TOMSK POLYTECHNIC UNIVERSITY

Lecture 7
Part 1. Optical Microscopy

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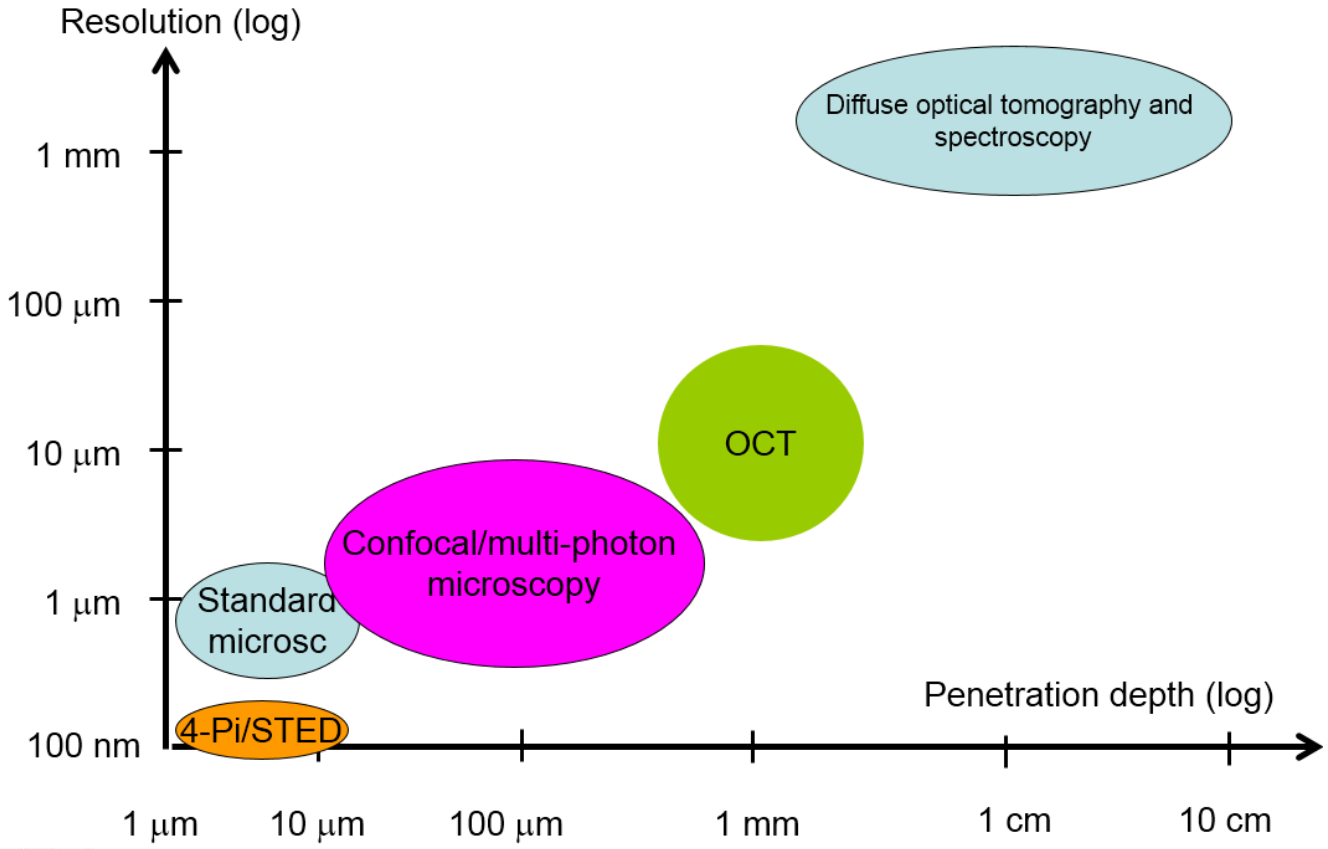
Optical Methods of Research

Main questions:

1) What is the required depth of penetration?
2) What is the acceptable resolution?
3) What type of information is needed?

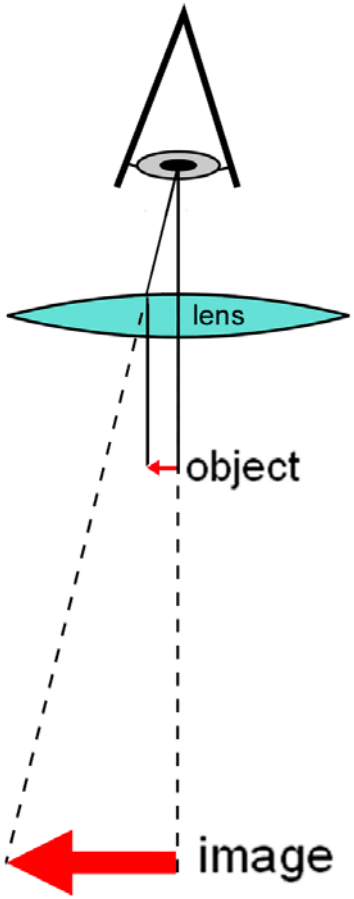


Optical Microscopy

The optical microscope or light microscope is a type of microscope, which uses visible light and a system of lenses to magnify images of small samples.

A simple microscope uses a lens or set of lenses to enlarge an object through angular magnification alone (Fig.1), giving the viewer an erect enlarged virtual image.

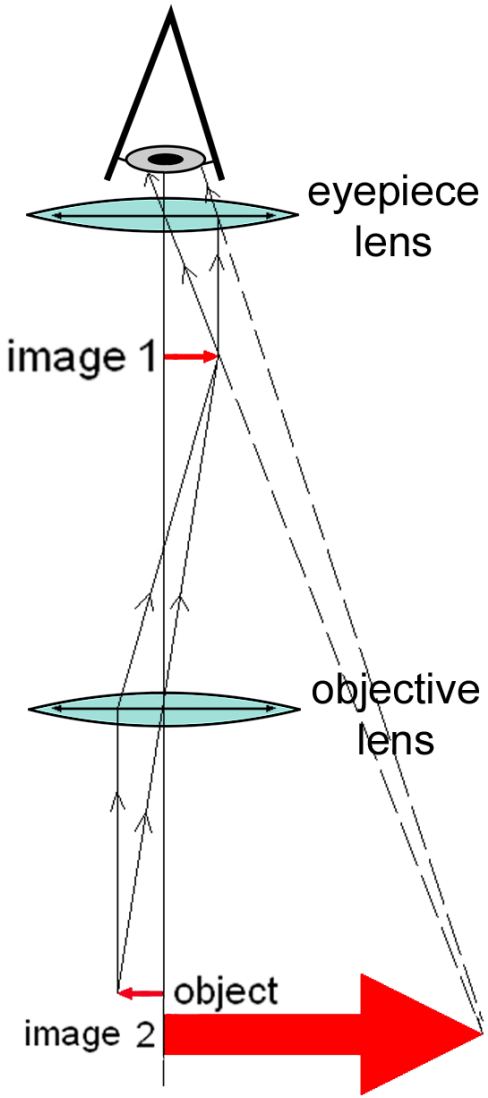
The magnification produced by a single lens is approximately $25/f$ where f is the focal length of the lens in centimeters. To increase the magnification the focal length must be reduced, which in practice requires the lens to have more sharply curved surfaces. A x10 magnifier is already a bulbous piece of glass and shows various defects in image formation.



Standard Microscopy

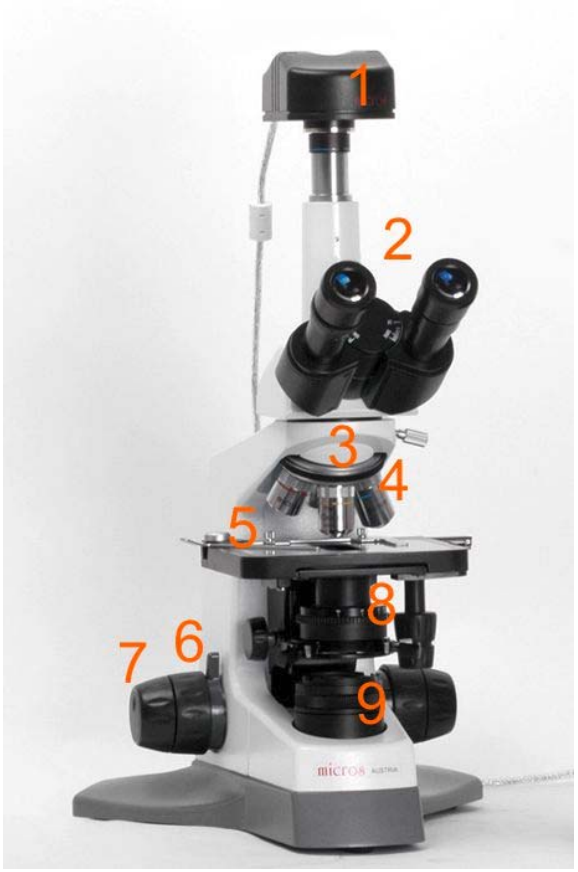
A compound microscope uses a lens close to the object being viewed to collect light (called the objective lens) which focuses a real image of the object inside the microscope (image 1).

