Optical Microscopy

Principles and Applications

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8/1/2018

Self Introduction

BE in Electrical Engineering from Tribhuvan University

Worked as a Telecom Engineer at Nepal Telecom

M.Sc. in Electrical Engineering from South Dakota State University

Ph.D. in Electrical Engineering from Boston University

Major Functions of the Microscope

- Illuminate
- Magnify
- Resolve features
- Generate Contrast
- Capture and display image

Pictures from MicroscopyU.com

Lecture Outline

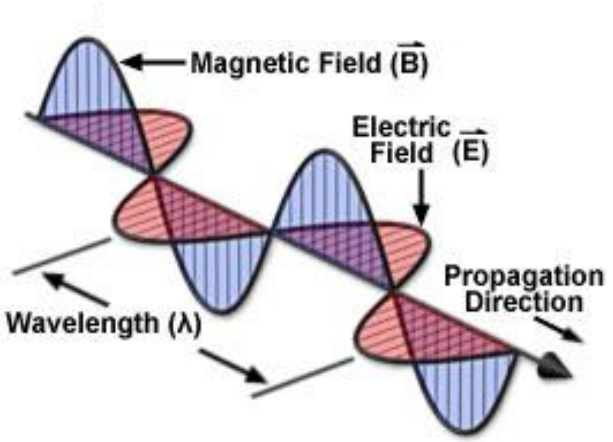
- Propagation, diffraction, and polarization
- Absorption, and scattering
- Wide-field imaging techniques
  - Bright-field/dark-field imaging,
  - Phase-contrast imaging, and
  - Differential interference contrast imaging
- Scanning imaging techniques
- Confocal detection
- Differential phase-gradient detection
- Non-linear imaging techniques
  - Multi-photon,
  - Second harmonic, and
  - Raman scattering

Light as photons, waves or rays

Light is an electromagnetic (EM) field in space-time.

Photon is the smallest, discrete quanta of EM field.

Rays are the propagation direction of the EM field.



Maxwell equations

$$\nabla \cdot E = 0, \quad \nabla \times E = -\mu \frac{\partial H}{\partial t}$$

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$$\nabla^2 E - \mu\varepsilon \frac{\partial^2 E}{\partial^2 t} = 0$$

Wave equation in Linear medium

$$\frac{n^2}{c^2} = \mu\varepsilon$$

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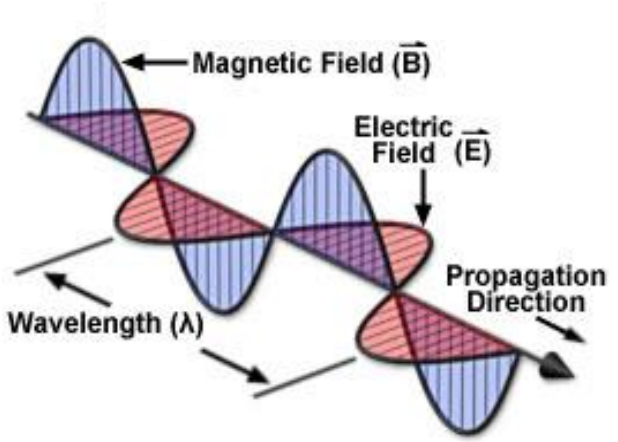

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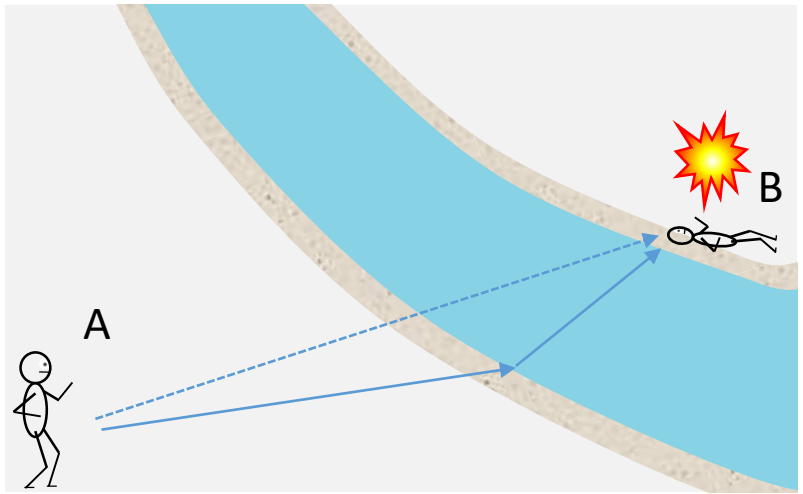
Wave equation in Linear medium

$$\frac{n^2}{c^2} = \mu\varepsilon$$

Ray Optics: Optical rays travelling between two points A and B follow a path such that the time of travel (or optical path-length) between two points is minimal relative to the neighboring paths.

$$\delta \int_A^B n(r) ds = 0$$

Light travels along the path of least time.



Light propagation

Wave equation $\nabla^2 E - \frac{1}{c^2}\frac{\partial^2 E}{\partial^2 t} = 0$

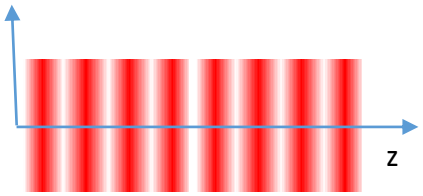
Helmholtz equation $\nabla^2 U + k^2 U = 0$

Solution $E(r,t) = a(r)e^{-i\boldsymbol{k}.\boldsymbol{r}}e^{i2\pi\nu t}$

$E(r,t) = U(r)e^{i2\pi\nu t}$

Plane wave:

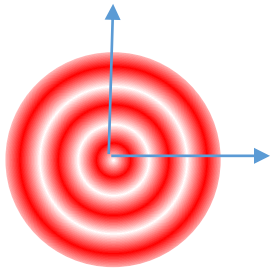
$U(r) = Ae^{-i\boldsymbol{k}.\boldsymbol{r}}$



Light from stars

Spherical wave:

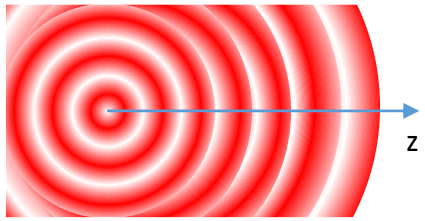
$U(r) = \frac{A}{r}e^{-ikr}$



Point source

Fresnel Approx. of Spherical wave:

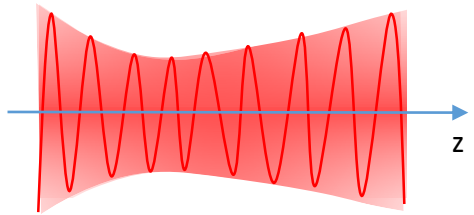
$U(r) \approx \frac{A}{r}e^{-ikz}e^{\left[-ik\frac{x^2+y^2}{2z}\right]}$



Point source at large distance

Paraxial wave:

$U(r) \approx A(r)e^{-ikz}$

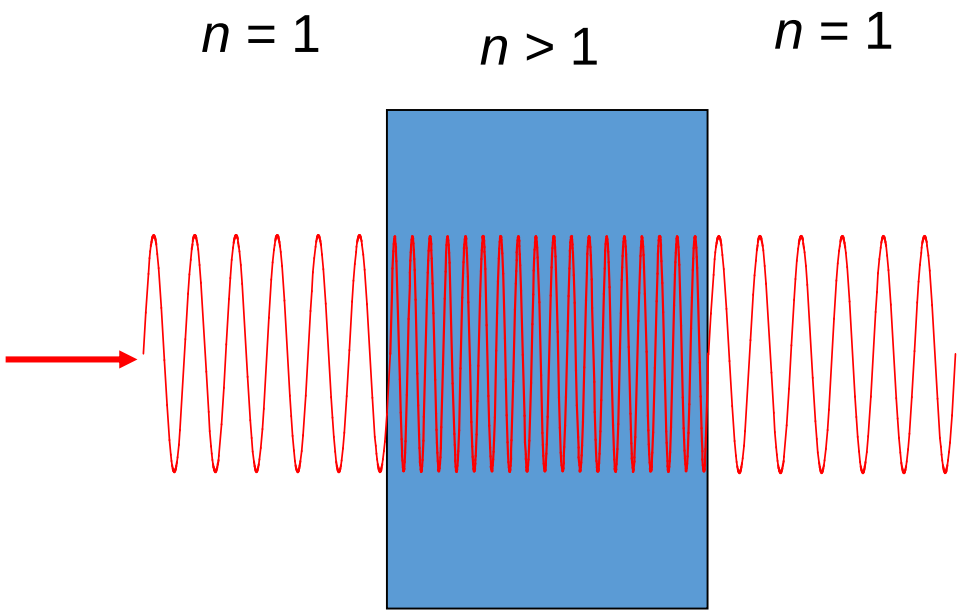


$\nabla^2 A - i2k\,A = 0$

Gaussian Beam

Light travels more slowly in matter

The speed ratio is the
Index of Refraction
(n=c/v)



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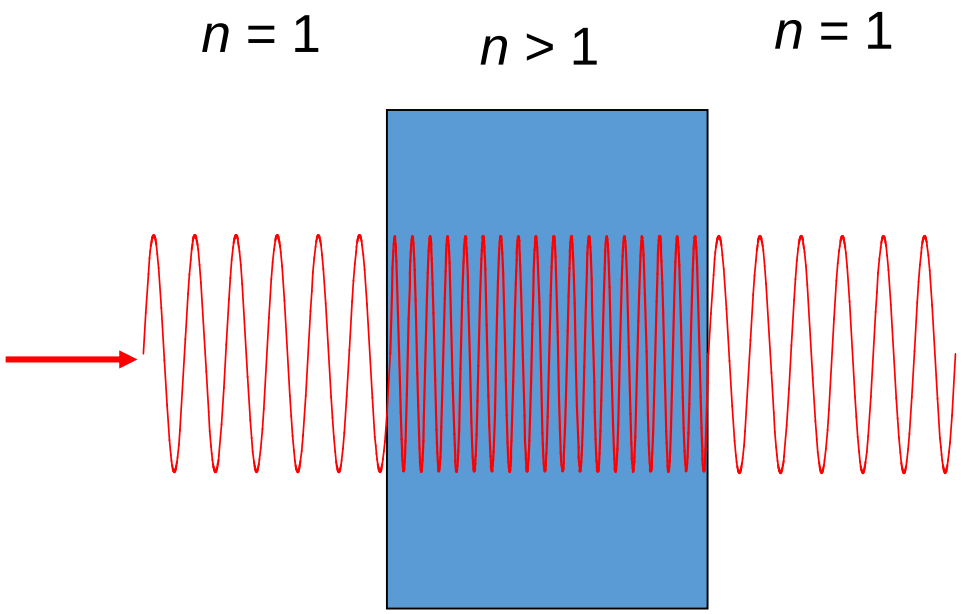
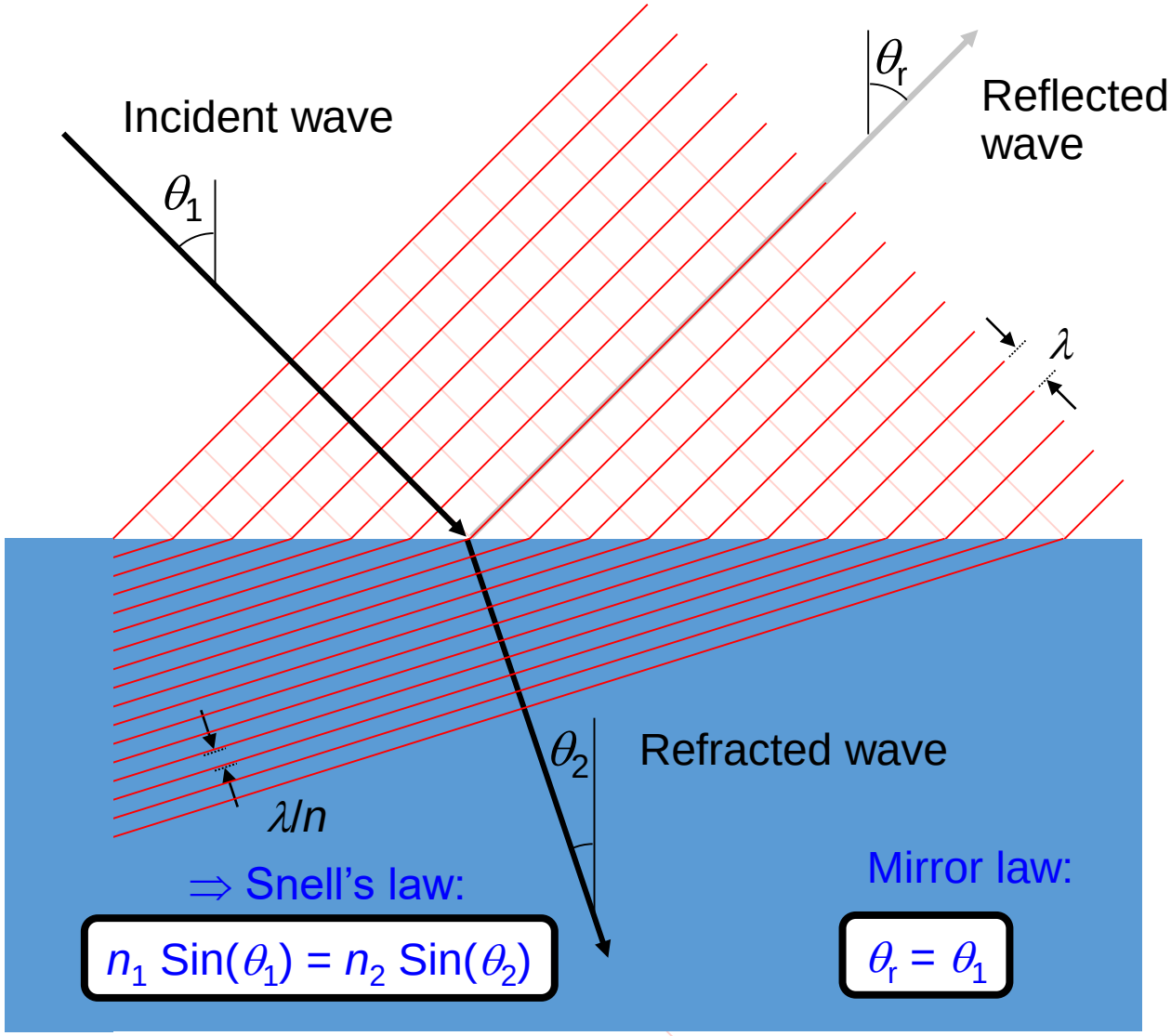
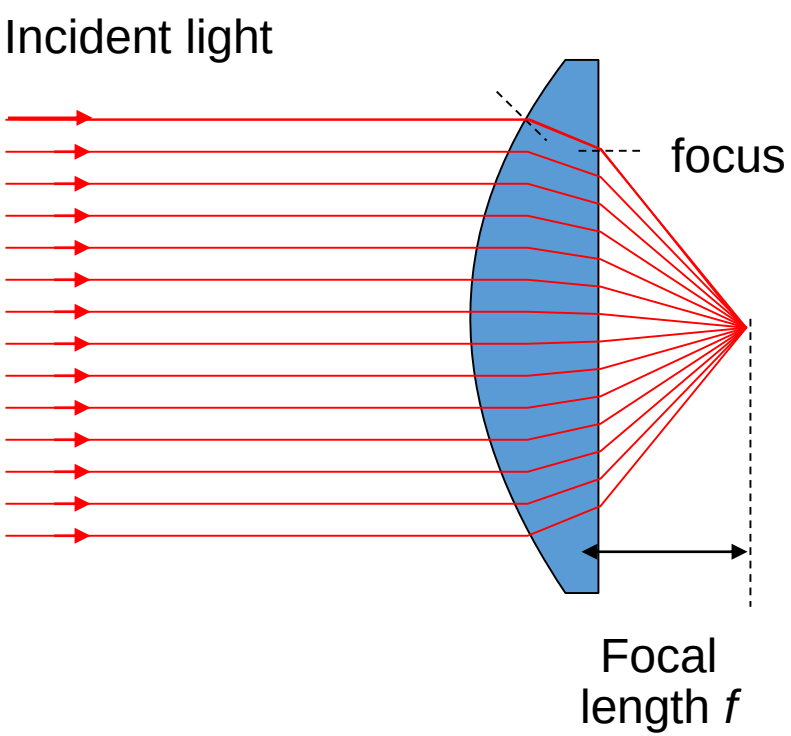
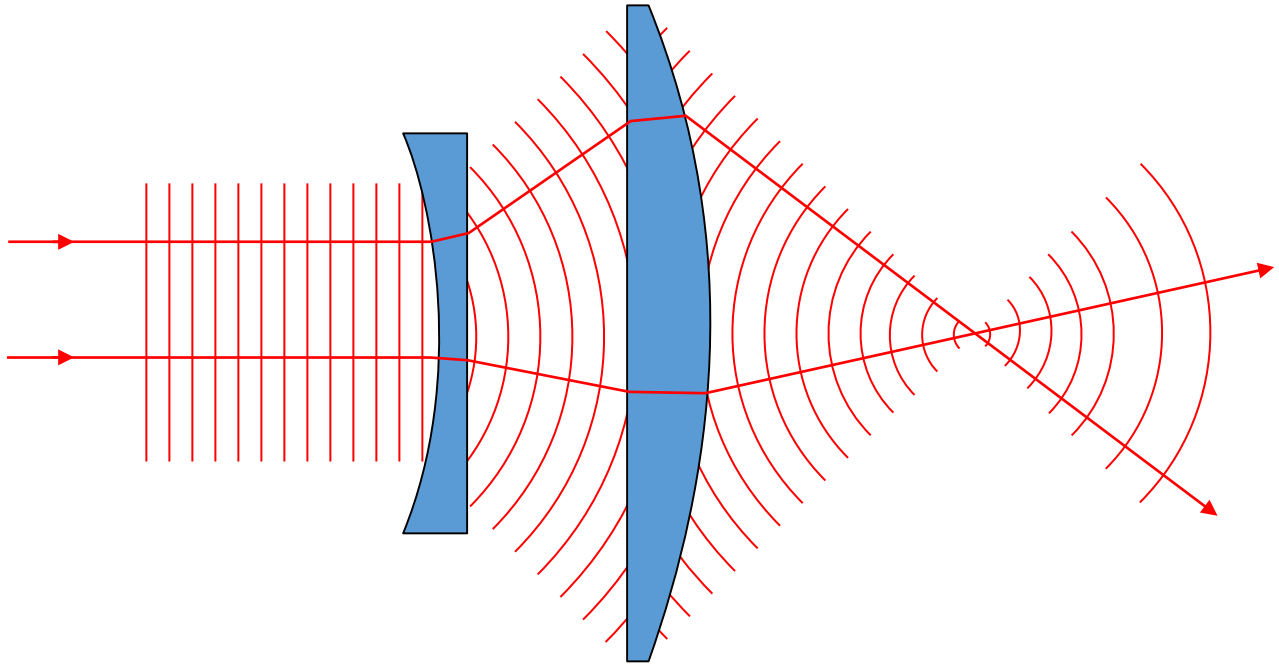




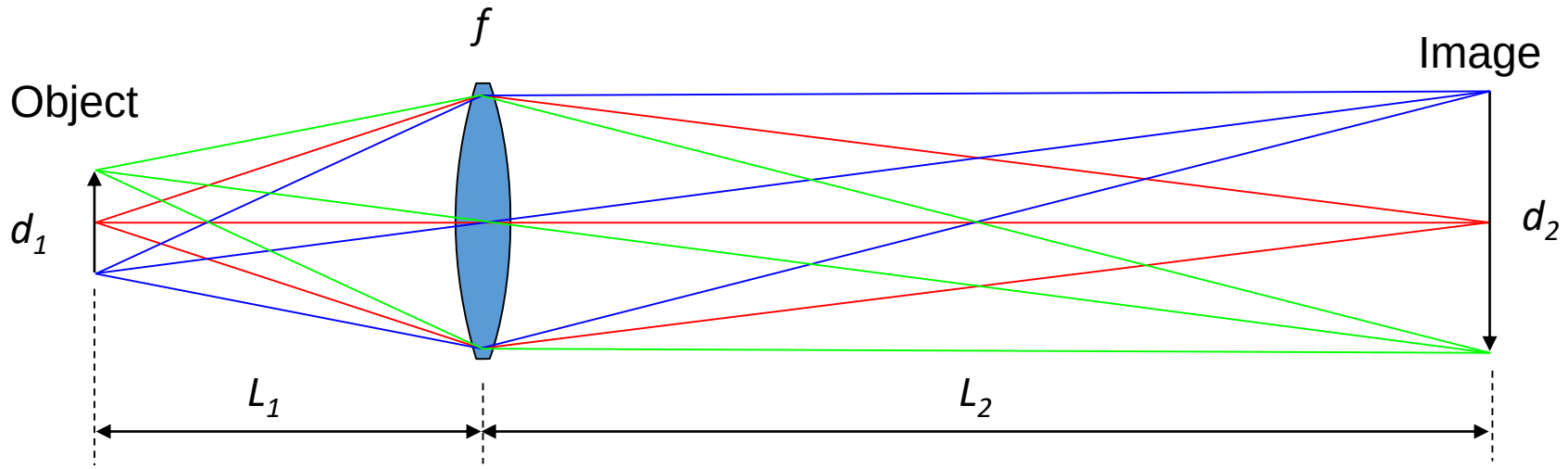
Lenses work by refraction





Rays are perpendicular
to wave fronts

Single lens Imaging

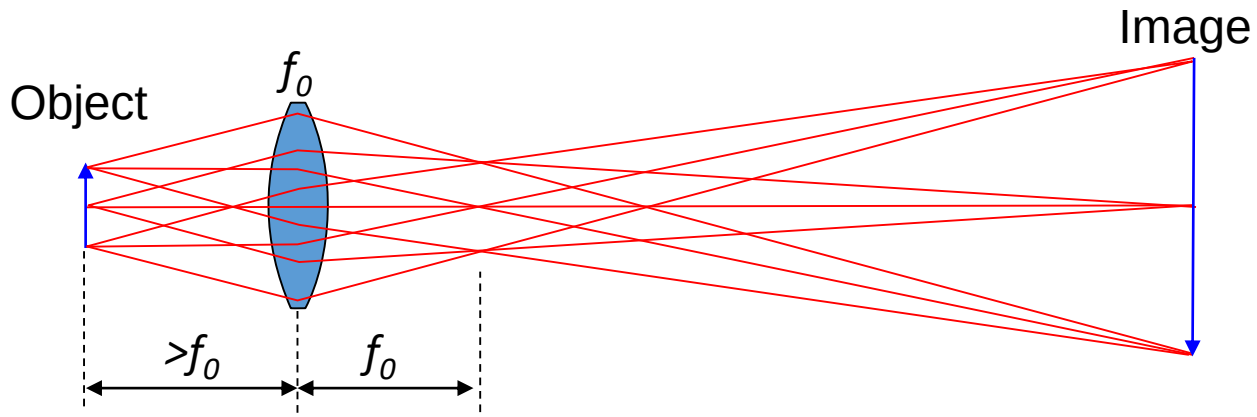


The lens law: $$\frac{1}{L_1}+\frac{1}{L_2}=\frac{1}{f}$$

Magnification: $$M=\frac{d_2}{d_1}=\frac{L_2}{L_1}$$

Finite vs. Infinite Conjugate Imaging

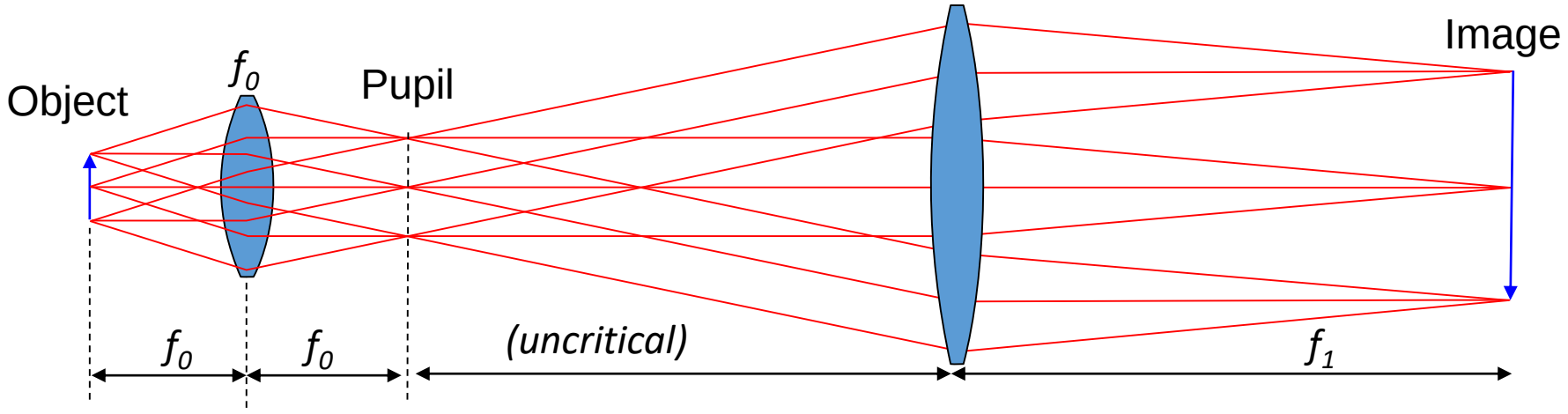
Finite conjugate imaging



Field-of-view (FOV) is determined by the size (optics diameter)of the lenses.

