FILTRATION

1. Ultrafiltration

Ultrafiltration (UF) is a variety of membrane filtration in which forces like pressure or concentration gradients lead to a separation through a semipermeable membrane. Suspended solids and solutes of high molecular weight are retained in the so-called retentate, while water and low molecular weight solutes pass through the membrane in the permeate. This separation process is used in industry and research for purifying and concentrating macromolecular (10^3 - 10^6 Da) solutions, especiallyprotein solutions. Ultrafiltration is not fundamentally different from microfiltration. Both of these separate based on size exclusion or particle capture. It is fundamentally different from membrane gas separation, which separate based on different amounts of absorption and different rates of diffusion. Ultrafiltration membranes are defined by the molecular weight cut-off(MWCO) of the membrane used. Ultrafiltration is applied in cross-flow or dead-end mode.

Principles

The basic operating principle of ultrafiltration uses a pressure induced separation of solutes from a solvent through a semi permeable membrane. The relationship between the applied pressure on the solution to be separated and the flux through the membrane is most commonly described by the Darcy equation:

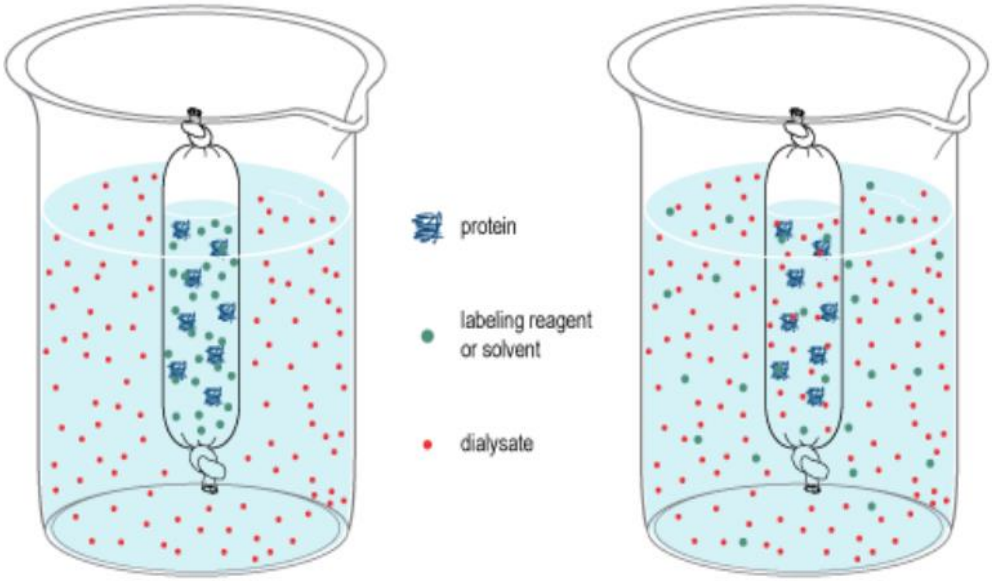
$$J = \frac{TMP}{\mu R_t}$$

where J is the flux (flow rate per membrane area),TMP is the transmembrane pressure (pressure difference between feed and permeate stream), μ is solvent viscosity, R_t is the total resistance (sum of membrane and fouling resistance).

DIALYSIS

Dialysis is the process of separating molecules in solution by the difference in their rates of diffusion through a semipermeable membrane, such asdialysis tubing.

Dialysis is a common laboratory technique that operates on the same principle as medical dialysis. In the context of life science research, the most common application of dialysis is for the removal of unwanted small molecules such as salts, reducing agents, or dyes from larger macromolecules such as proteins, DNA, orpolysaccharides. Dialysis is also commonly used for buffer exchange and drug binding studies.



Principles of Dialysis

Diffusion is the random, thermal movement of molecules in solution (Brownian motion) that leads to the net movement of molecules from an area of higher concentration to a lower concentration until equilibrium is reached. In dialysis, a sample and a buffer solution (called the dialysate) are separated by a semi-permeable membrane that causes differential diffusion patterns, thereby permitting the separation of molecules in both the sample and dialysate.

Due to the pore size of the membrane, large molecules in the sample cannot pass through the membrane, thereby restricting their diffusion from the sample chamber. By contrast, small molecules will freely diffuse across the membrane and obtain equilibrium across the entire solution volume, thereby changing the overall concentration of these molecules in the sample and dialysate (see dialysis figure at right). Once equilibrium is reached, the final concentration of molecules is dependent on the volumes of the solutions involved, and if the equilibrated dialysate is replaced (or exchanged) with fresh dialysate (see procedure below), diffusion will further reduce the concentration of the small molecules in the sample.

Dialysis can be used to either introduce or remove small molecules from a sample, because small molecules move freely across the membrane in both directions. This makes dialysis a useful technique for a variety of applications. See dialysis tubing for additional information on the history, properties, and manufacturing of semi-permeable membranes used for dialysis.

Dialysis Procedure

Equipment

Separating molecules in a solution by dialysis is a straightforward process. Other than the sample and dialysate buffer, all that is typically needed is:

Dialysis membrane in an appropriate format (e.g., tubing, cassette, etc.) and molecular weight cut-off (MWCO)

A container to hold the dialysate bufferThe ability to stir the solutions and control the temperature (optional)

General Protocol

A typical dialysis procedure for protein samples is as follows:

1. Prepare the membrane according to instructions
2. Load the sample into dialysis tubing, cassette or device
3. Place sample into an external chamber of dialysis buffer (with gentle stirring of the buffer)
4. Dialyze for 2 hours (at room temperature or 4 °C)
5. Change the dialysis buffer and dialyze for another 2 hours
6. Change the dialysis buffer and dialyze for 2 hours or overnight

The total volume of sample and dialysate determine the final equilibrium concentration of the small molecules on both sides of the membrane. By using the appropriate volume of dialysate and multiple exchanges of the buffer, the concentration of small contaminants within the sample can be decreased to acceptable or negligible levels. For example, when dialyzing 1mL of sample

against 200mL of dialysate, the concentration of unwanted dialyzable substances will be decreased 200-fold when equilibrium is attained. Following two additional buffer changes of 200mL each, the contaminant level in the sample will be reduced by a factor of 8×10^6 (200 x 200 x 200).

Variables and Protocol Optimization

Although dialyzing a sample is relatively simple, a universal dialysis procedure for all applications cannot be provided due to the following variables:

- The sample volume
- The size of the molecules being separated
- The membrane used
- The geometry of the membrane, which affects the diffusion distance

Additionally, the dialysis endpoint is somewhat subjective and application specific. Therefore, the general procedure might require optimization.

Dialysis Membranes and MWCO

Dialysis membranes are produced and characterized according to molecular-weight cutoff (MWCO) limits. While membranes with MWCOs ranging from 1-1,000,000 kDa are commercially available, membranes with MWCOs near 10 kDa are most commonly used. The MWCO of a membrane is the result of the number and average size of the pores created during production of the dialysis membrane. The MWCO typically refers to the smallest average molecular mass of a standard molecule that will not effectively diffuse across the membrane during extended dialysis. Thus, a dialysis membrane with a 10K MWCO will generally retain greater than 90% of a protein having a molecular mass of at least 10kDa.[3][4]

It is important to note that the MWCO of a membrane is not a sharply defined value. Molecules with mass near the MWCO limit of the membrane will diffuse across the membrane more slowly than molecules significantly smaller than the MWCO. In order for a molecule to rapidly diffuse across a membrane, it typically needs to be at least 20- to 50-times smaller than the MWCO rating of a membrane. Therefore, it is not practical to separate a 30kDa protein from a 10kDa protein using dialysis across a 20K rated dialysis membrane.

Dialysis membranes for laboratory use are typically made of a film of regenerated cellulose or cellulose esters. See reference for a review of cellulose membranes and manufacturing.[5]

Laboratory Dialysis Formats

Dialysis is generally performed in clipped bags of dialysis tubing or in a variety of formatted dialyzers. The choice of the dialysis set up used is largely dependent on the size of the sample

and the preference of the user. Dialysis tubing is the oldest and generally the least expensive format used for dialysis in the lab. Tubing is cut and sealed with a clip at one end, then filled and sealed with a clip on the other end. Tubing provides flexibility but has increased concerns regarding handling, sealing and sample recovery. Dialysis tubing is typically supplied either wet or dry in rolls or pleated telescoped tubes.

A wide variety of dialysis devices (or dialyzers) are available from several vendors. Dialyzers are designed for specific sample volume ranges and provide greater sample security and improved ease of use and performance for dialysis experiments over tubing. The most common preformatted dialyzers are Slide-A-Lyzer, Float-A-Lyzer, and the Pur-A-lyzer/D-Tube/GeBAflex Dialyzers product lines.

