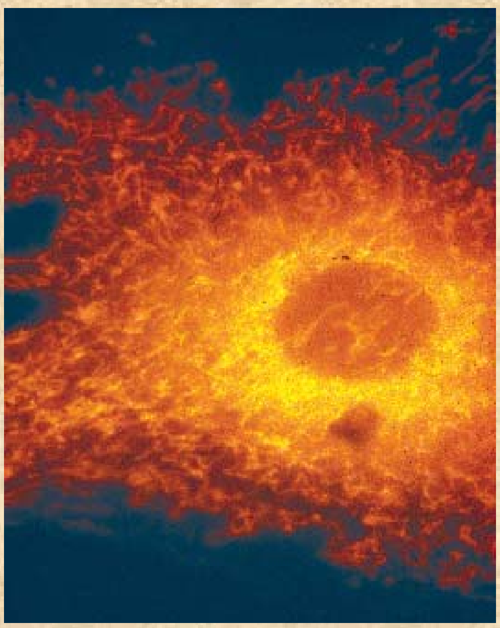
Day 3: Applications of Fluorescence Spectroscopy II

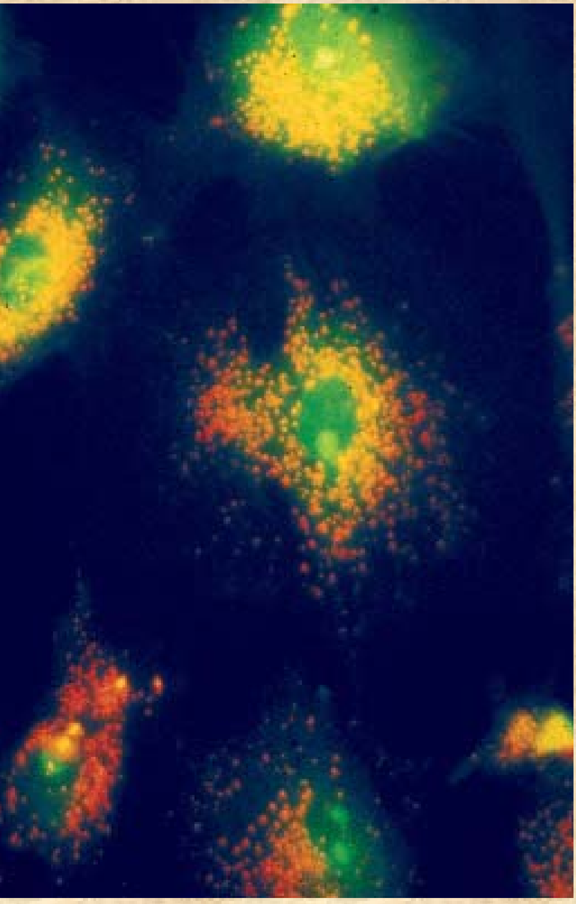
7. Confocal Fluorescence Microscopy

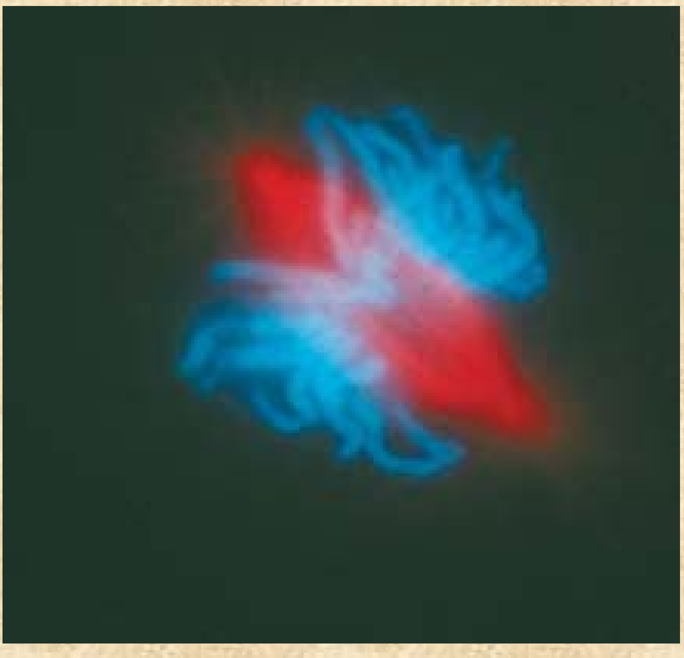
Instrumentation
Light Sources:
  One-photon and Multi-photon Excitation
Applications in Cells
Lifetime Imaging

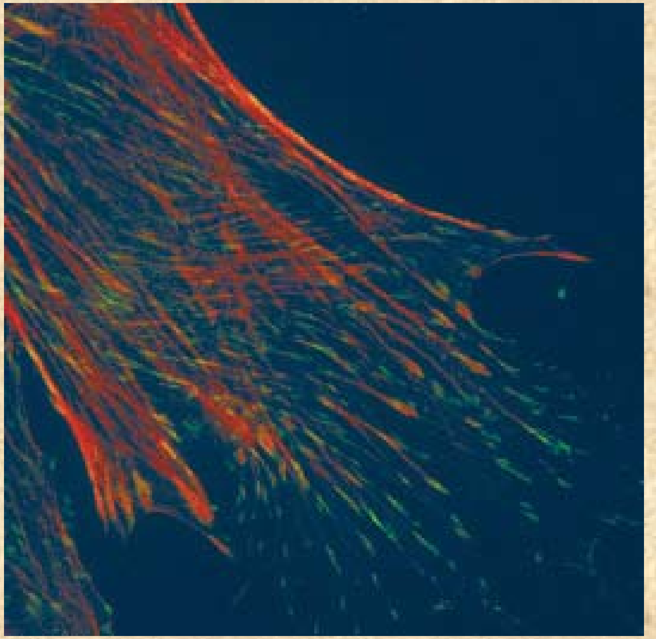
Figures acknowledgements: E.D. Salmon and K. Jacobson

Confocal microscopy images

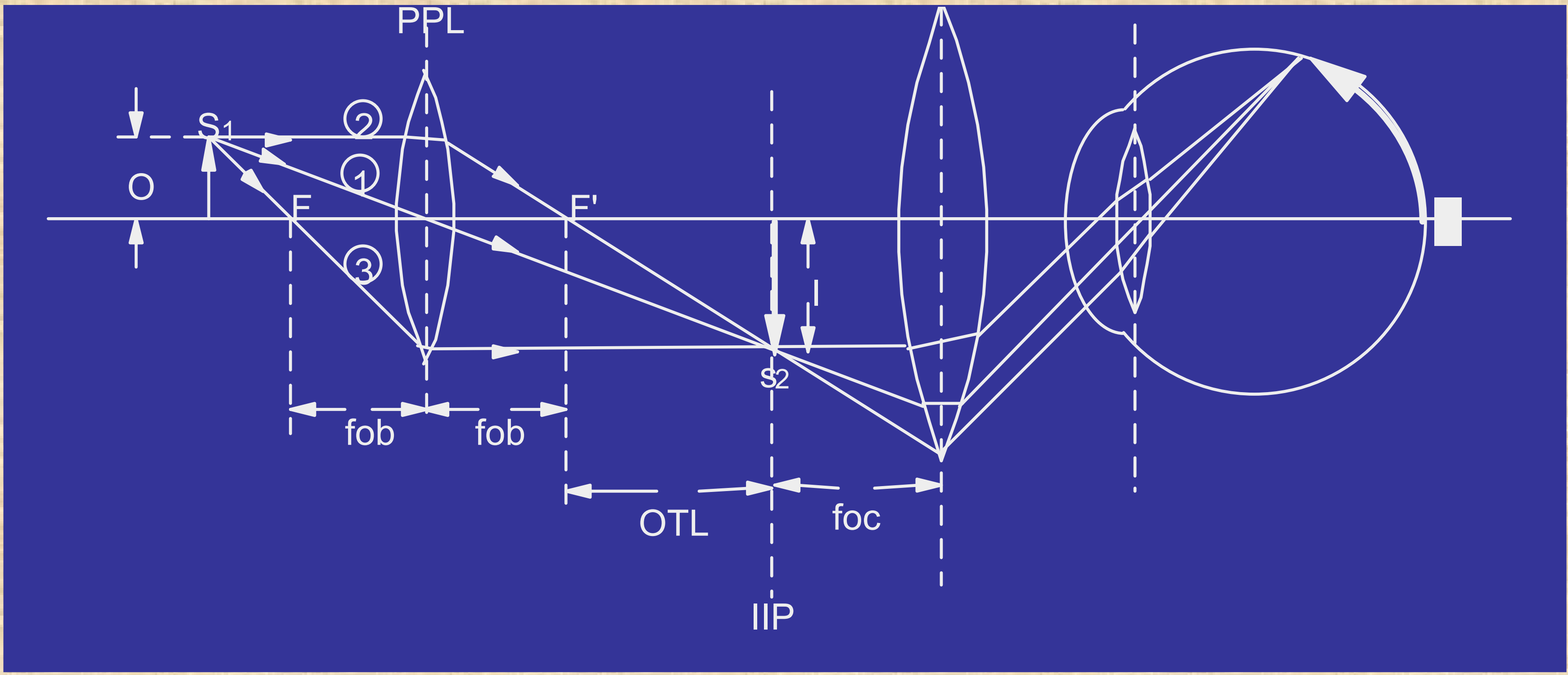








In the compound microscope the Finite Corrected Objective Forms a Real Image At the Ocular Front Focal Plane: The Primary or Intermediate Image Plane (IIP)



Conventional Optics

Objective with finite Focal Length

(Optical Tube Length, OTL, Typically 160 mm)

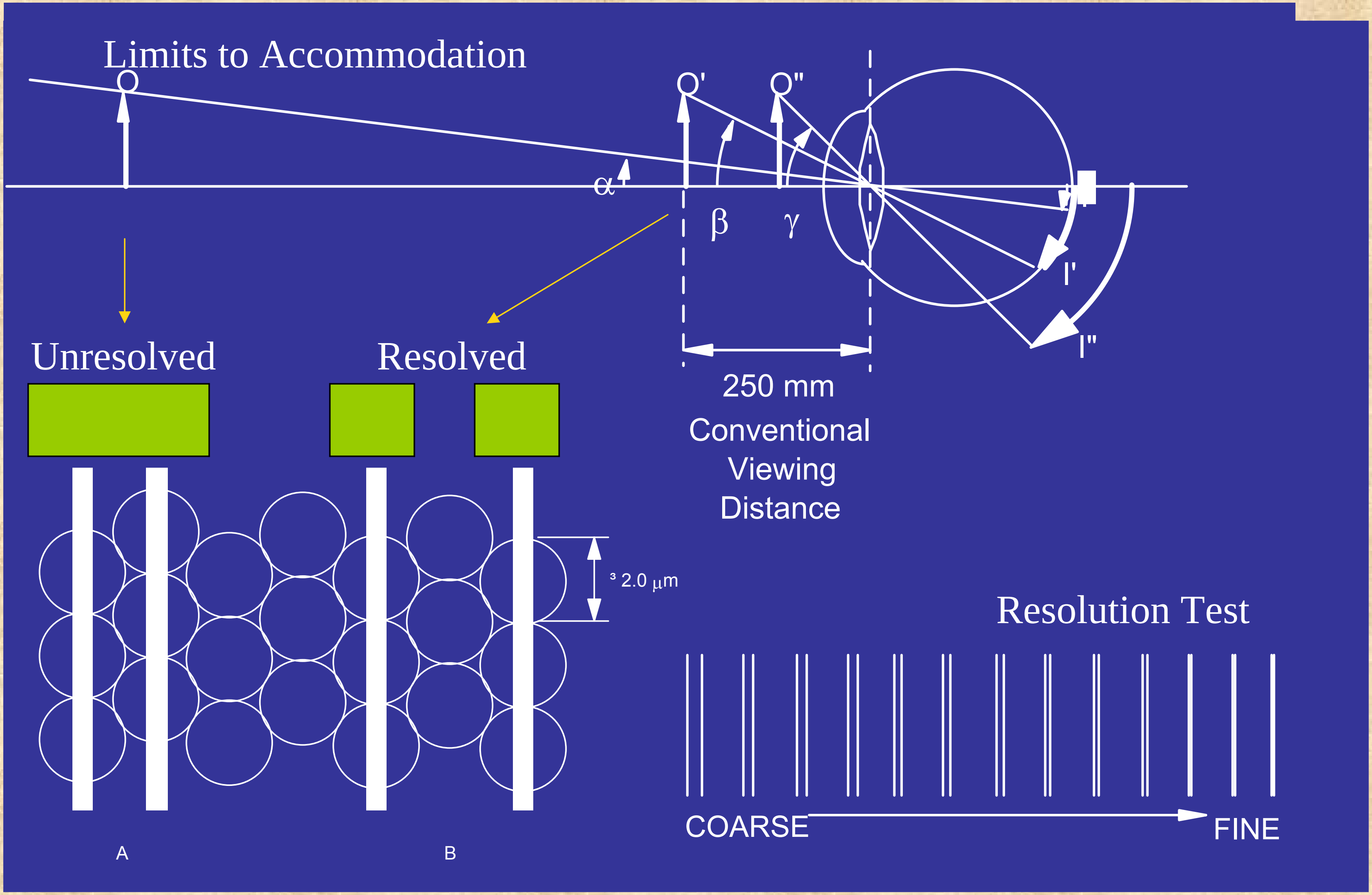
$M_{ob} = OTL/f_{ob}$

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



Limits to Accommodation

Unresolved

Resolved

250 mm
Conventional
Viewing
Distance

Resolution Test

COARSE

FINE

A word about infinity corrected optics and its advantages.

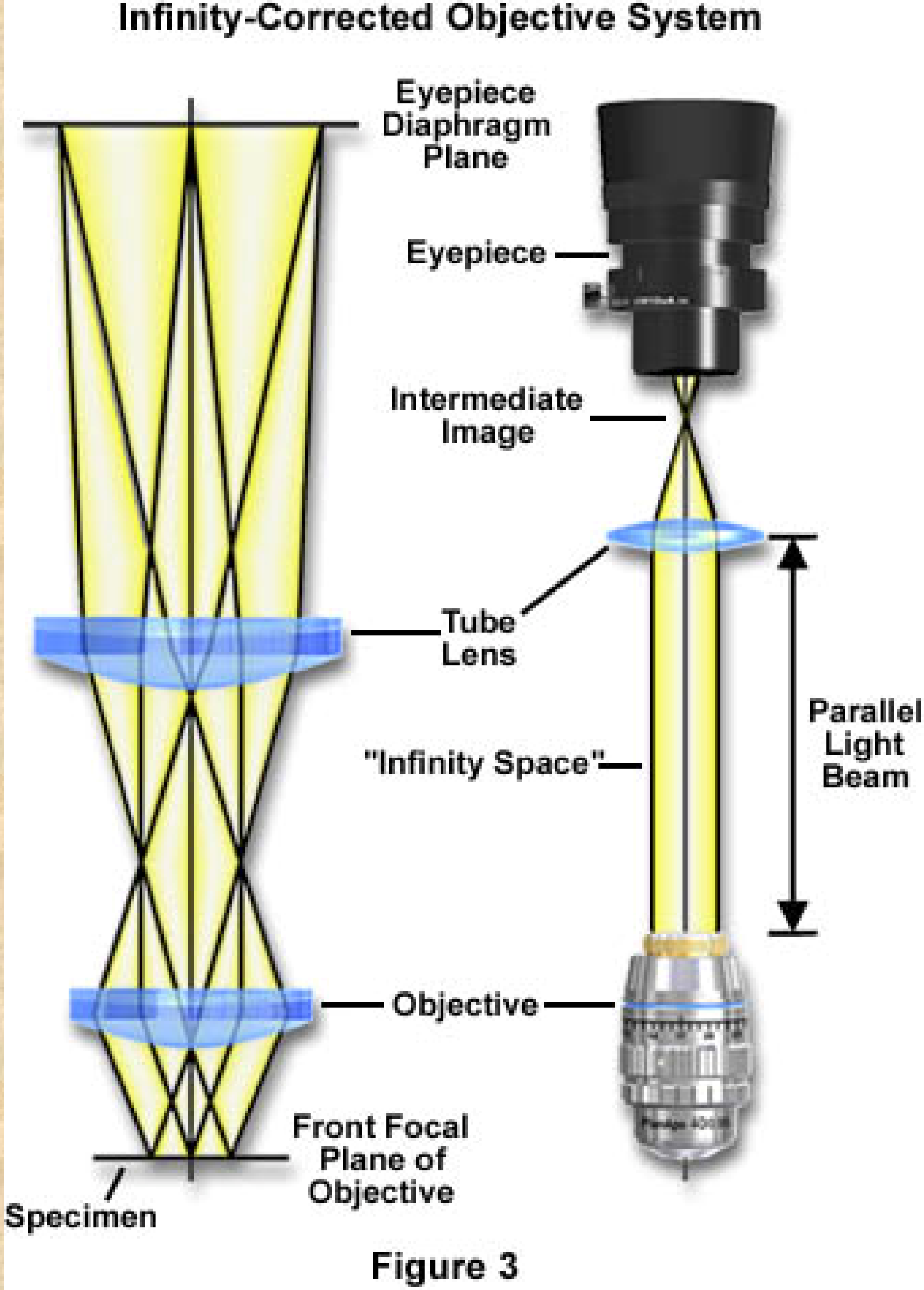
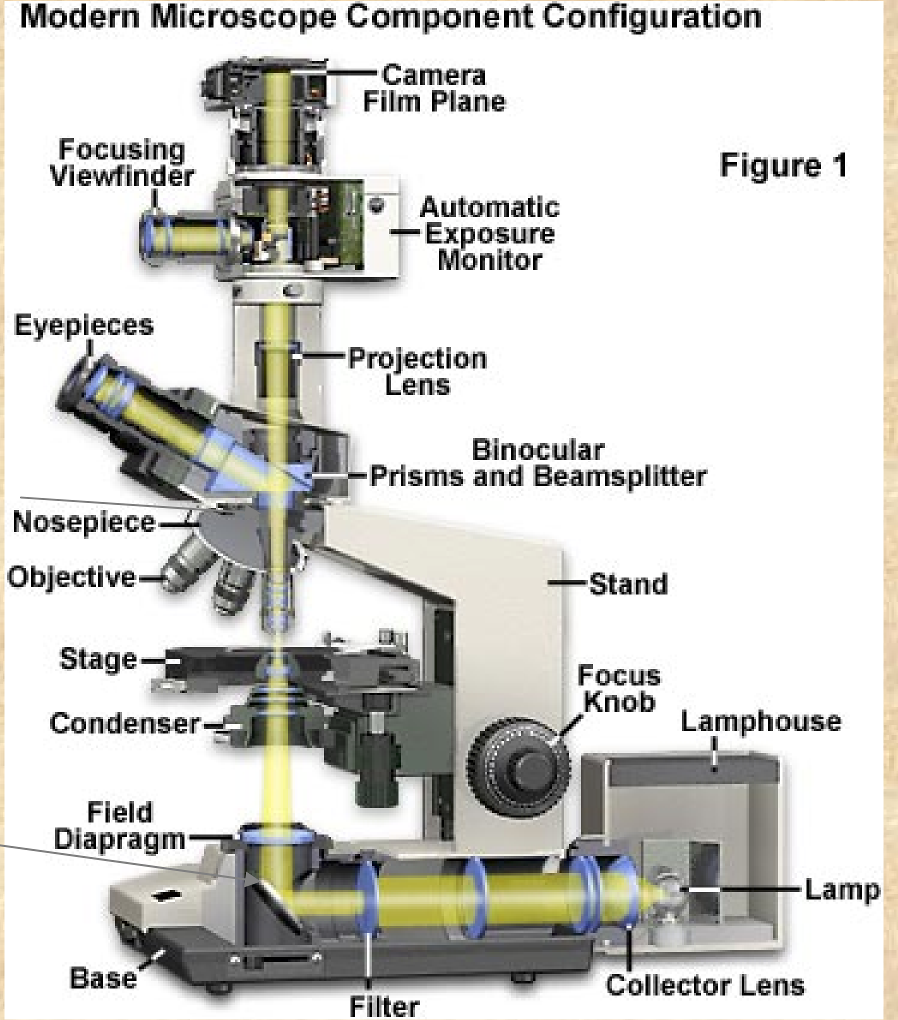


Figure 3

Modern microscope component identification

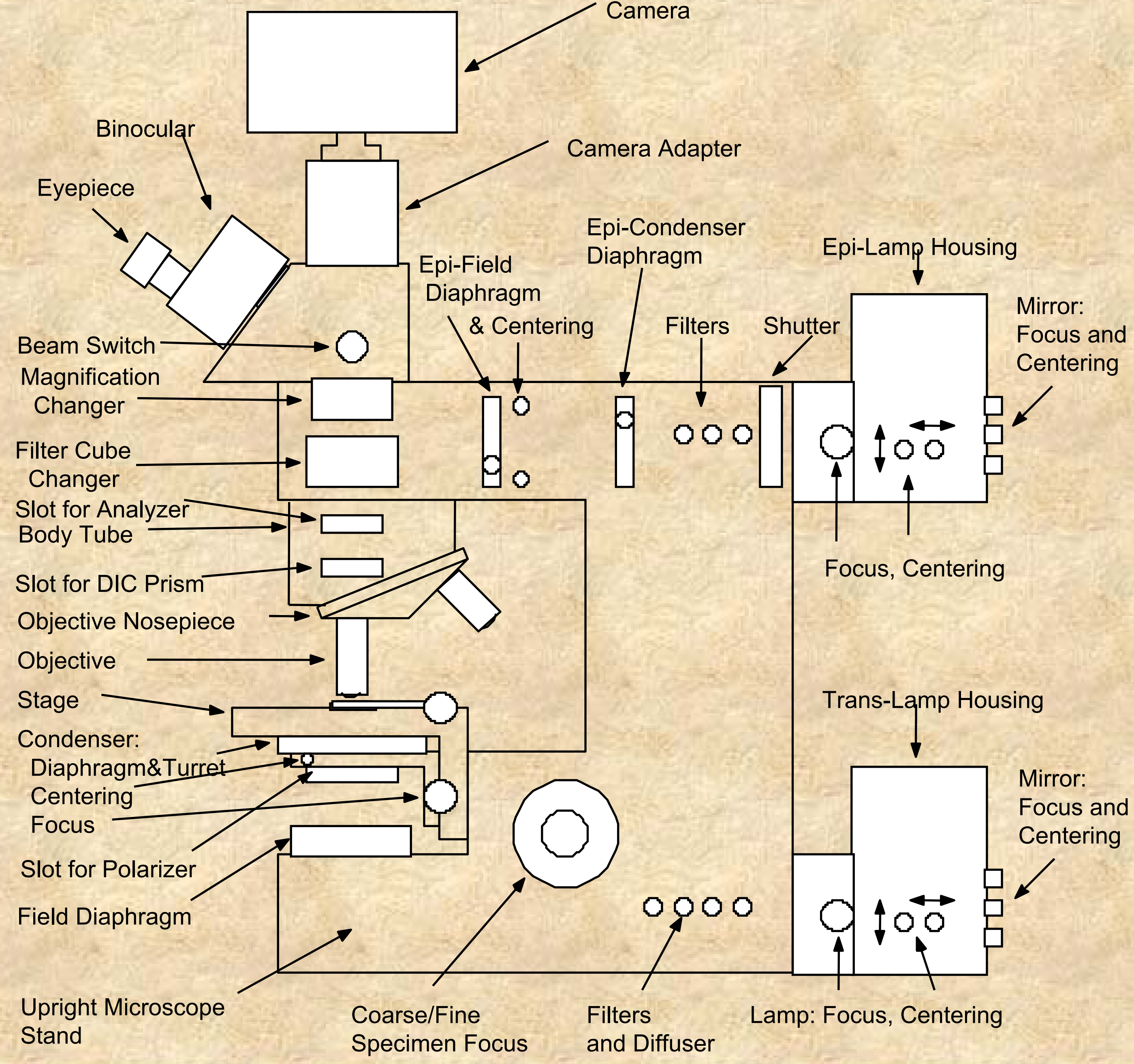


Prisms Used to Re-Direct Light In Imaging Path While Mirrors Are Used in Illumination Path

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)

MICROSCOPE COMPONENTS



Camera

Camera Adapter

Binocular

Eyepiece

Epi-Condenser Diaphragm

Epi-Lamp Housing

Epi-Field Diaphragm & Centering

Filters

Shutter

Mirror: Focus and Centering

Beam Switch

Magnification Changer

Filter Cube Changer

Slot for Analyzer

Body Tube

Slot for DIC Prism

Focus, Centering

Objective Nosepiece

Objective

Stage

Trans-Lamp Housing

Condenser:

Diaphragm&Turret

Centering

Focus

Mirror: Focus and Centering

Slot for Polarizer

Field Diaphragm

Upright Microscope Stand

Coarse/Fine Specimen Focus

Filters and Diffuser

Lamp: Focus, Centering

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

Common Objective Optical Correction Factors

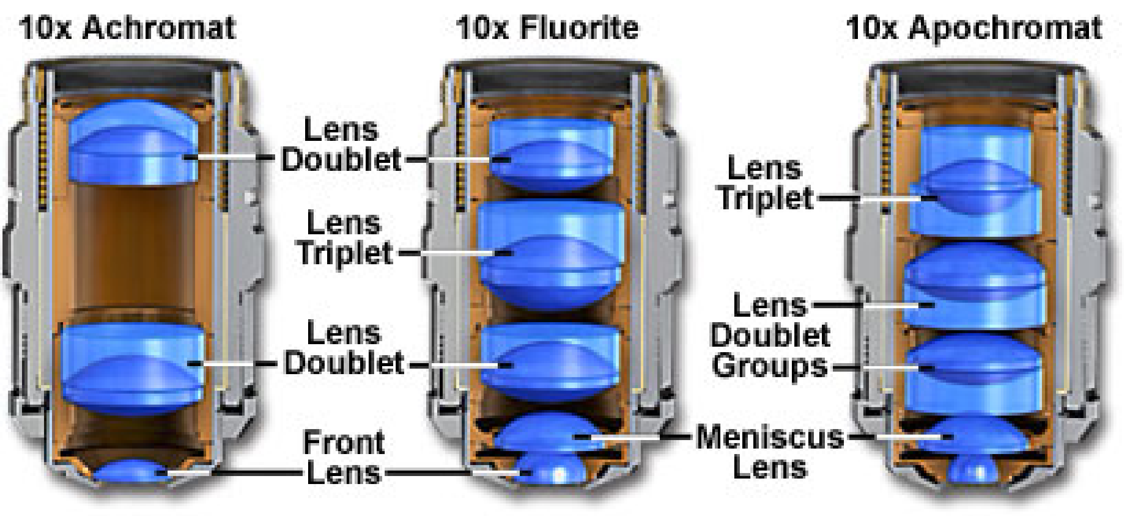


Figure 2

Chromatic and Mono-Chromatic Corrections

60x Plan Apochromat Objective

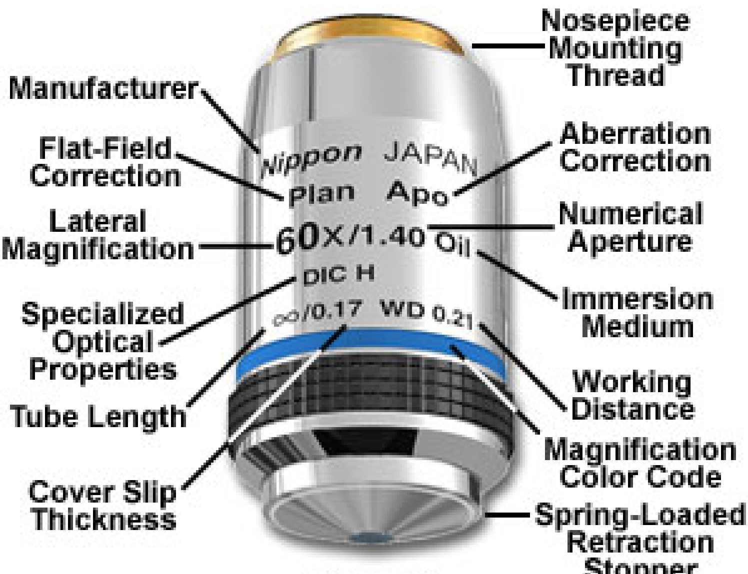
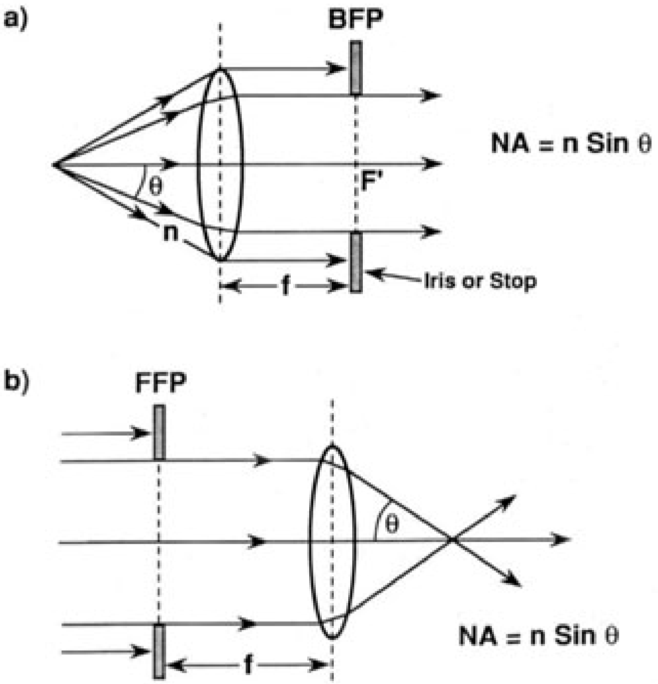


Figure 1

E.D. Salmon

What is numerical aperture (NA)?

