Biomedical Imaging & Analysis

Lecture 4, Part III. Fall 2014

Image Formation & Visualization (V):

Optical Imaging Systems.

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Thermal Noise

- Gaussian distribution:

  Given a Gaussian process, the probability of receiving *x* photons is given by

$$f(x, \mu, \sigma) = \frac{1}{\sigma\sqrt{2\pi}} e^{-\frac{(x-\mu)^2}{2\sigma^2}}$$

- Thermal energy in the silicon lattice causes Gaussian distributed noise.

Photon Shot Noise

- Law of physics
- Square root relationship between signal and noise
- Noise = square root of number of electrons
- Poisson distribution
- When photon noise exceeds system noise, data is photon (shot) noise limited

Poisson Image Noise (Shot Noise)

- Poisson distribution:

  Given a Poisson process, the probability of receiving n photons is given by

$$P_v(n) = \frac{\lambda^n e^{-\lambda}}{n!} \quad \approx N(\mu=\lambda, \sigma=\sqrt{\lambda})$$

$$\mu = \sigma^2 = \lambda \leftarrow \text{Average number of photons}$$

Poisson to Normal Distribution Approximation:
http://www.stat.ucla.edu/~dinov/courses_students.dir/Applets.dir/NormalApprox2PoissonApplet.html

- The process of photon counting in a CCD is statistically described by a Poisson distribution…

Signal-to-noise v/s **Contrast**-to-noise

- Signal-to-noise ratio (SNR)
- SNR provides a means to estimate the precision with which the signal S is measured
  - S: signal (or measurement variable)
  - σ: standard deviation of its measurement

$$SNR = \frac{S}{\sigma}$$

When is it is possible to have excellent SNR but poor CNR ..?

To discriminate two signals S_1 and S_2 we need more than just good signal to noise ratio. The ability to discriminate the two is assessed using the contrast to noise ratio…

Contrast-to-noise ratio (CNR)

S_1 and S_2 : two signals (or measurement variable) of two different tissues,

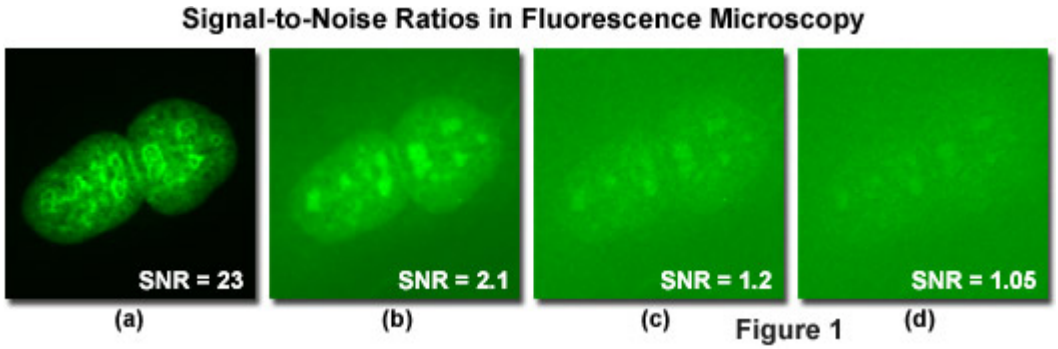
σ : standard deviation of their independent measurement (assumed here to be identical and statistically independent)

$$CNR = \frac{S_1 - S_2}{\sigma}$$

CNR provides a means to estimate the precision with which S_1 can be discriminated from S_2

Signal-to-Noise Ratio

- Signal-to-Noise ratio defines image quality.



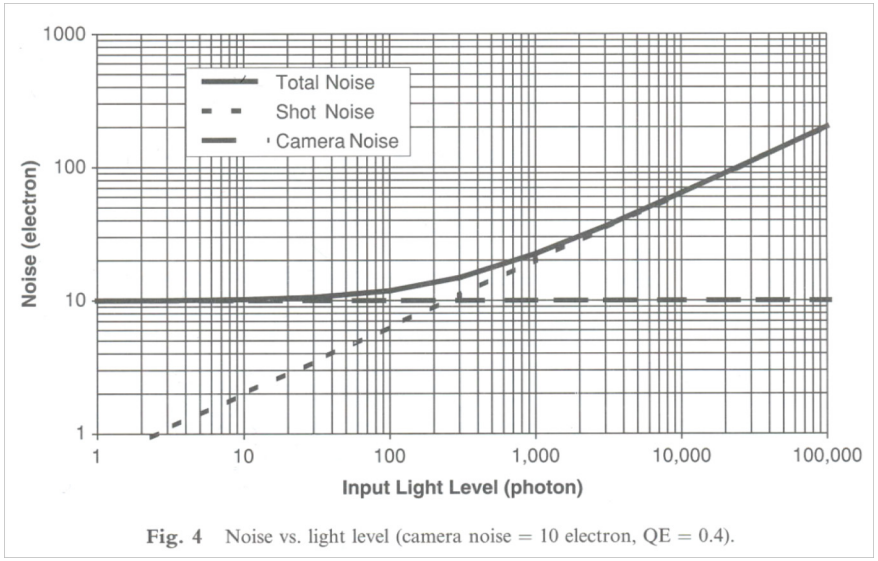


Fig. 4 Noise vs. light level (camera noise = 10 electron, QE = 0.4).

Optimizing SNR

- It is possible to optimize SNR by performing N repeated measurements S_i.
- Eg: 4 measurements improve the precision by two-fold.
- The precision of the average $\langle S\rangle=\sum S_i/N$ depends on the square root law

  - $S_i=S+\varepsilon_i$

  - where $\langle\varepsilon_i^2\rangle=\sigma^2$, $\langle\varepsilon_i\rangle=0$.

If S is the true signal (unknown):

  - $\langle S\rangle=\sum S_i/N=S+\sum \varepsilon_i/N$

$$\Delta S \equiv < S > - S = \frac{\sum \varepsilon_i}{N} \quad \Rightarrow \quad \Delta S^2 = \frac{\left(\sum \varepsilon_i\right)^2}{N^2}$$

$\langle\varepsilon_i\varepsilon_j\rangle=0$, $i\neq j$

$$\Delta S^2 = \frac{\left(\sum \varepsilon_i^{\,2}\right)}{N^2} + \frac{\overbrace{\left(\sum_{i\neq j} \varepsilon_i \varepsilon_j\right)}}{N^2}$$

$$\left\langle \Delta S^2 \right\rangle = \frac{\sum \left\langle \varepsilon_i^{\,2} \right\rangle}{N^2} = \frac{N\sigma^2}{N^2} = \frac{\sigma^2}{N}$$

$$\boxed{\left\langle \Delta S \right\rangle = \frac{\sigma}{\sqrt{N}}}$$

The Problem..?

