Introduction to Biomedical Engineering

Biomedical optics – imaging

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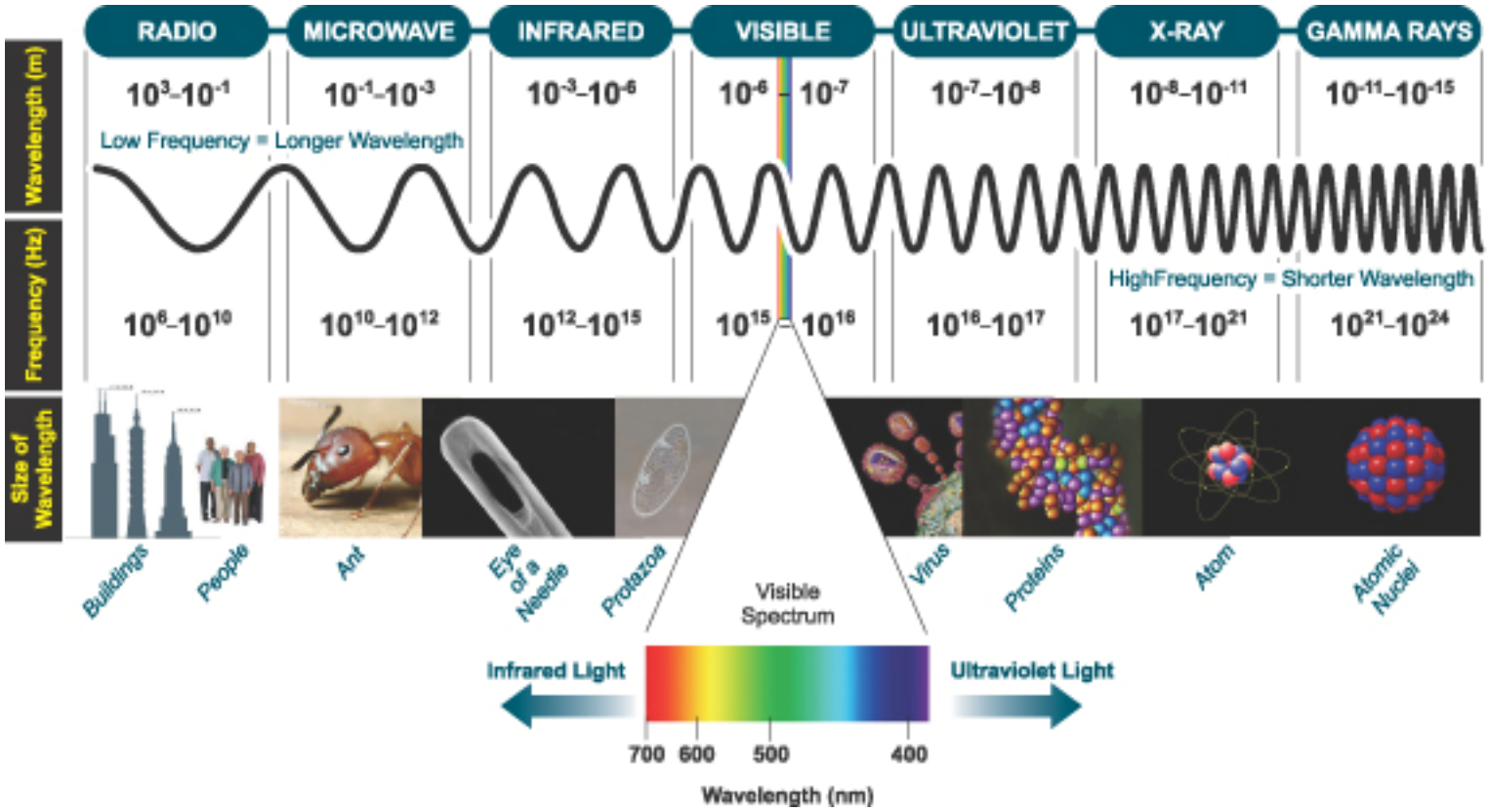
Outline

- Chapter 17: Biomedical optics and lasers
- Fundamentals of light
- Light-matter interaction
- Optical imaging
- Optical sensing (spectroscopy, fiber optic applications)
- Using light for manipulation
  - Laser tweezers, laser scissors
- Using light for therapeutics

Fundamentals of light

Classical view: electromagnetic (EM) wave



(visible) light occupies a very small range of the whole EM spectrum (400-700 nm or 0.4-0.7 μm)

Electromagnetic wave

Special case: plane wave (Transverse Electromagnetic wave)

$$\vec{E} = \vec{E}_0 e^{j(\omega t - kz)} \qquad \vec{E}_0 = E_x \hat{a}_x \qquad H_y = \frac{1}{\eta} E_x \qquad \eta = \sqrt{\frac{\mu}{\varepsilon}}$$

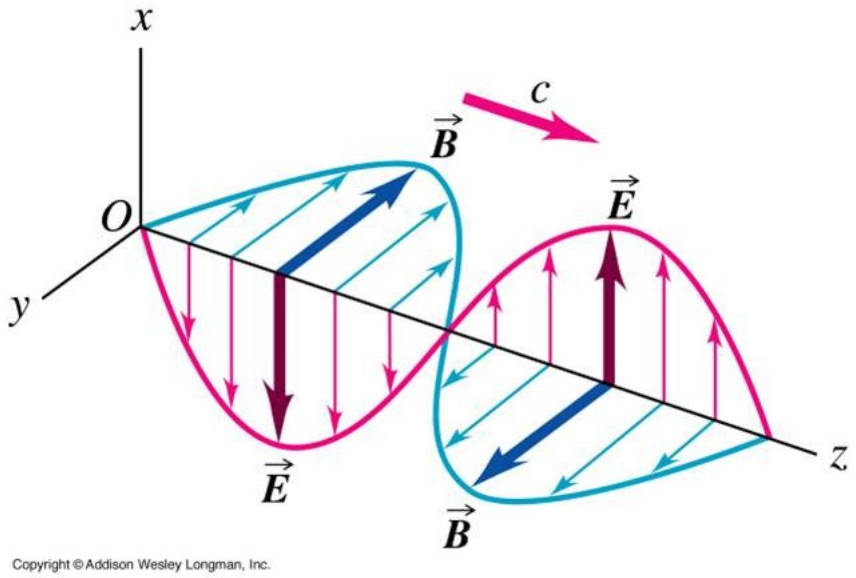
$$\vec{H} = \vec{H}_0 e^{j(\omega t - kz)} \qquad \vec{H}_0 = H_y \hat{a}_y$$

$$\omega = 2\pi f \qquad k = \frac{2\pi}{\lambda}$$

Speed of propagation:

$$c = \frac{1}{\sqrt{\varepsilon\mu}} = \frac{\omega}{k} = f\lambda$$

In free space c=3x10^8 m/s



Electromagnetic wave

The phase velocity of light in a medium with refractive index n

$$v_p = \frac{c}{n}$$ Where c is speed of light in free space

Average power density of the wave (along direction of propagation)

$$\left\langle \vec{E} \times \vec{H} \right\rangle = \frac{1}{2}\mathrm{Re}\left[\vec{E} \times \vec{H}^*\right]$$ (W/m^2)

Time average

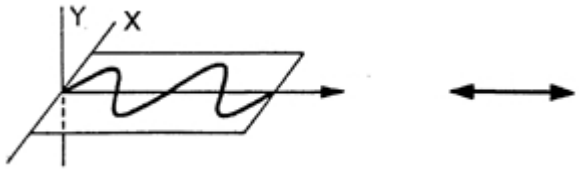
Total power of light (wave) is what we actually measure/observe
$\Rightarrow$ Integrate the above vector over cross section of detector

$$\therefore I_{measured} \propto \left|\vec{E}\right|^2$$ $\Rightarrow$ Square of amplitude of electric field

Polarization of light

- The electric field of a TEM wave oscillates along one direction (x-axis)

- This is a linearly polarized light and the direction of polarization is aligned with x-axis



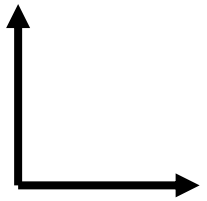
- Other polarization states of light exist such as circularly and elliptically polarized light

- Natural light source (sun light) and most lamps generate unpolarized light (direction of polarization is random)

- Light from a Laser source is linearly polarized

Polarization – examples

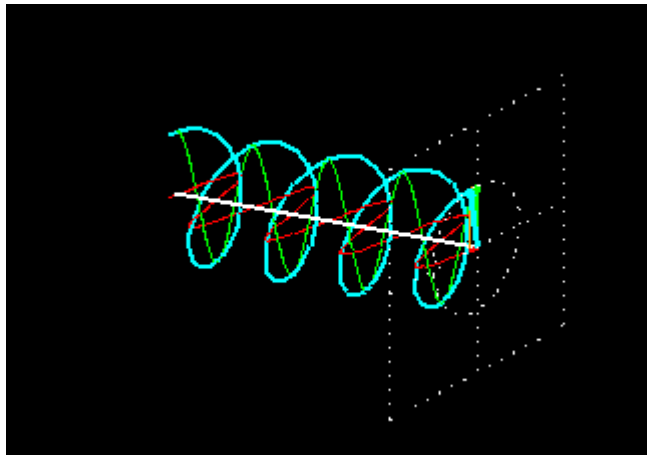
$$E_y = E_0 e^{j(\omega t - kz - \pi/2)}$$



$$E_x = E_0 e^{j(\omega t - kz)}$$

$$E_y = E_2 e^{j(\omega t - kz - \pi/2)}$$

$$E_x = E_1 e^{j(\omega t - kz)}$$



E1 ≠ E2 or phase difference ≠ π/2

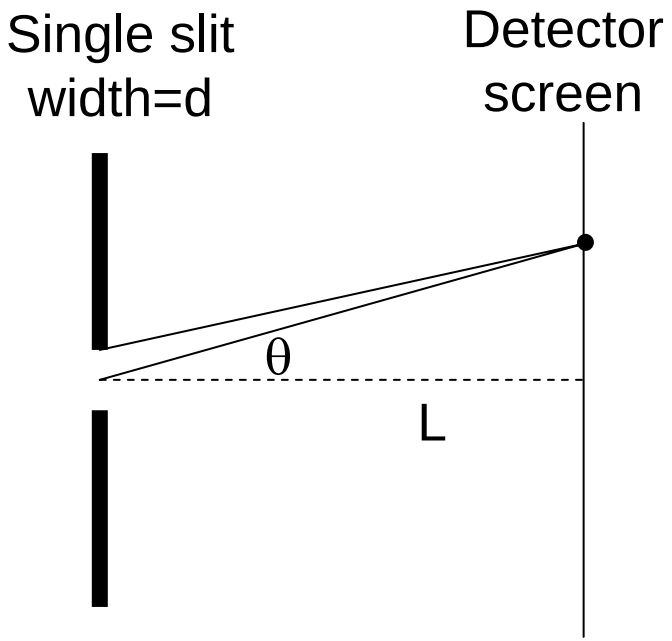
⇒ elliptical polarization

Diffraction of light

- Newton: light always travels in straight lines (1704) – correct on a large scale

- On a smaller scale (~λ), when light waves pass near a barrier, they tend to bend around the barrier and spread at oblique angles – **<u>diffraction</u>**

- Huygens principle: each point on a wavefront can be considered as a source of a new wave



Light path difference between light ray from center and light ray from top edge of the slit

$$\frac{d}{2}\sin\theta$$ If L>>d

There are infinite pairs of such light rays originating from the slit

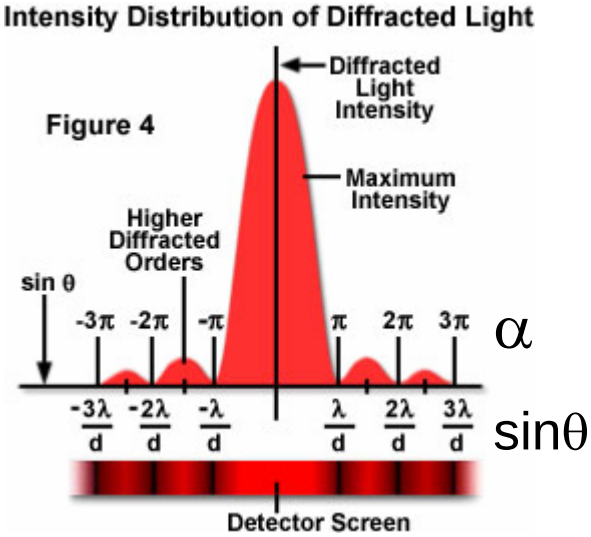
Diffraction of light

For the first intensity minimum at detection screen (destructive interference)

$$\frac{d}{2}\sin\theta = \frac{\lambda}{2} \qquad \sin\theta = \frac{\lambda}{d}$$

For the second intensity minimum at detection screen (destructive interference)

$$\frac{d}{2}\sin\theta = \lambda \qquad \sin\theta = \frac{2\lambda}{d}$$



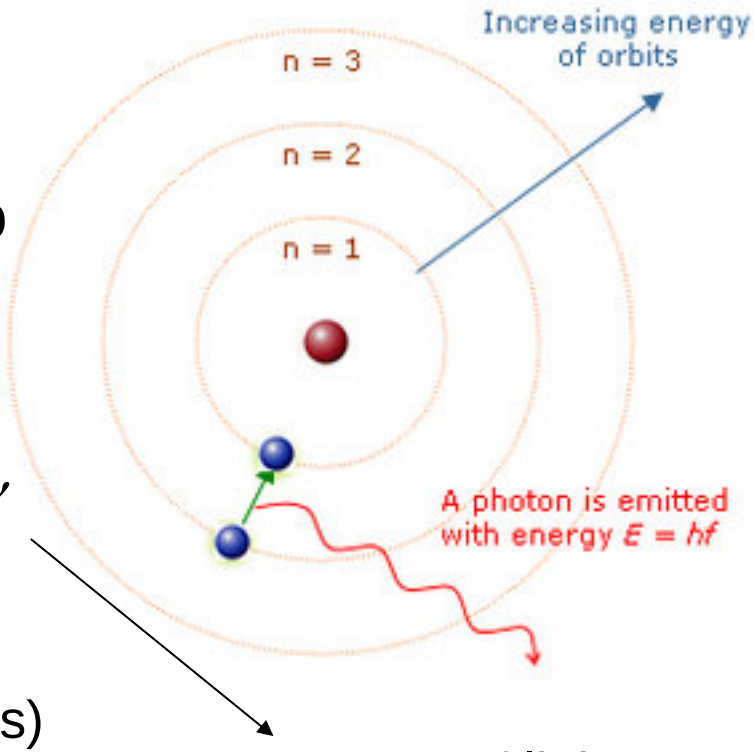
The intensity of interference at the detection screen

$$I \propto \frac{\sin^2\alpha}{\alpha^2} = \mathrm{sinc}^2(\alpha) \qquad \text{where} \quad \alpha = \frac{\pi d \sin\theta}{\lambda}$$

Modern physics – light as particles

Bohr's model of a hydrogen atom
- Electron can circle its nucleus in certain orbitals stably
- An atom emits or absorbs a photon of light when an electron jumps from one permitted orbit to another

$$E_{photon} = E_i - E_f = hcR\left(\frac{1}{n_f^2} - \frac{1}{n_i^2}\right) = h\nu$$



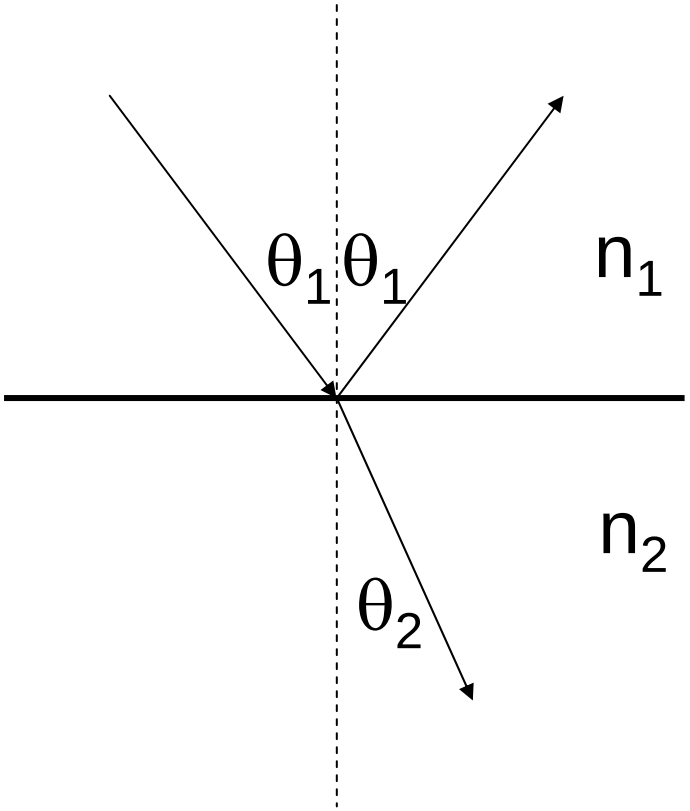
h: Planck's constant (6.626x10-34J·s)

R: Rydberg constant (1.097x10^7 m^{-1})

Light propagation at interface

Snell's law

$$n_1 \sin(\theta_1) = n_2 \sin(\theta_2)$$



Fresnel reflection coefficients: fraction of light reflected

Perpendicular polarization

$$R_s = \left[\frac{n_1 \cos(\theta_1) - n_2 \cos(\theta_2)}{n_1 \cos(\theta_1) + n_2 \cos(\theta_2)} \right]^2$$

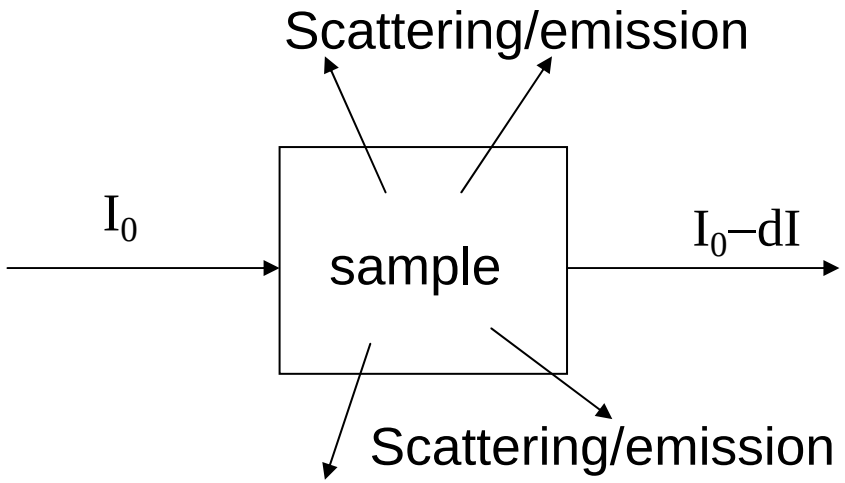
Parallel polarization

$$R_p = \left[\frac{n_1 \cos(\theta_2) - n_2 \cos(\theta_1)}{n_1 \cos(\theta_2) + n_2 \cos(\theta_1)} \right]^2$$

Normal incident

$$R = \left(\frac{n_1 - n_2}{n_1 + n_2} \right)^2$$

Light-matter interaction



Total loss of incident light
dI = absorption + scattering

Attenuation of incident light in biological samples

$$I(x) = I_0 \cdot e^{-\mu_t x} = I_0 \cdot e^{-(\mu_a + \mu_s)x}$$

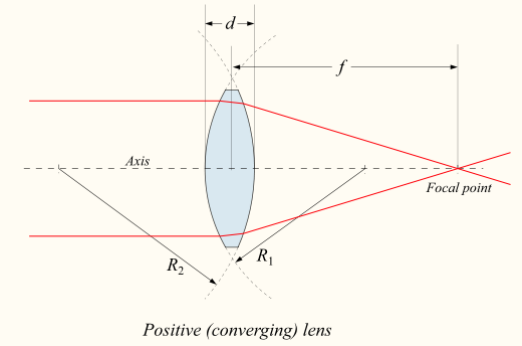
μ_t: attenuation coefficient
μ_a: absorption coefficient
μ_s: scattering coefficient

