

Bi177 - Lecture 5 Adding the Third Dimension

Wide-field Imaging

Point Spread Function

Deconvolution

Confocal Laser Scanning Microscopy

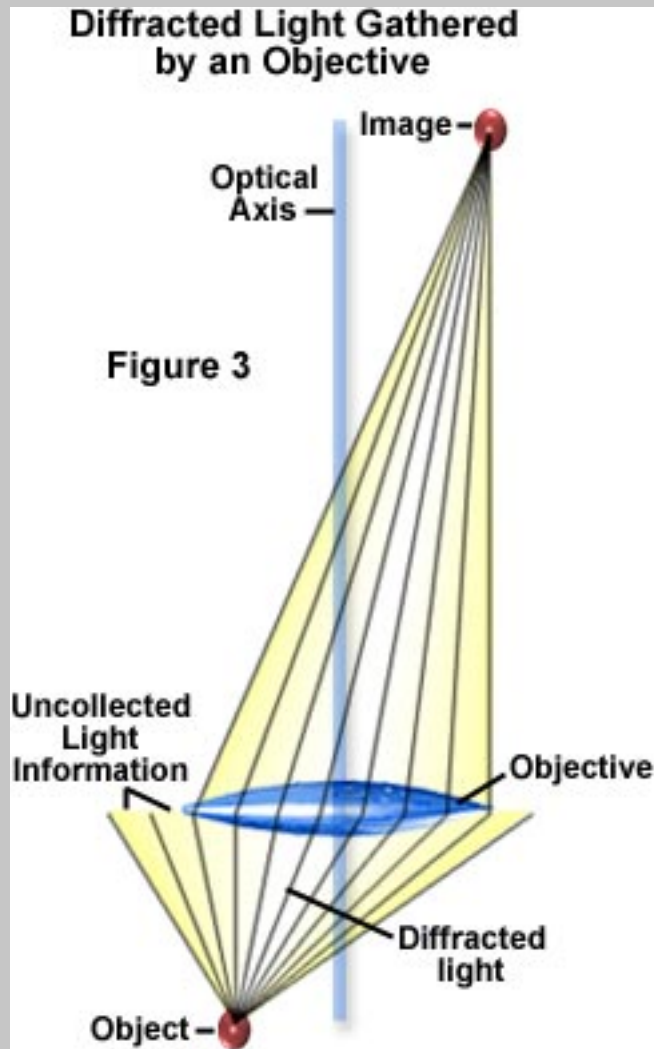
Confocal Aperture

Optical aberrations

Alternative Scanning Microscopy

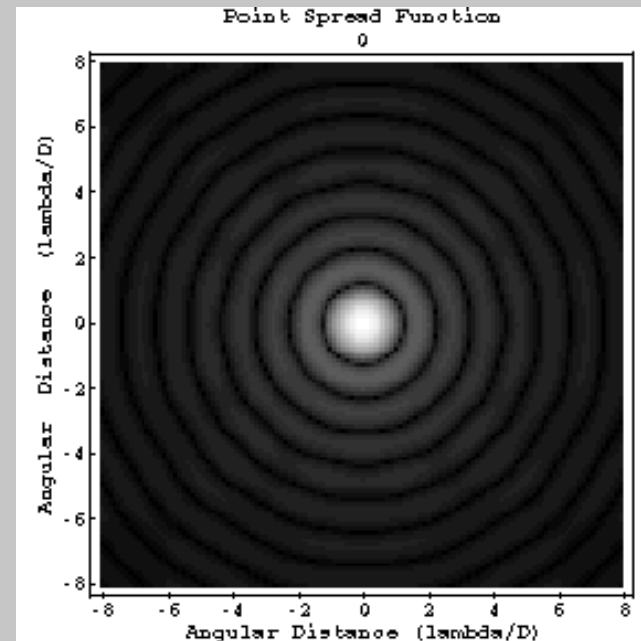
Two-photon Laser Scanning Microscopy

Widefield imaging: detail in the image from collecting diffracted light
Larger aperture = more diffraction peaks = higher resolution



Therefore, for any finite aperture:

1. diffraction limit in size of central maximum
2. Extended point spread function

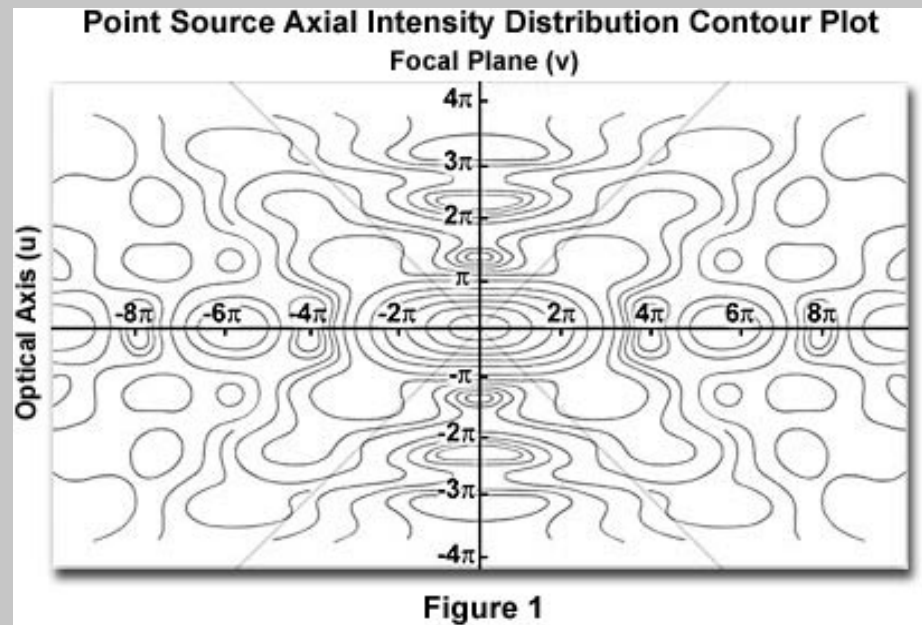
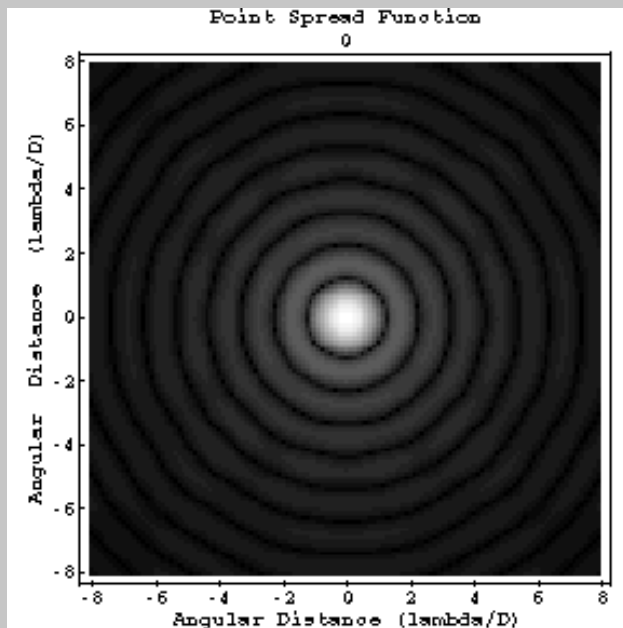


Point Spread Function: Image of a infinitely small object.

Point Spread Function is three dimensional

Image of subdiffraction limit spot

Subdiffraction limit spot



Thus, each spot in specimen will be blurred onto the sensor
(Aperture and “Missing Cone”)

To reduce contribution of the blurring to the image: Deconvolution

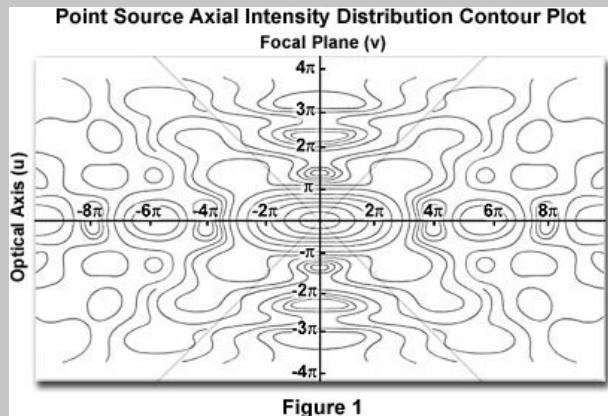
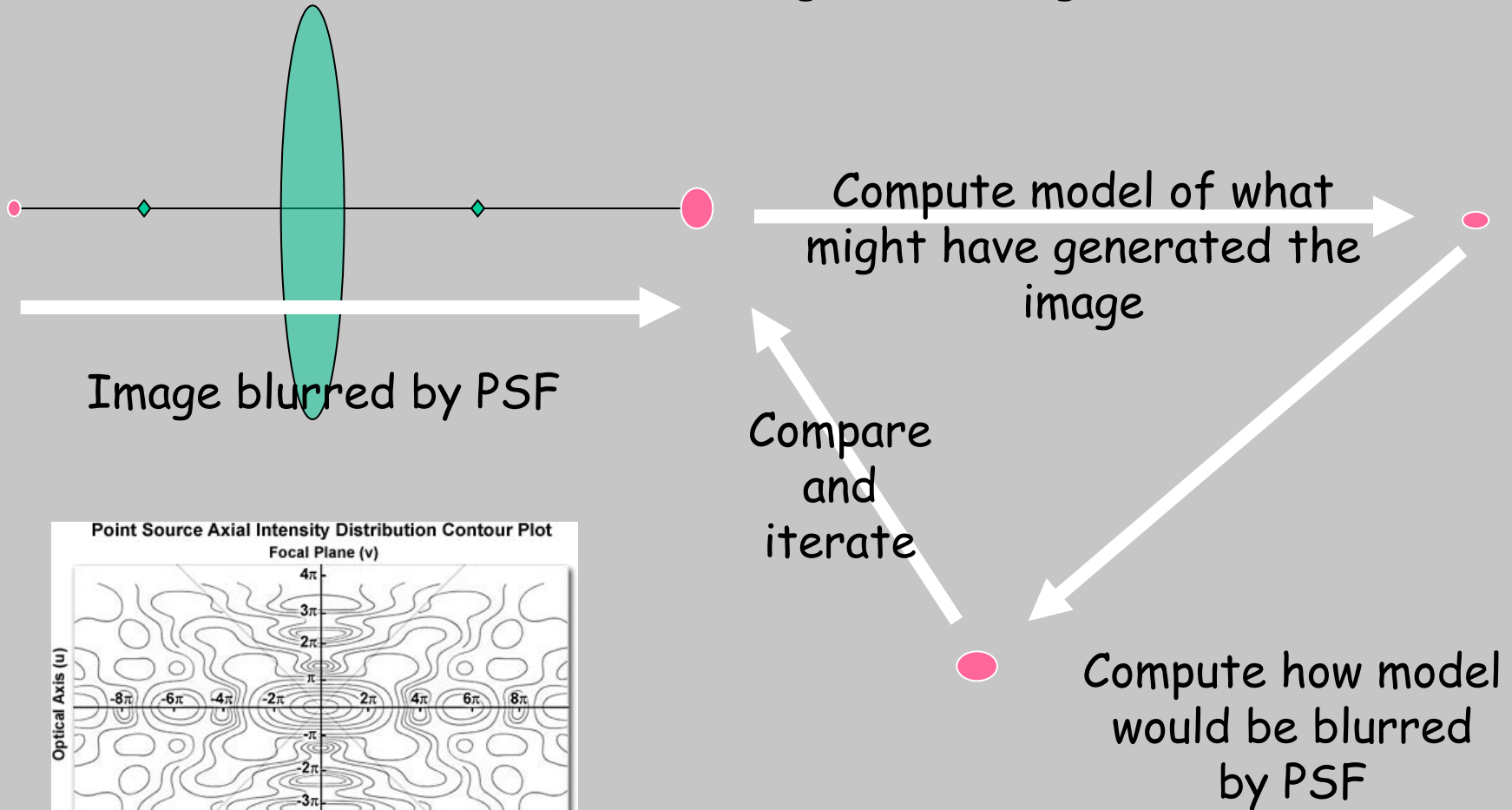


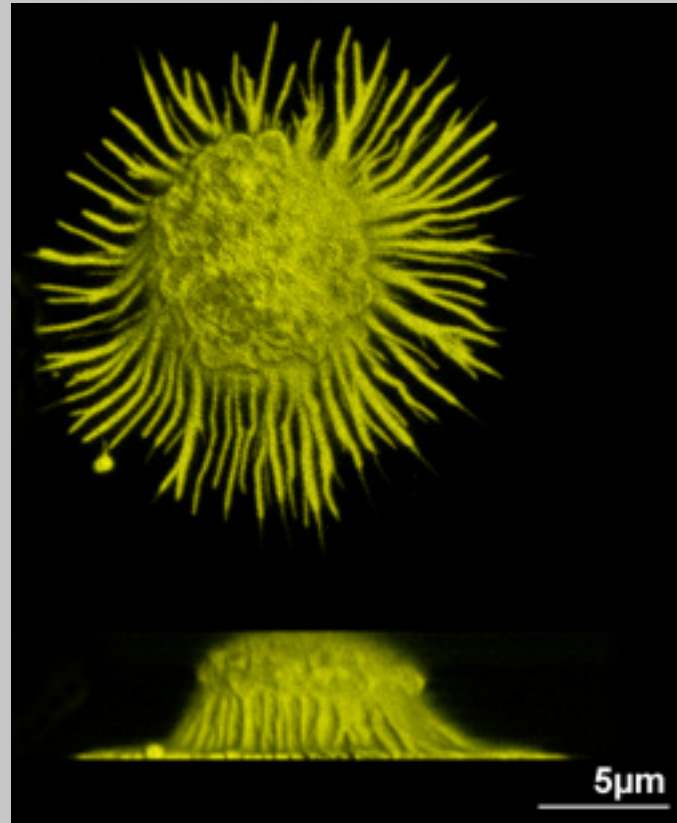
Image deconvolution

Inputs:

