Bioimage Informatics

Lecture 4, Spring 2012

Practical Issues in Bioimage Informatics (II)

Fundamentals of Fluorescence Microscopy (I)

Outline

- Useful software tools for microscopy images
- Basic image analysis concepts
- References

- A more detailed introduction to fluorescence
- Basic metrics of a microscope

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Formats of Microscopy Images (I)

- Control software is required to operate microscopes.
  - Example 1: Metamorph
    http://www.moleculardevices.com/pages/software/metamorph.html
  - Example 2: Nikon Element
    http://www.nis-elements.com/
  - Example 3: Micromanager (open source)
    http://www.micro-manager.org/
- Commercial software often uses proprietary image formats to save metadata.
  - Pros: convenient, optimized
  - Cons: difficult to exchange data
- Free viewing software is sometimes available.
  http://www.nis-elements.com/resources-downloads.html

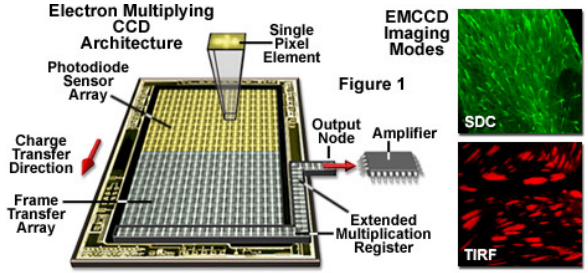
Formats of Microscopy Images (II)

- There are at least 50 proprietary image formats.

  Swedlow et al, Bioimage informatics for experimental biology, *Ann. Rev. Biophys.* 2009, 38: 327-346.

- TIFF is the most commonly used format for image analysis. http://partners.adobe.com/public/developer/tiff/index.html

- For bioimages, bit depth is normally > 8



- In general, image compression that changes pixel values should be avoided.

Free Software For Viewing High Bit-Depth Images

- Irfanview

  http://www.irfanview.com/

- ImageJ

  - Web: http://rsbweb.nih.gov/ij/
  - Initially started at NIH; Implemented using JAVA.
  - Provides basic bioimage viewing and analysis functions.
  - Many contributed plug-ins.

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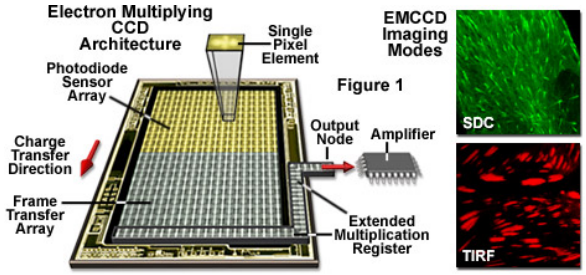
Demo: Basic Image Manipulation Functions

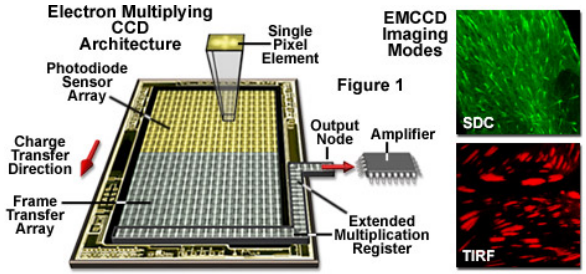
- Image read: *imread*
- Image write: *imwrite*
- Image file information: *imfinfo*
- Image pixel informaiton: *impixelinfo*



What is an Image?

- An image is a matrix.
  - Each pixel is an element of the matrix.
  - Computation on discrete data.

- An image is a surface.
  - Each pixel is point on the surface.
  - Computation on continuous data.



  - Example: calculate subpixel intensity using bilinear interpolation

- Read Chapters 1-3 of the MATLAB Image Processing Toolbox user guide.

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Image Processing vs Computer Vision

- Image processing normally refers to transformation from images to images.
  - Image enhancement
  - Image restoration
  - Image compression
  - Morphological image processing
  - …

- Computer vision aims to extract from images application-oriented information
  - Feature detection
  - Stereo vision
  - Robotic vision
  - Face recognition (HCI)
  - …

A Partial List of Computer Vision & Medical Image Analysis Journals

- IEEE Trans. Image Processing
- IEEE Trans. Pattern Analysis & Machine Intelligence (PAMI)
- International Journal of Computer Vision (IJCV)
- Computer Vision and Image Understanding
- Pattern Recognition

- IEEE Trans. Medical Imaging
- Medical Image Analysis

A Partial List of Related Conferences

- Image processing
  - IEEE Conference on Image Processing (ICIP)
- Computer vision
  - International Conference on Computer Vision (ICCV)
  - IEEE Conference on Computer Vision and Pattern Recognition (CVPR)
- Biomedical imaging
  - International Symposium on Biomedical Imaging (ISBI)

A Partial List of Microscopy Related Journals

- Journal of Microscopy

  http://www.wiley.com/bw/journal.asp?ref=0022-2720

- Biophysical Journal

  http://www.cell.com/biophysj/

- Nature Methods

  http://www.nature.com/nmeth/index.html

Literature Search Tools

- For image processing and computer vision references, use *IEEE xplore & ISI Web of Knowledge*.

