Technology Networks

Article

An Introduction to the Light Microscope, Light Microscopy Techniques and Applications

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Some of the most fundamental processes in nature occur at the microscopic scale, far beyond the limits of what we can see by eye, which motivates the development of technology that allows us to see beyond this limit. As early as the 4th century AD, people had discovered the basic concept of an optical lens, and by the 13th century, they were already using glass lenses to improve their eyesight and to magnify objects such as plants and insects to better understand them.[1] With time, these simple magnifying glasses developed into advanced optical systems, known as light microscopes, which allow us to see and understand the microscopic world beyond the limits of our perception. Today, light microscopy is a core technique in many areas of science and technology, including life sciences, biology, materials sciences, nanotechnology, industrial inspection, forensics and many more. In this

article, we will first explore the basic working principle of light microscopy. Building on this, we will discuss some more advanced forms of light microscopy that are commonly used today and compare their strengths and weaknesses for different applications.

What is light microscopy?

Light microscopy is used to make small structures and samples visible by providing a magnified image of how they interact with visible light, e.g., their absorption, reflection and scattering. This is useful to understand what the sample looks like and what it is made of, but also allows us to see processes of the microscopic world, such as how substances diffuse across a cell membrane.

Parts of a microscope and how a light microscope works

Fundamentally, a microscope comprises two subsystems: an illumination system to illuminate the sample and an imaging system that produces a magnified image of the light that has interacted with the sample, which can then be viewed by eye or using a camera system.

Early microscopes used an illumination system comprising sunlight that was collected and reflected onto the sample by a mirror. Today, most microscopes use artificial light sources such as light bulbs, light-emitting diodes (LEDs) or lasers to make more reliable and controllable illumination systems, which can be tailored to a given application. In these systems, light from the source is typically collected using a condenser lens and then shaped and optically filtered before being focused onto the sample. Shaping the light is essential to achieve high resolution and contrast, and often includes controlling the sample area that is illuminated and the angles at which light impinges on it. Optical filtering of the illumination light, using optical filters that modify its spectrum and polarization, can be used to highlight certain features of a sample, to improve the visibility of weak signatures or to observe a sample's fluorescence.

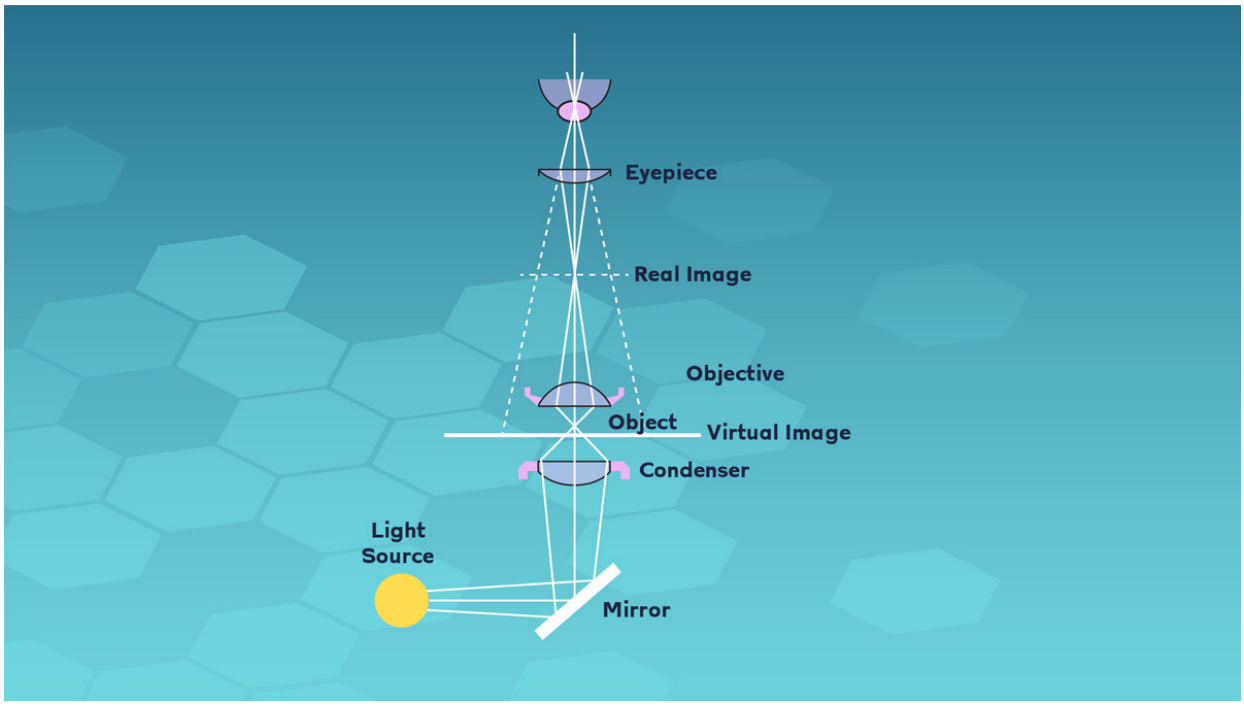

Figure 1: Basic compound microscope: Light from a source is focused onto the

sample (object) using a mirror and condenser lens. Light from the sample is collected by an objective, forming an intermediate image which is imaged again by the eyepiece and relayed to the eye, which sees a magnified image of the sample.

The imaging system collects illuminating light that has interacted with the sample and produces a magnified image that can be viewed (Figure 1). This is achieved using two main groups of optical elements: first, an objective lens that collects as much light from the sample as possible and second, an eyepiece lens which relays the collected light to the observer's eye or a camera system. The imaging system may also include elements such as apertures and filters that select certain portions of light from the sample, for example to see only light that has been scattered off the sample, or only light of a certain color or wavelength. As in the case of the illumination system, this type of filtering can be extremely useful to single out certain features of interest that would remain hidden when imaging all the light from the sample.

