Bright field Light Microscope

History of Microscopy: Overview

- The evolution of the Microbiology field put to perspective the need to identify, view, observe and understand microorganisms, including their structural morphologies and mechanisms. Microbiology's scope is to study organisms and minute agents that can only be examined and observed with a microscope.
- Although scientifically, the first simple microscope was discovered by two Dutch scientists, Zaccharias Janssen and his father, Hans who made spectacles, were the first to experiment with their lenses by combining lenses in a tube and observed that the objects that were nearby, appeared closer and larger. Despite not being included as a scientific discovery, this act paved the way for scientific evolution.
- From the History of Microbiology, Antony Van Lewnehoueek an amateur Microbiologist made the first simple microscope that enabled him to observe the presence of tiny living organisms in pond water that appeared like dots. His simple microscope was made up of a double convex glass lens that was held between two silver plates.
- The application of microscopy in Microbiology enhanced the visualization of cells and microorganisms by magnifying their images to make them larger.

The light microscope is also known as an optical microscope.

- A light microscope is a biology laboratory instrument or tool that uses visible light to detect and magnify very small objects, and enlarging them.
- They use lenses to focus light on the specimen, magnifying it thus producing an image. The specimen is normally placed close to the microscopic lens.
- Microscopic magnification varies greatly depending on the types and number of lenses that make up the microscope. Depending on the number of lenses,

  There are two types of microscopes

  i. Simple light microscope (it has low magnification because it uses a single lens) and t
  ii. The Compound light microscope (it has a higher magnification compared to the simple microscope because it uses at least two sets of lenses, an objective lens, and an eyepiece). The lenses are aligned in that; they can be able to bend light for efficient magnification of the image.
- The functioning of the light microscope is based on its ability to focus a beam of light through a specimen, which is very small and transparent, to produce an image. The image is then passed through one or two lenses for magnification for viewing. The transparency of the specimen allows easy and quick penetration of light. Specimens can vary from bacterial to cells and other microbial particles.

The modern types of Light Microscopes include:

1. Bright field Light Microscope
2. Phase Contrast Light Microscope
3. Dark-Field Light Microscope
4. Fluorescence Light Microscope

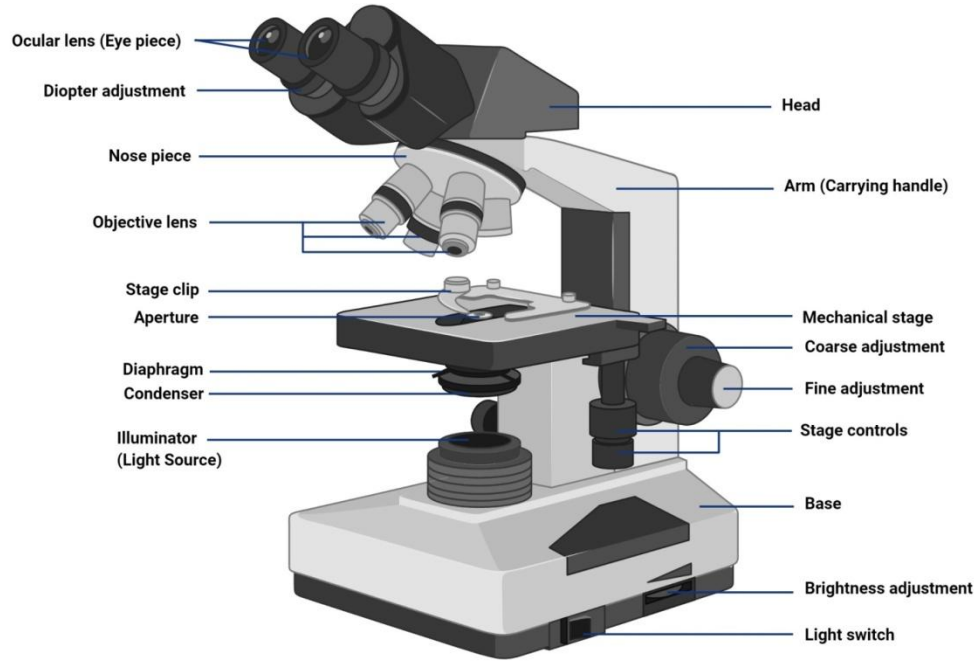
Bright field Light Microscope (Compound light microscope)

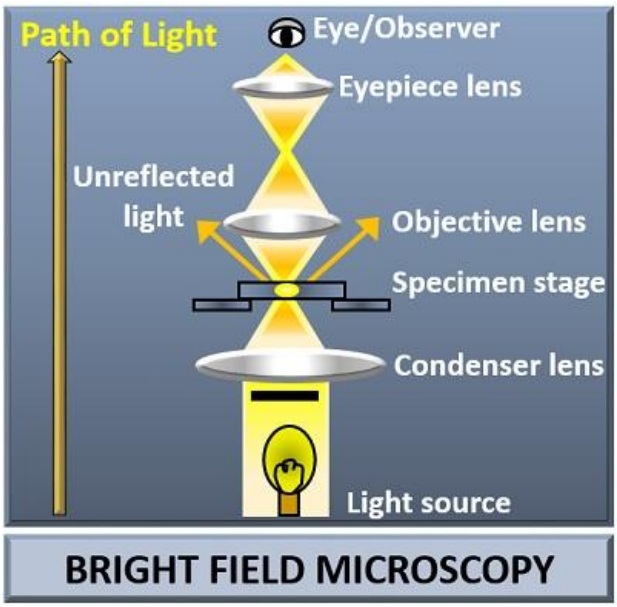
- This is the most basic optical Microscope used in microbiology laboratories which produces a dark image against a bright background. Made up of two lenses, it is widely used to view plant and animal cell organelles including some parasites such as *Paramecium* after staining with basic stains.
- Its functionality is based on being able to provide a high-resolution image, which highly depends on the proper use of the microscope. This means that an adequate amount of light will enable sufficient focusing of the image, to produce a quality image.
- It is also known as a compound light microscope.

Parts of a bright-field microscope (Compound light microscope)

Definition of Bright Field Microscopy

Bright field microscopy can define as the optical microscopy, which is the **simplest** of all the illumination techniques, wherein a smear, the **stained** or the **dense part** appear **darker** with a white or **brighter background**. It is a type of light microscopy, where a path of light is very simple, which requires a light source like





Principle of Bright field Microscope

For a specimen to be the focus and produce an image under the Bright field Microscope, the specimen must pass through a uniform beam of the illuminating light. Through differential absorption and differential refraction, the microscope will produce a contrasting image.

The specimens used are prepared initially by staining to introduce color for easy contracting characterization. The colored specimens will have a refractive index that will differentiate it from the surrounding, presenting a combination of absorption and refractive contrast.

The functioning of the microscope is based on its ability to produce a high-resolution image from an adequately provided light source, focused on the image, producing a high-quality image.

The specimen which is placed on a microscopic slide is viewed under oil immersion or/and covered with a cover slip.

Figure: Diagram of parts of a microscope

There are three structural parts of the microscope i.e. head, base, and arm.

1. **Head** – This is also known as the body, it carries the optical parts in the upper part of the microscope.
2. **Base** – It acts as microscopes support. It also carriers the microscopic illuminators.
3. **Arms** – This is the part connecting the base and to the head and the eyepiece tube to the base of the microscope. It gives support to the head of the microscope and it also used when carrying the microscope. Some high-quality microscopes have an articulated arm with more than one joint allowing more movement of the microscopic head for better viewing.

Optical parts of a microscope and their functions

The optical parts of the microscope are used to view, magnify, and produce an image from a specimen placed on a slide. These parts include:

1. **Eyepiece** – also known as the ocular. This is the part used to look through the microscope. It's found at the top of the microscope. Its standard magnification is 10x with an optional eyepiece having magnifications from 5X – 30X.

2. **Eyepiece tube** – its the eyepiece holder. It carries the eyepiece just above the objective lens. In some microscopes such as the binoculars, the eyepiece tube is flexible and can be rotated for maximum visualization, for variance in distance. For monocular microscopes, they are none flexible.

3. **Objective lenses** – These are the major lenses used for specimen visualization. They have a magnification power of 40x-100X. There are about 1- 4 objective lenses placed on one microscope, in that some are rare facing and others face forward. Each lens has its own magnification power.

4. **Nose piece** – also known as the revolving turret. It holds the objective lenses. It is movable hence it cal revolve the objective lenses depending on the magnification power of the lens.

5. **The Adjustment knobs** – These are knobs that are used to focus the microscope. There are two types of adjustment knobs i.e fine adjustment knobs and the coarse adjustment knobs.

6. **Stage** – This is the section on which the specimen is placed for viewing. They have stage clips hold the specimen slides in place. The most common stage is a mechanical stage, which allows the control of the slides by moving the slides using the mechanical knobs on the stage instead of moving it manually.

7. **Aperture** – This is a hole on the microscope stage, through which the transmitted light from the source reaches the stage.

8. **Microscopic illuminator** – This is the microscopes light source, located at the base. It is used instead of a mirror. it captures light from an external source of a low voltage of about 100v.

9. **Condenser** – These are lenses that are used to collect and focus light from the illuminator into the specimen. They are found under the stage next to the diaphragm of the microscope. They play a major role in ensuring clear sharp images are produced with a high magnification of 400X and above. The higher the magnification of the condenser, the more the image clarity. More sophisticated microscopes come with an Abbe condenser that has a high magnification of about 1000X.

10. **Diaphragm** – it's also known as the iris. It's found under the stage of the microscope and its primary role is to control the amount of light that reaches the specimen. Its an adjustable apparatus, hence controlling the light intensity and the size of the beam of light that gets to the specimen. For high-quality microscopes, the diaphragm comes attached with an Abbe condenser and combined they are able to control the light focus and light intensity that reaches the specimen.

11. **Condenser focus knob** – this is a knob that moves the condenser up or down thus controlling the focus of light on the specimen.

12. **Abbe Condenser** – this is a condenser specially designed on high-quality microscopes, which makes the condenser to be movable and allows very high magnification of above 400X. The high-quality microscopes normally have a high numerical aperture than that of the objective lenses.

13. **The rack stop** – It controls how far the stages should go preventing the objective lens from getting too close to the specimen slide which may damage the specimen. It is responsible for preventing the specimen slide from coming too far up and hit the objective lens.

