Designing a Microscopy Experiment

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Image from Susanne Rafelski, Marshall lab

The Chinese-menu view of imaging

Imaging Methods	**Contrasting techniques**
Wide-Field	Brightfield, phase, DIC
TIRF	Immunofluorescence
Laser-scanning confocal	Physiological dyes
Multi-point confocal	Fluorescent proteins
Multi-photon confocal	FRAP, FLIP, photoactivation
	FRET, FLIM

Experiment: Time Lapse, 3D, multi-point, multi-wavelength, …

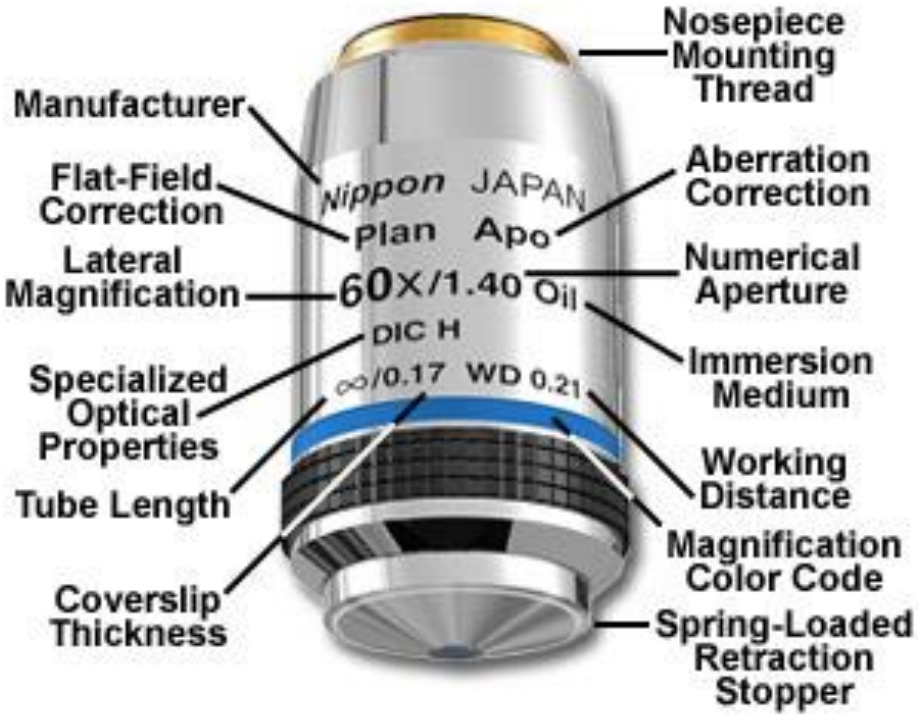
Standard microscope capabilities

Like all rules, these were made to be broken, but only if you have fancy equipment!

- Resolution: ~200nm in X and Y, 700 nm in Z
- Sensitivity: <100 photons
- Linear detection – quantification is possible
- Video rate acquisition
- 4-5 color imaging

By far the most important part:

the Objective Lens



Obviously, we care about the magnification. What other parameters are important?

Working Distance

In general, high NA lenses have short working distances

However, extra-long working distance objectives do exist

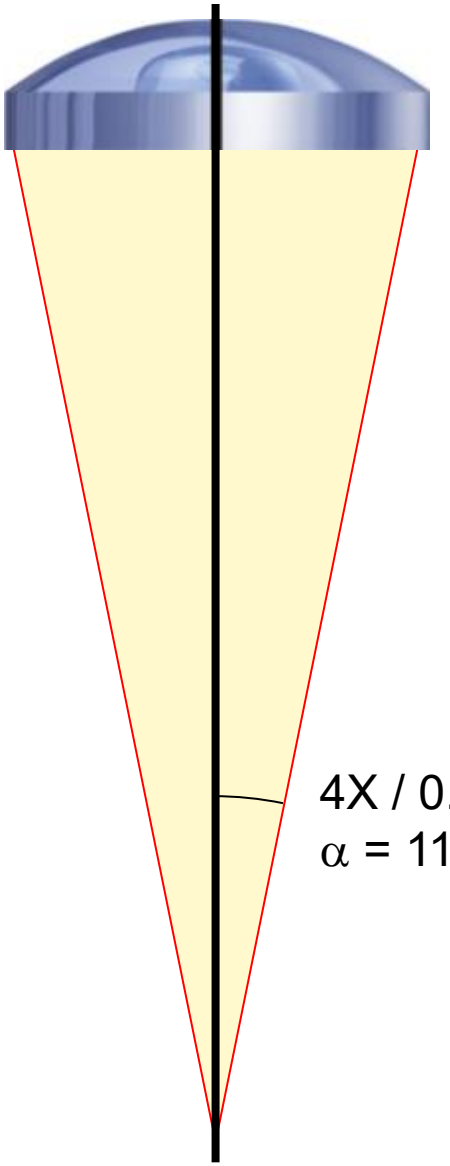
Some examples:

10x/0.3 WD = 15.2mm

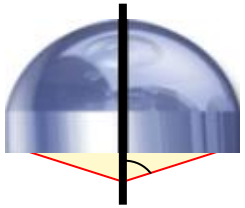
20x/0.75 WD = 1.0mm

100x/1.4 WD = 0.13mm

Numerical Aperture



4X / 0.20 NA
α = 11.5°



100X / 0.95 NA
α = 71.8°

$NA = n \sin(\alpha)$

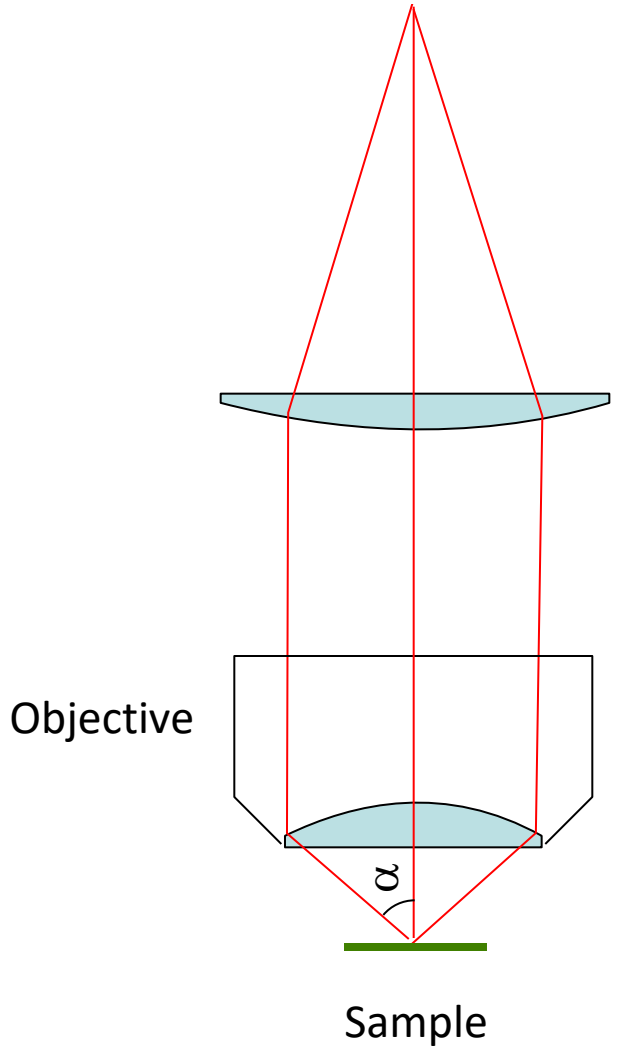
where

α = light gathering angle
n = refractive index of sample

Resolution of the Microscope

limited by the point-spread function

- Microscope objective collects a limited cone of light from the sample
- This limits the resolution achievable by the microscope
- Resolution can be measured by the blurring of a point object → the point-spread function



