Biotechnological instrumentation

Biotechnological instrumentation refers to the tools and machines used in biotechnology for analyzing, manipulating, and understanding biological systems. These instruments play a crucial role in research and development across industries such as medicine, agriculture, and environmental science. Essential equipment includes spectrophotometers, centrifuges, and PCR machines, each contributing to advancements in genetic engineering, drug discovery, and biological data analysis.

Definition

Biotechnological Instrumentation: Tools and devices employed in biotechnology for analyzing and manipulating biological materials.

I. INSTRUMENTS OF MICROSCOPY

1. THE LIGHT MICROSCOPE

The light microscopes use light to visualize images, which Many types is under this category who include:

- brightfield microscopes
- darkfield microscopes
- phase-contrast microscopes
- differential interference contrast microscopes,
- fluorescence microscopes,
- confocal scanning laser microscopes,
- two-photon microscopes.

1) Brightfield Microscopes

The brightfield microscope, is the most commonly used type of microscope, is a compound microscope with two or more lenses that produce a dark image on a bright background. Some brightfield microscopes are monocular (having a single eyepiece), though most newer brightfield microscopes are binocular (having two eyepieces); in either case, each eyepiece contains a lens called an ocular lens. The ocular lenses typically magnify images 10 times (10\times). At the other end of the body tube are a set of objective lenses on a rotating nosepiece. The magnification of these objective lenses typically ranges from 4\times to 100\times, with the magnification for each lens

designated on the metal casing of the lens. The ocular and objective lenses work together to create a magnified image. The total magnification is the product of the ocular magnification times the objective magnification:

Ocular magnification x objective magnification

For example, if a 40 objective lens is selected and the ocular lens is 10 , the total magnification would be:

(40x) (10x) = 400

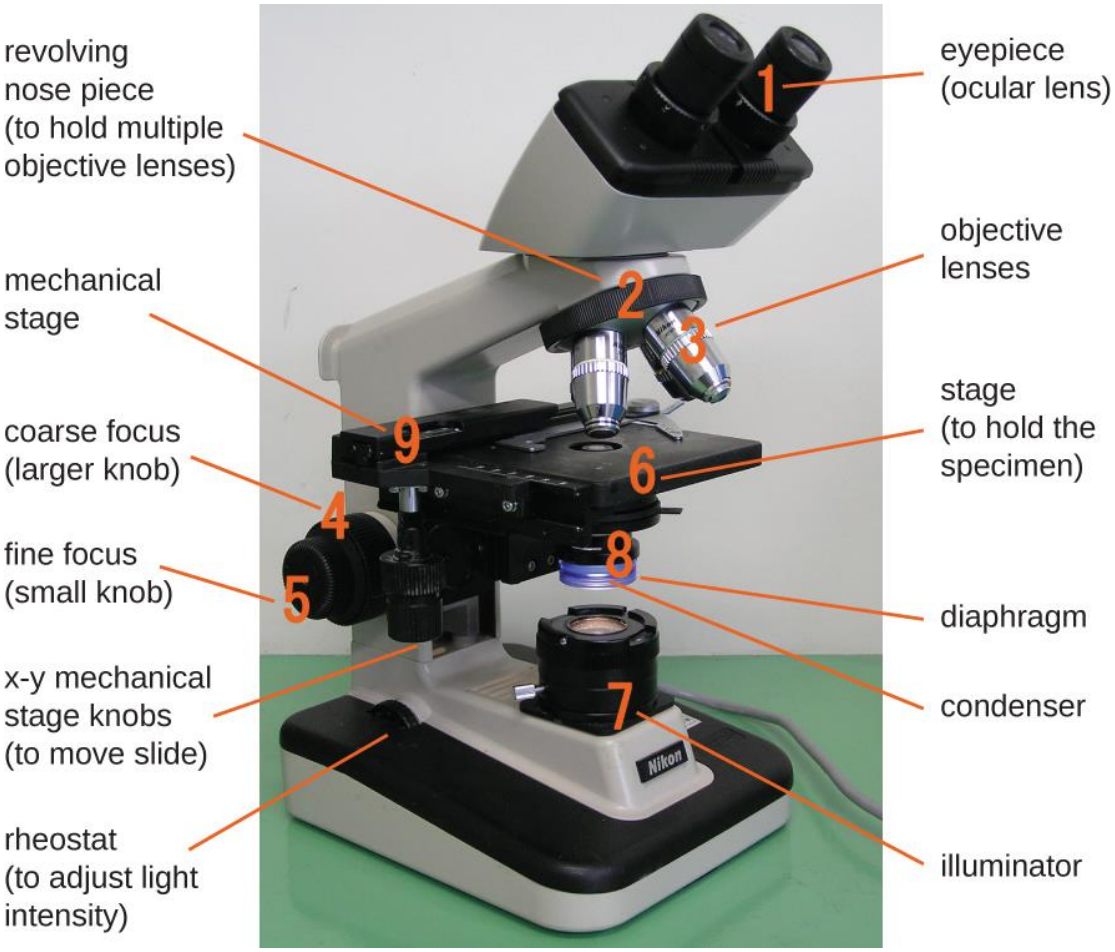

Fig 1: Components of a typical brightfield microscope.

https://ecampusontario.pressbooks.pub/app/uploads/sites/159/2018/07/OSC_Microbio_02_03_Brightfiel.jpg

a) application of microscopy

The specimen is placed on a glass slide, which is then clipped into place on the stage (a platform) of the microscope. Once the slide is secured the x-y mechanical stage knobs. These knobs move the slide on the surface of the stage, but do not raise or lower the stage. Once the specimen is centred over the light, the stage position can be raised or lowered to focus the image. The coarse focusing knob is used for large-scale movements with 4\times and 10\times objective lenses; the fine focusing knob is used for small-scale movements, especially with 40\times or 100\times objective lenses.