Overall, both the illumination and the imaging system play a key role in how well a light microscope performs. To get the best out of light microscopy in your application, it is essential to have a good understanding of how a basic light microscope works, and what variations exist today.

Simple and compound microscopes

A single lens can be used as a magnifying glass which increases the apparent size of an object when it is held close to the lens. Looking through the magnifying glass at the object, we see a magnified and virtual image of the object. This effect is used in simple microscopes, which consist of a single lens that images a sample held clamped into a frame and illuminated from below, as is shown in Figure 2. This type of microscope can achieve a magnification of typically 2-6 x, which is sufficient to study relatively large samples. However, achieving higher magnification and better image quality requires the use of more optical elements, which led to the development of the compound microscope (Figure 3).

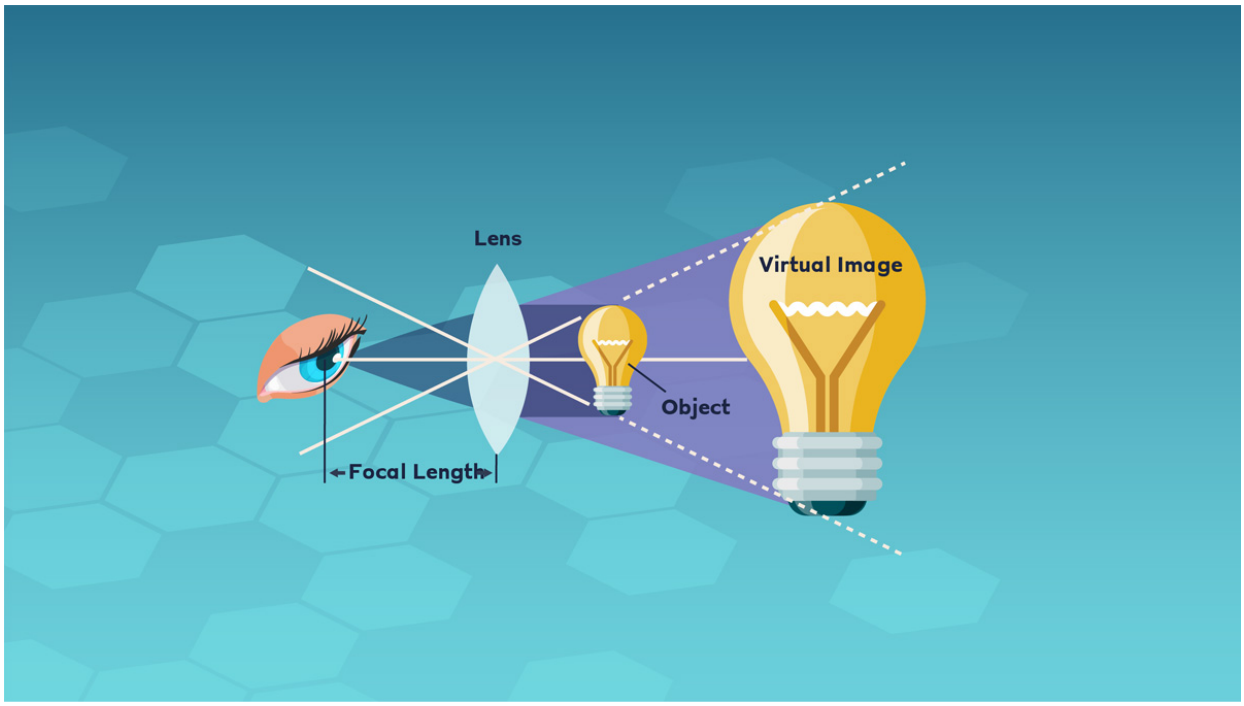

Figure 2: A single lens as a magnifying glass by creating a magnified, virtual image of an object placed close to it.

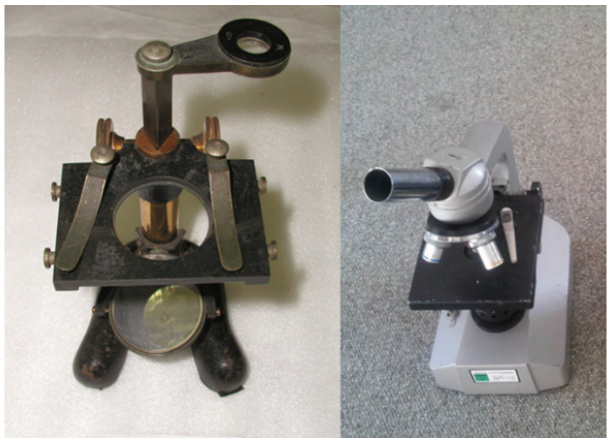

Figure 3: Left: Simple microscope. *Credit: Waters W./Auckland Museum. Reproduced under the Creative Commons Attribution 4.0 International (CC BY 4.0) license.* Right: Compound microscope. *Credit: Jacques from Cape Town, South Africa. Reproduced under the Creative Commons Attribution 2.0 Generic license. Cropped to highlight one instrument only.*

In a compound microscope, the sample is illuminated from the bottom to observe transmitted light, or from the top to observe reflected light. Light from the sample is collected by an optical system consisting of two main lens groups: the objective and the eyepiece, whose individual powers multiply to enable much higher magnifications than those achieved by a simple microscope. The objective collects

light from the sample and typically has a magnification of 40-100 x. Some compound microscopes feature multiple objective lenses on a rotating turret known as a “nose piece”, allowing the user to choose between different magnifications. The image from the objective is picked up by the eyepiece, which magnifies the image again and relays it to the user’s eye, with typical eyepieces having a magnification of 10 x. Therefore, the total magnification of a compound microscope, which is the product of the objective magnification and the eyepiece magnification, typically lies in the range of 400-1000 x.