A second lens or group of lenses (called the eyepiece) that gives the viewer an enlarged inverted virtual image of the object (image 2) then magnifies that image. The use of a compound objective/eyepiece combination allows for much higher magnification. Common compound microscopes often feature exchangeable objective lenses, allowing the user to quickly adjust the magnification. A compound microscope also enables more advanced illumination setups, such as phase contrast.



Trinocular microscope

Some of modern microscopes are trinocular. A trinocular microscope has two eyepieces like a binocular microscope and an additional third eye tube for connecting a microscope camera. They are therefore a binocular with a moving prism assembly in which light is directed either to the binocular assembly of the microscope or to the camera. The best models of this microscope will have at least three positions, allowing 100 percent of light to the binocular, 80 percent to camera and 20 percent to the binocular or simply a 100 percent to the camera.



The main components of modern optical microscope:

- Camera (1)
- Eyepiece (ocular lens) (2)
- Objective turret, revolver, or revolving nose piece (to hold multiple objective lenses) (3)
- Objective lenses (4)
- Stage (to hold the specimen) (5)
- Focus knobs (to move the stage): coarse adjustment (6), fine adjustment (7)
- Diaphragm and condenser (8)
- Light source (a light or a mirror) (9).

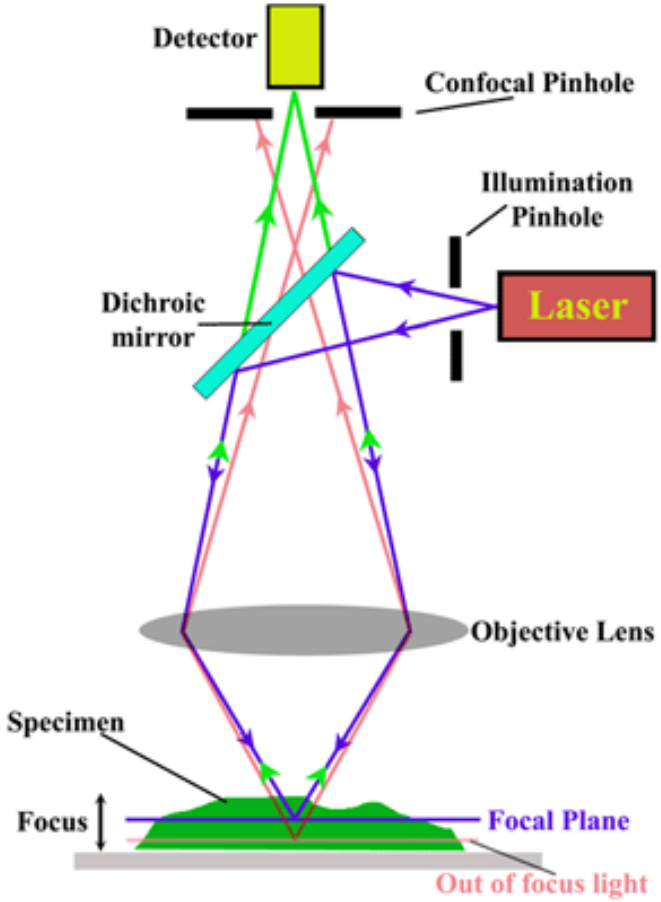
Confocal Microscopy

In traditional microscopy, the resolution that can be obtained is limited by the diffraction of light. The equation is:

$$D = \frac{\lambda}{2NA}$$

where D is the diffraction limit, λ is the wavelength of the light, and NA is the numerical aperture, or the refractive index of the medium multiplied by the sine of the angle of incidence.

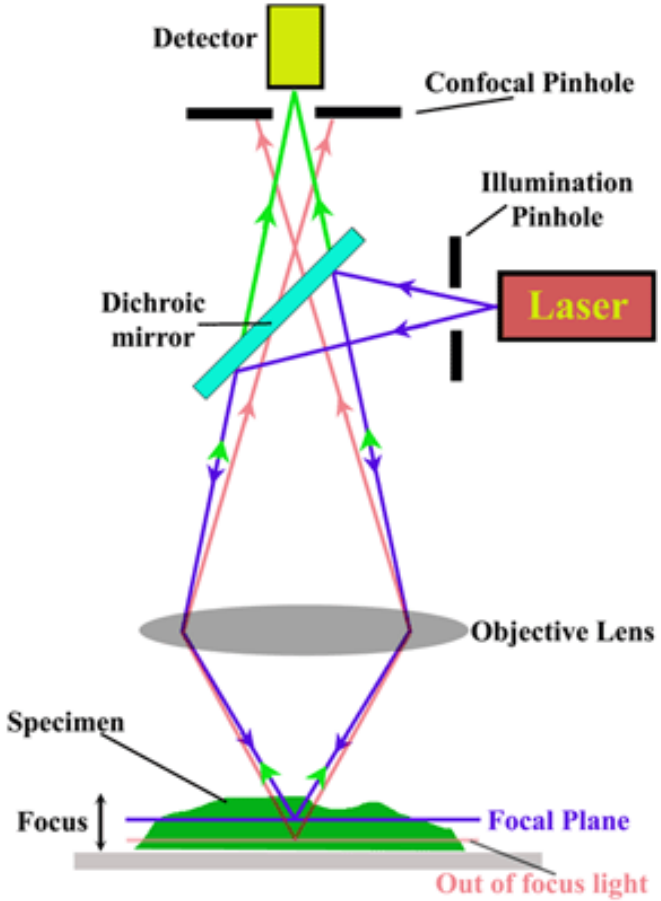
An important step in the evolution of modern optical imaging has been the introduction of confocal optics and the confocal laser-scanning microscope. In this method, a diffraction-limited excitation spot within the specimen is imaged onto a confocal aperture, which is positioned in front of a point detector.



Confocal Microscopy

Another problem of conventional light microscopy is out-of-focus blur degrading the image by obscuring important structures of interest, particularly in thick specimens. In conventional microscopy, not only is the plane of focus illuminated, but much of the specimen above and below this point is also illuminated resulting in out-of-focus blur from these areas. This out-of-focus light leads to a reduction in image contrast and a decrease in resolution.

In the confocal microscope, all out-of-focus structures are suppressed at image formation. This is obtained by an arrangement of diaphragms, which, at optically conjugated points of the path of rays, act as a point source and as a point detector respectively. The detection pinhole does not permit rays of light from out-of-focus points to pass through it. The wavelength of light, the numerical aperture of the objective and the diameter of the diaphragm affect the depth of the focal plane.



Confocal Microscopy

To obtain a full image, the point of light is moved across the specimen by scanning mirrors. The emitted/reflected light passing through the detector pinhole is transformed into electrical signals by a photomultiplier and displayed on a computer monitor.

Major improvements offered by a confocal microscope over the performance of a conventional microscope may be summarized as follows:

- Light rays from outside the focal plane will not be recorded.
- Defocusing does not create blurring, but gradually cuts out parts of the object as they move away from the focal plane. The practical consequence is that these parts become darker and eventually disappear. This feature is called optical sectioning.
- True, three-dimensional data sets can be recorded.
- Scanning the object in x/y-direction as well as in z-direction (along the optical axis) allows viewing the object from all sides.
- Due to the small dimension of the illuminating light spot in the focal plane, stray light and photo bleaching are minimized.
- By image processing, many slices can be superimposed giving an extended focus image. This can only be achieved in conventional microscopy by reduction of the aperture and thus sacrificing resolution.

Confocal Microscopy

Four types of confocal microscopes are commercially available:

Confocal laser scanning microscopes use multiple mirrors (typically 2 or 3 scanning linearly along the x-and the y-axis) to scan the laser across the sample and "descan" the image across a fixed pinhole and detector.

