RAMA UNIVERSITY

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DEPARTMENT OF BIOTECHNOLOGY
FACULTY OF ENGINEERING & TECHNOLOGY

LT.2. Dark Field microscopy & Phase Contrast microscopy

Content outline

1. Bright Field microscope
2. Phase contrast microscope

Dark Field microscopy

•This type of microscope are used to observe living, unstained cells and organisms simply by illuminating the specimen. In dark field microscopy, no direct light from the condenser enters the objective lens. The light is focused on specimen in such a way that only refracted or reflected light by specimen enters the objective and forms the image.

•The dark field condenser produces a circle of light. The light is at an extremely oblique angle to the surface of the slide. This oblique light comes to a focus on the specimen. It then diverges so strongly that no direct light enters the objective (figure 9.1). This type of illumination is a **hollow cone** of light.

•The numerical aperture of the condenser must be larger than the numerical aperture of the objective lens in order to prevent direct light from entering the objective lens. This is no problem for low magnification dry objectives if a 0.95 NA condenser is used. This is a problem however for high NA objectives. Here the condenser must have a very high NA such as 1.45 and be used with an objective of no more than 1.25 NA.

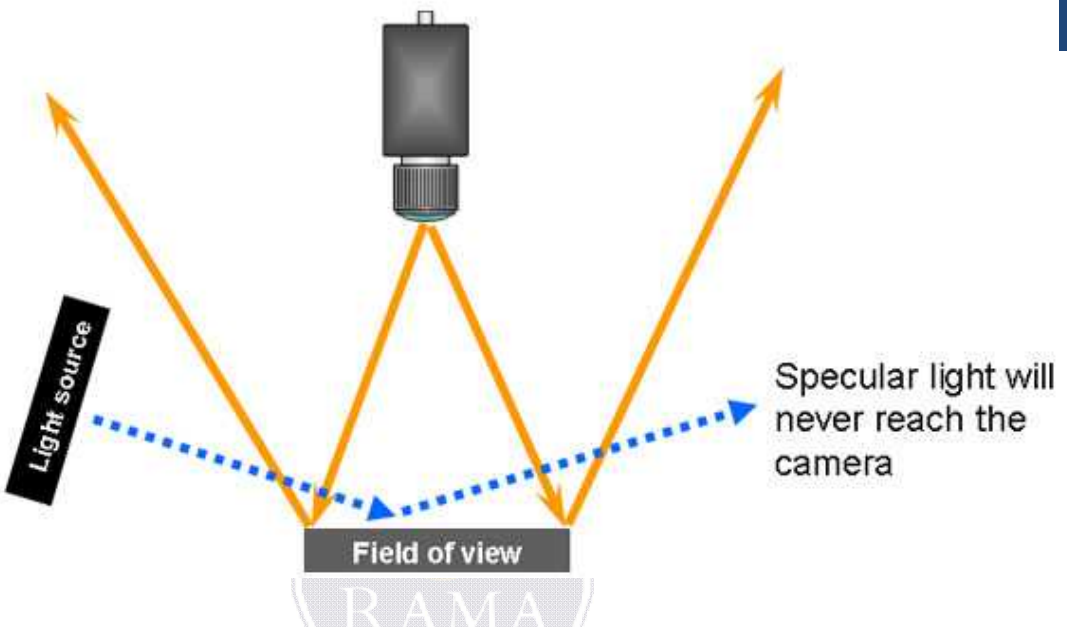
•The field surrounding a specimen appears black, while the object itself is brightly illuminated. Because the background is dark, this type of microscopy is called dark-field microscopy. Considerable internal structure is often visible in larger eucaryotic microorganisms. The dark-field microscope is used to identify bacteria like the thin and distinctively shaped *Treponema pallidum* , the causative agent of syphilis.

