Prof. Enrico Gratton - Lecture 6
Fluorescence Microscopy

Instrumentation

Light Sources:

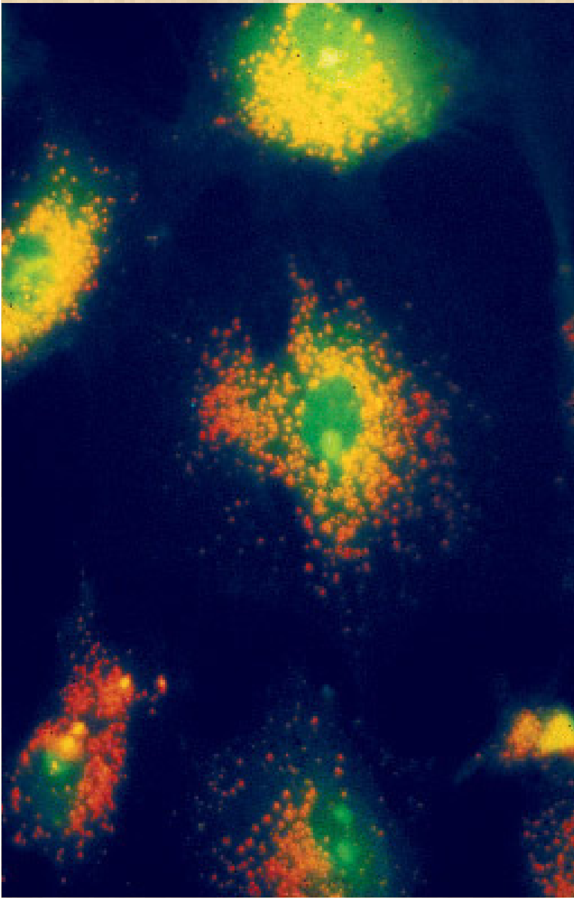
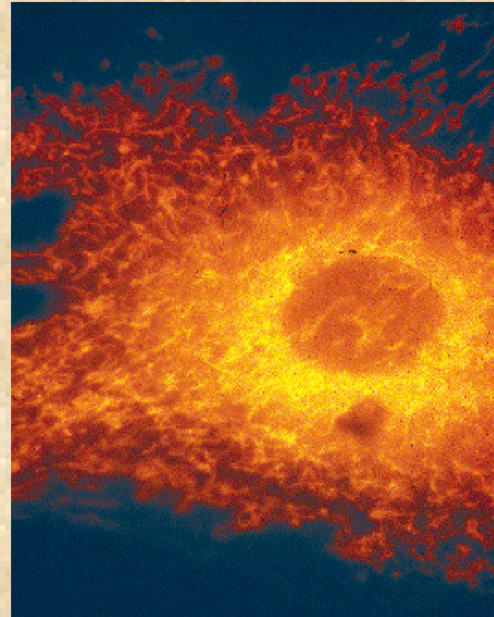
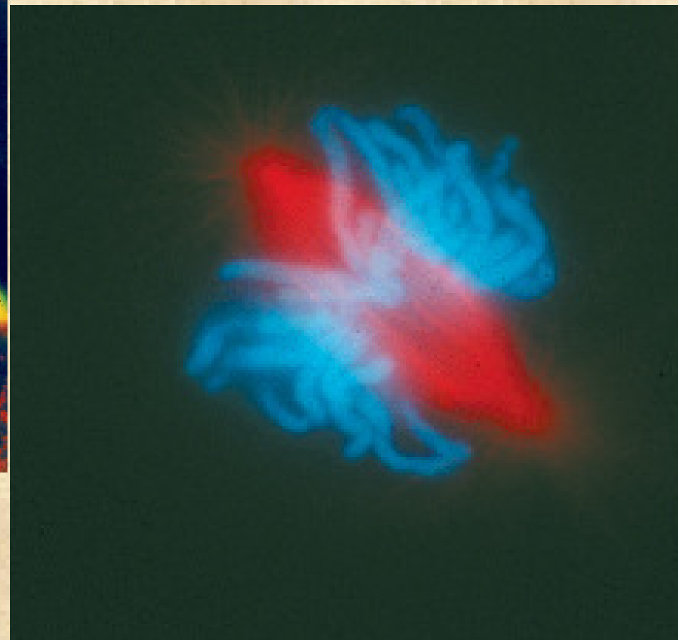
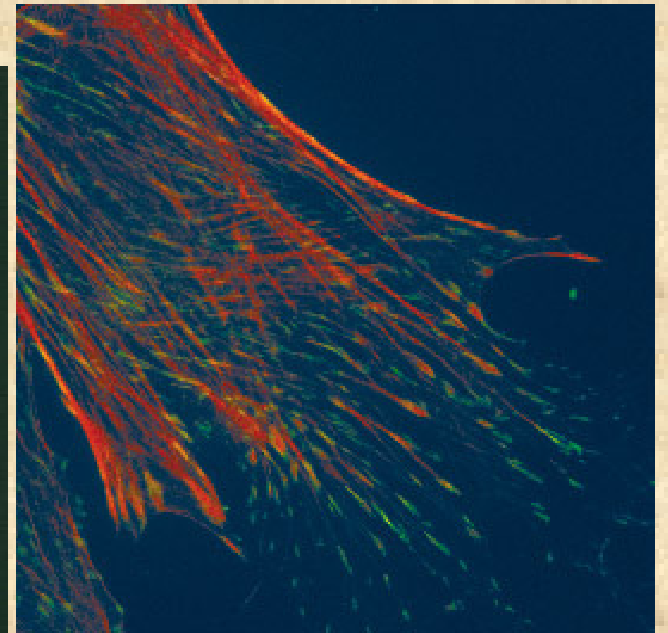
One-photon and Multi-photon Excitation

Applications in Cells

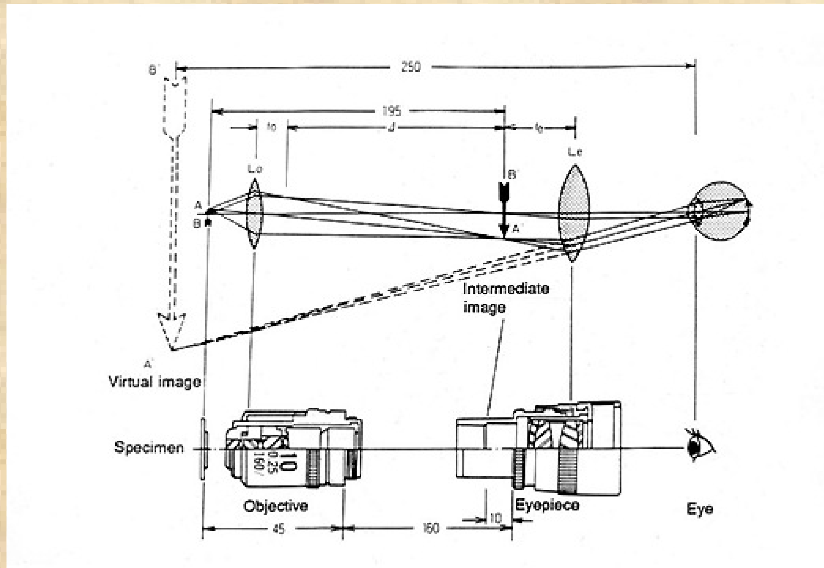
Lifetime Imaging

Figures acknowledgements: E.D. Salmon and K. Jacobson

Confocal microscopy images

In the compound microscope the Finite Corrected Objective Forms a Real Image At the Ocular Front Focal Plane: The Primary or Intermediate Image Plane (IIP)



Conventional Optics
Objective with finite Focal Length
(Optical Tube Length, OTL, Typically 160 mm)

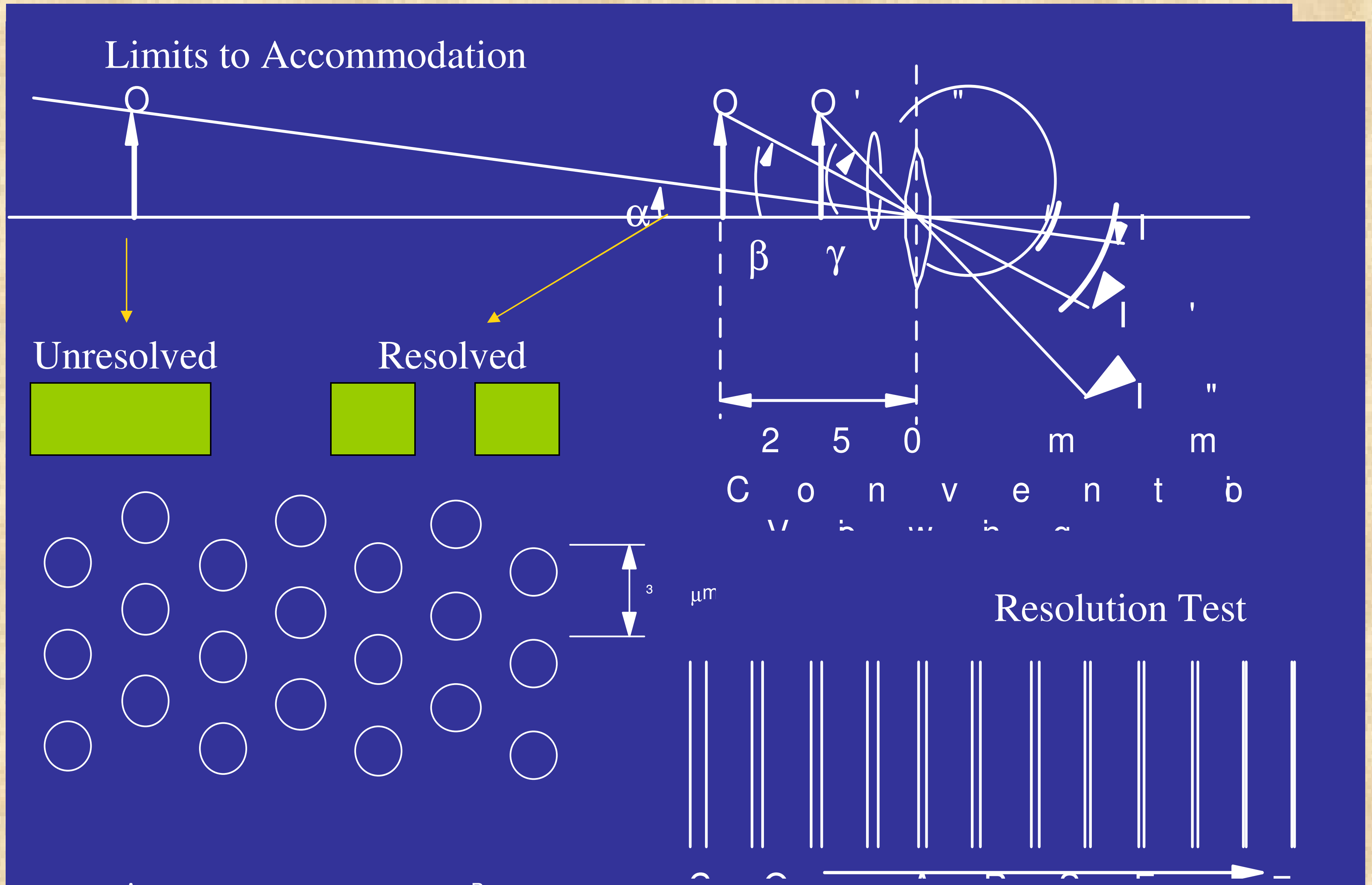
$M_{ob} = OTL/f_{ob}$

Total Magnification = $M_{ob} \times M_{oc} = OTL/f_{ob} \times 250mm/f_{oc}$

Why is the eyepiece necessary?

E.D. Salmon

Resolution Limitations of the Human Eye



A word about infinity corrected optics and its advantages.

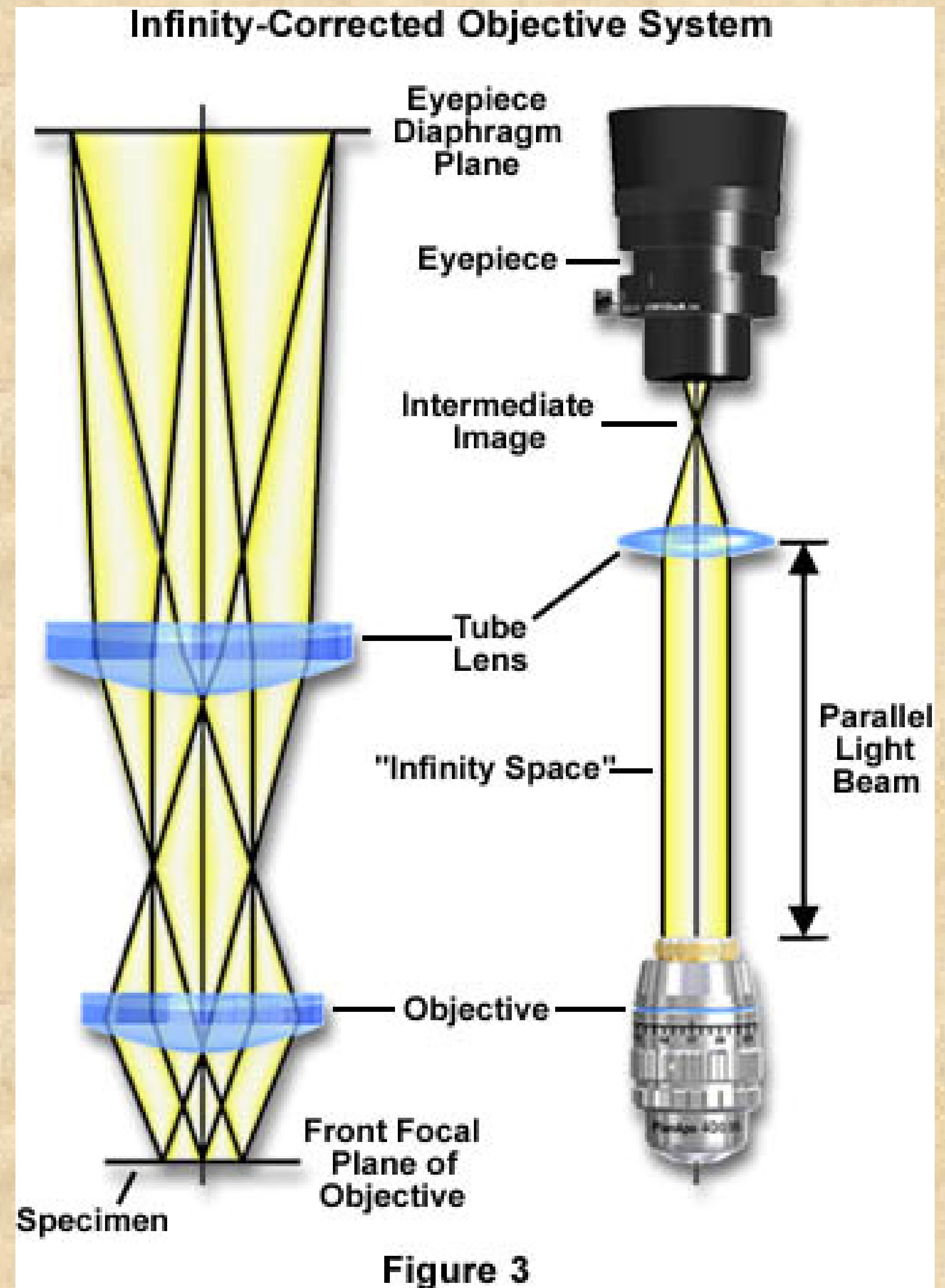
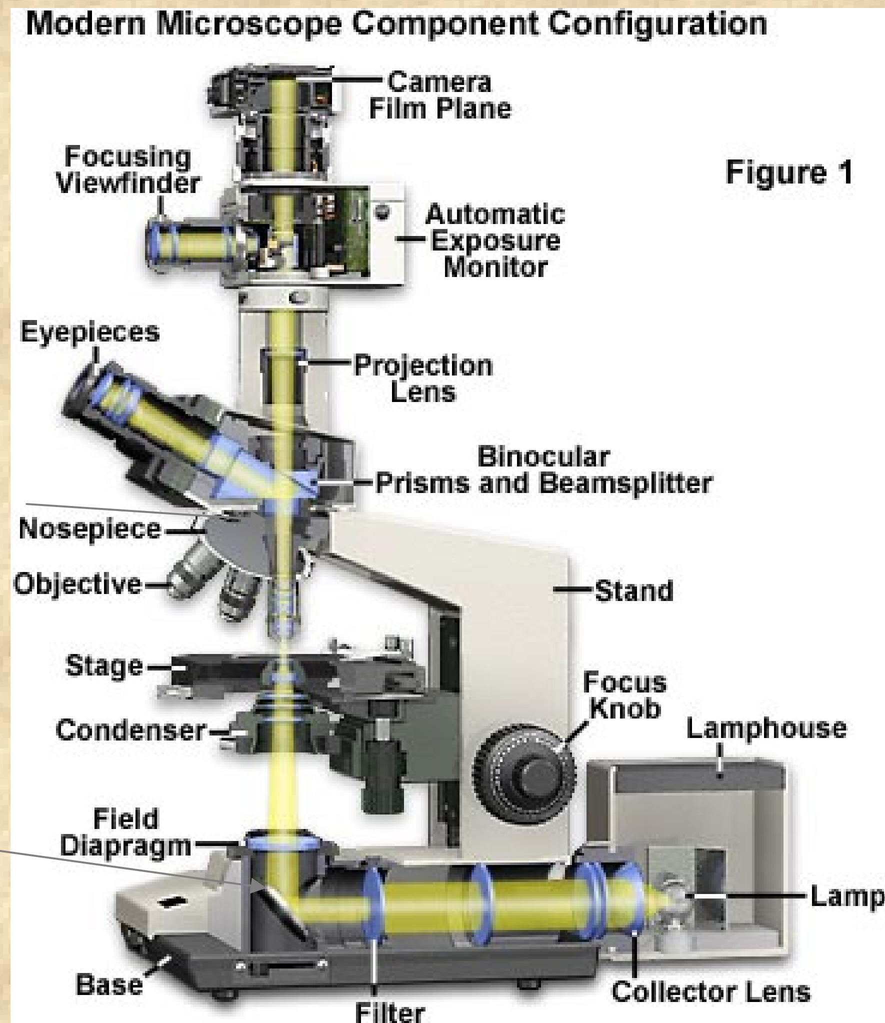