- 3-D image stack
- 3-D PSF (bead image)

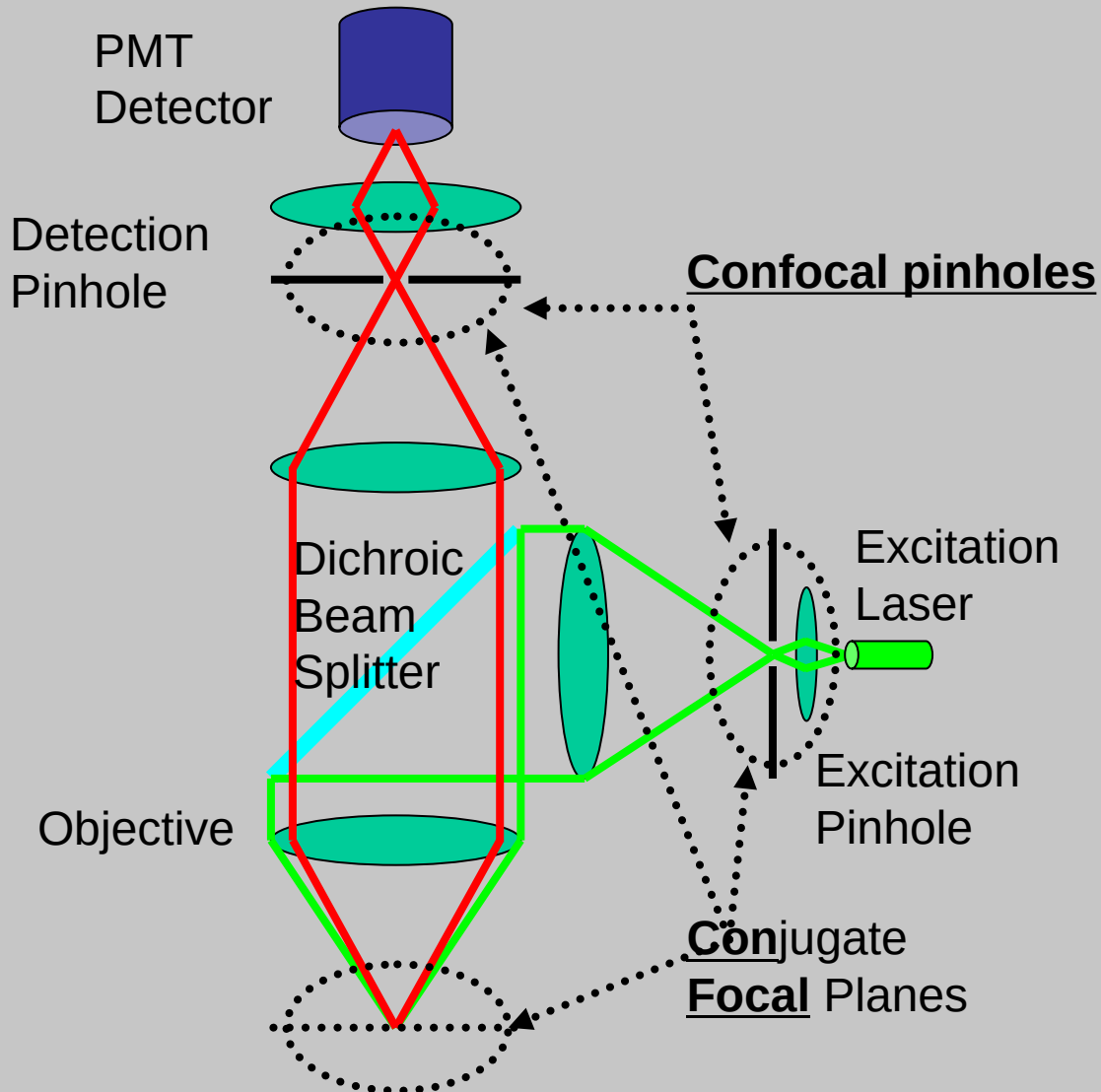
Requires:

- Time
- Computer memory



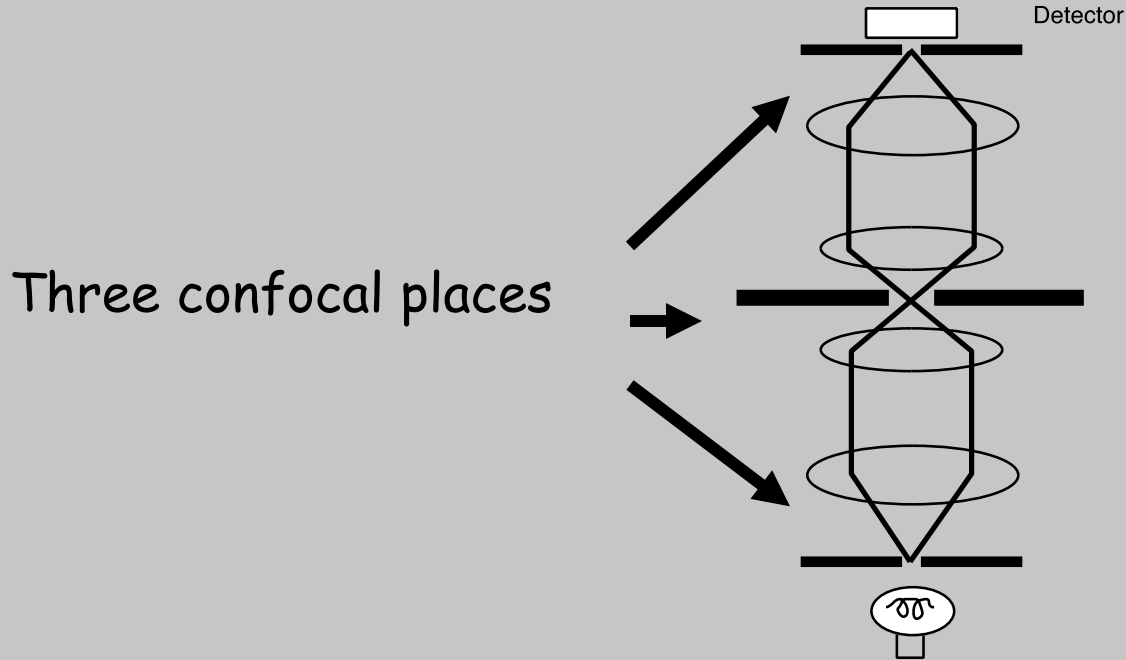
Note: z-axis blurring from the missing cone is minimized but not eliminated

How else to fill in the missing cone?
Need more data in the Z-axis --> Confocal microscopy

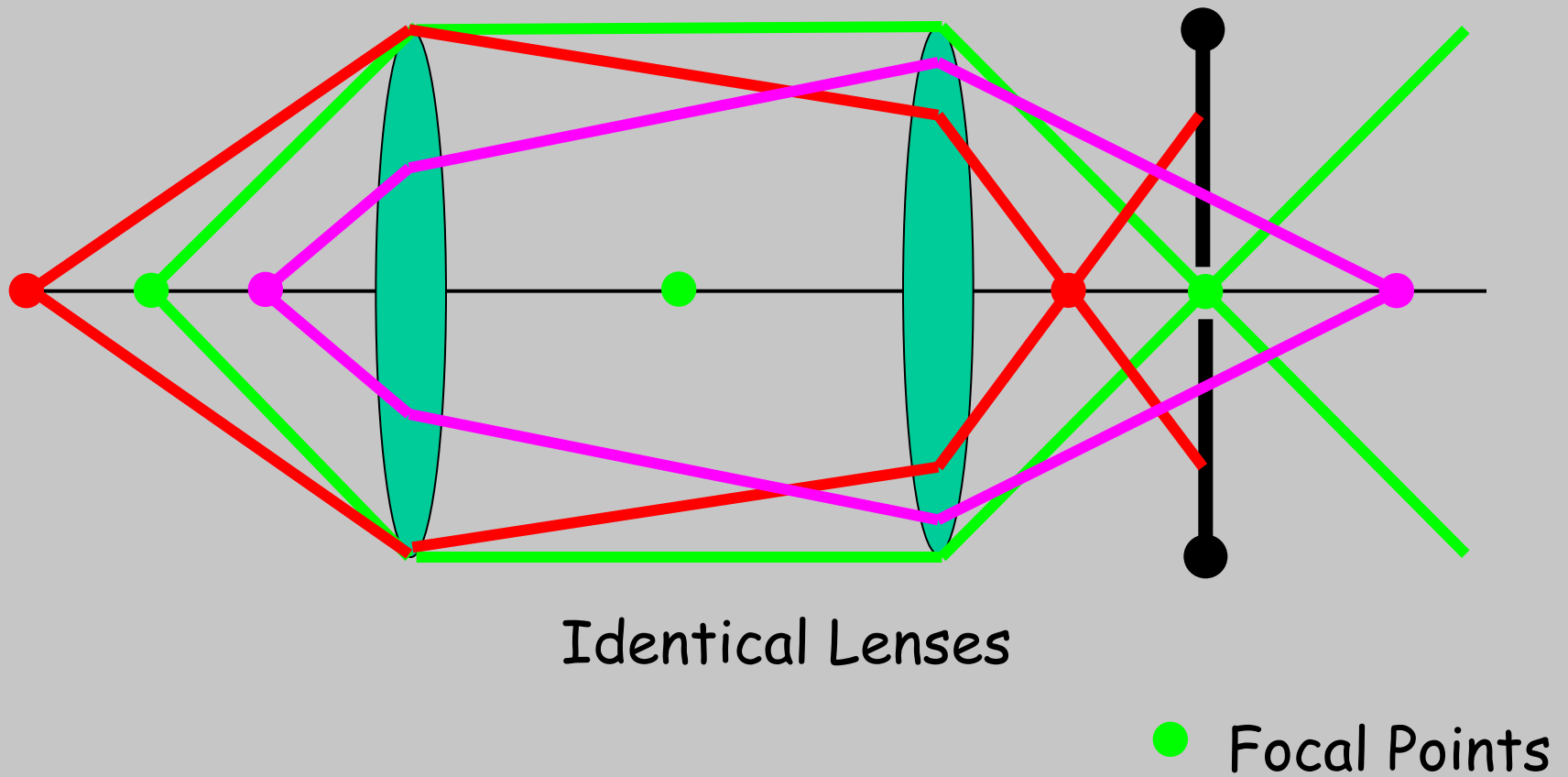


Confocal Microscopy

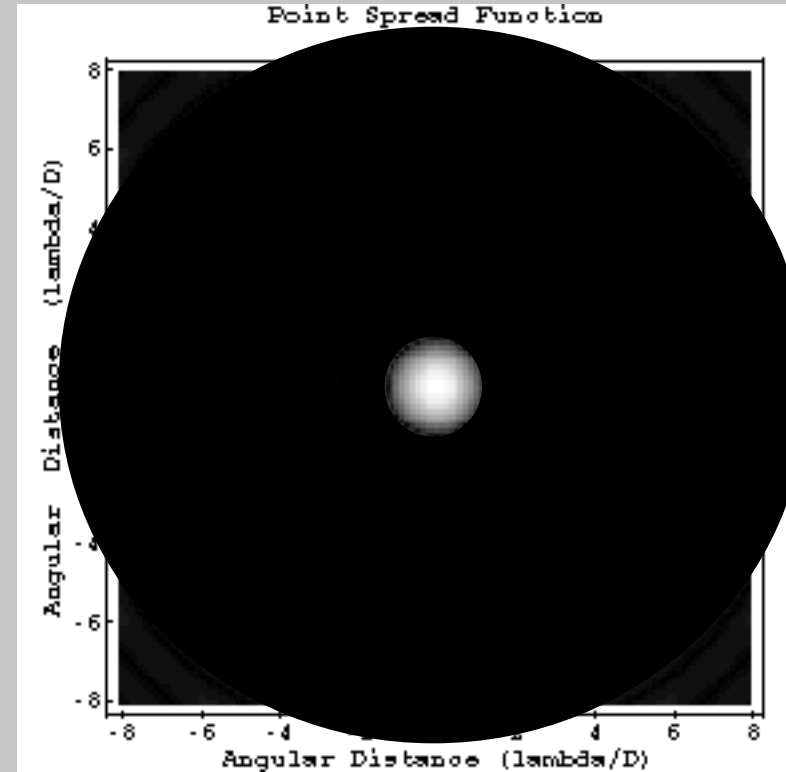
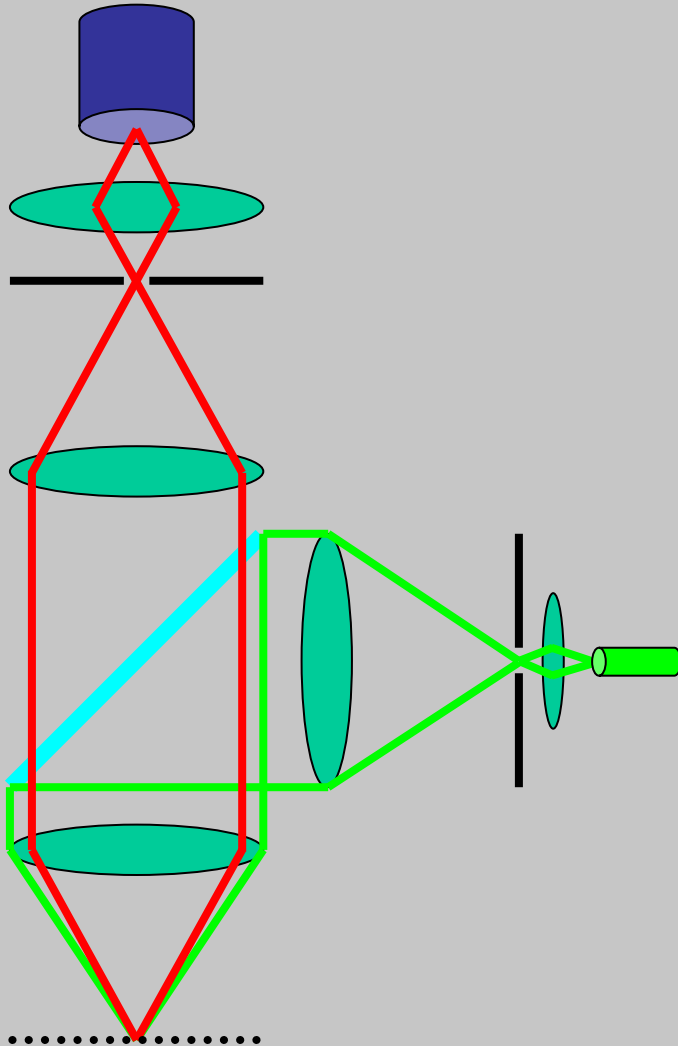
(Minsky, 1957)



