

# Welcome to the Imaging and Image Processing Tutorial

QBio<sup>^</sup>2 Bootcamp  
September 7, 8, 9, 2016

# The Plan

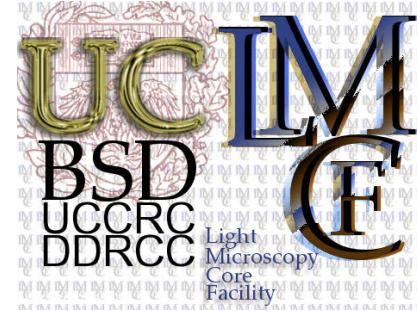
- 8:30 - 9:20am Lecture
- 9:20 - noon Rotate groups across the street to view samples under the microscope with Vicky. Groups / times are on the board. Every group gets 40 min. at the microscope.
- When you're not there, work here on the ImageJ problem set / go upstairs to LB302 for coffee / at least 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: advise from Christine and Hannah, Christine'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 Christine will go over problem sets on the screen, probably a couple of times so everyone gets to see the solutions
- 12:00pm Lunch

# A Field Guide to Resolution in Imaging

Christine Labno, PhD

Technical Director

Univ. of Chicago Light Microscopy Core Facility



# A Plug for the University Cores

- Office of Shared Research Facilities
- <http://osrf.uchicago.edu>
- 27 Cores including light microscopy, electron microscopy, sequencing, flow cytometry, transgenics and tissue processing
- “Centralized areas of technology and expertise” available to the entire University community
- Core Fair October 19, 2016 – FREE FOOD!!!



# What Do We Mean by “Resolution”?

Resolution – a. Originally: the effect of a telescope in making the stars of a nebula distinguishable by the eye. Later more widely: the process or capability of rendering distinguishable the component parts of an object or image; a measure of this, expressed as the smallest separation so distinguishable, or as the number of pixels that form an image; the degree of detail with which an image can be reproduced

b. The ability of a device to respond to small differences in input and to indicate or represent them accurately in output; a measure of this, expressed as the smallest difference so distinguishable. More widely: discrimination or distinction between things that are close together in space or time (OED)

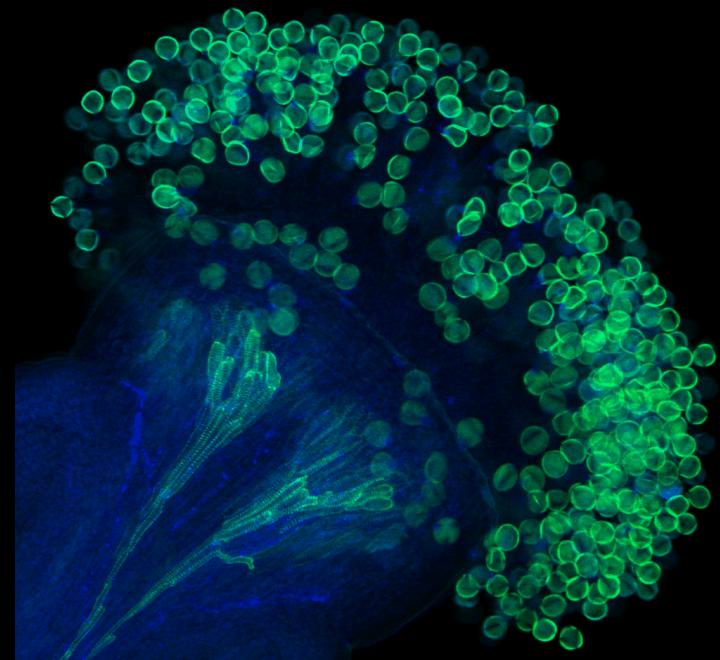
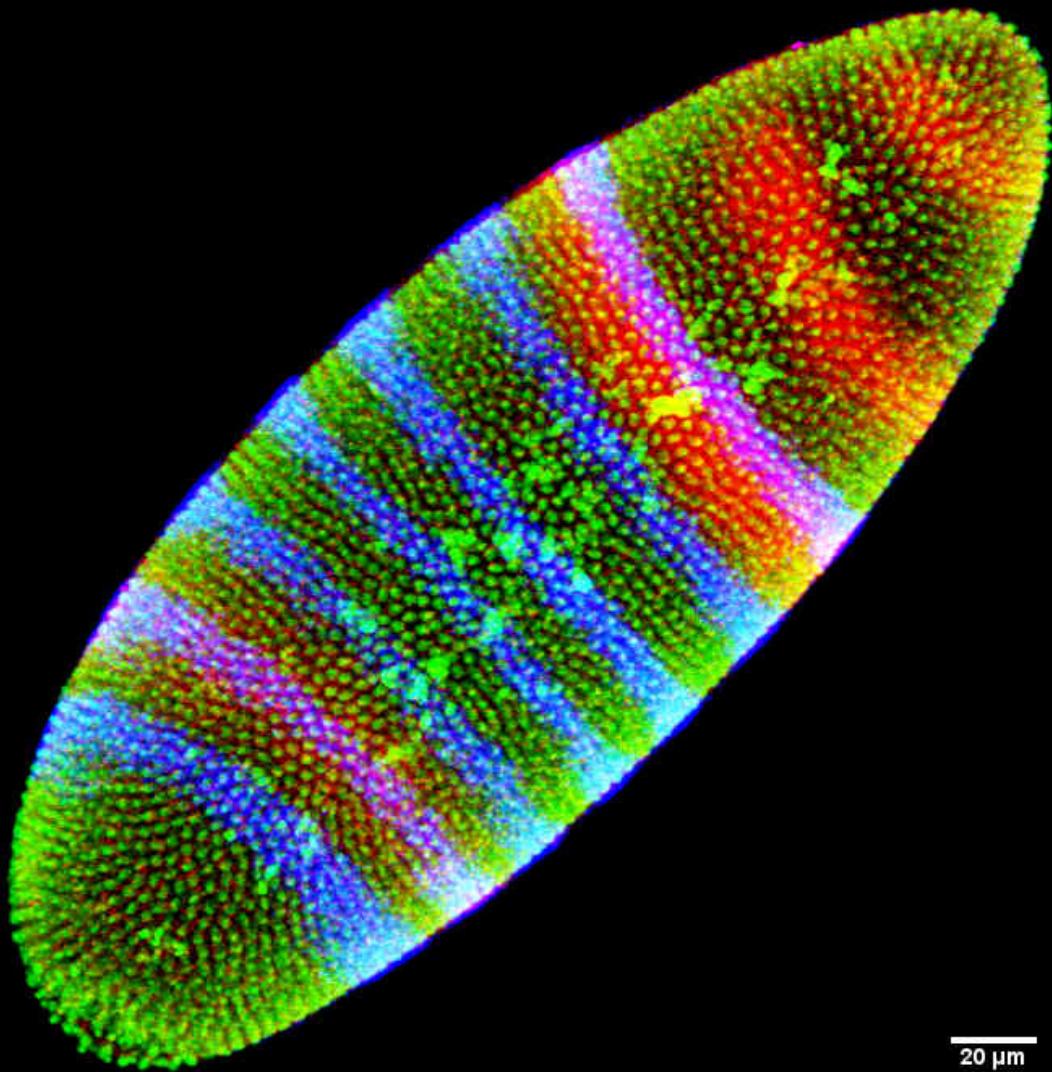


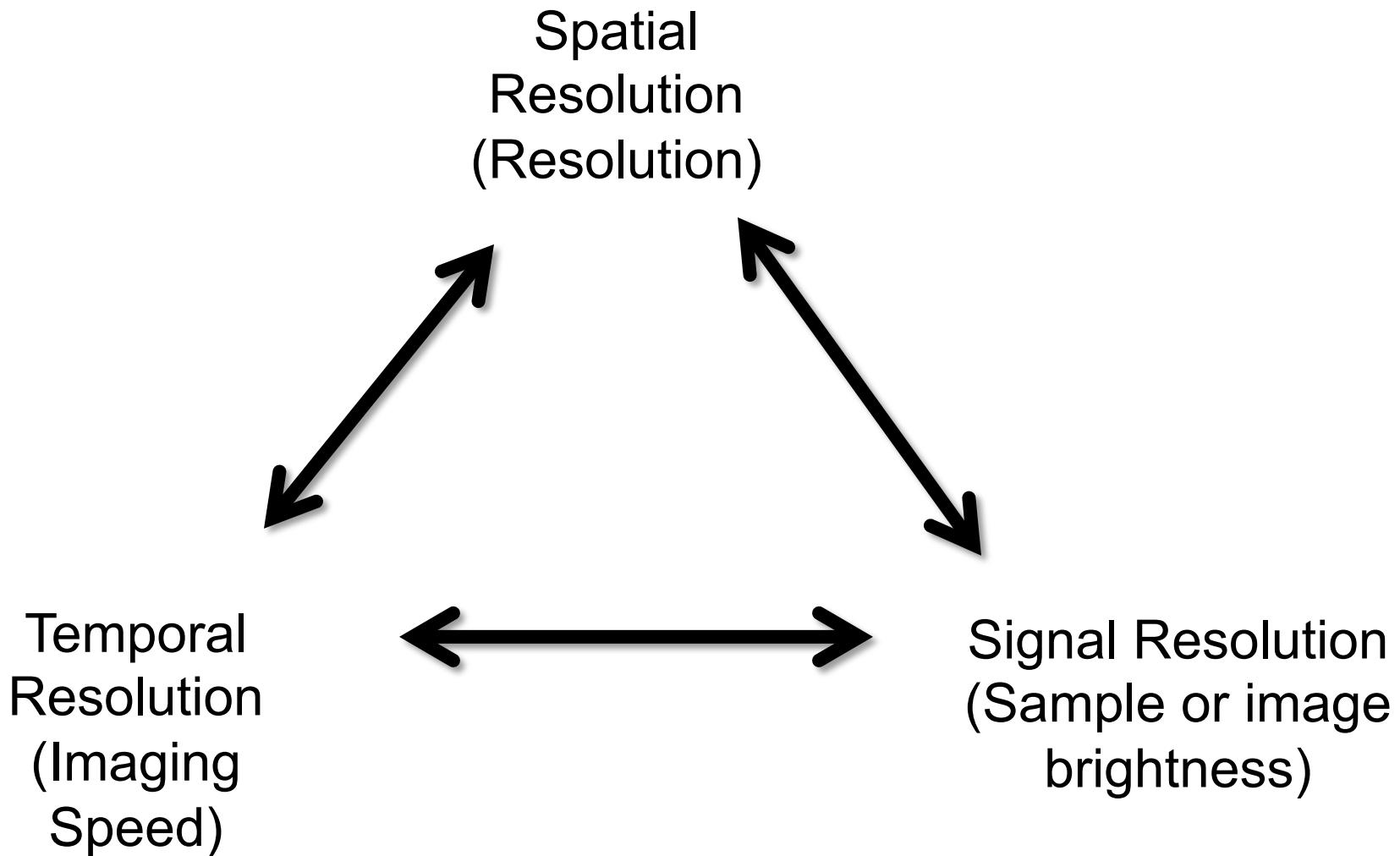
Image credit: Adam Hammond

# Three Kinds of Resolution



- Spatial – XY and Z, tells us where
- Signal – intensity, tells us how much
- Temporal – time, tells us when

# These Three Factors are Interdependent



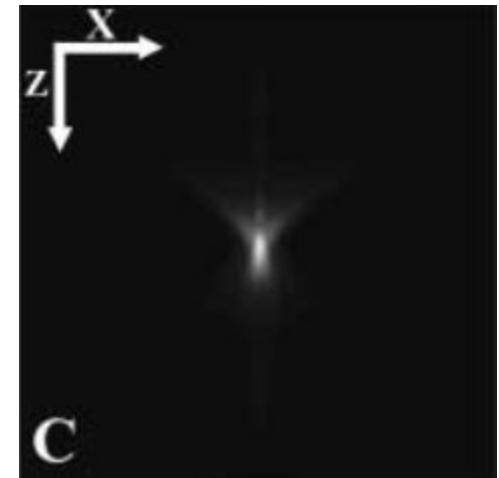
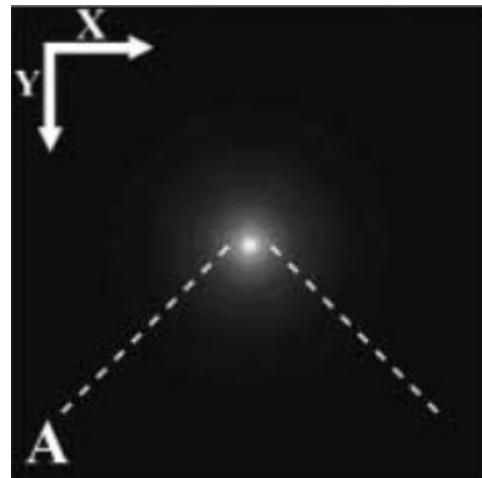
# Images are Representations of Objects



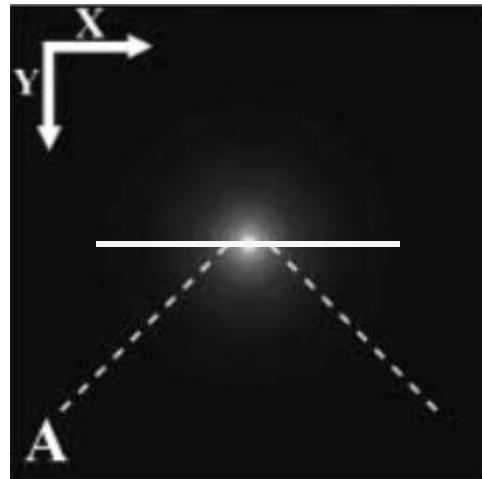
If it's not a pipe, what is it?

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

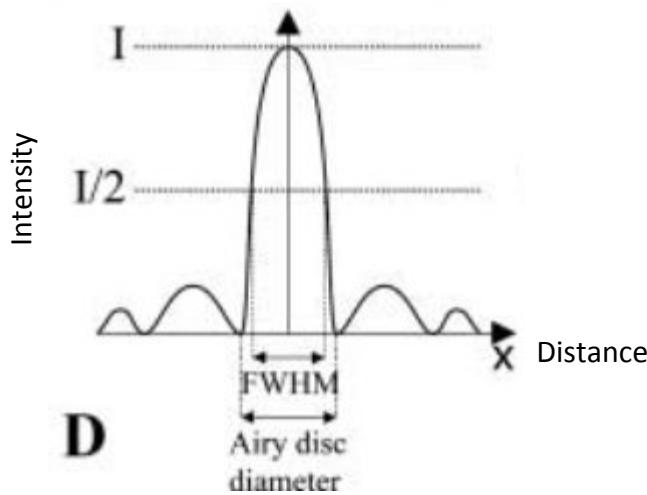
The image of a point is not a point, it is a pattern of light called a point spread function (PSF).



# The Airy Pattern and Airy Disk

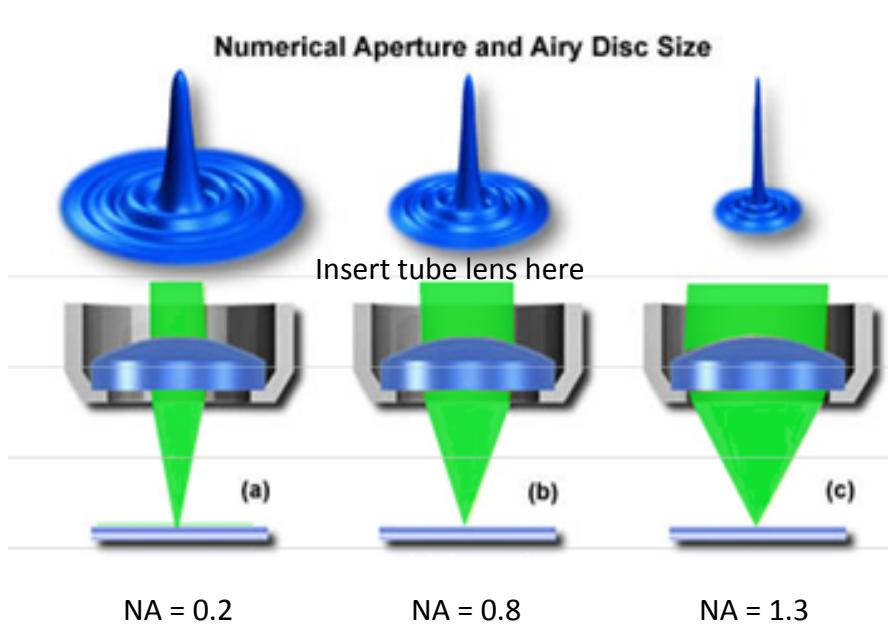


The better the spatial resolution of your system, the smaller the PSF/Airy pattern.



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

# What Determines the Size of the Airy Disk?



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

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

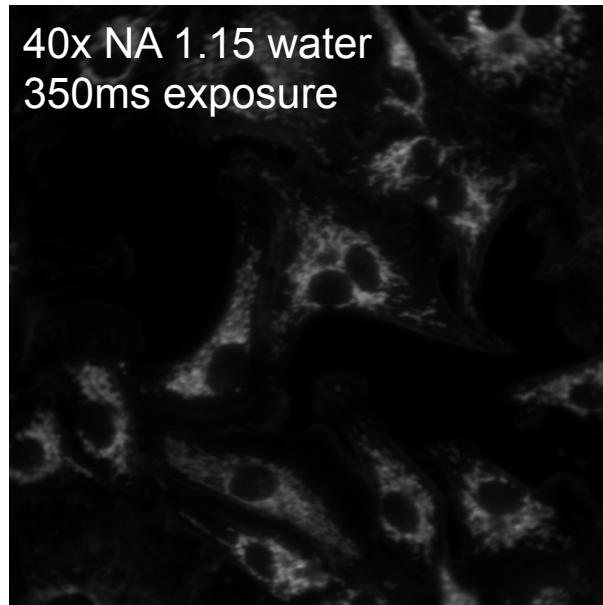
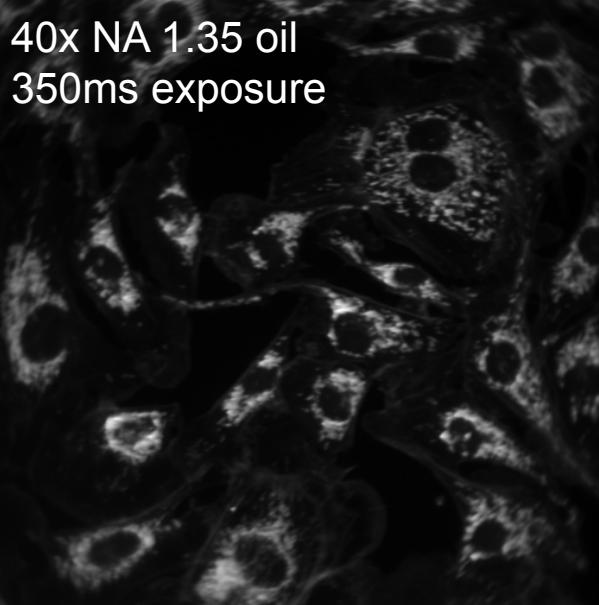
Where  $\lambda$  is the wavelength of light used for imaging

NA is the numerical aperture of the objective

These equations were first published by Ernst Abbe in 1873



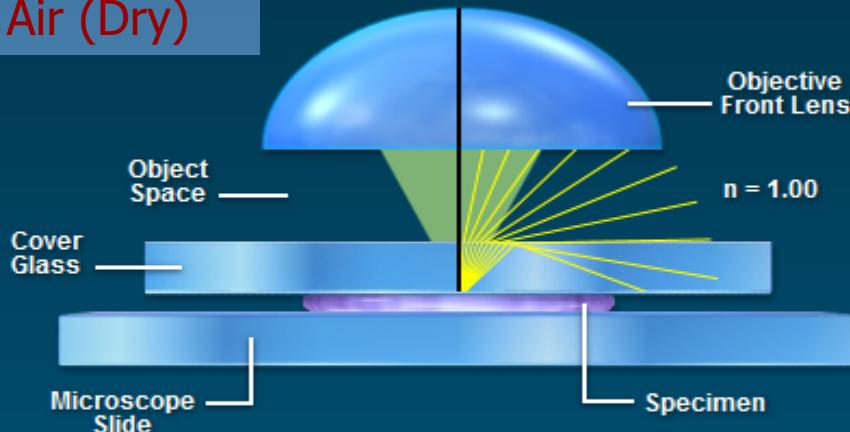
# Higher Numerical Aperture = Brighter, Sharper Pictures



# Immersion Media and the Light Path

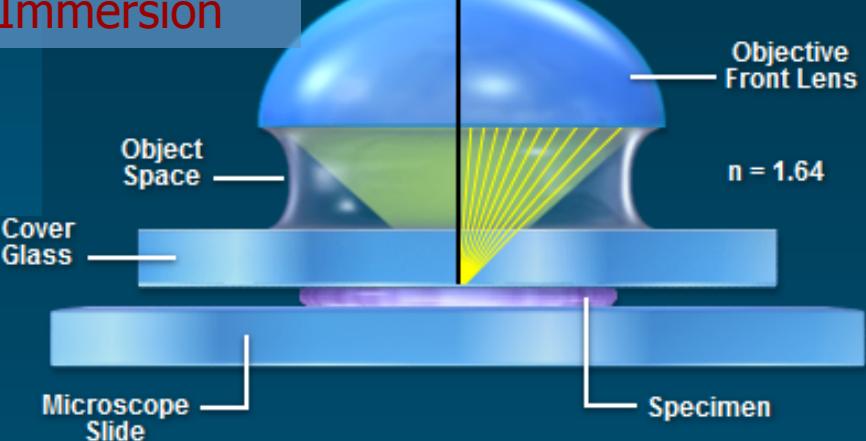
## Oil Immersion and Refractive Index

Air (Dry)



$$\begin{aligned} \text{Numerical Aperture (NA)} &= n \sin(\theta) \\ \text{Angular Aperture} &= \theta = 65^\circ \\ 0.90 &= 1.00 \sin(65^\circ) \end{aligned}$$

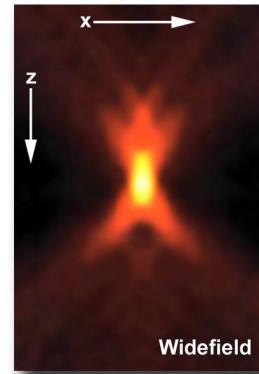
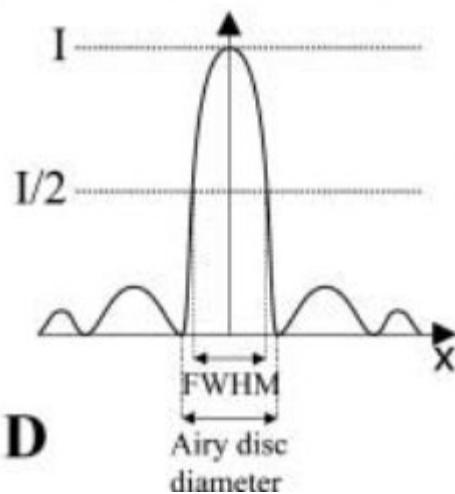
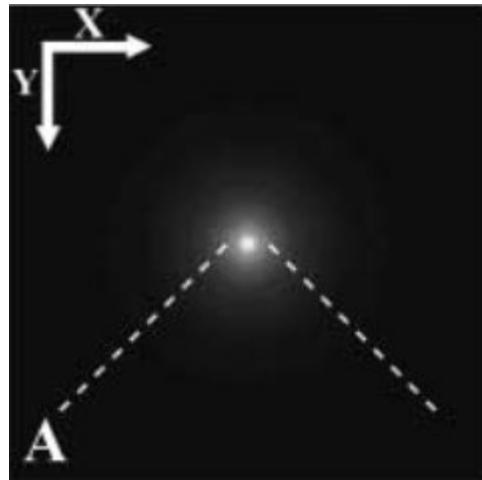
Immersion



$$\begin{aligned} \text{Numerical Aperture (NA)} &= n \sin(\theta) \\ \text{Angular Aperture} &= \theta = 65^\circ \\ 1.49 &= 1.64 \sin(65^\circ) \end{aligned}$$

