GEM4 Summer School OpenCourseWare
http://gem4.educommons.net/
http://www.gem4.org/

Lecture: "3-D Microscopy: Deconvolution, Confocal, Multiphoton" by Dr. Peter So.
Given August 10, 2006 during the GEM4 session at MIT in Cambridge, MA.

Please use the following citation format:

> So, Peter. "3-D Microscopy: Deconvolution, Confocal, Multiphoton." Lecture, GEM4 session at MIT, Cambridge, MA, August 10, 2006. http://gem4.educommons.net/ (accessed MM DD, YYYY). License: Creative Commons Attribution-Noncommercial-Share Alike.

Note: Please use the actual date you accessed this material in your citation.

3D Microscopy: Deconvolution, Confocal, Multiphoton

Images removed due to copyright restrictions.

Biological systems are inherently 3D!

Images removed due to copyright restrictions.
Cross-sections of the length and width of a human brain, and electron microscope images of neurons.

Biological processes also occur on multiple length scale

3D Microscopy

Deconvolution:

Hiraoka, Science, 1987

McNally, Methods, 1999

Confocal Microscopy:

Minsky, US Patent, 1961

Two-Photon Microscopy:

Sheppard et al., IEEE J of QE, 1978

Denk et al., Science, 1990

Understanding Optics: 4 simple rules of tracing light rays

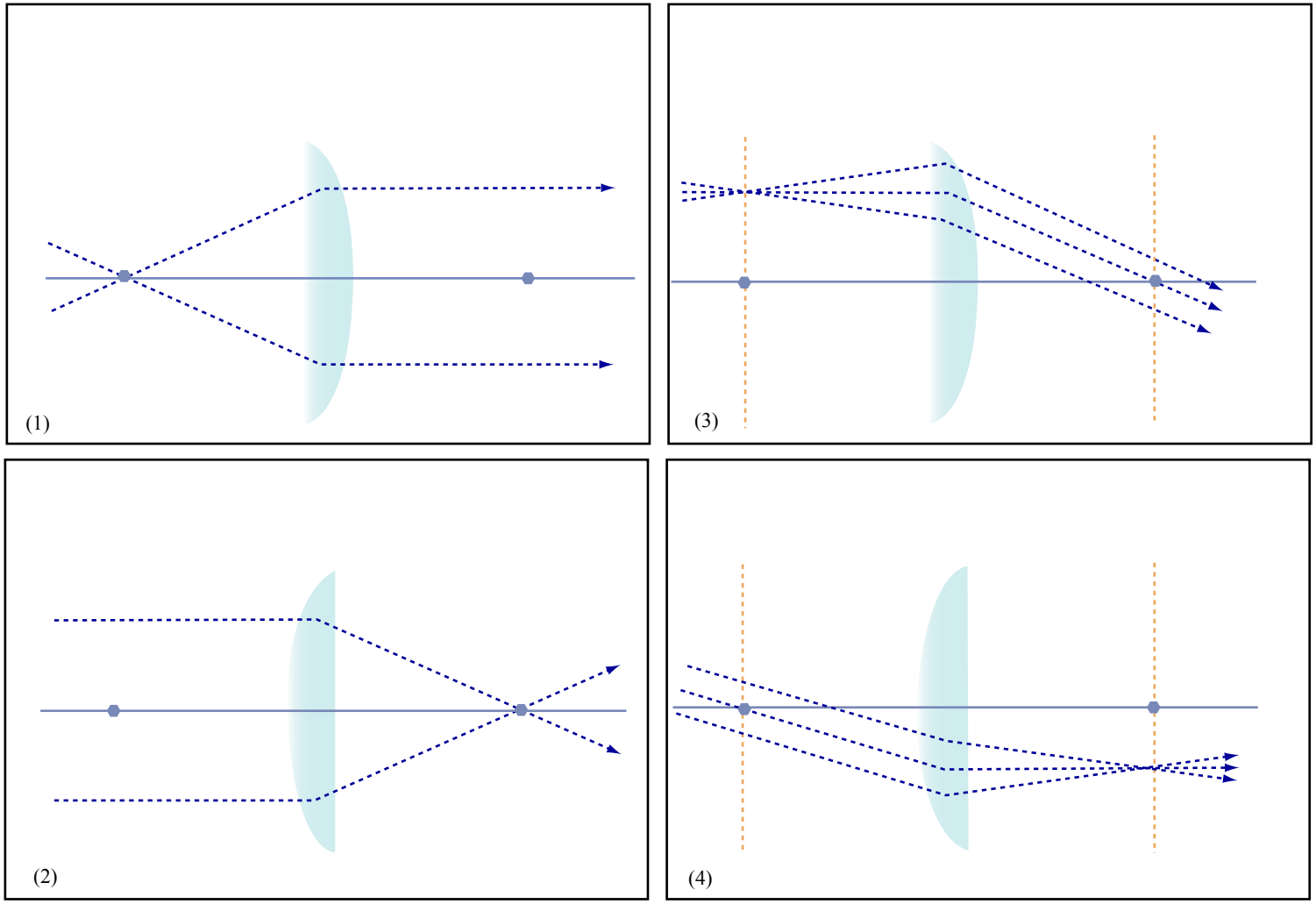


Figure by MIT OCW.

What is a microscope?

