607SM - Techniche avanzate di indagine microscopica

Advanced microscopy techniques – 6CFU, 2002/23, 1st semester

Part1:

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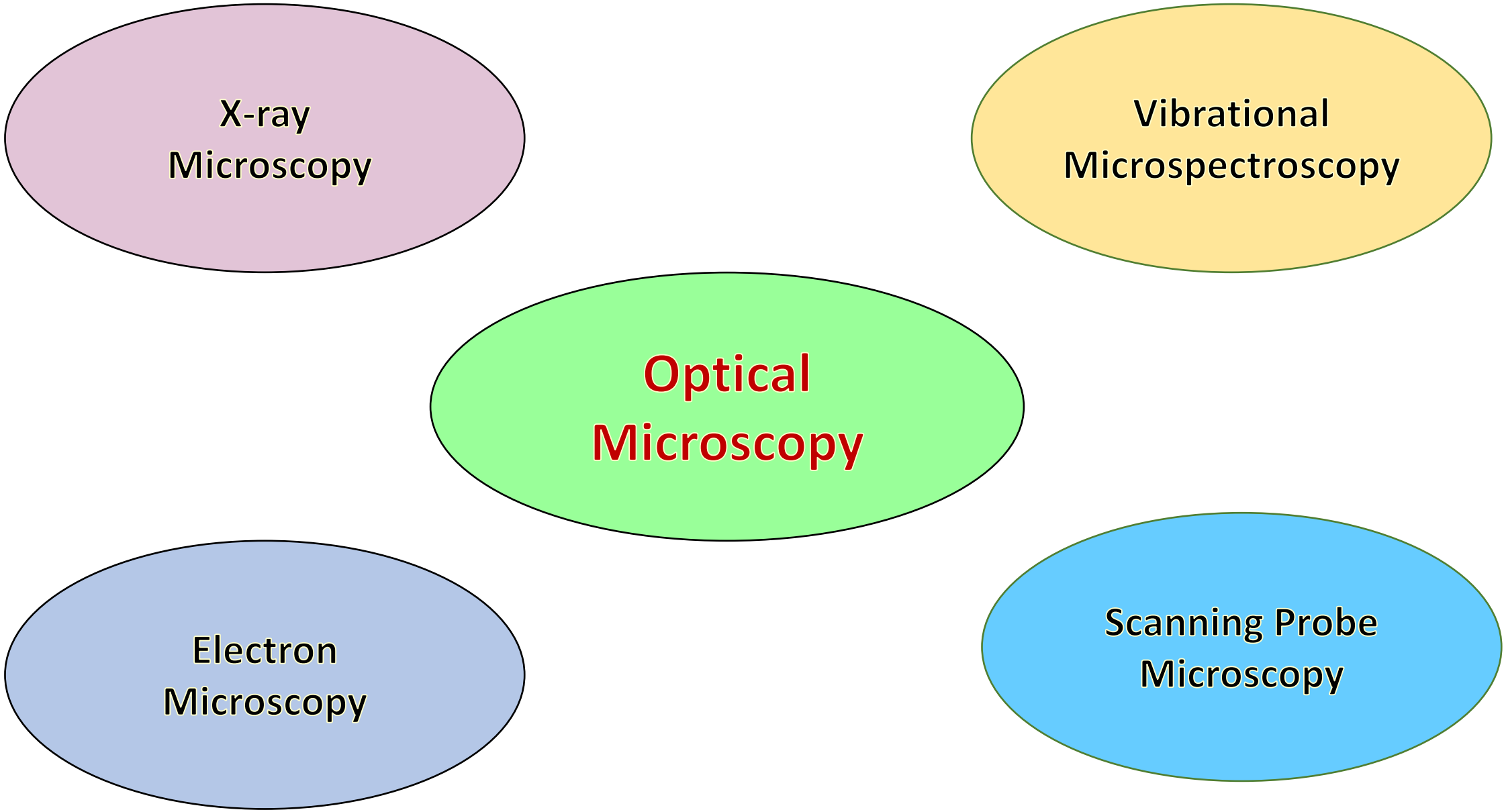
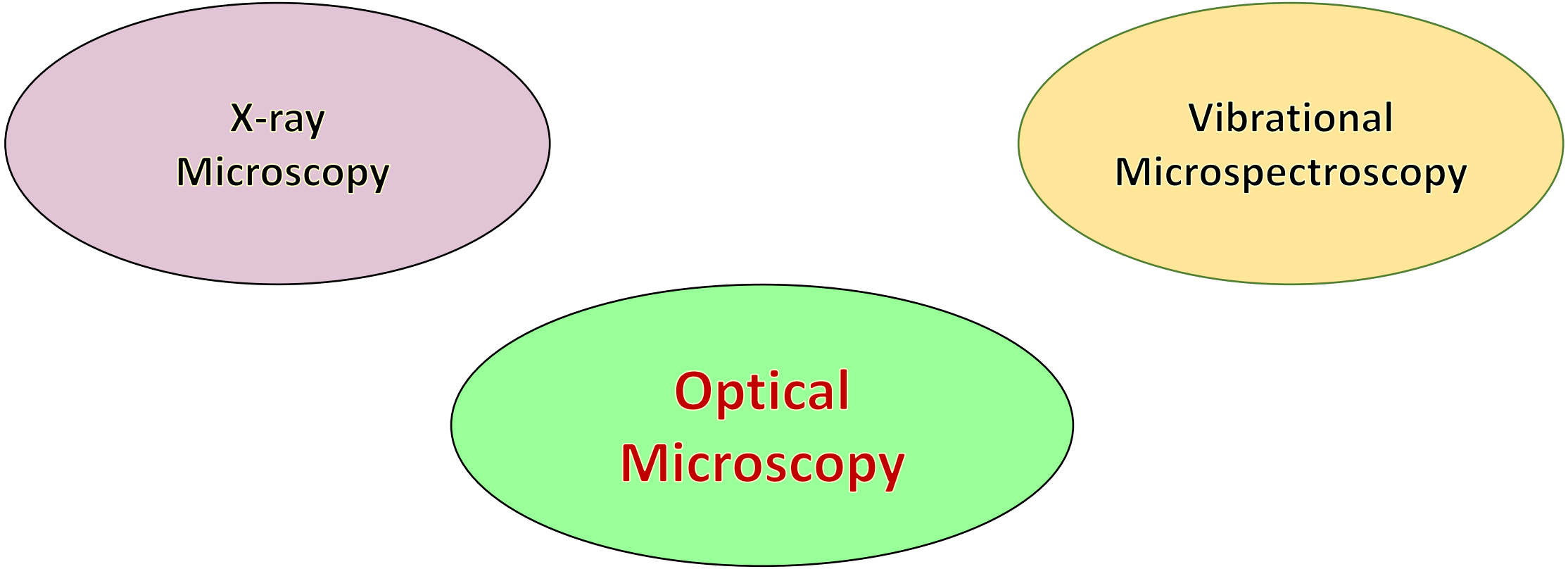
Tuesday 11-13, Aula 1C, Ed H3 + Thursday 14-16, Aula 5A EdH2-H2bis (colleg H3)

WHAT TYPE OF MICROSCOPIES

WILL WE DISCUSS ABOUT ?


X-ray
Microscopy
Vibrational
Microspectroscopy
Optical
Microscopy
Electron
Microscopy
Scanning Probe
Microscopy

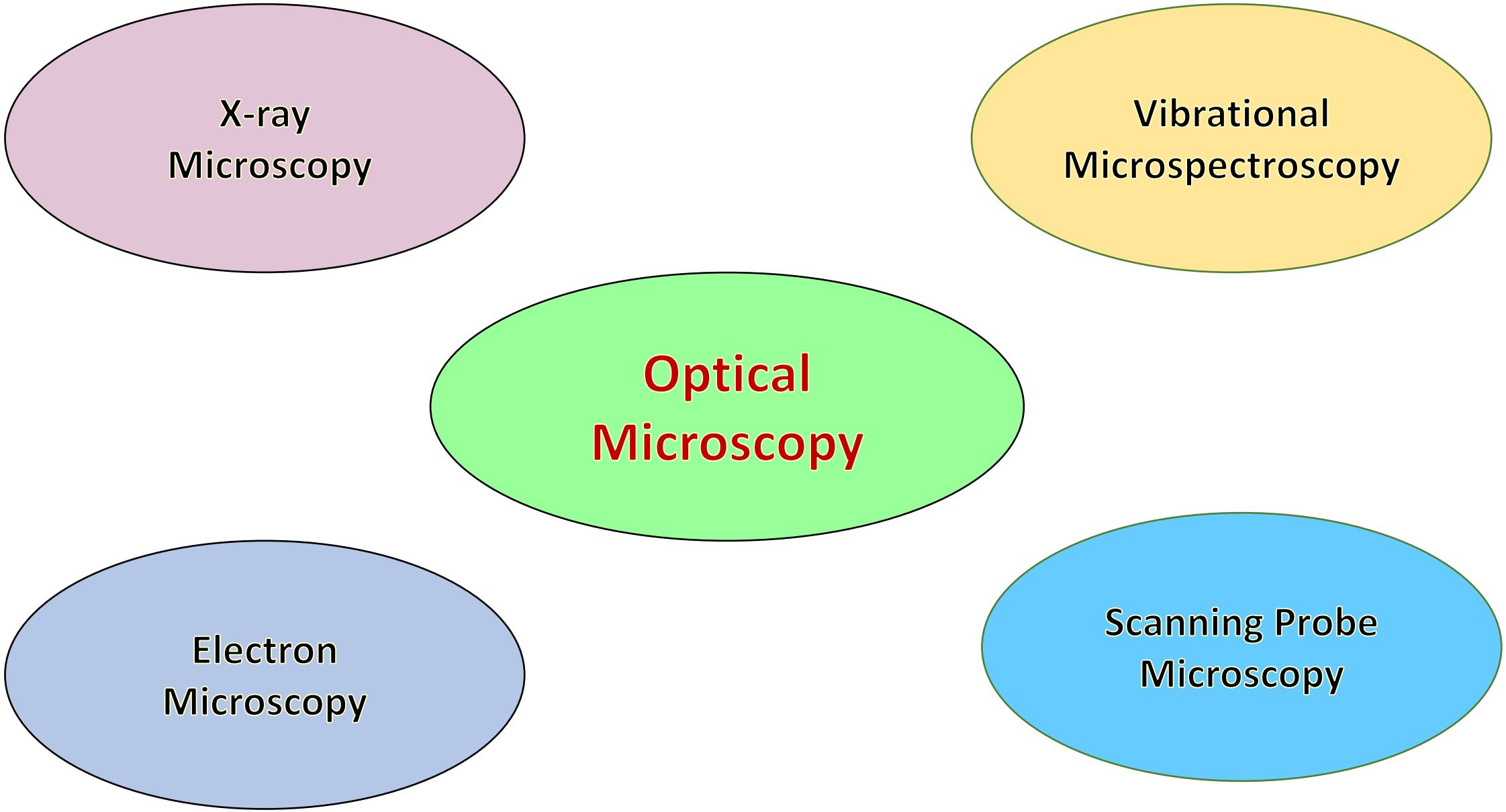


Electromagnetic Radiation (EMR)