Applications of Ultrafiltration

Industries such as chemical and pharmaceutical manufacturing, food and beverage processing, and waste water treatment, employ ultrafiltration in order to recycle flow or add value to later products. Blood dialysis also utilizes ultrafiltration.

Drinking water

UF can be used for the removal of particulates and macromolecules from raw water to produce potable water. It has been used to either replace existing secondary (coagulation, flocculation, sedimentation) and tertiary filtration (sand filtration and chlorination) systems employed in water treatment plants or as standalone systems in isolated regions with growing populations.[1] When treating water with high suspended solids, UF is often integrated into the process, utilising primary (screening, flotation, filtration) and some secondary treatments as pre-treatment stages.[2] UF processes are currently preferred over traditional treatment methods for the following reasons:No chemicals required (aside from cleaning)Constant product quality regardless of feed quality Compact plant size Capable of exceeding regulatory standards of water quality, achieving 90-100% pathogen removal UF processes are currently limited by the high cost incurred due to membrane fouling and replacement. Additional pretreatment of feed water is required to prevent excessive damage to the membrane units.In many cases UF is used for pre filtration in reverse osmosis (RO) plants to protect the RO membranes.

PROTEIN CONCENTRATION

UF is used extensively in the dairy industry; particularly in the processing of cheese whey to obtain whey protein concentrate (WPC) and lactose-rich permeate. In a single stage, a UF process is able to concentrate the whey 10-30 times the feed.
The original alternative to membrane filtration of whey was using steam heating followed by drum drying or spray drying. The product of these methods had limited applications due to its granulated texture and insolubility. Existing methods also had inconsistent product composition,

high capital and operating costs and due to the excessive heat used in drying would often denature some of the proteins. Compared to traditional methods, UF processes used for this application

- Are more energy efficient
- Have consistent product quality, 35-80% protein product depending on operating conditions
- Do not denature proteins as they use moderate operating conditions

The potential for fouling is widely discussed, being identified as a significant contributor to decline in productivity.[5][6][7] Cheese whey contains high concentrations of calcium phosphate which can potentially lead to scale deposits on the membrane surface. As a result substantial pretreatment must be implemented to balance pH and temperature of the feed to maintain solubility of calcium salts.[7]

Figure: 11 A selectively permeablemembrane can be mounted in acentrifuge tube. The buffer is forced through the membrane bycentrifugation, leaving the protein in the upper chamber.

OTHER APPLICATIONS

- Filtration of effluent from paper pulp mill
- Cheese manufacture, see ultrafiltered milk
- Removal of pathogens from milk
- Process and waste water treatment
- Enzyme recovery
- Fruit juice concentration and clarification
- Dialysis and other blood treatments
- Desalting and solvent-exchange of proteins (via diafiltration)

2. Laboratory grade manufacturing

REVERSE OSMOSIS

Reverse Osmosis (RO) Reverse Osmosis is a high pressure, energy-efficient means of de-watering process streams, concentration of low molecular weight compounds or clean-up of waste effluents. Common applications include pre-concentration of dairy or food streams prior to evaporation, polishing of evaporator condensate, and purification of process water.

In order to understand the term "reverse osmosis", the term "osmosis" must first be understood. To do this, the term "Chemical Potential" is defined first. It is the inequality of the chemical potential between, say, fresh and brackish waters that is the driving force for osmosis. A solution's chemical potential is determined by the following:

• Concentration of salts in the water

• The temperature of the water (expressed in absolute terms)

• The pressure of the solution

The concentration of salts in the water supply has an inverse effect on the chemical potential of the solution, whereas the temperature and pressure have a direct effect. Therefore, at constant temperature and pressure of a solution, increased salt content results in decreased chemical potential. This phenomenon is shown in Figure 12. This figure shows a vessel filled with waters of two different salt concentrations. These are separated by a semi-permeable membrane. A semi-permeable membrane is one that allows the passage of pure water, while preventing the passage of salts. The fresh water side has a higher chemical potential than the saline water side. Thus, pure water will pass through the membrane from the fresh water side to the more saline water side. This is the phenomenon called "osmosis". As the pure water passes to the saline water side, four things occur:

(1) the water on the saline side becomes less concentrated;

(2) the level of the solution on the saline side increases;

(3) the water on the fresh side becomes more concentrated; and

(4) the water level on the fresh water side decreases.

These occurrences result in increasing the chemical potential of the solution on the saline side of the membrane and decreasing the potential on the fresh water side. The flow of pure water from the fresh to the saline side will continue until a chemical potential equilibrium condition between the two sides is obtained (Figure 13). At this point, the flow of pure water stops. This is called "osmotic equilibrium". It follows that if the pressure on the saline side were now to be increased, the flow could be reversed. That is, flow across the membrane could be directed from the saline

side to the fresh water side. This is called "reverse osmosis" (Figure 14). Its occurrence is caused by the increased pressure applied to the saline side. The pressure required to begin flow is termed the "osmotic pressure". If this flow were allowed to continue unchanged, the pressure on the saline side would have to be continually increased because the chemical potential on the fresh water side would continue to increase. Thus, for the RO process to work at constant pressure, the concentration of the saline side must be kept constant. This is done by adding new water (feed water) to the saline side while continually extracting concentrated water from the saline side.

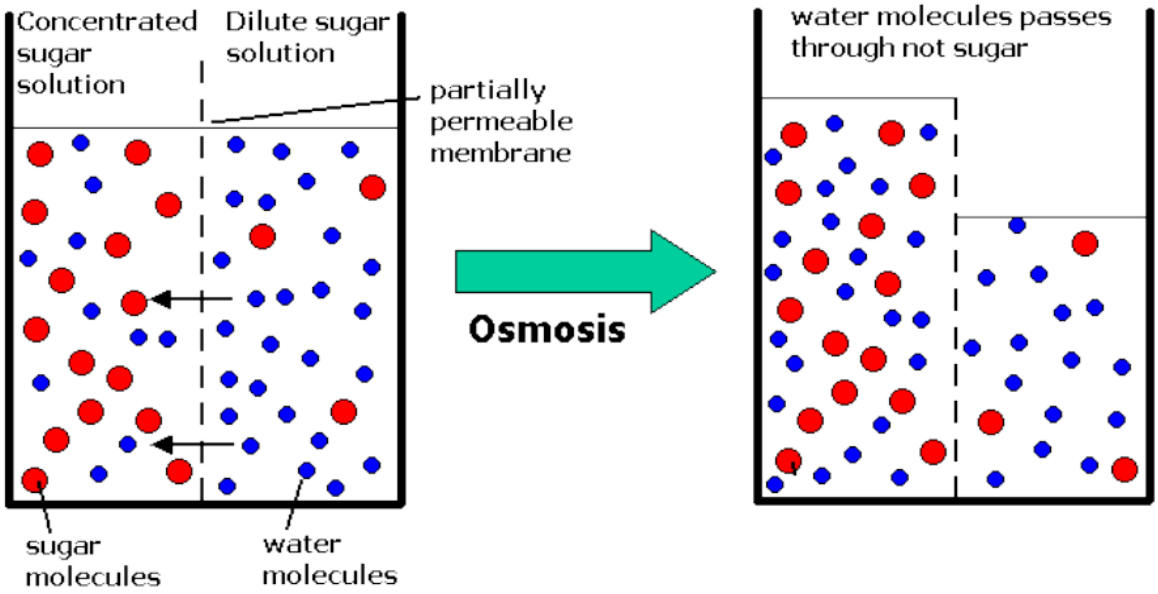
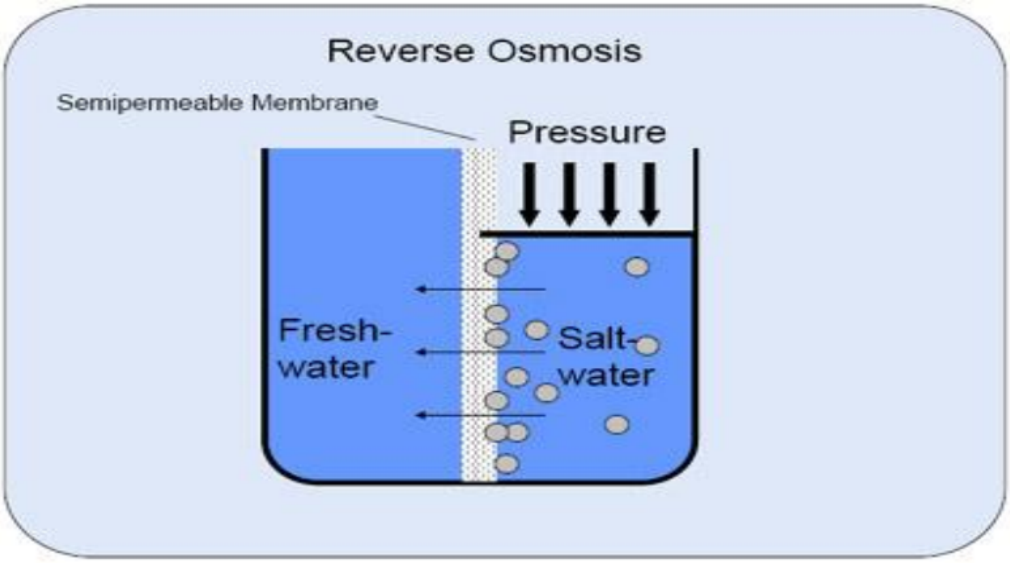