- For microscopy and related biological application references, use *PubMed*.

http://www.ncbi.nlm.nih.gov/pubmed/

A Partial List of Open Source Software Packages & Public Image Libraries

- OpenCV

  http://opencv.willowgarage.com/wiki/

- ITK (Insight Toolkit)

  http://www.itk.org/

- JCB data viewer

  http://jcb-dataviewer.rupress.org/jcb/

- Comments: to understand an image analysis technique
  - we must understand exactly how it works at the pixel level;
  - we must understand exactly how it is implemented.

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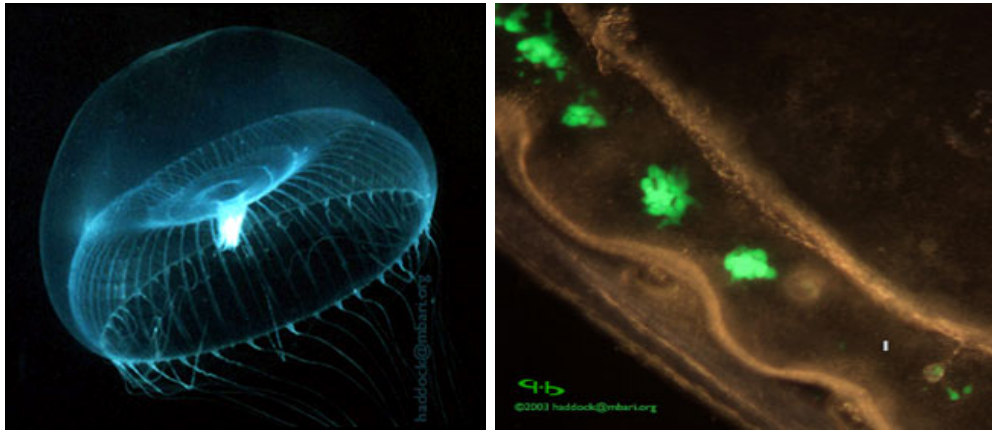
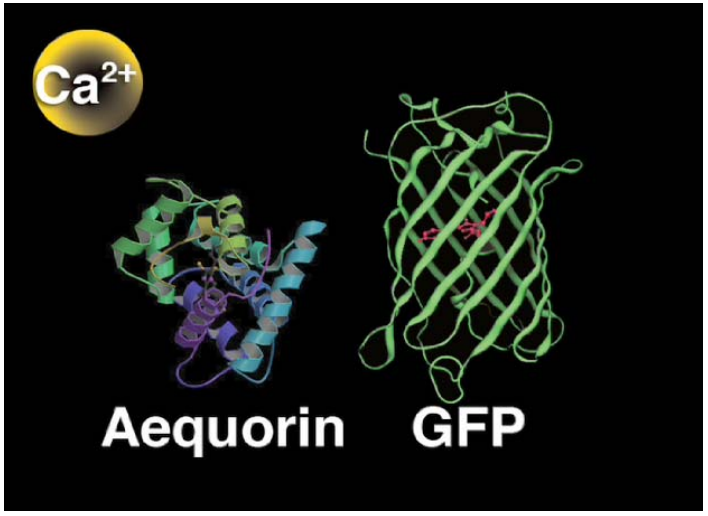
Green Fluorescence Protein



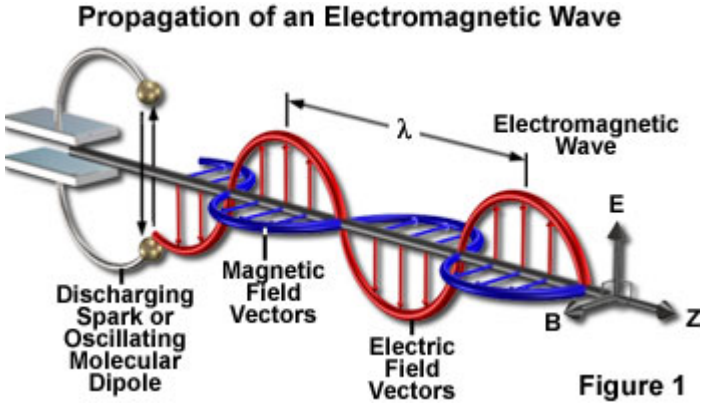
Jellyfish: *Aequorea victoria*



http://gfp.conncoll.edu/GFP-1.htm

What is Fluorescence

- Fluorescence is the property of some atoms and molecules to absorb light of certain wavelengths and to re-emit light at longer wavelengths.
- Molecules that can generate fluorescence are called fluorescent molecules or fluorophores.
- Light has characteristics of both particle and wave (particle-wave duality).
- A photon is a quantum of electromagnetic radiation and the unit particle of light.