# Increasing Resolution Beyond Widefield

- Use confocal microscopy



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

Widefield

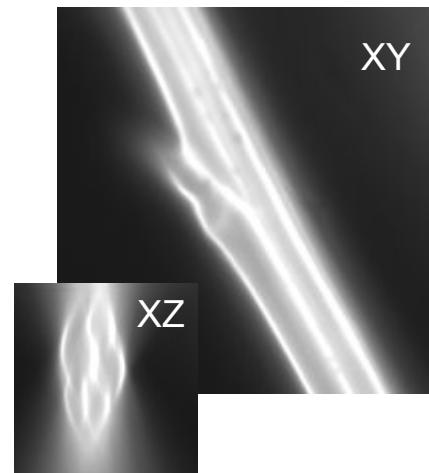


Image credits: (top) Claxton et al [www.olympusconfocal.com/theory/LSCMIntro.pdf](http://www.olympusconfocal.com/theory/LSCMIntro.pdf)  
(bottom) Olympus DSU spinning disk confocal

# Increasing Resolution Beyond Widefield

- Use confocal microscopy

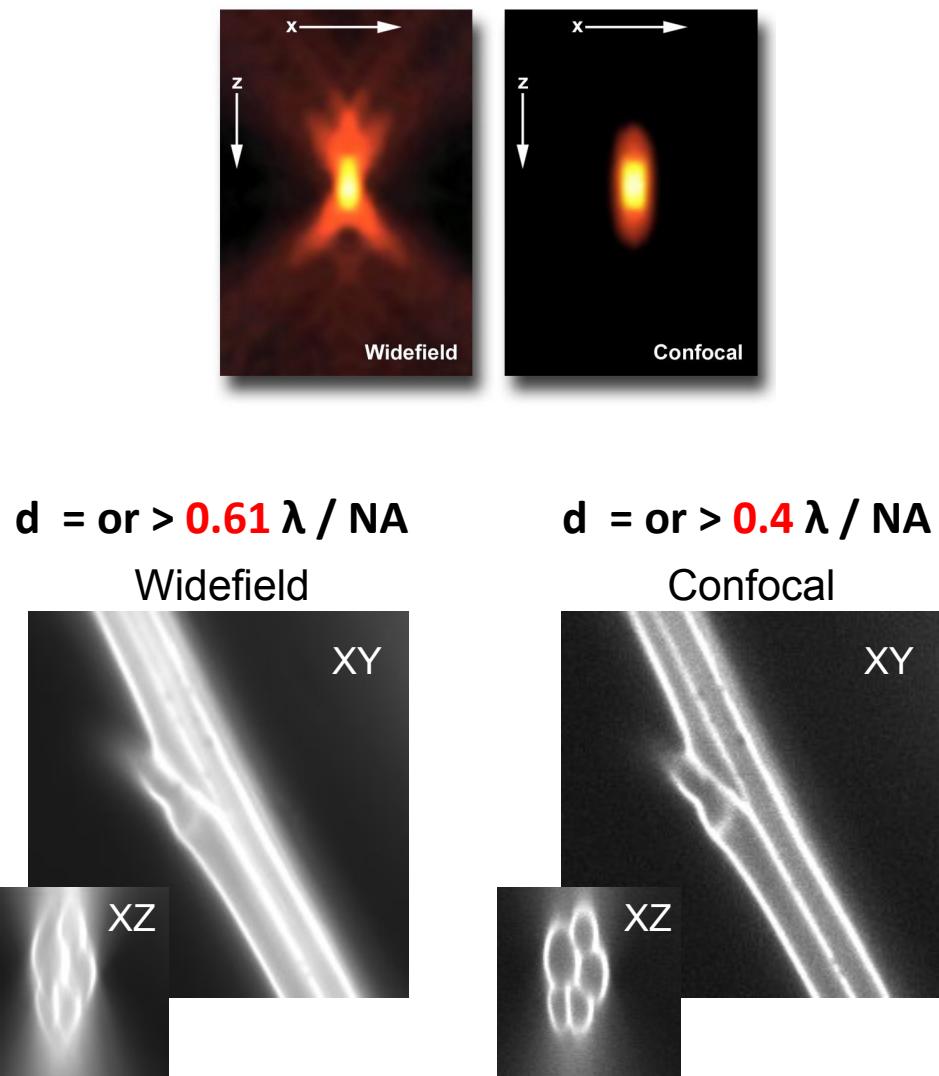
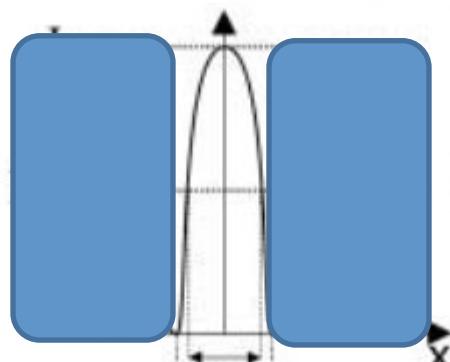
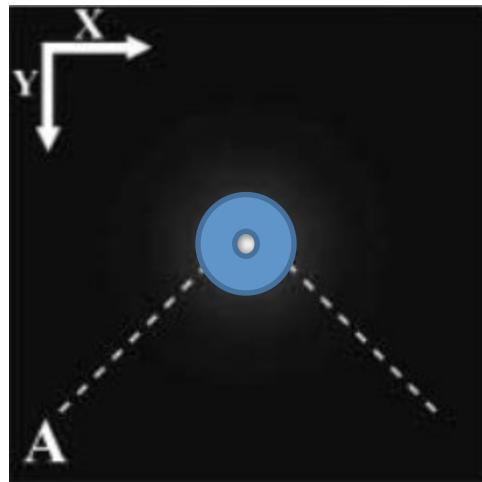
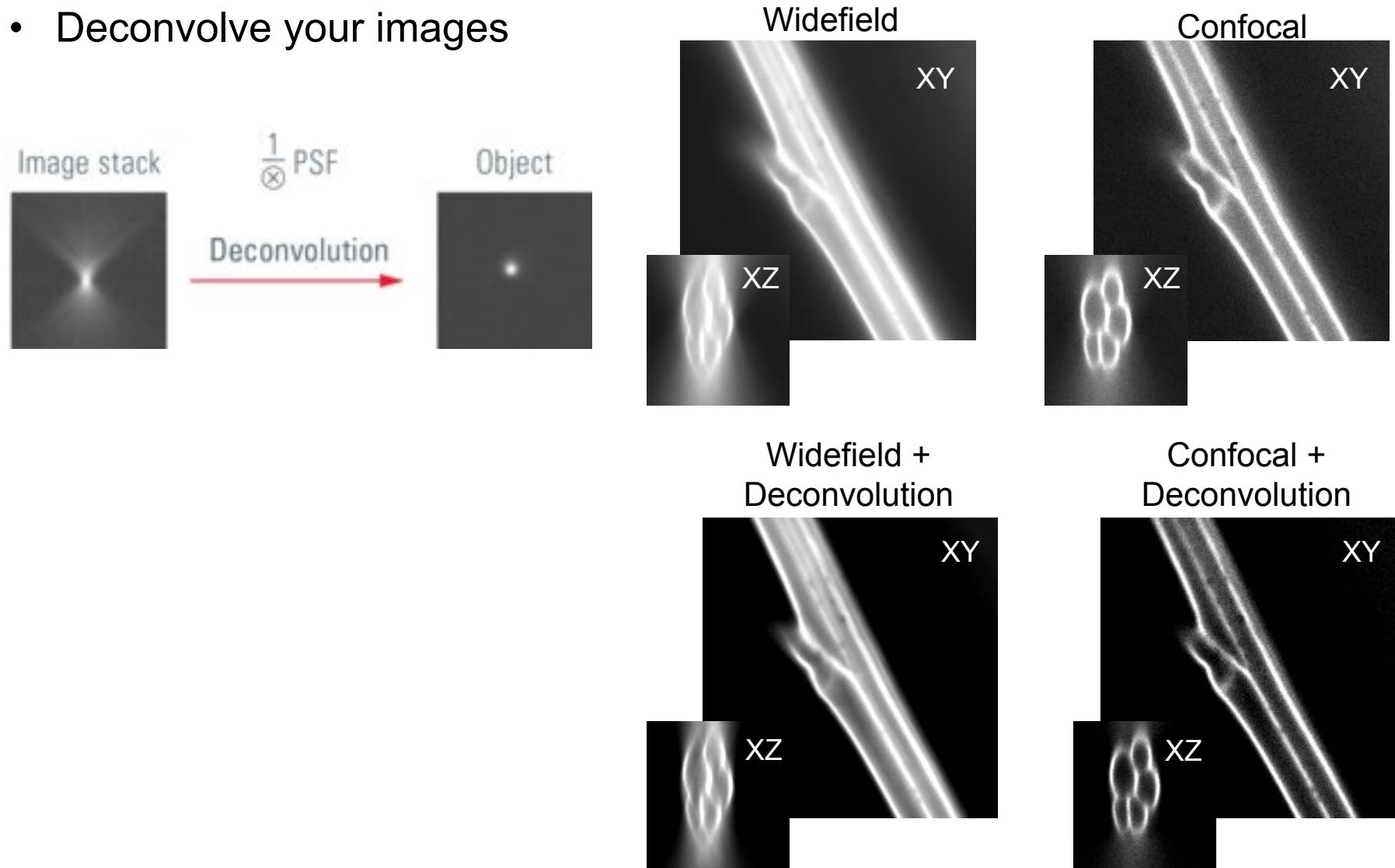


Image credits: (top) Claxton et al [www.olympusconfocal.com/theory/LSCMIntro.pdf](http://www.olympusconfocal.com/theory/LSCMIntro.pdf)  
(bottom) Olympus DSU spinning disk confocal

# Increasing Resolution Beyond Confocal

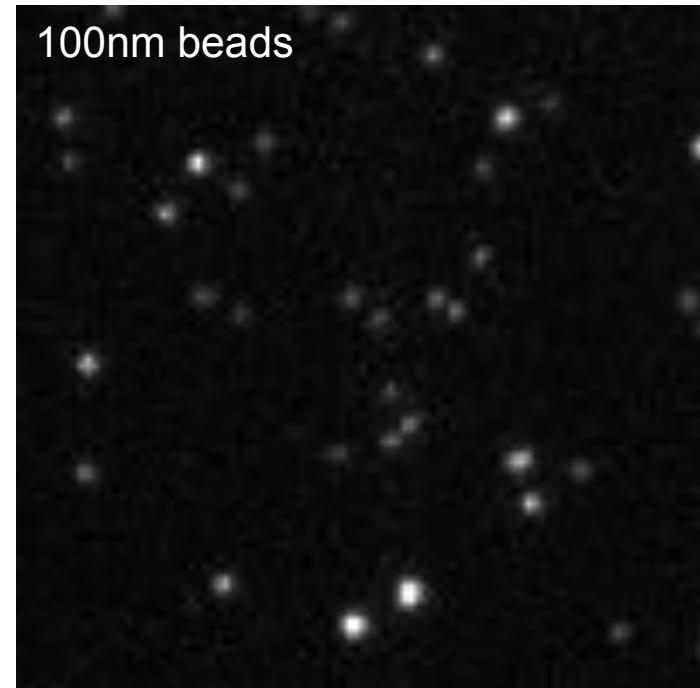
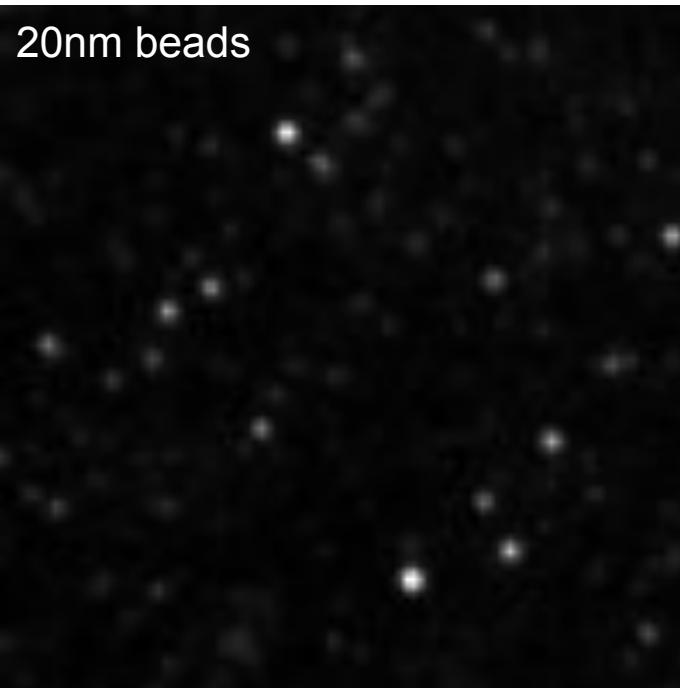
- Deconvolve your images



# 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**



# 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

**More info about these at the back of the .pdf**

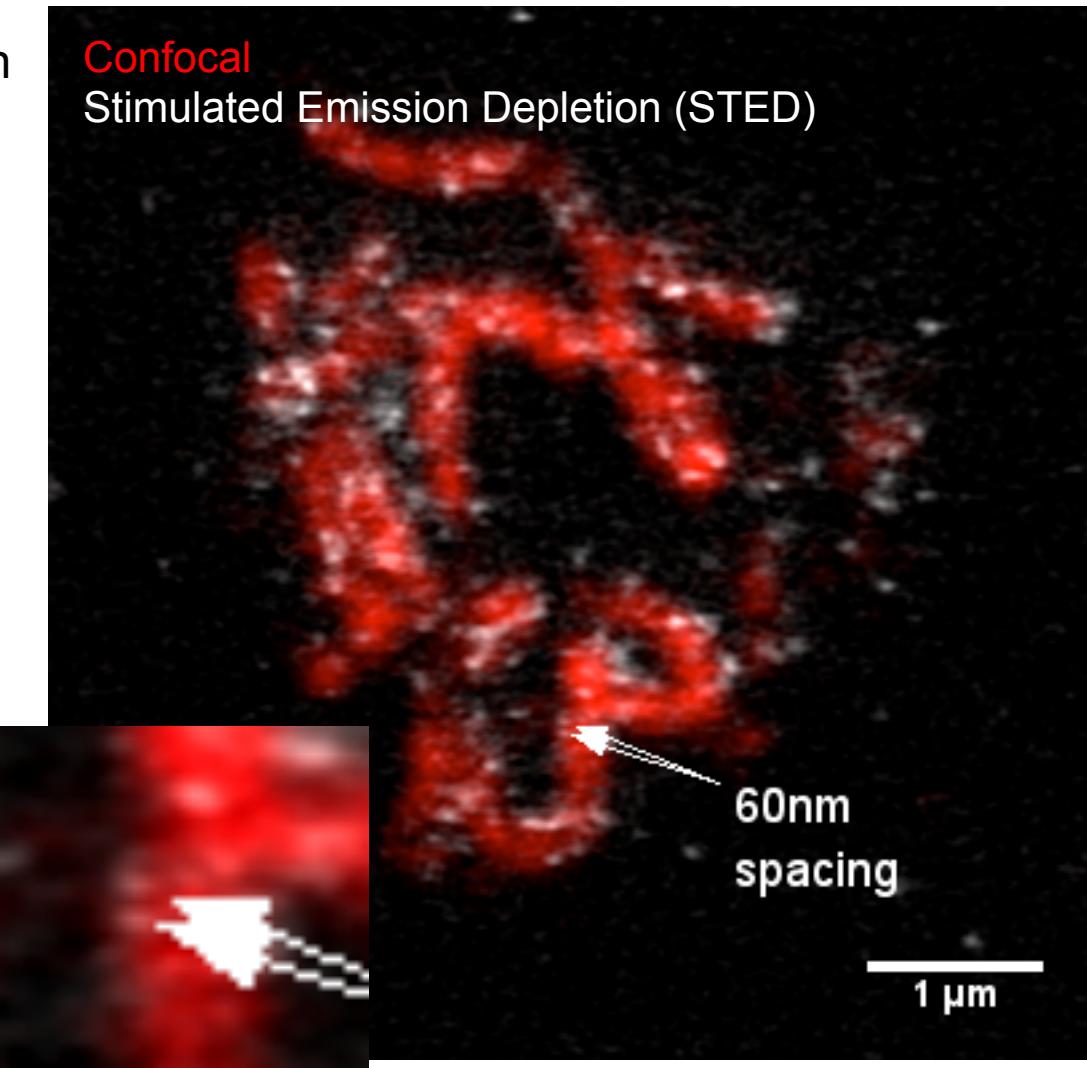
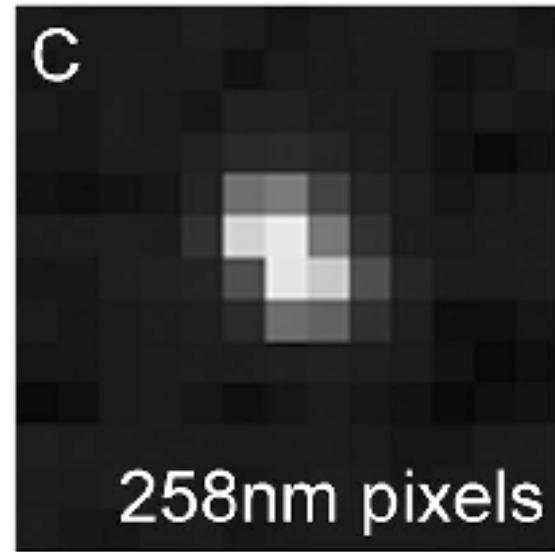
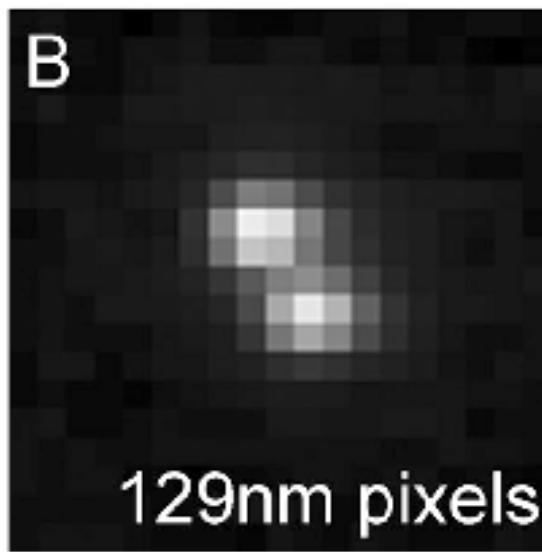
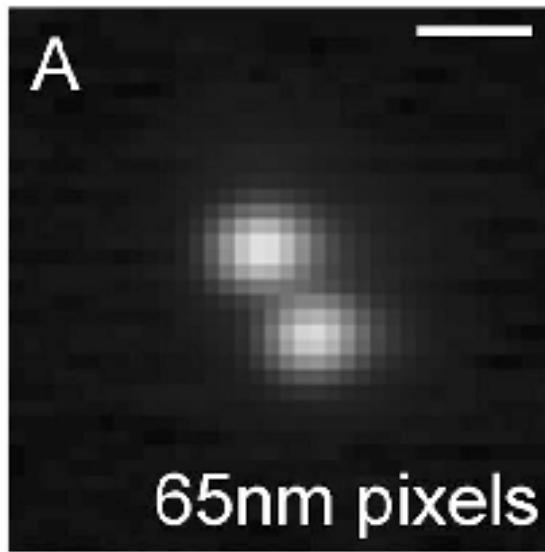


Image credits: V. Cloud, Bishop lab

# Digital Imaging – PSFs to Pixels

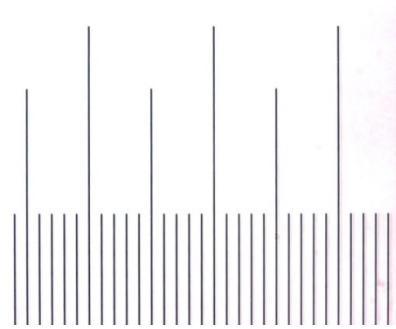
In order to do science, we must convert our observations into something we can share / quantify.

How we convert our PSFs to pixels effects resolution and is as important to accuracy of our measurements as our optical imaging parameters.



# What Determines Pixel Size?

Pixel size is determined by the size of the well on the camera chip and the magnification of the optical path



**Image pixel size =**  
**chip pixel size / mag. of optical path**

test - Position 1 - 1_C350.log			
File Edit Font			
Export Date-Time:	05/21/2013 13:14:42		
Capture Date-Time:	5/20/2013 15:38:12		
Z Planes:	1		
Time Points:	1		
Channels:	2		
Microns Per Pixel:	1.6		
Z Step Size Microns:	0		
IFD	X Position (um)	Y Position (um)	
0	13178.6	-4536.8	3622.92
0	13178.6	-4536.8	3622.92

1 pixel =

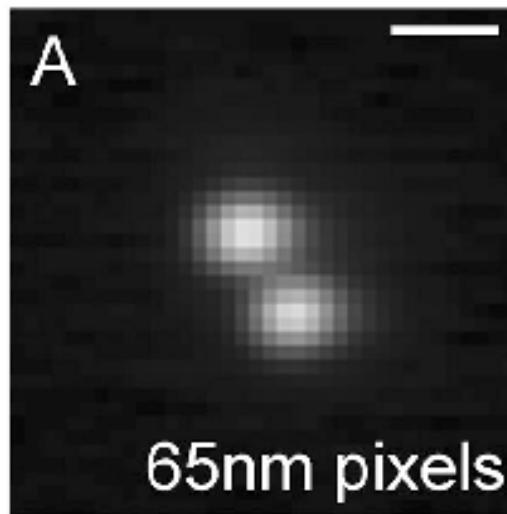
- 2x 8.00um
- 4x 4.00um
- 10x 1.60um
- 20x 0.800um
- 40x 0.400um
- 60x 0.270um
- 100x 0.160um
- 150x 0.107um

# The Nyquist-Shannon Theorem

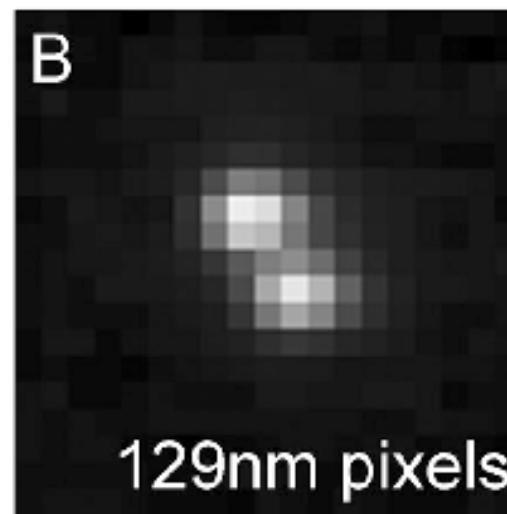
How do we know what pixel size is adequate for sampling our image?