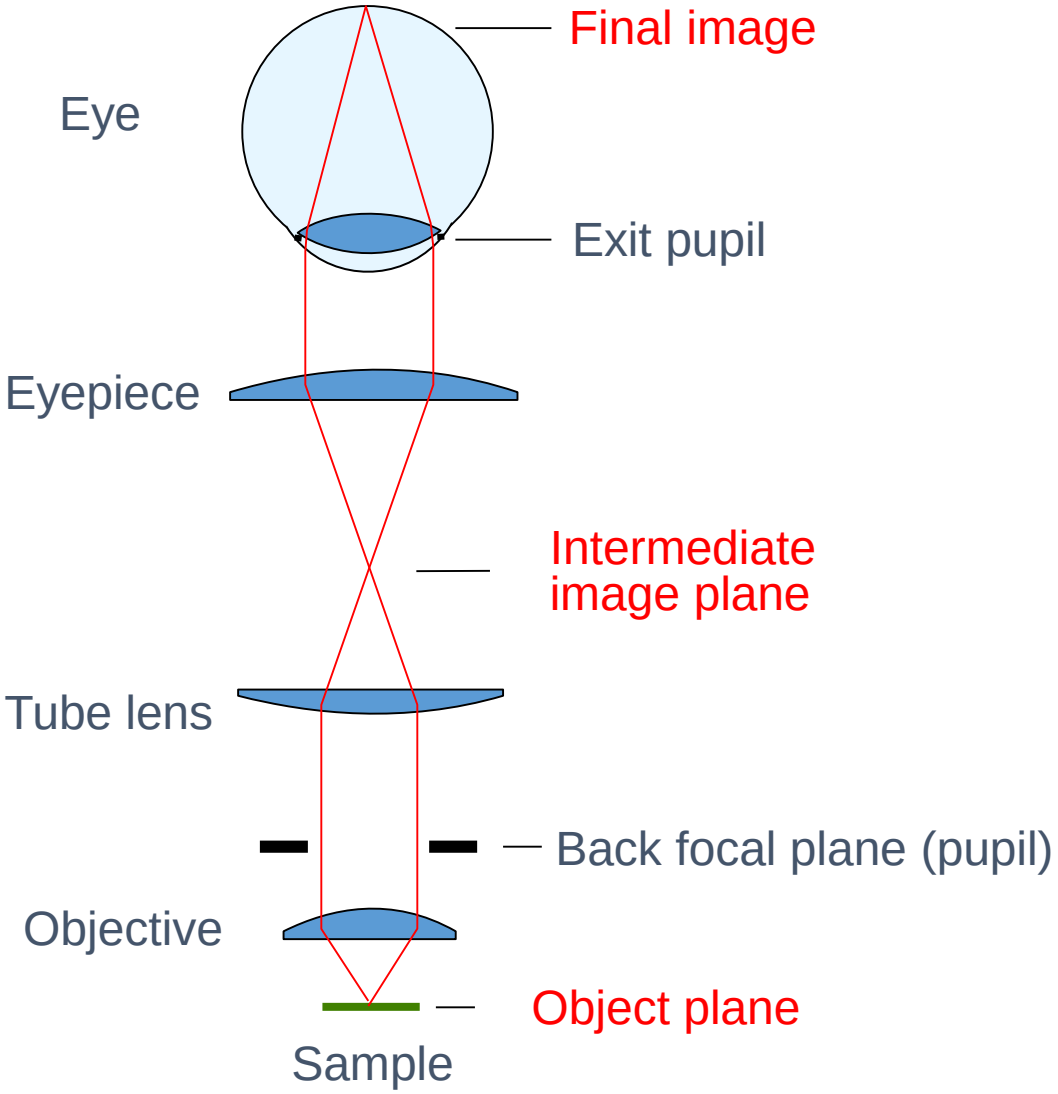
Infinite conjugate imaging

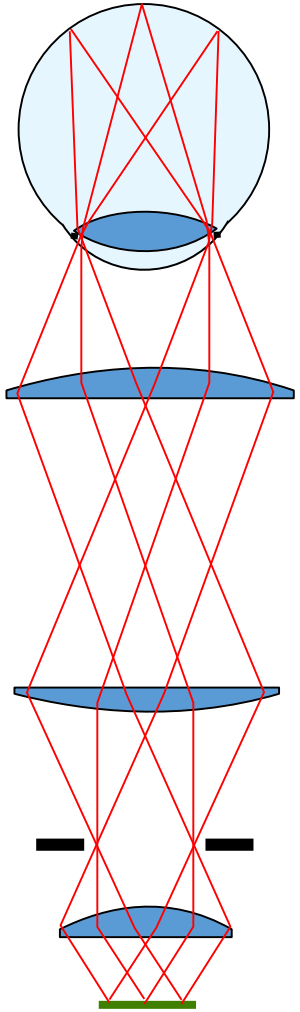
f_1 $\Rightarrow$ Need a ***tube lens***



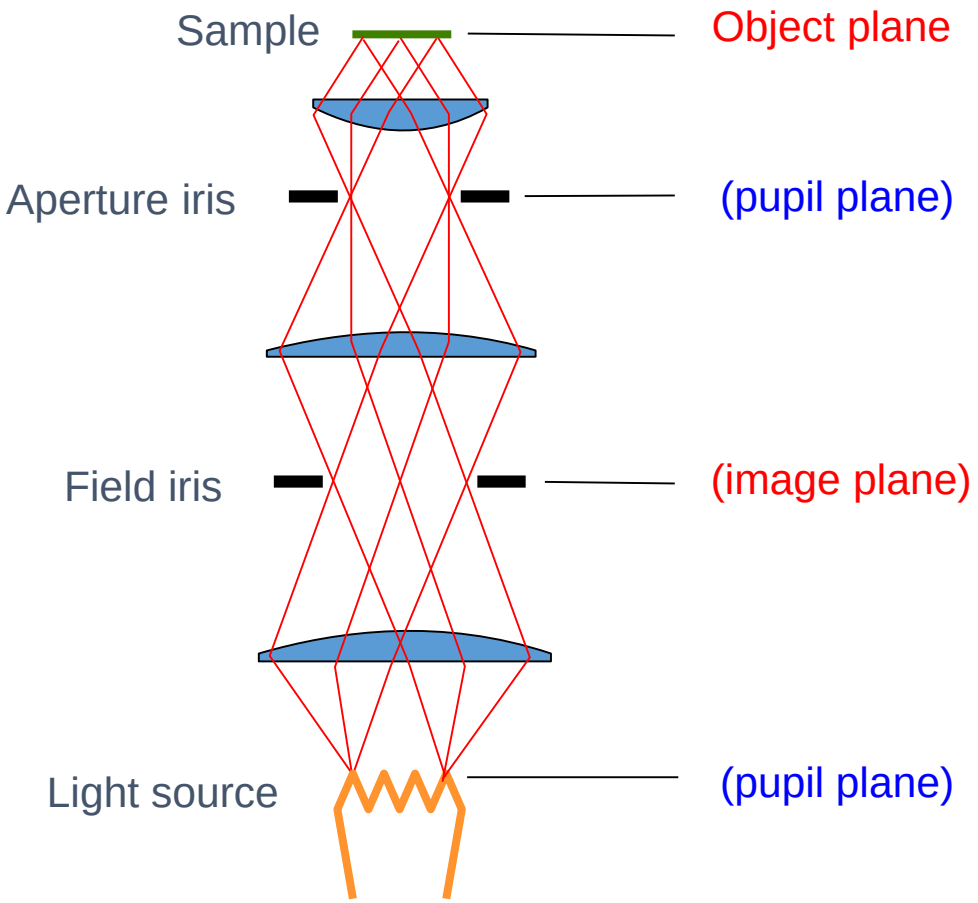
$$M = \frac{f_1}{f_o}$$

The Compound Microscope



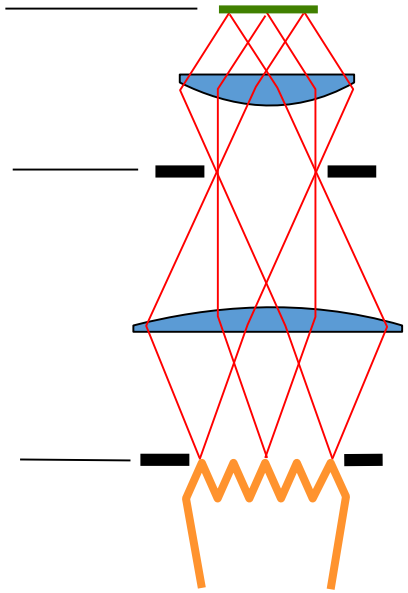


Köhler Illumination



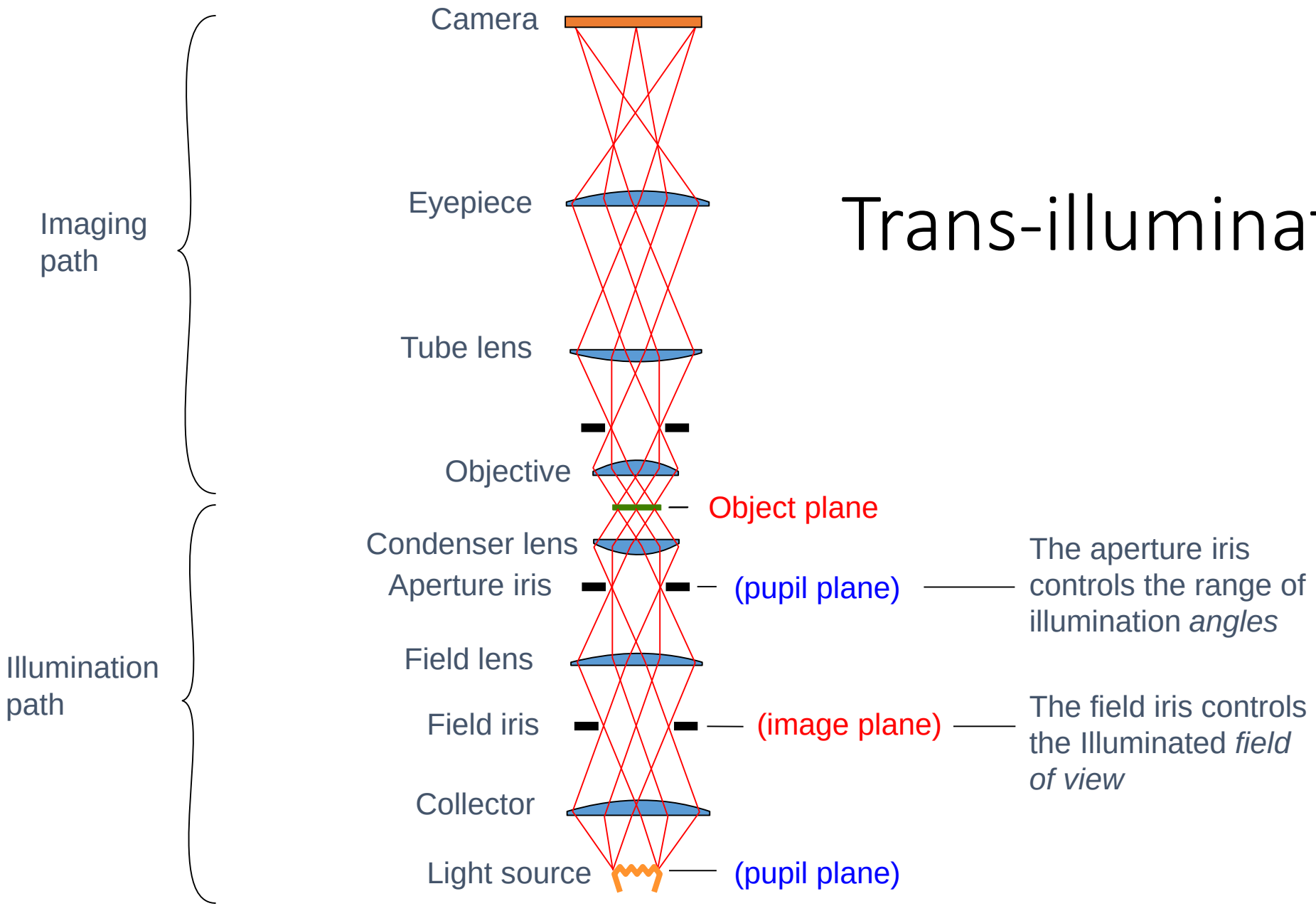
- Each light source point produces a parallel beam of light at the sample
- Uniform light intensity at the sample even if the light source is not uniform

Critical Illumination



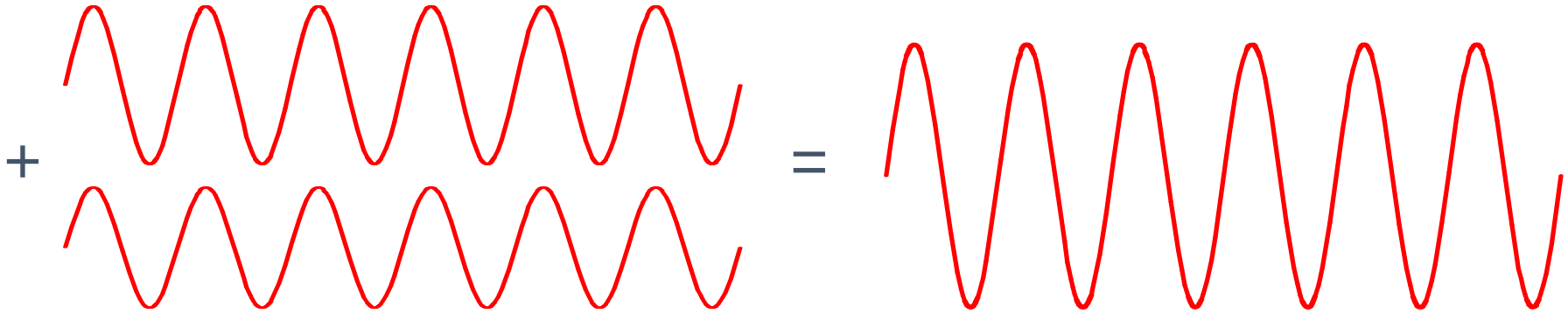
- The source is imaged onto the sample
- Usable only if the light source is perfectly uniform

Trans-illumination

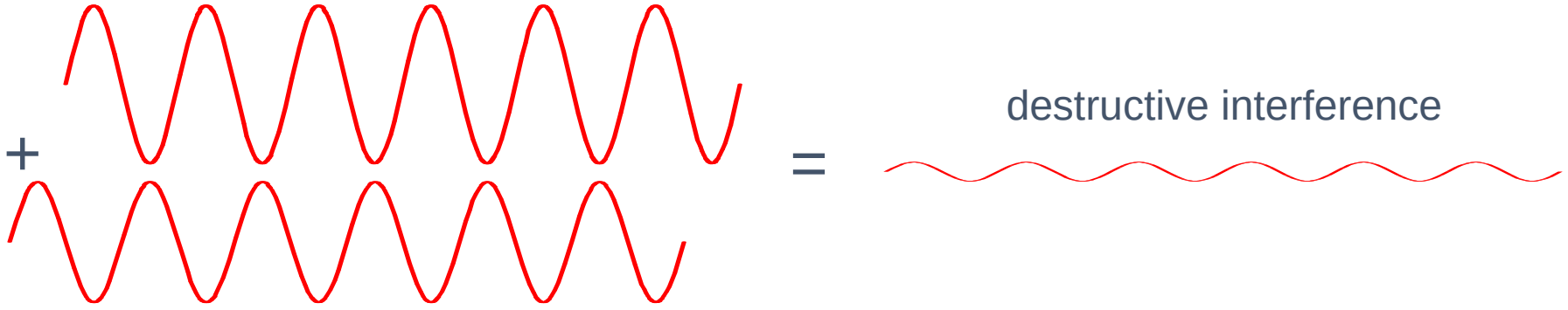


Interference

In phase



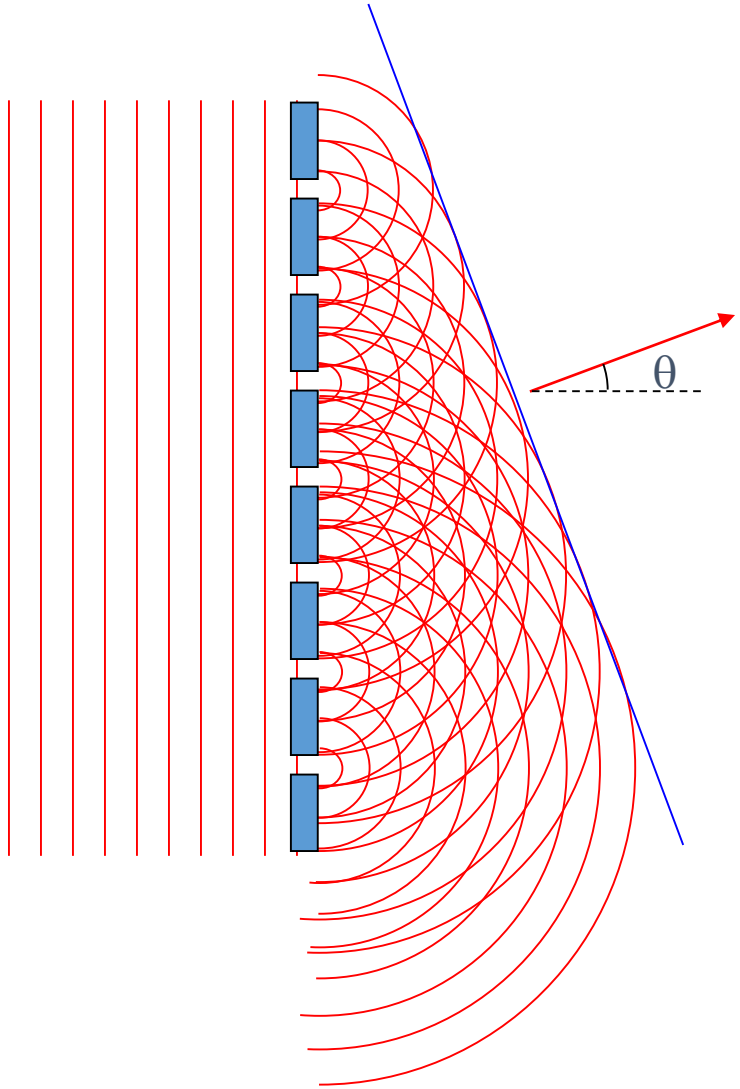
Opposite phase

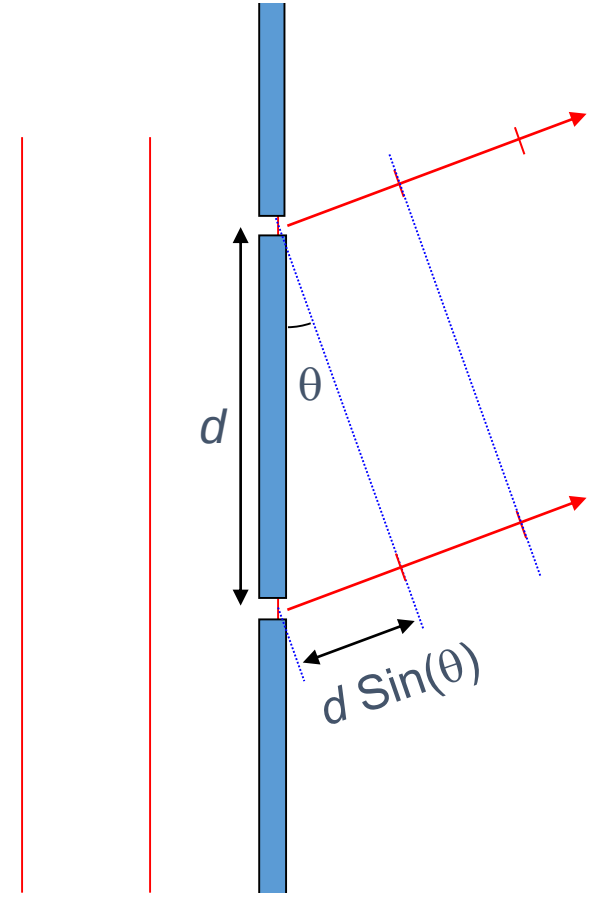


Diffraction by a periodic structure (grating)

Why is light diffract?

