



The Plan

- 8:30 - 9:20am Lecture
- 9:20 - noon Rotate groups across the street to view samples under the microscope with Vicki. Groups / times are on the board. Every group gets 40 min. at the microscope.
- When you're not there, work here on the ImageJ problem set / go upstairs for coffee / at least try to look like you're doing something productive
- 9:20 - 10:40am Attempt ImageJ problem sets in small groups (2-4 people) using any resources you want, including: advice from Patrick and Tas, Christine Labno's instruction manuals (pdfs from the GitHub), internet searches, and the built in command finder (press L when ImageJ is open). Tiny prizes will be awarded for complete solutions / really good efforts
- 10:40 - noon Patrick will go over problem sets on the screen, probably a couple of times so everyone gets to see the solutions
- 12:00pm Lunch

Remember to ask

How many people have experience with:

- Microscopy / imaging
- Image processing
- ImageJ

Introduction to digital imaging and microscopy

Patrick La Riviere, Ph.D.

Associate Professor,
Radiology and Medical Physics

building on slides from

Christine Labno, PhD
Assistant Technical Director
Univ. of Chicago Light Microscopy Core Facility

**"Discovery is to
see what everybody else has seen and
to think what nobody else has thought."**

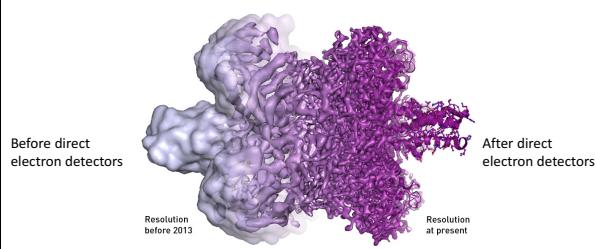


Albert Szent-Gyorgyi

Nobel Prize in Physiology/Medicine (1937)

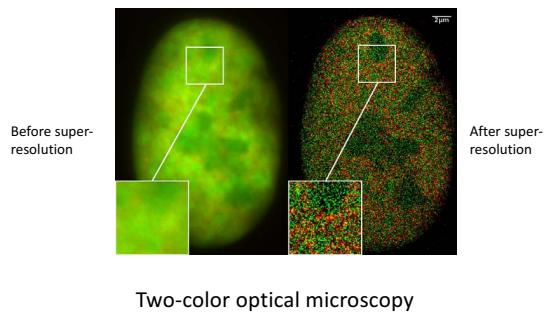
First year-round MBL scientist

**Seeing what nobody else has seen
is good too!**

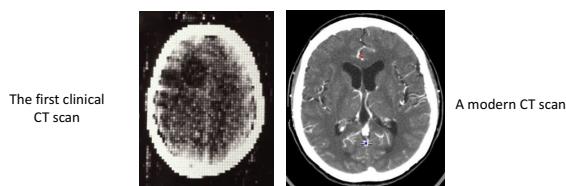


Cryo electron microscopy for protein structure

Seeing what nobody else has seen
is good too!



Seeing what nobody else has seen
is good too!



Computed tomography from 1972 to today

An image is a spatial map of a physical property

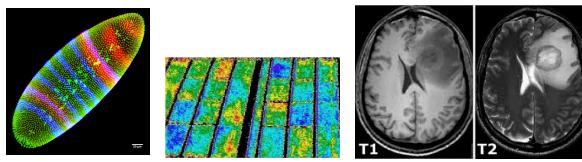


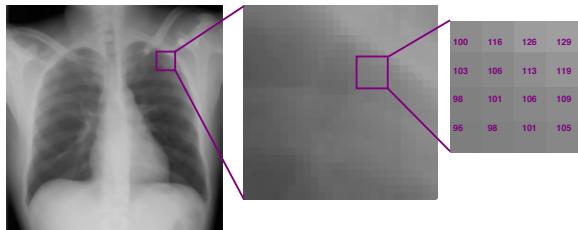
Image credit: Cecelia Miles, Kreitman lab