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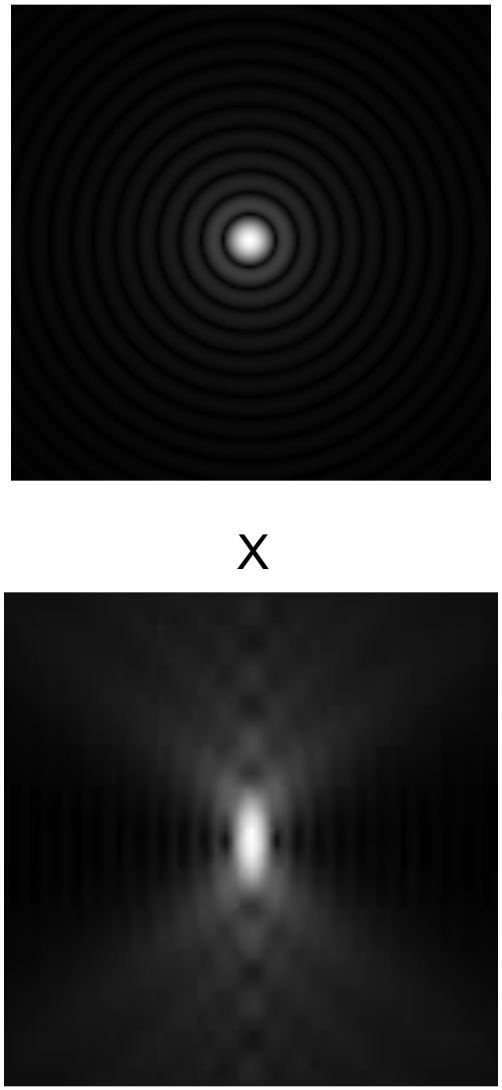


Resolution of the Microscope

limited by the point-spread function

XY, 0.61λ / NA
Z, λn / NA^2

Resolution for some common objectives, in nm:

NA	**X-Y**	Z
0.3	1017	16830
0.75	407	2690
0.95	321	1680
1.4	218	770

Light-gathering power

Light-gathering power goes as the square of NA

All things being equal, a higher NA lens will give a brighter image

Increasing magnification generally decreases brightness as light is spread out over more pixels

NA	**Brightness**
0.3	0.09
0.75	0.56
0.95	0.90
1.4	1.96

Choosing an objective

- Questions:
  - What resolution do you need?
  - How bright is your sample?

- For high resolution, you'll need high NA.
- For dim samples, you'll want high NA, regardless of resolution, to maximize light-gathering.
  - Dim, low-resolution samples (e.g. protein abundance in nucleus): bin camera to trade off resolution for brightness

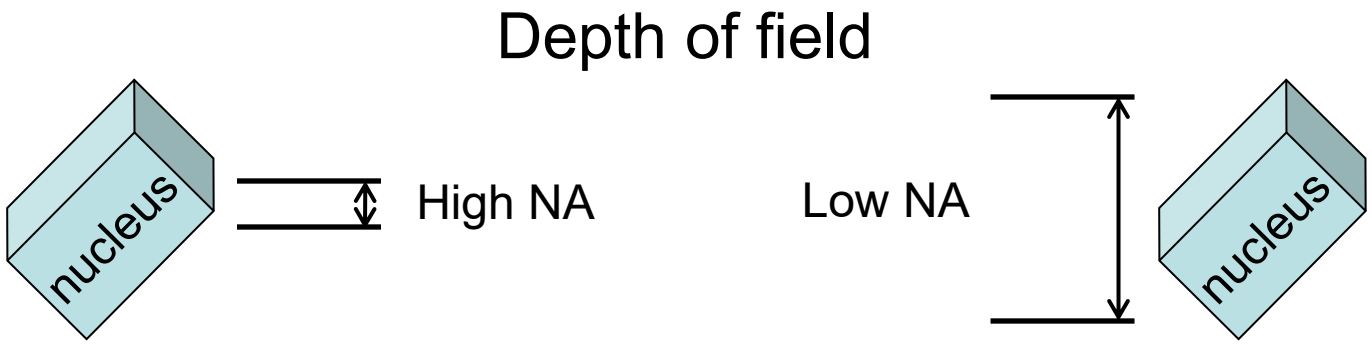
Choosing an objective

- Questions:
  - What resolution do you need?
  - How bright is your sample?

- When to use low NA?
  - Bright samples at low resolution / low magnification
  - If you need long working distance
  - If spherical aberration is a concern
  - If you want large depth of field to get whole structures in focus at once (avoid Z-stacks)

NA, Z-resolution, and Z-stacks

Idea – want to record total nuclear fluorescence



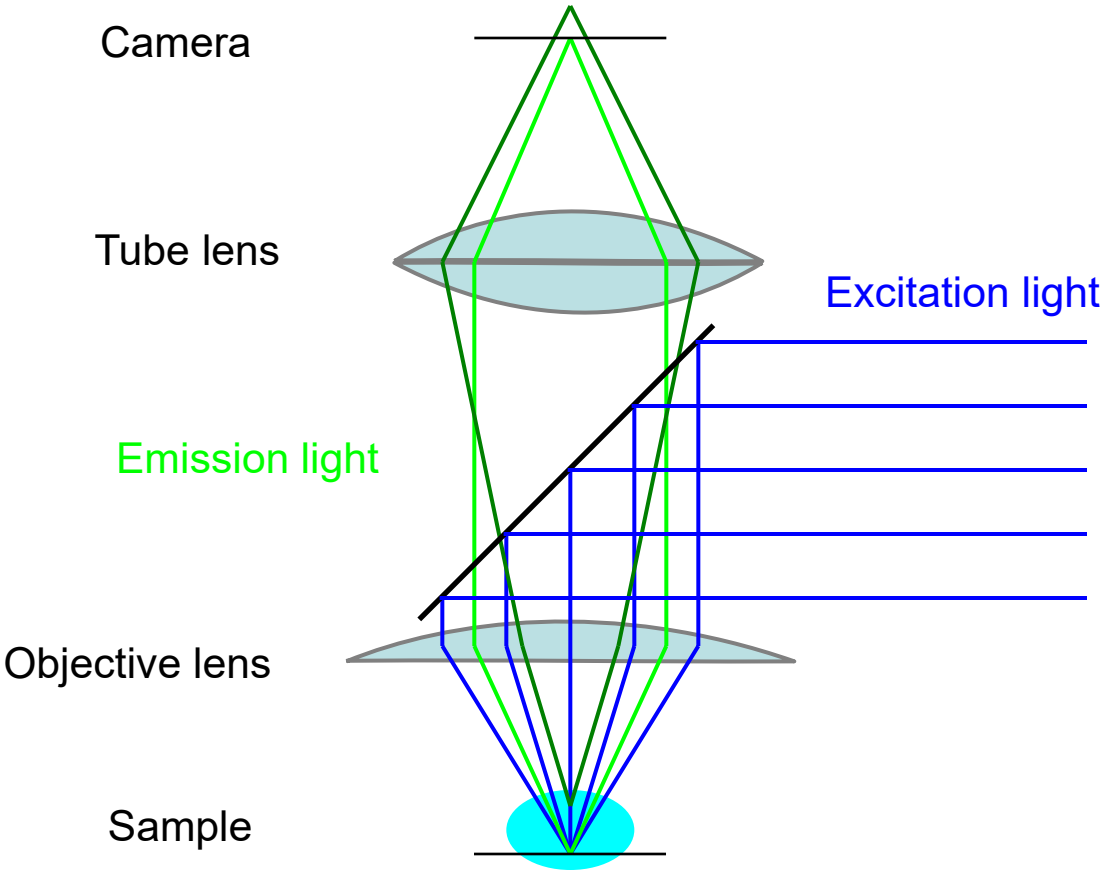
Need multiple Z-sections
to capture entire nucleus

