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### The Plan

- 8:30 - 9:20am Lecture
- 9:20 - noon Rotate groups across the street to view samples under the microscope with Vicky. 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 to LB302 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

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### Remember to ask

How many people have experience with:

- Microscopy / imaging
- Image processing
- ImageJ

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# 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

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“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

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Seeing what nobody else has seen  
is good too!

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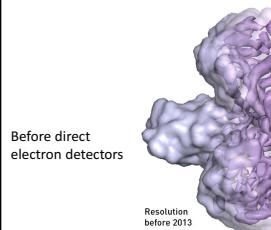
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Seeing what nobody else has seen  
is good too!



Cryo electron microscopy for protein structure

Image credit: Martin Hägglom

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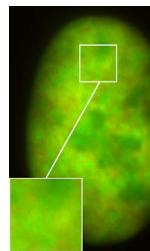
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Seeing what nobody else has seen  
is good too!



Two-color optical microscopy

Source: Gunkel et al, Biotechnology Journal, 2009, 4, 927-938.

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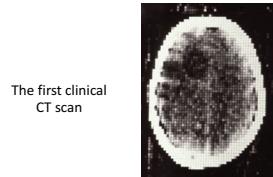
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Seeing what nobody else has seen  
is good too!



Computed tomography from 1972 to today

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An image is a spatial map of a physical property

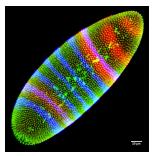
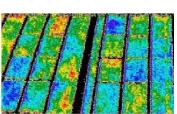
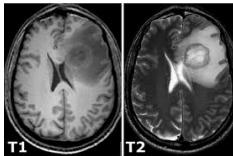


Image credit: Cecelia Miles, Kreiman lab



Water content of crop fields with thermal imaging



Nuclear relaxation times in MRI

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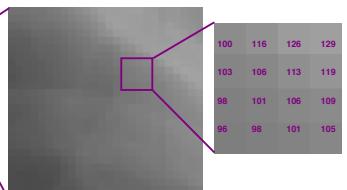
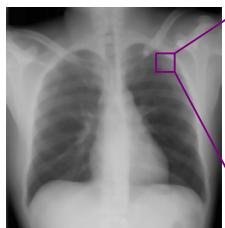
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All digital images are arrays of numbers



100 116 126 129  
103 106 113 119  
98 101 106 109  
96 98 101 105

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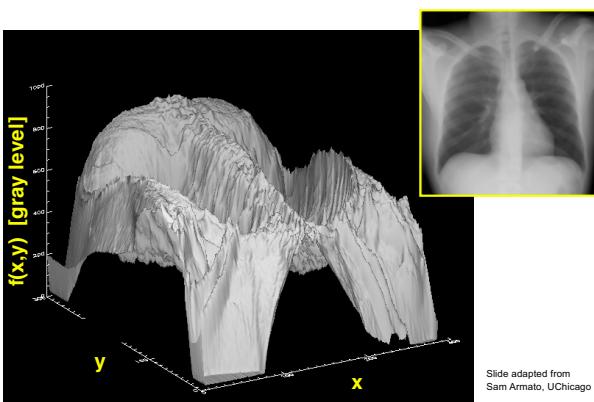
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You can think of them as functions or surfaces.



Slide adapted from Sam Armato, UChicago

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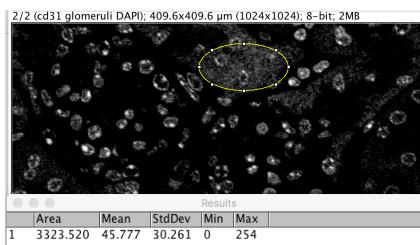
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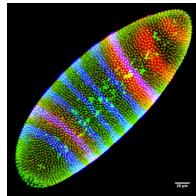
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You can always make quantitative measurements

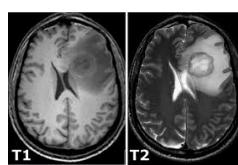


Here I have calculated the mean, standard deviation, and range of values in the oval-shaped region of interest (ROI).

But not all images are absolutely quantitative



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

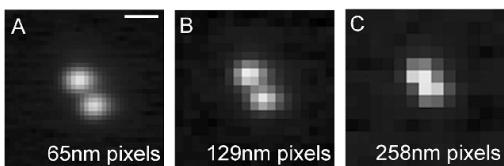


In a T1-weighted MR image, you  
can't extract absolute value of T1  
from pixel values. Only relative.

