MSc in Photonics & Europhotonics
Laser Systems and Applications 2017/2018

Applications of lasers in the life sciences

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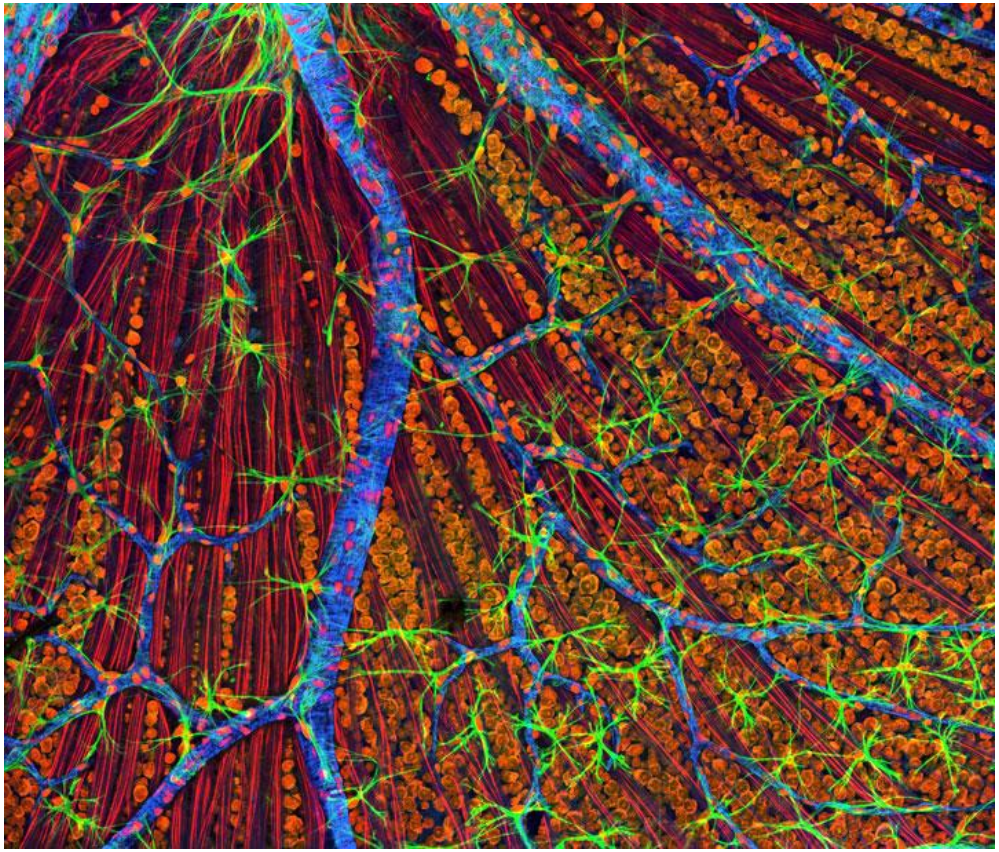
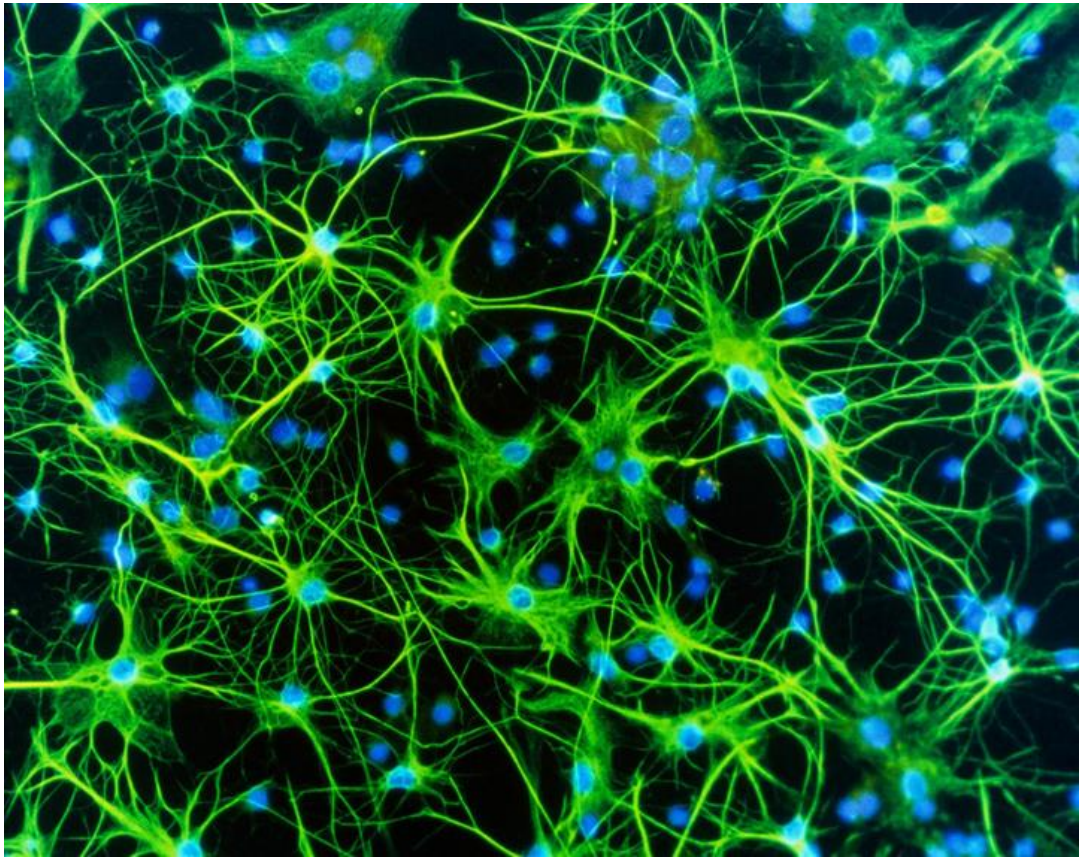
-BIOMEDICAL OPTICAL IMAGING



A laser scanning, or "confocal" microscope scans a sample point-by-point or line-by-line at once, assembling the pixel information to generate one image. This allows for a very high-resolution and high-contrast image in three dimensions. The image is from a **laser scanning microscope** of a **mouse retina**, where the cells have been stained with **fluorescent dye**.

http://lightexhibit.org



Astrocytes are the star-shaped cells found in spinal cord and the brain. In this image of astrocytes, the **nucleus** of each cell has been stained blue while the **cytoplasm** (the fluid that fills the cell) has been colored green. To achieve this, the process of immuno-fluorescence was used (antibodies are used to **attach fluorescent dyes to specific molecules in the cells**).

http://lightexhibit.org

Protozoa are single-celled animals found throughout the world in many different habitats. They play a key role in maintaining and balance of bacteria, algae, and other microbial life. This photograph illuminates one particular type of protozoa called vorticella. In this image, a technique called "**dark field microscopy**" was used. This technique blocks out the direct light from the source, so that **only light scattered** by the specimen is observed, enabling brilliant bright images to be seen again a dark background.

