



Development of the Optical Microscope

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Products: Cytation 5 Cell Imaging Multi-Mode Reader



An Optical Microscope commonly found in schools and universities all over the world.

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Ptolemy and Light Refraction

Optical microscopes generally work through the refraction of light rays. Refraction occurs when light passes from one medium to another of different optical density. One can see refraction by simply placing a long object, such as a straight stick in water such that a portion of the object is in the water and another portion in the air. According to our eyesight, the stick appears bent (Figure 1).



Figure 1. Refraction at work...

Of course the stick does not physically bend, but the light rays coming into our eyes do as they pass from the water (index of refraction = 1.33) into air (index of refraction = 1.00). Thus the stick appears bent! The Greco-Roman Claudius Ptolemy was the first to realize this physical phenomenon and actually tabulated scientific data into a table about 2000 years ago comparing the angle differences of a straight stick in air compared to water (see Figure 2).

Angle in Air	Angle in Water
10°	8°
20°	15.5°
30°	22.5°
40°	29°
50°	35°
60°	40.5°
70°	45.5°
80°	50°

Figure 2. Ptolemy's Table of Refraction

Islamic Polymaths and Optics

One thousand years ago, during the height of the Islamic Golden Age, a pair of Arabic polymaths took the science of optics far further. First Ibn Sahl extended Ptolemy's work on refraction to lenses and parabolic mirrors in his treatise "On Burning Mirrors and Lenses." This laid some ground work for Ibn al-Haytham who developed a seven-volume treatise on optics, *Kitab al-Manazir* (Book of Optics), written over a period of a decade. The legacy of this work is thought to be one of the first instances of documentation demonstrating the scientific method of observation based on empirical, measurable evidence – data!. A Latin translation was produced in the thirteenth century which was read by and greatly influenced a number of reputed Western scientists including Leonardo da Vinci and Galileo Galilei.

The First Microscope

While it is historically clear that Arab science fostered the scientific method in Europe during the Renaissance and beyond, it is not clear whether Ibn Sahl's and Ibn al-Haytham's works inspired the development of the first microscope. It is thought that the first microscope, consisting of a compound design with an eyepiece and objective lens, was developed around the end of the 16th century by either Hans and/or Zacharias Janssen. Zacharias was rather a dubious scientist/inventor as he was arrested a number of times for counterfeiting coins and his claims of invention are clouded in inconsistencies. Whatever the truth, a Janssen is widely purported to be the inventor of the first telescope, shown in Figure 3.



Figure 3. The Janssen microscope. The central tube allows for extension moving the objective away from the eyepiece allowing for magnification of between 3x – 10x.

The works of Antonie van Leeuwenhoek and Robert Hooke about a half century later are by contrast incontrovertible and much more significant. Each developed their own microscopes and used them to make new scientific discoveries, but Hooke was a true scientist; while van Leeuwenhoek was a cloth merchant.

Hooke and Micrographia

Hooke was a product of Christ Church, Oxford where he rubbed shoulders with such luminaries as Christopher Wren, the famed architect of St. Paul's cathedral in London. His contemporaries at the time were no other than Robert Boyle and Isaac Newton. During the same period as Newton, both derived fundamental Force equations defining aspects of mechanics:

Hooke's Law: $F = -kx$;

Newton's 2nd Law of Motion: $F = ma$.

But from a microscopy perspective, Hooke is famed for his *Micrographia* publication in the fledgling publication sponsored by the Royal Society – a science-funding organization lasting over 350 years that sponsors scientific research today to the tune of about £42M. Hooke's *Micrographia* contained illustrations of such common irritants as lice and fleas – vermin perhaps everyone was inflicted with the 17th century. Hooke's artistry together with his science illustrated these vermin (see Figure 4) to the delight of a wide audience, albeit still scratching ...