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

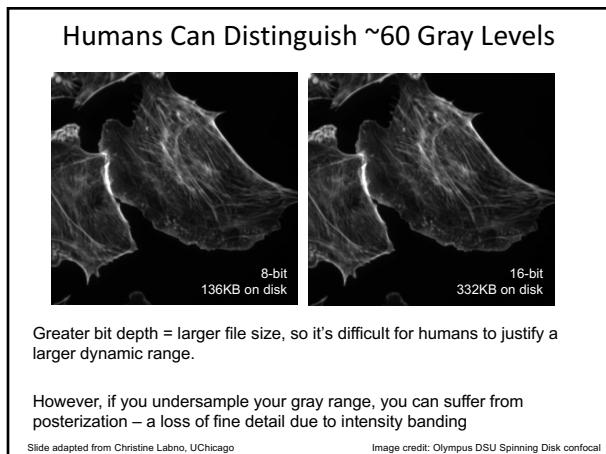
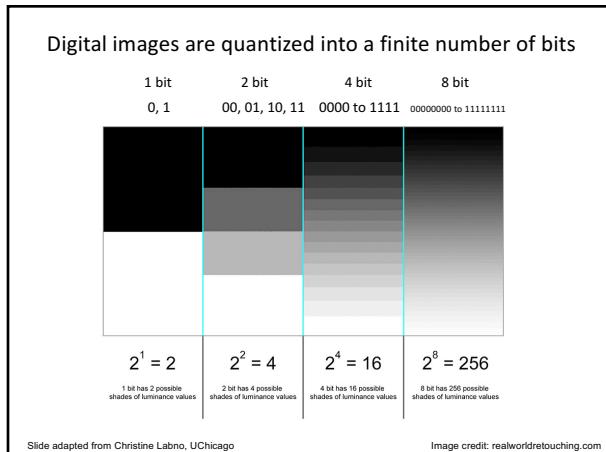
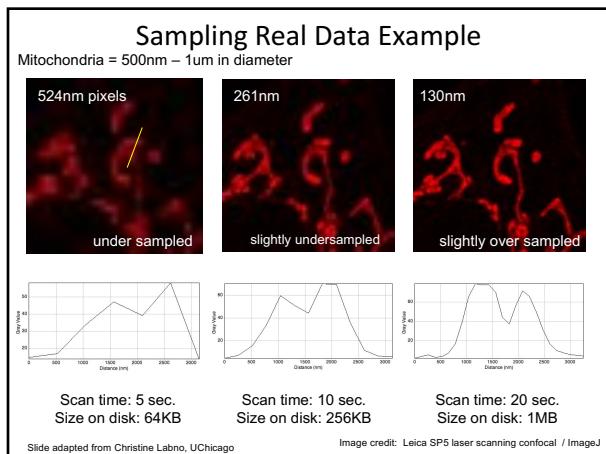
Digital images are sampled

- 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. (We'll learn why later)

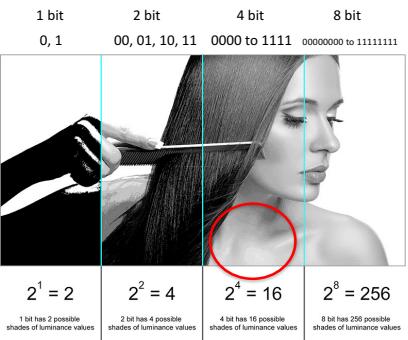


Slide adapted from Christine Labno, UChicago

Image credit: Vytas Bindokas, Light Microscopy Core



## What Happens When You Shrink Bit Depth?

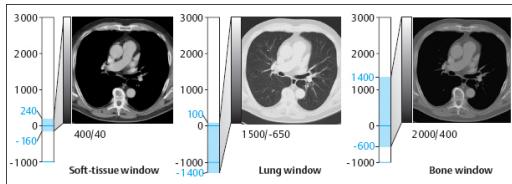


Slide adapted from Christine Labno, UChicago

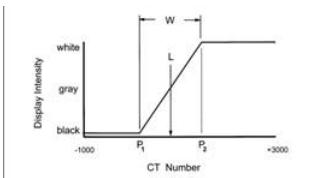
Image credit: realworldretouching.com

## So Eight is Enough?

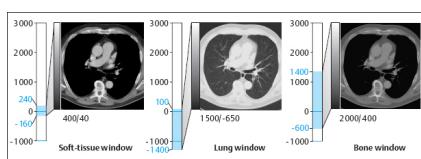
- No, no, no! Not always!
- Quantitatively, there may be statistically significant differences at higher precision.
- You can store more bits and change display mapping to bring out contrast!



