# MICRO-562

## Biomicroscopy II

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TA:
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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** | |

# Optical Microscopy

- **Wide-field microscopy methods:**
  - **Bright-field**
  - **Dark-field**
  - **Phase Contrast**
  - **Differential Interference Contrast (DIC or aka Nomarski)**
  - **Polarization**
  - **Fluorescence**

- **Large varieties of fluorescence microscopy methods exist:**

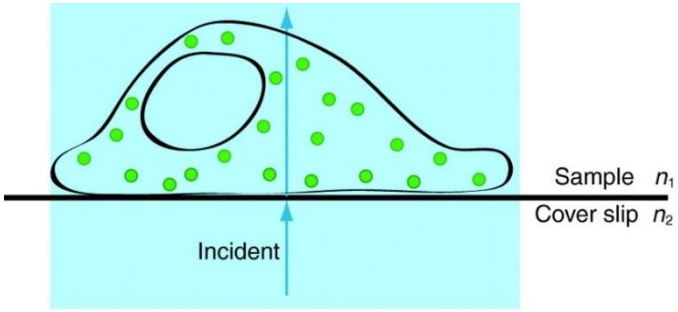
  Conventional fluorescence microscope *(i.e. epi-fluorescence)*
  FRET: Förster resonance energy transfer
  TIRF: Total Internal Reflection Fluorescence Microscopy
  FRAP: Fluorescence Recovery After Bleaching Microscopy
  Confocal Microscopy
  Super-resolution microscopy: STED, STORM, PALM…
  **Multiphoton (i.e. two-photon) or Nonlinear Microscopy**
  4-Pi Microscopy
  SIM: Structured illumination microscopy ……..

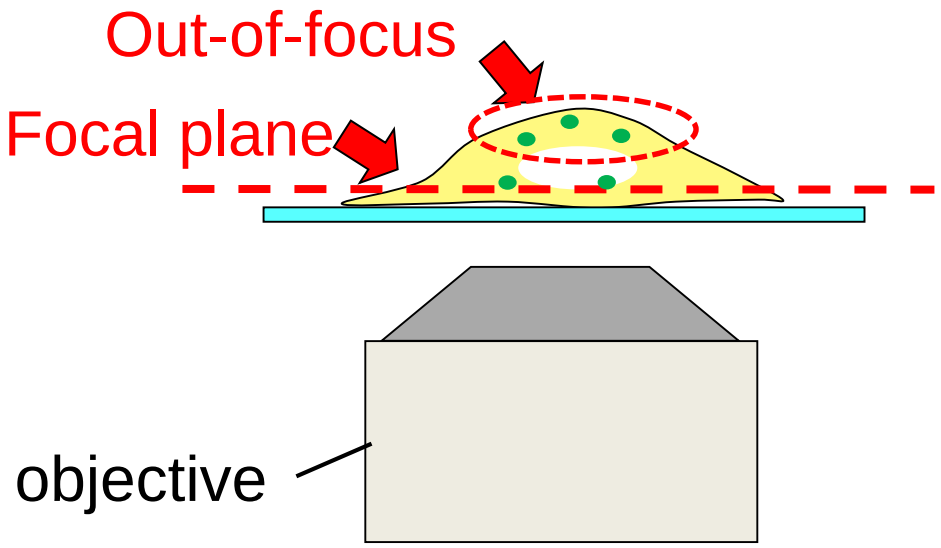
  ….. *and many more*

# Reminder: Limitations of conventional fluorescence microscopy

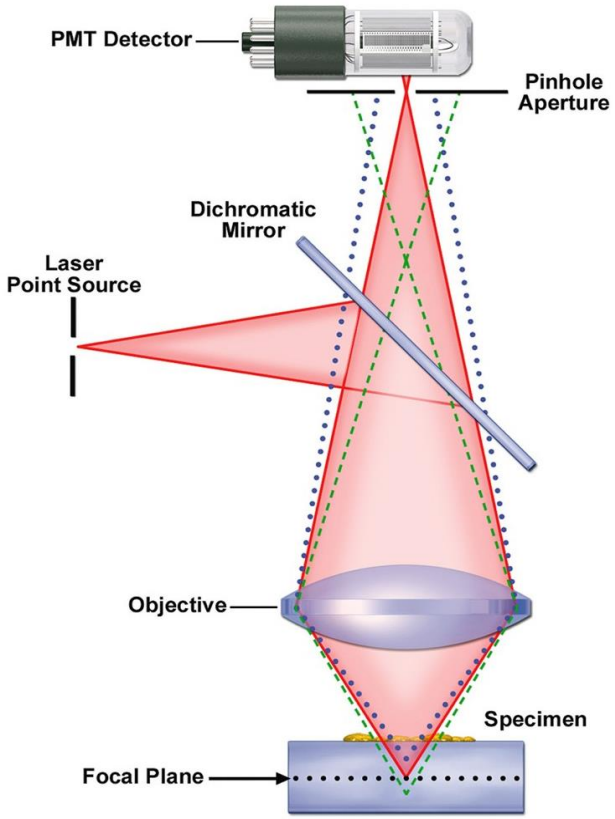
- In a standard fluorescence microscope, the excitation beam illuminates a wide field of the sample.
- If the sample is thick (i.e. rounded cells and tissue sections), fluorescence will be excited within the focal plane, but also within the planes above and below the focus.
- Some of this fluorescence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

**Confocal microscopy can solve this problem!**



# Reminder: Confocal Microscopy



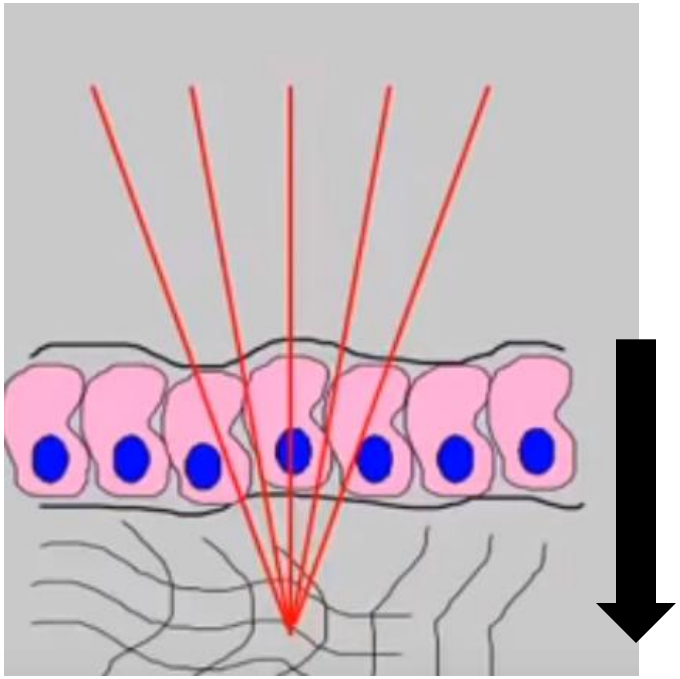

**Use a pinhole to block out-of-focus light**

The principal gain in confocal imaging is the ability to ***optically section*** through fluorescent **objects up to 10- to 50-$\mu$m thick**.

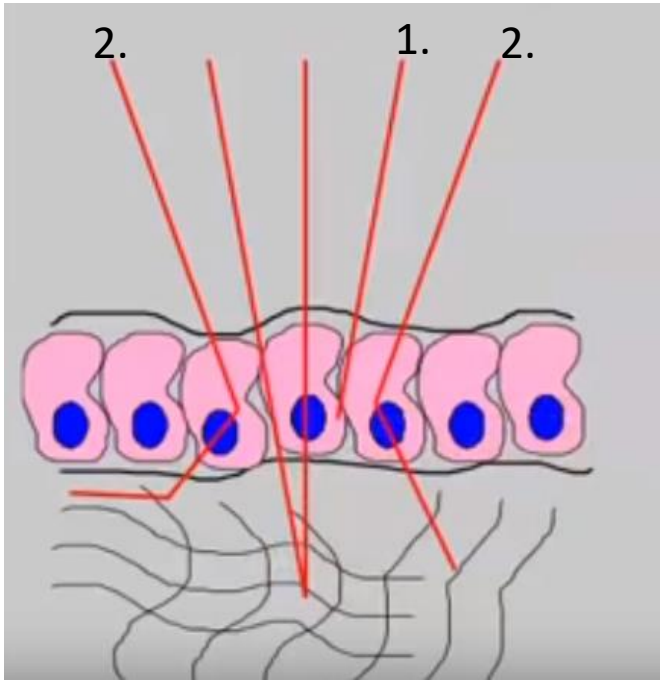
**Question: What does limit the imaging depth in confocal microscopy?**

# Imaging thick biological samples

**Ideal case,**
One should have arbitrarily deep tissue penetration of light.

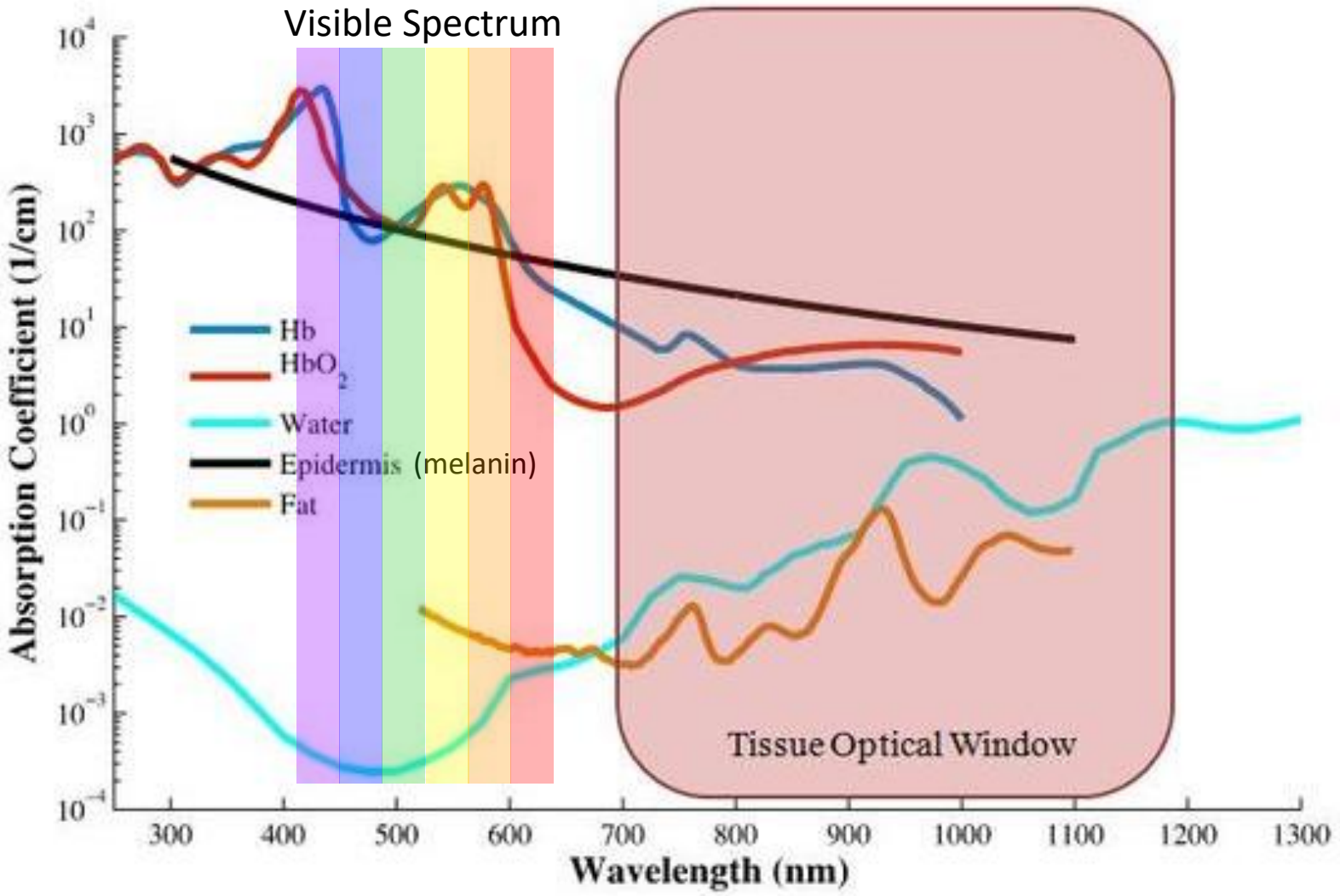


**In reality,**
Tissue penetration depth is limited by:
1. Absorption of light
2. Scattering of light



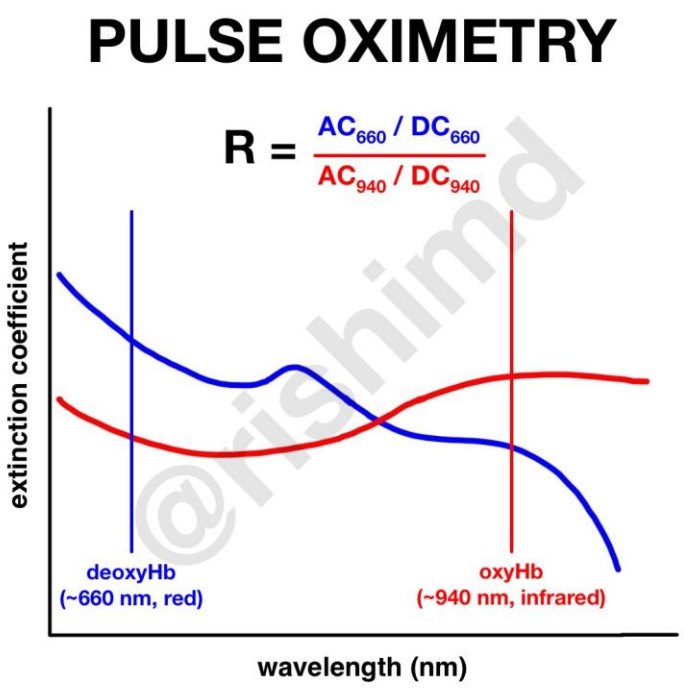

*Consider excitation beam (shown with five rays)*

# Absorption in Tissue




➔ **Visible spectrum** is not good to image tissues due to high absorption loss.

