



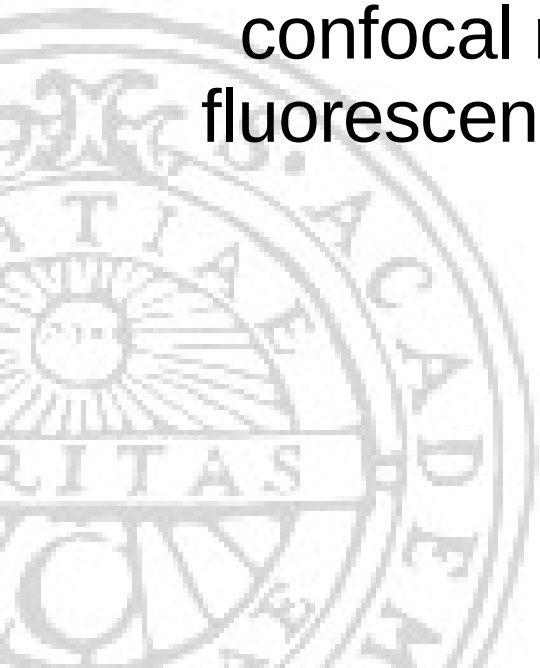
Centre for Image Analysis

Swedish University of Agricultural Sciences
Uppsala University

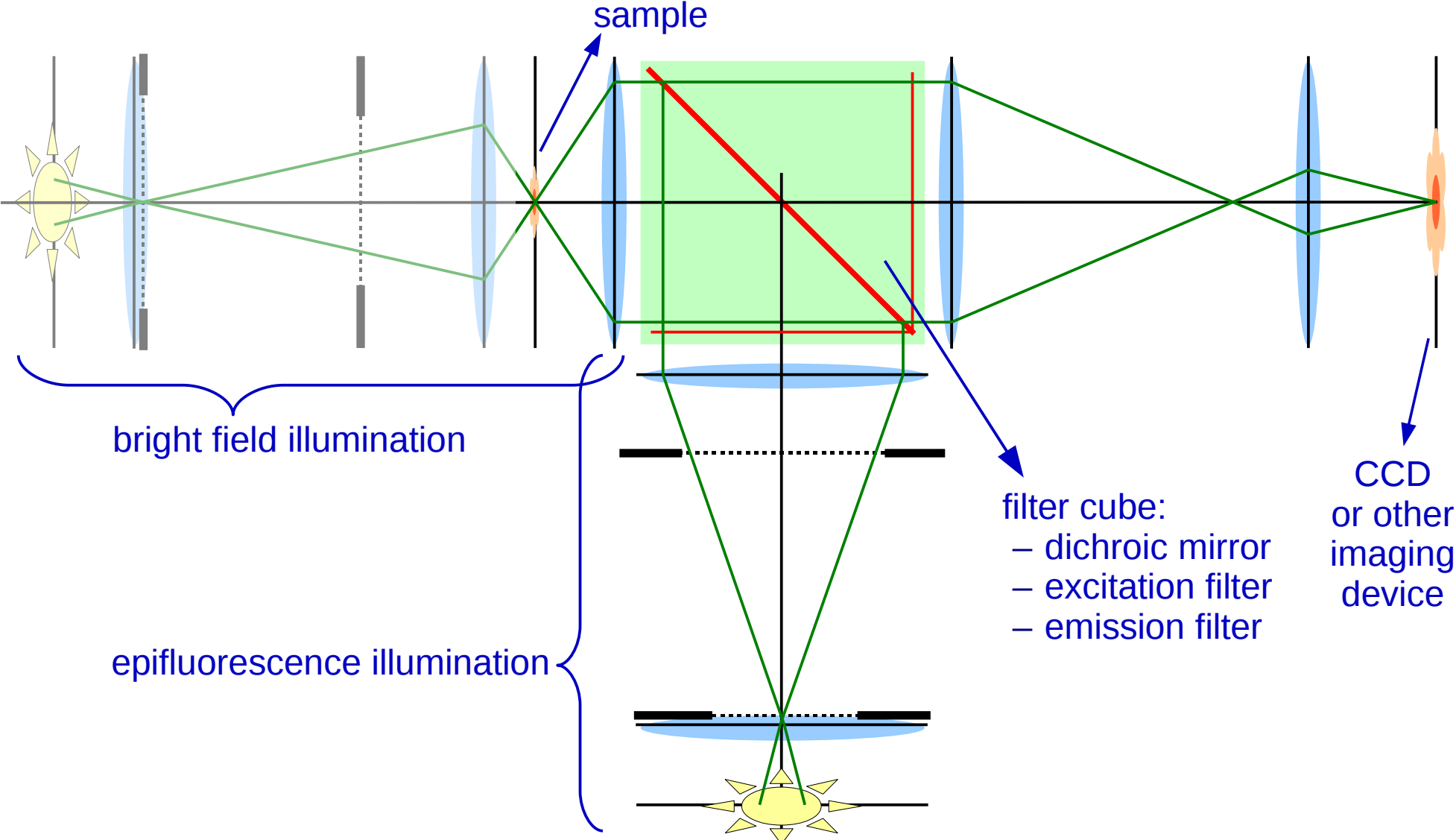
Digital Imaging Systems Confocal Microscopy

confocal microscopes, non-linear optics, advanced
fluorescence techniques, breaking the diffraction limit

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Repetition: The Epifluorescence Microscope



Contents

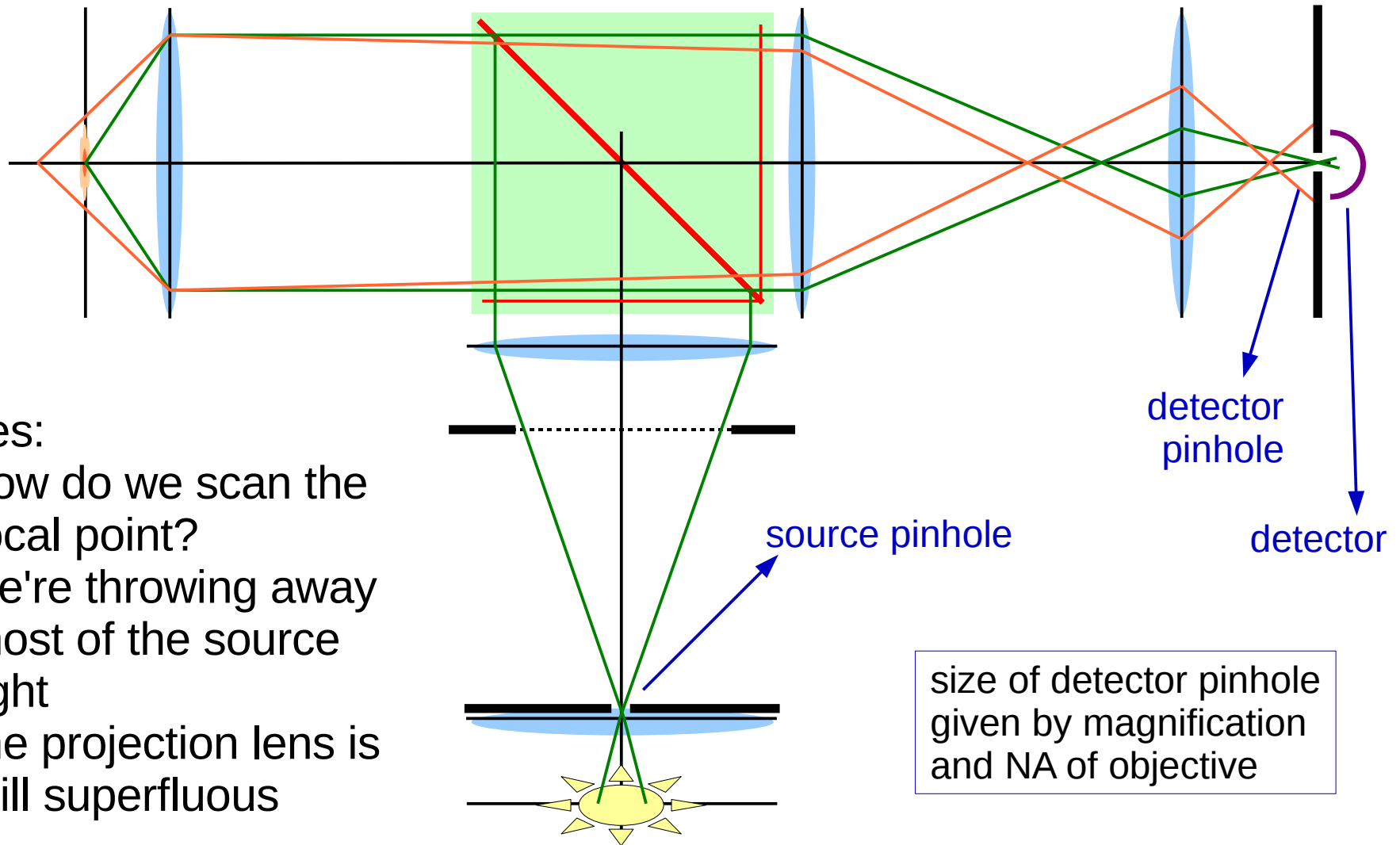
- The Confocal Microscope
 - Laser Scanning, Spinning Disks, et al.
- Multi-Photon Excitation
- Further Increasing the Resolution
 - 4Pi Microscopy
 - Breaking the Diffraction Limit: STED, PALM/STORM & TIRF
- Advanced Fluorescence Techniques:
 - FRAP, FRET & FLIM
- Other Optical Sectioning Techniques:
 - Wide Field Deconvolution
 - Selective Plane Illumination Microscopy

The Confocal Microscope

- Bright field / epifluorescence has strong out-of-focus signal
- This is OK for thin section specimens
- This is really bad when 3D information is needed

- Confocal microscopy filters out out-of-focus light
- To do so, it can only illuminate a single point at a time
- Scanning of 3D volume yields volumetric image

The Confocal Microscope

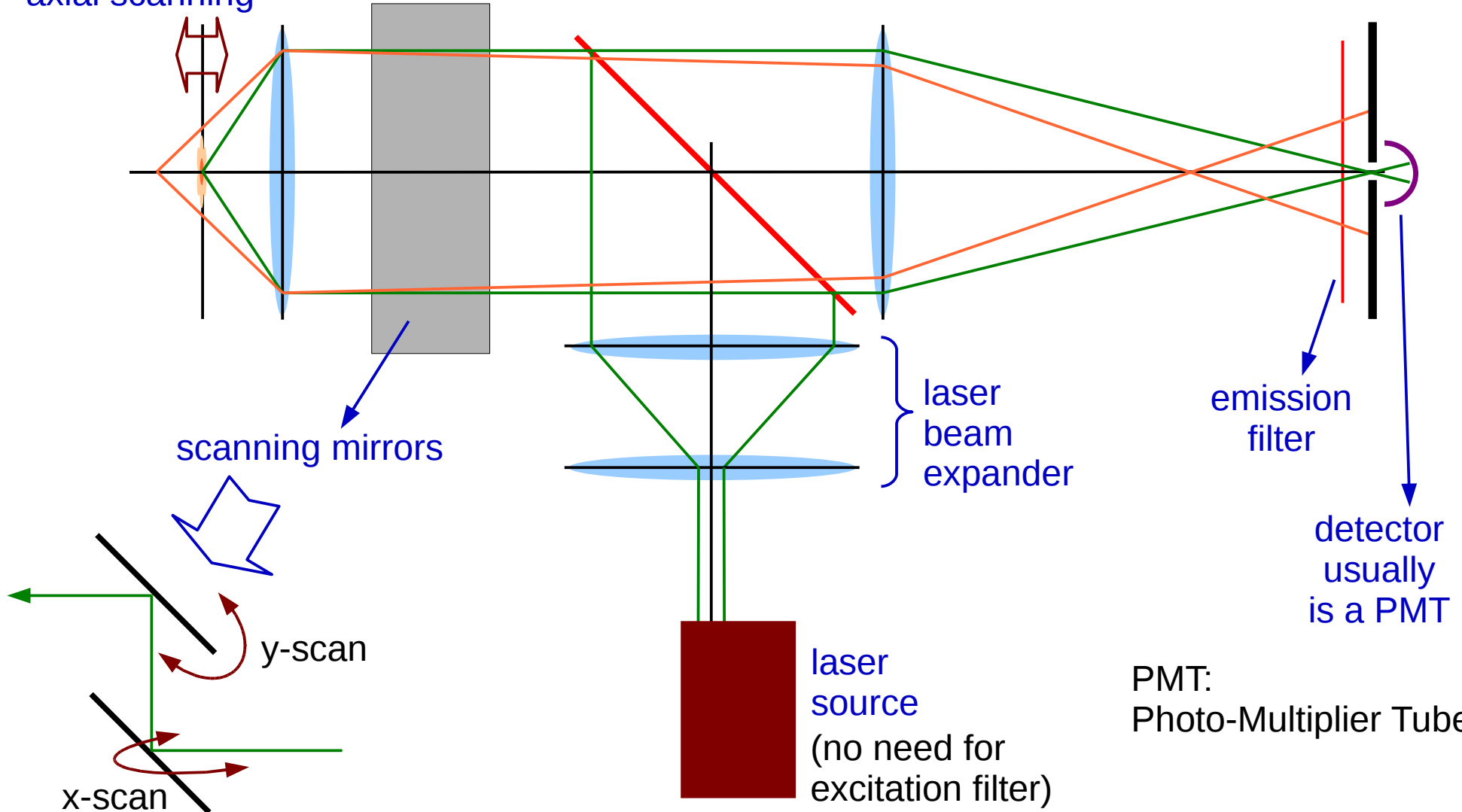


issues:

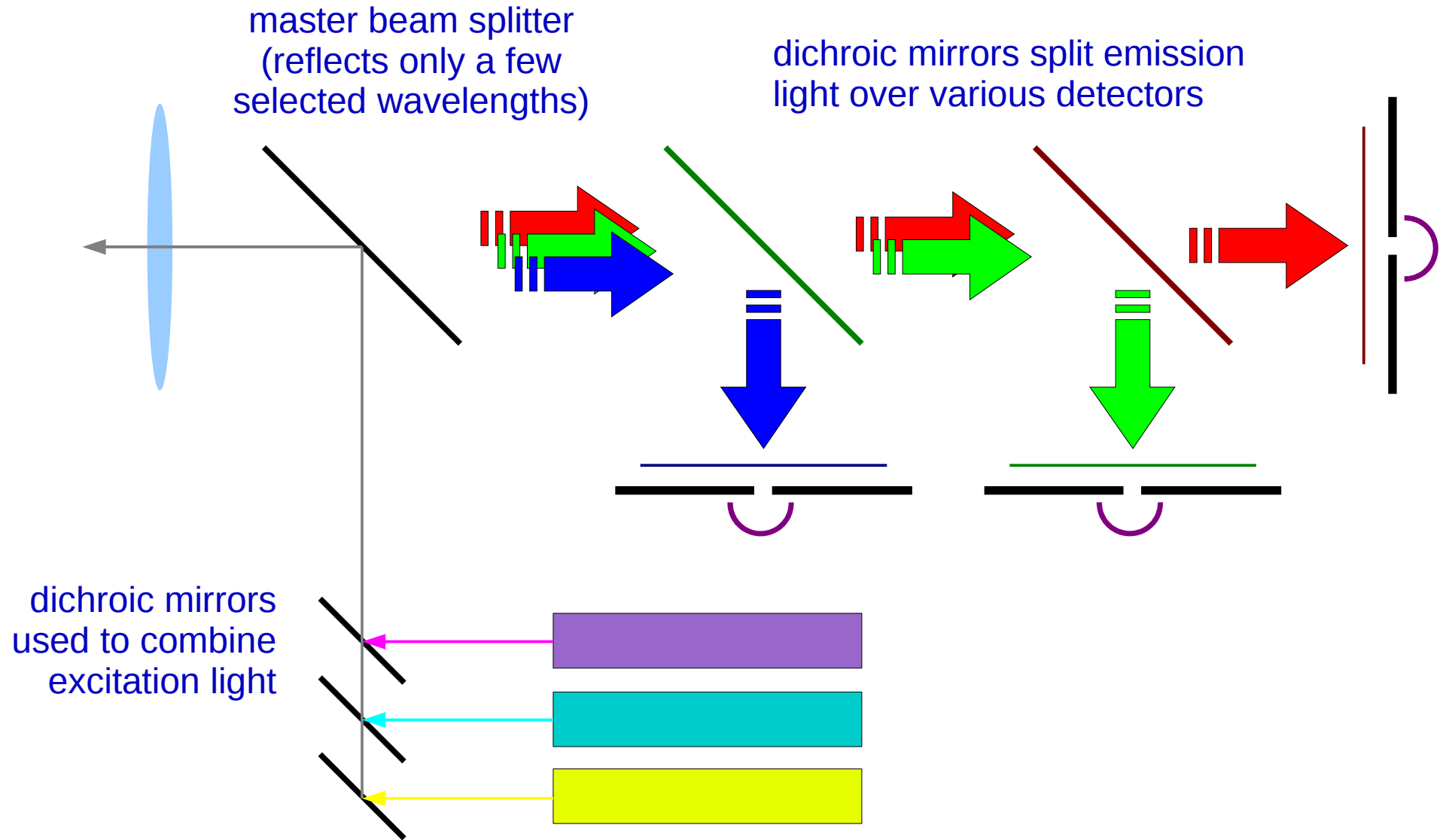
- how do we scan the focal point?
- we're throwing away most of the source light
- the projection lens is still superfluous

