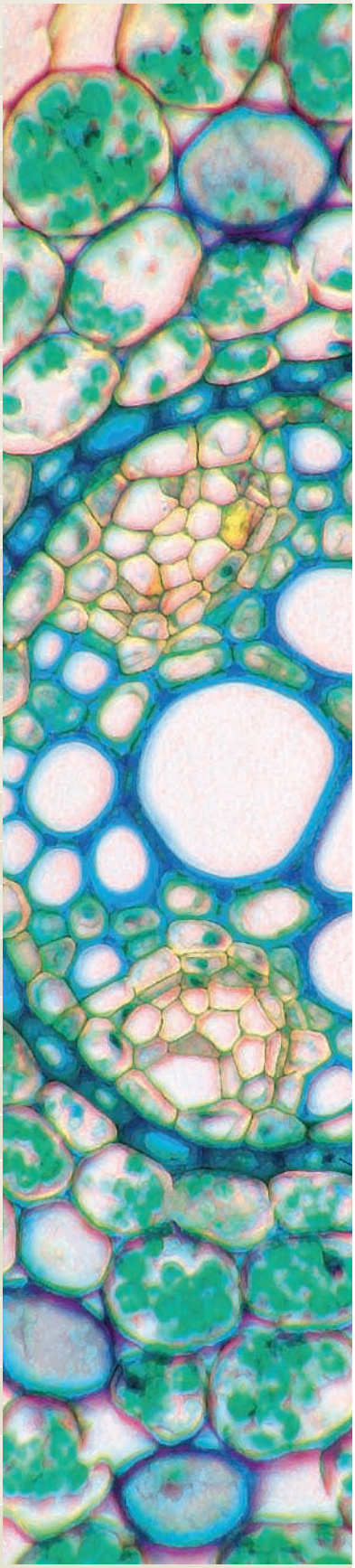
MILESTONES TIMELINE

1595	Invention of the microscope (Milestone 1)
1858	First histological stain (Milestone 2)
1871	Synthesis of fluorescein (Milestone 2)
1873	Diffraction limit theory (Milestone 3)
1911	First fluorescence microscopy (Milestone 4)
1929	First epifluorescence microscope (Milestone 4)
1935	Phase contrast microscopy (Milestone 5)
1939	Polarization microscopy (Milestone 6)
1942	Immunofluorescence (Milestone 7)
1955	Differential interference contrast (Milestone 8)
1961	Concept of confocal microscopy (Milestone 9)
1967	The dichroic mirror (Milestone 4)
1972	Fluorescence correlation spectroscopy (Milestone 10)
1976	FRAP (Milestone 10)
	FRET (Milestone 11)
1980	Calcium probes (Milestone 12)
1981	Video-enhancement differential interference contrast (Milestone 8)
	TIRF microscopy (Milestone 13)
1983	Deconvolution microscopy (Milestone 14)
1987	Realization of confocal microscopy (Milestone 9)
1990	Two-photon microscopy (Milestone 15)
1993	Light sheet microscopy (Milestone 16)
	Single molecule microscopy (Milestone 17)
1994	GFP (Milestone 18)
1997	Fluorescent protein-based biosensors (Milestone 19)
1999	Red fluorescent proteins (Milestone 20)
2000	Breaking the diffraction limit: STED (Milestone 21)
2002	Photoactivatable fluorescent proteins (Milestone 20)
2006	Breaking the diffraction limit: PALM/STORM (Milestone 21)



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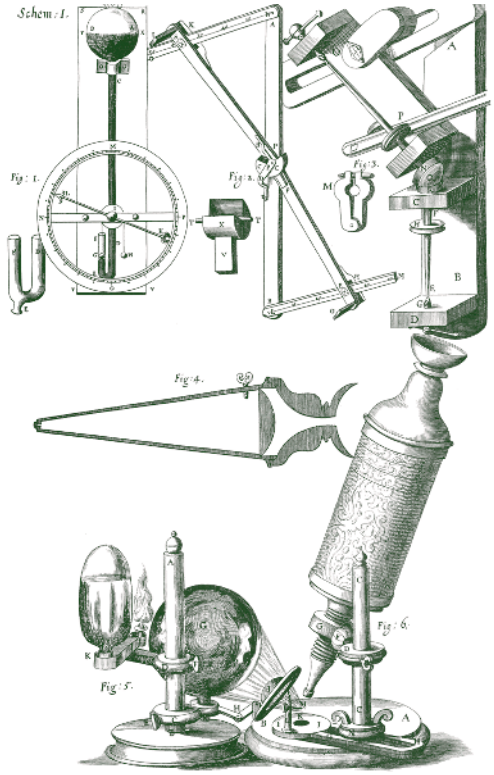
MILESTONES

MILESTONE 1

The beginning



Drawings of the instruments used by Robert Hooke. Image is reproduced from R. Hooke (1665).

> … and by the help of Microscopes, there is nothing so small as to escape our inquiry … *Robert Hooke*

If a microscope is defined as an instrument that enables the visualization of objects or structures that are usually invisible to the naked eye, then microscopes appeared at the end of the sixteenth century. The properties of concave and convex lenses were first described around the year 1000, although magnifiers in the form of lens-shaped crystals or glass may have been used much earlier. Spectacles were invented around 1300 and became widely used across Europe.

Letters by William Borel, the Dutch envoy to the court of France, mentions the Dutch spectacle-maker Hans Jansen and his son Zacharias as inventors of the first compound microscope in 1595. Their microscope had two lenses combined in a tube in which the magnification was varied by altering the distance between the lenses. However, the Jansens are not known to have published any observations through their microscope; instead, the two names associated with the origin of microscopy are Robert Hooke and Antonie van Leeuwenhoek.

Hooke's masterpiece, known widely as simply *Micrographia*, was published in 1665 and is the first book on microscopy by a scientist. At the time of *Micrographia*'s publication, Robert Hooke (1638–1703) was a Fellow of the Royal Society in London, holding the position of Curator of Experiments. His refined compound microscope, described and illustrated in the preface to his book, was a forerunner of modern microscopes — equipped with a stage, a light source and three optical lenses. *Micrographia* puts forward scientific theories on many subjects, as well as containing descriptions of biological and other specimens that are accompanied by beautiful drawings made by Hooke himself. Among these are drawings of a louse and a flea, the compound eye of a fly, seeds and plant sections. He observed the porous structure of cork and famously described the pores as "cells". Although these were not cells in the biological meaning of the word, the modern term derives from Hooke's usage. The book was enormously influential and demonstrated for the first time what microscopy could do for science.

Van Leeuwenhoek (1632–1723) was a Dutch draper with no formal scientific training, who became fascinated by the magnifying glasses used to count the threads in cloth. He learned how to grind and polish lenses and developed such techniques further. Van Leeuwenhoek made more than 500 simple microscopes, each containing a single, tiny convex lens that could resolve detail as small as 1 micrometre. He became extremely skilled in dissecting and mounting specimens and, although his microscopes were laborious to use, he was the first to describe sperm cells and life in droplets of water in the form of bacteria and protozoa. From 1673 onwards, he communicated his observations to the Royal Society in a series of letters, eventually being recognized for his careful observations when he was elected a member of the Royal Society in 1680.

The introduction of multiple lenses increased problems with spherical and chromatic aberration. Van Leeuwenhoek's simple instruments were, in fact, superior to compound microscopes such as those of Hooke, and many microscopists continued to use single lens microscopes until achromatic lenses (corrected for chromatic aberration) became widely available in the early nineteenth century.

Christina Karlsson Rosenthal, Associate Editor, Nature Cell Biology

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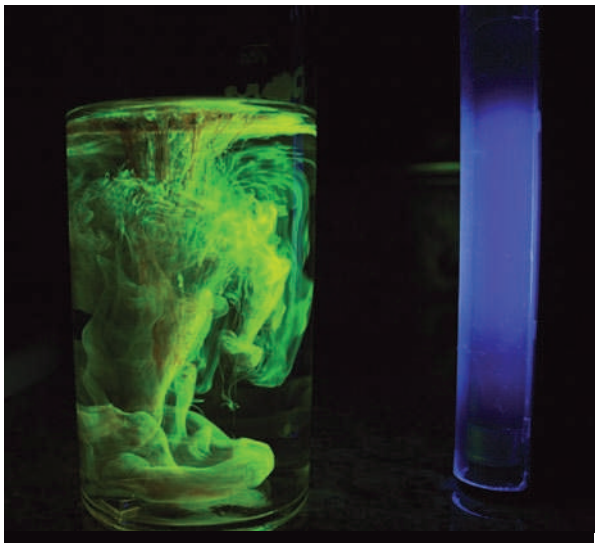
MILESTONE 2

Stains and fluorescent dyes

Robert Hooke may have been the first to describe the light microscopic appearance of a stained object, in the form of dyed wool and hair (see MILESTONE 1). And although others subsequently reported the use of staining solutions, any differential staining that was observed is thought to have been mostly accidental. Hartig and Osborne, who independently observed the colouring of the cell contents in plants, are sometimes cited as the discoverers of staining in microscopy, but neither contributed significantly to the development of the technique. Hartig even casually mentioned the use of carmine, a dye derived from female scale insects, in an 1854 paper.

> "… it was not long before Adolf von Bayer synthesized the first fluorescent dye …"

It was Joseph von Gerlach who, in 1858, while experimenting with solutions of carmine and leaving a section of brain tissue in a dilute carmine solution overnight, reported good differential staining of the nucleus and nuclear granules compared with little or no staining of the cytoplasm and intercellular substance. He concluded that previous staining solutions had been too concentrated, and also noted that the dye was absorbed by specific cellular elements and could not be washed out. Von Gerlach therefore deserves credit for recognizing the importance of staining and for carefully describing his staining method.



Fluorescein powder dropped into a solution of tap water under ultraviolet light, after approximately 15 seconds have elapsed. Image is reproduced with permission from Bricksnite and licensed under the Creative Commons Attribution 3.0 Unported License (http://creativecommons.org/licences/by/3.0/)

The late 1800s and early 1900s marked the discovery of many cytological phenomena and the development of new stains and synthetic dyes. The use of silver staining in cytology was pioneered by Camillo Golgi in 1873 and made famous by Santiago Ramón y Cajal's detailed neuroanatomical observations. The notion that basic and acidic dyes are histologically distinct was important for the development of the haematoxylin and eosin stain (Paul Mayer, 1896), which became a key diagnostic stain. An acidic dye (eosin) and a basic dye (methylene blue) also form the basis of the Giemsa stain (Gustav Giemsa, 1904), which is still used to diagnose malaria and other parasites. Robert Feulgen's discovery in 1924 that chromosomal material can be stained by a chemical reaction based on acid hydrolysis of DNA became a cornerstone of cytochemistry. The cytochemical staining of peroxidase activity, as reported by Graham *et al.*, was subsequently developed as an immunohistochemical approach by Sternberger *et al.* in 1970.

