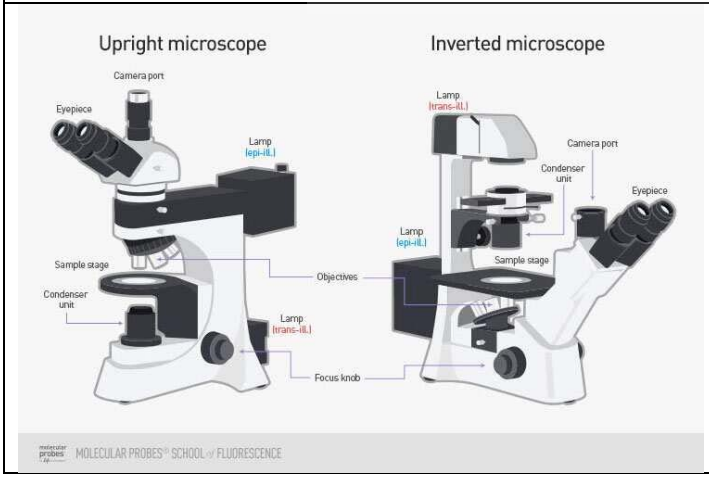
Introduction to microscopy

Microscopes are specialized optical instruments designed to produce magnified visual or photographic (including digital) images of objects or specimens that are too small to be seen with the naked eye. Collectively, this varied group of tools includes not only multiple-lens (compound microscope) designs featuring objectives and condensers,

A compound microscope consists of a group of lenses (called objective) which focuses a real image of the object inside the microscope. A second group of lenses (called eyepiece) magnifies this image and projects it on the retina of the eye. This compound optical system (objective - eyepiece) together with other mechanical components are the basics of a modern microscope

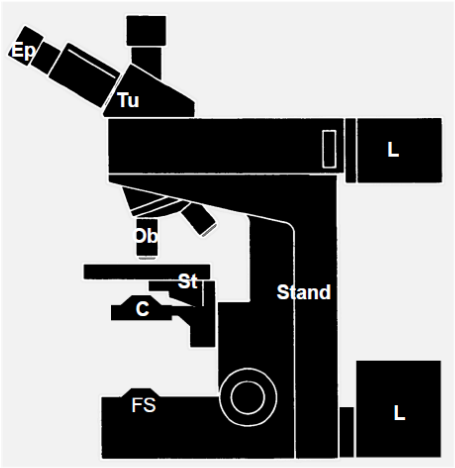
There are different types of microscopes available depend upon the magnification and applications

Types of microscopes

Compound microscopes	Stereo Microscopes
In an upright microscope, the light source and condenser are located beneath the stage and, therefore, the specimen. Light is transmitted through the sample from below, and it can then be viewed from above with an ocular lens. inverted microscopes have objectives below the stage where you put your sample.	.
	

Components in a microscope

- **Eyepiece:** in the tube, magnifies intermediate image and offers it to the eye
- **Tube:** contains tube lens, holds eyepieces
- **Objective:** magnifies specimen
- **Stage:** height adjustable (focus), holds specimen
- **Condenser:** homogeneous illumination of specimen
- **Lamp:** in or at stand
- **Stand:** stability, holds all components and keeps them in appropriate distances to each other
- **Field Stop:** avoids stray light



Aberrations in microscopy

In practice, the resolution of a microscope is lower than the theoretical resolution due to optical aberrations, improper illumination or bad alignment of the optics and components of the microscope. Despite corrections for chromatic and spherical aberrations, coma, astigmatism and distortion, the real maximum resolution of a microscope with a 100x objective is about 0.25 µm for 550 nm light rays