FIG. 2.1 Numerical aperture of collection (a), or illumination (b)



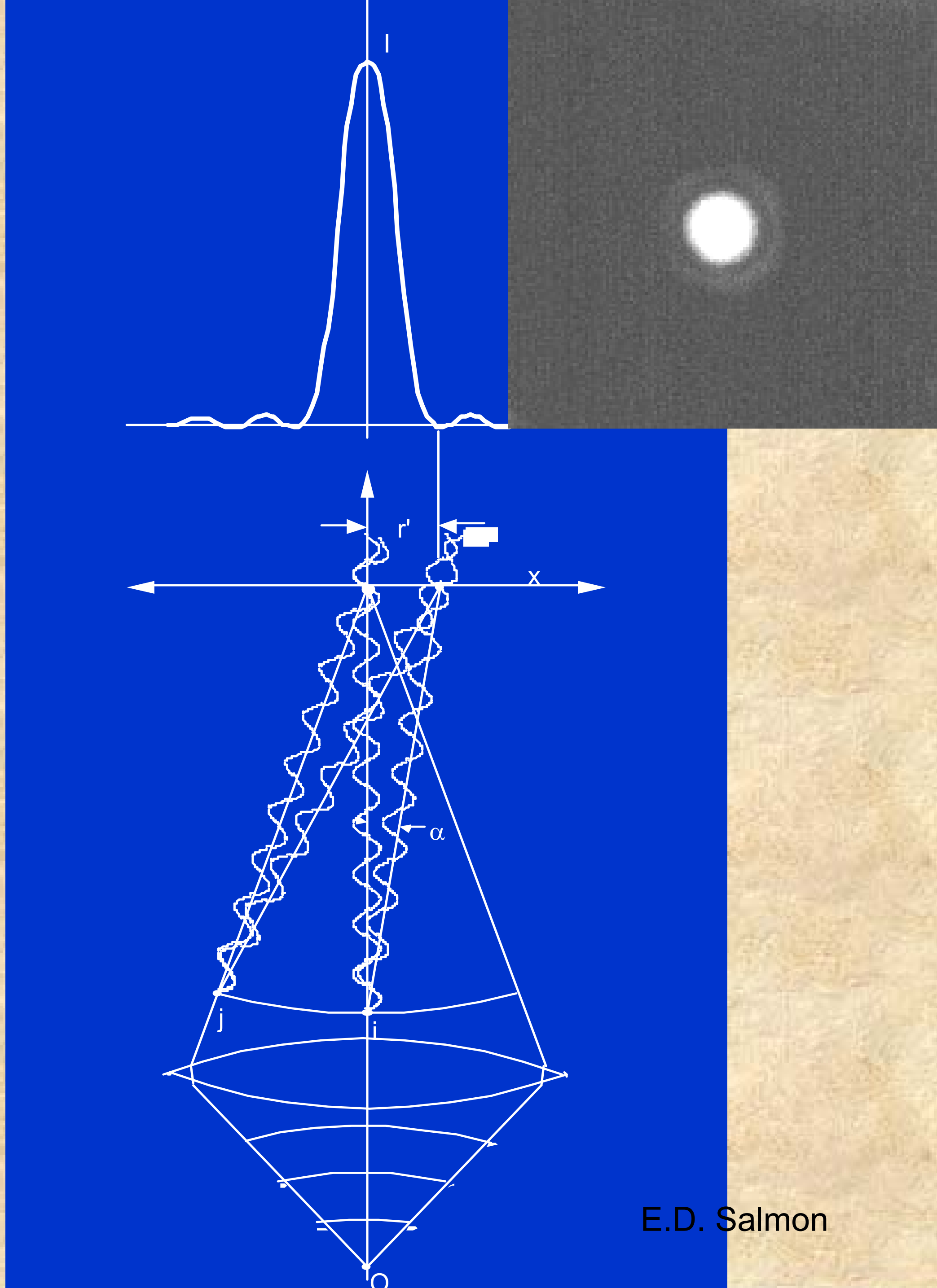
- Image Intensity: $I \sim NA_{obj}^2/M_{tot}^2$
- Image Lateral Resolution for Corrected Objective:

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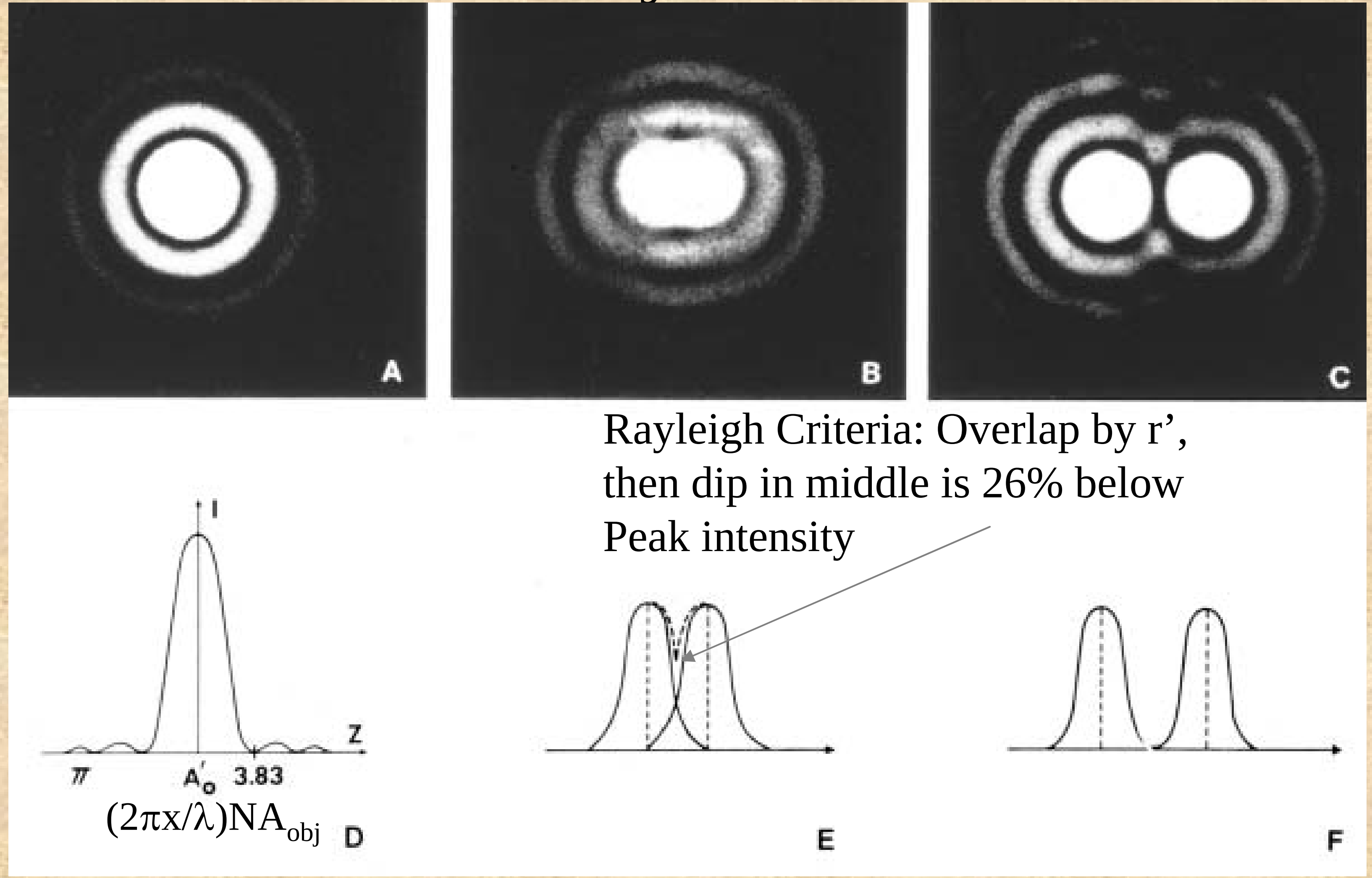
Lateral Resolution in Fluorescence Depends on Resolving Overlapping "Airy Disks"



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

$(2\pi x/\lambda)NA_{obj}$

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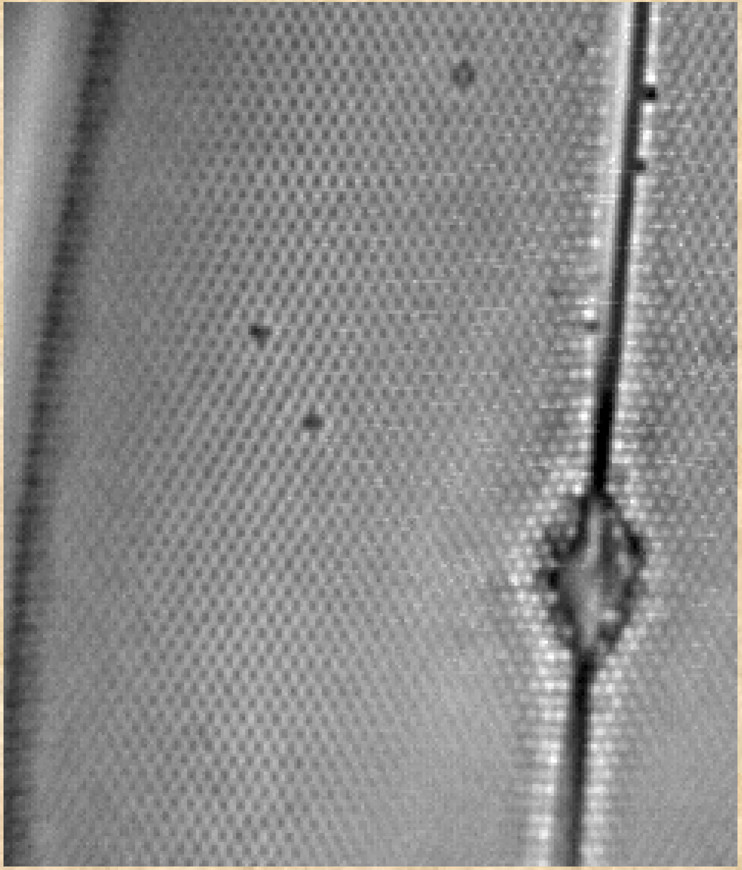
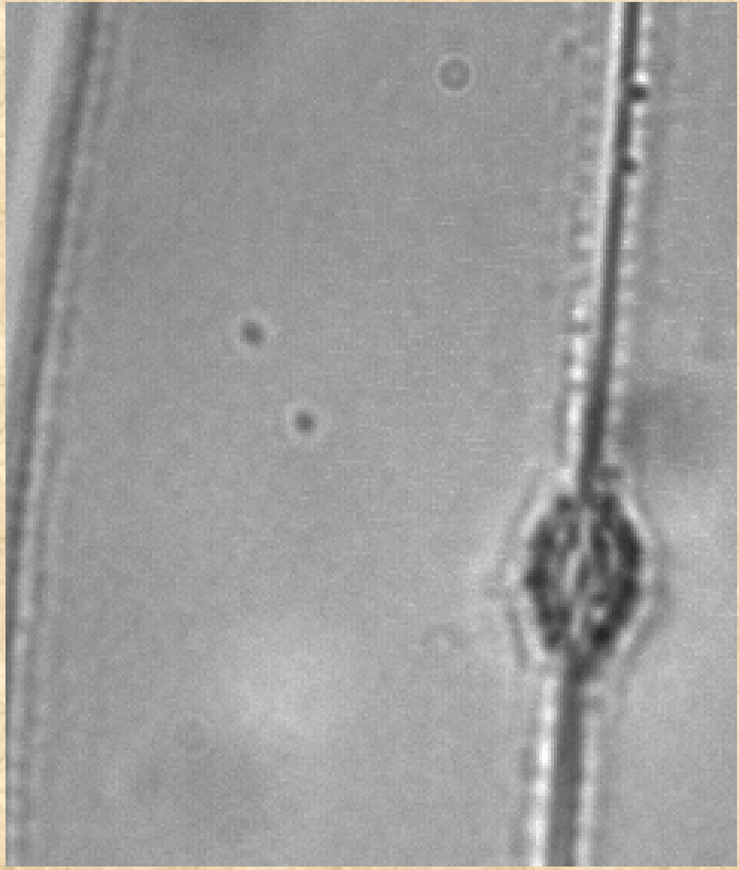
Resolution is better at shorter wavelengths, higher objective NA or higher condenser NA



High NA and/or shorter λ



Low NA and/or longer λ

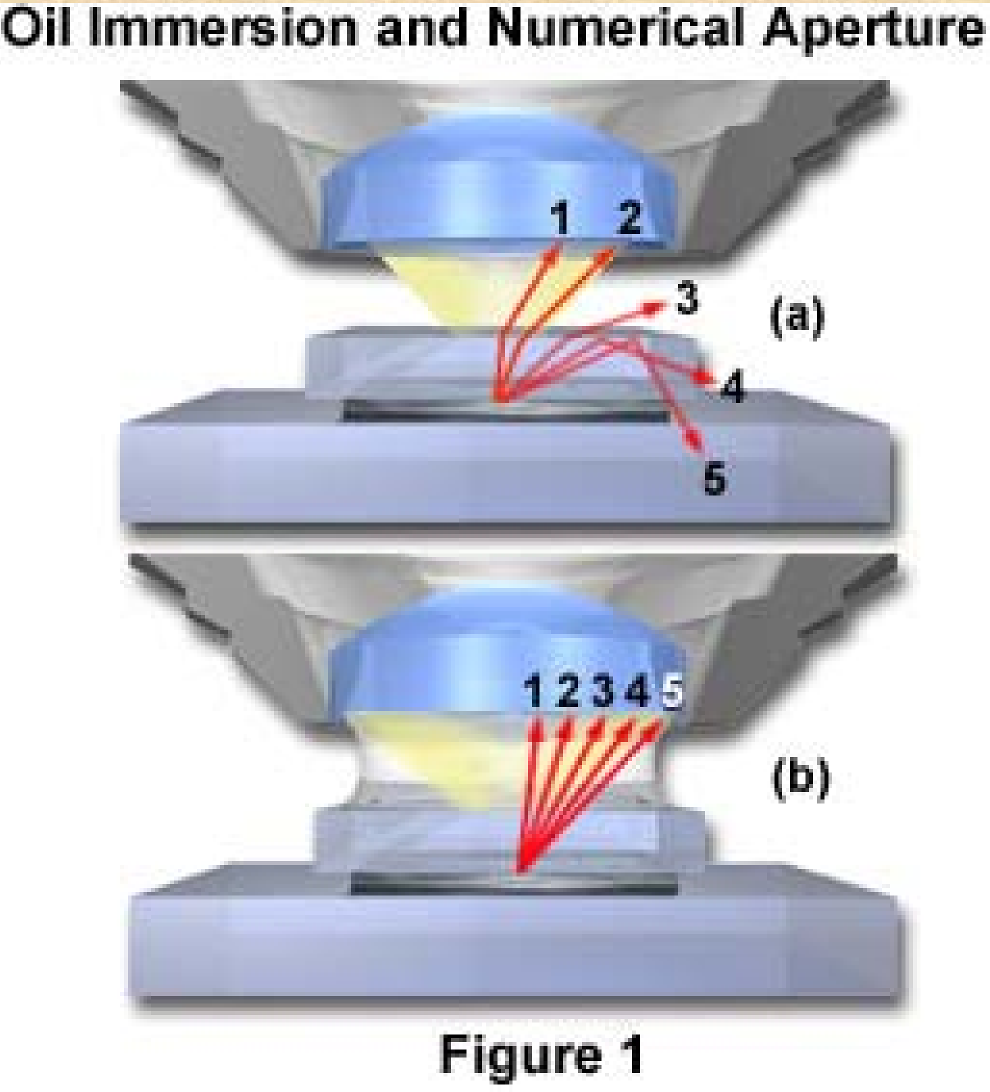
Rayleigh Criterion for the resolution of two adjacent spots:

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

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



Oil Immersion and Numerical Aperture

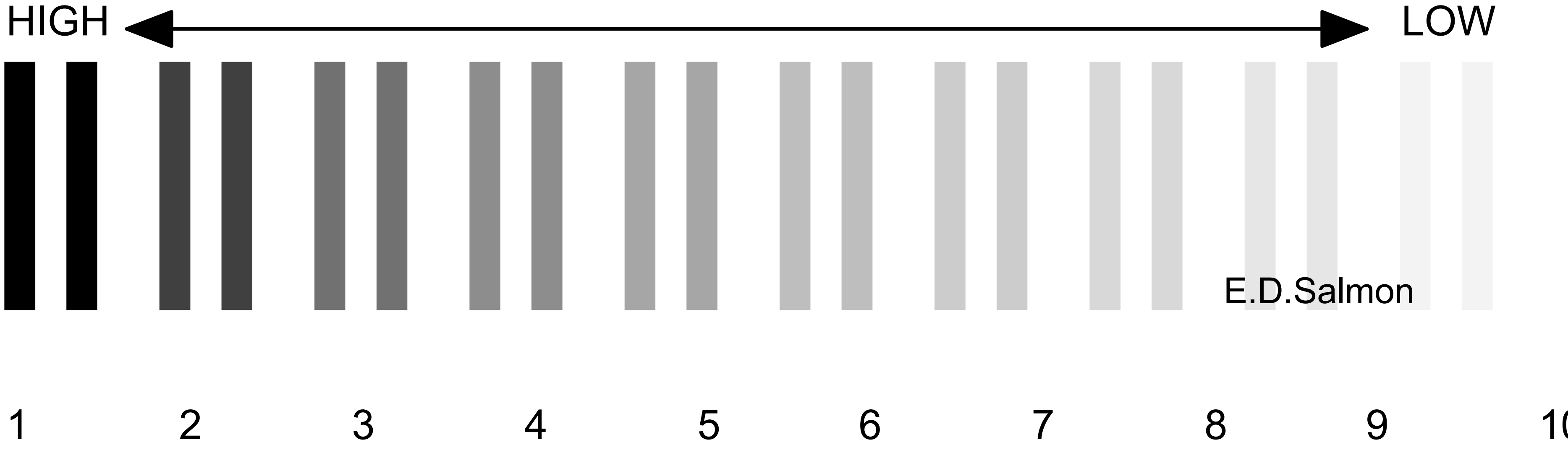
Figure 1

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

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.

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



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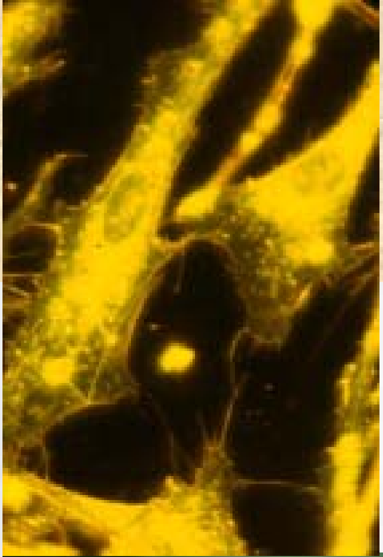
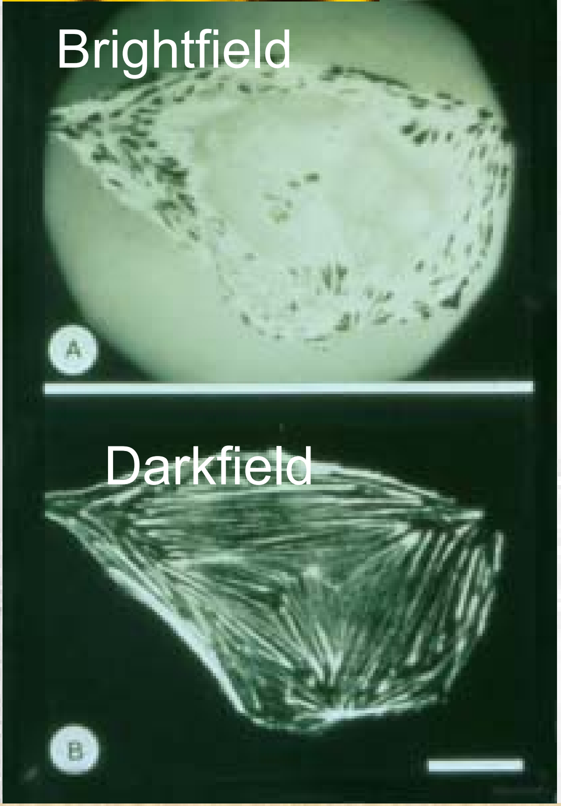
CONTRAST MODES OF LIGHT MICROSCOPY