Photon Energy

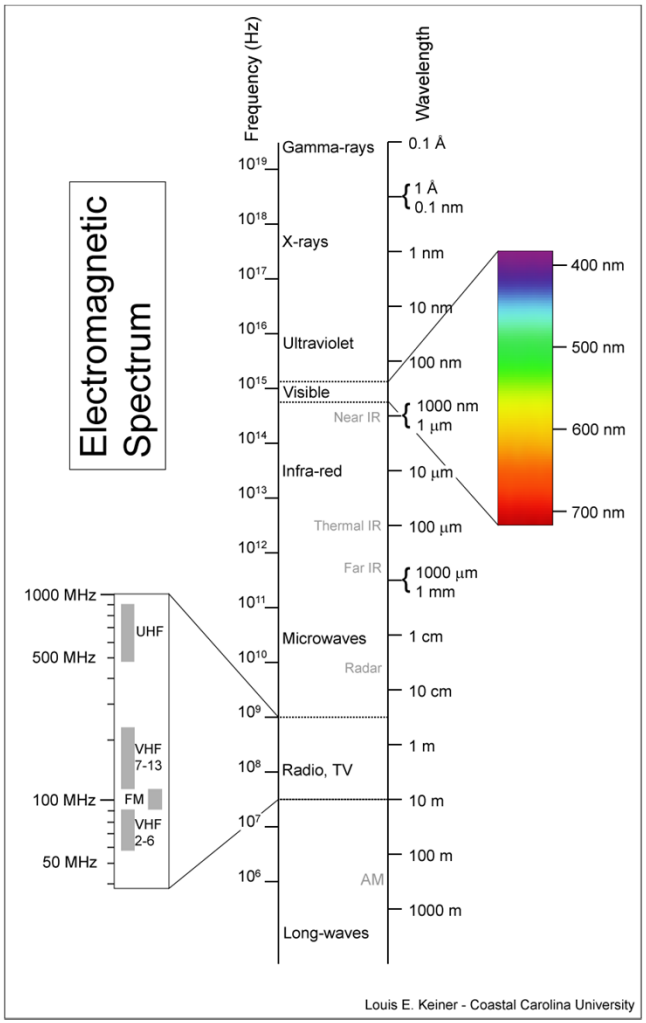
- Energy of a photon: Planck's law

$$E = h\nu = h\frac{c}{\lambda}$$

h: Planck's constant; 6.626×10^{-34}J·s
ν: frequency of light;
λ: wavelength of light
c: speed of light

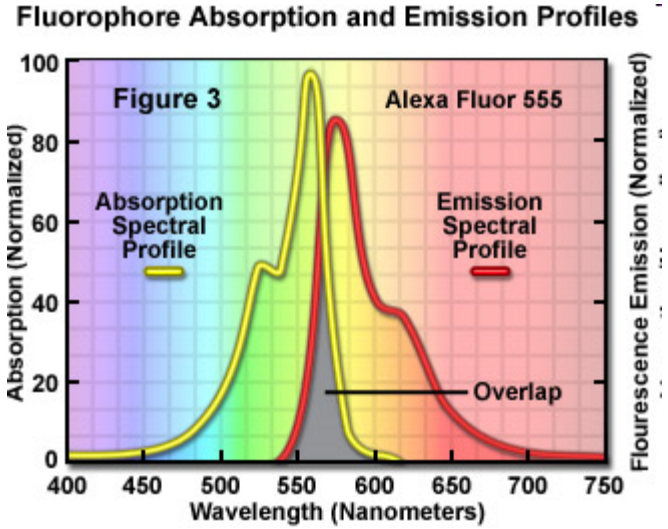
energy of a photon = 3.973×10^{-19}J at 500nm

- Shorter waves have higher energy.



Excitation & Emission Spectrum

- A fluorescent molecule can only absorb excitation light within a certain range of wavelengths (excitation spectrum).
- A fluorescent molecule can only emit light within a certain range of wavelengths (emission spectrum).
- Emission wavelengths are always longer due to internal energy loss.
- Emission spectrum is approximately a mirror image of excitation spectrum.



