It is not clear, however, whether and to what extent EU member states will fund pan-European infrastructures: the UK, Finland and Poland, among others, have earmarked some money for the establishment of ELIXIR—the infrastructure for biological information—and other projects, but this is far from what is needed and does not address the problem of long-term operation and maintenance, particularly in these difficult economic times. Moreover, coordinating support for the 13 projects recommended by the ESFRI remains a major challenge. “It is unprecedented to coordinate all these activities across 27 countries,” Baines remarked.

The main problem is that most EU member states alone cannot fund and support even a medium-sized research infrastructure

Mere coordination by the EC to organize support from individual member states might, therefore, not be enough. Bernd Pulverer, head of publications for EMBO, who moderated the hearing, enquired whether a European agency similar to the European Research Council (ERC), which funds basic research, would be a solution to the problem of guaranteeing long-term stability. Pero agreed that an agency that identifies needs and funds the establishment, maintenance and operation of pan-European infrastructures would be a viable solution, but he was not optimistic. “It would be the way forward to create a body at the EU level to coordinate funds and actions. Unfortunately, the time is not right,” he said. Given the economic crisis, various member states are not keen to contribute more money to the EU. Moreover, the ERC has not existed for long enough to convince the EP and ministers that additional funding for another agency for research would benefit the whole EU. Nevertheless, the EC is aware of the problem of long-term financial support, and has therefore included research infrastructures in its proposal for the next research framework.

Some MEPs at the hearing share the concerns of scientists about the viability of long-term funding. Vittorio Prodi expressed concern over nationalistic reflexes that would be an impediment to international research. Instead, he said the EU should focus on the added value of pan-European

research infrastructures and their potential for development. Even so, economic and other factors may well force the EU to take a more proactive role. “In times of global challenges, the best answer for the EU is to pull together and not go for nationalistic solutions,” Prodi said, “[and to] give the EU directly the resources that are needed.”

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New optics sheds light on biology

Advances in microscopy, genetics and biochemistry are together increasingly enabling scientists to watch molecules in action

Philip Hunter

Advances in light microscopy are about to transform molecular biology by enabling biologists to observe molecular processes *in vivo*. Although biochemical analysis or genomic sequencing, along with imaging techniques such as X-ray diffraction and electron microscopy, can provide detailed information about cellular structures and the products of particular processes, it is light microscopy that allows scientists to capture dynamic events in the cell. Basic research is not the only enterprise that benefits from improvements in microscopy; new optical techniques also have an increasing role in diagnosis and surgery—in particular for some cancers—to analyse tissue in real time.

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For decades, light microscopy was regarded as the poor relative of electron microscopy and X-ray crystallography, both of which enable much greater resolutions, down to atomic scales around 0.1 nm. Yet both techniques come at the expense of the object because they can only analyse fixed samples—crystals in the case of

X-ray crystallography and thin sections for electron microscopy. This has always been the advantage of light microscopy: to capture events in living systems. Now, various developments over the past two decades have come together to introduce real-time imaging. “Revolutionary improvements in laser light sources, detectors, or cameras, and the advent of computerized digital light and fluorescence microscopy have been essential for enabling real-time imaging and micro-endoscopy in living systems,” said Frank Chuang, associate director of research and education at the Centre for Biophotonics and Research at the University of California, Davis in Sacramento, USA.

The first major step for real-time light microscopy was the development of fluorescence methods for labelling proteins *in vivo*. These methods exploit the fact that fluorescent compounds absorb electromagnetic radiation at one wavelength and emit light at a different wavelength. A sample can be illuminated by electromagnetic radiation at the prescribed wavelength, and only the fluorescent components of the sample, as well as areas very close to them, are observed. Using fluorophores as markers for specific

proteins, scientists can obtain functional information as this allows them to track their movement.

The opening move was the discovery of the green fluorescence protein (GFP) in the jellyfish *Aequorea victoria* in the late 1970s (Tsien, 1998). GFP is composed of 238 amino-acid residues and gives off bright green fluorescence on exposure to blue light. Since its discovery, GFP has been further improved by protein design and has been applied to studies of proteins that are marked with GFP in bacteria, yeast and fish, as well as plant and mammalian cells. For these discoveries, Martin Chalfie, Osamu Shimomura and Roger Tsien received the Nobel Prize in Chemistry in 2008.