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



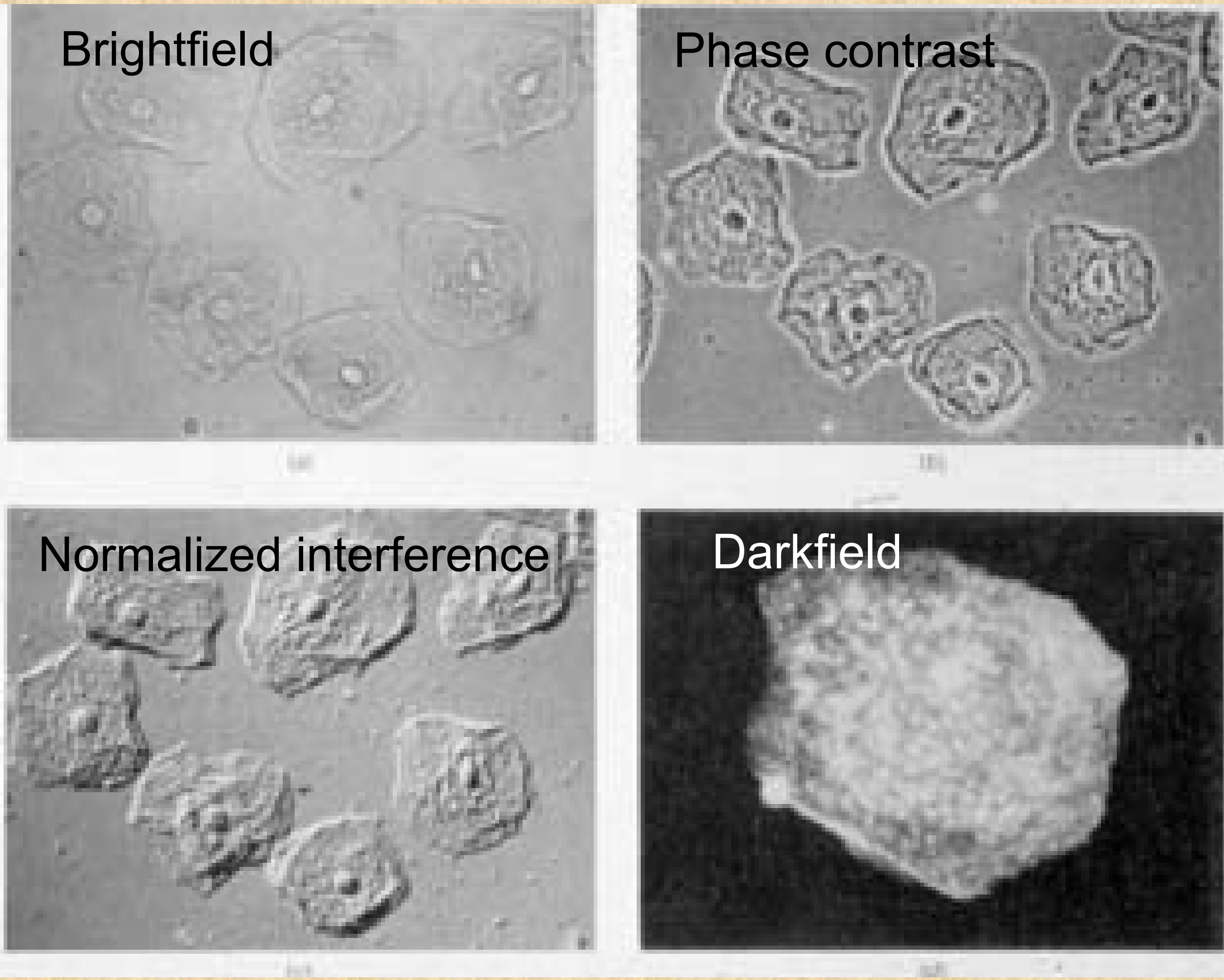
Fluorescence



Brightfield

Darkfield

Index of refraction



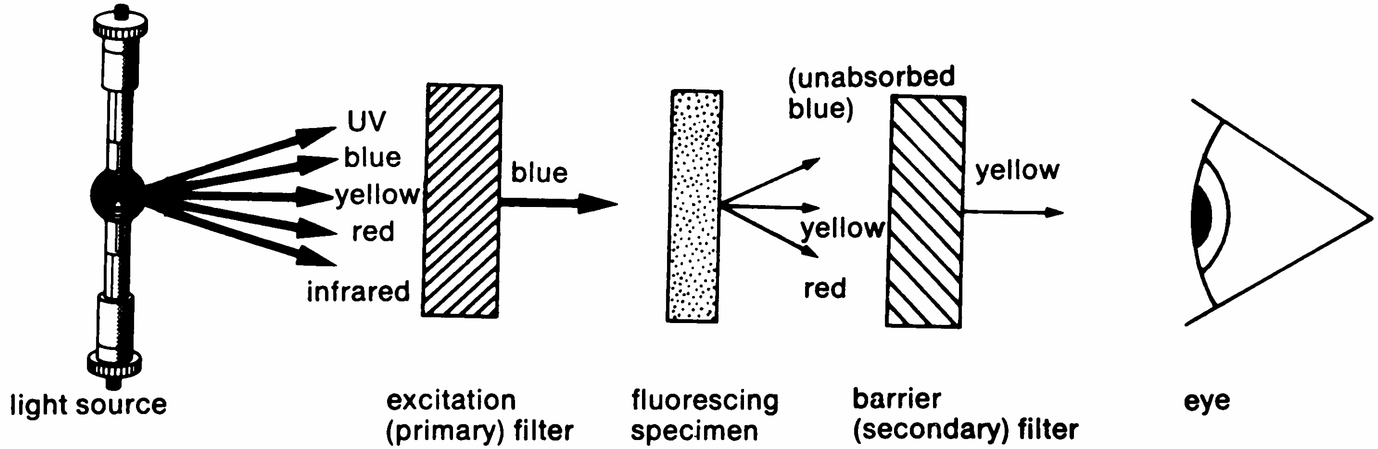
Brightfield

Phase contrast

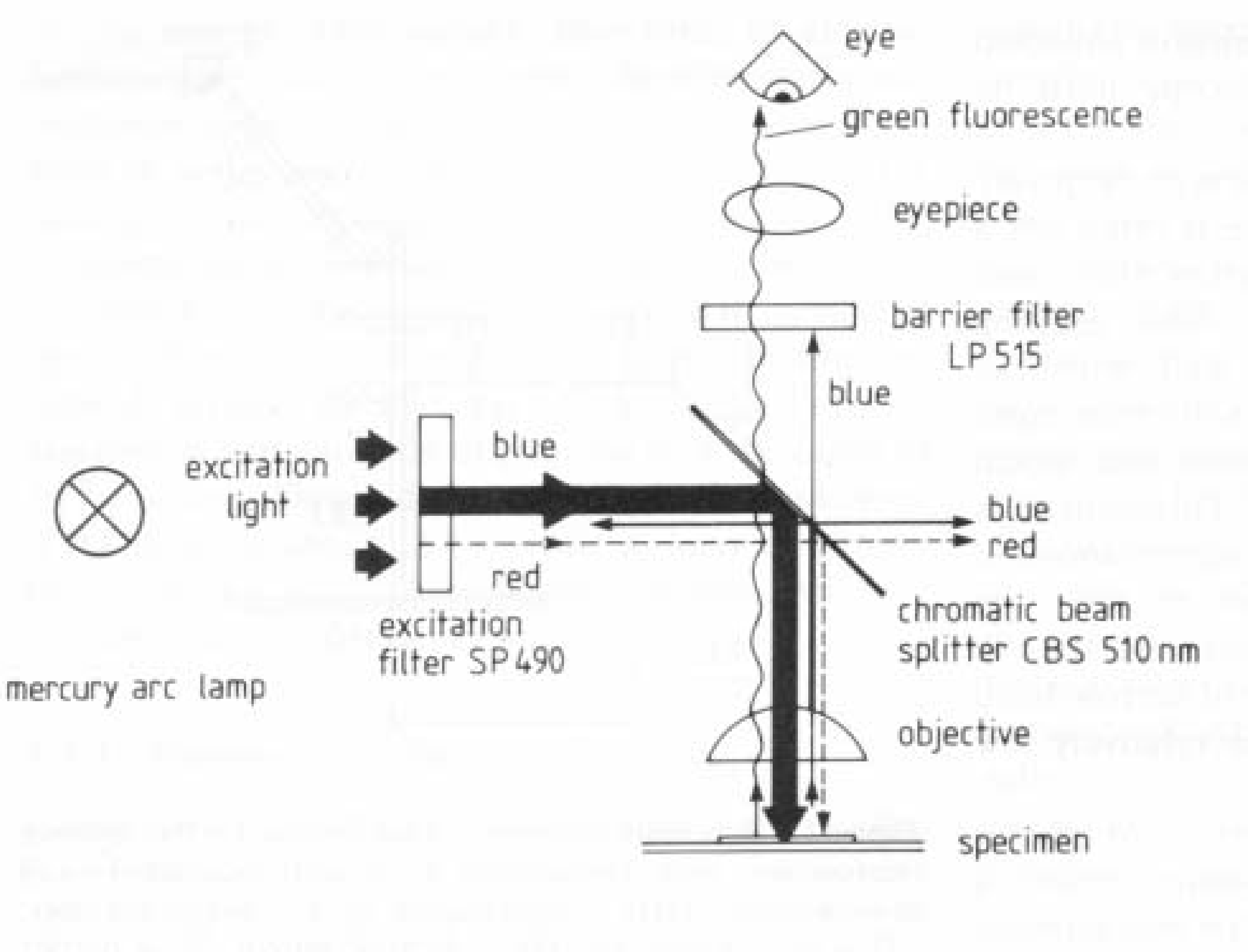
Normalized interference

Darkfield

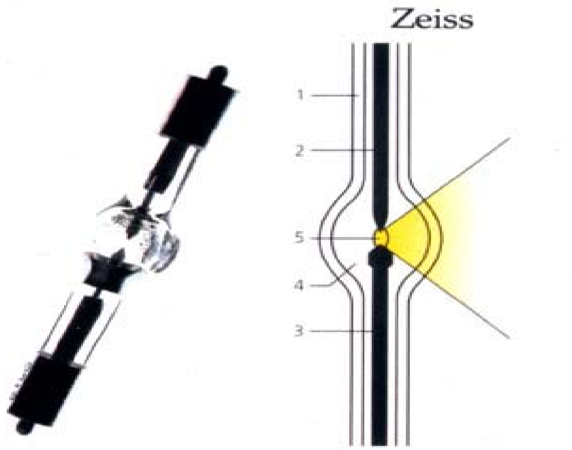
The microscope as a filter fluorometer with focusing optics

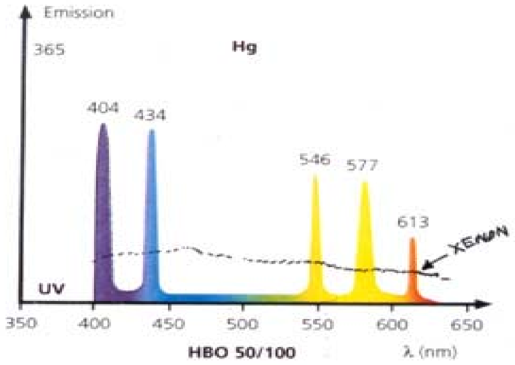


Basic design of the epi fluorescence microscope



Common non-laser light sources: arc lamps





	Type	**Wattage (W)**	**Luminous density (cd/cm²)**	**Arc size h x w (mm)**	**Lifetime (h)**
High-pressure Mercury lamps	HBO 50W/AC HBO 100W/2	50 100	30 000 170 000	1.0 x 0.3 0.25 x 0.25	100 200
High-pressure Xenon lamps	XBO 75W/2	75	40 000	0.5 x 0.25	400
Tungsten-Halogen lamps	12V 100W	100	4500	4.2 x 2.3	50

From C. Zeiss

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.

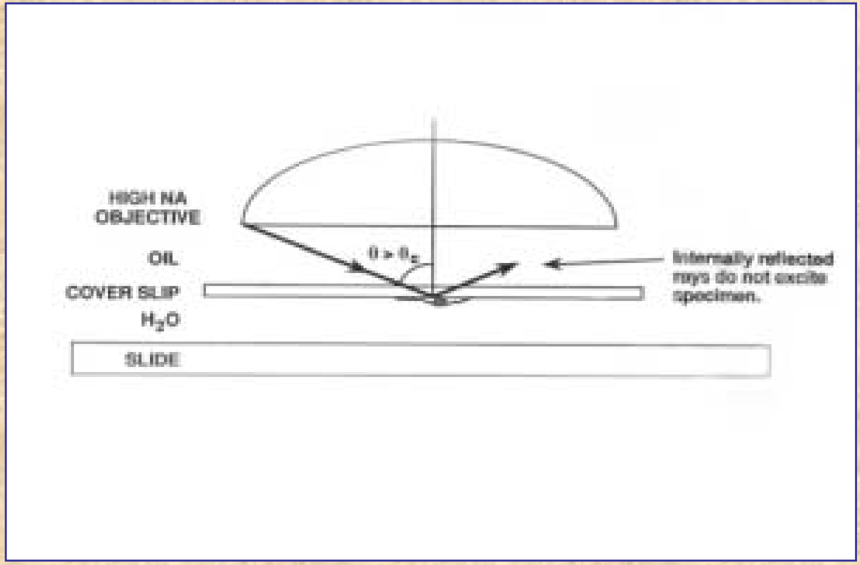
Negligible autofluorescence or solarization [color change upon prolonged illumination]

Maximizing image brightness (B)

$$\left.\begin{array}{l}\text{excitation efficiency} \sim (NA)^2 \\ \\ \text{collection efficiency} \sim (NA)^2\end{array}\right\} \Rightarrow B \sim (NA)^4$$

$$\text{also } B \sim \frac{1}{M^2} \quad \Rightarrow \quad B \sim \frac{(NA)^4}{M^2}, \text{ for } NA \leq 1.0$$

at high NA,



Filters

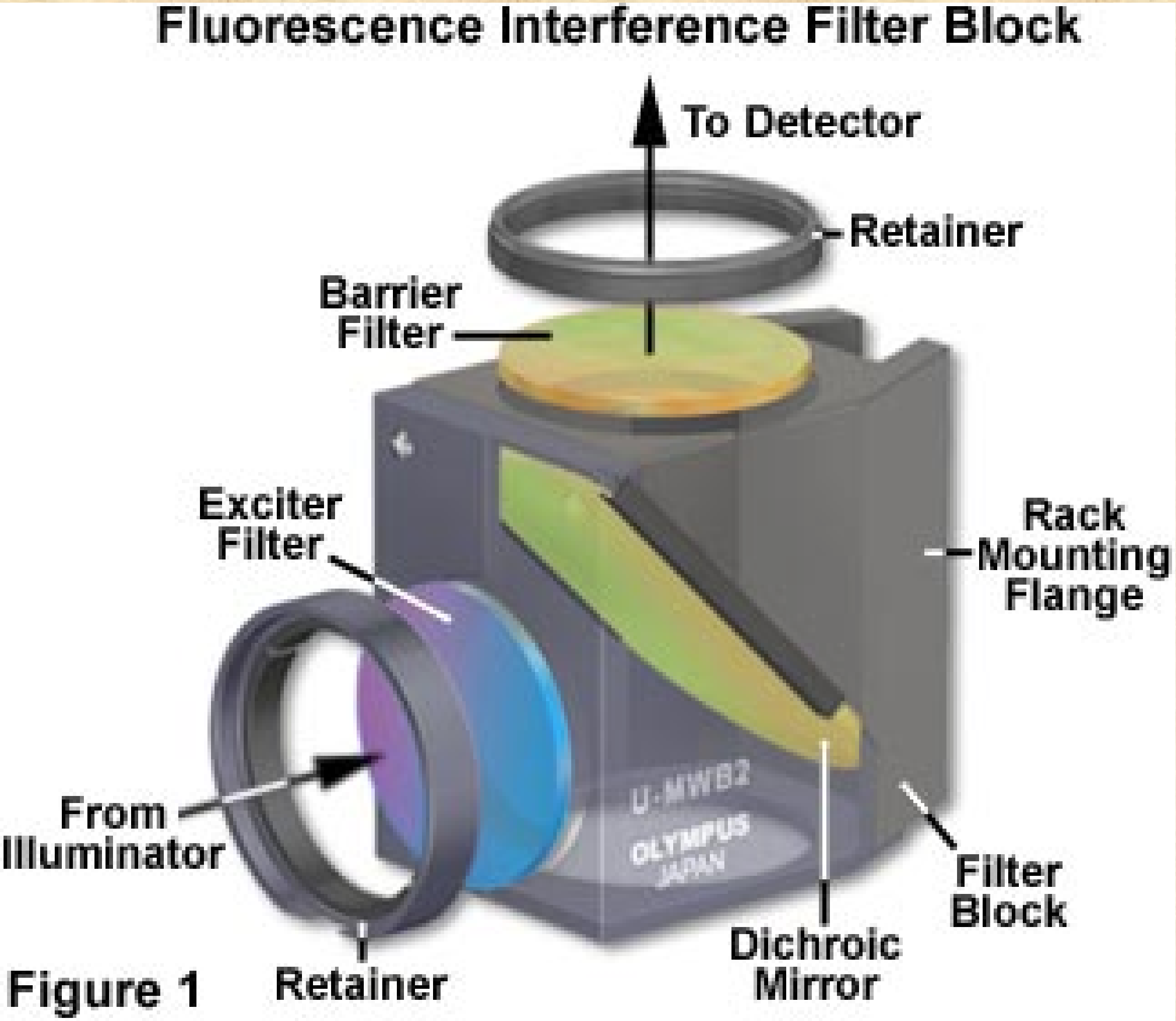
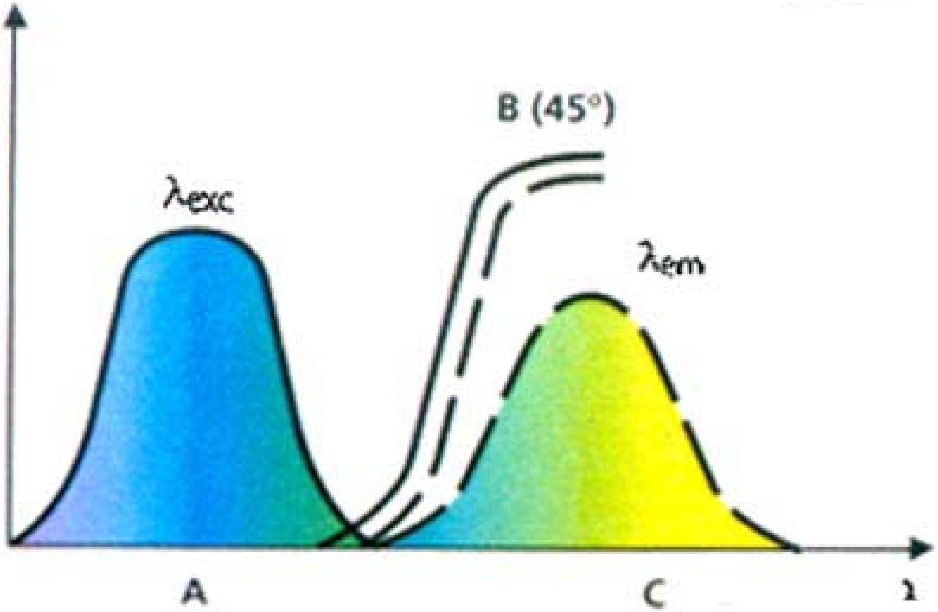
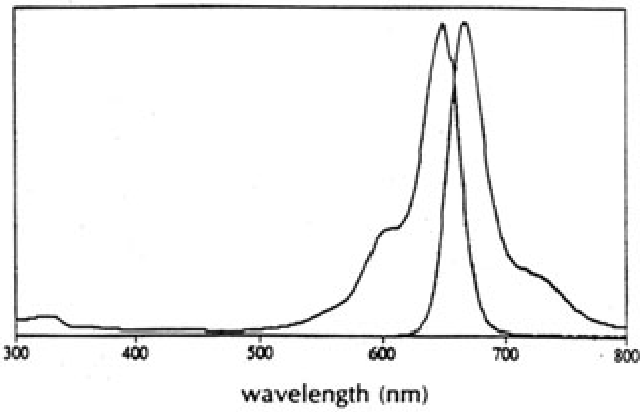


Figure 1

Zeiss



Cy5-methylamine conjugate



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

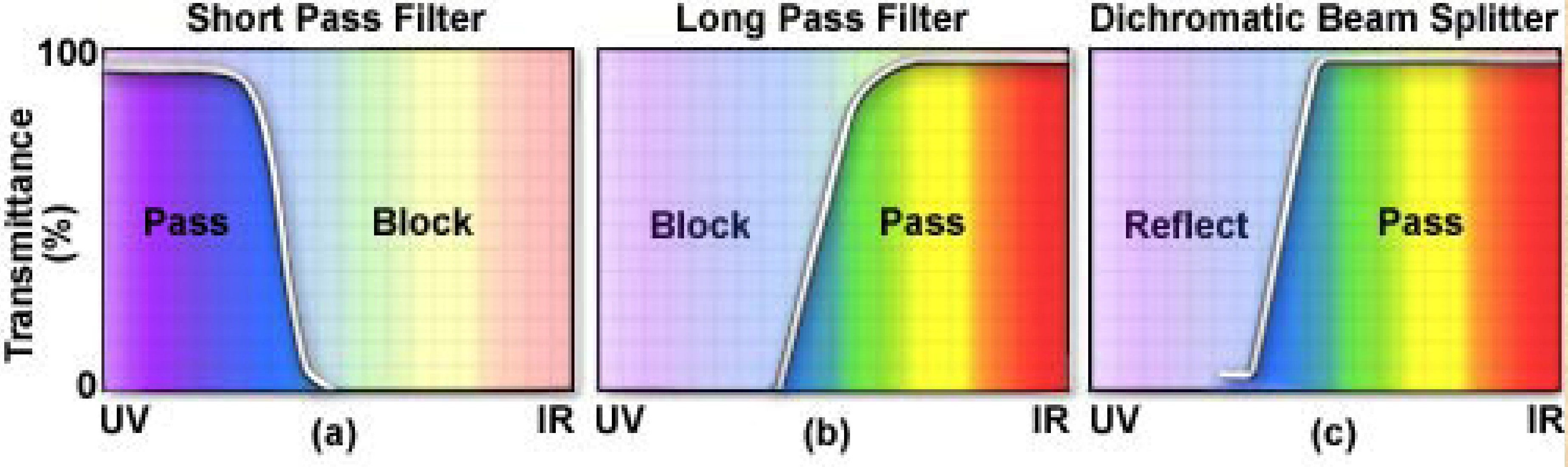


Figure 2

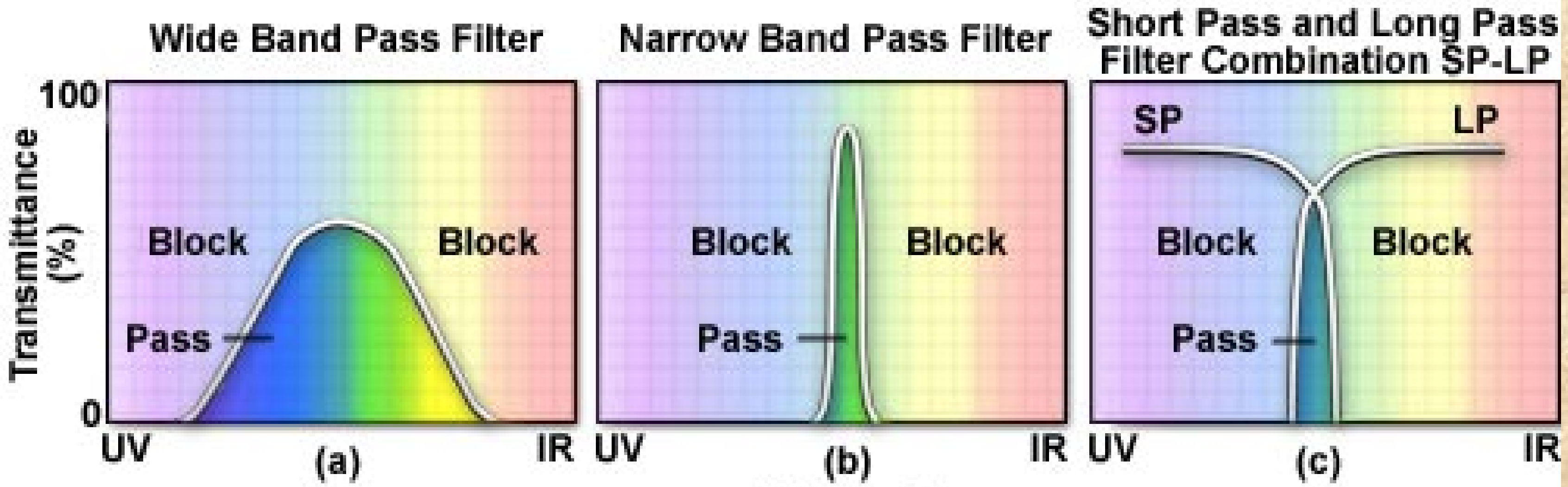
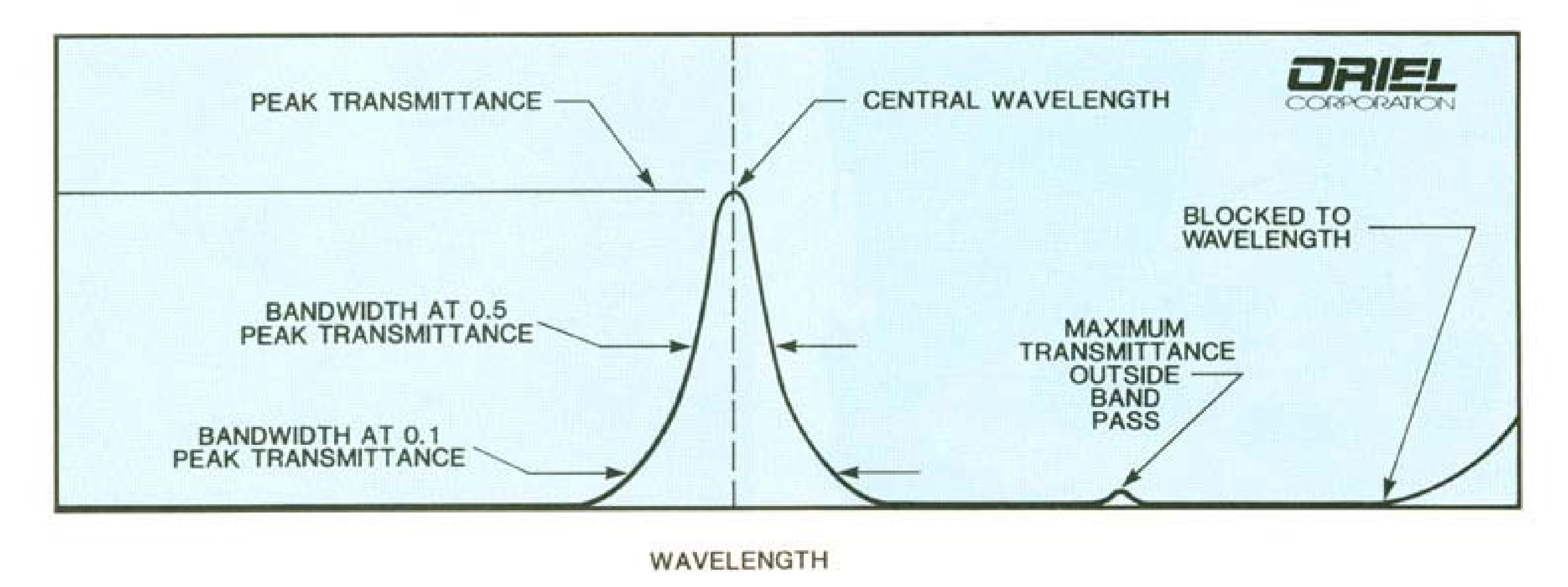


