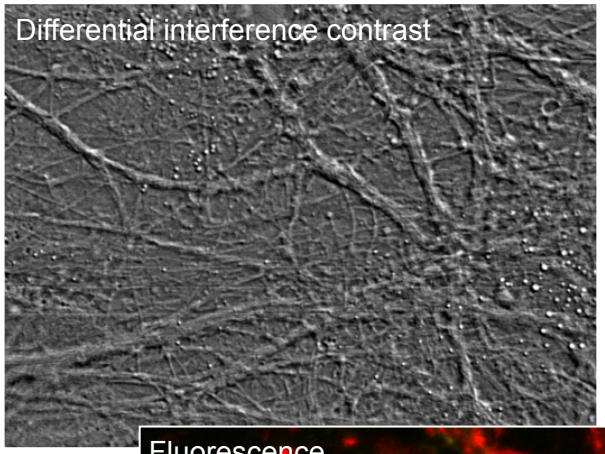


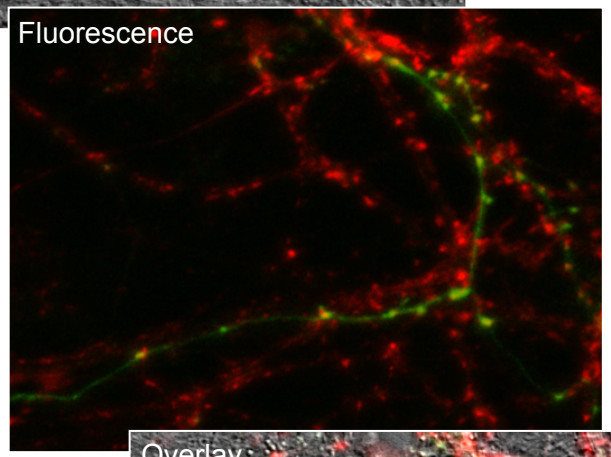
Spectral unmixing

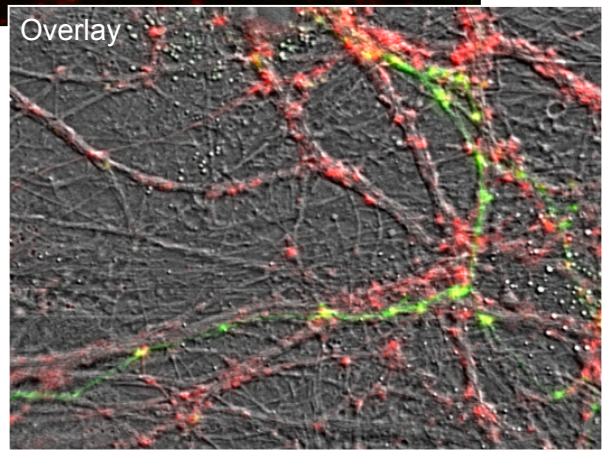


Combining fluorescence and transmitted light images

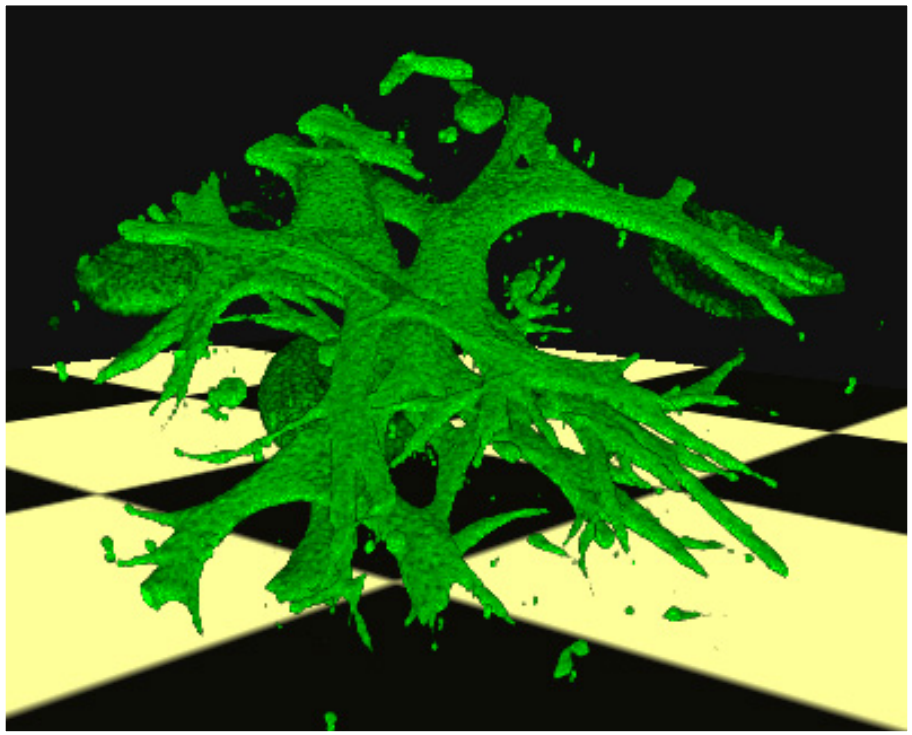


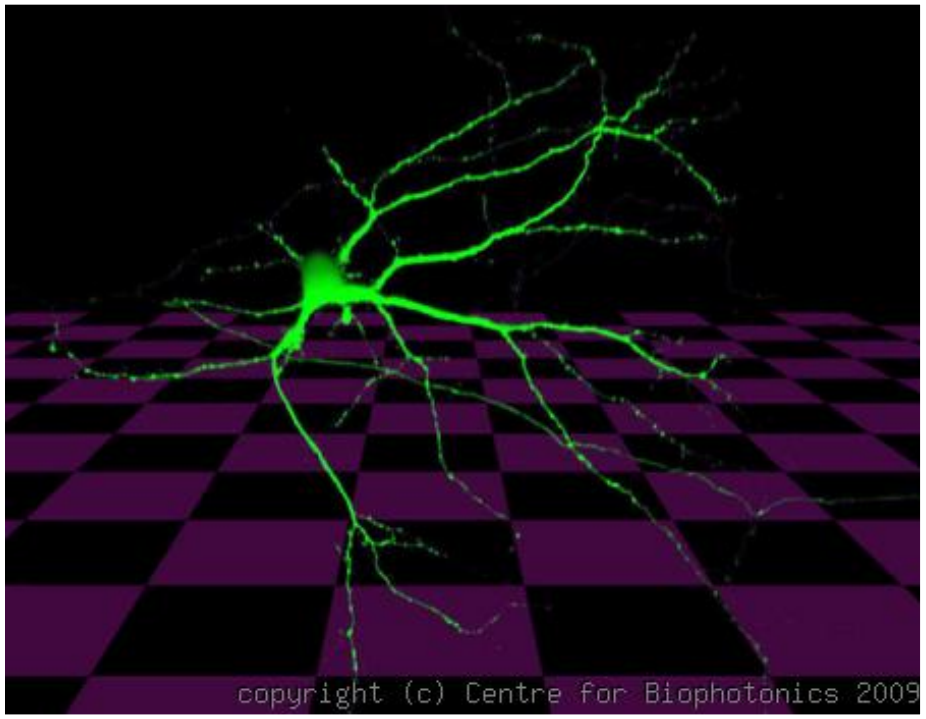




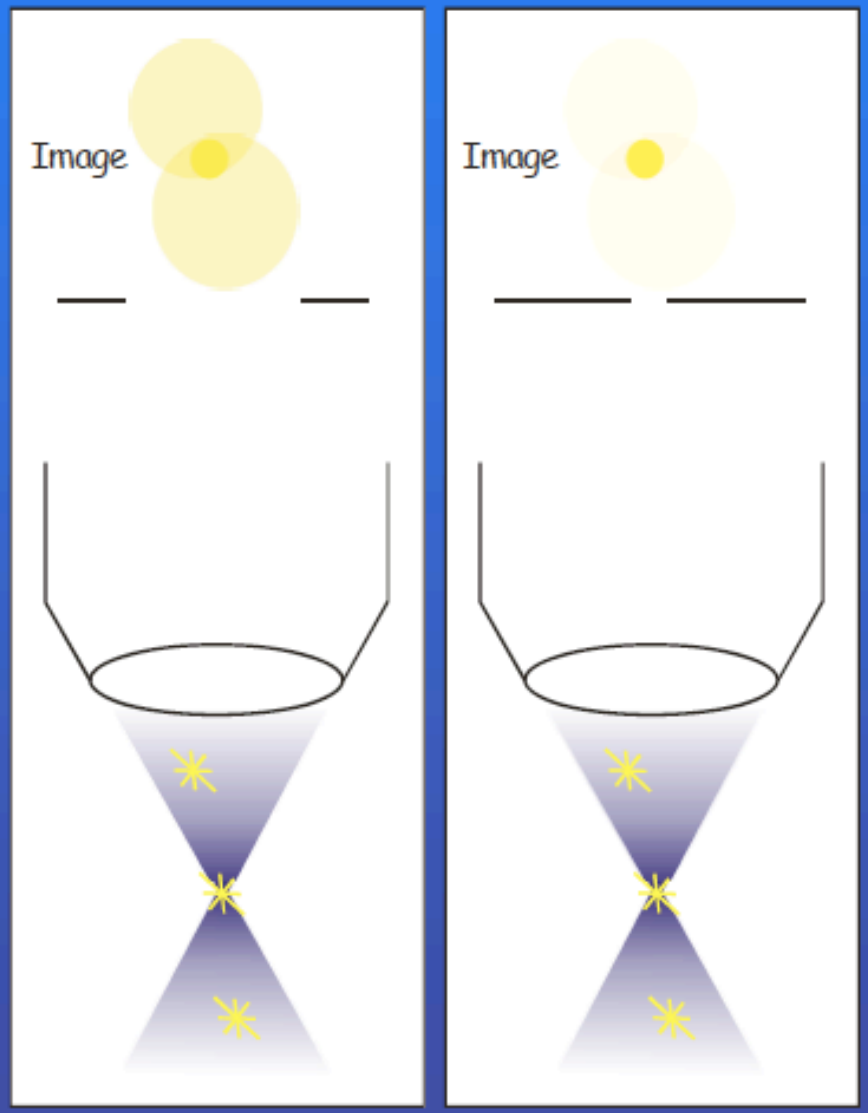


3D reconstruction and visualization





The excitation hour-glass

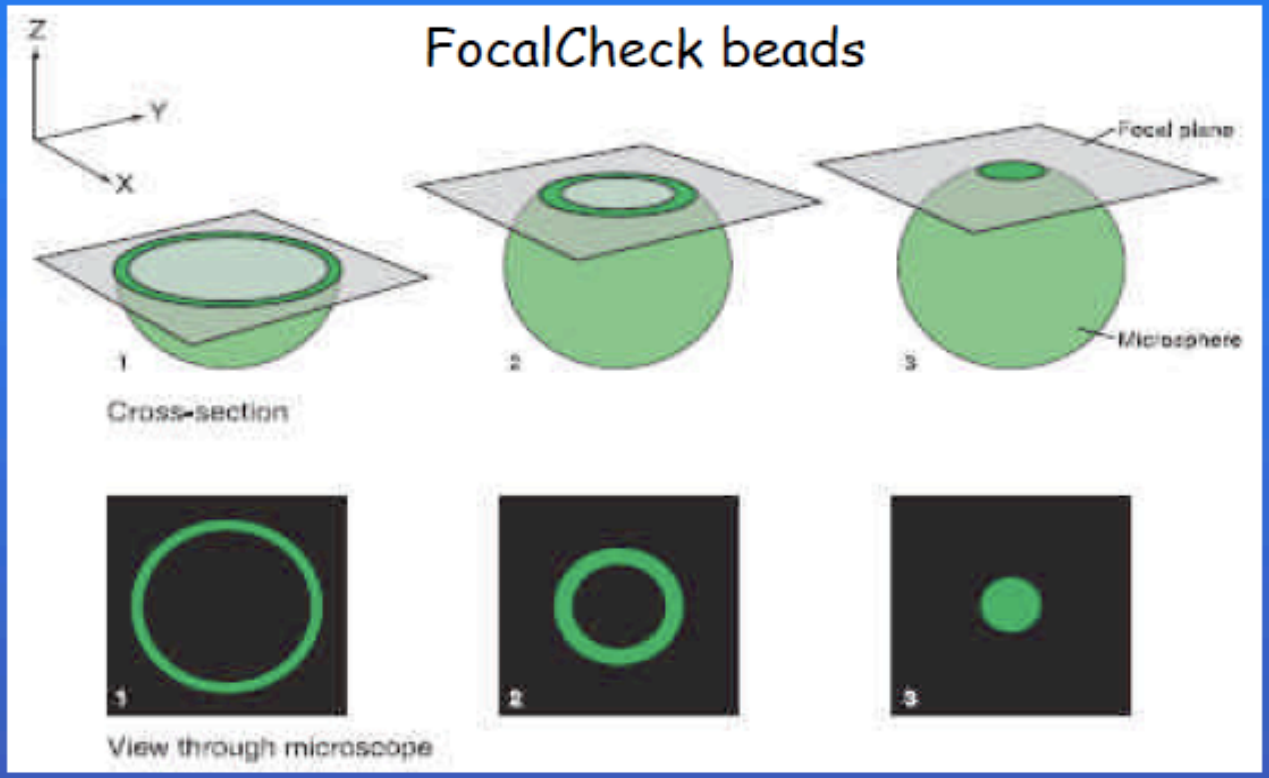


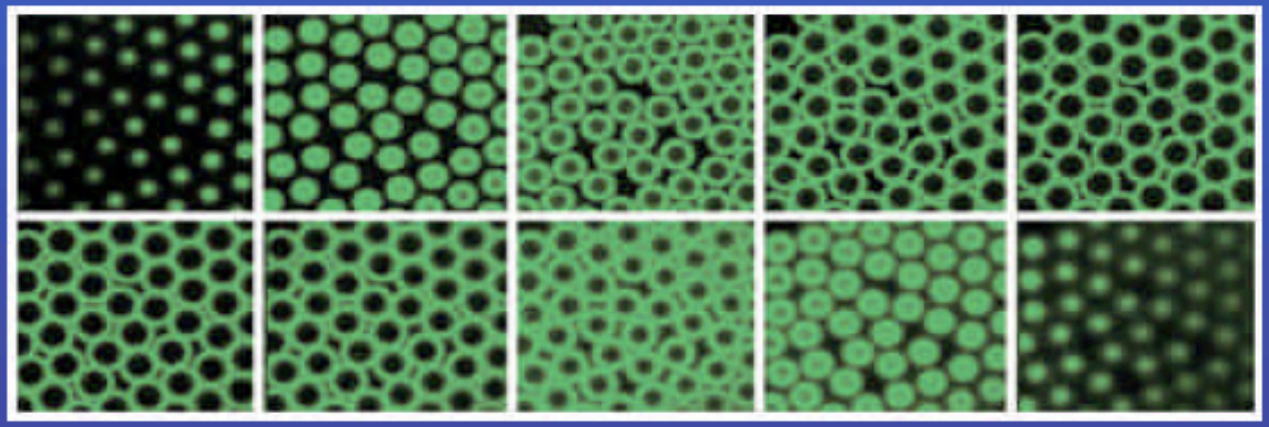
Light from out-of-focus objects is rejected

However,

Out of focus objects are excited!

Focal and scanning mechanism

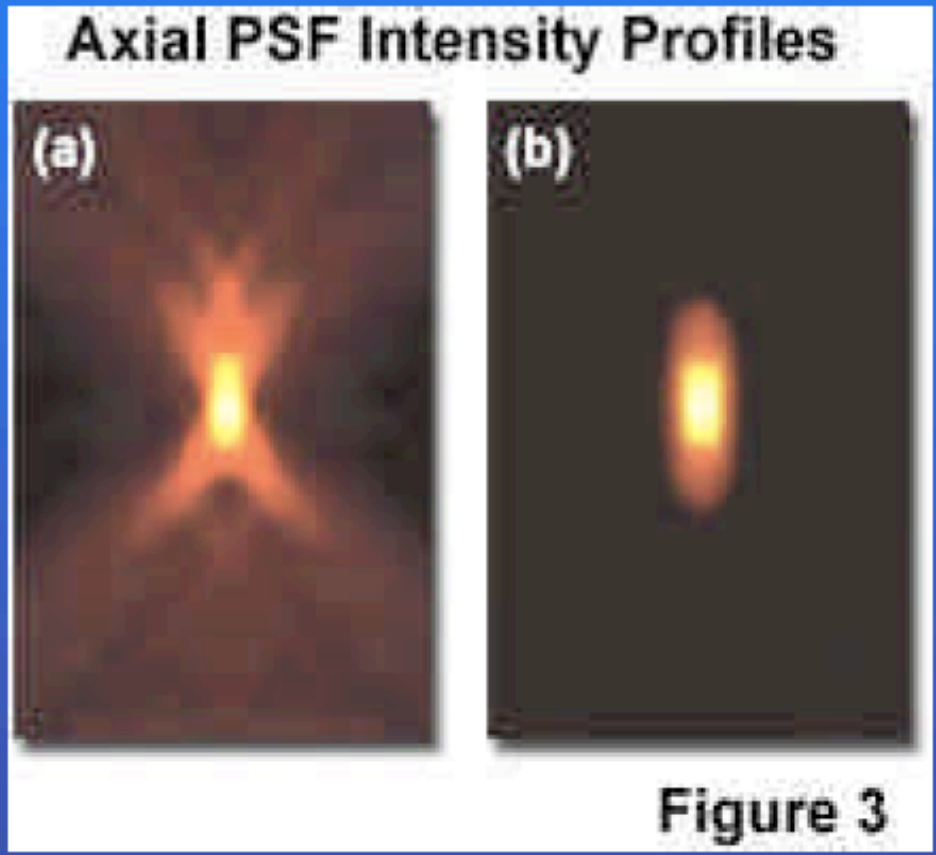




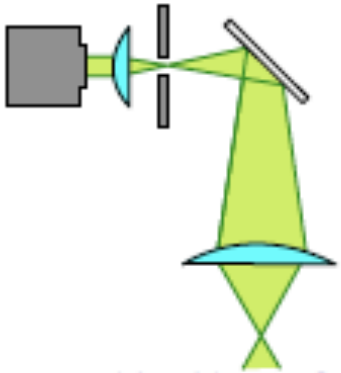
Effect of the pinhole on the point spread function

The PSF is the image of an infinitely small light source (in practice – an object smaller than the resolution of the optical system).

The confocal PSF is shorter in the Z direction, reflecting the removal of out-of-focus light.



PSF of Confocal Microscopy

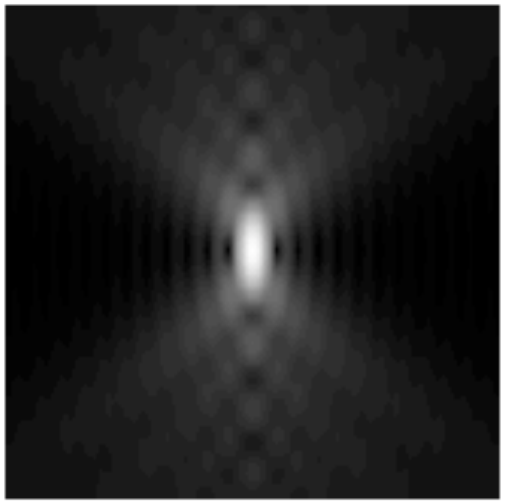


$$PSF_{confocal}(r) = Illumination(r) \times (Detector\ sensitivity)(r) = PSF_{exc}(r) \times PSF_{det}(r)$$

Excitation PSF

Detection PSF

Confocal PSF



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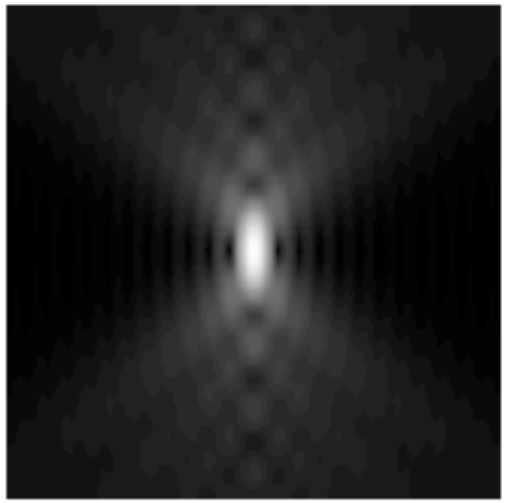

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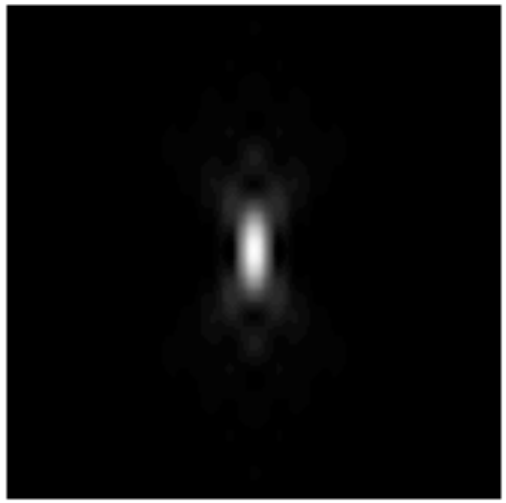
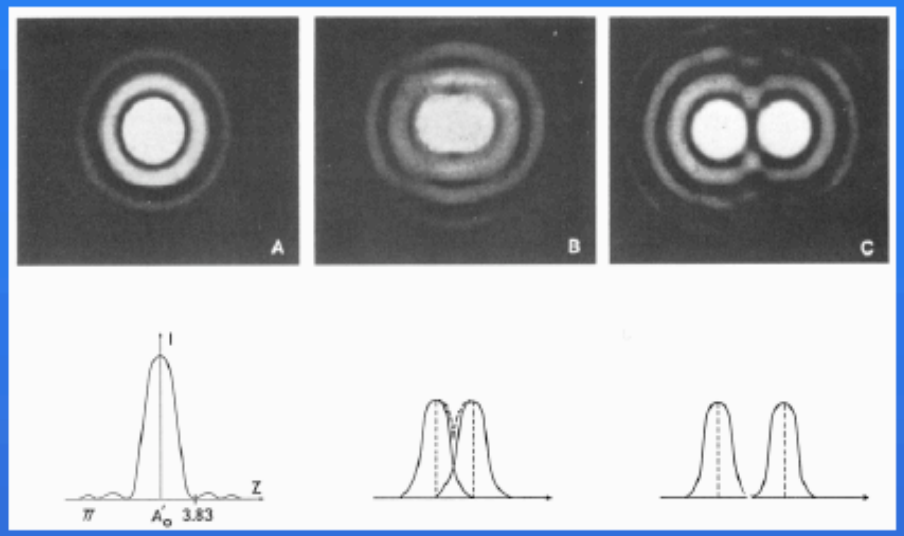


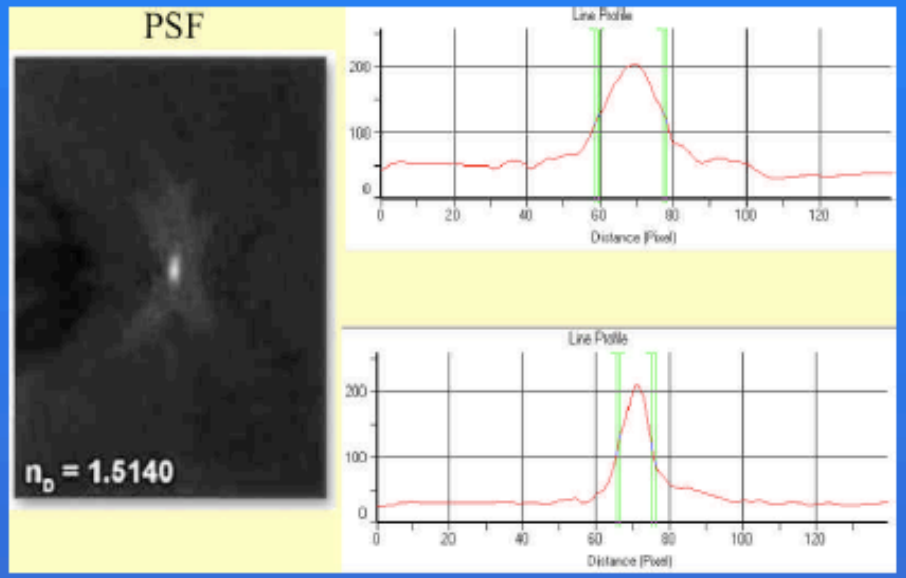
$$PSF_{confocal}(r) \approx (PSF_{widefield}(r))^2$$

Resolution issues

- Resolution is determined mostly by the objective's Numerical Aperture (NA).
  - Magnification is irrelevant when zoom is available, **No advantage to 100x over 60x lens of same NA.**
- The pinhole increases contrast by removing out-of-focus light.
- Scanning with a point increases resolution relative to conventional microscopy because both excitation and emission are diffraction-limited.