Fluorescent proteins in microscopy

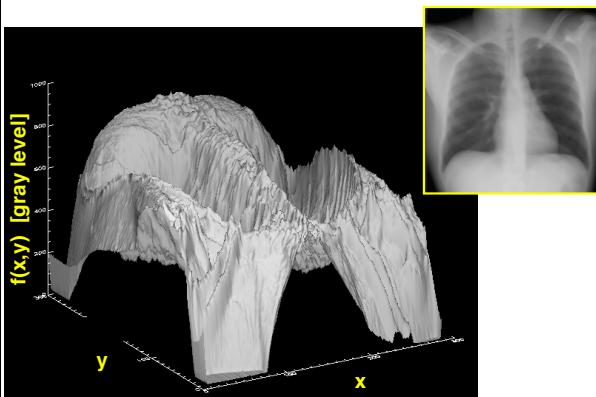
Water content of crop fields with thermal imaging

Nuclear relaxation times in MRI

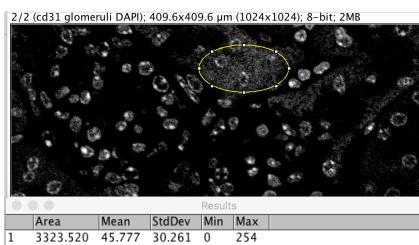
All digital images are arrays of numbers



You can think of them as functions or surfaces.

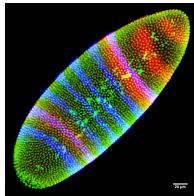


You can always make quantitative measurements

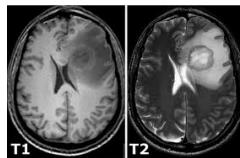


Here I have calculated the mean, standard deviation, and range of values in the oval-shaped region of interest (ROI).

But not all images are absolutely quantitative



Most microscope images don't give you absolute values of fluorescence nor of underlying molecules.



In a T1-weighted MR image, you can't extract absolute value of T1 from pixel values. Only relative.

You can quantify and compare within images and across images acquired under identical conditions. Be very careful comparing measurements ACROSS acquisition schemes.

Digital images are sampled

- They are stored on a finite array of pixels or voxels (3D pixels).
- Pixels too big: resolution suffers and aliasing artifacts appear.
- Pixels too small and you gain nothing.
- Shannon-Nyquist theorem says sample at half the inherent resolution of the optical system.

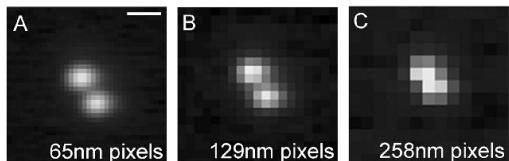
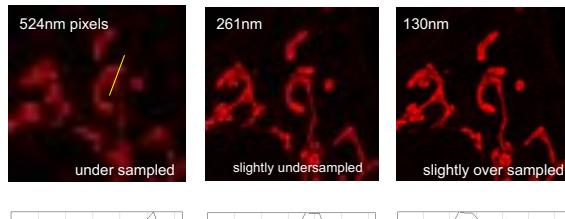


Image credit: Vytas Bindokas, Light Microscopy Core

Sampling Real Data Example

Mitochondria = 500nm – 1μm in diameter



524nm pixels
under sampled
Scan time: 5 sec.
Size on disk: 64KB

261nm
slightly undersampled
Scan time: 10 sec.
Size on disk: 256KB

130nm
slightly over sampled
Scan time: 20 sec.
Size on disk: 1MB

Image credit: Leica SP5 laser scanning confocal / ImageJ

Digital images are quantized into a finite number of bits

1 bit	2 bit	4 bit	8 bit
0, 1	00, 01, 10, 11	0000 to 1111	00000000 to 11111111
			

1 bit has 2 possible shades of luminance values

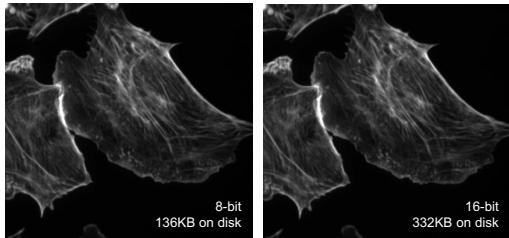
2 bit has 4 possible shades of luminance values

4 bit has 16 possible shades of luminance values

8 bit has 256 possible shades of luminance values

Image credit: realworldretouching.com

Humans Can Distinguish ~60 Gray Levels



Greater bit depth = larger file size, so it's difficult for humans to justify a larger dynamic range.

