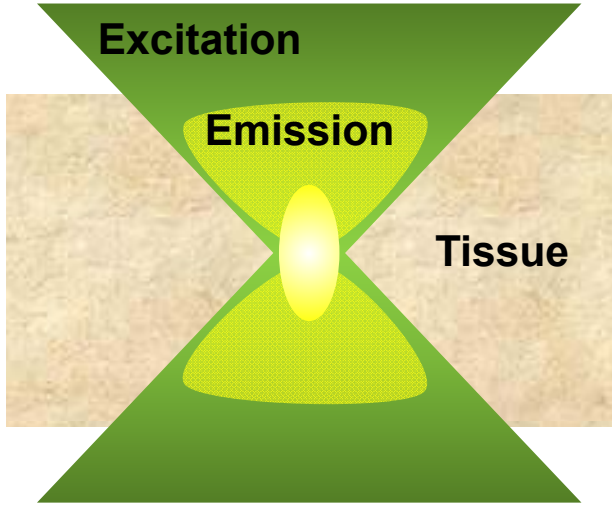
Optical Sectioning Microscopy

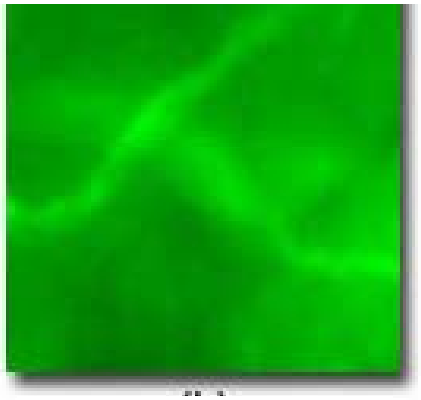
Confocal, Multi-Photon, SMI, SHG

Limitations of Microscopy

- **Resolution is limited in thick specimens**
  - Detection of out-of-focus light
  - The excitation beam illuminates uniformly a wide field of the sample.
  - If the sample is thick, fluorescence or scattering will be emitted within the focal plane, but also within planes above and below the focus.
  - Some of this light will be imaged onto the detector and will result in a defocused-looking image



Human medulla



Rabbit Muscle Fibers

Pollen Grain

Principles of Confocal Microscopy

- **Goal: Reject as much of the out-of-focus light as possible**
- **In confocal microscopy two pinholes are typically used:**
  - A pinhole in front of the illumination source → transmission only through a small area → imaged onto the focal plane of the specimen (i.e. only a point of the specimen is illuminated at one time)
  - Fluorescence or scattering from the focal point → imaged onto a confocal pinhole (right in front of the detector)
  - Out-of focus light can not go through the detector pinhole
- **Need to scan point onto the sample**
  - Either specimen is scanned past excitation beam or laser beam is scanned across specimen
  - For biological experiments, it is most common to scan the laser beam across focal plane using a combination of two galvanometric-driven mirrors

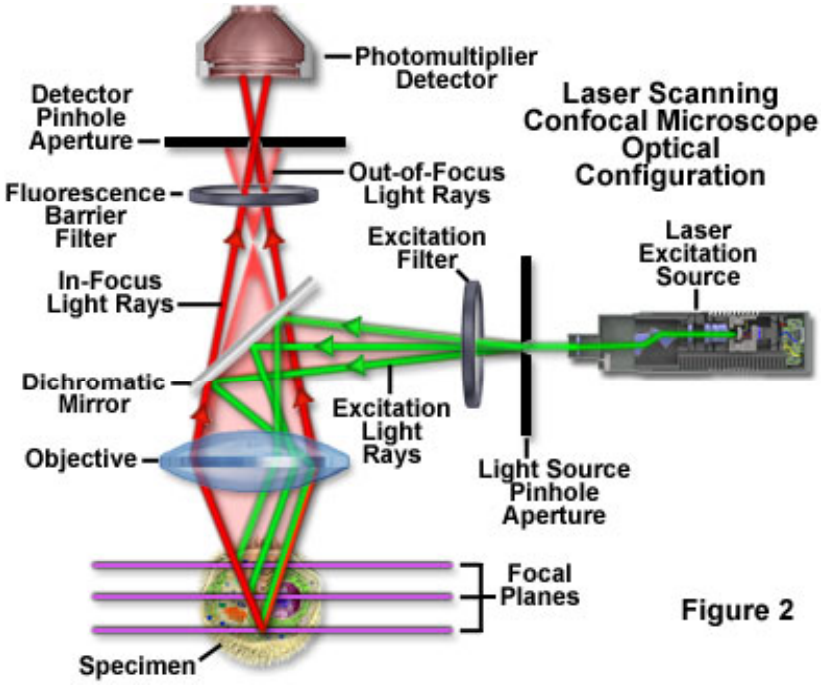
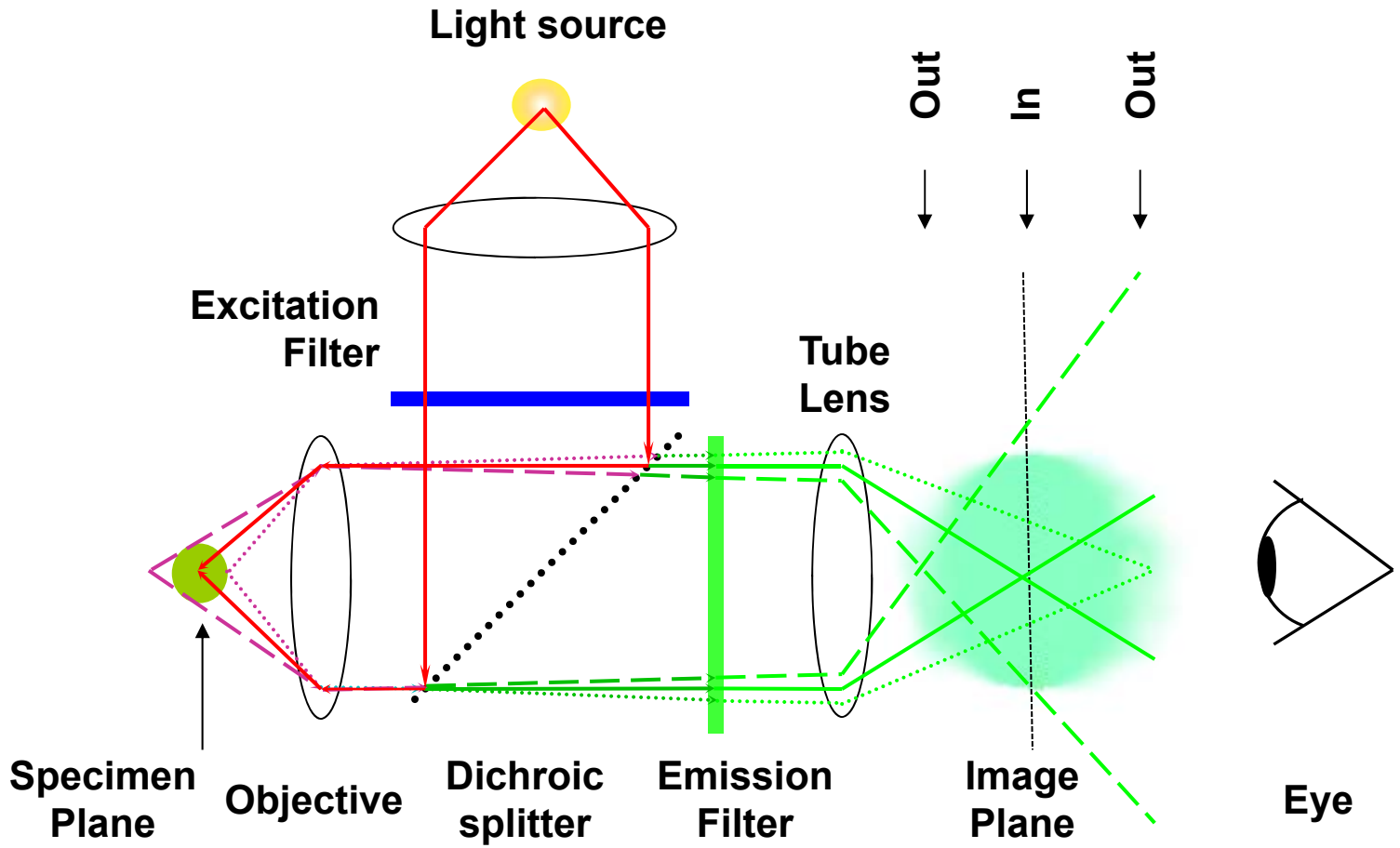
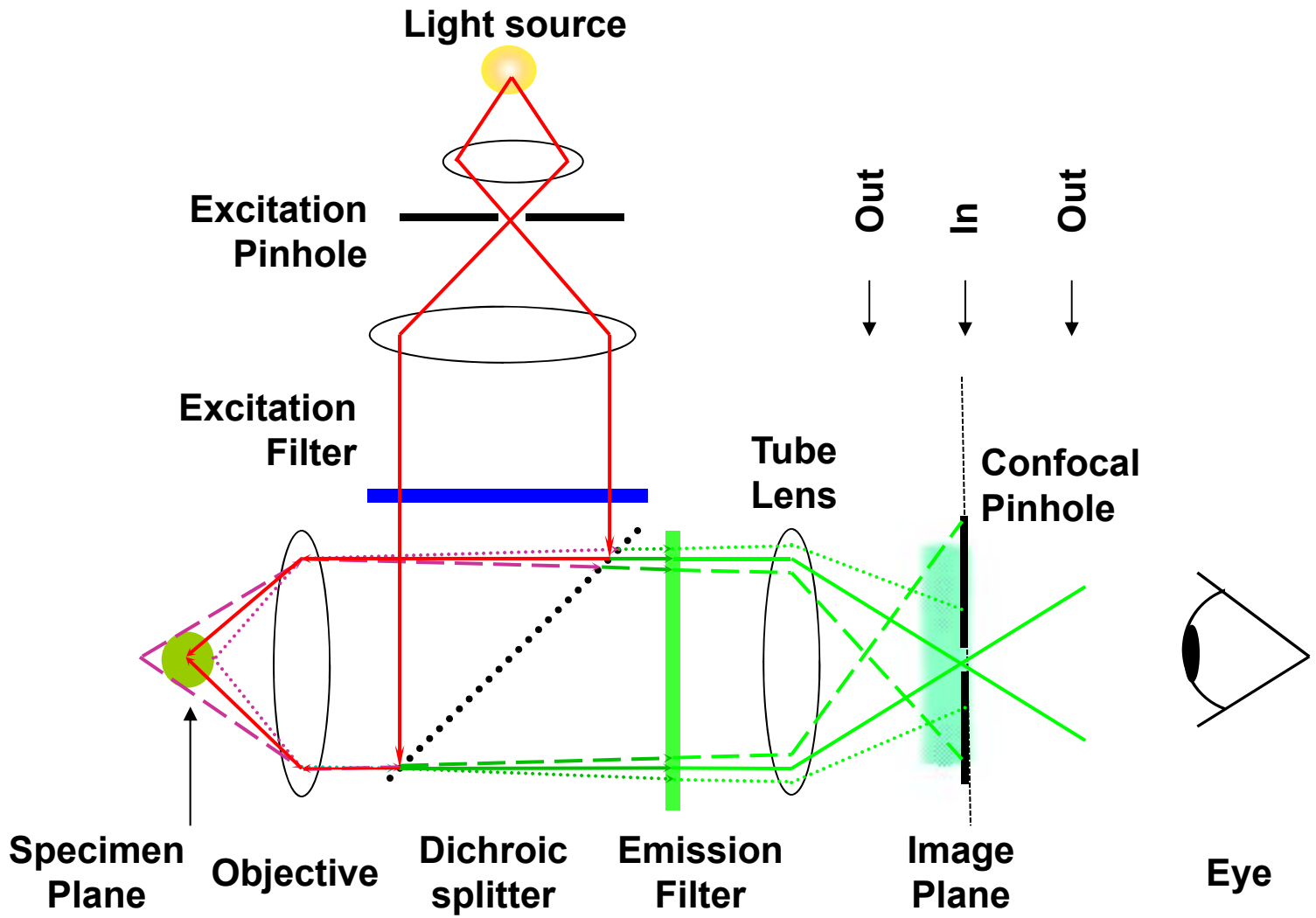


