MICRO-562

Biomicroscopy II

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Biomicroscopy-II Syllabus – (TENTATIVE)

Lecture 1	Course	Brief Review, Dark-Field
Lecture 2	Course	Phase Contrast & Polarization, Birefringence
Lecture 3	Course	DIC & Fluorescence Microscopy Techniques (FRET & TIRFM)
Lecture 4	Course	FRAP & Introduction to Confocal Microscopy
Lecture 5	Course	Advanced Microscopy-I
Lecture 6	Course	Advanced Microscopy-II
Lecture 7	TP	Practical: Intro to Wide-Field Microscopy
Lecture 8	TP	Practical: Transmission Techniques
Lecture 9	TP	Practical: Intro to Fluorescence
Lecture X	**EASTER**	
Lecture 10	TP	Practical: Intro to Confocal
Lecture 11	TP	Practical: Fluo/Conf
Lecture 12	TP	Practical: Fluo/Conf
Lecture 13	**EXAM**	
Lecture X	**HOLIDAY**	

Variety of fluorescence microscopy technique exists

- A strong feature of fluorescence microscopy is that the signals making up an image are “molecule-specific”.
- With the addition of time-lapse methods, it is possible to track time-dependent changes of molecules & dynamic molecular events.

A few examples of dynamic fluorescence imaging methods:

- **FRET (Förster resonance energy transfer)**
- **TIRFM (Total internal reflection fluorescence microscopy)**
- FRAP (Fluorescence recovery after photo bleaching)
- FLIM (Fluorescence lifetime imaging microscopy)
- FLIP (Fluorescence loss in photobleaching)
- FLAP (Fluorescence localization after photobleaching)
- FISH (Fluorescence in situ hybridization)
- FCS (Fluorescence correlation spectroscopy)

…

Reminder: Some Fluorescence Terms

Excitation (absorption) spectrum & its peak

Emission spectrum & its peak

Stoke's shift:

Extinction coefficient:

Quantum efficiency (yield):

Brightness: extinction coefficient * quantum yield

Fluorescence life-time:

Photostability:

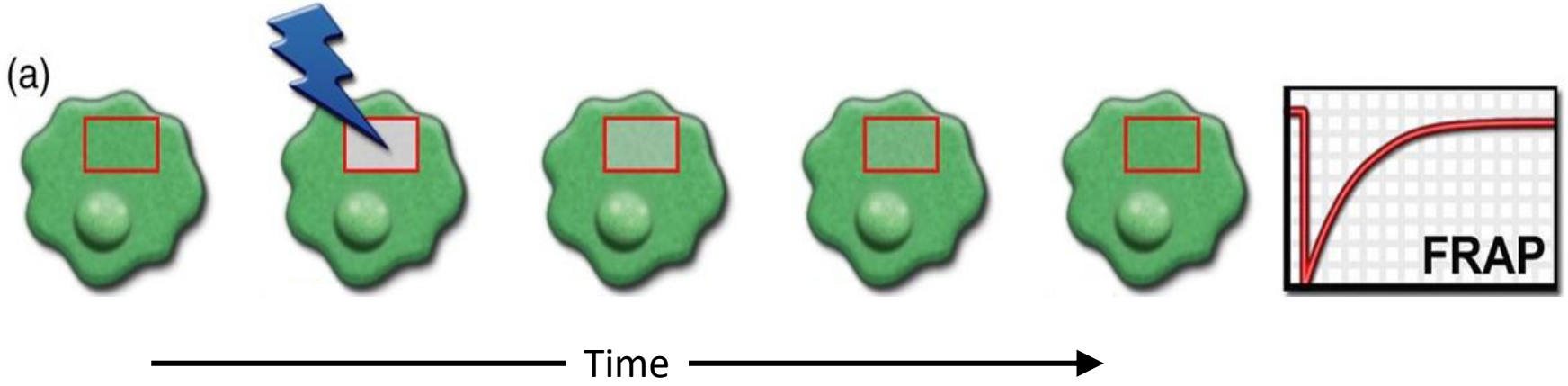
Blinking:

Quenching:

Photo-bleaching (also termed as *fading*):

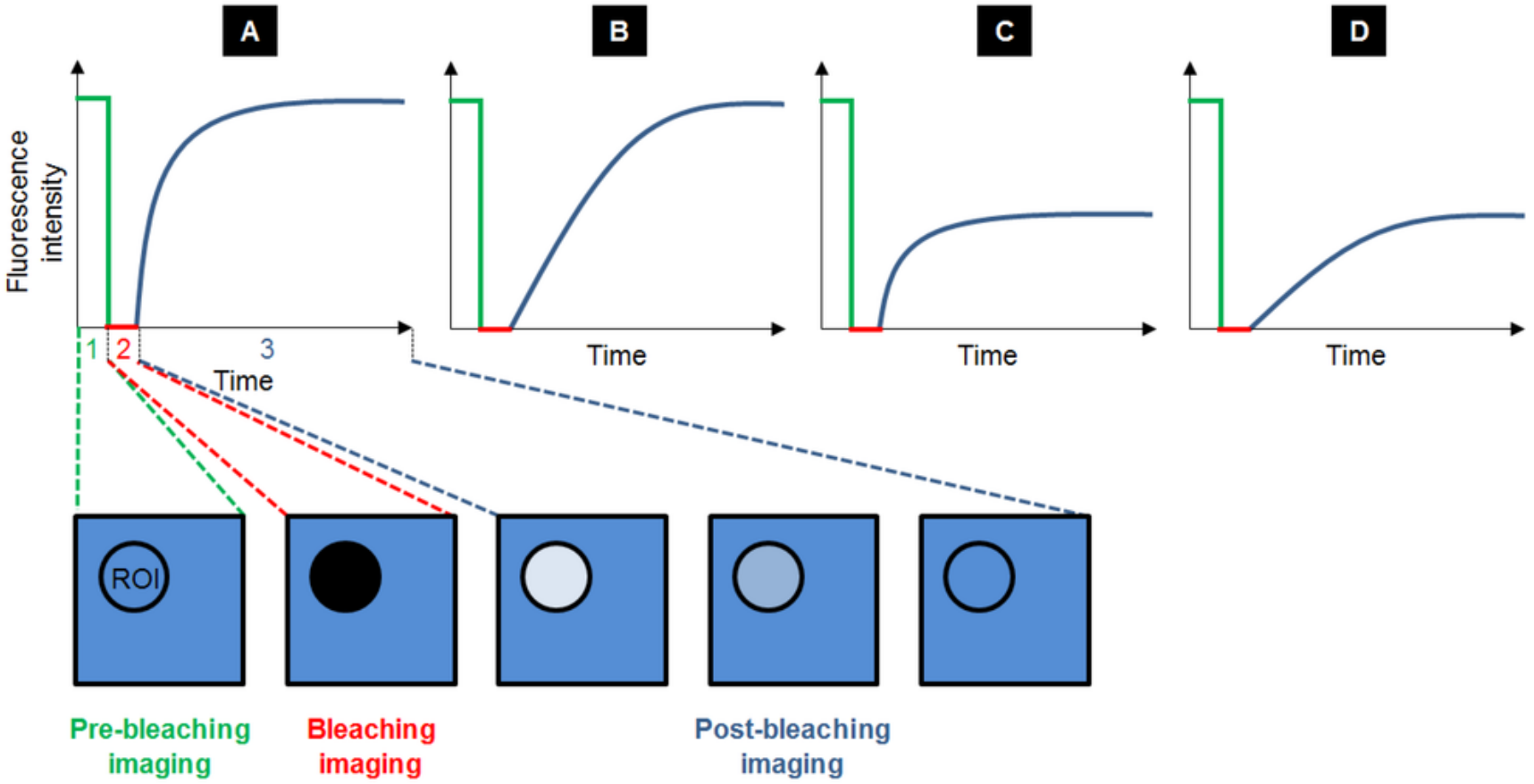
FRAP (**F**luorescence **R**ecovery **A**fter **P**hotobleaching)

- **Method principle:** Fluorescently tagged molecules at equilibrium in a structure are **photo-bleached** with brief exposure to an intense beam of light, and the scene is monitored over time to determine the half-time of recovery. Fluorescence recovery can be analysed by measuring fluorescence intensities or lifetime.



- **Application of the method :**
  - Useful in biochemical and biophysical studies of purified proteins for calculating the ***diffusion coefficient***, a parameter that contains information regarding the mass and shape of the molecule.
  - FRAP measurements in cells reveal the ***percent mobile fraction***, the fraction of a molecular species that is free in the cell and therefore able to participate in the dynamic equilibrium under study.

FRAP Recovery Kinetics



- **Typical recovery curves obtained after FRAP experiments:** the fluorescence intensity is measured before (step 1, in green), during (step 2, in red) and after photobleaching (step 3, in blue), while equilibrium is taking place.
- Several profiles can be observed depending on the velocity and the amplitude of the recovery. Recovery can be fast with a maximal recovery (**A**), or slow with a maximal recovery (**B**). It can also be fast with a limited recovery (**C**) or slow with a limited recovery (**D**).

Example: FRAP experiment with GFPs fused to an endoplasmic reticulum in a living cell

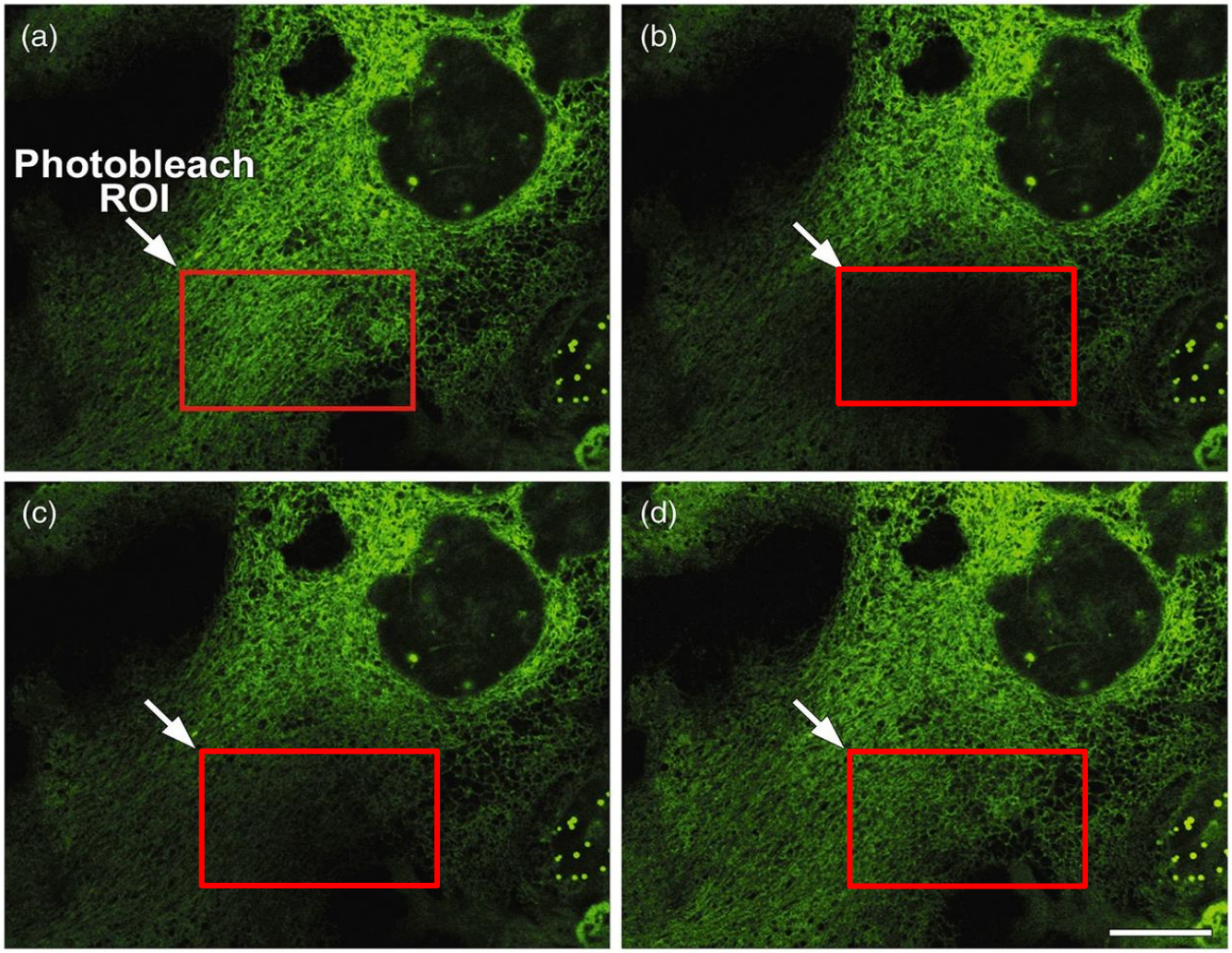
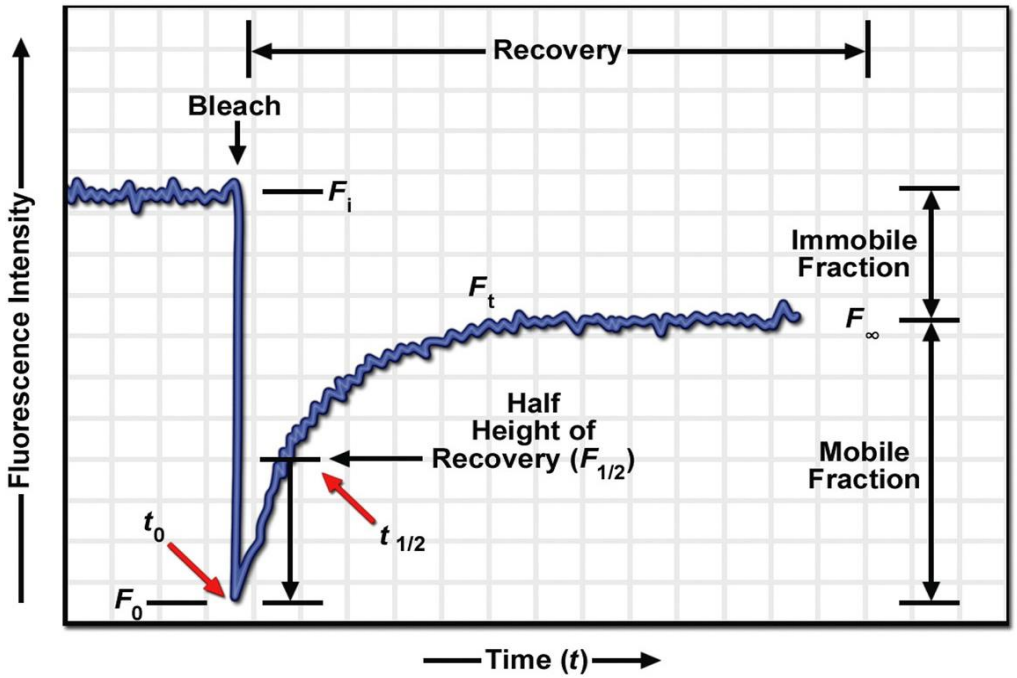


Image frames show (a) pre-bleach and the selected ROI (red rectangle)
(b) 2 seconds post-bleach (c) 5 seconds post-bleach (d) 50 seconds post-bleach. Bar = 10 μm.