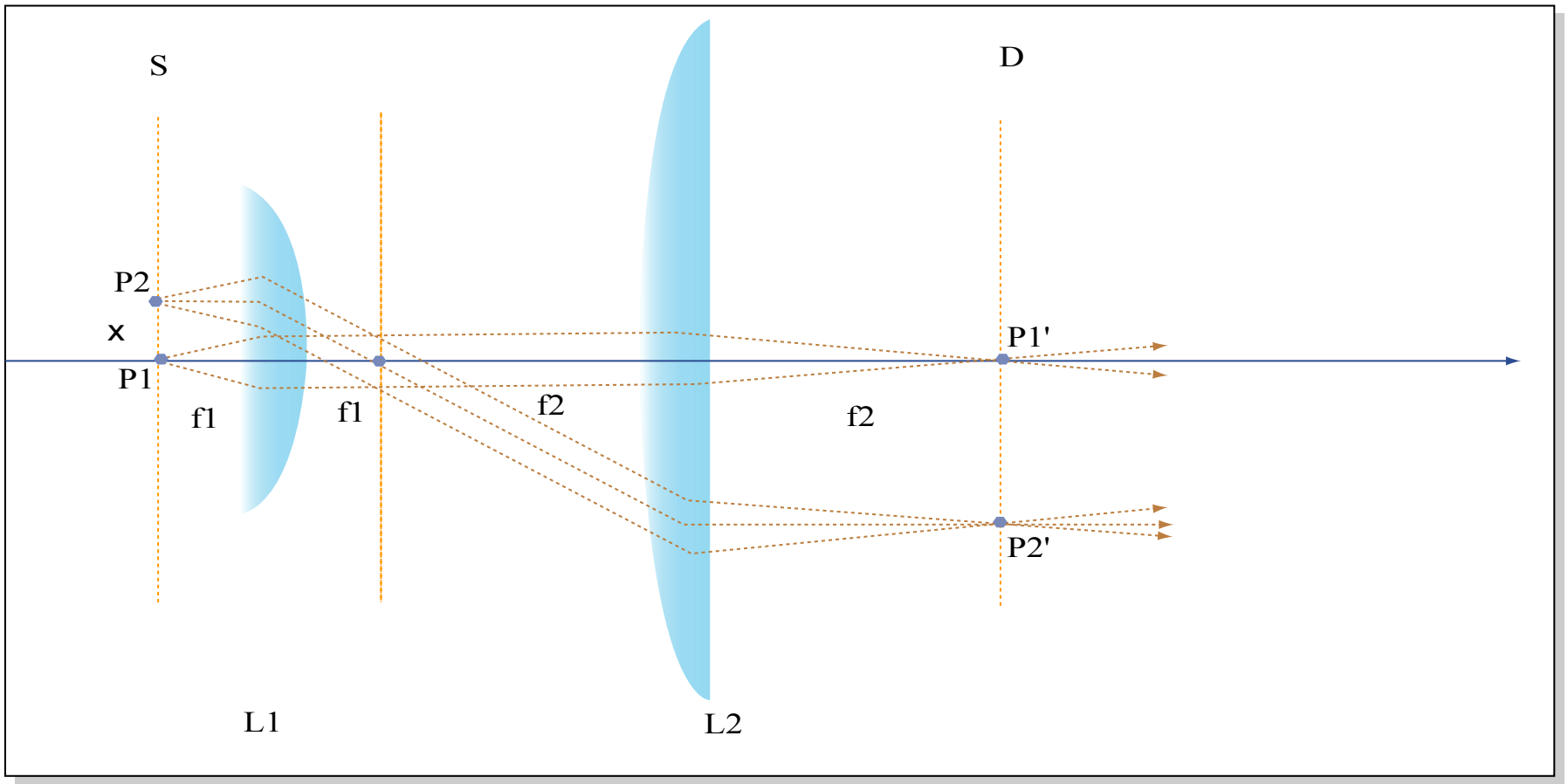


Figure by MIT OCW.

Magnification = f2/f1

This is a wide field microscopy

How light focus by a microscopy objective?

