

# Microscopy

CS/CME/BioE/Biophys/BMI 279

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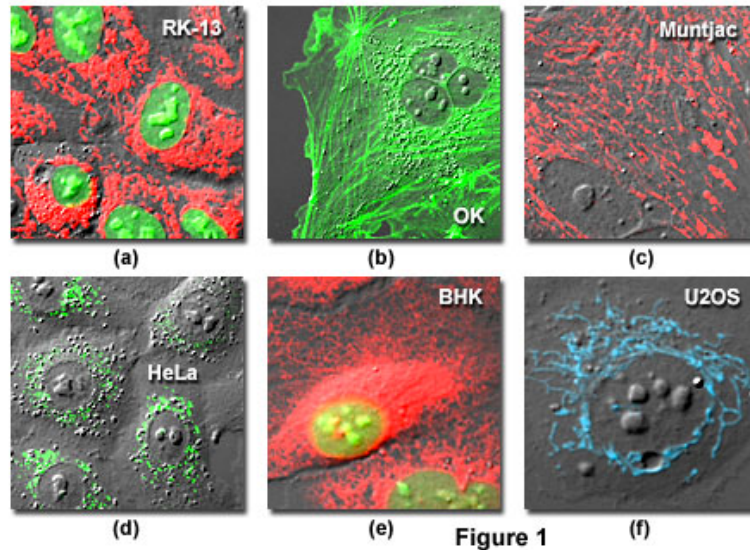
# Outline

- Microscopy: the basics
- Fluorescence microscopy
- Resolution limits
  - The diffraction limit
  - Beating the diffraction limit

# Microscopy: the basics

# Most of what we know about the structure of cells come from imaging

- Light microscopy, including fluorescence microscopy



<https://www.microscopyu.com/articles/livecellimaging/livecellmaintenance.html>

- Electron microscopy



<http://blog.library.gsu.edu/wp-content/uploads/2010/11/mtdna.jpg>

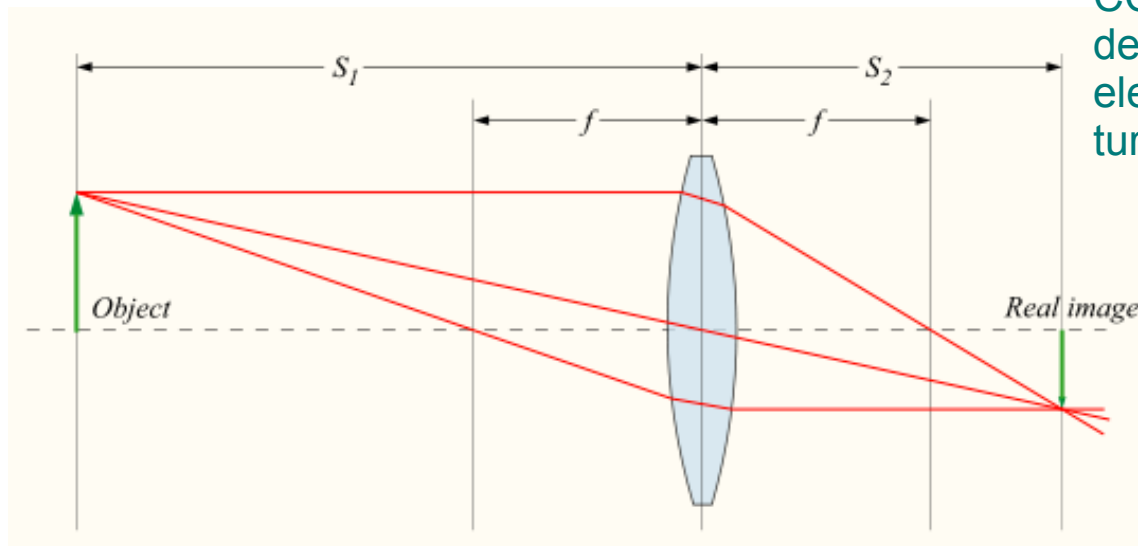
# Light microscopy

- Basic idea:
  - Shine light on a biological sample (i.e., one or more cells)
  - Measure the light that is *reflected* or *transmitted*
  - Use lenses
    - Why do we need lenses in a microscope?