FRAP Kinetic Analysis



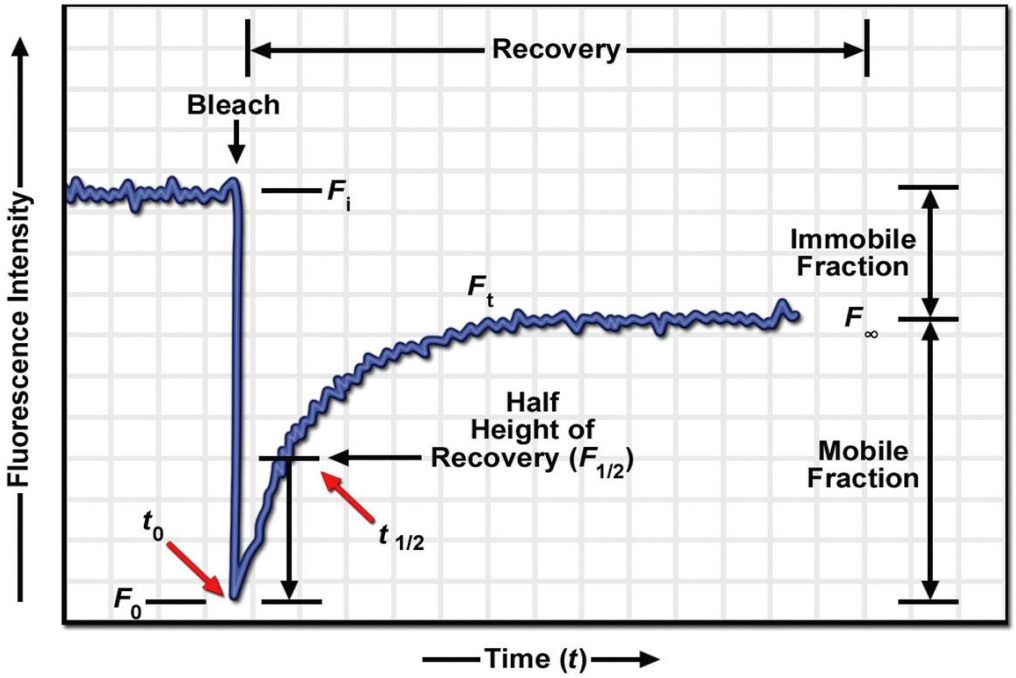
Fluorescence intensity is shown with: pre-bleach (F_i), immediately after photo-bleach (F_o), at fluorescence equilibrium (F_∞), at the half-time for recovery ($F_{1/2}$) and at time = t during recovery (F_t).

- **Mobile fraction** of molecules show the strength of recovery and calculated as: F_∞/F_i
  Usually F_∞ is less than the original level F_i. *This* occurs when:
  - → photobleaching remove part of the total fluorescence signal in the system
  - → or a fraction (aka immobile fraction) of molecules recovers at a much slower rate or don't recover at all

- **Immobile fraction** is calculated as $(F_i - F_\infty)/F_i$

- Percent recovery is calculated as:
  - → $\%\ Recovery = (F_\infty - F_o)/(F_i - F_o)$

FRAP Kinetic Analysis

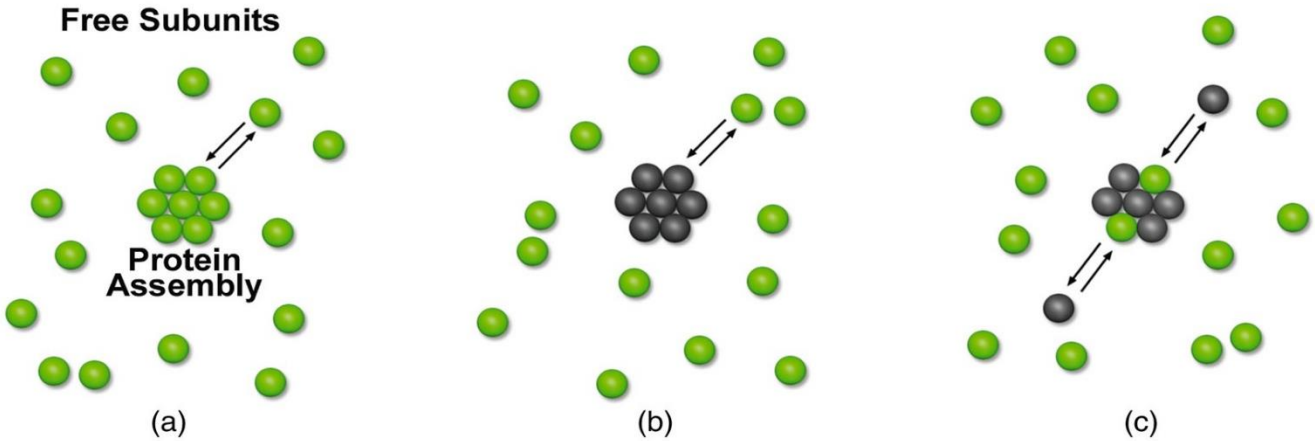


For simple first-order reactions, a basic approach is to fit the data to the exponential equation:

$$F_t = F_\infty - (F_\infty - F_i)e^{-kt}$$

Where F_t, F_i, and F_∞ are the fluorescence at time t during the recovery, the initial and final fluorescence, respectively. And, k is a constant.

Measurement of Diffusion Coefficients Using FRAP



- **FRAP can be use to measure diffusion coefficients (D) of fluorescently tagged macromolecules, such as protein labelled with a small fluorescent dye.**

- **The implementation of the method:**
  - A drop of labelled molecules form a thin film when sandwiched between a microscope slide and coverslip.
  - The laser beam spreads into a large spot (diameter 4—50 um), and FRAP curves are obtained.
  - The kinetics of the fluorescence recovery of the bleached zone is analyzed by various equations, such as the one that is developed by Axelrod et al (1976) to calculate D in a 2D system:

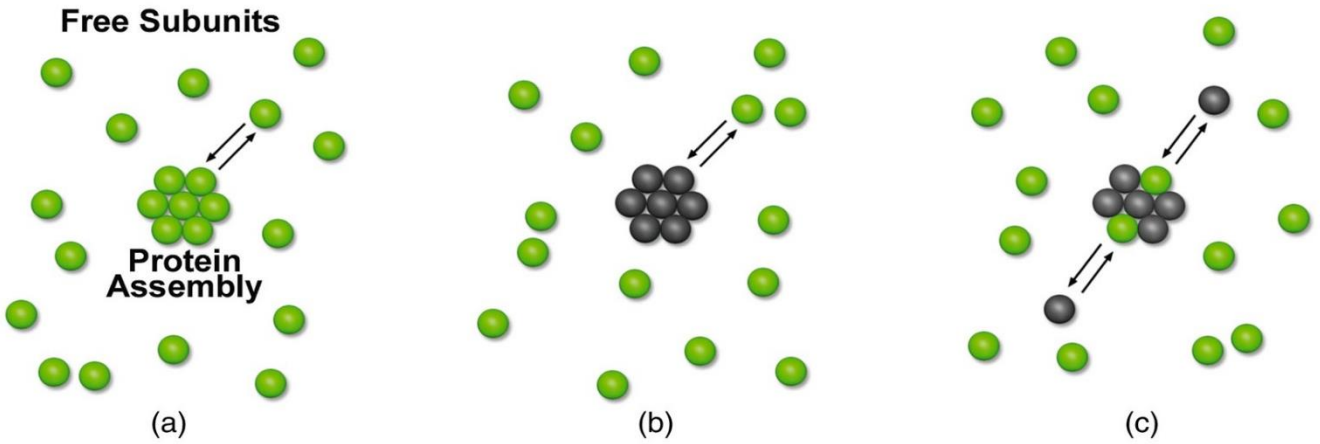
$$\tau_{1/2} = \omega^2/4D$$

Here ω is the radius of the focused laser beam, and $\tau_{1/2}$ is the fluorescence recovery time.

  - Other similar equations/fittings are available for calculating D more accurately.

Applications of FRAP in Biology

FRAP can be applied in cells to study the chemical equilibrium of binding and dissociation of molecules in organized structures, such as filament biopolymers, proteins and lipids composing cell membranes and organelle surfaces, nucleic acid-protein complexes, supramolecular assemblies.



FRAP Equipment

- Performed typically on a wide-field or confocal fluorescence microscope

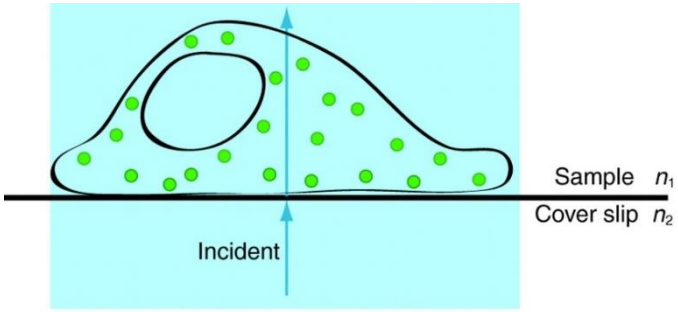
- A bright light sources, such a s a laser, for producing photobleaching.
  Example: a laser beam of around 200 mW can be useful.
  Caution: because of potential photodamage to the live cells, caution should be taken while performing FRAP on live cells.

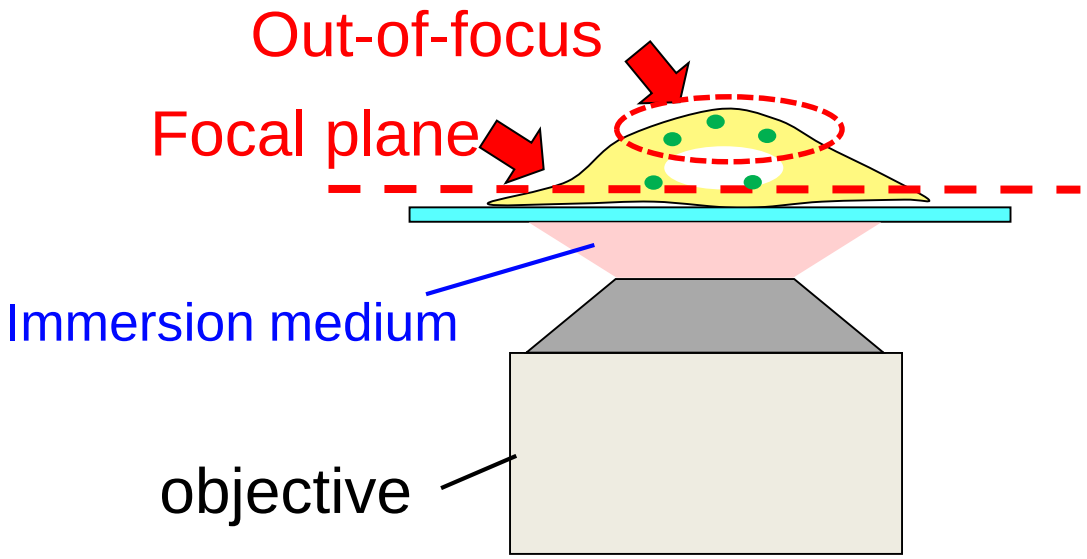
- The laser should be sufficiently powerful to induce 30-50% bleach in a short time period, about 5-100 ms, so that an appropriate photobleach signal is captured in the first recovery frame.

- Since molecules can diffuse across the diameter of a cell in about 2 seconds, it is important to keep the bleach time as short as possible and obtain the first recovery frame quickly.

Limitations of conventional fluorescence microscopy

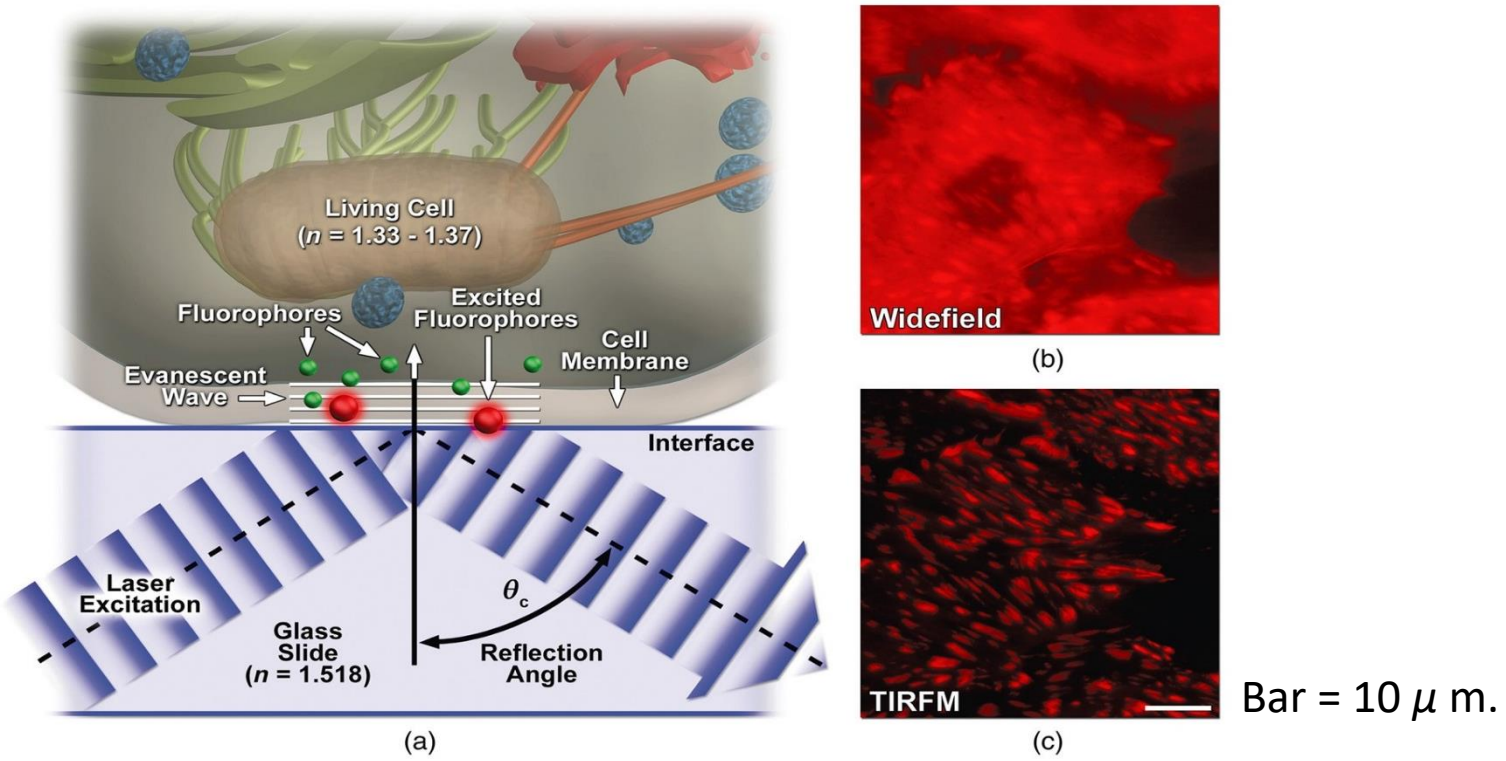
- In a standard fluorescence microscope, the excitation beam illuminates a wide field of the sample.
- If the sample is thick (i.e. rounded cells and tissue sections), fluorescence will be excited within the focal plane, but also within the planes above and below the focus.
- Some of this fluorescence will be imaged onto the detector and will result in a **defocused-looking image.**





Reminder - Total internal reflection fluorescence (TIRF) microscopy

- TIRF microscopy uses a specialized method of illumination to excite fluorescence in molecules that are located within the 100 nm of the surface of the coverslip to which specimens are attached.



