

It's all a question of image

Dave Roberts & Gianfranco Novarino

● History

A picture is worth a thousand words, as the saying goes. The ability to visualize microbial structures and processes is a central part of the communication and, in many cases, the understanding of our discipline.

Your eyes are forming an image of the letters on this page because you are able to resolve the adjacent parts of each letter into its black and white components and so discern its shape. If you were to move the page away from you, the letters would form smaller and smaller images on your retina until you were no longer able to resolve the black and white parts and only see the text as a grey blur. The angular separation of any two points on the page which can be separated by your eye is a measure of resolving power and is called the acuity. Its absolute limit is when the two points fall on adjacent retinal cells.

To see smaller objects, we can use lenses to form images which appear to be larger and thus are resolvable. Magnifying glasses are an example of this principle, well known for centuries. If it has been known for so long it is reasonable to ask whether today's microscopes are really better than those from the turn of the century or are they just easier to use? Is there any new physics in the modern instrument?

● Lens design and aberrations

The theory of microscope lenses and how to get the best from them was essentially worked out by Ernest Abbe working in the University of Jena, Germany. The formation of an image by a lens depends on the diffraction of light. School-level physics tells us how lenses work, but there are a number of imperfections in the image formed which are an inevitable consequence of the process of diffraction. For example, the diffraction of light depends on its wavelength, so that an image formed with white light has a slightly different place for each component colour, resulting in images having a coloured fringe around the edge, and generally reducing the sharpness of the perceived image. This is called chromatic aberration. There are a total of six basic kinds of aberration which are corrected by careful design of the component parts of the objective lens, all of which was well understood by the end of the 19th century.

● Resolution

Traditional microscopy was, and in purist circles still is, judged by the simple measure of resolution. Resolution is a question of how close together two objects can be placed and be perceived as two objects. It is almost entirely governed by the angle formed by the light from the edges of the objects as it enters your eye and, therefore, the wider the cone of light, the better the resolution. The light-gathering potential of the microscope objective is measured by its numerical aperture and the greater the numerical aperture, the higher the resolution. The downside of this relationship is that the higher the numerical aperture, the shallower the depth of field will be. Real specimens are almost always thicker than

the depth of field and parts of the specimen which are out of focus will clutter up and technically degrade the image. In real applications it is necessary to trade the greater depth of field of lower numerical apertures with the loss of resolution to get the best combination.

● Contrast

The capacity to resolve an object is of no use unless you can see it; that is, it has to be different from its background. Many microscope test objects are high-contrast, typically being completely opaque, which helps to separate the different components of lens performance. A number of ways to enhance the level of contrast have been developed.

Stains. Perhaps the most obvious way to increase contrast is to apply a stain. Surely every microbiologist must have done a Gram stain, the first stage of which, staining with crystal violet, demonstrates a clear increase in the contrast of the objects. Bacteria that were once translucent become an opaque purple and stand out clearly against their background. The inverse of this process, negative staining with, for example, nigrosin has the same general effect.

Completion of the Gram stain illustrates a second principle of staining, which is the capacity to use chemical reactions to discriminate morphologically similar objects. This is one area in which considerable progress continues to be made.

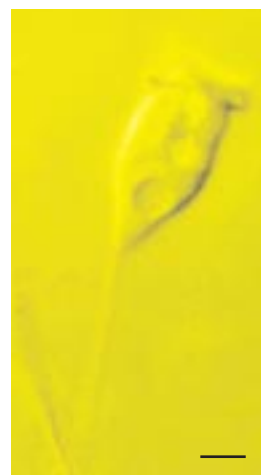
Dark field. When light interacts with a specimen it may do so in several ways. It may be occluded by an opaque specimen, resulting in a silhouette, absorbed at one or more wavelengths, resulting in a coloured image, or the light may be re-directed or scattered. Light scattering by small particles is known as the Tyndall effect and is the mechanism by which we see dust motes in a sunbeam. The objects being seen by this method are often too small to be resolvable directly. Dark-field microscopy works by arranging for the objective lens to collect scattered light so that the specimen is seen as a bright object against a dark background. In technical terms this improves the signal-to-noise ratio and gives a particularly powerful technique for detecting low-abundance objects.