Figure 2

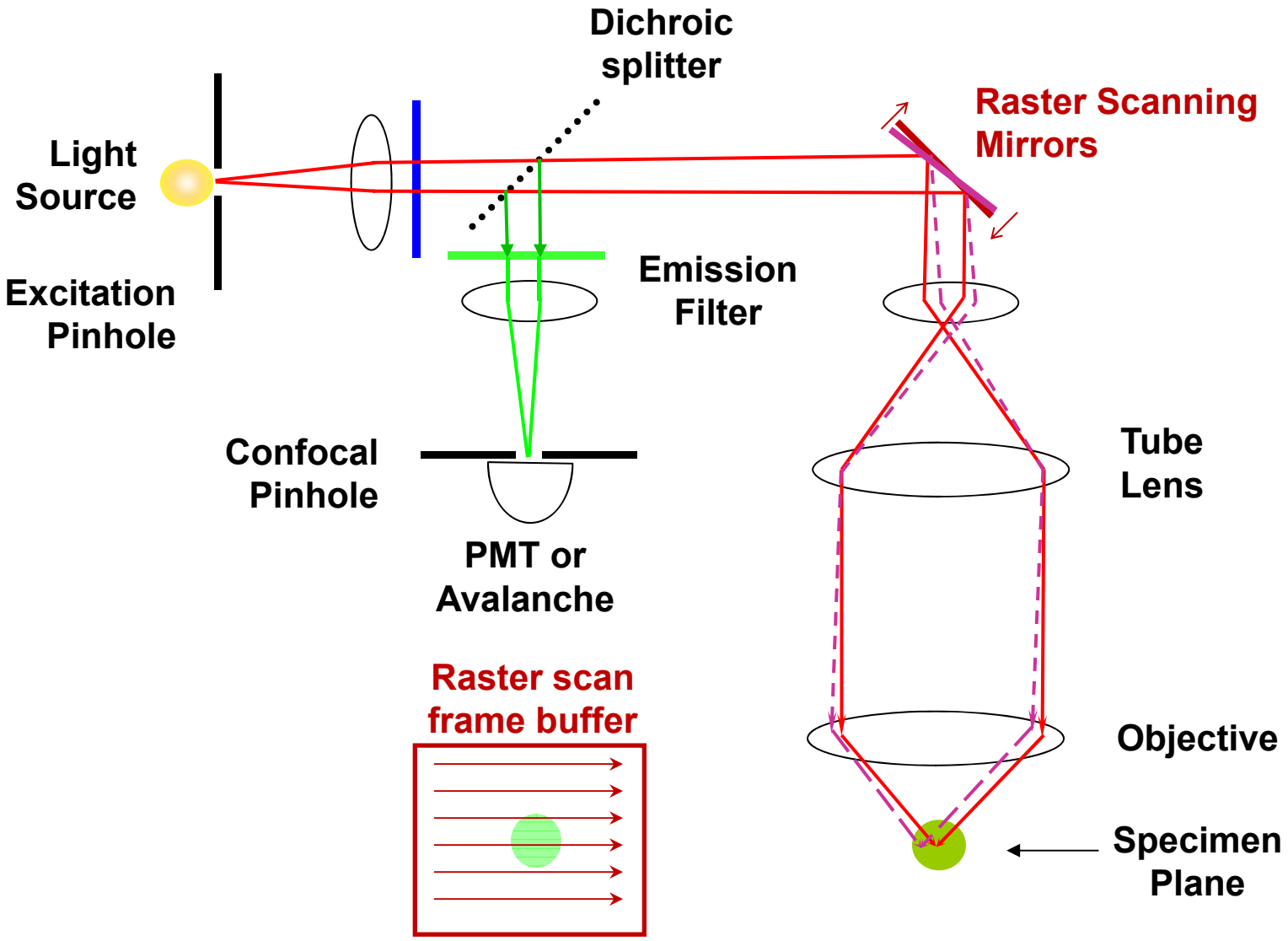
Principles of Confocal Microscopy



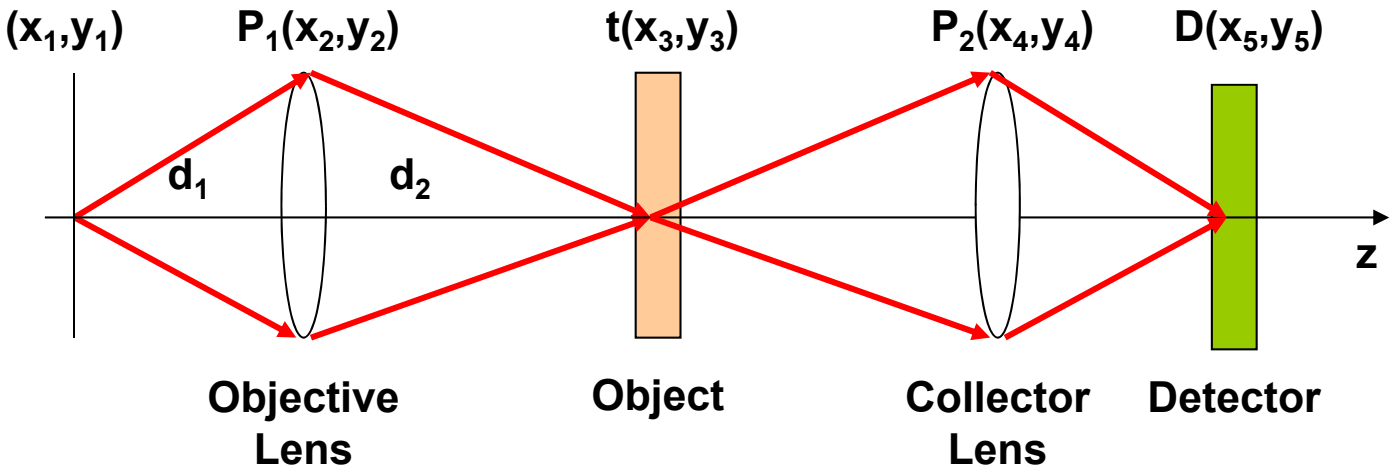
Principles of Confocal Microscopy



Principles of Confocal Microscopy



Principles of Confocal Microscopy



$$E(x_3, y_3) = \iint E(x_1, y_1) h\left(x_1 + \frac{x_3}{M}, y_1 + \frac{y_3}{M}\right) dxdy$$

$$h(x, y) = \iint P(x_2, y_2) \exp\left[\frac{2\pi j}{\lambda}(x_2 x + y_2 y)\right] dx_2 dy_2$$

$$I(x_3, y_3) = \left| h\left(\frac{x_3}{M}, \frac{y_3}{M}\right) \right|^2$$

(point source)

Principles of Confocal Microscopy

Transverse Response

$$I(v) = \left| 2\int_0^1 P(\rho) J_0(v\rho)\rho d\rho \right|^2$$

(circular aperture)

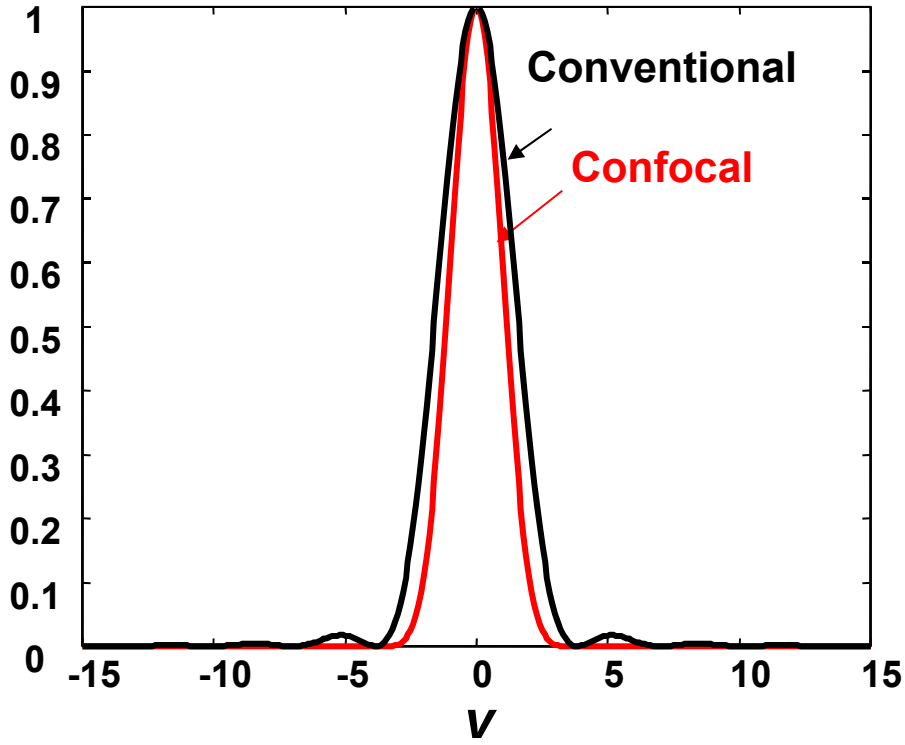
Conventional (Incoherent) Detection:

$$I = |h_1|^2 \otimes |t|^2 \qquad I(v) = \left(\frac{2J_1(v)}{v} \right)^2$$

Confocal (Coherent) Detection:

$$I = |h_1 \otimes t|^2 \qquad I(v) = \left(\frac{2J_1(v)}{v} \right)^4$$

$$v = \frac{2\pi}{\lambda} r_3 \sin a$$



Principles of Confocal Microscopy

Axial Response

$$h(u) = \iint P(\rho) \exp\left[\frac{j}{2} u\rho^2\right] J_0(v\rho)\, d\rho v\, dv$$

Conventional (Incoherent) Detection:

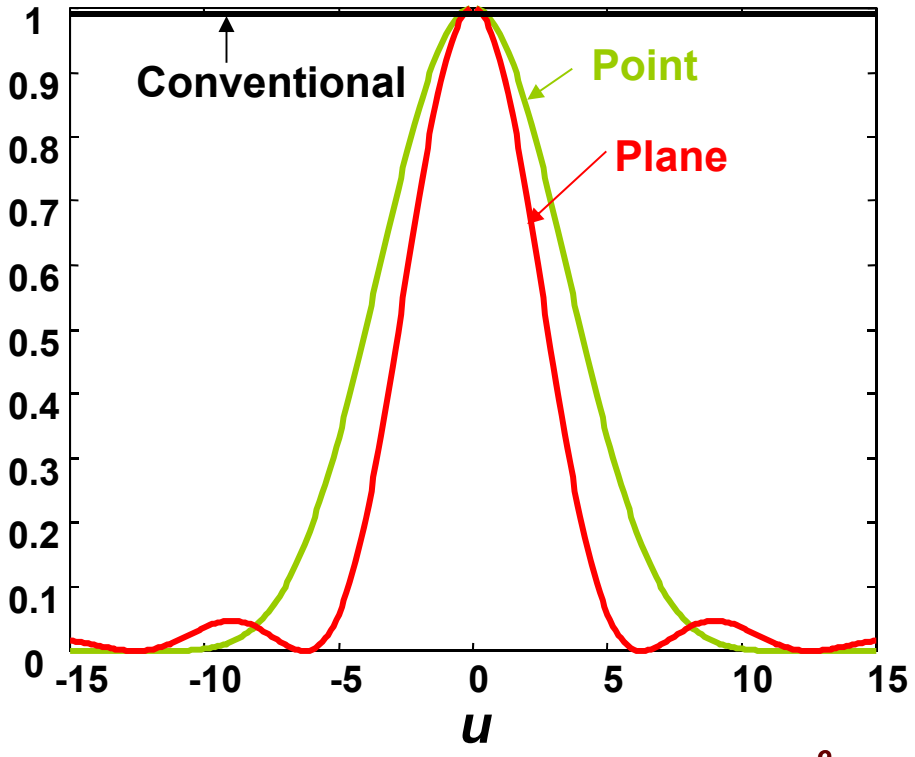
$$I(u) = \text{constant}$$

Confocal (Coherent) Detection:

$$I(u) = \left(\frac{\sin(u/4)}{u/4}\right)^4 \quad \text{(point)}$$

$$I(u) = \left(\frac{\sin(u/4)}{u/4}\right)^2 \quad \text{(plane)}$$

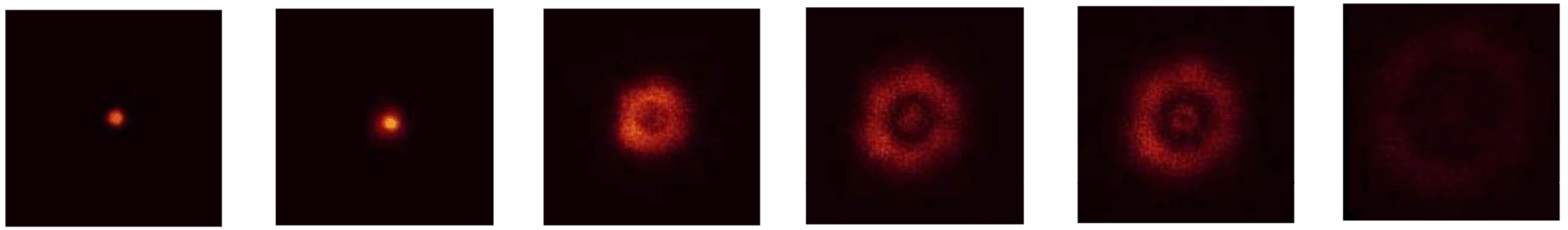
$$u = \frac{8\pi}{\lambda} z \sin^2(a/2)$$



Resolution

- **Airy Disk**
  - Resolution quickly deteriorates out of focus

0.5 μm bead Plan Apo 100x 1.4 NA oil



0.0 μm | 1.1 μm | 2.4 μm | 3.4 μm | 3.7 μm | 5.2 μm

Lateral Resolution

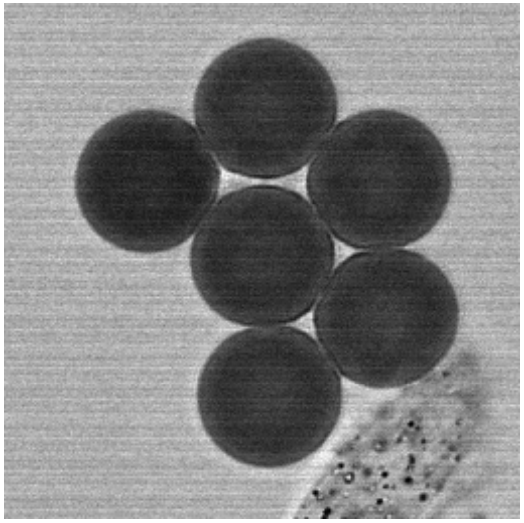
Wide field

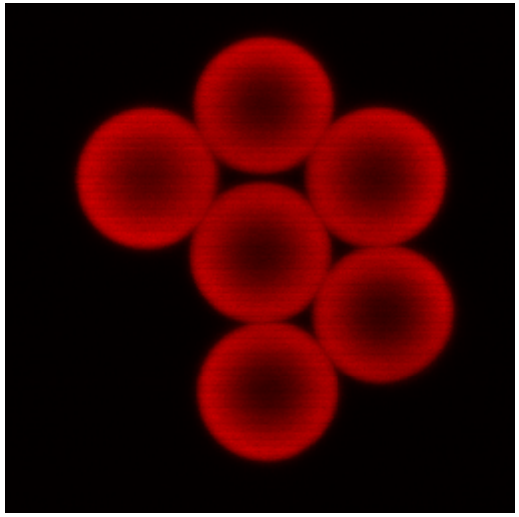
	Transverse	
	dy	**dy_{3db}**
Point Object	$dy = \frac{0.61\lambda}{NA}$	$dy_{3dB} = \frac{0.51\lambda}{NA}$
Edge Response (10-90%)	$dy = \frac{0.49\lambda}{NA}$	

Confocal

	Transverse	
	dy	**dy_{3db}**
Point Object	$dy = \frac{0.56\lambda}{NA}$	$dy_{3dB} = \frac{0.37\lambda}{NA}$
Plane Object	N/A	N/A
Edge Response (10-90%)	$dy = \frac{0.44\lambda}{NA}$	