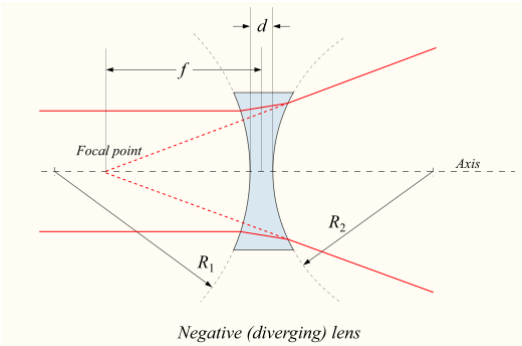
x: light path

Optical imaging – outline

- Fundamentals
  - Geometrical optics
  - Diffraction and resolution
- Light microscopy
  - Methods for illumination
  - Various techniques (brightfield, darkfield, phase contrast, fluorescence, confocal)
  - Digital imaging
- Optical coherence tomography

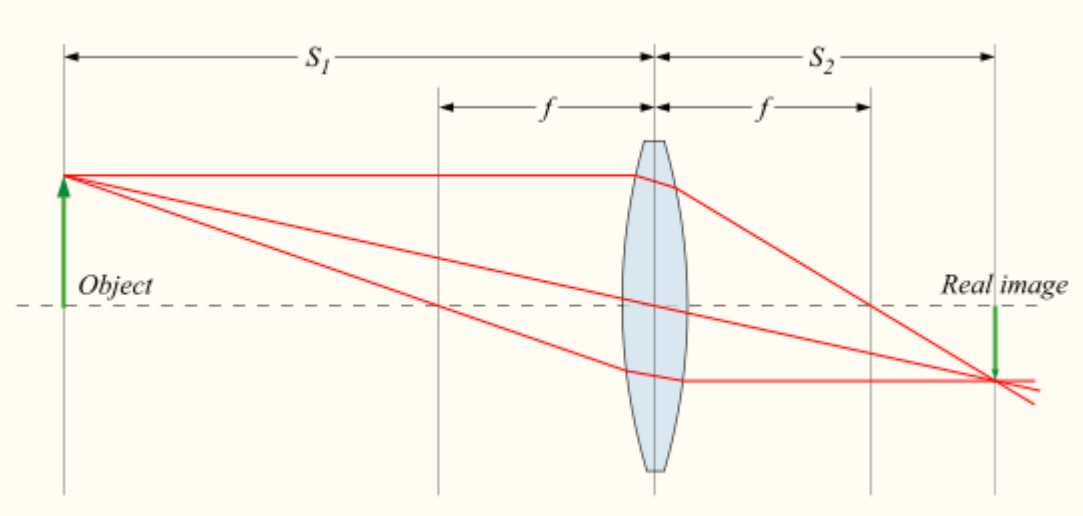
Geometrical optics – thin lens





f : Focal length of lens

Collimated $\Leftrightarrow$ point



thin lens equation

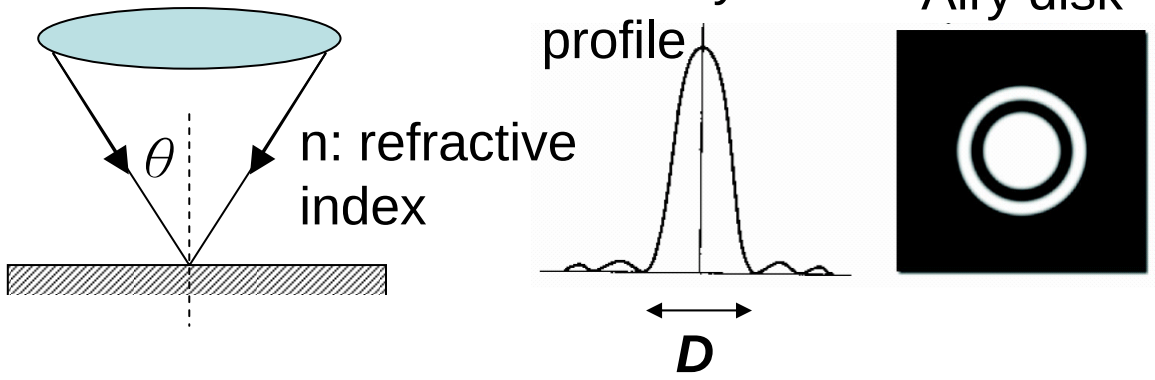
$$\frac{1}{S_1}+\frac{1}{S_2}=\frac{1}{f}$$

Magnification = $\frac{S_2}{S_1}$

[Optical Microscopy Primer demo]

Diffraction limit

The focused “point” has a finite area and certain spatial distribution due to the wave property of light



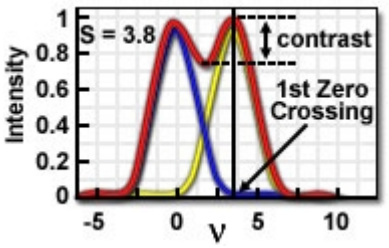
$$D = \frac{1.21\lambda}{n\sin\theta}$$ Width of the central band of the Airy disk

$$NA = n\sin\theta$$ Numerical aperture (NA) of the objective

$$\Delta d = \frac{0.61\lambda}{NA}$$ **resolution based on Rayleigh criterion**

Resolution (spatial)

Rayleigh criterion of resolution



Two point objects separated by $\Delta d = \frac{0.6\lambda}{NA}$

can be distinguished as two objects; the contrast is ~26%

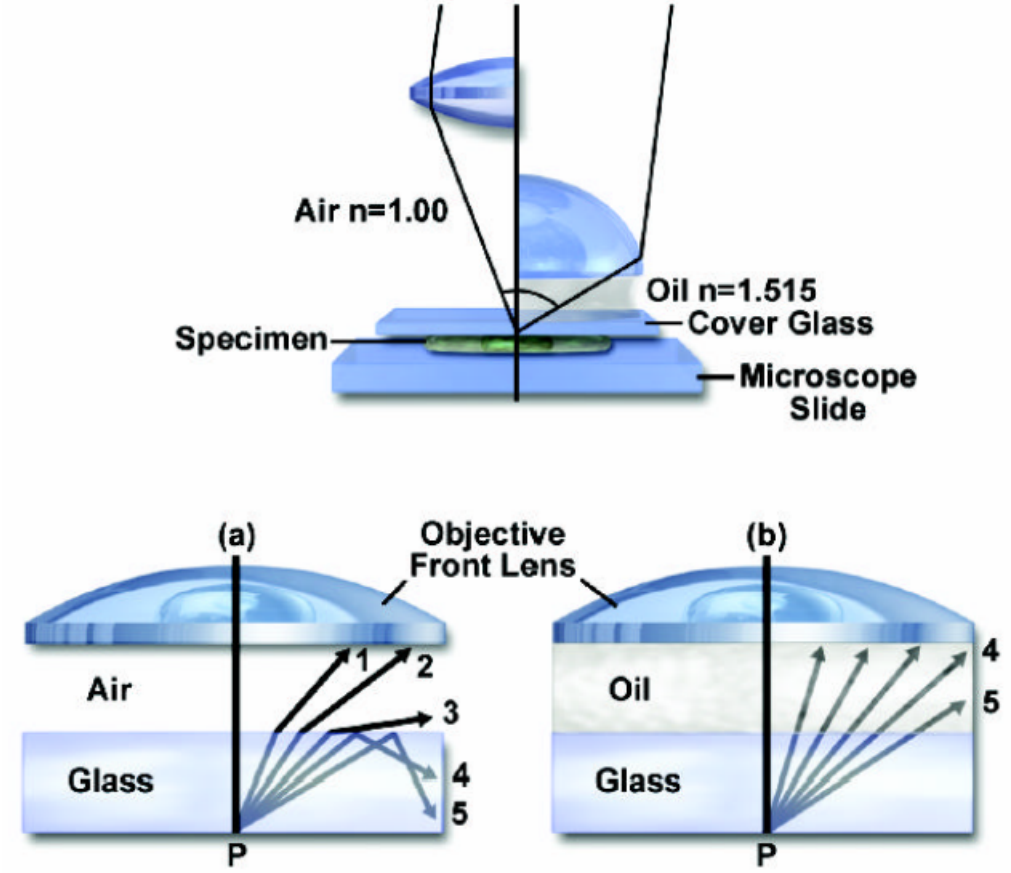
Example: resolution of a typical light microscope at λ = 550nm

	Objective Type					
	Plan Achromat		Plan Fluorite		Plan Apochromat	
Magnification	N.A.	Resolution (&microm)	N.A.	Resolution (&microm)	N.A.	Resolution (&microm)
4x	0.10	2.75	0.13	2.12	0.20	1.375
10x	0.25	1.10	0.30	0.92	0.45	0.61
20x	0.40	0.69	0.50	0.55	0.75	0.37
40x	0.65	0.42	0.75	0.37	0.95	0.29
60x	0.75	0.37	0.85	0.32	0.95	0.29
100x	1.25	0.22	1.30	0.21	1.40	0.20
N.A. = Numerical Aperture						

Size of biological cells ~ several to 20 μm

∴ Light microscopy has adequate resolution to study cells and tissue

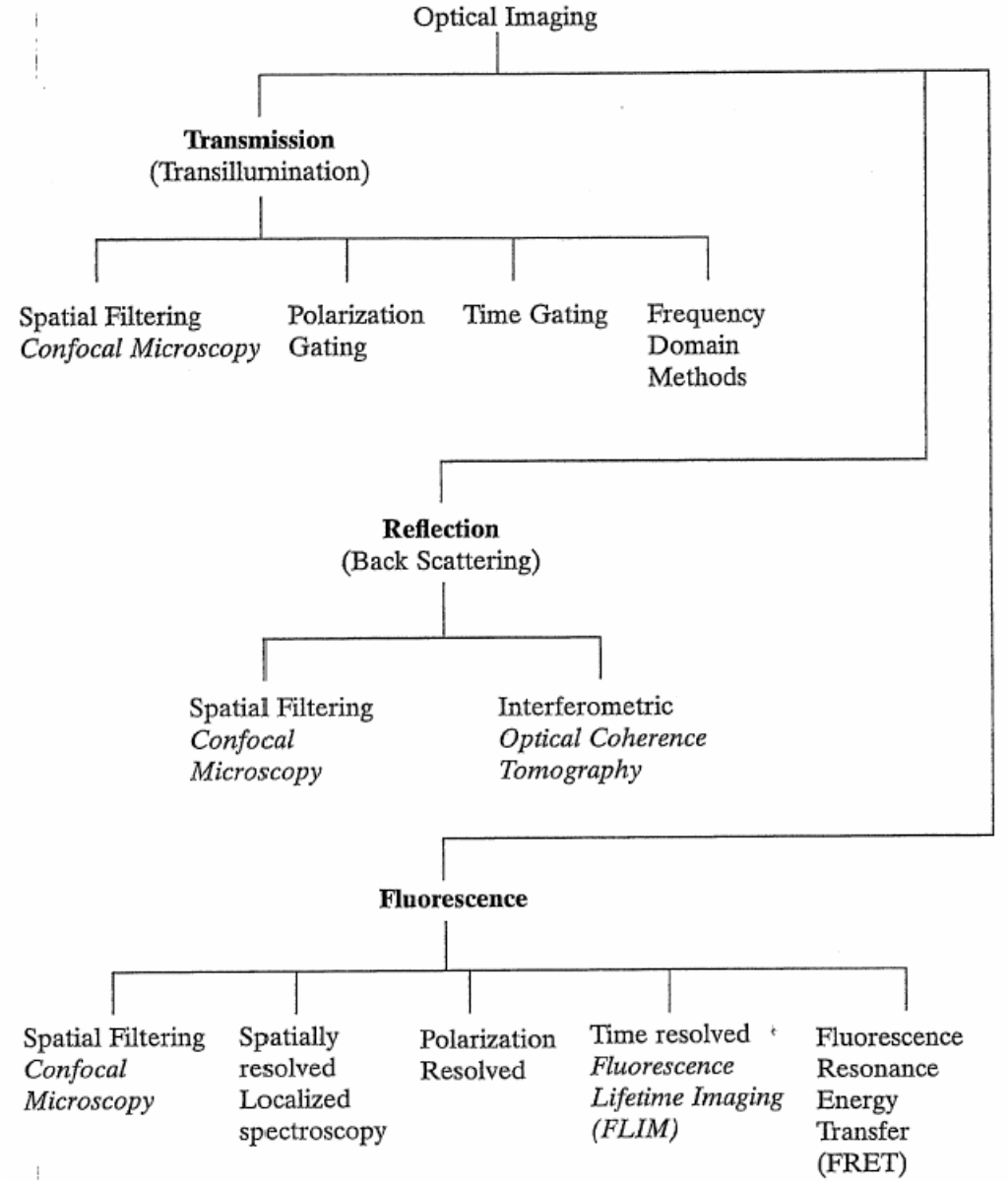
Immersion Objectives



Refractive index of
water = 1.33
index matching oil = 1.515

- Increase NA and hence improve resolution
- Reduce aberration due to refraction of light – if no cover glass is used, water immersion would be better

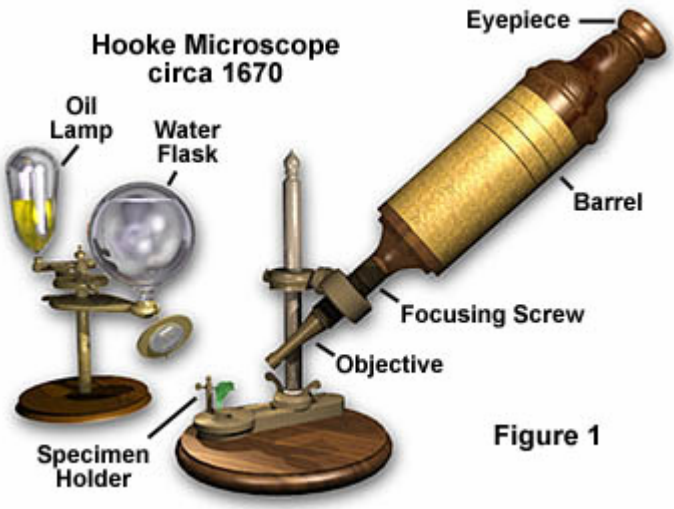
Optical imaging

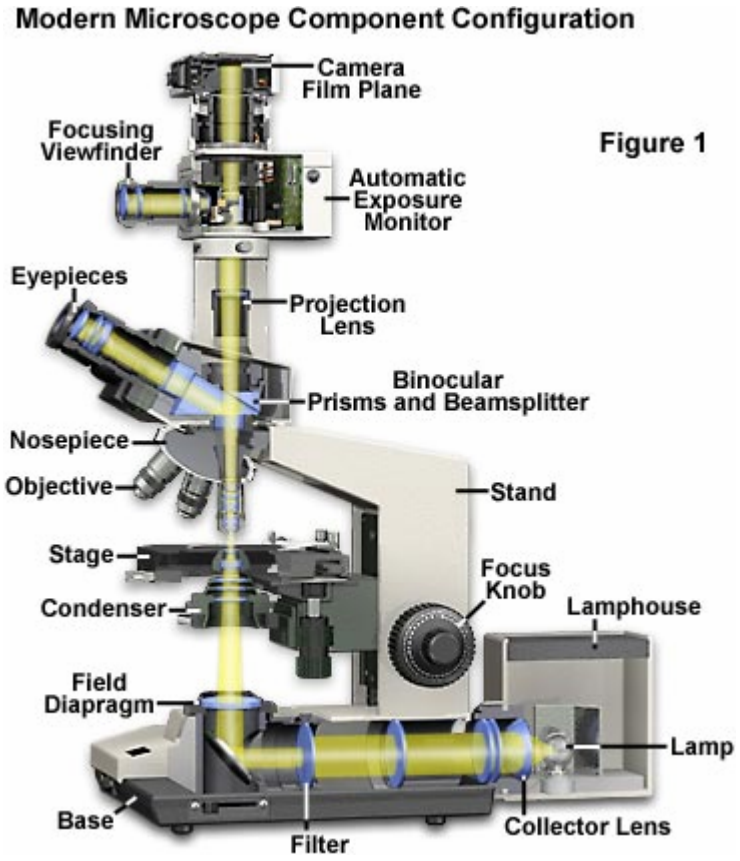


Optical imaging methods – categorized using light illuminating and detecting configurations

Note that confocal microscopy appears in all three types of illumination modes

Light microscopy

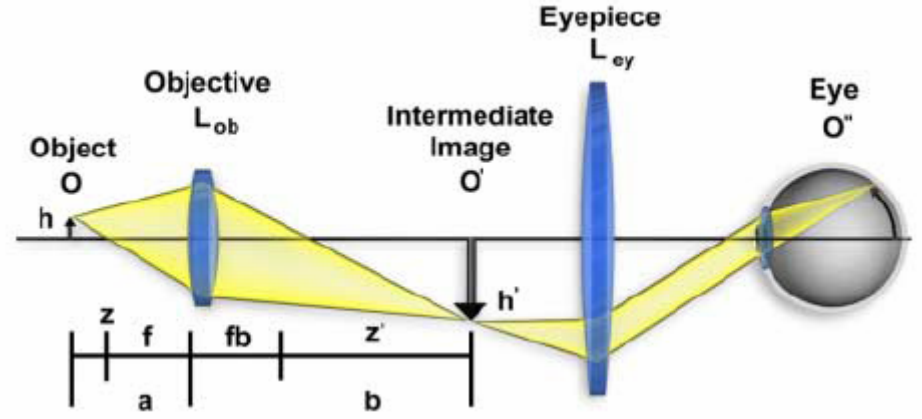




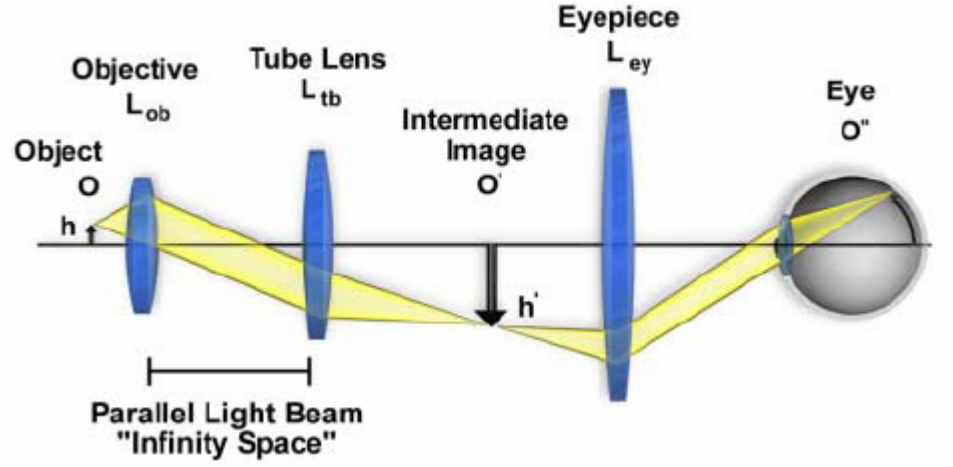
Basic operation is the same for over 300 years

