Designing a Microscopy Experiment

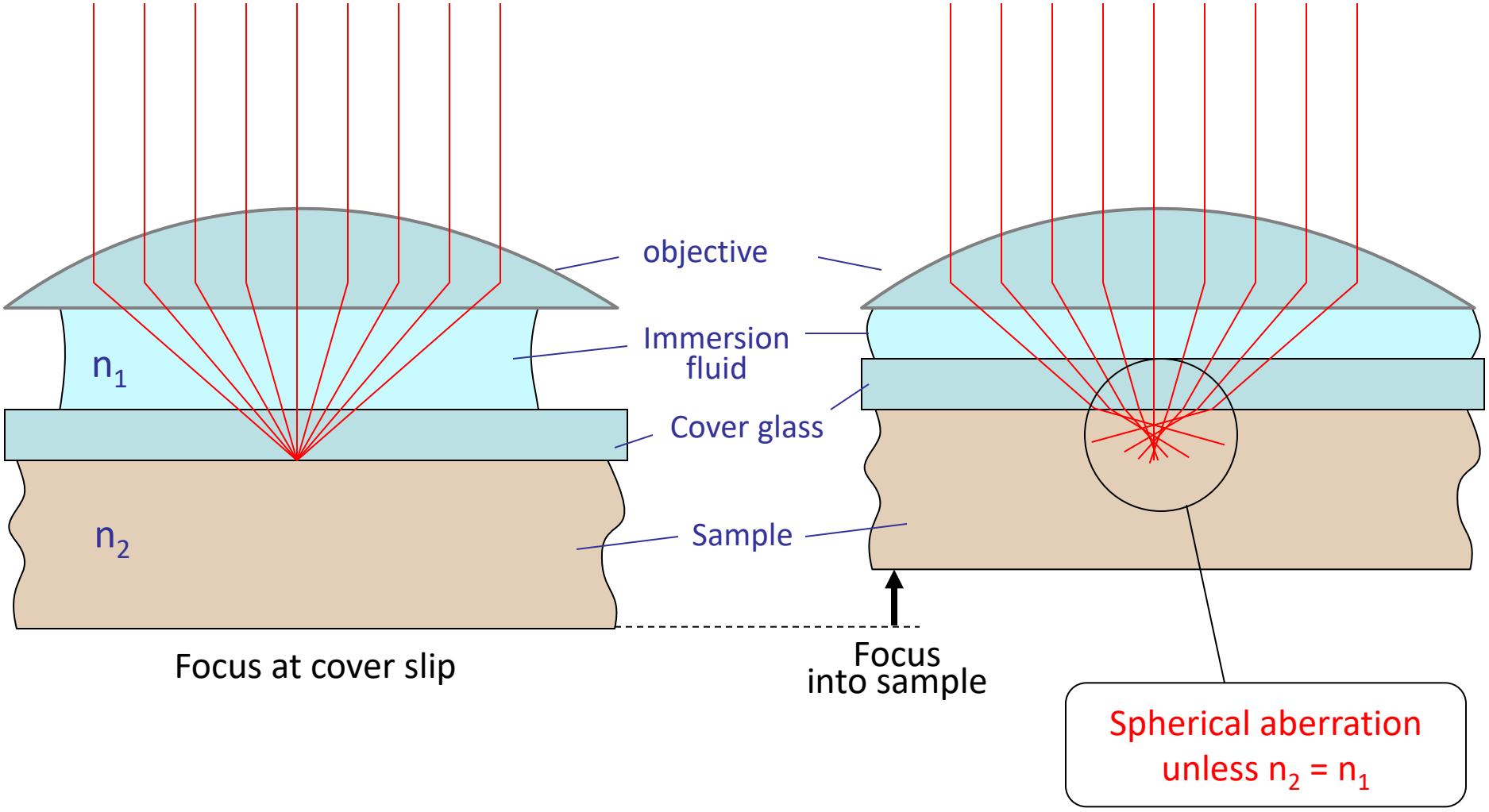
Kurt Thorn, PhD
Director, NIC@UCSF

Image from Susanne Rafelski, Marshall lab

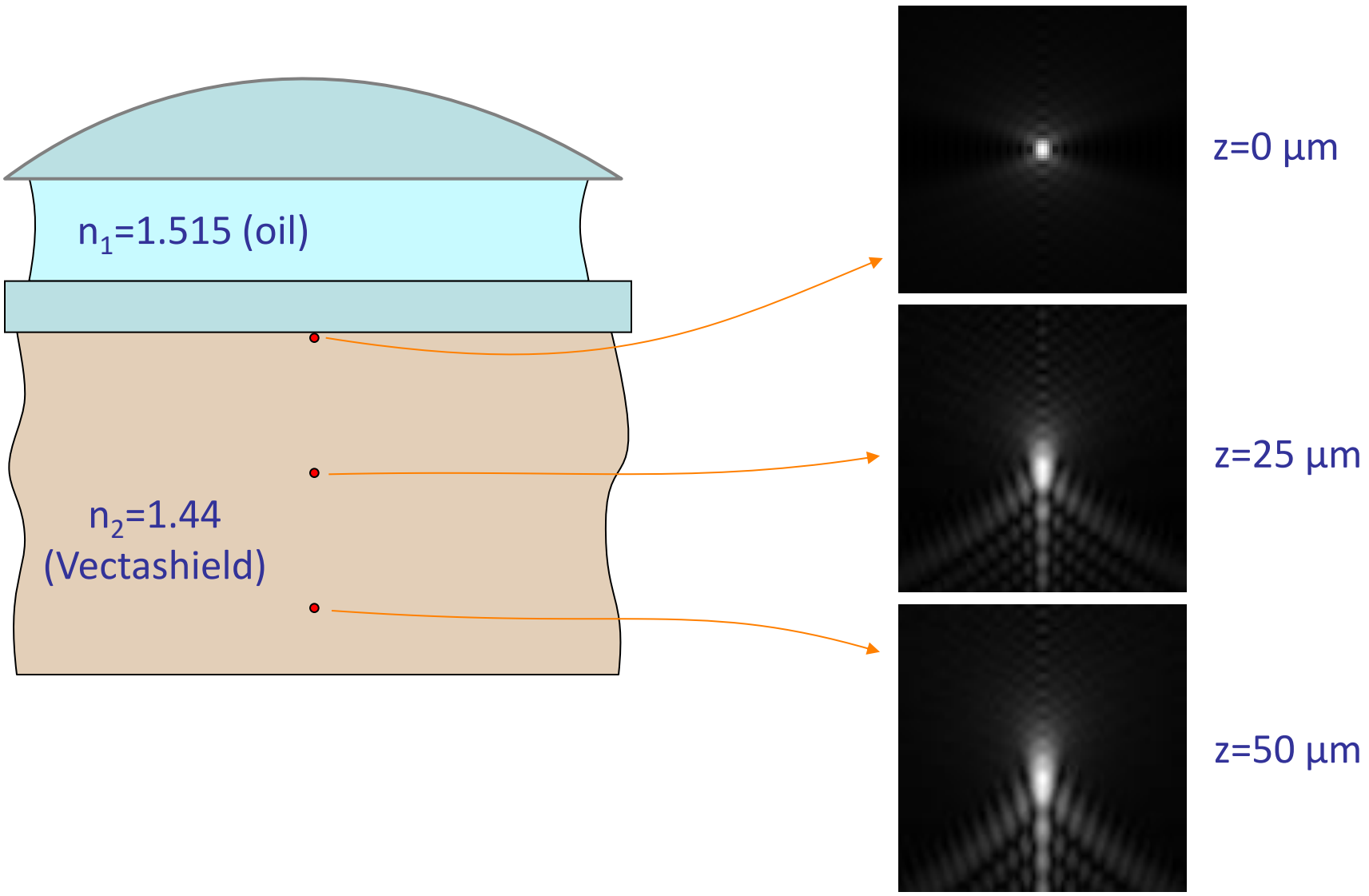
Sample preparation and mounting

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

Index Mismatch
& Spherical Aberration



Index Mismatch
& Spherical Aberration



What can you do about spherical aberration?