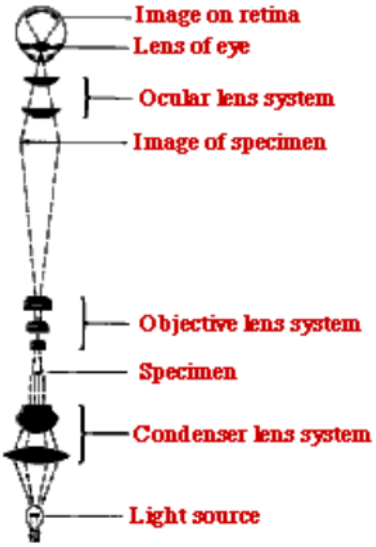
Figure 13 osmotic equilibrium



MICROSCOPY

BRIGHT FIELD MICROSCOPY

Light path of bright field microscope



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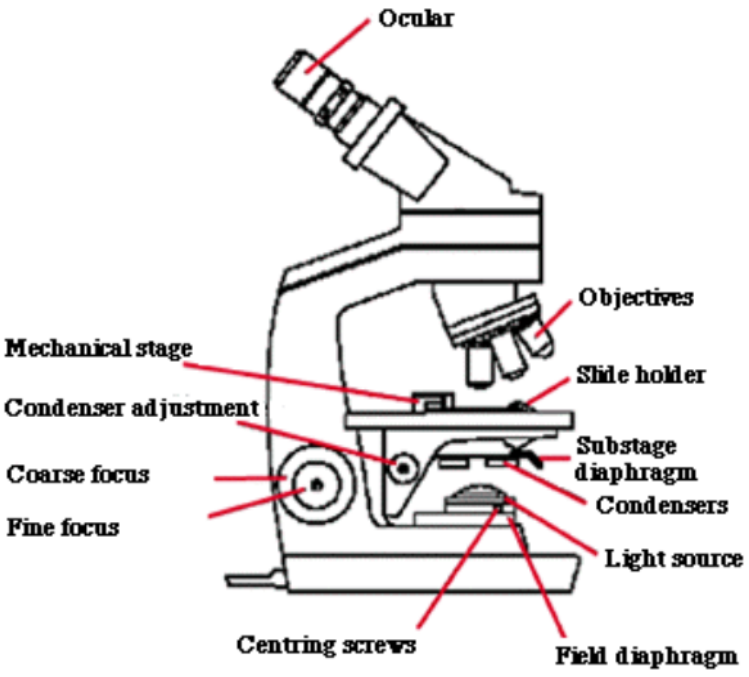

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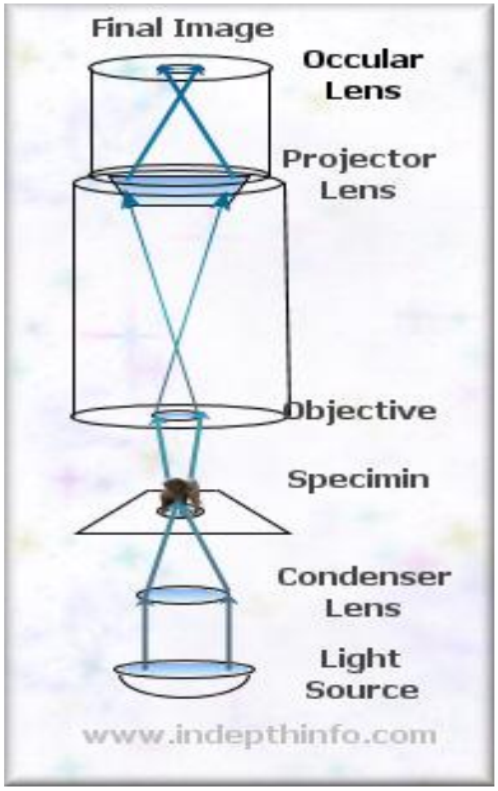
Bright field microscopy is the simplest of all the optical microscopy illumination techniques. Sample illumination is transmitted (i.e., illuminated from below and observed from above) white light and contrast in the sample is caused by absorbance of some of the transmitted light in dense areas of the sample. Bright field microscopy is the simplest of a range of techniques used for illumination of samples in light microscopes and its simplicity makes it a popular technique. The typical appearance of a bright field microscopy image is a dark sample on a bright background, hence the name.



Light path

The light path of a bright field microscope is extremely simple, no additional components are required beyond the normal light microscope setup. The light path therefore consists of:

light source

1. a transillumination light source, commonly a halogen lamp in the microscope stand; A **halogen lamp**, also known as a **tungsten halogen lamp** or **quartz iodine lamp**, is an incandescent lamp that has a small amount of a halogen such as iodine or bromine added. The combination of the halogen gas and the tungsten filament produces a **halogen cycle** chemical reaction which redeposits evaporated tungsten back onto the filament, increasing its life and maintaining the clarity of the envelope
2. a **condenser lens** which focuses light from the light source onto the sample. A **condenser** is one of the main components of the optical system of many transmitted light compound microscopes. A condenser is a lens that serves to concentrate light from the

illumination source that is in turn focused through the object and magnified by the objective lens.

3. **objective lens** : In an optical instrument, the **objective** is the optical element that gathers light from the object being observed and focuses the light rays to produce a real image. Objectives can be single lenses or mirrors, or combinations of several optical elements. Microscope objectives are characterized by two parameters: magnification and numerical aperture. The typically ranges are 4× , 10x , 40x and 100×.
4. **oculars** to view the sample image. An **eyepiece**, or **ocular lens**, is a type of lens that is attached to a variety of optical devices such as microscopes. It is so named because it is usually the lens that is closest to the eye when someone looks through the device. The objective lens or mirror collects light and brings it to focus creating an image. The eyepiece is placed near the focal point of the objective to magnify this image. The amount of magnification depends on the focal length of the eyepiece.

Magnification is the process of enlarging something only in appearance, not in physical size. Typically magnification is related to scaling up visuals or images to be able to see more detail, increasing resolution.

Resolving power is the ability of an imaging device to separate (i.e., to see as distinct) points of an object that are located at a small angular distance..

In optics, the **numerical aperture** (**NA**) of an optical system is a dimensionless number that characterizes the range of angles over which the system can accept or emit light. In most areas of optics, and especially in microscopy, the numerical aperture of an optical system such as an objective lens is defined by

$$\mathrm{NA} = n \sin\theta$$

where n is the index of refraction of the medium in which the lens is working (1.0 for air, 1.33 for pure water, and up to 1.56 for oils; see also list of refractive indices), and θ is the half-angle of the maximum cone of light that can enter or exit the lens. In general, this is the angle of the real marginal ray in the system

Working Performance

Bright field microscopy typically has low contrast with most biological samples as few absorb light to a great extent. Staining is often required to increase contrast, which prevents use on live cells in many situations. Bright field illumination is useful for samples which have an intrinsic colour, for example chloroplasts in plant cells.

Light is first emitted by the light **source** and is directed by the **condenser lens** on to the specimen, which might be a loose object, a prepared plate or almost anything. A microscope can even be applied to small parts of larger objects, though with a bit more difficulty. (The light does not absolutely need to originate below the specimen.)

The light from the specimen then passes through the objective lens. This lens is often selected from among three or four and is the main determinant for the level of magnification. It bends the light rays and in the case of this example sends them to a **projector lens**, which reverses their direction so that when the image reaches the eye it will not appear "upside-down". Not all microscopes have a projector lens, so the viewer may be seeing a reverse image. In these cases, when the slide is moved, it will appear to be moving in the opposite direction to the viewer.

The light rays then travel to the **oracular lens** or "eye piece". This is often a 10X magnification lens, meaning it magnifies the magnified image an additional ten times. The image is then projected into the eye. It is very seldom that a specimen is in focus the moment it is placed beneath a microscope. This means that some adjustment will have to be made. Unlike in telescopes, the focal length between lenses remains constant when adjusting the focus. The lens apparatus is brought closer to or further from the object. The focus adjustment is often along the neck of the tube containing the lenses, but it might just as well move the slide up and down. The best way to make this adjustment is to make a course adjustment so that it is too close to the object and then back off with the fine adjustment[2]. This helps to ensure that the specimen is not inadvertently smashed by the lens.