Demonstrated using geometrical optics

Finite tube length microscope

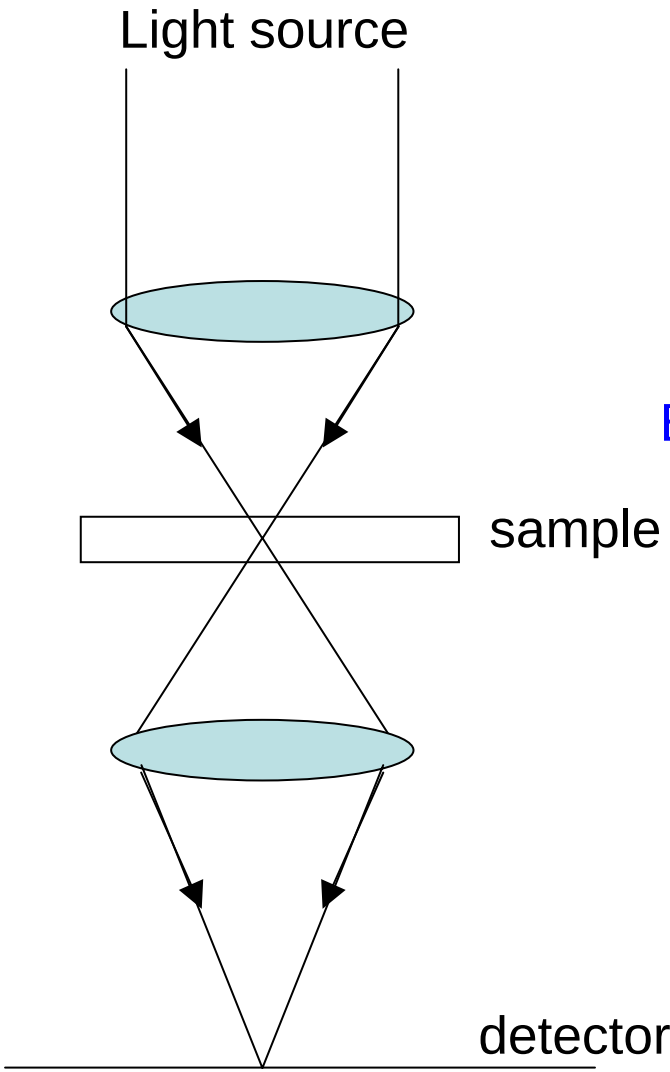


Infinity-corrected microscope



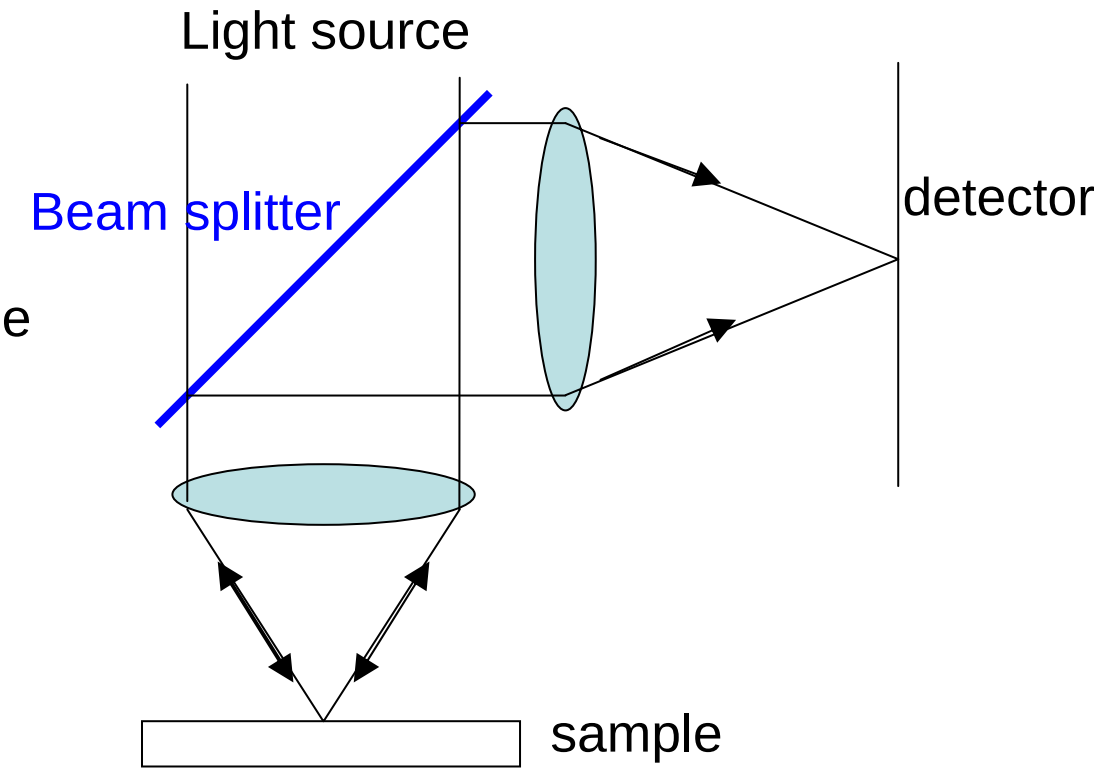
Illumination/observation modes

Transmission



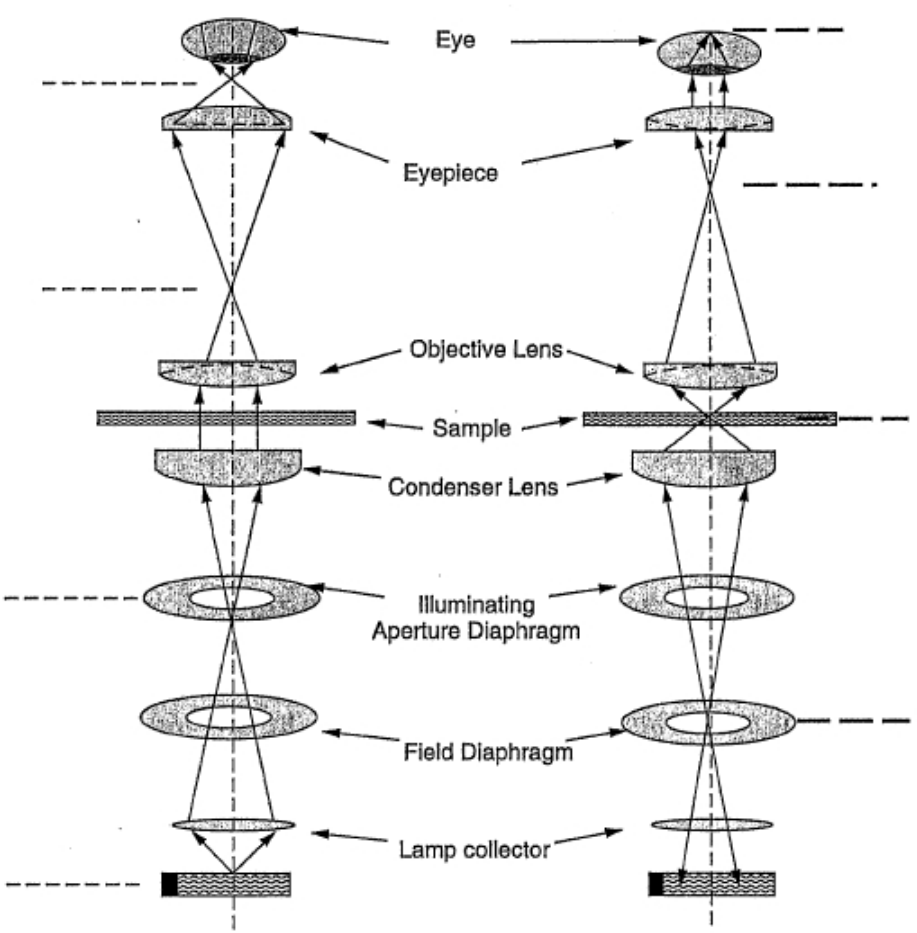
Reflection/Epi-fluorescence

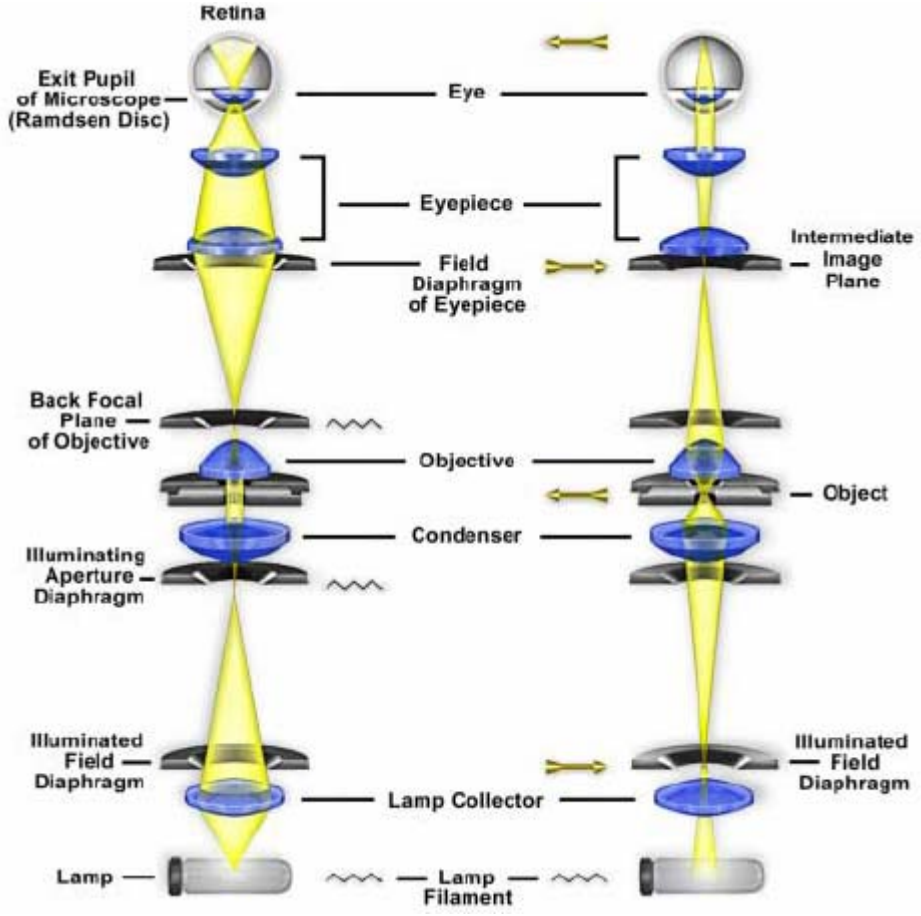
Use of a beam splitter to separate the illumination and detected light



Köhler illumination

Illumination Sample (imaging) Illumination Sample (imaging)



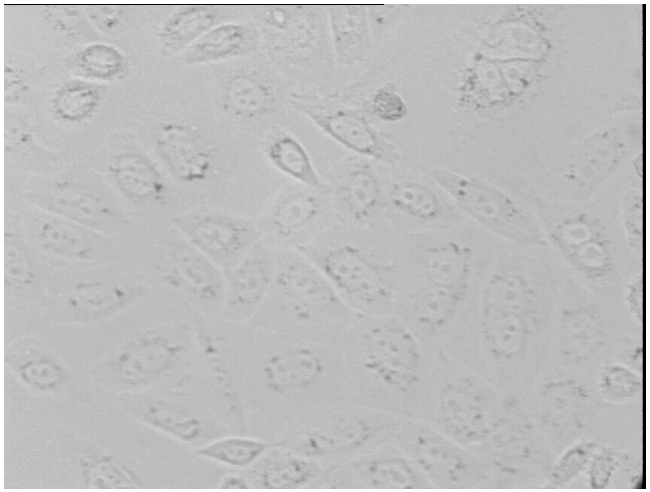


Conjugate planes: object-image pairs in image formation by a lens

Images of cells using different methods

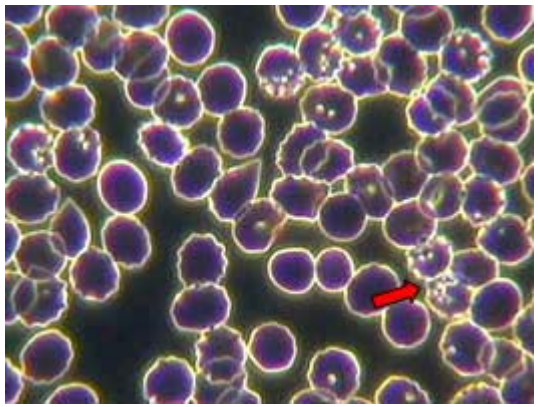
Where does contrast come from?

Brightfield (transmission)



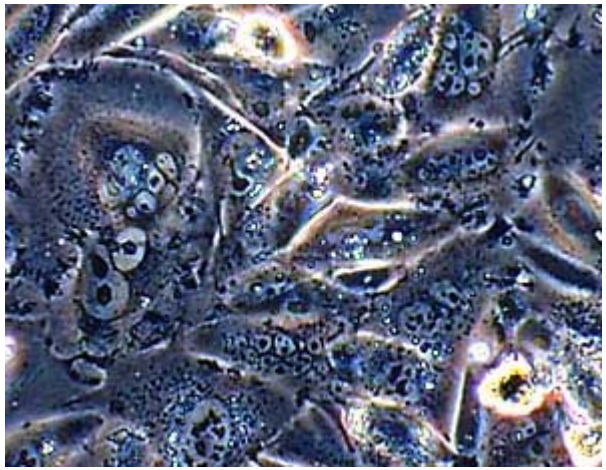
Chinese hamster ovary cells

Darkfield (transmission or reflection)



Red blood cells

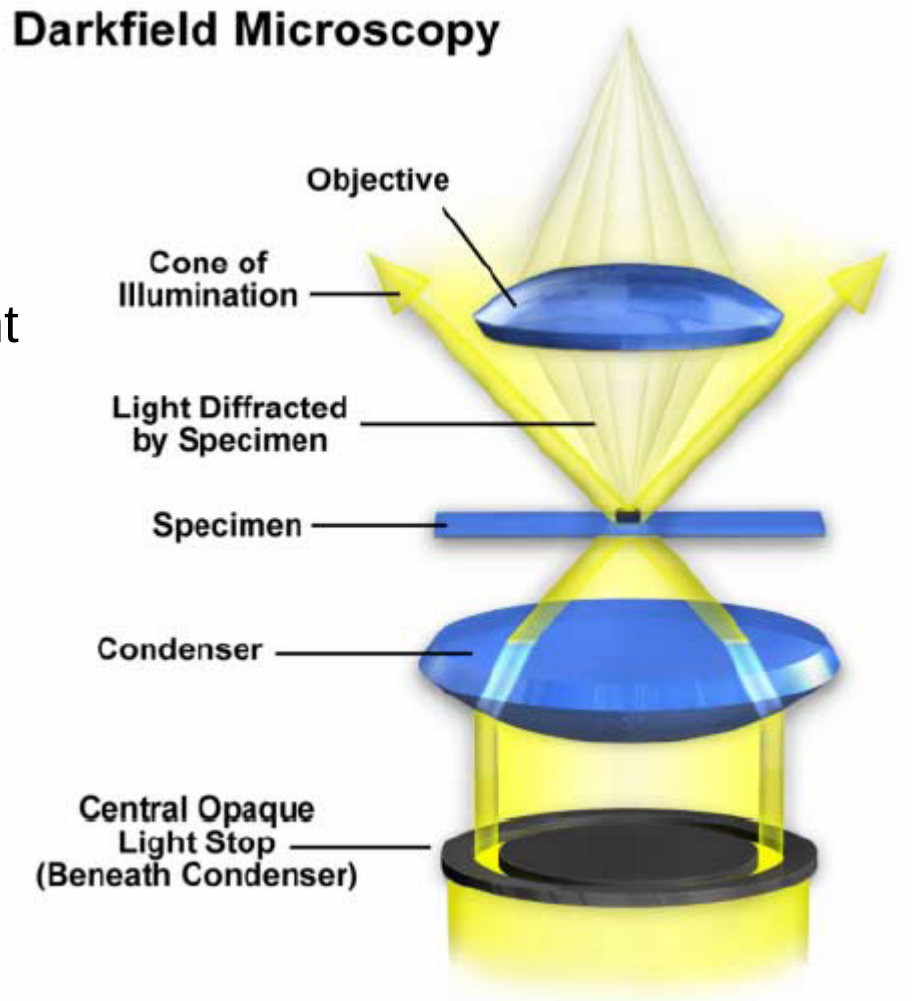
Phase contrast (transmission)



Darkfield microscopy

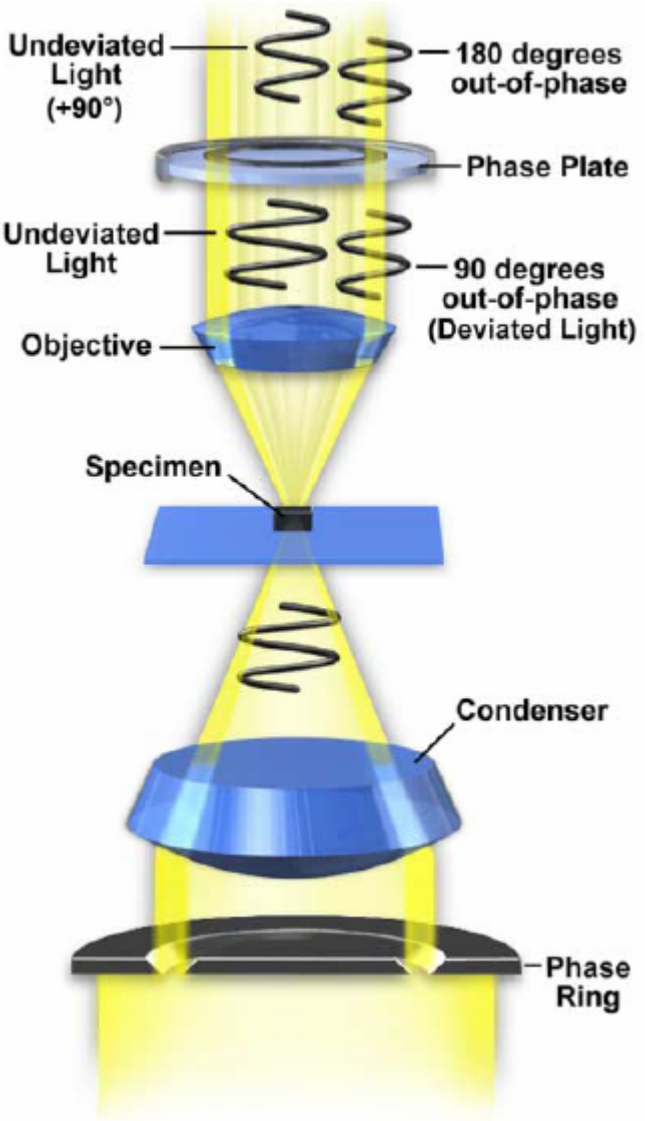
The image is “dark” when there is no specimen

- NA of the condenser is higher than that of the objective
- Central light rays are blocked by an opaque disk located at the front focal plane of the condenser
- Only light diffracted or scattered by the specimen can enter the objective and therefore be detected
- Bright objects against a dark background