# Absorption in Tissue



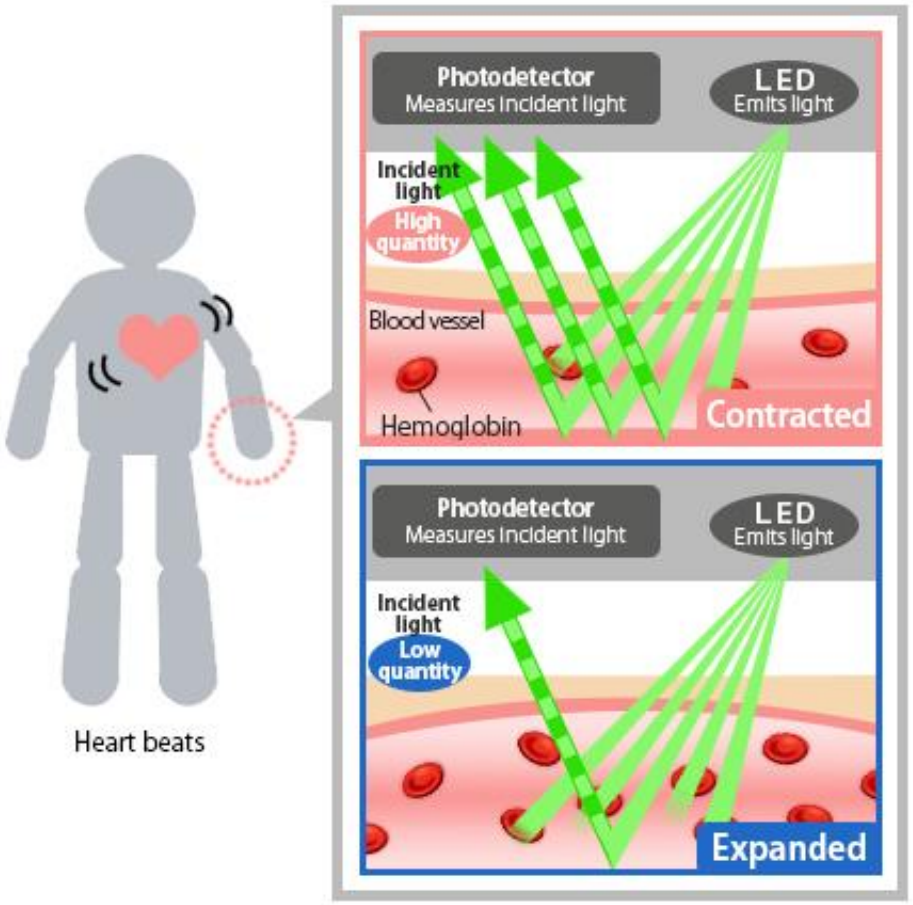


Extinction coefficient describes the degree to which oxyhemoglobin (oxyHb) and deoxyhemoglobin (deoxyHb) absorb light at a given wavelength.
At 660 nm (red), the deoxyHb to oxyHb ratio is maximized, whereas at 940 nm (infrared), the converse is true.

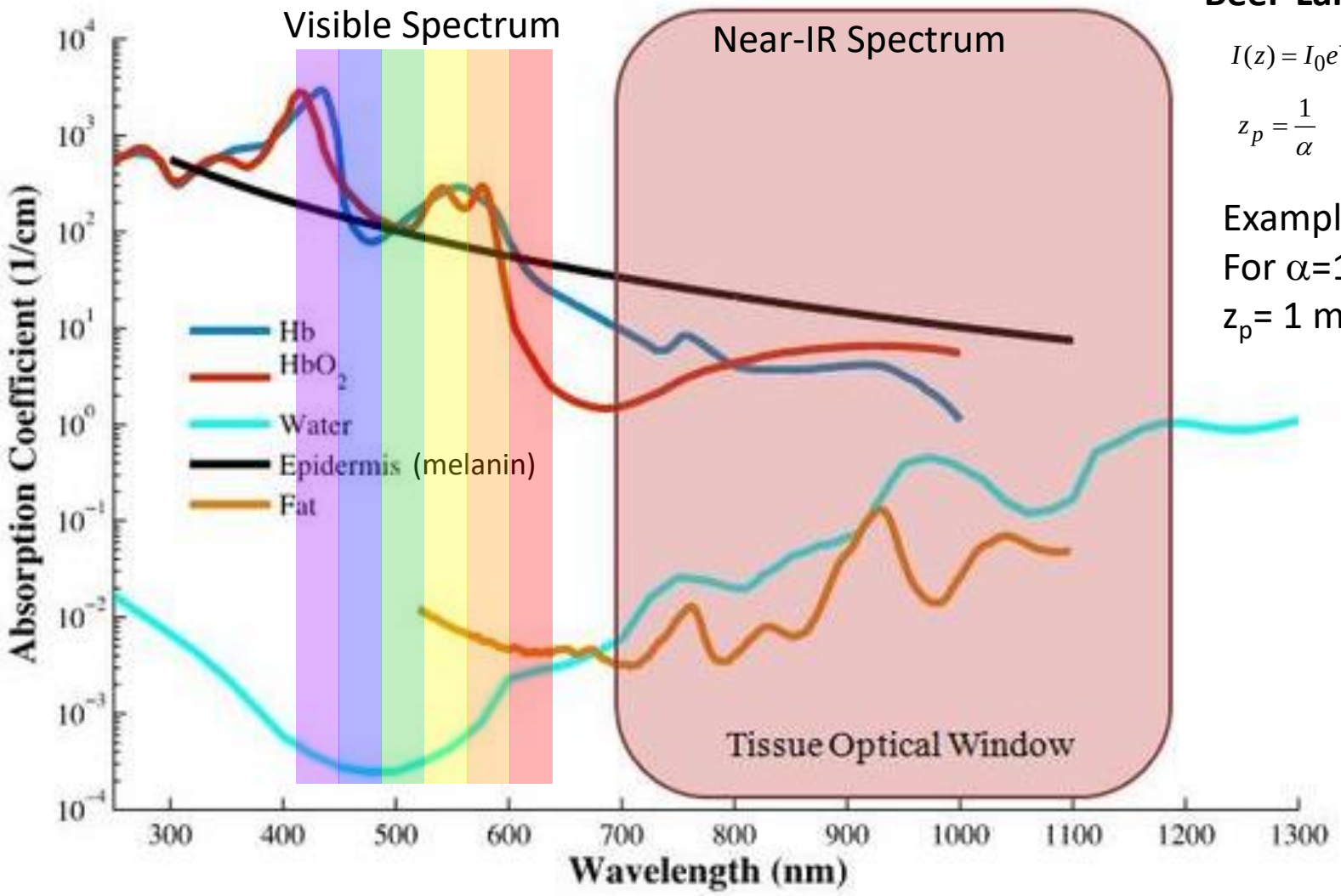
For accurate pulse ox reading (minimizing noise and amplifying the arterial signal), the reader picks up an arterial component ('AC') and a nonpulsatile component ('DC').

- The ratio ('R') of AC to DC at the 660 nm and 940 nm wavelengths is determined.
- This ratio is then correlated to a hemoglobin oxygen saturation based on a calibration curve derived from desaturation studies in healthy patients.
- SpO2 is unreliable below ~70% (because it's unethical to have volunteers desaturate below that. Mathematical models try to extrapolate desaturation < 70%.)

# Absorption in Tissue




# Absorption in Tissue




**Beer-Lambert Law**

$$I(z) = I_0 e^{-\alpha z}$$

$$z_p = \frac{1}{\alpha}$$

Example:
For $\alpha$=10/cm,
$z_p$= 1 mm

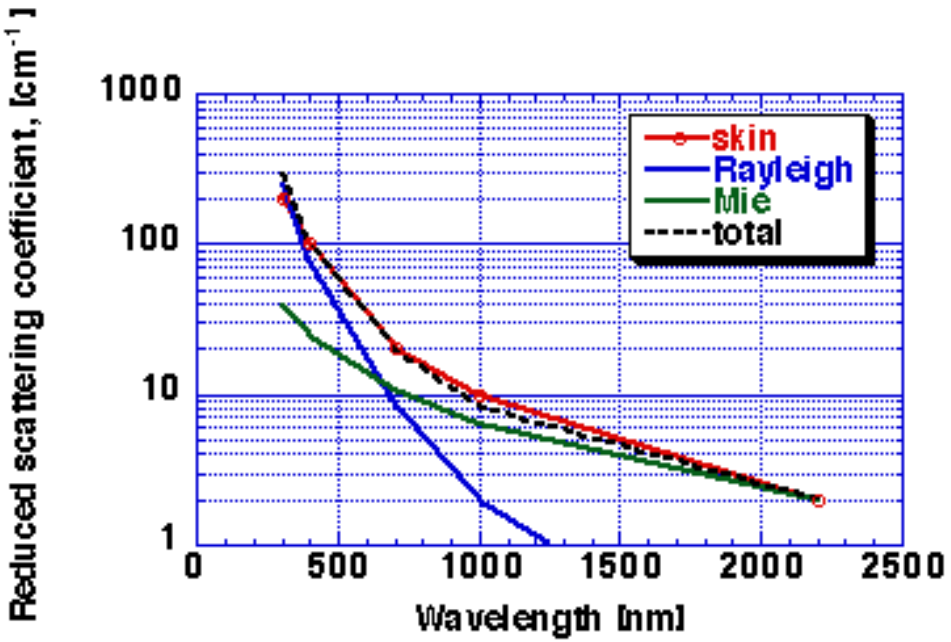
➔ **Visible spectrum** is not good to image tissues due to high absorption loss.
➔ Penetration will be better at **Near-IR spectrum**, indicated by tissue "**optical window**".

# Light Scattering

- Light scattering in tissues (from objects such as cell bodies, cell components etc..) weakens the beam exciting the specimen and also reduces the number of fluorescent photons reaching the detector.

- Major contributions are due to:
  - **Mie scattering** by the larger objects (i.e. collagen fibers) and
  - **Rayleigh scattering** by the small-scale structures.

  ➔The combination of both yields the observed scattering properties of dermis.




- In Rayleigh scattering, the amount of scattered light scales as:

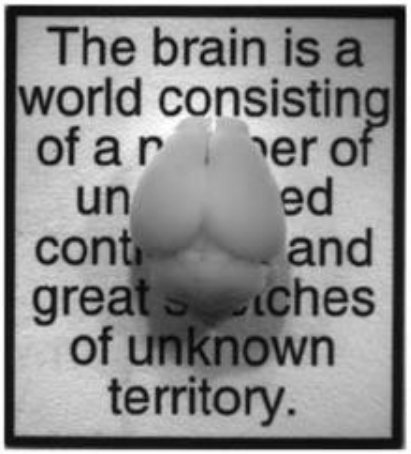
$$\propto \frac{1}{\lambda^4}$$

Eg. the scattering at 400 nm is 9.4 times stronger than at 700 nm!

