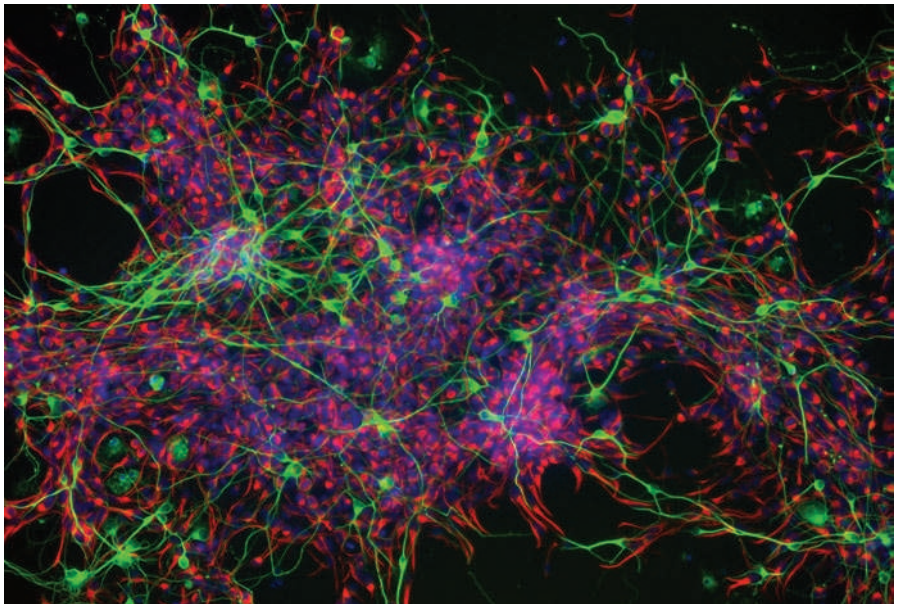
Taking a long, hard look

Long-term, live-cell imaging helps to settle long-running debates. Monya Baker investigates how the huge investment and time commitment is finally paying off.



S. GODERIE/TEMPLE LAB

Neural stem cells and their descendants showing neurons (green) and neural epithelial cells (red).

In the late 1980s, Sally Temple was studying neural development in mice at the University of Miami in Florida and needed a way to observe neural progenitor cells for days on end. At the time, no one had observed mammalian cells for more than a few hours, because the conditions that could be maintained under a microscope were too dry, cold and oxygen-rich to keep cells alive for long. Undeterred by the lack of precedent, Temple decided to build her own device that could monitor cells around the clock.

Temple, a developmental neuroscientist now at the Neural Stem Cell Institute in Rensselaer, New York, credits her husband Jeffrey Stern with the inspiration for her apparatus. "He said, 'if the cells are living well in the incubator, you have to put the microscope in the incubator'," she recalls. Although the idea was obvious to Stern — a vision researcher and co-founder (with Temple) of the Neural Stem Cell Institute — it seemed ludicrous to most cell biologists, who had long held the view that the humidity inside an incubator would ruin microscope optics.

Temple was also sceptical. But she found an abandoned microscope and decided it was worth a try. She outfitted the ageing microscope with a red filter (a piece of broken glass taped onto a Petri dish) to minimize the cells' exposure to more damaging, higher-energy light during her extended experiment. She then attached a camera and drilled a hole through the incubator to connect the camera to a Panasonic tape deck, which could record image data several times an hour for up to seven days.

Remarkably, the makeshift contraption worked. "We got some really neat data that showed to our surprise — I think to everyone's surprise — that the vertebrate brain had lineage trees that were similar to *Caenorhabditis elegans* and other invertebrates," says Temple[1].

But the discovery required as much luck as it did innovation. Temple's cobbled-together set-up could only keep track of what was under the microscope at a particular time, and the slide couldn't be moved around to find the most photogenic cells. If the few cells that were in the microscope's field of view had died or grown poorly, the whole project would never have panned out.