- Light is an EM field.
- Small aperture behaves like point source.
- Light from each point source propagates in all directions.
- Only in-phase field can propagate.

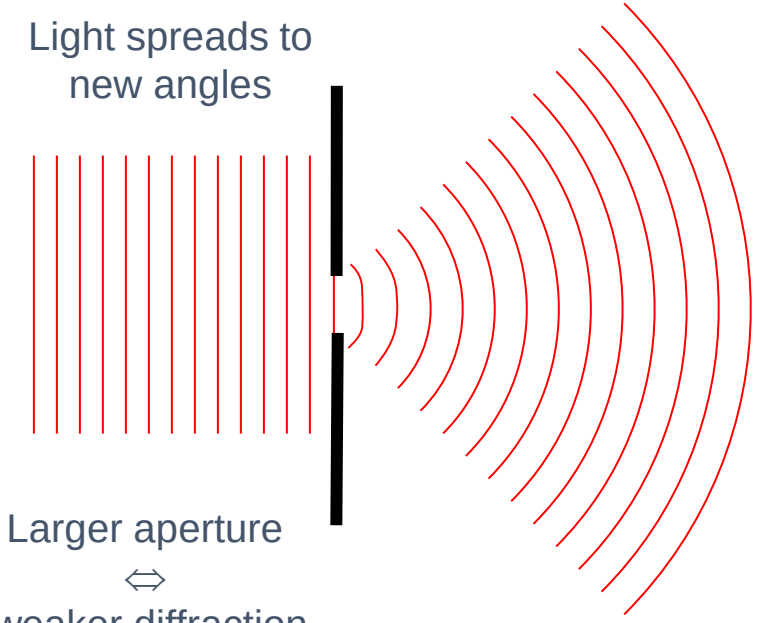




In phase if $d \sin(\theta) = m\lambda$ for some integer m.

Diffraction by an aperture

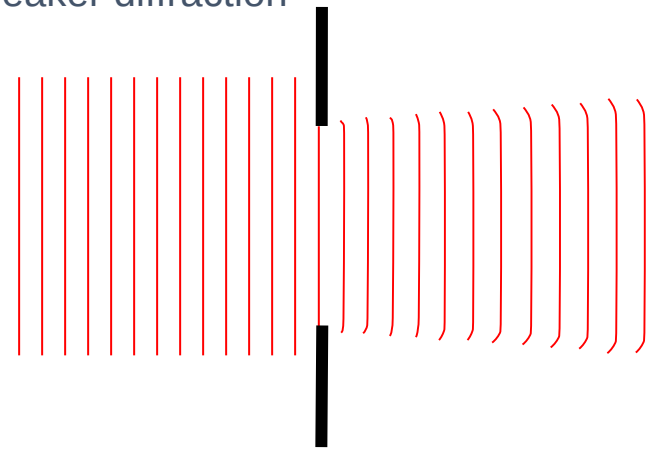
Light spreads to new angles

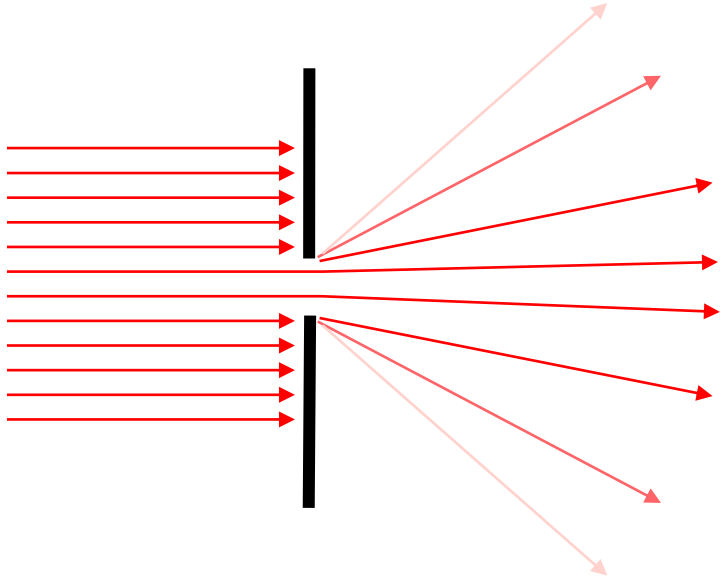


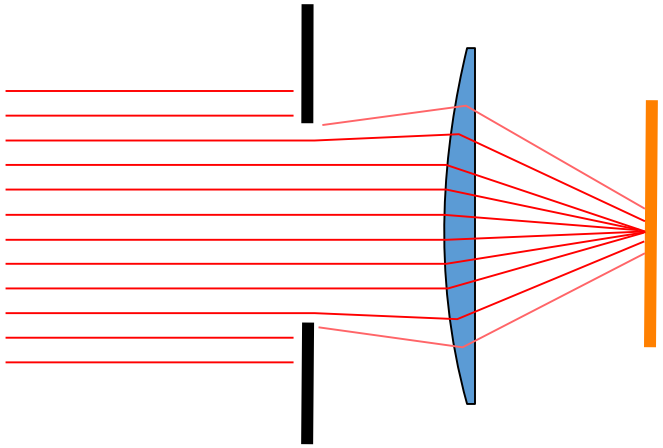
Larger aperture

⇔

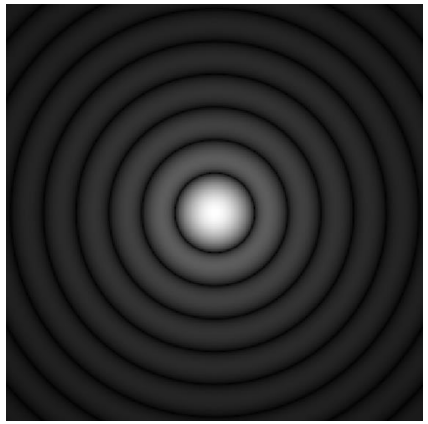
weaker diffraction







The pure, “far-field” diffraction pattern is formed at ∞ distance



It can be formed at a finite distance by a lens

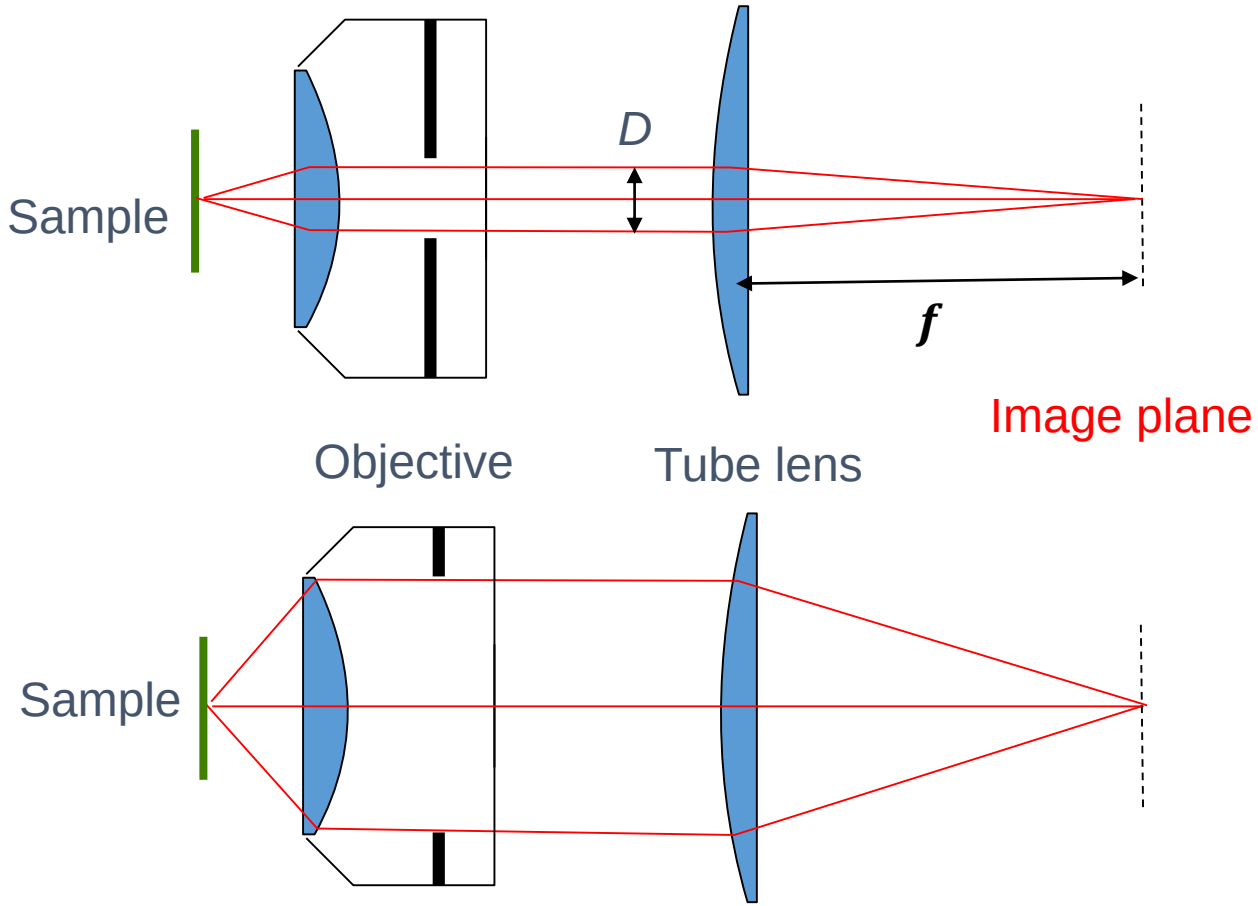
Any aperture produces a diffraction pattern

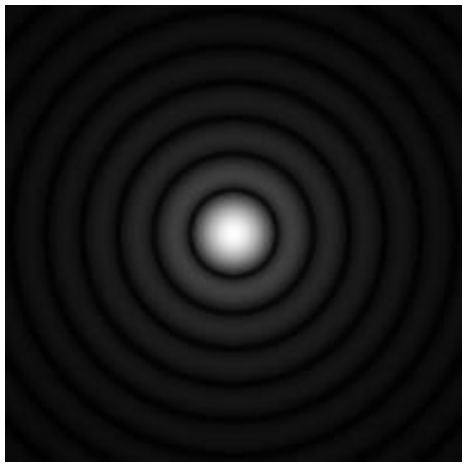
Point Spread function (PSF)

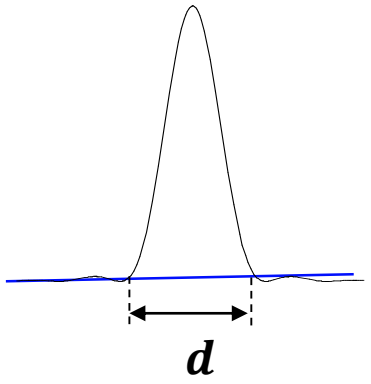
Diffraction spot
on image plane
= ***Point Spread Function***

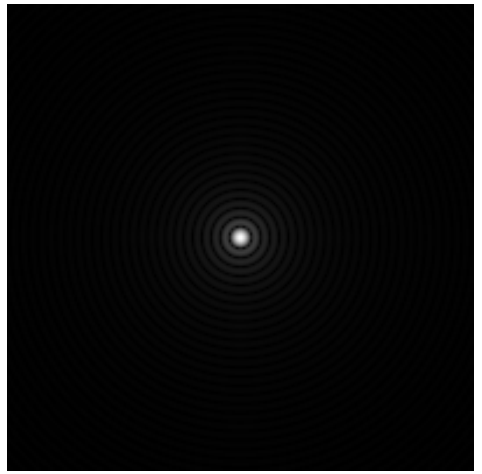
"Airy disk" diameter

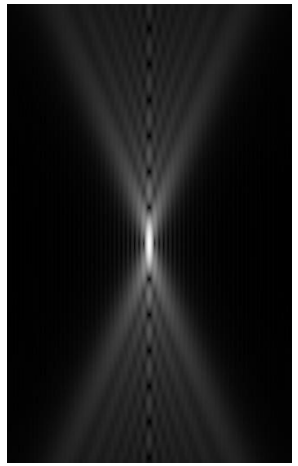
$$d = \frac{2.44\,\lambda\,f}{D}$$



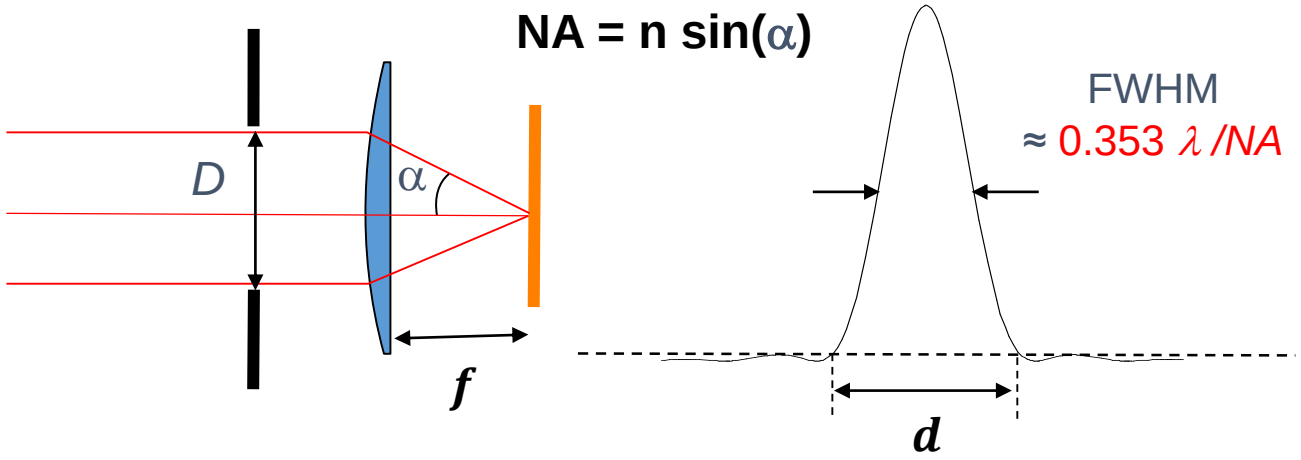






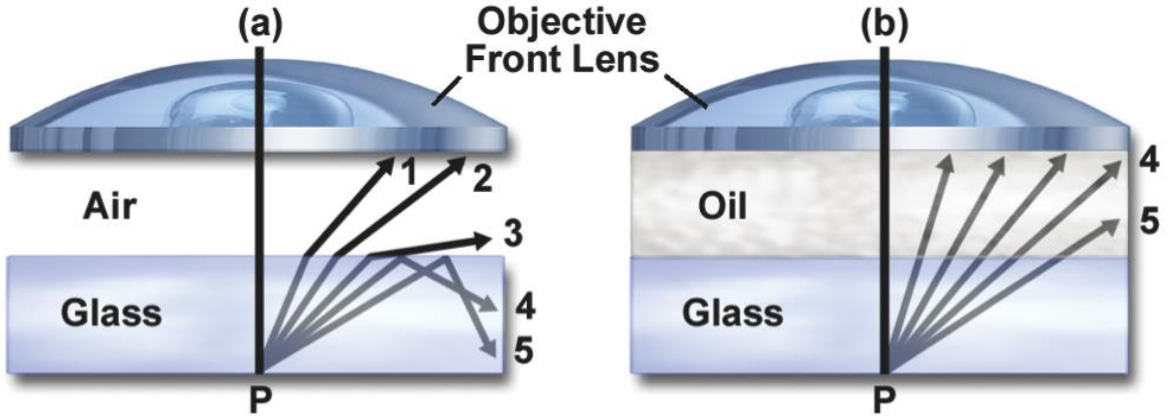


Numerical Aperture and Resolution



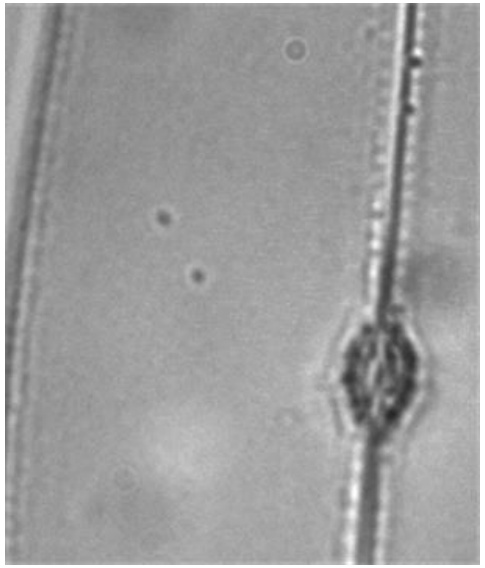
Resolution
≈ 0.61 λ / *NA*

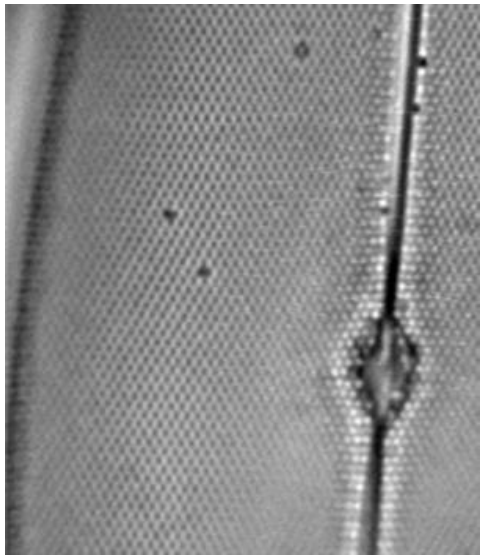
Axial Resolution
≈ 2 λ / NA^2



Oil immersion:
n ≈ 1.515
max NA ≈ **1.4**

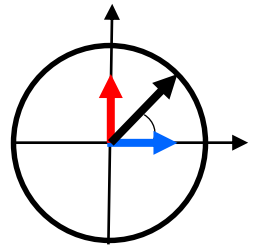
Water immersion:
n ≈ 1.33
max NA ≈ **1.2**





Source: MicroscopyU.com

Polarization



Light is a vector wave: it has not only field strength, but also field direction.

$$E(z,t) = \boldsymbol{A}e^{-ikz}e^{i2\pi\nu t}$$

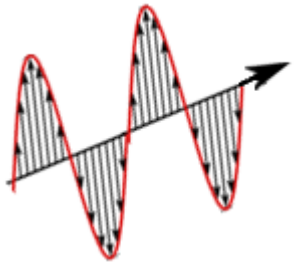
$$\boldsymbol{A} = A_x\hat{\boldsymbol{x}} + A_y\hat{\boldsymbol{y}}$$

$$E_x(z,t) = A_x e^{i(2\pi\nu t - kz)}$$

$$E_y(z,t) = A_y e^{i(2\pi\nu t - kz)}$$

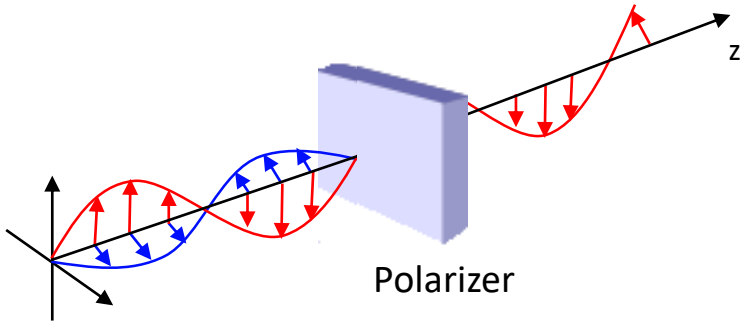
$$A_x = a_x e^{i\varphi_x}$$

$$A_y = a_y e^{i\varphi_y}$$



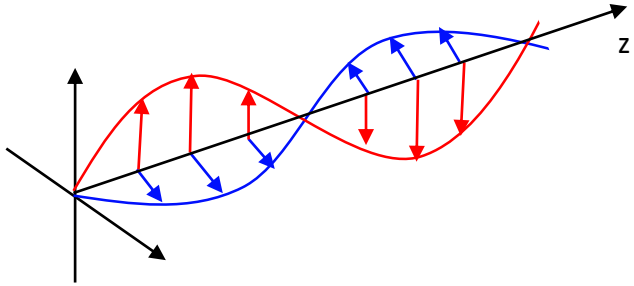
Polarizer

Polarizer allows propagation of only one component of electric field.



Linear polarization

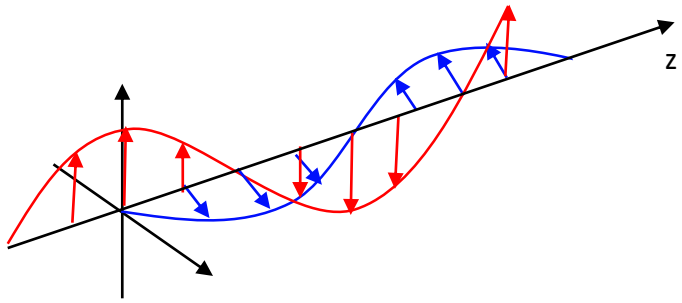
$$\varphi_x = \varphi_y$$



Circular polarization

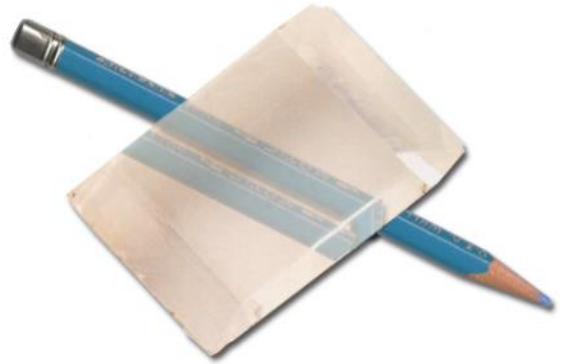
$$\varphi_x - \varphi_y = \pi/2$$

$$a_x = a_y$$

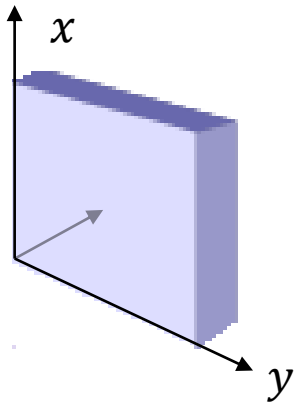


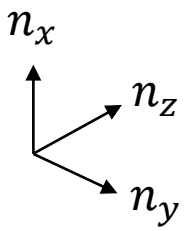
Polarization

Birefringent Crystal: Refractive index depends on the polarization and propagation direction of light.