The Nyquist-Shannon Sampling Theorem says that in a perfect world, a dataset with a given frequency can be accurately reconstructed if sampled at a rate greater than twice its frequency.

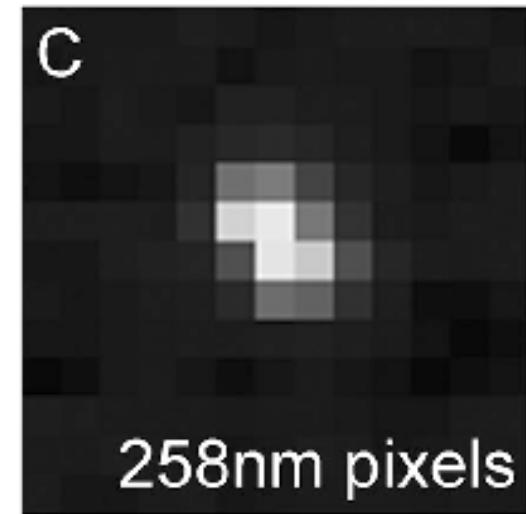
When applied to imaging, it is generally taken to mean that there should be no fewer than 2 pixels per PSF, or put another way, your pixel size should be no larger than half the size of your smallest resolvable feature.



65nm pixels



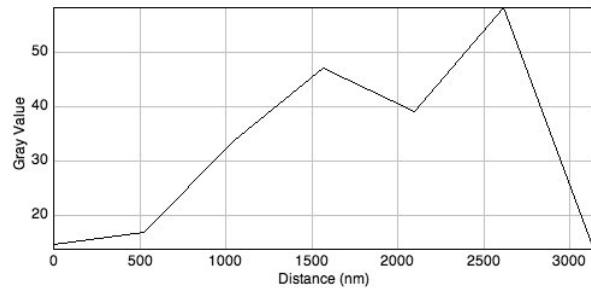
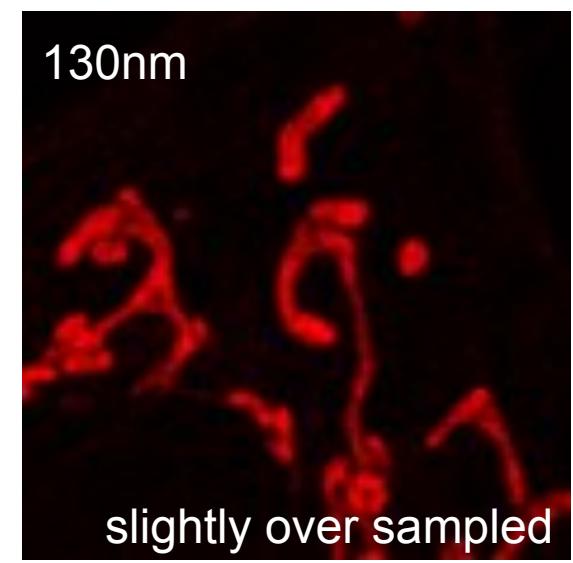
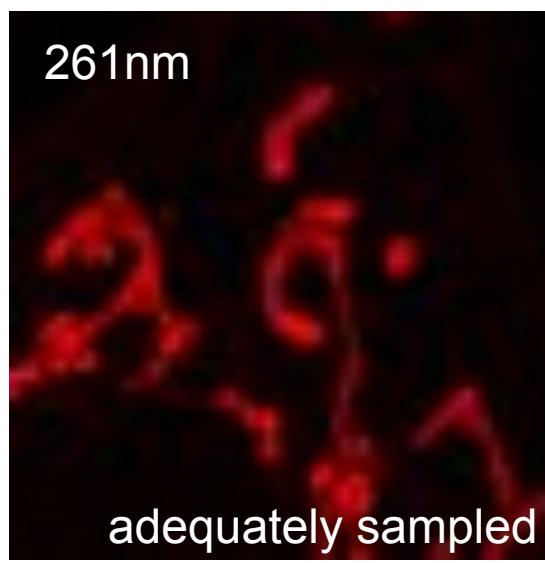
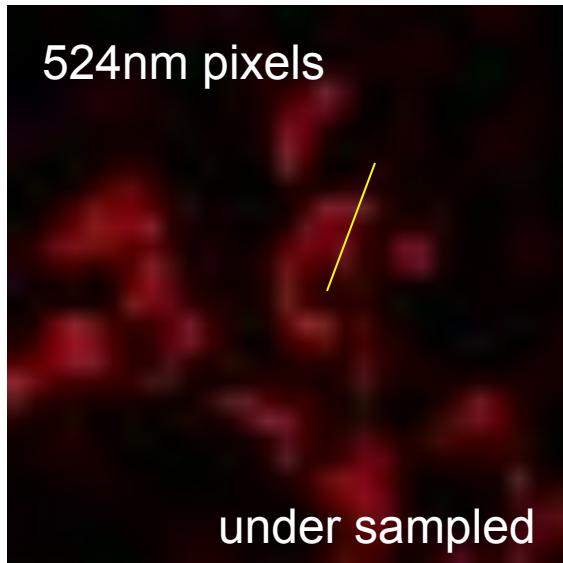
129nm pixels



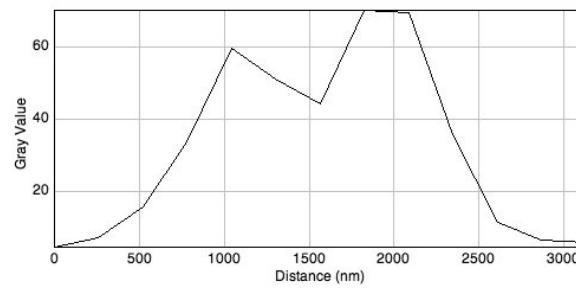
258nm pixels

# Real Data Example

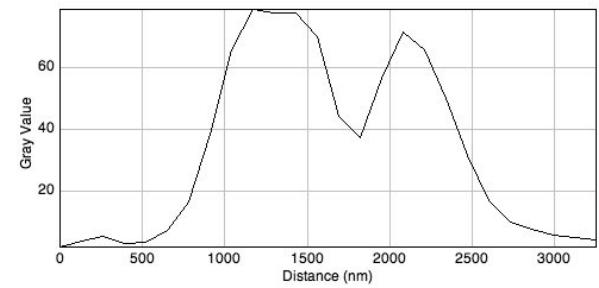
Mitochondria = 500nm – 1um in diameter



Scan time: 5 sec.  
Size on disk: 64KB



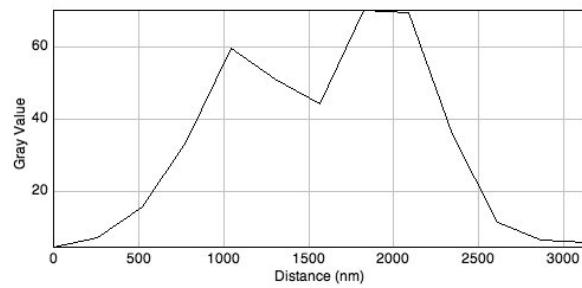
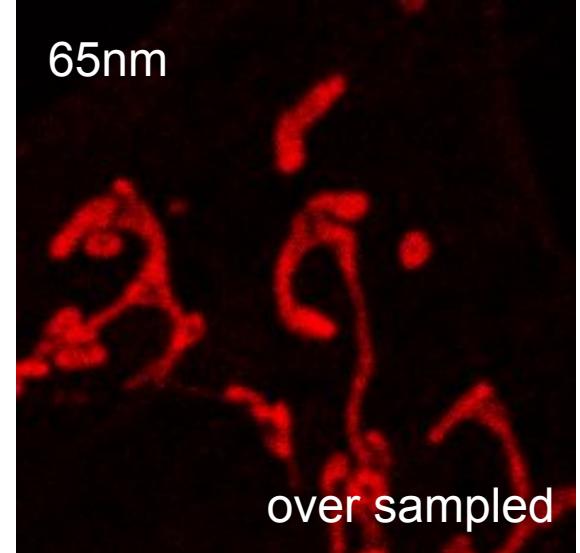
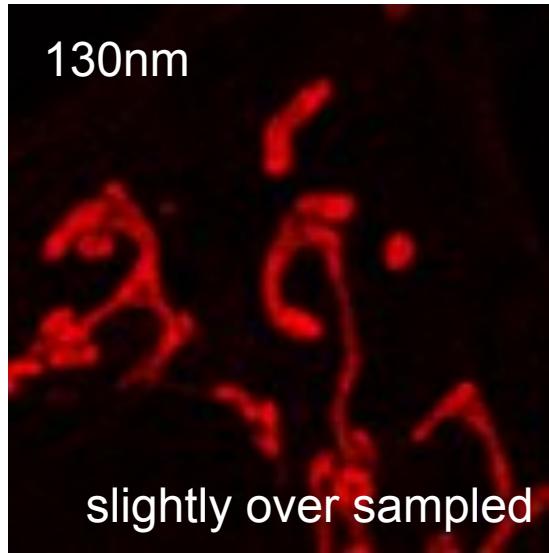
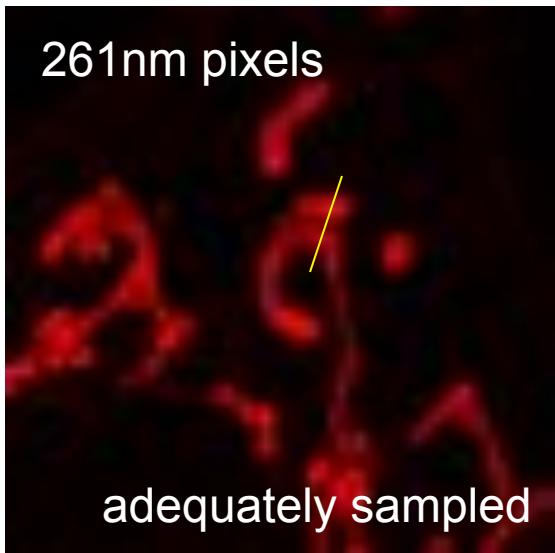
Scan time: 10 sec.  
Size on disk: 256KB



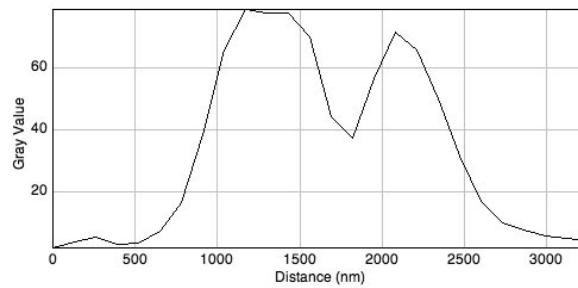
Scan time: 20 sec.  
Size on disk: 1MB

# The Perils of Massive Oversampling

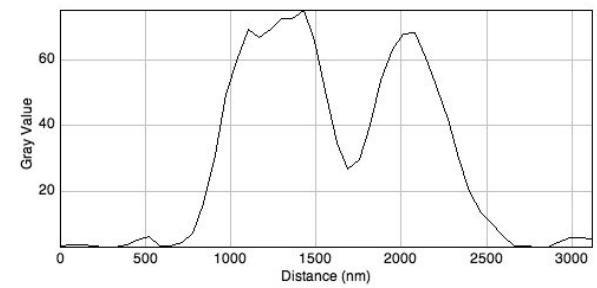
Mitochondria = 500nm – 1um in diameter



Scan time: 5 sec.  
Size on disk: 64KB



Scan time: 10 sec.  
Size on disk: 256KB

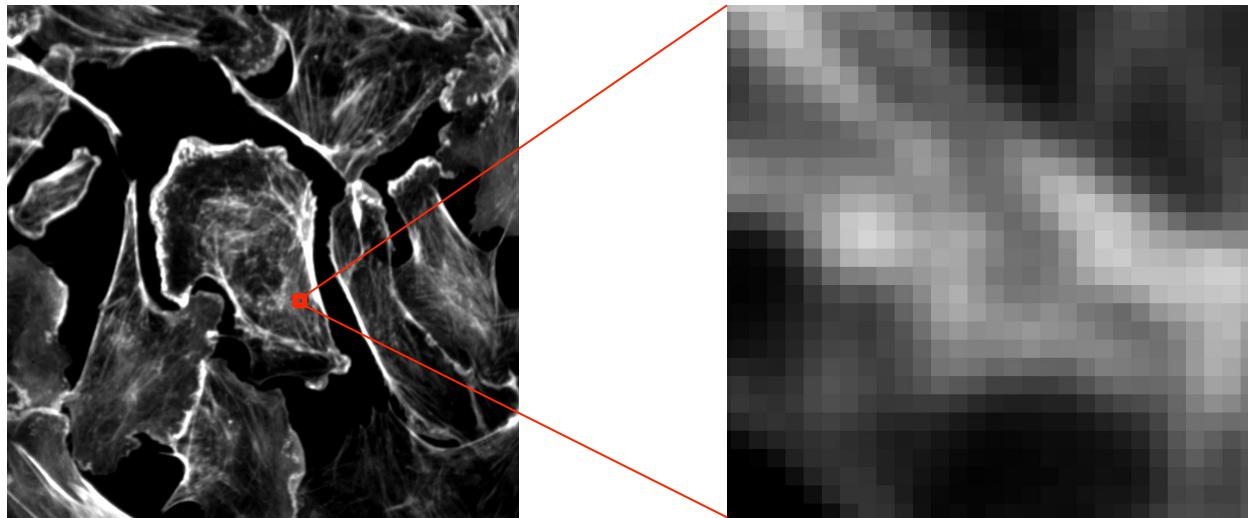


Scan time: 20 sec.  
Size on disk: 1MB

# Summary: Spatial Resolution

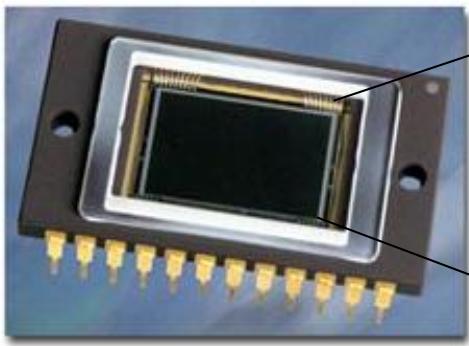
- Images are subject to distortion (convolution) by the optics of the imaging system, turning points into point spread functions
- Resolution is determined by the wavelength of light and the NA of the objective, and can be improved by things like confocal microscopy and deconvolution
- The absolute lower 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

# Imaging Also Collects Intensity Information

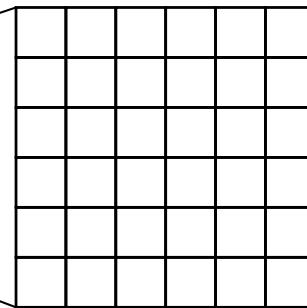


camera chip

pixel array



KAF-3200E



⋮

individual pixel

Metal Oxide Semiconductor (MOS) Capacitor

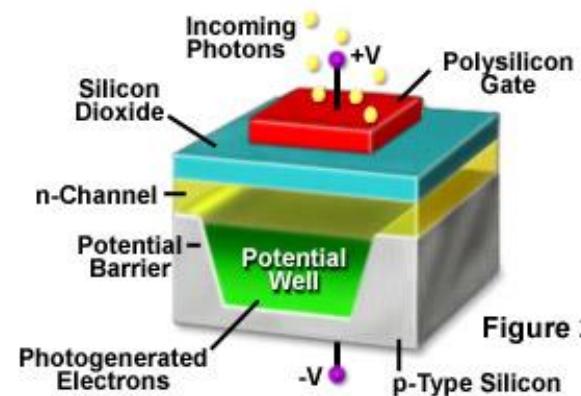
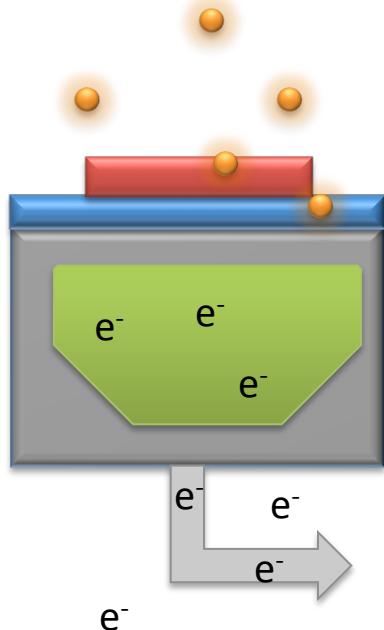


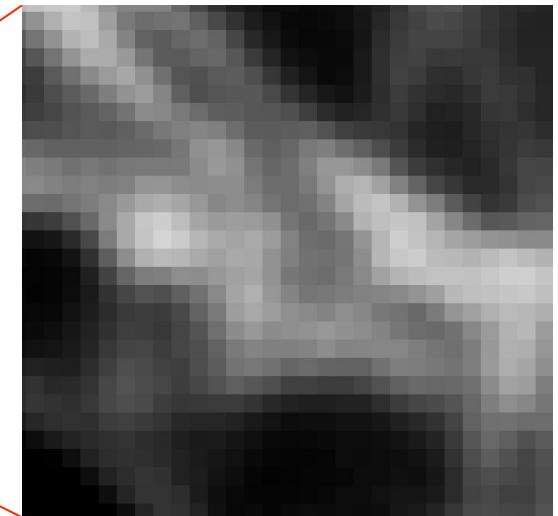
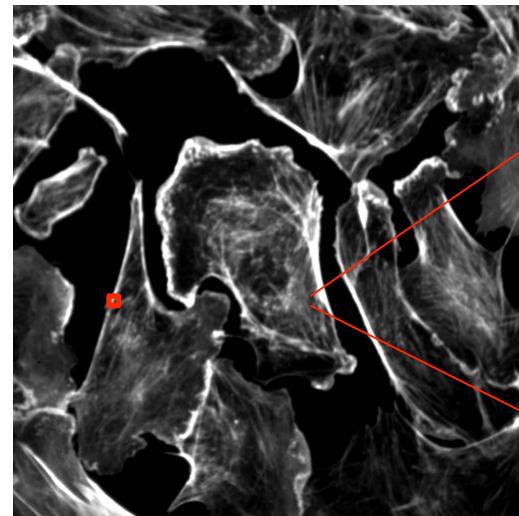
Figure 2

# Pixels also collect intensity information

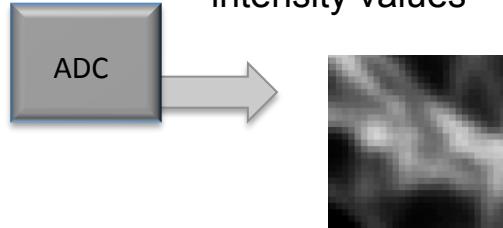
60-95% of photons which hit the chip are converted to signal ( $e^-$ )



Additional  $e^-$  (noise) generated by heat, current, etc.



Analog to digital conversion translates  $e^-$  into intensity values

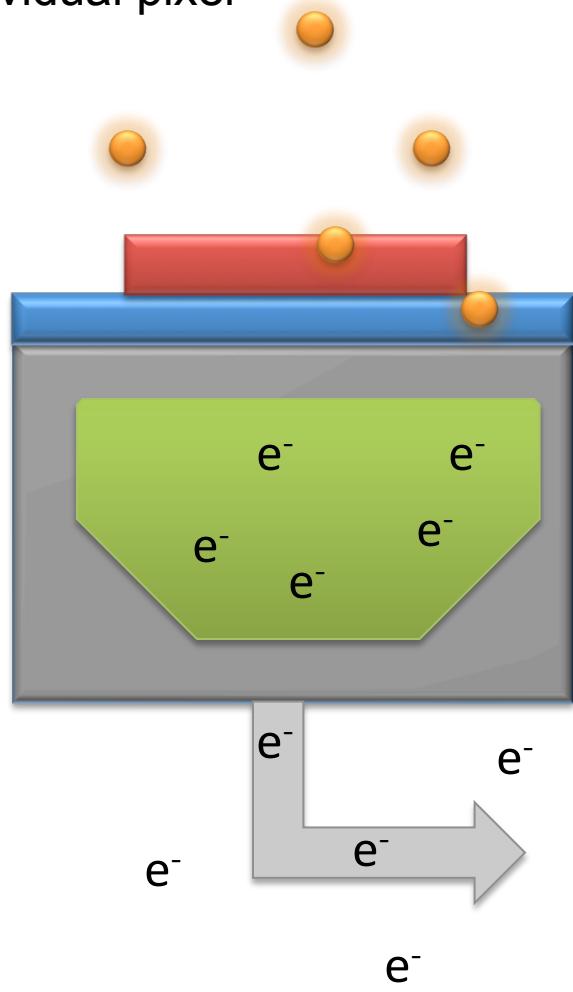


Conversion of photons to analog voltage ( $e^-$ ) to digital signal (gray values) is not a straightforward, linear process

What does this mean for you as a microscope user?

# How Does a Camera Convert Photons to Grey Levels? (The Simple Version)

Individual pixel



Incoming photons from the sample hit a semi-conducting metal film and some (never all) are converted to electrons.

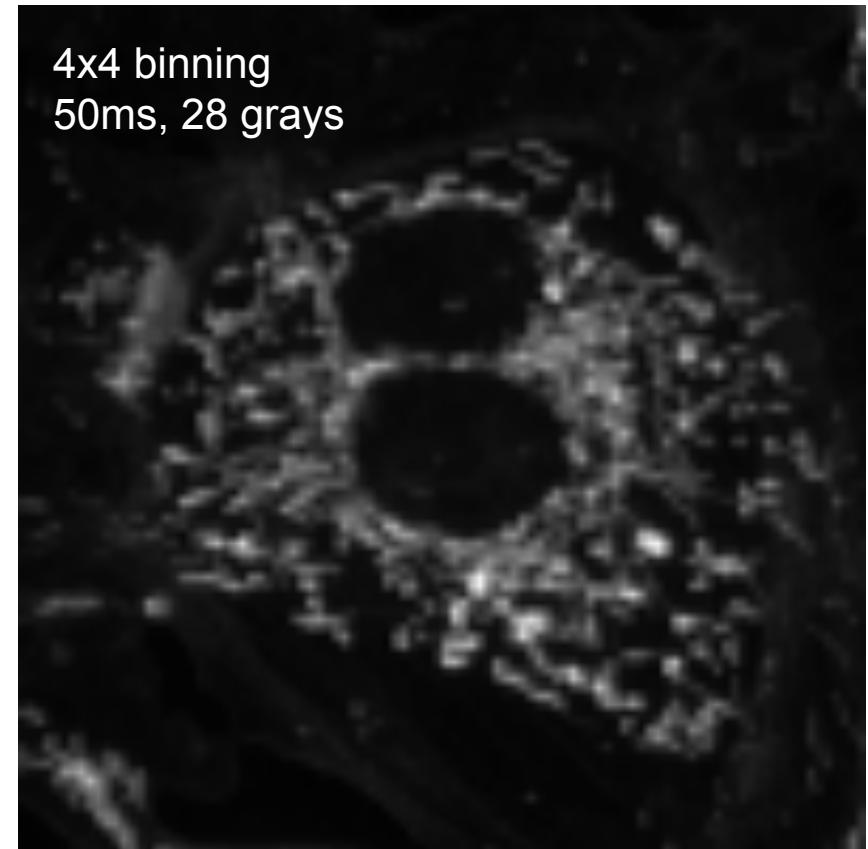
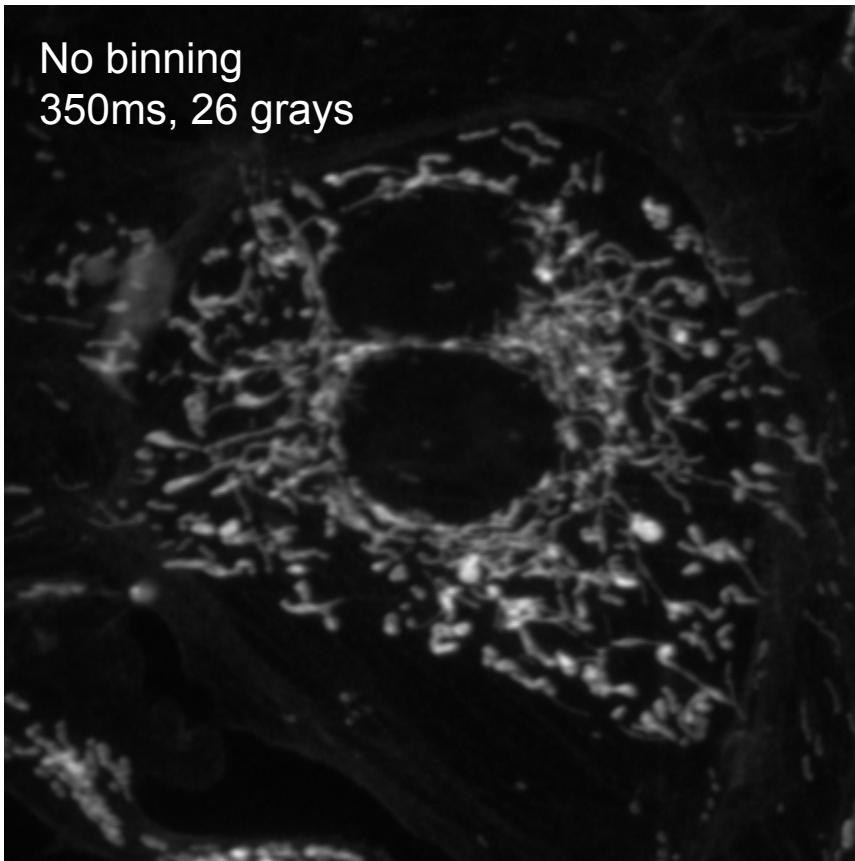
Additional electrons are generated in the chip by heat and the travel of the electrons themselves.

