Lecture 8.4:

Imaging Technology

Imaging Technology

- **Confocal imaging microscope**
- **Backscattered confocal imaging**
- **Dual/multi-photons excitation imaging**

Contents

- **Confocal laser scanning microscope (CLSM)**
- **Optical pathways**
- **Optical resolution**
- **Sampling rate (Nyquist Theorem)**
- **Reflection / backscattered imaging**
- **Multi-Photon Microscopy**

Optical Sectioning in Biological Microscopy

Conventional light microscopy doesn't work well on thick (> few microns) specimens

Fluorescently labeled sea urchin eggs

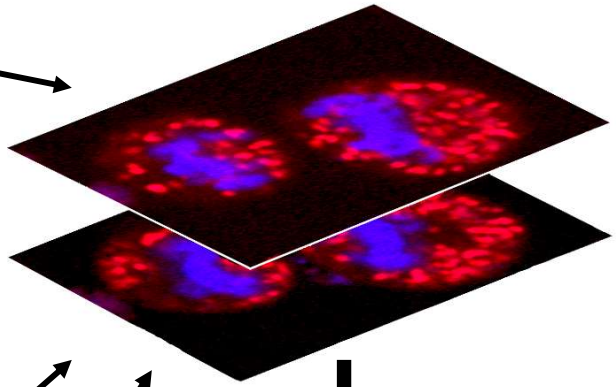
Live specimens

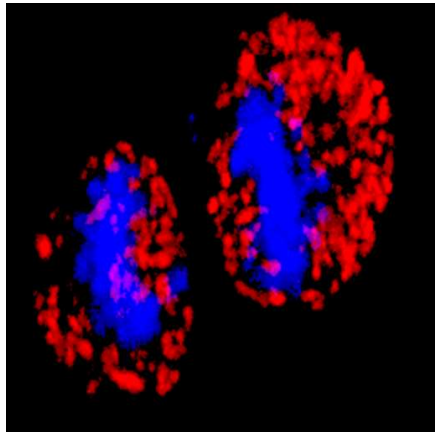
Widefield Fluorescence → Fixation and Physical Sectioning

Widefield Fluorescence → Deconvolution Methods (Computational)

Confocal Microscopy → Confocal Aperture

Multiphoton Microscopy → **nonlinear processes**





Learning Objectives

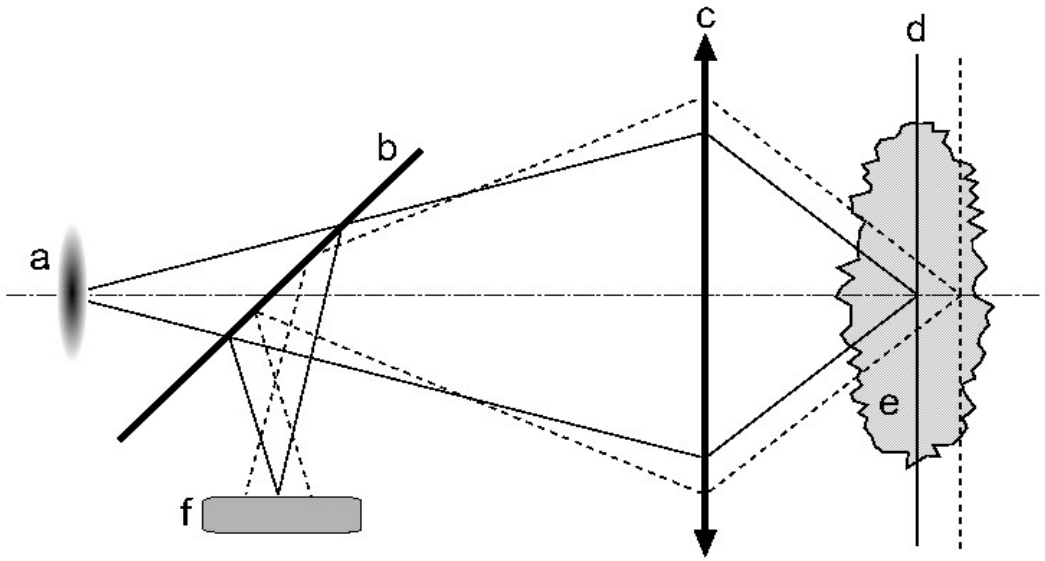
Understand:

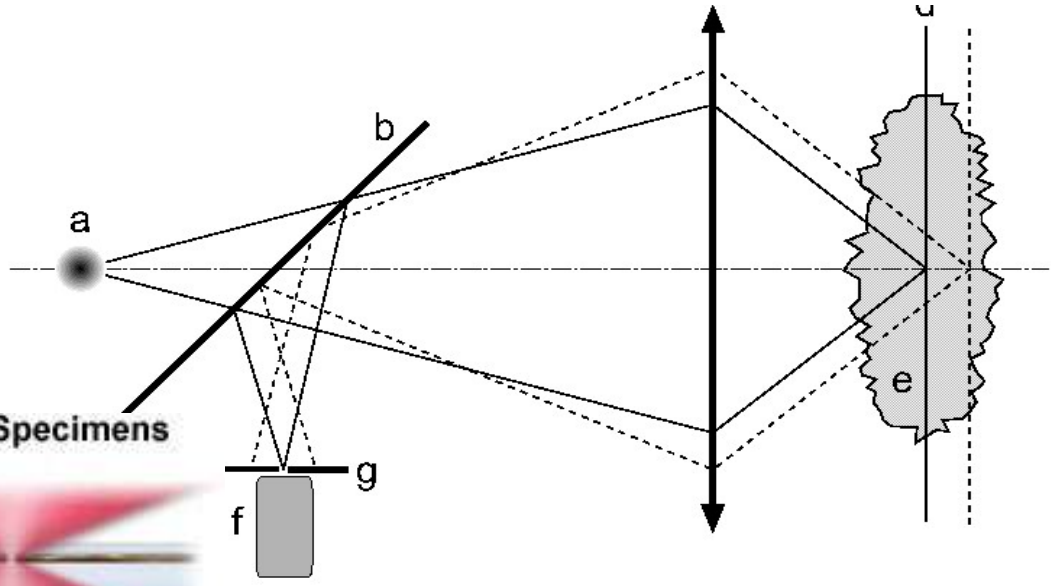
- **Components of a confocal microscope system**
- **Optical pathways used in systems**
- **Optical resolution - Airy disks**
- **Reflected light/backscatter imaging**

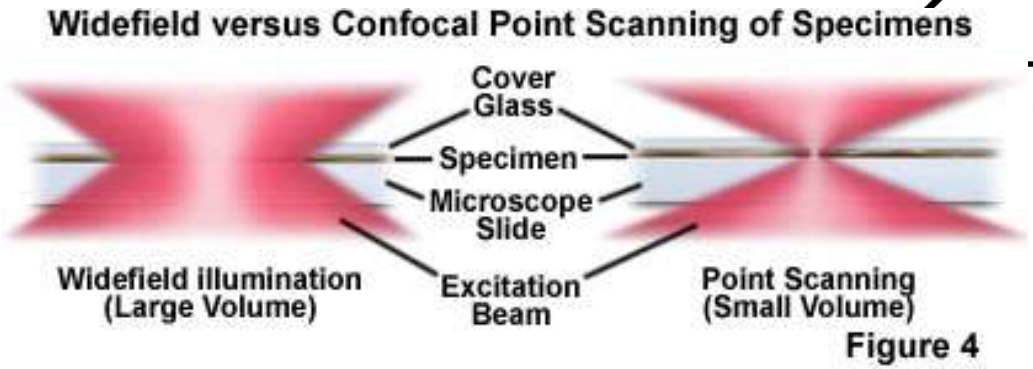
Benefits of Confocal Microscopy

- Reduced blurring of the image from light scattering
- Increased effective resolution
- Improved signal to noise ratio
- Clear examination of thick specimens
- Z-axis scanning
- Depth perception in Z-sectioned images
- Magnification can be adjusted electronically

Wide-field microscopy





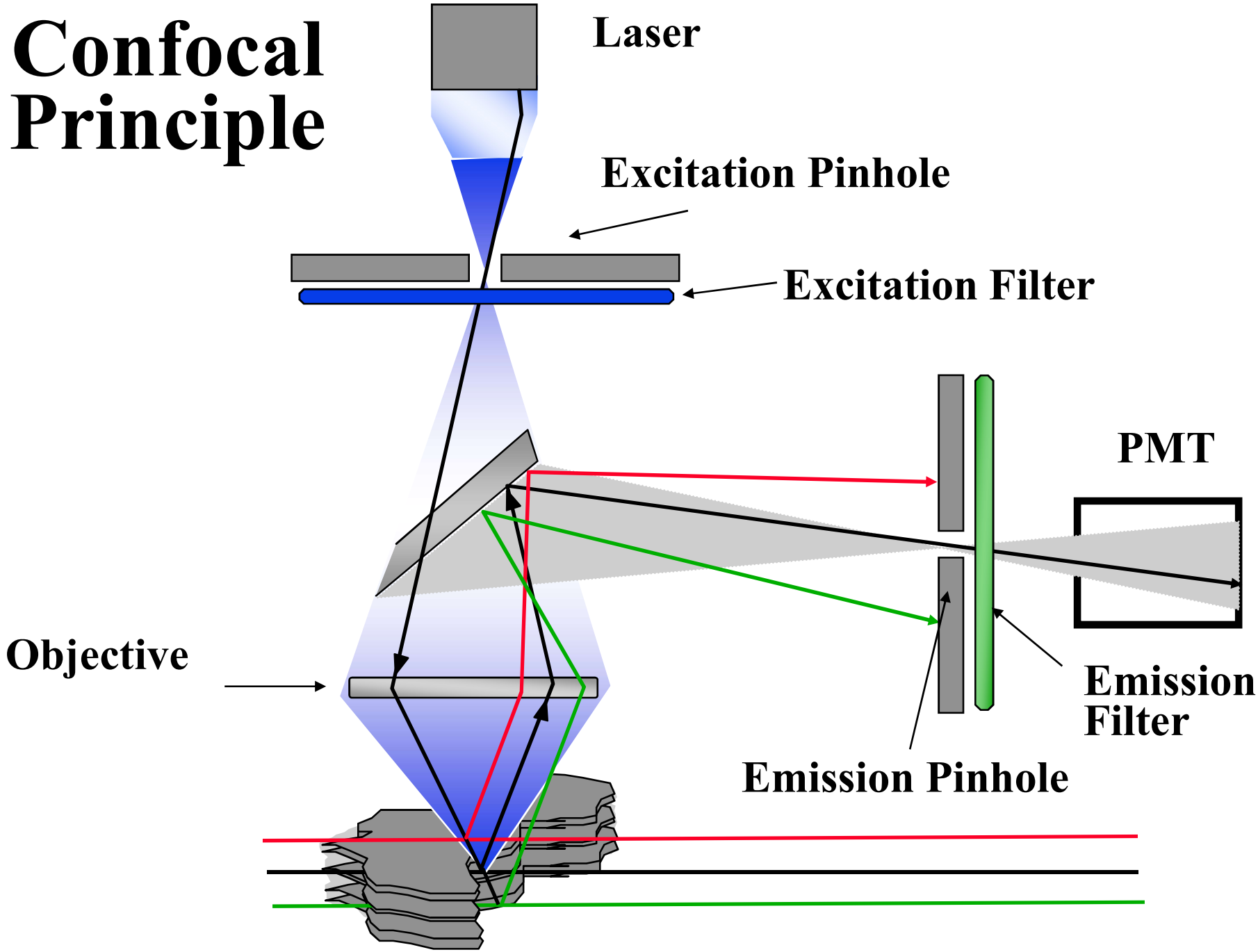


Confocal microscopy

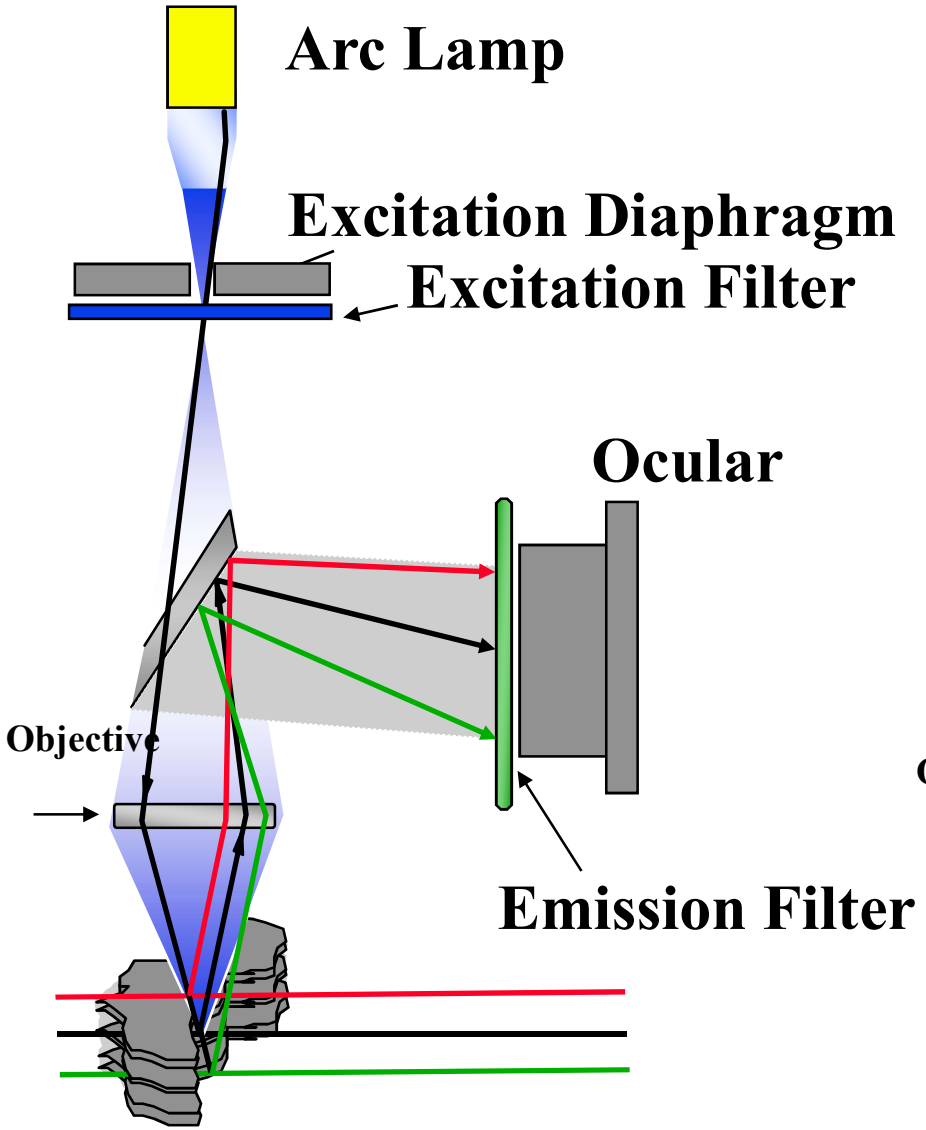
Basic principle

Light source of conventional microscope is field source. Image for each point from sample is interfered by diffraction or scattering. CLSM scans each point on focal plane through pinhole. The point on sample is illuminated and imaged at detector via. pinhole. The detector (PMT or CCD) accepts the image point-by-point. The point out off focal plane cannot be imaged at detector. Thus the final image is cross-section in horizontal of the sample. The shortage of image blur for conventional microscope is overcome accordingly.

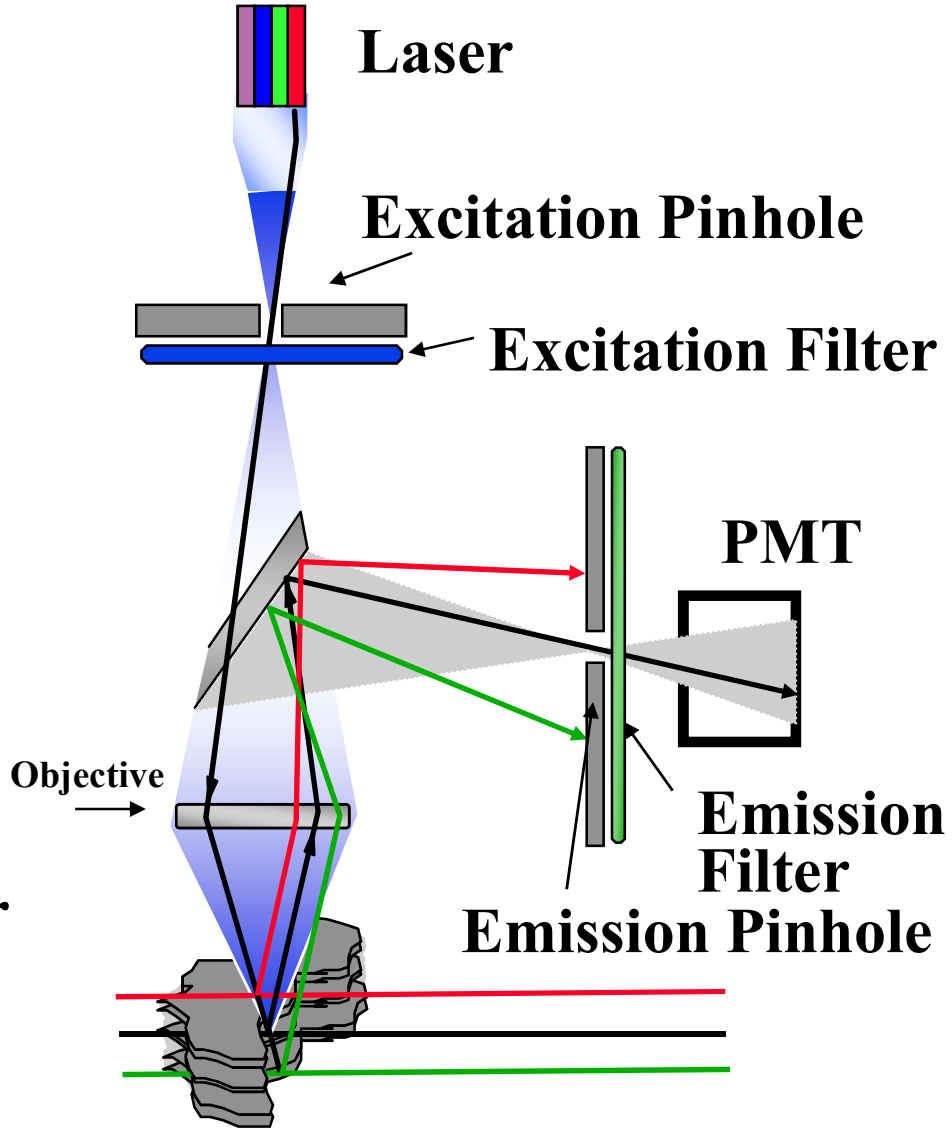
Confocal Principle



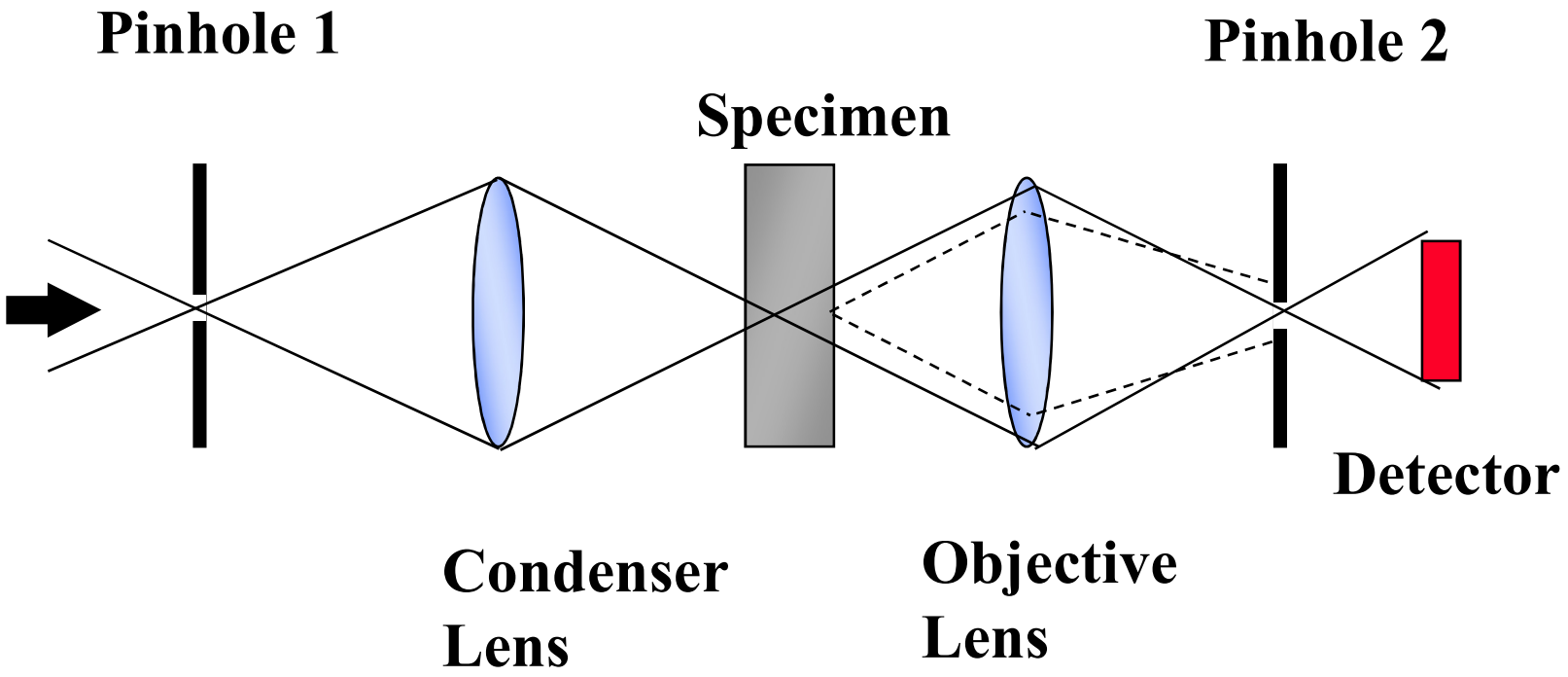
Fluorescent Microscope



Confocal Microscope



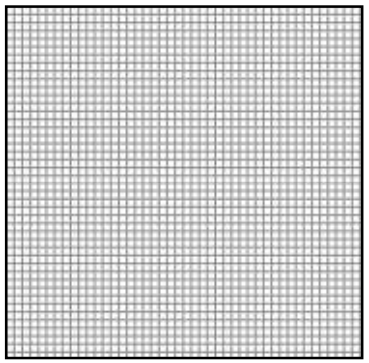
How a Confocal Image is Formed



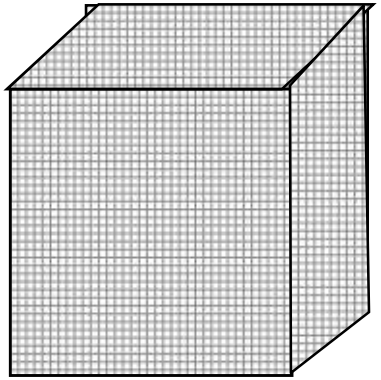
Modified from: Handbook of Biological Confocal Microscopy. J.B.Pawley, Plennum Press, 1989