Figure 3

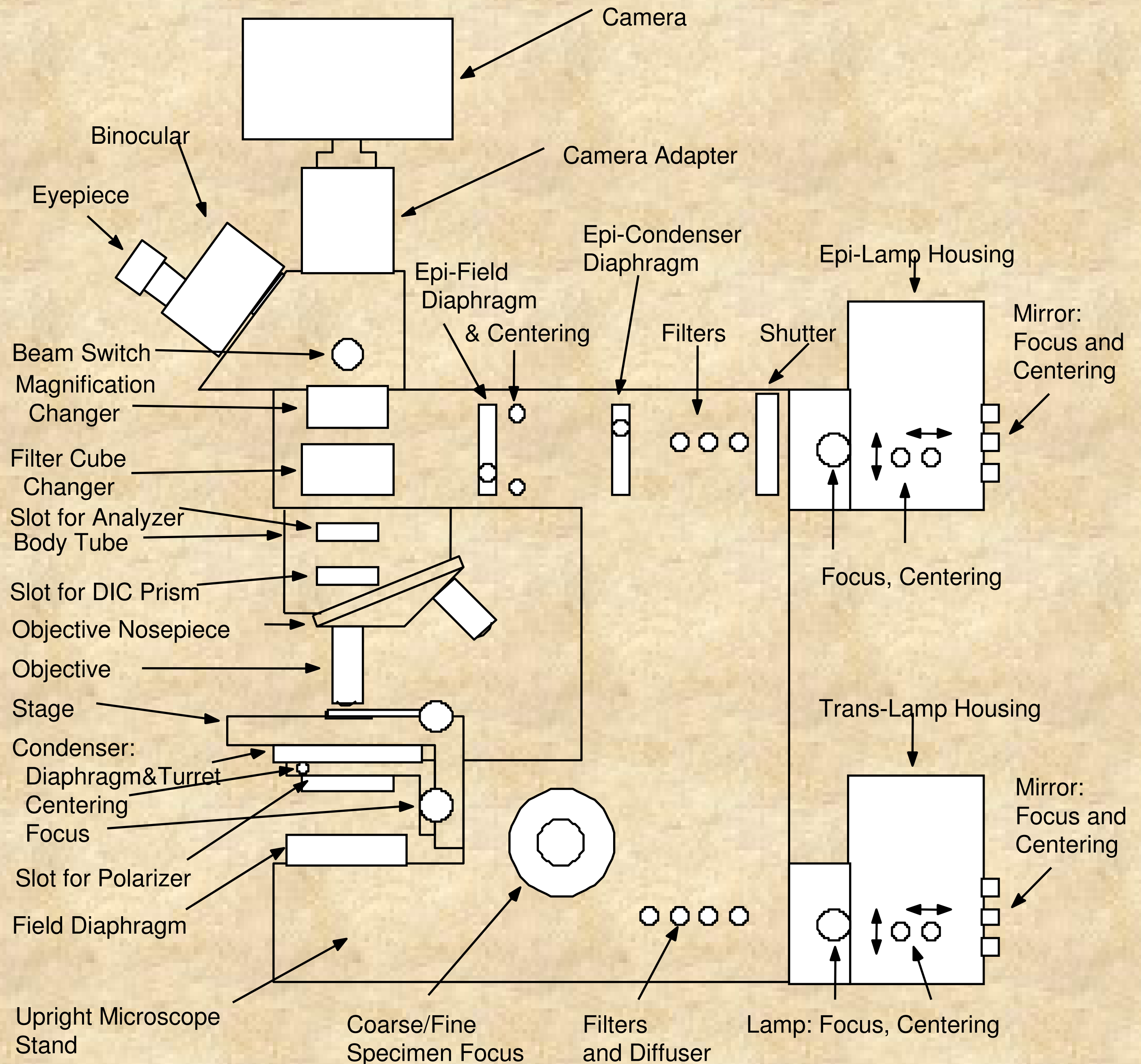
Modern microscope component identification

Prisms Used to Re-Direct Light In Imaging Path While Mirrors Are Used in Illumination Path



E.D.Salmon

Identify Major Components And Their Locations And Functions Within Modern Research Light Microscope (See Salmon And Canman, 2000, Current Protocols in Cell Biology, 4.1)



Key component: the objective

Achromats: corrected for chromatic aberration for red, blue

Fluorites: chromatically corrected for red, blue; spherically corrected for 2 colors

Apochromats: chromatically corrected for red, green & blue; spherically corrected for 2 colors

Plan-: further corrected to provide flat field

The 3 Classes of Objectives

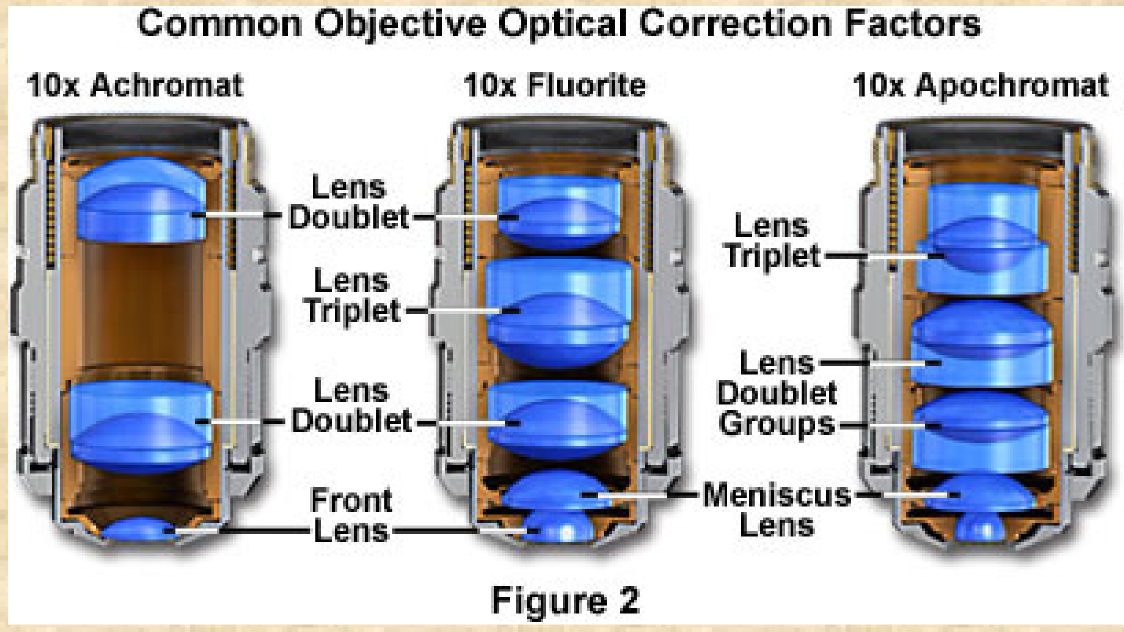


Figure 2

Chromatic and Mono-Chromatic Corrections

Figure 1

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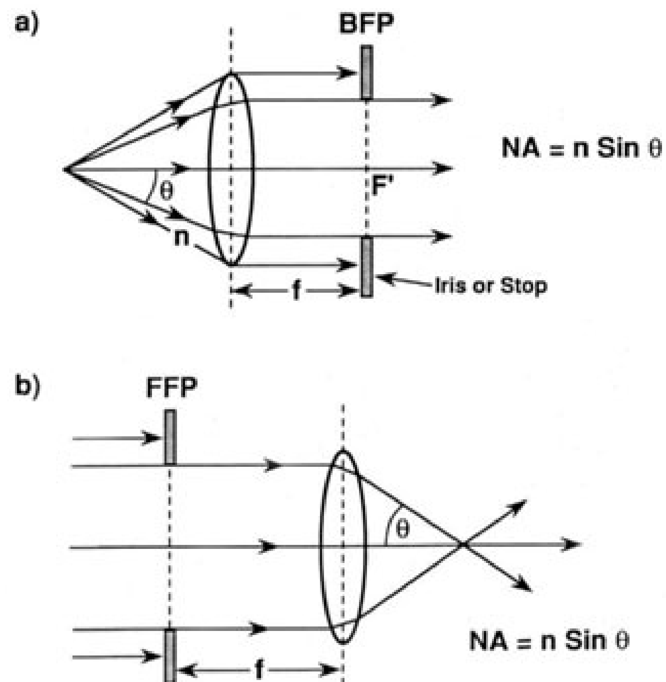
What is numerical aperture (NA)?



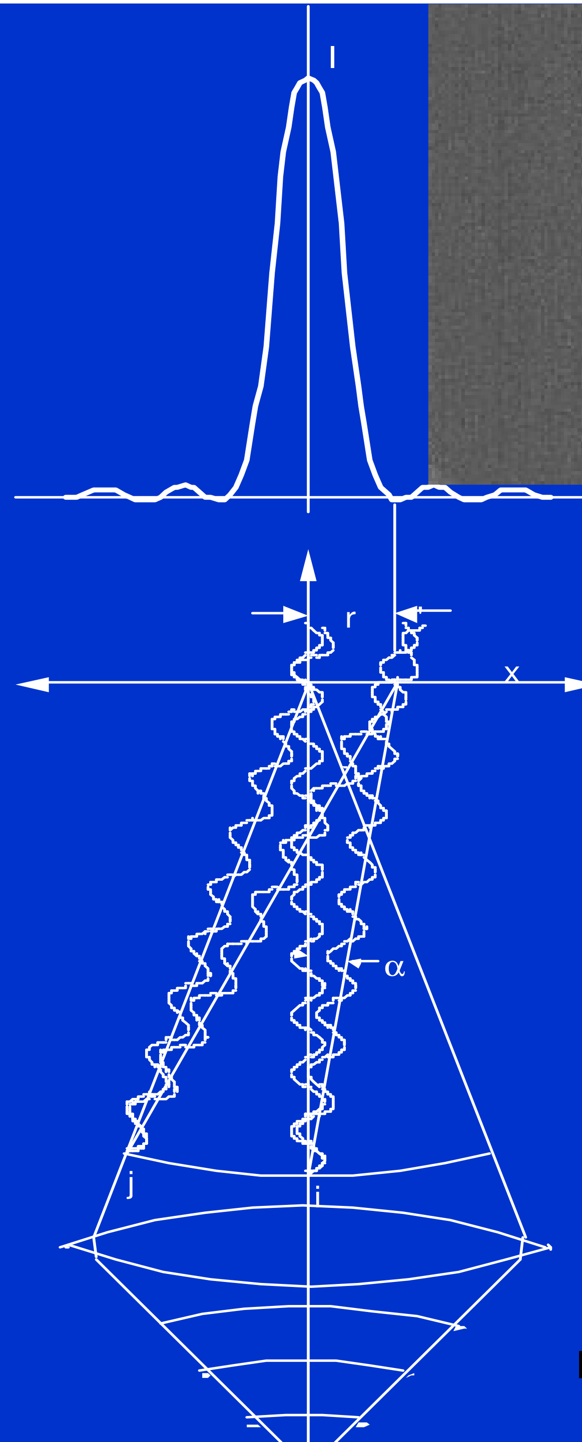
- Image Intensity: $I \sim NA_{obj}^2/M_{tot}^2$
- Image Lateral Resolution for Corrected Objective:

-Fluorescence: $r = 0.61\lambda/NA_{obj}$

-Trans-Illumination: $r = \lambda/(NA_{obj} + NA_{cond})$

Airy Disk Formation by Finite Objective Aperture:

The radius of the Airy Disk at the first minimum, r', occurs because of destructive interference; the diffraction angle, α, is given by: $\sin(\alpha) = 1.22\lambda/D$, where D = diameter of objective back aperture



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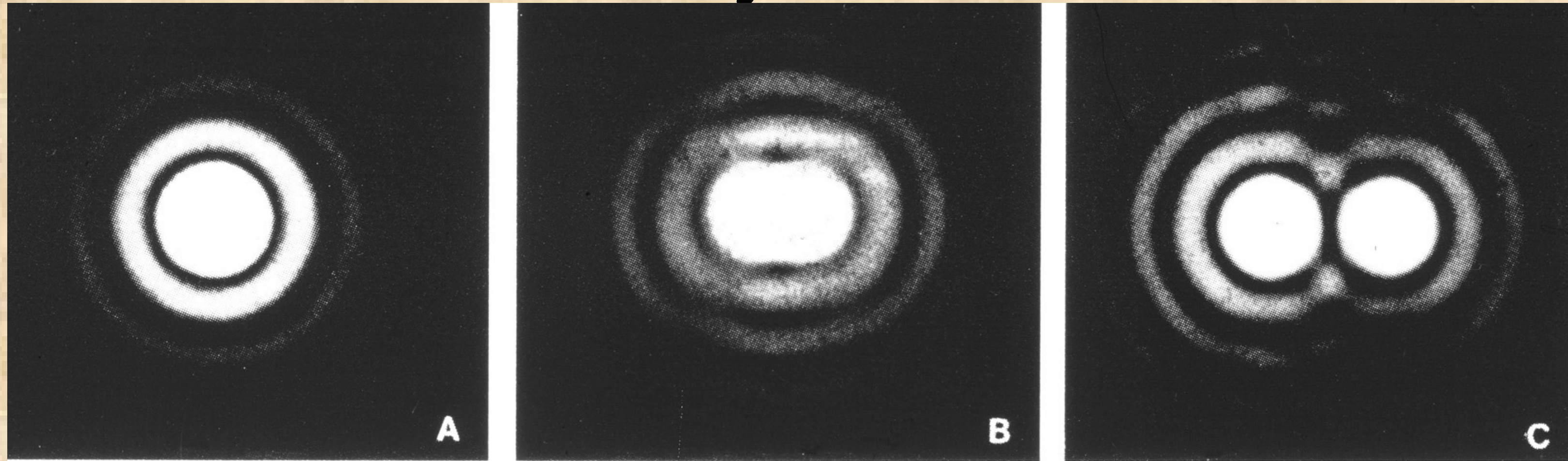
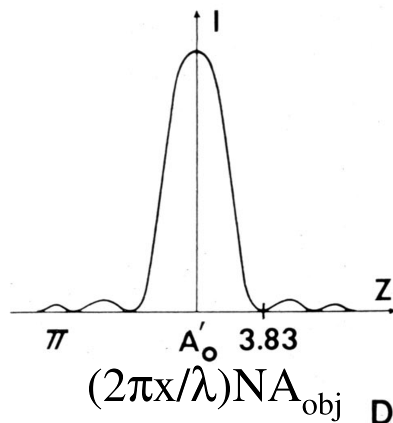
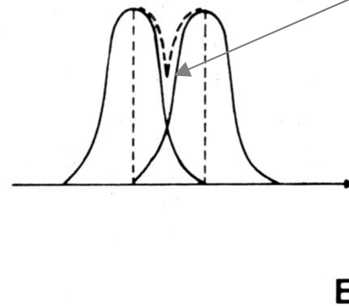
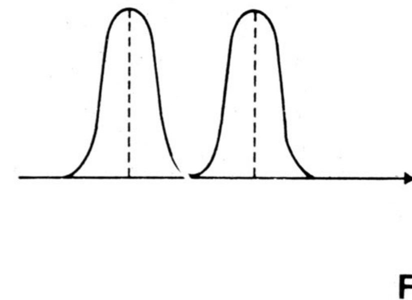
Lateral Resolution in Fluorescence Depends on Resolving Overlapping "Airy Disks"



Rayleigh Criteria: Overlap by r', then dip in middle is 26% below Peak intensity







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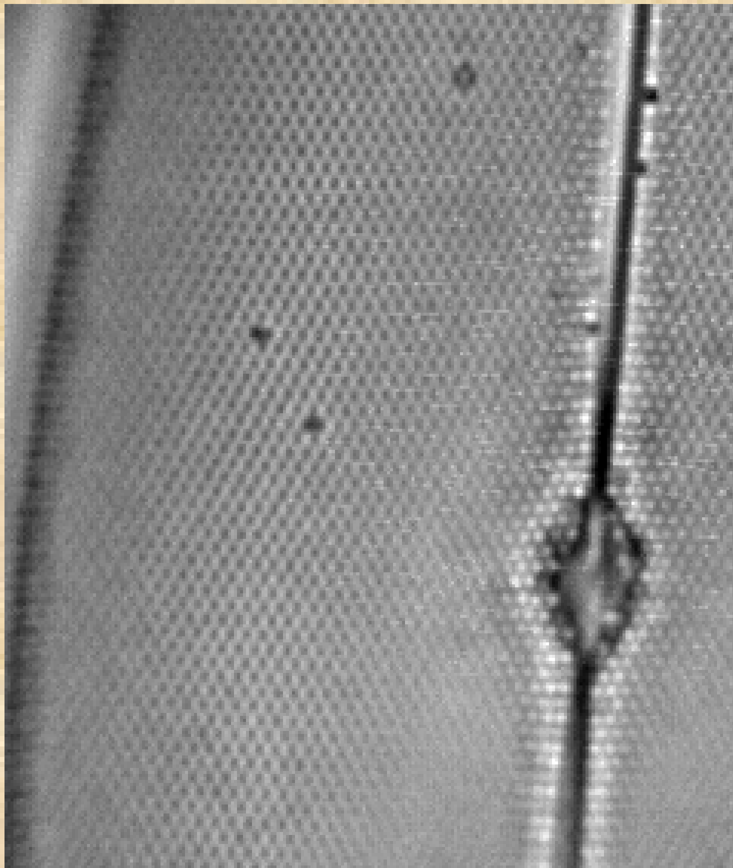
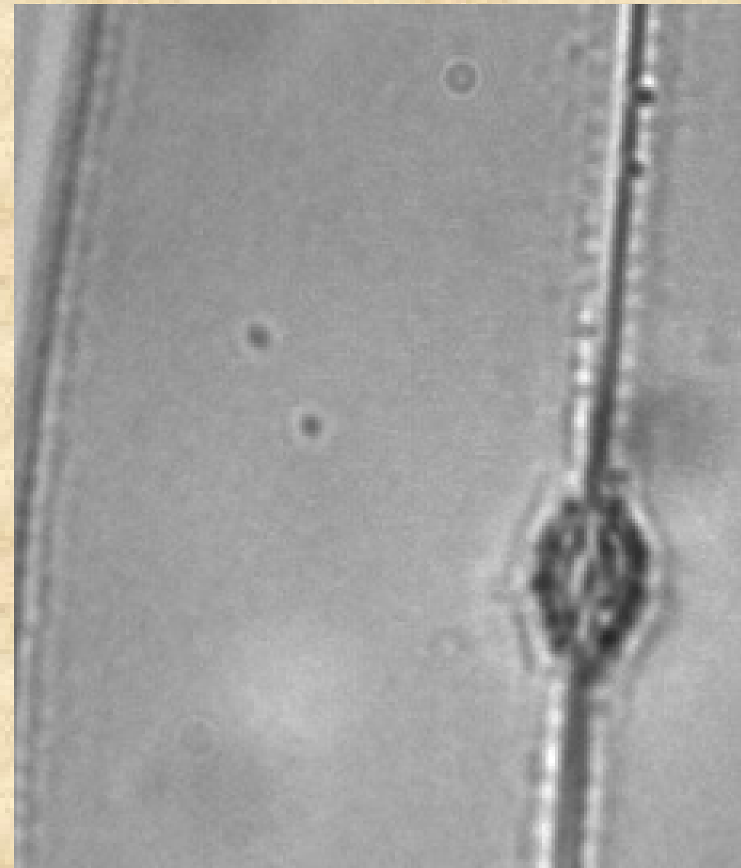
Resolution is better at shorter wavelengths, higher objective NA or higher condenser NA