(a) Cartoon of a cell on the surface of a coverslip labeled with fluorophores that are excited by the evanescent wave field from the reflected laser.
(b) Wide-field image of living cells expressing a red fluorescent protein fused to paxillin, a focal adhesion component.
(c) Same field of view in TIRF illumination. Note the higher contrast and S/N in the TIRF image.

TIRF microscopy is used to examine membrane proteins, cell signaling events, receptor clustering in membranes, focal adhesions, cytoskeletal components, and other membrane - associated molecules.

Limitations of conventional fluorescence microscopy

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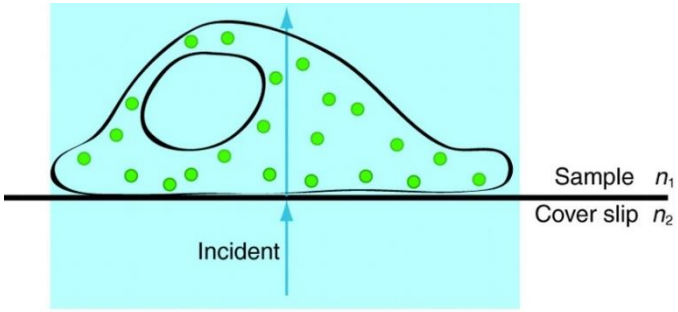
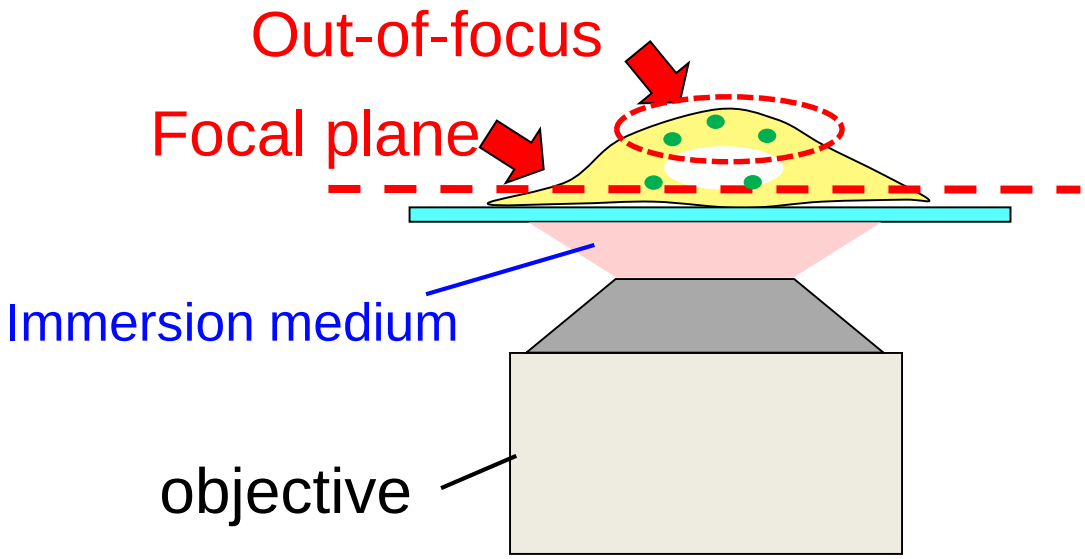
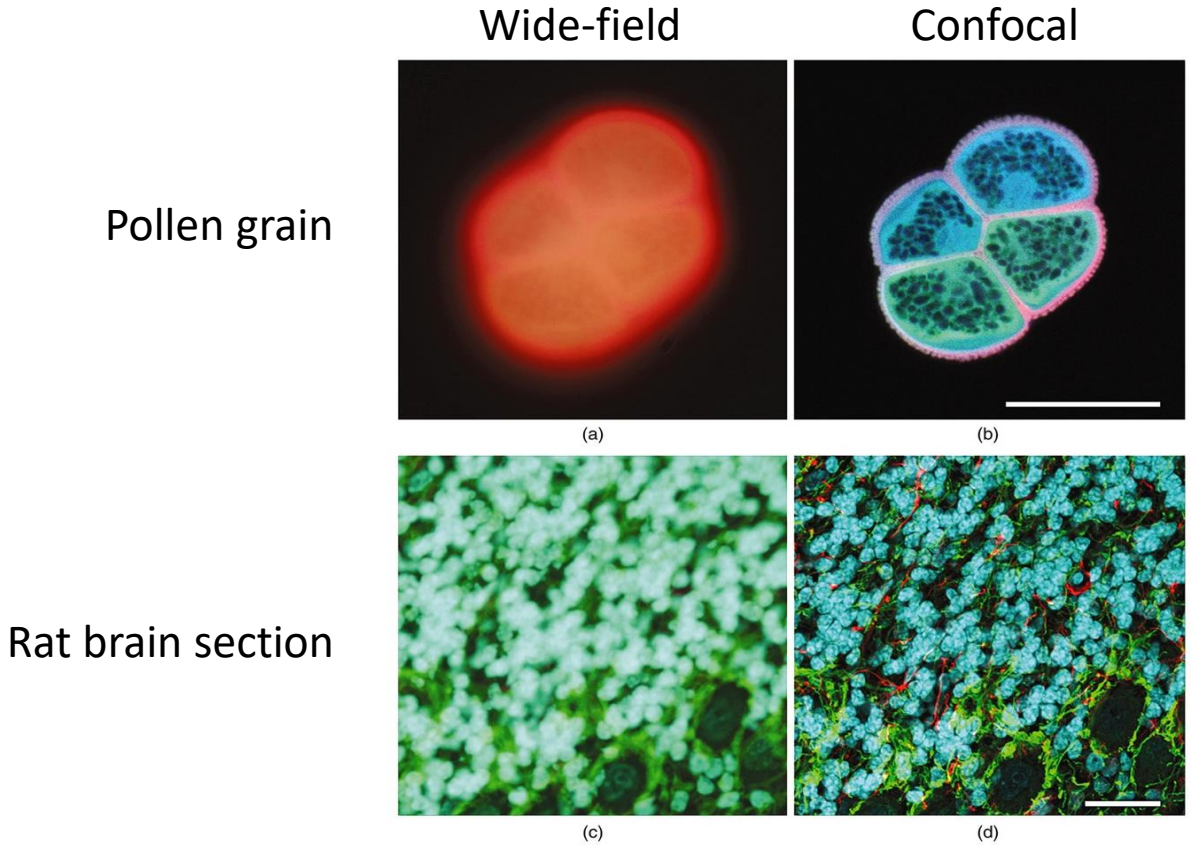
- Some of this fluorescence will be imaged onto the detector and will result in a **defocused-looking image.**

Confocal microscopy can solve this problem!





Elimination of out-of focus fluorescence yields superior images



- **(a,c) Wide-field fluorescence images** of autofluorescence in a pollen grain (**top row**) and a thick (30 μm) section of rat brain (**bottom row**) triple-labeled with Alexa Fluor 568 (glial fibrillary acidic protein; GFAP), Alexa Fluor 488 (neurofilaments), and DAPI (nuclei).
- **(b,d) Confocal optical sections** of the same samples shown in (a & c).

➔ Although wide-field images highlight the overall shape of the pollen grain and the outline of the nuclei in the brain section, the images are blurred by emission originating away from the focal plane.

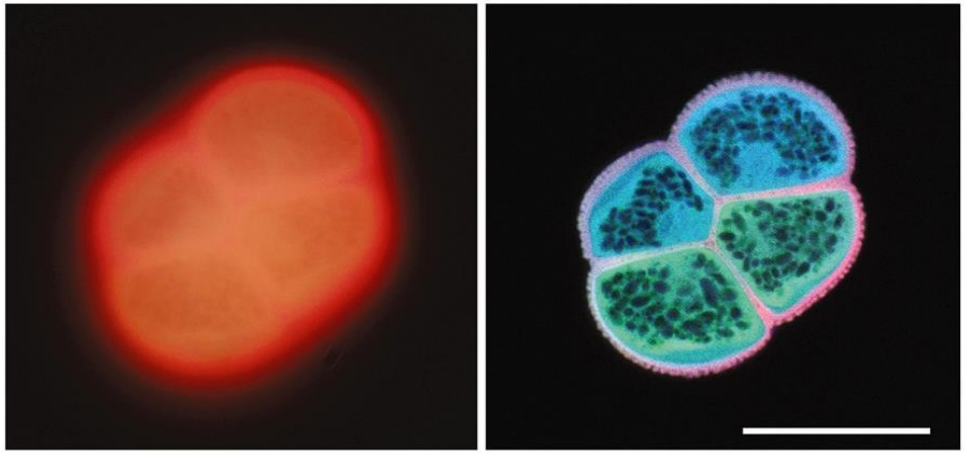
➔ **In contrast, confocal optical sections reveal fine structural details.**

Bars = 50 μm

Confocal Microscopy

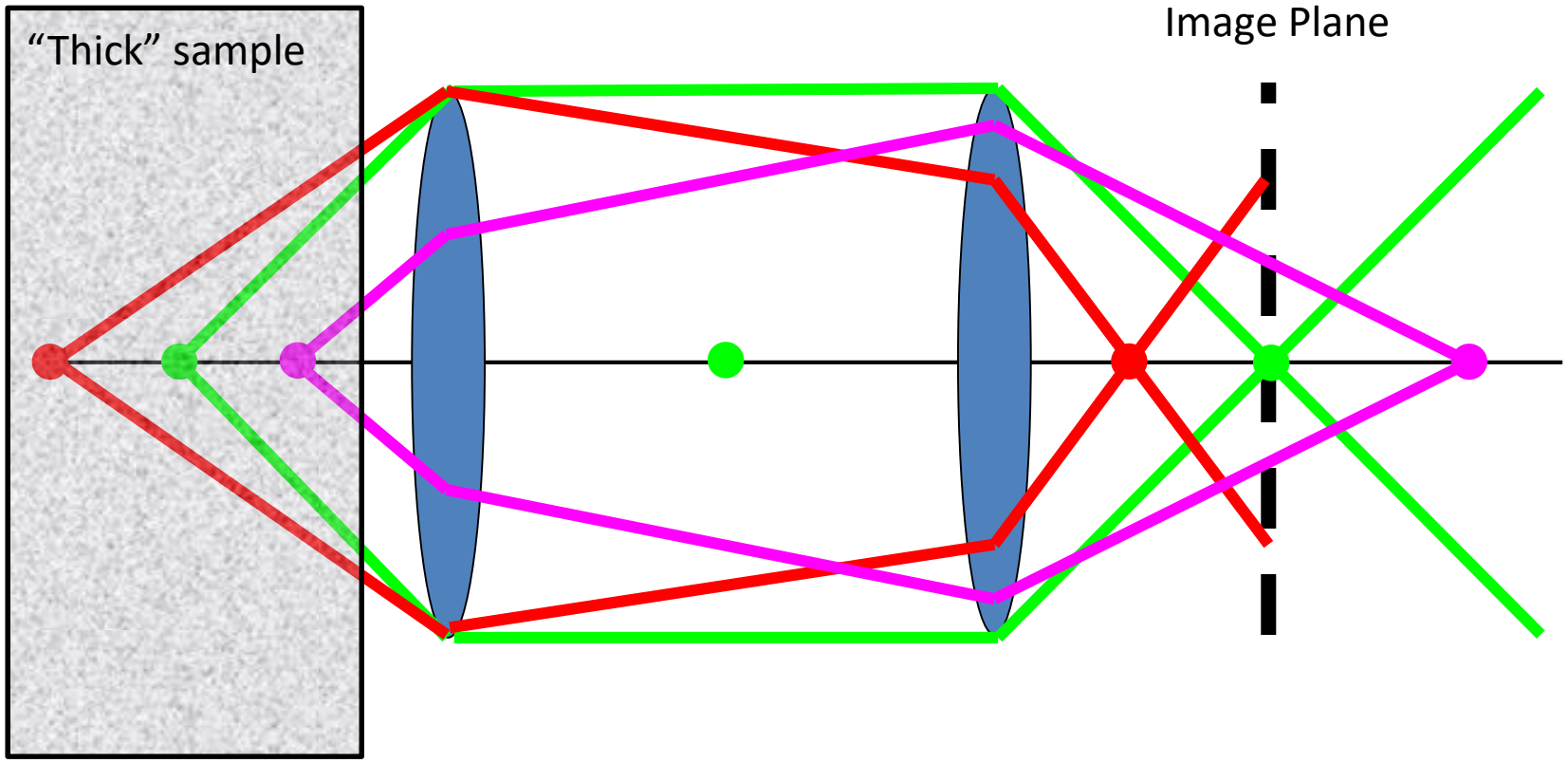
Confocal method can address some commonly asked questions about fluorescent microscope specimens, such as:

- Is fluorescent signal distributed on a membrane surface or contained throughout the cytoplasm as a soluble factor?
- What is the 3D structure of a specimen?

