Principles and Practice of Confocal Microscopy

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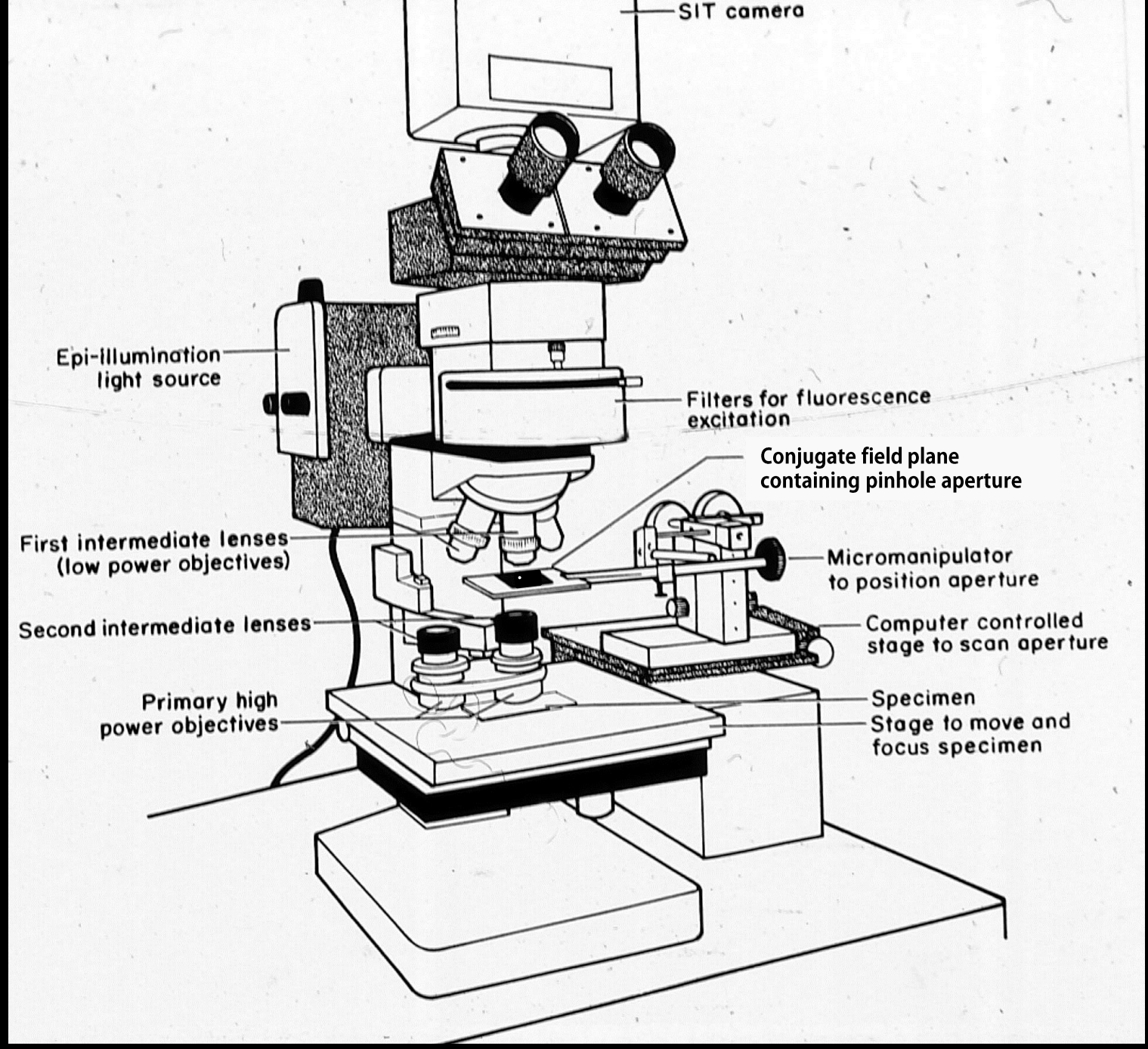
Rat Superior Cervical Ganglion

Closing the field diaphragm in the epi-illumination exciting light pathway

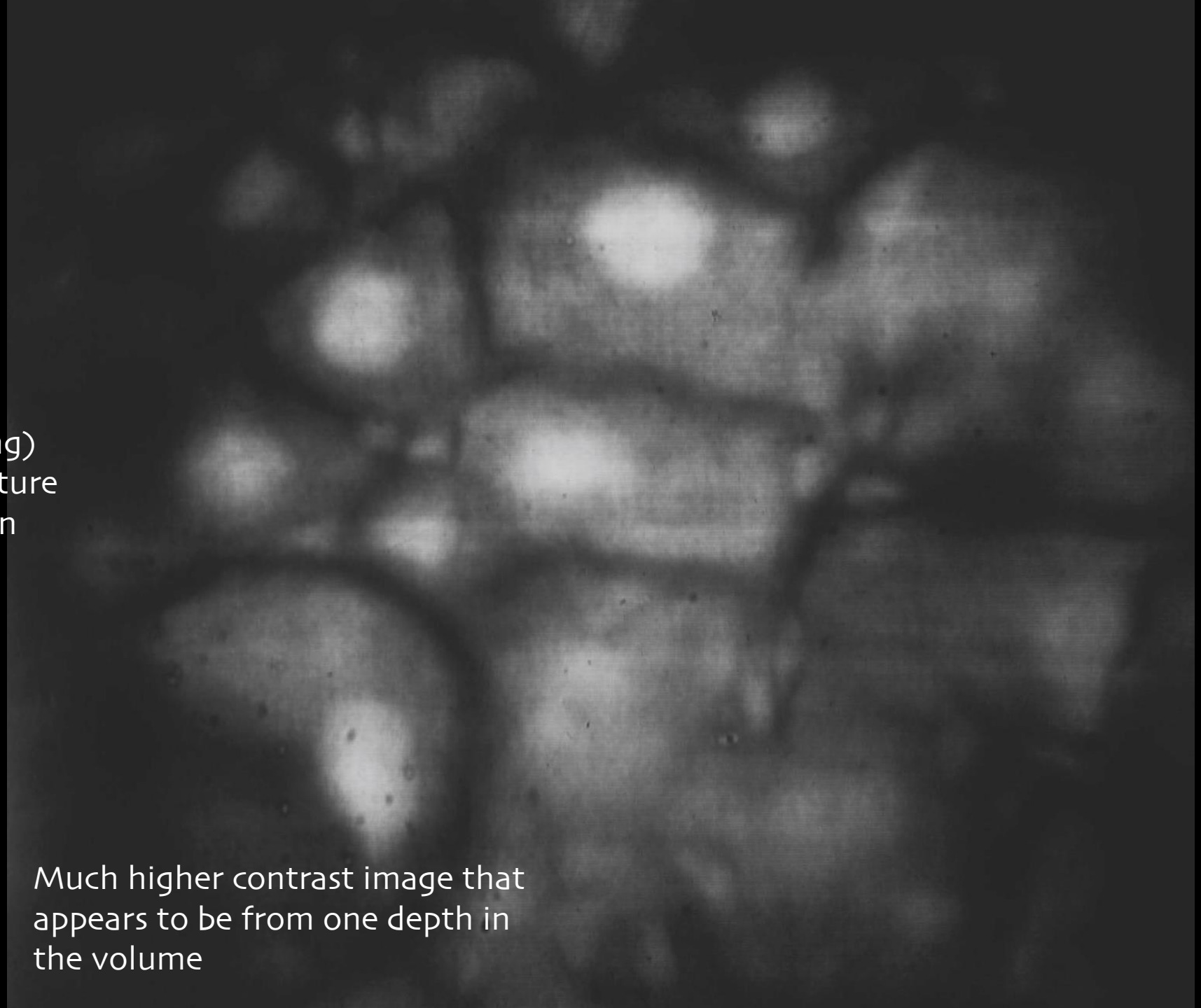
Rat Superior Cervical Ganglion

Closing the field diaphragm in
the epi-illumination exciting
light pathway



Lichtman, Sunderland and Wilkinson , 1989

Scanning (moving)
the pinhole aperture
in a raster pattern



Much higher contrast image that
appears to be from one depth in
the volume

widelfield

Medical Professor Improves Microscope

Reduces 'Scatter' Effect Of Light

By Robert Steyer
Of the Post-Dispatch Staff

For optical engineers who spend months or even years trying to create a better microscope, Dr. Jeff Lichtman's first invention must be both fascinating and infuriating.

Fascinating because Lichtman has found a way to sharpen the images of specimens examined under microscopes by reducing "scatter," a diffusion of light that Lichtman likens to fog being viewed through a car's headlights.

Infuriating because Lichtman is not an engineer — he's an associate professor of neurobiology at the Washington University School of Medicine. Doubly infuriating, no doubt, because it took him only a week to make something that will reduce "scatter" more cheaply than similar devices.

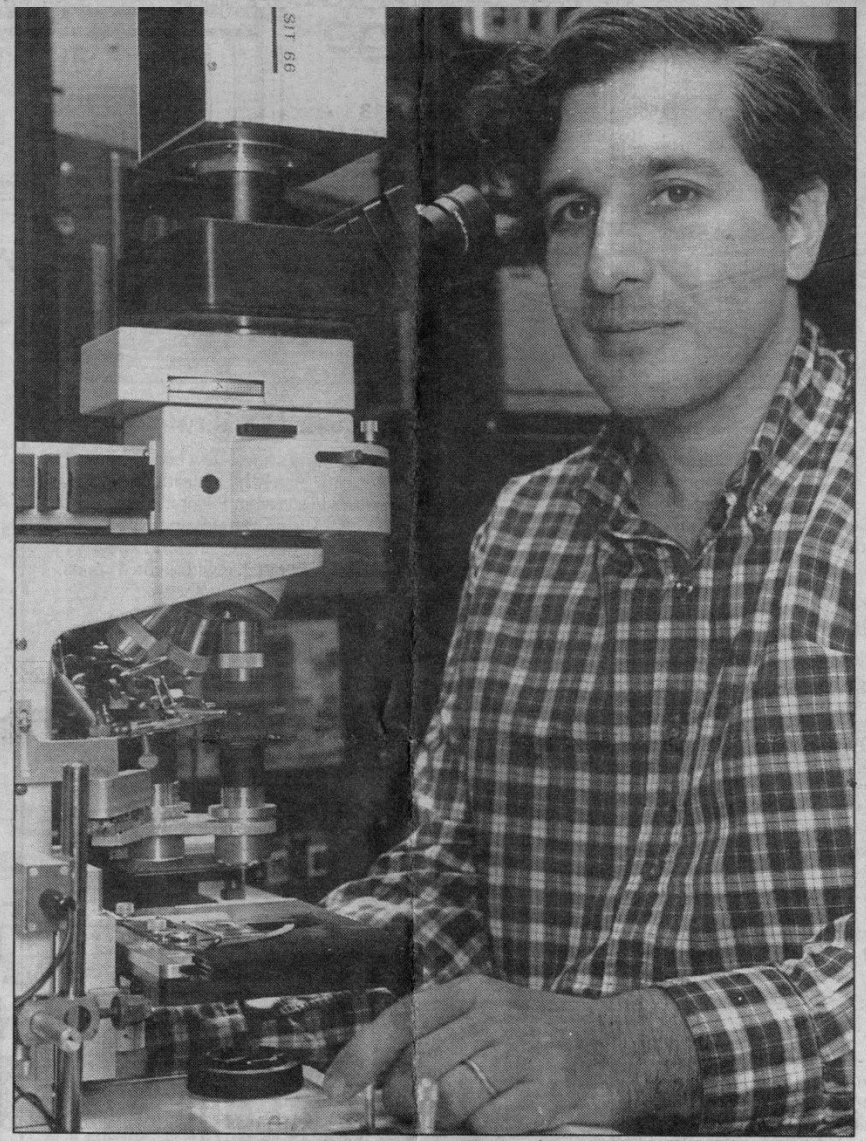
And Washington University officials who license academic research to companies say Lichtman's invention eventually may be used for checking semiconductor chip quality and for helping pathologists identify cancerous tumors. The university is in the final stage of licensing his invention to a Georgia microscope manufacturer.

Optical microscopes date back to the early 1600s, and Lichtman said most advances in this field were assumed to have been made by World War II. "Computers have been been used to improve the lenses, but the consensus has been that the resolution (clarity) limits had been reached," he said.

Lichtman, who specializes in studying how nerve terminals determine control of muscle fibers, said he became an inventor by accident.

In his research, he stains specimens to identify certain nerve endings, but he had problems with a standard microscope. The haze of scattered light reflected by the specimens made it difficult to distinguish pieces of nerve from the surrounding tissue. "Scatter" is especially troublesome when scientists look at living tissue or thick specimens.

So, Lichtman started tinkering with his mi-



Robert C. Holt Jr./Post-Dispatch

Dr. Jeff Lichtman in his office at the Washington University School of Medicine with microscope device he invented.

MICROSCOPE

FUNCTION: Used in studying cell biology.

PROBLEM: Traditional devices permit too much hazy light reflecting off specimens. This "scatter" hampers scientists' ability to distinguish tiny features in cells of tissue.

SOLUTION: A special attachment that removes "scatter" and improves clarity at a lower price than similar confocal microscopes.

INVENTOR: Dr. Jeff Lichtman, 37, associate professor of neurobiology at Washington University School of Medicine. Received M.D. and Ph.D. in neurobiology from Washington University.

PROSPECTS: Washington University close to a licensing deal with a microscope company. Production could start within six months after signing contract.

The company will pay product development expenses and patent application bills, and it will give royalty payments to the university based on sales. Leahey would not identify the company.

The university still must clear a patent hurdle caused by a patent for a confocal microscope that IBM received 10 years ago. Although IBM did not make a product, Washington University still may have to negotiate a license with IBM to pay what Leahey said is a "reasonable" royalty in order to manufacture Lichtman's device.

The university maintains, however, that Lichtman's invention is different than the one covered by the IBM patent. The university is asking the U.S. Patent Office for a separate patent for Lichtman's device.

Whatever the outcome, Lichtman will be "well compensated," Leahey said. So will William S. Sunderland, a former colleague of

Confocal Microscopy is a *scanning* technique

What does that mean? Not "widefield" deal with one part at a time

Potential advantages of scanning (mostly unrealized):

Better resolution (***Lukosz's principle)***

Stage scanning:

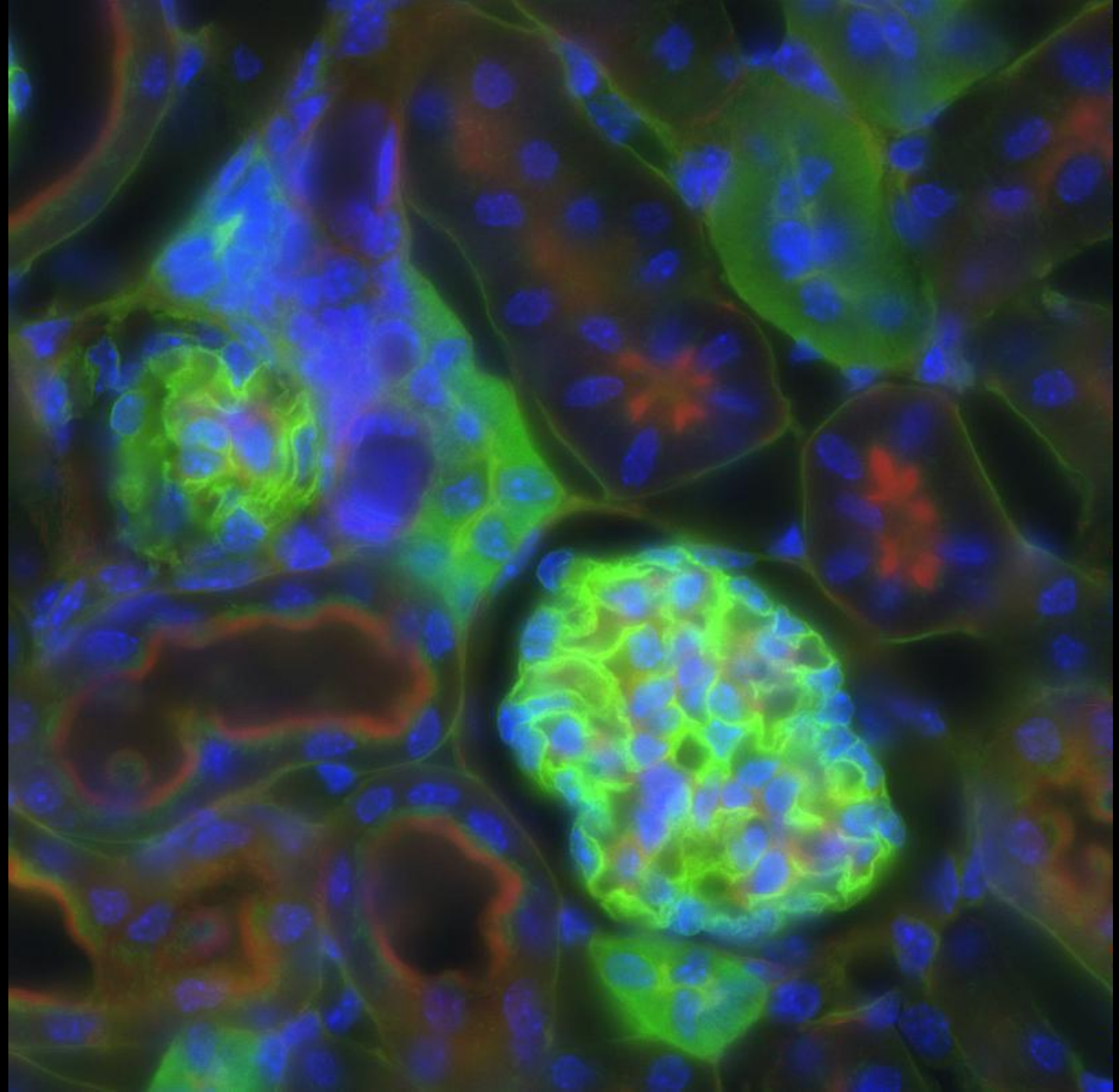
- Infinite image size
- Use center of objective (less aberrated)

Disadvantages:

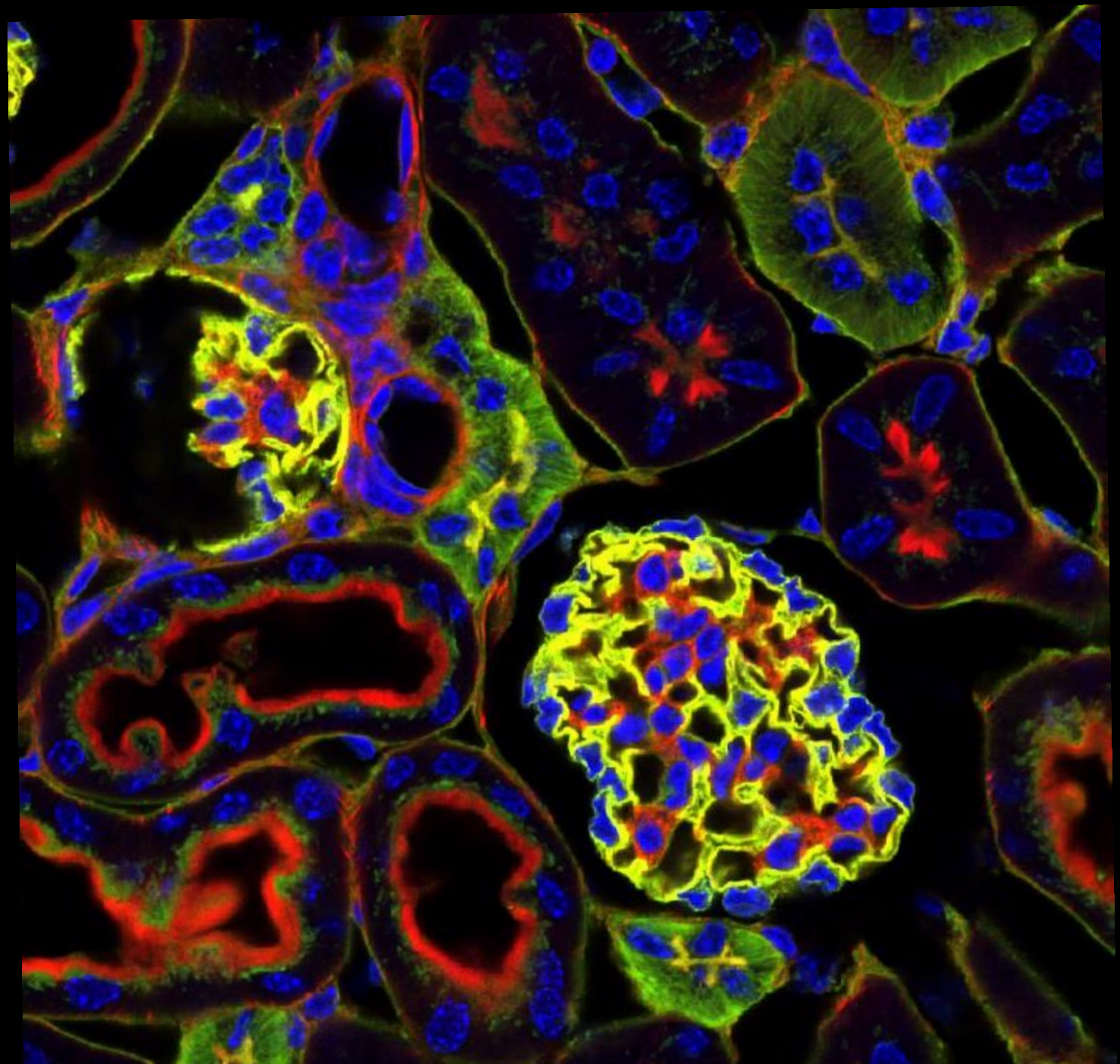
Slower than wide field

Often not direct view

Confocal Microscopy improves images both qualitatively and quantitatively



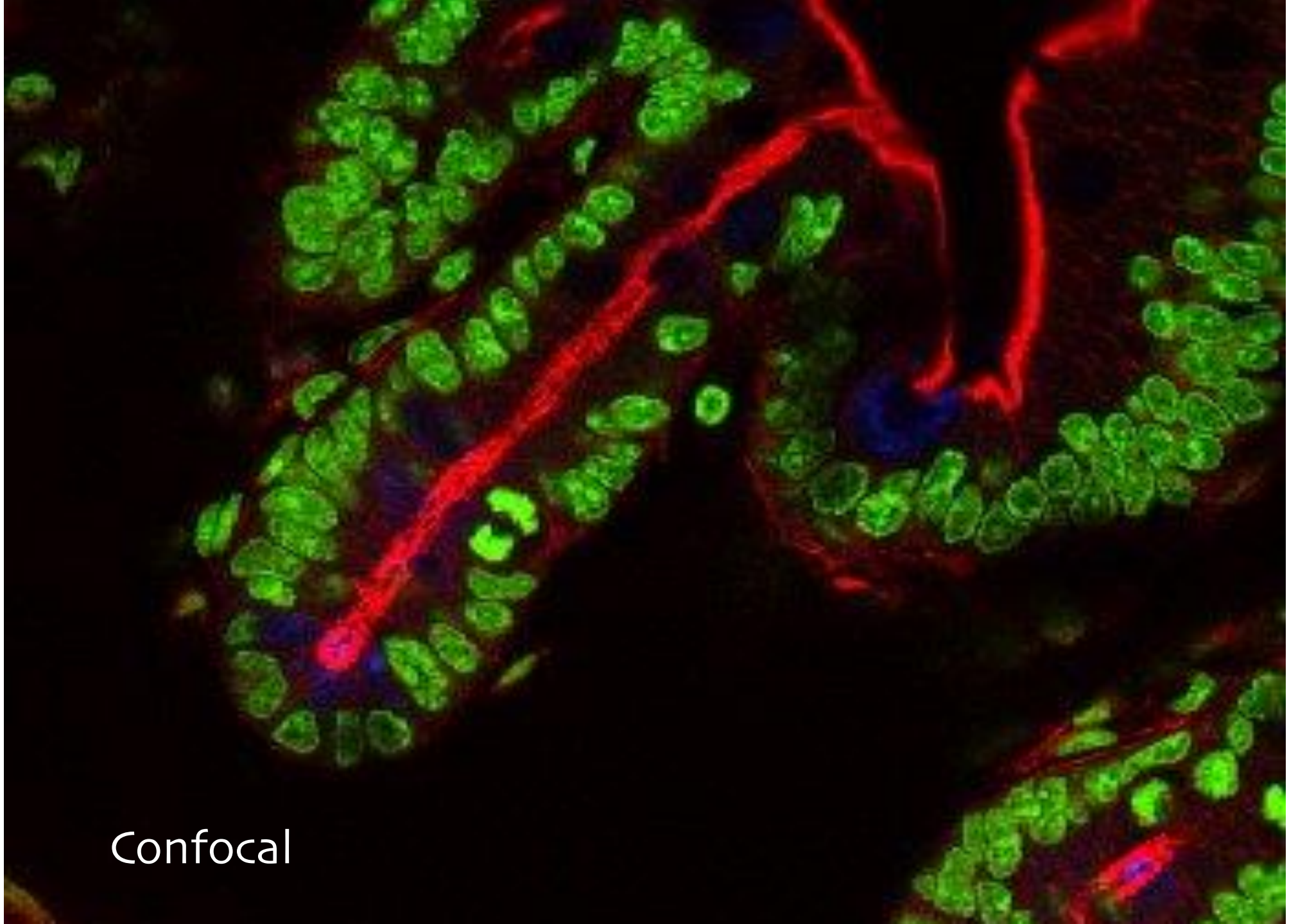
widefield



confocal


What's wrong with this image?

Dropped brightness :What’s wrong with this image?


Confocal

The confocal Image

3 Independent Improvements:

1) Better Contrast (by virtue of less scattered light in image)

2) Optical-sectioning (reduces contribution of out of focus blur to image which also improves contrast)

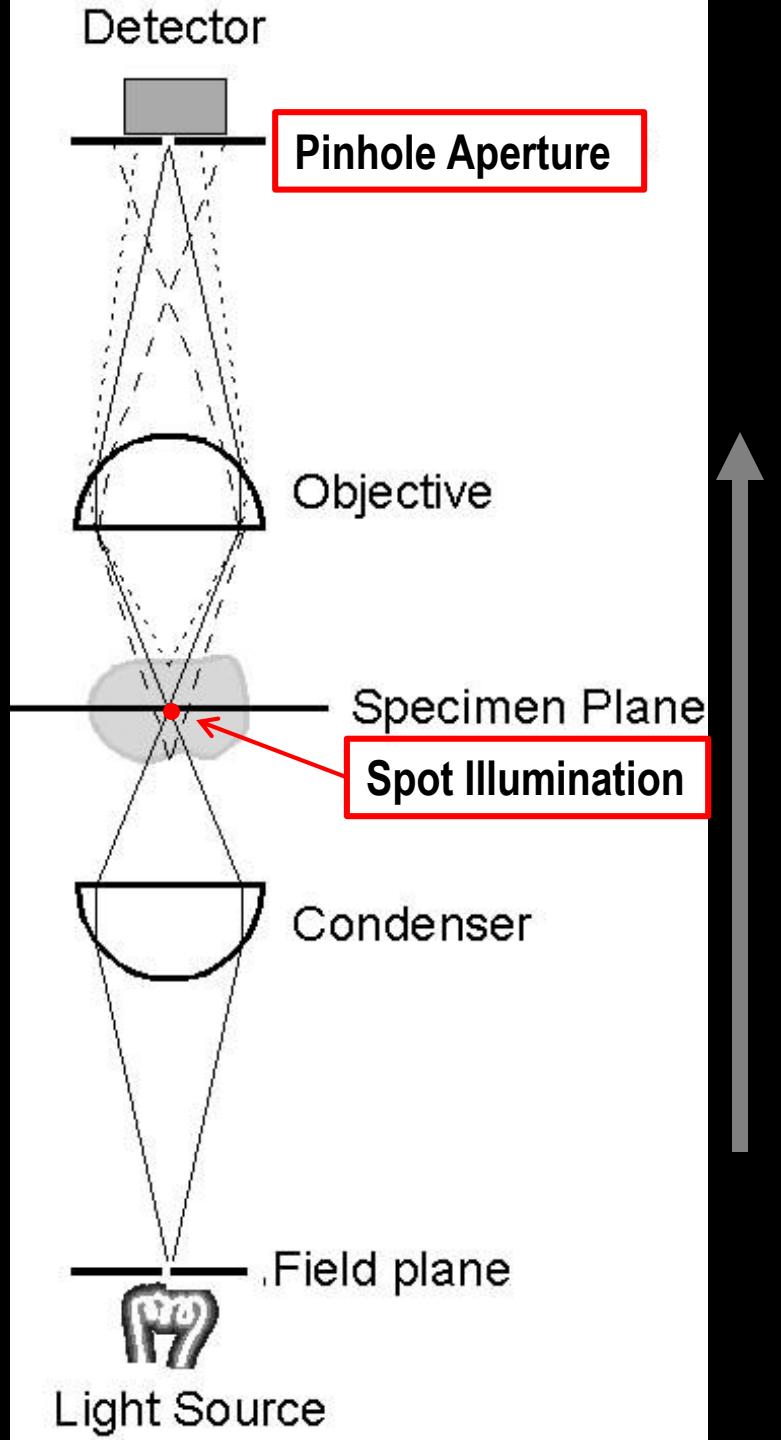
3) Resolution (mitigates the limitations based on diffraction of light)

Lots of ways to do confocal but all of them have the following three design principles:

1) illuminate only a small region of the image plane at a time (hourglass-shaped beam)

2) use an aperture in the return light path to only detect the light from the illuminated area

3) scan to generate an image

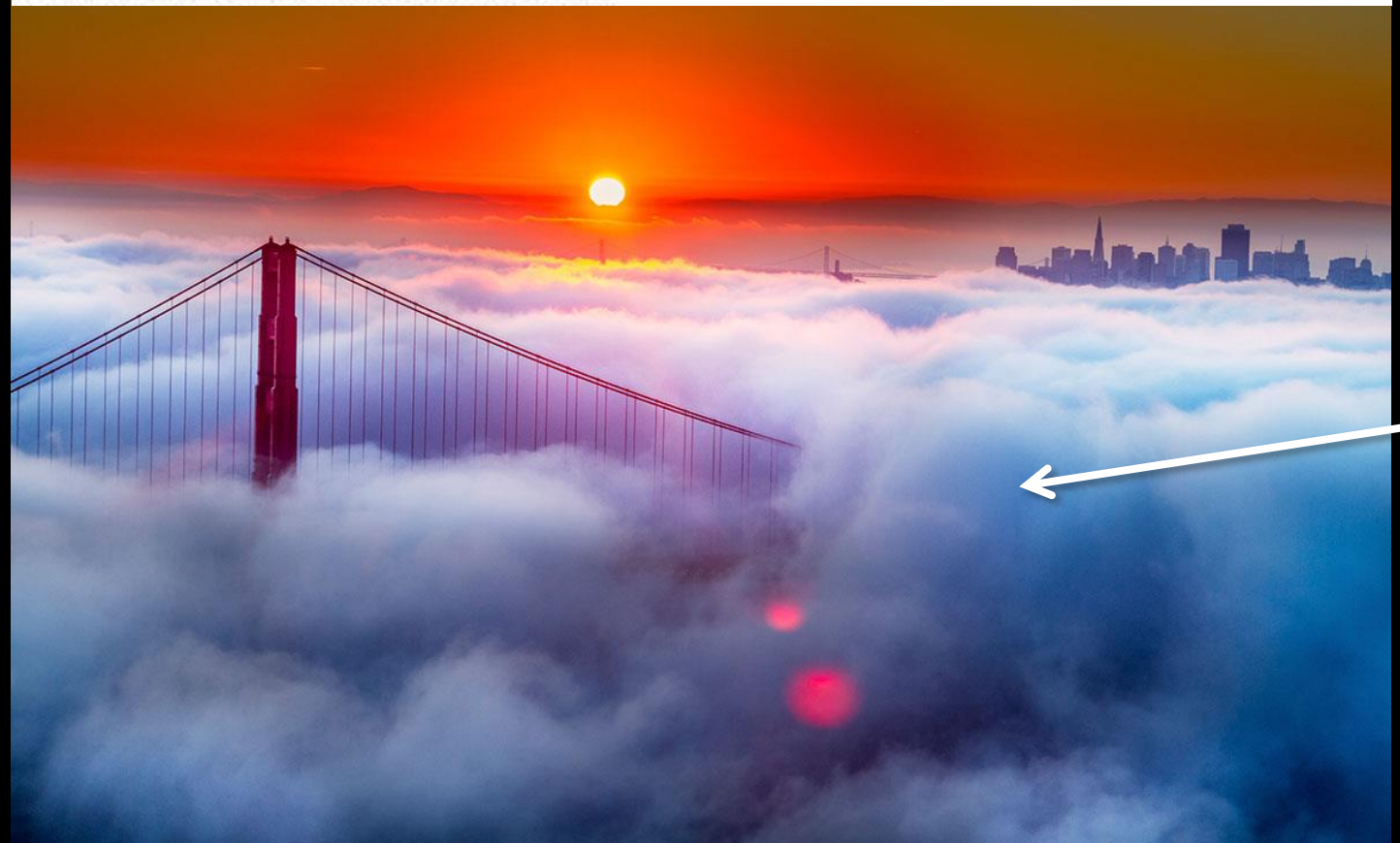


Light scatter

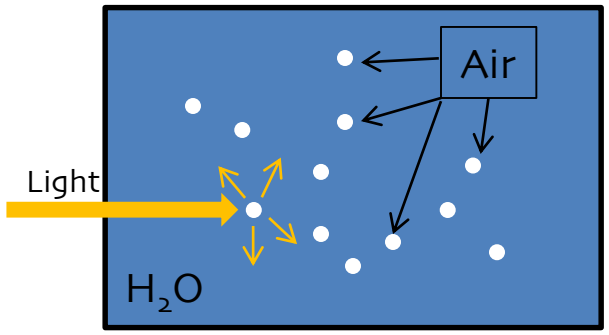
- What is it?
- What harm does it do?
- Why does it occur?
- How can we prevent it?

Scattering

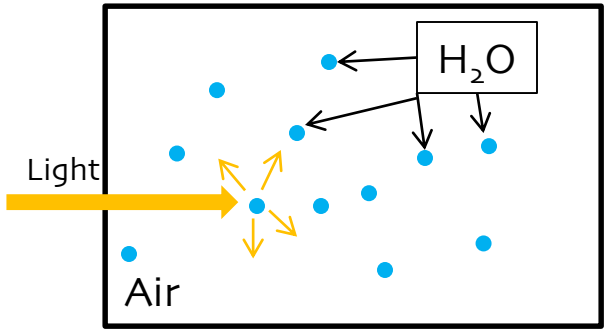
Air making
clear water cloudy



Water making
clear air cloudy



Cloudy water

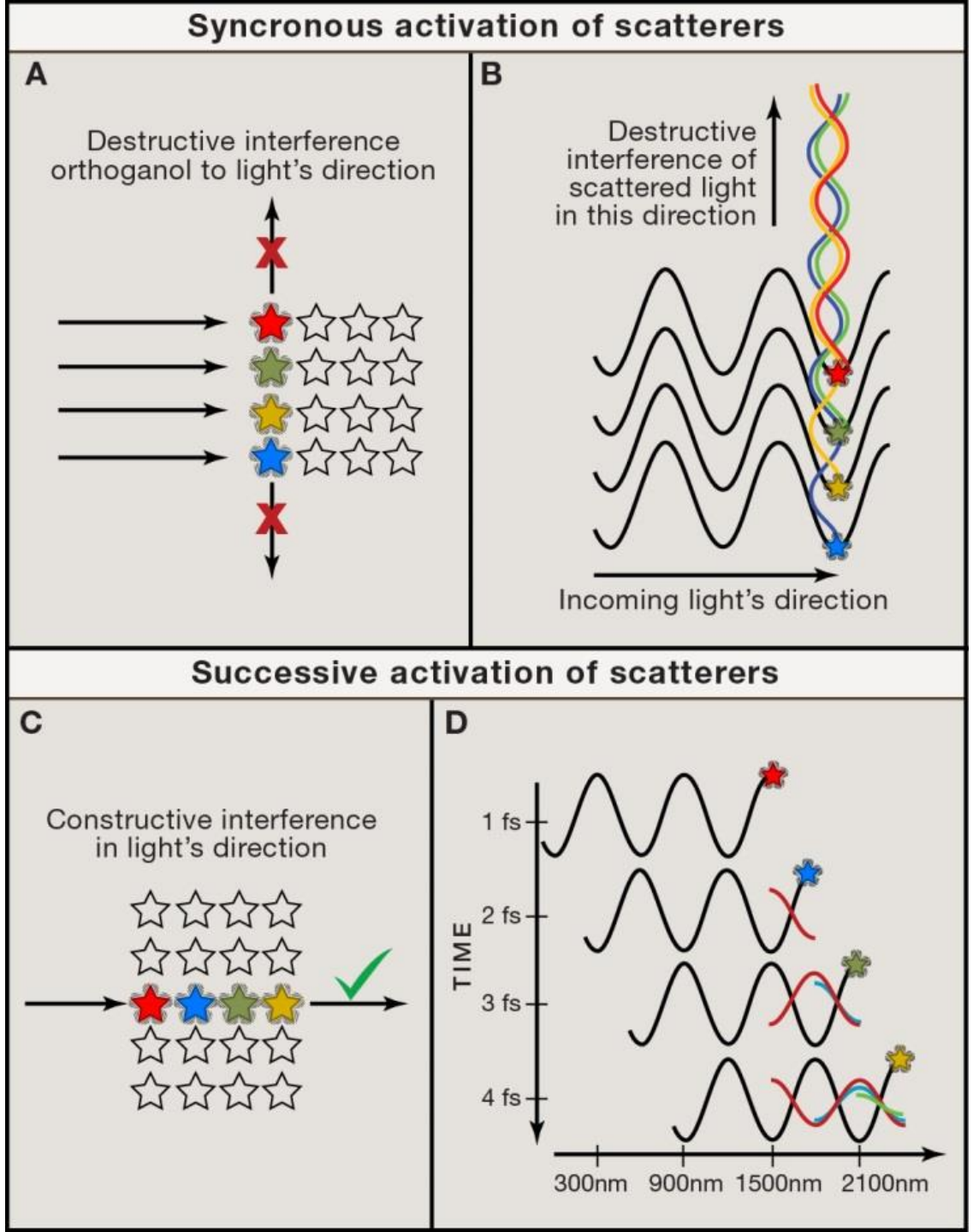


Cloudy air

The fact that clear spheres dispersed at low density (greater than the wavelengths of visible light between them) in a clear medium leads light to go every which way means that scatter is not explicable by simply saying some kinds of atoms scatter light.

Indeed even homogeneous **"non-scattering" media** (like pure air or water) where light exits continuing on along the same path it was going when it entered are in fact scattering the light. It is just that most of the **scattered light is annihilated** by interference because the scatters are very densely packed (much closer together than the wavelength of light).

Scattering



All interactions of light and matter that slow the speed of light (refractive index >1) scatter light

This includes clear materials (air, water, glass)

Light wave is absorbed by electron and re-emitted ~1fs later as a spherical wave (elastic scattering- no energy loss ≠ fluorescence emission). This is the fundamental cause of the refractive index!