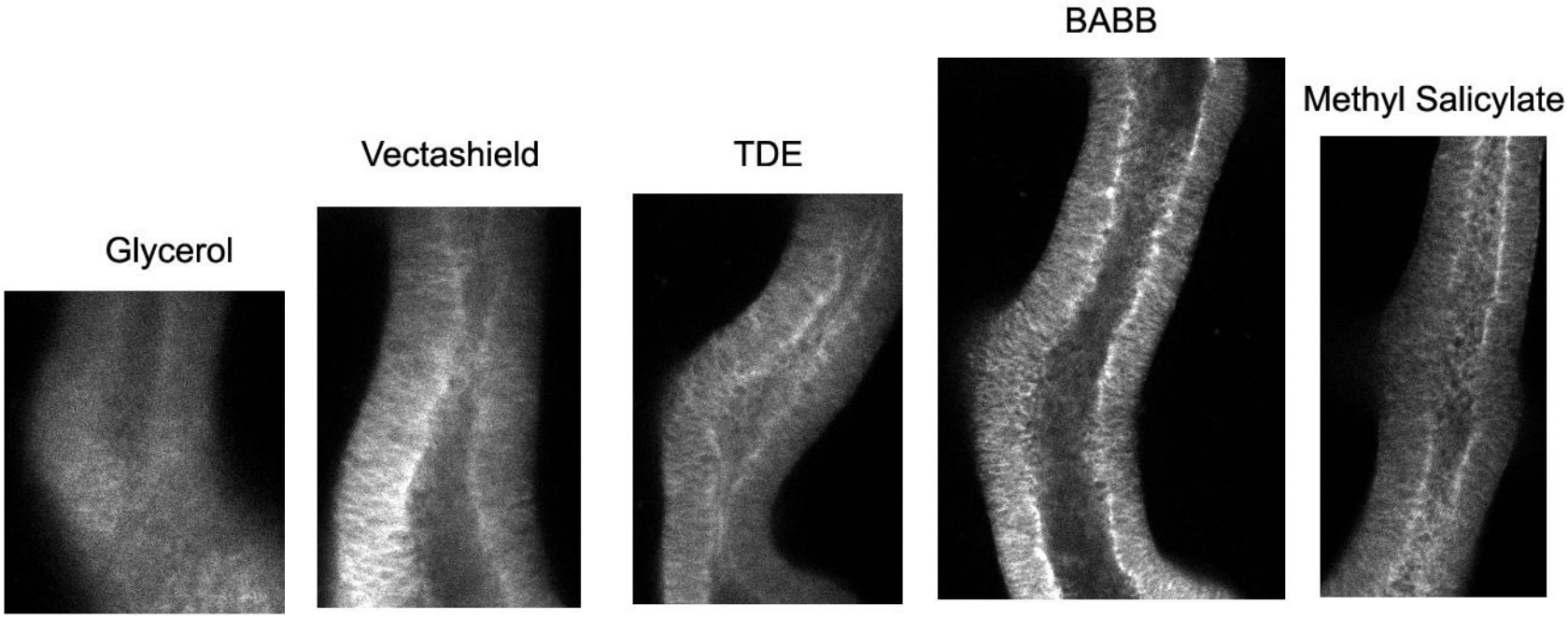
- Use 0.17 mm coverslips (~ #1.5)
- Work close to the coverslip
- Match lenses to the refractive index of your samples, and vice versa
  - For aqueous samples, use water immersion / water dipping lenses
  - For fixed samples and oil immersion lenses, mount your sample in a medium with n = 1.515
- Adjust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

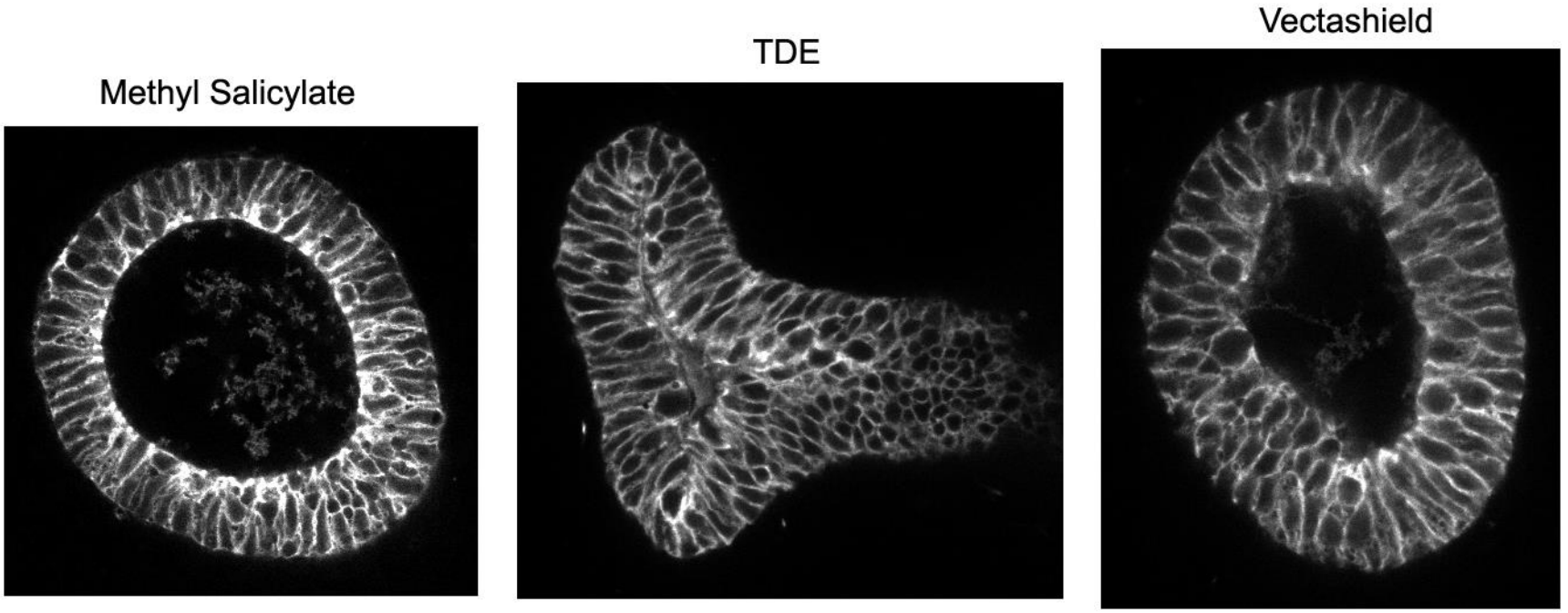


Samples imaged with 20x / 0.75 air objective on spectral confocal

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.