When images are magnified, they become dimmer because there is less light per unit area of image. Highly magnified images produced by microscopes, therefore, require intense lighting. In a brightfield microscope, this light is provided by an illuminator, which is typically a high-intensity bulb below the stage. Light from the illuminator passes up through condenser lens (located below the stage), which focuses all of the light rays on the specimen to maximize illumination. The position of the condenser can be optimized using the attached condenser focus knob; once the optimal distance is established, the condenser should not be moved to adjust the brightness. If less-than-maximal light levels are needed, the amount of light striking the specimen can be easily adjusted by opening or closing a diaphragm between the condenser and the specimen. In some cases, brightness can also be adjusted using the rheostat, a dimmer switch that controls the intensity of the illuminator.

A brightfield microscope creates an image by directing light from the illuminator at the specimen; this light is differentially transmitted, absorbed, reflected, or refracted by different structures. Different colours can behave differently as they interact with chromophores (pigments that absorb and reflect particular wavelengths of light) in parts of the specimen. Often, chromophores are artificially added to the specimen using stains, which serve to increase contrast and resolution. In general, structures in the specimen will appear darker, to various extents, than the bright background, creating maximally sharp images at magnifications up to about 1000 . Further magnification would create a larger image, but without increased resolution. This allows us to see objects as small as bacteria, which are visible at about 400 or so, but not smaller objects such as viruses.

At very high magnifications, resolution may be compromised when light passes through the small amount of air between the specimen and the lens. This is due to the large difference between the refractive indices of air and glass; the air scatters the light rays before they can be focused by the lens. To solve this problem, a drop of oil can be used to fill the space between the specimen and an oil immersion lens, a special lens designed to be used with immersion oils. Since the oil has a refractive index very similar to that of glass, it increases the maximum angle at which light leaving the specimen can strike the lens. This increases the light collected and, thus, the resolution of the image **(Figure 2**). A variety of oils can be used for different types of light.

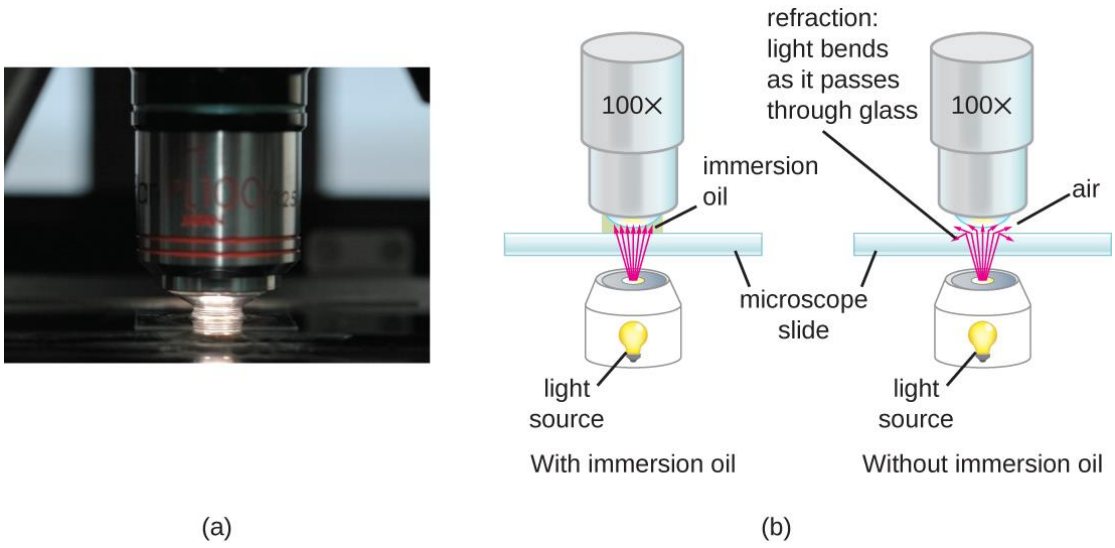

Fig 2: (a) Oil immersion lenses like this one are used to improve resolution. (b) Because immersion oil and glass have very similar refractive indices, there is a minimal amount of refraction before the light reaches the lens. Without immersion oil, light scatters as it passes through the air above the slide, degrading the resolution of the image. https://ecampusontario.pressbooks.pub/microbio/chapter/instruments-of-microscopy/

2) Darkfield Microscopy

A darkfield microscope is a brightfield microscope that has a small but significant modification to the condenser. A small, opaque disk (about 1 cm in diameter) is placed between the illuminator and the condenser lens. This opaque light stop, as the disk is called, blocks most of the light from the illuminator as it passes through the condenser

on its way to the objective lens, producing a hollow cone of light that is focused on the specimen. The only light that reaches the objective is light that has been refracted or reflected by structures in the specimen. The resulting image typically shows bright objects on a dark background (**Figure 3**).