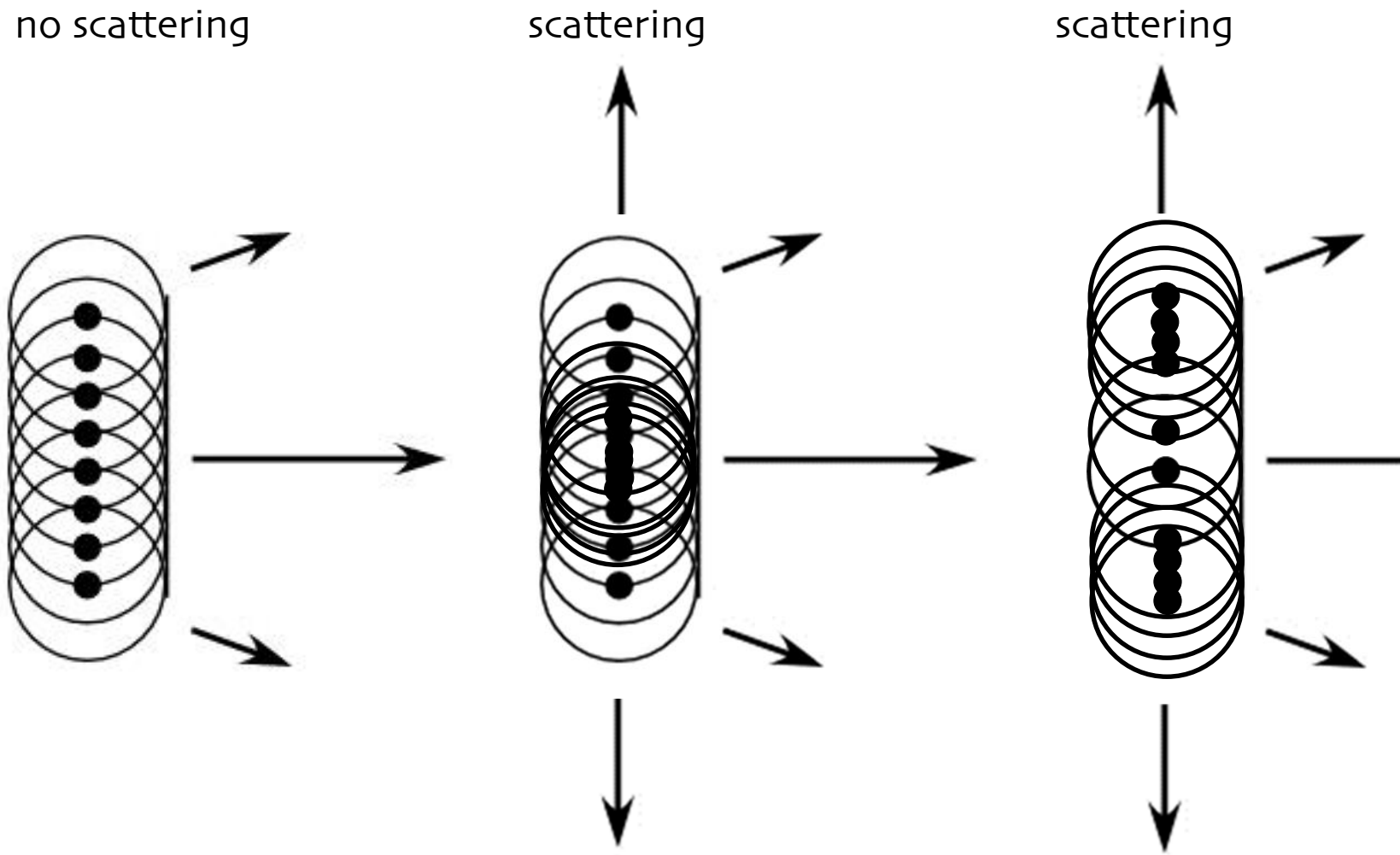
For clear materials:

Destructive interference orthogonal to light's original direction (from synchronous scatterers)

Constructive interference along the direction of the path from which light entered the medium (from sequential scatterers)

Richardson and Lichtman, Cell 2015

Huygens wavelets in a plane wave propagate in the forward direction only if their density is high and uniform

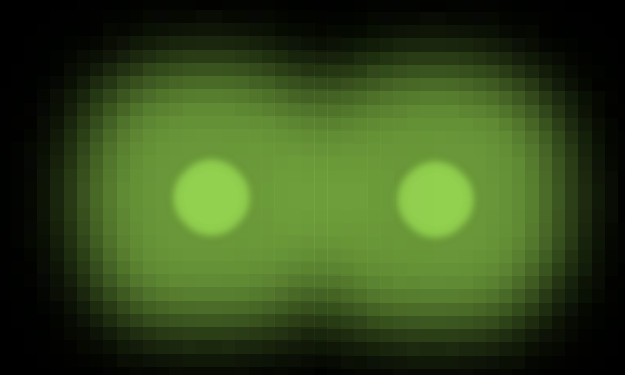


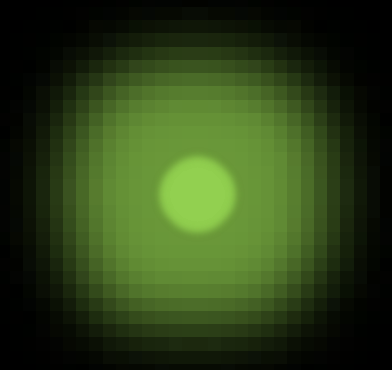
Uniform density of scatterers
pure water, air or glass

Regions of high density of scatterers
e.g., fog

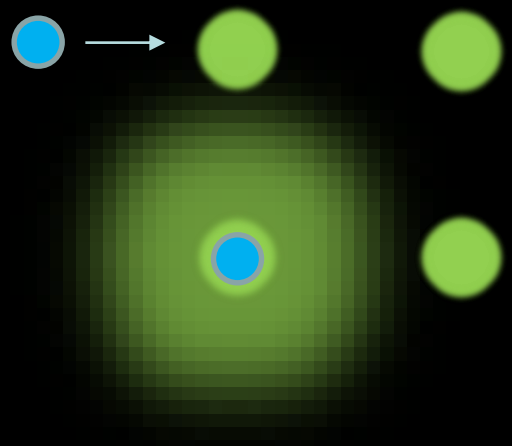
Regions of low density of scatterers
e.g., aerated water

Key therefore is to homogenize the scattering i.e. with "clearing" agents





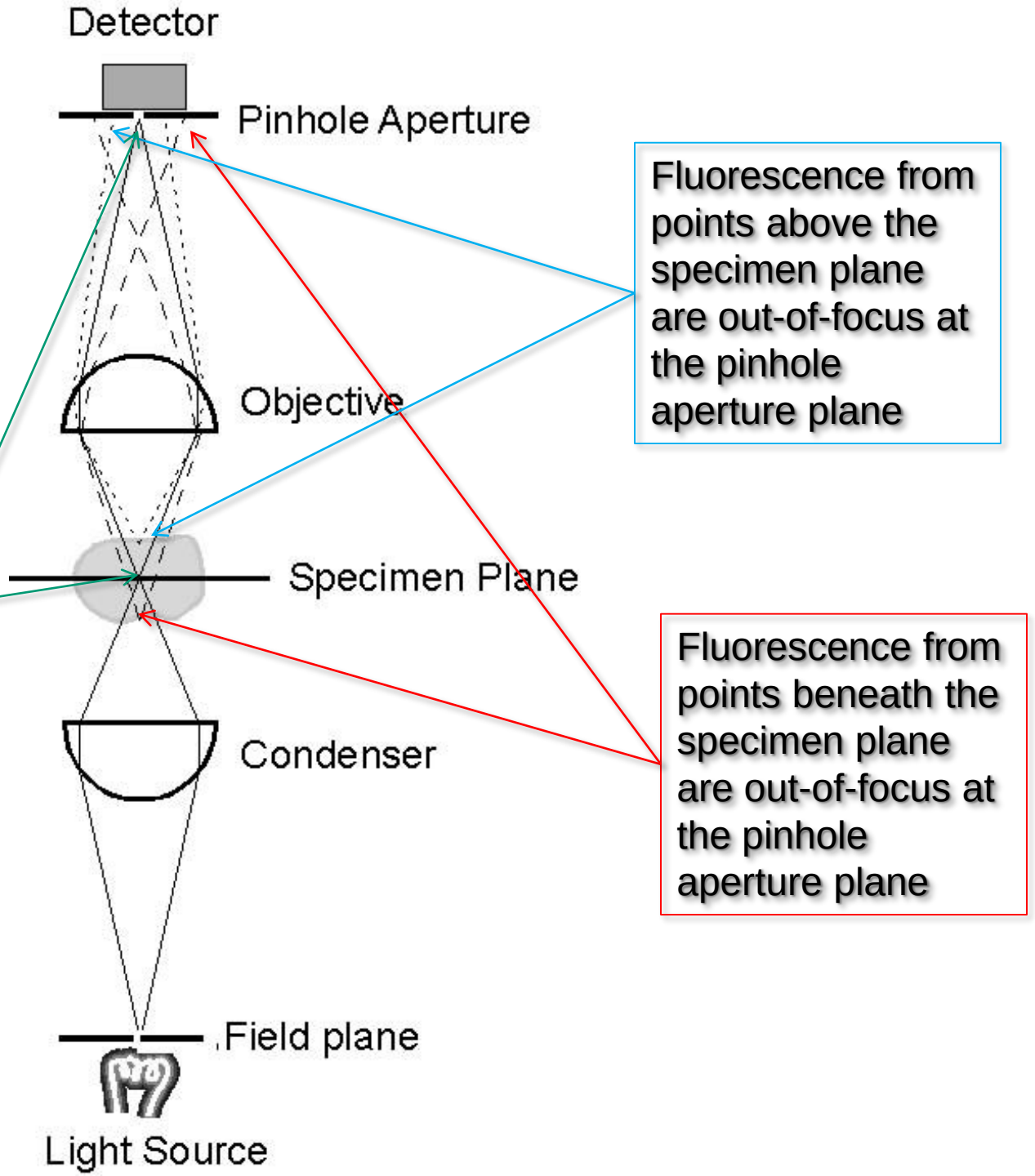
How confocal scanning improves contrast



No scatter when beam is not on object
&
No detection of scatter when beam is on object because the scatter is off the axis of the pinhole in the return light aperture

Confocal Image Improvements:

1) contrast
2) optical-sectioning
3) resolution



Fluorescence from points above the specimen plane are out-of-focus at the pinhole aperture plane

Fluorescence from points at the specimen plane are in focus at the pinhole aperture plane

Fluorescence from points beneath the specimen plane are out-of-focus at the pinhole aperture plane

Ways of looking at confocal optical section image data

Confocal filters out light from the image that is out-of-focus

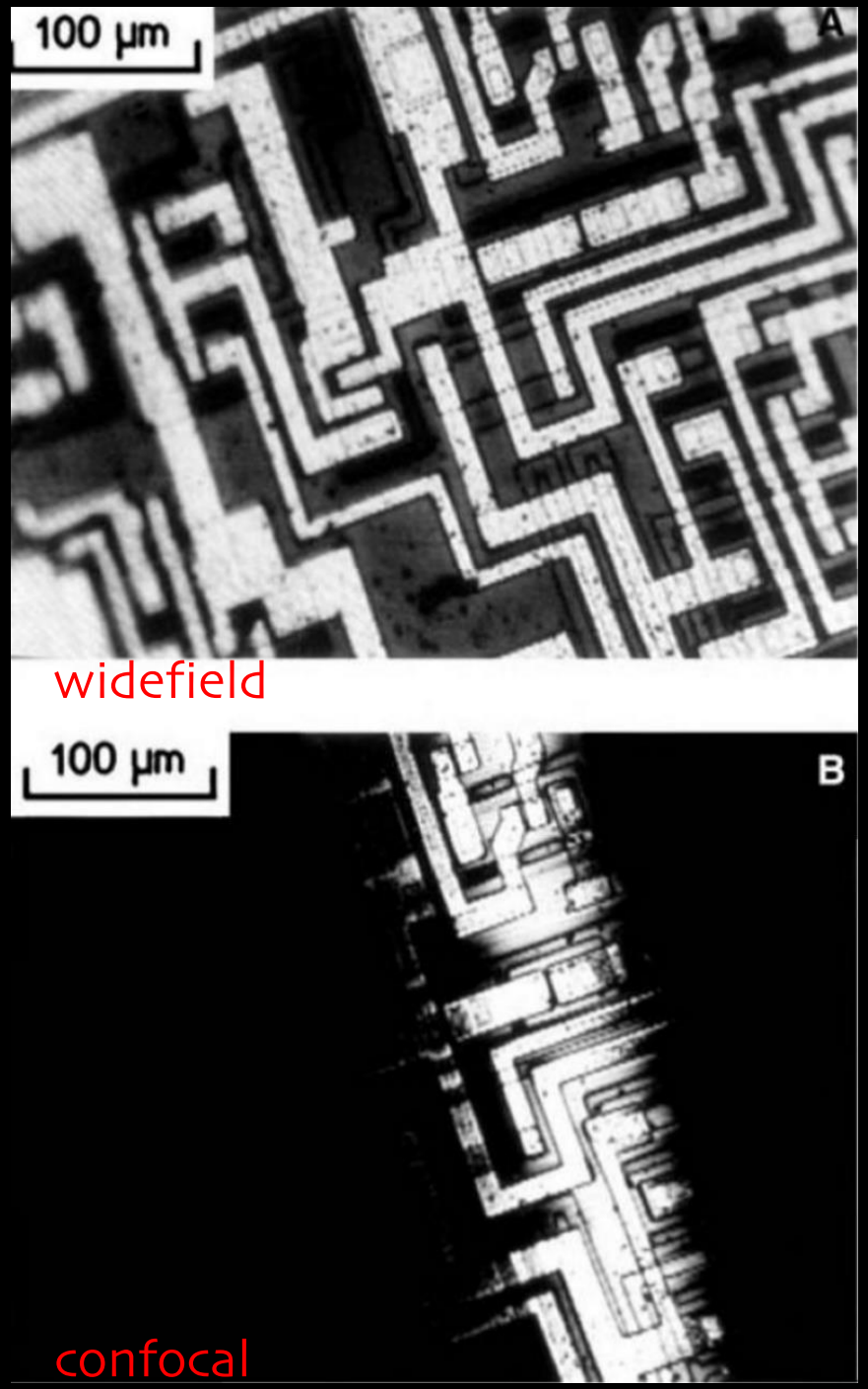
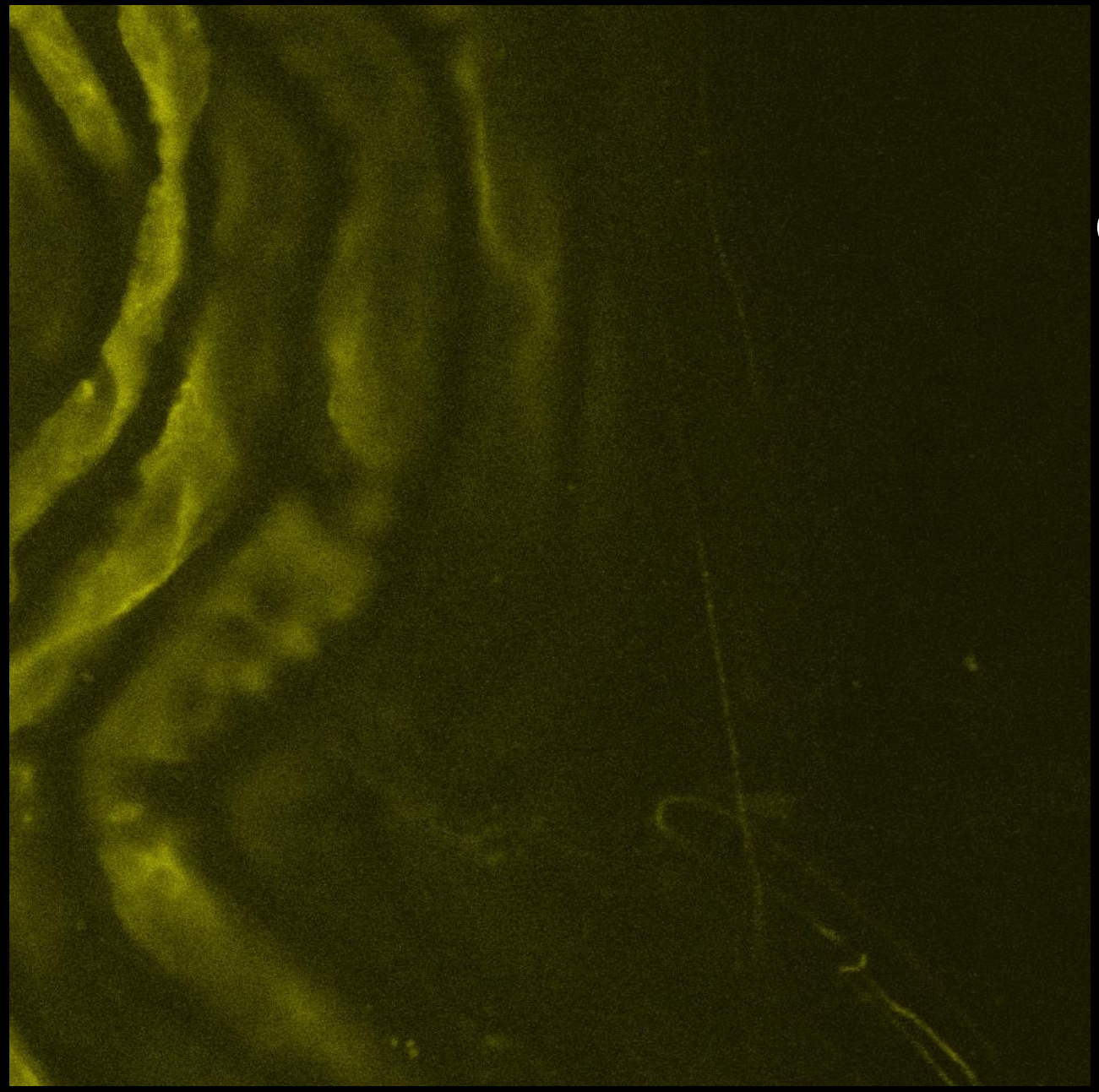


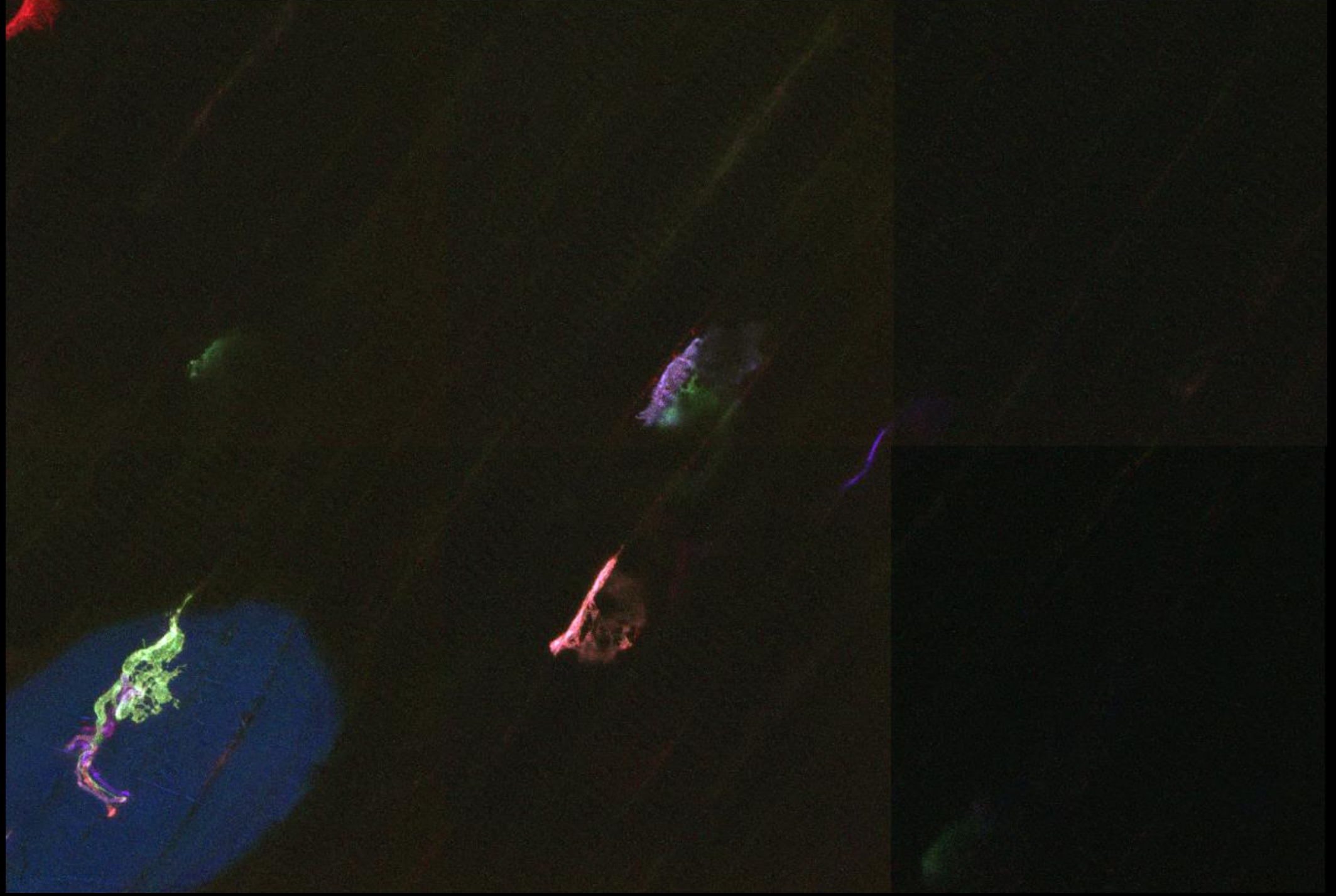
FIG. 5.2. (a) Conventional scanning microscope image of tilted specimen: the parts of the object out of the focal plane are blurred. (b) Confocal scanning microscope image of tilted specimen: only the part of the specimen in the focal plane is imaged strongly. 0·5 numerical aperture objective was used with a He–Ne (0·6328 µm wavelength) laser. A 10-µm diameter pinhole was used in the confocal mode.