Figure 3

Interference filter definitions



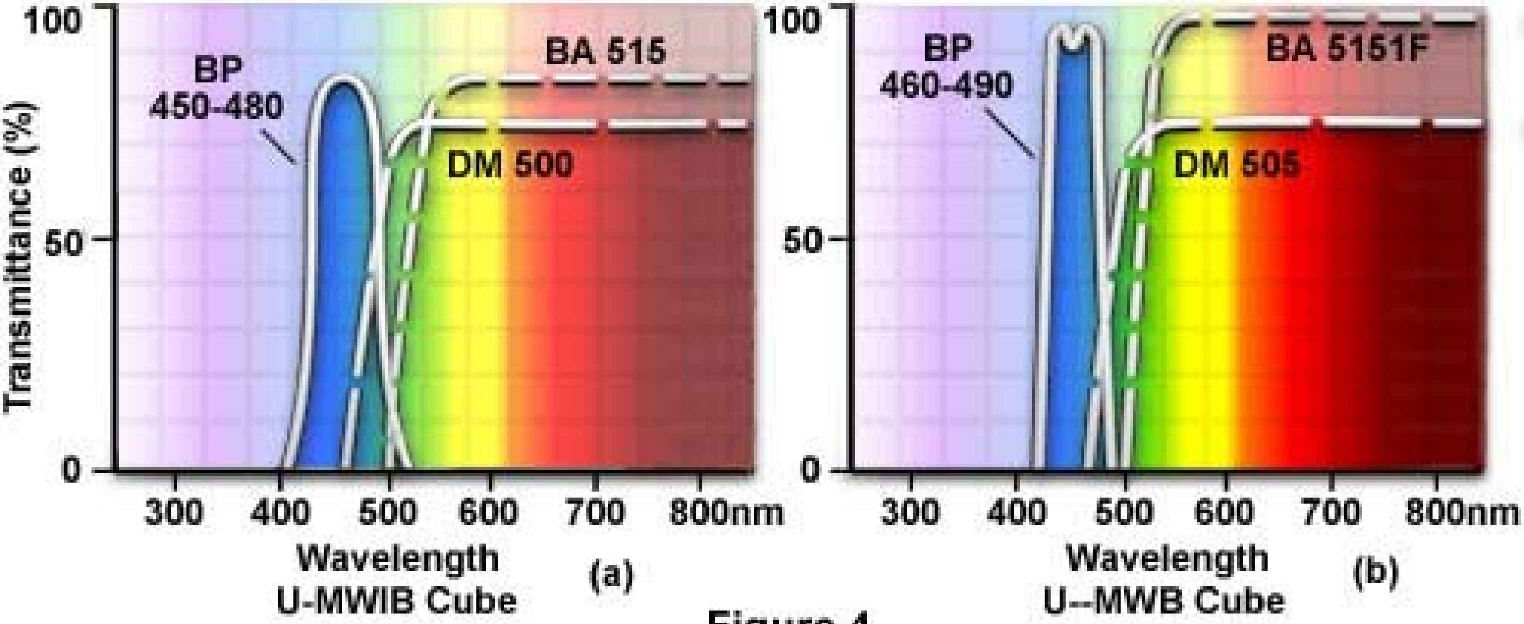


Figure 4

Filter cube designs employing long-pass emitter filters

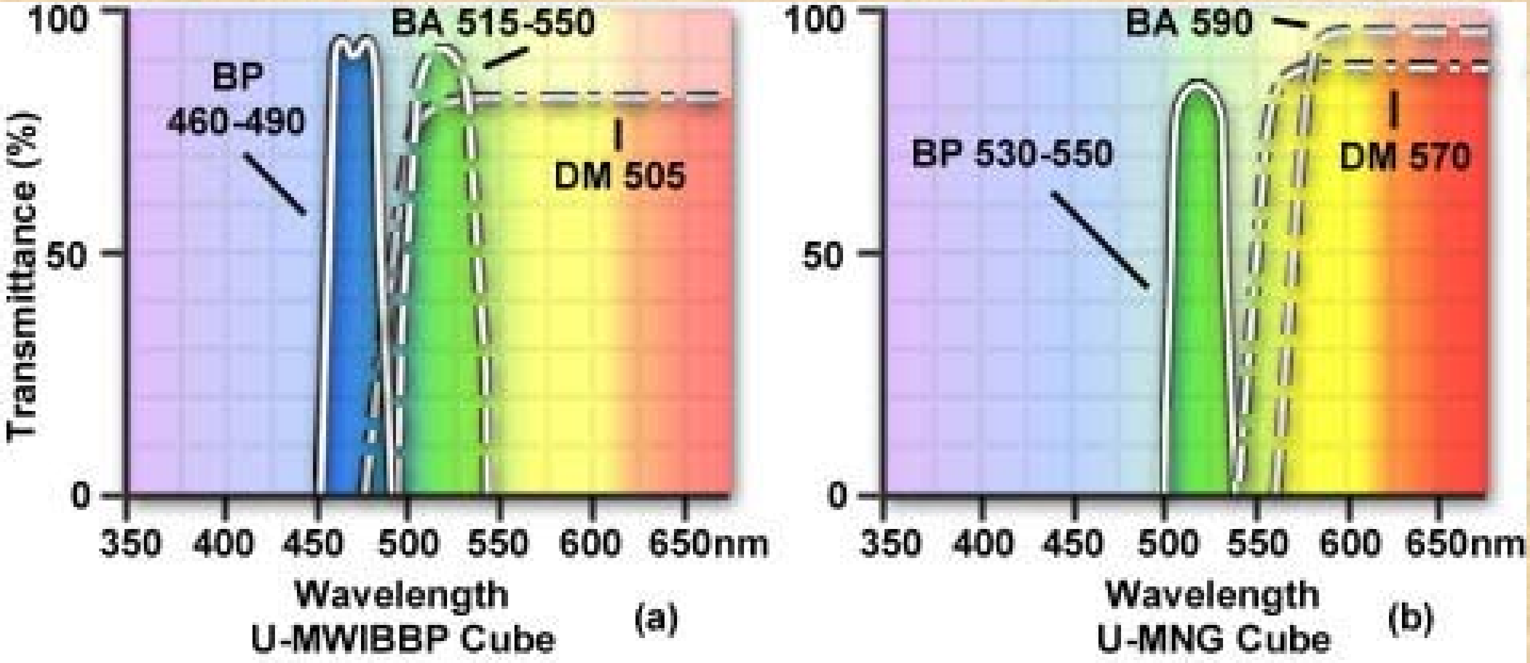
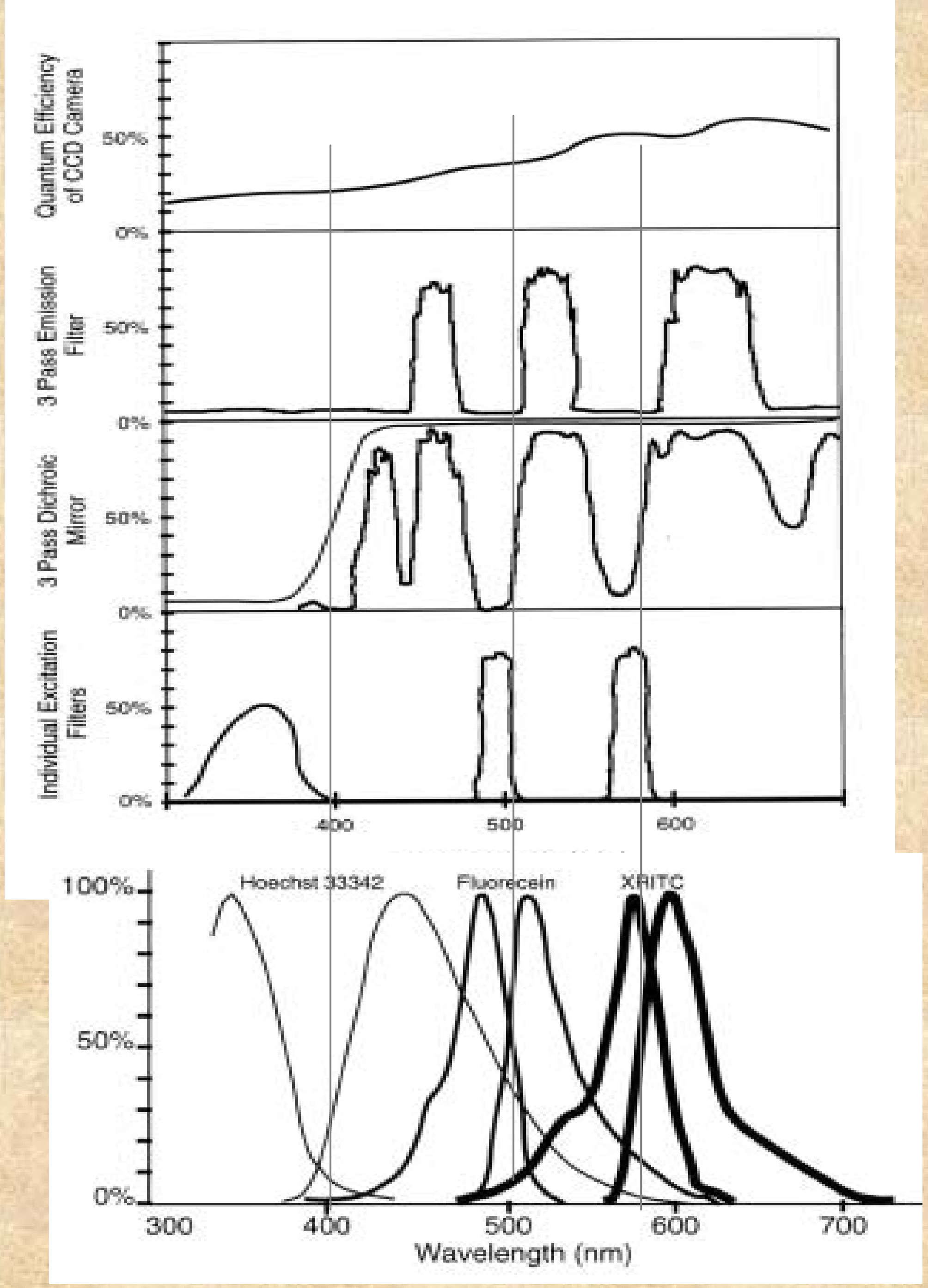


Figure 5

Filter cube designs employing band-pass emitter filters

Multiple Band-Pass Filters



From E.D. Salmon

Multi-Wavelength Immunofluorescence Microscopy

Bovine Pulmonary Artery Epithelial Cells

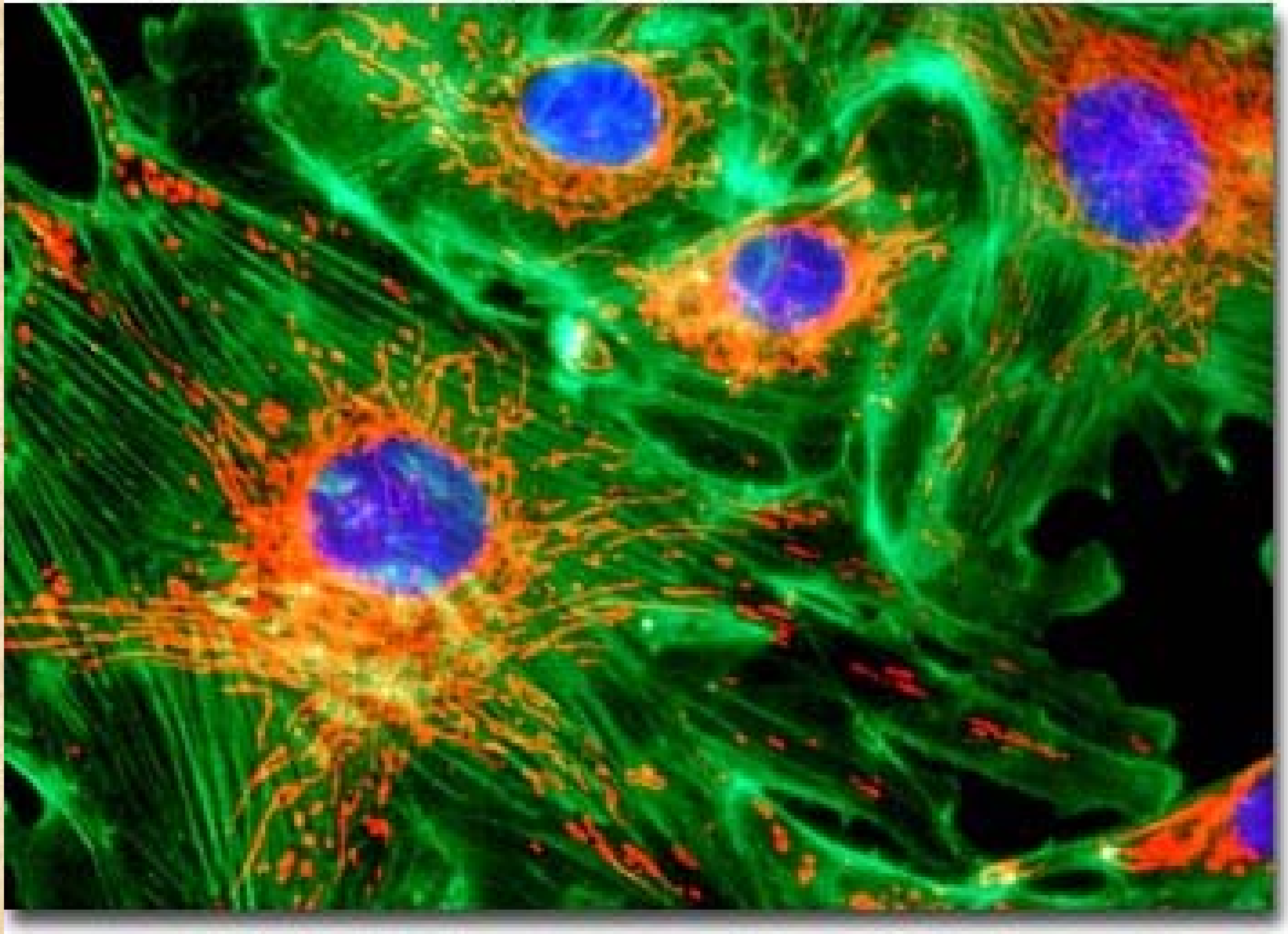
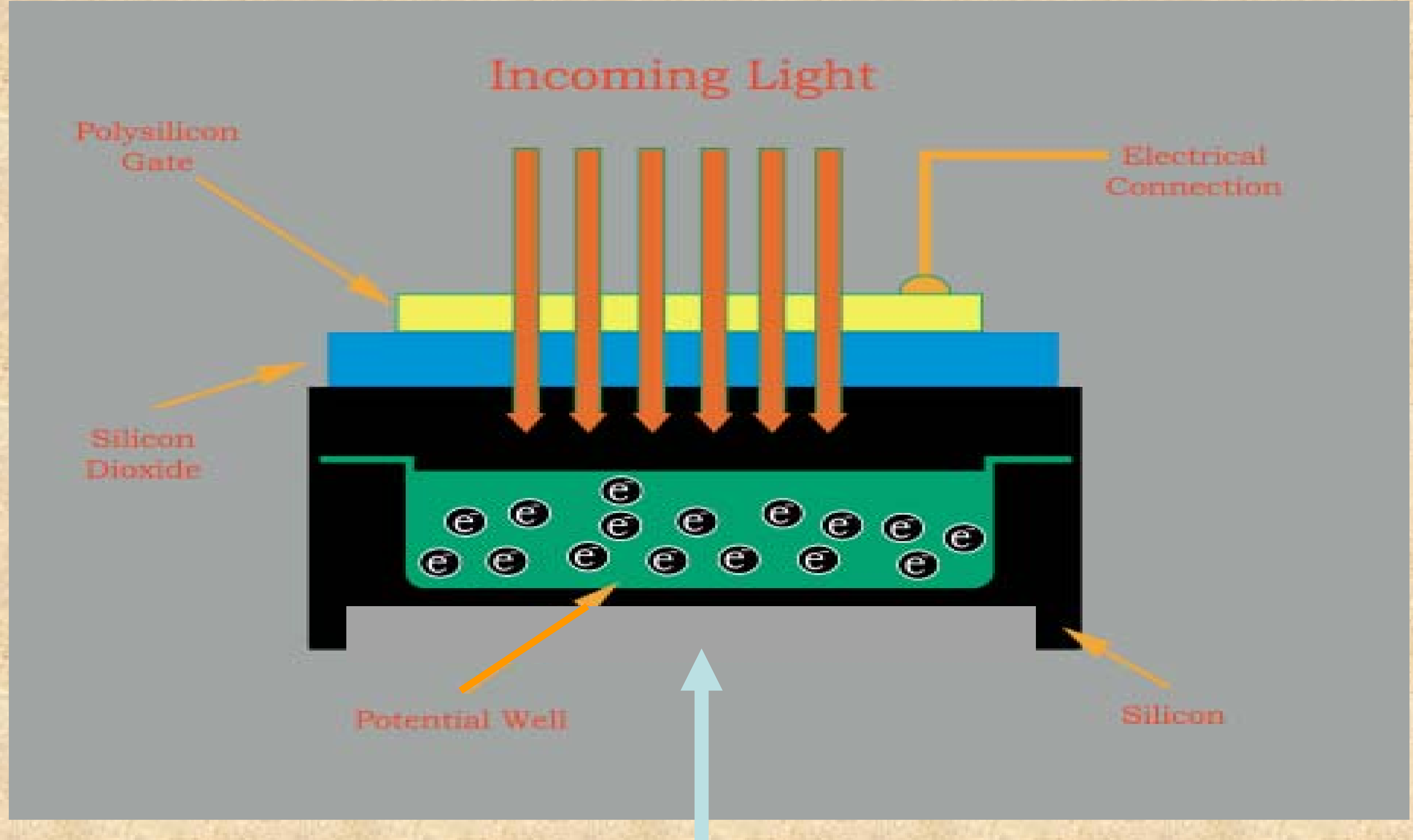


Figure 1

PIXELS
The building blocks of CCDs



Back thinned CCDs receive light from this side

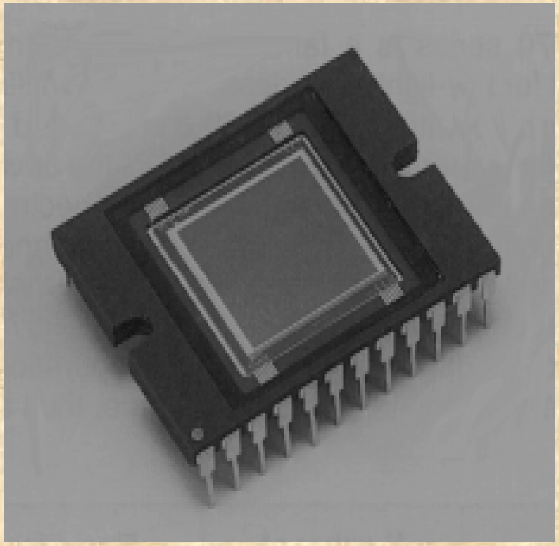
Primary Features of CCD

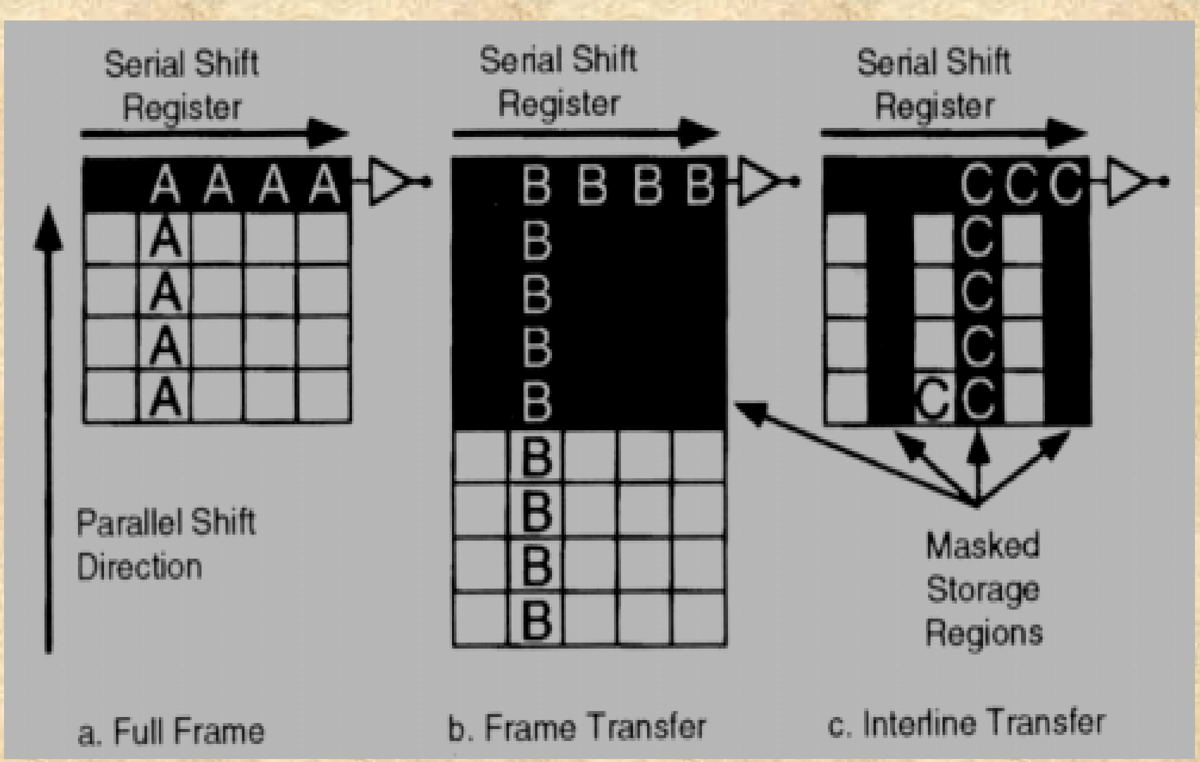
- Spatial resolution of the CCD array
  - Number of Pixels in X and Y
  - Center to Center Distance of Pixels in microns
- Full Well Capacity
  - Related to Physical size and electronic design
  - Determines Maximum Signal level possible
- Quantum Efficiency/Spectral Range
  - Determines the usefulness 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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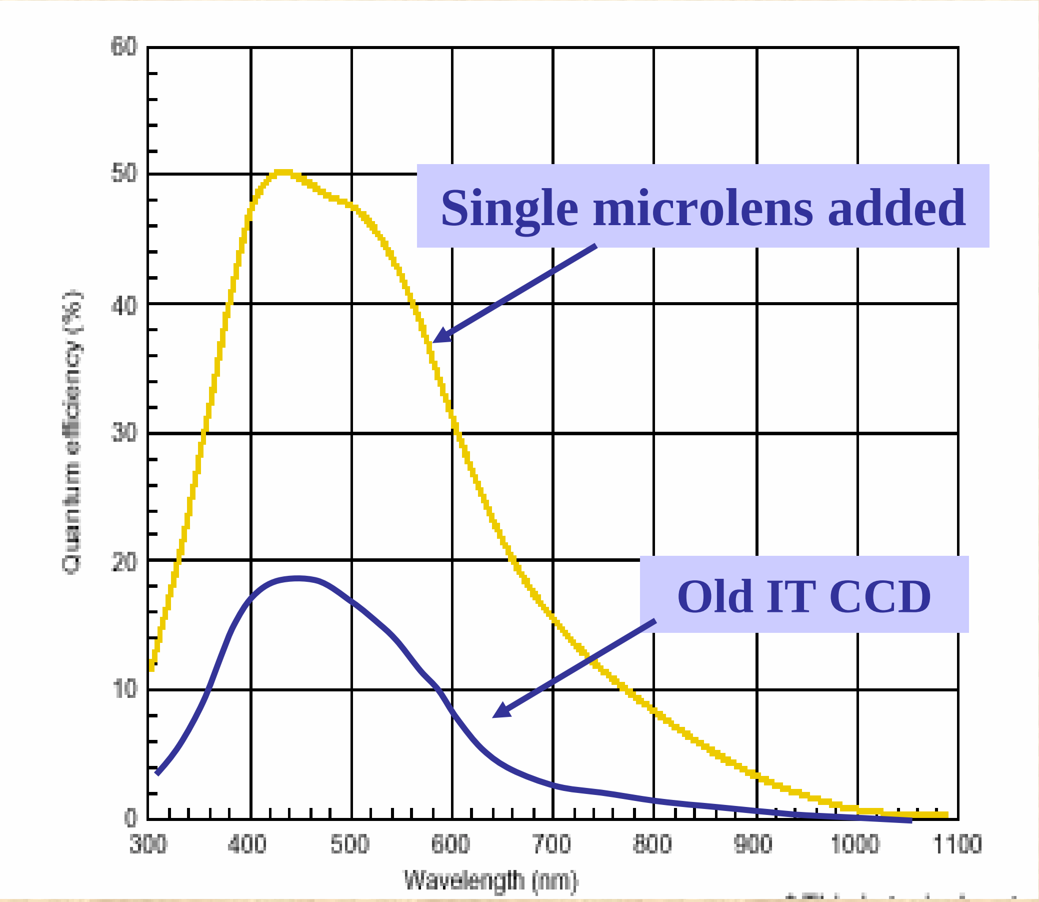
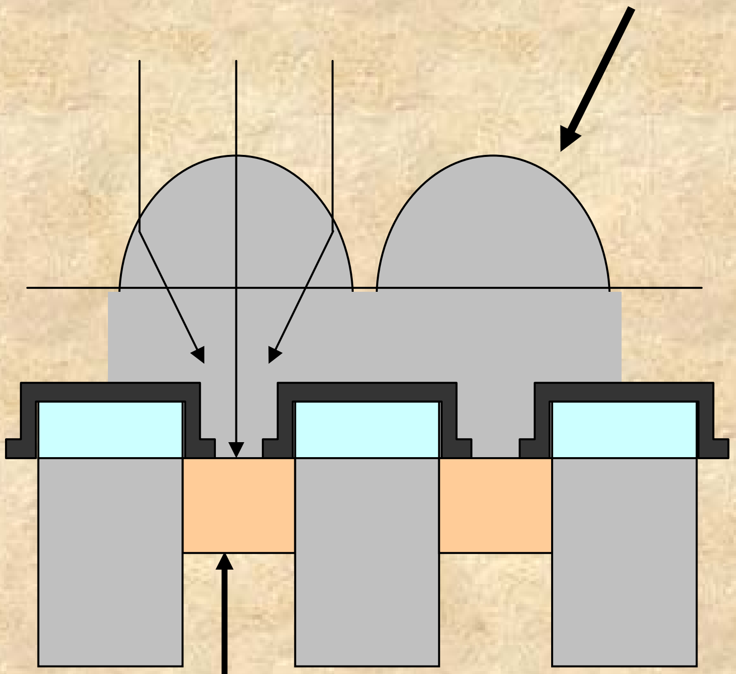
Improvements in Interline CCDs

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





Input light

Microlens

Open window

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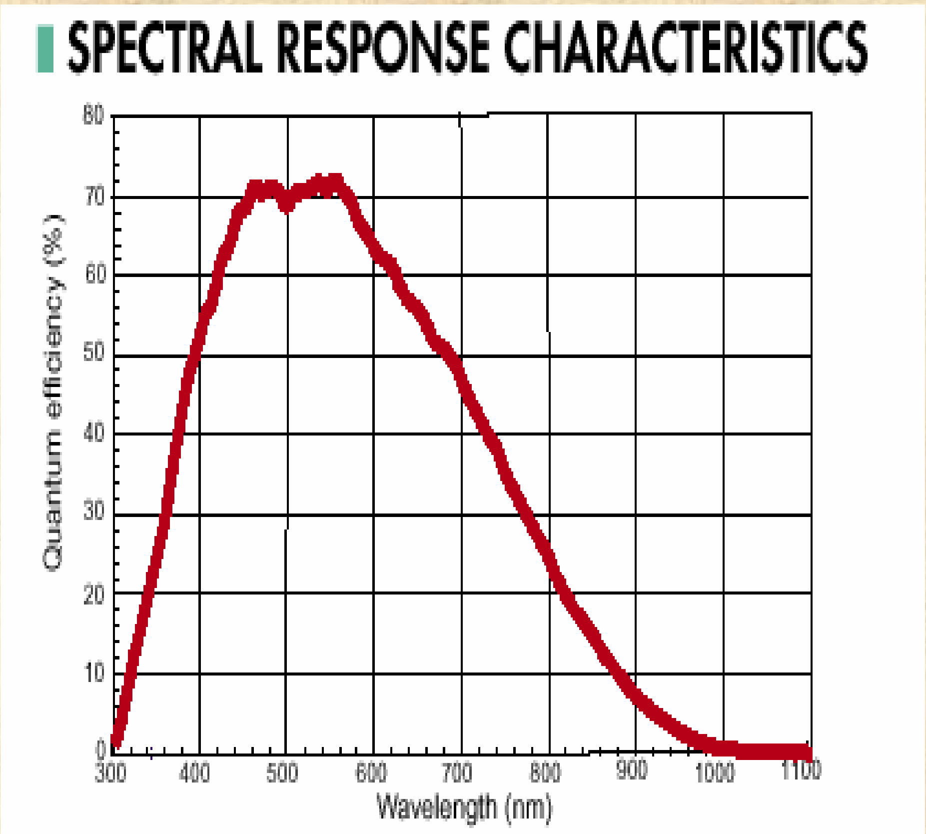
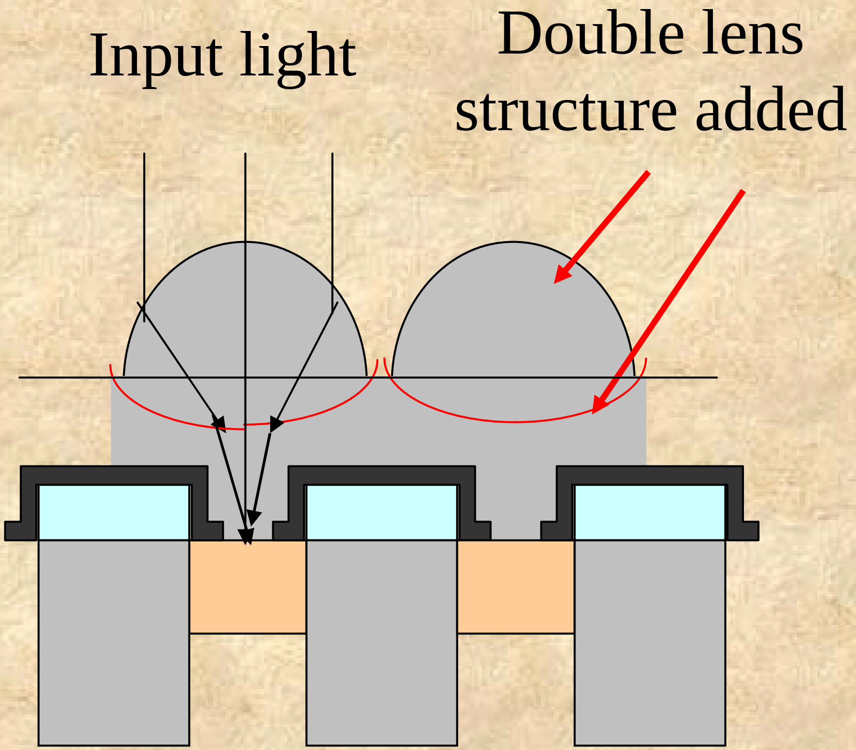
Latest Improvement to Interline CCDs