- Results in increased measurement time.

Slide adapted from EPFL, Fund BioImag 2013

Signal to Noise

- Standard CCD SNR Equation:

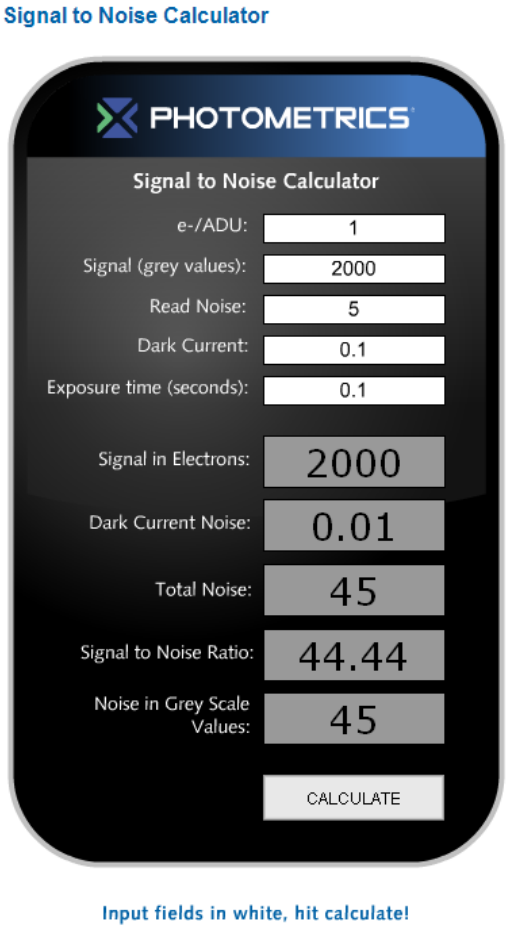
$$\bullet\ SNR = [S*QE] \div \sqrt{[S*QE^2 + D + \sigma_R^2]}$$

- S = Signal in Photons (converted to electrons by * QE)
- QE = Quantum Efficiency of light at that emission
- D = Dark Current Noise = Dark Current * Exposure Squared
- σ_R = Read Noise

- All values must be compared in electrons

Signal to Noise Calculators

- Many Signal to Noise calculators exist but a quick and easy one to use is at www.photomet.com - Select scientific imaging tools
- Simply enter your gain, signal, read noise, dark current and exposure time
- A good experiment is to see how varying dark from 2 to 0.001 effects a 100ms exposure



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.

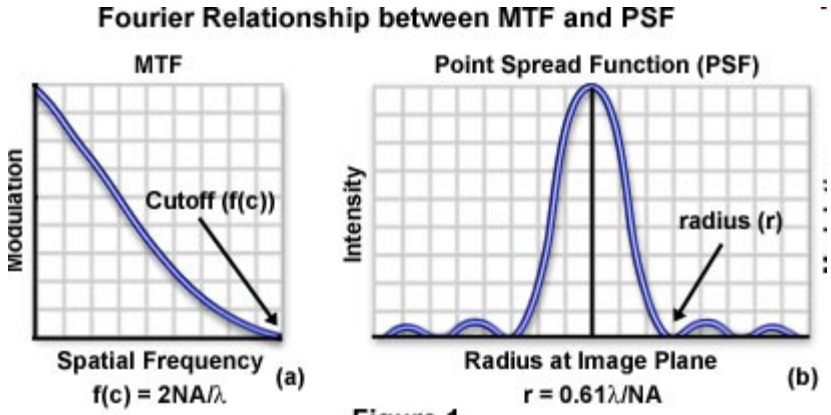
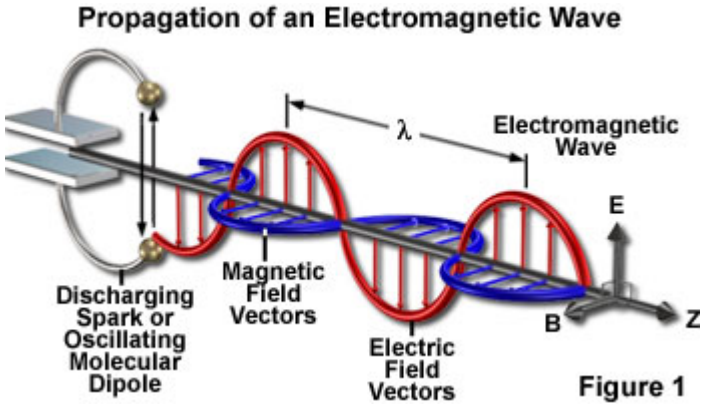
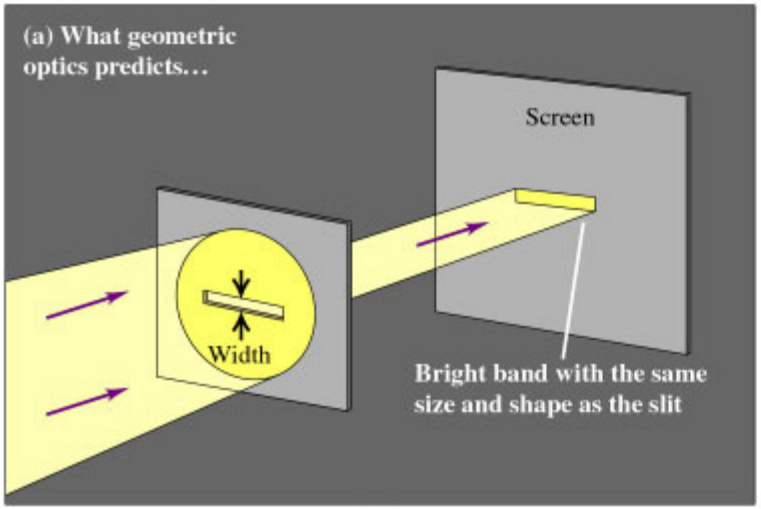


