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, …

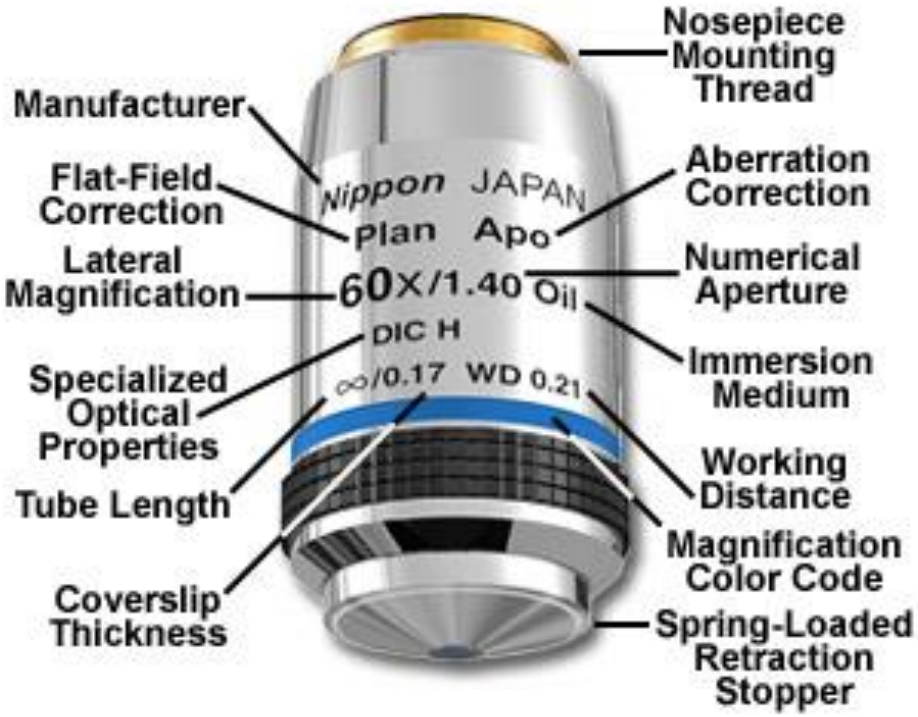
Standard microscope capabilities

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

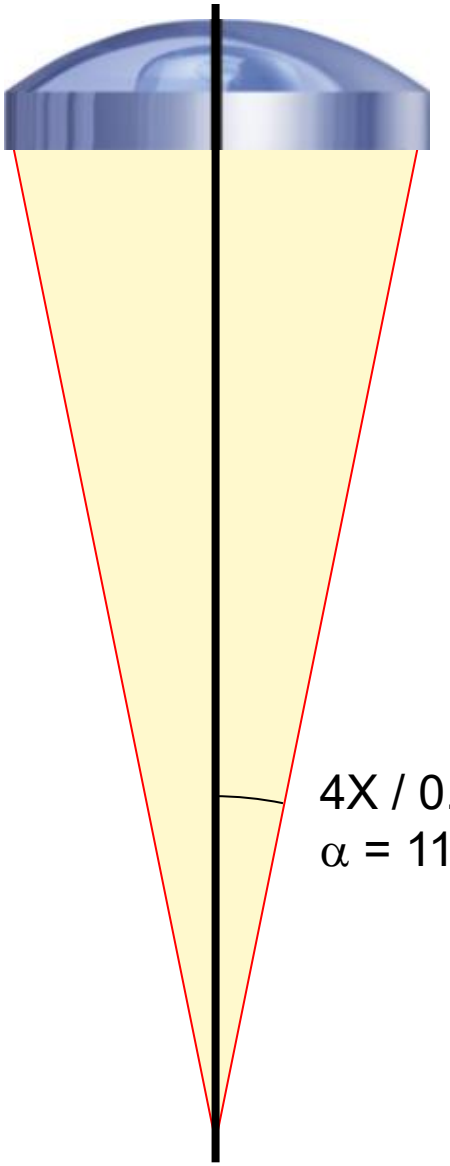
Some examples:

10x/0.3 WD = 15.2mm

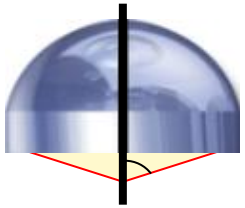
20x/0.75 WD = 1.0mm

100x/1.4 WD = 0.13mm

Numerical Aperture



4X / 0.20 NA
α = 11.5°



100X / 0.95 NA
α = 71.8°

$NA = n \sin(\alpha)$

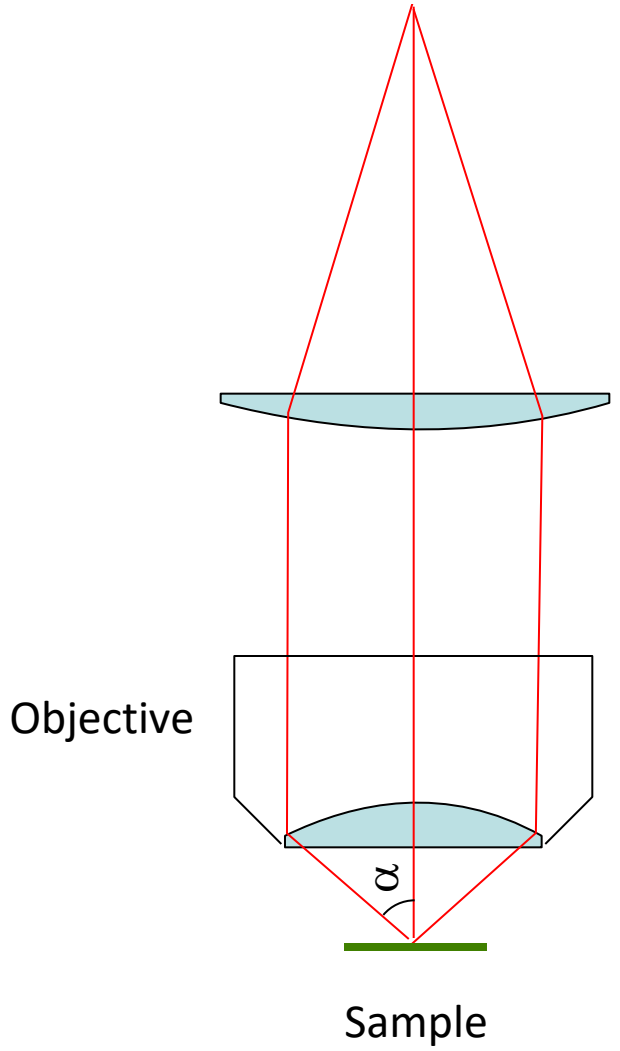
where

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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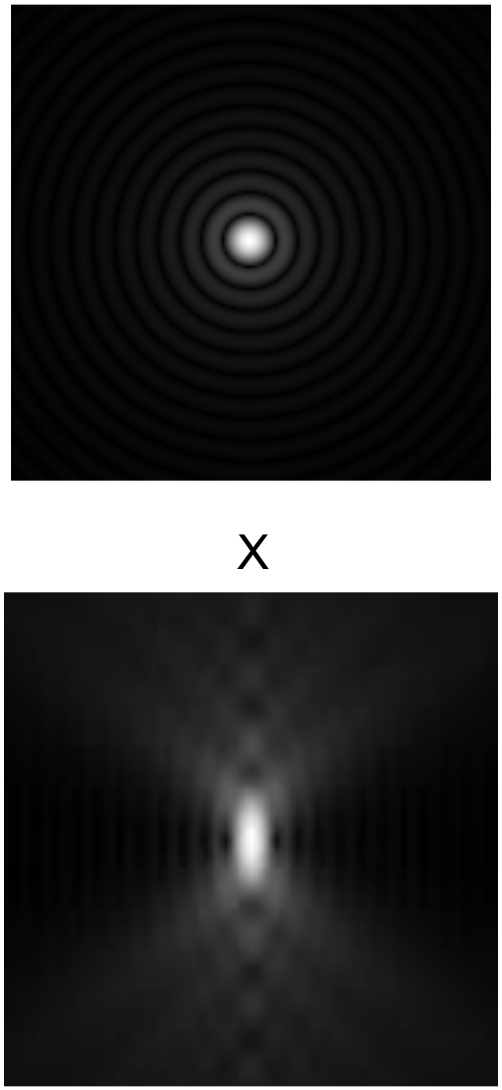