All of the signal and noise electrons are combined and converted to a voltage, which is then converted to an intensity value (gray level) by the analog to digital converter (ADC).

What  
does this  
mean for  
you?

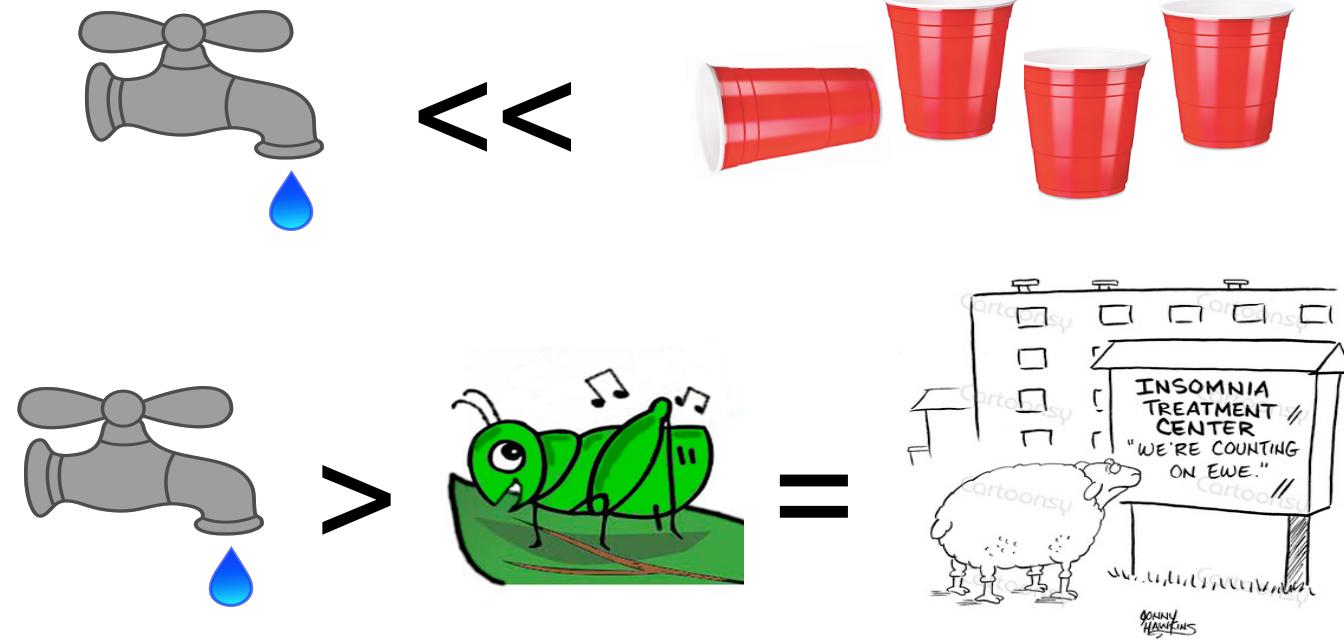
# Boosting Signal in Your Samples

- 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.



# Thing #1: Not All the Grays in Your Image are From Your Sample

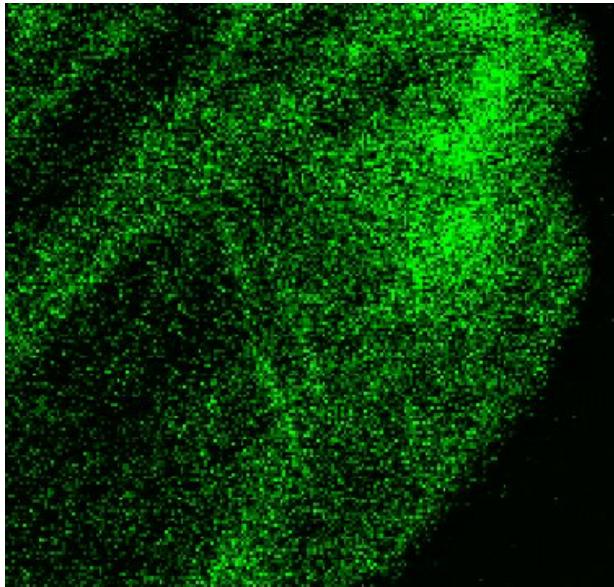
Non-signal grays are called noise. Noise is contributed by a variety of things, including the imaging process itself. It's impossible to eliminate noise, but we can minimize its contribution.



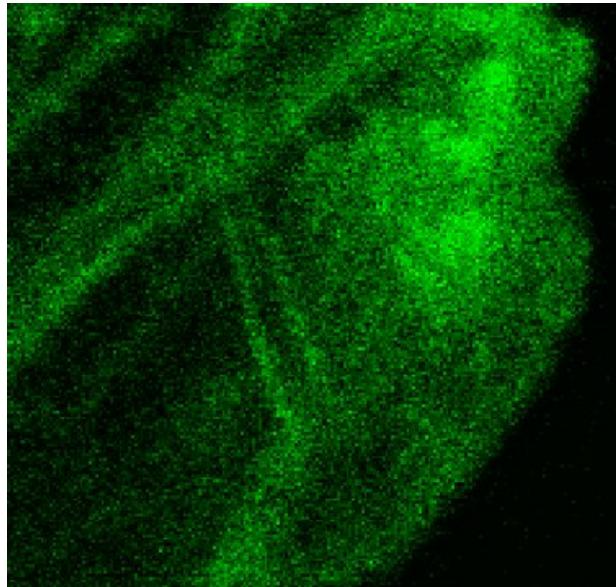
# Reducing the Contribution of Noise

- 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)

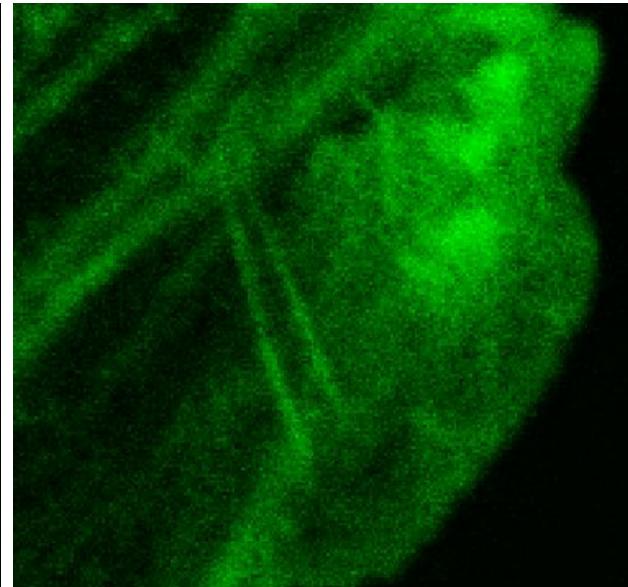
No line averaging  
0.5 second per frame



4 line averaging  
2 seconds per frame

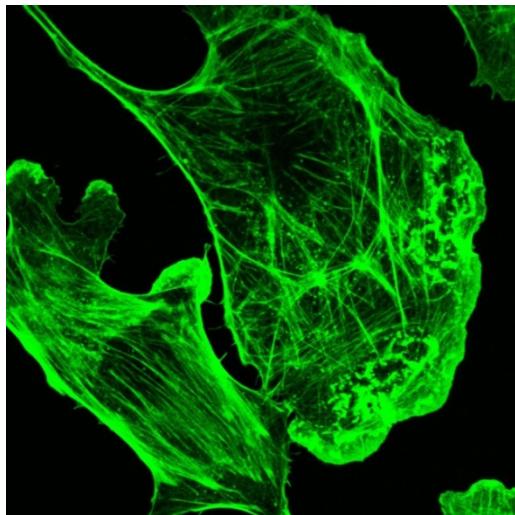


16 line averaging  
8 seconds per frame

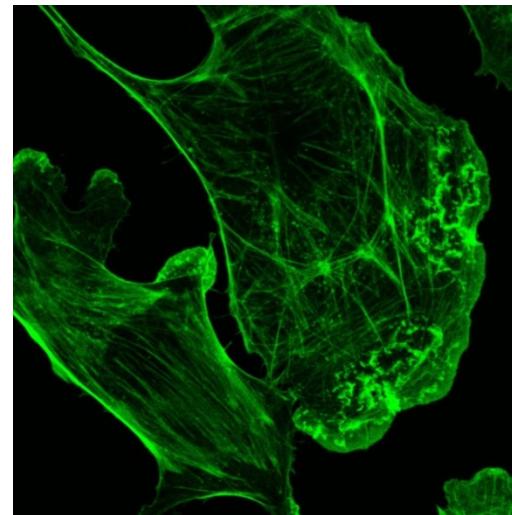


# Reducing the Contribution of Noise

- If two or more objectives with the same NA are available, use the one with the *lowest* magnification you can justify. This will collect more light



63x NA 1.45  
with 3.25x  
zoom  
  
Mean gray  
value: 113.7



100x NA 1.45  
with 2x zoom  
  
Mean gray  
value: 71.3 (a  
40% loss)

# Boosting Signal in Your Samples

- Use bright, photostable fluorophores such as the AlexaFluors and “enhanced” fluorescent proteins like eGFP.
- Titrate your staining reagents. Begin with three slides: the recommended dilution, +3x and -3x. Sans recommendation? Try 1:100, 1:300 and 1:900.
- Use a high quality mounting medium with anti-fade. Keep samples at 4C and away from bright light. Clean coverslips / dish bottoms.

# Thing #2: The Relationship Between Photons and Grays is NOT Straightforward

The relationship between the number of photons generated by your sample and the number of grays in your image is not 1:1.

It's difficult to say that A grays = B photons, and even more difficult to say that B photons = C amount of whatever it is you're labeling (protein, Ca++, etc).

So to day that you have A number of grays in an image is virtually meaningless. However, to say that you have A level of grays in Image 1, and after treatment or with a mutation or with no antibody you have B levels of gray in Image 2, as long as the two images were taken with the same parameters, values A and B can have meaning *relative to each other*.