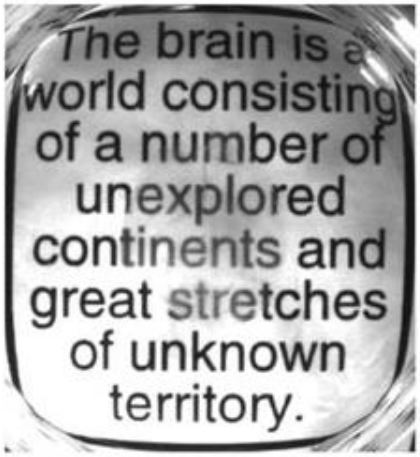
➔ **Imaging in the near-infrared spectrum minimizes both absorption & scattering.**

# Alternative Approach: Optical Clearing of Biological Tissue

**Before**




**After**




**The process replaces a scattering source (lipid) with an optically transparent hydrogel**.

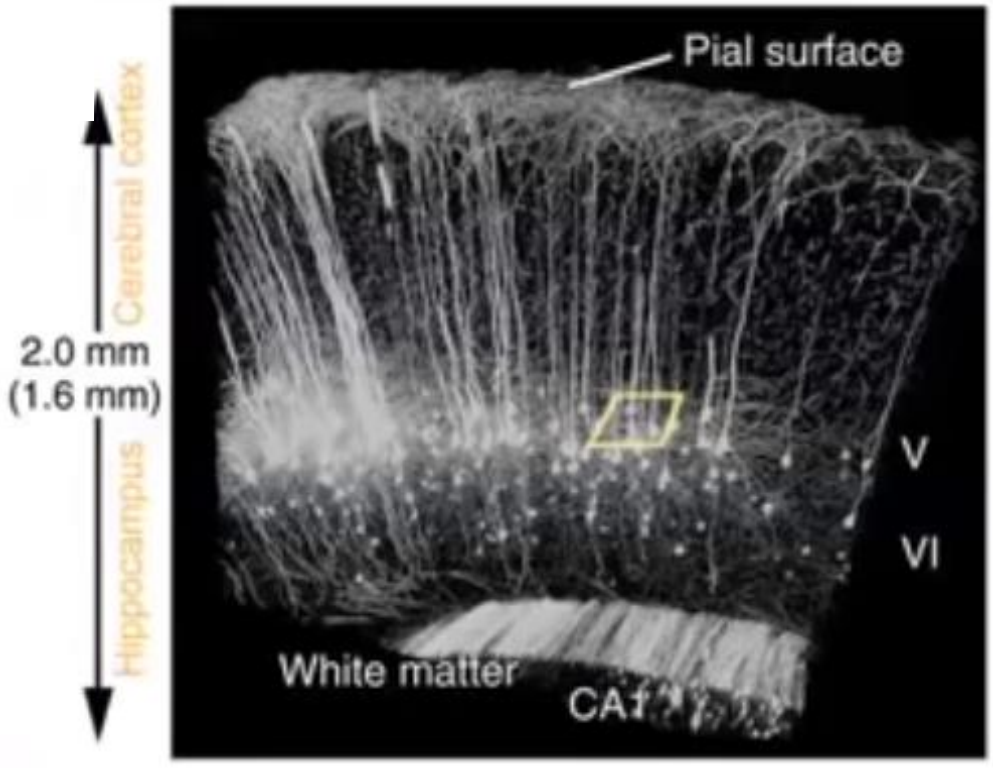
- Immerse the intact, postmortem brain in a hydrogel solution, which contains short individual molecules known as hydrogel monomers.
- The hydrogel monomers infuse the tissue and, when the brain is heated to about body temperature, begin to congeal into polymers that form a mesh throughout the brain and hold everything together. Everything, that is, except the lipids.
- This then allows the lipids to be extracted using an electric field in a process called electrophoresis.

The process is supposed to preserve the biochemistry of the brain so that what remains is a 3D, transparent brain with all of its neurons, axons, dendrites, synapses, proteins, nucleic acids and other important structures right where they should be.

# Alternative approach for deep tissue optical imaging

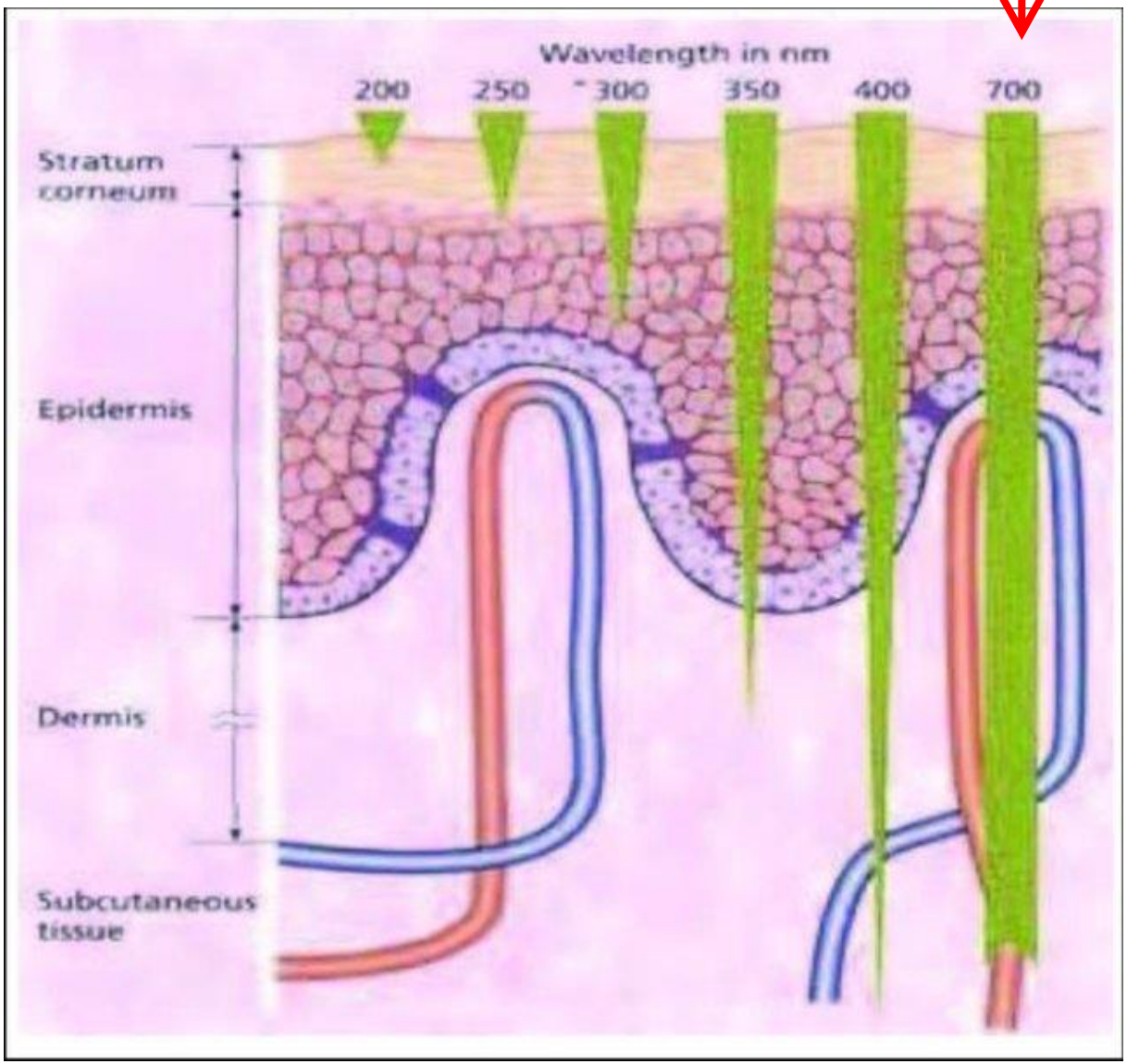
**“Clearing” scattering sources (lipids) chemically:**

- This approach is good for fixed samples.
- But, it is limited for live samples.

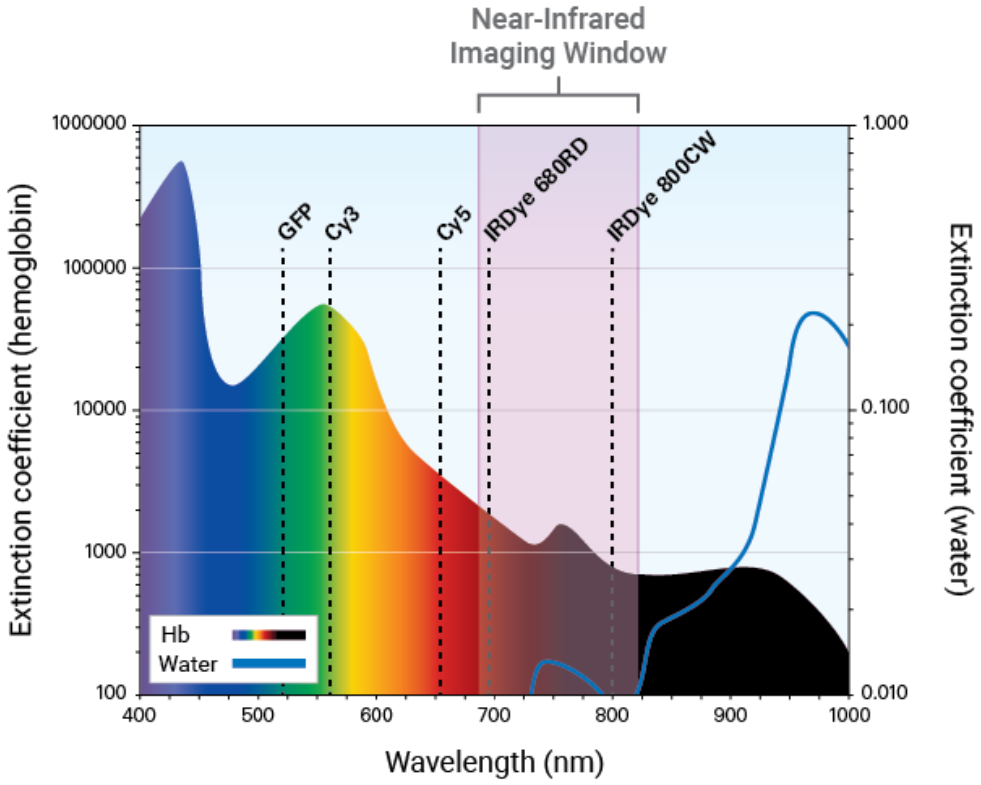


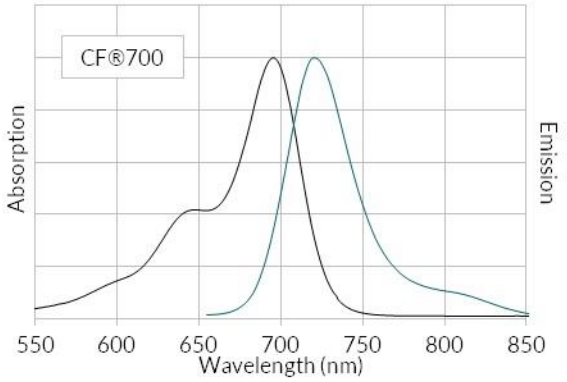
# Deep tissue optical imaging

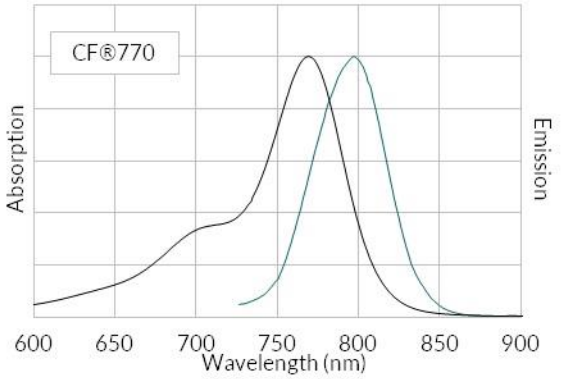
**Imaging in the Near-Infrared (Near-IR) for thick samples**