Whereas stains provide finite contrast by changing the light absorption properties of different cellular structures, fluorescence provides infinite contrast with the right equipment — although such equipment obviously did not exist when fluorescence was discovered, and is still being improved today. The earliest description of fluorescence is thought to date from the sixteenth century, when Nicolás Monardes reported the fluorescent properties of wood extract from *Lignum nephriticum* (Athanasius Kircher described similar observations nearly a century later). John Herschel's description of the fluorescent properties of quinine sulphate in 1845 is considered the 'modern' milestone for observing fluorescence and realizing what it was, together with George Stokes's 100-page monograph published in 1852 that describes a vast collection of fluorescent substances, from quinine sulphate to Oporto wine. Although David Brewster first used the term 'internal dispersion' to describe fluorescence phenomena in 1838, it was Stokes who coined the term 'fluorescence' to describe light emission induced during excitation.

With the development of the synthetic dye industry, it was not long before Adolf von Bayer synthesized the first fluorescent dye, fluorescein, in 1871. Paul Ehrlich used the fluorescent dye uranin (a sodium salt of fluorescein) in 1882 to determine the pathway of secretion of aqueous humour in the eye — representing the first use of a fluorescent dye in animal physiology. In 1914, not long after the first fluorescence microscope was developed (see MILESTONE 4), Stanislav von Provazek used fluorescent dyes at a microscopic level as a means to enhance the autofluorescence of cells and tissues — representing the first use of a fluorescent dye as a stain in cell biology.

Arianne Heinrichs, Chief Editor,
Nature Reviews Molecular Cell Biology

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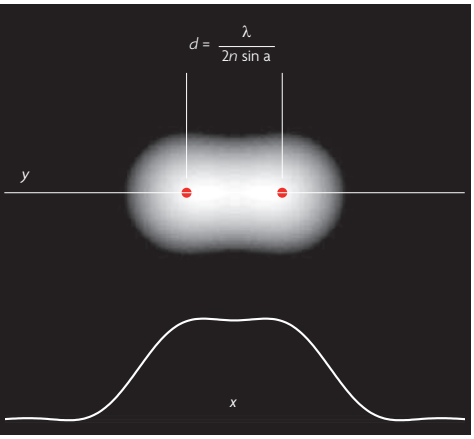
MILESTONE 3

Knowing the limit

Without light microscopy, our knowledge of 'little biology' would be severely impaired. The ability to observe migrating cells, examine the distribution of organelle populations or predict putative protein interactions (based on proximity) drive modern cell-biological research. However, these observations have physical limits, governed by the properties of light, which consequently restrict our view of this cellular world.

In light microscopy, when light passes through an opening it is diffracted, affecting the spatial resolution or, in other words, the smallest separation that two objects can have and still be discerned. When this opening is a lens, the diffraction pattern created by light passage through the illuminated circular aperture appears as an 'Airy disc', as described by George Airy in 1835. Later, the mathematical foundations for quantifying diffraction-limited microscopy were noticed by Emile Verdet but fully described and formalized by Ernst Abbe.



Blurred by diffraction, the image of two point objects (red) can just about be resolved at distance d. Line profile (bottom) quantifies the brightness along the direction of separation. Diagram courtesy of S. Hell, MPI Biophysical Chemistry, Göttingen, Germany.

In his landmark paper of 1873, Abbe reported that the smallest resolvable distance between two points using a conventional microscope may never be smaller than half the wavelength of the imaging light. Although no mathematical equations actually appear in the paper, Abbe stated in this and following papers that the resolution was limited by diffraction to half the wavelength modified by the refractive index of the medium and the angle of the cone of focused light. Based on the resulting equation, one can improve spatial resolution by using light with a shorter wavelength (for example, ultraviolet), but in biological samples, this is undesirable because of the greater potential for sample damage and increased light scattering within a tissue. Reciprocally, longer wavelengths improve tissue penetration at the expense of point separation.

Abbe's mathematical foundations of image formation and lens aberrations provided for the proper design of microscope lenses, accomplished in collaboration with Carl Zeiss and Otto Schott. Abbe's quantitative insights greatly enhanced the quality of microscope optics, contributing enormously to improved data collection and an enhanced user experience for the microscopist. Of course, the success of Abbe, Zeiss and Schott in designing lenses also had an enormous impact on the eventual success of the microscopy manufacturer Carl Zeiss itself. But besides these tangible legacies, another long-lasting legacy brought about by Abbe's work included the establishment of physical boundaries in imaging for quite some time. In fact, Abbe's study influenced the field so greatly that very few attempts were made to overcome the diffraction limit, despite the increasing necessity to enhance resolution and to improve the visualization of cellular structure. Rather, other techniques were developed to provide these data, such as replacing photons with electrons in the development of electron microscopy. Only more recently have scientists revisited Abbe's limit and successfully increased the resolution of light microscopy through a variety of innovative strategies (see MILESTONE 21).

Noah Gray, Senior Editor, Nature

> "… Abbe's study influenced the field so greatly that very few attempts were made to overcome the diffraction limit…"

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MILESTONE 4

The fluorescence microscope

Inspired by Ernst Abbe's finding that shorter wavelength light leads to higher resolution, August Köhler constructed the first ultraviolet (UV) microscope at Zeiss Optical Works in Jena, Germany, in 1904. The instrument used UV illumination from 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 the physicist Oskar Heimstädt who, in 1911, used this observation as the basis for the construction of the first successful fluorescence microscope.

Heimstädt noted two main challenges: one was to concentrate enough UV light on the sample to make it fluoresce; the other was to capture the emitted fluorescent light free of any noise. To address the former, he used cuvettes constructed by Hans Moritz Lehmann to eliminate all but the UV light from the white light generated by an arc lamp; to address the latter, he employed darkfield illumination that ensured no excitation light would enter the objective lens and provided high contrast for the fluorescence signal.

Although Heimstädt successfully imaged bacteria, he wasn't convinced that fluorescence microscopy would have a lasting impact. He concludes his paper with the words: "If and to what degree fluorescence microscopy will widen the possibilities of microscopic imaging only the future will show." In fact, the reliance on autofluorescence of the imaged object and the need for transmitted illumination and darkfield condensers limited the initial applications of the microscope.

Both hurdles were overcome during the next two decades, when the Austrian investigator Max Haitinger ▶

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> If and to what degree fluorescence microscopy will widen the possibilities of microscopic imaging only the future will show.
>
> *Oskar Heimstädt*

► together with other scientists developed the technique of secondary fluorescence, which involved applying exogenous fluorescent chemicals to samples. Haitinger also coined the term 'fluorochrome'.

Fluorochromes were essential to the use of fluorescence microscopy on living tissue, as was the development of the incident light (epi)-fluorescence microscope. Here, the light source lies on the same side of the sample as the objective, and excitation and emission light pass through the objective. This configuration allows more efficient sample excitation and the imaging of opaque objects.

The prototype of the epi-fluorescence microscope was designed in 1929 by German pharmacologist Philipp Ellinger and anatomist August Hirt. In what they called an 'intravital microscope' the excitation light passed through a series of filters before the right wavelength hit the objective lens and triggered emission of fluorescent light in the observed tissue. A yellow barrier filter between the objective and ocular prevented most reflected excitation light from interfering with observation. Ellinger and Hirt imaged kidney and liver tissue in rodents injected with the fluorochromes fluorescein and trypaflavin.

Another major advance came almost 40 years later, with the invention of dichromatic beamsplitters, or dichromatic mirrors. Instead of absorbing certain wavelengths, as traditional filters did, dichromatic filters reflect a narrow width of wavelengths while transmitting all others, allowing illumination of the sample with a precise wavelength and ensuring that no excitation light is transmitted to the ocular.

The Dutch scientist Johan Sebastiaan Ploem is closely associated with the development of these mirrors and described incident vertical illumination with dichromatic mirrors in 1967. Fixed at a 45° angle to the incoming excitation light, the mirror reflects up to 95% of the shorter wavelength excitation light but transmits longer wavelength light. The reflected beam of excitation light then enters the objective, which also functions as a condenser, ensuring even excitation of the sample. The emitted longer wavelength fluorescent light is collected by the objective, and passes back through the dichromatic mirror and a barrier filter to the eyepiece.

Dichromatic mirrors converted the epi-fluorescence microscope from a tool that could be used only by trained specialists to a universal and indispensable instrument for modern biology.

Nicole Rusk, Senior Editor,
Nature Methods

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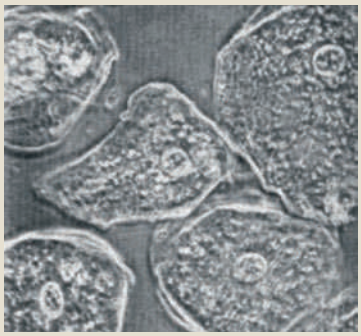
MILESTONE 5

Finding phase

In 1930, physicist Frits Zernike was studying the optics of diffraction gratings when his lab obtained a large concave grating. Owing to its size, Zernike had to position a small telescope 6 metres from its surface to visualize the light patterns created by the gratings. Normally, this resulted in a striped pattern, the effect of seeing a principal spectral line of direct light and weaker spurious lines of diffracted light to the left and right caused by slight imperfections associated with the gratings. But when Zernike focused his telescope on the gratings themselves, something surprising happened — the striped patterns disappeared. Determining why this occurred would change how microscopists look at cells and earn Zernike the 1953 Nobel Prize in Physics.

Through a series of experiments, Zernike came to understand that the direct light and the diffracted light were actually in different phases. When the telescope was focused on the gratings, the resulting image, created through interference, was rendered invisible because, unlike changes in amplitude or brightness, the eye cannot pick up phase differences. But Zernike knew that the phase differences contained information about the object of interest, and realized that he would need an adequate reference surface if he were to make use of it.