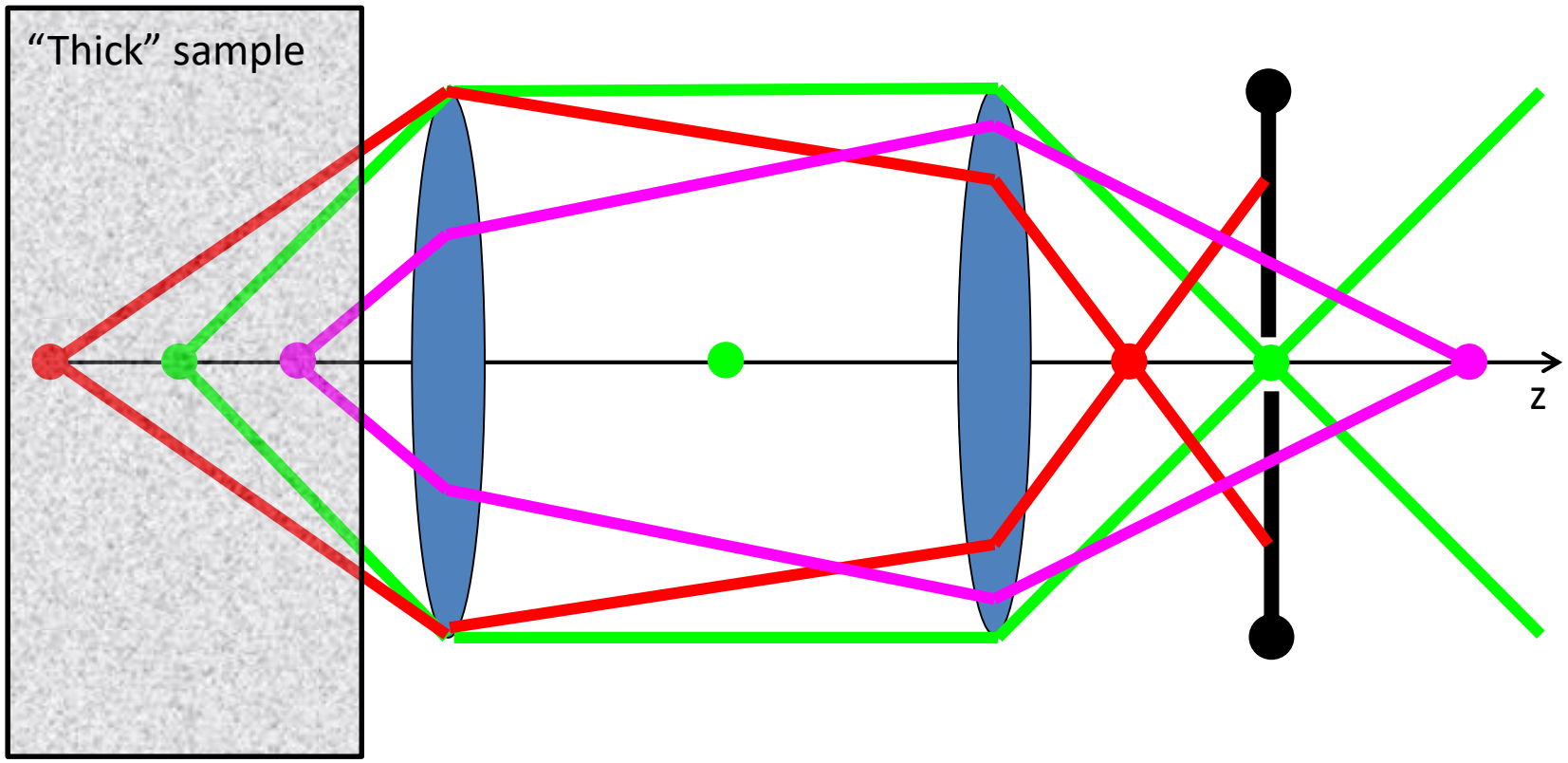


(a) (b)

Out-of-focus photons at the image plane



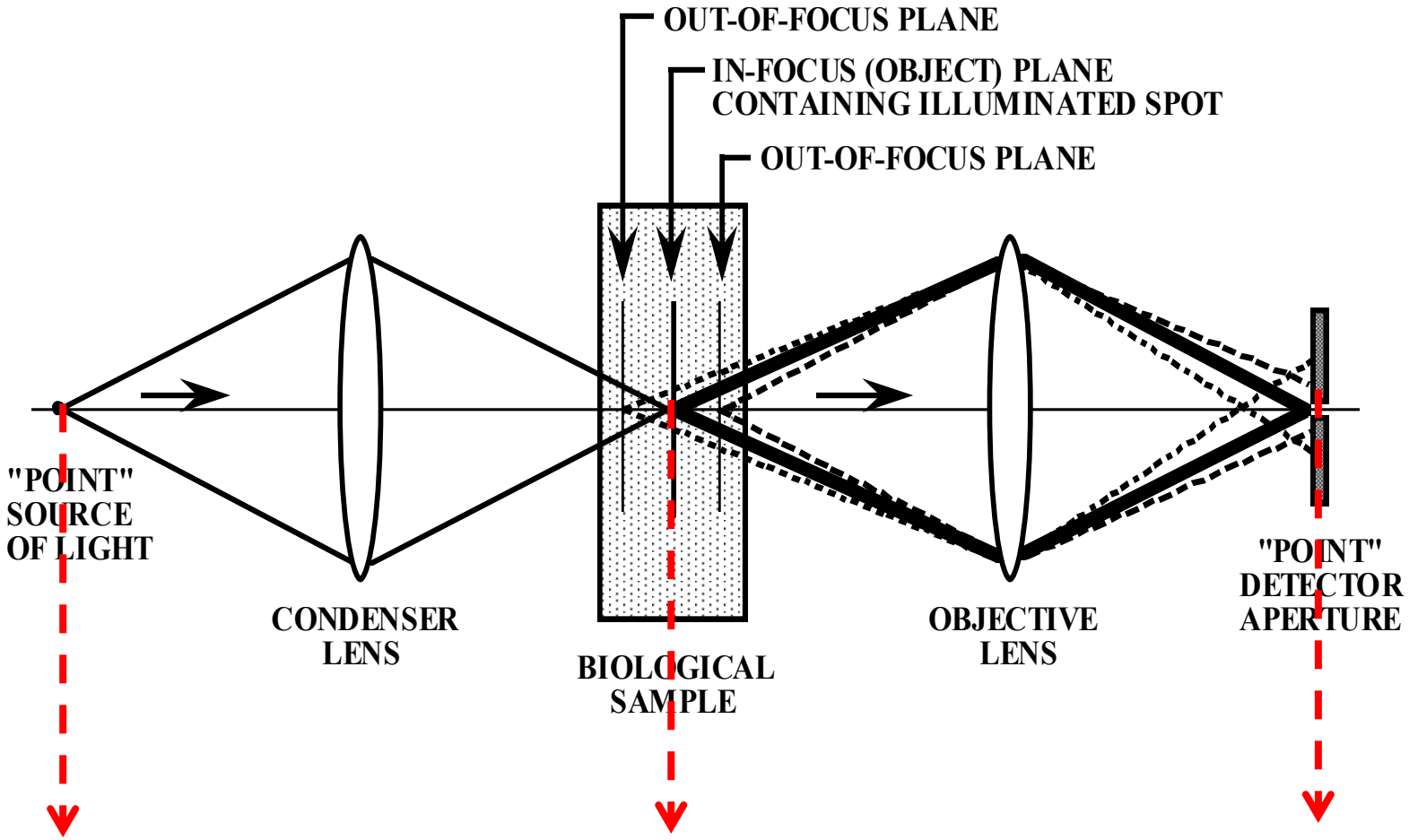
Pinhole: Axial (z-axis) filtering of out-of-focus photons



Principle of confocal microscopy

“Confocal” means that:

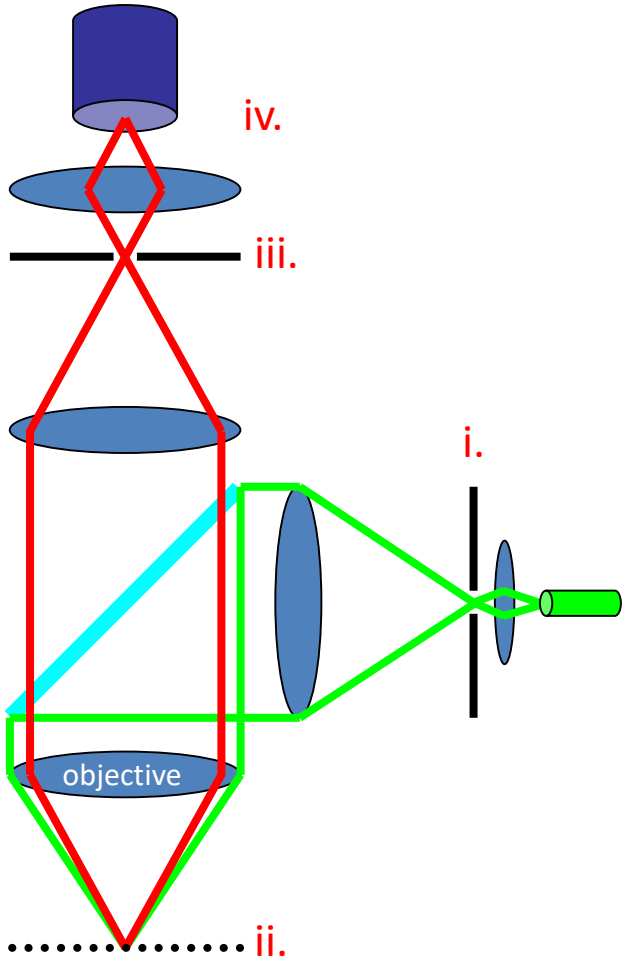
- Both illumination and detection focal planes coincide (i.e. they are conjugated).
- And, light from outside the focal region does not reach the detector.



How many conjugated focal planes are there in the following optical lay-out?

Principle of confocal microscopy

Epi-illumination configuration (objective lens also functions as the condenser lens):



Typically, two pinholes are used:

i) The 1st pinhole is placed in front of a coherent illumination source to allow transmission only through a small area (green rays)

ii) This illumination pinhole is imaged onto the focal plane of the specimen (green rays)

iii) Fluorescence excited at the focal plane is imaged onto a confocal pinhole placed right in front of the detector (red rays)

iv) Only fluorescence excited within the focal plane of the specimen will go through the detector pinhole (red rays)

Marvin Lee Minsky (1927–2016)

- He was an American cognitive scientist concerned largely with research of artificial intelligence (AI) & co-founder of MIT's AI laboratory.
- He developed and patented confocal imaging principle in **1957** while he was a post-doctoral fellow at Harvard University. His wanted to image connections among interneurons.

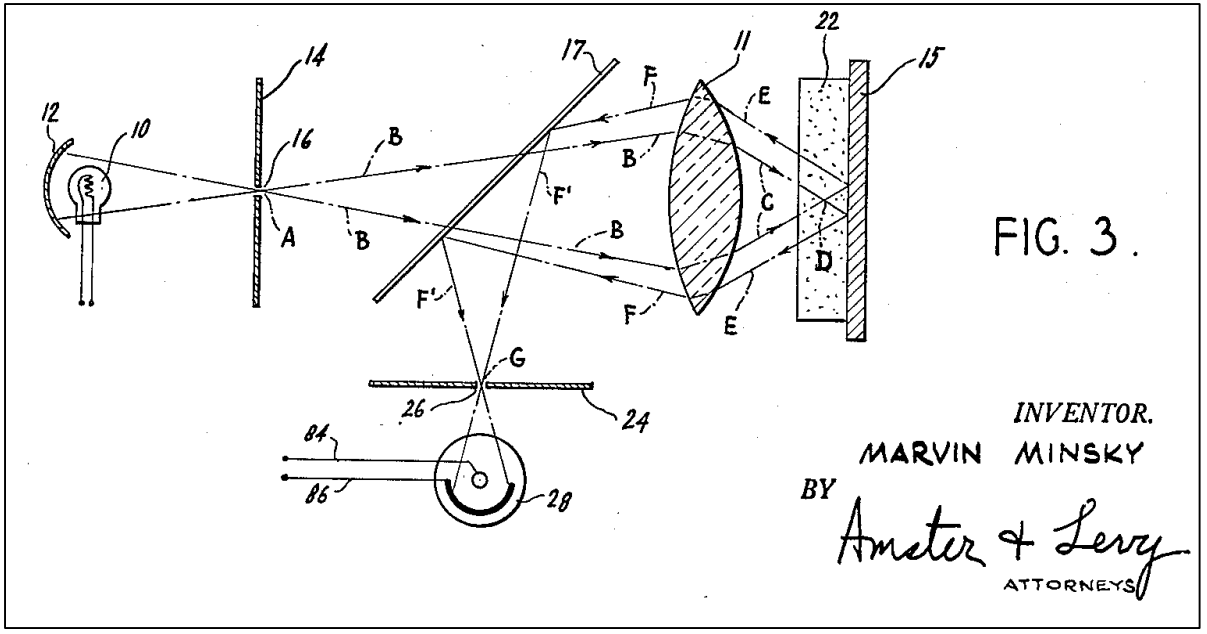
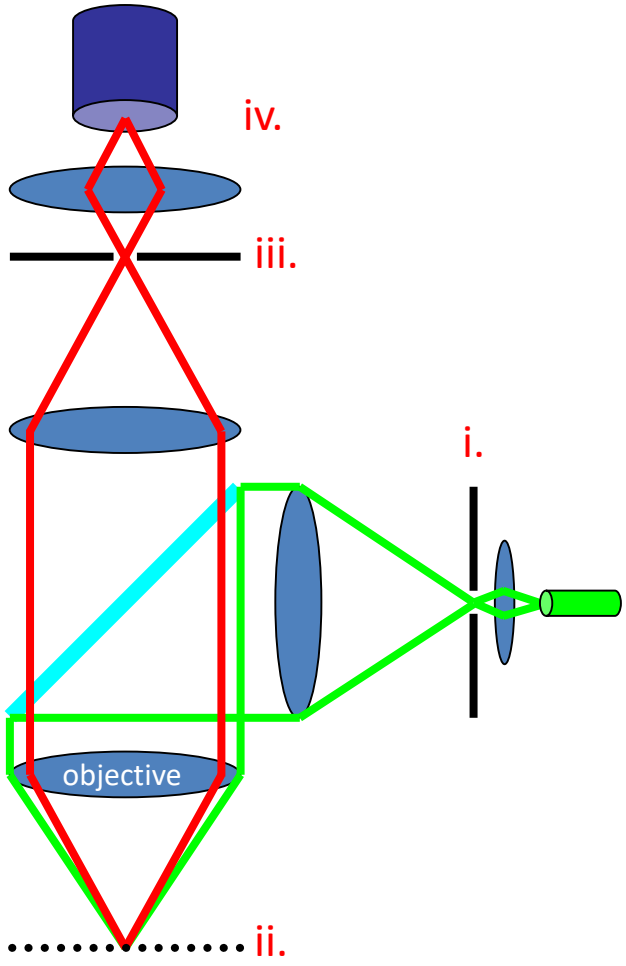


FIG. 3.

- The first commercial instruments appeared in **1987**.
- Since then, interest in confocal microscopy and improvements in the capacity of confocal imaging have increased at a rapid pace.