Microscopy Techniques

Non- Optical

Microscopy Techniques


X-ray
Microscopy
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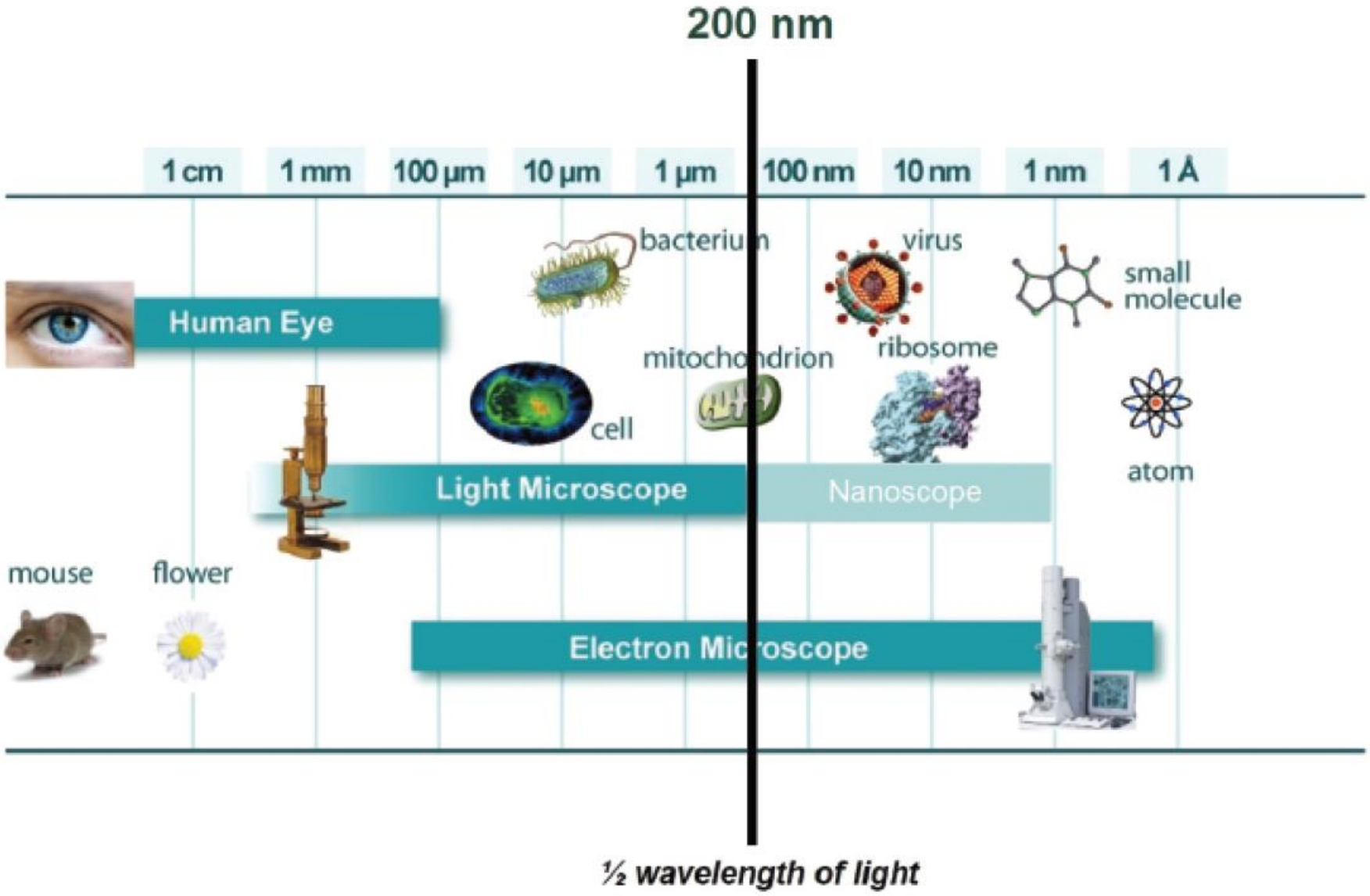
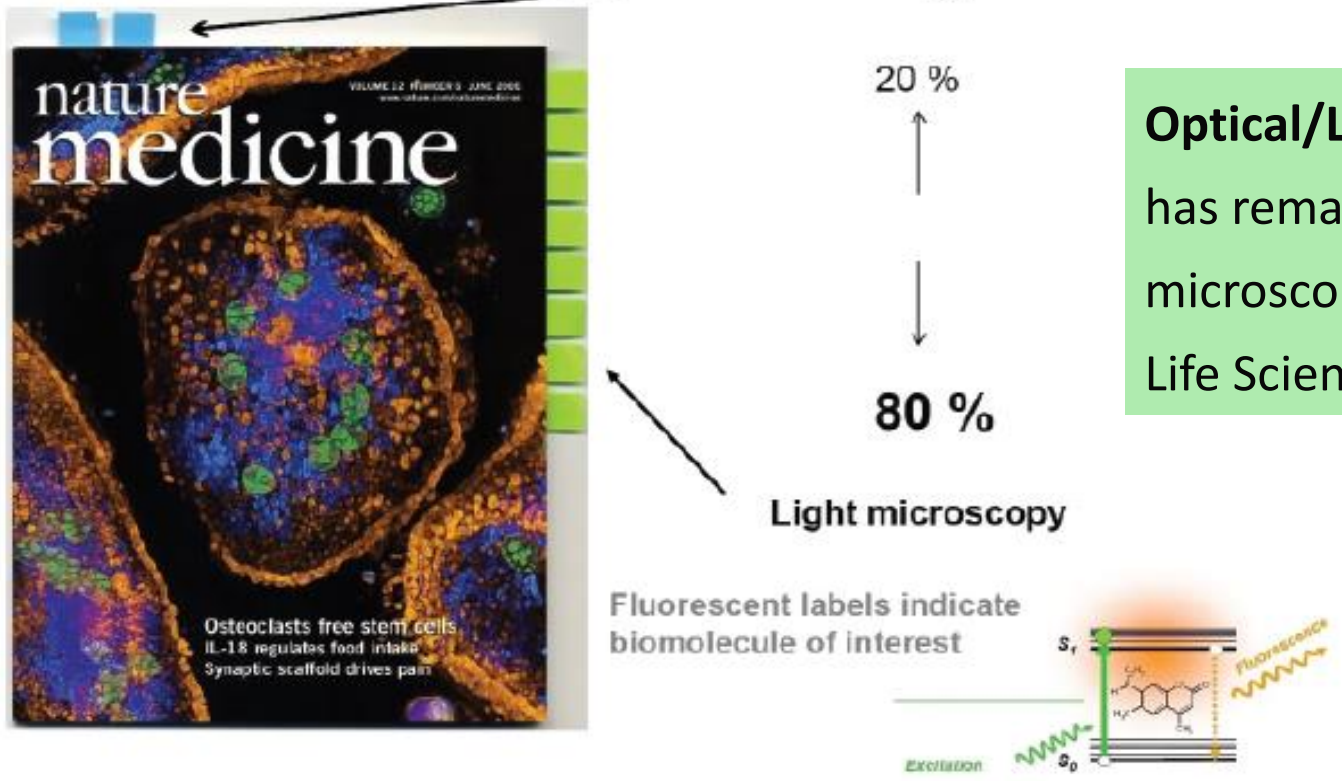
Scanning Probe
Microscopy

Length scales and spatial resolution limits



Why do we bother with the optical microscope, now that we have the electron microscope ?



Optical/Light microscopy
has remained the most popular microscopy technique in the Life Sciences

Optical microscopy is the only way in which we can look inside a living cell, or even living tissues, in three dimensions; it is minimally invasive.

When we look into a cell, we are usually interested in a certain species of proteins or other biomolecules, and we have to make them distinct from the rest we have to "highlight" those proteins → fluorescence.

S. W. Hell, Nobel Prize Lecture, Dec 2014

Novel microscopic methods to monitor cell biological processes of live or fixed cells without the **use** of any dye, stains, or other contrast agent.

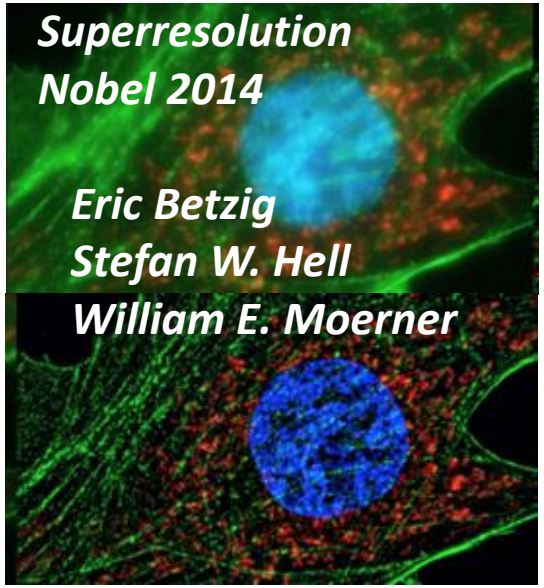
These methods are based on **spectral techniques** that detect inherent spectroscopic properties of biochemical constituents of cells.

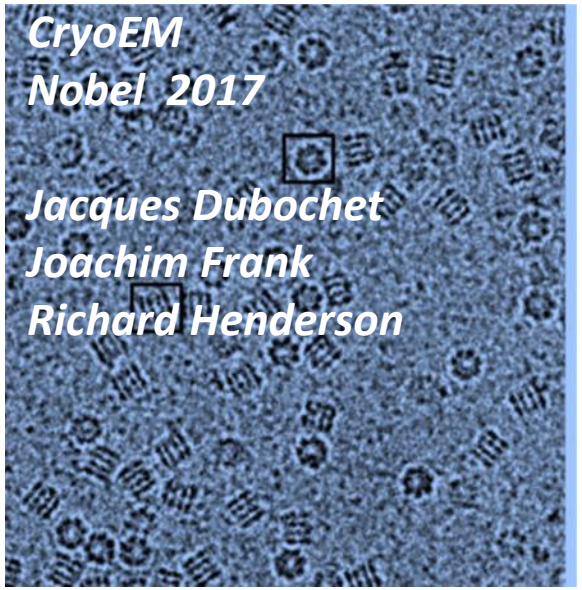
1. **Infrared micro-spectroscopy**, in which an average snapshot of a cell's biochemical composition is collected in fractions of a second, at 25 um resolution, is particularly suited for studying gross biochemical changes.
2. **Raman micro-spectroscopy**, is ideally suited to study variations of cellular composition on the scale of subcellular organelles.

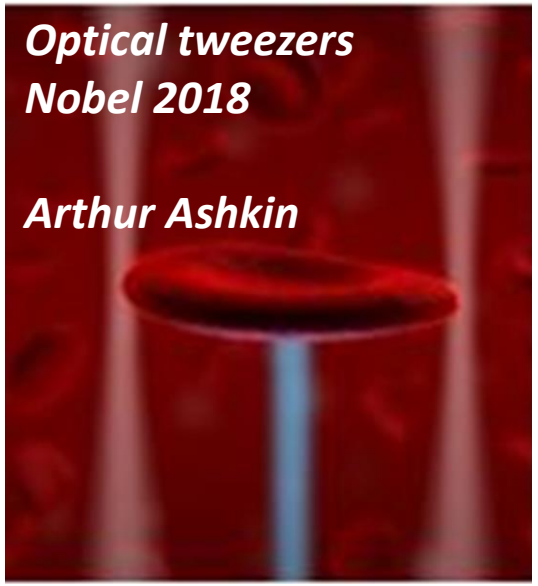
Advanced Microscopy

The microscopy plays a very important role in the study of biochemical mechanisms, at the tissue, cellular and subcellular level, and is constantly evolving.

In the last decade **3 Nobel Prizes** have been awarded for innovations in microscopic techniques.