Advantages

The name "brightfield" is derived from the fact that the specimen is dark and contrasted by the surrounding bright viewing field. Simple light microscopes are sometimes referred to as bright field microscopes.

Brightfield microscopy is very simple to use with fewer adjustments needed to be made to view specimens.

Some specimens can be viewed without staining and the optics used in the brightfield technique don’t alter the color of the specimen.

It is adaptable with new technology and optional pieces of equipment can be implemented with brightfield illumination to give versatility in the tasks it can perform.

Disadvantages

Certain disadvantages are inherent in any optical imaging technique.

- By using an aperture diaphragm for contrast, past a certain point, greater contrast adds distortion. However, employing an iris diaphragm will help compensate for this problem.
- Brightfield microscopy can't be used to observe living specimens of bacteria, although when using fixed specimens, bacteria have an optimum viewing magnification of 1000x.

Brightfield microscopy has very low contrast and most cells absolutely have to be stained to be seen; staining may introduce extraneous details into the specimen that should not be present.

Also, the user will need to be knowledgeable in proper staining techniques.

Lastly, this method requires a strong light source for high magnification applications and intense lighting can produce heat that will damage specimens or kill living microorganisms.

DARK FIELD MICROSCOPY

Dark field microscopy (dark ground microscopy) describes microscopy methods, in both light and electron microscopy, which exclude the unscattered beam from the image. As a result, the field around the specimen (i.e. where there is no specimen to scatter the beam) is generally dark.

Light microscopy applications

In optical microscopy, darkfield describes an illumination technique used to enhance the contrast in unstained samples. It works by illuminating the sample with light that will not be collected by the objective lens, and thus will not form part of the image. This produces the classic appearance of a dark, almost black, background with bright objects on it.

The light's path

The steps are illustrated in the figure where an upright microscope is used.

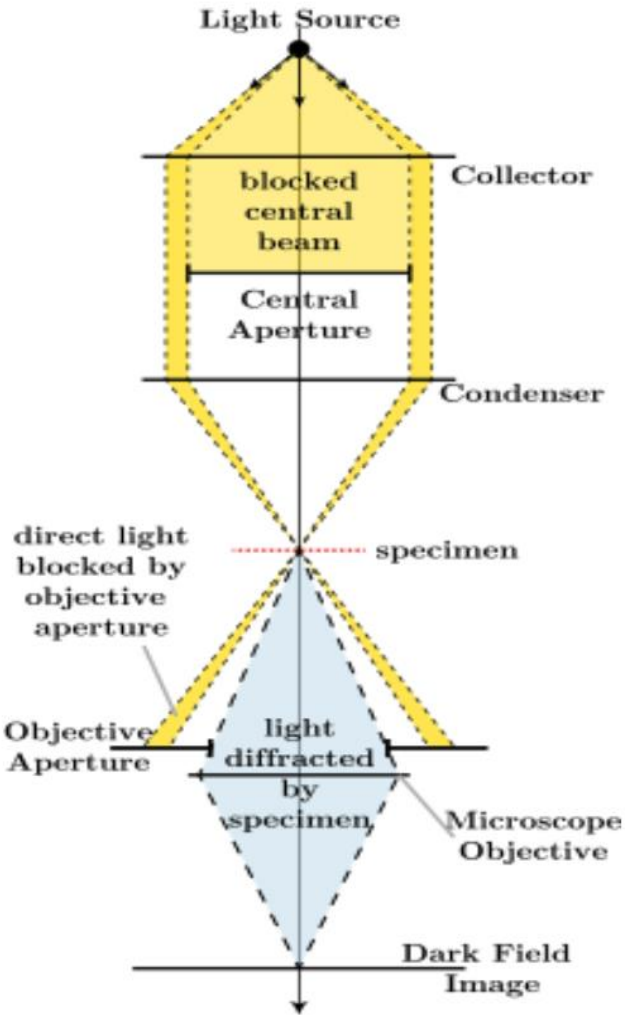


Diagram illustrating the light path through a dark field microscope.

1. Light enters the microscope for illumination of the sample.
2. A specially sized disc, the *patch stop* (see figure) blocks some light from the light source, leaving an outer ring of illumination. A wide phase annulus can also be reasonably substituted at low magnification.
3. The condenser lens focuses the light towards the sample.
4. The light enters the sample. Most is directly transmitted, while some is scattered from the sample.
5. The **scattered light** enters the objective lens, while the **directly transmitted light** simply misses the lens and is not collected due to a *direct illumination block* (see figure).
6. Only the scattered light goes on to produce the image, while the directly transmitted light is omitted.

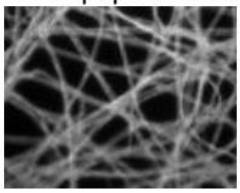
Advantages and disadvantages

Dark field microscopy produces an image with a dark background.

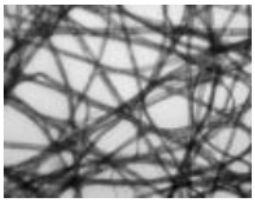
Dark field microscopy is a very simple yet effective technique and well suited for uses involving live and unstained biological samples, such as a smear from a tissue culture or individual water-borne single-celled organisms. Considering the simplicity of the setup, the quality of images obtained from this technique is impressive.

The main limitation of dark field microscopy is the low light levels seen in the final image. This means the sample must be very strongly illuminated, which can cause damage to the sample. Dark field microscopy techniques are almost entirely free of artifacts, due to the nature of the process. However the interpretation of dark field images must be done with great care as common dark features of bright field microscopy images may be invisible, and vice versa.

While the dark field image may first appear to be a negative of the bright field image, different effects are visible in each. In bright field microscopy, features are visible where either a shadow is cast on the surface by the incident light, or a part of the surface is less reflective, possibly by the presence of pits or scratches. Raised features that are too smooth to cast shadows will not appear in bright field images, but the light that reflects off the sides of the feature will be visible in the dark field images.

- Comparison of transillumination techniques used to generate contrast in a sample of tissue paper. 1.559 µm/pixel (when viewed at full resolution).
- 

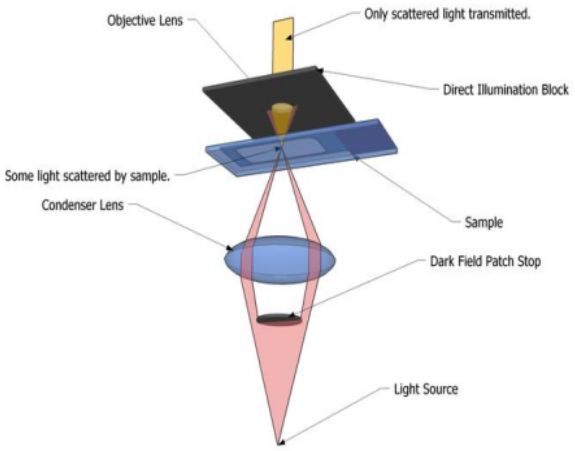
  Dark field illumination, sample contrast comes from light scattered by the sample.

- 

  Bright field illumination, sample contrast comes from absorbance of light in the sample.

Use in computing

Dark field microscopy has recently been used in computer mouse pointing devices, in order to allow an optical mouse to work on transparent glass by imaging microscopic flaws and dust on its surface.



PHASE CONTRAST MICROSCOPY

Phase contrast microscopy is an optical microscopy technique that converts phase shifts in light passing through a transparent specimen to brightness changes in the image. Phase shifts themselves are invisible, but become visible when shown as brightness variations.

When light waves travels through a medium other than vacuum, interaction with the medium causes the wave amplitude and phase to change in a manner dependent on properties of the medium. Changes in amplitude (brightness) arise from the scattering and absorption of light, which is often wavelength dependent and may give rise to colors. Photographic equipment and the human eye are only sensitive to amplitude variations. Without special arrangements, phase changes are therefore invisible. Yet, often these changes in phase carry important information.

History and Background Information

Frits Zernike, a Dutch physicist and mathematician, built the first phase contrast microscope in 1938.

It took some time before the scientific community recognized the potential of Zernike's discovery; he won the Nobel Prize in 1953 and the German-based company Zeiss began manufacturing his phase contrast microscope during World War II.