Resolution of the Microscope

limited by the point-spread function

Resolution: X-Y, 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

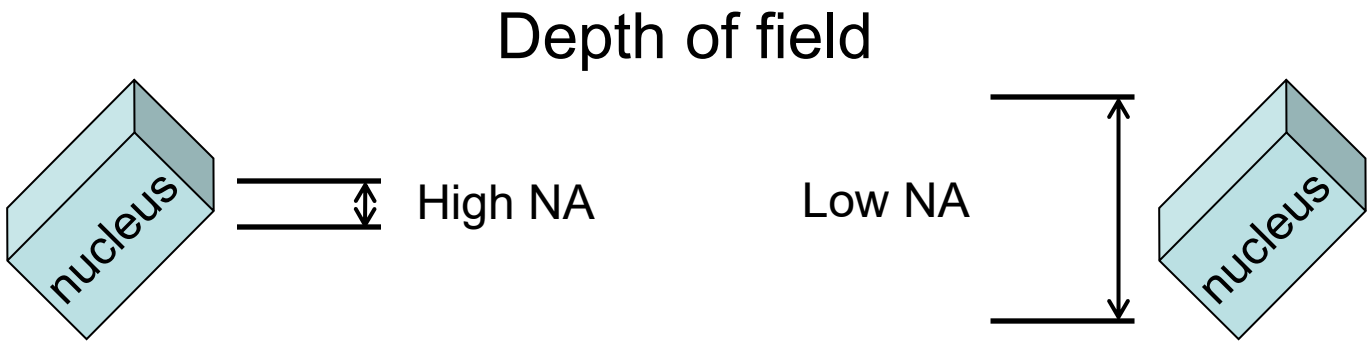
Choosing an objective

- 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



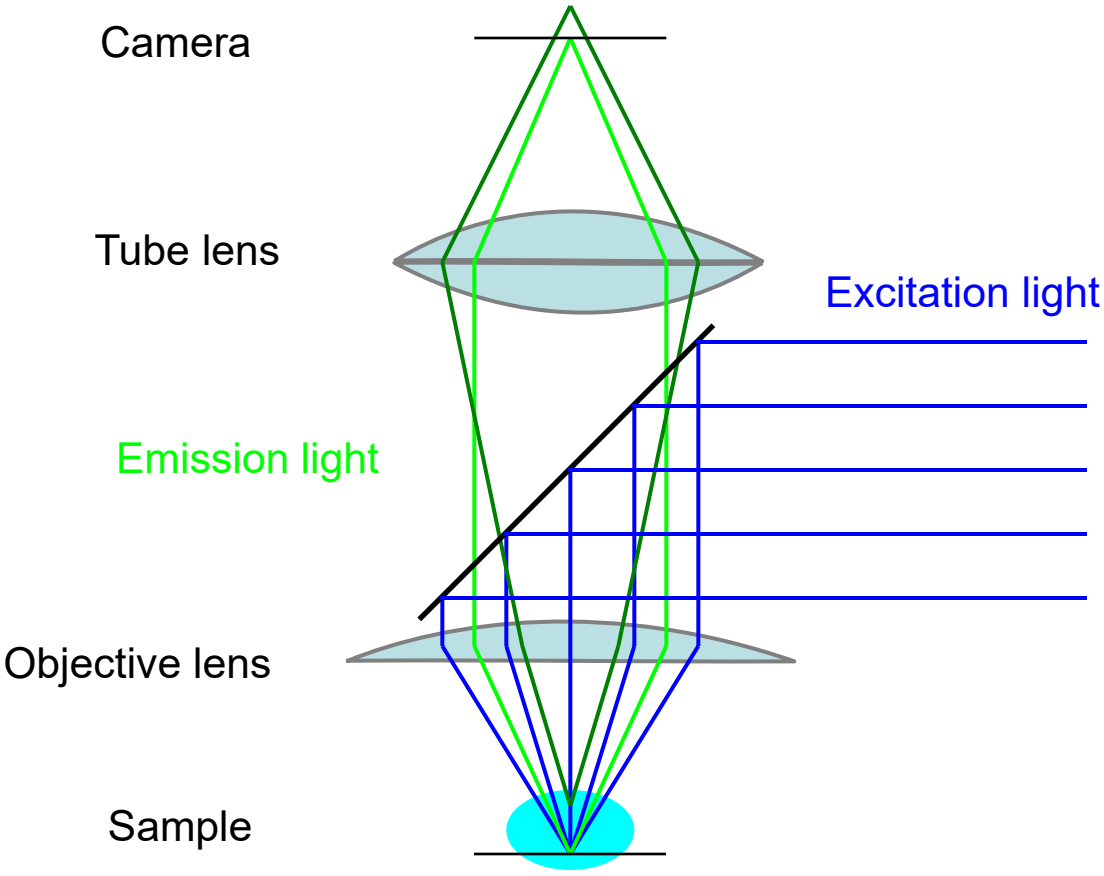
Need multiple Z-sections
to capture entire nucleus

Only need a single image
to capture entire nucleus

Confocal Microscopy

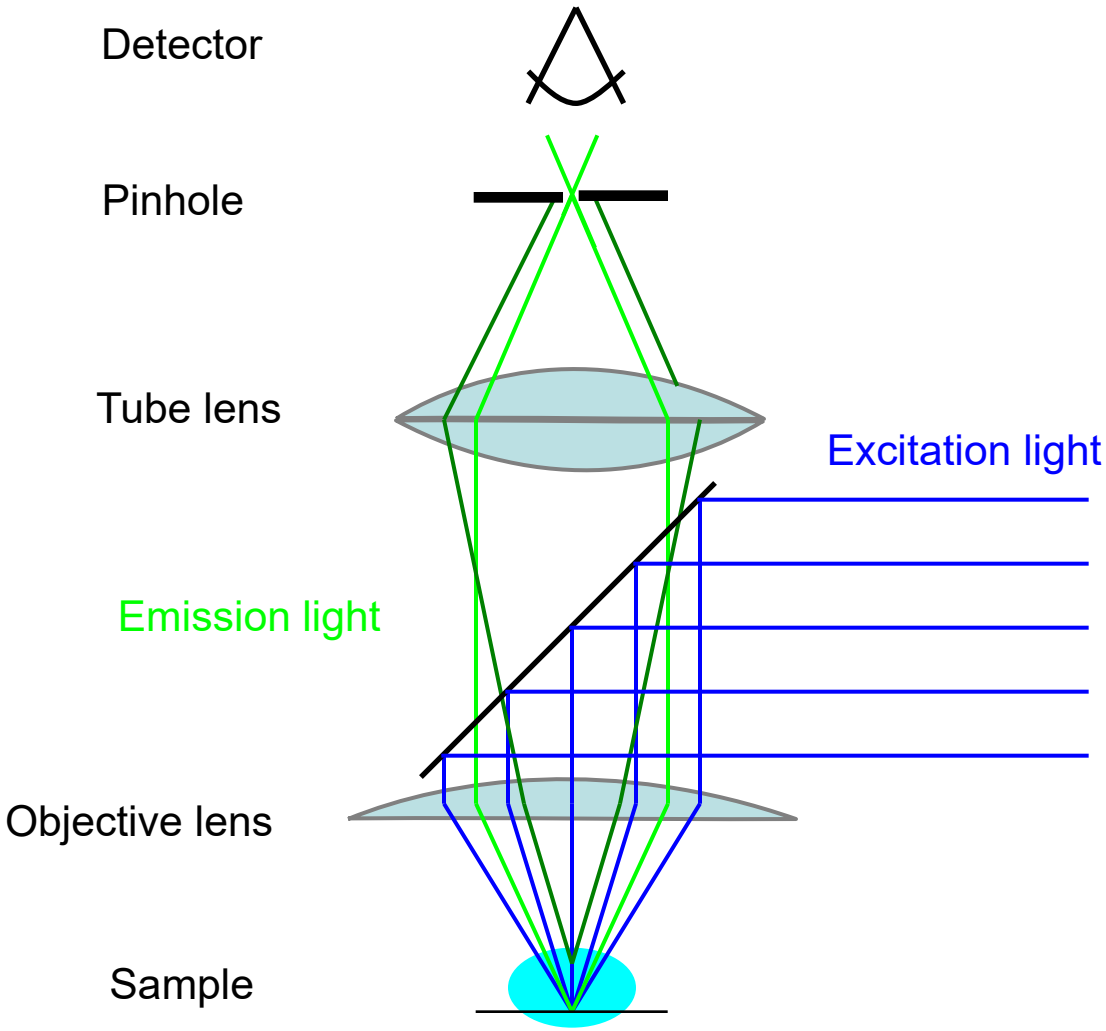
- Confocal microscopy has the same resolution as widefield, but eliminates out-of-focus light.
- This improves contrast for thick, heavily stained specimens.
- However, it usually comes at a cost in sensitivity.

Fluorescence Illumination of a single point

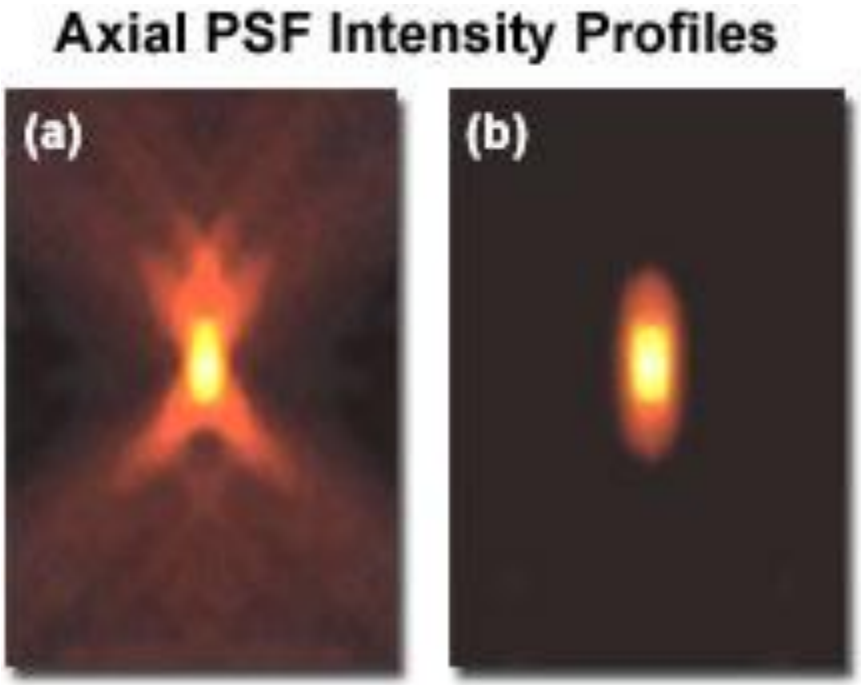


Problem – fluorescence is emitted along entire illuminated cone, not just at focus