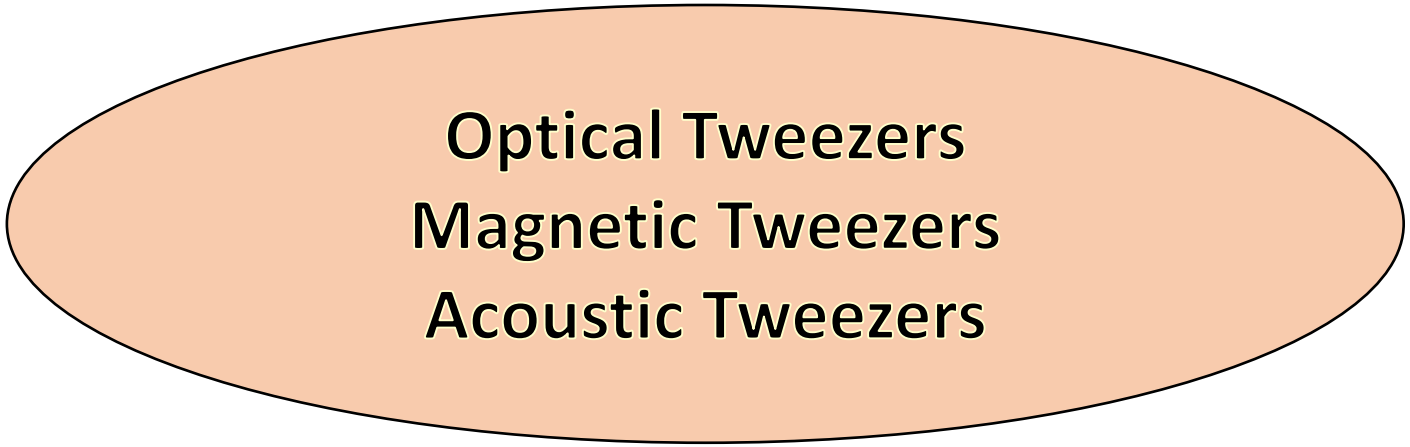




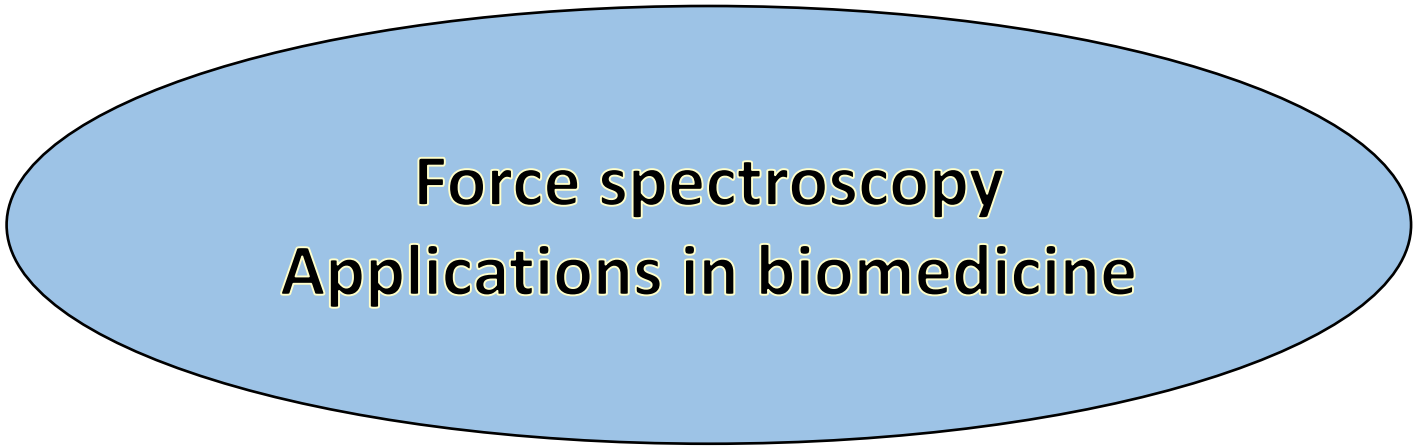


Various strategies have been developed to overcome the limit of diffraction of light and now the focus is on nanotechnology approaches aimed at revolutionizing microscopy → **nanoscopy**

Contact less micro and nanoparticle manipulation techniques



Single molecule and single cell biomechanics

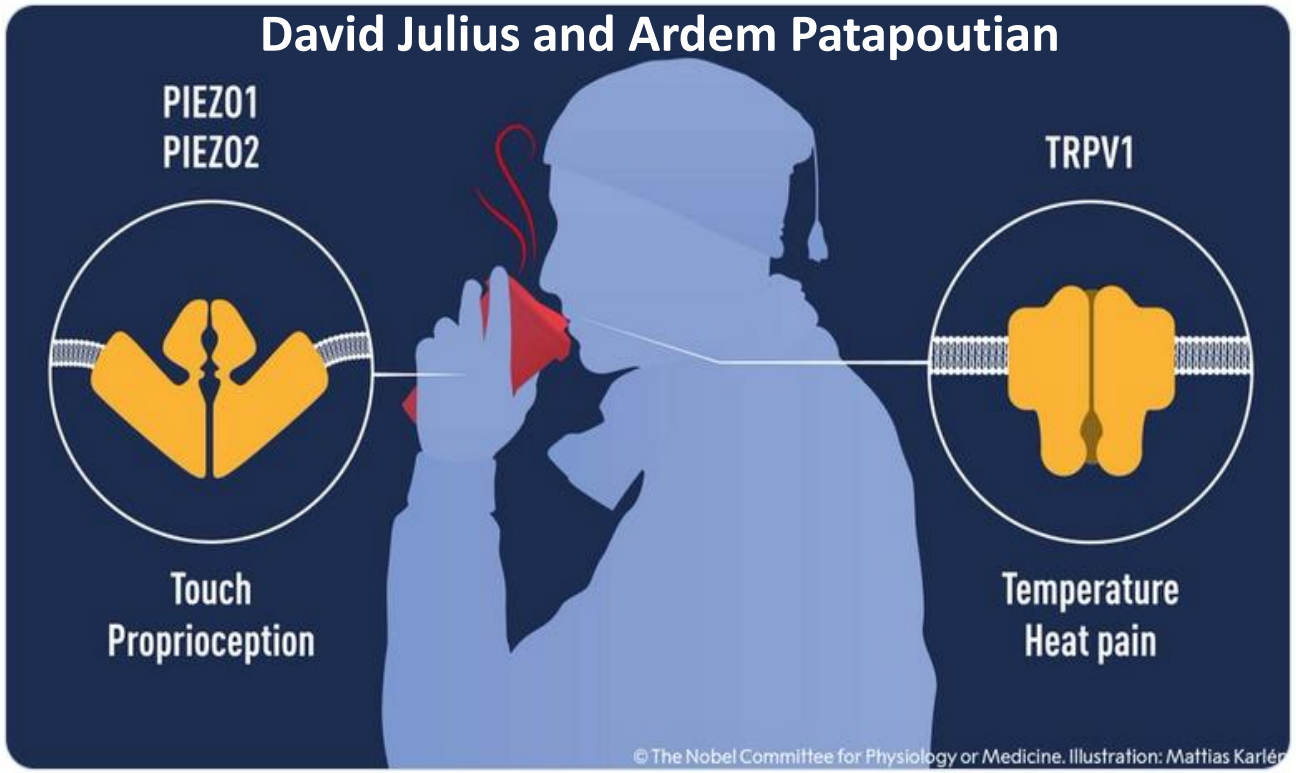


The Nobel Prize
@NobelPrize

Learn more about the 2021 #NobelPrize in Physiology or Medicine

Press release: nobelprize.org/prizes/medicin...
Advanced information: nobelprize.org/prizes/medicin...

David Julius and Ardem Patapoutian



607SM - TECNICHE AVANZATE DI INDAGINE MICROSCOPICA

Part 1 Dan Cojoc

1. Optical microscopy – 9h

1.1. Basics – 3h

- Why optical microscopy?
- Image formation; magnification and resolutiont;
- diffracted limited resolution;
- Optical abberations and image quality;
- Digital camera image acquisition (formats, properties, SNR)

1.2. Phase imaging techniques – 1h

- Phase contrast and differential interference contrast (DIC);
- Quantitative phase imaging: digital holographic microscopy.

1.3 Other techniques to image non-stained samples - 1h

- Dark field microscopy
- Polarization microscopy

1.4 Fluorescence microscopy - 4h

- Epifluorescence basics; confocal; two photons;
- Super-resolution techniques: STED. PALM, MULTIFLUX. SIM
- Other techniques: FRET, FRAP, FLIM

2. X-ray microscopy – 2.5h

2.1. Why X-ray microscopy ?
2.2. Image formation in X-ray microscopy, characteristics
2.3. X-ray microscope components and techniques
2.4. X-ray microscopy / microspectroscopy with Synchrotron Light and Free Electron Laser

3. Electron microscopy – 2.5h

3.1. Why Electron microscopy ?
3.2.Electron beam – specimen interaction for image formation in EM
3.3.Types of EM, resolution, depth of field, elemental imaging
3.4.Contrast formation and imaging in TEM and SEM, applications
3.5.Advanced EM techniques:
volume EM, protein localization, correlative microscopy and cryo EM

4. Vibrational spectrocopy and microscopy – 2h

4.1. Fourier Transform InfraRed FTIR microspectroscopy
4.2. Raman scattering microspectroscopy

5. Scanning probe microscopy – 3h

5.1. Scanning Tunneling Microscopy (STM) – principle
5.2. Atomic Force Microscopy (AFM)
5.3. Scanning Near field Optical Microscopy (SNOM)

6. Contact less manipulation techniques at molecular and cellular level – 2h

6.1. Optical Tweezers (OT) and scissors
6.2. Magnetic and acoustic tweezers

7. Single molecule and single cell biomechanics – 2h

7.1 Force spectroscopy: AFM vs Optical tweezers
7.2. FS Applications in biomedicine

8. Laboratory: Optical Tweezers; Digital Holographic Microscopy – 3h

Part 2. Agnes Thalhammer

A. Sample preparation - X-Ray and electron microscopy (1 h)

B. Optical microscopy: Applications in cellular biology and biotechnology (21 h + 6 h lab)

1. White light microscopy applications (1 h)

1.1. Which samples?

2. Immunofluorescence protocols and fluorescence microscopy applications (4 h + 2 h lab)

2.1. Fluorophores and Filters
2.2. Sample preparation: Fixing, blocking, antibodies, bleaching, quenching, antifade agents
2.3. Practical considerations to multichannel fluorescence labelling
2.4. Laboratory: White light and fluorescent microscopes (2 h)

3. Confocal, superresolution and multiphoton microscopy applications (4 h + 4 h lab)

3.1. SIM, FRET, FRAP
3.2. Choosing the right microscopy technique for your experiment
3.3. Laboratory: confocal, SIM superresolution and multiphoton microscopes (4 h)

4. Live imaging microscopy applications (6 h)

4.1. How to keep a live sample: labelling, perfusion, temperature, humidity, oxygenation, osmolarity, phototoxicity
4.2. The trinity of resolution-speed-sensitivity
4.3. Calcium-, voltage- and pH-sensitive dyes; consideration for data acquisition and analysis

5. Digital image processing and analysis (6 h)

5.1. Image display options (LUTs, brightness, contrast, filters, denoising, deconvolution, z-stack projections, overlay)
5.2. Quantitative microscopy (grey values, histogram, threshold, binary image, masks, intensity profile, particle analysis, colocalization, 3D reconstruction, time series analysis)

APPROACH

Lectures:

- Introduce the working principle and main characteristics of the technique
- Discuss examples of applications in Biotehnology, Biomedicine …