# Digital Display of Analog “Brightness” Data

1 bit

0, 1

2 bit

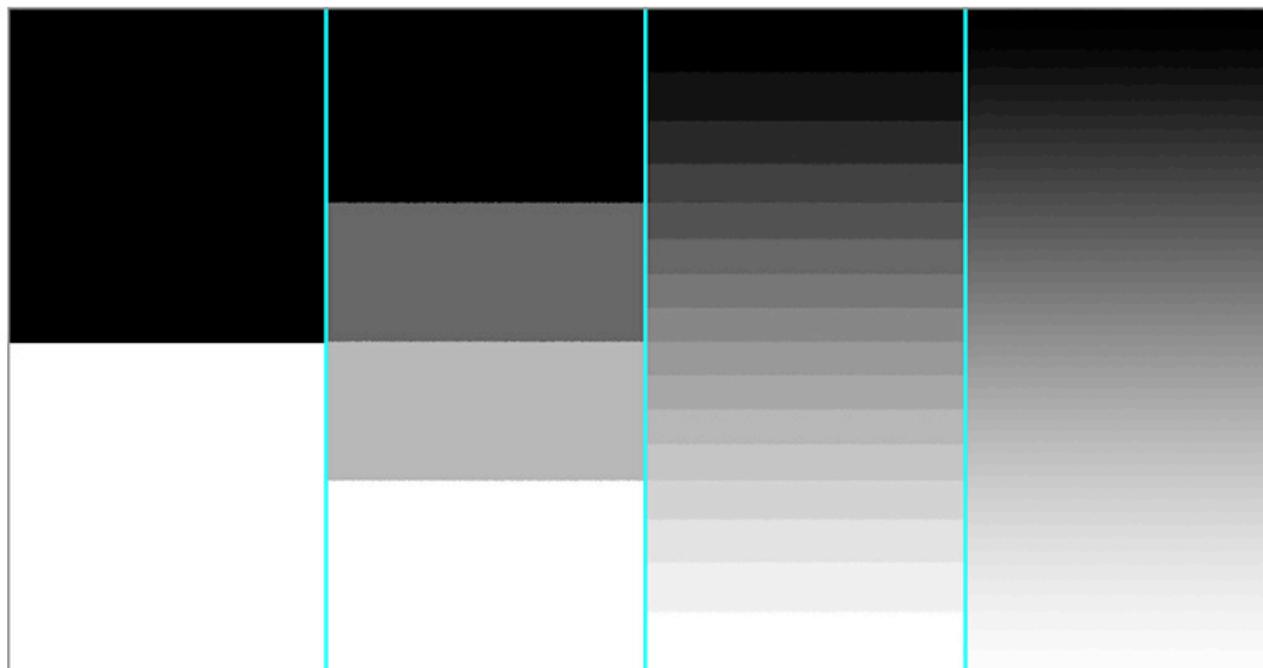
00, 01, 10, 11

4 bit

0000 to 1111

8 bit

00000000 to 11111111



$$2^1 = 2$$

1 bit has 2 possible  
shades of luminance values

$$2^2 = 4$$

2 bit has 4 possible  
shades of luminance values

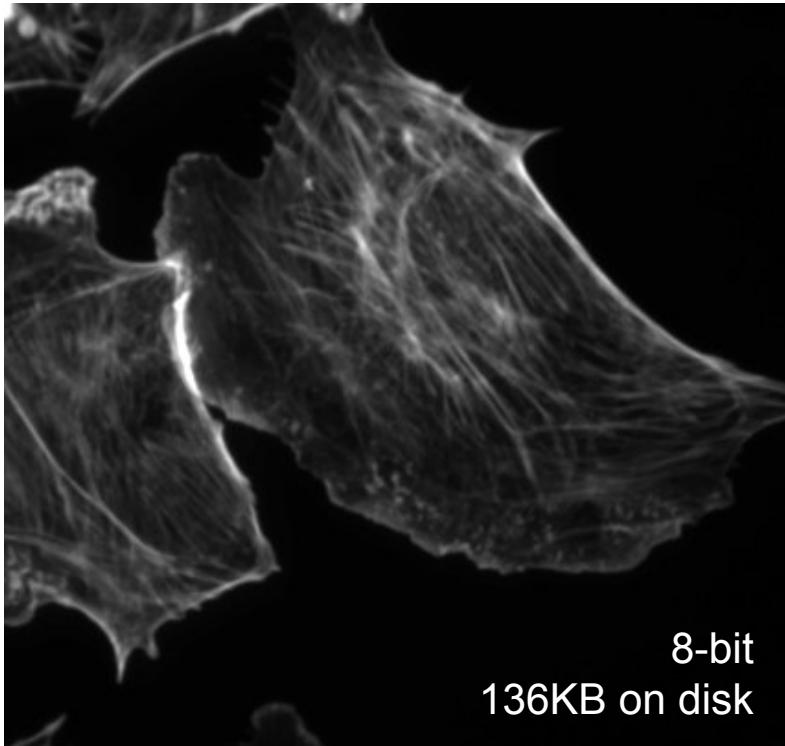
$$2^4 = 16$$

4 bit has 16 possible  
shades of luminance values

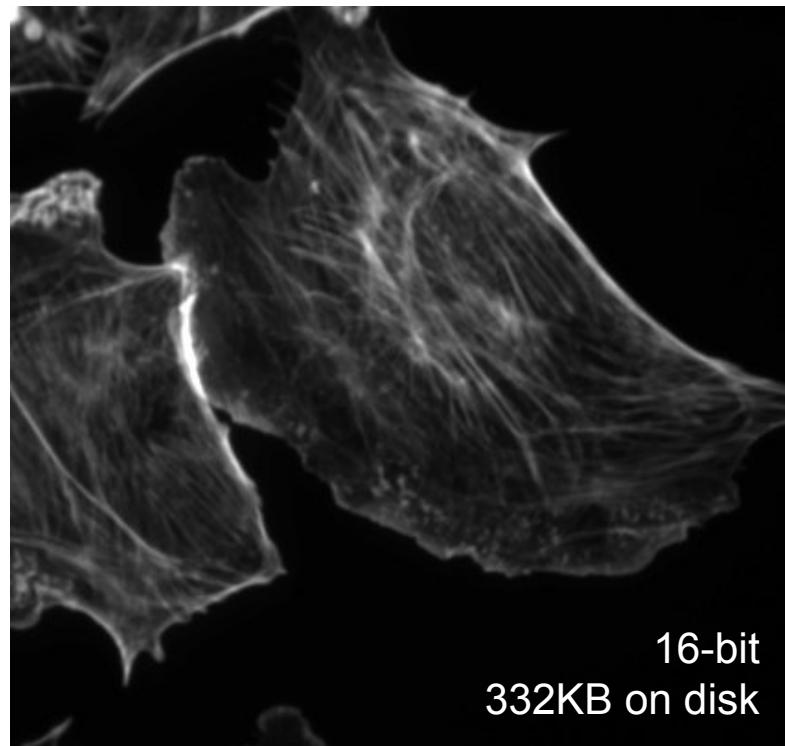
$$2^8 = 256$$

8 bit has 256 possible  
shades of luminance values

# Humans Can Distinguish ~60 Gray Levels



8-bit  
136KB on disk

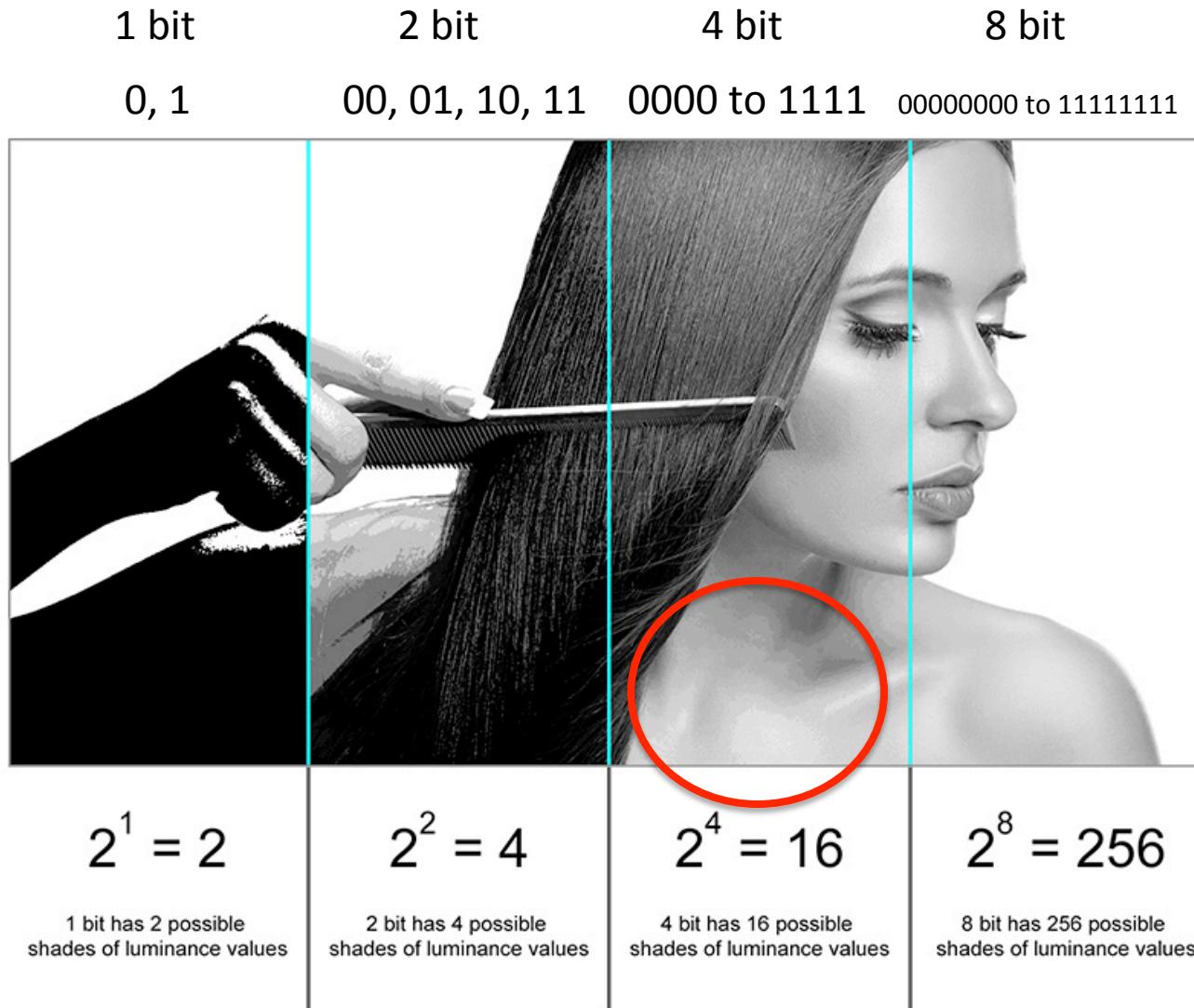


16-bit  
332KB on disk

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

# What Happens When You Shrink Bit Depth?



# If We Can't See, How Do We Know?

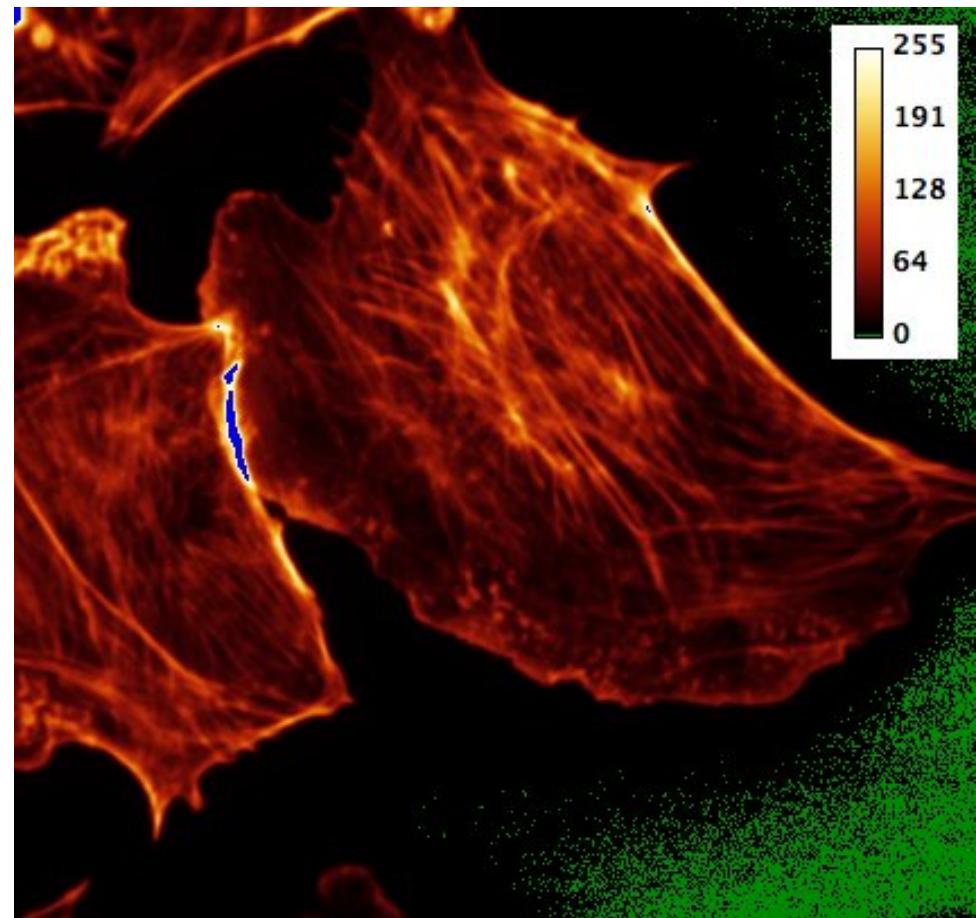
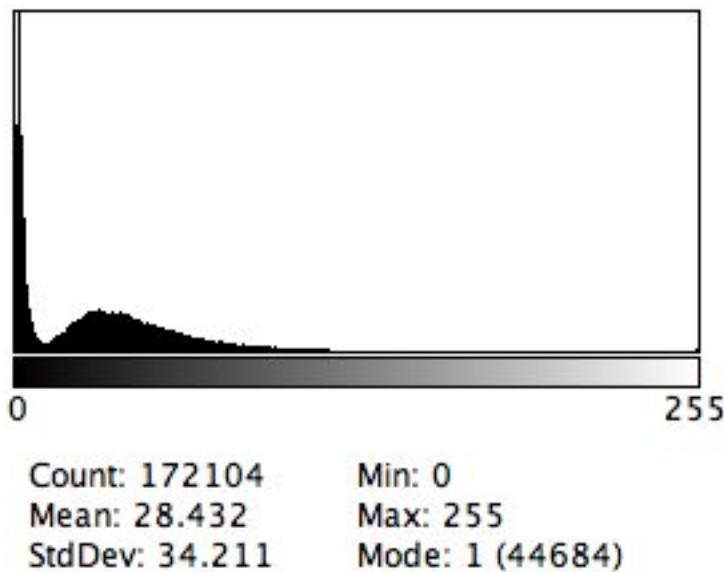
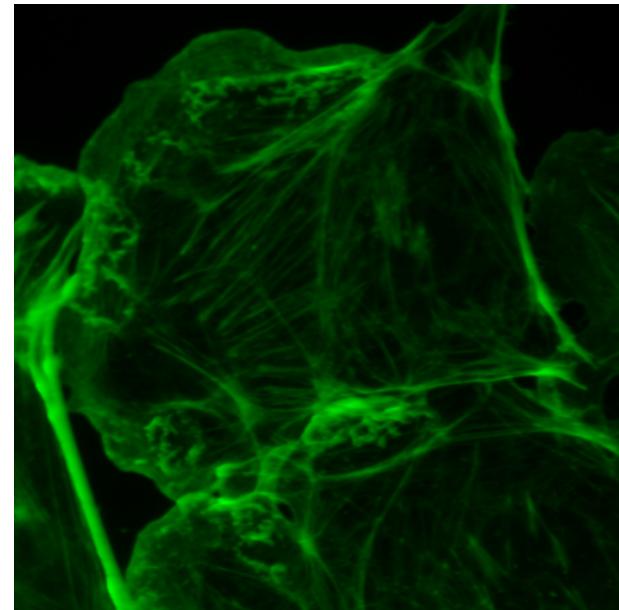


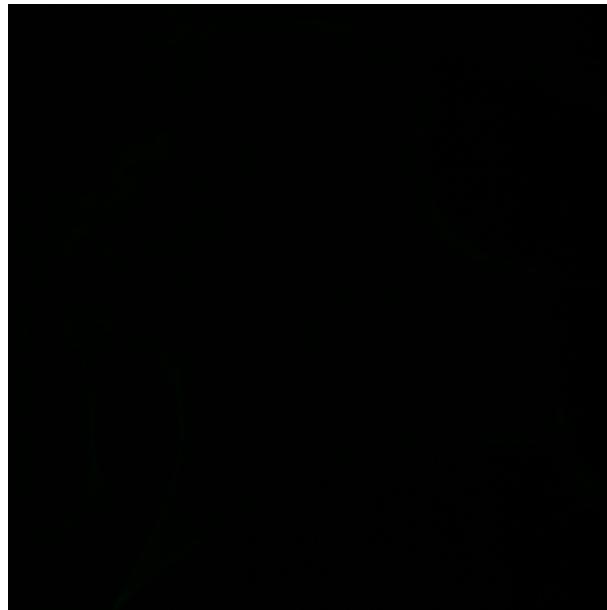
Image credit: Olympus DSU Spinning Disk confocal and ImageJ software

# How to Set Up Your Experiment

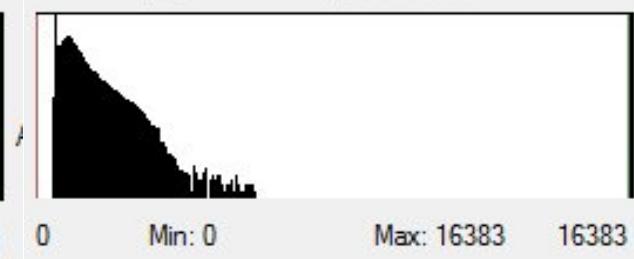
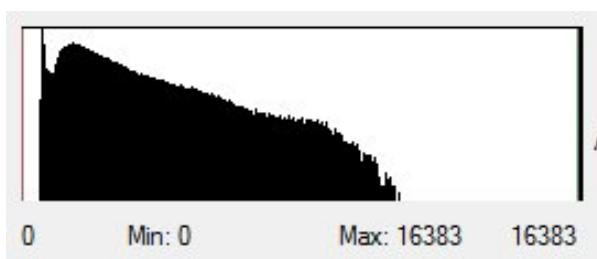
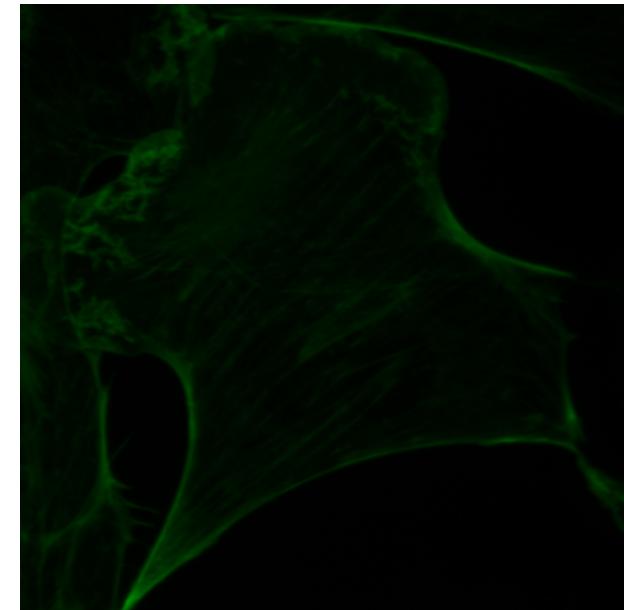
#1 Brightest Sample



#2 Secondary only

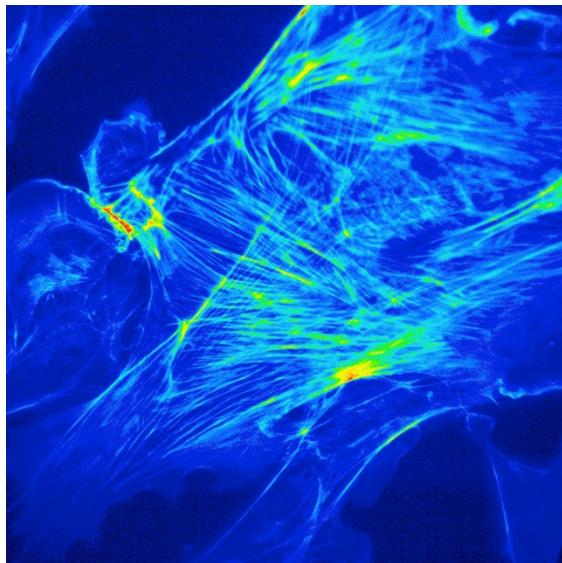


#3 Lower Concentration

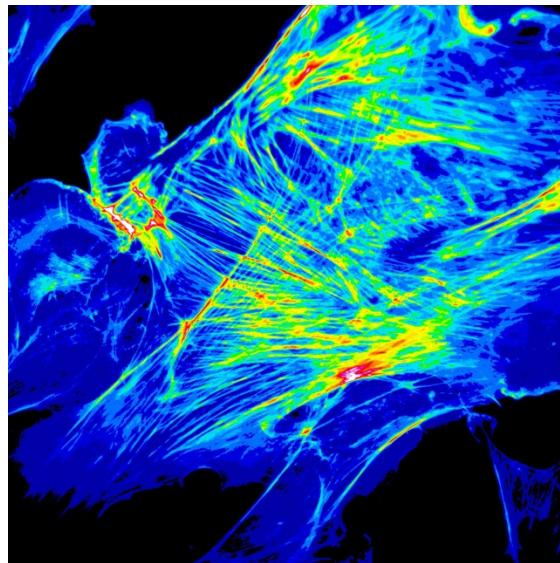


# Take Advantage of the Full Dynamic Range

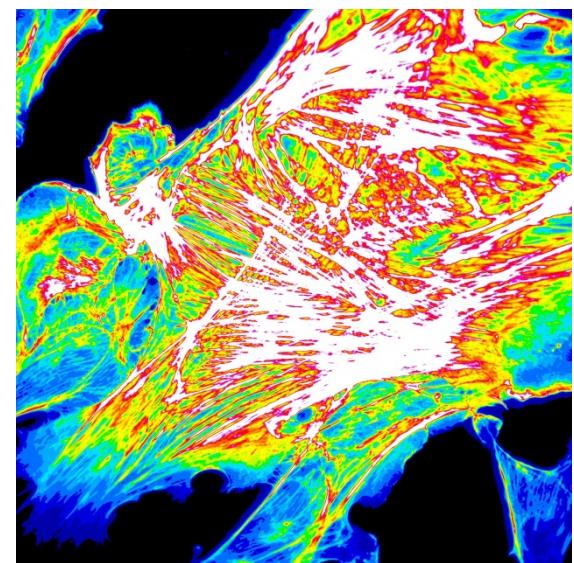
Low range



Good range

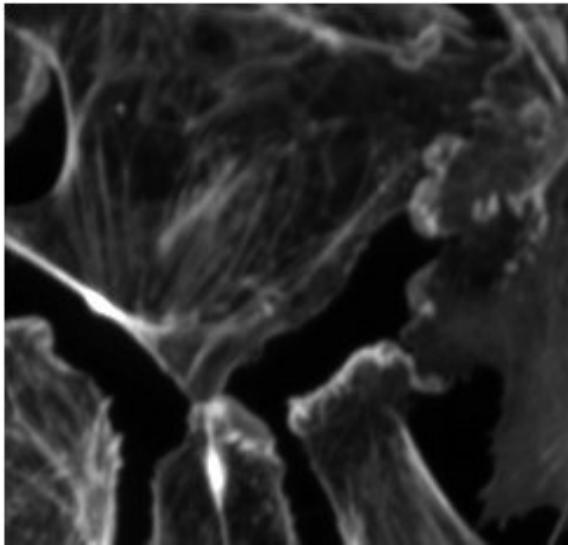


Saturation

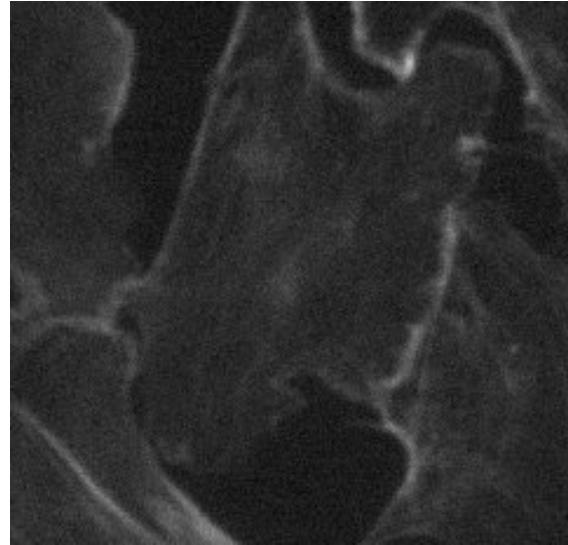


# Autoscale and Why it is Evil

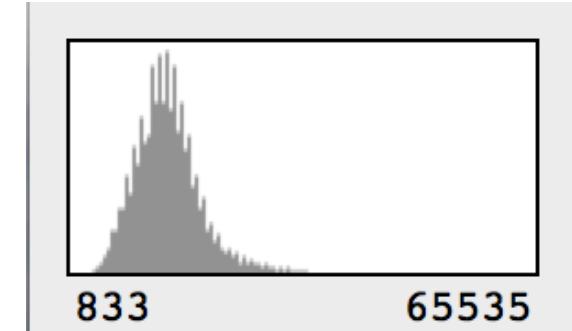
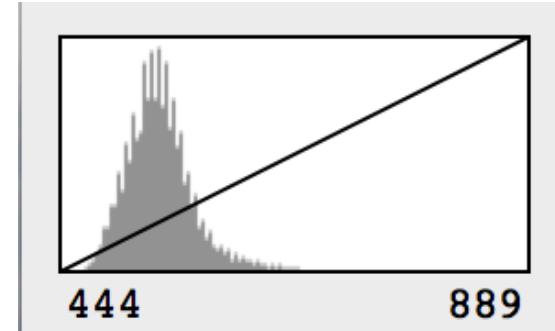
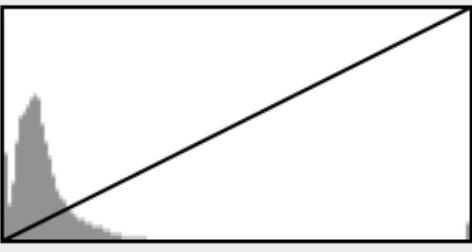
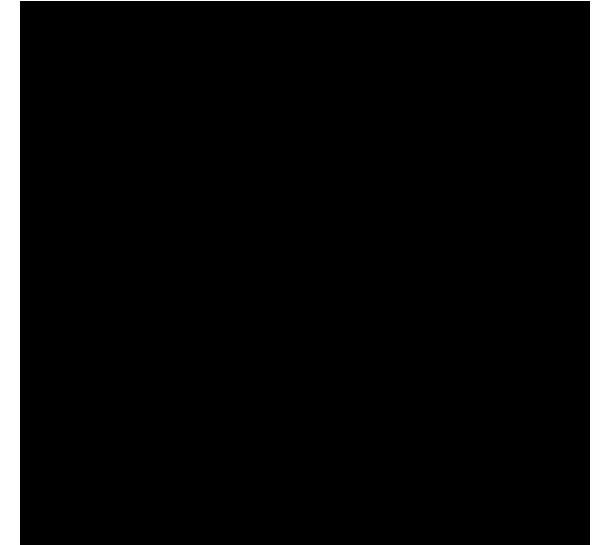
Experimental image



Secondary control  
(autoscaled)



Secondary control  
(properly scaled)



# 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.

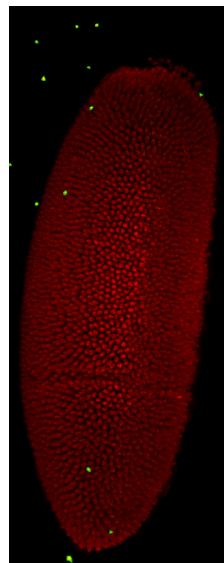
As a starting point, we can return to Nyquist-Shannon and use a sampling rate that is twice the period of the activity we are interested in capturing.



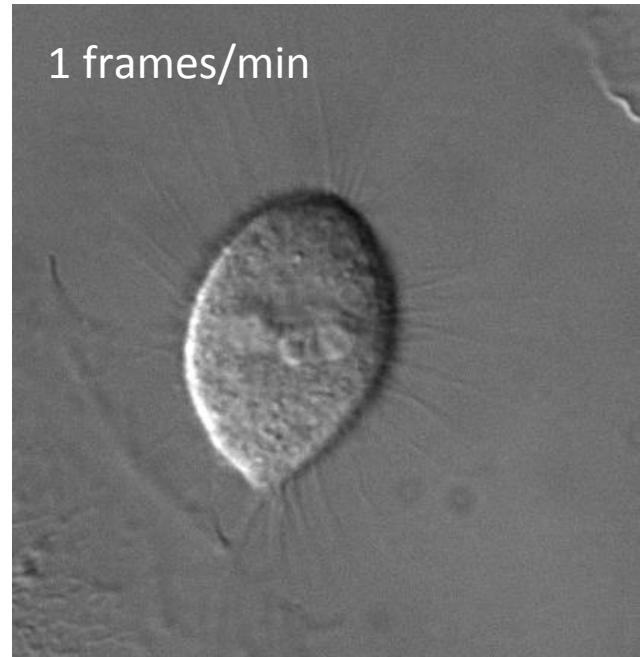
# Two Types of Live Sample Imaging

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.

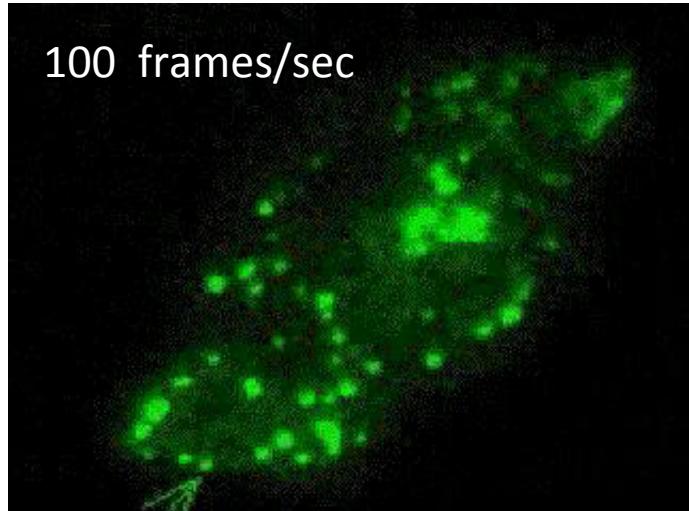
1 frame/  
20 min



1 frames/min



100 frames/sec

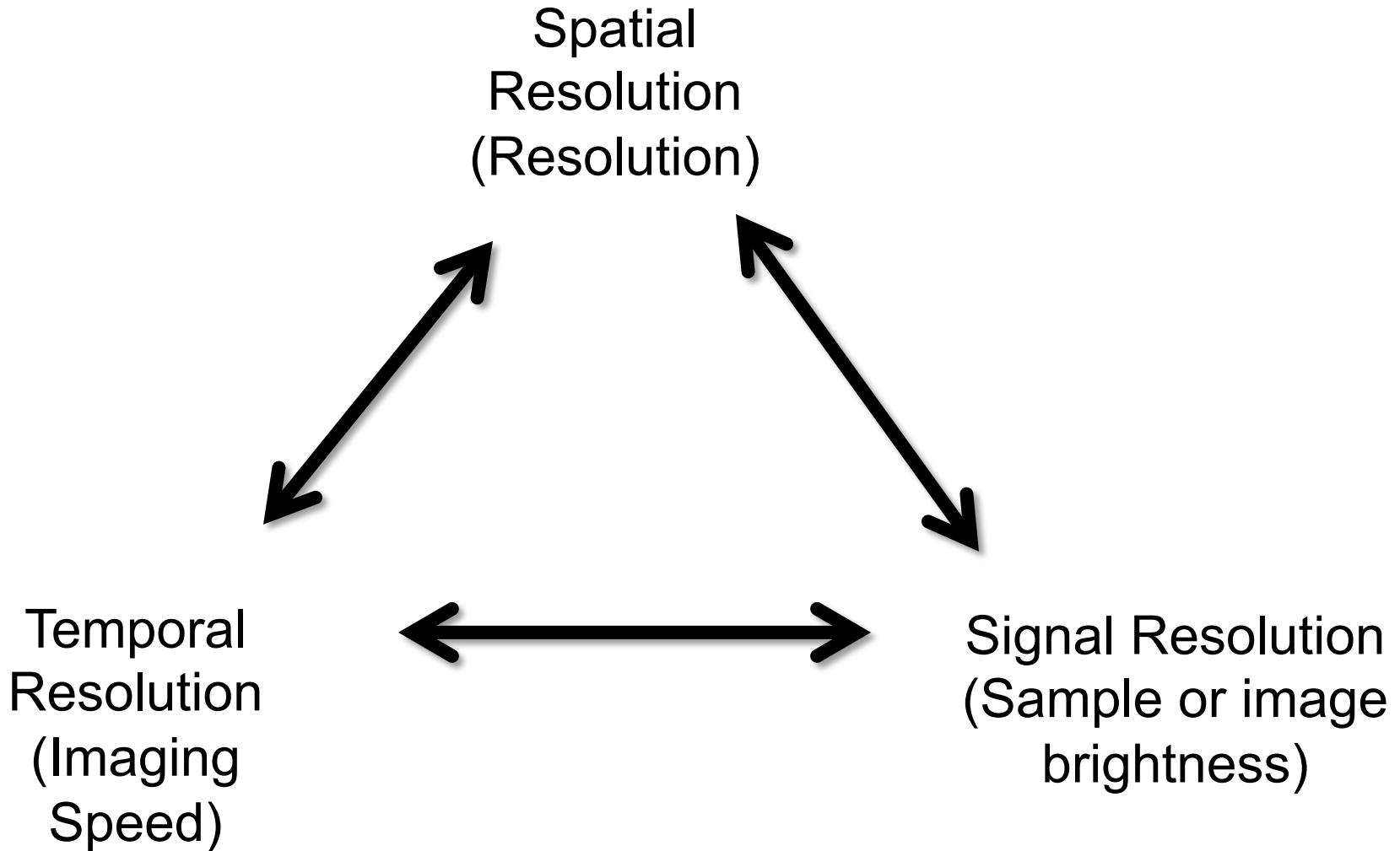


Streaming – Processes that happen at rates that can be visualized in real time (or faster). Most often taken as a stream of images as fast as the system can be made to run and with no pauses between images. Played back at a rate slower than collected.

