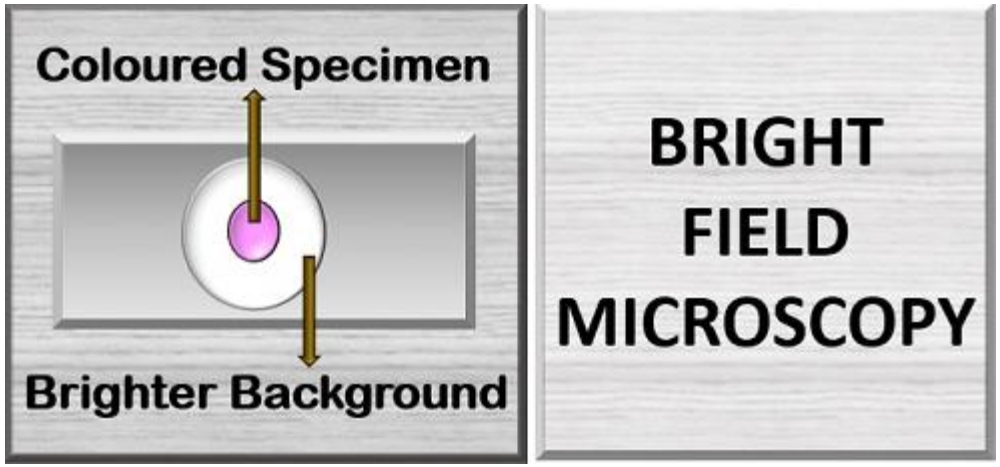
PH 301

ENGINEERING OPTICS

Lecture_Optical Systems_25

Light Microscopes

- **Bright field microscope**
- **Dark field microscope**
- **Phase contrast microscope**
- **Fluorescent microscope**



Light Microscopes

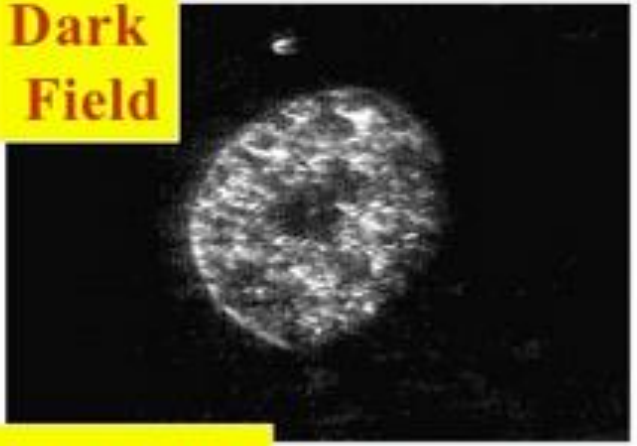
- **Bright field microscope** gives a magnified image of dark specimen with the colourless background.
- **Dark field microscope** excludes unscattered beam from the image. Field around the specimen is generally dark.
- **Phase contrast microscope** takes advantage of objects that alter phase of incident light.
- **Fluorescence microscope** takes advantage of inherently "**fluorescent material"** of biological object that can be fluorescently labeled.

"Advanced" Light Microscopic Methods

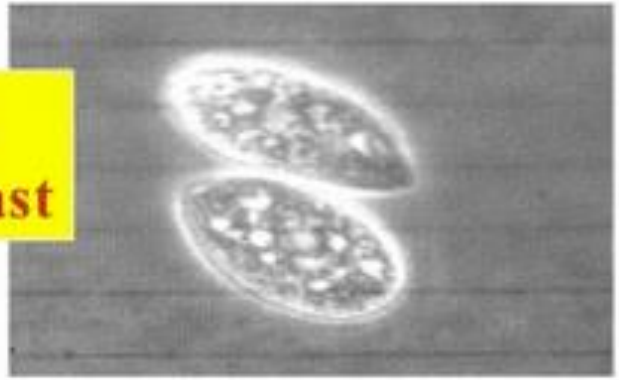
Single Cell Organism (*Tetrahymena*) observed with:

Bright Field

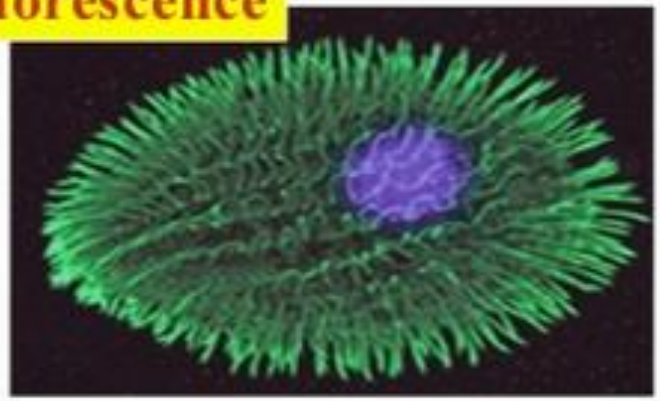
Dark Field



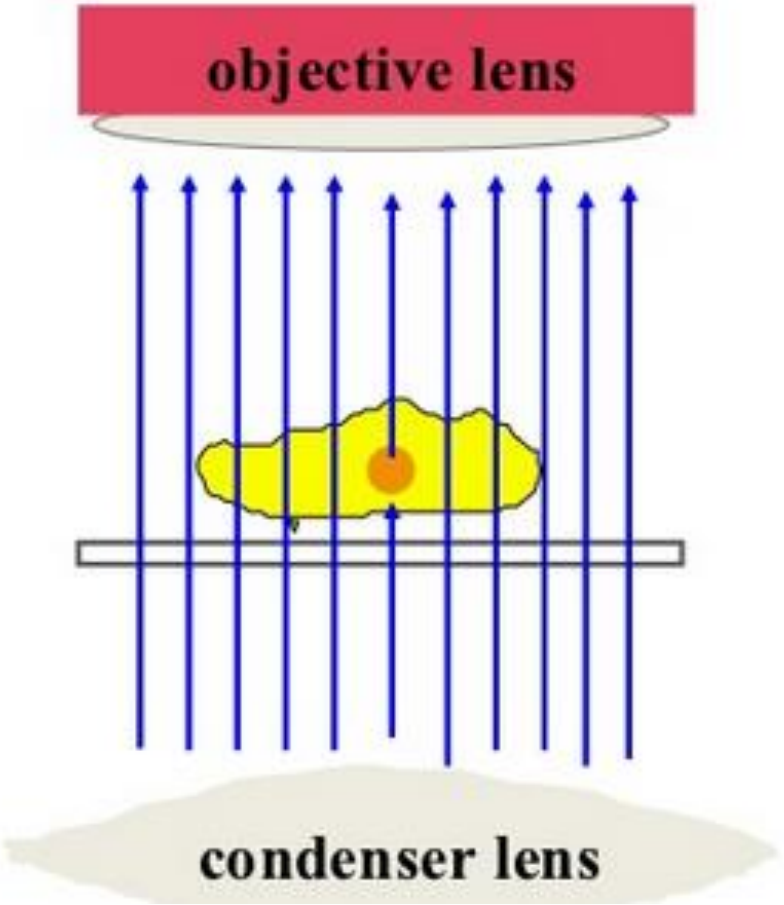
Phase Contrast



Fluorescence

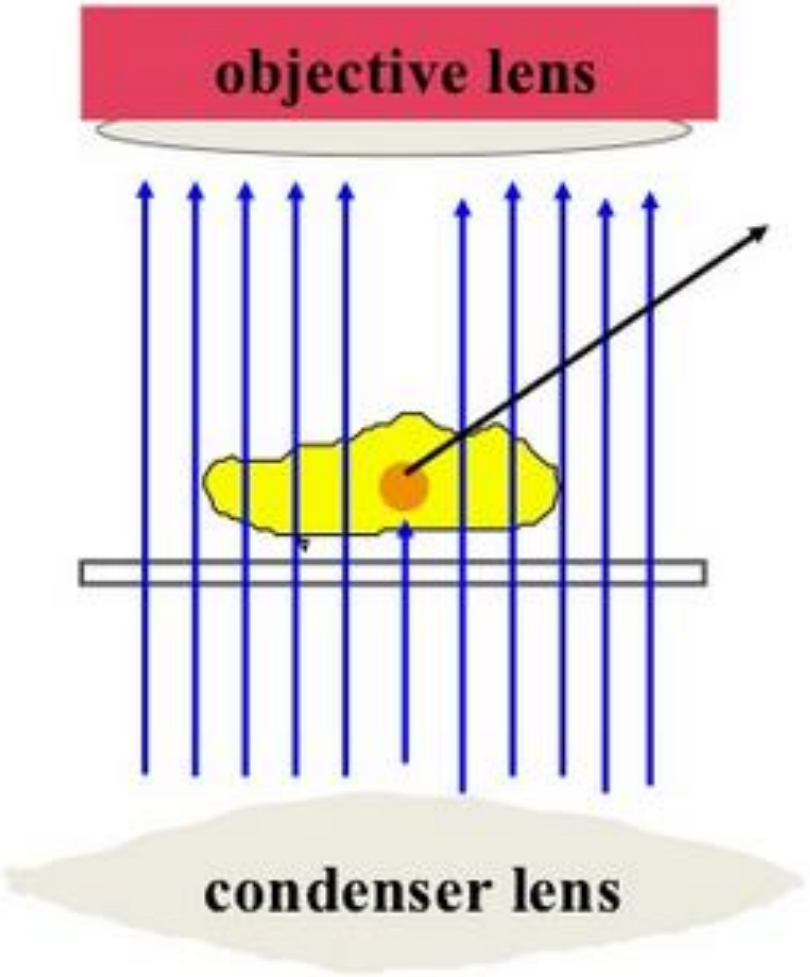


Recall that a specimen (e.g a live cell) with few structures that strongly absorb light provides little contrast with bright field microscopy



e.g., the single celled protist ***Tetrahymena***

Dark Field Microscopy: Some objects can alter the light path by diffraction & or light scattering



Scattering of Light

Scattering: small particles suspended in a medium of a different index of refraction diffuse a portion of the incident radiation in all directions.

With scattering, there is no energy transformation, but a change in the spatial distribution of the energy. Scattering, along with absorption, causes attenuation problems with radar & other measuring devices.

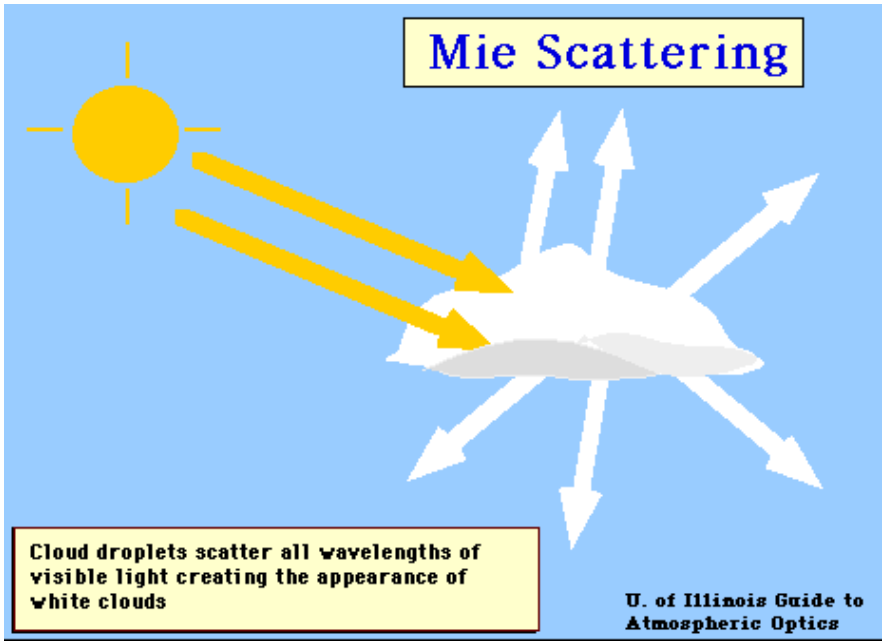
There are three different types of scattering:

- **Rayleigh scattering,**
- **Mie scattering, &**
- **Non-selective scattering.**

Rayleigh scattering mainly consists of scattering from atmospheric gases. This occurs when particles causing scattering are smaller in size than the wavelengths of radiation in contact with them. As the wavelength decreases, the amount of scattering increases.