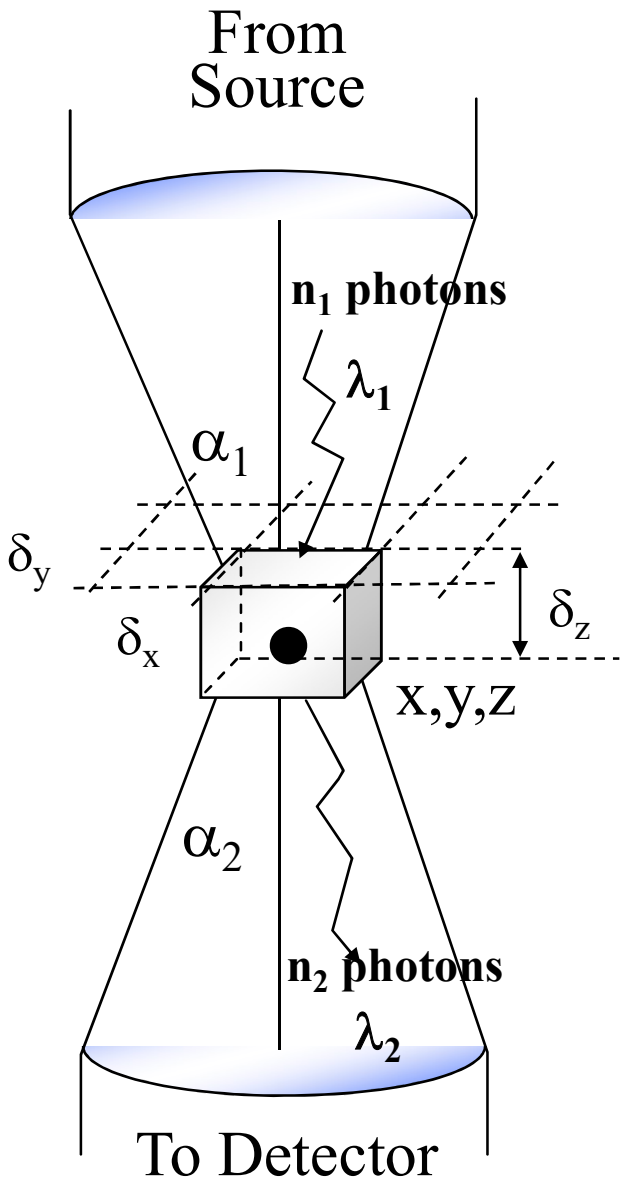
Fundamental Limitations of Confocal Microscopy

PIXEL
2D space



VOXEL
3D space





From: Handbook of Biological Confocal Microscopy. J.B.Pawley, Plennum Press, 1989

Confocal microscope vs. widefield microscope

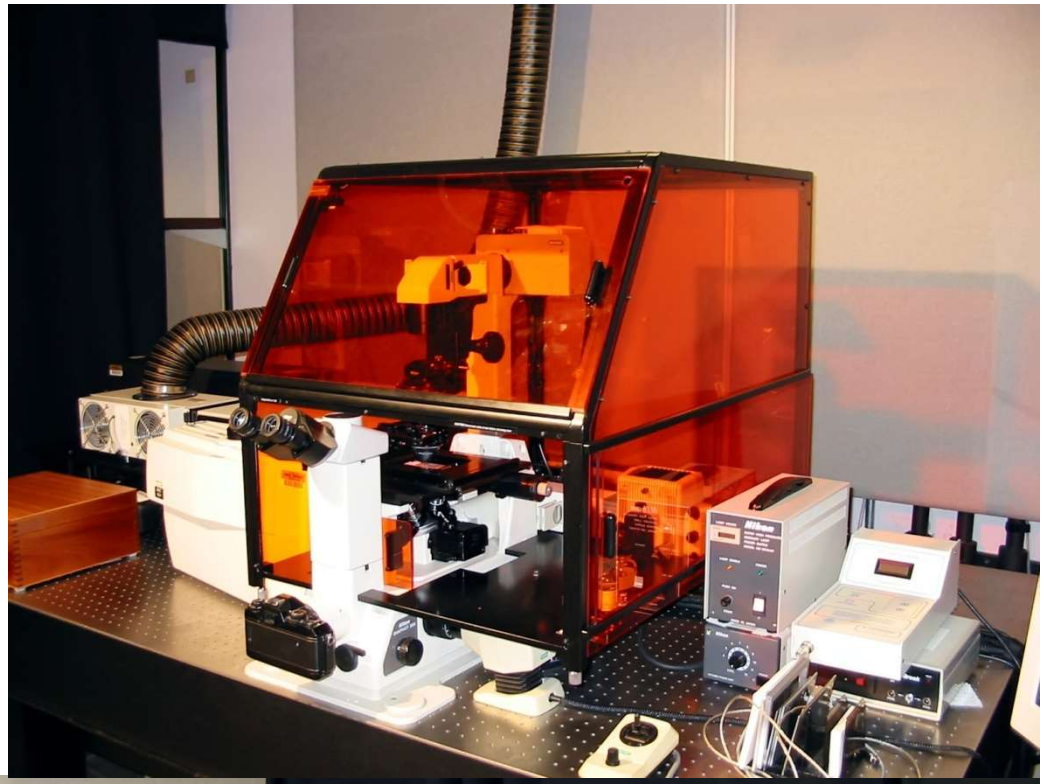
Widefield

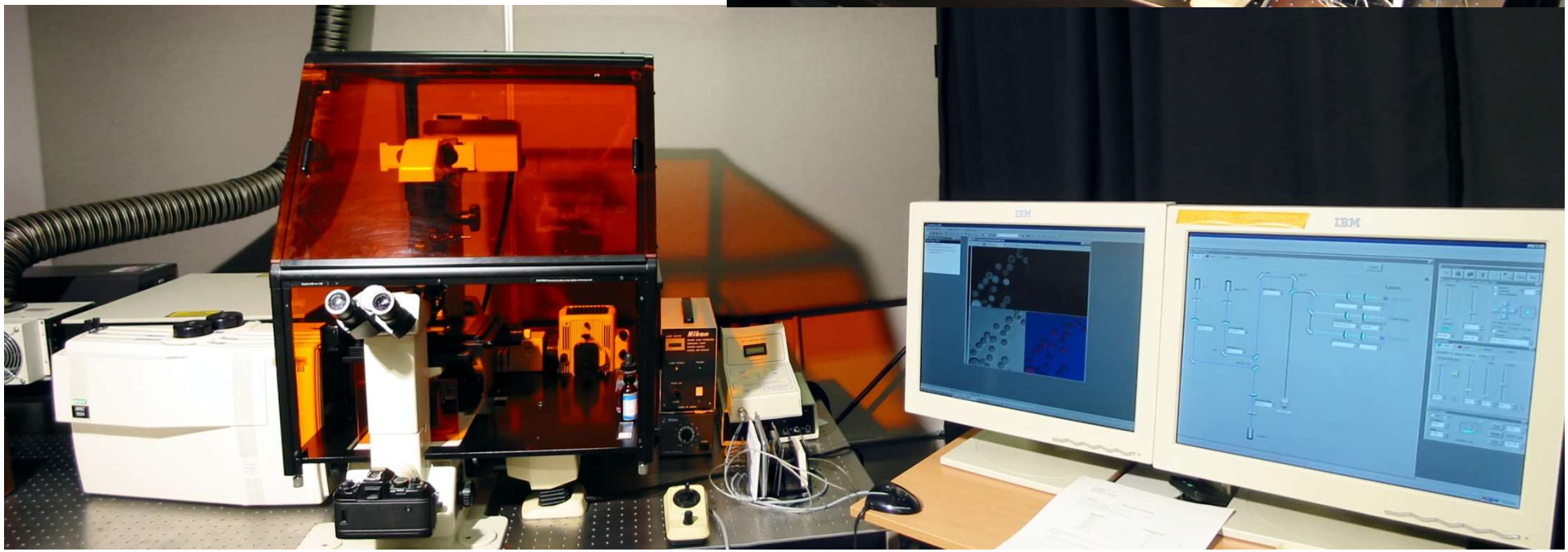
- The whole picture is taken at once
  - Eyepiece image
  - Potentially fast imaging
  - High photodamage
- High background noise (secondary fluorescence)
- "Cheap"

Confocal

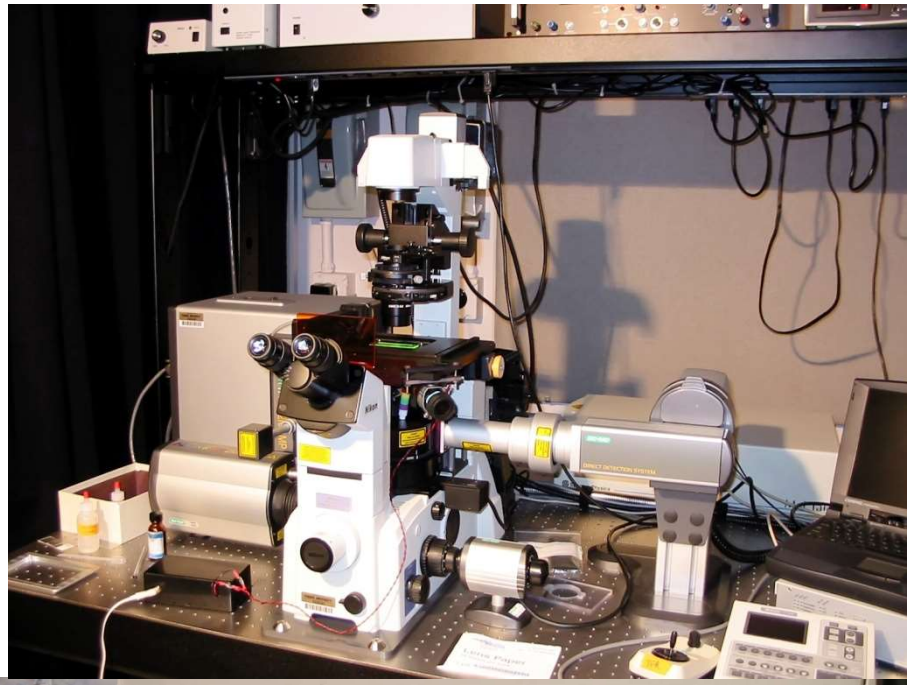
- Thin sections (.5-1.5µm)
- Max thickness ~50µm
- High contrast and definition
- Reduced photo-damaged
- Scanning
  - → "slow"
  - → "-" eyepieces
  - → digital zooming
- Limited number of laser colors
- expensive