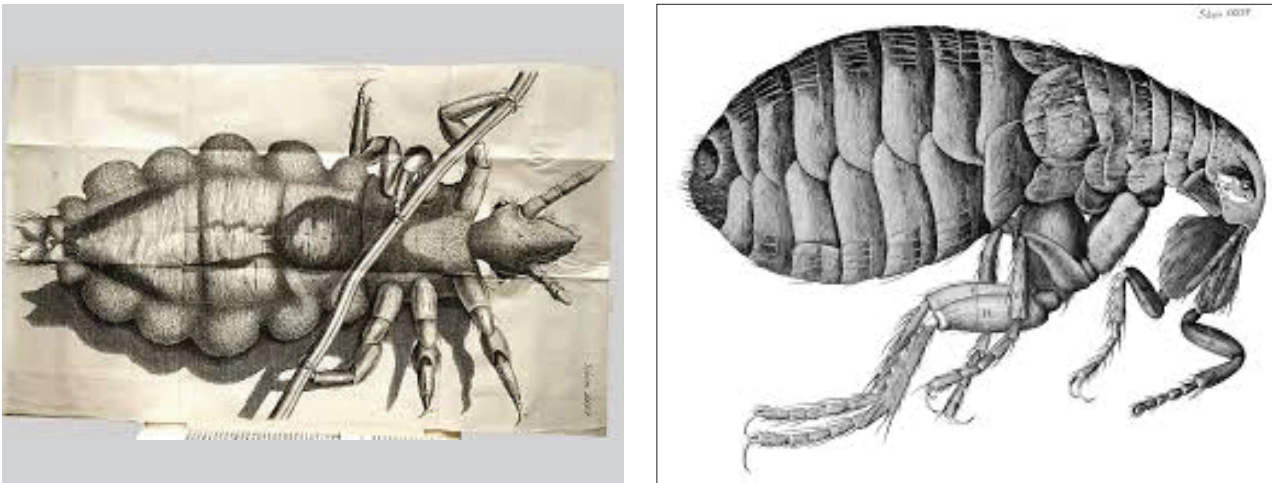


Figure 4. *Micrographia* hand drawn illustrations by Hooke: at left: a louse, at right: a flea.

Hooke's used a compound microscope to obtain these arresting images which his talent as an artist faithfully copied. His microscope provided a marginal improvement over the Janssen microscope by providing a magnification of about 30x. As part of his legacy, however is coining the term "cell" as describing the 'compartments' in cork visible under his microscope (see Figure 5).



Figure 5. A replica of Hooke's compound microscope: notice the thread near the base which allowed for focusing.

Van Leeuwenhoek and Animalcules

Van Leeuwenhoek was not a scientist by training. And yet his accomplishments in the science of microscopy, both from an ability to magnify and to discover new biology, outdistanced Hooke's contribution. And he didn't even use a compound microscope! Van Leeuwenhoek had a commercial interest in weave patterns in cloth and had read with avid interest Hooke's *Micrographia*. This interest and a familiarity with glass processing led to a rather unique ability in making high quality lenses that provided magnification above and beyond what Hooke could accomplish. It is not known how exactly he made his lenses, but it is thought that he used Hooke's described method of drawing heated soda lime rods and creating a spherical globule of glass by reheating one of the drawn ends (Figure 6). It was speculated that van Leeuwenhoek also ground these globules to provide the high magnification of his microscopes.

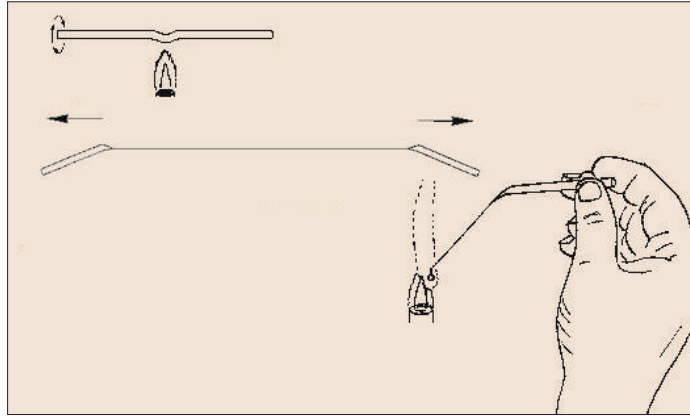


Figure 6. Hooke's recipe for lens making.