Magnification by Bright field Microscope

- The objective lenses are the main lenses used for focusing the image, on the condenser. This produces an enlarged clear image that is then magnified again by the eyepiece to form the primary image that is seen by the eyes.
- During imaging, the objective lenses remain parfocal in that, even when the objective lens has changed the image still remains focused. The image seen at the eyepiece is the enlarged clear image of the specimen, known as the virtual image.
- The magnification of the image is determined by the magnification of the objective against the magnification of the eyepiece lens. The objectives have a magnification power of 40x-1000x depending on the type of bright field microscope while the eyepiece lens has a standard magnification power of 10x.
- Therefore to calculate:

Total Magnification power = Magnification of the objective lens x Magnification of the eyepiece

- For example: if the magnification of the objective is 45x and that of the eyepiece is 10x, the total magnification of the specimen will be 450x.

Applications of the Bright Field Light Microscope (Compound light microscope)

Vastly used in Microbiology, this microscope is used to view fixed and live specimens that have been stained with basic stains. This gives contrast for easy visibility under the microscope. Therefore it can be used to identify basic bacteria cells and parasitic protozoan's such as *Paramecium.*

Some of its applications include:

1. Used to visualize and study the animal cells
2. Used to visualize and study plant cells.
3. Used to visualize and study the morphologies of bacterial cells
4. Used to identify parasitic protozoan's such as *Paramecium.*

Advantages of Bright field Microscope

- It is simple to use with few adjustments involved while viewing the image.
- It can be used to view both stained and unstained.
- The optics of the microscope do not alter the color of the specimen.
- The microscope can be adjusted and modified for better viewing such as installing a camera, to form a digital microscope or in the way image illumination is done such as by use of fluorochromes on the specimen and viewing under a dark environment, forming a dark field microscope.

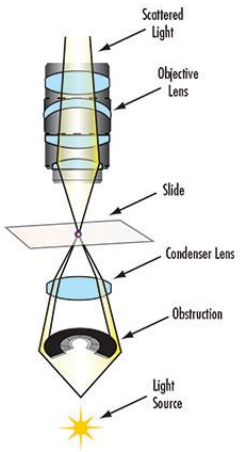
Disadvantages

1. The aperture diaphragm may cause great contrast which may distort the outcome of the image, therefore iris diaphragm is preferred.
2. It cannot be used to view live specimens such as bacterial cells. Only fixed specimens can be viewed under the bright field microscope.
3. Maximum magnification of the bright field microscope is 100x but modification can readjust the magnification to 1000x which is the optimum magnification of bacterial cells.
4. It has low contrast hence most specimens must be stained for them to be visualized.
5. Use of oil immersion may distort the image
6. The use of coverslip may damage the specimen
7. Staining may introduce extraneously unwanted details into the specimen or contaminate the specimen.
8. The microscope needs a strong light source for magnification and sometimes the light source may produce a lot of heat which may damage or kill the specimen.

Dark field Microscope

- Microbiology, the branch of science that has so vastly extended and expanded our knowledge of the living world, owes its existence to Antoni van Leeuwenhoek.
- In 1673, with the aid of a crude microscope consisting of a biconcave lens enclosed in two metal plates, Leeuwenhoek introduced the world to the existence of microbial forms of life.
- Over the years, microscopes have evolved from the simple, single-lens instrument of Leeuwenhoek, with a magnification of 300 X, to the present-day electron microscopes capable of magnifications greater than 250,000X.
- Microscopes are designated as either light microscopes or electron microscopes.

- Light microscopes use visible light or ultraviolet rays to illuminate specimens. They include **bright field**, dark field, phase-contrast, and fluorescent instruments.
- This is similar to the ordinary light microscope; however, the condenser system is modified so that the specimen is not illuminated directly.
- The condenser directs the light obliquely so that the light is deflected or scattered from the specimen, which then appears bright against a dark background.
- Living specimens may be observed more readily with dark field than with bright field microscopy.



Principle of the Dark field Microscope

- A dark field microscope is arranged so that the light source is blocked off, causing light to scatter as it hits the specimen.
- This is ideal for making objects with refractive values similar to the background appear bright against a dark background.
- When light hits an object, rays are scattered in all azimuths or directions. The design of the dark field microscope is such that it removes the dispersed light, or zeroth order, so that only the scattered beams hit the sample.
- The introduction of a condenser and/or stop below the stage ensures that these light rays will hit the specimen at different angles, rather than as a direct light source above/below the object.
- The result is a "cone of light" where rays are diffracted, reflected and/or refracted off the object, ultimately, allowing the individual to view a specimen in dark field.

1. The dark-ground microscopy makes use of the dark-ground microscope, a special type of compound light microscope.
2. The dark-field condenser with a central circular stop, which illuminates the object with a cone of light, is the most essential part of the dark-ground microscope.
3. This microscope uses reflected light instead of transmitted light used in the ordinary light microscope.
4. It prevents light from falling directly on the objective lens.
5. Light rays falling on the object are reflected or scattered onto the objective lens with the result that the microorganisms appear brightly stained against a dark background.

Uses of Dark field Microscope

The dark ground microscopy has the following uses:

- It is useful for the demonstration of very thin bacteria not visible under ordinary illumination since the reflection of the light makes them appear larger.
- This is a frequently used method for rapid demonstration of *Treponema pallidum* in clinical specimens.
- It is also useful for the demonstration of the motility of flagellated bacteria and protozoa.
- Dark field is used to study marine organisms such as algae, plankton, diatoms, insects, fibers, hairs, yeast and protozoa as well as some minerals and crystals, thin polymers and some ceramics.
- Dark field is used to study mounted cells and tissues.
- It is more useful in examining external details, such as outlines, edges, grain boundaries and surface defects than internal structure.

Advantages of Dark field Microscope

- Dark-field microscopy is a very simple yet effective technique.
- It is well suited for uses involving live and unstained biological samples, such as a smear from a tissue culture or individual, water-borne, single-celled organisms.
- Considering the simplicity of the setup, the quality of images obtained from this technique is impressive.
- Dark-field microscopy techniques are almost entirely free of artifacts, due to the nature of the process.
- A researcher can achieve a dark field by making modifications to his/her microscope.

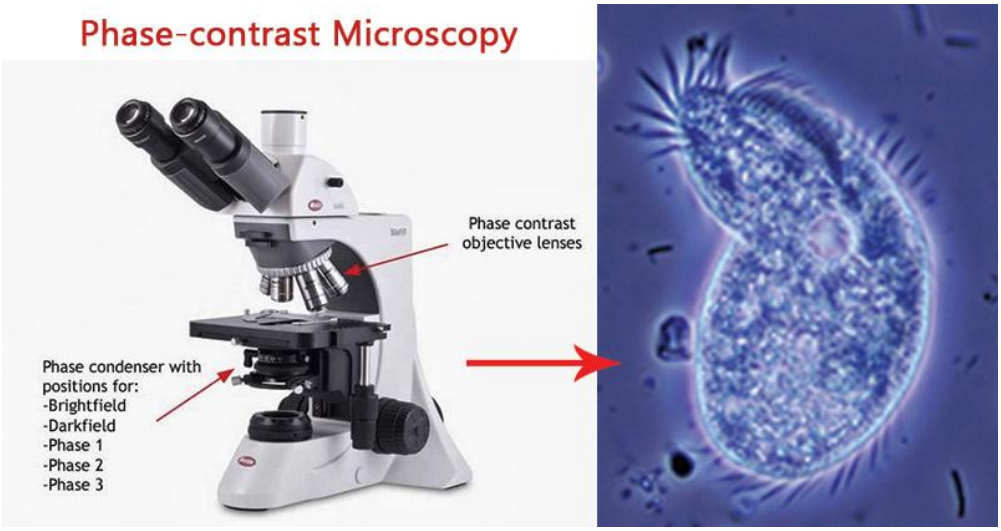
Limitations of Dark field Microscope

- The main limitation of dark-field microscopy is the low light levels seen in the final image.
- The sample must be very strongly illuminated, which can cause damage to the sample.

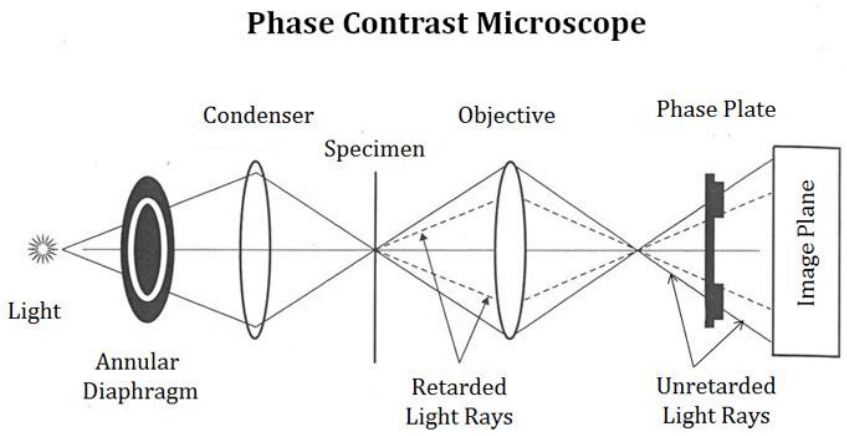
Phase contrast microscopy definition

- Phase-contrast microscopy is an optical microscopy technique that converts phase shifts in the light passing through a transparent specimen to brightness changes in the image.

- It was first described in 1934 by Dutch physicist Frits Zernike.



Principle of Phase contrast Microscopy



When light passes through cells, small phase shifts occur, which are invisible to the human eye. In a phase-contrast microscope, these phase shifts are converted into changes in amplitude, which can be observed as differences in image contrast.

The Working of Phase contrast Microscopy

- This is a type of optical microscope whereby small light deviations know as **phase shifts** occur during light penetration into the unstained specimen. These phase shifts are converted into the image to mean, when light passes through the opaque specimen, the phase shifts brighten the specimen forming an illuminated (bright) image in the background.
- The phase-contrast microscope produces high contrast images when using a transparent specimen more so those of microbial cultures, thin tissue fragments, cell tissues, and subcellular particles.