Sky appears blue. This is because blue light is scattered around four times as much as red light, & UV light is scattered about 16 times as much as red light.

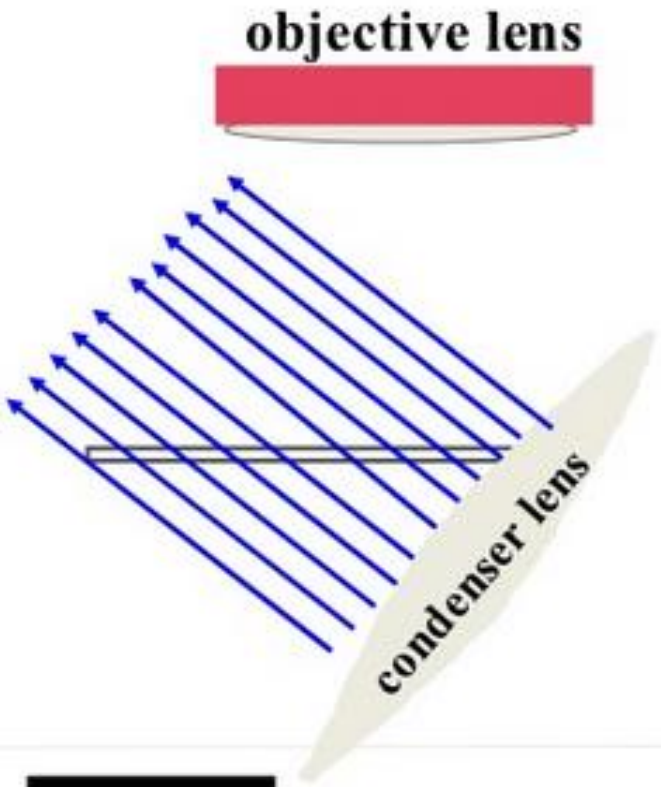
Rayleigh Scattering



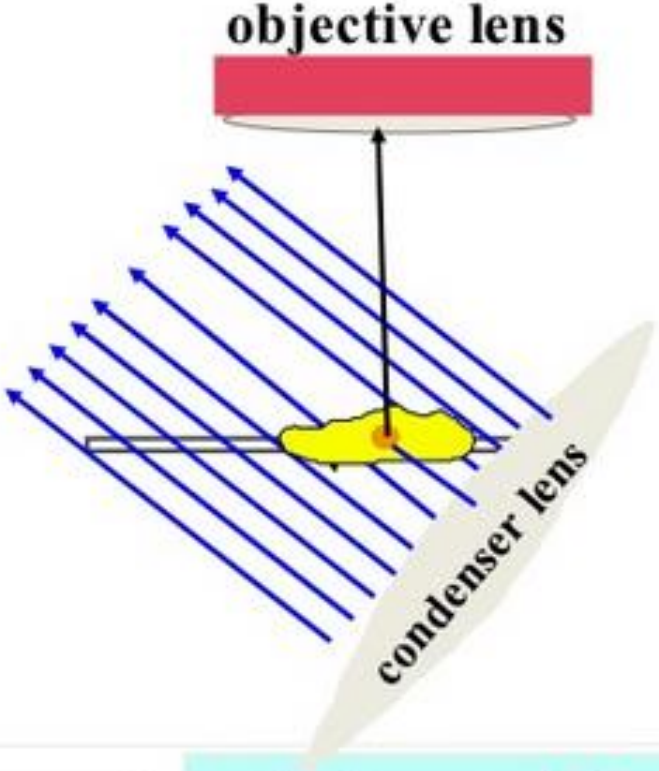
Mie scattering is caused by pollen, dust, smoke, water droplets, & other particles in lower portion of atmosphere. It occurs when particles causing scattering are larger than the wavelengths of radiation in contact with them. Mie scattering is responsible for the white appearance of the clouds

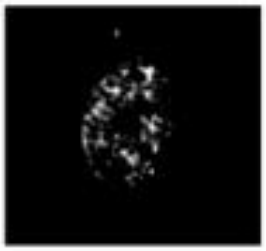
Non-selective scattering occurs in the lower portion of atmosphere when particles are much larger than the incident radiation. This is not wavelength dependent & is primary cause of haze.

If the light pathway is angled <u>without</u> a specimen the field is dark...



Dark Field seen





…but any object that diffracts light will be detected!

Dark Field Microscopy

- Most commonly employed light microscope allows light to pass through object -> Bright Field Microscopy
- Limitation of such microscope is that transparent & semitransparent objects are not readily visible (needs staining)
- Visibility -> Contrast between object & background
- Contrast can be greatly improved by creating a dark background.

Principle

- If aperture of condenser is opened completely & darkfield stop is inserted below condenser, light rays reaching object, forms a hollow cone.
- If a stop of suitable size is selected, all direct rays from condenser can be made pass outside object.
- Any object within this beam of light will reflect some light into objective & become visible.
- Method of illumination of object -> object become self illuminous against dark background -> Dark-field illumination.