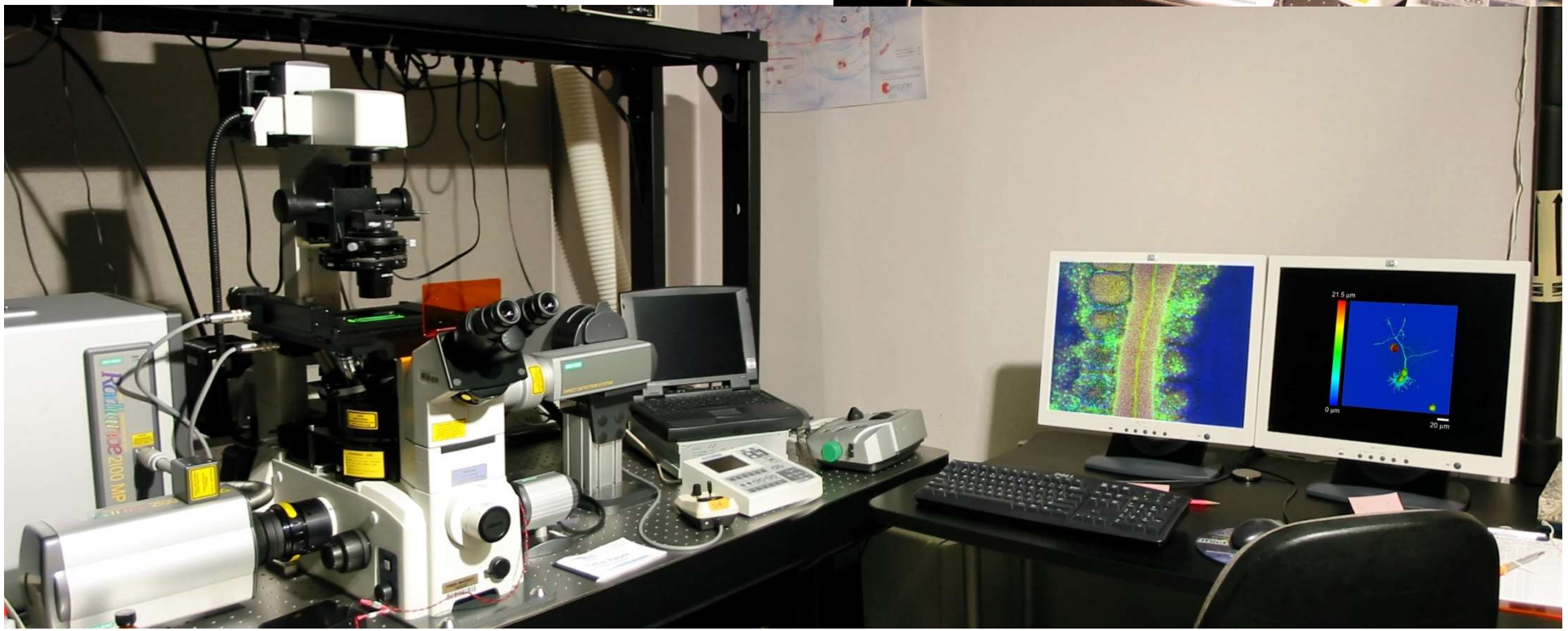
Bio-Rad MRC 1024 at Purdue



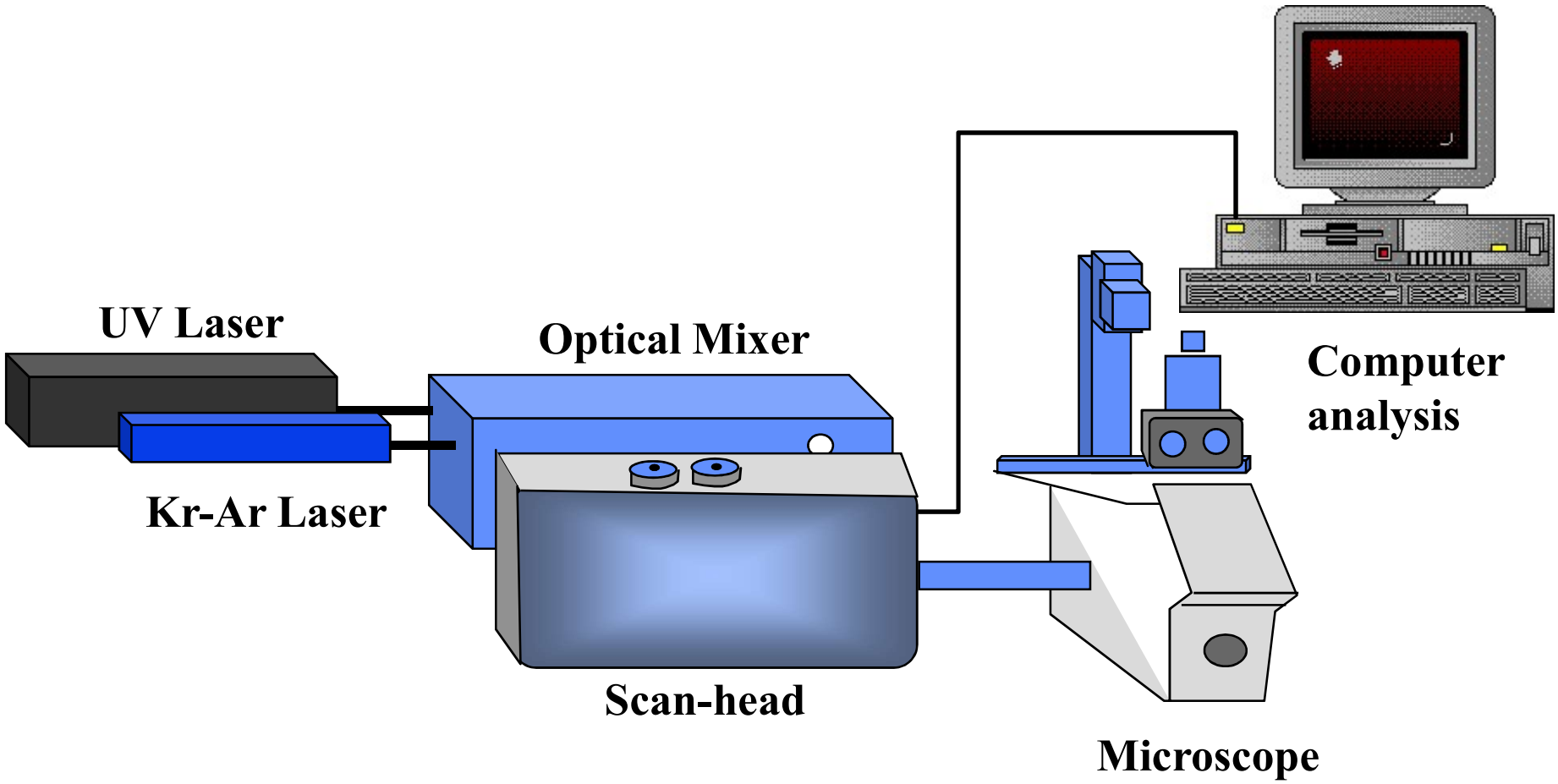


Radiance 2100MP at Purdue



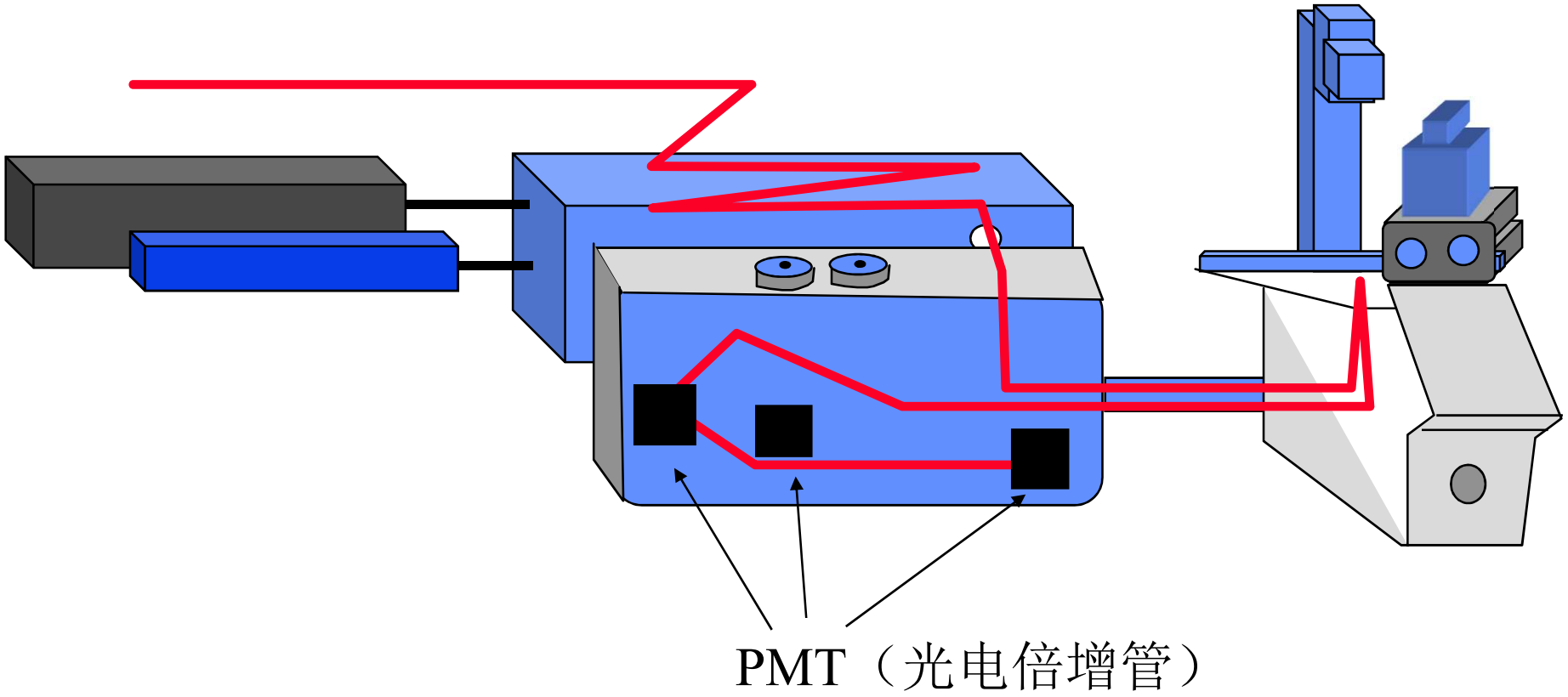


MRC 1024 System

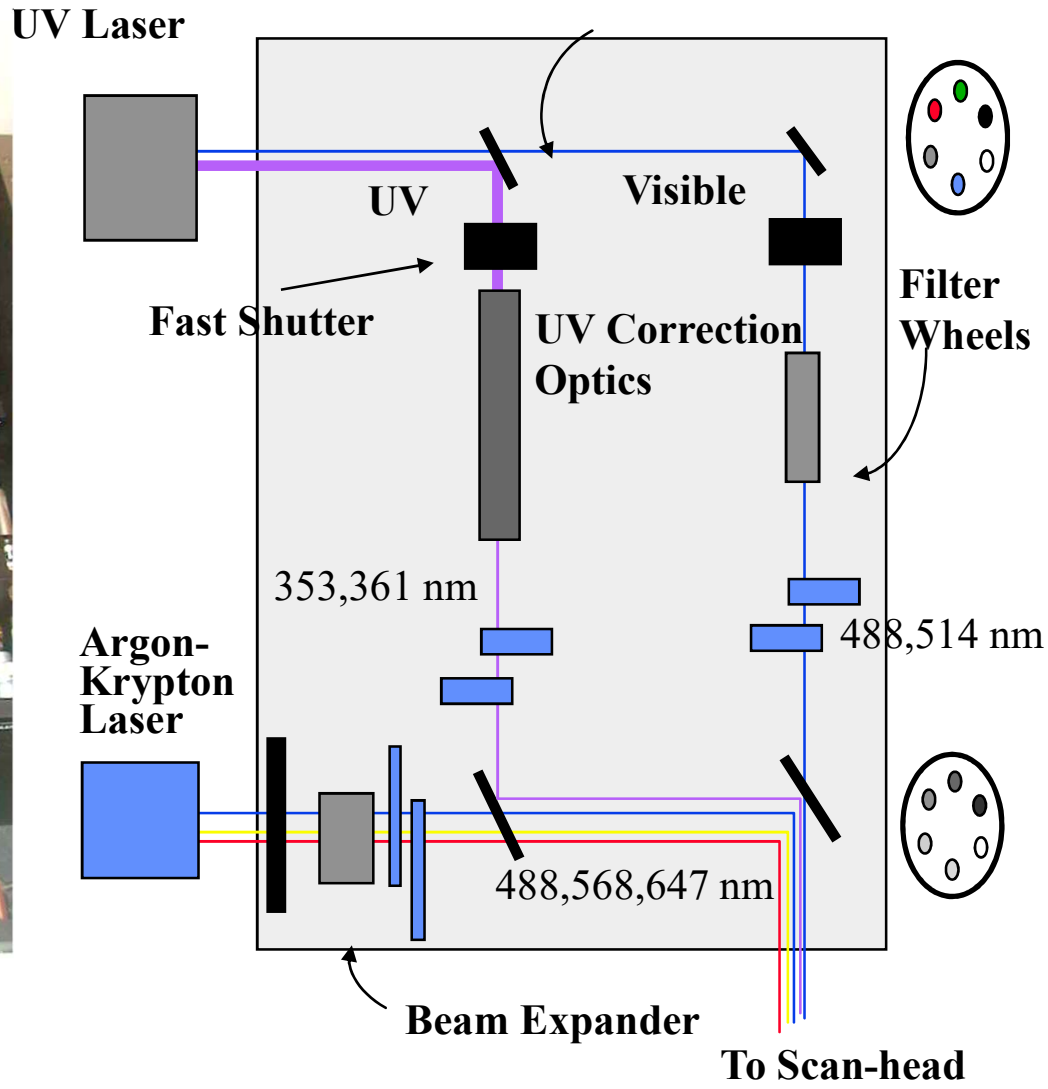


MRC 1024 System

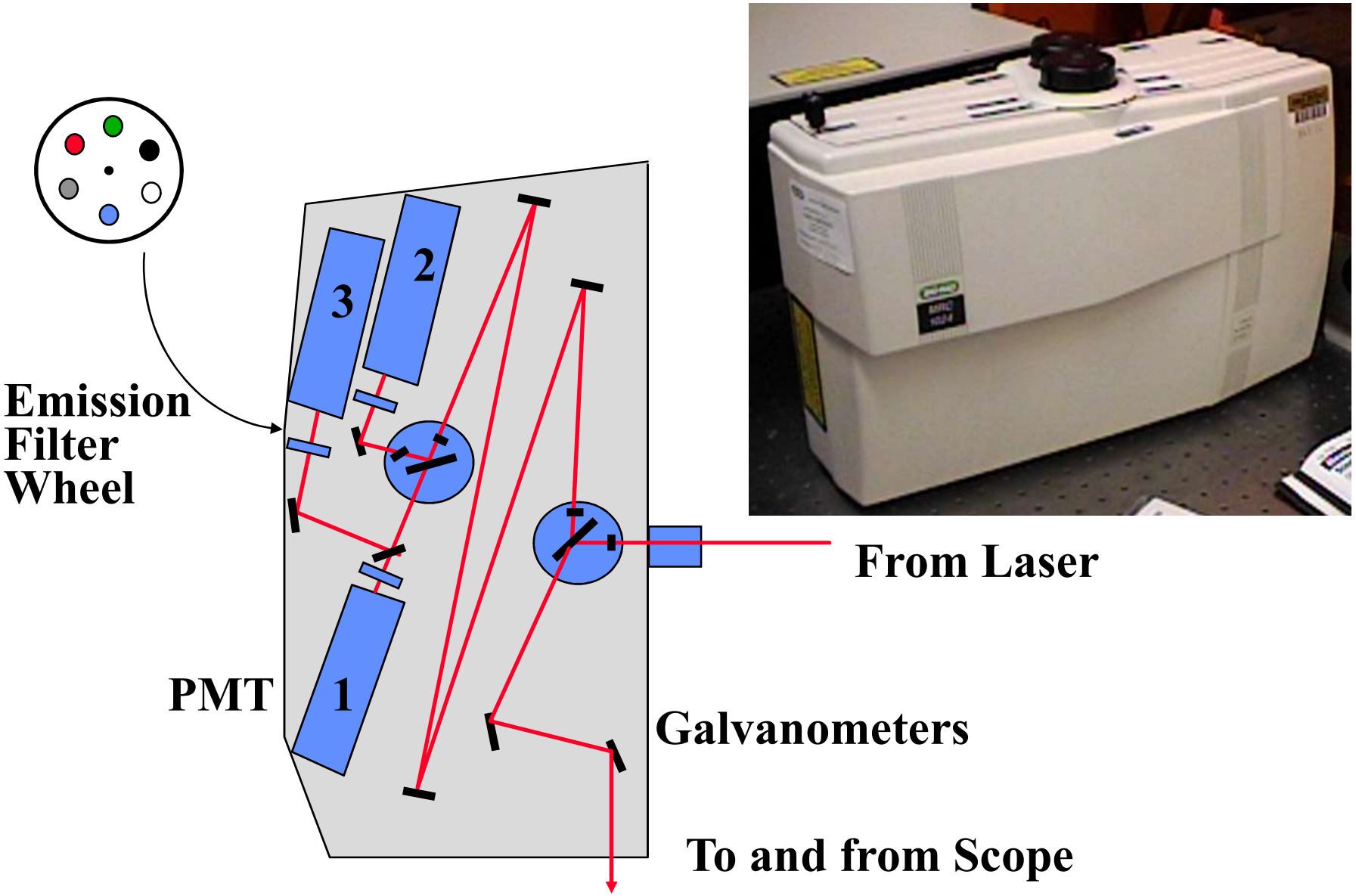
Light Path

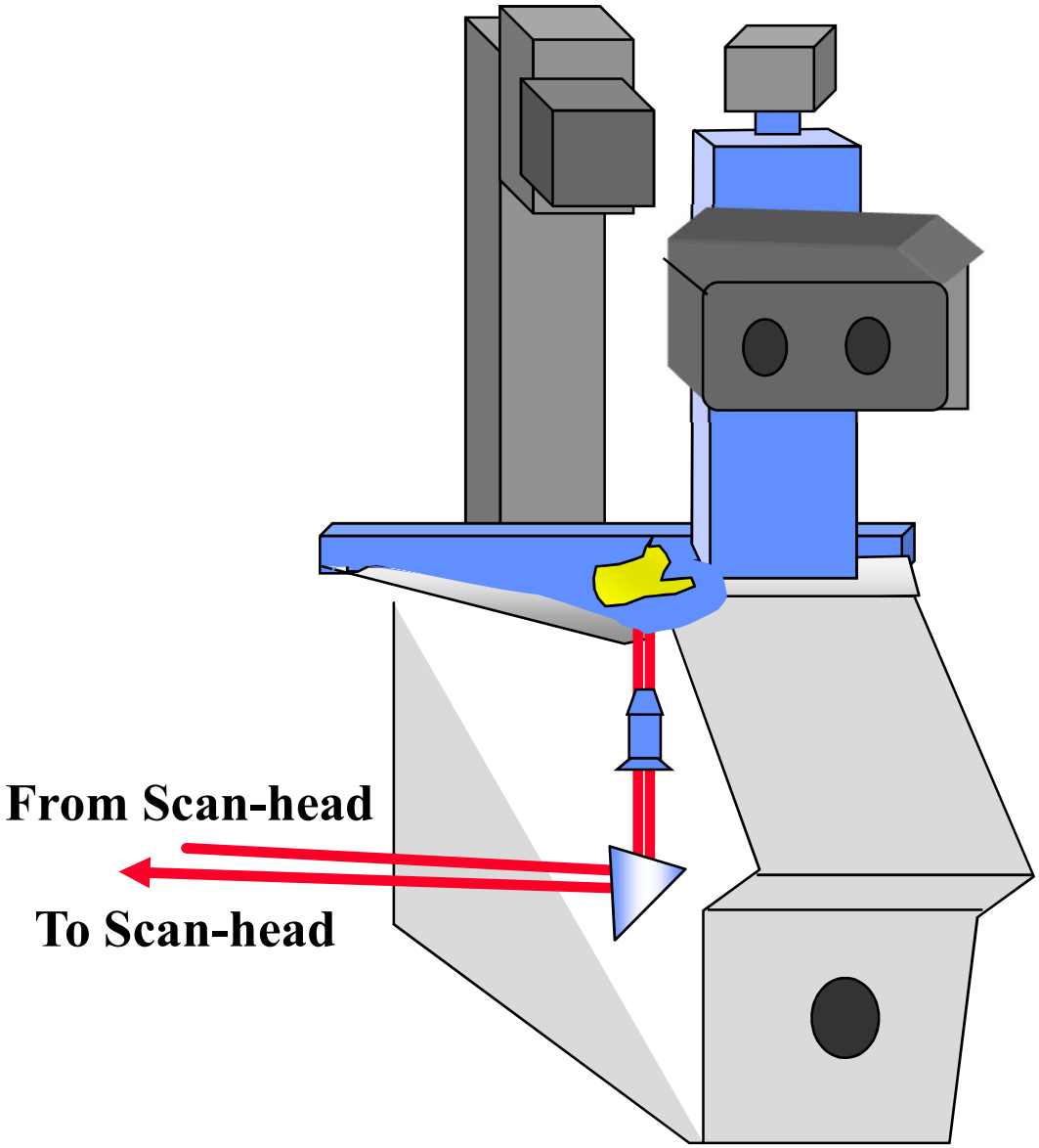


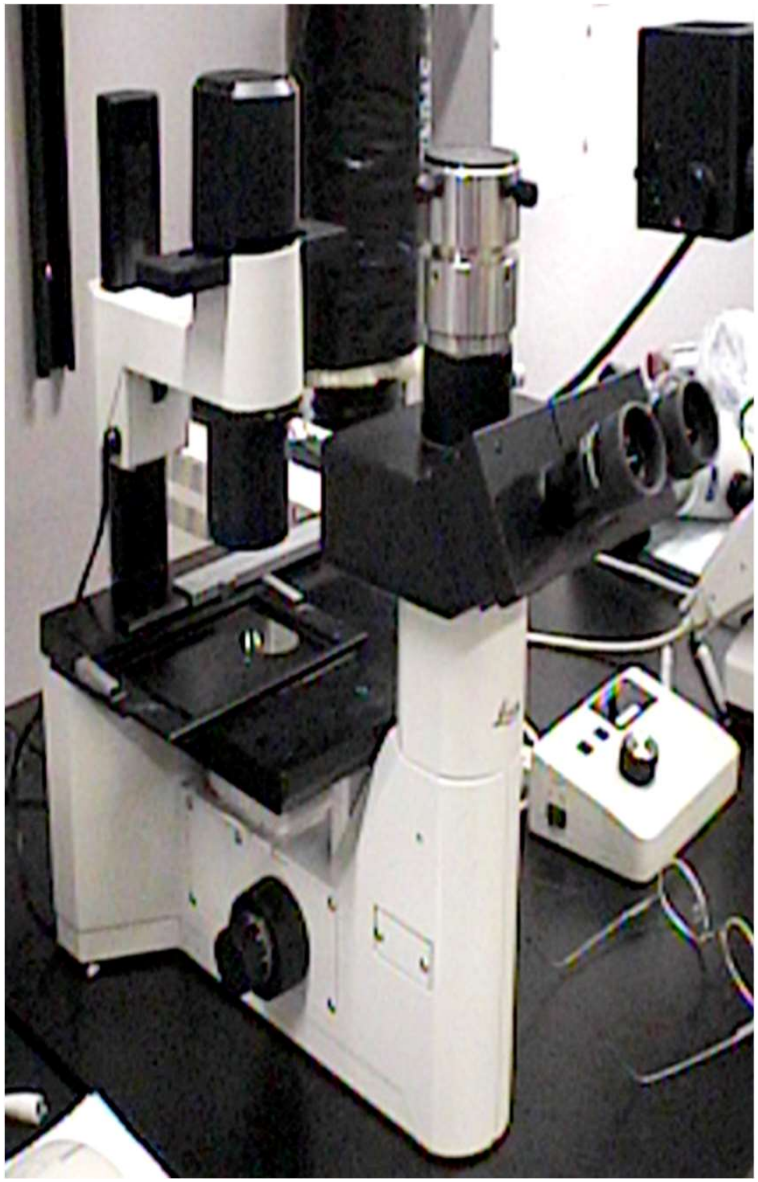
Optical Mixer - MRC 1024 UV



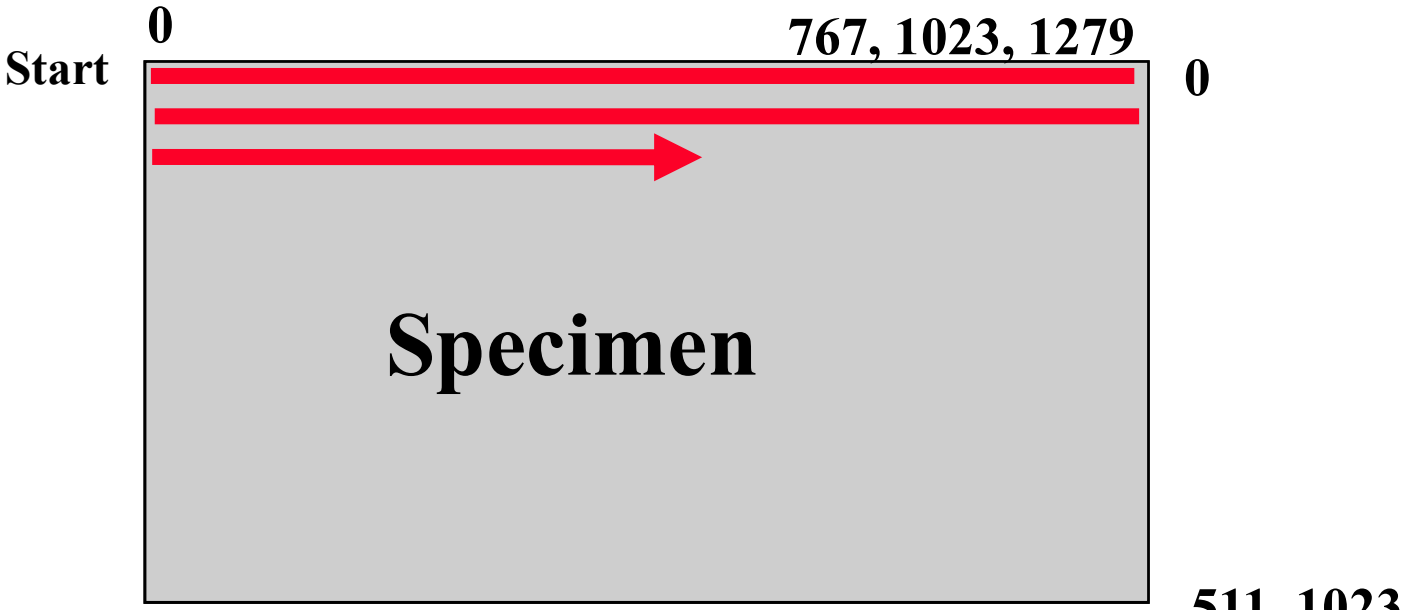
MRC 1024 Scan-head




From Scan-head
To Scan-head

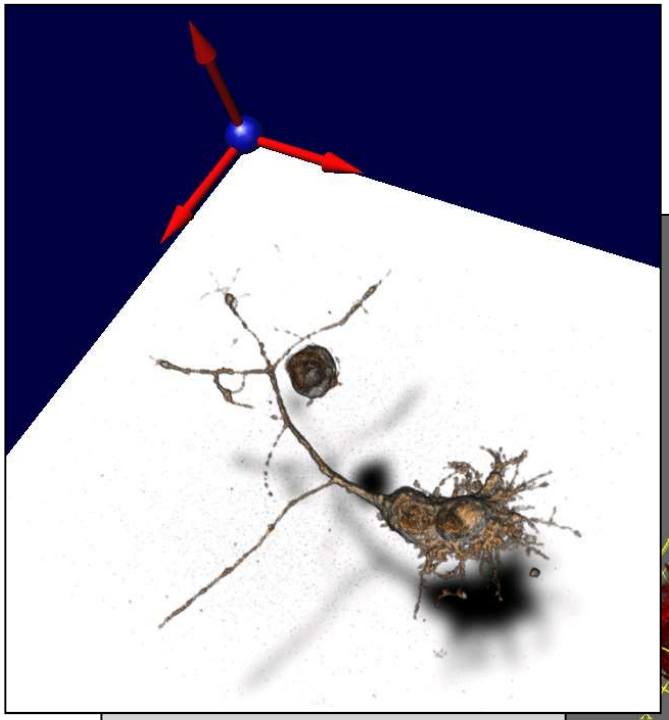


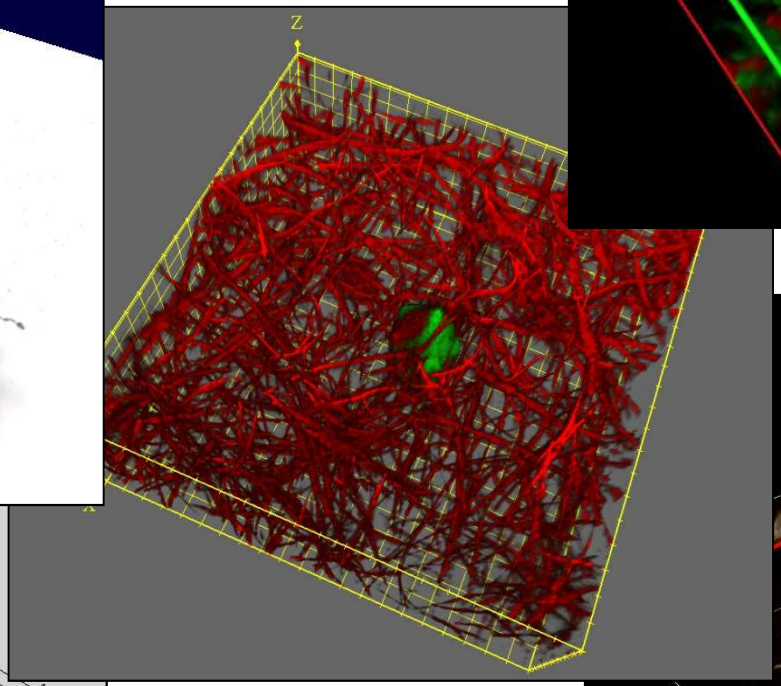
The Scan Path of the Laser Beam

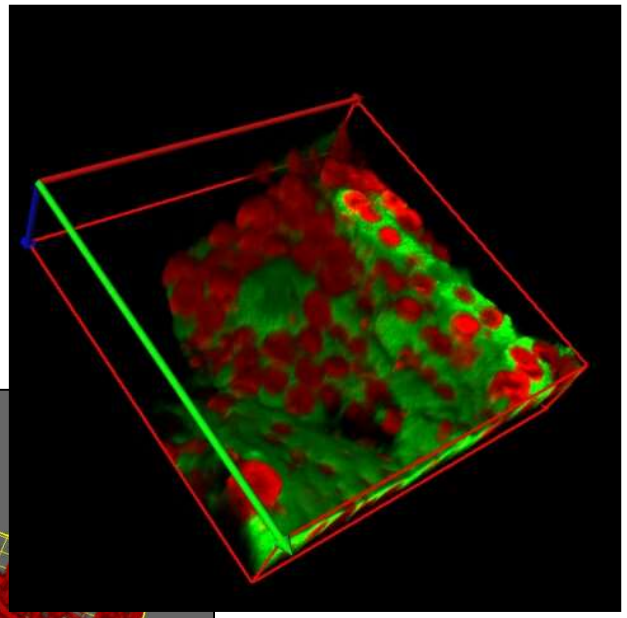


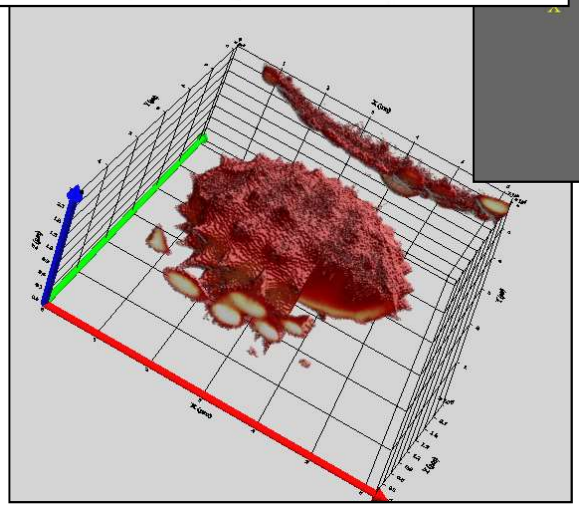
Frames/Sec	# Lines
1	512
2	256
4	128
8	64
16	32

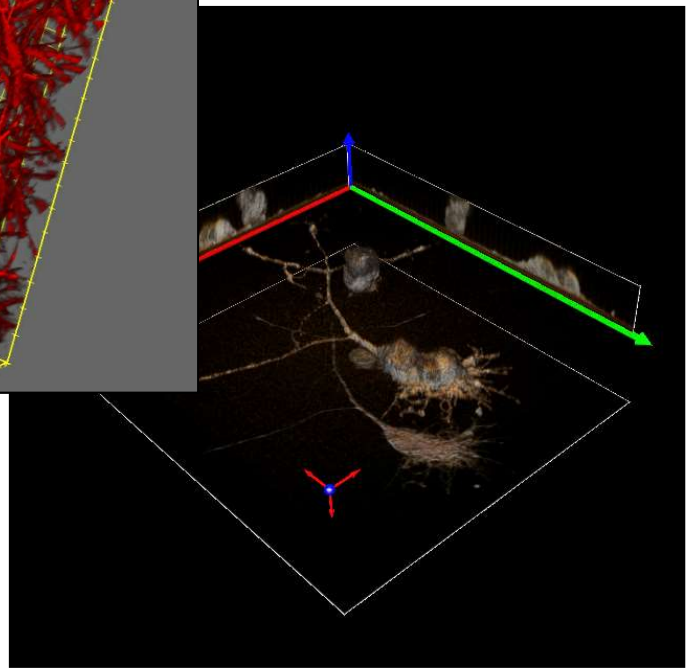
3D imaging using CLSM











CLSM has the following advantages vs. conventional optical microscope:

1.1 Using laser as light source due to its monochromatic, achromatic aberration is eliminated accordingly.

1.2 Diaphragm with pinhole blocks stray light, eliminates spherical aberration, and further reduces chromatic aberration.

1.3 Image of CLSM is re-constructed by the images from point-by-point scanning through the pinholes at both sides of light source and detector. No interference from diffraction and scattering.

1.4 Using computer for data collection and processing, the final reconstructed image has high resolution and sensitivity.