# Summary: Temporal Resolution

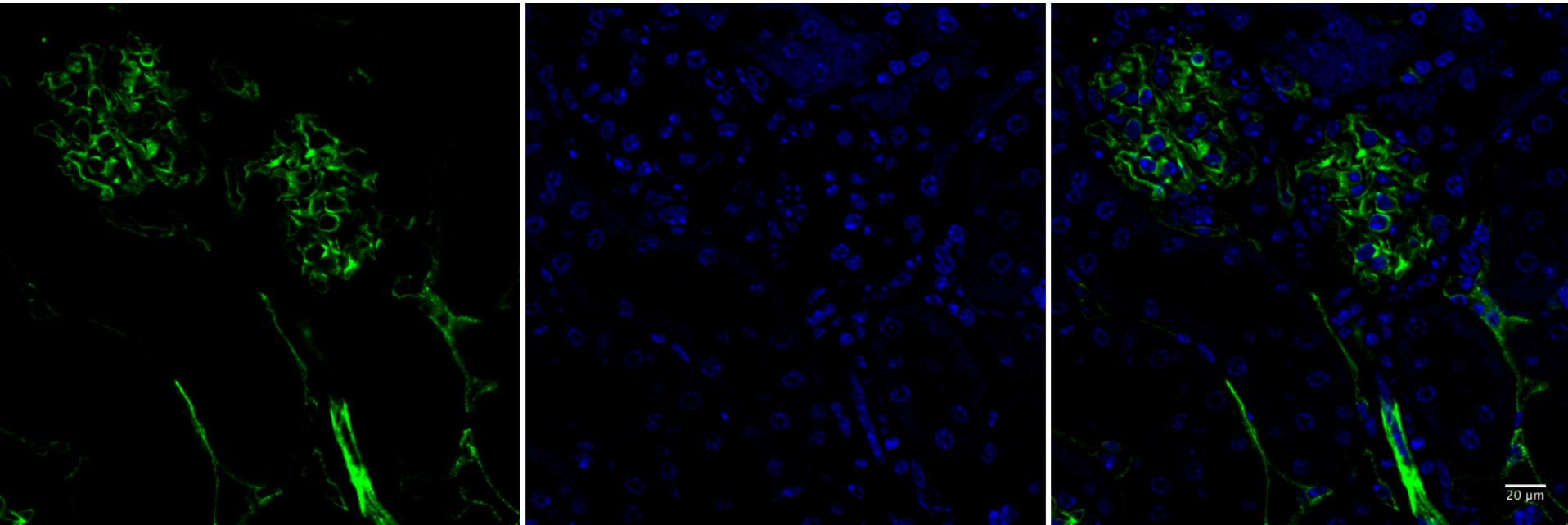
- Look in the literature to see what others have done in similar situations. Use that as a starting point
- For long processes, image more often than you think you should. You can always take out timepoints. Check in with the experiment often to make sure the sample hasn't died / moved / bleached / dried 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.



# Tradeoffs – A Real World Example

Granule exocytosis speed: 20 msec.

Camera speed: 15 frames / sec

Granule dia.: 300-350nm

Pixel size on chip: 6.45um

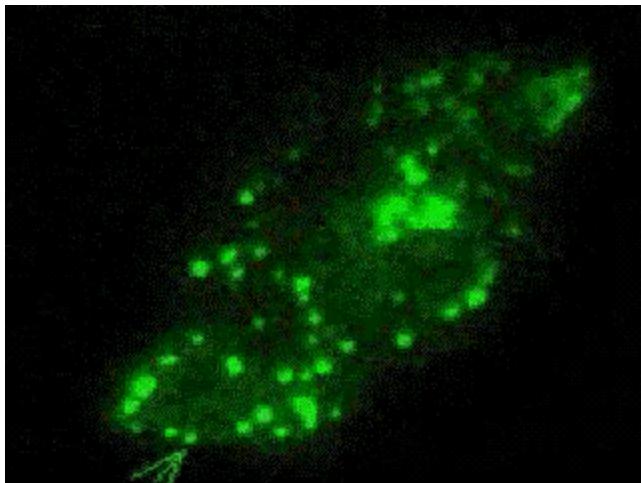
Objective: 60x NA 1.45

Actual pixel size: 107.5nm

Insulin granules are small and release their contents very quickly.

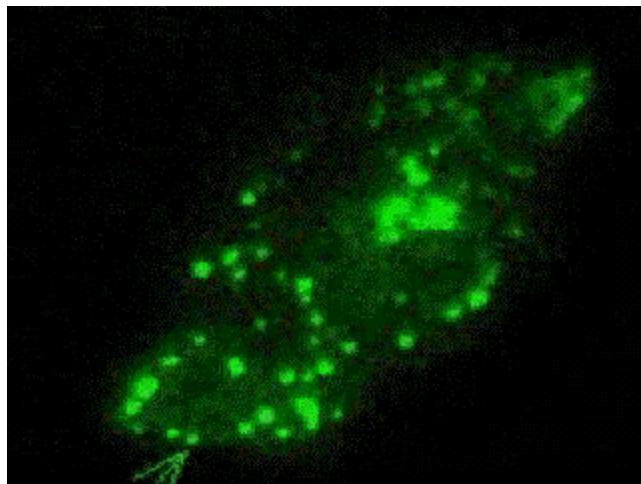
Release events last for approx. 20 msec, so for adequate sampling we need a frame rate of 2 frames every 20 msec, or 100 frames per second.

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.



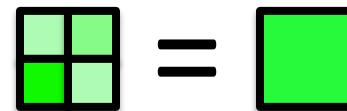
# Tradeoffs – A Real World Example

Granule exocytosis speed: 20 msec.  
Camera speed: 120 frames / sec  
Granule dia.: 300-350nm  
Pixel size on chip: 12.9um  
Objective: 60x NA 1.45  
Actual pixel size: 215nm



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

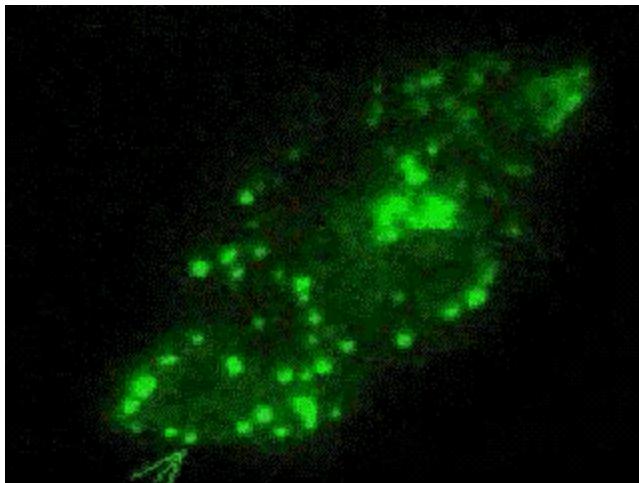
- 1) Crop the area being imaged from 1392 x 1040 down to 696 x 520 (1/4 of the chip) increasing readout speed twofold to 30 frames per second
- 2) Bin the pixels. A 2x2 bin reads out a group of 4 pixels as though they are 1.



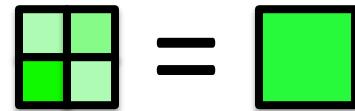
This binning increases imaging speed by 4x, giving us 120 frames / second.

# 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.

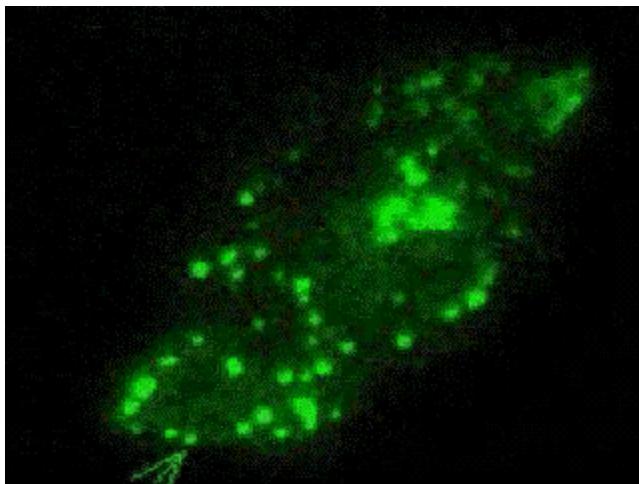


So we must increase our magnification until we can sample our granules adequately (if possible).

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

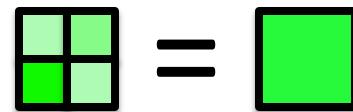
# Tradeoffs – A Real World Example

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.

# Abbe's Formula

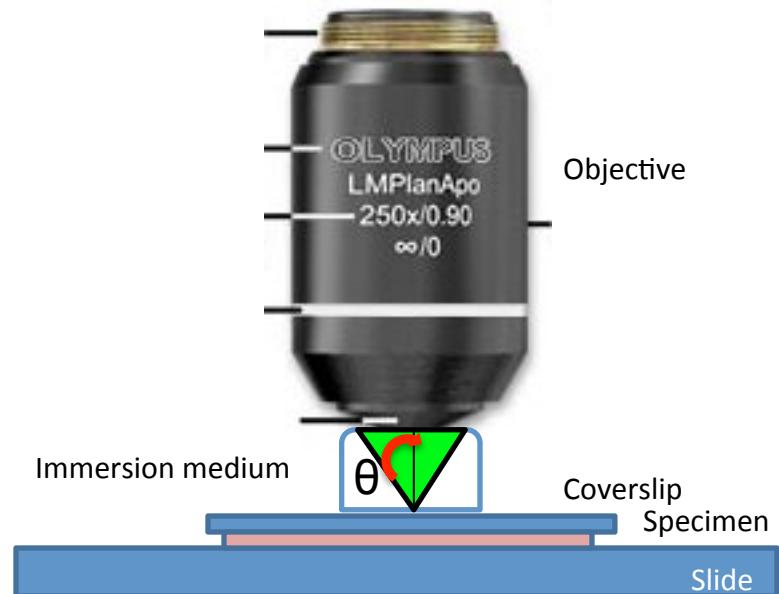
$$\text{Resolution (xy)} = \lambda / 2(n(\sin\theta))$$

$$\text{Resolution (z)} = 2\lambda / (n(\sin\theta))^2$$

Where  $\lambda$  is the wavelength of light

$n$  is the refractive index of the immersion medium used by the objective

$\theta$  is half of the angle of the cone of light collected by the objective (also known as the angular aperture).



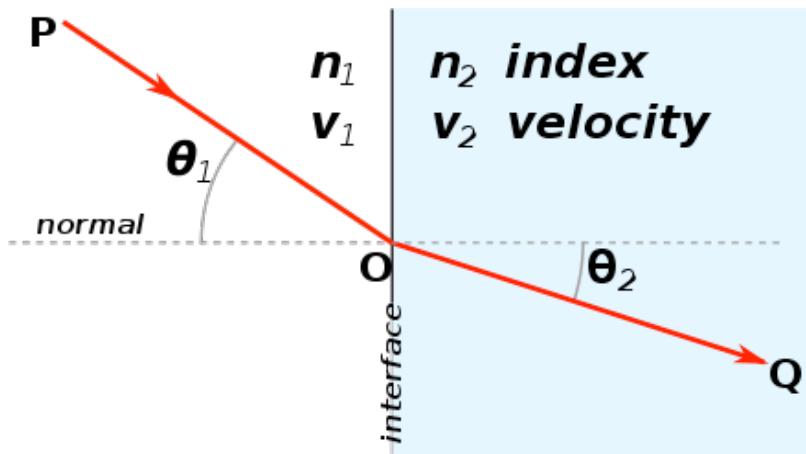
Abbe coined the term Numerical Aperture (NA) to represent  $n(\sin\theta)$ , so the equations can also be expressed as in the talk:

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

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

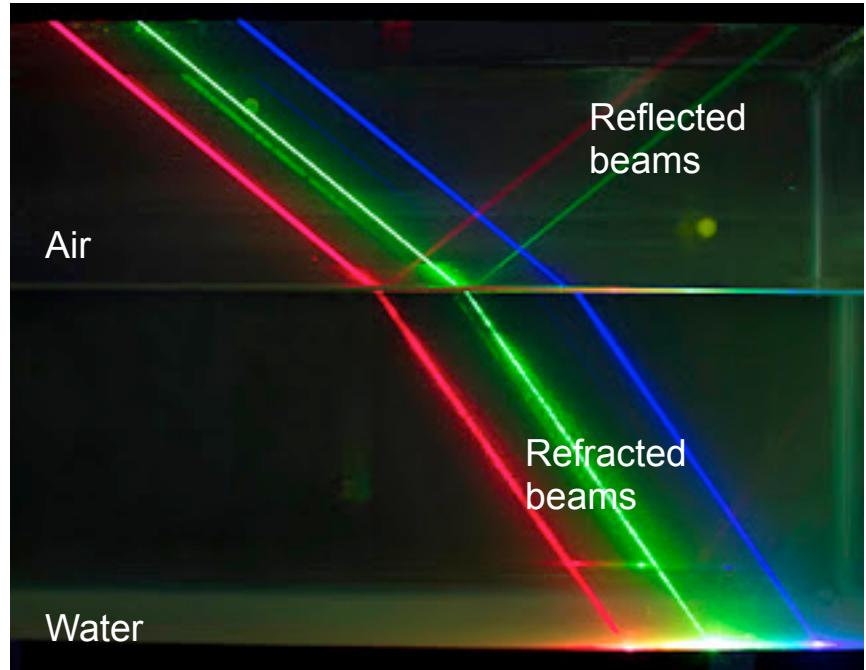
# Immersion Medium and the Light Path

Refraction – change in the direction of a wave when it passes between media of different densities.



$$\frac{\sin\theta_1}{\sin\theta_2} = \frac{v_1}{v_2} = \frac{n_2}{n_1}$$

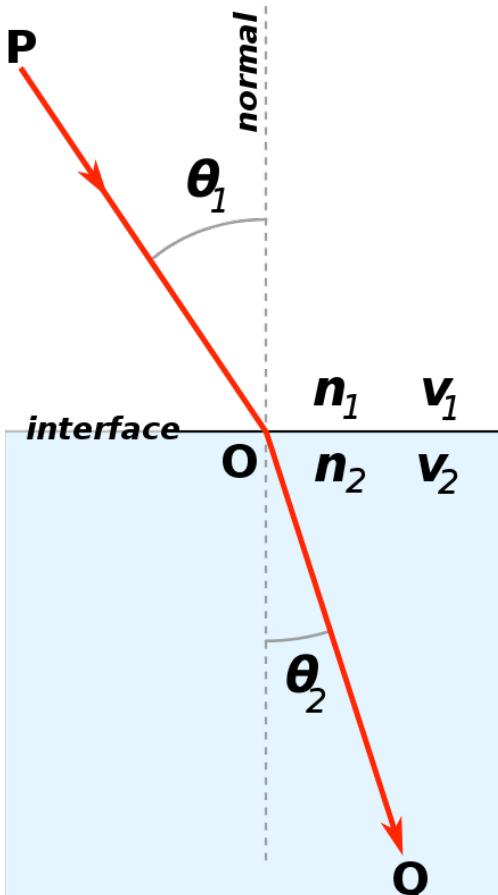
Refraction is described by Snell's law, which states that the ratio of the sines of the angle of incidence ( $\theta_1$  in this example) and the angle of refraction ( $\theta_2$ ) is equivalent to the ratio of the phase velocities in the two media or the reciprocal of the ratio of the refractive indices.



# Refractive Index Measurement

Refractive index measures how much a light ray is bent with respect to the normal upon entering a medium.

The normal is the path of light in a vacuum, which by definition has a refractive index of 1.000 and allows light to pass through without bending.



Using Snell's law, we can see that refractive index can be defined as the ratio of the speed of light in a vacuum and the phase velocity of the same light in the medium being measured.

$$\frac{n_2 \text{ (medium)}}{n_1 \text{ (vacuum)}} = \frac{v_1 \text{ (vacuum)}}{v_2 \text{ (medium)}}$$

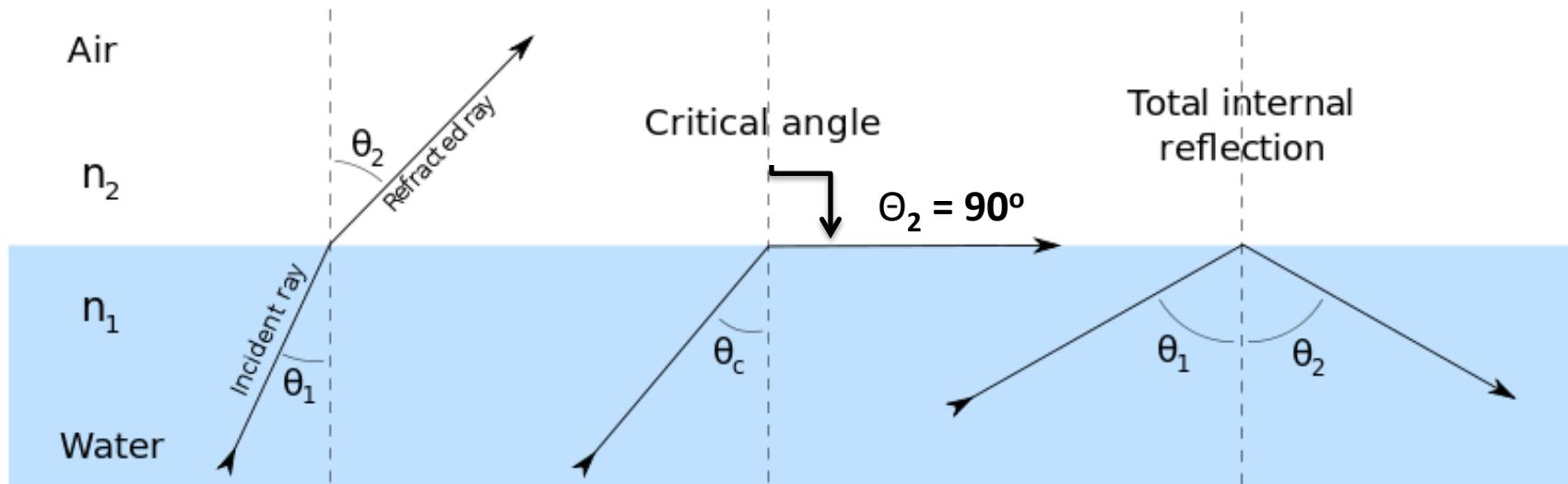
Since the wavelength (color) of the ray of light also influences the speed at which it travels through a medium, the standard refractive index for a material is measured using light at 589nm (sodium yellow).

# Total Internal Reflection - Snell's law at Extremes

When light travels from a medium of high refractive index (e.g. water or glass) to one with lower refractive index (e.g. air or cell medium) at an angle of incidence greater than the critical angle, Snell's law requires that the sine of the angle of refraction be greater than 1 and the angle be greater than  $90^\circ$ .

Since it is impossible for visible light to behave this way, none of the light refracts and all is reflected back into the medium of high refractive index. This is known as total internal reflection.

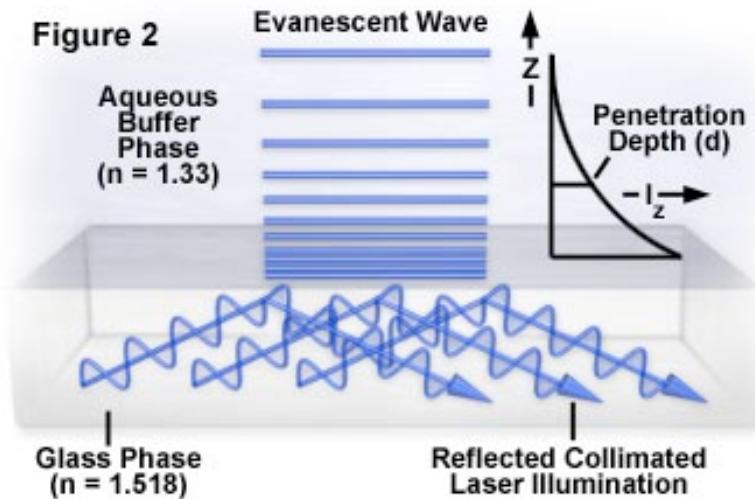
The critical angle  $\theta_{\text{crit}}$  is the value of  $\theta_1$  for which  $\theta_2$  equals  $90^\circ$ :



# Total Internal Reflection and Imaging

Evanescent Wave Exponential Intensity Decay

Figure 2

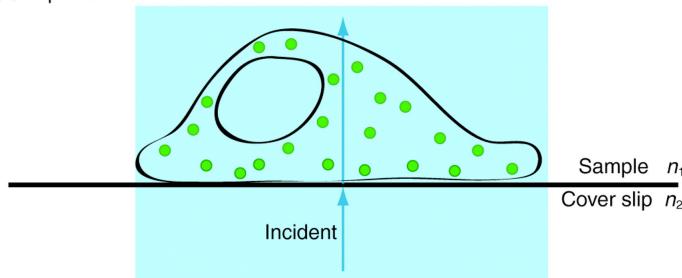


When light is made to travel through a coverslip (refractive index 1.52) at a high enough incident angle that it reflects back into the glass instead of passing through the buffer/sample, an evanescent wave with energy equivalent to that of the incident light is created.

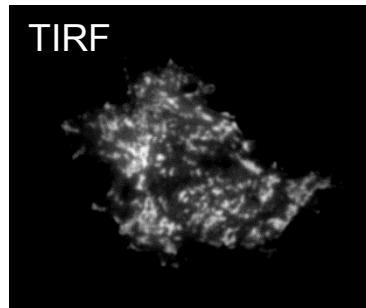
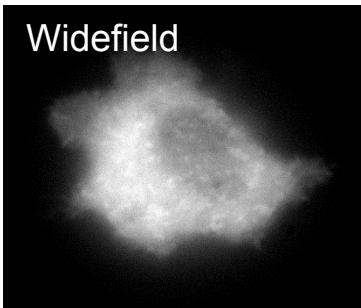
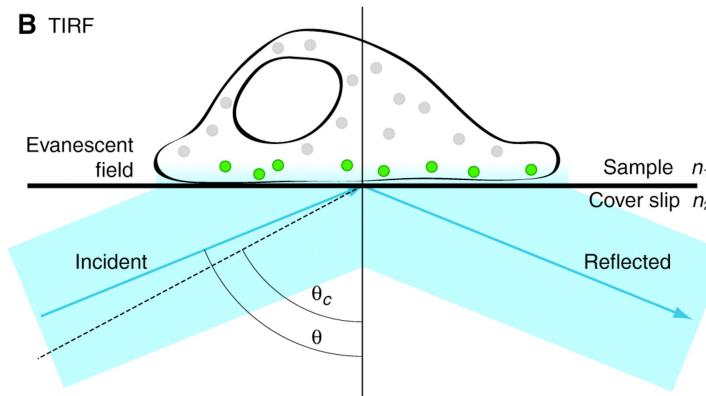
This evanescent wave is capable of exciting the same fluorophores as the original light wave, but unlike the light decays exponentially with distance.

# TIRF Microscopy

A Epifluorescence



B TIRF



Total internal reflection was first applied to fluorescence microscopy by Daniel Axelrod at the University of Michigan in the early 1980's.

In total internal reflection fluorescence (TIRF) microscopy, only fluorophores within the first 100nm of the sample are excited.

This is great when we want to view things close to the membrane, such as GFP-labeled focal adhesions.