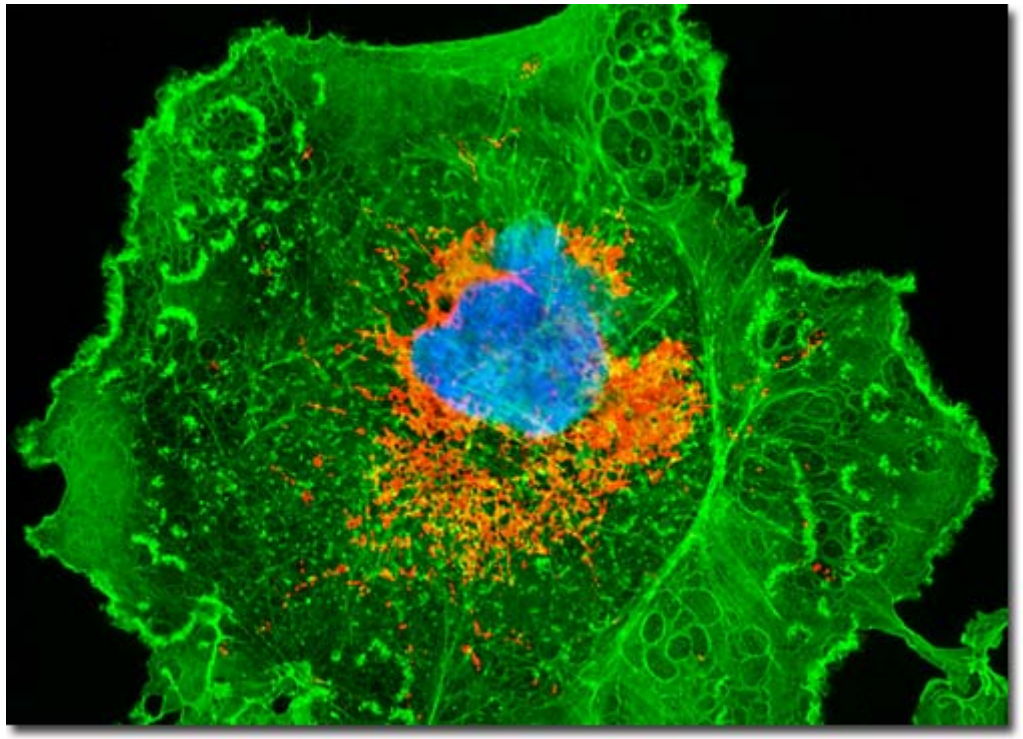
Phase contrast microscopy

- Light diffracted by the specimen is slowed (lag) by ~λ/4 (90°)
- Separate the diffracted and undiffracted light paths
- Introduce another λ/4 light path difference between the diffracted and undiffracted light
- Destructive interference at the image plane causes the specimen look darker



Fluorescence microscopy

Visualization and co-localization of targets of interest using fluorescent labels (using different detection channels)



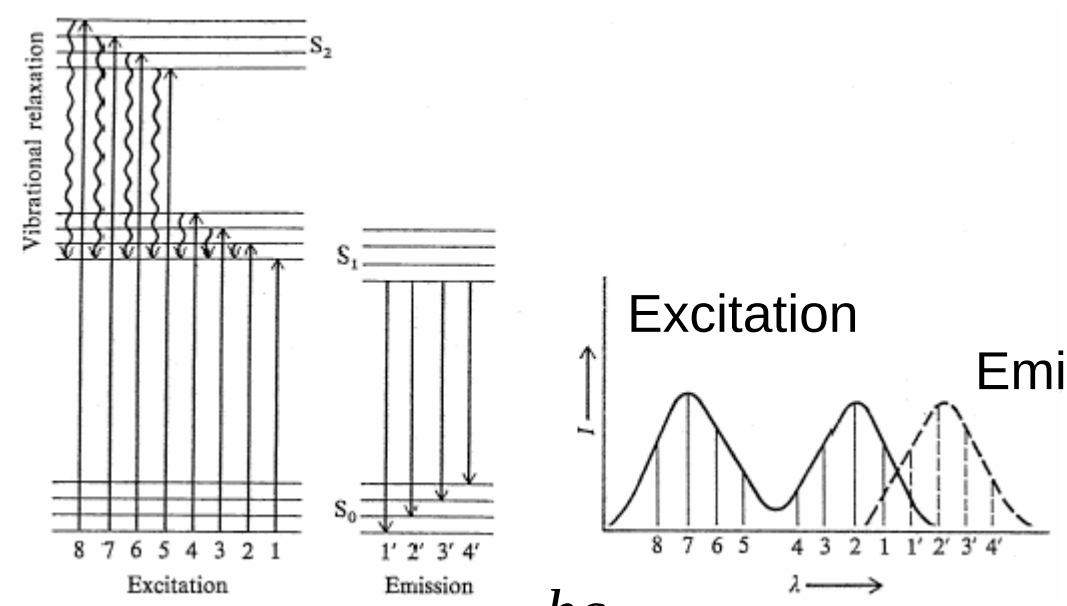
COS-1 African green monkey kidney fibroblast (pseudo-colored)
Red: mitochondria; Green: cytoskeletal f-actin;
Blue: nucleus

Fluorescence

Absorption of incident light
- Electrons are excited to higher energy level
Emission of fluorescence
- Some of the electrons returning to the ground energy level emit light (probability of emission is called quantum yield)

First excited electronic energy level

Ground (unexcited) electronic energy level

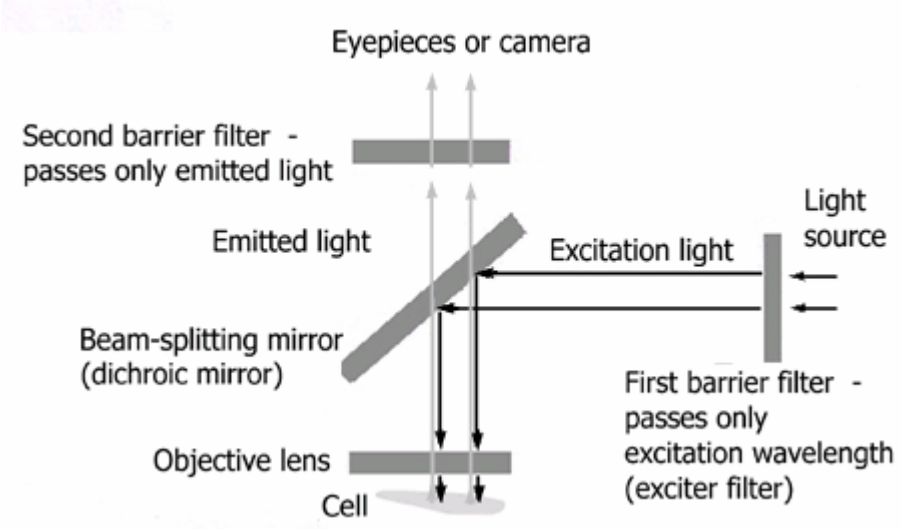


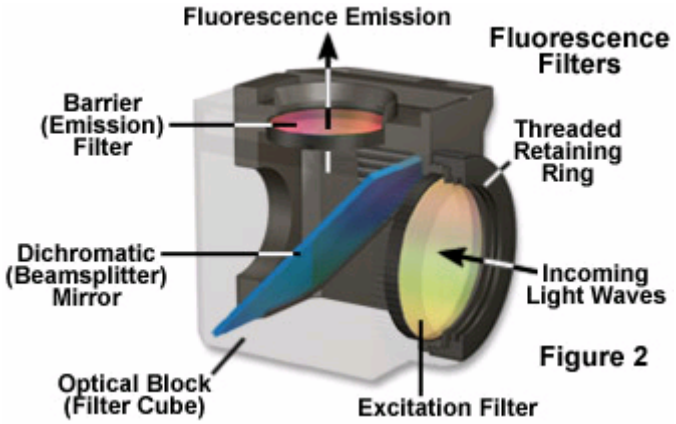
$$E_{initial} - E_{final} = h\nu = \frac{hc}{\lambda}$$

$\lambda_{Em} > \lambda_{Ex}$

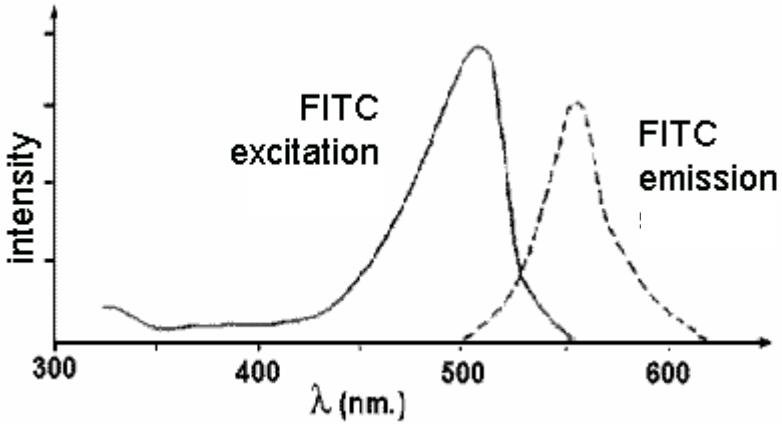
Fluorescence microscopy

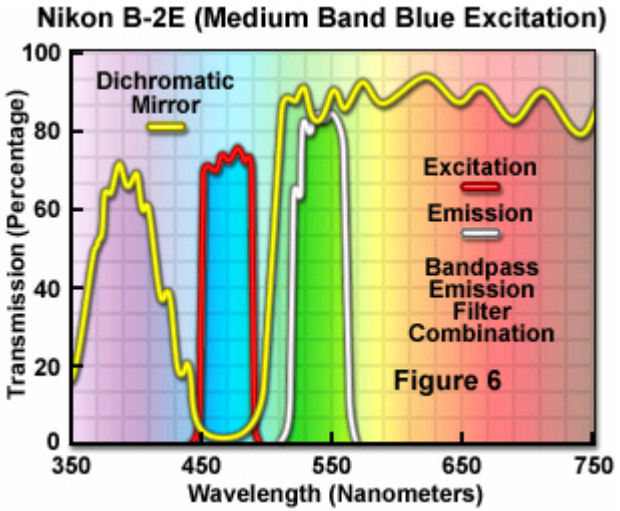
Epi-fluorescence configuration



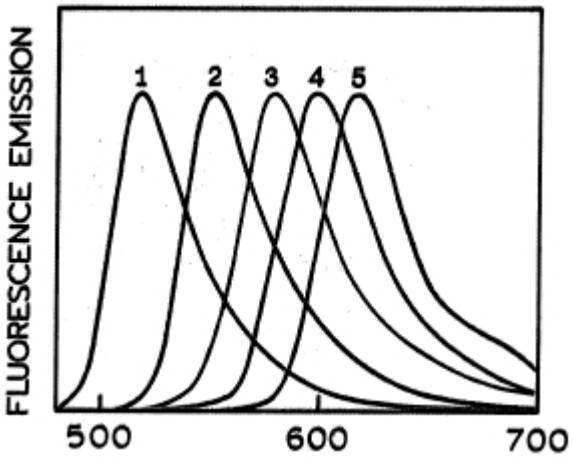


Filter selection example: fluorescein





Fluorescence of extrinsic dyes



1. Fluorescein
2. Rhodamine 6G
3. Tetramethylrhodamine
4. Lissamin rhodamine B
5. Texas Red

Other commercially available fluorescent labels

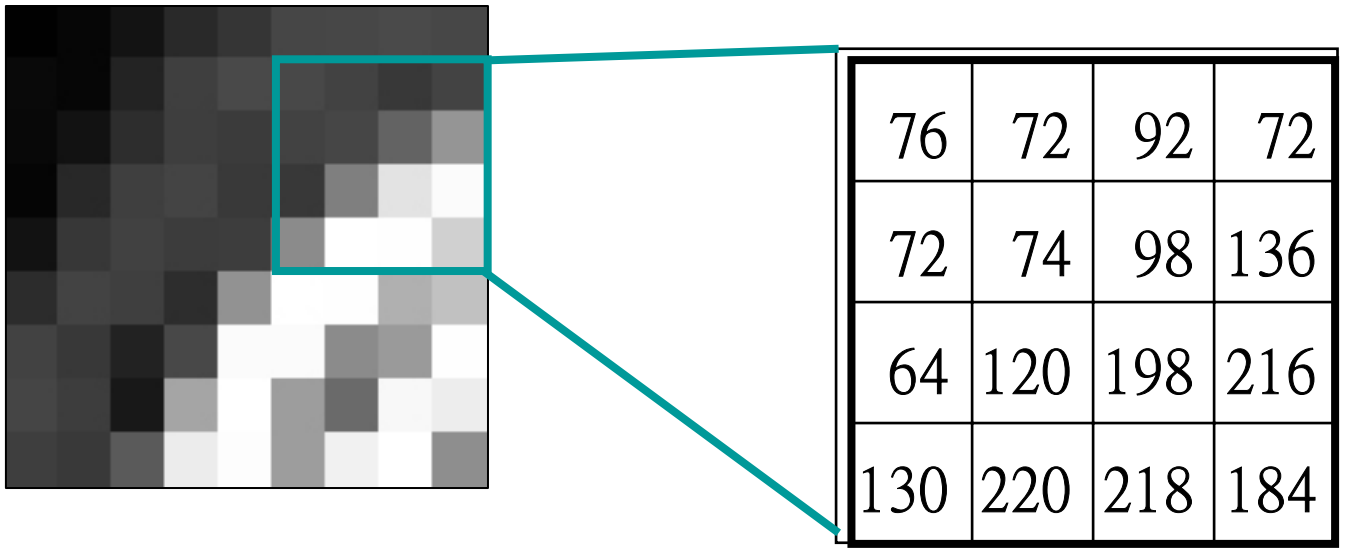
1. Alexa Fluor Dyes: conjugated with affinity reagent such as antibody
2. Cy3, Cy5: for the detection of nucleic acids
3. Quantum dots: semiconductor nanocrystals

More on microscopy

- Other optical microscopy techniques
  - Polarized light, differential interference contrast (DIC), near-field scanning etc.
- More techniques associated with fluorescence
  - Multi-photon excitation, total internal reflection, fluorescence lifetime imaging, fluorescence resonance energy transfer (FRET) etc.

Digital imaging

Recall that digital images are made of arrays of numbers; each element is called a pixel



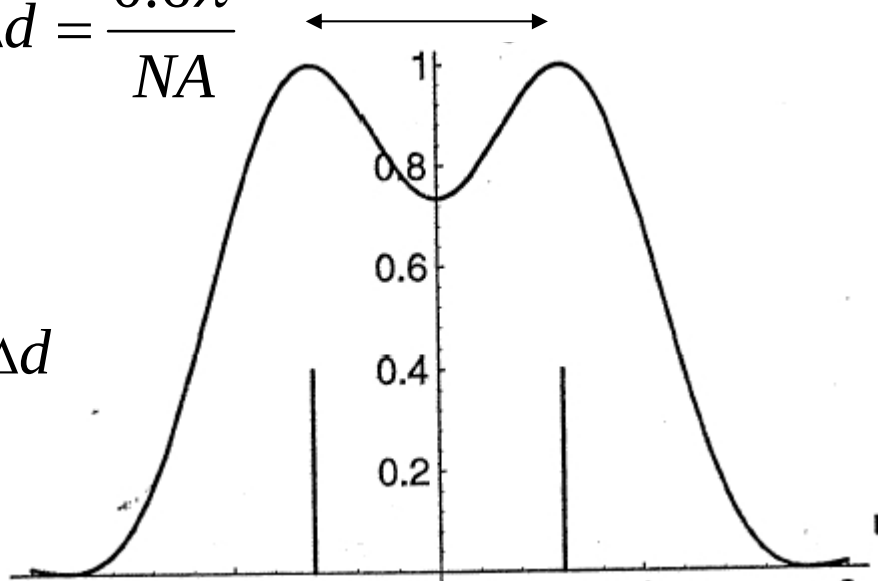
So taking a digital image is like sampling the original (continuous/analog) image in two-dimensional space

How “fast” (how many pixels per unit area) should we sample?

Sampling

Consider the resolution of the optical imaging system (based on Rayleigh criterion)

$$\Delta d = \frac{0.6\lambda}{NA}$$

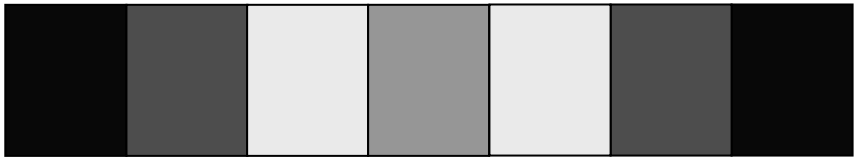


Assume we have 2 pixels within Δd

$\Rightarrow \Delta d = 2\Delta x$

Looks like one peak →

Looks like 2 peaks →



Δx

Sampling (cont.)

Therefore, we need to sample more than 2 pixels within $\Delta d \Rightarrow$ Nyquist sampling theorem

In practice, ratio of $\Delta d/\Delta x$ = 2.3~3

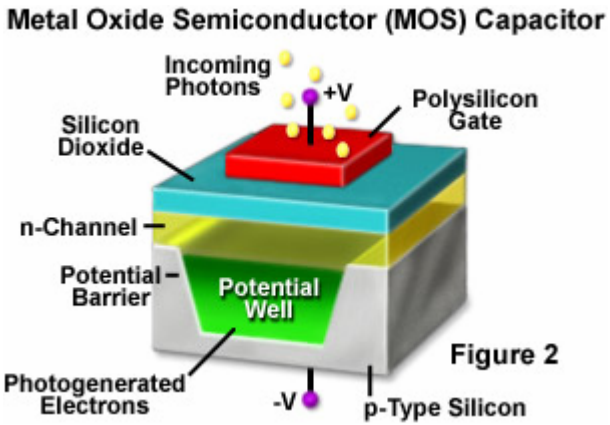
Since Δd is calculated at the object plane and the detector is located at the image plane $\Rightarrow$ add the contribution of the magnification M of the microscope

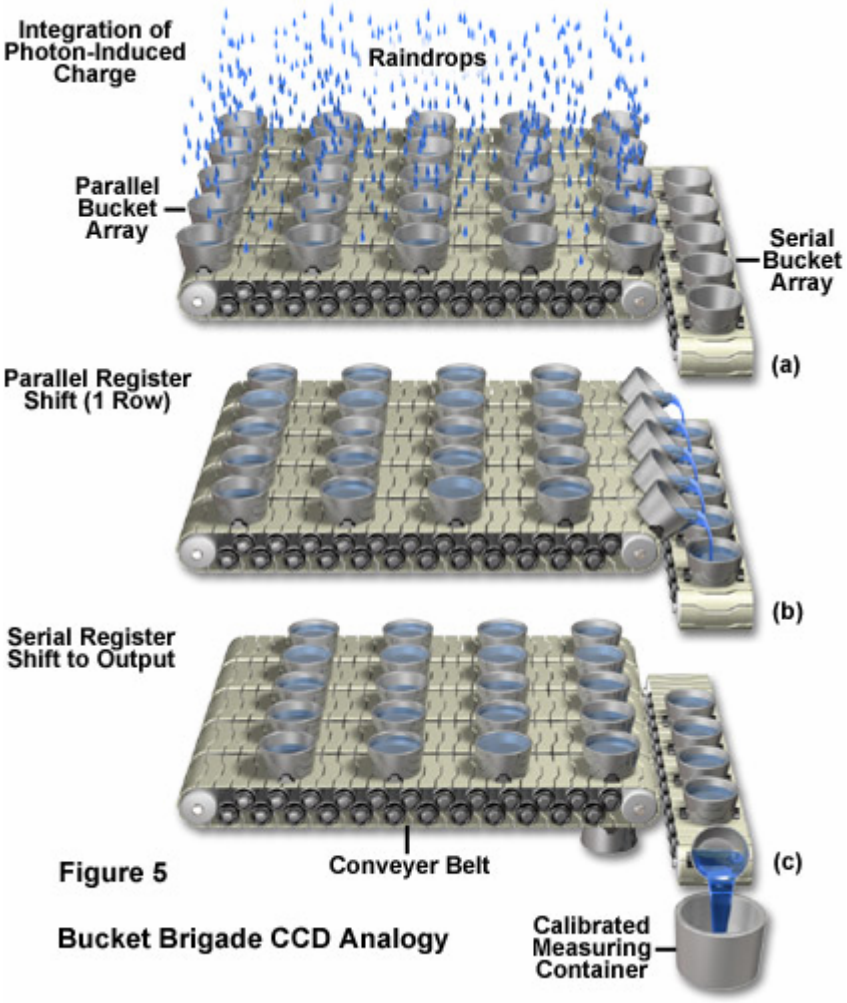
$$\frac{M\Delta d}{\Delta x} = 2.3 \sim 3$$