Van Leeuwenhoek lenses provided an astounding magnification of up to about 300x, representing about 10 times higher than Hooke's! And the whole device could easily be hidden in the palm of one's hand (Figure 7). It resembled a magnifying glass more than a microscope.

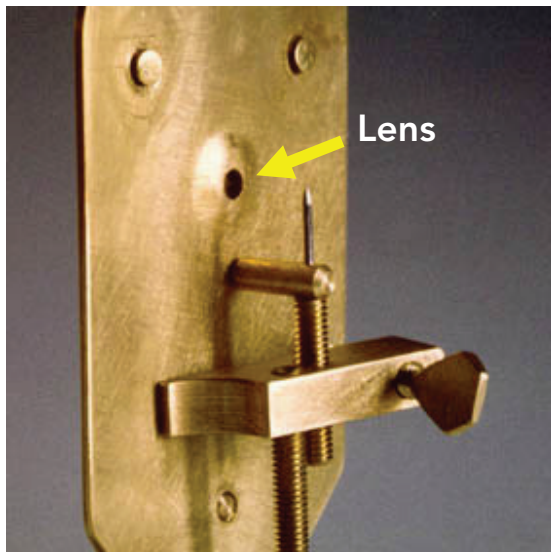


Figure 7A. Close-up of the device.

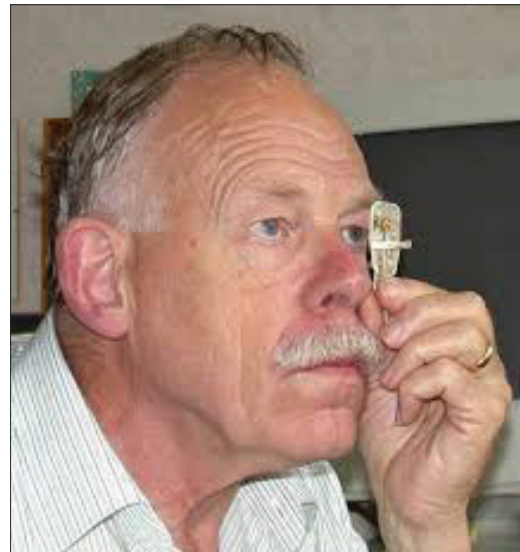


Figure 7B. A modern day user.

With the aid of the magnification provided by his microscope, van Leeuwenhoek made startling discoveries, which he described in a series of letters to the Royal Society rather than publish. Two of his first letters described “animalcules” which he found in a drop of pond water and in the scum of his teeth. We know these microorganisms now as infusoria. Van Leeuwenhoek was amazed at the number of these animalcules in such small samples. To quote him, “There are more of these in my mouth than men in the whole kingdom.” But at the time, scientists at the Royal Society believed that van Leeuwenhoek must have made experimental errors as such small living creatures living in water, or worse, in people’s mouths was deemed preposterous. The Royal Society convened a special committee to investigate the work which ultimately returned a complete vindication for van Leeuwenhoek. Three years later, he was elected to the Royal Society. In all, van Leeuwenhoek wrote 190 letters to the Royal Society describing his microscopic findings, including white and red blood cells, spermatozoa, muscle fibers and aspects of cellular structure. Yet he never once published any result. The letters remain in the Royal Society Library.

Abbe Limit

Van Leeuwenhoek was extremely secretive of his method for lens making and his secrets died with him. Microscopy was largely limited over the next couple of centuries to a resolving power not much dissimilar to Hooke’s compound microscope. Ernst Abbe in the late 19th century did much to improve the ability of microscopes to magnify objects. He worked for Carl Zeiss as an optical theorist, and eventually became a partner in Zeiss’s firm. He invented the apochromatic lens, which reduces both chromatic and spherical aberrations in lenses used in microscopes. He also postulated the Abbe Sine Condition, which details the necessary condition of a lens to form a sharp image. He is perhaps best known for developing a clearer theoretical understanding of the inherent limits of magnification, known as the Abbe Limit or Resolution Limit of microscopy which is determined by the wavelength of light and the numerical aperture of the lens used:

$$d = \frac{\lambda}{2NA}, \text{ where } d \text{ is the spatial resolution possible, } \lambda \text{ is the wavelength of light and } NA \text{ is the numerical aperture.}$$