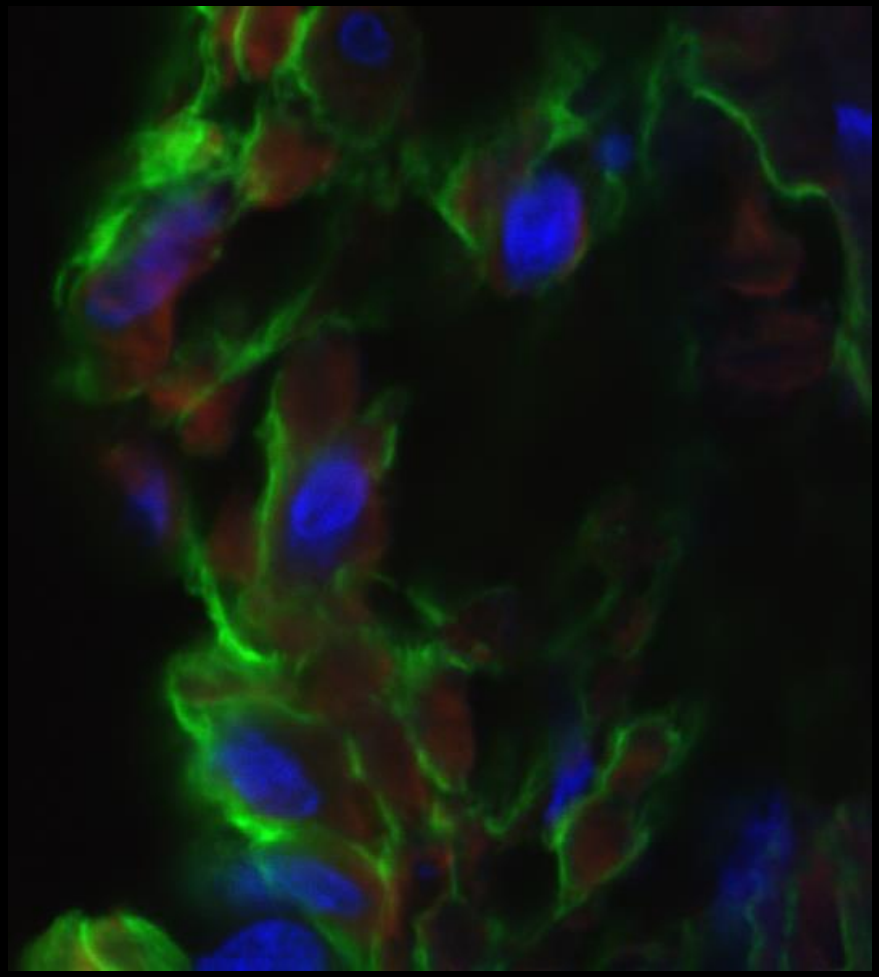
The confocal microscope



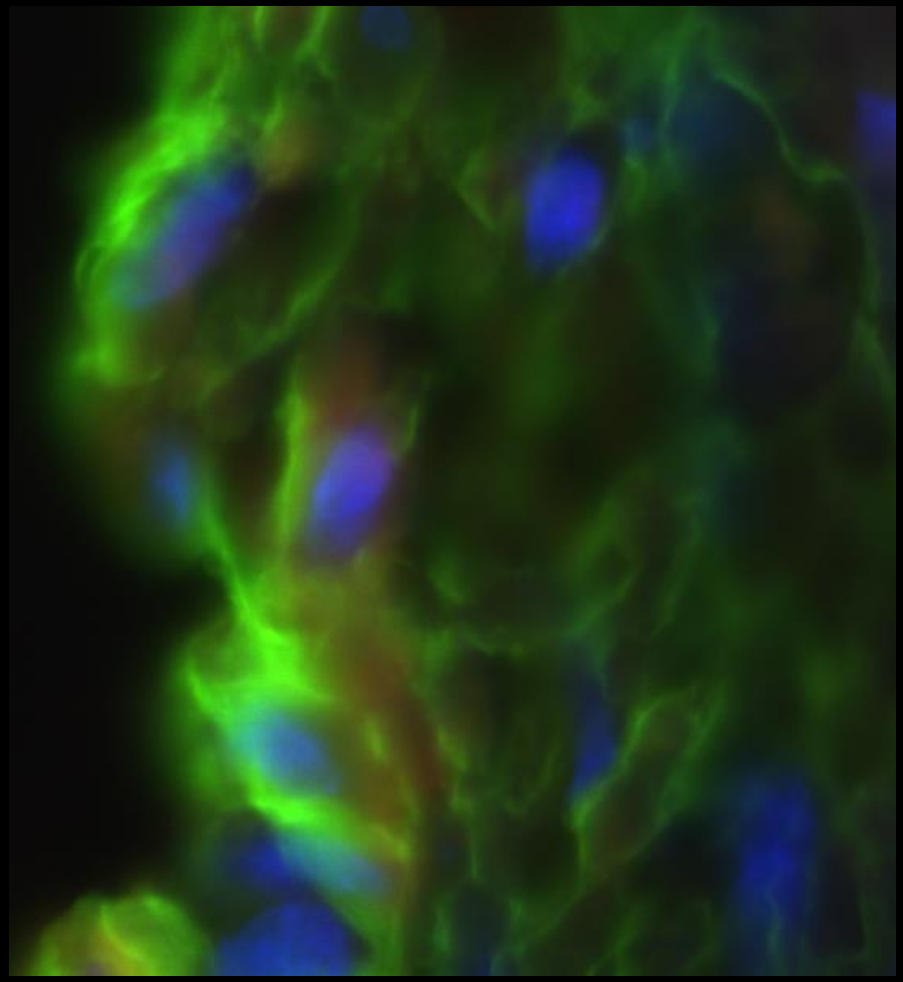
What do you get?