Phase Object

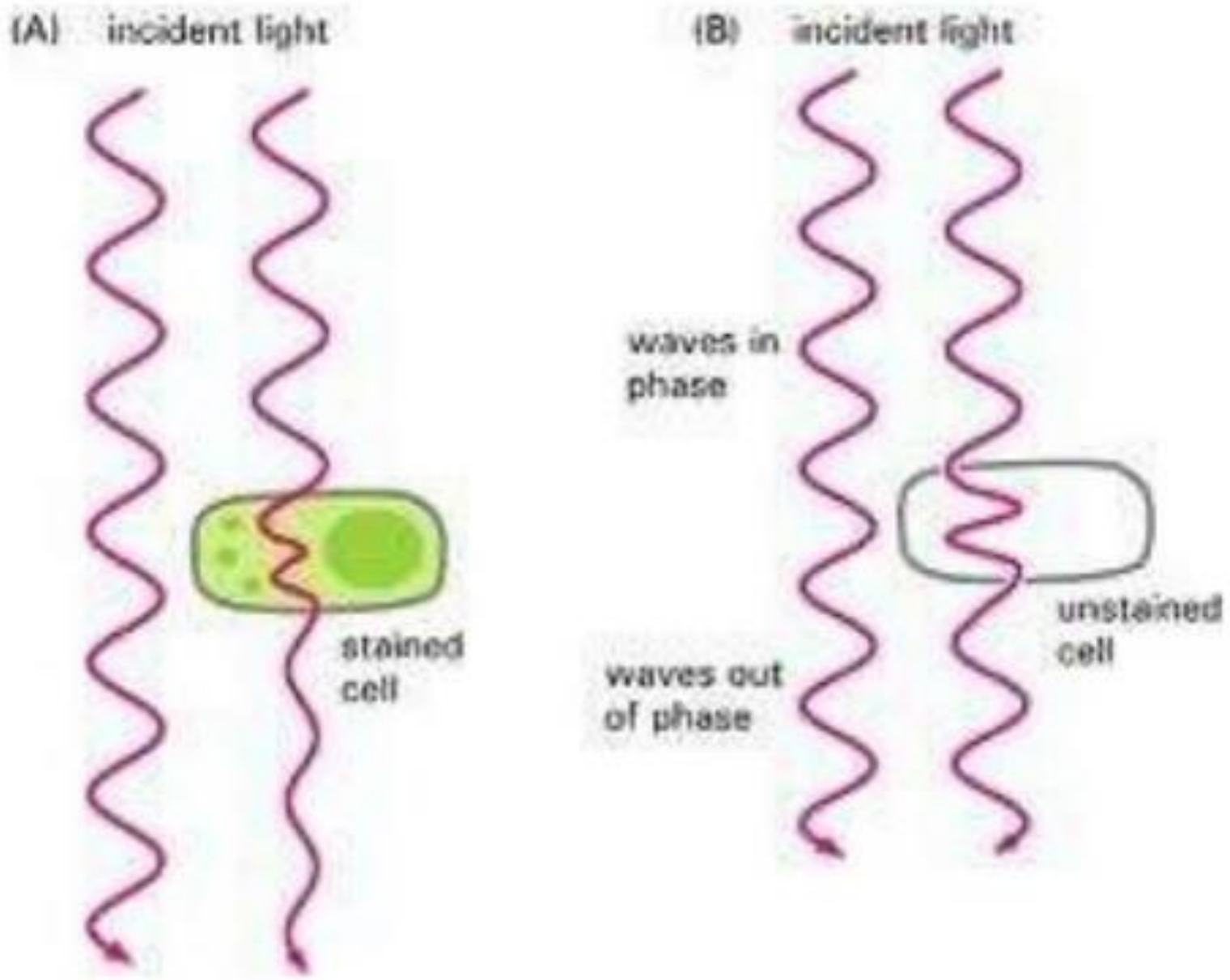
- **Object is completely transparent but has an optical thickness which varies from point to point.**
- **It introduces phase differences between disturbances which pass through different parts of it.**
- **Consequently, disturbances immediately behind object, & in conjugate image plane, have same amplitude at all points but will show variations in phase from point to point.**
- **Human eye is sensitive to intensity only & cannot detect changes of phase so that field of view appears uniformly bright.**

Phase Contrast Microscopy

- **Phase contrast microscopy is a special adaptation of light microscopy & help obtain a clear picture of living or unstained cells.**
- **Adaptors convert minute difference in phase changes in transmitted light due to refractive indices of all cell organelles into perceptible shades of grey**
- **This allows organelles of living cell to become visible with fair contrast in them.**

Principle

- **Different wavelength of light rays detect differences in colours.**
- **Different shades of grey are distinguished to our eyes due to differences in amplitude of light rays.**
- **Phase contrast microscope converts invisible small phase changes caused by cell component into visible intensity changes.**
- **Phase changes are caused by biological material through which light ray passes. If a material is absorbent, it causes the ray to undergo a change in amplitude, which is distinguished by our eyes. Ex. Light passing through window glass & without them.**


(A) incident light
stained cell
(B) incident light
waves in phase
unstained cell
waves out of phase

Consider three different materials & their effects on light.