Jablonski Diagram

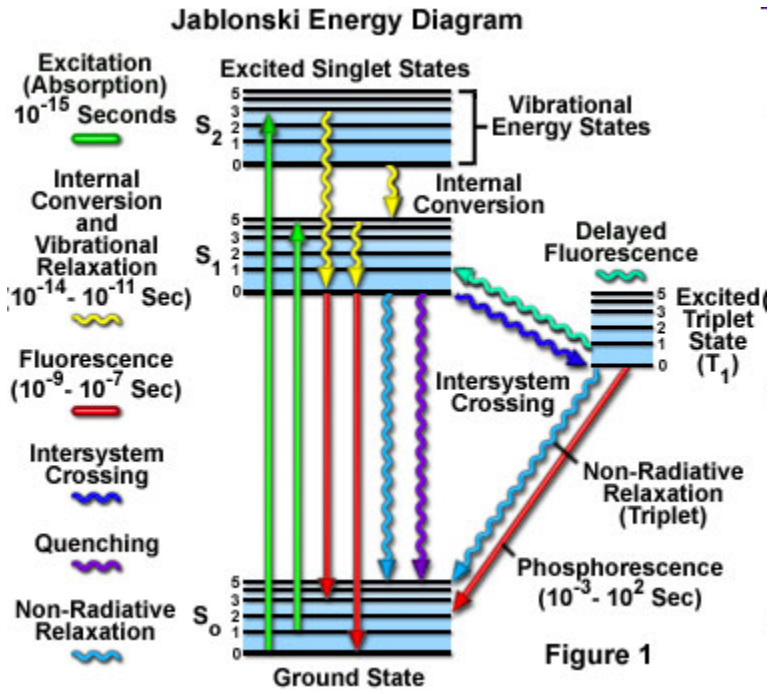
- Generation of fluorescence
  1. Absorption of light (10^{-15} sec)

  2. Relaxation to the lowest singlet state (10^{-11} sec)
     - a process called internal conversion
     - happens due to collision with solvent molecules

  3. Stay in the lowest singlet state for 10^{-9} sec

  4. Relaxation to the ground state produces a photon

- Each fluorescent molecule can repeat this process hundreds to thousands of times before photobleaching.



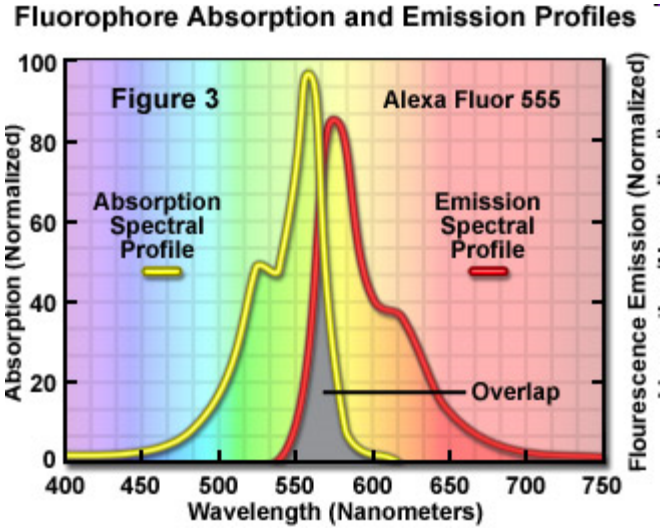
Jablonski Diagram Demo

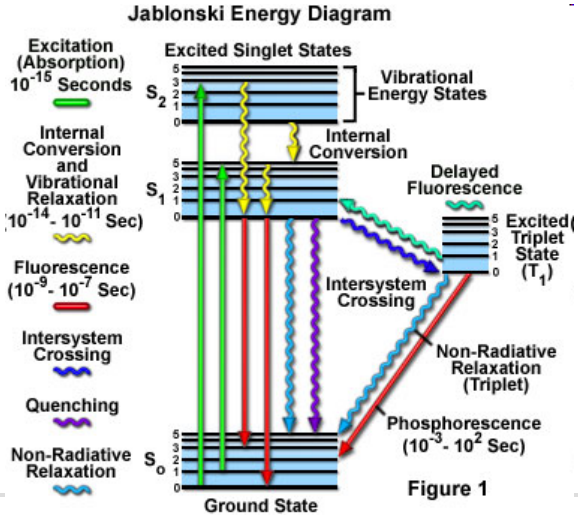
- Different events may occur.
- Without excited triplet state
  - Fluorescence emission
  - Non-radiative relaxation:
    Not due to collision. Will result in heat generation
  - Quenching (again, non-radiative)
    Non-radiative relaxation due to collision with other molecules in solution
- With excited triplet state
  - Phosphorescence emission
  - Delayed fluorescence
  - Triplet non-radiative relaxation