Pinhole: Axial Filtering

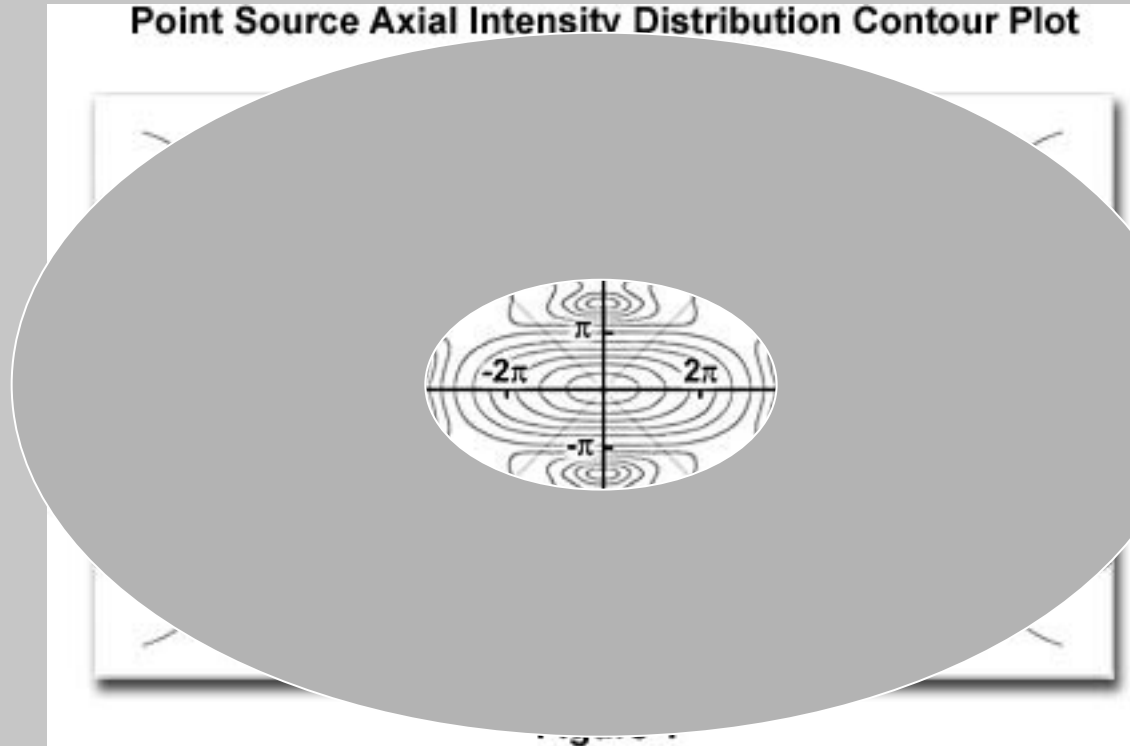
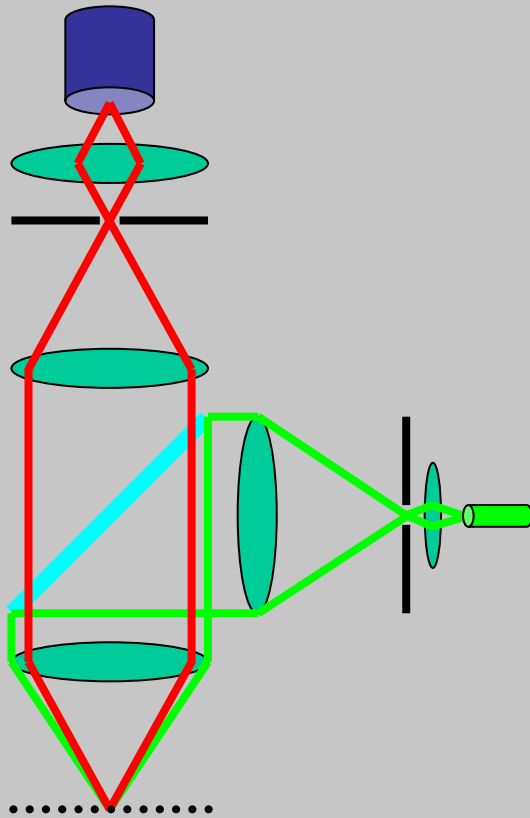


Aperture trims the PSF: increased resolution in XY plane



Cost: Loss of light

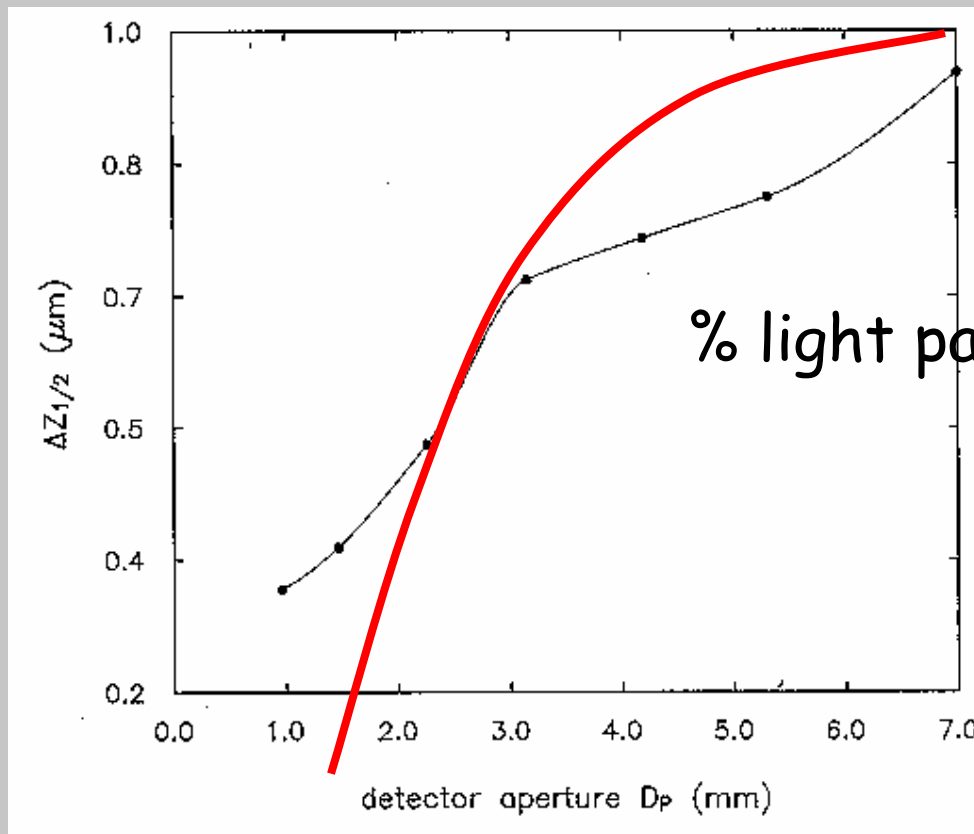
Aperture trims the PSF: increased resolution in Z direction



But at a cost in brightness:

- Thinner section means less labeled material in image
- Aperture rejects some in focus light
- Subtle scattering or distortion rejects more light

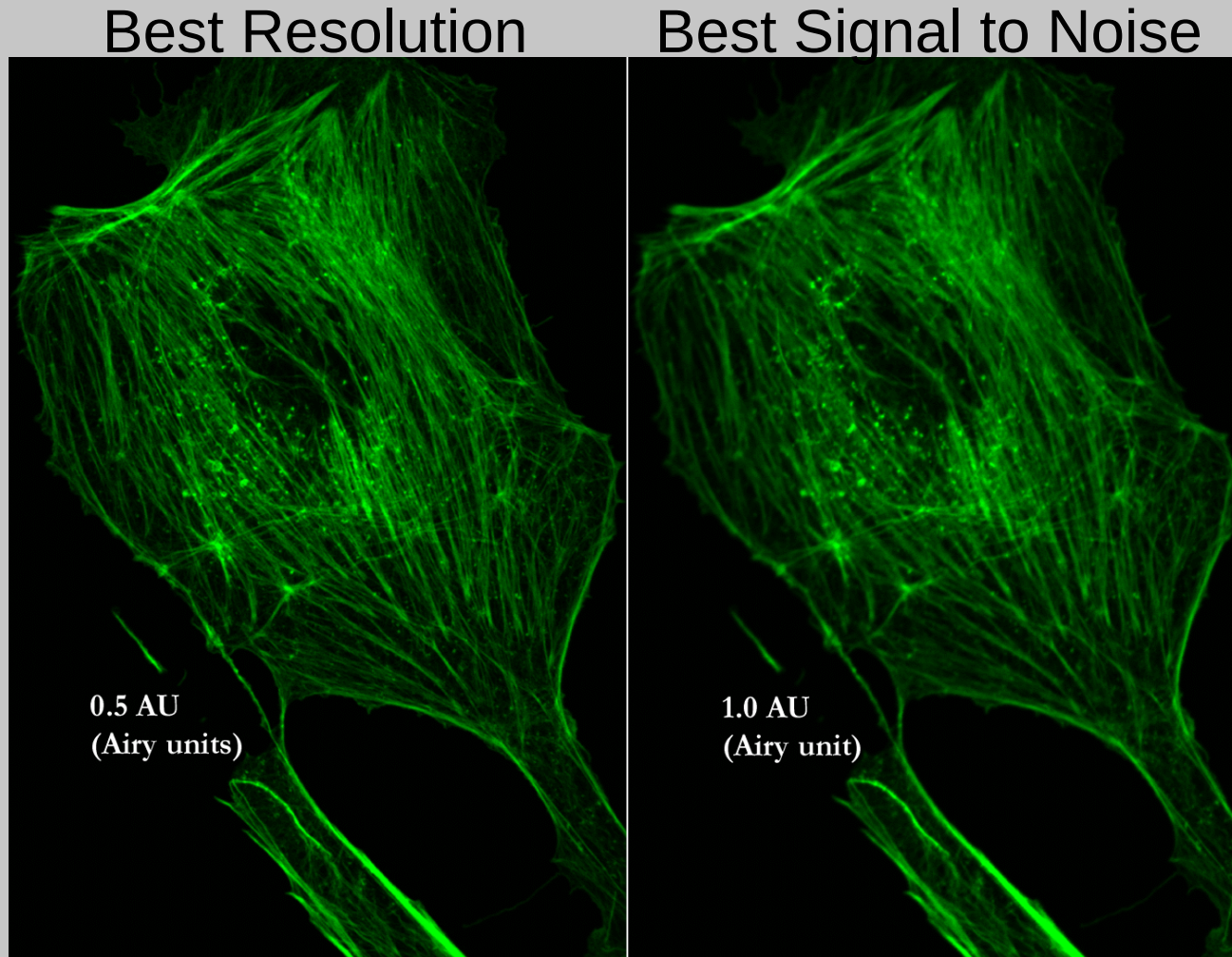
Optical section thickness vs pinhole size



% light passed by aperture

Apparent brightness will be the product of these two!!

Resolution, Signal and Pinhole Diameter



<http://depts.washington.edu/keck/leica/pinhole.htm>

Why does confocal add depth discrimination?

