Quantitative
Imaging
@ Berkeley

More Than Just a Pretty Picture: Acquiring Images for Analysis

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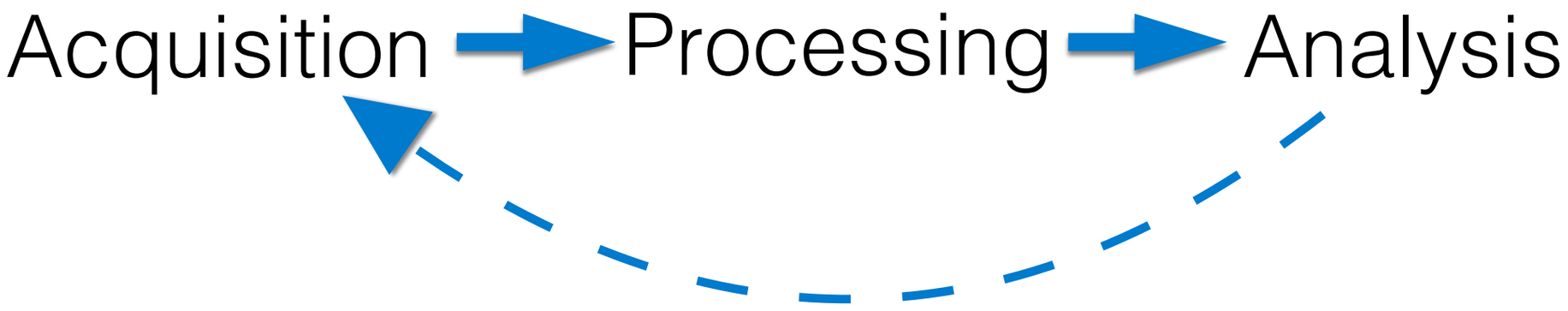
Goals

- Overview of best practices and controls for quantitative imaging
- Help with experimental design
- Lay a foundation for upcoming discussions on image processing & analysis

Quantitative Imaging Workflow



Ideal to go through this workflow before bulk acquisition because re-imaging is:

- Time-consuming
- Costly
- Sometimes impossible (precious and/or photobleached samples)

Outline

- Quick overview of detectors - which one is best for your experiment?
- Explain importance of the signal to noise ratio (SNR) and how to achieve a high SNR
- Survey of main types of quantitative imaging experiments - potential pitfalls, best practices

Know Your Detector!

- Analog to digital converters
- Considerations: speed, resolution, quantum efficiency, cost, noise, bit depth
- Array detectors (cameras) vs. Point detectors (PMTs)

Array Detectors (Cameras)

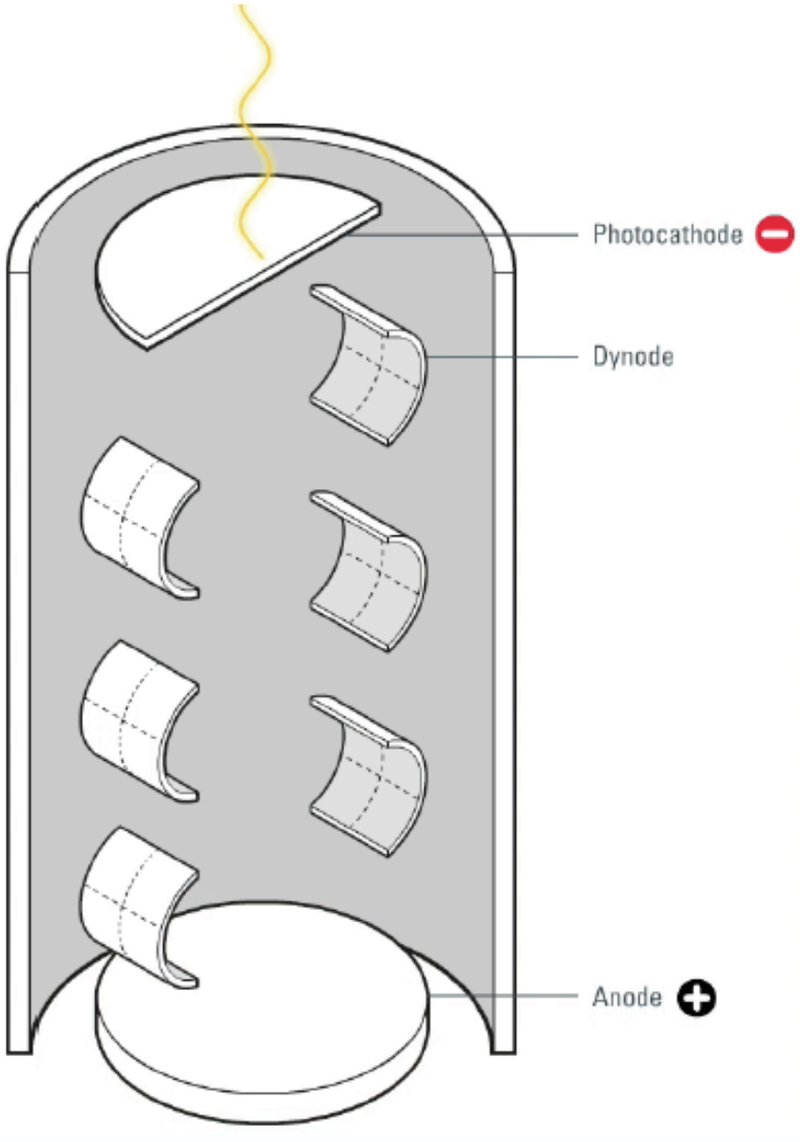
- **Interline CCD** (charge-coupled device): standard camera on most systems
- **EMCCD** (electron-multiplied CCD)
  - Pros: amplifies low signals, fast, higher QE
  - Cons: multiplication is noisy, large chip sizes, expensive
- **sCMOS** (scientific complementary metal-oxide semiconductor)
  - Pros: VERY fast (parallel amplification + transfer), low read noise, high frame rates, large FOV
  - Cons: each pixel has its own gain, offset, and read noise, potential rolling shutter artifact