Confocal vs. Widefield



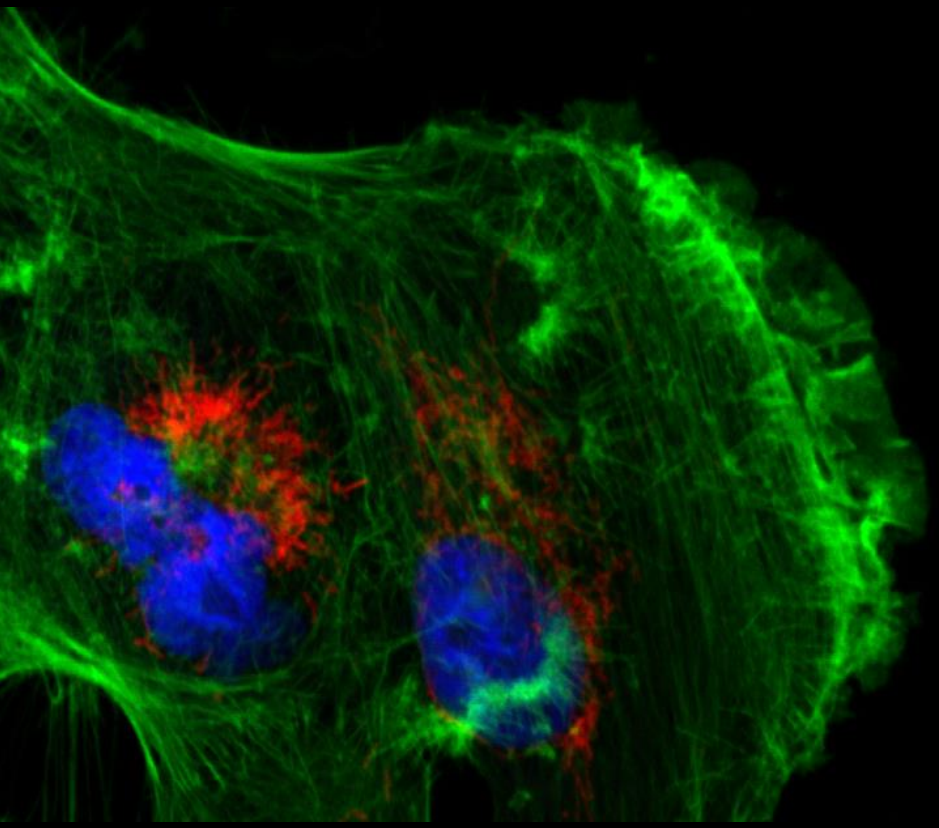
Confocal



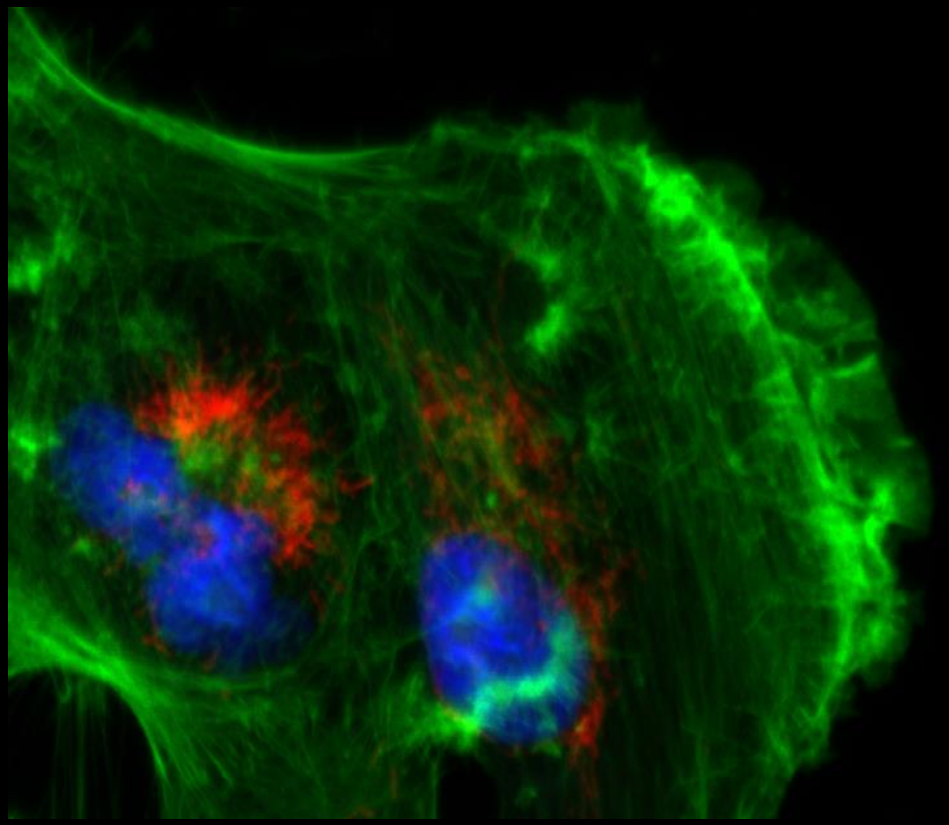
Widefield

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

Confocal vs. Widefield



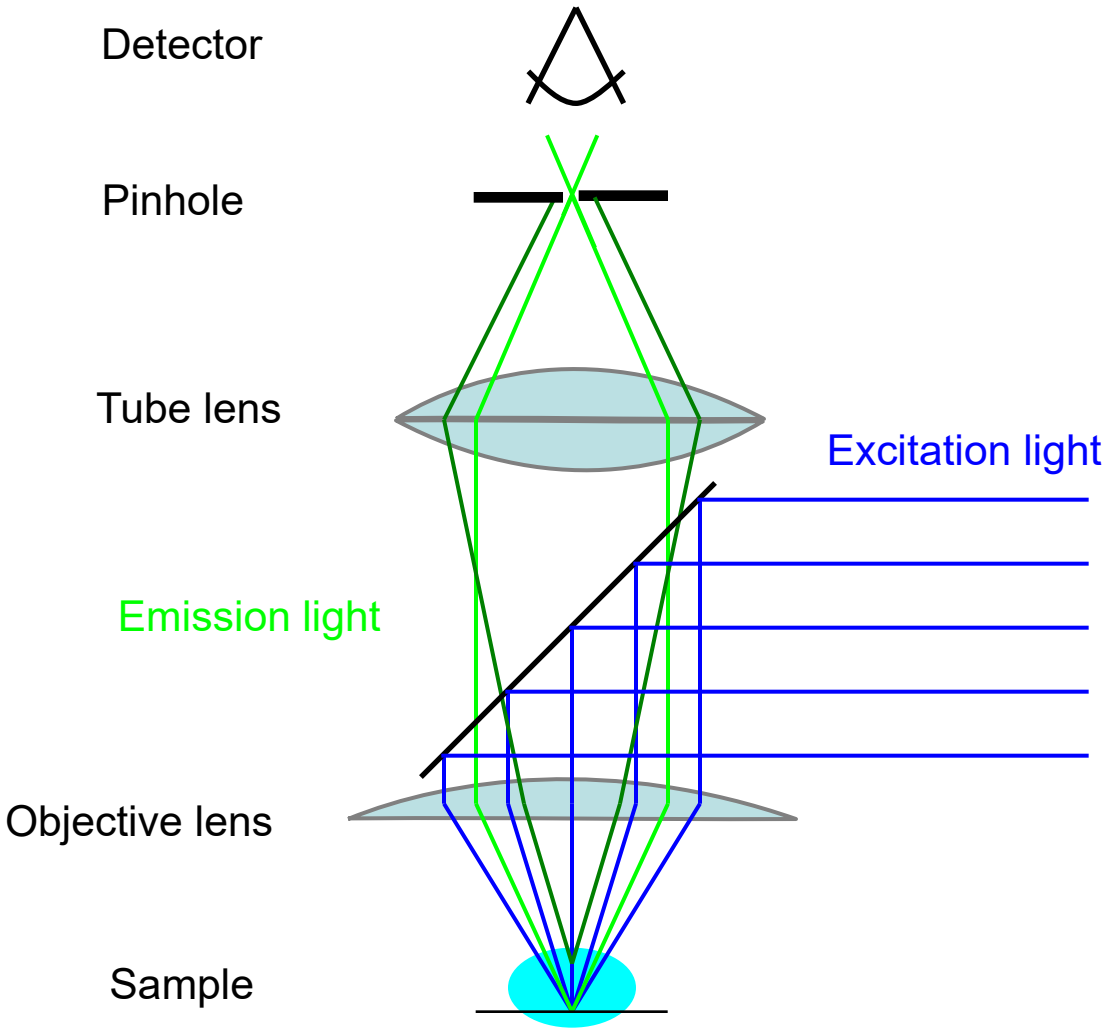
Confocal



Widefield

Tissue culture cell with 60x / 1.4NA objective

The confocal microscope

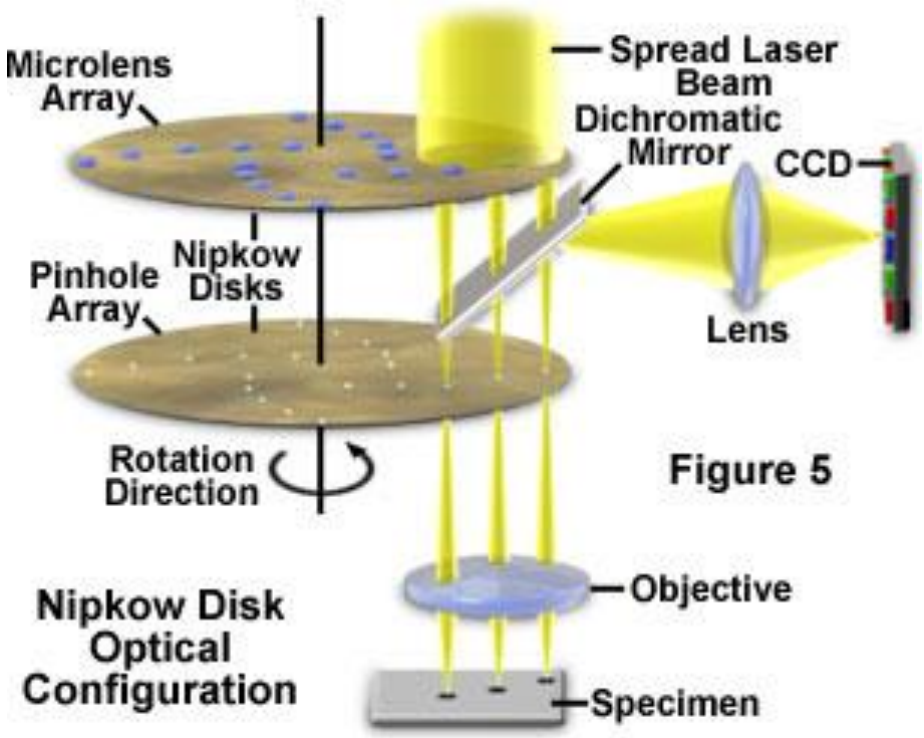


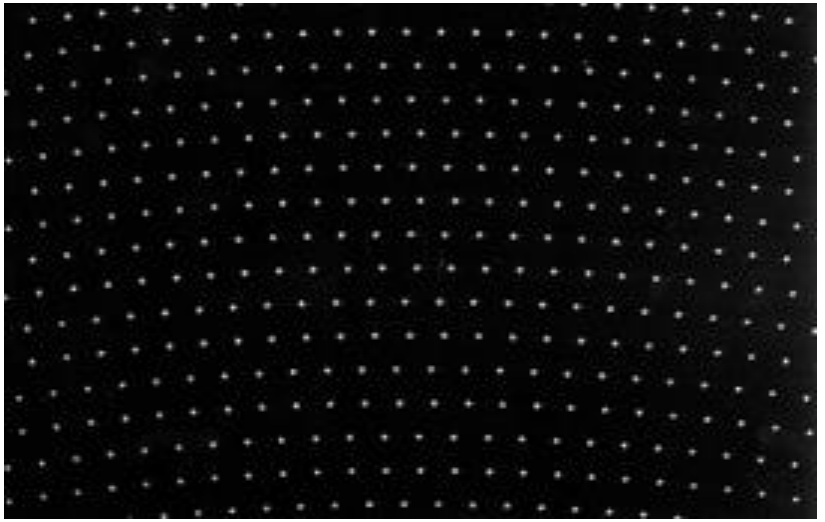
Scan excitation spot point-by-point to build up image

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

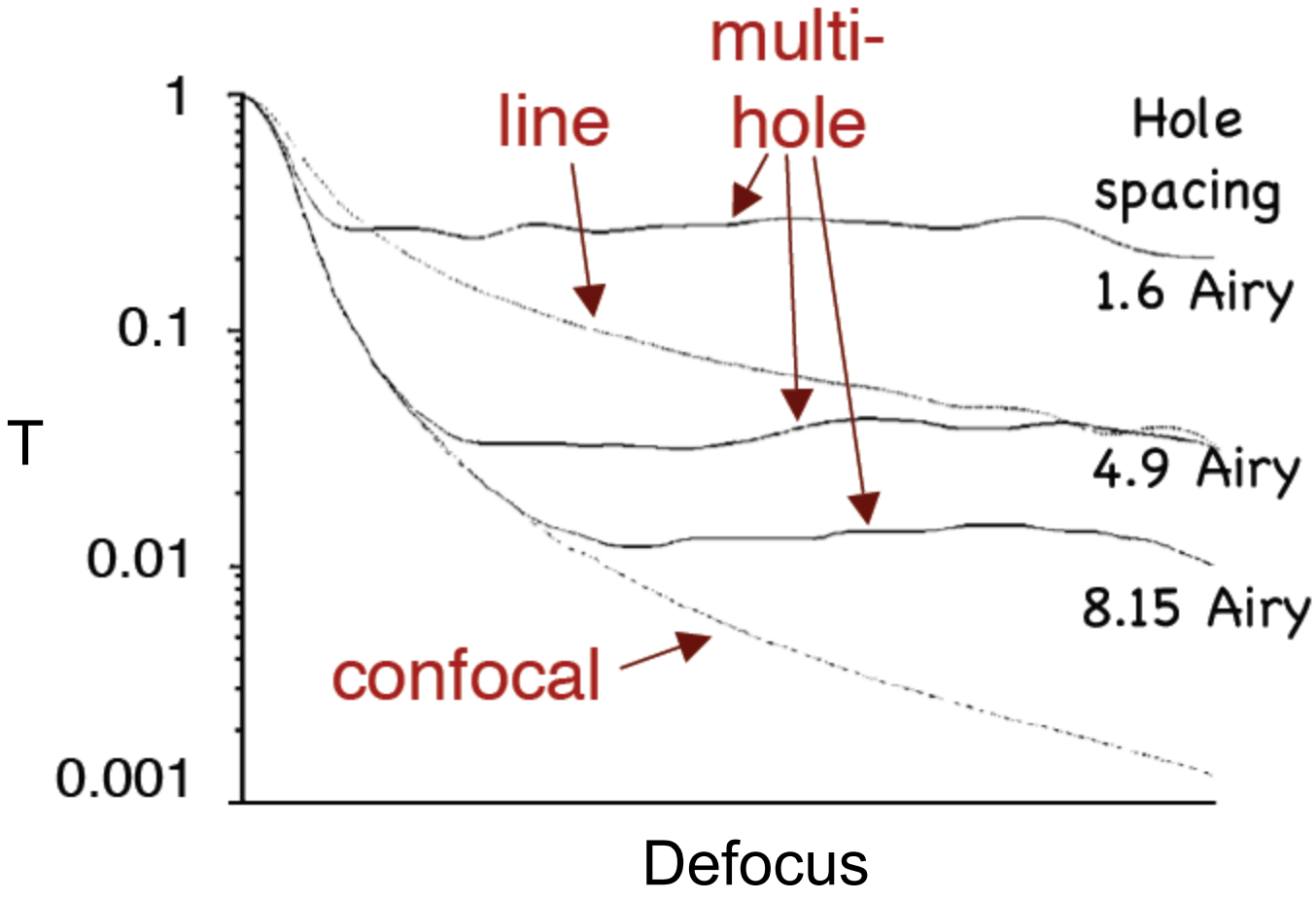
Solution:
Use multiple pinholes and a camera

Spinning Disk Confocal





Out-of-focus rejection

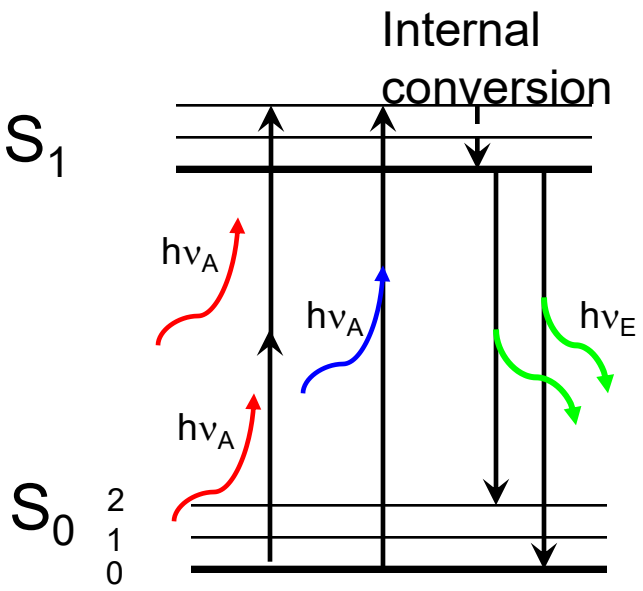


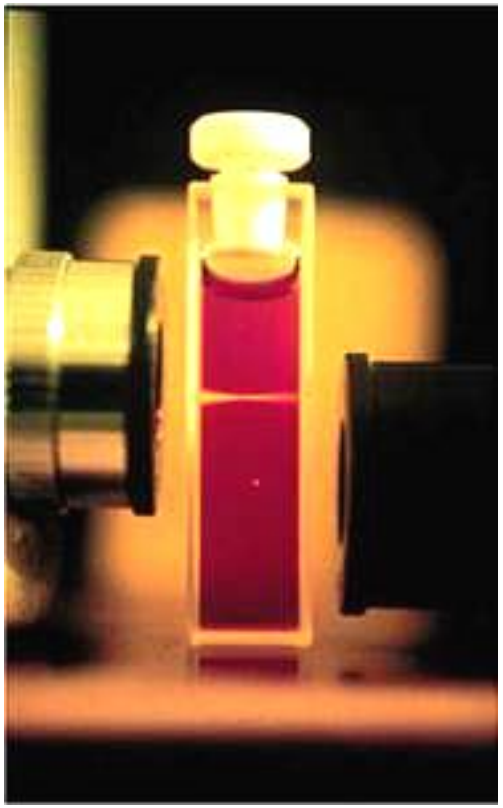
Relative Sensitivity

- 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

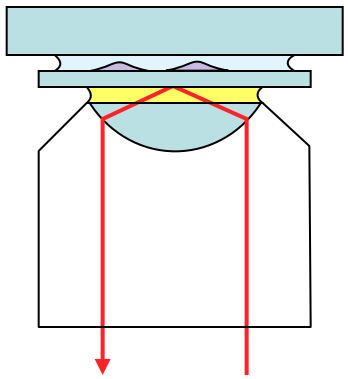
Multi-photon excitation does not excite out-of-focus light, so you can get rid of pinhole