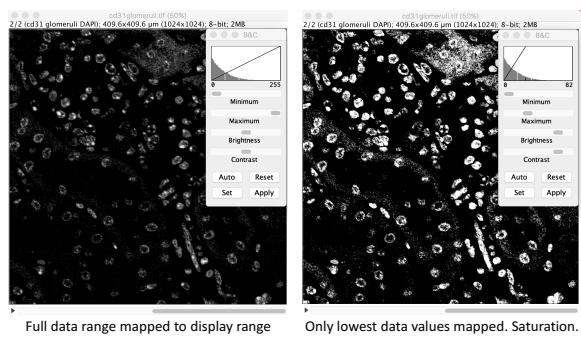
## Display mapping



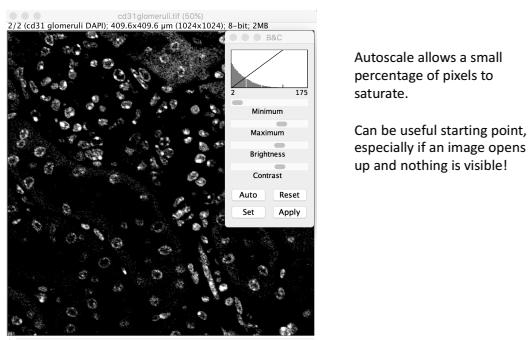
A transformation between stored pixel value and display pixel brightness.



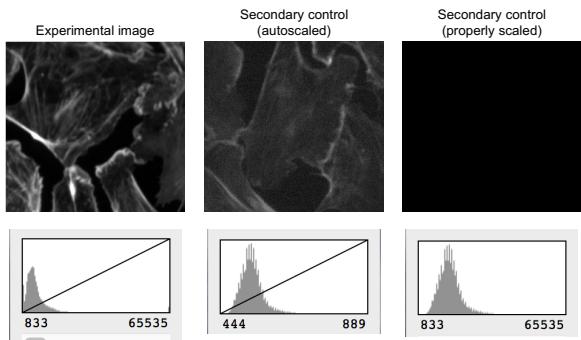
## ImageJ Window/Level example



## ImageJ autoscale



## Dangers of autoscale



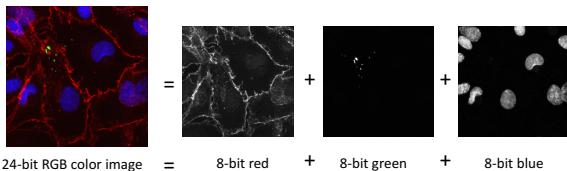
Slide adapted from Christine Labno, UChicago

Image credit: Olympus DSU confocal with histograms from ImageJ

## 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.

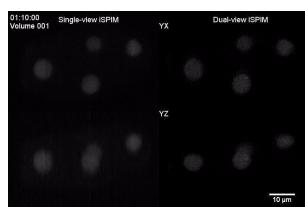


If you collected your raw data as 12-bit or 16-bits per channel, converting to RGB causes a LOSS of information, from 4096 or 16536 down to 256 grays

Slide adapted from Christine Labno, UChicago

Image credit: T. Mirzajalilova

## Why care about storage? Images are the biggest big data



13-hour movie of a worm embryo.

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

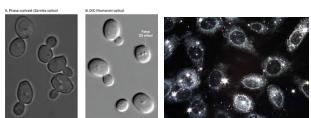
2 Terabytes of data at the end of the experiment=> equivalent of 600,000 photographs!

Data from Hari Shroff, NIH

## Kinds of optical microscopes

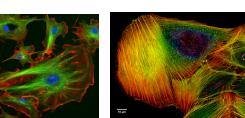
### Label-free

- Transmission
- Phase contrast
- Differential interference contrast
- Darkfield

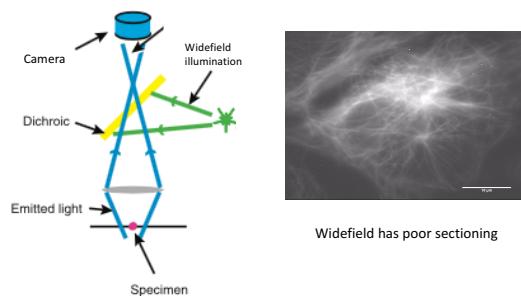


### Fluorescence

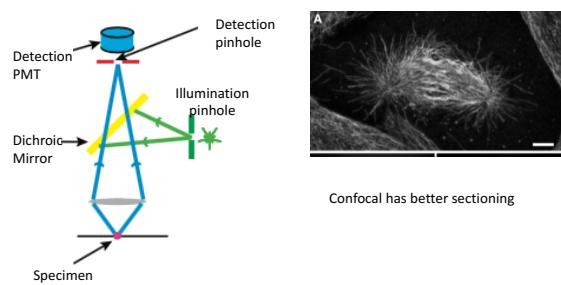
- Widefield
- Confocal
- Total internal reflection
- Two-photon
- Light sheet