2D Airy Disk diffraction pattern



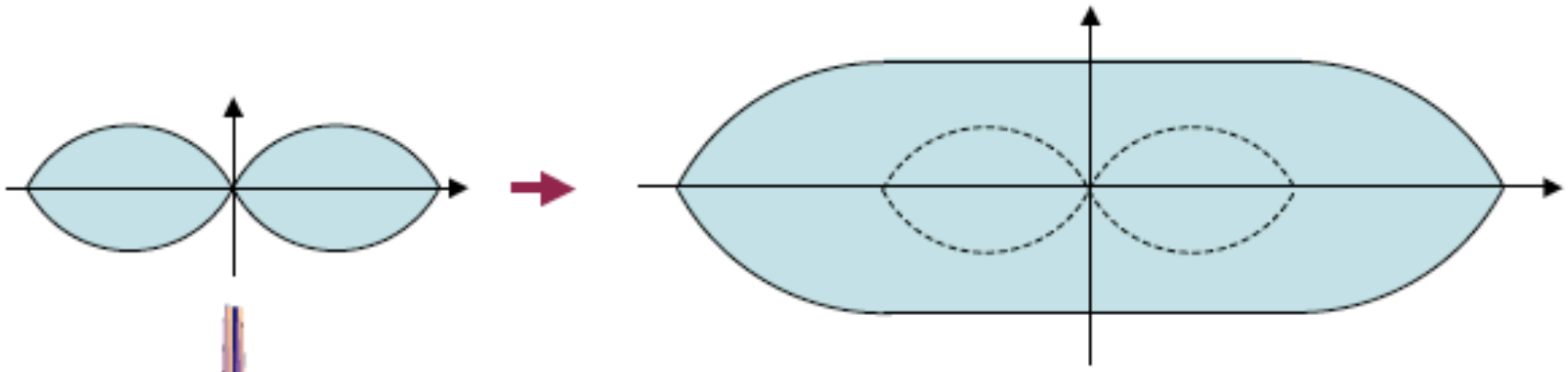
- Lateral resolution Airy Disk diameter: 0.61λ/NA (Rayleigh criterion). In a confocal: 0.4λ/NA.
  - Lateral typical limit: 250nm
- Axial resolution; Section thickness $2\eta\lambda/(NA)^2$
  - Axial typical limit: 500- 1000nm
- Deconvolution can increase resolution

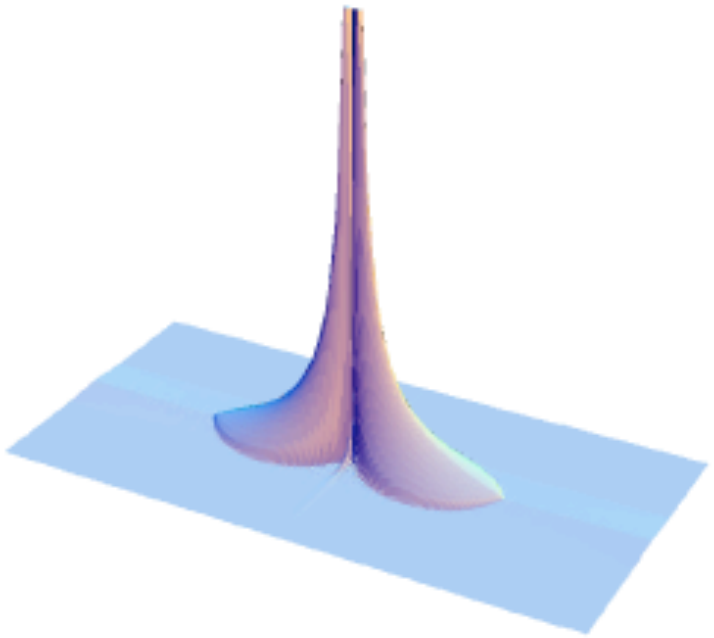
OTF of Confocal Microscopy

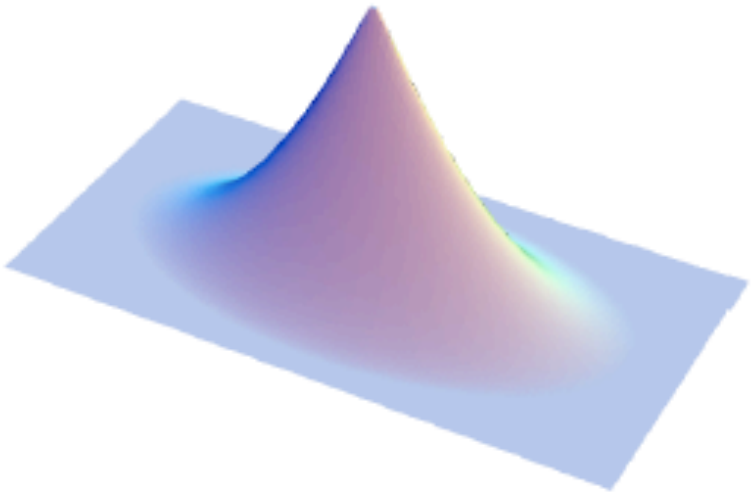
$$PSF_{confocal}(r) = PSF_{exc}(r) \times PSF_{det}(r)$$

$$\Rightarrow$$

$$OTF_{confocal}(k) = OTF_{exc}(k) \otimes OTF_{det}(k)$$





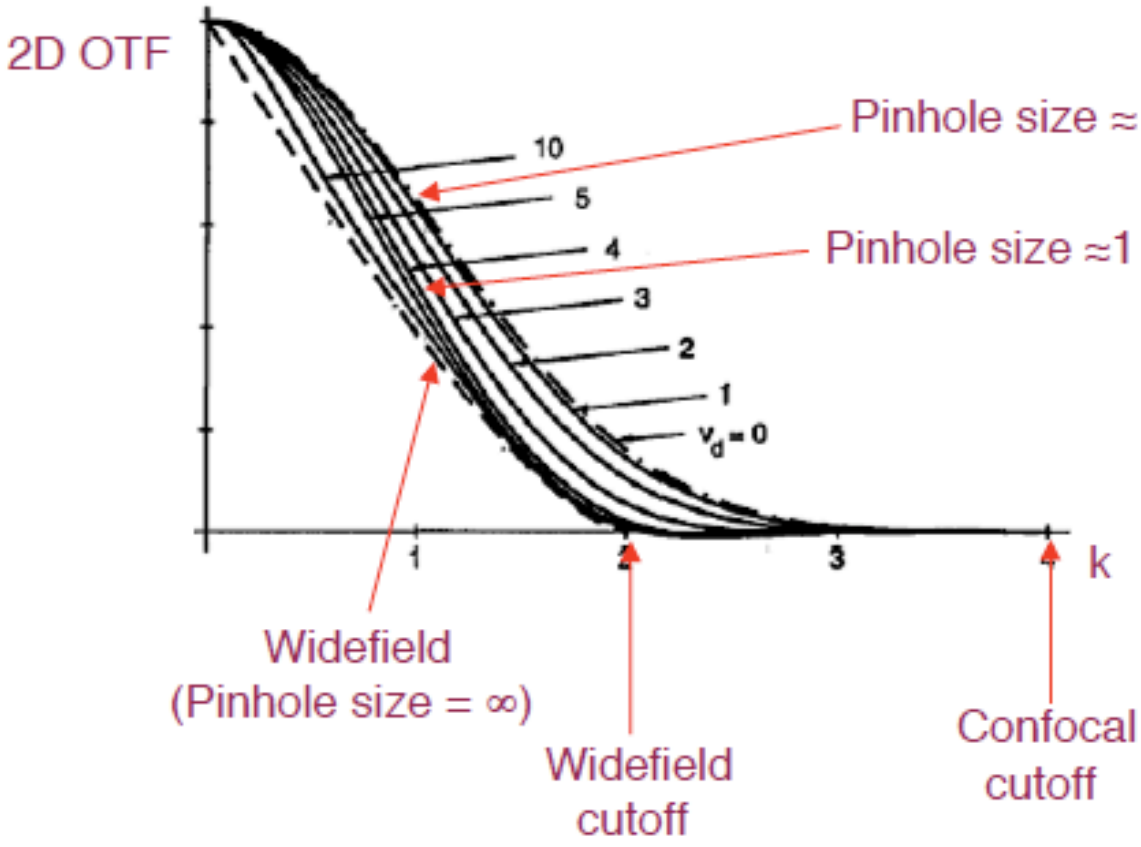


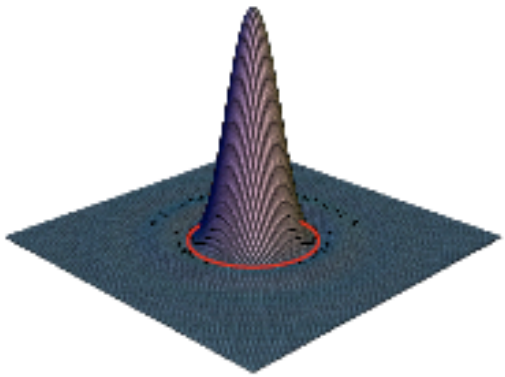
Confocal 2D OTF vs Pinhole size

Lateral FWHM
Point object

Axial FWHM
Thin layer

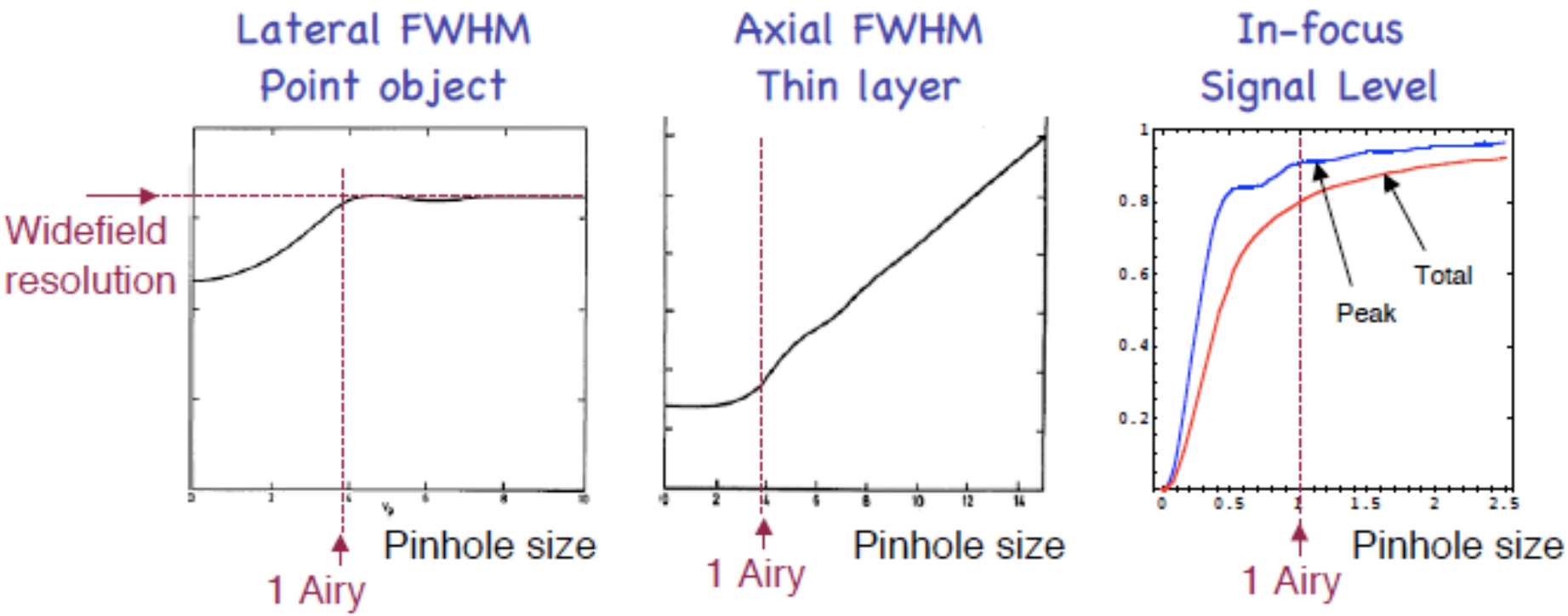
Signal Level
Point source





The Airy disk

Pinhole size, Resolution and Signal



Need a pinhole size ≈ 1 Airy disk diameter to get full signal
Then you get ≈ *no* lateral resolution improvement
But you *do* get near-ideal axial sectioning

The saturation speed limit

Acquires *one pixel at a time*

Frame time = pixel dwell time × pixel number

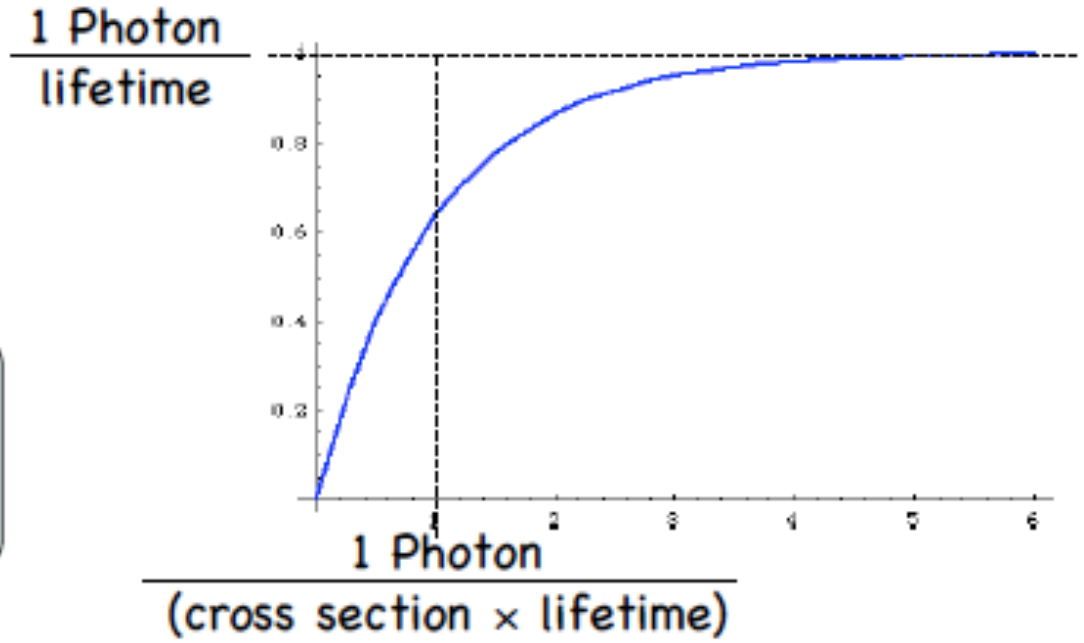
To decrease dwell time, must *increase emission rate*
to maintain signal level

⇒ Must *increase illumination intensity*

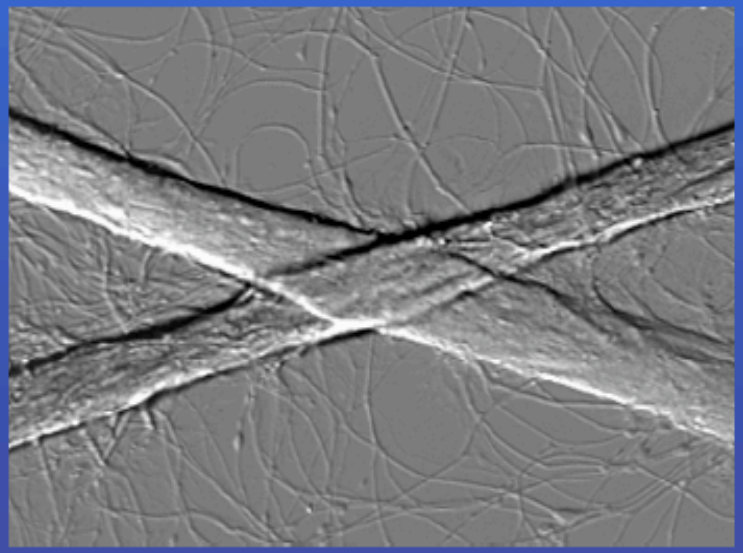
Where's the limit?

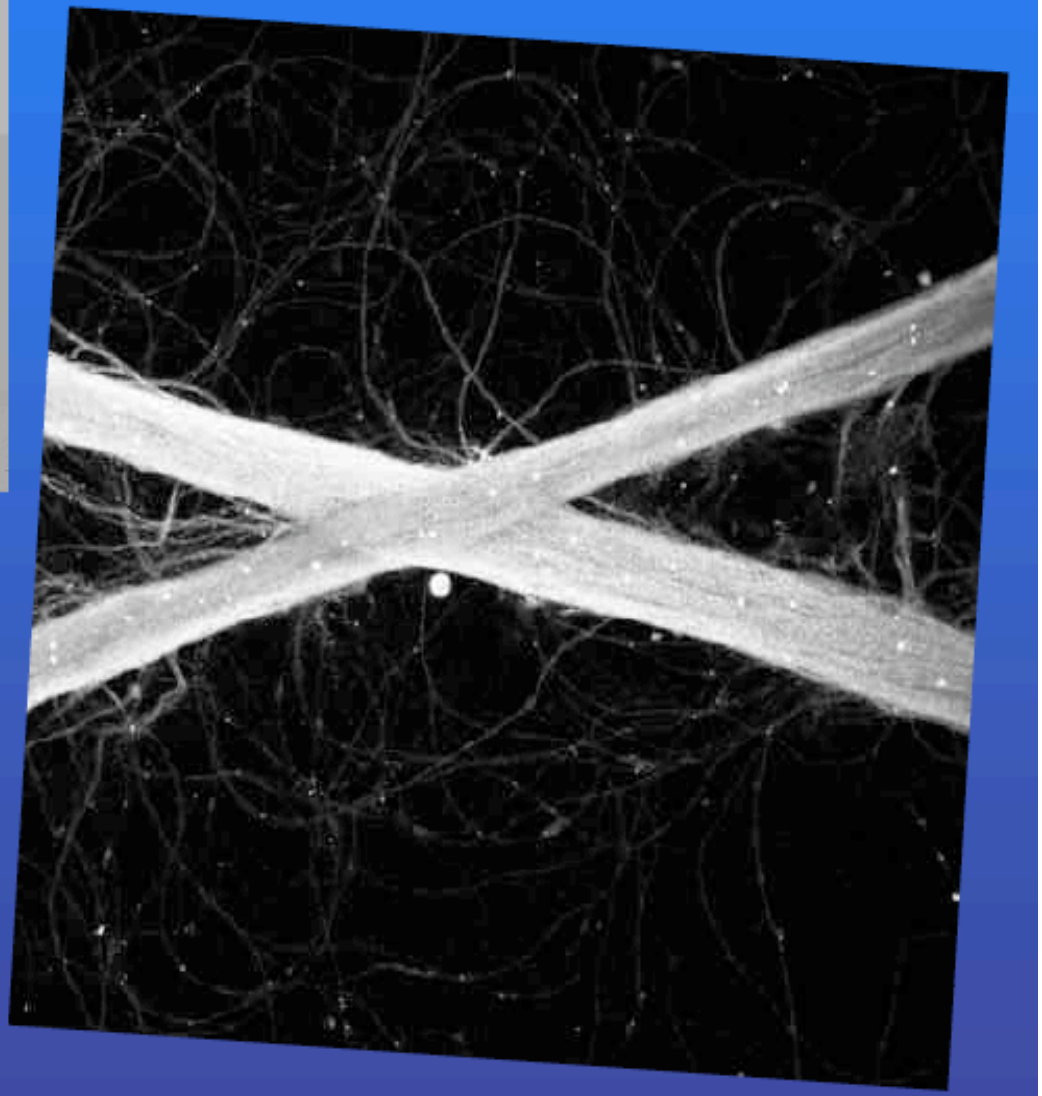
- Phototoxicity?
- Saturation

Point scanning microscopes have a maximum speed imposed by saturation



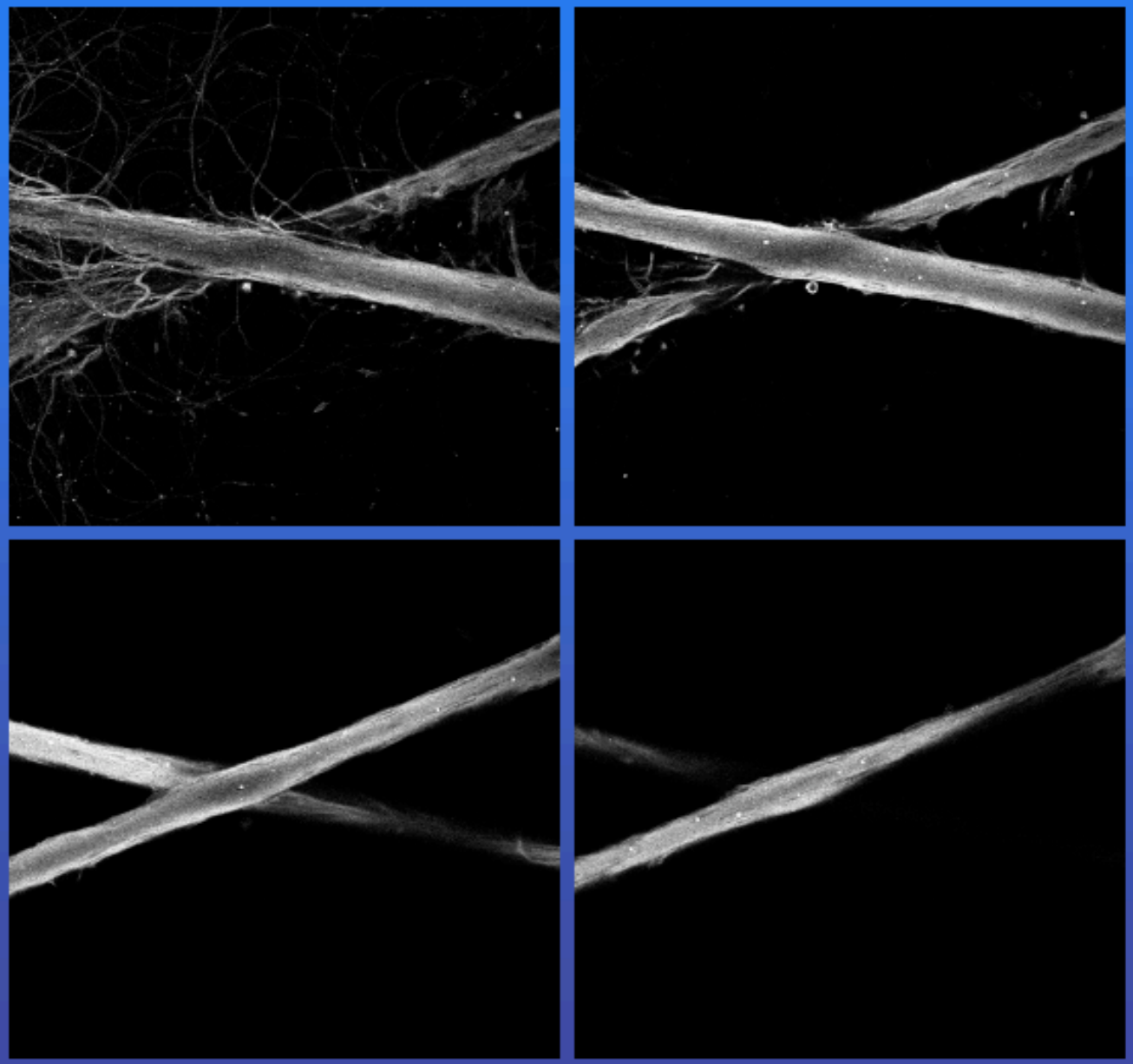
Example of optical sectioning



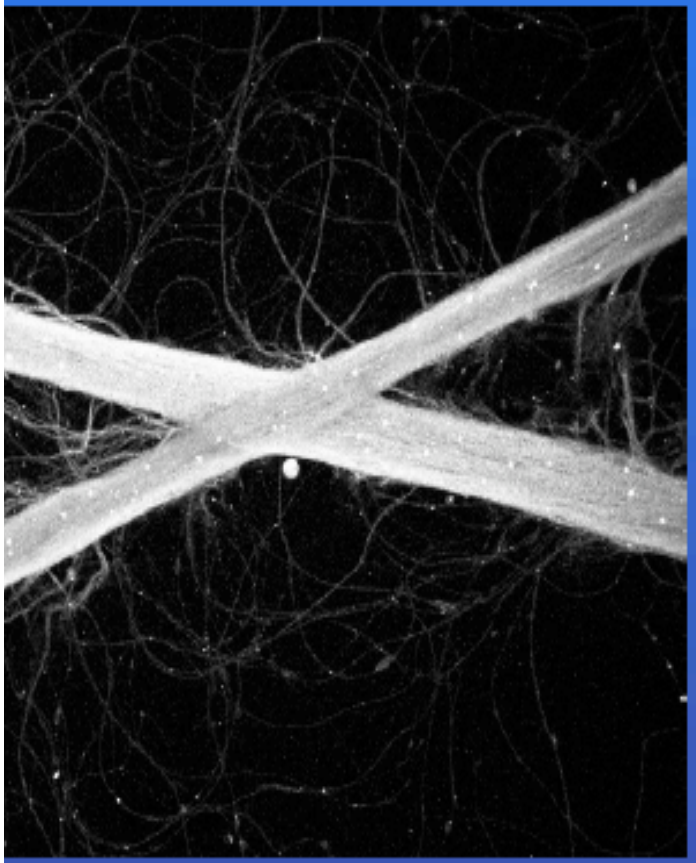


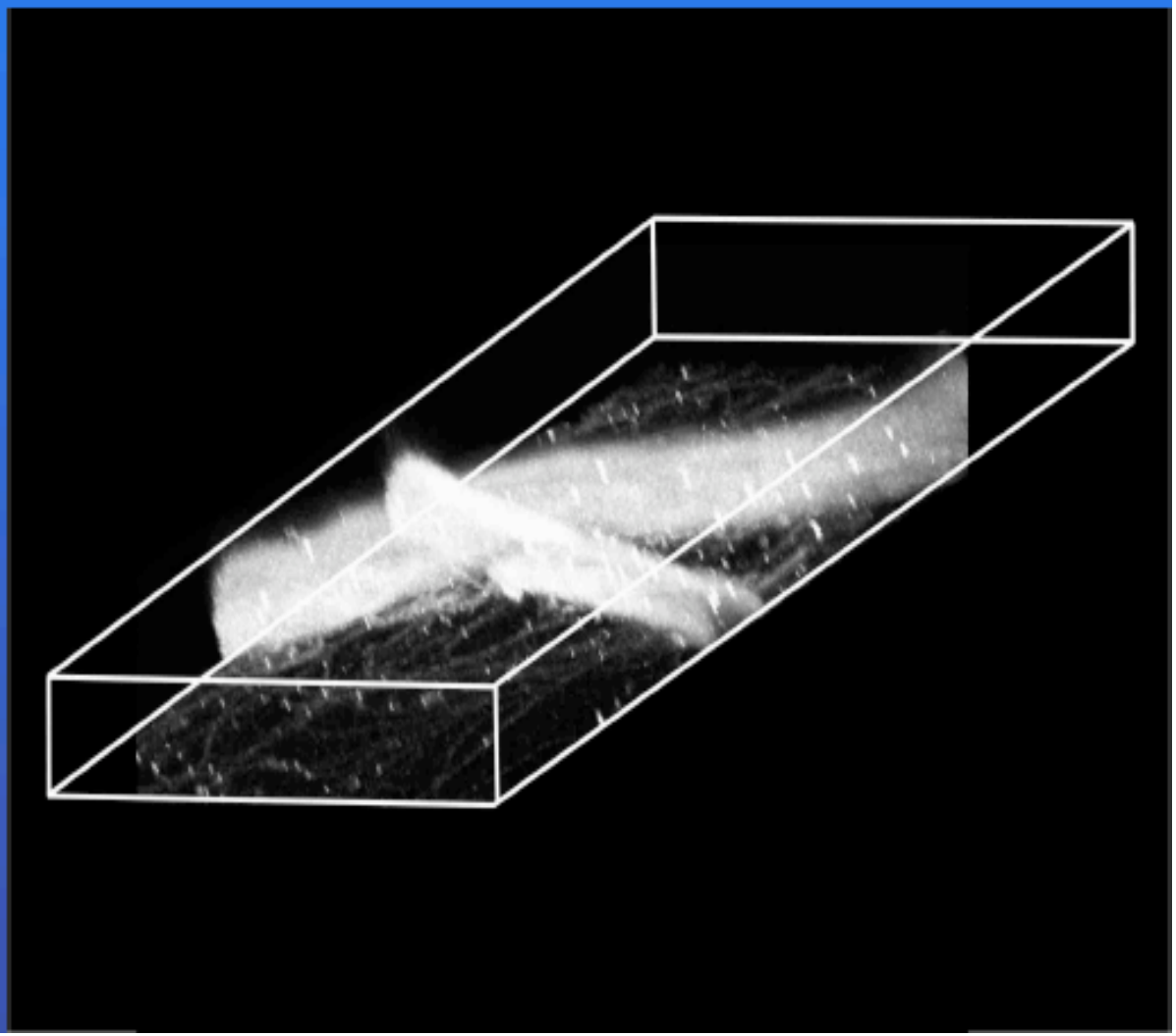
Which is above?