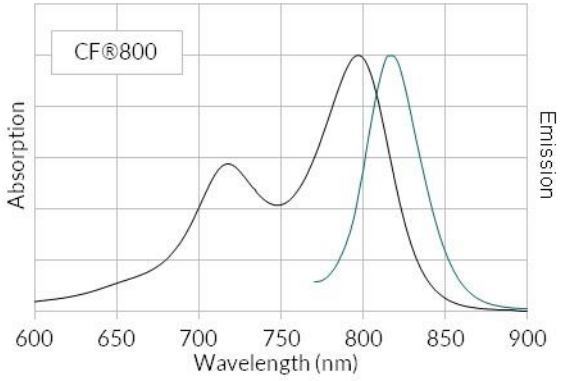


# Imaging in the Near-Infrared with **Near-IR Dyes**



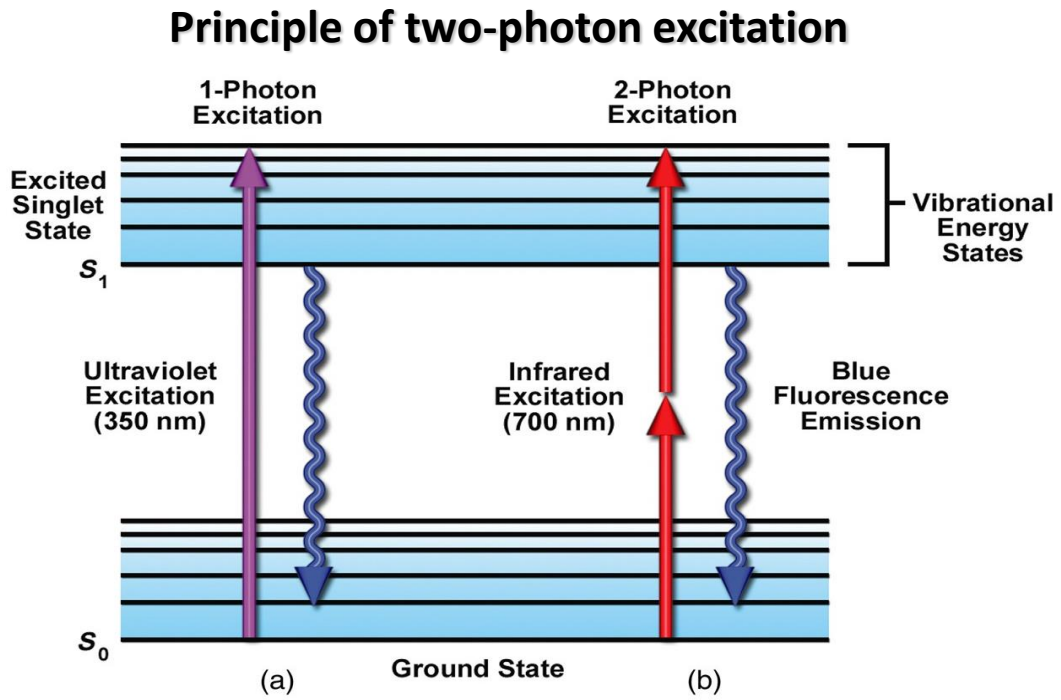

- Near-IR dyes are commonly used **for whole-animal imaging (i.e. tumors in mouse)**

- …But, limited choice of near-IR dyes are available compared to the “standard” dyes that operate in the visible spectrum.
- Also, standard dyes have very good optical characteristics (brightness, stability..)
- How can we leverage “standard” dyes?

# Imaging in the **Near-Infrared** with **Two-Photon** Microscopy

**In two-photon microscopy:**

➔ Use two "Near-IR" photons to do the work of one "visible" photon and image with "standard dyes" at the Near-IR spectrum

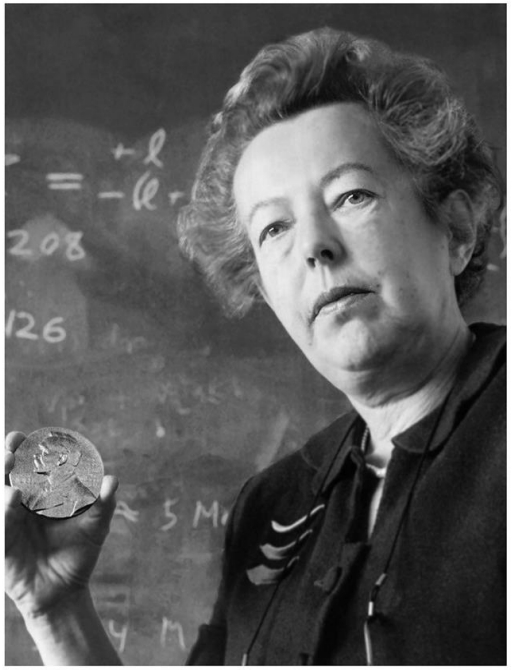



**Jablonski diagram showing one- and two-photon modes of fluorescence excitation.**

**(a) In one-photon excitation,** fluorophores excited to the singlet excited state by absorption of a **single photon** of a specified energy (**purple arrow**) can return to the ground state through emission of a ***lower energy*** **photon** and vibrational energy (**wavy blue arrow**).

**(b) In two-photon imaging with an Near-IR/IR laser**, **two photons**, each with half the required energy, are absorbed simultaneously (red arrows), and the emitted fluorescent photon can have ***higher energy*** than the excitation photons (**wavy blue arrow**).

# Two-photon absorption and microscopy



**Maria Göppert-Mayer (1906 – 1972)**

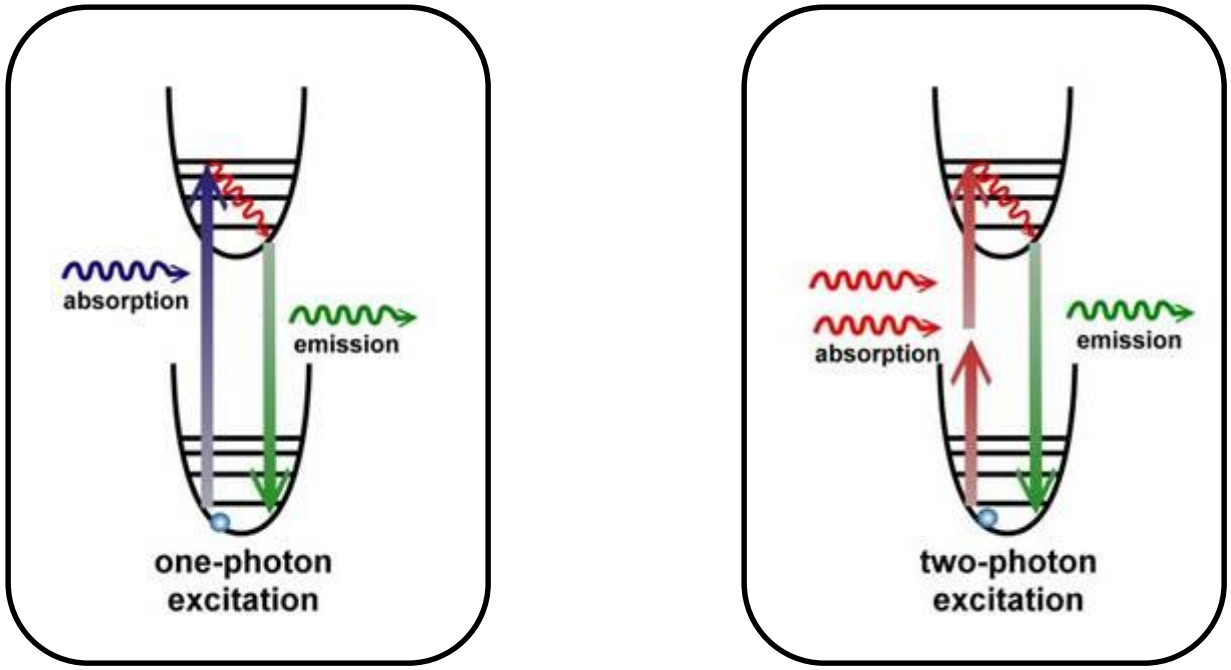
- In her doctoral thesis in physics (1931 in Göttingen, Germany), she described the **theory of two-photon absorption process by atoms**.
- The phenomenon was observed ~30 years later after development of the laser.
- She received **the Nobel Prize in physics in 1963** for extensive contributions to the shell nuclear model of the atom.
- She is the second woman to win Nobel prize in physics (after Marie Salomea Skłodowska Curie in **1903) after 60 years**!

**Winfried Denk**

- Pioneered **two-photon microscopy** in **1990** while he was a was doing his PhD in the lab of Watt W. Webb at Cornell University.
- He patented it together with James Stickler and Watt Webb.

➔Two-photon microscopy has since revolutionized fluorescence microscopy by enabling imaging of living tissue to greater depth (up to a millimeter) than was previously possible.

# Single versus Two-Photon Excitation

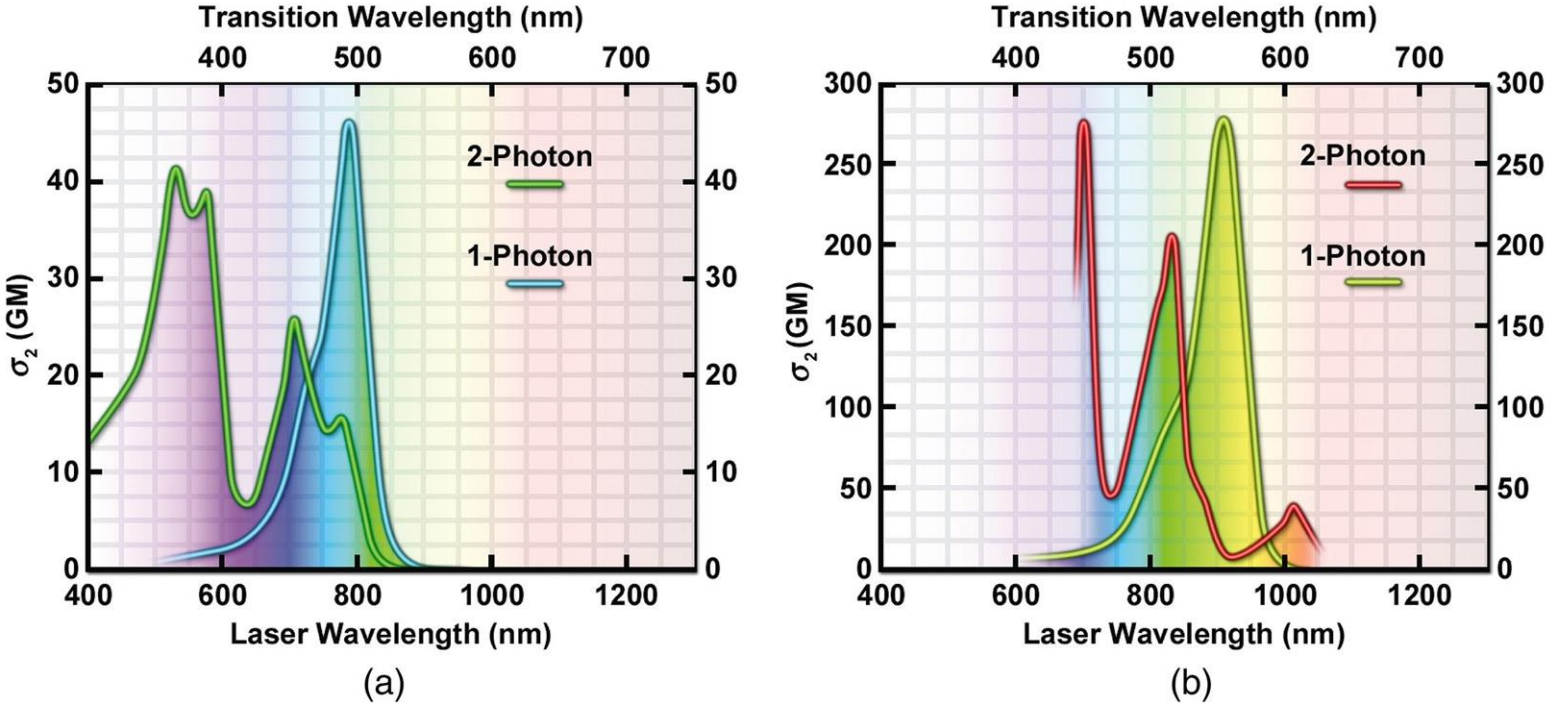



- To estimate the wavelength required to stimulate a fluorescent probe by the two-photon process, one can double the one-photon absorption maximum.
- In reality, excitation spectra are typically blue-shifted compared with the expected values and they can be rather complex, sometimes bearing little resemblance to the one-photon spectra.