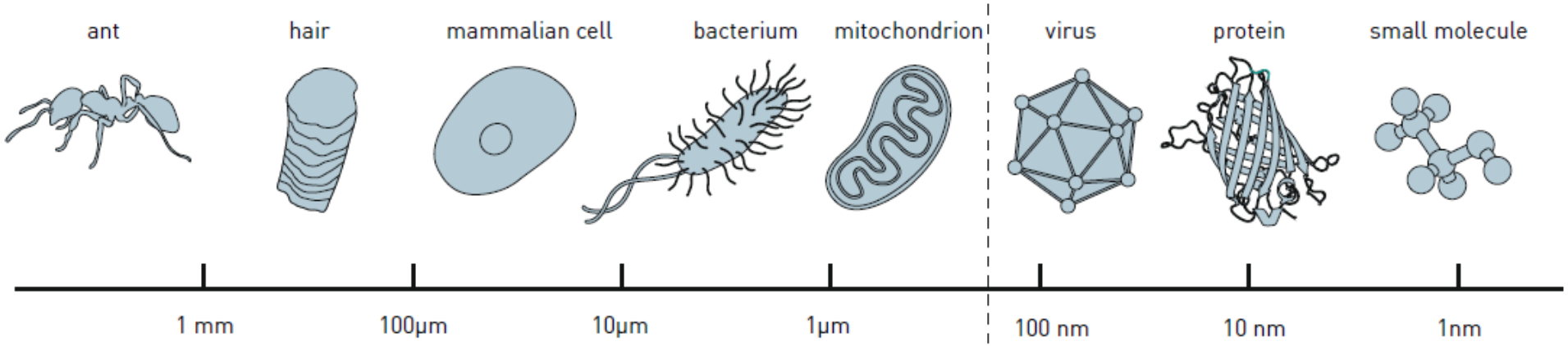
Optical imaging: advantages & drawbacks

- Advantages:
  - Relatively low cost.
  - Wide range of spatial resolution: molecular to tissue information.
  - Time-resolved measurements.

- Drawback:
  - Limited depth penetration: about 10-fold loss in photon intensity for every centimeter of tissue depth.

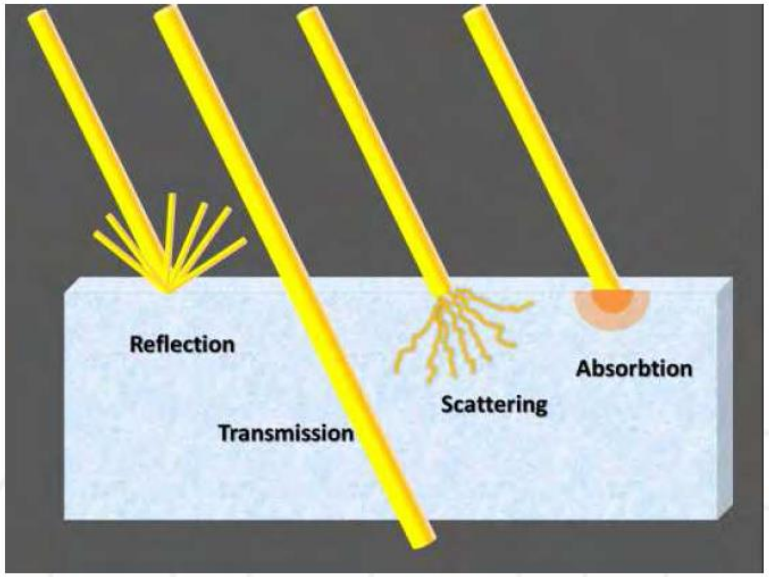
The classical limit for visualizing the biological world

- The spatial resolution of optical microscopy is limited by the diffraction of light to $d \sim \lambda/(2NA)$ NA=numerical aperture
- Considering green light ~500 nm and NA of 1 d~ 250 nm (small compared to cells, but large compared to viruses, proteins and molecules).