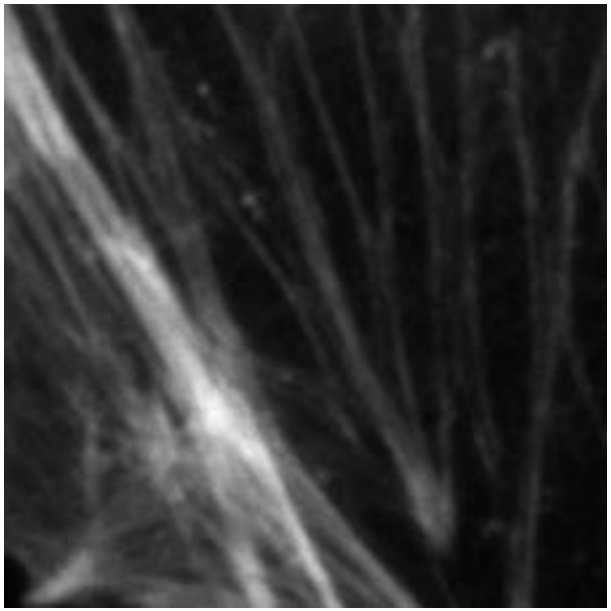
- Consider new RFP variants: mRuby, mApple

Time and noise - tradeoffs

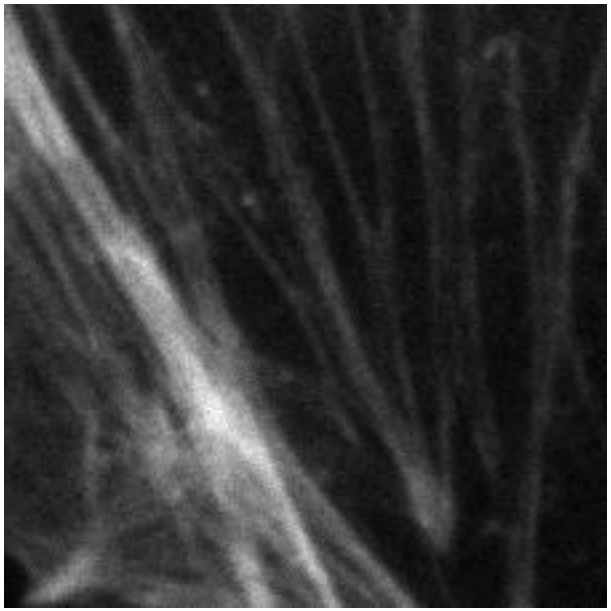
- The number of photons collected by the camera generally determines the amount of noise in your image
- Noise = square root (# of photons)
- Doubling signal to noise ratio requires 4-fold increase in exposure

What does this look like?

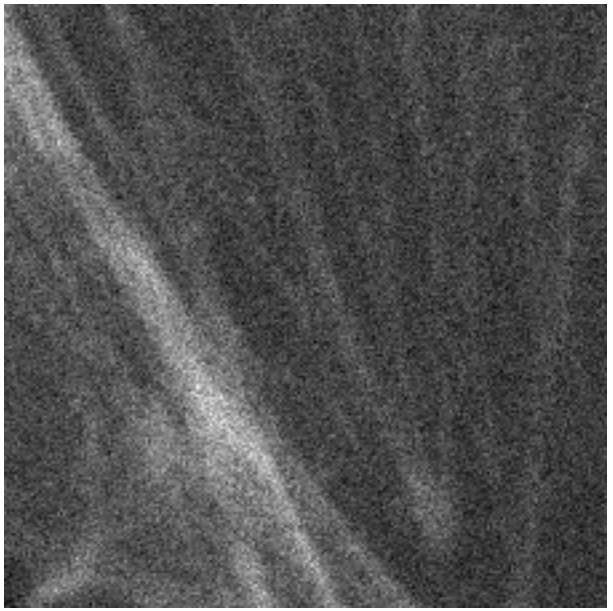
With 5 e^- camera read noise



1000 photons / pixel



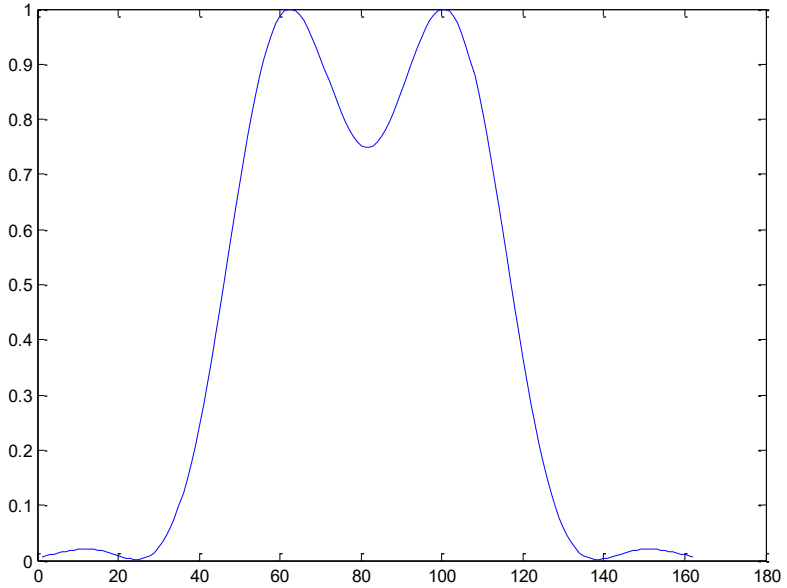
100 photons / pixel



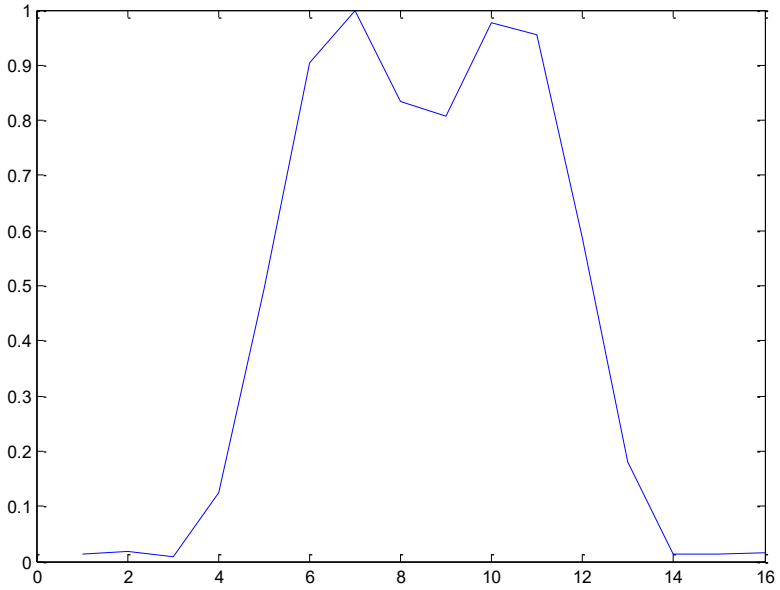
10 photons / pixel

Noise and resolution

Theoretical perfect data



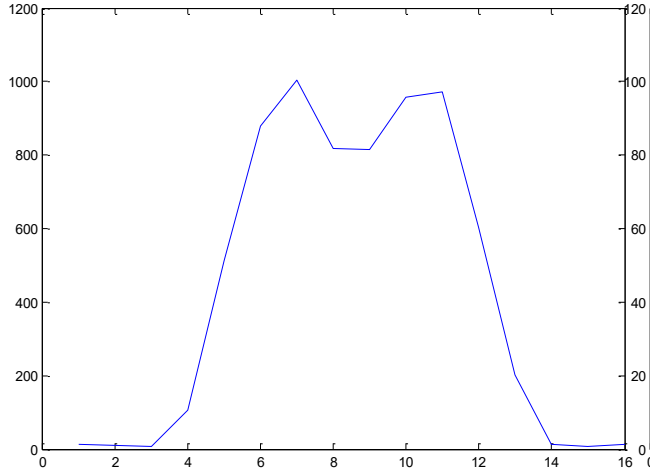
Two spots separated by diffraction limit



