

Optical Microscopy Basics

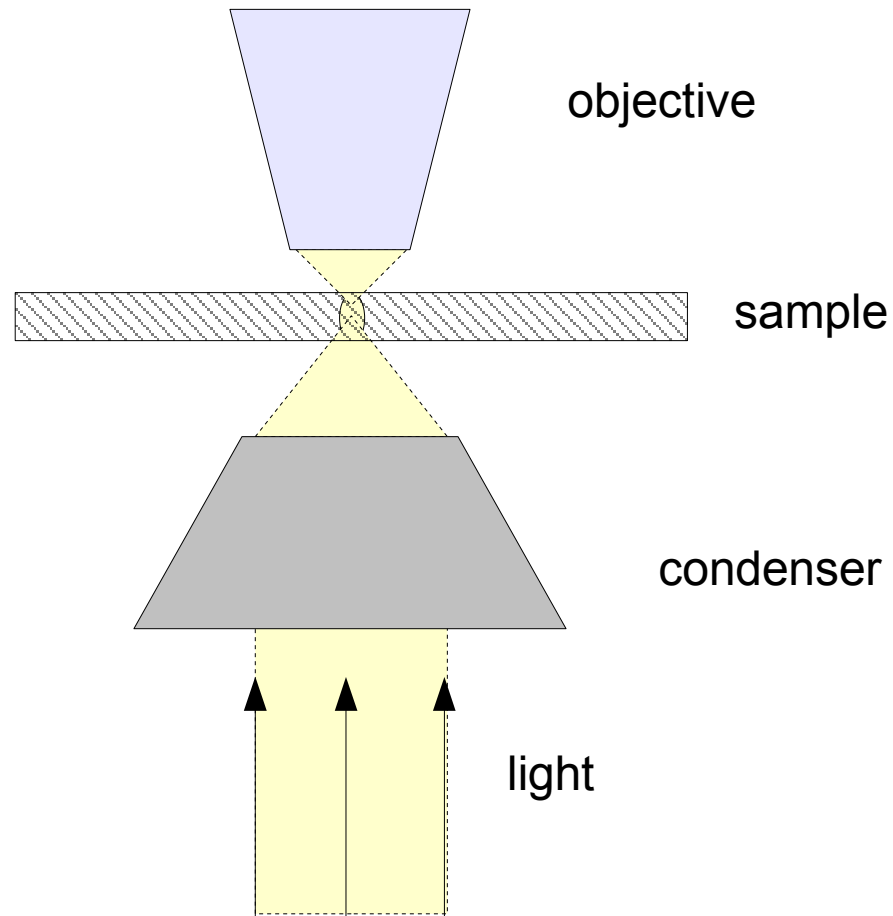
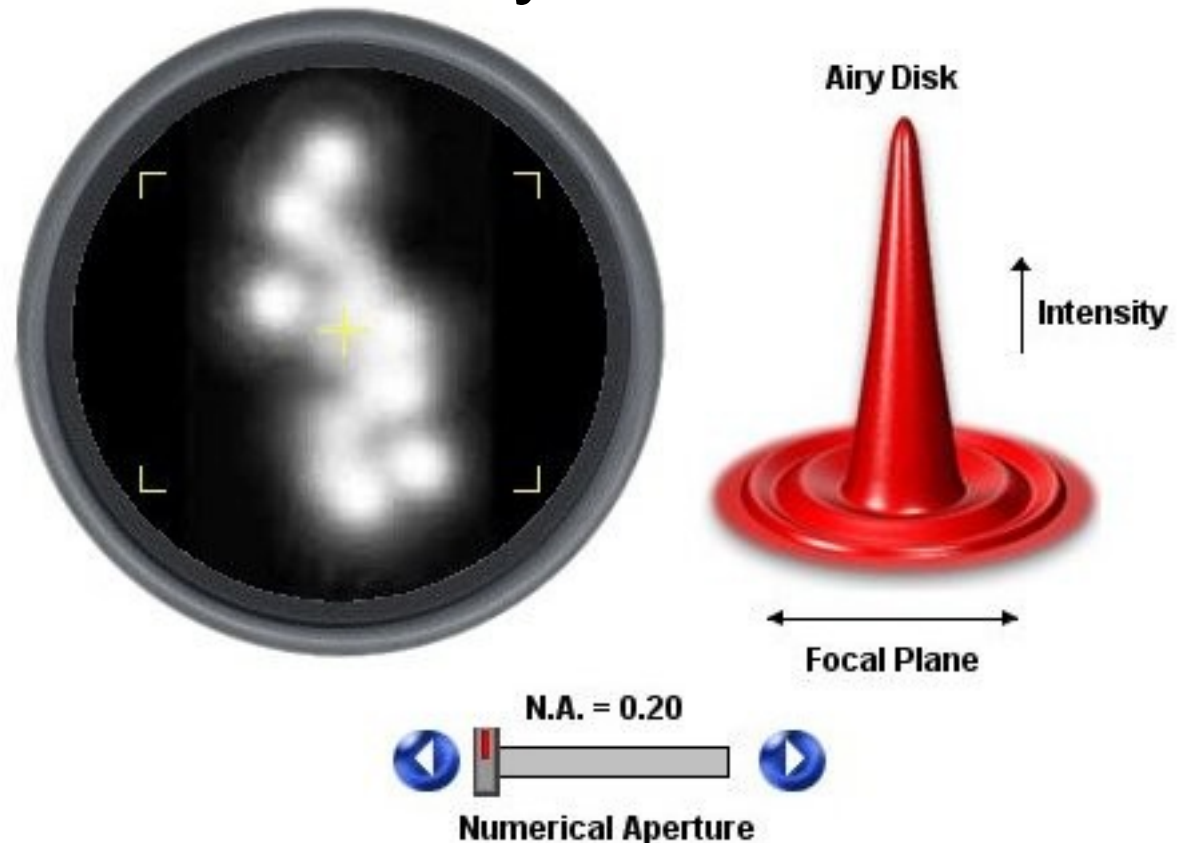


Image Resolution

When a point source is focused to a spot (for now assume an ideal lens), the intensity profile in the image plane is an “Airy disk”