The smallest feature size that can be observed with a standard light microscope is set by the optical wavelength (λ) used and the resolving power of the microscope objective, defined by its numerical aperture (NA), with the maximum value of NA = 1 for an objective in air. The resolution limit, which defines the smallest feature size (r) that can be distinguished is given by the Rayleigh criterion[2] as:

$$r = 0.61 \times (\lambda/NA)$$

For example, using green light with a wavelength of 550 nm and an objective with a typical NA of 0.7, a standard light microscope can resolve features down to a limit of $0.61 \times (550\ nm)/0.7 \approx 480\ nm$, which is sufficient to observe cells (typically 10 µm size), but not enough to observe details of smaller organisms, e.g. viruses (typically 250-400 nm). To image smaller features, more advanced and expensive objectives with higher NA and shorter wavelengths can be used, but this may not be practical for all applications.

In standard compound microscopes (Figure 4a), the sample (often on a glass slide) is held on a stage that can be moved manually or electronically for higher precision, and the illumination system is in the lower part of the microscope, while the imaging system is above the sample. However, the microscope body can usually also be adapted to particular uses. For example, stereo microscopes (Figure 4b) feature two eyepieces at a slight angle to each other, allowing the user to see a slightly three-dimensional image. In many biology applications, an inverted microscope design (Figure 4c) is used, where both the illumination system and the imaging optics are below the sample stage to facilitate placing e.g., containers of cell cultures onto it. Finally, comparison microscopes (Figure 4d) were often used in forensics, for example to compare fingerprints or bullets by eye before the advent of digital microscopy, which allowed images to be saved and compared.

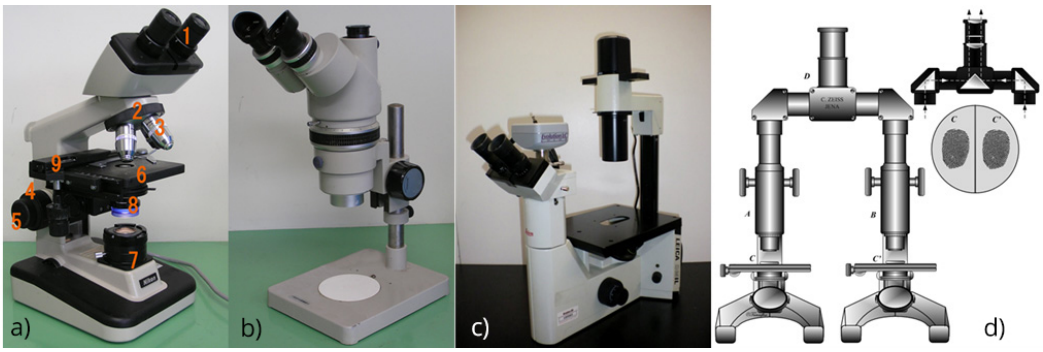

Figure 4: Compound microscope bodies. a) Standard upright microscope indicating (1) eyepiece (ocular lens), (2) objective turret, revolver, or revolving nose piece (to hold multiple objective lenses), (3) objective lenses, focus knobs (to move the stage) (4) coarse adjustment, (5) Fine adjustment, (6) Stage (to hold the specimen), (7) Light source (a light or a mirror), (8) Diaphragm and condenser, (9) Mechanical stage. *Credit: GcG(jawp).* b) Stereo microscope. *Credit: GcG(jawp).* c) Inverted microscope. *Credit: Kitmondo LAB. Reproduced under the Creative Commons Attribution 2.0 Generic license.* d) Comparison microscope. *Credit: Tamasflex. Reproduced under the Creative Commons Attribution-Share Alike 3.0 Unported license.*

Types of light microscopy

In the following, we will present a selection of different light microscopy techniques available today, discuss their main operating principles and the strengths and weaknesses of each technique.

Bright field microscopy

Bright field microscopy (BFM) is the simplest form of light microscopy, where the sample is illuminated from above or below, and light transmitted through or reflected from it is collected to form an image that can be viewed. Contrast and color in the image are formed because absorption and reflection vary over the area of the sample. BFM was the first type of light microscopy developed and uses a relatively simple optical setup, which allowed early scientists to study microorganisms and cells in transmission. Today, it is still very useful for the same purposes, and is also widely used to study other partially transparent samples such as thin materials in transmission mode (Figure 5), or microelectronics and

other small structures in reflection mode. However, the magnification of BFM is limited to 1300 x and it is not suitable for imaging highly transparent samples.

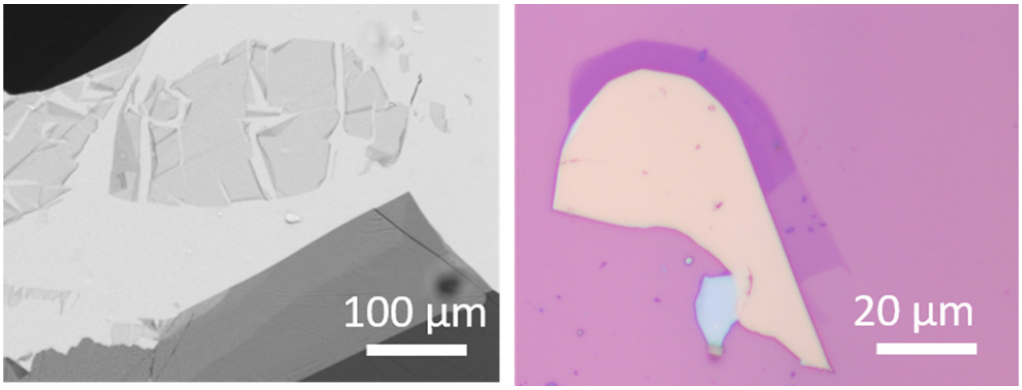

Figure 5: Bright field microscopy. Left: Transmission mode - flakes of graphite (dark grey) and graphene (lightest grey) as seen in a bright field microscope. Here, the difference in brightness seen on the image is proportional to the thickness of the graphite layer. Right: Reflection mode - flakes of graphene and graphite on a SiO_2 surface. Small surface contaminants are also visible. *Credit: Author.*

Dark field microscopy

Dark field microscopy is a technique where only light that is scattered by the sample is collected. This is achieved by adding apertures that block the illuminating light from being imaged directly, such that only illumination light that is scattered by the sample is seen. In this way, dark field microscopy highlights small structures that scatter light (Figure 6), and can be very useful to reveal features not visible in BFM without having to modify the sample in any way. However, as the only light that is seen in the final image is light that has been scattered, dark field images may be quite dark and require high illumination powers, which may damage the sample.