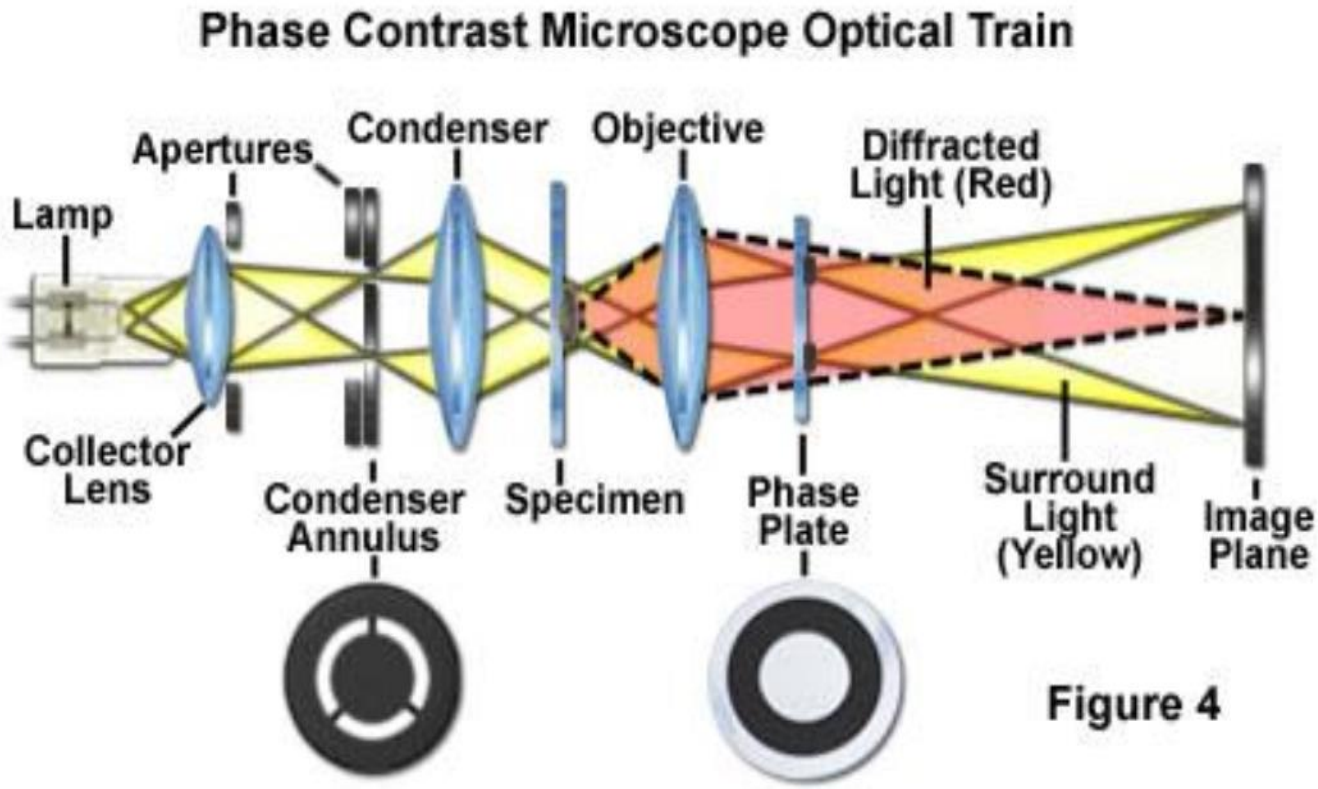
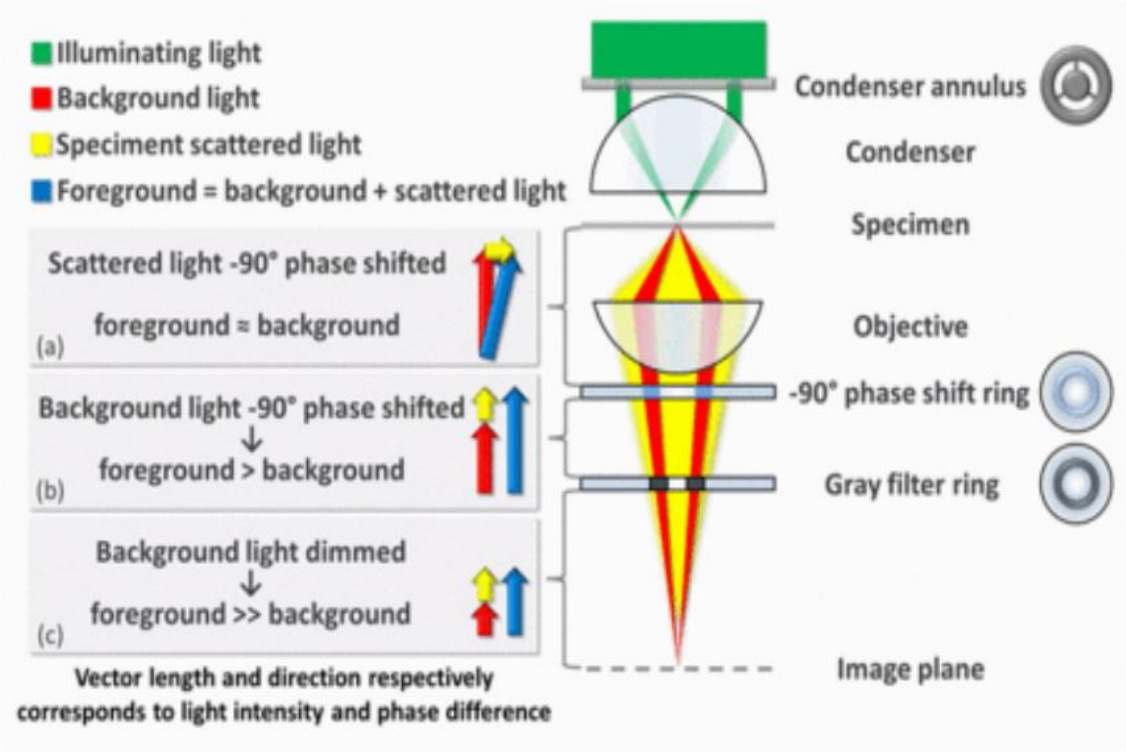


Figure 4

Working principle



The basic principle to make phase changes visible in phase contrast microscopy is to separate the illuminating background light from the specimen scattered light, which make up the foreground details, and to manipulate these differently.

The ring shaped illuminating light (green) that passes the condenser annulus is focused on the specimen by the condenser. Some of the illuminating light is scattered by the specimen (yellow). The remaining light is unaffected by the specimen and form the background light (red). When observing unstained biological specimen, the scattered light is weak and typically phase shifted by -90° — relative to the background light. This leads to that the foreground (blue vector) and the background (red vector) nearly have the same intensity, resulting in a low image contrast (a).

In a phase contrast microscope, the image contrast is improved in two steps. The background light is phase shifted -90° by passing it through a phase shift ring. This eliminates the phase difference between the background and the scattered light, leading to an increased intensity difference between foreground and background (b). To further increase contrast, the background is dimmed by a gray filter ring (c). Some of the scattered light will be phase shifted and dimmed by the rings. However, the background light is affected to a much greater extent, which creates the phase contrast effect.

The above describes *negative phase contrast*. In its *positive* form, the background light is instead phase shifted by +90°. The background light will thus be 180° out of phase relative to the scattered light. This results in that the scattered light will be subtracted from the background light in (b) to form an image where the foreground is darker than the background, as shown in Figure 1. [3][4][5][6][7][8]

Applications in Microscopy

The possible applications of Zernike's phase contrast microscope in microscopy are evident in the fields of molecular and cellular biology, microbiology and medical research.

Specimens that can be observed and studied include live microorganisms such as protozoa, erythrocytes, bacteria, molds and sperm, thin tissue slices, lithographic patterns, fibers, glass fragments and sub-cellular particles such as nuclei and organelles.

Advantages

The advantages of the phase contrast microscope include:

- The capacity to observe living cells and, as such, the ability to examine cells in a natural state
- Observing a living organism in its natural state and/or environment can provide far more information than specimens that need to be killed, fixed or stain to view under a microscope
- High-contrast, high-resolution images
- Ideal for studying and interpreting thin specimens
- Ability to combine with other means of observation, such as fluorescence
- Modern phase contrast microscopes, with CCD or CMOS computer devices, can capture photo and/or video images

In addition, advances to the phase contrast microscope, especially those that incorporate technology, enable a scientist to hone in on minute internal structures of a particle and can even detect a mere small number of protein molecules.

Disadvantages

Disadvantages and limitations of phase contrast:

- Annuli or rings limit the aperture to some extent, which decreases resolution
- This method of observation is not ideal for thick organisms or particles
- Thick specimens can appear distorted
- Images may appear grey or green, if white or green lights are used, respectively, resulting in poor photomicrography
- Shade-off and halo effect, referred to a phase artifacts
- Shade-off occurs with larger particles, results in a steady reduction of contrast moving from the center of the object toward its edges
- Halo effect, where images are often surrounded by bright areas, which obscure details along the perimeter of the specimen

Modern advances and techniques provide solutions to some of these confines, such as the halo effect.