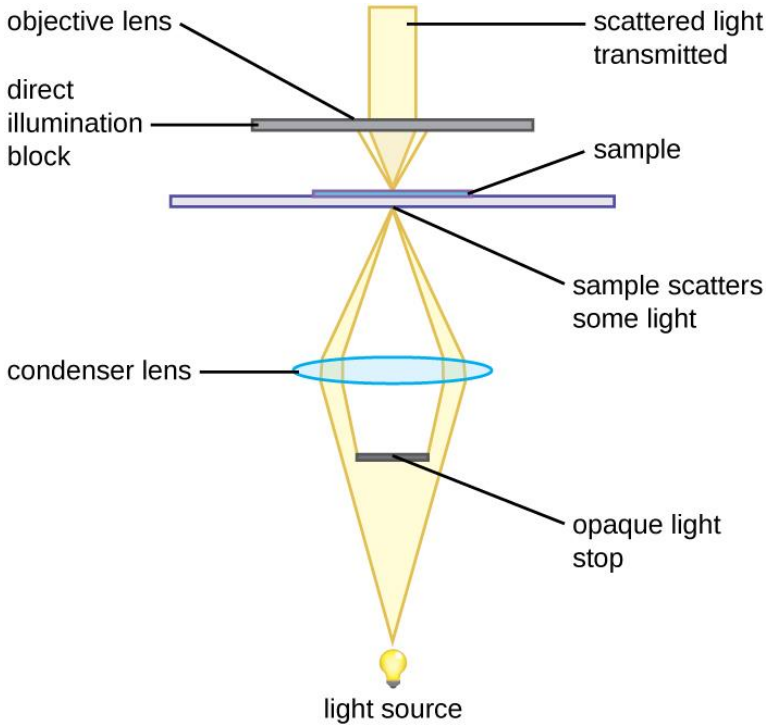

Fig 3: An opaque light stop inserted into a brightfield microscope is used to produce a darkfield image. The light stop blocks light traveling directly from the illuminator to the objective lens, allowing only light reflected or refracted off the specimen to reach the eye.

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This darkfield provides a high degree of contrast and allows samples to stand out against complex backgrounds with little or no effort.

Samples with very low contrast inherent to brightfield microscopy shine on the darkfield. Darkfield illumination is particularly suitable for observing tiny living aquatic organisms, diatoms, small insects, bones, fibers, hair, unstained bacteria, yeast, tissue culture cells, and protozoa. (figure 4)

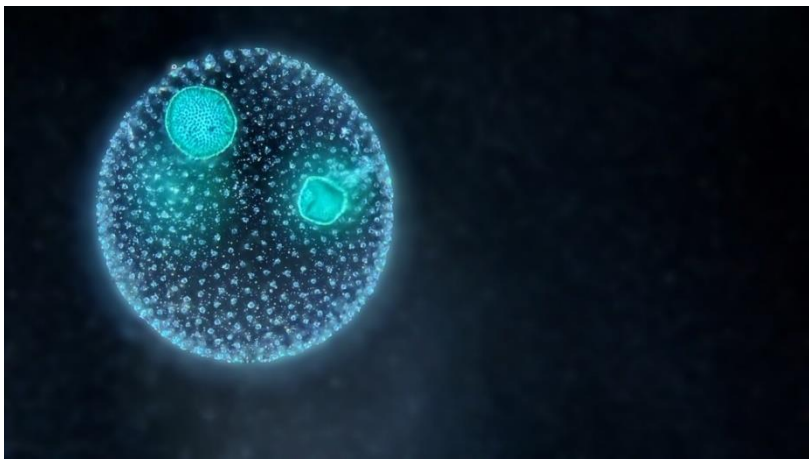

Fig 4: A colony of green planktonic algae Volvox under the dark field microscope. Image Credit: *D. Kucharski K. Kucharska/Shutterstock.com* (*https://d36nqgmw98q4v5.cloudfront.net/image-handler/ts/20221019080205/ri/750/src/images/Article_Images/ImageForArticle_23355_16661809246465900.jpg*)

3) Phase-Contrast Microscopes

Phase-contrast microscopes use refraction and interference caused by structures in a specimen to create high-contrast, high-resolution images without staining. It is the oldest and simplest type of microscope that creates an image by altering the wavelengths of light rays passing through the specimen. To create altered wavelength paths, an annular stop is used in the condenser. The annular stop produces a hollow cone of light that is focused on the specimen before reaching the objective lens. The objective contains a phase plate containing a phase ring. As a result, light traveling directly from the illuminator passes through the phase ring while light refracted or reflected by the specimen passes through the plate. This causes waves traveling through the ring to be about one-half of a wavelength out of phase with those passing through the plate. Because waves have peaks and troughs, they can add together (if in phase together) or cancel each other out (if out of phase). When the wavelengths are out of phase, wave troughs will cancel out wave peaks, which is called destructive interference. Structures that refract light then appear dark against a bright background of only unrefracted light. More generally, structures that differ in features such as refractive index will differ in levels of darkness (**Figure 5**).