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GFP can be spliced into the genome next to the gene of the protein of interest, which is then expressed as a fluorescent fusion protein. GFP can also be introduced into target cells to analyse, for example, brain circuitry or the entry of viruses into cells during infection. "That was definitely a turning point [...] and people can now capture not only stills but also dynamic scenarios of these proteins, to observe how they are moving, and where they are localized in living samples ranging from bacteria to entire living animals," commented Michael Knop, professor of molecular biology at Heidelberg University, Germany, who applies live-cell microscopic imaging to studying protein function in meiosis.

One problem though was that in traditional so-called wide-field light microscopy—in which the light source illuminates the whole sample—background light interferes with the area of interest. This was addressed by the confocal microscope, which eliminates the background by shining light through a pinhole so that it can be focused on a small target zone. This improves optical resolution immensely, but at the expense of signal intensity, because most of the light is blocked at the pinhole. Images have to be exposed for longer periods or at increased intensity, which causes greater photo damage to the object.

This problem was eventually addressed by another important advance for light

microscopy, the development of fast, ultra-sensitive charged-coupled-device (CCD) chips for photodetection. "This really started 10–12 years ago when a new generation of CCDs allowed the construction of cameras that could be used for very low light detection," said Knop. CCD cameras help to reduce photo damage to samples and capture images more quickly, which increases the scope for making time-lapsed movies.

These improvements led the way for other developments, notably the light-sheet microscope in 2003. It was pioneered at the European Molecular Biology Laboratory in Heidelberg, Germany, by a group headed by Ernst Stelzer, now Professor in Advanced Light Microscopy at the University of Frankfurt, Germany. The microscope works on the basis of illuminating the specimen from the side rather than from above; a sheet of light exposes only the narrow slice on which the microscope is focused at the time. Light-sheet microscopy involves two independently operated lenses for illumination and detection. By focusing the light only on the narrow optical section within the focal plane of observation, it further reduces photo damage or photo-bleaching.

The other goal of light-microscopy development is improving resolution, which is needed to observe individual proteins and detail at the molecular level

Light-sheet microscopy has since been combined with other techniques for improving contrast and manipulating specimens to create even better three-dimensional images. It enabled the dramatic *in vivo* recording of embryonic development in a zebrafish, from the early 32-cell stage to the neurulation period, when cells start to differentiate to form organs. Individual cells were visualized with 60–90 seconds between samples, and four-megapixel image frames were recorded at a rate of five per second. This recording speed was important because cell movement could then be tracked with sufficient accuracy to study migration. The work showed that light-sheet microscopy exposed the embryo to 200 times less energy than a conventional microscope and 5,000–6,000 times less than a confocal fluorescence microscope (Keller *et al*, 2008).

The other major goal of light-microscopy development is improving resolution, which is needed to observe individual proteins and detail at the molecular level. It requires overcoming the diffraction-limit rule, which states that the resolution cannot be better than half the wavelength of light, around 200 nm, which is insufficient even to observe organelles within cells. This limit occurs because the wavelength defines the minimum spot size that can be resolved from a single ray of light. Any smaller details will be blurred by interference from light emitted from neighbouring points in the sample.

Even when a sample is being observed *in vivo*, the preparation—involving excitation with light—is not normal

However, ways have been found to extract information from samples at higher resolution. One such technique is called STED (stimulated emission depletion) fluorescence microscopy, described in 1994 by Stefan Hell at the Max Planck Institute for Biophysical Chemistry in Göttingen, Germany (Hell & Wichmann, 1994). STED is based on the idea of blocking light that is emitted from around the point of interest, to prevent interference. It combines the normal pulse of light that stimulates fluorescence with a second pulse tuned to the frequency of the excited radiation, which cancels it out. This second pulse is directed close to the focal point from a doughnut-shaped emitter. By transmitting the second cancelling pulse from the rim of the doughnut only, the light is focused onto a ring around the focal point so that only a small area in the centre receives the pulse that generates fluorescence. This creates a coherent spot of emitted light corresponding to the molecules in the centre of the ring only, which yields a sharp image at a resolution beyond the diffraction limit. Furthermore, by increasing the intensity of the cancelling light that is emitted from the rim of the doughnut, the diameter of the coherent spot in the middle can be reduced to further increase resolution, in principle, down to the size of a single atom. In practice, one of the highest resolutions obtained so far is 2.6 nm, which is in the range of the size of individual proteins (Rittweger *et al*, 2009).