# Lenses in microscopy

- The lenses in a microscope do two things:
  - Magnify the image
  - *Focus* the image, so that much of the light coming from a particular point in the sample ends up focusing on a particular point on either your retina or a sensor (e.g., CCD)
- You need a lens to form a clear image, even if you have a very high-resolution sensor



CCD (charge-coupled device) is an array of electronic elements that turn photon into charges

# Fluorescence microscopy

# Fluorescence microscopy: basic idea

- Suppose we want to know where a particular type of protein is located in the cell, or how these proteins move around
- We can't do this by simply looking through a microscope, because:
  - We (usually) don't have sufficient resolution
  - The protein of interest doesn't look different from the ones around it
- If only the protein would glow!
- Can we get a protein (or other molecule of interest) to glow?

# Fluorescence microscopy: basic idea

- Make the molecules of interest glow
- Attach a fluorophore (fluorescent molecule) to the molecule of interest
- When you shine light of a particular wavelength on a fluorophore, it emits light of a different wavelength For visible light, different wavelength corresponds to different color
  - Additional advantage: not only does the molecule glow, the light it emits has a different wavelength than the incident illumination, making it easier to isolate

The light emitted by fluorescent molecule will be of longer wavelength (lower frequency) which corresponds to lower energy level than the incidence light.

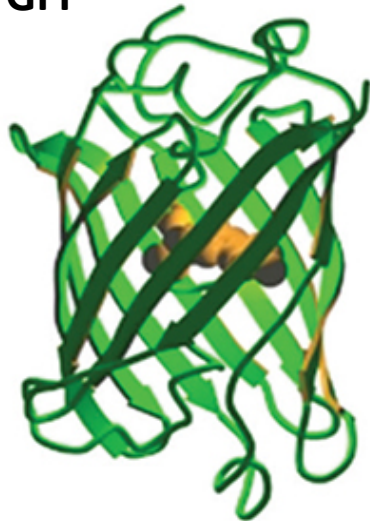
Fluorescent molecule can capture photons at particular energy level to go to an excited quantum state. When dropping back down, it emits photons of a lower frequency which has less energy.

# Fluorophores

- Fluorophores can themselves be either proteins or much smaller molecules Most widely used small molecule fluorophores is Alexa fluors
  - Among the most widely used is green fluorescent protein (GFP) GFP can be found naturally in some jellyfish

The Nobel Prize in Chemistry 2008 was awarded jointly to Osamu Shimomura, Martin Chalfie and Roger Y. Tsien *"for the discovery and development of the green fluorescent protein, GFP"*.

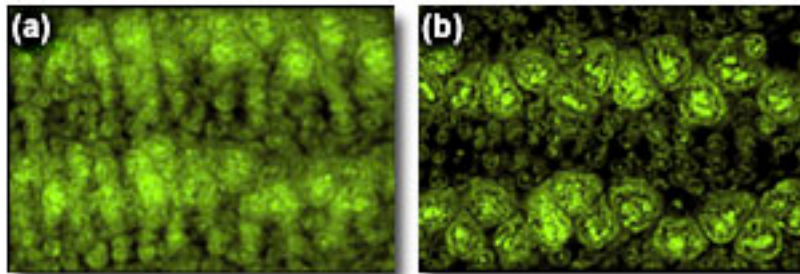
GFP



# Fluorescence microscopy images

- There are many types of fluorescence microscopy: wide-field, confocal, TIRF (total internal reflectance fluorescence), etc.
  - You're not responsible for knowing them