Only need a single image
to capture entire nucleus

Confocal Microscopy

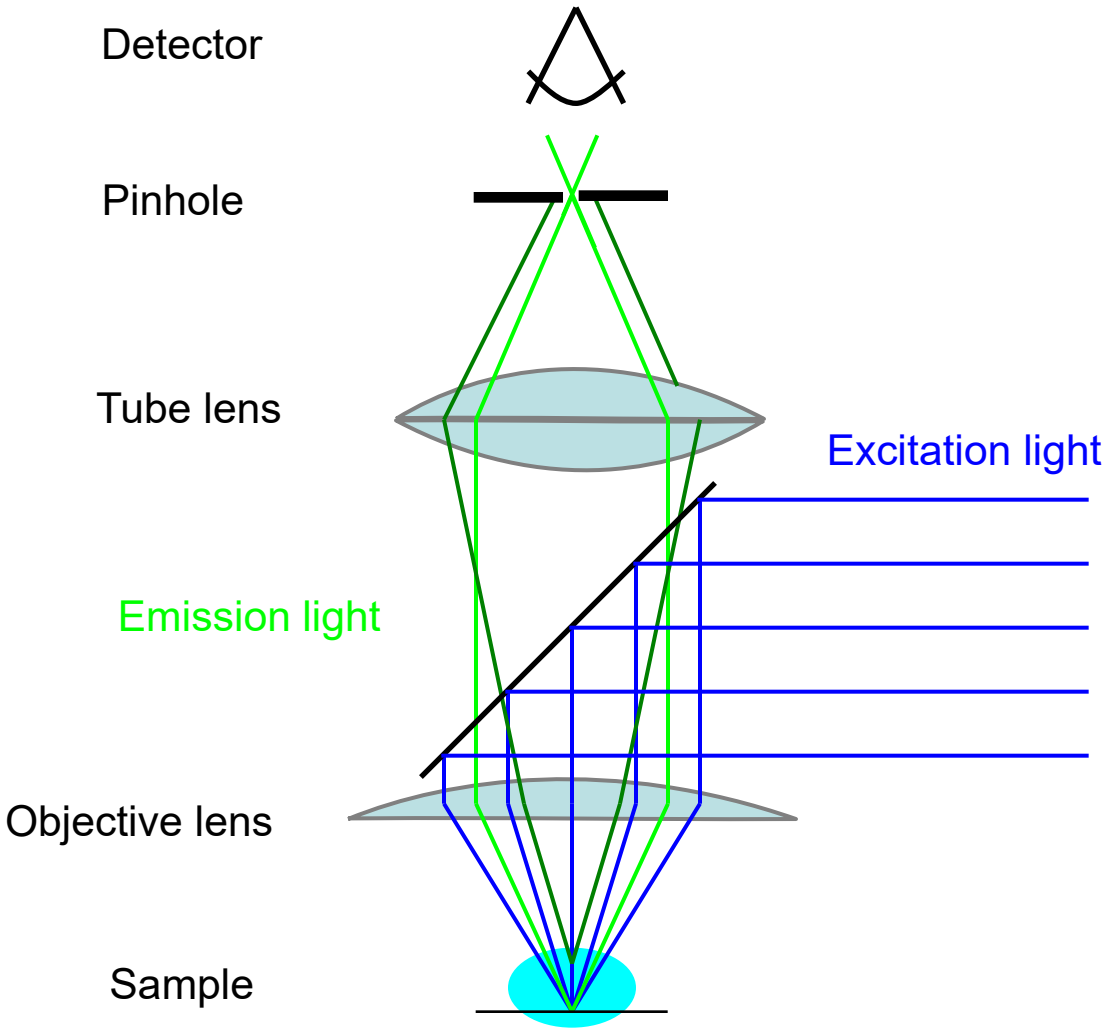
- Confocal microscopy has the same resolution as widefield, but eliminates out-of-focus light.
- This improves contrast for thick, heavily stained specimens.
- However, it usually comes at a cost in sensitivity.

Fluorescence Illumination of a single point

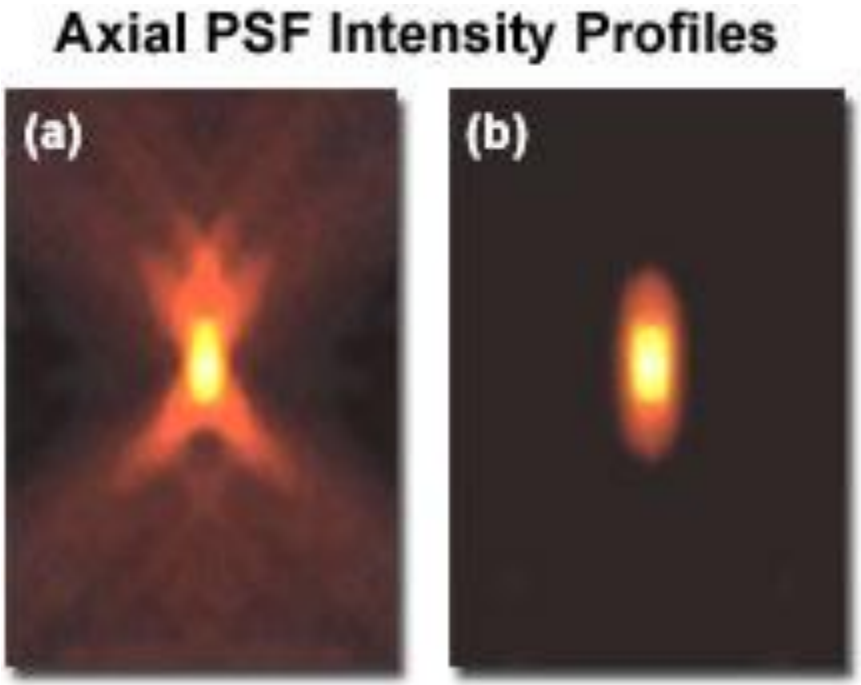


Problem – fluorescence is emitted along entire illuminated cone, not just at focus