Although STED and light-sheet microscopy—combined with other techniques and tools—promise to revolutionize cell biology by providing image sequences at higher resolutions and shorter timescales, they also bring new challenges or accentuate existing ones. The two main issues concern disturbing the system being observed and introducing visual artefacts that cannot be immediately identified, because the images are constructed, at least partly, by computers. This boils down to one fundamental problem: how to tell whether the image reflects the system being observed under normal environmental conditions. Even when a sample is being observed *in vivo*, the preparation—involving excitation with light—is not normal. “This is a major issue, because when you basically set up conditions to detect a protein of interest, you also interfere with the system,” commented Knop. “One of the typical problems in researching mammalian cells for example is that many proteins are over expressed when people label them.”

In the case of the zebrafish embryo, it might seem as though normal development has occurred, but even when a healthy animal is produced it is impossible to be sure that some details of the mechanisms involved

have not been changed by the observational process. This can be addressed by validating findings obtained by high-resolution optical microscopy in other ways. “Depending on the organism there are different options,” said Knop. “In yeast we can use genetics.”

...the increasing power and resolution of light microscopy also increases the sensitivity of the observations to small fluctuations in the environment

Furthermore, the increasing power and resolution of light microscopy also increases the sensitivity of the observations to small fluctuations in the environment. This was an issue for Peter Sudbery at the University of Sheffield in the UK, who uses a microscope called Deltavision to study the growth of the fungus *Candida*. “*Candida* only forms hyphae at 37°C, and even a small drop in temperature changes its behaviour,” he said. “Also small fluctuations in temperature of less than 0.1°C cause the image to lose focus. So while the Deltavision can be programmed to make time-lapse movies with the exposures taken automatically at preset intervals, for videos

longer than a few minutes the microscope has to be manually monitored constantly to check the focus and temperature.”

Although artefacts and environmental control are important issues at the cutting edge of research near the limits of resolution, they are fortunately less critical for medical applications. Here, resolution at the cell level is usually not necessary; the main issue lies in developing sufficiently small systems that can accurately and conveniently observe the region of interest during diagnosis and surgery.

The most prevalent application in medical imaging is for tumour detection—both during diagnosis and surgery—either to replace traditional biopsy, which is invasive and time-consuming, or to replace direct visual inspection, which can be inaccurate. One method is near-infra-red (NIR) microscopy, in which the patient ingests compounds that cause tumour cells to emit radiation in the NIR spectrum in response to excitation. This can either be used to identify specific tumours or general cancers, according to Stijn Keereweere, research fellow at the Erasmus Medical Centre in Rotterdam, the Netherlands. “Agents can target specific epitopes which differ between

various tumours, but can also be targeted against general characteristics, such as MMP [matrix metalloproteinase] activity or increased glucose uptake," said Keereweer. He added that by mounting cameras on laparoscopic probes, the technique could be extended to internal abdominal-cancer surgery, as well as for neck and head cancers when the observation can be performed by external systems.

The advantage of these optical systems is that the surgical margin required to be sure that all malignant tissue has been removed can be reduced, thereby minimizing risk and injury to the patient. One of the hurdles for this technique is regulatory approval of the tumour-specific agents that are needed to elicit the fluorescence.

However, there are emerging techniques that do not require such agents, by using conventional confocal laser microscopy without fluorescence. A study at the Garvan Institute of Medical Research in Sydney, Australia, showed that *in vivo* analysis of surface and near-surface tissue could be performed successfully in real time by using a contrast agent administered intravenously to show structural features (Nguyen *et al*, 2009).

Techniques that avoid the need for patients to ingest an agent—for contrast or to generate fluorescence—would be ideal

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as they would reduce preparation time. This can be achieved by exploiting auto-fluorescence instead of adding fluorescent compounds, and has been shown to successfully identify oesophageal cancer, according to Bevin Lin at the Department of Biomedical Engineering at the University of California Davis. "Regarding oesophageal carcinoma, current screening methods can take 20–30 minutes," he said. "There is a need for a rapid method of imaging that is less complicated and faster than topical or intravenous contrast agents. Therefore, significant advantages of auto-fluorescence microscopy include the elimination of contrast agents, preparation time, and complex instrumentation, which could directly translate to reduced cost and improved patient care." As Lin pointed out, regulatory approval from the US Food and Drug Administration is still required for the endoscopy system, but he believes that there is great potential for optical systems. "Hopefully, optical methods will become as clinically mainstream as traditional methods," he said.

There is similar expectation that real-time optical imaging will become as widely used in research once teething troubles

associated with recognition of artefacts and disturbances to the systems under observation are overcome. "Effective, affordable and usable super-resolution imaging that could be used on living, non-fixed cells with GFP and variants would transform the whole of cell biology," said Sudbery.

CONFLICT OF INTEREST

The author declares that he has no conflict of interest.

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