Single optical sections

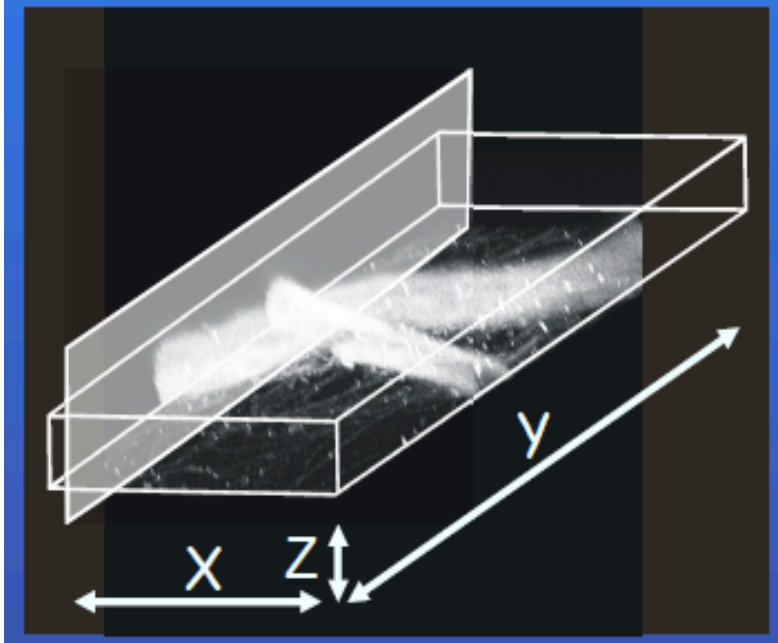


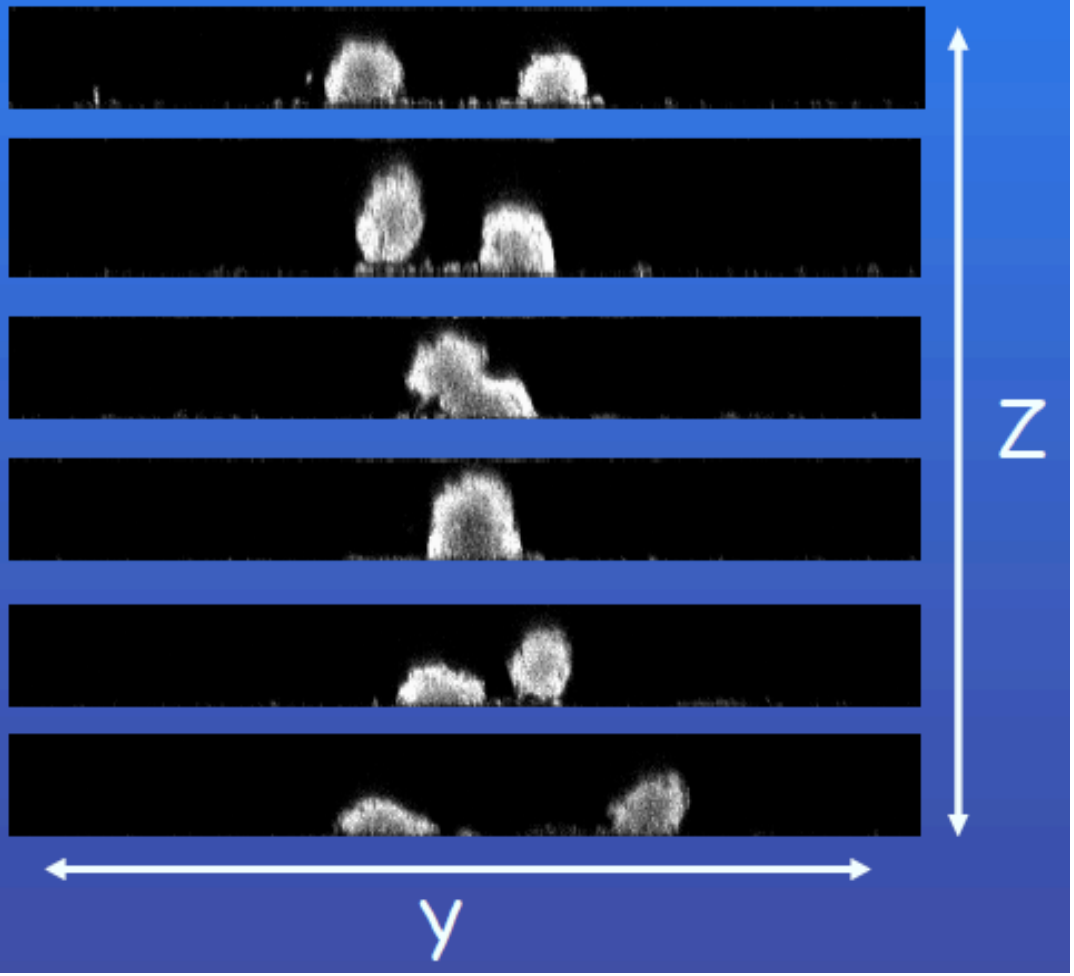
The data cube



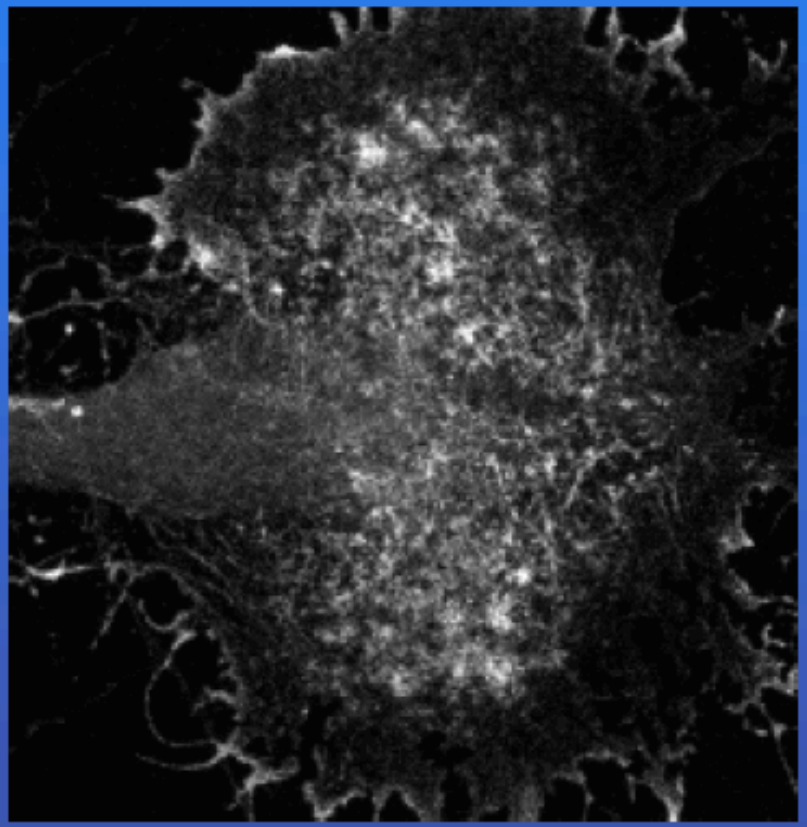


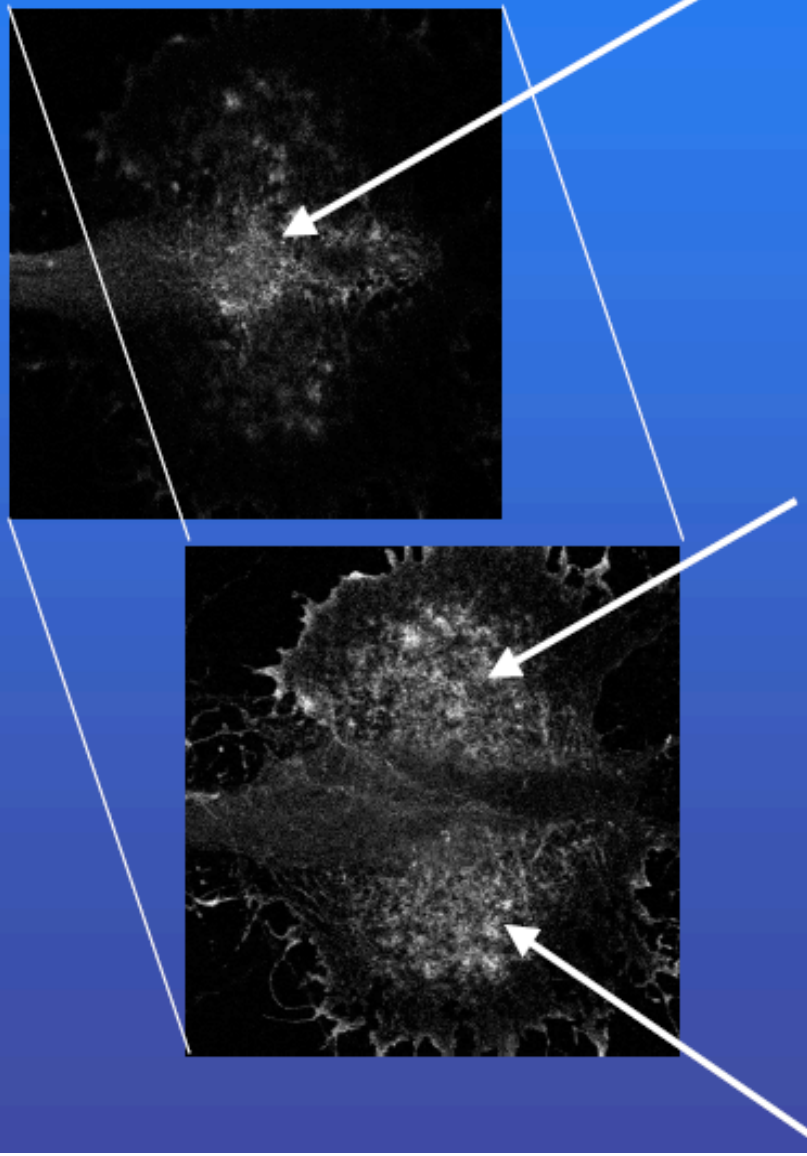
Re-sectioning





Projecting to see everything in focus





I. Increase excitation intensity

Let more laser light through…

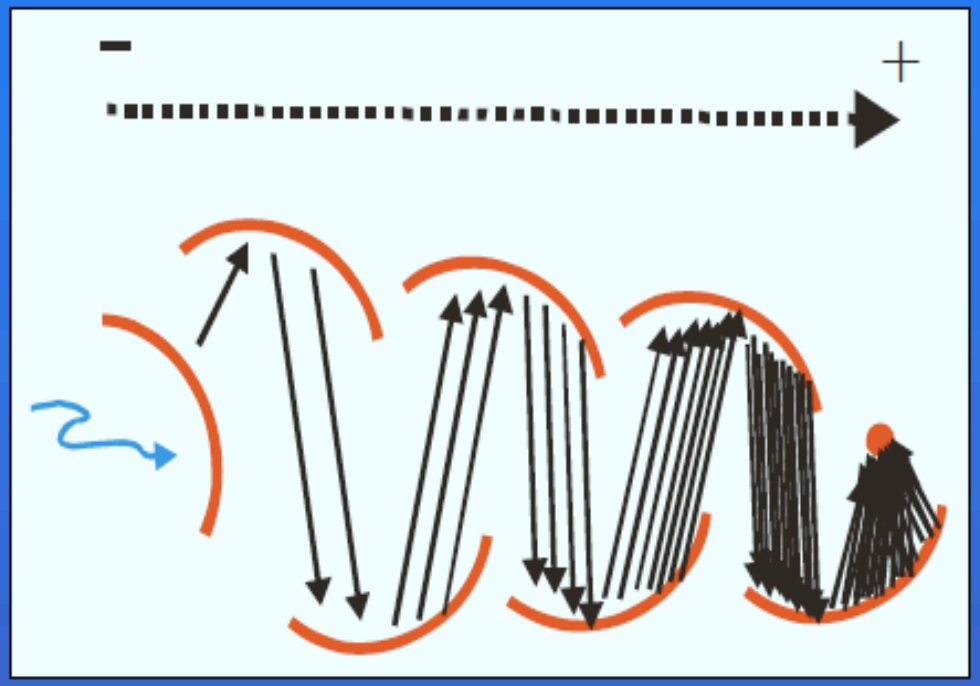
Pro:

+ More photons will be emitted, reducing SNR.
+ Relatively linear.

Con:

- Increased Photobleaching
- Increased Photo damage
- Possible Dye saturation: If light flux is strong, excitation photons may arrive before fluorophore emits, resulting in non-linearity.

II. Increase voltage on the PMT



Pro:

+ Does not affect acquisition time or sample exposure time.

Con:

- Not linear, should be taken into account in quantitative experiments!
- Increases non-Poisson noise

III. Increase pixel dwell time
IV. Average Filtering
V. Summation

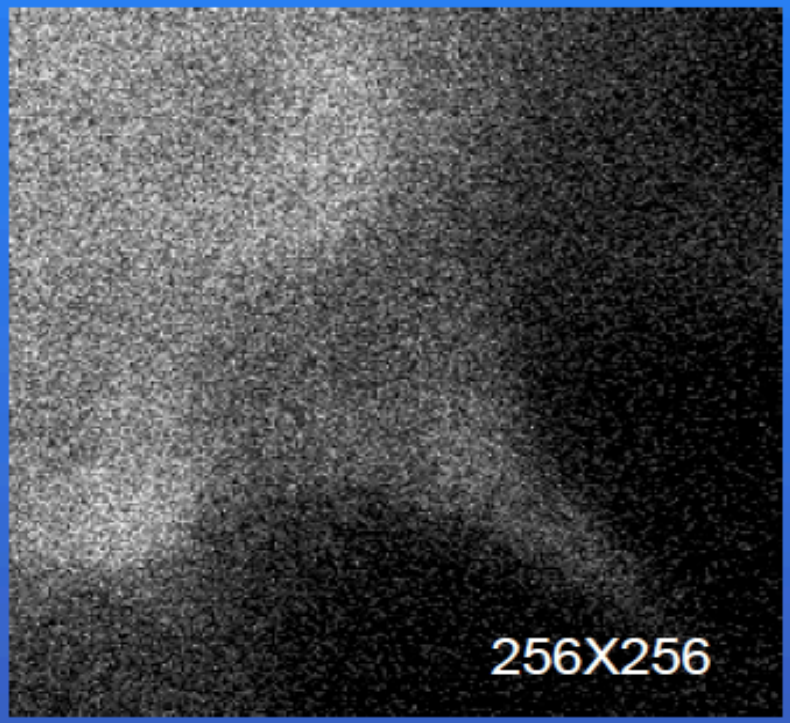
Pro:

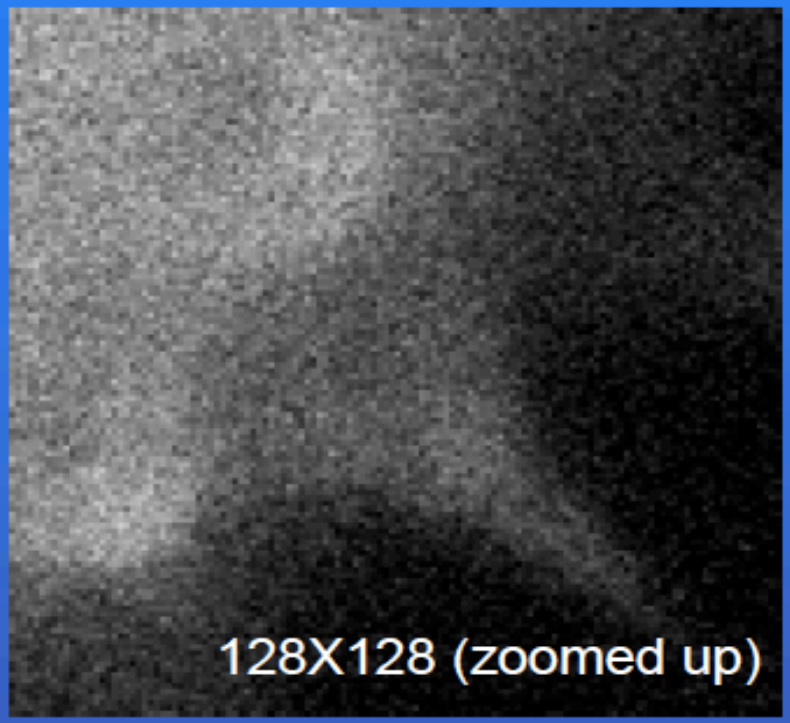
+ More photons are collected
+ Signal to noise ratio is improved

Con:

- Increases Photobleaching
- Increases Photo damage
- Increases scan time

VI. Binning of pixels





Pro:

Increases SNR without elongating scan time

Does not increase sample exposure

Con:

Image resolution is lowered

=> Should be considered, since over-sampling is common...

VII. Open the pinhole

Pro:

+ Increase photon detection without elongating exposure or scan time.

Con:

- Loose confocal effect.
- Non-linear, sample dependent.

Ask yourself: How thin does the section really need to be?

VIII. Play with emission filters

Expanding the acceptable range of emission wavelengths will increase number of detected photons.

Pro:

+ No effect on scan time or length of exposure.

Con:

- Unintended photons, emitted from other fluorophores or from auto-fluorescence, may contaminate the image.

Axial Chromatic mismatch

- Immersion medium should be matched to mounting medium. In live cell work water immersion objectives should be considered.
- When Stoke's Shift is large, focal planes of excitation and emission may differ !!! Detection may be hampered!

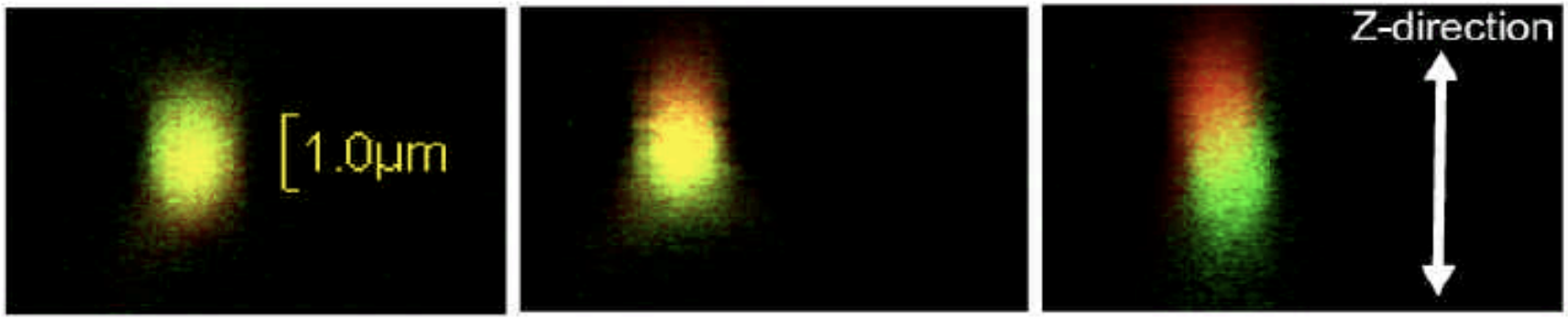
Axial chromatic mismatch

OLYMPUS
Your Vision, Our Future

FluoView FV1000

New UIS2 objectives

- Wide range chromatic aberration corrected

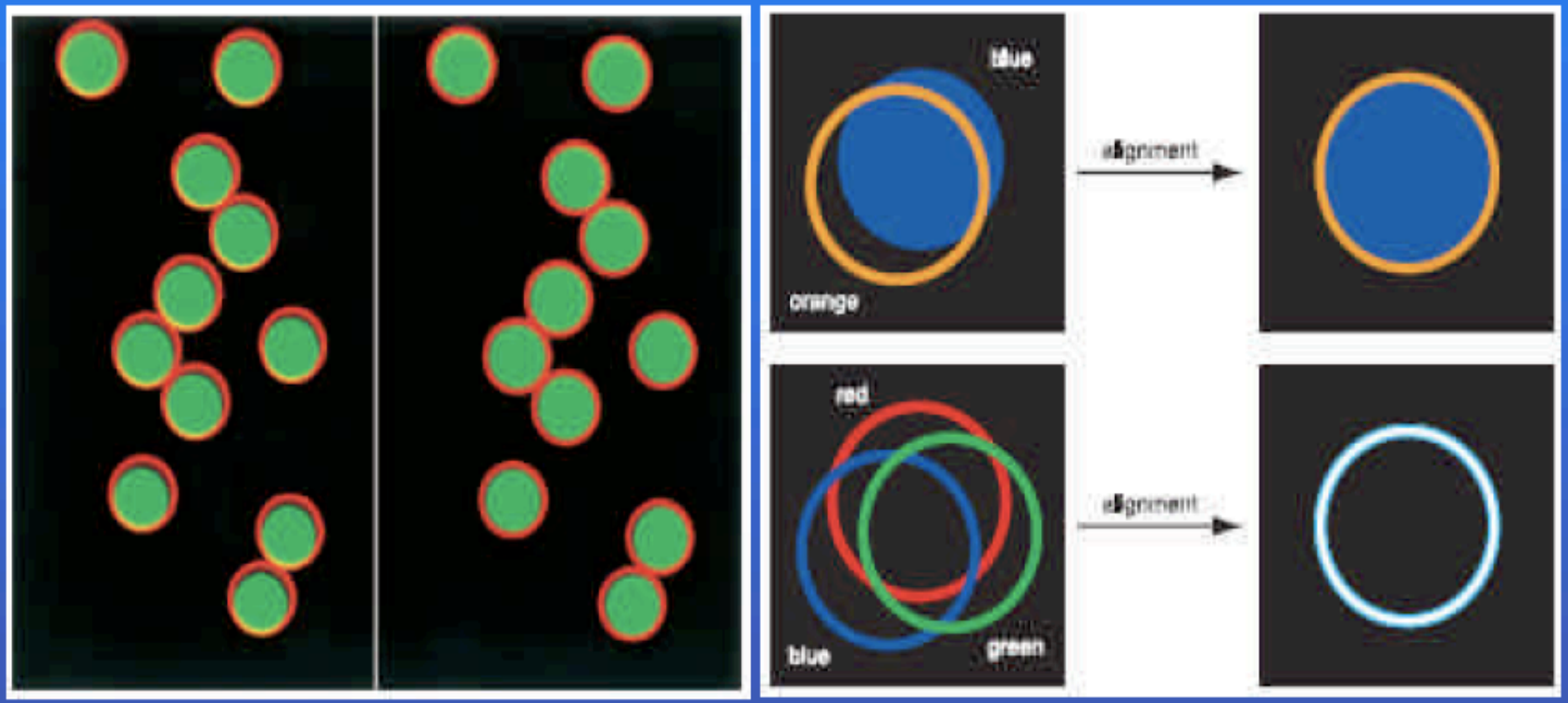


SAPO APO Fluorite

Fluorescence beads (1micron)

