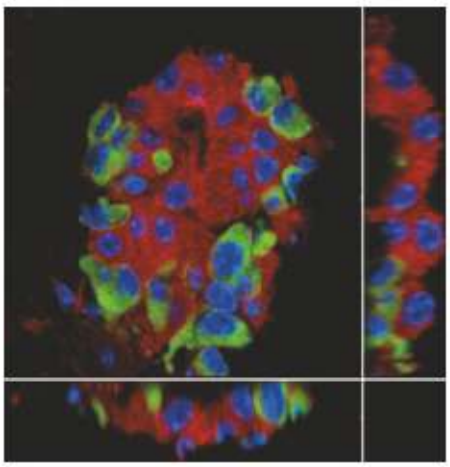
The big picture

Over the past ten years, microscopy has been transformed from slice, stain and fix, to the capacity to view living cells and even whole organisms in real time. Lisa Melton looks at what's on offer.

When it was introduced in the late 1980s, confocal laser-scanning microscopy opened up a new high-resolution world to biologists. But researchers are becoming more demanding of their microscopes. They expect not just to see a clearer image, but to monitor dynamic protein networks within cells, map the kinetics of intracellular organelles and track calcium signalling. Microscope developers have responded by producing confocal microscope systems that have dramatically improved scanning speeds, greater resolution and the capacity to see detail in live cells labelled with multiple colours.

Researchers traditionally struggled to obtain sharp images from multi-stained specimens, the main obstacle being spectral emission overlap from different fluorescent probes. Nikon's recently launched Digital Eclipse C1 Spectral Imaging confocal system collects high-resolution data from the 400–750 nanometres range with a single pass of the laser. A mathematical process unmixes closely overlapping spectral data to produce clean images with no cross-talk, even for notoriously difficult reds. "We use chromatic aberration-free objectives, so everything towards either end of the spectra focuses at the same point," says Chay Keogh, marketing manager for Nikon, UK in Kingston upon Thames. "By eliminating the need for multiple scans, specimen damage is kept to a minimum."

NIKON



Pancreatic cells imaged in the Digital Eclipse.

A rival system from Olympus, the Fluoview FV1000, is a confocal laser-scanning microscope with two independent, synchronized laser scanners in a single instrument. While one laser provides high-resolution confocal images, the second scanner, the SIM scanner, simultaneously stimulates the sample. This makes the FV1000 an ideal choice for live-cell applications such as fluorescence recovery after photobleaching (FRAP), fluorescence loss in photobleaching (FLIP), uncaging, or photoactivation and photoconversion. "It offers, for the first time, the opportunity to study the kinetics of rapid cellular reactions after laser stimulation, without a time lag, because you don't have to stop imaging when using laser light for stimulation," points out Martin Tewinkel, business manager at Olympus Life and Material Science Europa in Hamburg, Germany. "Such studies of rapid cellular responses can provide important insights into how various cellular mechanisms operate."

Another high-speed, high-resolution confocal imaging system is the Nipkow-disk-based Ultraview ERS from PerkinElmer of Boston, Massachusetts, which offers a choice of cameras for different applications: a standard interline CCD detector for the highest resolution with slower processes or bright samples that withstand higher laser intensities, or an electron-multiplying CCD detector for

AN ILLUMINATING BREAKTHROUGH

As more biologists become interested in imaging the big picture, a new microscopy technique is being developed that will enable them to look much deeper into living organisms than ever before.

Selective plane illumination microscopy (SPIM) is a timely breakthrough, according to physicist Jan Huisken, now at the University of California, San Francisco. Huisken co-developed the technology with colleagues when he was at the European Molecular Biology Laboratory in Heidelberg, Germany. "People are moving away from looking at single cells in a Petri dish to studying whole organs in an intact embryo," he says.

"In biology there has always been a limit — you couldn't image whole systems from living embryos," says Huisken. With SPIM, it is possible to completely image living specimens 2–3 millimetres in size. The process of organogenesis — the formation of organs such as eyes and brain — can be followed, or gene and protein expression patterns tracked over days. The system can also track three-dimensional cell cultures over time, and is fast enough for researchers to watch the heartbeat of a living fish. "The fish heart beats two to five times a second. You have to be fast enough to record that movement without blurring the image, and a high-speed camera in SPIM shooting 70 frames a second can achieve it," says Huisken.