High NA and/or shorter λ



Low NA and/or longer λ

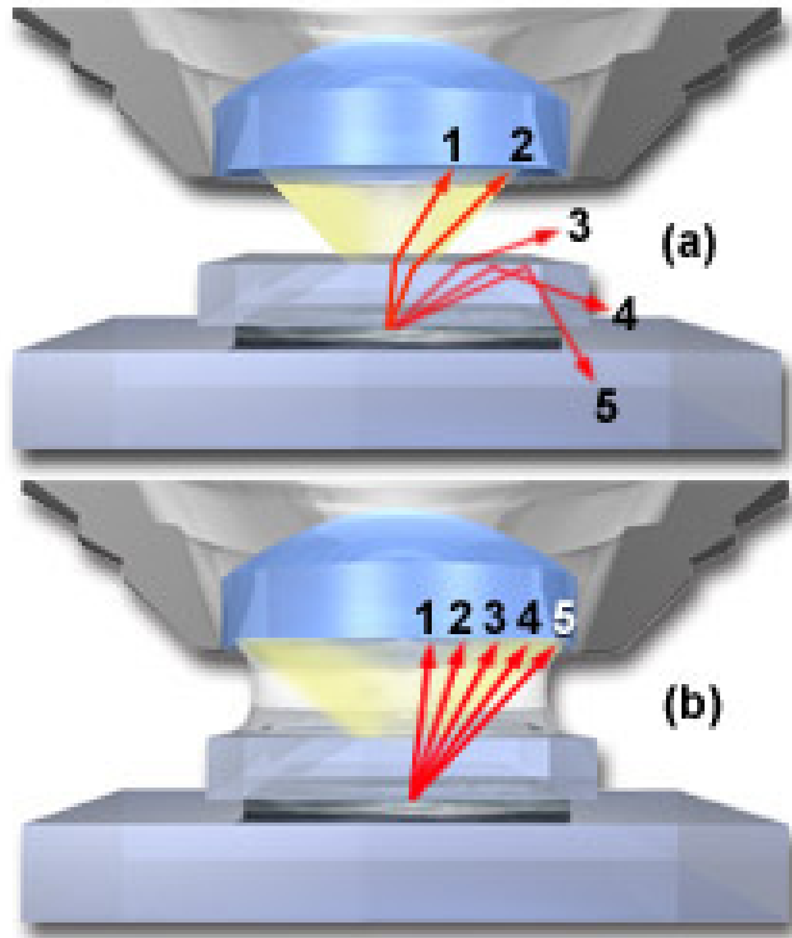
Rayleigh Criterion for the resolution of two adjacent spots:

$$P_{lim} = 0.61\ \lambda_o / NA_{obj}$$

Examples: (λ_o = 550 nm)

	Mag	f(mm)	n	a	NA	P_{lim} (μm)	($NA_{cond}=NA_{obj}$)
high dry	10x	16	1.00	15	0.25	1.10	
	40x	4	1.00	40	0.65	0.42	
oil	100x	1.6	1.52	61	1.33	0.204	
	63x	2.5	1.52	67.5	1.40	0.196	

Why oil immersion lenses have greater resolution

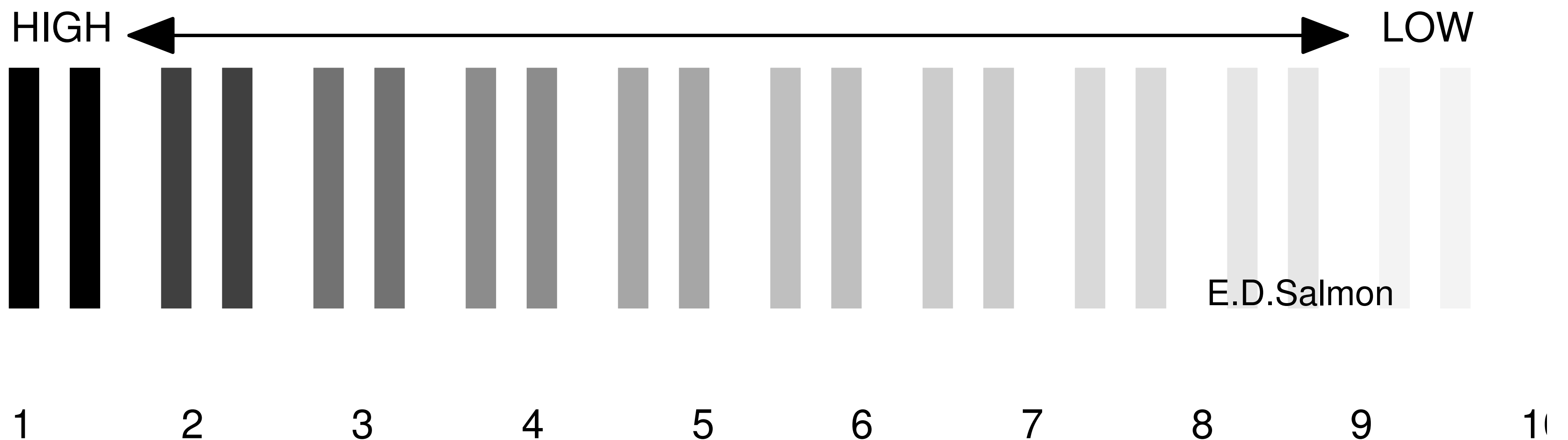


$$D = 0.61\ \lambda \cos \alpha / n(NA)^2$$

Low power,	NA~ 0.25	D~ 8 μm
Hi, dry,	NA~0.5	D~ 2 μm
Oil immersion,	NA~ 1.3	D~0.4 μm

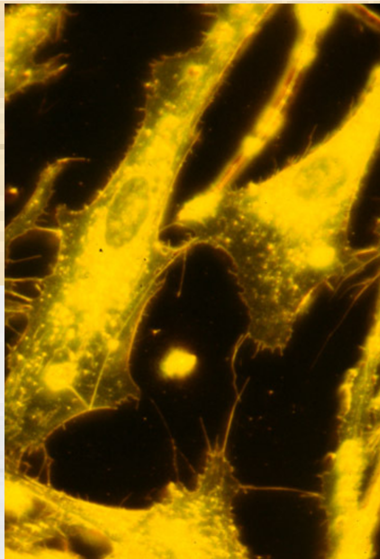
Contrast : All the resolution in the world won't do you any good, if there is no contrast to visualize the specimen.

$$\text{CONTRAST} = (I_{sp} - I_{bg})/I_{bg}$$



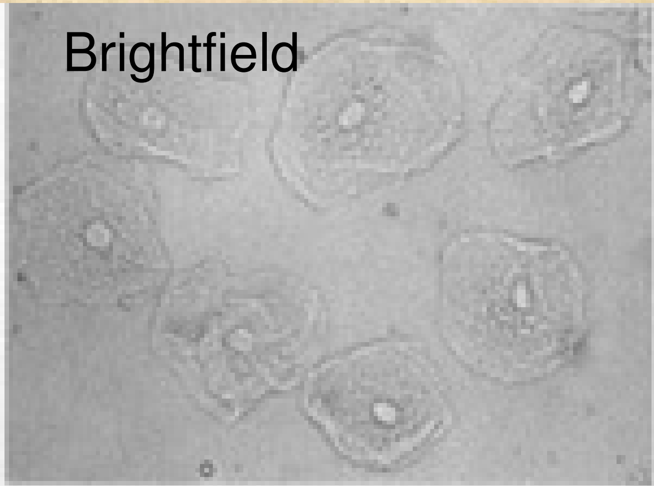
CONTRAST MODES OF LIGHT MICROSCOPY

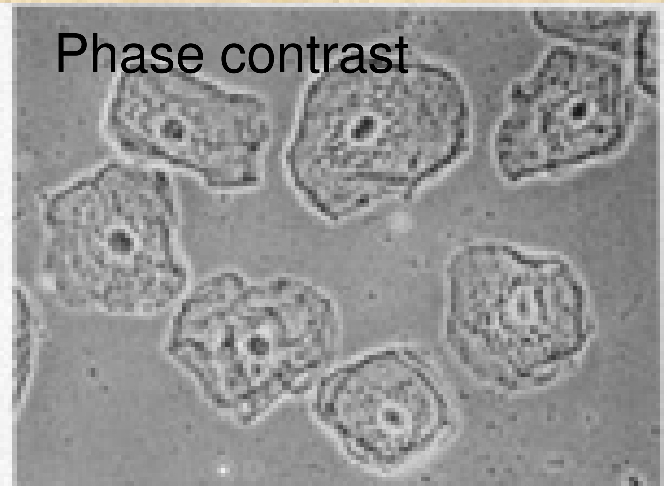
MODE	MECHANISM OF CONTRAST
Brightfield	Absorption of light
Phase contrast	Optical path length (index, density)
DIC	Rate of change of optical path
Widefield fluorescence	Absorption of light, quantum yield of fluorophore
Confocal fluorescence	same as fluorescence
Darkfield	light scattering by edges in specimen
Interference reflection contrast	interference between reflections from ventral cell surface and substratum
Polarization	Extinction between crossed polars caused by specimen birefringence

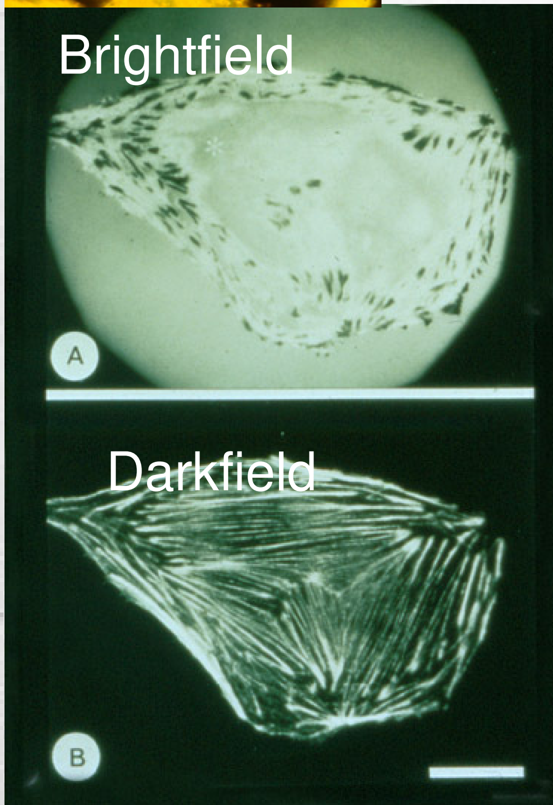


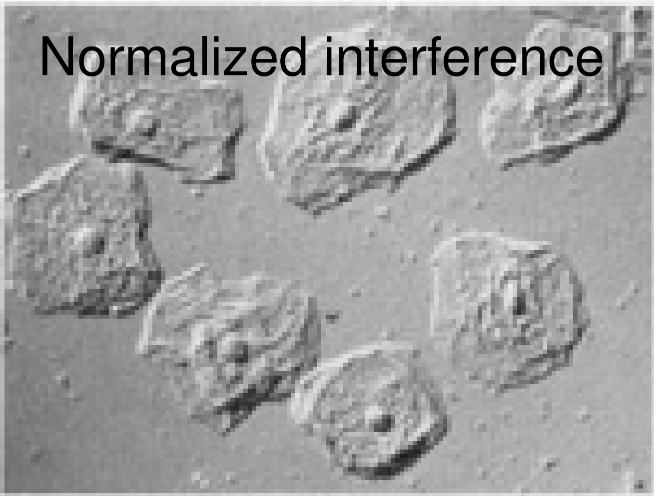
Fluorescence

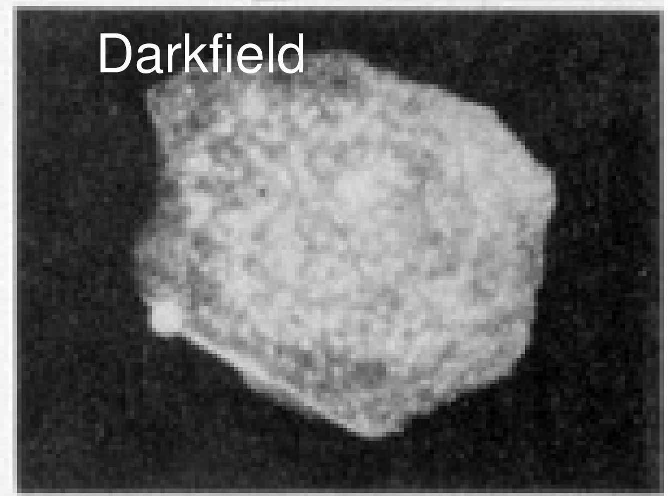
Index of refraction



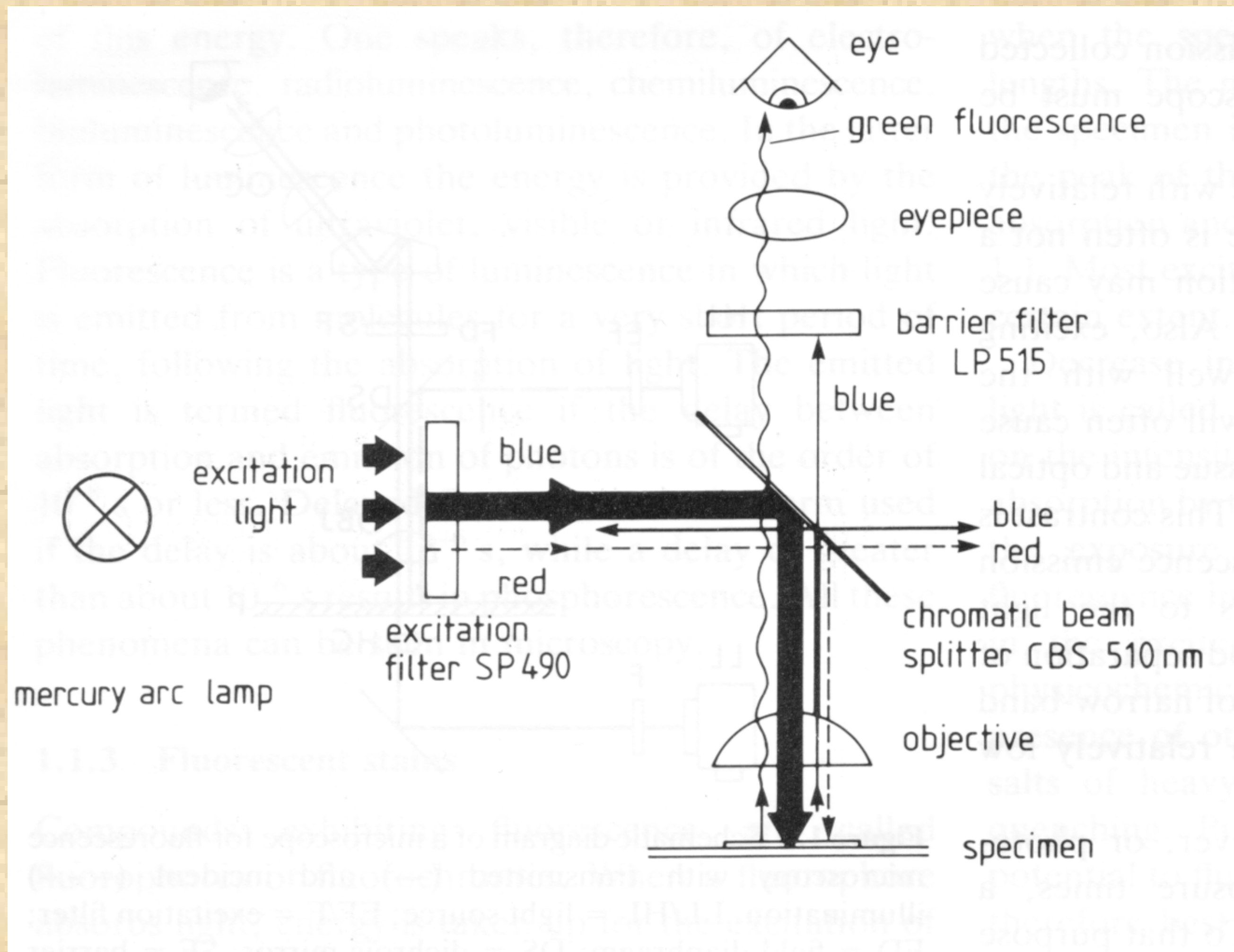








Basic design of the epi fluorescence microscope



Objectives

High transmittance

Fluorite lenses: $\lambda > 350$ nm [ok for FURA]

Quartz lenses: $\lambda < 350$ nm

Employ simple, non plan lenses to minimize internal elements.