Apodized phase contrast utilizes amplitude filters that contain neutral density films to minimize the halo effect. Essentially, this is attempting to reverse the definition achieved through phase contrast annuli, but the halo effect can never be eliminated completely.

The pros that phase contrast has brought to the field of microscopy far exceed its limitations. This is easily seen with the myriad of advances in the fields of cellular and microbiology as well as in medical and veterinary sciences.

Conclusion

The **phase contrast microscope** opened up an entire world of microscopy, providing incredible definition and clarity of particles never seen before.

PHASE CONTRAST

Phase contrast microscopy imparts contrast to unstained biological material by transforming phase differences of light caused by differences in refractive index between cellular components into differences in amplitude of light, i.e., light and dark areas, which can be observed. As light rays pass through areas within the tissue of different optical path (refractive index and geometric path length) they may be retarded in phase by up to ¼Ü but will remain unchanged in amplitude. Since the eye cannot discern phase differences, a mechanism for transforming phase changes into amplitude changes is required.

In the early 1950s Zernike[13] discovered the method by which phase differences can be transformed into amplitude differences. Zernike invented what is now known as positive or dark phase contrast. An alternate method, negative or bright phase contrast, was subsequently developed and has supplanted Zernike's original approach. In positive phase contrast the object (e.g., cell component) appears darker than the surrounding background. In negative phase contrast the object appears brighter than the background.

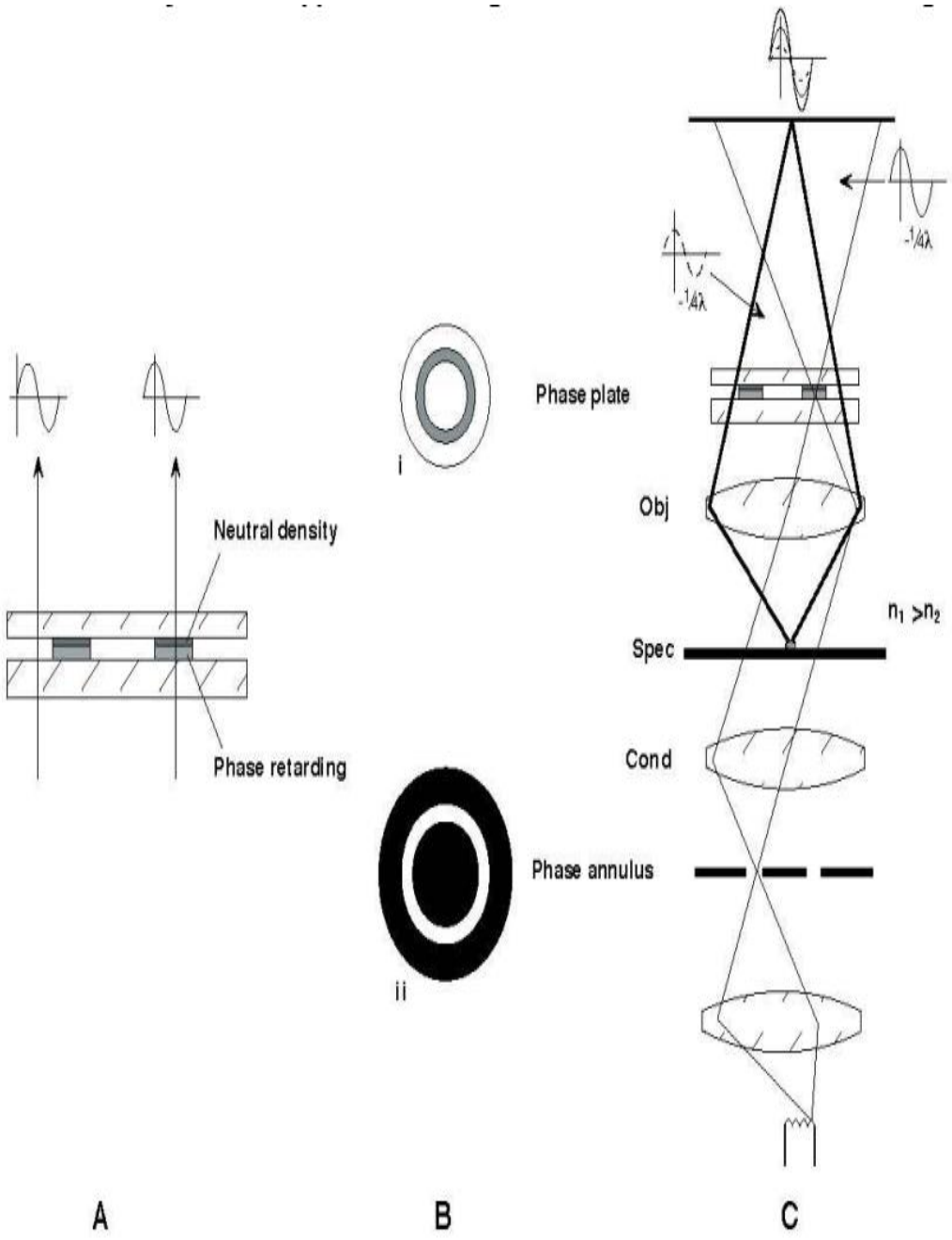
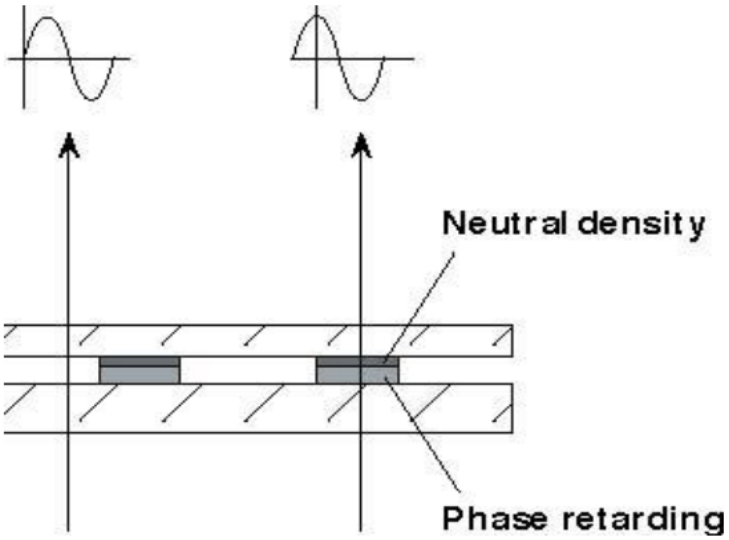
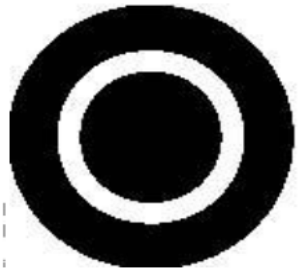


Figure 2-3 Zernike negative phase contrast. A: Construction of the phase plate showing the relative phase shifts induced by the phase rings. B: Face view of the phase annulus (*ii*) and phase plate (*i*). C: The optical path for phase contrast illumination. **Cond**: condenser lens; **Spec**: specimen plane; **Obj**: objective lens; n_1, n_2: refractive index of the sample and background, respectively. (Figure C redrawn from Françon, 1968.)





Phase annulus

How phase contrast works

A compound microscope equipped for negative phase contrast has two additional components: a "phase plate" that retards light exactly ¼ wavelengthin a centered, ring-shaped area located at the back focal plane of the objective lens and a matching "phase annulus" in the condenser consisting of a clear ring on a black field (Figure 2-3B). The presence of the annulus and matching phase plate causes the direct (unmodified background) light to pass only through the phase ring and thus be retarded ¼Ü. Because the light intensity of the diffracted light will be slightly diminished by absorption within the specimen, a neutral density coating on top of the phase ring attenuates undiffracted, background light to balance total illumination.

The light rays interacting with the specimen, on the other hand, diffract away from the sample as spherical waves that do not impinge on the phasing areas of the phase plate to any appreciable degree, but are focused by the objective onto the image plane. Due to a difference in optical path

between the specimen and the surrounding medium, the refracted waves will be retarded in phase up to 1/4 wavelength.

Thus, in the correctly adjusted phase contrast microscope, there are two possible light paths. Light that does not interact with the specimen is collected by the objective, passes through the

phase plate ring, and is retarded exactly ¼ wavelength. The phase shift is not detectable by the eye so the resulting image on the image plane in the microscope appears as a normal bright background.

Conversely, light that passes through the specimen may be diffracted by edges and local irregularities within the tissue. This refracted light will be retarded in phase by up to ¼Ü. The diffracted light will diverge from the object, fill the back focal plane of the objective, and be resolved on the image plane within the microscope. Since the background light is restricted to, and attenuated by, the relatively small annular phase plate, the light diffracted by the specimen can assume a significant role in image construction at the image plane.