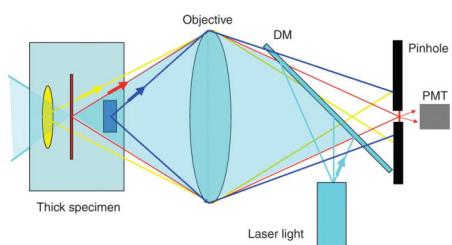
## Widefield fluorescence microscopy



## Confocal fluorescence microscopy

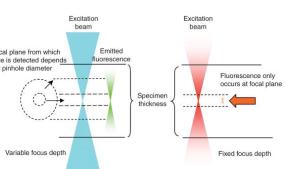
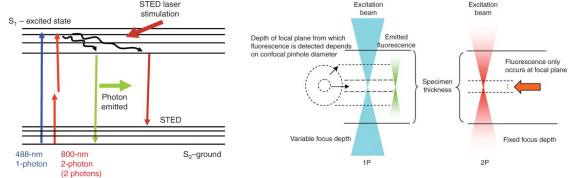


## Confocal principle



From Cold Spring Harb Protoc; doi:10.1101/pdb.top071795

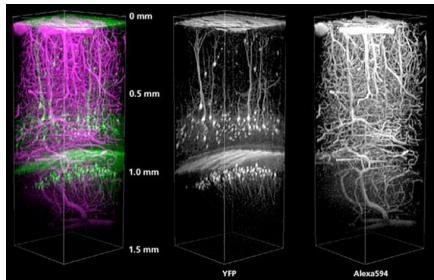
## Two-photon microscope



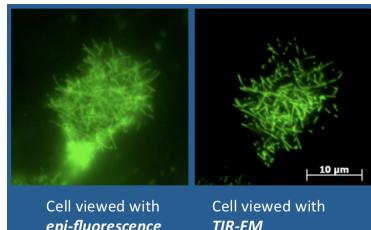
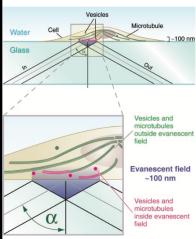
Two photon microscopes can image deeper into scattering tissue.

From Cold Spring Harb Protoc; doi:10.1101/pdb.top071795

## Two-photon microscope

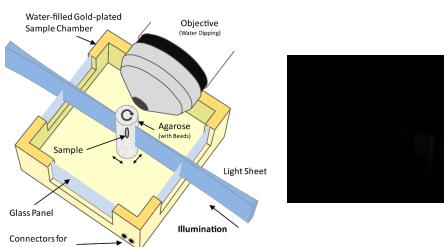


## TIRF microscopy



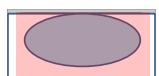
TIRF microscopes give excellent sectioning right at the cover slip.

## Light sheet microscopy

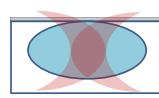


Light sheet microscopes are very dose efficient.

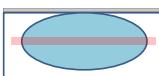
## How to optimally image fluorescence?



**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

Slide adapted from Hari Shroff

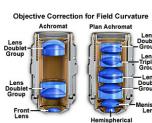
## Computational imaging: computing to form images

Better microscopes used to mean better glass



Leuwenhoek microscope  
c. late 1600s

Modern corrected  
objective lenses



Now better microscopes = glass + math

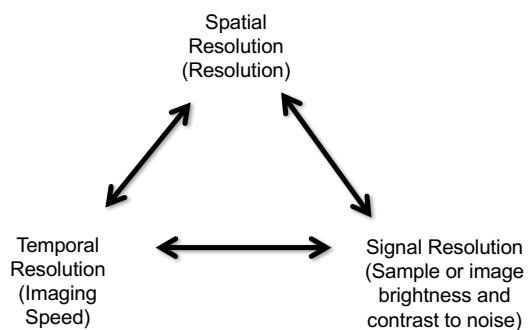


Test object      Structured illumination of test object

Using math to extend the resolution



### 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).

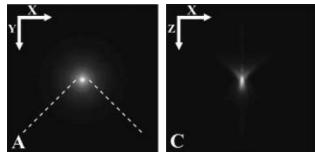
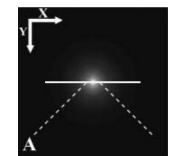
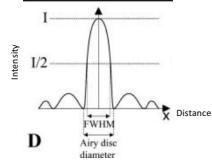