- The principle behind the working of the phase-contrast microscope is the use of an optical method to transform a specimen into an amplitude image, that's viewed by the eyepiece of the microscope.
- The PCM can be used to view unstained cells also known as the **phase objects**, which means that the morphology of the cell is maintained and the cells can be observed in their natural state, in high contrast and efficient clarity. This is because if the specimens are stained and fixed, they kill most cells, a characteristic that is uniquely undone by the brightfield light microscope.
- The shifts that occur during light penetration, become converted to changes in amplitude which causes the image contrast.
- Coupled with contrast-enhancing elements such as fluorescence, they produce better visuals of the specimens' image.

Parts of the Phase Contrast Microscope

The instrumentation of the Phase Contrast Microscope is based on its light pathways from receiving the source of light to the visualization of the image.

Therefore it's sequentially made up of:

- Light source (Mercury arc lamp)
- Collective lens
- Aperture
- Condenser
- Condenser annular

Parts of Phase contrast Microscopy

Phase-contrast microscopy is basically a specially designed light microscope with all the basic parts in addition to which an annular phase plate and annular diaphragm are fitted.

The annular diaphragm

- It is situated below the condenser.
- It is made up of a circular disc having a circular annular groove.
- The light rays are allowed to pass through the annular groove.
- Through the annular groove of the annular diaphragm, the light rays fall on the specimen or object to be studied.
- At the back focal plane of the objective develops an image.
- The annular phase plate is placed at this back focal plane.

The phase plate

- It is either a negative phase plate having a thick circular area or a positive phase plate having a thin circular groove.

- This thick or thin area in the phase plate is called the conjugate area.
- The phase plate is a transparent disc.
- With the help of the annular diaphragm and the phase plate, the phase contrast is obtained in this microscope.
- This is obtained by separating the direct rays from the diffracted rays.
- The direct light rays pass through the annular groove whereas the diffracted light rays pass through the region outside the groove.
- Depending upon the different refractive indices of different cell components, the object to be studied shows a different degree of contrast in this microscope.

Applications of Phase contrast Microscopy

To produce high-contrast images of transparent specimens, such as

1. living cells (usually in culture),
2. microorganisms,
3. thin tissue slices,
4. lithographic patterns,
5. fibers,
6. latex dispersions,
7. glass fragments, and
8. subcellular particles (including nuclei and other organelles).

Applications of phase-contrast microscopy in biological research are numerous.

Advantages

- Living cells can be observed in their natural state without previous fixation or labeling.
- It makes a highly transparent object more visible.
- No special preparation of fixation or staining etc. is needed.
- PCM saves a lot of time.
- Examining intracellular components of living cells at relatively high resolution. eg: The dynamic motility of **mitochondria**, mitotic chromosomes & vacuoles.
- PCM helps to study living cells and how they proliferate through cell division.
- Phase-contrast optical components can be added to virtually any brightfield microscope.

Limitations

- Phase-contrast condensers and objective lenses add considerable cost to a microscope, and so phase contrast is often not used in teaching labs except perhaps in classes in the health professions.
- To use phase-contrast the light path must be aligned.
- Generally, more light is needed for phase contrast than for corresponding bright-field viewing, since the technique is based on the diminishment of the brightness of most objects.

Fluorescence Microscope: Principle, Types and Applications

Fluorescence microscopy is a type of light microscope that works on the principle of fluorescence. A substance is said to be fluorescent when it absorbs the energy of invisible shorter wavelength radiation (such as UV light) and emits longer wavelength radiation of visible light (such as green or red light). This phenomenon, also termed fluorescence, is widely used in clinical and diagnostic settings for the rapid detection of microorganisms, antibodies, and many other substances.

When fluorescence microscopy is used for the detection of antigen-antibody reaction, it is known as **immunofluorescence.**

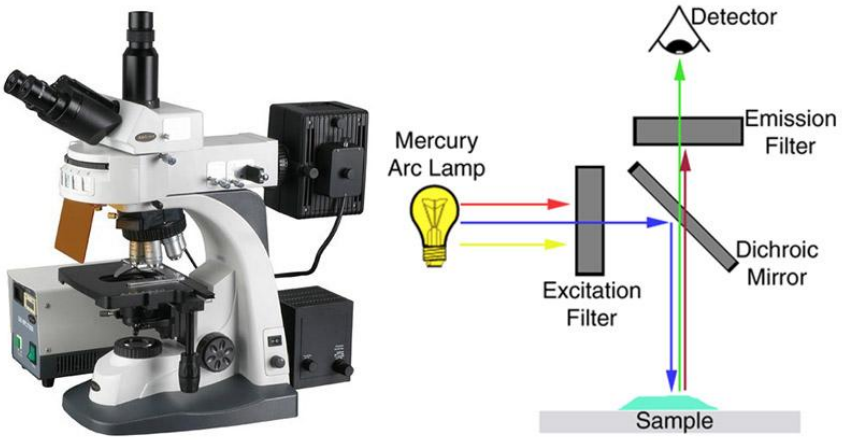
Components of a Fluorescence Microscope:

Fluorescence microscopes as other light microscope has a

1. **Light source**: Xenon arc lamp or mercury-vapor lamp are common; power LED and lasers are used in more advanced forms.
2. **A set of optical filters:** Optical filters include a set of a compatible excitation filter, emission filter, and dichroic beam splitter;
    - ❖ An **excitation filter** selects the wavelengths to excite a particular dye within the specimen.
    - ❖ A **dichroic beam splitter/ dichroic mirror** reflects light in the excitation band and transmit light in the emission band, enabling the classic epifluorescence incident light illumination.
    - ❖ An **emission filter** serves as a kind of quality control by letting only the wavelengths of interest emitted by the fluorophore pass through.
3. **Darkfield condenser**: It provides a black background against which the fluorescent objects glow.

The filters are often plugged in together in a filter cube (compound microscopes) or in a flat holder (mainly stereo microscopes).

Principle: To observe the sample through a fluorescence microscope, it should be first labeled with a fluorescent dyes/substance known as a fluorophore. Working mechanism of Fluorescence Microscope Higher energy light shorter wavelength of lights (UV rays or blue light) generated from mercury vapor arc lamp passes through the **excitation filter** which allows only the short wavelength of light to pass through and removes all other non-specific wavelengths of light. The filtered light is reflected by the **dichroic filter** and falls on the **sample** (i.e. fluorophore-labeled). The fluorochrome **absorbs shorter wavelength rays** and emits rays of longer wavelength (lower energy) that passes through the **emission filter.** The emission filter blocks (suppresses) any residual excitation light and passes the desired longer emission wavelengths to the **detector.** Thus the microscope forms glowing images of the fluorochrome-labeled microorganisms against a dark background.



To the observer, the background is dark, as there is no visible light and only the labelled specimen (cells, microorganisms etc.) appear bright (fluoresce).

Types of Fluorescence Microscopes

There are various types of fluorescence microscopes. Some of the common types are:

1. **Epifluorescence microscopes**: The most common type of fluorescence microscope in which, excitation of the fluorophore and detection of the fluorescence are done through the same light path (i.e. through the objective).
2. **Confocal microscope**: In this type of fluorescence microscope, high-resolution imaging of thick specimens (without physical sectioning) can be analyzed using fluorescent-labeled dye.
3. **Multiphoton microscope:** In this type of microscope, multiphoton fluorescence excitation results in the capture of high-resolution three-dimensional images of specimen tagged with highly specific fluorophores.
4. **Total internal reflection fluorescence (TIRF) microscope**: Total internal reflection fluorescence microscopy (**TIRFM**) exploits the unique properties of an induced evanescent wave or field in a limited specimen region immediately adjacent to the interface between two media having different refractive indices.

Applications of Fluorescence Microscope

Fluorescence microscopy is widely used in diagnostic microbiology and in microbial ecology (for enumerating bacteria in natural environments).

Fluorescent antibody-stained specimen showing numerous, *Toxoplasma* sp. tachyzoite (Image credit: CDC)

1. Detection of **acid-fast bacilli (AFB)** in sputum or CSF when stained with auramine fluorescent dye.

2. Detection of ***Trichomonas vaginalis***, intracellular gonococci, and other parasites when stained by acridine orange.
3. In **immunodiagnosis** of infectious diseases, using both direct and indirect antibody techniques.

Limitations of Fluorescence Microscope

1. Fluorophores gradually lose their ability to fluoresce as they are illuminated in a process called **photobleaching**. Photobleaching can severely limit the time over which a sample can be observed by fluorescence microscopy. However, several techniques exist to reduce photobleaching such as the use of more robust fluorophores, by minimizing illumination, or by using photoreactive scavenger chemicals.
2. Fluorescence microscopy has enabled analysis of live cells; but fluorescent molecules generate reactive chemical species under illumination that enhances the **phototoxic effect**, to which live cells are susceptible.
3. Fluorescence microscopy **only allows observation of the specific structures** which have been labeled for fluorescence. For example, observing a tissue sample prepared with a fluorescent DNA stain by fluorescence microscopy only reveals the organization of the DNA within the cells and reveals nothing else about the cell morphologies.

Polarizing Microscope

What is a polarizing or petrographic microscope?

The polarizing microscope or "petrographic microscope" is used mainly in geological studies for geological specimens but also in medicine and biology. This type of microscope differs from the normal one by using a polarized light, in which the light waves vibrate in one direction. Unlike the ones from normal light that vibrate in random directions. It's used in anisotropic materials (like minerals) because of their birefringent optical properties – they have several refractive indices. When studying a specimen the light has to pass through a polarizer (polarizing filter) and then in some cases through an analyzer – to increase the quality of image contrast

A petrographic microscope is usually a modified compound microscope, although stereo microscopes can also be altered to achieve polarization.

This microscope differs from others because it contains the following components:

- A polarizer and analyzer
- A circular rotating stage
- Special plates or filters placed between the object and light path.
- Bertrand lens

A polarizer is a filter that only allows specific light waves or vibrations to pass through it and focus them in a single plane.

An analyzer, mainly used as a second polarizer located above the sample, determines the quantity and the direction of the light that illuminates a sample.