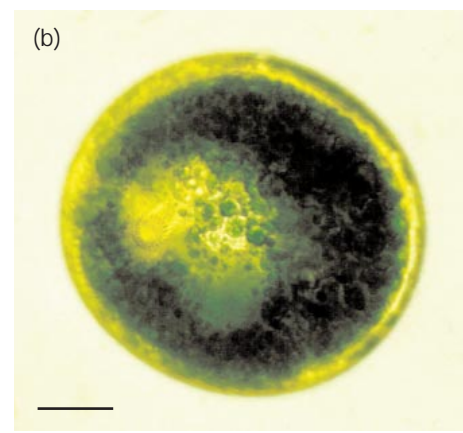
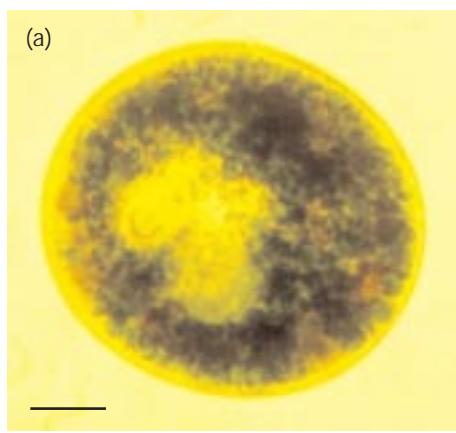
Phase contrast. Many microbes are essentially hyaline but that does not mean they have no effect on the light which passes through them, which may be changed in phase or polarity, although neither change is visible to the eye. In



ABOVE:
Two species of the penicilline ciliate *Frantonia* [*F. vernalis* (green) and *F. leucas*] from the sediment of Esthwaite Water. The black background indicates that the microscope was set up for dark-field illumination. Each cell is about 200 μm long.
COURTESY BLAND FINLAY, INSTITUTE OF FRESHWATER ECOLOGY

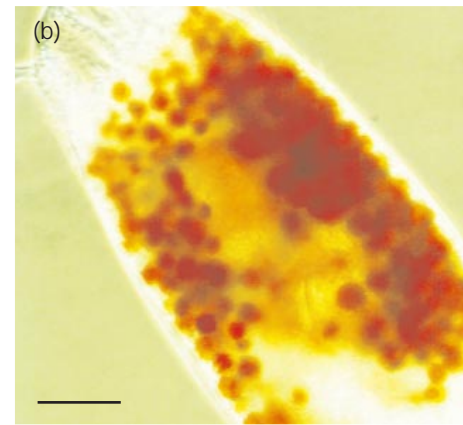
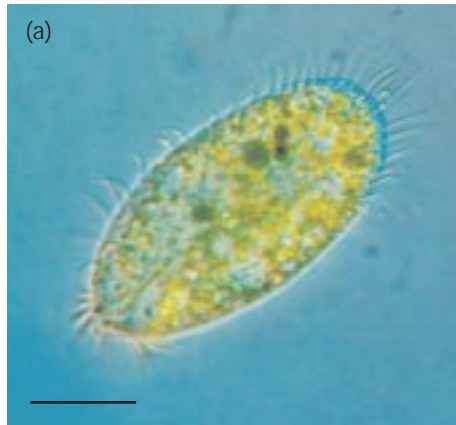
BELOW:
The sessile peritrich *Vorticella similis* photographed under DIC. Note the illusion of oblique illumination caused by the brightening of the left side and the darkening of the right edge. The round object in the lower portion of the cell is the contractile vacuole.
Bar, 100 μm .
COURTESY NICOLA MILLER, SGM



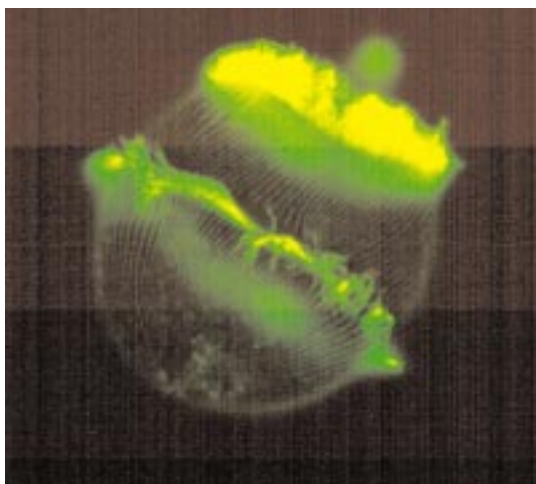


UPPER ROW:
The ciliate *Nassula aurea*. (a) In bright field, Köhler illumination, and (b) with DIC. These images show that the two systems show different aspects of the cell and that DIC is not always the automatic choice of illumination system. Bars, 100 μm .
COURTESY NICOLA MILLER, SGM

LOWER ROW:
(a) An unidentified species of the stichotrich ciliate genus *Oxytricha* photographed under DIC. The blue background colour is an artifact of the DIC configuration. The cirri clearly visible around the edge of the cell are compound cilia and because of their size are best seen under DIC. (b) The stichotrich ciliate *Stylonychia mytilus* photographed under phase contrast. Note the loss of detail, particularly at the anterior (top) end of the cell due to swamping by the halo. Bars, 100 μm .
COURTESY NICOLA MILLER, SGM