http://micro.magnet.fsu.edu/primer/java/jablonski/jabintro/index.html

Reading Absorption/Excitation Spectra

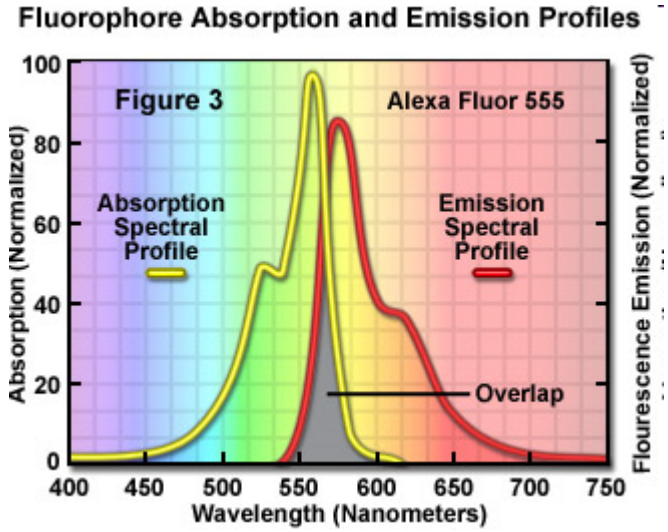
- Absorption spectrum shows usable excitation wavelength band and relative strengths of each wavelength.
- Emission spectrum shows emission wavelength band and relative strengths of each wavelength.
- Peaks reflect different energy states (electron orbitals).
- The mirror image rule reflects the same set of energy states experienced in excitation and emission.

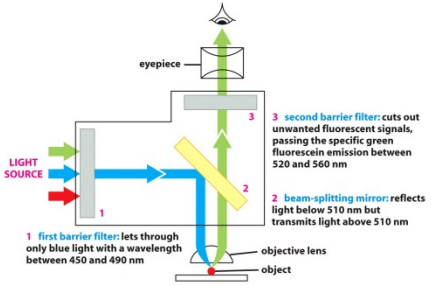


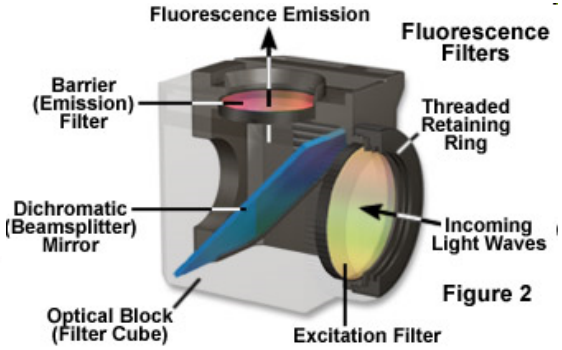


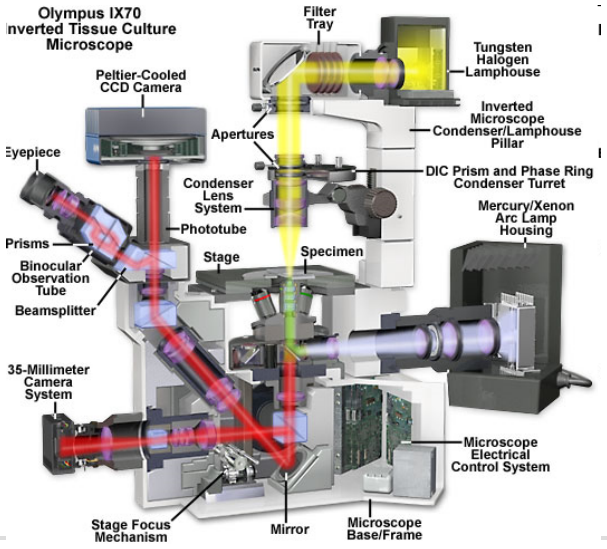
Stokes Shift

- Stokes shift: emission is shifted to longer wavelengths compared to excitation.
- Stokes shift reflects the loss of energy in fluorescence generation.
- Stokes shift makes it possible to detect low number of photons that are separated from large numbers of excitation photons.









Characterizing Fluorophores

- Fluorescence lifetime: the characteristic time that a molecule remains in an excited state before returning to the ground state.

  - Typically on the order of 10^{-9} sec

- Quantum yield

$$Q = \frac{\text{Photon emitted}}{\text{Photon absorbed}}$$

  - e.g. Cy3 4%; Cy5 27%; Fluorescin 95%

Quenching & Photobleaching (Demo)

- Quenching: a generic term that refers to non-radiative relaxation due to collision with other molecules in solution.

  - Quenching can be actively induced.