Abbe could produce microscopes with objective lens systems with numerical apertures that approached 1, once he started collaborating with glass chemist Otto Schott and using techniques such as water immersion to improve NA, and thus achieve a spatial resolution of about half the wavelength of light used for illumination. This resolution was a fraction of a micron and allowed for significantly higher magnification than that achieved by van Leeuwenhoek. Furthermore, Zeiss and Abbe produced microscopes that could be afforded by many scientists in the late 19th century, opening up microscopy-based research broadly.



Figure 8. Zeiss microscope ca. 1877.

Zernicke and Phase Contrast

The Zeiss microscope pictured above is also termed a brightfield microscope where white light is used to illuminate the sample. The ability to view different features in the sample is provided by differences in optical density in the sample: features that absorb more light will be darker; those that absorb less, lighter. The differing optical densities provide contrast in the sample which we can discern. The optics of a brightfield microscope are relatively simple and thus inexpensive and were widely found in many laboratories throughout the 20th century and today. Yet some samples absorb light only poorly or differences in optical density across the sample are small, such that poor contrast in the microscopic image is achieved, leading to difficulties in resolving any features in the sample. This is certainly true with trying to image cells. The advances by Abbe allowed microscopes to possess the resolving power to view these tiny units of life, typically ranging in size from about a micron (i.e. a bacterium) to several decades of microns (typical mammalian cell), but cellular structures were difficult to view due to the lack of significant optical density throughout the cell resulting in poor contrast.

Light transmitting through a sample in a brightfield microscope may be absorbed, scattered or phase shifted. This latter process is caused by light being slowed as it passes through a different medium. Building on Abbe's work, the Dutch scientist Frits Zernicke developed a brightfield microscope just before the Second World War in 1938 that measured phase shifts as illuminating light passed through the sample. At the time, it appeared everyone was preoccupied with the threat of war in Europe and Zernicke's development was not recognized for its ability to vastly improve the imaging contrast of transparent samples (Figure 9). Ironically, it was the German war effort that recognized the significance of Zernicke's achievement and made a number of perfected phase contrast microscopes which demonstrated the true utility of the technique: the ability to monitor live cells over time. After the war, most microscope manufacturing companies adopted the technique and Zernicke's innovation was recognized with the 1953 Nobel Prize in Physics.

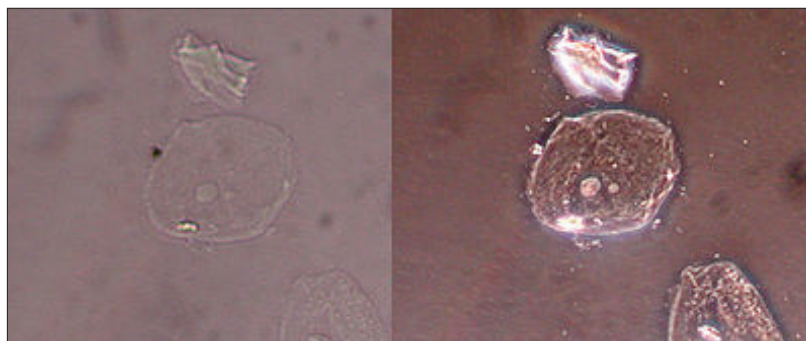


Figure 9. Image comparison of a cell – left: brightfield, right: phase contrast.

Fluorescence Microscopy

Before the phase contrast microscope, cells had been visualized through the use of various stains. Nuclear staining began more than 150 years ago followed by silver staining and at the turn of the 20th century, hematoxylin and eosin, basic and acidic stains respectively, were used to differentially stain the nucleus and cytoplasm of cells. During this same time period starting with George Stokes, fluorescent properties of substances were being investigated. While stains can provide good contrast in samples by the introduction of chromophores that absorb light, much higher contrast in samples can be achieved through the use of fluorescence since the quantity of emission is proportional to the intensity of the light source. The trick of course, is having the right light source.