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

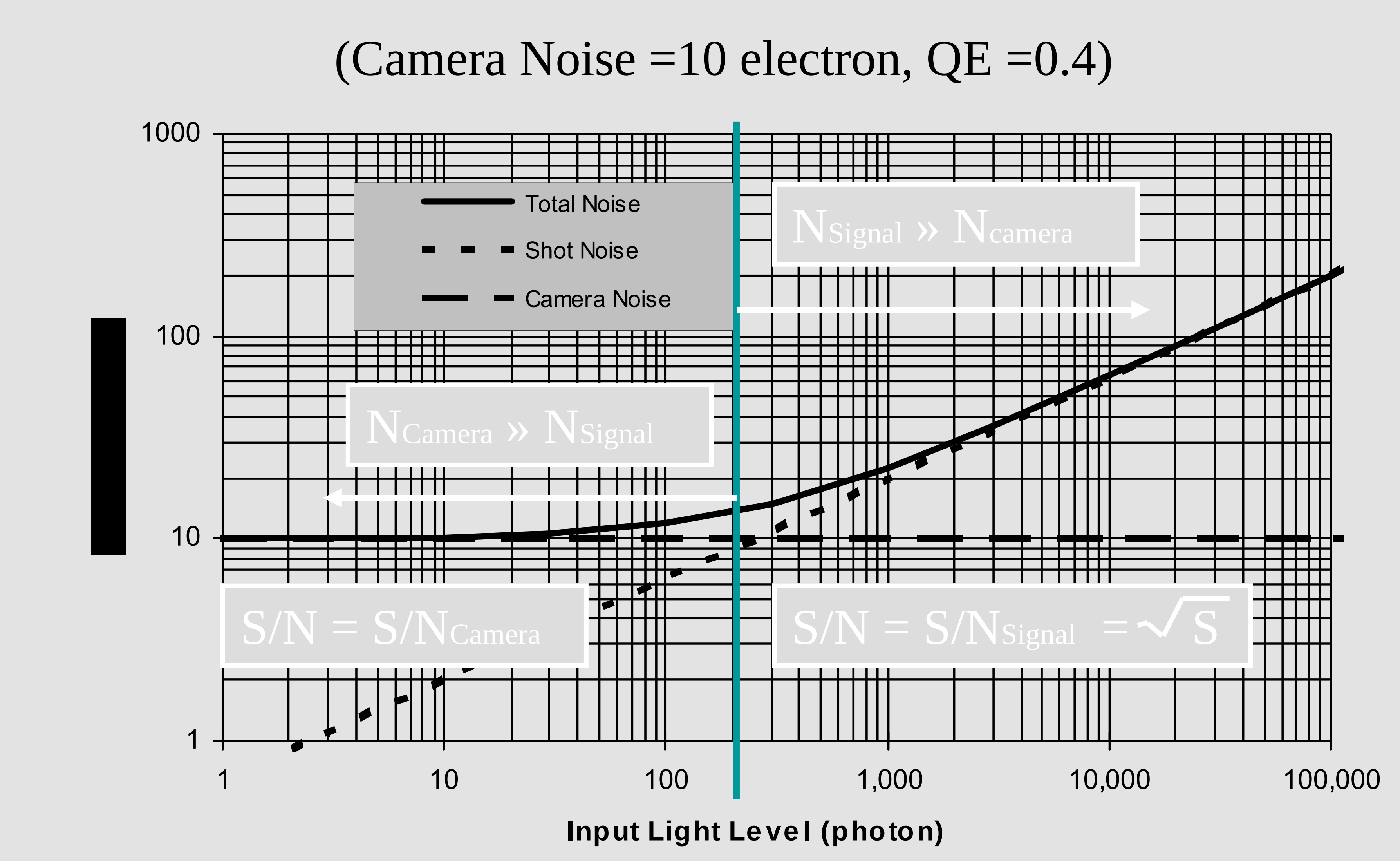



Input light

Double lens structure added

B. Moomaw, Hamamatsu Corp.

Noise as a function of incident camera illumination



(Camera Noise =10 electron, QE =0.4)

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. ($Qy_{bleaching}$ ~ 3E-5)
- At low excitation intensities, pb 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

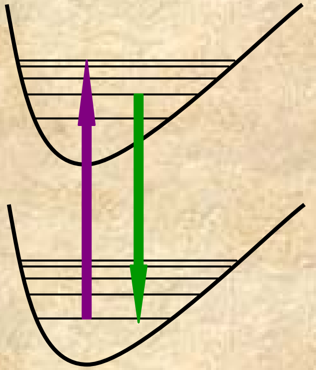
Live Cell Considerations

- 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

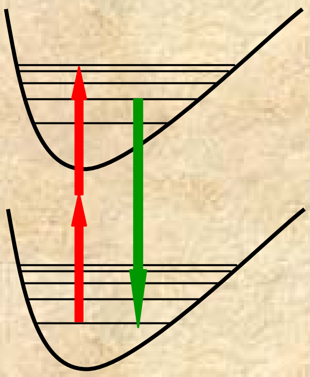
Adapted from E.D. Salmon

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

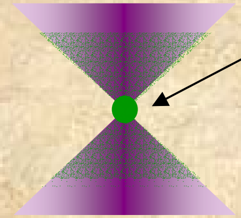
1 - Photon



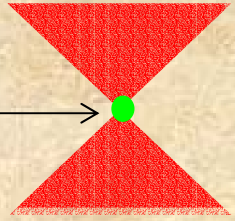
2 - Photon



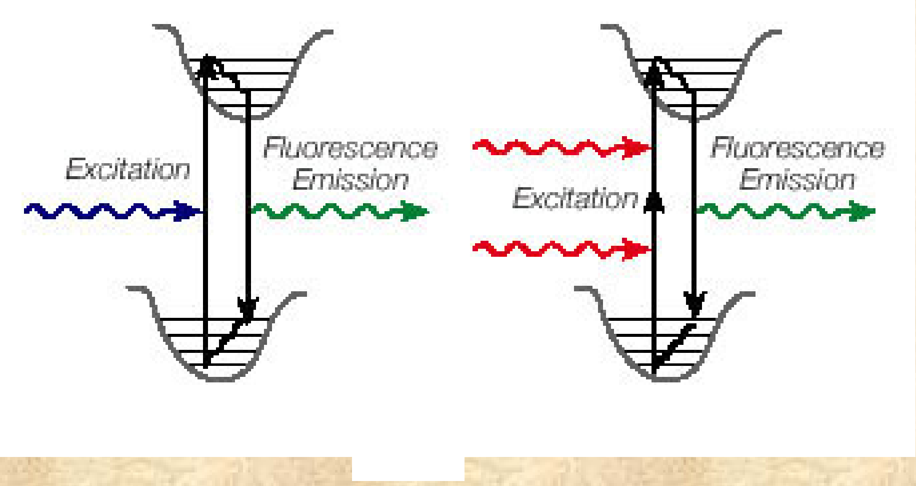
Defined by the pinhole size, wavelength, magnification and numerical aperture of the objective



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

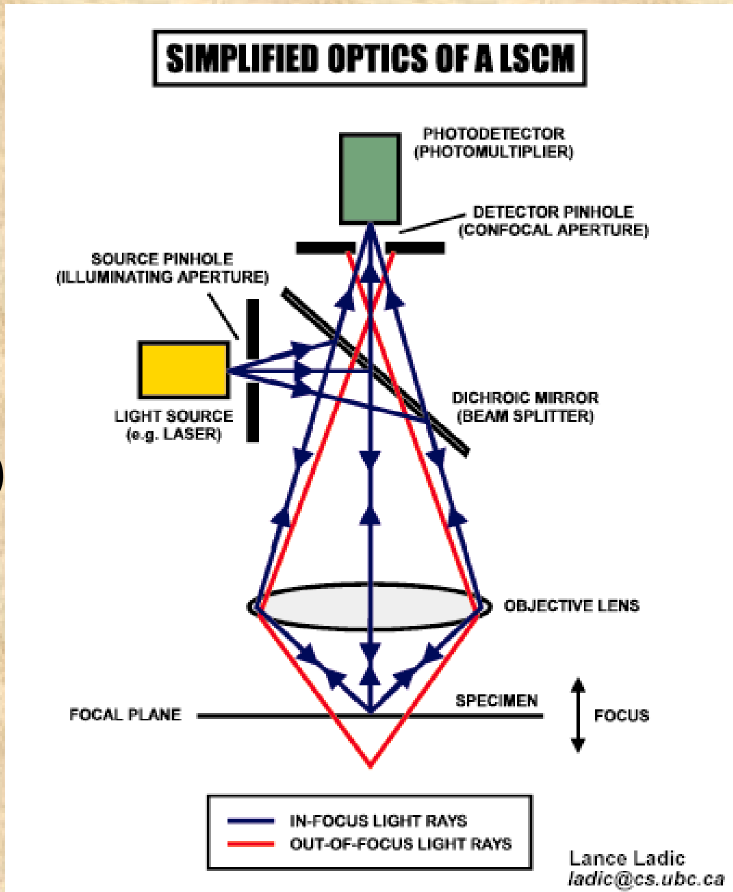
Methods to produce a confocal or small volume

(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)

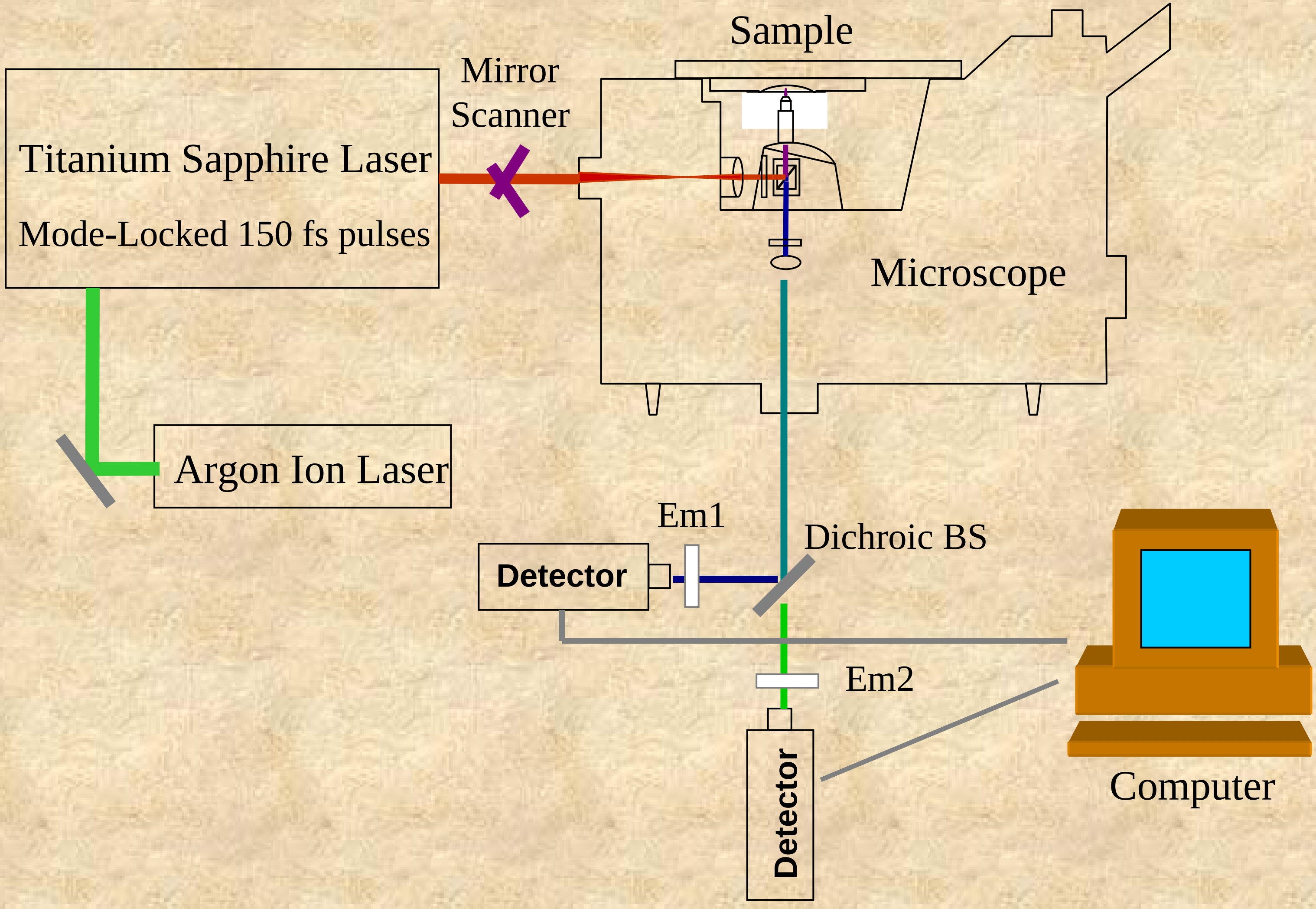
- Nanofabrication
- Local field enhancement
- Near-field effects



Lance Ladic
ladic@cs.ubc.ca

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

Two-Photon, Scanning, FCS Microscope



Laser technology needed for two-photon excitation

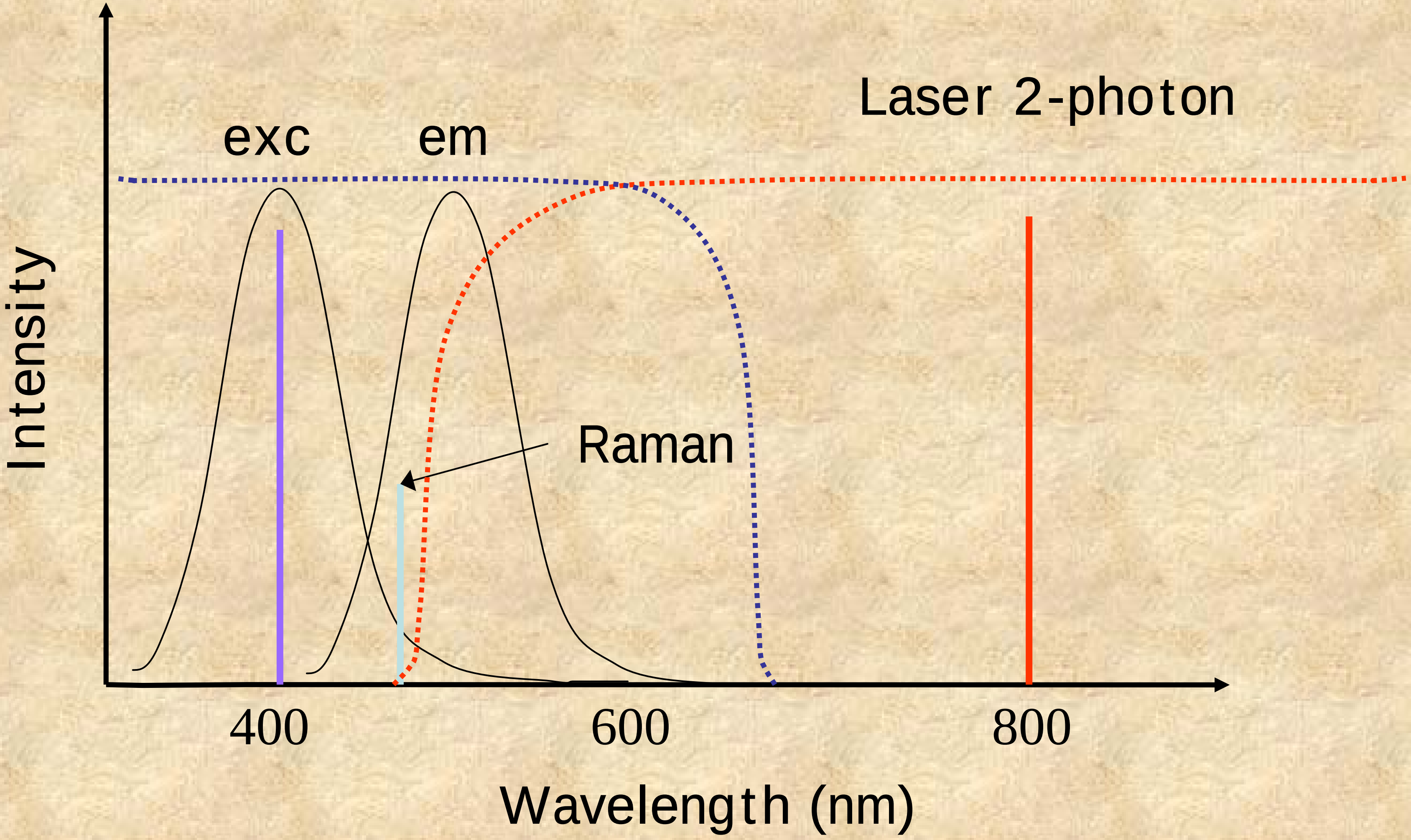
Ti:Sapphire lasers have pulse duration of about 100 fs
Average power is about 1 W at 80 MHz repetition rate
About 12.5 nJ per pulse (about 125 kW peak-power)
Two-photon cross sections are typically about

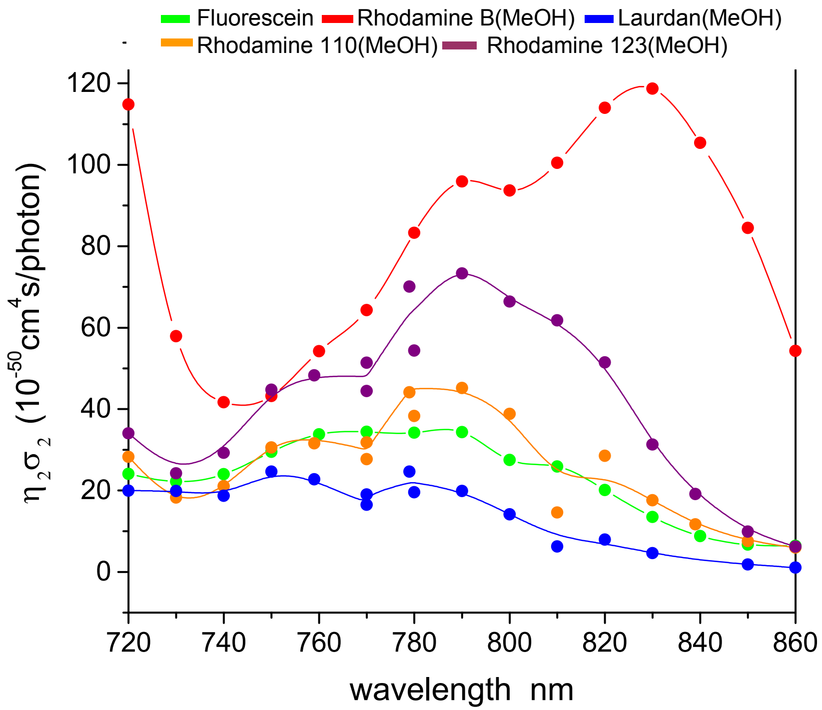
$\delta=10^{-50}$ cm^{4} sec photon^{-1} molecule-1

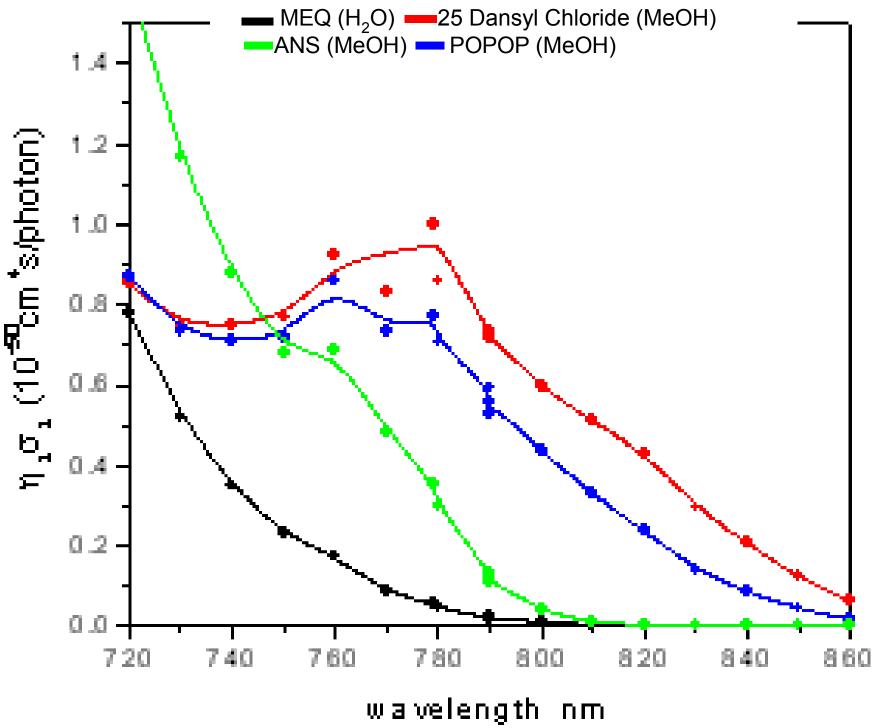
Enough power to saturate absorption in a diffraction limited spot

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

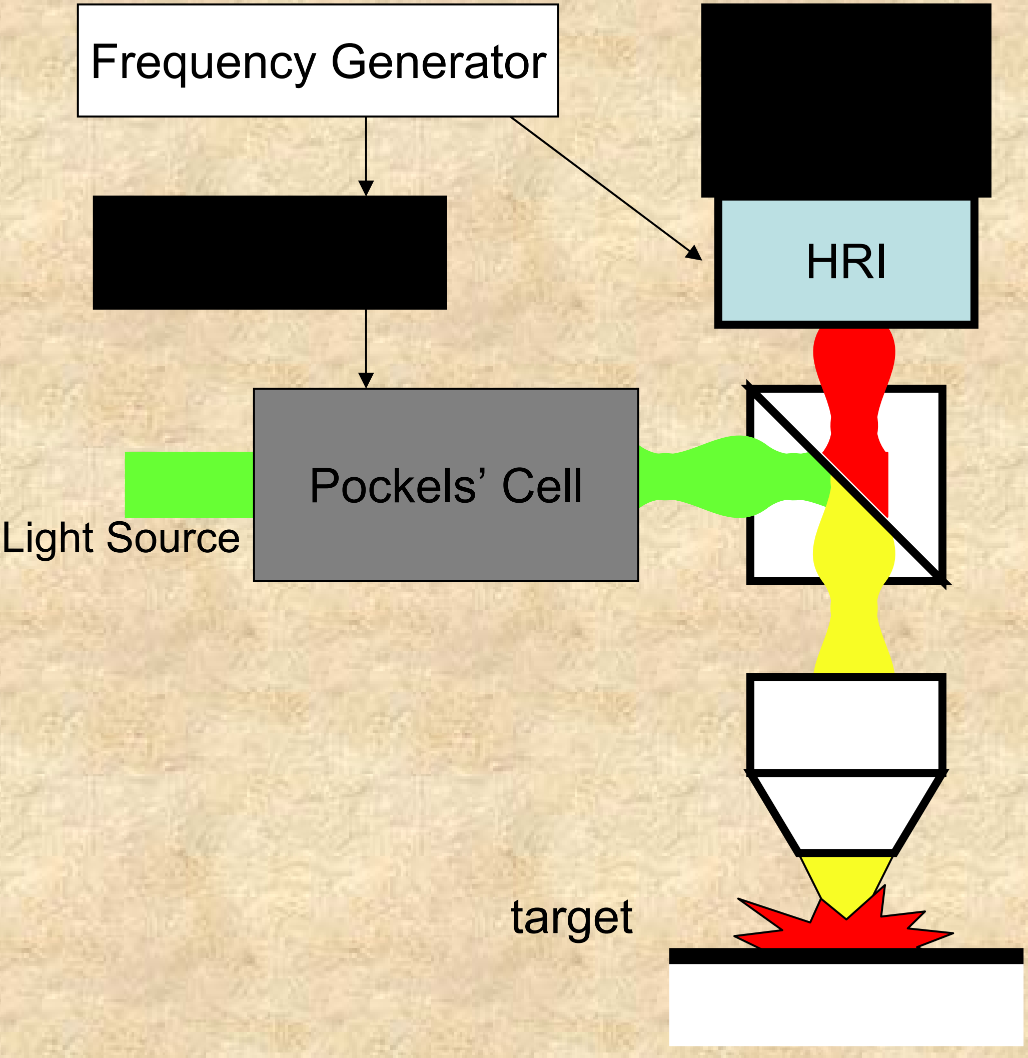
n_a	Photon pairs absorbed per laser pulse
p	Average power
τ	pulse duration
f	laser repetition frequency
A	Numerical aperture
λ	Laser wavelength
d	cross-section

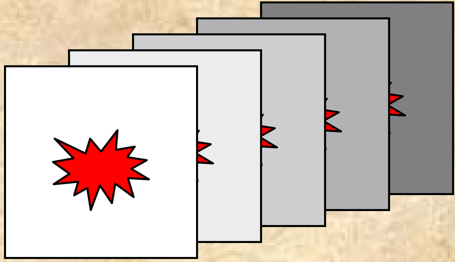

Laser 2-photon
exc
em
Intensity
Raman
400
600
800
Wavelength (nm)


Fluorescein
Rhodamine B(MeOH)
Laurdan(MeOH)
Rhodamine 110(MeOH)
Rhodamine 123(MeOH)
$\eta_2\sigma_2$ (10^{-50}cm^{4}s/photon)
120
100
80
60
40
20
0
720
740
760
780
800
820
840
860
wavelength nm

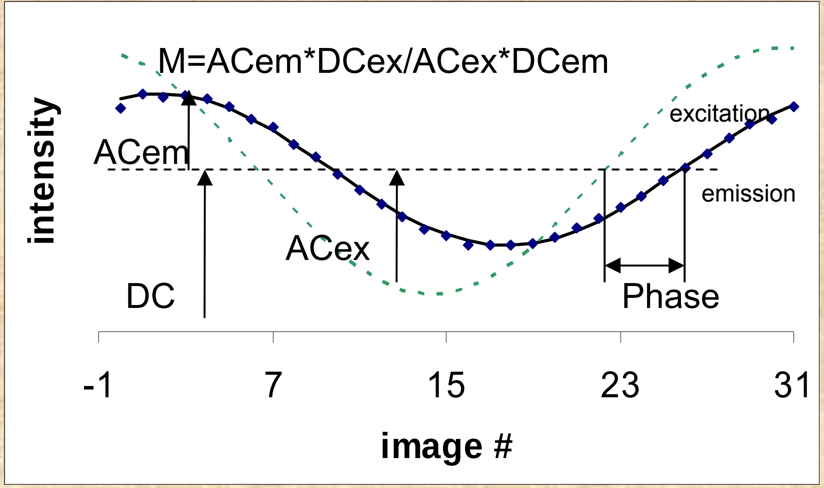

MEQ (H_2O)
25 Dansyl Chloride (MeOH)
ANS (MeOH)
POPOP (MeOH)
$\eta_1\sigma_1$ (10 cm^{4}s/photon)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
720
740
760
780
800
820
840
860
wavelength nm