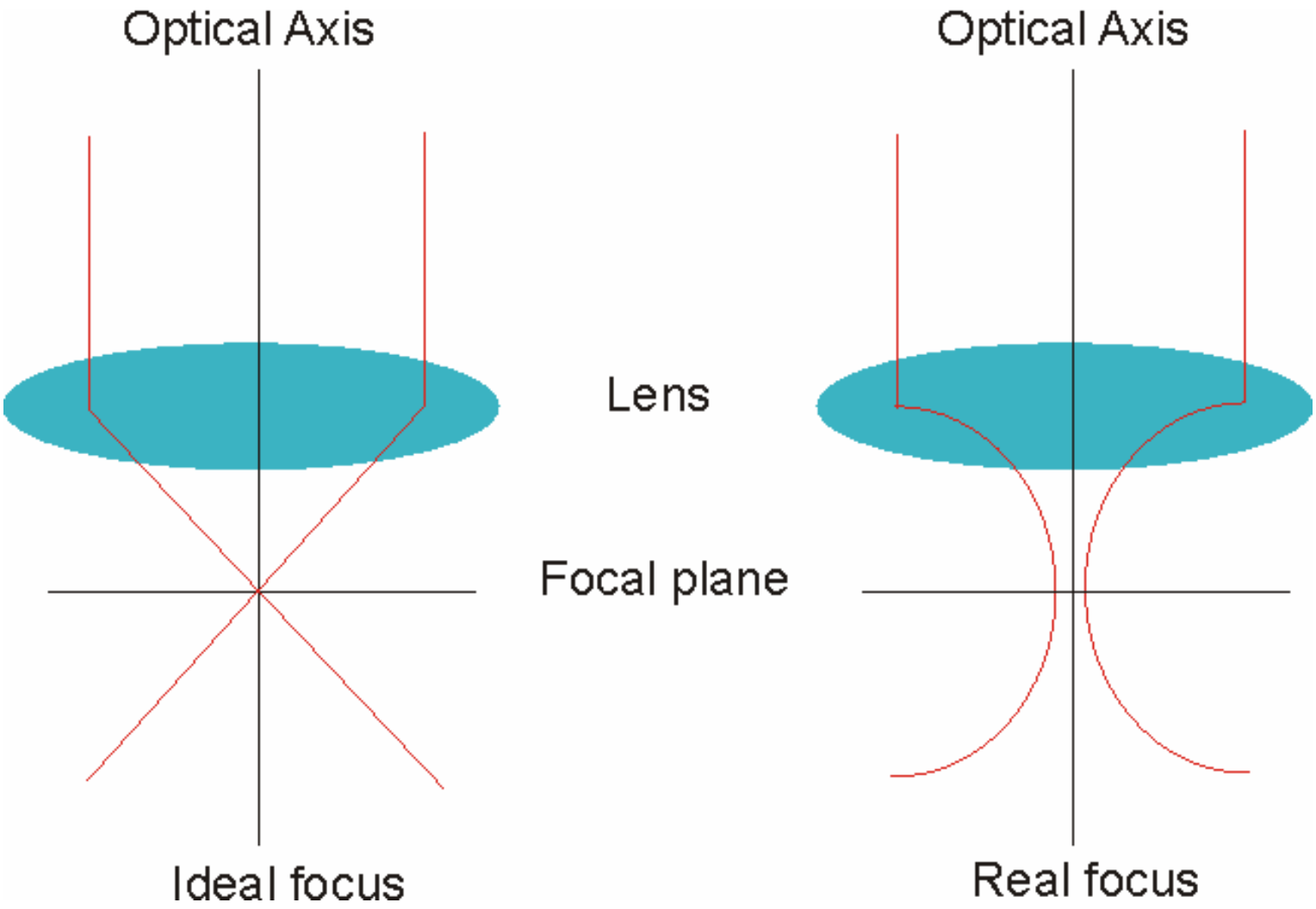


Image removed due to copyright restrictions.
Two-slit diffraction pattern.

Interference & Diffraction Effects are Important at the Focus

Experimentally Measuring the Light Distribution at Focus

What we observe?

(1)Radial resolution
--the lateral dimension is NOT infinitely small

(2) Axial resolution
--light is generated above & below the focal plane

Images removed due to copyright restrictions.

See Fig. 1 in McNally, et al. "Three-Dimensional Imaging by Deconvolution Microscopy." *Methods* 19 (1999): 373-385.

McNally, Methods, 1999

Point Spread Function – Image of an Ideal Point

Lateral Dimension: Airy function

$$PSF(kr) \propto [\frac{2J_1(kr)}{kr}]^2$$

$k = \frac{2\pi}{\lambda}$ is the wave number

$$\text{FWHM} \approx \frac{\lambda}{2}$$ Resolution

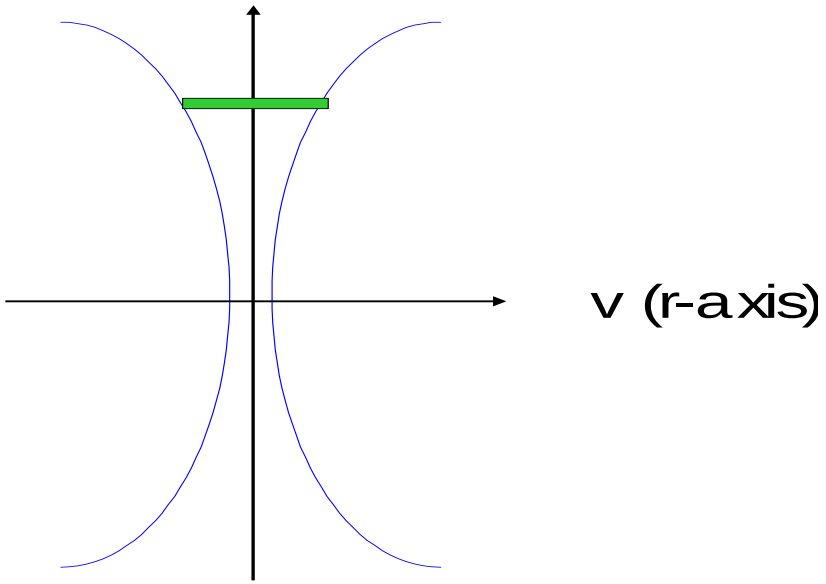
Images removed due to copyright restrictions.

Axial Dimension : Sinc function

$$PSF(kz) \propto [\frac{\sin(kz)}{(kz)}]^2$$

Depth discrimination

For a uniform specimen, we can ask how much fluorescence is generated at each z-section above and below the focal plane assuming that negligible amount of light is absorbed throughout.



$$F_{z-\sec}(u) \equiv 2\pi \int_0^\infty PSF(u,v)v\,dv$$

Ans: Photon number at each z-section is the same (little absorption) →

The amount of light generated at each z-section is the same!

$$F_{z-\sec}(u) = \text{ Constant}$$

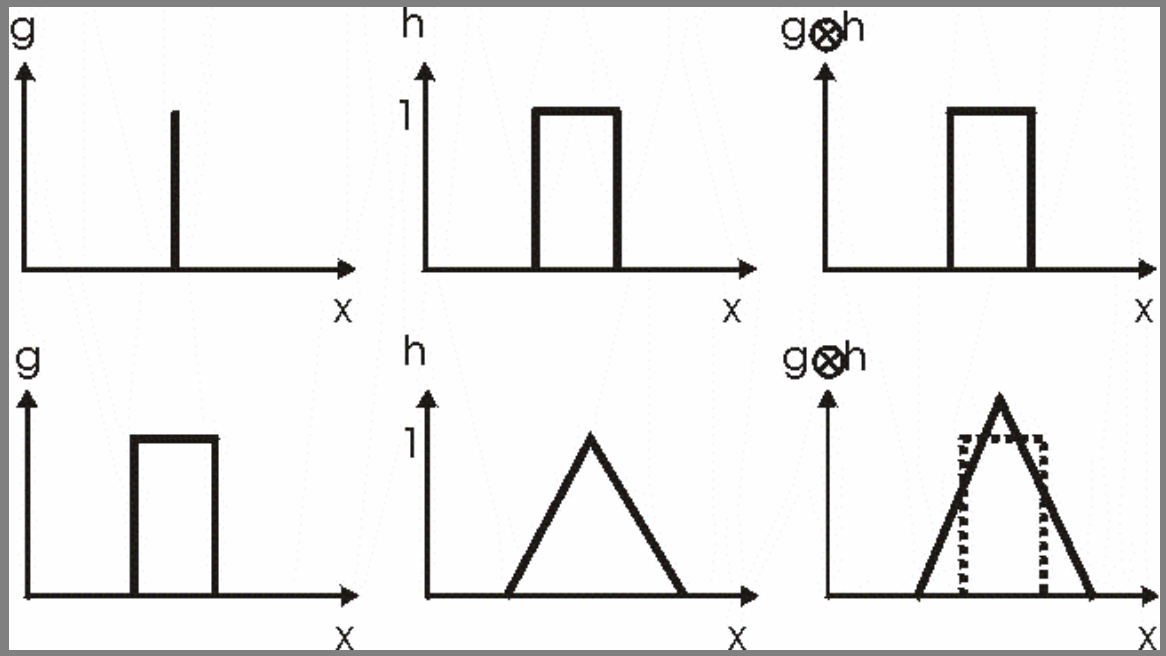
There is no depth discrimination!!!

