MICROSCOPY

Dr. Bincy Joseph
Assistant Professor
PGIVER, Jaipur

MICROSCOPY

- Microbiology usually is concerned with organisms so small that they cannot be seen distinctly with the unaided eye
- Because of the nature of this discipline, the microscope is of crucial importance
- Thus it is important to understand how the microscope works and the way in which specimens are prepared for examination.

LENSES AND BENDING OF LIGHT

- When a ray of light passes from one medium to another, **refraction occurs—that is, the ray is bent at the interface**
- The **refractive index is a measure of how greatly a substance** slows the velocity of light
- The direction and magnitude of bending is determined by the refractive indices of the two media forming the interface
- When light passes from air into glass, a medium with a greater refractive index, it is slowed and bent toward the normal, a line perpendicular to the surface

Lenses and bending of light

- As light leaves glass and returns to air, a medium with a lower refractive index, it accelerates and is bent away from the normal
- Thus a prism bends light because glass has a different refractive index from air, and the light strikes its surface at an angle

Lenses and bending of light

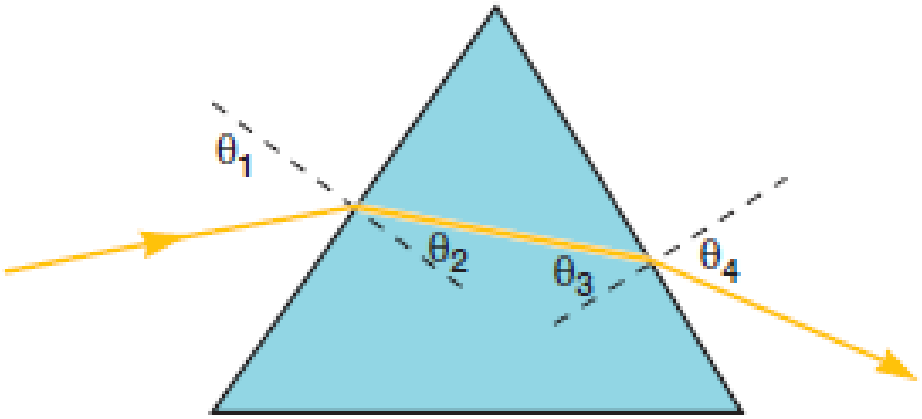


Figure 2.1 The Bending of Light by a Prism. Normals (lines perpendicular to the surface of the prism) are indicated by dashed lines. As light enters the glass, it is bent toward the first normal (angle θ_2 is less than θ_1). When light leaves the glass and returns to air, it is bent away from the second normal (θ_4 is greater than θ_3). As a result the prism bends light passing through it.

Lenses and bending of light

- Lenses act like a collection of prisms operating as a unit
- When the light source is distant so that parallel rays of light strike the lens,a convex lens will focus these rays at a specific point, the **focal Point (F)**
- **The distance between the center of the lens** and the focal point is called the **focal length (*f*)**

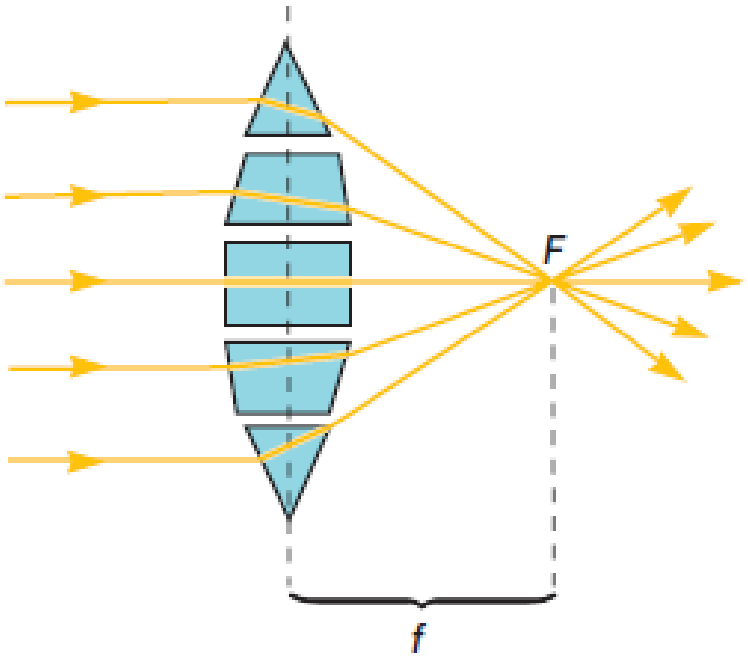


Figure 2.2 Lens Function. A lens functions somewhat like a collection of prisms. Light rays from a distant source are focused at the focal point *F*. The focal point lies a distance *f*, the focal length, from the lens center.