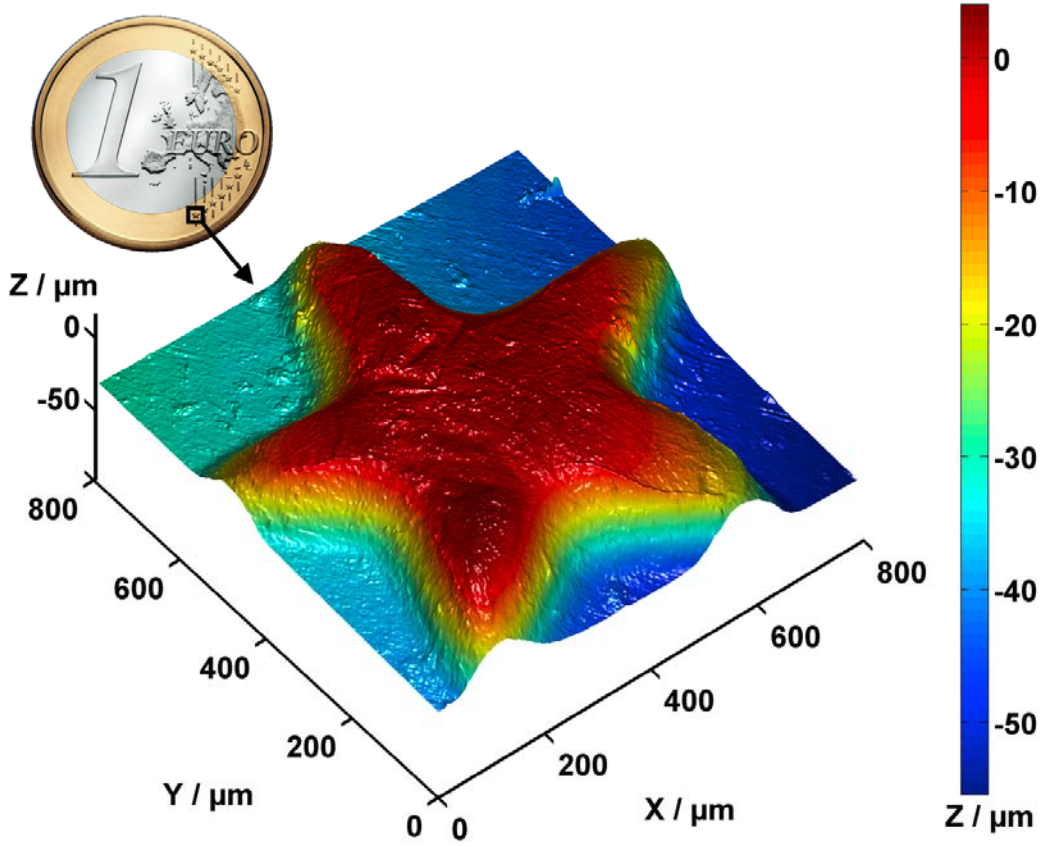
Spinning-disk (Nipkow disk) confocal microscopes use a series of moving pinholes on a disc to scan spots of light. Since a series of pinholes scans an area in parallel, each pinhole is allowed to hover over a specific area for a longer amount of time thereby reducing the excitation energy needed to illuminate a sample.

Microlens enhanced or dual spinning disk confocal microscopes work under the same principles as spinning-disk confocal microscopes except a second spinning disk containing micro-lenses is placed before the spinning disk containing the pinholes. Every pinhole has an associated micro-lens.

Programmable array microscopes (PAM) use an electronically controlled spatial light modulator (SLM) that produces a set of moving pinholes. The SLM contains microelectromechanical mirrors or liquid crystal components. The image is usually acquired by a charge coupled device (CCD) camera.

Confocal Microscopy

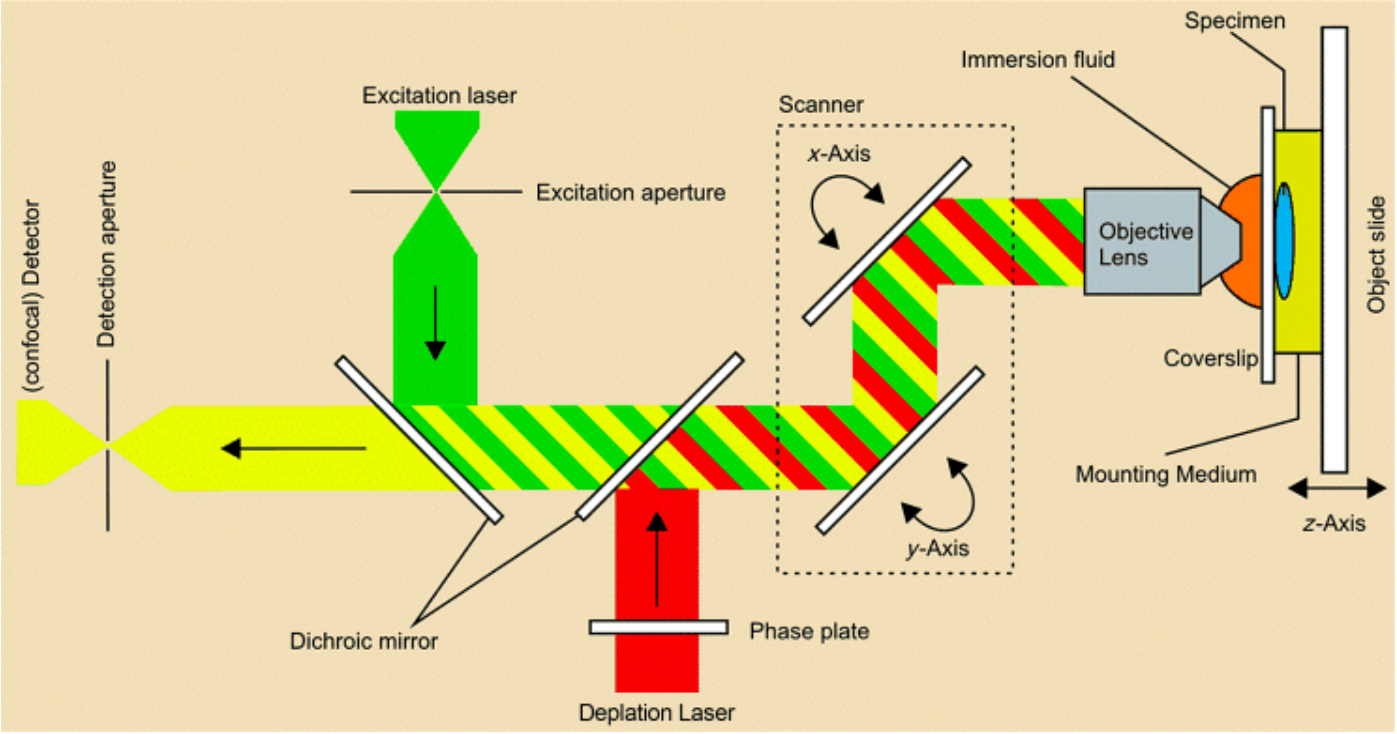
Partial surface profile of a 1-Euro coin, measured with a Nipkow disk confocal microscope. Three dimensional representation of a 1-euro coin's surface detail: One of the stars embossed on one side of the coin is shown. The 3d profile has been measured with a confocal white light microscope. The lateral range of the measurement is 800 µm by 800 µm (0.8 mm by 0.8 mm), the vertical range is approximately 60 µm (0.06 mm). The colors of the surface show the height according to the scale on the right side of the image.



https://en.wikipedia.org/wiki/Confocal_microscopy

STED / Fluorescence Microscopy

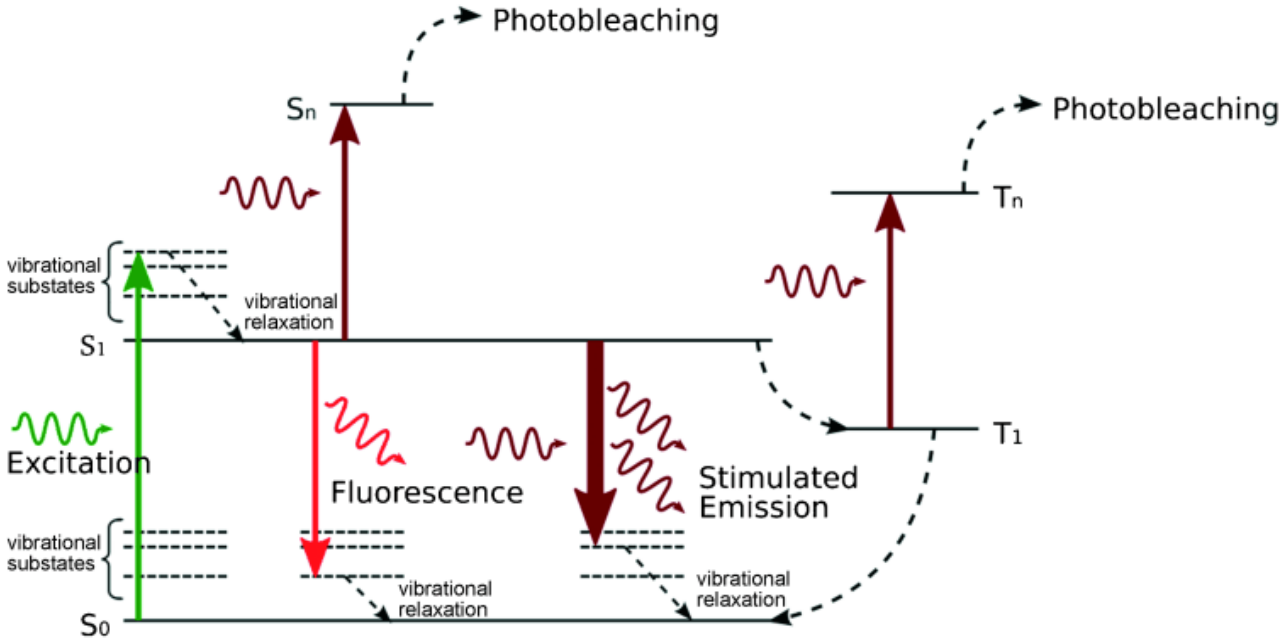
The spectroscopic methods implemented in modern confocal microscopes encompass among others time-resolved spectroscopy (FLIM, fluorescence lifetime imaging), photoselection and polarization anisotropy measurements and fluorescence correlation spectroscopy.



