MICRO-562

Biomicroscopy II

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TA:
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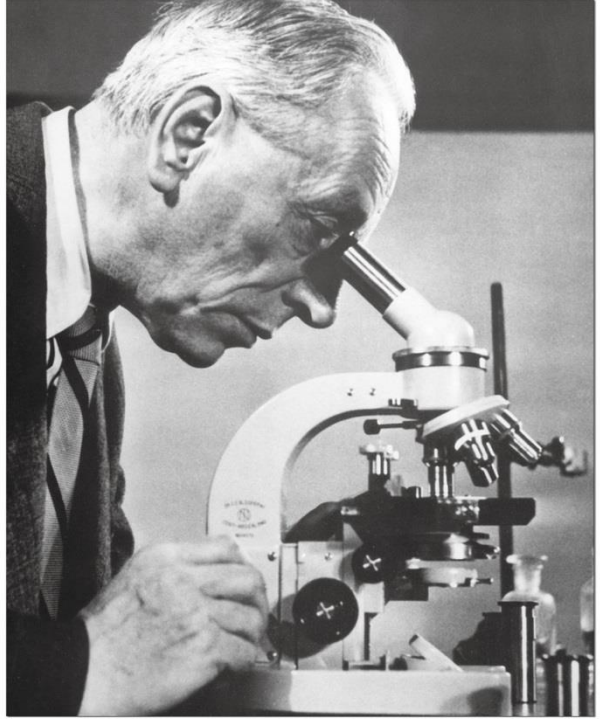
Biomicroscopy-II Syllabus – (TENTATIVE)

Lecture 1	Course	Brief Review, Dark-Field
Lecture 2	Course	Phase Contrast & Polarization, Birefringence
Lecture 3	Course	DIC & Fluorescence Microscopy Techniques
Lecture 4	Course	Introduction to Confocal Microscopy
Lecture 5	Course	Advanced Microscopy-I
Lecture 6	Course	Advanced Microscopy-II
Lecture 7	TP	Practical: Intro to Wide-Field Microscopy
Lecture 8	TP	Practical: Transmission Techniques
Lecture 9	TP	Practical: Intro to Fluorescence
Lecture X	**EASTER**	
Lecture 10	TP	Practical: Intro to Confocal
Lecture 11	TP	Practical: Fluo/Conf
Lecture 12	TP	Practical: Fluo/Conf
Lecture 13	**EXAM**	
Lecture X	**HOLIDAY**	

Contrast in microscopy

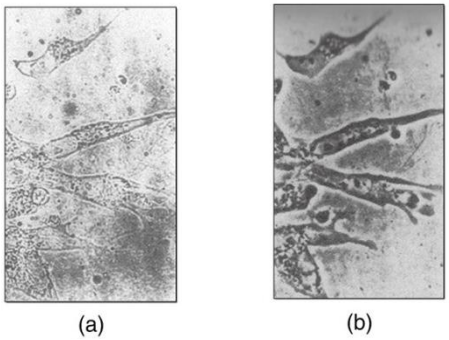
- For transparent specimens, adjust the contrast level:
  - **Bright field microscope** (low contrast)
  - **Stained specimen**
  - **Dark field microscopy**
  - **Phase contrast microscope**
  - Polarization microscopy
  - Differential Interference Contrast (DIC) microscopy
  - Fluorescence microscopy

Reminder:Phase-Contrast Microscopy



In the 1930s, Frits Zernike, a Dutch physicist created an optical design that could transform phase differences to amplitude differences, thus making objects appear as if they had been "optically" stained without any dye and label.

➔ **"Label-free" "high-contrast" microscopy**



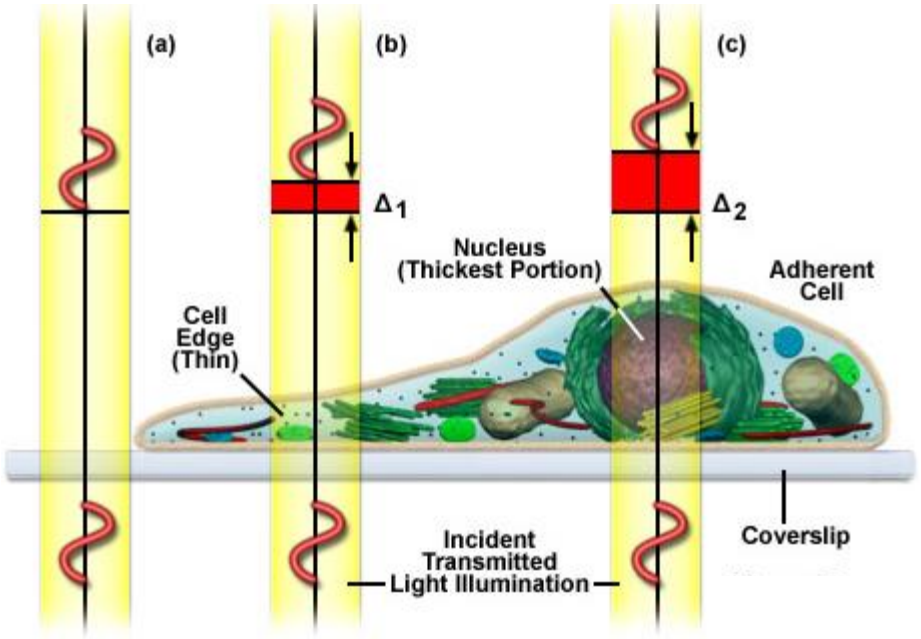
Living tissue culture cells as seen with
(a) bright-field
(b) phase contrast

- The Zeiss in Jena introduced **phase contrast objectives & accessories** in 1942, which transformed research in biology and medicine.
- The development of phase contrast microscopy is a brilliant example of how basic research in optics leads to a practical solution for viewing unstained transparent samples.
- For his invention and theory of image formation, Zernike won the Nobel Prize in Physics in 1953.

Reminder: Phase in Microscopy

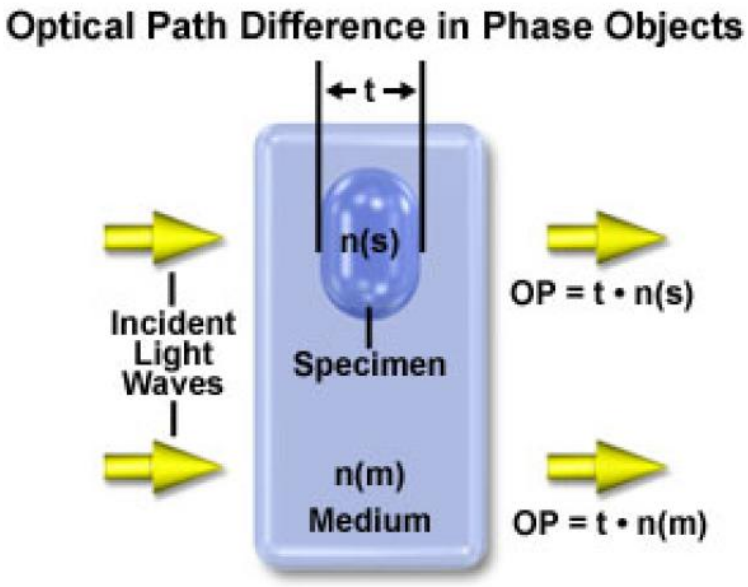
- Cells have higher refractive index (n) than water.
- In higher (n) medium (i.e. cells):
  - Light moves slower (v=c/n)
  - Light has shorter wavelength ($\lambda = \lambda_o/n$)
  - Light is delayed wrt free space

    ➔ **phase-retarded (φ)**



How to exploit this for optical imaging?

Reminder: Optical Path Length and Phase Shift



Optical Path :

$$OP = t \times n$$

Phase shift due to *Optical Path Difference*:

$$\delta = \frac{2\pi}{\lambda}\Delta(OP) = \frac{2\pi}{\lambda}\Delta(OP_s - OP_m)$$

$$= \frac{2\pi}{\lambda}t(n_s - n_m)$$

- ***t*** is the sample thickness → Typical cell in monolayer is ~5μm
- n_s is the refractive index of sample → Most cell has index ~1.36
- n_m is the refractive index of medium → Cell medium index is ~1.335

Calculate optical path difference: Ans: ~125 nm

For visible light illumination with λ_{green} ~ 500 nm

- What is the ratio of optical path difference to wavelength ? Ans: $\lambda/4$
- What is the amount of phase shift ? Ans: $\pi/2$ or 90^o

Phase in Bright-Field Microscopy

Upon transit through a phase object, the transmitted beam is divided into two components:

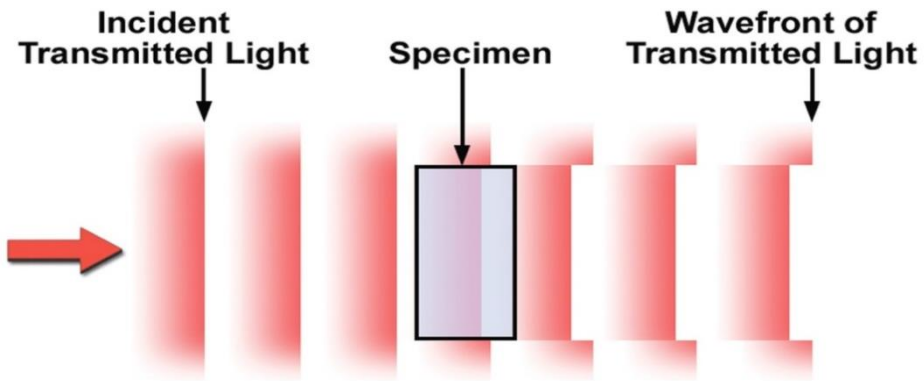
1) Undeviated (0^{th} order) wave or **surround wave (S wave)** that does NOT interact with the object
2) Deviated or **diffracted wave (D wave)** that gets scattered in different directions.

- Typically only a minority of incident waves are diffracted by a biological object (i.e. cell).
- In this notation, surround wave represents "background".

3) Both **S** wave and **D** wave are collected by the objective and focused in the image plane, where they ***interfere*** and generate the resultant **particle wave (P wave)** to form the image of the object**.**

- Thus, the relationship between these three waves are described as:

$$\vec{P} = \vec{D} + \vec{S}$$



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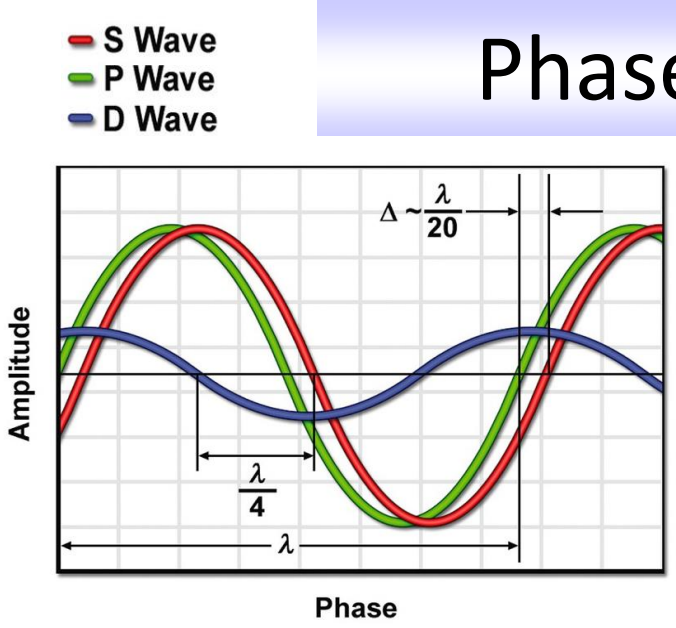
→ **Detection** of the object depends on the **intensities**, hence on the **amplitudes** of the **P wave** & **S** wave.

→ **Only when the amplitudes of the P wave (i.e. object signal) and S wave (i.e. background signal) are significantly different in the image plane, then we can see the object by the microscope.**

Phase in Bright-Field Microscopy



S = Surrounding (background)
D = Diffracted
P = Particle

(P= S + D) ~ (S)

Here, **S wave**, **D wave** and **P wave** are represented as ***sine waves*** of a given λ in the image plane (for simplicity).

- **S wave** and **P wave**, whose relative intensity determines the visual contrast, are shown in **red** & **green**.
- **D wave**, which is not directly observed in the image is shown in **blue**.

→ **D wave** is lower in amplitude than the **S wave**, because diffracted light is minority.
→ Let's assume **D wave** is "**retarded**" in phase by λ/4 relative to the **S wave** due to its interaction with the object.

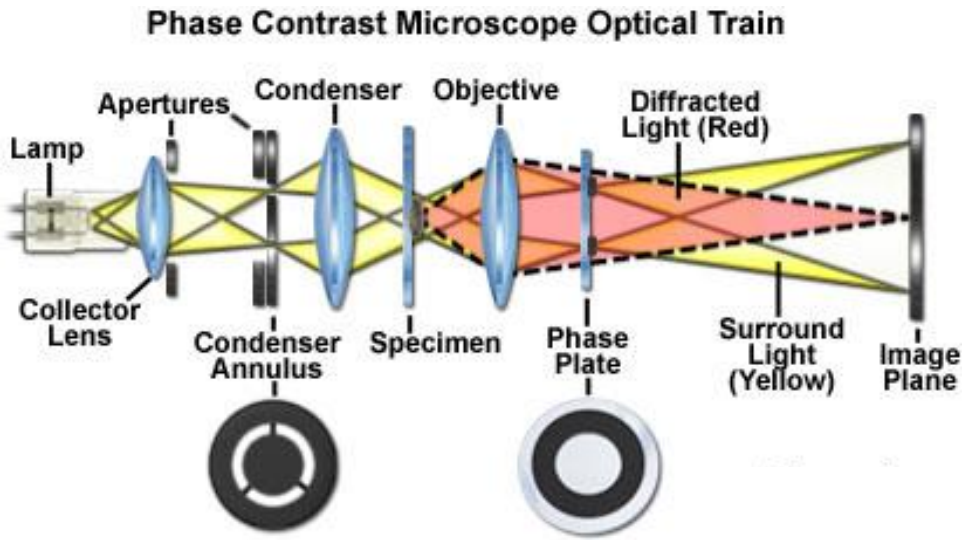
- The **P wave** resulting from the interference between the **D wave** and **S wave** is "**retarded**" relative to the **S wave** by only a small amount (in this example we used λ/20) and **P wave** has an amplitude similar to that of the **S** wave.

→ Since the **S wave** and **P wave** have nearly same amplitude, in regular bright-field microscope there will be no contrast in the image, and the object will be invisible.

Key Concepts of Phase-Contrast Microscopy

Step 1) First, spatially isolate the surround wave (**S**) & diffracted wave (**D**) emerging from sample plane so that they occupy different locations in the diffraction plane at the rear aperture of the objective.