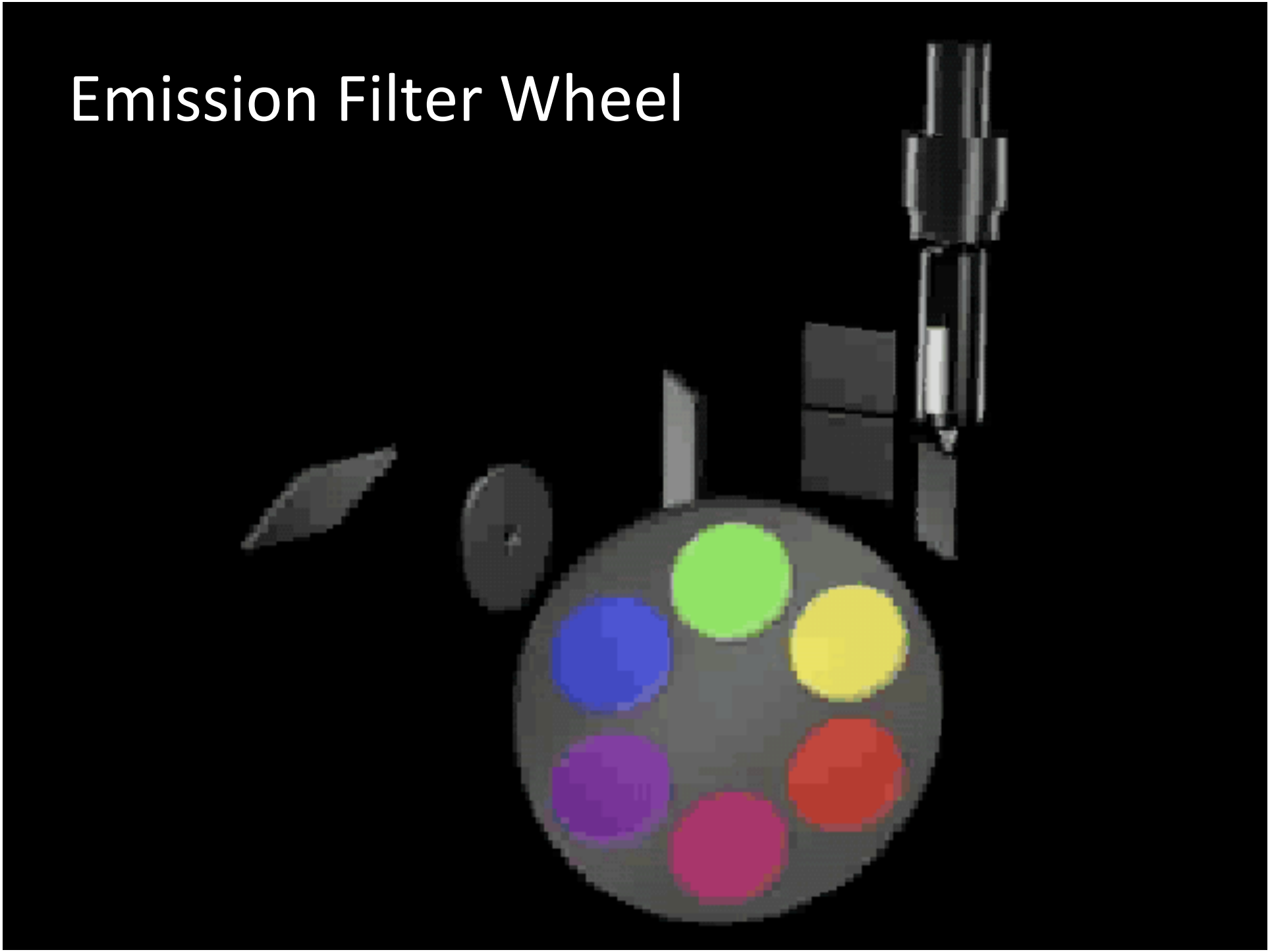
FluoView: 488nm/633nm excitation

Chromatic-match calibration



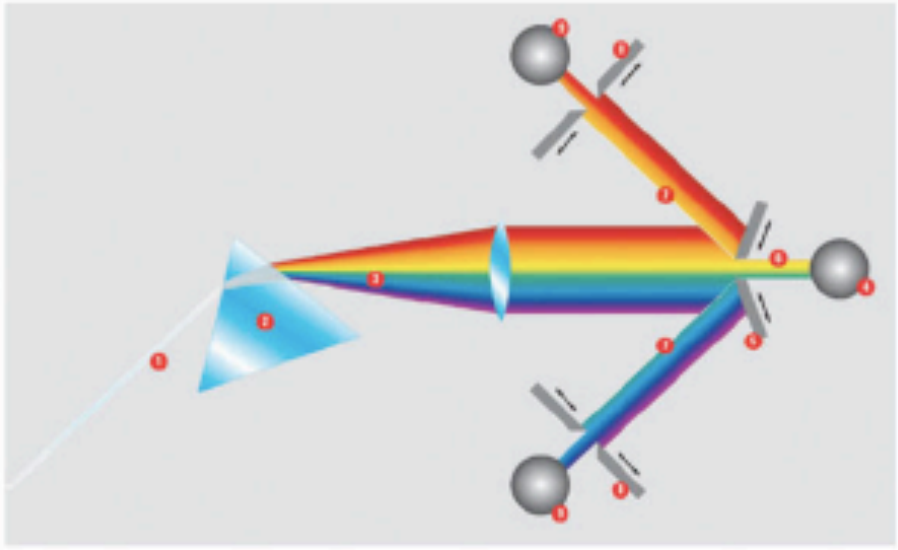
Using multi-colored FocalCheck beads to correct channel alignment

Emission Filter Wheel



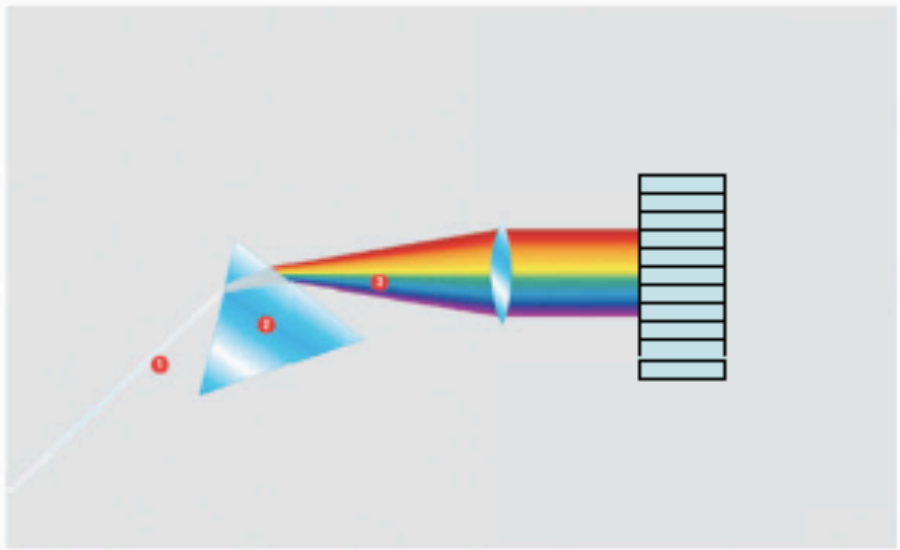
"Spectral" Detection

Prism + slits as tunable "emission filters"

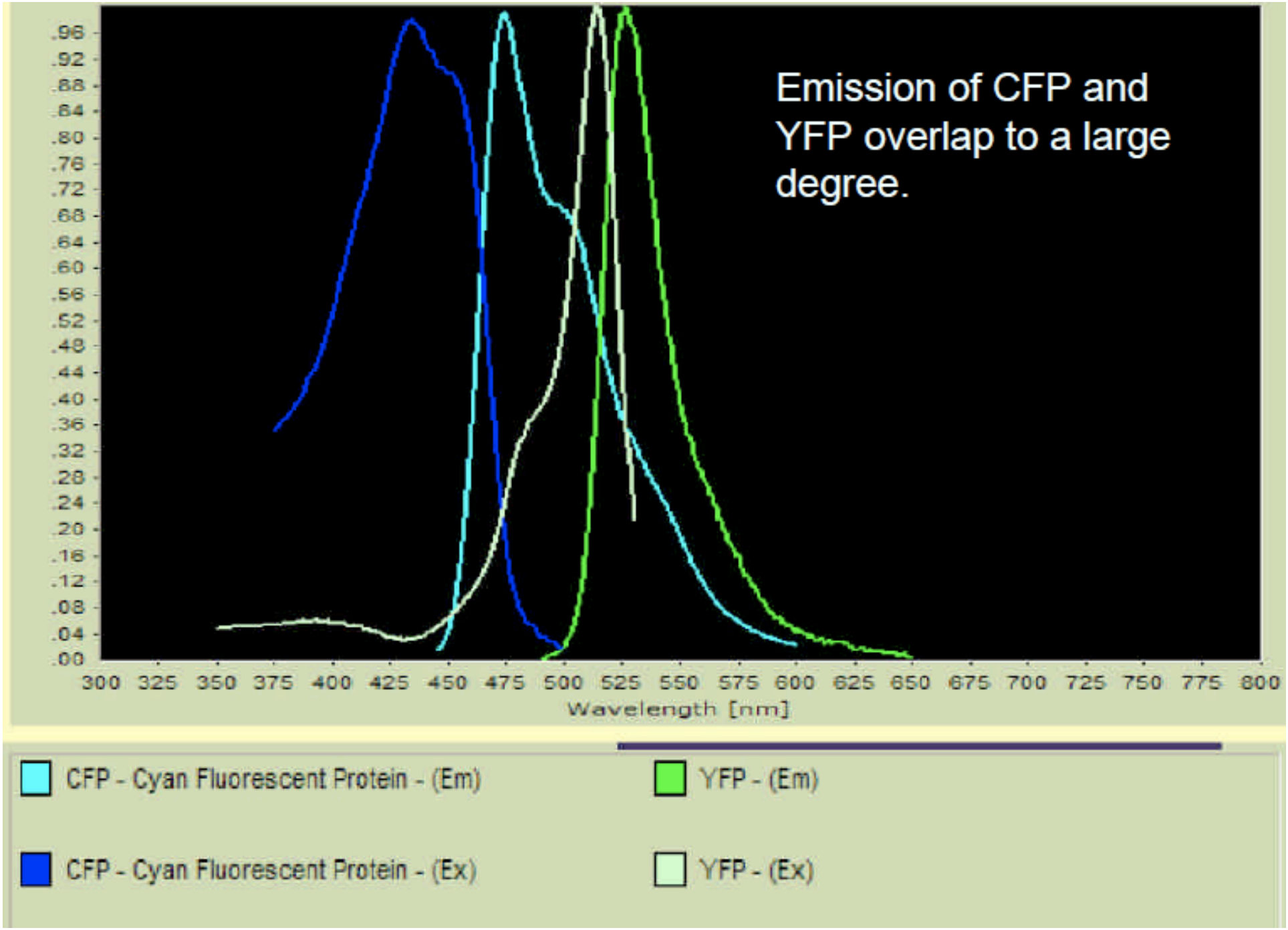


(Leica SP series)

Prism + photo-multiplier array



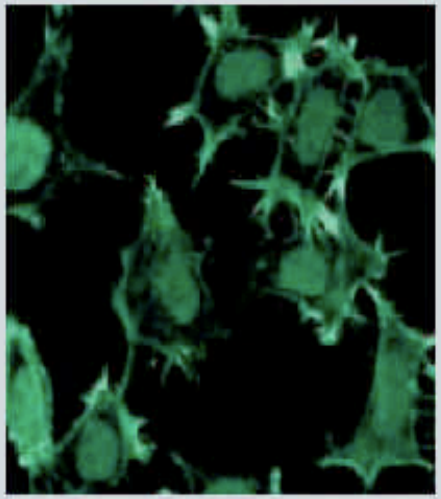
(Zeiss LSM 510 Meta)
(32 channels)


Emission of CFP and YFP overlap to a large degree.
.96
.92
.88
.84
.80
.76
.72
.68
.64
.60
.56
.52
.48
.44
.40
.36
.32
.28
.24
.20
.16
.12
.08
.04
.00
300 325 350 375 400 425 450 475 500 525 550 575 600 625 650 675 700 725 750 775 800
Wavelength [nm]
CFP - Cyan Fluorescent Protein - (Em)
YFP - (Em)
CFP - Cyan Fluorescent Protein - (Ex)
YFP - (Ex)

Spectral unmixing/fingerprinting

Separation of GFP and Alexa 488 spectra

GFP expressed in HeLa cell nuclei and actin stained with Alexa 488. Excitation wavelength 488 nm.



Combined 32 channel True Color image obtained with 2.5 nm wavelength resolution in 493-570.5 nm range

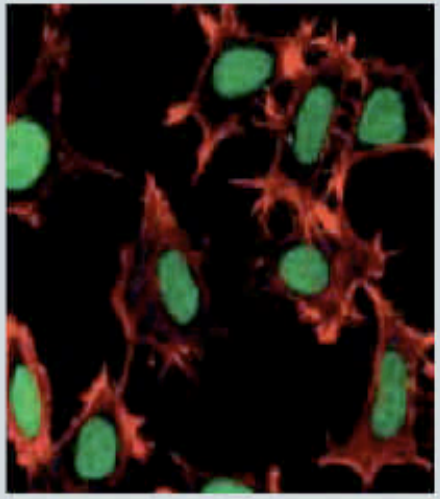
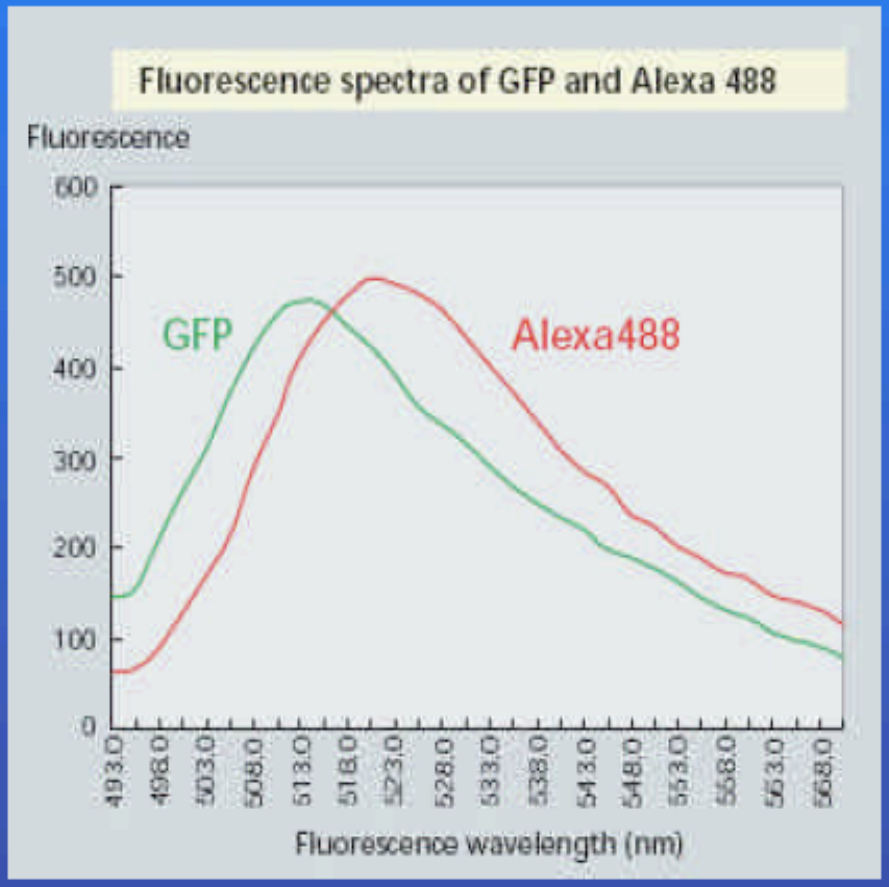


Image with separated spectra after using unmixing software



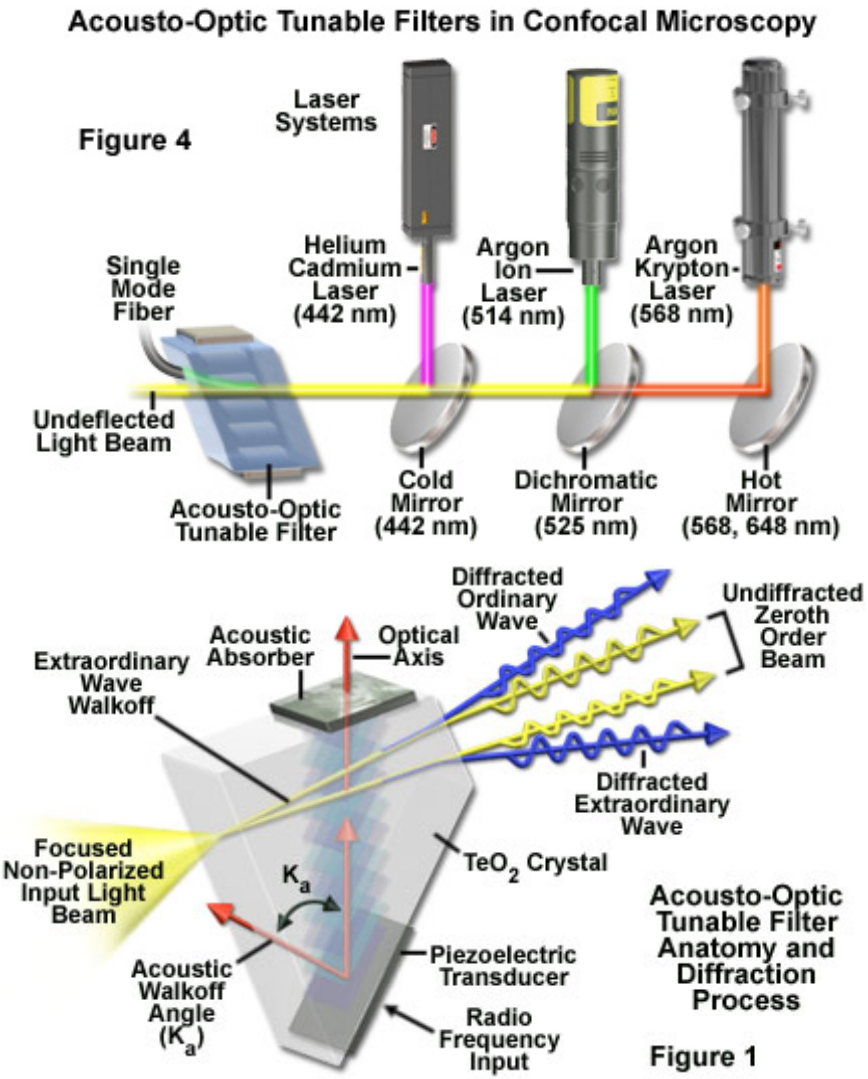
Multi-color imaging

Discrete filters: (Squencial or simultaneous)
- Narrower for multicolor to reduce cross-talk
- Color decomposition with multiple exposures
- 4-5 colors can be resolved

Spectral Imaging:
- Records the whole emission spectrum
- 3D data block for each 2D image
- Each fluorophore can be deconvolved
  - if no spectral shifts
  - if background and auto-fluorescence << signal
- In practice 5-6 colors can be resolved

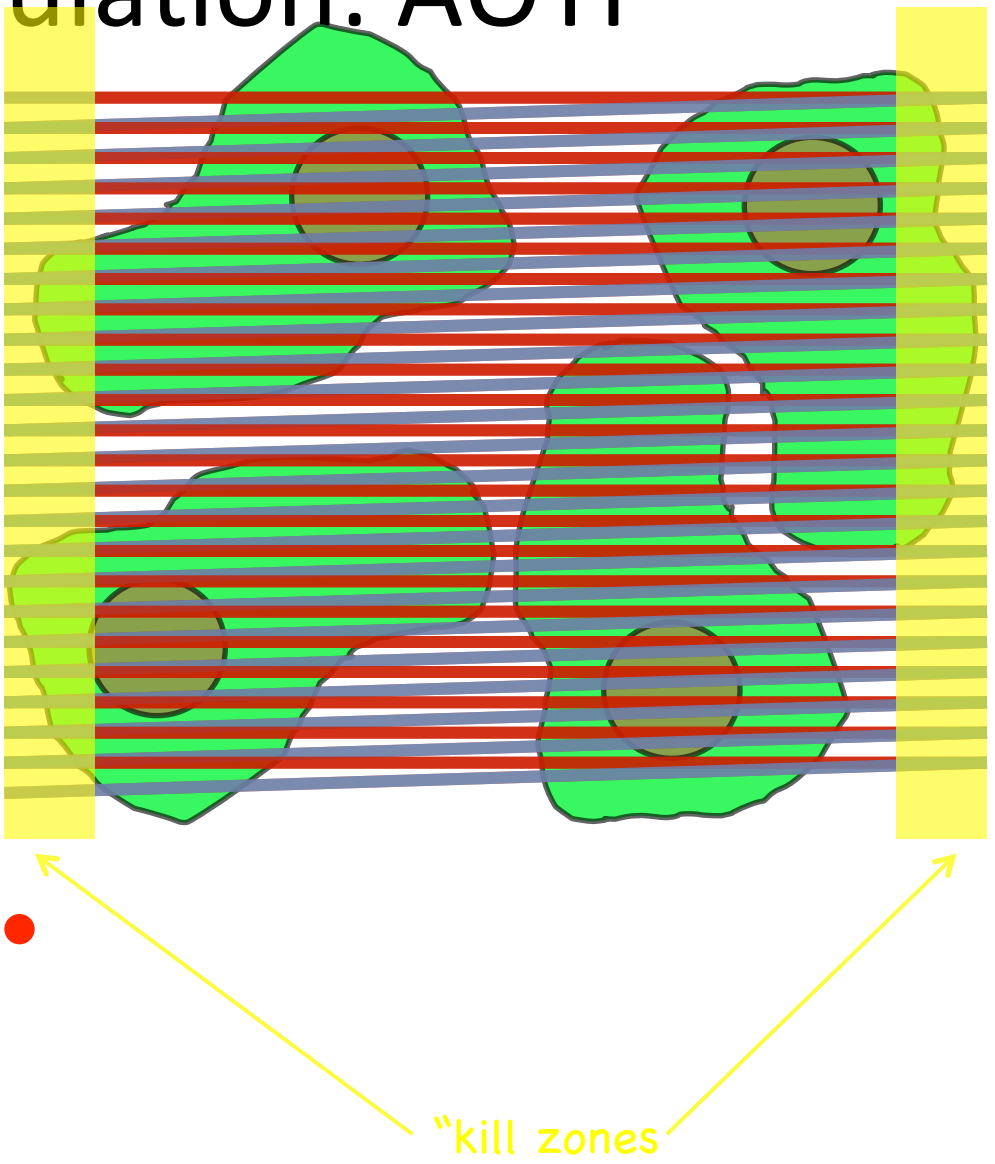
Modulation: AOTF

- AOTF = Acousto-Optic Tunable Filter
- Used to separate a particular wavelength from a mixture of wavelengths
- Also used to modulate the intensity of the selected wavelength
- Multiple wavelengths can be selected simultaneously and controlled separately
- Switching time on the order of microseconds
- Can also be used to illuminate regions of interest (ROIs) while scanning
- Made of a tellurium dioxide crystal, that is "bombarded" by an acoustic (periodic pressure) wave
- As a result the crystal lattice structure is alternately compressed and relaxed converting it into a grating that diffracts light

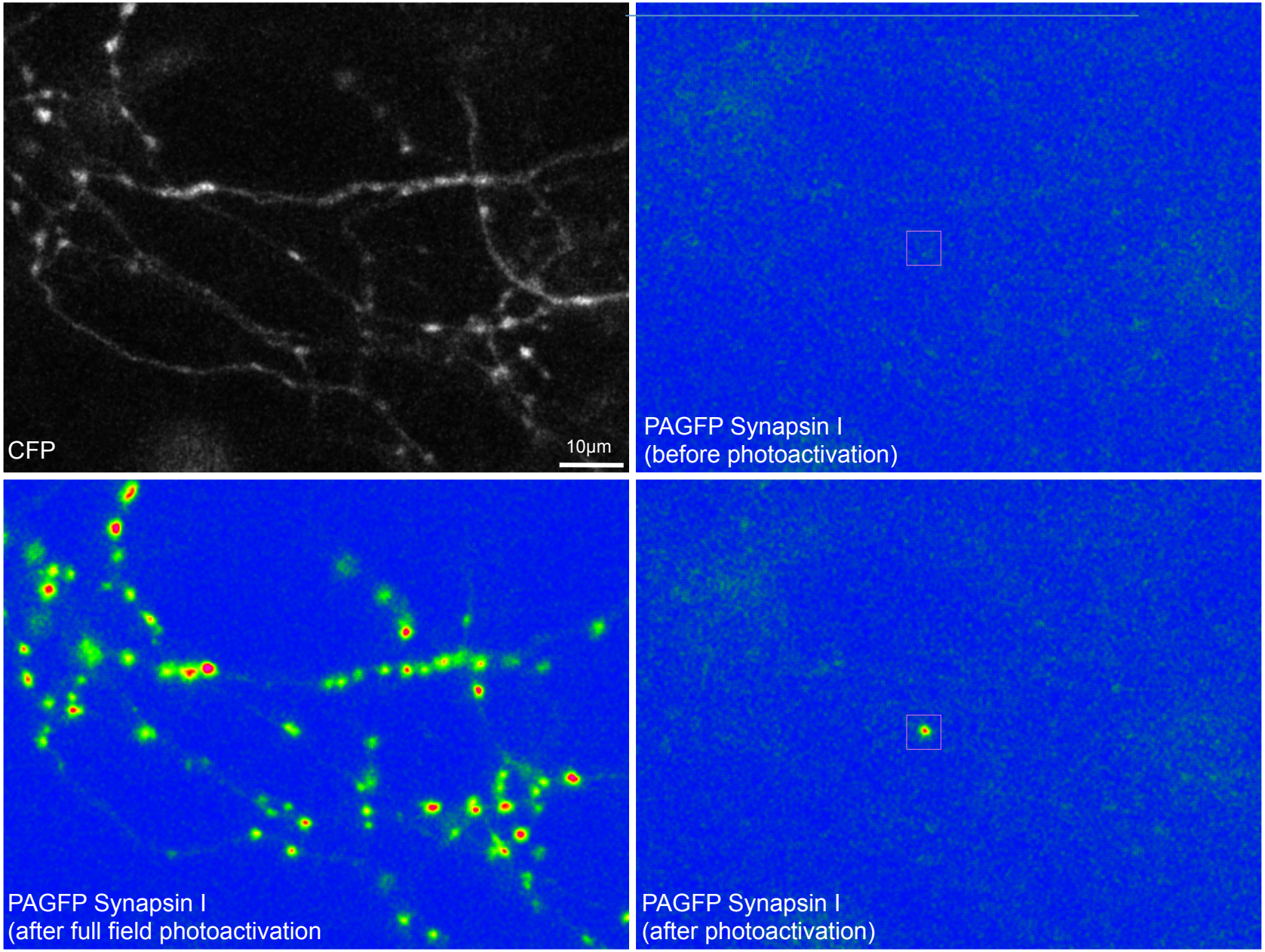


Modulation: AOTF

Crucial function:
Turn of laser beam when data is not collected - avoid unnecessary exposure