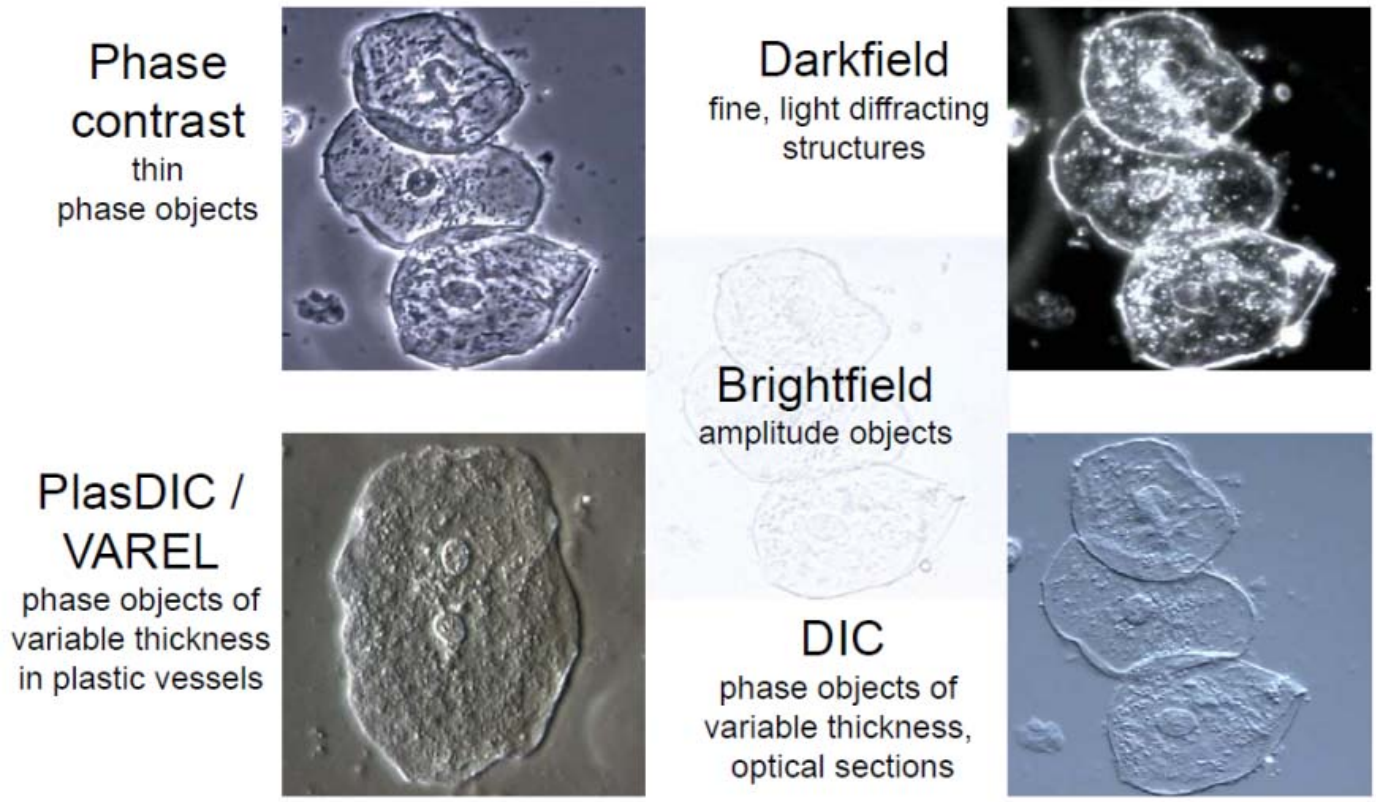
1. Chromatic aberration: Difference in focus of different colors caused by difference in refractive indices for each wavelength
2. Spherical aberration: Difference in focus caused by defects or irregularities on the surface of a lens
3. Astigmatism: Rays traveling through two perpendicular planes having different focal planes
4. Coma aberration: Variation in magnification over the entrance pupil
5. Distortion: Stretching of an image as a variation in magnification across the lens field

Objectives

Microscope objectives are perhaps the most important components of an optical microscope because they are responsible for primary image formation and play a central role in determining the quality of images that the microscope is capable of producing. Objectives are also instrumental in determining the magnification of a particular specimen and the resolution under which fine specimen detail can be observed in the microscope.

1. Achromatic: This objective brings red and blue light to a common focus, and is corrected for spherical aberrations for green. It is excellent for black and white viewing and not corrected for Chromatic aberrations
2. Plan achromat: Corrected for Spherical aberrations, these objectives produces a flat image across the field of view
3. Fluorite or semi-apochromat objectives–These lenses are chromatically corrected for red and blue, and the green focus is also close. They are spherically corrected for blue and green. This objective is better suited for color viewing or recording than achromatic objectives.
4. Apochromatic: It is chromatically adjusted for four colors (deep blue, blue, green and red) and spherically corrected for deep blue, blue and sometimes green. This is the best choice for color viewing. These have a higher numerical aperture (N.A.) than achromats or fluorites.