Nikon Website

<http://www.microscopyu.com/tutorials/java/imageformation/airyna/>

Image Resolution

Two adjacent points (when illuminated and imaged by identical lenses) can barely be resolved when the centres of their Airy disks are separated by a distance r

$$r = 1.22 \frac{\lambda_0}{2 NA_{obj}}$$

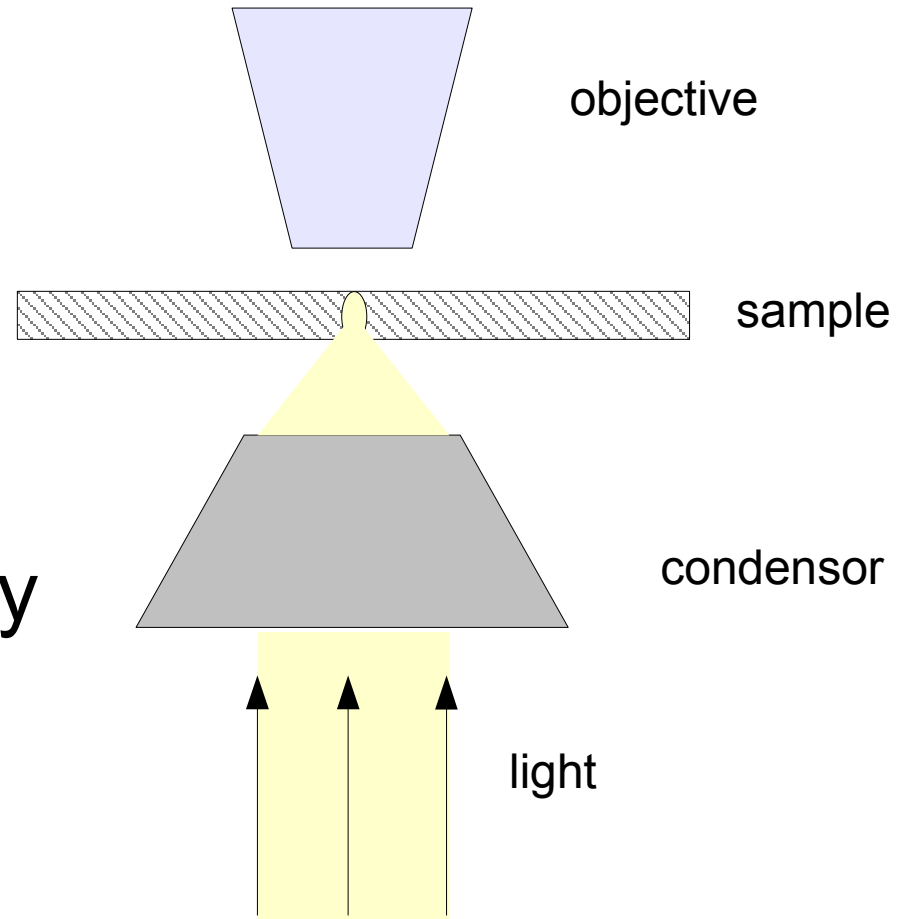
$NA_{obj} = n \sin \theta$ is the “numerical aperture”

n is lens refractive index and

θ is the half-cone angle of light captured by the objective lens

Image Resolution

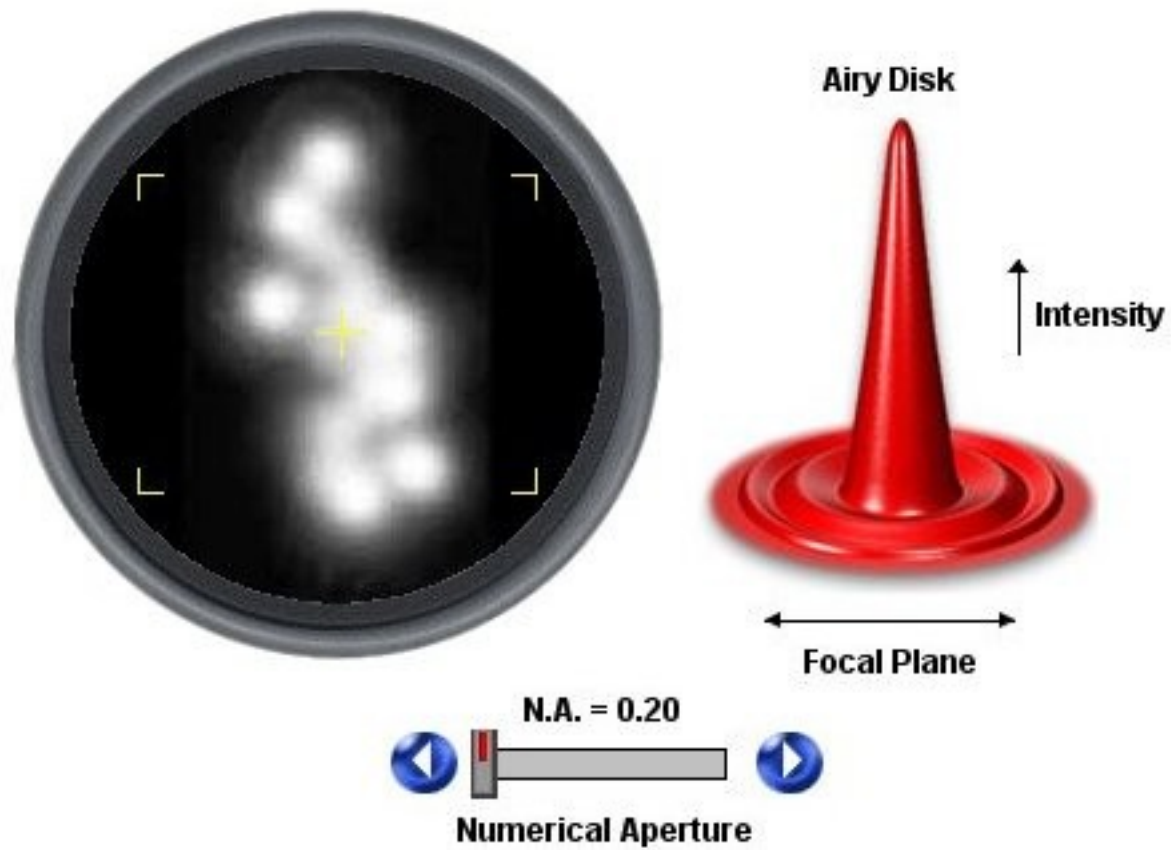
More generally, when illuminated by a condensor and imaged by an objective lens, the resolution might be poorer:



$$r = 1.22 \frac{\lambda_0}{NA_{con} + NA_{obj}}$$

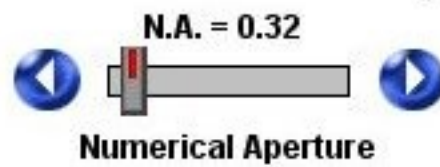
A Microscope Image

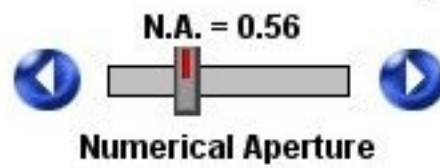
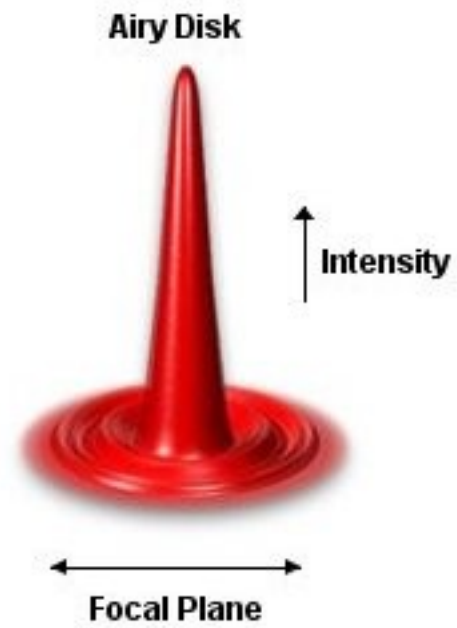
All specimens viewed through a microscope can be thought to be made up of a series of points, which become imaged as Airy disks. Therefore the smaller the radius of the Airy disk, the greater the resolution.

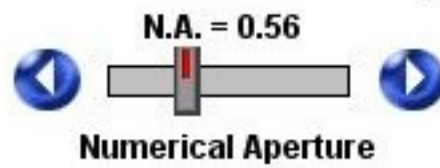


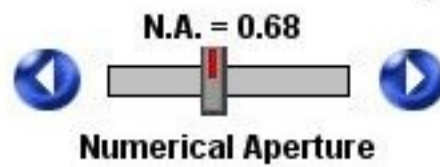
Nikon Website

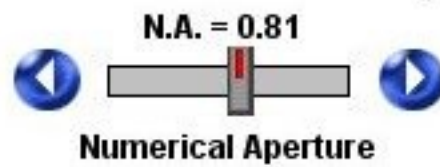
<http://www.microscopyu.com/tutorials/java/imageformation/airyna/>

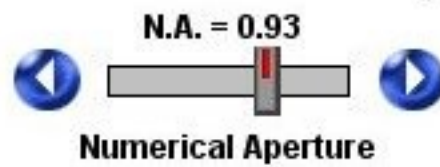














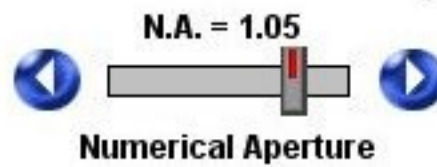
Airy Disk



Intensity



Focal Plane



Numerical Aperture



Airy Disk

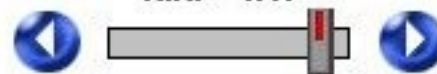


Intensity

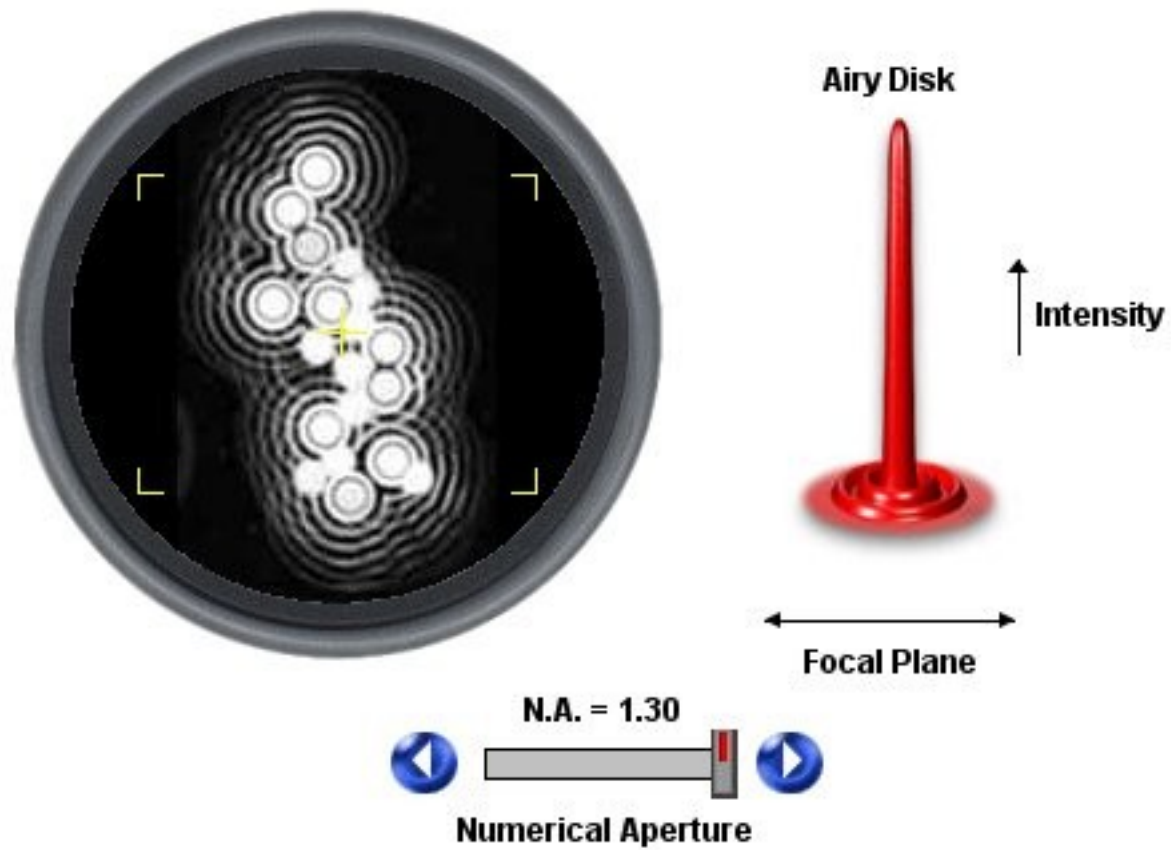


Focal Plane

N.A. = 1.17



Numerical Aperture



Nikon Website

<http://www.microscopyu.com/tutorials/java/imageformation/airyna/>

The diffraction limit

Lets say the objective lens has a numerical aperture of 1.45. Then

$$r = 1.22 \frac{\lambda_0}{2 NA_{obj}} = 0.2 \lambda_0 \geq 100\text{nm}$$

Practically speaking, about 200 nm.