August Köhler, who became famous for Köhler illumination, also constructed the first ultraviolet (UV) microscope at Zeiss in 1904. His intent was to improve spatial resolution as the Abbe Limit dictates resolution is improved by using smaller wavelengths of illumination. The source used was a cadmium arc lamp and allowed photographic reproduction of objects at twice the resolution of visible light microscopes. Köhler also noted that some objects emitted light of longer wavelength on illumination with UV, but it was Oskar Heimstädt who used this observation as the basis for the construction of the first successful fluorescence microscope in 1910.

At the time, this microscope was more of a curiosity than an effective scientific tool due to the reliance on intrinsic fluorescence from the sample and background signal limitations in the microscope design. Heimstädt himself concluded in his publication that the future of fluorescence microscopy was uncertain.

Happily, the limitations to Heimstädt's work were resolved over the next several decades. A veritable cornucopia of fluorescent probes have been developed specifically for cell biology purposes including intercalating stains, reactive dyes, fluorescently labeled antibodies (based on the pioneering work of Albert Coons) and the family of green fluorescent proteins (largely due to the work of Roger Tsien). With these probes, almost any biomolecule in a cell can be imaged and its function studied. Instrument background signal limitations were resolved by two developments: first, an epi-fluorescence geometry was developed by Ellinger and Hirt in 1929; followed by the development of dichroic mirrors in the sixties, primarily by Johan Ploem, that transmitted some wavelengths of light while reflecting others. Together, these two hardware innovations coupled with the availability of fluorescent probes make the fluorescent microscope an indispensable tool for the cell biologist.

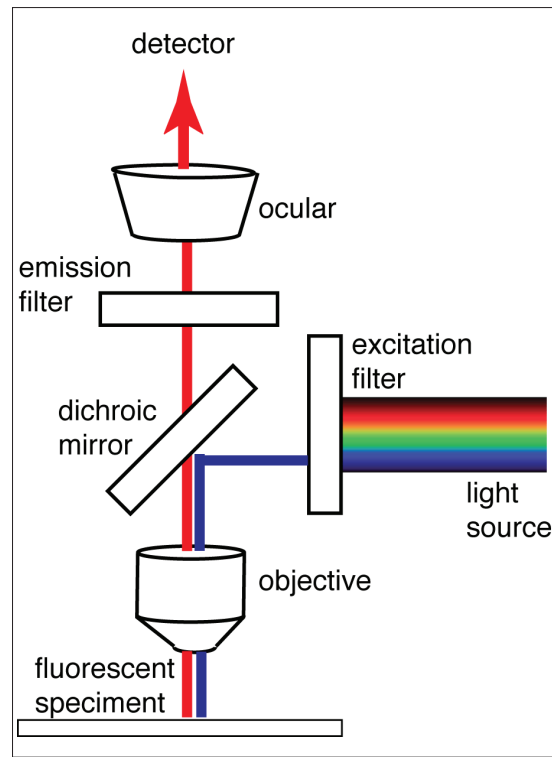


Figure 10. A basic fluorescence microscope demonstrating an epi-fluorescence design coupled with spectral filters isolating excitation and emission light and a dichroic mirror that reflects excitation light (blue) onto the sample and away from the detector at the same time as allowing emission light (red) to pass through to the detector.

Confocal Microscopy

Cell biology is three dimensional. When pushing the Abbe Limit to see ever finer structure in cells and tissue slices, images can appear out of focus due to the wide field of illumination and depth of field limitations of microscope objectives. In 1955, Marvin Minsky found a way around this out of focus problem common to wide field fluorescence microscopes. His basic premise was to restrict both the field of illumination of the sample together with the depth of detection within the sample using a pair of pinholes.