Negligible auto-fluorescence or solarization [color change upon prolonged illumination]

Maximizing image brightness (B)

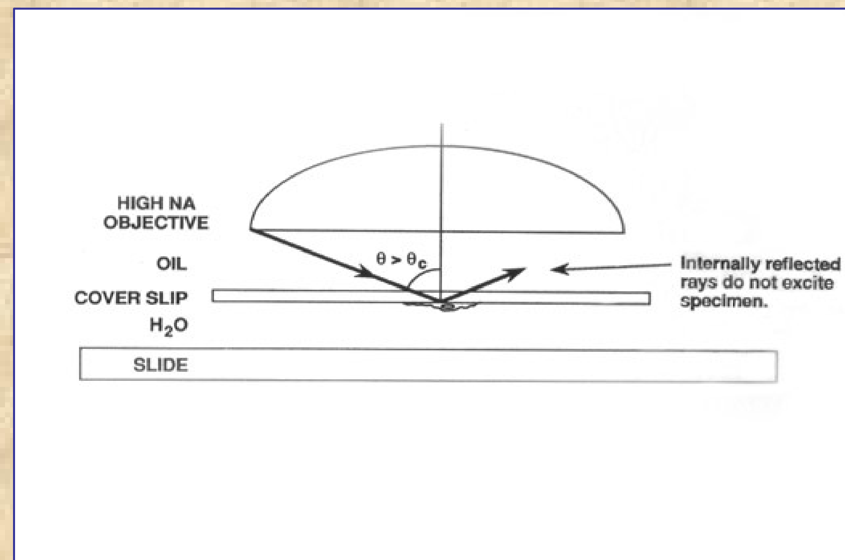
excitation efficiency ~ $(NA)^2$

collection efficiency ~ $(NA)^2$

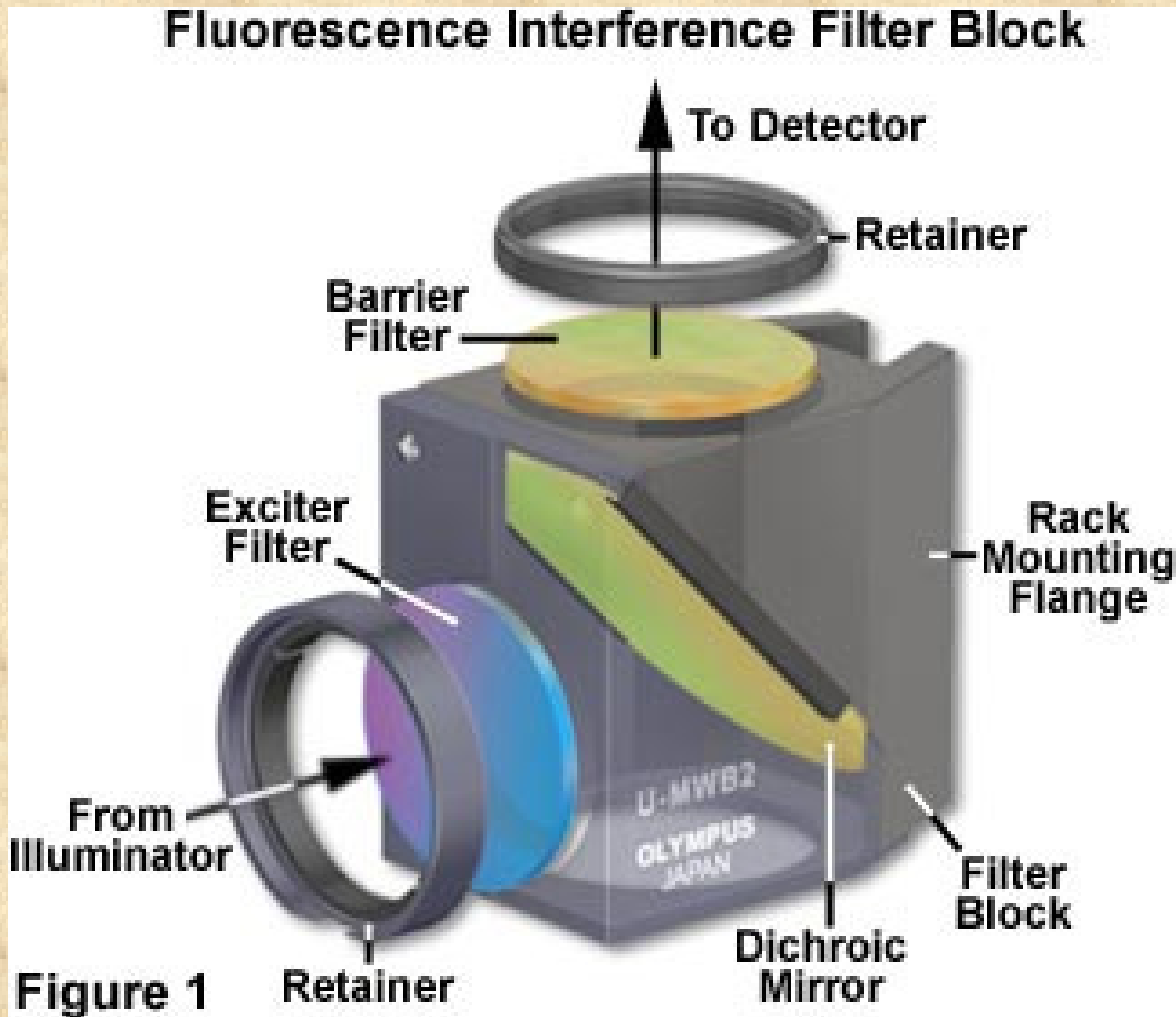
=> $B \sim (NA)^4$

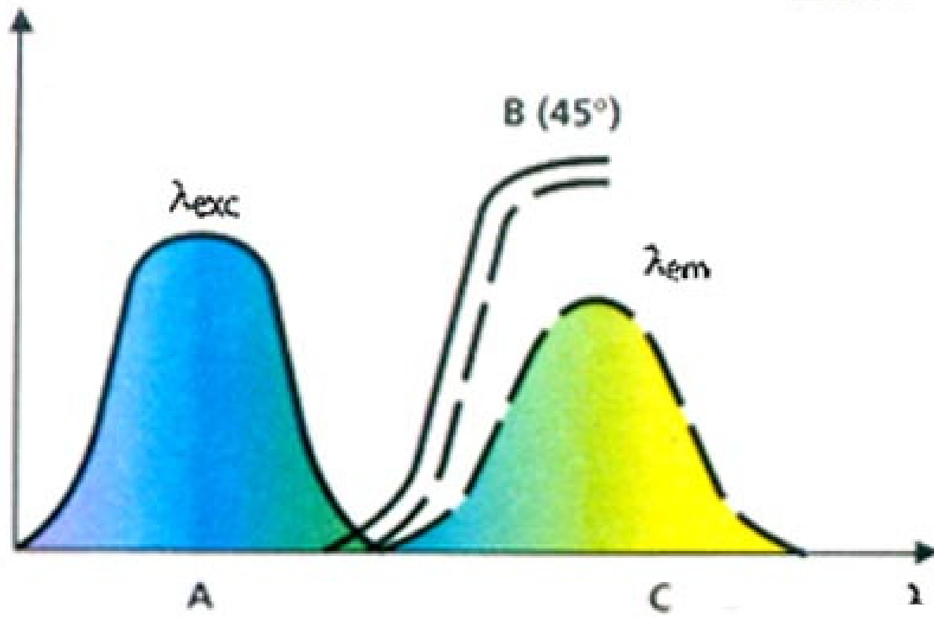
also $B \sim \dfrac{1}{M^2}$ => $B \sim \dfrac{(NA)^4}{M^2}$, for $NA \leq 1.0$

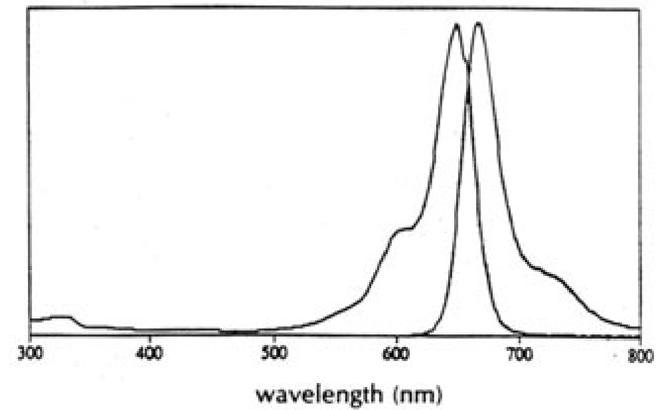
at high NA,



Filters

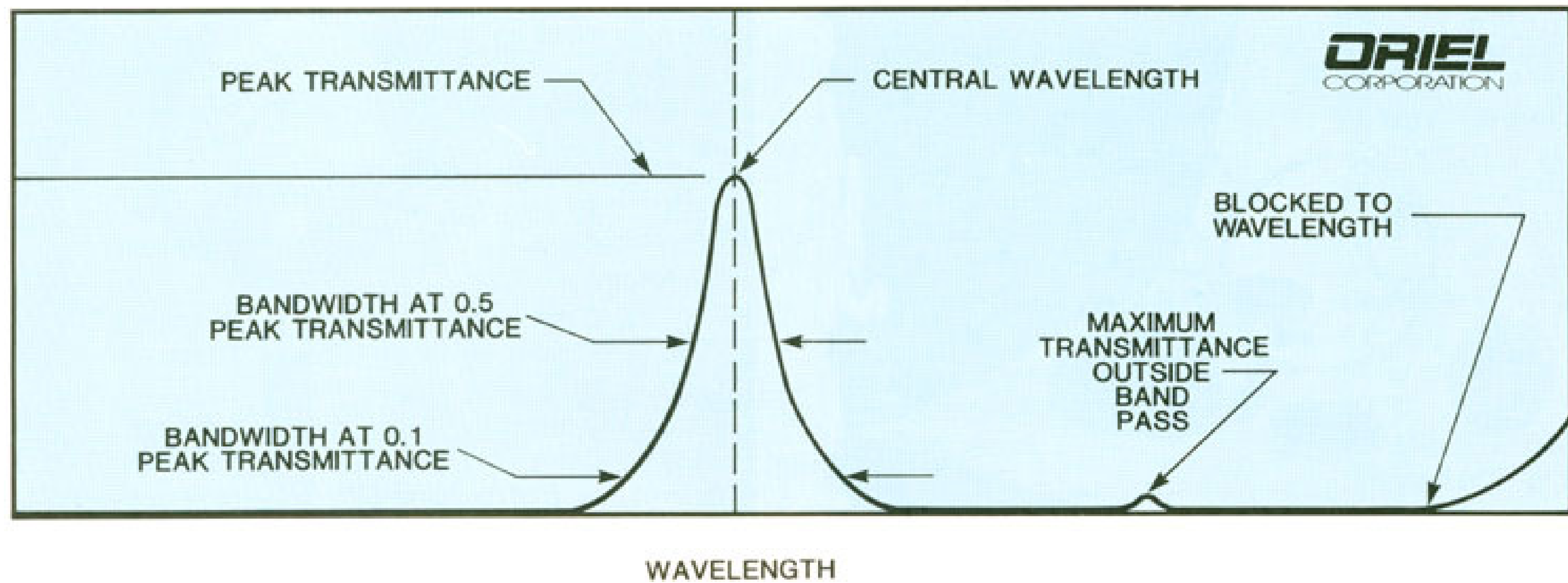






Abs. max.	652 nm
Ext. max.	> 200,000 $M^{-1}cm^{-1}$
Fluor. max.	667 nm
Q. Y. (Ab,N~2)	0.28