Negative phase-contrast micrograph of epithelial cells from the mouth. Image is reproduced, with permission, from L. C. Martin *Nature* **159**, 827–830 © (1947) Macmillan Publishers Ltd. All rights reserved

Zernike then recalled the 1900 work of Lord Rayleigh, who described an operation to make shallow etchings in glass with very dilute hydrofluoric acid. Using this approach, Zernike created 'phase strips', in which the direct light hit a thin etching on a glass surface while the diffracted light passed through the glass. The result was to shift the direct light by 90°, making it darker than a uniform background of coherent light. When Zernike added a phase strip to his telescope, the striped pattern on the grating reappeared.

Zernike immediately saw the potential of translating his discovery to the world of microscopy, where transparent objects found in cells had previously been compared to gratings. By placing his phase strip in the focal plane of a microscope, similarly to the way in which he had used it with the telescope, Zernike was able to visualize otherwise transparent objects without the need for a stain. Zernike published a description of his method, which he coined 'phase contrast', in 1935.

Shortly after his discovery, Zernike excitedly demonstrated the method to microscope maker Carl Zeiss, who were less than enthusiastic about its potential at first glance. It took another 10 years before companies started manufacturing microscopes with the ability to perform phase contrast, but today most high-end microscope systems have this capability, enabling researchers to see cellular structures in real time without the need for specialized stains or dyes.

Nathan Blow, Technology Editor,
Nature and Nature Methods

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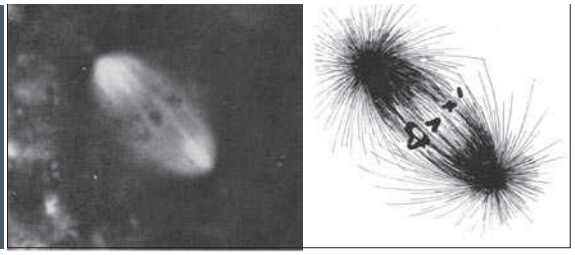
MILESTONE 6

Seeing is believing: subcellular structures brought to life



First maturation division spindle in a living oocyte of the marine annelid *Chaetopterus pergamentaceous* imaged with a polarization microscope (left) and traced the projected image negative (right). Image is reproduced, with permission, from S. Inoué © 1953.

The development of fluorescence microscopy technologies has made live-cell imaging routine for the current generation of cell biologists. However, the ability to visualize the cell's architecture was not taken for granted until the 1950s. Although images of cells and subcellular organelles had been produced as early as the mid-nineteenth century, they originated from fixed material. As a result, the existence of three-dimensional macromolecular structures within cells had remained a source of controversy.

The view of the cell's internal organization was revolutionized in the 1940s and 1950s with techniques that were initially developed to image the mitotic spindle. Until then, the observed assembly of polymers into fibres was believed to be an artefact originating from fixation and staining treatments that induced protein coagulation and gave the spindle a fibrous appearance. The first evidence of fibrous organization in the living spindle came from W. J. Schmidt's observations of developing sea-urchin eggs made in 1937, and reinterpreted in 1939, with a polarizing microscope. This apparatus exploits the optical anisotropy of transparent materials to reveal detailed information about the internal structure of samples — information that was not available with any other optical microscopy technique at the time. Schmidt reported that spindles have positive birefringence — a measure of optical anisotropy — a feature that he concluded to be caused by aligned protein units. However, although football-shaped spindles were visible in Schmidt's pictures, his microscope did not provide adequate resolution to see discrete fibres.

The definitive visual demonstration of the existence of spindle fibres in untreated living cells was given by Shinya Inoué more than a decade later. Inoué built his first polarizing microscope by hand in post-war Japan and managed to repeat Schmidt's observations. However, it was only later at Princeton University in New Jersey, USA, that he built an improved polarizing microscope that could image weakly birefringent structures. In 1953 this led to the publication of the structure of the metaphase and anaphase spindles in oocytes of the marine annelid *Chaetopterus pergamentaceous* and in *Lilium longiflorum* pollen mother cells: "With the increased resolution and sensitivity achieved by the new instrument, I have been able to observe the detailed structure of the spindle in living cells and to follow its change during mitosis," he wrote. Further observations led Inoué to suggest how microtubule dynamics generate the force for chromosome movement and also to deduce the arrangement of DNA and chromatin folding in living sperm.

Schmidt's and Inoué's findings were the first demonstrations of the structural complexity of the cell interior and showed the great potential of live-cell imaging for understanding complex biological processes. Polarization microscopy offers certain advantages over fluorescence microscopy in that it is label-free and therefore a non-invasive mode of imaging that can be used, for example, to monitor the viability of *in vitro* fertilized oocytes. Furthermore, the development of the 'pol-scope' in 1995 overcomes the limitation of previous microscopes in that the polarization contrast is independent of the orientation of the sample.

Kim Baumann, Associate Editor,
Cell Migration Gateway

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MILESTONE 7

Recipe for fluorescent antibodies

> … the basic idea remains unchanged…

During a seminar in 1961, Albert Coons humbly mentioned that "the hour of the fluorescent antibody" had started. Nearly 70 years since Coons and colleagues published the first report describing the use of fluorescent antibodies, immunofluorescence staining remains a cornerstone of cell biology.

The idea to make antibodies visible was conceived to visualize group A haemolytic streptococci in the Aschoff nodules that characterize rheumatic fever. Initially, Coons and his colleagues repeated the work of John Marrack, who had successfully modified antibodies with tetrazotized benzidine to produce coloured antibodies without disturbing their antigen-binding properties. These coloured antibodies were too faint to be detected in dilute solutions, so Coons *et al.* turned to a fluorescent compound, anthracence isothiocyanate. They obtained brightly fluorescent antibodies that could agglutinate bacteria, indicating that the conjugation had not affected the antibodies' binding properties. However, when they tested the fluorescence in tissue sections, they discovered that human and mouse tissues autofluoresce at the same wavelength as anthracence.

Coons and his co-workers then turned to fluorescein, which was a known fluorophore. However, a form of fluorescein that could be ►

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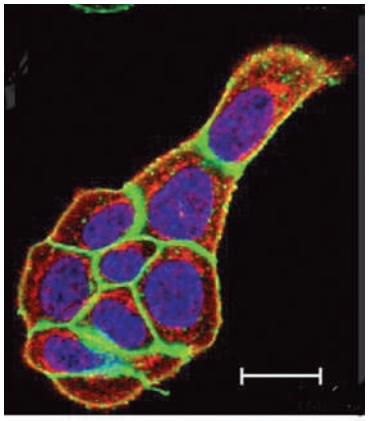
► conjugated to protein, fluorescein-iso-cyanate, had to be made from scratch. In collaboration with colleagues at Harvard University in Boston, Massachusetts, Coons obtained a crude preparation of this compound and used it to label an anti-*Pneumococcus* strain 3 serum. The labelling was performed by mixing the antiserum and fluorescein-iso-cyanate, dissolved in dioxane, in a buffer of saline and sodium carbonate at pH 9.0 — a protocol that is almost unchanged today. The antibodies remained specific for *Pneumococcus* strain 3, and *Pneumococcus* strain 2 did not become fluorescent when incubated with the antibodies. To test whether the antibodies could detect bacteria in tissues, organs of mice infected with *Pneumococcus* strain 3 were harvested and stained with the fluorescent antibodies. When Coons and colleagues were able to see bright fluorescent patches in the organs of the infected, but not uninfected, animals, the era of immunofluorescence microscopy was born.



The location of the extracellular domain (green) and the intracellular domain (red) of the epithelial cell-adhesion molecule (EpCam) was revealed by double-label immunofluorescence. FaDu cells were stained with primary antibodies against the different parts of EpCam. The primary antibodies were visualized with fluorescently labelled secondary antibodies that specifically recognize one of the two primary antibodies. Scale bar, 18.83 µm. Image is reproduced, with permission, from D. Maetzel *et al. Nature Cell Biol.* **11**, 162–171 © (2009) Macmillan Publishers Ltd. All rights reserved.

Many improvements have followed, primarily in the lenses, microscopes and cameras that the field relies on, as well as through the introduction of fluorophores with different, non-overlapping excitation and emission spectra. The resolution has moved from the tissue to the intracellular level, and the technique is now used for subcellular localization in even the smallest cells. However, the basic idea remains unchanged, almost seven decades after its first description.

Christiaan van Ooij, Chief Editor,
Nature Reviews Microbiology

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MILESTONE 8

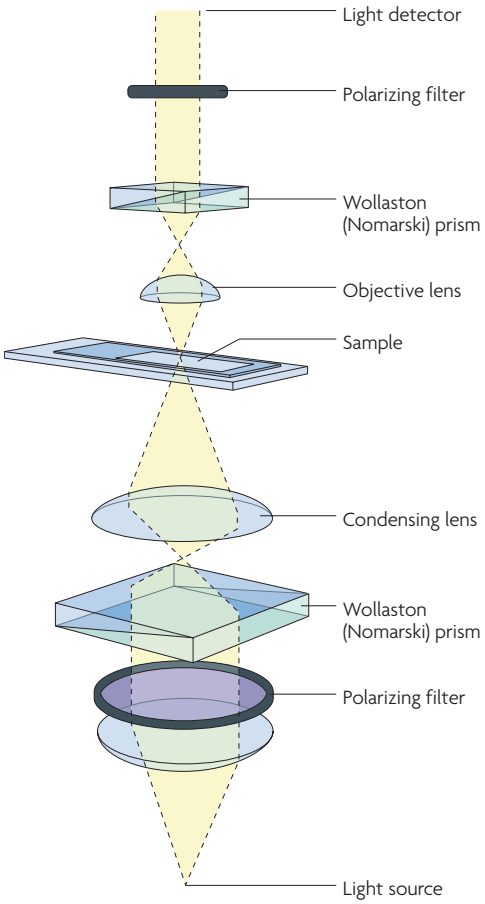
Contrast by interference

In the 1950s, differential-interference contrast (DIC) systems were designed as an alternative technique to phase contrast (see MILESTONE 5). In DIC, polarized light is separated into two beams, which take slightly different paths through a sample depending on its optical density. When the beams are recombined, their interference reveals interfaces between regions of different thickness and/or refractive index and gives the illusion of a three-dimensional image. DIC allows for high-resolution imaging of unstained and living cells and organisms, and for 'optical sectioning' of thick samples. Another advantage is that there is no 'halo' artefact in DIC images, unlike that in phase-contrast images.

> The full potential of DIC was realized only when video cameras entered the stage.