Figure 1

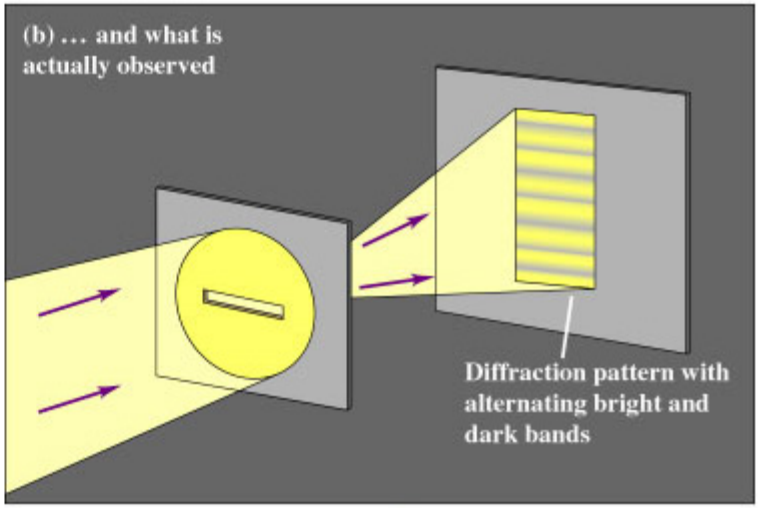
Optics: Some Basics (III)

- 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.





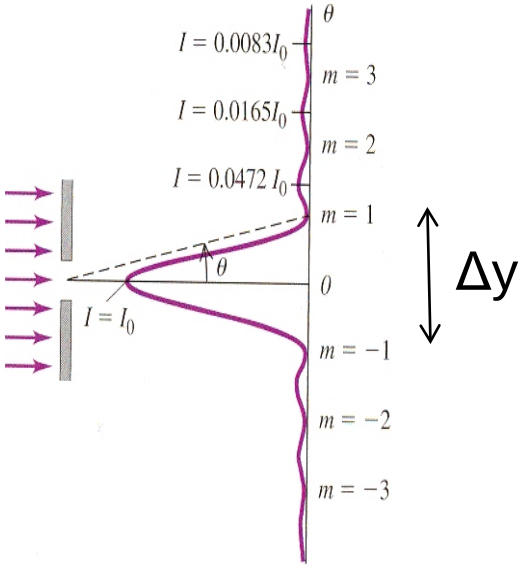
INCORRECT



CORRECT

Sinc Function: Width of central maximum

- We can define the width of the central maximum to be the distance between the m = +1 minimum and the m=-1 minimum, below.
- This pattern is called a Point Spread Function (PSF).
- It is modelled mathematically as the Sinc Function = (sinθ) / θ
- **Airy-Disc** in 2D.



Intensity distribution

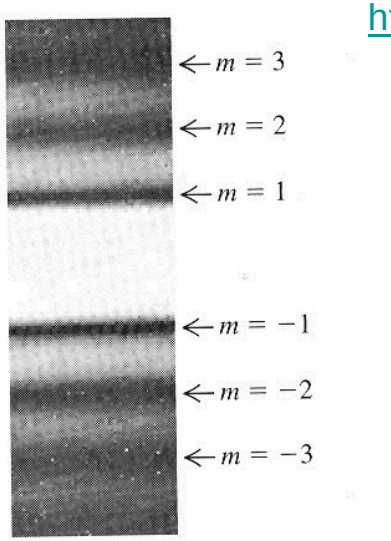


image of diffraction pattern

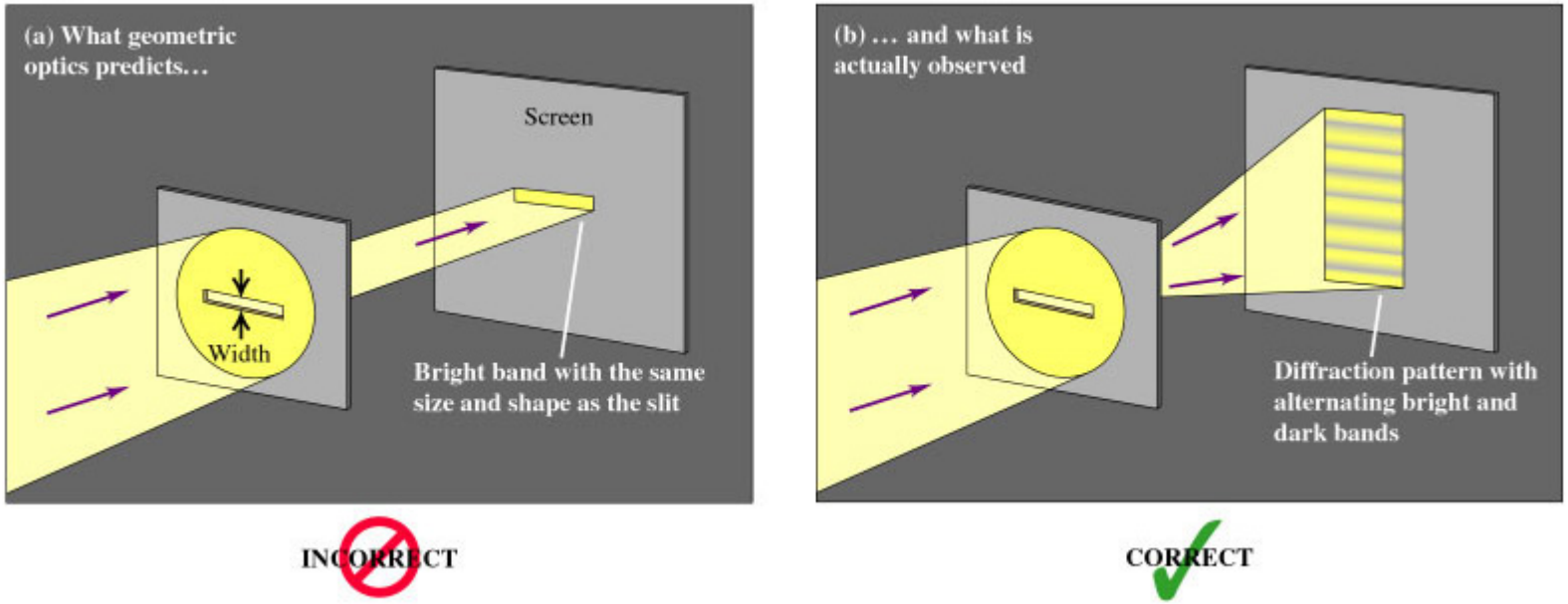
http://mathworld.wolfram.com/SincFunction.html

$$\sin\theta = \frac{\lambda}{a} \Rightarrow \theta = \frac{\lambda}{a}$$

$$\Delta y = \frac{R\lambda}{a} - - \frac{R\lambda}{a} = \frac{2R\lambda}{a}$$

Narrower the slit, the more the diffraction pattern “spreads out”

Conclusions on Single Slit Diffraction – when does light behave like a wave..?