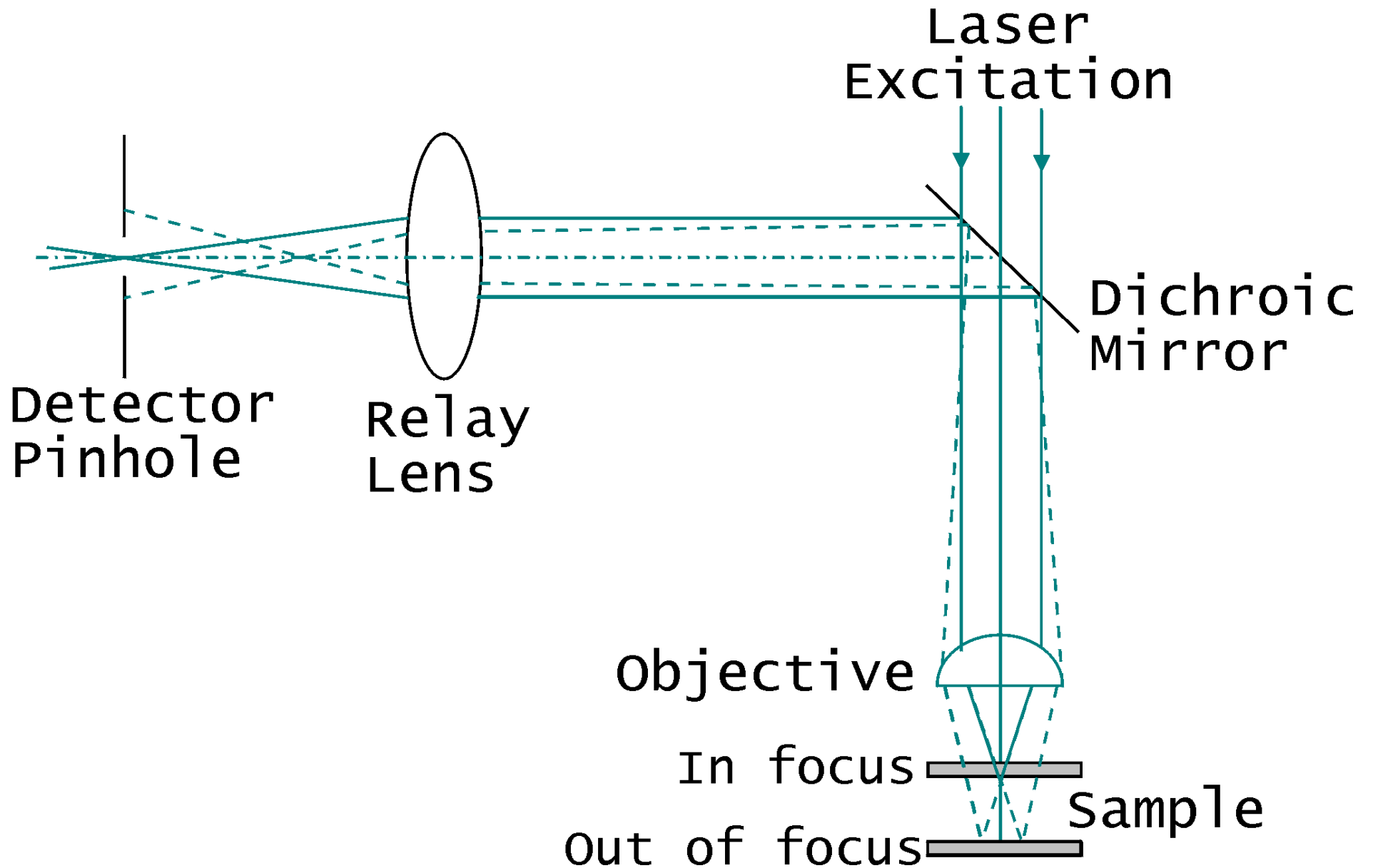
Unless you image using photons of lower wavelengths than the visible.

Confocal Microscopy

Rather than illuminate with a low NA condenser; one aims for ideal diffraction limit imaging:

image a single illuminated point, and scan that point across the sample to build up the image.