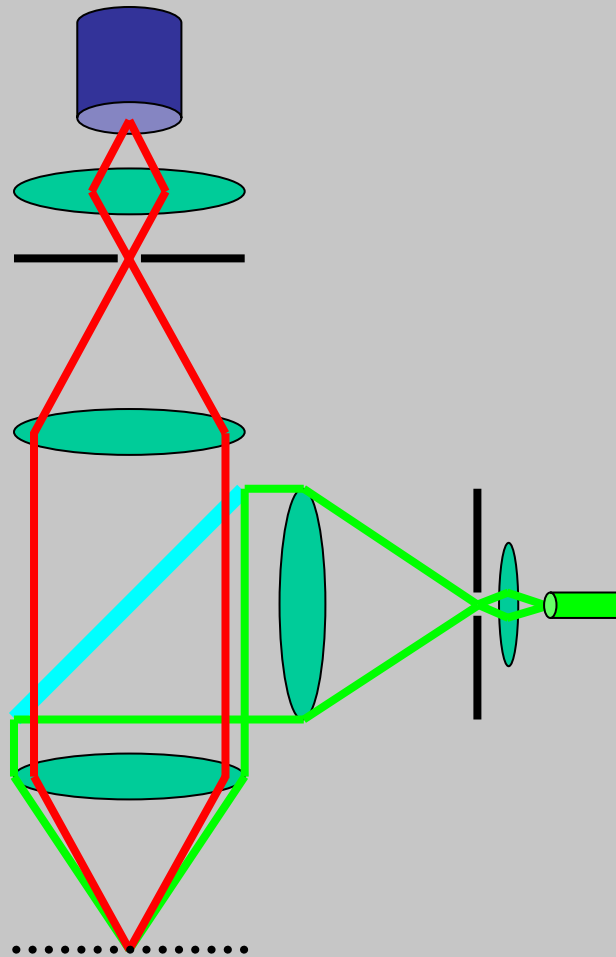
Light projected on a single spot in the specimen

Good: excitation falls off by the distance from the focus squared

Spatial filter in front of the detector

Good: detection falls off by the distance from the focus squared

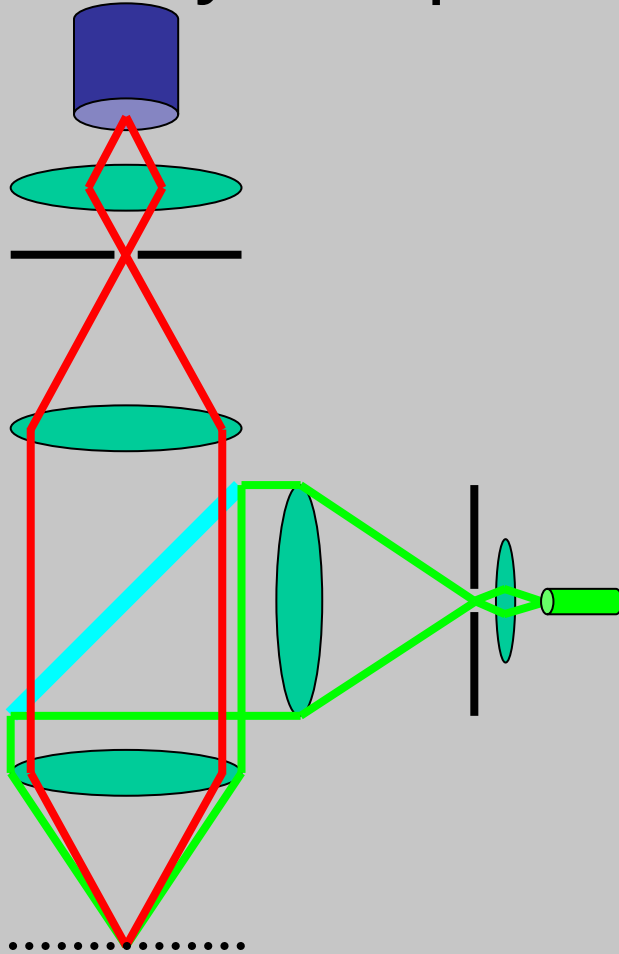
Bad: illumination of regions that are not used to generate an image



Optical sectioning

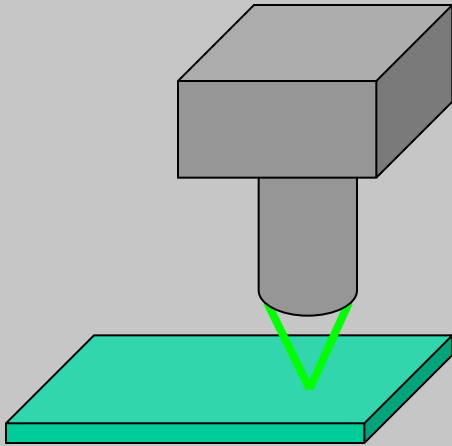
Combined, sensitivity falls off by (distance from the focus)⁴

But this arrangement generates an “image” of only one point in the specimen



- Only a single point is imaged at a time.
- Detector signal must be decoded by a computer to reconstruct image.
- Imaging point needs to be scanned somehow.

Scan Specimen



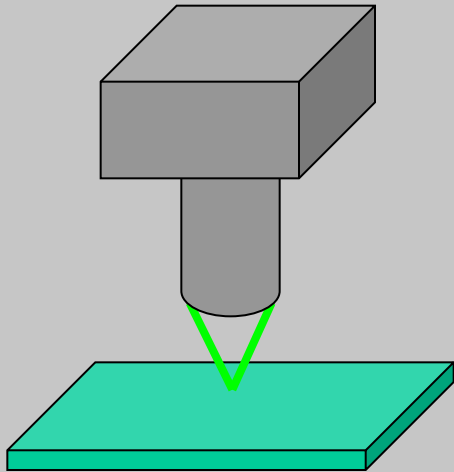
Good:

- Microscope works on axis
- Best correction for optical aberrations
- Most uniform light collection efficiency

Bad:

- Slow
- Sloshes specimen

Scan Microscope Head



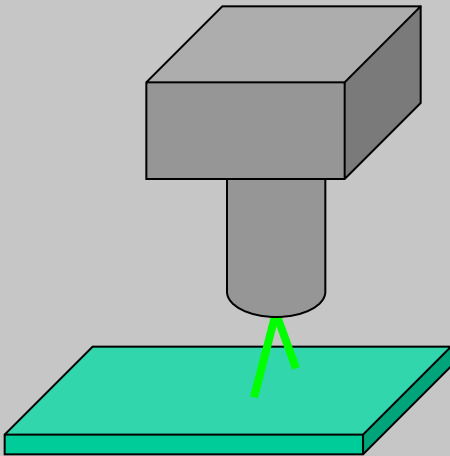
Good:

- Specimen doesn't move
- Microscope works on axis
- Best correction for optical aberrations
- Most uniform light collection efficiency

Bad:

- Slow
- Optics can be more complicated

Scan Laser



Good:

- Faster
- Specimen moves slowly—less sloshing

Bad:

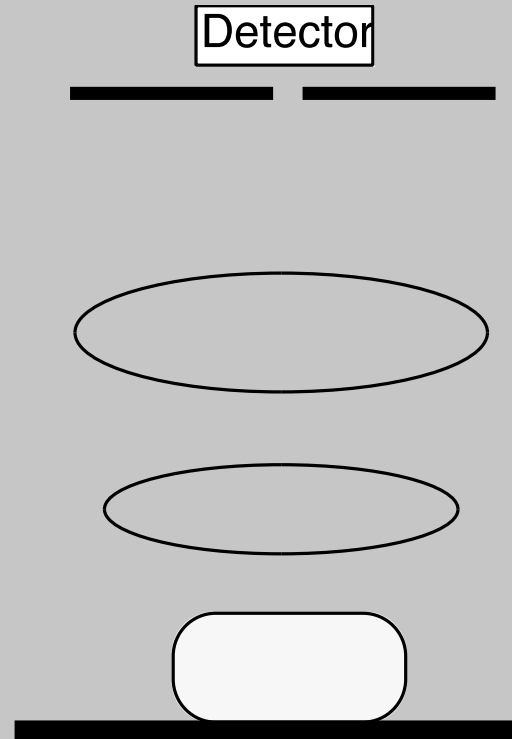
- Very high requirements on objective
- Light collection may be non-uniform off-axis
- More complicated

Optical Aberrations: Imperfections in optical systems

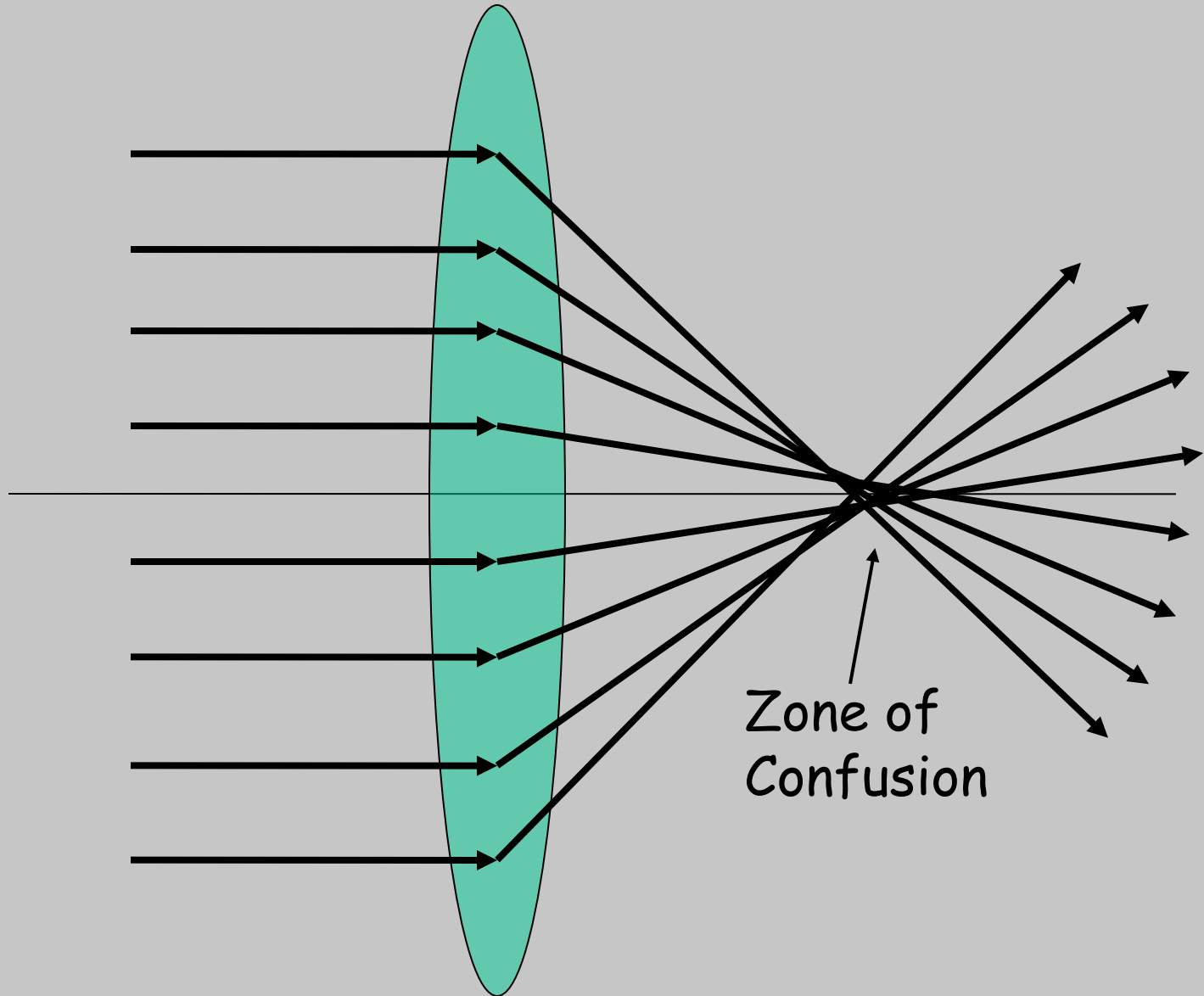
Chromatic (blue=shorter wavelength)