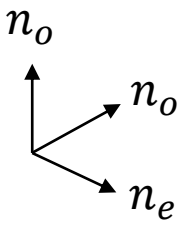
Calcite crystal





$$\nabla n = n_y - n_x$$

Uniaxial crystal: There is a single direction governing the optical anisotropy. All other directions perpendicular to it are optically equivalent.

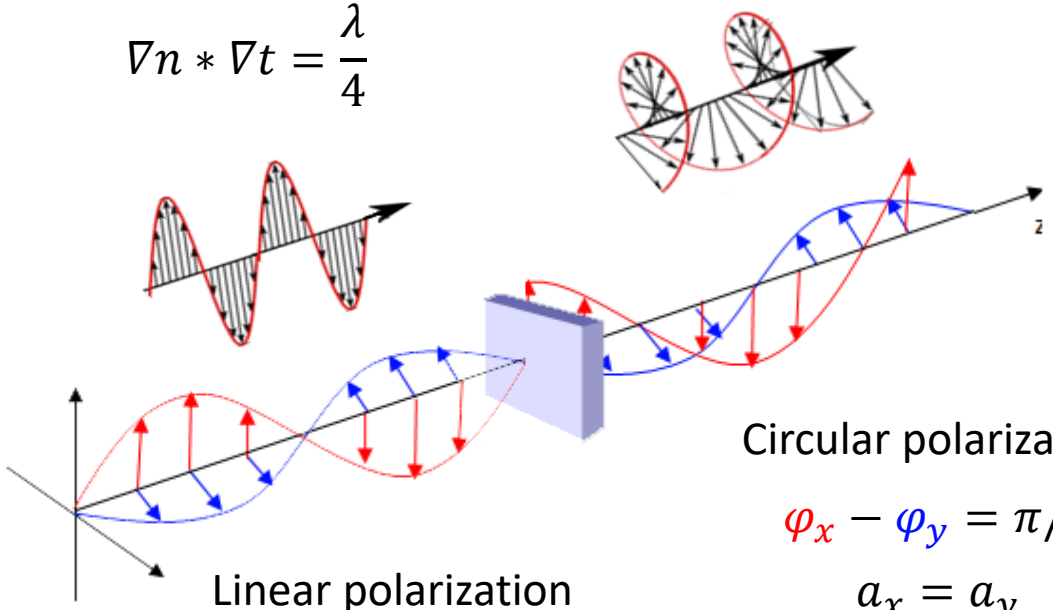


$$\nabla n = n_e - n_o$$

n_o is ordinary index
n_e is extra-ordinary index

Quarter Waveplate

$$\nabla n * \nabla t = \frac{\lambda}{4}$$



Linear polarization

$\varphi_x = \varphi_y$

$a_x = a_y$

Circular polarization

$\varphi_x - \varphi_y = \pi/2$

$a_x = a_y$

Light Scattering and Absorption

Scattering of illumination light by the tissue limits our ability to image deeper.

$$I_b = I_i \; e^{-\frac{L}{l_e}}$$

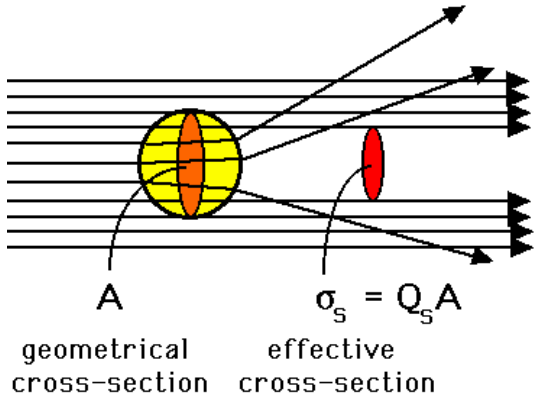
Beer – Lambert Law

l_e attenuation length

$$\frac{1}{l_e} = \frac{1}{l_s} + \frac{1}{l_a}$$

l_s scattering mean free path

l_a absorption length

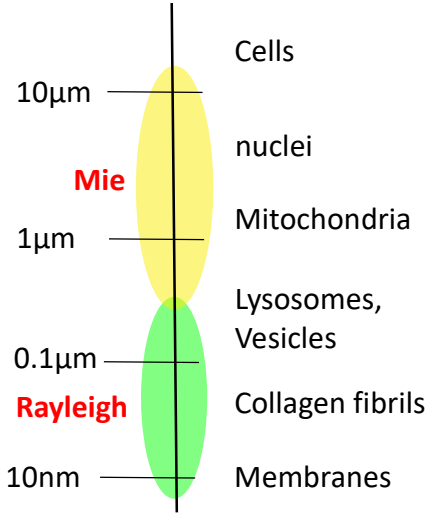


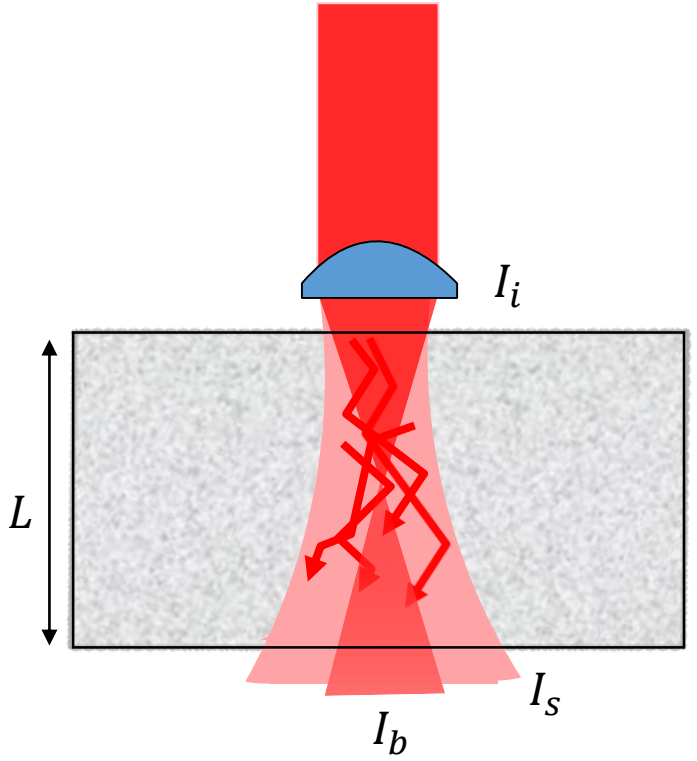
$$\frac{1}{l_s} = \mu_s = \rho_s \sigma_s$$

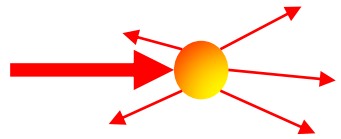
μ_s scattering coefficient

ρ_s volume density

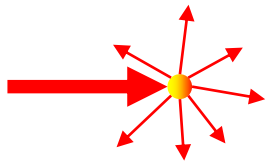
Q_s scattering efficiency



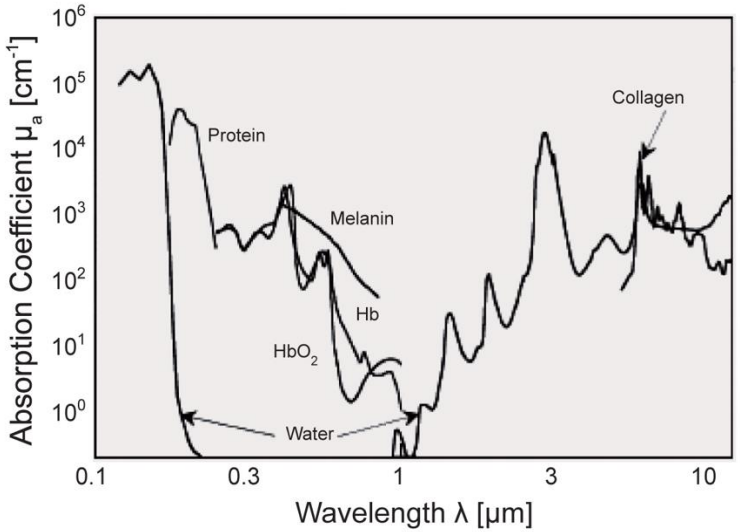




Mie Scattering



Rayleigh Scattering



Wide-field imaging techniques

Wide-field microscopy illuminates whole sample at all times and image is taken by camera..

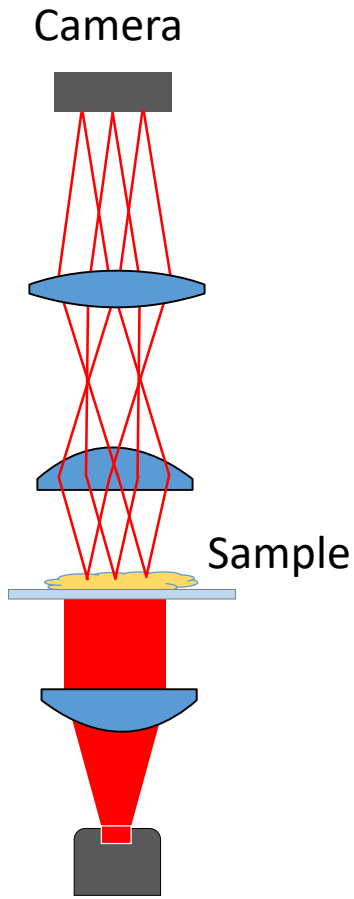
…..

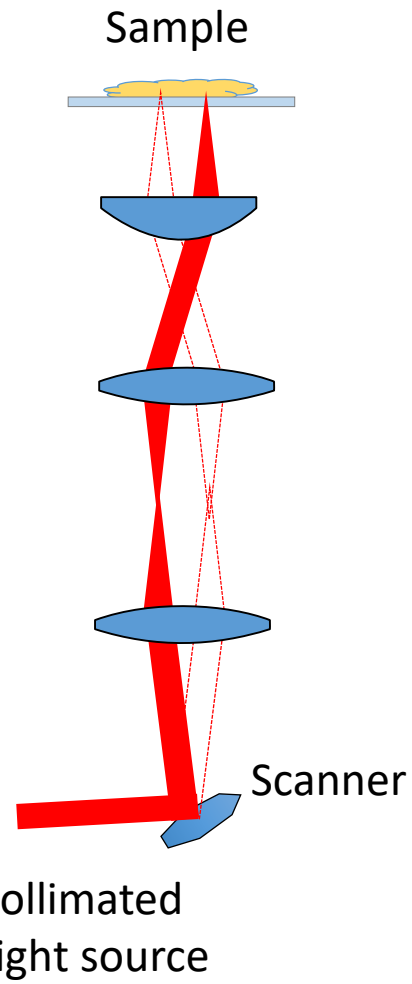
while in confocal microscopy, only a single focal spot is illuminated and recorded at a time.

Illumination sources: halogen lamp, metal halide lamps, or LED.

Detection: directly by eyes, or with a digital camera.

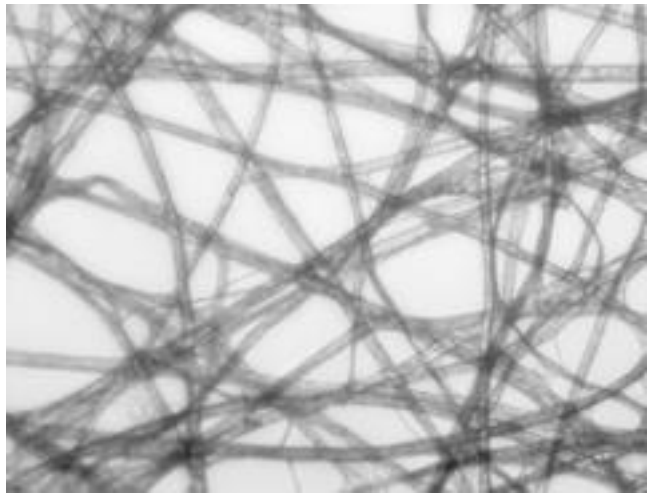
Contrast methods: Phase Contrast, Differential Interference Contrast (DIC), Fluorescence



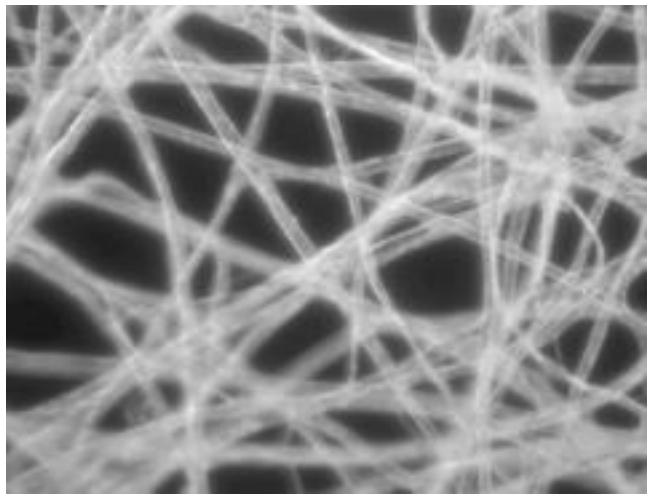


Dark-field microscopy

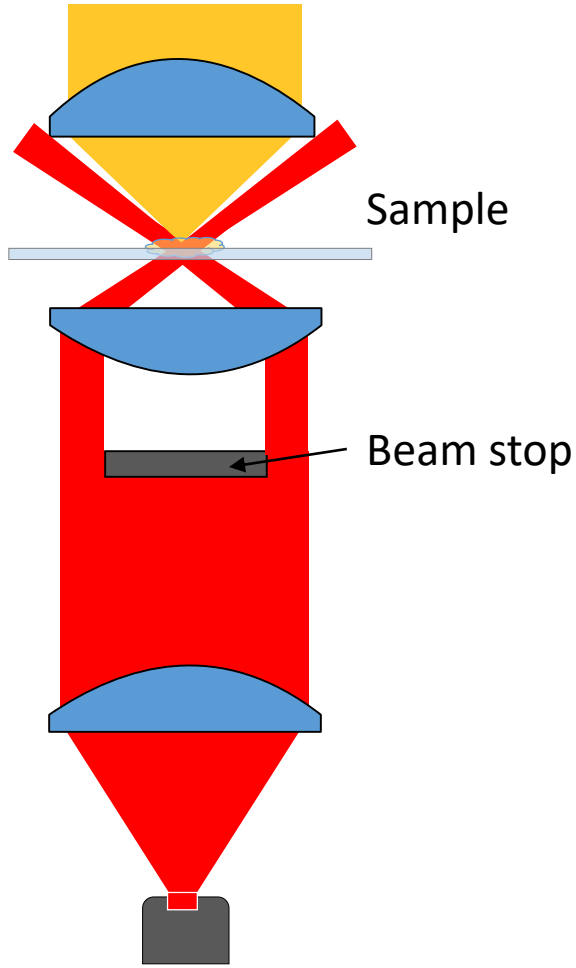
- In Dark-field microscopy, any un-scattered beam is excluded from the image, as a result, the field around the specimen is dark
- It is well suited for uses involving live and unstained biological samples.



Bright-field



Dark-field

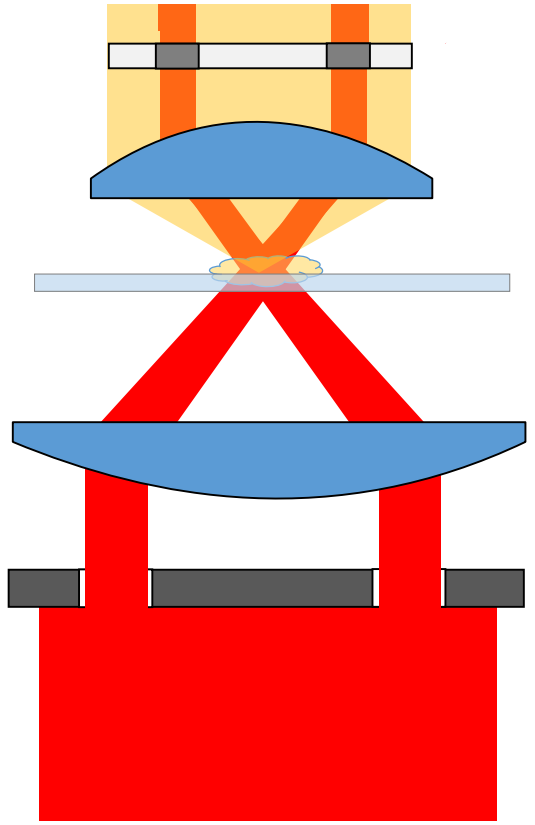


Phase-contrast microscopy

- Phase contrast is an optical contrast technique for making unstained transparent objects visible under the optical microscope.
- An annulus aperture is placed in the front focal plane of the condenser and limits the angle of the penetrating light waves.
- A phase plate is placed in the back focal plane of the objective
- The light waves which are not interacting with the specimen are focused as a bright ring in the back focal plane of the objective.

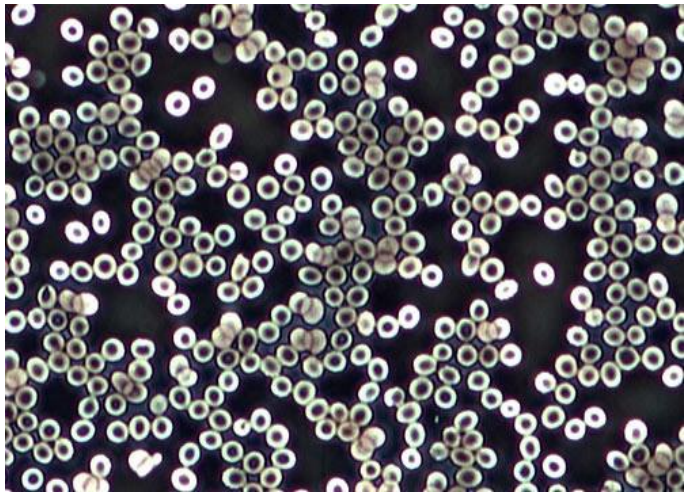
Phase plate

Annulus aperture

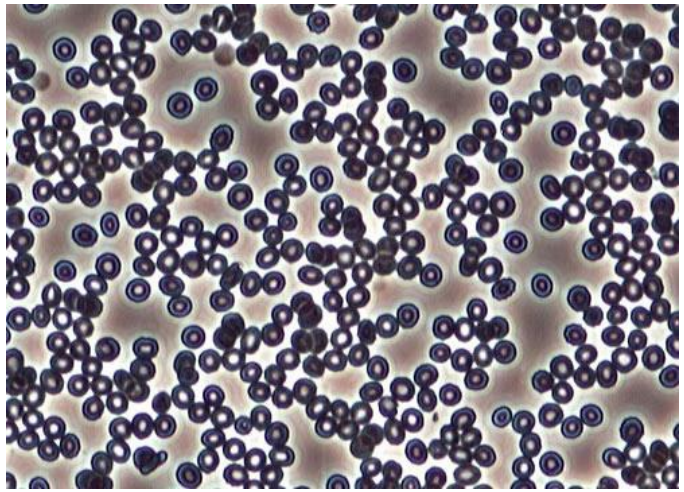


Phase-contrast microscopy

- Phase plate changes the phase by λ/4 and dim the light.
- Scattered light is phase shifted by -λ/4.
- Phase shift in scattered light is caused by the differences in optical path length in the specimen.
- Phase contrast is generated via interference.



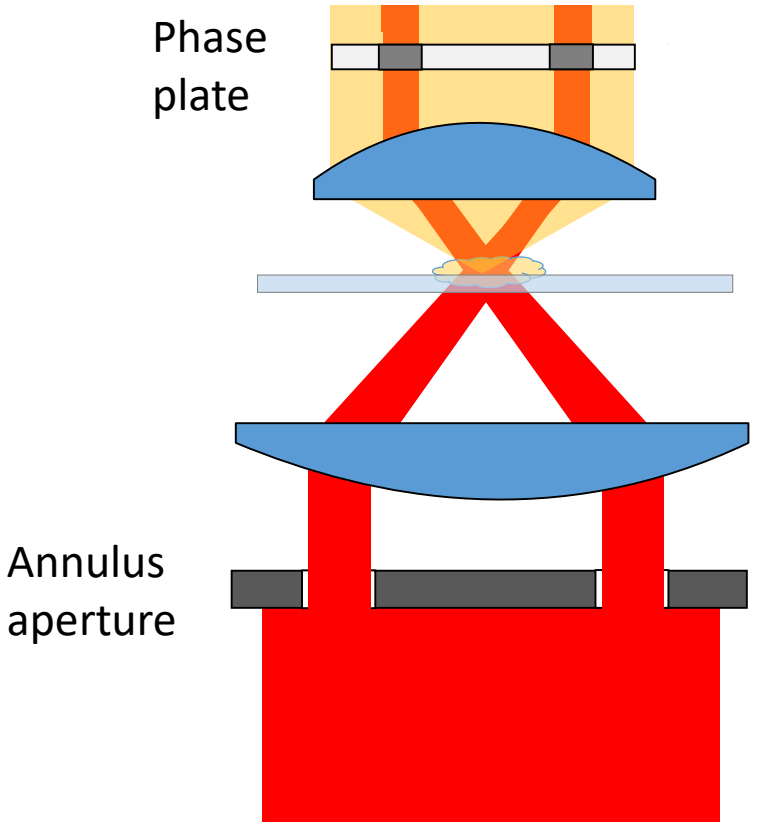
Negative Phase shift

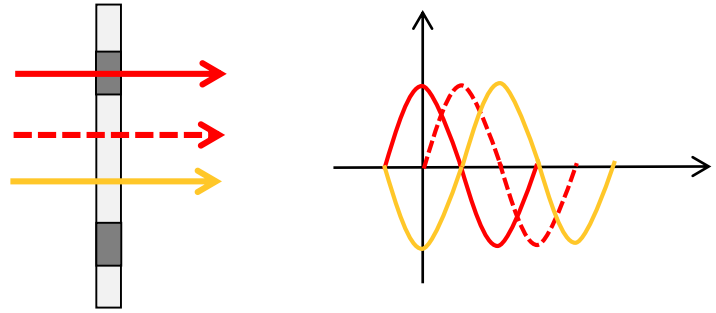


Positive Phase shift

Human Blood cells

Source: MicroscopyU.com

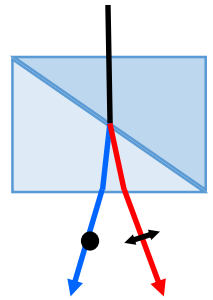




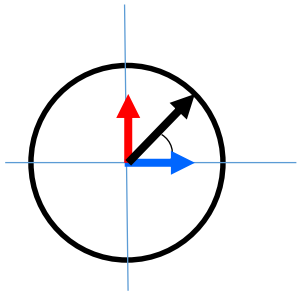
Differential interference contrast (DIC)

DIC microscopy is a technique which uses gradients in the optical path length or phase shifts to make phase objects visible under the light microscope.