Optical path and location of the sample in a conventional laser-scanning microscope. The laser beam scans the sample in xy direction first. The focal plane can be adjusted by variation of the distance between sample and objective (axial, along the z-axis). With a depletion laser, the configuration corresponds to a STED microscope.

STED / Fluorescence Microscopy

The basic idea behind STED microscopy is the spatially selective deactivation of fluorophores, preventing their participation in image formation:

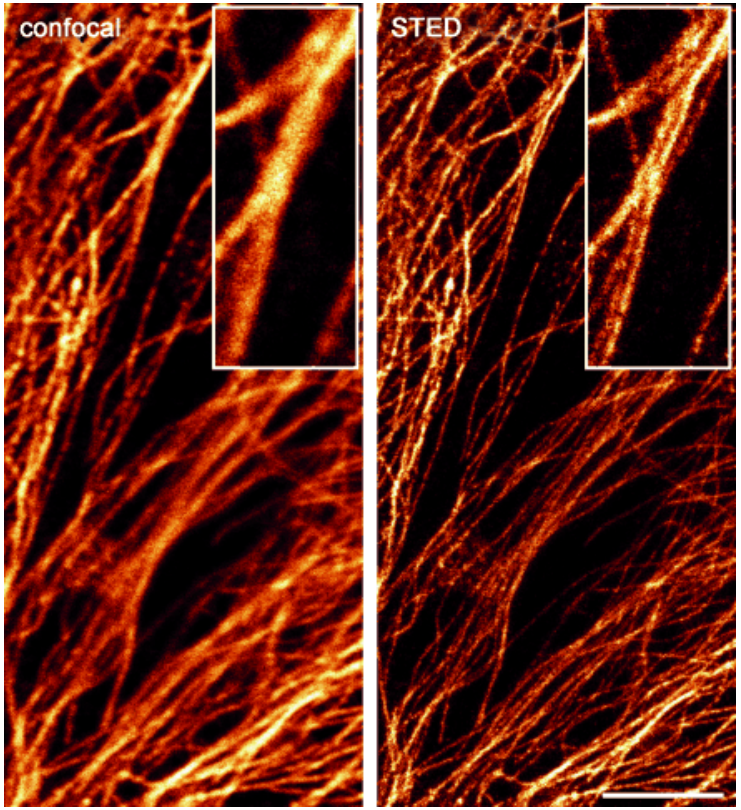


After initial excitation from the S0 to the S1 state and vibrational relaxation, fluorophores can emit fluorescence photons. Alternatively, the fluorescence can be silenced by driving a stimulated emission transition into a higher vibrational substrate of the electronic ground state S0. The red-shifted stimulated emission light can be spectrally separated during detection. The bleaching pathways indicated in the diagram are addressed in the text.

STED / Fluorescence Microscopy

Structural Analyses by Means of STED Microscopy

STED microscopy has opened up a field of application for fluorescence microscopy that had previously been an exclusive domain of electron microscopy: The analysis of protein structure and distribution on the sub-organelle level. In proof-of-principle studies, cytoskeletal filaments are most often used as a standard to demonstrate superresolution. Neurofilaments, actin and, most frequently, tubulin are routinely imaged for comparisons of confocal and STED resolving power.



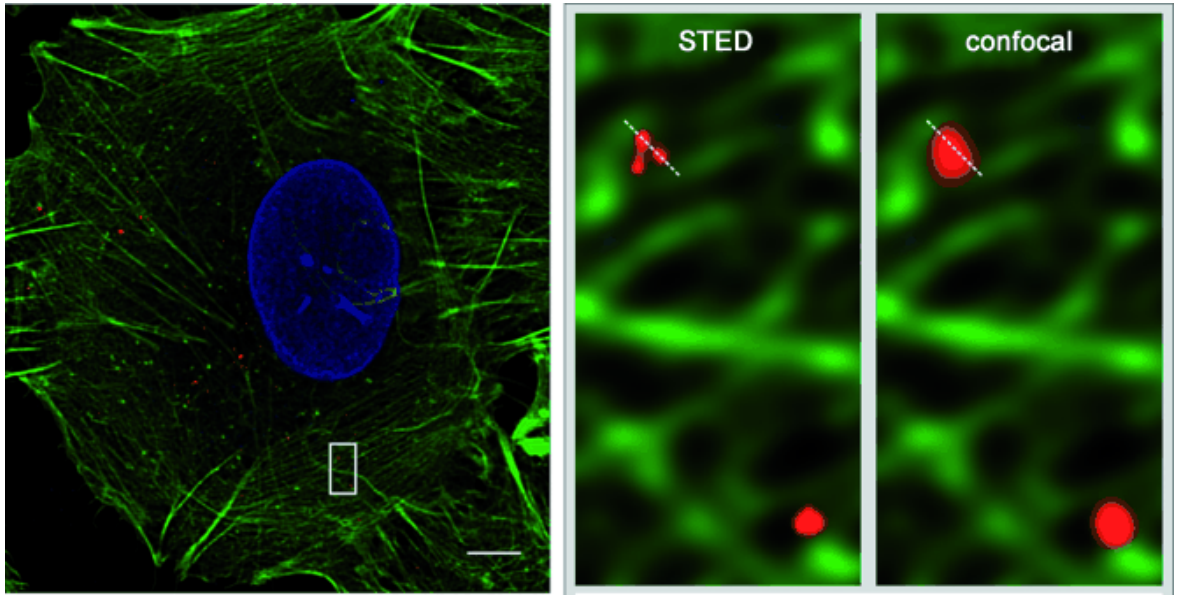
Microtubules of a Caco-2 cell stained with Atto647N. Comparison of confocal mode (left) and STED mode (right) at about 80 nm lateral resolution. Some large, ill-defined structures from the confocal recording emerge as bundles of multiple filaments after the depletion laser is turned on. Scale bar: 5 µm.

STED / Fluorescence Microscopy

Live-Cell STED Microscopy

Combining STED microscopy with fluorescence correlation spectroscopy (FCS), is possible to reach nanometer resolution.
Fluorescent proteins, which can be expressed in a site-specific manner fused to target proteins, provide the versatility to visualize almost any organelle or protein distribution in a living cell. This feature has been implemented for STED microscopy. After the principle had been proven, the method was shown to work at 50 nm lateral resolution

A549 cells after 48 h exposure to 80 nm silica particles. The particles have been internalized and have started to migrate towards the nucleus. The enlarged comparison between confocal and STED recording as seen. The distance of 180 nm is between two of the three clustered particles, which is below the limit of confocal resolution.



Green: actin stained with Alexa 488, blue: lamin B stained with Alexa 546 (both confocal), red: 80 nm silica particles fully labeled with Atto647N (STED). Scale bar: 5 µm.