Spherical

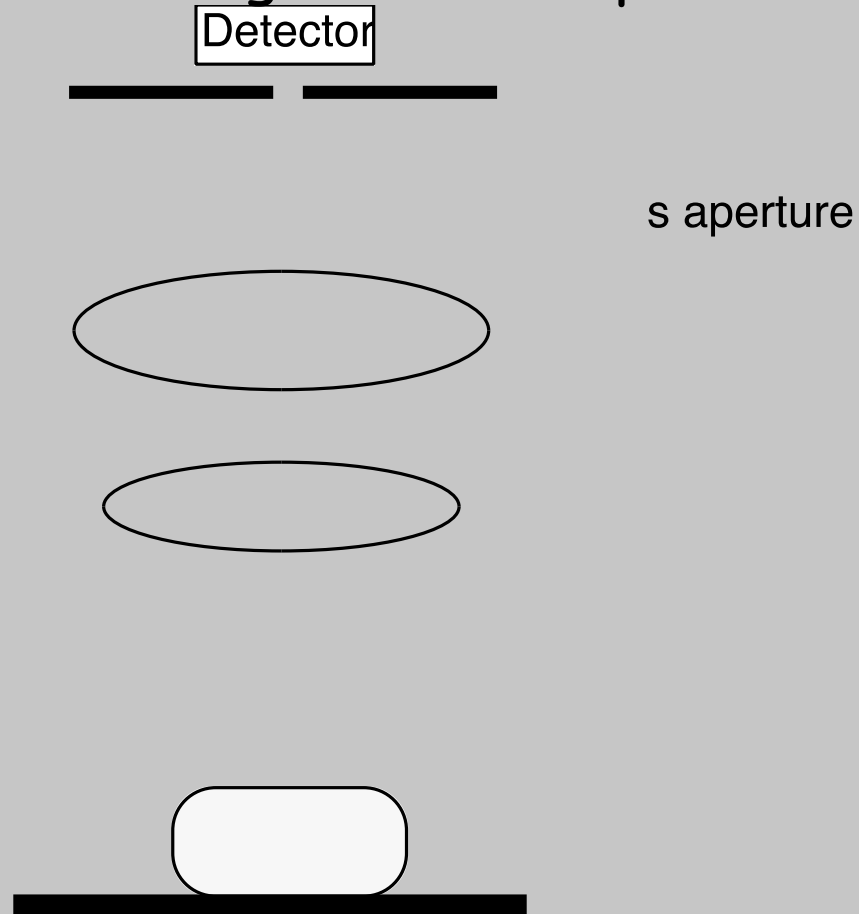
Curvature of field



Spherical Aberration

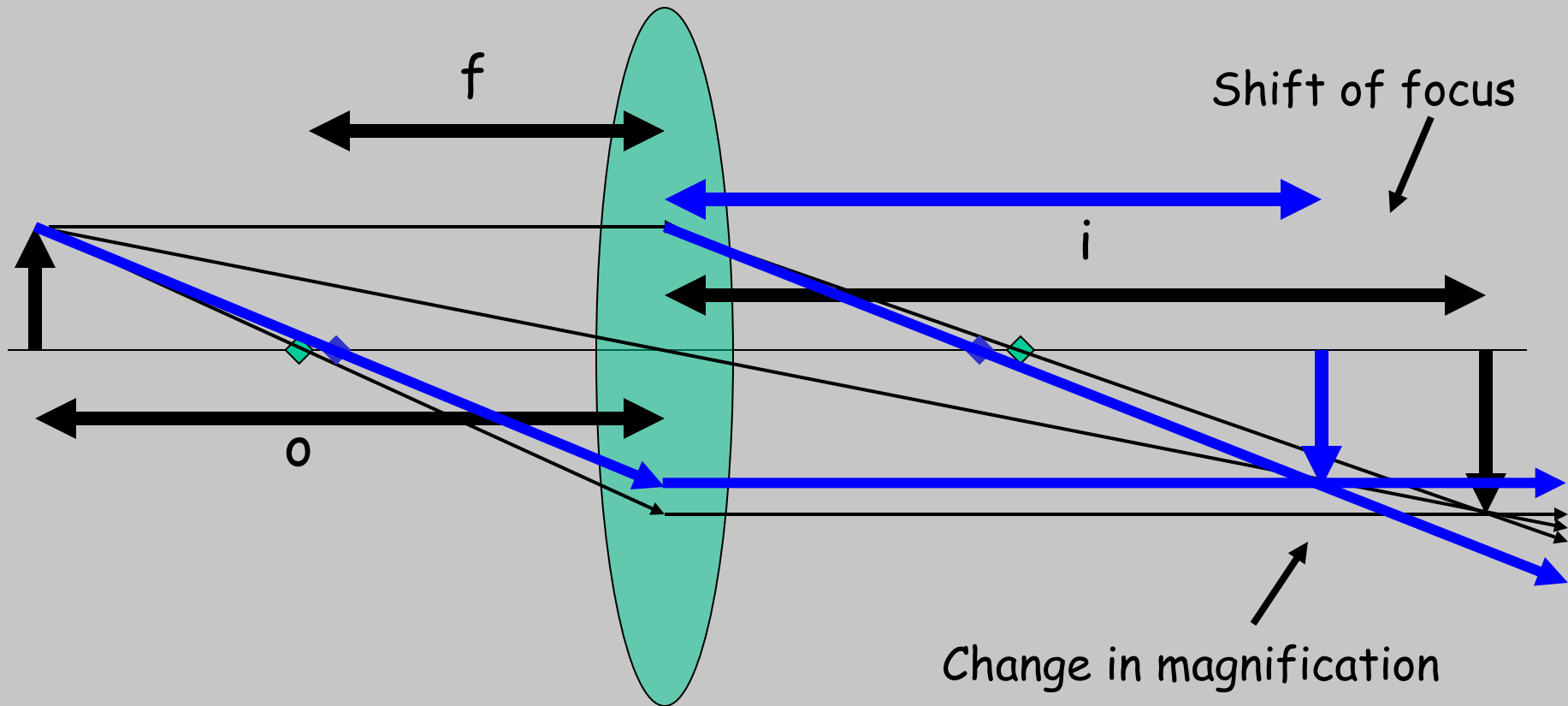


Spherical aberration: Light misses aperture (and defocused)

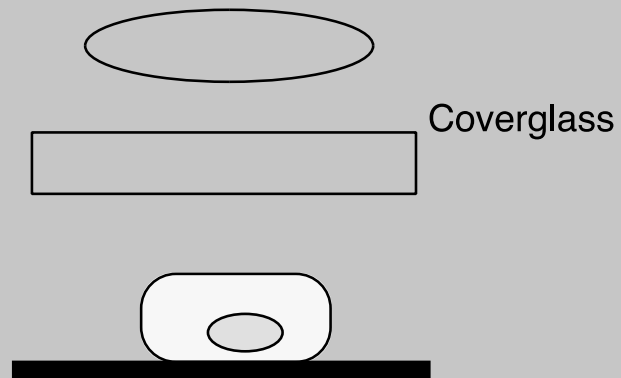


Higher index of refraction results in shorter f

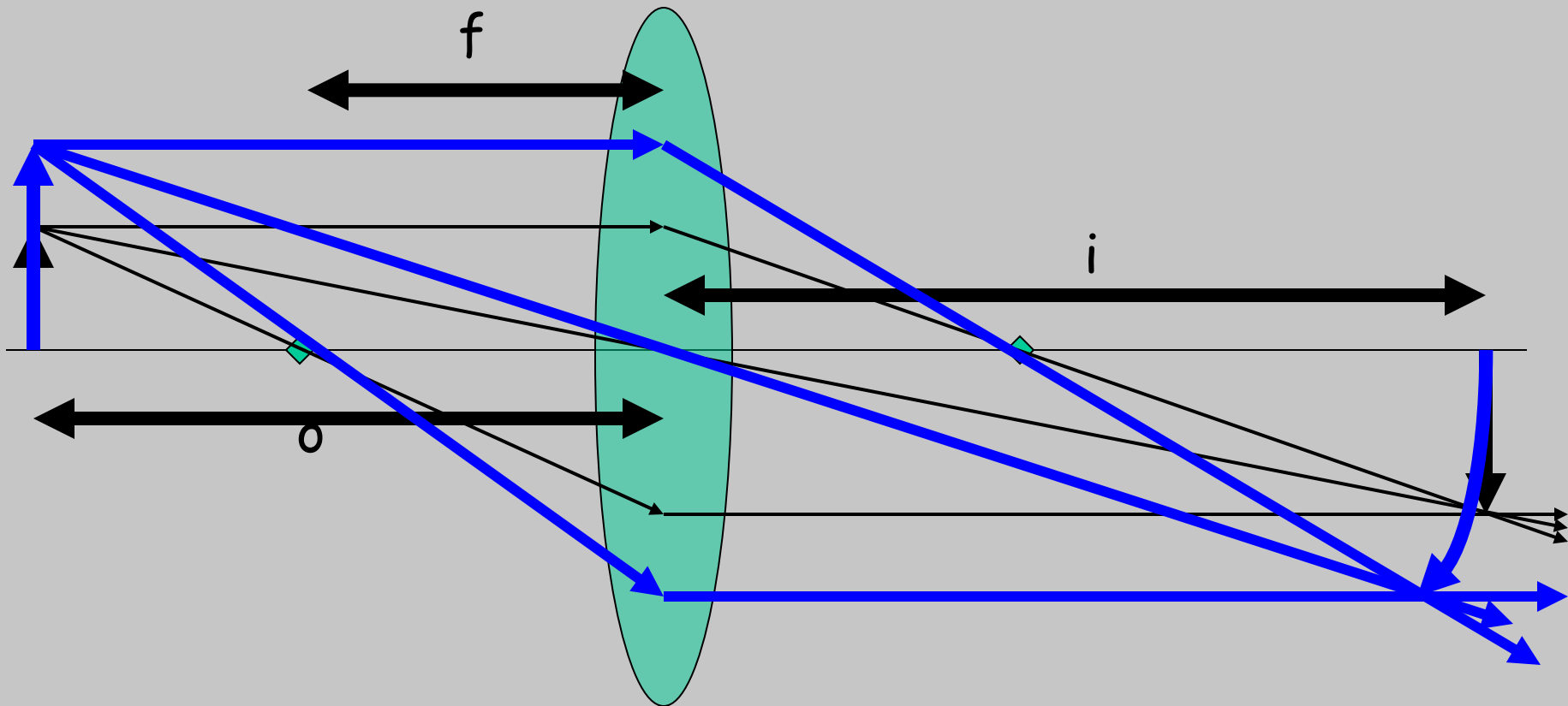
→ Chromatic Aberration
Lateral (magnification)
Axial (focus shift)



Lateral chromatic aberration - light misses aperture

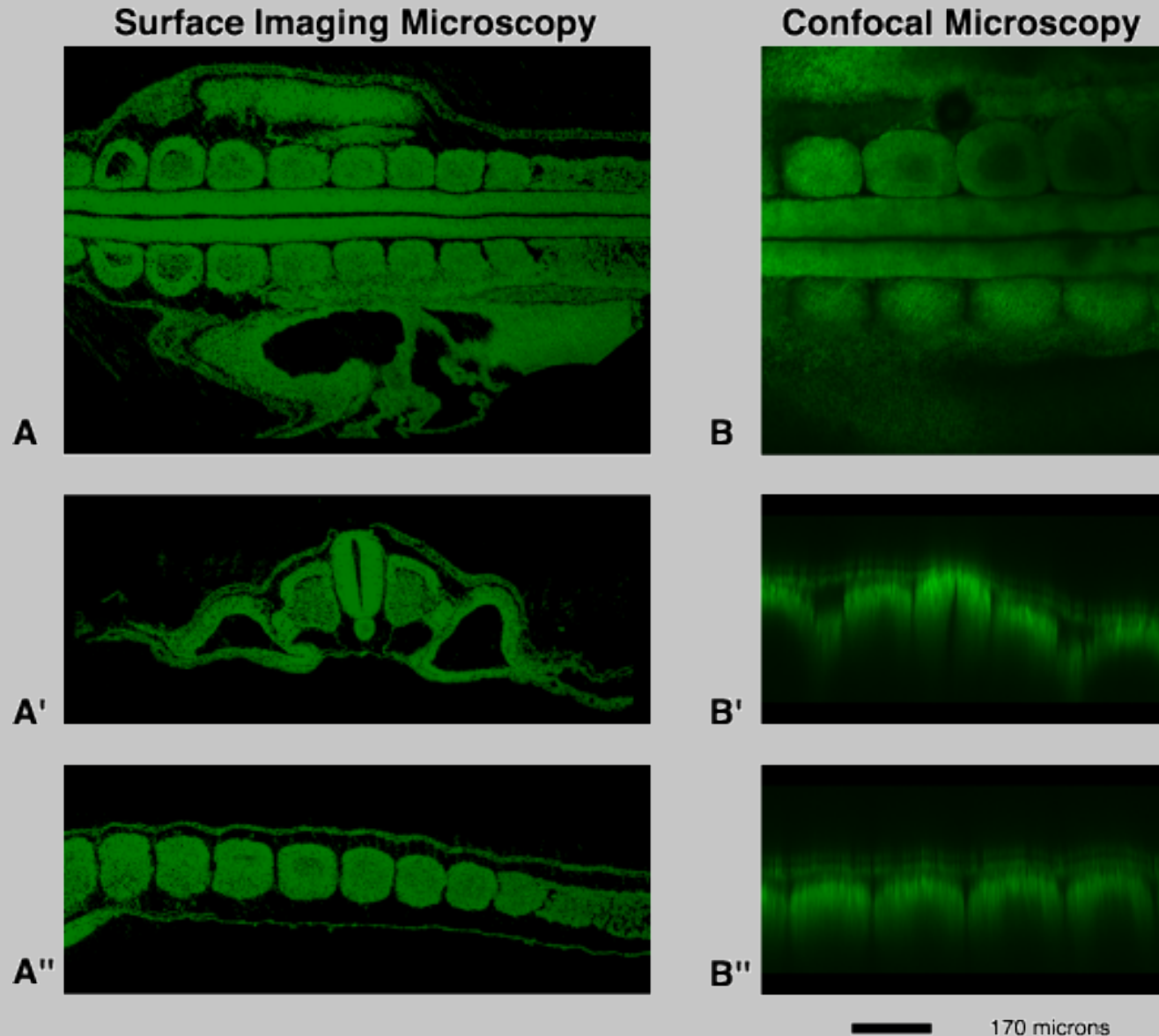


Curvature of field: Flat object does not project a flat image



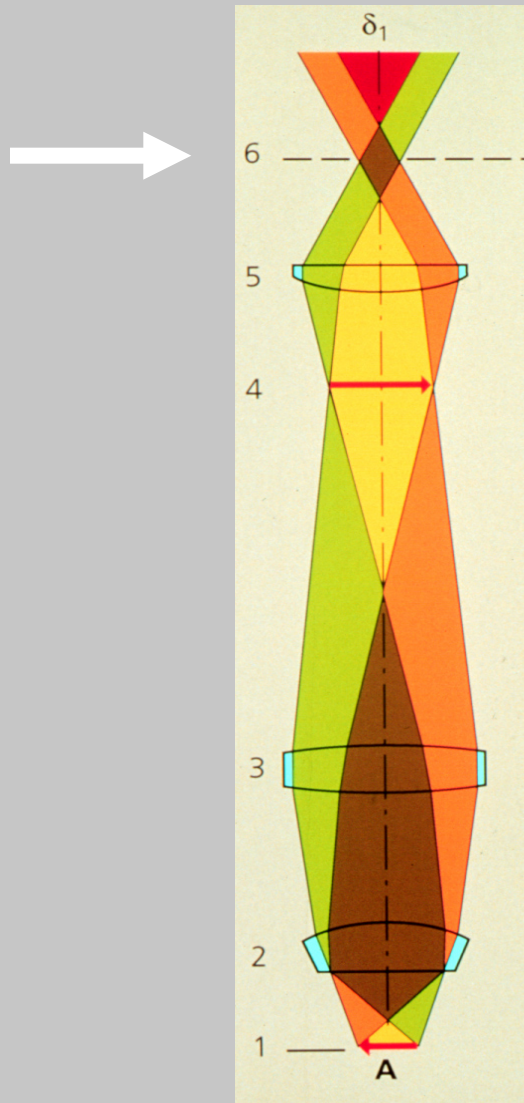
Results in a “port hole” image: dimmer at edges

Aberrations result in loss of signal and soft focus at depth

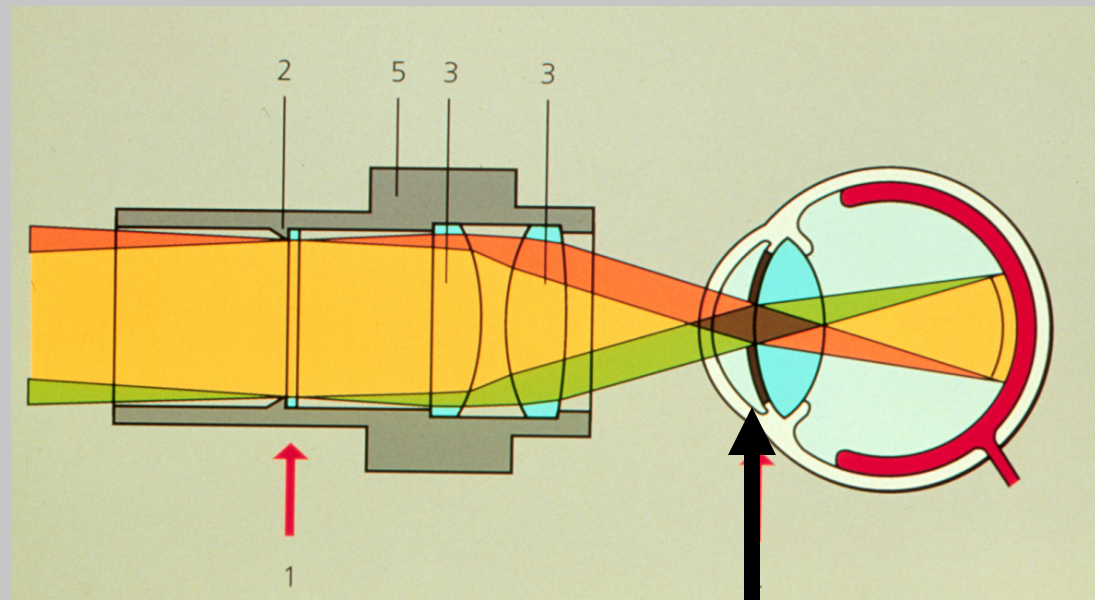


How to scan the laser beam?