This basic concept is illustrated in Figure 11. The pinhole used with the excitation source restricts the illumination of the sample to a point defined by the size of the pinhole aperture. Additionally, the second pinhole placed in front of the detector restricts the light reaching the detector to only the focal plane. Thus the two pinholes working together effectively restrict the field of view of the sample fluorescence to a point on the focal plane. Minsky then proposed that to create an image of the specimen with the desired field of view, a series of images would be raster-scanned to create a composite image.

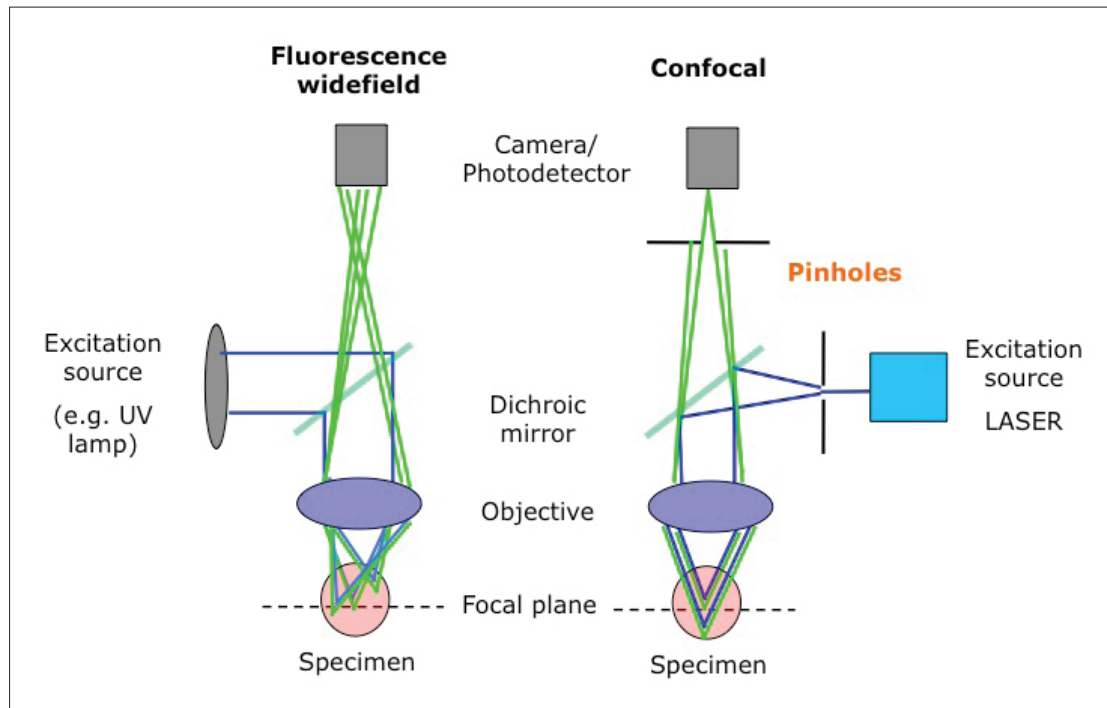


Figure 11. Comparison of wide field with confocal fluorescence. Two pin holes used in the confocal geometry restrict the field of view to a single point on the focal plane.

Minsky patented this concept for confocal microscopy in 1961. Unfortunately for Minsky, technology did not catch-up with his concept until his patent had expired. At issue with Minsky's concept was the restriction of view caused by the pair of pinholes. At the time of his patent, there was no light source of sufficient power to generate adequate fluorescence signal to noise for the confocal concept to work. The advent of the first lasers in the '70's solved the problem and the first commercial instruments became available in 1987. With the development of software that allowed three dimensional views of cells and tissues (see Figure 12) raster scanning multiple focal planes (i.e. slices) through the object, laser scanning confocal microscopy took off.