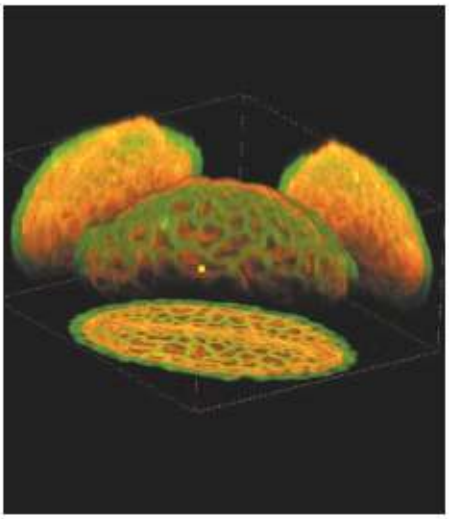
Jan Huisken: plane illumination means microscopy can now go live.

The illumination in SPIM comes from a sheet of laser light 2–8 micrometres thick, which optically sections the sample while micromotors systematically move and turn it in different directions to illuminate successive planes. The fluorescence-detection system is at right angles to the light sheet.

For developmental research, the successive layer-by-layer images are merged by an image-processing algorithm to produce three-dimensional movies of growing embryos. SPIM has higher and more isotropic resolution compared with scanning technologies and can achieve greater penetration depth. Whereas confocals can only reach 100 to 200 micrometres deep, SPIM resolves structures inside the fish embryo at 500 micrometres and deeper when more views are combined.

Another advantage is that the embryos being studied can be kept alive in a medium-filled chamber for a few days while under observation; and the chamber is ideal for adding drugs to follow their impact on the embryo.

Although Huisken says that it would be possible to build your own SPIM system, for those who don't feel up to tackling the job Carl Zeiss of Jena, Germany, will be commercializing this remarkable new technology within the next three years. L.M.



OLYMPUS

3D rendering with the Olympus Fluoview.

highly dynamic processes, very dim fluorescence, or light-sensitive samples.

For researchers who want speed but don't need the high resolution of a confocal microscope, Olympus has designed live-cell imaging systems that can be fitted to Olympus's upright BX or inverted IX series wide-field microscopes — the relatively inexpensive cell^M imaging station and the high-end station cell^R. The cell^R takes ten multicolour images per second at full resolution and can be used to track cell growth, metabolic transport and signal transduction in real time. "It's always a trade-off: the quality of the data on the one hand and speed on the other," explains Christian Seel, head of information transfer management at Olympus BioSystems in Munich. The key to speed is finely synchronized illumination and camera controls. The high-intensity coloured light is switched off the specimen immediately after taking each photo, reducing photo-damage to a minimum, says Seel. Time-lapse series are stored by the imaging software and presented as movies or charts.

Developers at Carl Zeiss believe there is no need to sacrifice resolution for ultra-fast cellular dynamics with the confocal imaging system LSM 5 LIVE. "Our main motivation was to develop an instrument dedicated to fast live-cell imaging, with a much higher speed than any other system available today while maintaining a very good confocal resolution," says Richard Ankerhold, director of advanced development at Carl Zeiss in Jena, Germany. Dynamic interactions can be viewed at different scales — a group of interacting molecules, a complete cell, a developing organ, or even an entire zebrafish embryo, notes Ankerhold. LSM 5 LIVE operates even on weakly fluorescent specimens and collects up to 120 confocal images per second at a resolution of 512 × 512 pixels, scanning about 20 times faster than a traditional confocal system.

Developmental biologist Mary Dickinson and her group at the California Institute of Technology in Pasadena have capitalized on the instrument's fast-frame recording to image erythroblasts rushing through the heart of an 8-day-old mouse embryo, and to produce a time-lapse series of the beating heart of a zebrafish embryo. This technology is expensive, however, and at present only affordable by large research institutes. Alternative optical approaches to imaging embryos for developmental research are selective plane illumination microscopy and optical projection tomography microscopy (see 'An illuminating breakthrough', page 775, and 'Optical tomography for embryos', below).