What is Convolution?

Recall the definition of convolution:

$$g(t) \otimes h(t) = \int_{-\infty}^{\infty} g(\tau) h(t-\tau) d\tau$$

Graphical explanation of convolution:



Convolution is a smearing operation

What is the effect of finite side PSF on imaging?

$$I(\vec{r}) = O(\vec{r}) \otimes PSF(\vec{r})$$

The finite size point spread function implies that images are “blurred” in 3D!!!

Images removed due to copyright restrictions.
See Fig. 2 in McNally, et al. "Three-Dimensional Imaging by Deconvolution Microscopy. " *Methods* 19 (1999): 373-385.

McNally, Methods, 1999

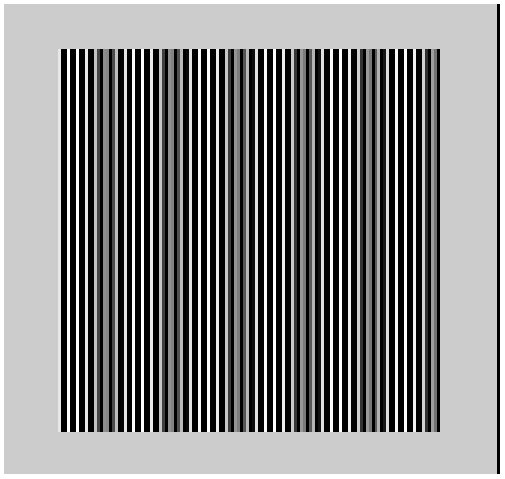
A View of Resolution and Depth Discrimination
In terms of Spatial Frequency

2D Fourier Transform

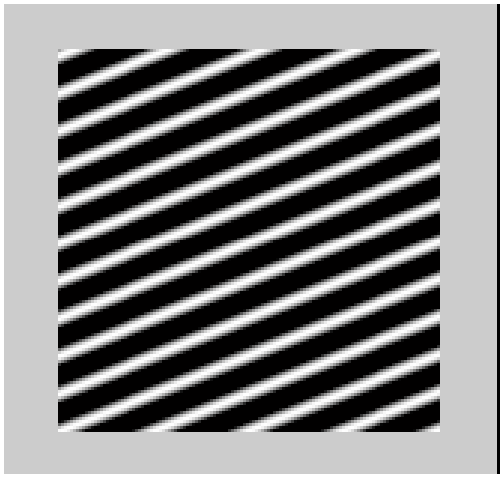
$$\tilde{I}(\vec{k}) = \int_{-\infty}^{\infty}\int_{-\infty}^{\infty} I(x,y,z)\exp[-2\pi i(k_x x + k_y y + k_z z)]dxdydz$$

Power Spectrum $\tilde{P}(\vec{k}) = \left|\tilde{I}(\vec{k})\right|^2$

Two dimensional examples



High frequency



Low frequency

Convolution Theorem

$$\Im(g \otimes h)(f) = \tilde{g}(f)\tilde{h}(f)$$

Proof in 1-D

$$\int_{-\infty}^{\infty} g \otimes h(t) e^{-i2\pi ft} dt = \int_{-\infty}^{\infty}\int_{-\infty}^{\infty} g(\tau)h(t-\tau)d\tau e^{-i2\pi ft} dt$$

$$= \int_{-\infty}^{\infty} d\tau g(\tau) e^{-i2\pi f\tau} \left(\int_{-\infty}^{\infty} dt h(t-\tau) e^{-i2\pi f(t-\tau)} \right)$$

$$= \int_{-\infty}^{\infty} d\tau g(\tau) e^{-i2\pi f\tau} \left(\int_{-\infty}^{\infty} dt' h(t') e^{-i2\pi f(t')} \right)$$

$$= \tilde{g}(f)\tilde{h}(f)$$

where $t' = t - \tau \quad dt' = dt$

Fourier transform of the convolution of two functions is the product of the Fourier transforms of two functions