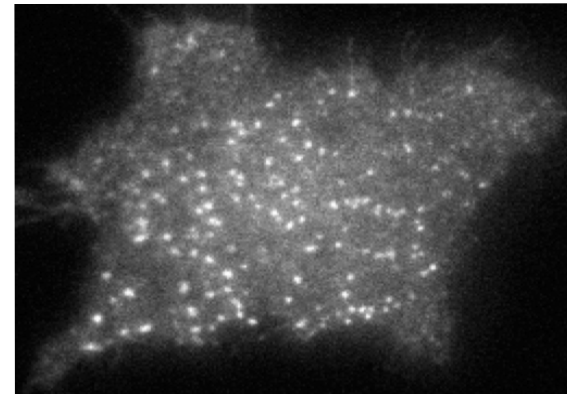
Butterfly Wing Epithelium



Wide-field

Confocal

TIRF



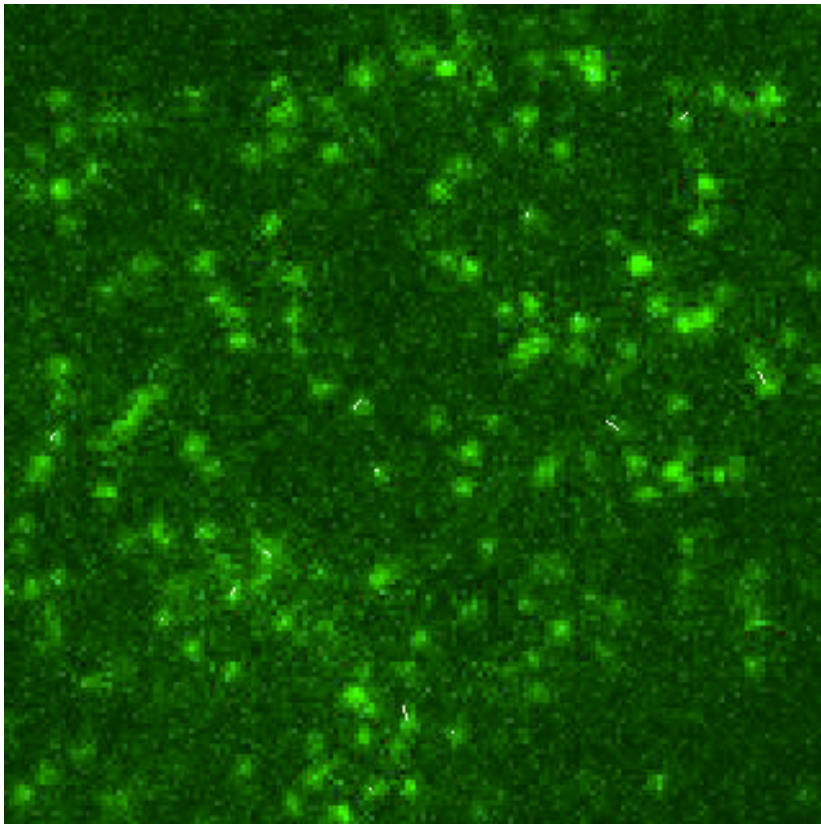
Von Zastrow lab, UCSF

<http://www.microscopyu.com/articles/confocal/confocalintrobasics.html>

Analyzing this data quantitatively involves the types of image analysis we discussed in previous lectures, and more

# Single-molecule tracking

- If the density of fluorescent molecules is sufficiently low, we can track individual molecules
  - Doing this well is a challenging computational problem