In this way it is possible to observe living cells and organisms with adequate contrast and resolution.



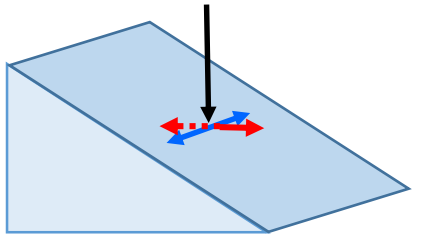
s-pol. p-pol.



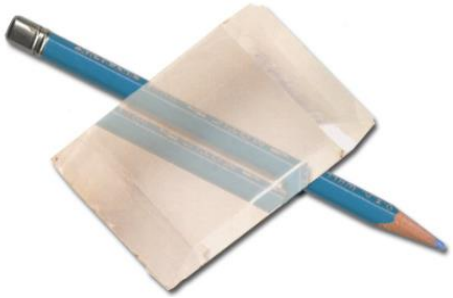
45° polarized

The polarized light is dispersed into two distinct light rays with an orthogonal plane of polarization using a Wollaston prism.

These two light rays are extremely near to each other.



Wollaston Prism



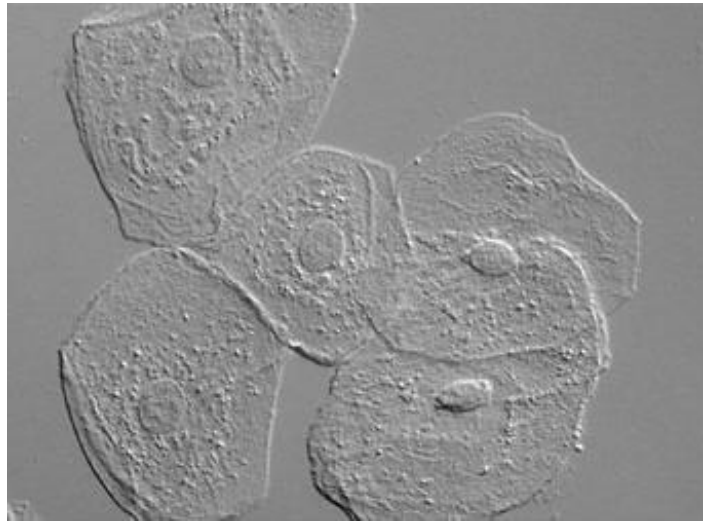
Differential interference contrast (DIC)

The two rays experience different phase shifts from the specimen.

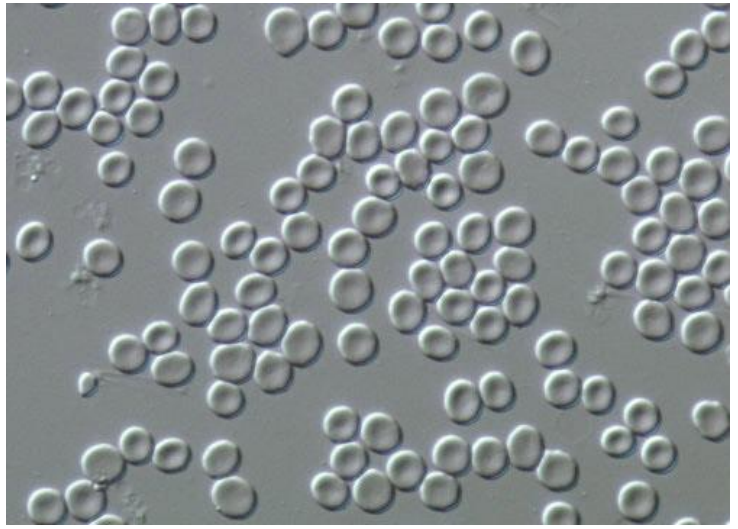
After recombination they interfere with each other producing interference contrast.

Images are relief-like, have a shadow cast, and no halo artifacts.

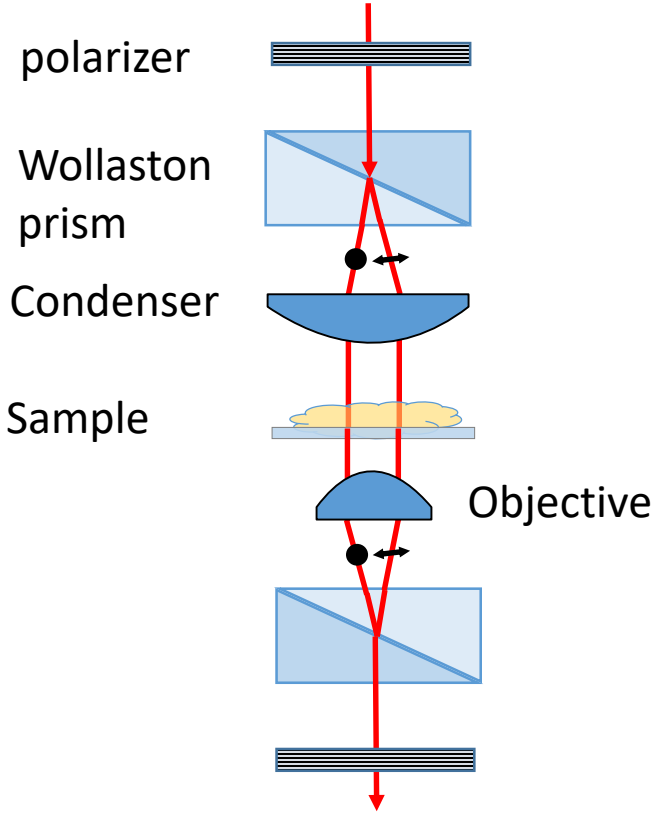
Relatively thick specimens can be imaged due to the possibility of optical sectioning.



Cheek Epithelial cells



Human Blood cells

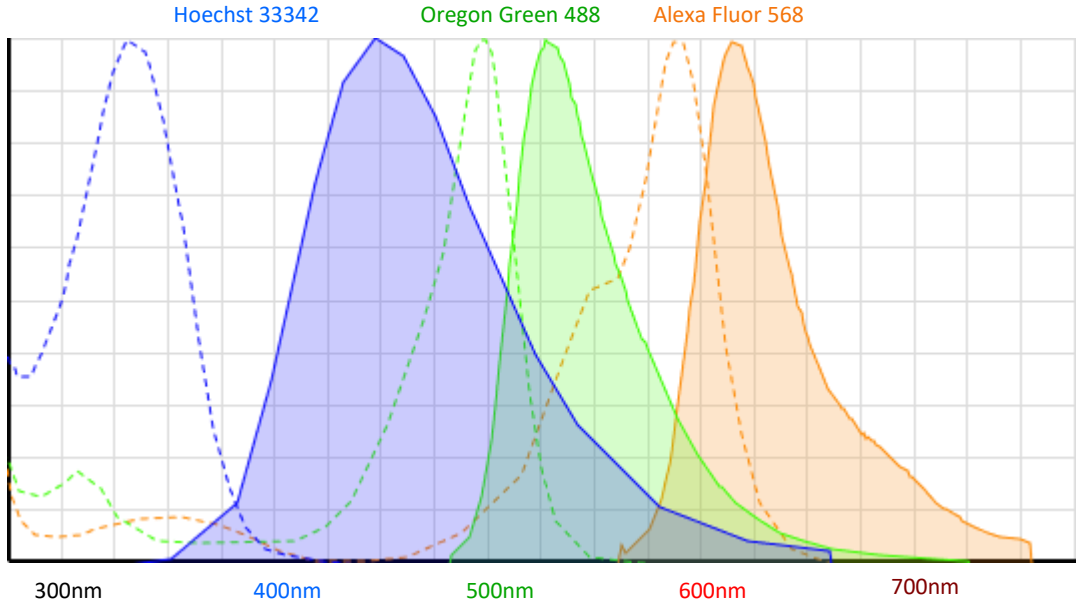


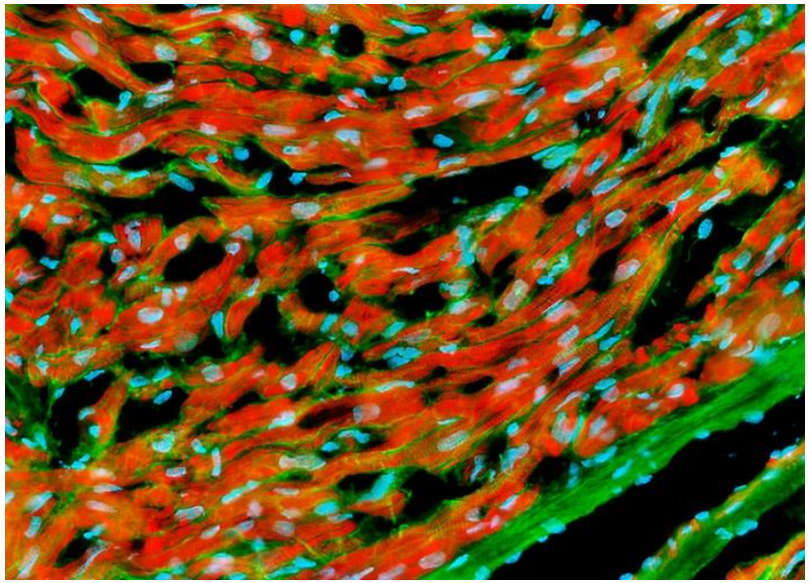
Source: microscopyU.com
micro.magnet.fsu.edu

Fluorescence imaging

Fluorescence microscopy uses fluorescence and phosphorescence of biochemical compounds as a contrast mechanism.

Fluorescent proteins and dyes have been powerful tools to visualize cellular components of cells.



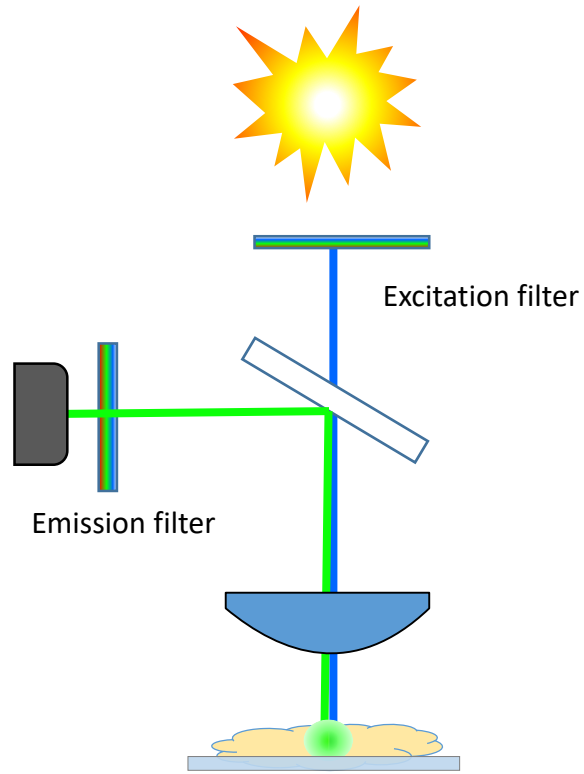


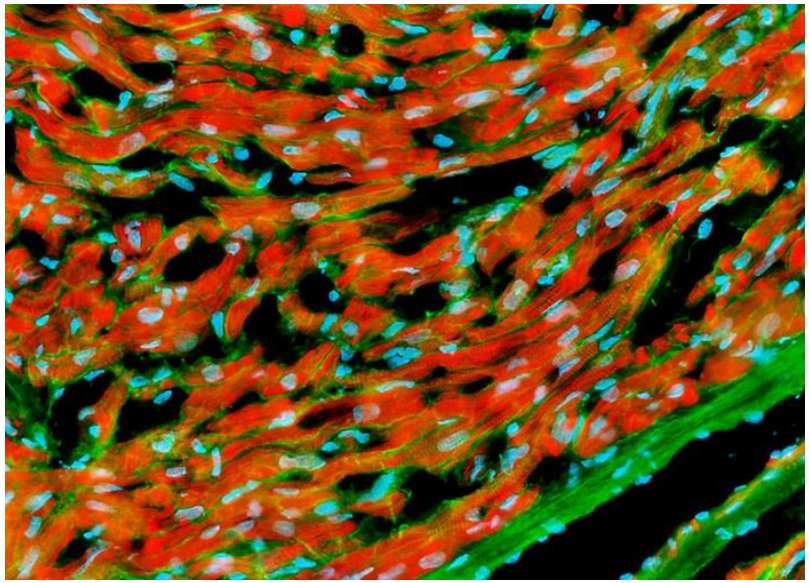
Rat Heart Tissue Labeled with Alexa Fluor 568 (for F-actin), Oregon Green 488 (for N-acetylglucosamine and N-acetylneuraminic), and Hoechst 342 (nucleus)

Source: MicroscopyU.com

Fluorescence imaging

- Excitation filter selects specific wavelengths for illumination.
- Fluorophore emits light of longer wavelengths.
- Fluorescence light can be collected by the illumination objective lens (epifluorescence).
- Fluorescence light is separated from the strong illumination light by spectral emission filters.



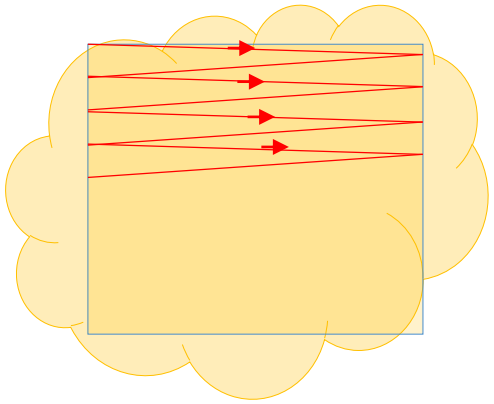


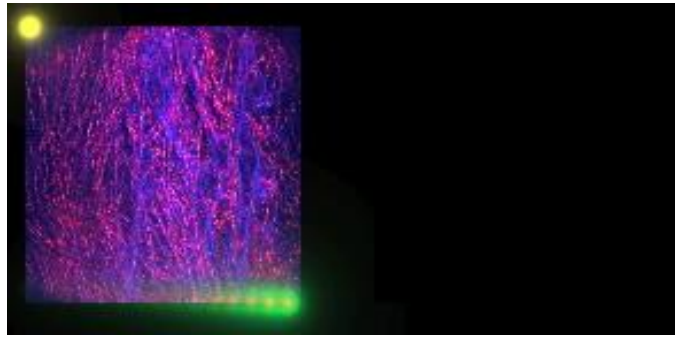
Rat Heart Tissue Labeled with Alexa Fluor 568 (for F-actin), Oregon Green 488 (for N-acetylglucosamine and N-acetylneuraminic), and Hoechst 342 (nucleus)

Source: MicroscopyU.com

Scanning Imaging Microscopy

- In scanning microcopy one focal point is illuminated at a time.
- Illumination point is raster scanned using beam scanner.
- Image is formed serially (pixel by pixel) by a single pixel detector.

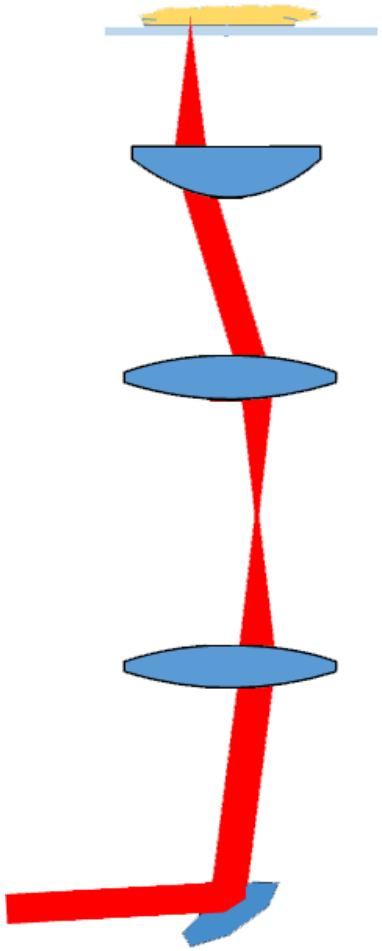




It provides better background rejection and optical sectioning.

Optical sectioning:

- Confocal pinhole
- Differential detection
- Non-linear techniques



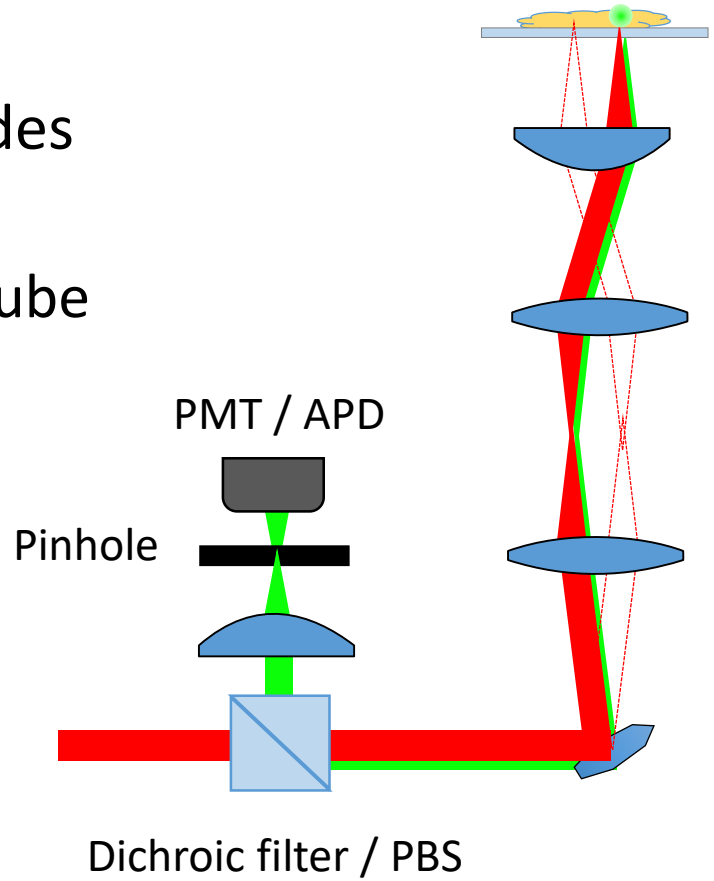
Beam scanning

Scanning Confocal Microscopy

- Confocal scanning microscopy has a pinhole in its return path after scanners.
- Pinhole is placed at image plane.
- The Pinhole **rejects background light** and provides **optical sectioning**.
- Avalanche Photodiode (APD) or Photomultiplier Tube (PMT) are used for light detection.

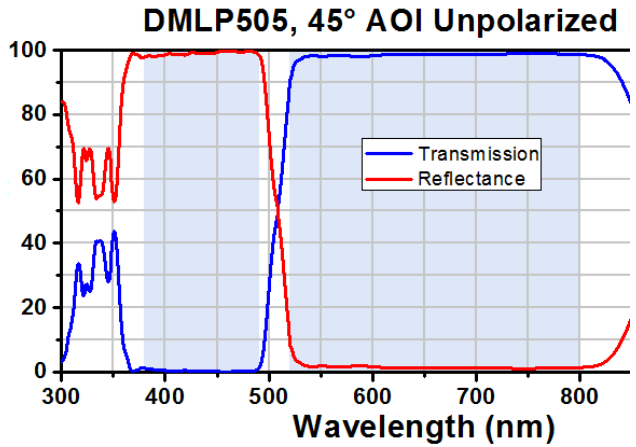
Confocal Scanning → Confocal reflectance

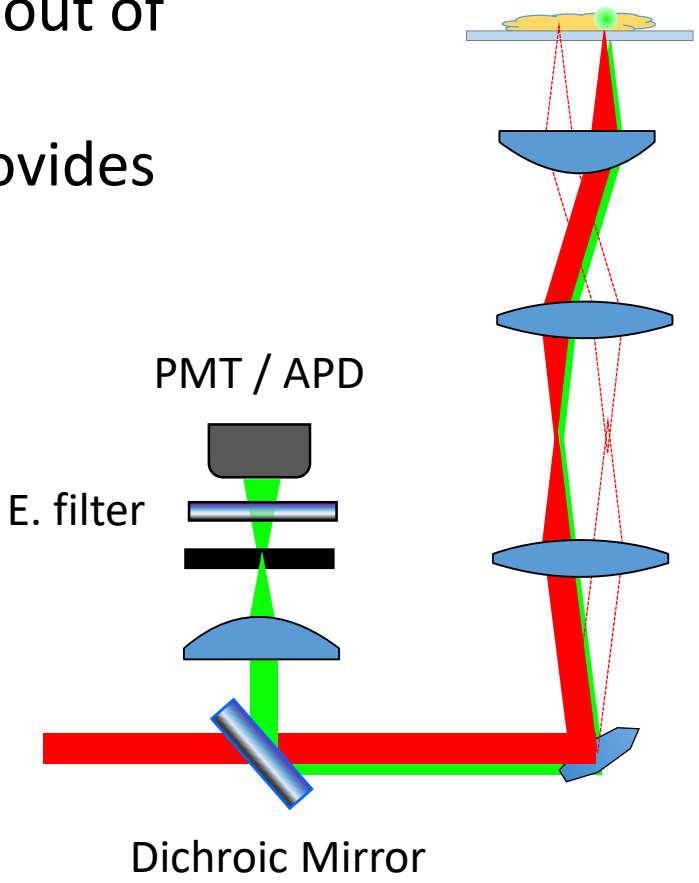
Confocal Scanning → Confocal fluorescence



Confocal Fluorescence Microscopy

- Fluorescence light is separated from the illumination light by a **dichroic filter**.
- **Fluorescence filter** provides further rejection of out of band light.
- Pinhole rejects background fluorescence and provides optical sectioning.