Place galvometer mirror at the telentric point



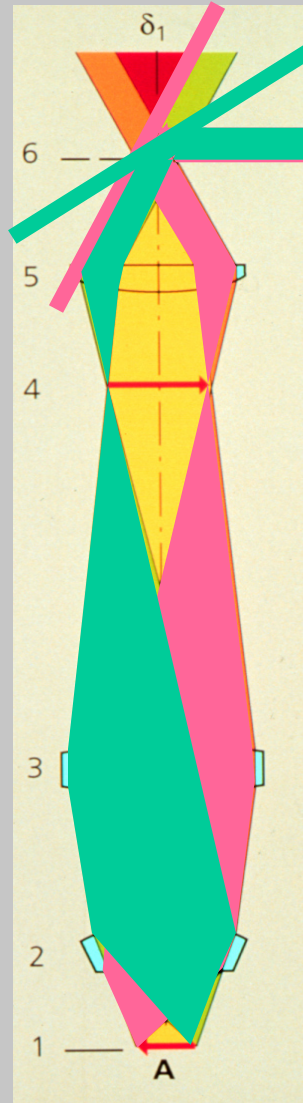
All light travels through the same zone
Angle at which the light travels dictates the
position in the specimen plane



Telentric Plane

Position is critical

Place galvanometer mirror AT the telentric point



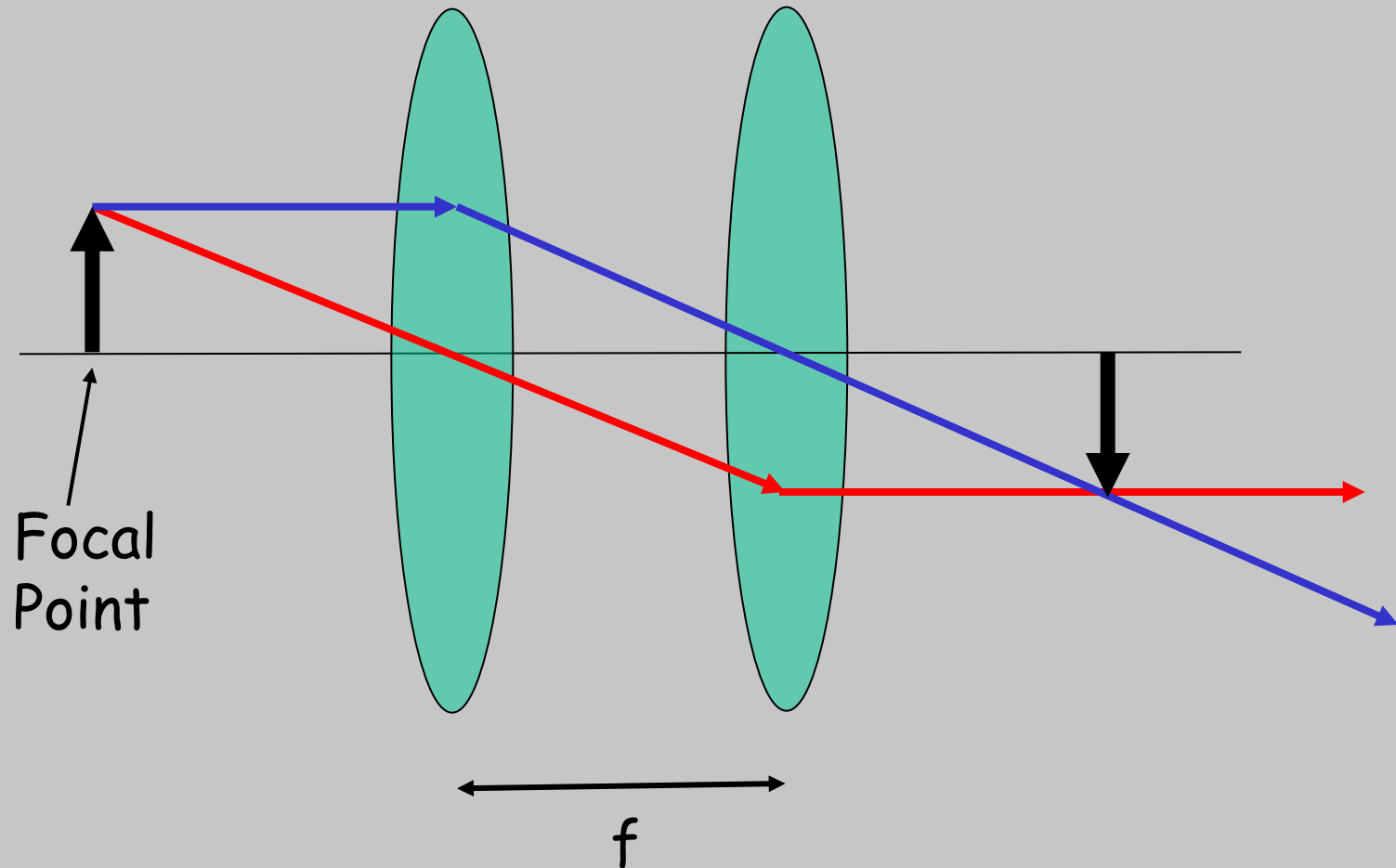
laser

If not at the point,
Spherical aberration results

How can two mirrors be at
the same point??

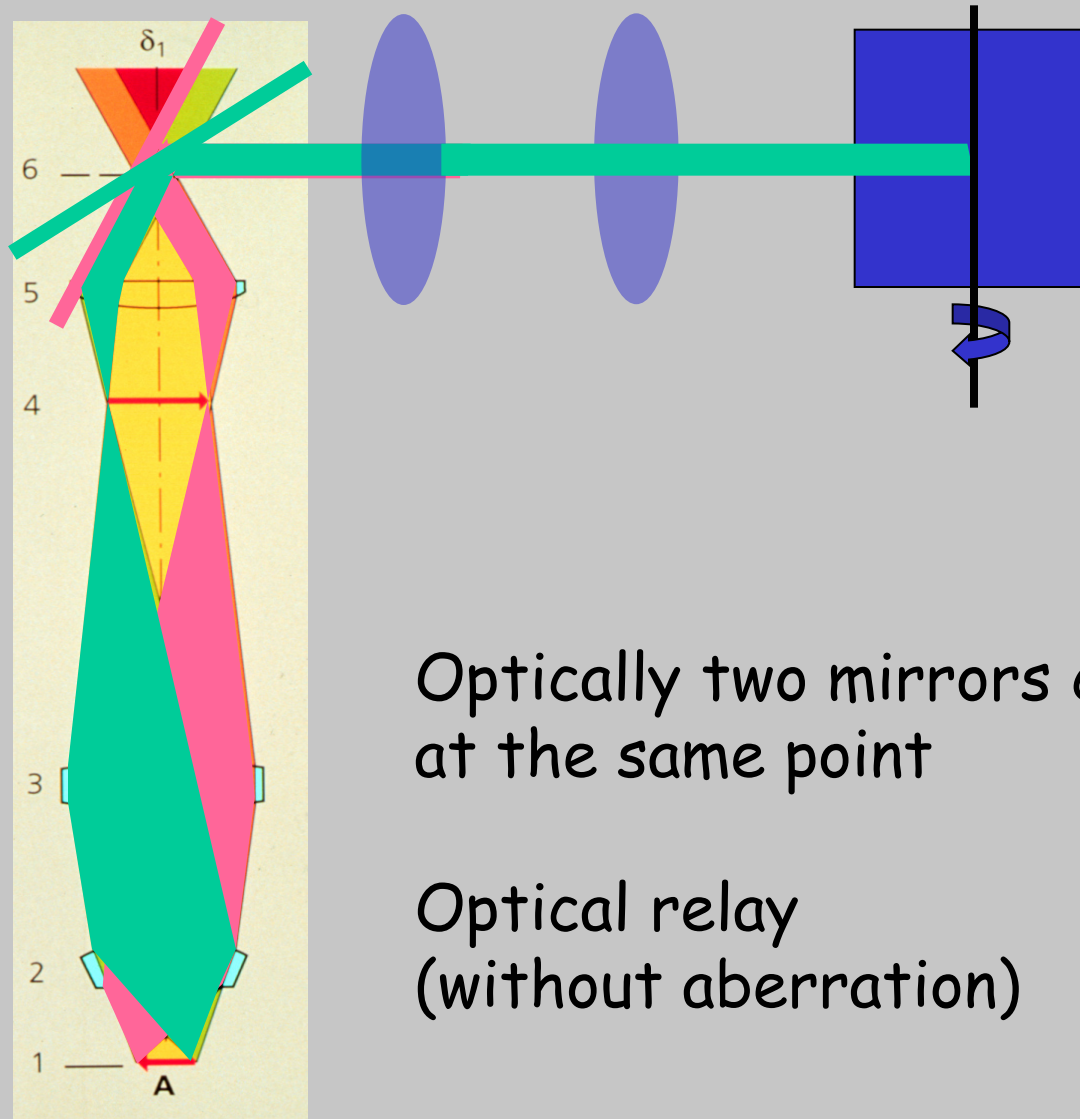
Optical relay
(without aberration)

1:1 Image relay



Position is critical

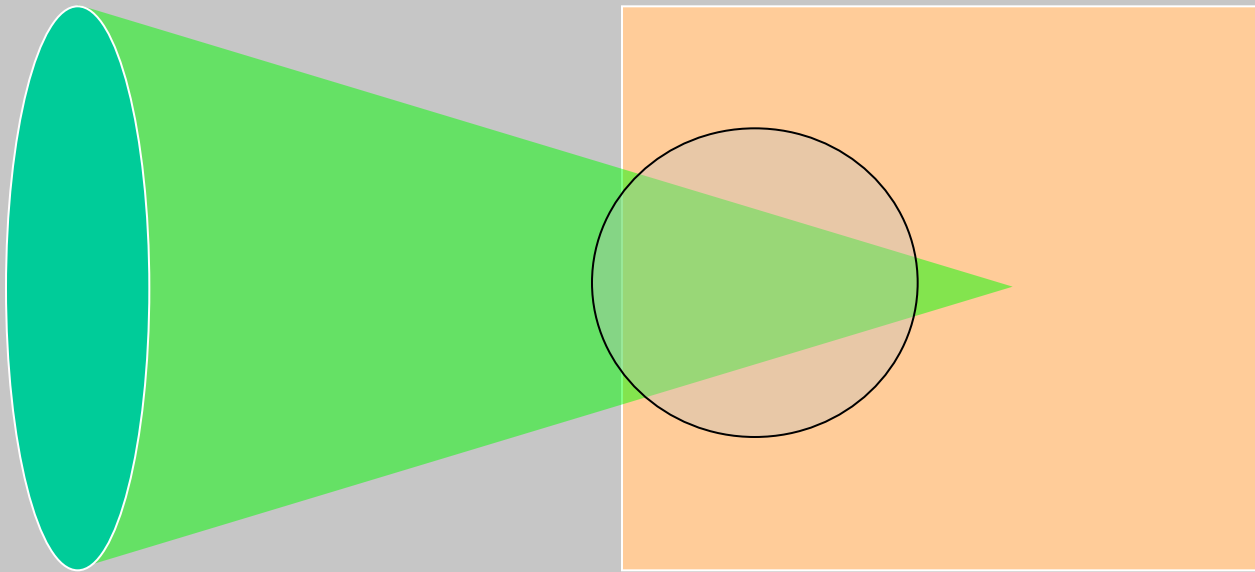
Place galvanometer mirrors AT the telentric point