The Light Microscope

- Bright-field, Dark-field, Phase-contrast, and Fluorescence microscopes are most commonly used
- **Compound microscope**: the magnified image by objective lens is further magnified by eyepiece lense

Bright field microscope

- The ordinary microscope is called a **bright-field microscope because** it forms a dark image against a brighter background.
- The microscope consists of a sturdy metal body or stand composed of a base and an arm to which the remaining parts are attached
- **A light source, either a mirror or an electric illuminator,** is located in the base
- Two focusing knobs, the fine and coarse adjustment knobs, are located on the arm and can move either the stage or the nosepiece to focus the image.

Bright field microscope

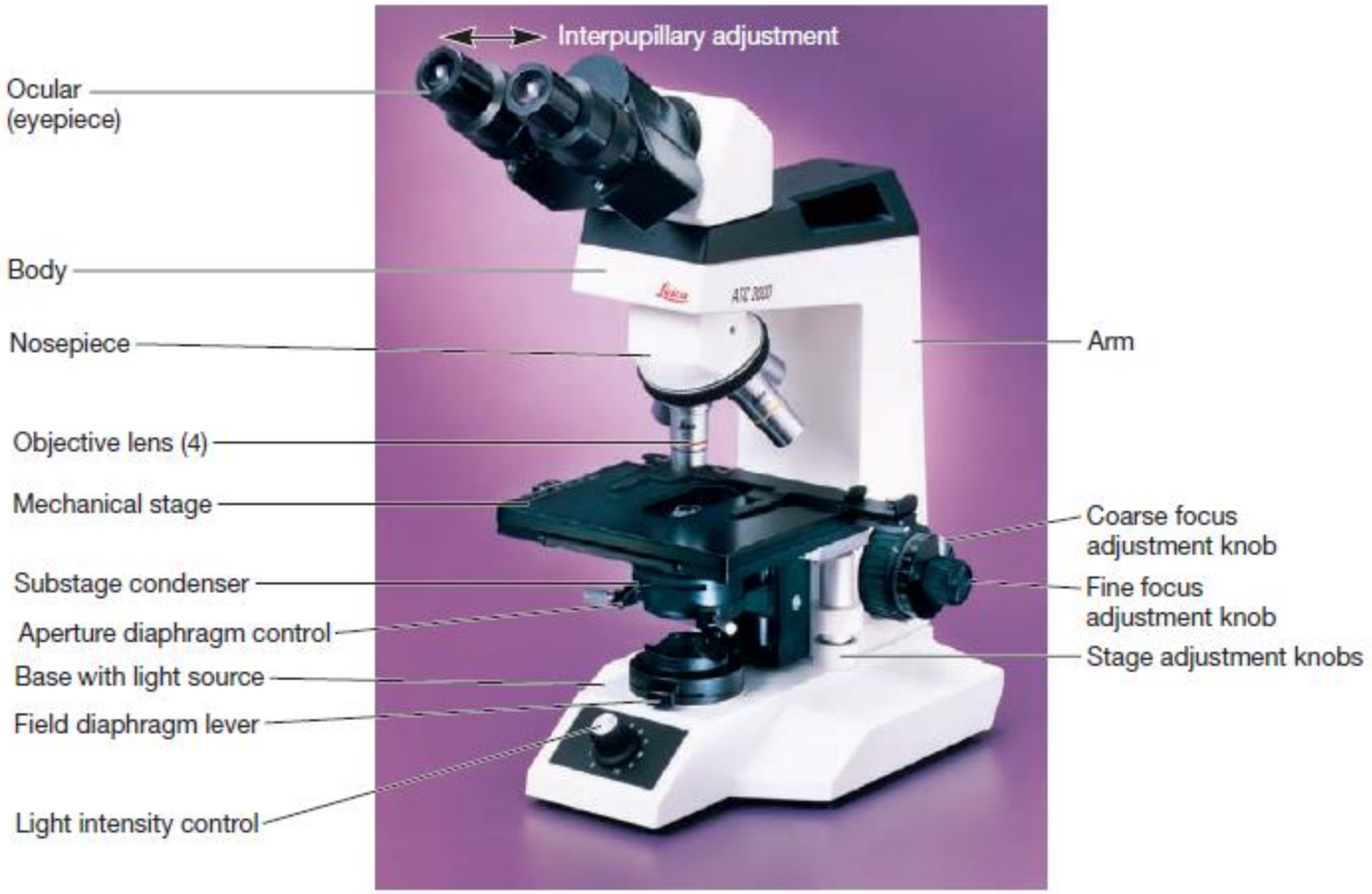
- The stage is positioned about halfway up the arm and holds microscope slides by either simple slide clips or a mechanical stage clip
- A mechanical stage allows the operator to move a slide around smoothly during viewing by use of stage control knobs
- The **substage condenser is mounted within or beneath the stage** and focuses a cone of light on the slide
- Its position often is fixed in simpler microscopes but can be adjusted vertically in more advanced models.