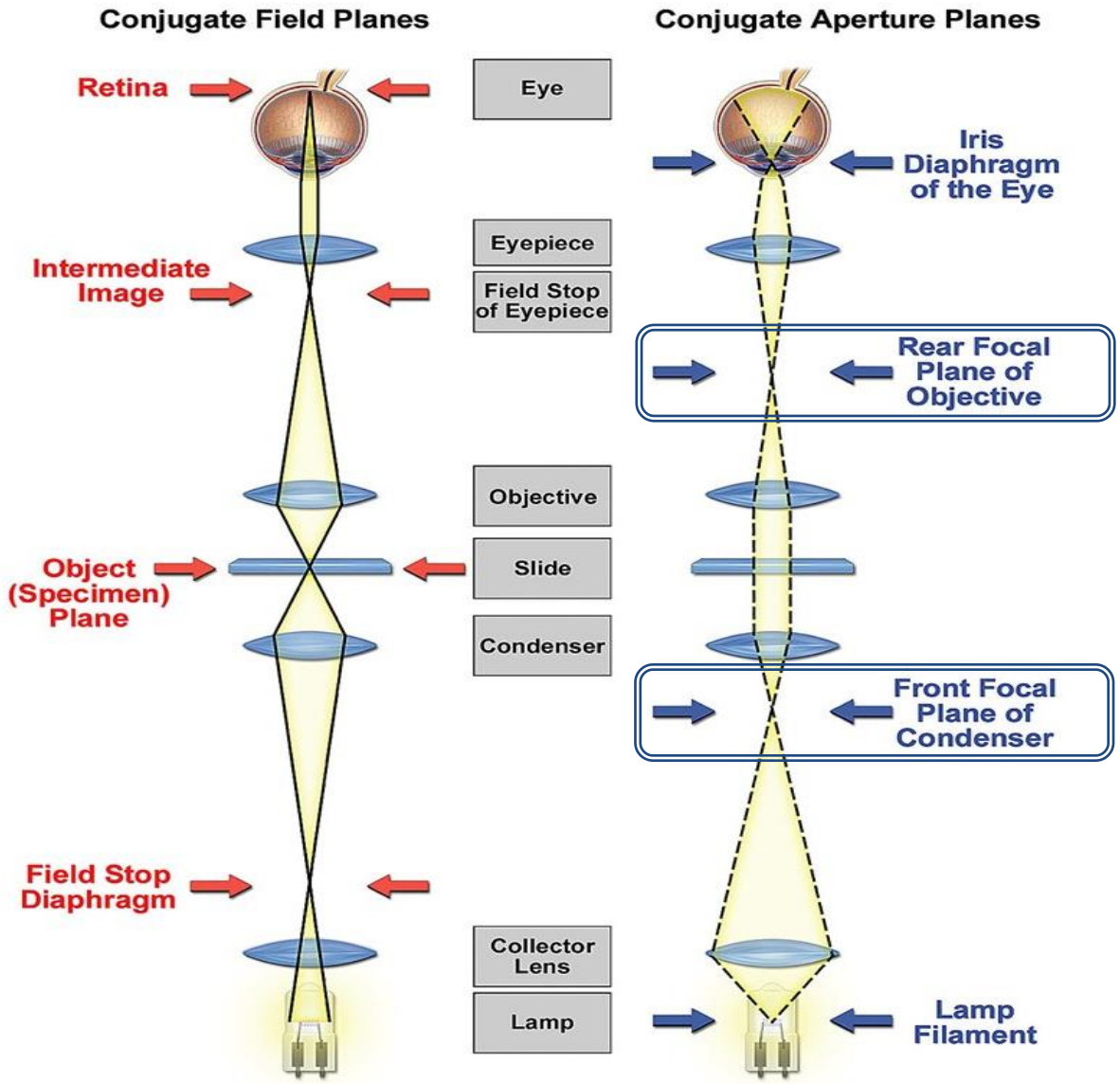
Step 2) Then, advance the phase and reduce the amplitude of the surround wave (**S**) in order to maximize the difference in the amplitude between the **P** wave and **S** wave.



To implement step 1 use a **condenser annulus**, an opaque black plate with a transparent annulus, positioned at the front focal plane of the condenser.

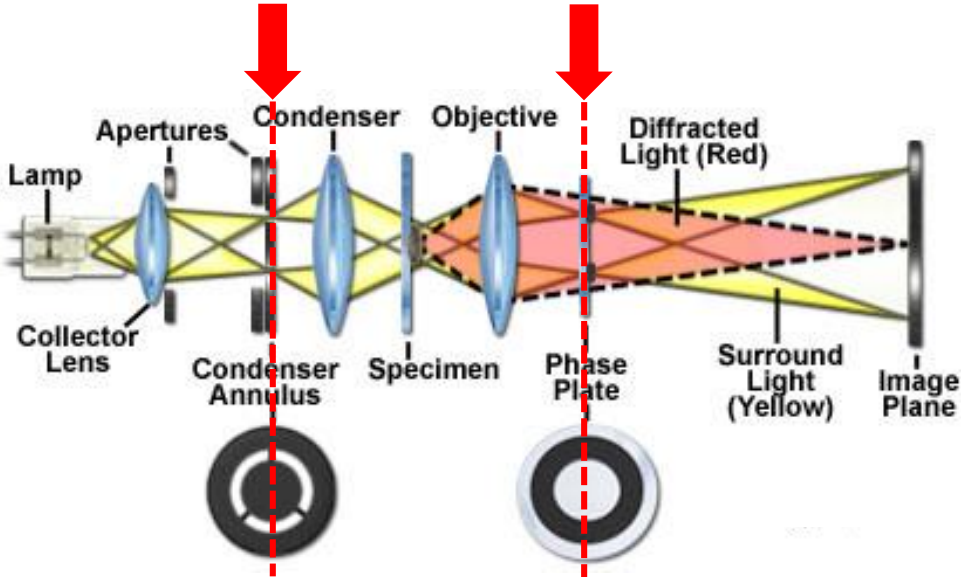
Remember: *Under Kohler illumination the condenser's front focal plane is conjugate to the objective's rear focal plane.*

Reminder: Bright-Field Kohler Illumination



Step-1 of Phase-Contrast Microscopy

Step 1) Isolate the surround wave (**S**) and diffracted wave (**D**) emerging from sample plane so that they occupy different locations in the diffraction plane at the rear aperture of the objective



For step 1: Use a **condenser annulus**, an opaque black plate with a transparent annulus, positioned at the front focal plane of the condenser).

Given that condenser's front focal plane is conjugate to the objective's rear focal plane:

- **S-wave** (indicated above by **yellow shading**) that do NOT interact with the specimen are focused as a bright ring (corresponding to a bright image of the condenser annulus) at the rear focal plane of the objective (a.k.a. diffraction plane of the objective).
- On the other hand, **D-wave** (indicated by **red shading**) diffracted by specimen cover the entire rear focal plane.

1st key concept is achieved:

➔ **Surround wave (S) and diffracted wave (D) are spatially separated at the rear focal plane!**

This can allow us to selectively manipulate the phase of the either waves.

Step-2 of Phase-Contrast Microscopy

Step 2) Advance the phase and reduce the amplitude of the surround wave (**S**) wrt diffracted wave (**D**) in order to maximize the difference in the amplitude between the **P wave** and **S wave**.

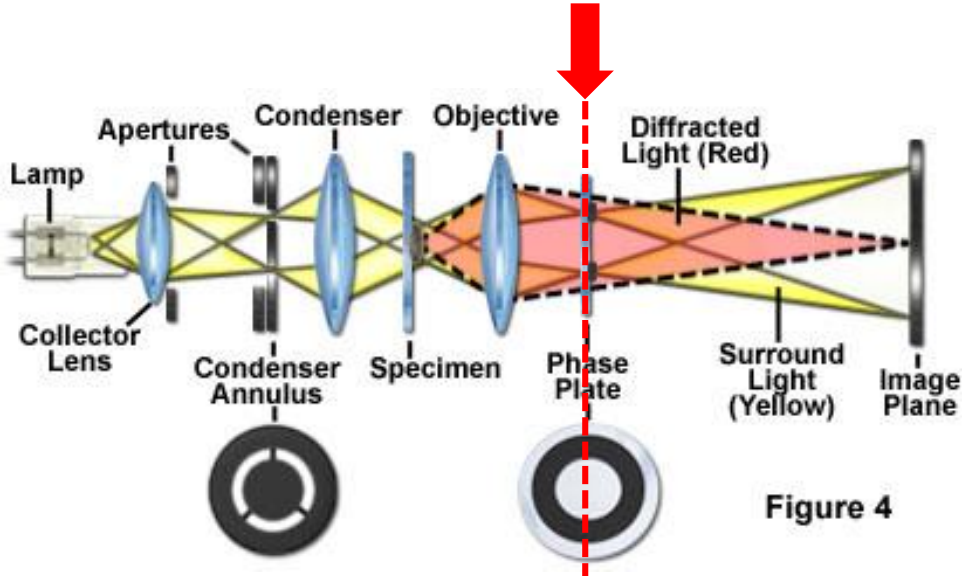
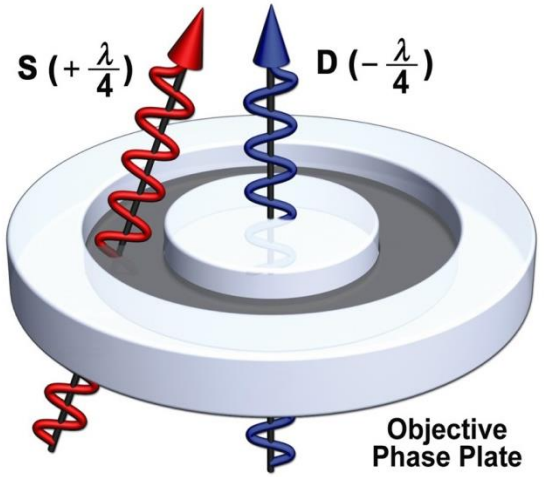


Figure 4



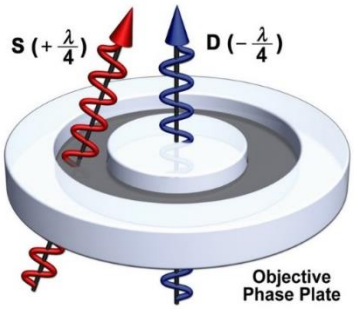
For step-2: Insert a **phase plate** at the rear focal plane of the objective, in order to selectively alter the phase & amplitude of the **S-wave** wrt **D-wave**.

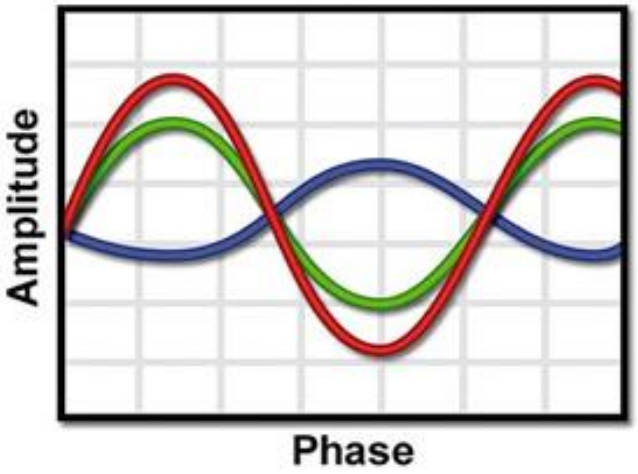
- As an example:
  - The phase plate can be a glass slab with an etched ring of reduced thickness to selectively "***advance***" the phase of the **S-wave** by λ/4.
  - The etched ring can also be coated with a partially absorbing film to reduce the amplitude of **S-wave** for a higher contrast.

- As we have mentioned before, **D-wave** has been assumed to be "***retarded***" by **λ/4** by the object.

Phase-Contrast Microscopy

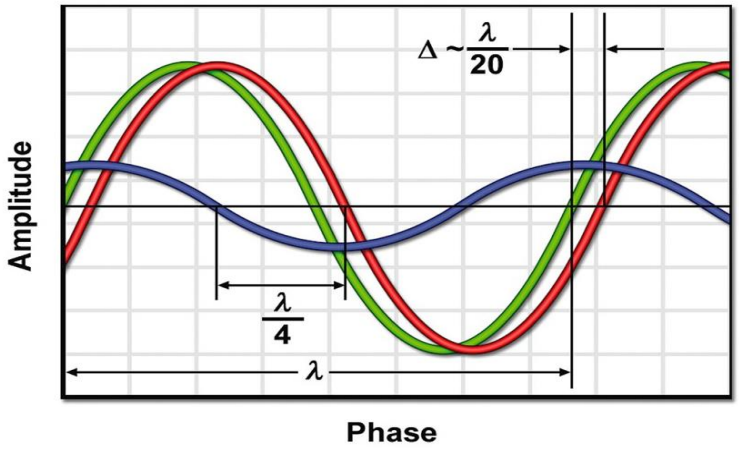
Positive phase plate allows to selectively **"advance"** the phase of the **S-wave** relative to that of the **D-wave**.





Compare to the conventional bright-field microscopy scheme:

P ~ S



- **Key concept 2 (using a positive phase plate):** With the "**advancement**" of **S-wave**, the **net phase shift between S wave & D wave is λ/2 = +λ/4 – (–λ/4)**.

- **Their destructive interference gives an amplitude contrast between S-wave and the resulting P-wave ➔ allowing contrast (thus visibility) of the P-wave w.r.t. S-wave**

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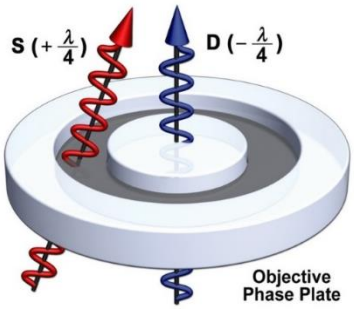


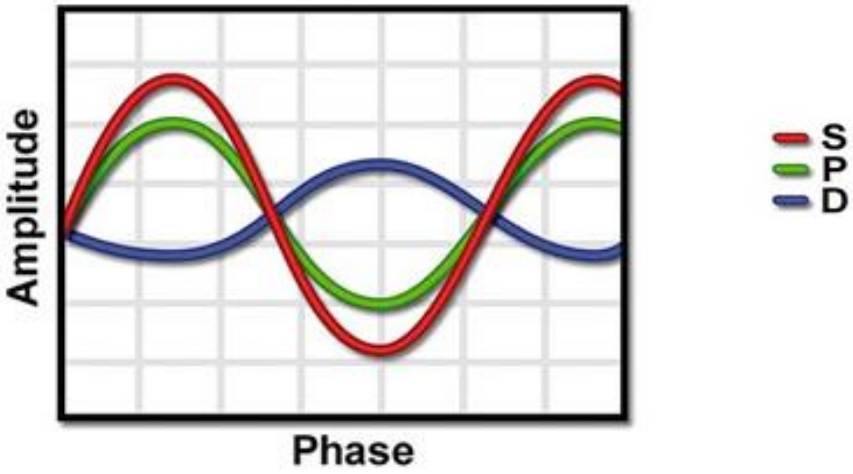
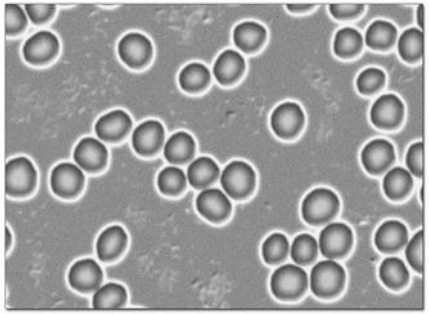


Image effect in phase contrast microscopy:



Erythrocytes with positive phase contrast

- Objects having a higher refractive index that the surrounding medium appears dark.
- Objects having a lower refractive index than the surrounding medium appear bright.

- **Key concept 2 (using a positive phase plate):** With the "**advancement**" of **S-wave**, the **net phase shift between S wave & D wave is λ/2 = +λ/4 – (–λ/4)**.