However, if you undersample your gray range, you can suffer from posterization – a loss of fine detail due to intensity banding

Image credit: Olympus DSU Spinning Disk confocal

What Happens When You Shrink Bit Depth?

1 bit 2 bit 4 bit 8 bit

0, 1 00, 01, 10, 11 0000 to 1111 00000000 to 11111111

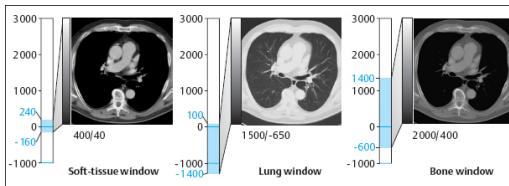
$2^1 = 2$ $2^2 = 4$ $2^4 = 16$ $2^8 = 256$

1 bit has 2 possible shades of luminance values
2 bit has 4 possible shades of luminance values
4 bit has 16 possible shades of luminance values
8 bit has 256 possible shades of luminance values

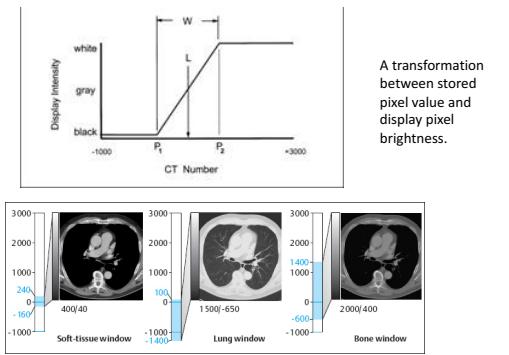
Image credit: realworldretouching.com

So Eight is Enough?

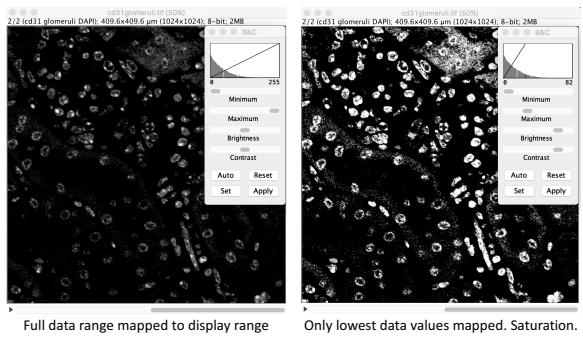
- No, no, no! Not always!
- Quantitatively, there may be statistically significant differences at higher precision.
- You can store more bits and change display mapping to bring out contrast!



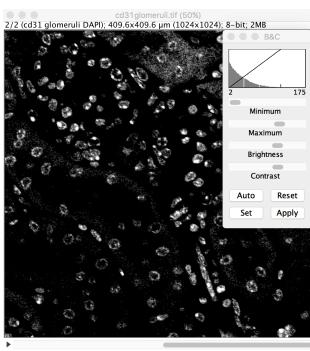
Display mapping



ImageJ Window/Level example



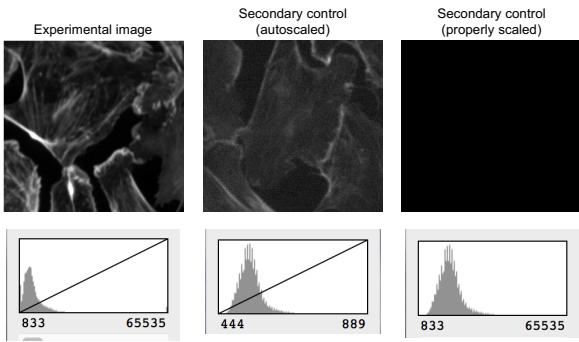
ImageJ autoscale



Autoscale allows a small percentage of pixels to saturate.

Can be useful starting point, especially if an image opens up and nothing is visible!