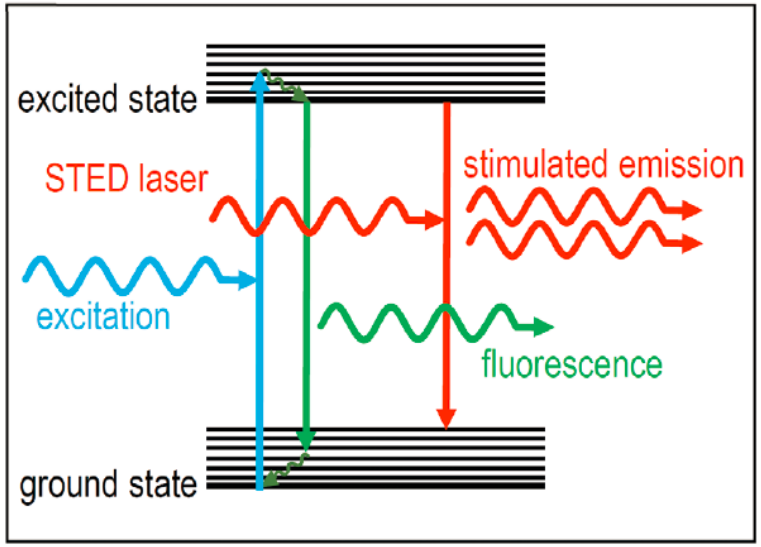


Main operation principles

- Techniques based on light transport



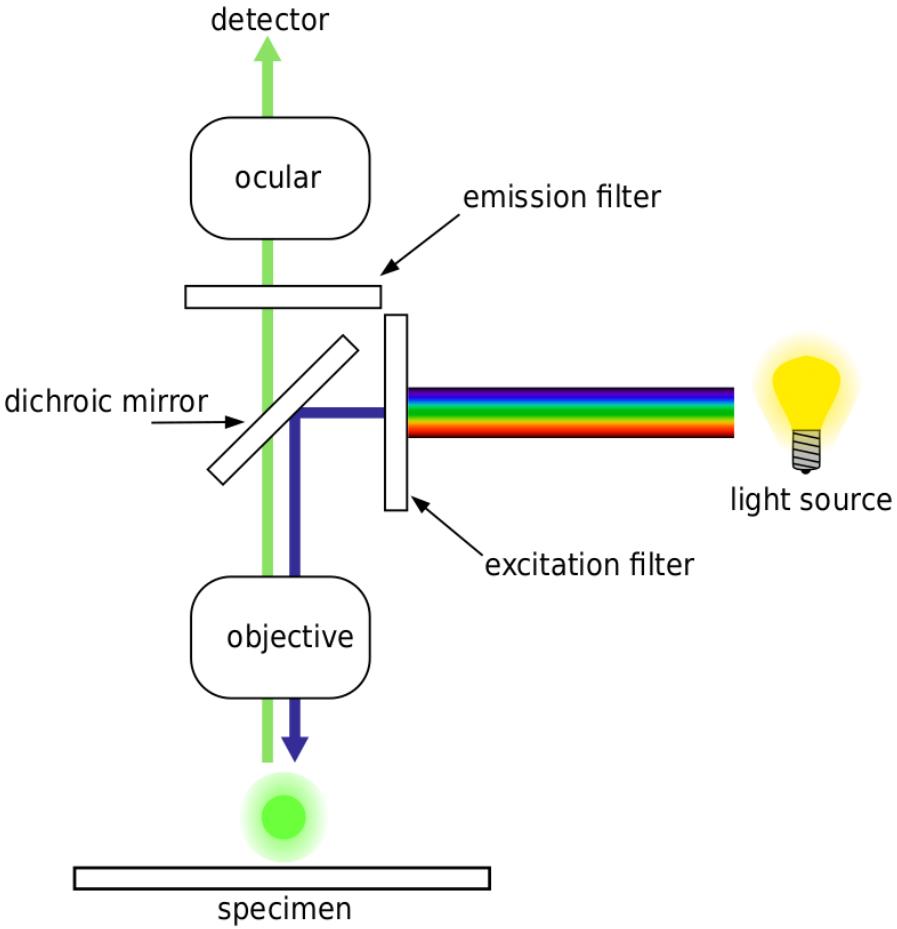
- Techniques based on fluorescence



Fluorophore (or fluorochrome): molecule that absorbs light and re-emits light upon light excitation

Fluorescent techniques

- Usually the emitted light has a **longer wavelength** (red-shifted - lower energy), than the absorbed radiation (excitation light).
- Light sources: high-power LEDs and lasers.
- The illumination light needs to be separated from the much weaker emitted fluorescence through the use of a spectral filter.



Fluorescent proteins (photoproteins)

Source: Wikipedia

- The most widely used fluorescent proteins are derivatives of a native protein from the jellyfish Aequorea victoria.
- Nontoxic
- This jellyfish is capable of producing flashes of blue light by a quick release of calcium (Ca2+) which interacts with photoprotein aequorin.
- The blue light produced is in turn transduced to green by the now famous **green fluorescent protein** (GFP).

The discovery of the GFP was awarded with the **2008 Nobel Prize in Chemistry** (Roger Y. Tsien, Osamu Shimomura, and Martin Chalfie)

Intrinsic and extrinsic fluorophores

- Some proteins or small molecules in cells are naturally fluorescent (intrinsic fluorescence or autofluorescence).
- In nature most molecules of interest are non-fluorescent or their intrinsic florescence is very weak.
- Alternatively, proteins, nucleic acids, lipids or small molecules can be "labelled" with an extrinsic fluorophore, a fluorescent dye which can be a small molecule, protein or quantum dot.
- Genetically engineered GFPs emit different colors and be used simultaneously to monitor independent events in cells.

- **GloFish**: genetically engineered fluorescent fish. Now sold in bright red, green, orange-yellow, etc. One of the first genetically modified animals to become publicly available. Sold only in the US (source: Wikipedia)

Confocal microscopy (also known as Laser Scanning Microscopy, LSM)

- Signals generated by the sample outside of the optical focus are physically blocked by an aperture, preventing their detection.
- Uses point illumination and a pinhole in an optically conjugate plane in front of the detector to eliminate out-of-focus signal.
- Capturing multiple 2D images at different depths in a sample enables the reconstruction of 3D structures (a process known as optical sectioning).
- Video: https://upload.wikimedia.org/wikipedia/commons/thumb/a/a6/Fluorescent_and_confocal_microscopes.ogv/330px--Fluorescent_and_confocal_microscopes.ogv.jpg

Non-fluorescence techniques

- **Raman spectroscopy**: is based on inelastic (Raman) scattering of laser light (visible, near infrared, or near ultraviolet). The laser light interacts with molecular vibrations, phonons or other excitations in the system, resulting in a shift of the photons' energy that gives information about the molecular vibrational modes.
- **Photo-acoustic**: when laser light strikes the target tissue, some of the energy is converted to heat by the cells, resulting in a mechanical wave that can be processed into an image.

  Because different types of tissue absorb laser energy at different rates, lasers can be tuned to target specific tissues.

Tissue-level imaging: tomography

- Optical tomography: creates a digital volumetric model of an object by reconstructing images made **from light transmitted and scattered through an object**.