Optically two mirrors can be
at the same point

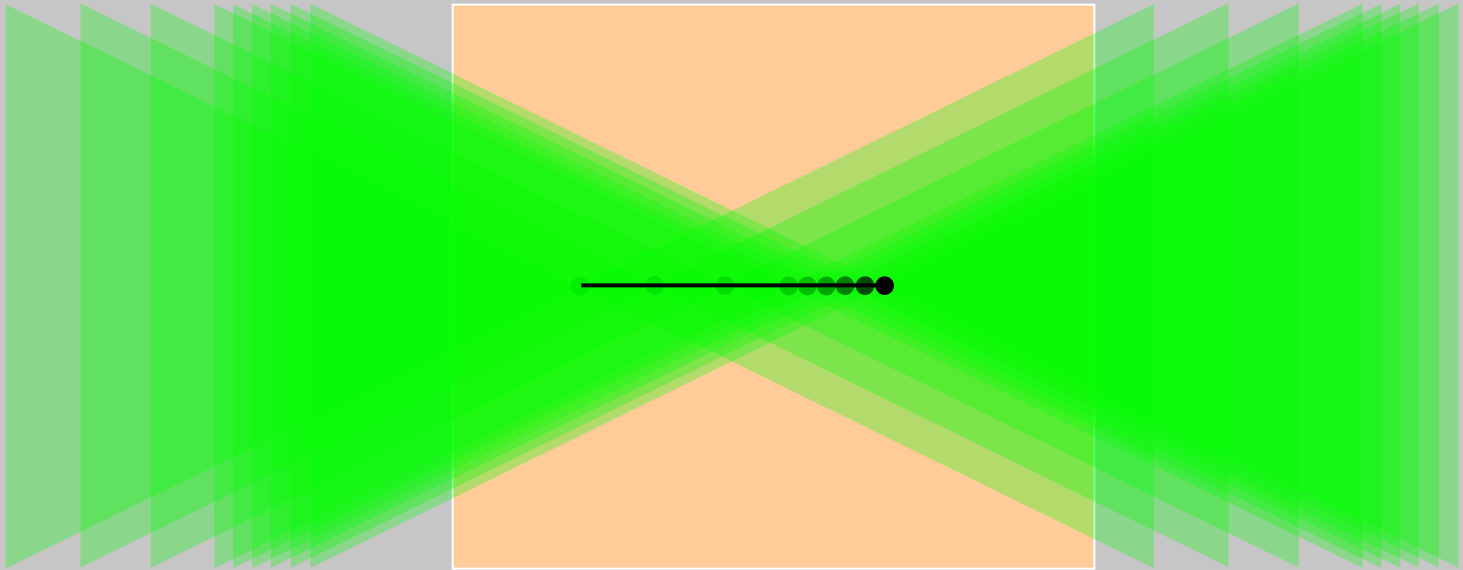
Optical relay
(without aberration)

Limitations: Phototoxicity



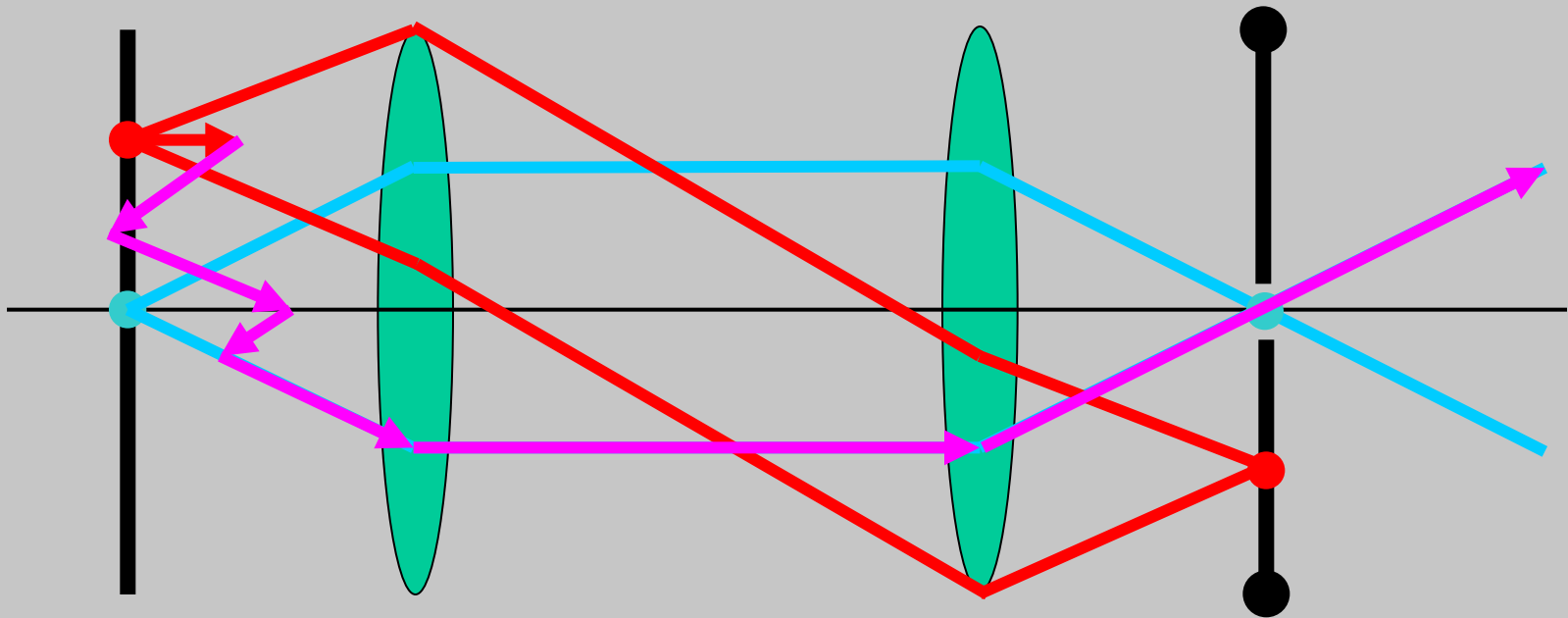
- Sample is continuously exposed to light.
- Weaker signal within sample requires stronger excitation and causes more toxicity.

Limitations: Photobleaching

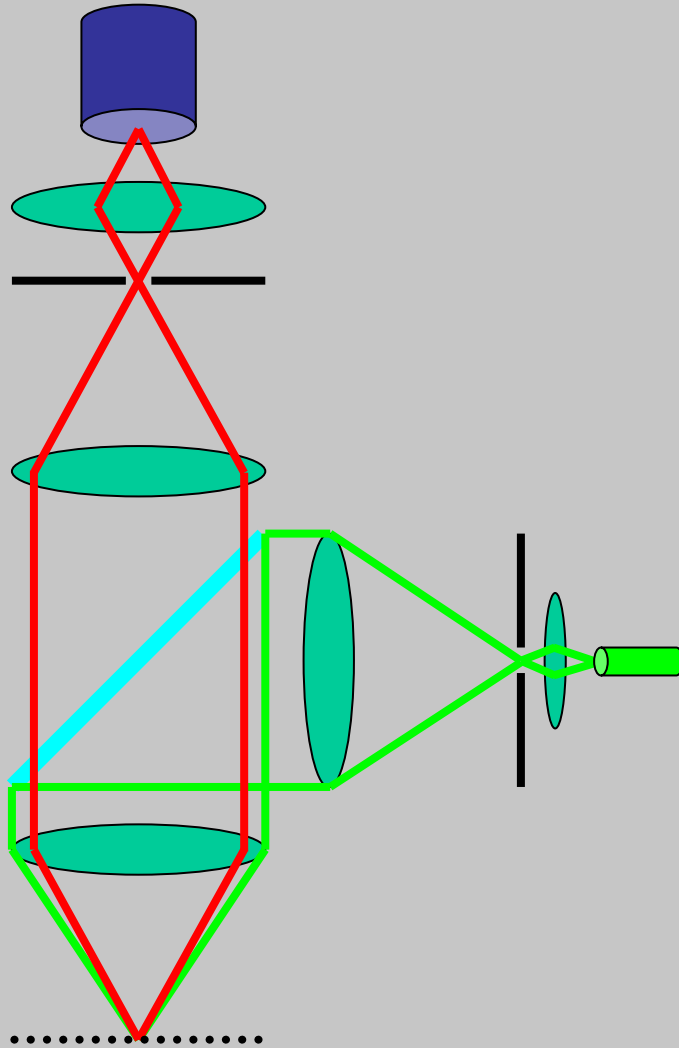


- Scanning causes repeated exposure above and below.

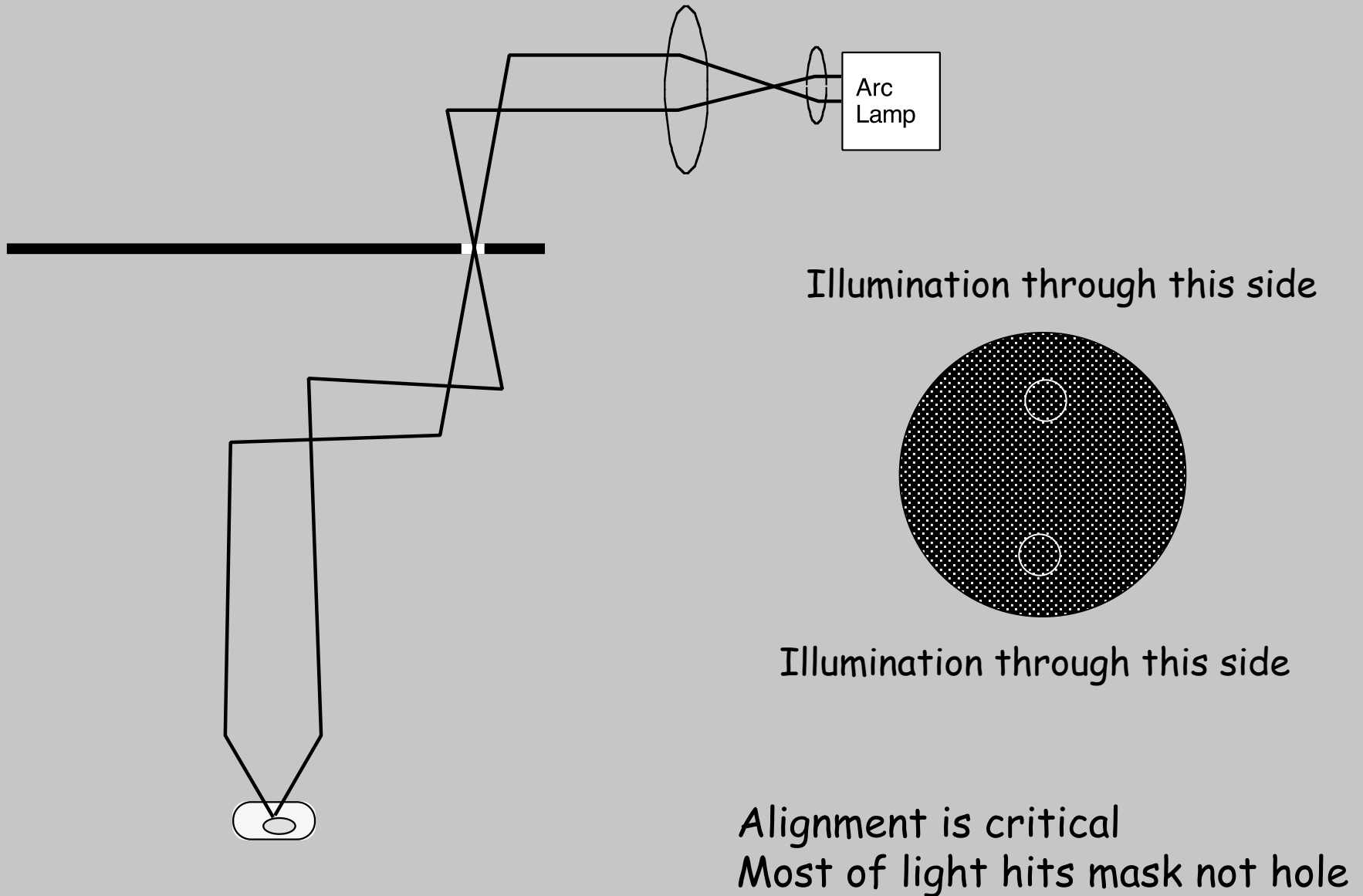
Loss of sectioning by Scattering



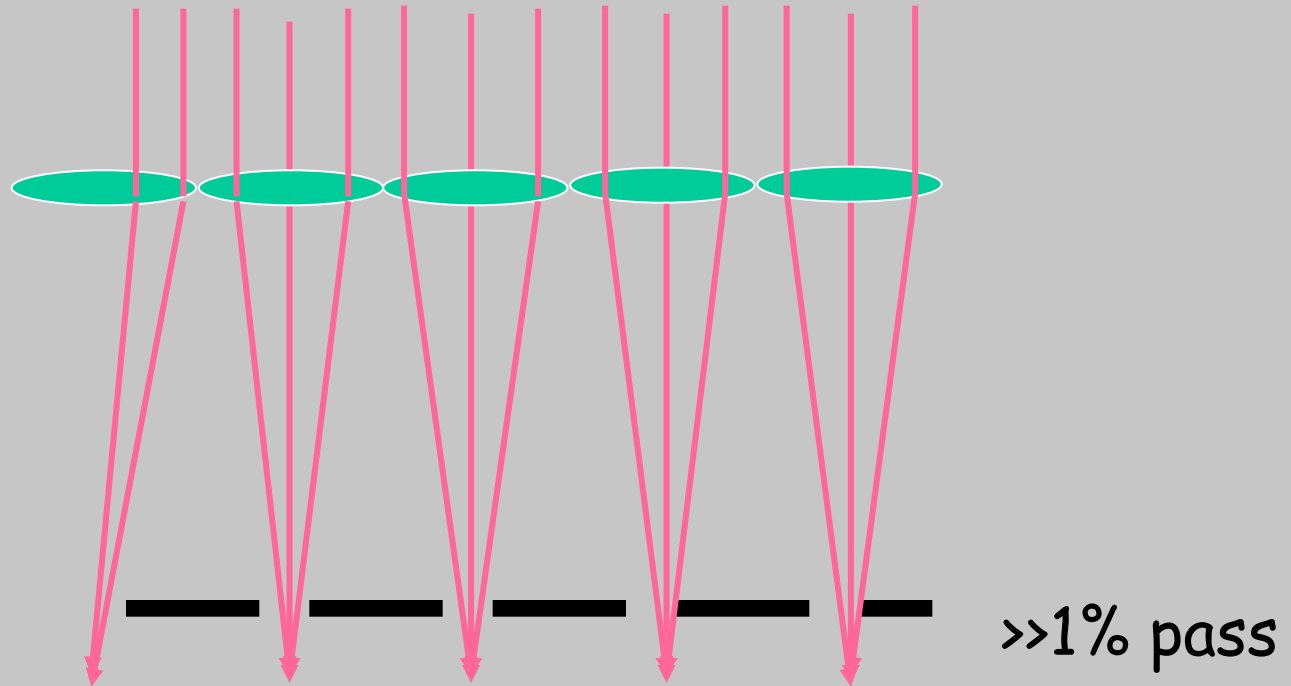
How else to do confocal microscopy?