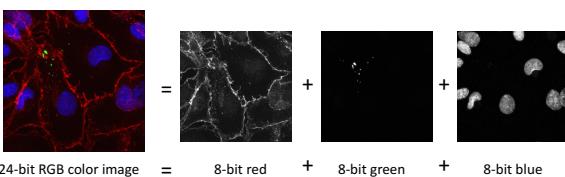
Dangers of autoscale



A Word on Color vs. Grayscale

Most microscopy images are taken with grayscale cameras. The different wavelengths are captured one at a time using various filters, and then combined with false colors to reproduce the colors of the original sample.

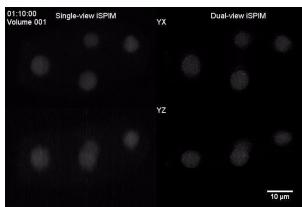
Color images are lovely, but not great for storing scientific information. Color images are 24-bit, which sounds great until you consider that each color image is made up of three channels: red, green and blue. Each channel therefore only gets 8-bits worth of information encoded.



If you collected your raw data as 12-bit or 16-bits per channel, converting to RGB causes a LOSS of information, from 4096 or 16536 down to 256 grays

Image credit: T. Mirzapouraiva

Images are the biggest big data



13-hour movie of a worm embryo.

Microscope acquires a 3D volume every second for 13 hours.

2 Terabytes of data at the end of the experiment=> equivalent of 600,000 photographs!

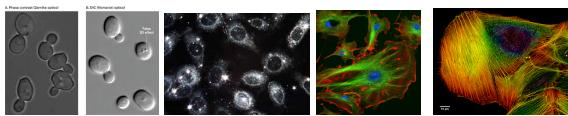
Kinds of optical microscopes

Label-free

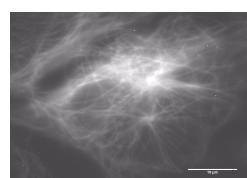
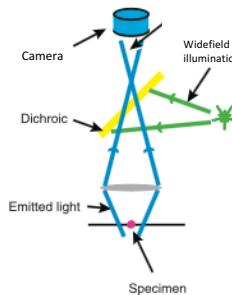
- Transmission
- Phase contrast
- Differential interference contrast
- Darkfield

Fluorescence

- Widefield
- Confocal
- Total internal reflection
- Two-photon
- Light sheet

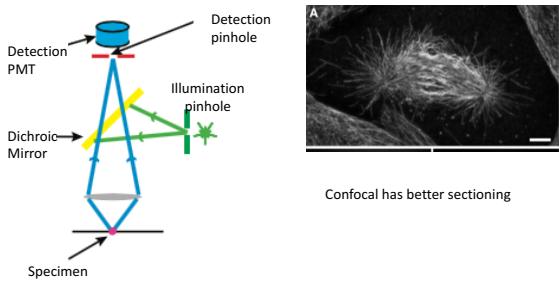


Widefield fluorescence microscopy

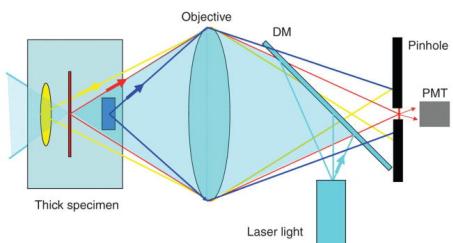


Widefield has poor sectioning

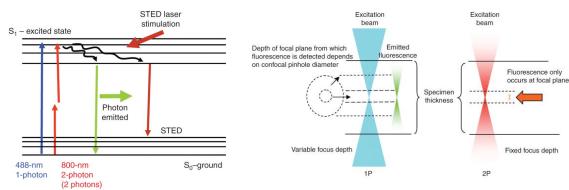
Confocal fluorescence microscopy



Confocal principle

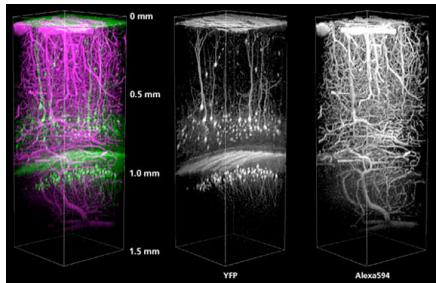


Two-photon microscope

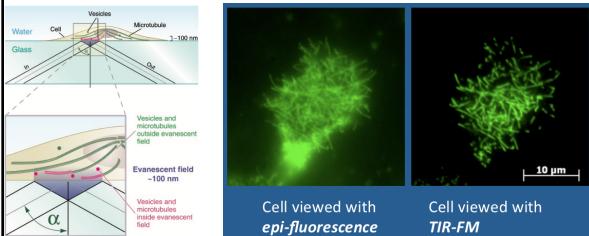


Two photon microscopes can image deeper into scattering tissue.