In regular (epifluorescence) microscopy, the signal from the entire population of the protein is seen. In TIRF, only the membrane proximal portion is illuminated.

# Structured Illumination Microscopy (SIM)

In order to understand how SIM works, we must go back to our discussion of how the objective's numerical aperture creates the Airy pattern

One of the earliest superresolution techniques, SIM was pioneered by Mats G. L. Gustafsson at UCSF / HHMI Janelia Farm

In this method, instead of a solid beam of light illuminating the sample, the excitation light is passed through a grating to create a striped pattern (thus giving *structure* to the *illumination*).

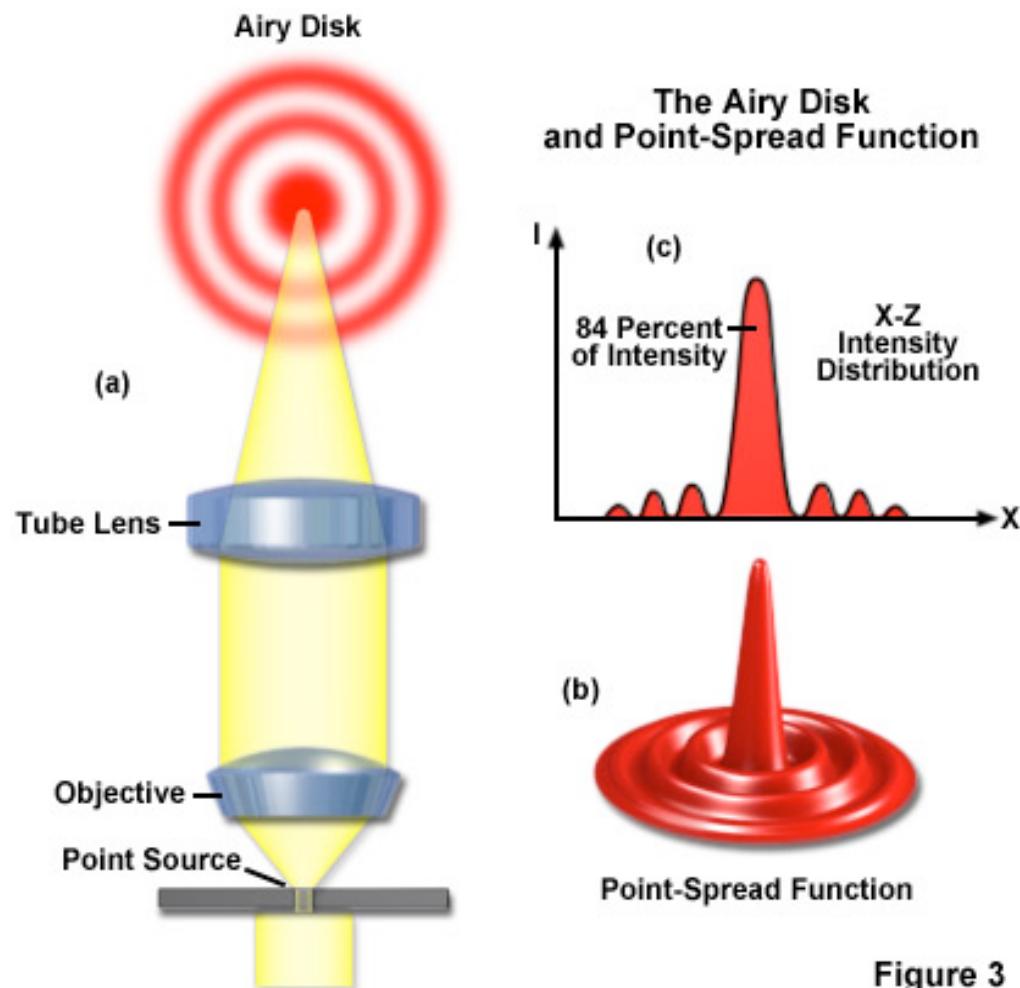
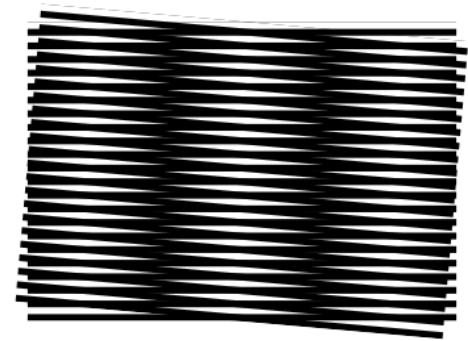
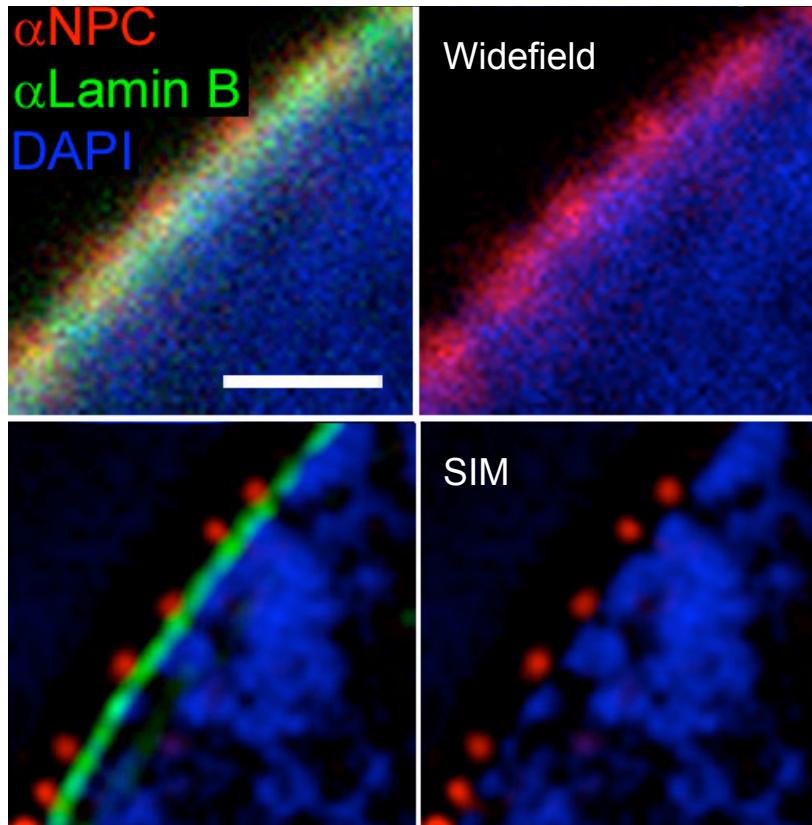


Figure 3

# Structured Illumination Microscopy (SIM)

When the patterned excitation light is superimposed on the Airy pattern created by the sample, a Moire effect occurs. This creates a new, more detailed pattern. Measuring the fringes of the pattern allows us to gather information that we would not otherwise be able to observe. This information is found in what is known as reciprocal space

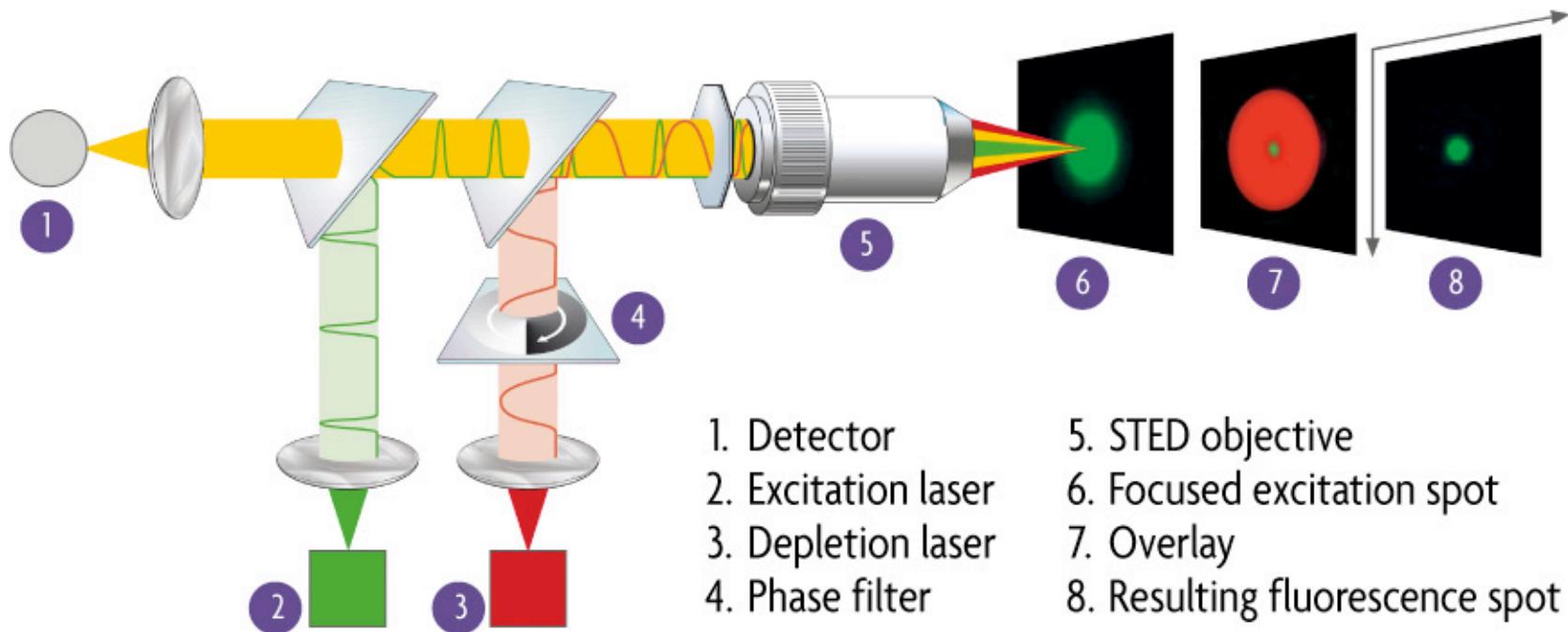


The patterned light is both shifted laterally and rotated across the sample several times so that a mathematical process call a Fourier transform can be used to deconstruct the patterns in reciprocal space. This gives us access to the information about fine details hidden in the reciprocal space. We can then use these fine details to improve the resolution of our image.

# Structured Illumination Microscopy (SIM)

- Resolution improvement is 2-fold over confocal – 100nm laterally (XY) and 200nm axially (Z)
- Common fluorophores and fluoroproteins can be used. Unmodified staining protocols from confocal can usually be used as well
- Machines are generally built on a widefield base. They are easy to use and image reconstruction is done automatically through the imaging software
- Image reconstruction can be computationally intensive, requiring a non-trivial amount of time and a capable processor
- Fast and gentle enough to do timelapse imaging of live samples (cell division) but not fast enough for live streaming (insulin granule release)

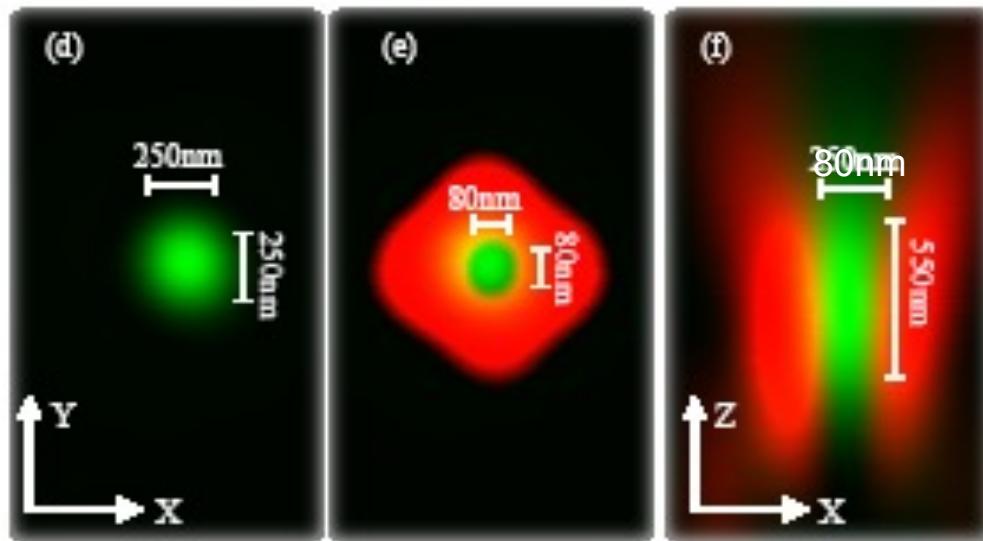
# STimulated Emission Depletion (STED)



## Principle of the Leica TCW STED microscopy.

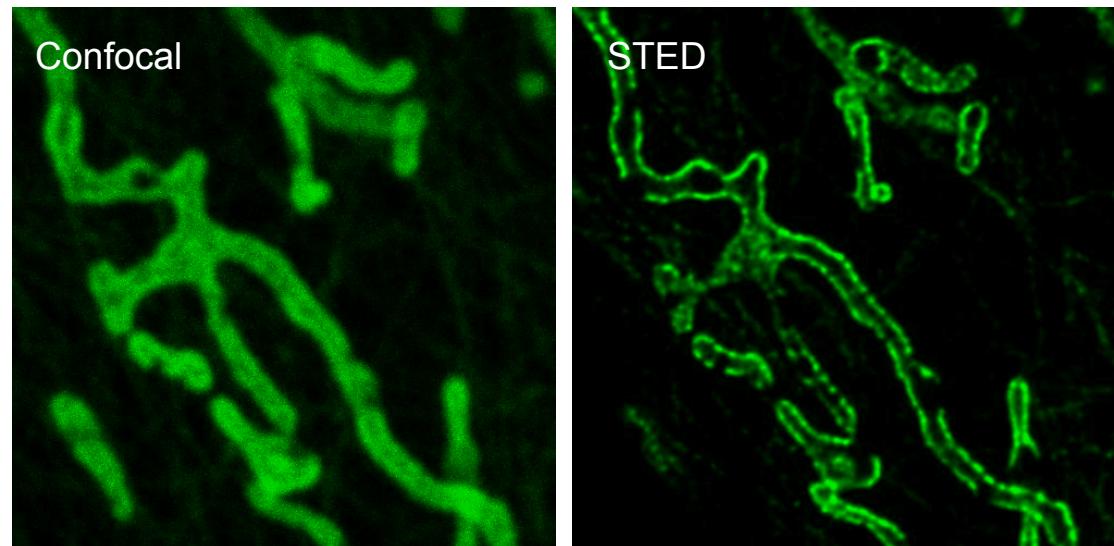
The key to resolution enhancement in STED is downsizing of the fluorescent spot used to scan the sample, which is achieved through use of an upconverted confocal laser scanning microscope that utilizes two lasers. The first laser (2, green) excites the fluorophores of the sample the same way as a conventional confocal system. These pulses are directly followed by a pair of perpendicularly polarized beams from a red-shifted stimulating depletion laser – the STED pulse (3, red). This induces a depletion of the excited dye molecules, which de-excites them before they can emit any fluorescent light. Due to the depletion beam's doughnut-like shape (7), fluorescence is inhibited only in the outer regions of the illuminated spot. The result is a small, tightly focused, super-resolution spot that is scanned across the sample (8). This smaller spot overcomes the resolution-limiting effects of diffraction, resulting in more accurate scanning. Diagram courtesy of Leica Microsystems, Germany.

# STimulated Emission Depletion (STED)



Two lasers are used almost simultaneously to achieve STED microscopy. The first beam excites the fluorophore, creating the usual diffraction limited PSF. The second, red shifted beam is shaped like a donut and depletes the emission in the outer portion of the PSF, leaving behind a smaller PSF.

Early versions of STED only depleted in xy, as shown above. Newer versions of STED feature 3D donuts, adding z resolution improvement. Multiple excitation / depletion laser pairs can be used to create multi-color images.



# STimulated Emission Depletion (STED)

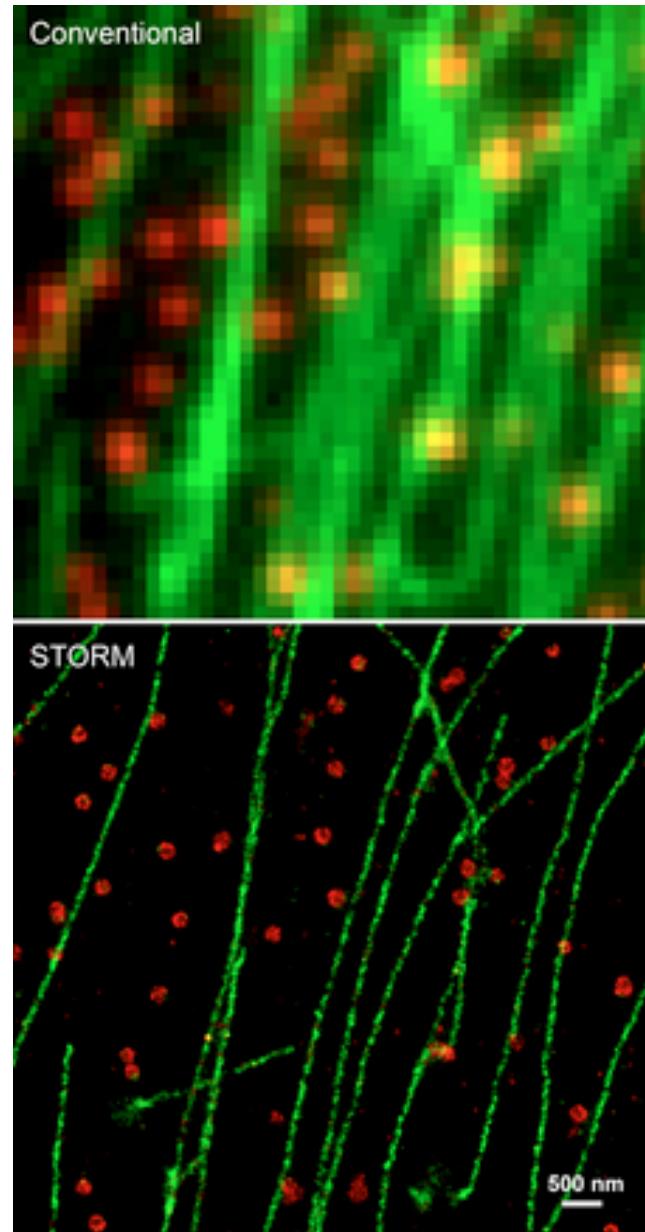
- Early systems offer 4-fold improvement over confocal in xy – down to 50nm – but no improvement in z resolution
- Newer systems offer a 3D depletion donut, giving improvements in z (70nm) as well as xy resolution
- High quality fluorophores and some fluoroproteins can be used. DAPI is not recommended. High quality mounting medium is essential
- Machines are generally built on a confocal base. They are easy to use and there is no image reconstruction, superresolution results are shown in real time
- The fastest of the big three methods in terms of imaging time
- Live sample imaging is possible but the depletion donut is high-energy. Samples can suffer large amounts of photo damage

# STORM and Other Localization Techniques



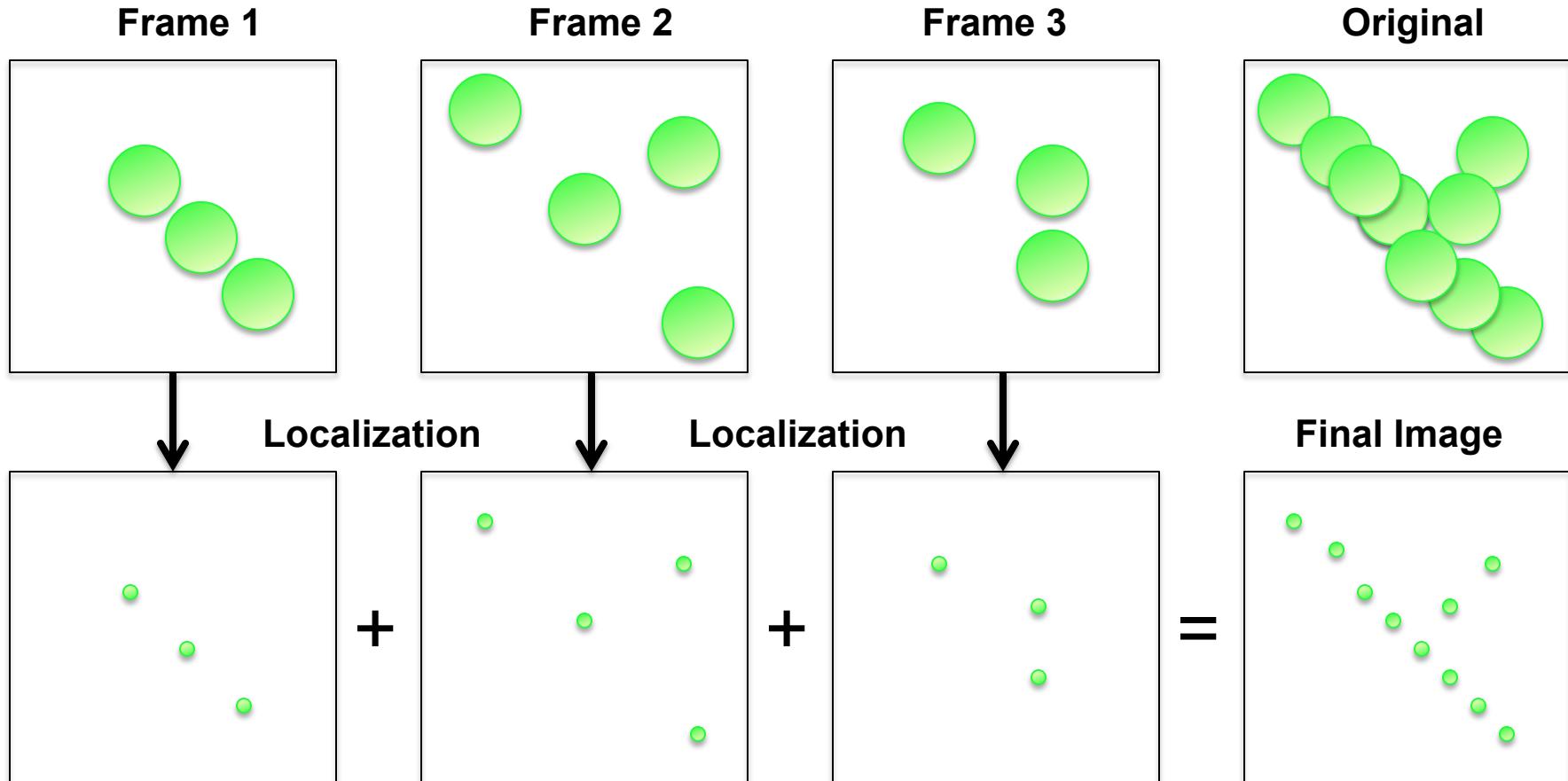
Localization techniques for superres imaging rely on capturing a handful of diffraction limited points, then using a mathematical formula to find and map the centers of those points, allowing for precision localization of labeled molecules down to 20nm lateral resolution. Images are built a few points at a time, much like a Seurat painting, and are thus also known as “pointillist” techniques.

Image credits (left): “A Sunday Afternoon on the Island of La Grande Jatte” by Georges-Pierre Seurat, The Art Institute of Chicago (right): X. Zhuang lab, HHMI at Harvard University



# STORM and Other Localization Techniques

Here is an illustration of the basic principle of all localization techniques. In the first frame, three fluorophores along a branched microtubule are excited (top) and localized (bottom) with a Gaussian fitting algorithm. In the second frame, different molecules are excited and localized. The process repeats over thousands of frames, building the final image over the course of several minutes.