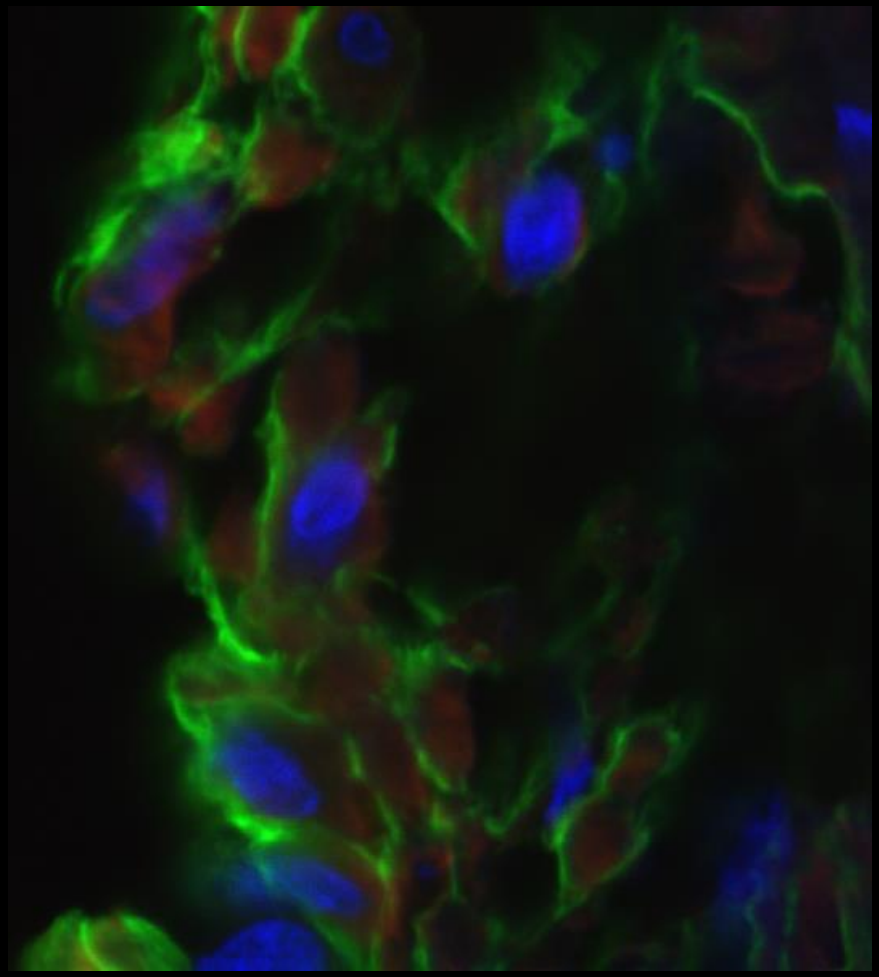
The confocal microscope



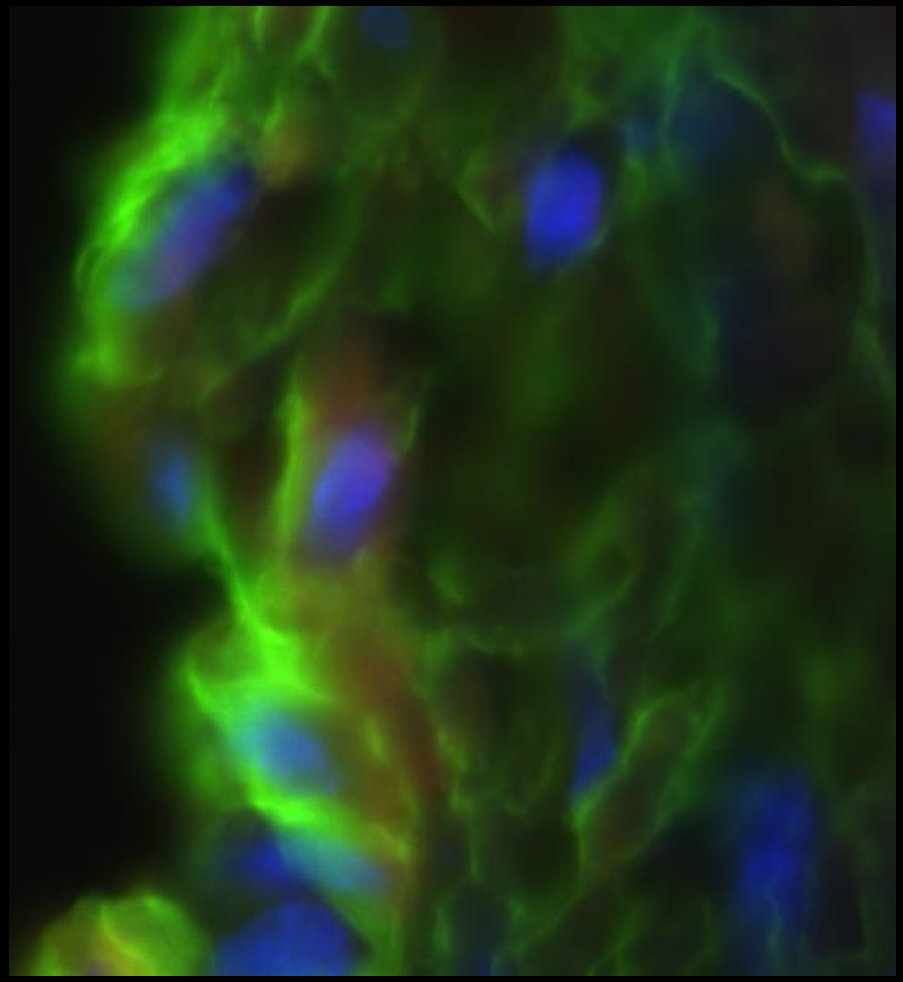
What do you get?