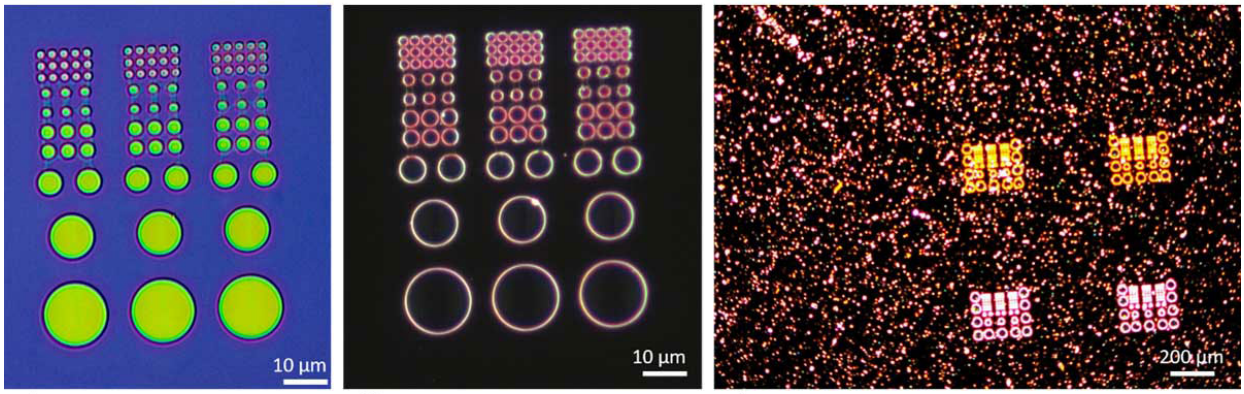

Figure 6: Bright field and dark field imaging. a) Polymer microstructure in bright field illumination. b) Dark field image of the same structure as in a), highlighting scattering from edges and surface contamination. c) Similar structures as in a) and b), covered by nanocrystals of 100-300 nm diameter. Only light scattered by the nanocrystals is observed, while the background light is strongly suppressed. *Credit: Author.*

Phase contrast microscopy

Phase contrast microscopy (PCM) is a technique that visualizes changes of optical phase caused by variations of the sample's optical path length. This enables imaging of transparent samples that create little or no contrast in BFM, such as cells (Figure 7), for example. Because optical phase shifts are not readily observable by eye, phase-contrast microscopy requires additional optical components that convert phase shifts caused by the sample into visible changes of brightness in the final image. This requires manipulation of both the illumination system and the imaging system using apertures and filters. These shape and selectively phase shift both the light from the sample, which carries the phase information of interest, and the illumination light such that they constructively interfere on the eye or detector to create a visible image.

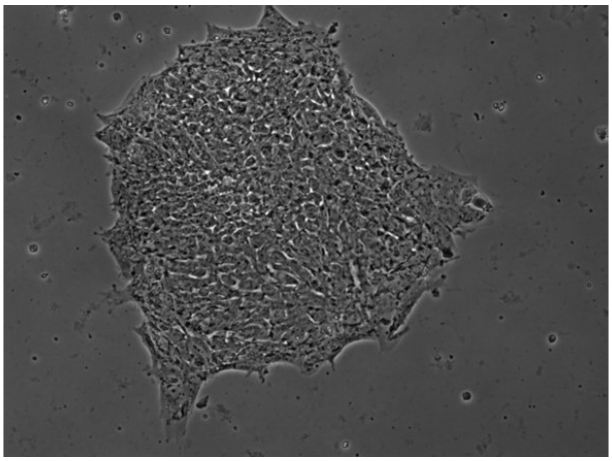

Figure 7: Phase contrast microscopy of a human embryonic stem cell colony. *Credit Sabrina Lin, Prue Talbot, Stem Cell Center University of California, Riverside.*

Differential interference contrast microscopy

Differential interference contrast microscopy (DICM) enables visualization of transparent samples such as live, unstained cells by converting optical phase due to variations of the sample's optical path length to visible contrast by interference, similarly to PCM. However, compared to PCM, DICM can achieve higher resolution images and clarity and image artefacts introduced by the optics required for PCM are reduced. In DICM, the illuminating beam is polarized by a linear polarizer and its polarization rotated such that it is split into two polarized beams with perpendicular polarization and a small (typically below 1 μm)[3] separation between them. After traversing the sample, both beams are recombined such that they interfere with each other. This creates an image with contrast proportional to the *difference* in optical phase between the two polarized beams, hence the name "differential" interference microscopy. Images produced by DICM appear three-dimensional related to the direction of displacement between the sampling beams, which leads to the edges of the sample having bright or dark areas depending on the sign of the optical phase difference between the two (Figure 8).

Figure 8: Differential interference contrast microscopy. Left: Schematic setup for DICM. Right: Live adult *Caenorhabditis elegans* (*C. elegans*) nematode imaged by DICM. *Credit: Bob Goldstein, Cell Image Library. Reproduced under a Creative Commons Attribution 3.0 Unported license (CC BY 3.0).*

Polarized light microscopy

In polarized light microscopy, the sample is illuminated with polarized light and the detection of the light is also sensitive to polarization. To achieve this, polarizers are used to control both the illumination light polarization and to restrict the polarization detected by the imaging system to only one specific polarization. Often, the illumination and detection polarizations are set to be perpendicular so that unwanted background illumination light that did not interact with the sample is strongly suppressed. This configuration requires a birefringent sample that introduces a rotation of the illumination light polarization angle such that it can be detected by the imaging system, for example to observe the birefringence of crystals and variations of thickness and refractive index across them (Figure 9).