All the major microscope manufacturers now offer a new generation of devices built for live-cell imaging, complete with computerized incubation chambers and microscope stages. Jochen Tham, global marketing and communications director at Carl Zeiss MicroImaging in Thornwood, New York, tracks what he describes as some of the major developments at his company: multilayered incubation, cooling and heating, computer control of environmental parameters, integration of microscope-controlling software and image-acquisition software, incubators for super-resolution and total internal reflection, and control over oxygen levels to mimic physiological conditions, to name just a few.

Such systems are helping researchers come to a more complete understanding of how functional cells and tissues develop. "Unless you can actually watch everything that is happening from the first cell up to the developing progeny, you have no idea how [cell development] actually plays out," says Michel Cayouette, a developmental neurobiologist at the Clinical Research Institute of Montréal, Canada, whose work has revealed a way to predict how retinal progenitor cells divide[2], information that could help to produce cells for treating blindness. Cayouette compares studying cell differentiation to watching ice hockey: how much could he learn about the game if all he knew was the final score?

Scientists have valid reasons for avoiding long-term imaging experiments. They require expensive equipment that gets tied up for days or weeks at a time and are prone to time-consuming false starts. As an experiment runs its course, all aspects of keeping cells alive and in focus get harder: cells move, routine handling becomes disruptive and computer hard drives fill up.

Any one of these unpredictable events can derail an experiment, says Cayouette. "You can't say, 'I'll try this and at the end of the week, I'll figure out what

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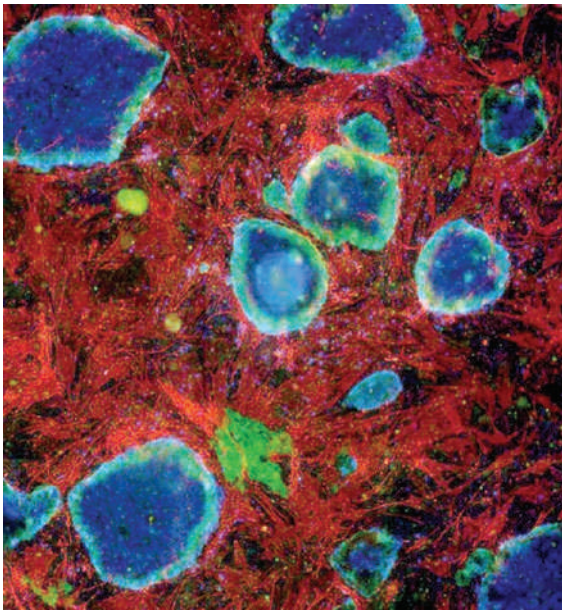
Sally Temple (inset) now uses a fully automated incubation system, but her first time-lapse set-ups relied on microscopes inside incubators.



T. SCHLAEGER/CHILDREN'S HOSPITAL BOSTON

Human embryonic stem cells on mouse embryonic fibroblasts.

to do.'" So researchers are planning ahead, and devising new systems for tracking cells in real time.

Getting answers

Beyond offering a lens onto new biology, long-term imaging studies are also beginning to resolve long-standing debates in developmental and cell biology. For example, Timm Schroeder, a stem-cell biologist at the Helmholtz Centre in Munich, Germany, led a team that used continuous imaging to distinguish between two competing hypotheses about the role of cytokines in blood development.

One view held by some immunologists is that cytokines — regulatory proteins found in the immune system — cause cells to take on new fates; another theory is that cytokines help certain cell types but not others to survive. Both ideas would ultimately result in the same blood cells, but the path by which the cells got there would be radically different. As such, the two hypotheses would hold vastly different implications for treating diseases or generating blood in the laboratory.

To tease apart the actual mechanism, Schroeder's group took pictures of mouse blood cells every two to three minutes for several days. Because the researchers did not observe extensive cell death, their time-lapse film firmly supported the active instruction over the passive-survival hypothesis[3]. Long-term images make "a big, big difference", says Schroeder. "You can say, 'this is how it was', not 'this is how it probably was'."