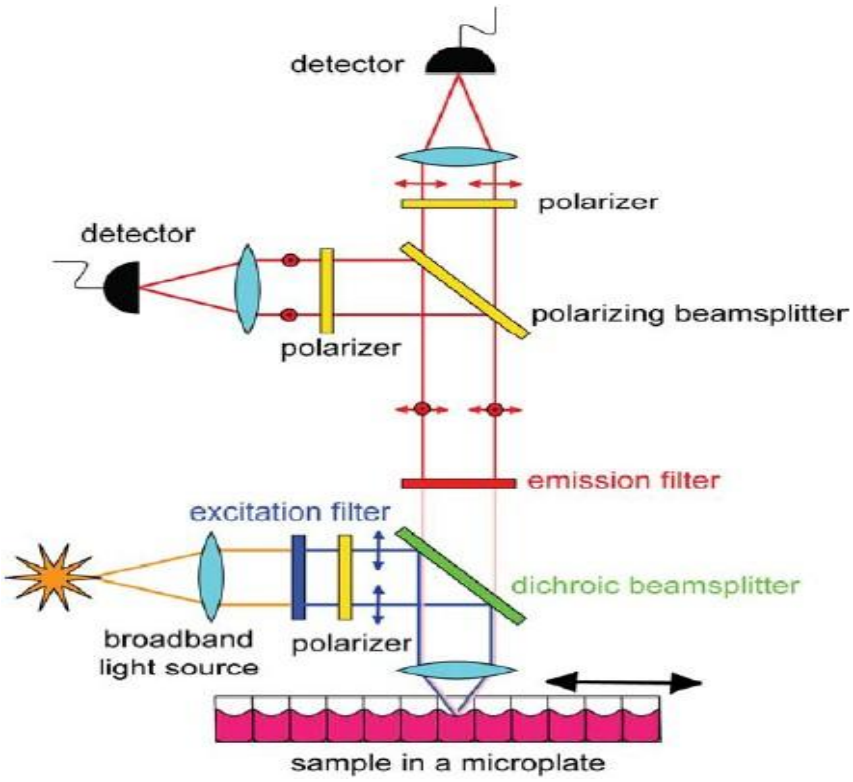
Due to the use of these filters, the polarized light waves vibrate in one single direction, instead of the normal ones that vibrate in random directions.

In this way the polarized light is more concentrated and then more efficient to the study of minerals, for example.

By changing the relationship of the polarizer and the analyzer, it's possible to determine the amount of absorbance, reflection and refraction of the light through the microscope.

Pathway of Light

- The light passes through a polarizing filter called the polarizer (the polarizer is fixed in an east to west vibrational way, but it can be rotated if necessary. There is one more polarizing filter called the analyzer. It is usually situated above the objectives and can be moved in and out of the optical path).
- Passes through the birefringent specimen. The polarizer is usually fixed in an east to west vibrational direction, but it can be rotated as required. There is one more polarizing filter called the analyzer. It is usually situated above the objectives and can be moved in and out of the optical path.



Applications of Polarized Light Microscopy

In polarized light microscopy, plane polarized light is passed through a double refracting material and then collected using a second polarizing filter to generate a high-contrast image. This technique finds application in several fields, such as medicine, basic biology, industry, and to study rock minerals.

Medical Applications

Gout diagnosis

Gout occurs because of the deposition of urate crystals in the synovial fluid of the joints which causes inflammation and pain. Polarized microcopy is used to examine synovial fluid for the diagnosis of gout. Urate crystals causing gout have negative elongated optical features, while pyrophosphoric acids which cause pseudo-gout have positive optical features. These differences in the crystal direction and the interference colour on polarized light microscopy are used to differentiate these crystals.

Amyloid protein examination

Amyloid proteins are created by abnormal metabolism of proteins which leads to their aggregation within cells. These proteins may be deposited in various organs, such as the spleen, liver, kidney, brain among others. These aggregates are not observed in normal cells. Using polarized light, these amyloid structures can be visualised. The presence of amyloid proteins is ascertained by the appearance of a bright green color on polarization light microscopy.

Applications in Basic Biology

Polarized light microscopy is used to visualize several birefringent or double-refractive structures in the body, including teeth, striated bone, muscle tissue, neurons, spindles, and actomyosin fibers. These structures can be visualized with great contrast by adding a dye; however, as these are living structures, this step causes cell death. Thus, this technique offers a non-invasive method of high-contrast imaging for these tissues and cells. Polarized light microscopy does not require any contrast agent or dye, can be performed in a non-invasive manner, and generates high-contrast images.

Examination of Rocks

Polarized light microscopy can be used to examine rock structures and their optical characteristics. This method can also be used to identify minerals inside rocks. Two methods are used for this purpose.

Transmitted polarizing microscopy

In this method, a rock cutter is used to cut a thin slice of rock. One side is polished and the specimen is fixed on to a glass slide using an adhesive. Subsequently, the opposite side is also polished and fixed to a cover glass using balsam.

Immersion method

In this method, the rock is ground to a fine powder and then sprinkled on a glass slide. The powder is surrounded with an immersion liquid and then a cover slip is added. However, in

this method the structure of the rock is destroyed. The advantage is that it does not require the preparation of thin rock sections.

Applications in Industry

Liquid crystals

Liquid crystals are materials with properties in between liquids and solids. Polarizing microscopes are used to detect peculiar optical patterns and phase defects in liquid crystals. They can also be used to determine if a crystal is optically positive or negative. Liquid crystal retardation can also be measured using polarized microscopes.

Macromolecular materials

Spherulites are transparent, double refractive materials of spherical shape, belonging to the class called macromolecular crystals. Polarized microscopes can be used to determine their density and size which in turn determines the strength and transparency.

Food chemicals

Polarized microscopes can be used to determine the properties of emulsions of butter and cream as they possess optical anisotropy. Thus, using this method, any deviations in the emulsion conditions or impurities can be determined.

Glass and ceramics

Polarized microscopy can be used to determine the quality of and defects in glass and ceramics by identifying their colour, shape, diffraction index, and crystal impurities.

Metals

Metal inspection for composition, surface impurities and properties is possible using polarizing microscopy on a polished metal sample.

Important Applications

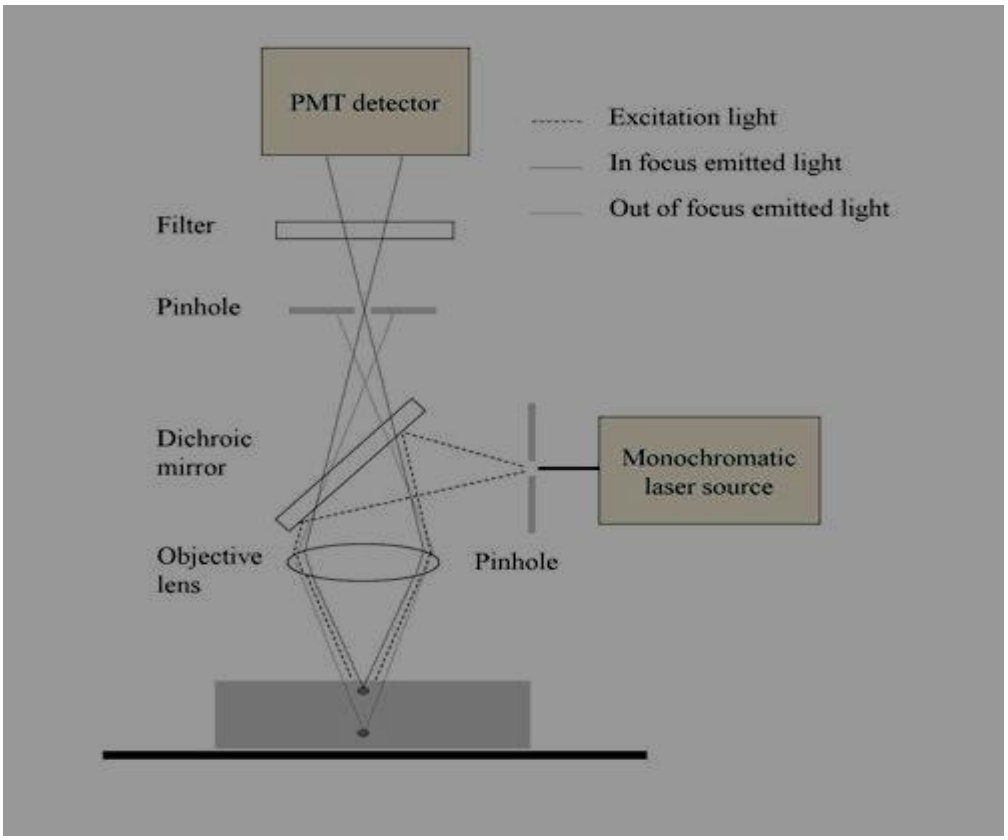
- It is mostly used in the field of geology to study rocks and minerals.
- Besides that can also be used in medicine, chemistry, biology and some times in metallurgy.
- It is the best choice to study materials like minerals, polymers, ceramics, wood, urea.
- Substances of natural and synthetic fibers with those birefringent properties, cellophane, and also botanical and insect specimens and fish scales can study.
- With polarizing microscopy it is possible to determine the color absorption, structure, composition and refraction of light in isotropic (gases and liquids – one refractive index) and anisotropic substances.
- This technique finds application in several fields, such as, industry, and to study rock minerals.

CONFOCAL MICROSCOPE

Laser Scanning Basics and Applications

A confocal microscope, on the other hand, uses light emitted by a laser for fluorescence emission. Apart from the different light sources, the manner in which light is transmitted is different between the two techniques.

The following is a diagrammatic representation of light pathways in a confocal microscope configuration:



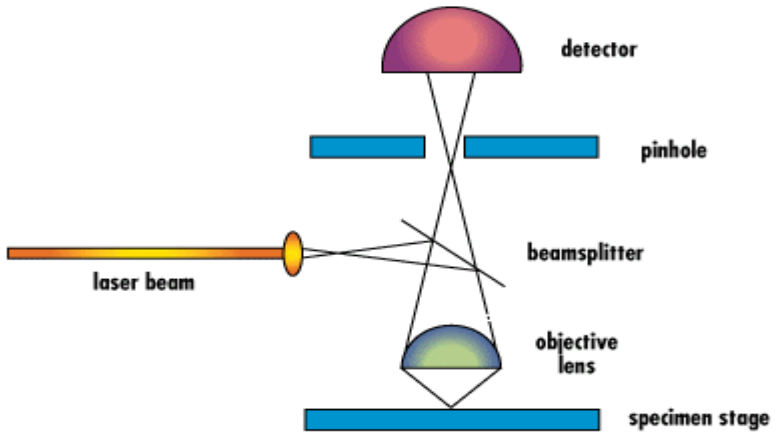


Figure 1 -- Components Needed for HazeBuster

In a confocal microscope, a laser system acts as the excitation source. As such, it emits a coherent light which passes through a pinhole aperture situated at the conjugate plane before reaching the dichromatic mirror. The laser is then reflected by the mirror and directed towards the specimen in a defined focal plane where a secondary fluorescence is emitted.