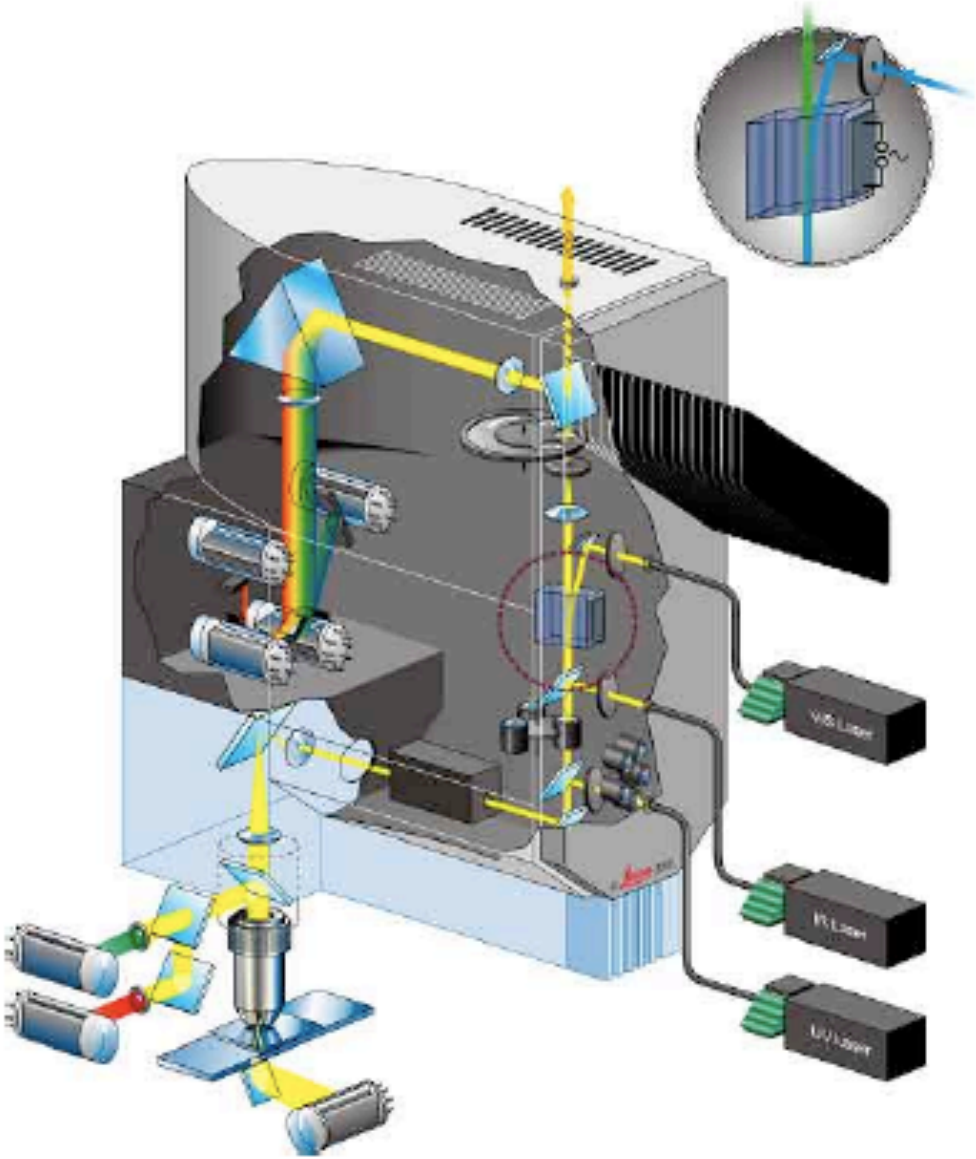


Modulation: AOTF

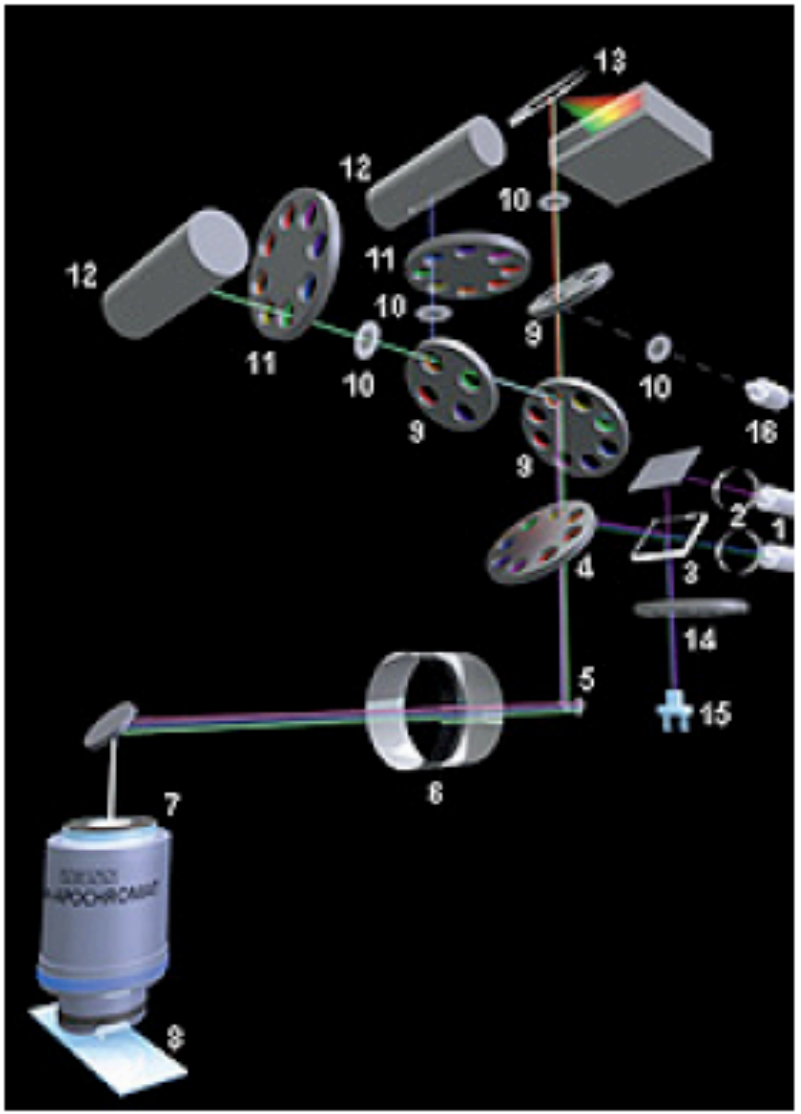


Confocal Laser Scanning Units

Leica TCS SP2 AOBS

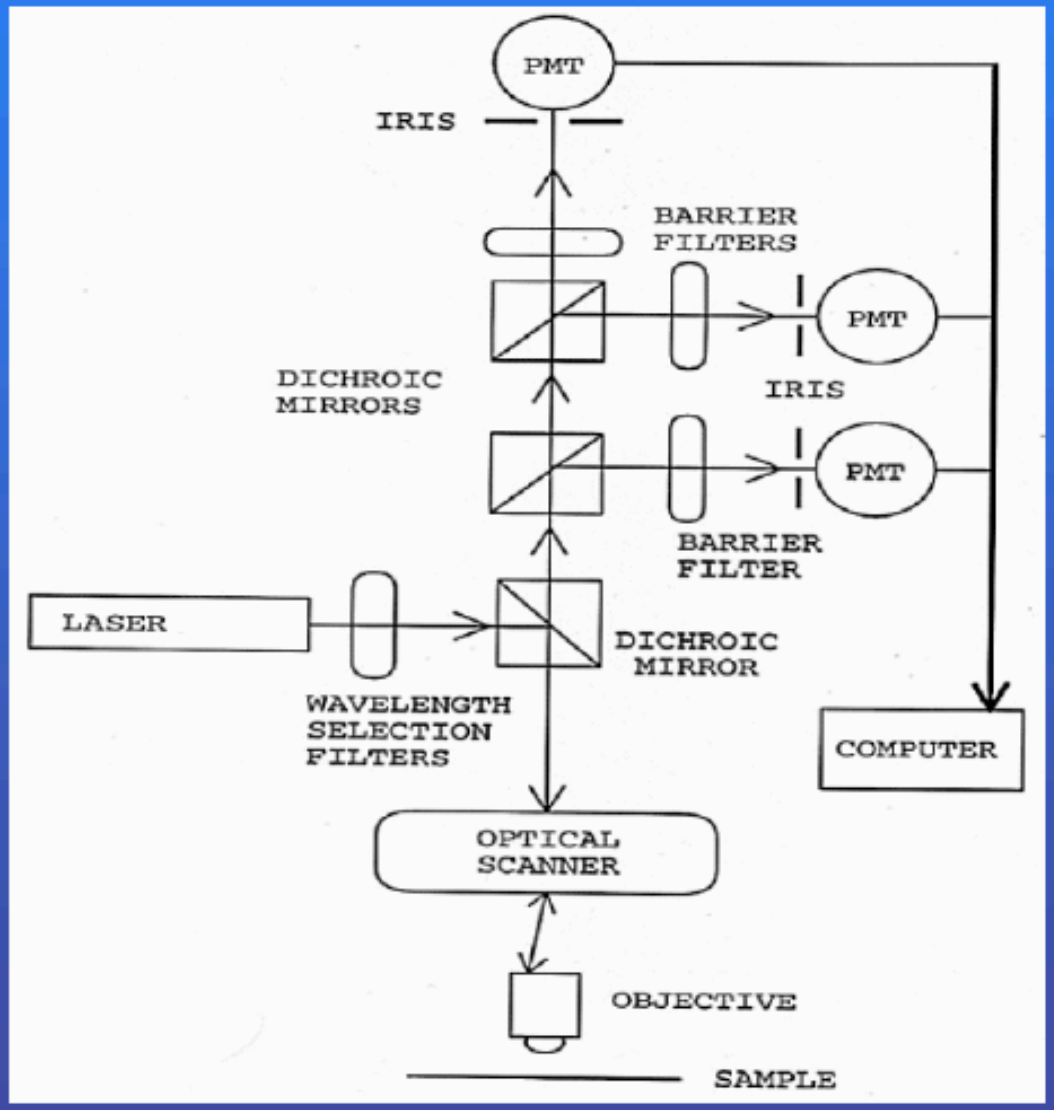


Zeiss LSM 510 Meta



Schematic diagram of a LSCM

(laser scanning confocal microscope)

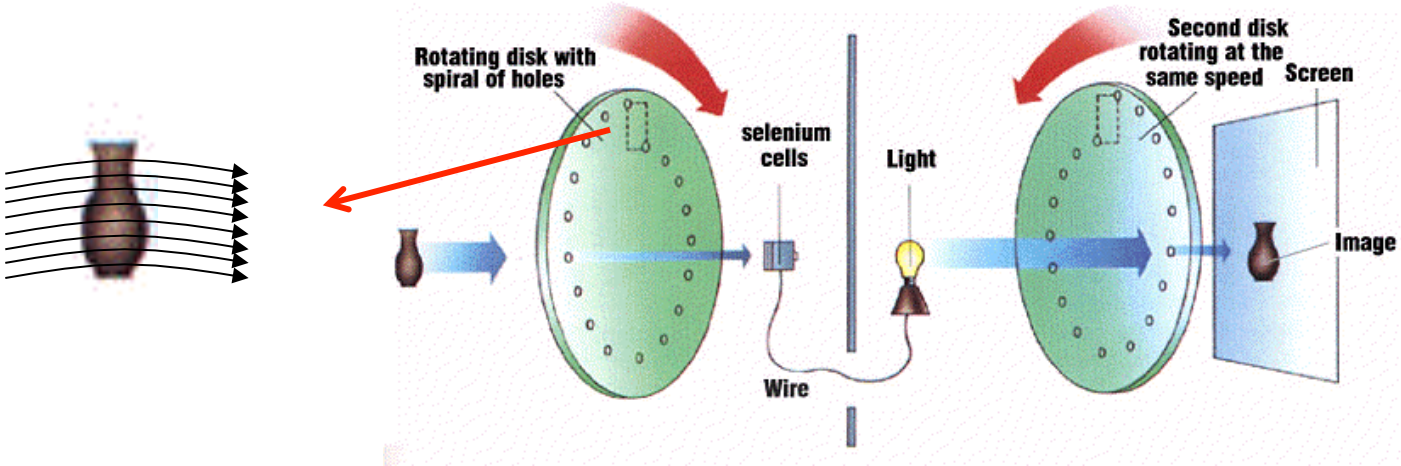


Spinning Disk Confocal

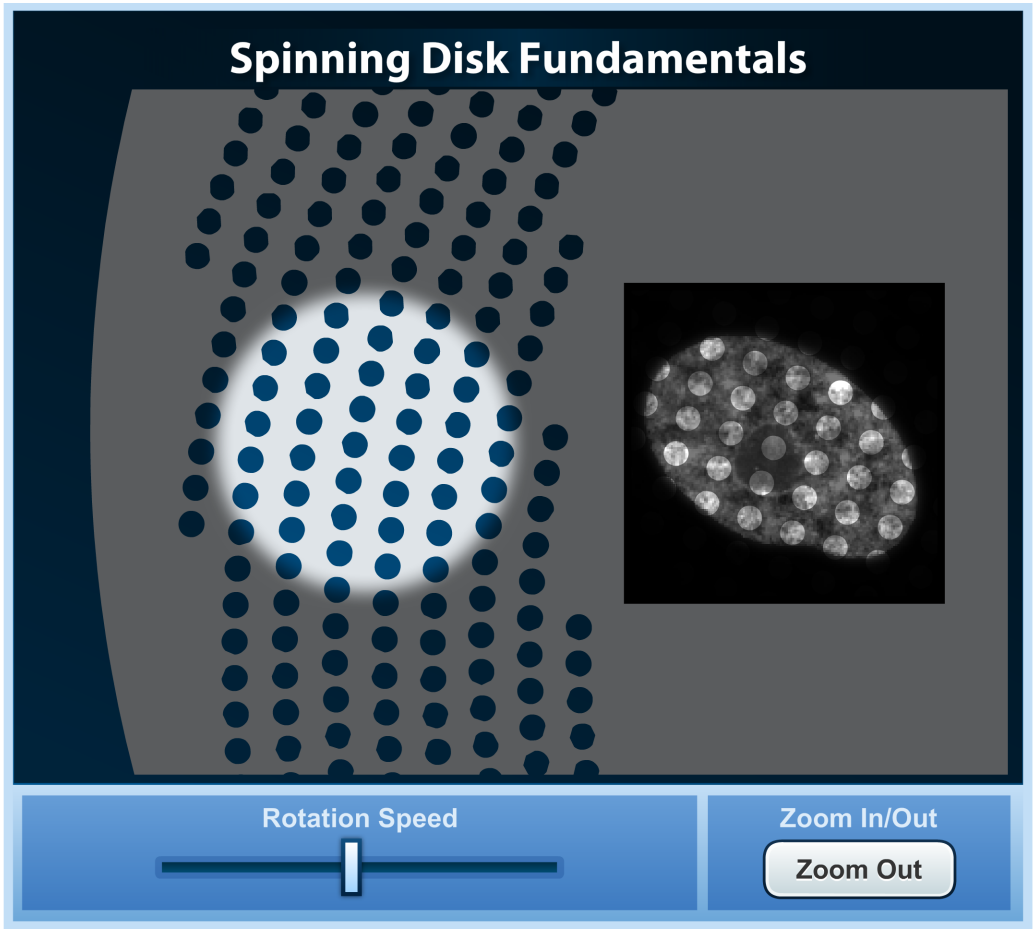
Spinning disk (Nipkow) confocal

- *Laser scanning confocal microscopes have two major shortcomings*
  - *Slow scan rates (limited by scanner galvanometer mechanics)*
  - *Light concentrated in time and space (scanning spot) – leading to fluorophore saturation and extended photobleaching*
- *An alternative – spinning disk confocal microscopy (Nipkow disks)*
- *(Historically, spinning disks predated laser scanning systems…)*
- *Fast scan rates (up to 1000 images/sec)*
- *Allows use of high QE detectors (i.e. EMCCDs)*
- *Less bleaching*

Paul Gottlieb Nipkow

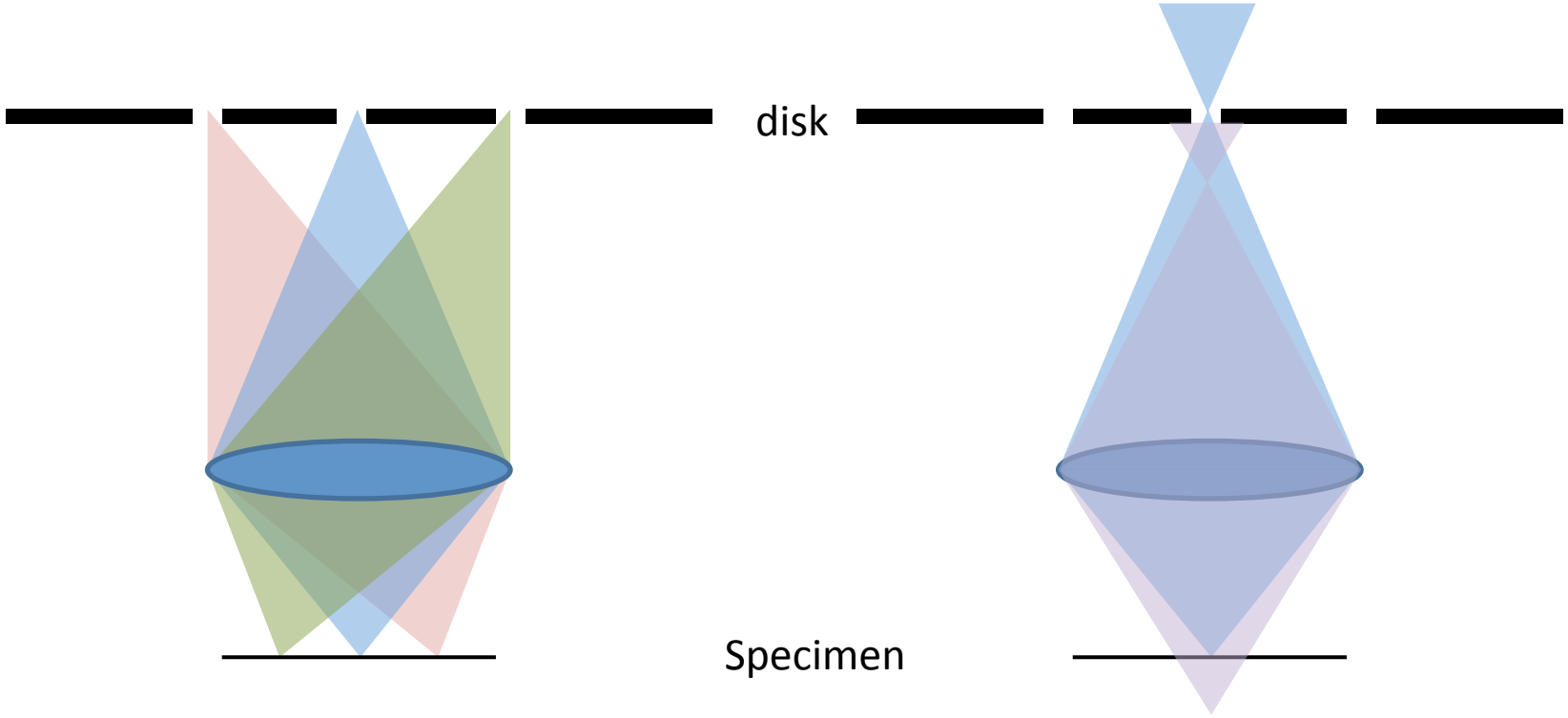


Spinning disk confocal



Spinning disk confocal

Multiple spots generated by multiple pinholes
Each pinhole serves as a miniature confocal system



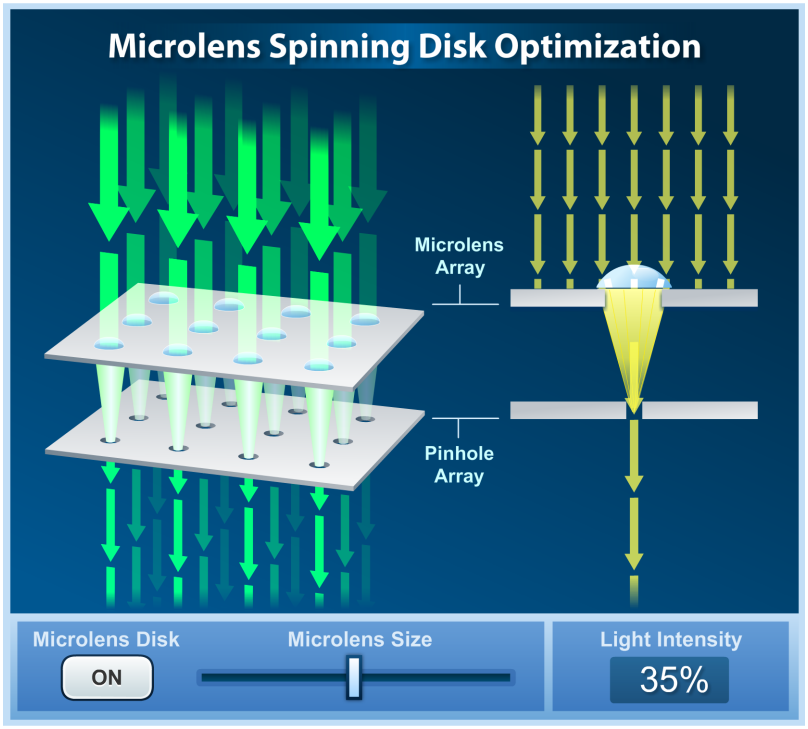
Spinning disk confocal

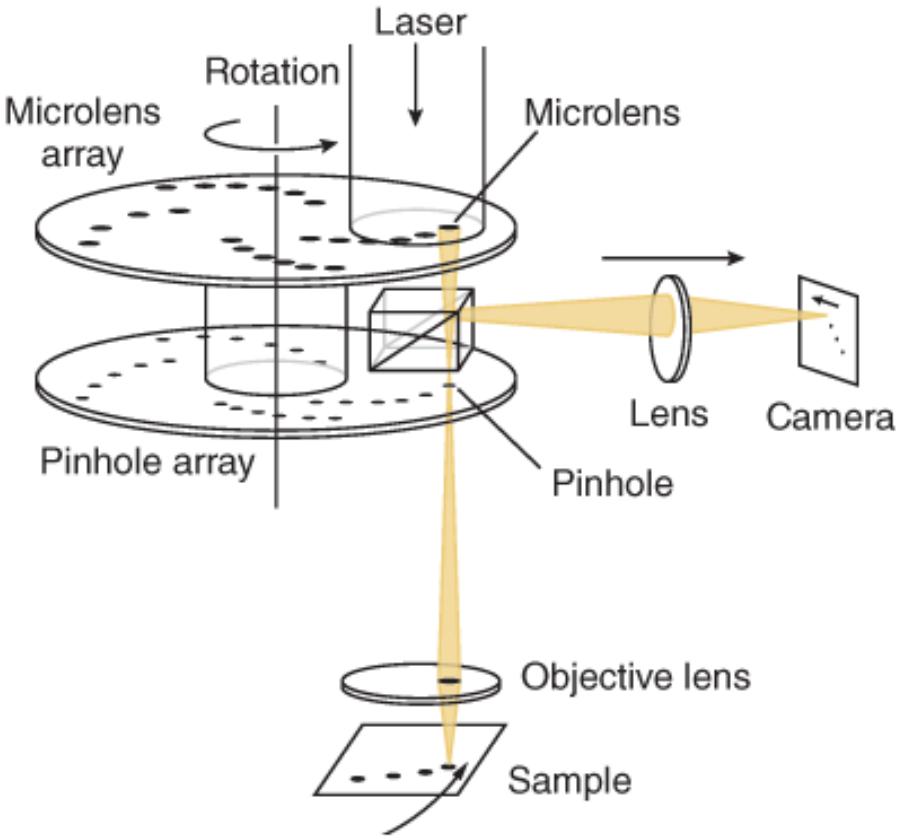
Problem:

- *Only a minuscule amount of light passes through pinholes*

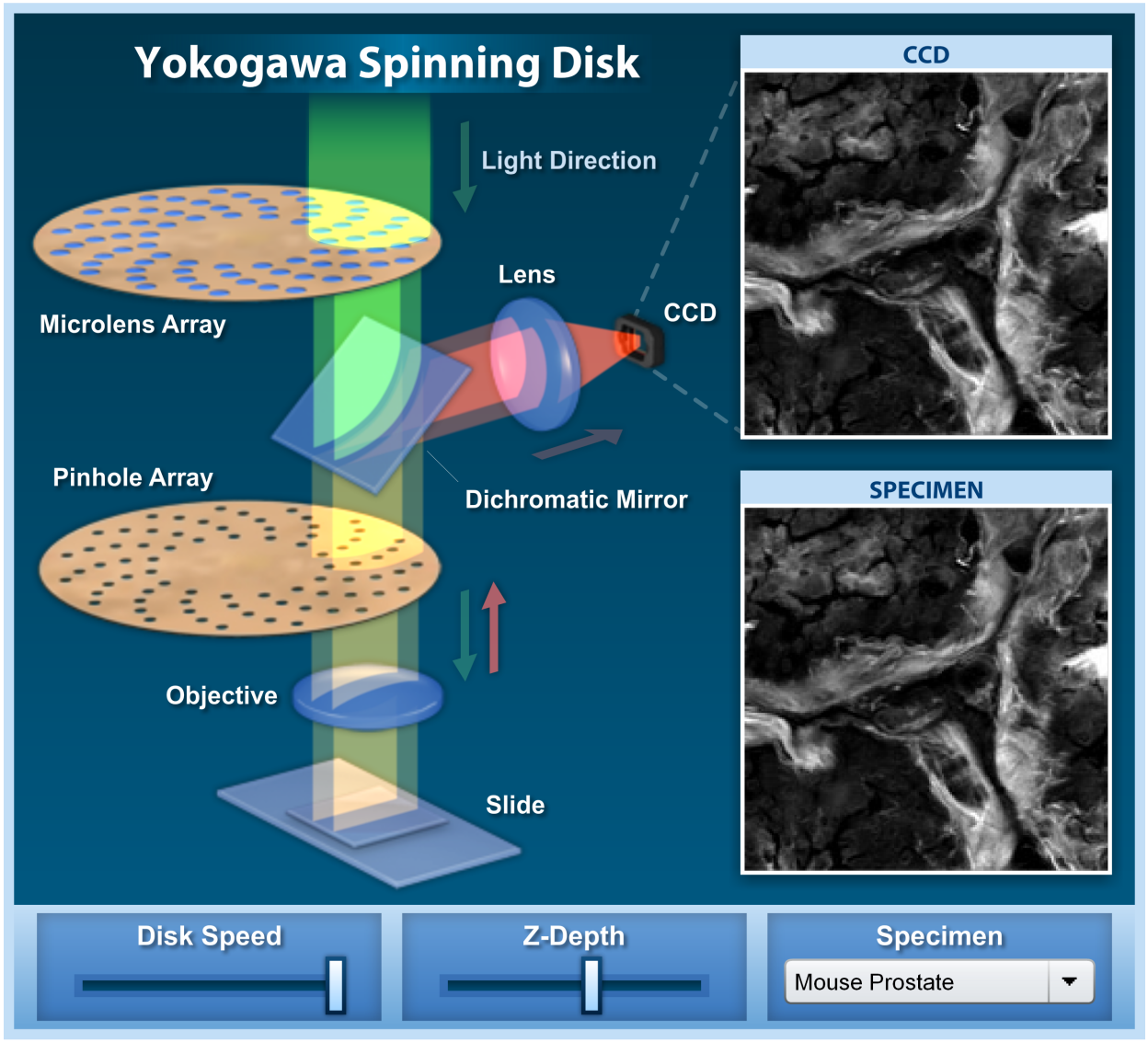
Solution:

- *The Yokogawa Microlens-enhanced Nipkow Disk*





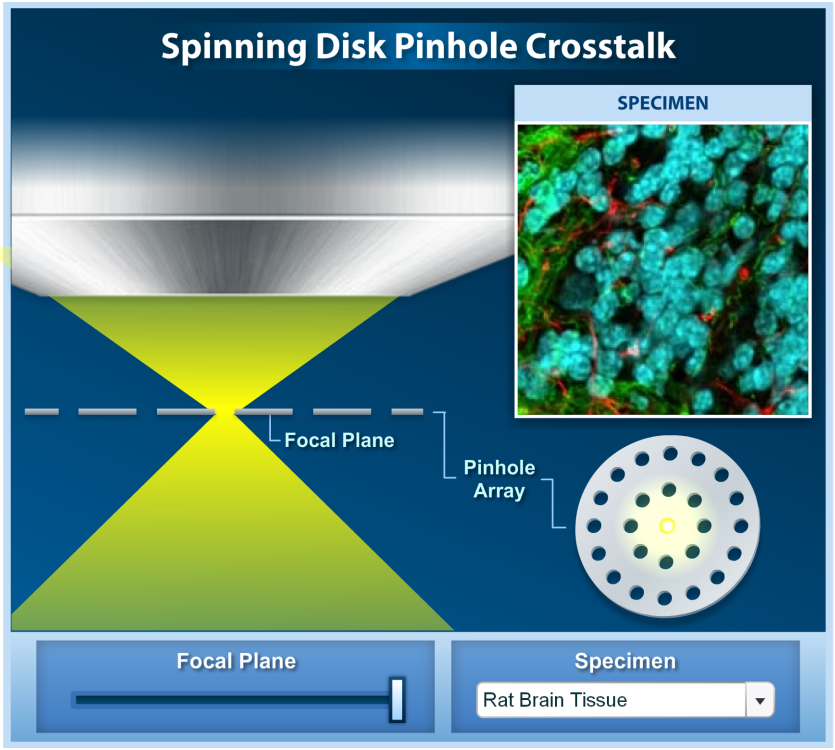
Spinning disk confocal



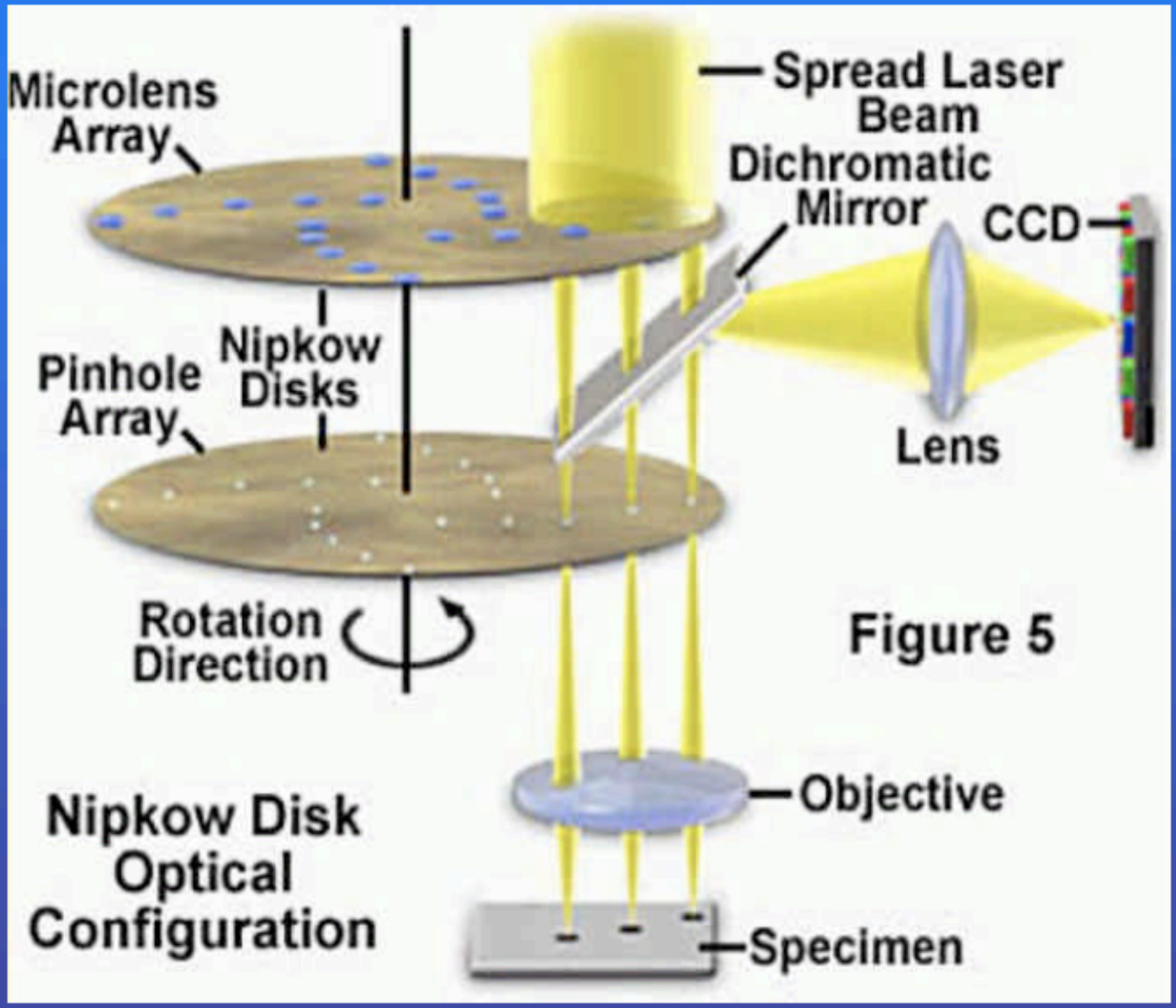
Spinning disk confocal

Not everything is perfect:

- *Complicated (and expensive) system*
- *Axial resolution hampered by pinhole crosstalk*
- *Multiple emission channels and spectral unmixing – difficult (multiple CCDs, registration)*
- *Although high frame rates are possible in principle, in practice multiple frames are accumulated/averaged to obtain useful data*



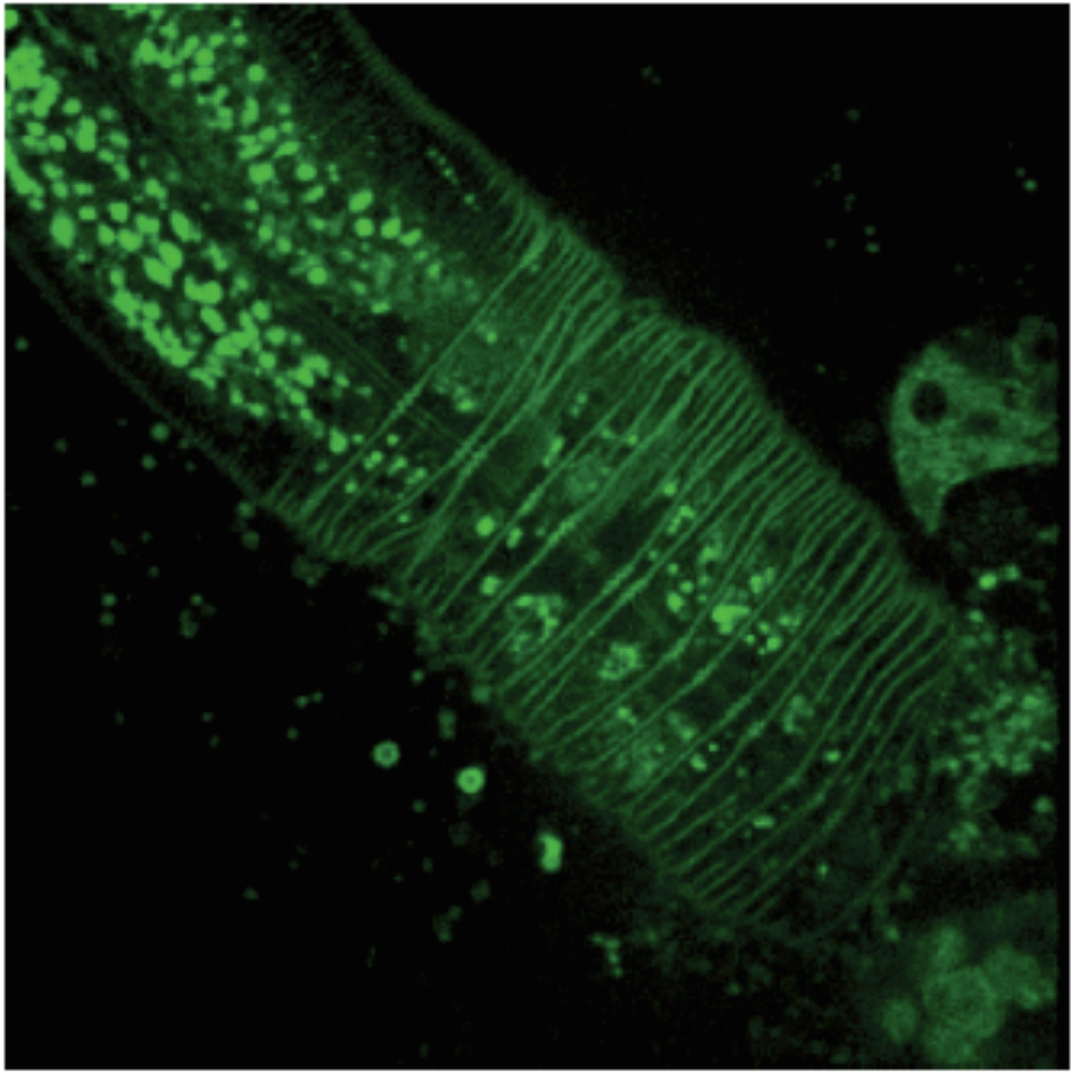
Nipkow Disk Confocal



- Fast (360Hz)
- Uses sensitive CCD as detector
- Saturation not an issue
- Directly viewable
- Possibly gentler on live cells

Less flexible:
Fixed size pinholes
No zooming, ROIs etc.

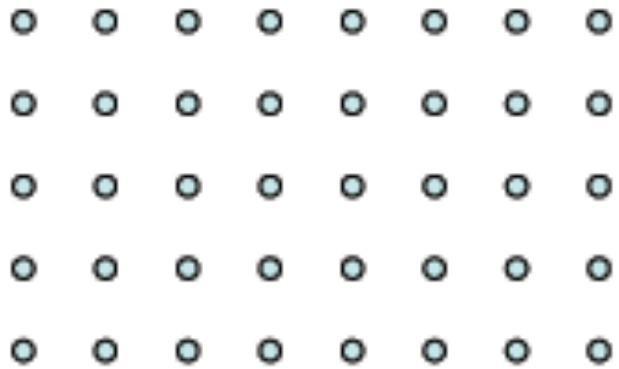
Spinning disk example



How go faster?

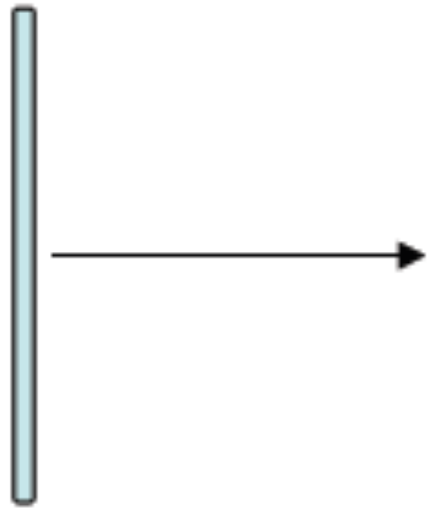
Multi-point Confocal Microscopy

Multiple pinholes
at the same time



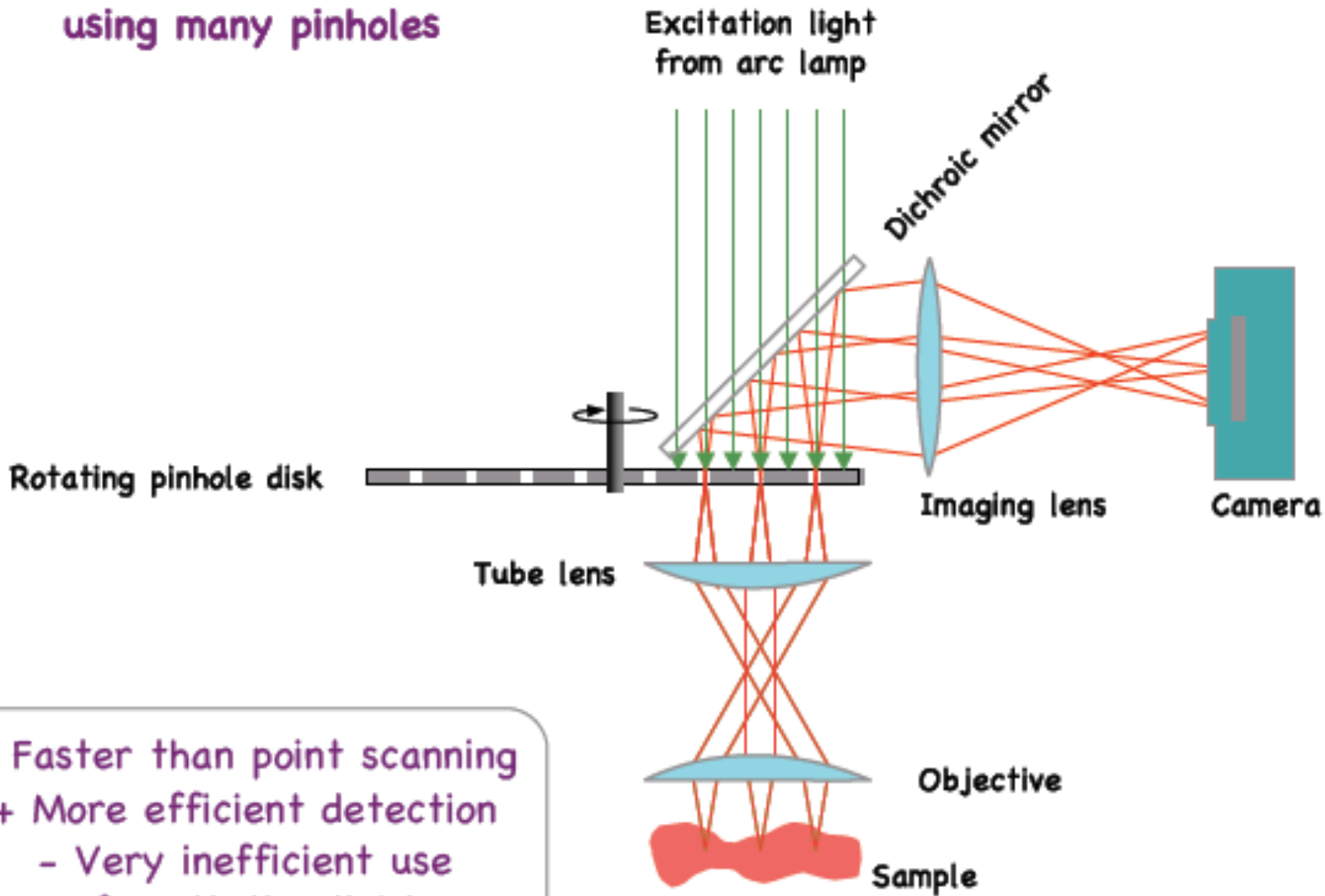
or

Scan a slit in 1D,
not a point in 2D



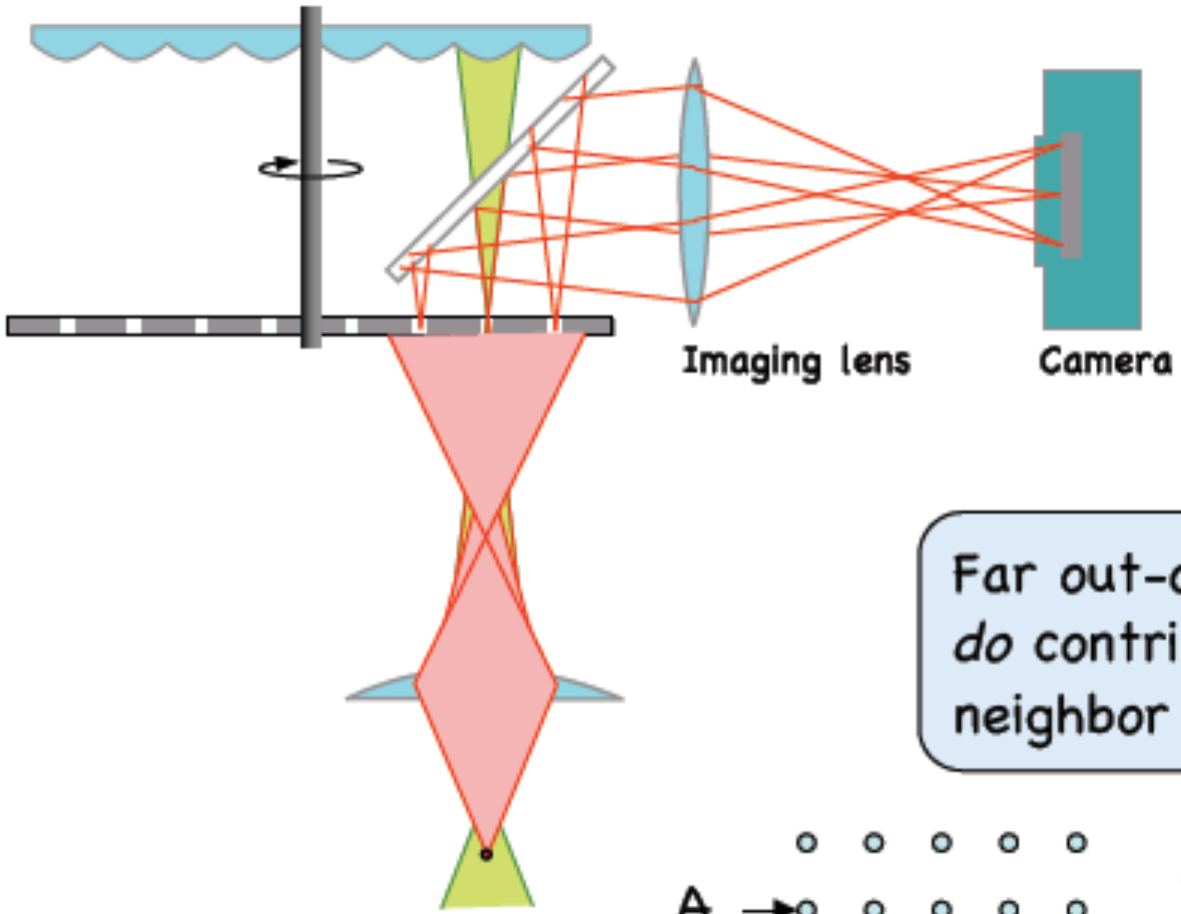
Spinning disk confocal

Scan many points in parallel
using many pinholes

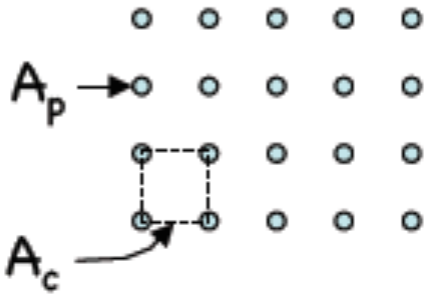


+ Faster than point scanning
+ More efficient detection
- Very inefficient use
of excitation light

Spinning disk drawback: Crosstalk

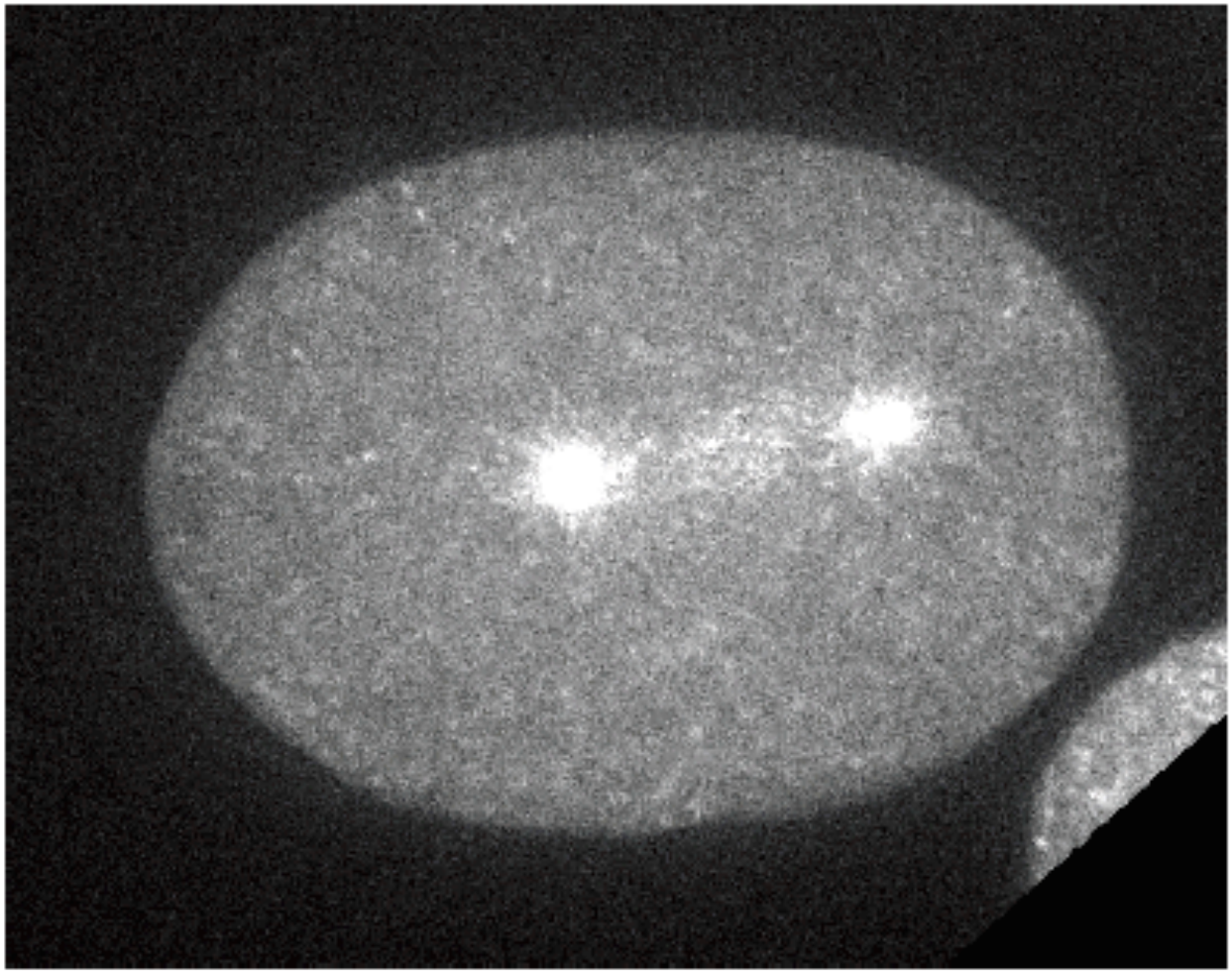


Far out-of focus sections *do* contribute blur through neighbor pinholes



Crosstalk level
≈ pinhole area fraction
= A_p/A_c ≈ 5% (Yokugawa)

Spinning disk



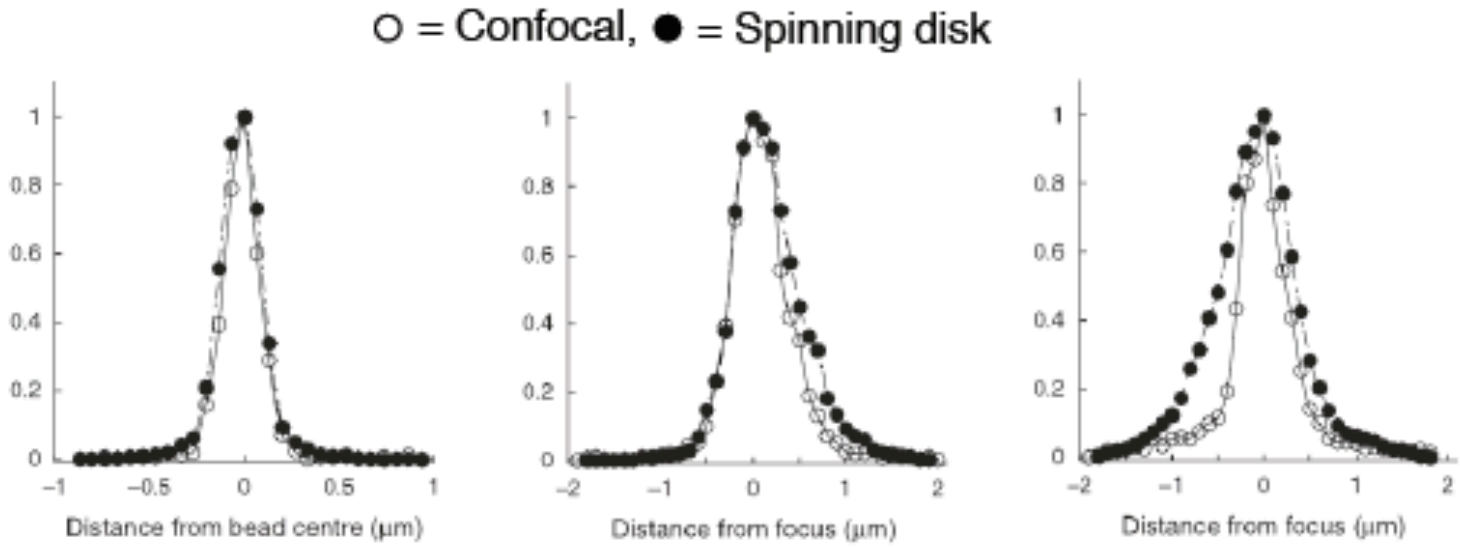
Dynamics of GFP-EBP-1 (a microtubule plus-end binding protein)
during the first cell division of a C. elegans embryo. 1-s exposure times at 2-s intervals

Fumio Motegi & Asako Sugimoto, RIKEN Center for Developmental Biology)

Spinning disk drawback: Inflexibility

Hard to exchange dichroics?