# STORM and Other Localization Techniques

There are a variety of ways to label molecules with fluorophores or fluoroproteins which blink on and off. The most often used are:

## Stochastic Optical Reconstruction

**(STORM)** – Pioneered by Xiaowei Zhuang at Harvard University. Requires molecules to be double-labeled with two fluorophores (Cy3 and Cy5 for example). All Cy5 molecules are switched “off” with a strong red laser pulse. A green laser pulse excites a random handful of Cy3 molecules, causing them to transfer their energy to their partner Cy5 molecules and turning the Cy5 “on” again. These Cy5 molecules are then recorded with low energy red light and localized before photobleaching. The process is repeated until the majority of the molecules have been recorded.

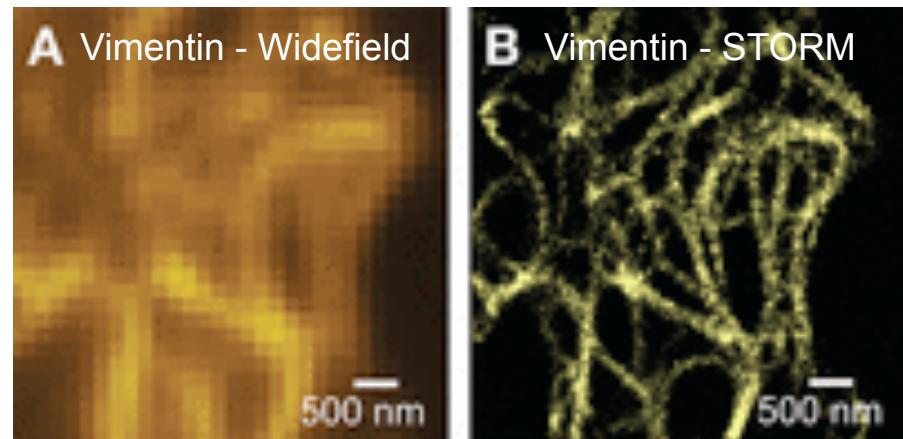
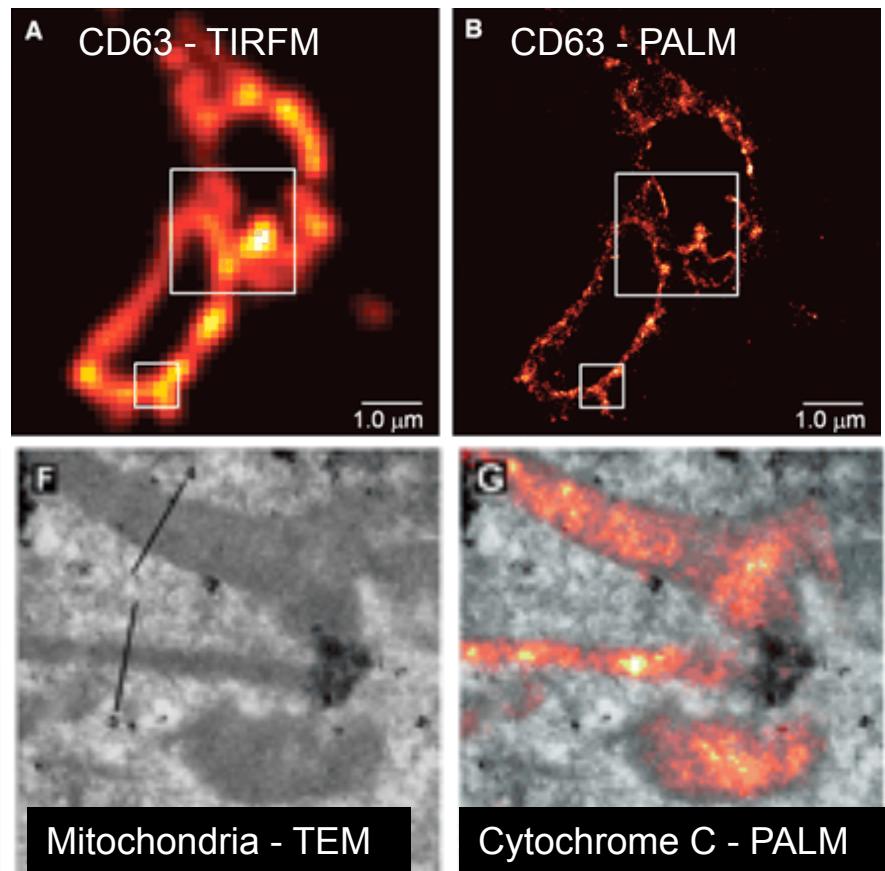
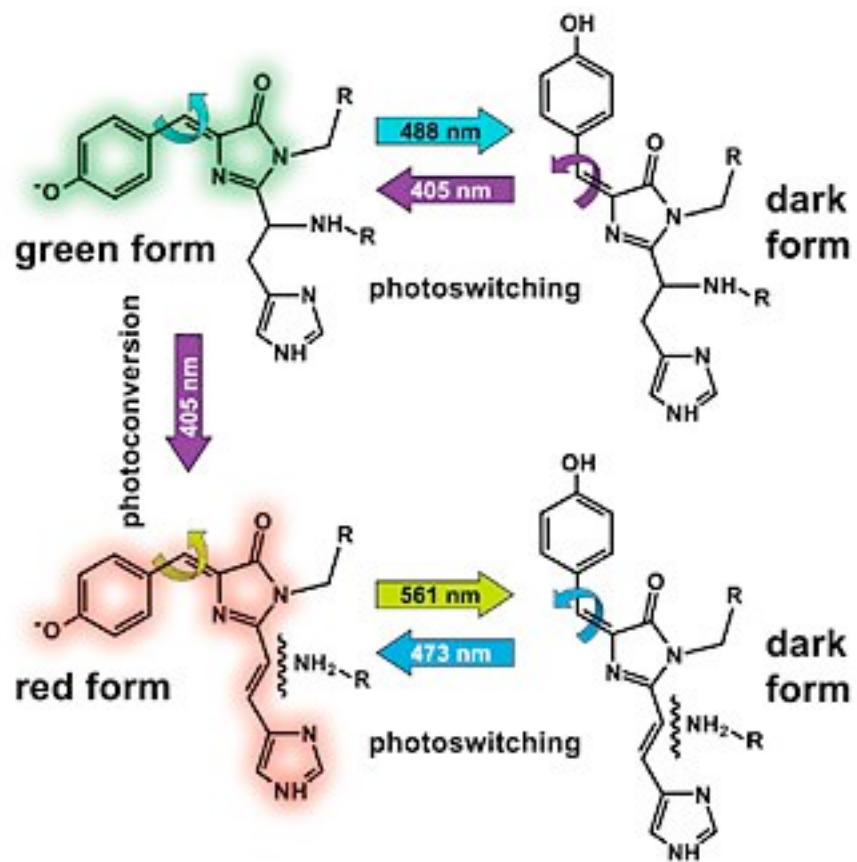


Image credits: Bates M, Jones SA and Zhuang X, Cold Spring Harbor Protocols 2013.

# STORM and Other Localization Techniques

**PhotoActivated Localization Microscopy (PALM)** – Pioneered by Eric Betzig and Harald Hess at the HHMI Janelia Farm campus. Uses photoactivatable or photoconvertible fluorescent proteins such as DRONPA or Kaede. Controlling the state of individual fluoroproteins allows for capture and localization of single molecule fluorescence.



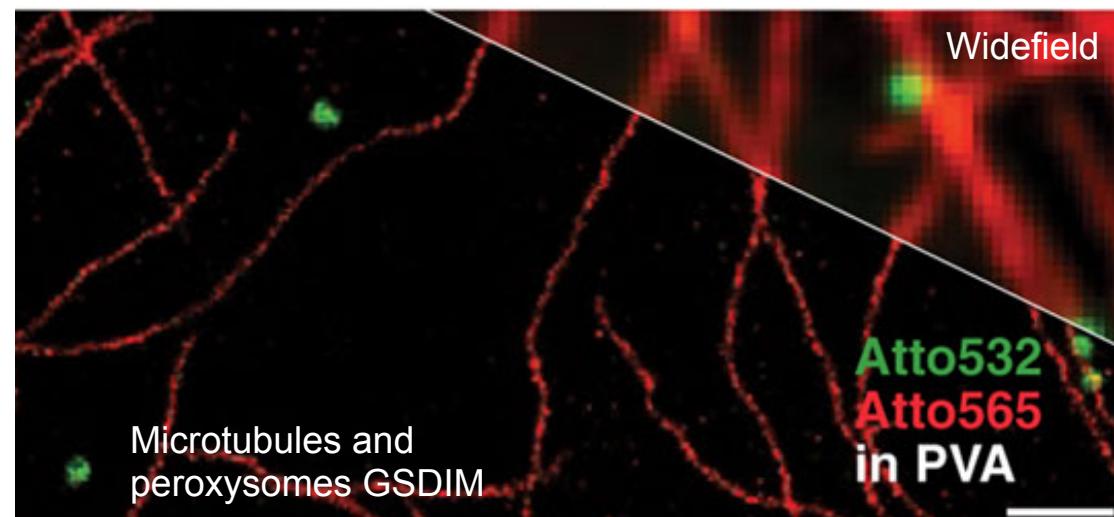
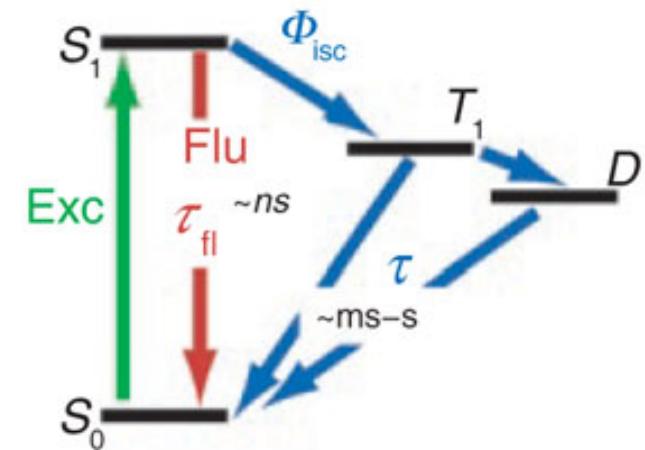
# STORM and Other Localization Techniques

## Ground State Depletion with Individual Molecule

**return (GSDIM)** – Pioneered by Stefan Hell at the Max Planck Institute for Biophysical Chemistry.

Uses large amounts of excitation light to drive fluorophores from the ground state ( $S_0$ ) through the excited state (green line to  $S_1$ ) and into a long-lived, high energy, non-fluorescent “dark” state (blue lines to D). Once all fluorophores are in a dark state (i.e. the ground state is depleted) the excitation light is turned down.

Random molecules return to the ground state (blue lines to  $S_0$ ) and are excited again (green line to  $S_1$ ). Some of these emit light while returning to ground state (red line to  $S_0$ ) while others go dark again. When individual molecules fluoresce, they are imaged and localized.

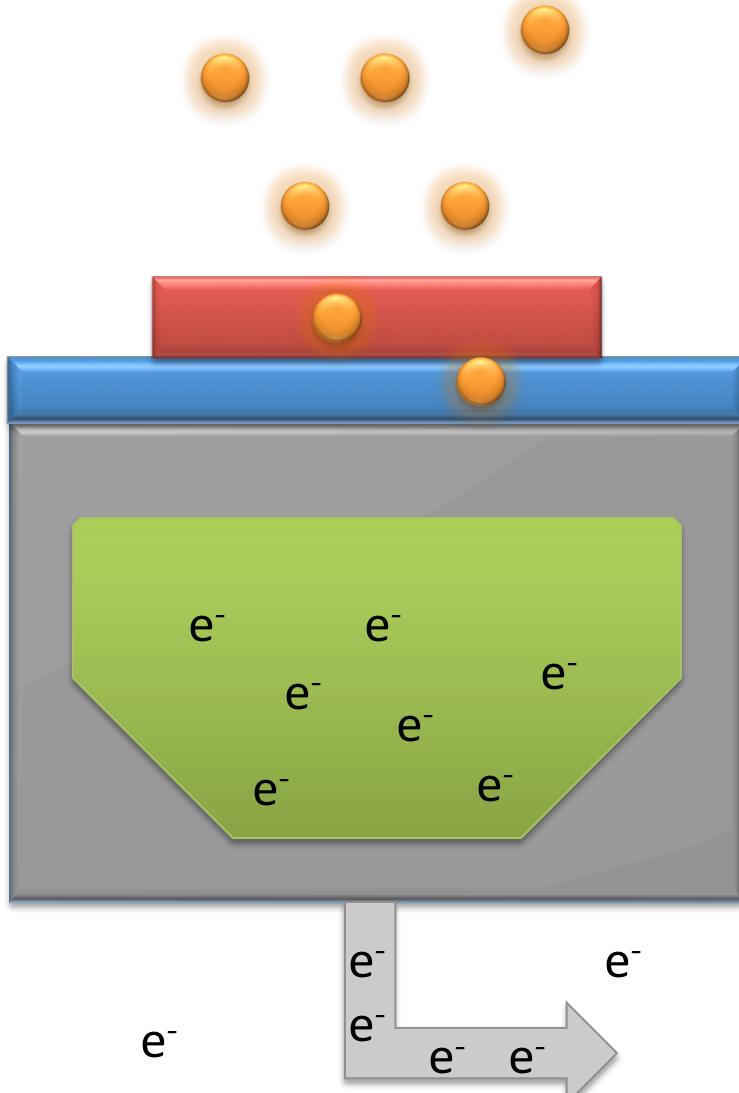


# STORM and Other Localization Techniques

- These techniques offer the highest 3D resolution yet achieved with light microscopy, down to 20nm lateral (xy) and 50 nm axial (z)
- Rely on activation of a handful of diffraction limited points, followed by computational localization of the centers of those points. This is the slowest and most computationally intense of the big three superresolution techniques
- Images are built one channel at a time over several minutes. Movement alters localization, so live sample imaging is not possible
- Techniques such as true STORM and PALM require special fluorophores or fluoroproteins. GSD can use common high-quality fluorophores but still requires special sample preparation techniques
- Machines are built on a widefield / TIRF platform and have a steeper learning curve than either SIM or STED

# How Does a Camera Convert Photons to Grey Levels? (With More Detail)

Individual pixel



Incoming photons

↓ Exposure time

Semiconducting metal

↓ Quantum efficiency

Production of electrons

↓ Photon shot noise

Electrons fill the potential well, building charge proportional to the # of photons

↓ Dark current (noise)

Well empties when charge passed to another section of the chip

↓ Read noise

Which passes the information on through an ADC to the computer

# Signal to Noise Ratio Definitions

Quantum efficiency (QE) – when talking about cameras or other photovoltaic devices this represents the % of photons hitting the detector which are converted to electrons. QE for scientific cameras ranges from 65% - 95%, depending on the semiconducting materials used and the way the well is manufactured. The higher the QE the higher the cost.

Full well depth – the total number of electrons that can be recorded per pixel. Well depth increases as pixel area increases, so larger pixels have greater capacity, but also collect photons from a larger area of the sample.

Photon shot noise – the “error” added to the signal when photons interacting with the semiconducting metal are converted to electrons. Equal to the square root of the number of photons collected.

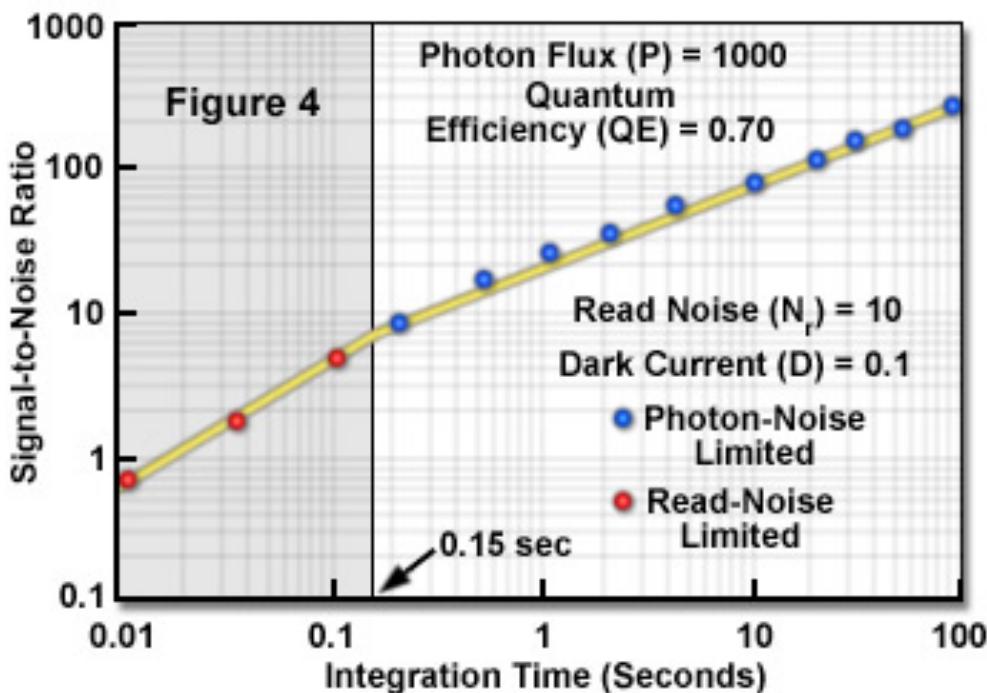
Dark current – electrons which are spontaneously generated through thermal motion. Strongly dependent on temperature, so the more you can chill your camera the less dark current you will have. Ranges from 10-20 electrons per pixel in a high quality, room temperature CCD to 2-5 electrons per pixel in a chilled CCD. Also, the longer your exposure time, the bigger the effect of dark current (more time for thermally generated electrons to build up). This type of noise usually makes the smallest contribution to total noise.

Read noise – Introduced as the signal travels though the chip to the ADC (analog to digital converter). Lowest level possible is dependent on the way the camera was manufactured, but for all cameras read noise increases at a rate proportional to the square root of the imaging speed, so the faster you image, the more readout noise there will be.

# Signal to Noise Improves with Integration Time

$$\text{SNR} = \# \text{ of photons} / \sqrt{\# \text{ of photons}}$$
$$= \sqrt{\# \text{ of photons}}$$

Signal-to-Noise Variation with Integration Time



With a properly cooled camera, dark current (heat) makes a tiny contribution

When photon numbers are low, read noise (electron movement) dominates

When photon numbers are high, photon shot noise (photon to e- conversion “error”) dominates

In order to double your SNR, you must collect 4x more photons

# A Word on Color vs. Grayscale

Some scientific images are collected with color cameras, and the accuracy of the representation of those colors is important to the interpretation of the image.

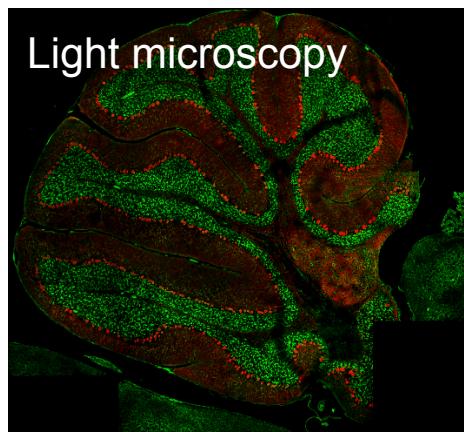
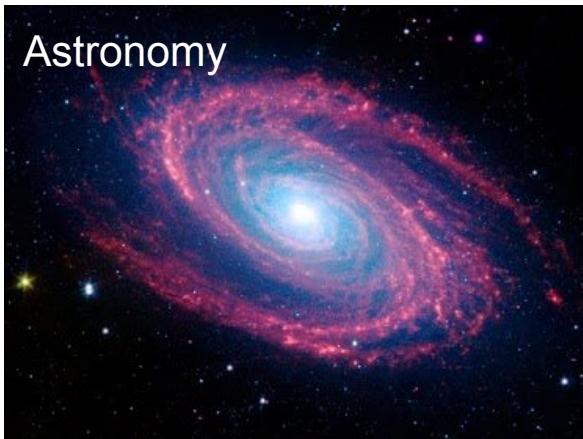
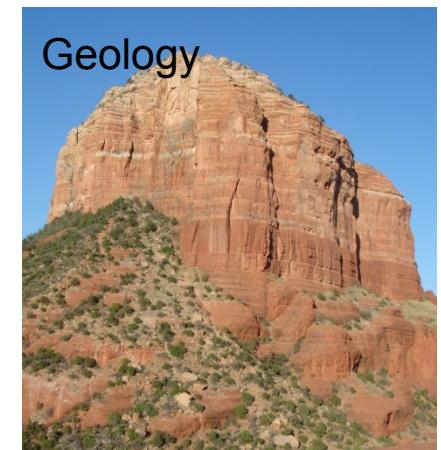


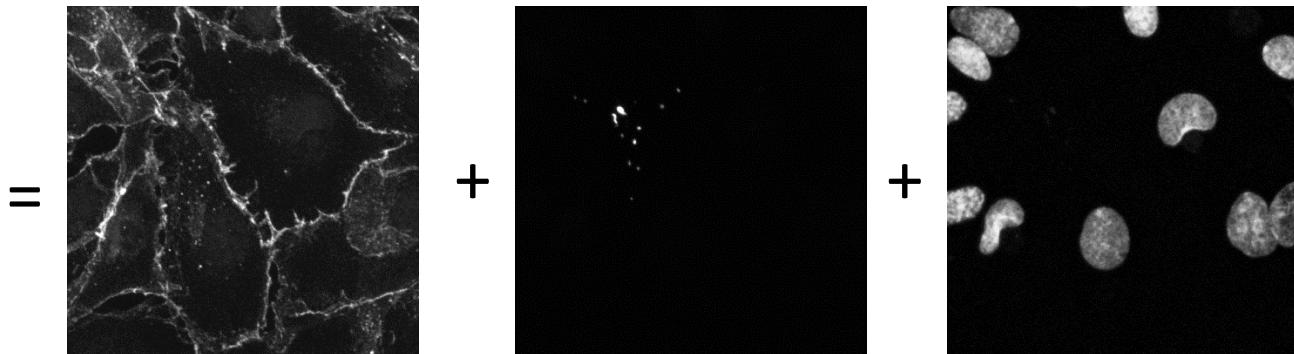
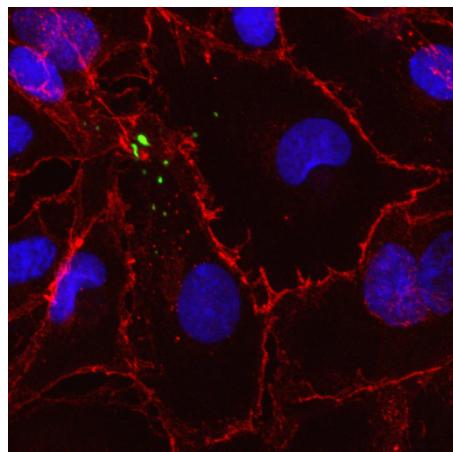
Image credits: Perkin Elmer Pannoramic (sic) whole slide scanner (histology); Sedona, Arizona (geology); NASA Spitzer telescope (astronomy) and Olympus IX81 (light microscopy)

Other scientific images are collected with grayscale cameras, or with instruments which detect wavelengths outside what the human eye can perceive. Colors in these images are applied after the fact, and are more to aid our understanding of the image than to represent the actual colors present in the object.

# 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.



$$\text{24-bit RGB color image} = \text{8-bit red} + \text{8-bit green} + \text{8-bit blue}$$

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