Δx is the pixel size of the detector. For a typical charge-coupled device (CCD) camera, pixel size is 4μm ~ 20μm

Charge-coupled device (CCD)

Basically a 2D array of photodiodes





- Photons generate electron/hole pairs during certain exposure or integration time
- During read-out, accumulated charges are shifted row by row
- Then charges are converted to voltage and digitized sequentially
- This time-varying signal is used to reconstruct 2D images

Light microscopy summary

- High (sub-micron) spatial resolution
- High sensitivity but with some restrictions
- Real-time imaging (given enough signal)
- Non-invasive but only works on thin slices of samples
- Minimally destructive (energy $\propto$ frequency of radiation)

For thicker specimen such as tissues, we can use confocal microscopy or optical coherence tomography

Confocal microscopy

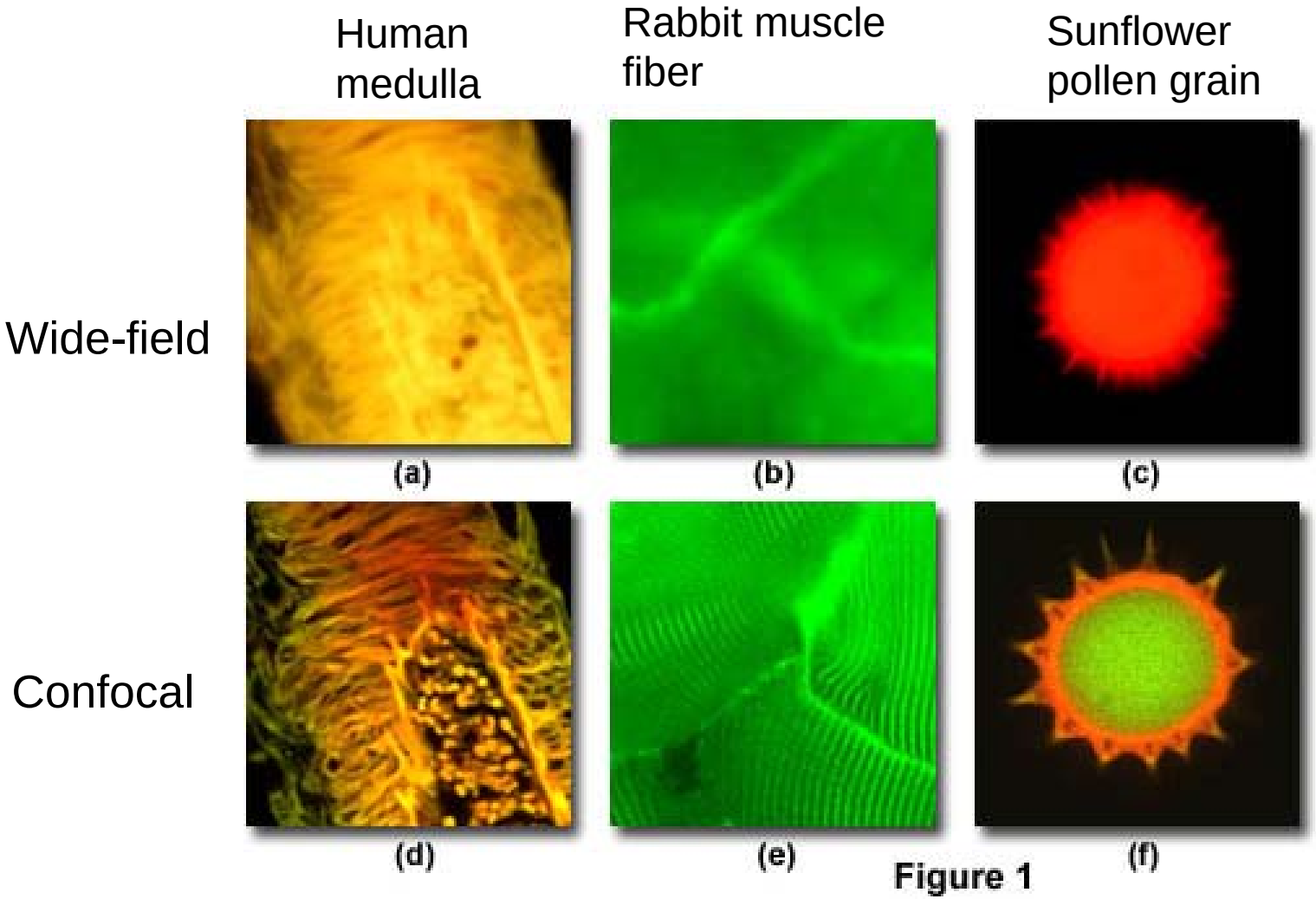
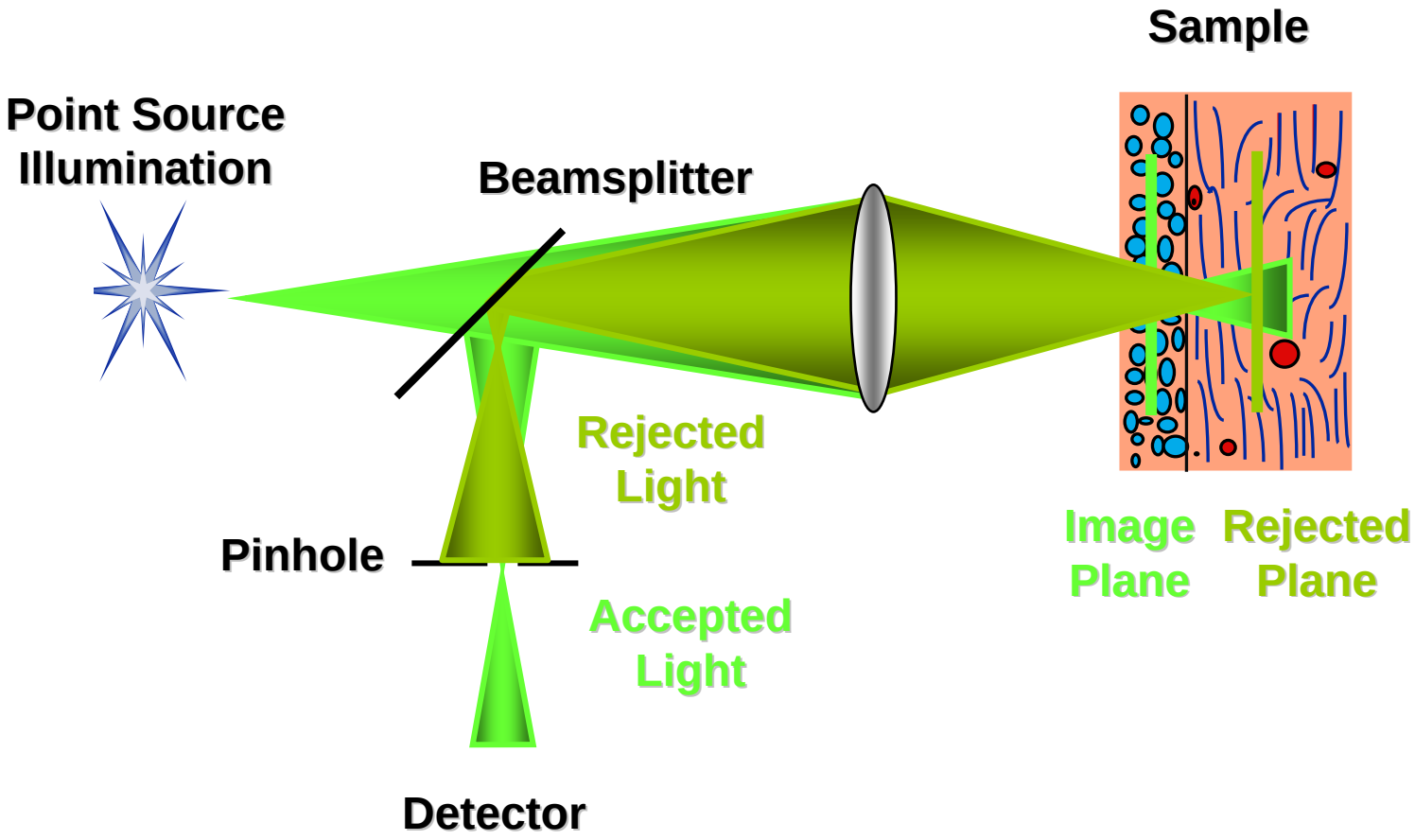


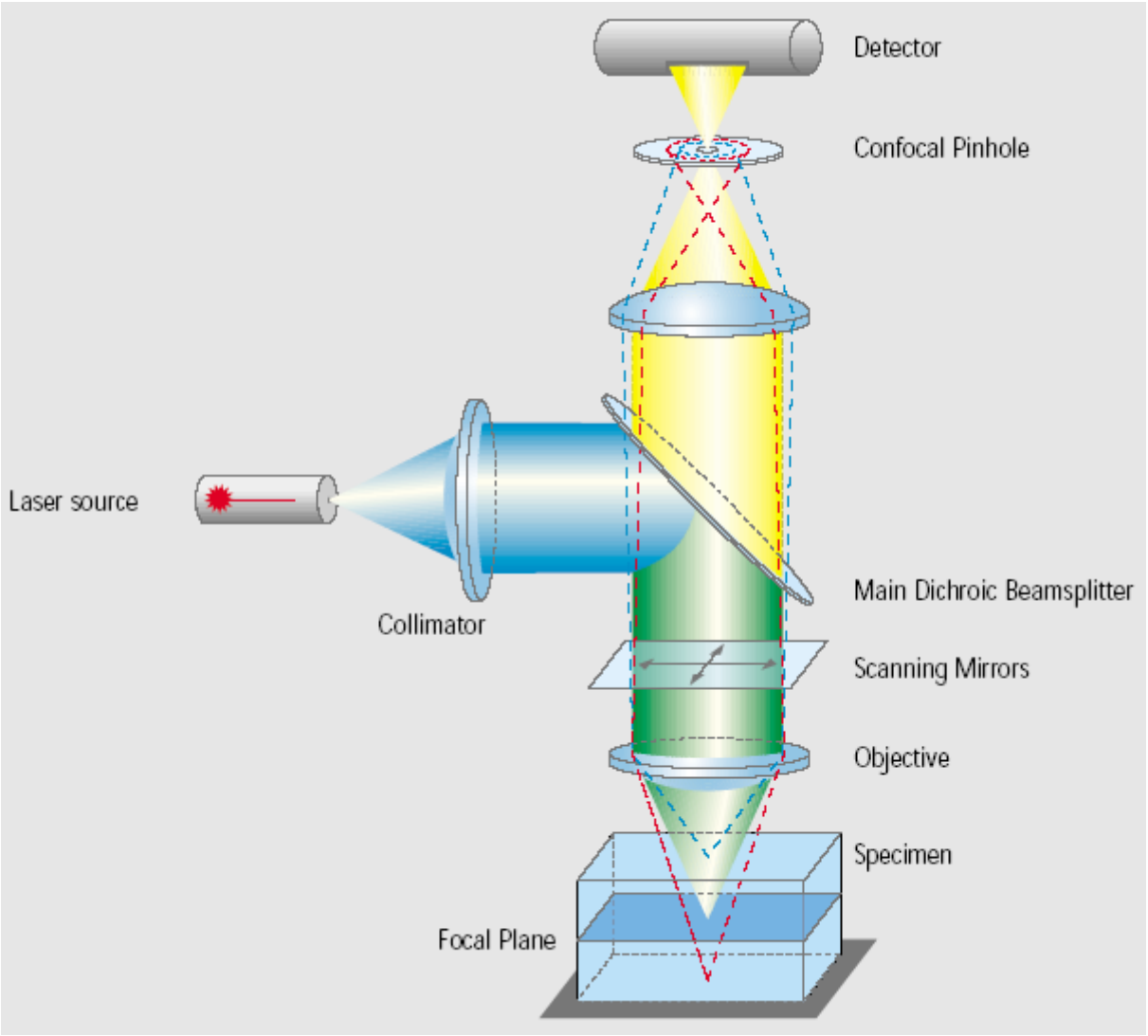
Figure 1

Confocal microscopy has the ability to reject light from out-of-focus planes $\Rightarrow$ Optical sectioning (most useful in thick samples)

Confocal microscopy (cont.)



Confocal microscopy (cont.)



Confocal microscopy (cont.)

Confocal imaging principle:
- Point illumination and detection
- Use of a pinhole (spatial filter) to isolate and detect signal from a small volume in the sample
- Scanning in 2D or 3D to get images

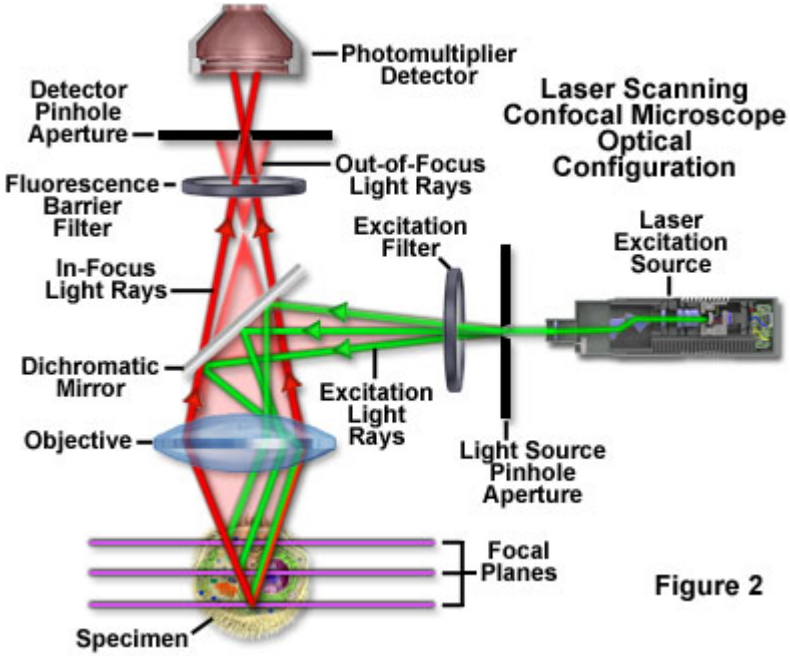
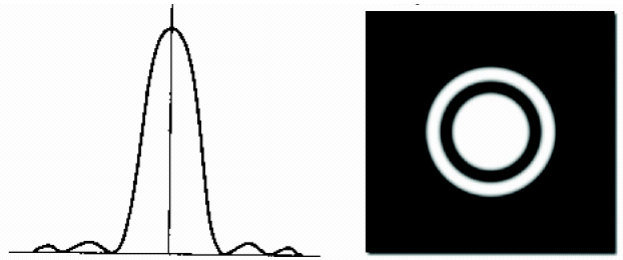
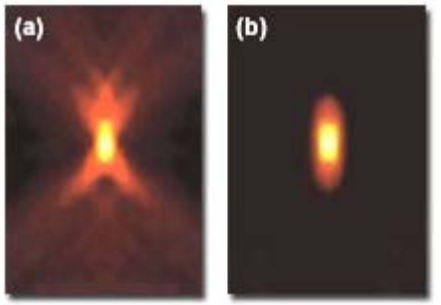


Figure 2

Point spread function: intensity profile measured from a point object; recall the Airy disk at the focal plane



Pinhole aperture as a spatial filter

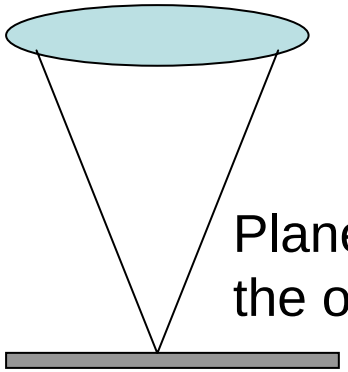


Depth of image

Wide-field Confocal

Optical sectioning (axial response)

More importantly, the spatial filter in confocal microscopy removes contributions from out-of-focus objects ⇒ only signal from a thin layer of the sample is detected (optical sectioning)

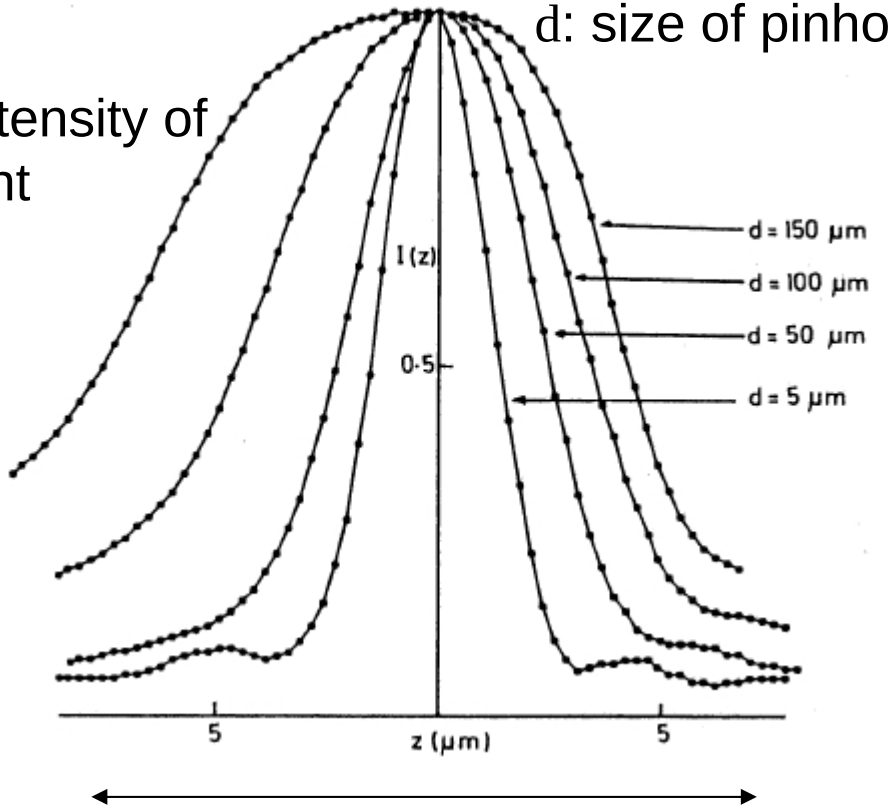


Plane reflector as the object

Scan the object along z through focus

d: size of pinhole

I(z): Total intensity of reflected light



Axial displacement of a plane reflector

Confocal microscopy – resolution

Lateral resolution

Light microscope: $\Delta d = \frac{0.6\lambda}{NA}$

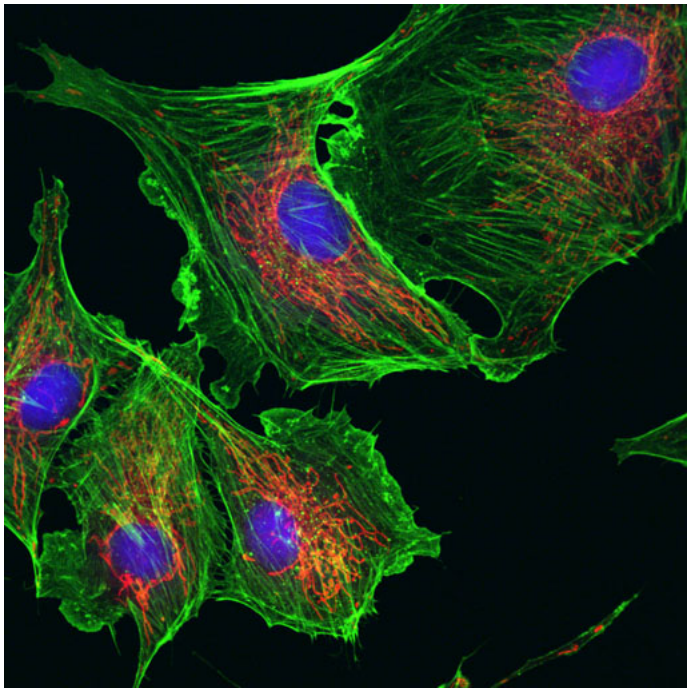
Confocal microscope: $\Delta d = \frac{0.4\lambda}{NA}$