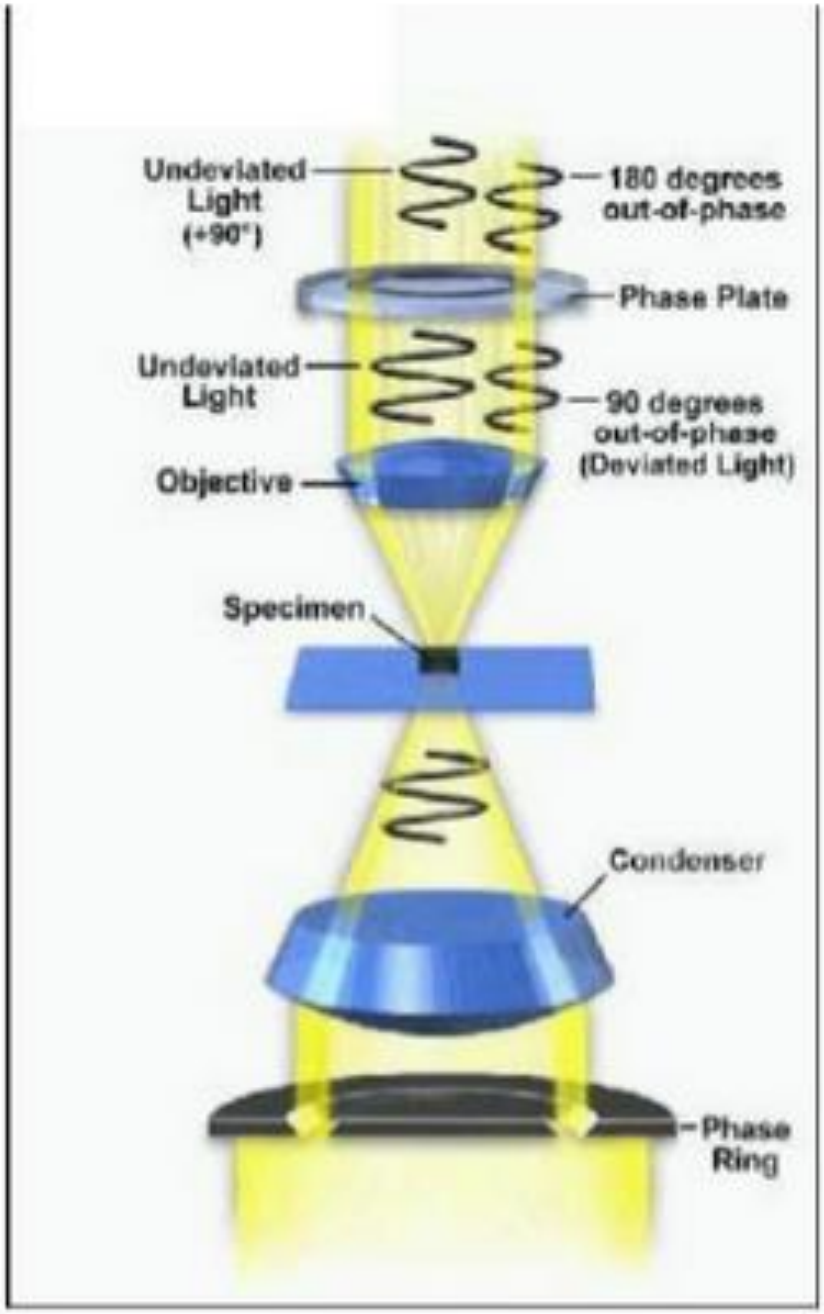
1. **Transparent & non-absorbent material with higher refractive index,**
2. **Transparent & non-absorbent but thicker, &**
3. **Transparent & absorbent.**

Interference

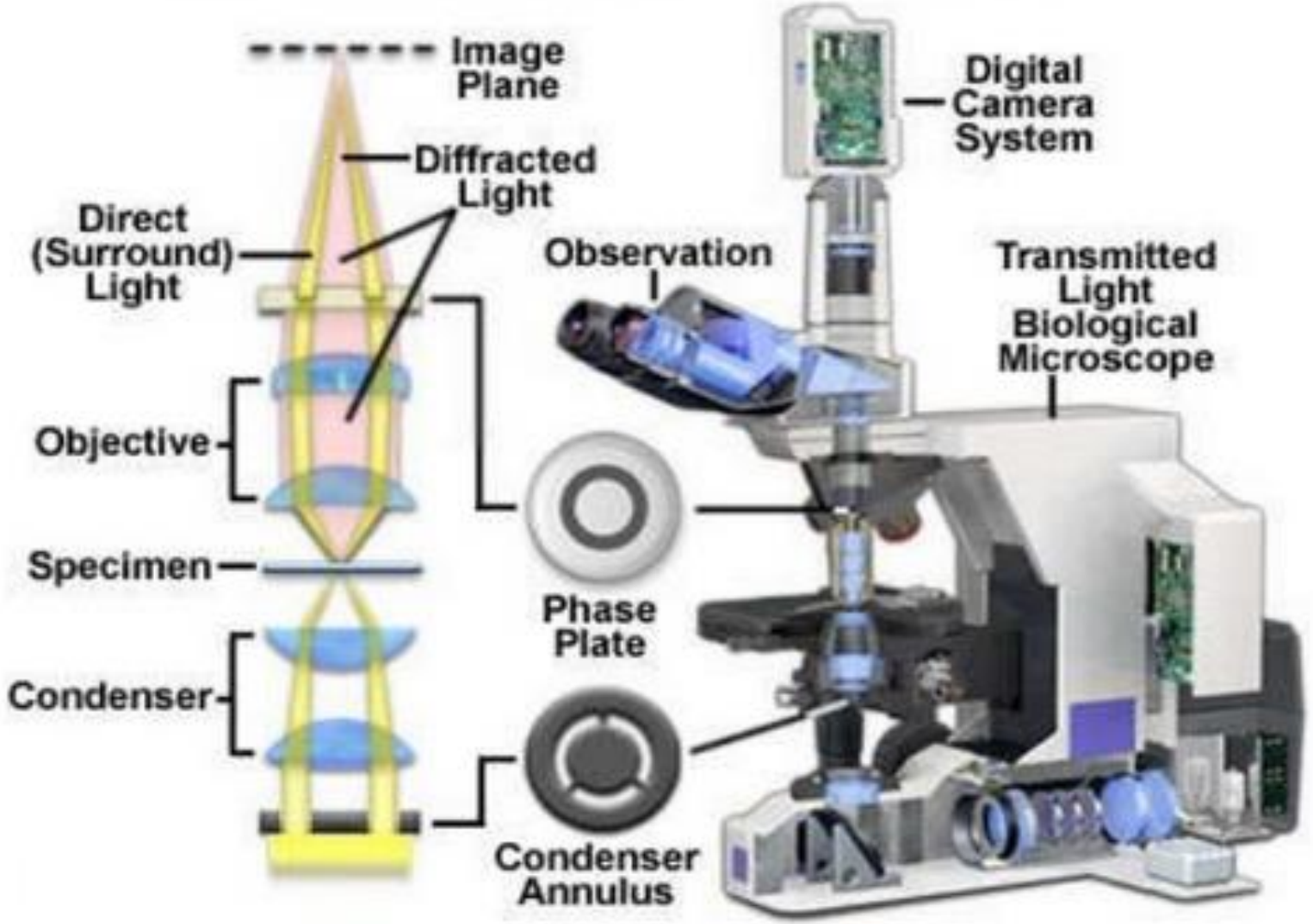
1. **Light rays undergo a phase change depending upon refractive index of transparent material.**
2. **Phase change is in direct proportion to thickness of material.**
3. **Light rays undergo change in amplitude when it passes through an absorbent material.**