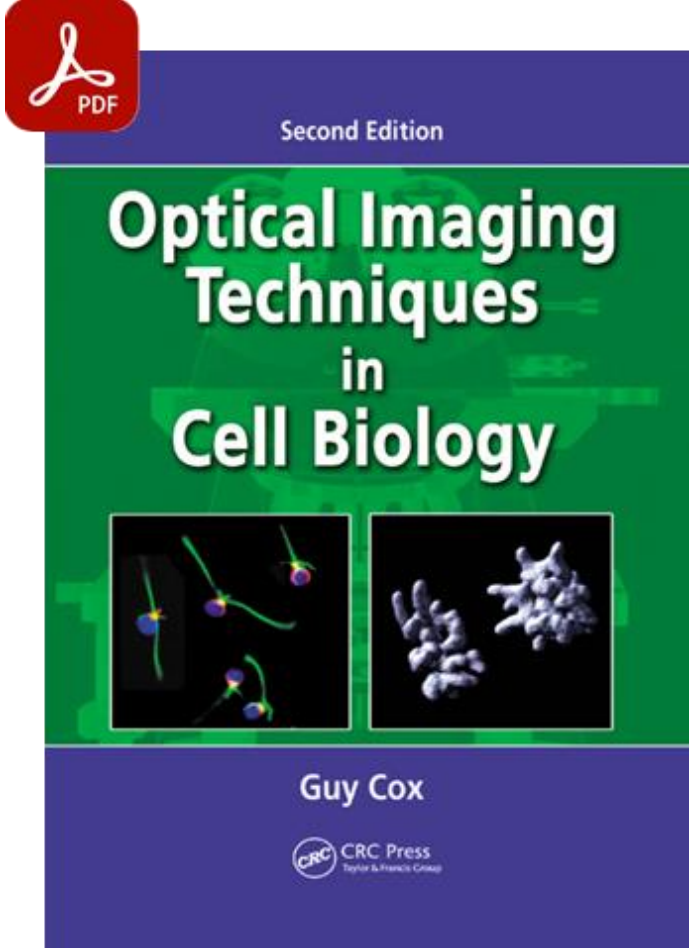
Laboratory:

Optical microscopy, e-microscopy – dr. Agnes Thalhammer (22-24 h) Univ

Optical Tweezers and Digital Holographic Microscopy – dr. Dan Cojoc (4h) CNR-IOM

References:

- Lectures slides – pdf – upload to noodle
- Papers, web links – indicated / downloaded – mentioned during the lectures
- Books – pdf downloaded , mentioned during the lectures

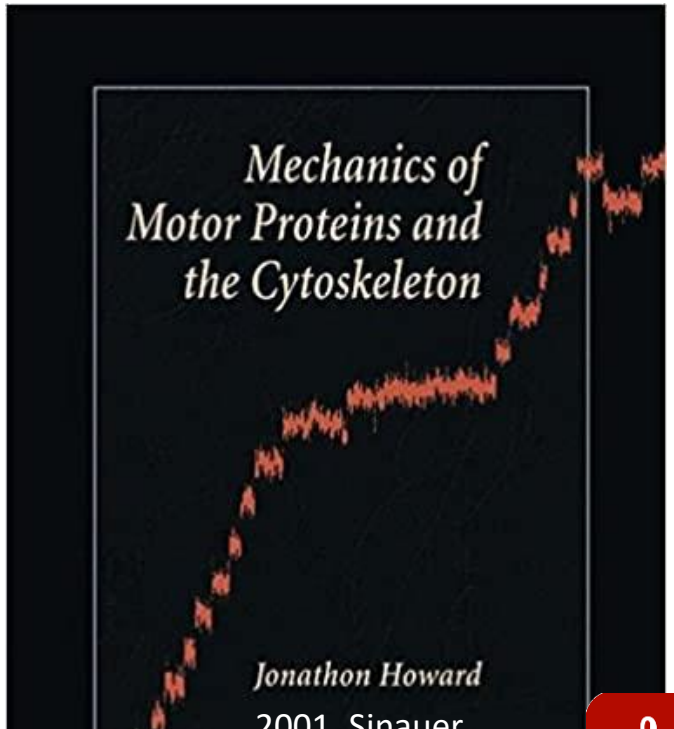


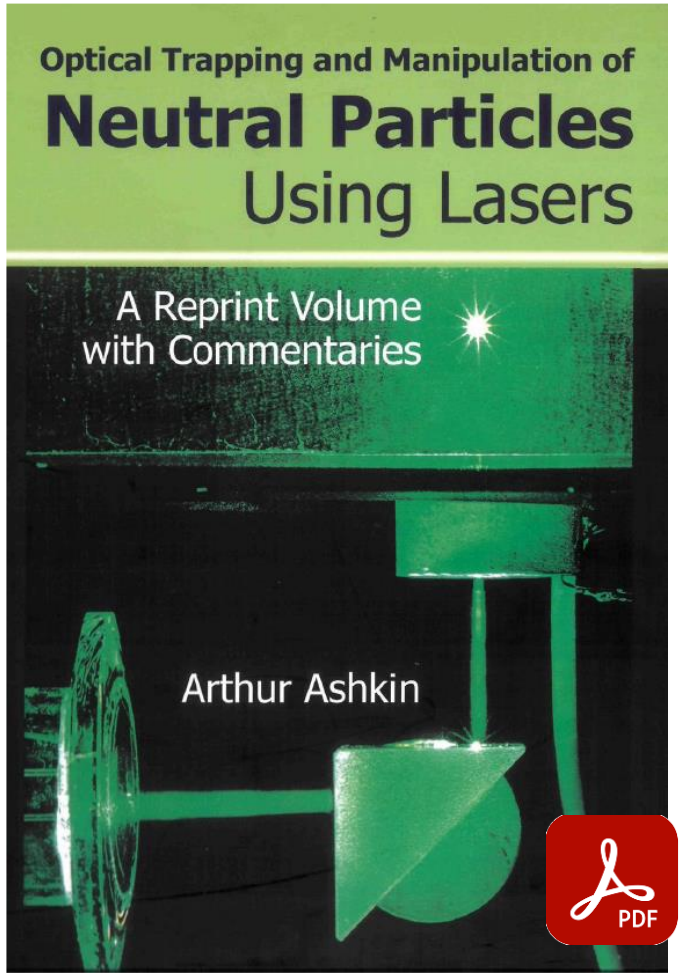
Principles of Fluorescence Spectroscopy

Third Edition

2006, Springer

Joseph R. Lakowicz
University of Maryland School of Medicine
Baltimore, Maryland, USA





And others

EXAM

ORAL

- Present / discuss a microscopy technique indicated by teacher 2 days before exam, with example of application of the technique published in literature (searched and chosen by student);

  ppt slides, max 20 min

- Answer questions related to the technique presented

- Answer general questions related to other techniques presented during the course

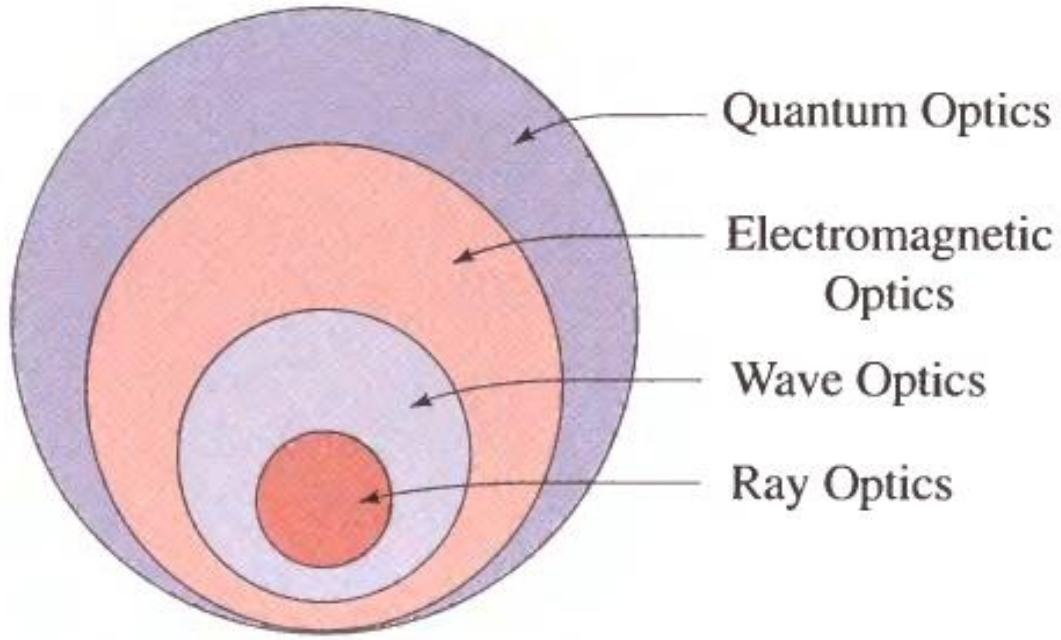
Note

- Al the material of the course should be studied, not only the topic indicated for exam; this requires more time than 2 days preparation

- Students are kindly invited to study during the year and address questions/explanations

REVIEWING BRIEFLY SOME PROPERTIES OF LIGHT

1. Light as an Electro Magnetic wave – Wave Optics
2. Ray Optics
3. Particle-wave duality of light

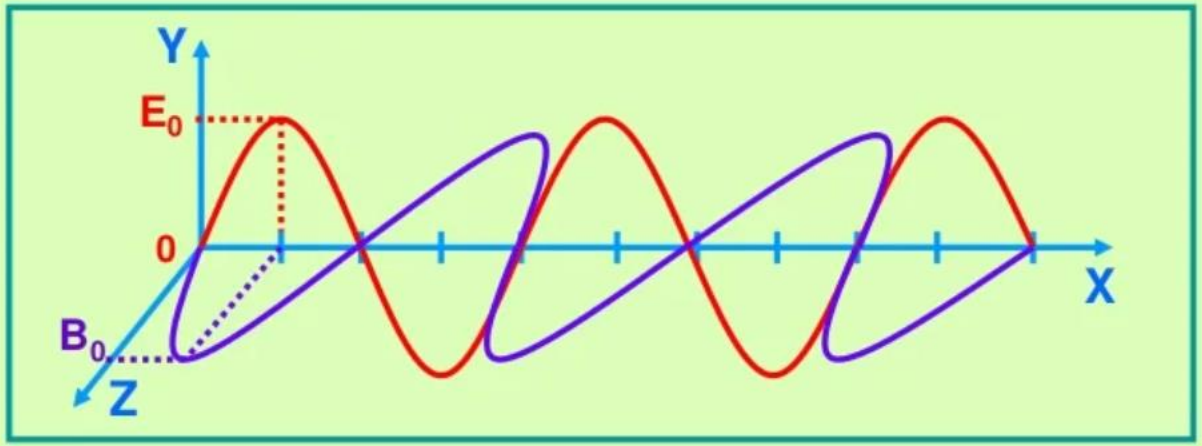


WAVE OPTICS - I

1. Electromagnetic Wave
2. Wavefront
3. Huygens' Principle
4. Reflection of Light based on Huygens' Principle
5. Refraction of Light based on Huygens' Principle
6. Behaviour of Wavefront in a Mirror, Lens and Prism
7. Coherent Sources
8. Interference
9. Young's Double Slit Experiment
10. Colours in Thin Films

https://www.slideshare.net/RameshMeena12/class-12th-physics-wave-optics-ppt

Electromagnetic Wave:



1. Variations in both electric and magnetic fields occur simultaneously. Therefore, they attain their maxima and minima at the same place and at the same time.
2. The direction of electric and magnetic fields are mutually perpendicular to each other and as well as to the direction of propagation of wave.
3. The speed of electromagnetic wave depends entirely on the electric and magnetic properties of the medium, in which the wave travels and not on the amplitudes of their variations.