Confocal vs. Widefield



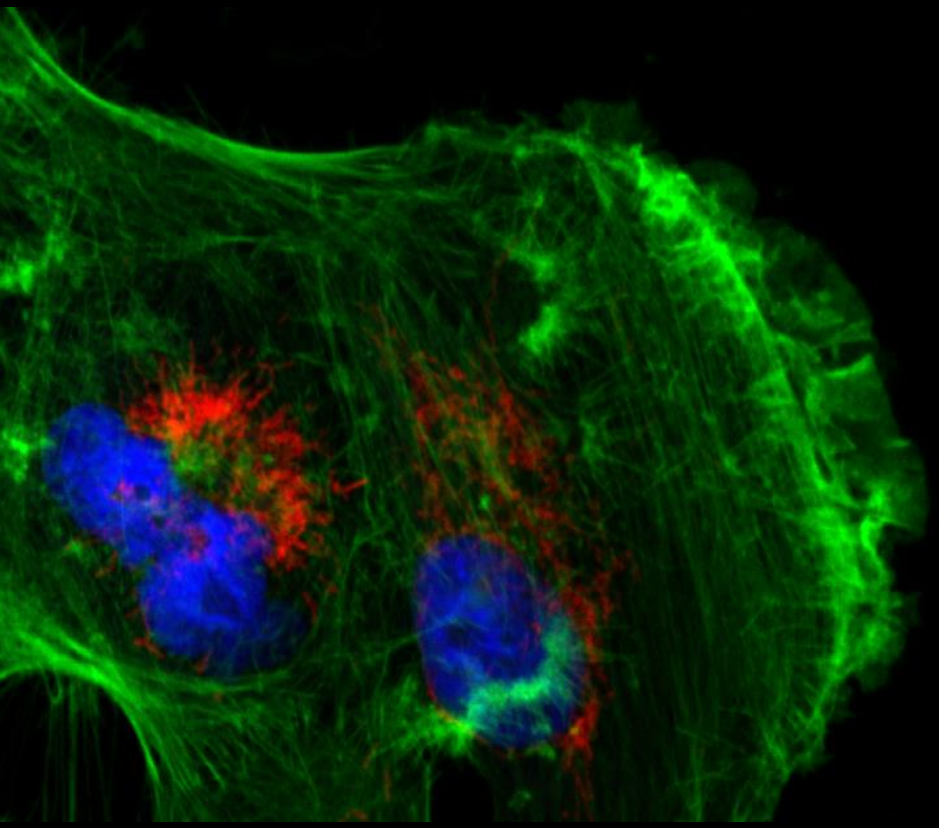
Confocal



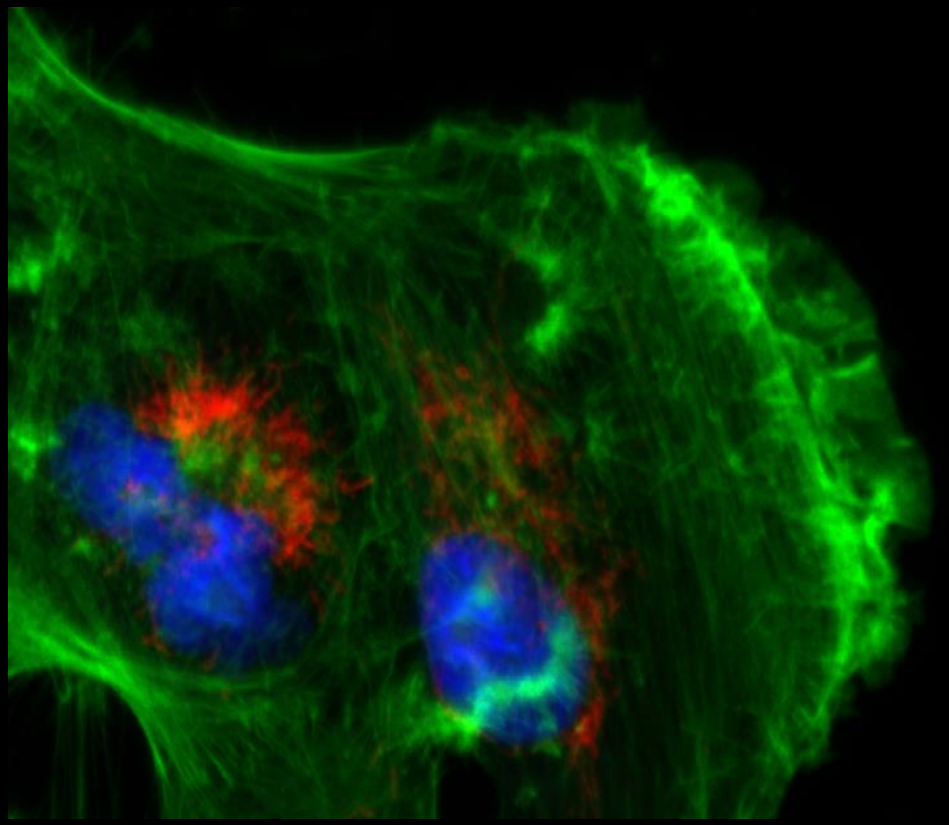
Widefield

20 μm rat intestine section recorded with 60x / 1.4NA objective

Confocal vs. Widefield



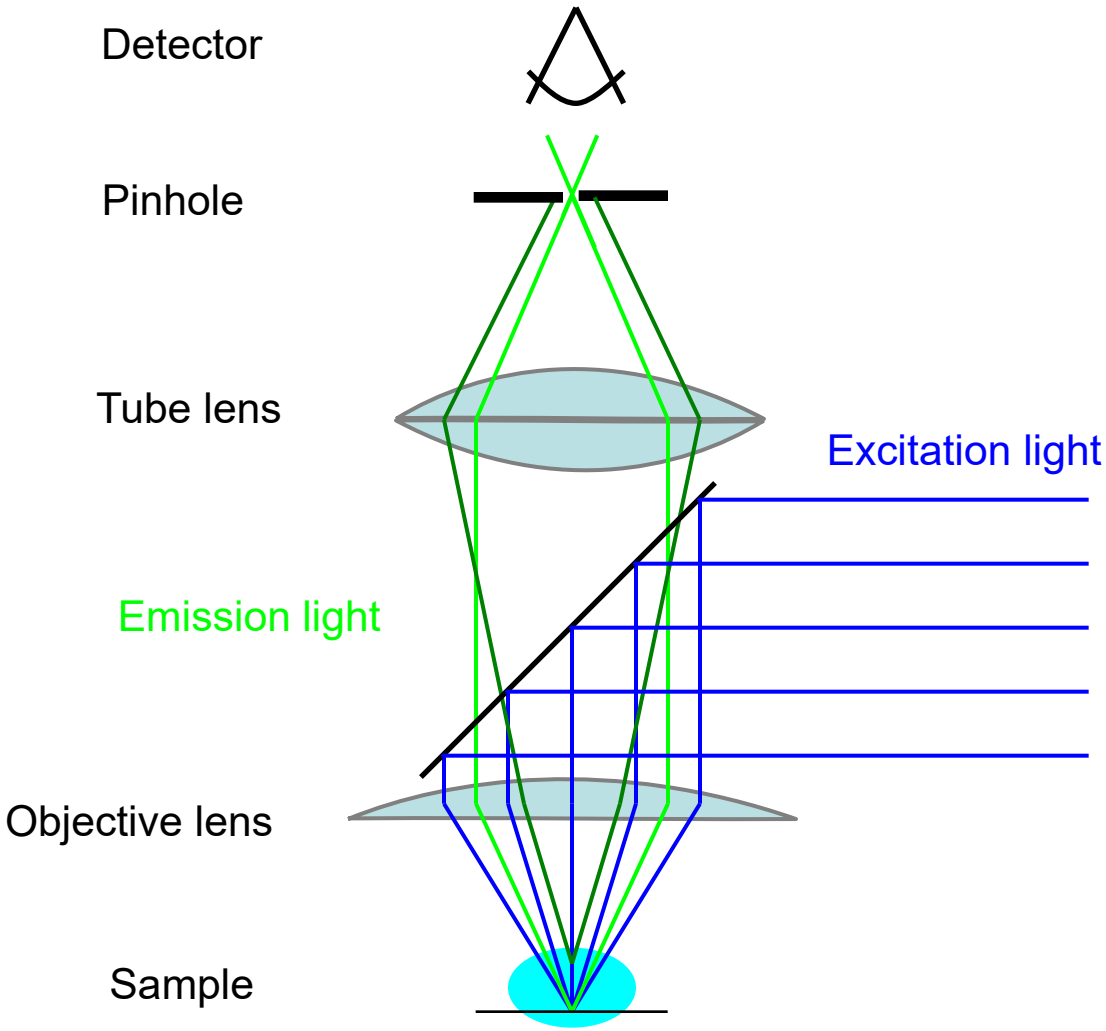
Confocal



Widefield

Tissue culture cell with 60x / 1.4NA objective