In the first DIC systems such as those of Smith in 1955, one of the Wollaston prisms that were used as beam splitters was located at or near the focal plane of the objective, where it was often inaccessible. The French physicist Georges Nomarski, who described the theoretical basis for DIC, modified the Wollaston prisms so that they could be physically located away from the focal planes, making the system more practical to use. This design was then developed commercially by microscopy manufacturer Carl Zeiss and its theory and potential applications were described by Allen, David and Nomarski in 1969.

The full potential of DIC was realized only when video cameras entered the stage. In 1981, two papers demonstrated that changing camera gain and offset could increase resolution and contrast in the image. Of course, the video camera also enabled researchers to follow events ►



The components of the basic differential interference contrast microscope set-up. Adapted with permission from Richard Wheeler and licensed under the GNU Free Documentation License (http://commons.wikimedia.org/wiki/Commons:GNU_Free_Documentation_License).

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► over time. Allen, Allen and Travis exemplified the possibilities of the technique by visualizing the dynamic movement of microtubules in the marine protist *Allogromia*. Inoué demonstrated the dramatic improvement of both polarized light and DIC imaging by video enhancement.

Video-enhanced DIC (VE-DIC) was subsequently used in numerous studies of dynamic behaviour within the cell at high resolution. For example, the technique was used to discover the fast transport of membrane vesicles, some 50–100 nanometres in diameter, along microtubules within neuronal axons and, subsequently, in *in vitro* motility assays that revealed the microtubule motors that pull this vesicle cargo (see Further Reading).

Christina Karlsson Rosenthal, Associate Editor, Nature Cell Biology

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CITING THE MILESTONES
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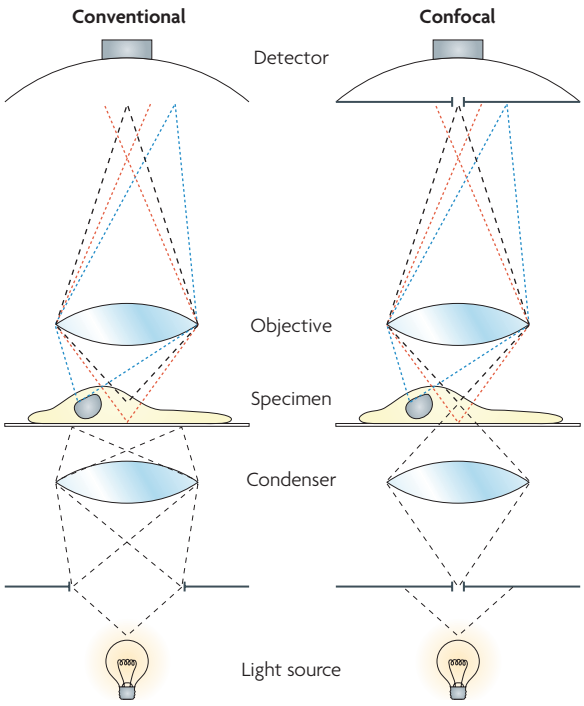
MILESTONE 9

Seeing the wood for the trees



In a conventional microscope (left), a large region of the specimen is illuminated by the light source and condenser. The detector forms an image from both the in-focus light (black lines) and the out-of-focus light (red and blue lines). Conversely, a confocal microscope (right) restricts both the illumination and the light that can reach the detector by introducing two pinholes, so only the in-focus light is detected (black lines). Adapted, with permission, from Spector D. L. & Goldman, R. D. *Basic Methods in Microscopy* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, 2006).

During the 1960s, researchers worked to overcome a debilitating feature of epifluorescence microscopy — background signals. Efforts began in both America and Czechoslovakia to do away with these unwanted signals, and the result was the advent of confocal microscopy.

The resolution achievable with conventional microscopy can be negated by out-of-focus information. Confocal microscopy avoids this issue by restricting both the field of illumination and the light reaching the objective to a single point in the same focal plane. An image of the region of interest is then built up by raster scanning through the sample — either by moving the sample, or by moving a laser beam or pinhole disk. By excluding the out-of-focus light, the image contrast is increased and finer detail can be seen.

The initial patent for a confocal microscope was granted to Marvin Minsky — better known for his pioneering work in Artificial Intelligence — who was working at Harvard University in 1961. Shortly thereafter, Mojmir Petrán of the Charles University in Prague, Czechoslovakia, began a collaboration with David Egger and Robert Galambos at Yale University. Back home, Petrán went on to build a pocket-size pinhole scanning microscope; he then returned to Yale where, in 1967, he and Egger reported unstained images of brain and ganglion cells using reflected light. In 1981, the design of Petrán's microscope was discussed in a theoretical analysis of conventional versus confocal microscopes by Colin Sheppard and Tony Wilson, who proposed a theory on how to combine "the resolution and depth discrimination improvements of confocal microscopy with the ease of operation of the conventional microscope".

Several follow-up studies made a key adjustment to the confocal design — the use of laser beams. This allowed faster scanning and higher resolution, but, most importantly, provided the illumination needed to obtain fluorescence images. Two papers published in 1987 used such instruments in the first key applications of confocal ►

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> If I could have only one single apparatus in the lab, it would be a point scanner. *Kees Jalink*

► microscopy to cell biology. In the first study, John White, William Bradshaw Amos and Michael Fordham, working at the Medical Research Council laboratory in Cambridge, UK, used their laser scanning confocal microscope to compare images of various cells and tissues — from the endoplasmic reticulum of plasmacytoma cells to the mitotic spindles of worm embryos — produced by epifluorescence versus confocal techniques. The second study came from Gerrit van Meer and colleagues, working in Kai Simon's laboratory at the European Molecular Biology Laboratory in Heidelberg, Germany, who used a laser scanning confocal microscope developed by Wijnaendts-van Resandt *et al.* together with fluorescent ceramide labels to follow the transport of newly synthesized sphingolipids in epithelial cells. In this way, the advantages of confocal microscopy for analysing subcellular processes became clear.

By revealing clear images in thick tissue samples, confocal microscopes obviated the need for tissue sectioning, and allowed tissues to be imaged under more physiological conditions. Together with the discovery of green fluorescent protein (see MILESTONE 18), this ushered in the era of *in vivo* imaging.

Alison Schuldt, Senior Editor,
Nature Cell Biology

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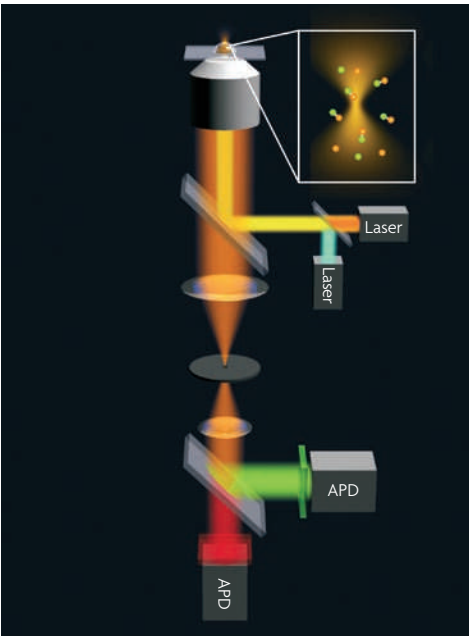
MILESTONE 10

FCS and FRAP: illuminating cellular processes



Principle of dual-colour fluorescence cross-correlation spectroscopy (dcFCCS). Intensity fluctuations from two spectrally distinct species of labelled molecule (red and green) in the focal volume are recorded by two different avalanche photodiode (APD) detectors. Co-diffusion of molecules that are tightly linked to each other induces coincident fluctuations in both channels and, consequently, non-negative cross correlation. The cross-correlation amplitudes directly reflect on the degree of interactions. Image courtesy of P. Schwille, Technical University of Dresden, Dresden, Germany.

By the late 1960s, fluorescence imaging was routinely used in a number of applications. But in 1972, the Webb laboratory put fluorescence microscopy to a novel use: measuring reaction kinetics. Neither ethidium bromide (EtBr) nor DNA fluoresces independently, but in complex the two emit an orange glow under ultraviolet light. By measuring the fluctuations in fluorescence of EtBr binding to DNA, Magde *et al.* calculated the thermodynamics of complex formation and diffusion at equilibrium, and derived precise mathematical formulae explaining their interdependencies.

Between 1972 and now, this technique, termed fluorescence correlation spectroscopy (FCS), has been applied to measure the kinetics of chemical and biological reactions, diffusion and flow. The rates of diffusion and flow were measured by detecting the stochastic movement of fluorophores into and out of a focused laser beam. Detection techniques have now improved, making it possible to quantify the dynamics of single molecules (Rigler and Widengren, 1990). The widespread adoption of FCS was initially constrained by the requirement for extensive calibration measurements and specialized equipment, but FCS modules for confocal microscopes are now available.

> …the Webb laboratory put fluorescence microscopy to a novel use: measuring reaction kinetics.

Twenty-five years after the initial studies using FCS, the development of fluorescence cross-correlation spectroscopy (FCCS) permitted the measurement of intermolecular interactions. In 1997, Schwille *et al.* labelled oligonucleotides with two discrete fluorophores — red and green — and measured the kinetics of red–green complex formation to deduce average annealing time. This technique has since been used to measure protein–protein interactions, including dynamic co-localization, trafficking and caspase-mediated cleavage.

FCS could be used to measure the diffusion of membrane proteins, but a technique called fluorescence recovery after photobleaching (FRAP) enabled researchers to elucidate the movement of proteins within and between cellular membranes at micrometre-scale resolution. In 1974, Bähr and colleagues ►

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► photobleached half of an erythrocyte plasma membrane labelled with fluorescein isothiocyanate (FITC) and found that membrane proteins did not diffuse rapidly from the unbleached area into the bleached area. The robustness of FRAP, and its utility in a range of applications, permitted Axelrod *et al.* to measure intracellular membrane trafficking just 2 years later. Examining the recovery curves of photobleached samples allowed the authors to infer whether recovery was accomplished by lateral diffusion, flow from intracellular compartments or a combination of the two. The development of precise mobility-rate formulae also permitted the comparison of diffusion and flow between heterogeneous samples. Today, FRAP is widely used to study trafficking within and between subcellular compartments and to quantitate the binding and disassociation of membrane proteins *in vivo*.