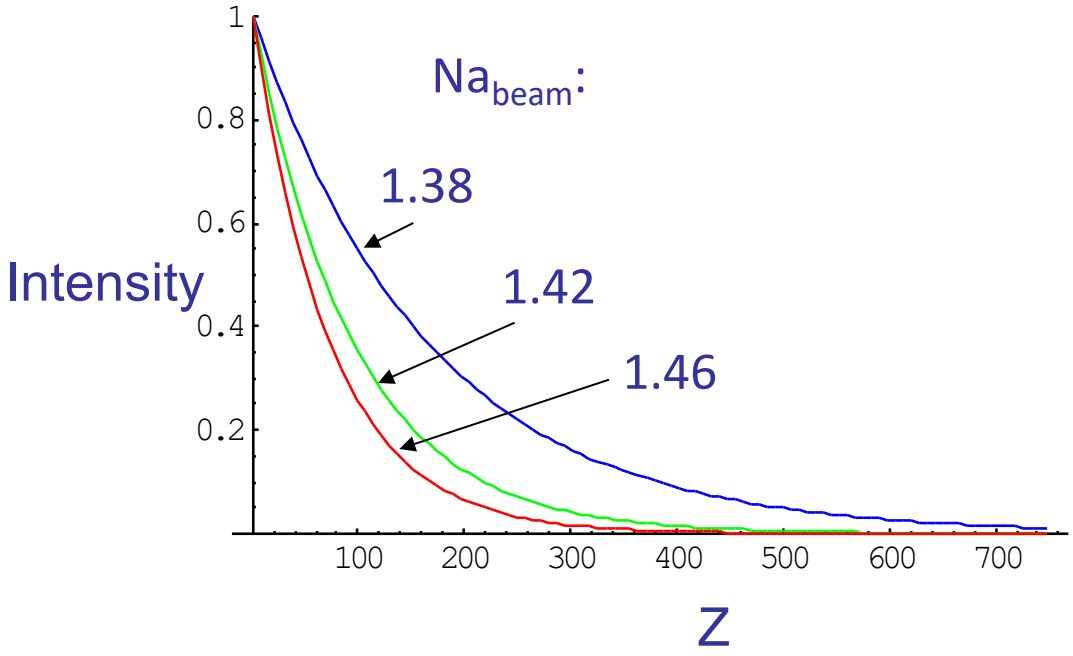
Total Internal Reflection: TIRF

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

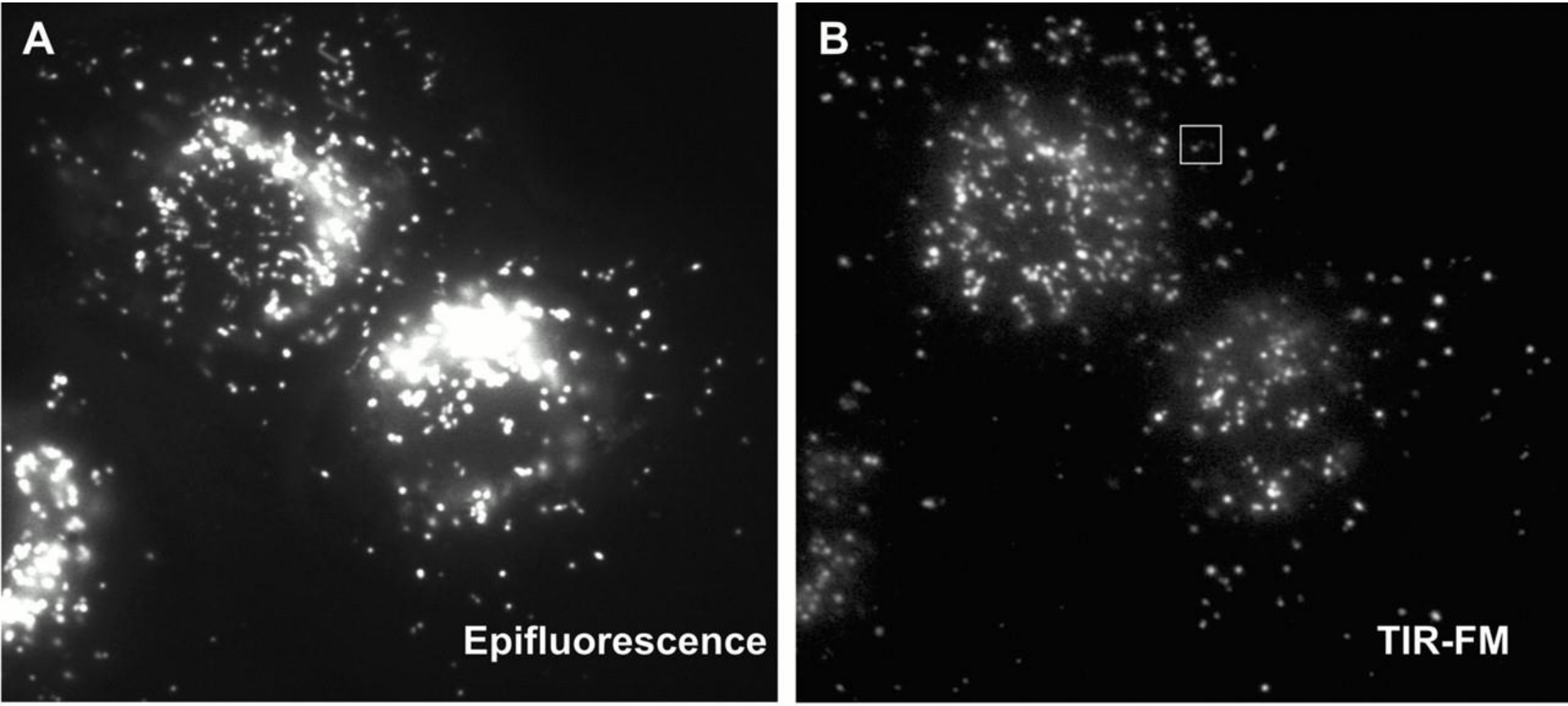
Illuminate through the objective

Sample on cover glass



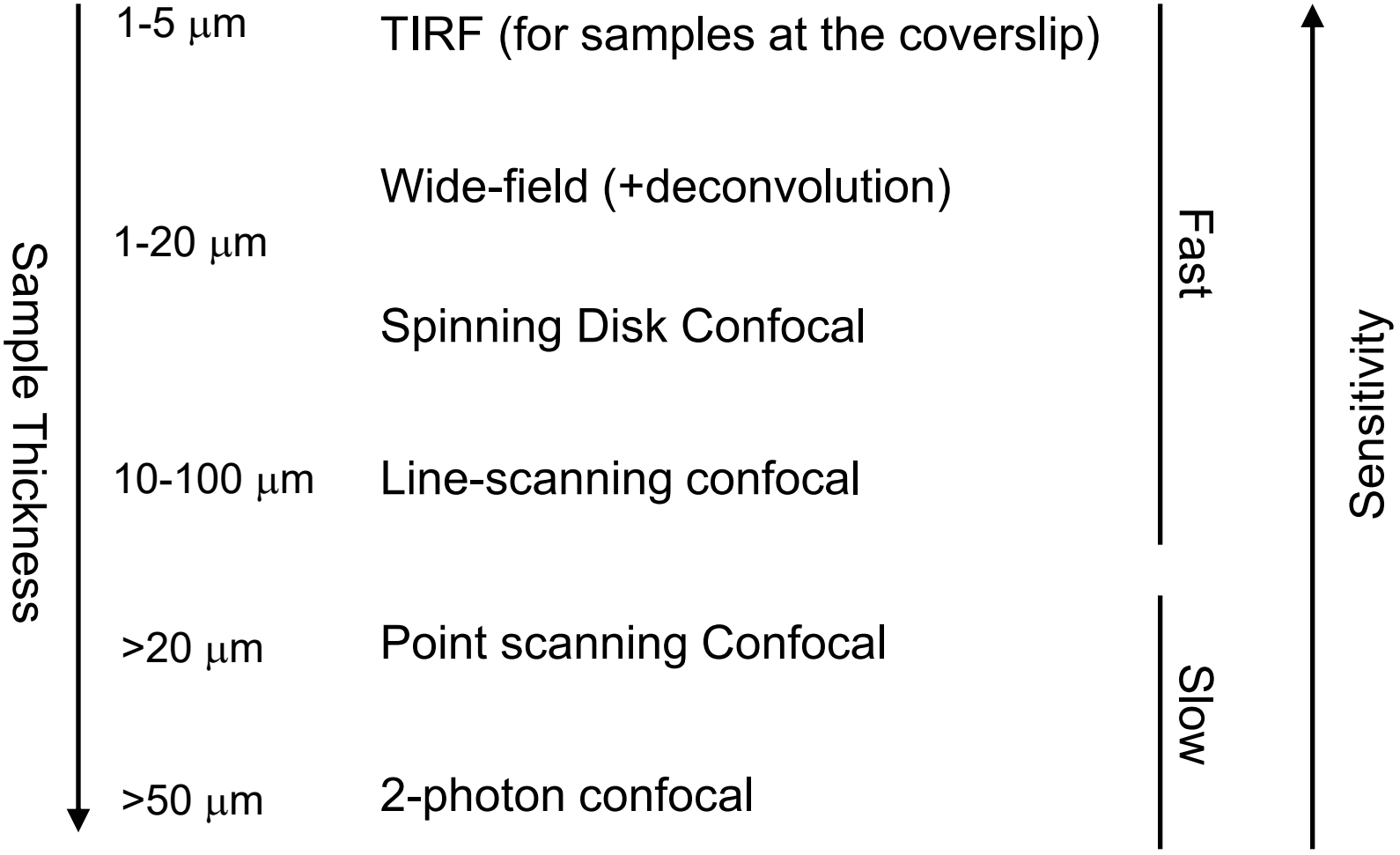


Epifluorescence vs. TIRF



Jaiswal et al 2002; cells loaded with FITC-dextran

Which imaging technique should I use?