Tandem spinning disk scanner



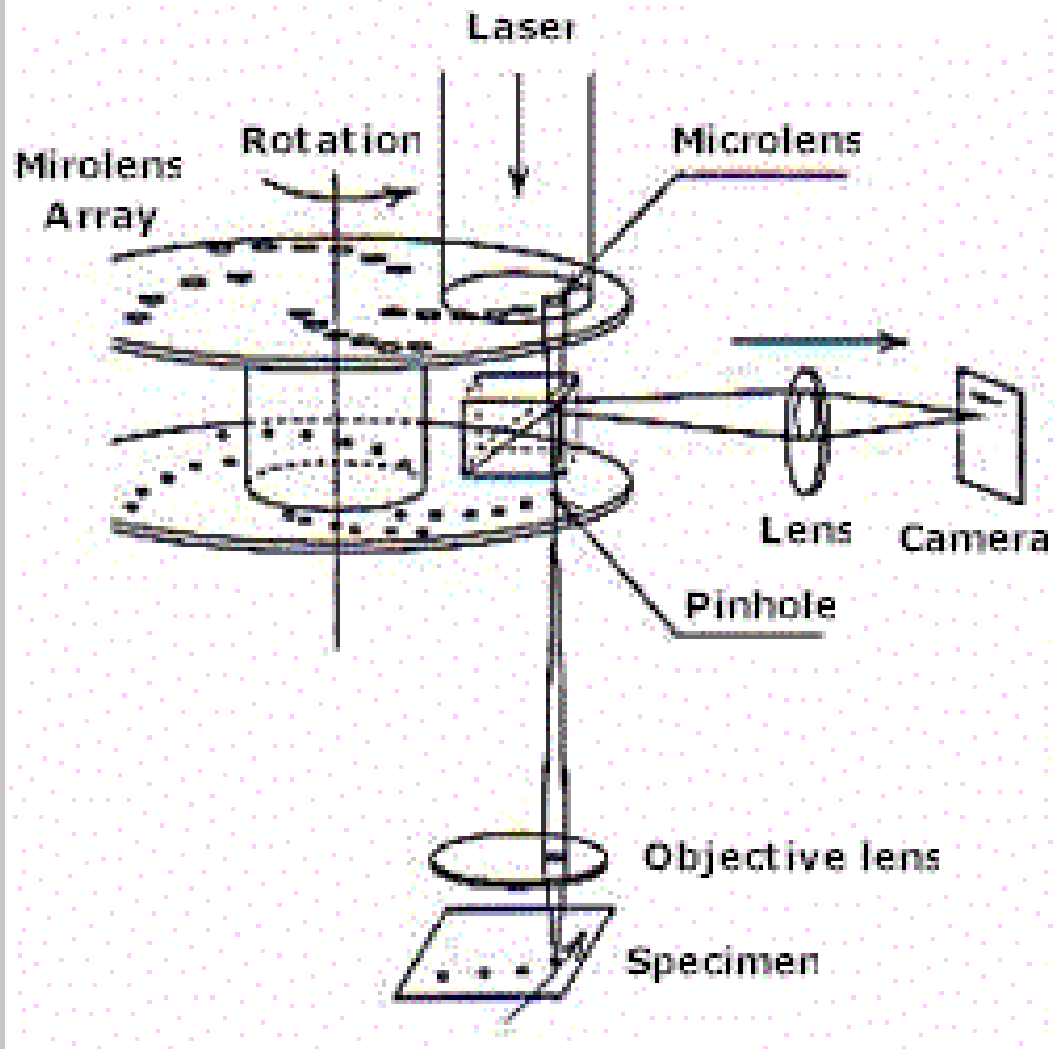
Nipkow disk with lenslets



Yokogawa

Nipkow disk with lenslets

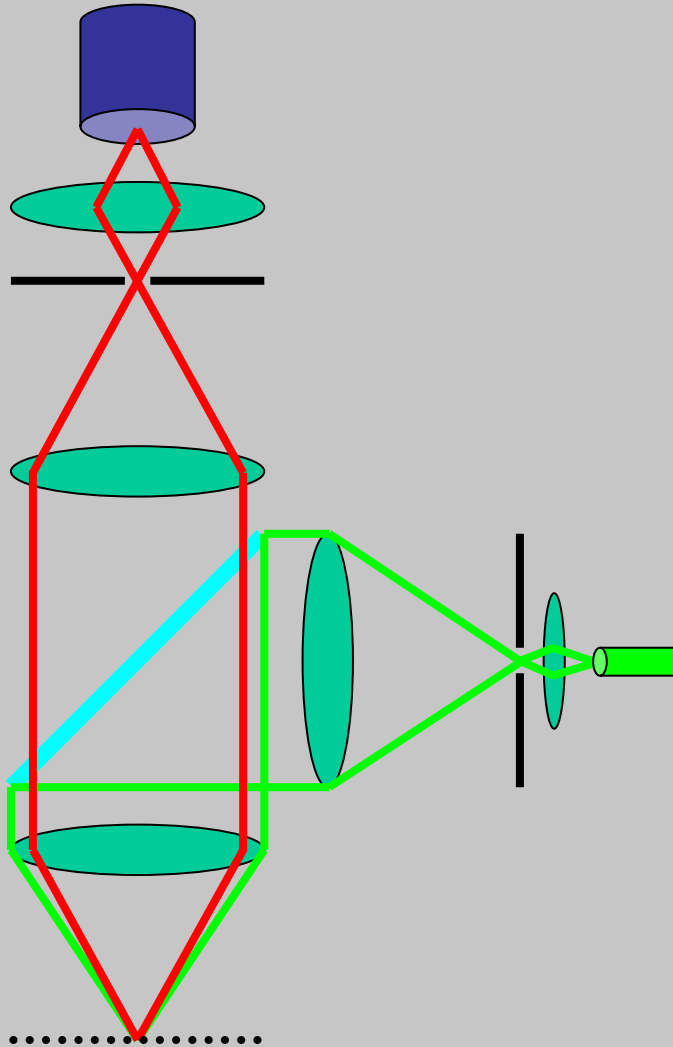
Construction of QLC100



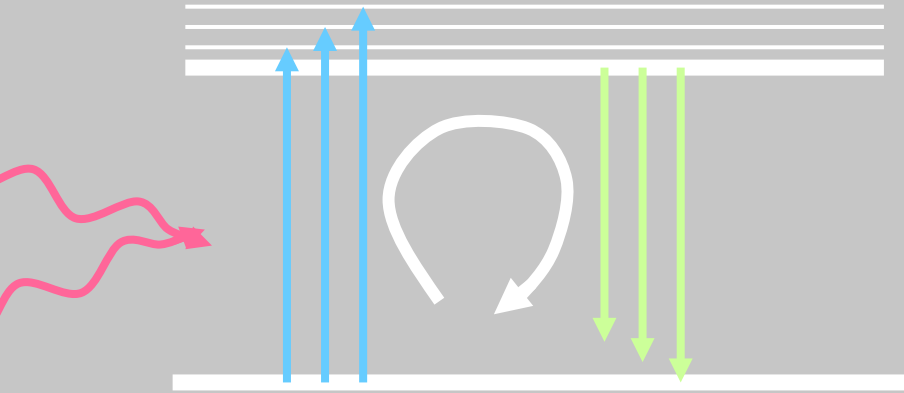
Yokogawa

Optical sectioning without an aperture?

Two-Photon laser-scanning microscopy



Two-Photon Excited Fluorescence



Very low probability: required intense pulsed laser light
Requires two photons: excitation is a function of (exciting light)²

Exciting light falls off by (distance from focus)²

Thus, Emission falls off by (distance from focus)⁴

--> Optical Sectioning without a confocal aperture!!

TPLSM depth discrimination by selective excitation

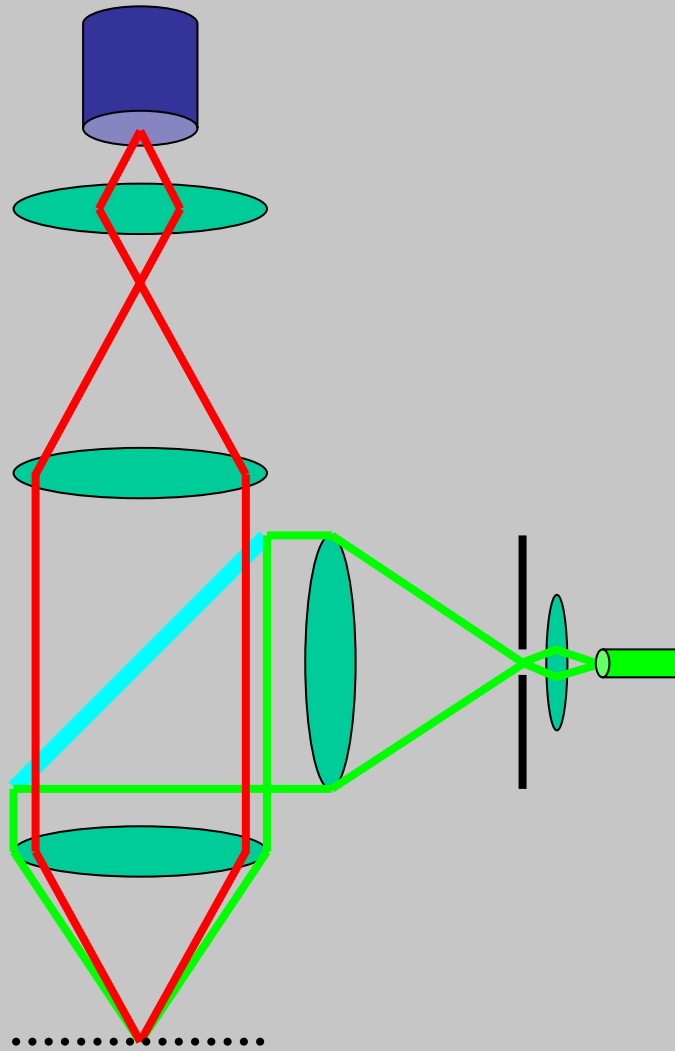
Light projected on a single spot in the specimen

Good: illumination falls off by the distance from the focus squared

And
Excitation depends on the square of the intensity

Optical sectioning

Combined, sensitivity falls off by (distance from the focus)⁴



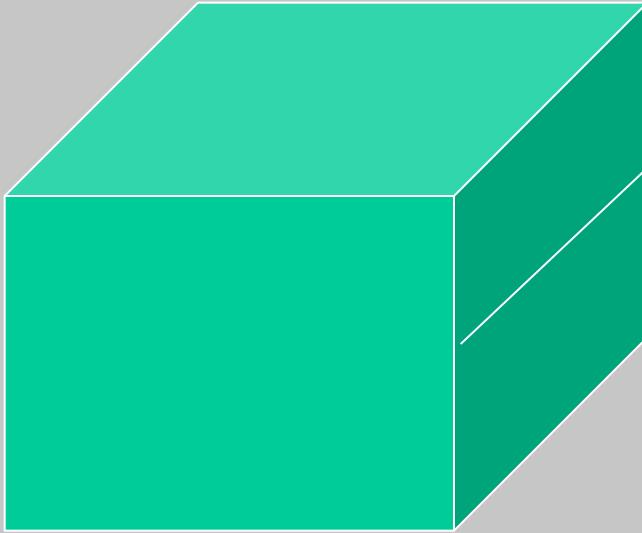
~~Spatial filter in front of the detector~~

~~Good: detection falls off by the distance from the focus squared~~

~~Bad: illumination of regions that are not used to generate an image~~

Evil test case (David Piston)

Deconvolution vs Confocal

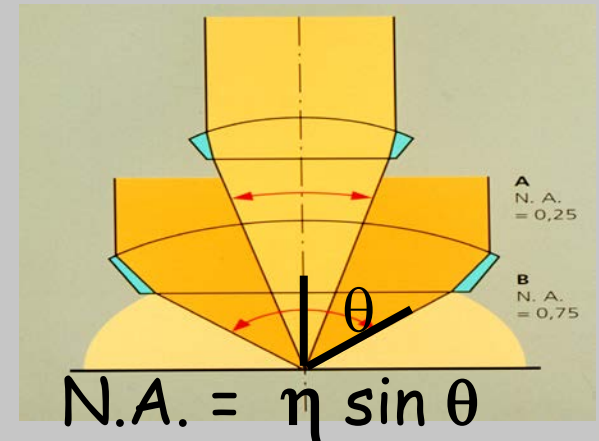


Fluorescent plastic cube



Bleach one plane
using TPLSM

N.A. and image brightness



Epifluorescence

Brightness = fn (NA^4 / magnification²)

10x 0.5 NA is 8 times brighter than 10x 0.3NA

But time matters

Pulses are very short

$$100\text{fsec} \times c = 30\mu\text{m}$$