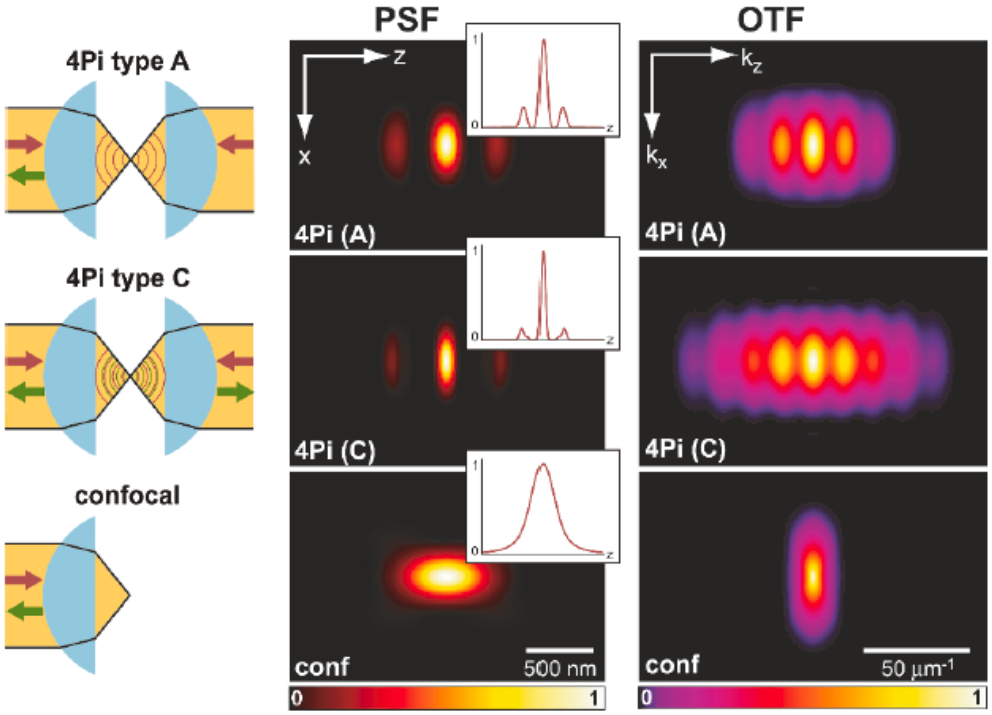
STED / Fluorescence Microscopy

4Pi Microscopy

A 4Pi microscope is a laser scanning fluorescence microscope with an improved axial resolution. The typical value of 500–700 nm can be improved to 100–150 nm, which corresponds to an almost spherical focal spot with 5–7 times less volume than that of standard confocal microscopy.

In Type A 4Pi microscopy, the sample placed between two coverslips is coherently illuminated by two opposing objective lenses focusing a pulsed infrared laser beam into the same spot. The 2PE fluorescence is collected from a single side and focused onto a (variable) confocal pinhole.

In Type C 4Pi microscopy, the fluorescence is collected by both lenses and coherently combined at the detector. Due to the combination of the interference in excitation and detection, the Type C mode exhibits lower sidelobes and a by ~30% increased axial resolution compared to Type A.

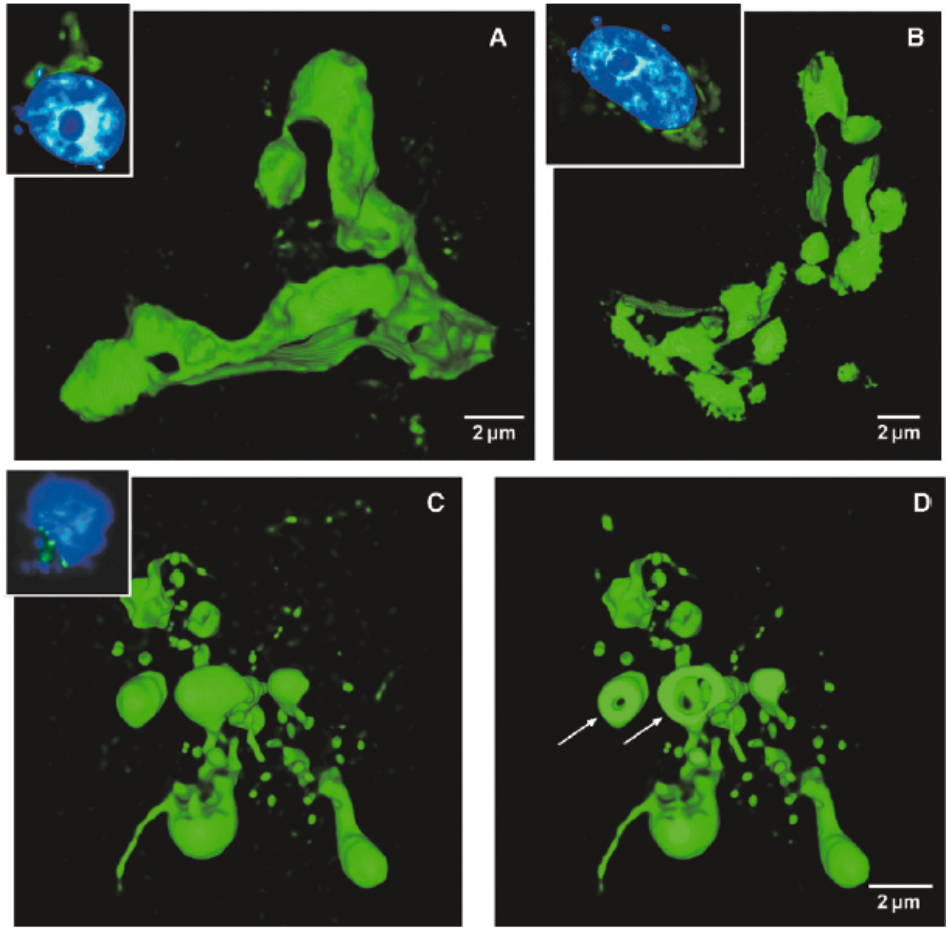


PSF - point spread function, OTF – optical transfer function

STED / Fluorescence Microscopy

4Pi Microscopy

Golgi apparatus as obtained through 4Pi confocal imaging of GalT-EGFP (A) and 2-OSTEGFP (B) in living Vero cells at ~100 nm axial resolution. The inset in the upper left-hand corner of Figure displays a conventional overview xy-image of the GalTEGFP–labeled Golgi apparatus located close to the nucleus counterstained with the fluorophore Hoechst. The central panel of Fig. displays the corresponding 3D surface-rendered image at 100 nm resolution. The 3D image clearly reveals ribbons, fractioned stacks, as well as smaller tubular and vesicular sub-compartments.

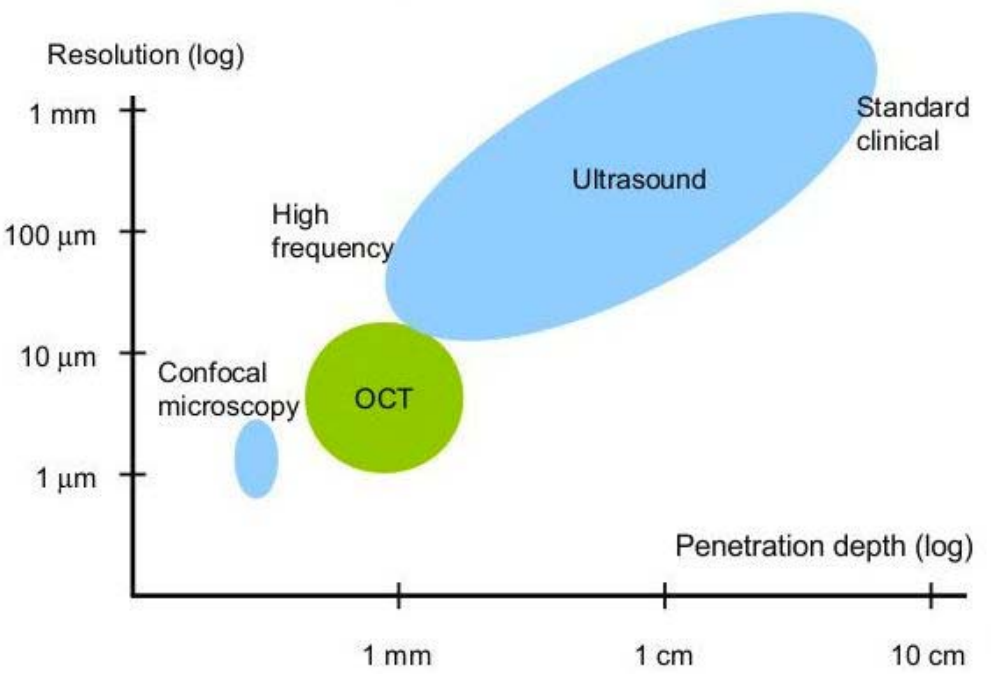


The data clearly pinpoint the ability of the 4Pi microcope to resolve small structures like the cavities which become apparent when the upper caps of the balloon-like structures are removed (D)

Optical coherence tomography (OCT)

Advantages:

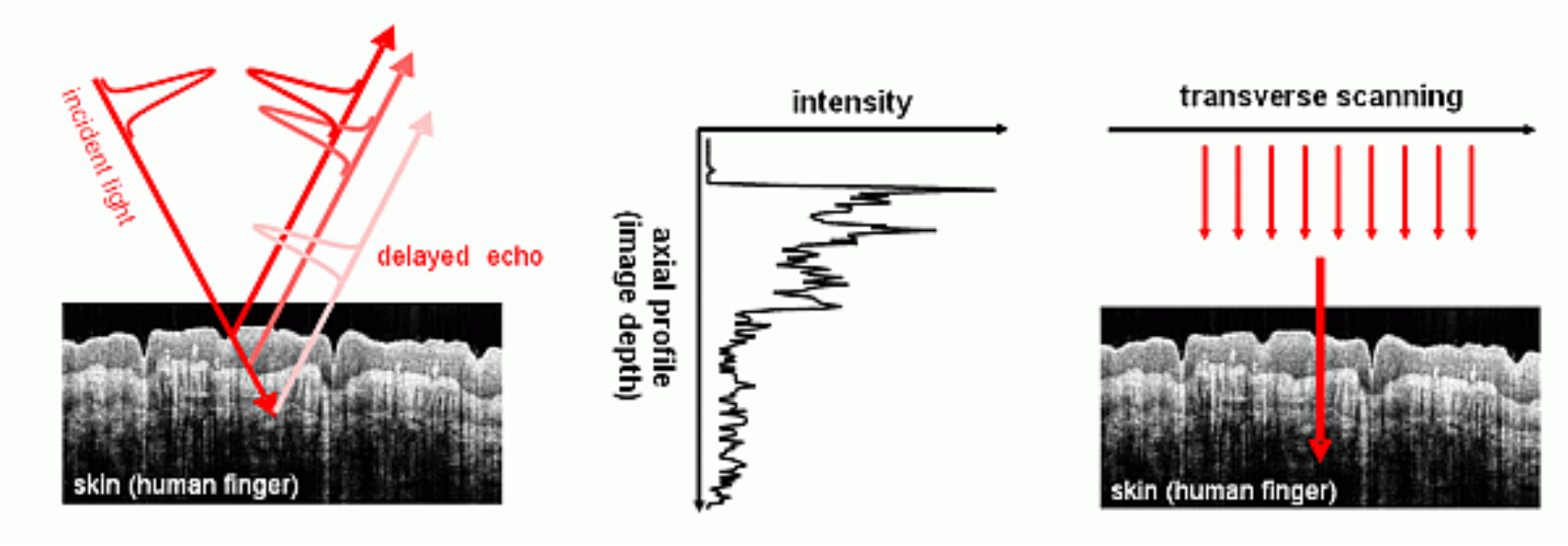
- Image resolutions of 1 to 15 µm can be achieved – one to two orders of magnitude higher than conventional ultrasound
- Imaging can be performed in situ and nondestructively. High-speed, real-time imaging is possible with acquisition rates of several frames per second.
- OCT enables “optical biopsy,” the imaging of tissue structure or pathology on resolution scales approaching that of histopathology, with imaging performed in situ and in real time, without the need to excise specimens and process them as in standard excisional biopsy and histopathology.
- OCT technology can be fiber-optically based and use components developed for the telecommunications industry. Thus, it takes advantage of a well-established technology base.



- OCT can be interfaced with a wide range of imaging delivery systems and imaging probes. Image information is generated in electronic form enabling image processing and analysis as well as electronic transmission, storage, and retrieval.
- OCT systems can be engineered to be compact and low cost, suitable for applications in research, manufacturing, or the clinic.

OCT Principle of Operation

OCT performs imaging by measuring the echo time delay and magnitude of backreflected or backscattering light. Cross-sectional images are constructed by performing axial measurements of the echo time delay and magnitude of backscattered or backreflected light at different transverse positions. This results in a two-dimensional data set that represents the backscattering in a cross-sectional plane of the material or tissue being imaged.



In OCT, measurements of distance and microstructure are performed by directing a light beam onto the material or tissue and measuring the backreflecting and backscattering light from internal microstructural features as shown in Fig. below. For conceptual purposes, it is possible to visualize the operation of OCT by thinking of the light beam as composed of short optical pulses. However, although OCT may be performed using short pulse light, most OCT systems operate using continuous wave, short coherence length light.

Resolution and Sensitivity of Optical Coherence Tomography

In contrast to conventional microscopy, in OCT the mechanisms that govern the axial and transverse image resolution are decoupled. The coherence length of the light source determines the axial resolution in OCT imaging, and high axial resolution can be achieved independent of the beam focusing conditions.
For a source with a Gaussian spectral distribution, the axial resolution *Δz* is given as

$$\Delta z = \frac{2\ln 2}{\pi} \cdot \frac{\lambda_0^2}{\Delta\lambda} \approx 0.44 \cdot \frac{\lambda_0^2}{\Delta\lambda}$$

where Δz and Δλ are the full-widths-at-half-maximum of the autocorrelation function and power spectrum, respectively, and λ is the source center wavelength. Broad bandwidth optical sources are required to achieve high axial resolution.

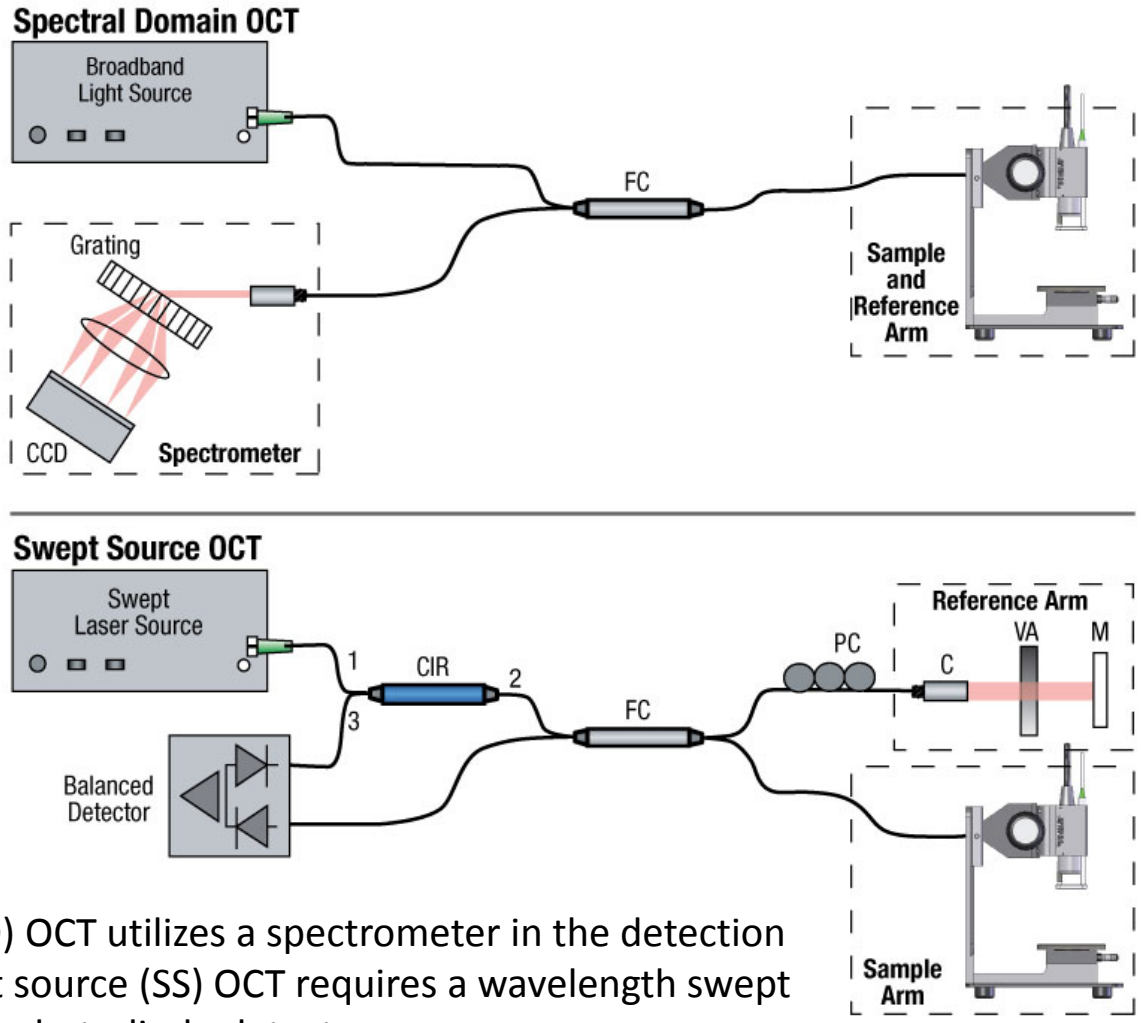
The transverse resolution in OCT imaging is the same as for conventional optical microscopy and is determined by the focusing properties of an optical beam. The minimum spot size to which an optical beam may be focused is inversely proportional to the numerical aperture or the angle of focus of the beam. The transverse resolution is given as:

$$\Delta x = \frac{4\lambda}{\pi} \cdot \frac{f}{d}$$

where d is the spot size on the objective lens and f is its focal length. High transverse resolution can be obtained by using a large numerical aperture and focusing the beam to a small spot size.