10 um beads – xy view

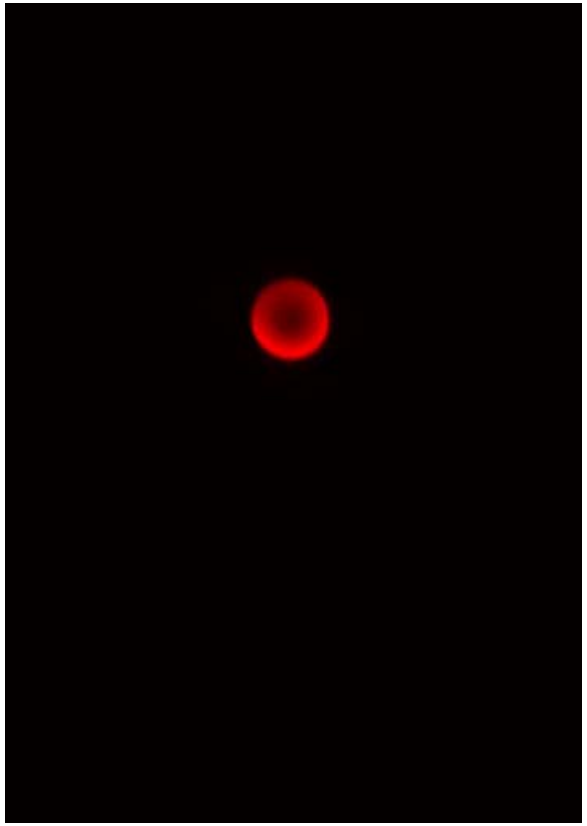




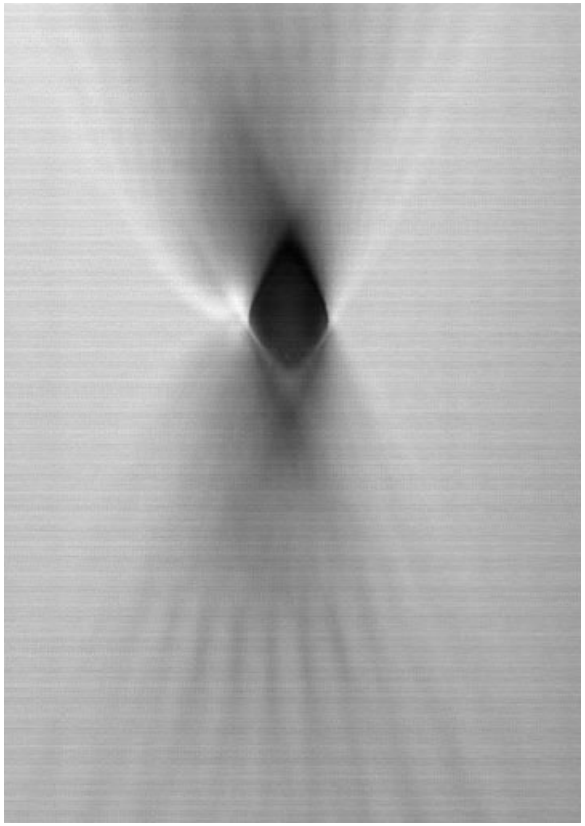
Axial Resolution

10 um bead – xz side view

Confocal



Wide field



Axial Resolution

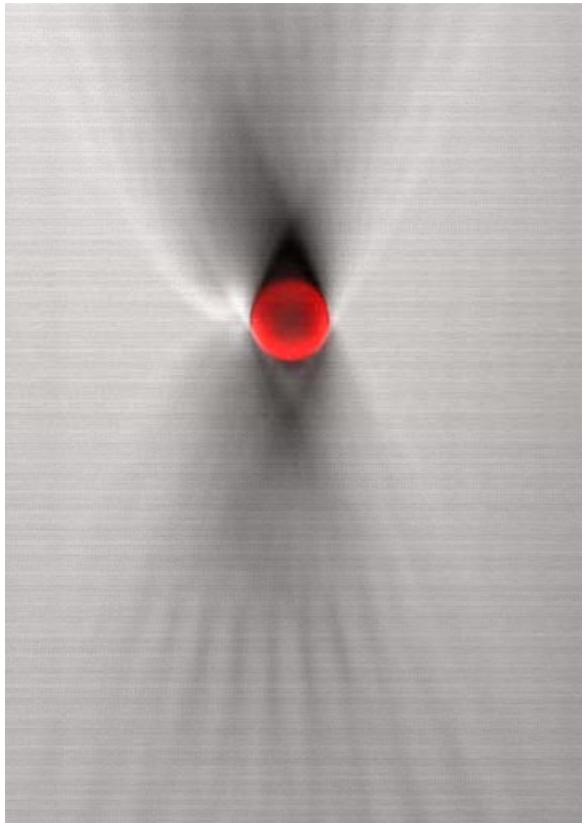
Wide field

	Axial	
	dz	dz_{3db}
Point Object	N/A	
Edge Response (10-90%)		

Confocal

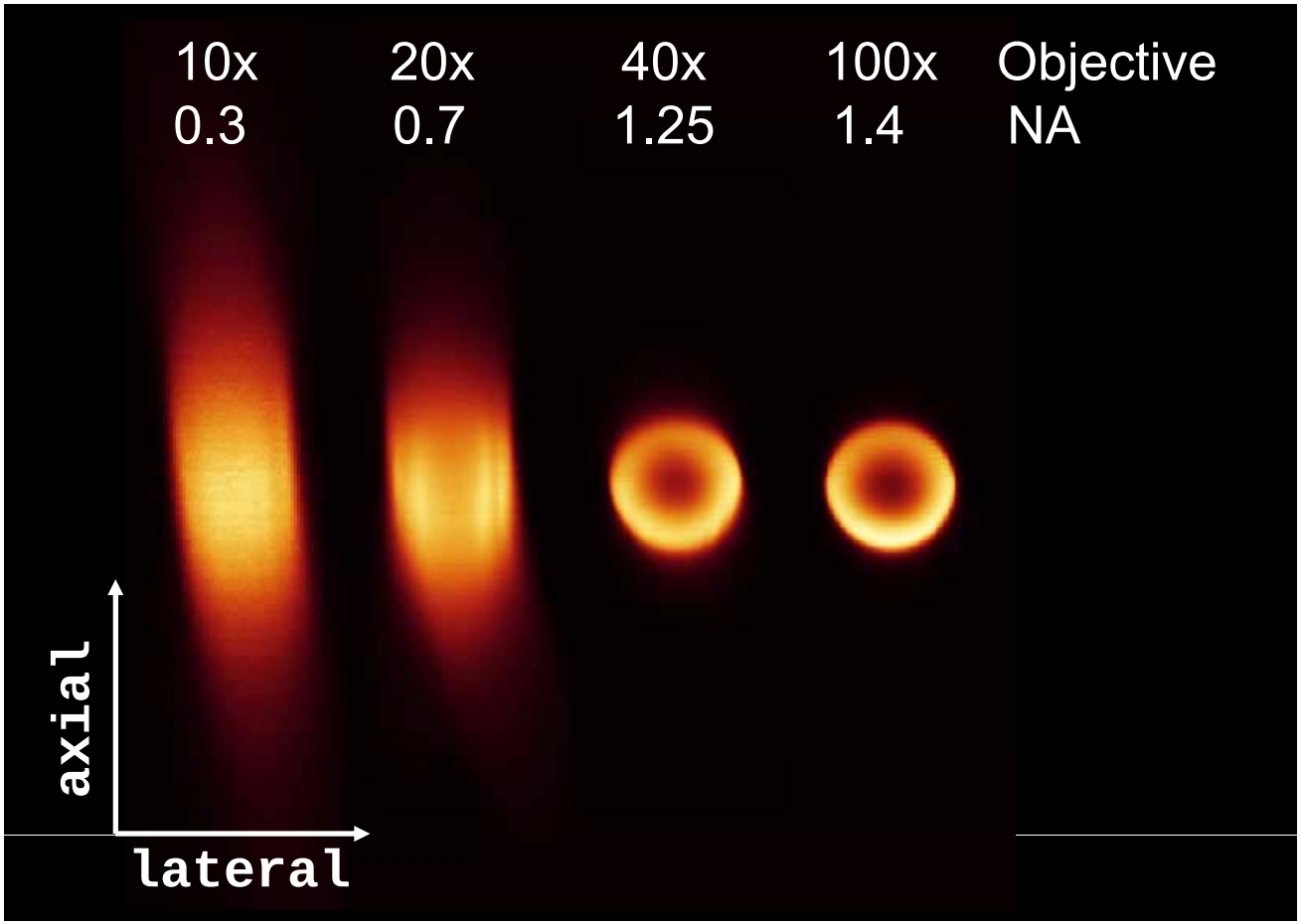
	Axial	
	dz	dz_{3db}
Point Object	$dz = \frac{0.89\lambda}{n(1-\cos\vartheta_o)}$	$dz_{3dB} = \frac{0.62\lambda}{n(1-\cos\vartheta_o)} \approx \frac{1.24n\lambda}{NA^2}$ (for large NA the approximation results in a 2-6% error)
Plane Object	$dz = \frac{0.72\lambda}{n(1-\cos\vartheta_o)}$	$dz_{3dB} = \frac{0.45\lambda}{n(1-\cos\vartheta_o)} \approx \frac{0.90n\lambda}{NA^2}$ (for large NA the approximation results in a 2-6% error)
Edge Response (10-90%)	N/A	

10 um bead – xz side view



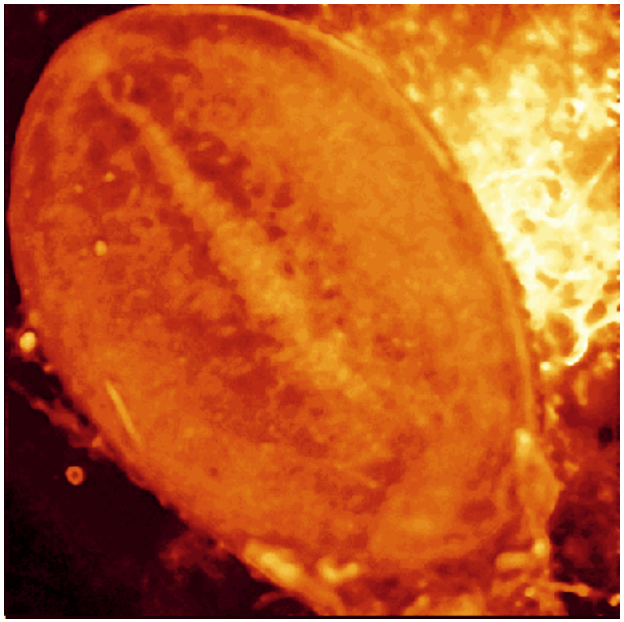
Axial Resolution

- **Resolution vs. NA**

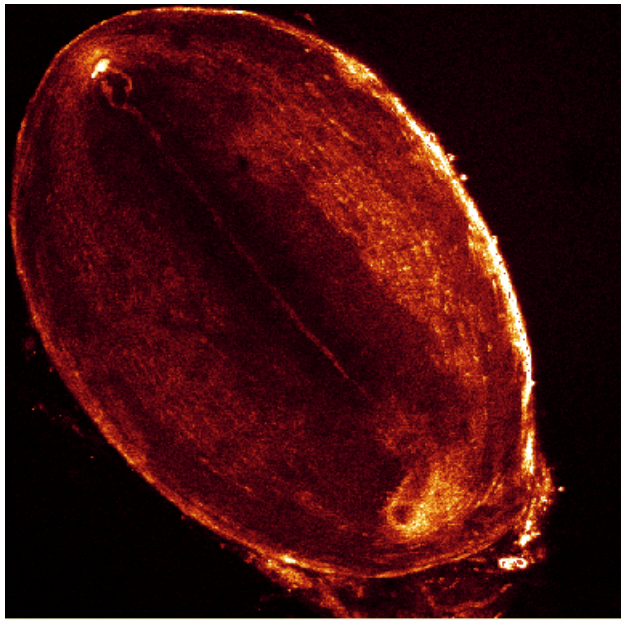


Wide Filed vs. Confocal

Pacinian Corpuscle 10X NA 0.3 FM 1-43



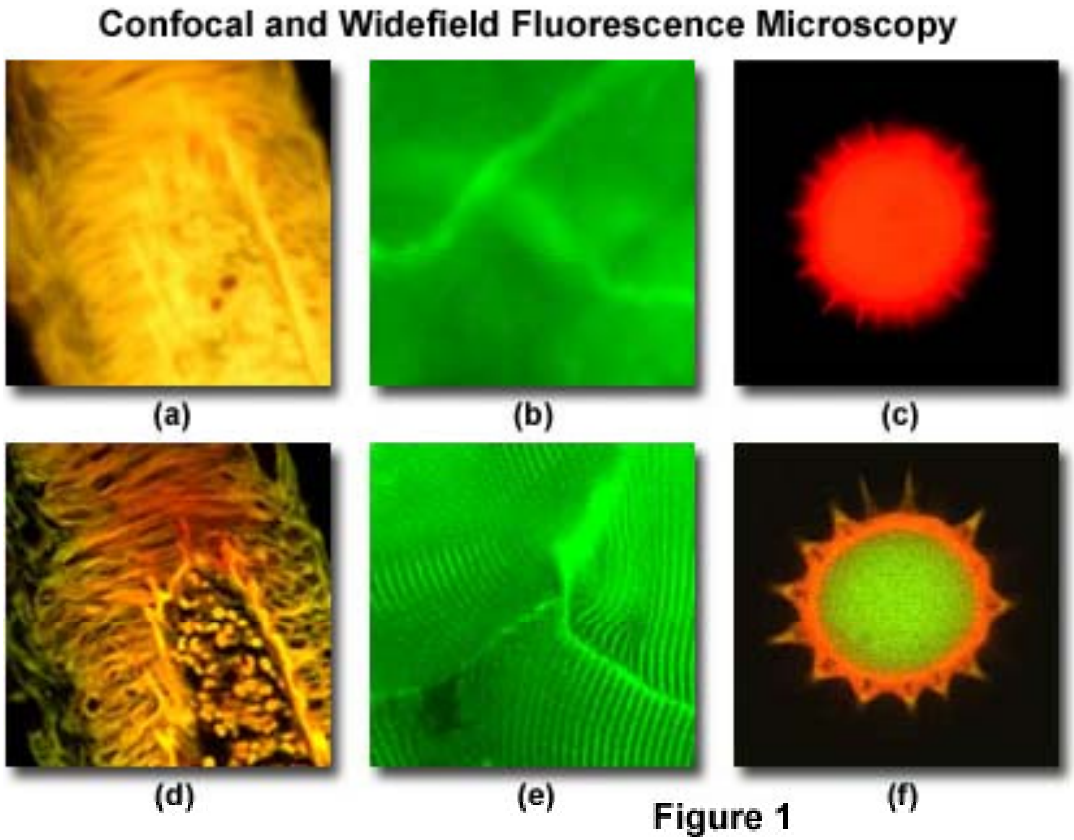
Wide field
~150 um thick view
Glare
Out of focus
Low resolution