Principle of confocal microscopy

Epi-illumination configuration (objective lens = condenser lens):

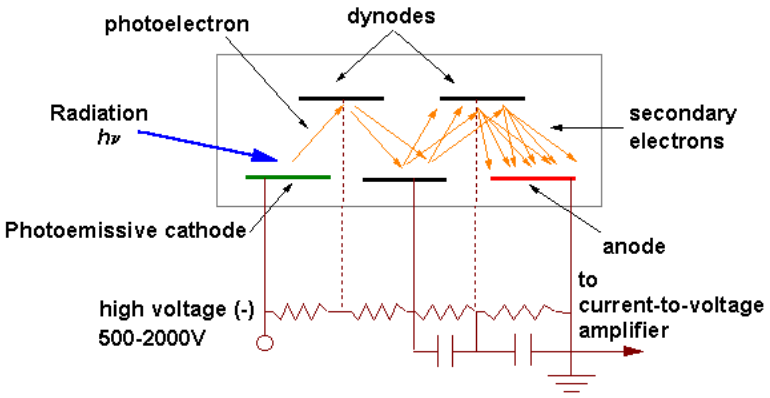


How does the image look like in a confocal microscopy?

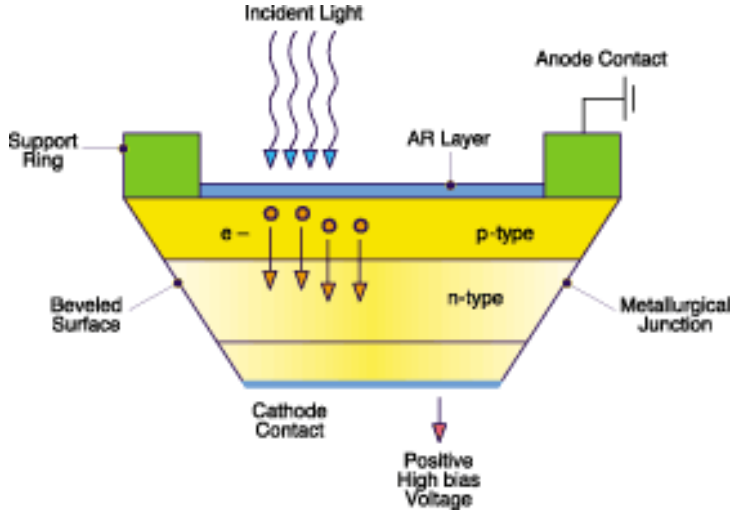
- Confocal arrangement generates a **“data” from only one point** in the specimen.
- The **confocal point needs to be scanned** to **generate an image** of an extended object.

Detectors for confocal microscopy

Photo-multiplier Tube (PMT)

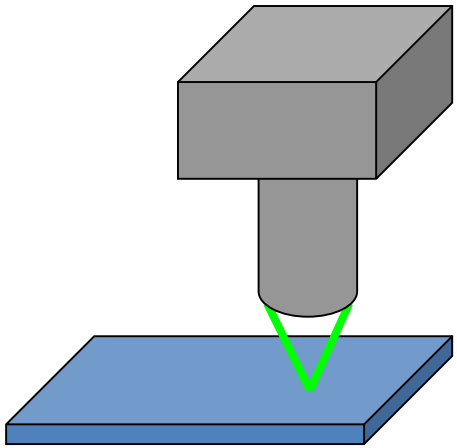


Avalanche PhotoDiode (APD)



Both can work under single-photon counting mode.

Option 1: Scan Specimen (i.e. Stage)



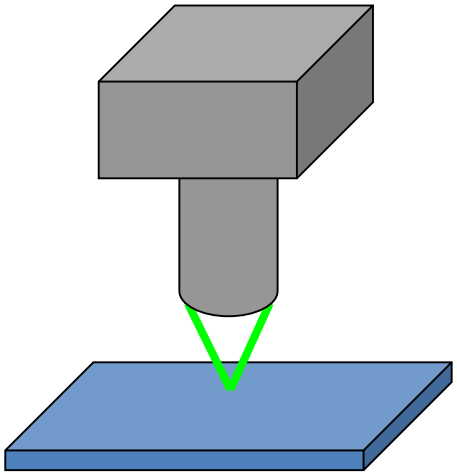
Good:

- Microscope works on axis
- Best correction for optical aberrations
- Most uniform light collection efficiency

Bad:

- Slow
- Requires sample movement - sloshes the specimen

Option 2: Scan Microscope Head



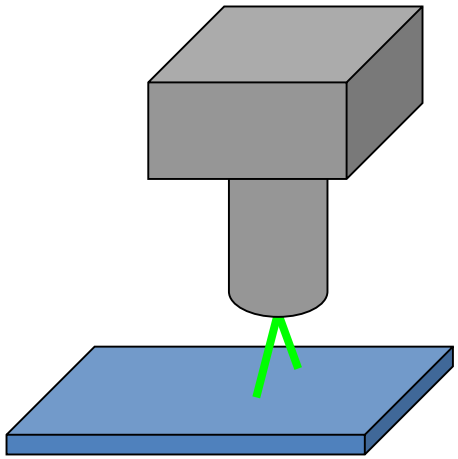
Good:

- Specimen doesn’t move

Bad:

- Optics is more complicated
- Slow

Option 3: Scan Laser



Good:

- Still require specimen movement but slowly (i.e less sloshing)
- Faster

Bad:

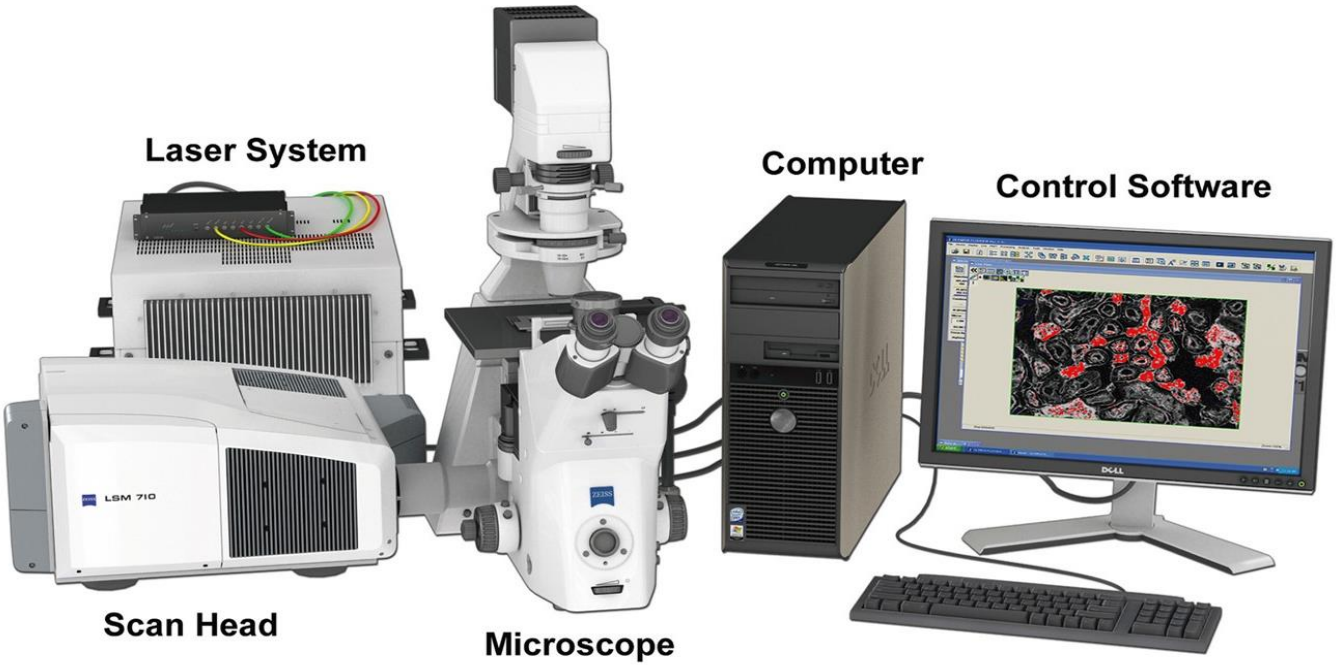
- High requirements on objective
- Optics is complicated
- Light collection may be non-uniform

For biological experiments, it is **more common** to **scan the excitation laser** across the focal plane.

Commonly used confocal terminology ….

- LSCM
  - Laser Scanning Confocal Microscopy
- CLSM
  - Confocal Laser Scanning Microscopy
- CSLM
  - Confocal Scanning Laser Microscopy
- LSM
  - Laser Scanning Microscopy

Basic components of a laser scanning confocal microscopy

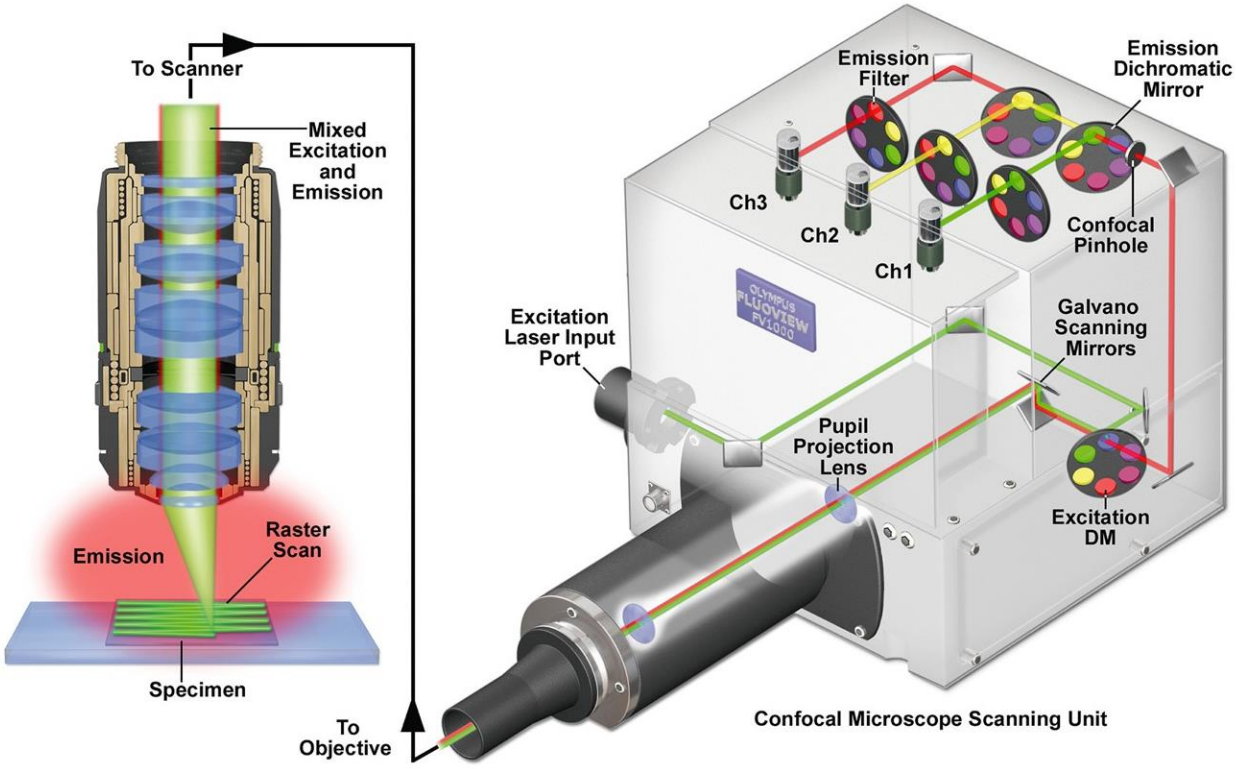


Input: **Laser system** provides a beam of light that is scanned across the specimen by the **scan head** under the control of a computer.

Output: Scan head directs fluorescence signals from the specimen to its pinhole and **detector** (i.e. a photomultiplier tube - PMT).

Image processing: The computer holds the image in a buffer until it is processed and displayed on a computer monitor.

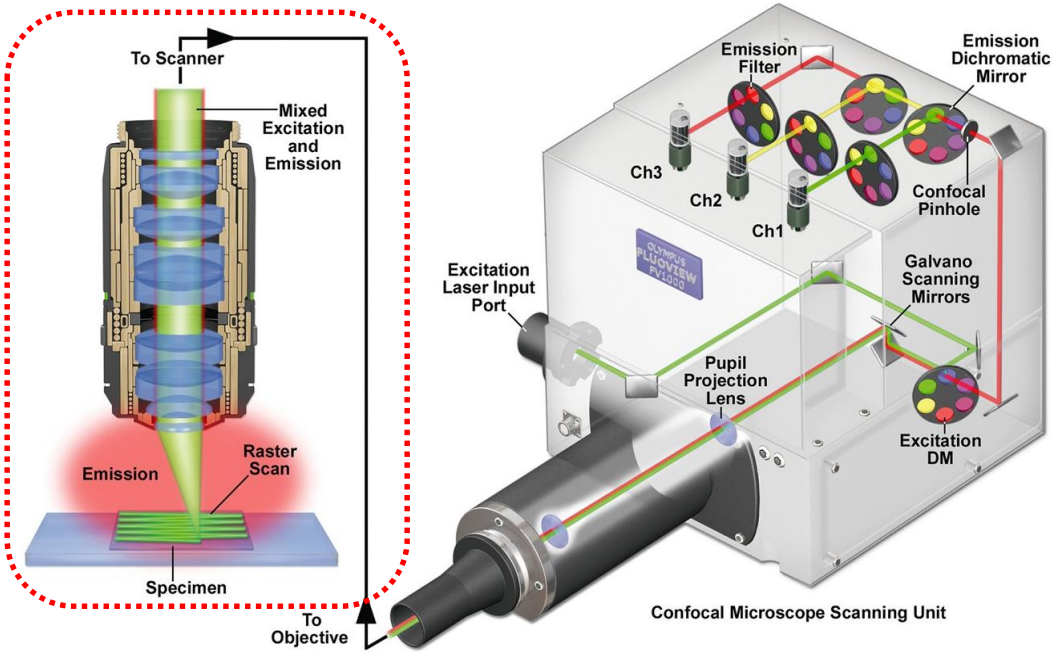
Confocal Microscopy with Scanning Mirrors



<u>Above scheme is epi-illumination:</u> Light source and detector are both on the same side of the specimen plane and separated from it by the **objective, which functions in dual duty as both a condenser and objective**.

- The components of fluorescence **filter sets** (excitation DM, emission dichroic mirror, & emission filter) perform the function of filter cube as in wide-field fluorescence microscopy.