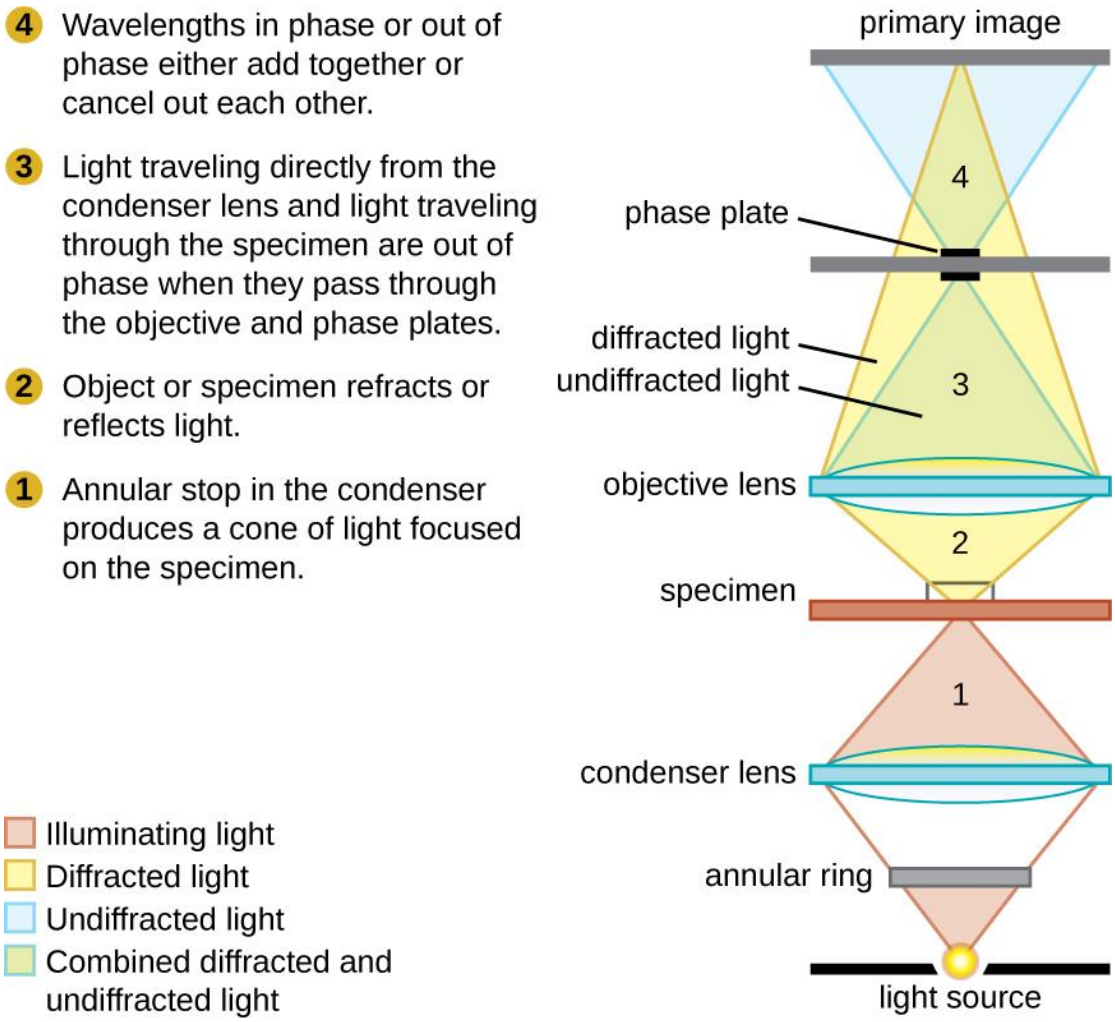

Fig 5: This diagram of a phase-contrast microscope illustrates phase differences between light passing through the object and background. These differences are produced by passing the rays through different parts of a phase plate. The light rays are superimposed in the image plane, producing contrast due to their interference. https://ecampusontario.pressbooks.pub/app/uploads/sites/159/2018/07/OSC_Microbio_02_03_Phasecontr.jpg

Because it increases contrast without requiring stains, phase-contrast microscopy is often used to observe live specimens. Certain structures, such as organelles in eukaryotic cells and endospores in prokaryotic cells, are especially well visualized with phase-contrast microscopy. (figure 6)

Fig 6: Photographie d'un cellule épithéliale de joue vue par un Microscope à contraste de phase. https://upload.wikimedia.org/wikipedia/commons/thumb/7/7e/Cheek_cell_phase_contrast.jpg/220px-Cheek_cell_phase_contrast.jpg

4) Differential Interference Contrast Microscopes

In a Differential Interference Contrast Microscopes, two beams of light are created in which the direction of wave movement (polarization) differs. Once the beams pass through either the specimen or specimen-free space, they are recombined and effects of the specimens cause differences in the interference patterns generated by the combining of the beams. This results in high-contrast images of living organisms with a three-dimensional appearance. These microscopes are especially useful in distinguishing structures within live, unstained specimens (Figure 3.2).

Fig 2: A DIC image of *Fonsecaea pedrosoi* grown on modified Leonian's agar. This fungus causes chromoblastomycosis, a chronic skin infection common in tropical and subtropical climates. https://ecampusontario.pressbooks.pub/app/uploads/sites/159/2018/07/OSC_Microbio_02_03_DICmicro.jpg