Researchers working in the fast-paced field of stem-cell reprogramming have also been keen to track how cells take on desired fates. Under most experimental set-ups, the early events are the hardest to follow. But a team led by George Daley and Thorsten Schlaeger, stem-cell biologists at the Children's Hospital Boston in Massachusetts, used long-term imaging to reveal the history of rare, reprogrammed cells.

To identify the presence of expected molecules on cell surfaces, the researchers added fluorescently tagged antibodies to the culture media as the cells grew into the colonies characteristic of induced pluripotent stem (iPS) cells. Meanwhile, they set their microscope to scan the cells constantly, making a complete survey of the 4-square-centimetre area every two or three days for about two weeks. After assessing which colonies produced high-quality iPS cells, the team could go back to the images to identify the cell clusters that gave rise to fully reprogrammed cells, even though each group of iPS cells took up as little as 0.0003% of the scanned area[4].

Learning the hallmarks of iPS cells as they undergo reprogramming could not only yield better methods for growing patient-specific stem cells, but also prevent weeks of wasted effort (and costs) in animal experiments, says Schlaeger.

Come to light

These types of study are starting to shed light on hitherto unsolvable biological problems, such as why some patterns of cell division contribute to cancer and which progenitor cells give rise to blood, sperm, neurons or other tissue types. But researchers using live-cell imaging have to be careful not to shed too much light — quite literally — because illuminating cells for long durations can damage cells or alter their behaviour. Schroeder's advice to biologists is to take the worst image possible to get the necessary data. "If you're pushing the envelope, you should aim for having healthy cells rather than the best images," he says. Beautiful images, he notes, often make for unhealthy cells.

So instead of continuous snapshots, researchers often rely on taking pictures at less frequent intervals. Many factors contribute to the imaging

HELMHOLTZ CENTRE MUNICH

Timm Schroeder observes cells to disinguish between competing hypotheses.

A LONG-TERM LIVE-CELL COMMITMENT

The decision to undertake a long-term imaging project is not trivial. Experts suggest questions that researchers should ask themselves before starting out.

How frequently do you need to take an image?
Tracking individual cells often requires taking an image every few minutes. The more dense and mobile the cells are, the less time can elapse between images. For example, Michel Cayouette at the Clinical Research Institute of Montréal, Canada, takes images of retinal progenitor cells every seven minutes until they develop into neurons, at which stage he slows the rate of image acquisition to roughly once an hour.

Can your cells survive the experiment?
Repeated imaging can harm cells, especially when the imaging requires high-energy light. But the tolerance of different cells for fluorescence varies widely. Blood-forming stem cells are generally more robust than neural stem cells, for example, and thus can be imaged more frequently without affecting cell behaviour, notes Tannishtha Reya, a stem-cell biologist at Duke University in Durham, North Carolina.

Can you keep calm?
Inexperienced researchers sometimes set up their long-term microscope systems in the middle of a heavily trafficked work station or worse, under ventilation systems. Such disturbances can easily overwhelm a system's ability to maintain stable conditions and can cause obfuscating artefacts, cautions Cayouette.

Can you follow your cells?
Following cells in culture gets complicated once cells start crawling under and over each other. To track individual cells at low densities, labelling nuclei with Hoeschst often works well, says Thorsten Schlaeger at the Children's Hospital Boston in Massachusetts, although he cautions that some cells stain poorly, and non-toxic genetic labels can work better. If cells must be grown at high density, consider mixing in a few labelled cells and tracking just these.

Are you computationally prepared?
Crunching through large data sets can easily go beyond the capacity of standard lab computers, and a single experiment can completely fill a computer's hard drive. Researchers need appropriate servers and back-up systems. A dedicated informatics set-up and the help of a programmer are "highly desirable", says Schlaeger. M.B.

method used — the types of cell and how robust they are, what features need to be followed, even the size of the image files that will be collected (see 'A long-term live-cell commitment').