Wave is propagating along X – axis with speed $c = 1 / \sqrt{\mu_0 \varepsilon_0}$

For discussion of optical property of EM wave, more significance is given to Electric Field, E. Therefore, Electric Field is called 'light vector'.

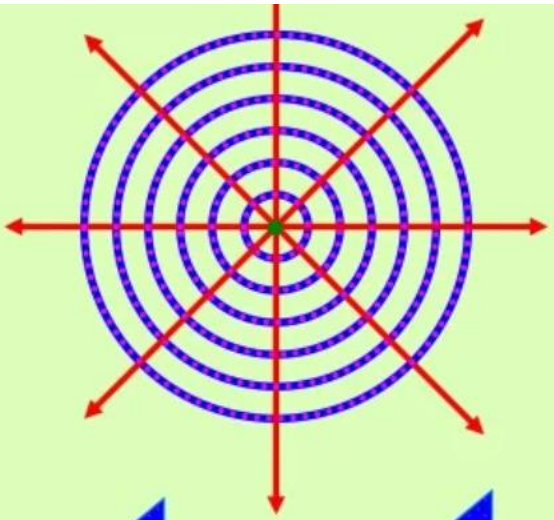
Wavefront:

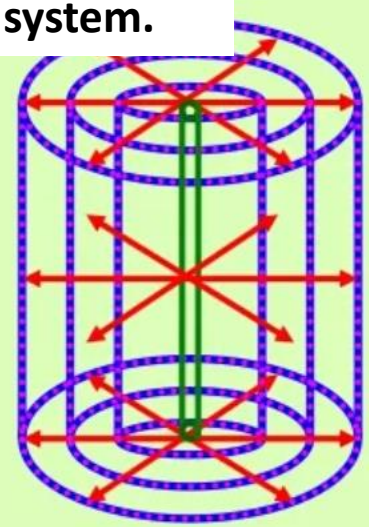
A wavelet is the point of disturbance due to propagation of light.

A wavefront is the locus of points (wavelets) having the same phase of oscillations.

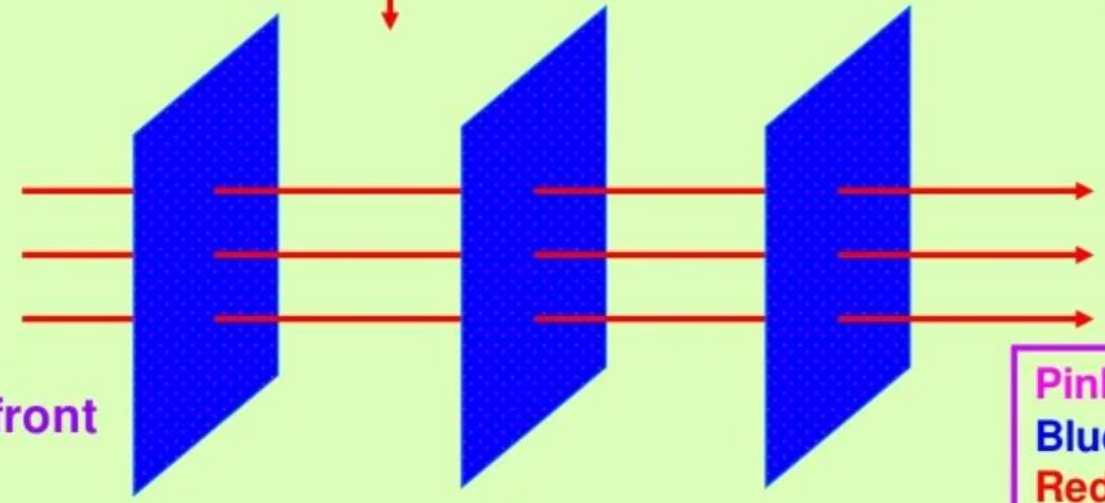
We define a ray as the path along which light energy is transmitted from one point to another in an optical system.

Spherical Wavefront from a point source





Cylindrical Wavefront from a linear source



Plane Wavefront

Pink Dots – Wavelets
Blue Envelope– Wavefront
Red Line – Ray

Huygens' Construction or Huygens' Principle of Secondary Wavelets:

(Wavelets - Red dots on the wavefront)

1. Each point on a wavefront acts as a fresh source of disturbance of light.
2. The new wavefront at any time later is obtained by taking the forward envelope of all the secondary wavelets at that time.

Note: Backward wavefront is rejected. Why?

Amplitude of secondary wavelet is proportional to ½ (1+cosθ). Obviously, for the backward wavelet θ = 180° and (1+cosθ) is 0.

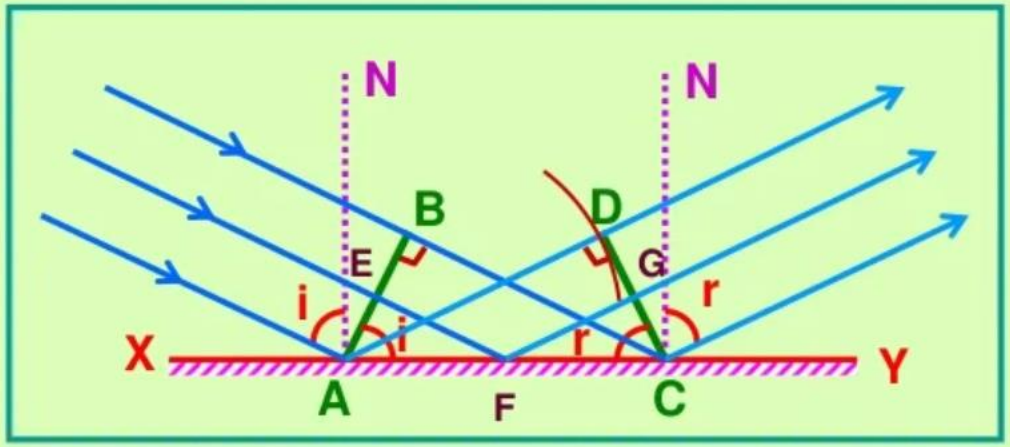
Laws of Reflection at a Plane Surface (On Huygens' Principle):

If c be the speed of light, t be the time taken by light to go from B to C or A to D or E to G through F, then

$$t = \frac{EF}{c} + \frac{FG}{c}$$

$$t = \frac{AF \sin i}{c} + \frac{FC \sin r}{c}$$

$$t = \frac{AC \sin r + AF (\sin i - \sin r)}{c}$$



AB – Incident wavefront
CD – Reflected wavefront
XY – Reflecting surface

For rays of light from different parts on the incident wavefront, the values of AF are different. But light from different points of the incident wavefront should take the same time to reach the corresponding points on the reflected wavefront.

So, t should not depend upon AF. This is possible only if $\sin i - \sin r = 0$.

i.e. $\sin i = \sin r$ or $i = r$

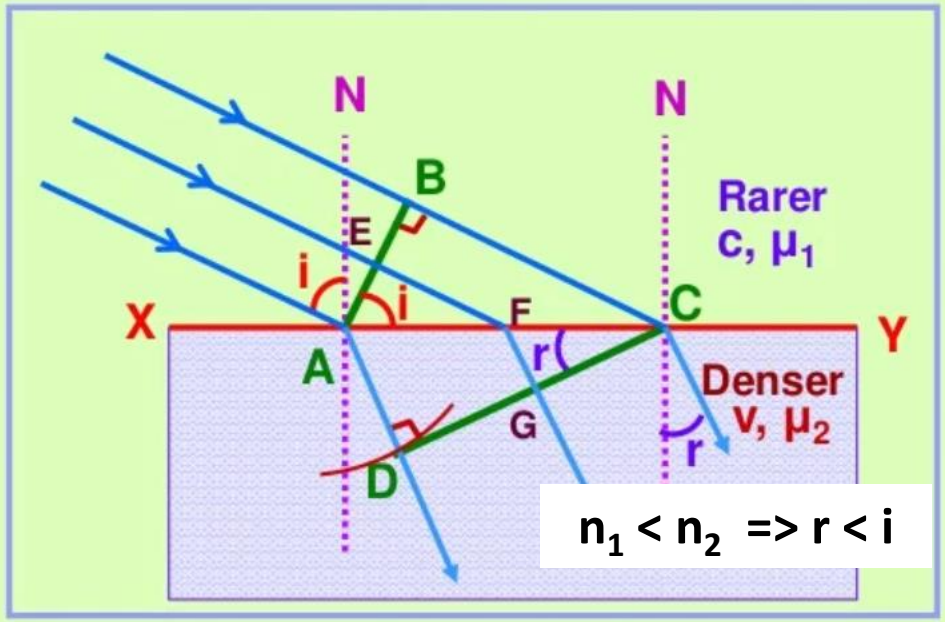
Laws of Refraction at a Plane Surface (On Huygens' Principle):

If c be the speed of light, t be the time taken by light to go from B to C or A to D or E to G through F, then

$$t = \frac{EF}{c} + \frac{FG}{v}$$

$$t = \frac{AF \sin i}{c} + \frac{FC \sin r}{v}$$

$$t = \frac{AC \sin r}{v} + AF\left(\frac{\sin i}{c} - \frac{\sin r}{v}\right)$$



AB – Incident wavefront
CD – Refracted wavefront
XY – Refracting surface

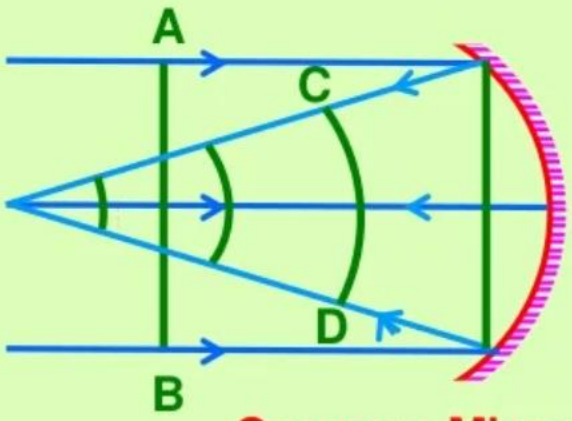
For rays of light from different parts on the incident wavefront, the values of AF are different. But light from different points of the incident wavefront should take the same time to reach the corresponding points on the refracted wavefront.