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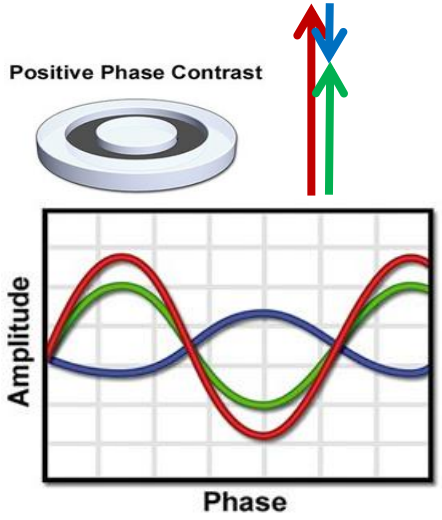
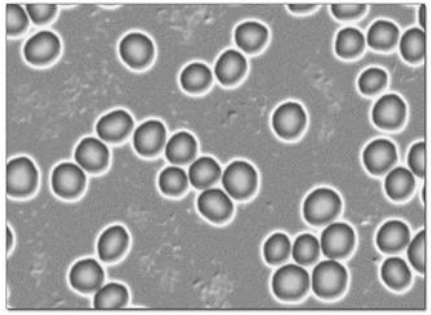
Phase-Contrast Microscopy



<u>Positive phase contrast</u> allows to selectively "<u>advance</u>" the phase of the S-wave relative to that of the D-wave.

- Objects having a *higher* refractive index that the surrounding medium appears *dark*.
- Objects having a lower refractive index than the surrounding medium appear bright.



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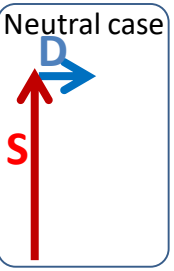
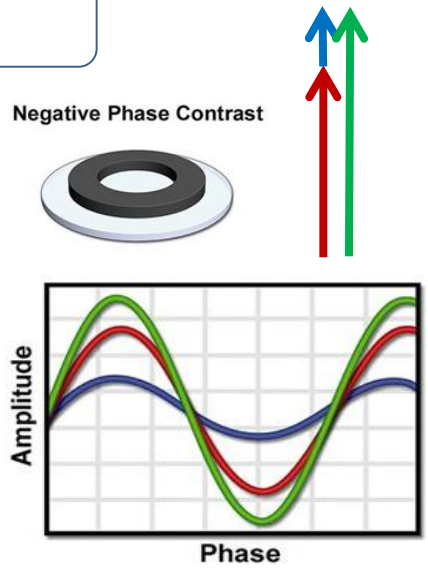


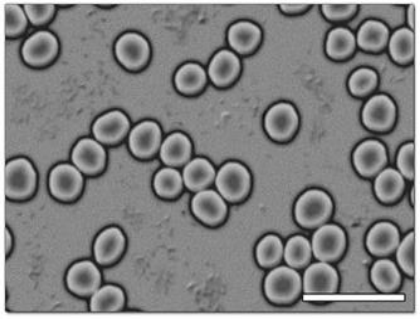
total= $+\lambda/4 - (-\lambda/4) = \lambda/2$ ➔ "destructive"

Phase contrast differentially enhances the contrast of the edges!



<u>Negative phase contrast</u> allows to selectively "<u>retard</u>" the phase of the S-wave relative to that of the D-wave.

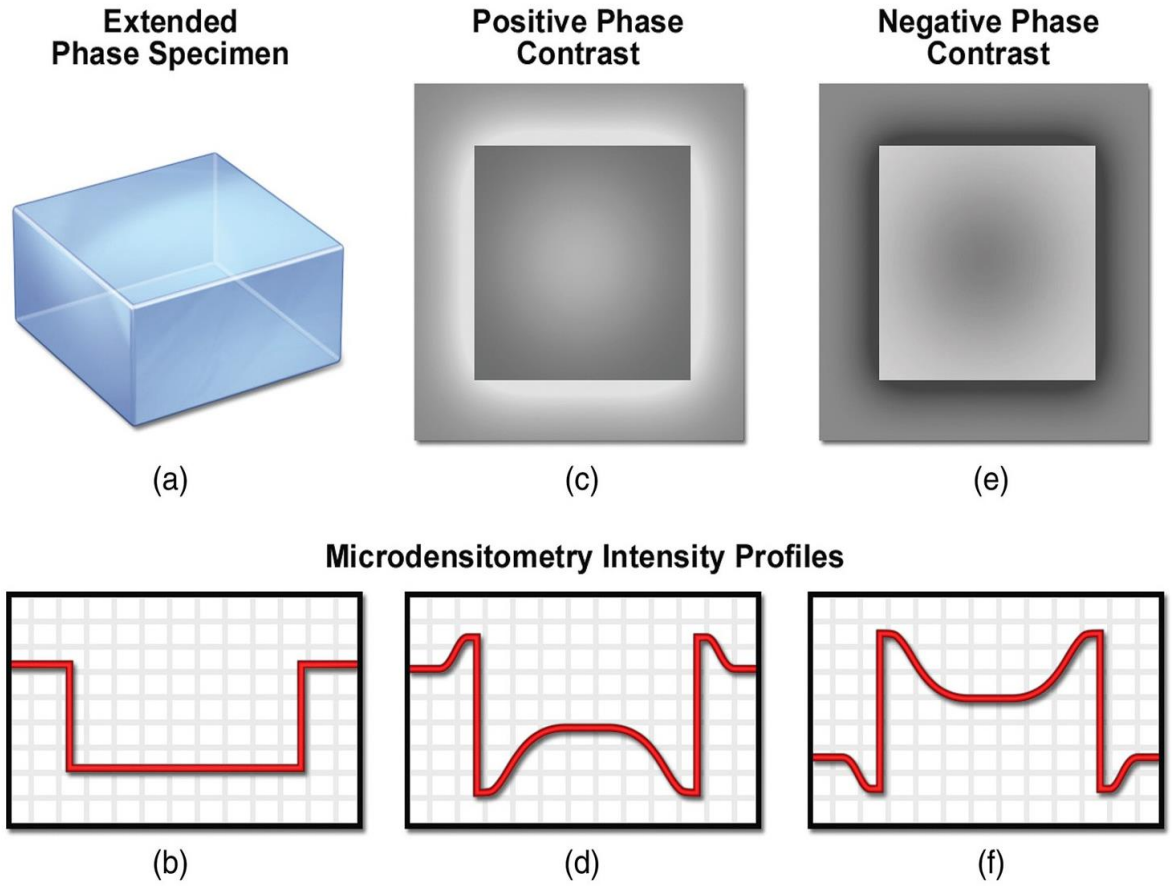
- Objects having a *higher* refractive index that the surrounding medium appears *bright*.



Erythrocytes with negative phase contrast

total= $-\lambda/4 - (-\lambda/4) = 0$ ➔ "constructive"

Interpreting images in phase-contrast microscopy

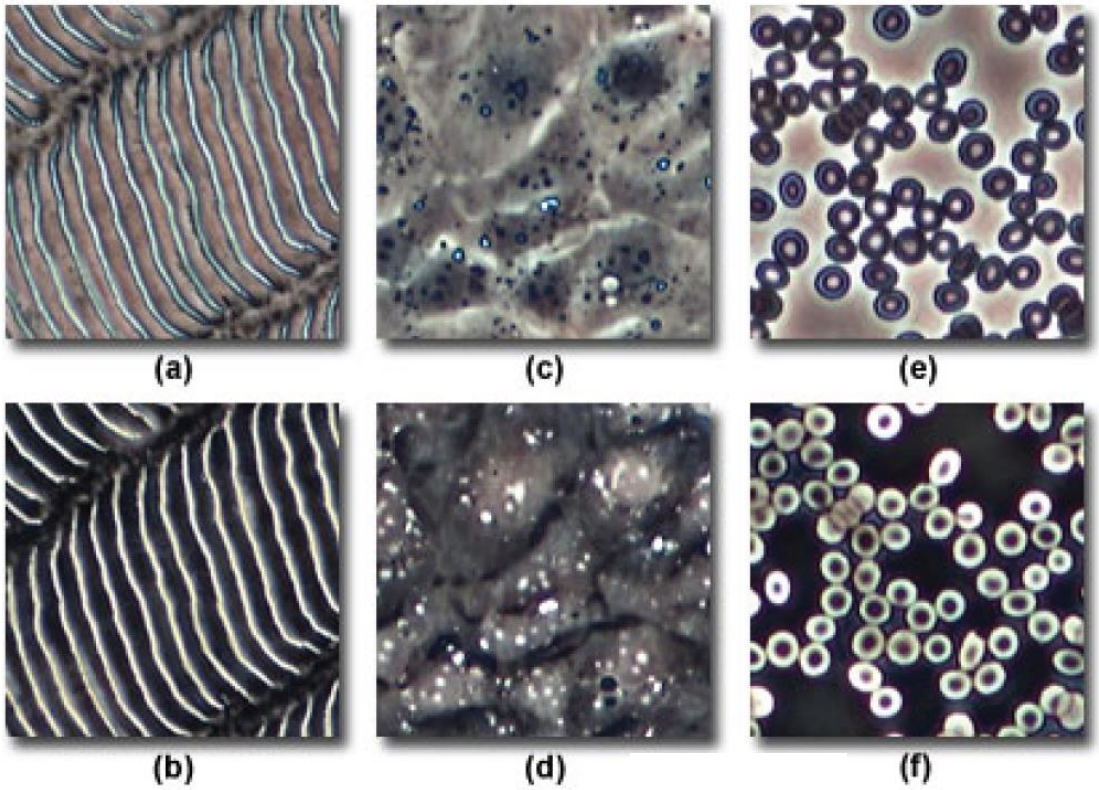


The effects of shade-off & halos

Shade-off: image of a phase object with constant optical path length does not necessarily appear uniformly dark or light. If the object is large enough, the light intensity in the center may reach to that of the surrounding.

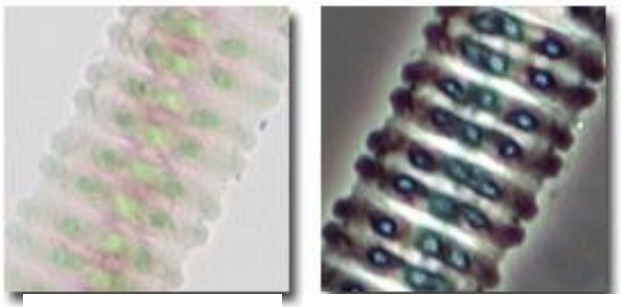
Phase halos: always surround phase objects and may appear dark or light.

Example Images with Phase-Contrast Microscopy



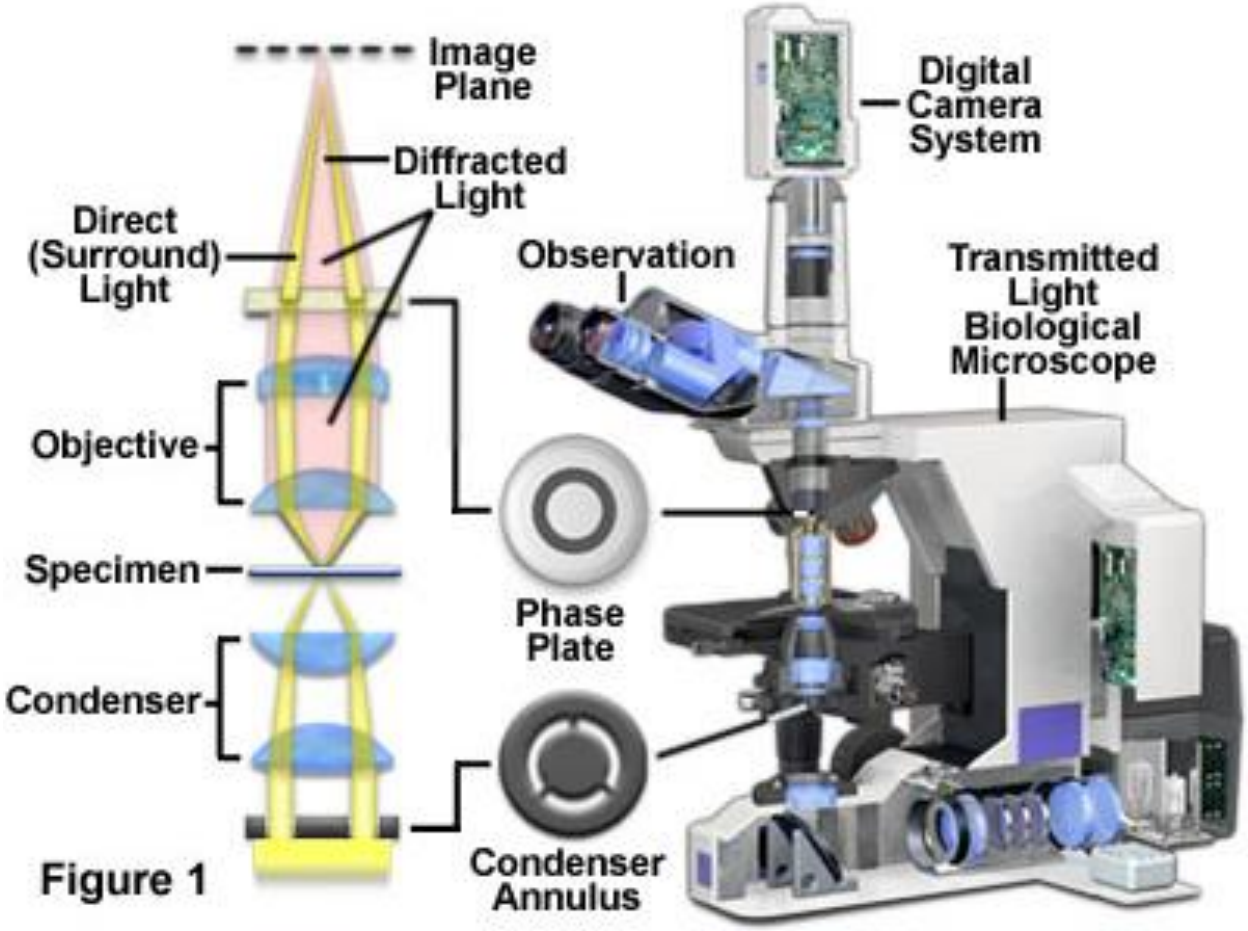
Positive phase contrast

Negative phase contrast



Bright-field Phase-contrast

Phase-Contrast Microscopy Implementation



1. Condenser annulus: this accessory replaces the variable diaphragm in front of the condenser

2. Phase Plate (phase contrast ring): This accessory can be in the form of a glass plate and mounted in or near the rear focal plane of the objective. In some cases, the lens surface at this plane is etched by acid, resulting in a modified objective that is different than regular objectives.