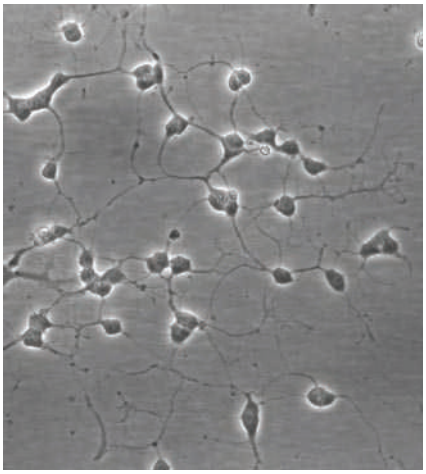
The most important decision is usually what type of light to use. Fluorescent tags can be linked to the expression of particular proteins to indicate specific biological activity or the production of an oncogene, for example. But too much fluorescence-activating light can trigger damage that prevents cells from growing — a phenomenon known as phototoxicity.

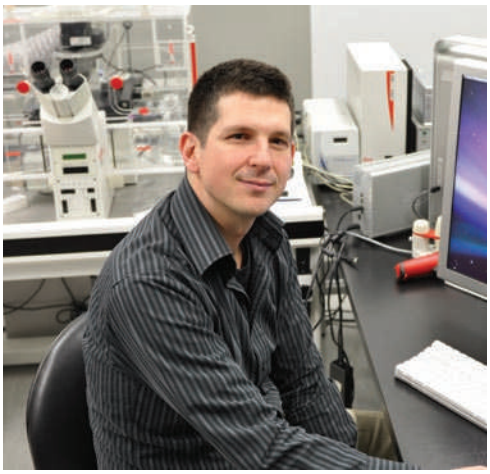
Even with cells that resist phototoxicity, there are image-processing considerations. If viewed too often, fluorescent proteins can fade and become invisible. Overcoming such photobleaching requires the subtraction of background fluorescence and correcting for the fact that because the sample is not perfectly flat, levels of illumination vary across the field of view.

Unlike fluorescence microscopy, phase-contrast imaging produces black and white pictures using less damaging wavelengths of light. But the technique reveals only the general cell shape, rather than the presence of a particular protein. Researchers often combine the two methods by, for example, taking a fluorescent image every hour and a phase-contrast image every ten minutes, but even that compromise must be planned carefully. Some instruments that are great for fluorescence microscopy perform less effectively at bright-field microscopy, explains Schlaeger.

Keeping watch

Keeping cells alive requires a much more delicate balancing act than finding the right mix or amount of photons. Even if cells aren't perturbed by the imaging set-up, they still need fresh culture media and the removal of waste products. Plus, any continuous imaging study that lasts for more than an hour will probably require a system with built-in environmental controls for temperature, humidity and gas concentration — and maintaining the right conditions for cells often requires special care for microscopes.





CAYOUETTE LAB (LEFT); H. LAMBIN (RIGHT)/IRCM

Michel Cayouette (right) uses long-term imaging to predict which retinal progenitors will produce neurons.

For example, to avoid creating air currents that could blur an image when cells are kept at standard 37 °C, microscopes for studying live cells have to maintain the lens at the same temperature as the culture. Environmental chambers are available for all high-end microscopes, either from the microscope manufacturer or from third parties, but none works as well as an incubator, say researchers. Temple and her graduate students nicknamed an early model 'the Sahara' because it caused cells to dry out so quickly.

The commercially available products have improved, says Jin-Wu Tsai, who studies cultured brain slices in Arnold Kriegstein's lab at the University of California, San Francisco. "A few years ago, we built our own incubator on top of the microscope. Now you have lots of options," he says. With these newer commercial tools, "we can just keep the culture dish on the stage of the microscope, and the software allows us to take images every ten or fifteen minutes".

Although he doesn't have to transfer samples, Tsai is still tethered to his microscope, keeping watch on its confocal image. The brain slices he studies flatten over time, making the image go out of focus, and it doesn't matter how healthy the cells are if the data collected from them aren't usable. Prototype microscopes with autofocus have been introduced. They might be reliable for flat culture, Tsai says, but he doesn't trust them to work in thick brain slices yet.