Laser Scanning Confocal

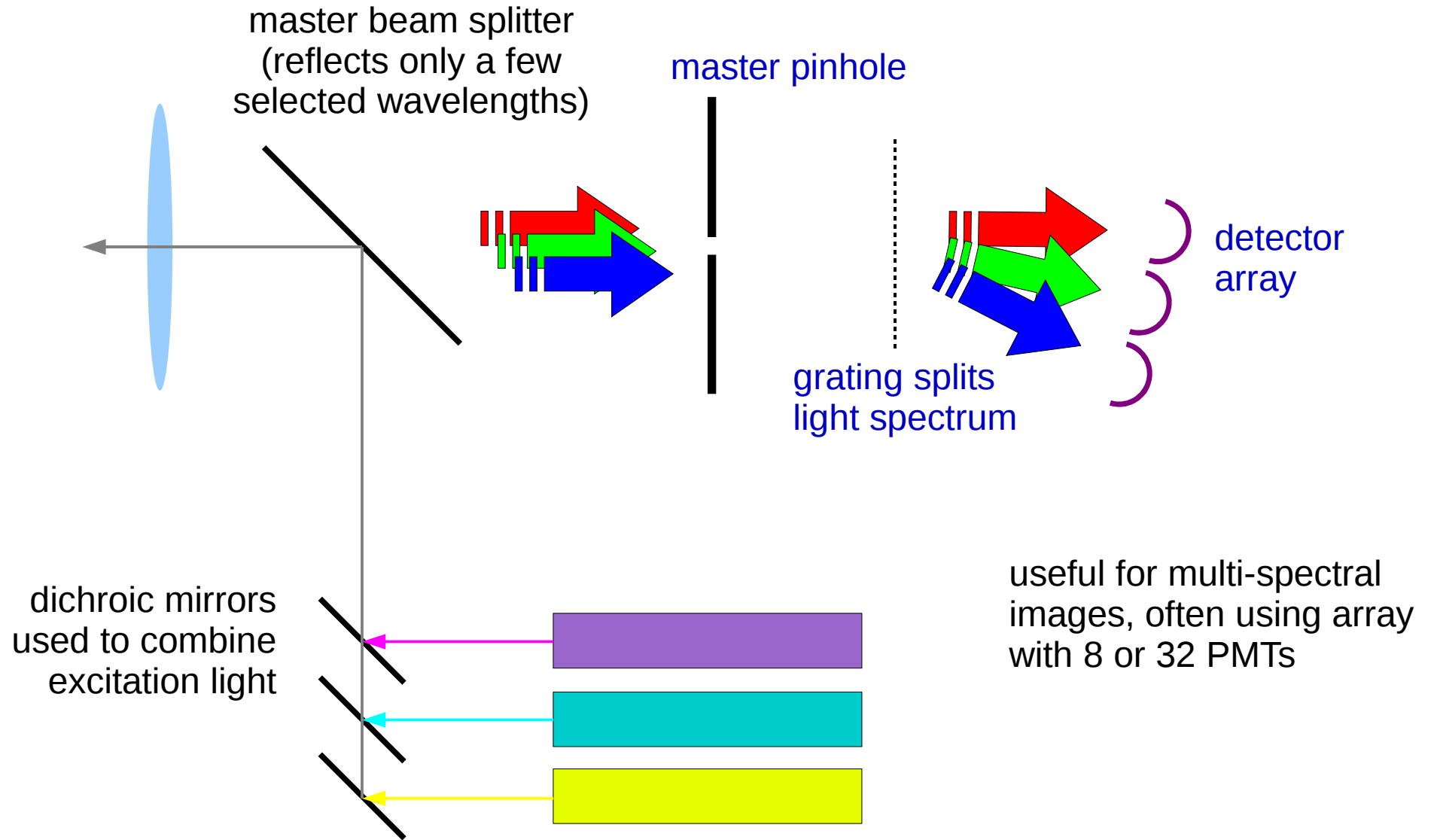
move stage for axial scanning



Multi-Colour Detection

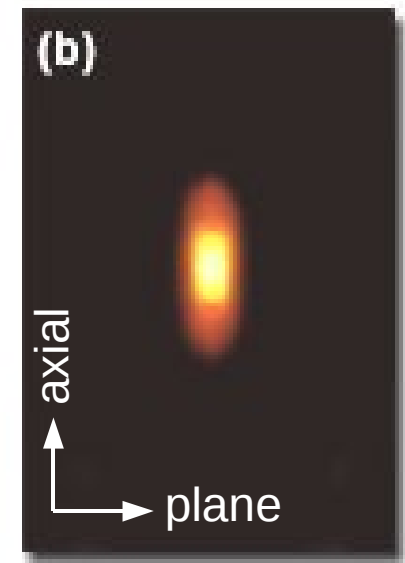
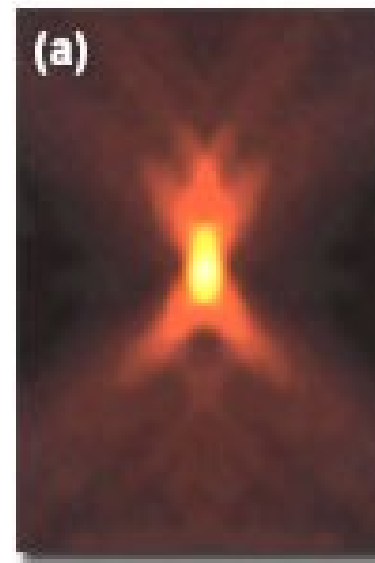
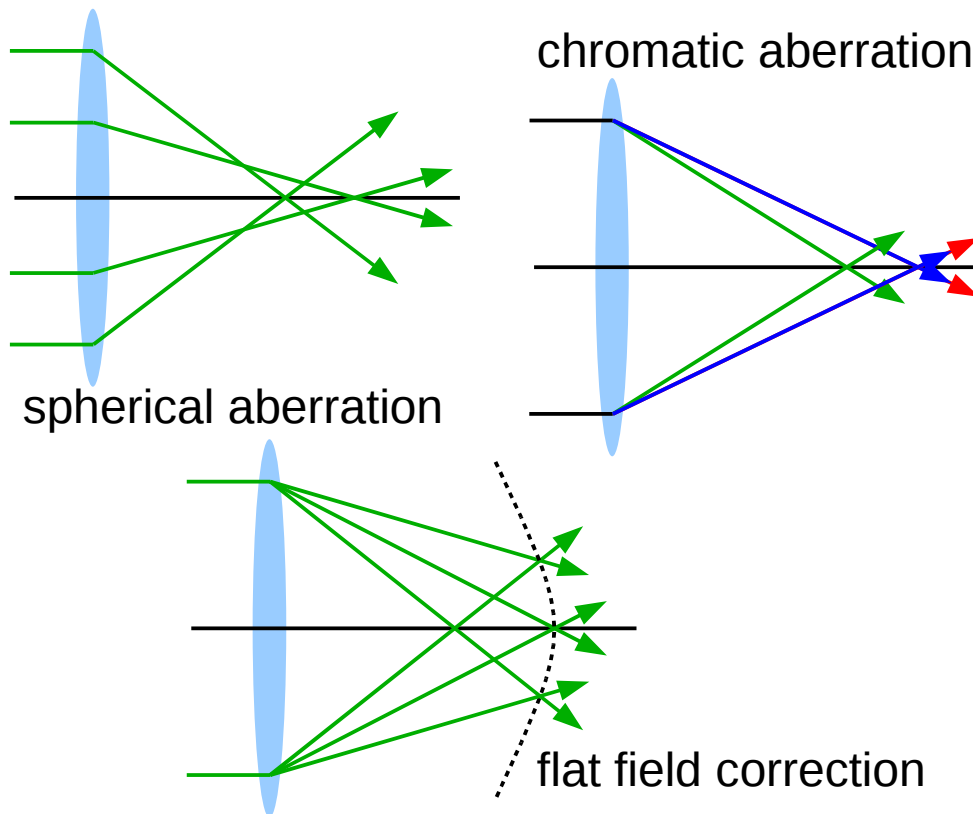


Multi-Colour Detection



The 3D PSF

- Confocal PSF is about 3 or 5 times as extended axially (z) as in the plane (x-y)
- In-plane resolution is approximately the same as wide field at focal plane



wide field

confocal

Faster Confocal Microscopy

- Scanning a volume one dot at a time is slow
- Solutions:
 - Scan many dots at once:
 - multi-focal scanning confocal
 - spinning disk confocal
 - Scan one line at once:
 - line scanning confocal
- Caveats:
 - Focal points need to be separated enough to uphold confocal principle

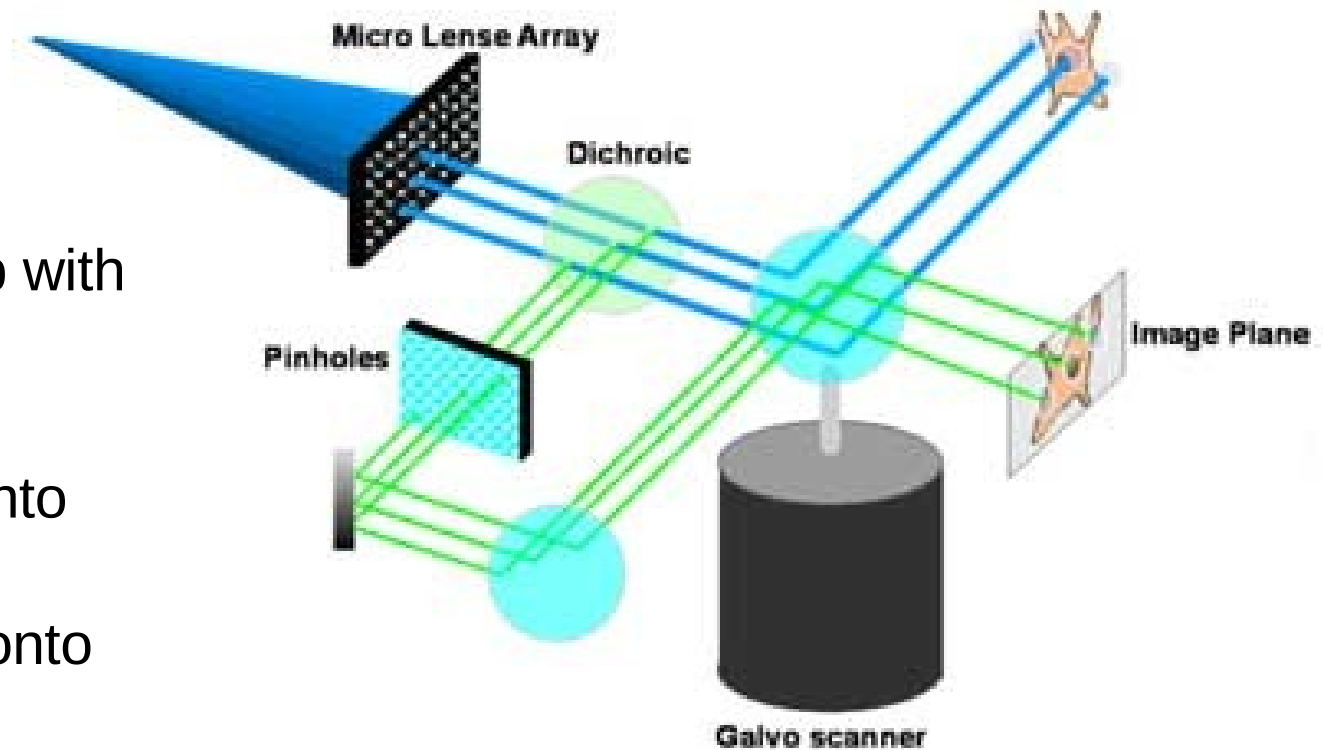
Multi-Focal Scanning Confocal

Visitron Systems VT-Infinity 2D array scanner

Micro lens array lined up with pinhole array

Scanning mirror to:

- scan excitation light onto sample
- descan fluorescence onto pinholes



Spinning Disk Confocal Microscope

Yokogawa CSU10

Disk spins 1800 rpm

Scans entire focal plane 30 times a second (video rate)

CCD array or our eyes can see the confocal image

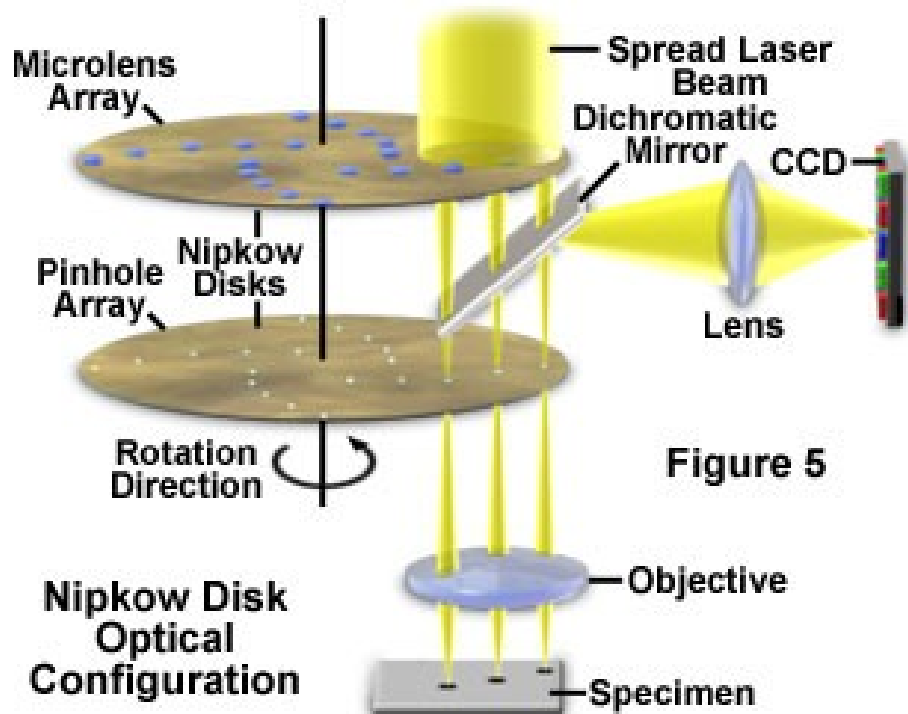
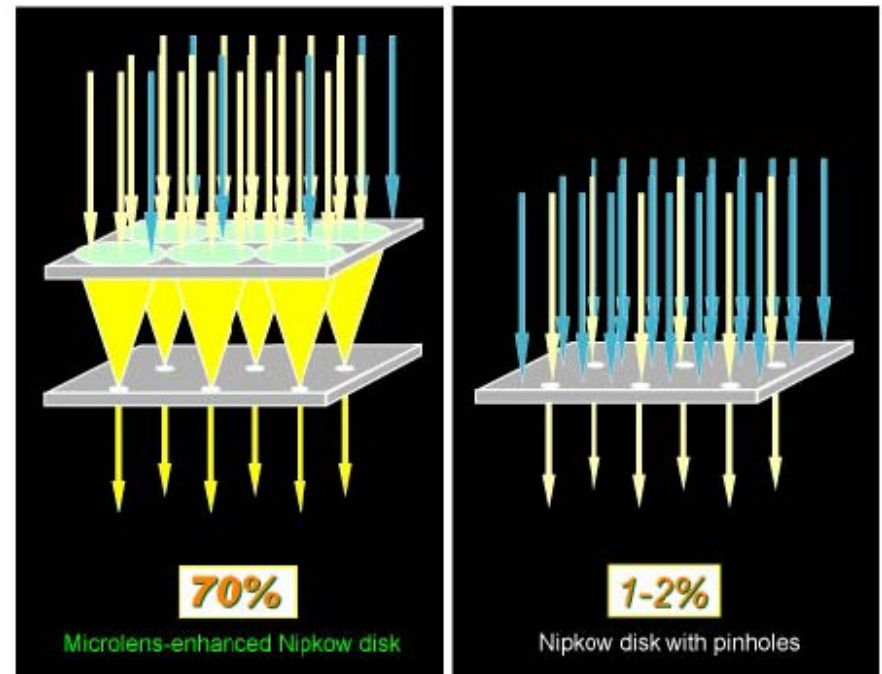