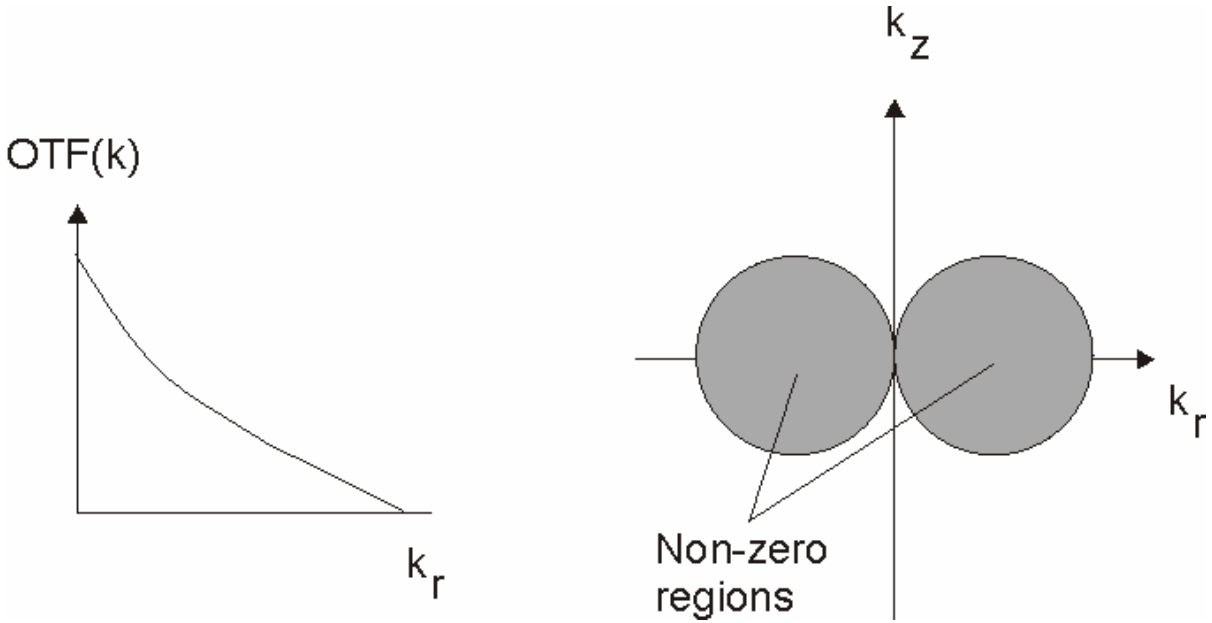
Resolution and Discrimination in Frequency Domain

$$I(\vec{r}) = O(\vec{r}) \otimes PSF(\vec{r})$$

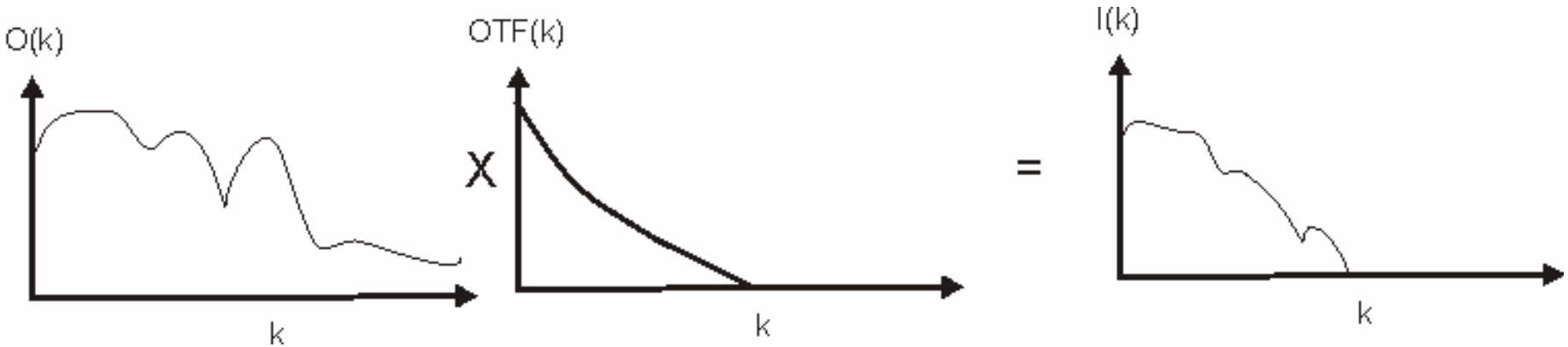
$$\tilde{I}(\vec{k}) = \tilde{O}(\vec{k}) \cdot OTF(\vec{k})$$

Goes from convolution
To simple multiplication

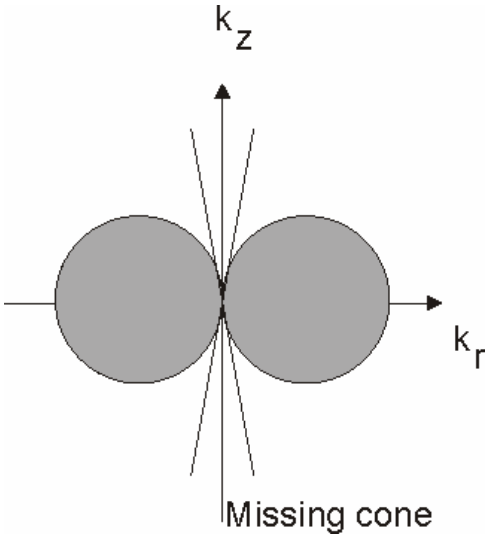
Optical transfer function, OTF, is the Fourier transform of PSF.
How does it looks like?



Effect of OTF on Image – Loss of Frequency Content



Effects: (1) lower amplitude at high frequency
(2) completely loss of information at high frequency



Missing all info along kz axis.
"Missing cone" is the origin of no depth discrimination

Deconvolution Microscopy

What is Deconvolution Microscopy?

$$\tilde{I}(\vec{k}) = \tilde{O}(\vec{k}) \cdot OTF(\vec{k})$$ Convolution

$$\tilde{O}(\vec{k}) = \tilde{I}(\vec{k}) \cdot OTF(\vec{k})^{-1}$$

$$O(\vec{r}) = \mathrm{F}^{-1}[\tilde{O}(\vec{k})]$$ Deconvolution

What is the problem of this procedure?

OTF is zero at high frequency…. Divide by 0???

There are many possible “O” given “I” and “OTF”
This belongs to a class of “ill posted problem”

The “art” of deconvolution is to find constrains that allow the best estimate of “O”. An example of these constraints is positivity

Application of Deconvolution I

Image removed due to copyright restrictions.
See Fig. 3 in McNally, et al. "Three-Dimensional Imaging by Deconvolution Microscopy." *Methods* 19 (1999): 373-385.