BELOW:
The ciliate *Didinium nasutum* labelled with anti- α -tubulin tagged with fluorescein and photographed under UV light. The tubulin in the cytoskeleton and in the cilia has taken the label revealing the two girdles of cilia and the longitudinal organization of the cytoskeletal fibrils. The cell is 140 μm long.
COURTESY SUE HOPE, NATURAL HISTORY MUSEUM



practice polarized light microscopes are most commonly found in a mineralogical context and are uncommon in biology, but Frits Zernike was awarded the 1953 Nobel Physics Prize for the phase-contrast microscope which allowed changes in phase to be made visible. Essentially this is done by making the light passing through an object interfere with light passing through the background. This interference may be additive, causing a brightening, or subtractive, causing a darkening in the image. The cell may be alive and moving and otherwise unstained. This technique is probably best used in microbiology to observe fine projections, such as eukaryotic flagella which are exceedingly difficult to observe in life by any other technique. The disadvantage of phase contrast is the halo which it creates around an object which can be so bright as to obscure detail.

Interference contrast. Strictly, all microscopes form their images through interference, but in the interference contrast microscope the optics are arranged so that the interference is most sensitive to the rate of change of the optical properties, hence the system is known as differential interference contrast or DIC. The optical principle of this method was first described in the late 19th century by Jamin but it was the work of Nomarski in the 1950s that made the technique easily available to the biological laboratory.

DIC images are difficult to interpret because the effect of the interference is as though the specimen was obliquely illuminated creating a pseudo 3-D appearance. The contrast effects are, however, demonstrating differences in the optical path properties, not the actual thickness. Real cells do not often look like fried eggs with a protruding nucleus.

Fluorescence. UV light was first used as a microscope illumination source in 1904 by Köhler, in an effort to improve spatial resolution. Instead, the far more useful property of specimen fluorescence was discovered. Like dark-field illumination, the fact that most materials do not fluoresce greatly improves the ability to study those that do.

However, like phase contrast, the halo generated by a strongly fluorescent object can obscure more weakly fluorescing objects nearby. Many fluorochromes also bleach when they are exposed to UV light and so fluoresce for only a very short time. The use of scanning high intensity light sources, typically lasers, can help to overcome this problem.

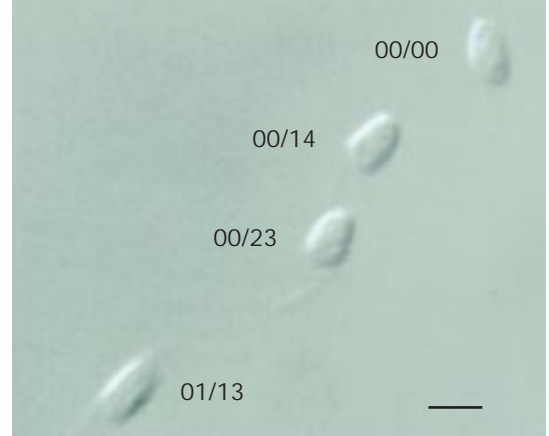
● Depth of field

The problem of depth of field is faced by those who want to observe complete systems rather than thin sections. The parts of the specimen which are out of focus exist as a diffuse blur which detracts from the clarity of the in-focus image.

Holography. In principle it is possible to take a holographic image of a drop of water which could then be re-created by a projector and studied using a conventional microscope. To our knowledge, there is no published record of this ever having been done. The formation of the holographic plate is a process of convolution which cannot readily be reversed by computer because of the complexity and, particularly, the size of the calculations necessary.

Confocal microscopy. The new-kid-on-the-block is still the confocal microscope, which once again has its roots in the 19th century. The true microscope, i.e. an instrument capable of forming a real-time image, was patented by Minsky in 1957 and mechanical instruments were produced a decade later. In the late 1970s the advent of comparatively low-cost digital computers and lasers made the instruments more widely available and by the early 1990s it was a practical proposition to have them in biological laboratories.

The principle of the confocal microscope is to use an optical stop, a small hole, at the objective's primary focus. The effect of this is to allow only in-focus light rays to pass through. To turn this pin-point into an image it has to be scanned over the specimen. This takes real time, of course, and renders the system of limited value to moving (live) specimens. The removal of out-of-focus information from the resulting image results in what is known as an optical section and repeated sections can be built up within a computer into an effective solid model.