Various contrasting techniques



Bright field microscopy

In bright-field microscopy, illumination light is transmitted through the sample and the contrast is generated by the absorption of light in dense areas of the specimen. The limitations of bright-field microscopy include low contrast for weakly absorbing samples and low resolution due to the blurry appearance of out-of-focus material.

Phase contrast microscopy

Phase contrast microscopy, is a contrast-enhancing optical technique that can be utilized to produce high-contrast images of transparent specimens, such as living cells (usually in culture), microorganisms, thin tissue slices, lithographic patterns, fibers, latex dispersions, glass fragments, and subcellular particles

Dark field microscopy

The condensing system for dark-field illumination utilizes a central circular disk stop that prevents direct condenser rays from entering the objective lens. Only those rays that have been suitably scattered by the object (by reflection, refraction or diffraction) enter the objective lens to generate the final image. Object detail responsible for the scattering appears bright on a dark background or field.

Differential Interference Contrast

The technique produces a monochromatic shadow-cast image that effectively displays the gradient of optical paths for both high and low spatial frequencies present in the specimen. Those regions of the specimen where the optical paths increase along a reference direction appear brighter (or darker), while regions where the path differences decrease appear in reverse contrast. As the gradient of optical path difference grows steeper, image contrast is dramatically increased.

Fluorescence Microscopy

Fluorescence is the phenomenon of subsequent specific emission of photons by a fluorophore (chemical compound) after the absorption of specific excitation photons.

Fluorophores are used as a dye for staining cells, tissues or materials that can be observed as markers under a fluorescence microscope

The excitation light of a microscope for fluorescence is provided with either a mercury vapor light source or a monochrome LED. The selection of the specific fluorophore excitation light is done by a so-called emission interference filter that is mounted in a cube together with a dichroic mirror (beam splitter) and the emission filter. The filtered excitation light is further reflected by the dichroic mirror through the objectives towards the sample

The fluorophore(s) inside the specimen will absorb this excitation energy with some efficiency (quantum yield) and will start re-emitting the so-called emission energy, visible light with longer wave lengths than the absorbed light.

The emission light from the specimen that fluoresces will be collected by the objective, pass the dichroic mirror and is separated from excitation and other unwanted light

wavelengths by the emission filter. The filtered emission light is further projected to the eyepieces or/and camera imaging sensor

Microscope Parameters

The numerical aperture

The numerical aperture of a microscope objective is a measure of its ability to gather light and resolve fine specimen detail at a fixed object distance. Image-forming light waves pass through the specimen and enter the objective in an inverted cone. A longitudinal slice of this cone of light reveals the angular aperture, a value that is determined by the focal length of the objective

Resolution

The resolving power of an objective lens is measured by its ability to differentiate two lines or points in an object. The greater the resolving power, the smaller the minimum distance between two lines or points that can still be distinguished. The larger the N.A., the higher the resolving power.

The following formula is generally used for determing resolution.

$$\varepsilon = 0.61 \times \frac{\lambda}{N.A.} \quad \text{(Reyleigh formula)}$$

λ : Wavelength

λ =0.55μm is used for visible light

N.A.: Objective lens N.A.

Example MPLFLN100×(N.A. 0.90)、λ =0.55μm

$$\varepsilon = 0.61 \times \frac{\lambda}{N.A.} = \frac{0.3355}{N.A.} = \frac{0.3355}{0.90} = 0.37\mu m$$

FOV (Field of View)

Field of view (FOV) is the visible area when looking through the microscope eyepiece (eyepiece FOV) or camera (camera FOV) and is usually expressed as a diameter

CALCULATING FOV OF AN EYEPIECE

The FOV through an eyepiece is equal to:

$$FOV = \frac{\text{Field Number}}{\text{Object Magnification}}$$

Field number (FN) is usually engraved on the eyepiece as a figure next to the magnification and expressed in mm, e.g. WF 10x/18. A 10x/18 eyepiece with a 40x objective will have a FOV = 18 mm / 40 = 0.45 mm or 450 nm