Figure 5



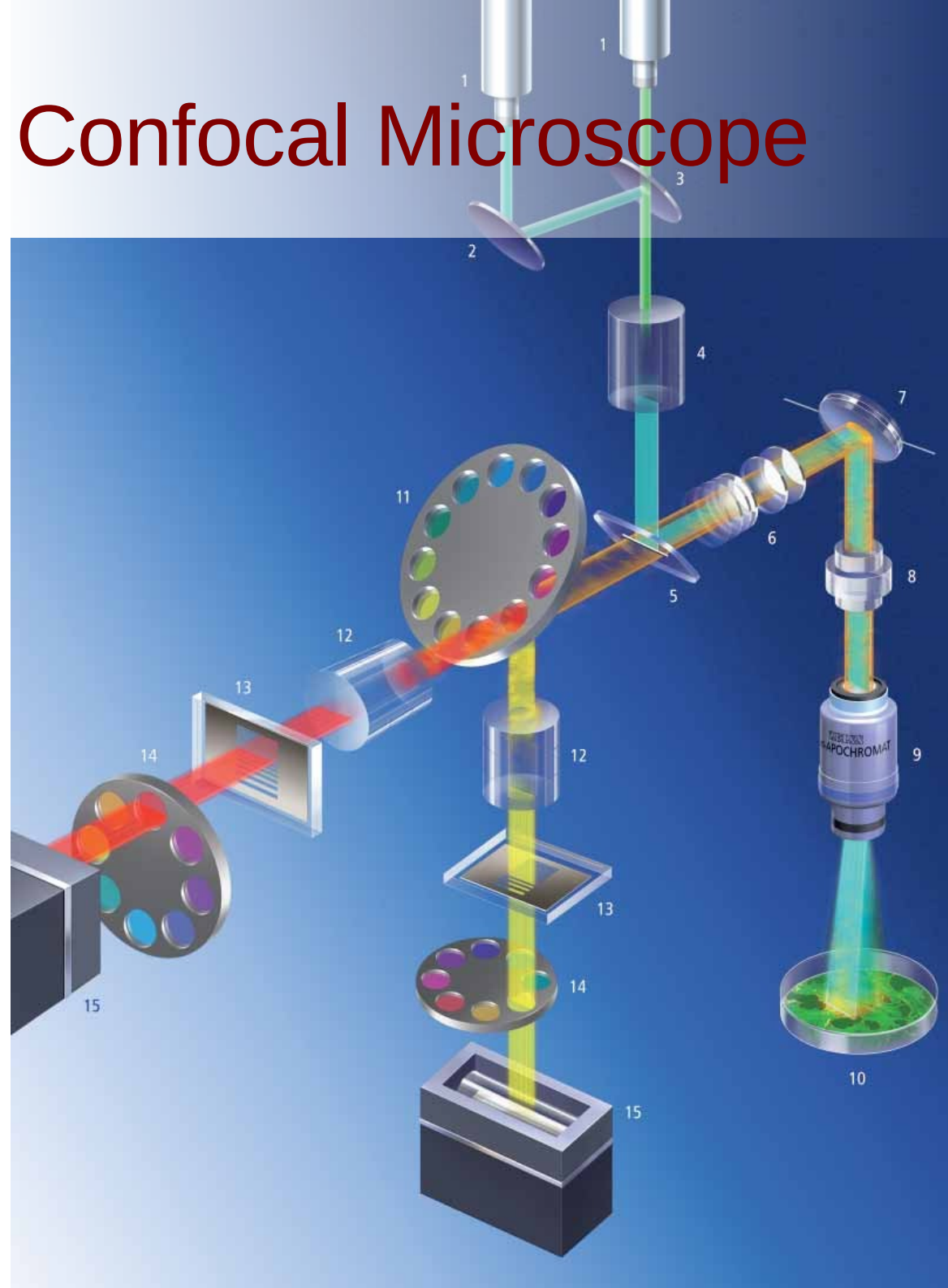
Line Scanning Confocal Microscope

Zeiss LSM 5 LIVE

Just like normal confocal but:

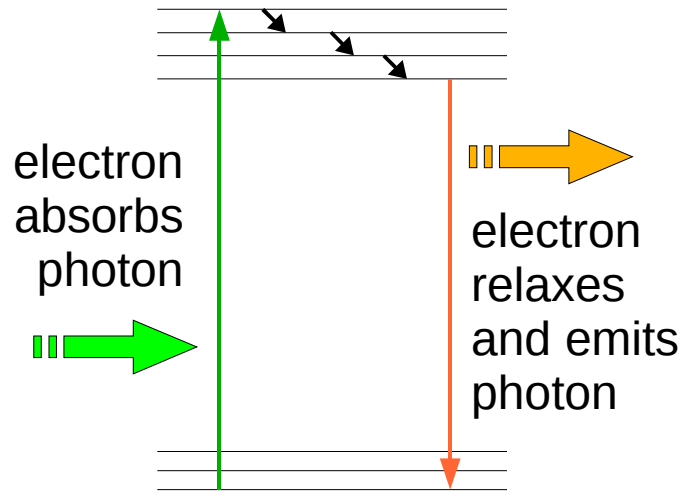
- slits instead of pinholes
- linear CCD detector instead of PMT

axial resolution not as good as true confocal microscope



Multi-Photon Excitation

Multi-Photon Excitation



Jablonski energy diagram

photon energy $\propto 1/\lambda$

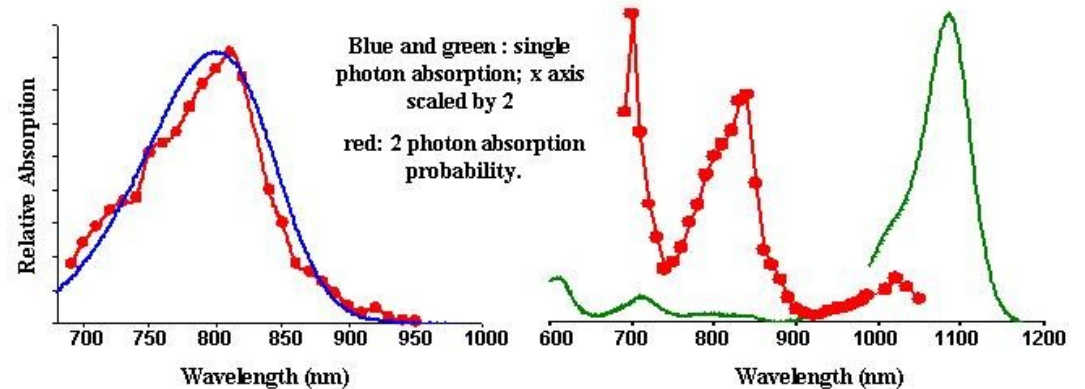
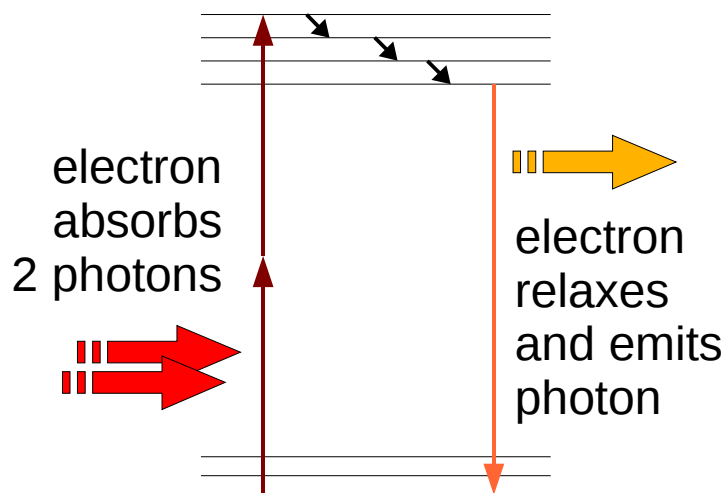
1 photon (λ) \approx 2 photons (2λ) \approx 3 photons (3λ)

The peak absorption is often at twice the single photon peak.

Example: Coumarin

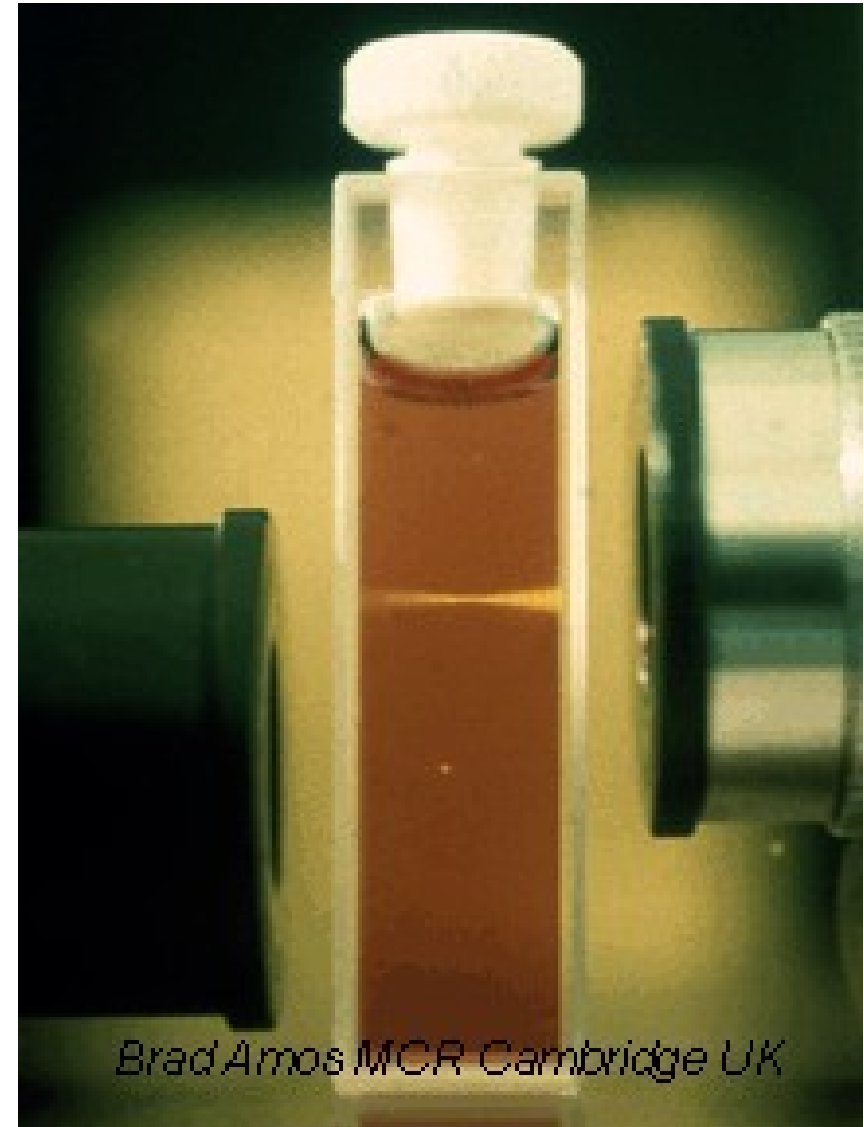
... and often it's not.

Example: Rhodamine.



Multi-Photon Excitation

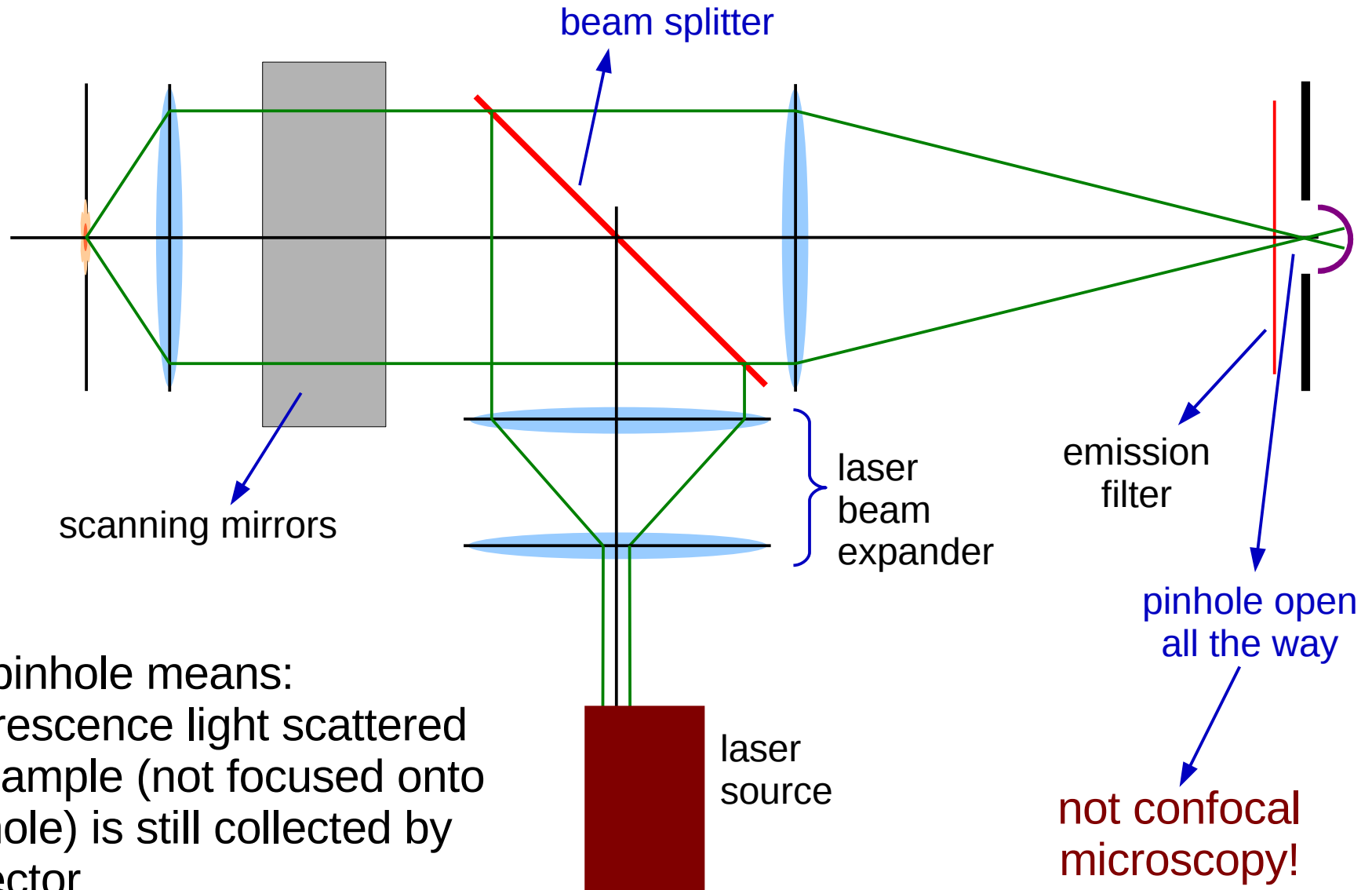
- Photon absorption probability proportional to square of photon density
 - (“Non-linear optics”)
- Fluorescence excitation only at focal point
 - No out-of-focus light to filter with a confocal pinhole
- Huge photon density required
 - Needs pulsed laser