Alignment of Phase-Contrast Microscopy

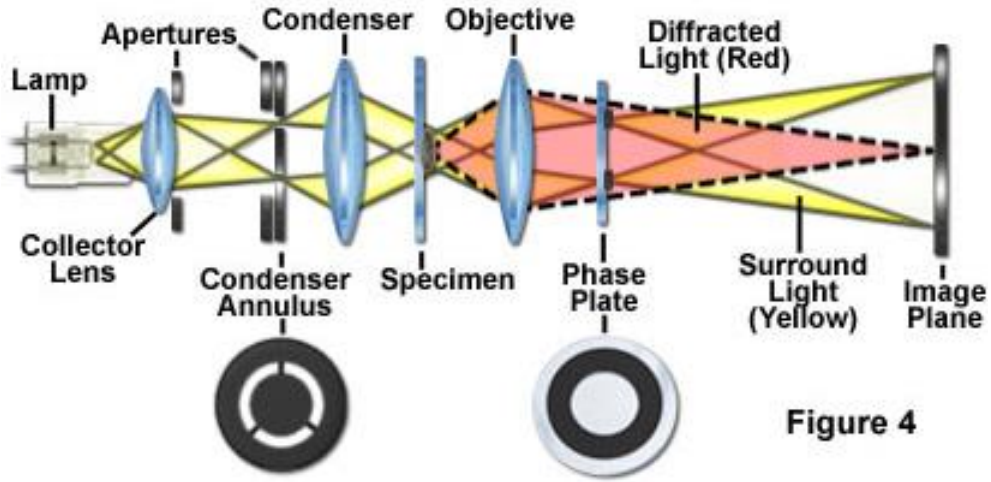
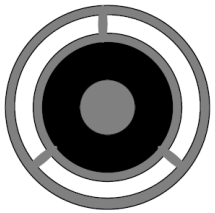
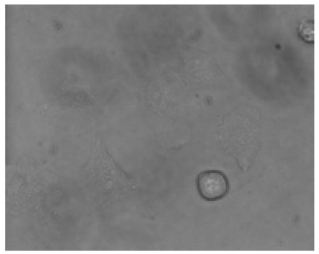


Figure 4

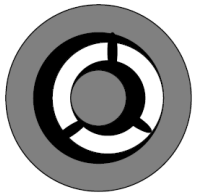
- Correct distance needs to be adjusted.
  (much strict alignment than Kohler Illumination, especially at high magnification)
- Phase contrast ring of the objective must match to the illumination ring of the condenser.

Wrong phase contrast ring

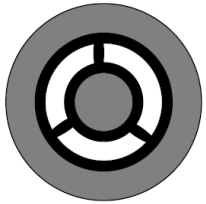


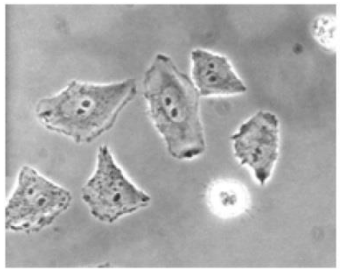


Phase contrast ring not centered



Kohler Illumination & aligned phase contrast





Contrast in microscopy

- For transparent specimens, adjust the contrast level:
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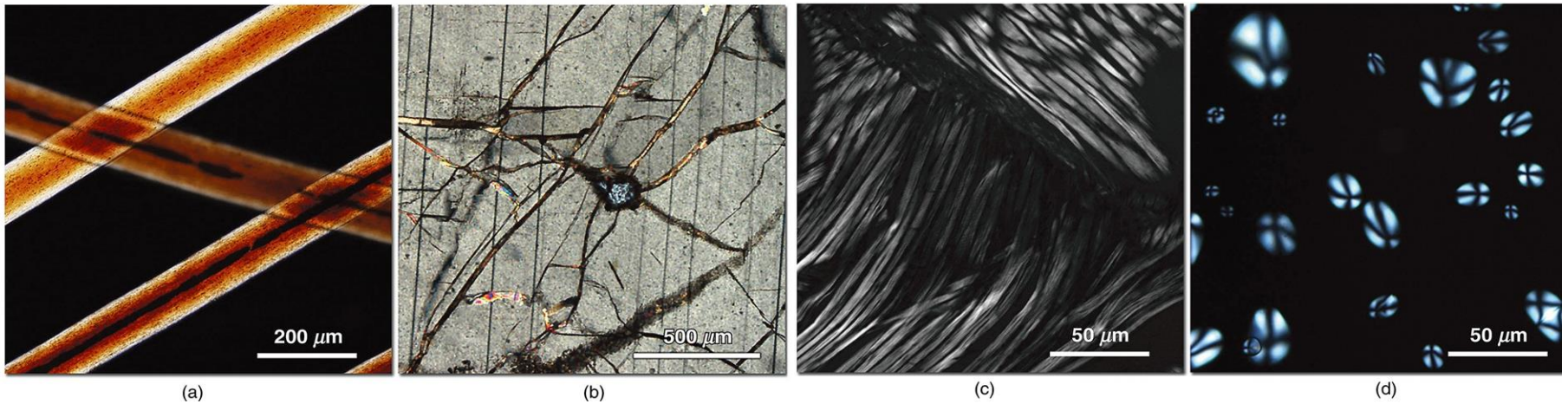
  - **Stained specimen**
  - **Dark field microscopy**
  - **Phase contrast microscope**
  - **Polarization microscopy**
  - Differential Interference Contrast (DIC) microscopy
  - Fluorescence microscopy

Polarization in Microscopy

Polarization microscopy: enables to see to a unique class of molecularly ordered objects (a.k.a. birefringent objects) that become “visible” upon illumination with polarized light.

Examples of materials imaged with polarized light:
revealing molecular orders in samples (biological, mineral)



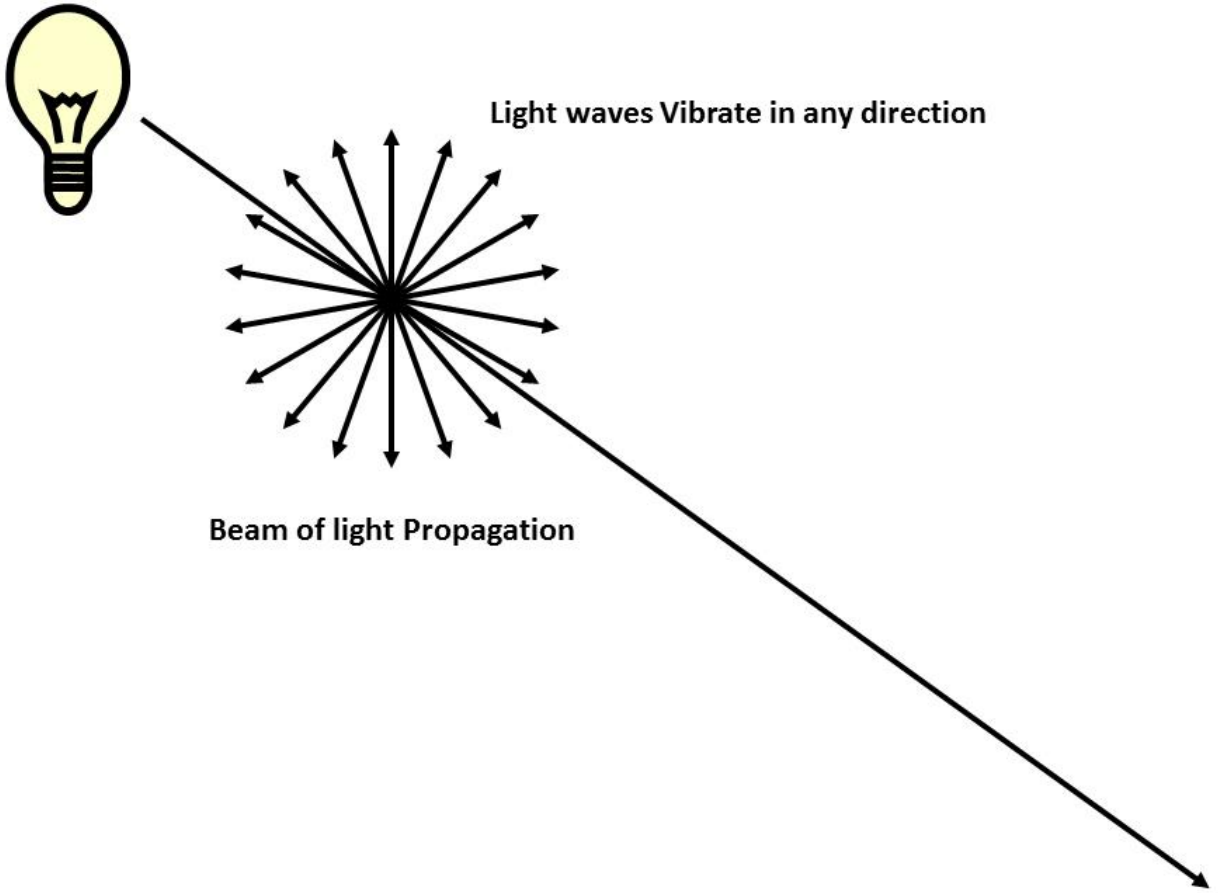
(a) Human hair
(b) Polished section of the mineral - camptonite
(c) Rabbit smooth muscle
(d) Potato starch

Although we can observe high-contrast images of molecularly ordered objects using a polarizing microscope, naked eye has no ability to distinguish a polarized light from a randomly polarized light.

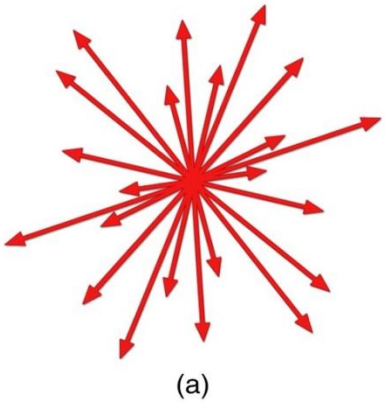
Unpolarized Light

- Light from majority of illuminators used in regular light microscopy is "**unpolarized**".

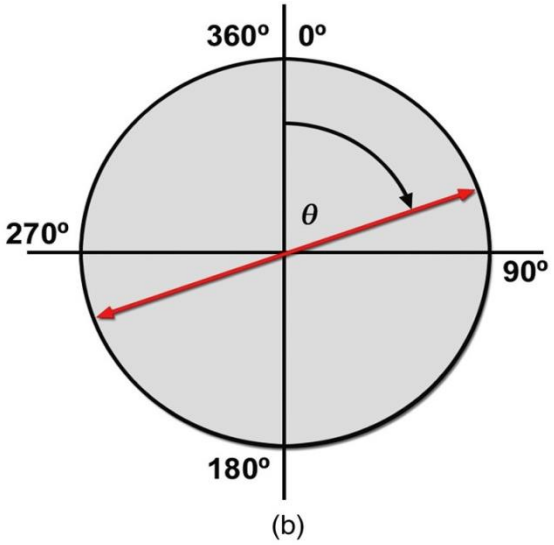
➔ This corresponds that E-vectors of different rays for an unpolarized light vibrate at all possible angles w.r.t. the axis propagation.



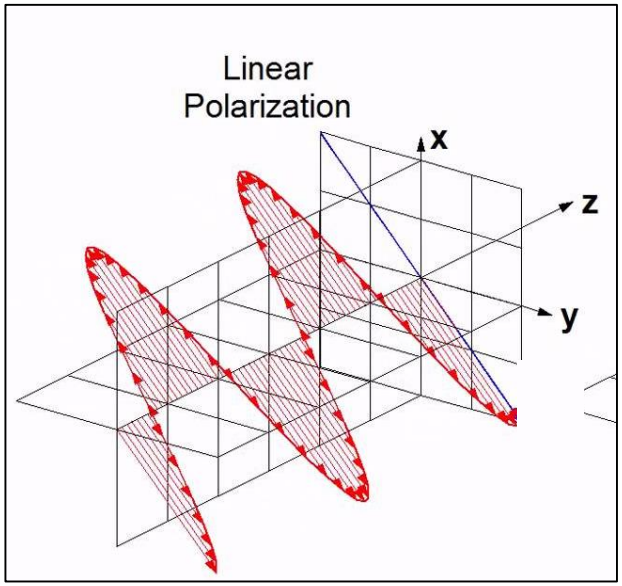
Linearly Polarized Light



(a)



(b)



(a) The light from majority of illuminators used in regular light microscopy is **"unpolarized"**:

➔The E-vectors of different rays vibrate at all possible angles w.r.t. the axis propagation

(b) For **"linearly polarized light",** the E-vectors of all rays vibrate in the same plane at a specific direction.

➔ Since the plane of vibration can occur at any angle, to describe the orientation of the plane in a beam cross-section, we describe the tilt angle (θ) relative to a fixed reference plane designated as 0^o.

Representing Linearly Polarized E-field

For any (general) polarization:

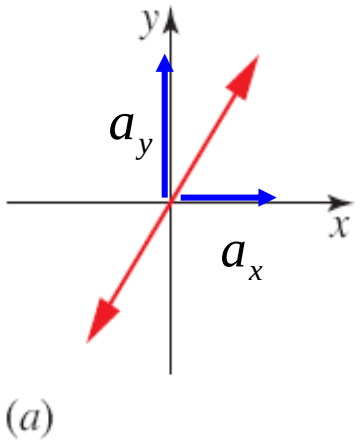
$$E_x = a_x \cos[\omega(t - z/c_o) + \delta_x]$$
$$E_y = a_y \cos[\omega(t - z/c_o) + \delta_y]$$

For linear polarization: $\delta_x = \delta_y = 0$

$$E_x = a_x \cos[\omega(t - z/c_o)]$$
$$E_y = a_y \cos[\omega(t - z/c_o)]$$

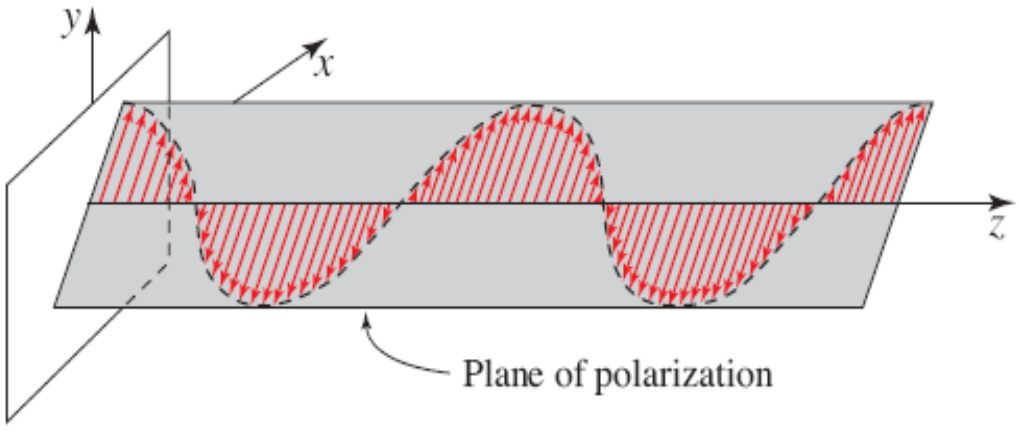
$$E_y = (a_y/a_x)E_x$$

For a fixed point in *z-axis*



(a)