The confocal microscope

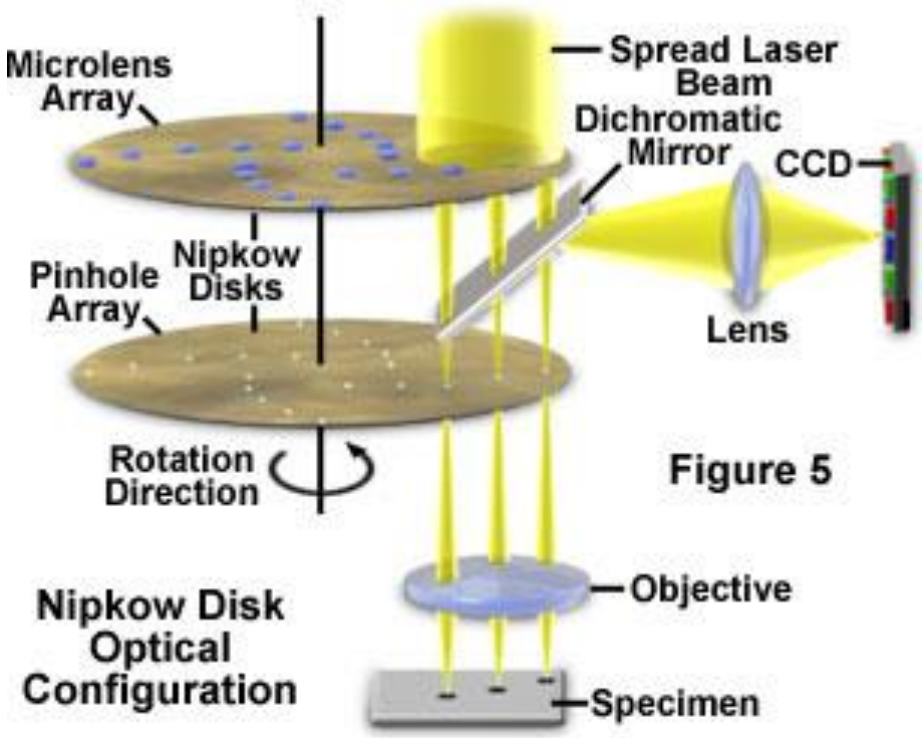


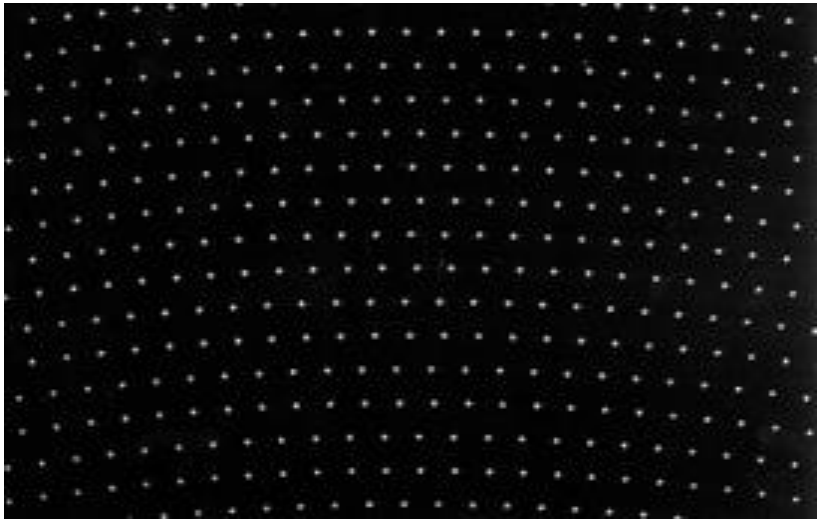
Scan excitation spot point-by-point to build up image

Problems:
Slow (~1 sec to acquire an image)
Low light efficiency (due to use of PMT as detector)

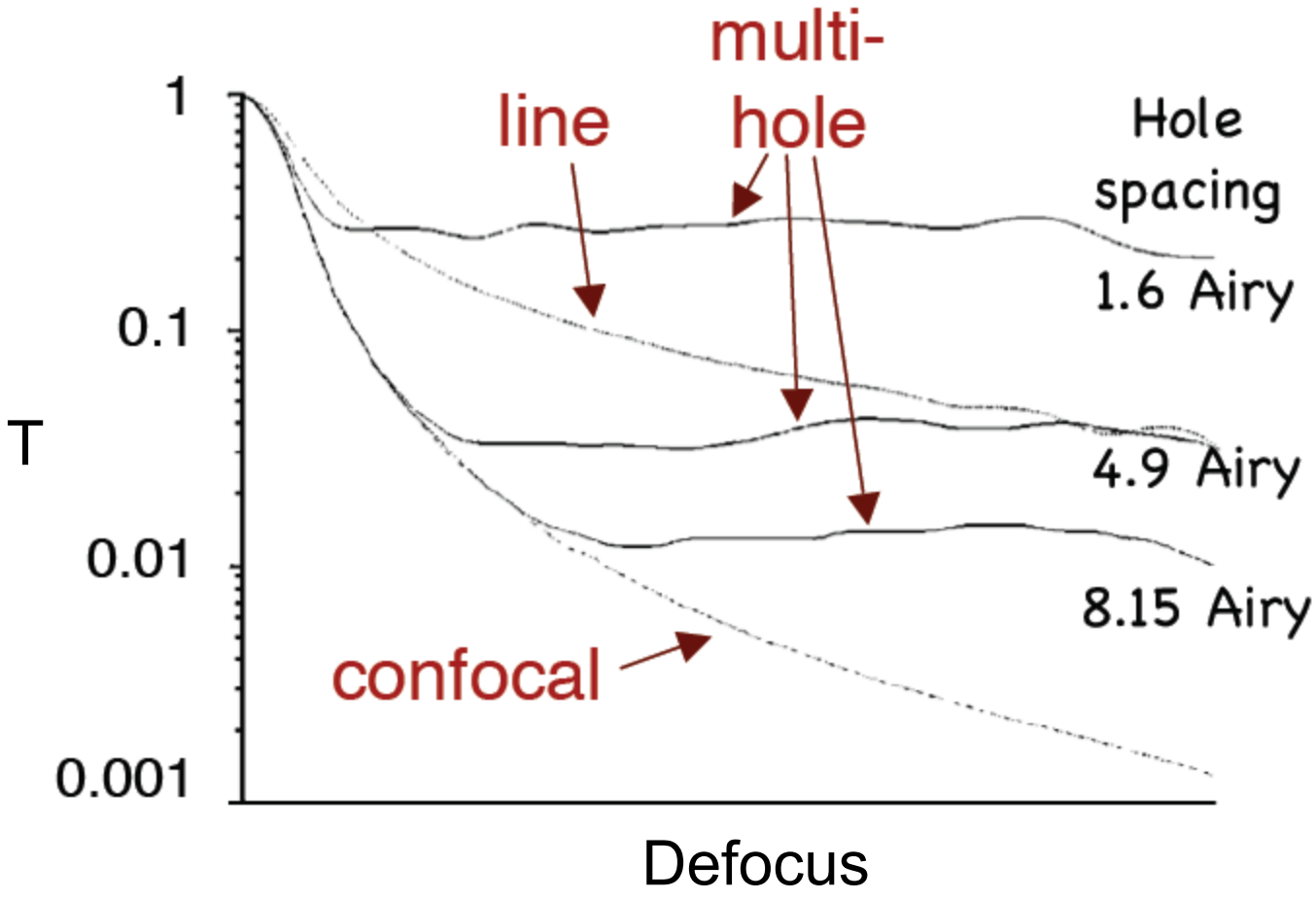
Solution:
Use multiple pinholes and a camera