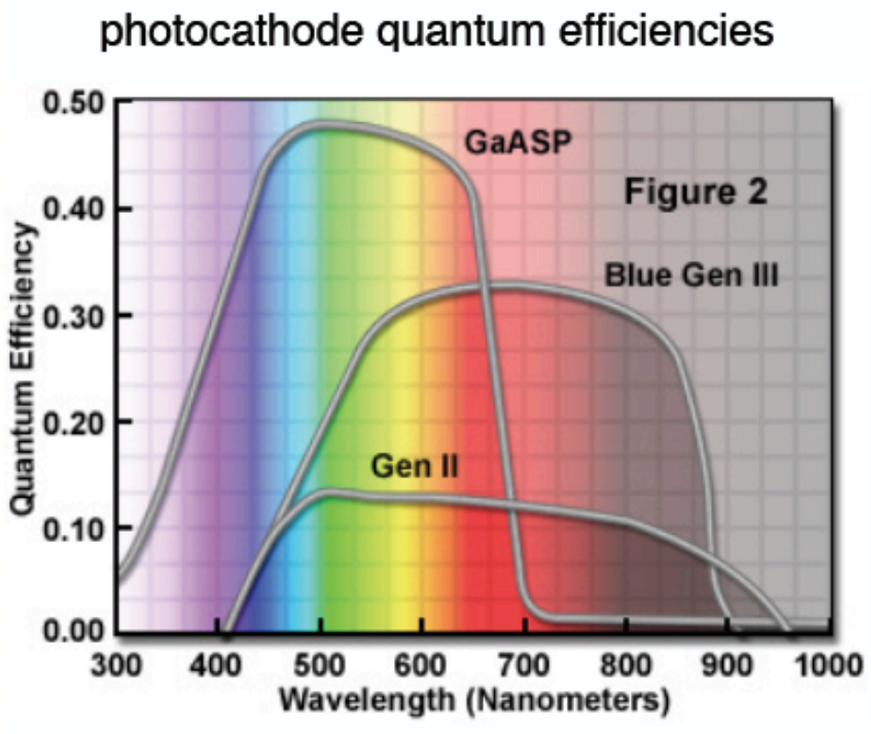
Rolling Shutter Effect

https://www.youtube.com/watch?v=17PSgsRlO9Q

Point Detectors (PMTs)

- Spatial resolution is determined by sampling rate (user defined)
- QE is much lower than most camera systems
- GaAsP = more sensitive
- Sources of noise: heat, gain