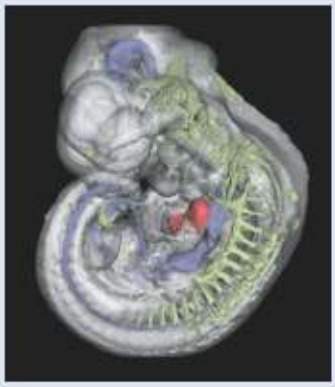
With calcium-sensitive dyes such as Fura-2 you can visualize spikes, waves and oscillations of calcium in living cells. An instrument dedicated to imaging intracellular ion kinetics — and with a price tag within the reach of most research labs — is the InCyt Imaging system from Intracellular Imaging of Cincinnati, Ohio, co-founded by Eric Gruenstein, director of the Center for Image Analysis at the University of Cincinnati.

Starting at US$30,000, it includes a fluorescence microscope, low-light-level CCD camera, filter changer and image-processing computer. "It's a very cost-effective and feature-rich solution for ion imaging and cell kinetics," says Tim Fletcher, product specialist at Image Solutions of Preston, the UK distributors for Intracellular Imaging. InCyt has been tailored to image and quantify calcium, but can measure other ions, and works with all commonly used dyes. Designed by cell biologists, the software follows the logical flow of an experiment. Images can be displayed in real time or saved and played back as an animated sequence. "The InCyt system needs very little training, and researchers pick it up quickly," says Fletcher.

OPTICAL TOMOGRAPHY FOR EMBRYOS

Developmental biologist James Sharpe became acutely aware of the drawbacks of traditional imaging methods when trying to map three-dimensional gene and protein expression patterns in the 1990s. Reconstructing images from hundreds of serial sections is not only laborious but inevitably introduces distortions. "Even in an expert lab, it takes weeks of work to reconstruct a single specimen," Sharpe points out.

To get over the problem Sharpe, who is at the MRC Human Genetics Unit in Edinburgh, UK, developed a 3D-imaging system, which he called optical projection tomography (OPT) microscopy. This approach follows the principles of the X-ray CT scanner but uses light, and so works in a very different way from confocal microscopy or SPIM, which directly image a series of optical sections. OPT is carried out on fixed specimens, and it takes 5 to 10 minutes to image an entire embryo. The specimen is rotated while the images are taken. "This new technique works in conjunction with normal microscope optics, making it cheaper than confocal and much cheaper than microscopic magnetic resonance imaging [mMRI], and therefore accessible to many more biologists", says Sharpe, who helped to found Bioptonics, the vehicle for the commercialization of OPT technology run by MRC Technology, the UK Medical Research Council's technology transfer branch. The Edinburgh-based company offers an OPT scanning service and is developing the scanners, which will be ready for sale early next year.



OPT reveals the nervous system (green) and gut (blue) in a mouse embryo.

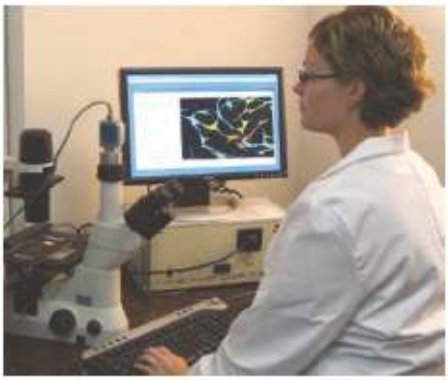
OPT can produce fluorescent or transmission three-dimensional images of specimens between 1 and 15 millimetres in size and computationally sections the images to reveal internal structures. Mouse, rat, chicken and zebrafish embryos fit the OPT size bracket, as does the adult fruitfly. Staining methods such as LacZ and alkaline phosphatase, which produce a non-fluorescent coloured stain, can be visualized with OPT, as well as fluorescent signals and unstained embryos.

"It fills an imaging gap by allowing analysis of specimens that are too large for confocal but too small for MRI," Sharpe says. "In addition, because it is an optical technique, OPT can extract more detail from tissues than mMRI, which cannot take advantage of fluorescent gene labelling techniques." This optical bonus can be exploited for applications outside developmental biology, including phenotyping and gene-expression analysis in adult mouse tissues.

The principle of projection tomography had not been used for optical microscopy, and there were no commercial machines. "I built the machine using parts from an old cell sorter and other equipment lying around the lab," says Sharpe, who also developed the software to turn the raw data into a 3D view of the embryo that can be rotated and sectioned to show different aspects. L.M.

SHARPE

INTRACELLULAR IMAGING



Calcium signals: the InCyt imaging system.