Spinning Disk Confocal





Out-of-focus rejection

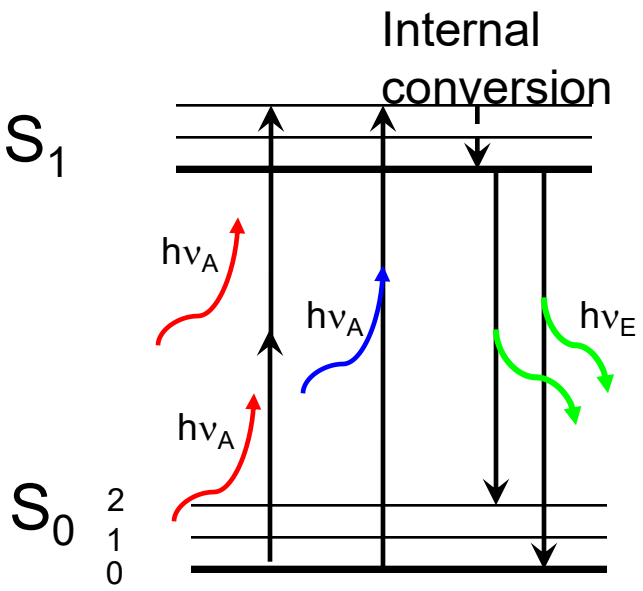


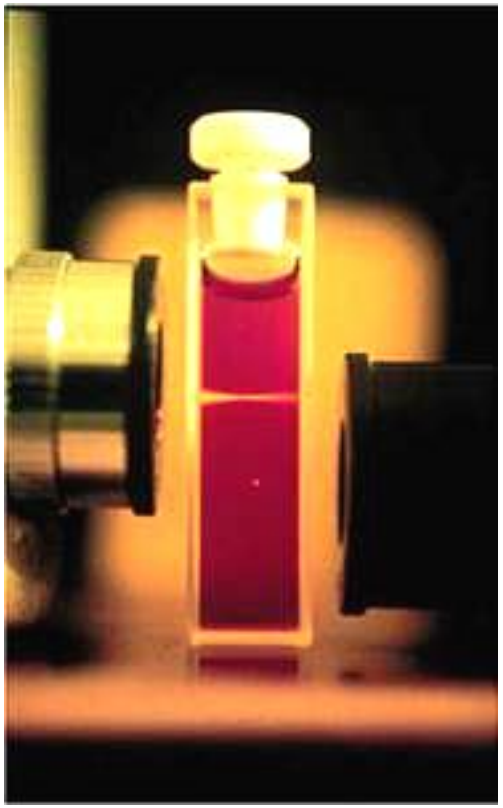
Relative Sensitivity

- Widefield 100
- Spinning-Disk Confocal 25
- Laser-scanning Confocal 1

- See Murray JM et al, J. Microscopy 2007 vol. 228 p390-405

Multi-photon excitation





Brad Amos, MRC, Cambridge

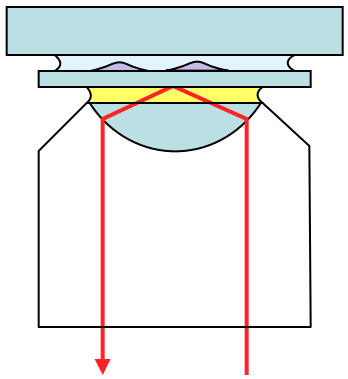
Multi-photon excitation does not excite out-of-focus light, so you can get rid of pinhole