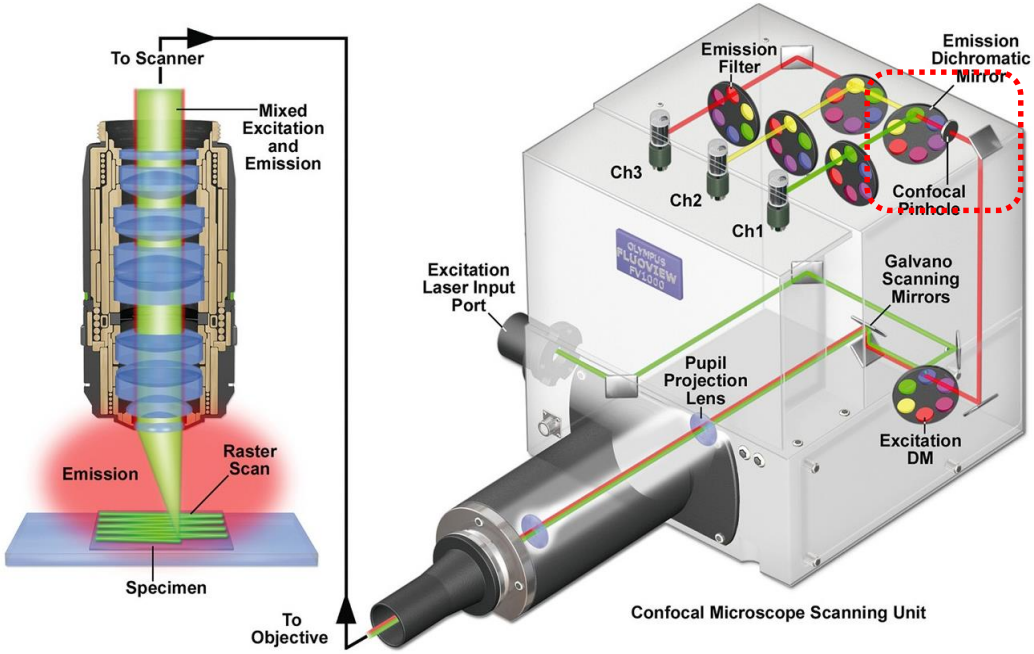
CSLM –1

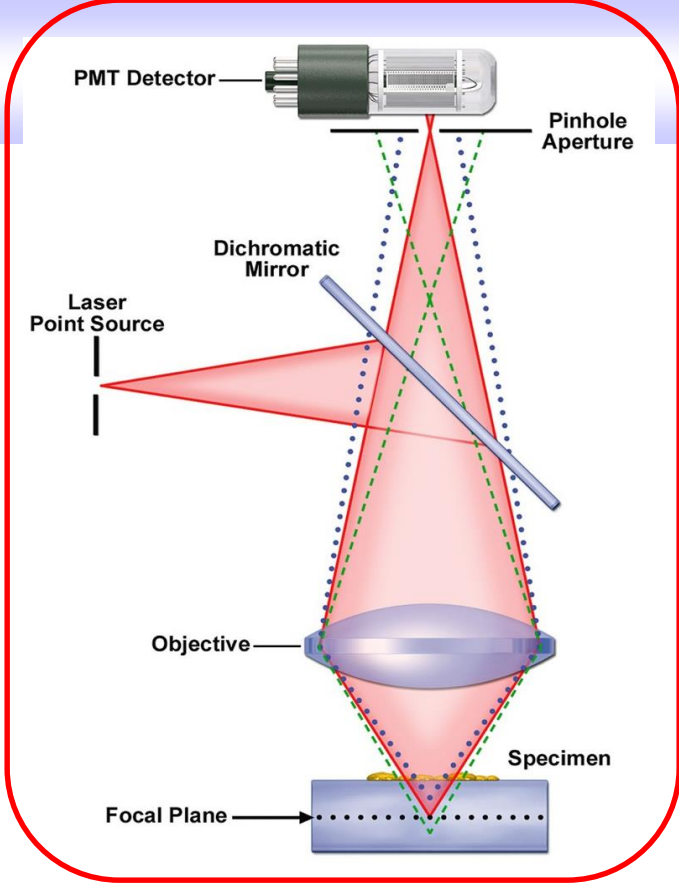


- The laser beam is expanded to fill the rear aperture of the objective and forms an intense diffraction-limited spot.
- The excitation spot is scanned from side-to-side and from top-to-bottom over the specimen in a pattern of ***raster scanning***.

➔ This procedure is called ***point scanning***.

CSLM –2

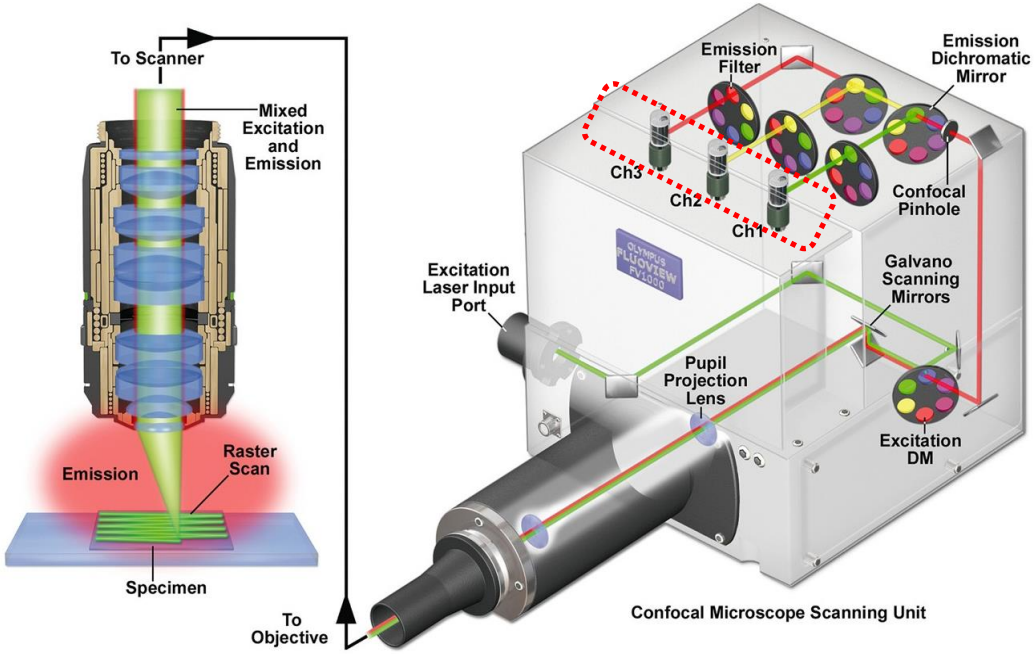




- The heart of confocal optics is **the *pinhole aperture***:
  - Receives fluorescent photons from the illuminated focused spot in the raster
  - Largely excludes fluorescence signals from objects above and below the focal plane, which, being out of focus, arrive at the pinhole as diffuse extended disks.
  - Because the size of the disk of an out-of-focus object is spread out over such a large area, only a small fraction of light from out-of-focus objects passes through the pinhole.
  - The pinhole also eliminates much of the stray light in the optical system.

➔ The pinhole is optically confocal with (and conjugate to) the specimen plane. The pin-hole acts as a ***spatial filter*** at the conjugate image plane.

CSLM – 3

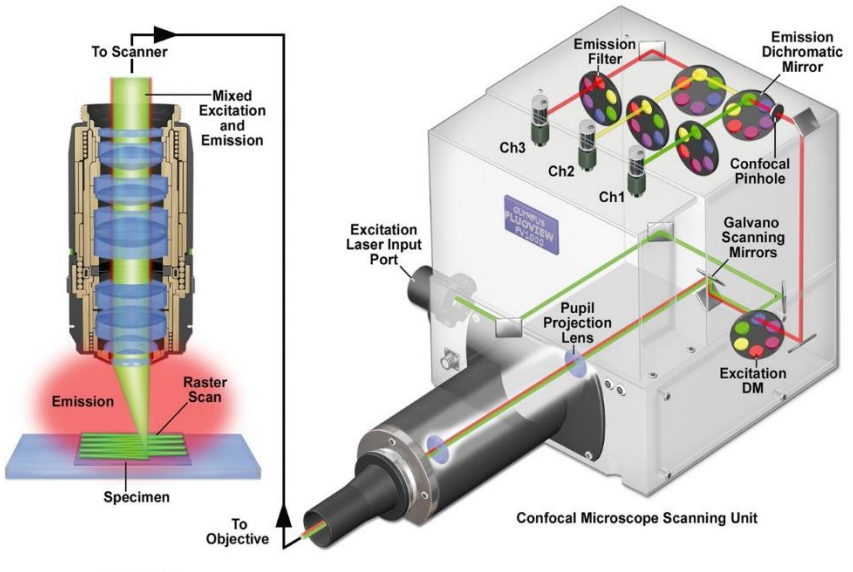


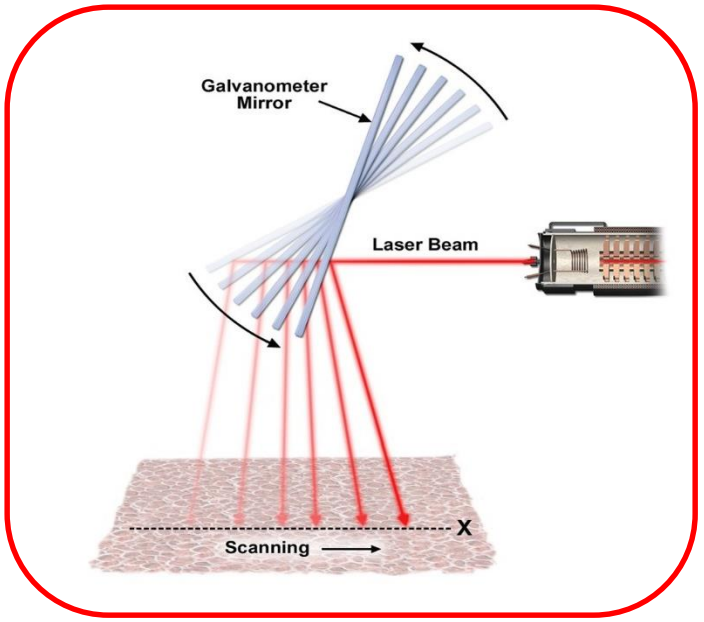
- The signal can be detected by multiple PMTs corresponding to different colors/channels, $ch_{1,2..}$.

➔ PMT does not see an image, but produces a voltage that corresponds to the intensity of incident fluorescent photons.

➔ Computer digitizes the signal and displays it on the monitor.

CSLM – 4



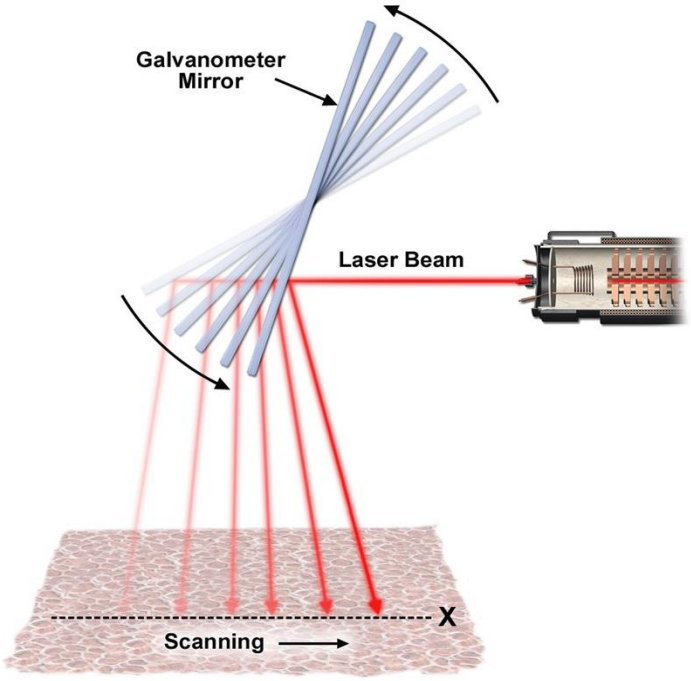


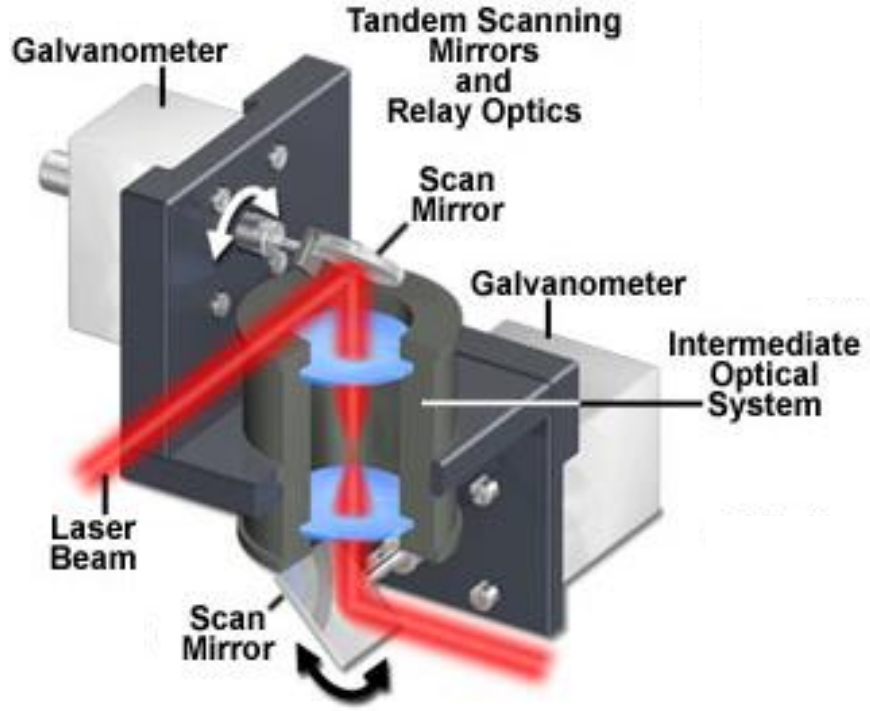
- To generate an image of an extended specimen, the **laser beam is scanned across the object by a raster pattern** using **two high-speed vibrating mirrors driven by galvanometer motors**.

- Two mirrors oscillate in mutually perpendicular directions.
  - Because the speed of the galvanometer mirrors is negligible relative to the speed of light, fluorescent light follows the same light path on its return and is brought to the same position on the optical axis as the original excitation laser beam.
  - This process is called ***descanning***.

- The fluorescent light then passes through the excitation dichromatic mirror and becomes focused at the confocal pinhole.
  - Because *descanning* is instantaneous, the image in the pinhole remains steady and does not move back and forth like the beam in the plane of the specimen.
  - However, the focused spot varies in intensity over time as the spot excites different locations in the specimen.