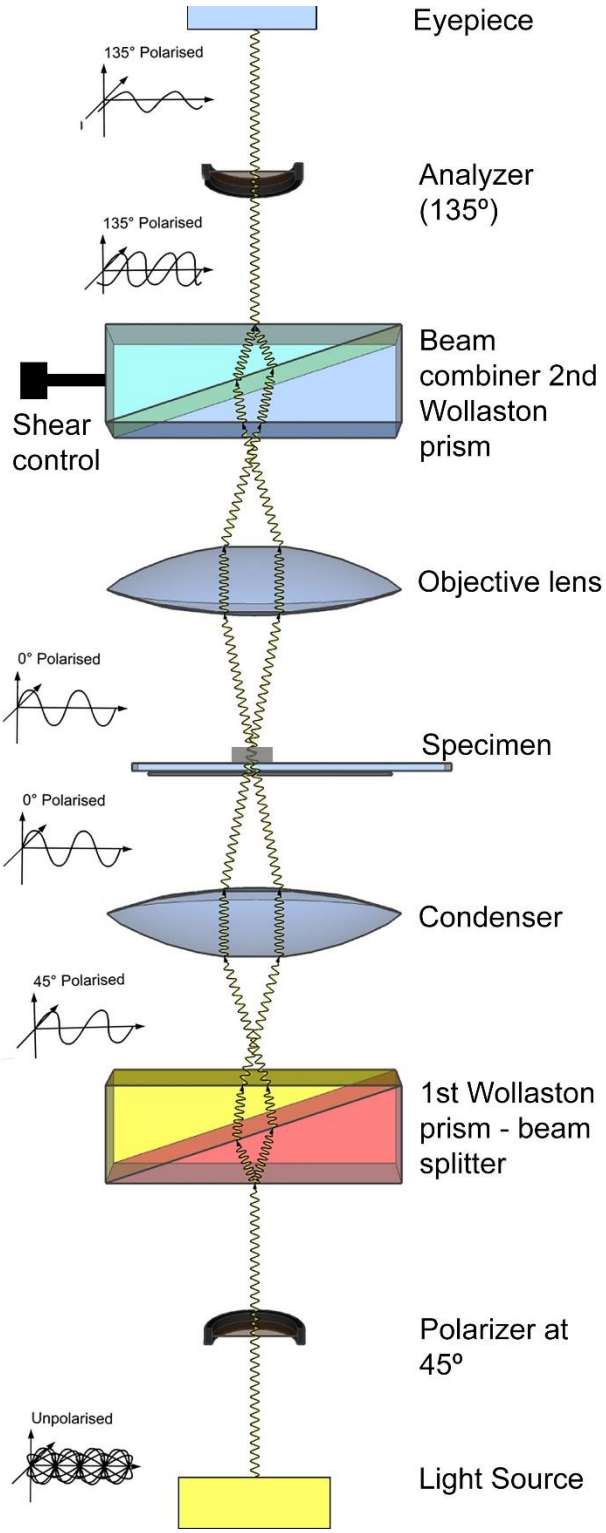

Fig 3 : *Differential Interference contrast (DIC) microscope. Diagram modified - the original i by Richard Wheeler (Zephyris) – Wikpedia Public Domain https://commons.wikimedia.org/wiki/File:DIC_Light_Path.png*

5) Fluorescence Microscopes

A fluorescence microscope uses fluorescent chromophores called fluorochromes, which are capable of absorbing energy from a light source and then emitting this energy as visible light.

The microscope transmits an excitation light, toward the specimen; the chromophores absorb the excitation light and emit visible light with longer wavelengths. The excitation light is then filtered out (in part because ultraviolet light is harmful to the eyes) so that only visible light passes through the ocular lens. This produces an image of the specimen in bright colours against a dark background.

Fluorescence microscopes are especially useful in clinical microbiology. They can be used to identify pathogens, to find particular species within an environment, or to find the locations of particular molecules and structures within a cell. Approaches have also been developed to distinguish living from dead cells using fluorescence microscopy based upon whether they take up particular fluorochromes. Sometimes, multiple fluorochromes are used on the same specimen to show different structures or features.

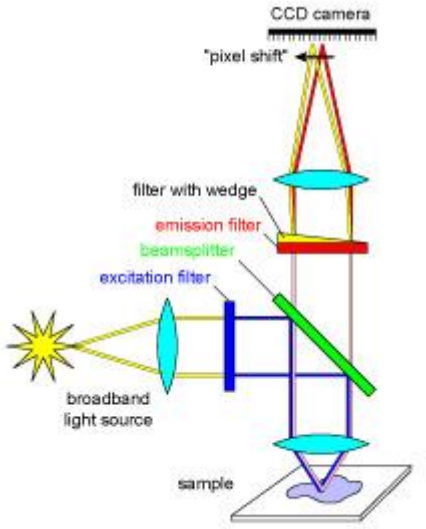

Fig 4: *In an epifluorescence microscope, a wedge angle on the dichroic or emitter causes a beam deviation (gold path) that results in pixel shift. The wedge and beam deviation angles are exaggerated for illustration.* https://www.idex-

hs.com/images/default-source/view-all-resources/filter-best-practice/pixelshift-fig-4848edc4f-a59a-4c92-bfe5-08d7e5da44ae.jpg?Status=Master&sfvrsn=a1735ceb_3