Two-photon microscope

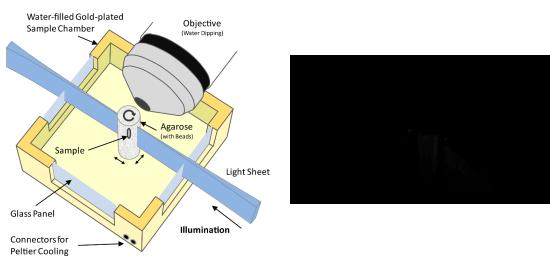


TIRF microscopy



TIRF microscopes give excellent sectioning right at the cover slip.

Light sheet microscopy

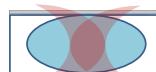


Light sheet microscopes are very dose efficient.

How to optimally image fluorescence?



Widefield illumination
Fast
No optical sectioning
Volumetric photobleaching/damage



Confocal illumination
Slow
Optical sectioning
Volumetric photobleaching/damage



Light-sheet illumination, better solution
Fast
Optical sectioning
Photobleaching/damage confined to imaging plane

Key concept: match excitation/detection volume

Computational imaging: computing to form images

Better microscopes used to mean better glass



Leiden microscope
c. late 1600s

Now better microscopes = glass + math



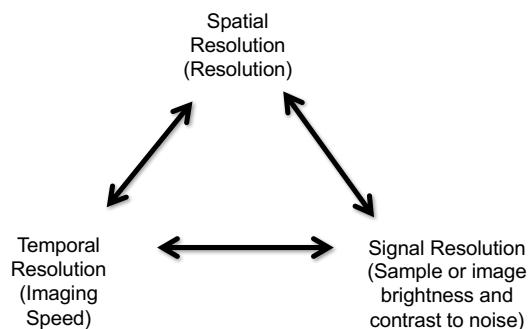
Test object Structured illumination of test object Using math to extend the resolution

Modern corrected objective lenses



Objective Correction for Field Curvature
Achromat Plan Achromat
Lens Doublet Group Lens Doublet Group Lens Doublet Group
Front Lens Hemispherical Front Lens

Image quality tradeoffs



Images are Representations of Objects



If it's not a pipe, what is it?

It's an IMAGE of a pipe. Not the object itself but a representation of the object.

The image of a point is not a point, it is a pattern of light called a point spread function (PSF).

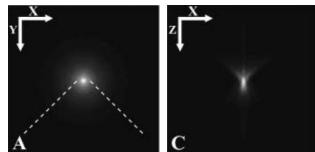
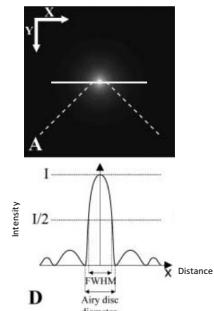


Image credits: (top) René Magritte "The Treachery (Treason) of Images" (bottom) Bolte and Corderieres (2006) J. Microscopy v. 224 pt3, pg. 214

The point spread function, aka Airy pattern

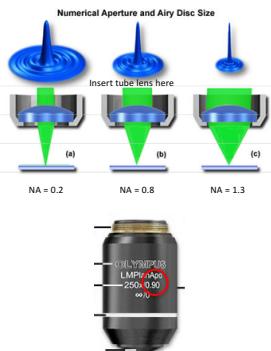


The better the spatial resolution of your system, the smaller the PSF/Airy pattern.

Measurement of the full width at half maximum (FWHM) intensity is one way to determine resolution.