Gray Level & Pixelation

- **Analogous to intensity range**

  For computer images each pixel is assigned a value. If the image is 8 bit, there are 2^8 or 256 levels of intensity If the image is 10 bit there are 1024 levels, 12 bit 4096 levels etc.

- The intensity analogue of a pixel is its grey level which shows up as brightness.

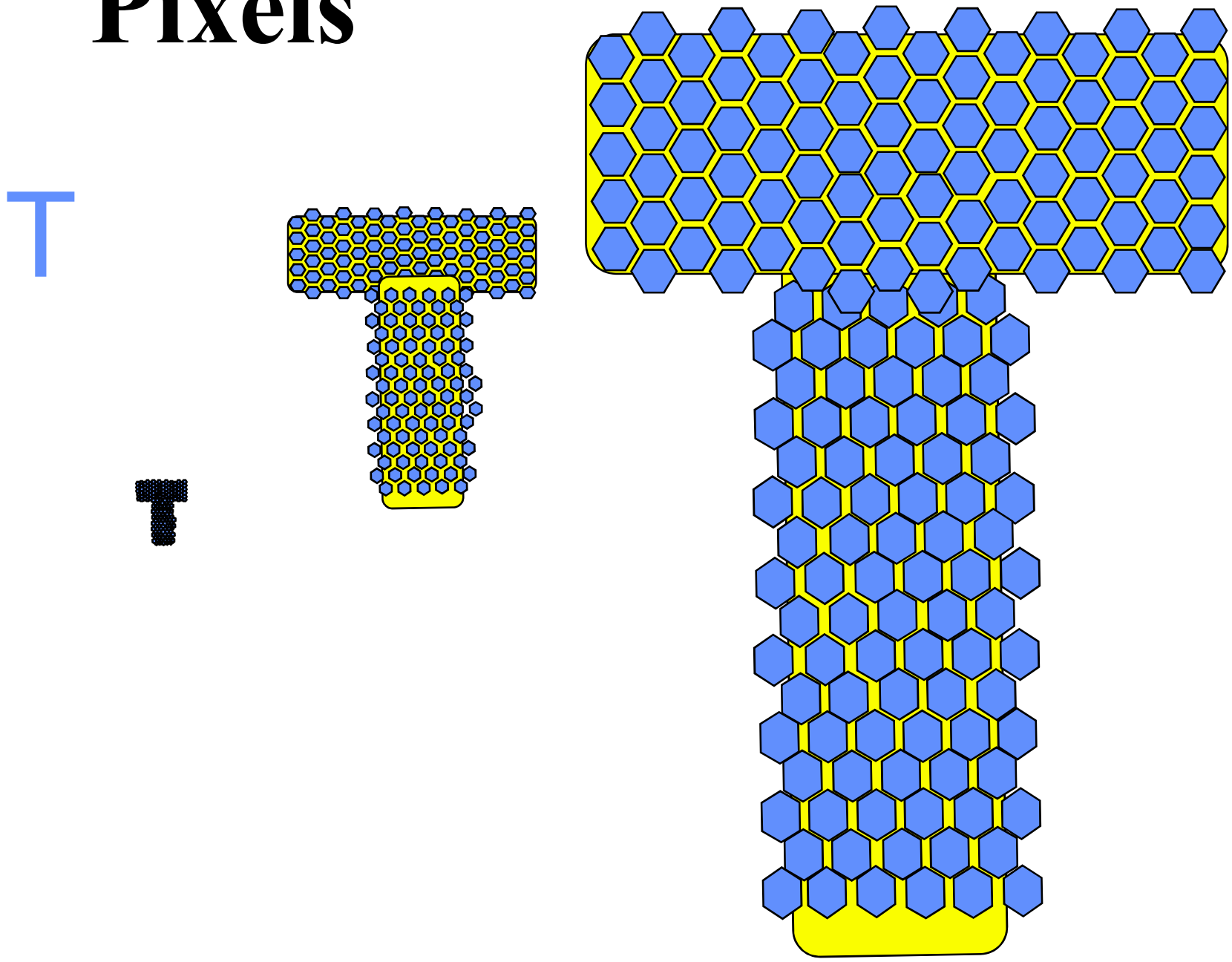
- The display will determine the possible resolution since on a TV screen, the image can only be displayed based upon the number of elements in the display. Of course, it is impossible to increase the resolution of an image by attributing more “pixels” to it than were collected in the original collection!

Pixels

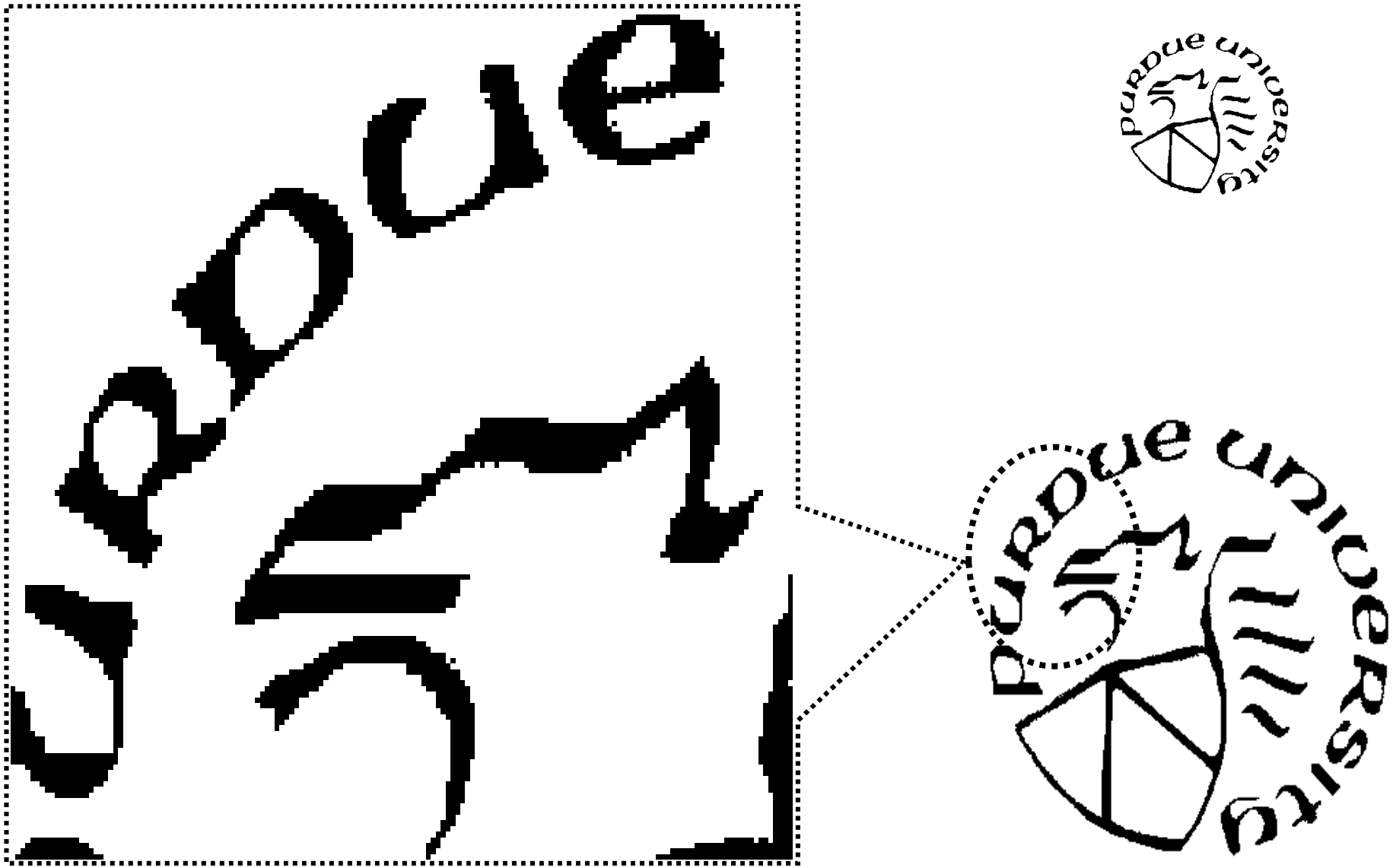
- **Pixels & image structure**

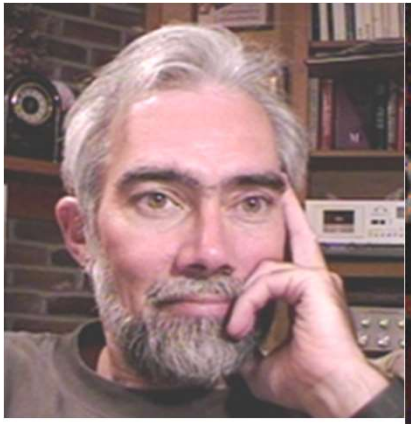
  Hardcopy usually compromises pixel representation. With 20/20 vision you can distinguish dots 1 arc second apart (300 μm at 1 m) so 300 DPS on a page is fine. So at 100 m, you could use dots 300 mm in size and get the same effect! Thus an image need only be parsimonius, i.e., it only needs to show what is necessary to provide the expected image.

Pixels

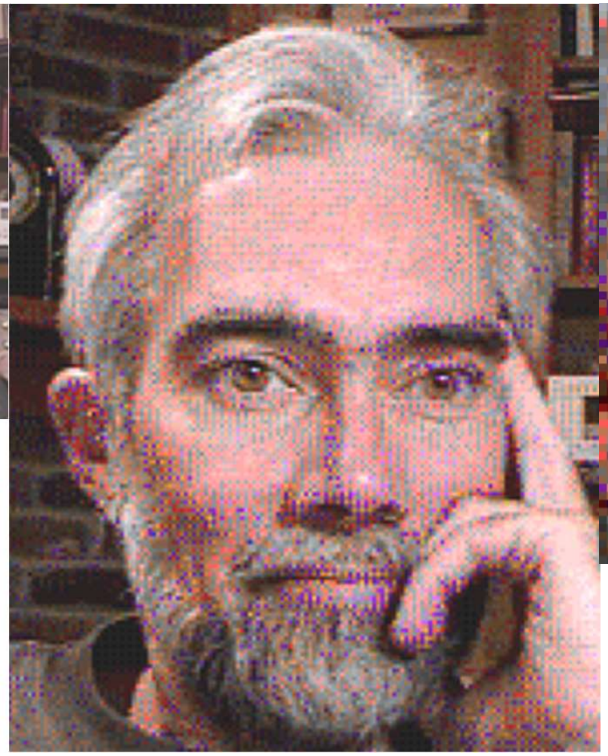


Magnification beyond acceptable limits

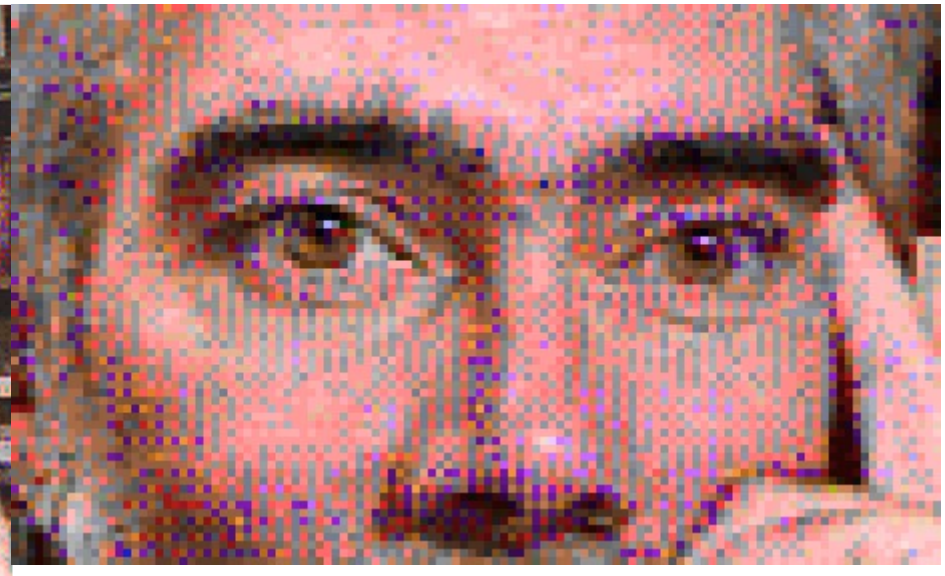




320x240 x 24



Zoom x 2

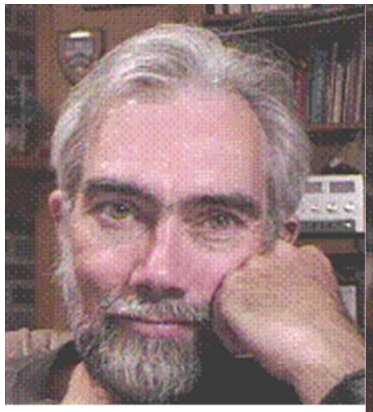


Zoom x 4

The final image appears to be very "boxy" this is known as "pixilation".