Interference filter definitions



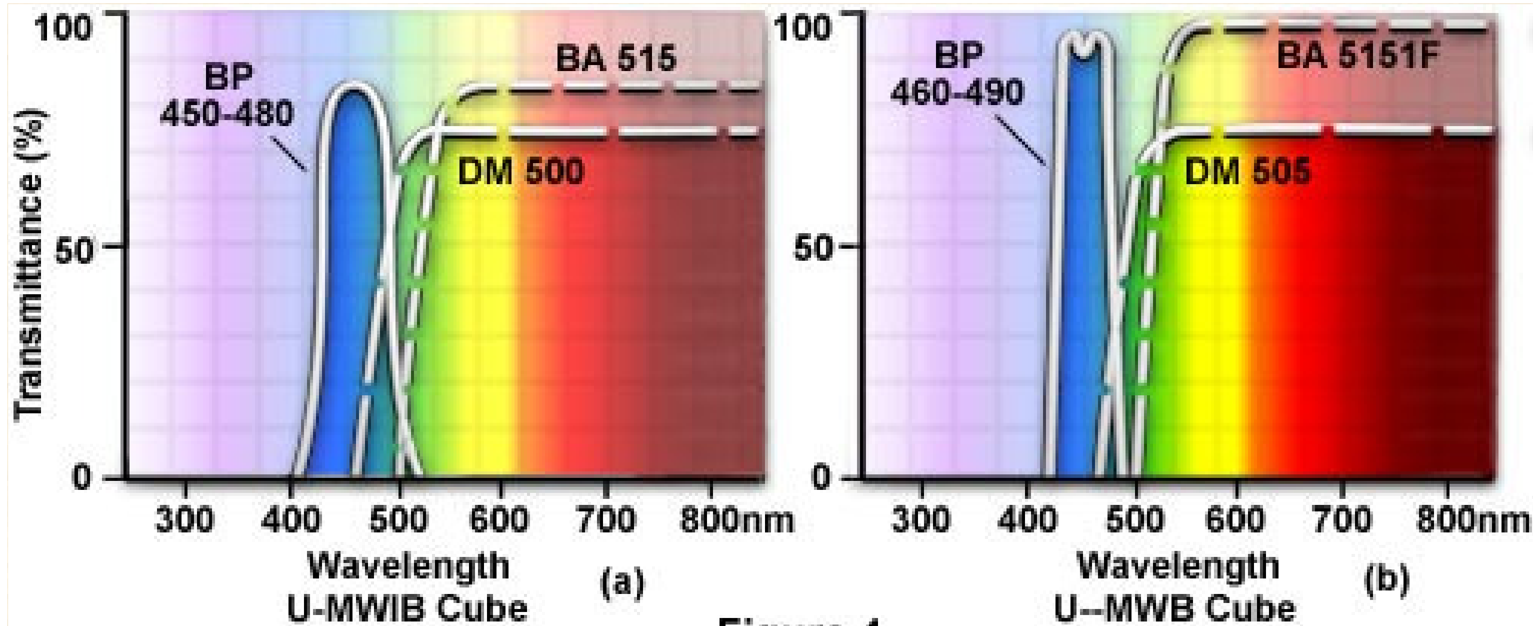


Figure 4

Filter cube designs employing long-pass emitter filters

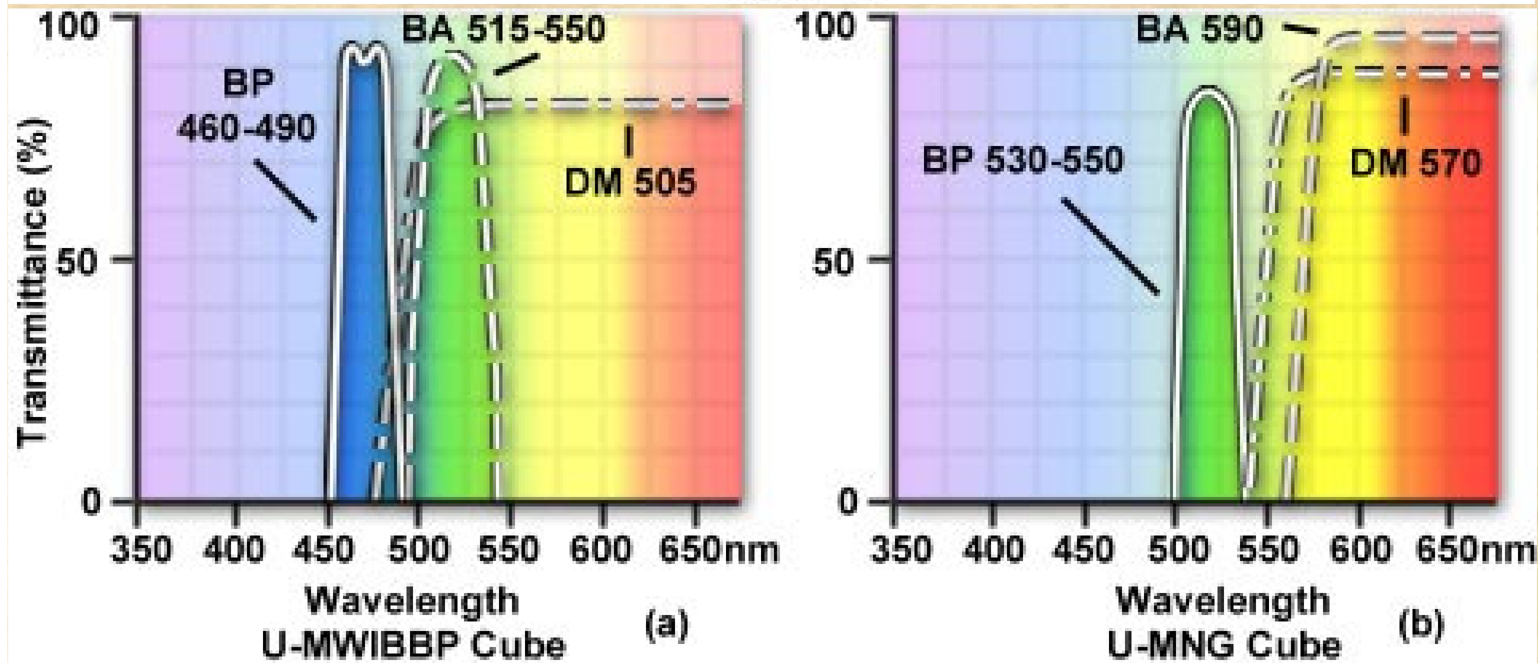


Figure 5

Filter cube designs employing band-pass emitter filters

Multi-Wavelength Immunofluorescence Microscopy

Bovine Pulmonary Artery Epithelial Cells

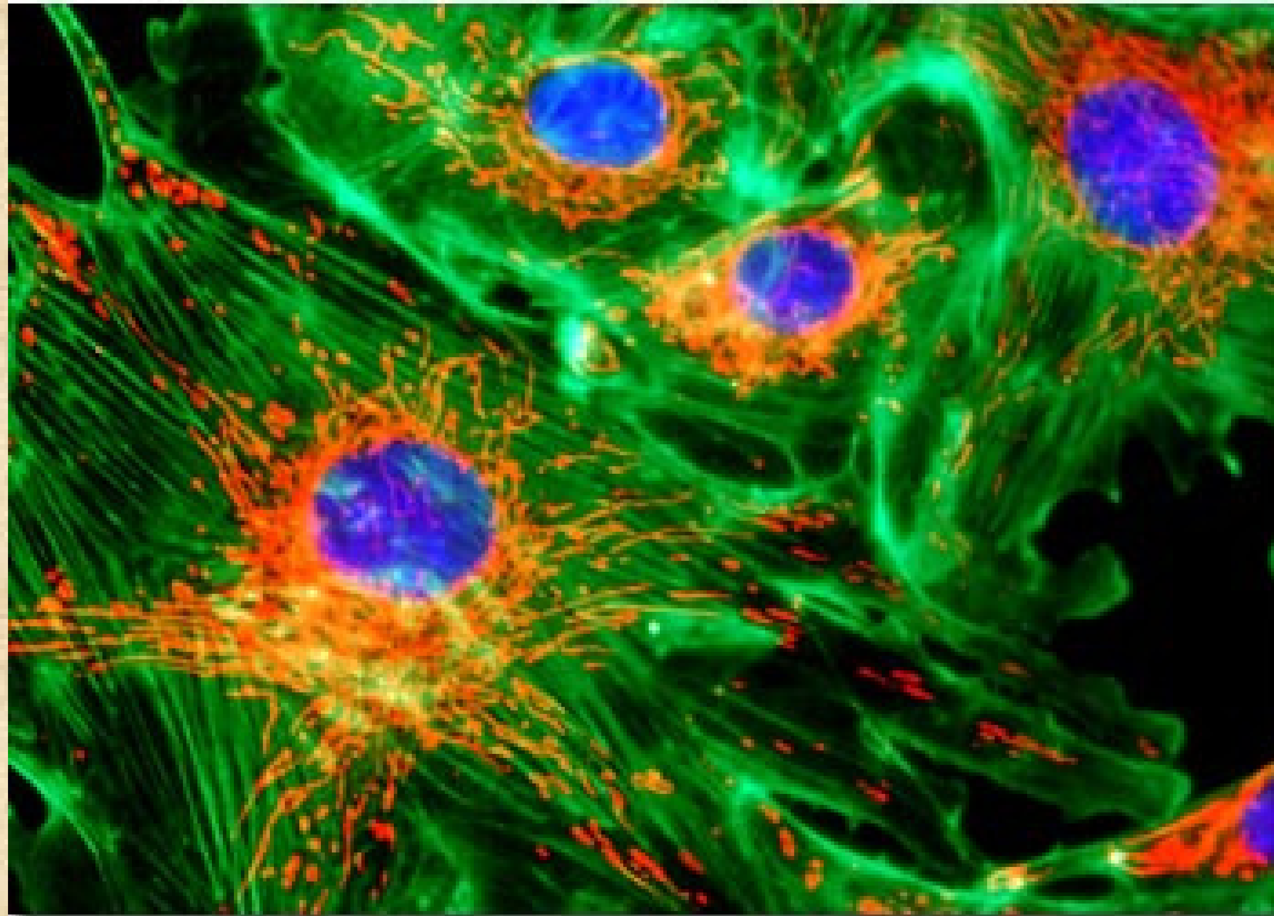
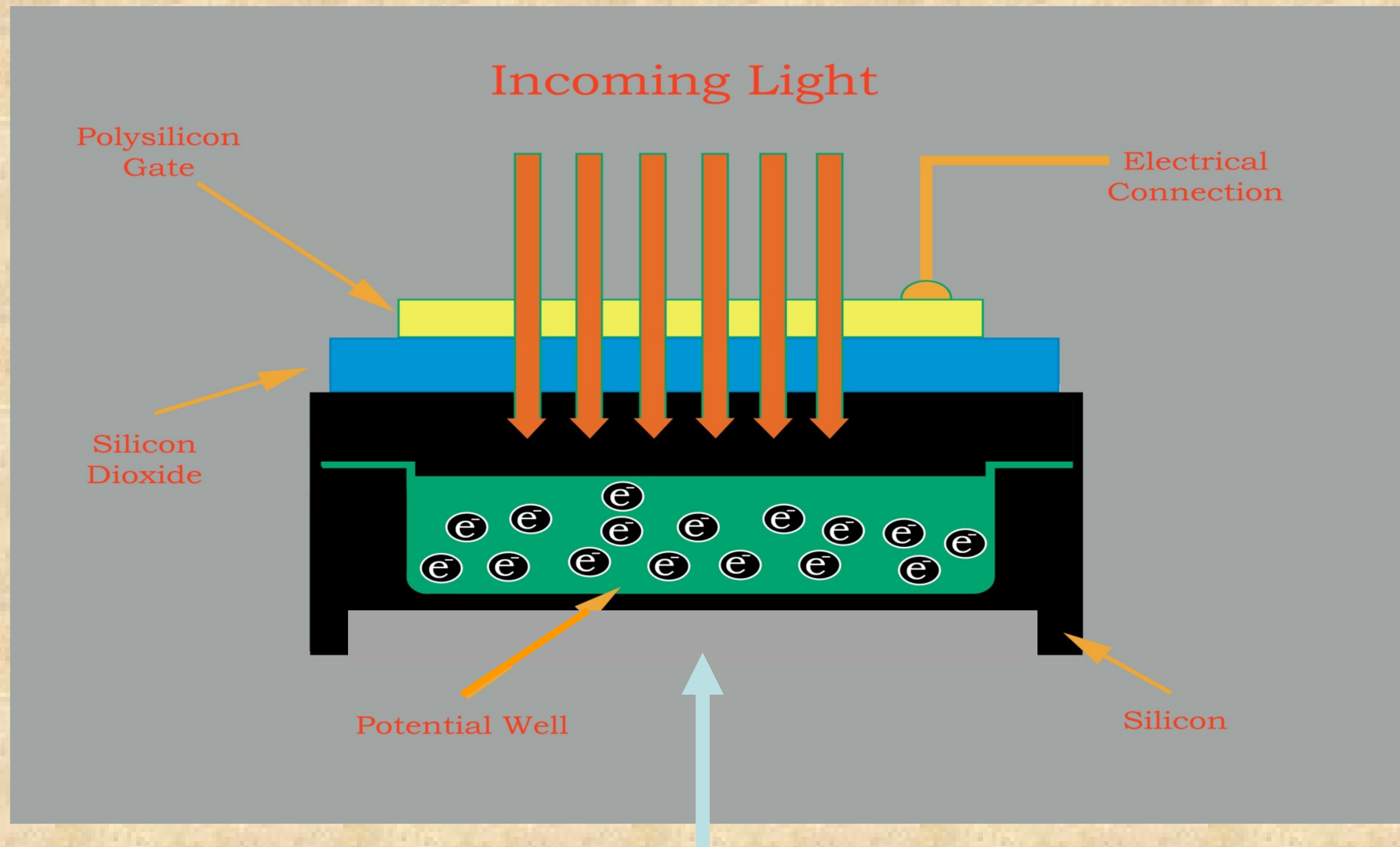


Figure 1

PIXELS
The building blocks of CCDs



Back thinned CCDs receive light from this side

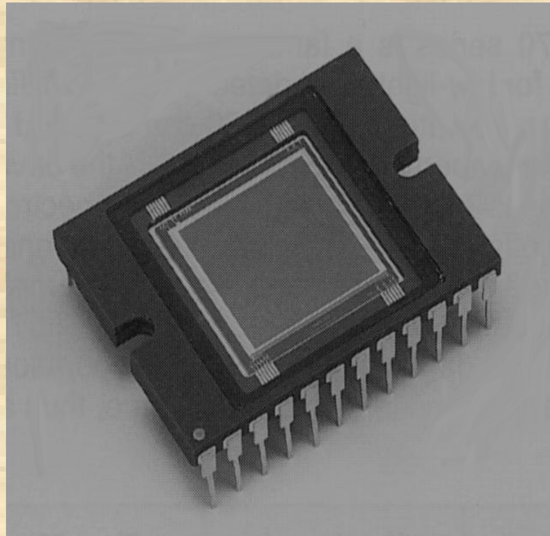
Primary Features of CCD

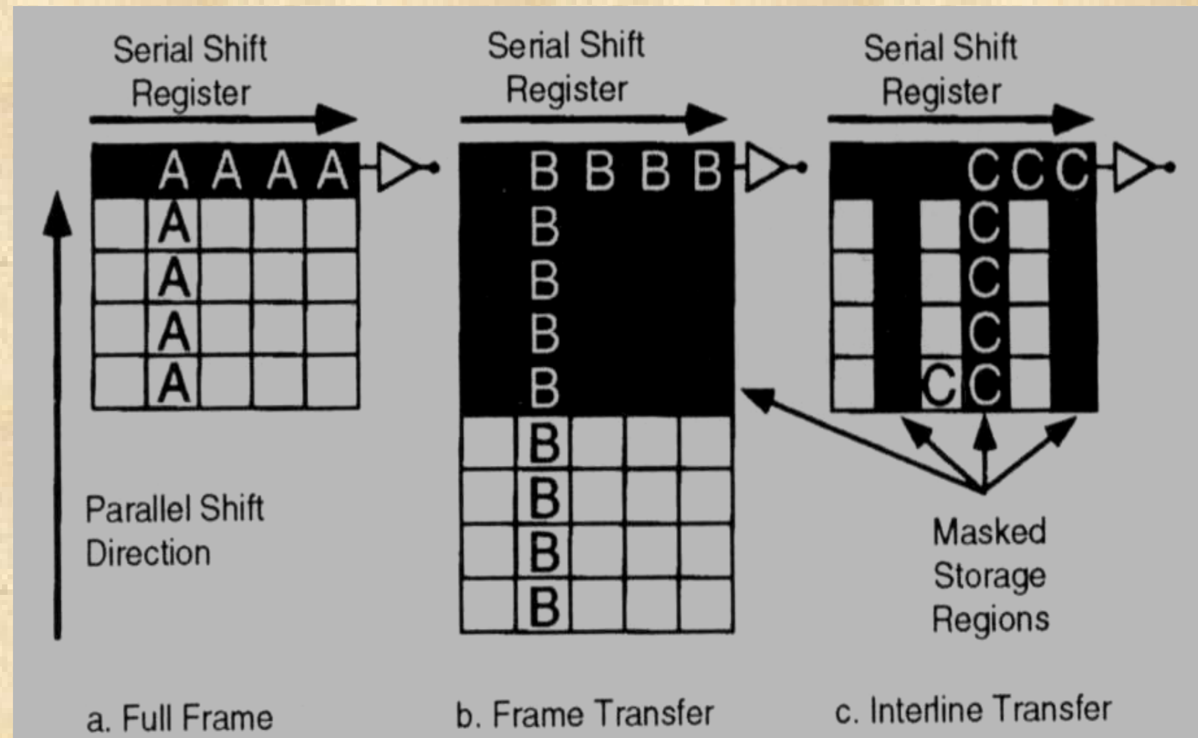
- **Spatial resolution of the CCD array**
  - **Number of Pixels in X and Y**
  - **Center to Center Distance of Pixels in microns**
- **Full Well Capacity**
  - **Related to Physical size and electronic design**
  - **Determines Maximum Signal level possible**
- **Quantum Efficiency/Spectral Range**
  - **Determines the usefulness of the camera**
  - **Major influence on exposure time**
- **Camera Noise**
  - **The limiting feature in low light applications**
  - **Influenced by Readout Speed / Readout Noise**
  - **Influenced by Dark Current / Time**
- **CCD Chip Design**
  - **Influences Total Frame Rate**
    - **Exposure time plus Readout time**
  - **Total Photon Efficiency**
    - **Quantum Efficiency and Exposure Cycle**

B. Moomaw, Hamamatsu Corp.

Types of CCD Detectors

- CCD Cameras - 3 Primary Designs





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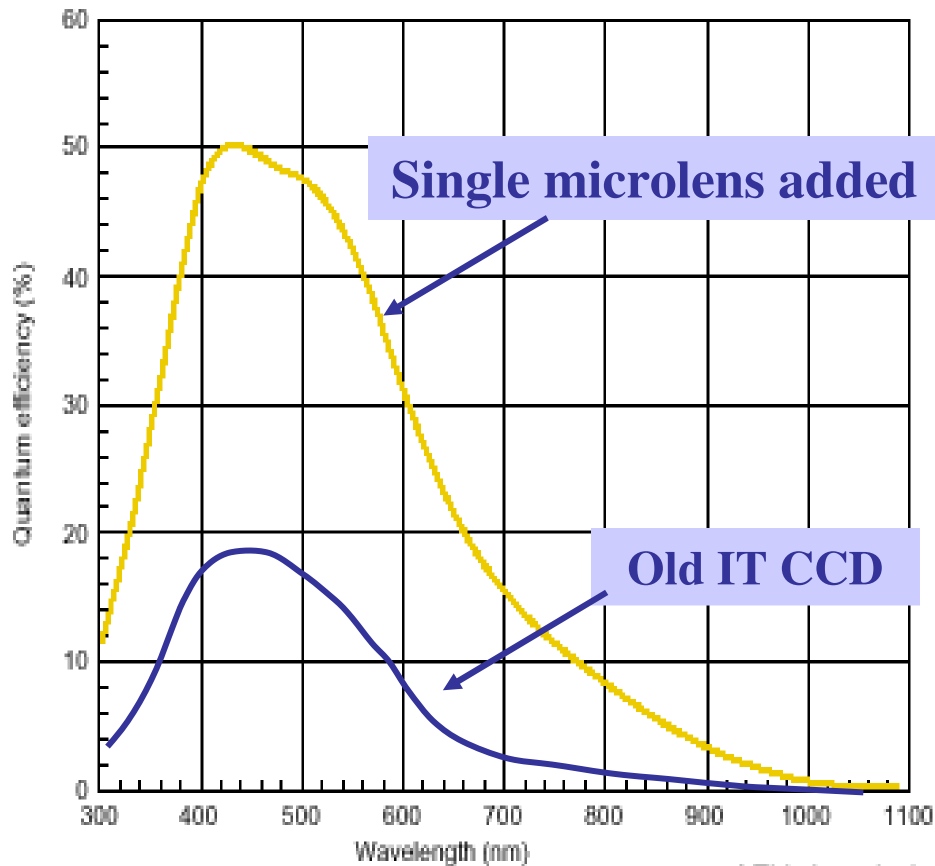
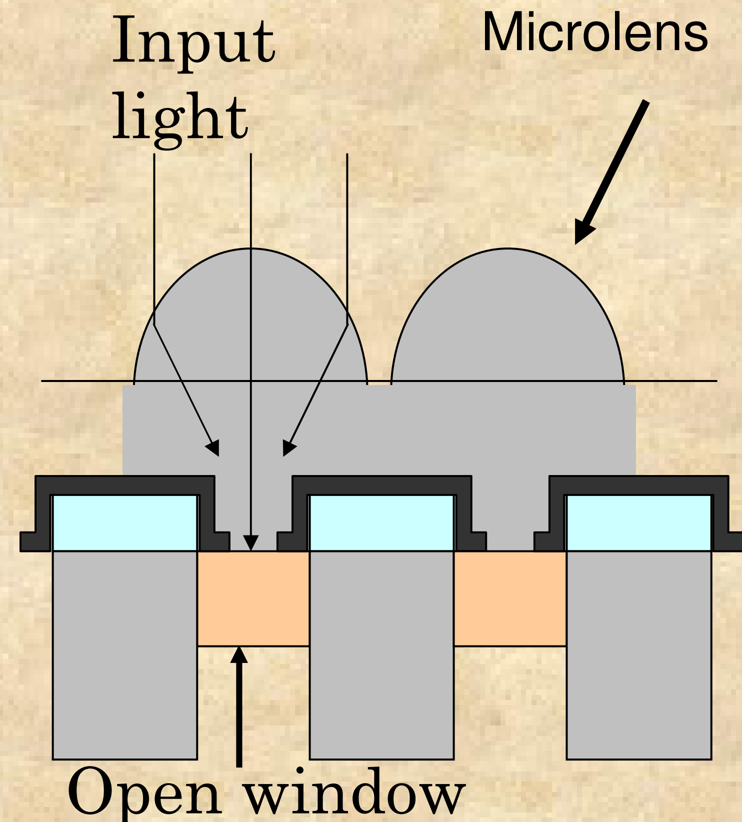
Improvements in Interline CCDs

- Effective Q.E. was greatly increased by Microlens technology.





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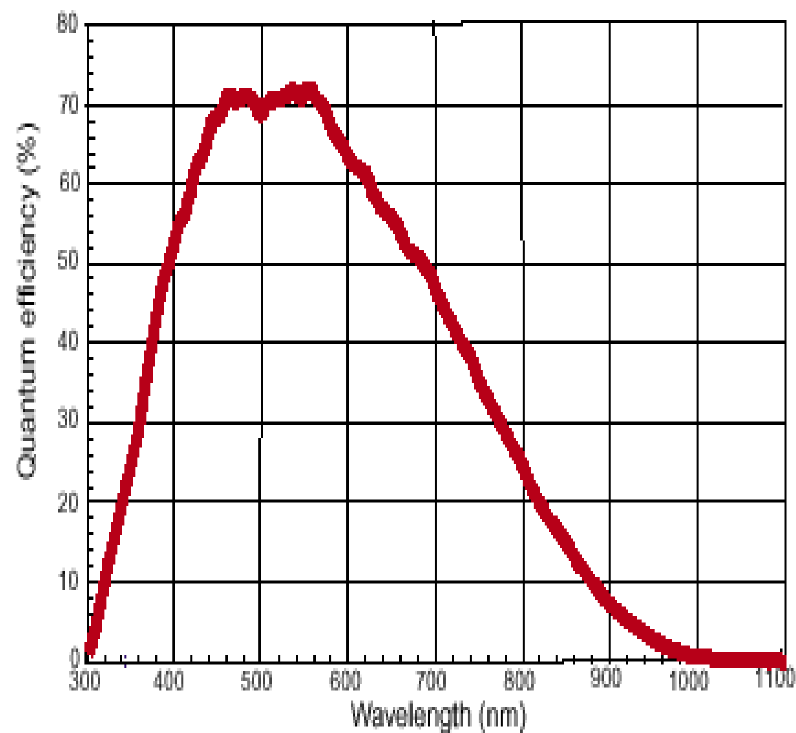
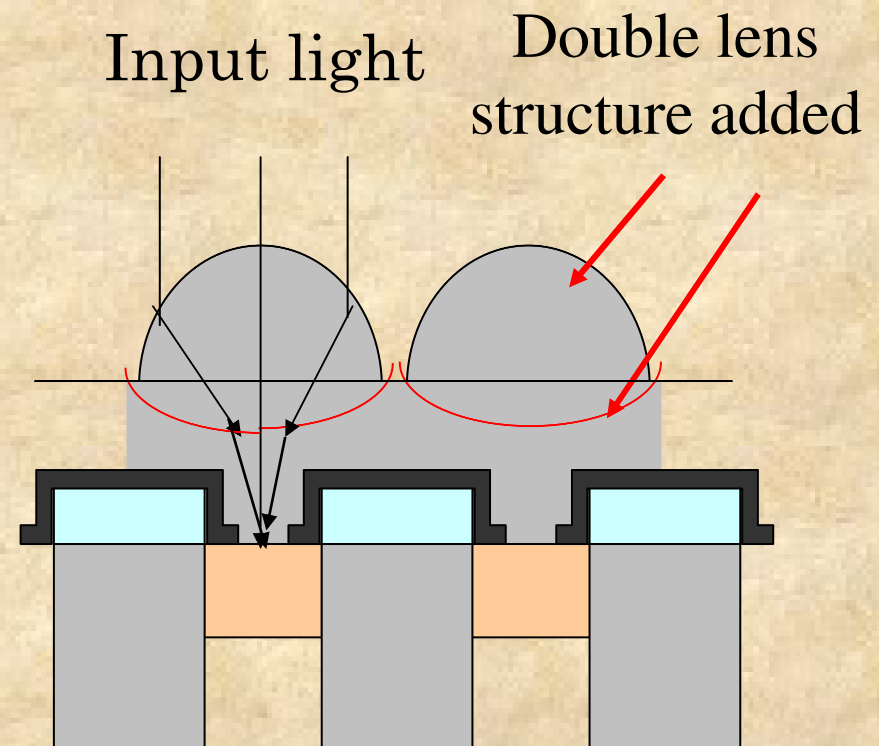
Latest Improvement to Interline CCDs

- Latest double micro lens structure improved the CCD open ratio up to 80% and Q.E. to over 70%!