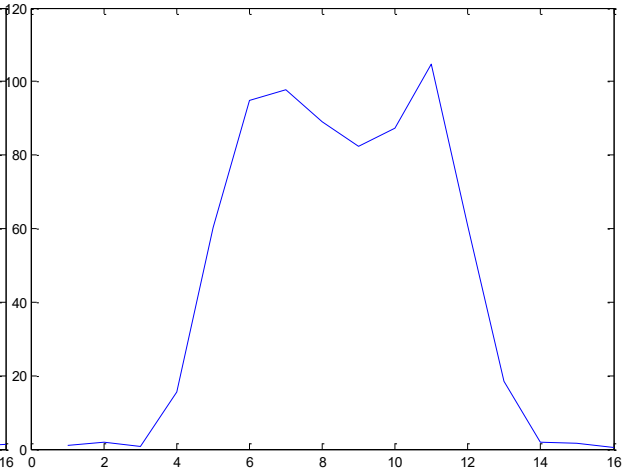
Slightly oversampled

Noise and resolution

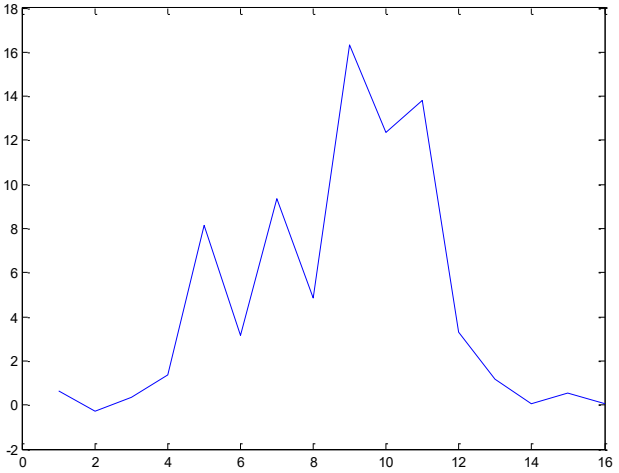
With shot noise



1000 ph/pixel at peak



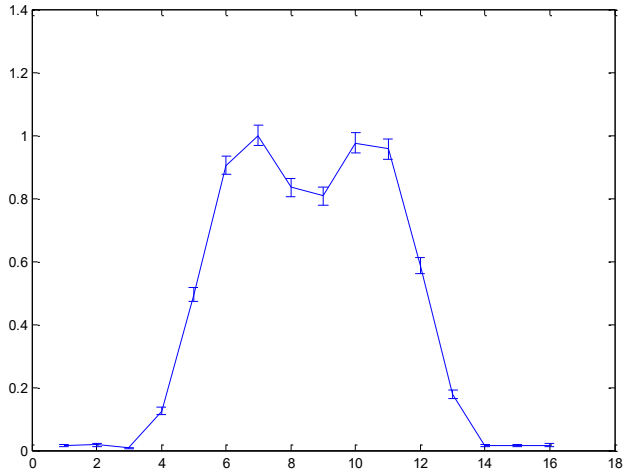
100 ph/pixel at peak



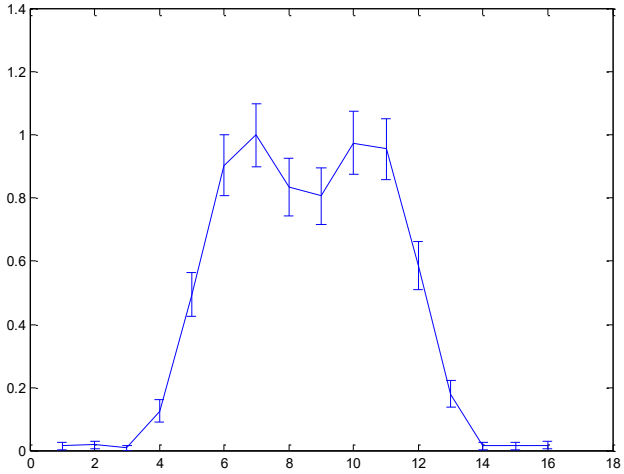
10 ph/pixel at peak

Noise and resolution

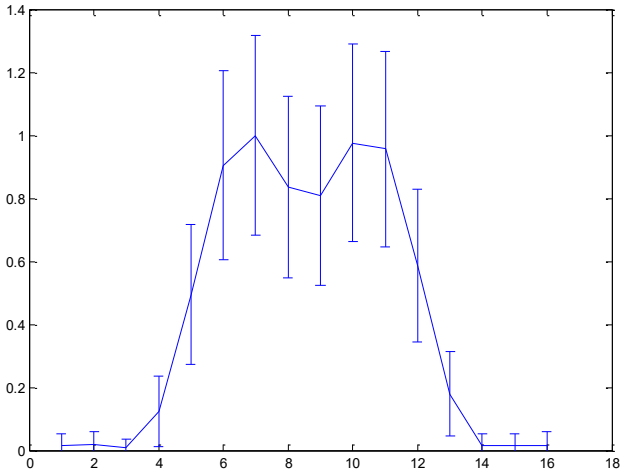
Expected error bars with shot noise



1000 ph/pixel at peak



100 ph/pixel at peak



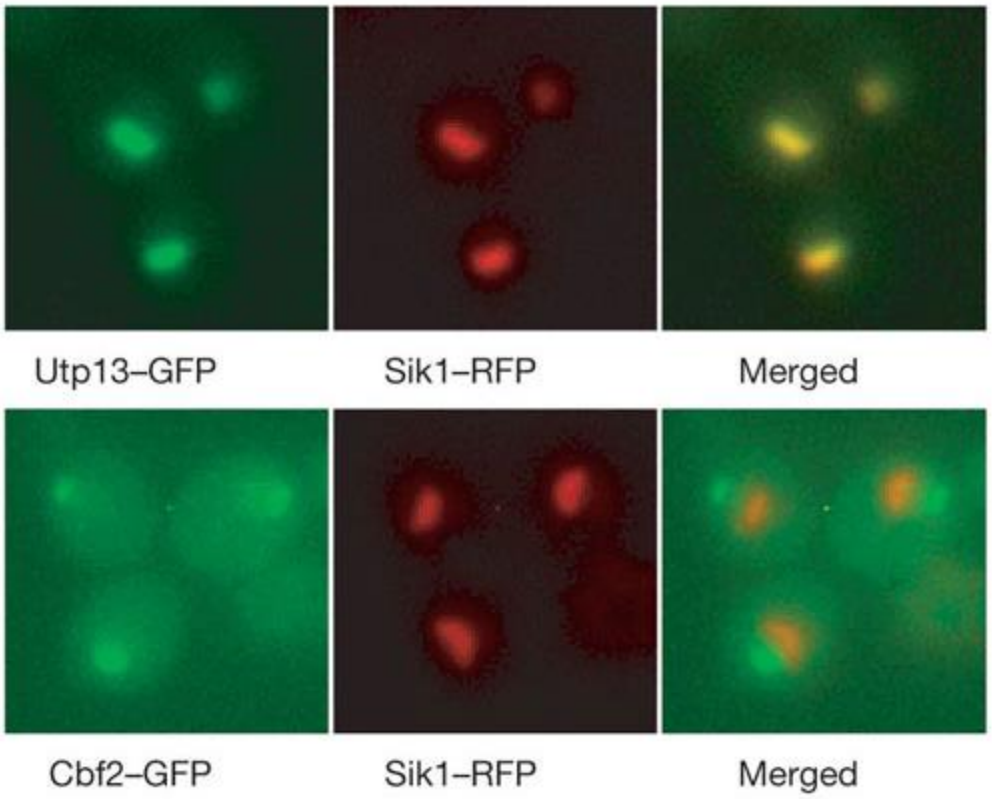
10 ph/pixel at peak

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!