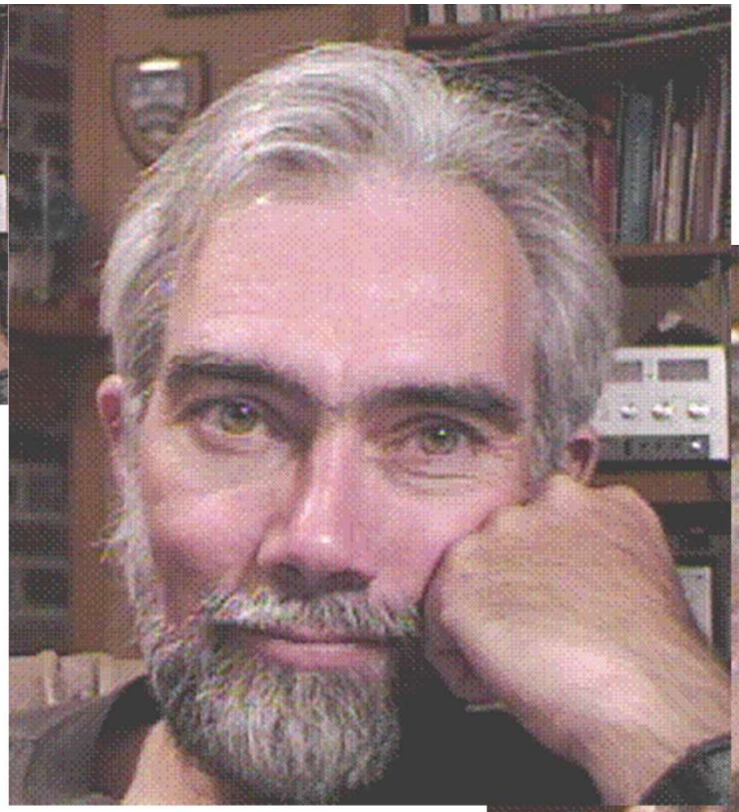
Magnifying with inadequate information. This is known as "empty magnification" because there are insufficient data points.

Zoom x 8



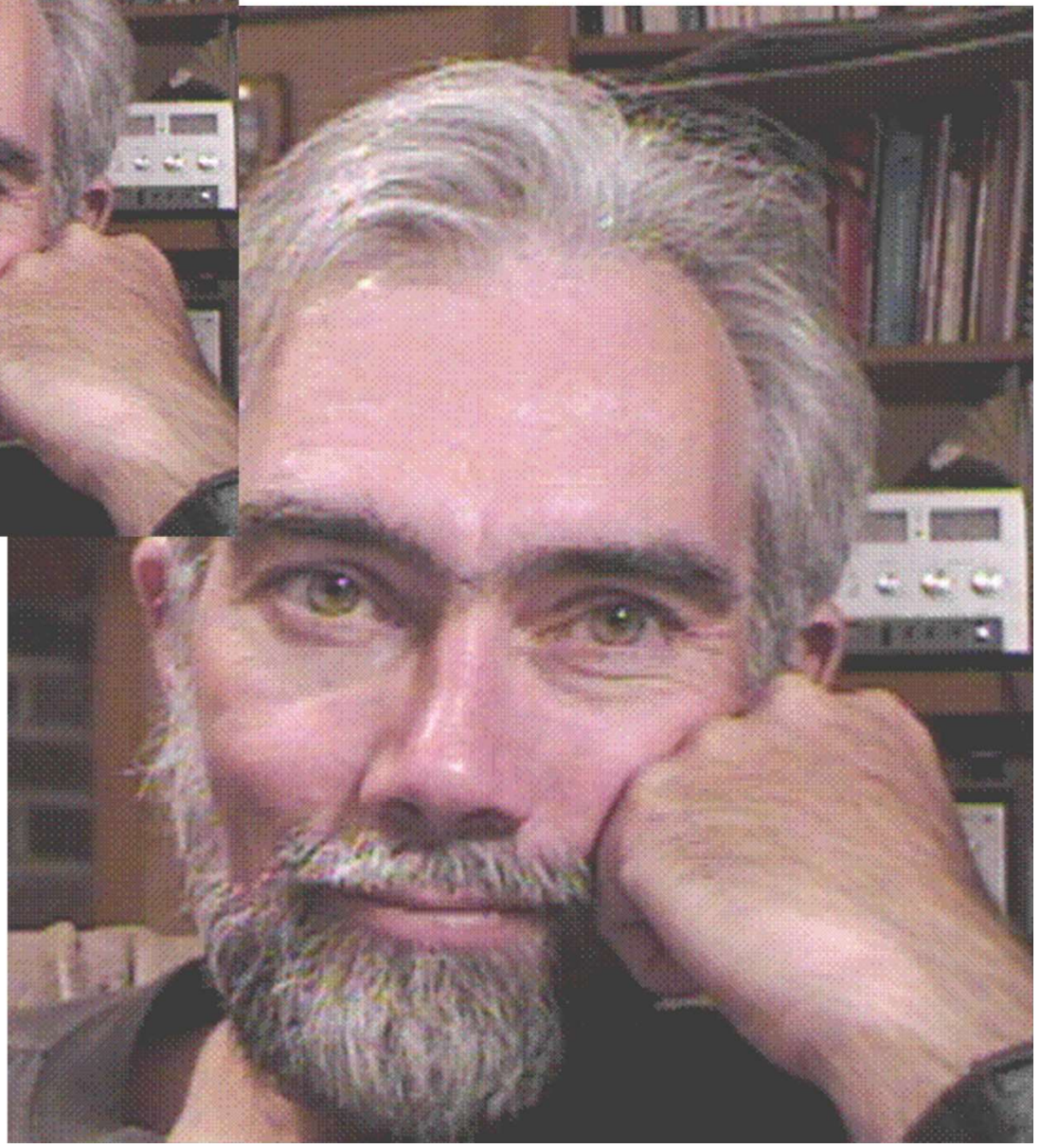
180x200x8

(288,000) 1X



361x400x8

(1,155,200) 2x

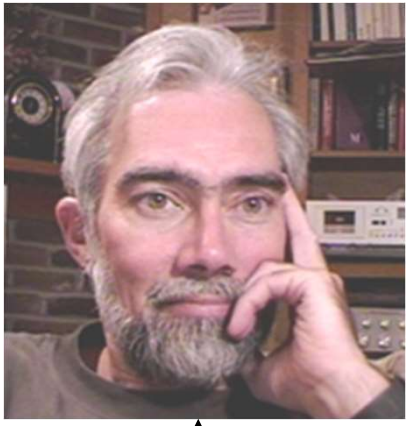


541x600x8

(2,596,800) 1.5x)

Socrates?….well perhaps not...

Magnifying with adequate information. Here, the original image was collected with many more pixels - so the magnified image looks better!



320 x 240 x 24

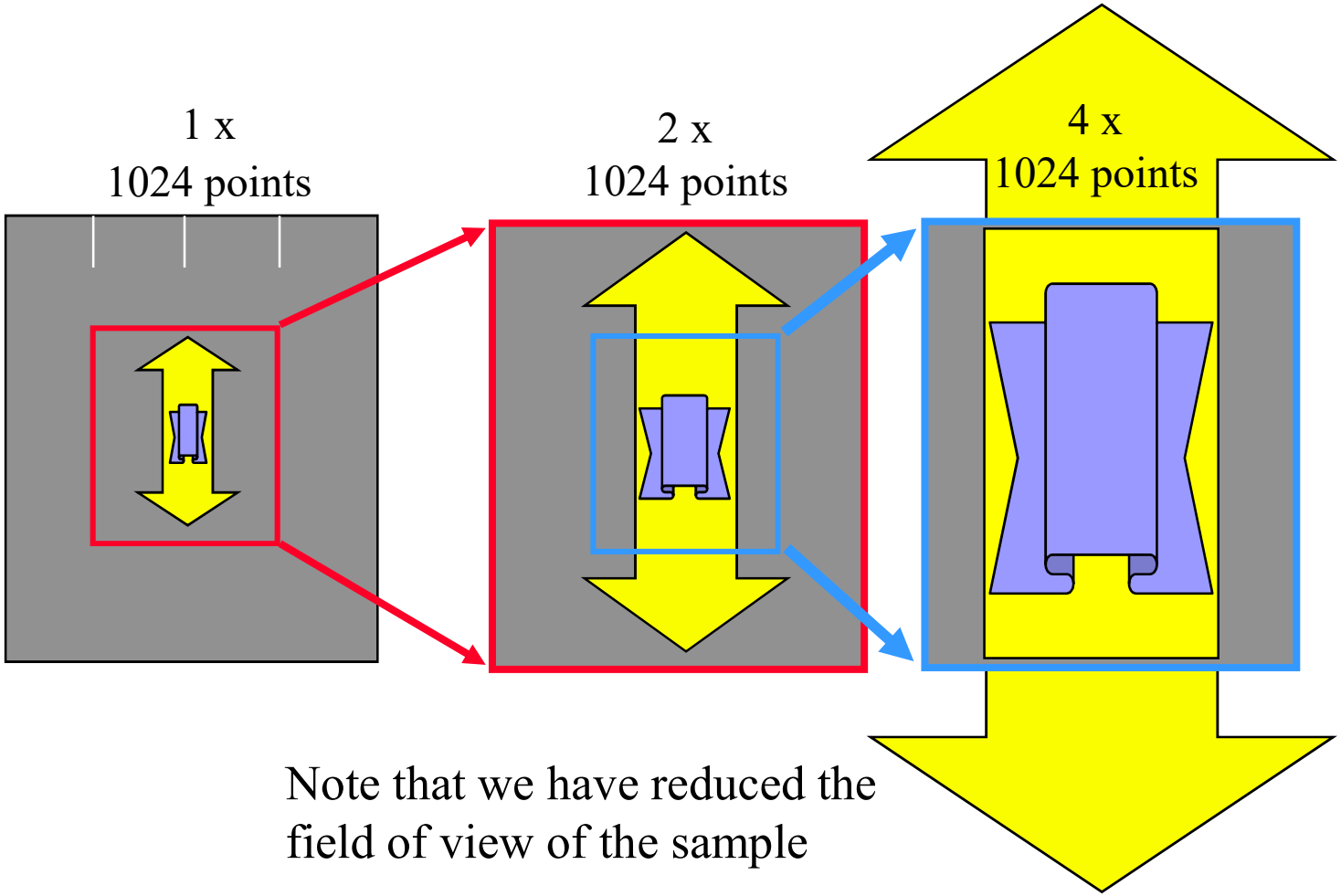
Originals collected at high resolution - compared to a low resolution image magnified

1500 x 1125 x 24

Sampling Theory

- The Nyquist Theorem
  - Nyquest theory describes the sampling frequency (f) required to represent the true identity of the sample.
  - *i.e.,* how many times must you sample an image to know that your sample truly represents the image?
  - In other words to capture the periodic components of frequency f in a signal we need to sample at least $2f$ times
- Nyquist claimed that the rate was $2f$. It has been determined that in reality the rate is $2.3f$ - in essence you must sample at least 2 times the highest frequency.
- For example in audio, to capture the 22 kHz in the digitized signal, we need to sample at least 44.1 kHz (unless of course you can't hear 22k Hz and then you don't need 44.1 kHz!)

Digital Zoom



Note that we have reduced the field of view of the sample

Note: There will only be a single zoom value where optimal resolution can be collected.

Imaging Technology

- **Confocal imaging microscope**
- **Backscattered confocal imaging**
- **Dual/multi-photons excitation imaging**

Backscattered/Reflection Imaging

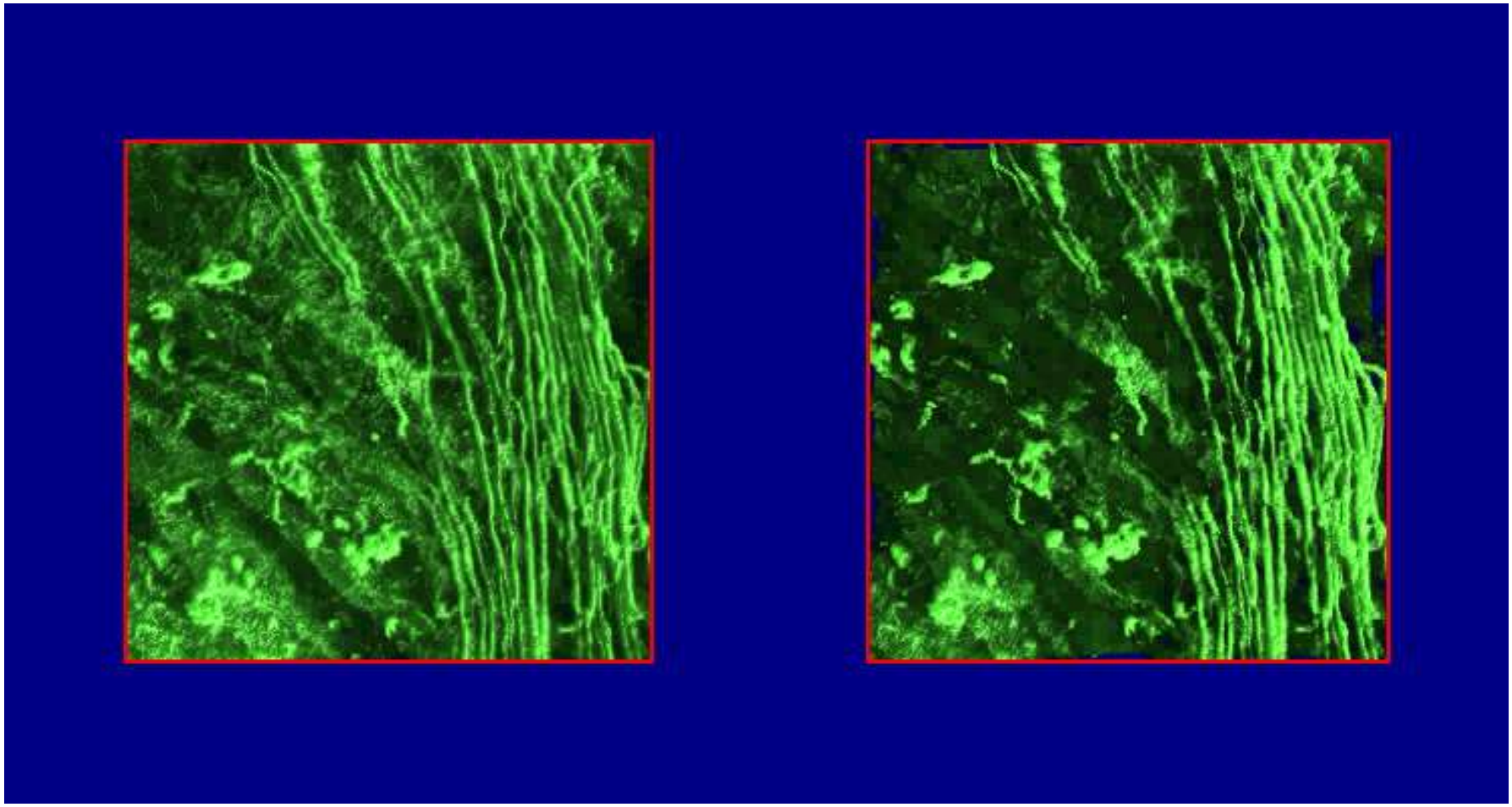
Backscattered light imaging

Same wavelength as excitation

Advantages: no photobleaching since not using a photo-probe (note: does not mean no possible damage to specimen)

Problems: optical reflections from components of microscope

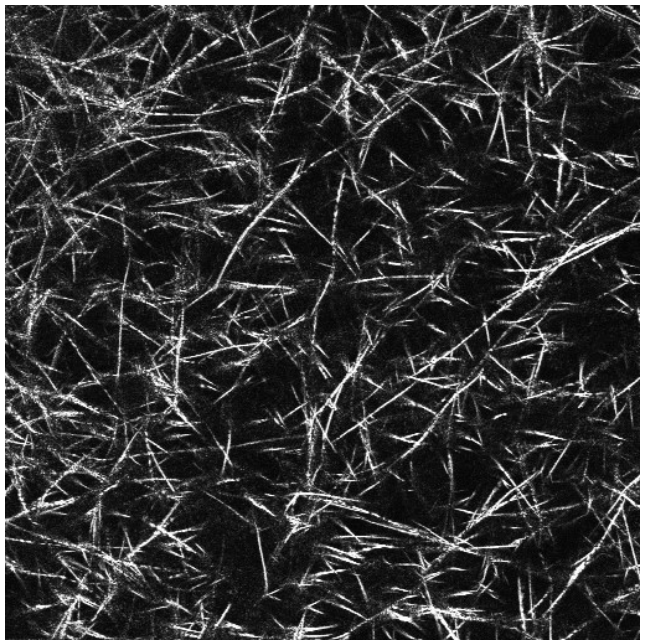
CLSM can employ autofluorescence!
Example: topography of SIS