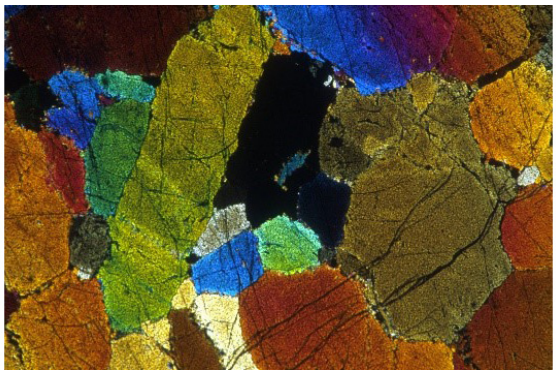

Figure 9: Polarization microscopy. Photomicrograph of olivine adcumulate, formed by the accumulation of crystals with different birefringence. Variations of thickness and refractive index across the sample result in different colors. *Credit: R. Hill, CSIRO.*

Fluorescence microscopy

Fluorescence microscopy is used to image samples that fluoresce, that is, they emit long-wavelength light when illuminated with light of a shorter wavelength. Examples include biological samples that are intrinsically fluorescent or have been labeled with a fluorescent marker, as well as single molecules and other nanoscale fluorophores. The technique employs a combination of optical filters that block short-wavelength illumination light but let longer-wavelength sample fluorescence pass, such that the final image only shows the fluorescent parts of a sample (Figure 10). This allows singling out and visualization of the distribution of fluorescent particles or cells of interest that have been stained with a dye from within a sample consisting of many other non-fluorescent particles. At the same time, fluorescence microscopy can also overcome the resolution limit of traditional optical microscopes by tagging particles that are smaller than this limit. For example viruses can be tagged with fluorescent markers to reveal their location[4] or a fluorescent protein, such as green fluorescent protein,[5] may be expressed in the case of biological samples. Combined with various novel forms of sample illumination, this advantage of fluorescence microscopy enables “super resolution” microscopy techniques[6] that break the resolution limits of traditional light microscopy. One of the main limitations of fluorescence microscopy is photobleaching, where the markers or particles cease to fluoresce because the process of absorbing the illumination light eventually alters their structure, such that they no longer emit light.

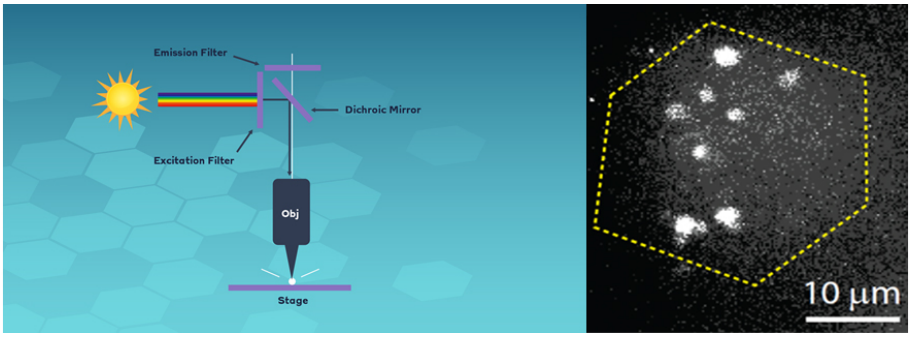

Figure 10: Fluorescence microscopy. Left: Working principle - illumination light is filtered by a short-pass excitation filter and reflected towards the sample by a dichroic mirror. Fluorescence from the sample passes the dichroic mirror and is additionally filtered by an emission filter to remove residual excitation light in the image. Right: Fluorescence image of molecules hosted in an organic crystal (crystal outline shown dashed yellow). The background is not completely dark due to fluorescence from other molecules and the crystal material. *Credit: Author.*

Immunofluorescence microscopy

Immunofluorescence microscopy is a specific variation of fluorescence microscopy that is mainly used in microbiology to visualize the location of biomolecules within a cell. Here, antibodies tagged with fluorescent markers or that are intrinsically fluorescent bind to the biomolecules of interest, revealing their location.[7]

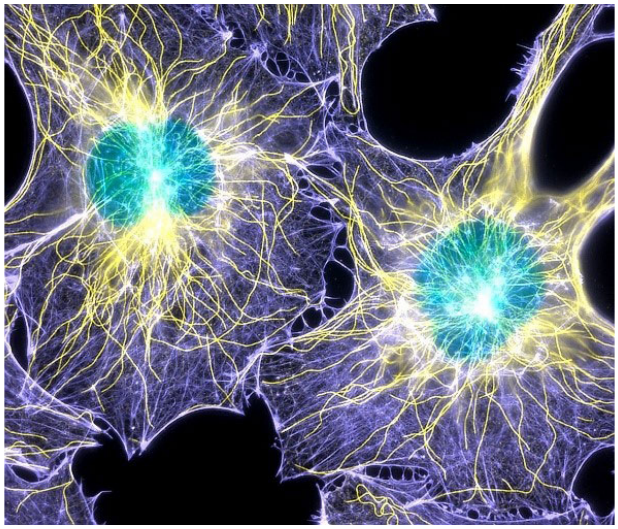

Figure 11: Immunofluorescence microscopy. Two interphase cells with immunofluorescence labeling of actin filaments (purple), microtubules (yellow), and nuclei (green). *Credit: Torsten Wittmann, NIGMS Image Gallery.*

Confocal microscopy

Confocal microscopy is a microscopy technique that images scattering or

fluorescence from a sample point by point. Instead of illuminating and imaging the whole sample at once, an illumination spot originating from a point-like source is scanned over the sample area and only light from that point is detected with a sensitive detector, producing a 2D image. This approach allows imaging of samples with weak signals at high resolution because unwanted background signals from beyond the sampling point are efficiently suppressed. Here, the wavelength and objective used limits the size of the imaging spot in all three dimensions. This allows 2D images to be made at different depths within the sample by moving the objective to different distances from the sample. These 2D image "slices" can then be combined to create a 3D image of the sample, which is not possible with other wide field microscopy techniques discussed and also allows measurement of the sample dimensions in 3D. These advantages come at the cost of not being able to take an image in one shot, but instead having to build up the image point by point, which can be time-consuming and inhibits real-time imaging of a sample (Figure 12).