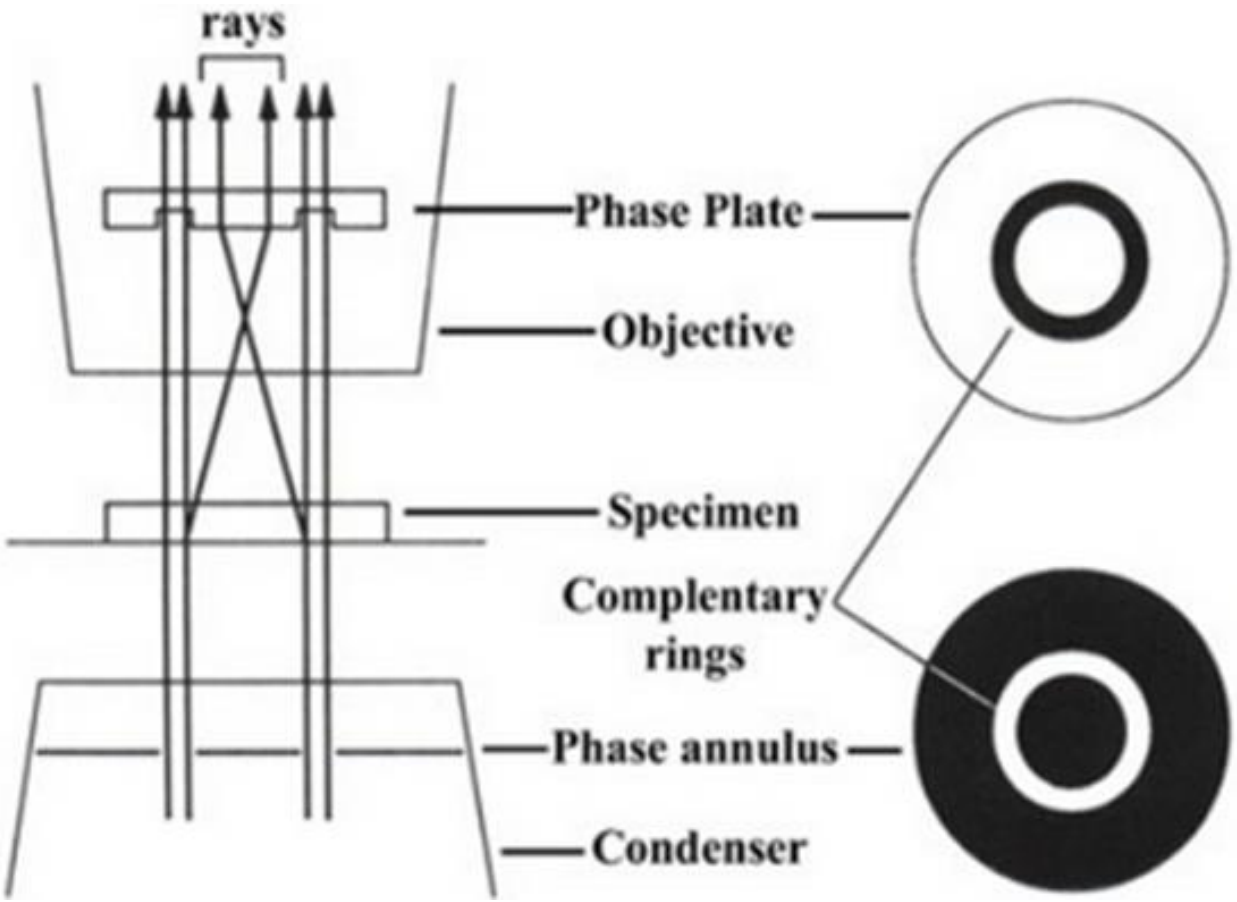
- More refractive index & thickness, more change in phase.
- If biological material absorbs light ray they show contrast but living cell generally do not absorb light ray.
- Cells & their components don't show phase change.
- Value of phase change is ¼ of wavelength of light. But this phase change is not distinguished by our eyes.

Principle behind phase contrast microscope is to convert undistinguishable phase change into distinguishable phase change in terms of variation of contrast, with the help of two adaptors – annular diaphragm & annular phase plate.