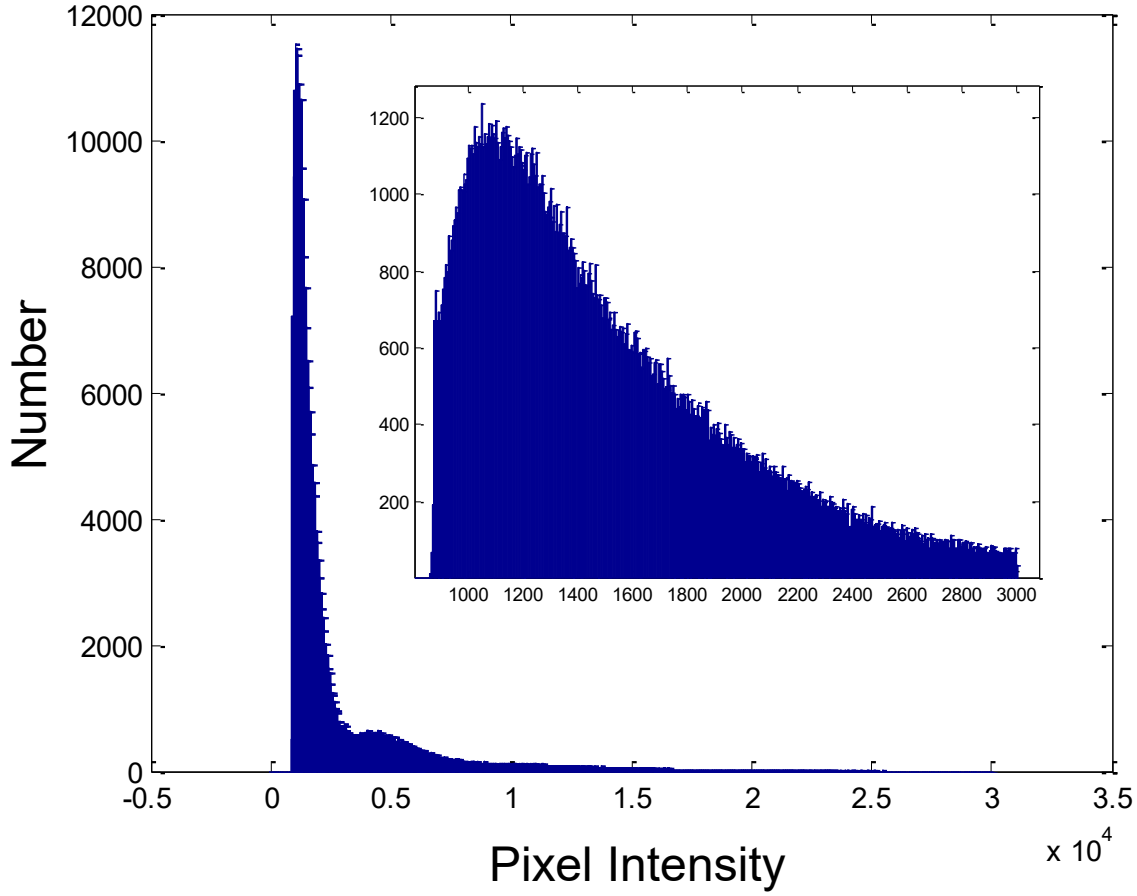
If you care about it, you should measure it!

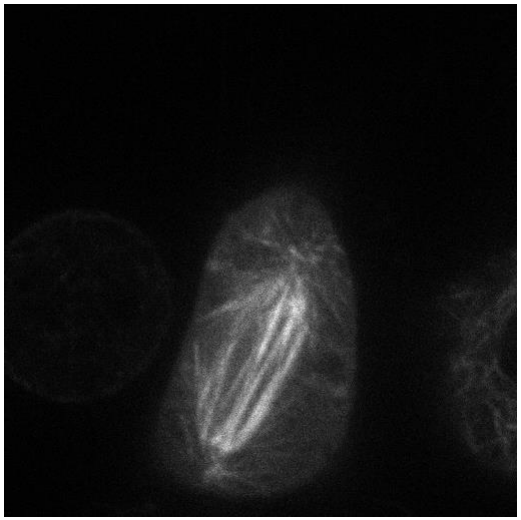
- Spatial uniformity
  - Illumination and detection is not uniform over the field of view of the microscope.
  - Can be measured and corrected with a shading image.
  - Photobleaching may make this hard
- Temporal uniformity
  - Lamp power and alignment fluctuates from day to day
  - Can measure
  -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

Background correction

- Cameras have a non-zero offset
- There can also be background fluorescence due to media autofluorescence, etc.
- Want to correct for this by background subtraction
  - Camera dark image
  - Estimate background from image