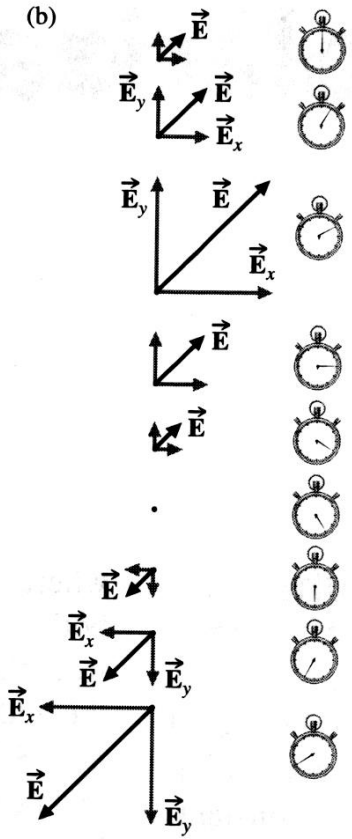
For a fixed *time*



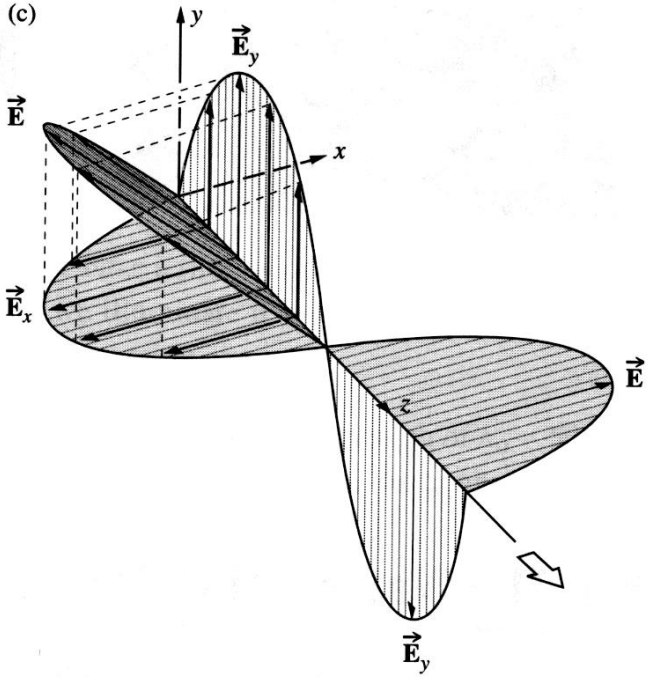
(b)

A Special Case: 45° Linear Polarization

For a fixed point in *z*

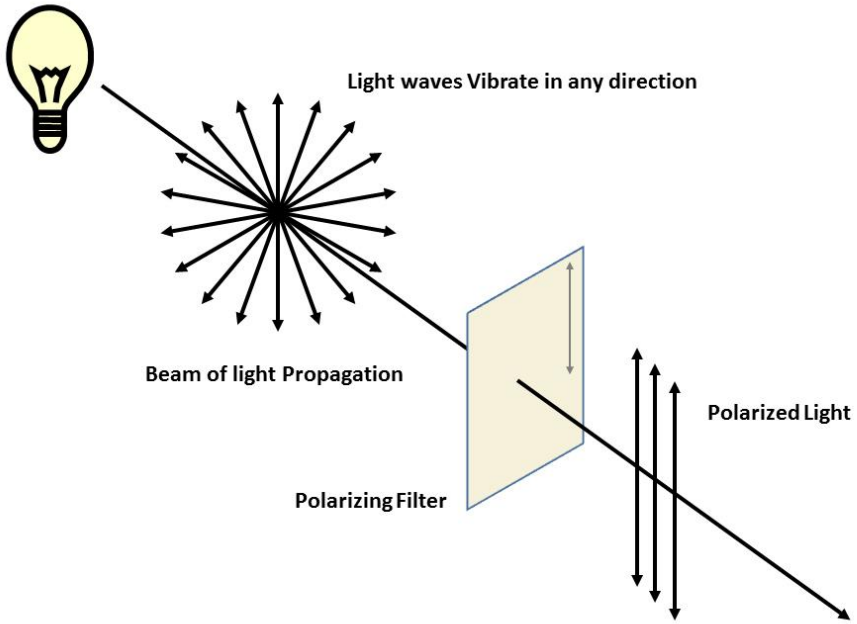


For a fixed *time, take a snapshot in x-y-z*

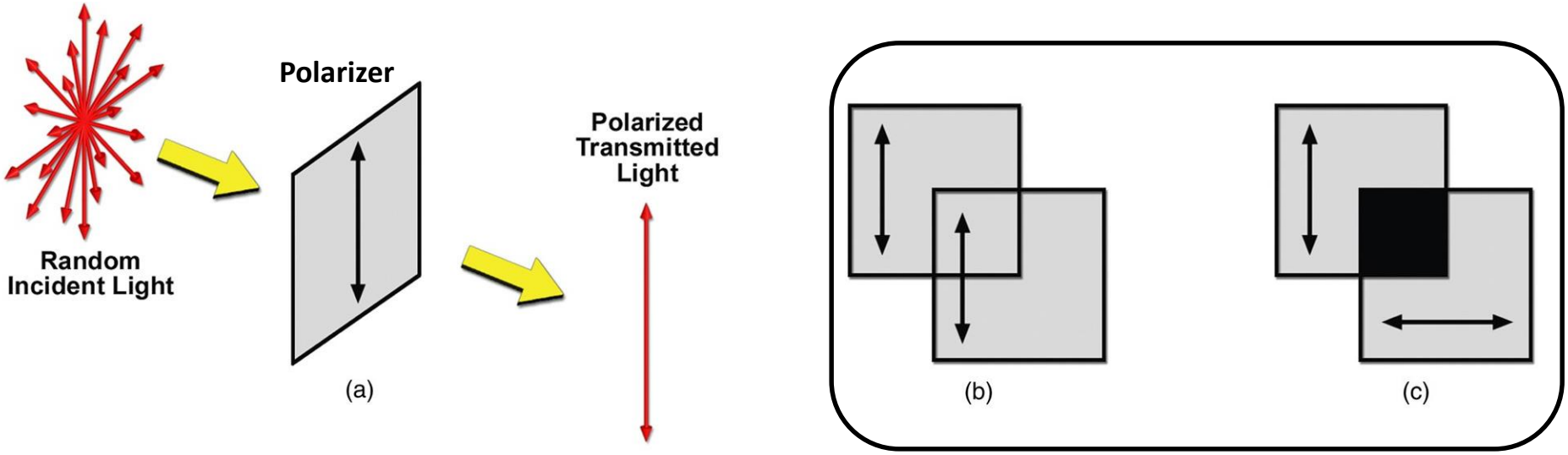


Linear Polarizer: a common component in microscopy

- *A device that produces polarized light is called **polarizer.***
- *When it is used to determine the plane of vibration, the same device is called **analyzer***



Polarized light



(a) In a, a polaroid sheet generates linearly polarized light. In this case only the rays whose E-vectors vibrate in a plane parallel with the transmission axis of the polarizer are transmitted as a linearly polarized beam; other rays are partially transmitted or blocked.

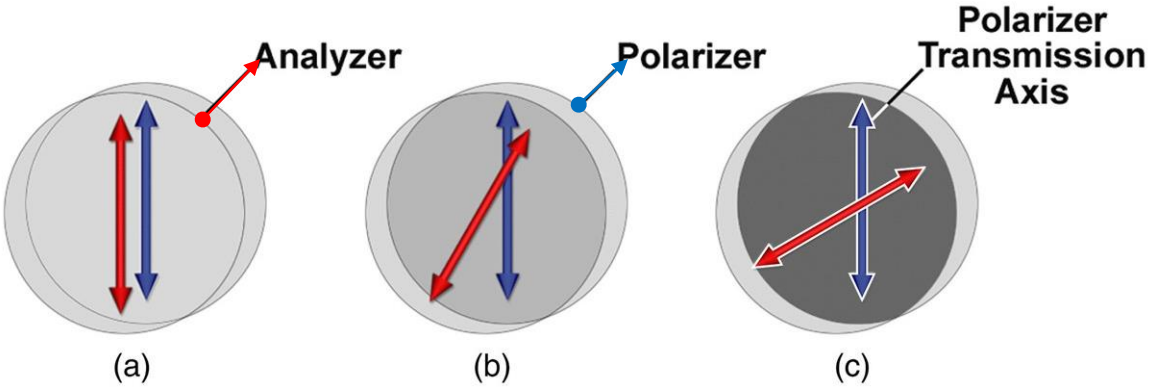
(b) In b, a second polarizer (a.k.a. analyzer) can transmit light from the first polarizer if its transmission axis is parallel to that of the first polarizer.

(c) Transmission is blocked if the transmission axes of the two polarizers are crossed with each other.

The extent at which incident random light is extinguished by two cross polarizers is called **extinction factor**:

- It is defined as the ratio of transmitted light when they are in parallel and in crossed orientations.
- Extinction factors of 10^3-10^5 or greater are required for polarization microscopy.

Polarizer & Analyzer Pair

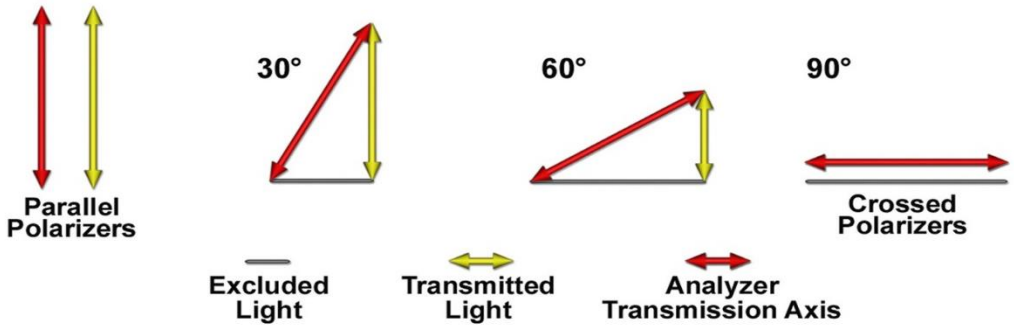


The percent of light transmission through two overlapping linear polarizers depends on the relative orientation of their transmission axes (red & blue arrows).

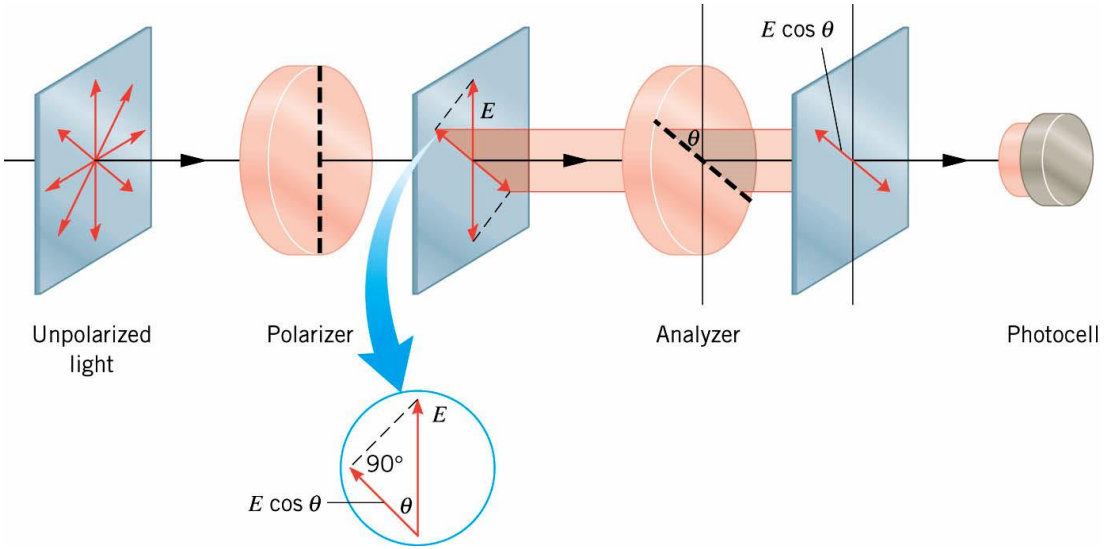
Lets consider a fixed **polarizer (blue)** and a rotating **analyzer (red):**

- Varying of azimuthal angles (between the transmission axis of the polarizers) performs vector analysis.
- The amplitude of the transmitted ray (yellow) is equal to the vertical vector component.

Example: In **(d),** when the transmission axes are crossed at 90°, transmission is suppressed.



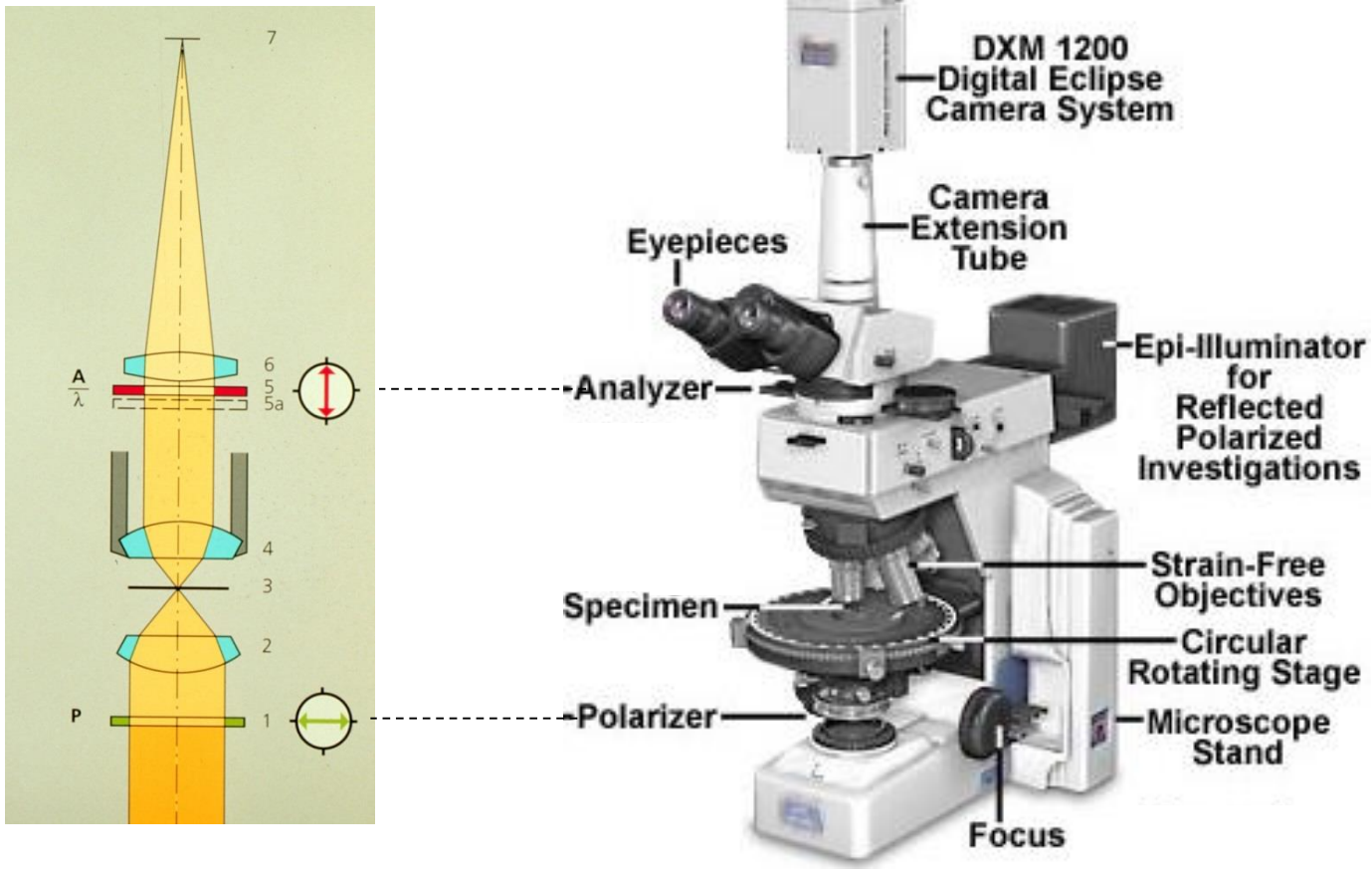
Polarizer/Analyzer Pair



Malus Law:

$$I = I_0 \cos^2 \theta$$

Polarized Light Microscope Configuration



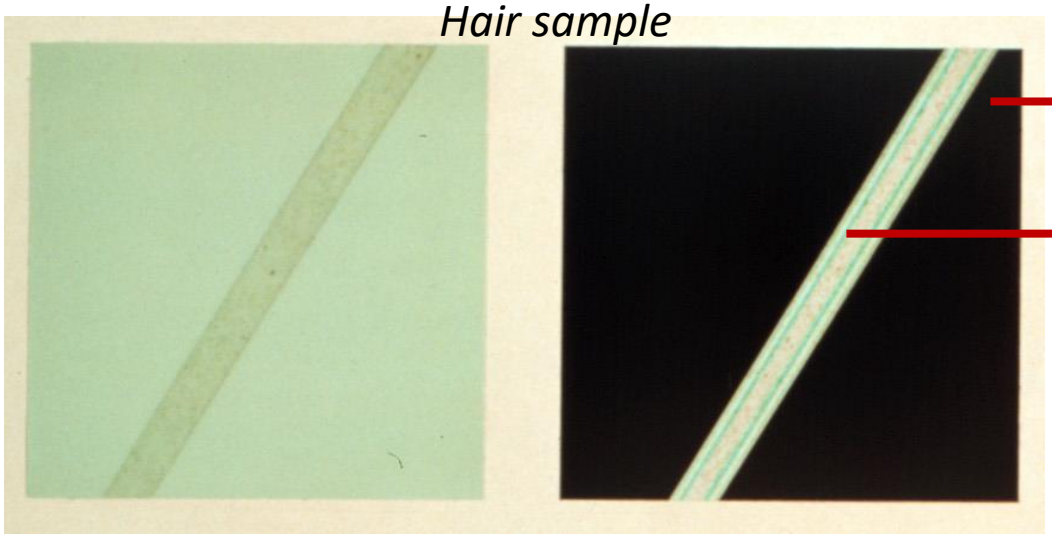
Step 1: As shown in the left diagram, there is a polarizer in position (1) and an analyzer in position (5).