The “geometrical” picture breaks down when slit width becomes comparable with wavelength .
Clearly, diffraction is relevant to microscopes with small apertures!

The 3-D wave equation is $\nabla^2 f(x,y,z,t) = \frac{1}{v^2}\frac{\partial^2 f(x,y,z,t)}{\partial t^2}$

One particular "traveling wave" solution to this is often written

$$\tilde{f}_1(x,y,z,t) = \tilde{A}\, e^{i(\vec{k}\cdot\vec{r}-\omega t)}, \text{ where } \tilde{A} = A\, e^{i\delta}.$$

This wave travels in the **k** direction (do you see why?)

This wave has wavelength lambda= 2π/|**k**| (do you see why?)

This wave has period 2π/ω (do you see why?)

This wave has speed v = ω/|**k**| (do you see why?)

What is the real form of this wave?

A) $A\cos(kx-\omega t)$ B) $A\cos(kx-\omega t+\delta)$

C) $A\cos(\vec{\mathbf{k}}\cdot\vec{\mathbf{r}}-\omega t)$ D) $A\cos(\vec{\mathbf{k}}\cdot\vec{\mathbf{r}}-\omega t+\delta)$

E) More than one of these/other/???

What does this mean for the particle nature of light..?

Attempting to localizing photons in the y-direction to a slit of width a leads to a spread of y-momenta of at least h/a.

$$\overline{p} = \overline{p}_x + \overline{p}_y$$

$$p_y = \theta p_x = p_x \frac{\lambda}{a} = p_x \frac{h}{p_x a} = \frac{h}{a}$$

- So, the more we seek to localize a photon (ie define its position) by shrinking the slit width, *a*, the more spread (uncertainty) we induce in its momentum:
  - In this case, we have $\Delta p_y \Delta y \sim h$

Microscope Image Formation

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

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

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

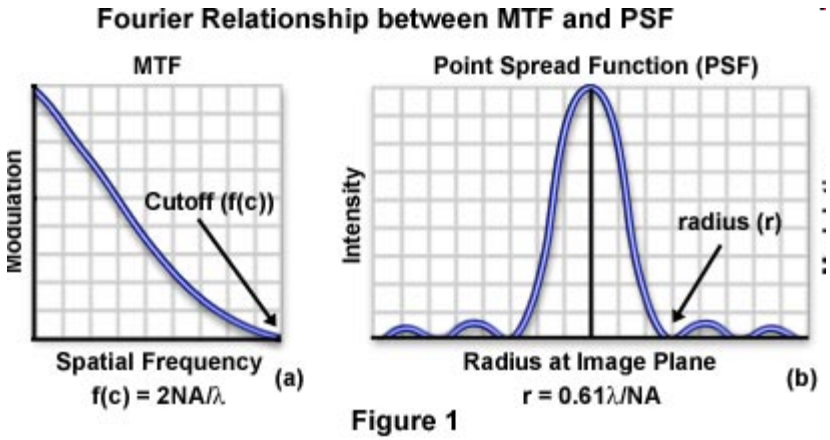
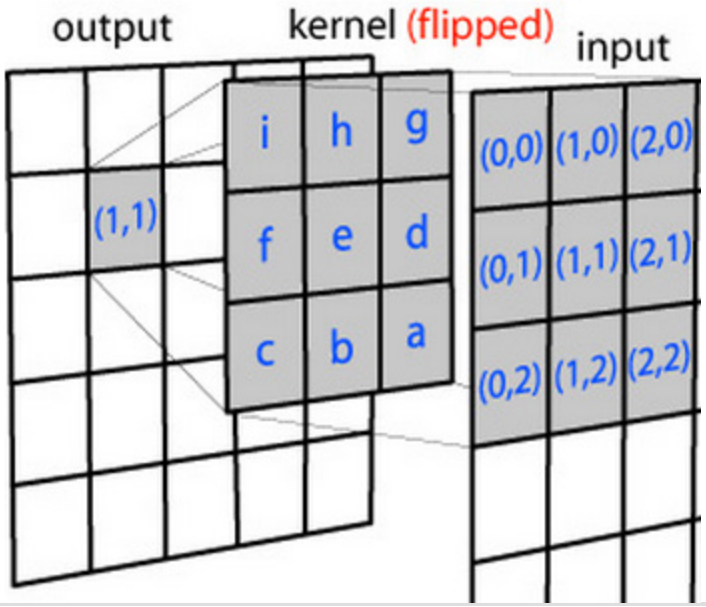


Figure 1

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

The Convolution Operator – what happens to an input 1D image function, f(x,y) as it passes through an aperture ..?

- Simple example of convolution of input image (matrix, S) and impulse response (kernel) in 2D spatial. *Y[n] = (S * h) [n].*
- Notice that the kernel matrix is flipped both horizontal and vertical direction before multiplying the overlapped input data.



n \ m	-1	0	1
-1	a	b	c
0	d	e	f
1	g	h	i

$$y[1,1] = \sum_{j=-\infty}^{\infty}\sum_{i=-\infty}^{\infty} x[i,j]\cdot h[1-i,1-j]$$

$$\begin{aligned} = \; & x[0,0]\cdot h[1,1] + x[1,0]\cdot h[0,1] + x[2,0]\cdot h[-1,1] \\ & + x[0,1]\cdot h[1,0] + x[1,1]\cdot h[0,0] + x[2,1]\cdot h[-1,0] \\ & + x[0,2]\cdot h[1,-1] + x[1,2]\cdot h[0,-1] + x[2,2]\cdot h[-1,-1] \end{aligned}$$