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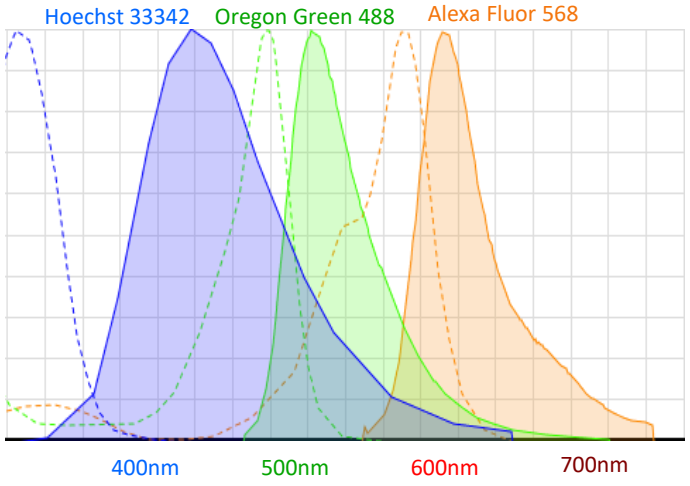
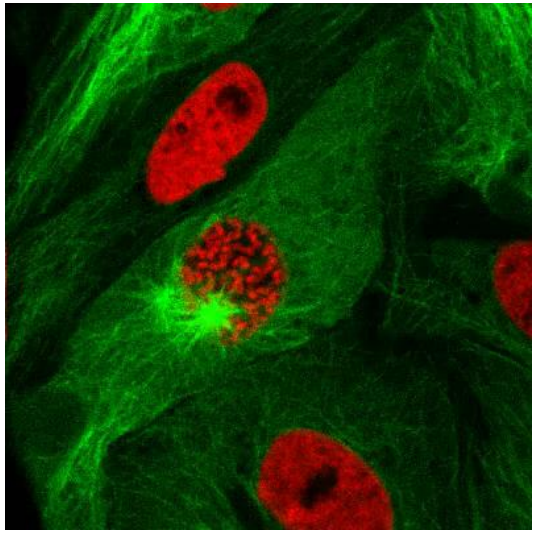
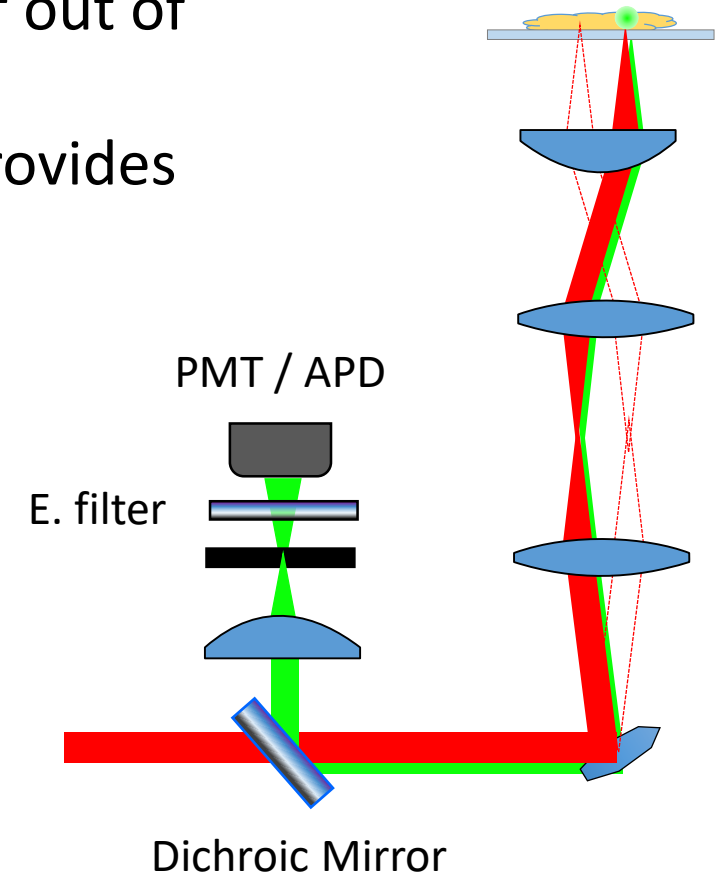


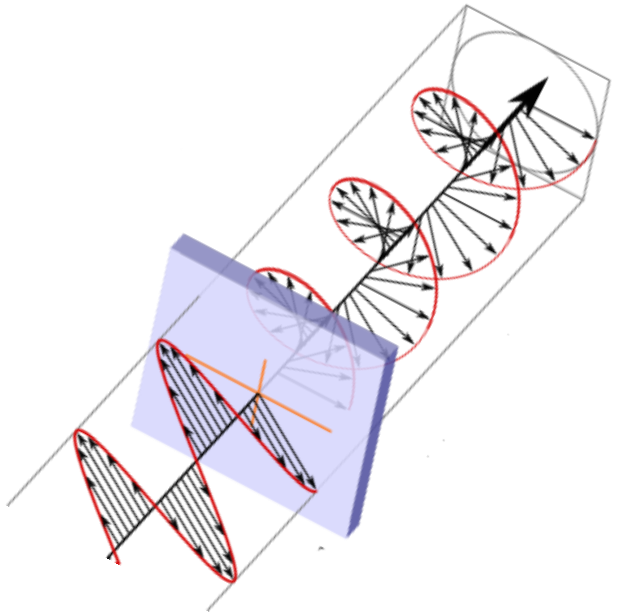


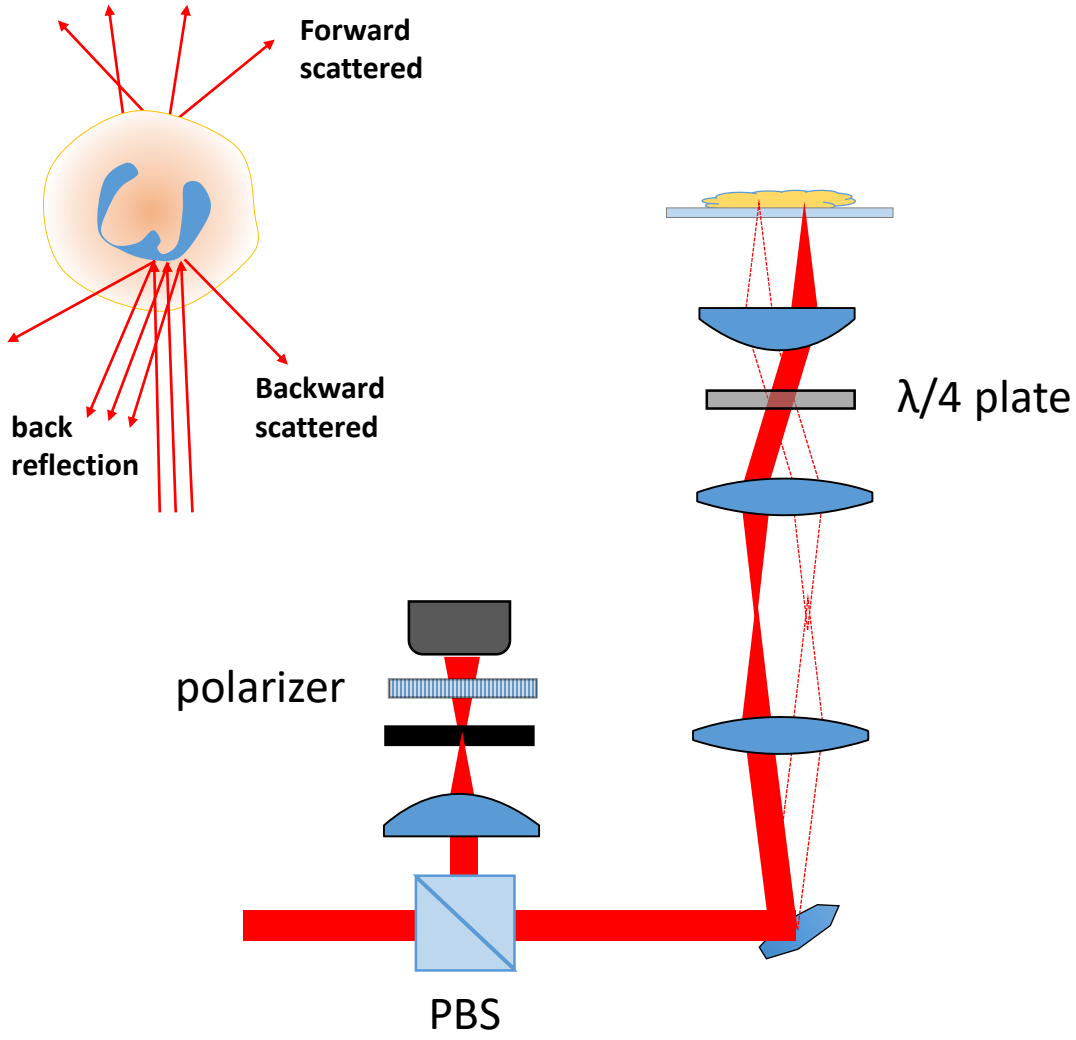
Pig kidney epithelial cells labeled with EGFP and mCherry (source: microscopyu.com)



Confocal Reflectance Microscopy

- Confocal reflectance detects a sharp index variation in tissue.
- Non-confocal light is rejected using a λ/4 plate and polarization beam splitter (PBS).





Confocal Reflectance Microscopy

- Confocal reflectance detects a sharp refractive index variation in tissue.
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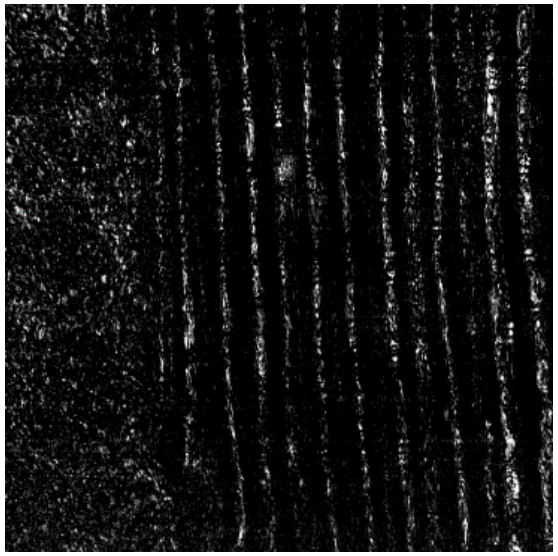
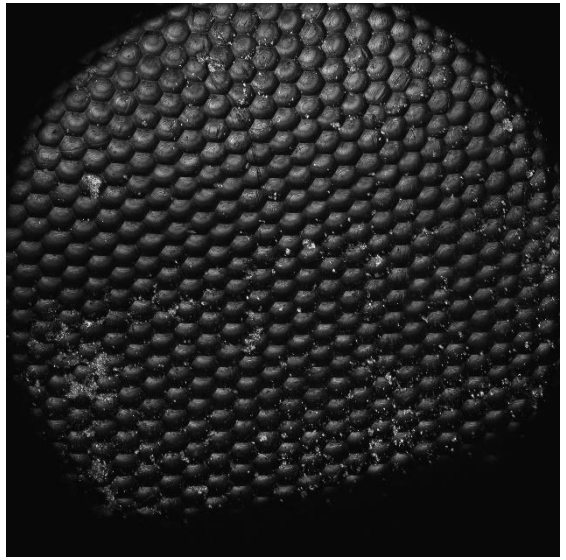
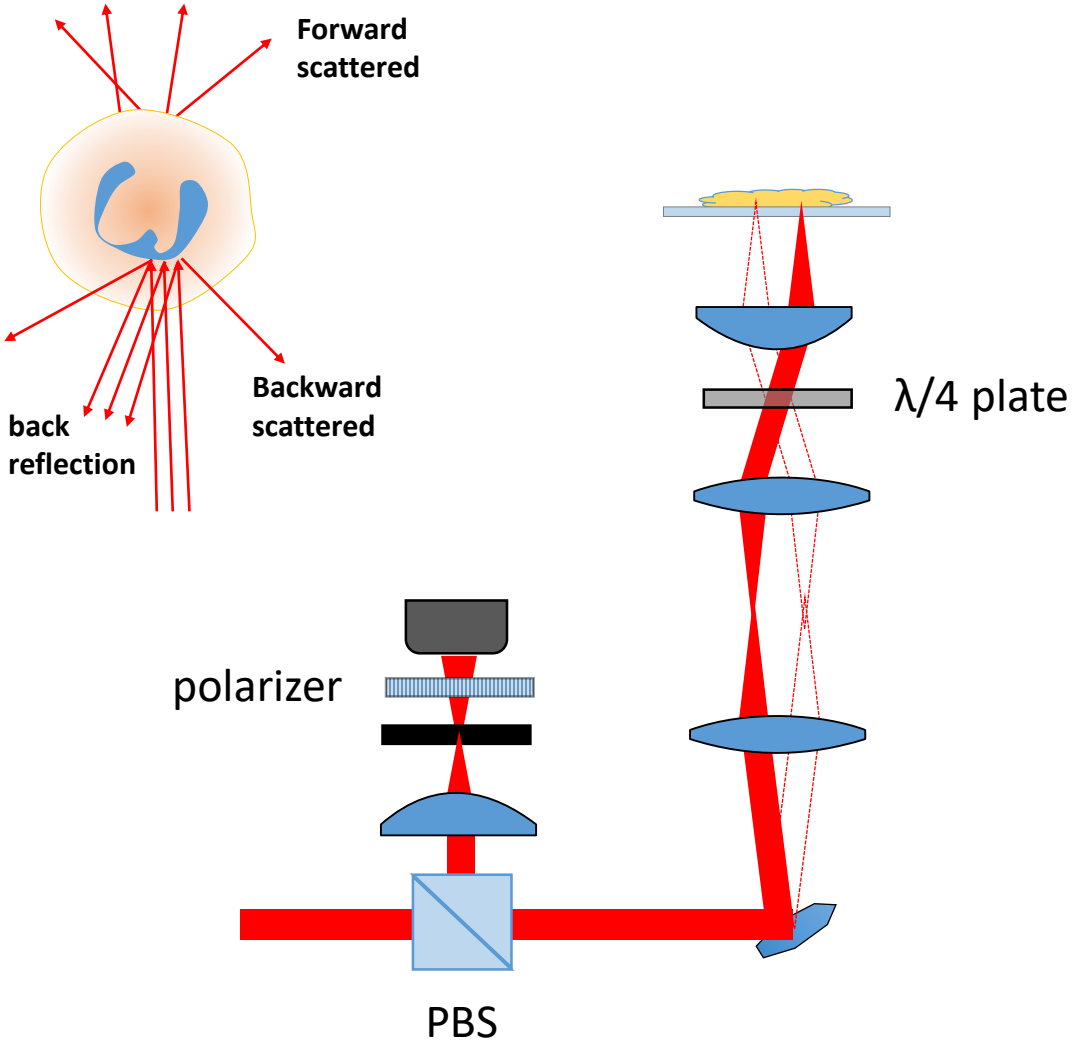


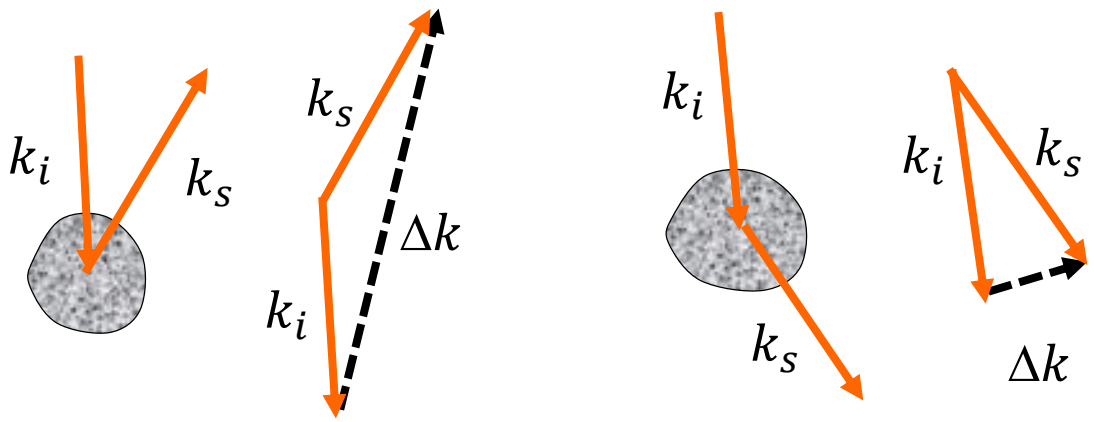
Mouse spinal cord

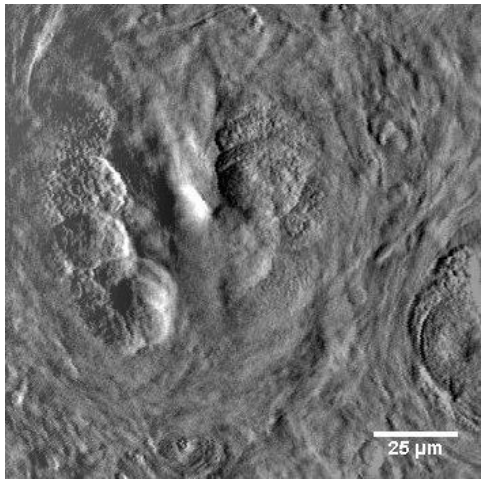


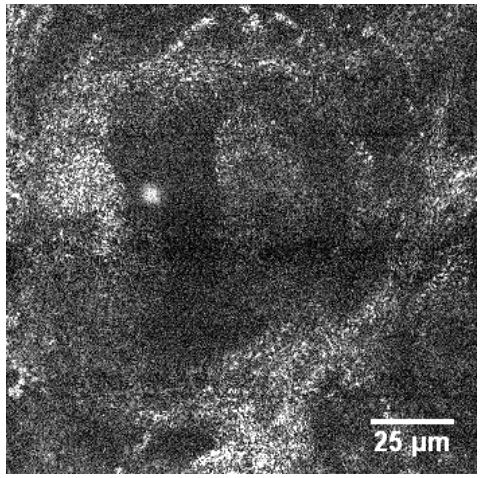
Dragonfly Eye *(source: thorlabs.com)*

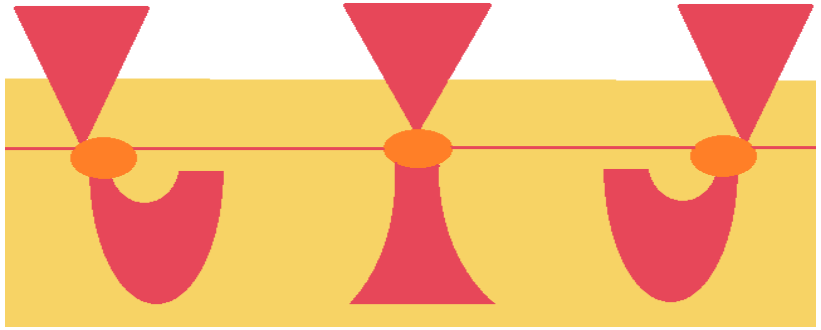


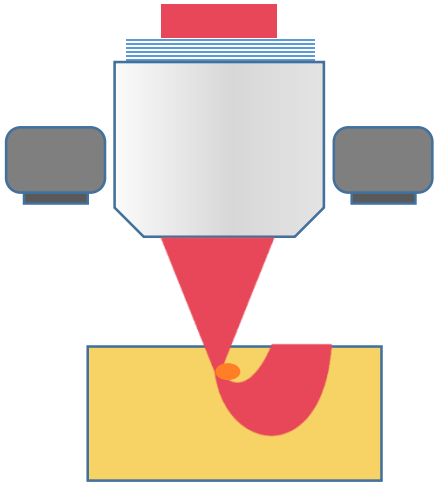
Differential phase-gradient detection







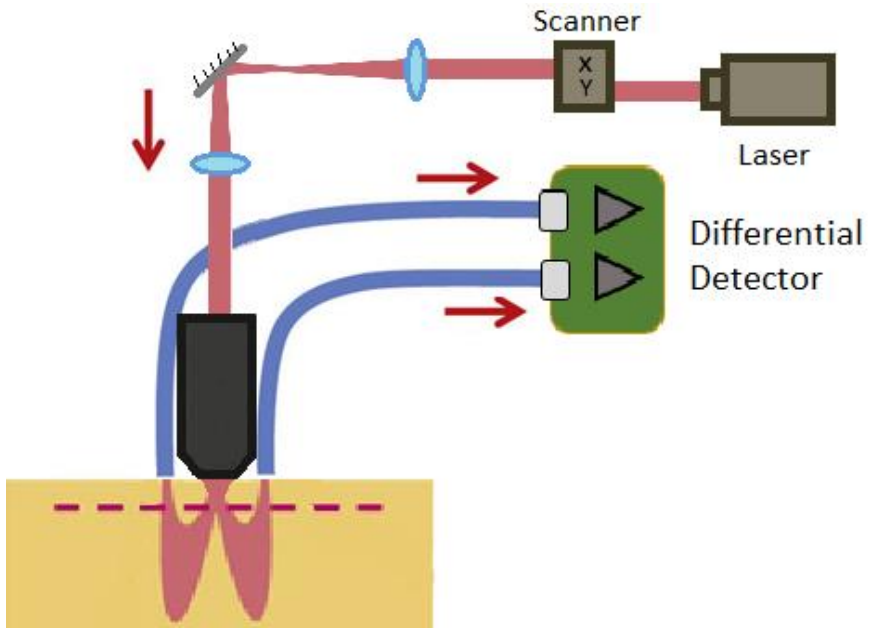


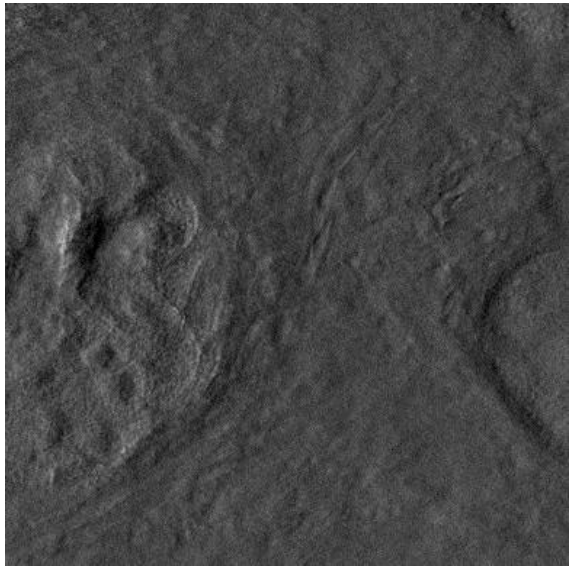


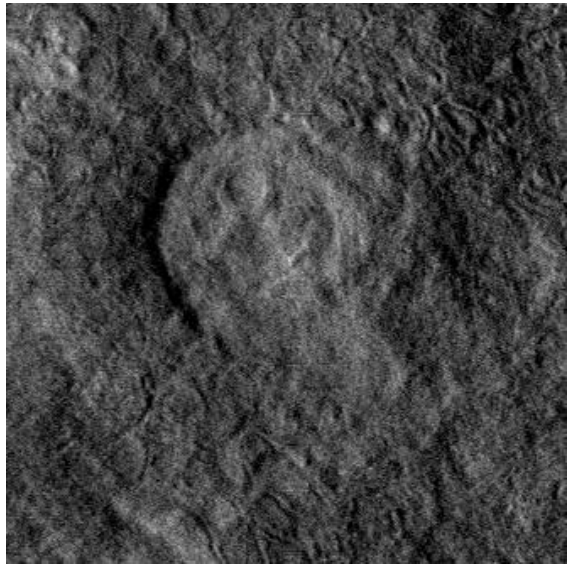
Label-free contrast enhancement.