450 nm FOV with 10x/18 eyepiece and S40x objective

Micrometer stage 1 mm/100

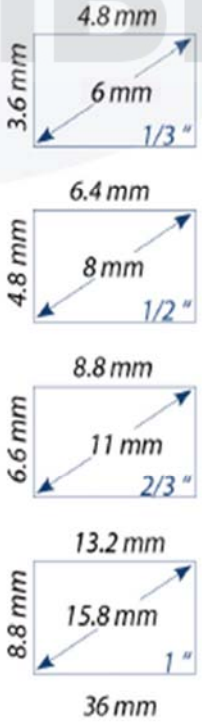
900 nm FOV with 10x/18 eyepiece and S20x objective

Micrometer stage 1 mm/100

FIELD OF VIEW WITH CAMERA

Most commercially available microscope digital cameras use 1/3, 1/2- or 2/3-inch rectangular sensors. A few will use a 1 inch camera sensor

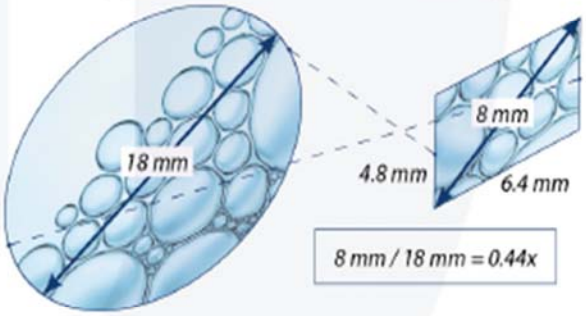
As a consequence, a rectangular camera sensor cannot capture the circular FOV that exits from a eyepiece, microscope third tube or photo port



EYEPIECE VERSUS CAMERA FOV

Circular FOV through a WF10x/18 eyepiece

1/2 " camera sensor and its rectangular FOV



Furthermore, the circular eyepiece FOV is much larger than the camera FOV and thus the microscope FOV must be "reduced" with a so-called "relay" lens or "photo-adapter" to fit the camera FOV. However - in order to avoid vignetting (dark shadows in the corners of an image), the circular microscope FOV must just be projected outside the image sensor area. Subsequently the camera FOV will always be smaller than the microscope FOV by 50 to 60%

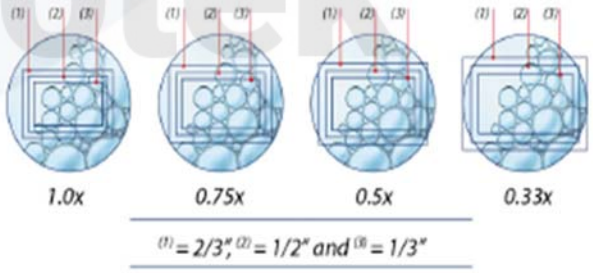


Figure: different camera sensor sizes (2/3, 1/2, 1/3 inch) with different "demagnifying" lenses (C-mount photo-adapters) 0.33x, 0.50x, 0,75x and 1.0x

IN ORDER TO OBTAIN THE DESIRED FIELD OF VIEW WITHOUT VIGNETTING USE:

a 0.33 x photo adapter for cameras with a 1/3" sensor
a 0.50 x photo adapter for cameras with a 1/2" sensor
a 0.75 photo adapters for cameras with a 2/3" sensor
a 1.0x or 1.2x photo adapters for cameras with a 1"
or larger camera sensor

Digital microscopy

A camera is a device that records images, three types of cameras in use:

1. SLR: Used for conventional film-based photochemistry.
2. Analog: Analog continuous output which is mainly used for live image display and also for digitization through Frame grabber cards.
3. Digital: Digitizes the analog output of the sensor with on board digitizer built-in the camera itself.

Digital imaging starts with the conversion of light (photons) into electrons (Photo-Electric Effect).