- Typically one of them is in a rotating mount (which is the polarizer on the right illustration).
- With no sample on the stage, you rotate the mount until the total transmitted light extinguishes ➔ This corresponds to **cross-polarization** and the **background becomes dark**.

Step 2: Then, put the sample on the stage. Only the specimens that can rotate the plane of polarization will result in light to reach the detector…thus only these **samples will become visible behind a dark background** → Such samples are called **birefringent!**

Polarized Light Microscopy Images

Hair sample



Background does not rotate polarization → it is dark.

Sample rotates polarization→ it becomes “visible” (bright).

Bright-field microscopy

Polarized light microscopy

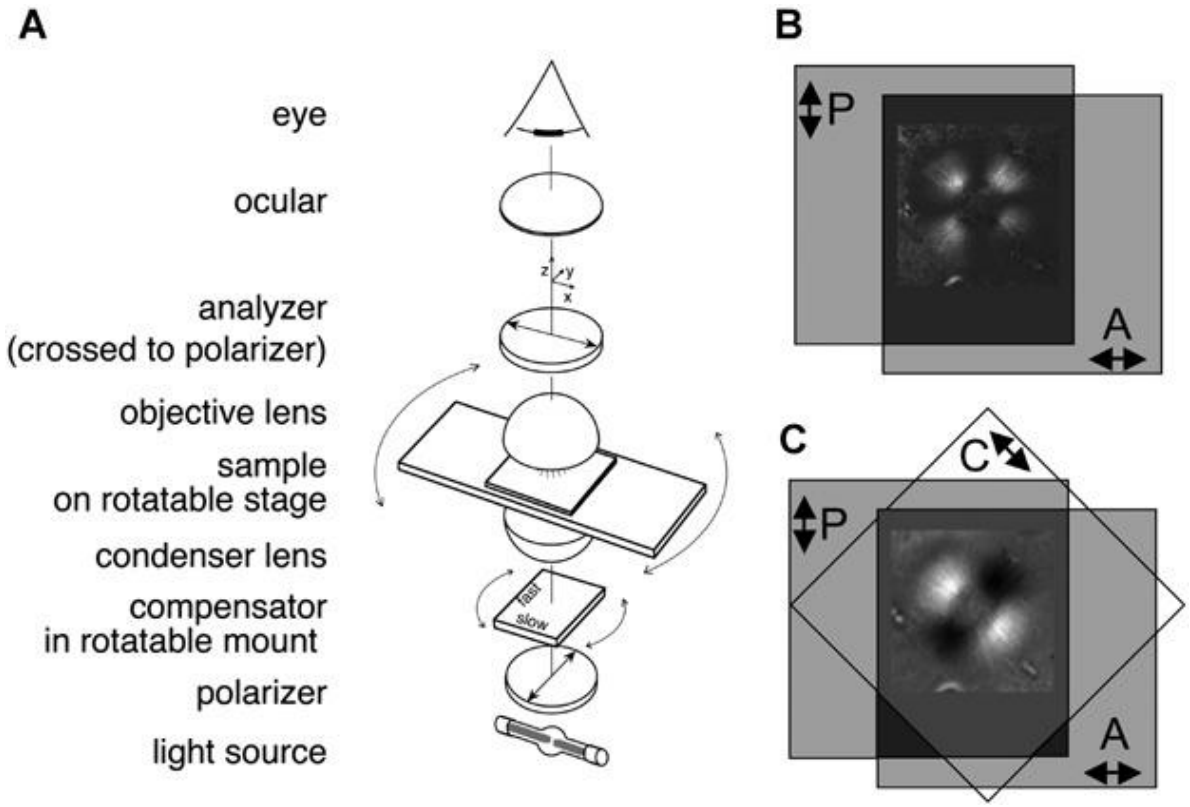
Polarized Light Microscopy in Biology

Figure 9.1

Polarized light image of meiosis in primary spermatocytes from the crane fly, *Nephrotoma suturalis*. (Image courtesy of Rudolf Oldenbourg, Marine Biological Laboratory, Woods Hole, MA.)

Polarized Light Microscopy in Biology

Example: Use of polarized light microscopy in developmental biology



**Molecular Reproduction Devel., Volume: 82, Issue: 7-8, Pages: 548-562,
First published: 30 July 2013, DOI: (10.1002/mrd.22221)**

Contrast in microscopy

- For transparent specimens, adjust the contrast level:
  - **Bright field microscope** (low contrast)
  - **Stained specimen**
  - **Dark field microscopy**
  - **Phase contrast microscope**
  - **Polarization microscopy**
  - **Differential Interference Contrast (DIC) microscopy**
  - Fluorescence microscopy (very high contrast)

Birefringence in Optics

- **Birefringent means double refraction:**
  - Certain transparent crystals and minerals such as quartz, calcite, rutile, tourmaline
  - Most **molecularly ordered biological structure**s such as lipid bilayers, bundles of microtubule and actin filaments, plant cell walls, chromosomes from certain organisms, chloroplast etc…

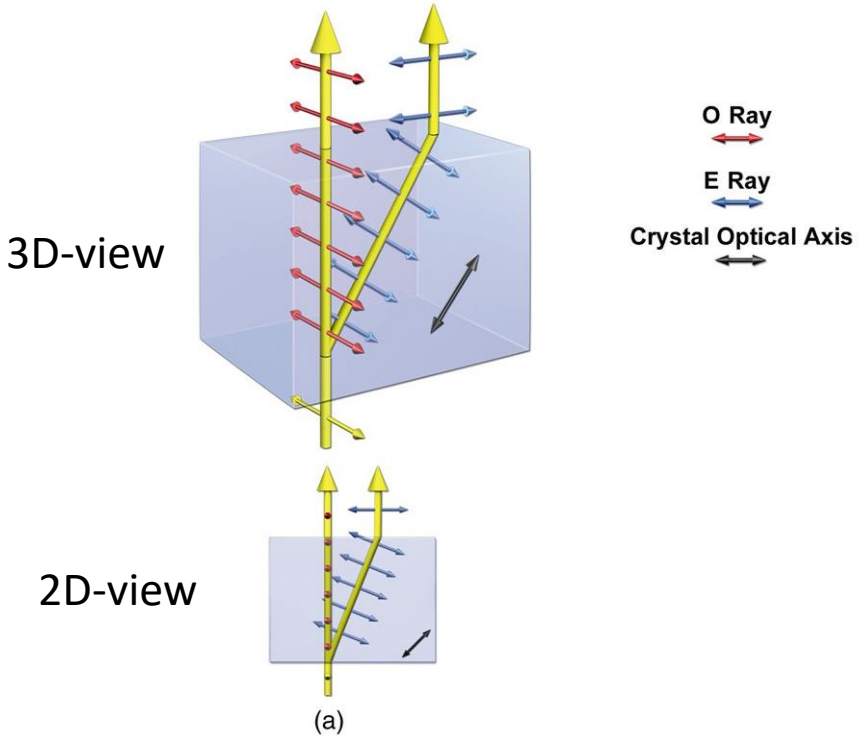
Demonstration:

- When letters in a printed page are viewed through a birefringent crystal (e.g. calcite), then each letter appears double:

As the crystal is rotated, the lighter gray image rotates around the darker gray image.

- Birefringence is due to the fact that birefringent materials **split an incident ray into two components** (extraordinary and ordinary rays) that traverse different path through the crystal and emerge as two separate rays.
- This occurs because atoms in such crystals are ordered in a precise geometrical arrangement and result in **refractive index values that are different depending on the light propagation direction.**

Birefringence: ordinary ray & extra-ordinary ray



(a) When a light beam is incident on a birefringent crystal, it splits into two rays that follow separate paths:

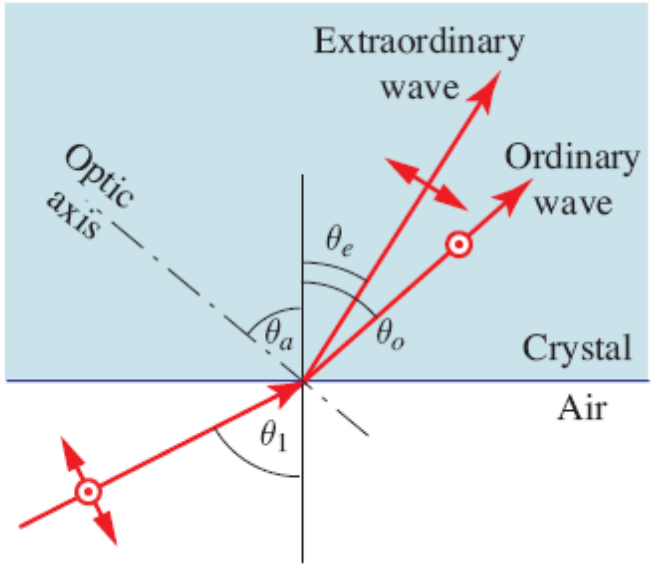
- **Ordinary ray** or **O-Ray** observes the regular law of refraction .
- **Extraordinary ray** or **E-Ray** travels along a different path.
- For every ray entering the crystal, there is **a pair of O-Ray and E-Ray that emerges**, each of which is linearly polarized.
- The E-field vectors of these two rays vibrate in mutually perpendicular planes!

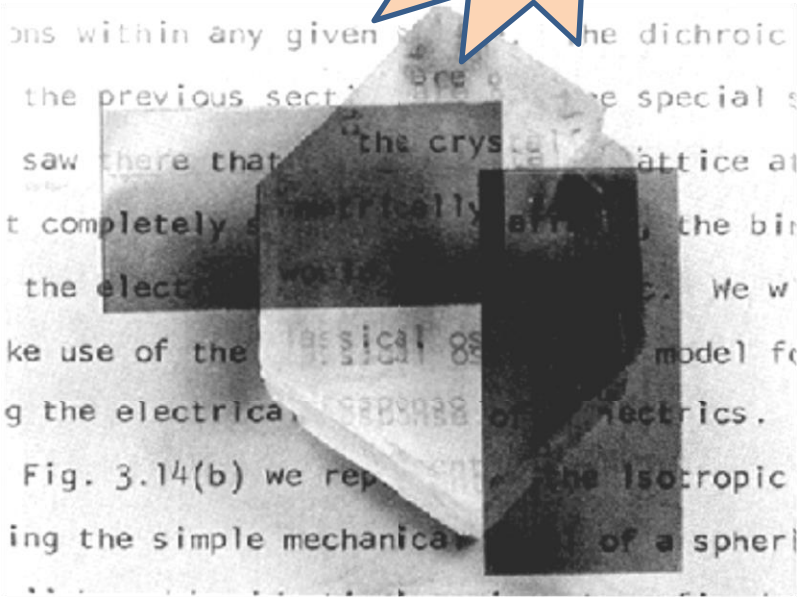
→ Exiting two rays **have different polarization**

→ Response depends to the orientation of optical axis

Calcite – birefringent material

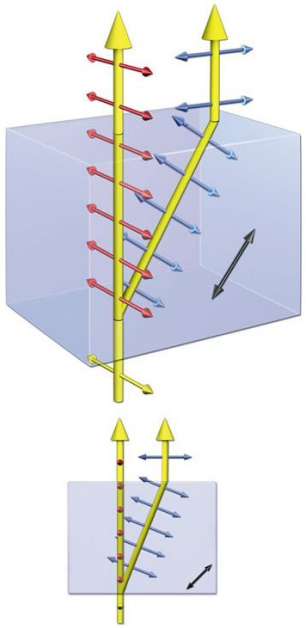
Polarized

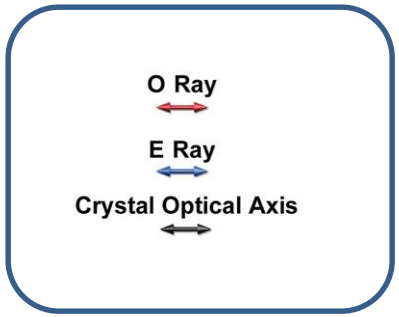




Birefringence: ordinary ray & extra-ordinary ray

Case 1



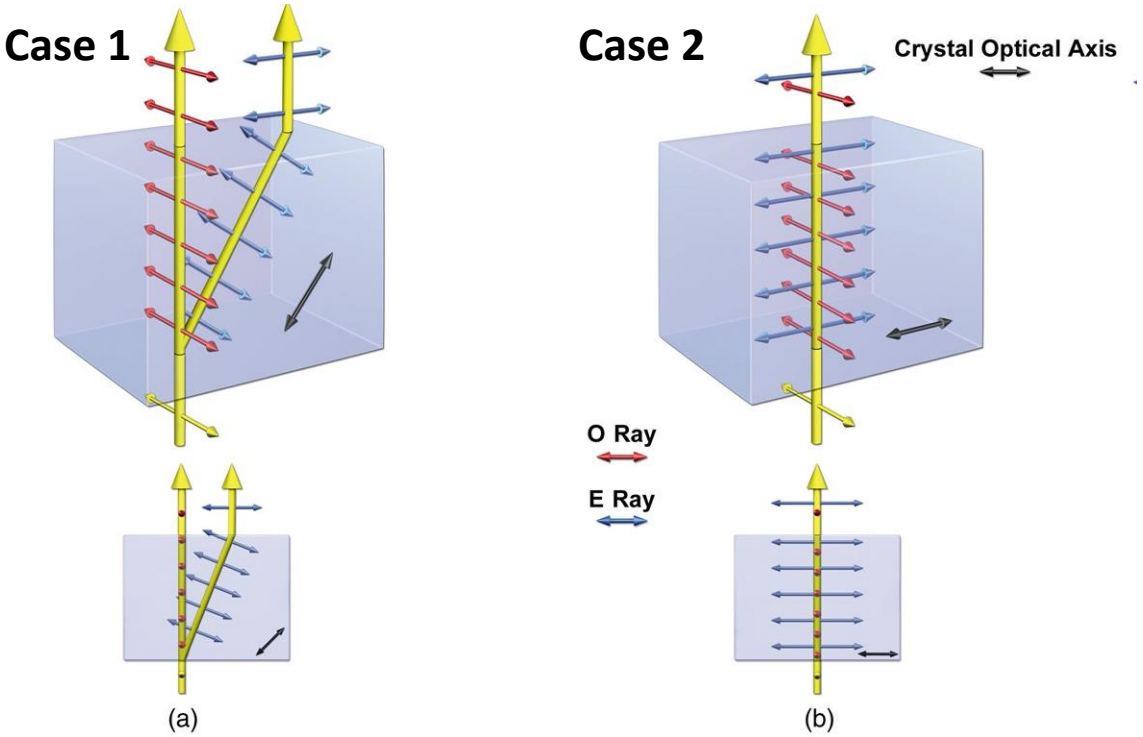


Birefringent materials contain a single unique axis known as the <u>crystal axis</u>.
- If incidence beam is **not perpendicular/parallel** to crystal axis (as shown in panel a), the two rays exit the crystal with a **physical separation**.

a) For this case, light splits into two rays that follow separate paths:

- Ordinary ray (**O-ray**) observes the regular law of refraction
- Extraordinary ray (**E-ray**) travels along a different path.
- For every incident ray entering the crystal, there is a pair of **O-ray** and **E-ray** that emerges, each of which is linearly polarized.
- The E-field vectors of these two rays vibrate in mutually perpendicular planes.