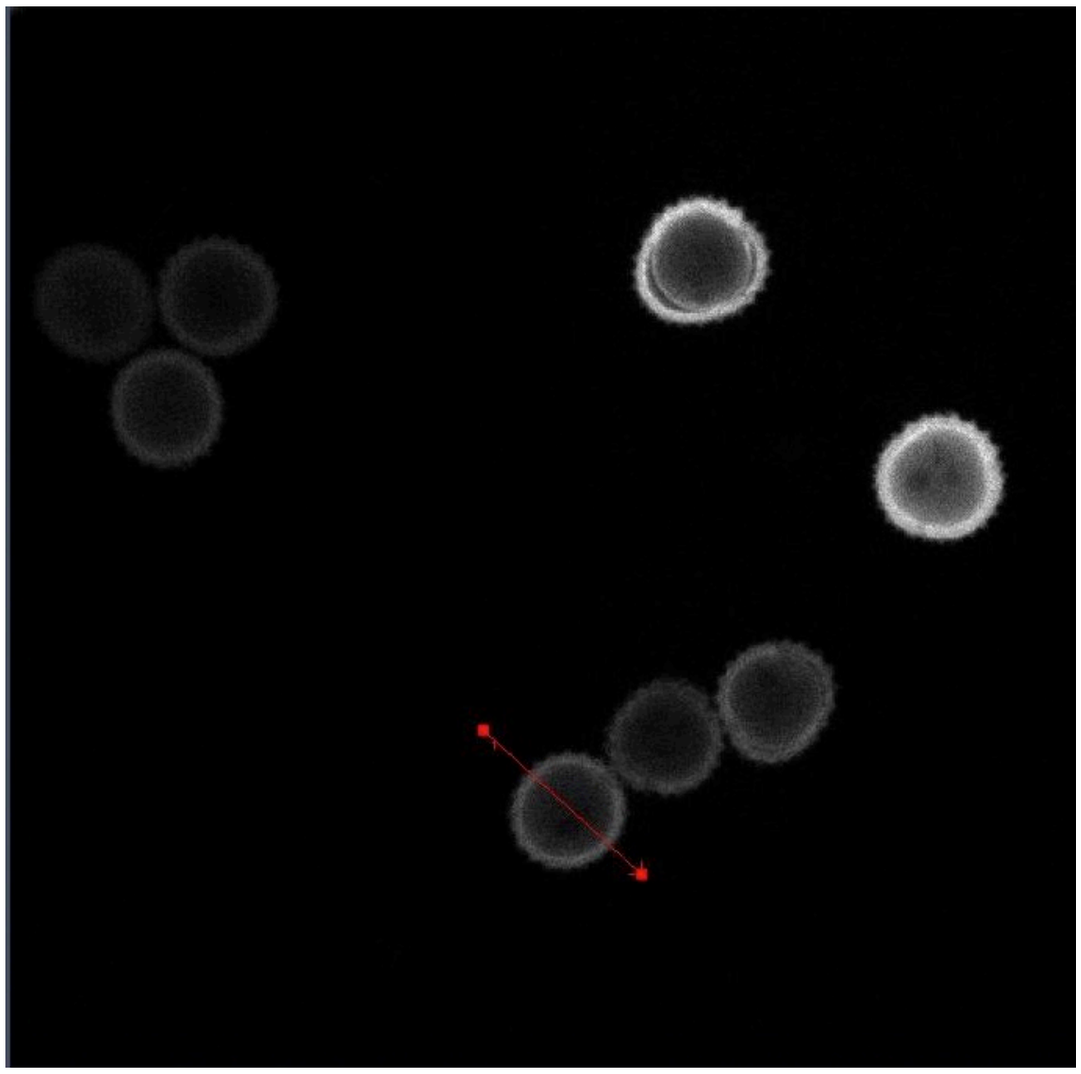


What is Quantitative Imaging?

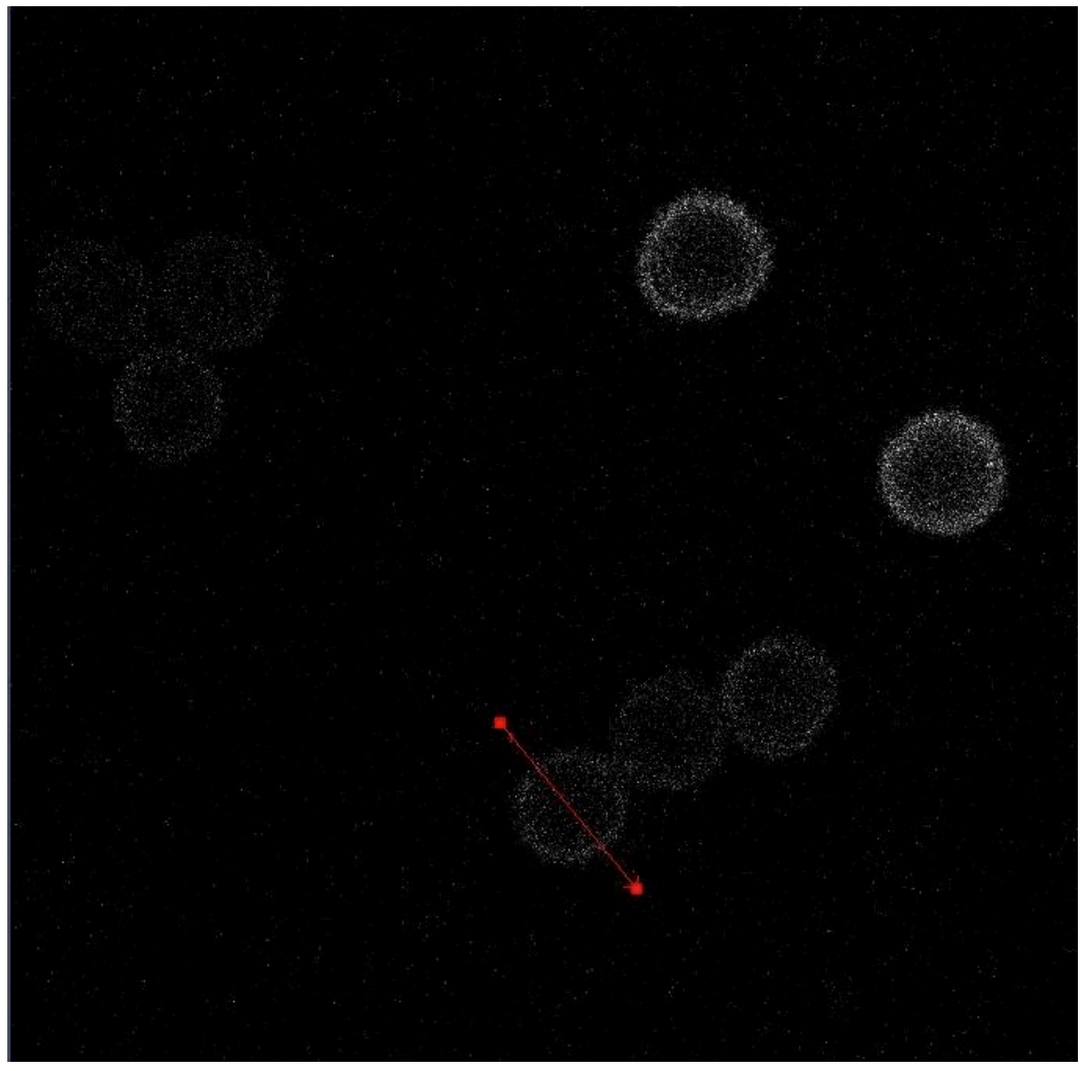
- Quanta = numbers = data, information
- Examples:
  - Where & how many?
  - Colocalization
  - Dynamics
  - Intensity
- In an ideal world, we can use semi-automated or automated means to perform analysis, which requires good signal-to-noise (SNR)