In addition to keeping tabs on the brain slices themselves, Tsai has to monitor every facet of the experiment. If the conditions are just slightly off — say, a shift in pH, or a slight increase in carbon dioxide — the neurons stop

A SOFTWARE SPOT

Computerized robotics are already easing lab-based wet tasks such as feeding cells and changing media. Several vendors now sell programs that can track cells in flat culture, keeping them in focus and in the field of view.

The latest version of the Nikon BioStation CT can 'memorize' the positions of non-motile cells before a plate is removed for media exchange, and can then continue tracking them when the plate is replaced, avoiding the 'image jiggle' that would disrupt statistical analysis, says Ned Jastromb, a senior application manager at Nikon Instruments in Melville, New York. It also integrates a calendar function with a robotic system that slides culture plates in and out of an imaging area on schedule, allowing one instrument to run several long-term experiments.

But software is poised to solve a wider range of problems. By combining a fast image-acquisition program with a noise-reducing algorithm that compares consecutive images, John Sedat at the University of California, San Francisco, and his colleagues decreased the amount of light needed to image yeast cell division by several orders of magnitude[5]. Advances in fully automated cell identification and tracking, and modern continuous cell-imaging techniques can outperform traditional manual methods[6]. Historically, software advances have spread slowly because programs designed to follow a particular cell type tend not to recognize other types, says Andrew Cohen, a computer engineer at the University of Wisconsin–Milwaukee.

More broadly, Cohen says he may be on the cusp of solving a problem that plagues many live-cell imaging experiments. Many software programs work only when cells are sparse. That limits the technology because some cells can grow only in dense cultures, and some cells divide many times before producing the desired cell types, in which case a single cell produces hundreds of daughters. By the time the most interesting cells appears, it is impossible to tell which cells they came from. Recently, Cohen found that an algorithm he originally wrote to follow hundreds of organelles within a single cell can be applied to trace neural stem-cell fate. "Our ability to track very high-density image sequences is going to improve very rapidly," he says.

Larger advances, however, may come less from improvement in technology than from biologists' awareness of what software can do, says Cohen. "Sometimes the biologists start out just wanting to characterize data, and they don't think about the big questions they can ask." M.B.

growing. And there is still no way to change media under the microscope and maintain sterile conditions, he says. Nonetheless, Tsai and his colleagues have been able to keep brain slices alive for more than a week, after which time bacteria have started to grow.

Conditions also need to be tightly controlled to minimize artefacts and experiment-ruining variability. Over the run of a protracted experiment, subtle differences in culture conditions can start to look like cell behaviour, notes Alfred Bahnson, a biologist at Kairos Instruments in Pittsburgh, Pennsylvania, which manufactures optically accessible environmental chambers and other long-term imaging products. Movement that appears to be cell migration, says Bahnson, might instead be cells moving downhill or following slight temperature gradients.

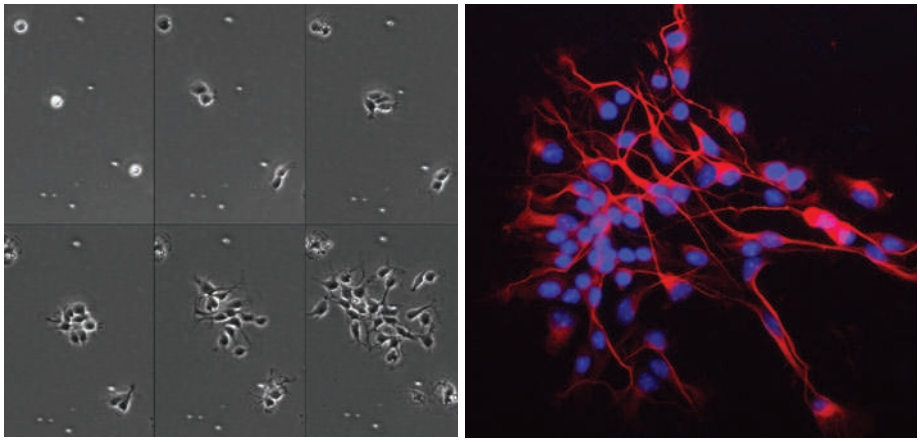
Coordinating accessories

Those imaging issues are typical, says Keith Bogdon, an adviser with consulting company Coalesce Corporation in Larkspur, California, who has researched live-cell imaging products. Often features to control temperature or pH conflict with features for positioning or focusing cells in the microscope, particularly if researchers want to compare several experimental conditions. "There are a lot of wires and tubes coming out of the plates," says Bogdon, and it is difficult to design a chamber so that cells can be both monitored and unperturbed.