FLIM: Instrument Diagram





Phase shifted images



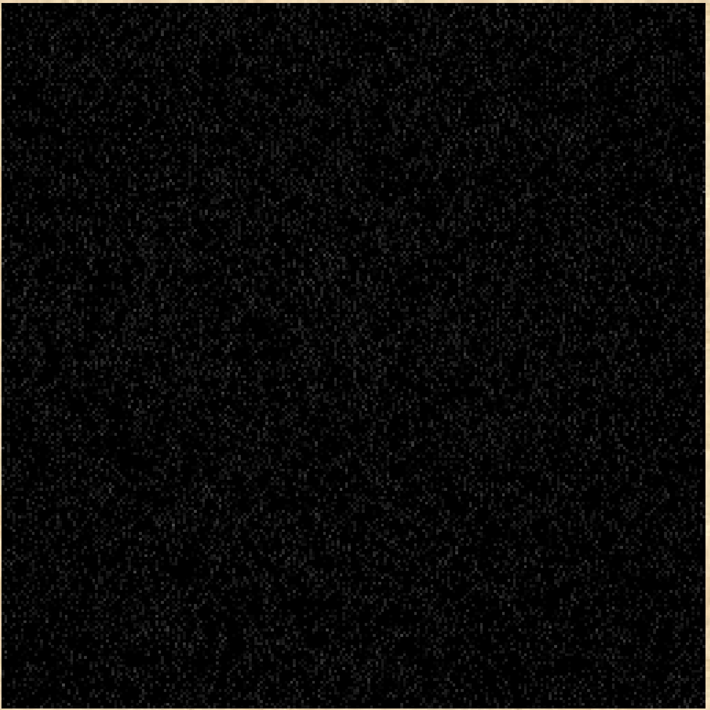
Current FLIM Performance

- 300 to 800 nm excitation wavelength
- 350 to 800 nm emission wavelength
- Frequency range is 20 to 140 MHz, corresponding to 10^{-7} to 10^{-10} s
- Deep (>80%) total modulation (higher S/N)
- 90 frames/sec image capture = 30 frames/sec lifetime image capture
- Real-time paletted display of lifetime images as well as real-time histograms, averages, and traces
- Post-analysis software suite for data extraction, visualization, and rendering
- Multi-lifetime fitting algorithms and visualization

Endoscope Images

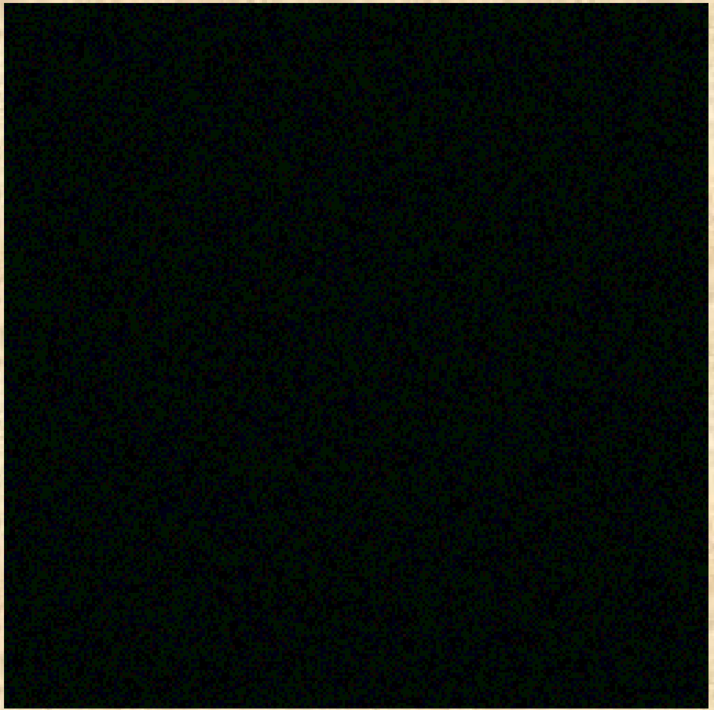
Intensity only

30 fps



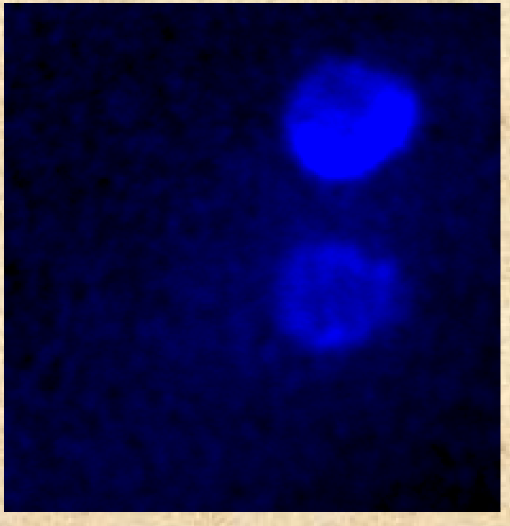
Lifetime Image

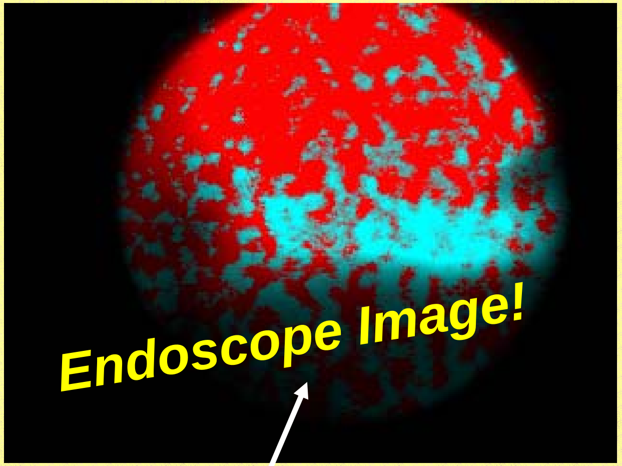
5 fps



These are lifetime images taken through an endoscope. Demonstrated is the fast lifetime imaging capabilities of the system.

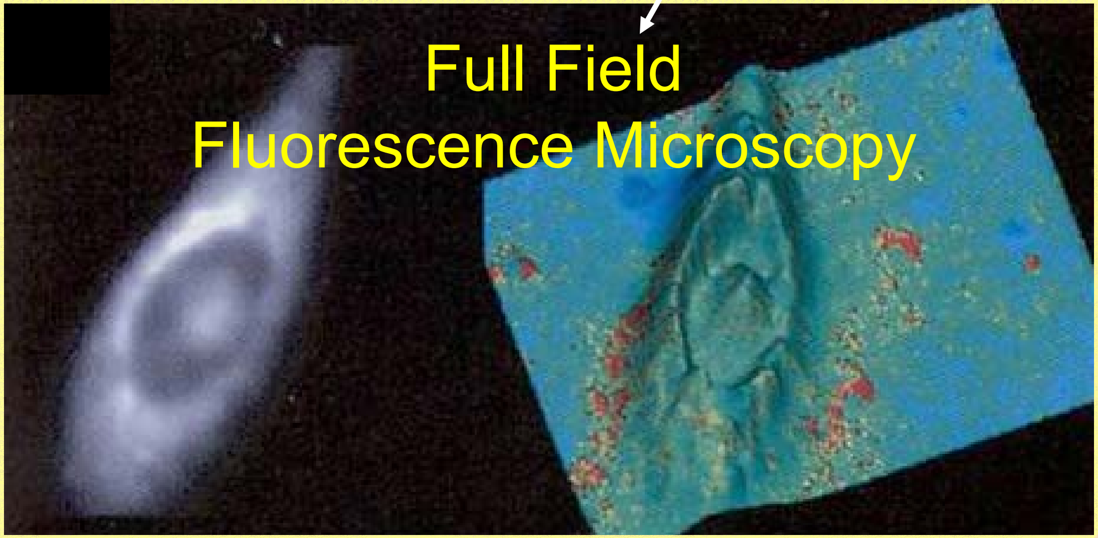
DNA Chip Analysis





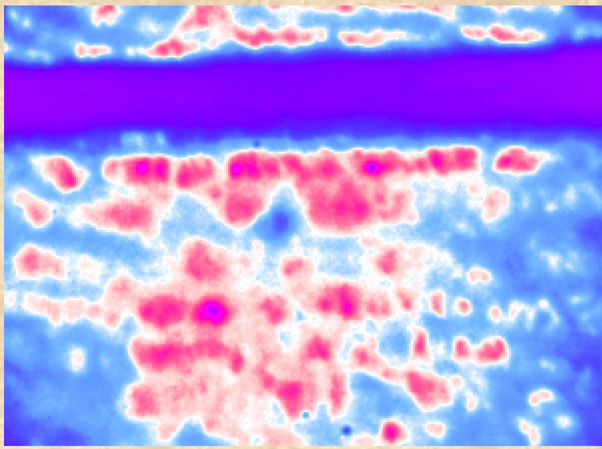
Endoscope Image!

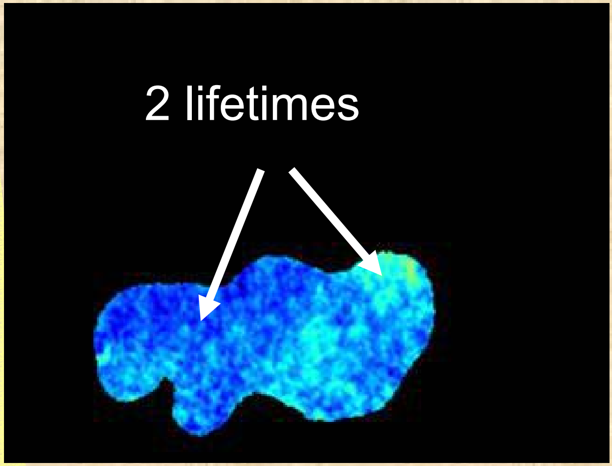
Tumor Detection



Full Field
Fluorescence Microscopy

***In Vivo* Biology**
Photosynthesis
Plant stress

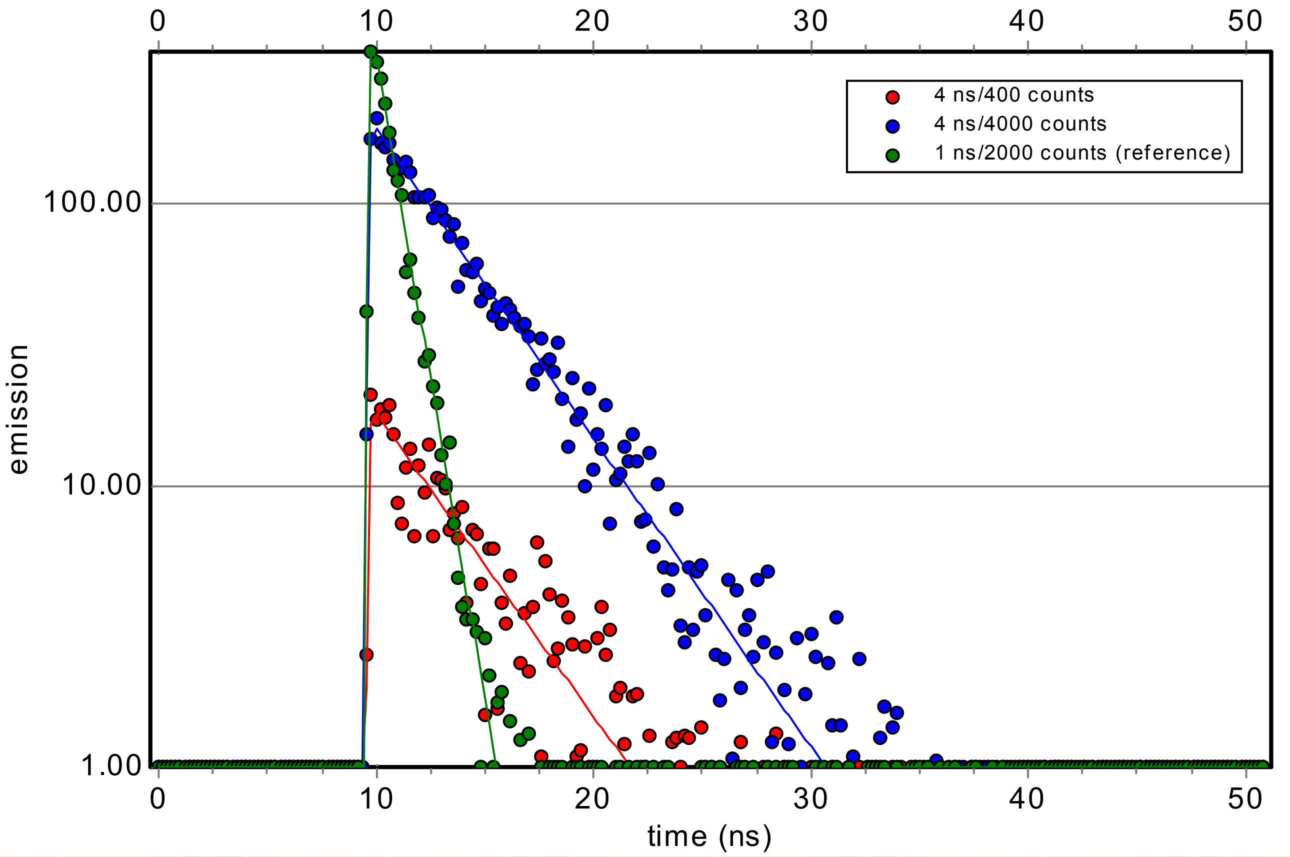




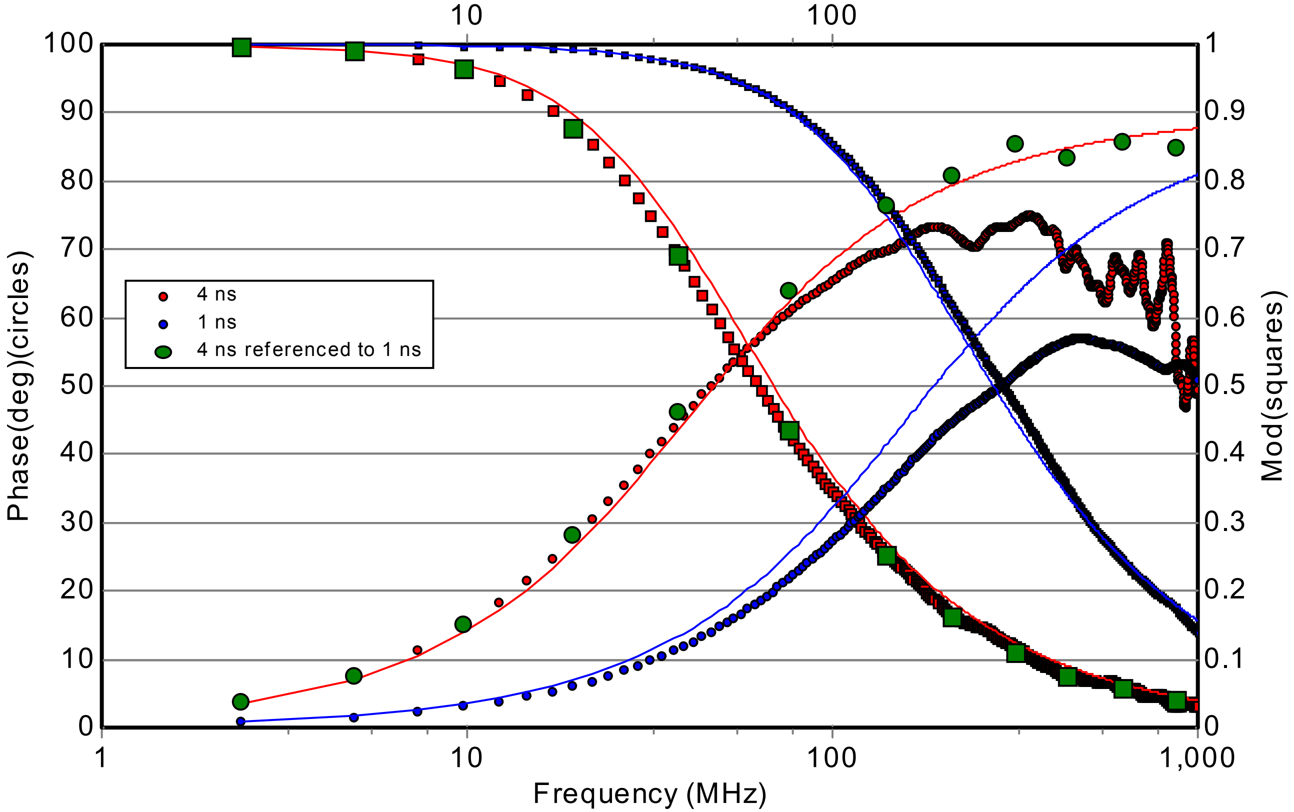
2 lifetimes

Confocal or two-photon FLIM

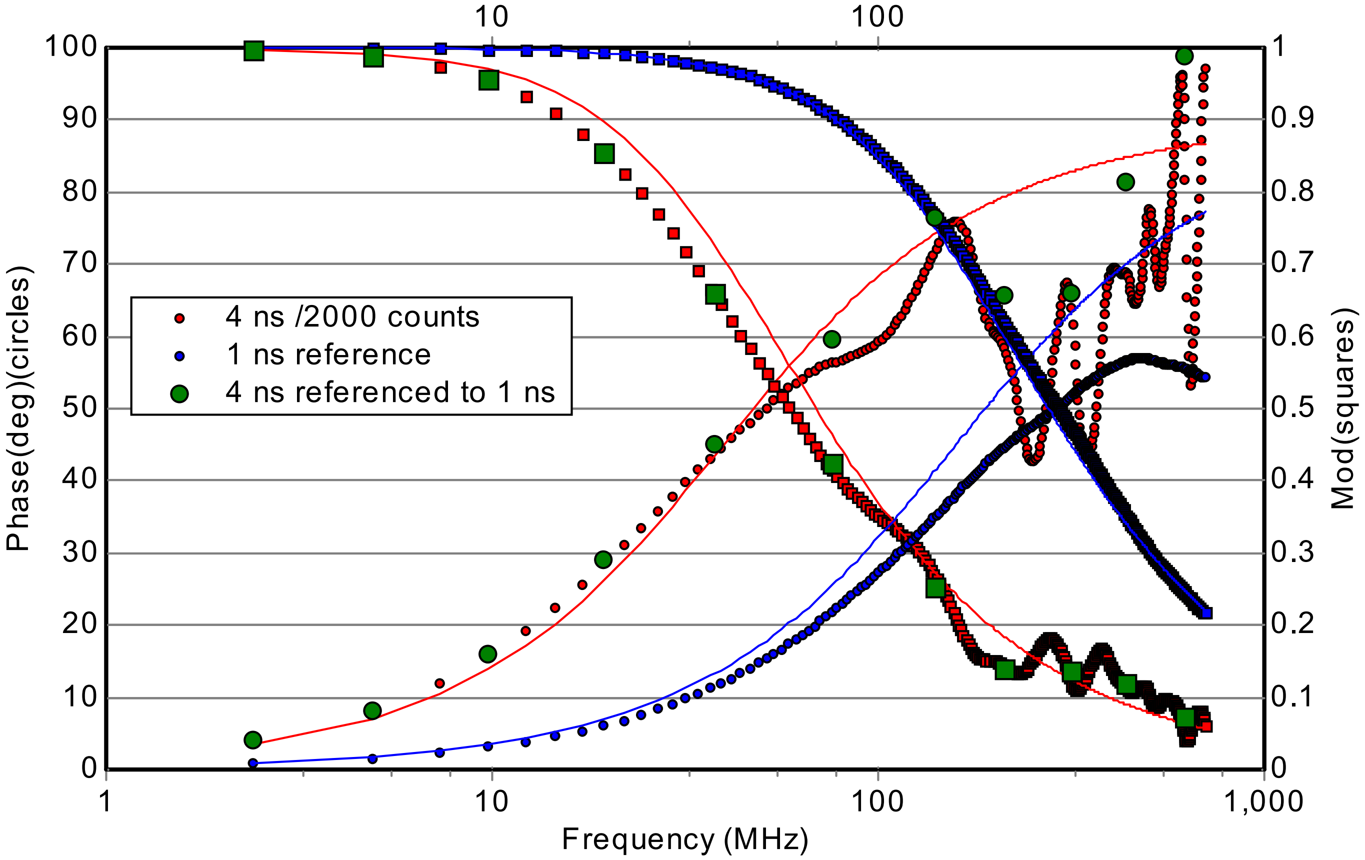
Same as lifetime in the cuvette but at every pixel only few photons are collected



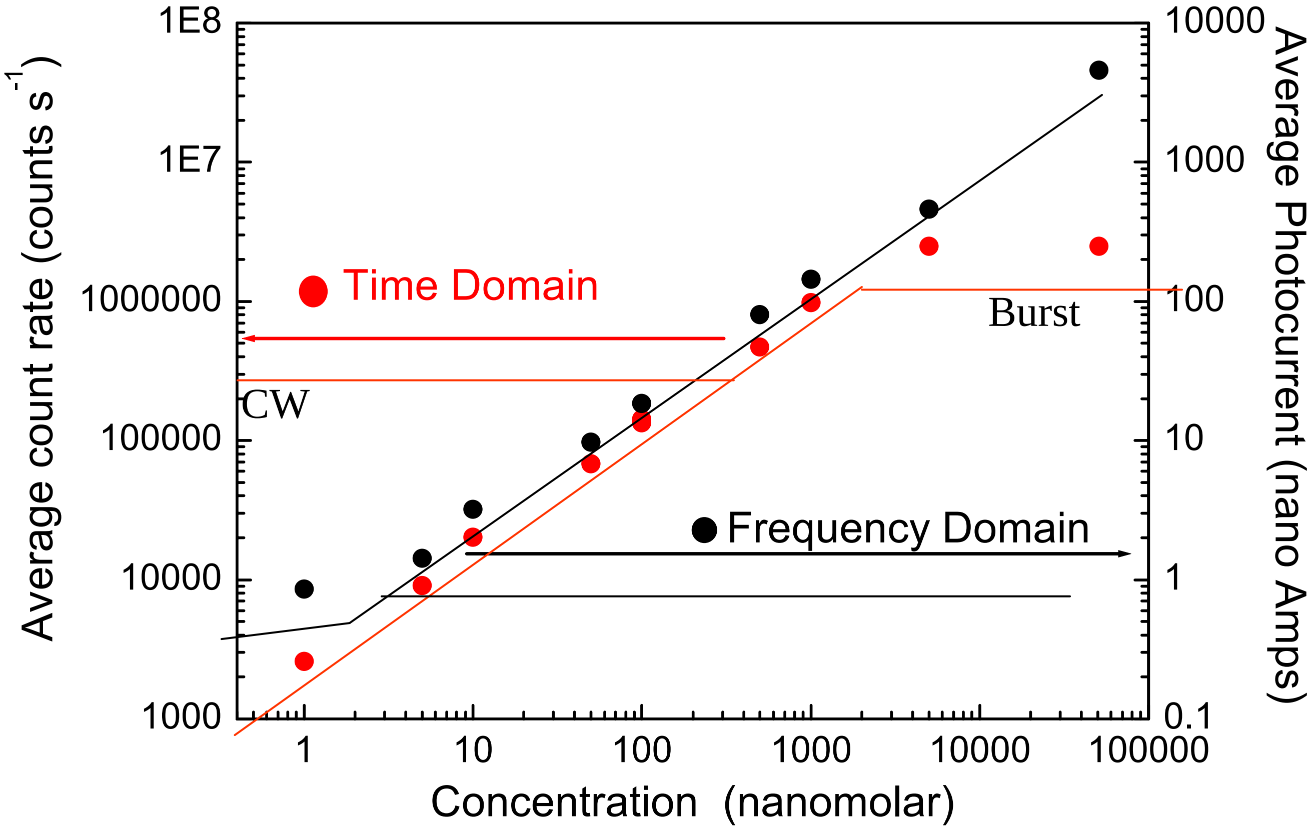
Frequency-Domain analysis simplifies time-domain lifetime evaluation



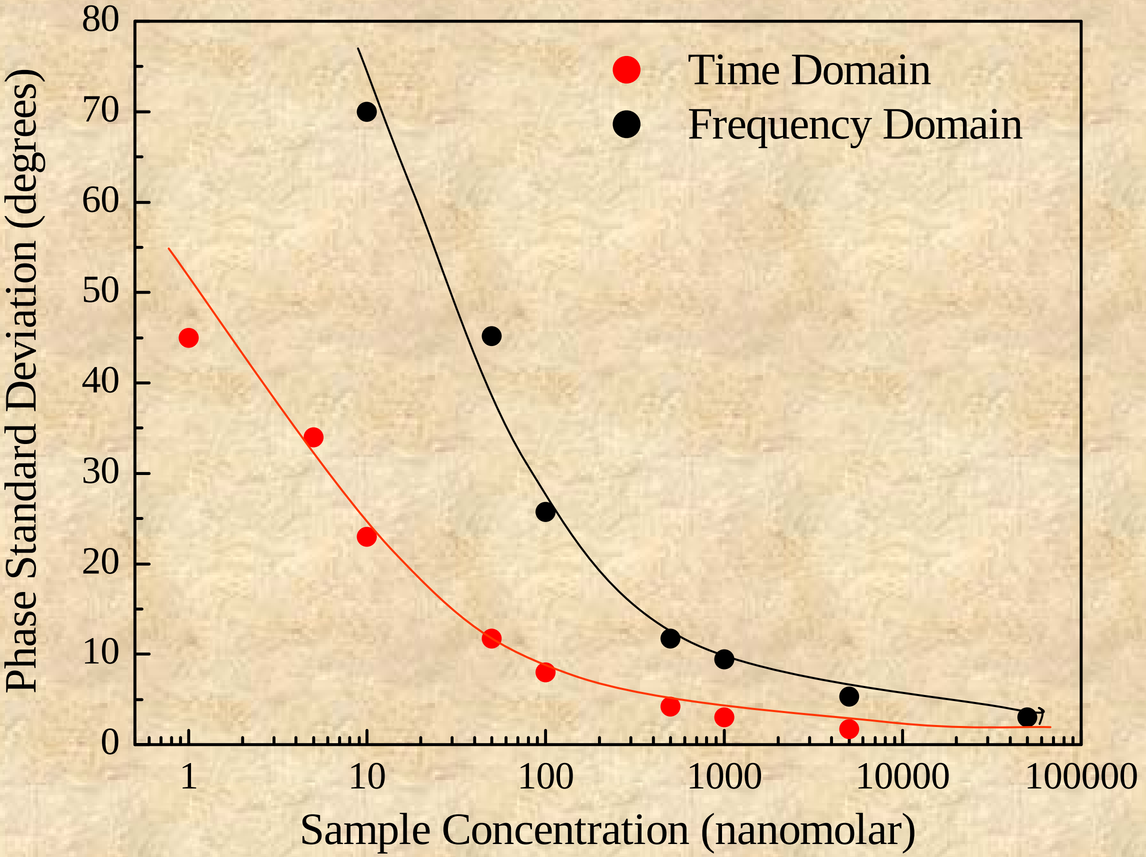
The harmonic content of time-domain data is limited!