- Photobleaching: a fluorophore loses the ability to generate fluorescence due to photon-induced modification or damage.
  - Can be minimized
  - Can be actively induced.

http://micro.magnet.fsu.edu/primer/java/fluorescence/photobleaching/index.html

Useful References

- Lakowicz JR, *Principles of fluorescence spectroscopy*, Springer, 2006.

- Herman B, *Fluorescence microscopy*, 2nd ed., Taylor & Francis, 1998.

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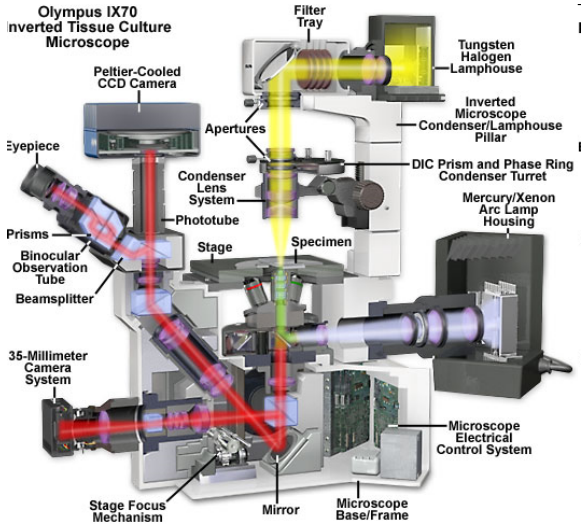
Performance Metrics of a Fluorescence Microscope

- Resolution: the smallest distance that can be resolved.
- Field of view: the area of a specimen that can be observed and recorded in an image.
- Depth-of-field: the axial distance (depth) in the specimen that appears in focus in an image.
- Light collection power: it determines image brightness.

Microscope as a Linear System



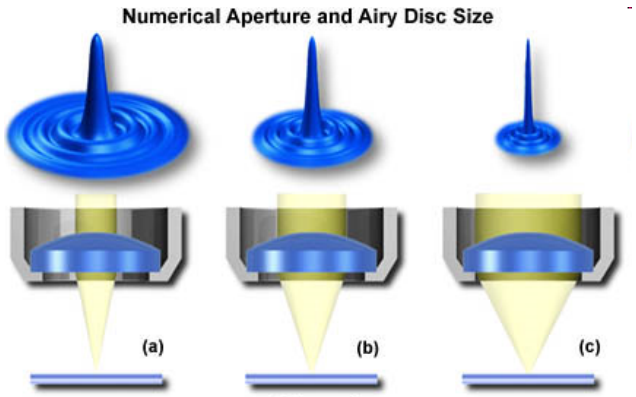


Figure 4

- A light microscope can be considered as a linear system.

http://micro.magnet.fsu.edu/primer/java/imageformation/airydiskformation/index.html

Airy Disk

- Airy (after George Biddell Airy) disk is the diffraction pattern of a point feature under a circular aperture.

- It has the following form

$$y = \left[\frac{2J_1(x)}{x} \right]^2$$

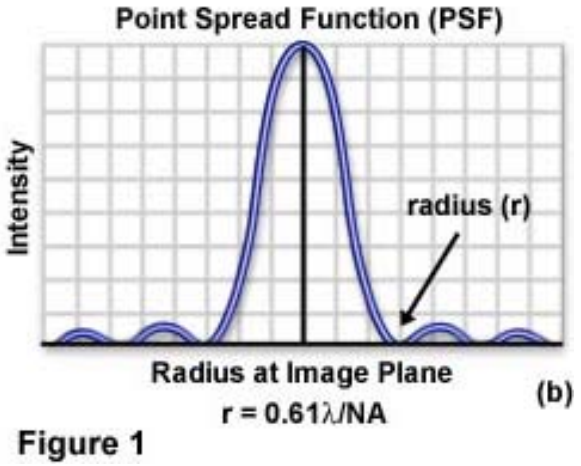


Figure 1

$J_1(x)$ is a Bessel function of the first kind.

- Detailed derivation is given in

  Born & Wolf, Principles of Optics, 7th ed., pp. 439-441.

Microscope Image Formation

- The impulse response of the microscope is called its point spread function (PSF).
- The transfer function of a microscope is called its optical transfer function (OTF).
- The PSF has the shape of an Airy Disk.