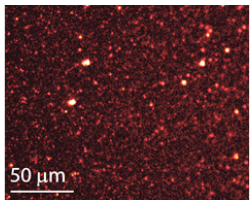

Figure 12: Confocal fluorescence image of single molecule fluorescence. Small spots correspond to fluorescence from individual molecules, while larger spots correspond to clusters of molecules. The fluorescence background here is much weaker than in a simple fluorescence microscope image, as seen by the dark areas between bright spots. *Credit: Author.*

Two-photon microscopy

Two-photon microscopy (TPM) is a variation of fluorescence microscopy that uses two-photon absorption to excite fluorescence instead of single photon excitation. Here, fluorescence is excited by absorbing a combination of two photons of approximately half the energy required for excitation by a single photon. For example, a fluorophore that is normally excited by single blue photons could be excited by two near-infrared photons in this scheme. In TPM, the image is built up point by point just as in confocal microscopy, that is, the two-photon excitation spot is scanned across the sample and sample fluorescence detected by a sensitive

detector. The large difference in excitation and fluorescence energy leads to multiple advantages compared to traditional fluorescence microscopy: first, it allows the use of longer excitation wavelengths which scatter less within the sample and thus penetrate more deeply to allow imaging of a sample below its surface and creation of 3D sample images. At the same time, photobleaching is greatly reduced as the excitation energy is much lower, which is useful for fragile samples. Fluorescence background from around the excitation spot is also greatly reduced because efficient two-photon absorption only occurs at the focus of the excitation beam, such that fluorescence from small portions of a sample can be observed (Figure 13).

A disadvantage of TPM is that the probability of two-photon absorption is much lower than single-photon absorption and thus requires high-intensity illumination such as pulsed lasers to achieve a practical fluorescence signal intensity.

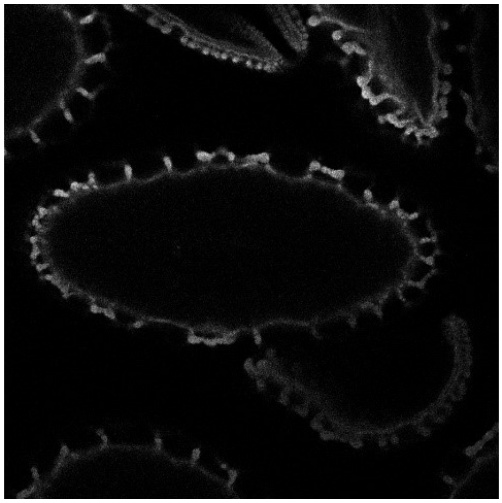

Figure 13: Two-photon microscopy. Thin optical section of pollen, showing fluorescence mostly form the outer layers. *Credit: Michael Cammer, Cell Image Library.*

Light sheet microscopy

Light sheet microscopy is a form of fluorescence microscopy where the sample is illuminated by a thin “sheet” of light perpendicular to the direction of observation, such that only a thin section (typically a few micrometers) of the sample is imaged.[8] By taking a sequence of images while the sample is rotated in the light

sheet, a 3D image can be formed. This requires the sample to be mostly transparent, which is why this technique is often used to form 3D images of small, transparent biological structures such as cells, embryos and organisms such as zebra fish[9] (Figure 14).

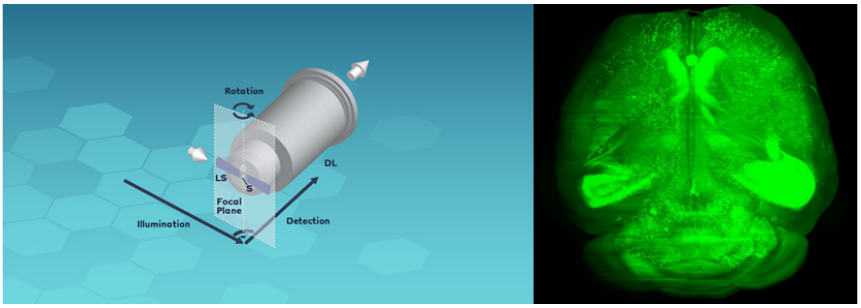

Figure 14: Light sheet microscopy. Left: Working principle. Right: Fluorescence image of a mouse brain taken with light sheet microscopy by fluorescence imaging. *Credit: Fenerliabi11, Wikimedia Commons. Reproduced under the Creative Commons Attribution-ShareAlike 4.0 International license (CC BY-SA 4.0).*

Total internal reflection fluorescence microscopy

Total internal reflection fluorescence (TIRF) is a fluorescence microscopy technique that allows 2D fluorescence images to be made of an extremely thin (approximately 100 nm thick) sample slice.[10] This is achieved by exciting the fluorescence of the sample by evanescent fields of the illuminating light, which occur when it undergoes total internal reflection at a boundary between two materials of different refractive index (n). Evanescent fields have the same wavelength as the illuminating light but are tightly bound to the interface. In TIRF microscopy, the excitation light typically undergoes total internal reflection at the interface between a glass slide (n = 1.52) and the aqueous medium (n = 1.35) the sample is dispersed in. The intensity of the evanescent field falls off exponentially with distance from the interface, such that only fluorophores close the interface are observed in the final image. This also leads to a strong suppression of fluorescence background from areas outside the slice, which allows weak fluorescence signals to be picked up, for example when localizing single molecules. This makes TIRF extremely useful to observe the weak signal of fluorescent proteins (Figure 15) involved in intercellular interactions, but also requires the

sample to be dispersed in an aqueous medium, which may limit the types of samples that can be measured.