Circular Aperture - Airy Disk Diffraction Pattern

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

- It has the following form

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

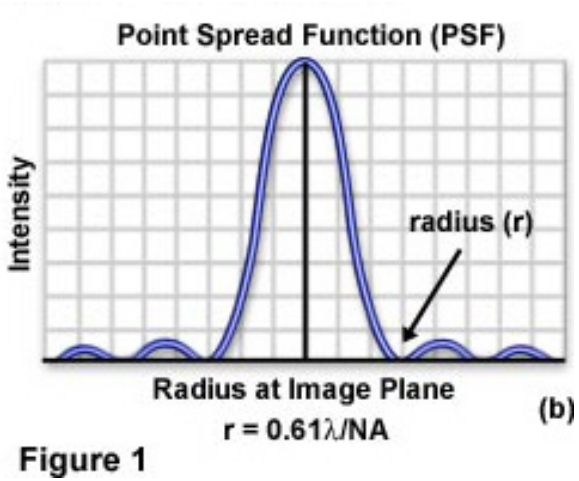


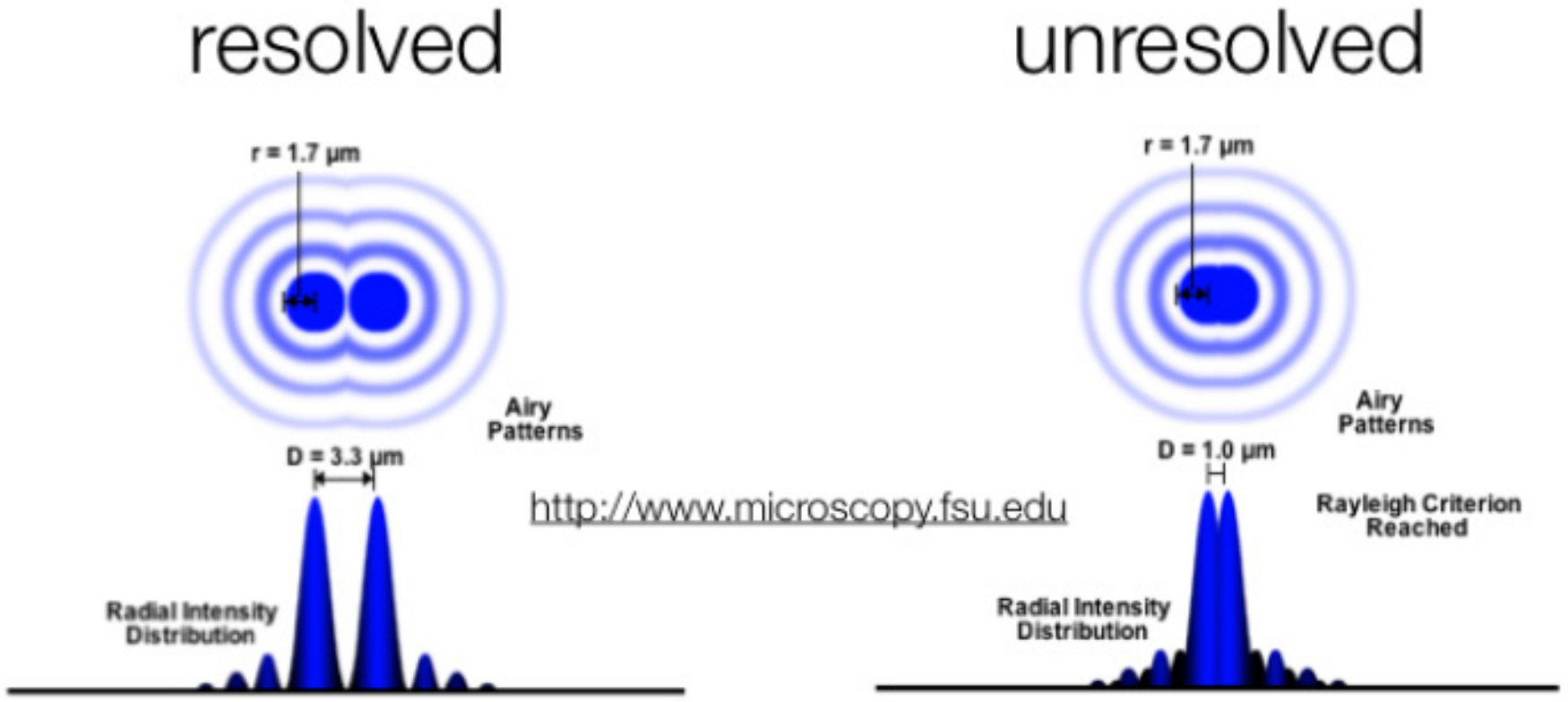
Figure 1

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

- Detailed derivation is given in

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

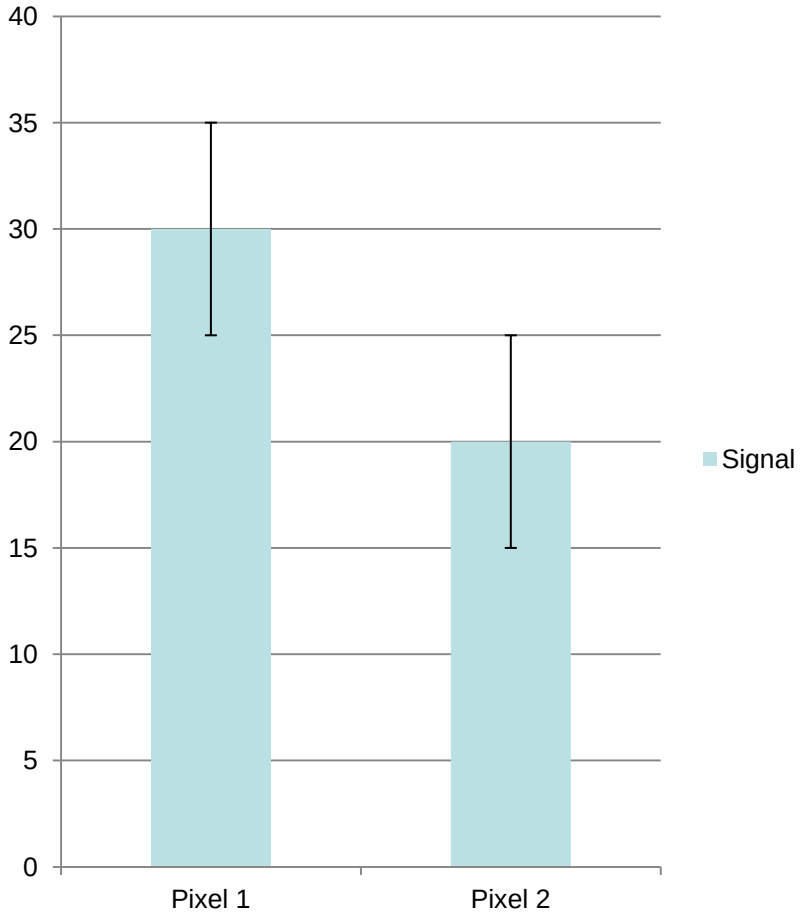
Different Definition of Light Microscopy Resolution Limit (Demo)