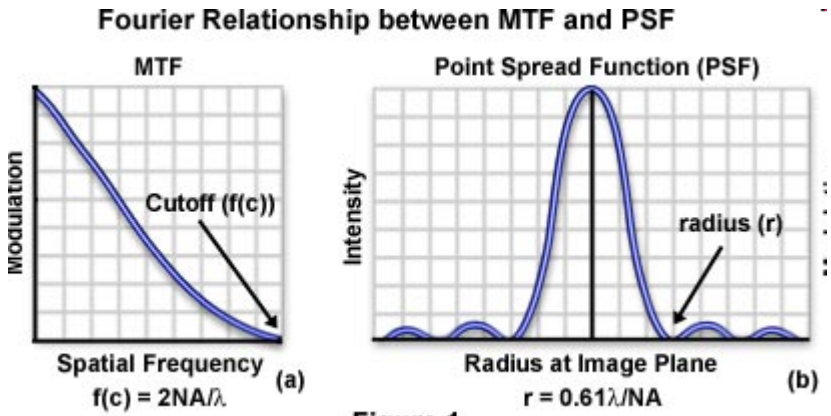
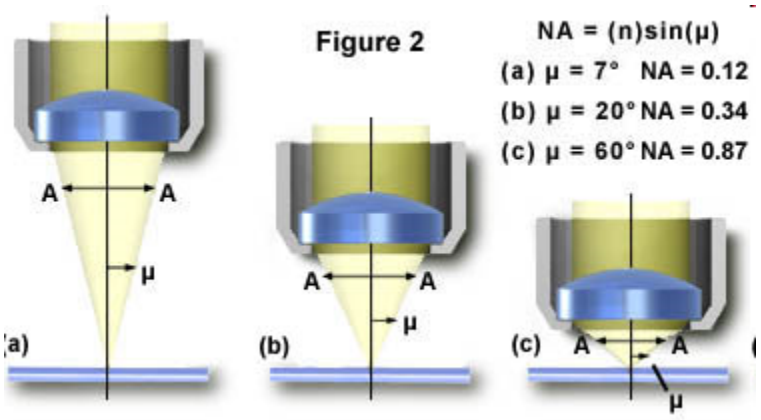


Figure 1

Numerical Aperture

- Numerical aperture (NA) determines microscope resolution and light collection power.



$$NA = n \cdot \sin \mu$$

n: refractive index of the medium between the lens and the specimen

μ: half of the angular aperture

Microscope Image Formation

- Microscope image formation can be modeled as a convolution with the PSF.

$$I(x,y) = O(x,y) \otimes psf(x,y)$$

$$F\{I(x,y)\} = F\{O(x,y)\} \cdot F\{psf(x,y)\}$$

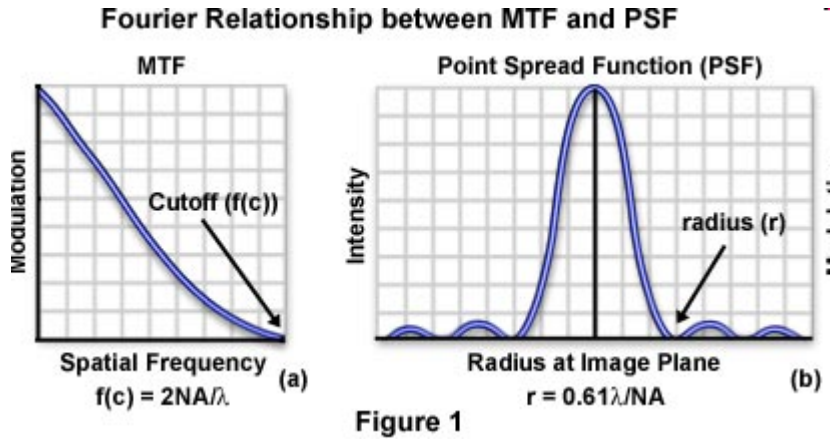
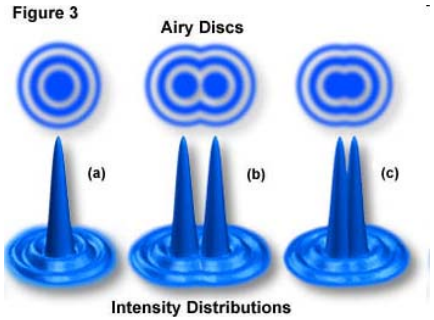


Figure 1

http://micro.magnet.fsu.edu/primer/java/mtf/airydisksize/index.html

Different Definition of Light Microscopy Resolution Limit (Demo)

- Rayleigh limit $D = \frac{0.61\lambda}{NA}$



- Sparrow limit $D = \frac{0.47\lambda}{NA}$

http://www.microscopy.fsu.edu/primer/java/imageformation/rayleighdisks/index.html

Field of View (Demo)

- Field of view: the region that is visible under a microscope

- If characterized in diameter

$$D \propto \frac{\text{Field diaphragm diameter}}{M}$$

- If characterized in area

$$S \propto \frac{\text{Field diaphragm diameter}^2}{M^2}$$

http://micro.magnet.fsu.edu/primer/java/microscopy/diaphragm/index.html

Depth-of-Field

- Depth-of-field: the axial distance (depth) in the specimen that appears in focus in the image.