Birefringence – Crystal Optical Axis

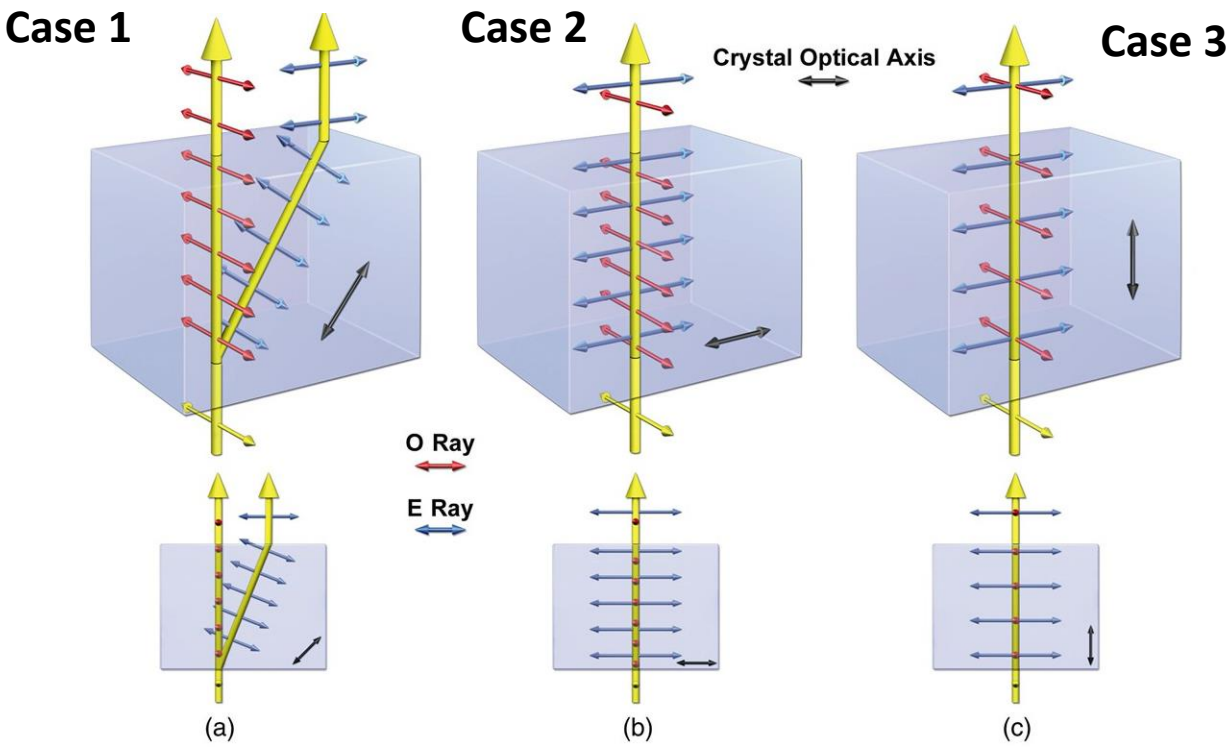


If incident beam is perpendicular to the crystal axis (shown in panel b), light splits into **O-Ray** and **E-Ray**, but their trajectories coincide.

b) For this case

- **O-Ray** and **E-Ray** emerge at the **same location** on the crystal surface, but due to experience of different optical path lengths, they are **phase shifted**. Emerging light exits as elliptically polarized.
- This geometry pertains to most biological specimens that are examined in a polarizing microscope.

Birefringence – Crystal Optical Axis

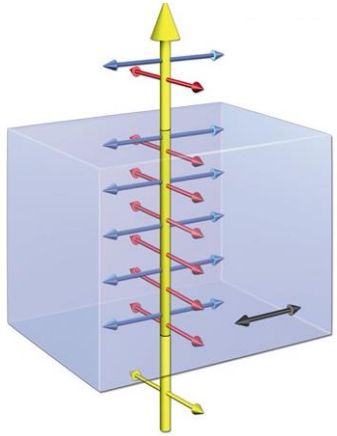


If incident ray follows a trajectory parallel to the crystal axis (as shown in panel c):
- O-Ray and **E-Ray** emerge at the **same location** on the crystal surface and with the **same phase**. Thus, light stays linearly polarized.

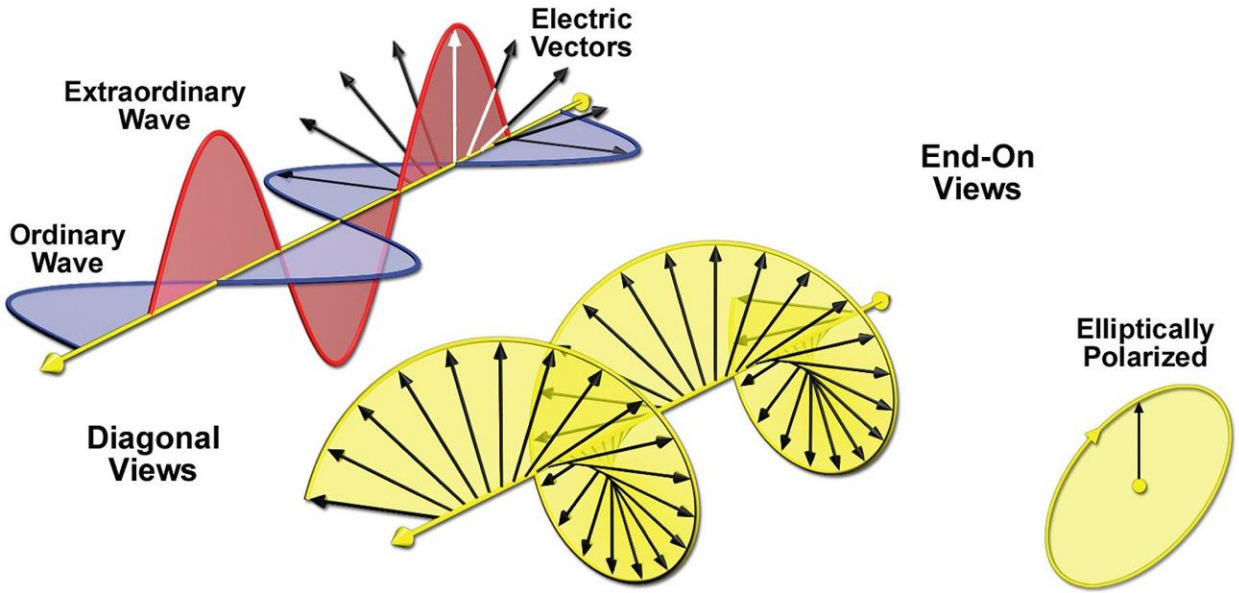
Generation of elliptically polarized light by birefringent specimen

For birefringent samples that are mounted on a microscope with their optical axis perpendicular to the incident beam:

- **O-Ray** and **E-Ray** follow the same trajectory as the incident ray, with one ray lagging behind the other and exhibiting a phase difference.
- The mutually perpendicular orientation of the two E vectors and phase difference between the two rays result in ***elliptically polarized light***.



Case 2



Remember: Representing Linearly Polarized E-field

For any (general) polarization:

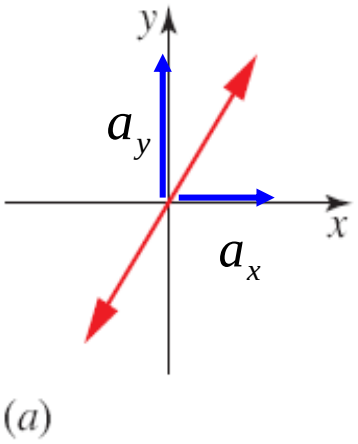
$$E_x = a_x \cos[\omega(t - z/c_o) + \delta_x]$$
$$E_y = a_y \cos[\omega(t - z/c_o) + \delta_y]$$

For linear polarization: $\delta_x = \delta_y = 0$

$$E_x = a_x \cos[\omega(t - z/c_o)]$$
$$E_y = a_y \cos[\omega(t - z/c_o)]$$

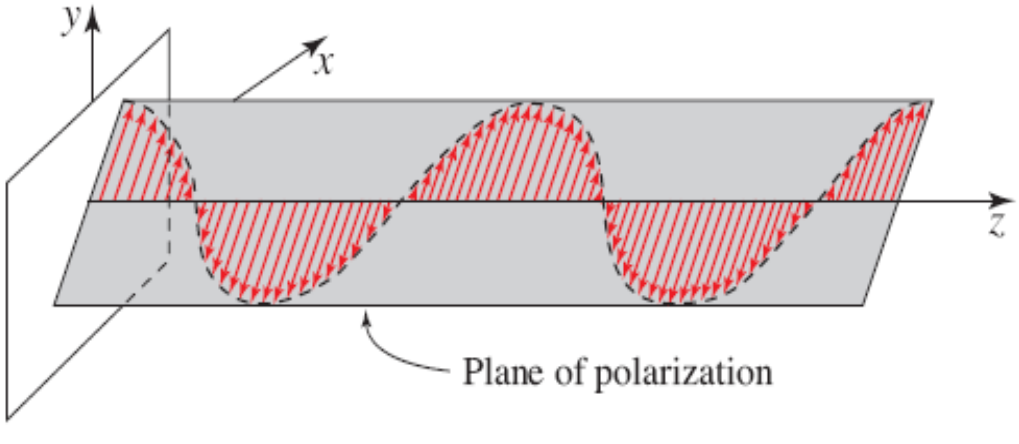
$$E_y = (a_y/a_x)E_x$$

For a fixed point in *z-axis*



(a)

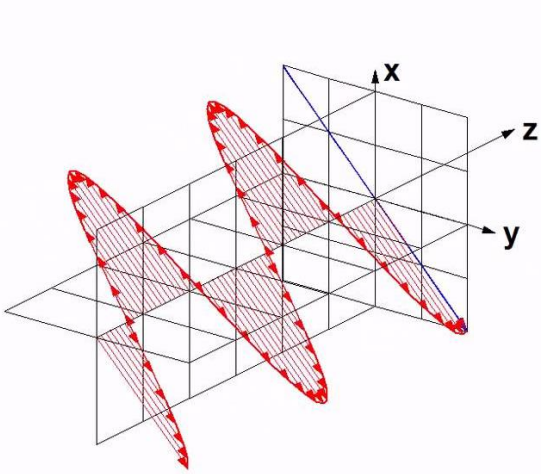
For a fixed *time*

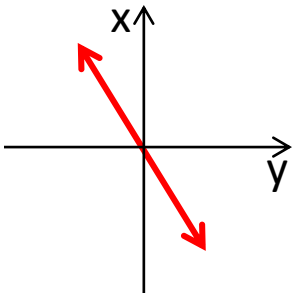


(b)