In 1976, Koppel *et al.* undertook a side-by-side comparison of FCS and FRAP, and noted that each system has distinct advantages and disadvantages. Whereas FCS can provide information about single molecules, its potential to photobleach samples limits the length and scope of experiments, and the experimental set-up is sensitive to perturbation. Unlike FCS, FRAP can detect immobile elements in cell membranes and can also be applied across populations of living cells, but the required photobleaching step can irrevocably damage delicate samples. Nonetheless, the development and refinement of these complementary techniques ushered in a new era of fluorescence microscopy and supported additional applications of fluorescent probes.

Emily J. Chenette, Associate Editor,
UCSD-Nature Signaling Gateway

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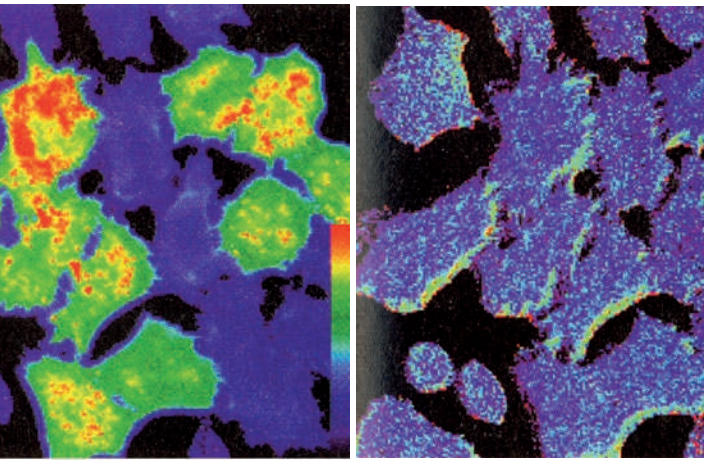
MILESTONE 11

Time to start FRETting



Förster (or fluorescence) resonance energy transfer microscopy has been used to address questions about the existence of lipid rafts. Image is reproduced, with permission, from R. Varma & S. Mayor © (1998) Macmillan Publishers Ltd. All rights reserved.

The theory behind Förster resonance energy transfer (FRET) — or fluorescence resonance energy transfer, as it is also known — was first formally proposed in the 1940s by Theodor Förster, who showed that electronic excitation energy can be transferred from a donor fluorophore to an acceptor chromophore in close proximity, with an efficiency dependent on the inverse of the sixth power of the distance separating the two. This relatively simple equation would turn out to have a considerable impact on biology, and on microscopy in particular.

Förster's proposal was confirmed experimentally in 1967 by Lubert Stryer and Richard Haugland, who demonstrated that the phenomenon of resonance energy transfer could be used as a 'spectroscopic ruler' to determine the distance between two chromophores. These findings paved the way for using FRET to observe interactions and conformational dynamics in purified proteins *in vitro*.

But Förster's theory also led to another application: mapping protein distributions and interactions in living cells with high resolution using a fluorescence microscope. Fernandez and Berlin first showed in 1976 that the concept of FRET could be used to examine the dynamic distribution of receptor complexes on the cell surface. This was a substantial advance for the field because it facilitated the determination of receptor distribution with much higher spatial resolution than with standard fluorescence tagging. However, unlike the authors of work to come, Fernandez and Berlin did not show any microscopy images.

FRET is uniquely suited to imaging protein–protein

> “FRET is uniquely suited to imaging protein–protein interactions in cells…”

interactions in cells because for a change to be seen in the FRET signal the two fluorophores must be in very close proximity. The first papers demonstrating this application of the technique were published in the mid-1990s, after fluorescence microscope technology had improved and the confocal fluorescence microscope had become available. In 1993, Brian Bacskai, Roger Tsien and their colleagues used FRET and confocal fluorescence microscopy to follow the fate of the catalytic and regulatory subunits of cyclic AMP-dependent protein kinase, as well as free cAMP, in response to stimulation of sensory neurons from a marine snail with serotonin.

Thomas Jovin's group soon followed with the pioneering of FRET microscopy techniques that improved the sensitivity and accuracy of FRET so as to allow quantitative measurements of protein interactions ►

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► in cells. In 1995, Jovin and colleagues introduced donor photobleaching and fluorescence lifetime imaging microscopy (FLIM), which they used to study epidermal-growth-factor receptor oligomerization on single cells. They introduced acceptor photobleaching in 1996, which they used to follow the subcellular localization and oligomerization state of the cholera toxin in cells.

Since these landmark developments, the importance of FRET as a microscopy technique has been borne out in its application to challenging biological questions, such as the existence of lipid rafts. A wide variety of FRET-based biosensors are now available for detecting small molecules and enzyme activities in cells. And the technology continues to evolve, as novel donor–acceptor pairs are developed, new methods are introduced and instrumentation improves.

Allison Doerr, Associate Editor,
Nature Methods

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MILESTONE 12

Calcium, a messenger under the spotlight

"It is scarcely necessary any longer to stress the importance of intracellular free Ca^{2+} as a second messenger" reads the opening line of Roger Tsien's 1980 paper, reporting for the first time the synthesis of rationally designed fluorescent Ca^{2+} probes for intracellular use. Indeed, studies on calcium date back to the late nineteenth century, underscoring its significance. Reliable methods to measure intracellular Ca^{2+} concentrations first appeared in the 1960s, but it was not until the 1980s that the rapid expansion of new Ca^{2+}-measurement techniques opened the way to spatial and temporal analysis of the complex dynamics of Ca^{2+} concentrations in living cells.

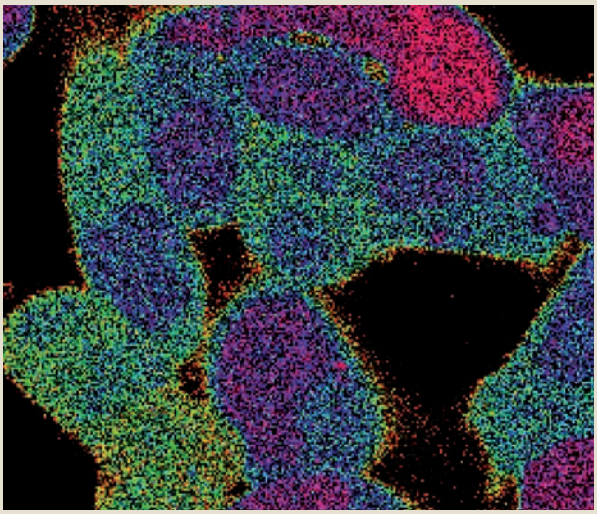
In his original paper, Tsien introduced a new generation of fluorescent polycarboxylate dyes derived from the selective Ca^{2+} chelator ethylene-glycoltetraacetic acid (EGTA). Despite their ground-breaking potential, the prototype BAPTA and its derivative Quin2 had relatively limited application owing to restrictions imposed by their absorption spectrum, low selectivity and fluorescence intensity. However, all of the polycarboxylate dyes currently available are derived from this original design, the flexibility of which enabled the synthesis of many improved dyes within a few years of the original report.

In 1985, Tsien and his colleagues introduced six new indicators, among which Fura-2 and Indo-1 were the most successful. Compared with Quin2, the new dyes showed much stronger fluorescence, higher wavelength shift on Ca^{2+} binding, weaker affinity for Ca^{2+} and better selectivity against other ions. Soon after, high-resolution digital imaging of single smooth muscle cells labelled with Fura-2 allowed Tsien, Fredric Fay and their co-workers to perform unprecedented spatiotemporal measurements of Ca^{2+} gradients in subcellular compartments, revealing the differential regulation of Ca^{2+} concentrations in the cytoplasm, nucleus and sarcoplasmic reticulum of these contracting cells.

Critical to the widespread popularity of the polycarboxylate Ca^{2+} indicators was the synthesis of their acetoxymethyl esters, which are membrane permeable and allow non-disruptive loading and efficient trapping of the dyes in intact cells once the esterifying groups are hydrolysed (Tsien, 1981). One disadvantage of the Fura-2 family of dyes was that they required excitation at ultraviolet wavelengths, which limited their range of potential applications. In 1989, Tsien and colleagues further developed the visible-wavelength indicators Rhod-2, Fluo-2 and Fluo-3, which are among the most widely used non-ratiometric Ca^{2+} indicators so far, thanks in part to their general applicability to many cell types, including neurons.

Besides their crucial role in dogma-changing discoveries, synthetic Ca^{2+} indicators have driven technological advances such as fast cameras and automated filter wheels, and their uses have been further expanded by the concomitant development of imaging technologies such as fluorescence lifetime imaging and multiphoton microscopy. Today, fluorescent calcium dyes remain key to studies that combine imaging with electrophysiological analyses.

Silvia Grisendi, Associate Editor,
Nature Cell Biology



Carbachol-evoked Ca^{2+} signals in human embryonic kidney cells recorded using the fluorescent indicator Fluo-4. Warmer colours denote a higher Ca^{2+} concentration. Image courtesy of Z. Ding and C. W. Taylor, University of Cambridge, UK.

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> "… Ca^{2+} measurement techniques opened the way to spatial and temporal analysis …"

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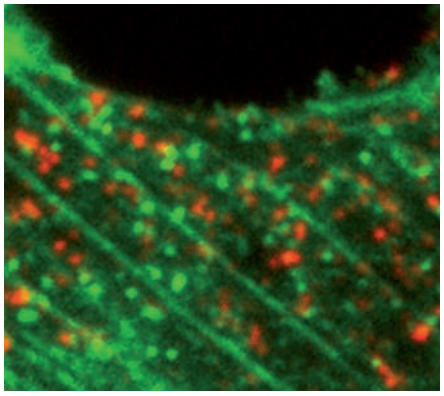
MILESTONE 13

TIRF: imaging at the cellular edge

If you want to visualize what happens at a biological interface or on the surface of a cell, it is vital to eliminate the background that comprises all the stuff that sits beyond that first layer. This is exactly the approach used in total internal reflection fluorescence microscopy (TIRF). Building on studies from the late 1800s on the detection of scattered photons from TIR excitation, Daniel Axelrod built a microscope in which a laser beam hits, at an angle, a glass slide — on the other side of which the sample is placed. The incident angle is greater than the critical angle, and therefore the beam is completely reflected back into the slide. At the same time, a tiny part of the beam called an evanescent wave manages to penetrate a few hundred nanometres into the sample and can stimulate fluorescence in a biological medium that is tagged with chromophores.