$$d_{tot} = \frac{\lambda \cdot n}{NA^2} + \frac{n}{M \cdot NA} e$$

n: refractive index of the medium between the lens and the specimen

λ: emission wavelength

M: magnification

NA: numerical aperture

e: smallest resolvable distance in the image plane

Image Intensity: Light Collecting Power

- For transmitted and reflected light

$$I \propto \frac{\mathrm{NA}^2}{M^2}$$

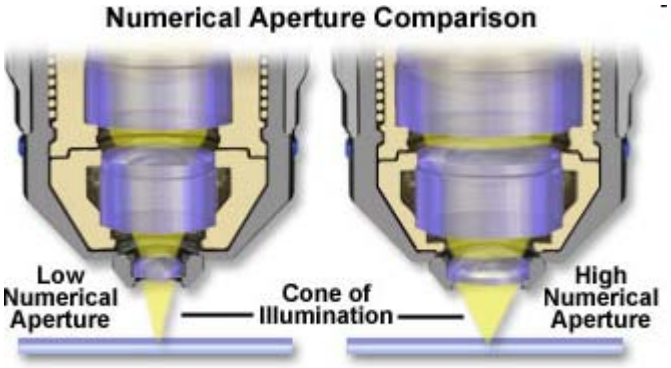
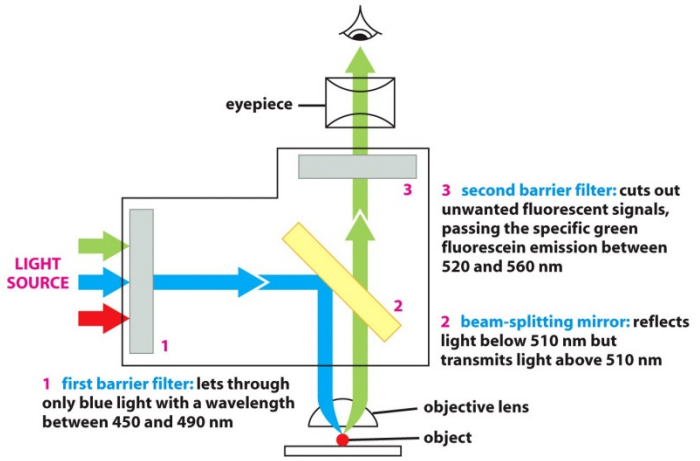


Figure 2

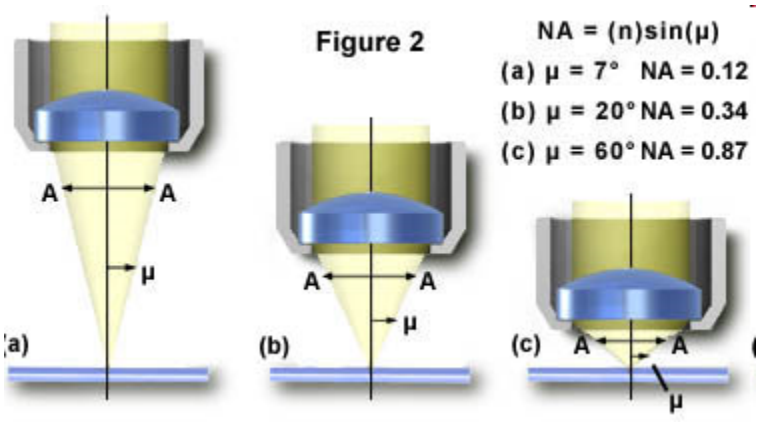
- For fluorescence

$$I \propto \frac{\mathrm{NA}^4}{M^2}$$



Working Distance

- The distance between the objective lens and the specimen.
- Working distance does not directly influence imaging but may determine how images can be collected.



Summary: High Resolution Microscopy

- Size of cellular features are typically on the scale of a micron or smaller.

- To resolve such features require

  - Shorter wavelength (electron microscopy)
  - High numerical aperture (resolution)
  - High magnification (spatial sampling)

$$D = \frac{0.61\lambda}{NA}$$

Summary: High Resolution Microscopy

- Higher magnification and higher numerical aperture mean

  - Smaller field of view $S \propto \frac{\text{Field diaphragm diameter}^2}{M^2}$

  - Smaller depth of field $d_{tot} = \frac{\lambda \cdot n}{NA^2} + \frac{n}{M \cdot NA} e$

  - Lower light collection power $I \propto \frac{\text{NA}^2}{M^2}$

  - Smaller working distance

Questions?