Light from both possible paths (background path and specimen path) will interact at the image plane resulting in wave interference where light from the specimen interacts with light from the objective phase plate. In negative phase contrast, constructive interference occurs at the image plane. The results of this interference are bright areas in the specimen image that correspond to refractive index differences in the specimen itself (organelles, cell walls, etc.) set against a background of "normal" intensity derived from nonrefracted light.

Optical path and the "phase halo"

Phase contrast objects always have a "halo" of light (either bright around dark objects or dark surrounding bright objects), which is the result of diffracted light passing through the phase ring as well as the nonphase areas and interacting at the image plane. This halo, also referred to as shading-off, is representative only of light diffraction and interference and not of the optical path of the sample itself. That is, the halo adds artificial structure to the specimen. Intensities seen at the image plane are the result of optical path difference (refractive index plus geometric distance) within the specimen and may not necessarily represent the actual structure of the specimen.

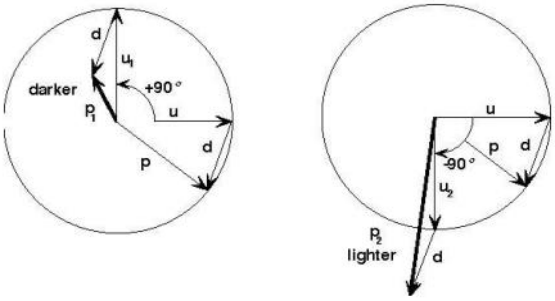
Setting up a phase contrast microscope

In practice adjustment of the microscope for phase contrast is simple. Objectives equipped with a phase plate are coded "Ph1," "Ph2," or "Ph3" depending on the lens magnification and the size of the phase rings. Phase objectives must be matched to the appropriately sized annular diaphragm in the condenser by rotating the condenser to the Ph1, Ph2, or Ph3 position. By placing a lens (Amici-Bertrand lens or 'phase telescope') that focuses on the back focal plane of the objective into

[14]A vector diagram helps explain the phase relationships that exist in phase contrast microscopy. Amplitude is represented by the length and phase by the angle of the arrow.

The light diffracted by a nonabsorbing microscopic object with a higher refractive index than the surrounding medium will be retarded in phase usually only a small amount. The phased light (p) may be treated as the summation of incident light (u) plus a new vector (d) that has a higher phase shift. With most biological material, d may be shifted in phase up to ±¼Ü.

Under normal circumstances u and p are indistinguishable at the image plane because their amplitudes are equal (or nearly so) and thus a phase object is invisible. However, by artificially advancing (**A**; u1) or retarding (**B**; u2) background light ¼Ü (±90°), a vector can be created from the sum of u1 or u2 plus the diffracted light vector d. This new vector is either shorter (p1) or longer (p2) than the background light vector (u1 or u2) and thus is visible as either a dark or light spot (respectively) on the image plane.



AB

Vector diagram of the phase relationships between background and object light in a phase contrast microscope.

FLUORESCENCE MICROSCOPE

An upright fluorescence microscope (Olympus BX61) with the fluorescent filter cube turret above the objective lenses, coupled with a digital camera.

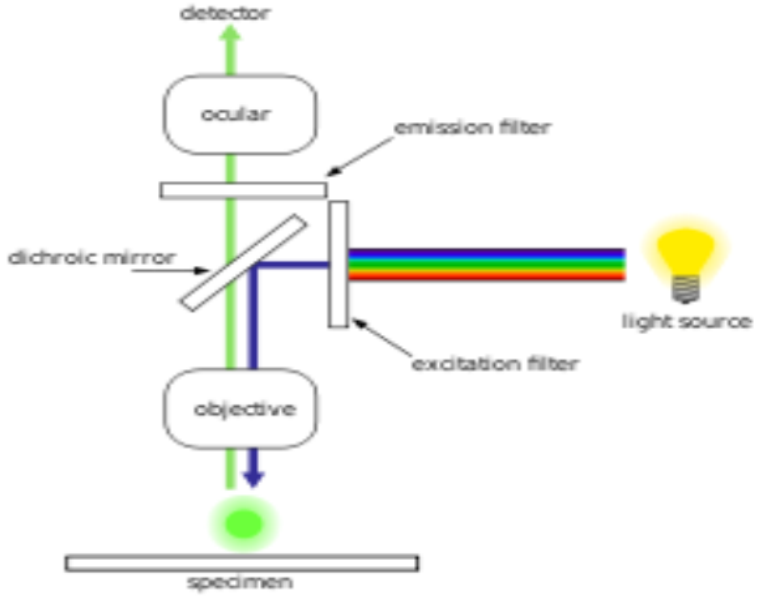
A **fluorescence microscope** is an optical microscope that uses fluorescence and phosphorescence instead of, or in addition to, reflection and absorption to study properties of organic or inorganic substances.[1][2] The "fluorescence microscope" refers to any microscope that uses fluorescence to generate an image, whether it is a more simple set up like an epifluorescence microscope, or a more complicated design such as a confocal microscope, which uses optical sectioning to get better resolution of the fluorescent image.

Principle

The specimen is illuminated with light of a specific wavelength (or wavelengths) which is absorbed by the fluorophores, causing them to emit light of longer wavelengths (i.e., of a different color than the absorbed light). The illumination light is separated from the much weaker emitted fluorescence through the use of a spectral emission filter. Typical components of a fluorescence microscope are a light source (xenon arc lamp or mercury-vapor lamp are common; more advanced forms are high-power LEDs and lasers), the excitation filter, the dichroic mirror (or dichroic beamsplitter), and the emission filter (see figure below). The filters and the dichroic are chosen to match the spectral excitation and emission characteristics of the fluorophore used to label the specimen.[1] In this manner, the distribution of a single fluorophore (color) is imaged at a time. Multi-color images of several types of fluorophores must be composed by combining several single-color images.[1]

Most fluorescence microscopes in use are epifluorescence microscopes, where excitation of the fluorophore and detection of the fluorescence are done through the same light path (i.e. through the objective). These microscopes are widely used in biology and are the basis for more advanced microscope designs, such as the confocal microscope and the total internal reflection fluorescence microscope (TIRF).

Fluorescence microscopy



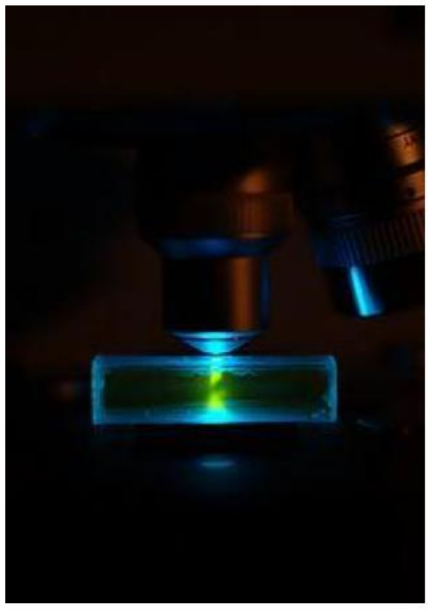
Schematic of a fluorescence microscope.

The majority of fluorescence microscopes, especially those used in the life sciences, are of the epifluorescence design shown in the diagram. Light of the excitation wavelength is focused on the specimen through the objective lens. The fluorescence emitted by the specimen is focused to the detector by the same objective that is used for the excitation which for greatest sensitivity will have a very high numerical aperture. Since most of the excitation light is transmitted through the specimen, only reflected excitatory light reaches the objective together with the emitted light and the epifluorescence method therefore gives a high signal to noise ratio. An additional barrier filter between the objective and the detector can filter out the remaining excitation light from fluorescent light.

Light sources

Fluorescence microscopy requires intense, near-monochromatic, illumination which some widespread light sources, like halogen lamps cannot provide. Four main types of light source are used, including xenon arc lamps or mercury-vapor lamps with an excitation filter, lasers, supercontinuum sources, and high-power LEDs. Lasers are most widely used for more complex fluorescence microscopy techniques like confocal microscopy and total internal reflection fluorescence microscopy while xenon lamps, and mercury lamps, and LEDs with a dichroic excitation filter are commonly used for widefield epifluorescence microscopes.

Sample preparation



A sample of herring sperm stained with SYBR green in a cuvette illuminated by blue light in an epifluorescence microscope. The SYBR green in the sample binds to the herring sperm DNA and, once bound, fluoresces giving off green light when illuminated by blue light.