Fixed pinhole size, optimized for a single objective



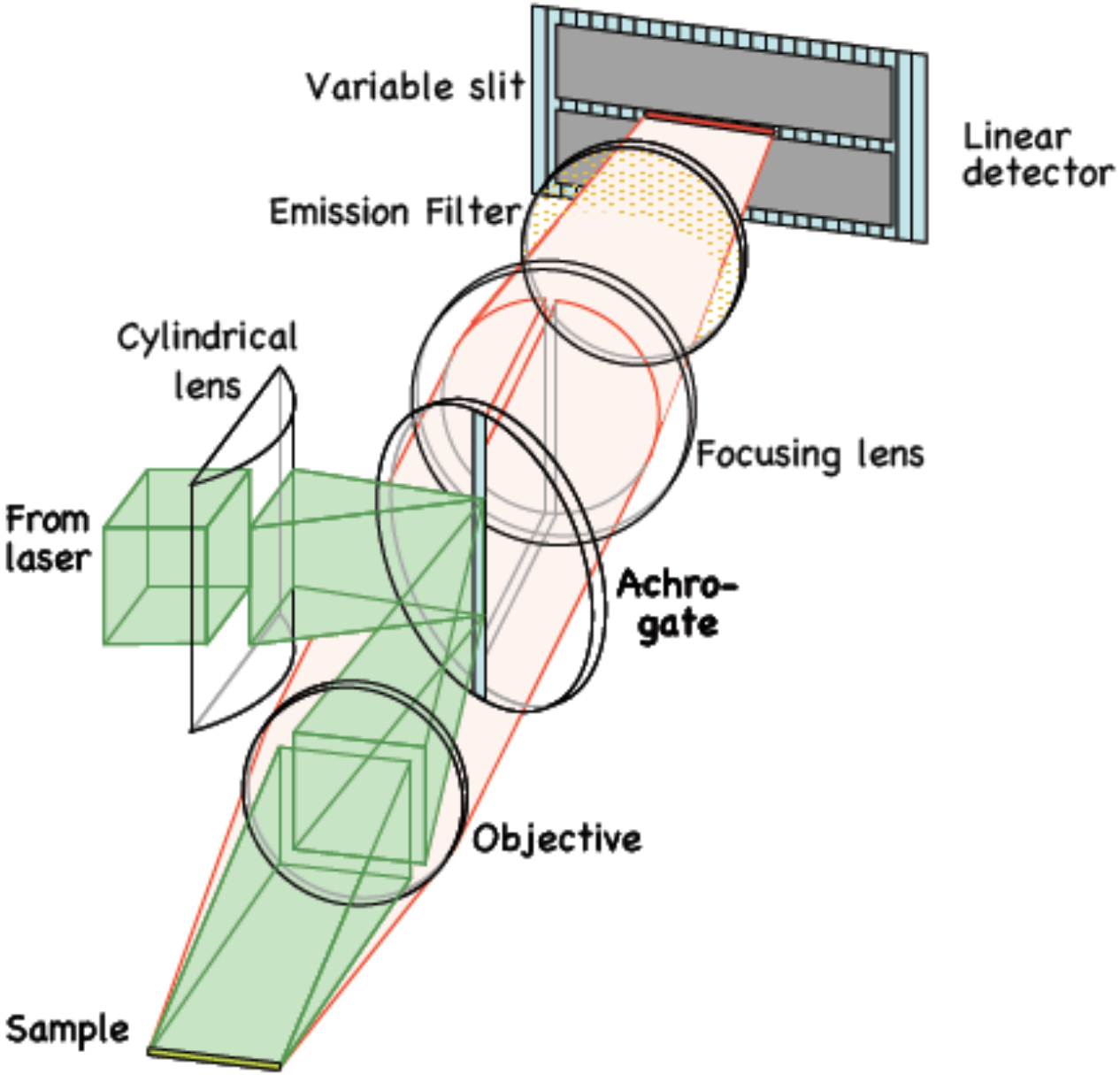
Lateral PSF
100X/1.4
(Good)

Axial PSF
100X/1.4
(Good)

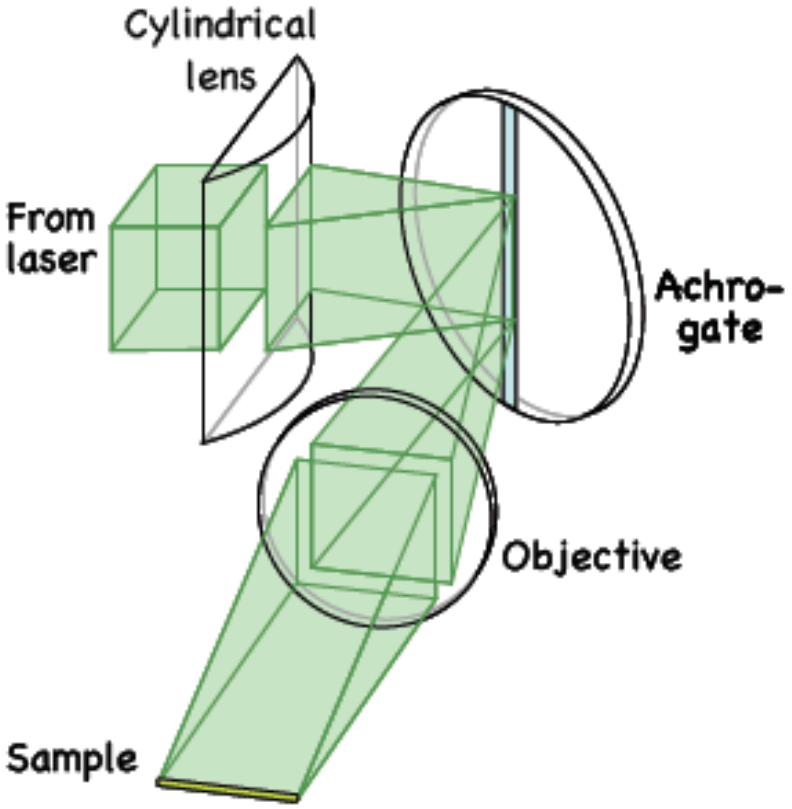
Axial PSF
60X/1.4
(Worse)

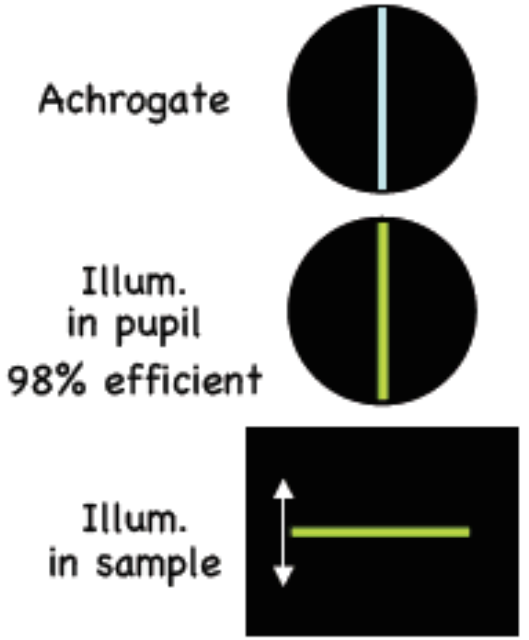
E. Wang, C. M. Babbey & K. W. Dunn, J. Microsc. **218**, 148 (2005)

A Line-scanning confocal microscope: Zeiss 5-Live

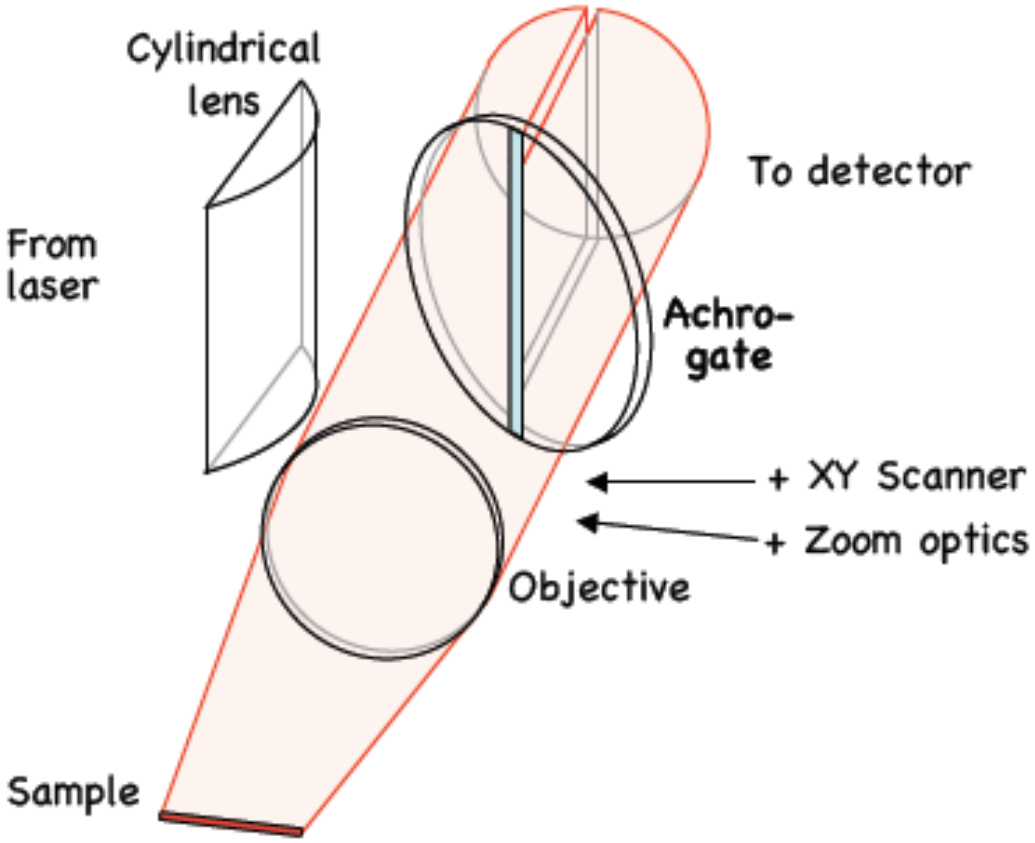


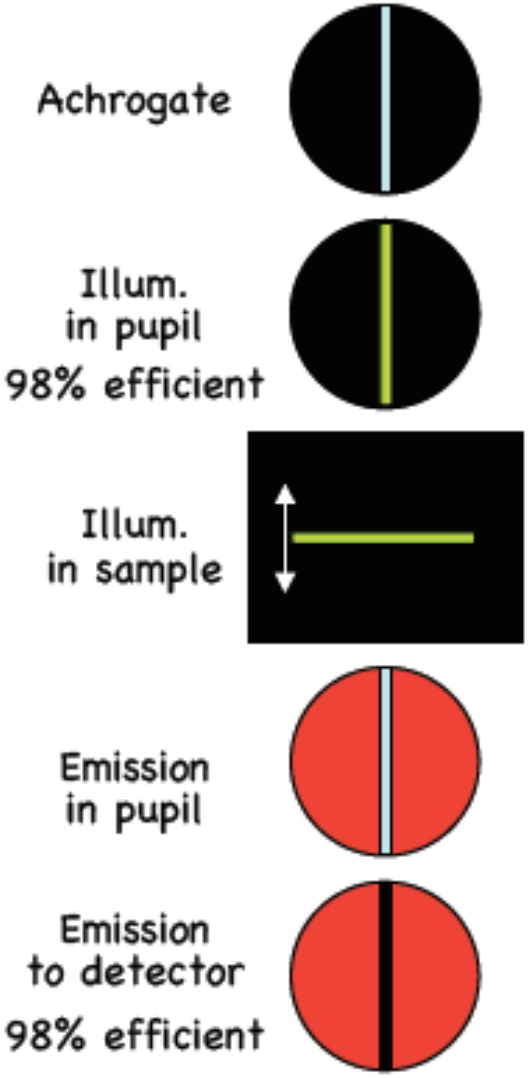
A Line-scanning confocal microscope: Zeiss 5-Live



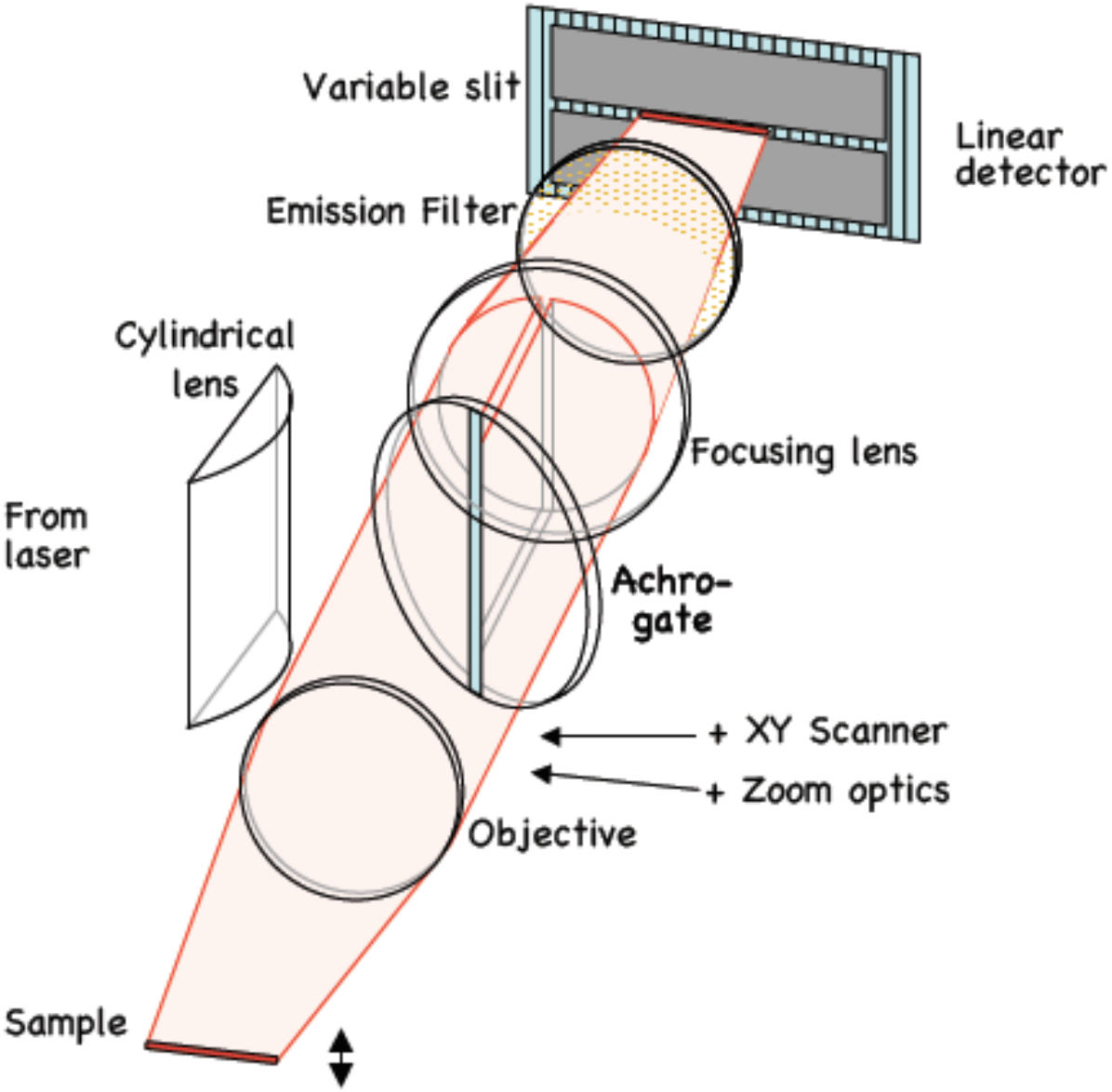


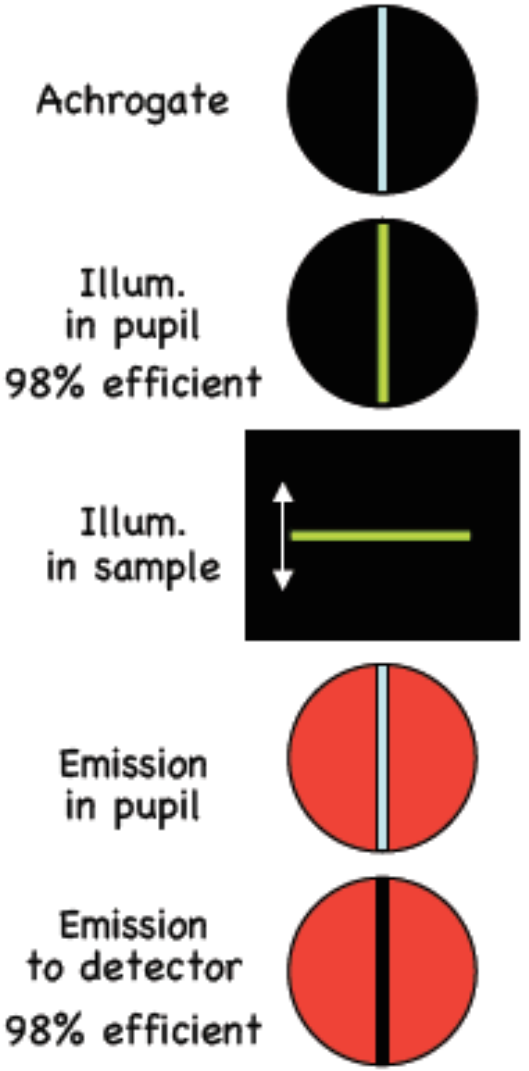
A Line-scanning confocal microscope: Zeiss 5-Live





A Line-scanning confocal microscope: Zeiss 5-Live





Line-Scanning vs Multi-Spot Scanning

Line

- Confocal in one dimension only
- No crosstalk

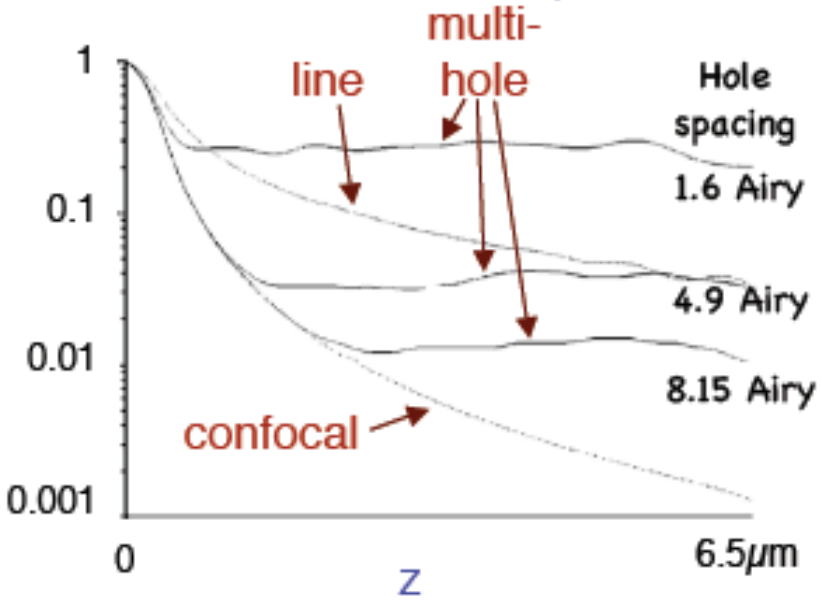
  ⇒
- Confocal y, conventional x resolution
- Semi-confocal sectioning

Multi-spot

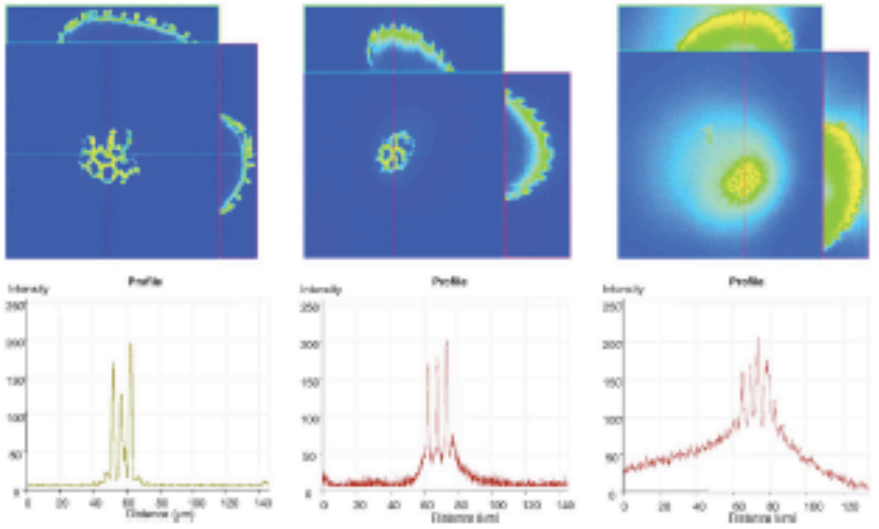
- Confocal in two dimensions
- Crosstalk between pinholes

  ⇒
- Confocal xy resolution
- Confocal z sectioning near focus
- No more sectioning far from focus (blur suppressed by finite factor)

Thin layer axial response

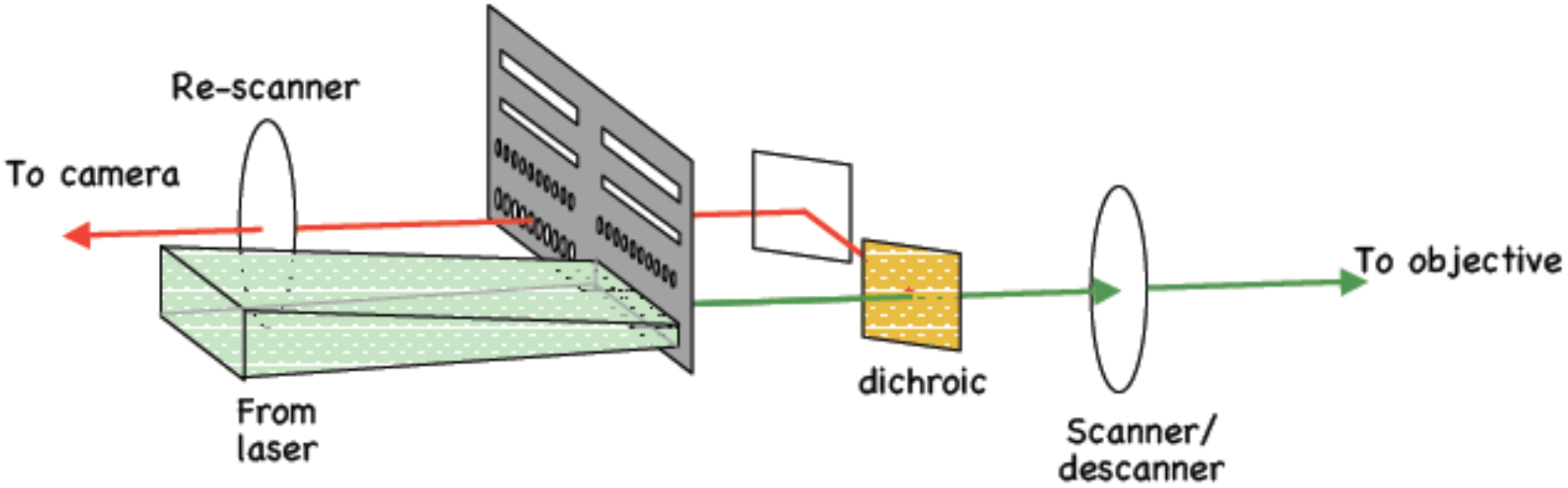


Large (>100 μm) pollen grains

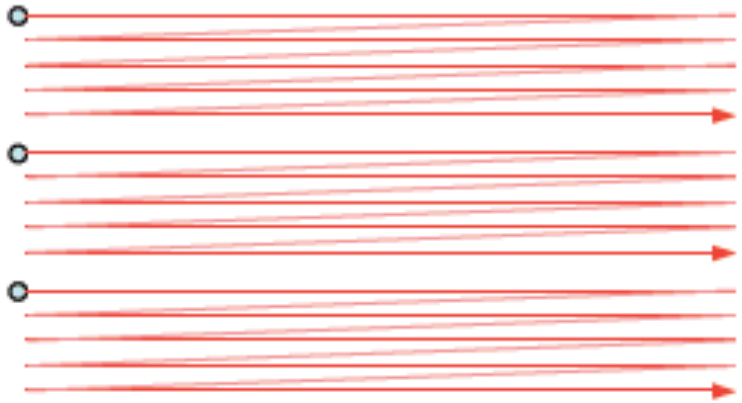


Nikon "Swept-field" microscopy

Multi-spot *or* line-scan

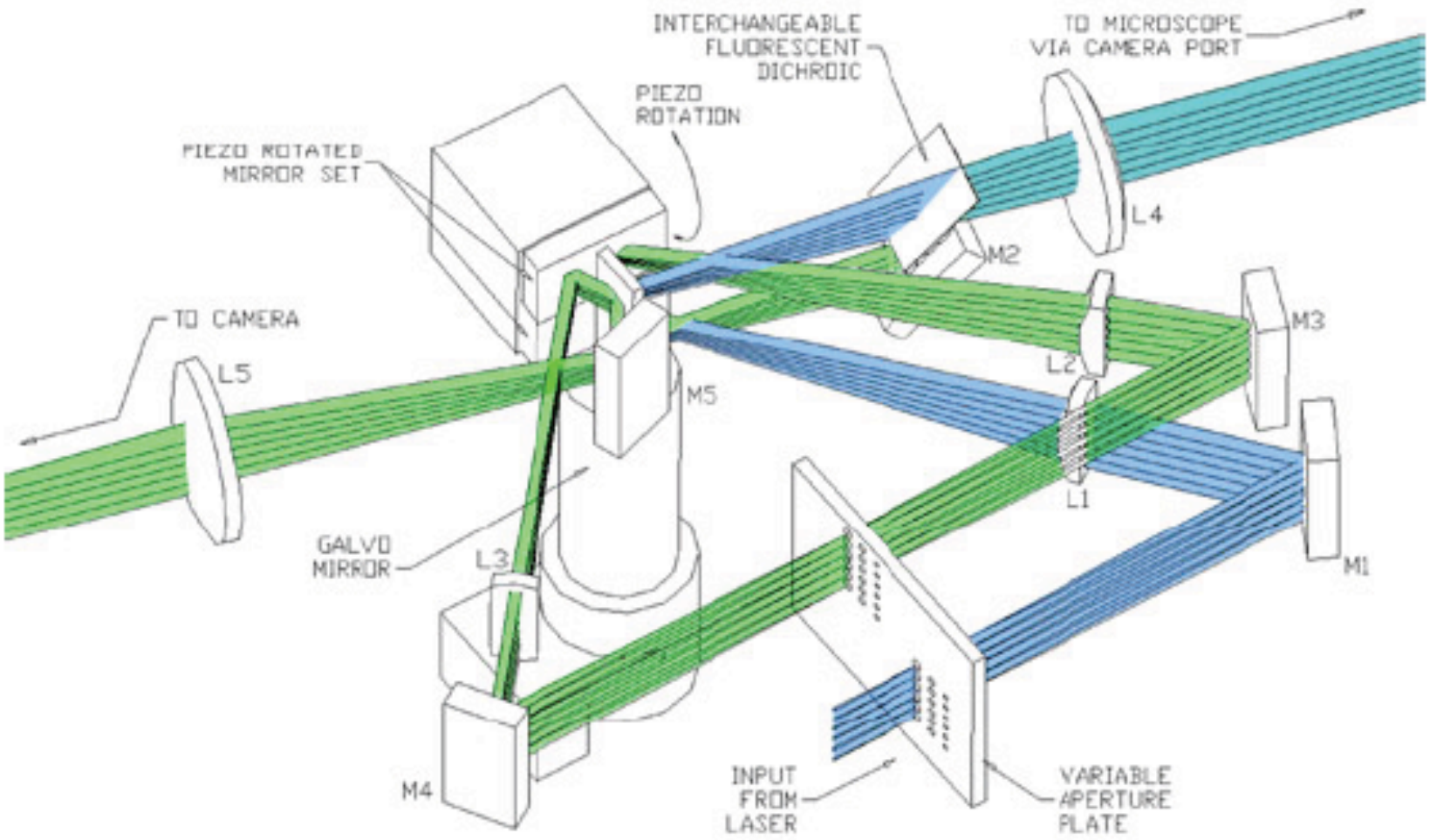


Row of 32 pinholes, or slit
No microlenses
Synch scan and camera
→ 1 scan/exposure
4 pinhole sizes, two line widths
260 Hz in pinhole mode
2600 Hz in slit mode
(if camera is that fast)



Q: How synch the scanners?