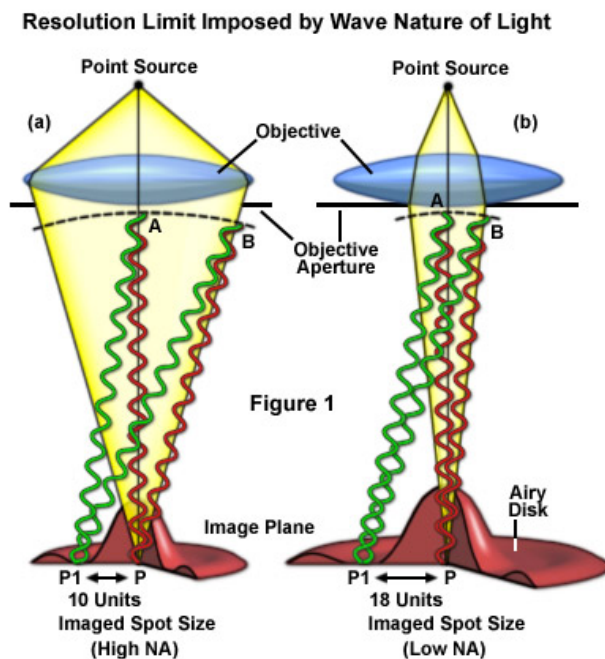
# Resolution limits

Resolution limits

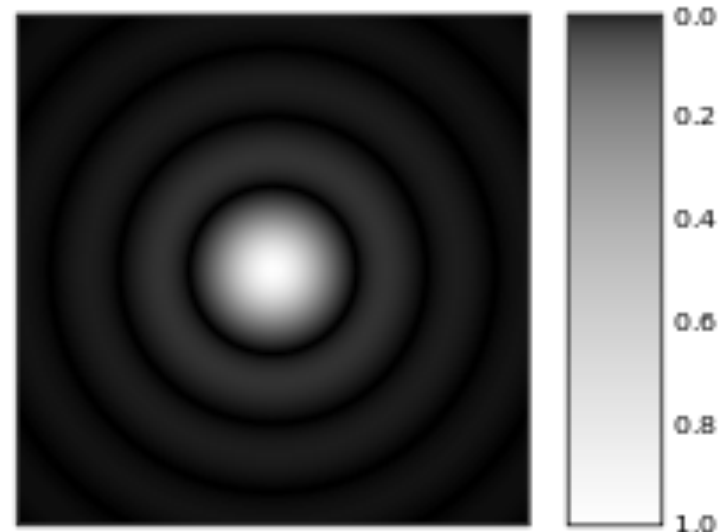
**The diffraction limit**

# A limit on focusing light

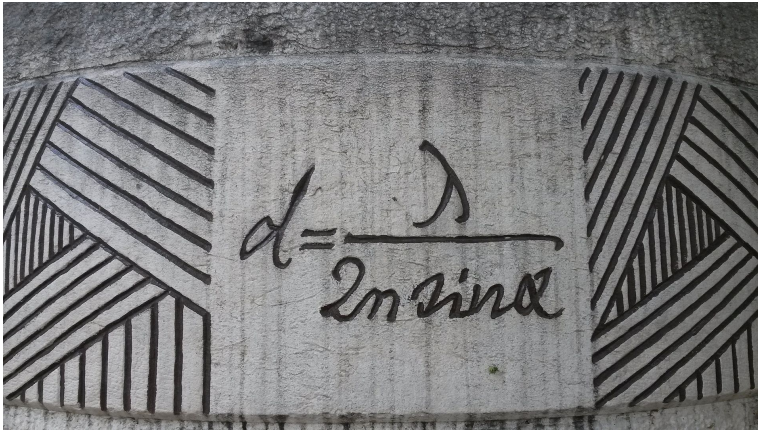
- The physics of light—in particular, the fact that it is a wave—impose a fundamental limit on how well a lens can focus it
- The light from a single point in space will not focus to a single point
- Instead, it will focus to a disk-like pattern called an “Airy pattern”
  - This means the observed image will be slightly blurred
  - In fact, we can think of the observed image as the true image convolved with the Airy pattern. This constitutes a low-pass filter.



Airy pattern



# The diffraction limit



[http://en.wikipedia.org/wiki/STED\\_microscopy#mediaviewer/File:Ernst-Abbe-Denkmal\\_Jena\\_F%C3%BCrstengraben\\_-\\_20140802\\_125708.jpg](http://en.wikipedia.org/wiki/STED_microscopy#mediaviewer/File:Ernst-Abbe-Denkmal_Jena_F%C3%BCrstengraben_-_20140802_125708.jpg)

- This limit on how well one can focus light is known as “the diffraction limit”
  - It’s literally “written in stone” in Jena, Germany (on a memorial to Ernst Abbe, who published it in 1873)
- The radius  $d$  of the Airy disk (the central spot of the Airy pattern) is proportional to the wavelength  $\lambda$  of the light
- It also depends on some other parameters that determine the “numerical aperture” ( $n \sin \theta$ )
  - You don’t need to worry about this
  - It’s usually between 0.1 and 1

The numerical aperture relates to the range of angles over which your lens system will capture incoming light

# The bottom line

Red light has longer wavelength/lower energy. Blue light has shorter wavelength/higher energy.