Bright field microscope

- The curved upper part of the arm holds the body assembly, to which a nosepiece and one or more **eyepieces or ocular lenses** are attached.
- More advanced microscopes have eyepieces for both eyes and are called binocular microscopes
- The nosepiece holds three to five **objective lenses of** differing magnifying power and can be rotated to position any objective beneath the body assembly
- Ideally a microscope should be **parfocal—that is, the image should remain in focus when objectives** are changed.

Bright field microscope

- The image one sees when viewing a specimen with a compound microscope is created by the objective and ocular lenses working together
- Light from the illuminated specimen is focused by the objective lens, creating an enlarged image within the microscope
- The ocular lens further magnifies this primary image
- The total magnification is calculated by multiplying the objective and eyepiece magnifications together.
- For example, if a 45 objective is used with a 10x eyepiece, the overall magnification of the specimen will be 450.


Interpupillary adjustment
Ocular (eyepiece)
Body
Nosepiece
Objective lens (4)
Mechanical stage
Substage condenser
Aperture diaphragm control
Base with light source
Field diaphragm lever
Light intensity control
Arm
Coarse focus adjustment knob
Fine focus adjustment knob
Stage adjustment knobs

MICROSCOPIC RESOLUTION

- **Resolution is the ability of a** lens to separate or distinguish between small objects that are close together
- The Abbé equation states that the minimal distance *(d) between two* objects that reveals them as separate entities depends on the
  - wavelength of light (λ) used to illuminate the specimen
  - **numerical aperture of the lens (*n sinθ*), *which is the ability*** of the lens to gather light.

$$d = \frac{0.5\lambda}{n \sin \theta}$$

Microscopic resolution

- As *d becomes smaller, the resolution increases, and finer detail* can be discerned in a specimen
- *d becomes smaller as the wavelength* of light used decreases and as the numerical aperture (NA) increases.
- Thus the greatest resolution is obtained using a lens with the largest possible NA and light of the shortest wavelength, light at the blue end of the visible spectrum

Microscopic resolution

- It is defined by two components:n is the refractive index of the medium in which the lens works (e.g., air) and θ is 1/2 the angle of the cone of light entering an objective
- If the cone of light has a very wide angle and spreads out rapidly after passing through a specimen, closely packed objects appear widely separated and are resolved
- The angle of the cone of light that can enter a lens depends on the refractive index (n) of the medium in which the lens works, as well as upon the objective itself
- The refractive index for air is 1.00 and sin θ cannot be greater than 1 (the maximum is 90° and sin 90° is 1.00)

Numerical aperture

- The only practical way to raise the numerical
- aperture above 1.00, and therefore achieve higher resolution, is to increase the refractive index with immersion oil, a colorless liquid with the same refractive index as glass
- **If air is replaced** with immersion oil, many light rays that did not enter the objective due to reflection and refraction at the surfaces of the objective lens and slide will now do so
- **An increase in numerical** aperture and resolution results