Confocal
~10 um thick
High contrast
Low background
Good resolution

Wide Filed vs. Confocal

- **Elimination of out-of focus fluorescence yields superior images**



http://www.olympusfluoview.com/theory/confocalintro.html

Wide Filed vs. Confocal

- **Confocal Advantages**
  - Reduction of background
  - Control of depth of field → Optical sectioning, 3d imaging
  - Improved resolution

Pollen Grain Serial Optical Sections by Confocal Microscopy

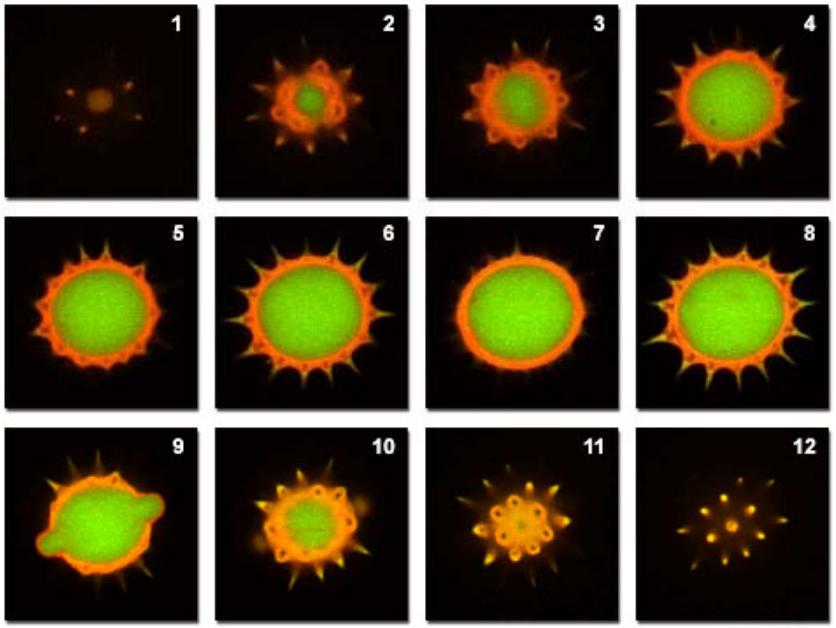


Figure 6

Three-Dimensional Volume Renders from Confocal Optical Sections

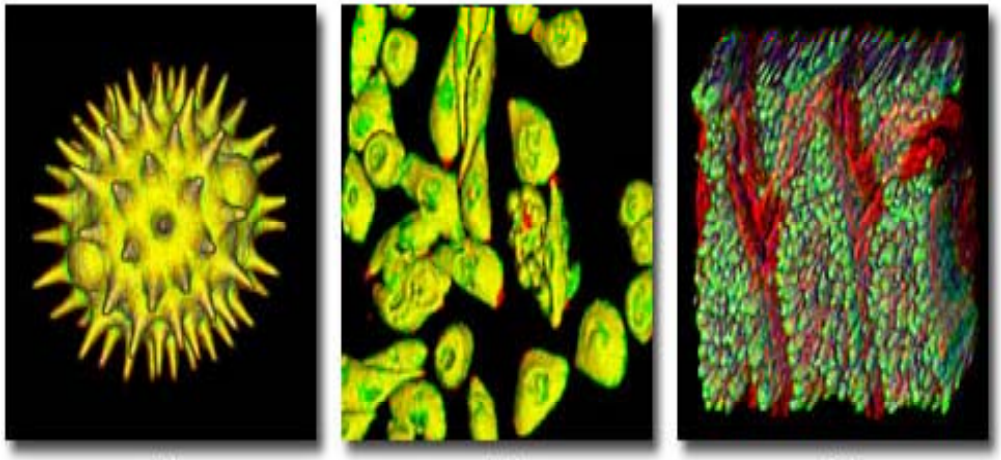


Figure 7

Pollen grain

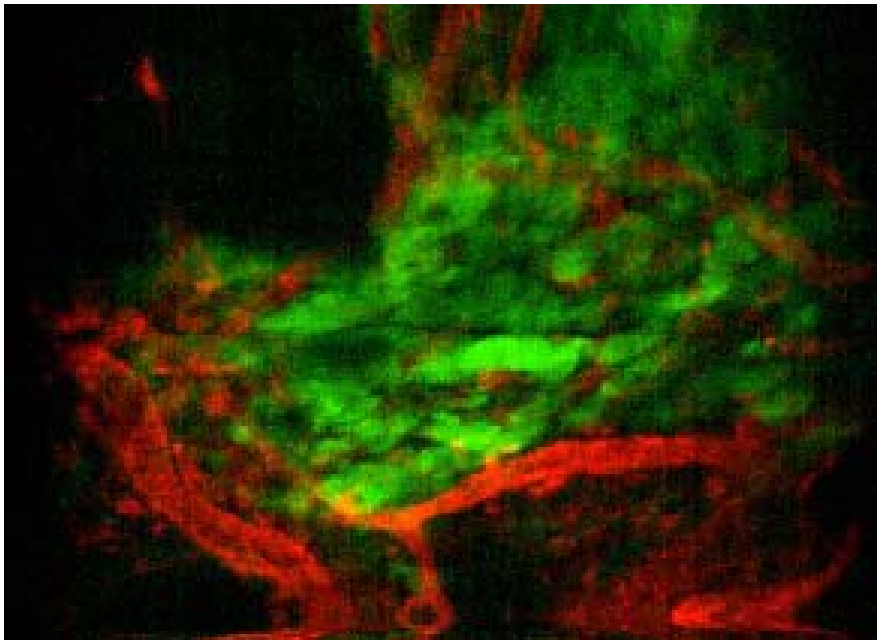
Hamster ovary cells

Mouse intestine

http://www.olympusfluoview.com/java/scanningmodes/index.html

Applications

- **Applications**
  - Cellular imaging
  - Developmental biology
  - Cancer imaging
- **In vivo depth-resolved imaging is possible**
  - Tumor cells grown subcutaneously in mice, expressing Green Fluorescent Protein
  - Blood vessels stained with Cy5-conjugated anti-PECAM antibody
  - Study interactions of tumor cells with their environment and potential factors/drugs that affect processes, such as tumor growth or metastasis



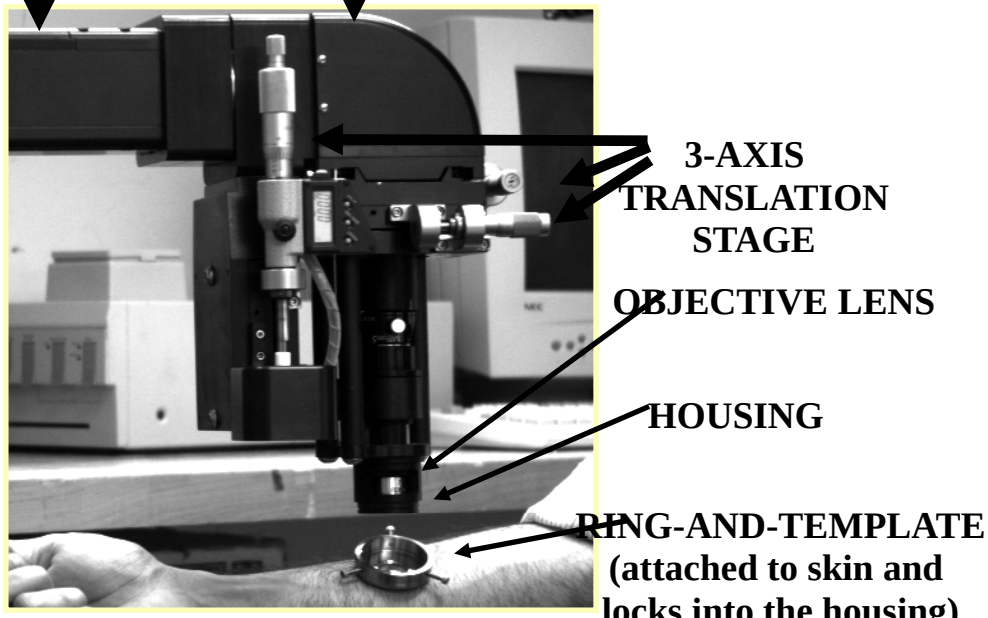
Applications

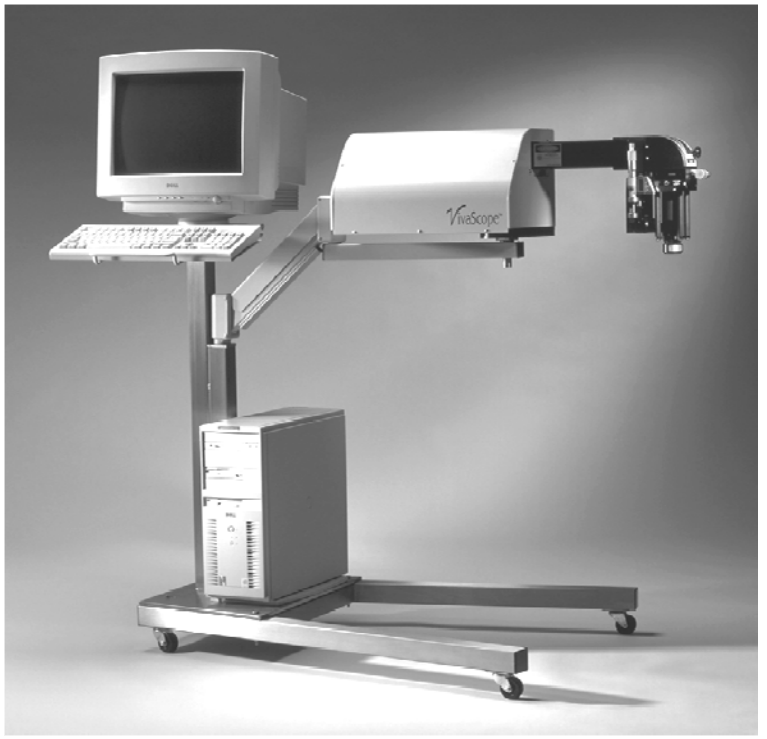
- **Developmental Biology**
  - Zebra fish embryo wholemount
    - Neurons (green)
    - Cell adhesion molecule (red)