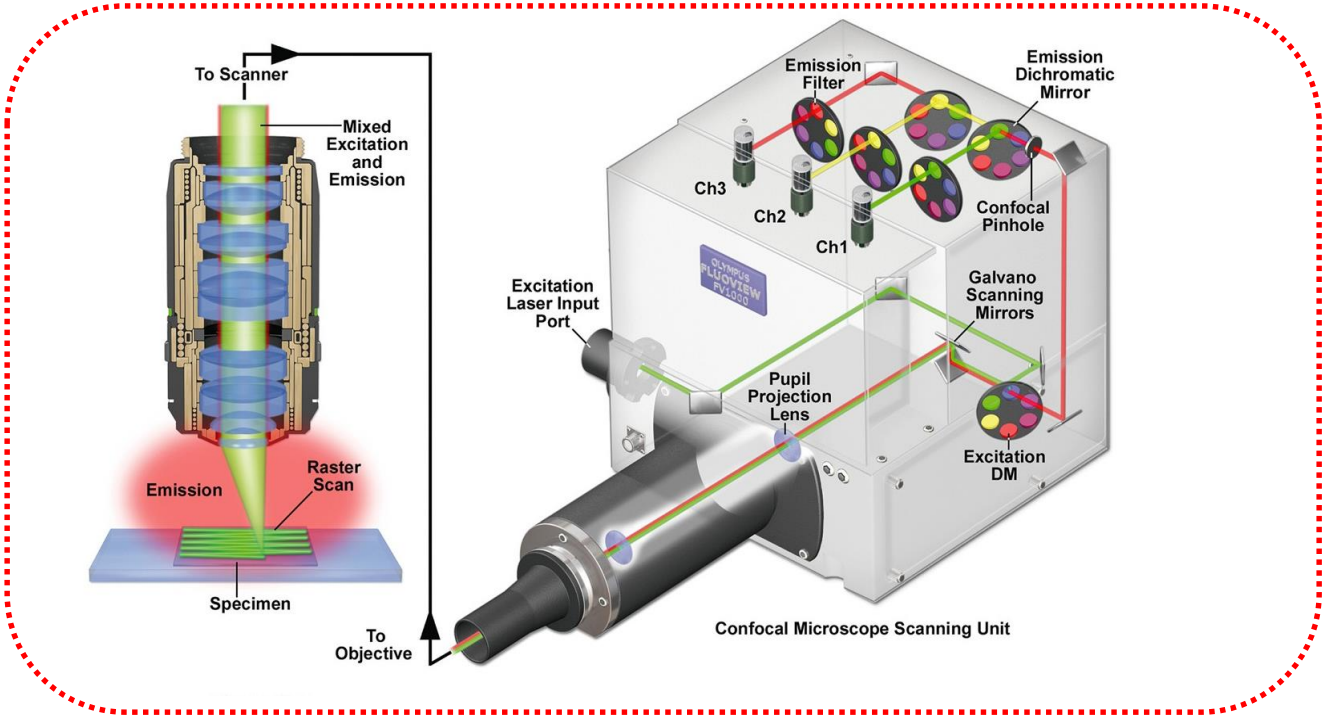
CSLM – 5





- Mirrors are on the magnets & their scanning is much faster than the stage scanning (1’000 x)
- Mirrors vibrate in mutually perpendicular axis within the confocal scan head.
  - **One mirrors controls scanning along the x-axis while the other controls along the y-axis**.
  - **Both mirrors are controlled to generate a pattern of a raster on the specimen**.
- The speed & angular extend of the deflection of the mirrors are controlled to regulate the scanning rate & the extend of the scan.

CSLM – 6



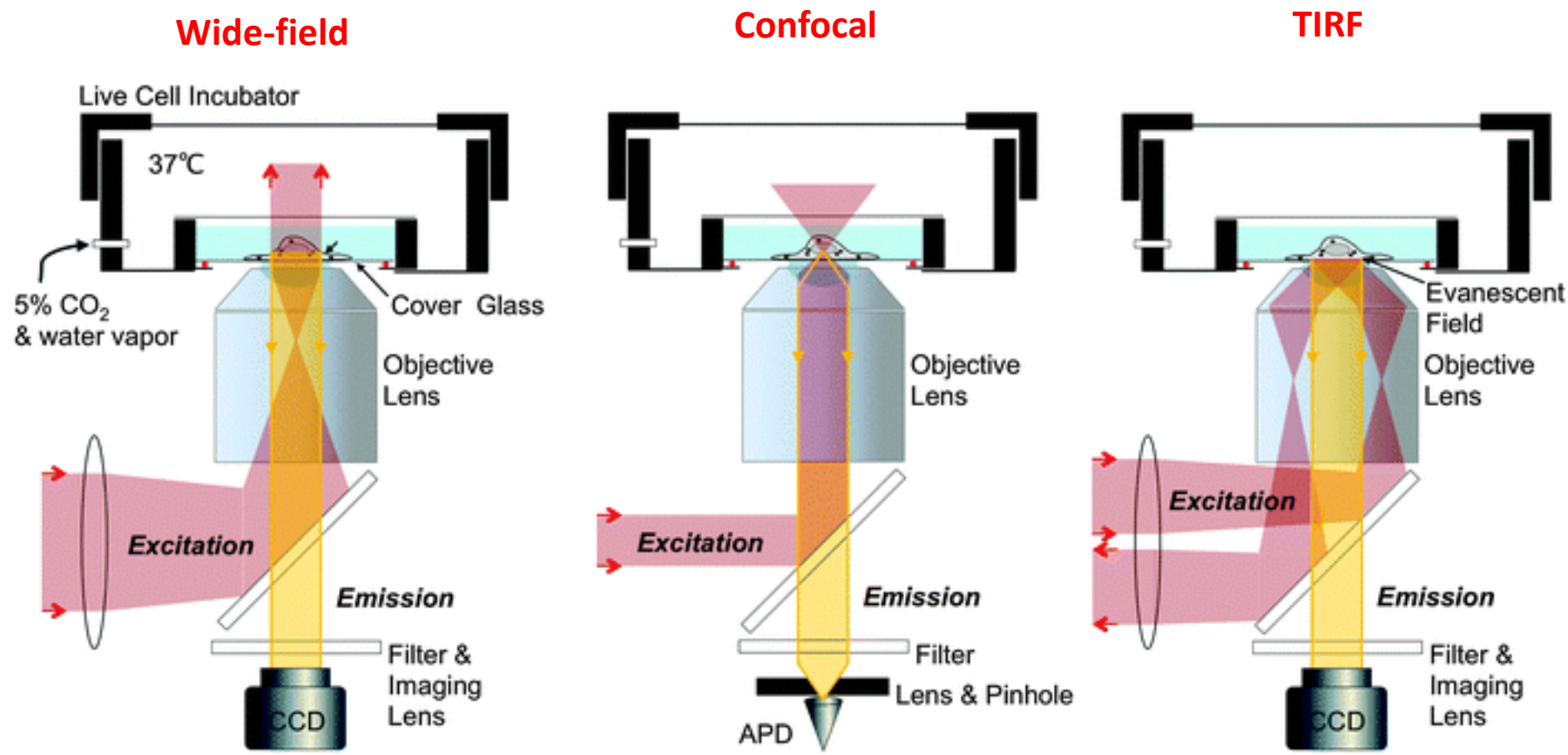
- Fluctuations in light intensity are converted into a continuously changing voltage (an analog signal) by the PMT detector.

  - The analog signal is digitized at regular time intervals by an analog-to-digital converter to generate pixels (digital picture elements) that are stored in an image frame buffer board and are displayed on a computer monitor.

➔ Thus, **a confocal image of an object is reconstructed from photon signals that are built up over time and displayed on the computer monitor**.

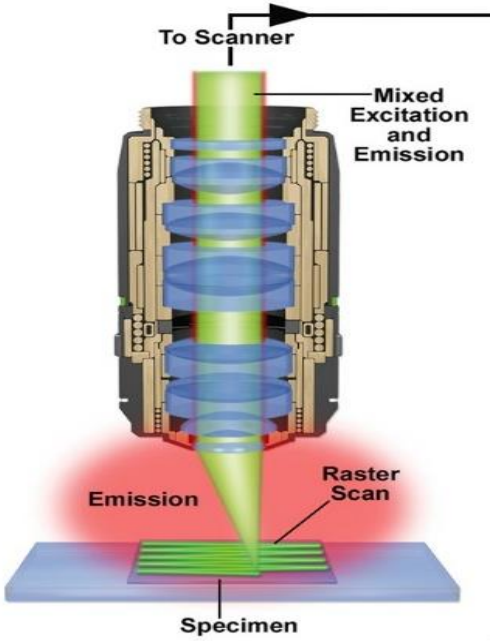
➔ The confocal image does not exist as a real image that can be seen by the eye in the microscope.

Wide-field vs confocal vs TIRF on an inverted microscope

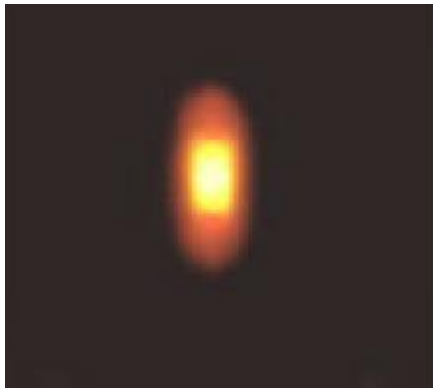


Advantages of confocal over wide-field

- The main advantage of confocal imaging is the ability to ***optically section*** through fluorescent objects up to 10- to 50-μm thick.



PSF



- Based upon a fine optical depth and the ability to generate successive registered focal planes by accurate movement of the focus position between subsequent images, one can build what is called a "**z-series**" (**z-stack**).

Sectioning in z-direction with confocal microscopy

➔ With image stacks representing different focal planes spaced at regular intervals along the optical axis (such that *z*-axis), the specimen can be displayed in a variety of ways.

➔ For example:

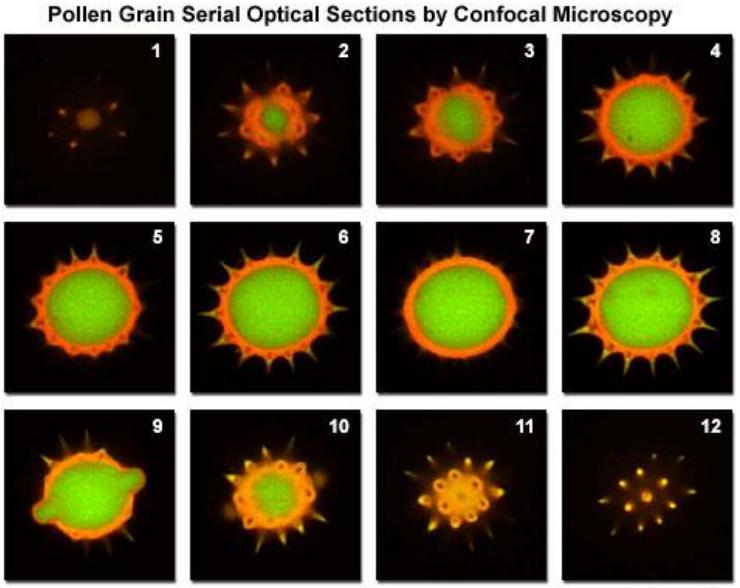
- ***3D views*** of an object can be obtained using a *z*-stack of confocal images and computer software.
  - Most confocal software programs can display an object in 3D at different angular perspectives with rotation around the *x*-, *y*- , or *z*-axis, or a combination of axes.
  - This mode of viewing is valuable for examining complex 3D objects, such as details in thick tissue sections.

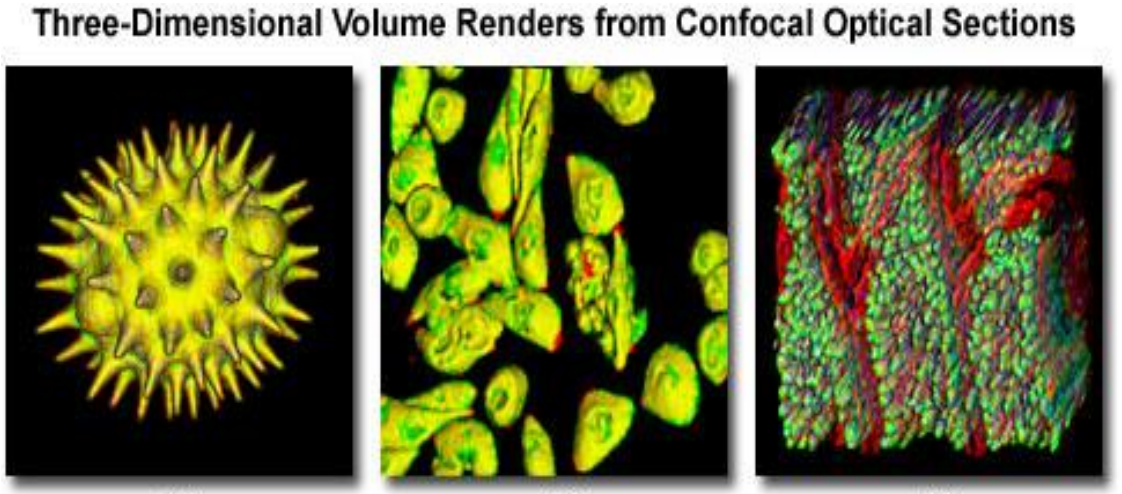
- ***x–z* or *y–z cross-sectional views*** can be generated by most confocal software programs.
  - The object appears as if it had been cut transversely in a plane oriented parallel to the optical axis.

- ***5D views***, including information in *x*, *y*, and *z* dimensions, in a **timed** sequence, and in multiple **colors**.
  - Such sequences can be displayed as a 3D, multicolor movie in real time or time-lapse mode.
  - 5D viewing will become more convenient as computer processing speed and storage capacity continue to improve.

Example: Sectioning in z-direction with confocal microscopy

A thick specimen can be ***optically scanned*** in three dimensions and the images can be processed to yield cross-sections along plane of interest, three dimensional composites and animations.