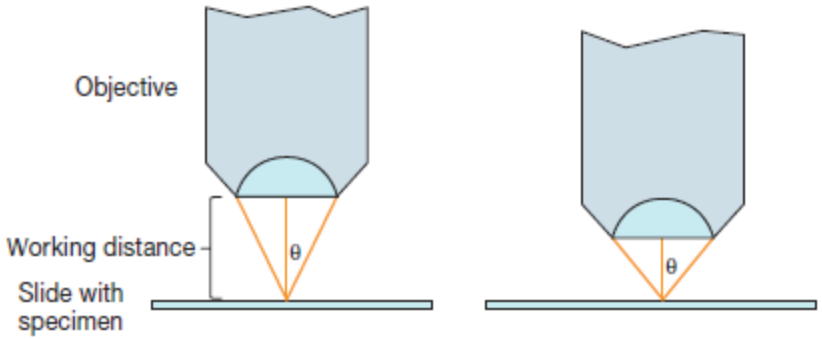


Figure 2.5 Numerical Aperture in Microscopy. The angular aperture θ is 1/2 the angle of the cone of light that enters a lens from a specimen, and the numerical aperture is $n \sin \theta$. In the right-hand illustration the lens has larger angular and numerical apertures; its resolution is greater and its working distance smaller.

Numerical aperture

- Numerical aperture is related to another characteristic of an objective lens, the working distance.
- The **working distance of an** objective is the distance between the front surface of the lens and the surface of the cover glass (if one is used) or the specimen when it is in sharp focus
- Objectives with large numerical apertures and great resolving power have short working distances

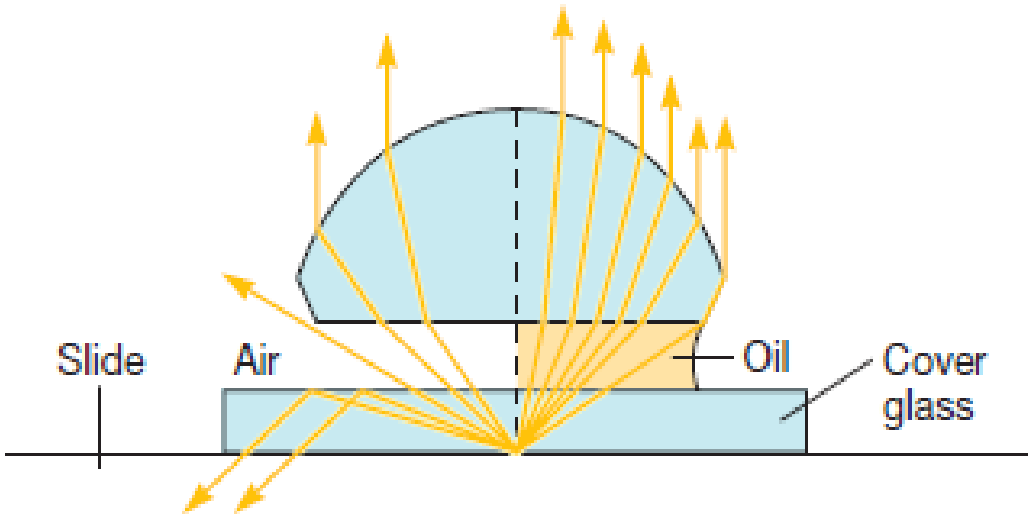


Figure 2.6 The Oil Immersion Objective. An oil immersion objective operating in air and with immersion oil.