Monika Marks, Martin Bastmeyer
University of Konstanz

Applications

- In Vivo Reflectance Confocal Microscopy of human skin

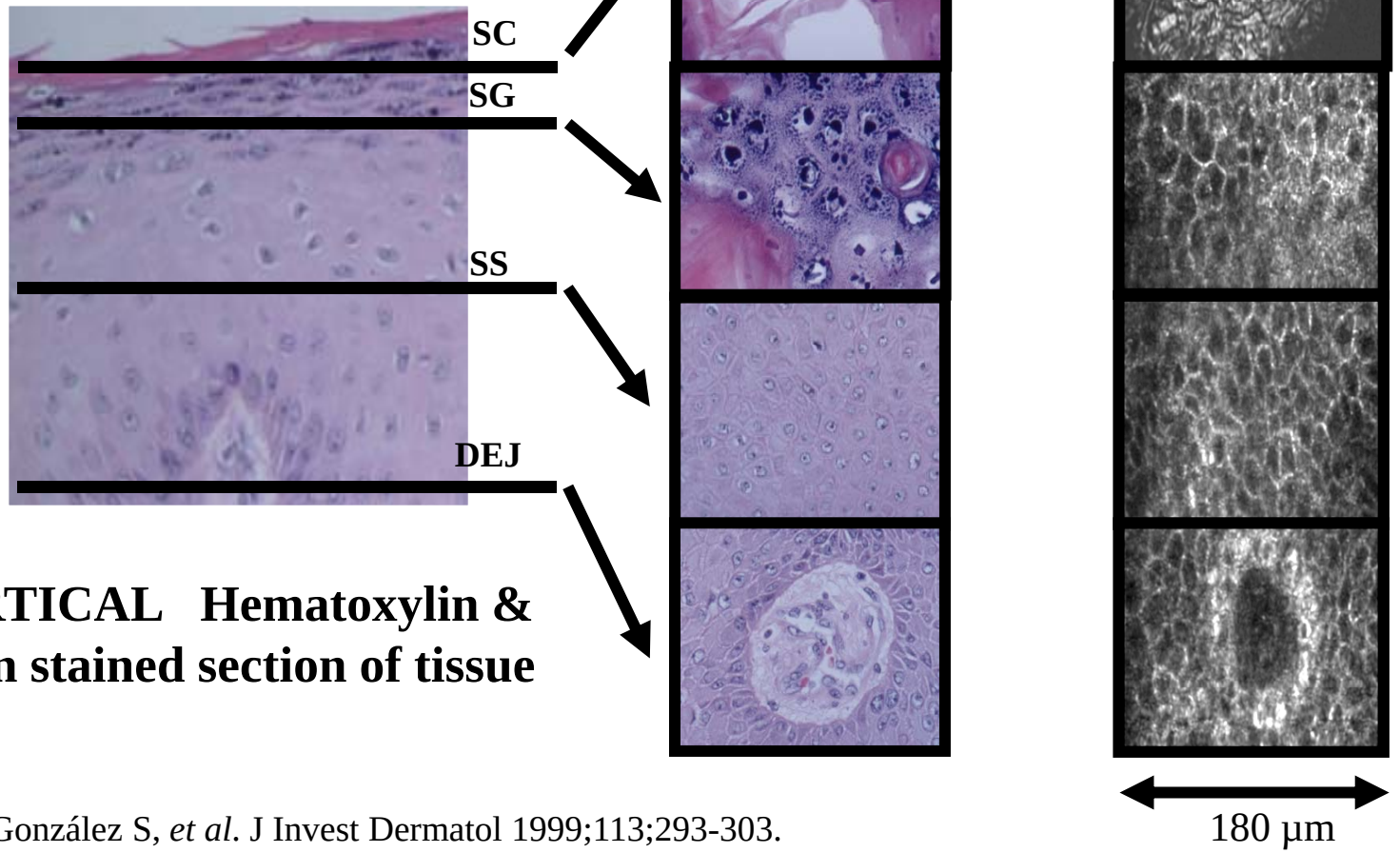




VivaScope by Lucid

Applications

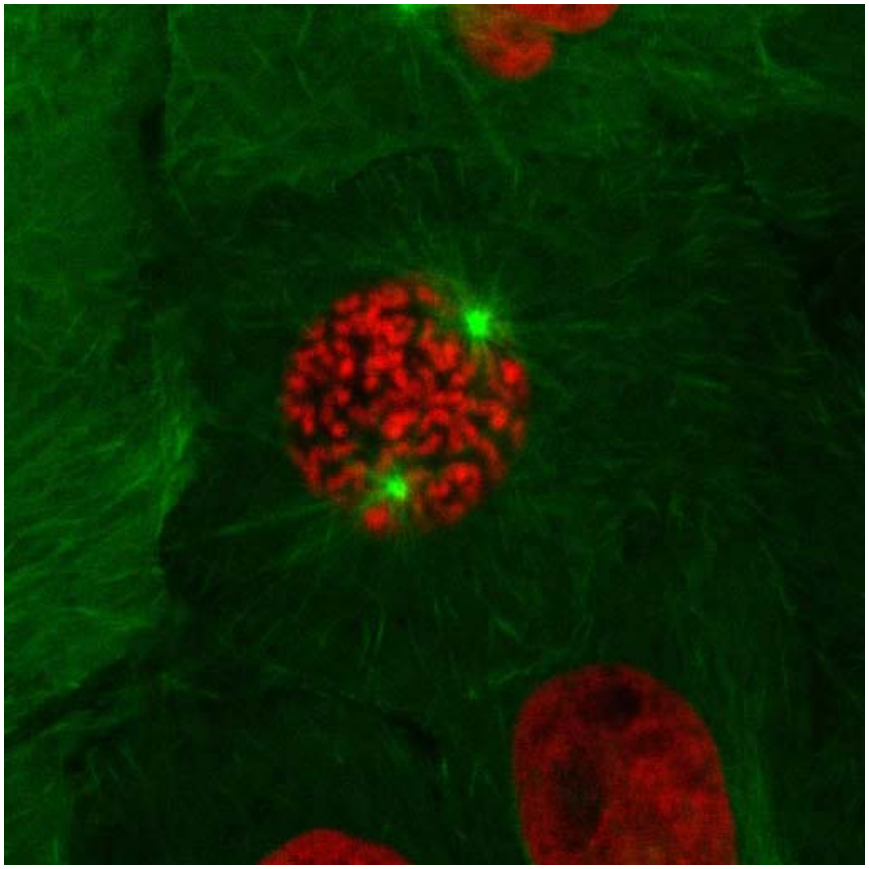
- **Reflectance-mode Confocal Microscopy**
  - Live Normal Skin



Rajadhyaksha M, González S, *et al.* J Invest Dermatol 1999;113;293-303.

Applications

- **Video rate microscopy captures dynamic interactions**
  - Monitor cell-cell, cell-environment interactions in natural environment
  - Understand animal and human biology and processes involved in disease development
  - Monitor dynamic interactions

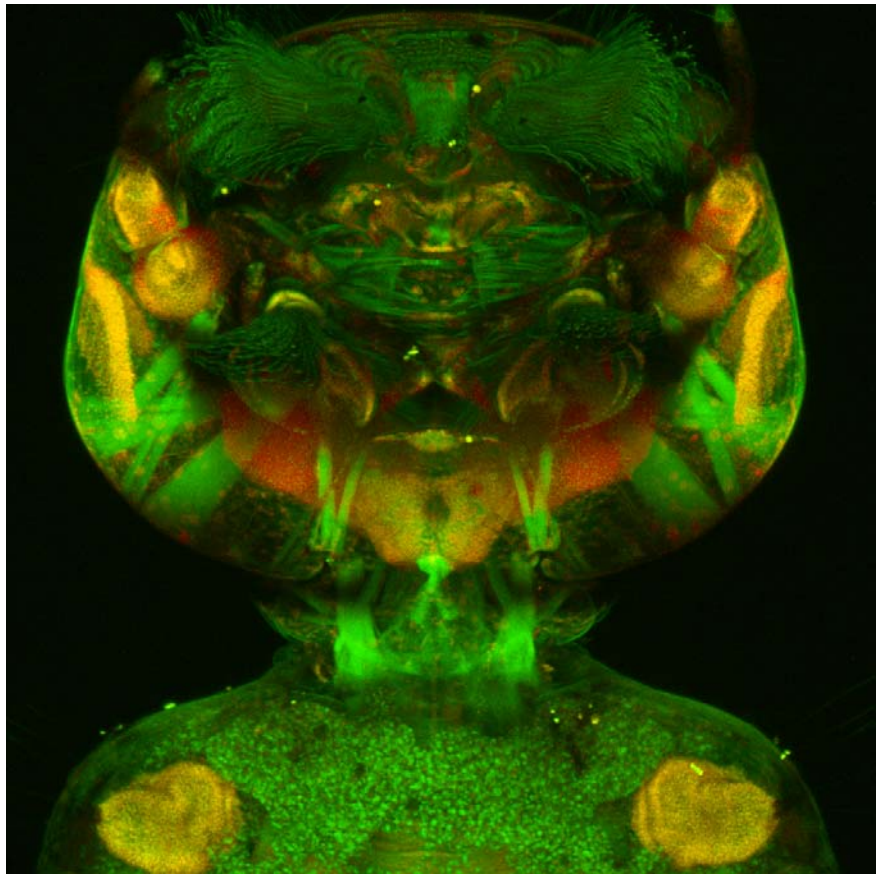


Mitosis in Pig Kidney Epithelial Cells

http://www.microscopyu.com/moviegallery/c1si/mitosiseb3/index.html

Limitations of Confocal Microscopy

- **But confocal microscopy has limitations:**
  - Efficiency of light collection limited by pinhole.
  - Scanning slows the image generation.
  - Scanning hard to implement endoscopically
  - Scattering in tissue limits depth of the section that can be imaged.
  - There are engineering tricks to partially compensate for these problems, but only partially.
    - (e.g., replacing the pinhole with multiple pinholes, or a line scan, gives more light but increases the problems from scattering.)



Hydroethidine R. Zucker, EPA

Microscope Resolution Limit

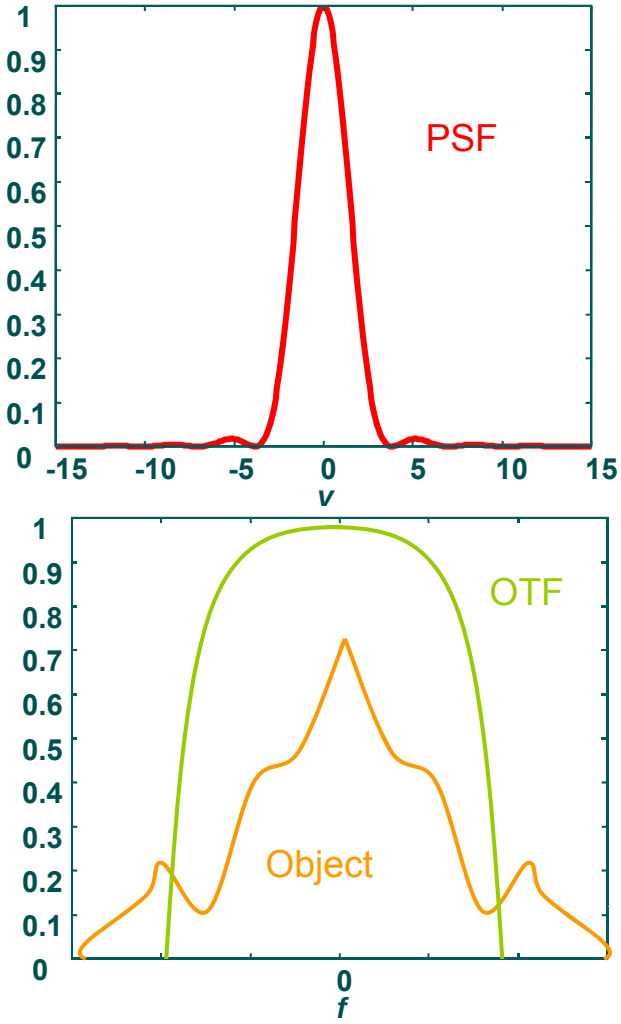
- **Resolution**
  - Fundamentally limited (λ)
  - PSF / OTF
- **For a conventional microscope**

$$I = |h_1|^2 \otimes |t|^2 \qquad I(v) = \left(\frac{2J_1(v)}{v} \right)^2$$

$$v = \frac{2\pi}{\lambda} r_3 \sin a$$

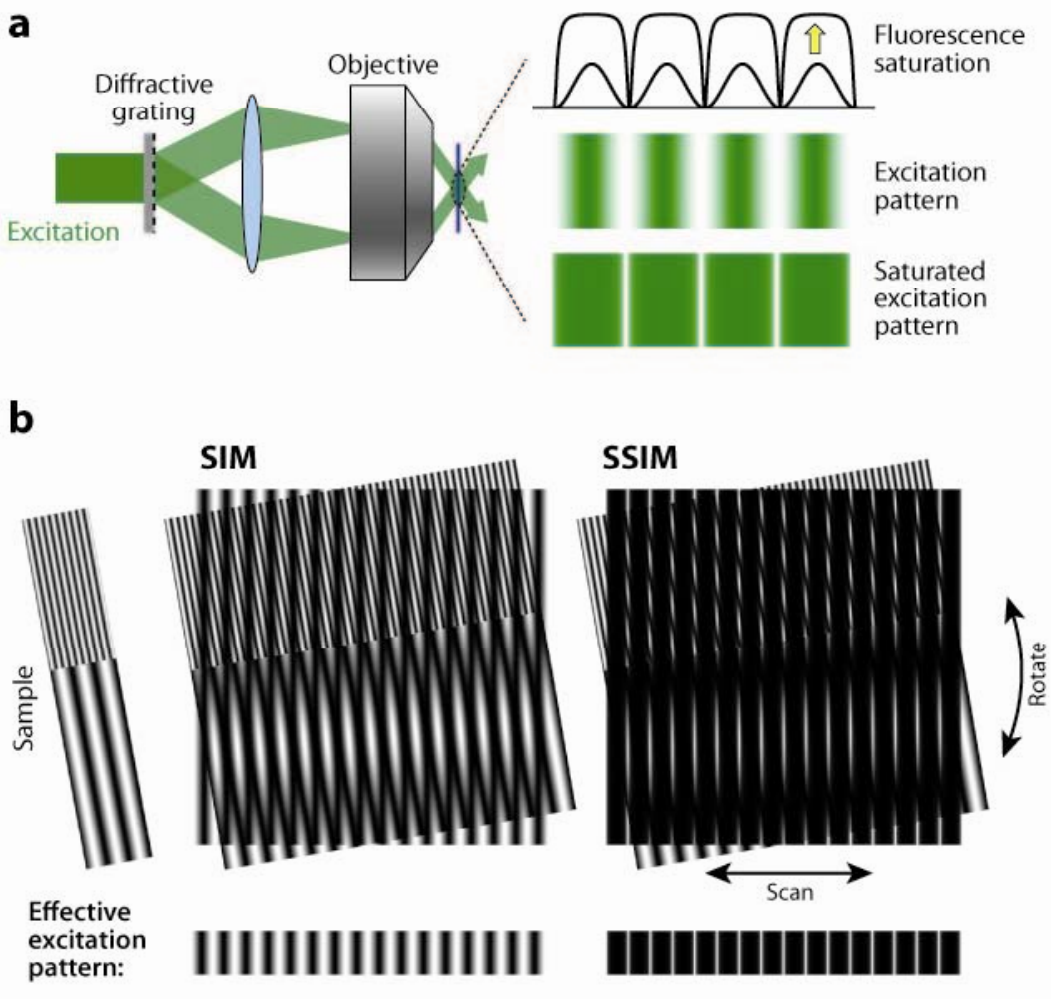
(Assuming circular aperture)

- **Lateral resolution improvement?**
  - Spatially modulated illumination (SMI)



Spatially Modulated Illumination

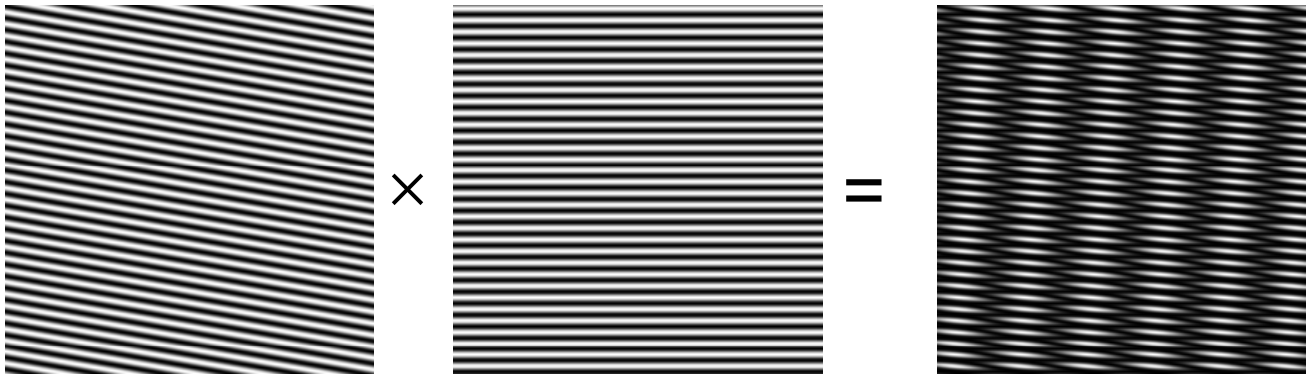
- **Structured or Modulated Illumination**
  - A diffractive grating in the excitation path splits the light into two beams → interference after the objective → sinusoidal illumination pattern
- **Moiré pattern**
  - Sinusoidal illumination patterns is applied to a sample → a moiré pattern at a significantly lower spatial frequency than that of the sample → can be imaged by the microscope
  - Multiple images from scanning and rotating the excitation pattern → used to reconstruct the sample structure.
  - SMI introduces a high-frequency component into the excitation pattern, allowing features far below the diffraction limit to be resolved



Huang, Bo, Mark Bates, and Xiaowei Zhuang. 2009. "Super-Resolution Fluorescence Microscopy." *Annual Review of Biochemistry* 78 (1): 993-1016. doi:10.1146/annurev.biochem.77.061906.092014.