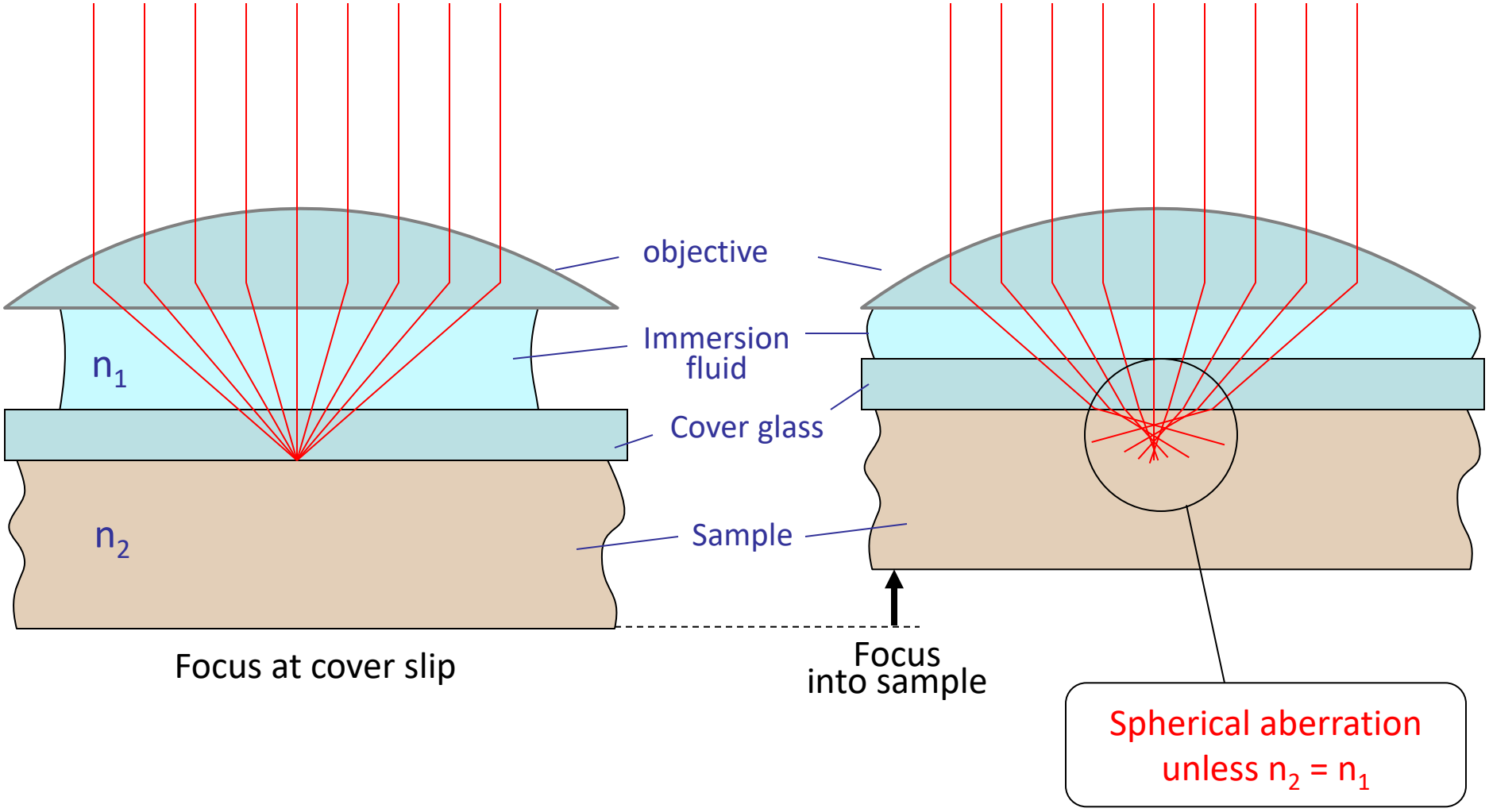
Microscope choice

- 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

Sample preparation and mounting

- Mounting media serve several purposes:
  - Stabilizing the sample
  - Preventing photobleaching
  - Clearing the sample
  - Matching refractive index

Index Mismatch
& Spherical Aberration



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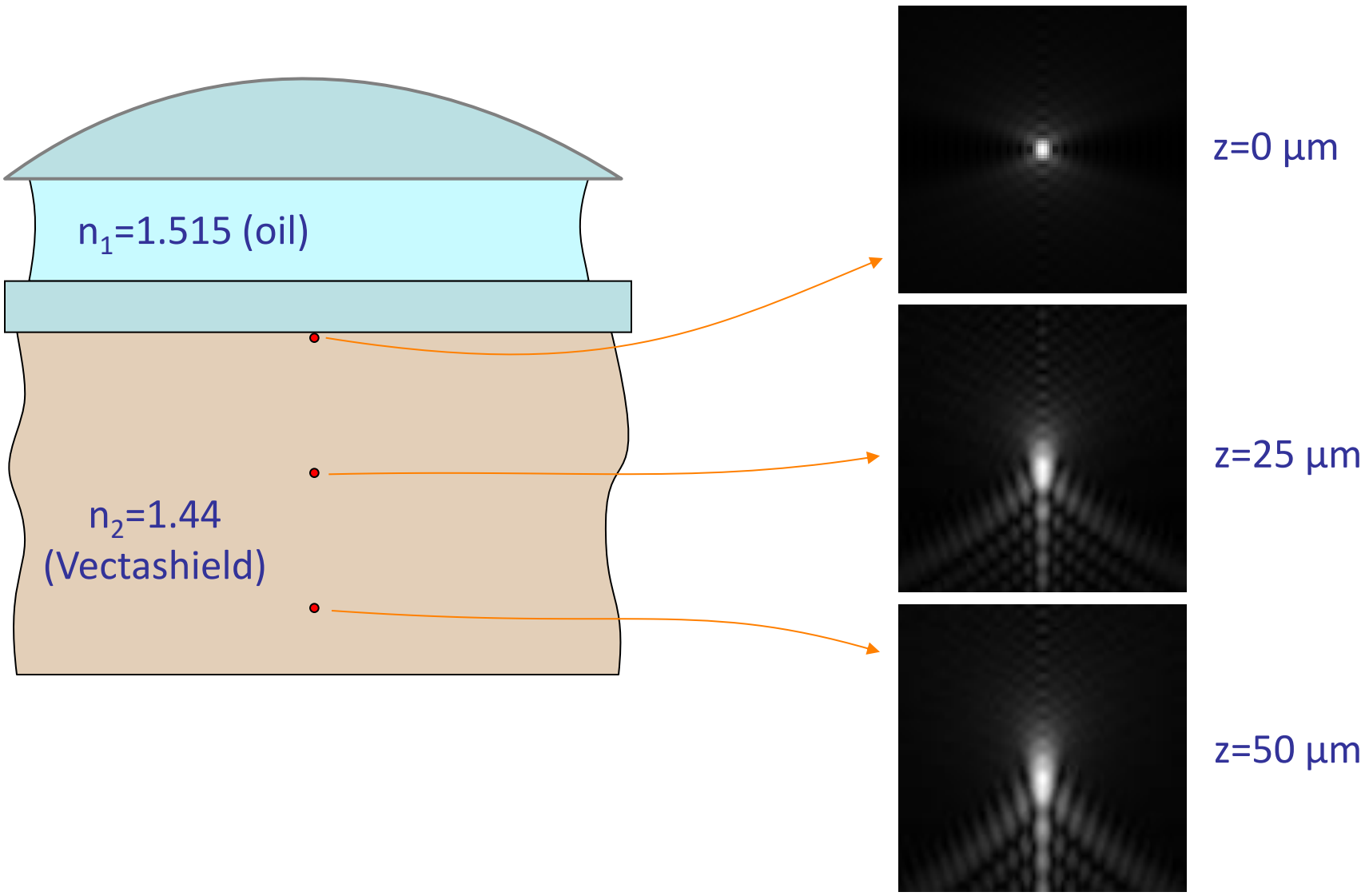


What can you do about spherical aberration?

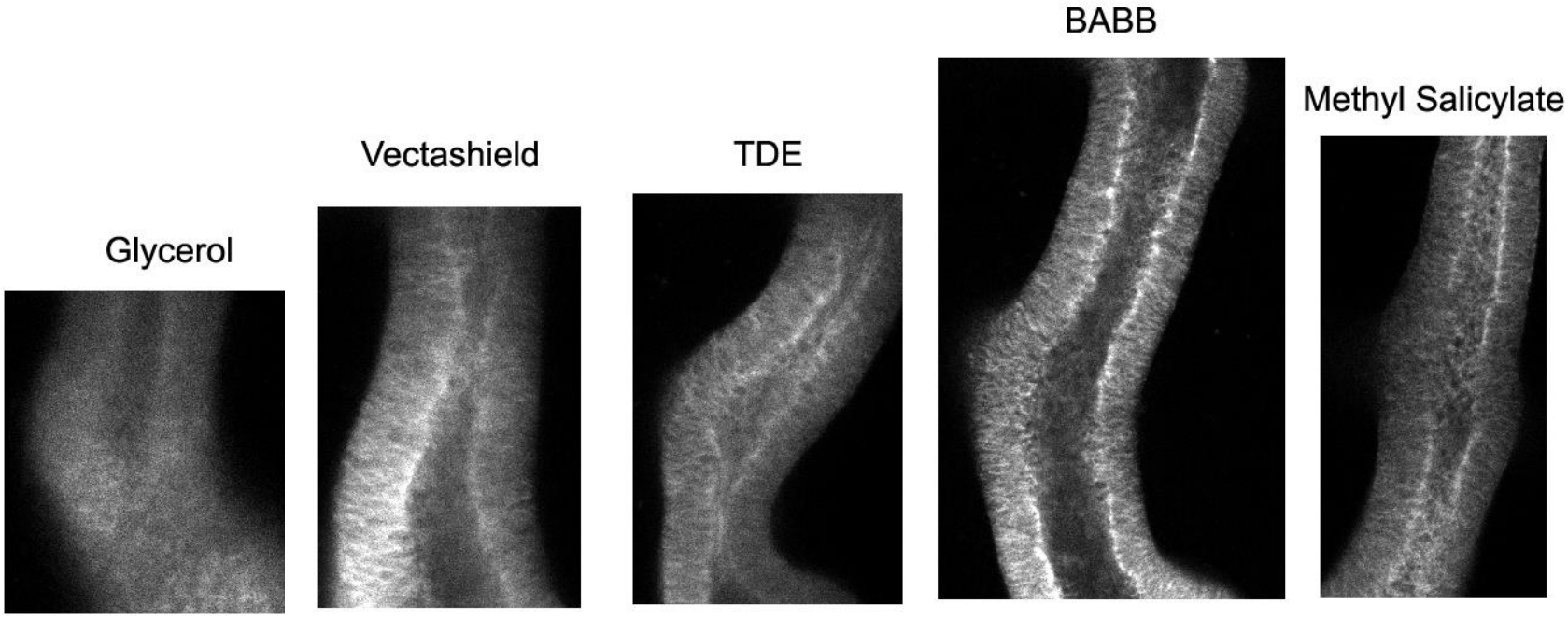
- Use 0.17 mm coverslips (~ #1.5)
- Work close to the coverslip
- Match lenses to the refractive index of your samples, and vice versa
  - For aqueous samples, use water immersion / water dipping lenses
  - For fixed samples and oil immersion lenses, mount your sample in a medium with n = 1.515
- Adjust objective correction collar when available
- Use lower NA lenses