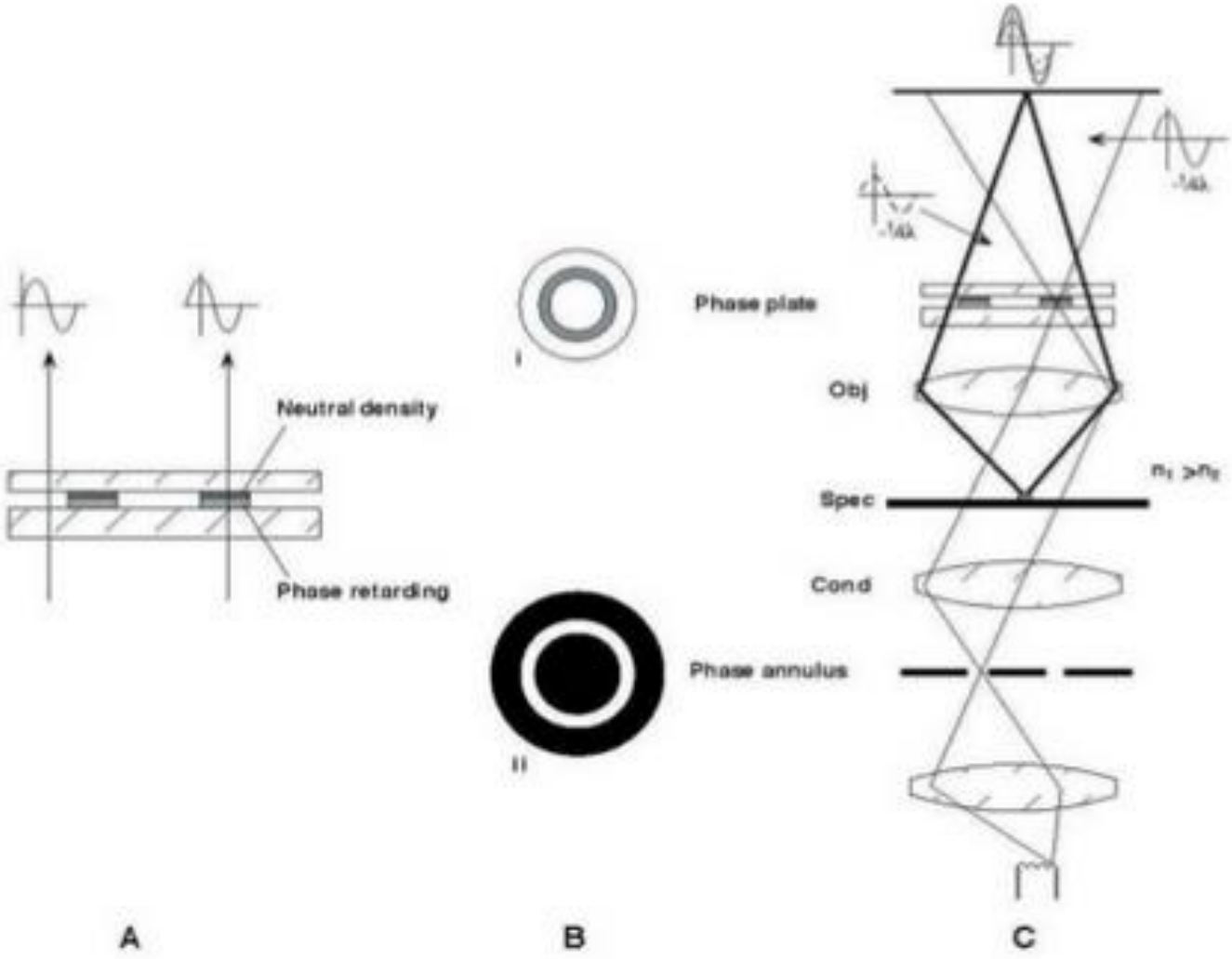

Undeviated Light (+90°)
180 degrees out-of-phase
Phase Plate
Undeviated Light
90 degrees out-of-phase (Deviated Light)
Objective
Specimen
Condenser
Phase Ring