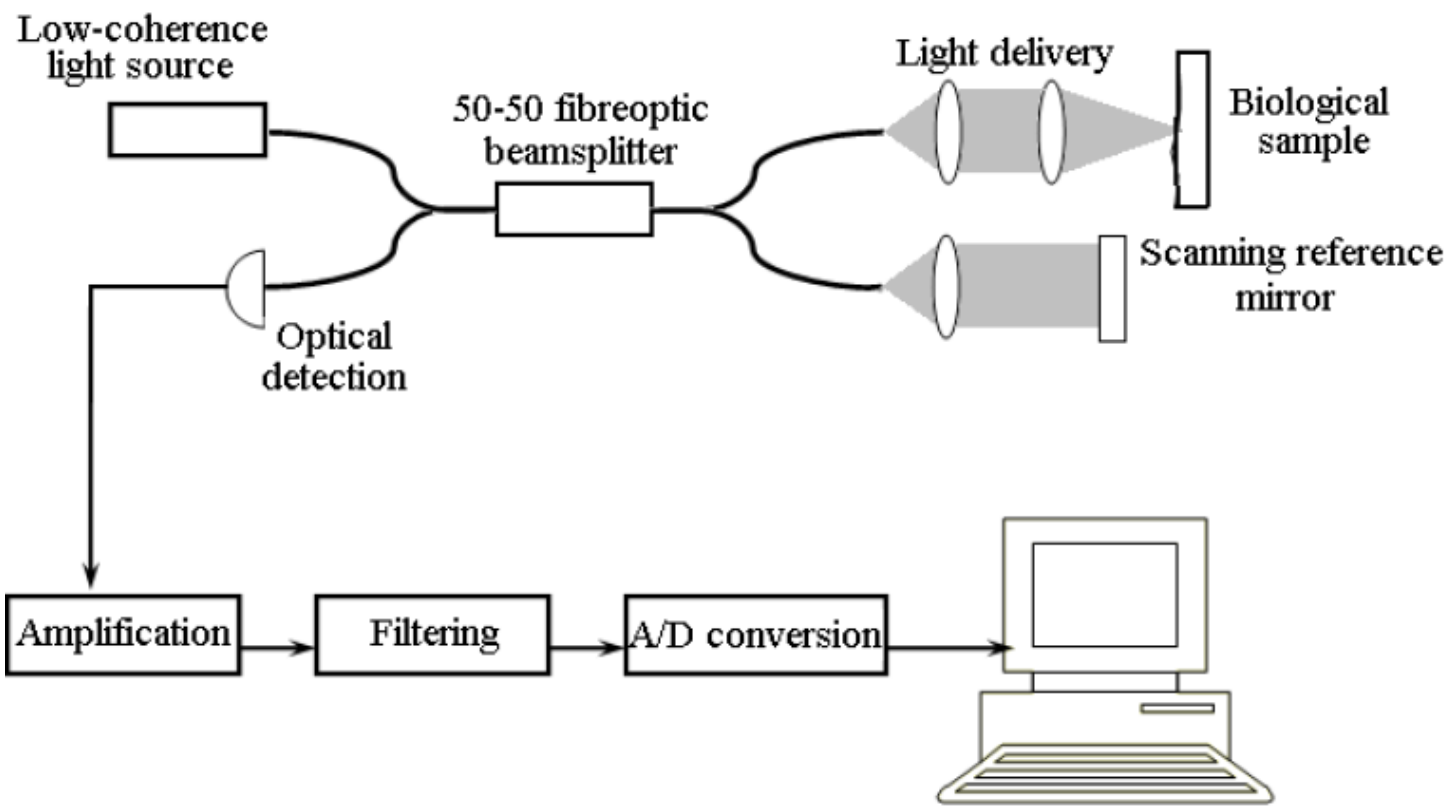
- A tomogram (2-D slice) is produced from image reconstruction algorithms. 3-D reconstructions are made by scanning a detector across the sample.

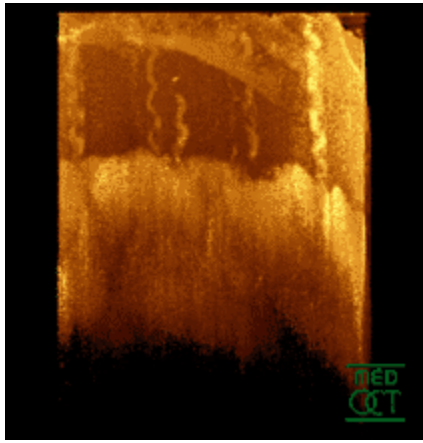
- Many types of tomography for many types of imaging.

Optical coherence tomography (OCT) = optical ultrasound

- Imaging technique that uses pulses of **NIR light** into the tissue (NIR light allow deep penetration); the resultant optical scattering is processed to create high-resolution images at the micrometer scale.
- While ultrasound provides a resolution of tenths of millimeter, OCT can achieve orders-of-magnitude finer resolution (better than 10 µm).
- **Broadband light sources**: superluminescent LEDs, ultrashort pulsed lasers (femtosecond), and supercontinuum lasers.
- Applications: Ophthalmic and tissue imaging that require micrometer resolution and millimeter penetration depth.

OCT setup





OCT of a a fingertip
Source: wikipedia

OCT [videos](#) produced by the Association for Research in Vision and Ophthalmology

OCT operation principle

- OCT is based on low-coherence interferometry.
- In conventional interferometry with long coherence length (i.e., laser interferometry), interference of light occurs over a distance of meters. In OCT, this interference distance is shortened to micrometers (due to the broad-bandwidth light source).
- Light in an OCT system is broken into two arms—a sample arm (containing the item of interest) and a reference arm (usually a mirror).
- The combination of reflected light from the sample arm and reference light from the reference arm gives rise to an interference pattern, but light that is outside the short coherence length will not interfere.

Comparison of biomedical imaging techniques

- Medical ultrasonography and magnetic resonance imaging (MRI): whole body but low resolution imaging capability (typically a fraction of a millimeter)

- Confocal microscopy: resolutions well below 1 micrometer (i.e. sub-cellular), between 0 and 100 micrometers in depth

- OCT can probe as deep as 500 micrometers with resolution around 10 micrometers in lateral and a few micrometers in depth.

	Δf	Primary contrast	Δz	δz	δx	Speed
	Hz		*mm*	*μm*	*μm*	*Mvx/s*
Photoacoustic microscopy	50 M	Optical absorption	3	15	45	0.5
Photoacoustic tomography	5 M	Optical absorption	50	700	700	0.5
Confocal microscopy		Fluorescence, scattering	0.2	3-20	0.3-3	10-100
Two-photon microscopy		Fluorescence	0.5-1.0	1-10	0.3-3	10-100
Optical coherence tomography	300 T	Optical scattering	1-2	0.5-10	1-10	20-4.000
Scanning Laser Acoustic Microscopy	300 M	Ultrasonic scattering	1-2	20	20	10
Acoustic microscopy	50 M	Ultrasonic scattering	20	20-100	80-160	0.1
Ultrasonography	5 M	Ultrasonic scattering	60	300	300	1

Comparison of contrast mechanisms, penetration depth (Δz), axial resolution (δz), lateral resolution (δx=δy) and imaging speed.

Source: wikipedia

FOCUS ON TWO EXAMPLES:

- SUPER-RESOLUTION FLUORESCENCE MICROSCOPY

- PHOTOACOUSTIC TOMOGRAPHY/MICROSCOPY

“Super-resolution” fluorescence microscopy

- Fluorescence microscopy allows imaging cells and tissues.
- Various “super-resolution” optical imaging techniques:
  - STED: stimulated emission depletion microscopy
  - STORM: stochastic optical reconstruction microscopy
  - PALM: photo-activated localization microscopy
  - FRET: fluorescence resonance energy transfer
  - Etc.
- Tens nm resolution in the far field has been achieved, allowing probing molecular interactions on the nm scale.