A: Reuse same scanners three times!

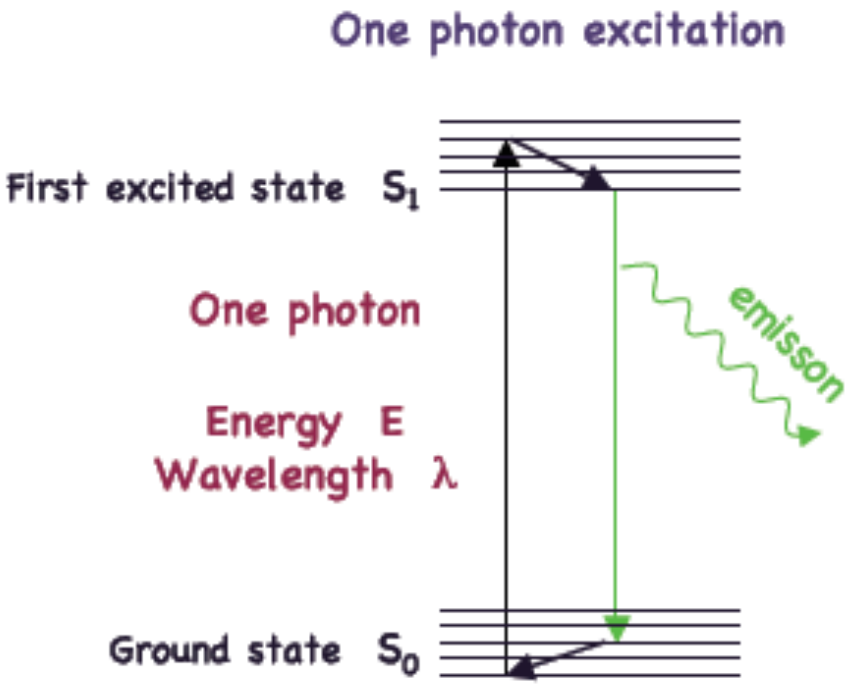


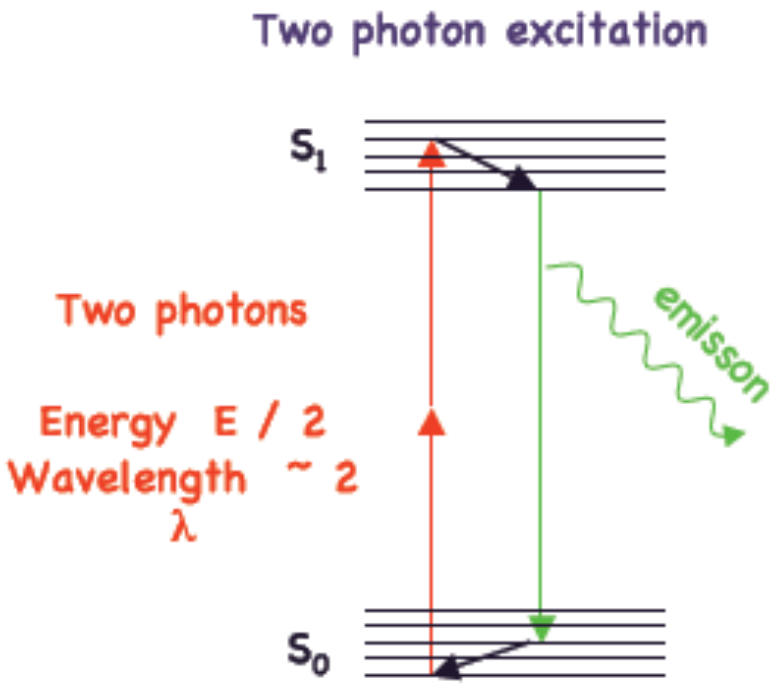
Scanner by Prairie Technologies

Multiphoton Confocal

Two- (and multi-) photon microscopy

Use two excitation photons to do the work of one





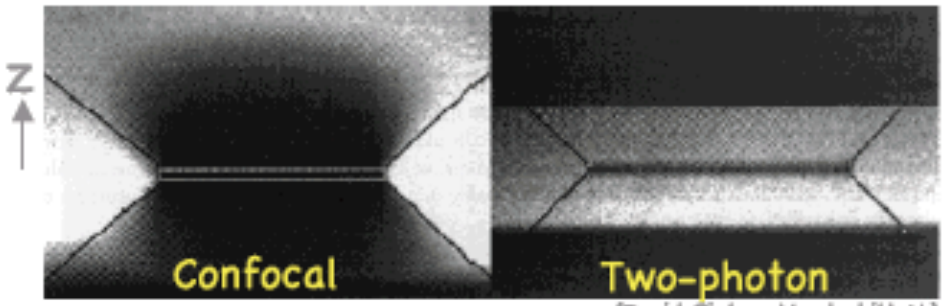
Excitation ∝ (illumination intensity)²

Excitation takes place ~only here at the intensity maximum in the focal plane

Multi-photon microscopy, continued

Strengths:

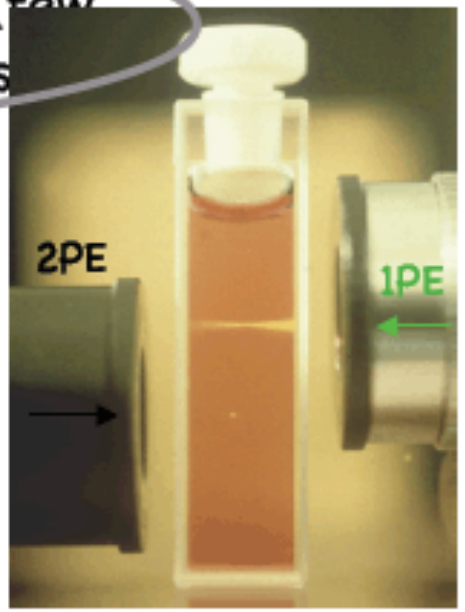
+ Bleaching of the dye is confined to the focal plane



(David Piston, Vanderbilt U.)

+ True 3D imaging (no missing cone) even without pinhole

+ Longer λ → less scattering → can look deep (few 100 μm) into scattering samples (e.g. brain slices)



(Brad Amos, MRC, Cambridge)

+ Useful for well-defined bleaching or activation

Drawbacks:

- Femtosecond lasers cost ~ $200,000
- Non-linear sample damage

Multi-photon fluorescence Requires ultra-short pulses

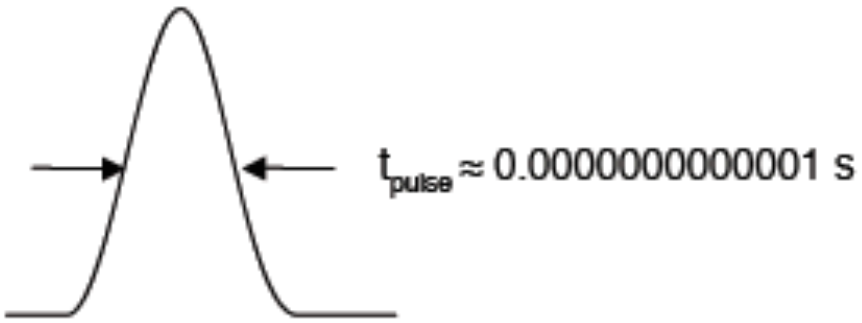
Emission $\propto$ Intensity^2

At a given average intensity, signal goes up if we concentrate the energy into very short pulses

Typical pulse length 50 fs – 2 ps

Need expensive ultra-fast lasers (Ti-sapphire)

Typically tunable, 700-1000 nm



Maintaining the pulse length

Short pulse ⇒ Wide wavelength bandwidth

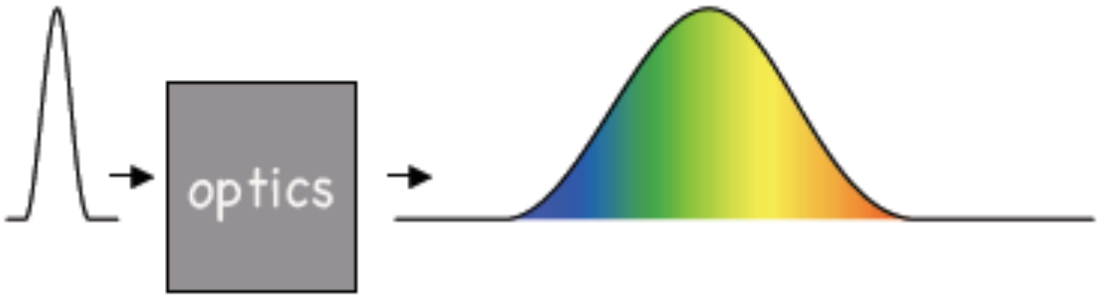
Phase changes between wavelengths mess up the pulse shape

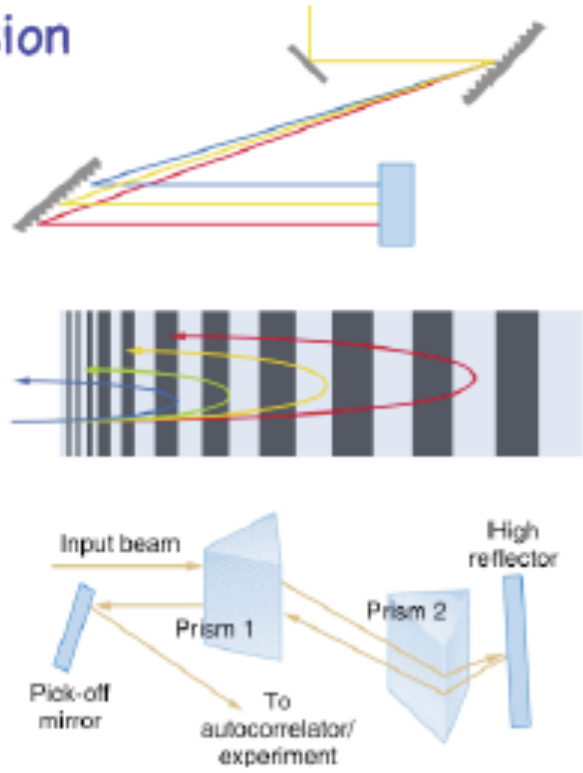
⇒ Dispersion broadens the pulse
(Group velocity dispersion, GDV)

Objectives and microscope optics have dispersion

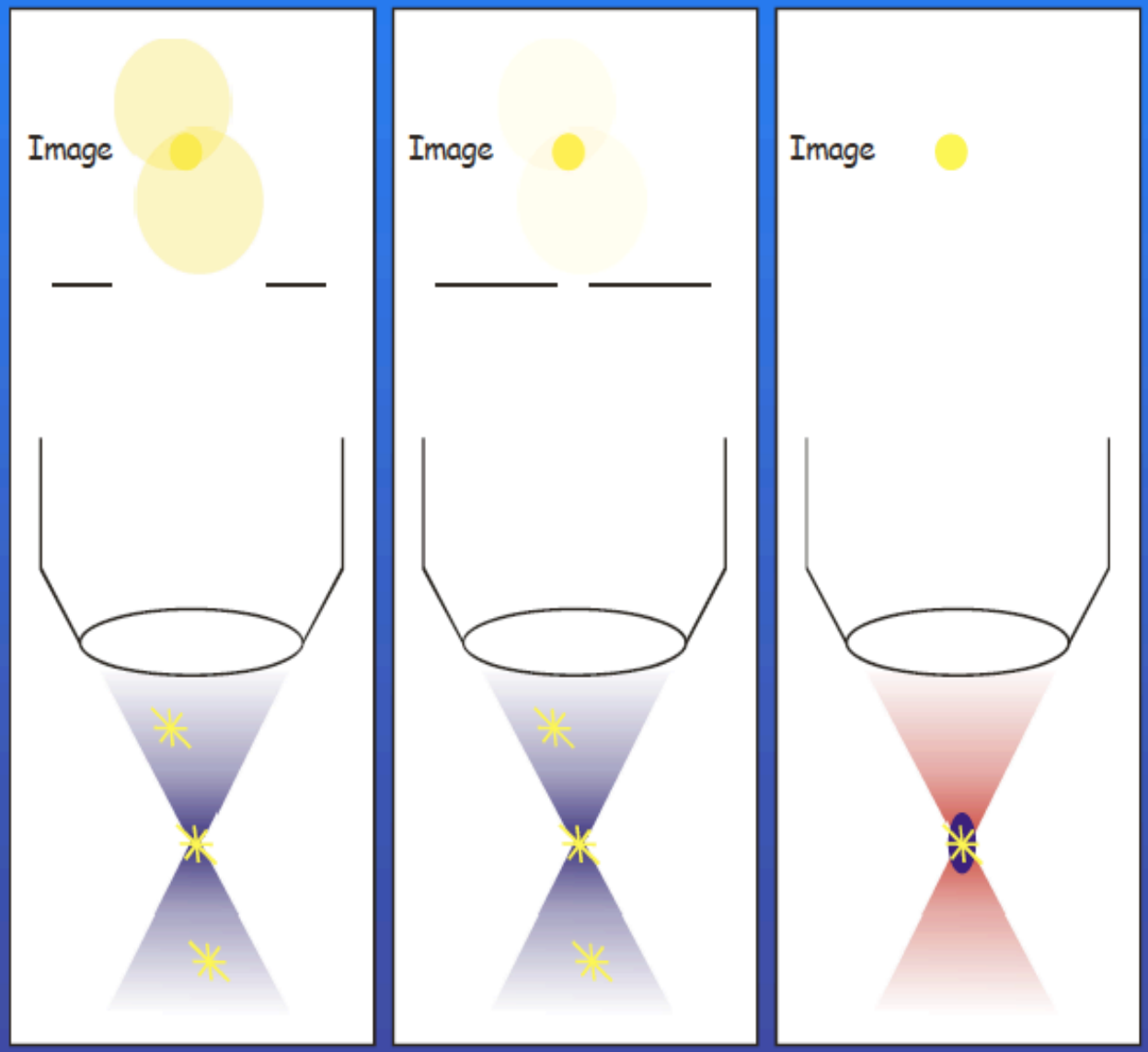
Fibers have lots of dispersion

May need to pre-compensate
by imposing opposite dispersion





Multi-photon confocal microscopy



Multiphoton confocal microscopy

- *A relatively new technique that had some unique advantages*
  - *Excitation of fluorescent molecules occurs at focal plane only, hence:*
    - *Much less bleaching and photo damage*
    - *Pinhole not needed – all emitted light is used*
  - *Based on IR light – much better tissue penetration (hundreds of micrometers)*
  - *Requires special pulsed lasers (very expensive), and special optics*
  - *Mainly used for in vivo imaging*
  - *Lower resolution than single photon confocal microscopy*
  - *Use of multiple fluorophors is tricky.*

Multiphoton confocal

- *Basic idea: use 2 or 3 low energy photons (long wavelength) to simultaneously excite a fluorescent molecule*
- *As this is a very low probability event, it will occur in significant amounts only where photon flux is concentrated enormously*
  - *in space – at focal plane*
  - *In time – by concentrating all energy into incredibly brief pulses (~100 femtosec or 10^{-13} sec) repeated at 80 -100 megahertz*
- *For 2P excitation,*

  Fluorescence α (power) ^{2}

  and

  $(power)_d = 1/d^2 \cdot (power)_{focal\ point}$
- *Therefore, excitation drops with the 4^{th} power of distance from focal plane*

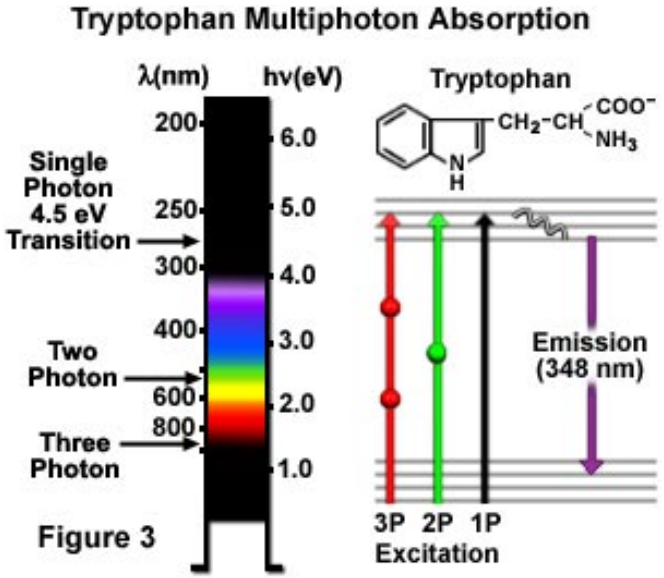


Figure 3

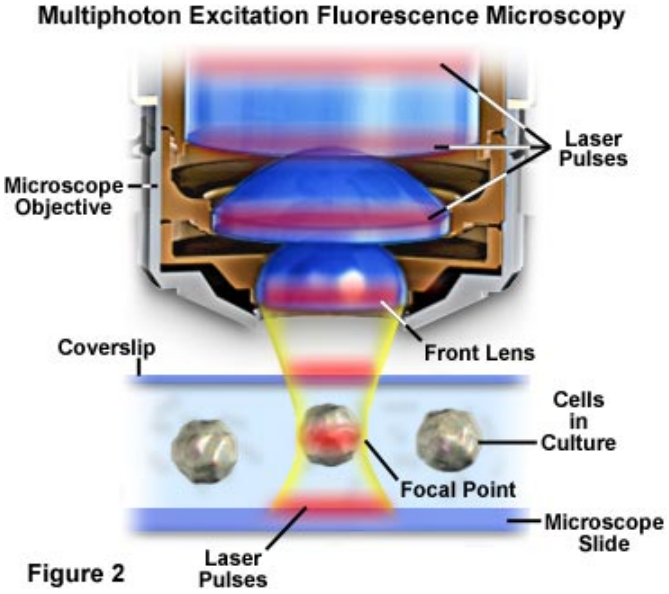


Figure 2

Penetration depth

- Scattering $\propto 1/\text{wavelength}^4$! 

  Doubled excitation wavelength ⇒ 1/16 the scattering

- Don't care about scattering of the emission light

  Intrinsic sectioning ⇒ don't need a pinhole

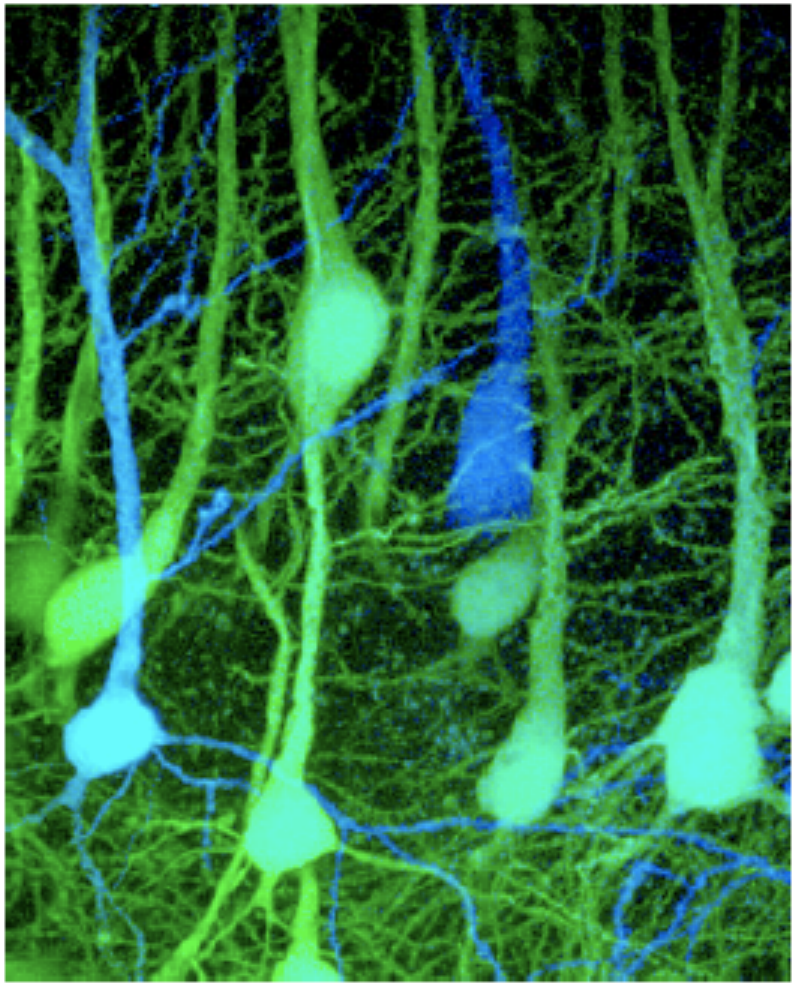
  Point scanning ⇒ don't need a camera

  ⇒ Can collect all photons coming out

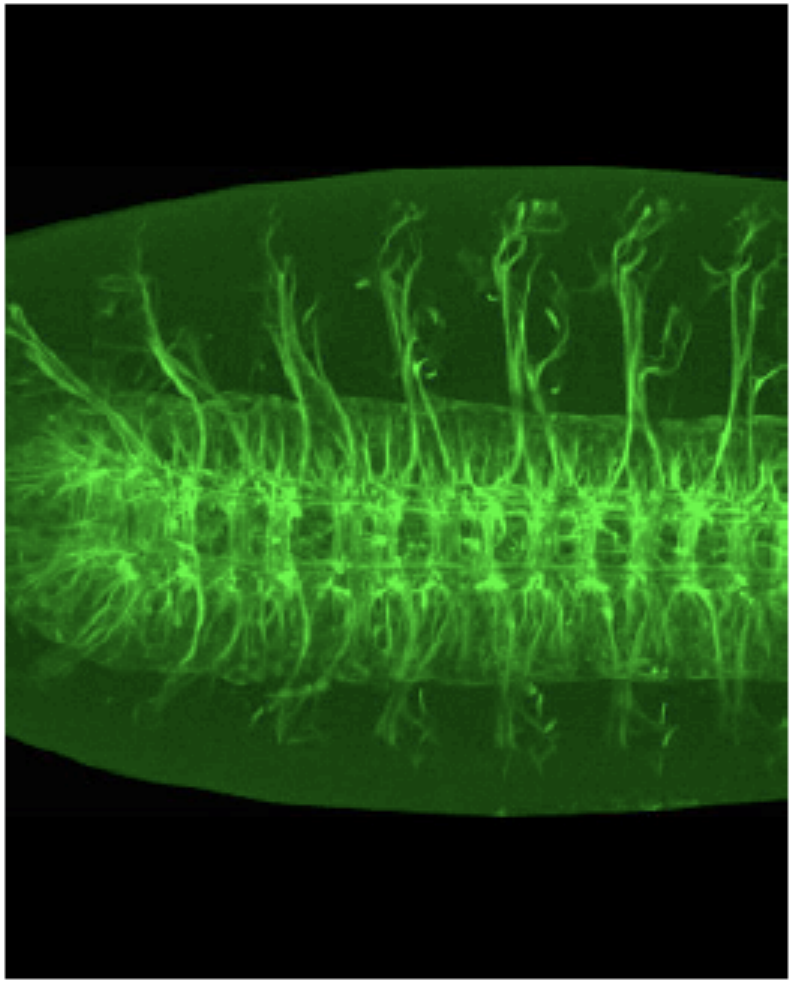
  (can even add front + back side emission)

⇒

Multi-photon microscopy



Brain slice from doubly transgenic mouse expressing CFP and GFP in subsets of cortical neurons



Drosophila embryo expressing tau-GFP in neurons (under the ElaV promoter)

Brand, van Roessel, and Denk, Cold Spring Harbor Imaging Course

SOF