Measurement Confidence

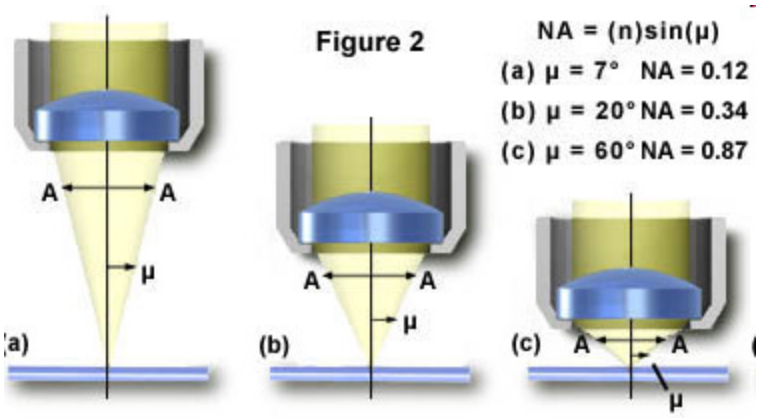
(based on calibration of ‘noise’)

- If we have 2 pixels next to each other - one has a value of 30 and the other 20, we will assume noise is 5 electrons
- Q, Can we tell them apart?
- A, No – the error bars overlap and so we have no confidence in the measurement
- The signal change that can be detected with confidence is calculated using a confidence level calculation



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

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

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

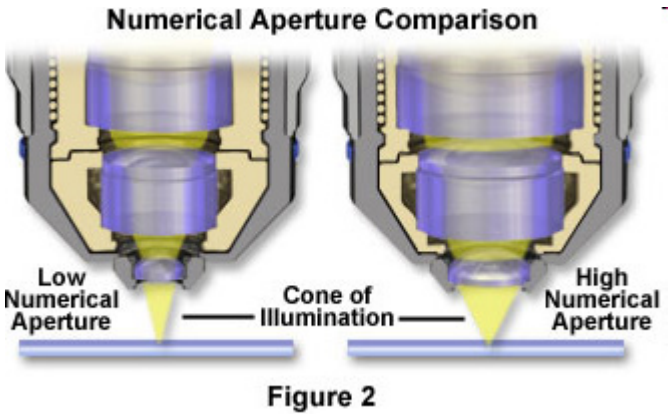
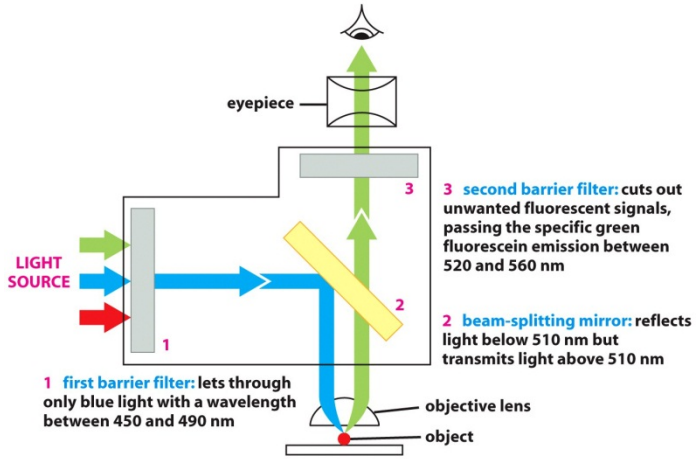


Figure 2

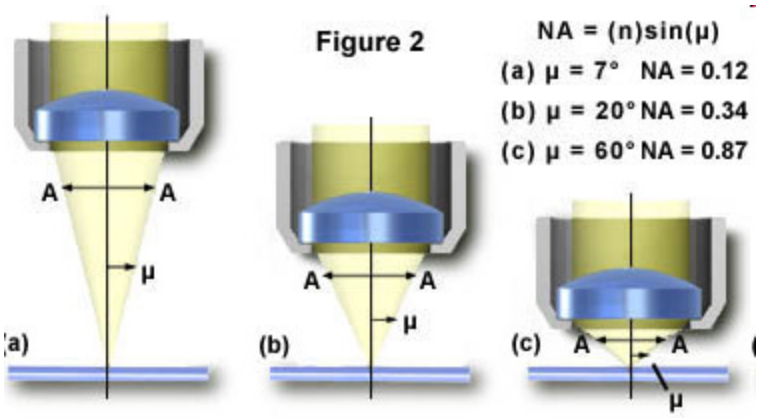
- For fluorescence

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



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)

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

- Resolution (D) improves (gets smaller) if λ ↓ or n↑ or α↑ (parameters affecting 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

Objective Specifications

Figure 1

Why Oil immersion medium..?

☺

Checking your Math!

Assuming that sin α = 0.95 (α = 71.8°)

Maximum resolution by Rayleigh Limit:

$$d_{min} = 0.61\ \lambda\ /\ N.A.$$

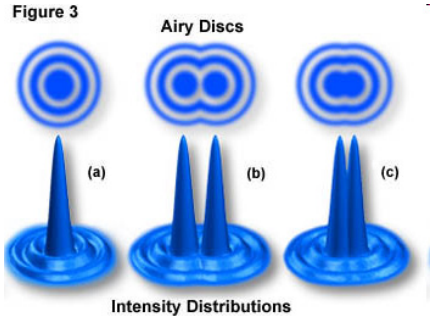
Wavelength		Air (n= 1)	Oil (n = 1.515)
Red	650 nm	0.42 µm	0.28 µm
Yellow	600 nm	0.39 µm	0.25 µm
Green	550 nm	0.35 µm	0.23 µm
Blue	475 nm	0.31 µm	0.20 µm
Violet	400 nm	0.27 µm	0.17 µm

Resolution $_{air}$ **Resolution $_{oil}$**

d_{min} = (0.61)(0.52)/1.4 = 0.23 µm

Beyond the Rayleigh Limit…

Is the Rayleigh limit (200 nm) unbeatable by an optical system..??