- The maximum theoretical resolving power of a microscope with an oil immersion objective (numerical aperture of 1.25) and blue-green light

is approximately 0.2 m.

$$d = \frac{(0.5)(530\ \text{nm})}{1.25} = 212\ \text{nm or } 0.2\mu\text{m}$$

Table 2.2 The Properties of Microscope Objectives

Property	Objective			
	Scanning	Low Power	High Power	Oil Immersion
Magnification	4×	10×	40–45×	90–100×
Numerical aperture	0.10	0.25	0.55–0.65	1.25–1.4
Approximate focal length (*f*)	40 mm	16 mm	4 mm	1.8–2.0 mm
Working distance	17-20 mm	4-8 mm	0.5–0.7 mm	0.1 mm
Approximate resolving power with light of 450 nm (blue light)	2.3 μm	0.9 μm	0.35 μm	0.18 μm

THE DARK FIELD MICROSCOPE

- To observe living,unstained cells and organisms
- A hollow cone of light is focused on the specimen in such a way that unreflected and unrefracted rays do not enter the objective
- Only light that has been reflected or refracted by the specimen forms an image
- **The field** surrounding a specimen appears black, while the object itself is brightly illuminated
- ***The dark-field microscope*** can reveal considerable internal structure in larger eucaryotic icroorganisms
- *It also is used to identify certain bacteria*
- like the thin and distinctively shaped *Treponema pallidum the causative agent of syphilis, leptospira and other spirochaetes*

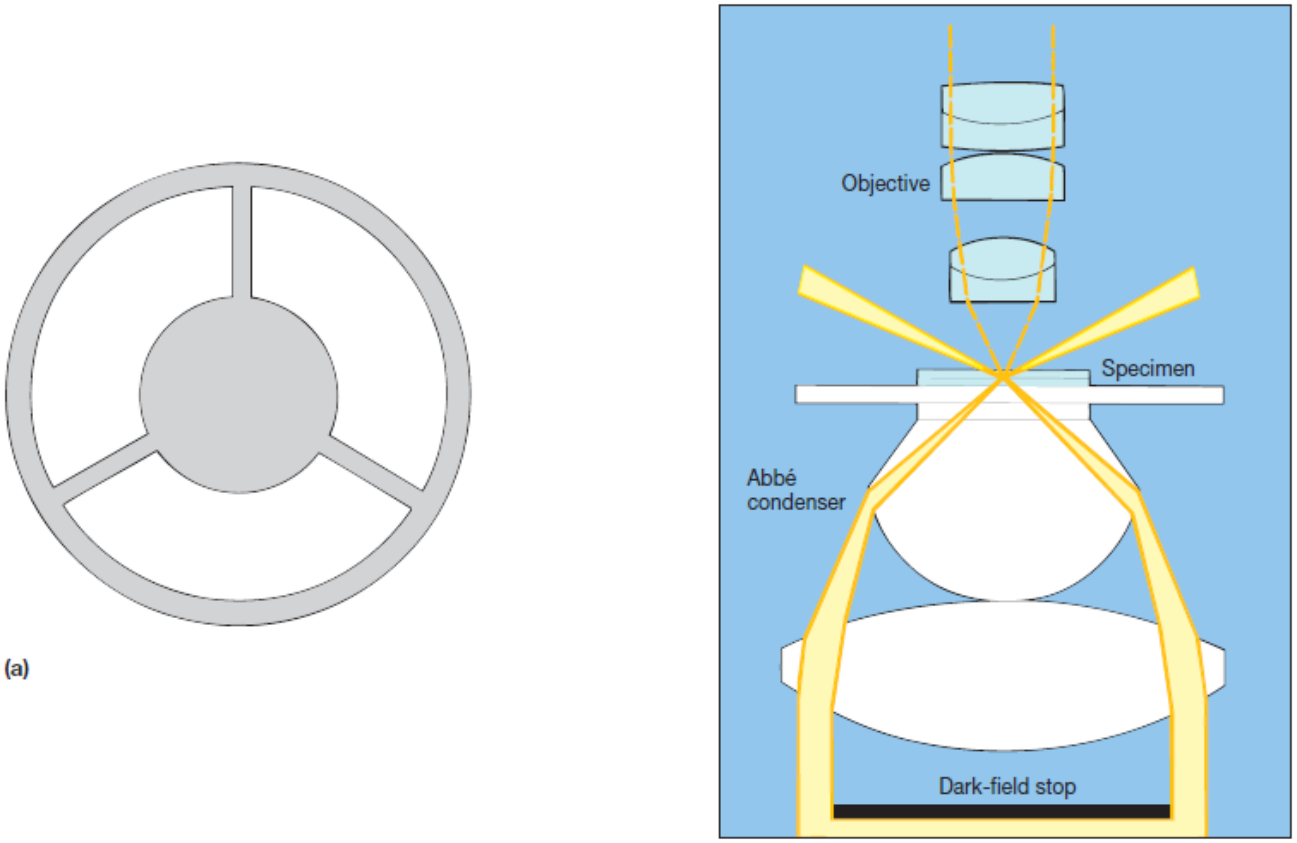
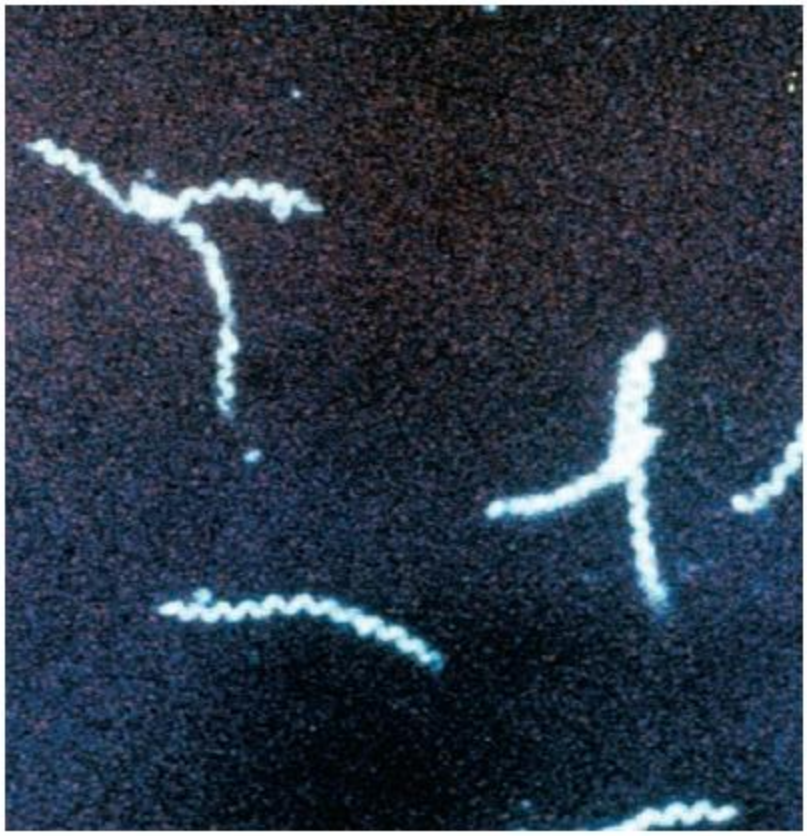