- Resolution limit of a light microscope:
  - The wavelength of visible light is 400–700 nm
  - A light microscope can't distinguish points that are closer than 200 nm
- Many cellular structures are smaller than this. A protein is just a few nm across.

Resolution limits

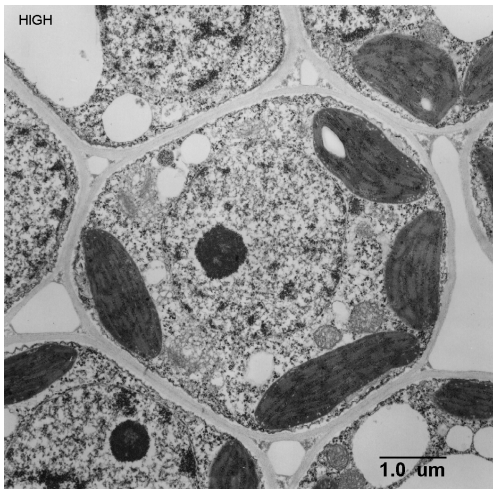
**Beating the diffraction limit**

# Option 1: Decrease the wavelength

- Higher-frequency radiation (e.g., x-rays) has shorter wavelengths and thus allows higher resolution
  - It also damages the sample more
- It's possible to image with electrons, which have a *much* shorter wavelength ( $\sim .1$  nm) You need vacuum and cold environment for electron microscopy. That's why you can't use it on living cells since they will die in that kind of environment
  - Electron microscopy can thus achieve much higher resolution
  - Disadvantages: can't use living cells, and molecules of interest won't glow

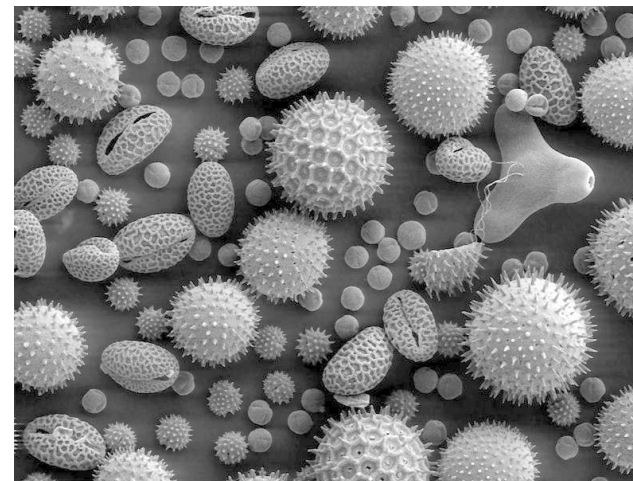
## Transmission electron microscopy

You shine electrons through the sample, and image electrons coming from the other side



[http://www.cas.miamioh.edu/~meicenrd/ANATOMY/Ch2\\_Ultrastructure/Tempcell.htm](http://www.cas.miamioh.edu/~meicenrd/ANATOMY/Ch2_Ultrastructure/Tempcell.htm)

## Scanning electron microscopy



The actual image recorded by scanning electron microscopy is actually 1D, even though the image makes it look like it is 3D

[http://www.newscientist.com/data/images/ns/cms/dn14136/dn14136-1\\_788.jpg](http://www.newscientist.com/data/images/ns/cms/dn14136/dn14136-1_788.jpg)

# Option 2: super-resolution fluorescence microscopy

- A number of recently developed techniques achieve resolution well beyond the diffraction limit
  - This requires violating some of the assumptions of that limit
- I'll briefly describe the most popular of these techniques, known alternately as STORM (stochastic optical reconstruction microscopy) or PALM (photoactivation localization microscopy)

STORM and PALM differ by the type of the fluorophores being used, but conceptually they do the same thing

The Nobel Prize in Chemistry 2014 was awarded jointly to Eric Betzig, Stefan W. Hell and William E. Moerner *"for the development of super-resolved fluorescence microscopy"*.

You're not responsible for this

# STORM/PALM

- If we have only a few fluorophores in an image, we can localize them very accurately
- Thus by getting only a few fluorophores to turn on at a time, identifying their locations in each image, and combining that information (computationally) across many images, we can build a composite image of very high resolution