Confocal Microscopy



Contrast

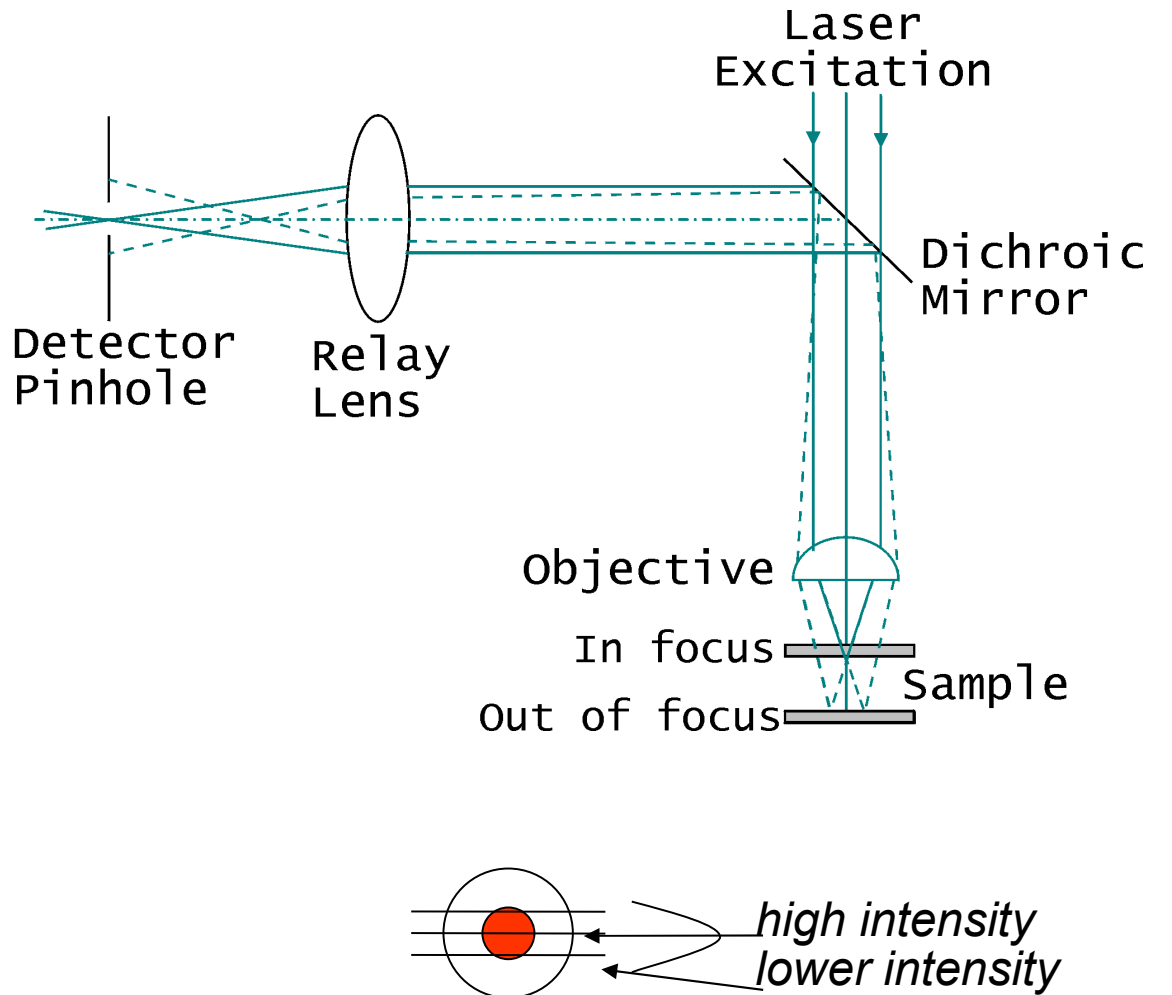
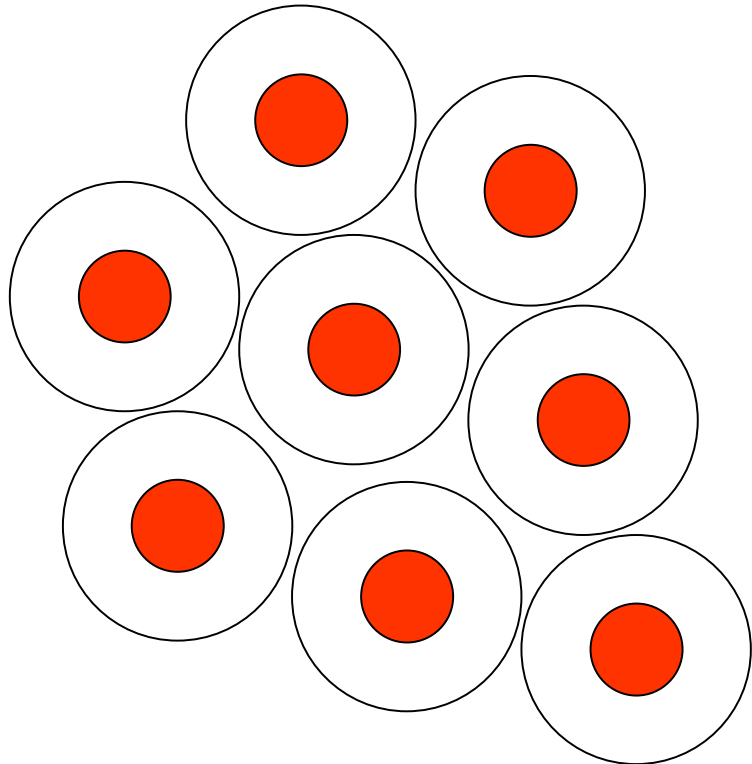
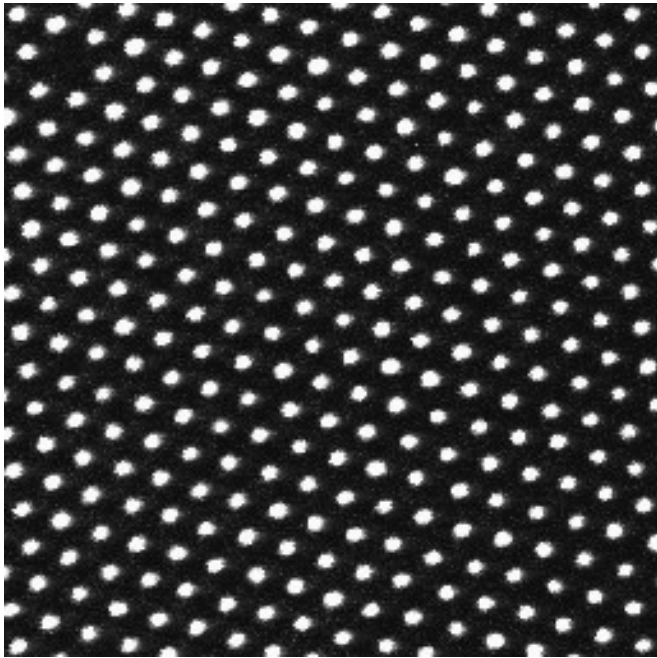
Even if two objects can be resolved by the optics, the contrast of the image is determined by:

- Light level and signal to noise
- Detector sensitivity and “dynamic range”

Fluorescence as contrast enhancement

The excitation wavelength varies from the emission wavelength. You label the species of interest with a fluorochrome, which absorbs light at a particular wavelength (undergoes a transition in electronic states), and emits at a lower wavelength.

Fluorescent 3D Confocal Microscopy



Green Fluorescent Protein (GFP)

2008: “This year's Nobel Prize in Chemistry rewards the initial discovery of GFP and a series of important developments which have led to its use as a tagging tool in bioscience.”