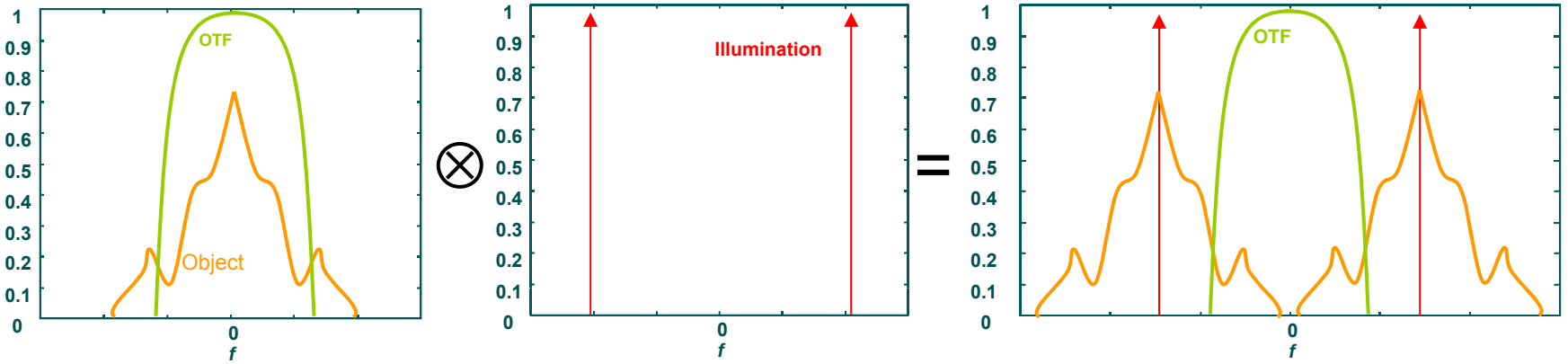
Spatially Modulated Illumination

Spatial Domain



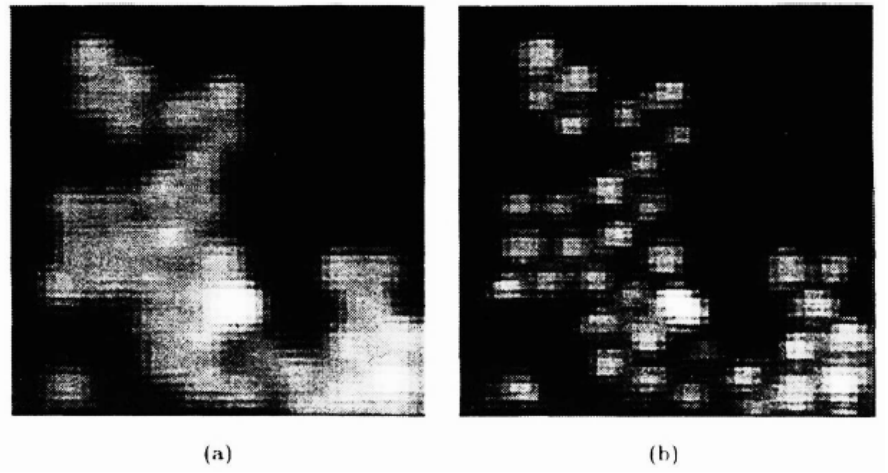
Moiré fringes

Frequency Domain



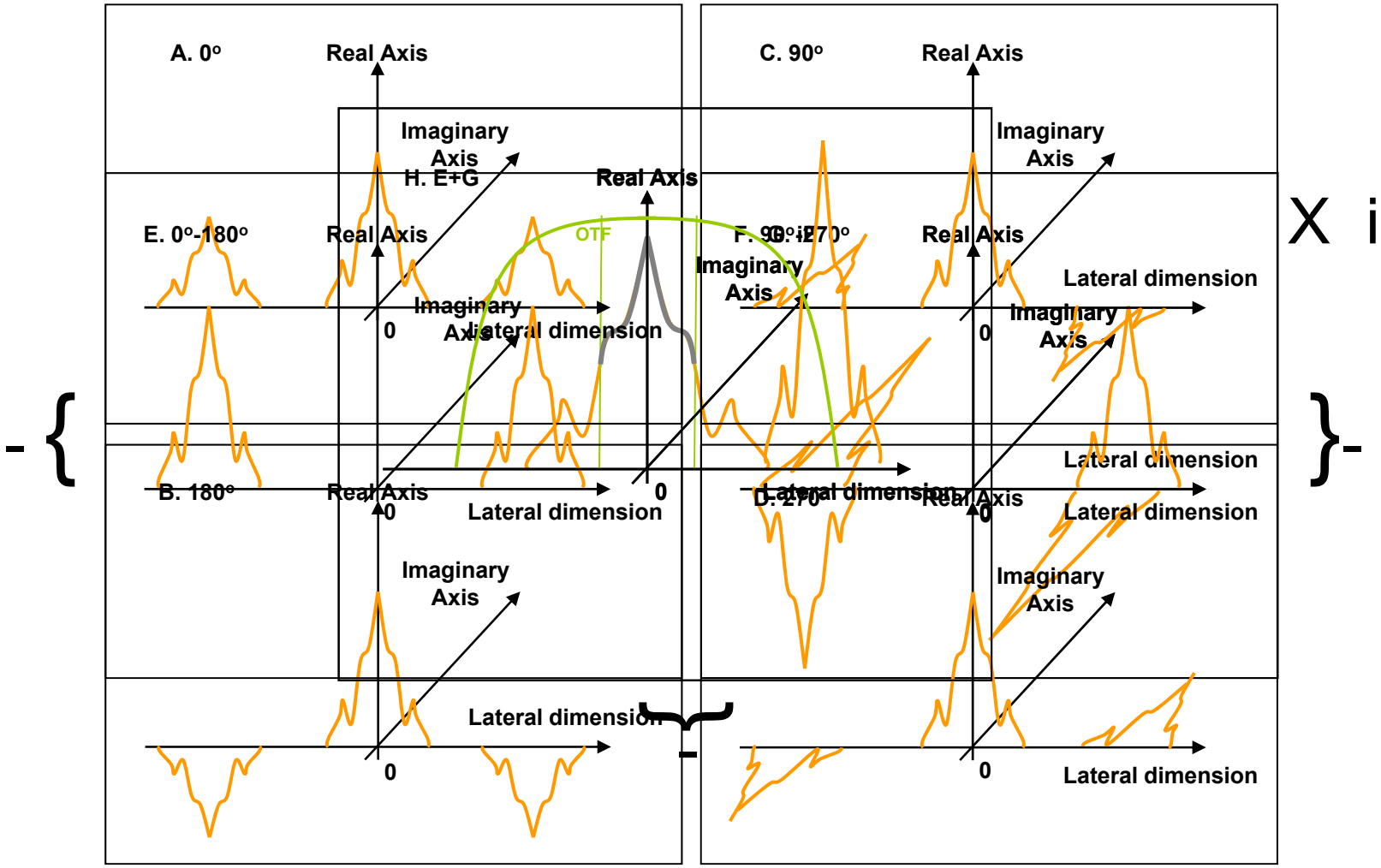
Spatially Modulated Illumination

- **Image reconstruction**
  - Illumination at 0, 90, 180, 270 degrees relative phase shift
  - Elimination of DC terms and aliasing
    - Simple additions, subtractions and conjugations
  - Restoration of low frequencies
  - Repeat for x and y modulation and combine



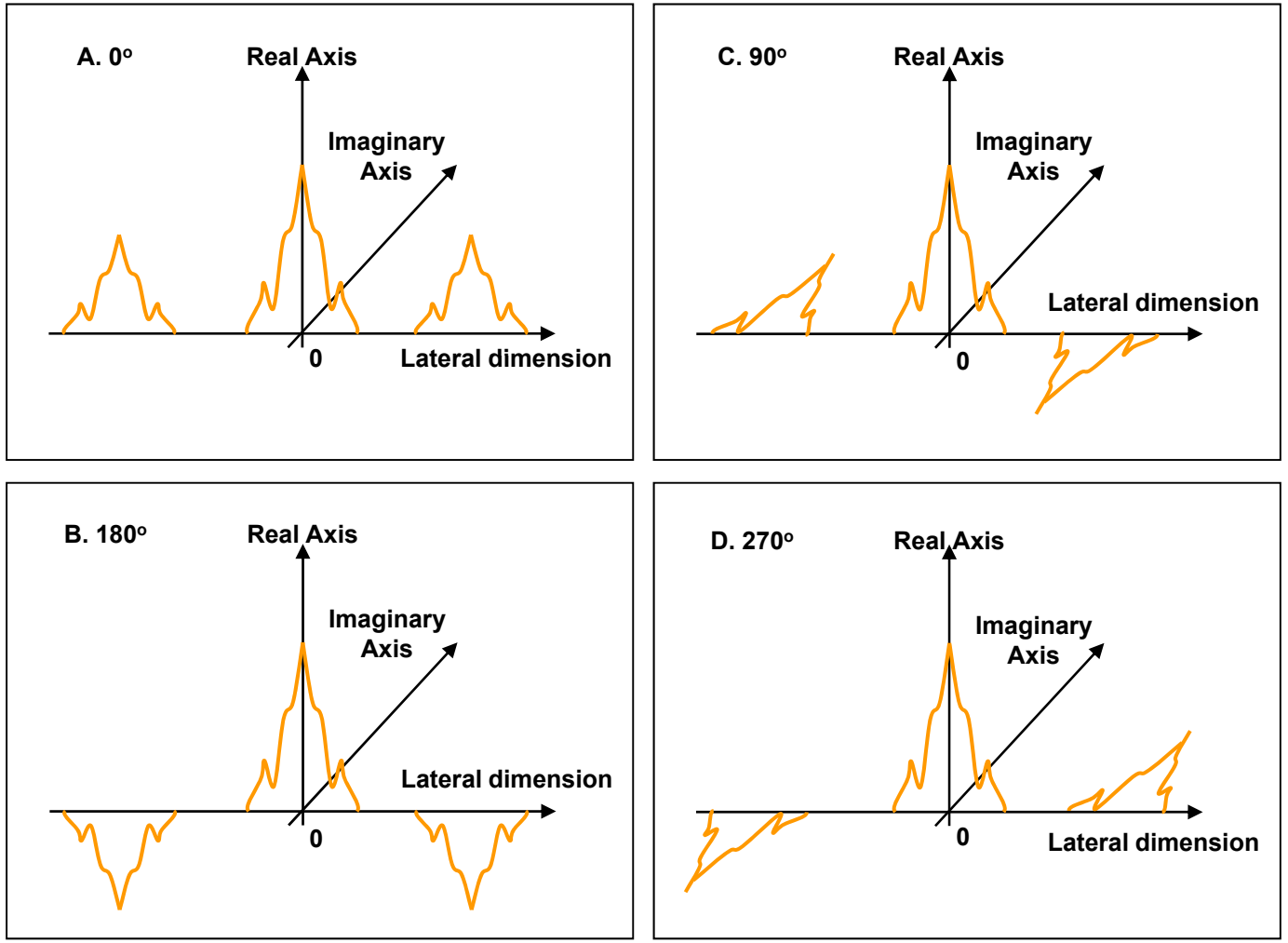
A cluster of microscopical quartz heads (416 nm in diameter with a 200 nm fluorescent core)
R. Heintzmann & C. Cremer, Laterally Modulated Excitation Microscopy: Improvement of resolution by using a diffraction grating. SPIE Proceedings Vol. 3568, 1998