- Nevertheless, as an approximation we can say that dyes excited in the 400–500 nm range require ~800-1000 nm IR excitation for two-photon fluorescence.

# Two-photon **absorption spectra** for commonly used fluorescence probes

- Two-photon absorption spectra of small fluorophores are more irregular.
- The spectra for larger molecules like fluorescent proteins are more regular.



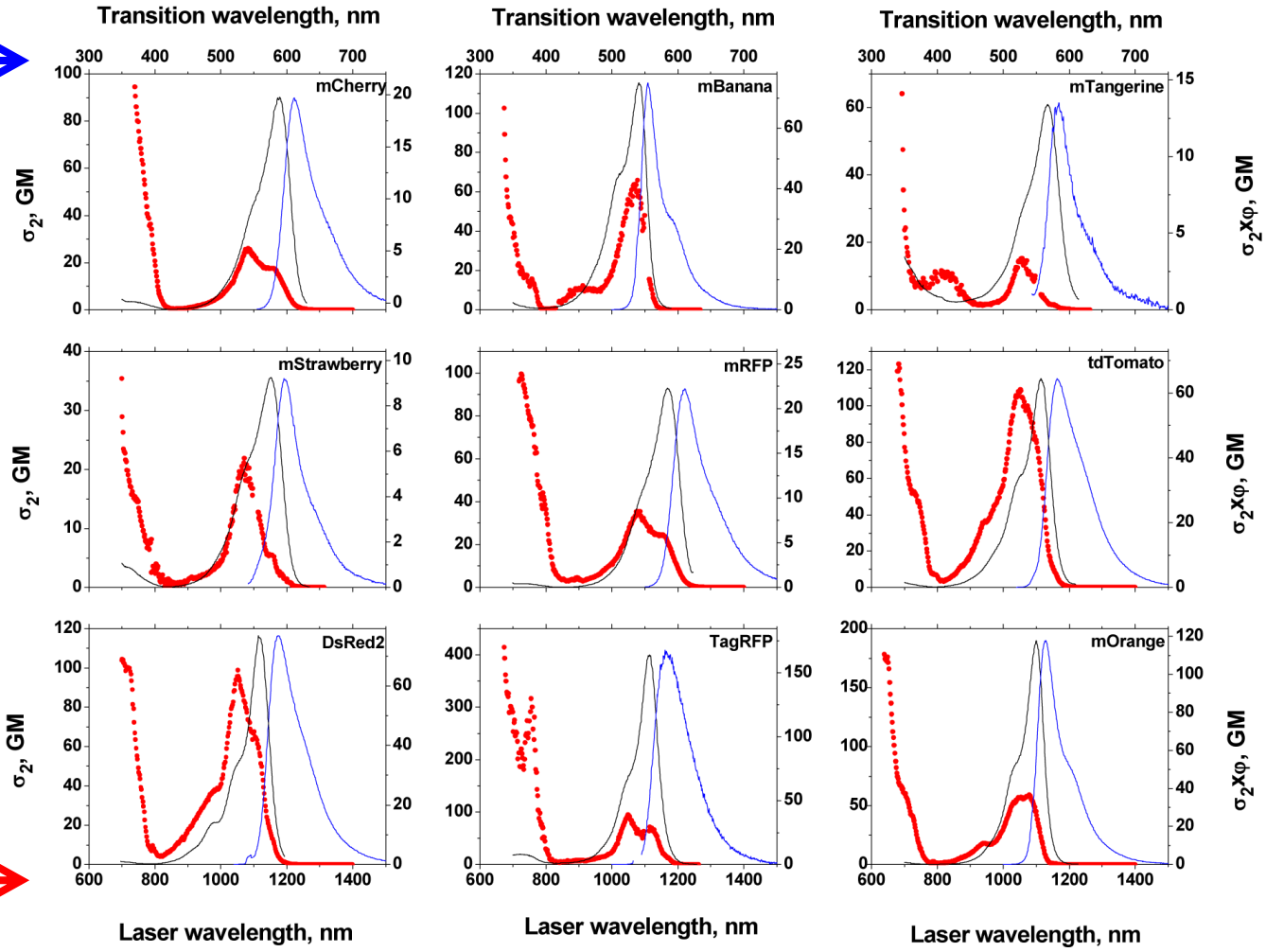

One- and two-photon **absorption spectra** of **small probes**: (a) **fluorescein** and (b) **rhodamine-b**.
(Fluorescence **emission spectra**, not shown, **are the same** for both one- and two–photon mechanisms.)

# Two-photon absorption spectra for some **fluorescent proteins:**

x-axis for 1PA process

**Pay attention to x & y axes!**

x-axis for 2PA process




**Two-photon absorption (2PA) spectra** of some fluorescent proteins **(red dots)** shown along with **one-photon fluorescence absorption** (**black curve**) and **fluorescence emission** (**blue curve**) spectra.

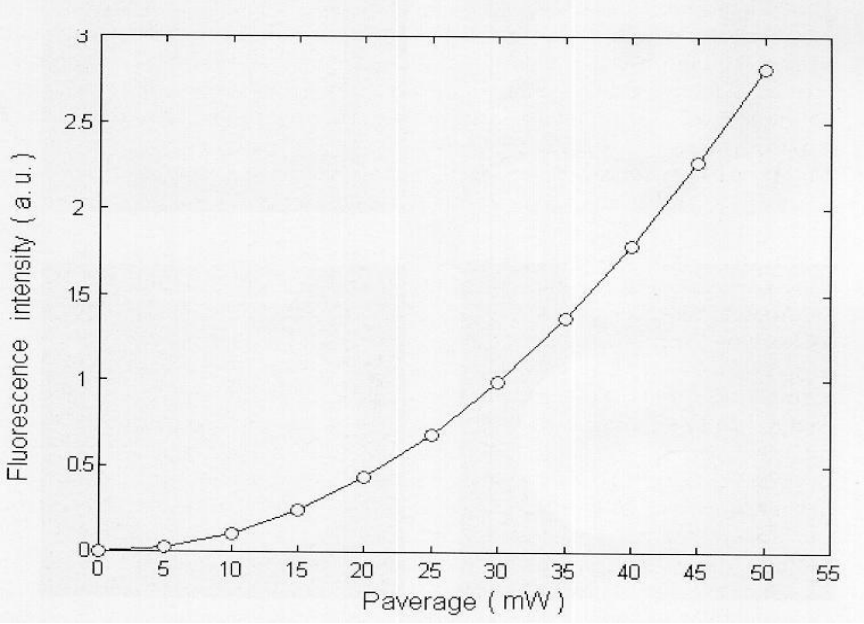
- Bottom x-axis is for 2PA process
- Top x-axis is for 1 PA process
- Left y-axis represents the two-photon cross-section values (in units of GM)
- Right y-axis represents the two-photon brightness

# Two-photon is a nonlinear optical process

- **One-photon fluorescence excitation shows a linear response** to illumination intensity and fluorescence molecules are not excited by longer or shorter wavelengths outside their absorption (excitation) spectra.

- In contrast, in a **two-photon process**, two photons, usually of the same wavelength and each with half the energy required to excite a fluorescent molecule, are **absorbed ~simultaneously** and have the same effect as a single photon of half the wavelength and twice the energy (frequency).

**Quadratic dependence:** $I_{2p} \propto P^2$




- Because two-photon excitation depends on two simultaneously absorbed photons, stimulation to the excited state thus the **brightness of the sample** (i.e fluorescence emission intensity) is **proportional to the square of the excitation light intensity**.
- Therefore, two-photon is a **second-order, nonlinear process**.

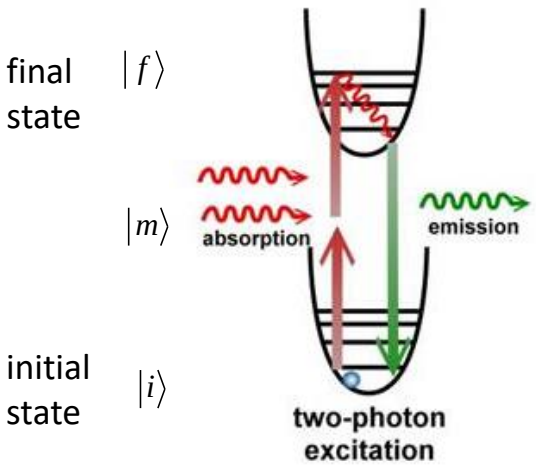
# Two-photon absorption process

Two-photon excitation process is linked to principles of **quantum mechanics**.

Multi-photon transition **probability** (P):

$$P \sim \left| \sum_m \frac{\langle f | \vec{E_r} \cdot \vec{r} | m \rangle \langle m | \vec{E_r} \cdot \vec{r} | i \rangle}{\varepsilon_r - \varepsilon_{mi}} \right|^2$$

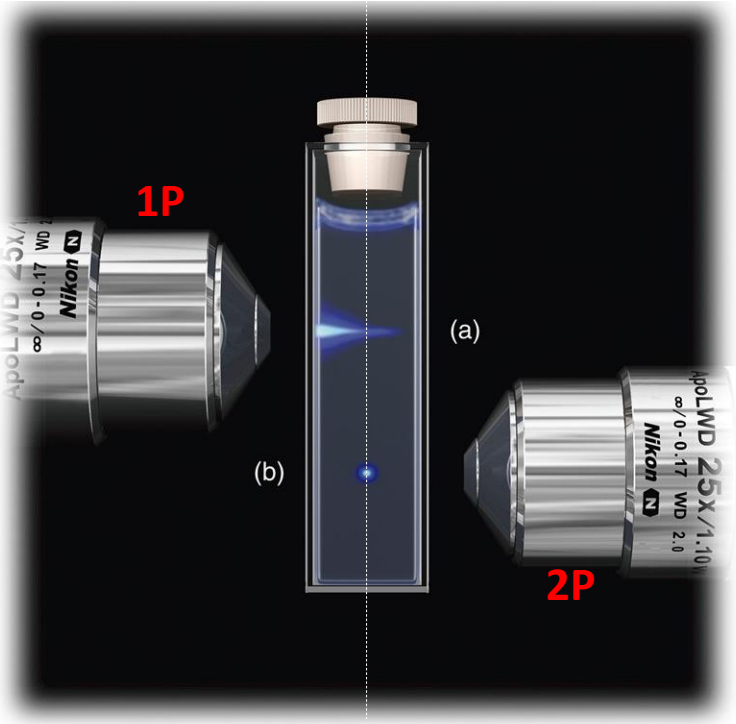
➔ One needs to ***multiply the probability of absorbing two photons ~simultaneously***.




- It is not convenient to describe two-photon absorption in terms of Beer's law and molar extinction coefficients.
- Instead, two-photon absorption is related to the ***probability of molecular excitation*** in the electric field of light and is described by the term, ***molecular two-photon cross-section, σ***.
- In practice, σ is given for a particular wavelength in GM unit, where 1 GM = $10^{-50}$cm$^4$.s.photon$^{-1}$.

# Localization of excitation

- The key feature of two-photon absorption process for microscopy is that it provides ***localization of excitation***, which is the confinement of fluorescence excitation to a tiny volume at the focal point.
- This feature makes T2A process extremely valuable for deep tissue imaging.




**With 2P:**

- There is no out-of-focus light.
- We only get excitation from where the beam is most tightly focused.

➔ Localization of excitation

**(a) One-photon excitation** of fluorescein dye in a cuvette by a focused 488-nm beam of a laser shows an extended path of excitation above and below the focal plane.
**(b) Two-photon excitation** of the same dye with a 960–nm near-IR beam shows just a single isolated point.

# Localization of excitation with two-photon versus one-photon:

Total fluorescence ($\Phi$) is given as the product of absorption rate ($\phi$) and the area (A) of the excitation zone at a distance (z) from the focal plane:

$$\Phi = \phi . \ A$$

The key difference between 1P and 2P excitation is that in 1P the absorption rate ($\phi$) is linearly proportional to intensity (I), but in 2P it is **quadratically (nonlinearly)** proportional ($I^2$).