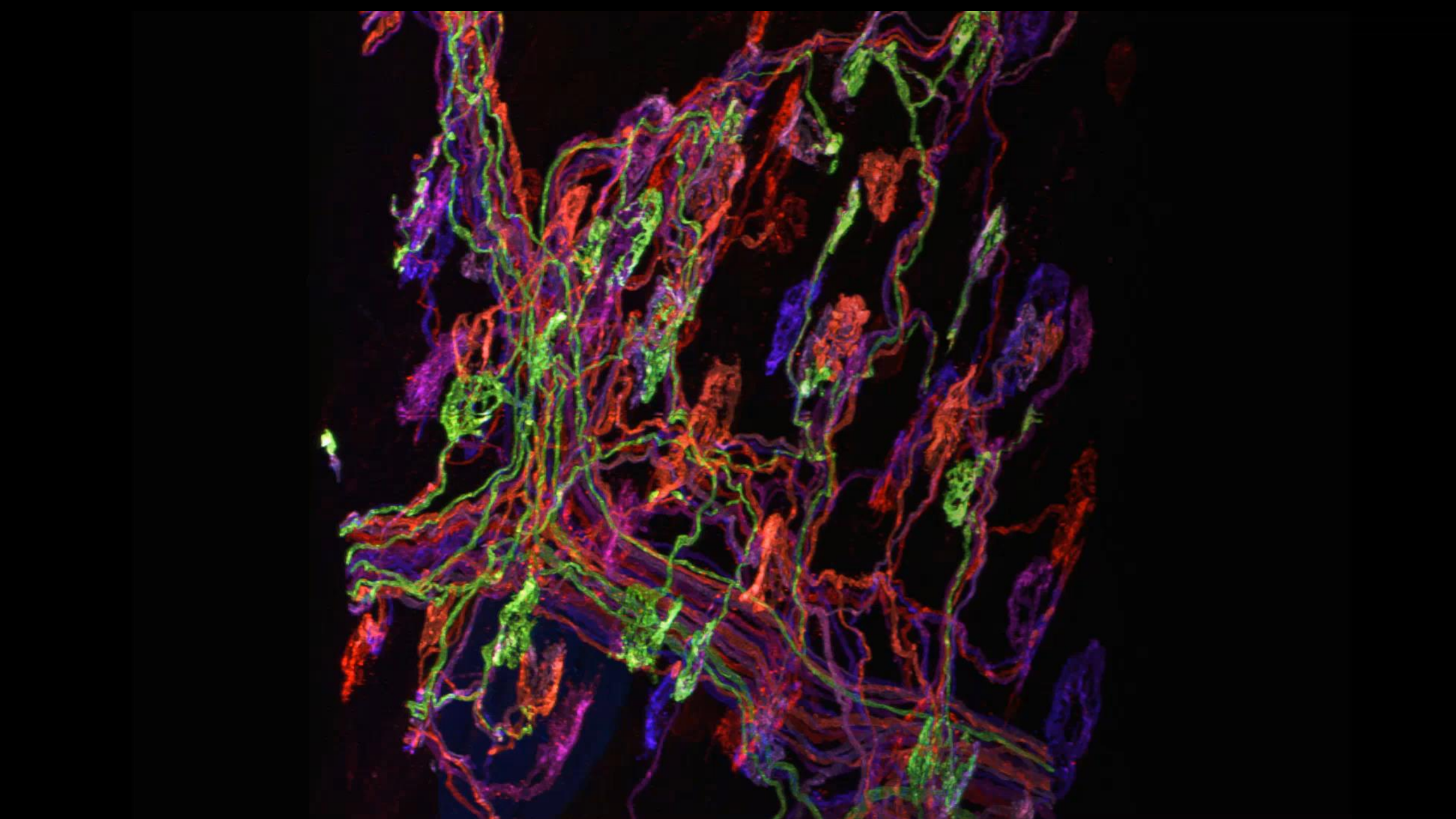
From Wilson and Sheppard,
Theory and Practice of Scanning Optical Microscopy



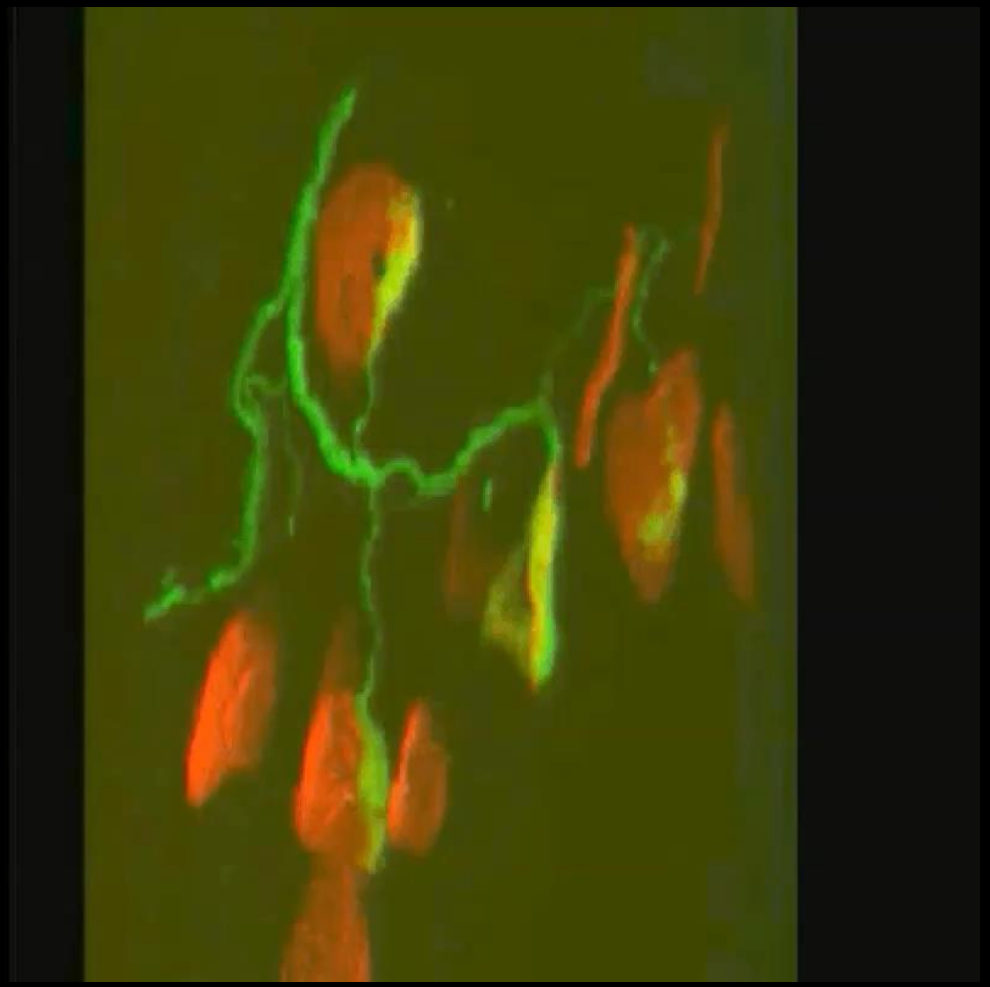
A confocal "stack": a set of images from successive depths in a volume of tissue

Nyquist limited imaging of the axons in a mouse peripheral nerve from a YFP-16 transgenic XFP mouse



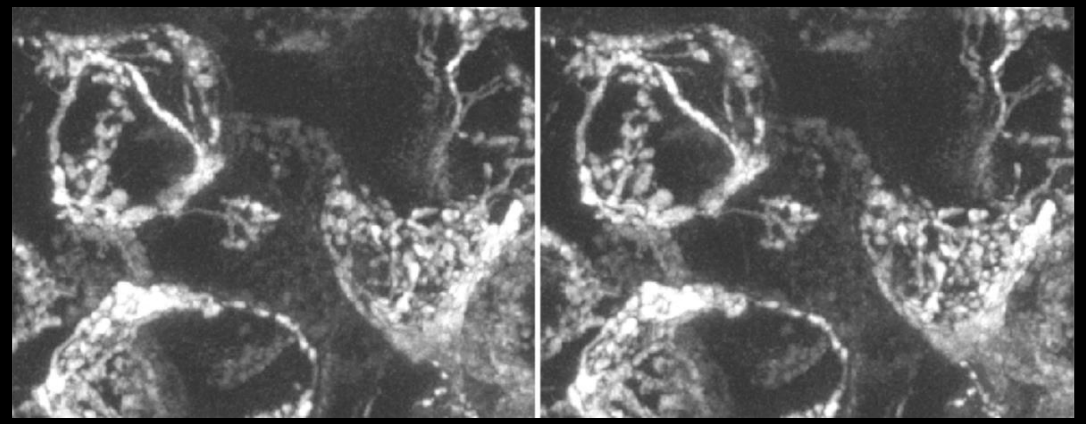


“Maximum intensity” projections in a tilt series



Keller-Peck, Walsh, Gan, Feng, Sanes , Lichtman 2001

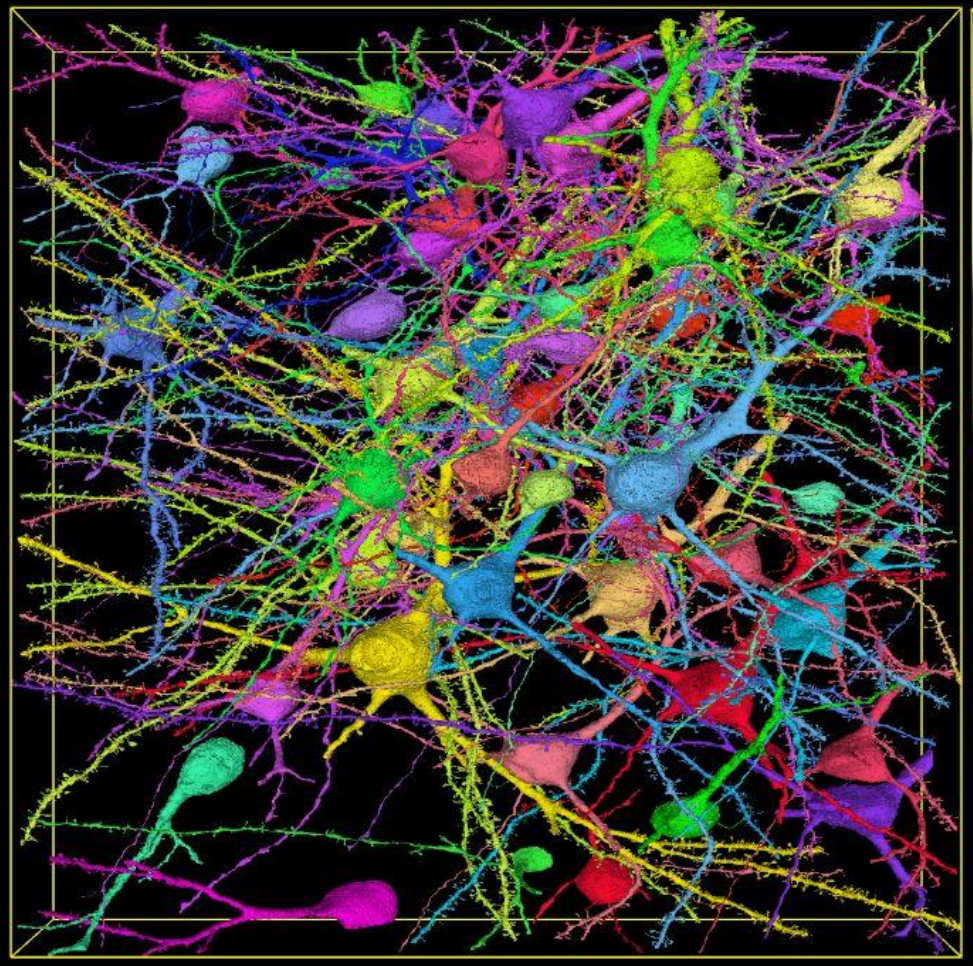
"Stereo Pair" : view each of the two frames in a different eye (crossed eye viewing is left frame in right eye and vice versa; walleye is seeing left panel with left eye and vice versa)



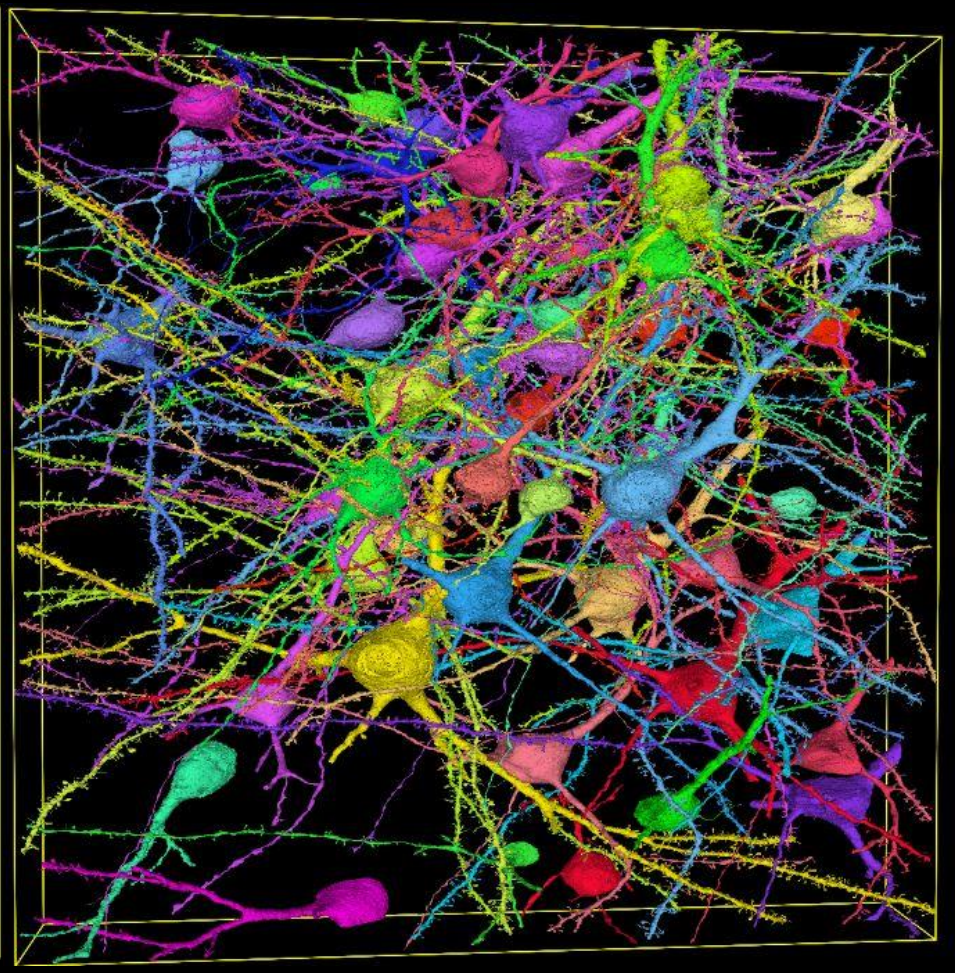
(this skill takes practice to master- but is worth the trouble)

Gan, Kwon, Feng, Sanes, Lichtman, 2003

Advanced Stereopair

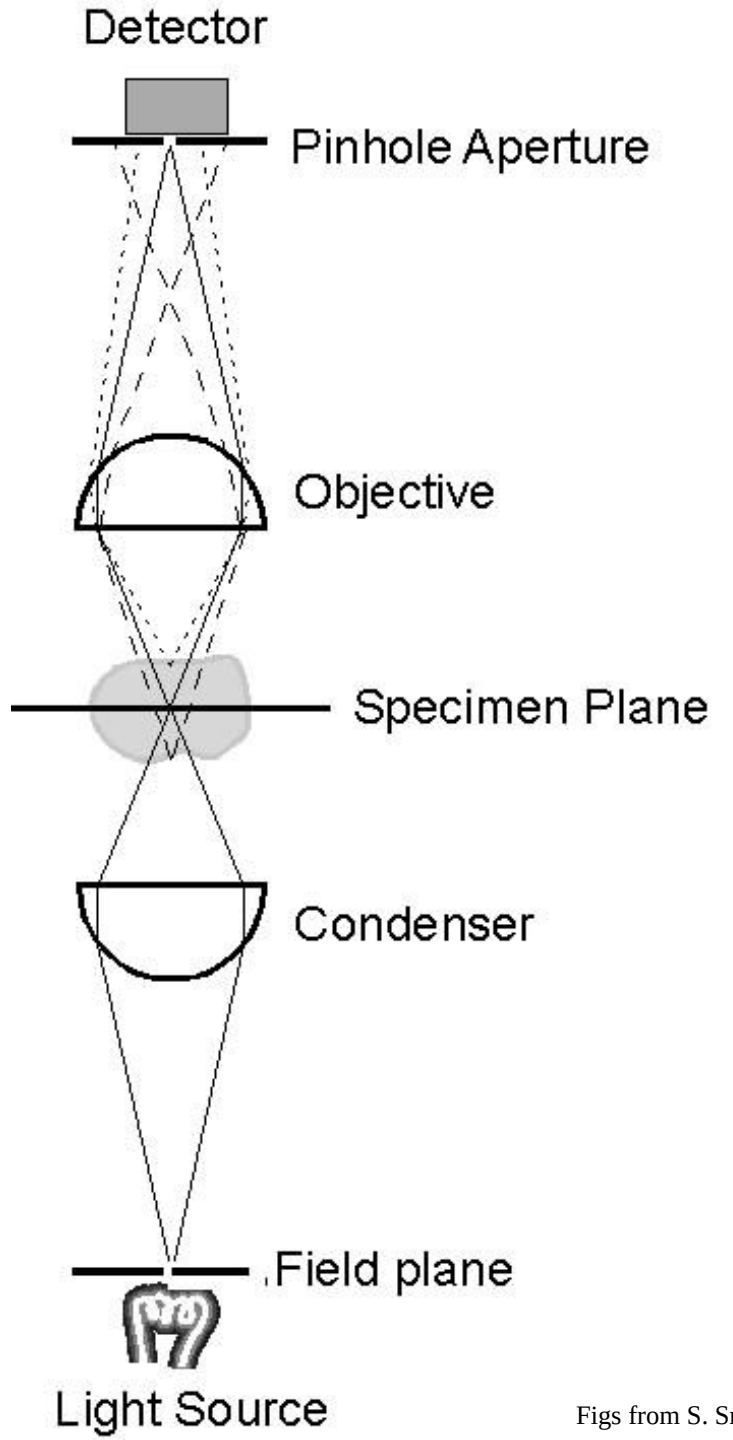


Advanced Stereopair



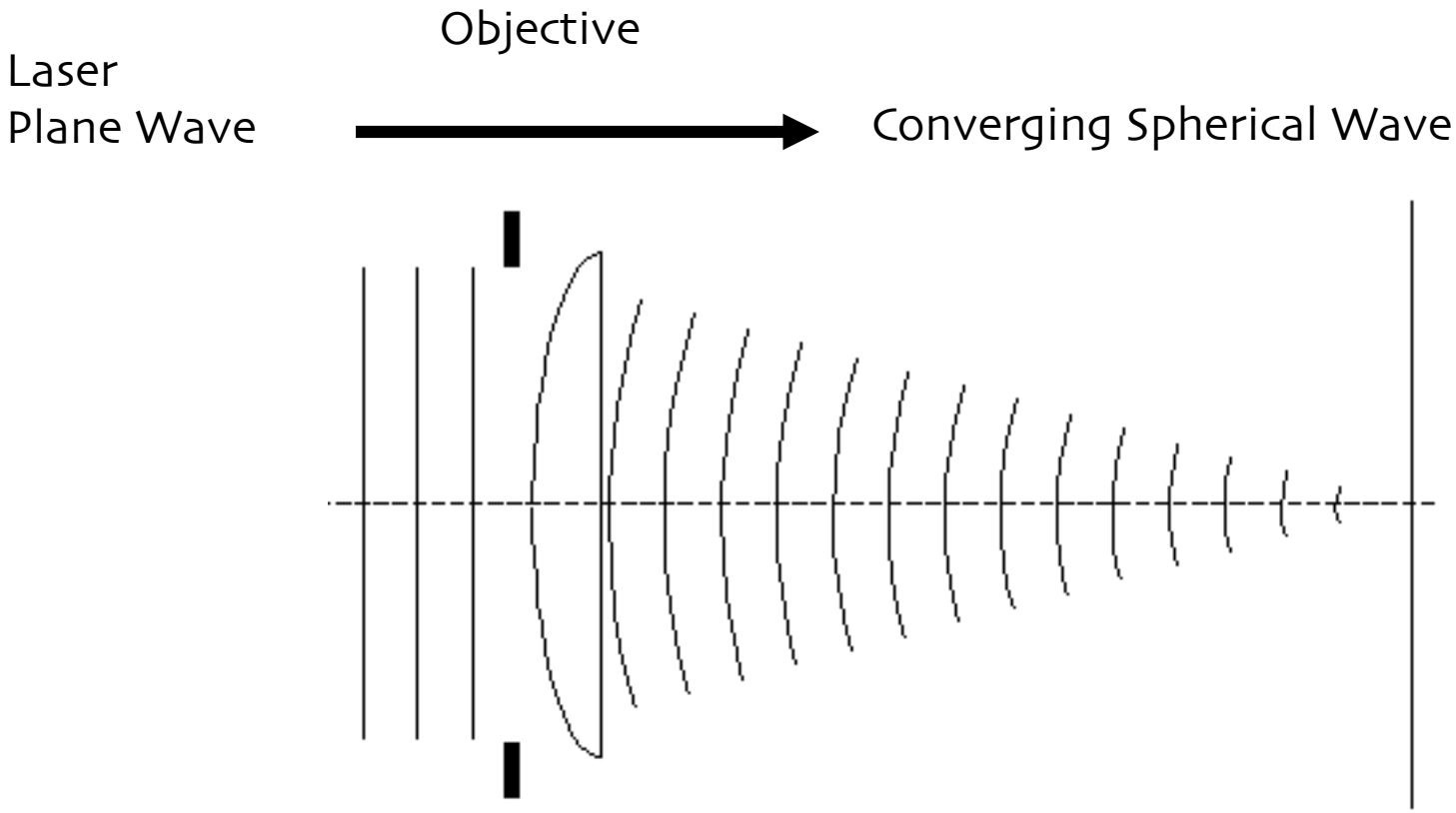
Confocal Image Improvements:

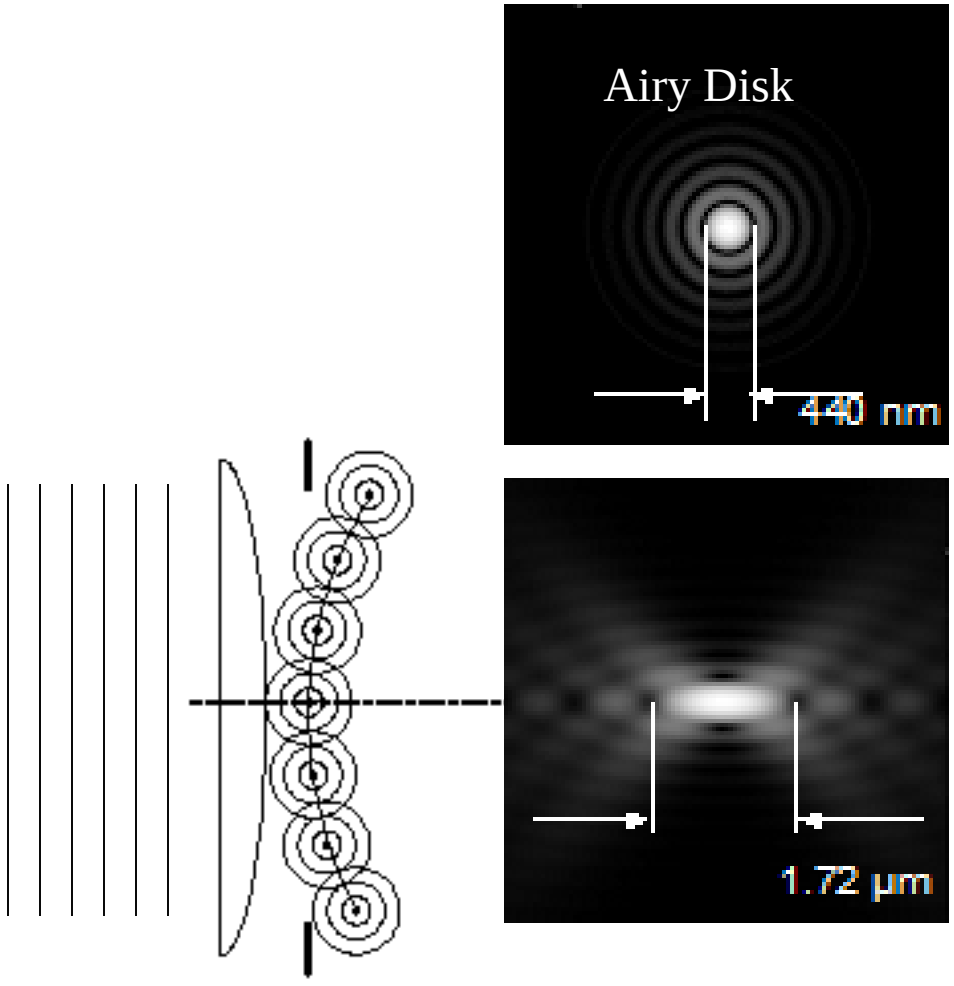
1) contrast
2) optical-sectioning
3) resolution



Figs from S. Smith, G. Augustine, J. Conchello, J Lichtman, in prep

Derivation of the PSF: interference pattern of wavelets from a converging spherical wave



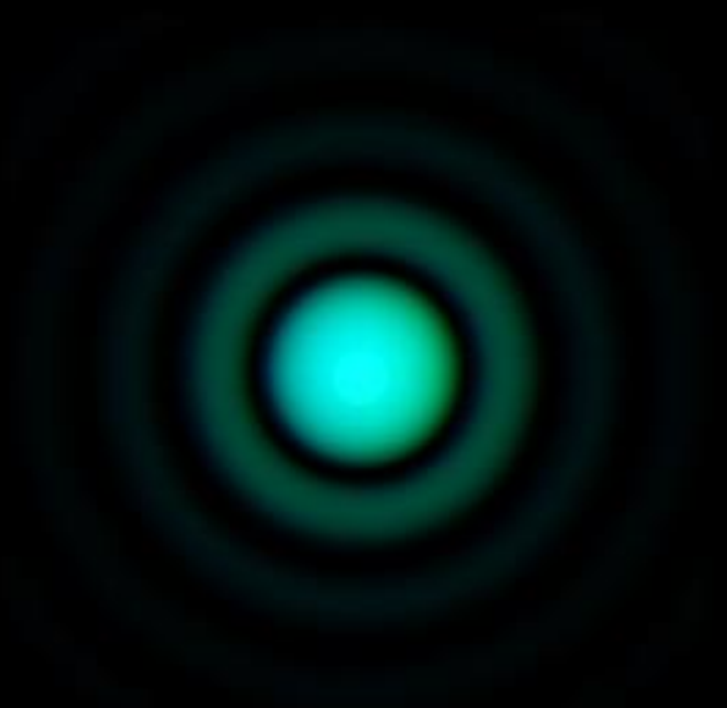


XY

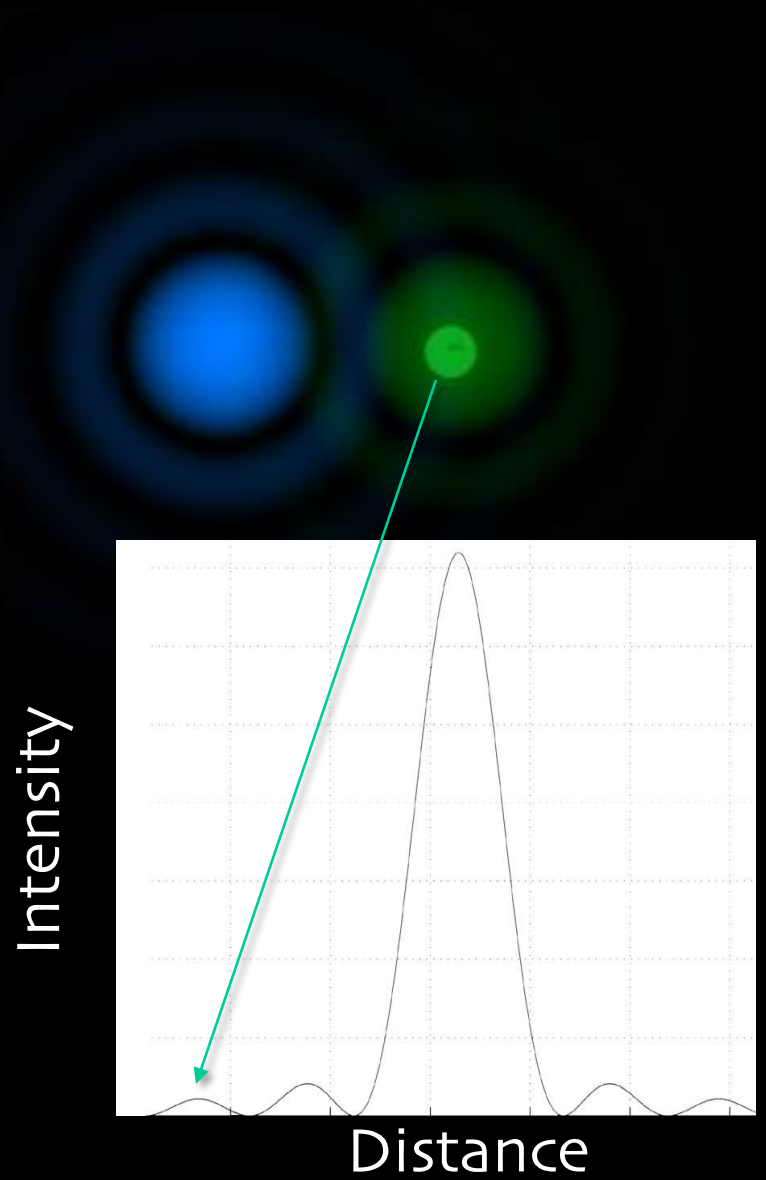
XZ

Plane Wave → Point Spread Function

Now in slow motion…



Pinhole wide open:
detector sees all
green light



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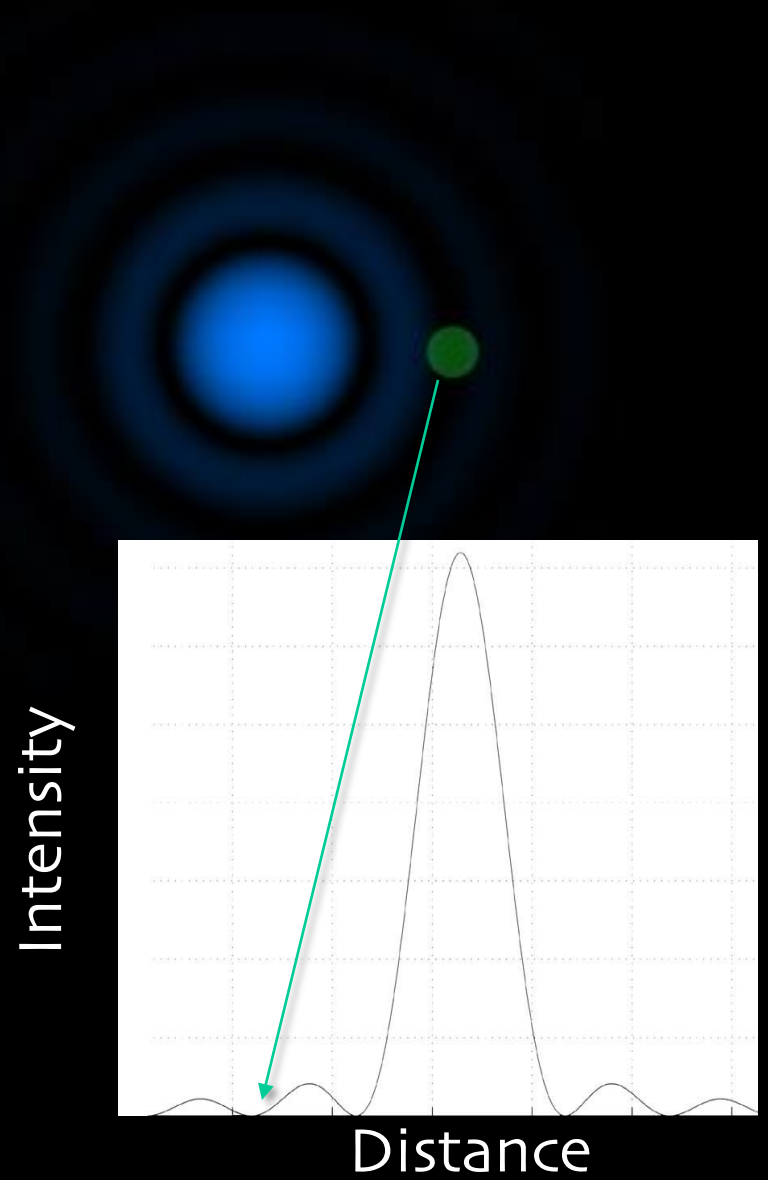

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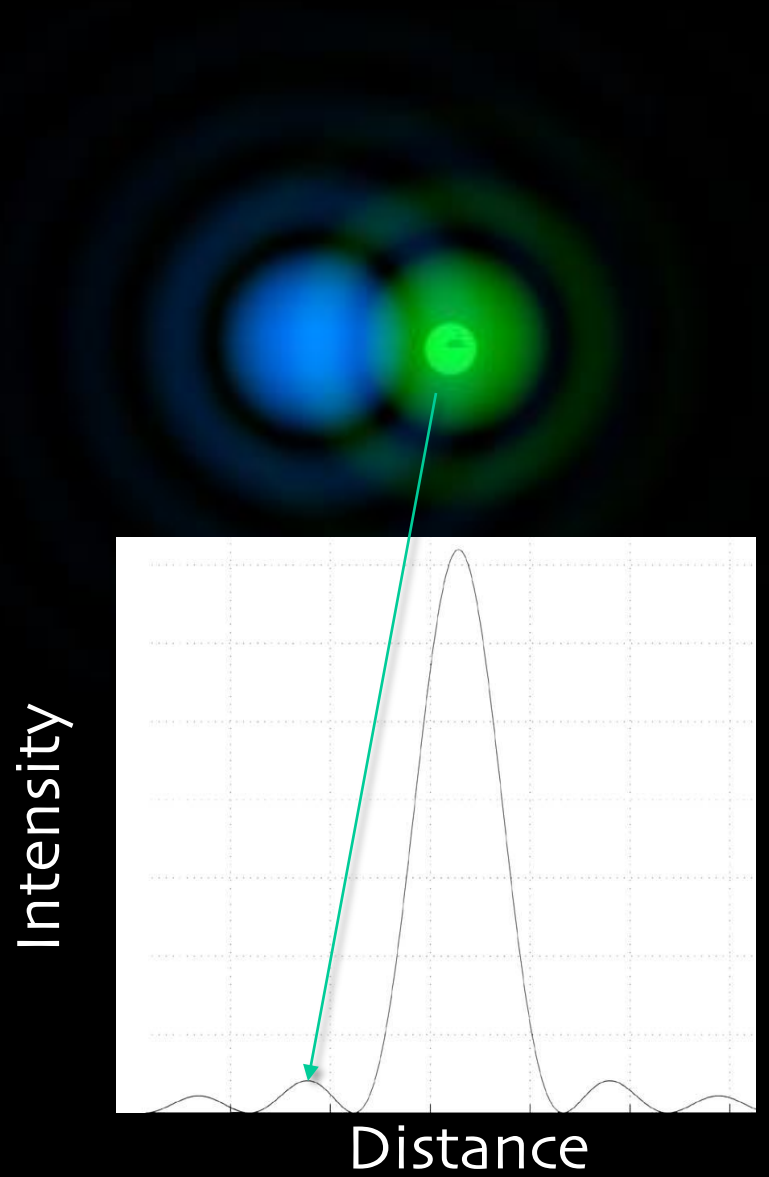

Pinhole wide open:
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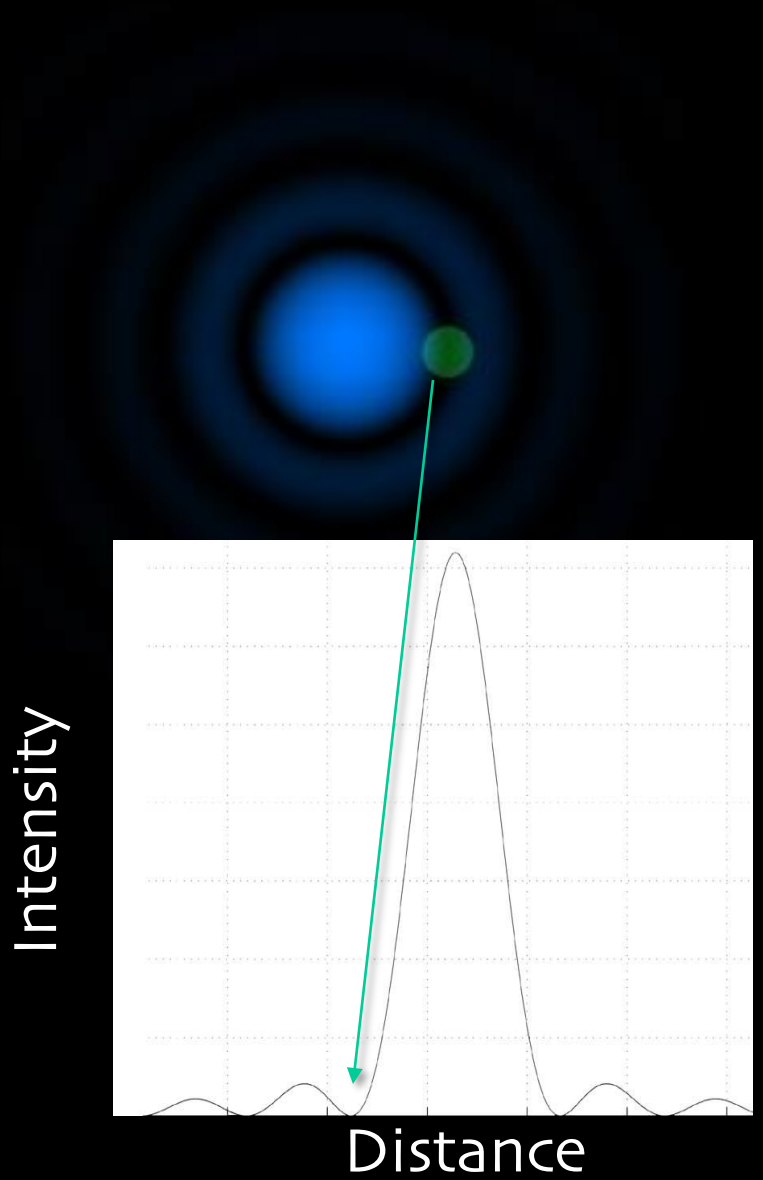
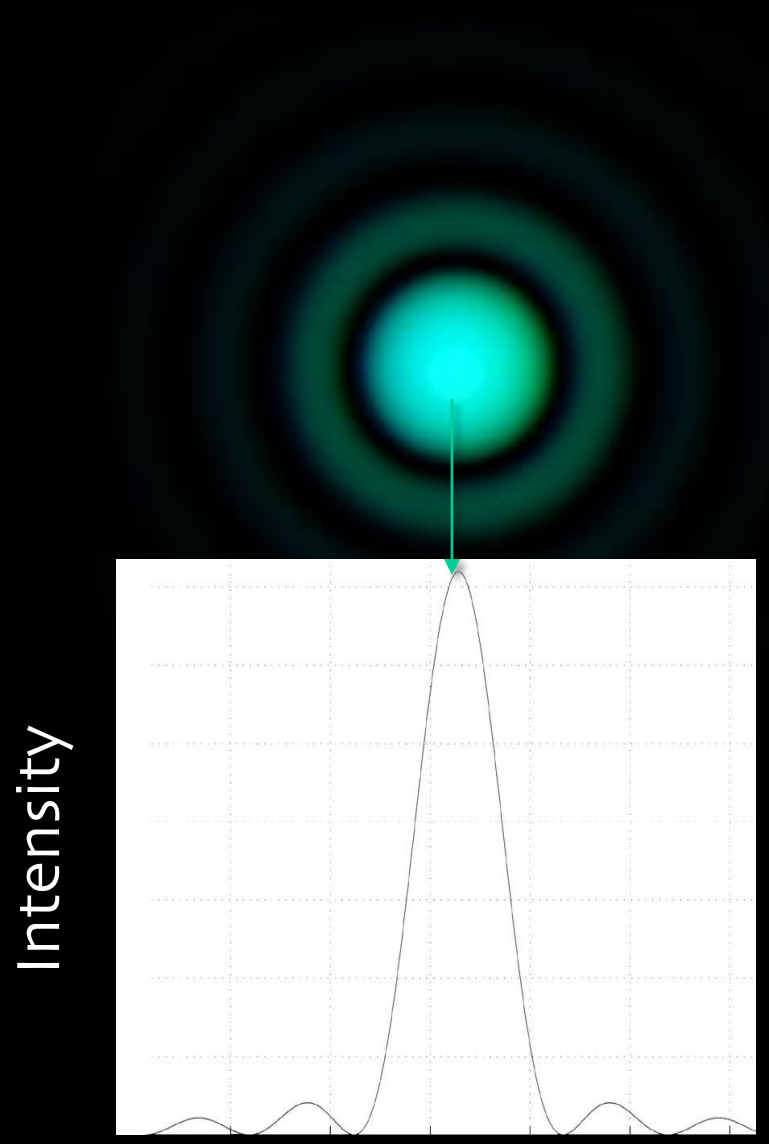
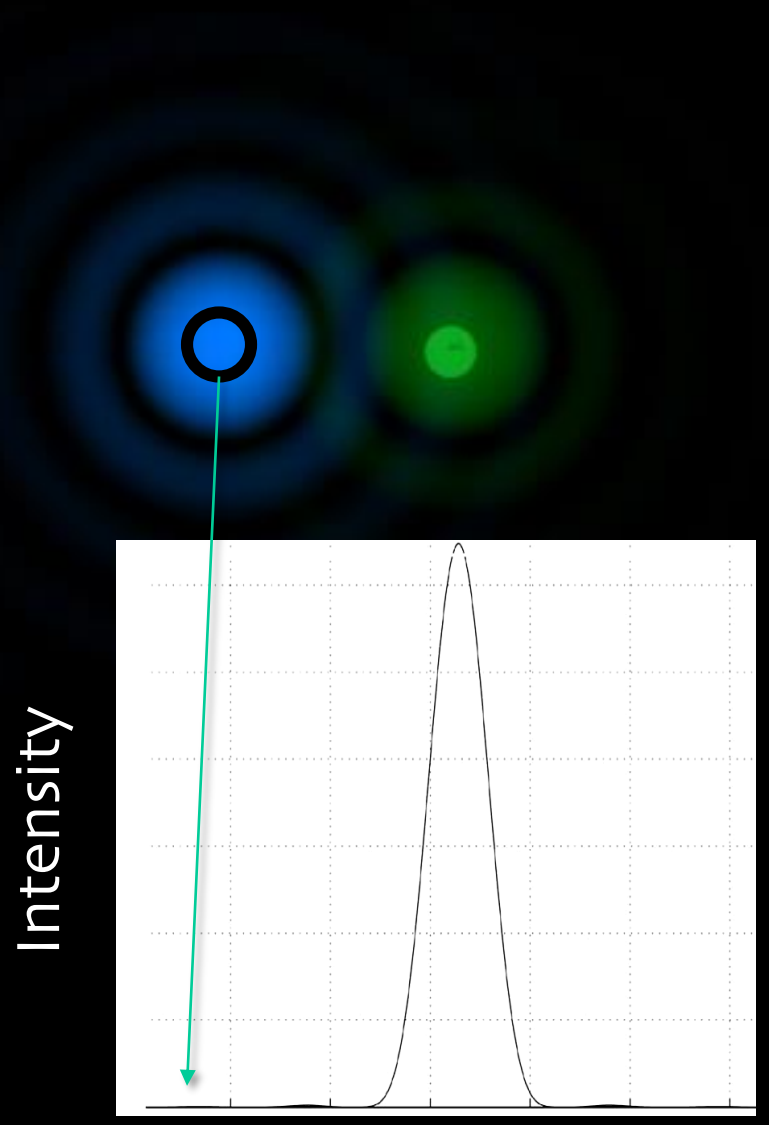