SMI Image Reconstruction



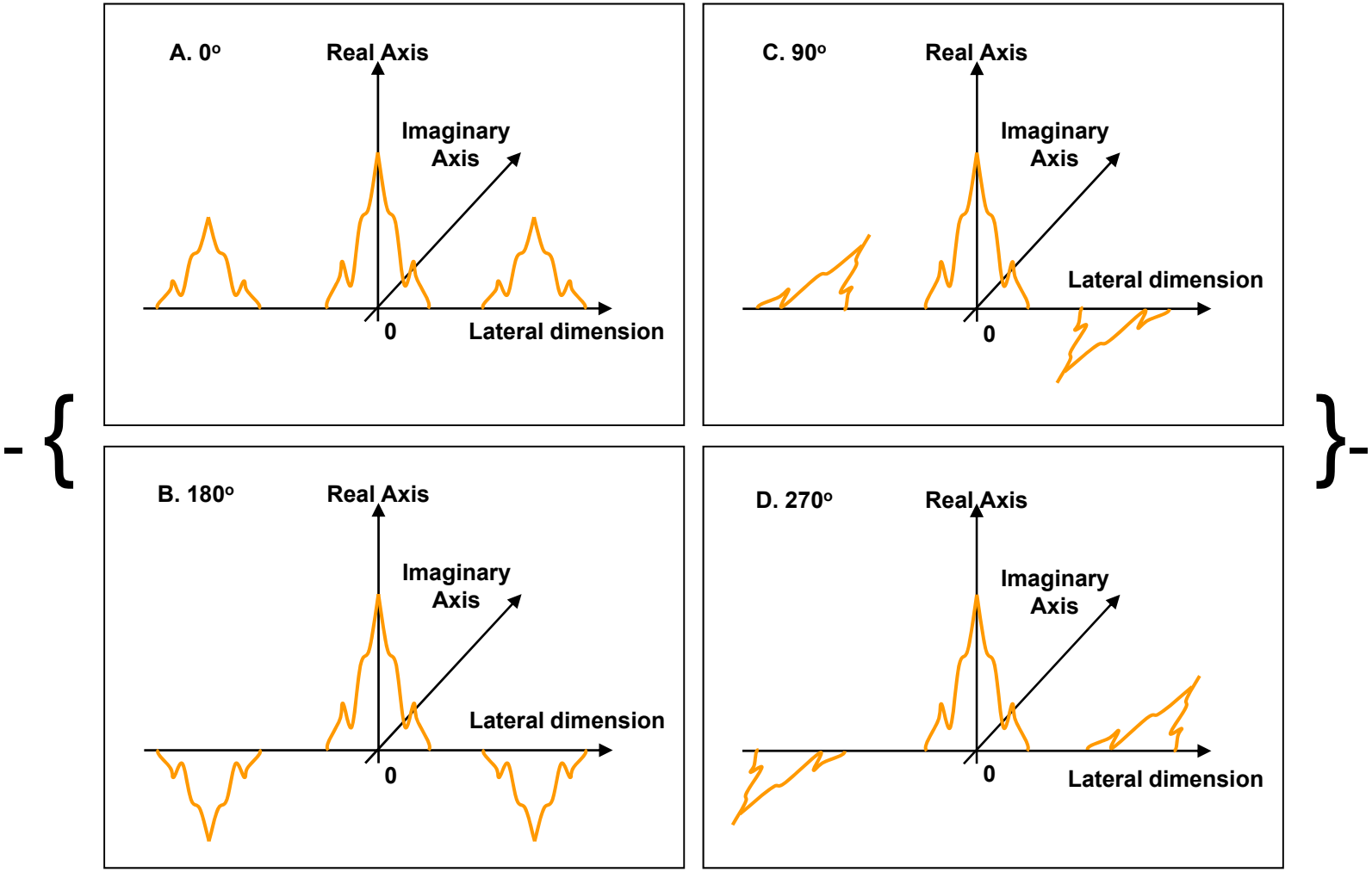
SMI Image Reconstruction

Illumination at different phases



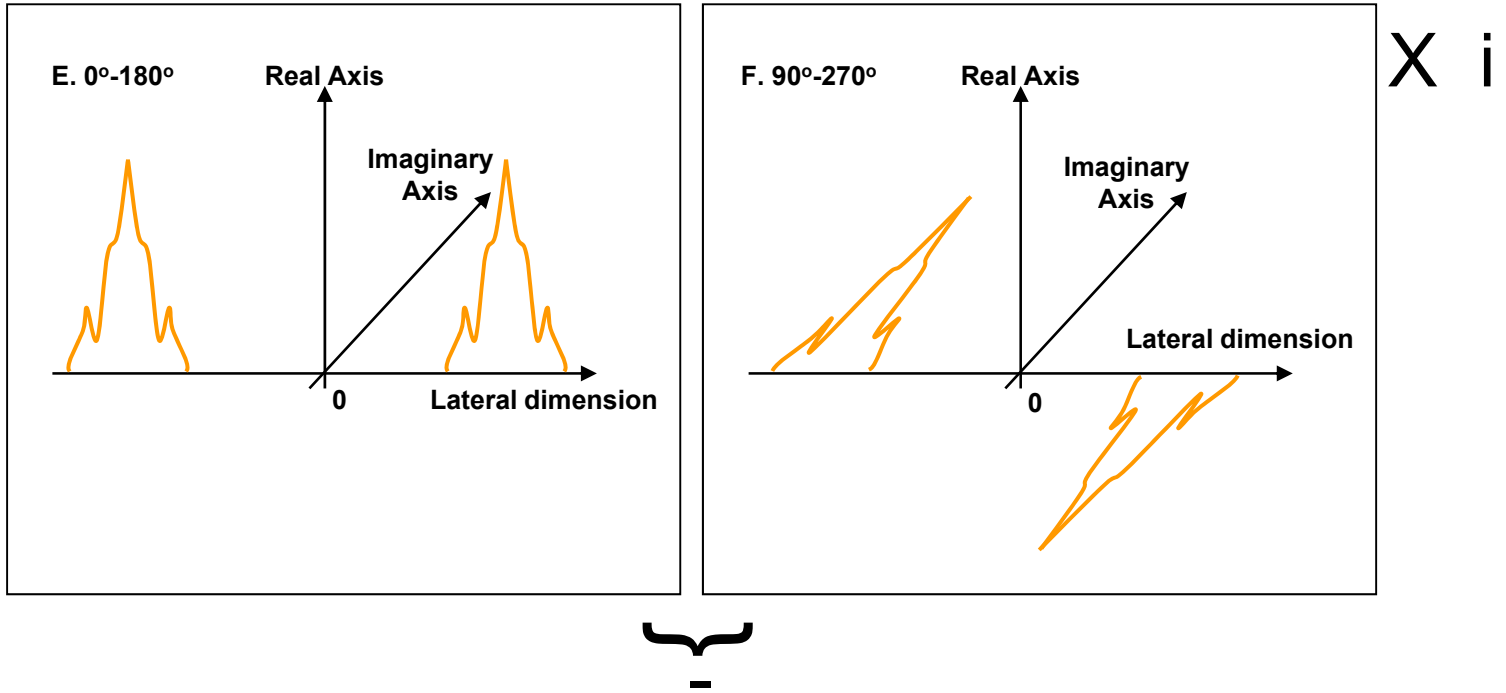
SMI Image Reconstruction

Elimination of DC terms



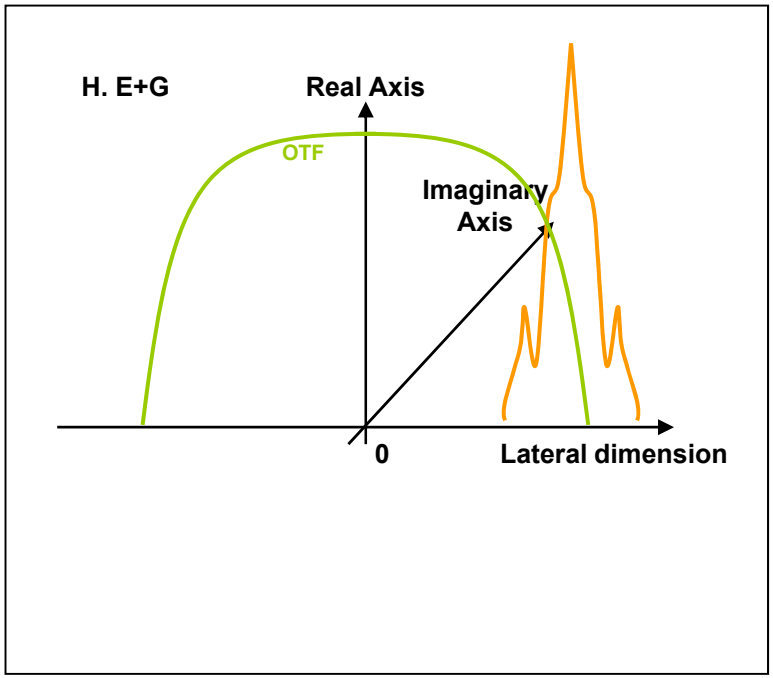
SMI Image Reconstruction

Elimination of aliasing



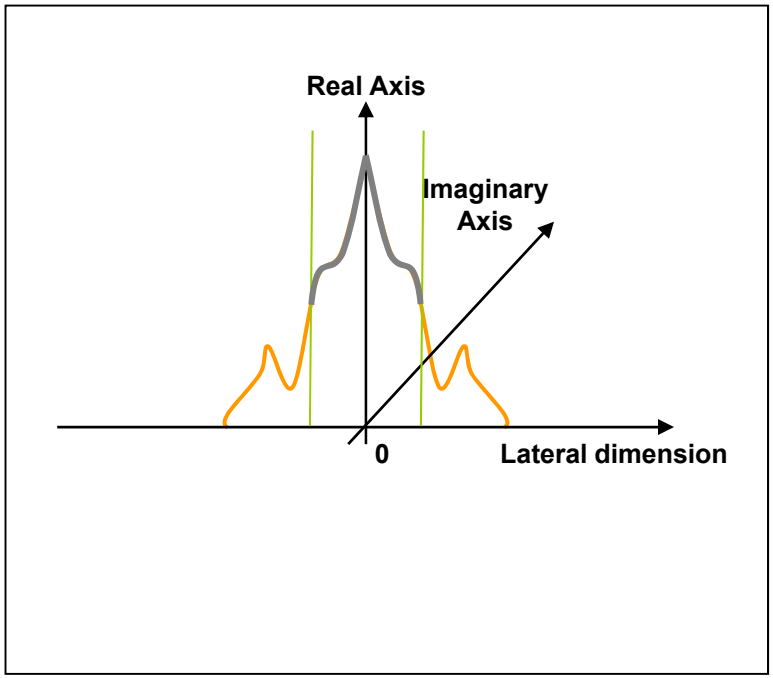
SMI Image Reconstruction

Elimination of DC terms and aliasing



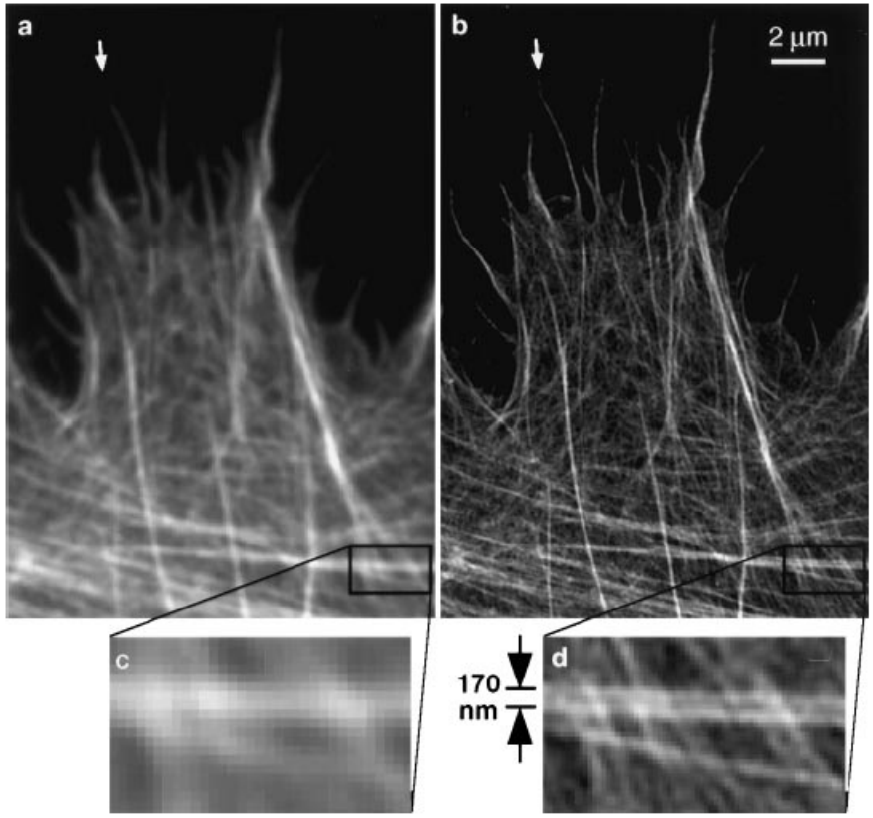
SMI Image Reconstruction

Shift and Restoration of Low Frequencies



Spatially Modulated Illumination

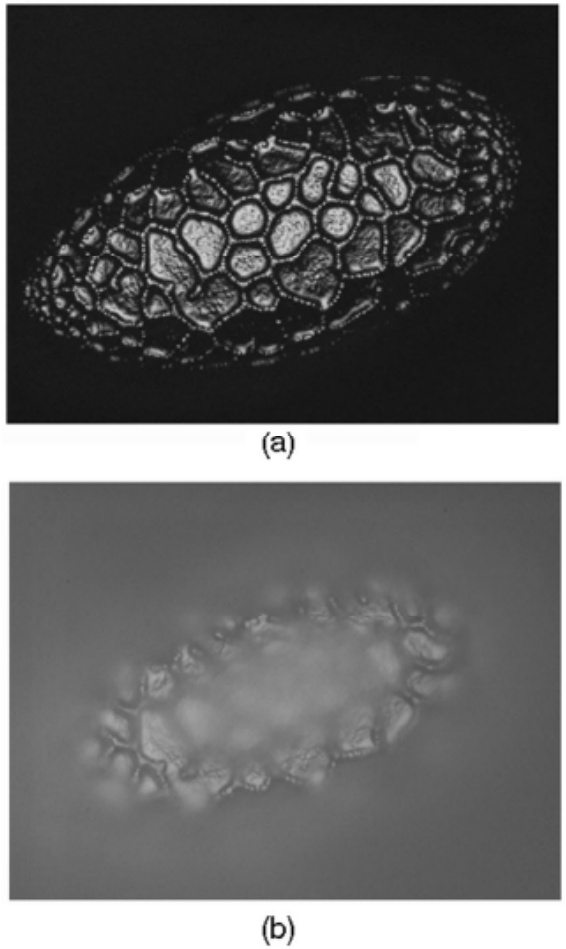
- **Epi-luminescence fluorescence SMI microscopy**
  - Line-patterned phase grating
    - Secondary image plane of fluorescence microscope.
  - Demagnified image
    - Line spacing close to the diffraction limit of the objective lens
  - Illumination pattern control
    - Rotation and lateral translation of the grating



The actin cytoskeleton of a cell. Conventional (a, c) and spatially modulated illumination (b, d) microscopy.
M. G. L. GUSTAFSSON, Journal of Microscopy, Vol. 198, Pt 2, May 2000, pp. 82-87.
University of California San Francisco

Spatially Modulated Illumination

- **Optical sectioning with SMI microscopy**
  - A single-spatial-frequency grid pattern (coarse)
  - Images taken at three spatial positions
    - Relative spatial phases 0, 2π/3, 4π/3)
  - Optically sectioned images
    - Substantially similar to confocal microscopy
  - Processed in real time



Autofocus image of lily pollen grain (a) and conventional image (b)
M. A. A. Neil, R. Juskaitis, and T. Wilson, Optics Letters, Vol. 22, Dec. 1997, 1905-7

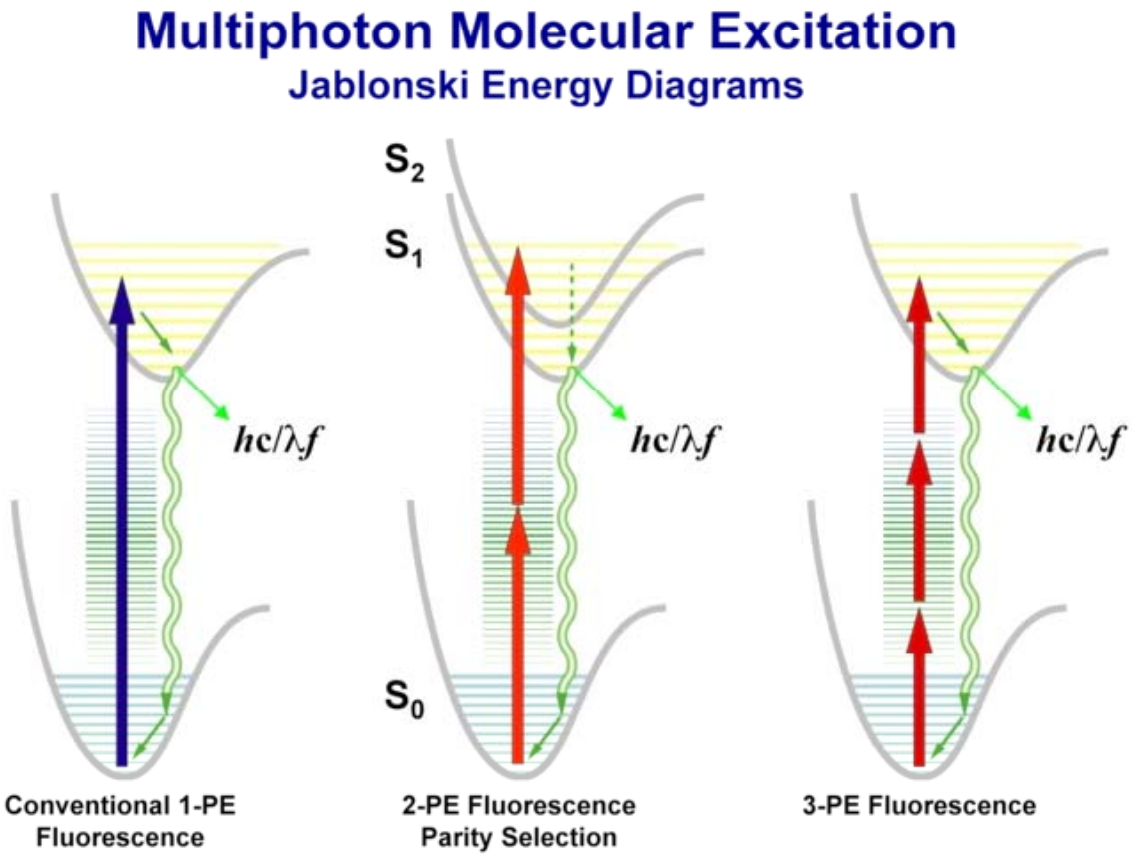
Spatially Modulated Illumination

- **Advantages**
  - Simple design
  - Better signal to noise than confocal
  - Works well for thin specimens
- **Disadvantages**
  - For, thicker specimens → excitation of fluorophores in other parts of the cell by adjacent standing waves
    - It is possible to mathematically deconvolve, but difficult!
  - Requires more processing power

A detailed 3D image of the nucleus of a mouse cell. The picture was captured using a new imaging microscope technique called three-dimensional structured illumination, described in the journal Science.