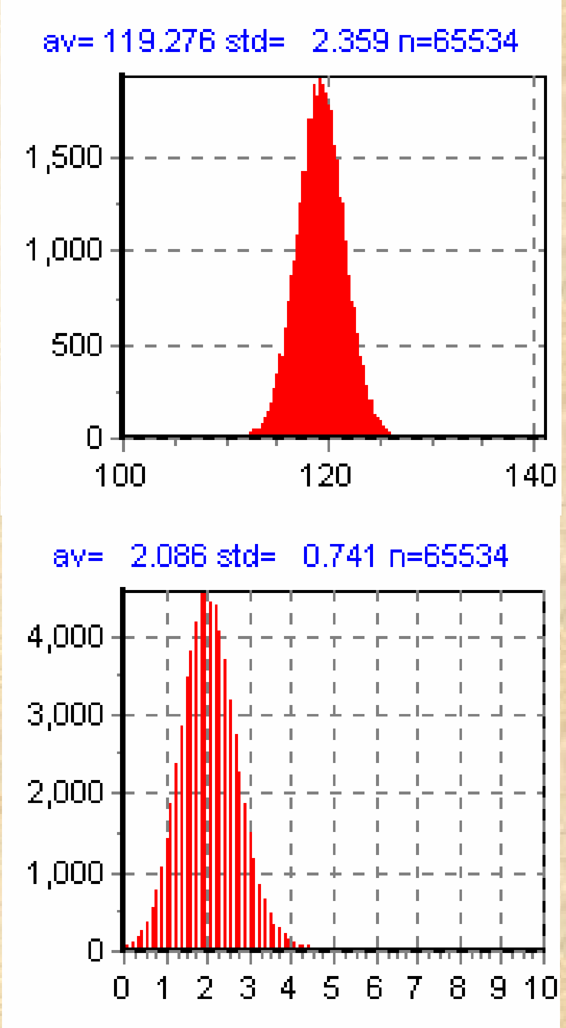


Regions of linearity of time-domain and frequency-domain

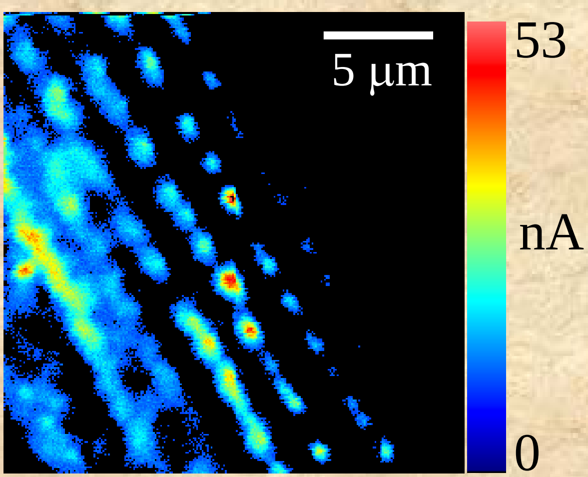


Measurement uncertainties in the time-domain and frequency-domain

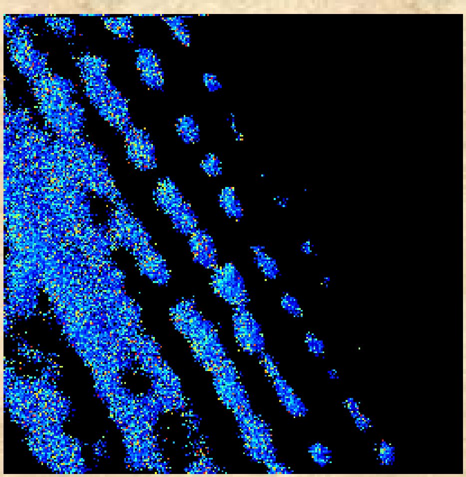




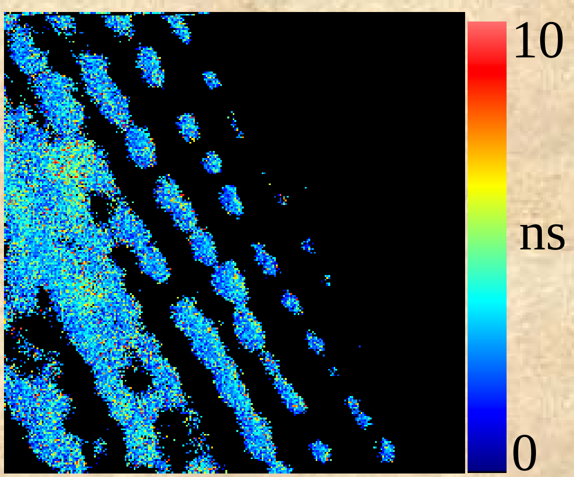
Frequency-domain FLIM in *C. elegans*



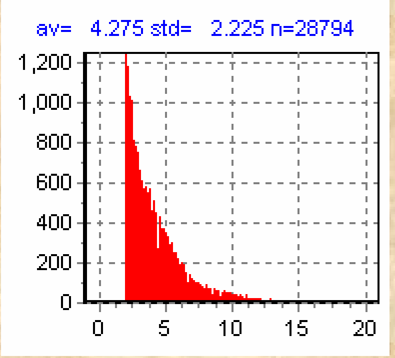
Intensity
(photocurrent)

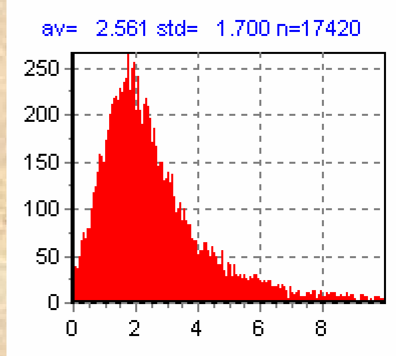


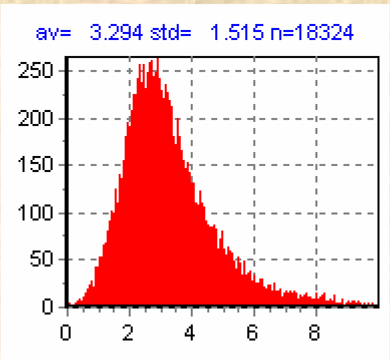
τ_{phase}
lifetime



$\tau_{modulation}$
lifetime

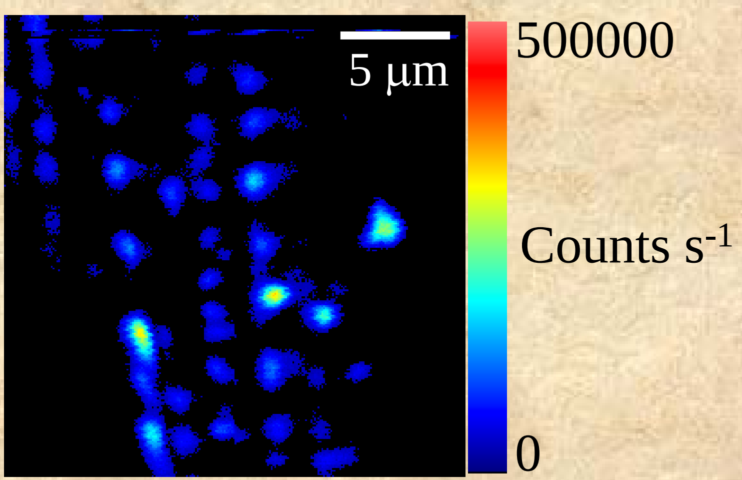




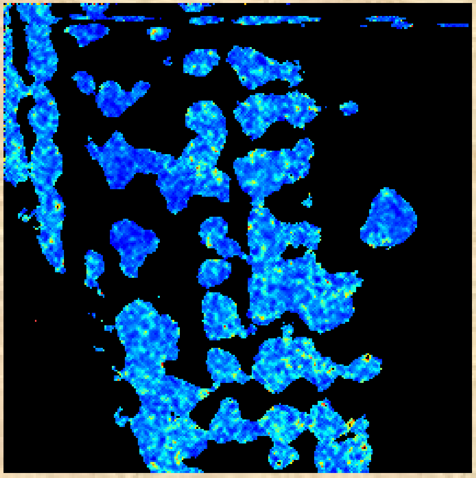


80 MHz modulation frequency, threshold at 2 nA
Pixel time = 0.8 msec

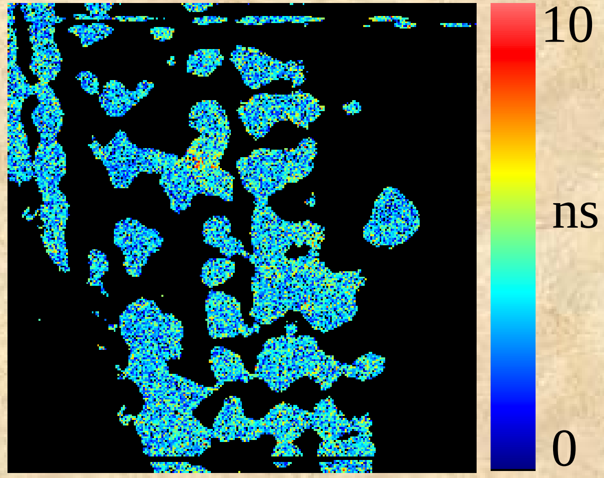
Time-domain FLIM in *C. elegans*



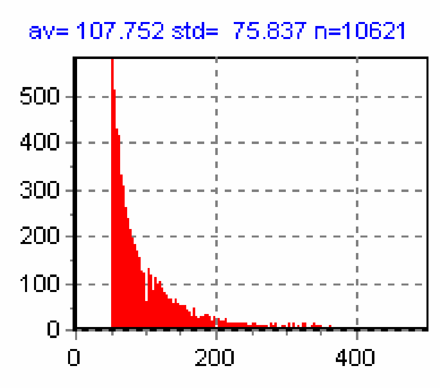
Intensity
(photon counts)

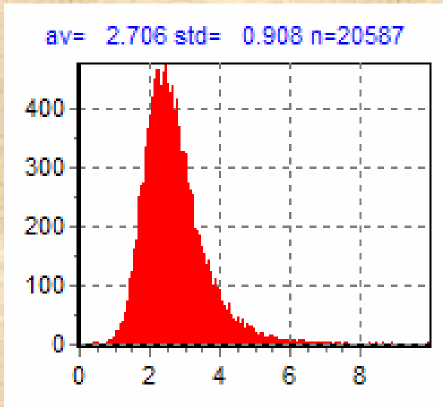


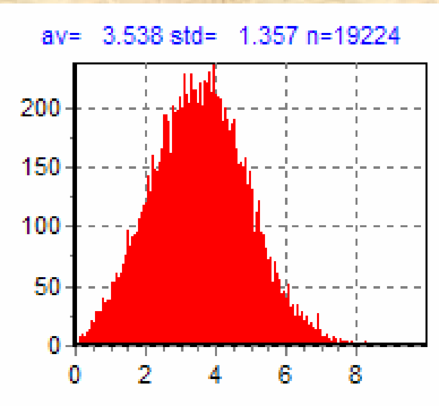
τ_{phase}
lifetime



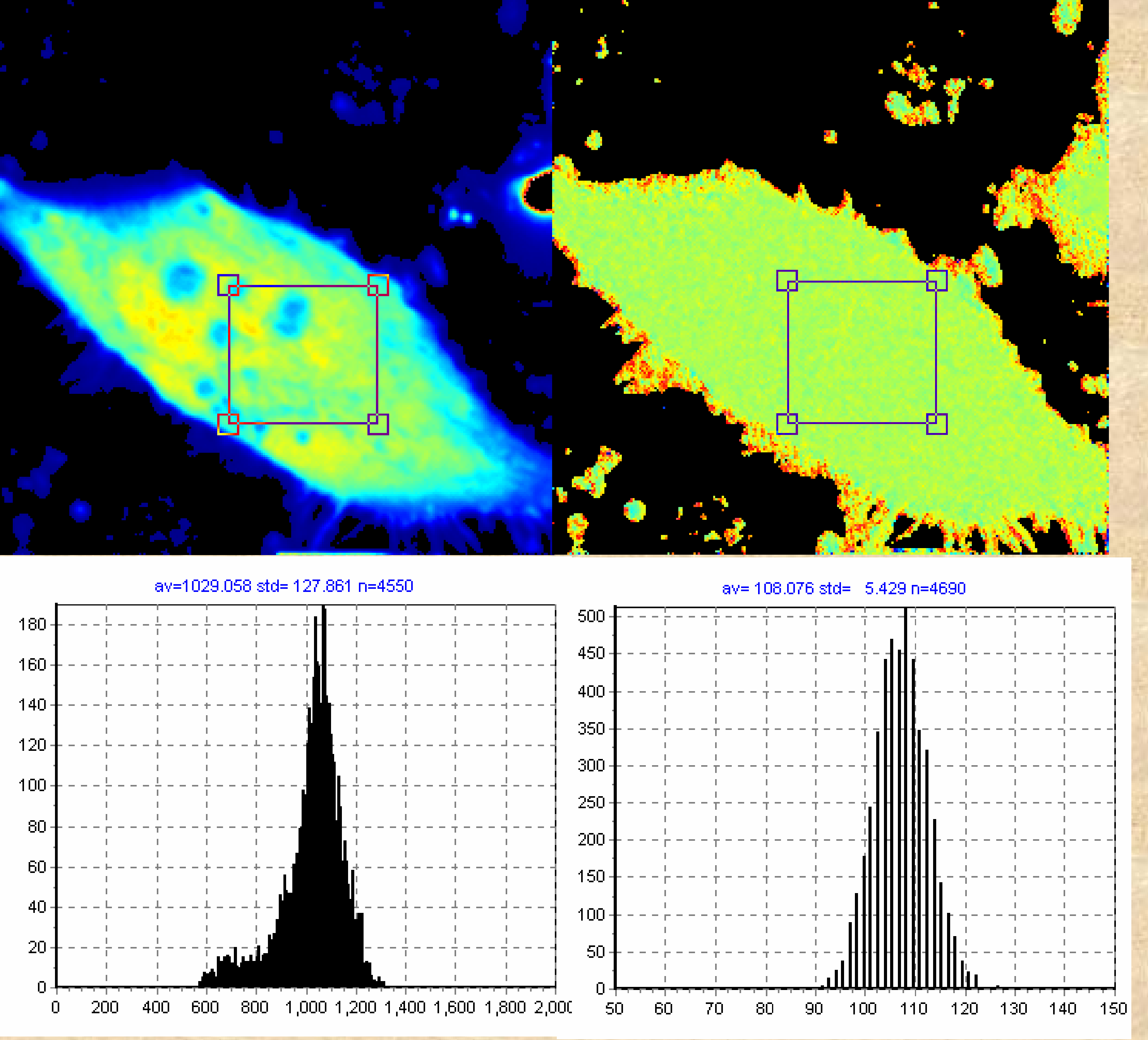
$\tau_{modulation}$
lifetime



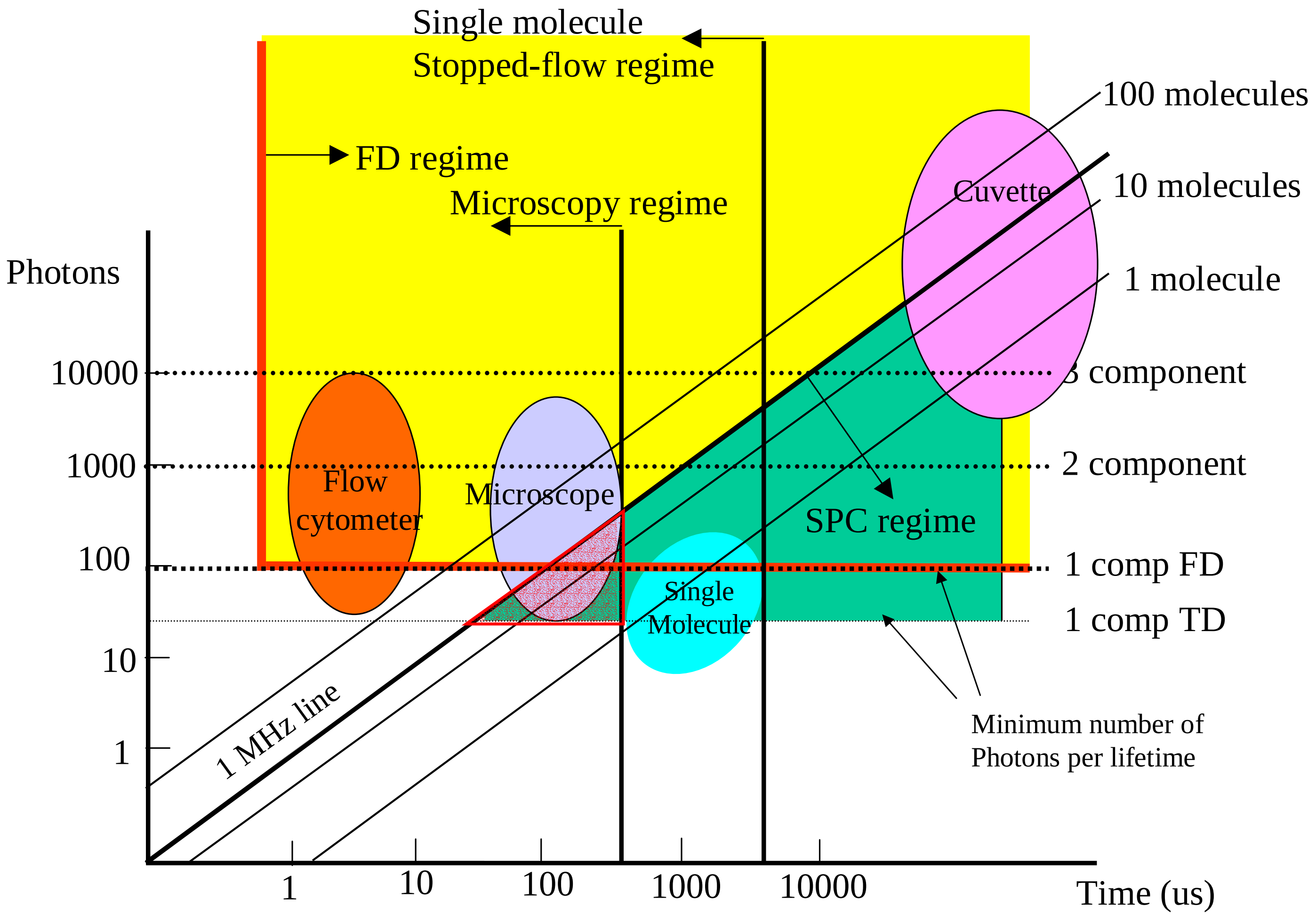




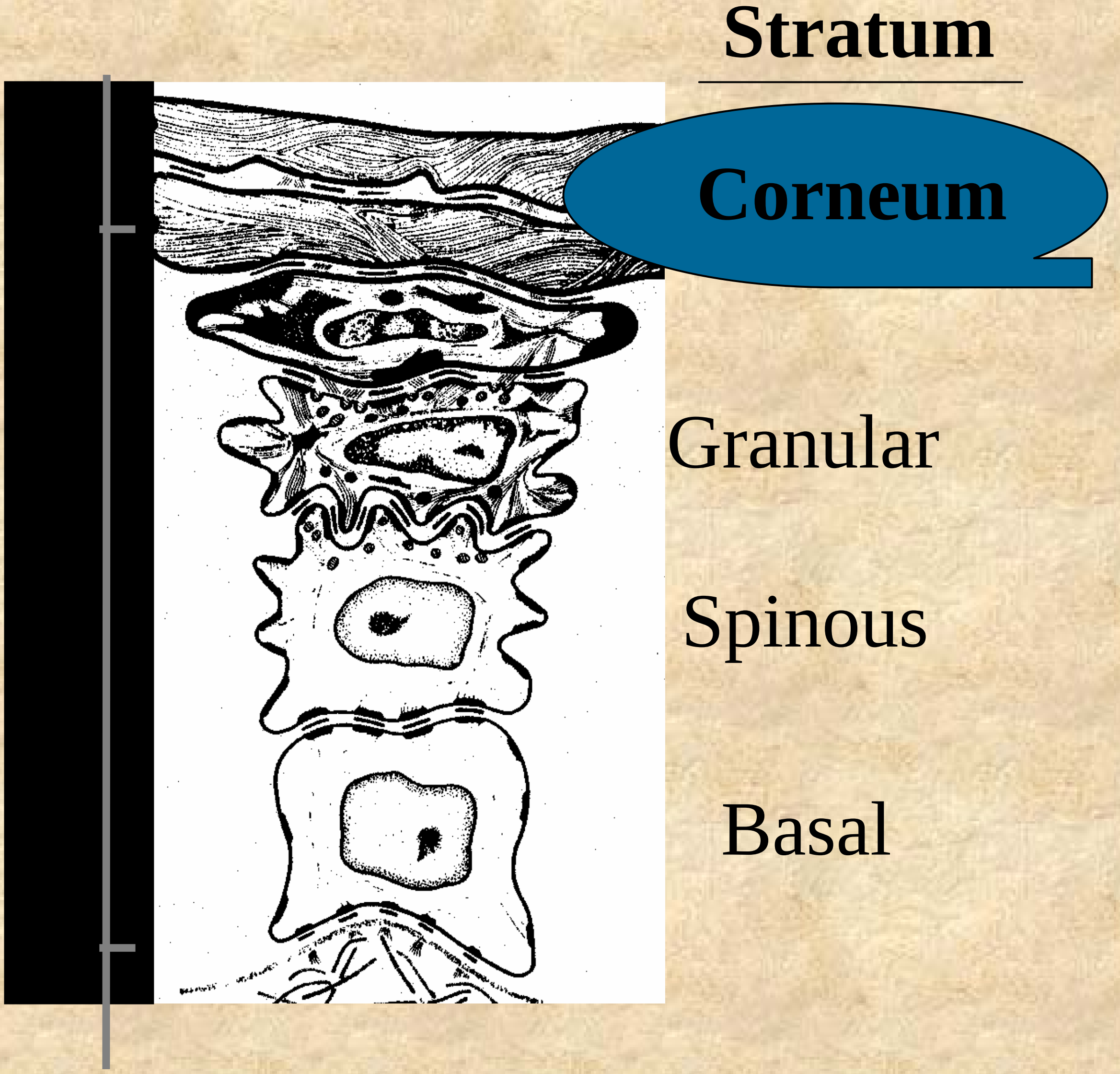
Laser repetition 80 MHz, threshold at 30 counts
Pixel time = 1msec


av=1029.058 std= 127.861 n=4550
0
20
40
60
80
100
120
140
160
180
0
200
400
600
800
1,000
1,200
1,400
1,600
1,800
2,00
av= 108.076 std= 5.429 n=4690
0
50
100
150
200
250
300
350
400
450
500
50
60
70
80
90
100
110
120
130
140
150

Lifetime Measurements limits



Measuring pH in the Stratum Corneum (SC)

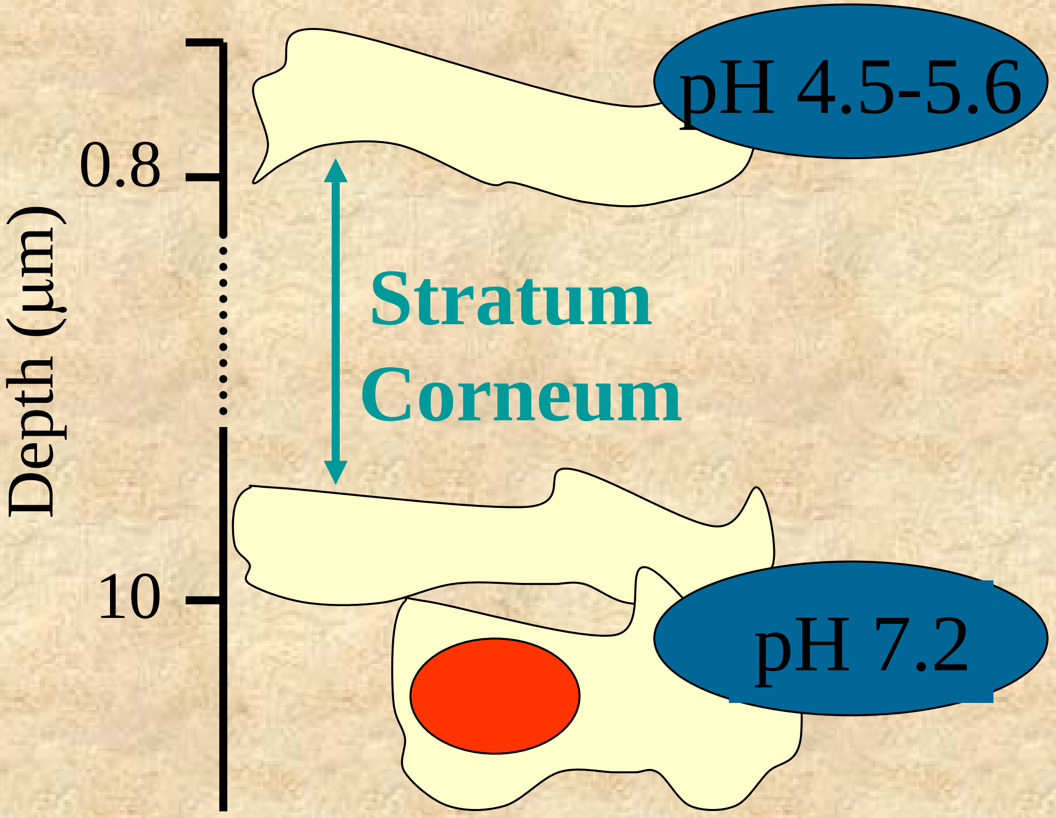


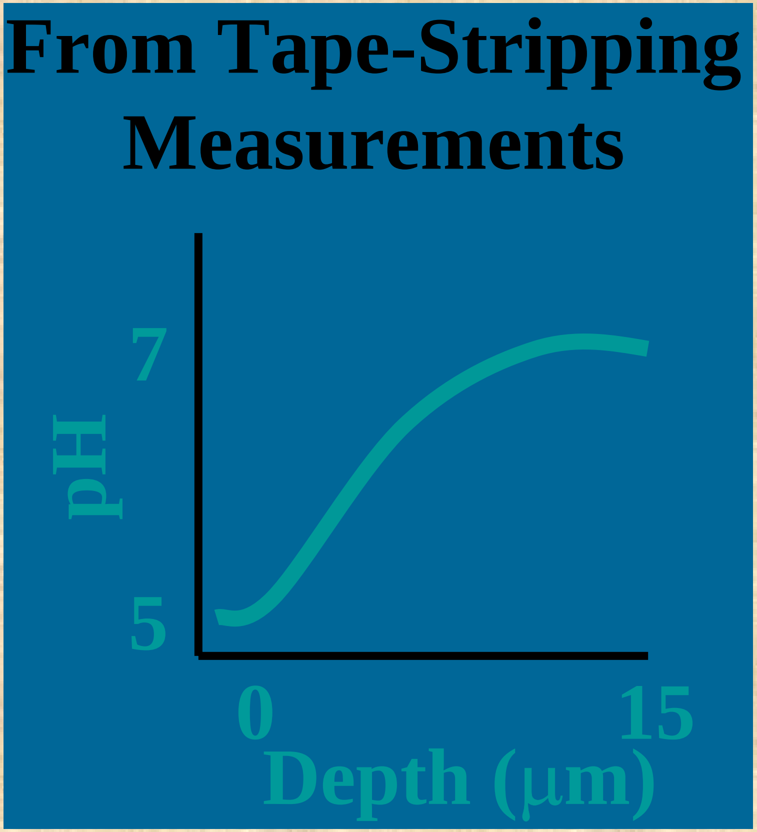
Barrier Function is provided by the SC and is greatly influenced by SC acidic pH

Correct pH Affects

Diaper Rash
Exzema
Drug Penetration
“The Patch”

pH Increases with SC Depth




From Tape-Stripping Measurements

Acta Dermatol. Venereol. 74:375-379 (1994)