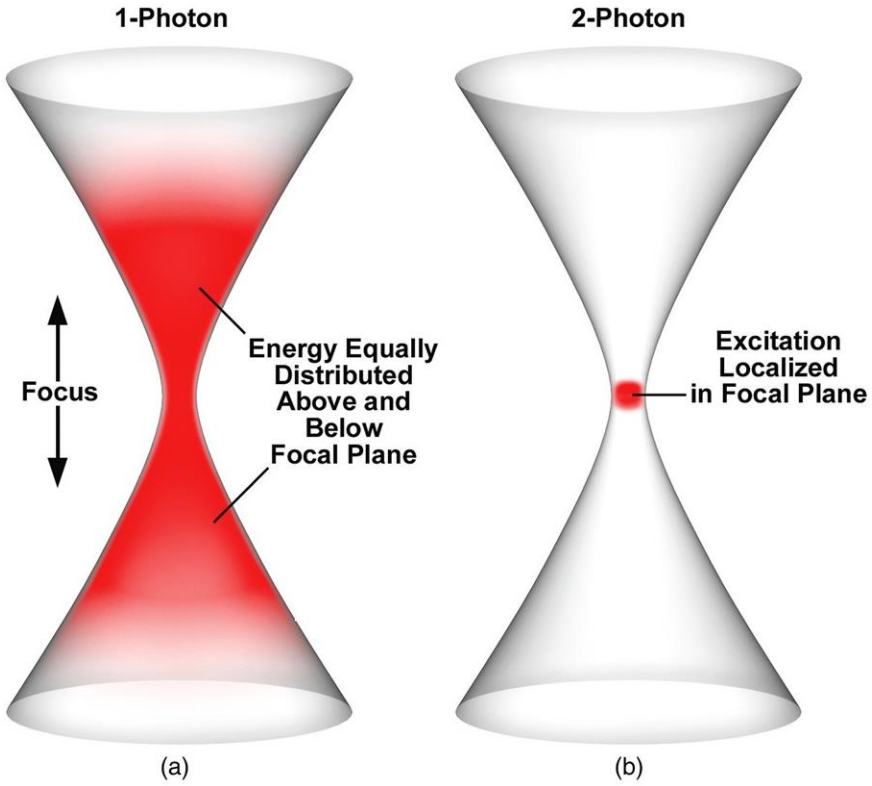
| Terms | 1 Photon | 2 Photon |
|---|---|---|
| Area **A** of illuminated spot | $A \sim z^2$ | $A \sim z^2$ |
| Intensity **I** ($W/m^2$) | $I \sim 1/z^2$ | $I \sim 1/z^2$ |
| Absorption rate $\phi$ | $\phi \sim I \sim 1/z^2$ | $\phi \sim I^2 \sim 1/z^4$ |
| Total fluorescence $\Phi$ | $\Phi \sim 1$ | $\Phi \sim 1/z^2$ |

Equally distributed below and above the focal plane.

Decreases rapidly away from the focal plane, thus provides strong localization!

**Note:** For a typical objective of NA =1, the distance along the z-axis from the focal plane equals the radius of the illuminated plane. Therefore, the illuminated cross-section area is taken as $A \sim z^2$.

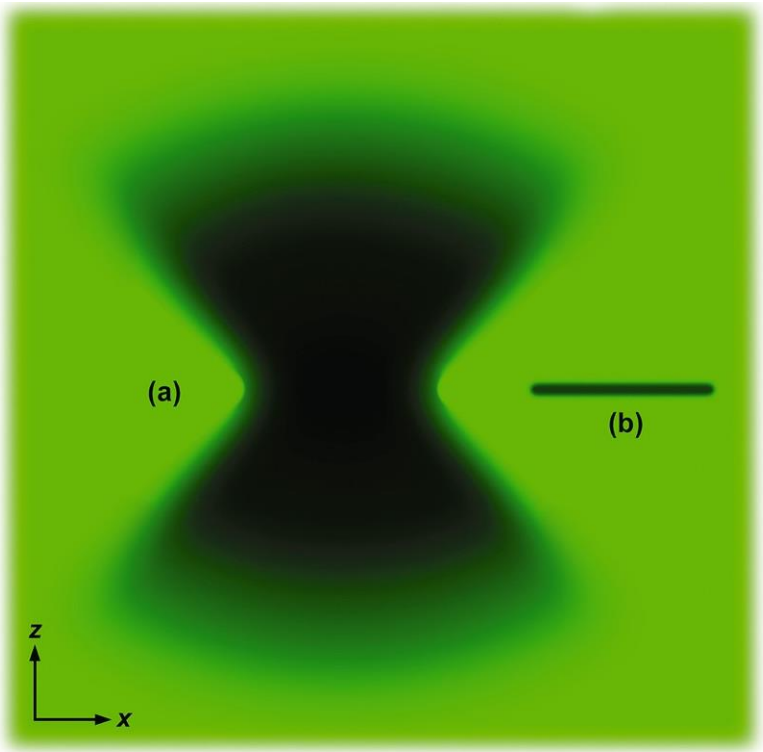
# Localization of excitation with two-photon versus one-photon:




(a) Light path in a 1P microscope showing fluorescence constricted at the focal plane but occupying all planes above and below the focus and showing the same total amount of fluorescence in each plane.

(b) Light path in a 2P microscope shows fluorescence excitation is localized (and limited) to the focal plane.

# Localization of excitation with two-photon versus one-photon:

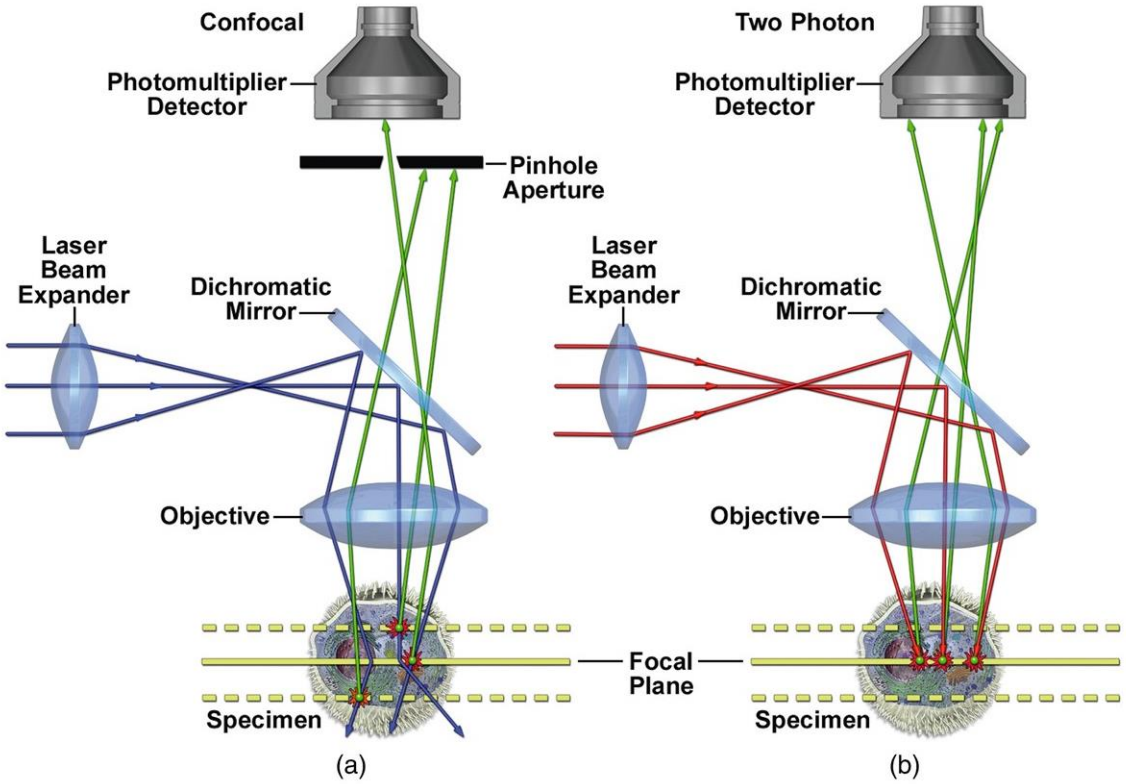



**Visualization of the "localization of excitation"** by a microscope objective in a fluorescent substrate for **confocal microscope (a) and two-photon microscope (b).** It is achieved by photobleaching an area of interest at the same depth of focus of a rhodamine-containing plastic substrate.

**(a)** For the confocal microscope, the plastic block was scanned to produce an image stack through the bleached area and the stack is displayed in the *x*-*z* plane perpendicular to the surface of the block. The obtained confocal image highlights two cones meeting in the focal plane, which is controlled by the numerical aperture of the objective.

**(b)** In contrast, two–photon excitation bleached a sharply defined line in the focal plane.

# Single versus Two-Photon Imaging

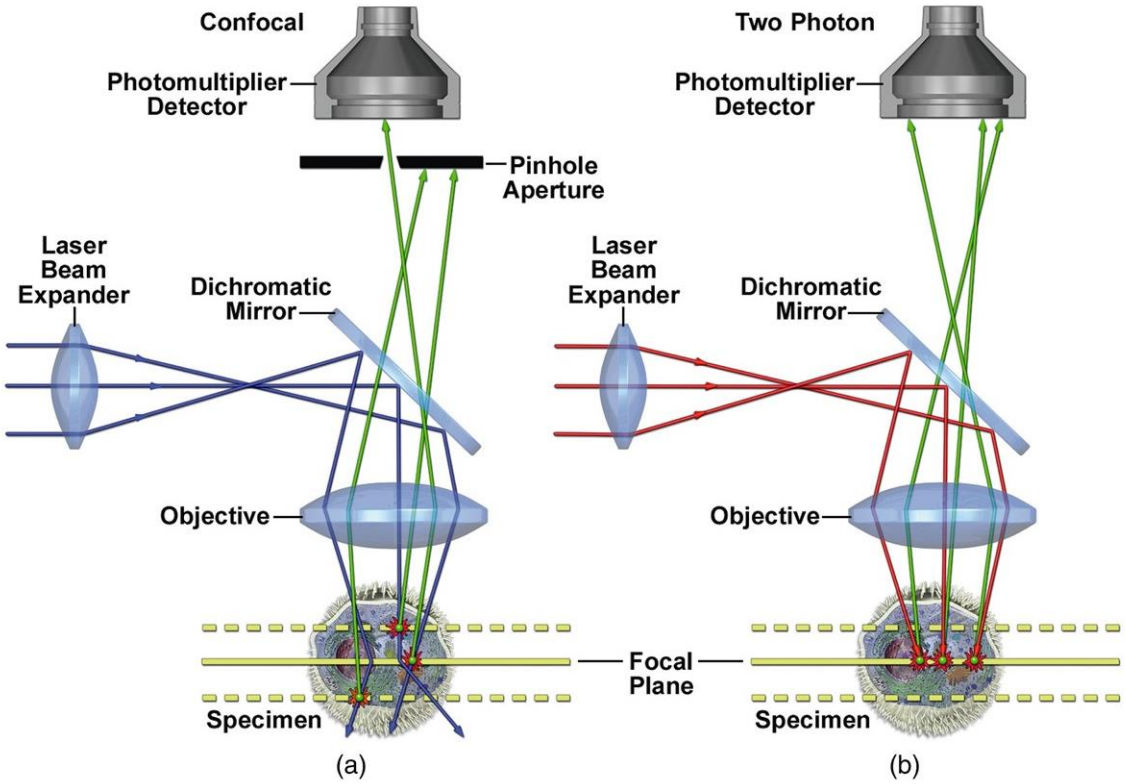



**Light scattering limits in fluorescence imaging.**

**(a) In confocal imaging**, scattering reduces excitation of the specimen (blue arrows) and also diverts confocal fluorescent rays (green arrows) from the pinhole, thus it cuts significantly the signal strength.

**(b) In contrast, for two-photon imaging** with near-IR rays, scattering of excitation rays (red arrows) is reduced, and fluorescence emission (green arrows) comes from a single point, thus removes the need for the pinhole.

# Single versus Two-Photon Imaging




Advantages of 2P imaging:

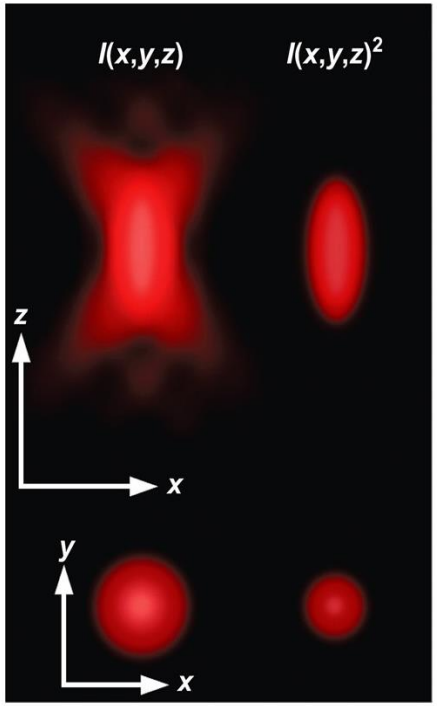
- We can have optical sectioning (z-control) but **without requiring pinhole in front of the detector.**
- No pin-hole means we can collect all of the emitted photons, thus receive “higher number of photons” (stronger signal levels).
- Two-photon microscopy is particular useful to image thick tissues. **It works for thicker specimens than what confocal can image.**

# Two-photon versus one-photon confocal microscopy

- **In confocal,** to eliminate out-of-focus light (and thus have a good z-accuracy) we need to use pin-hole. Consequently, in confocal the focal volume is defined by approximately the point-spread function times a detection pinhole.