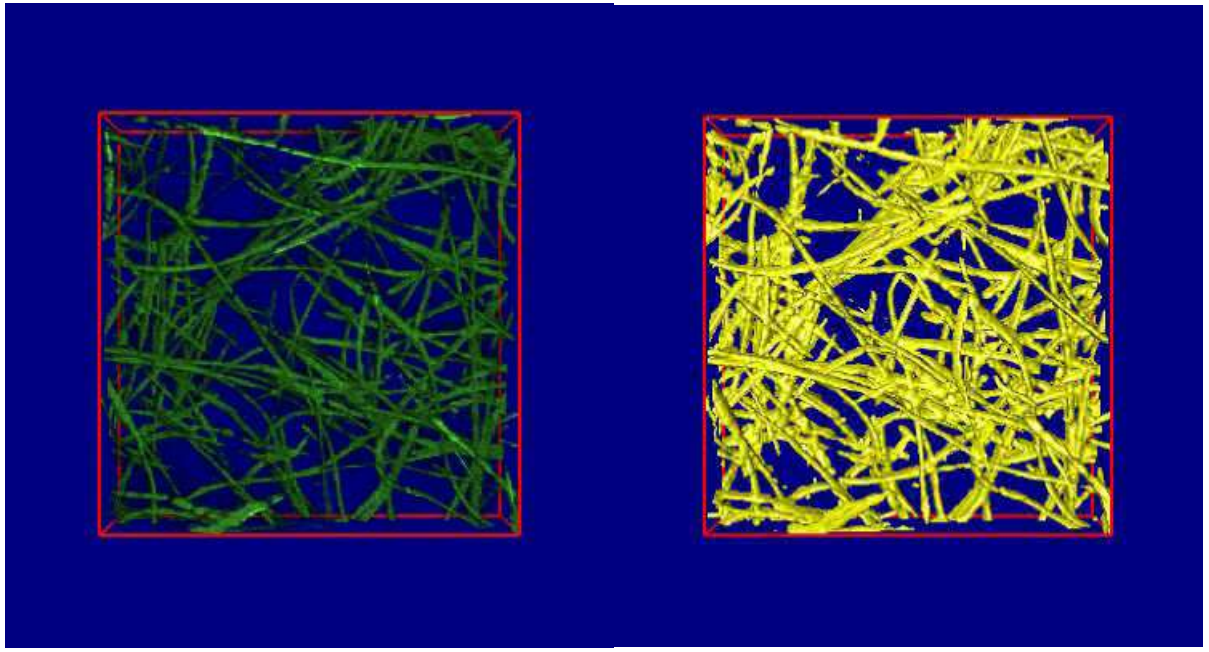
Raw image

Deconvolved image

Backscattered light imaging – another CLSM modality

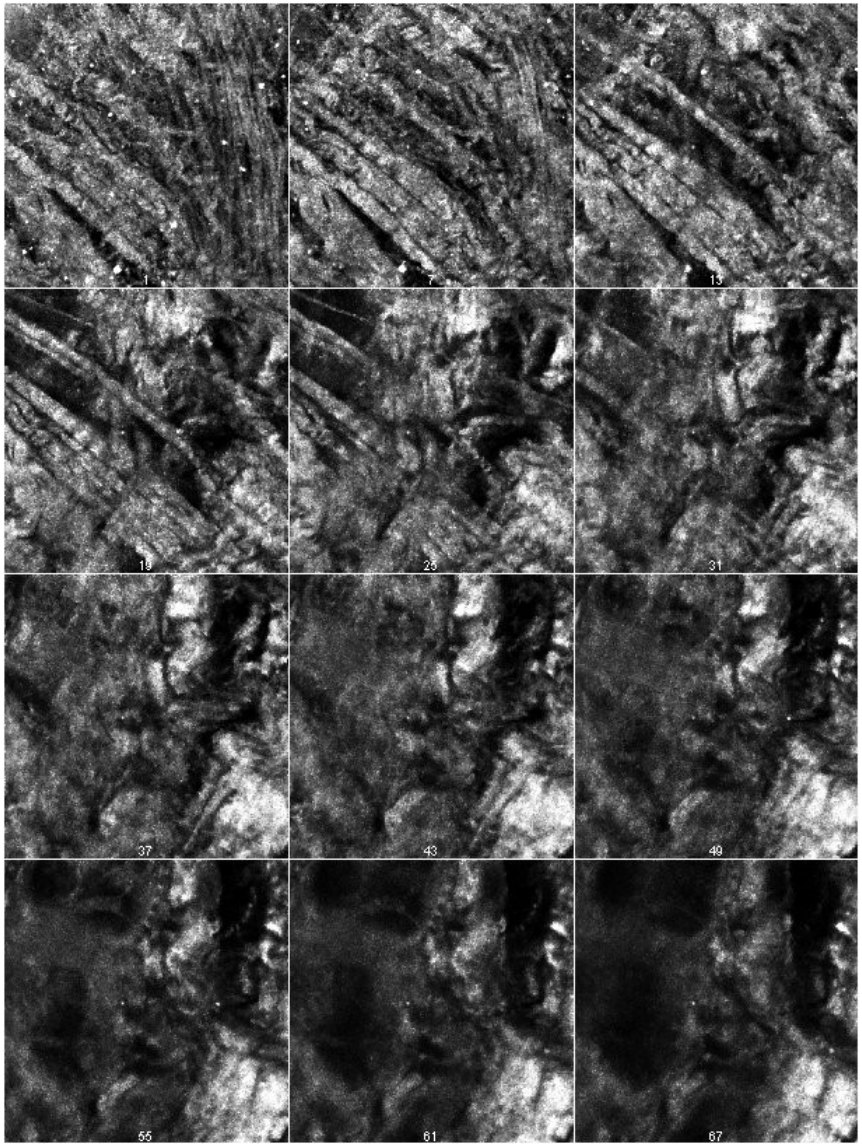


Collagen gel – an optical section

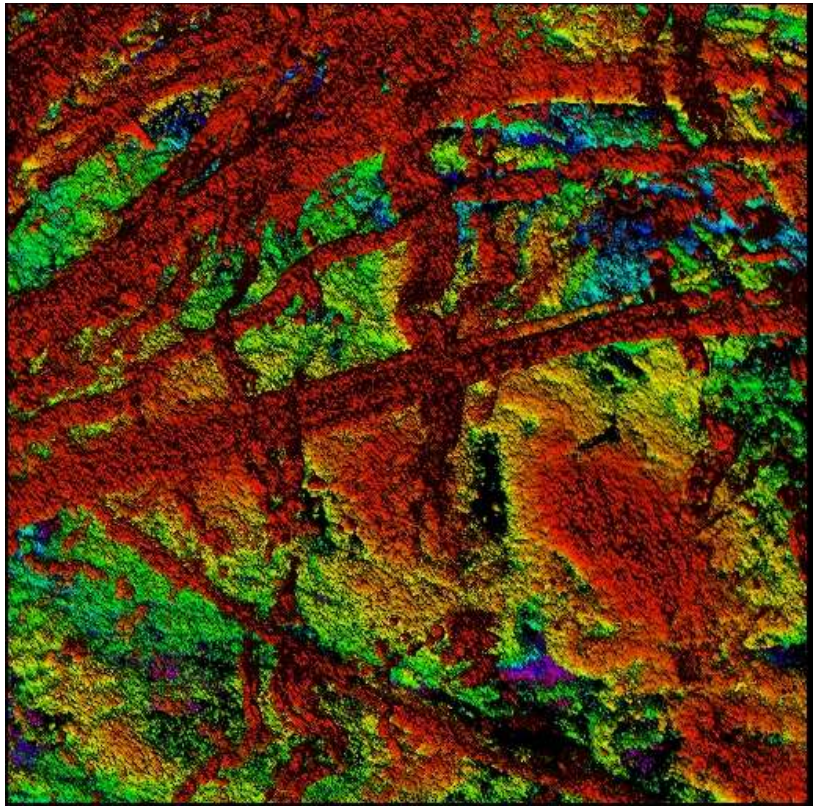


Collagen gel (ECM model) visualized by BSL-confocal microscopy. Volume- (left) and surface-rendering (right) of a confocal dataset.

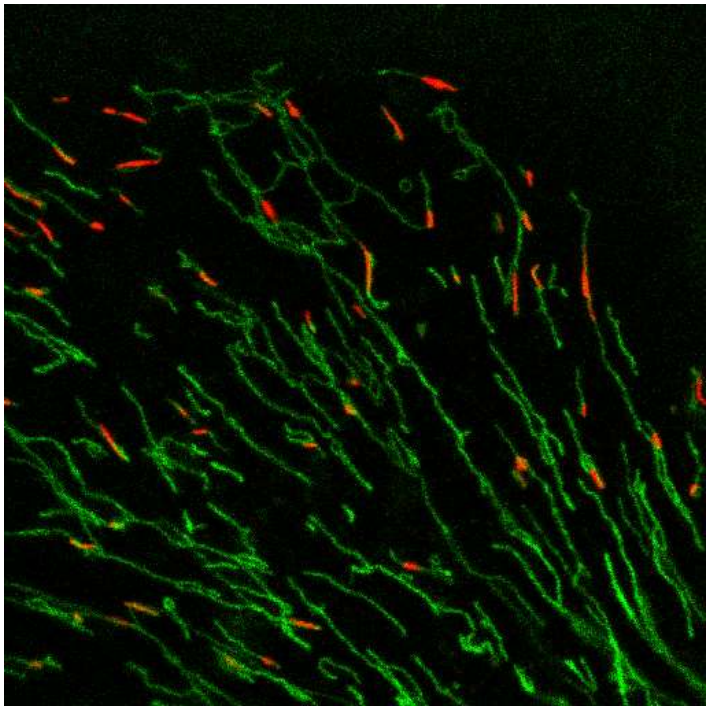
Backscattered light: tissue imaging

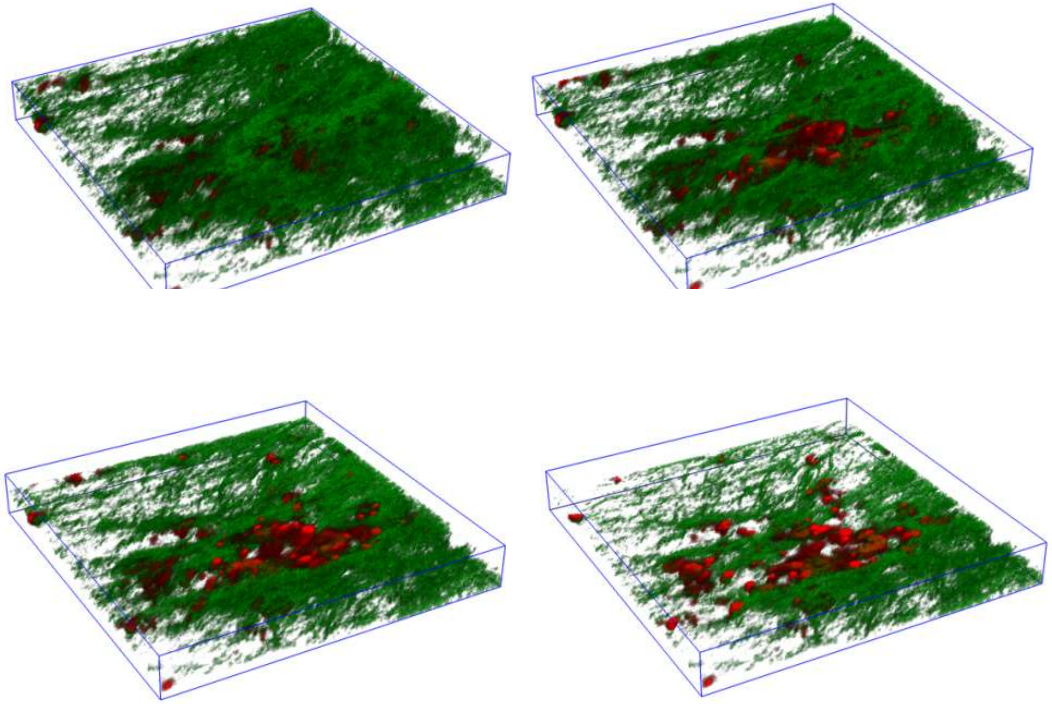


Left: Optical sections of SIS visualized with BSL-confocal technique. Bottom: color-coded height map revealing the topography of height map of SIS



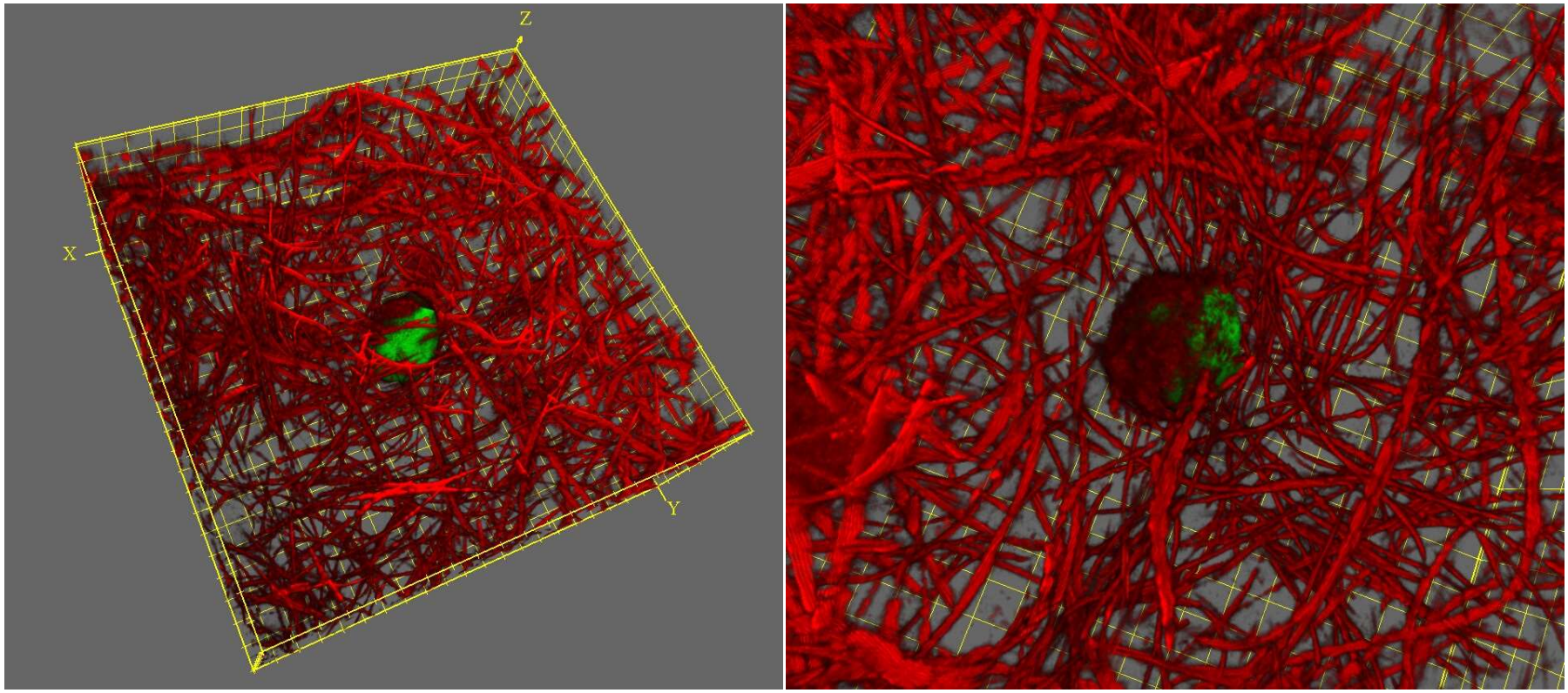
Example: BSL and AF signals combined





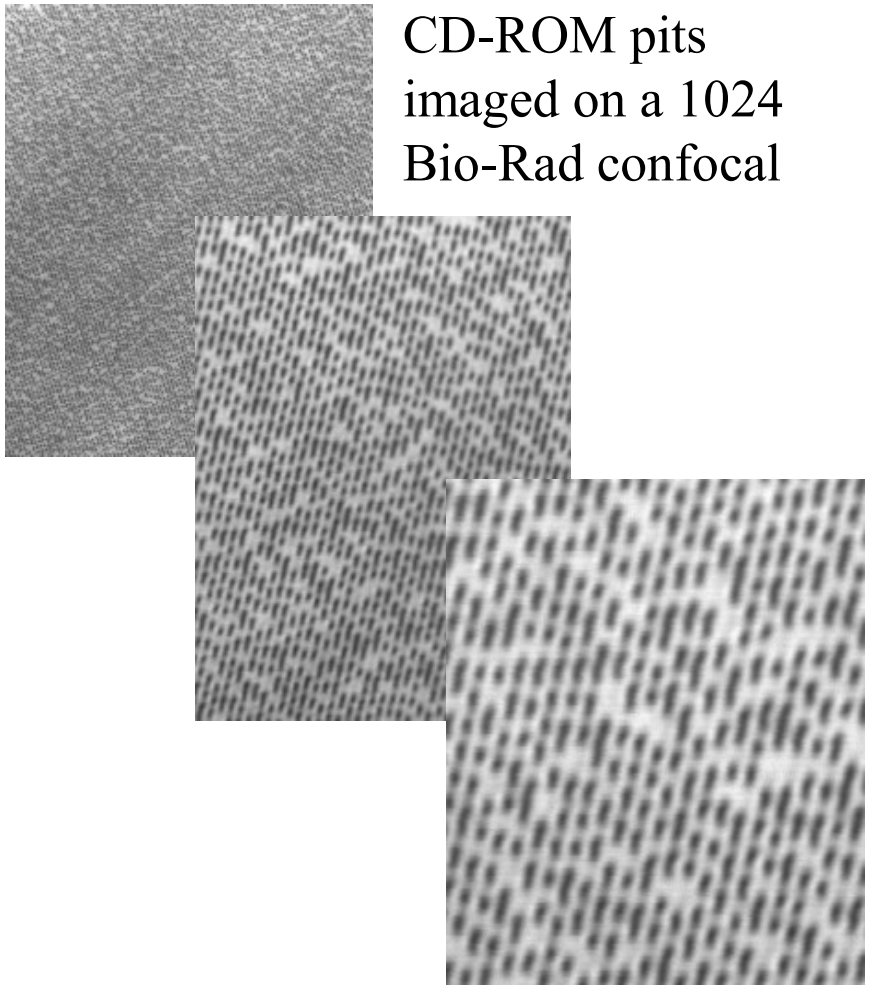
HepG2 cells grow embedded within a collagen matrix (animation)

Backscattered light and autofluorescence signals combined: collagen gel & $HepG_2$ cells

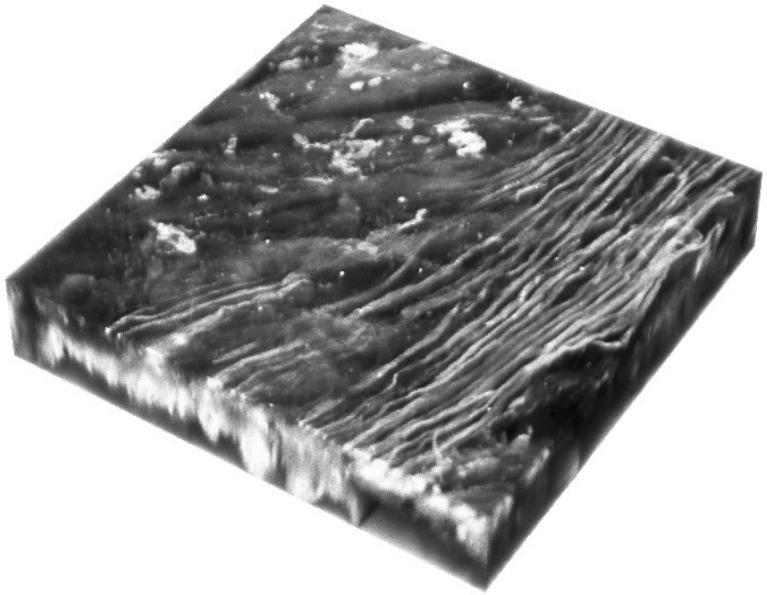


Reflected light images

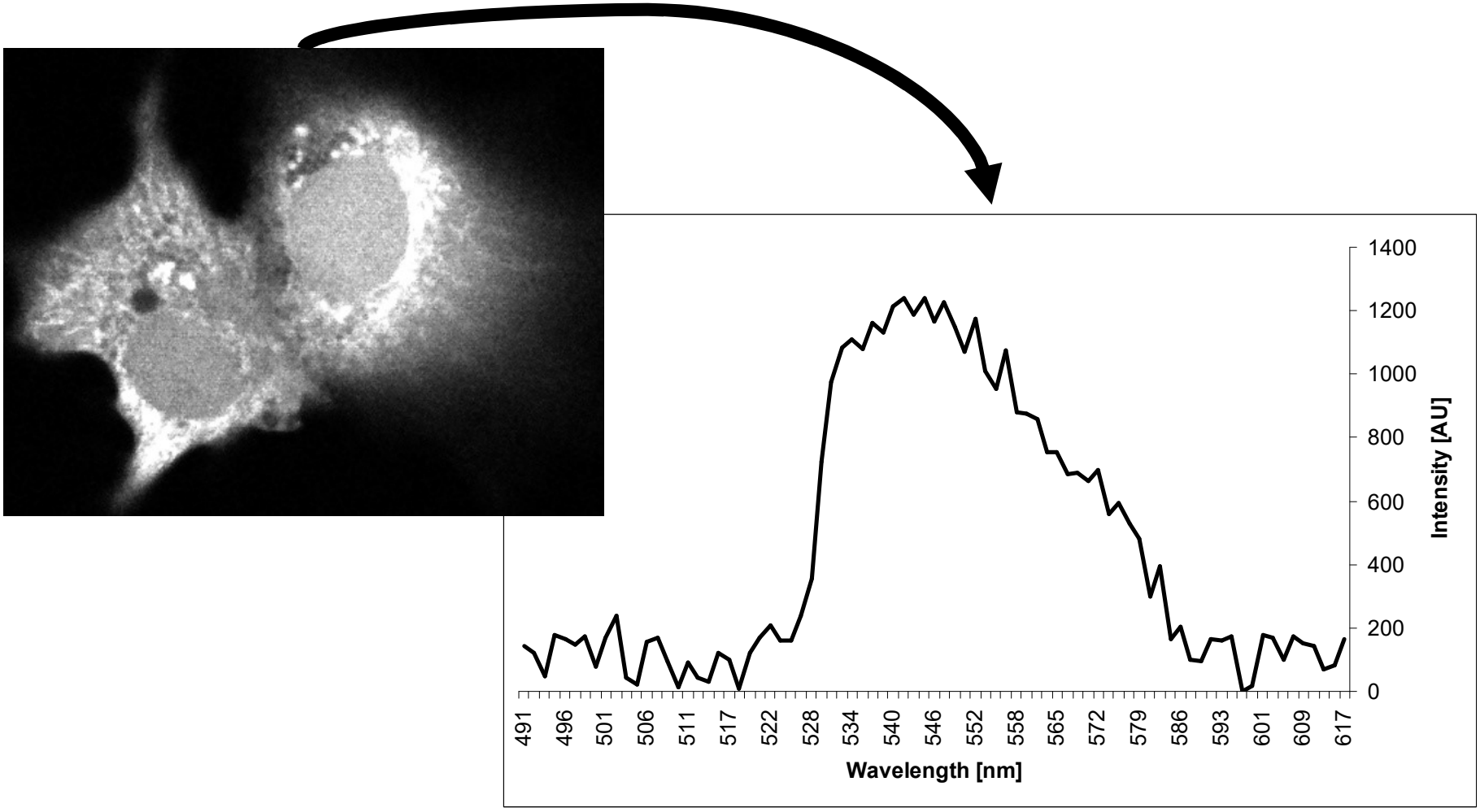
CD-ROM pits
imaged on a 1024
Bio-Rad confocal



Collagen imaged by
a 1024 Confocal



Imaging spectroscopy



Imaging Technology

- Confocal imaging microscope
- Backscattered confocal imaging
- Dual/multi-photons excitation imaging

CONCEPT

Two-photon excitation microscopy is a fluorescence imaging technique that allows imaging of living tissue up to about one millimeter in depth. It differs from traditional fluorescence microscopy, in which the excitation wavelength is shorter than the emission wavelength, as the wavelengths of the two exciting photons are longer than the wavelength of the resulting emitted light. Two-photon excitation microscopy typically uses near-infrared excitation light which can also excite fluorescent dyes.