It is important for in-vivo imaging in humans.

Differential phase-gradient detection





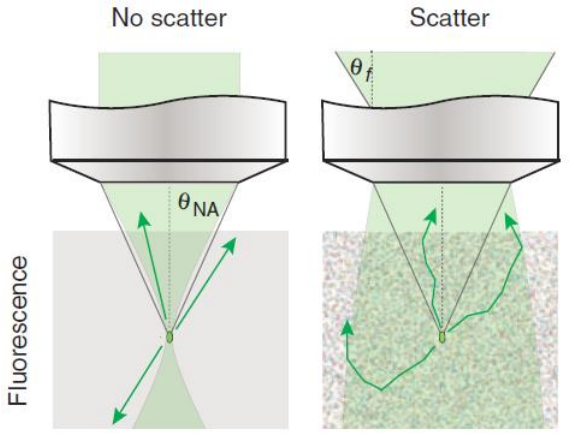


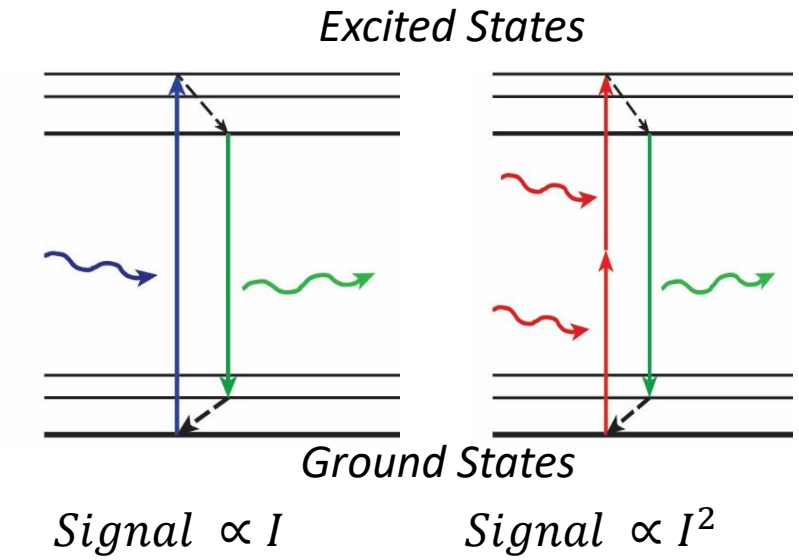
Non-linear microscopy

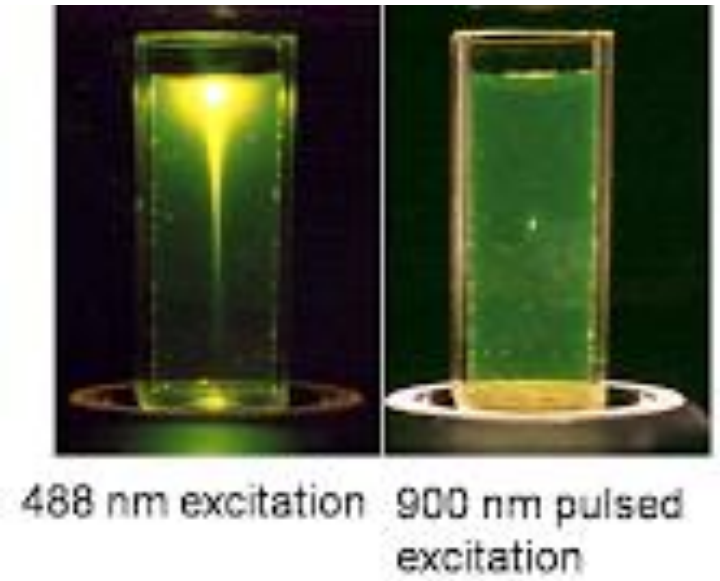
Optical sectioning is provided by the **higher probability** of multiphoton absorption or harmonic generation.

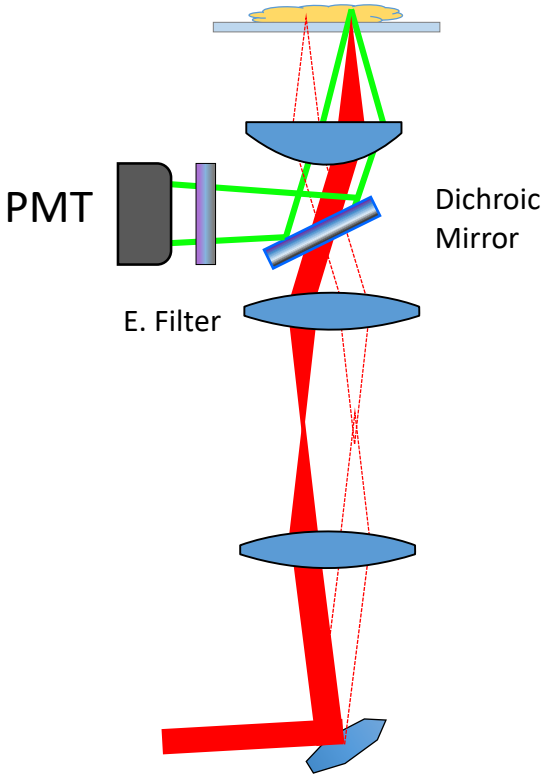
Multiphoton absorption or higher order harmonic generation is proportional to the square (or third power) of intensity.

At focal plane, intensity of light is highest.









Multi-photon microscopy

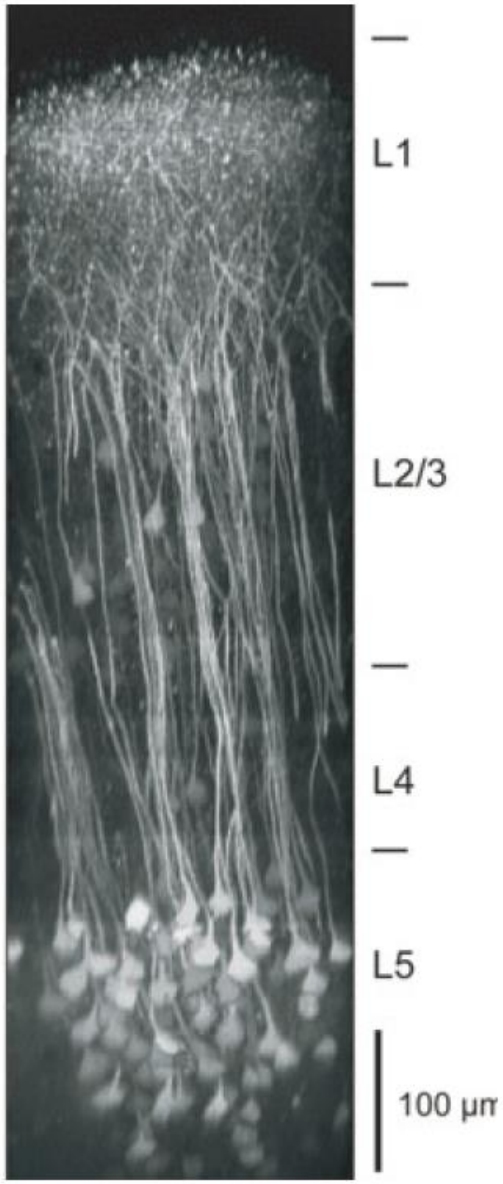
Two-photon absorption cross-section is very low, therefore, **it needs very high photon density**.

Femto-second pulsed laser provides the photon density without damaging tissue.

Imaging depth is limited by scattering. Longer wavelengths have lower scattering.

For a 150fs, 80MHz pulsed laser the intensity at focus is **100,000 times** higher than the same power CW laser.

Ti-sapphire tunable lasers are the most common source of two-photon excitation.
680nm-1080nm, 150fs, 3W

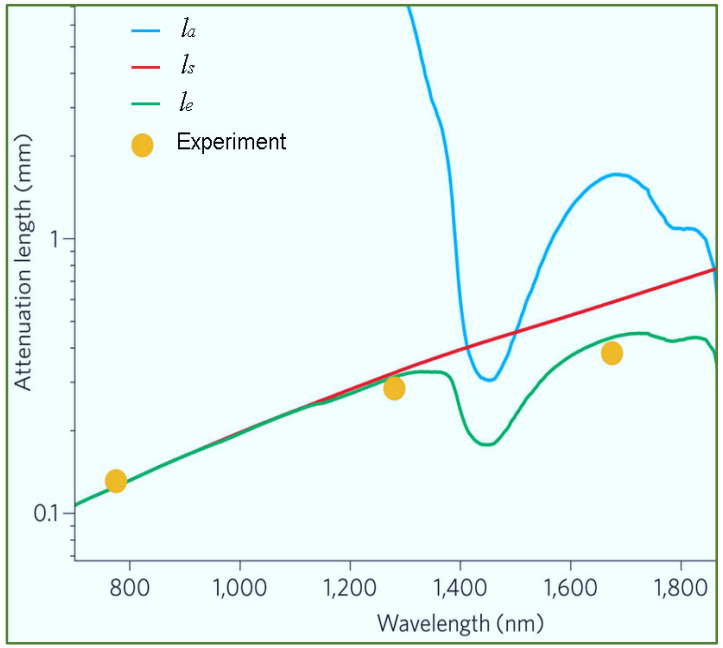


Expressing Clomeleon (Denk 2005 Nat. method

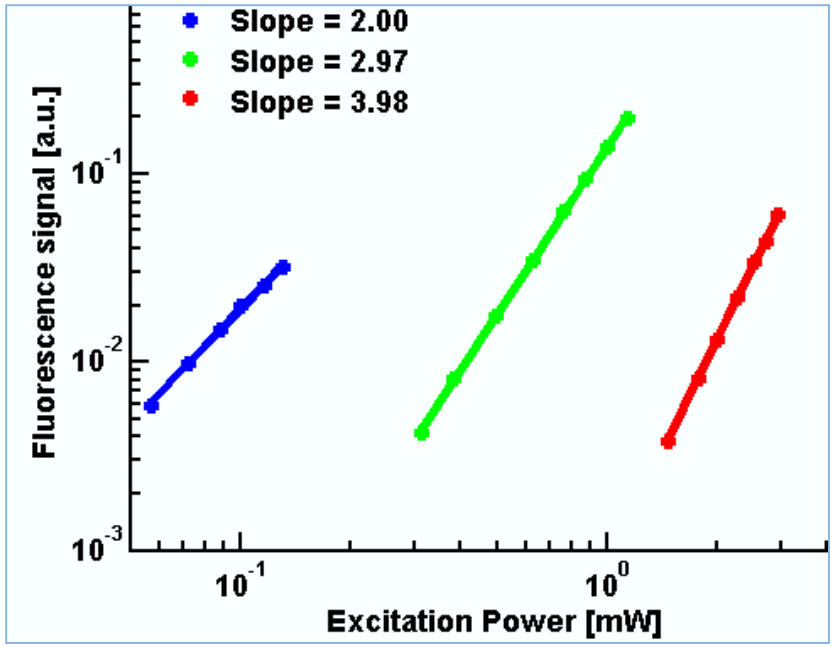
Three-photon microscopy

Three-photon absorption cross-section is even lower, however, due to the third order non-linearity **SNR is much higher**.

Imaging depth is greater due to longer wavelength of excitation light.

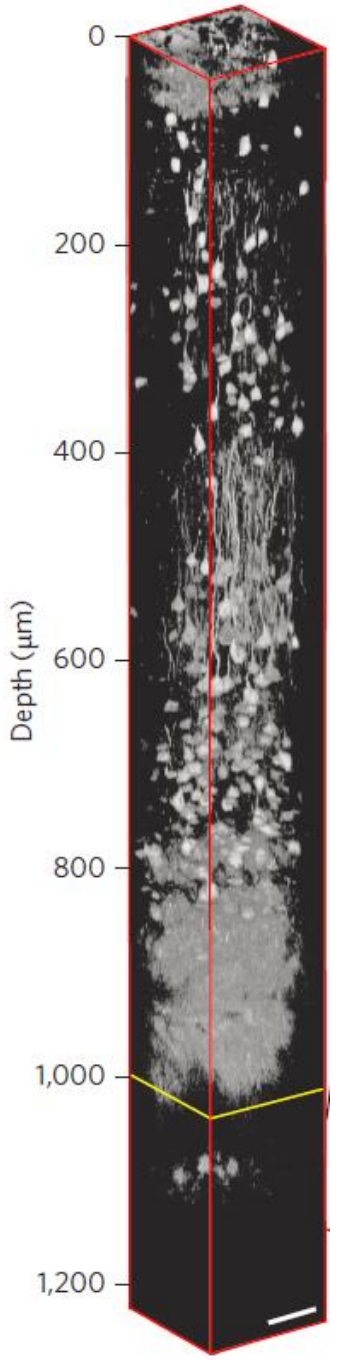


Source: Xu 2013, Nat. Phot.



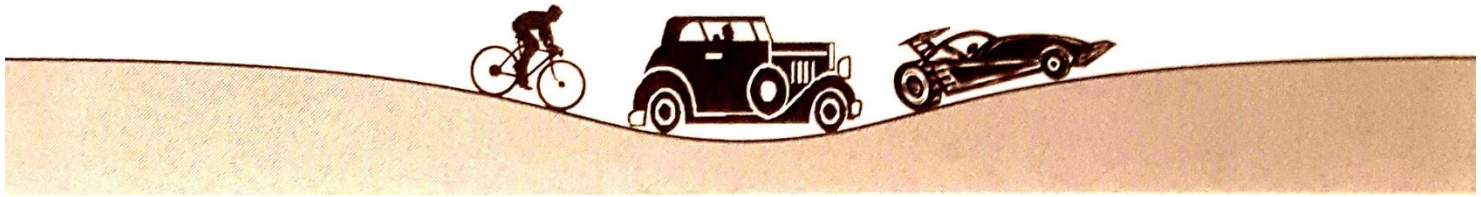
2P Alexa Fluor® 790
3P Sulforhodamine 101
4P Fluorescein

Needs a long wavelength femto-second laser source.

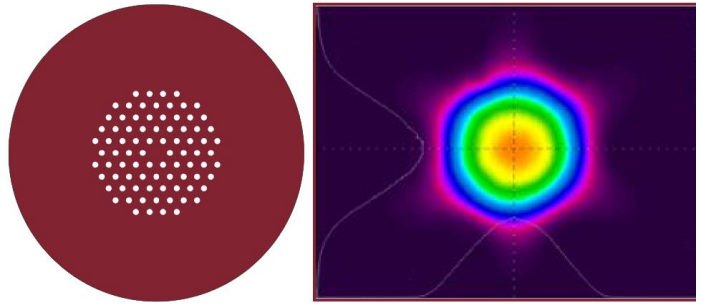


RFP-labelled neurons
Source: Horton 2013 Nat. Phot.

Self phase modulation & Soliton generation



Soliton is a locally stable solution of nonlinear differential equation



Large mode area (LMA) photonic crystal fiber (PCF) fibers can have soliton and single mode even at large power.

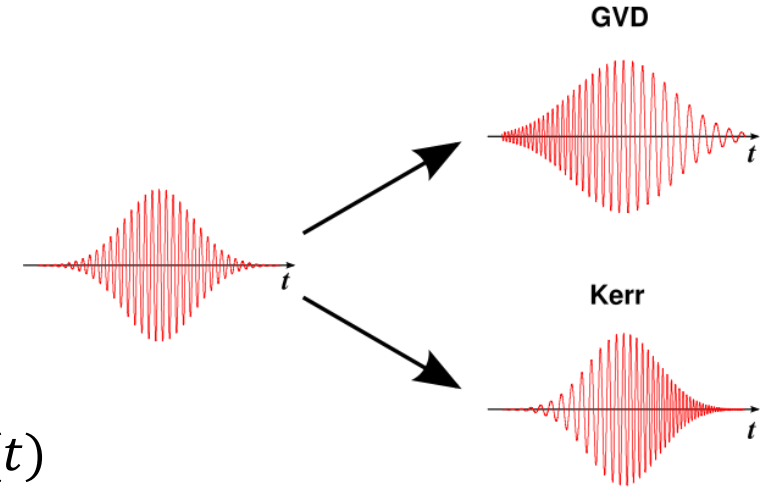
Electric field propagating in non-linear medium shows **optical Kerr** effect, that is, the refractive index changes due to the electric field intensity.

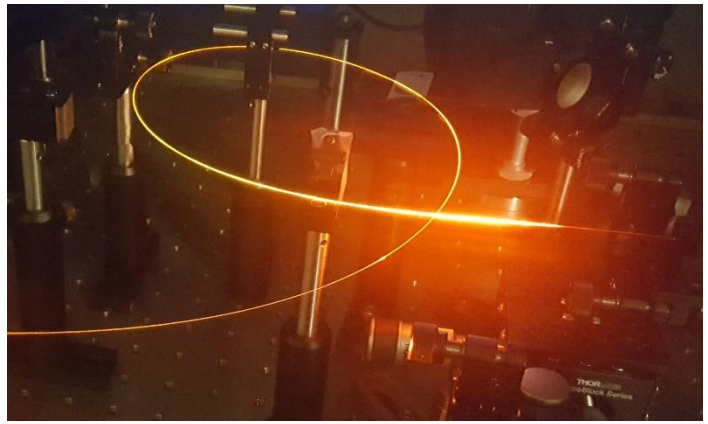
$$n(I) = n_o + n_2 I,$$

$$E(z,t) = A_m e^{i\boldsymbol{\varphi}(\boldsymbol{t},\boldsymbol{z})}$$

$$\varphi(t,z) = \omega_o t - k_o n(t) z$$

$$\omega(t) = \frac{\partial \varphi(t)}{\partial t} = \omega_o - k_o n_2 z \frac{\partial I(t)}{\partial t}$$

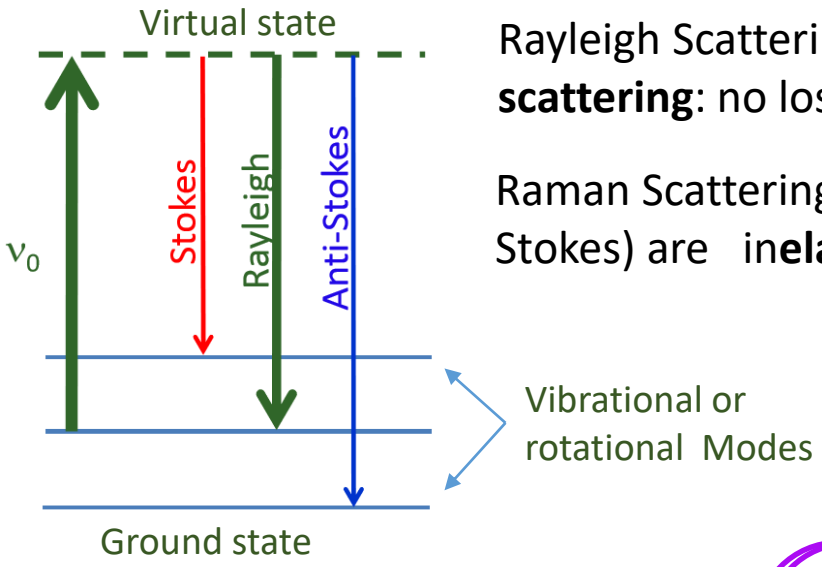




Supercontinuum from 1550nm laser

Soliton Self frequency shifting (SSFS)

Due to intrapulse **Raman scattering**, the blue portion of the soliton spectrum pumps the red portion of the spectrum, causing a continuous redshift in the soliton spectrum

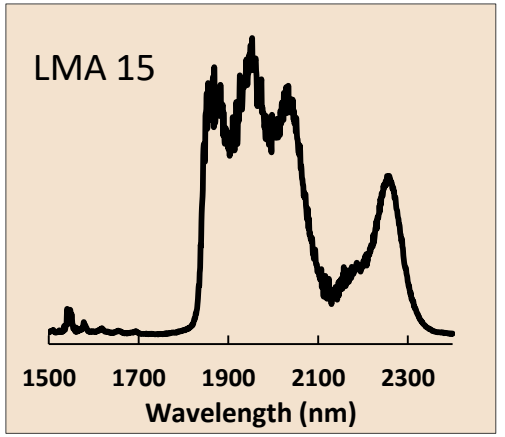


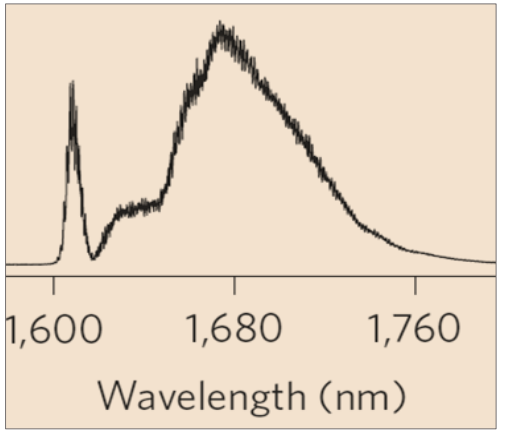
Rayleigh Scattering is an **elastic scattering**: no loss or gain

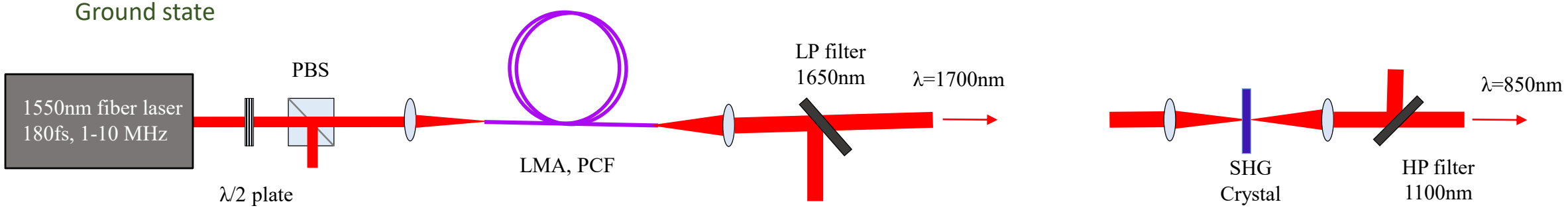
Raman Scattering (Stokes and Anti-Stokes) are in**elastic scattering**

$$\frac{\partial \nu}{\partial z} \propto \frac{h(\tau)}{\tau^4}$$

$h(\tau)$: Raman gain function
τ : temporal width of pulse

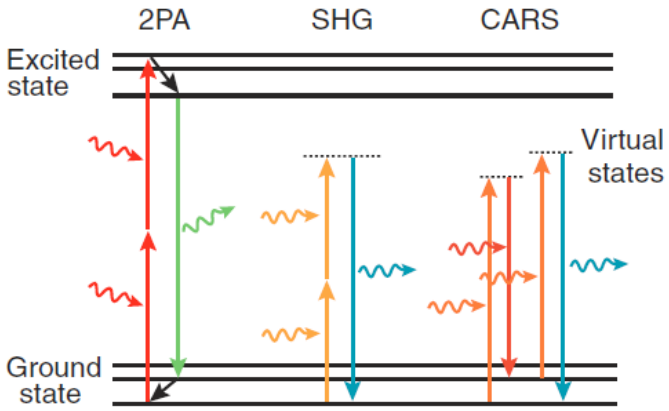






Second harmonics generation

Second harmonic generation (SHG) is a non-linear optical process in which two photons with same wavelength interact with a non-linear material and generate a new photon with twice the energy.