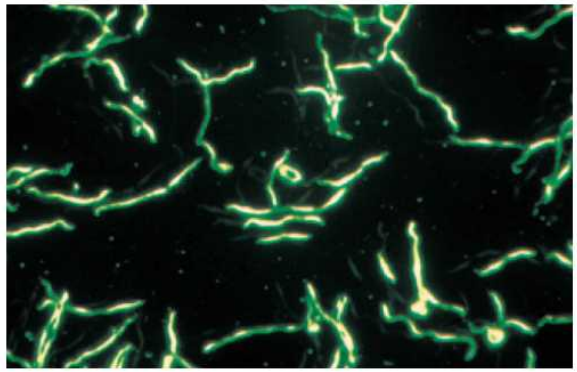
Dark Field Lighting:

Diffused light is reflected into the camera; specular light is reflected away

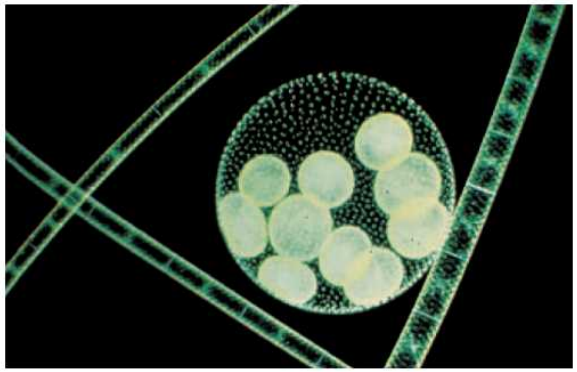
Light source is outside the “W”

Light is reflected away except for textured surfaced and elevation change





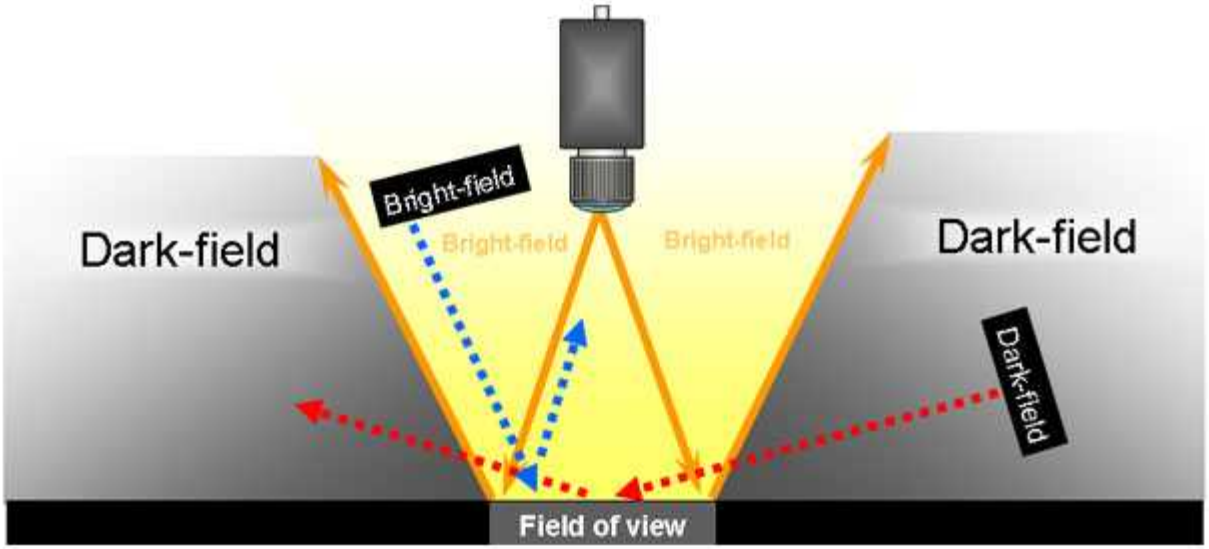
(a)



(b)

(a) *Treponema pallidum,* the spirochete that causes syphilis; dark-field microscopy (500). **(b)** *Volvox* and *Spirogyra;* dark-field microscopy (175). Note daughter colonies within the mature *Volvox* colony (center) and the spiral chloroplasts of *Spirogyra* (left and right). Please note the dark background around the specimen.