Two photon excited fluorescence microscopy has similarities to confocal laser scanning microscopy. Both use focused laser beams scanned in a raster pattern to generate images, and both have an optical sectioning effect. Unlike confocal microscopes, multiphoton microscopes do not contain pinhole apertures that give confocal microscopes their optical sectioning quality. The optical sectioning produced by multiphoton microscopes is a result of the point spread function: the multiphoton point spread function is typically dumbbell-shaped (longer in the x-y plane), compared to the upright rugby-ball shaped point spread function of confocal microscopes.

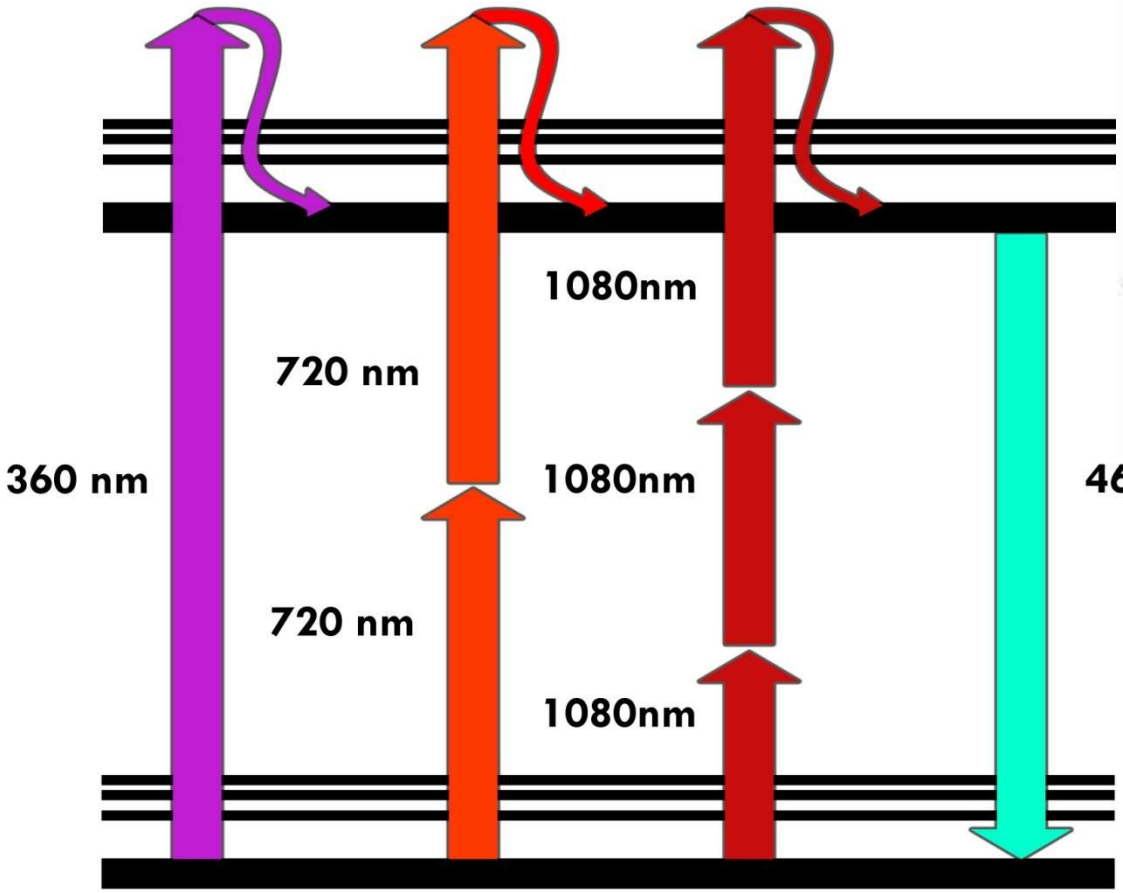
The purpose of employing the two-photon effect is that the axial spread of the point spread function is substantially lower than for single-photon excitation. As a result, the resolution along the z dimension is improved, allowing for thin optical sections to be cut.

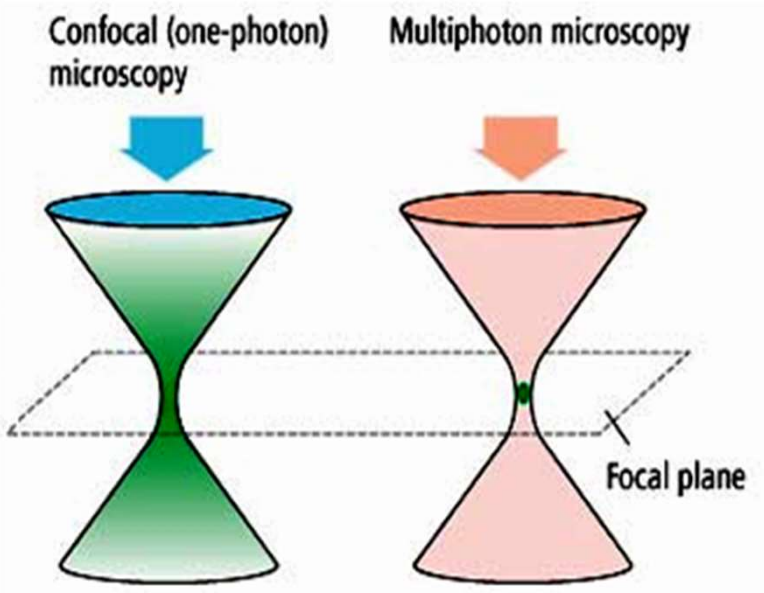
ADVANTAGES:

- deeper tissue penetration,
- efficient light detection,
- reduced photobleachin

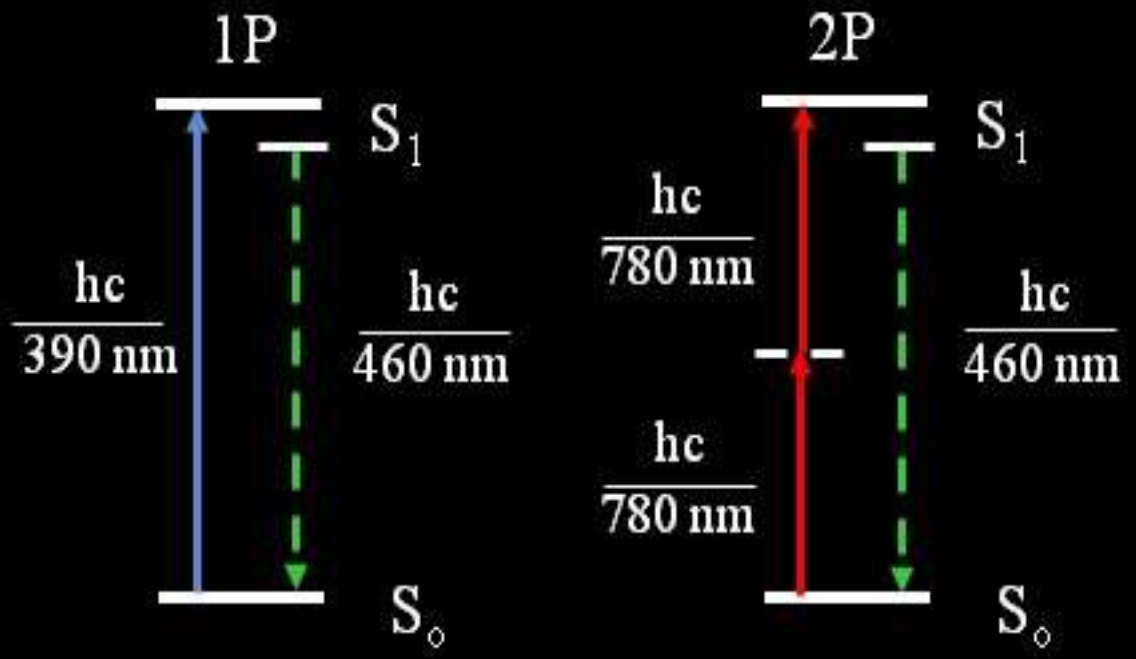
Multi-Photon Microscopy

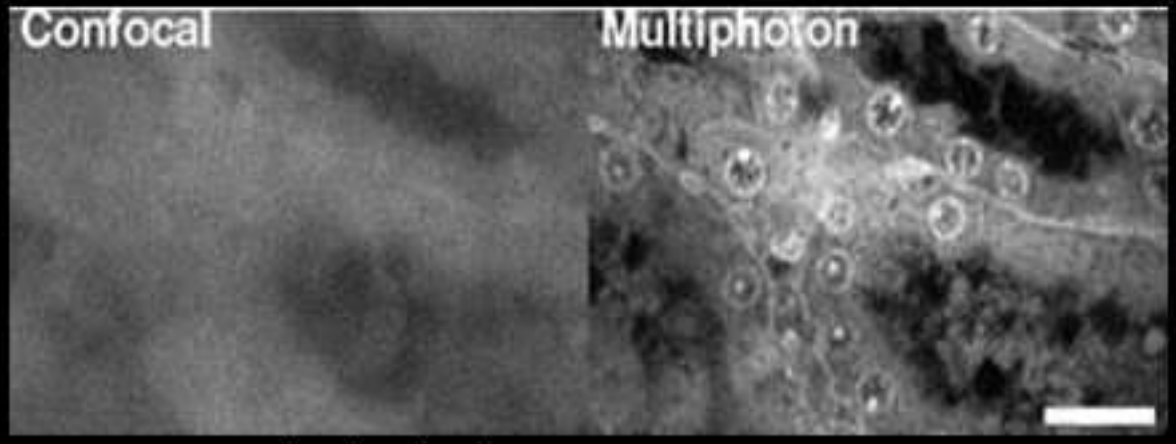
Resolution is improved (in comparison to the same excitation wavelength)



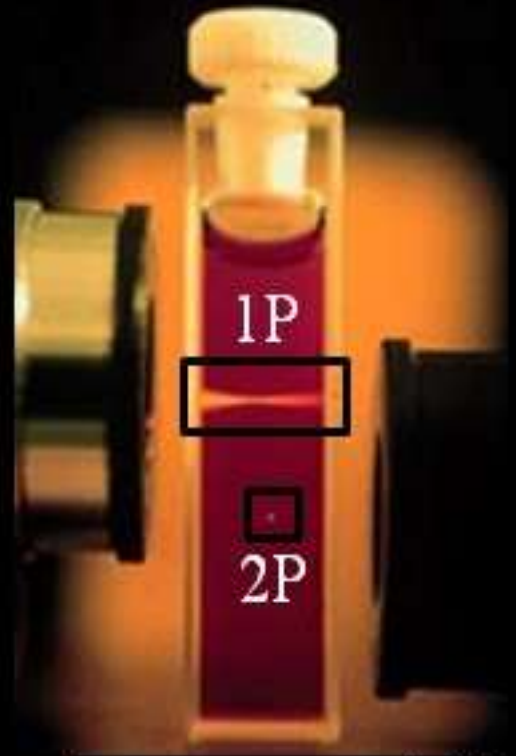


Multi-Photon Microscopy





Centonze, Biophysical J. 1998

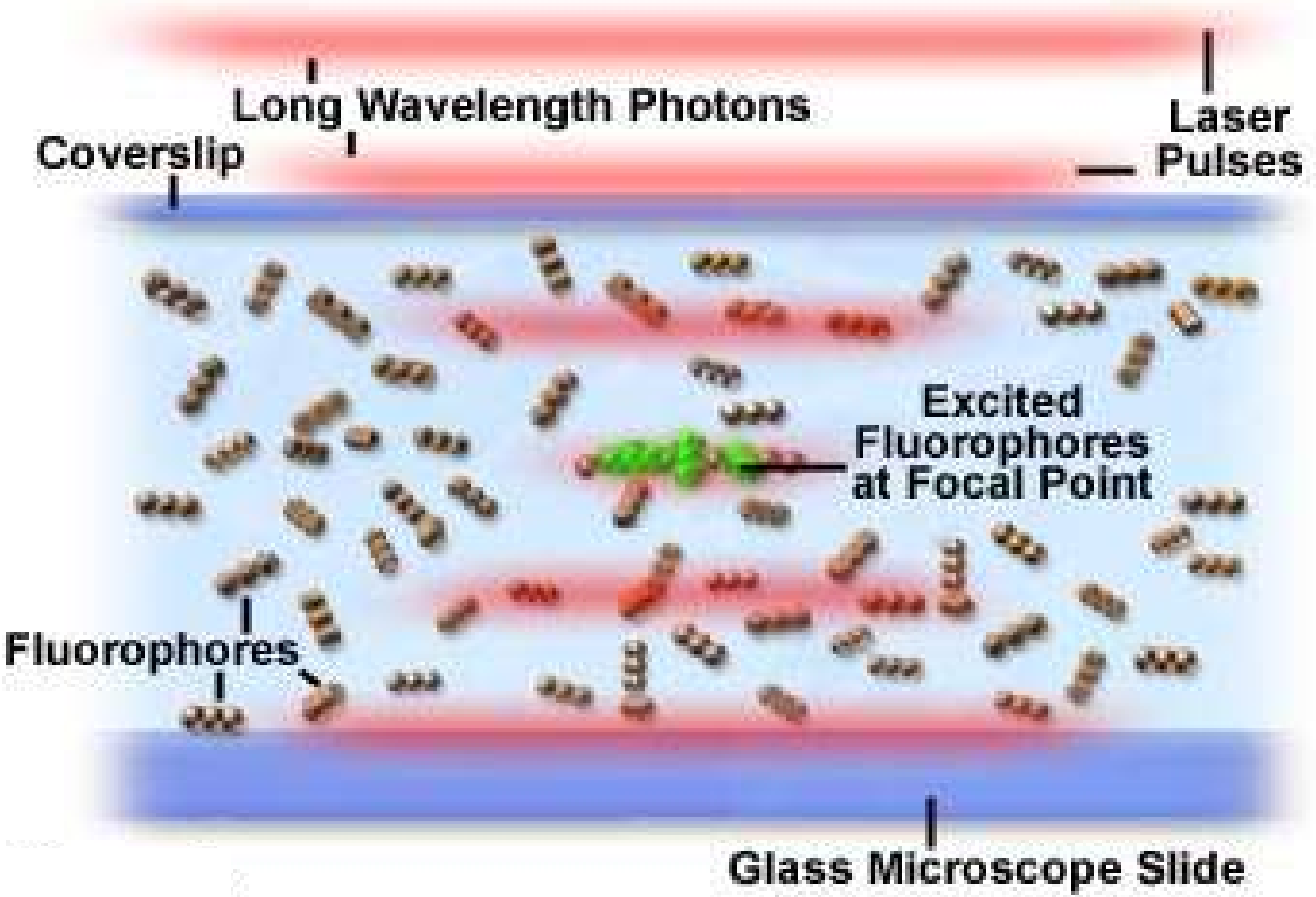


Brad Amos MRC, Cambridge

Viability

Penetration depth

Fluorophore Excitation in Multiphoton Microscopy



Multi-Photon Microscopy: IR Lasers

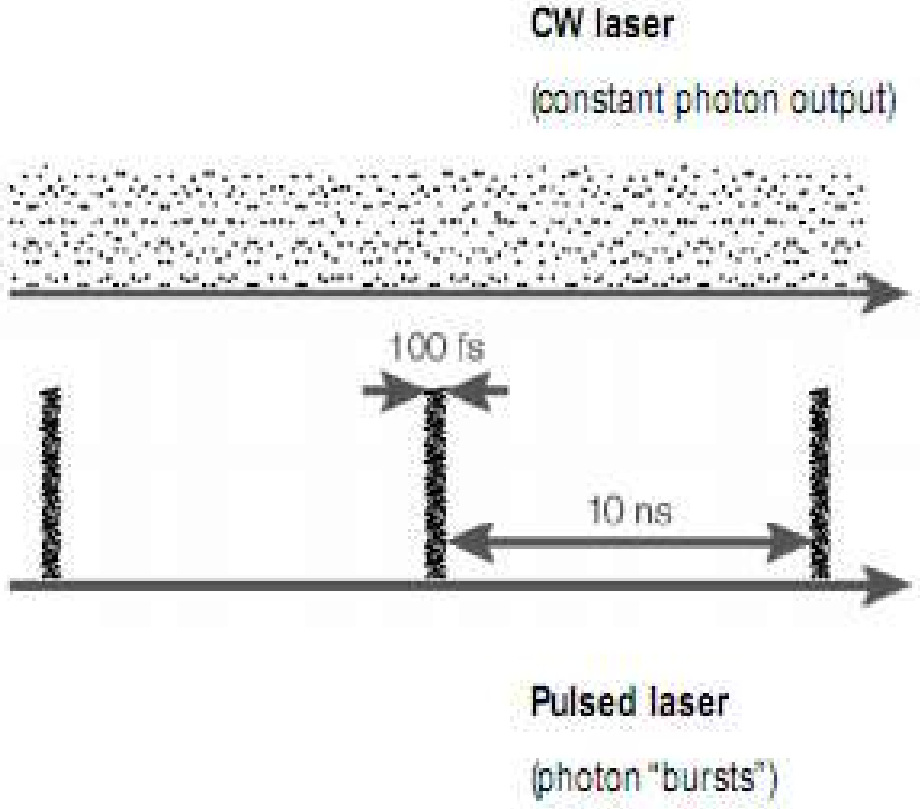
Fluorochrome excitation width pulsed IR lasers

CW laser (continuous wave) for LSM

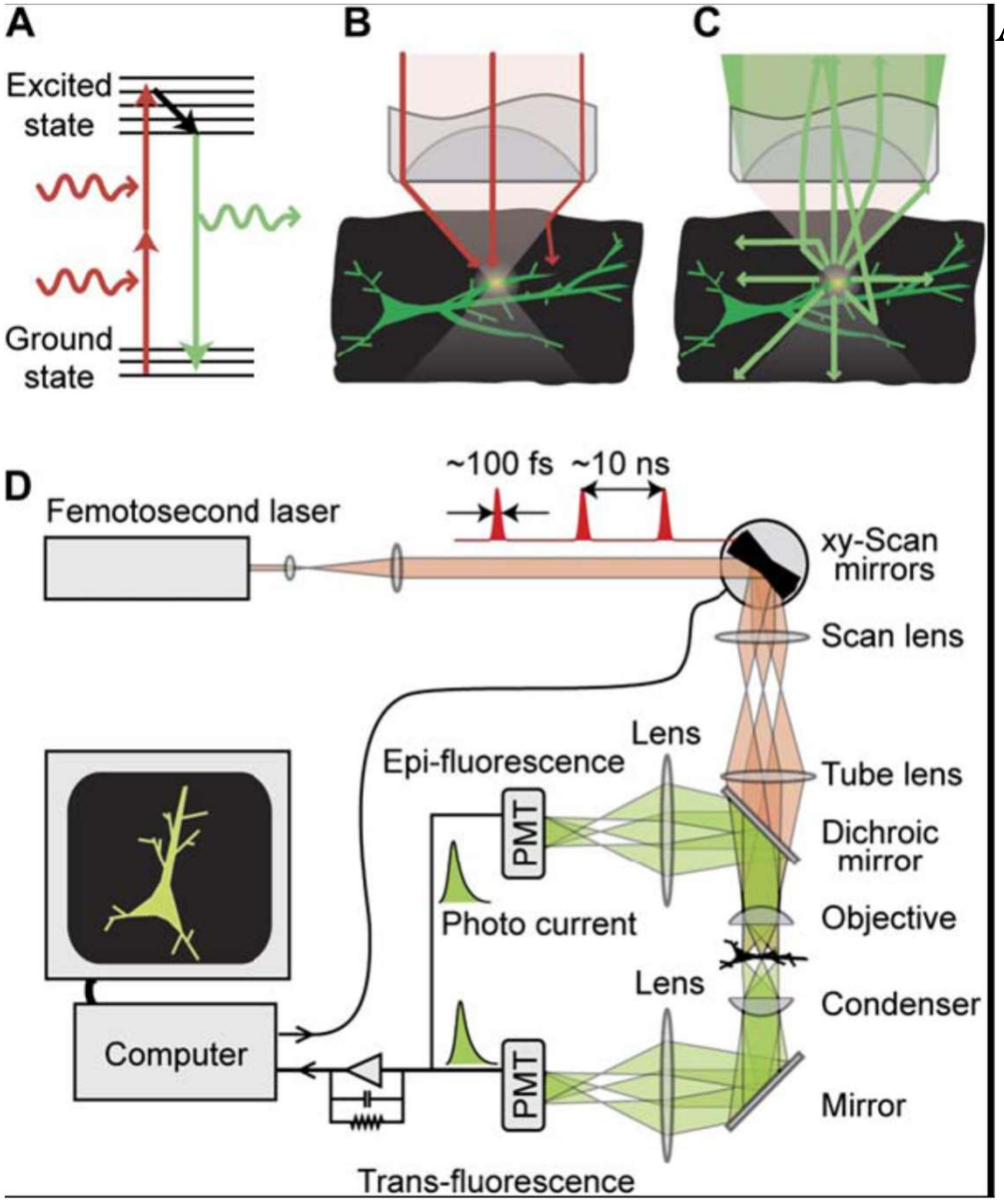
Continuous emission with low average power (e.g. 30mW Argon laser, 1mW HeNe543)

Pulsed laser

Ultra short pulses with a very high peak power (0,5 W average power with 100 fs pulses correspond to a peak power of 65.8 kW).