Pinhole wide open:
detector sees all
green light



Pinhole closed down:
detector only sees
green light that aligns
with center of
exciting light (blue) PSF



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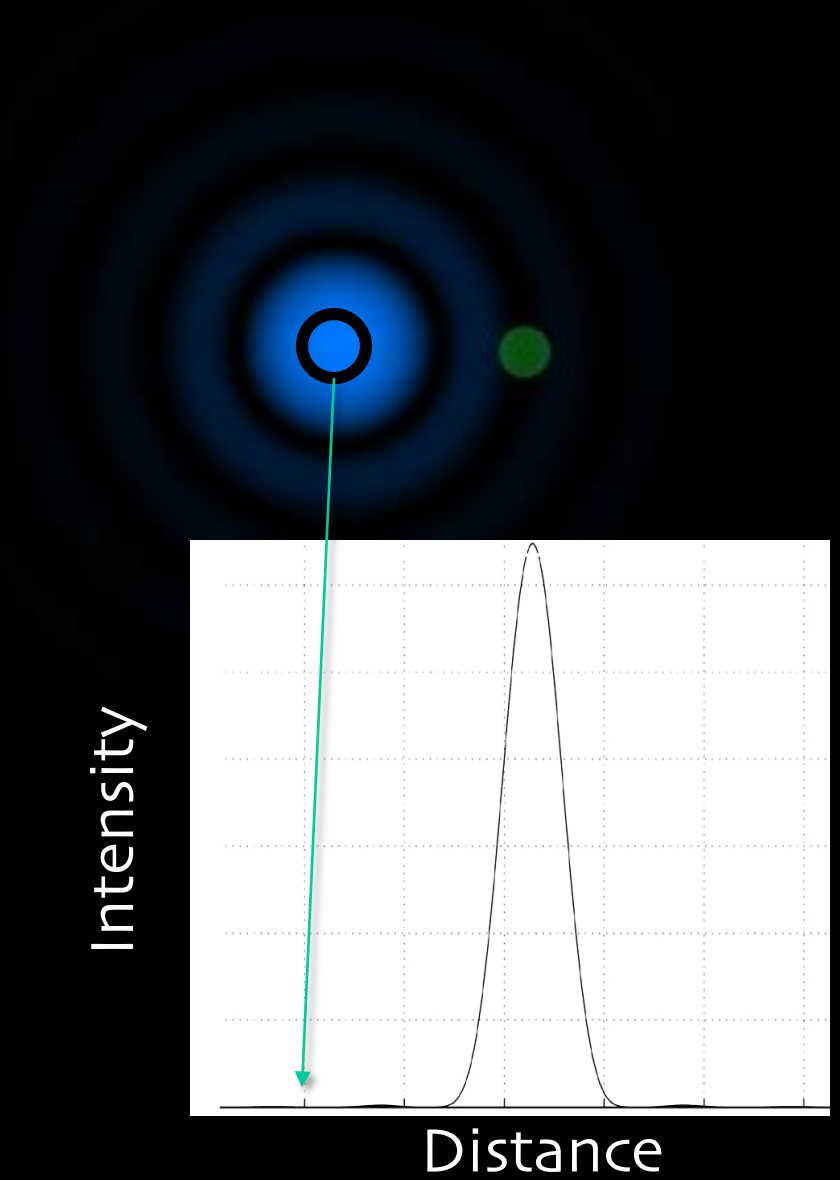

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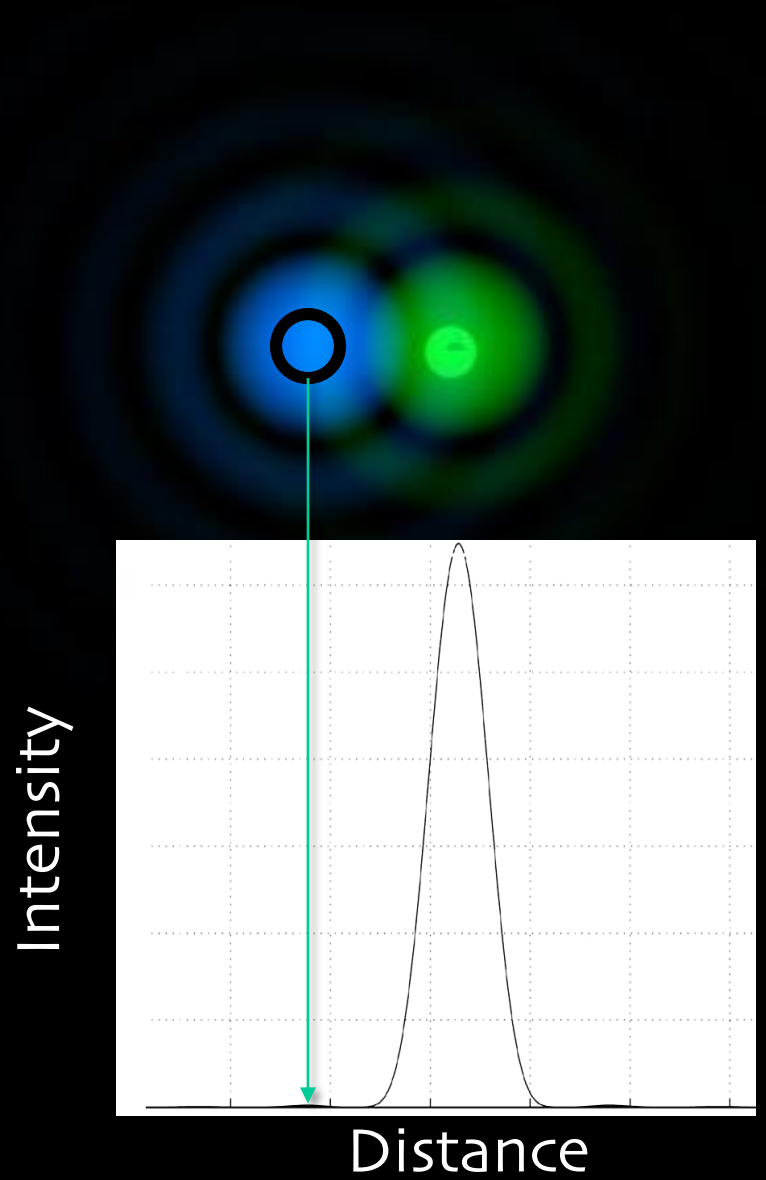

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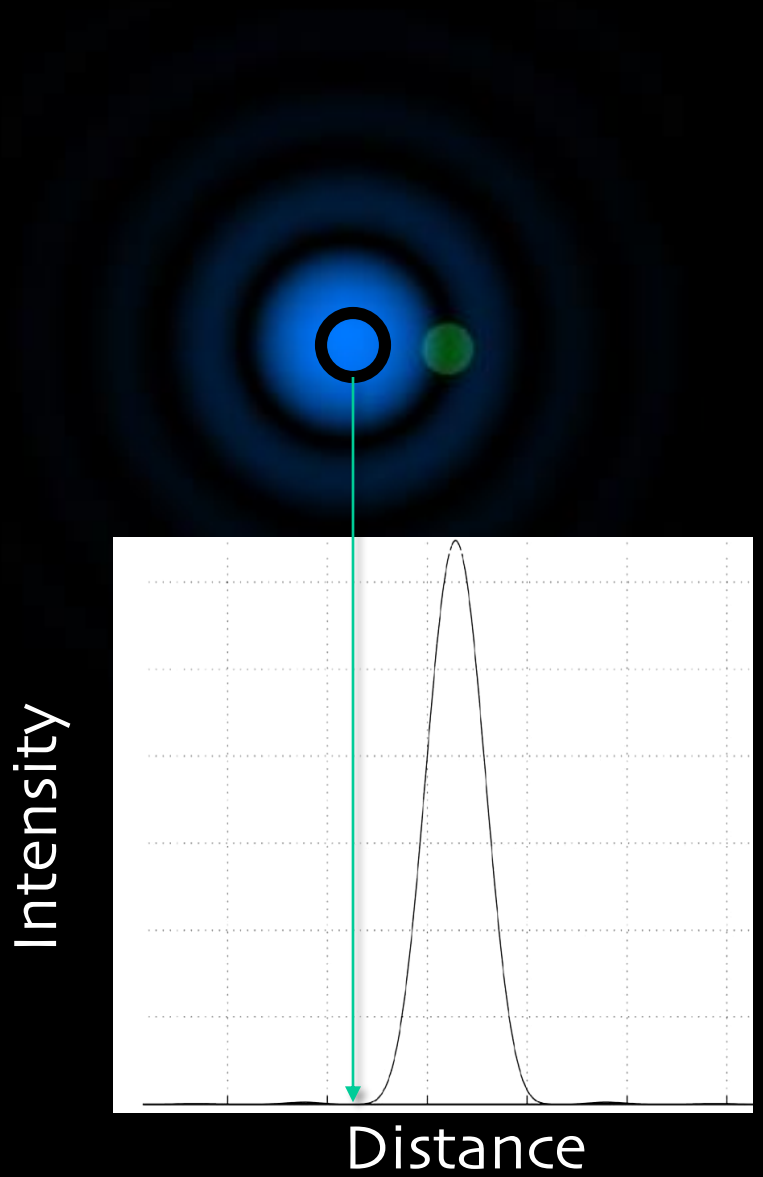

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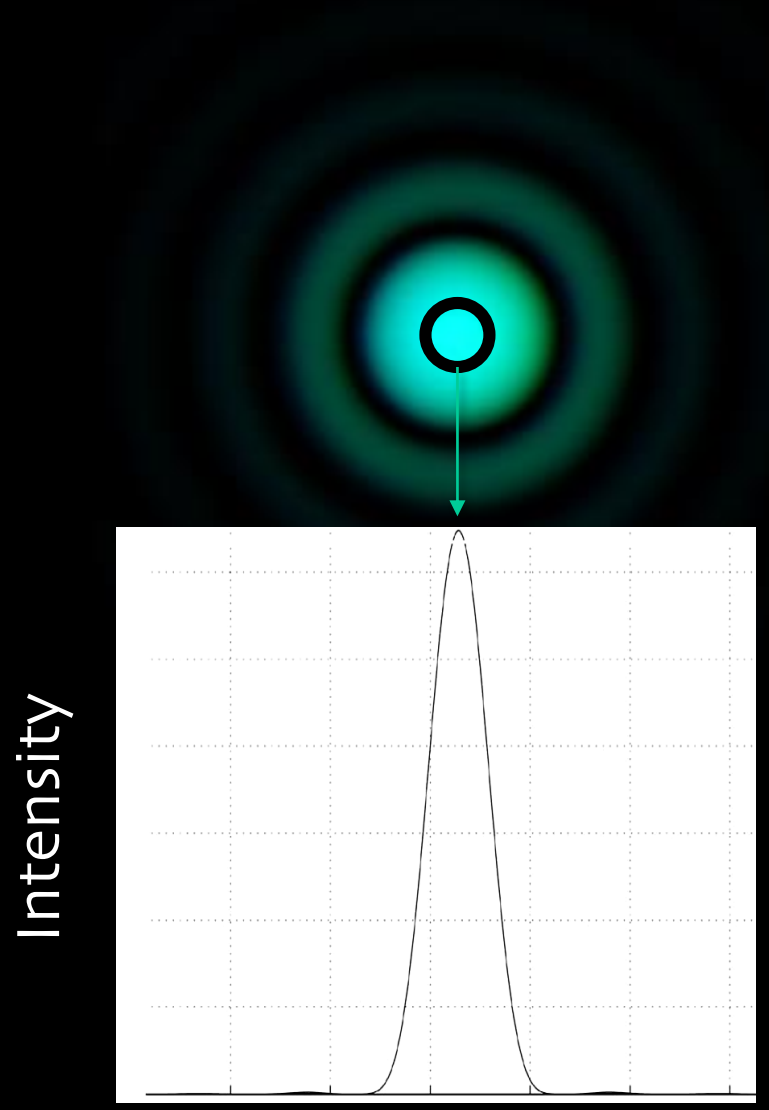
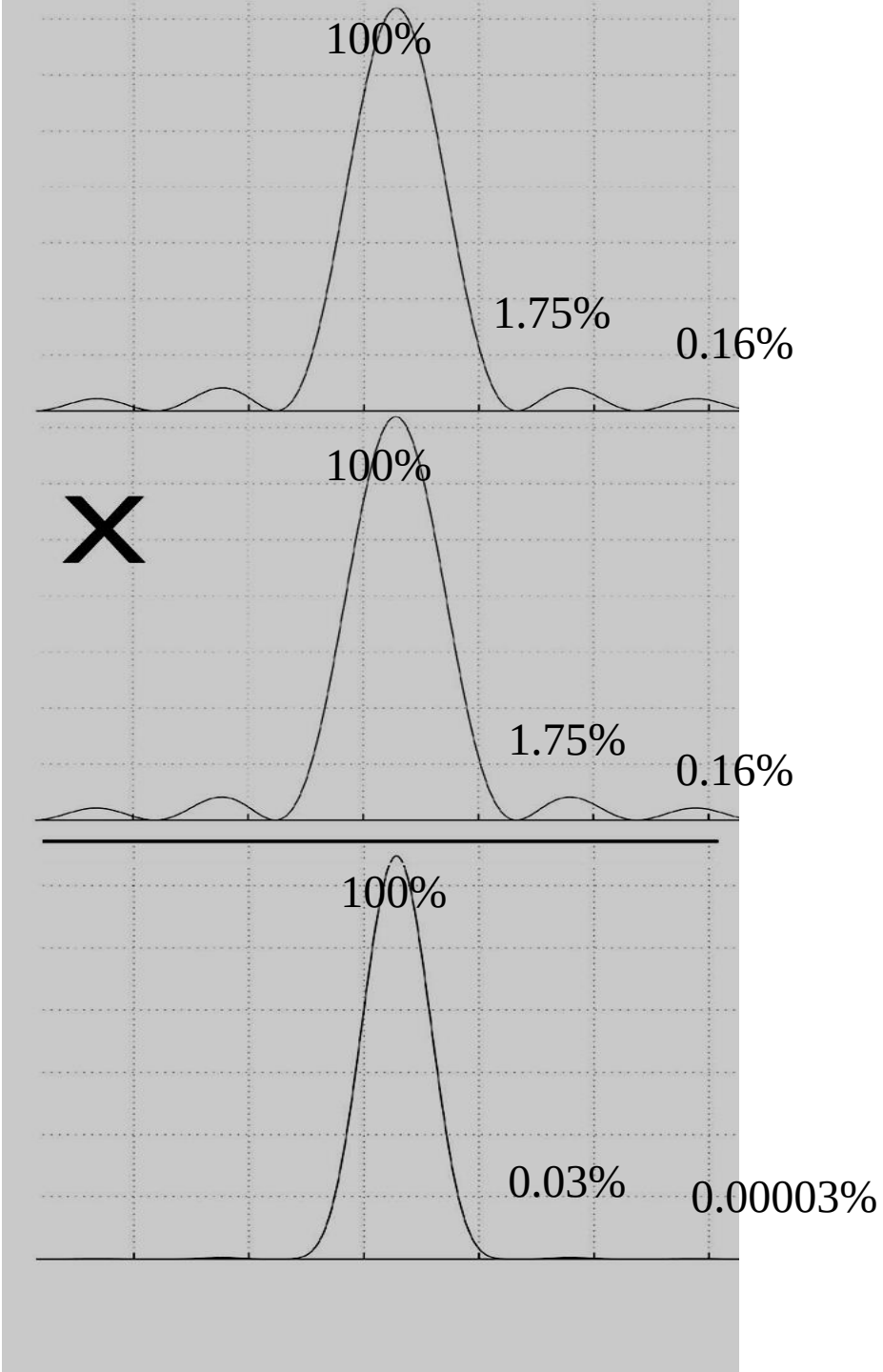


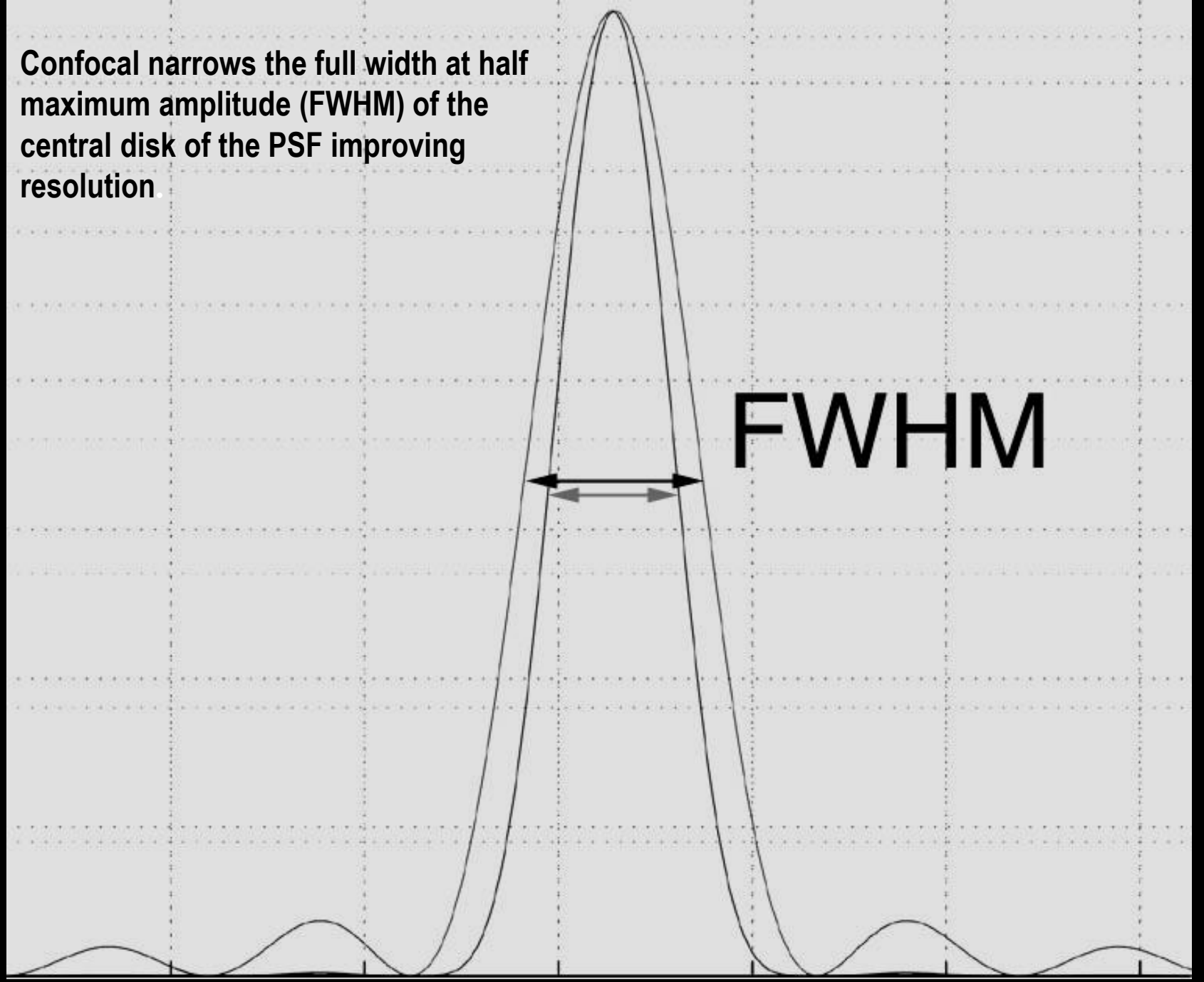
Detector PSF

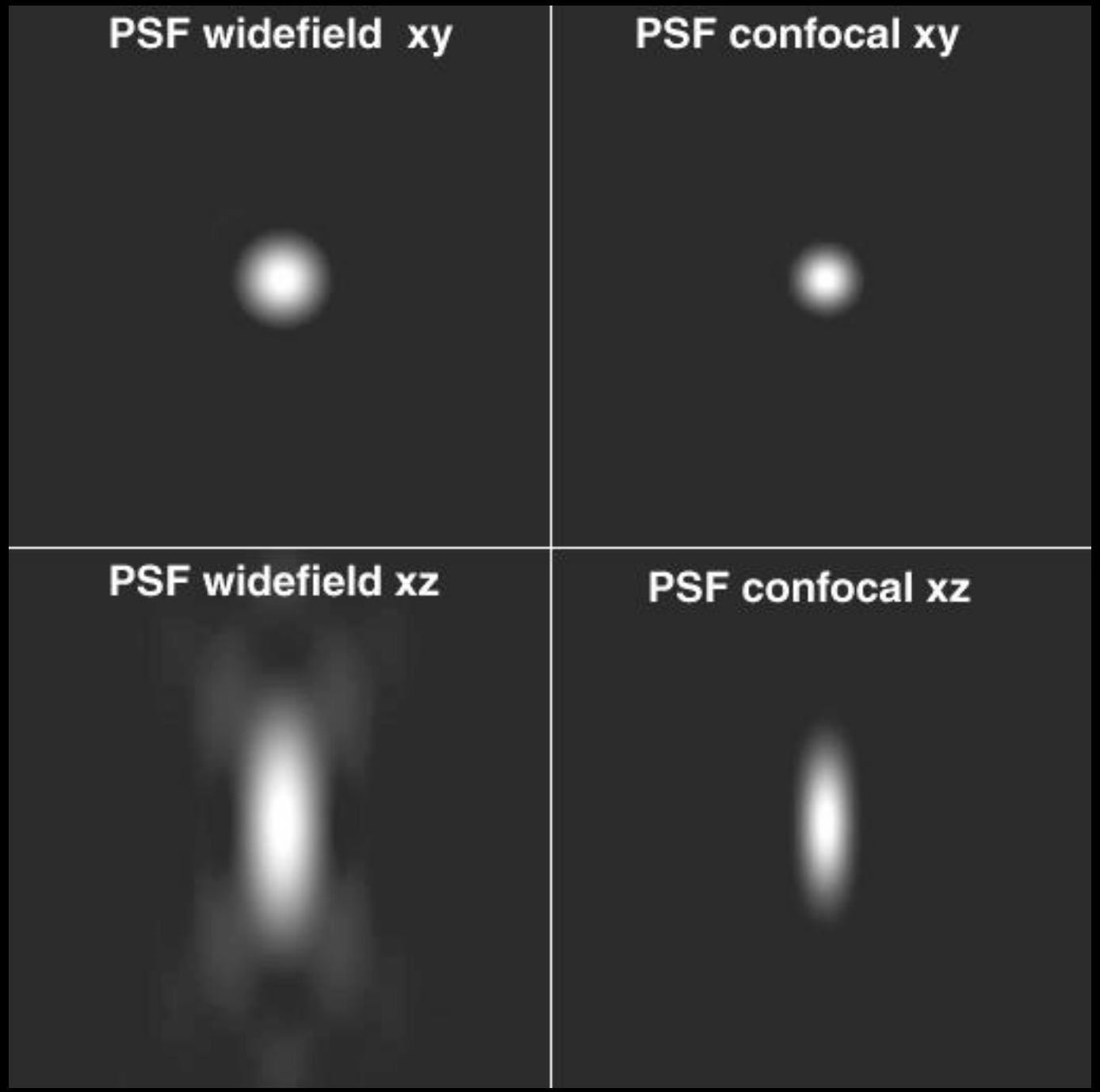
Excitation PSF

Resultant confocal PSF is product of 2 PSFs



Confocal narrows the full width at half maximum amplitude (FWHM) of the central disk of the PSF improving resolution.