Application of Deconvolution II

Raw images deconvoluted by 3 different methods

Depending on deconvolution algorithm chosen different “features” and “artifacts” are seen

Image removed due to copyright restrictions.
See Fig. 7 in McNally, et al. "Three-Dimensional Imaging by Deconvolution Microscopy." *Methods* 19 (1999): 373-385.

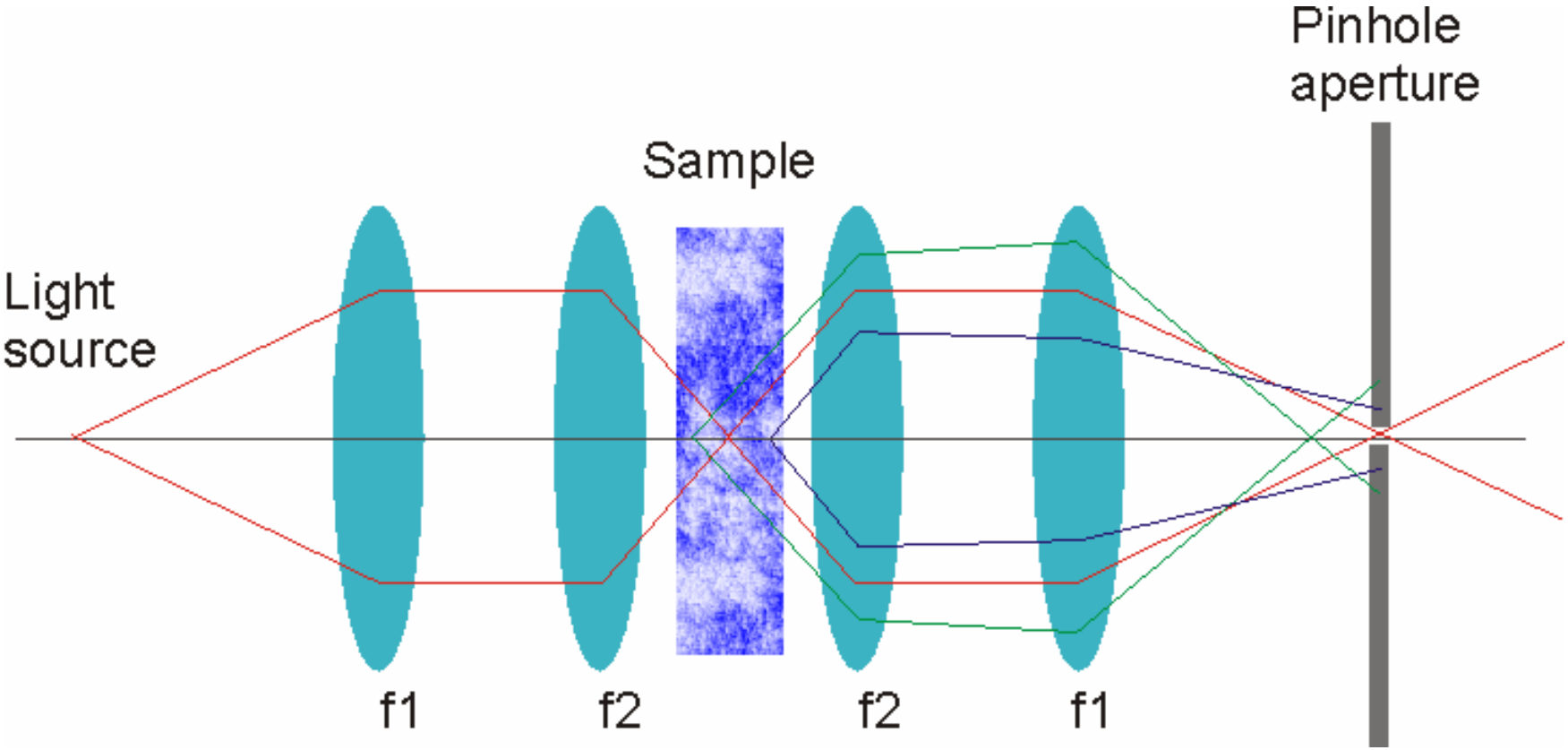
Confocal Microscopy

The Invention of Confocal Microscopy

Confocal microscopy is invented by Prof. Melvin Minsky of MIT in about 1950s.

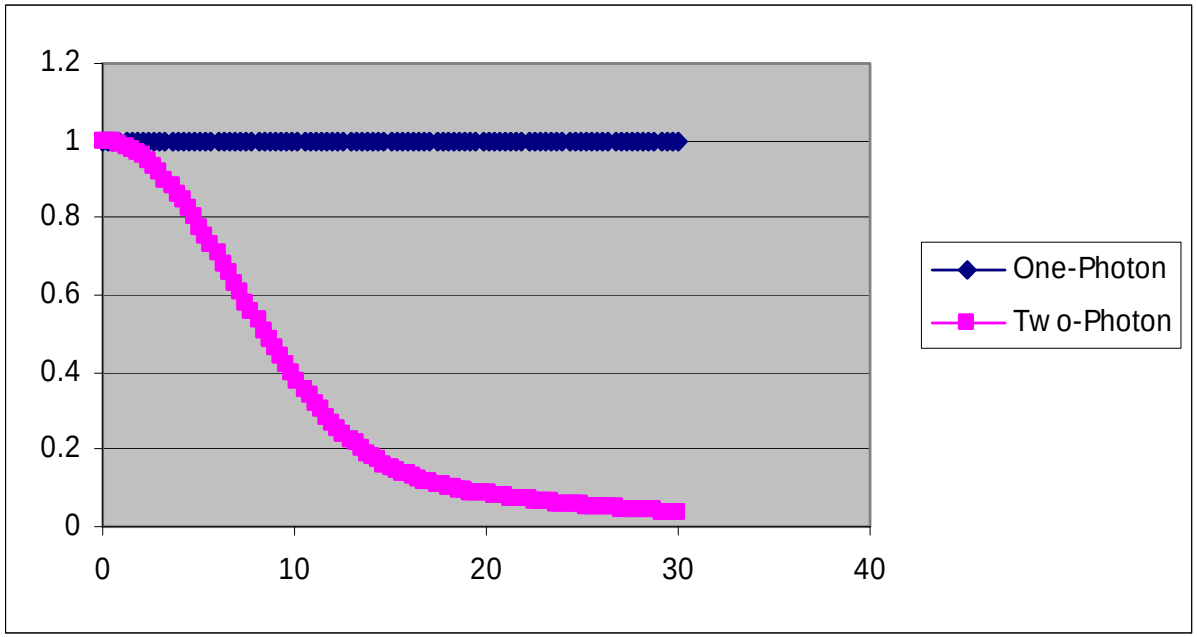
Images removed due to copyright restrictions.
Scans of the patent documents for the confocal microscope.