The Importance of High SNR

Pollen grains imaged with Trinity
20X W/1.0, 3X Zoom, 488 Laser

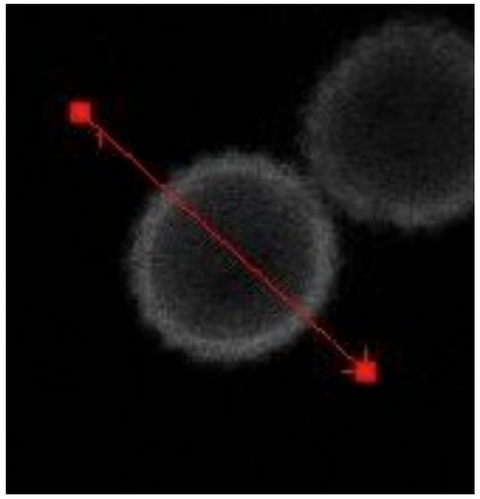


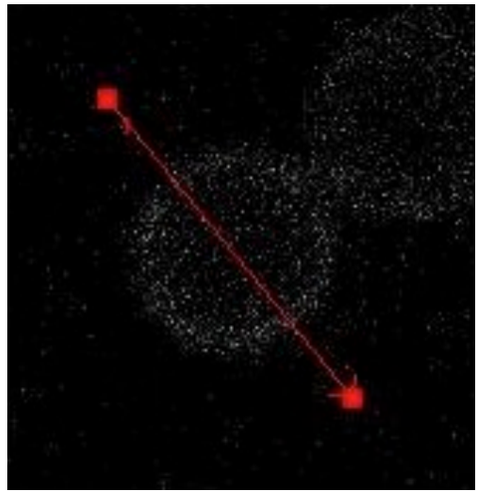
2% Laser, Pinhole 1AU, Gain: 620

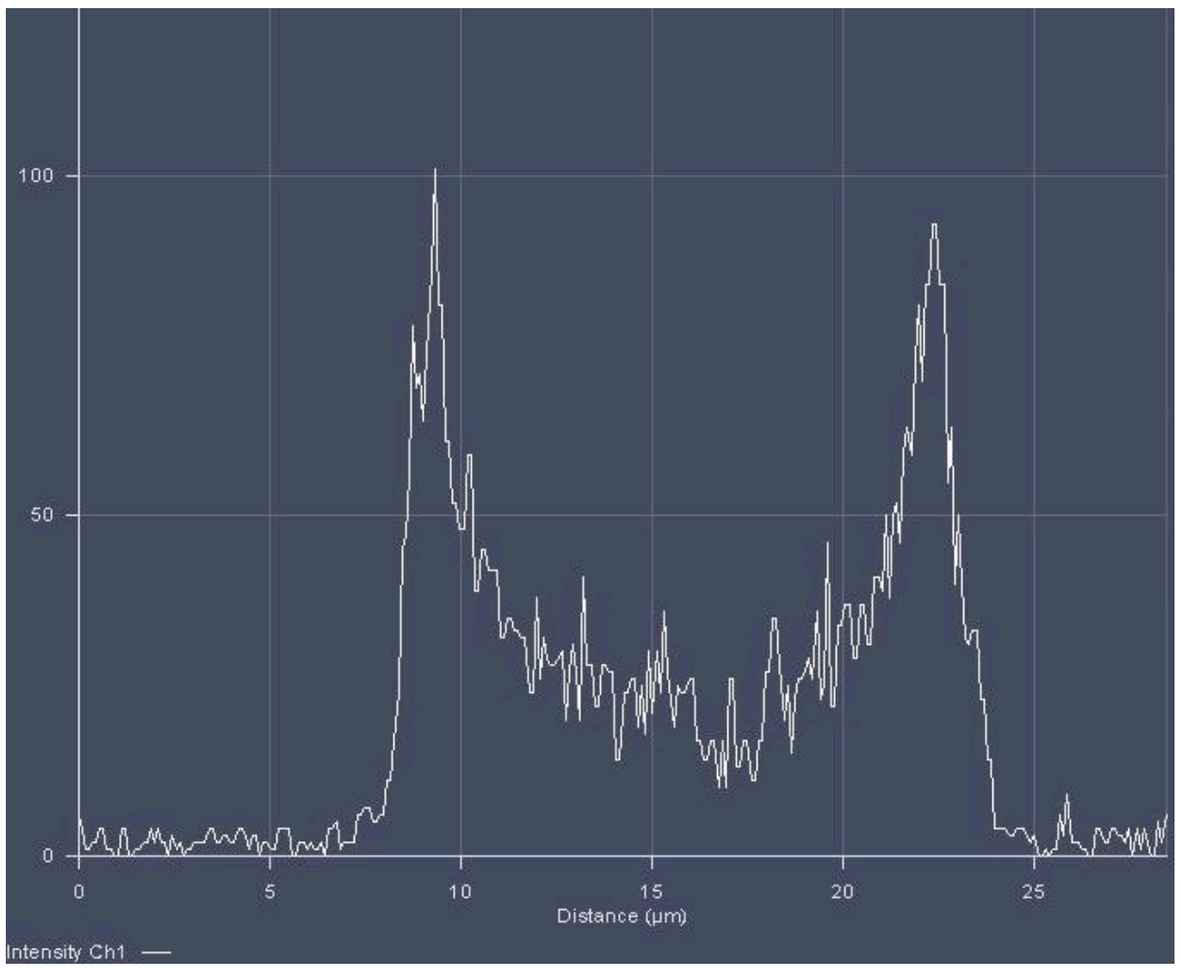


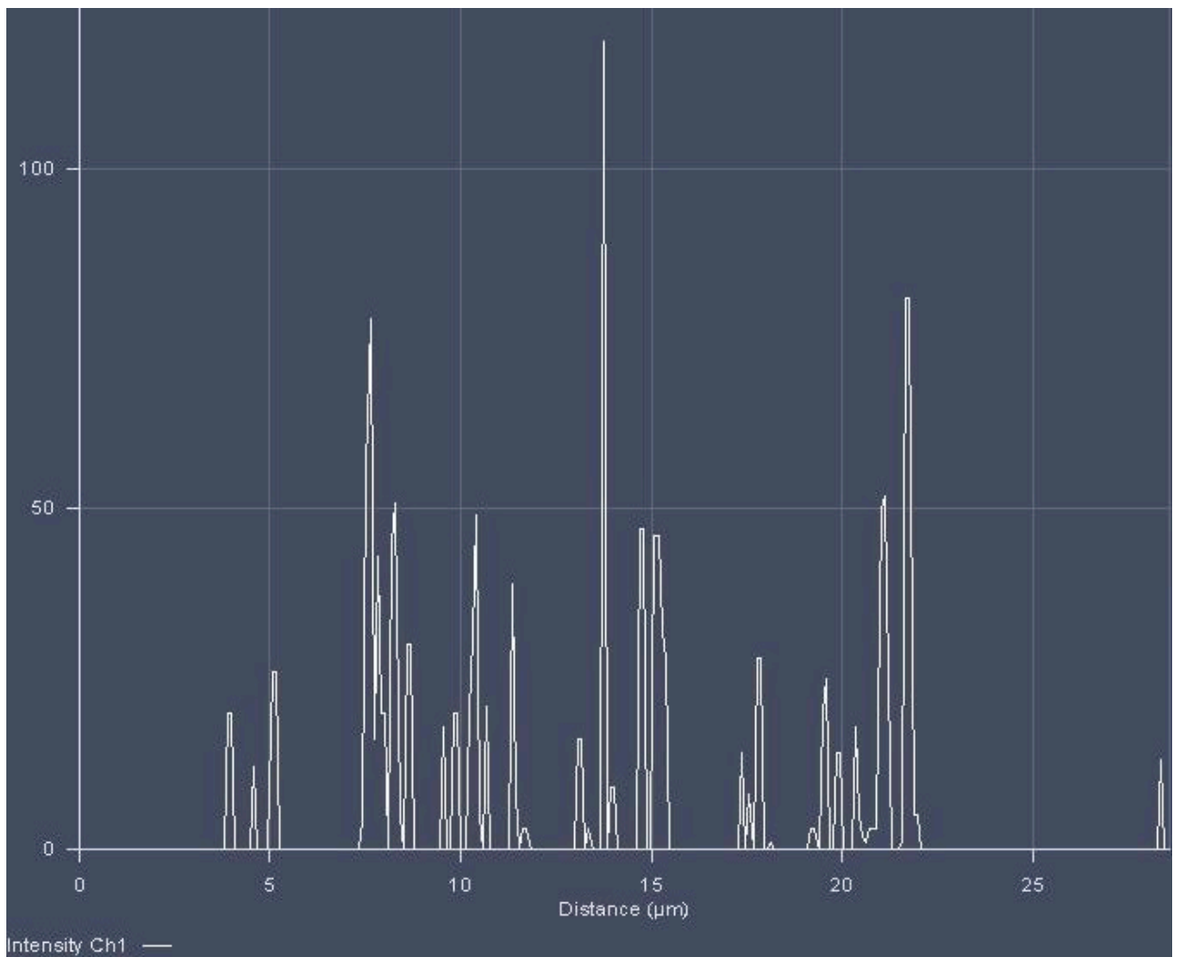
0.05% Laser, Pinhole <1AU, Gain: 950

The Importance of High SNR









Optimizing SNR: Increasing Signal

- Increase light intensity
- Increase exposure time or pixel dwell
- Troubleshoot staining or transfection protocol
- Choose a photostable, efficient dye
- Choose the best objective for your needs:
  - Lowest magnification to see your objects of interest
  - High NA
  - Immersion > Air

Optimizing SNR: Increasing Signal (continued)

- Choose a more sensitive detector
- If you don't need optical sectioning, use a widefield system
- Turn off all room lights
- Bin pixels if using a camera system
- Open pinhole if using a confocal (but only if it suits your experiment)
- Increase gain on PMT or EMCCD (but will also increase noise after a certain threshold!)

Optimizing SNR: Decreasing Noise

- Avoid lengthy exposure times, which increases Poisson or shot noise

- Decrease background fluorescence (avoid DAPI in Permount, phenol red, test different serums and fixatives, use a different fluorescent protein construct)

- Subtract autofluorescence with linear unmixing

Quantitative Imaging: Best Acquisition Practices

- Optimize SNR
- Controls
  - No stain/transfection control
  - No primary antibody control
  - Photobleaching
  - Correctly aligned optics
  - Live imaging: monitor cell health
- Counterstains can be useful! (example: DAPI)

Quantitative Imaging: Best Acquisition Practices

Specific Examples:

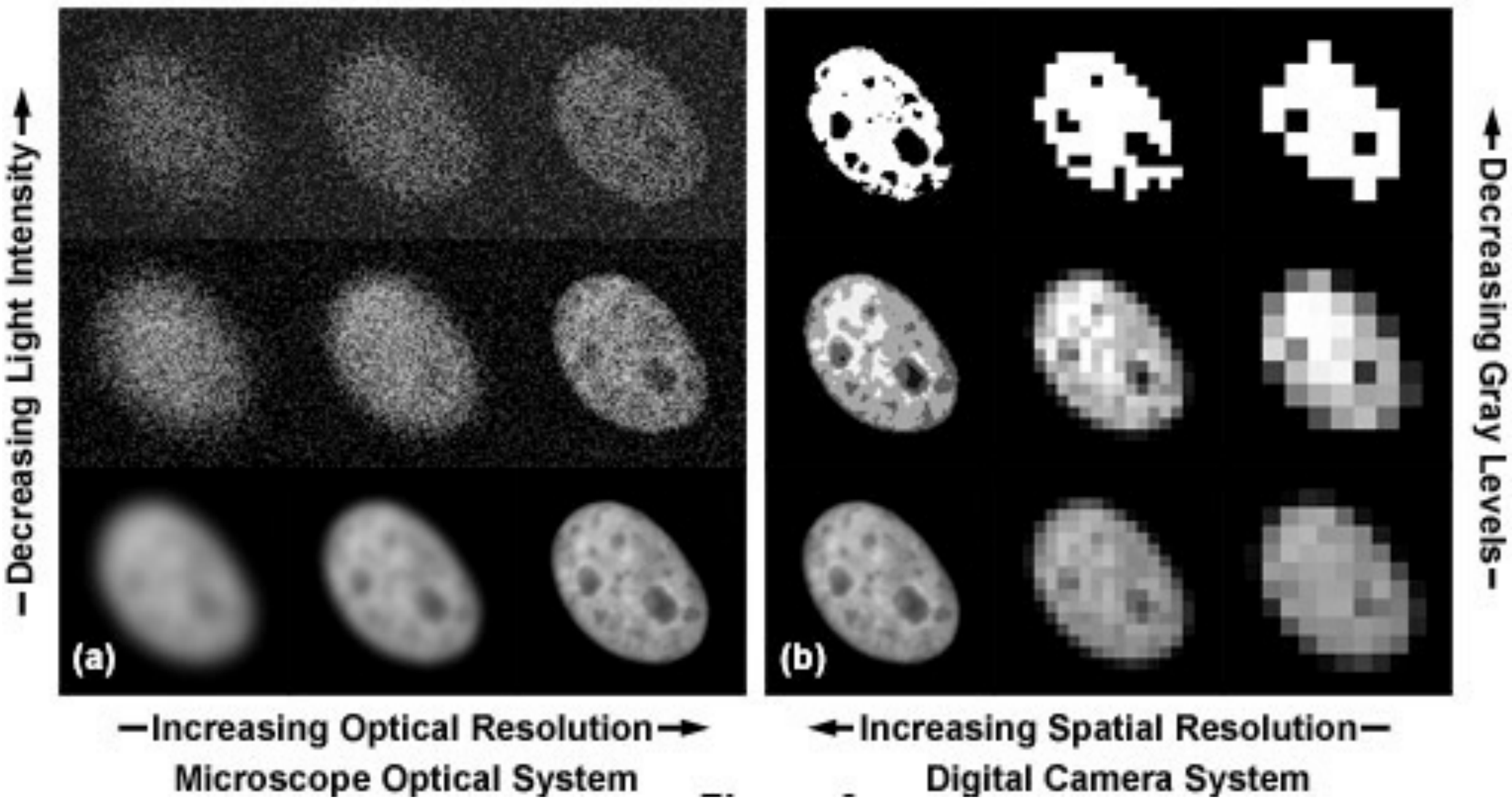
- Where & how many?
- Colocalization
- Dynamics
- Intensity

Where & How Many?
Importance of Resolution

- Lateral resolution: $\Delta x,y = 0.61\lambda / NA_{obj}$
- Axial resolution: $\Delta z = 2\lambda \mathbf{n} / (NA_{obj})^2$

- To increase resolution:
  - Increase NA
  - Decrease wavelength
  - Match detector resolution with optical resolution
  - Increase axial resolution by closing down pinhole

Intensity, Sampling, & Resolution



https://www.microscopyu.com/articles/livecellimaging/imagingsystems.html

Colocalization

- Look out for: registration shift, bleed through, difference in intensity levels due to light source/ optics

- Select suitable dyes (matching filters, dichroics, laser lines, etc.)