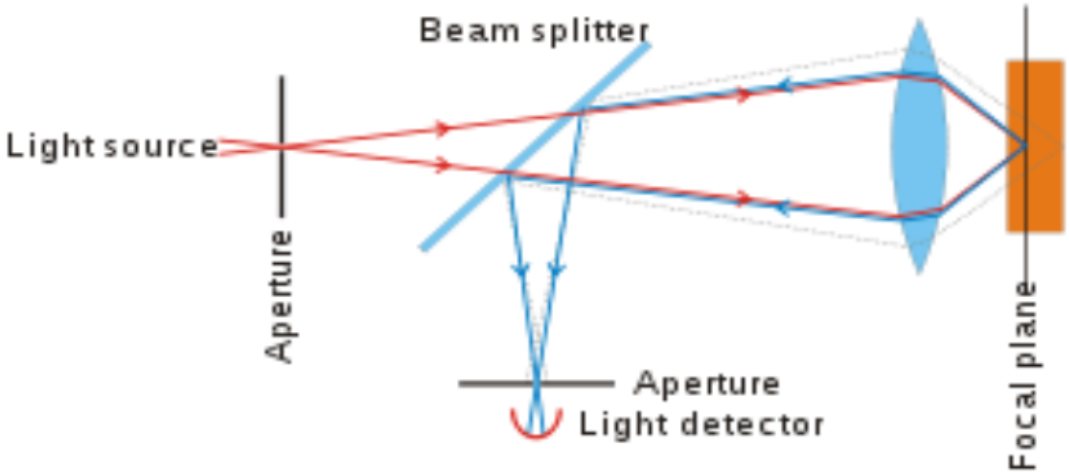
In order for a sample to be suitable for fluorescence microscopy it must be fluorescent. There are several methods of creating a fluorescent sample; the main techniques are labelling with fluorescent stains or, in the case of biological samples, expression of a fluorescent protein. Alternatively the intrinsic fluorescence of a sample (i.e., autofluorescence) can be used.[1] In the life sciences fluorescence microscopy is a powerful tool which allows the specific and sensitive staining of a specimen in order to detect the distribution of proteins or other molecules of interest. As a result there is a diverse range of techniques for fluorescent staining of biological samples.

Biological fluorescent stains

Many fluorescent stains have been designed for a range of biological molecules. Some of these are small molecules which are intrinsically fluorescent and bind a biological molecule of interest. Major examples of these are nucleic acid stains like DAPI and Hoechst (excited by UV wavelength light) and DRAQ5 and DRAQ7 (optimally excited by red light) which all bind the minor groove of DNA, thus labelling the nuclei of cells. Others are drugs or toxins which bind specific cellular structures and have been derivatised with a fluorescent reporter. A major example of this class of fluorescent stain is phalloidin which is used to stain actin fibres in mammalian cells.

There are many fluorescent molecules called fluorophores or fluorochromes such as fluorescein, Alexa Fluors or DyLight 488, which can be chemically linked to a different molecule which binds the target of interest within the sample.

CONFOCAL MICROSCOPY

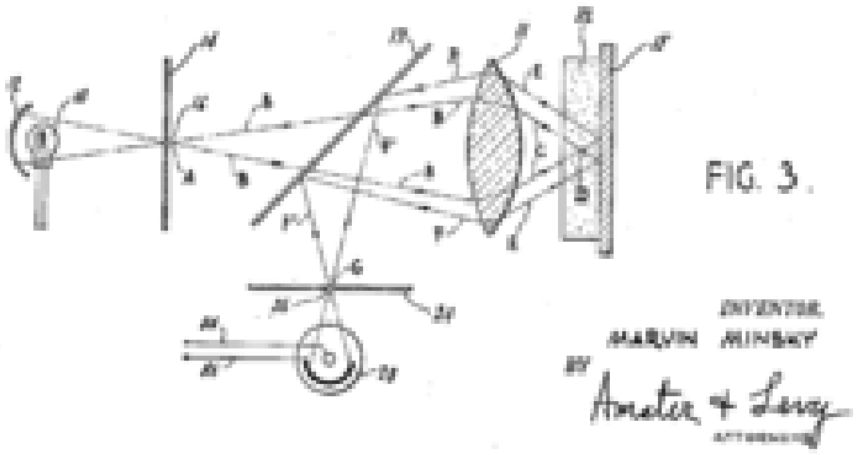


Principle of confocal microscopy

Confocal microscopy is an optical imaging technique used to increase optical resolution and contrast of a micrograph by using point illumination and a spatial pinhole to eliminate out-of-focus light in specimens that are thicker than the focal plane.[1] It enables the reconstruction of three-dimensional structures from the obtained images. This technique has gained popularity in the scientific and industrial communities and typical applications are in life sciences, semiconductor inspection and materials science.

Contents

Basic concept



Confocal point sensor principle from Minsky's patent

The principle of confocal imaging was patented in 1957 by Marvin Minsky[2][3] and aims to overcome some limitations of traditional wide-field fluorescence microscopes. In a conventional (i.e., wide-field) fluorescence microscope, the entire specimen is flooded evenly in light from a light source. All parts of the specimen in the optical path are excited at the same time and the resulting fluorescence is detected by the microscope's photodetector or camera including a large unfocused background part. In contrast, a confocal microscope uses point illumination (see Point Spread Function) and a pinhole in an optically conjugate plane in front of the detector to eliminate out-of-focus signal - the name "confocal" stems from this configuration. As only light produced by fluorescence very close to the focal plane can be detected, the image's optical resolution, particularly in the sample depth direction, is much better than that of wide-field microscopes. However, as much of the light from sample fluorescence is blocked at the pinhole, this increased resolution is at the cost of decreased signal intensity – so long exposures are often required.

As only one point in the sample is illuminated at a time, 2D or 3D imaging requires scanning over a regular raster (i.e., a rectangular pattern of parallel scanning lines) in the specimen. The achievable thickness of the focal plane is defined mostly by the wavelength of the used light divided by the numerical aperture of the objective lens, but also by the optical properties of the specimen. The thin optical sectioning possible makes these types of microscopes particularly good at 3D imaging and surface profiling of samples.

Techniques used for horizontal scanning

Three types of confocal microscopes are commercially available:

- **Confocal laser scanning microscopes** use multiple mirrors (typically 2 or 3 scanning linearly along the x and the y axis) to scan the laser across the sample and "descan" the image across a fixed pinhole and detector.

- **Spinning-disk (Nipkow disk) confocal microscopes** use a series of moving pinholes on a disc to scan spot of light.
- **Programmable Array Microscopes (PAM)** use an electronically controlled spatial light modulator (SLM) that produces a set of moving pinholes. The SLM is a device containing an array of pixels with some property (opacity, reflectivity or optical rotation) of the individual pixels that can be adjusted electronically. The SLM contains microelectromechanical mirrors or liquid crystal components. The image is usually acquired by a CCD camera.

Each of these classes of confocal microscope have particular advantages and disadvantages. Most systems are either optimized for recording speed (i.e. video capture) or high spatial resolution. Confocal laser scanning microscopes can have a programmable sampling density and very high resolutions while Nipkow and PAM use a fixed sampling density defined by the camera's resolution. Imaging frame rates are typically slower for single point laser scanning systems than spinning-disk or PAM systems. Commercial spinning-disk confocal microscopes achieve frame rates of over 50 per second[4] – a desirable feature for dynamic observations such as live cell imaging. In practice, Nipkow and PAM allow multiple pinholes scanning the same area in parallel[5] as long as the pinholes are sufficiently far apart. Cutting-edge development of confocal laser scanning microscopy now allows better than standard video rate (60 frames/second) imaging by using multiple microelectromechanical systems-based scanning mirrors.

Confocal X-ray fluorescence imaging is a newer technique that allows control over depth, in addition to horizontal and vertical aiming, for example, when analyzing buried layers in a painting.[6]

Variants and enhancements

Improving axial resolution

The point spread function of the pinhole is an ellipsoid, several times as long as it is wide. This limits the axial resolution of the microscope. One technique of overcoming this is 4π microscopy where incident and or emitted light are allowed to interfere from both above and below the sample to reduce the volume of the ellipsoid. An alternative technique is **confocal theta microscopy**. In this technique the cone of illuminating light and detected light are at an angle to each other (best results when they are perpendicular). The intersection of the two PSFs gives a much smaller effective sample volume. From this evolved the single plane illumination microscope.

Super resolution

There are confocal variants that achieve resolution below the diffraction limit such as STED microscopy.

Low-temperature operability

To image samples at low temperature, two main approaches have been used, both based on the laser scanning confocal microscopy architecture. One approach is to use a continuous flow cryostat: only the sample is at low temperature and it is optically addressed through a transparent window.[7] Another possible approach is to have part of the optics (especially the microscope objective) in a cryogenic storage dewar.[8] This second approach, although more cumbersome, guarantees better mechanical stability and avoids the losses due to the window.