Femto-Second Pulsed Lasers

- Typically used Ti:Sapphire (titanium-sapphire) laser
 - Mode-locked oscillator
 - Tunable 650-1100 nm wavelength (red to near infrared)
 - 10-1000 fs pulse duration (ultrashort pulses)
- Short pulses means:
 - High photon density but low average energy
 - High bandwidth (many wavelengths in laser line)

2-Photon Laser Scanning Microscopy



No pinhole means:
fluorescence light scattered
by sample (not focused onto
pinhole) is still collected by
detector

Advantages of Multi-Photon Microscopy

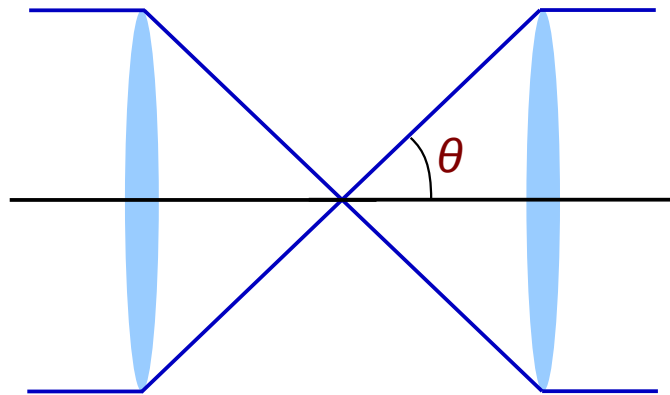
- Excitation with longer wavelength
 - Less scattering means deeper penetration and higher axial resolution
 - Scattered fluorescent light still collected because no pinhole
- Excitation only at focal point
 - No excitation in “cone of light” means less photobleaching and less phototoxicity
- Broad laser spectrum
 - Many fluorophores excited by same laser line
- Disadvantages:
 - Expensive laser
 - Lower x-y resolution (because of wavelength of laser light)

Further Increasing Resolution

4Pi Microscopy

- Diffraction limit given by numerical aperture (NA)
 - $NA = n \sin(\theta)$
- Increase resolution by either:
 - increasing refractive index (impractical)
 - increasing aperture angle (limited to $\theta \approx 74^\circ$)

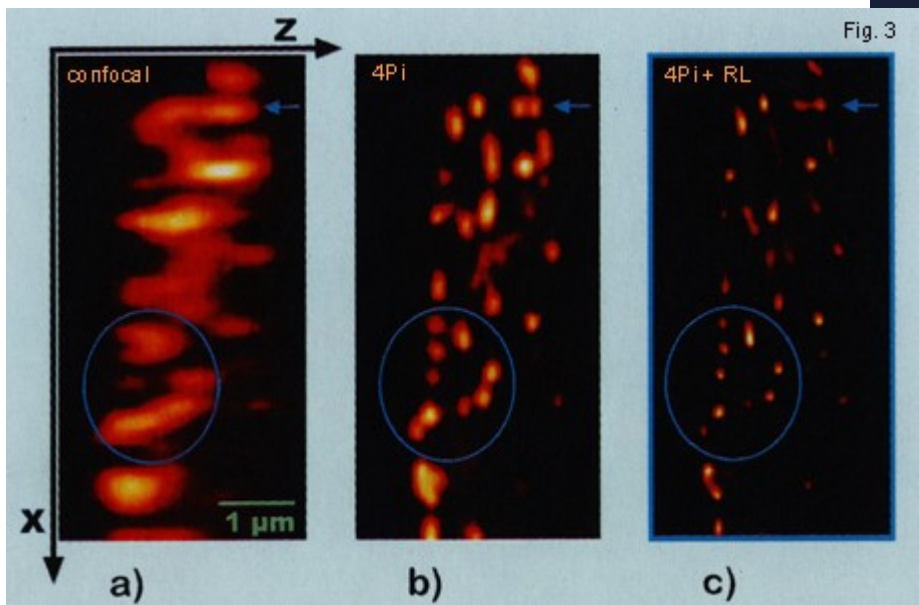
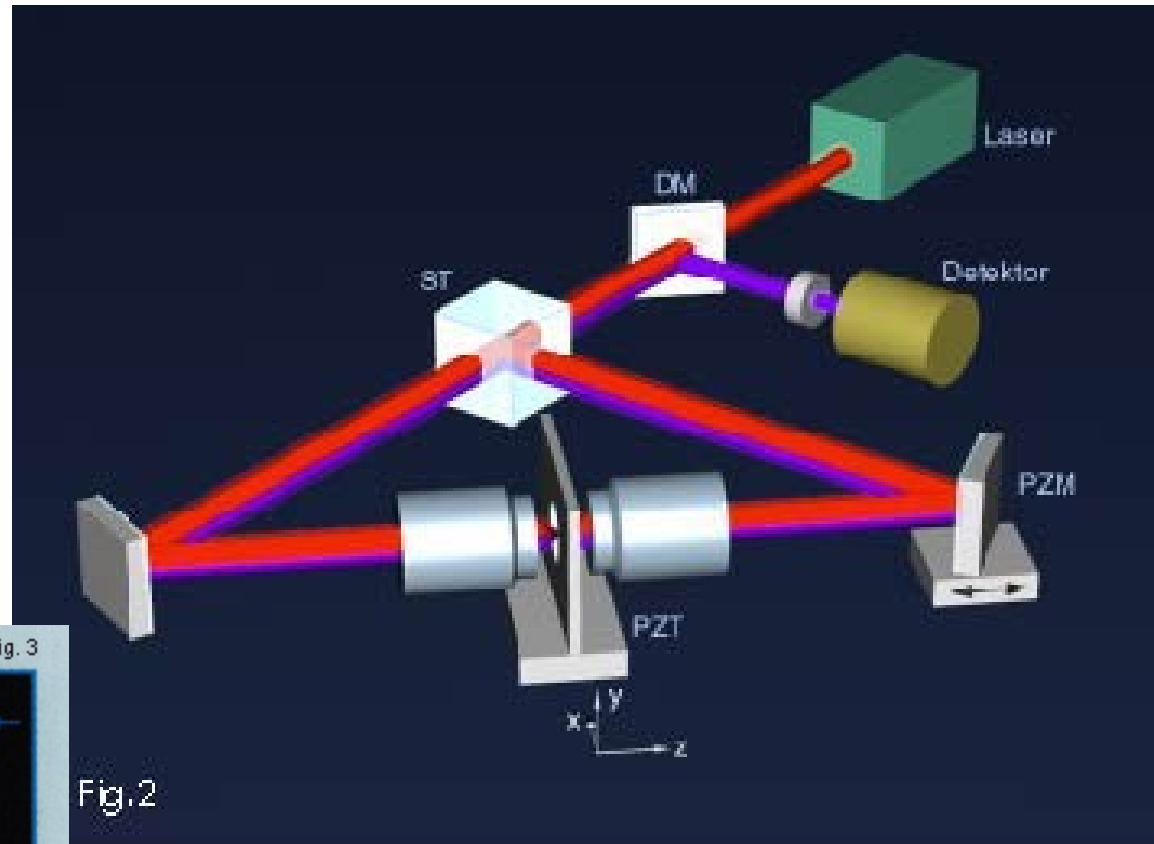
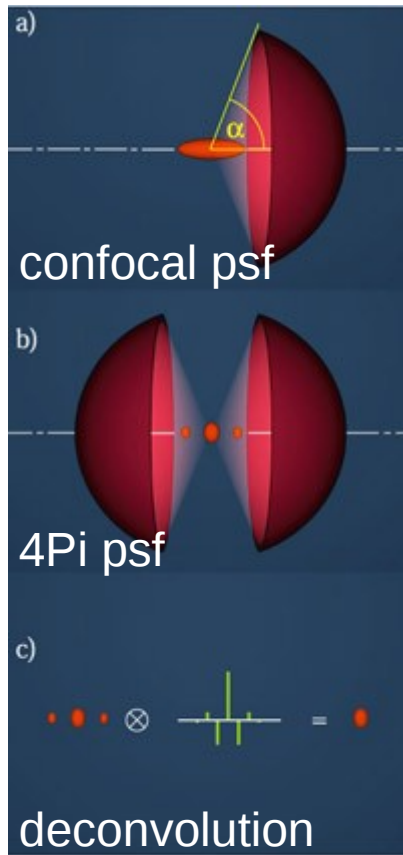
or is it?



two objectives
focused on same point
on common light path (coherent)

4π solid angle
=
full sphere

4Pi Microscopy



Breaking the Diffraction Limit

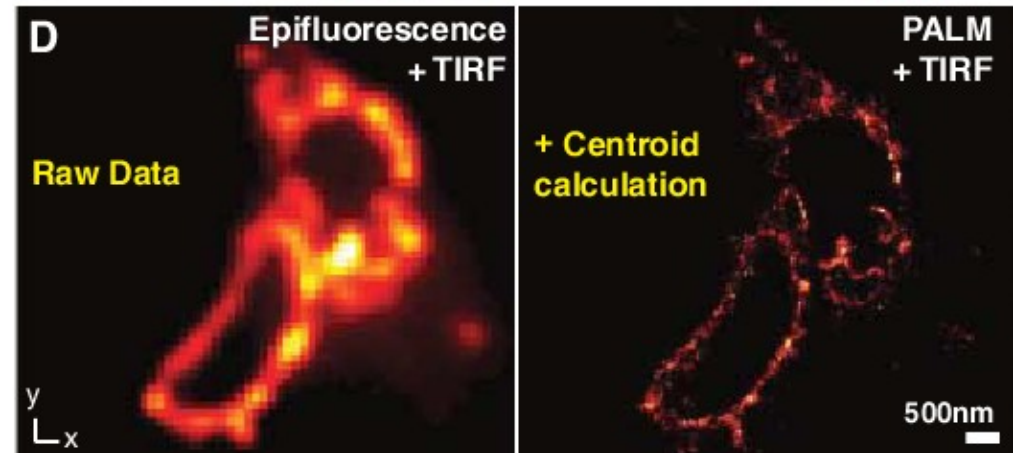
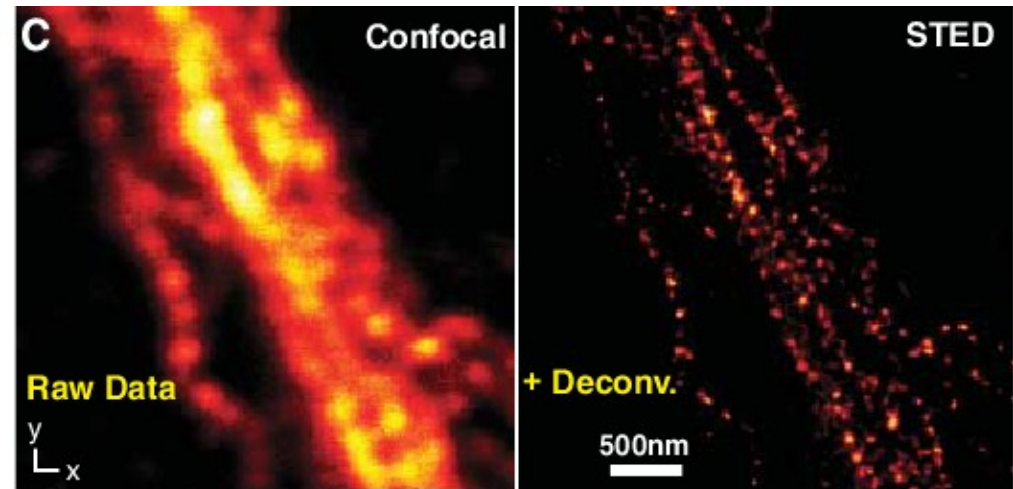
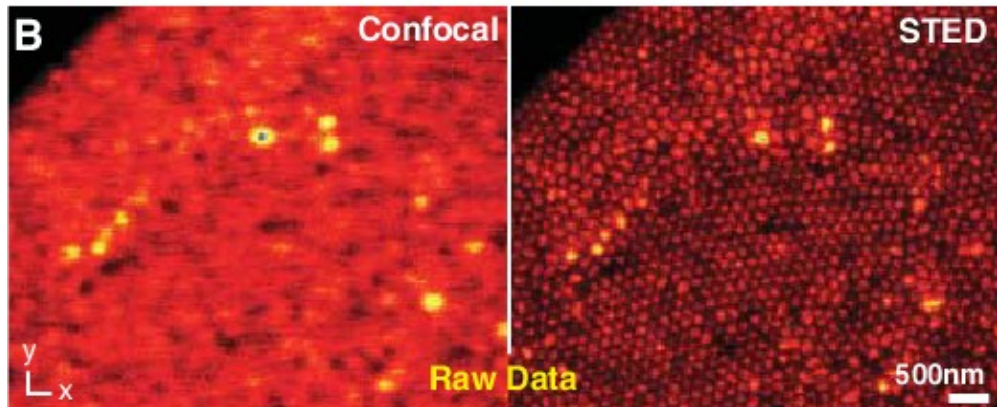
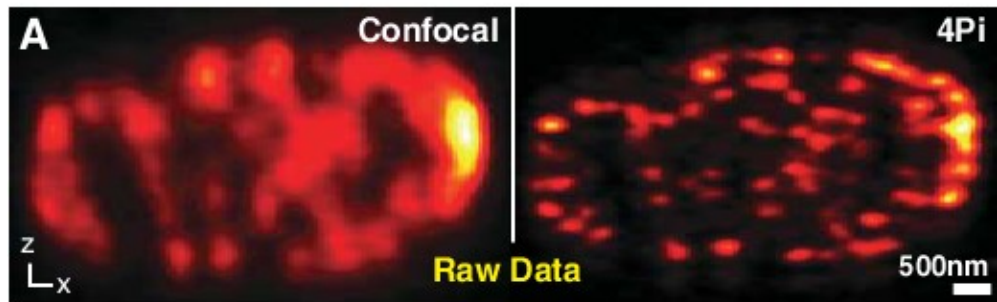
- STED: Stimulated Emission Depletion
- PALM: Photo-Activated Localization Microscopy
- STORM: Stochastic Optical Reconstruction Microscopy
- TIRF: Total Internal Reflection Fluorescence

The diffraction limit causes the PSF.

The maximal theoretical resolution of a microscope is given by the wavelength of the light.

All these techniques excite fluorescence only in a sub-resolution spot. The detector still sees the same PSF, but we know where that light comes from!