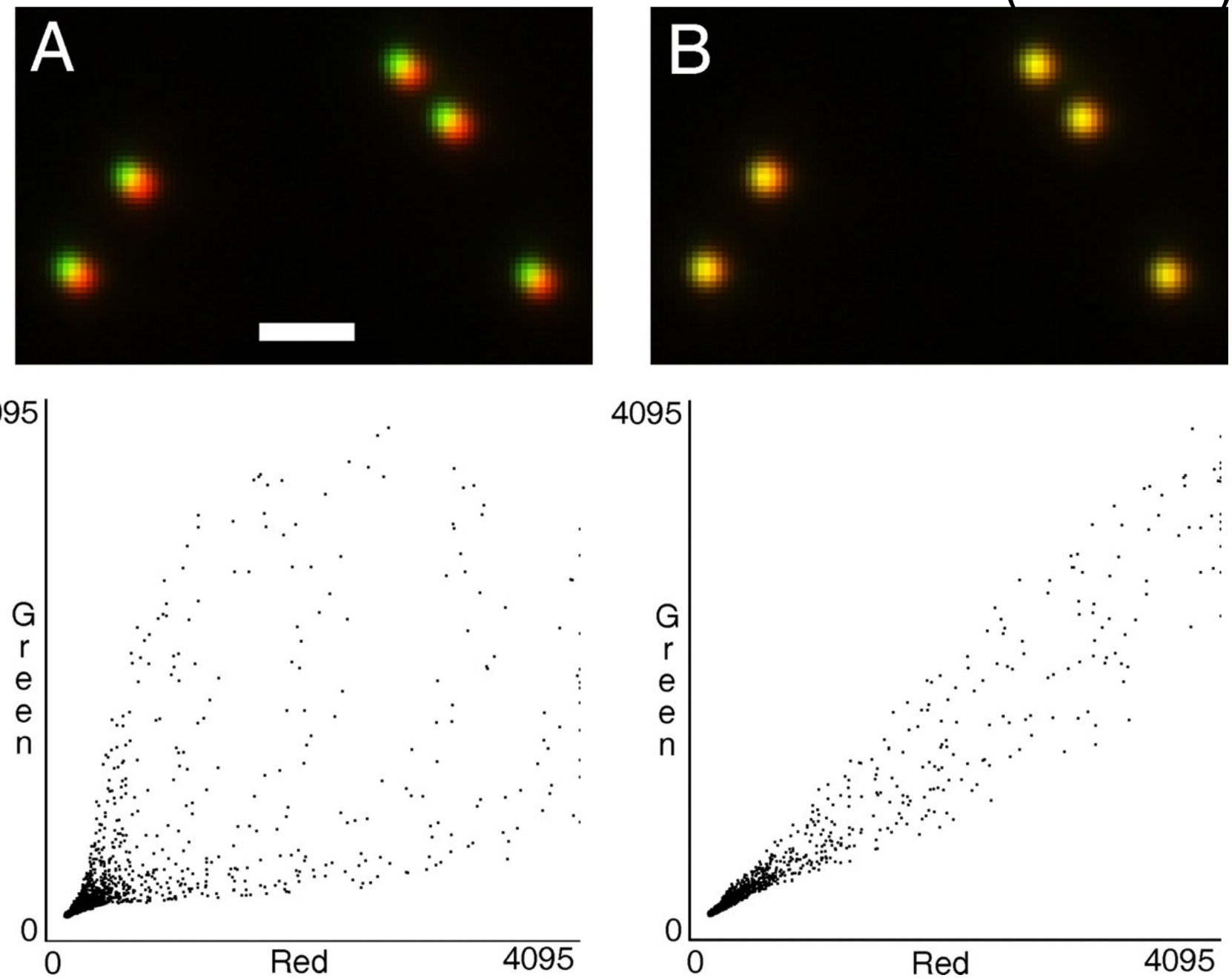
- Avoid switching filters if possible (mechanical offset)

- Control experiment: multi-colored beads

Colocalization Control: Multi-colored Beads

No correction

Shift-corrected (software)