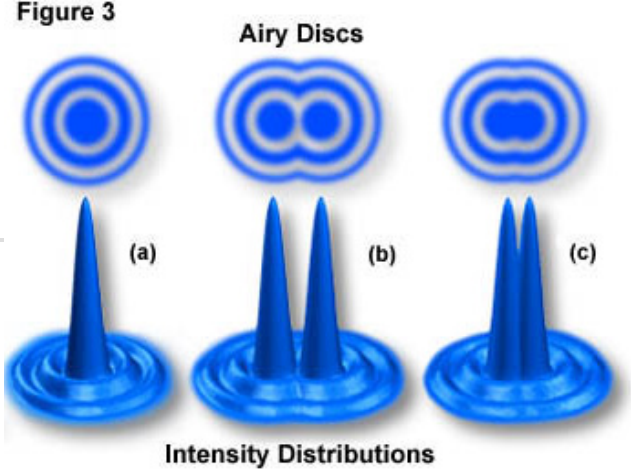
Some Perspective - a recap:

The resolution of far field instruments is fundamentally limited by the wave nature of light. A practical definition of the resolving power is the well known Rayleigh criterion.

It states that two points are just resolved if their lateral separation equals the radius of the first dark ring of the Airy pattern.

For green light (wavelength 540 nm) and objectives with a numerical aperture (NA) of 1.4, this criterion imposes a limit of 240 nm

Beyond the Rayleigh Limit…



We've been beating the Rayleigh limit for a while now…

- **Fluorescence microscopy**, a resolution beyond the common Rayleigh limit can be achieved by means of a nonuniform excitation pattern that contains high spatial frequency components.
- In scanning **confocal fluorescence microscopy (CFM),** this pattern is a small light spot that is scanned over the specimen, theoretically yielding a 1.4-fold improved resolution compared with standard fluorescence microscopy.

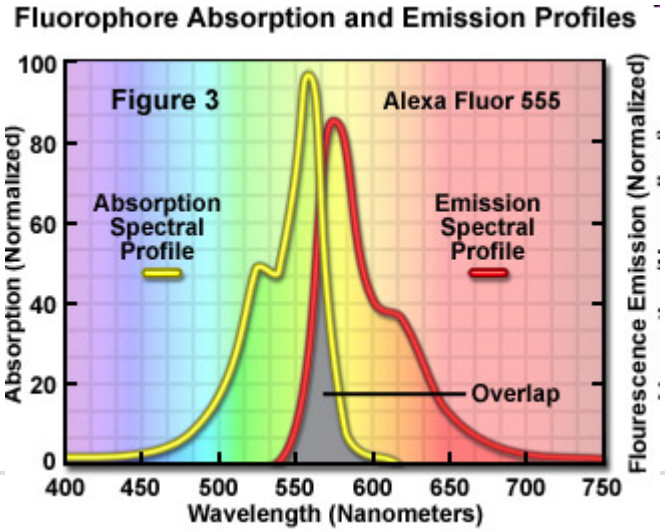
True optical resolution beyond the Rayleigh limit achieved by standing wave illumination
7232–7236 | PNAS | June 20, 2000 | vol. 97 | no. 13
*Jan T. Frohn, Helmut F. Knapp, and Andreas Stemmer**

This paper defines harmonic excitation light microscopy (HELM) to **outperform the lateral resolving power of CFM by a factor of 1.5 and additionally avoid disadvantages of scanning methods.**

Distinguishing Cell Components:
Leveraging Absorption/Excitation Spectra

- Measurement/interpretation of the excitation (absorption) spectrum
  - Shining light at different wavelengths
  - For each wavelength, measure the output light at the peak wavelength of the emission wavelength – known based on *Fluorescence properties* of *fluorophores*.

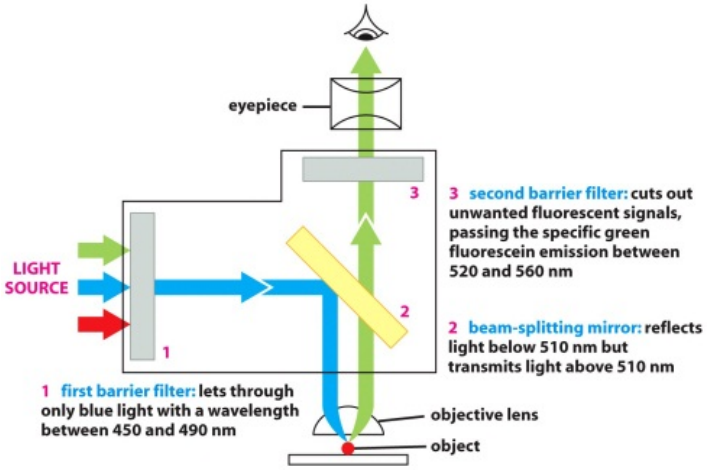
- Measurement/interpretation of the emission spectrum
  - Shining light at the peak excitation wavelength
  - Measure the emission over the range of emission wavelengths

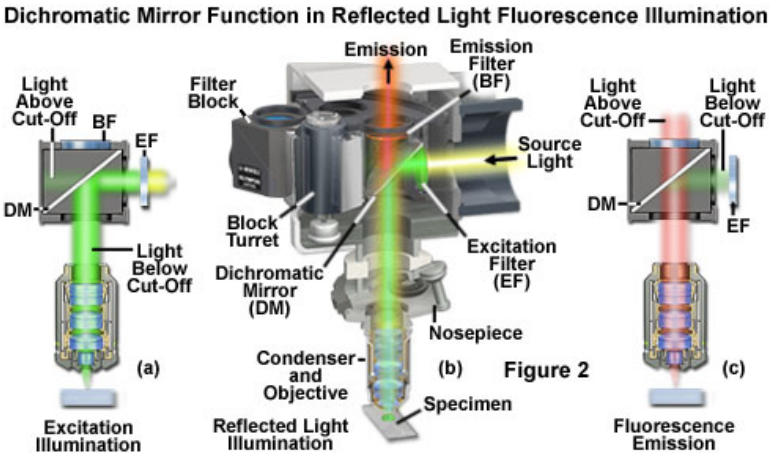


Stokes shift: emission is shifted to longer wavelengths compared to excitation.