Estimating background from image

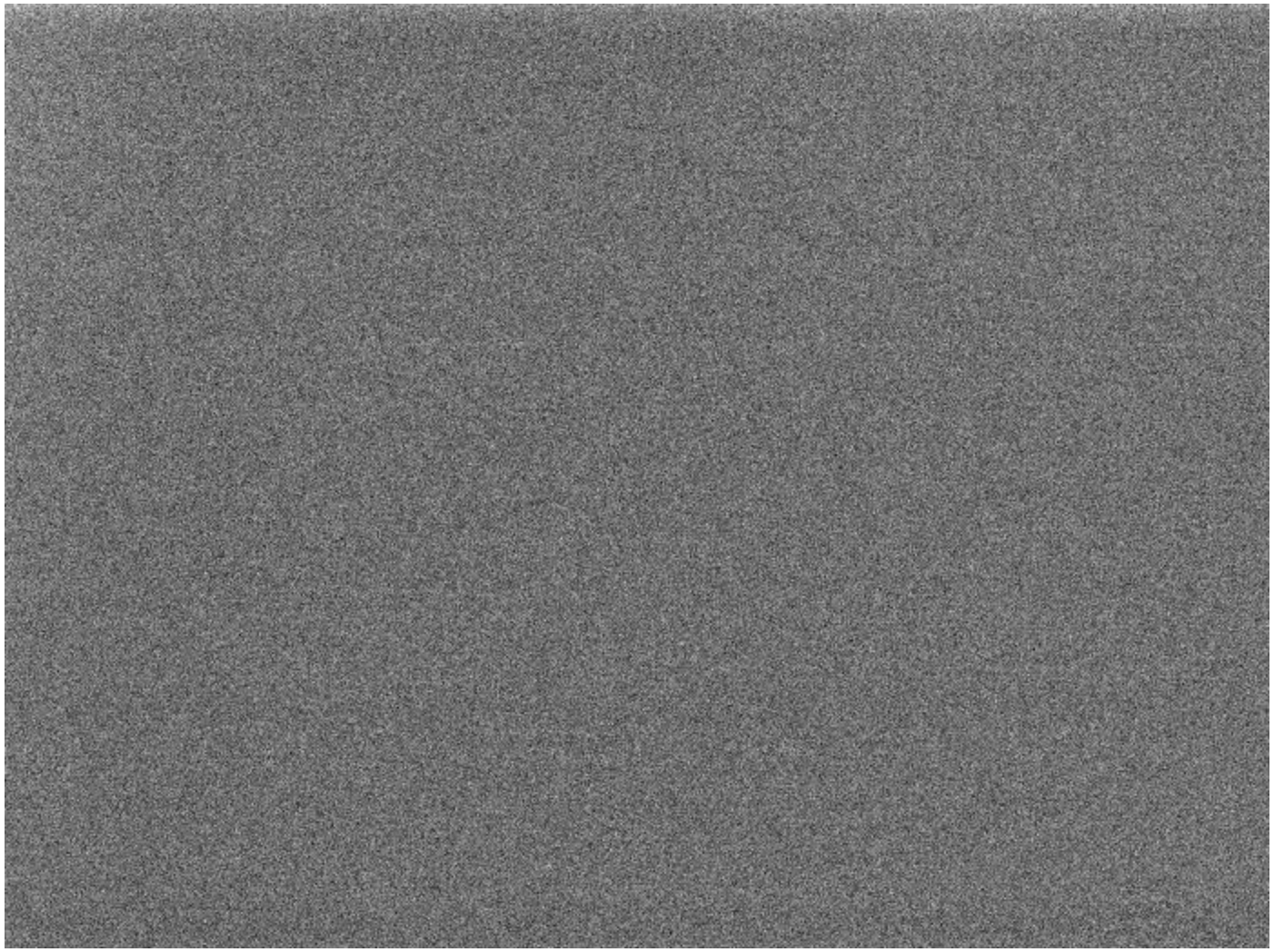




Dark image

- Acquired with no light going to the camera
  - Allows you to measure instrument background
  - Can detect what's real background autofluorescence

Dark image



Shading correction

- Measure and correct for nonuniformity in illumination and detection
- Image a uniform fluorescent sample

Shading correction

Correction procedure

$I_{meas} = I_{true} * Shading + Dark$

$I_{true} = (I_{meas} – Dark) / Shading$

Good to do on all images

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

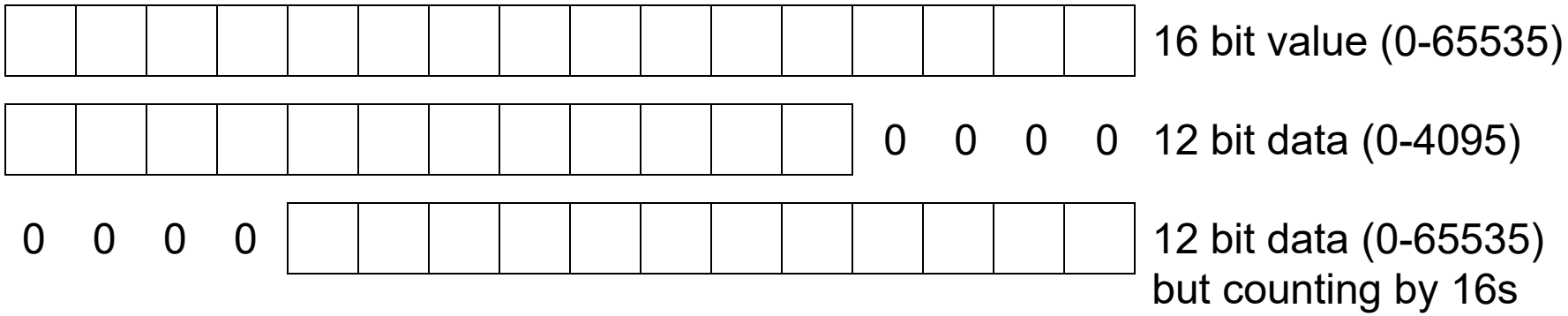
File Formats and Bitdepth

- Digital cameras have a specified bitdepth = number of gray levels they can record

- 8-bit $\rightarrow 2^8 = 256$ gray levels
- 10-bit $\rightarrow 2^{10} = 1024$ gray levels
- 12-bit $\rightarrow 2^{12} = 4096$ gray levels
- 14-bit $\rightarrow 2^{14} = 16384$ gray levels
- 16-bit $\rightarrow 2^{16} = 65536$ gray levels

Bitdepth and file formats

- Standard imaging formats, like tiff, are always 8 or 16 bit (because 8 bits = 1 byte)



Color Images

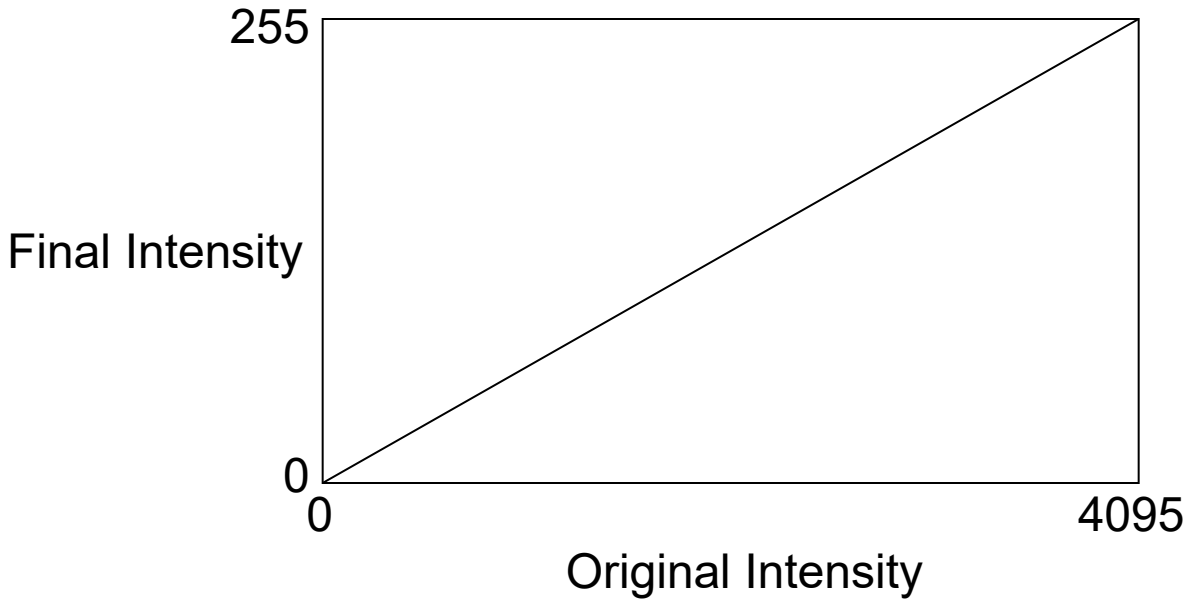
- Color images are made up of three gray scale images, one for each of red, green, and blue
- Can be 8 or 16 bits per channel

File Formats

- Most portable: TIFF
  - 8 or 16-bit, lossless, supports grayscale or RGB
- Most metadata: Manufacturer format (nd2, ids, etc.)
  - Lossless, supports full bitdepth
  - Custom formats often support multidimensional images
  - Not so portable
- OK: JPEG2000
  - Not so common
- Bad: JPEG, GIF, BMP, etc.
  - Lossy and / or 8-bit