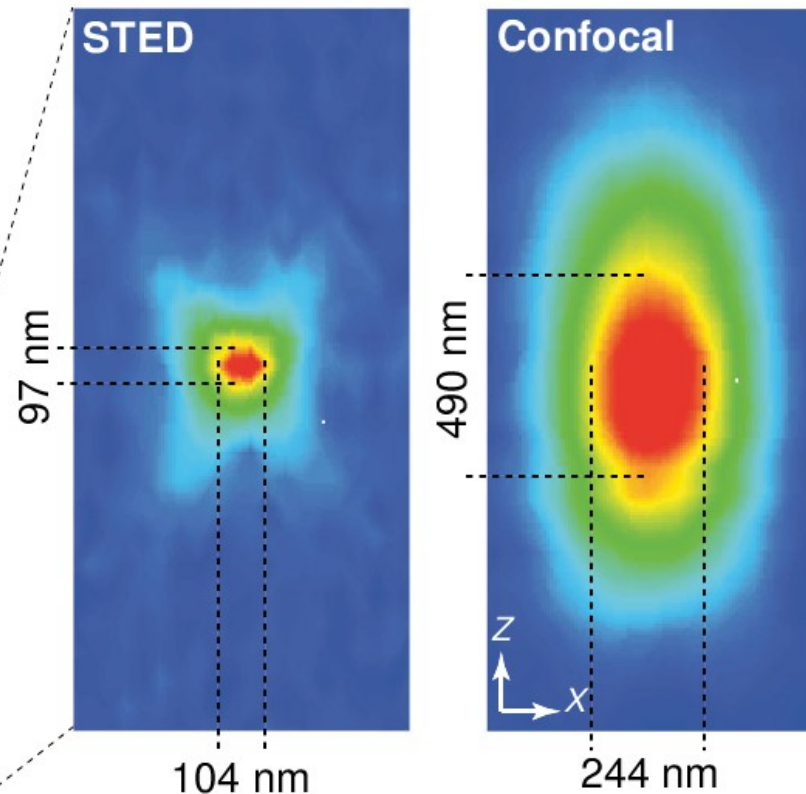
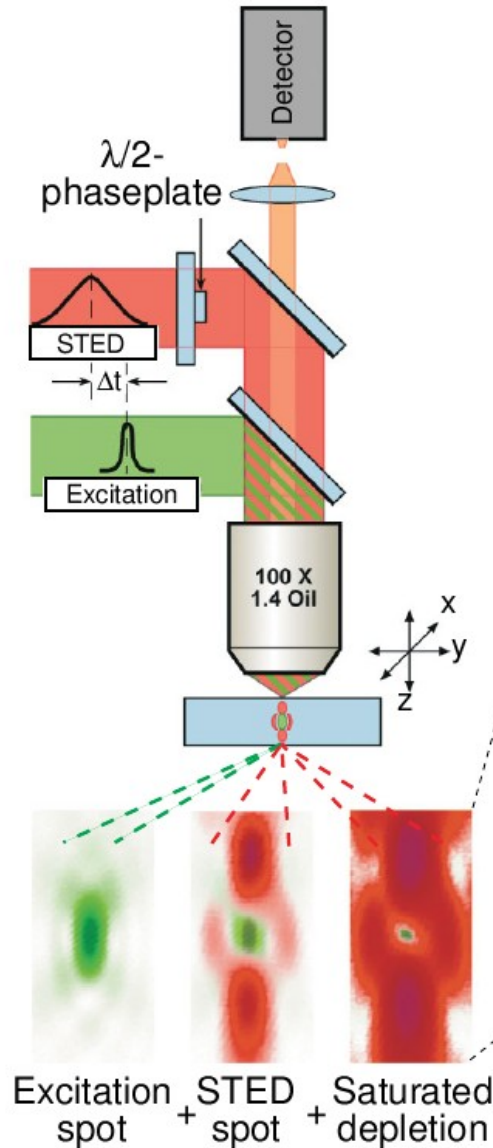
Breaking the Diffraction Limit



from: Stefan W. Hell, Science 316:1153-1158, May 2007.

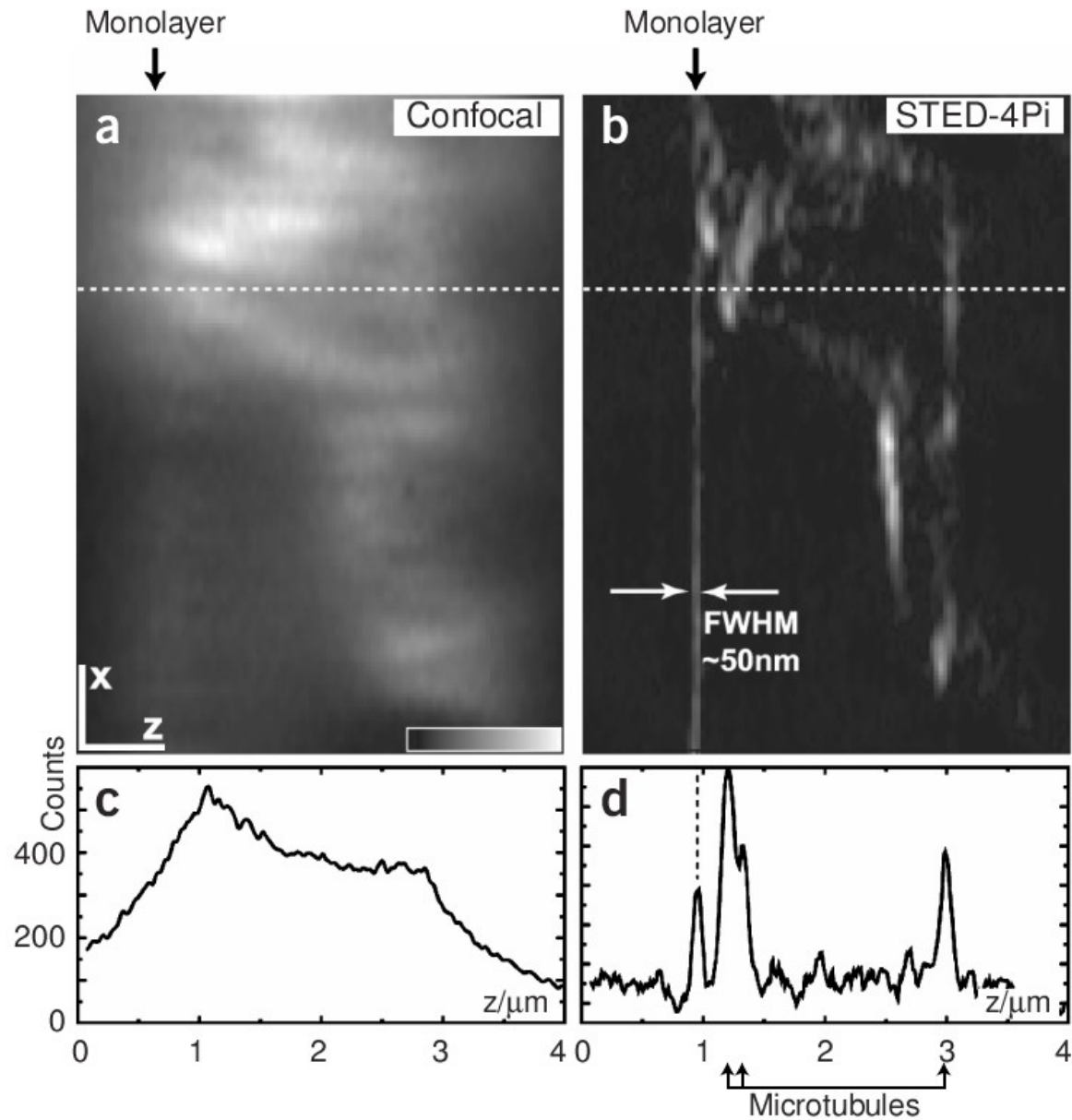
STED: Stimulated Emission Depletion

stimulated emission:
forcing excited fluorescent molecules to relax



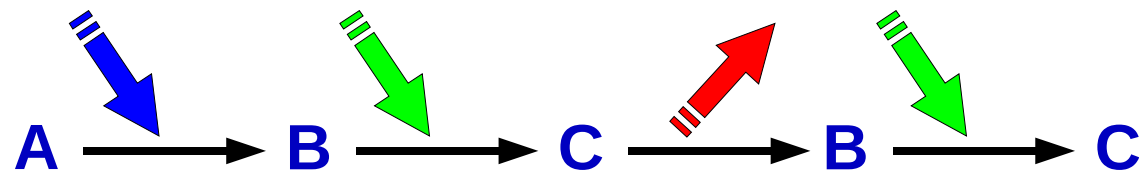
nonlinearities
introduced by
saturation can
(in theory)
reduce spot size
without limits

STED: Stimulated Emission Depletion



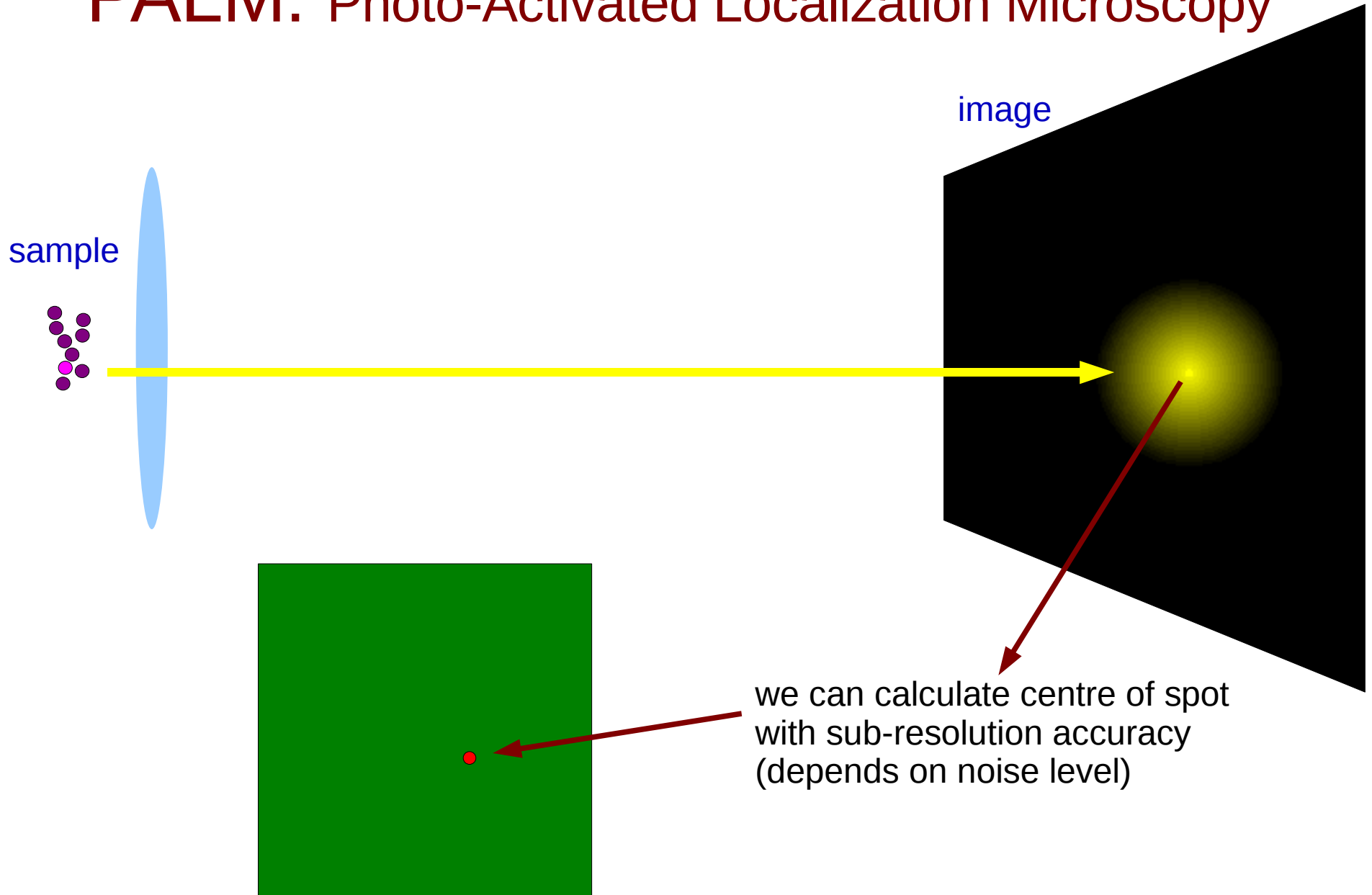
PALM: Photo-Activated Localization Microscopy

- a.k.a. STORM: Stochastic Optical Reconstruction Microscopy
- Based on photo-switchable fluorescent probes, which have 3 states:
 - A: inert (non-fluorescent)
 - B: relaxed (fluorescent)
 - C: excited (ready to emit a photon λ_3)

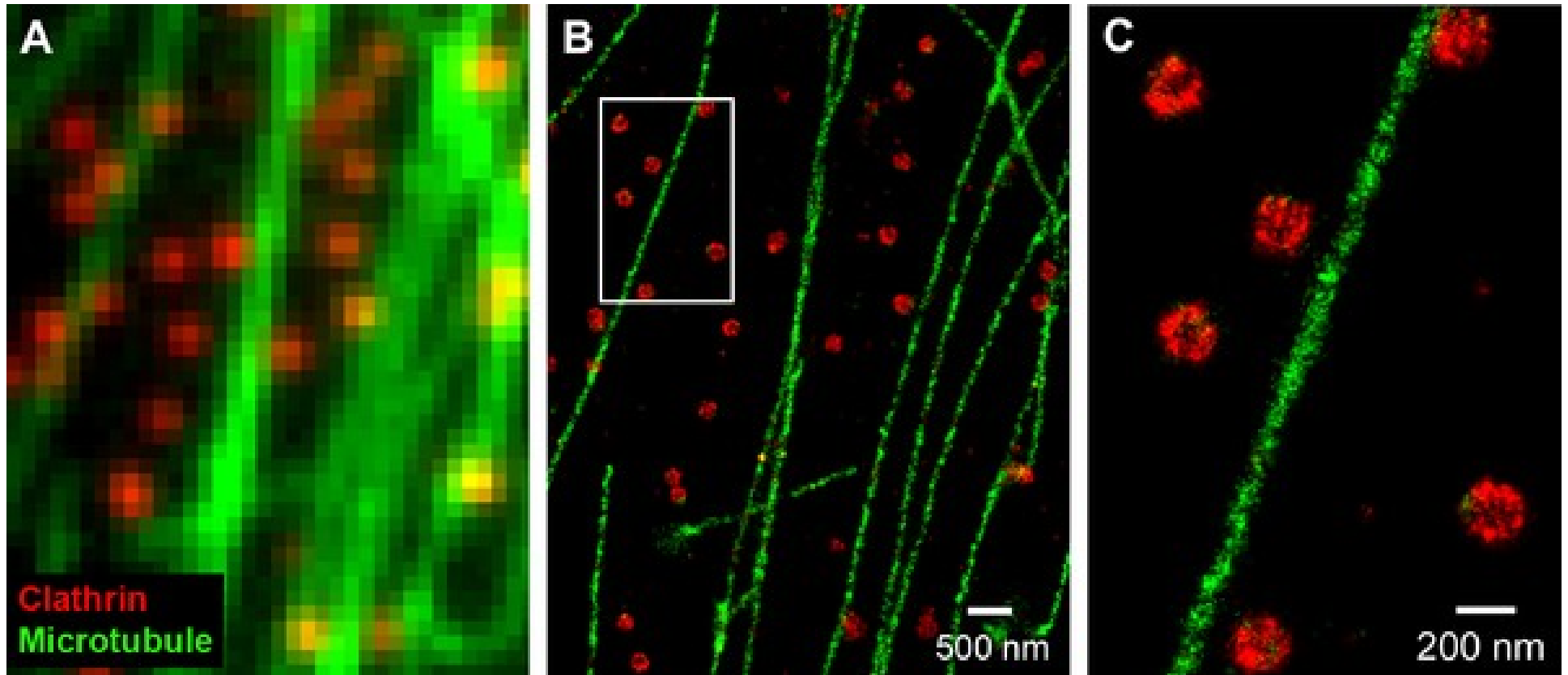


- Inert molecules brought to relaxed state by light λ_1
- Relaxed molecules excited by light λ_2
- Photobleached molecules cannot be recovered