So, t should not depend upon AF. This is possible only

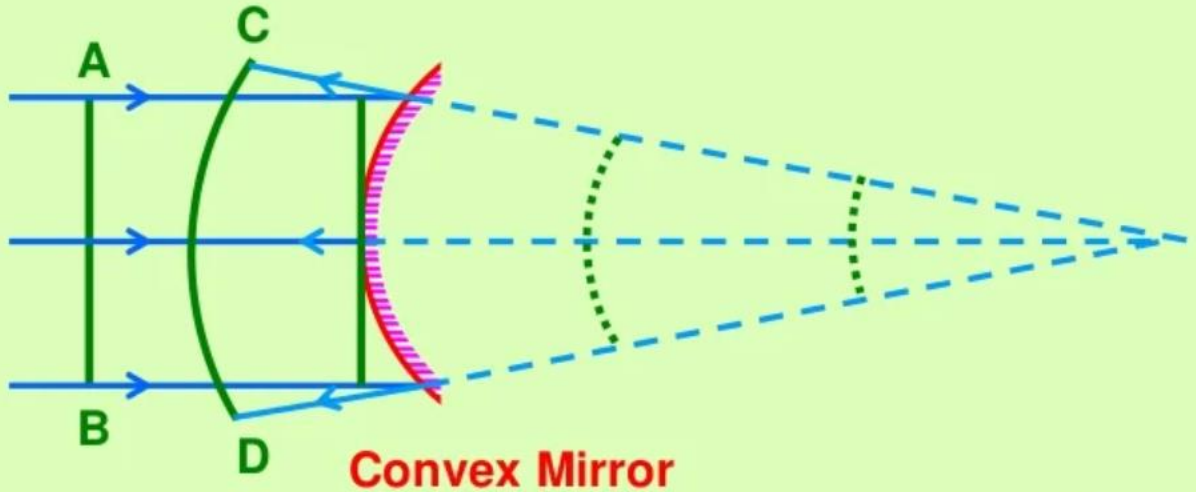
$n_1 \sin i = n_2 \sin r$

if $\frac{\sin i}{c} - \frac{\sin r}{v} = 0$ or $\frac{\sin i}{c} = \frac{\sin r}{v}$ or $\frac{\sin i}{\sin r} = \frac{c}{v} = \mu$

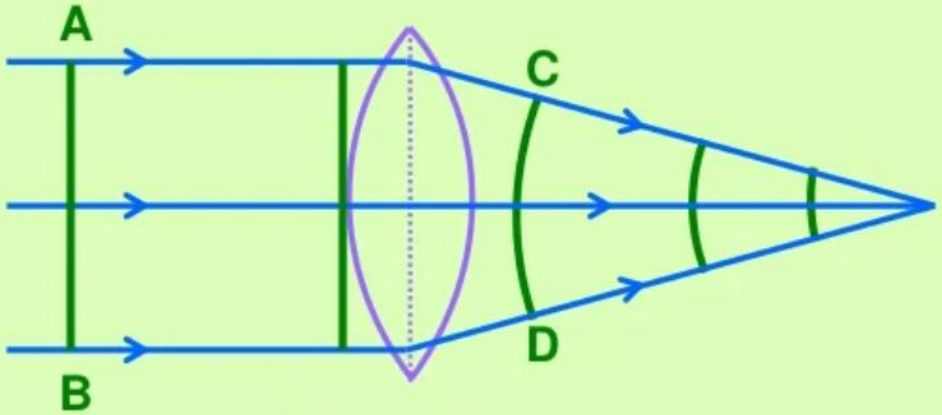
Behaviour of a Plane Wavefront in a Concave Mirror, Convex Mirror, Convex Lens, Concave Lens and Prism:



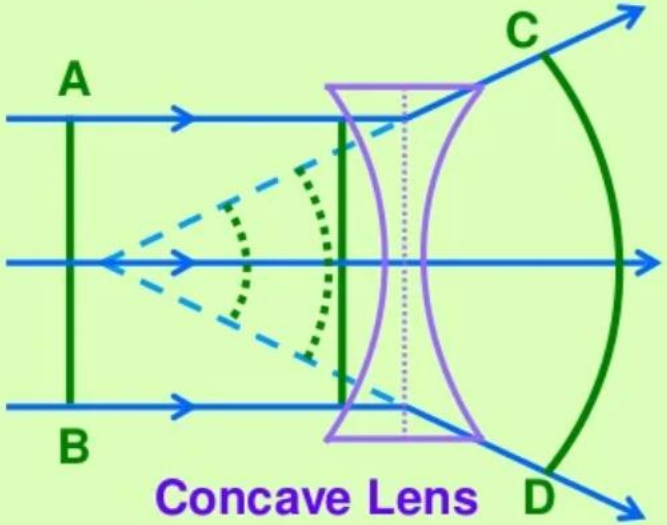
Concave Mirror



Convex Mirror



Convex Lens

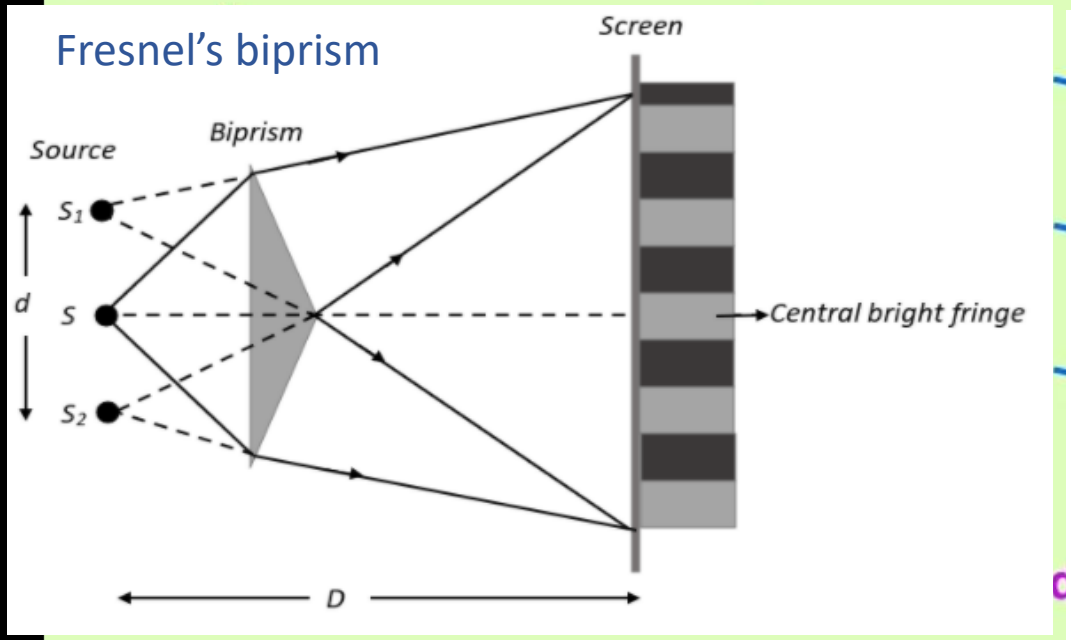


Concave Lens

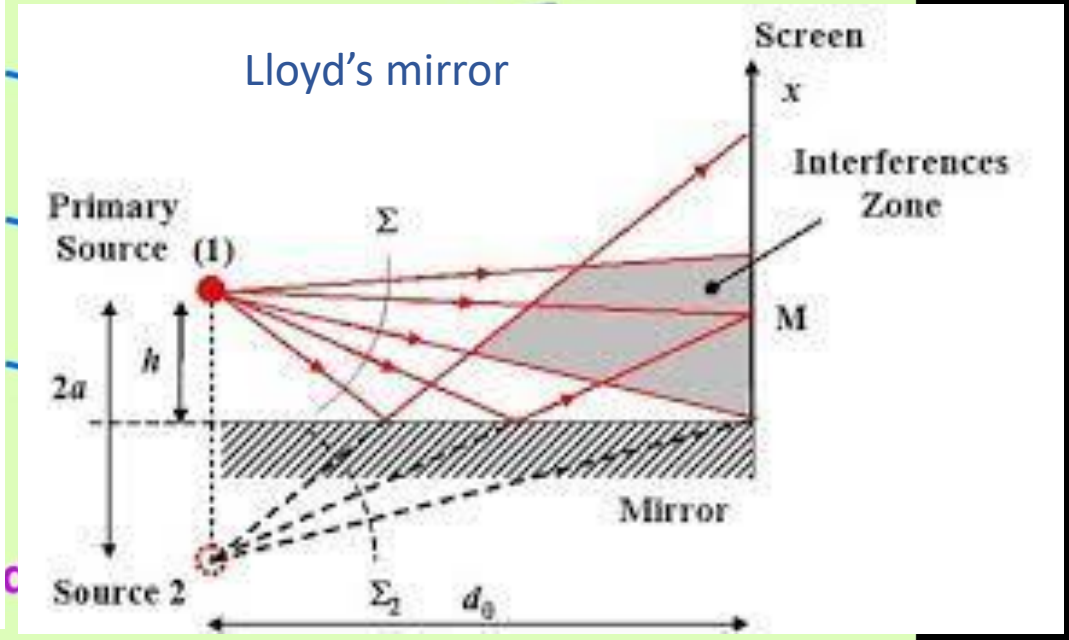
AB – Incident wavefront

CD – Reflected / Refracted wavefront

Fresnel's biprism



Lloyd's mirror

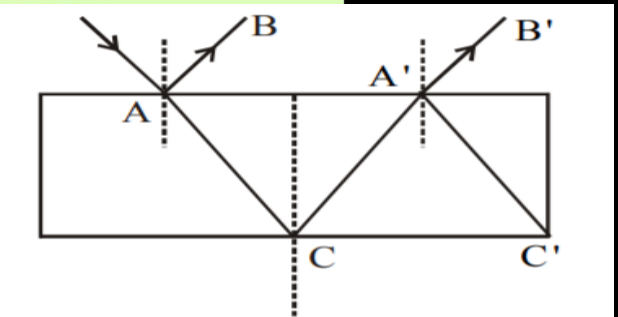


Coherent Sources:

Coherent Sources of light are those sources of light which emit light waves of same wavelength, same frequency and in same phase or having constant phase difference.

Coherent sources can be produced by two methods:

1. **By division of wavefront (Young's Double Slit Experiment, Fresnel's Biprism and Lloyd's Mirror)**
2. **By division of amplitude (Partial reflection or refraction)**



SEE THE EXPLANATION AT THE BLACKBOARD FOR CALCULATING THE INTENSITY DISTRIBUTION OF THE INTERFERECE PATTERN

Theory of Interference of Waves:

$E_1 = a \sin \omega t$

$E_2 = b \sin (\omega t + \Phi)$

The waves are with same speed, wavelength, frequency, time period, nearly equal amplitudes, travelling in the same direction with constant phase difference of Φ.

ω is the angular frequency of the waves, a,b are the amplitudes and E_1, E_2 are the instantaneous values of Electric displacement.

Applying superposition principle, the magnitude of the resultant displacement of the waves is $E = E_1 + E_2$

$$E = a \sin \omega t + b \sin (\omega t + \Phi)$$

$$E = (a + b \cos \Phi) \sin \omega t + b \sin \Phi \cos \omega t$$

Putting $a + b \cos \Phi = A \cos \theta$

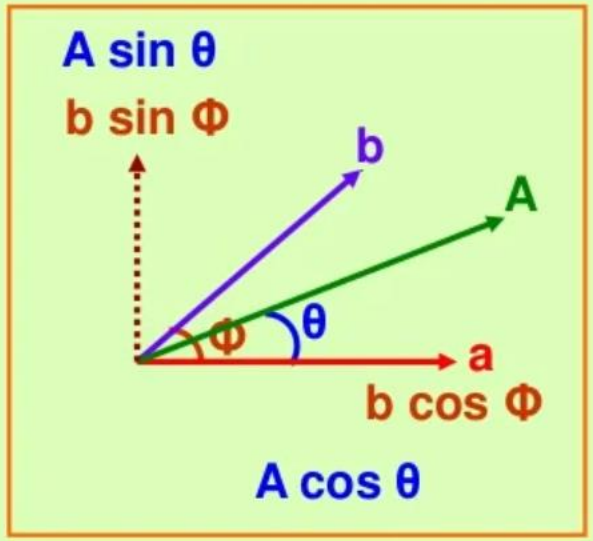
$b \sin \Phi = A \sin \theta$

We get $E = A \sin (\omega t + \theta)$

(where E is the resultant displacement, A is the resultant amplitude and θ is the resultant phase difference)

$$A = \sqrt{(a^2 + b^2 + 2ab \cos \Phi)}$$

$$\tan \theta = \frac{b \sin \Phi}{a + b \cos \Phi}$$



SEE THE EXPLANATION AT THE BLACKBOARD FOR CALCULATING THE INTENSITY DISTRIBUTION OF THE INTERFERECE PATTERN

$$A = \sqrt{(a^2 + b^2 + 2ab \cos \Phi)}$$

Intensity I is proportional to the square of the amplitude of the wave.