Optical Coherence Tomography Technology

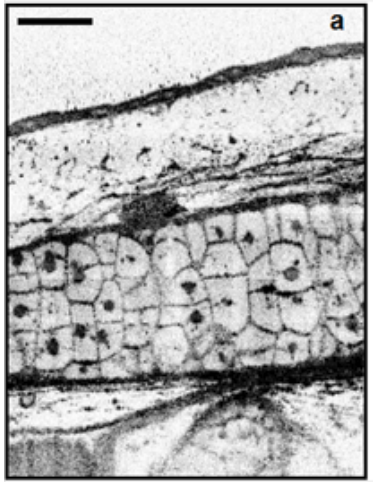
Optical coherence tomography has the advantage that it can be implemented using compact fiber-optic components and integrated with a wide range of medical instruments.
OCT schemes use a fiber-optic Michelson-type interferometer principle.

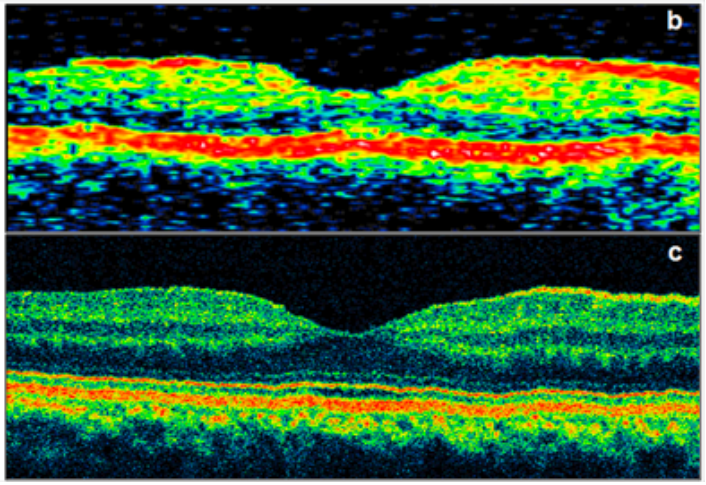


Spectral domain (SD) OCT utilizes a spectrometer in the detection arm, whereas swept source (SS) OCT requires a wavelength swept laser and a standard photodiode detector.

Applications of Optical Coherence Tomography

- Optical coherence tomography imaging in ophthalmology;
- Optical Biopsy – the in situ, real-time imaging of tissue morphology, imaging where excisional biopsy is hazardous or impossible;
- Detecting early neoplastic changes;
- Guiding surgical intervention.

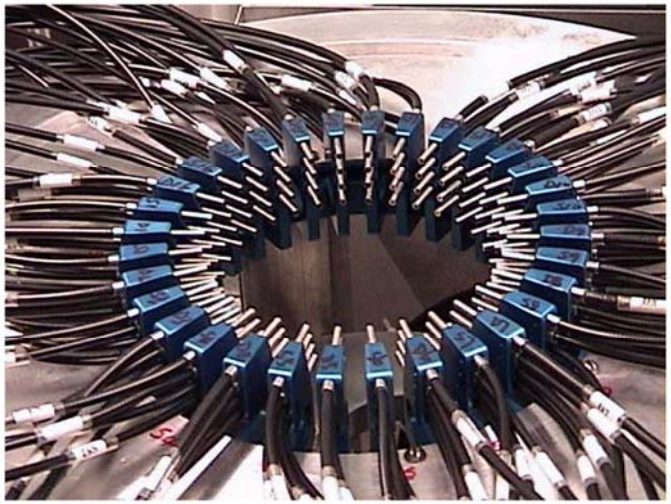




Optical Coherence tomograph for retina diagnostics.

Diffuse Optical Tomography and Imaging

Diffuse Optical Tomography (DOT) and Imaging (DOI) are non-invasive techniques that utilize light in the near infrared spectral region to measure the optical properties of physiological tissue. The techniques rely on the object under study being at least partially light-transmitting or translucent, so it works best on soft tissues such as breast and brain tissue. By monitoring spatial-temporal variations in the light absorption and scattering properties of tissue, regional variations in oxy- and deoxy-hemoglobin concentration as well as cellular scattering can be imaged.



The prototype of optical tomograph
http://www.nature.com/nphoton/journal/v8/n6/full/nphoton.2014.107.html

Applications:

- breast cancer detection
- brain function
- oxygen consumption by muscles
- arthritis
- atherosclerosis
- pulse oximeter
- billirubin test for neonates.

Diffuse Optical Tomography and Imaging

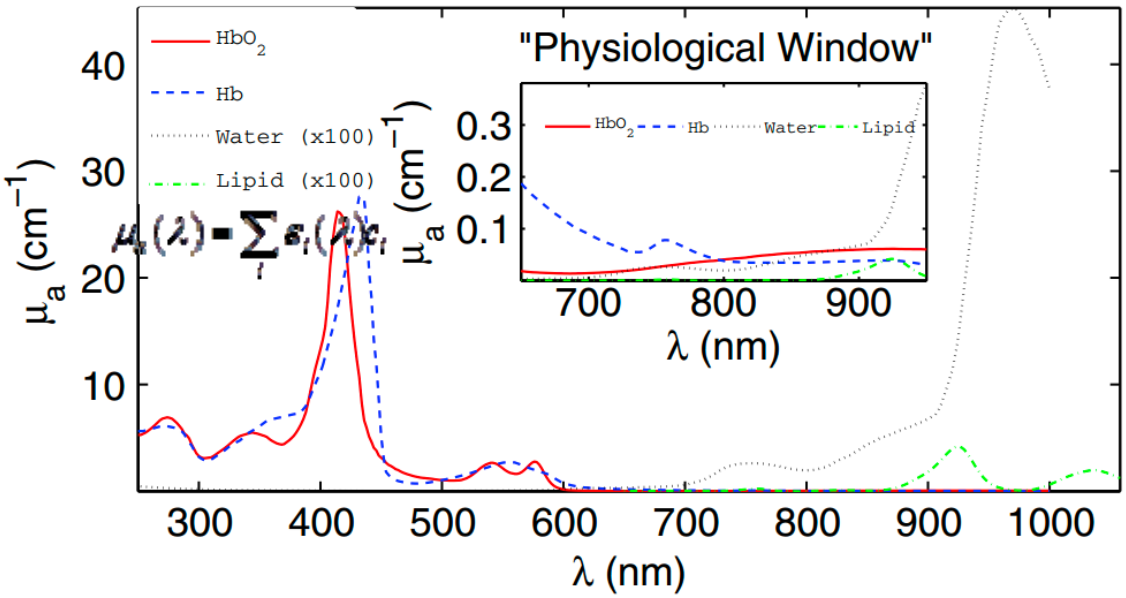
Optimal Wavelengths

Determination of tissue chromophore concentrations requires the separation of tissue absorption from tissue scattering at more than one optical wavelength.

The tissue absorption depends linearly on the concentrations of tissue chromophores):

$$\mu_a(\lambda) = \sum_i \varepsilon_i(\lambda) c_i$$

where $\varepsilon_i(\lambda)$ is the wavelength-dependent extinction coefficient and c_i the concentration of the i-th chromophore.



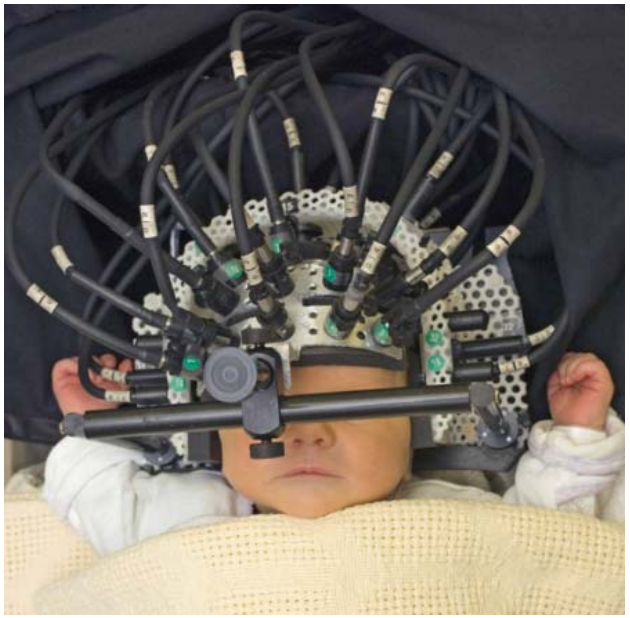
Absorption (μ_a) spectra of major tissue chromophores over a large wavelength range. The inset shows the so-called 'physiological window' in the near-infrared where water and hemoglobin absorption are relatively low. In this part of the spectrum, light can penetrate several centimeters in tissue.