Principle of Confocal Microscopy

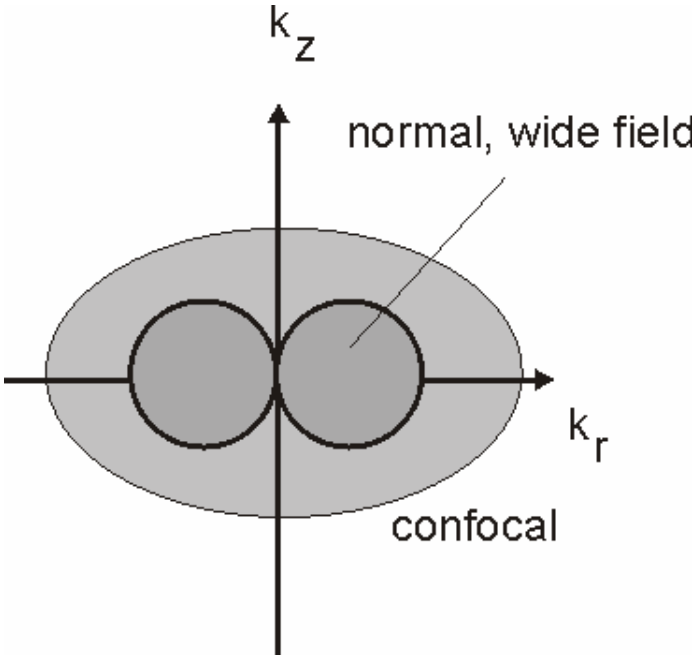


Information comes from only a single point. Needs to move the light or move the sample!

Depth discrimination



OTF



Point Spread Function – Image of an Ideal Point

Lateral Dimension: Airy function

$$PSF_c(kr) \propto [\frac{2J_1(kr)}{kr}]^4$$

Axial Dimension : Sinc function

$$PSF_c(kz) \propto [\frac{\sin(kz)}{(kz)}]^4$$

Images removed due to copyright restrictions.

The PSF of confocal is the square of the PSF of wide field microscopy

$$F_{z-\sec}(u) = 2\pi \int_0^\infty PSF_c(u,v) v dv$$

$$= 2\pi \int_0^\infty PSF^2(u,v) v dv \neq \text{ constant}$$

Early Demonstration of Confocal Microscopy in Biological Imaging

Images removed due to copyright restrictions.

See Fig. 1 and 3 in White, J. G., W. B. Amos, and M. Fordham. "An Evaluation of Confocal Versus Conventional Imaging of Biological Structures by Fluorescence Light Microscopy." *Journal of Cell Biology* 105 (1987): 41-48.

Tandem Scanning Confocal Microscope

Image removed due to copyright restrictions.
Illustration of Nipkow disk.

Image removed due to copyright restrictions.
Illustration of Petran's multiple-beam confocal microscope utilizing a Nipkow disk, circa the late 1960's.

Image removed due to copyright restrictions.

A Model Tandem Confocal Microscope Utilizing Yokogawa Scan Head

Images removed due to copyright restrictions.

Image removed due to copyright restrictions.
See Fig. 2: http://www.yokogawa.com/rd/pdf/TR/rd-tr-r00033-005.pdf