- In contrast, **in 2P excitation**, the focal volume is defined by approximately the point-spread function times itself.

➔ **Thus, 2P microscopy can achieve nearly the same point-spread function as confocal BUT without a pinhole.**



# Resolution in Two-Photon Microscopy

For objective NAs > 0.7, lateral resolution and axial resolution are calculated as:

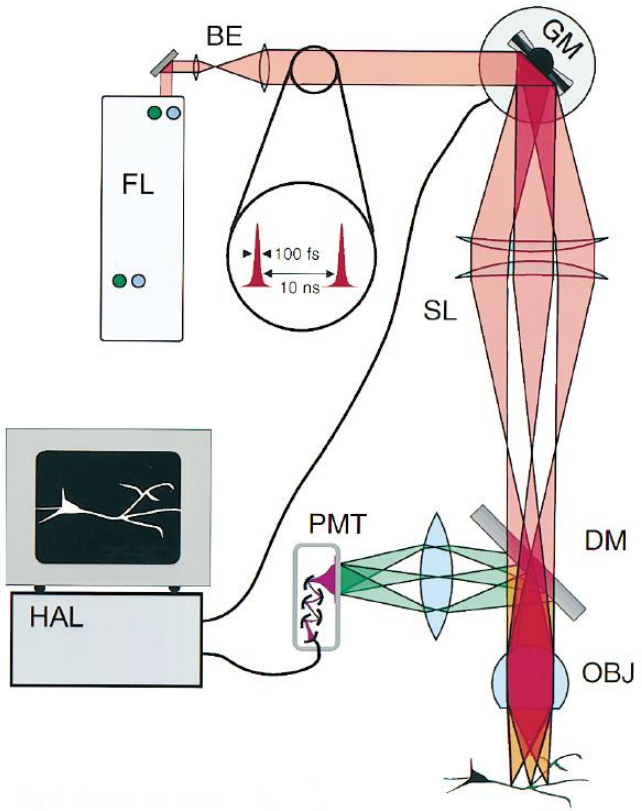
$$\omega_{xy} = 0.325\lambda/\sqrt{2}\ \mathrm{NA}^{0.91},\ \text{and}$$

$$\omega_z = [0.532\lambda/\sqrt{2}]\cdot[1/(n-\sqrt{(n^2-\mathrm{NA}^2)}],$$

**Example:** For a 1.0 NA water immersion objective at 800 nm and n=1.333, the values of $\omega_{xy}$ and $\omega_z$ are ~0.2 and 0.66 μm, respectively.

- It is important to highlight that despite the advantages of the intensity squared relationship for resolution, for a given dye like fluorescein in a thin specimen, **similar resolution will be obtained using a confocal microscope and shorter wavelengths**.
- **Unique advantage of two-photon imaging is therefore not on the resolution, but on its ability to localize excitation and image deep in specimens.**
- In **thick sections and tissues**, two-photon imaging can produce clearer images.

# Image formation by raster scanning

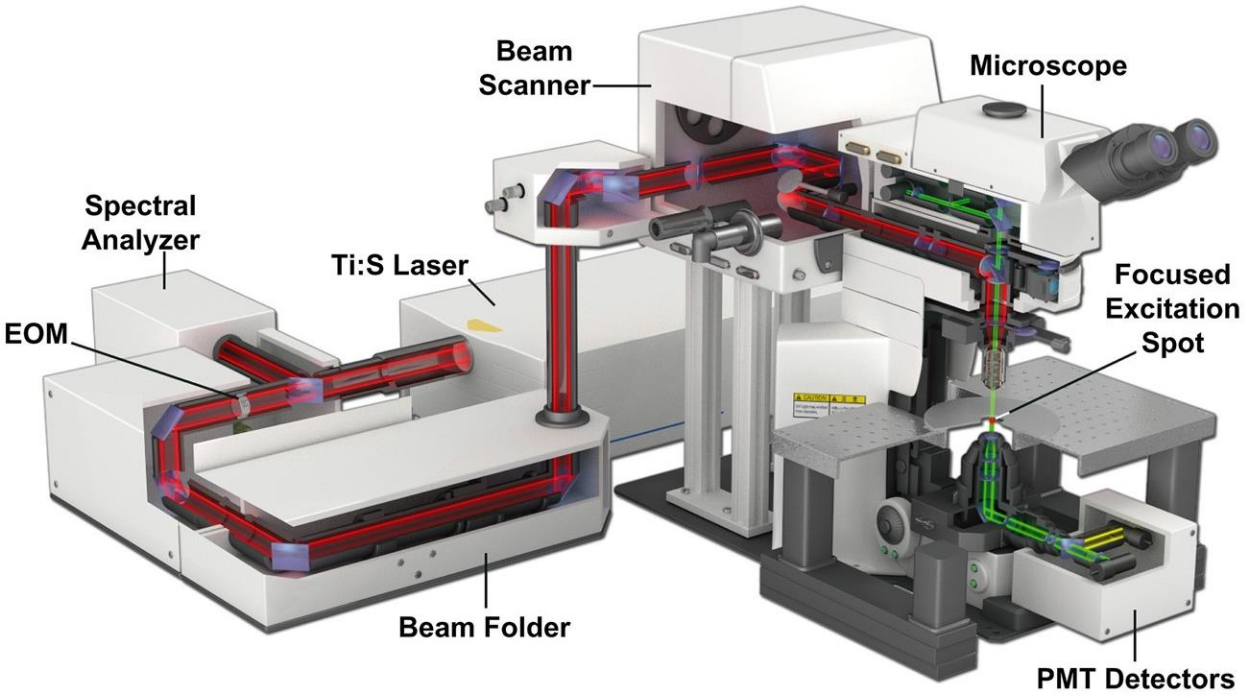



➔ Excitation path is same as confocal but detection is simpler than confocal as it does not require pinhole.

(FL) femtosecond mode-locked laser
(BE) beam expander
(GM) pair of galvanometer scanning mirrors
(SL) scan-lens intermediate optics
(DM) dichroic mirror
(OBJ) objective lens
(PMT) photomultiplier detector
(HAL) computer

The instrument set-up might be dedicated for two-photon imaging system, or, alternatively, it might be incorporated into a general-purpose instrument equipped for wide-field or confocal fluorescence imaging with raster scanning.

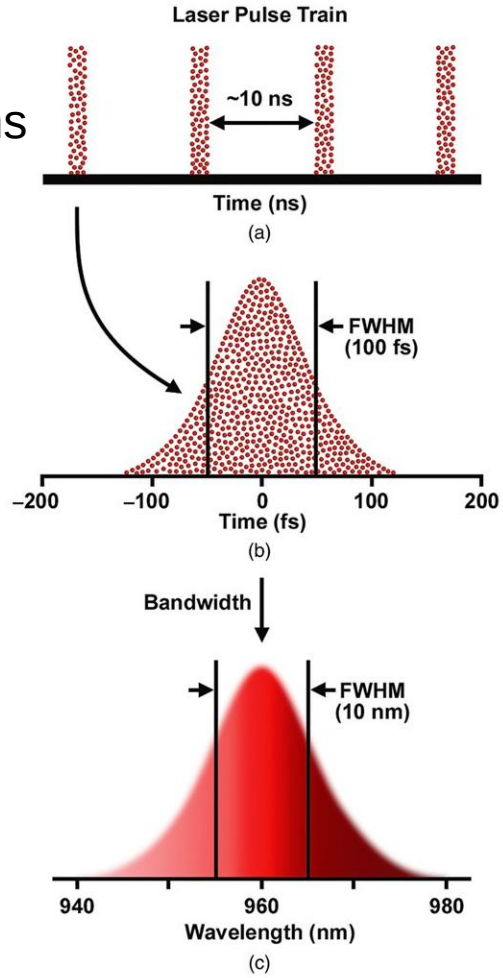
# Equipment configuration for two-photon microscopy.



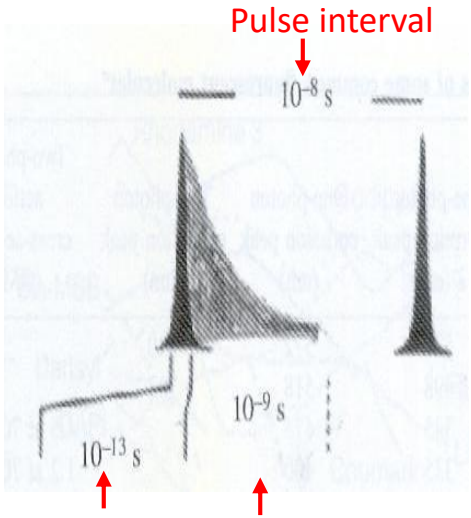

- **A titanium:sapphire (Ti:S) laser producing a near-infrared beam** is typically used.
- The laser beam is expanded to fill the rear aperture of the objective to form a focused point on the specimen, and the specimen is scanned in a raster pattern.
- Two-photon fluorescence emission is reflected by appropriate dichromatic mirrors and captured by photomultiplier tube detectors.
- The spectral analyzer allows checking of the laser beam for proper spectral output.
- The electric optical modulator (EOM) controls intensity or blocks the beam.

# Light source in two-photon microscopy is a **mode-locked fs NIR laser.**

pulsed
near-IR beams




Relevant time scales:




(a) Pulse frequency of 80 MHz corresponds to a pulse interval of ~10 ns

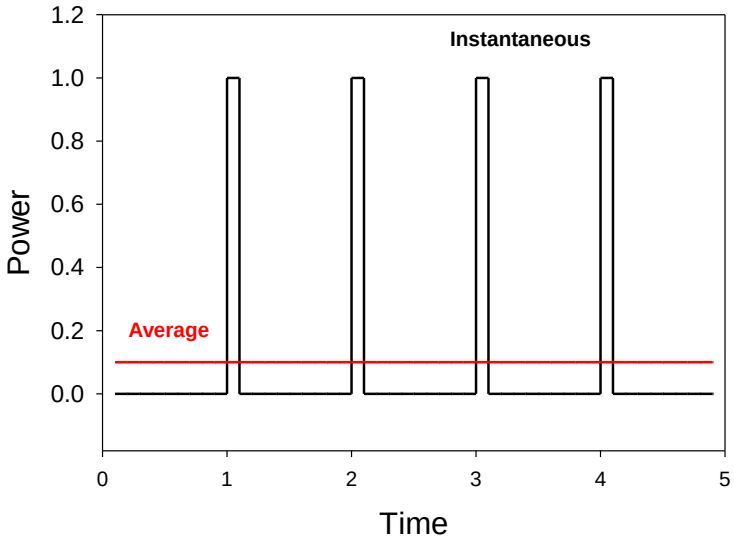
(b) Pulse width is ~100 femtosecond (FWHM in time domain)

(c) The spectral bandwidth of each pulse is in the range of 10–20 nm. And, the laser can be tuned from 680 nm to 1100 nm. This tuning range fits well to the optical window of tissues/biology.

# Example: A mode-locked fs NIR laser source in two-photon microscopy

Despite with a low average power, the peak power of an ultrafast laser pulse can be very high.