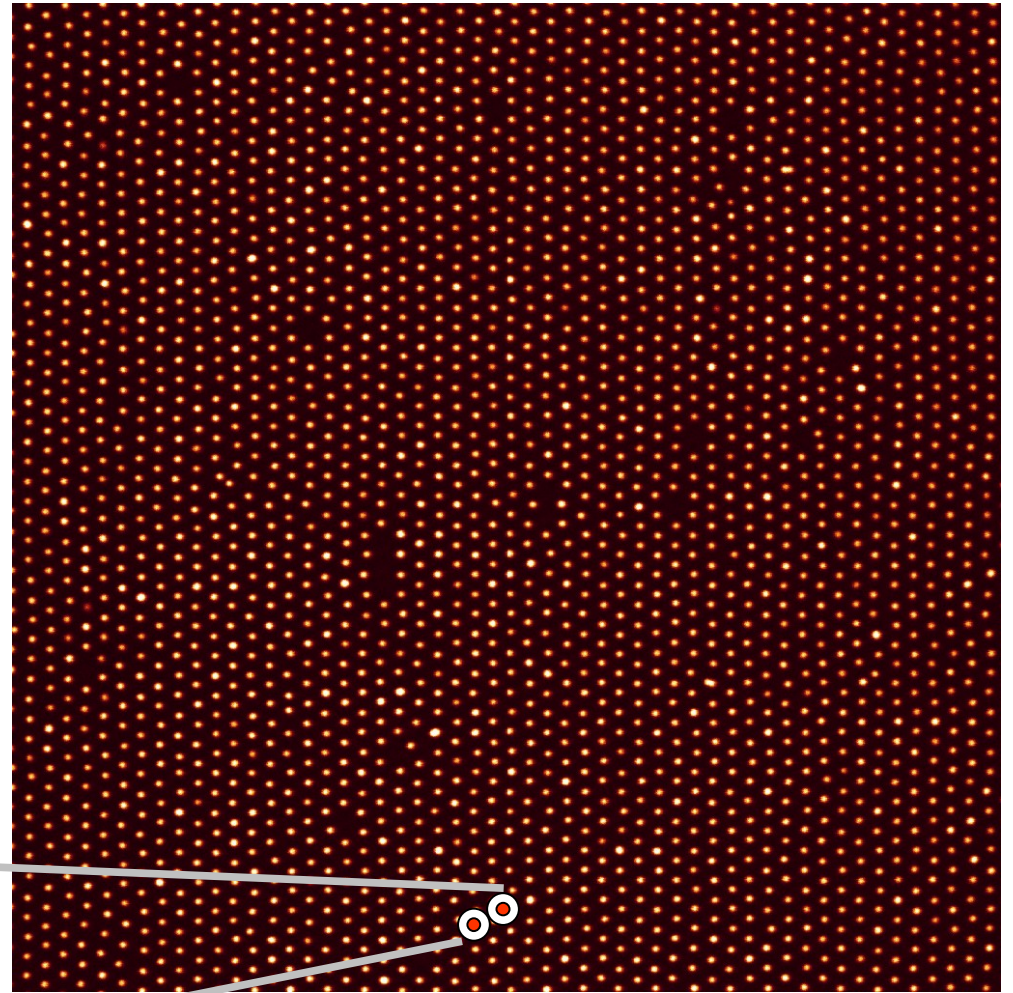
http://nobelprize.org/nobel_prizes/chemistry/laureates/2008/press.html

Osamu Shimomura first isolated GFP from the jellyfish *Aequorea victoria*, which drifts with the currents off the west coast of North America. He discovered that this protein glowed bright green under ultraviolet light.

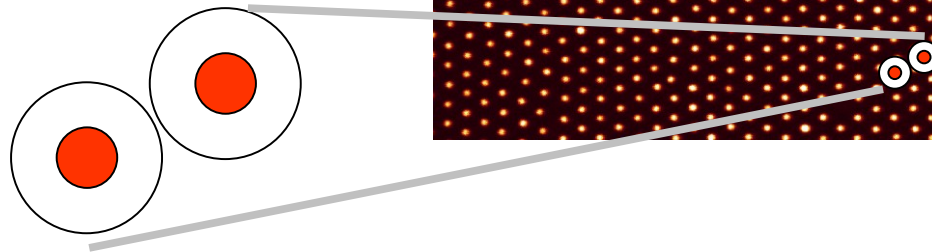
Martin Chalfie demonstrated the value of GFP as a luminous genetic tag for various biological phenomena. In one of his first experiments, he coloured six individual cells in the transparent roundworm *Caenorhabditis elegans* with the aid of GFP.

Roger Y. Tsien contributed to our general understanding of how GFP fluoresces. He also extended the colour palette beyond green allowing researchers to give various proteins and cells different colours. This enables scientists to follow several different biological processes at the same time.