Clathrin-coated pits (red), actin stress fibres (parallel green lines) and local foci of actin polymerization (green dots) near the plasma membrane of a fibroblast. Image courtesy of W. Almers, Vollum Institute, Portland, Oregon, USA.

The evanescent wave penetrates the sample only for some 200 nm and its intensity decreases exponentially, thereby producing exquisite z-axis sensitivity. Restricting illumination to a thin section at the interface of the sample and the glass slide eliminates background fluorescence from deeper in the sample and allows longer imaging periods by limiting bleaching of chromophores that can move into or out of the illuminated layer during imaging. In 1981, Axelrod demonstrated the imaging capabilities of the technique by visualizing cellular focal adhesions on a glass slide.

In the mid-1990s, Yanagida and colleagues took the thin illumination layer provided by TIRF to its logical consequence and performed single-molecule detection of biological events in aqueous solutions and in real time. They reported individual ATP turnover events by a single myosin molecule in 1995. This setup predated confocal microscopy by several years, and to a large extent it is much simpler and less expensive because it can be easily fitted on to conventional inverted fluorescence microscopes.

In 2000, Almers and colleagues used the high z-sensitivity of TIRF to image the process of exocytosis of synaptic vesicles, and visualized the vesicles approaching the plasma membrane and unloading their contents. In the same year, Axelrod and colleagues similarly imaged the fusion of post-Golgi carriers with the plasma membrane. These studies are also early examples of through-the-objective TIRF, which, through the use of high numerical aperture objectives, further simplifies the setup and sample handling. Given the number of important events that take place at the cellular membrane, including endocytosis, exocytosis, receptor activation and ion transport, having a technique that can access this layer has set the stage for what can be done with TIRF at present.

Stefano Tonzani, Associate Editor, Nature

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MILESTONE 14

Imaging without the blur

The distribution and structure of molecules within the cell are central to their functions. By enabling high-resolution three-dimensional (3D) imaging of the cell's interior, the application of deconvolution algorithms to microscopy has made a vital contribution to our understanding of cell biology.

By the 1980s, fluorescent probes for many specific cellular molecules were available. However, visualizing their distribution in relatively thick specimens was tricky. Researchers hoped to reconstruct 3D images from stacks of 'optical sections' — images captured at consecutive focal planes through a thick object. However, interpreting the information in any given focal plane was complicated by the presence of out-of-focus fluorescence from neighbouring planes.

In 1983, Agard and Sedat brought deconvolution — a method to eliminate out-of-focus fluorescence or restore it to its original source — to the attention of cell biologists. Deconvolution algorithms use knowledge of the microscope's optical properties to model the process by which imaging distorts the 'real' object to produce the raw image. In several rounds of iterative computation, estimates of the object are entered into the equation and a comparison of the calculated outcome to the raw image is used to improve the estimate, until the object's true properties are determined. In this way, Agard and Sedat obtained the first high-resolution images of the structure and organization of ▶

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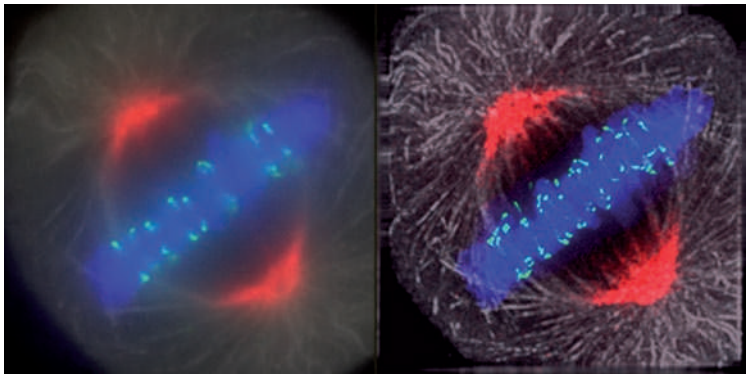

A HeLa cell in metaphase, before (left) or after (right) deconvolution. Staining shows DNA (blue), microtubules (white), centrosomes (green) and the centrosomal protein ASPM (red). Images courtesy of A. Ferrand, University of Dundee, Dundee, UK.

► fluorescently labelled chromatin in the nuclei of *Drosophila melanogaster* salivary glands.

Numerous deconvolution algorithms exist (see Further Reading); however, another key advance was made in 1989, when Fay and colleagues were examining the molecular basis of force generation in smooth muscles. Basic deconvolution algorithms can amplify noise in the images, making it difficult to narrow the range of possible estimates to determine the real object. To circumvent this problem, the authors introduced constraints to the object estimates: they eliminated estimates in which the object was not 'regular' (or smooth) or in which fluorescence levels were less than zero, a physically impossible situation. This yielded high-resolution images that enabled the quantitative analysis of α-actinin distribution in smooth muscle cells.

Initially, deconvolution was limited to those researchers who had access to the large computing facilities required to perform the intensive calculations. However, as desktop computers became more powerful, cell biologists quickly realized the advantages of deconvolution, particularly for long-term, live-cell imaging in which reduced light intensities are important and confocal microscopy techniques (see MILESTONE 9) are limited. The contribution of deconvolution to the visualization of organelles and cellular macromolecules has thus been central to our current understanding of many cellular processes.

Katherine Whalley, Senior Editor,
Nature Reviews Neuroscience

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> These papers … demonstrated the high level of 3D macromolecular structure present in cells and the structural complexity of the cell interior.
> *Jason Swedlow*

MILESTONE 15

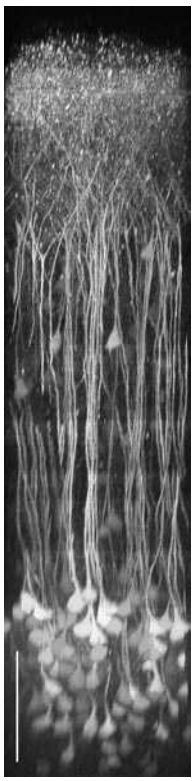
It takes two photons to tango

A microscopist's dream is to be able to visualize phenomena occurring in cells deep inside tissues with minimum photodamage to the live samples. The achievement of this goal is hampered by the presence of out-of-focus flare, which blurs images obtained with conventional epifluorescence microscopes. In confocal laser scanning microscopy (see MILESTONE 9), out-of-focus background is eliminated by a pinhole, but the large excitation volume means that light scattering limits the extent to which this background can be reduced. In two-photon microscopy, excitation is confined to the focal volume, so fluorescence detection is less sensitive to light-scattering issues.

> … provides clear imaging at a ten times greater depth than a normal confocal microscope.

Two-photon absorption is an old concept in quantum optics and was first described by Maria Göppert-Mayer in her doctoral dissertation in 1931. Thirty years later, Kaiser and Garrett observed the phenomenon in crystals.

Two-photon excitation results from the almost simultaneous absorption of two photons by a molecule. The wavelength of the excitation light needs to be about twice that required for single-photon excitation because photon energy is inversely proportional ►



Maximum-intensity side projection of a two-photon image stack obtained in a transgenic mouse expressing Clomeleon, a genetically encoded chloride indicator, preferentially in deep layer 5 pyramidal cells. Data were taken with a 40x, NA 0.8 water immersion objective. Scale bar, 100 µm. Image courtesy of F. Helmchen and T. Kuner, University of Zurich, Switzerland.

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► to the wavelength. In two-photon microscopy, a laser beam is focused on a sample so as to increase the probability of the fluorophore becoming excited, and the laser focus is scanned across a field of view to create an image. This only became feasible in 1990, when mode-locked lasers became available that could deliver photon densities a million times higher than those of conventional lasers, in ultrashort pulses with typical durations in the femtosecond range.

Denk, Strickler and Webb were the first to use this method to image chromosomes in live cultured cells. But Denk *et al.* soon adapted it to image neurons *in situ*, and the full potential of the technique became apparent when Svoboda *et al.* used it in 1997 to measure sensory-stimulus-induced dendritic calcium dynamics in pyramidal neurons in intact rat brain.

By confining excitation to the focal volume, two-photon laser scanning fluorescence microscopy allows the correct assignment of photons to the spatial position from which they originated. The use of long excitation wavelengths and the reduced sensitivity to scattering associated with excitation confinement provides clear imaging at a ten times greater depth than a normal confocal microscope. In addition, two-photon excitation greatly diminishes photobleaching and photodamage, making it ideal for imaging living cells buried deep inside intact embryos or organs.

Nathalie Le Bot, Senior Editor,
Nature Cell Biology

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MILESTONE 16

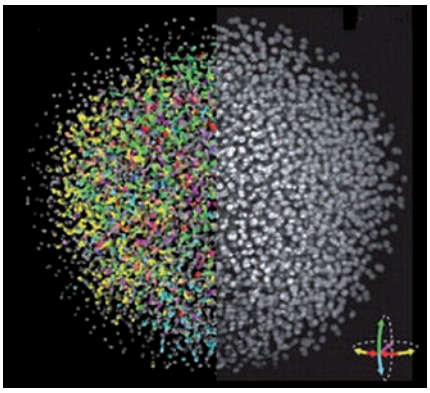
Seeing the light, perpendicularly

Despite tremendous advances in light microscopy, visualizing large biological structures in three dimensions (3D) has been problematic. Images from thin histological sections do not convey enough spatial information, and sectioning cannot be used with live specimens. Furthermore, non-destructive, optical sectioning (for example, by confocal microscopy) offers a limited imaging depth for large samples, and the axial ('z') resolution is inherently much lower than the lateral resolution. A simple solution might overcome these issues: illuminating the specimen at a 90° angle with a light sheet.

The idea of illuminating samples from the side with a light sheet was first described in 1903, but was not explored until Voie *et al.* pioneered the same principle in 1993 to develop orthogonal-plane fluorescence optical sectioning (ORFOS). In this approach, the specimen is optically sectioned by a thin light sheet that is produced by a cylindrical macroscopic lens. The light sheet is projected on to one plane of a transparent, fluorescently labelled specimen, perpendicularly to the detection axis. Because only one plane of the specimen is illuminated, no light is emitted from regions outside the focal plane and, therefore, there are no out-of-focus components in the image. By rotating their hamster cochlea specimen, Voie *et al.* collected serial images at different angles and reconstructed a 3D representation of the cochlea, showing the fine internal structures in great detail.