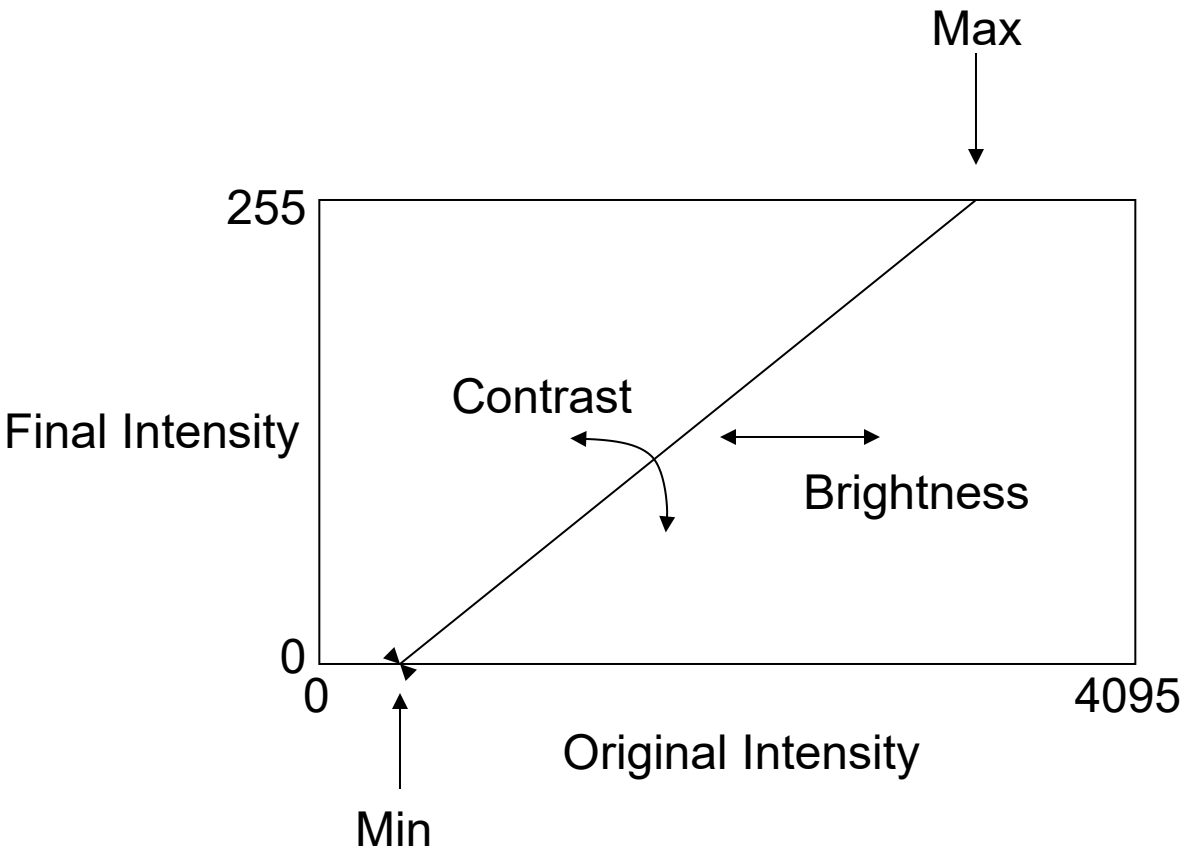
Intensity scaling

- Computer screens are 8-bit
- Publishers also want 8-bit files

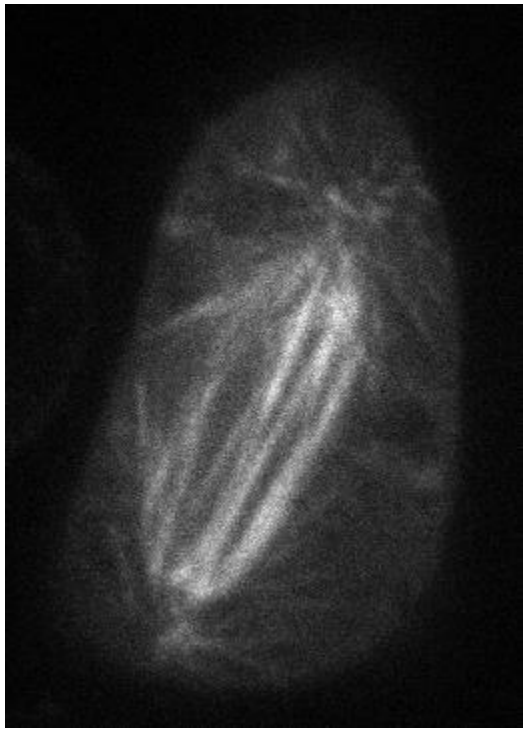


You lose information in this process – values 4080-4095 all end up as 255

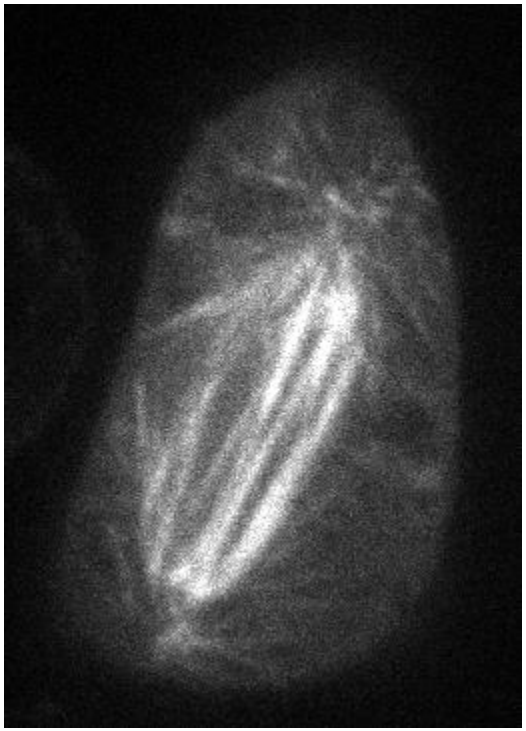
Intensity scaling



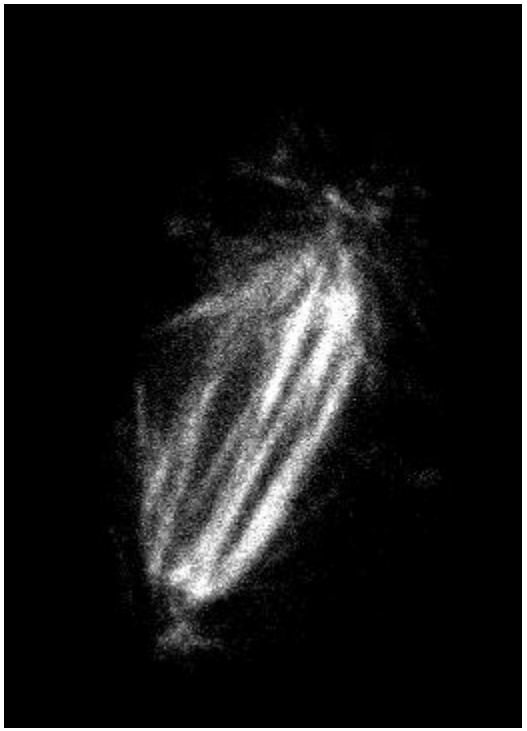
Effect of scaling



Scaled to min / max
(874 / 25438)