Axial resolution

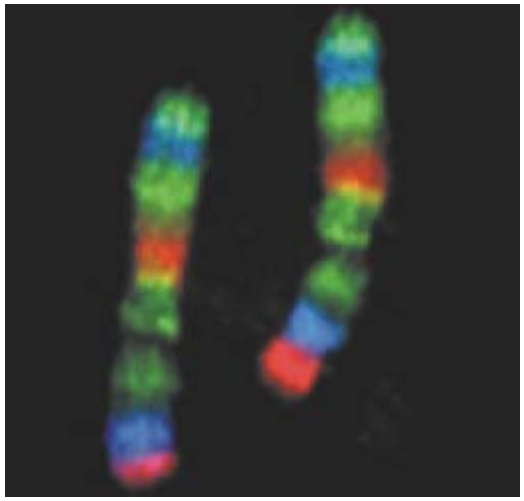
Confocal microscope: $\Delta z = \frac{1.4n\lambda}{NA^2}$

for an ideal infinitely small pinhole

Confocal imaging in life science



Mitotracker (red), Bodipy: F-actin (green), and DAPI (blue): nuclei

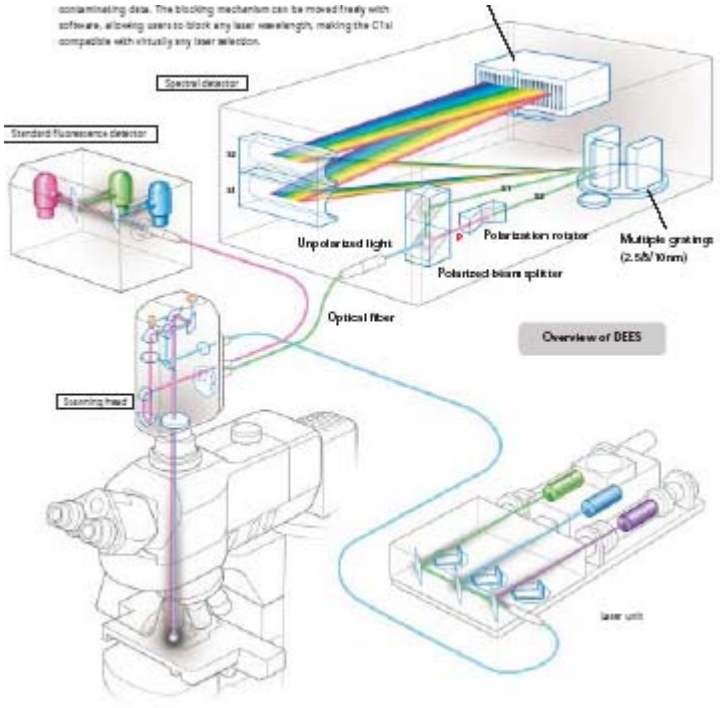


Multi-fluorescence in-situ hybridization (multi-FISH or MISH)

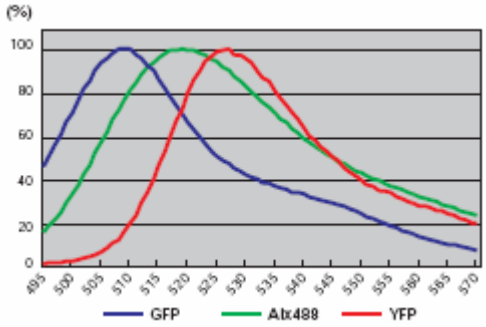
Confocal microscopy – spectral imaging

Nikon C1si

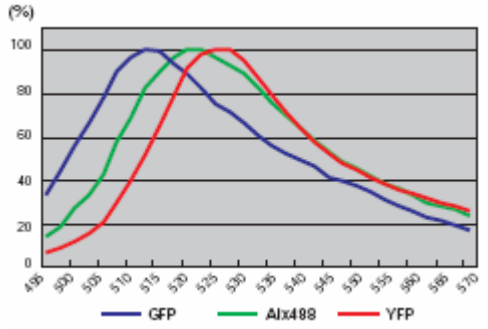
32 spectral detection channels



Emission spectra provided by the probe manufacturer



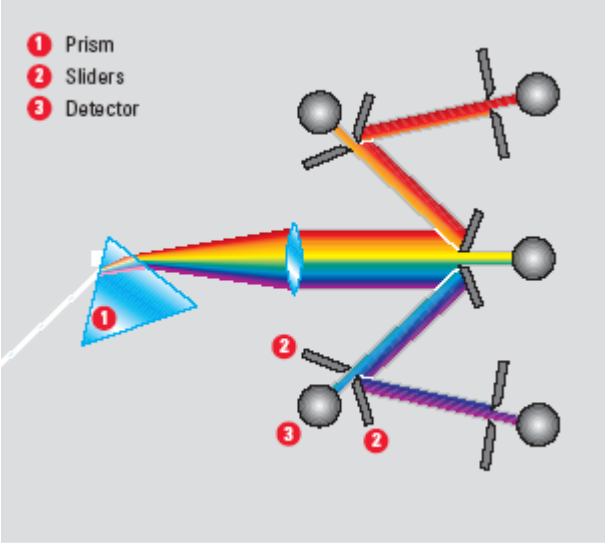
Measured emission spectra



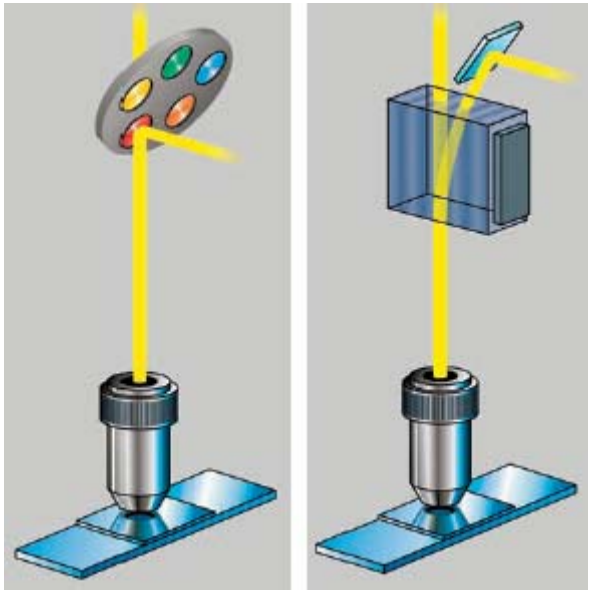
Confocal microscopy – spectral imaging

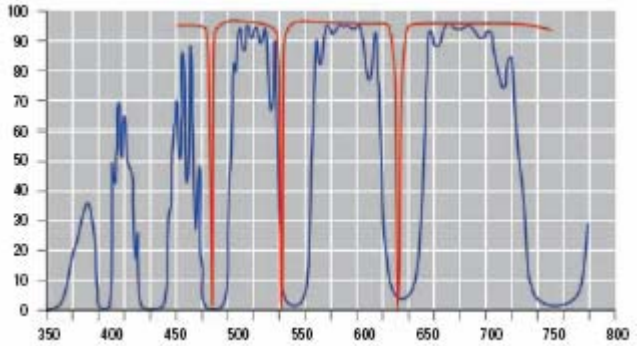
Leica TCS SP5

5-channel detection



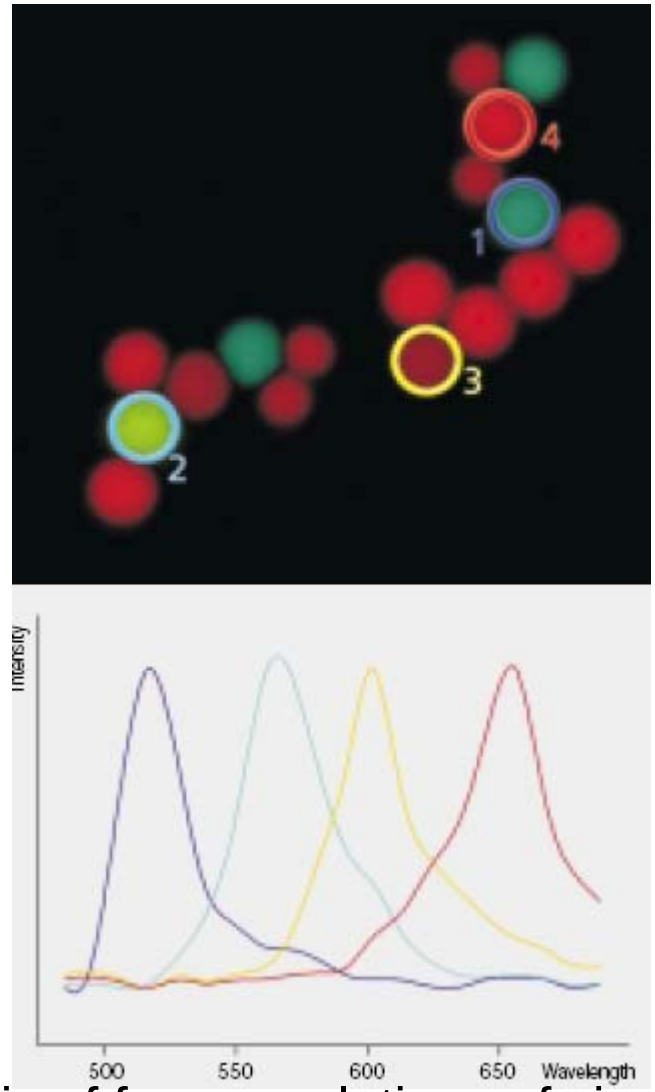
Conventional (left) and Acousto optical beam splitter (AOBS)





Transmission of conventional (blue) and AOBS (red)

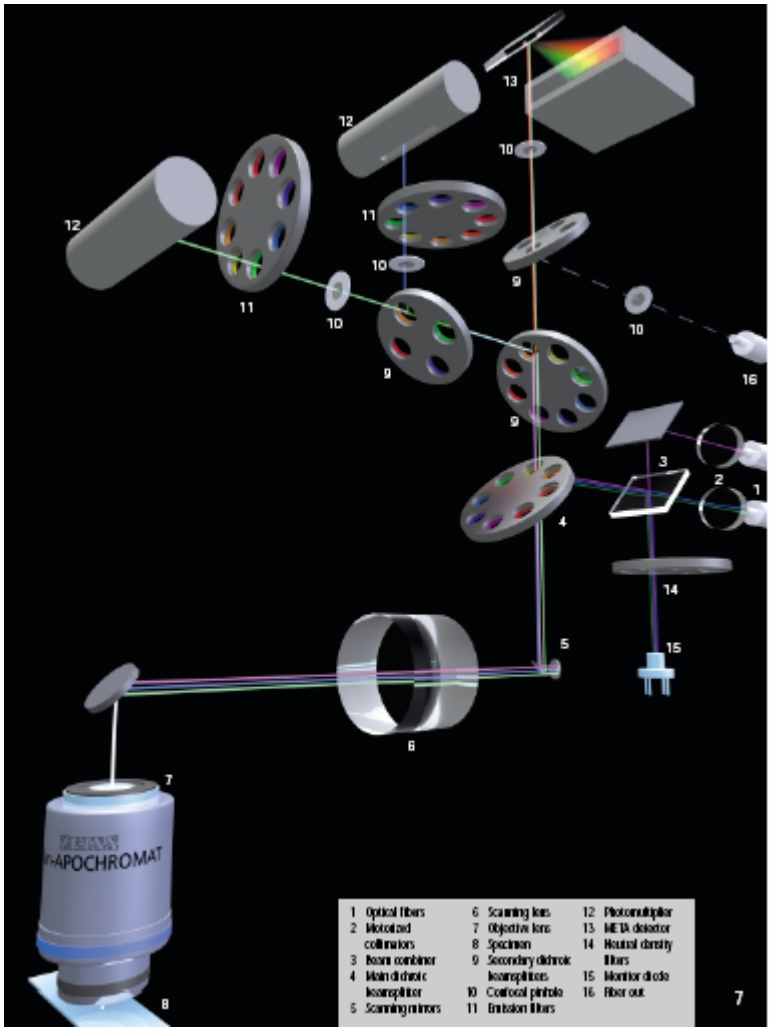
Confocal microscopy – spectral imaging



Mix of four populations of single-labeled fluorescent beads

Zeiss LSM 510 META

32 spectral detection channels

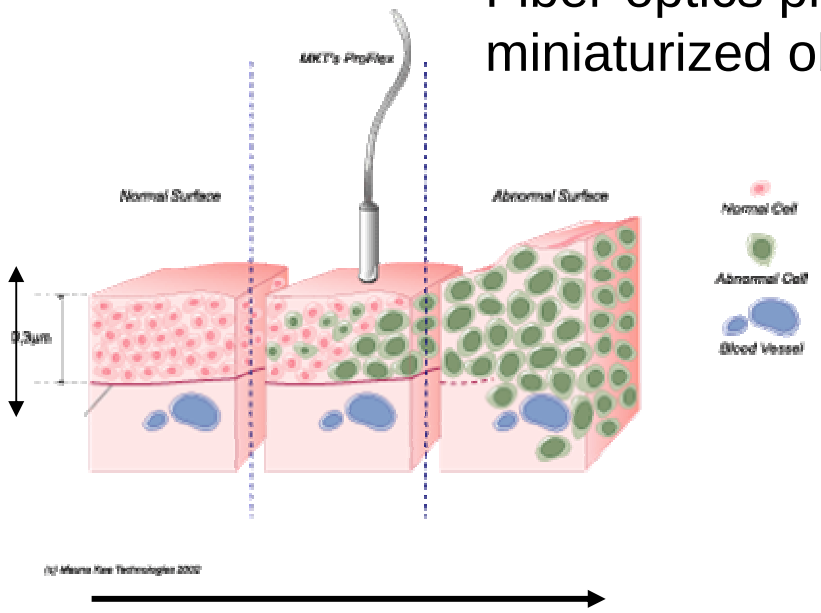


Confocal imaging *in vivo*

Scanning mechanism to achieve 3D imaging

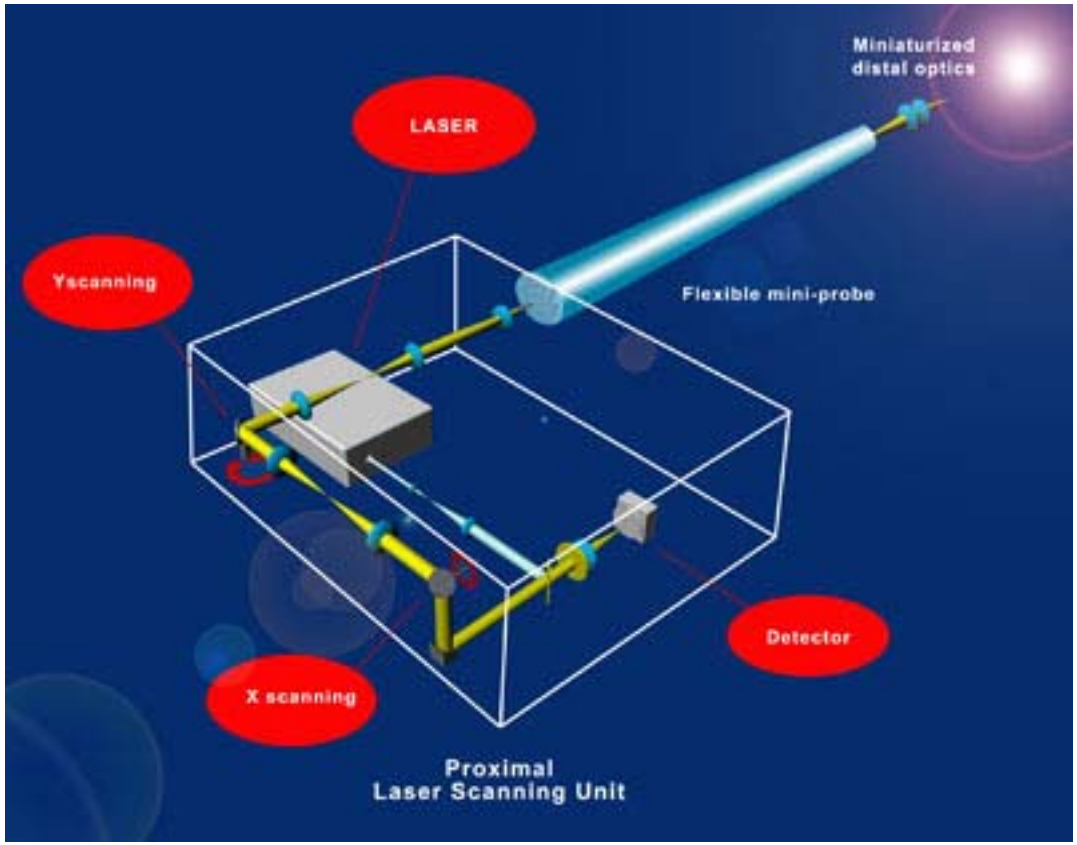
Fiber-optics probe with miniaturized objective

Thickness of <u>epithelial tissue</u> 0.3~0.5 mm

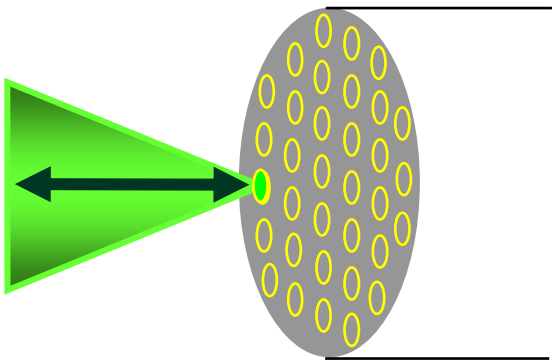


Progressing of cancer

Confocal imaging *in vivo* (cont.)