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.





Confocal Fluorescence Microscopy

- Measurement/interpretation of the excitation (absorption) spectrum
  - Shining light at different wavelengths

- Point by Point Imaging
  - Suppresses out of focus points and gives better clarity

19.2.1 Confocal Microscopy

Confocal microscopy enables microscopic imaging of untreated tissue without previous fixation and preparation of slices and thus meets some of the operational ease requirements as well as the resolution requirement. The technical principle is based on point-by-point imaging. In a laser scanning confocal microscope, a laser beam is focused by the objective lens into a small focal volume within the imaged sample. A mixture of emitted fluorescence light as well as reflected laser light from the illuminated spot is then recollected by the objective lens. The detector aperture obstructs the light that is not coming from the focal point. This suppresses the effect of out-of-focus points. Depending on the imaging mode, the detector either measures the fluorescence light or the reflected light. The measured signal represents only 1 pixel in the resulting image. In order to get a complete image and perform dynamic imaging, the imaged sample has to be scanned in the horizontal plane for 2-D imaging as well as the vertical plane for 3-D imaging.

Confocal microscopy can be adapted for in vivo and in situ imaging by schematically inserting a fiber optics link between the laser source and the objective lens.

From text: Microscopic Image Analysis for Life Science Applications

Image **Formation** & **Visualization**

- ~~System's model of an Imaging Device.~~
- ~~Noise formation and propagation.~~
- ~~Signal to Noise v/s Contrast to Noise~~
  - ~~Image bit depth~~
  - ~~Windowing & Leveling~~
- ~~Software for Image Visualization~~
  - ~~ImageJ, ITK-SNAP, Paraview~~
- ~~Physics of Imaging~~
  - ~~X-ray & CT~~
    - ~~Tomographic Reconstruction Techniques~~
  - ~~Fluoroscopy~~
  - ~~MRI~~
  - ~~Optical Imaging~~
    - ~~Resolution limits~~
    - ~~Point Spread Functions~~

Whats next..?

- ~~Image **Formation**~~
- **Basic** Image Processing
  - Image Rendering – Marching Cubes, Surface vs Volume Rendering Ray Tracing etc.
  - Linear filtering
  - Singular Value Decomposition & Image Compression (Gilbert Strang)
  - Applications in Machine Vision & Feature Extraction
  - Feature Classification
- **Advanced** Image Processing Topics
  - Rotation, Translation (posing) and Scaling + What is Invariance.
  - Shape Analysis, Registration, Mutual Information
  - Optical Flow & Physics based image processing / Regularization
  - Shape and Appearance Models

BIA 2014

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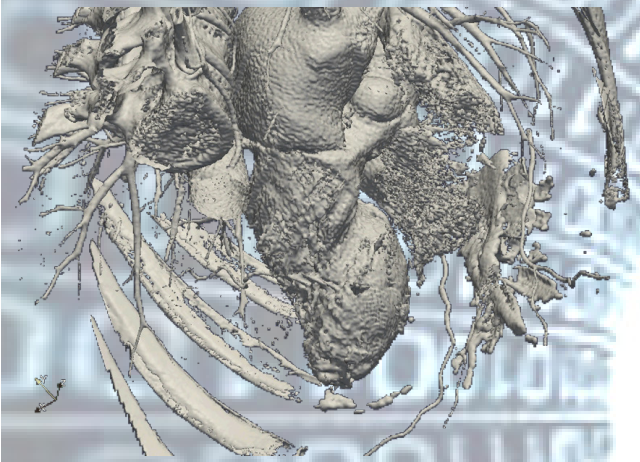

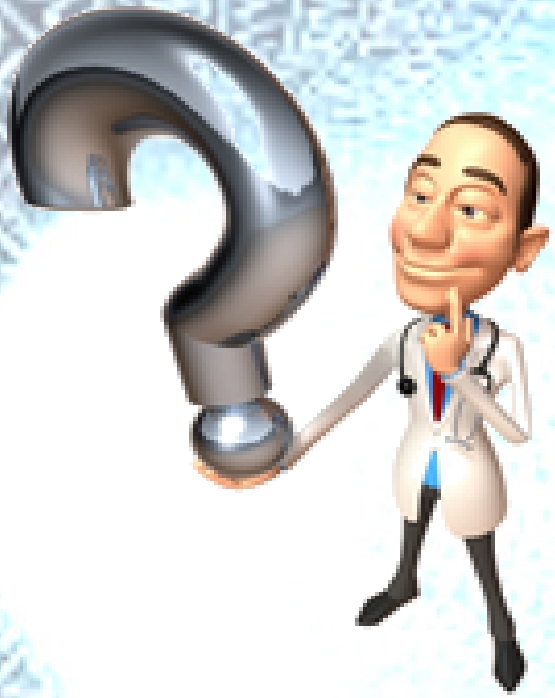

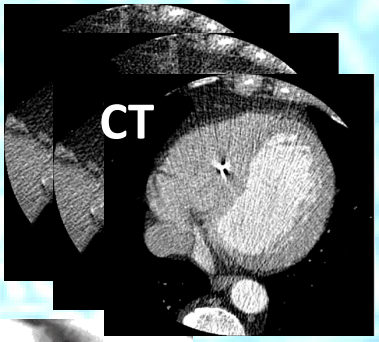

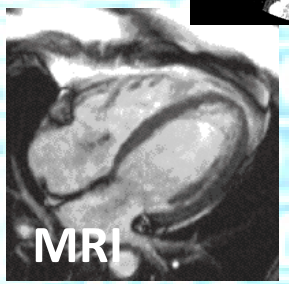

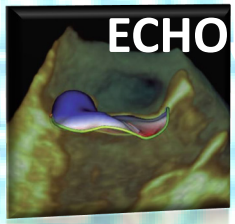

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