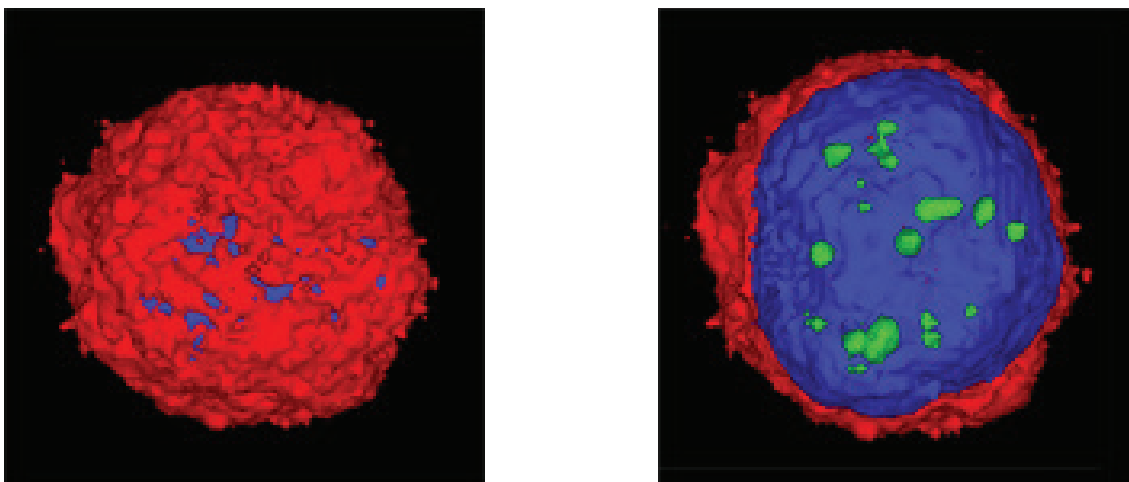


Figure 12. Two views of a single U937 cell imaged with a confocal microscope: at left is a 3D representation of the whole cell (a total of 54 slices at 0.3 micron intervals were recorded); at right, the cytosol (red) has been electronically peeled away exposing the nucleus (blue) and nucleoid bodies (green).

Digital Microscopy

The digital age began with the invention of the transistor in 1947. Moore's law over the last forty years has accurately predicted the ability to pack more and more transistors into integrated circuits boosting computing power while at the same time reducing cost of manufacture. Today, most technologies are digitally-based – and microscopy is no exception. The first digital microscope was developed by the Japanese company Hirox, where oculars were replaced with a digital camera and monitor. The magnification of a conventional light microscope with oculars is the product of the magnification from the eye piece and objective. Eyepiece oculars typically provide 10x magnification, while the highest power objectives can provide 100x. Thus these microscopes can magnify objects about 1,000x such that a 20 μm ID cell can appear to be 2 cm in size viewed through the oculars. Magnification is quite different with a digital microscope where the magnified digital image can be zoomed, displayed on computer monitors and even projected. Using a standard projector, the digital image of a cell can be easily projected on a screen or wall as a 2 m wide image, providing a magnification of 100,000x.

Other attractive attributes of digital microscopes are monitor displays where multiple users can view images simultaneously and image portability where captured images can be downloaded as TIF files and viewed away from the microscope on anyone's laptop. Modern digital microscopes use automated routines for image capture and image analysis making microscopy a very simple process for the research and industrial scientist. These microscopes are typically not immediately recognizable as they have no oculars and often no visible translation stages or focusing knobs. They are also able to handle sample vessels other than the microscope slide. Petri dishes, culture flasks and microplates of various densities (6- to 384-well densities) are now enabled.

BioTek's family of digital microscopes includes both Cytation™ 3 and 5. These instruments not only perform microscopy, but also cell-based assays quantified by a wide number of optical detection technologies including spectrophotometry, fluorescence and luminescence, common to the PMT-based microplate reader. Our latest member of the product family, Cytation 5 (see Figure 13) incorporates many of the developments discussed in this treatise, including fluorescence, color and phase contrast microscopy.



Figure 13. Cytation 5 Cell Imaging Multi-Mode Reader. This digital microscope provides wide field fluorescence and brightfield, color brightfield for histology applications (H&E staining) and phase contrast for imaging of cells without staining or fluorescent probes.

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