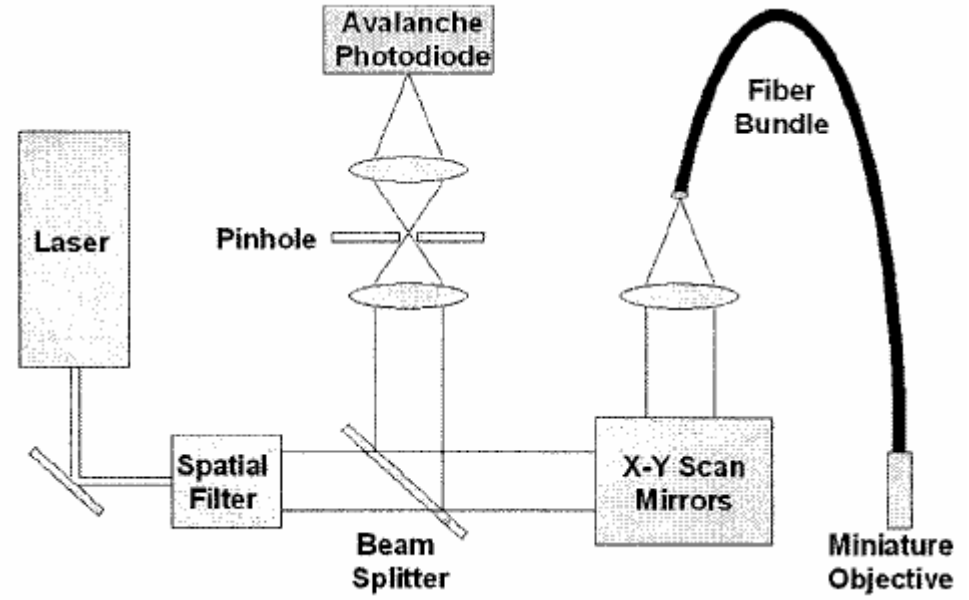
Focused onto one fiber at a time



Fiber Bundle

Confocal imaging *in vivo*

Use optical fibers to transfer illumination light and reflected or fluorescence light to and from the target; more on fiber-optics later



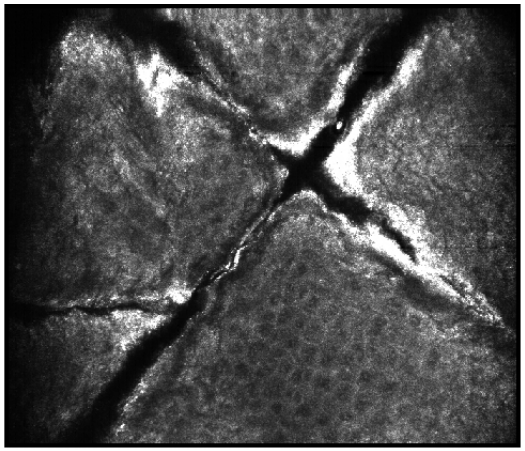
Endoscopic probes (Mauna Kea Technologies)

Both reflectance and fluorescence modes have been demonstrated

Confocal imaging *in vivo*

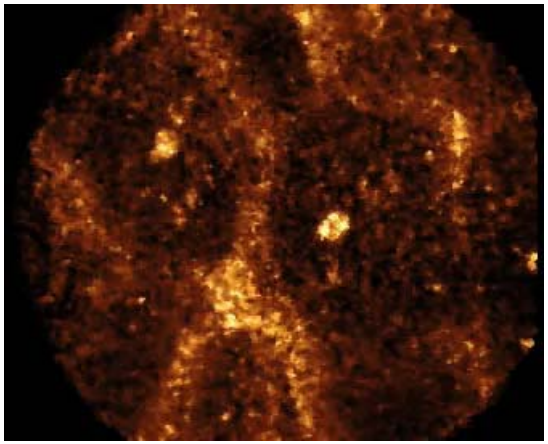
Confocal imaging using reflected light from tissue

skin[1]

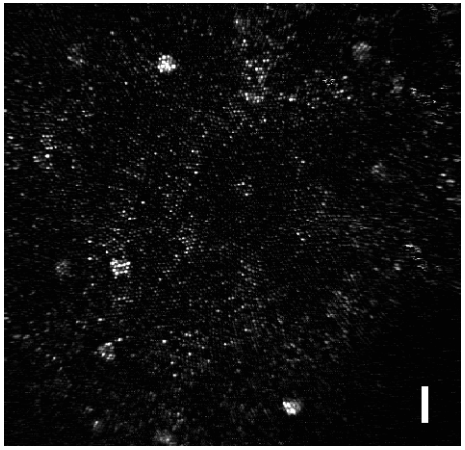


In Vivo VivaScope® image showing an en face optical section of the stratum corneum and granular cells. The dark "cross" in the image is a fold or wrinkle in the skin.

Oral cavity[2]



Uterine cervix[3]



[1]Lucid, Inc. http://www.lucid-tech.com/index.shtml

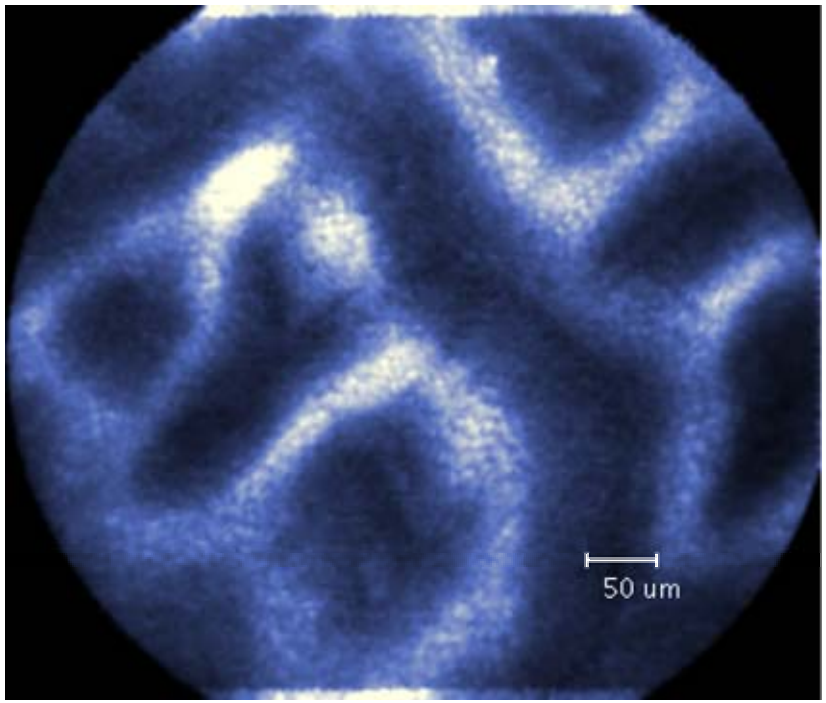
[2]Mauna Kea Technologies, http://www.maunakeatech.com/index.php

[3]Sung et al. Optics Express **11**, 3171-3181 (2003)

Confocal imaging *in vivo*

Confocal images of various tissue (fluorescence from topically applied dyes)

Gastric metaplasia in Barrett's esophagus (IV injection of 10% fluorescein)



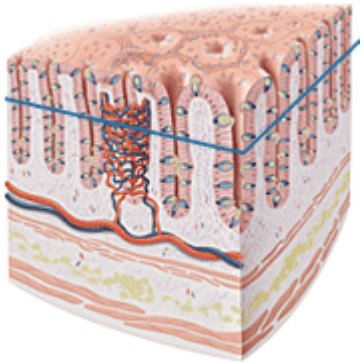
Colonic mucosa
(topical application of 0.05% fluorescein)

Cardia mucosa
(topical application of 0.25% cresyl violet)

Mauna Kea Technologies, http://www.maunakeatech.com/index.php

Confocal imaging *in vivo*

rectum

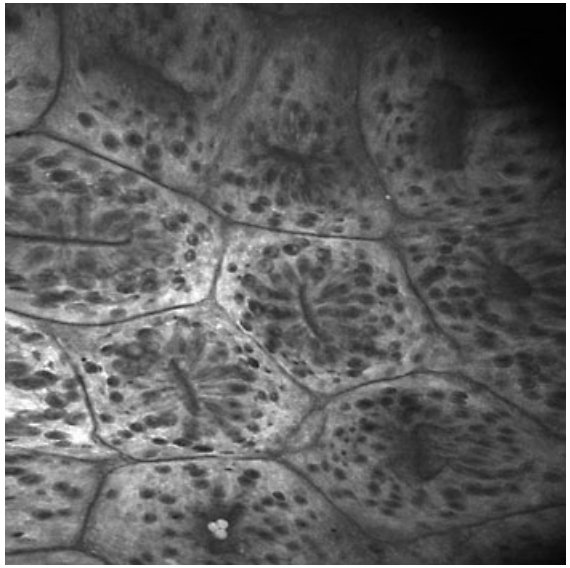


1

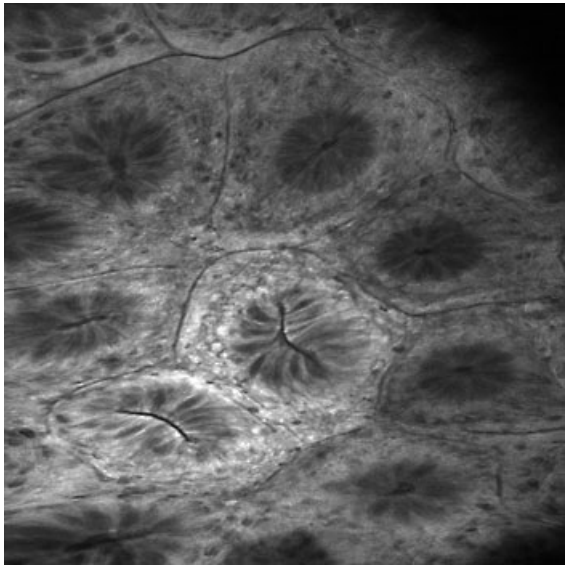
4

(IV administration of 10% fluorescein sodium)

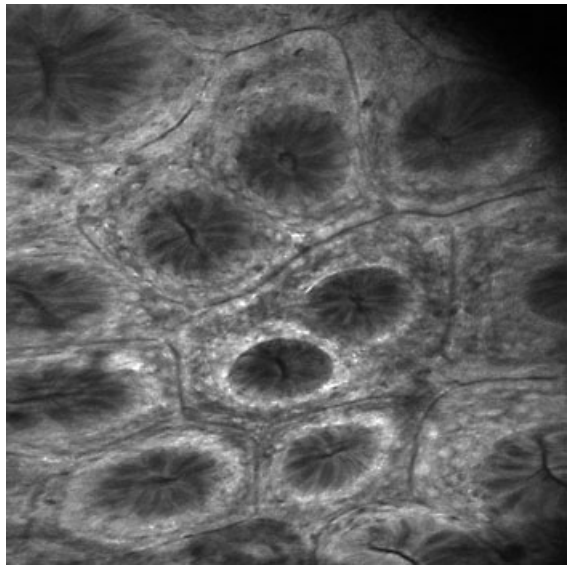
1



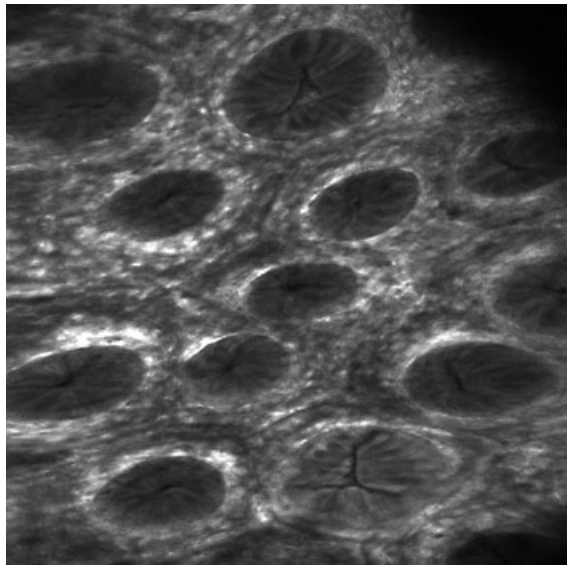
2



3



4



Optiscan, http://www.optiscan.com/index.asp

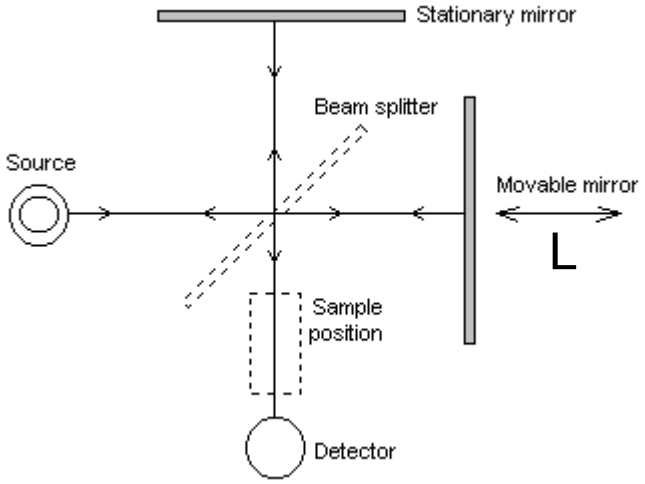
Optical coherence tomography

Similar to ultrasound: signal is reflected from surfaces within sample

- Speed of light is much faster than speed of sound, so we can not measure time-of-flight directly

- Use interference between two light waves to measure light path difference $\Rightarrow$ interferometry

Michelson interferometer



First consider single wavelength:

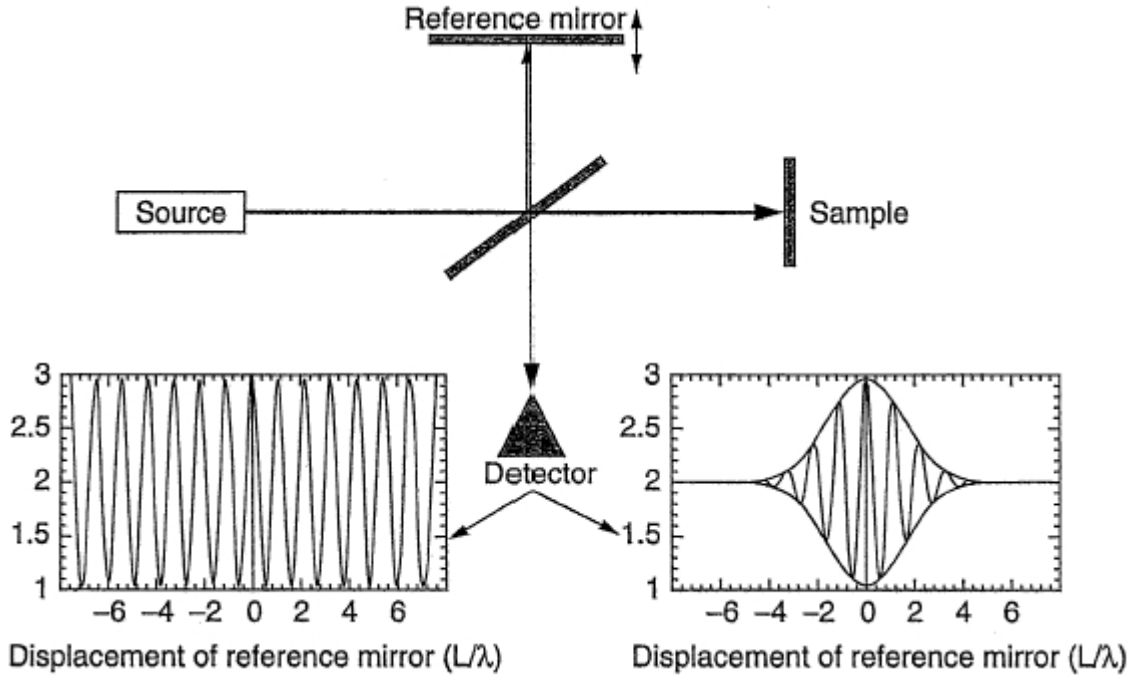
Light path difference = 2L

Phase difference = $\frac{2\pi}{\lambda} \cdot 2L = \frac{4\pi L}{\lambda}$

The intensity of interference

$$I = I_1 + I_2 + 2\sqrt{I_1 I_2} \cdot \cos(\frac{4\pi L}{\lambda})$$

OCT (cont.)



Coherent light

Low coherence light

- With broadband illumination (multiple wavelengths), constructive interference happens only when light paths match exactly (mirror displacement = 0)
- When light path difference gets larger, light with different wavelengths will also differ in relative phase

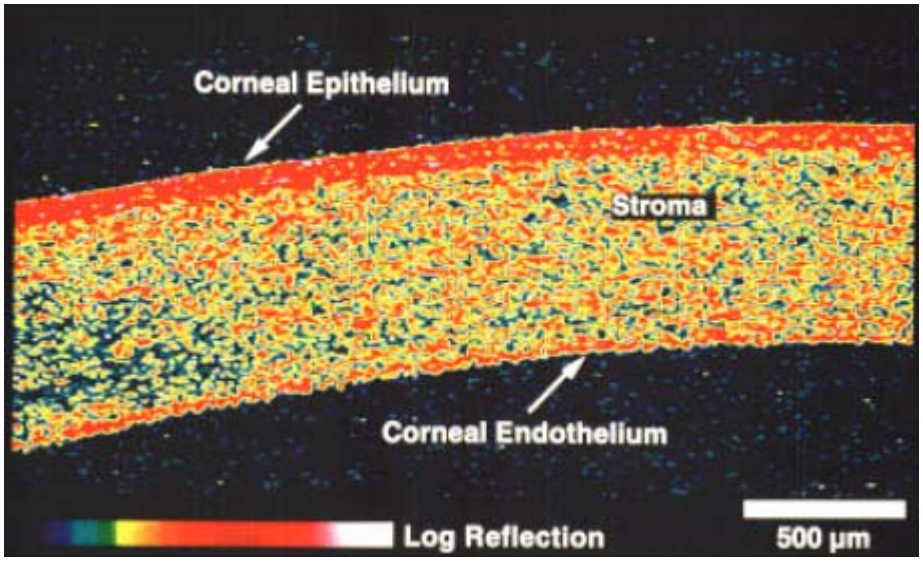
OCT image formation

- Low-coherence interferometry can be used to identify the location of interface (origin of reflected signal) and measure its intensity within the sample by scanning the reference mirror

- This is similar to one scan line in B-mode ultrasound; to get a 2D image, we need to scan the line across the sample

Scanning of the sample relative to the system

Depth (scanning of the reference mirror)



OCT image of cornea

OCT (cont.)