Image credit: Bolte and Corderieres (2006) J. Microscopy v. 224 pt3, pg. 214

What Determines the Size of the Airy Disk?



$$\text{Resolution (xy)} = d = \lambda / 2\text{NA}$$

$$\text{Resolution (z)} = d = 2\lambda / \text{NA}^2$$

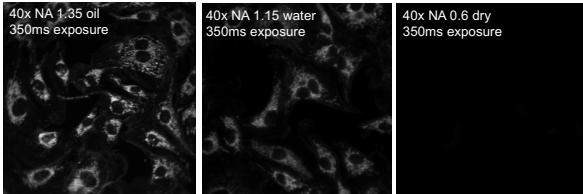
Where λ is the wavelength of light used for imaging

NA is the numerical aperture of the objective

These equations were first published by Ernst Abbe in 1873

Image credit: Olympus Microscopy Resource Center

Higher Numerical Aperture = Brighter, Sharper Pictures



Increasing Resolution Beyond Widefield

- Use confocal microscopy

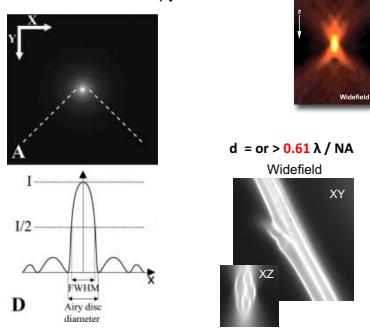


Image credits: (top) Claxton et al www.olympusconfocal.com/theory/LSCMintro.pdf
(bottom) Olympus DSU spinning disk confocal

Increasing Resolution Beyond Widefield

- Use confocal microscopy

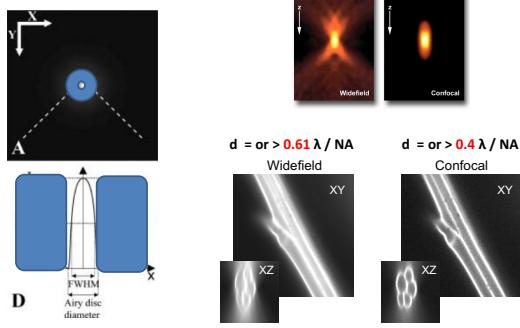
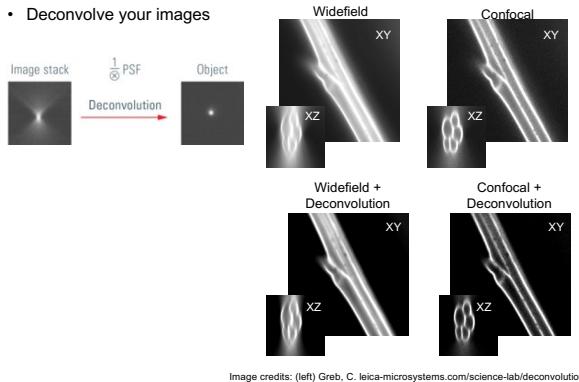


Image credits: (top) Claxton et al www.olympusconfocal.com/theory/LSCMintro.pdf
(bottom) Olympus DSU spinning disk confocal

Increasing Resolution Beyond Confocal

- Deconvolve your images



The Abbe Diffraction Limit / Barrier

Even when all parameters are optimal, there is an absolute lower limit to resolution in light microscopy, imposed by the wave nature of light itself.

Resolution limit (xy) = 200nm
Resolution limit (z) = 400nm

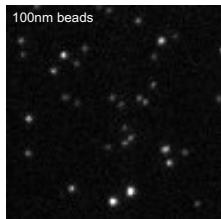
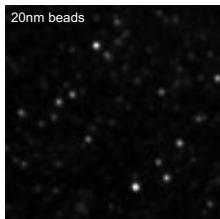


Image credits: Olympus DSU confocal

New Ways Around the Diffraction Barrier

SIM – Structured Illumination Microscopy

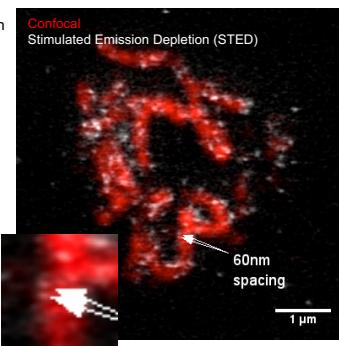
Pioneered by Mats G.L. Gustafsson
100nm lateral(XY), 150nm axial(Z)