PALM: Photo-Activated Localization Microscopy



PALM: Photo-Activated Localization Microscopy



source: zhuang.harvard.edu

TIRF: Total Internal Reflection Fluorescence

- Again, selectively illuminating only a portion of the sample:
 - Illuminating a ~100 nm-thick region just below the cover slip
 - Confocal microscope has max 1000 nm axial resolution
- Typically used to examine processes on the cell membrane

Cell Focal Adhesions in Widefield and TIR Fluorescence

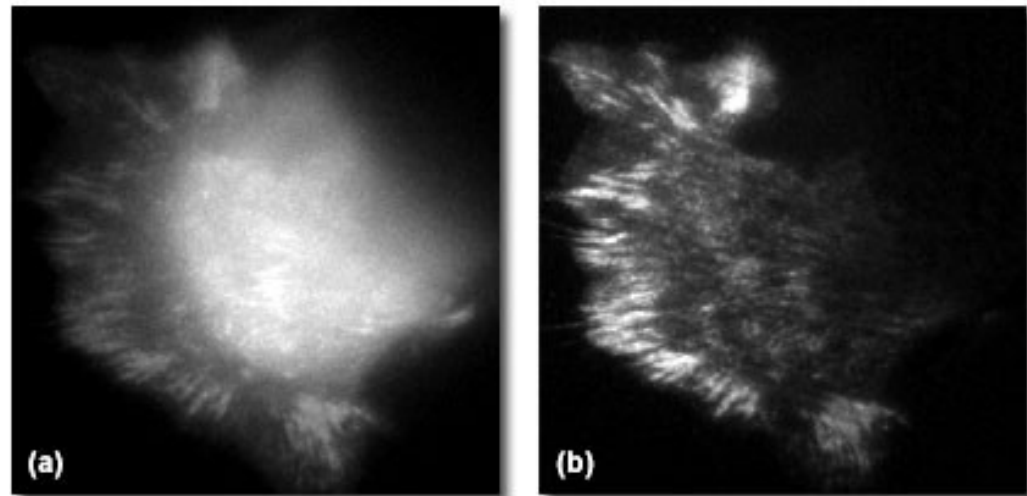
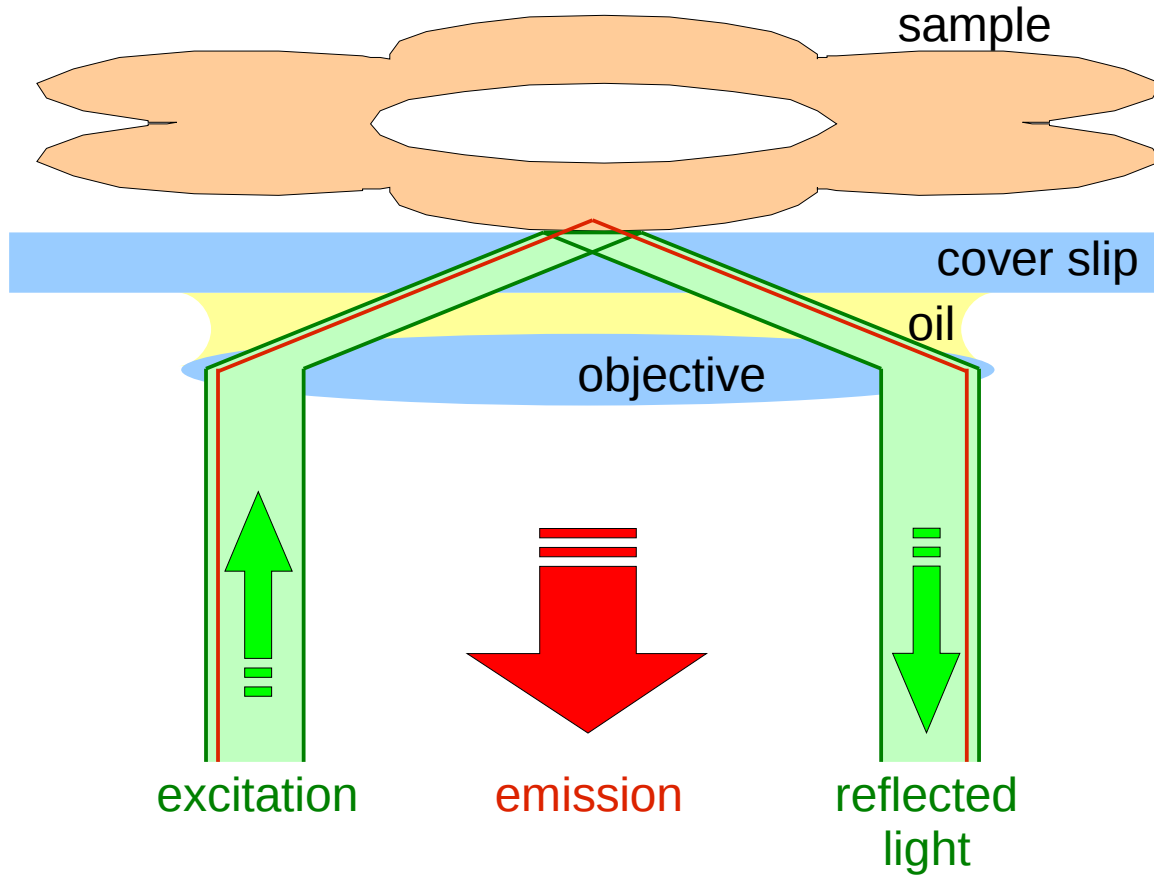
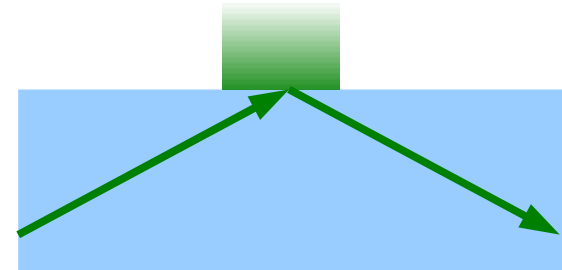


Figure 5

TIRF: Total Internal Reflection Fluorescence



evanescent wave



evanescent wave decays exponentially

requires very high NA lens (1.4 or higher)



Advanced Fluorescence Techniques

Advanced Fluorescence Techniques

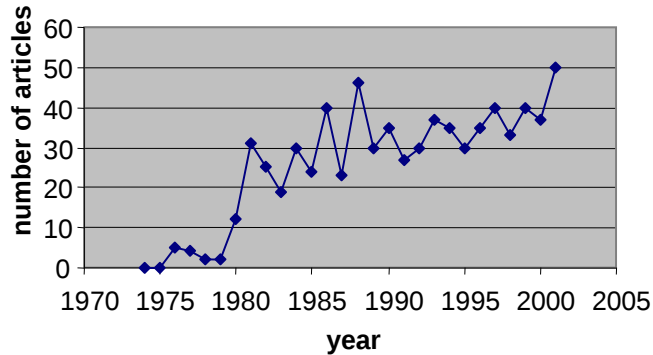
- FRAP: Fluorescence Recovery After Photo-Bleaching
 - Study diffusion and transport of protein
- FRET: Fluorescence Resonance Energy Transfer
 - Study protein interactions and changes in conformation
- FLIM: Fluorescence Lifetime Imaging Microscopy
 - Study changes in quantum efficiency

These techniques allow for more information than just “where is the fluorescent label.”

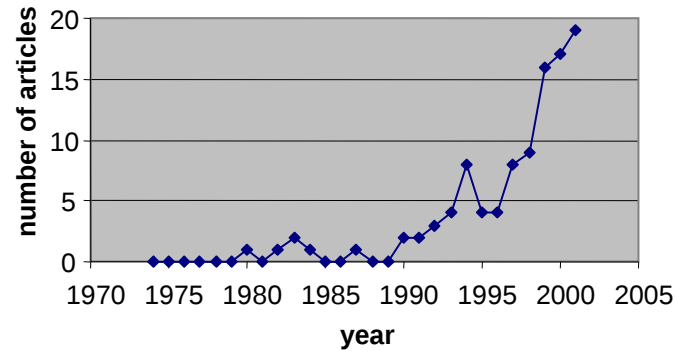
Dramatic trend towards F-techniques

source: PubMed
search by
Sylvain Costes

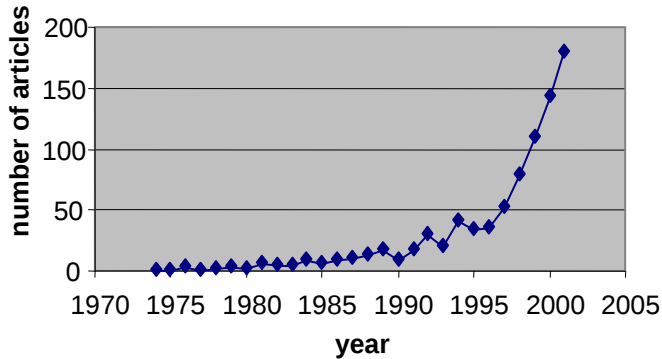
FRAP (fluorescence recovery after photobleaching)



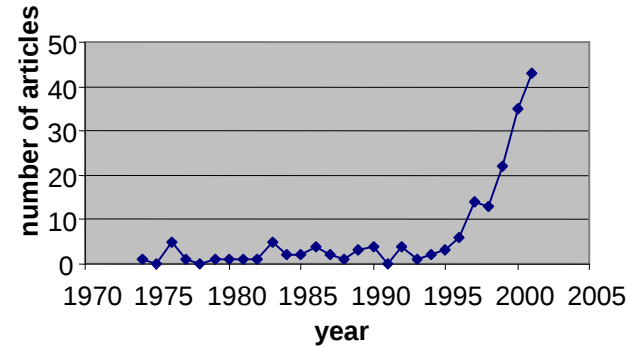
FLIM (fluorescence Lifetime Imaging)



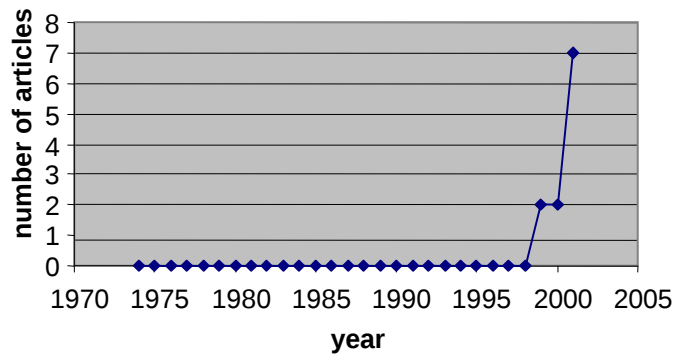
FRET (Fluorescence resonance energy transfer)



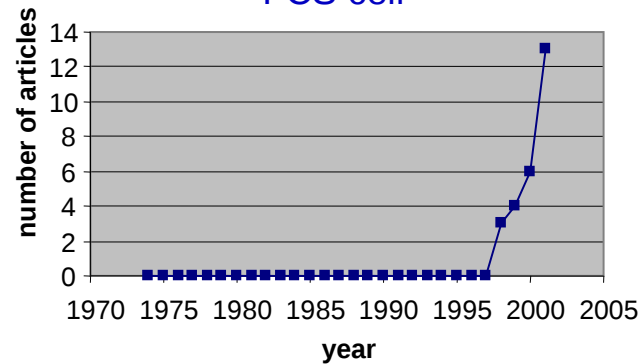
FCS (Fluorescence Correlation Spectroscopy)



FLIP (fluorescence loss in photobleaching)



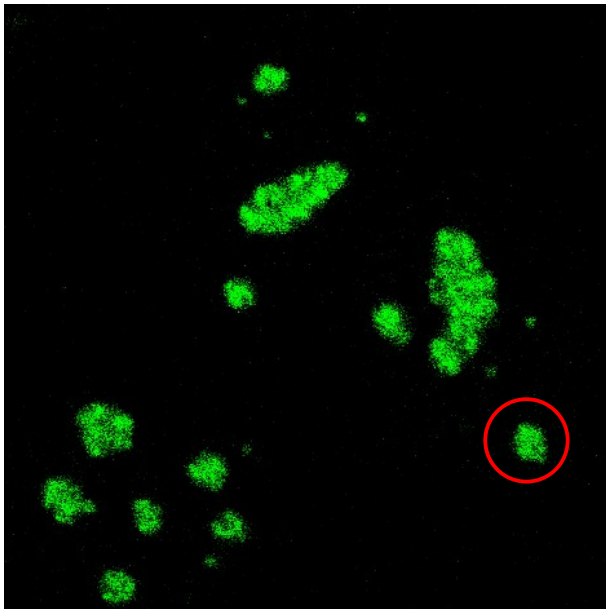
FCS cell



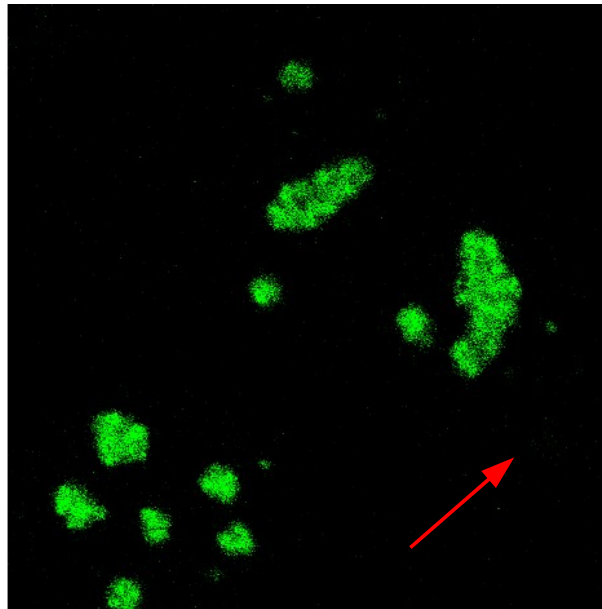
FRAP: Fluorescence Recovery After Photo-Bleaching

- Used to study transport of molecules, diffusion, etc.
- Photobleach a region in the image by high-power laser scanning
- Observe region using low-power laser scanning
- Measure speed of fluorescence recovery
- Bleached molecules never become fluorescent again: new fluorescence caused only by molecules coming into the region