STORM imaging of microtubules in a mammalian cell

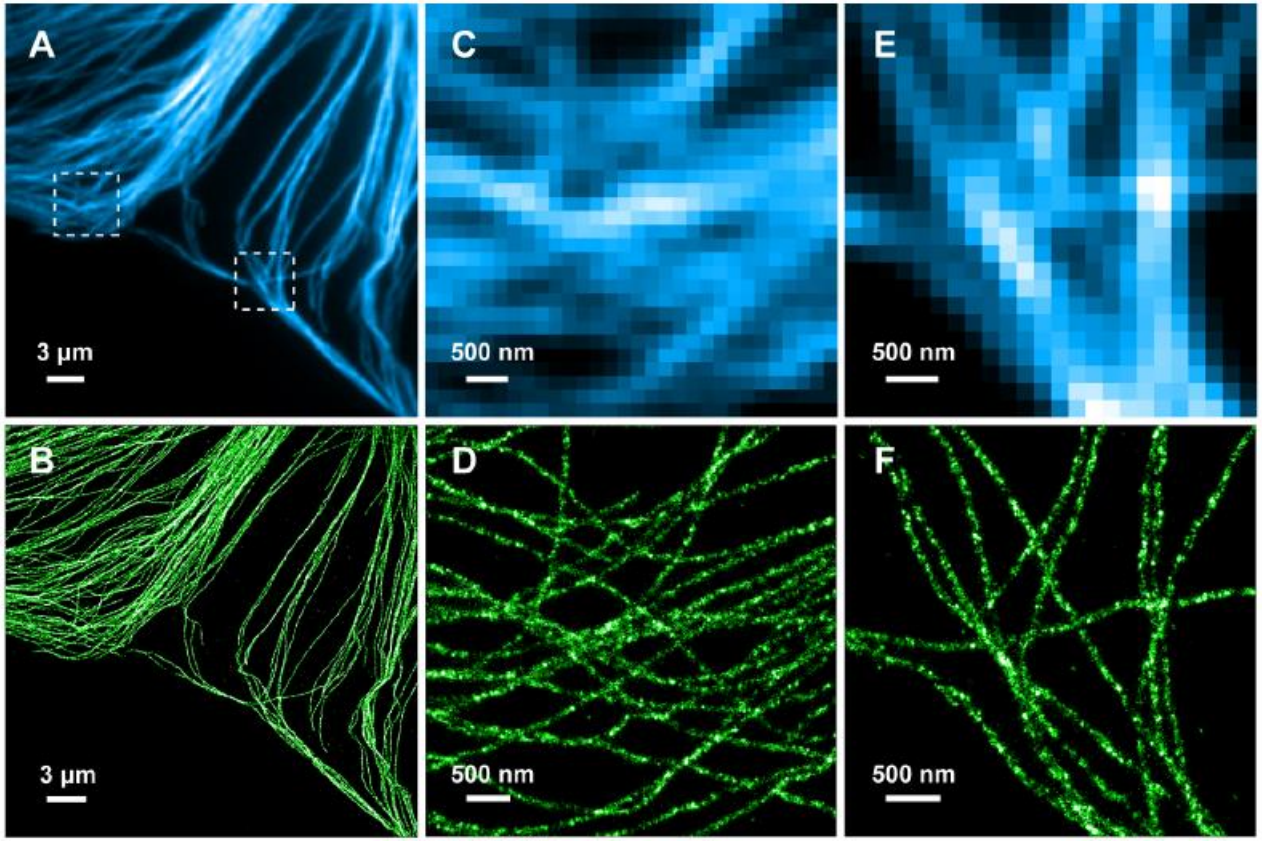
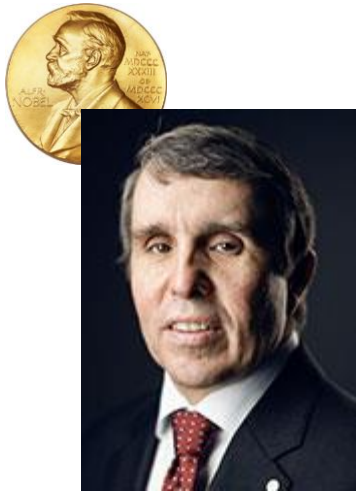


Photo-switchable dyes:
A red laser (657nm), excited the molecules to fluorescent state, that then quickly switched into a non-fluorescent, dark state.
A green laser pulse (532 nm) led to reactivation of the dyes back to the fluorescent state

Bates et al, Science 317, 1749 – 1753 (2007)

The Nobel Prize in Chemistry 2014



Eric Betzig

Stefan W. Hell

William E. Moerner

"*for the development of <u>super-resolved fluorescence microscopy</u>*".

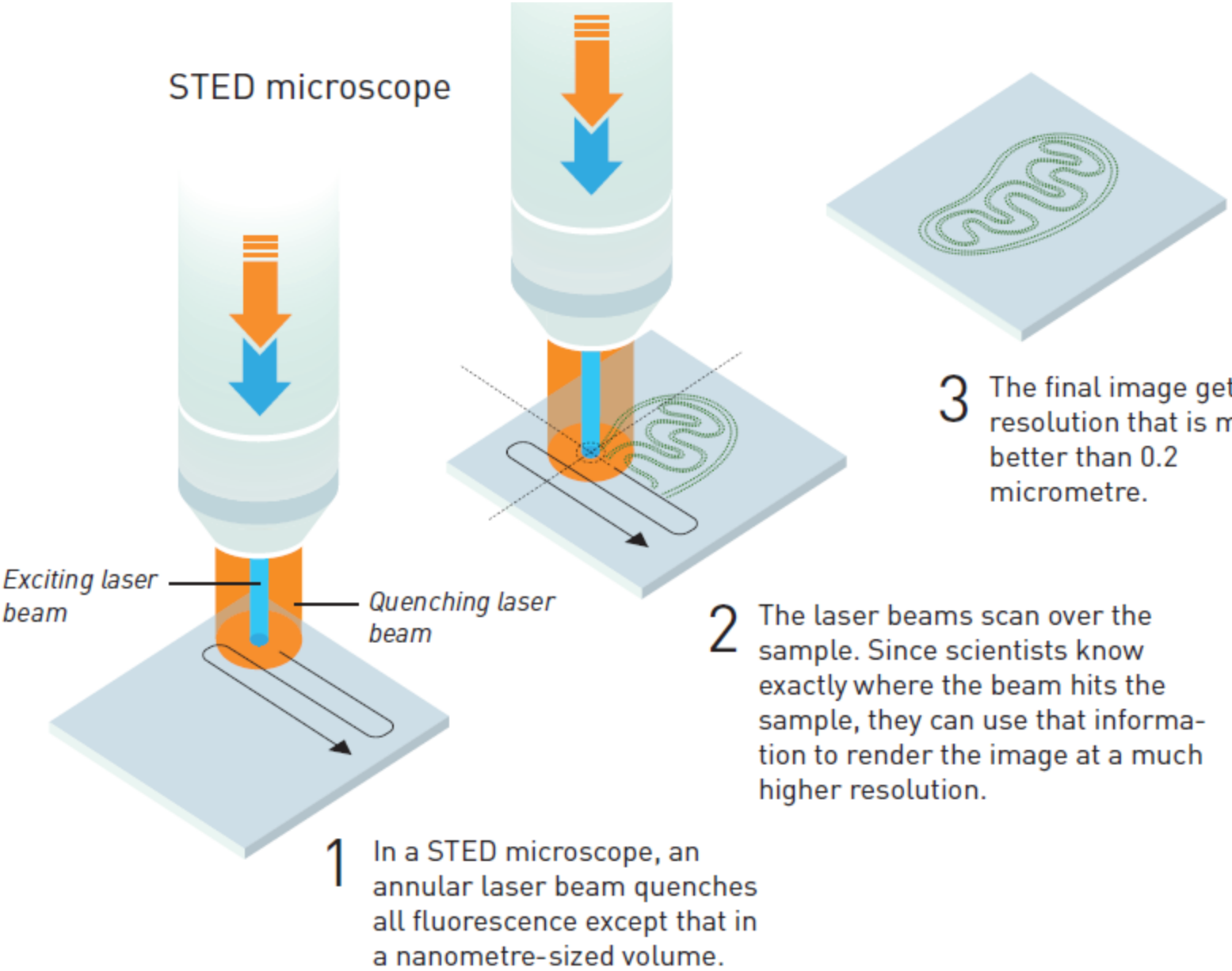
United Nations Educational, Scientific and Cultural Organization