Difference between Dark field & Bright Field microscopy



Reflected: light is the same angle as the source

Bright field: light is reflected **into** the camera

Dark field: light is reflected **away** from the camera

Phase Contrast Microscope

Phase contrast microscopy

•Phase contrast microscopy, first described in 1934 by Dutch physicist Frits Zernike, is a contrast-enhancing optical technique that can be utilized to produce high-contrast images of transparent specimens, such as living cells (usually in culture), microorganisms, thin tissue slices, lithographic patterns, fibers, latex dispersions, glass fragments, and subcellular particles (including nuclei and other organelles).

•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.

•Phase-contrast microscopy is especially useful for studying microbial motility, determining the shape of living cells, and detecting bacterial components such as endospores and inclusion bodies that contain poly-β-hydroxybutyrate, polymetaphosphate,sulfur, or other substances.

➢One of the major advantages of phase contrast microscopy is that living cells can be examined in their natural state without previously being killed, fixed, and stained.

A typical Phase Contrast Microscope

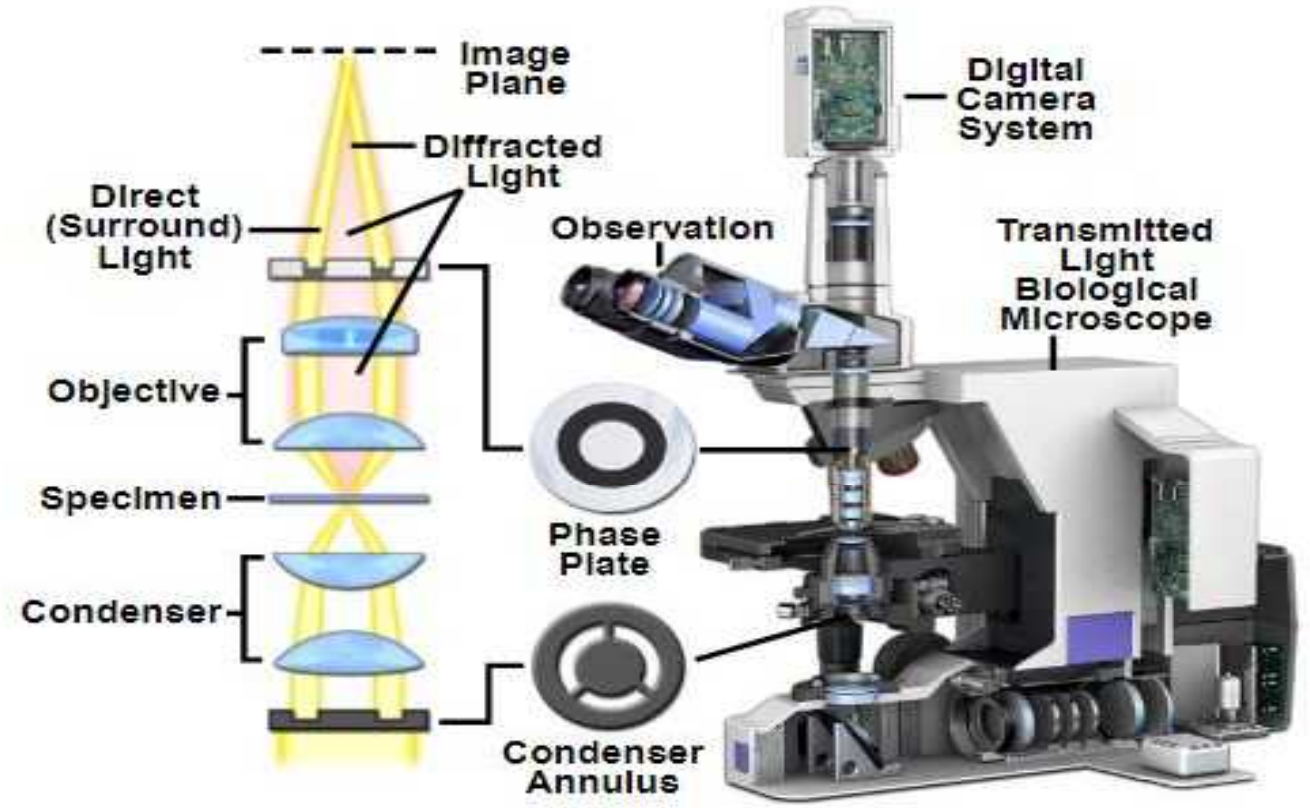
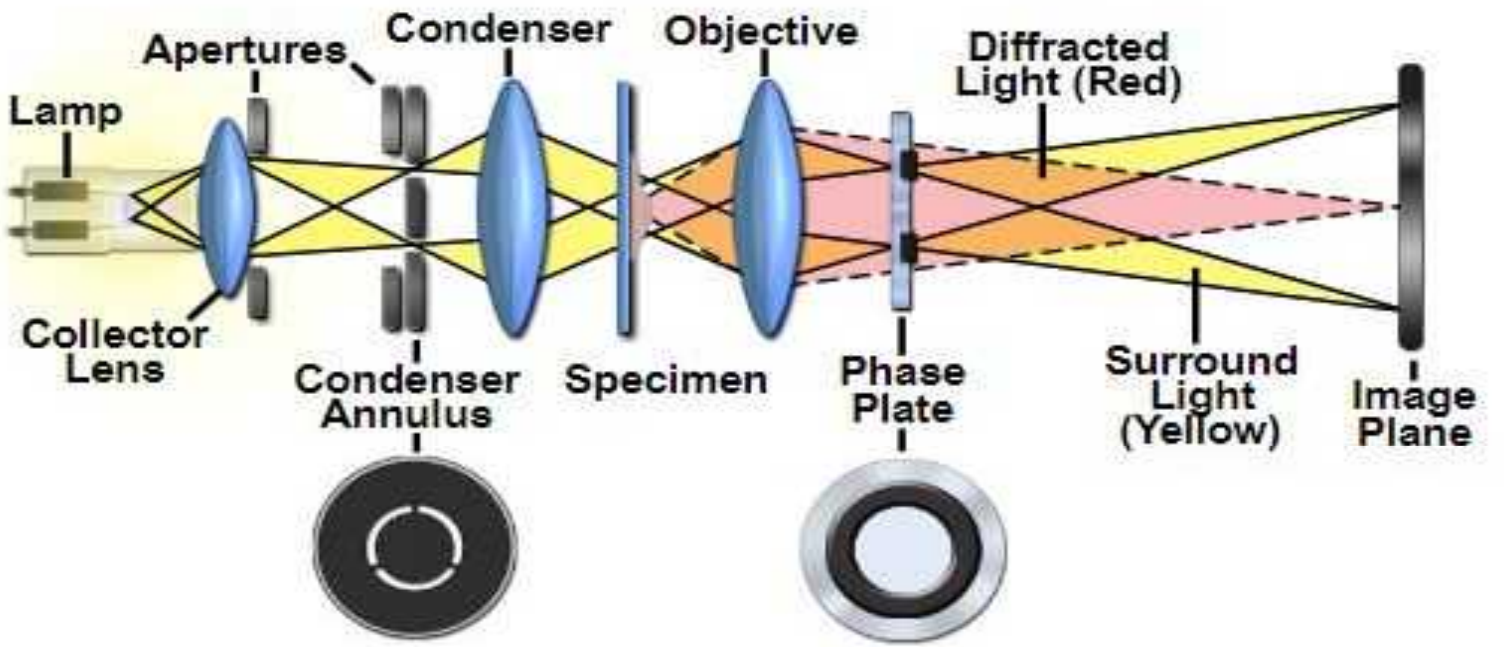