Multi-photon Microscopy

- At very high photon densities, it becomes possible for two or more photons to be simultaneously absorbed
- Each multiple absorption induces a molecular excitation of a magnitude equivalent to the sum of the absorbed photon energies



http://www.aep.cornell.edu/drbio/MPE/mpe.html

Basic principles of multi-photon excitation

- **Multi-photon excitation is a nonlinear process**
  - Because two photons are required for each excitation, the rate of two-photon absorption depends on the square of the instantaneous intensity.
  - Because of the large intensities required, high power lasers providing very short pulses (~100 fs) are used, so that peak intensity is high, but average power doesn't damage the specimen.
  - We have photon flux densities sufficiently high for multiple photons to arrive "simultaneously" (in 10^{-15} s) at an excitable molecule (of 10^{-16} cm^2 cross section) only at the focus point of a beam.

- **The probability that a given fluorophore at the center of a focused beam absorbs a photon pair during a single pulse is**

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

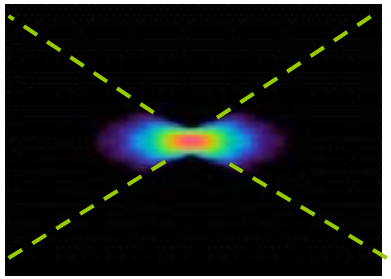
δ is the two-photon absorption cross-section

$\langle P \rangle$ is the average power

NA is numerical aperture

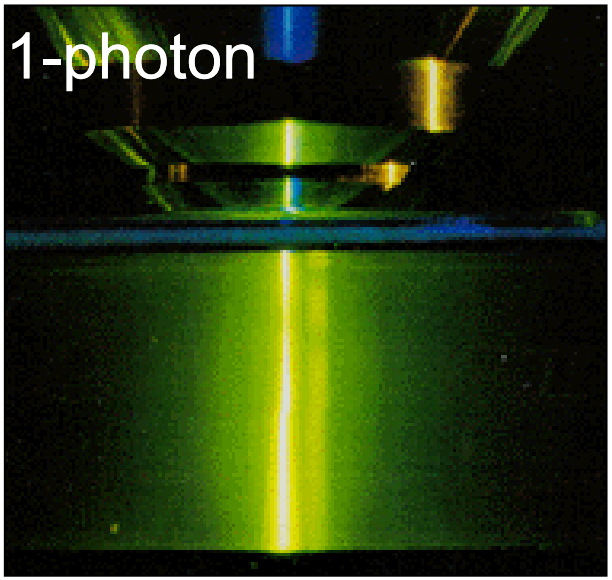
F_p is the repetition frequency

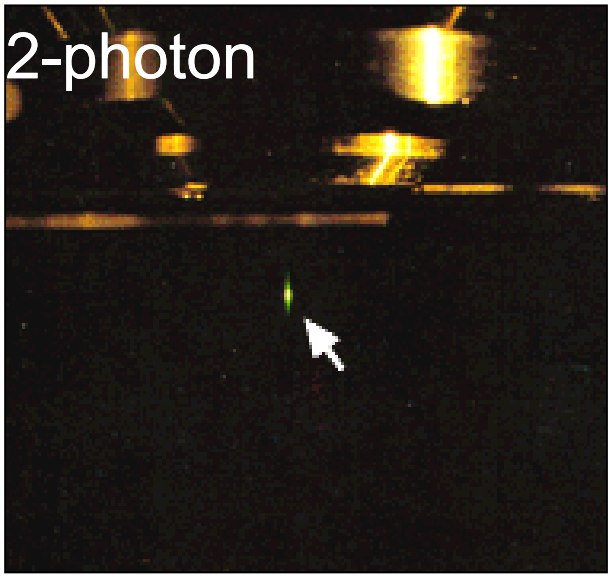
$\xi = \frac{\langle p^2 \rangle}{\langle p \rangle^2}$ is known as the two-photon advantage



Advantages of multi-photon excitation

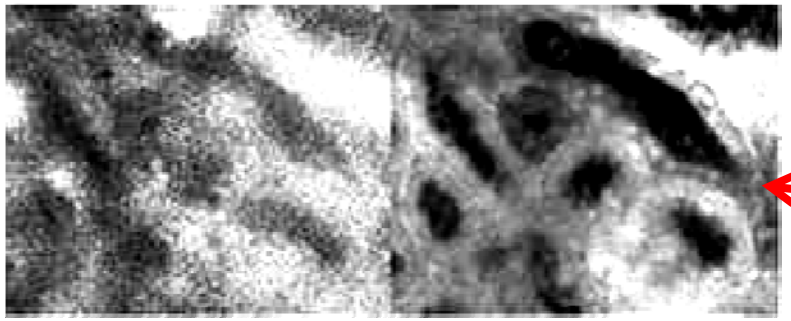
- **Built in confocality**
  - With a single-photon source excitation occurs throughout the beam profile
  - With a two-photon source excitation events are limited to the beam focus
  - Focal point restriction of excitation automatically provides 3-dimensionally resolved submicron information
- **Photodamage is restricted to the focal plane**

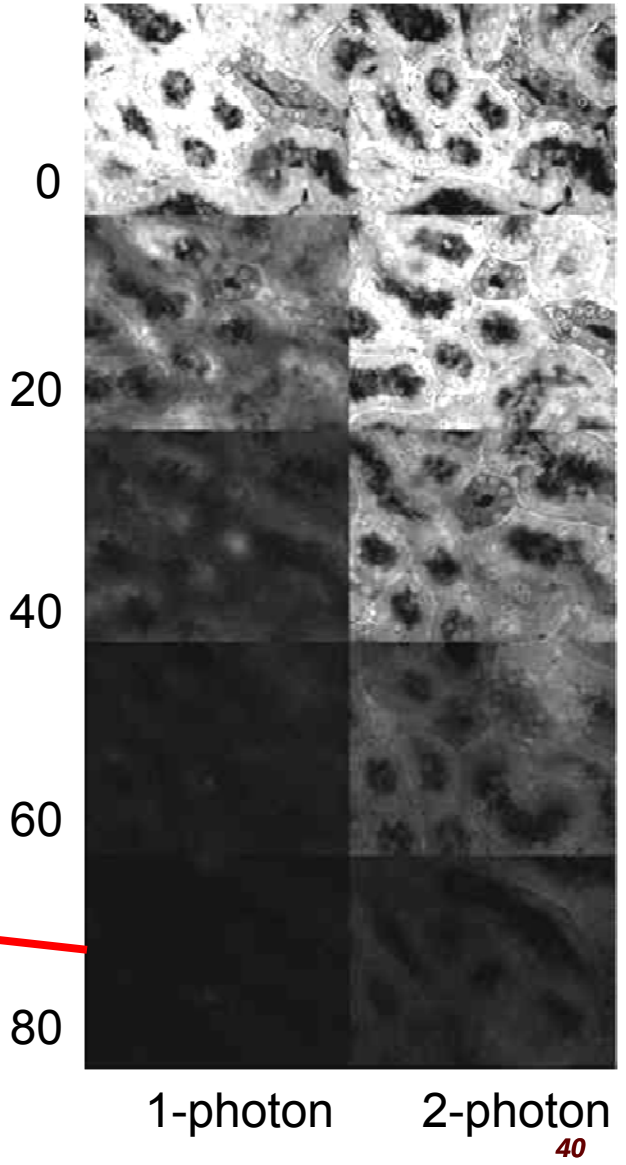




Advantages of multi-photon excitation

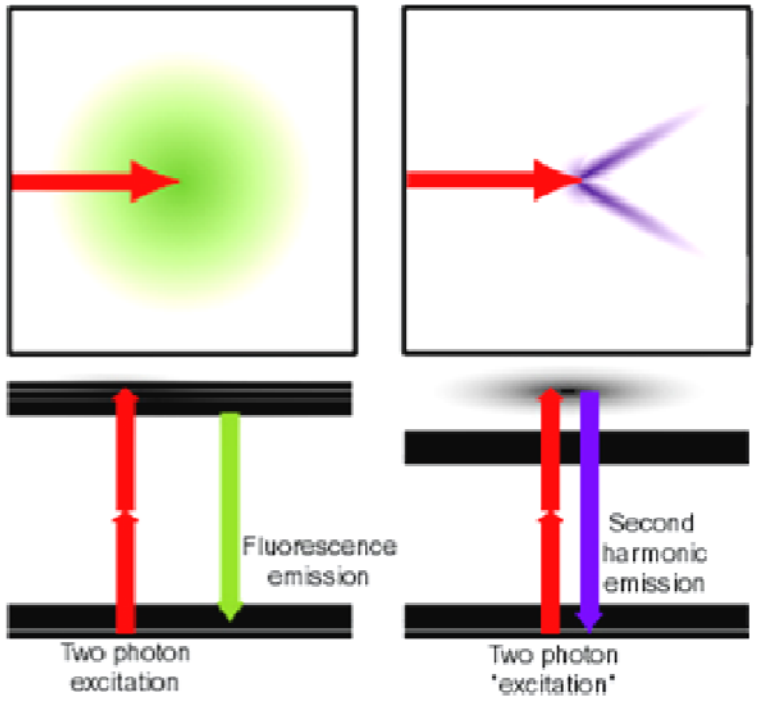
- **ot necessary to refocus the fluorescence through an aperture**
  - Simpler, more efficient optical detection design → stronger signal
  - Scattering in thick specimens degrades signal to a smaller extent
- **UV absorbing molecules can be excited using visible/NIR wavelength ranges**
  - Better penetration(2-400 mm)
  - Enables measurements from intact cells in a proper physiologicalenvironment.





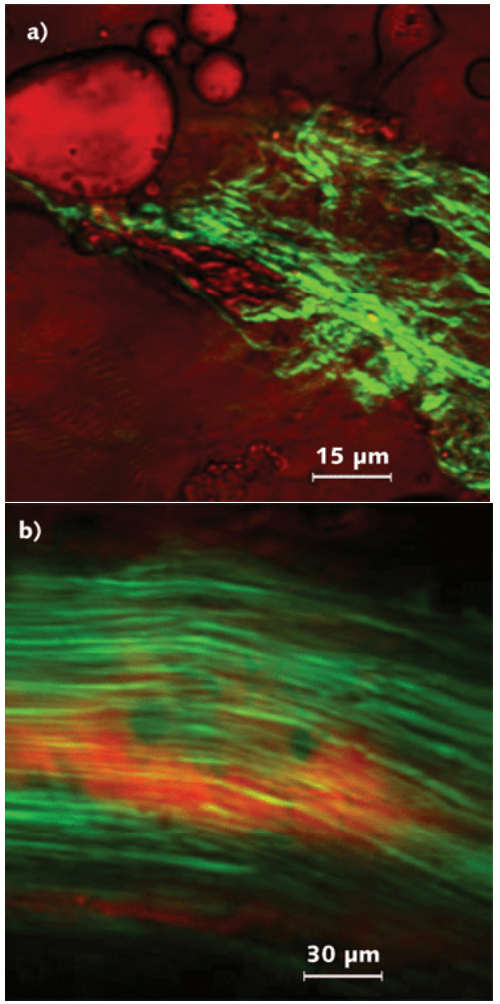
Second Harmonic Generation (SHG)

- **SHG (also called frequency doubling)**
  - A nonlinear optical process
  - Can be thought off as the scattering equivalent of two-photon excited fluorescence
  - Photons interacting with a nonlinear material are effectively "combined" to form new photons with twice the energy
    - The emitted photons are at exactly half of the wavelength of the incident radiation (
  - As excitation I changes, emitted SHG signal I also changes
- **SHG signal**
  - Phase matched to the incident radiation
  - Emitted in a highly directional fashion
    - Depends on the size, shape and refractive index of the scatterers
    - Remember: fluorescence is incoherent and isotropic



Second Harmonic Generation (SHG)

- **Instrumentation**
  - A short-pulse laser such as a femtosecond laser
  - A set of appropriate filters
    - The excitation light can be easily separated from the emitted, frequency-doubled SHG signal.
- **Very high axial and lateral resolution**
- **SHG microscopy has been used for extensive studies of the tissues consisting primarily of collagen**



Coherent anti-Stokes Raman scattering (CARS, red) and second harmonic generation (SHG, green) microscopy of tissue from a) bovine muscle and b) rodent tail.

Newport Application Note 36, Newport Technology & Applications Center (2007).