STED – STimulated Emission Depletion

Pioneered by Stefan Hell
50nm lateral, 100-400nm axial

STORM – STochastic Optical Reconstruction Microscopy and other "pointillist" techniques

Based on work by Eric Betzig + Harald Hess
20nm lateral, 50nm axial

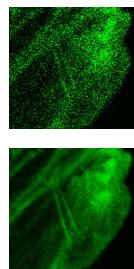


Summary: Spatial Resolution

- Images are subject to distortion (convolution) by the optics of the imaging system, turning points into point spread functions
- Resolution is determined by the wavelength of light and the NA of the objective, and can be improved by things like confocal microscopy and deconvolution
- The absolute lower limit of resolution with light microscopy is 200nm in xy and 400 nm in z, although a series of Nobel prize-winning techniques are allowing us to achieve resolution up to 10-fold better
- Pixel size is determined by the image capture device and the magnification of the optical path
- 2.5-3 pixels per smallest resolvable feature is adequate sampling. Beware both over- and under-sampling

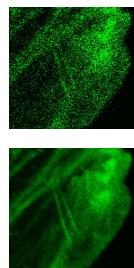
Signal resolution, aka contrast-to-noise ratio

- Your sample needs to be bright enough and have enough difference between bright and dark that there is useful contrast.
- How much contrast is enough?
- It needs to be large compared to the noise (random fluctuations) in the image.



Sources of noise

- Photo counting statistics (aka shot noise).
 - Follows Poisson statistics, so variance = mean number detected counts.
$$SNR = \frac{MEAN}{\sqrt{VARIANCE}} = \frac{N}{\sqrt{N}} = \sqrt{N}$$
 - So more counts means better SNR.
- Electronic readout noise. Often on the order of 1-5 photons worth of noise.
 - Not important unless really dim sample or short exposure.
 - EM-CCD gets above this noise floor but amplifies shot noise.



Reducing the Contribution of Noise

- Choose a high quality detection system, preferably one that is cooled
 - Increase your exposure time (in the case of a camera-based system) or use line / frame averaging (on a detector based system)

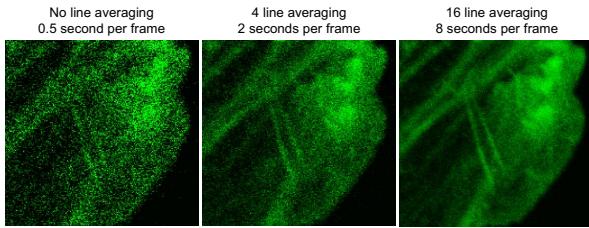


Image credit: Leica SP2 confocal

Boosting Signal in Your Samples

- And finally, bin your pixels. A 2×2 bin will add the signal of 4 pixels together, boosting signal (and increasing imaging speed) but sacrificing spatial resolution.

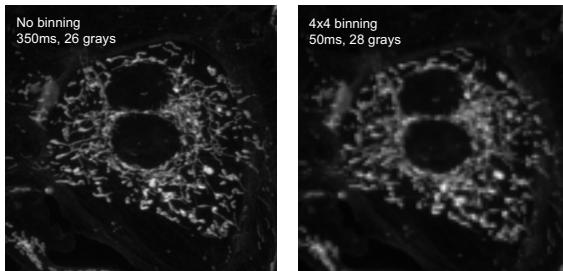


Image credit: Olympus DSU confocal

Summary: Signal Resolution

- Cameras and other photon detection devices are means of translating analog values (photons, voltages) into digital values (grays)
 - The grays in an image are a mix of signal and noise. It's impossible to eliminate noise, but we can reduce its contribution
 - Due to the inexact conversion of sample concentration to photons to voltage to grays, the gray levels in an image only have meaning relative to the gray levels in another image taken with the same parameters
 - To create an image dataset which can be measured and compared: Generate an image of your brightest sample such that the signal covers about 80-85% of your dynamic range. Avoid saturation. Keep parameters constant as you image your sample set.

Adding the Fourth Dimension: Time

As with spatial and signal resolution, temporal resolution is a matter of adequate sampling.

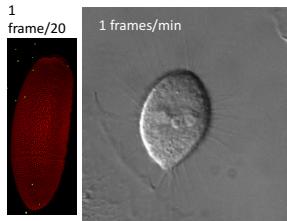
Choose an imaging speed that allows accurate recording of the events of interest without damaging the sample or generating a giant dataset.