Clearing

- Clearing media dissolve lipids to make samples more transparent
- Can be important for thick samples and tissues

- Commonly used:
  - BABB = 1:2 Benzyl Alchohol : Benzyl Benzoate
  - Methyl Salicylate

Sample Preparation

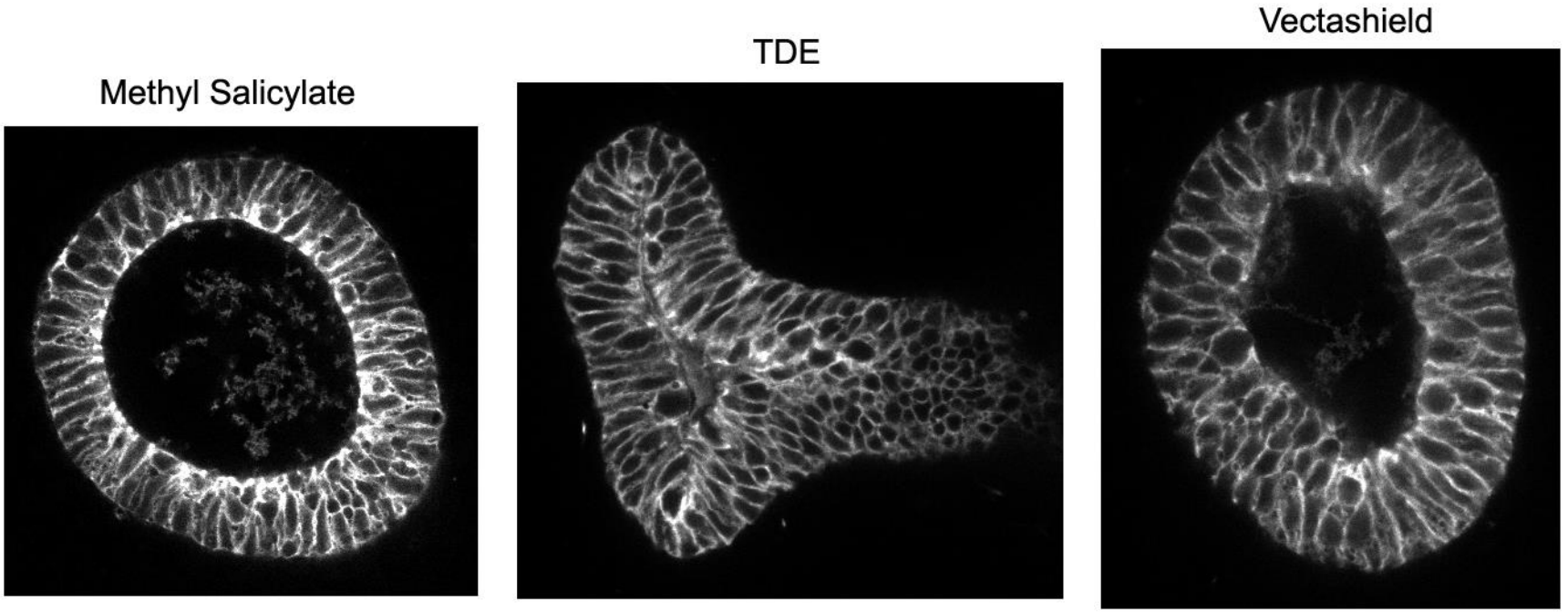


Samples imaged with 20x / 0.75 air objective on spectral confocal

Sections acquired ~ 50 μm into tissue

Embryonic mouse lungs; samples from Nan Tang, Martin Lab

Sample Preparation



Samples imaged with 40x / 1.3 oil objective on spectral confocal

Sections acquired ~ 50 μm into tissue

Embryonic mouse lungs; samples from Nan Tang, Martin Lab

Dye choices – Fixed samples

- Common filter set is DAPI / FITC / Rhodamine / Cy5
- Dye choices:
  - DAPI / Hoechst / Alexa 350 / Alexa 405
  - Alexa 488
  - Rhodamine / Alexa 546 / Alexa 568
  - Cy5 / Alexa 647 / Atto 647
- More than four colors probably requires special filters or spectral imaging.

Dye choices – Live samples

- Common filter sets: GFP / mCherry, CFP / YFP, CFP / YFP / RFP

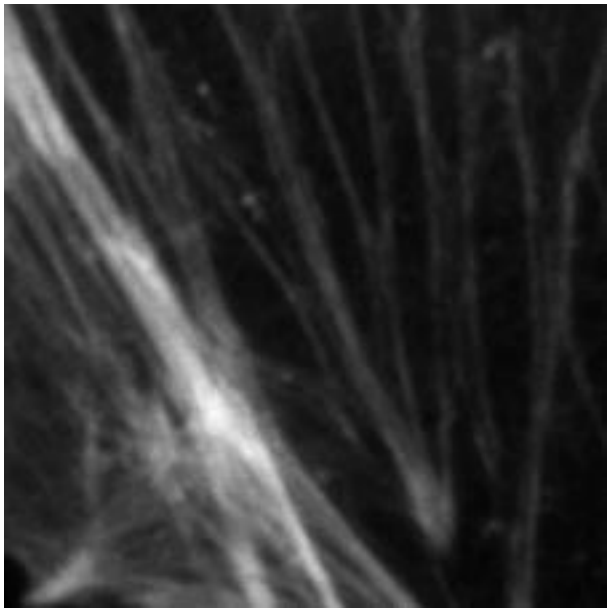
- Two-color choice: GFP / mCherry
- Three-color: CFP / GFP / mCherry or CFP / YFP / mCherry or BFP / GFP / mCherry
- Four-color: BFP / CFP / YFP / mCherry or Sapphire / CFP / YFP / mCherry
- Five-plus colors: possible but tricky, probably requires custom filters or spectral imaging.

Time and noise - tradeoffs

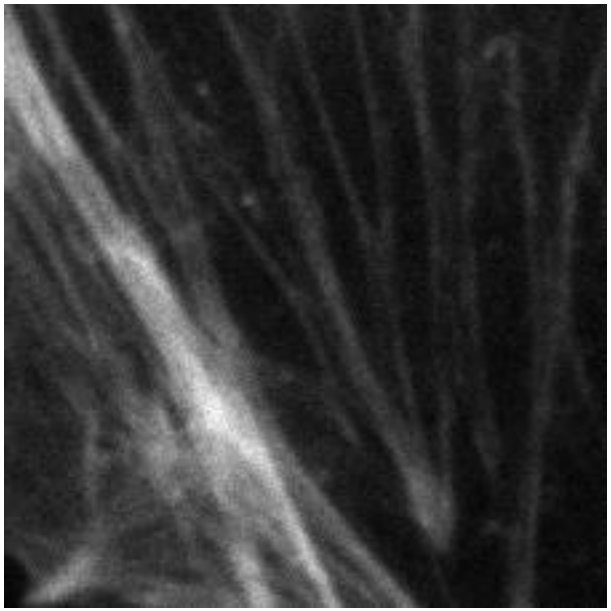
- The number of photons collected by the camera generally determines the amount of noise in your image
- Noise = square root (# of photons)
- Doubling signal to noise ratio requires 4-fold increase in exposure

What does this look like?