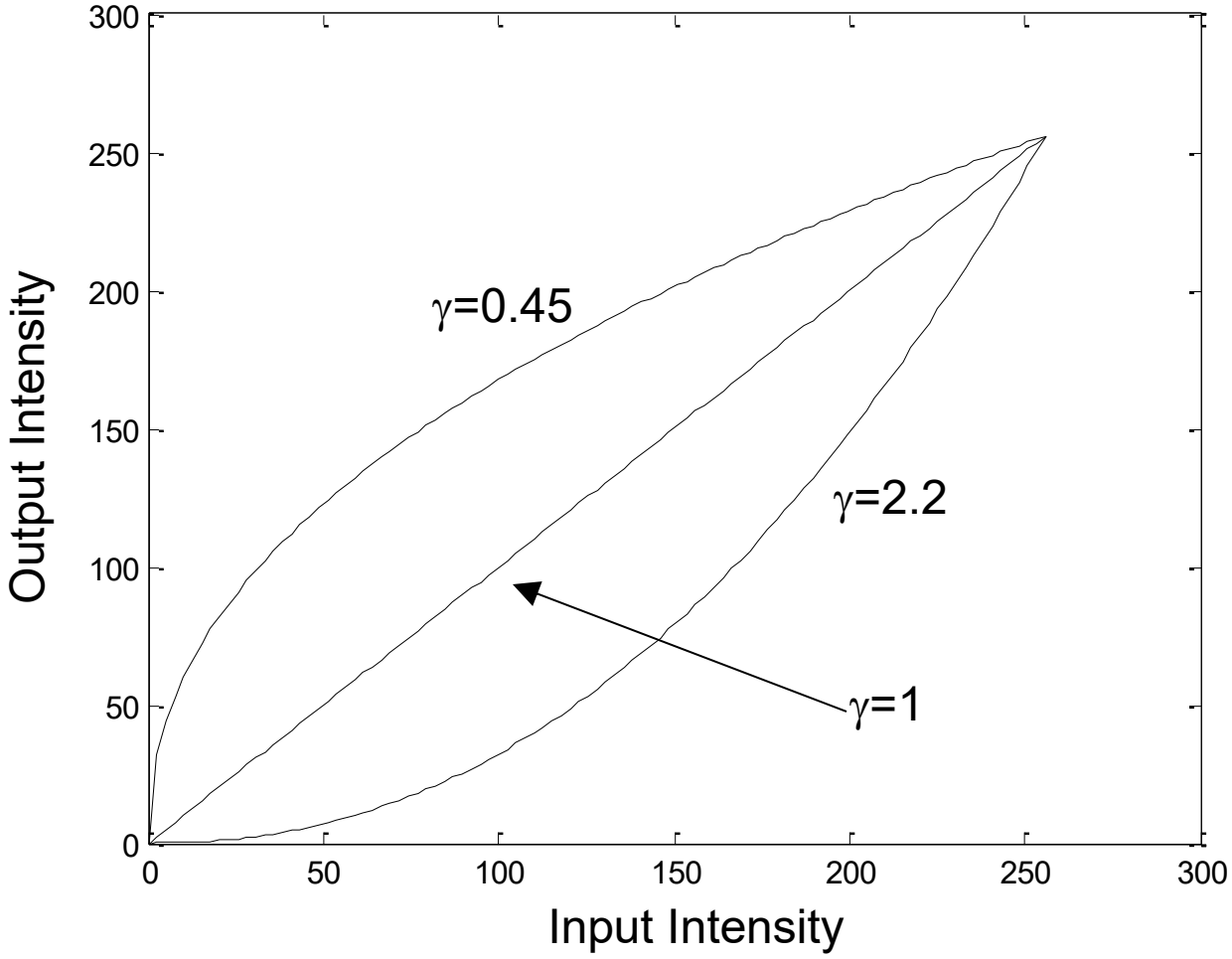
(874 / 19200)



(6400 / 18432)

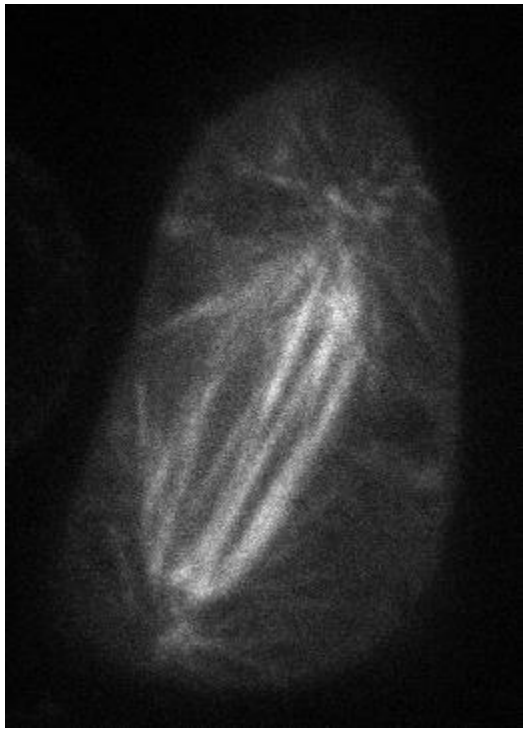
Drosophila S2 cell with mCherry-tubulin (Nico Stuurman)

Gamma correction

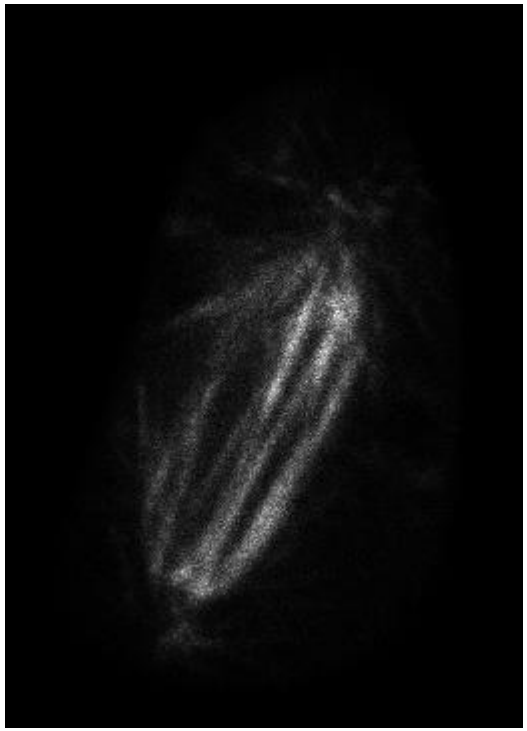


Other contrast stretching transforms….

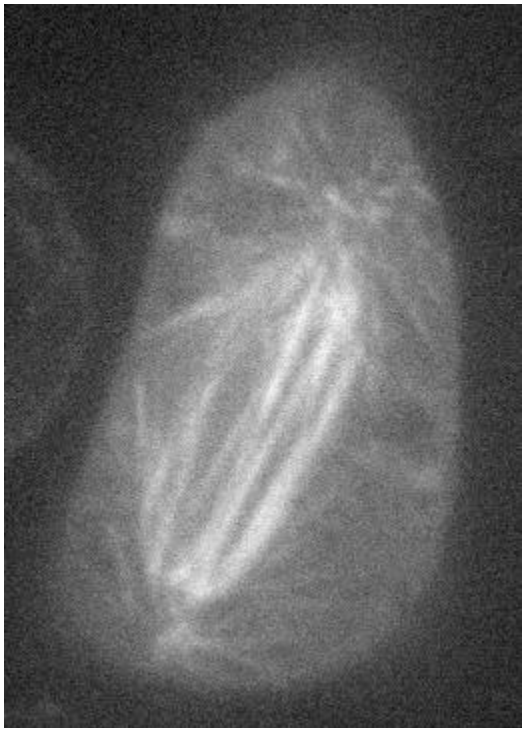
Effect of gamma



Scaled to min / max
(874 / 25438), $\gamma = 1$



Scaled to min / max
(874 / 25438), $\gamma = 2.16$



Scaled to min / max
(874 / 25438), $\gamma = 0.45$

What are acceptable image manipulations?

- JCB has the best guidelines
  - http://jcb.rupress.org/misc/ifora.shtml#image_aquisition
  - http://jcb.rupress.org/cgi/content/full/166/1/1
- Brightness and contrast adjustments ok, so long as done over whole image and don't obscure or eliminate background
- Nonlinear adjustments (like gamma) must be disclosed
- No cutting and pasting of regions within an image (e.g. individual cells)

References

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