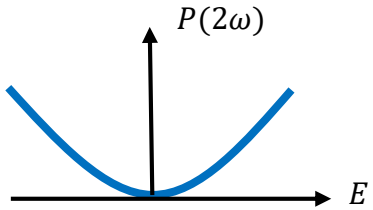
$$\boldsymbol{D} = \varepsilon_o \boldsymbol{E} + \boldsymbol{P}, \quad \boldsymbol{P} = \varepsilon_o \chi \boldsymbol{E},$$

$$P = \varepsilon_o \chi^{(1)} E^1 + \varepsilon_o \chi^{(2)} E^2 + \varepsilon_o \chi^{(3)} E^3 \ldots$$

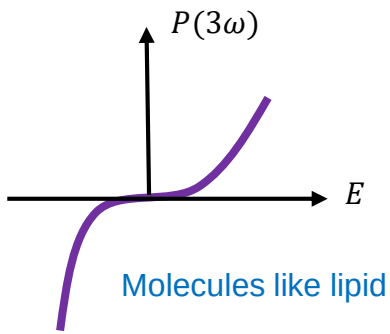
P : polarization density
ε_o: electric permittivity
χ : electric susceptibility

Second Harmonic

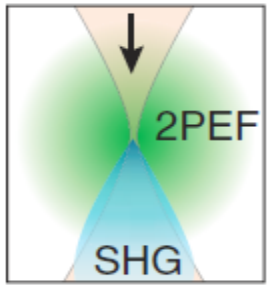
Third Harmonic



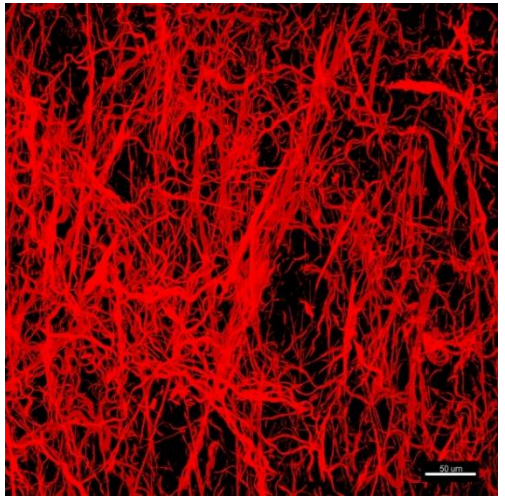
Non-centrosymmetric (***polar or chiral***) molecules e.g. Collagen, Bone



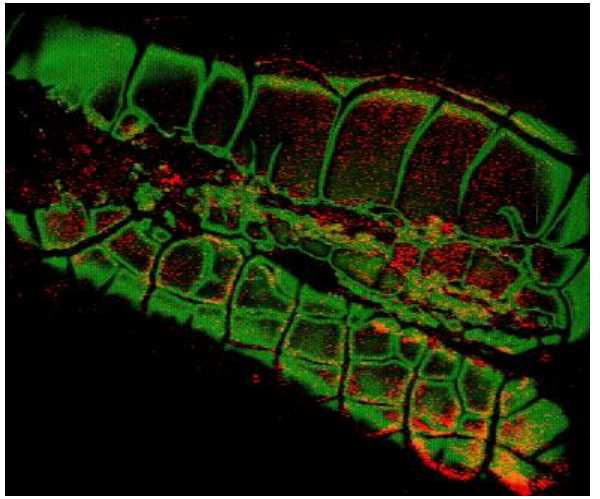
Molecules like lipid



Must match both the frequency and the phase

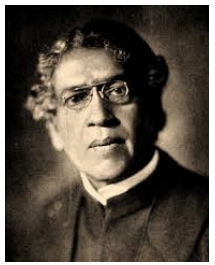
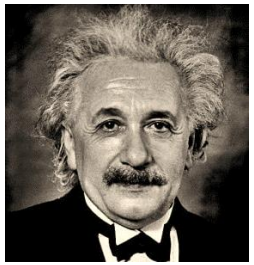


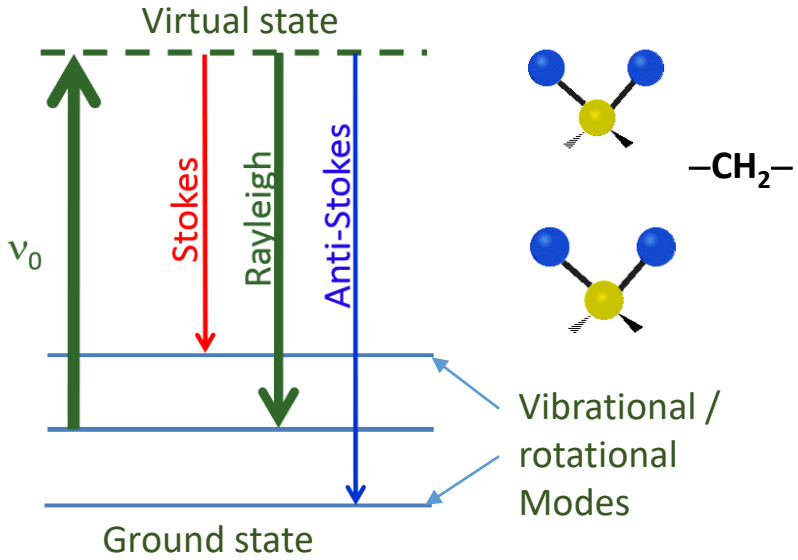
Mouse skin collagen fiber

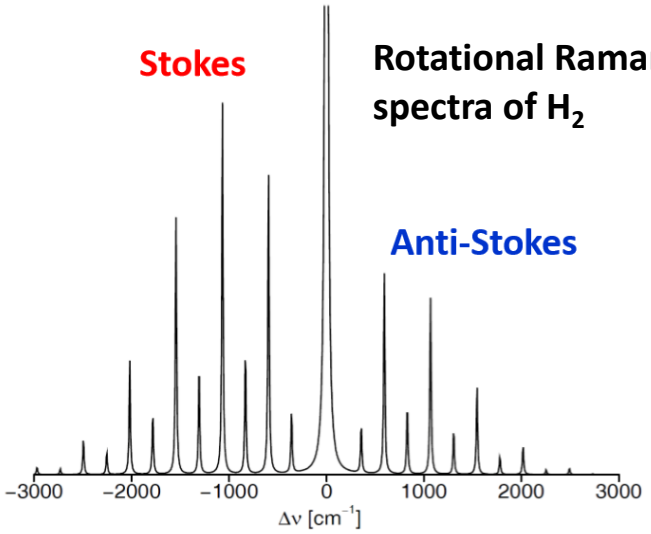


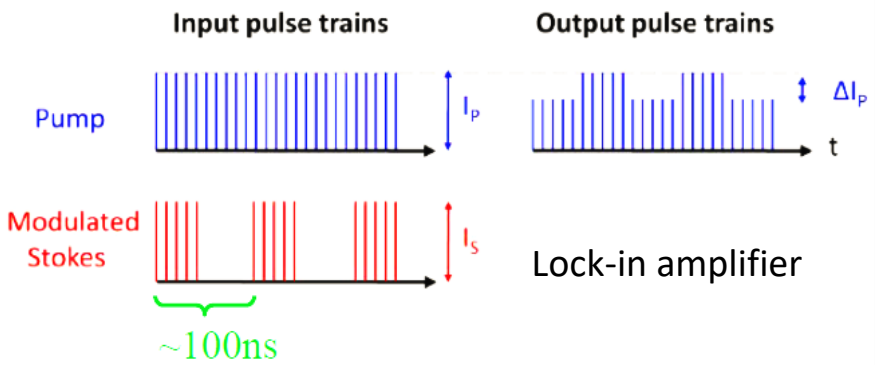
BBO crystals on glass slide

Raman scattering

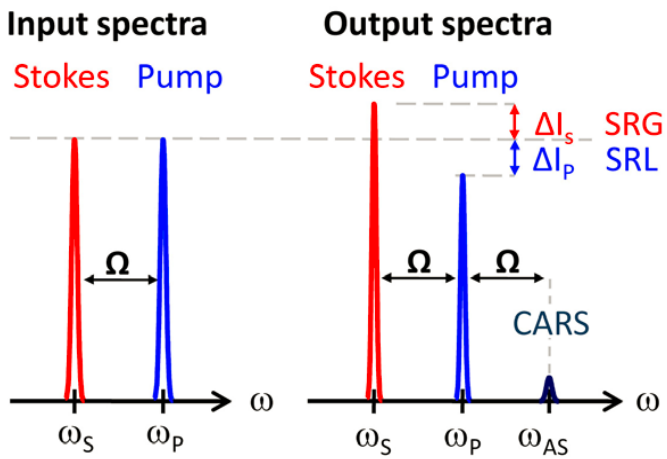










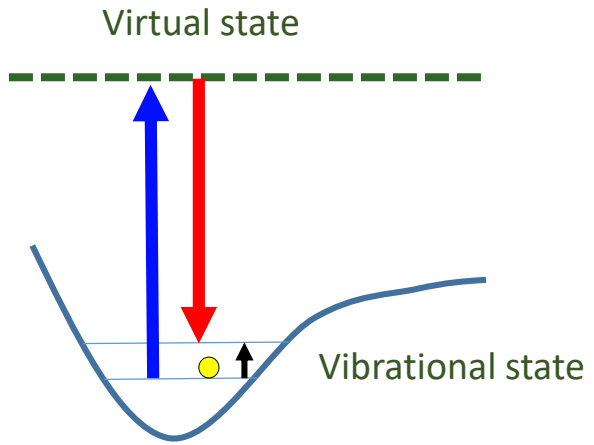
Lock-in amplifier



<u>Bose-Einstein Statistics</u>

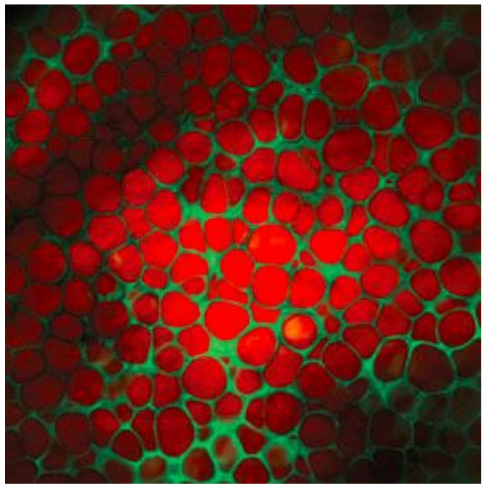
If N photons occupy a given state, the transition rates into that state are proportional to (N+1).

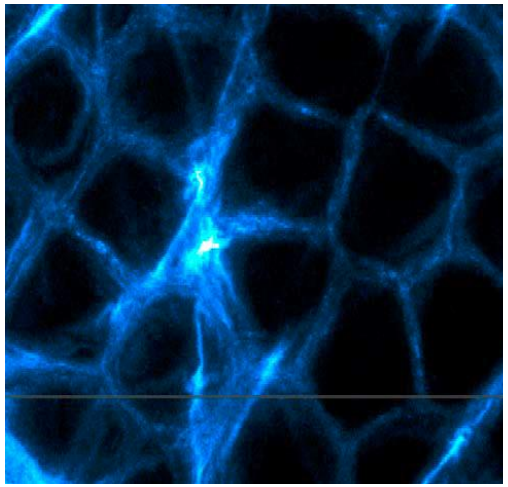
$$\langle n+1|a^+|n\rangle = \sqrt{n+1}$$

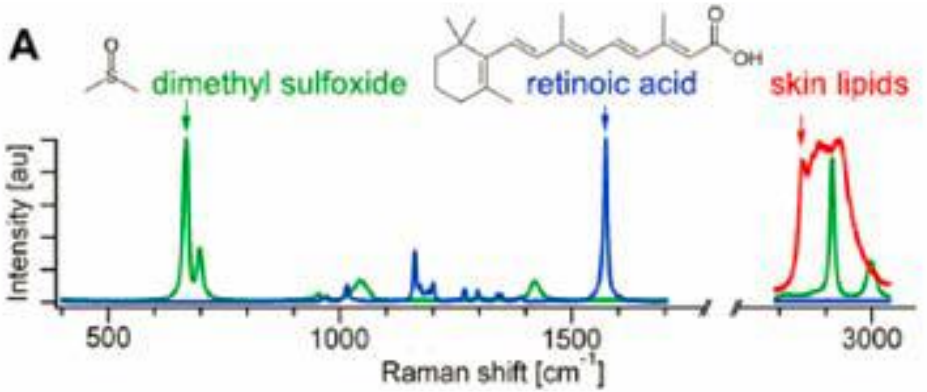


$$\frac{rate_{stim.}}{rate_{spon.}} = n_{Stokes} + 1$$

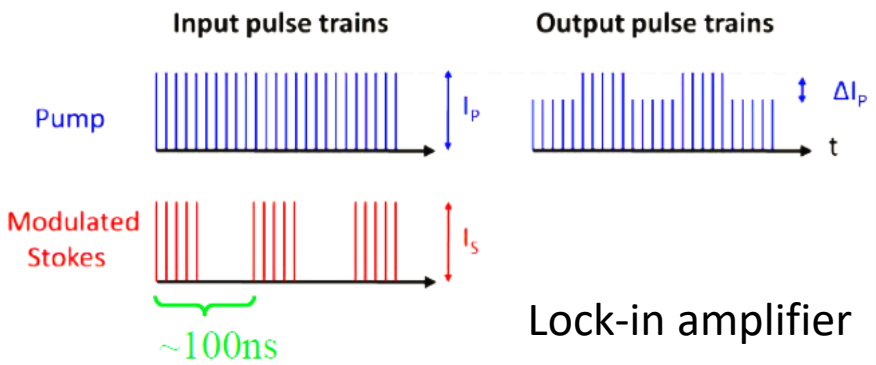
Stimulated Raman scattering (SRS)



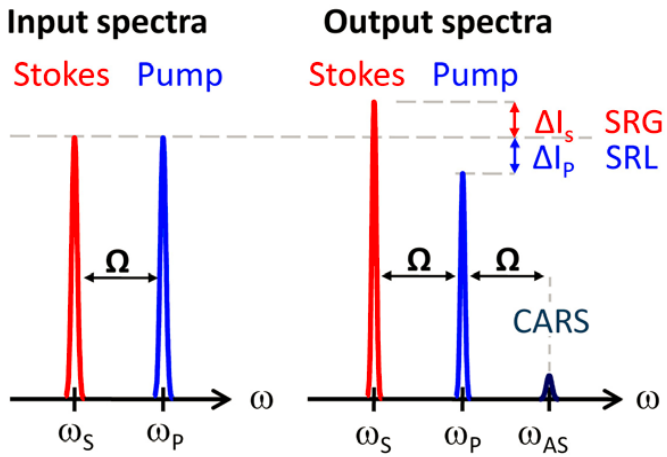


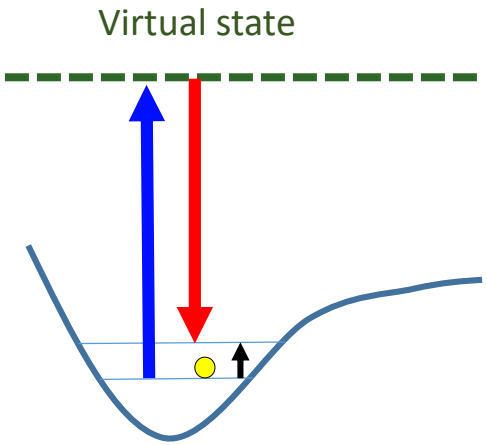


Source: Freudiger Science (2008)



Lock-in amplifier

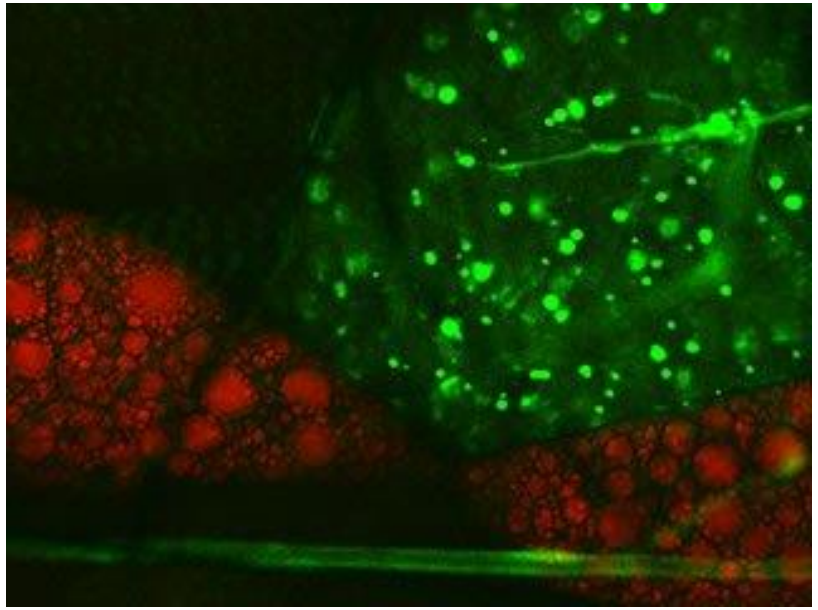




$$\frac{rate_{stim.}}{rate_{spon.}} = n_{Stokes} + 1$$

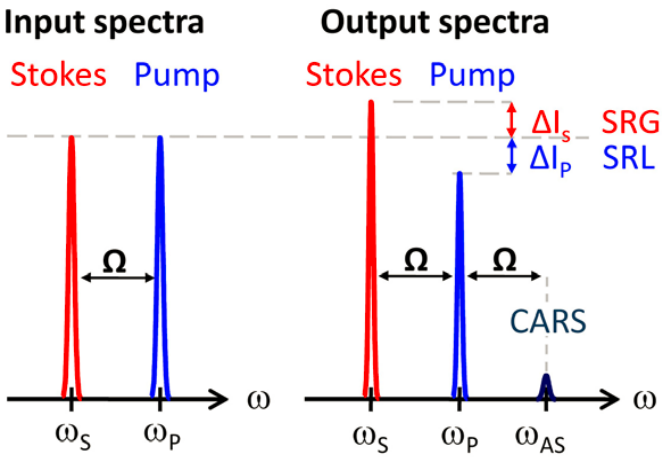
Coherent Anti-Stokes Raman Scattering (CARS)

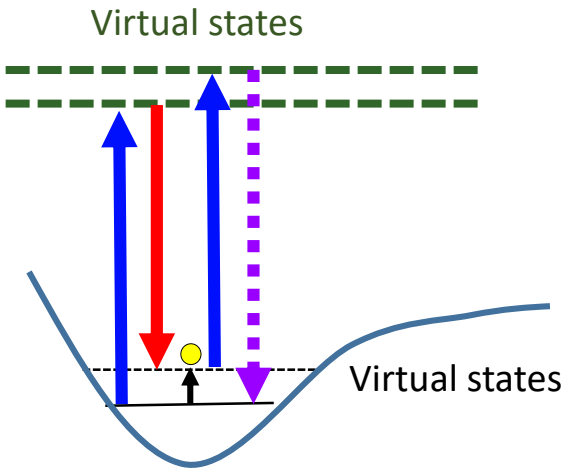
CARS is a third-order nonlinear process that involves a pump beam and a Stokes beam.



In vivo imaging of a larvae of a fruit fly (Drosophila melanogaster). Fat cells shown in red (816 nm) and auto fluorescence in green.

Source: leica-microsystems.com





Third-order non-linear parametric process

CARS anti-Stokes frequency: $\omega_{AS} = 2\omega_p - \omega_s$

Vibrational contrast created at frequency: $\nabla\omega = \omega_p - \omega_s$

Thank you!

Questions?