Polarized Light in General

Linear Polarization

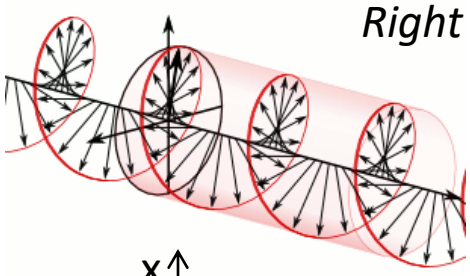


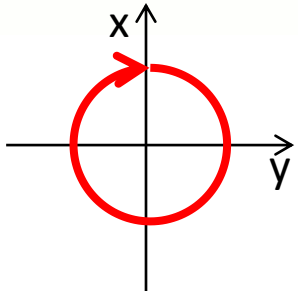


$For\ \delta_y - \delta_x = 0$

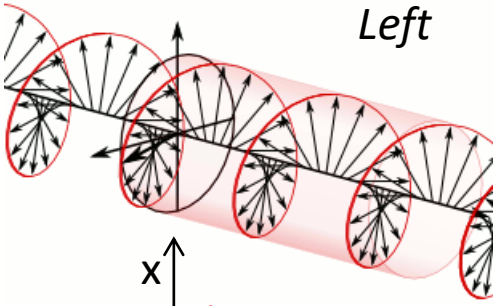
Circular Polarization

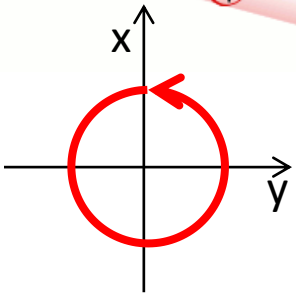
Right





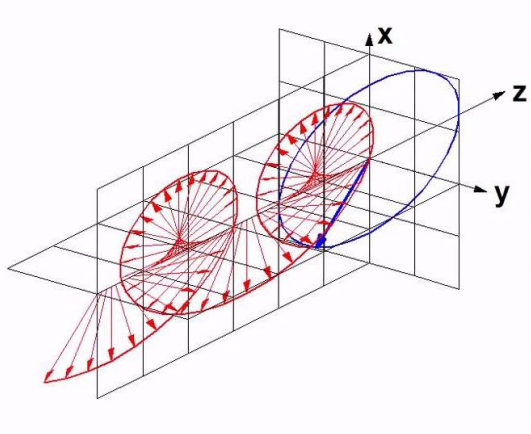
Left



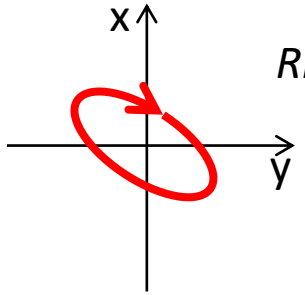


$For\ \delta_y - \delta_x = \mp\frac{\pi}{2}\ and\ a_x = a_y$

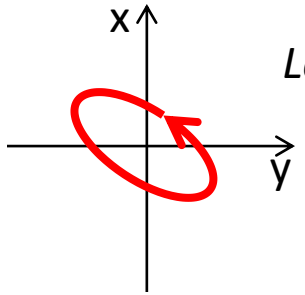
Elliptical Polarization



Right

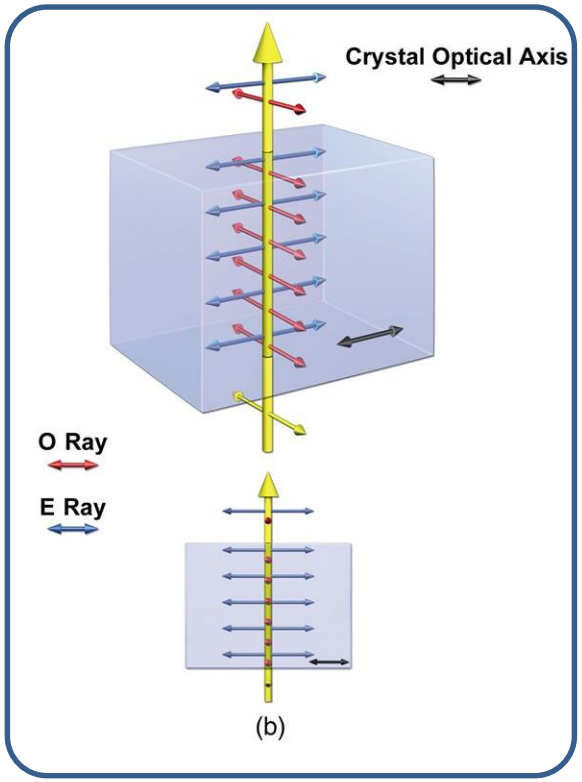


Left



$For\ \delta_y - \delta_x \neq 0\ and\ a_x \neq a_y$

Generation of elliptically polarized light by birefringent specimen

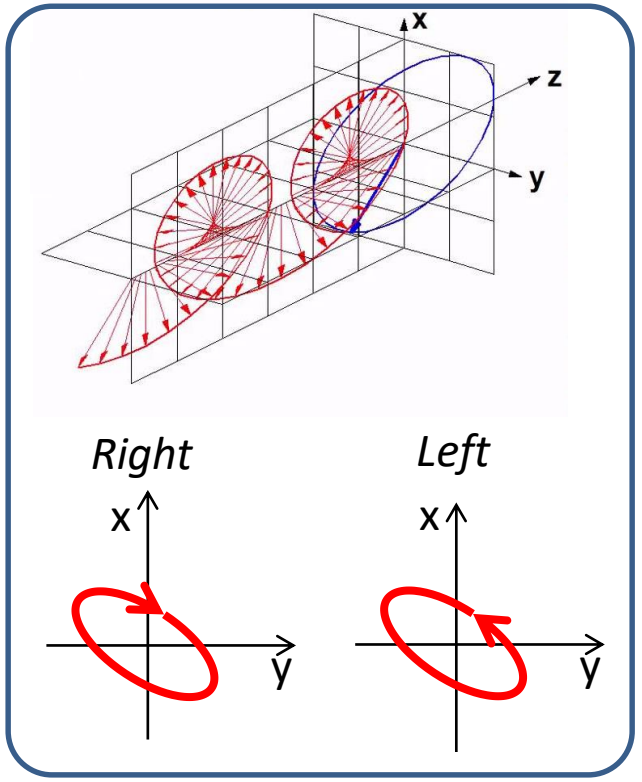


Elliptical Polarization

$$E_x = a_x \cos[\omega(t - z/c_o) + \delta_x]$$

$$E_y = a_y \cos[\omega(t - z/c_o) + \delta_y]$$

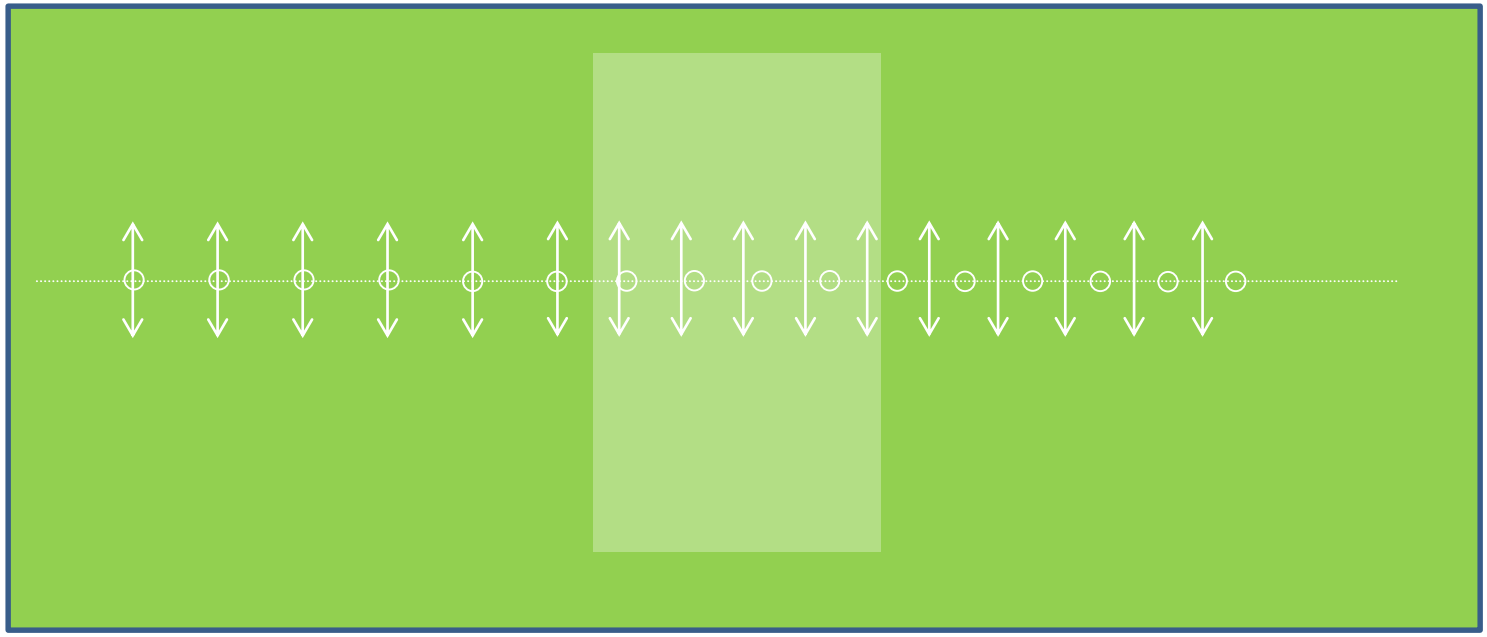
$$\delta_x - \delta_y \neq 0$$

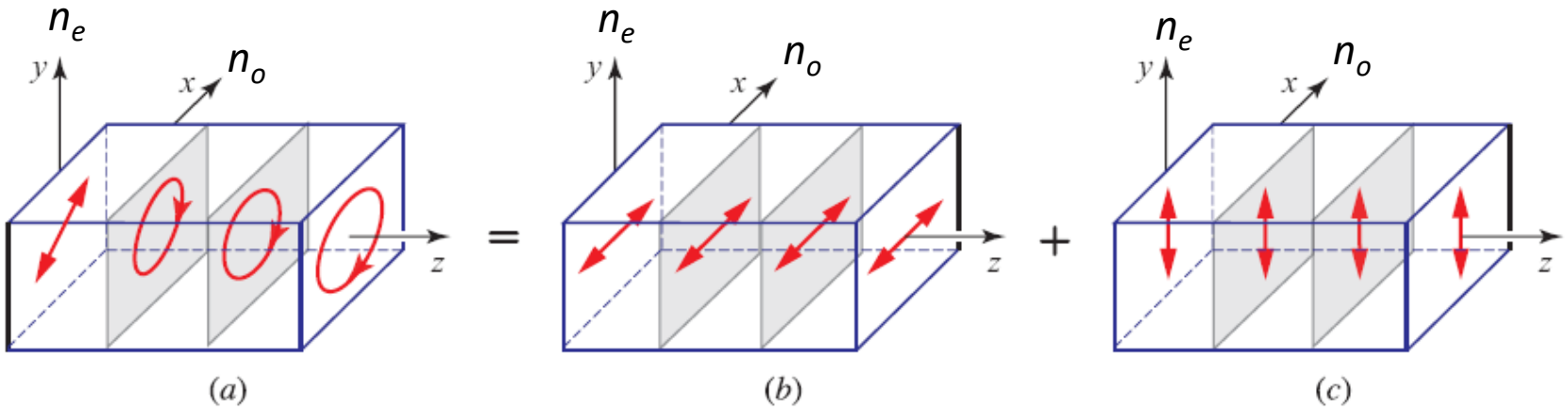


(b) Incident beam that is perpendicular to the optical axis is split into **O-Ray** and **E-Ray**, but their trajectories coincide.

- At this unique angle of incidence, **O-Ray** and **E-Ray** emerge at the same location on the crystal surface, but due to experiencing different optical path lengths, they are **phase shifted**.
- This geometry pertains to most biological specimens that are examined in polarization microscopy.

Generation of elliptically polarized light by birefringent specimen





$$\delta = \delta_y - \delta_x = k_0 n_e t - k_0 n_o t = k_0 \left| n_e - n_o \right| t = \frac{2\pi}{\lambda_o} \left| n_e - n_o \right| t$$

Birefringent Materials

The difference between the two indices, **n_o and n_e,** determines the amount of:

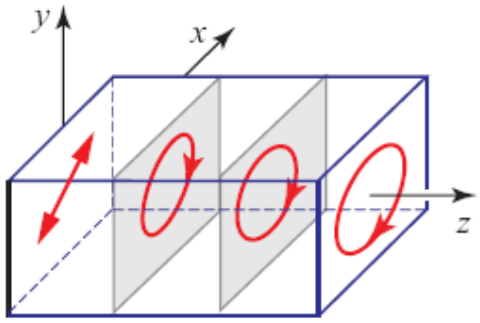
- Phase shift (δ)
- Physical splitting of E-Ray & O-Ray (when the incident light is not ***//*** or $\perp$ to the optical axis)

TABLE 8.1 Refractive indices of some uniaxial birefringent crystals ($\lambda_0 = 589.3$ nm).

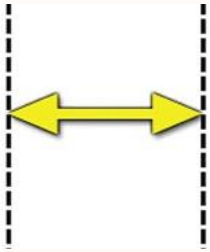
Crystal	n_o	n_e
Tourmaline	1.669	1.638
Calcite	1.6584	1.4864
Quartz	1.5443	1.5534
Sodium nitrate	1.5854	1.3369
Ice	1.309	1.313
Rutile (TiO_2)	2.616	2.903

Generation of elliptically polarized light by birefringent specimen

Depending on the amount of birefringence (n_e-n_o), material thickness (t) and wavelength of light (λ), a linearly polarized light will become elliptically polarized at the exit ➔



Let's consider the initial incident light is polarized along y axis:

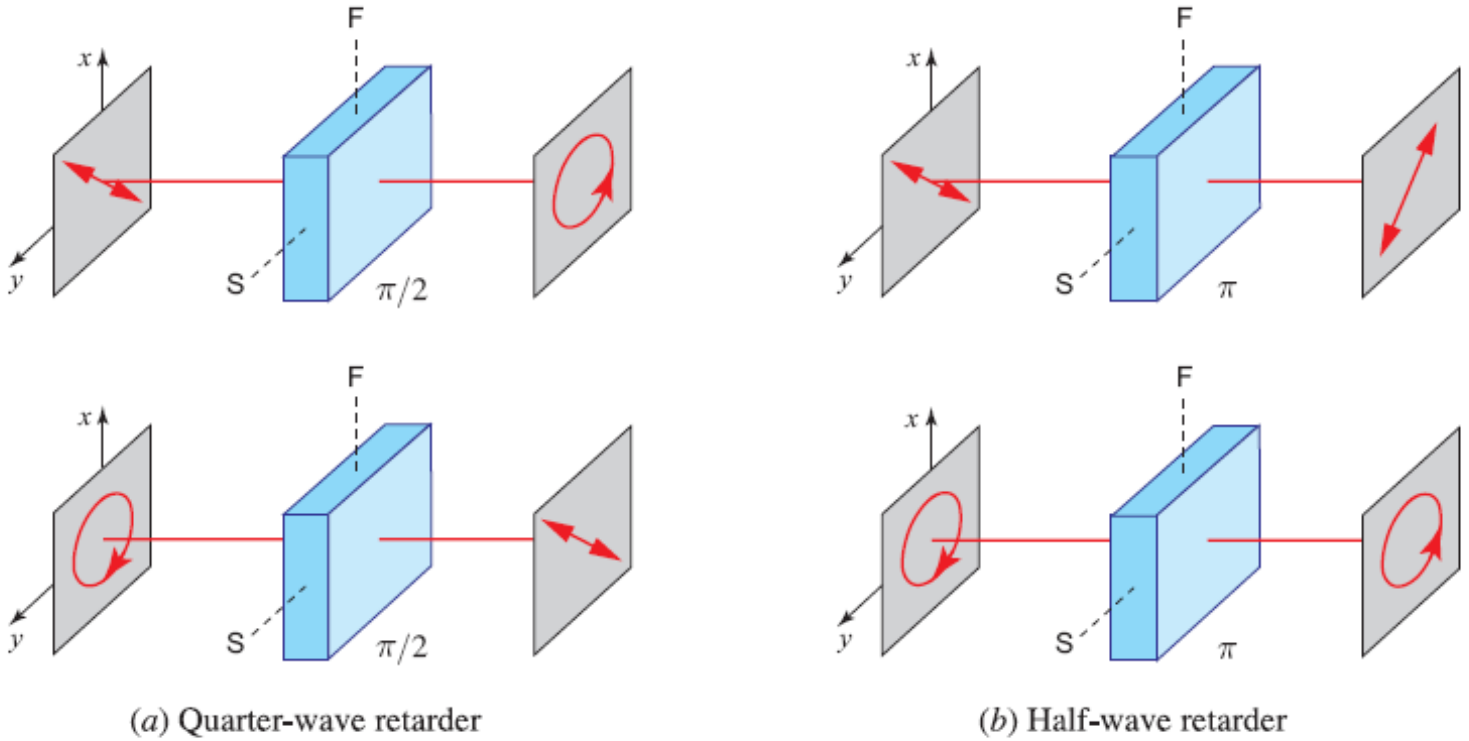
Amount of phase retardation (δ):	$\mathbf{0\ (m2\pi)}$	$\pi/4$	$\boldsymbol{\pi/2}$	$3\pi/4$	$\boldsymbol{\pi}$	$5\pi/4$	$3\pi/2$	$7\pi/4$
Amount of relative retardation (Γ):	$\mathbf{0\ (m\lambda)}$	$\lambda/8$	$\boldsymbol{\lambda/4}$	$3\lambda/8$	$\boldsymbol{\lambda/2}$	$5\lambda/8$	$3\lambda/4$	$7\lambda/8$
Polarization of output light:								

Stays same

Linear becomes circular

Still linear but orthogonal

Examples of Retarders



(*a*) Quarter-wave retarder (*b*) Half-wave retarder

Figure 6.1-8 Operations of quarter-wave $(\pi/2)$ and half-wave (π) retarders on several particular states of polarization are shown in (*a*) and (*b*), respectively. F and S represent the fast and slow axes of the retarder, respectively.