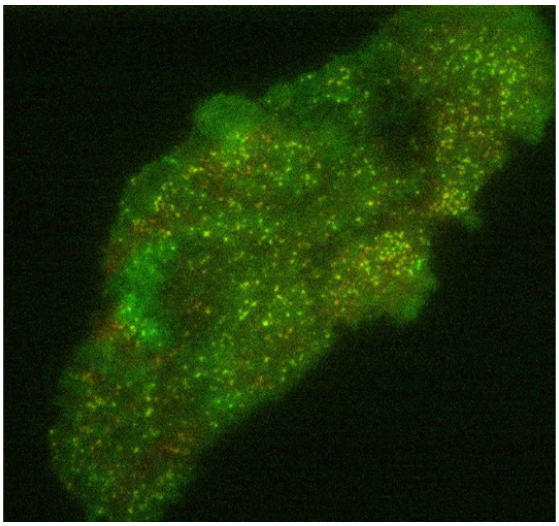

Figure 15: TIRF image showing protein fluorescence in a cultured retinal pigment epithelial cell. Each pixel corresponds to 67 nm. *Credit: Allen Liu, Sandra L. Schmid, Cell Image Library.*

Expansion microscopy

The basic concept behind expansion microscopy is to increase the size of a sample that would normally require high-resolution microscopes such that it can be imaged with standard microscopy techniques, in particular fluorescence microscopy. This works well for preserved specimens such as biomolecules, cells, bacteria and slices of tissue, which can be expanded equally in all dimensions (isotropically) by up to 50 x using a chemical process[11] shown in Figure 16. Expanding the samples allows isolation of individual features of interest that are normally hidden and can make them transparent allowing their interior to be imaged.

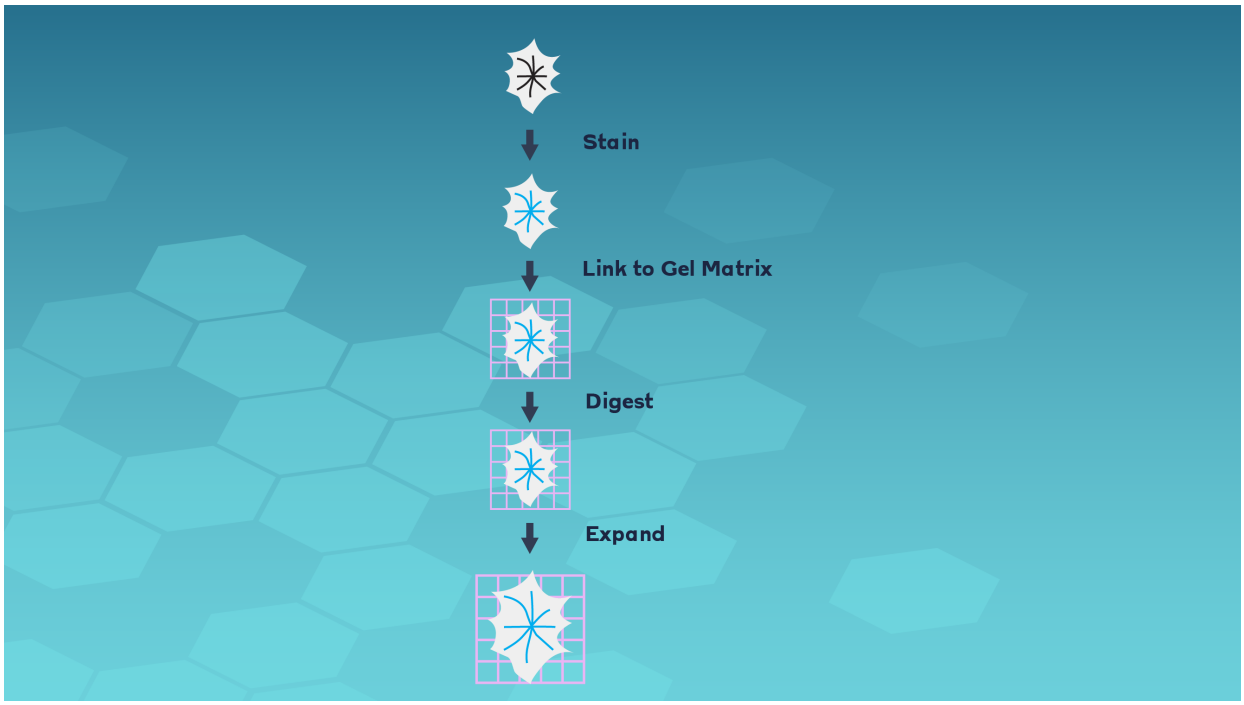

Figure 16: Sample preparation for expansion microscopy. A cell is first stained and then linked to a polymer gel matrix. The cell structure itself is then dissolved (digested), allowing the stained parts to expand isotropically with the gel, allowing the stained structure to be imaged with more detail.

Deconvolution in light microscopy

Beyond adapting the optical system to a specific use case, modern light microscopy also takes advantage of digital image processing, such as image deconvolution.[12] This technique allows the increase of spatial resolution as well as the localization precision of images taken with an optical microscope by compensating for blurring that is inherent to the optical system itself. This blurring can be measured in a calibration step and can then later be used to deconvolve the image, thereby reducing the blurring. By combining of high-performance optics with advanced image processing, digital microscopy[13] can push the limits of resolution to see ever further into the microscopic world.