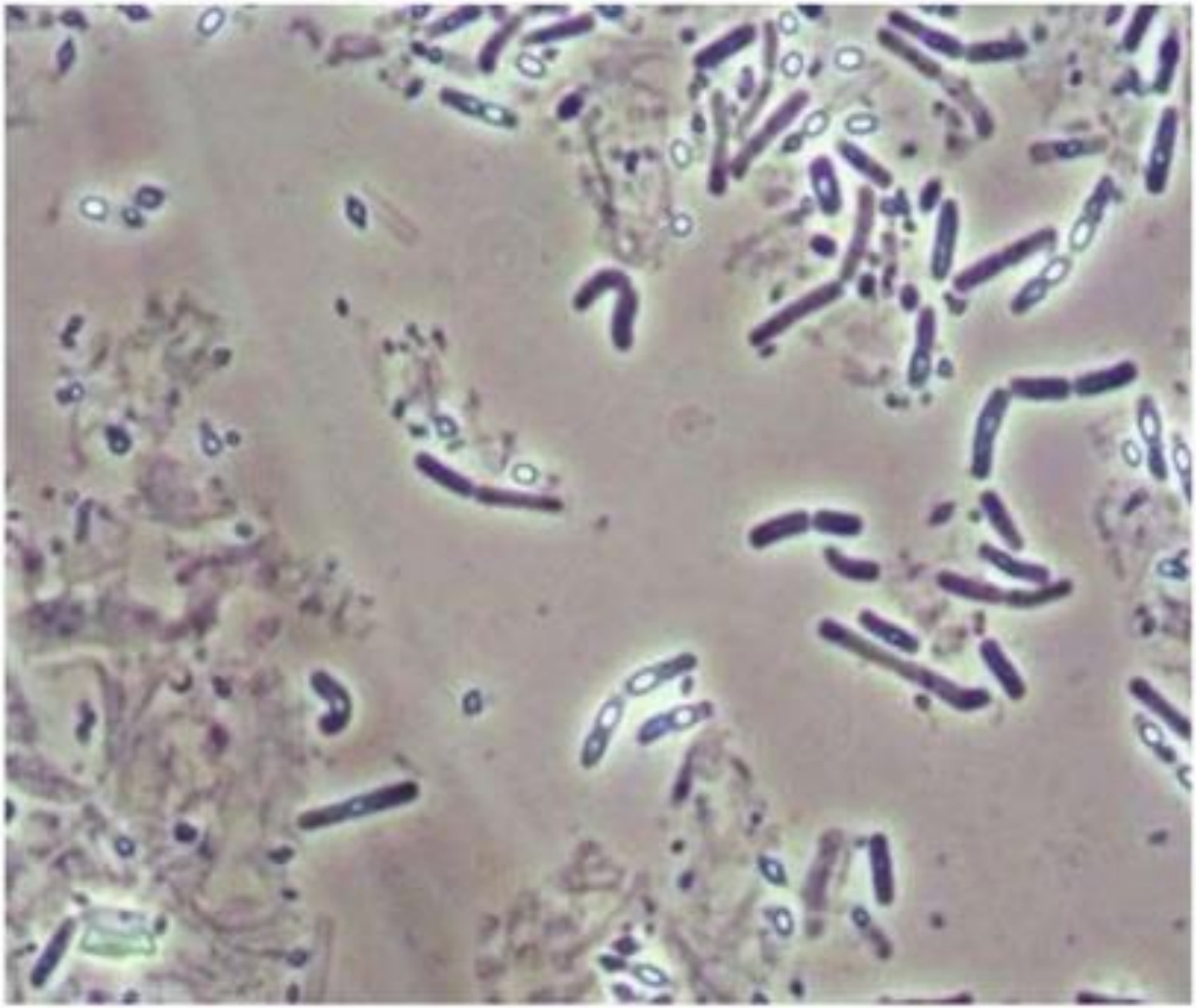
Phase Contrast Microscope Configuration





Phase contrast is obtained with the help of annular diaphragm by separating central & direct rays from diffracted rays.


Neutral density
Phase retarding
Phase plate
Obj
Spec
Cond
Phase annulus
A
B
C



Phase Imaging

Consider a transparent object with amplitude transmittance,

$$t_A(x,y) = \exp[i\phi(x,y)]$$

Expanding *exp*[*iφ*(*x*,*y*)],

$$e^{i\phi(x,y)} = 1 + i\phi(x,y) - \frac{1}{2}\phi^2(x,y) - \frac{1}{6}i\phi^3(x,y) + \frac{1}{24}\phi^4(x,y) + ...$$

For mathematical simplicity, assuming object has a magnitude of unity & finite extent of entrance & exit pupils are neglected. Also, there is a necessary condition to achieve linearity between phase-shift & intensity. The condition is that the variable part of the object-induced phase-shift, $\Delta\varphi(x,y)$, should be small compared with 2π radians. Applying approximation to amplitude transmittance,

$$t_A(x,y) = e^{i\phi_0} e^{i\Delta\phi} \approx e^{i\phi_0}[1 + i\Delta\phi]$$

$$I \approx \left|1 + i\Delta\phi(x,y)\right|^2 \approx 1$$

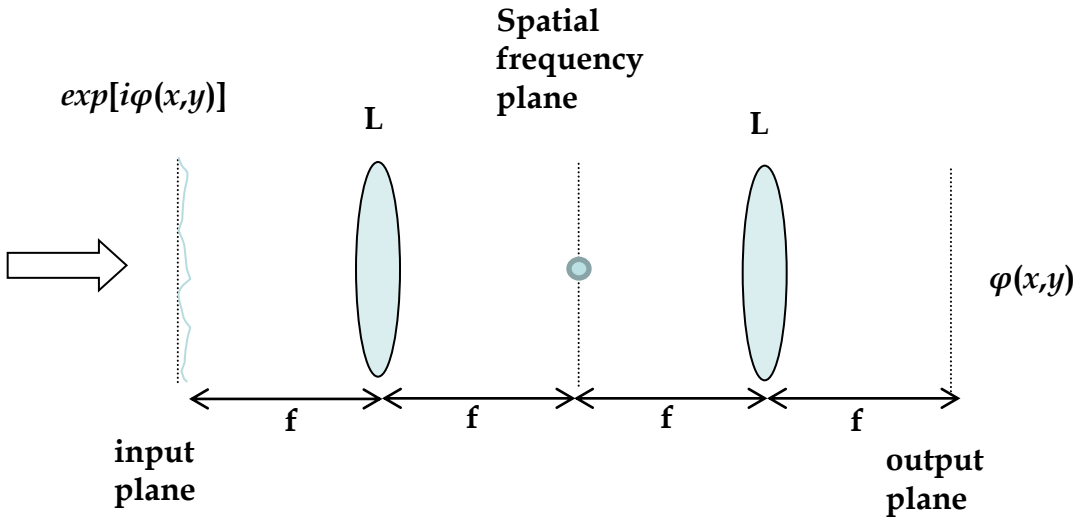
Phase-changing plate consists of a glass substrate on which a small transparent dielectric dot is deposited. Dot is centered on optical axis in focal plane & has a thickness & index of refraction such that it should change phase of focused light by either $\pi/2$ radians or $3\pi/2$ radians relative to phase retardation of diffracted light. If phase retardation is by $\pi/2$ radians, intensity in image plane becomes,

$$I \approx \left|\exp[i(\pi/2)] + i\Delta\phi(x,y)\right|^2 = \left|i\{1+\Delta\phi(x,y)\}\right|^2 \approx 1 + 2\Delta\phi(x,y)$$

Image intensity has become linearly related to variations of phase-shift $\Delta\varphi(x,y)$. This situation is referred to as positive phase contrast. If the phase retardation is by $3\pi/2$ radians, intensity in image plane becomes,

$$I \approx \left|\exp[i(3\pi/2)] + i\Delta\phi(x,y)\right|^2 = \left|-i\{1-\Delta\phi(x,y)\}\right|^2 \approx 1 - 2\Delta\phi(x,y)$$

This case is referred to as negative phase contrast.



Arrangement for phase contrast