Image credits: (top) René Magritte "The Treachery (Treason) of Images" (bottom) Bolte and Corderieres (2006) J. Microscopy v. 224 pt3, pg. 214

### The point spread function, aka Airy pattern



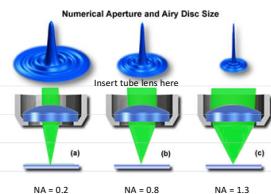
The better the spatial resolution of your system, the smaller the PSF/Airy pattern.



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?



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

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

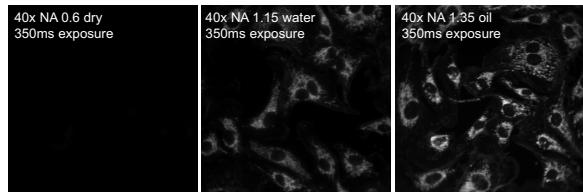
Where  $\lambda$  is the wavelength of light used for imaging

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

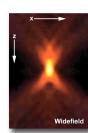
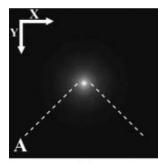


Increasing NA

Slide adapted from Christine Labno, UChicago

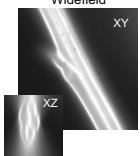
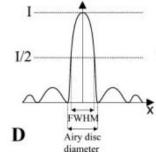
## Increasing Resolution Beyond Widefield

- Use confocal microscopy



$$d = \text{or} > 0.61 \lambda / \text{NA}$$

Widefield



Slide adapted from Christine Labno, UChicago

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

**A**

**D**

$d = \text{or} > 0.61 \lambda / \text{NA}$  Widefield

$d = \text{or} > 0.4 \lambda / \text{NA}$  Confocal

FWHM  
Airy disc diameter

Slide adapted from Christine Labno, UChicago 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

Image stack  $\frac{1}{\otimes} \text{PSF}$  Object

Deconvolution  $\rightarrow$

Widefield XY XZ

Confocal XY XZ

Widefield + Deconvolution XY XZ

Confocal + Deconvolution XY XZ

Slide adapted from Christine Labno, UChicago Image credits: (left) Greb, C. leica-microsystems.com/science-lab/deconvolution/

### 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**

20nm beads

100nm beads

Slide adapted from Christine Labno, UChicago 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

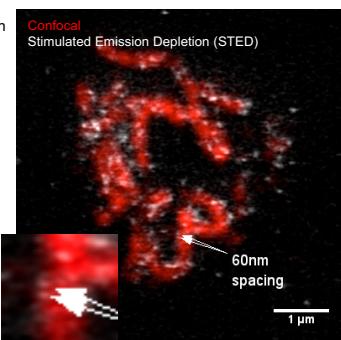
Pioneered by Stefan Hell  
50nm lateral, 100-400nm axial

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

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

Slide adapted from Christine Labno, UChicago

Image credits: V. Cloud, Bishop lab

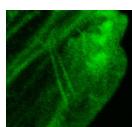
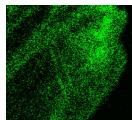


## Summary: Spatial Resolution

- 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

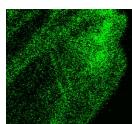
- Your sample needs to be bright enough and have enough difference between bright and dark that there is useful contrast.
- How much contrast is enough?
- It needs to be large compared to the noise (random fluctuations) in the image.



## Sources of noise

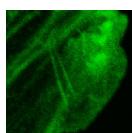
- Photo counting statistics (aka shot noise).
  - Follows Poisson statistics, so variance = mean number detected counts.

$$SNR = \frac{MEAN}{\sqrt{VARIANCE}} = \frac{N}{\sqrt{N}} = \sqrt{N}$$



– 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)

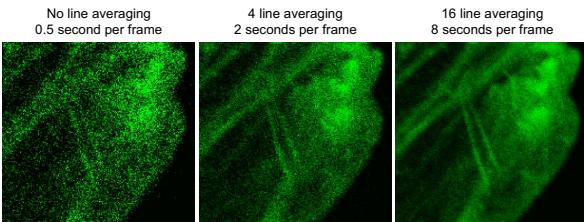


Image credit: Leica SP2 confocal

## Boosting Signal in Your Samples

- And finally, bin your pixels. A 2x2 bin will add the signal of 4 pixels together, boosting signal (and increasing imaging speed) but sacrificing spatial resolution.