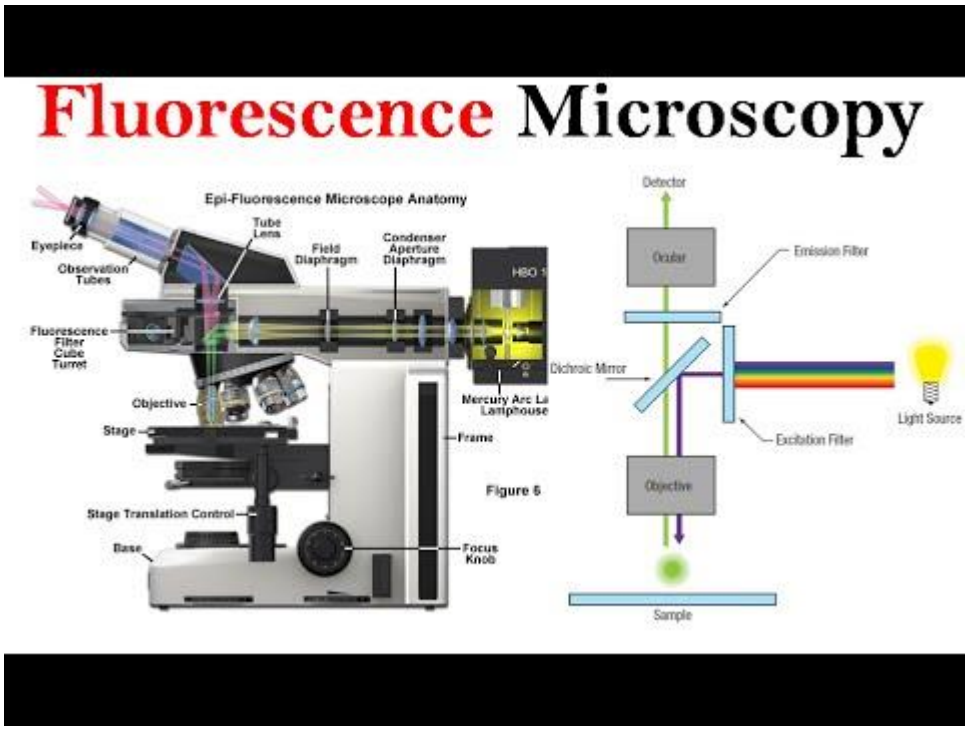

Fig 5: Epi-fluorescences microscopy. http://zeiss-campus.magnet.fsu.edu/articles/basics/fluorescence.html.

Fig 6: A fluorescence microscopy image of a mitochondrion. https://img.freepik.com/premium-photo/fluorescence-microscopy-

image-mitochondrion-showing-distribution-mitochondria-within_216520-6846.jpg?w=826

6) Confocal Microscopes

a confocal microscope uses a laser to scan multiple z-planes successively. This produces numerous two-dimensional, high-resolution images at various depths, which can be constructed into a three-dimensional image by a computer. As with fluorescence microscopes, fluorescent stains are generally used to increase contrast and resolution. Image clarity is further enhanced by a narrow aperture that eliminates any light that is not from the z-plane. Confocal microscopes are thus very useful for examining thick specimens such as biofilms, which can be examined alive and unfixed .

a. Principle of confocal microscopes

Similar to the widefield microscope, the confocal microscope uses fluorescence optics. Instead of illuminating the whole sample at once, laser light is focused onto a defined spot at a specific depth within the sample. This leads to the emission of fluorescent light at exactly this point. A pinhole inside the optical pathway cuts off signals that are out of focus, thus allowing only the fluorescence signals from the illuminated spot to enter the light detector.

By scanning the specimen in a raster pattern, images of one single optical plane are created. 3D objects can be visualized by scanning several optical planes and stacking them using a suitable microscopy deconvolution software (z-stack). It is also possible to analyze multicolor immunofluorescence stainings using state-of-the-art confocal microscopes that include several lasers and emission/excitation filters.