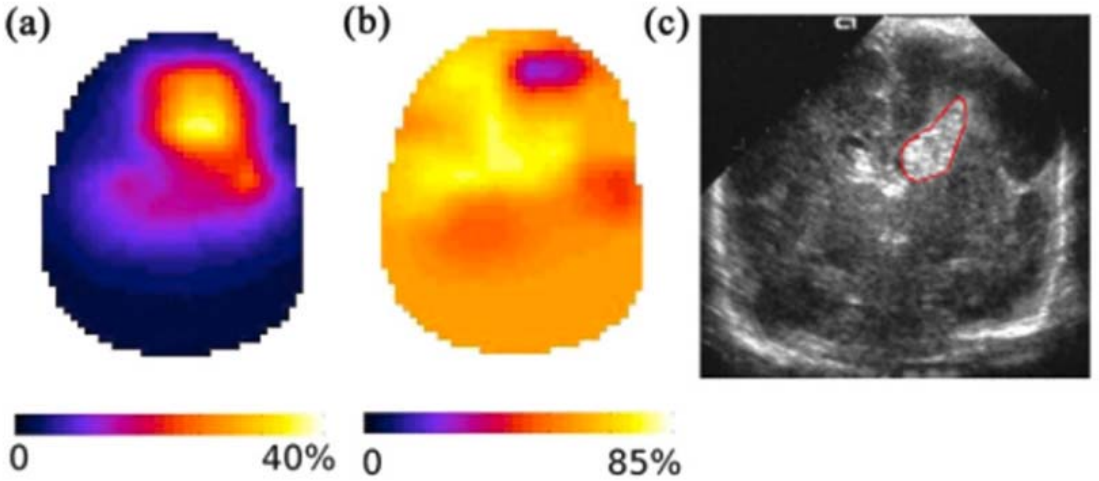
Practically two variants are commonly used:

- a pair at 660–760 nm and 830 nm
- 780 and 830 nm.

Diffuse Optical Tomography and Imaging

Full Head, 3D Tomography in Neonates

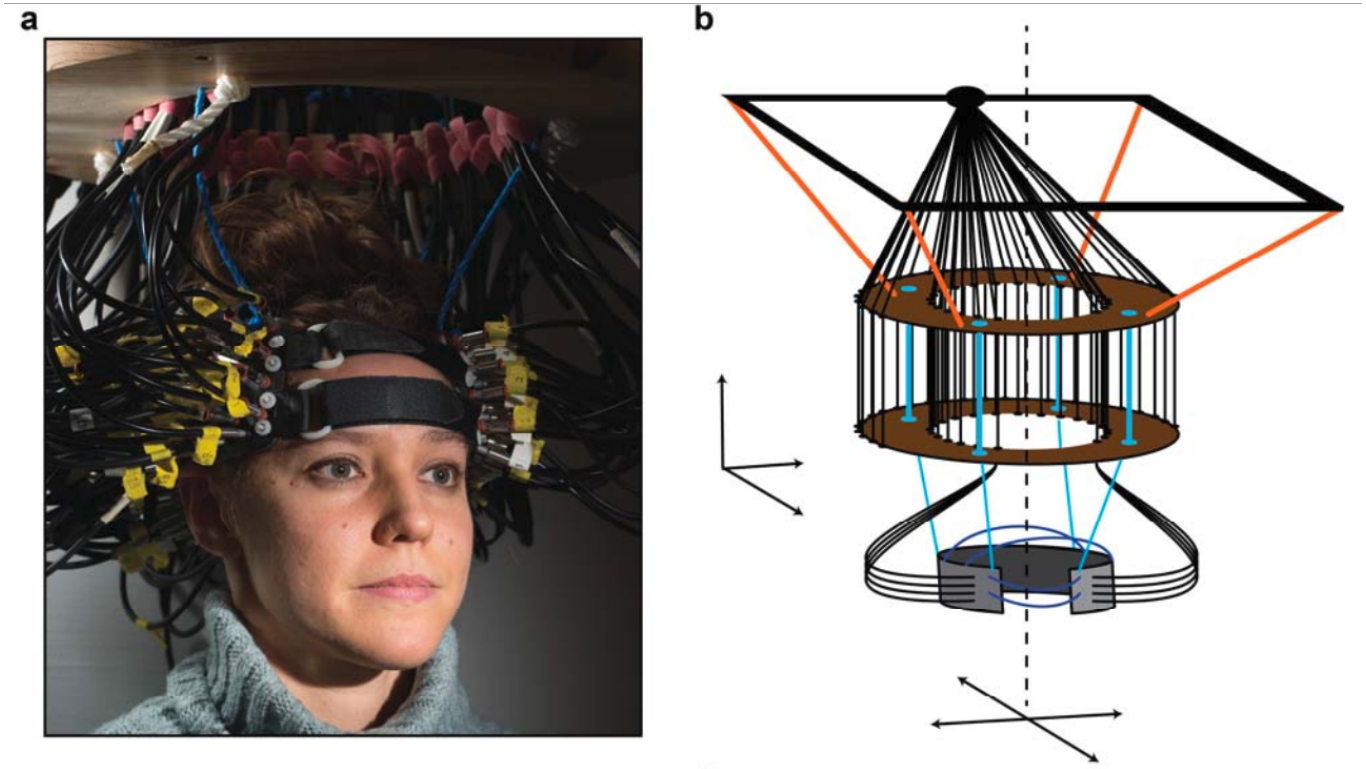




Placement of probes on an infant. (Lower) Coronal sections showing (a) blood volume, (b) blood oxygen saturation and (c) the corresponding ultrasound image. [Hebden J.C., Austin T. Optical tomography of the neonatal brain // European Journal of Radiology 17(11) 2926 (2007)].

Diffuse Optical Tomography and Imaging

High-Definition DOT

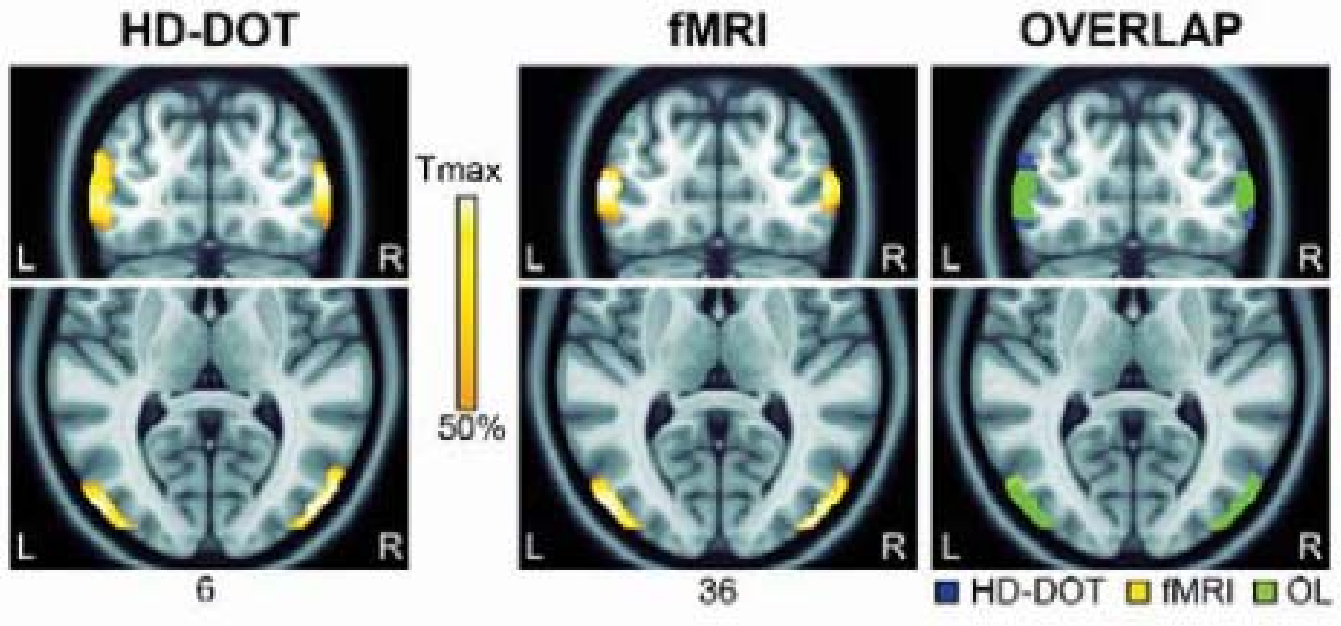


HD-DOT cap design

Adam T. Eggebrecht, Silvina L. Ferradal, Amy Robichaux-Viehoever, Mahlega S. Hassanpour, Hamid Dehghani, Abraham Z. Snyder, Tamara Hershey, Joseph P. Culver Mapping distributed brain function and networks with diffuse optical tomography // Nature Photonics. 2014. Vol. 8. P. 448-454.

Diffuse Optical Tomography and Imaging

High-Definition DOT



Authors have demonstrated several advances in HD-DOT functional neuroimaging:

- mapping hierarchically organized responses to single word stimuli,
- mapping multiple higher-order RSNs, and
- functional neuroimaging of patients in whom MRI is precluded.

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Thank you for your attention!