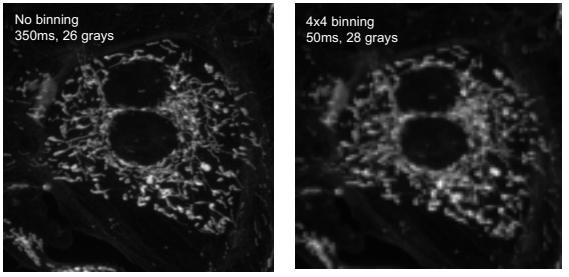


Image credit: Olympus DSU confocal

## 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.

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## 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.




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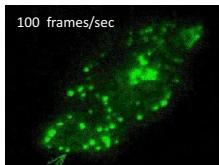
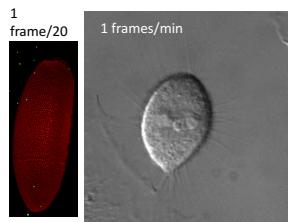
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## Two Types of Live Sample Imaging

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.

Image credits upper right: BioPhysics grad students, Hammond lab, upper left: Misha Ludwig, Kreitman/White labs, lower: Li Ma, Phillipson lab

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### 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

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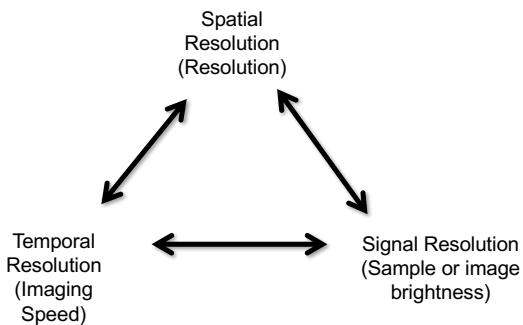


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### Optimization: Pick Two, Sacrifice the Third




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### 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.

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## 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

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## Tradeoffs – A Real World Example

Granule exocytosis speed: 20 msec.

Camera speed: 15 frames / sec

Granule dia.: 300-350nm

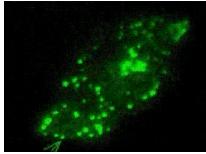
Pixel size on chip: 6.45μm

Objective: 60x NA 1.45

Actual pixel size: 107.5nm

Insulin granules are small and release their contents very quickly.

Release events last for approx. 20 msec, so for adequate sampling we need a frame rate of 2 frames every 20 msec, or 100 frames per second.



The camera that we used (Retiga EXi Blue from Q Imaging) has a maximum capture rate of 15 frames per second -- not fast enough to adequately sample our granule exocytosis.

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## Tradeoffs – A Real World Example

Granule exocytosis speed: 20 msec.

Camera speed: 120 frames / sec

Granule dia.: 300-350nm

Pixel size on chip: 12.9μm

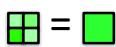
Objective: 60x NA 1.45

Actual pixel size: 215nm

In order to image more quickly, we can do two things:

- 1) Crop the area being imaged from 1392 x 1040 down to 696 x 520 (1/4 of the chip) increasing readout speed twofold to 30 frames per second

- 2) Bin the pixels. A 2x2 bin reads out a group of 4 pixels as though they are 1.



This binning increases imaging speed by 4x, giving us 120 frames / second.

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## Tradeoffs – A Real World Example

Granule exocytosis speed: 20 msec.

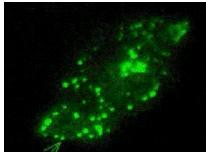
Camera speed: 120 frames / sec

Granule size: 300-350nm

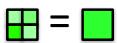
Pixel size on chip: 12.9um

Objective: ???

Actual pixel size: need  $\leq$  150nm



Unfortunately, binning increases our pixel size on chip from 6.45um to 12.9um, decreasing our resolution.



So we must increase our magnification until we can sample our granules adequately (if possible).

Given granule size and pixel size on chip, how much magnification do we need to sample adequately?

## 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

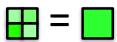
Obj.: 100x NA 1.4 or 150x NA 1.4

Actual pixel size: 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