International Year of Light 2015

Beating the diffraction limit via **stimulated emission** of **fluorescent molecules**

- Stimulate Emission Depletion (**STED**) microscopy (developed by **Stephan Hell**):

  molecules are first excited by a focused laser beam and then de-excited by a second laser with a doughnut-shaped focus, so that only few molecules in the center of the doughnut remain in their excited state and their fluorescence serves as measure signal.


STED microscope
Exciting laser beam
Quenching laser beam
1 In a STED microscope, an annular laser beam quenches all fluorescence except that in a nanometre-sized volume.
2 The laser beams scan over the sample. Since scientists know exactly where the beam hits the sample, they can use that information to render the image at a much higher resolution.
3 The final image gets a resolution that is much better than 0.2 micrometre.

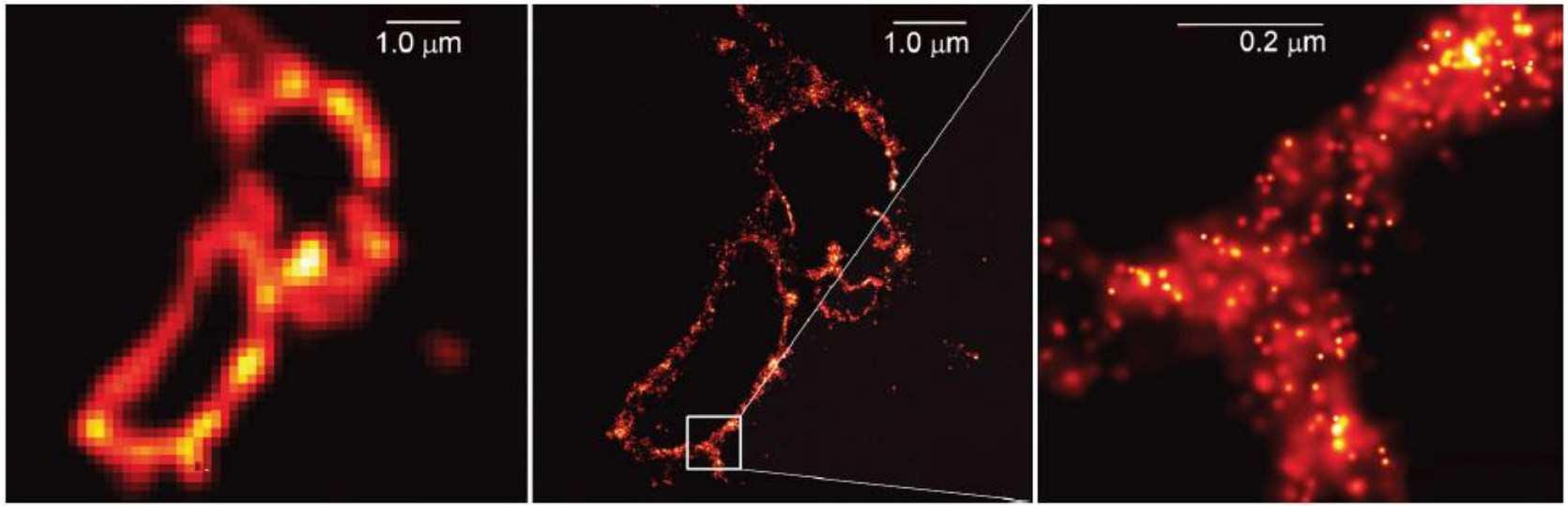
The path to single-molecule microscopy

- **William E. Moerner** discovered that it is possible to optically control fluorescence of single molecules.
- The fluorescence of one variant of a *green fluorescent protein* (GFP) could be turned on and off at will.
- The strong green color of a GFP protein appears under blue and ultraviolet light.
- When Moerner excited the protein with light of wavelength 488 nanometres the protein began to fluoresce, but after a while it faded.
- Regardless of the amount of light he then directed at the protein, the fluorescence was dead.
- But light of wavelength 405 nm could bring the protein back to life again.
- When the protein was reactivated, it once again fluoresced at 488 nm.