With each SC layer

- **Is pH uniform?**
- **Do pockets of variable pH exist?**

Goal: Measure pH on the cellular level

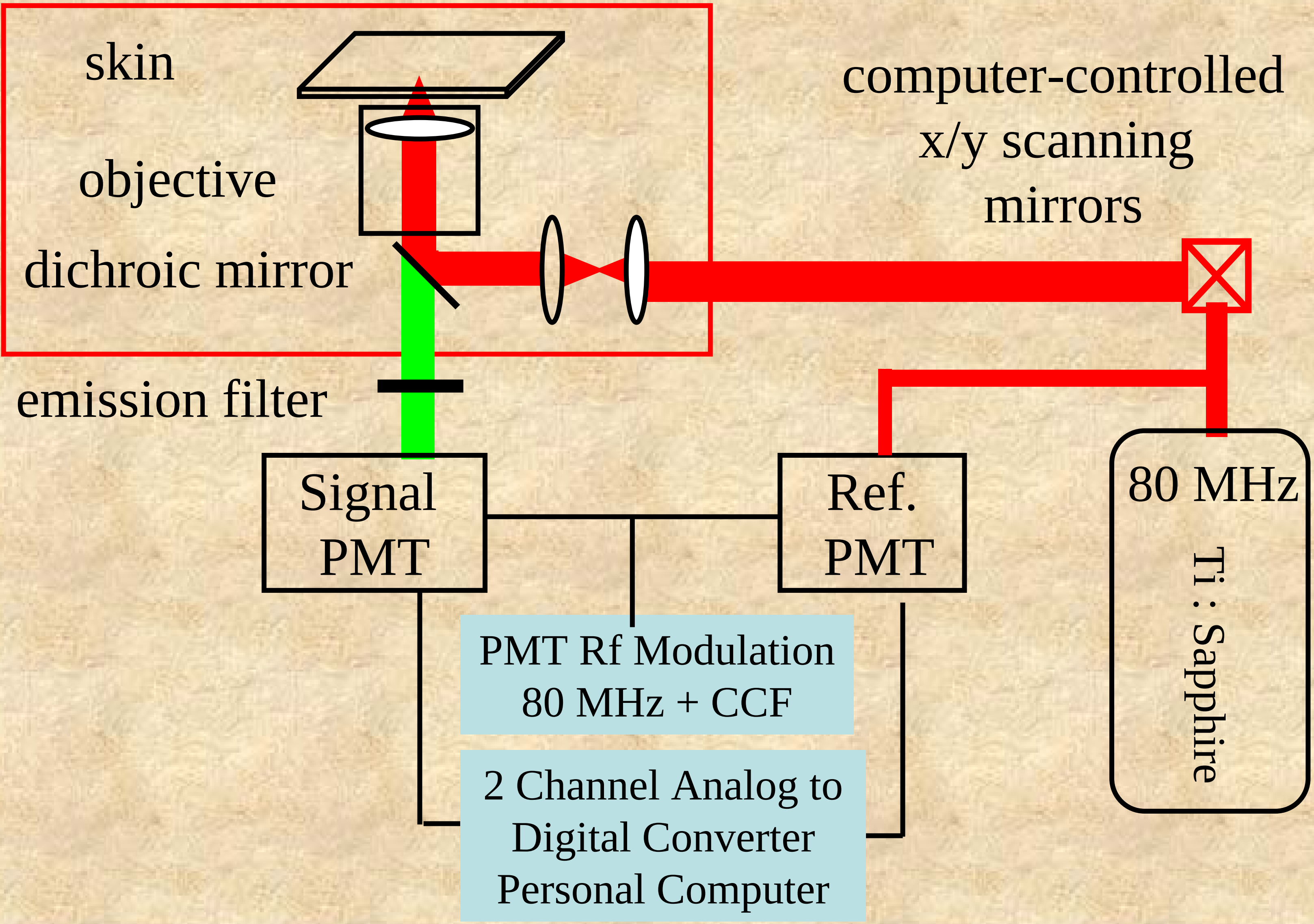
- **Protocol**

1. Two-Photon Fluorescence Lifetime Imaging

Depth penetration
Submicron spatial resolution
Single excitation wavelength
Insensitive to inhomogeneous labeling
Fast
Little photodamage

Instrumentation

Zeiss S100 Axiovert microscope



skin

objective

dichroic mirror

emission filter

computer-controlled
x/y scanning
mirrors

Signal
PMT

Ref.
PMT

80 MHz

Ti : Sapphire

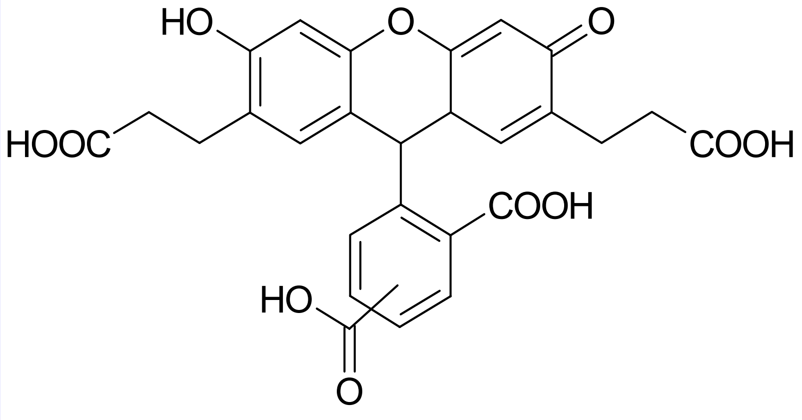
PMT Rf Modulation
80 MHz + CCF

2 Channel Analog to
Digital Converter
Personal Computer

- **Protocol** ***(cont'd)***

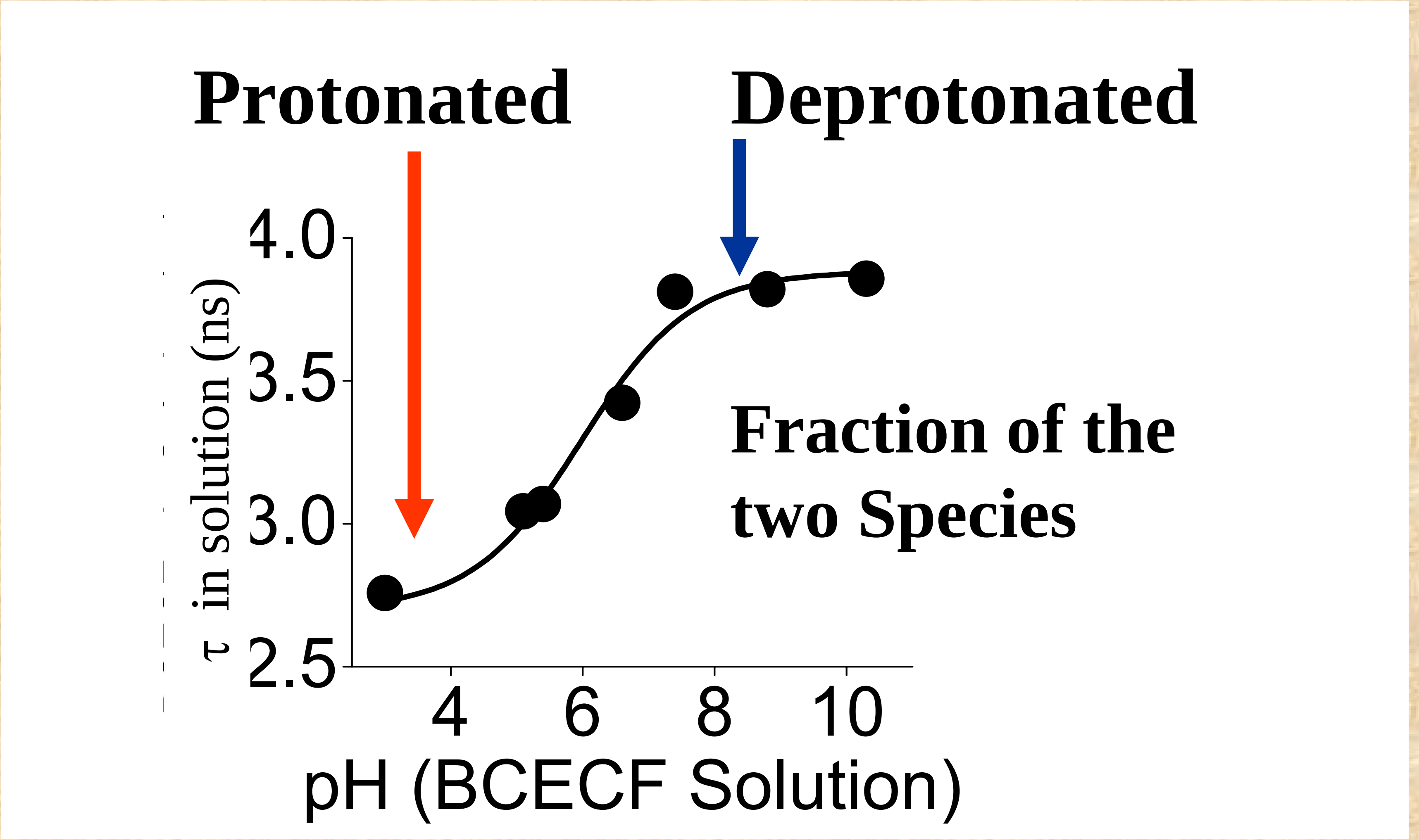
2. Mouse skin w/ the lifetime-sensitive pH probe BCECF

Lifetime Measurements:
Insensitive to Inhomogeneous Labeling



BCECF

Two Different Species Affect BCECF's τ with pH



Protonated

Deprotonated

Fraction of the two Species

Measure the weighted average of the two species

Calculating pH from Lifetime Data

Henderson-Hasselbach using Species Fraction obtained from linear fitting procedure

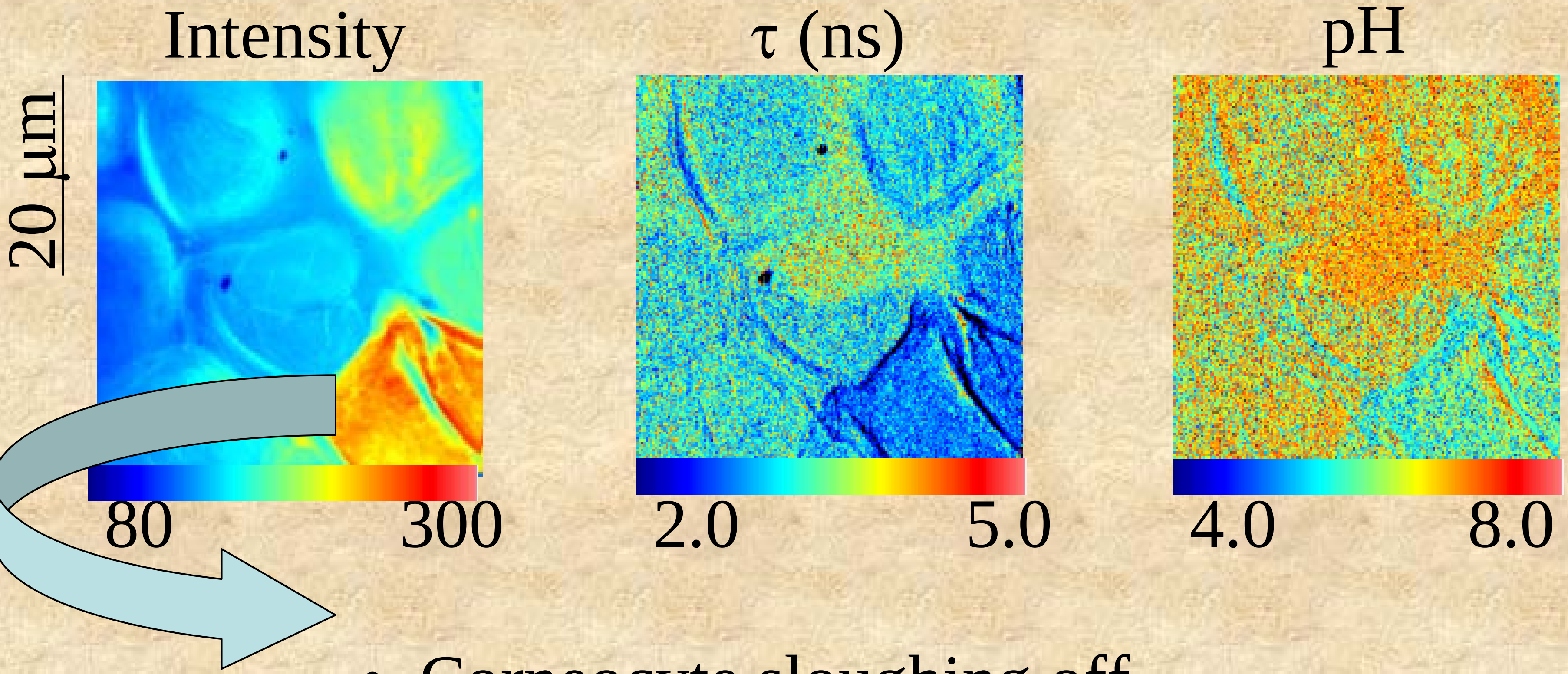
$$\mathrm{pH} = \mathrm{pK}_{\mathrm{a}} + \mathrm{Log}\frac{F_{\mathrm{BCECF}^{-}}}{F_{\mathrm{HBCECF}}}$$

Ion Concentrations are replaced with Species Fraction determined from lifetime data

F Species Fraction

Data: Stratum Corneum Surface

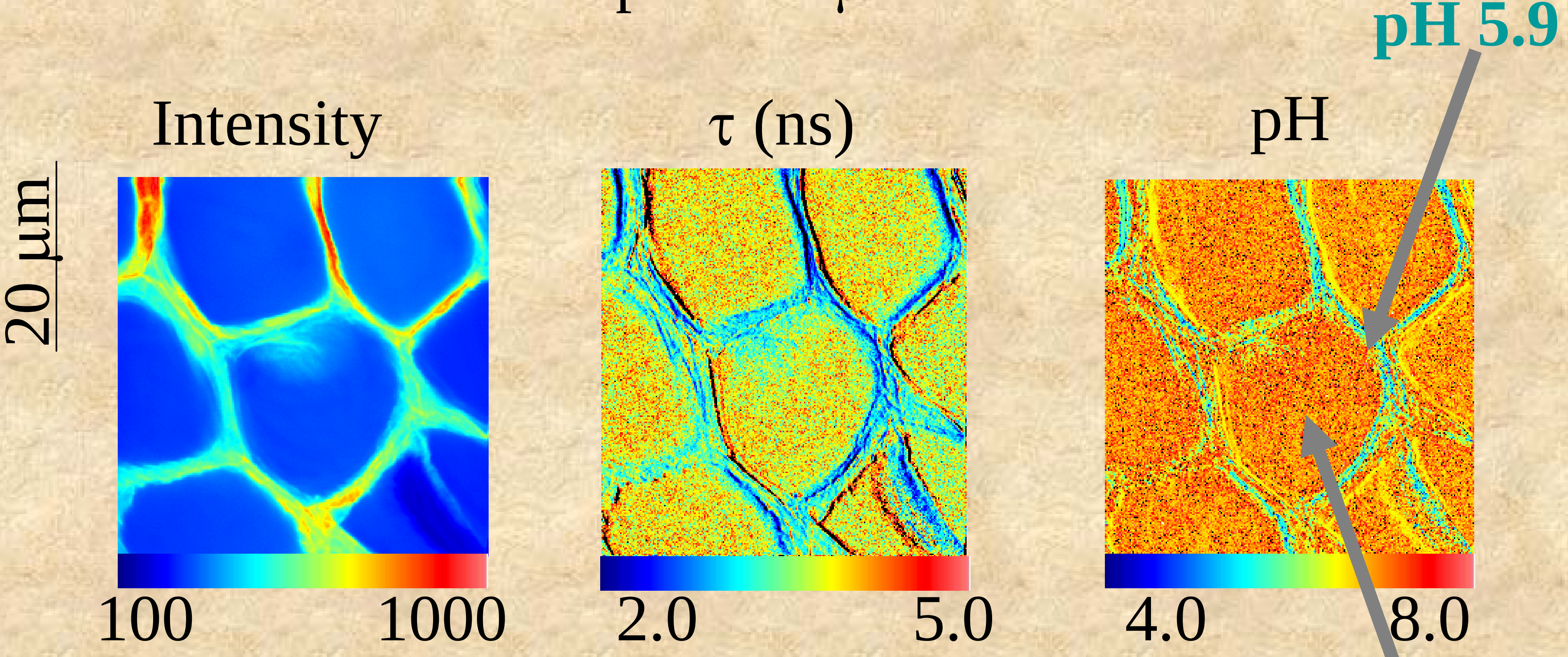
Depth: 0 μm



- Corneocyte sloughing off
- Intensity does not correlate with τ, pH
- **High Intensity does not indicate high pH**

Data: Middle Stratum Corneum

Depth: 5.1 µm



pH 5.9

pH 7

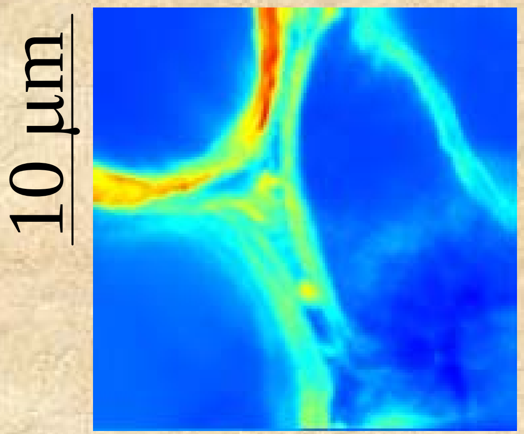
- Low pH found in extracellular matrix
- Corneocytes are neutral

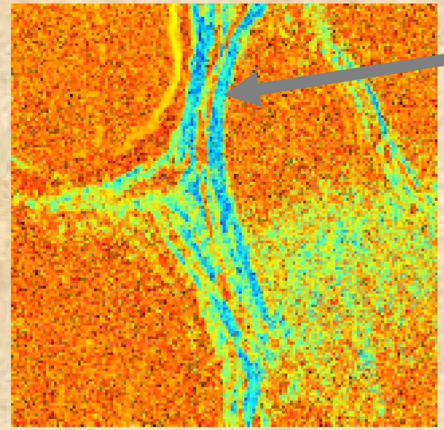
Data: Middle SC & Upper Granular

Intensity

pH

Depth:
6 µm

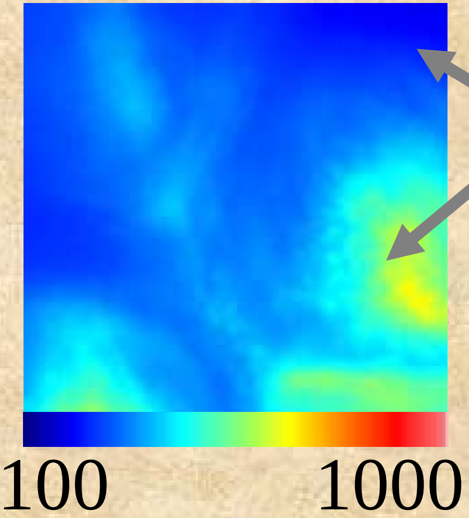




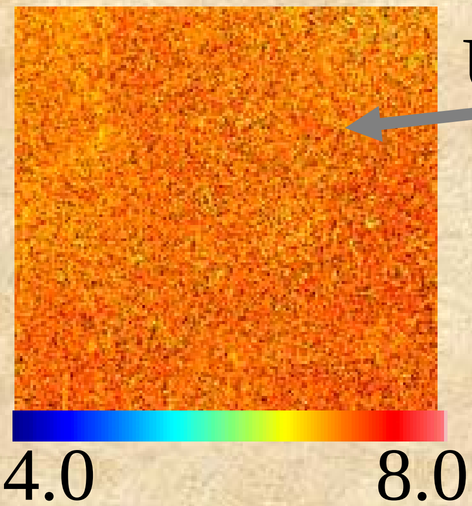
Acidic
Pockets

……

Depth:
17 µm



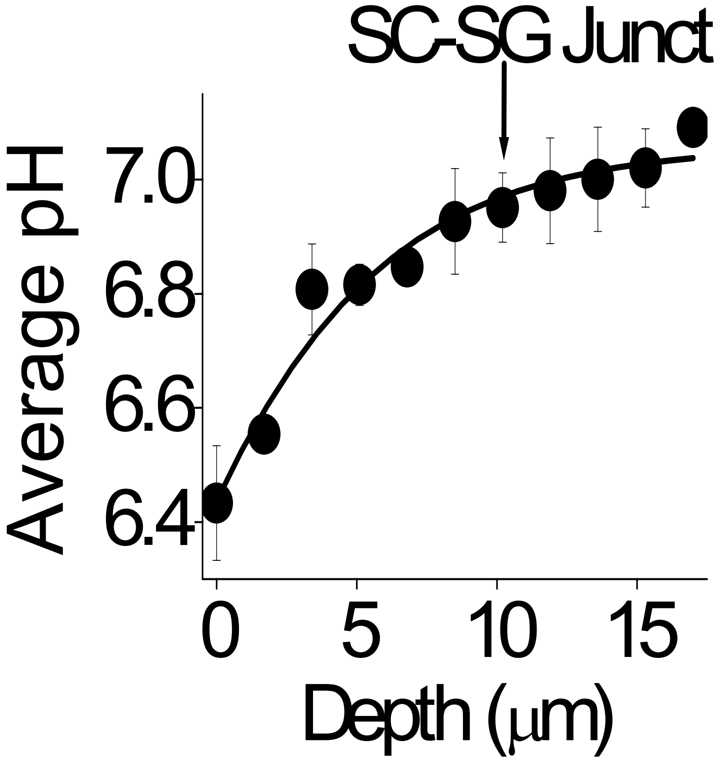
Uneven
Emission



Uniform
pH

Image –vs– Tape-Stripping Data

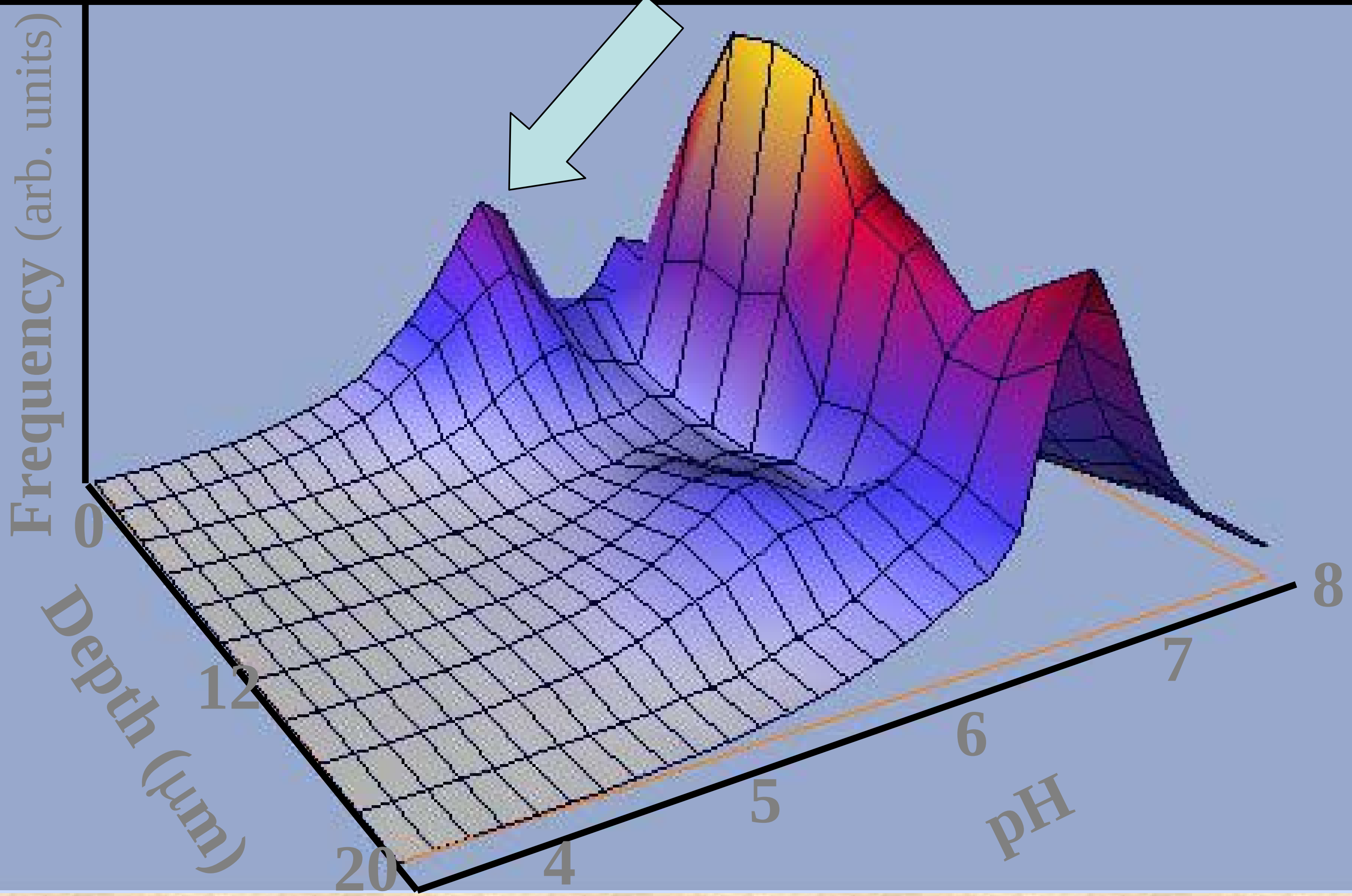
Average pH at Each Depth



- **Average pH data are identical to tape-stripping measurements**

- **Images show pH gradient is due to acidic pockets within extracellular matrix**

Histogram Shows Two pH Values Present

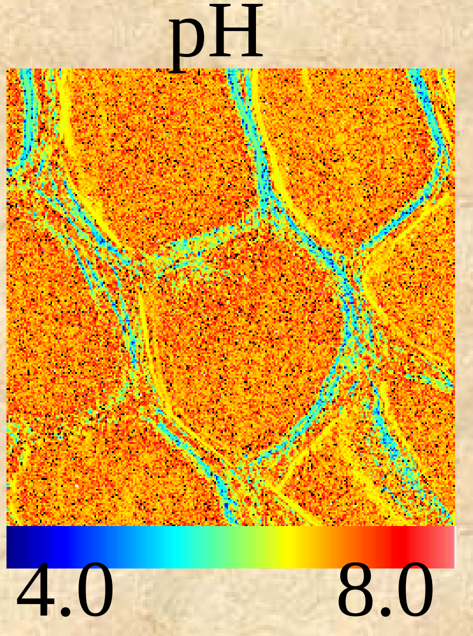


Number of Pockets w/ Acidic pH Decreases w/ depth

Summary of pH Experiments

1. Stratum Corneum is acidic due to pH pockets in extracellular matrix

2. TP-FLIM can be used to detect pH within skin



- Mechanisms: Origins of pH are studied to improve barrier function understanding

  Na$^+$/H$^+$ Antiporter **J. Biol. Chem. 277, 47399-47406 (2002)**

  Sebum, sweat

1. Two-photon fluorescence microscopy can generate useful data and images in HUMAN tissue, which is the ultimate goal for the

  - Medical/Biomedical
  - Pharmaceutical
  - Cosmetic industry

communities.

2. Next Goal: More specific targets within the skin.

General References

- Salmon, E. D. and J. C. Canman. 1998. Proper Alignment and Adjustment of the Light Microscope. Current Protocols in Cell Biology 4.1.1-4.1.26, John Wiley and Sons, N.Y.
- Murphy, D. 2001. Fundamentals of Light Microscopy and Electronic Imaging. Wiley-Liss, N.Y.
- Keller, H.E. 1995. Objective lenses for confocal microscopy. In “Handbook of biological confocal microsocpy”, J.B.Pawley ed. , Plenum Press, N.Y.

On line resource:

Molecular Expressions, a Microscope Primer at:

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