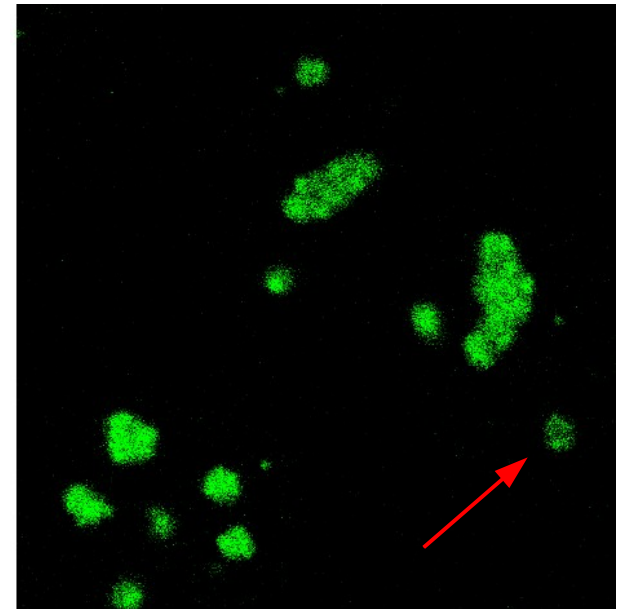
FRAP: Fluorescence Recovery After Photo-Bleaching



before bleaching

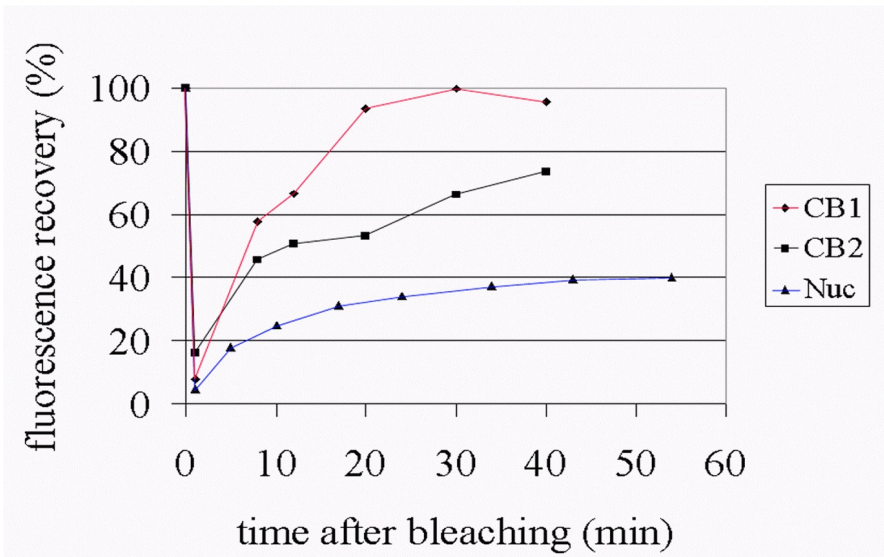


t = 30 sec

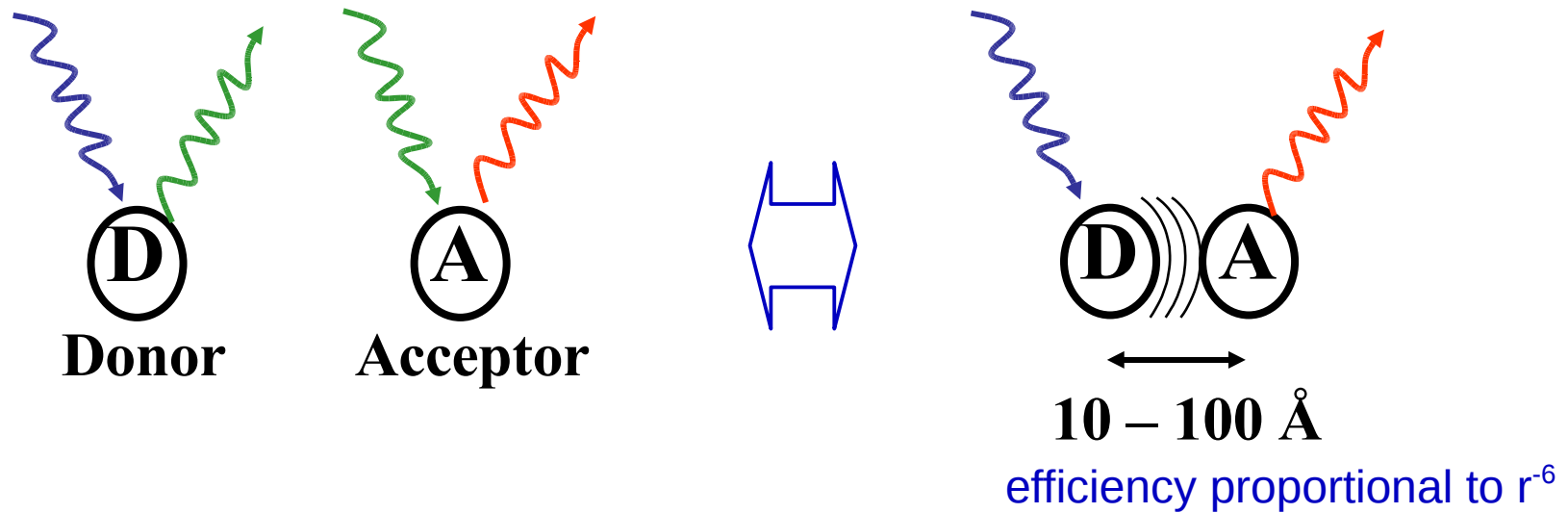


t = 40 min

mobility of GFP-fib in
nucleoli vs Cajal body



FRET: Fluorescence Resonance Energy Transfer

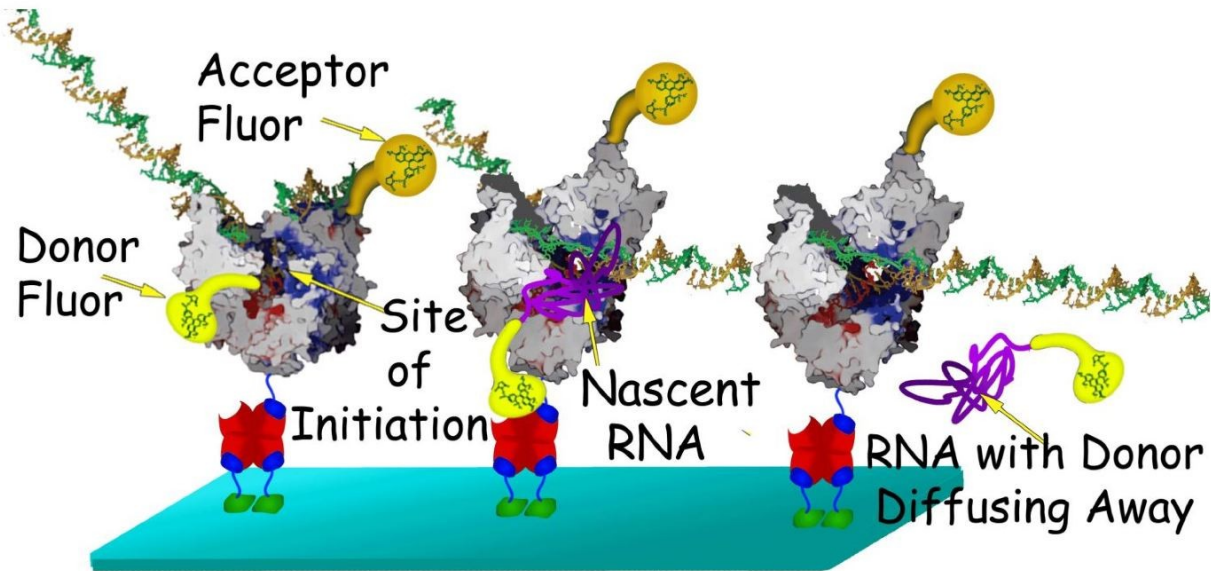


Förster radius is distance at which FRET happens with 50% efficiency

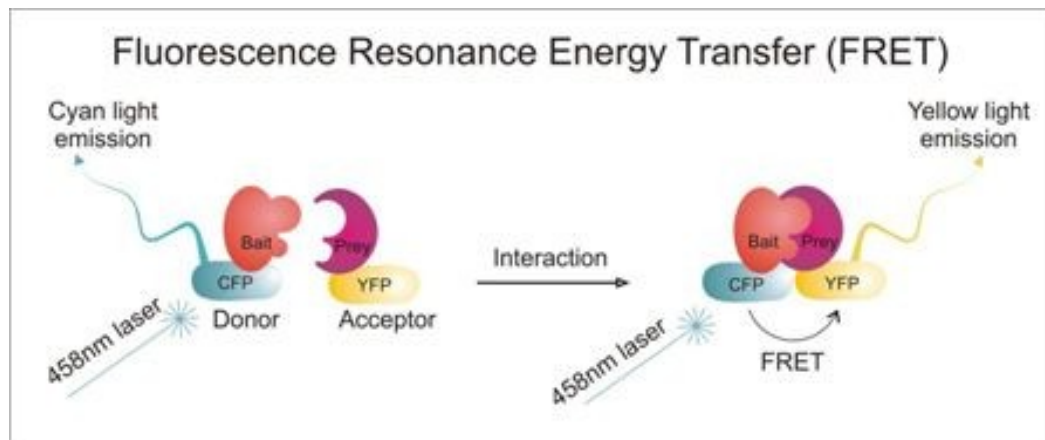
FRET shows functional interaction

Co-localization measurements only say whether molecules are close together

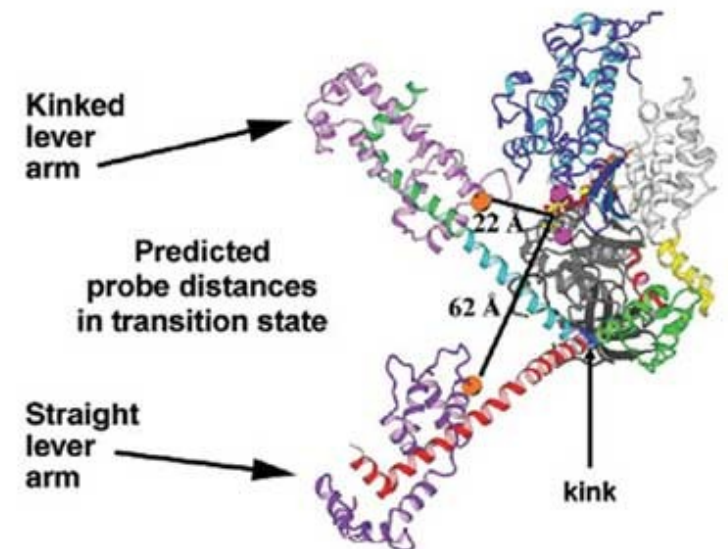
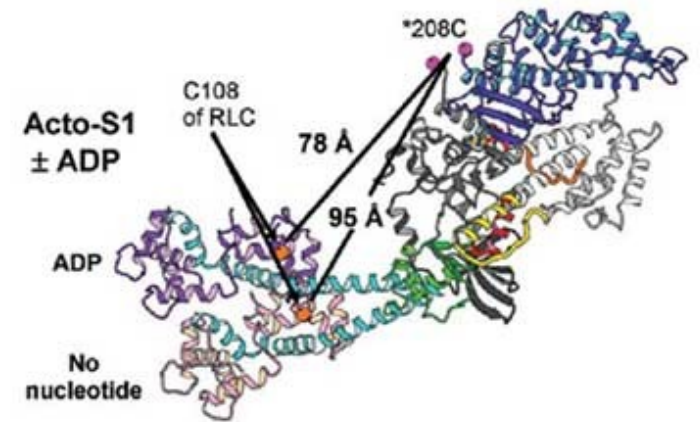
FRET: Fluorescence Resonance Energy Transfer



source: www.wfu.edu/~macoskjc/



source: www.pri.wur.nl



source: Nature Structural Biology 10:402-408 (2003)

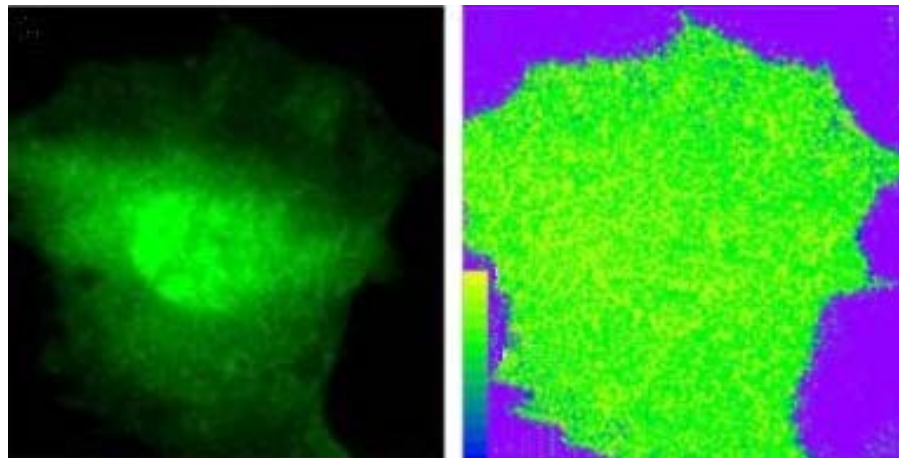
FLIM: Fluorescence Lifetime Imaging Microscopy

- Fluorescent lifetime is dependent on:
 - fluorophore
 - pH
 - concentration of ions
 - concentration of oxygen
 - protein binding
- Fluorescent lifetime is independent of:
 - fluorophore concentration
 - photobleaching
 - light scattering
 - excitation light intensity

FLIM: Fluorescence Lifetime Imaging Microscopy

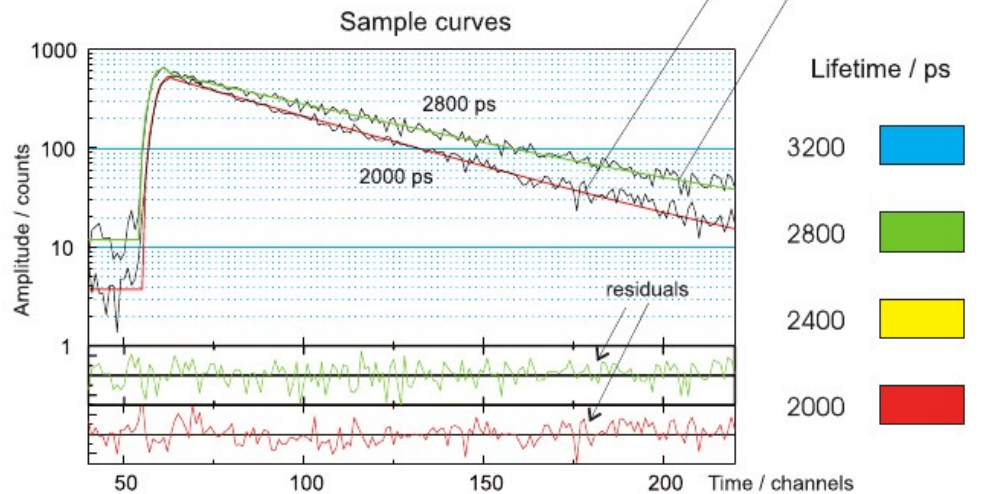
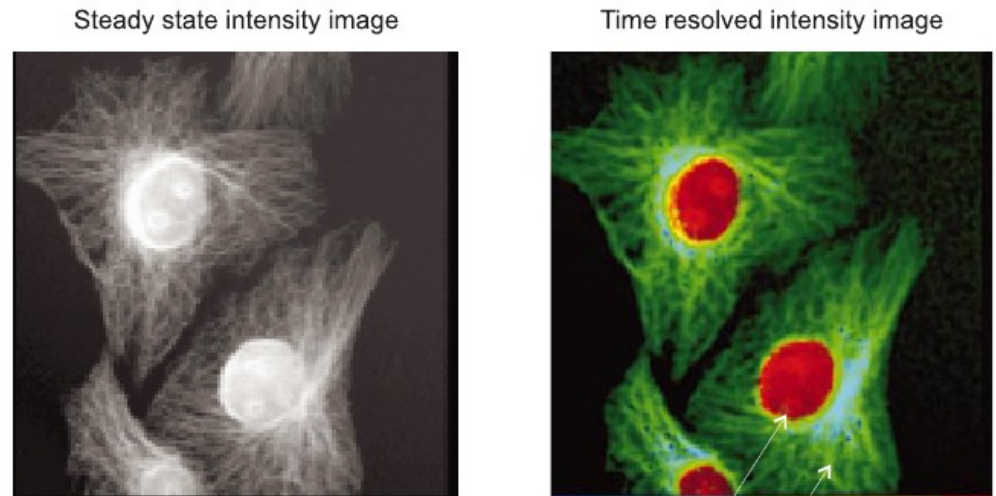
FLIM can be used to:

- measure environment of dye (e.g. local oxygen concentration)
- measure FRET more accurately
- separate different fluorophores
- distinguish autofluorescence