As a starting point, we can return to Nyquist-Shannon and use a sampling rate that is twice the period of the activity we are interested in capturing.



Two Types of Live Sample Imaging

Time-lapse – Processes that take minutes, hours or days to complete. Most often taken as a series of images with a pause between each image. Played back at a rate faster than collected.



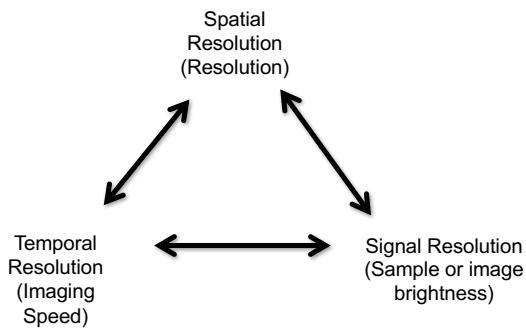
Streaming – Processes that happen at rates that can be visualized in real time (or faster). Most often taken as a stream of images as fast as the system can be made to run and with no pauses between images. Played back at a rate slower than collected.

A microscopy image showing numerous small, bright green spots distributed across a dark background. These spots represent individual cells or fluorescently labeled structures being tracked over time.

Summary: Temporal Resolution

- Look in the literature to see what others have done in similar situations. Use that as a starting point
 - For long processes, image more often than you think you should. You can always take out timepoints. Check in with the experiment often to make sure the sample hasn't died / moved / bleached / dried out / etc. Refine in the next round to maximize sample viability and minimize the size of the dataset
 - For fast processes, determine how light sensitive your sample is and aim for being as gentle as possible. You can't get data from a dead sample. Find ways to increase your speed (lower exposure time, binning). Refine on the next round to maximize the ability to track the activity you're looking for

Optimization: Pick Two, Sacrifice the Third



Tips for Image Processing

- Keep your raw images and all metadata in their original formats. Reviewers and journal editors can ask to see your original data.
- Do minimal processing – if your images are not good enough, go back and refine your staining and imaging first, rather than looking for post-processing solutions.
- Process every pixel in the image the same way (no selective erasing or non-linear adjustments like gamma). Process every image in the dataset the same way also.
- Don't leave your analysis until the end! You wouldn't stain a bunch of samples without imaging to see how they worked. Don't take a bunch of images without processing to see if they give you quality data! Analyze each preliminary experiment as you do it and use the results to inform your next experiment.

ImageJ Problem Sets

- To search for a command in ImageJ/Fiji, hit the I key on your keyboard to pop up the Command Finder
- For set one with the color figure, use the Basics guide in your Imaging GitHub "readings" folder
- For set two with the pancreatic islet, use the Intermediate guide
- You may search the web or use any other resources you can find

Tradeoffs – A Real World Example

Granule exocytosis speed: 20 msec.

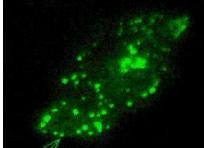
Camera speed: 120 frames / sec

Granule size: 300-350nm

Pixel size on chip: 12.9um

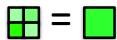
Obj.: 100x NA 1.4 or 150x NA 1.4
Actual pixel size: 138nm or 86nm

Actual pixel size: 129nm or 86nm



We would need a minimum magnification of 86x ($12.9 \text{ um} / x = 0.15 \text{ um}$) to get the sampling we need.

We have a 100x NA 1.4 and a 150x NA 1.45, both of which collect fewer photons than the 60x NA 1.45 we could have used without binning. So our sample will appear to be dimmer, unless we use more light. However, if we use more light, we can kill the sample.



Fortunately, binning has a side benefit of increasing brightness, as the photons hitting the four pixels are ADDED (not averaged) together to produce the new gray value.

A Plug for the University Cores

- Office of Shared Research Facilities
 - <http://osrf.uchicago.edu>
 - 27 Cores including light microscopy, electron microscopy, small animal imaging, flow cytometry, transgenics, tissue processing and biostatistics
 - “Centralized areas of technology and expertise” available to the entire University community