So, $I \alpha A^2$ i.e. $I \alpha (a^2 + b^2 + 2ab \cos \Phi)$

Condition for Constructive Interference of Waves:

For constructive interference, I should be maximum which is possible only if $\cos \Phi = +1$.

i.e. $\Phi = 2n\pi$ where n = 0, 1, 2, 3, ...

Corresponding path difference is $\Delta = (\lambda / 2\pi) \times 2n\pi$

$$\Delta = n\lambda$$

$$I_{max} \alpha (a + b)^2$$

Condition for Destructive Interference of Waves:

For destructive interference, I should be minimum which is possible only if $\cos \Phi = -1$.

i.e. $\Phi = (2n + 1)\pi$ where n = 0, 1, 2, 3, ...

Corresponding path difference is $\Delta = (\lambda / 2\pi) \times (2n + 1)\pi$

$$\Delta = (2n + 1)\lambda / 2$$

$$I_{min} \alpha (a - b)^2$$

Comparison of intensities of maxima and minima:

$$I_{max} \alpha (a + b)^2$$

$$I_{min} \alpha (a - b)^2$$

$$\frac{I_{max}}{I_{min}} = \frac{(a + b)^2}{(a - b)^2} = \frac{(a/b + 1)^2}{(a/b - 1)^2}$$

$$\frac{I_{max}}{I_{min}} = \frac{(r + 1)^2}{(r - 1)^2}$$

where r = a / b (ratio of the amplitudes)

Relation between Intensity (I), Amplitude (a) of the wave and Width (w) of the slit:

$$I \alpha a^2$$

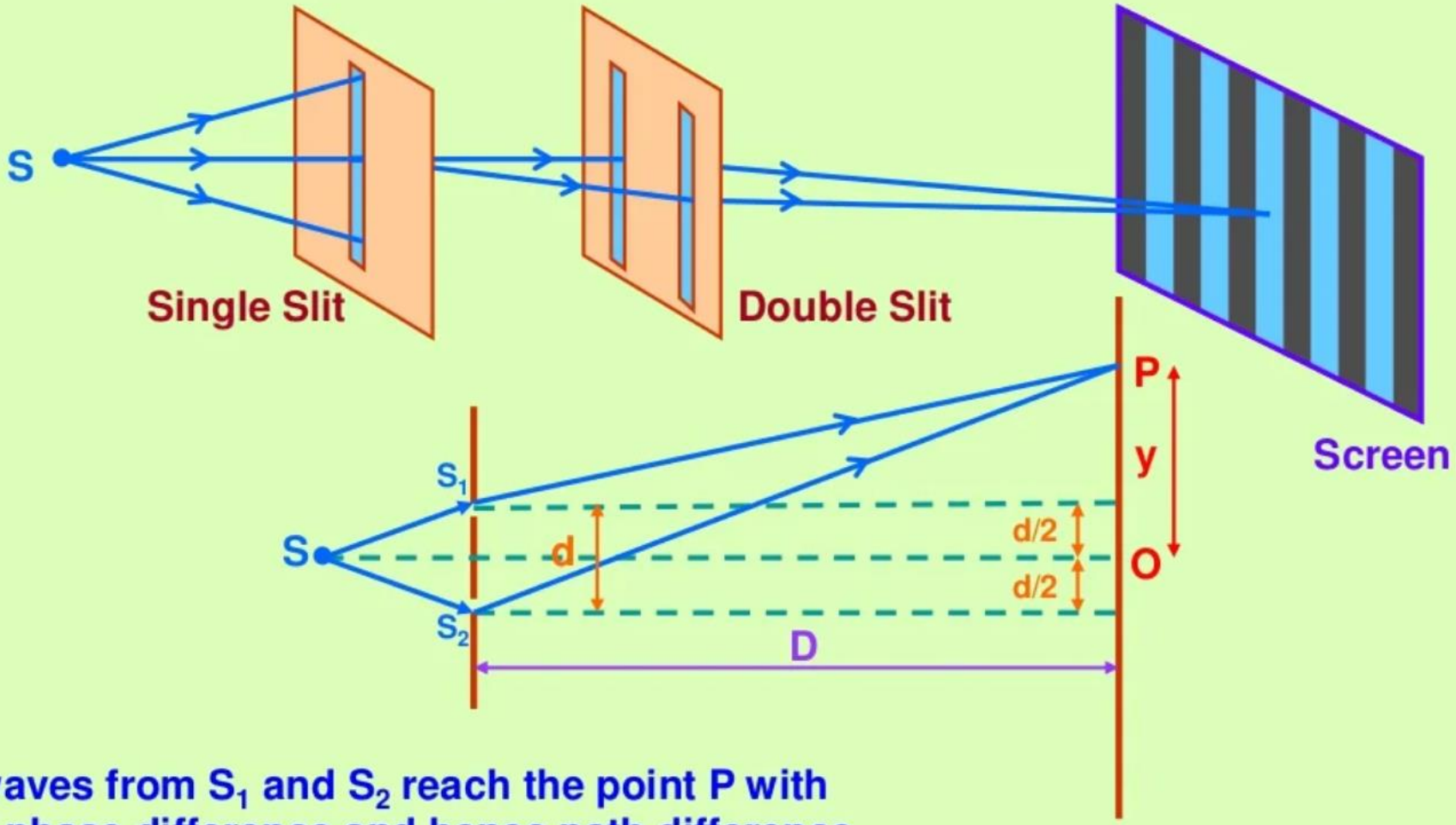
$$a \alpha \sqrt{w}$$

$$\frac{I_1}{I_2} = \frac{(a_1)^2}{(a_2)^2} = \frac{w_1}{w_2}$$

This experiment confirms that light is wave like.

It was also demonstrated using electrons !!!

Young's Double Slit Experiment:



The waves from S_1 and S_2 reach the point P with some phase difference and hence path difference

$\Delta = S_2P - S_1P$

$S_2P^2 - S_1P^2 = [D^2 + \{y + (d/2)\}^2] - [D^2 + \{y - (d/2)\}^2]$

$(S_2P - S_1P)(S_2P + S_1P) = 2\,yd$ $\qquad$ $\Delta\,(2D) = 2\,yd$ $\qquad$ $\Delta = yd / D$

Positions of Bright Fringes:

For a bright fringe at P,

$\Delta = yd / D = n\lambda$

where n = 0, 1, 2, 3, …

$y = n D \lambda / d$

For n = 0, $y_0 = 0$

For n = 1, $y_1 = D \lambda / d$

For n = 2, $y_2 = 2 D \lambda / d$ ……

For n = n, $y_n = n D \lambda / d$

Positions of Dark Fringes:

For a dark fringe at P,

$\Delta = yd / D = (2n+1)\lambda/2$

where n = 0, 1, 2, 3, …

$y = (2n+1) D \lambda / 2d$

For n = 0, $y_0' = D \lambda / 2d$

For n = 1, $y_1' = 3D \lambda / 2d$

For n = 2, $y_2' = 5D \lambda / 2d$ …..

For n = n, $y_n' = (2n+1)D \lambda / 2d$

Expression for Dark Fringe Width:

$\beta_D = y_n - y_{n-1}$

$= n D \lambda / d - (n - 1) D \lambda / d$

$= D \lambda / d$

Expression for Bright Fringe Width:

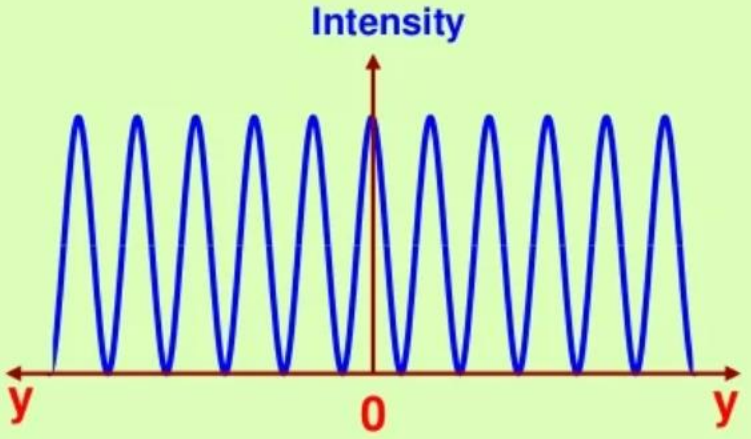
$\beta_B = y_n' - y_{n-1}'$

$= (2n+1) D \lambda / 2d - \{2(n-1)+1\} D \lambda / 2d$

$= D \lambda / d$

The expressions for fringe width show that the fringes are equally spaced on the screen.

Distribution of Intensity:



Suppose the two interfering waves have same amplitude say 'a', then

$I_{max} \alpha (a+a)^2$ i.e. $I_{max} \alpha 4a^2$

All the bright fringes have this same intensity.

$I_{min} = 0$

All the dark fringes have zero intensity.

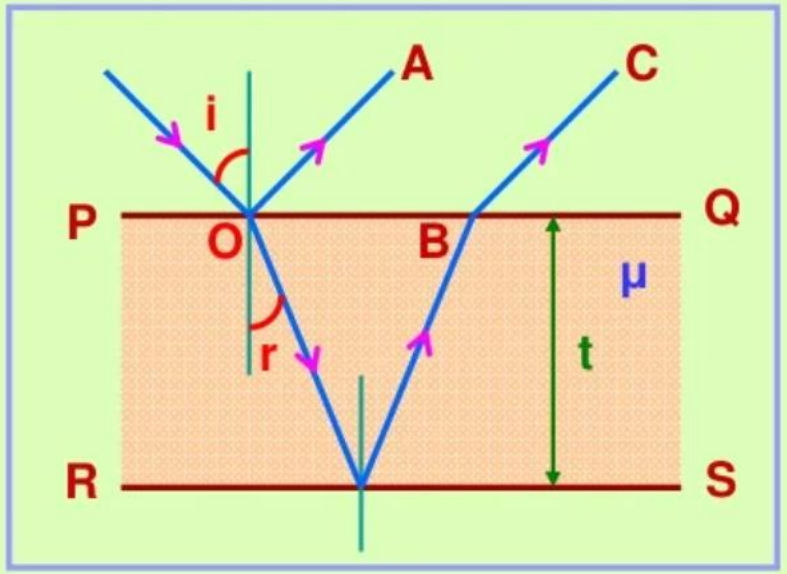
Conditions for sustained interference:

1. The two sources producing interference must be coherent.
2. The two interfering wave trains must have the same plane of polarisation.
3. The two sources must be very close to each other and the pattern must be observed at a larger distance to have sufficient width of the fringe. ($D \lambda / d$)
4. The sources must be monochromatic. Otherwise, the fringes of different colours will overlap.
5. The two waves must be having same amplitude for better contrast between bright and dark fringes.

Colours in Thin Films:

It can be proved that the path difference between the light partially reflected from PQ and that from partially transmitted and then reflected from RS is

$$\Delta = 2\mu t \cos r$$



Since there is a reflection at O, the ray OA suffers an additional phase difference of π and hence the corresponding path difference of λ/2.