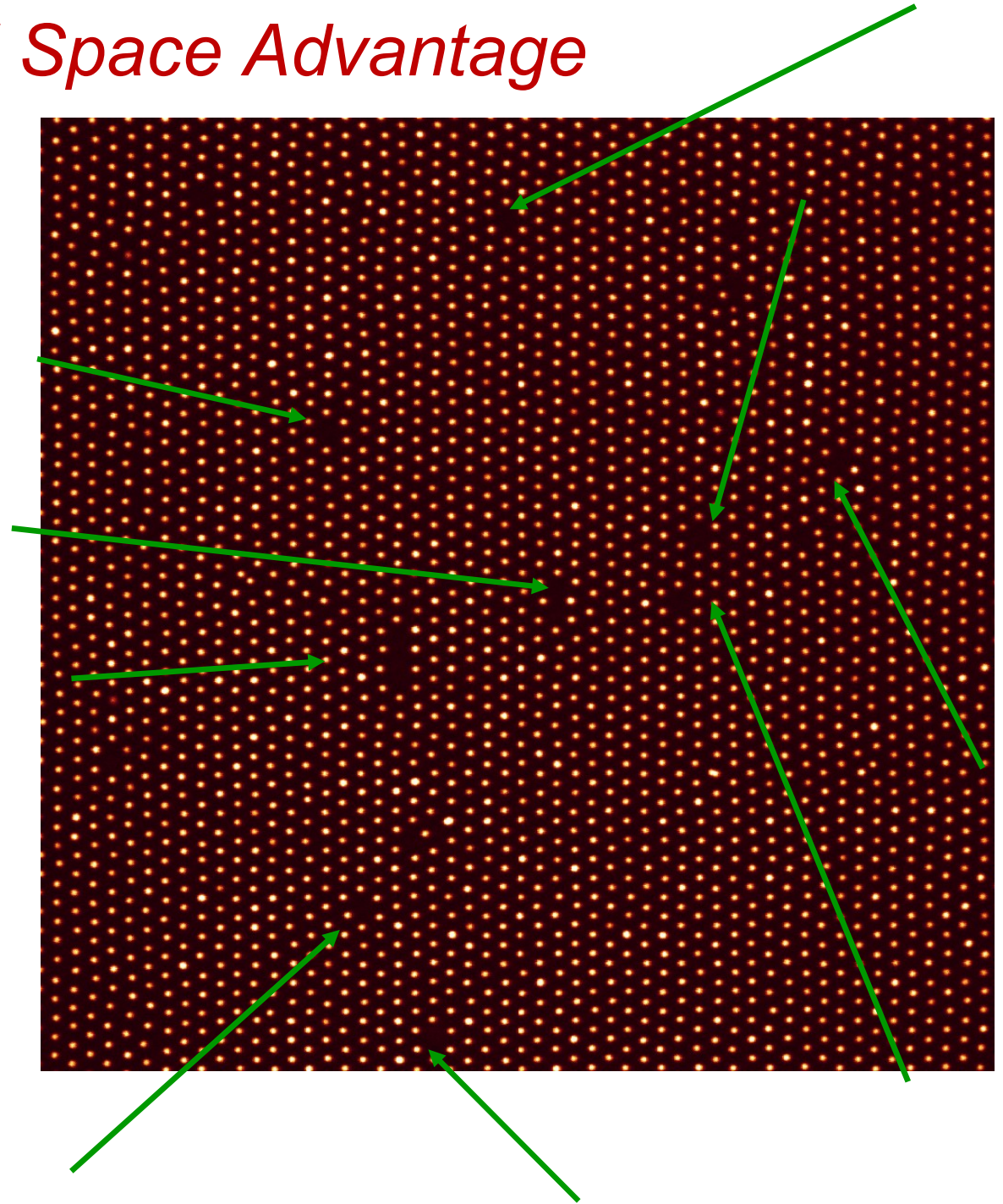
Fluorescence laser scanning confocal imaging of colloidal crystals



Fluorescent
core--non-fluorescent
shell silica spheres



The Real Space Advantage



Optical Microscopy

- Set up is simple (compared to neutron scattering)
 - Intuitive
- You can easily find things that you are not looking for.

But you cannot go below the diffraction limit.

Near-field Scanning Optical Microscopy

Near field: if you make the aperture smaller than the diffraction limit, and you detect in close proximity to the aperture, you can defeat the diffraction limit (Ahmad talk). But it is restricted to surfaces.

Also coming next week (Kris Poduska): (non-optical) scanning probe microscopies can also image surfaces to atomic resolution.

But today we want to look into materials.

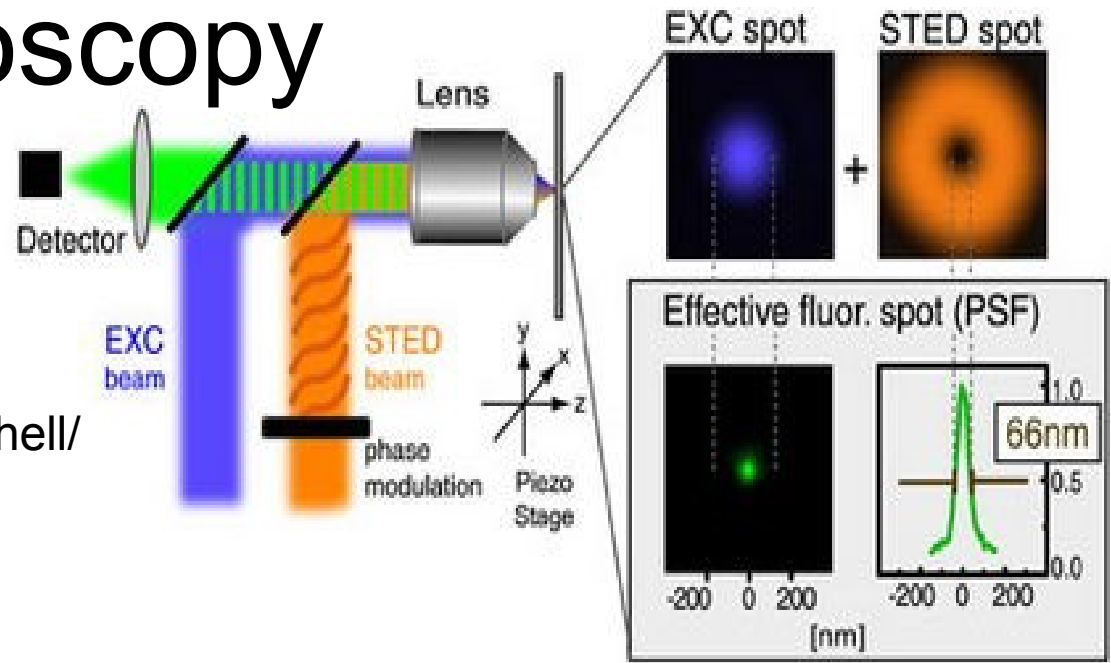
Two-photon microscopy

An enhancement of the confocal advantage.

If you have a femtosecond pulsed laser source, you can promote a molecule to an excited electronic state with two photons of half the energy of the transition.

Excitation probability depends quadratically on laser intensity, so a smaller volume is excited.