Although this method offered enormous potential for visualizing large specimens in detail, it was not developed commercially and was used only by a few research groups — most notably Fuchs *et al.*, who in 2002 developed a light sheet system using a microscope lens to visualize aquatic bacteria in their natural setting. In 2004, however, Stelzer and colleagues developed a light sheet microscopy application with even broader appeal, which could be used to image both fixed and live embryos. Termed selective plane illumination microscopy (SPIM), the technique offers 3D images at high resolution with impressive penetration depth of live embryos. Furthermore, phototoxicity over time is reduced because the thin sheet of light illuminates only one plane of the embryo. This helped to overcome a major challenge: visualizing embryogenesis over long periods of time. Indeed, in 2008 Stelzer and co-workers reported an improved version of SPIM that could image the development of zebrafish embryos over 24 hours and track every cell nucleus, thereby obtaining a complete map of cell division and cell migration patterns.

> "… the technique offers 3D images at high resolution with impressive penetration depth of live embryos."



Images of embryo development obtained with digital scanned laser light sheet fluorescence microscopy (DSLM), an improved version of selective plane illumination microscopy (SPIM), both of which were developed by the Stelzer laboratory. The digital embryo (left half) shows patterns of cell migration in different directions: dorsal migration (cyan), ventral migration (green), migration towards the body axis (red) or away from it (yellow), and migration towards the yolk (pink). Image is reproduced, with permission, from P. J. Keller *et al.* © (2008) American Association for the Advancement of Science.

With the Stelzer team now collaborating with microscope manufacturer Carl Zeiss to improve this method and develop a commercially available product, light sheet microscopy will no doubt further our understanding of biological processes in 3D.

Rachel David, Assistant Editor, Nature Reviews Molecular Cell Biology and Nature Reviews Microbiology

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MILESTONE 17

Single molecules in the dark

The potential to visualize bright fluorescent molecules on a dark background using fluorescence microscopy lit up the field by providing molecular specificity and image contrast unparalleled by other dyes. Theory suggested that these properties should permit the detection and imaging of single molecules. A first glimpse of the possibilities came in 1976, when Thomas Hirshfeld used a fluorescence microscope to detect single molecules bound by dozens of fluorophores as they passed through a thin layer of illumination. But technical limitations hindered progress. Although the next two decades witnessed the detection of single fluorophores at low temperatures, or while transiting a highly focused laser beam (see MILESTONE 10), such methods were incapable of repeatedly imaging identified molecules in ambient environments.

In 1993, this situation changed markedly when Eric Betzig and Robert Chichester reported the first repetitive imaging of single fluorophores at room temperature with a new technique called near-field scanning optical microscopy (NSOM) that repeatedly scans an extremely small optical probe over a sample. This provided molecule-scale spatial localization and information on molecular orientation. The potential biological applications of single-molecule imaging captured the imaginations of microscopists and biologists alike, but because of its invasiveness and complexity NSOM proved largely unsuitable for complex biological samples.

Although a number of people continued to try to improve NSOM

> … Eric Betzig and Robert Chichester reported the first repetitive imaging of single fluorophores at room temperature …

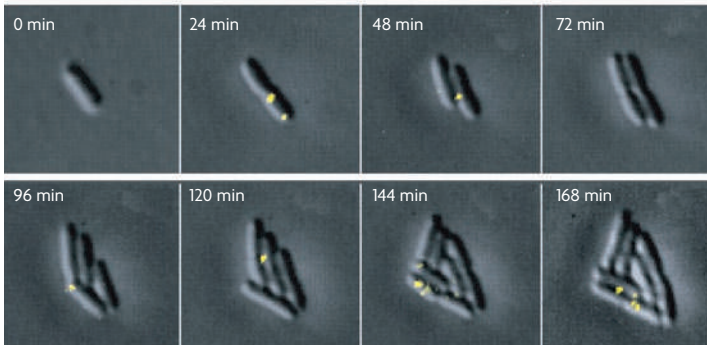
— including its use for Förster (or fluorescence) resonance energy transfer (FRET) as a molecular ruler for single molecules — others turned to alternatives. In 1995, Toshio Yanagida and colleagues used an optimized implementation of total internal reflection fluorescence microscopy (TIRF) to image single myosin molecules labelled with one fluorophore and to detect the turnover of ATP labelled with a different fluorophore. Unlike previous methods using immobilized molecules, this work showed that TIRF could image many molecules in aqueous solution for periods of several seconds before bleaching occurred, and demonstrated the suitability of this method for true biological applications.

Although TIRF would come to dominate most single-molecule imaging applications, it was not the first technique to image the movement of single fluorophores. This first was claimed by conventional epifluorescence microscopy (see MILESTONE 4) with a highly sensitive charge-coupled device (CCD) camera. Hansgeorg Schindler and his co-workers adapted the method of Jeff Gelles *et al.* for single-particle tracking based on Gaussian-peak fitting to determine the positions of individual fluorophore-labelled lipids in a membrane with an accuracy of about 15 nanometres and to track their movements over time.

Up until this point, single-molecule fluorescence microscopy applications had an almost proof-of-principle feel to them, and for the most part novel biological insights were limited. But in 1998, Sunney Xie and colleagues used the intrinsic on–off fluorescence switching of flavin adenine dinucleotide in the active site of cholesterol oxidase to reveal that enzyme activity is influenced by a form of molecular memory residing in conformational changes in the protein. This behaviour was completely unanticipated from ensemble experiments and showed unequivocally that single-molecule microscopy could shed new light on seemingly well-characterized systems. Similar enzymatic-turnover assays developed by other groups eventually led to the sequencing of individual DNA molecules.

Methods continued to be developed and refined as highlighted by a number of groundbreaking studies on ribozymes, molecular motors and gene expression in single living cells as scientists pushed the limits of technology and imagination.

Daniel Evanko, Editor, Nature Methods



Time-lapse images of *Escherichia coli* cells showing single-molecule imaging of the expression of single fluorescent fusion proteins. The fluorescent molecules are photobleached in the intervals between each image. Image is reproduced, with permission, from J. Yu *et al.* © (2006) American Association for the Advancement of Science.

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MILESTONE 18

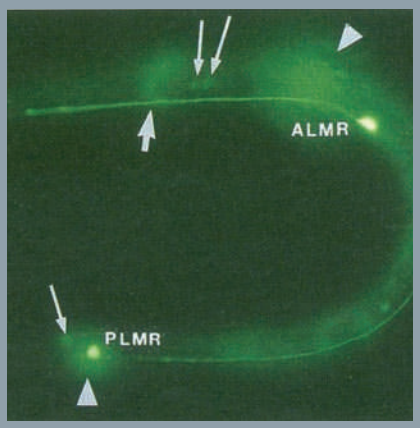
GFP: the green revolution

In 1994, Chalfie *et al.* published a report in *Science* showing that the green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* could be used as a marker for protein localization and expression in living bacteria and worm cells, in the absence of any auxiliary factors from *A. victoria*. This demonstration of GFP as a tool to study proteins *in vivo* fundamentally altered the nature and scope of the issues that could be addressed by cell biologists.

GFP was first discovered fortuitously in 1962 by Shimomura and colleagues during the purification of the bioluminescent protein aequorin from *A. victoria*. Subsequent purification, crystallization and reconstitution of energy transfer *in vitro* from aequorin to GFP by Morise and colleagues in 1974 provided insight into the fluorescent properties of GFP, which was shown to emit green light on energy transfer from aequorin.

Whether GFP would need aequorin and possibly other factors from the jellyfish to fluoresce in heterologous systems remained an open question for many years. In 1992, 30 years after its discovery, Prasher *et al.* cloned the gene encoding GFP, paving the way for experiments to assess its utility as an *in vivo* tag for proteins. Two years later, Chalfie *et al.* showed that GFP could fluoresce when expressed in bacteria and worm cells. In the worm, GFP was expressed from the promoter of a gene that encoded β-tubulin. Its spatial and temporal expression in specific neurons of the worm mimicked that of the endogenous β-tubulin gene, thus proving that GFP could be a faithful marker for monitoring gene-expression patterns. Soon thereafter, Roger Tsien's laboratory engineered native GFP to become brighter, more photostable and excitable at a wavelength that matched that of conventional microscope filter sets, increasing its practical usability (as summarized in his 1998 review). The next breakthrough in GFP technology came with the development of GFP variants to produce blue, cyan and yellow fluorescent proteins (also described in Tsien's review), thus enabling imaging experiments that could follow multiple tagged proteins in cells and organisms.

Almost half a century after GFP was discovered, the 2008 Nobel Prize in Chemistry was awarded to Osamu Shimomura, Martin Chalfie and Roger Tsien "for the discovery and development of the green fluorescent protein, GFP", acknowledging the seminal nature of this discovery.



Cell bodies of two touch-receptor neurons from the worm *Caenorhabditis elegans* are labelled with green fluorescent protein expressed from the gene encoding β-tubulin. Image is reproduced, with permission, from M. Chalfie *et al.* © (1994) American Association for the Advancement of Science.

Sowmya Swaminathan, Senior Editor,
Nature Cell Biology

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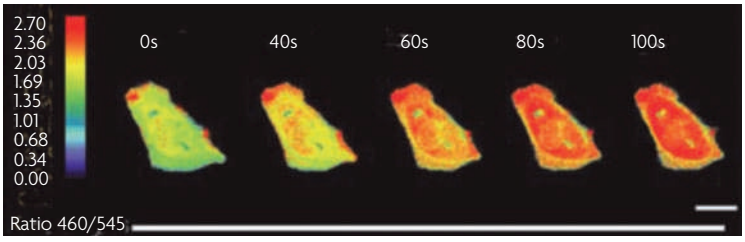
MILESTONE 19

Sensing second messengers

> These two Ca^{2+} biosensors were the first genetically encoded molecular sensors.

The discovery of green fluorescent protein (GFP) as a probe for visualizing proteins *in vivo* (see MILESTONE 18) laid the foundation for even more sophisticated microscopy techniques. GFP mutants with shifted excitation or emission wavelengths that emit in blue, cyan, green and yellow can serve as donors and acceptors for Förster (or fluorescence) resonance energy transfer (FRET) — energy transfer between two fluorophores that depends on their proximity and orientation, and allows the acceptor fluorophore to emit at a longer wavelength. In 1997, two groups used FRET to engineer biosensors that measure changes in Ca^{2+} concentrations in living cells.