PSF widefield xy
PSF confocal xy
PSF widefield xz
PSF confocal xz

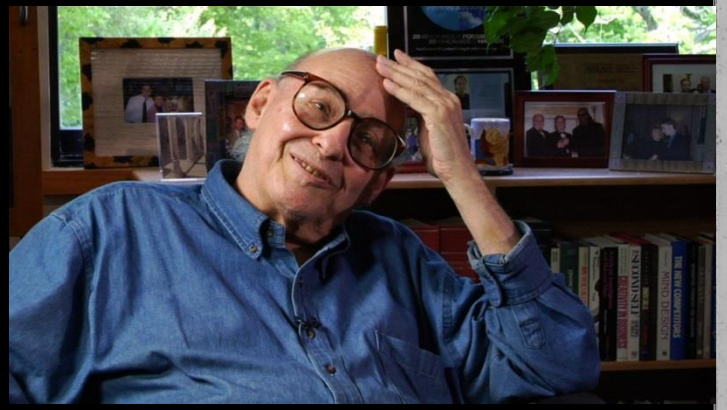
Three ways of doing confocal:

Stage scanning (now rare);

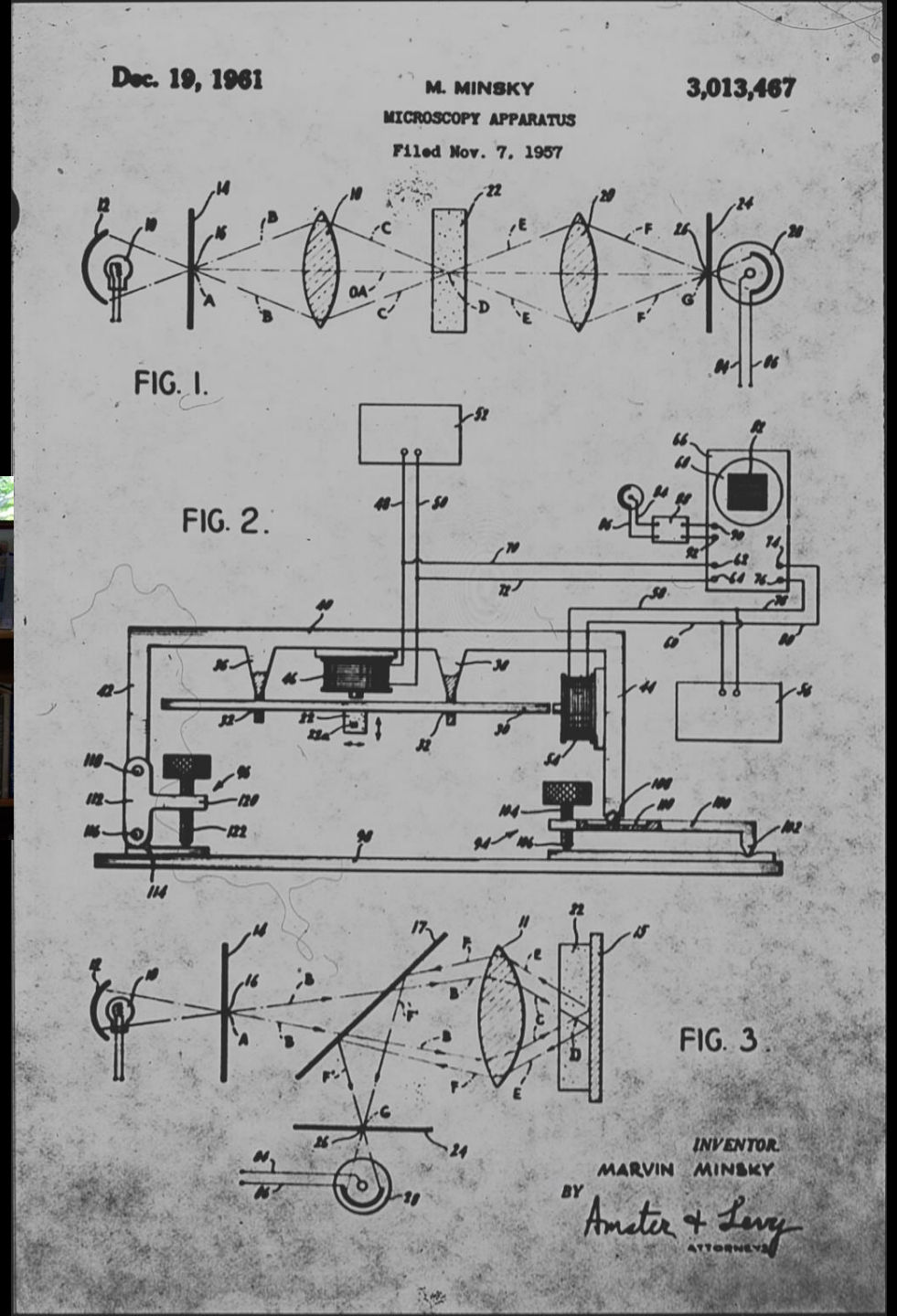
Aperture scanning (spinning disc);

Mirror scanning (laser scanning scopes)

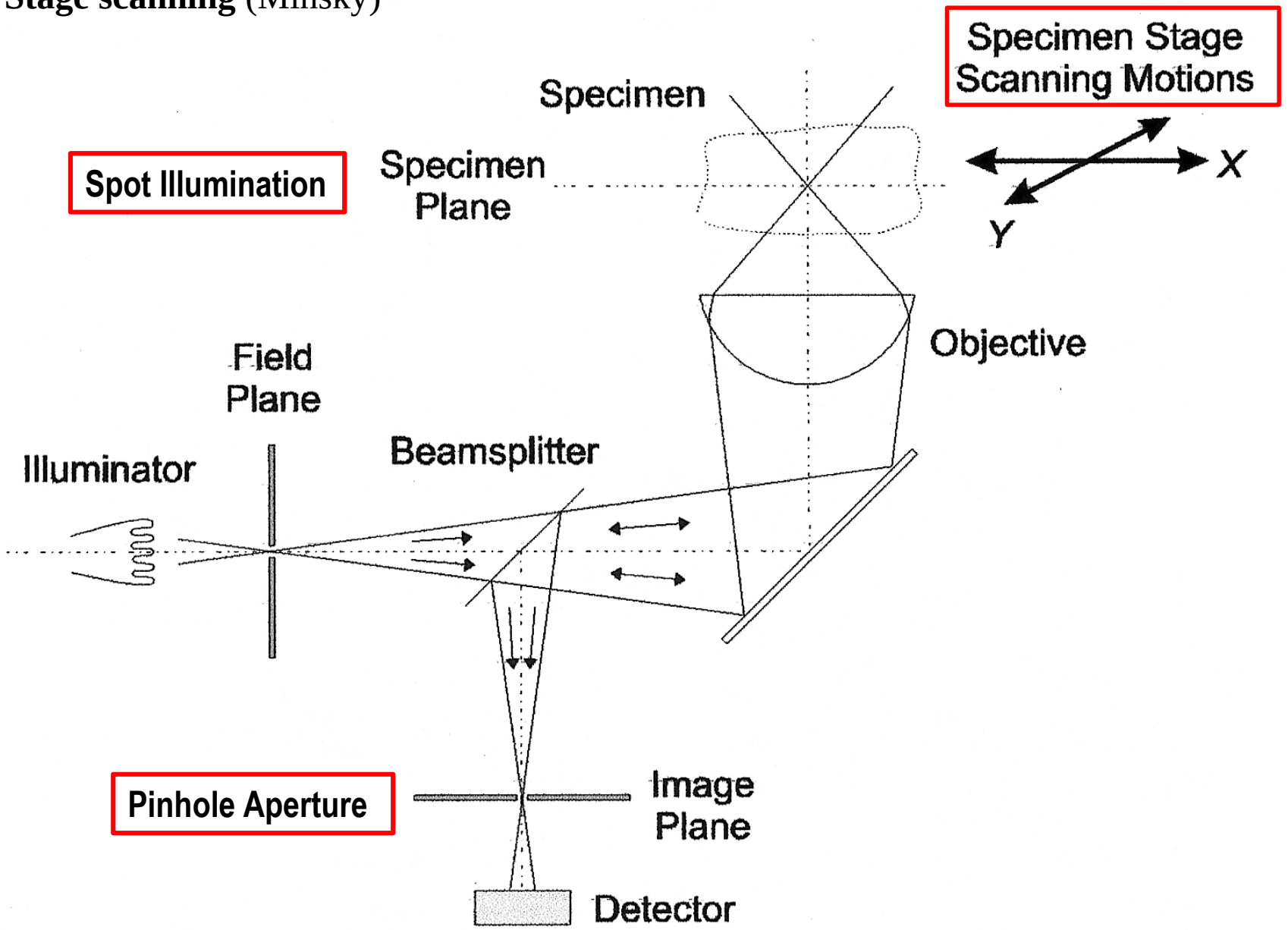
1st confocal:
Stage scanning
by Marvin Minsky
while a Harvard Junior Fellow



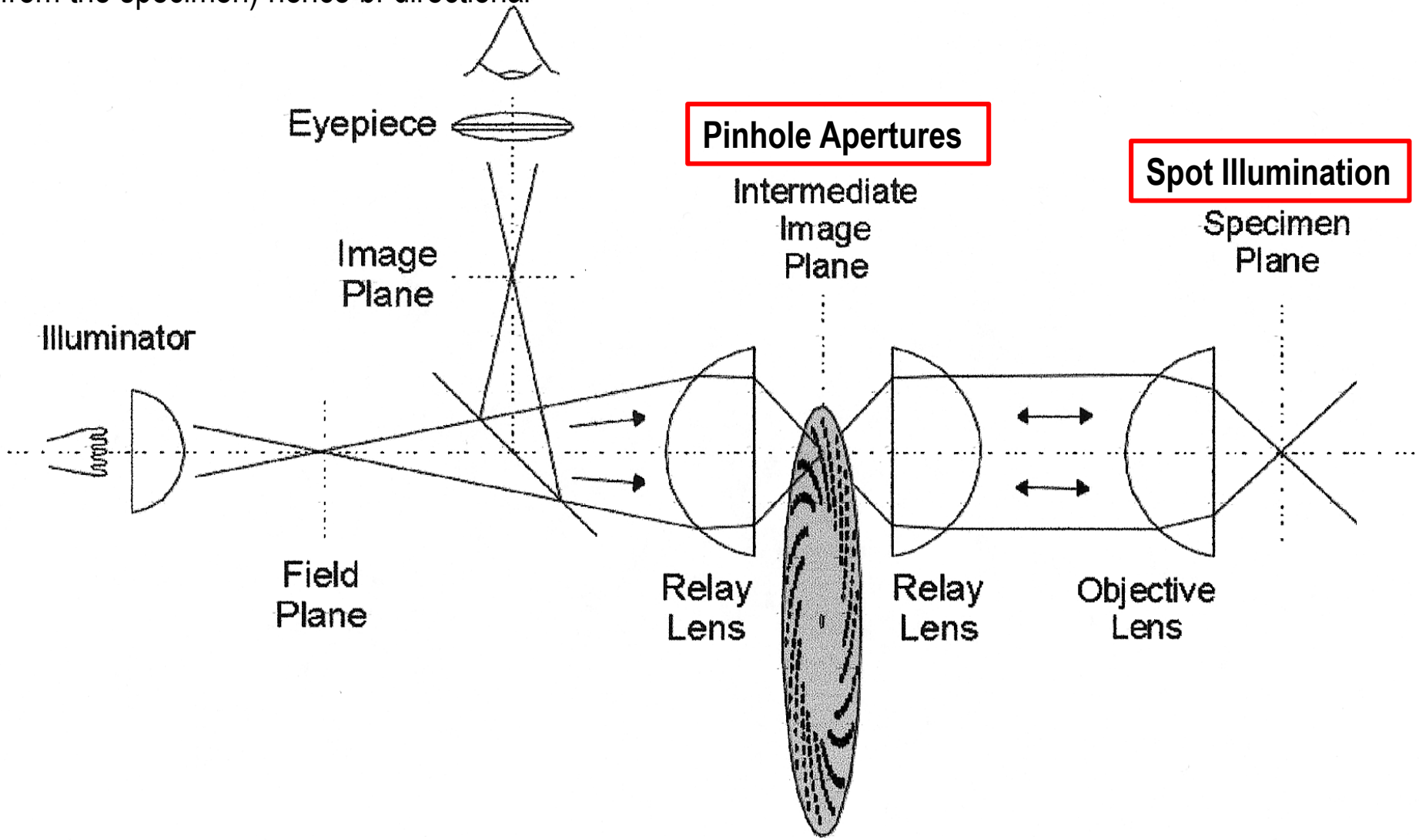
M. Minsky
1927-2016



Stage scanning (Minsky)



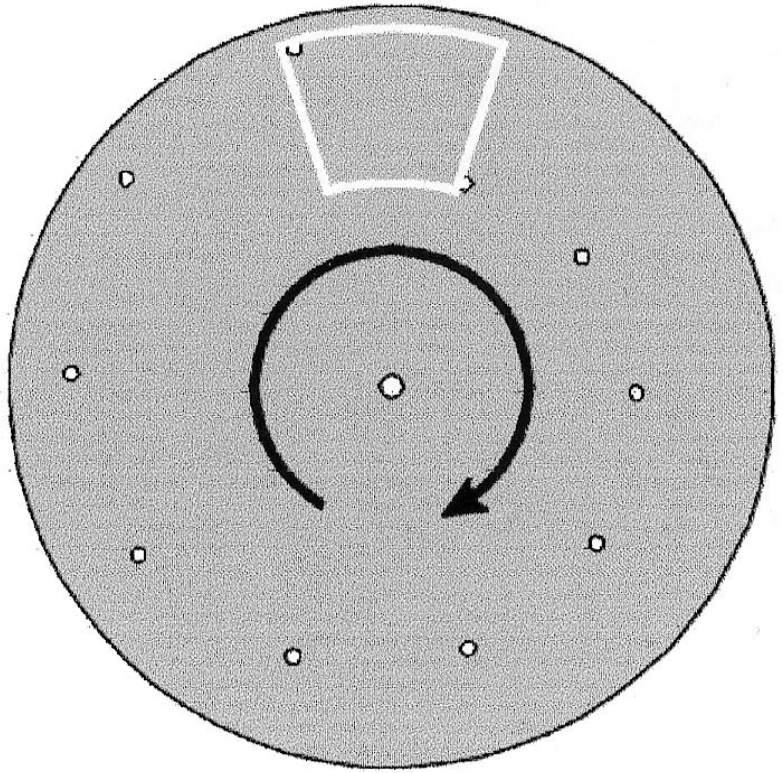
Single “bi-directional” **aperture scanning** (Lichtman, Kino). Apertures serve to both generate spot illumination (light going to the specimen) and to only detect light from the illuminated spots (light coming from the specimen) hence bi-directional



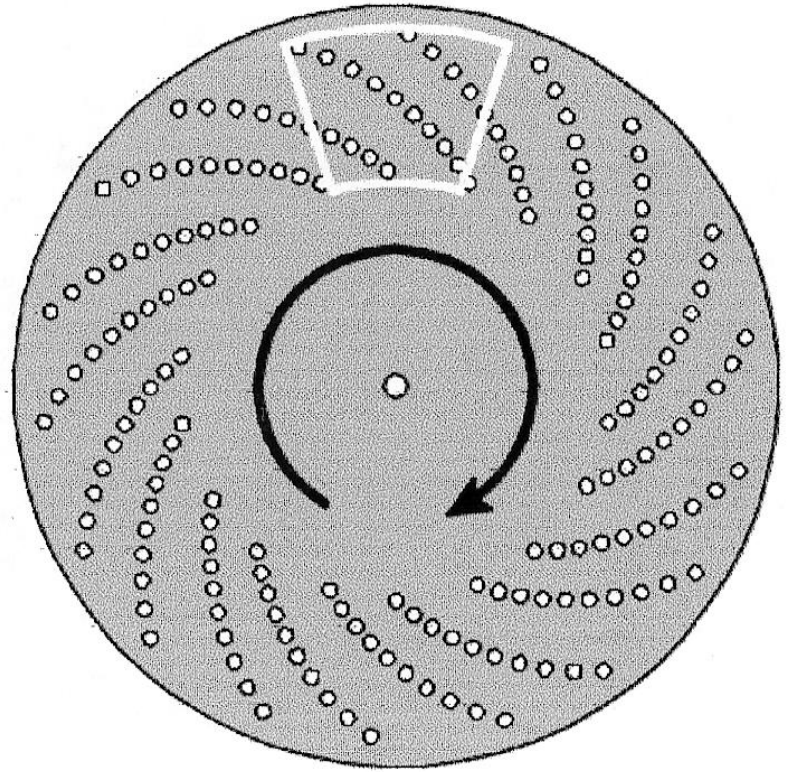
Rotation of apertures scans specimen

What is a the advantage to more pinholes?