$\Rightarrow$ individual molecules act like tiny lamps with switches

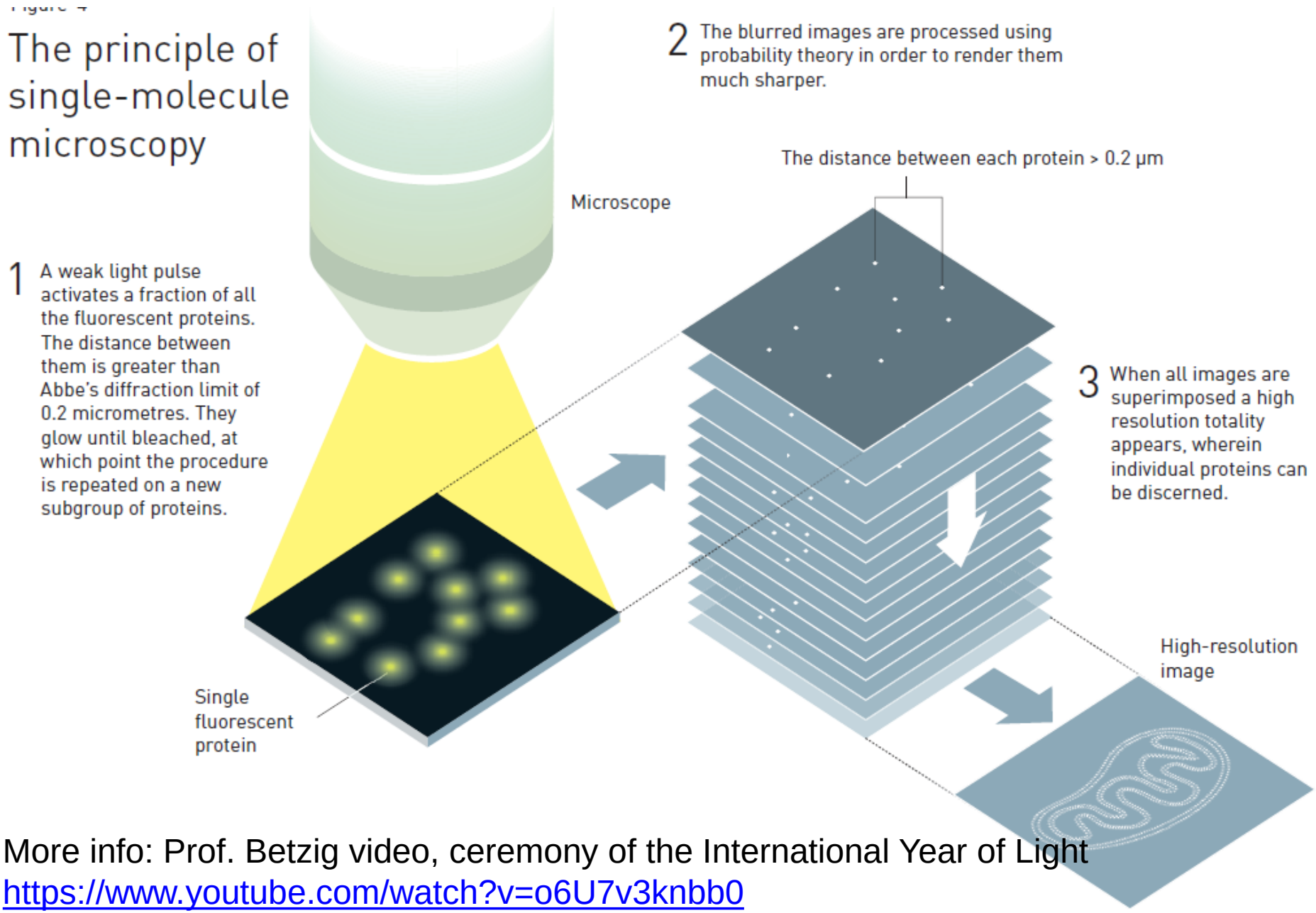
And by using light pulses

- **Eric Betzig**: a super-resolution image can be obtained by using fluorescent molecules that fluoresce at different times & <u>superimposing the images</u>.



conventional microscopy single-molecule microscopy

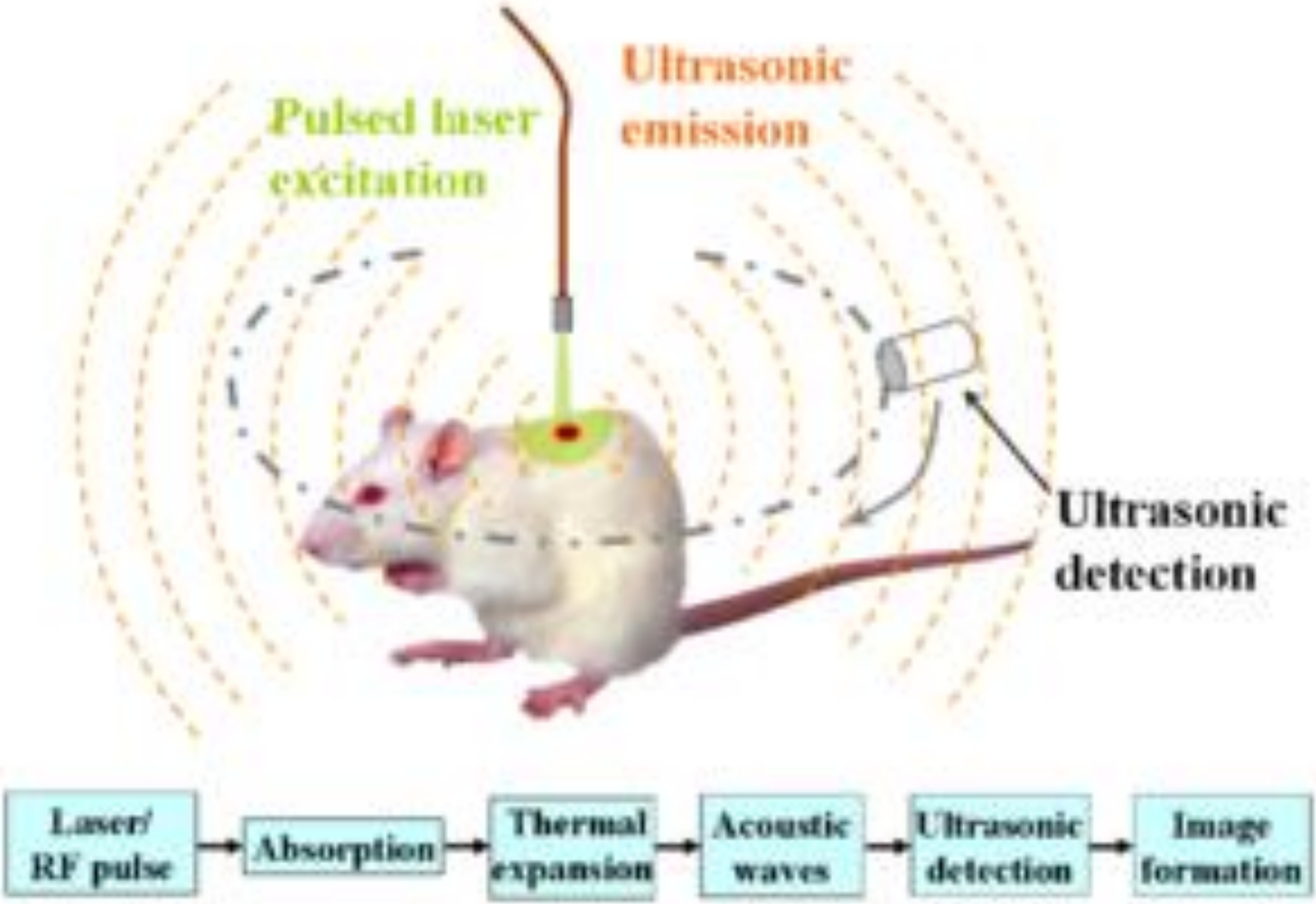
Science 313:1642–1645 (2006)