GFP intensity

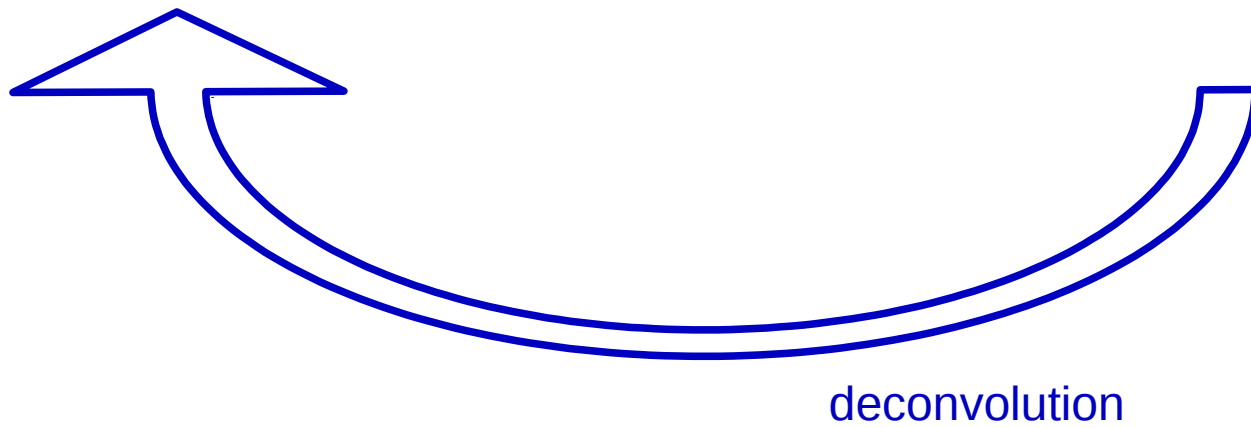
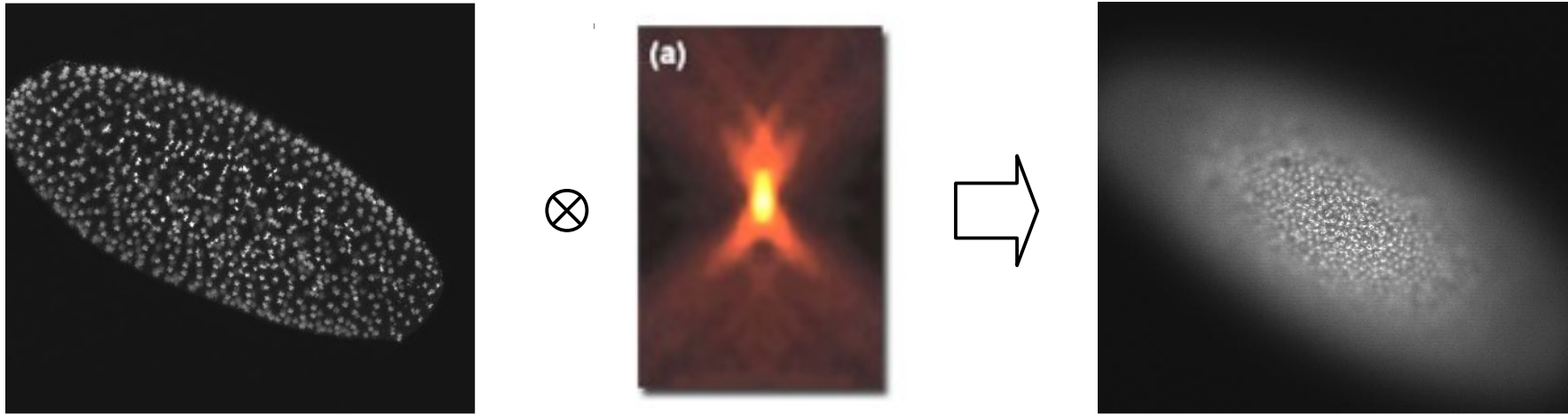
GFP lifetime



source: Becker & Hickl GmbH

Other Optical Sectioning Techniques

Wide Field Deconvolution



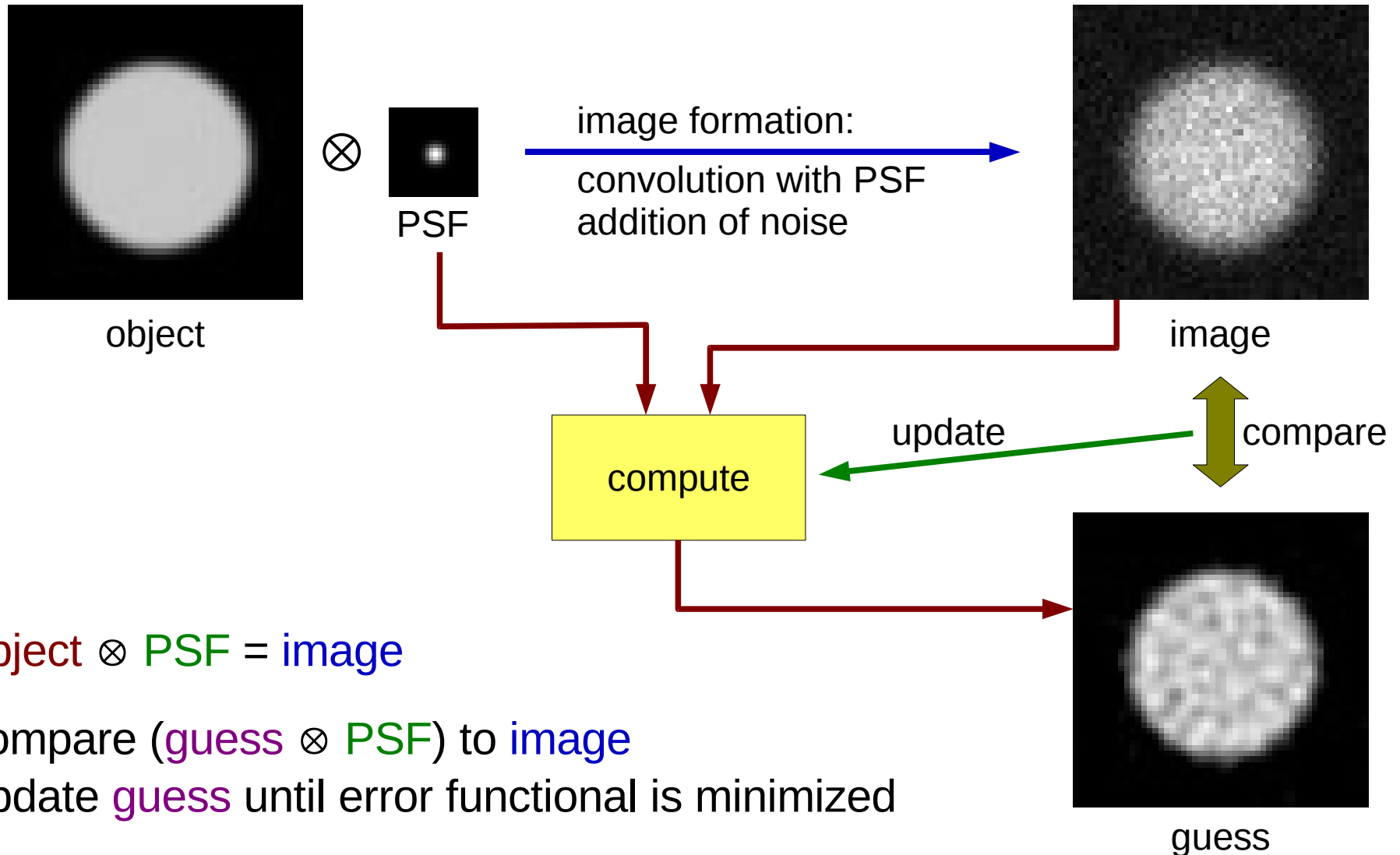
Deconvolution Algorithms

- Nearest-Neighbour (or multi-neighbour)
 - Subtract blurred version of neighbouring slices
- Inverse Filtering
 - e.g. Wiener Filter, Tikhonov-Miller Regularization, etc.
 - These are linear methods
 - Estimate of PSF is needed
- Non-Linear Iterative Restoration
 - Richardson-Lucy, ICTM, Carrington, etc.
 - Estimate of PSF and noise statistics are needed
- Blind Deconvolution
 - Also non-linear iterative restoration, but PSF is estimated at the same time

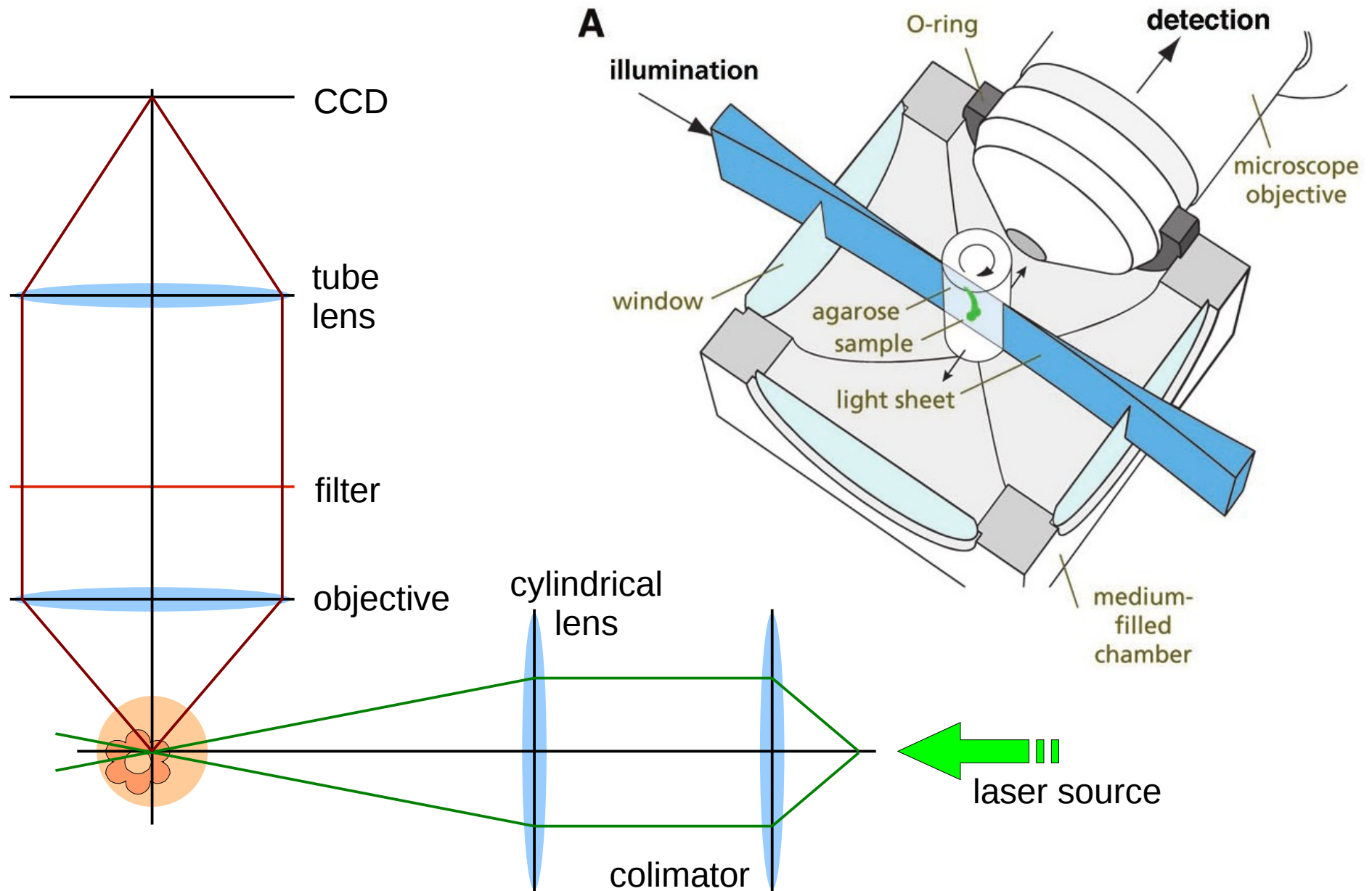
PSF for Deconvolution

- Theoretical PSF
 - Calculated based on diffraction theory
 - Knowledge of microscope parameters required
 - Noise-free
 - Does not take aberrations into account
- Measured PSF
 - By imaging fluorescent beads of known size
 - Lots of measurements necessary to avoid noise in data
 - Sometimes radial symmetry and axial symmetry enforced to reduce noise (ignoring spherical aberration)
- Blind Deconvolution does not need an accurate PSF
 - PSF is estimated from the same image

Non-Linear Iterative Restoration

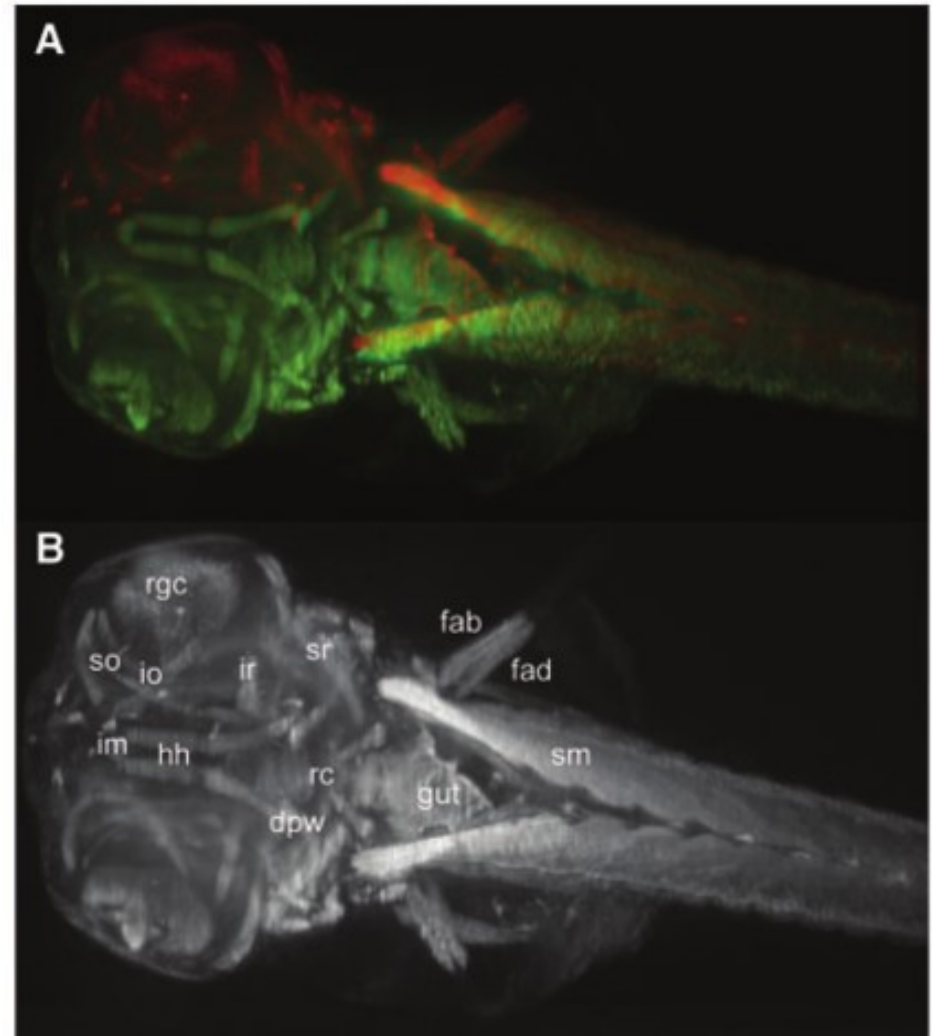


Selective Plane Illumination Microscopy



Selective Plane Illumination Microscopy

- Light comes from one side
 - Shadow cast across field of view
 - Attenuation with depth happens in two directions
- Sample holder is cylinder
 - It is possible to illuminate from both sides
 - It is possible to rotate cylinder, record and combine many 3D images
- Low to medium resolution
- Slice thickness not uniform



source: Jan Huisken et al., Science 305 (2004)

Further Reading

- Microscopy U, Microscopy Primer and Olympus Microscopy Resource Center
 - <http://www.microscopyu.com/>
 - <http://microscopy.fsu.edu/>
 - <http://www.olympusmicro.com/>
- The Handbook (on fluorescent probes)
 - <http://probes.invitrogen.com/handbook/>
- Far-Field Optical Nanoscopy
 - Stefan W. Hell, Science 316:1153-1158, May 2007.
- Lifetime Imaging Techniques for Optical Microscopy
 - Wolfgang Becker & Axel Bergmann, Becker & Hickl GmbH, Berlin