For the rays OA and BC to interfere constructively (Bright fringe), the path difference must be $(n + ½)\ \lambda$

So, $2\mu t \cos r = (n + ½)\ \lambda$

For the rays OA and BC to interfere destructively (Dark fringe), the path difference must be $n\lambda$

So, $2\mu t \cos r = n\ \lambda$

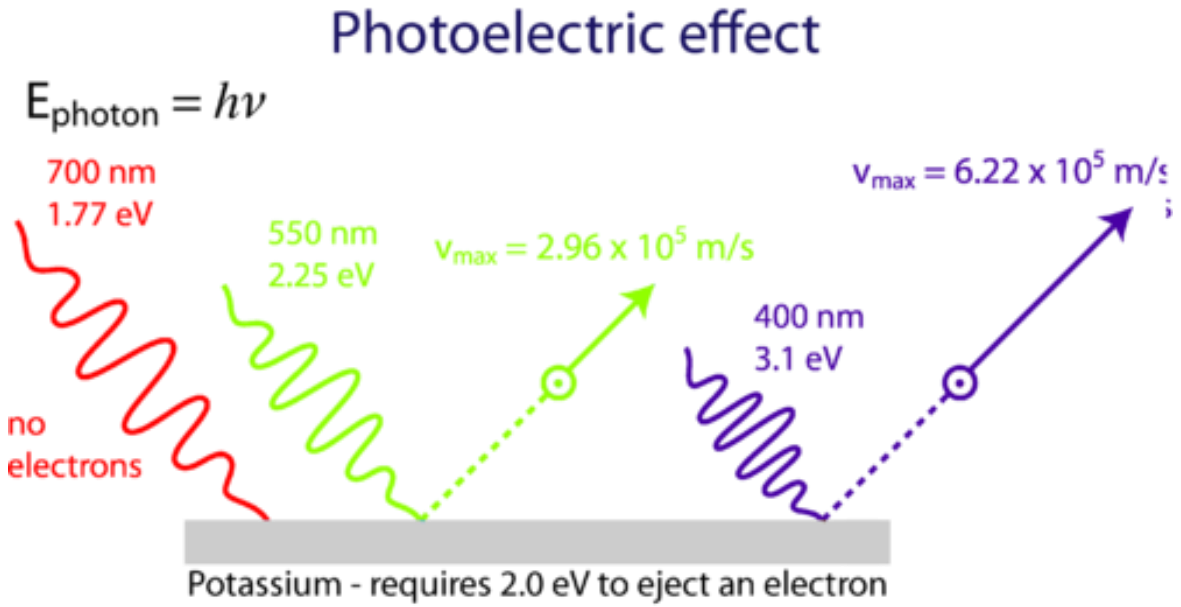
When white light from the sun falls on thin layer of oil spread over water in the rainy season, beautiful rainbow colours are formed due to interference of light.

End of Wave Optics - I

Light/photons as particles

The **photoelectric effect** is the emission of electrons when EM radiation, such as light, hits a material. Electrons emitted in this manner are called photoelectrons.
Classical electromagnetism predicts that continuous light waves transfer energy to electrons, which would then be emitted when they accumulate enough energy. Thus, an alteration in the intensity of light would theoretically change the kinetic energy of the emitted electrons, with sufficiently dim light resulting in a delayed emission. The experimental results instead show that electrons are dislodged **only when the light exceeds a certain frequency**—regardless of the light's intensity or duration of exposure.

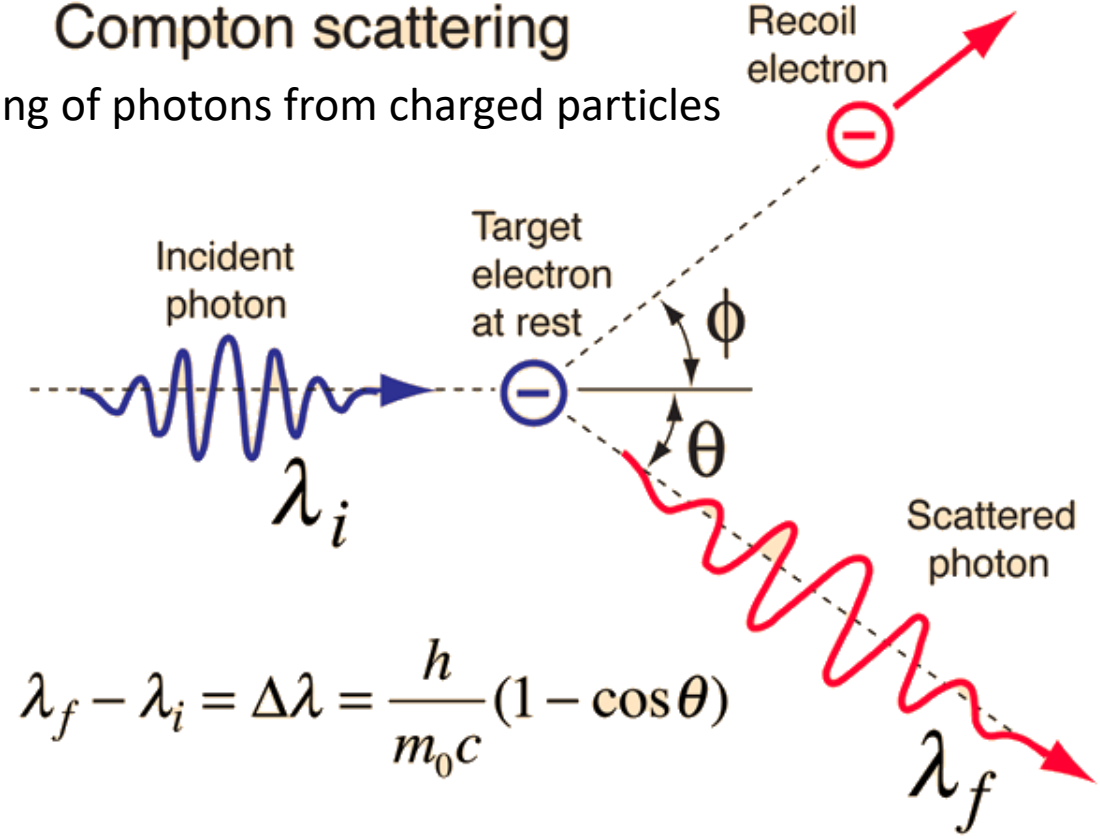
1 eV =
1,60218e-19 J



Because a low-frequency beam at a high intensity does not build up the energy required to produce photoelectrons, as would be the case if light's energy accumulated over time from a continuous wave, **Albert Einstein proposed that a beam of light is not a wave propagating through space, but a swarm of discrete energy packets, known as photons.**

Compton scattering

The scattering of photons from charged particles



Compton scattering interaction occurs with essentially unbound electrons, with transfer of energy shared between recoil electron and scattered photon, with energy exchange.

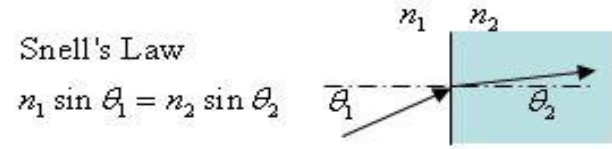
Compton scattering, discovered by Arthur Holly Compton, is the scattering of a high frequency photon after an interaction with a stationary charged particle, usually an electron. If it results in a decrease in energy (increase in wavelength) of the photon (which may be an X-ray or gamma ray photon), it is called the Compton effect.

http://hyperphysics.phy-astr.gsu.edu/

Optics

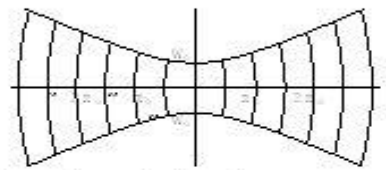
Ray Optics (Geometrical Optics)

- Focus on **location** & **direction** of light rays
- Limit of Wave Optics where $\lambda \to 0$



Wave Optics (Gaussian Beam)

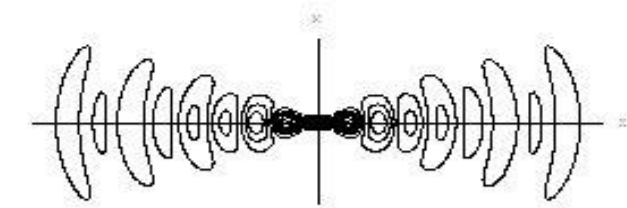
- Scalar wave theory
  (Single scalar wavefunction describes light)



$$U(\mathbf{r}) = A\frac{W_0}{W(z)}\exp\left[-\frac{\rho^2}{W^2(z)}\right]\exp\left[-jkz - jk\frac{\rho^2}{2R(z)} + j\zeta(z)\right]$$

E&M Optics (Geometrical Optics)

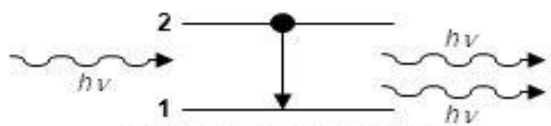
- Two mutually coupled vector waves (**E** & **M**)



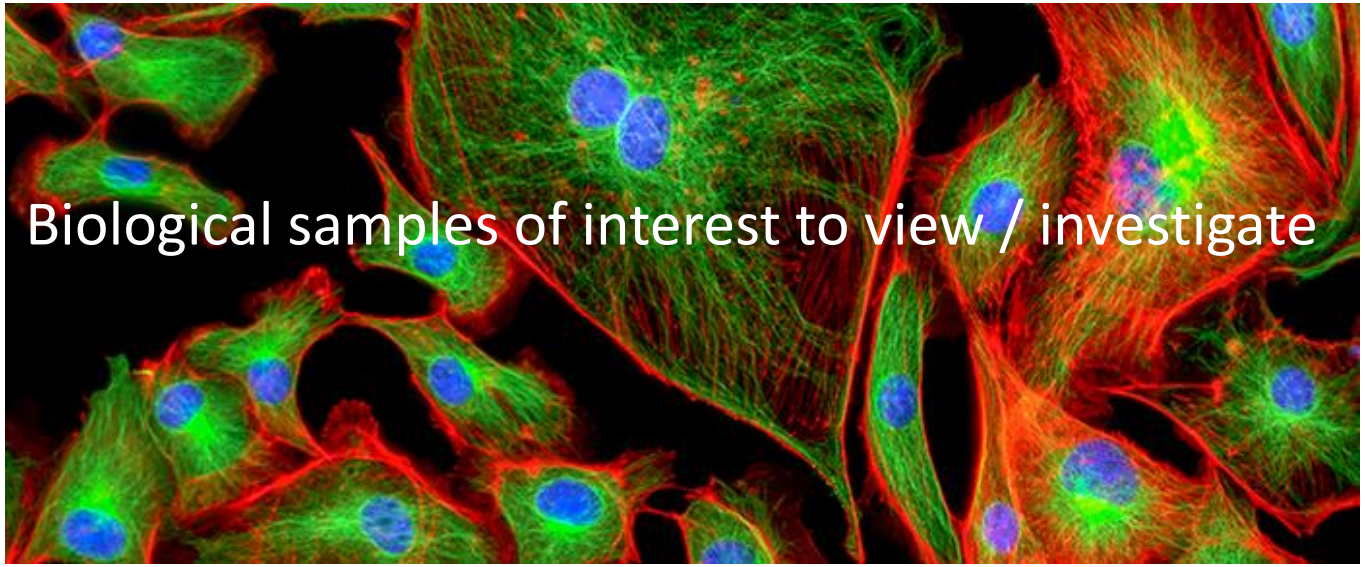
E-field of Gaussian Beam

Quantum Optics (Photon Optics)

- Describes certain optical phenomena that are characteristically quantum mechanical



Stimulated Emission



The microscopy techniques

The microscopy techniques used for biological sample rely on different physical principles but mostly are explained by Ray Optics and Wave Optics