The axial resolution is determined by the distance over which light from sample and reference can still interfere with each other $\Rightarrow$ coherence length, which is related to the bandwidth of the light source

Axial resolution

$$l_C / 2 = \frac{2 \ln 2}{\pi} \cdot \frac{\lambda^2}{\Delta\lambda}$$

Generally axial resolution is 4-20 μm

l_C: coherence length

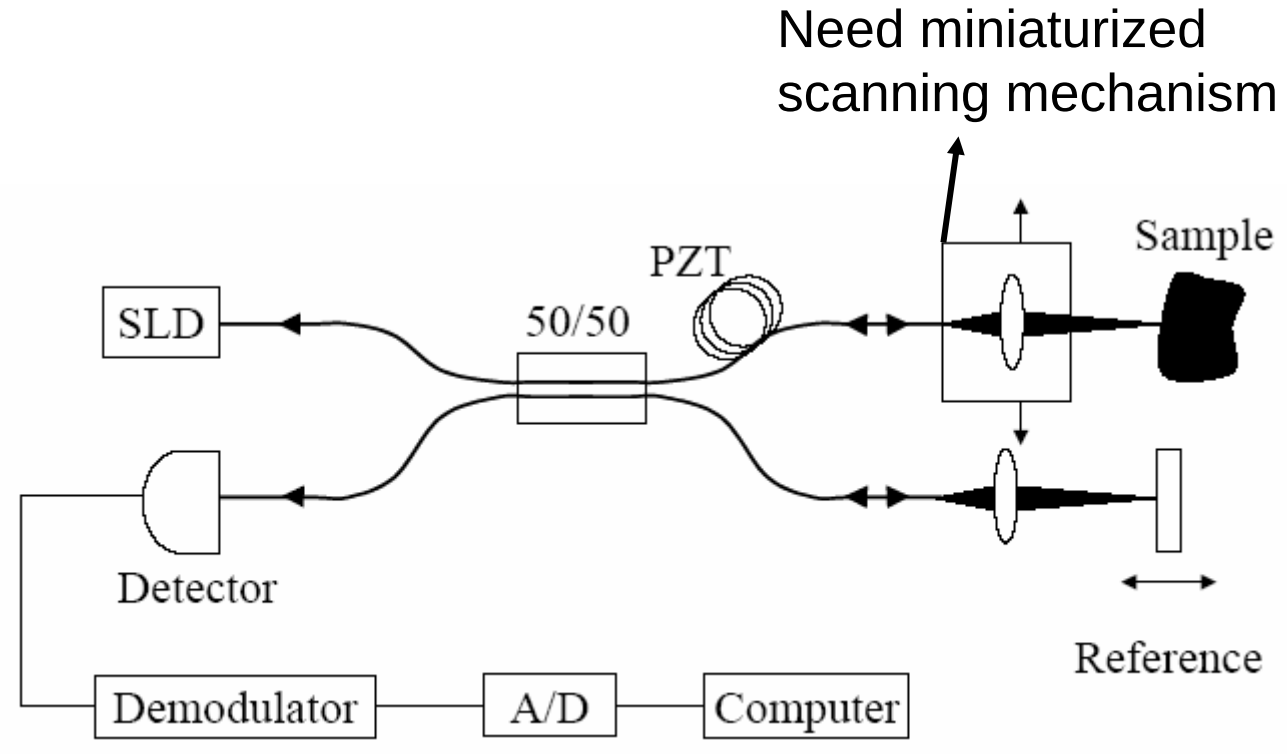
$\Delta\lambda$ is the bandwidth of the light source

λ is the center wavelength

Lateral resolution is determined by the spot size and is usually **larger** than axial resolution

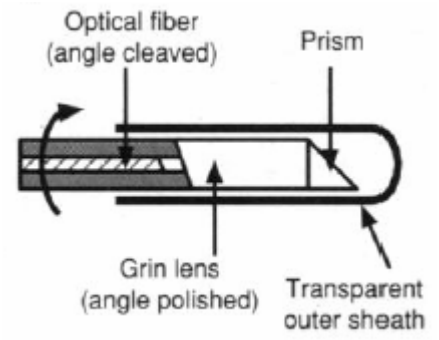
OCT (cont.)

Fiber optic components to make miniaturized probe for *in vivo* imaging



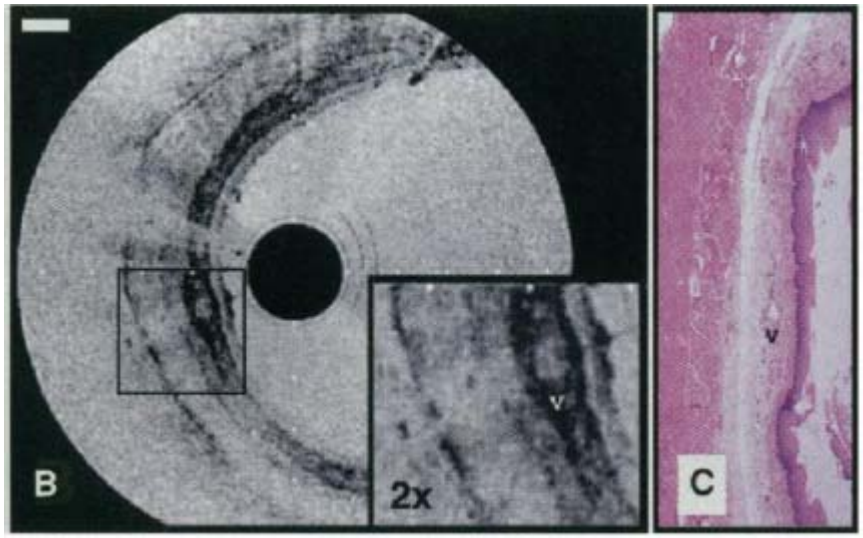
OCT – *in vivo* imaging

One design of scanning



1mm

OCT image of rabbit esophagus *in vivo*
Scale bar = 500 μm



Axial resolution = 10 μm

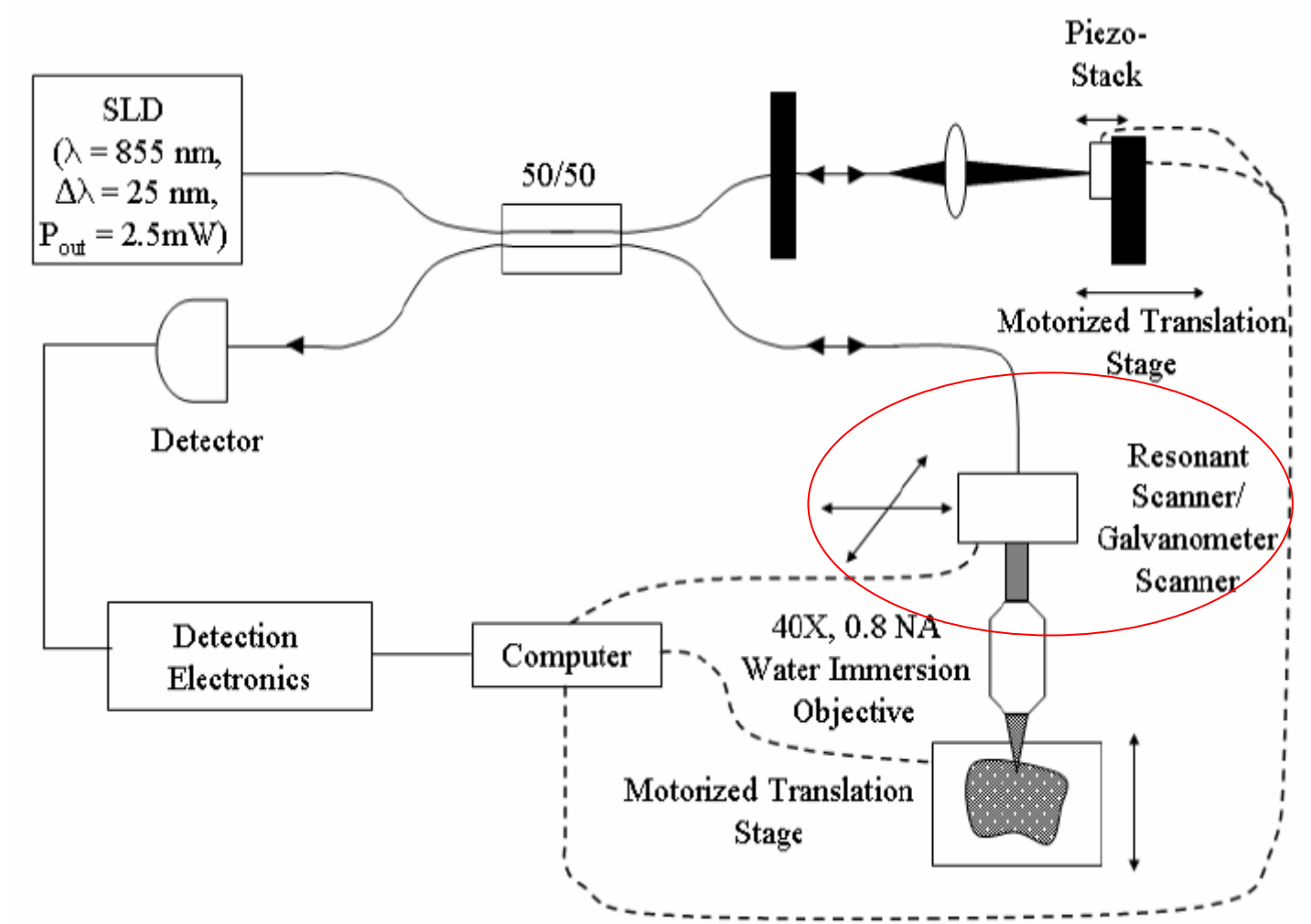
Rotates 4 revolutions per second

Focused spot diameter = 40 μm

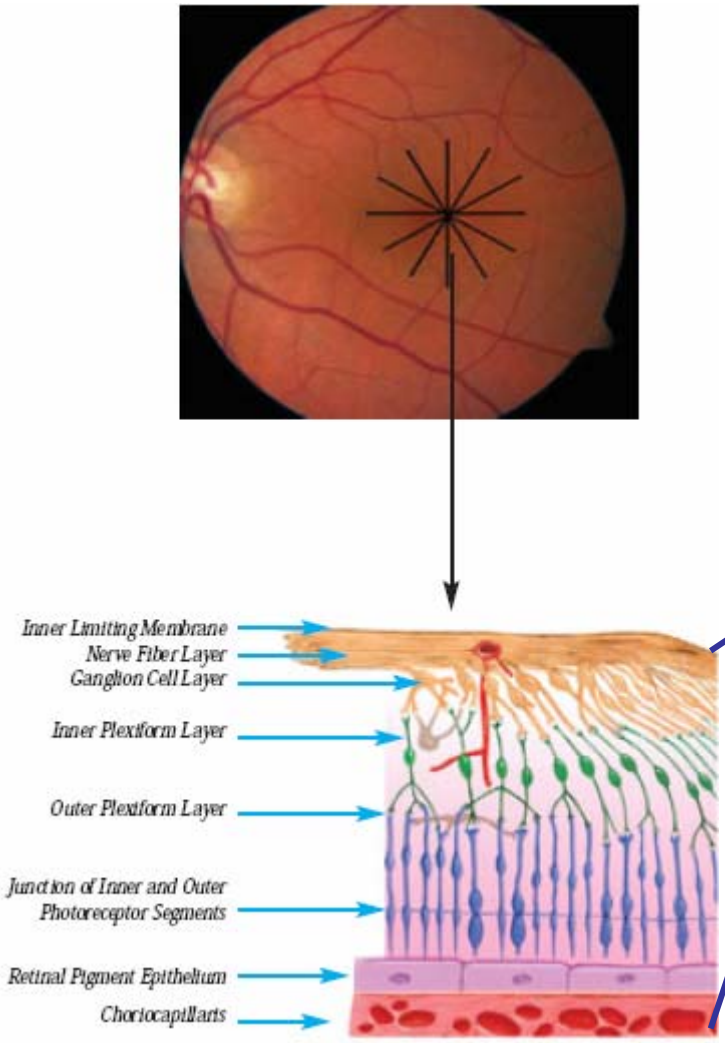
Science **276**, 2037-2039, 27 June 1997

Optical coherence microscopy

Scanning in both x and y directions to make 3D images

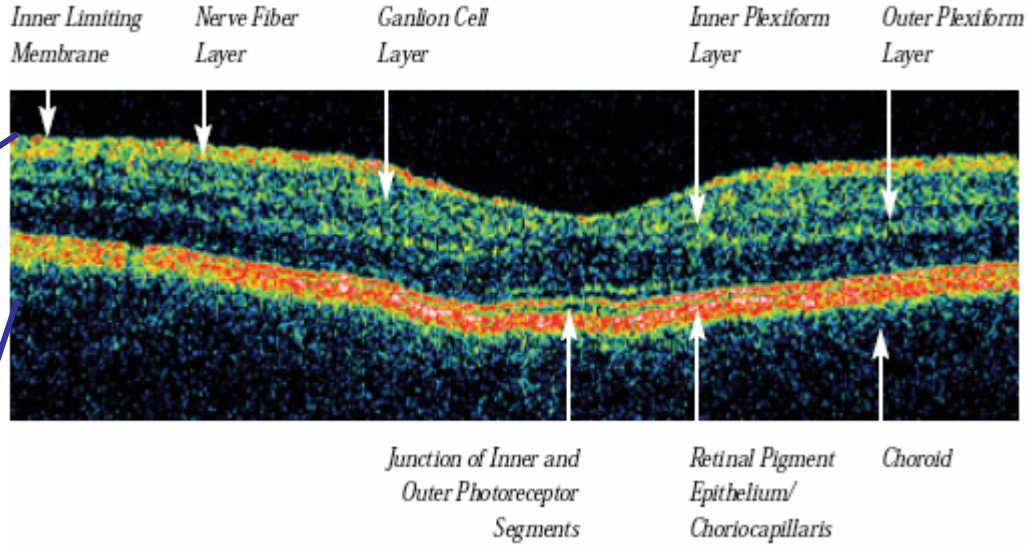


OCT imaging of retina



Zeiss Stratus OCT™

10μm axial and 20 μm lateral resolution



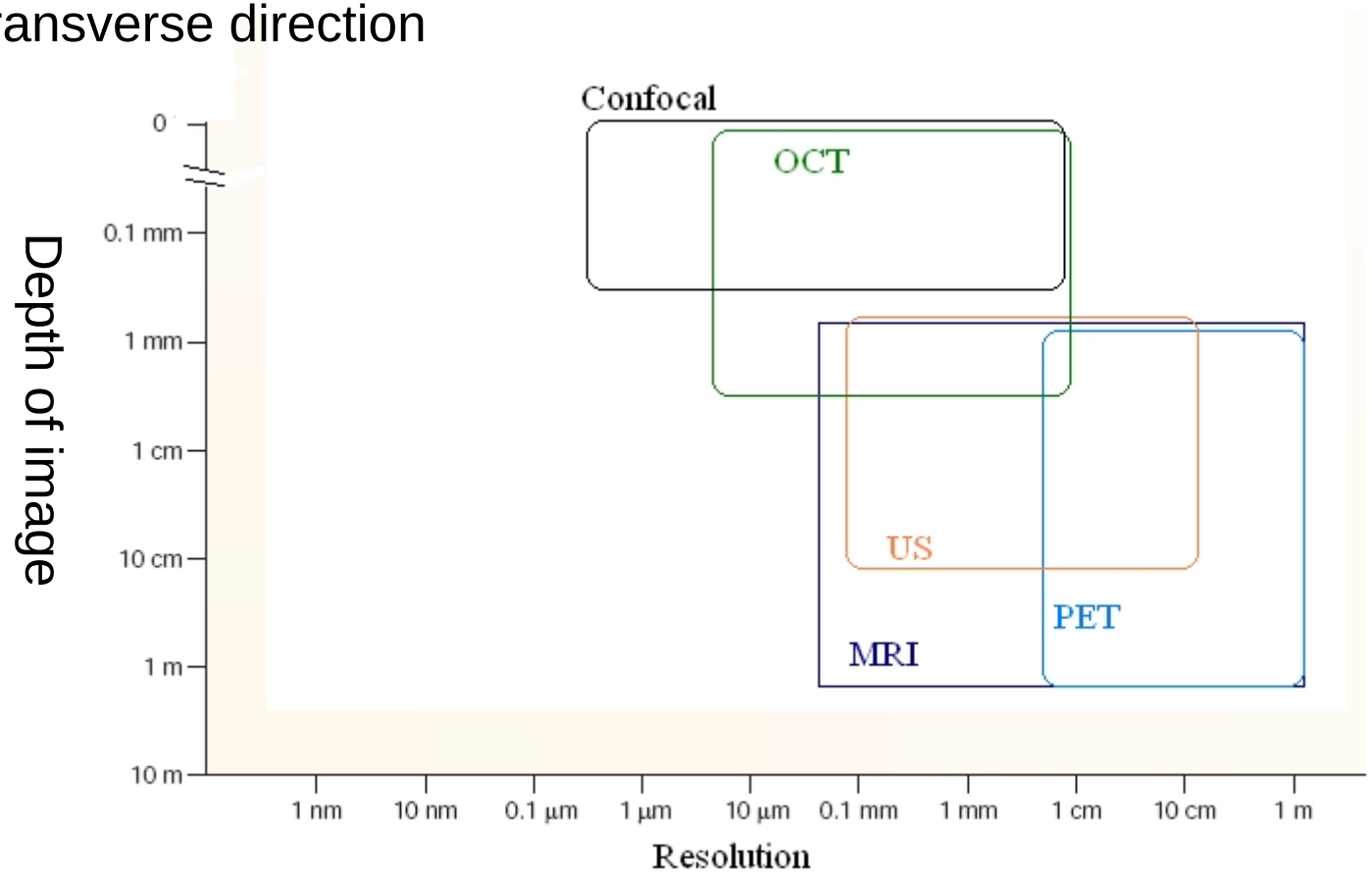
Zeiss Visante™ OCT Anterior segment and cornea imaging

More on OCT

- Many variations of techniques exist for OCT: frequency-domain, polarization-sensitive OCT, spectroscopic OCT, full-field OCT, optical coherence microscopy (OCM), etc.
- Very active R&D for commercialization in applications such as diagnostics

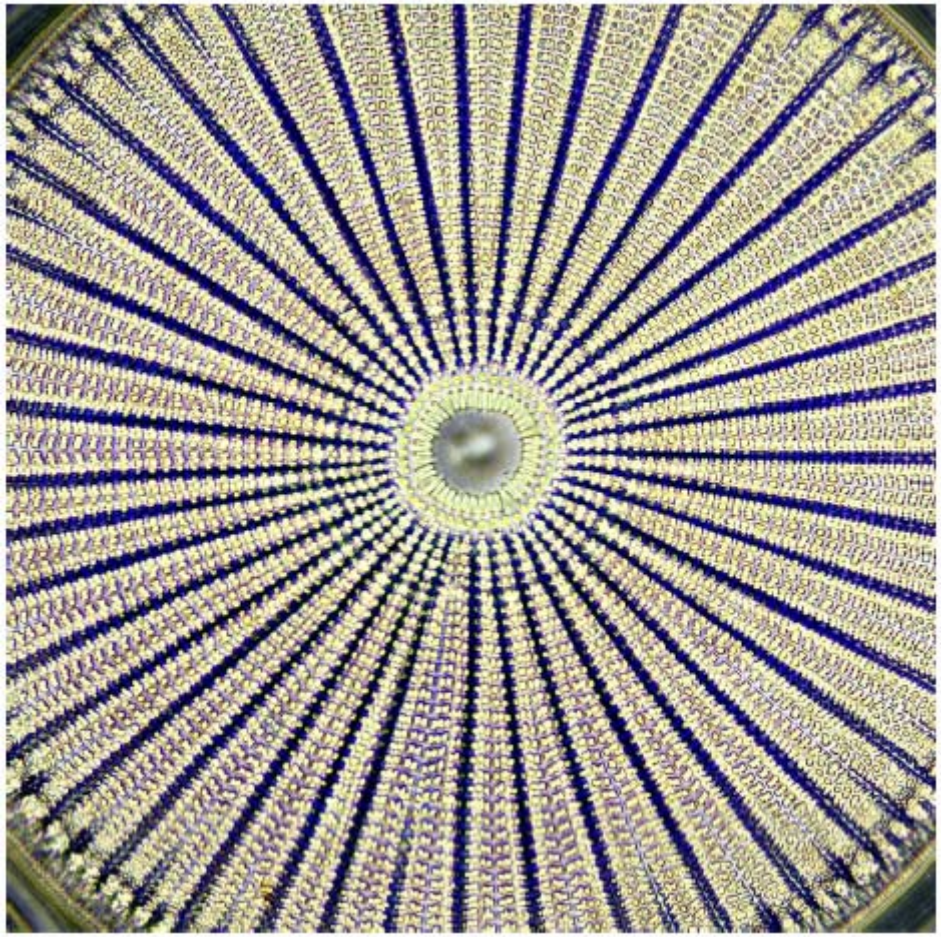
Compare confocal and OCT to other medical imaging methods

* For OCT, resolution in depth is usually better than resolution in transverse direction



Adapted from *Nature cell biology*, ss16-ss21, Sep 2003

Comments are welcome!



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- Mauna Kea Technologies, http://www.maunakeatech.com/index.php
- Introduction to Biophotonics, by Paras N. Prasad
  - Ch7 Bioimaging: Principles and Techniques
- Handbook of Biological Confocal Microscopy, 2nd ed., edited by James B. Pawley
  - Ch11 The Role of the Pinhole in Confocal Imaging System