More info: Prof. Betzig video, ceremony of the International Year of Light
https://www.youtube.com/watch?v=o6U7v3knbb0

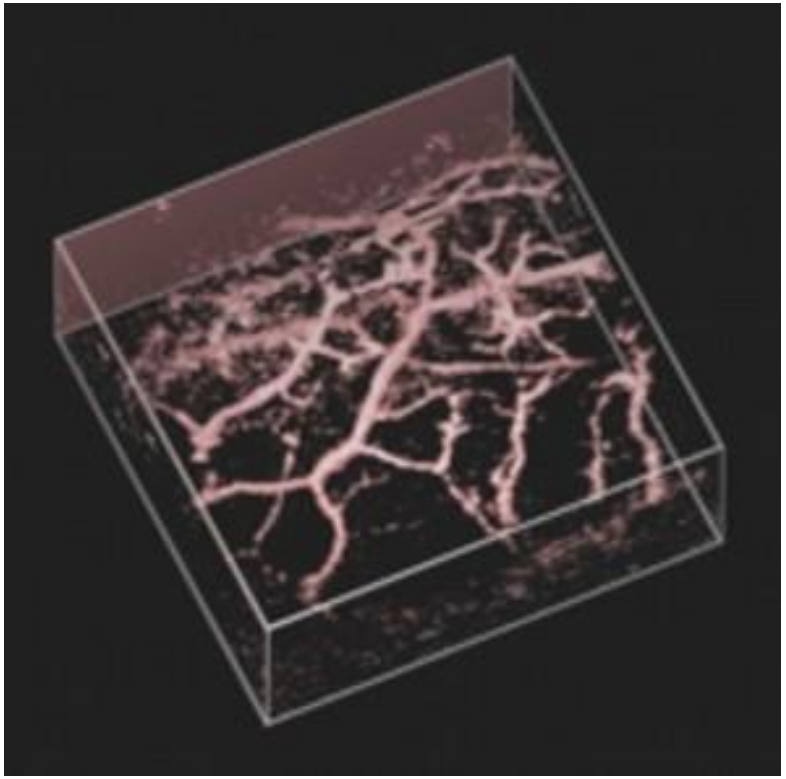
Photoacoustic tomography

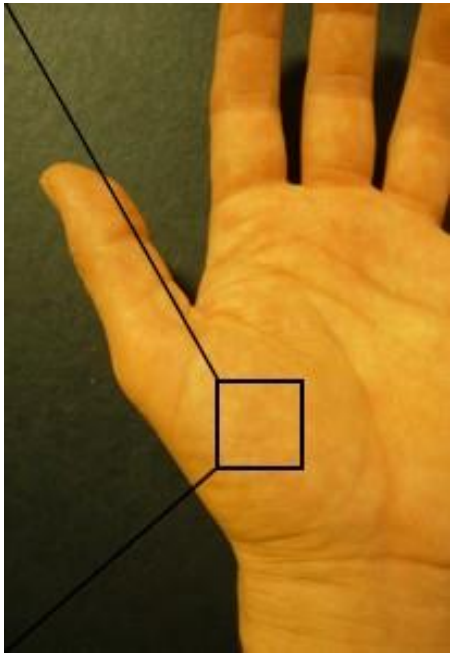
- Non-invasive imaging based on the photo-acoustic (PA) effect.
- **Optical pulses** are delivered into the tissue. Part of the energy is absorbed and converted into heat, leading to transient thermo-elastic expansion and thus, ultrasonic emission.
- The generated acoustic waves allow visualising the internal structure and function of soft tissues.
- The PA signal depends on optical properties, thermal diffusivity/expansion and elastic properties.
- Returning ultrasound is detected at tissue surface by a transducer (single transducer, a line array, or a circular array can be used for detection).
- Since blood has orders of magnitude higher absorption than surrounding tissues, there is sufficient endogenous contrast for photoacoustic imaging to visualize blood vessels.

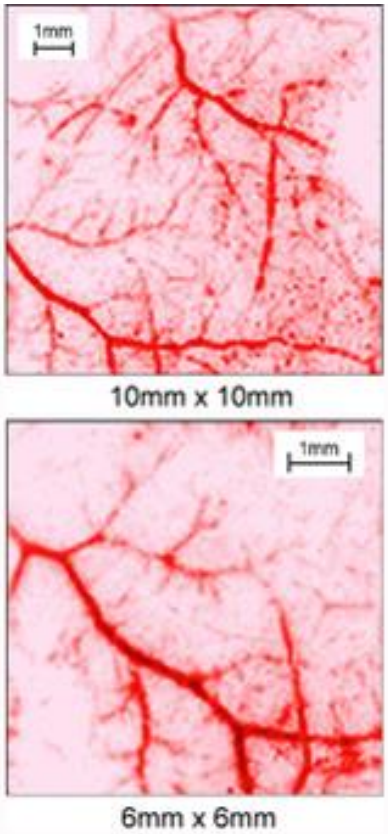


Source: Wikipedia

Photoacoustic visualizes blood vessels







Volume rendered *in vivo* photoacoustic image of the vascular anatomy in the palm of the hand. Image volume: 20mm x 20mm x 6mm. Excitation wavelength λ=670nm. Incident fluence: 8mJ/cm^2. No signal averaging was used. Image acquisition time: 15 minutes.

Source: Photoacoustic Imaging Group, Univ. College London

TF

- Fluorophores absorb light of a specific wavelength and re-emit light at a longer wavelength.
- Fluorescent techniques are based on illuminating the sample with light of a specific wavelength, which is absorbed and causes the emission of fluorescence light of shorter wavelength.
- The green fluorescent protein (GFP) is a protein that exhibits green fluorescence when exposed to light in the blue to ultraviolet range.
- OCT is based on optical scattering of tissue and uses low-coherence light.
- Conventional wide-field microscopes allow obtaining high-quality images of sub-wavelength structures.
- Laser Scanning Microscopy allows the visualization of 3D structures.
- In photoacoustic imaging, light pulses are delivered into the sample, where part of the energy is absorbed and re-emitted as ultrasound.

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