The whole animal

For some imaging applications, getting a picture from inside the living body is the goal (see 'Optical biopsies', below). And for others, a shift from imaging *in vitro* to the whole living animal is desirable. "Many pathways interact on a systems level, that is, the immune system and the endocrine system," says Pam Contag, co-founder of imaging company Xenogen in Alameda, California. Xenogen puts together non-invasive optical imaging systems with transgenic mice and rats containing the required luciferase-tagged genes. The Xenogen IVIS 200 will image either bioluminescence or fluorescence, and its adjustable field of view makes it versatile enough to image single cells at high resolution or up to five anaesthetized mice at a time. Biophotonic imaging has proved a success with the pharmaceutical industry to test drug candidates and make more educated predictions. "In drug discovery, the cell is used as the gold standard, but you don't treat single cells, you treat the whole person," Contag insists. "Our goal is to make animal models to be predictive for what happens in humans."

Lightools of Encinitas, California, concentrates on whole-body imaging of animals carrying fluorescent-tagged genes. "We can zoom in and out from a couple of centimetres to 10–15 centimetres in the field of view," says John Fox, Lightool's president. The company has recently introduced two novelties that are proving popular. One is a device for simultaneously viewing green and red fluorescent proteins (GFP and RFP) in transgenic animals, with independent controls for each fluorophore. "It allows you to turn up the RFP excitation and turn down the GFP — which is usually brighter — to obtain a more balanced image," says Fox.

The second is the Pan-A-See-Ya panoramic imaging system, which gives a 270° view of the animal in one evenly illuminated image. "It's like being able to see round the corner," says Fox. A target such as a fluorescent tumour can be viewed from two different angles at the same time with a single camera. The process is even fast enough to allow animals to be imaged without anaesthesia.

Breaking the resolution barrier

Abbe's law, postulating that optical resolution is impossible below 200 nanometres, went unchallenged for 120 years. Until recently, that is, when physicist Stefan Hell, a director of the Max Planck Institute for Biophysical Chemistry in Göttingen, Germany, established a new law that promises greater resolution in fluorescence microscopy. The first commercial application of his new ideas is the 4Pi fluorescence confocal imaging system, created in cooperation with Leica Microsystems in Mannheim, Germany.

The Leica TCS 4Pi improves resolution of fluorescent images to 110 nanometres along the *z* axis. Martin Hoppe, marketing manager for Leica Microsystems, says that the system addresses a resolution gap between optical and electron microscopes. "That's what I see when I offer this system to researchers," he says. Structures down to 110 nanometres can now be resolved in living cells — a malaria parasite can now be localized precisely inside a red blood cell, for example.

For life-sciences researchers, the advantage of the TCS 4Pi over electron microscopy is that specimens can be kept alive and fluorescent stains can be used. "People don't have to redo their staining techniques," says Hoppe.

The remarkable gain in sharpness is due to the use of two opposing lenses with high numerical aperture to illuminate a single focal spot. The two wavefronts of the opposing beams interfere constructively at the focal point, which gives much higher resolution.

But at US$1,000,000, the TCS 4Pi doesn't come cheap. "What drives up the cost is the combination of precision mechanics, interferometer optics and state-of-the-art electronics. Also, the 4Pi objectives have to be paired, this makes the manufacturing yield very low," Hoppe points out.

OPTICAL BIOPSIES

MAUNA KEA TECHNOLOGIES

One approach to *in situ* observation in the living body is the Cell-vizio miniaturized fibre-optic confocal fluorescence microscopy system developed by Mauna Kea Technologies of Paris, France. The core technology is the equivalent of a confocal microscope on the tip of a flexible fibre optic. "It's the smallest microscope in the world: less than one-third of a millimetre in diameter," says Benjamin Abrat, general manager and co-founder of Mauna Kea Technologies. This degree of miniaturization allows *in situ* exploration of peripheral nerves, blood capillaries, internal organs such as the colon or bladder, or deep in the brain, with minimal disturbance.