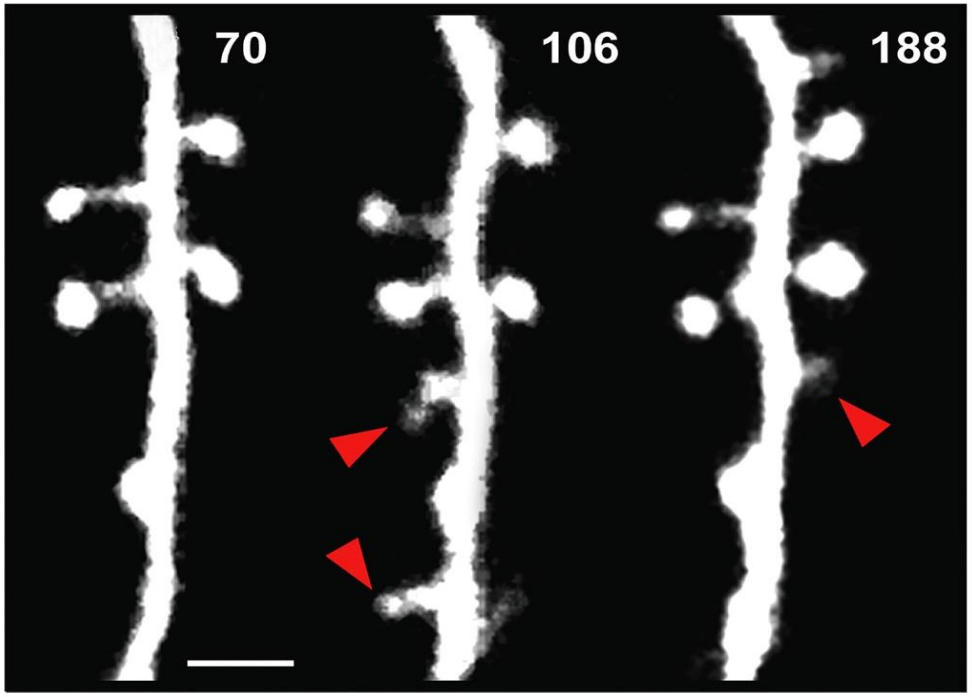
- Let's consider a laser beam which is focused on a diffraction-limited spot has an average power at the specimen of 10 mW (0.01 W).
- If we assume a spot size of ~450x450 nm then the area is $2 \times 10^{-9}$ $cm^2$
- Average laser power in the spot is:
  0.01 W /($2 \times 10^{-9}$ $cm^2$) = $5 \times 10^6$ $W/cm^2$
- Laser is "on" for *100 femtoseconds* in every *10 nanosecond* interval; therefore, the pulse duration to gap duration ratio = $10^{-5}$




- Thus, for this example the peak power in the spot when the laser is "on" is **$5 \times 10^{11}$ $W/cm^2$**

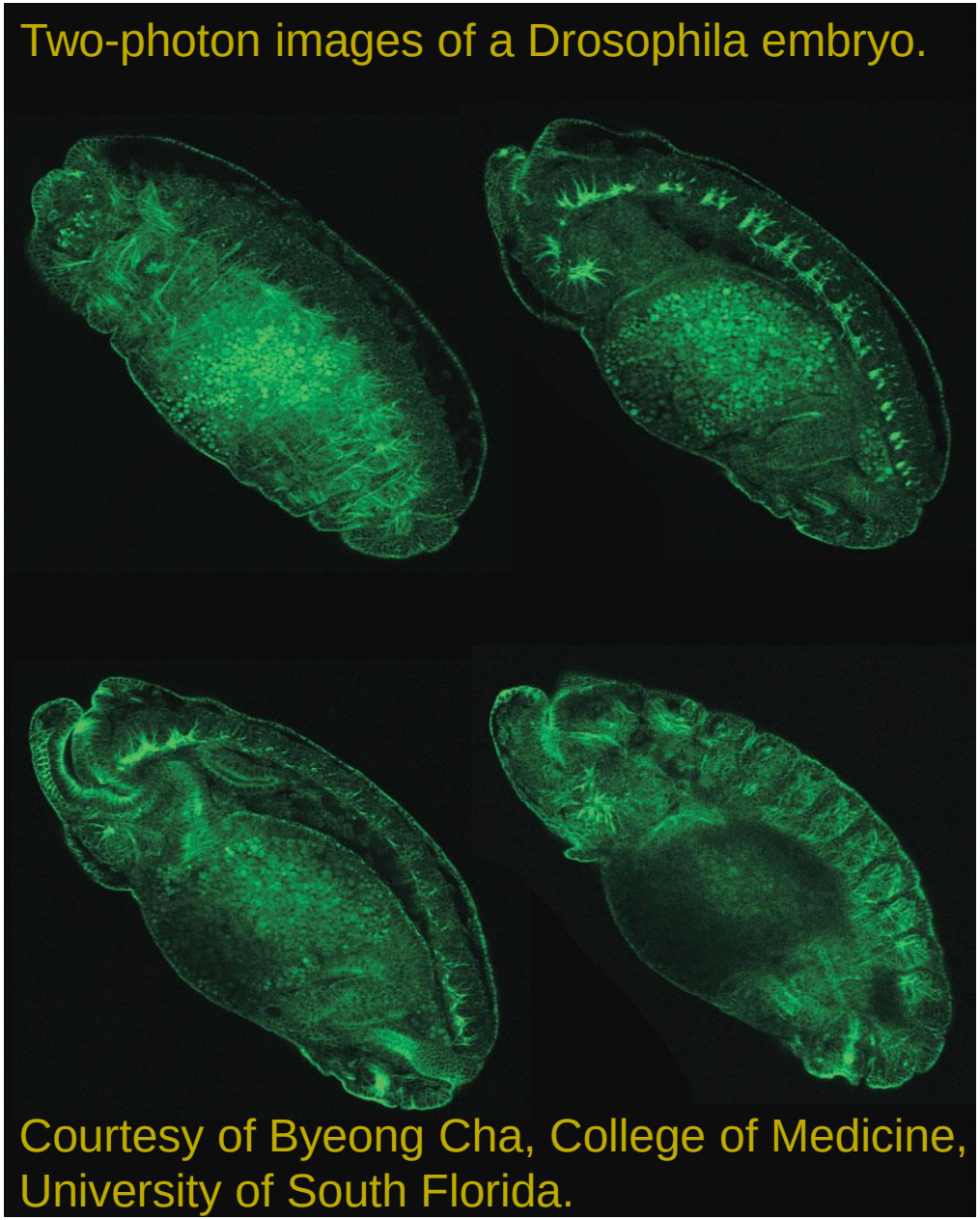
# Examples and Applications of Two–Photon Fluorescence Microscopy - 1

- Two-photon excitation microscopy is generally **less phototoxic** than confocal microscopy, as demonstrated by **time-lapse studies of deep brain imaging, embryo development**, and other systems.



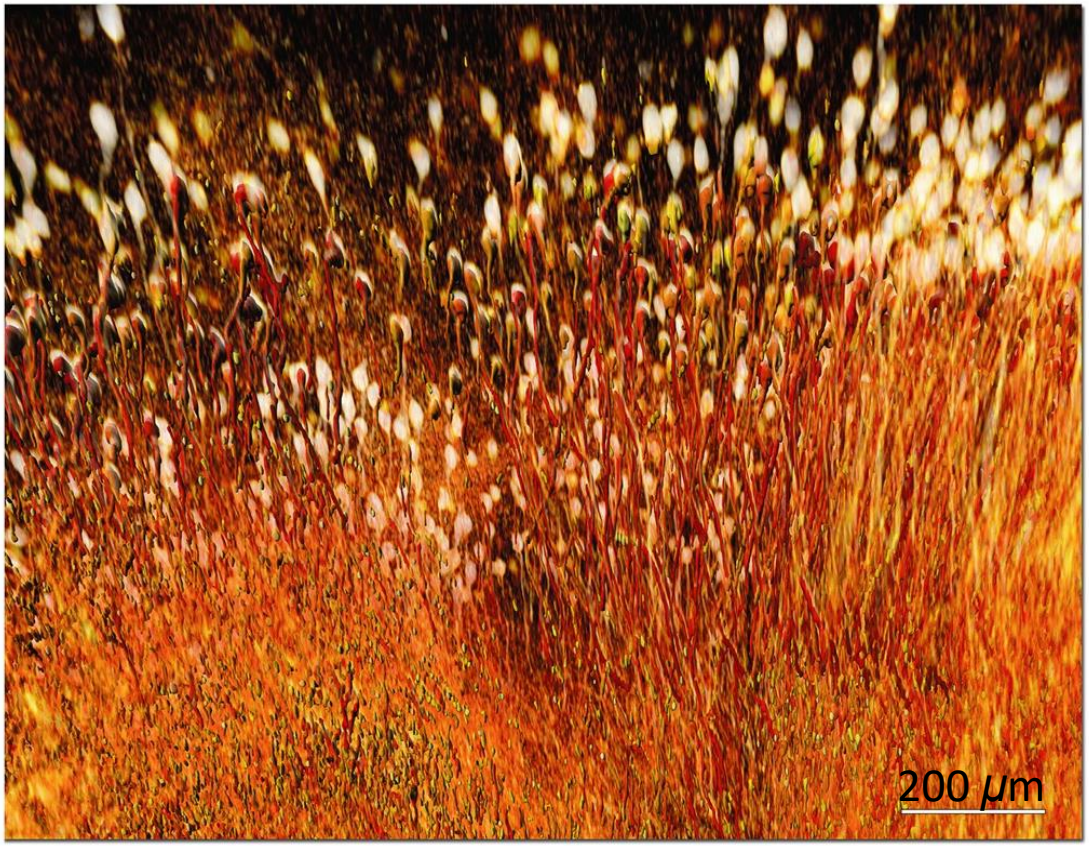

- ***In vivo* deep brain imaging, over a period of 4 months**, of dendritic spines by two–photon microscopy.
- Dendritic spines expressing EGFP persisted and new spines appeared (red arrowheads) on mouse pyramidal cell neurons.
- White numbers in the upper corner indicate the postnatal days on which images were captured. Bar = 2 μ m.

# Examples and Applications of Two–Photon Fluorescence Microscopy - 2



# Examples and Applications of Two–Photon Fluorescence Microscopy - 3

## Two-photon imaging allows deep tissue imaging

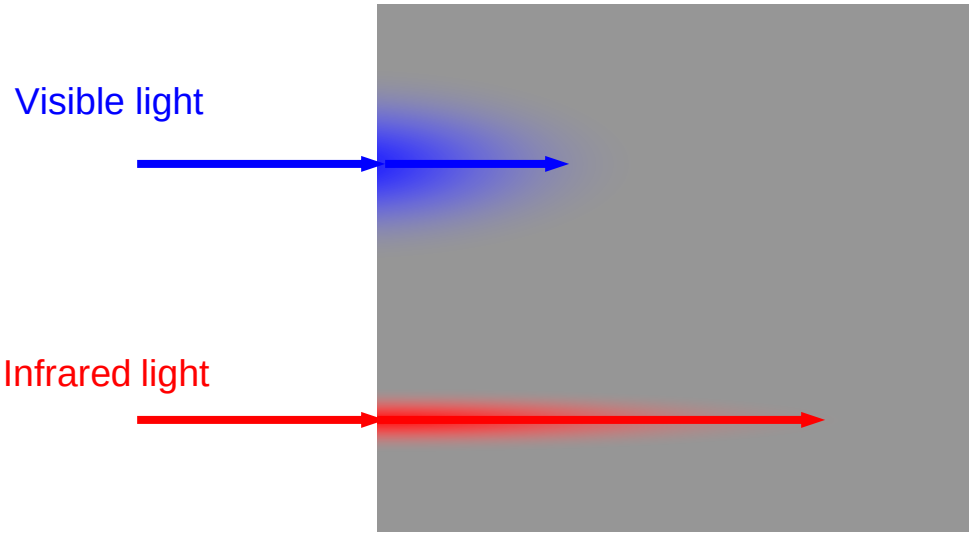



- Reconstructed tiled *z*-stack from **a 3.5 - mm thick section of mouse brain** hippocampus tissue expressing enhanced yellow fluorescent protein (EYFP).
- High-quality images are obtained at **depths up to 500 *μ*m**, and useful information is still obtained at depths of up to 800 *μ*m and beyond.

Bar = 200 *μ* m. (Olympus America Inc)

# Summary: Advantages of two photon imaging

- Better in depth penetration because photons with longer wavelengths (e.g. in near-IR spectrum) result in less scattering and absorption losses compared to photons in UV-Vis spectrum.
- Suppression of out-of-focus fluorescence because of inherent non-linear process (namely “localization of excitation”) with 2PA
- Less complicated set-up and higher signal than confocal because of no pinhole requirement (and this is due to the suppressed out-of-focus fluorescence & less scattering)
- Less phototoxicity for the tissue
- Less photobleaching of the dye
- Less photodamage of the dye



# Some Limitations of of Two Photon Microscopy

1. Two photon imaging has depth limit with out of focus light (background) > **1000 μm**
   *Theer, Hasan, Denk. Opt Lett. 2003*
2. *fs-IR lasers are expensive (100- 200K $)*
3. *Scanner frame rate is relatively slower*