In a similar fashion to widefield epifluorescence microscopy, the fluorescence is then emitted from different points of the specimen along the same focal plane and focused at the detector pinhole aperture before reaching the photomultiplier detector.

Here, it is worth noting that like in widefield fluorescence microscopy, the confocal principle may also employ the use of filters used in fluorescence microscopy (an excitation filter as well as a fluorescence barrier filter). Here, these components serve similar functions to those found in wide-field fluorescence microscopy. For this reason, the technique is sometimes referred to as epi-illumination scanning confocal microscopy.

In a confocal microscope set-up, the scan head is at the heart of the system.

Components:

- External laser sources
- Dichromatic mirror
- Pinhole apertures
- Photomultiplier
- Fluorescence filters
- Raster scanning mirror system

Apart from the laser sources in confocal microscopy, the pinhole apertures are some of the most important parts of the microscope. The first pinhole aperture (light source pinhole aperture/illuminating aperture) is located between the light/laser source and mirror.

Also known as a spatial filter pinhole, this pinhole is conveniently incorporated into the beam expander which helps produce a uniform illumination beam. Typically, the ideal size of this aperture is relatively large compared to the size of confocal aperture needed to achieve true confocal operation.

Light from this aperture is then reflected by the mirror and directed to the sample/specimen which in turn, produces the secondary fluorescence. From the specimen, the secondary fluorescence is first collected by the objective lens before reaching the mirrors (galvanometer mirrors) where it is descanned (fluorescence emission passes through the galvanometer mirror system towards the detector).

From here, the light may pass through emission filters (barrier filters) and consequently reach the second pinhole aperture (confocal aperture). Apart from the barrier filter, this aperture plays an important role in excluding out-of-focus fluorescence signals (emissions that do not originate from the focal plane) to ensure that only the signals from the illuminated spot enter the detector.

It is worth noting that in this set-up, the whole sample is not illuminated at once. Rather, the laser light is controlled in a manner that ensures that only a given spot at a specified depth within the specimen is illuminated.

As a result, fluorescent light is only emitted from this point. The out-of-focus signals from the features located above/below the focal plane are excluded from passing through the aperture thus only allowing a small fraction of light to pass through.

By rejecting the out-of-focus and stray light, the pinhole contributes to the high signal-to-noise ratio of the images produced.

As compared to widefield microscope, images in this system are formed by scanning a focused beam across a given area in a raster pattern. Here, the scanning process is controlled by high-speed oscillating mirrors with one mirror moving the beam along the x l During scanning, fluorescence emission is collected by the objective lens and passed through the optical system before reaching the detector. Once the emission passes the pinhole aperture, it has to be converted into an analog electrical signal by the photomultiplier so as to be converted into pixels in the scanning unit.

Given that the image is reconstructed point by point using this technique, it cannot be observed through the eyepiece because it does not exist as a real image.

Confocal microscopy can be performed using the epi-fluorescence mode of brightfield reflection mode depending on the sample being visualized. For instance, whereas the reflection microscopy mode is suitable for imaging surface and multilayer structures, the fluorescence mode is ideal for thick specimens.

Fluorescence Microscopy Vs Confocal Microscopy

Apart from the differences in the working principles of the two techniques, differences can also be identified in their strengths and limitations. For instance, fluorescence microscopy is very sensitive which allows for the detection of single-molecules. Moreover, unique optical properties of molecules contribute to high specificity among these microscopes.

They also have a number of limitations including susceptibility to autofluorescence, biocompatibility issues and the fact that fluorophores tend to lose their ability to fluoresce following illumination. On the other hand, fluorescent reporter proteins and fluorescent molecules tend to cause phototoxic effects on live cells.

With regards to confocal microscopy, one of the biggest advantages is that it's possible to control the depth of field by serially producing thin optical sections of thick specimens. By obtaining optical slices from increasing depth of the sample, it becomes possible to produce a 3D image of the sample.

In fluorescence microscopy, on the other hand, images of samples that are over 5 micrometers in thickness appear blurry (indistinct) given that some parts of the sample tend to be outside the focal plane. Therefore, when it comes to visualizing thicker specimen, confocal microscopy is the superior technique.

Advantage of confocal microscopy include:

- Specific wavelengths are used to excite the specimen and produce fluorescence
- By eliminating lateral interference, the technique is able to improve image contrast
- Light is collected from a single focal plane

Disadvantages of confocal microscopy include:

- High-intensity laser irradiation used in confocal microscopy is harmful to live cells (however, some of the newer models in the market have been able to address this issue)
- The signal to noise ratio in confocal microscopy tends to increase sensitivity to noise
- The detector pinhole tends to reduce signal strength
- The technique is time-consuming and requires good training

Applications:

Confocal microscopy is broadly used to resolve the detailed structure of specific objects within the cell. Similar to widefield fluorescence microscopy, various components of living and fixed cells or tissue sections can be specifically labeled using immunofluorescence, for example, and then visualized in high resolution.

As a distinctive feature, confocal microscopy enables the creation of sharp images of the exact plane of focus, without any disturbing fluorescent light from the background or other regions of the specimen. Therefore, structures within thicker objects can be conveniently visualized using confocal microscopy. Furthermore, by stacking several images from different optical planes, 3D structures can be analyzed. The sample penetration depth is limited, however, when using confocal microscopy. Thicker objects, like large spheroids, organoids, tissue, and small animals, should instead be optimally imaged using two-photon microscopy or LSFM.

Fluorescence **I**n **S**itu **H**ybridization

(**F**luorescence **I**n **S**itu **H**ybridization) is a molecular genetic technique applied in cytogenetic examinations to detect small chromosomal rearrangements.

☑ **FISH - Technique: Basic steps**

- Preparation of target DNA: DNA of metaphase or interphase cells are denatured into single-stranded DNA

- A DNA probe, corresponding to a specific chromosomal DNA sequence is labeled with a specific fluorophore

- Hybridization: Target DNA and the labeled DNA sequence are hybridized in situ to fixed metaphase or prometaphase chromosome spreads on a glass slide

- Each probe has the possibility of hybridizing specifically to two sister chromatids. The probe, marking a specific sequence of the chromosome is then visualized

Labeling

It is possible to either label the probe directly or indirectly. Direct labeling involves a labeled and modified nucleotide (often 2' deoxyuridine 5' triphosphate), which is directly incorporated into the probe. In deoxyuridine 5' triphosphate), which is directly incorporated into the probe. In indirect labeling, the DNA is labeled with a fluorophore to make the signal visible (not being incorporated into DNA). Indirect labeling can involve e.g. a hapten

1. Centromeric (satellite probes)
2. Locus specific probes
3. Whole chromosome painting probes (e.g. used in mFISH)

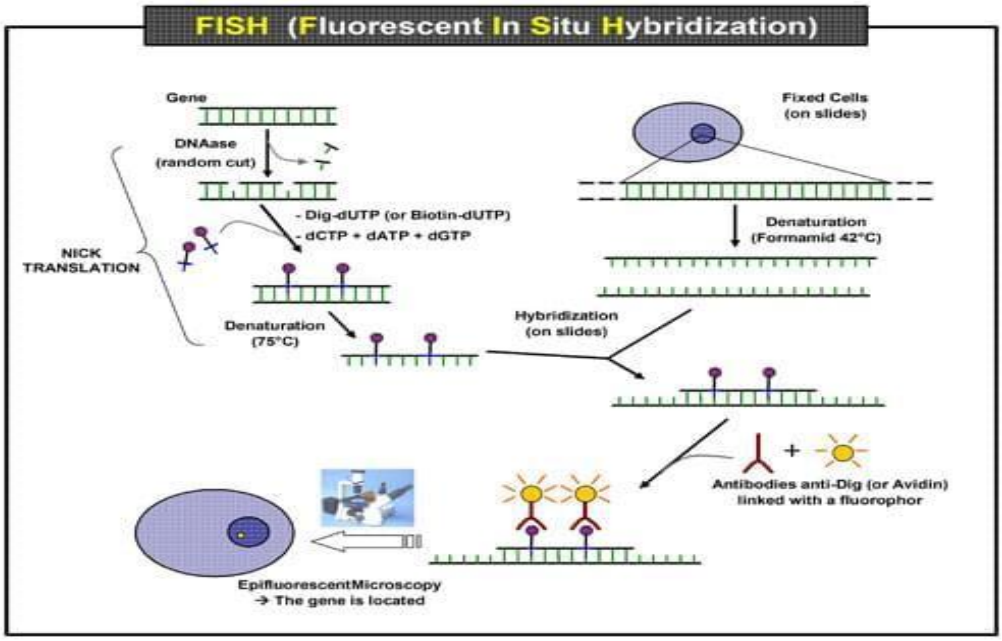


Fig. 1 Scheme of the principle of the FISH experiment to localize a gene in the nucleus.

How does FISH work?

FISH is useful, for example, to help a researcher identify where a particular gene falls within an individual's chromosomes.
Here'showitworks: Make a probe complementary to the known sequence. When making the

probe, label it with a fluorescent marker, e.g. fluorescein, by incorporating nucleotides that have the marker attached to them.

Put the chromosomes on a microscope slide and denature them. Denature the probe and add it to the microscope slide, allowing the probe hybridize to its complementary site.

Wash off the excess probe and observe the chromosomes under a fluorescent microscope. The probe will show as one or more fluorescent signals in the microscope, depending on how many sites it can hybridize to.