Two photons of different wavelengths: **S**timulated **E**mission **D**epletion microscopy

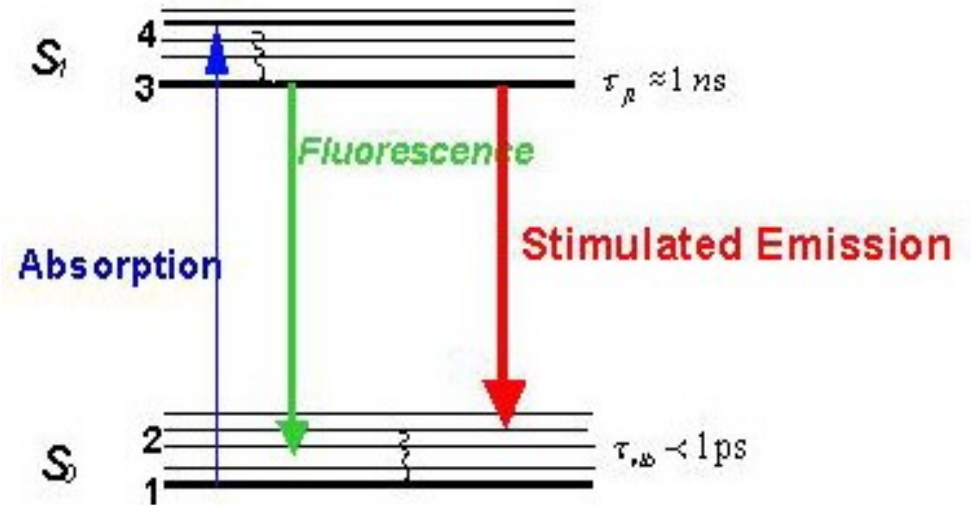


Website of Group of Stefan Hell
<http://www.mpibpc.mpg.de/groups/hell/>

- The EXC beam excites the fluorescent molecule in a spot
- The STED beam arrive picoseconds later and quenches the fluorescent markers before they fluoresce
- The STED beam is doughnut shaped and so quenches the fluorescence of the EXC spot preferentially on the outside (thus reducing the spot size).

Two photons of different wavelengths: **S**timulated **E**mission **D**epletion microscopy

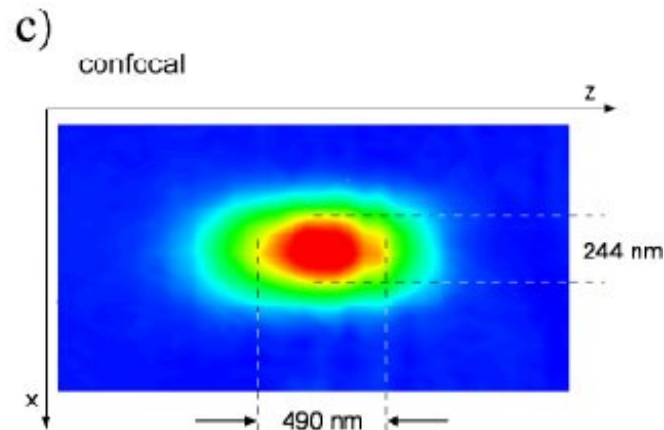
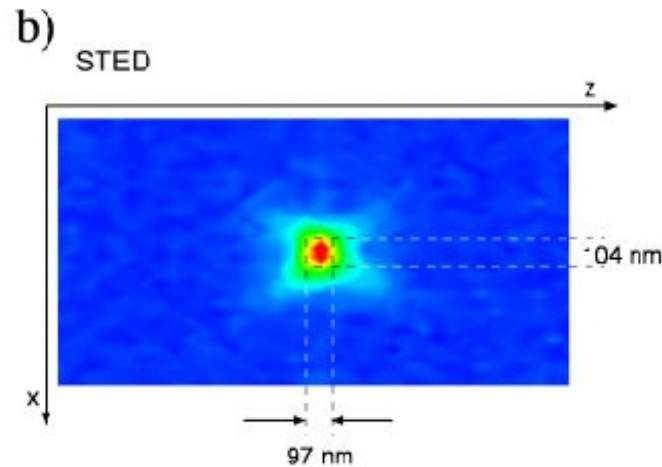
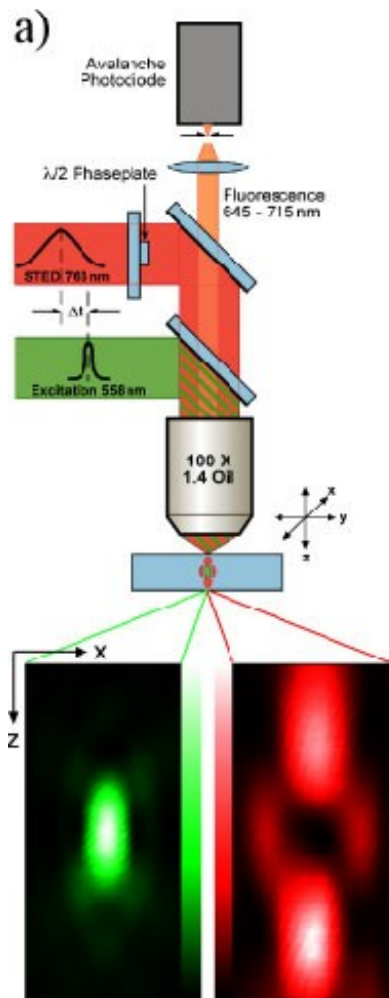
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STED is capable of nanoscale imaging.

Hein, B., K. I. Willig, S. W. Hell (2008): "Stimulated emission depletion (STED) nanoscopy of a fluorescent protein-labeled organelle inside a living cell". Proc. Natl. Acad. Sci. USA 105 (38), 14271-14276.



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Single molecule imaging and spectroscopy

W.E. Moerner, D. P. Fromm, Rev. Sci. Inst. 2003

- Laser is used to excite an electronic transition that is resonant with the optical wavelength
- Dilution: The molecule of interest is in ultra-dilute concentrations so there is only one fluorescent molecule in the excitation volume
- SNR: The signal-to-noise ratio for the single-molecule signal should be greater than unity for a reasonable averaging time.

Single molecule imaging and spectroscopy

W.E. Moerner, D. P. Fromm, Rev. Sci. Inst. 2003

Dilution:

IF the probed volume is 10 cubic microns, the concentration should be 10^{-10} moles per litre!

Single molecule imaging and spectroscopy

W.E. Moerner, D. P. Fromm, Rev. Sci. Inst. 2003

SNR:

Should minimize sample volume because other optical contributions (Raman scattering) is a noise contribution in this case.

(Another story: one can also use the Raman signal as contrast for imaging).

Single molecule imaging and spectroscopy

W.E. Moerner, D. P. Fromm, Rev. Sci. Inst. 2003

SNR: Detectors with high quantum efficiency and low dark counts

- *Photomultiplier tubes (PMT). QE~20%*
- *Avalanche photo-diodes (APD). This is the semiconductor version of a PMT. QE~60%*
- *Single-photon avalanche photodiodes (SPAD). QE ~ 95% in the infrared!*