Two-photon microscopy



Absorption probability at the focus

$$p_a = \delta \langle P \rangle^2 F_p^{-1} \left(\frac{NA^2}{2hc\lambda} \right)^2 \xi$$

C –concentration of fluorophors
δ – absorbing section of two-photons
F_p – repeat frequency
ξ – superior factor(determined by the pulse of laser)
η – refractive index
$\langle P \rangle^2$ – average photon density

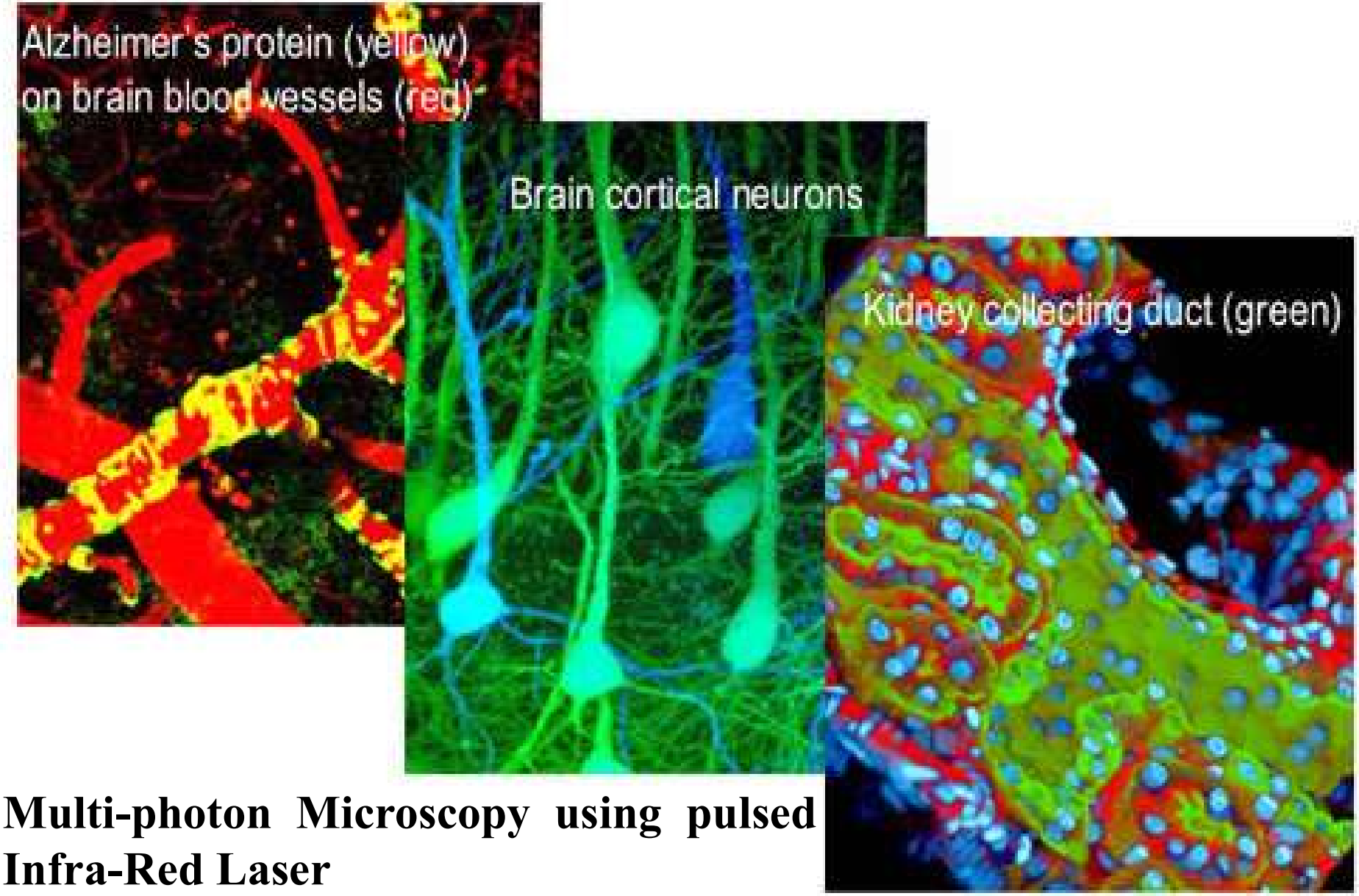
Total absorption probability

$$P_a = \frac{\delta C \langle P \rangle^2 \eta \xi}{2\pi hc}$$

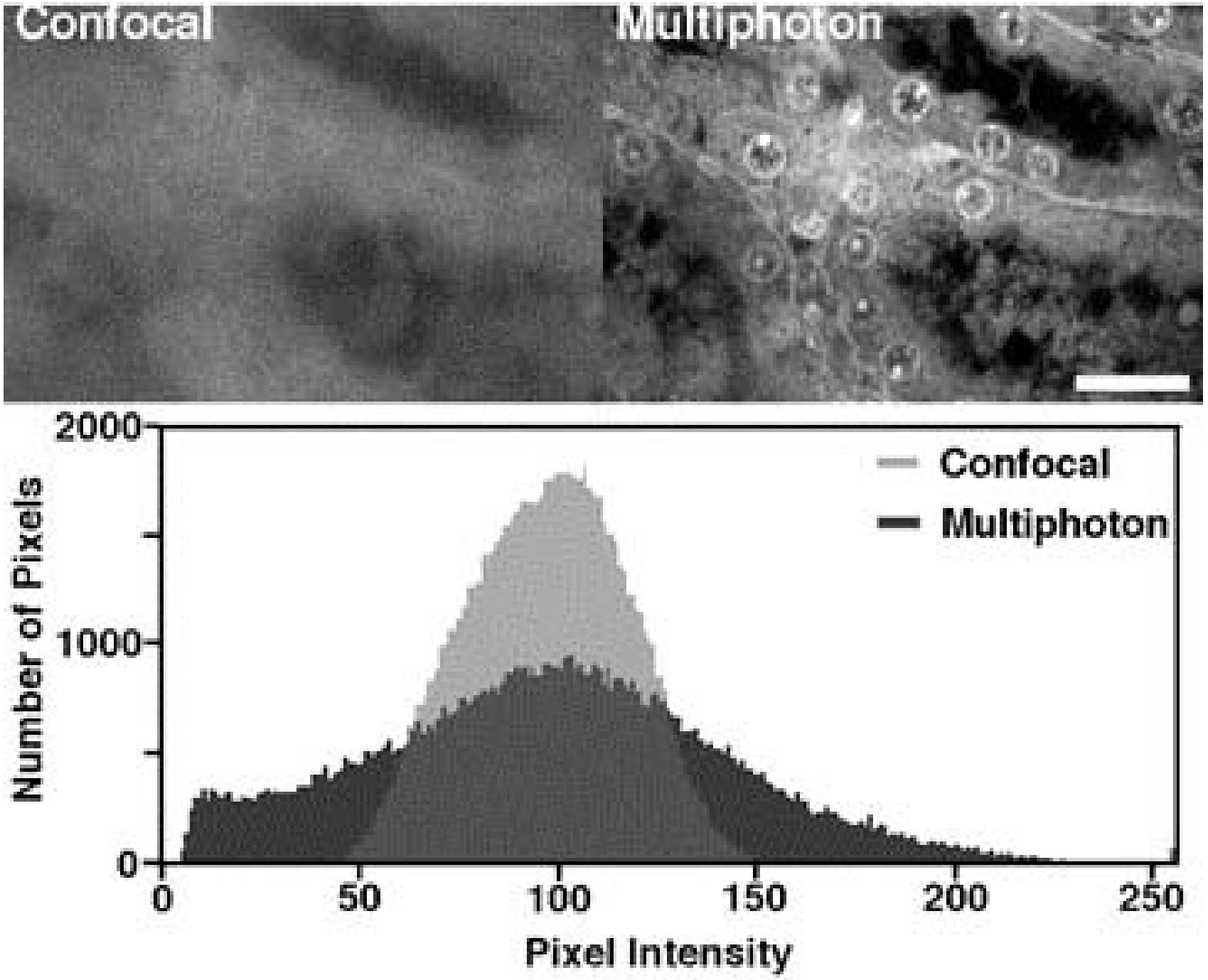
Svoboda & Yasuda, 2006.

Multiphoton microscopy

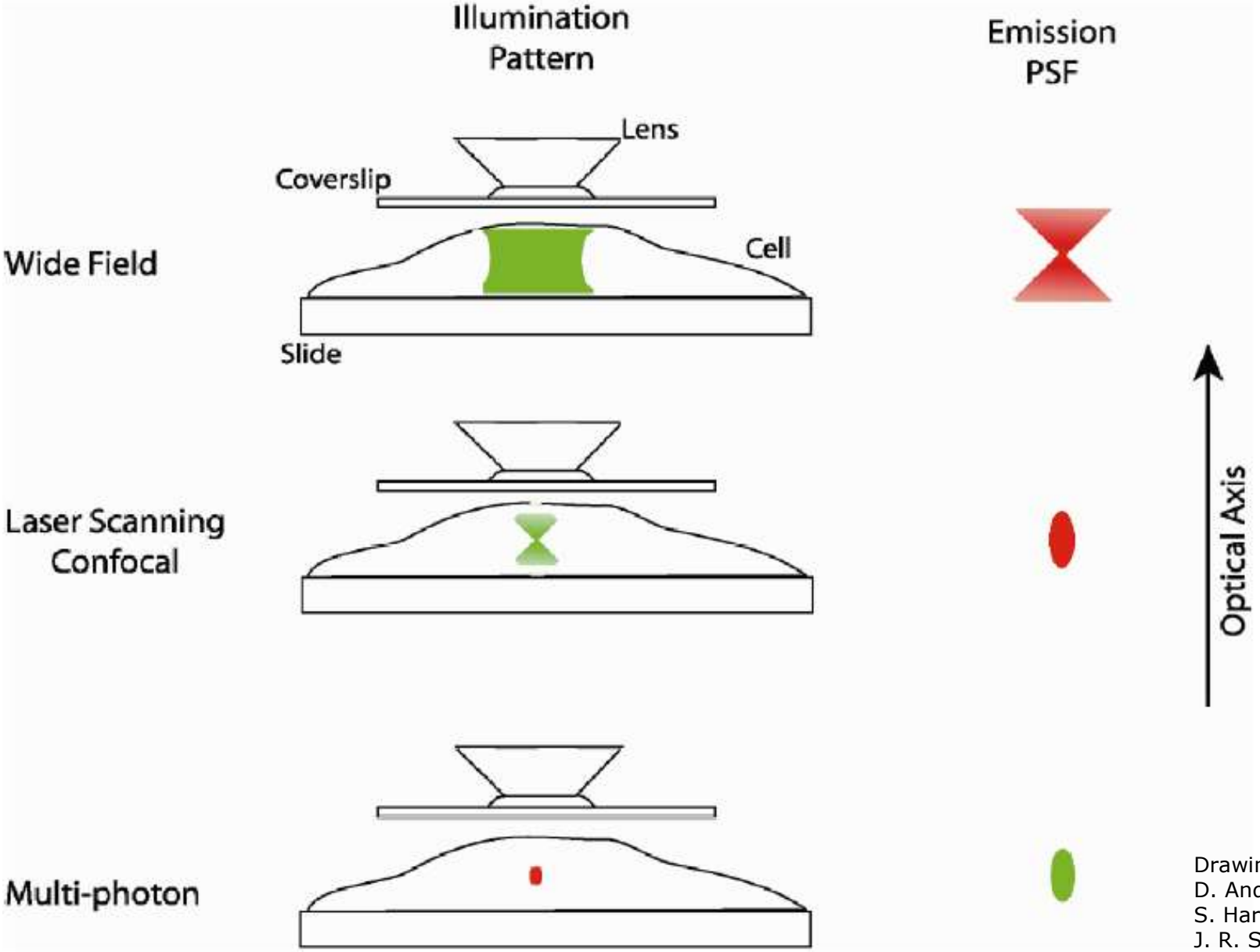
Multiphoton microscopy



Multi-photon Microscopy using pulsed Infra-Red Laser


Confocal
Multiphoton
2000
1000
0
Number of Pixels
Confocal
Multiphoton
0
50
100
150
200
250
Pixel Intensity

Wide-field vs confocal vs 2-p



Drawing by P. D. Andrews, I. S. Harper and J. R. Swedlow

Multiphoton microscopy is a powerful tool

1. True “Molecular Imaging,” with single-molecule sensitivity

2. Wealth of indicators capable of specific targeting
    - -Conventional dyes
    - -GFPs
    - -Intrinsic fluorescence & second harmonic generation
3. Sub-micron resolution
4. Optical sectioning in thick, turbid media
5. Wide variety of biological and clinical applications
    - -Gene expression
    - -Protein interactions
    - -Calcium concentrations
    - -Neural activity
    - -Disease diagnosis
    - -Optical biopsy

Advantages of Multi-photon Excitation

In addition to limiting photo bleaching and photo damage to the image plane, multi-photon excitation has several other advantages:

- Near-IR light scatters less than blue light in many biological samples, cause less damage than short-wavelength lasers.

- More efficient light collection
  - Deeper imaging into scattering tissue
  - Better looking images; greater effective resolution
  - Unaffected by chromatic aberrations

- Can excite dyes in their UV absorption bands
  - Can use wide range of useful UV dyes
  - Good for multicolor imaging

Reduction in phototoxic effects

- Phototoxic effects can be problematic when imaging fluorescent probes in vivo. Non-radiative transitions of the fluorophore to the ground state can give rise to highly reactive and destructive singlet oxygens. However, unlike confocal or wide-field fluorescence imaging, multiphoton imaging only excites fluorophore in the plane of the optical section being imaged. Therefore there is no fluorophore excitation in the bulk of the specimen. This considerably reduces the photo generation of toxic products.

- All the emitted photons from multi-photon excitation can be used for imaging (in principle) therefore no confocal blocking apertures or descanning optics have to be used allowing for a simpler, more light efficient optical design.

- UV flourophores may be excited using a lens that is not corrected for UV as these wavelengths never have to pass through the lens.

Limitations of multi-photon excitation

- ✓ Slightly lower resolution with a given fluorophore when compared to confocal imaging. This loss in resolution can be eliminated by the use of a confocal aperture at the expense of a loss in signal.

- ✓ Thermal damage can occur in a specimen if it contains chromophores that absorb the excitation wavelengths, such as the pigment melanin.

- ✓ Only works with fluorescence imaging.

- ✓ Currently rather expensive.

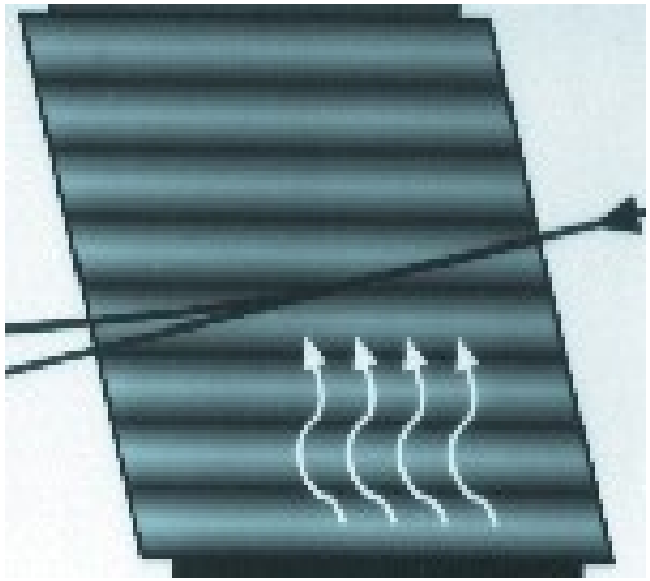
Issues for good confocal imaging

- **Axial Resolution**
  - Must determine the FWHM (full width half maximum) intensity values of a vertical section of beads
- **Field Flatness**
  - Must be able to collect a flat field image over a specimen - or z-axis information will be inaccurate
- **Chromatic Aberration**
  - must test across an entire field that emission is constant and not collecting radial or tangential artifacts due to chromatic aberration in objectives
- **Z-drive precision and accuracy**
  - must be able to reproducibility measure distance through a specimen - tenths of microns will make a big difference over 50 microns

THE END

Spectral CLSM (Leica)

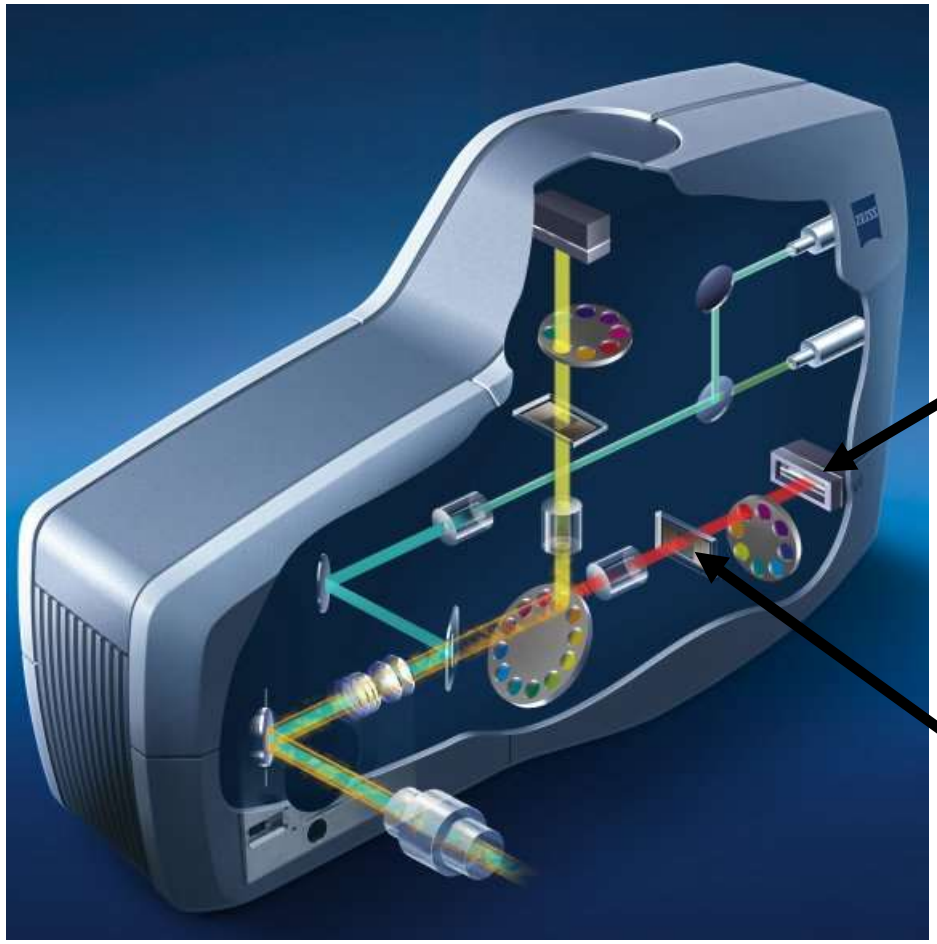
Fluorescence signal is dispersed on a prism, to provide spectral analysis capability.



Excitation via acousto-optical tunable filter:

- Free selection of excitation
- Individual line attenuation
- Less crosstalk
- Less fading

Spectral CLSM (Zeiss)



Zeiss LSM 5 *LIVE*

A new system concept of optics and electronics