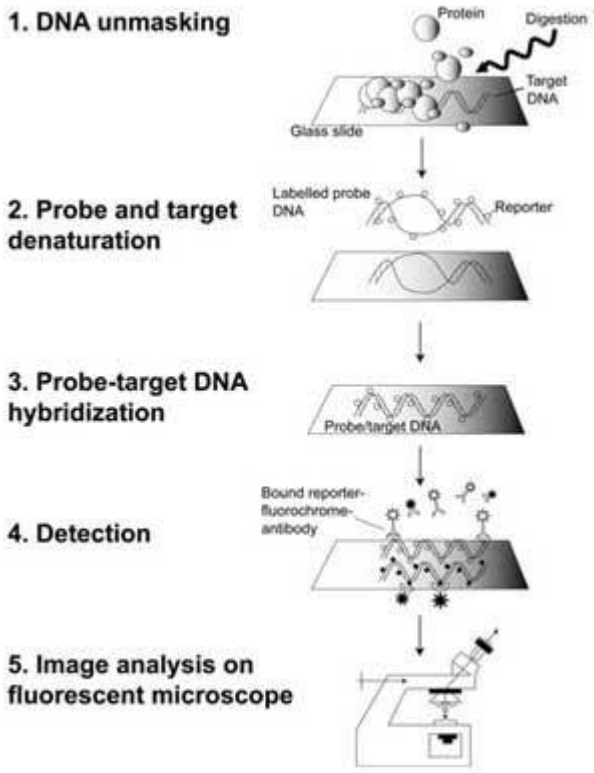


Fig. 2 The five basic steps of FISH. (Oliveira and French 2005)

What is FISH used for?

FISH is widely used for several diagnostic applications: identification of numerical and structural abnormalities, characterization of marker chromosomes, monitoring the effects of therapy, detection of minimal residual disease, tracking the origin of cells after bone marrow transplantation, identification of regions of deletion or amplification, detection of chromosome abnormalities in non-dividing or terminally differentiated cells, determination of lineage involvement of clonal cells, etc. Moreover it has many applications in research: identification of non-random chromosome rearrangements, identification of translocation molecular breakpoint, identification of commonly deleted regions, gene mapping, characterization of somatic cells hybrids, identification of amplified genes, study the

mechanism of rearrangements. FISH is also used to compare the genomes of two biological species to deduce evolutionary relationships.

How many types of probes for FISH?
Generally, researchers use three different types of FISH probes, each of which has a different application:

Locus specific probes bind to a particular region of a chromosome. This type of probe is useful when researchers have isolated a small portion of a gene and want to determine on which chromosome the gene is located.

Alphoid or centromeric repeat probes are generated from repetitive sequences found in the middle of each chromosome. Researchers use these probes to determine whether an individual has the correct number of chromosomes. These probes can also be used in combination with "locus specific probes" to determine whether an individual is missing genetic material from a particular chromosome.

Whole chromosome probes are actually collections of smaller probes, each of which binds to a different sequence along the length of a given chromosome. Using multiple probes labeled with a mixture of different fluorescent dyes, scientists are able to label each chromosome in its own unique color. The resulting full-color map of the chromosome is known as a spectral karyotype. Whole chromosome probes are particularly useful for examining chromosomal abnormalities, for example, when a piece of one chromosome is attached to the end of another chromosome.

Chromosome/Cell Preparation: cell chromosome FISH (FISH on interphase, metaphase and cultured cells), tissue chromosome FISH (FISH on formalin-fixed, paraffin-embedded tissue or cell slides) and RNA-FISH (FISH to study intracellular RNA localization, RNA processing, quantitation). We performed numerous tests aimed to improve the efficiency of cytogenetic slide preparation and to increase FISH signals. Several modifications of the general protocol resulted in better chromosome spreading, better chromosome morphology and shorter hybridization times, while yielding brighter FISH signals.

Probes Design, Labeling, Purification and Test: FISH probes are synthetic pieces of DNA that couple with a fluorescent indicator, so that the chromosomes or genes that they bind to can be directly visualized under our imaging analysis system. Our well-established standard operating procedure allows efficient optimization of labeling a variety of fluorescent probes. Also, we can provide experimental services to meet your special needs. Customized probes are available as well. The choice of directly or indirectly labeled SpectrumOrange, SpectrumGreen, or SpectrumAqua probes is dependent upon the use and combination of other fluorophores in the FISH assay.

- Whole chromosome painting probes (WPP)
- Chromosome arm painting probes (APP)
- Chromosome terminal band painting probes (TPP)
- Chromosome enumeration probes (CEP)
- Chromosome subtelomere probes (CSP)
- Chromosome loci specific probes (CLP)

***In Situ* Hybridization:** We can optimize the FISH hybridization conditions and provide a full array of FISH services.

Result Interpretation: Our FISH imaging analysis system consists of a fluorescent microscope, a charge-coupled-device ("CCD") camera and imaging analysis software. All three components are provided by our component suppliers with internal modifications. Through the use of FISH probes, the imaging analysis system enables medical practitioners to detect and localize the presence or absence of specific DNA sequences in chromosomes.

Which chromosomal aberrations can be identified? (Application of FISH)

- translocations (balanced and unbalanced)
- copy numer changes
- additions
- deletions
- insertions
- inversions
- identifies chromosomal origins
- can identify specific p/q arms/bands

Prenatal Diagnosis: Our optimized prenatal diagnosis detection kits offer innovative technologies and standard methods that are applied to the diagnosis of fetal disorders. Cancer, Prenatal, and Biology Research usethis FISH.

- Trisomy
- Turner Syndrome
- Trisomy X
- XYY Syndrome
- DiGeorge Syndrome
- Triploidy

Cancer Diagnosis and Prognosis: Many cancer diagnosis testing kits are available in our company. You can also custom other cancer diagnosis probes according to your needs.

- Bladder Cancer
- Breast Cancer
- Cervical Cancer
- Chronic Lymphocytic Leukemia
- Chronic Granulocytic Leukemia

- Multiple Myeloma

Advantages

- Simple, easy, fast and repeatable
- Sensitive
- Competitive price
- Easy to process multiple slides simultaneously
- High quality

Transmission Electron Microscope (TEM)

This is a powerful electron microscope that uses a beam of electrons to focus on a specimen producing a highly magnified and detailed image of the specimen.

The magnification power is over 2 million times better than that of the **light microscope**, producing the image of the specimen which enables easy characterization of the image in its morphological features, compositions and crystallization information is also detailed.

Among all microscopes both light and electron microscopes, TEM are the most powerful microscopes used in laboratories. It can magnify a mall particle of about 2nm, and therefore they have a resolution limit of 0.2um.

Principle of Transmission Electron Microscope (TEM)

The working principle of the Transmission Electron Microscope (TEM) is similar to the light microscope. The major difference is that light microscopes use light rays to focus and produce an image while the TEM uses a beam of electrons to focus on the specimen, to produce an image.

Electrons have a shorter wavelength in comparison to light which has a long wavelength. The mechanism of a light microscope is that an increase in resolution power decreases the wavelength of the light, but in the TEM, when the electron illuminates the specimen, the resolution power increases increasing the wavelength of the electron transmission. The wavelength of the electrons is about 0.005nm which is 100,000X shorter than that of light, hence TEM has better resolution than that of the light microscope, of about 1000times.

This can accurately be stated that the TEM can be used to detail the internal structures of the smallest particles like a virion particle.

Parts of Transmission Electron Microscope (TEM)

Their working mechanism is enabled by the high-resolution power they produce which allows it to be used in a wide variety of fields. It has three working parts which include:

1. Electron gun
2. Image producing system
3. Image recording system

Electron gun

- This is the part of the Transmission Electron Microscope responsible for producing electron beams.
- Electrons are produced by a cathode that is a tungsten filament that is V-shaped and it is normally heated. The tungsten filament is covered by a control grid known as a Wehnelt cylinder made up of a central hole which lies columnar to the tube. The cathode lies on top of or below the cylindrical column hole. The cathode and the control grid are negatively charged with an end of the anode which is disk-shaped that also has an axial hole.
- When electrons are transmitted from the cathode, they pass through the columnar aperture (hole) to the anode at high voltage with constant energy, which is efficient for focusing the specimen to produce an accurately defined image.
- It also has the condenser lens system which works to focus the electron beam on the specimen by controlling the energy intensity and the column hole of the electron gun. The TEM uses two condenser lenses to converge the beam of electrons to the specimen. The two condenser lens each function to produce an image i.e the first lens which has strong magnification, produces a smaller image of the specimen, to the second condenser lens, directing the image to the objectives.

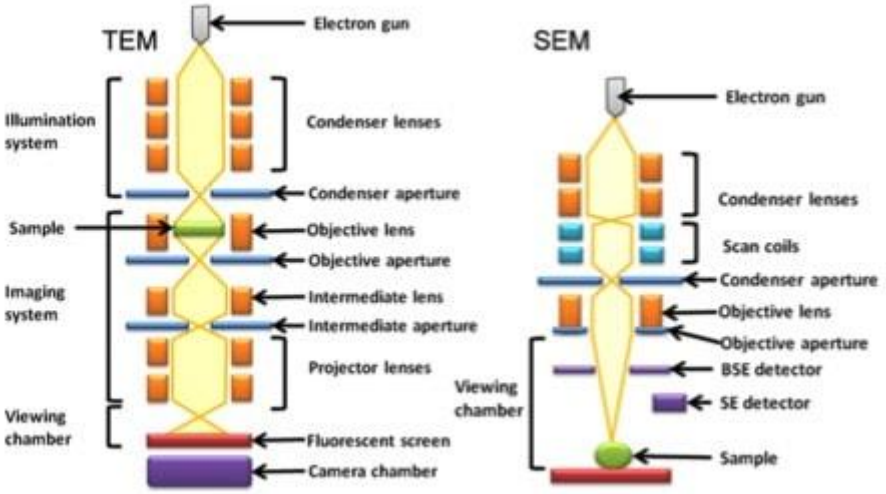
Image- Producing system

- Its made up of the objective lens, a movable stage or holding the specimen, intermediate and projector lenses. They function by focusing the passing electrons through the specimen forming a highly magnified image.
- The objective has a short focal length of about 1-5mm and it produces an intermediate image from the condenser which are transmitted to the projector lenses for magnification.
- The projector lenses are of two types, i.e the intermediate lens which allows great magnification of the image and the projector lens which gives a generally greater magnification over the intermediate lens.
- To produce efficient high standard images, the objectives and the projector lenses need high power supplies with high stability for the highest standard of resolution.