Figure 2.7 Dark-Field Microscopy. The simplest way to convert a microscope to dark-field microscopy is to place (**a**) a dark-field stop underneath (**b**) the condenser lens system. The condenser then produces a hollow cone of light so that the only light entering the objective comes from the specimen.



Phase contrast microscope

- Unpigmented living cells are not clearly visible in the brightfield microscope because there is little difference in contrast between the cells and water
- A **phase-contrast microscope converts slight differences** in refractive index and cell density into easily detected variations in light intensity and is an excellent way to observe living cells
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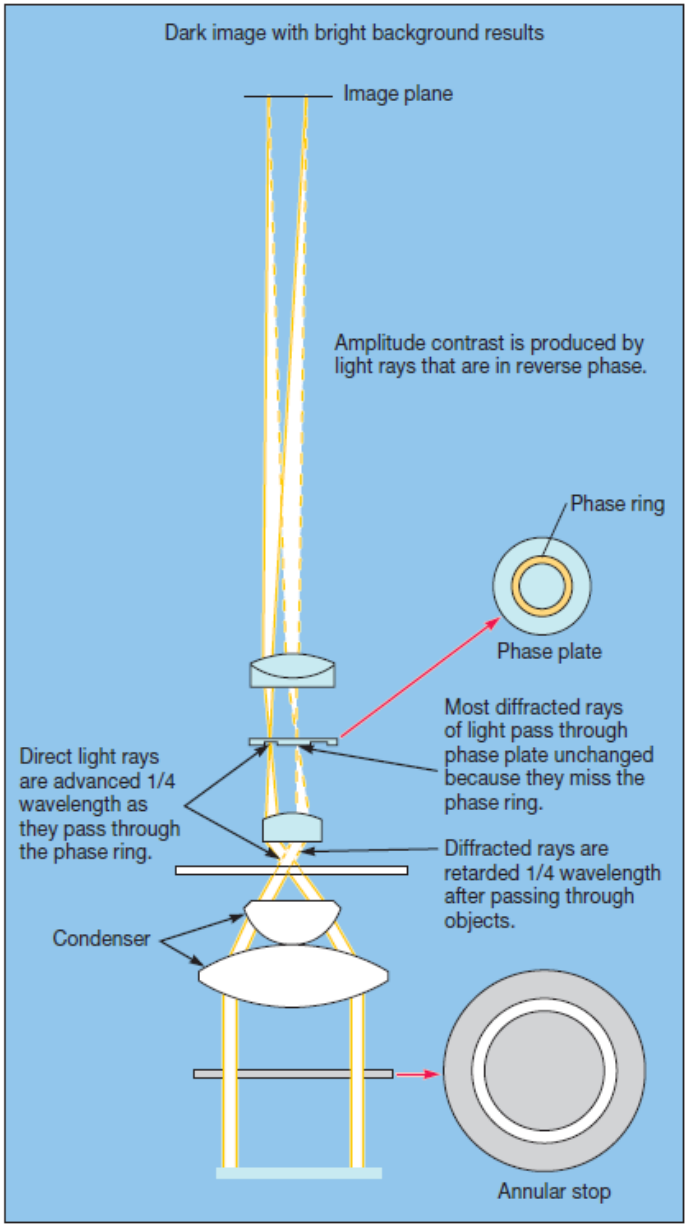


Figure 2.9 Phase-Contrast Microscopy. The optics of a dark-phase-contrast microscope.

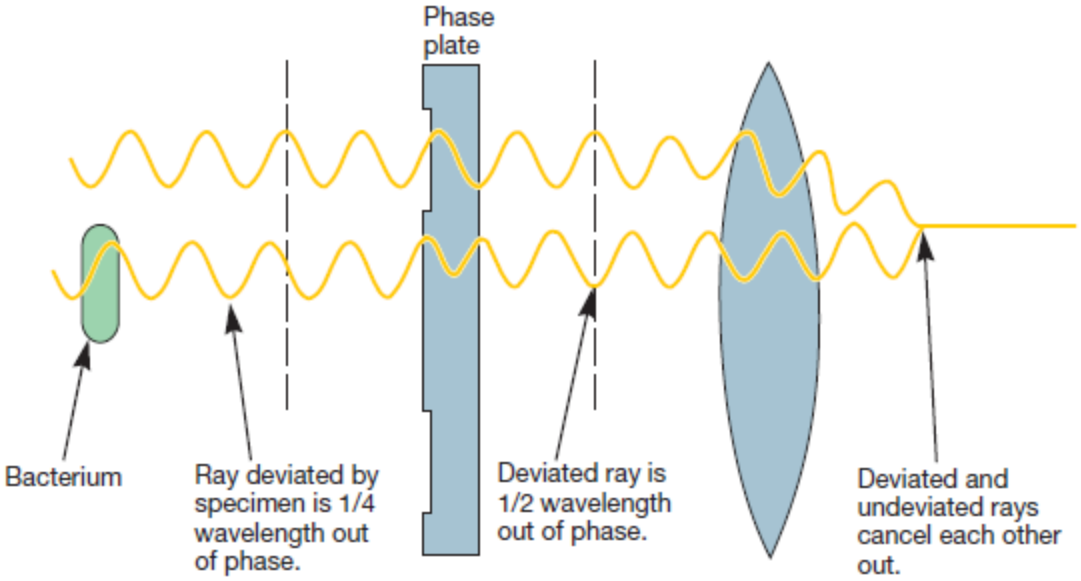


Figure 2.10 The Production of Contrast in Phase Microscopy. The behavior of deviated and undeviated or undiffracted light rays in the dark-phase-contrast microscope. Because the light rays tend to cancel each other out, the image of the specimen will be dark against a brighter background.