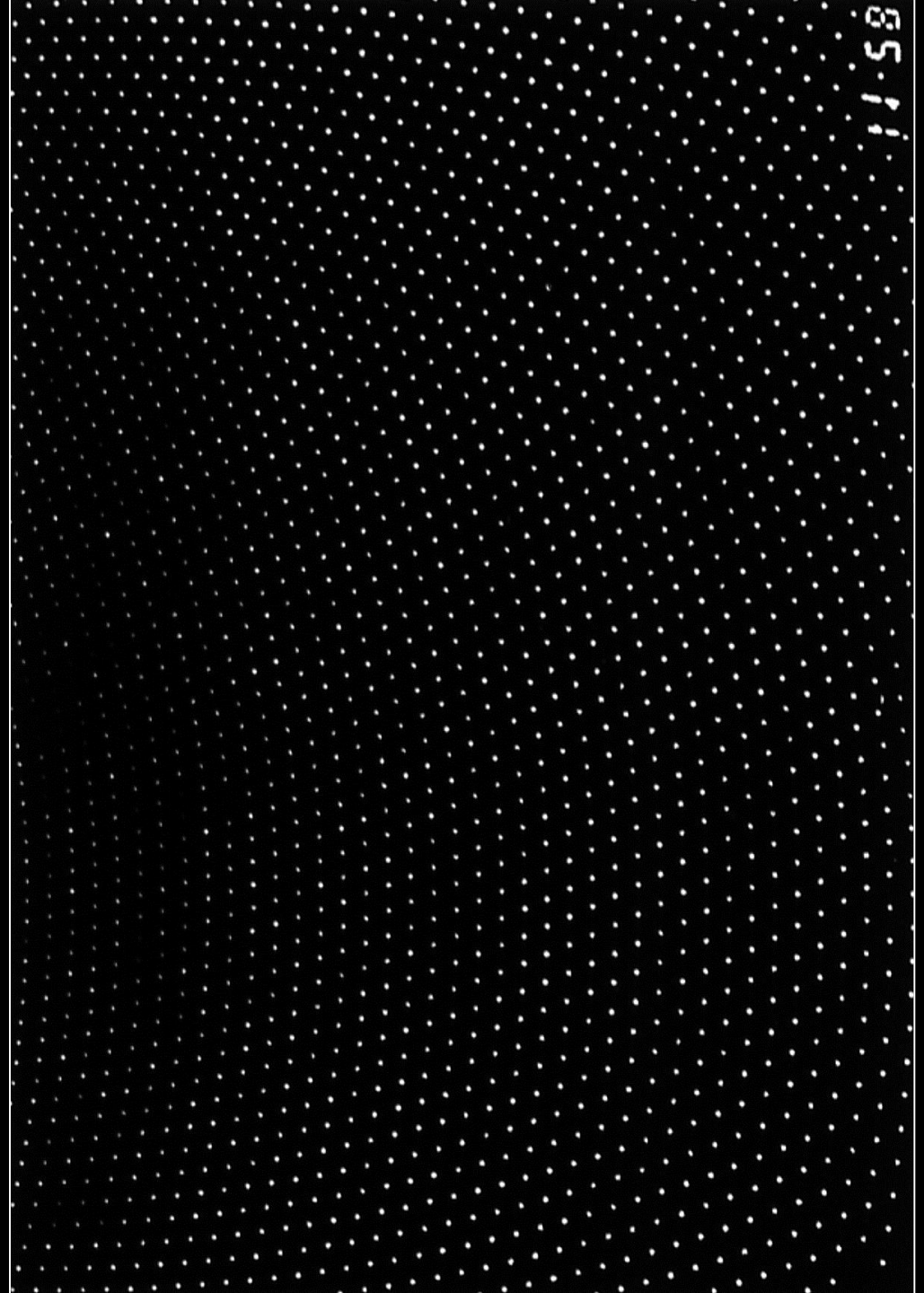
Nipkow Scanning Disk



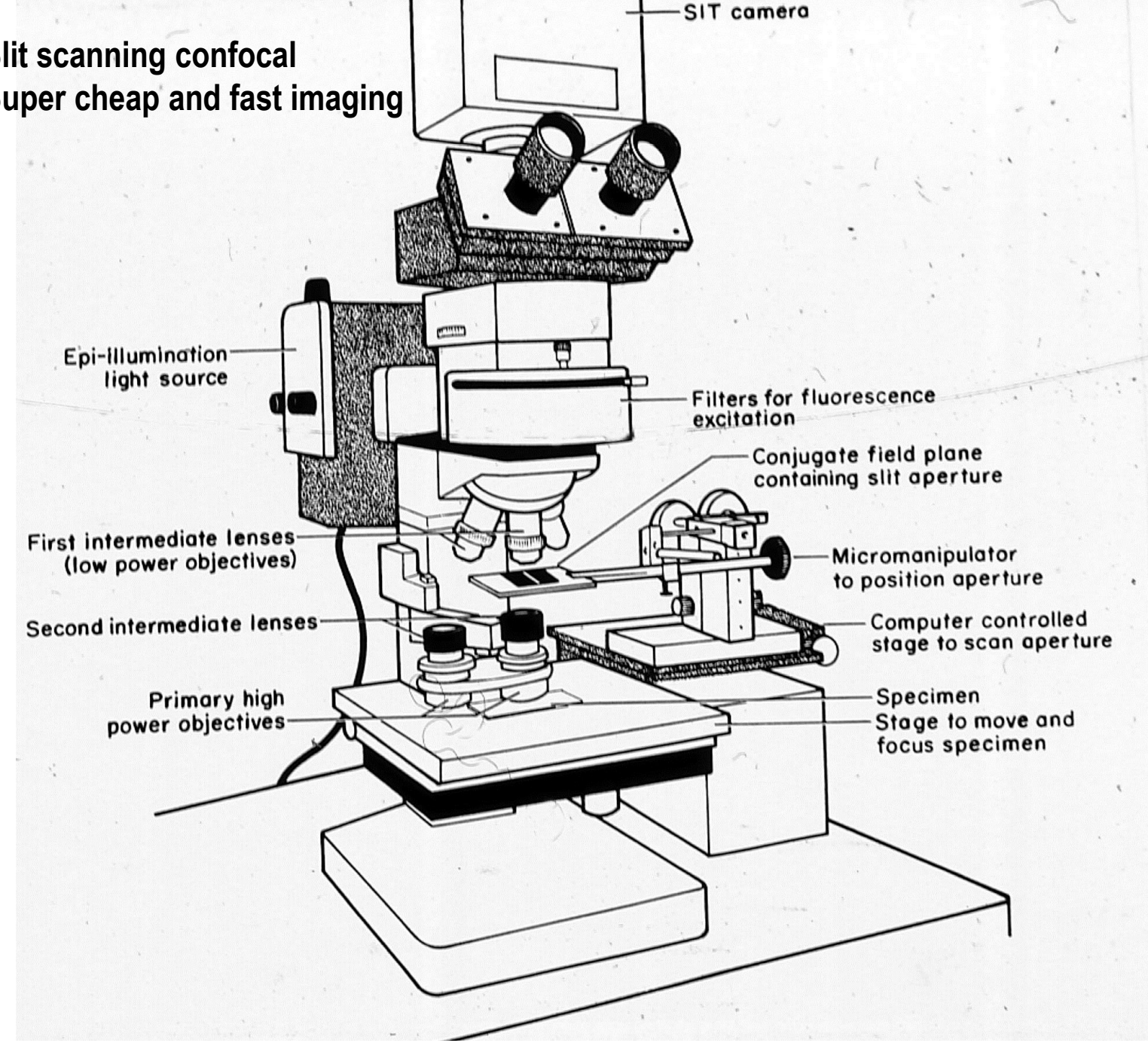
Nipkow Multiplexing Disk



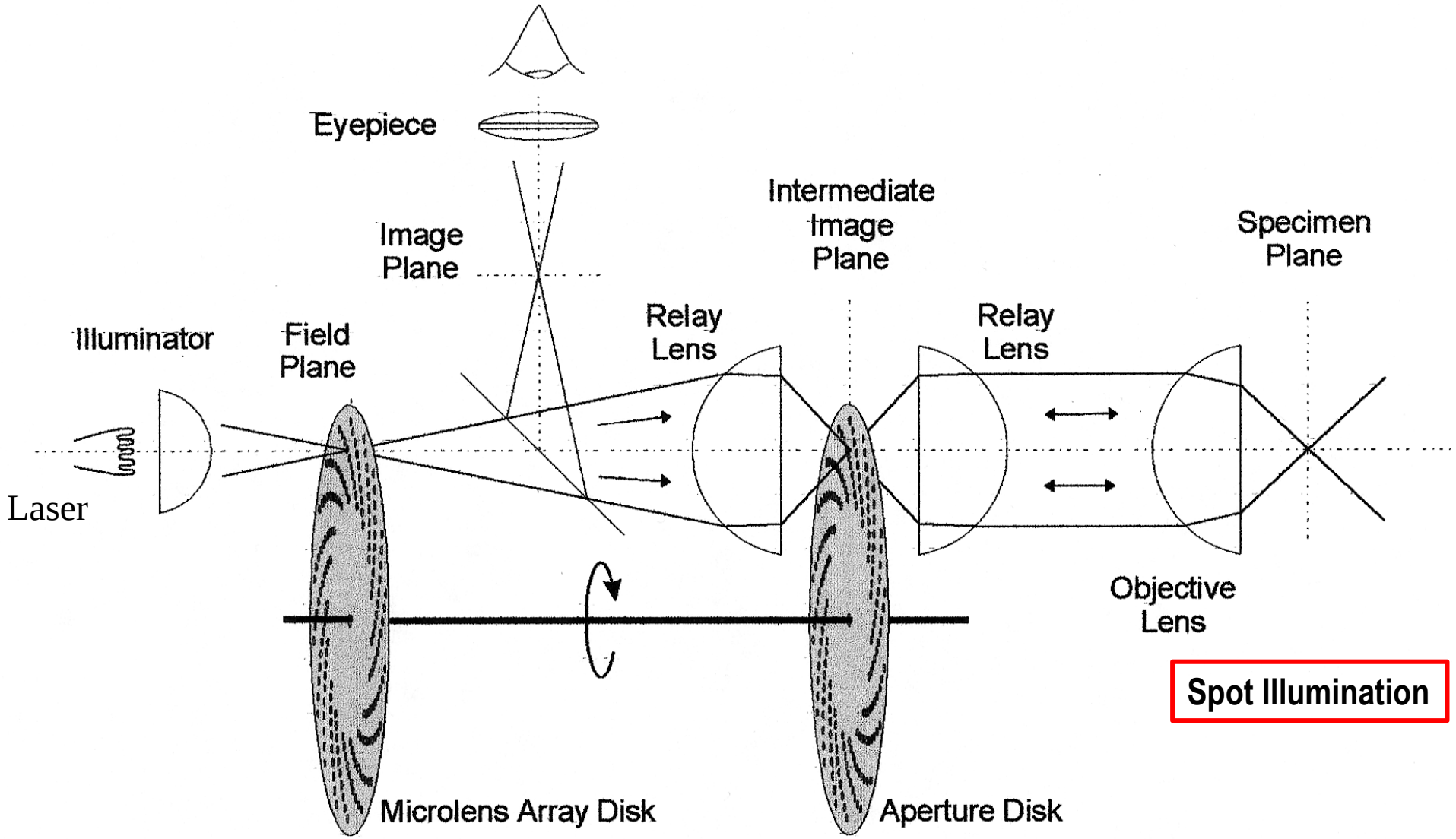
Actual Nipkow disk



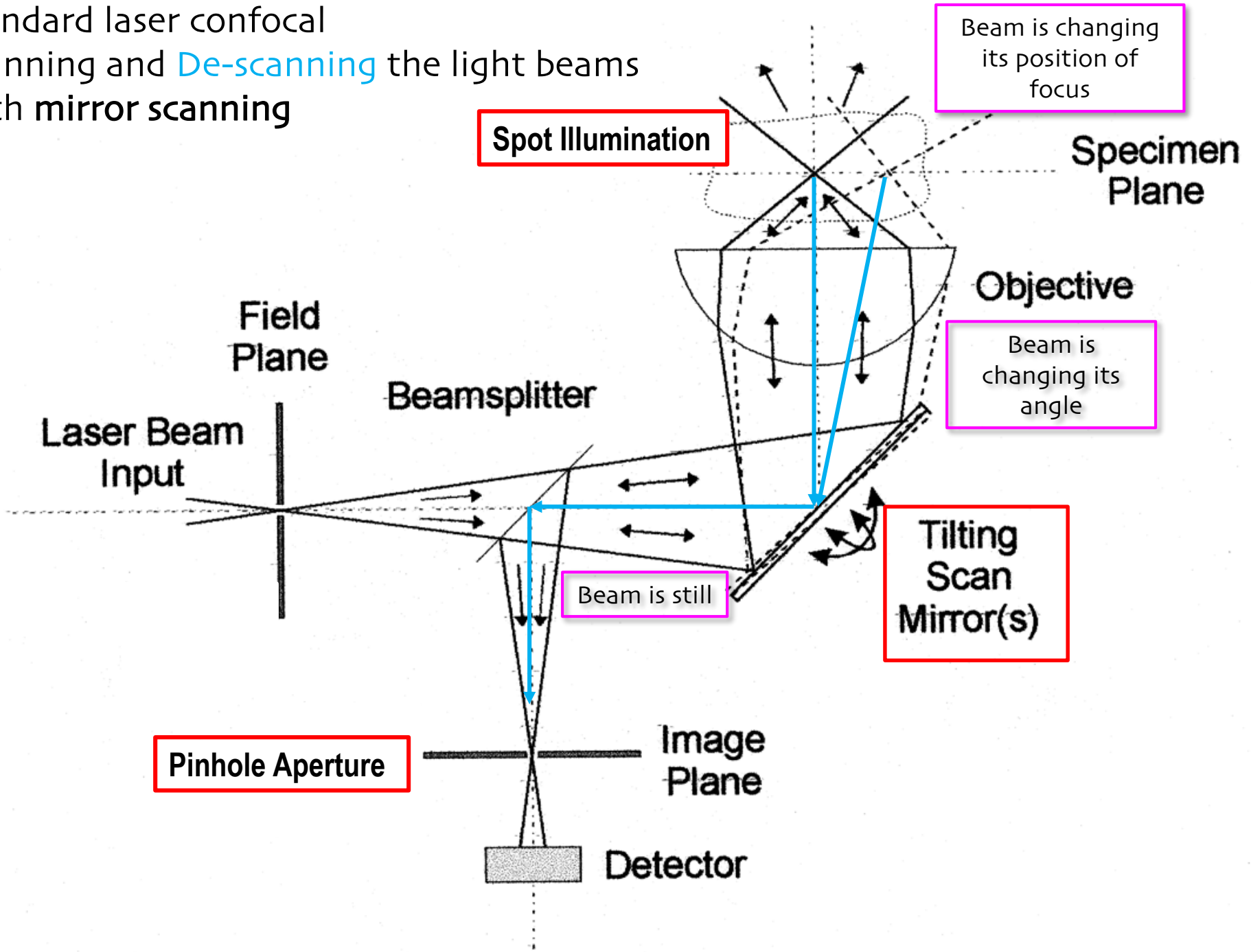
Slit scanning confocal
Super cheap and fast imaging



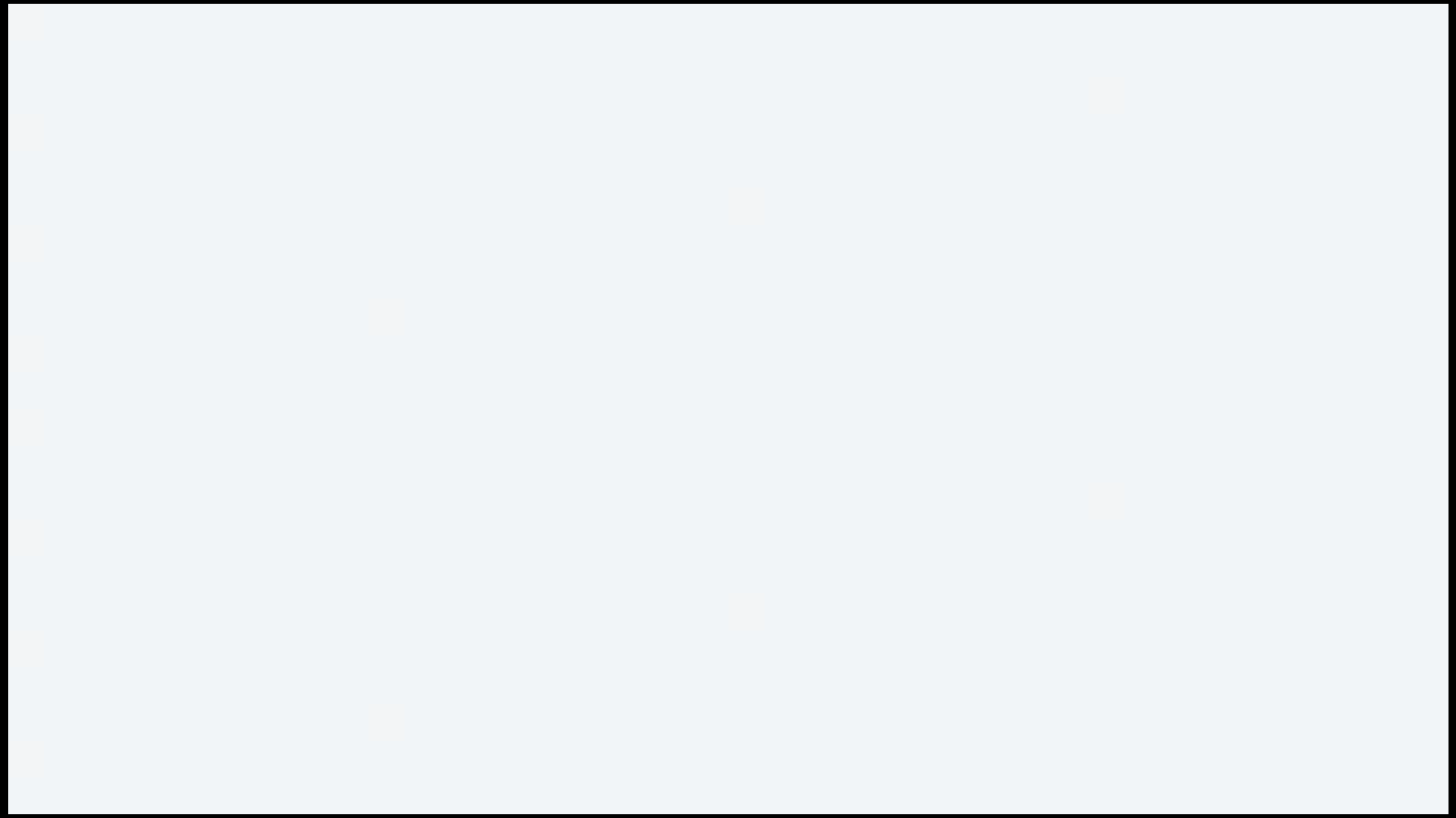
"Microlens" assisted single bi-directional aperture scanning (Yokagawa)



Standard laser confocal
Scanning and De-scanning the light beams
with **mirror scanning**



The newest confocal on the market



CONFOCAL CAVEAT #1:

Limited Number of Fluorophore Molecules

As the laser is focused into a very small spot most confocals will permit the intensity to be high enough to saturate the fluorophore.

Effect: As you add light the fluorophores in the focal plane won't get brighter- but what will get brighter?

CONFOCAL CAVEAT #2:

Intersystem crossing

A typical fluorophore such as fluorescein is in excited state for ~ 4.5 ns, has a high QE (~ 0.9) with a 30 fold lower probability of intersystem crossing (to triplet state; low QE= ~ 0.03) ***BUT*** the lifetime of the triplet state is orders of magnitude longer ~ μS,

Effect:: An accumulation of molecules in the triplet state. For example 1 mW laser illumination of fluorescein via a 1.25 NA lens focused to a .25 um spot causes 63% of the molecules within the beam to be excited at once- after just 190 ns: 81% in triplet state, 12% in excited state, 7% in ground state so fluorescence emission is way down. Moreover the triplet state is associated with free radical formation which causes toxicity and permanent bleaching.

CONFOCAL CAVEAT #3

Bleaching is not plane specific

Although the intensity of the laser excitation is maximal in the waist of the beam, the scanning means that, in the specimen plane, spots will be only intermittently illuminated. Above and below the specimen plane the hour glass beam is broader and the sample remains within the beam for greater proportion of the scan time- this effect completely offsets the drop in the intensity on the broader part of the beam.

The effect: The number of exciting photons that can cause bleaching is the same for the out of focus planes and the in focus plane. What does this mean for the acquisition of 3D stacks?

CONFOCAL CAVEAT #4

Scatter In and Scatter Out

Light both on its way in and way out the preparation may interact with matter and be scattered. The scattering is dependent on wavelength (Rayleigh scattering is inversely proportional to the 4^{th} power of λ. So some of the exciting light doesn't make it to the focal plane and some of the fluorescence emission is scattered on the way out.

Effect: Poor excitation at deep sections and as a result of that plus the scatter on the way out poor fluorescence signal making it back through the pinhole.

Possible solutions to these problems

- Use the minimal amount of light (less than 1% of the "power" settings on most laser scanning microscopes
- Clearing so deep scattering is not an issue
- The "ideal" microscope might use long wavelength light to penetrate deeply and decrease scatter of the illumination light (think red sunset and blue sky), illuminate with short pulses (femtoseconds), not use a pinhole on the return light path but still get optical sectioning, and only bleach the section that is imaged.- Too implausible?
- (No, 2Photon!)
- An even more ideal microscope would not bleach any plane except the focal plane and do so in widefield for speed: single plane or light sheet

Optional Reading and Viewing:

Fundamentals of Light Microscopy and Electronic Imaging. Douglas B. Murphy Michael Davidson . Wiley-Blackwell; 2nd edition (2012).

iBioMicroscopy. http://www.ibiology.org/ibioeducation/taking-courses/ibiology-microscopy-course/image-formation.html (video of some of this material by me and many others)

Handbook of Biological Confocal Microscopy. 3rd ed. James B. Pawley. Reissue: Springer 2006 -*Excellent treatment*

Optical sectioning microscopy. Conchello JA, Lichtman JW. Nat Methods. 2005 Dec;2(12):920-31. Review. PMID: 16299477

Fluorescence microscopy. Lichtman JW, Conchello JA. Nat Methods. 2005 Dec;2(12):910-9. Review. PMID: 16299476 (I am told is pretty good review- very popular)

High-resolution imaging of synaptic structure with a simple confocal microscope. Lichtman JW, Sunderland WJ, Wilkinson RS. New Biol. 1989 1:75-82.

Theoretical analysis of a rotating-disk partially confocal scanning microscope. Conchello JA, Lichtman JW. Appl Opt. 1994; 33:585-96. oi: 10.1364/AO.33.000585. (for aficionados)