Image-Recording System

- Its made up of the fluorescent screen used to view and to focus on the image. They also have a digital camera that permanently records the images captured after viewing.
- They have a vacuum system that prevents the bombardment or collision of electrons with air molecules disrupting their movement and ability to focus. A vacuumed system facilitates the straight movement of electrons to the image.
- The vacuumed system is made up of a pump, gauge, valves and a power supply.
- The image that is formed is called a monochromatic image, which is greyish or black and white. The image must be visible to the human eye, and therefore, the electrons are allowed to pass through a fluorescent screen fixed at the base of the microscope.

- The image can also be captured digitally and displayed on a computer and stored in a JPEG or TIFF format. During the storage, the image can be manipulated from its monochromatic state to a colored image depending on the recording apparatus eg use of pixel cameras can store the image in color.
- The presence of colored images allows easy visualization, identification, and characterization of the images.



How does a Transmission Electron Microscope (TEM) work?

From the instrumentation described, the working mechanism is a sequential process of the parts of the TEM mentioned above. To mean:

- A heated tungsten filament in the electron gun produces electrons that get focus on the specimen by the condenser lenses.
- Magnetic lenses are used to focus the beam of electrons of the specimen. By the assistance offered by the column tube of the condenser lens into the vacuum creating a clear image, the vacuum allows electrons to produce a clear image without collision with any air molecules which may deflect them.
- On reaching the specimen, the specimen scatters the electrons focusing them on the magnetic lenses forming a large clear image, and if it passes through a fluorescent screen it forms a polychromatic image.
- The denser the specimen, the more the electrons are scattered forming a darker image because fewer electron reaches the screen for visualization while thinner, more transparent specimens appear brighter.

Preparation of specimen for visualization by TEM

The specimen to be viewed under the TEM must undergo a special preparation technique to enable visualization and creation of a clear image.

- Electrons are easily absorbed and easily scattered on solid elements, showing poor visualization for thick specimens. And therefore, very thin specimens are used for

accurate and clear visualization forming a clear image as well. The specimen should be about 20-100nm thin and 0.025-0.1nm diameter, as small as that of a bacterial cell. Thin specimens allow interaction with electrons in a vacuumed space, are able to maintain their innate structure.

- To get thin slice specimens, the specimen is first fixed on a plastic material with glutaraldehyde or osmium tetraoxide. These chemical agents stabilize the structure of the cell and maintain its originality. The addition of an organic solvent like alcohol such as ethanol will dehydrate the cell completely for embedding the specimen to the plastics.
- The specimen is then permeated by adding an unpolymerized liquid epoxy plastic making it hardened like a solid block. This is where thin sections are cut from using a glass knife with a piece of special equipment known as an ultramicrotome.
- The specimen is then stained appropriately (with the appropriate stain) for the uniform scattering of electrons. The thin sections are then soaked in heavy metallic elements such as lead citrate and uranyl acetate allowing the lean and aluminum ions to bind to the cell structures. This forms an opaque layer against the electrons on the cell structures to increase contrast.
- The stained thin sections are then mounted on copper grids for viewing.
- The primary staining techniques that are applied for viewing under the TEM is Negative staining coupled with heavy metallic elements coating. The metallic coating scatters electrons which appears on the photographic film while uncoated sections and used to study bacterial, viral cell morphologies and structures.

Freeze-itching treatment:

To reduce the possible dangers of artifacts, freeze-itching is used especially for the treatment of microbial cells, unlike chemical fixation, dehydration, and embedding, where most specimens get contaminated.

- Microbial cell organelles undergo special treatment known as Freeze-itching whereby the specimens are prepared with liquid nitrogen and then warmed at -100°C in a vacuum chamber.
- The sections are then cut with a precooled knife in liquid nitrogen at -196°C. After warming up the sectioned specimen in a high vacuum for about 2 minutes, it can then coated ith platinum and carbon layer forming replicas.
- These are then be viewed under the TEM displaying more detailed internal structures of the cell in 3D.
- This step of treatment with Liquid nitrogen is known as freeze-itching.

Advantages of Transmission Electron Microscope (TEM)

1. It has a very powerful magnification of about 2 million times that of the Light microscope.
2. It can be used for a variety of applications ranging from basic Biology to Nanotechnology, to education and industrial uses.
3. It can be used to acquire vast information on compounds and their structures.

4. It produces very efficient, high-quality images with high clarity.
5. It can produce permanent images.
6. It is easy to train and use the Transmission Electron Microscope

LIMITATIONS

1. Generally, the TEMs are very expensive to purchase
2. They are very big to handle.
3. The preparation of specimens to be viewed under the TEM is very tedious.
4. The use of chemical fixations, dehydrators, and embedments can cause the dangers of artifacts.
5. They are laborious to maintain.
6. It requires a constant inflow of voltage to operate.
7. They are extremely sensitive to vibrations and electro-magnetic movements hence they are used in isolated areas, where they are not exposed.
8. It produces monochromatic images, unless they use a fluorescent screen at the end of visualization.

Scanning Electron Microscope (SEM)

- A Scanning Electron Microscope (SEM) is a type of electron microscope that images a sample by scanning it with a high energy beam of electrons in a raster scan pattern.
- The electrons interact with the atoms that make up the sample producing signals that contain information about the sample's surface topography, composition, and other properties such as electrical conductivity.
- Scanning electron microscopy is used for inspecting topographies of specimens at very high
  magnifications using a piece of equipment called the scanning electron microscope.
- SEM magnifications can go to more than 300,000 X but most semiconductor manufacturing
  applications require magnifications of less than 3,000 X only.

Advantages of SEM

- It gives detailed 3d and topographical imaging and the versatile information.
- This works very fast.
- Modern SEMs allow for the generation data ni digital form.
- Most SEM samples require minimal preparation actions.
- They are easy to operate and has user-friendly interfaces.
- They are used in a variety of industrial applications to analyze surfaces of solid objects.
- Some modern SEMs are able to generate digital data that can be portable.
- It is easy to acquire data from the SEM, within a short period of time of about 5 minutes.

Applications of the Scanning Electron Microscope (SEM)

It is used in a variety of fields including Industrial uses, nanoscience studies, Biomedical studies, Microbiology

1. Used for spot chemical analysis in energy-Dispersive X-ray Spectroscopy.
2. Used in the analysis of cosmetic components which are very tiny in size.
3. Used to study the filament structures of microorganisms.
4. Used to study the topography of elements used in industries.

Disadvantages

- SEMs are expensive and large.
- Special training is required to operate an SEM.
- SEMs are limited to solid samples.
- SEMs carry a small risk of radiation exposure associated with the electrons that scatter from beneath the sample surface.

A **Scanning Electron Microscope** (SEM) is a powerful magnification tool that utilizes focused beams of electrons to obtain information.

The high-resolution, three-dimensional images produced by SEMs provide topographical, morphological and compositional information makes them invaluable in a variety of science and industry applications.

SEM Properties

The Scanning Electron Microscope developed by professor Dr. Charles Oatlev with the assistance of graduate students in the 1950s, are one of the three types of electron microscopes (EM).

Electron microscopes utilize the same basic principles as light microscopes, but focus beams of energetic electrons rather than photons, to magnify an object.

SEMs consist of the following components:

- Electron Source
- Thermionic Gun
- Field Emission Gun
- Electromagnetic and/or Electrostatic Lenses
- Vacuum chamber
- Sample chamber and stage
- Computer
- Detectors (one or more)
- Secondary Electron Detector (SED)
- Backscatter Detector
- Diffracted Backscatter Detector (EBSD)
- X-ray Detector (EDS)

In addition, SEMs require a stable power supply, vacuum and cooling system, vibration-free space and need to be housed in an area that isolates the instrument from ambient magnetic and electric fields.

SEM Imaging/working principle

A Scanning Electron Microscope provides details surface information by tracing a sample in a raster pattern with an electron beam.

The process begins with an electron gun generating a beam of energetic electrons down the column and onto a series of electromagnetic lenses.

These lenses are tubes, wrapped in coil and referred to as solenoids.

The coils are adjusted to focus the incident electron beam onto the sample; these adjustments cause fluctuations in the voltage, increasing/decreasing the speed in which the electrons come in contact with the specimen surface.

Controlled via computer, the SEM operator can adjust the beam to control magnification as well as determine the surface area to be scanned.

The beam is focused onto the stage, where a solid sample is placed. Most samples require some preparation before being placed in the vacuum chamber.

Of the variety of different preparation processes, the two most commonly used prior to SEM analysis are sputter coating for non-conductive samples and dehydration of most biological specimens.

In addition, all samples need to be able to handle the low pressure inside the vacuum chamber.

The interaction between the incident electrons and the surface of the sample is determined by the acceleration rate of incident electrons, which carry significant amounts of kinetic energy before focused onto the sample.

When the incident electrons come in contact with the sample, energetic electrons are released from the surface of the sample. The scatter patterns made by the interaction yields information on size, shape, texture and composition of the sample.

A variety of detectors are used to attract different types of scattered electrons, including secondary and backscattered electrons as well as x-rays.

Backscatter electrons are incidental electrons reflected backwards; images provide composition data related to element and compound detection. Although topographic information can be obtained using a backscatter detector, it is not as accurate as an SED.

Diffracted backscatter electrons determine crystalline structures as well as the orientation of minerals and micro-fabrics.

X-rays, emitted from beneath the sample surface, can provide element and mineral information.

SEM produces black and white, three-dimensional images.

Image magnification can be up to 10 nanometers and, although it is not as powerful as its TEM counterpart, the intense interactions that take place on the surface of the specimen provide a greater depth of view, higher-resolution and, ultimately, a more detailed surface picture.

Atomic Force Microscopy

An **atomic force microscope** is a type of high resolution scanning probe microscope that has a resolution that you can measure in fractions of a nanometer.

It was pioneered in 1986 by Nobel Prize Winner Gerd Binnig along with Calvin Quate and Christoph Gerber.

Atomic force microscopy (**AFM**) or **scanning force microscopy** (**SFM**) is a very-high-resolution type of scanning probe microscopy (SPM), with demonstrated resolution on the order of fractions of a nanometer, more than 1000 times better than the optical diffraction limit.