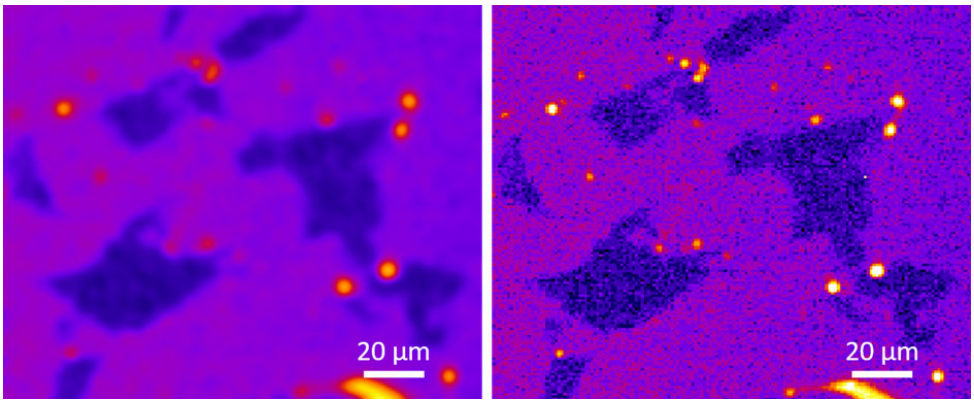

Figure 17: Image deconvolution. Left: Original fluorescence image. Right: Image after deconvolution, showing increased detail. *Credit: Author.*

Light microscopy vs electron microscopy

Light microscopy typically uses wavelengths of light in the visible spectrum, which inherently limits it spatial resolution due to the Rayleigh criterion to approximately half the wavelength used (approximately 200 nm at best). However, even when using objectives with high NA and advanced image processing, this fundamental limit cannot be overcome. Instead, observing smaller structures requires the use of electromagnetic radiation of shorter wavelength. This is the underlying principle of electron microscopy, where electrons are used to illuminate the sample instead of visible light. Electrons have an associated wavelength which is much shorter than visible light, which allows magnifications of up to 10,000,000 x to be achieved, such that even single atoms can be resolved.[14]

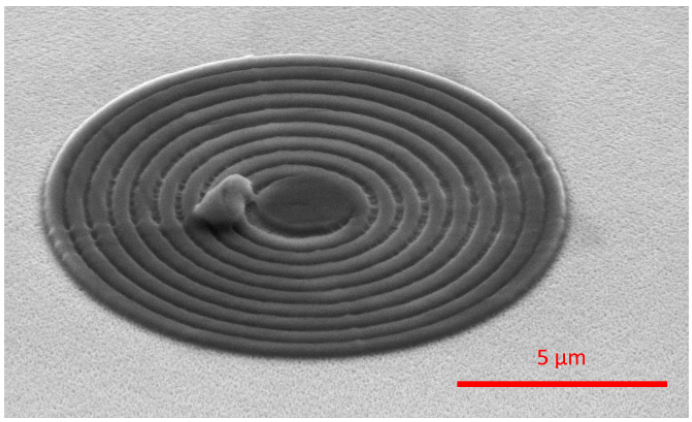

Figure 18: Scanning electron microscope image at 15,000 x magnification of a nanocrystal in a concentric polymer structure. Even fine details such as the pores

of the substrate are resolved. *Credit: Author.*

Summary and conclusion

Light microscopy is a powerful tool for examining small samples across a large range of applications. By adapting the illumination and imaging technique used to the specific use case, high-resolution images can be obtained, providing insight into microscopic structures and processes in the sample. In this article, we have discussed the features, strengths and weaknesses of various light microscopy techniques, which differ in the way light impinges and is collected.

Light microscopy techniques comparison table

Technique	Advantages	Limitations	Typical applications
Bright field microscopy	Relatively simple setup with few optical elements	Low contrast, fully transparent objects cannot be imaged directly and may require staining	Imaging colored or stained samples[15] and partially transparent materials[16]
Dark field microscopy	Reveals small structures and surface roughness, allows imaging of unstained samples	High illumination power required can damage the sample, only scattering image features seen	Imaging particles in cells,[17] surface inspection[18]
Phase contrast	Enables imaging of	Complex optical	Tracking cell

microscopy	transparent samples	setup, high illumination power required can damage the sample, generally darker images	motion,[19] imaging larvae[20]
Differential interference contrast microscopy	Higher resolution than PCM	Complex optical setup, high illumination power required can damage the sample, generally darker images	High resolution imaging of live, unstained cells[21] and nanoparticles[22]
Polarized light microscopy	Strong background suppression from non-birefringent areas of a sample, allows measurement of sample thickness and birefringence	Requires a birefringent sample	Imaging collagen,[23] revealing grain boundaries in crystals[24]
Fluorescence microscopy	Allows individual fluorophores and particular areas of interest in a sample to be singled out, can overcome the resolution limit	Requires a fluorescent sample and a sensitive detector, photobleaching can diminish signal	Imaging cell components, single molecules, proteins[25]
Immunofluorescence microscopy	Visualize specific biomolecules using antibody targeting	Extensive sample preparation, requires a fluorescent sample, photobleaching	Identifying and tracking cells[26] and proteins[27]
Confocal	Low background	Slow imaging speed,	3D cell imaging,

microscopy	signal, possible to create 3D images	requires a complicated optical system	imaging samples with weak fluorescence signals, surface profiling[28].
Two-photon microscopy	Deep sample penetration, low background signal, less photobleaching	Slow imaging speed, requires a complicated optical system and high-power illumination	Neuroscience,[29] deep tissue imaging[30]
Light sheet microscopy	Images only an extremely thin slice of the sample, can create 3D images by rotating the sample	Slow imaging speed, requires a complicated optical system	3D imaging of cells and organisms[8]
Total internal reflection fluorescence microscopy	Strong background suppression, extremely fine vertical sectioning	Imaging limited to thin area of sample, requires a complicated optical system, sample needs to be in aqueous medium	Single molecule imaging,[31] imaging molecular trafficking[32]
Expansion microscopy	Increases effective resolution of standard fluorescence microscopy	Requires chemical processing of the sample, not suitable for live samples	High resolution imaging of biological samples[11]

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