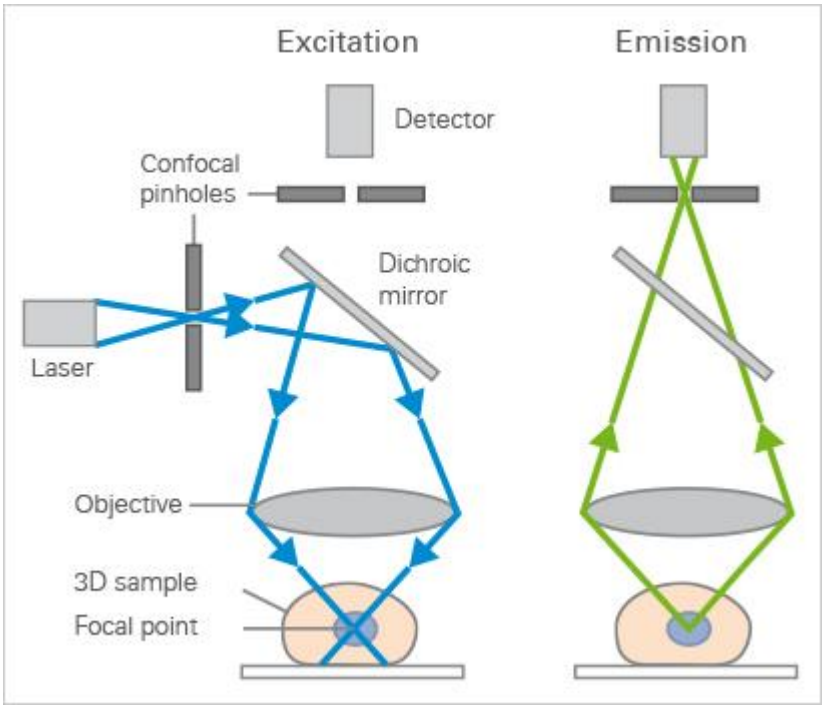

Fig 6: *Excitation and emission light pathways in a basic confocal microscope configuration.*
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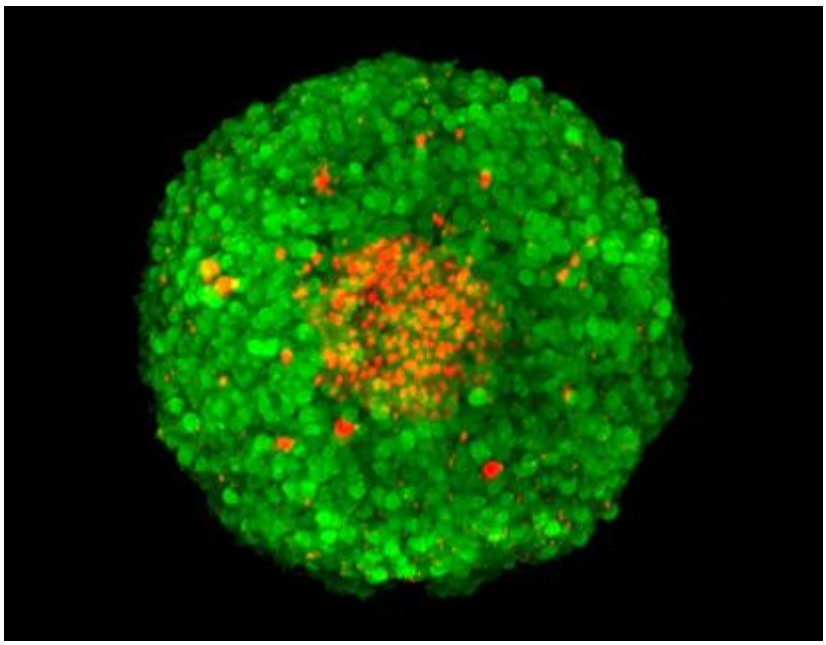

Fig 7 : *Z-stack of an FDA/PI-stained MCF-7 spheroid, acquisition by confocal microscopy. Green: FDA-stained living cells. Red: PI-stained dead cells in the necrotic center of the spheroid.*

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7) Two-Photon Microscopes

The two-photon microscope uses a scanning technique, fluorochromes, and long-wavelength light (such as infrared) to visualize specimens. The low energy associated with the long-wavelength light means that two photons must strike a location at the same time to excite the fluorochrome. The low energy of the excitation light is less damaging to cells, and the long wavelength of the excitation light more easily penetrates deep into thick specimens. This makes the two-photon microscope useful for examining living cells within intact tissues—brain slices, embryos, whole organs, and even entire animals.

Currently, use of two-photon microscopes is limited to advanced clinical and research laboratories because of the high costs of the instruments. A single two-photon microscope typically costs between $300,000 US and $500,000 US, and the lasers used to excite the dyes used on specimens are also very expensive. However, as technology improves, two-photon microscopes may become more readily available in clinical settings.

7.1. Principle of Two-Photon Microscopes

Just like widefield or confocal fluorescence microscopy, two-photon microscopy is based on fluorophore excitation, which results in the emission of light. In classic fluorescence microscopy, a fluorophore is excited by absorbing one single photon of a certain wavelength. When using two-photon microscopy, two or three photons of a higher wavelength do the work of one: When they hit the fluorophore at the very same time (typically within several femtoseconds), they are absorbed, resulting in fluorophore excitation and emission of light.

In this process, photons combine their energy, which allows low-energy infrared photons to excite standard fluorophores, such as GFP. The infrared light penetrates tissue more deeply than the standard excitation light used in fluorescence microscopy. Due to its low energy level, infrared light is less damaging, and therefore especially useful when working with living samples.

In order to increase the likelihood that two photons hit the fluorophore simultaneously, lasers with very high intensity are needed. Their infrared light only leads to excitation in the focus of the objective, because only in this area, the critical number of photons per time and space is reached. Therefore, all emitted light comes from one focal point in the specimen, strongly reducing background noise. The image is created just as in confocal microscopy: The laser scans across the sample, recording the image intensity point by point.

2. Microscope Maintenance: Best Practices

Even a very powerful microscope cannot deliver high-resolution images if it is not properly cleaned and maintained. Since lenses are carefully designed and manufactured to refract light with a high degree of precision, even a slightly dirty or scratched lens will refract light in unintended ways, degrading the image of the specimen. In addition, microscopes are rather delicate instruments, and great care must be taken to avoid damaging parts and surfaces. Among other things, proper care of a microscope includes the following:

- cleaning the lenses with lens paper
- not allowing lenses to contact the slide (e.g., by rapidly changing the focus)
- protecting the bulb (if there is one) from breakage
- not pushing an objective into a slide
- not using the coarse focusing knob when using the 40× or greater objective lenses
- only using immersion oil with a specialized oil objective, usually the 100× objective
- cleaning oil from immersion lenses after using the microscope
- cleaning any oil accidentally transferred from other lenses
- covering the microscope or placing it in a cabinet when not in use

3. Electron Microscopy

The maximum theoretical resolution of images created by light microscopes is ultimately limited by the wavelengths of visible light. Most light microscopes can only magnify 1000 , and a few can magnify up to 1500 , but this does not begin to approach the magnifying power of an electron microscope **(EM)**, which uses short-wavelength electron beams rather than light to increase magnification and resolution.

Electrons, like electromagnetic radiation, can behave as waves, but with wavelengths of 0.005 nm, they can produce much better resolution than visible light. An EM can produce a sharp image that is magnified up to 100,000 . Thus, EMs can resolve sub-cellular structures as well as some molecular structures (e.g., single strands of DNA); however, electron microscopy cannot be used on living material because of the methods needed to prepare the specimens.

There are two basic types of EM:

- the transmission electron microscope (TEM)
- the scanning electron microscope (SEM).

The TEM is somewhat analogous to the brightfield light microscope in terms of the way it functions. However, it uses an electron beam from above the specimen that is focused using a magnetic lens (rather than a glass lens) and projected through the specimen onto a detector. Electrons pass through the specimen, and then the detector captures the image

For electrons to pass through the specimen in a TEM, the specimen must be extremely thin (20–100 nm thick). The image is produced because of varying opacity in various parts of the specimen. This opacity can be enhanced by staining the specimen with materials such as heavy metals, which are electron dense. TEM requires that the beam and specimen be in a vacuum and that the specimen be very thin and dehydrated. The specific steps needed to prepare a specimen for observation under an EM are discussed in detail in the next section.