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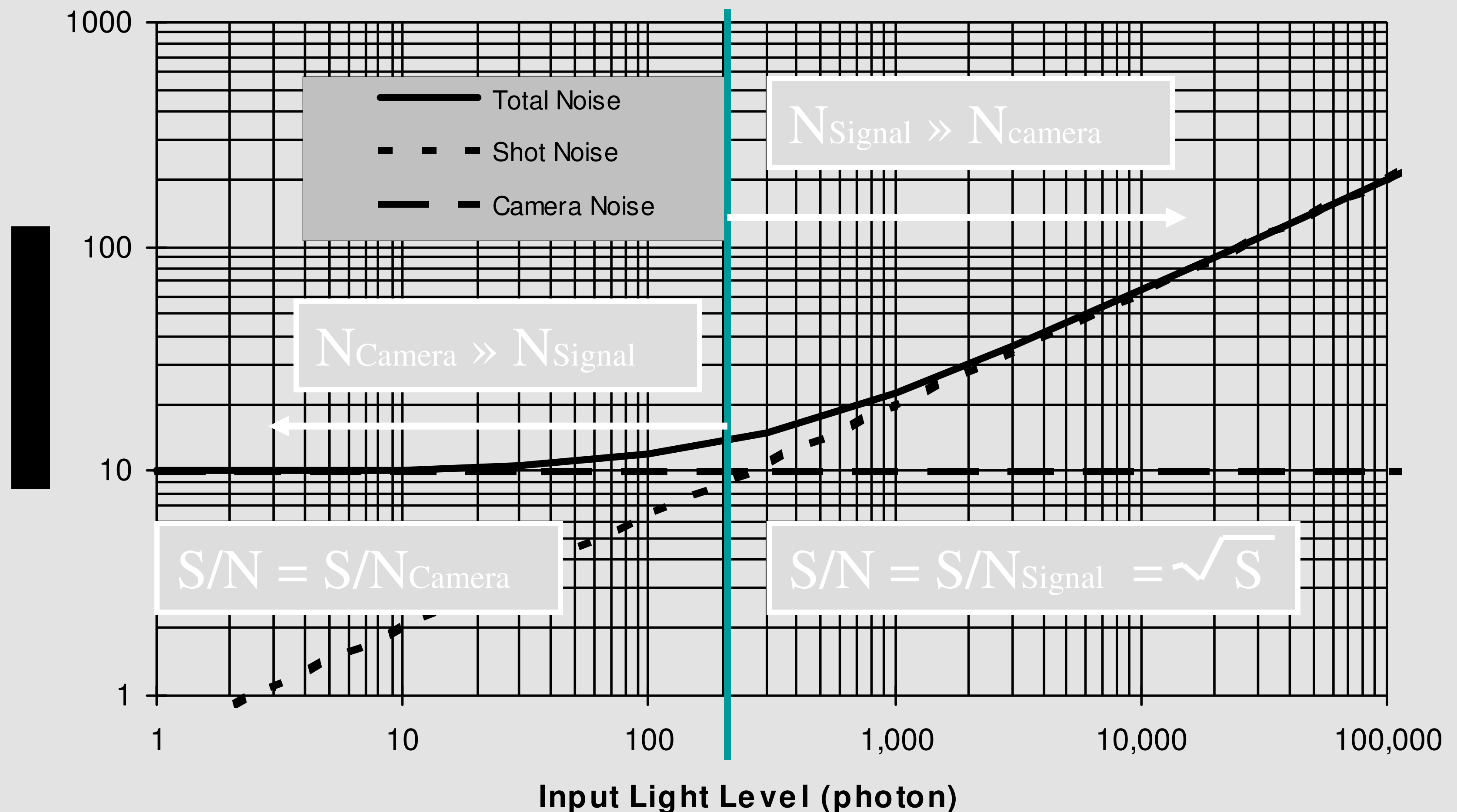
Noise as a function of incident camera illumination



COMMON SOURCES OF AUTOFLUORESCENCE

Autofluorescent Source	Typical Emission Wavelength (nm)	Typical Excitation Wavelength (nm)
Flavins	520 to 560	380 to 490
NADH and NADPH	440 to 470	360 to 390
Lipofuscins	430 to 670	360 to 490
Advanced glycation end-products (AGEs)	385 to 450	320 to 370
Elastin and collagen	470 to 520	440 to 480
Lignin	530	488
Chlorophyll	685 (740)	488

From Biophotonics International

Photobleaching

- *Photochemical lifetime:* fluorescein will undergo 30-40,000 emissions before bleaching. $(QY_{bleaching} \sim 3*10^{-5})$

- At low excitation intensities, photobleaching occurs but at lower rate.

- Bleaching is often photodynamic--involves light and oxygen.

Parameters for Maximizing Sensitivity

- Use High Objective NA and Lowest Magnification:

  $I_{fl} \sim I_{il} NA_{obj}^4 / M_{tot}^2$

  -Buy the newest objective: select for best efficiency
- Close Field Diaphragm down as far as possible
- Use high efficiency filters
- Use as few optical components as possible
- Match magnification to camera resolution:

  M_{Max} = 3*Pixel Size of Detector/Optical Resolution

  E.g.: 3*7 μm/[0.6 *520nm/1.4] = 91X
- Reduce Photobleaching
- Use High Quantum Efficiency Detector in Camera

Adapted from E.D.Salmon

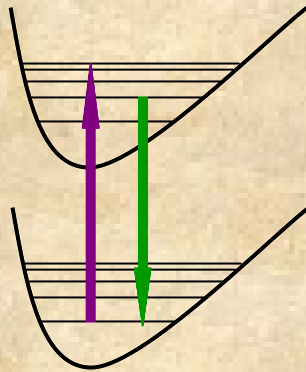
Live Cell Considerations

- Minimize photobleaching and photodamage (shutters)
- Use heat reflection filters for live cell imaging
- Image quality: Maximize sensitivity and signal to noise (high transmission efficiency optics and high quantum efficiency detector)
- Phase Contrast is Convenient to Use with Epi-Fluorescence
  - Use shutters to switch between fluorescence and phase
  - Phase ring absorbs ~ 15% of emission and slightly reduces resolution by enlarging the PSF

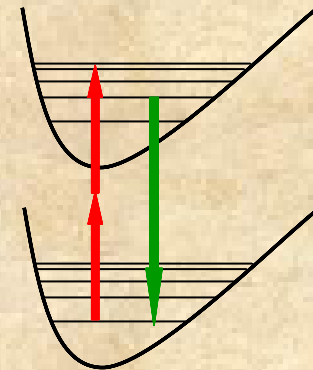
Adapted from E.D. Salmon

Defining Our Observation Volume: One- & Two-Photon Excitation.

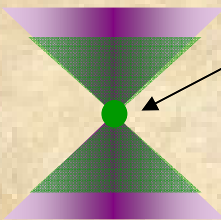
1 - Photon

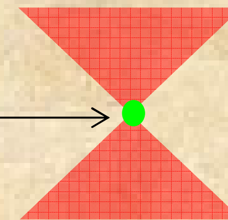


2 - Photon



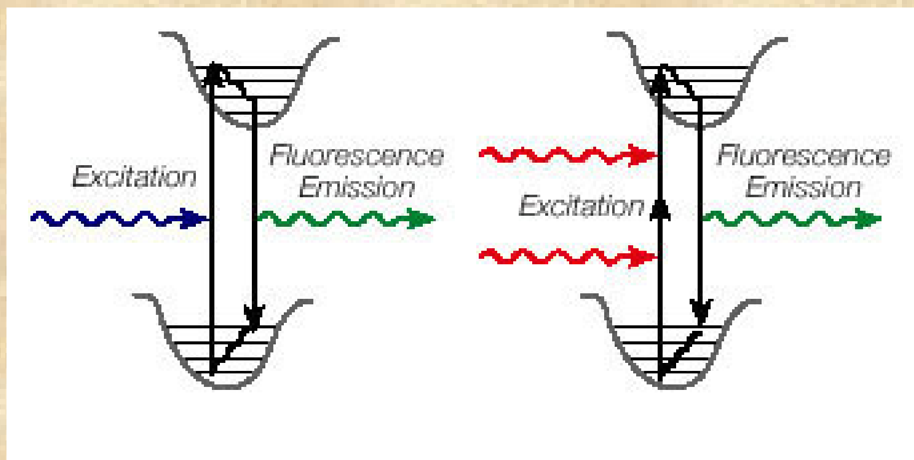
Defined by the pinhole size, wavelength, magnification and numerical aperture of the objective





Approximately 1 um^3

Defined by the wavelength and numerical aperture of the objective



Brad Amos
MRC, Cambridge, UK

Advantages of two-photon excitation

3-D sectioning effect
Absence of photo bleaching in out of focus regions
Large separation of excitation and emission
No Raman from the solvent
Deep penetration in tissues
Single wavelength of excitation for many dyes
High polarization

Why confocal detection?

Molecules are small, why to observe a large volume?

- Enhance signal to background ratio
- Define a well-defined and reproducible volume

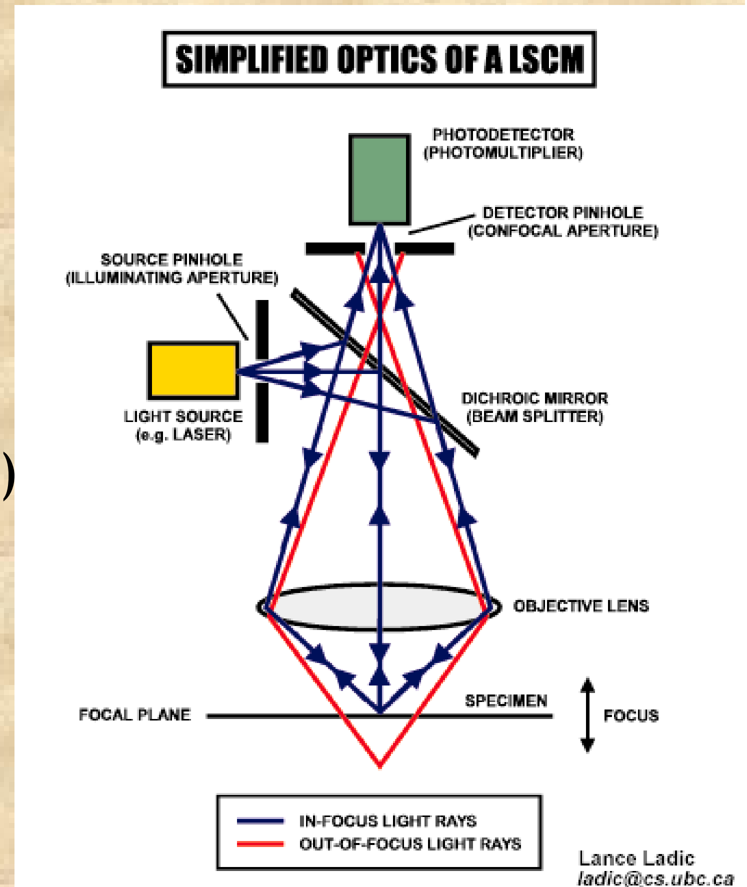
Methods to produce a confocal or small volume

(limited by the wavelength of light to about 0.1 fL)

- Confocal pinhole
- Multiphoton effects
  - 2-photon excitation (TPE)
  - Second-harmonic generation (SGH)
  - Stimulated emission
  - Four-way mixing (CARS)

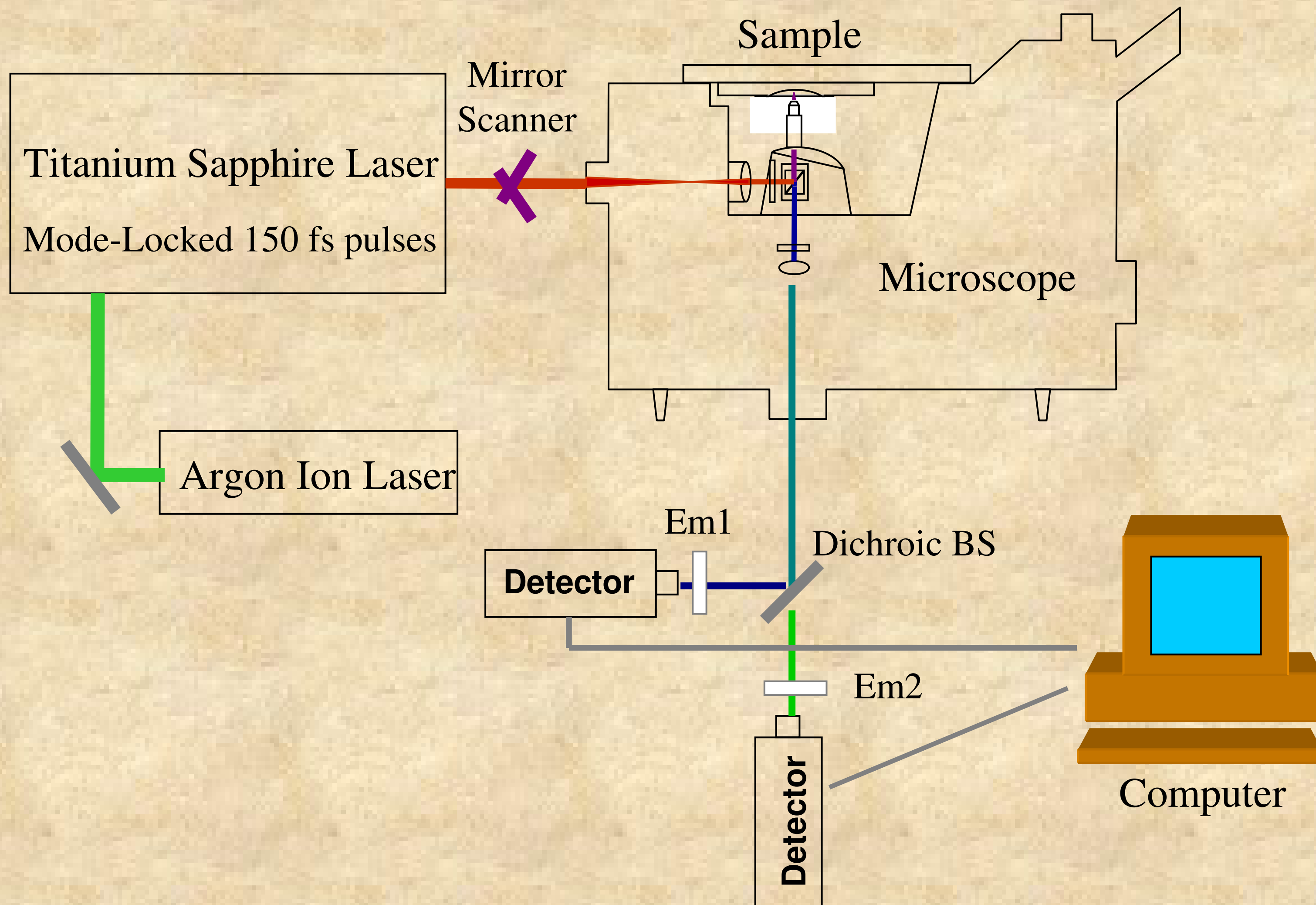
(not limited by light, not applicable to cells)

- Nanofabrication
- Local field enhancement
- Near-field effects



How does one create an observation volume and collect the data?

Two-Photon, Scanning, FCS Microscope



Laser technology needed for two-photon excitation

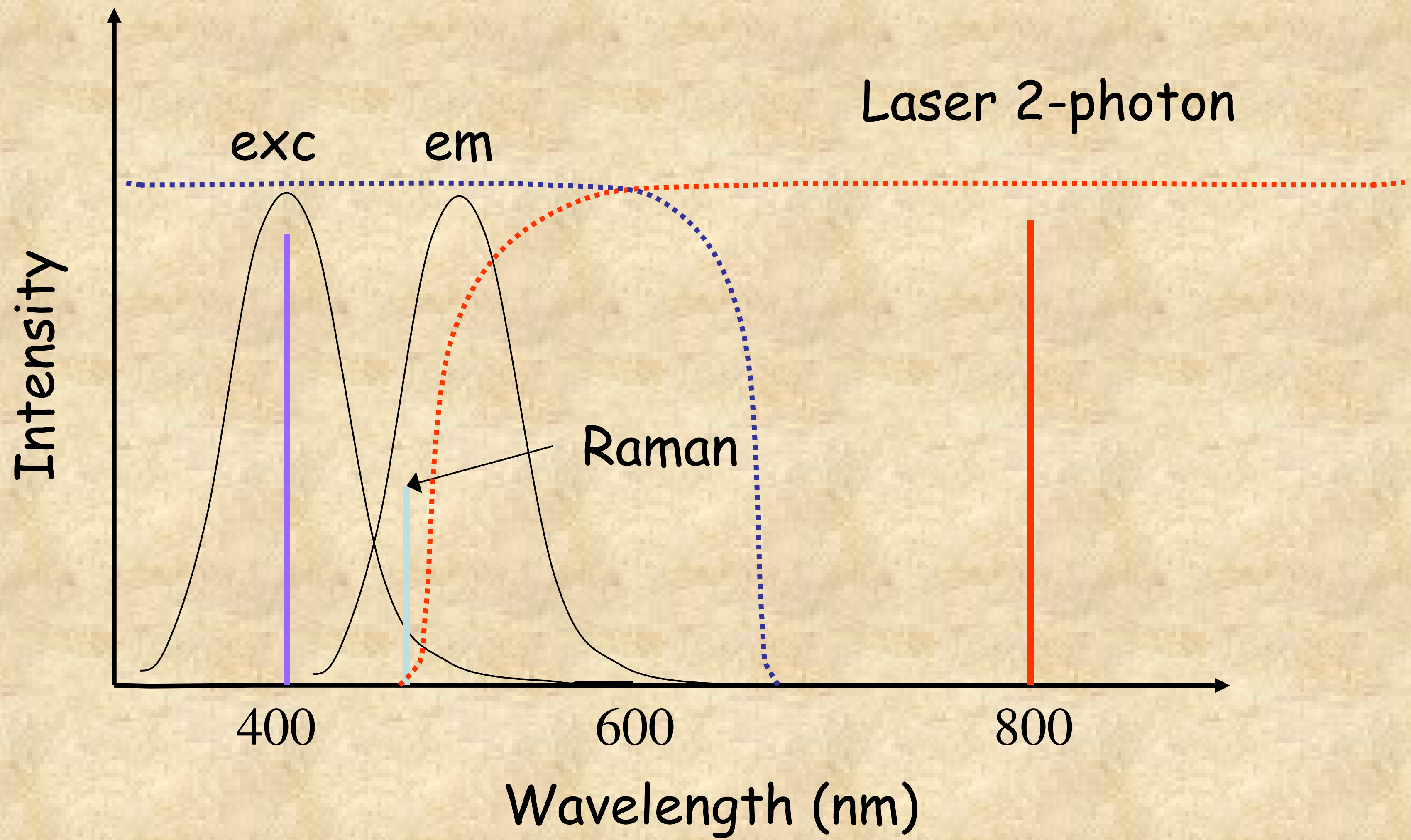
Ti:Sapphire lasers have pulse duration of about 100 fs
Average power is about 1 W at 80 MHz repetition rate
About 12.5 nJ per pulse (about 125 kW peak-power)
Two-photon cross sections are typically about