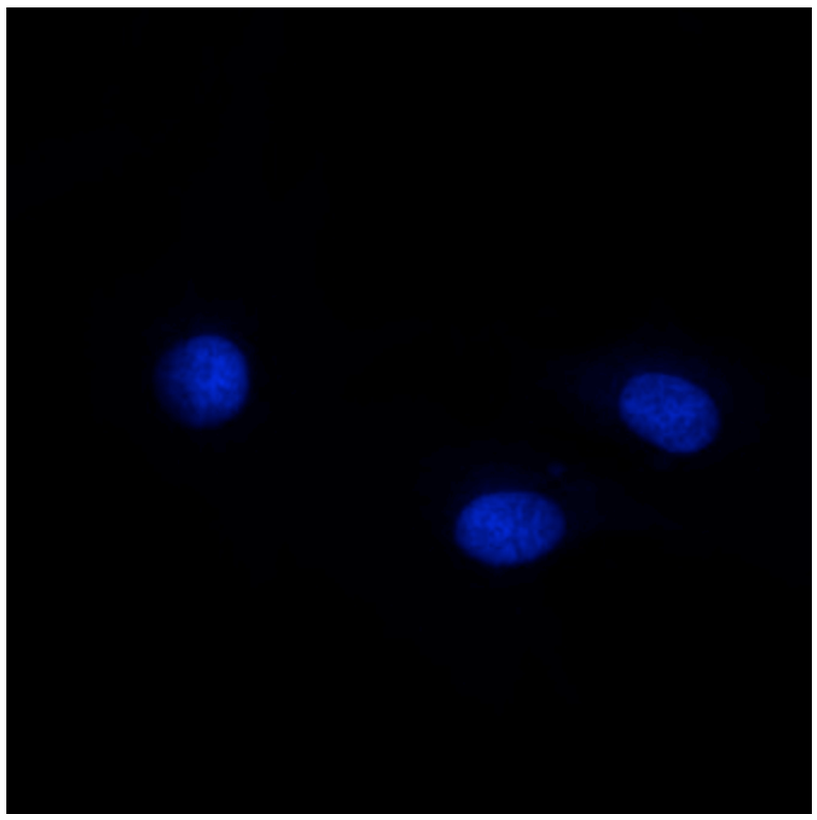
100 nm
Tetraspeck Beads
FITC, TRITC

Waters JC, *J Cell Biol* 2009;185:1135-1148

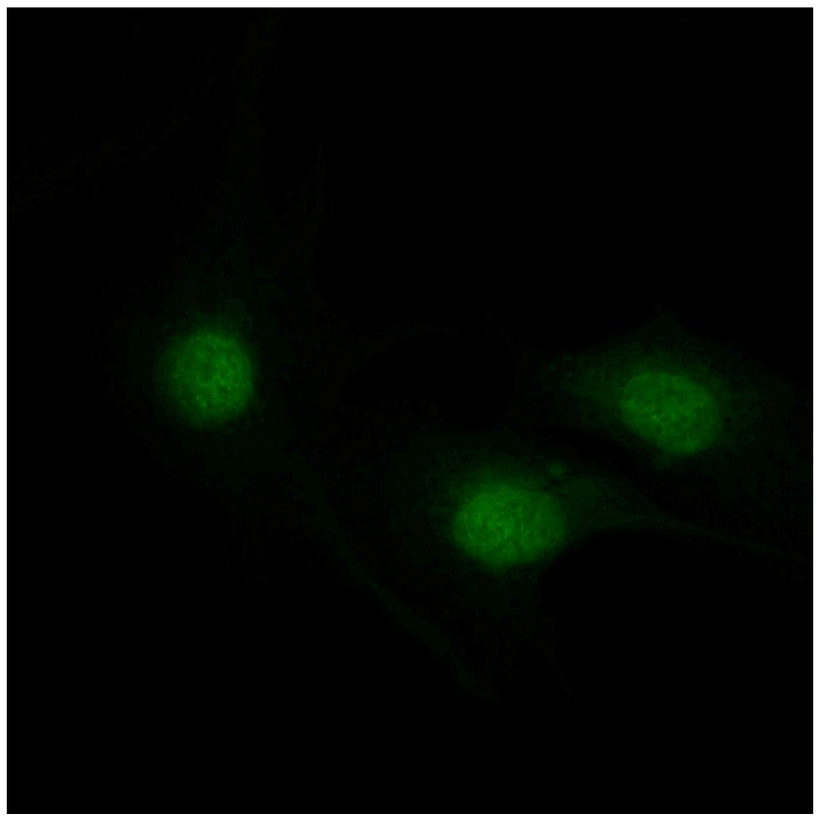
Multi-Channel Imaging:
Beware of Bleedthrough

- 405 nm laser excitation only -

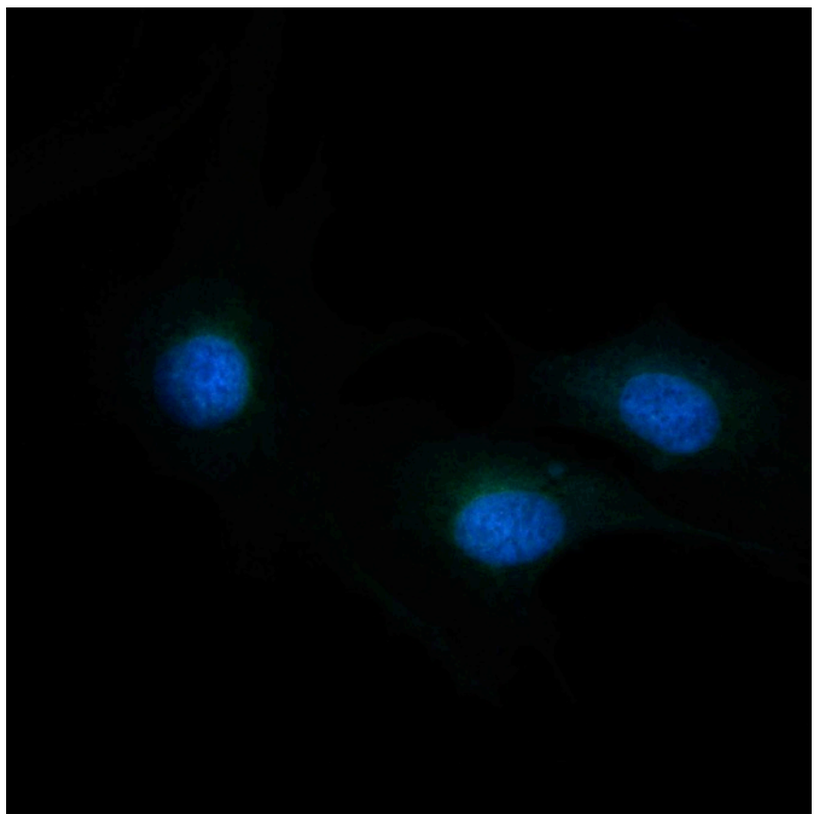
DAPI



Alexa 488



Overlay



Time-lapse Imaging: Temporal Resolution

- Decrease FOV
- Decrease exposure time
- Confocal: bidirectional scanning, decrease resolution
- Camera systems: bin, set on stream mode
- Choose a faster camera (e.g., sCMOS)
- Some cameras and detectors have a faster area
- Use software that directly controls camera (vs. using acquisition software that can cost you time)

Intensity Measurements/ Comparisons

- Most tricky of quantitative imaging experiments - need to do proper controls!
  - Internal controls within each image
  - Calibration sample at the beginning of each imaging session
- Avoid saturation
- Consider your detector - how much read noise, dark noise is inherent in your system?
- Illumination: Is it even and stable? Check over imaging session and over time.

Summary

- Consider the goals of your imaging and try out a workflow before you acquire all of your images. This is a reiterative process.

- Think about the best controls, dyes, microscope, and detectors for your experiment.

- Regardless of your experimental question, always strive to achieve high SNR.

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Next Open MIC:

Image Processing

Holly Aaron

Tuesday, May 24th

4:00 p.m.

Room TBA