Atomic Force Microscopy

One of the most important tools for imaging on the nanometer scale, **Atomic Force Microscopy** uses a cantilever with a sharp probe that scans the surface of the specimen.

When the tip of the probe travels near to a surface, the forces between the tip and sample deflect the cantilever according to Hooke's law.

Atomic force microscopy will measure a number of different forces depending on the situation and the sample that you want to measure.

As well as the forces, other microscopes can include a probe that performs more specialized measurements, such as temperature.

The force deflects the cantilever, and this changes the reflection of a laser beam that shines on the top surface of the cantilever onto an array of photodiodes. The variation of the laser beam is a measure of the applied forces.

Contact and Non-Contact Modes

There are two primary modes of operation for an atomic force microscope, namely contact mode and non-contact mode depending on whether the cantilever vibrates during the operation.

In **contact mode**, the cantilever drags across the sample surface and it uses the deflection of the cantilever to measure the contours of the surface.

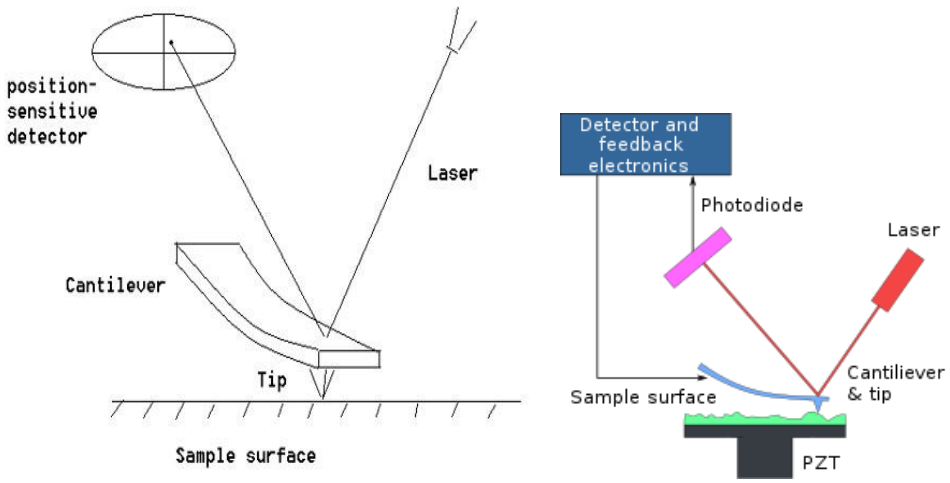
To eliminate the noise and drift that can affect a static signal, low stiffness cantilevers are used, but this allows strong attractive forces to pull the tip to the surface. To eliminate this attraction, the tip is in contact with the surface where the overall force is repulsive.

In **non-contact mode**, the tip vibrates slightly above its resonance frequency and does not contact the surface of the sample. Any long range forces, like van der Waals forces, decreases the resonant frequency of the cantilever.

A feedback loop system helps to maintain the oscillation amplitude constant by changing the distance from the tip to the sample. Recording the distance between the tip and sample at each point allows the software to construct a topographic image of the sample surface.

Most samples will form a layer of moisture on the surface if stored at ambient conditions, and this can make it difficult to measure the sample accurately.

If the probe tip is close enough to detect the short-range forces then it is close enough to stick to the moisture. One way around this is tapping, or dynamic contact mode.



Tapping Mode

In tapping mode, the cantilever uses a piezoelectric element mounted on the top to oscillate it at near to its resonance frequency with an amplitude of up to 200nm.

The forces cause the amplitude to decrease as the tip gets close to the surface, and the height of the cantilever adjusts to keep the amplitude constant.

This tapping results in less damage to the sample than contact mode and is more accurate than non-contact mode when moisture is present on a sample.

Applications

The AFM has been applied to problems in a wide range of disciplines of the natural sciences, including solid-state physics, semiconductor science and technology, molecular engineering, polymer chemistry and physics, surface chemistry, molecular biology, cell biology, and medicine.

Applications in the field of **solid state physics** include

- ✓ the identification of atoms at a surface,
- ✓ the evaluation of interactions between a specific atom and its neighboring atoms, and
- ✓ the study of changes in physical properties arising from changes in an atomic arrangement through atomic manipulation.

In molecular biology, AFM can be used to study the structure and mechanical properties of protein complexes and assemblies. For example, AFM has been used to image microtubules and measure their stiffness.

In cellular biology, AFM can be used to attempt to **distinguish cancer cells** and normal cells based on a hardness of cells, and to evaluate interactions between a specific cell and its neighboring cells in a competitive culture system. AFM can also be used to indent cells, to study how they regulate the stiffness or shape of the cell membrane or wall.

In some variations, electric potentials can also be scanned using conducting cantilevers. In more advanced versions, currents can be passed through the tip to probe the electrical conductivity or transport of the underlying surface, but this is a challenging task with few research groups reporting consistent data (as of 2004).

AFM Advantages

- The atomic force microscope is a powerful tool that is invaluable if you want to measure incredibly small samples with a great degree of accuracy.
- Unlike rival technologies it does not require either a vacuum or the sample to undergo treatment that might damage it.
- At the limits of operation however, researchers have demonstrated atomic resolution in high vacuum and even liquid environments.

AFM Disadvantages

- One of the major downsides is the single scan image size, which is of the order of 150x150 micrometers, compared with millimeters for a scanning electron microscope.
- Another disadvantage is the relatively slow scan time, which can lead to thermal drift on the sample.
- As the technology matures, researchers are relying on there being progress instrumentally, requiring improved signal-to-noise ratio, decreased thermal drift, and better detection and control of tip-sample forces, including the use of sharp probes.

- Novel solutions are steadily improving these performance issues.

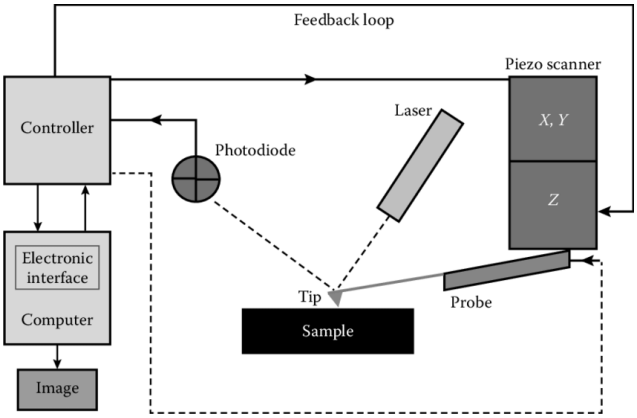


Diagram of AFM

Video Microscopes/videomicrography

Video microscopes provide a live feed image directly to a computer, TV or a LCD projector. There are several different setups for video microscopes which differ mostly based on the type of camera and the frames per second that the camera provides. The main goal for a video microscope is typically a smooth real-time video image that does not jump and is fluid

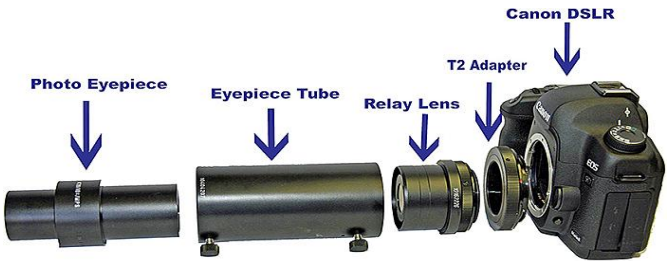
Video Microscope

High quality live feed **video microscopes** utilize a microscope video camera that provides a live video directly to a TV or LCD projector with a fast frame rate. Many video microscopes offer high quality HD (high definjition) output images and are commonly used in teaching environments. Video microscopes connect to the TV or projector through either an HDMI cable or a VGA cable. Some of the more advanced HD cameras also offer image capture to a microSD card. Video microscopes can have either a detachable camera or a built-in camera.

Digital Microscope With Video Capability

Many digital microscopes use a microscope digital camera that connects to the USB port on a computer, and they offer video capture. However, the frame rate on these USB videos is not as high quality as a video camera provides because the frames per second that are captured are much lower. A microscope digital camera is a better choice when the primary focus is capturing images and possibly making measurements, with only the occasional need for

video capture. Because of the lower frames per second on many microscope digital cameras, the live image feed is often not as smooth as one offered by a video microscope. For example, if you were soldering printed circuit boards under a microscope and needed to view quick real-time movements, a digital USB camera would not be the best choice for a fast frame rate.



This function is available on a growing number of DSLRs available on the market and allows a real-time image to be displayed on the camera screen or, if coupled to a computer with appropriate software, on the computer screen. Live View makes focusing much more reliable than viewing through the camera eyepiece; most DSLRs do not have interchangeable focus screens designed for finding accurate focus at high magnifications. The 'refresh' rate of Live View is much lower than for video and therefore it is less useful for tracking and focussing rapidly-moving objects under the microscope; in these instances coupling the camera to a relay lens system with focusing telescope is to be preferred.

For the microscopist, silent Live View mode is desirable for higher magnification photomicrography. The ability to record video. Several DSLRs released in the last year now have the ability to digitally record high-quality video. This can be of great use to the microscopist working with live specimens or dynamic processes such as crystallisation.

Reference :

- https://microbenotes.com/compound-microscope-principle-instrumentation-and-applications/
- https://microbenotes.com/darkfield-microscopy/
- https://microbenotes.com/phase-contrast-microscopy/
- https://microbenotes.com/fluorescence-microscope-principleinstrumentation-applications-advantages-limitations/
- https://en.wikipedia.org/wiki/Polarized_light_microscopy
- https://microbenotes.com/confocal-microscope/
- https://www.creative-biolabs.com/fluorescent-in-situ-hybridization-FISH.html
- https://geneticeducation.co.in/a-brief-introduction-to-fish-fluorescence-in-situ-hybridization/
- https://www.microscopyu.com/applications/fluorescence-in-situ-hybridization-fish/fluorescence-in-situ-hybridization
- https://www.wikilectures.eu/w/Fluorescence_in_situ_hybridization
- https://en.wikipedia.org/wiki/Atomic_force_microscopy

- https://www.microscopeworld.com/t-video_microscopes.aspx