C. Elegans

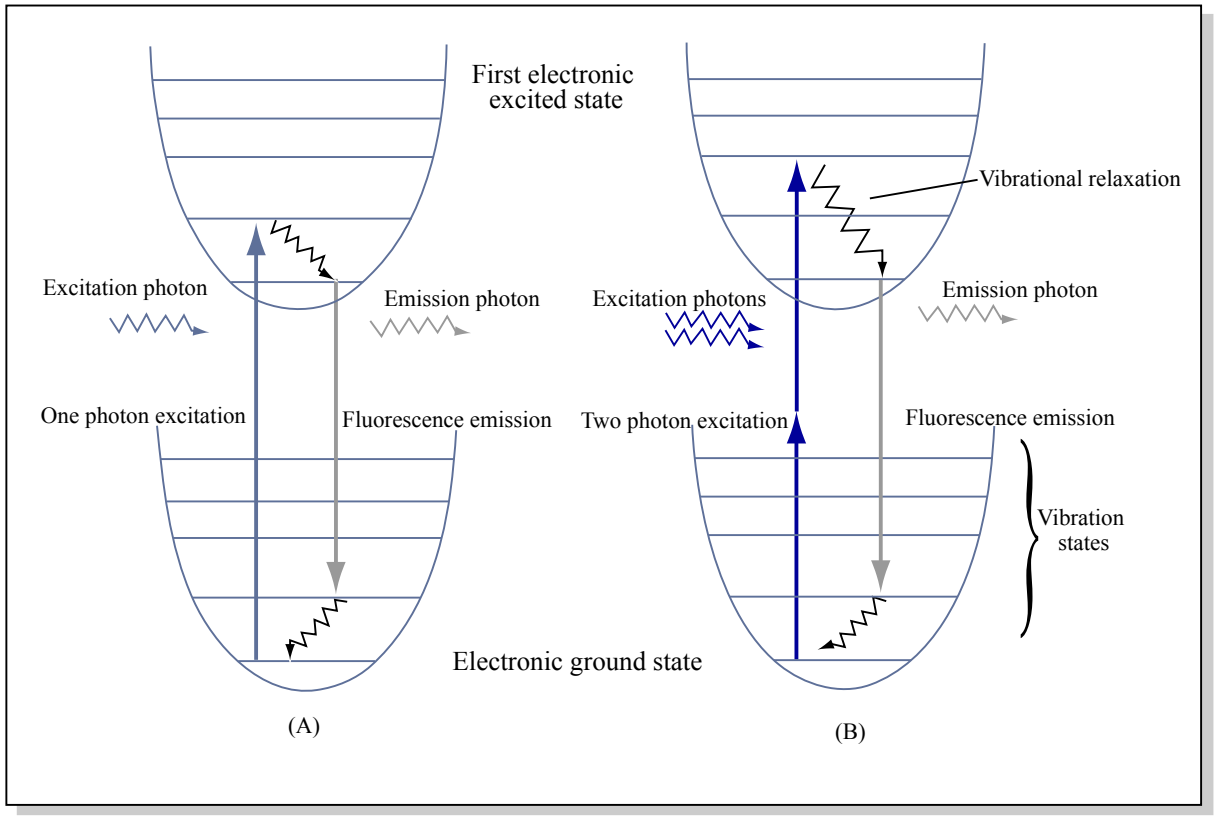
Eliminate light throughput
Issue by spinning both
a plate of lenslets and
nother plate of pinholes

Images removed due to copyright restrictions.

Calcium events in nerve fiber

Multiphoton Microscopy

Two-Photon Excitation Microscopy



Images removed due to copyright restrictions.

Figure by MIT OCW.

A comparison of two-photon and confocal microscopes

(1) Confocal microscopes have better resolution than two-photon microscopes without confocal detection.

(2) Two-photon microscope results in less photodamage in biological specimens.
The seminal work by the White group in U. Wisconsin on the development of *c. elegans* and hamsters provides some of the best demonstration.
After embryos have been continuously imaged for over hours,
live specimens are born after implantation.

(3) Two-photon microscope provides better penetration into highly scattering tissue specimen.
Infrared light has lower absorption and lower scattering in turbid media.

Images removed due to copyright restrictions.

Examples of applications of two-photon confocal microscopy

- 3D reconstruction of skin structures from a mouse ear tissue punch
- In vivo imaging of neuronal development
- 3D quantification of blood flow in solid tumors
- Quantifying and understanding genetically induced cardiac hypertrophy

Figure by MIT OCW.

3D Multiple Particle Tracking with Video Rate Two-Photon Microscopy

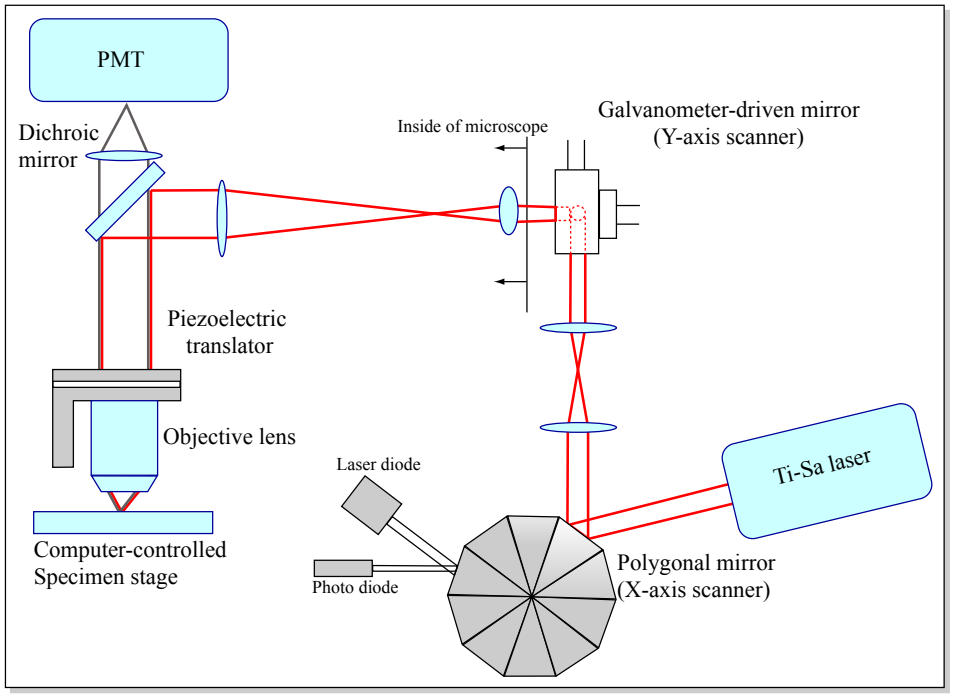


Figure by MIT OCW.

In collaboration with Ki Hean Kim (MIT)

Imaging of
myocyte contraction --
R6G labeled mitochondria

Image removed due to copyright restrictions.

In collaboration with J. Lammerding,
H. Huang, K. Kim, R. Kamm, R. Lee
(MIT and Brigham & Women's Hospital)

A Comparison of The Three 3D Imaging Methods with Wide Field

	Wide field	Deconvolution	Confocal	Multiphoton
Resolution	NA	Better (depend on SNR)	Better	Similar
3D	No	Yes	Yes	Yes
Imaging depth	--	-	+	++
Uncertainty	+	--	+	+
Cost	$	$$	$$$$	$$$$$