Even after the microscope, environmental chamber, and other equipment are brought together so that cells stay alive, the length of experiments can still be a problem. Most labs and core facilities aren't designed to accommodate experiments that tie up equipment for weeks at a time. If a researcher works out too late that some parameters need to be tweaked, it could take months to book the necessary time with the microscope again.

NIKON

Nikon BioStation CT has software and robotics to allow several scientists to conduct experiments.



S. GODERIE/TEMPLE LAB

Stills from a film of an individual cell dividing into dozens of neurons (left), and many neural cells produced from a single neural stem cell (right).

And long-term imaging is costly in money as well as time. Renting equipment from a core facility at, say, US$20 an hour, 24 hours a day for a week or more could make even a single experiment pricey. "If you do this for half a year," says Schroeder, "you've paid more for renting than for buying." But buying equipment with a six-figure price tag is not always easy. "It's a classic chicken and egg scenario," says Bogdon. "How can researchers get into the area if they don't have the clout to justify funding?"

Still, researchers report that commercial offerings in software, cell incubation, and visual systems have expanded greatly. Microscope systems used for live-cell imaging for time periods of more than 24 hours are produced by companies including BD Biosciences in San Jose, California; Essen BioSciences in Ann Arbor, Michgian; GE Healthcare in Waukesha, Wisconsin; Leica in Wetzlar, Germany; Molecular Devices in Sunnyvale, California; Nikon in Melville, New York; Olympus in Center Valley, Pennsylvania; PerkinElmer in Waltham, Massachussetts; and Zeiss. Each system comes with its own proprietary software and storage options, and additional software is available, all of which tends to require considerable expertise (see 'A software spot').

Molecular Devices offers MetaMorph imaging analysis software; the imaging-processing software MatLab from MathWorks in Natick, Massachusetts, can be purchased with add-on image analysis tools. Volocity from PerkinElmer is popular. ImageJ from the US National Institutes of Health and Cell Profiler from the Broad Institute in Cambridge, Massachusetts, are both freely available and widely used. Several companies, such as Oko Lab in Ottaviano, Italy, Tokai Hit in Shizuoka-ken, Japan, and WaferGen Biosystems in Fremont, California, sell environmentally controlled microscope slides or other equipment to control conditions on the microscope stage.

Still, a microscope accessory that solves one problem often creates another, says Schroeder. An incubator that fits to the stage of one microscope may not provide the kind of surface that a particular cell type grows on, or tubing that fits an incubator may not attach to a media pump. A computer program names files with a four-digit code, limiting experiments to less than ten-thousand images. And so on. Each problem is trivial individually, says Schroeder, but collectively they sap researchers' motivation and take up time that could be spent on experiments. Anyone who wants to conduct long-term live-cell experiments needs to be ready to spend a long time tinkering with equipment, he says. But the benefits are repeatedly proving worth the hassle, says Cayouette. "The technique is becoming increasingly user-friendly. More and more people are trying to do these long-term imaging studies."

Temple predicts that the results of such studies will be profound. "We've just forgotten the fourth dimension in so many of these analyses," she says. "Now that we've got time, we can finally start to understand." ■

Monya Baker is technology editor for *Nature* and *Nature Methods*.

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Correction

The Technology Feature 'The gatekeepers revealed' (*Nature* **465,** 823–826; 2010) stated that the crystal structure for the A2A adenosine receptor and similar receptors had been solved using an unmodified protein, but referenced the structure of a protein stabilized with T4 lysozyme. Structures for the unmodified receptors have not been published.