$\delta = 10^{-50}$ cm^4 sec photon^{-1} molecule-1

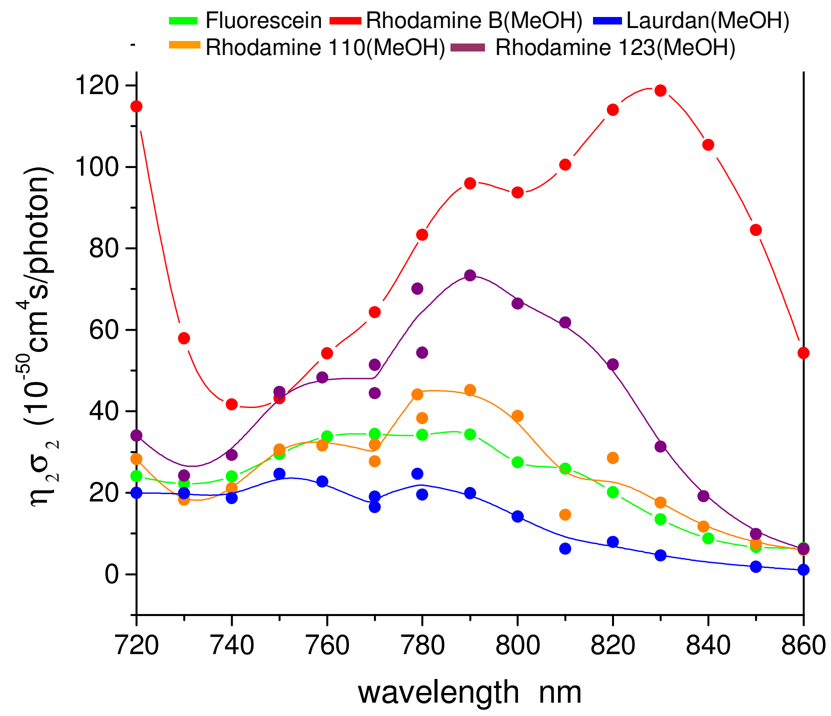
Enough power to saturate absorption in a diffraction limited spot

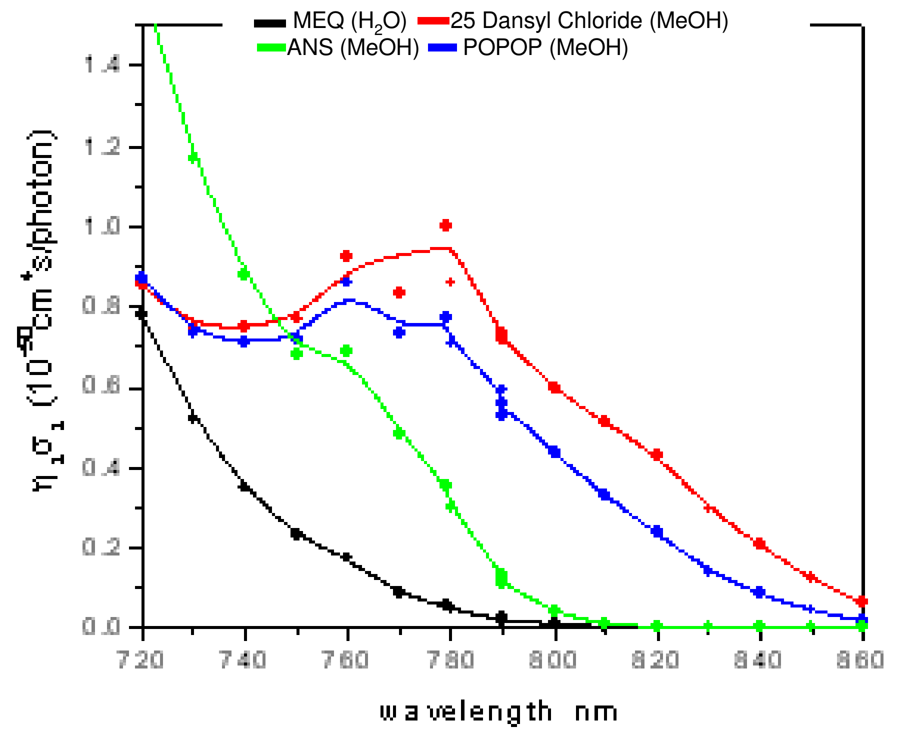
$$n_a \approx \frac{d}{\tau} \left(\frac{p\pi A^2}{fhc\lambda} \right)^2$$

- n_a Photon pairs absorbed per laser pulse
- p Average power
- τ pulse duration
- f laser repetition frequency
- A Numerical aperture
- λ Laser wavelength
- d cross-section



Laser 2-photon
exc
em
Intensity
Raman
400
600
800
Wavelength (nm)


Fluorescein
Rhodamine B(MeOH)
Laurdan(MeOH)
Rhodamine 110(MeOH)
Rhodamine 123(MeOH)
$\eta_2\sigma_2$ (10^{-50}cm^{4}s/photon)
wavelength nm


MEQ (H_2O)
25 Dansyl Chloride (MeOH)
ANS (MeOH)
POPOP (MeOH)
$\eta_1\sigma_1$
wavelength nm

Optical resolution and super-resolution

The Abbe (Ernest Abbe) principle establishes that the maximum resolution that can be achieved using light of wavelength l is given by

$$\Delta r = \frac{\lambda}{2n\sin(\alpha)}$$

For λ=500nm, n=1.5 and α=45degrees Δr=236nm

N sinα is called the Numerical Aperture. NA can be as large as 1.50, so that a resolution of λ/3 can be achieved

We call super-resolution any method that goes beyond the Abbe's limit

Approaches to super-resolution

•Mathematical methods

•Physical methods

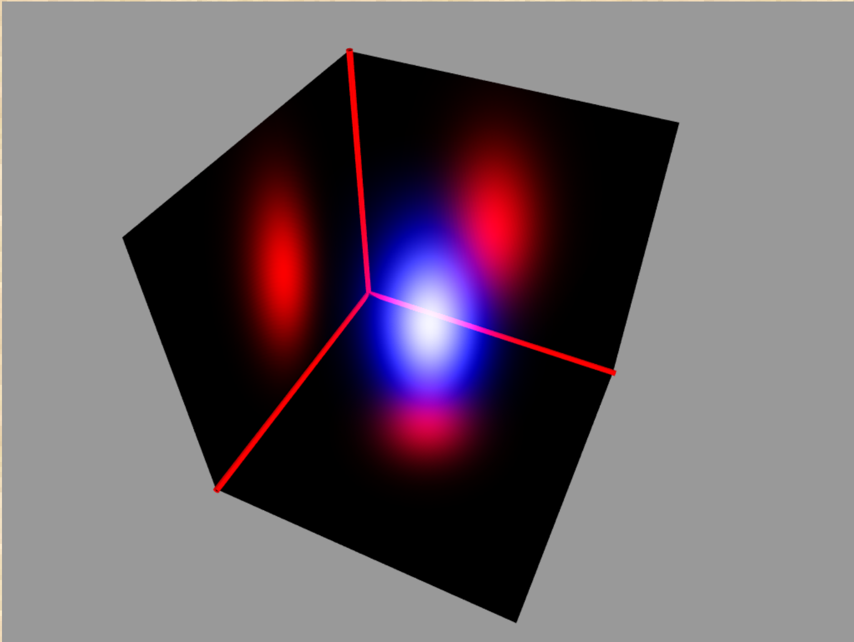
Non-linear optics

Center-of-mass determination

Optical principle: the image is the convolution of the optical point-spread function (PSF) with the real image

The PSF is the image of an ideal point. In the microscope, a very small point gives an image which is determined by the diffraction of light

Examples of PSF



$$F(x, y, z) = I_0 I(z) e^{-\frac{2(x^2+y^2)}{w_0^{\ 2}}}$$

$$I(z) = Exp\left[-\frac{2z^2}{w_{0z}^{\ 2}}\right]$$ Gaussian z

$$I(z) = \frac{1}{1+(\frac{z}{w_{oz}})^2}$$ Lorentzian z

For the 2-photon case, these expression must be squared

Mathematical deconvolution

Principle: if we know the PSF, we can "deconvolve" the effect of the PSF in the generation of the image.

There are various mathematical approaches to this process. The net result is an image that shows very high resolu … thods provide resolution … ending on the … ge

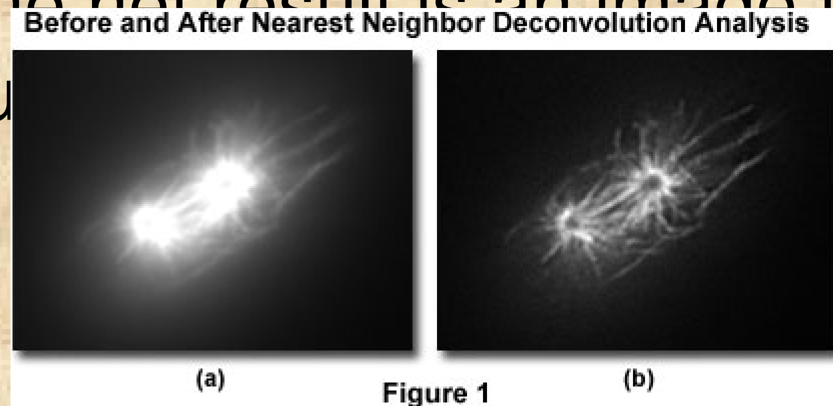


Figure 1

Example of 2-D next neighbor deblurring algorithm

2-D and 3_D reconstruction algorithms

3-D reconstruction algorithms are designed to re assign a intensity to the proper pixel. This is a true 3-D process. The mathematics is quite complex.

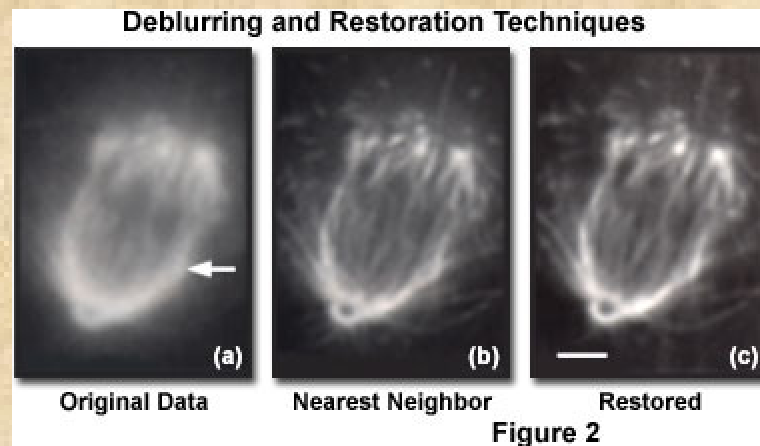
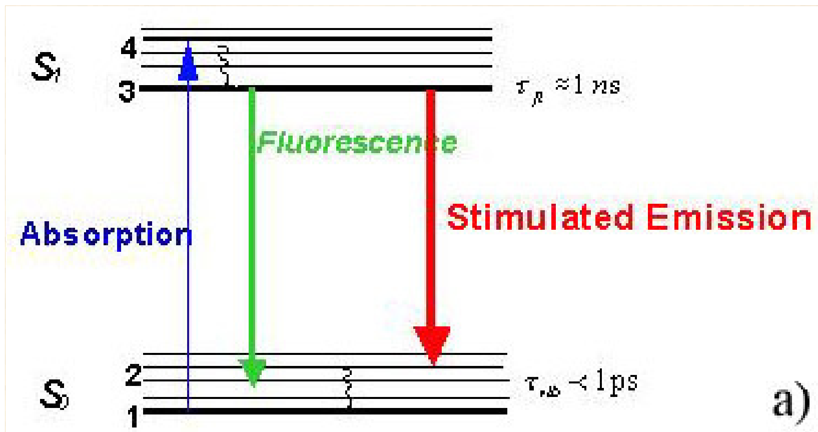


Figure 2

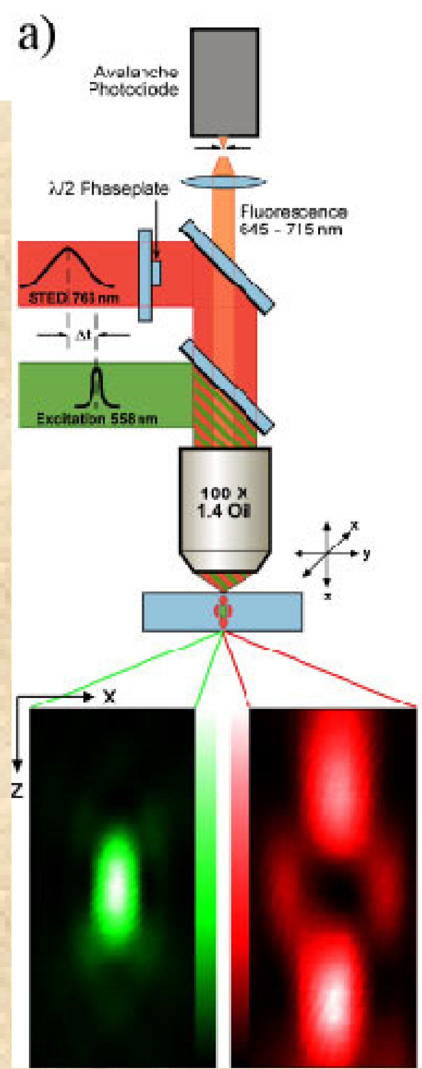
Methods based on physical principles

Decreasing the size of the PSF

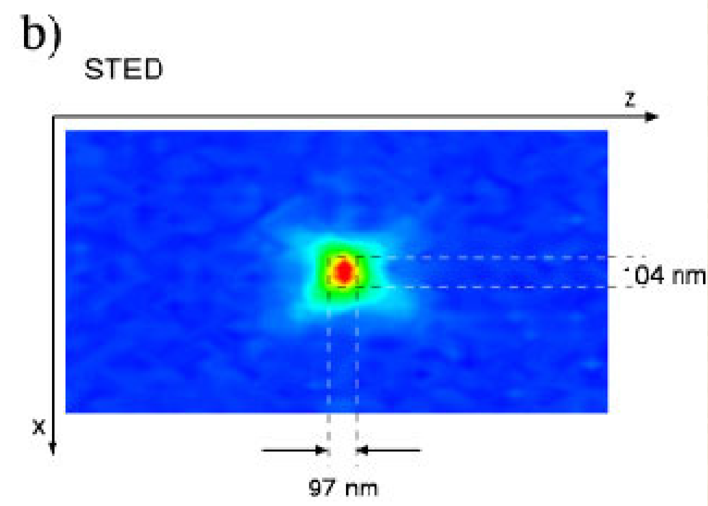
The perhaps most straightforward way to sharpen the fluorescence focal spot is to selectively inhibit the fluorescence at its outer part [1, 2]. If this is applied to an otherwise diffraction-limited spot, one would expect that the diffraction barrier can be overcome since scanning with a smaller fluorescent spot signifies increased spatial resolution. A phenomenon that stops fluorescence (=spontaneous emission) is that of stimulated emission. This is one of the key ingredients of the Stimulated Emission Depletion (STED-) microscope. However, STED by itself could not really break the diffraction barrier since the beams with which STED is accomplished are diffraction-limited as well. Therefore the real physical ingredient for breaking the diffraction barrier is the saturation of the fluorescence inhibition by stimulated emission, as we will argue below.