ABOVE:
Swimming path of the chrysoomonad flagellate *Spumella elongata* illustrated using digital film. A conventional video clip was recorded using DIC optics, a CCD camera and a U-Matic SP VTR. The clip was converted into digital format using a Radius digital film card on a Macintosh computer. Single frames (duration 1/25 s) were extracted from the digital clips and assembled into a single still-image file. The numbers on the left give the time in seconds/frames. Bar, 5 μ m.
COURTESY G. NOVARINO, NATURAL HISTORY MUSEUM

The loss of total light by the very small confocal aperture has to be compensated for either by use of a photomultiplier or by an enormous increase in the intensity of illumination. With very intense illumination it is necessary to be able to discriminate the illumination (background) from the interference pattern that is the image. This is done most effectively by employing fluorescence to shift the wavelength of the light to be observed and to filter out the illumination source, exactly as in a standard fluorescence microscope. If the illumination source is suitably concentrated, normally a focused laser, then only the region of instantaneous interest is illuminated which considerably reduces the impact of photo-bleaching.

Deconvolution. The combination of in-focus and out-of-focus information is called convolution and is well defined mathematically. It is possible to achieve the confocal effect with a normal microscope by reversing the process, which can be done if you have a sufficiently powerful computer and you know a great deal about the optics of the instrument. The latter issue is the real stumbling block. In practice, however, microscope images can be greatly improved by deconvolution.

● Time and the movies

There is more on the collective mind of modern biologists than mere description: the recognition of the dynamic nature of biological processes and interactions demands systems capable of producing images of changing specimens. The halo effect in phase contrast, for instance, rather limits its usefulness in the case of actively swimming flagellates, when DIC is a more appropriate choice. If the cells slow down then phase contrast once again becomes the method of choice.

● Cameras

The original microscopes were intended to permit visual observations. Film cameras were added later to allow the images to be recorded, although anyone experienced with a microscope will know that the information captured on film is often only a fraction of that available down the eye-pieces.

The first constraint is the amount of light. Film emulsions trade the amount of light needed to expose them against the grain size, i.e. the resolution, of the photographic image. Advances in the chemistry of emulsion production and processing, especially of colour emulsions, have greatly relieved this limitation. Nonetheless, for motile cells the length of time needed for exposure inevitably leads to motion-blur. The use of a flash tube is still the best way to overcome this problem, to deliver a high-intensity burst of light of very short duration. Sadly few modern microscopes are, or can be, equipped with flash tubes.

● Video

The amount of available light has been a limiting factor in the use of cine film in microscopy, but the arrival of

affordable video cameras in the 1980s brought a new technique to microscopy. The first video cameras were based on tube technology and were prone to a number of problems in terms of accuracy and resolution. These days tubes are used only in specialized circumstances.

The charge-coupled device (CCD) is a semi-conductor array which accumulates charge when a photo lands on one unit. These charges are read off at a standard time and the system is re-set for the next image. The data are run together into a standard TV signal in most cameras which is passed to a video recorder or a computer digitizer. In the latter case, the digitizer board has to try and dismantle the signal back into the cell units of which it was first composed. One problem with CCD cameras is that the array accumulates random signal as a result of thermal noise. This can be considerably reduced, and the sensitivity of the camera consequentially increased, by cooling the chip.

The tumbling price of fast personal computers and large storage devices has now opened the field of digital video as a routine application in the laboratory to record and study dynamic processes under the microscope. There have, as yet, been comparatively few published studies in microbiology exploiting the potential of this technique.

● Image analysis

Once you have a digital image, then many forms of image processing are now possible and these techniques are a powerful addition to the microscopist's arsenal. Apart from deconvolution and work on 3-D images, there has been very little progress in image analysis methods since the early 1980s. These techniques are, however, rather mathematical and tend to be driven from the command line of a computer. Few things seem to put biologists off more effectively, so progress in computer-assisted microscopy has largely come with improvements in software design making it easier to use.

● Developments

It should, we hope, be clear from the above that very little new physics has entered the field of microscopy since the development of phase contrast. Nonetheless, today's instruments will, in most but not all circumstances, out-perform their turn-of-the-century equivalents.

It is in the field of technology, of new combinations of known physics, chemistry and mathematics that microscopy is developing now. Since we started with a cliché, we should end with one too – *you ain't seen nuffin' yet!*

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Further reading

The Royal Microscopical Society (RMS) handbook series is recommended, particularly the titles listed below.

Bradbury, S. & Bracegirdle, B. (1998). *Introduction to Light Microscopy. Microscopy Handbook Series*, 94 pp. Oxford: BIOS Scientific Publishers, in association with the RMS.

Bradbury, S. & Evannett, P. (1996). *Contrast Techniques in Light Microscopy. Microscopy Handbook Series*, 118 pp. Oxford: BIOS Scientific Publishers, in association with the RMS.

Gall, J. G. (1996). *Views of the Cell. A Pictorial History.* American Society for Cell Biology.

Sheppard, C. & Shotton, D. (1997). *Confocal Laser Scanning Microscopy. Microscopy Handbook Series*, 106 pp. Oxford: BIOS Scientific Publishers, in association with the RMS.