Cell-vizio can image subcellular structures and at 12 frames a second is fast enough to observe cellular dynamics. Its excitation wavelength of 488 nanometres is compatible with a range of fluorophores and with GFP and YFP transgenic animals. Used at present for small-animal research, Cell-vizio is currently being evaluated for clinical use in humans, and the company is working on European Union CE Mark and US Food and Drug Administration approval. "The most promising applications are in cancer diagnosis," says Abrat. "The technology can enable optical biopsies — seeing at the cellular level without removing tissue," he says. Conventional excisional biopsy suffers from high false negatives due to sampling error. "With our system, you can precisely locate diseased areas," Abrat points out. The miniaturized optical catheter could simply be placed into the operating channel of an ordinary endoscope.

Fibre-optic microscopes like the Cell-vizio can look inside organs.

Optical coherence tomography (OCT) is another powerful imaging technology that can function as an 'optical biopsy'. It uses near infrared light, which reflects off the internal microstructure of biological tissues similarly to ultrasound, but gives far higher resolution, and is already in clinical use in opthalmology for diagnosing glaucoma. With the aim of extending OCT to other clinical applications, LightLab in Westford, Massachusetts, has designed a system that delivers infrared radiation to the target site through a single optical fibre, which could be threaded alongside a catheter. In collaboration with the University of Maryland, Baltimore, LightLab's research programme is expanding into cancer, in particular oesophagal and pulmonary cancer. The devices are being tested in animals for spinal cord imaging and the placement of deep-brain stimulation electrodes for treating Parkinson's disease (see *Nature* 436, 18-19; 2005). "The overall aim of OCT is to replace the biopsy. But we are not there yet," says Joseph Schmitt, chief technological officer at LightLab. "For the moment, the first step will be to guide the biopsy, to guide the resection, and to help make therapeutic decisions." L.M.

A world of colour

Quantum dots may be the newest kid on the block (see 'Quantum dots keep on glowing', below), but since its cloning a decade ago, the green fluorescent protein has become one of the most powerful molecular tools in the cell biology tool-box. Either by itself or as part of a fusion protein, GFP is used to visualize proteins inside living cells in a vast number of applications, from detecting gene expression to tracking cell fate in developing embryos.

The range of fluorescent proteins from jellyfish now includes red (RFP), cyan (CFP) and yellow (YFP) relatives of GFP. CFP and YFP, in particular, make a suitable contrasting pair for multicolour imaging for differential gene expression and protein localization.

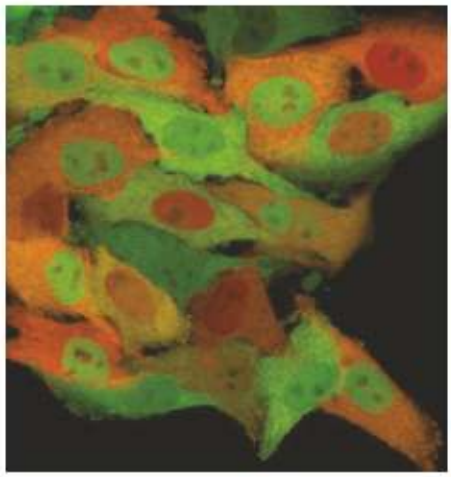
Species other than jellyfish are now being mined to expand the colour palette. The DsRed–Monomer fluorescent protein, an engineered variety of a protein from the sea anemone *Discosoma*, was recently launched by BD Biosciences Clontech of Mountain View, California, now part of Shiga-based Japanese life-sciences supply company Takara Bio.

"What is driving users' interest towards red is a better signal-to-noise ratio, because the background fluorescence from the culture medium is in the green range," explains Andrew Farmer, director of cellular and molecular biology for Clontech Business Research. The monomeric form of the new protein is an added advantage, particularly for subcellular labelling. "The DsRed–Monomer is less likely to misbehave than the tetrameric reds, which can sometimes disrupt the function of the fusion protein," says Farmer.

Stony corals have yielded a remarkable collection of new fluorescent proteins. The CoralHue range was originally isolated by Atsushi Miyawaki at the RIKEN Brain Science Institute in Saitama, Japan. Kaede (Japanese for maple leaf), the first of the family, is a brilliant green fluorescent protein that changes colour to a stable red when exposed to a short pulse of ultraviolet or violet light. Miyawaki's team have used Kaede to study hippocampal neuronal connections. Cultured neurons are labelled with green Kaede by gene transfection then, with a focused violet light pulse, a single cell body is illuminated. As the red spots spread rapidly throughout the cell's cytosol, all the nerve-cell processes, including an axon and illuminated dendrites, stand out from the green background, delineating the neuron and its multiple contact sites.