Phase contrast microscope

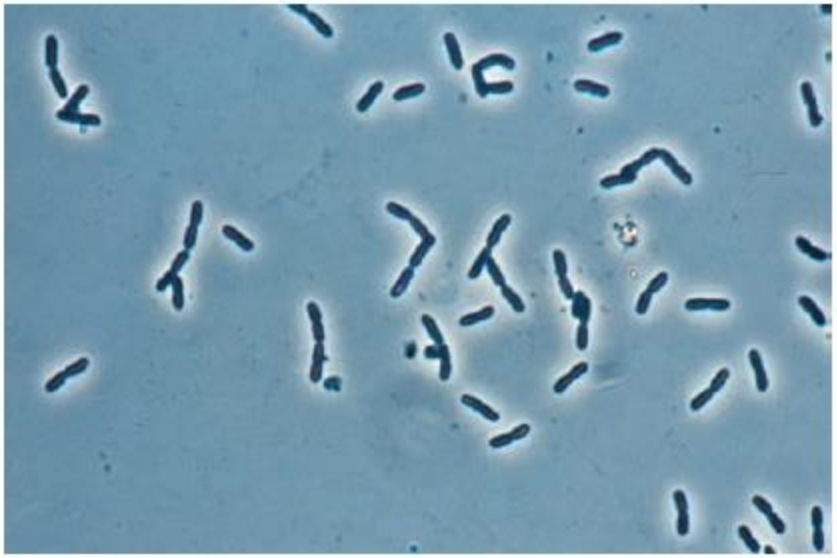
- The condenser of a phase-contrast microscope has an annular stop, an opaque disk with a thin transparent ring, which produces a hollow cone of light
- **As this cone passes through a** cell, some light rays are bent due to variations in density and refractive index within the specimen and are retarded by about ¼ wavelength
- The deviated light is focused to form an image of the object
- Undeviated light rays strike a phase ring in the phase plate,a special optical disk located in the objective, while the deviated rays miss the ring and pass through the rest of the plate.

Phase contrast microscopy

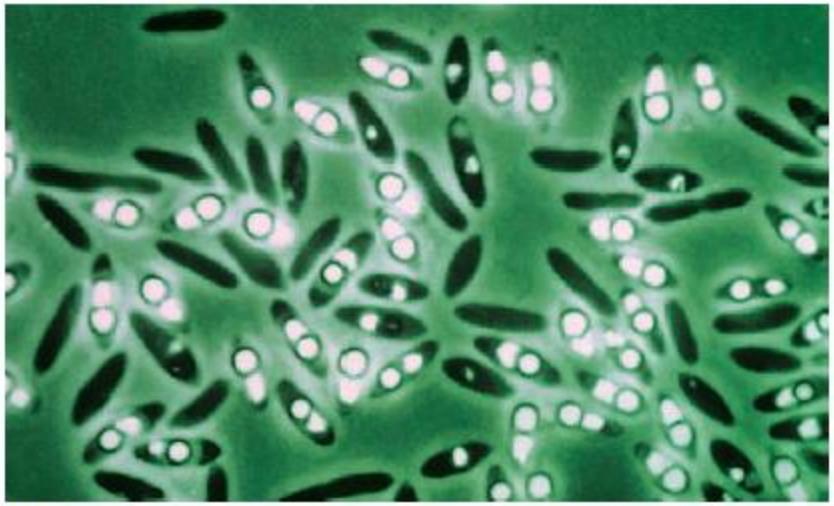
- If the phase ring is constructed in such a way that the undeviated light passing through it is advanced by 1/4 wavelength, the deviated and undeviated waves will be about 1/2 wavelength out of phase and will cancel each other when they come together to form an image
- The background, formed by undeviated light, is bright, while the unstained object appears dark and well-defined
- This type of microscopy is called dark-phase-contrast microscopy

Phase contrast microscopy

- Phase-contrast microscopy is especially useful for studyingmicrobial motility, determining the shape of living cells, and detectingbacterial components such as endospores and inclusion bodies



(c) *Pseudomonas:* phase-contrast microscopy



(d) *Desulfotomaculum:* phase-contrast microscopy

Thank you