- Light (photons) hits image sensors charge coupled device (CCD) or complementary metal oxide Digital imaging starts with the conversion of light (photons) into electrons (Photo-Electric Effect).
- Light (photons) hits image sensors charge coupled device (CCD) or complementary metal oxide semiconductor (CMOS) and are held as small charge.
- Silicon, a semiconductor is the primary material for the sensitive surface.
- Charges are then converted into voltage proportional to amount of charge held. Voltage is then converted

CCD camera vs CMOS

For many years, the charge-coupled device (CCD) has been the best imaging sensor scientists could choose for their microscopes. A CCD sensor consists of a two-dimensional grid of metal-oxide-semiconductor capacitors. Like miniaturized rain buckets, the capacitors collect incoming photons and convert them into electrical current, which is sequentially read out and reassembled into a picture.

However, a newer technology called the complementary metal-oxide-semiconductor (CMOS) sensor, also known as the active-pixel sensor, has emerged in the microscope optics marketplace. The pixels in a CMOS sensor are slightly more complicated than those in a CCD, because each pixel contains not just a photodiode, but also a set of tiny transistors to amplify the light-generated electrical signal and pass it along for processing.

The advantages of CMOS sensors, however, outweigh the added complexity of the individual pixels. CMOS sensors are faster than their CCD counterparts, which allows for higher video frame rates. CMOS imagers provide higher dynamic range and require less current and voltage to operate. The active pixels also have a higher quantum efficiency (a measure of how well the device converts photons to electrons)

Fortunately, the market's embrace of CMOS means that end users of imaging systems, including the overall biphotonic market, can benefit from ongoing improvements to the technology. The image quality of today's CMOS sensors exceeds that of CCD sensors and will only get better.

Performance	CCD	CMOS
Responsivity	Moderate	Slightly better
Dynamic Range	High	Moderate
Uniformity	High	Low to Moderate
Uniform Shuttering	Fast, common	Poor
Uniformity	High	Low to Moderate
Speed	Moderate to High	Higher
Windowing	Limited	Extensive
Antiblooming	High to none	High
Biasing and Clocking	Multiple, higher voltage	Single, low-voltage

Camera Properties

Resolution: Sample detail requires resolving above human eye's resolving ability, fineness of detail plays important role in significance of the result.

Dynamic Range: The ability of the sensor to reproduce the range of colors used to build the image.

Sensitivity: The ability of the sensor to capture enough photons to produce electrons.

Basic softwares for Image capturing

- To control the camera parameters (Exposure, gain…)
- To acquire images
- For adding basic annotations for captured images
- To do basic image editing/ Image enhancing (Brightness, Contrast etc)
- Convert and Save the images in required format (.jpg, tiff etc)

Common software modules in microscopy

- EDF (extended depth of field) This works by combining the best focused parts of a number of images taken at different focus levels into a composite image.

- Panorama : This is to stitch together a series of photographs of different parts of the sample under the microscope for a large field which cannot covered under microscope as a single frame

- Multichannel: This is for Adding (Merging)different channels which is captured separately In Fluorescence microscopy. Due to high sensitivity and dynamic range, monochrome cameras are commonly used to capture the images, Multichannel software can add the required colour (pseudo colour) depends on the dye which is used in the sample

- Automatic image stitch(mosaic): When microscope slides are too large to be acquired as single images, multiple overlapping fields of view (FOVs) are acquired and composed into a large single image. The reconstruction of the original slide is performed by a software module that aligns acquired images with their adjacent images. This operation is called image mosaicing or stitching

- Z stack (motorized): Stacking is the process of combining a series of digital images of the same subject area, taken at different focal planes, into a single image with great depth of field, using a computer program

- Time lapse: Tool for real-time imaging of living cells. The frames are captured at a regular interval of time / continues as video and single images in various time points can be exported as a movie

- Deconvolution: Is an image processing technique used to improve the contrast and resolution of fluorescent images captured using an widefield optical microscope. Deconvolution is a mathematical transformation of image data that reduces out of focus light or blur. Blurring is a significant source of image degradation in three-dimensional (3D) widefield fluorescence microscopy

- Colocalization: During the examination and digital recording of multiply labeled fluorescent specimens, two or more of the emission signals can often overlap in the final image due to their close proximity within the microscopic structure, this effect is known as colocalization. The degree of fluorophore colocalization in a specimen is measured by comparing color values for the equivalent pixel position in each of the acquired images