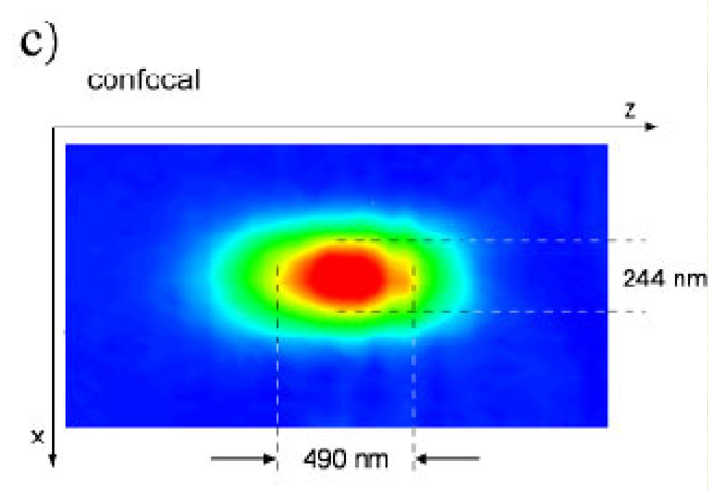
S1
4
3
τfl ≈1 ns
Fluorescence
Stimulated Emission
Absorption
2
S0
1
τvib < 1ps



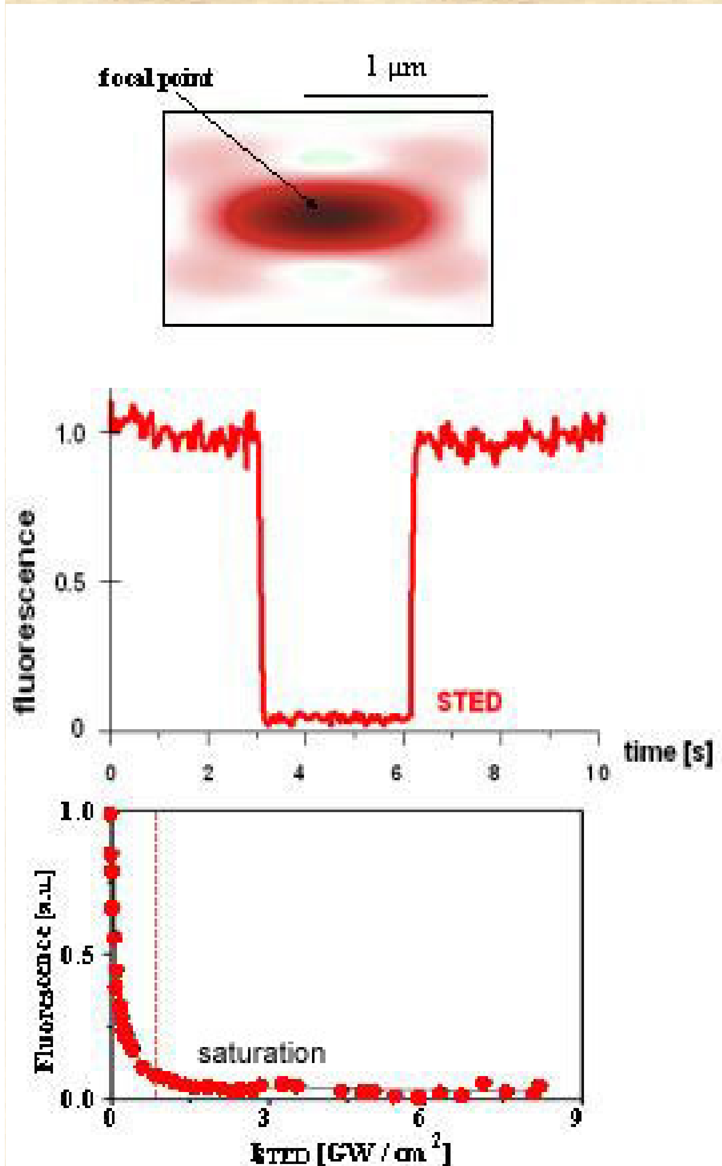
a)
Avalanche Photodiode
λ/2 Phaseplate
Fluorescence 645 - 715 nm
STED 765 nm
Δt
Excitation 558 nm
100 X 1.4 Oil
x
y
z
x
z



b)
STED
z
x
104 nm
97 nm



c)
confocal
z
x
244 nm
490 nm

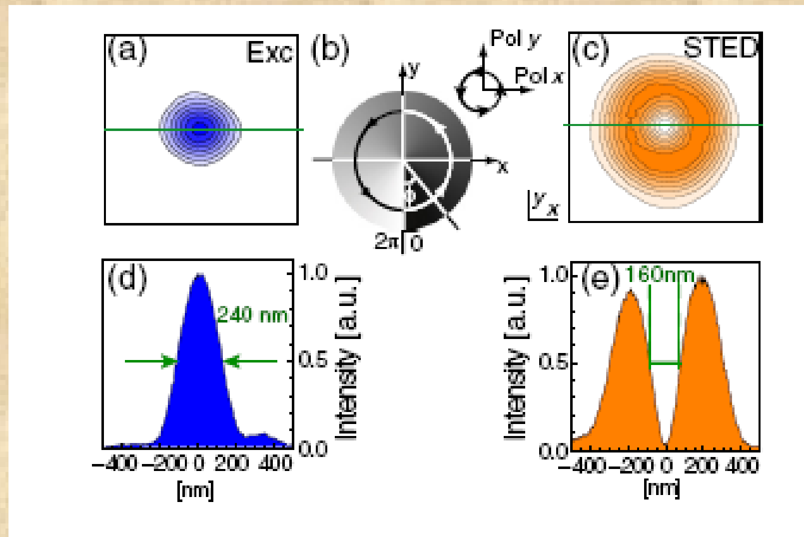


No resolution limit: By increasing the STED pulse intensity, the depletion becomes complete at the spot's periphery and increasingly more effective towards the middle. At the doughnut hole, however, the fluorescence is ideally not affected at all. Therefore, by increasing the intensity of the doughnut-shaped STED-pulse, the fluorescent spot can be progressively narrowed down, in theory, even to the size of a molecule. This concept signifies a fundamental breaking of the diffraction barrier. The essential ingredient is the saturated reduction of the fluorescence (= depletion) at any coordinate but the focal point.

$$\Delta r \cong \frac{\lambda}{2n \sin\alpha\sqrt{1+\zeta}},$$

ξ is the saturation factor of the depletion

The donut shape is obtained with a variable phase lens



Example of STED images

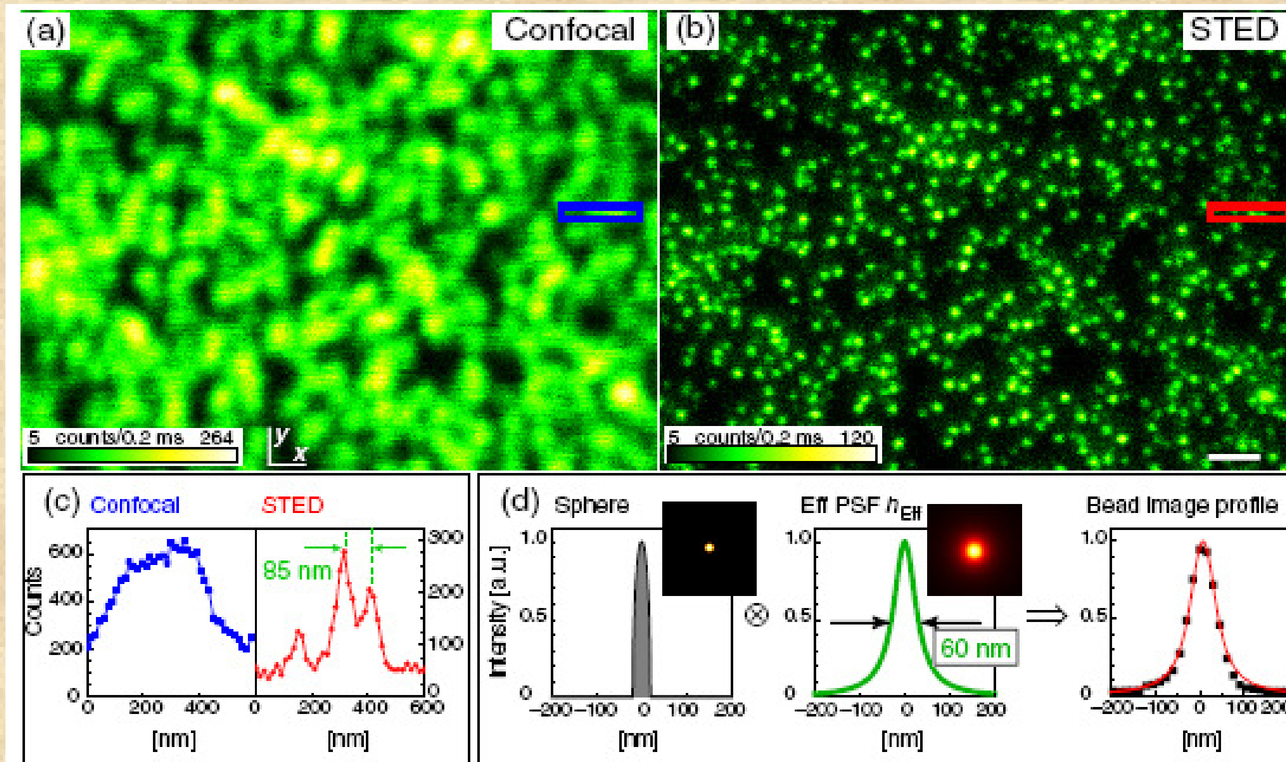


Figure 2. Subdiffraction resolution in the far-field. (a) Confocal versus (b) corresponding STED image of dispersed 40 nm beads. Scale bar = 500 nm (c) shows intensity profiles along the lines indicated in (a, b) (sum of 5 lines). Panel

Comparison of deconvolution with STED

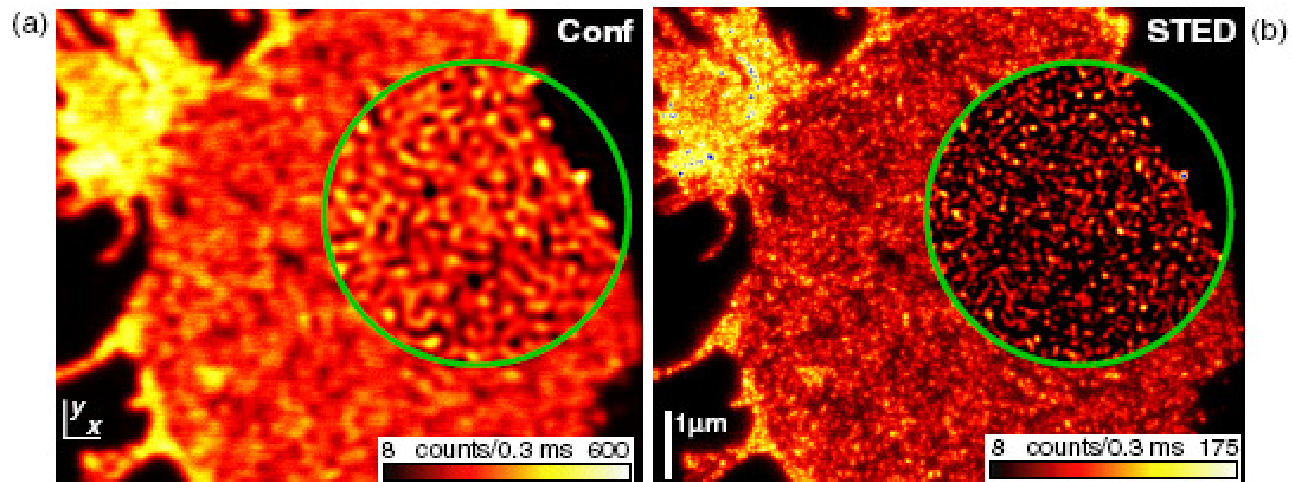


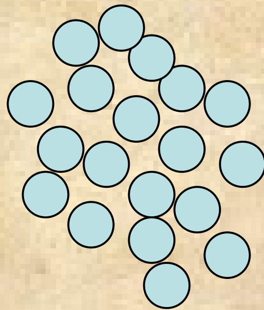
Figure 4. Revealing the nanopattern of the SNARE protein SNAP-25 on the plasma membrane of a mammalian cell. Confocal (a) versus STED image (b) of the antibody-tagged proteins. The encircled areas show linearly deconvolved data. STED microscopy provides a substantial leap forward in the imaging of protein self-assembly; here it reveals for the first time that SNAP-25 is ordered in clusters of <60 nm average size. (See also movie 2.)

The center of mass approach

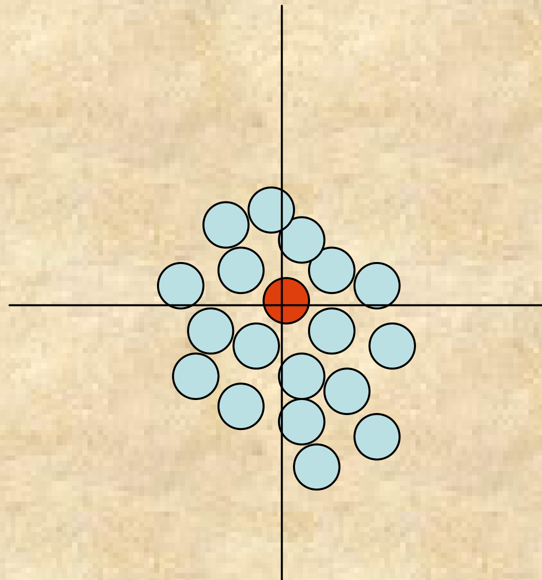
Principle: The center of mass of the emission of a particle can be obtained with very high precision, only dependent on the signal-to-noise ratio of the image.

If we can determine the position of one particle (molecule) at a time, we can achieve image resolution that is only determined by the S/N and not by the microscope resolution

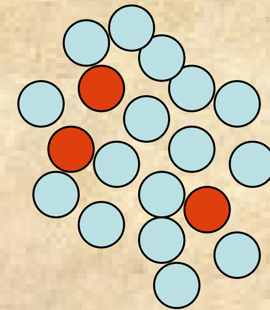
The principle of molecular imaging



Molecules in a cluster

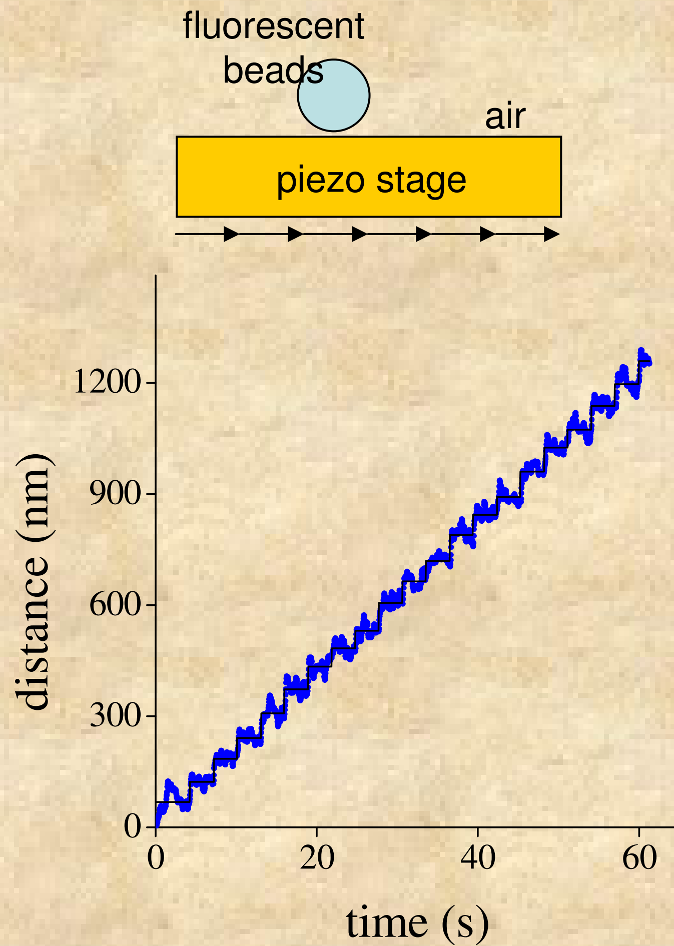


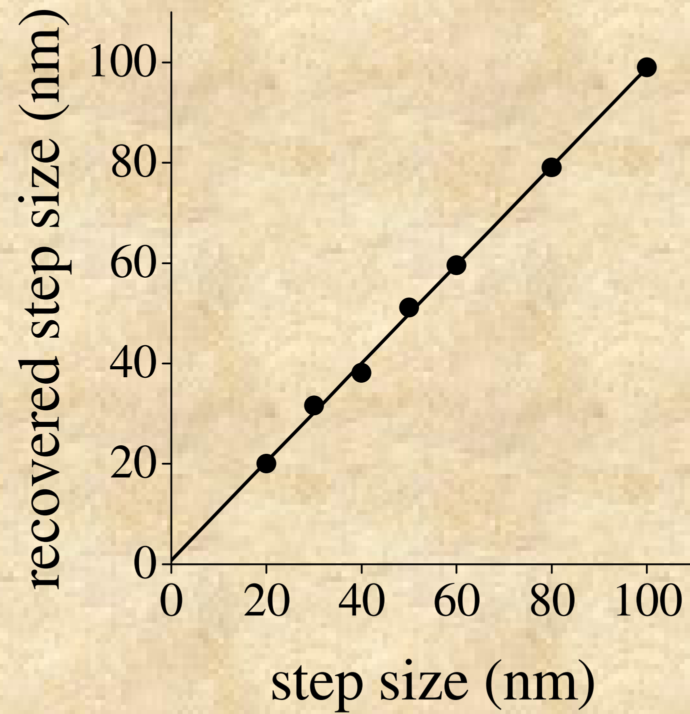
If only one of the molecules emits light, its position can be determined with great precision



If the operation could be repeated for each molecule, we could image the entire cluster

Determining the position of particles

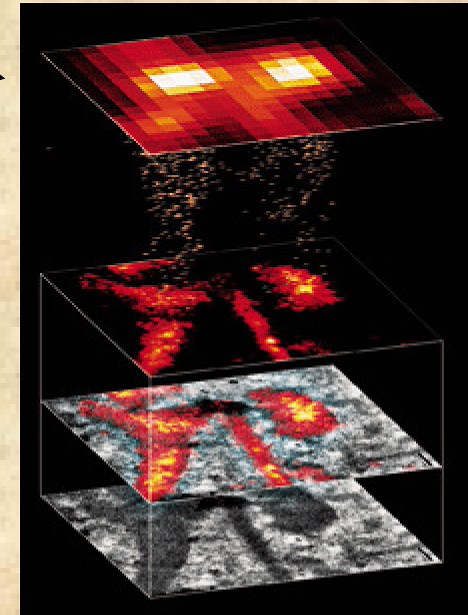




Confocal image

Determining the centroid of the emission one molecule at a time

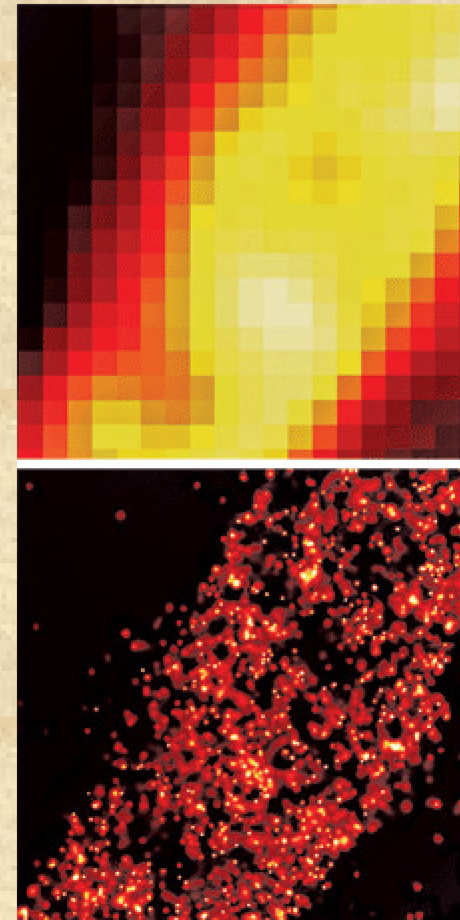
Projection of the image



The problem is how to make just one molecule to emit?

- Some molecules blink
- Molecules can be turned on and off by light

CELLULAR POINTILLISM At high resolution, conventional fluorescence microscopy picks up an amorphous distribution of signals from tagged molecules at the spot where a cell's cytoskeleton connects with an external surface (left). PALM resolves individual signals into a more revealing "star field" of signals from individual tagged molecules (right).



From Eric Betzig

General References

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On line resource:

Molecular Expressions, a Microscope Primer at:

http://www.microscopy.fsu.edu/primer/index.html