Image source: Google

Partially coherent illumination produced by the tungsten-halogen lamp is directed through a collector lens and focused on a specialized annulus (labeled **condenser annulus**) positioned in the substage condenser front focal plane. Wavefronts passing through the annulus illuminate the specimen and either undeviated or are diffracted and retarded in phase by structures and phase gradients present in the specimen. Undeviated and diffracted light collected by the objective is segregated at the rear focal plane by a **phase plate** and focused at the intermediate image plane to form the final phase contrast image observed in the eyepieces. Light waves that are diffracted and shifted in phase by the specimen (termed a **phase object**) can be transformed by phase contrast into amplitude differences that are observable in the eyepieces. Large, extended specimens are also easily visualized with phase contrast optics due to diffraction and scattering phenomena that occur at the edges of these objects. The background, formed by undeviated light, isbright, while the unstained object appears dark and well-defined. This type of microscopy is called **dark-phase-contrast microscopy.**

Ray diagram of Phase Contrast Microscope



The most important concept underlying the design of a phase contrast microscope is the segregation of surround and diffracted wavefronts emerging from the specimen, which are projected onto different locations in the objective rear focal plane (the **diffraction** plane at the objective rear aperture). In addition, the amplitude of the surround (undeviated) light must be reduced and the phase advanced or retarded (by a quarter wavelength) in order to maximize differences in intensity between the specimen and background in the image plane. The mechanism for generating relative phase retardation is a two-step process, with the diffracted waves being retarded in phase by a quarter wavelength at the specimen, while the surround waves are advanced (or retarded) in phase by a phase plate positioned in or very near the objective rear focal plane. Only two specialized accessories are required to convert a brightfield microscope for phase contrast observation. A specially designed annular diaphragm, which is matched in diameter and optically conjugate to an internal phase plate residing in the objective rear focal plane, is placed in the condenser front focal plane.

The **condenser annulus** is typically constructed as an opaque flat-black (light absorbing) plate with a transparent annular ring, which is positioned in the front focal plane (aperture) of the condenser so the specimen can be illuminated by defocused, parallel light wavefronts emanating from the ring. The microscope condenser images the annular diaphragm at infinity, while the objective produces an image at the rear focal plane.

The Differential Interference Contrast Microscope

The differential interference contrast (DIC) microscope is similar to the phase-contrast microscope in that it creates an image by detecting differences in refractive indices and thickness. Two beams of plane polarized light at right angles to each other are generated by prisms. In one design, the object beam passes through the specimen, while the reference beam passes through a clear area of the slide. After passing through the specimen, the two beams are combined and interfere with each other to form an image. A live, unstained specimen appears brightly colored and three-dimensional Structures such as cell walls, endospores, granules, vacuoles, and eucaryotic nuclei are clearly visible.

Test your understanding

1.Bright- field microscope forms a

a. a bright image against a brighter background

b. a dark image against a brighter background

c. a bright image against a darker background

d. None of these

2.Which of the following is used to visualise live cells?

Scanning Elecctron microscope

Transmission Electron Microscope

Phase Ccontrast microscope

All of the above

3.Phase-contrast microscopy is especially useful for studying

a. microbial motility

b. determining the shape of living cells

c. Detection of endospores

d. All of the above

Reference & Further Reading

1. Wilson, K, Walker, J., Principles and Techniques of Practical Biochemistry. 5th Ed. - Cambridge University Press,. Cambridge 1999.
2. Biotechniques, Theory & Practice: Second Edition by SVS Rana, Rustogi Publications.
3. Biochemical Methods of Analysis, Saroj Dua And Neera Garg : Narosa Publishing House, New Delhi.
4. Bioanalytical Techniques, M.L. Srivastava, Narosa Publishing House, New Delhi.