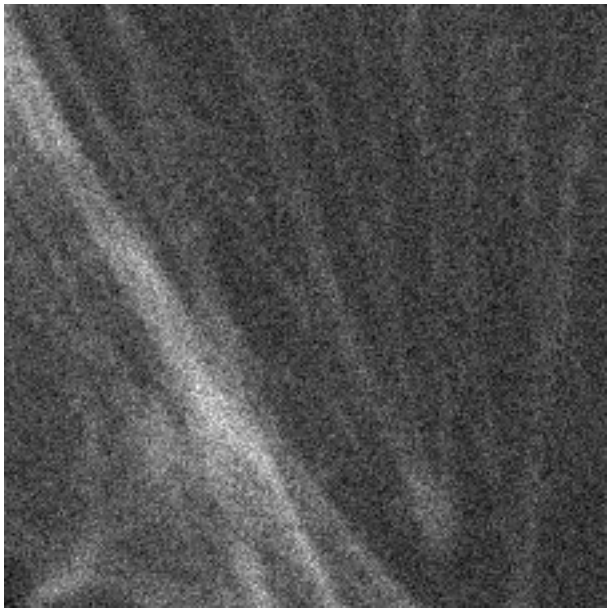
With 5 e^- camera read noise



1000 photons / pixel



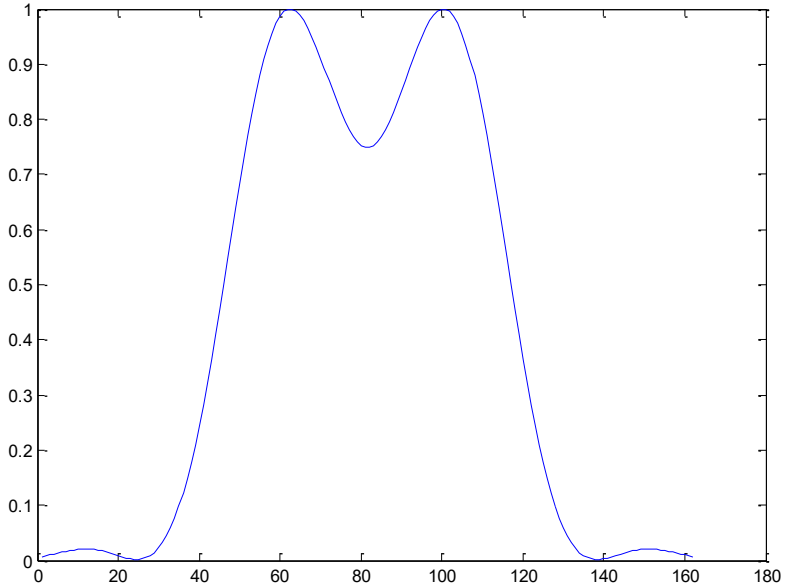
100 photons / pixel



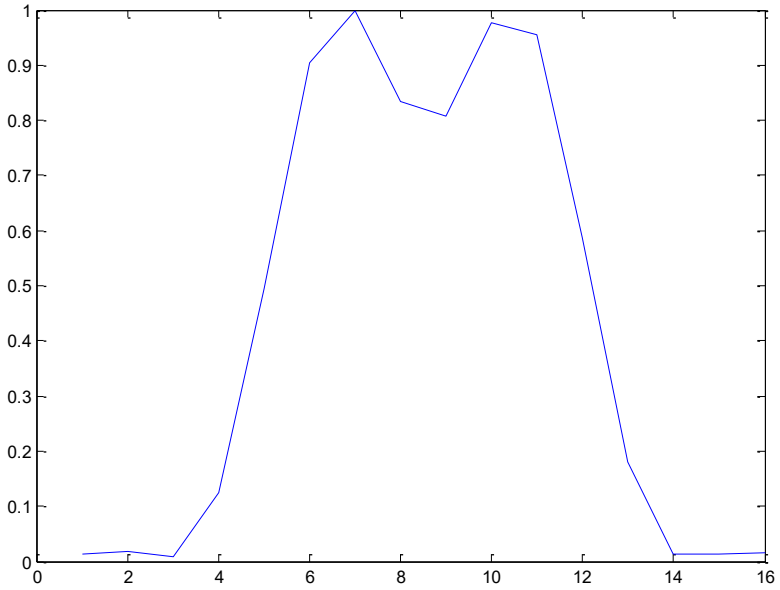
10 photons / pixel

Noise and resolution

Theoretical perfect data



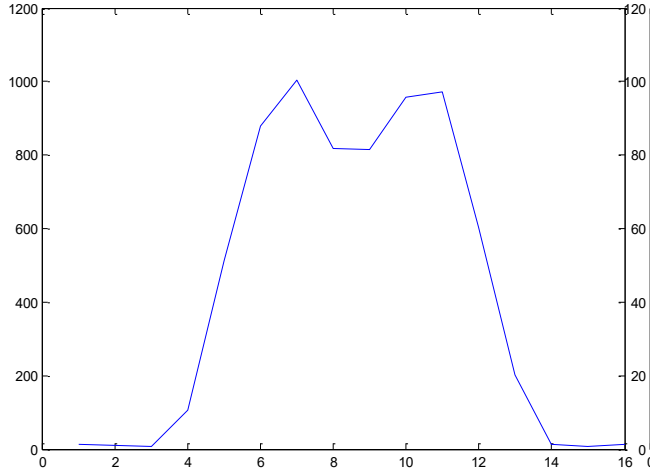
Two spots separated by diffraction limit



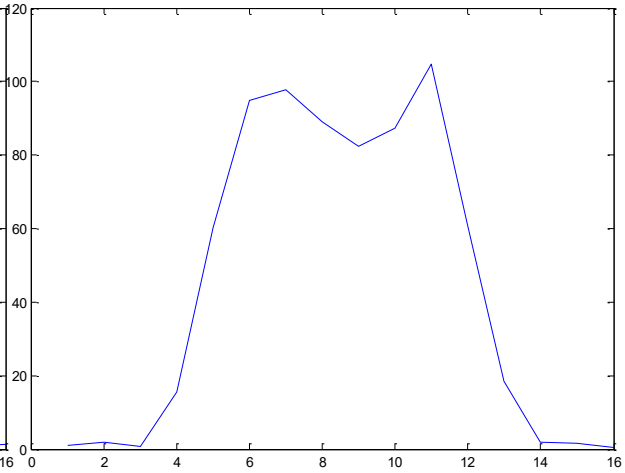
Slightly oversampled

Noise and resolution

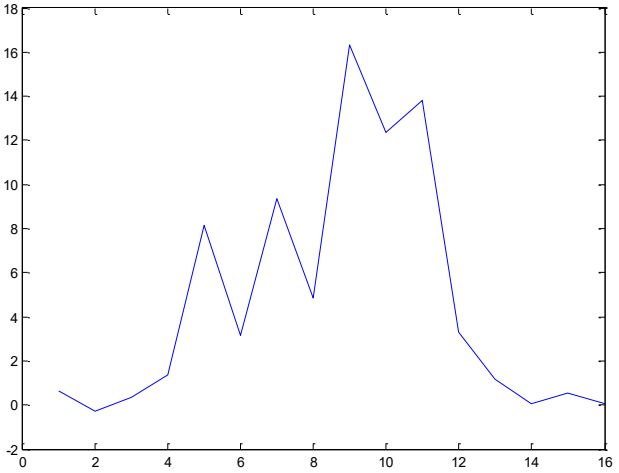
With shot noise



1000 ph/pixel at peak



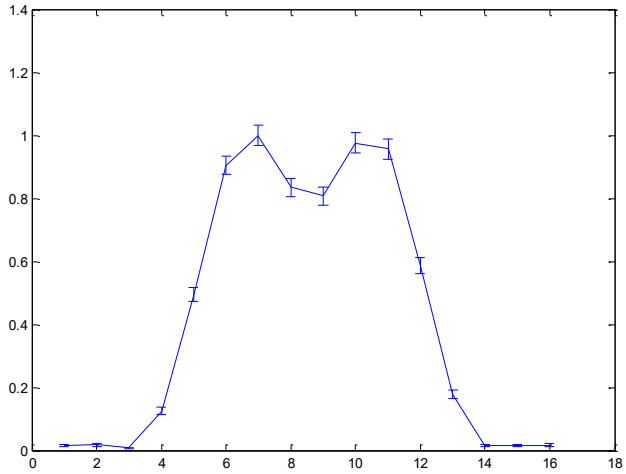
100 ph/pixel at peak



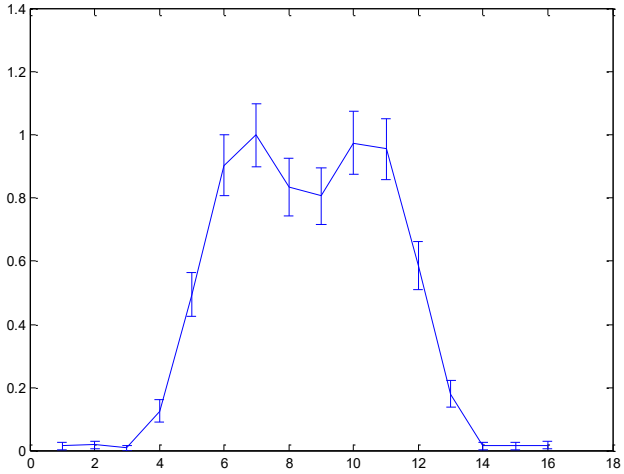
10 ph/pixel at peak

Noise and resolution

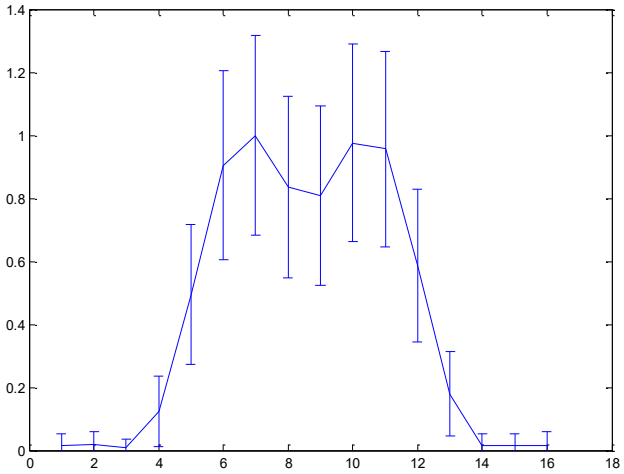
Expected error bars with shot noise



1000 ph/pixel at peak



100 ph/pixel at peak



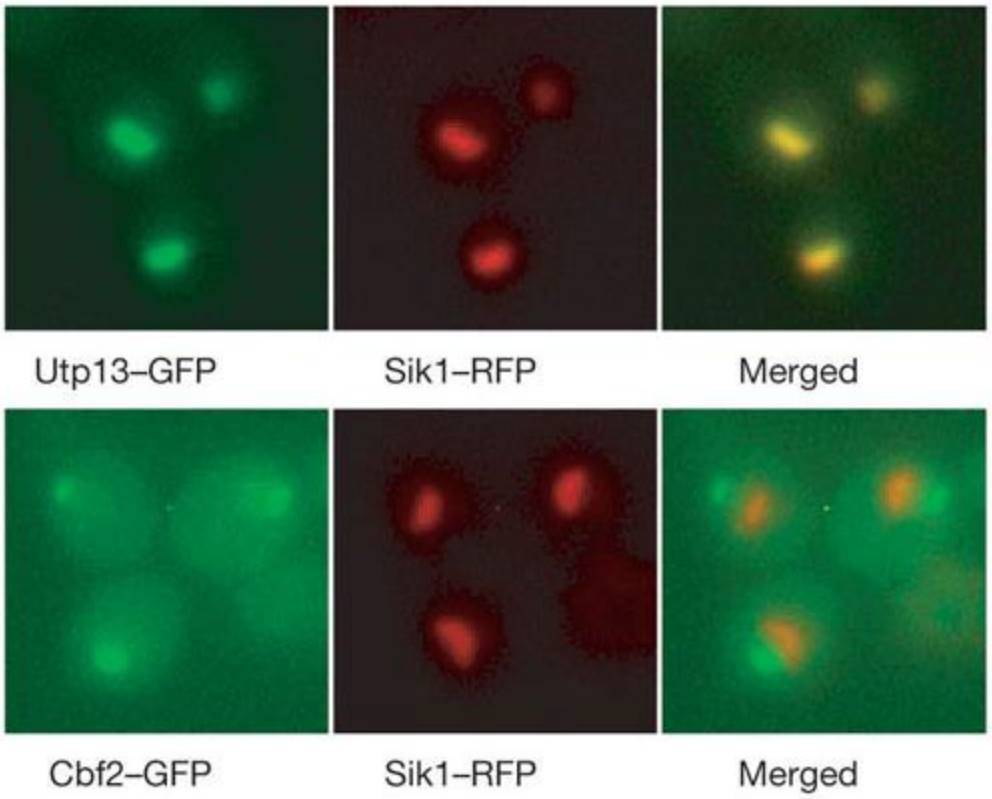
10 ph/pixel at peak

Noise and resolution

- High resolution and precise quantitation both require lots of light
- This means bright samples or long exposures
- This may cause problems with photobleaching and phototoxicity

- Be aware of potential tradeoffs between precision, speed, and photobleaching

Colocalization



Measures co-occurrence within the resolution limit of the microscope.

Does not say anything about molecular interaction

Huh et al. 2003

Nothing beats good data

- Think about what data you need before you take it.
- Do you need
  - Time resolution?
  - Spatial resolution?
  - Intensity resolution?
  - Day-to-day reproducibility?
  - Spatial uniformity?

- You can fix a lot of problems with post-processing, but it's better to fix problems in the data collection!

If you care about it, you should measure it!

- Spatial uniformity
  - Illumination and detection is not uniform over the field of view of the microscope.
  - Can be measured and corrected with a shading image.
  - Photobleaching may make this hard
- Temporal uniformity
  - Lamp power and alignment fluctuates from day to day
  - Can measure
  - But best to do experiments same day / same session

Think about data storage

- Databases are good, but cumbersome
- Save in manufacturer's native format so metadata is preserved
- If not using a database, systematic file names and notes on sample identity are a good idea

References

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