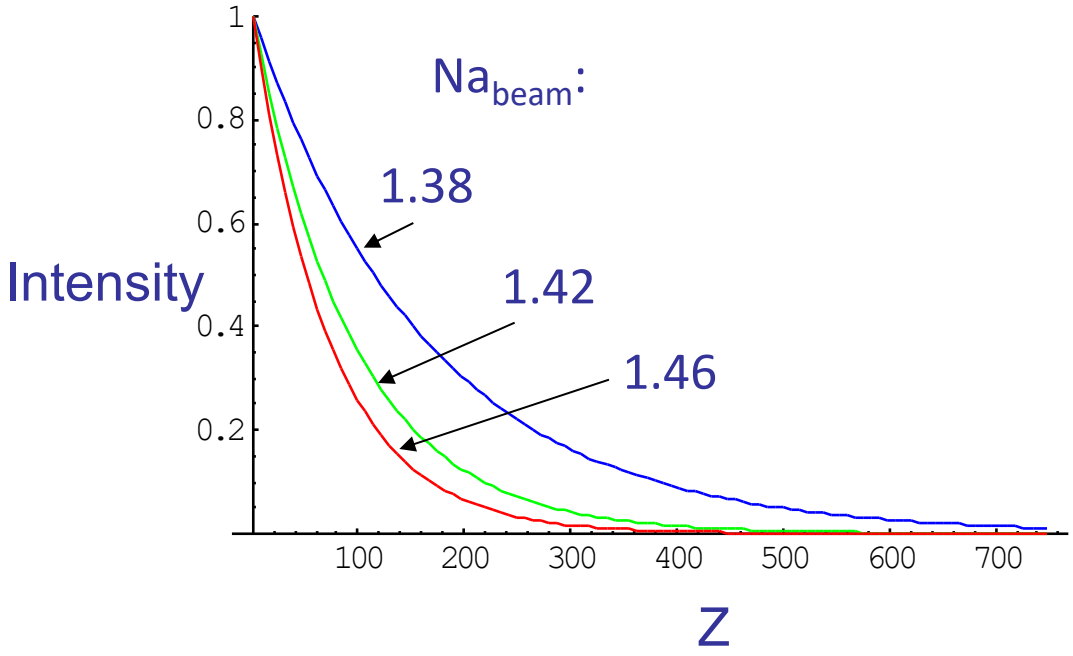
Total Internal Reflection: TIRF

Thinnest optical sections:
Images ~100nm section adjacent to coverslip

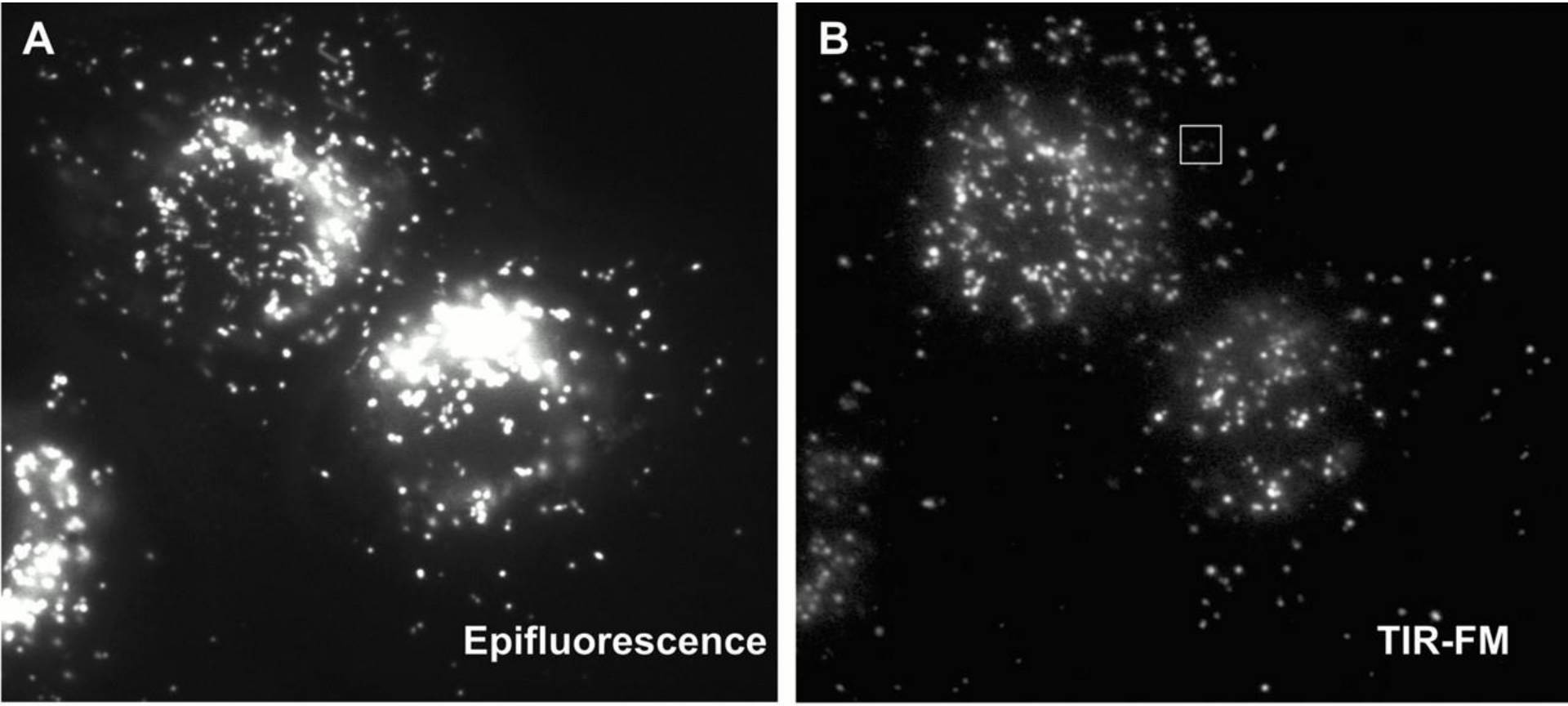
Illuminate through the objective

Sample on cover glass



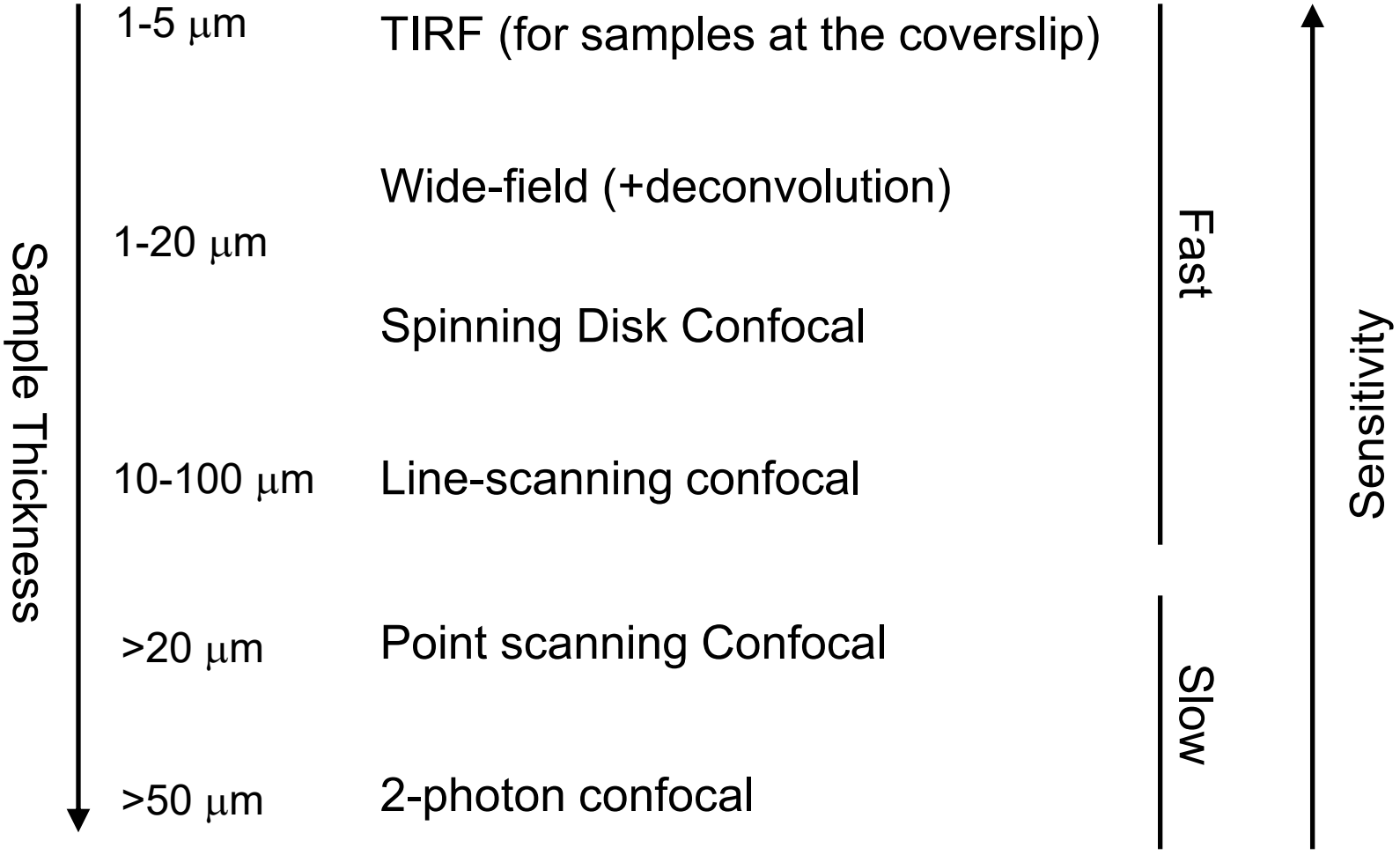


Epifluorescence vs. TIRF



Jaiswal et al 2002; cells loaded with FITC-dextran

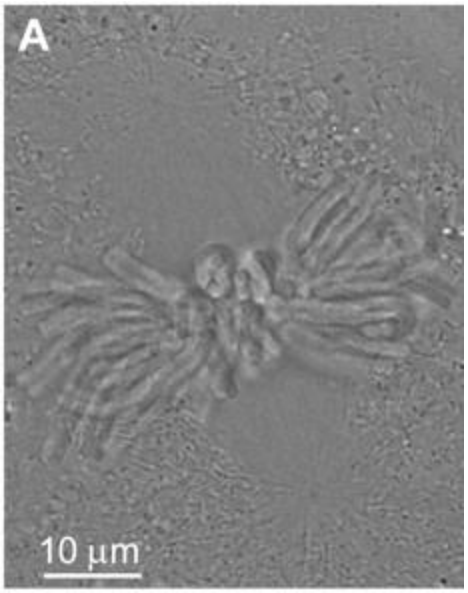
Which imaging technique should I use?



Microscope choice

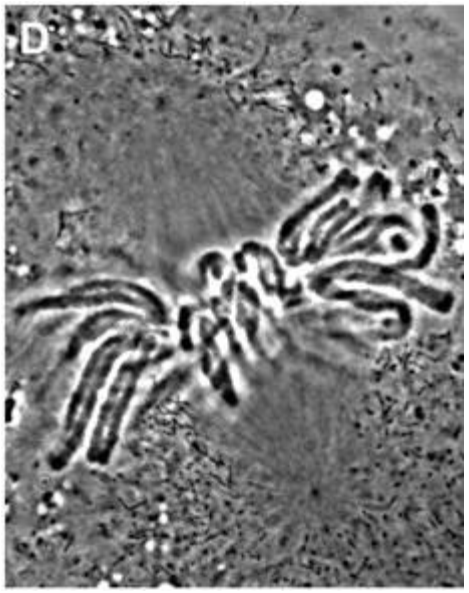
- Epifluorescence – routine work, low magnification, or thin samples where you don't need high-resolution 3D reconstruction
- TIRF – samples at the membrane or otherwise at the coverslip surface; very high signal-to-noise; single molecule imaging
- Spinning Disk Confocal – Live tissue culture cells, yeast, etc, or thin (<30 μm) tissue sections when you need 3D reconstructions
- Laser-Scanning Confocal – Thick tissues or specimens

Brightfield Contrasting Techniques



Brightfield

DIC



Phase Contrast

Reider and Khodjakov *Science* **300**: 91-96 (2003)

Brightfield Contrasting Techniques

- Both DIC and Phase Contrast can be acquired with fluorescence, but may interfere
- Phase Contrast: lose ~5% of fluorescence emission, possibility of increased background due to scatter
- DIC: prisms result in splitting of emission light, causing a reduction in resolution

References

- Slides: http://nic.ucsf.edu/edu.html