A recent addition to the CoralHue family is Kusabira orange, the first monomeric true-orange fluorescent protein. "Its greatest value is in combination with Midoriishi–Cyan for fluorescence resonance excitation transfer analysis," says Suzan Oberle, product manager for MBL International of Woburn, Massachusetts, which distributes the CoralHue range. The CoralHue pair is brighter and shows better spectral separation of donor and acceptor signals than the widely used CFP and YFP pair.

For FRAP and FLIP applications, CoralHue Dronpa green bleaches following excitation at 500 nanometres but completely regains its bright green fluorescence after minimal irradiation at 400 nanometres, without losing signal intensity. The switching can be repeated without losing brightness. Miyawaki's group has used this reversible protein highlighting technique to track proteins shuttling across the nuclear membrane after cell stimulation.



R. ANDO & H. MIZUNO

Violet light makes HeLa cells expressing Kaede fluorescent protein change from green to red.

Another photo-switchable fluorescent protein is produced by Evrogen, a biotechnology company based in Moscow, Russia. Their cyan-to-green photo-converting protein PS-CFP2, gives a 2,000-fold increase in the green-to-cyan fluorescence ratio, making it the highest-contrast monomeric photoactivatable fluorescent protein so far.

The microscope may be 400 years old, but microscopy is refusing to show its age. High-resolution live imaging is giving researchers a whole new look at the biological world. ■

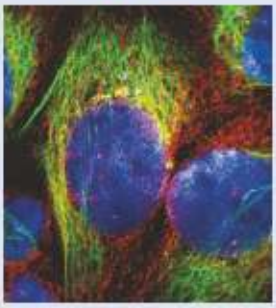
Lisa Melton is science writer in residence at the Novartis Foundation in London.

QUANTUM DOTS KEEP ON GLOWING

Further developments in imaging may well hinge on extending the range of labels. Fluorescent semiconductor nanocrystals — 'quantum dots' — are one answer. In 1998, Marcel Bruchez left the University of California in Berkeley to co-found Quantum Dot Corporation in Hayward, California. "Quantum dot nanoparticles solved a lot of the existing problems in fluorescence detection," he says. They fluoresce up to 100 times more brightly than organic dyes and are very stable. They can be fine-tuned to glow in almost any colour, each colour emitting in a narrow spectral window and thus avoiding colour overlap. And all quantum dot colours can be excited simultaneously with a single light source.

The company has just launched the Mosaic Assay System, an imaging system that uses combinations of its quantum dots to analyse the simultaneous expression of 100 genes in high-throughput mode. A similar system for cellular and protein analysis is promised soon. Quantum Dot's largest customers are in drug discovery, but business in academia is flourishing. "The technology has captured people's imaginations. A lot of researchers have incredibly innovative ideas of what they can do with it," says Bruchez.

Tracking cancer is one example. At the Beth Israel Deaconess Medical Center in Boston, quantum dots are being used in preclinical studies in pigs to map sentinel lymph nodes. "It is possible to label the cancer and see where the cancer cells spread and where they stop in the lymphatic system," says George Dunbar, chief executive officer at Quantum Dot. The next step is to test the long-term safety of these materials in animals to determine whether they could be used in people.



QUANTUM DOT CORPORATION

Quantum dots provide long-lasting colour.

This question is also being addressed by quantum-dot developer Evident Technologies of Troy, New York. The latest version of its EviTag quantum dots has an outer lipid layer to help ensure biocompatibility. Another useful property of quantum dots is that they can be tailored to glow in the near infrared, allowing scientists to see right through the tissue. "The colours last longer than organic dyes in the near infrared, and near infrared transmits through tissue without scattering," says Steven Talbot, Evident's chief marketing officer.

Evident's new indium phosphide deep red EviTags can be tuned to emit at near infrared wavelengths, so that light absorption from neighbouring tissue is avoided and reasonable signal-to-noise images generated.

Using a 4000IS scanner from Kodak of Rochester, New York, Evident has developed a label suitable for a dual-mode optical and X-ray imaging system. "You can see the bones of the mice, but also the fluorescence coming out from the quantum dots attached to the tumour cells," says Talbot. Although the question of toxicity is always on biologists' minds, Talbot is reassuring: "Several groups have been studying cytotoxicity in live cells and haven't yet seen any bad effect." L.M.