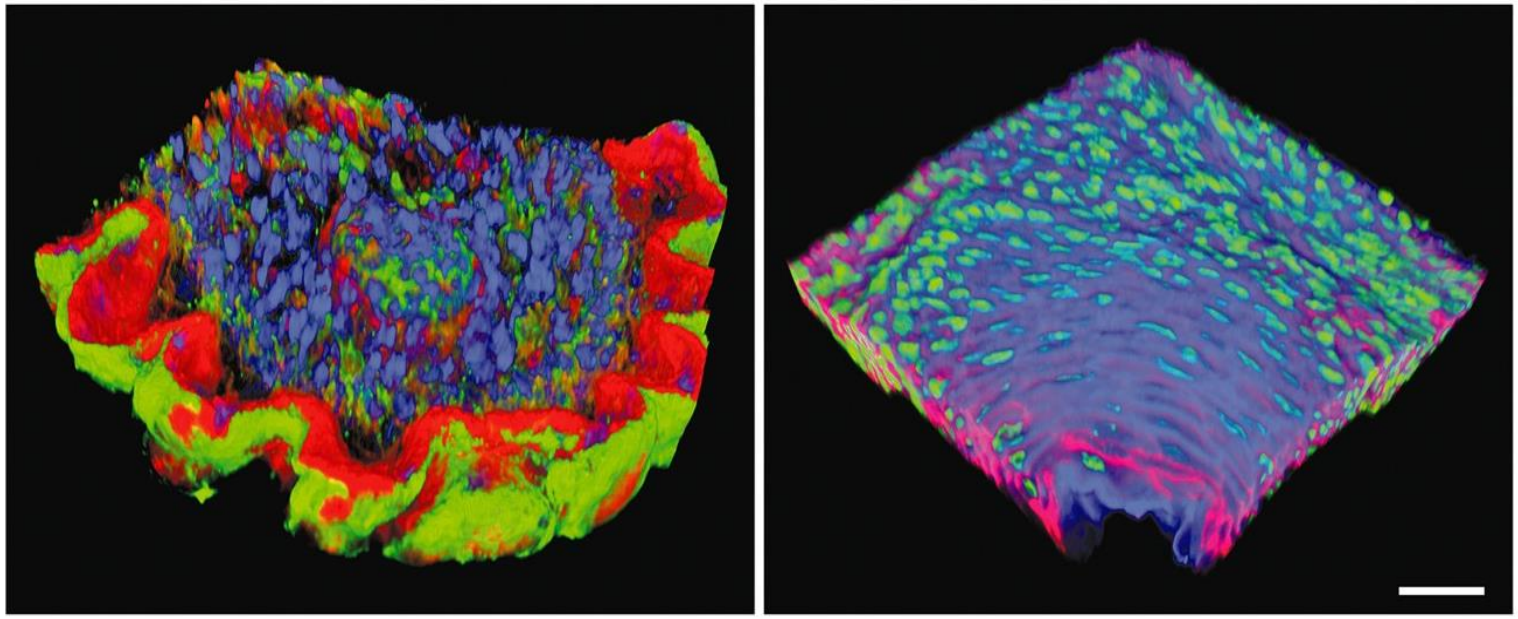
Pollen grain

Hamster ovary cells

Mouse intestine

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

Example: 3D view with confocal imaging



(a) (b)

3D views of triple-stained rodent embryo tissue:

(a) Mouse embryo (17 day) section (30 *μ*m) stained with Alexa Fluor 488 conjugated to wheat germ agglutinin (labels glycoproteins), Alexa Fluor 568 conjugated to phalloidin (filamentous actin), and DAPI (nucleus).

b) Rat embryo (19 day) section (30 *μ*m) immunofluorescently stained with Alexa Fluor 350 (glycoproteins), Alexa Fluor 568 (actin), and SYTOX Green (nuclei).

Scale bar = 20*μ*m.

Quality of confocal image depends on factors such as:

- ***Spatial resolution:***
  - Describes the smallest resolvable distance between two points in an image, including in the image plane and along z-axis.
  - It depends on multiple parameters including wavelength, NA of the objective and settings of the confocal scanning head (i.e. pinhole size).

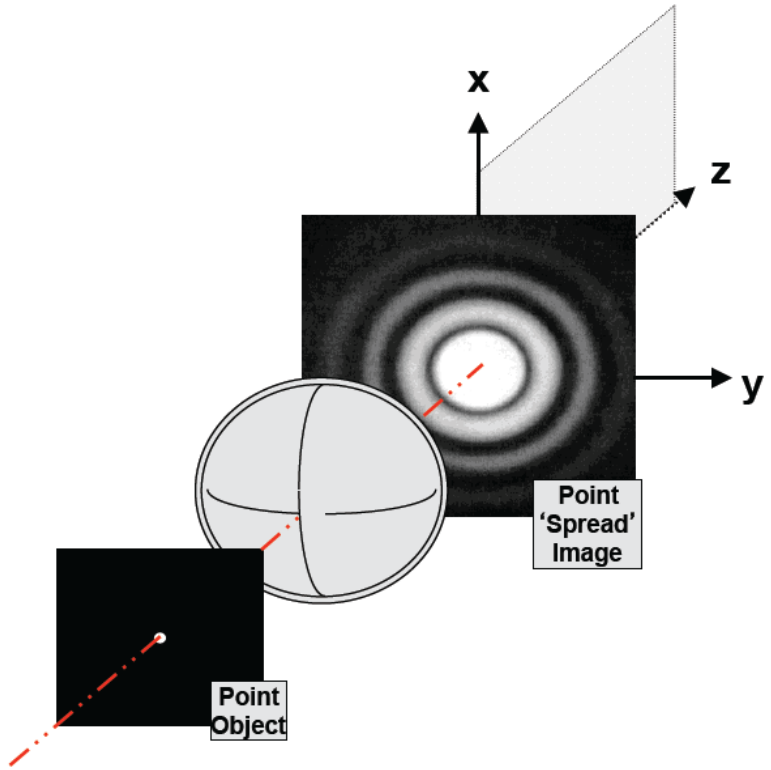
- ***Temporal resolution:***
  - Depends on multiple components such as raster scan rate and the processing of the detector, analog-to-digital converter and computer.
  - Frames are typically captured at a rate of 1–2 per second (fps) for a 512×512 pixel image, but faster rates (~100 fps) can be acquired for images of limited size.

- ***Dynamic range:***
  - Describes the resolution of light intensity in the image and it is defined as the number of gray levels that are assigned to an image by the A-D converter.

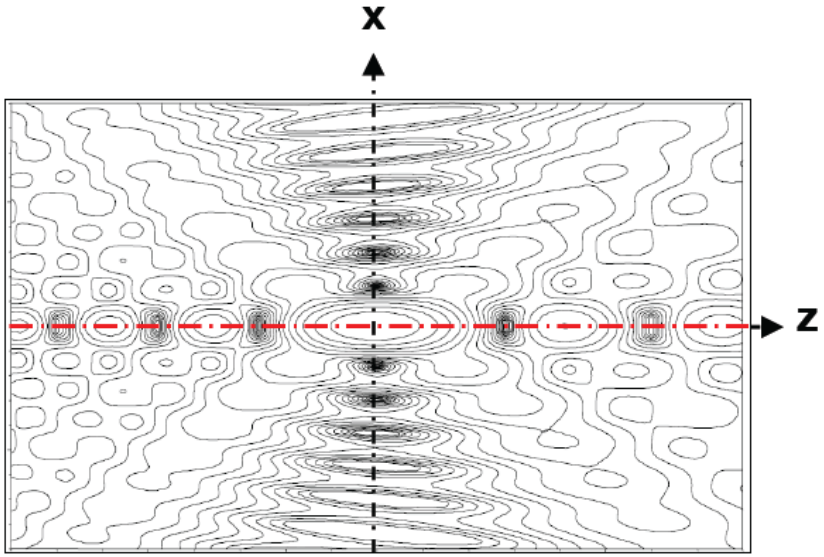
- ***Signal-to-background ratio***:
  - Defines the degree of visibility (clarity) of an image.
  - Depends on the signal amplitude of the object and its background, and on the electronic noise of the imaging system.

Resolution in confocal microscopy

Lateral resolution:
Point spread function in xy-plane



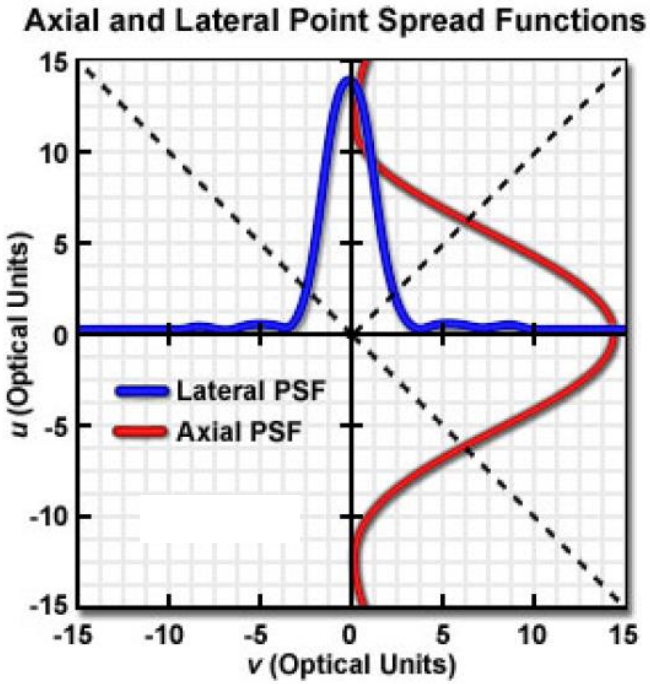
Axial resolution:
Point spread function in xz-plane (or yz plane)

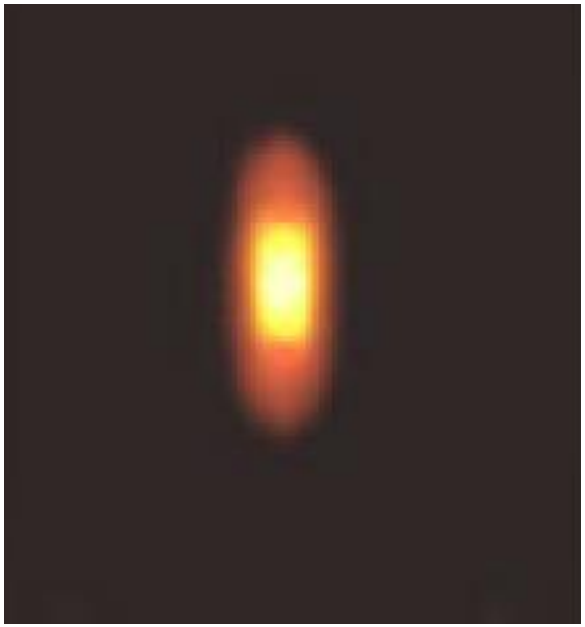


Comparison of axial PSF and lateral PSF

- According to reference R. Webb in Pawley (2006), resolution is estimated as:

$$d_{x,y} \approx 0.4\,\lambda/NA \;\; (lateral\ PSF)$$
$$d_z \approx 1.4\,\lambda n/NA^2 \;\; (axial\ PSF)$$



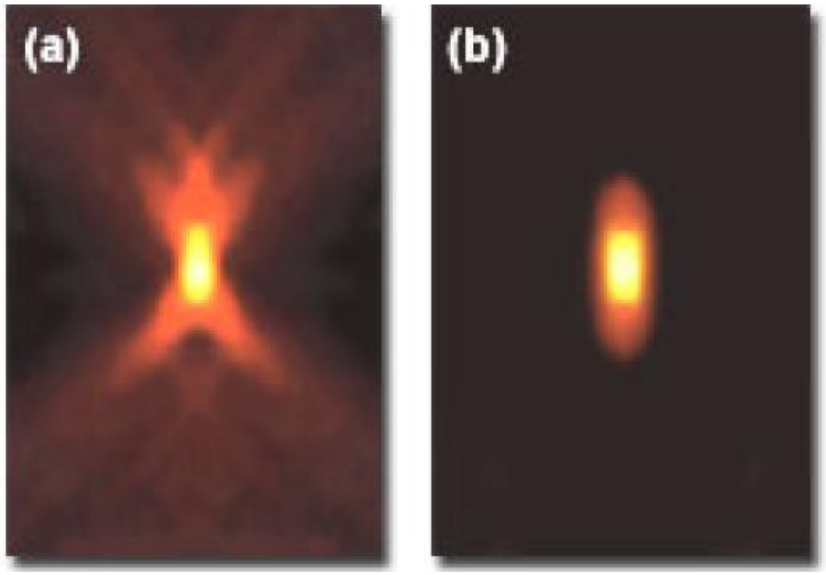


Axial PSF is ~2-3 time larger than lateral PSF.

Comparison of axial-PSF for wide-field vs confocal

Axial PSF intensity profiles for:

Wide-field **Confocal**



➔ Wide-field has limited axial resolution.

Confocal aperture - resolution

- Resolution depends on the NA of the objective & wavelength.
- It also depends on the size of the pinhole aperture, scan-rate, ...

•Decreasing the pinhole size rejects more out of focus light, therefore improves contrast and resolution.

•Decreasing the pinhole will increase x-y (lateral) resolution (better than wide-field)

•Decreasing pinhole size decreases the amount of the Airy disk that reaches to the detector. This results in less light from each point being collected.

- **Generally, collecting the diameter of 1 Airy disk is considered optimal.**
- **This setting collects about 85% of light.**

Extreme limits:

- **Pinhole is completely open: ~wide-field resolution**
- **Pinhole is closed: no image**

Why is the pinhole's diameter variable?

- For general confocal imaging, the recommendation is to just transmit the inner part of the diffraction limited spot by the pinhole. This inner part is called the "**Airy disc**".
- The size of Airy disc depends on the wavelength, numerical aperture NA, magnification of the objective lens and magnification of the internal optics of the microscope.
- Consequently, the required pinhole diameter is different for various colors and for different objective lenses which have different NA and/or magnification.
  ➔Using a pinhole with variable diameter gives flexibility.

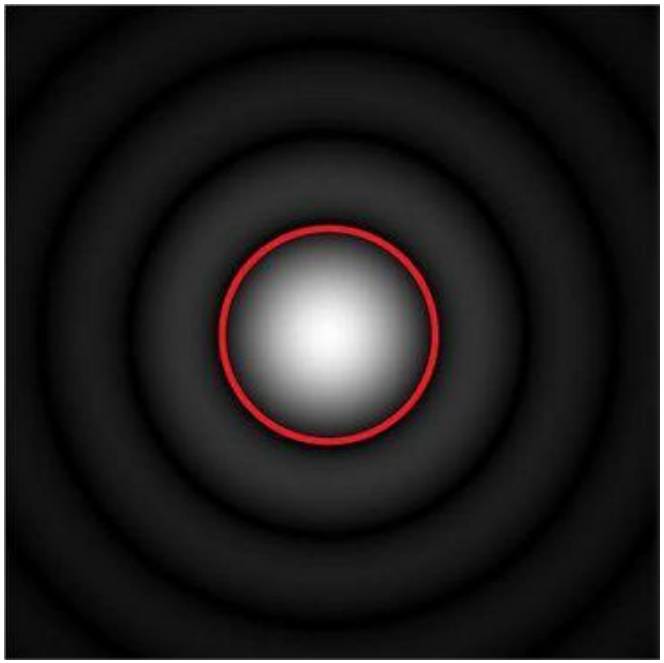


Figure: The red circle indicates a circular pinhole adjusted to 1 AU (**AU = "Airy Unit").**
This pinhole will cut out the inner disk of the diffraction pattern and generate an optical sectioning.

Image quality trade-offs

- Optical performance in the confocal microscope is affected by several variables, all of which must be evaluated and controlled by the microscopy user.
- It almost never happens that time, space, and intensity are all resolved optimally for a given combination of specimen and microscope.
- Generally, it is necessary to make compromises.

For example:

Parameter	Effect
↑ Pinhole diameter	↑ Intensity ↓ Spatial resolution
↑ Objective NA	↑ Intensity ↑ Spatial resolution
↑ Scan rate	↓ Intensity ↑ Temporal resolution

➔ Recognizing how to balance these parameters requires knowledge and experience and it is the key to successful confocal imaging.