SEMs form images of surfaces of specimens, usually from electrons that are knocked off of specimens by a beam of electrons. This can create highly detailed images with a three-dimensional appearance that are displayed on a monitor . Typically, specimens are dried and prepared with fixatives that reduce artifacts, such as shrivelling, that can

be produced by drying, before being sputter-coated with a thin layer of metal such as gold. Whereas transmission electron microscopy requires very thin sections and allows one to see internal structures such as organelles and the interior of membranes, scanning electron microscopy can be used to view the surfaces of larger objects (such as a pollen grain) as well as the surfaces of very small samples. Some EMs can magnify an image up to 2,000,000

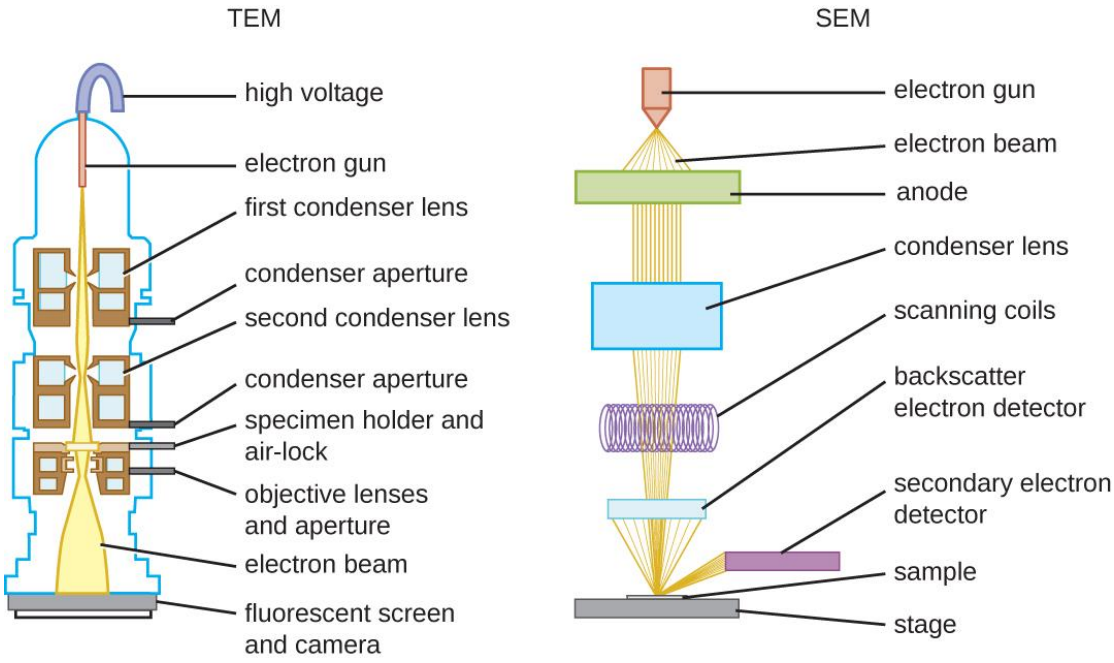

Fig 8: These schematic illustrations compare the components of transmission electron microscopes and scanning electron microscopes.

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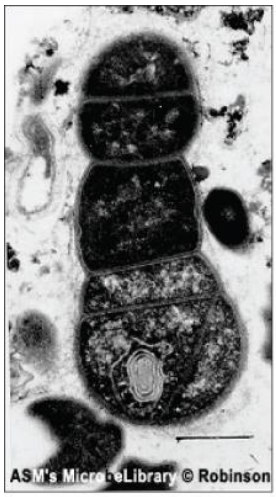
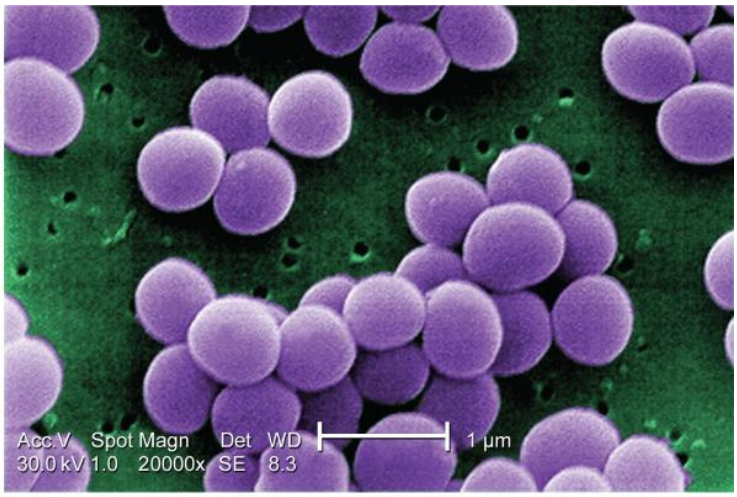


(a)



(b)

Fig 9: (a) This TEM image of cells in a biofilm shows well-defined internal structures of the cells because of varying levels of opacity in the specimen. (b) This colour-enhanced SEM image of the bacterium *Staphylococcus aureus* illustrates the ability of scanning electron microscopy to render three-dimensional images of the surface structure of cells. (credit a: modification of work by American Society for Microbiology; credit b: modification of work by Centers for Disease Control and Prevention).
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