Ca^{2+} binds to calmodulin (CaM), changing its conformation and allowing it to bind to target proteins. Miyawaki *et al.* made a construct encoding blue GFP, CaM, the CaM-binding peptide of myosin light-chain kinase (M13) and green GFP. On binding by Ca^{2+}, CaM wraps around M13, decreasing the distance between the GFP proteins and allowing FRET. In bacteria, the presence of Ca^{2+} was visualized as a change in emission on violet excitation from 445 nm to 510 nm, and ▶



Förster (or fluorescence) resonance energy transfer (FRET) between subunits of protein kinase A (PKA) tagged with green fluorescent protein is disrupted after cyclic AMP stimulation (time in seconds), which causes PKA subunits to dissociate. Image is reproduced, with permission, from M. Zaccolo *et al.* © (2000) Macmillan Publishers Ltd. All rights reserved.

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this biosensor reported Ca^{2+} concentrations from 10^{-7} to 10^{-4} M. Independently, and in parallel, Romoser *et al.* linked blue- and green-emitting GFPs through M13 to detect Ca^{2+}. In the absence of Ca^{2+}, excitation of blue GFP causes the green acceptor to fluoresce at 505 nm as a result of FRET. Binding of Ca^{2+} and CaM to M13 increases the inter-fluorophore distance, eliminates FRET and causes donor emission at 448 nm. Fluorescence responses to Ca^{2+} in cells were enhanced by co-expressing CaM with the biosensor. These two Ca^{2+} biosensors were the first genetically encoded molecular sensors.

In 2001, Nakai *et al.* developed a Ca^{2+} biosensor with a higher signal-to-noise ratio that used just one GFP protein. They positioned a GFP variant between M13 and CaM so that Ca^{2+}–CaM binding induced a conformational change in GFP that increased the intensity of its fluorescence. Fluorescence output from this high-affinity Ca^{2+} probe was enhanced when Ca^{2+} concentrations were increased in myotubes.

Another advance was made in 2000 when, using a biosensor for cyclic AMP, Zaccolo *et al.* showed that FRET can measure protein–protein interactions. The authors linked green GFP and blue GFP to catalytic and regulatory subunits of protein kinase A (PKA), respectively, and co-expressed these constructs in cells. Excitation at 380 nm resulted in FRET, which suggested that these subunits interacted *in vivo*. Physiological stimulation of cAMP induced a loss of FRET consistent with its ability to dissociate PKA subunits. Thus, this biosensor detects physiological changes in cAMP level and validates FRET as a tool for studying protein–protein interactions. Today, fluorescent biosensors allow protein activity, protein interactions and small-molecule signals to be sensed and quantified with high spatial and temporal resolution.

Katharine H. Wrighton, Associate Editor,
Nature Reviews Molecular Cell Biology

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MILESTONE 20

Beyond GFP: DsRed and PA-GFP

> …the next day the sample had turned red.

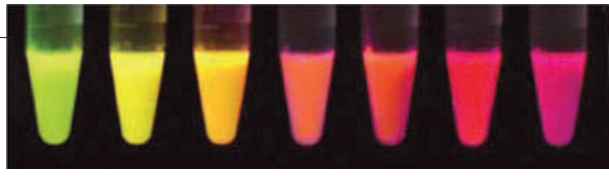
Following the isolation of green fluorescent protein (GFP) from the luminescent jellyfish *Aequorea victoria* in 1962 and its first application as a fluorescent probe in 1994 (see MILESTONE 18), the race was on to expand the colour palette of fluorescent proteins and to produce new probes suitable for studying dynamic cellular processes.

Blue, cyan and yellow fluorescent proteins were quickly obtained by modifying GFP, but variants emitting in the orange and red spectra (which would allow multicolour imaging experiments and be less phototoxic than other colours owing to their longer excitation wavelengths) remained elusive. A breakthrough occurred in 1999, when Matz *et al.* proposed that the fluorescent property of GPF-like proteins might not necessarily be linked to bioluminescence and searched for them in non-bioluminescent Anthozoa species — reef corals that exhibit bright fluorescent colours. This led to the cloning of the first red fluorescent protein (known as DsRed, for *Discosoma* sp. red). Injection of mRNAs encoding DsRed or a green variant into blastomeres of *Xenopus laevis* embryos revealed regions that were marked with individual proteins (red or green) or both proteins (yellow) at the tadpole stage. This represented the first double-labelling experiment.

Three years later, Patterson *et al.* generated the first photoactivatable GFP (PA-GFP), which turned out to be a crucial advance for super-resolution fluorescence microscopy approaches. The PA-GFP variant contains a Thr-to-His mutation that makes it devoid of green fluorescence until it is activated by violet light (390–415 nm). This leads to a 100-fold increase in green fluorescence (with an emission peak at 504 nm), which is ideal for rapidly marking a selected population of molecules within cells and following their kinetics over time. Indeed, Patterson *et al.* demonstrated the value of PA-GFP by tagging a lysosomal membrane protein with it and showing that it moved between lysosomes in a microtubule-dependent manner.

Later that year, Ando *et al.* cloned a gene encoding a bright GFP from the coral *Trachyphylla geoffroy* and accidentally left an aliquot of the expressed protein on the bench: the next day the sample had turned red. The fluorescent protein, named Kaede (Japanese for maple leaf), changed to red when irradiated with ultraviolet (350–400 nm) or violet light. Using a focused ultraviolet pulse, the authors highlighted a single neuron in red among many green ones expressing Kaede, thereby showing the protein's potential use as an optical marker.

Despite these advances, the available fluorescent proteins emitting in the orange and red regions were not optimal for multicolour live imaging (in terms of



Purified fluorescent proteins are shown in fluorescence (from left to right, mHoneydew, mBanana, mOrange, tdTomato, mTangerine, mStrawberry and mCherry). Image is reproduced, with permission, from N. C. Shaner *et al.* © (2004) Macmillan Publishers Ltd. All rights reserved.

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brightness and photostability, for example). However, the use of directed protein evolution by Shaner *et al.* generated red, orange and yellow fluorescent proteins with improved properties. These proteins, which were named after fruits that bear colours similar to the emission profiles (for example, mStrawberry, mCherry and mBanana), are monomeric, tolerant to amino-terminal fusions, have faster maturation rates and are brighter than previously identified red-shifted monomeric fluorescent proteins. The availability of fluorescent proteins in many colours, and the ability of some to be photoactivated (PA-GFP) or photoconverted (Kaede), has revolutionized live-cell imaging.

Francesca Cesari, Associate Editor, Nature Reviews Molecular Cell Biology

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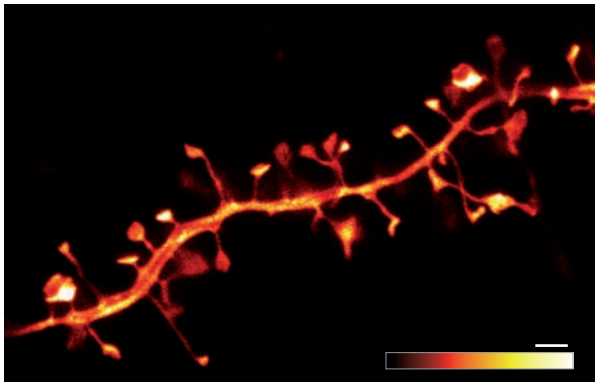
MILESTONE 21

Light microscopy at the limit



Stimulated-emission-depletion image recorded from a living neuron of an organotypical hippocampal slice, showing dendritic spines in unprecedented detail. The neuron is expressing yellow fluorescent protein. Image courtesy of K. Willig, V. Nägerl, N. Urban, T. Bonhoeffer & S. W. Hell, MPI Biophysical Chemistry, Göttingen, Germany

> It became necessary to 'break' the diffraction barrier.

For most of the twentieth century, it was held that far-field light microscopy could not resolve objects closer than 150–200 nanometres (see MILESTONE 3). This is because light diffracts as it passes through different media, so that the light emanating from a point is detected as emerging from a larger volume. Light microscopy was therefore said to be 'diffraction-limited'.

Yet many cellular processes take place within these diffraction-limited distances, at length scales of tens to hundreds of nanometres (nm). To be able to visualize these processes, therefore, microscopy with substantially improved resolution was needed. It became necessary to 'break' the diffraction barrier.

After early work that achieved the maximum theoretical resolution — including the standing-wave illumination reported by Fred Lanni and Jans Taylor and the two-photon 4Pi microscope of Stefan Hell and Ernst Stelzer — Hell showed, first theoretically and then experimentally, that a stimulated-emission-depletion (STED) microscope can break the diffraction barrier. To do this, the excitation focal spot is shrunk to a very small size by depleting the fluorophores at its rim through stimulated emission with a doughnut-shaped STED beam of red-shifted light. The tiny spot is then scanned over the sample to generate a sub-diffraction image. In the first experimental implementation of STED, which included imaging of live yeast and bacteria, resolution was improved both axially and laterally and reached about 100 nm. The resolution has since been improved further, allowing the technique to be used to show that the synaptic vesicle-associated protein synaptotagmin remains clustered on the neuronal plasma membrane after exocytosis.

While STED was being demonstrated, Mats Gustafsson was developing an approach called structured illumination microscopy (SIM). In SIM, the excitation light is structured in a controlled pattern, which renders normally unresolvable information accessible after image processing. Although still diffraction-limited, SIM improved lateral resolution by about twofold and produced clearer images of biological structures. The technique has since been extended to three dimensions using 3D structured light; moreover, it enables the diffraction barrier to be broken, using nonlinear processes.

Finally, by exploiting the known ability to localize single molecules with nanometre precision, several single-molecule approaches to achieving sub-diffraction resolution were developed. The pioneers of these methods — Eric Betzig and Harald Hess for photoactivated localization microscopy (PALM), Xiaowei Zhuang for stochastic optical reconstruction microscopy (STORM) and Samuel Hess for fluorescence photoactivation localization microscopy (FPALM) — used the combination of photoactivatable or photoswitchable fluorophores and high-accuracy single-molecule localization to break the diffraction barrier. Although the details differ, all three methods are based on a single principle: a sparse subset of fluorophores is switched on at any one time, and each molecule is localized with high accuracy. These fluorophores are then switched off and the process is repeated with another subset until enough information has been collected to generate a sub-diffraction image.

The hope for super-resolution microscopy is that visualizing cells at this unprecedented scale will yield unprecedented insights. Although the application of these methods to biological questions has begun, the full realization of their potential is still to come. Their extension to imaging in all three dimensions and within living cells — capabilities that are developing rapidly — will be a crucial part of this process.

Natalie de Souza, Associate Editor, Nature Methods

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