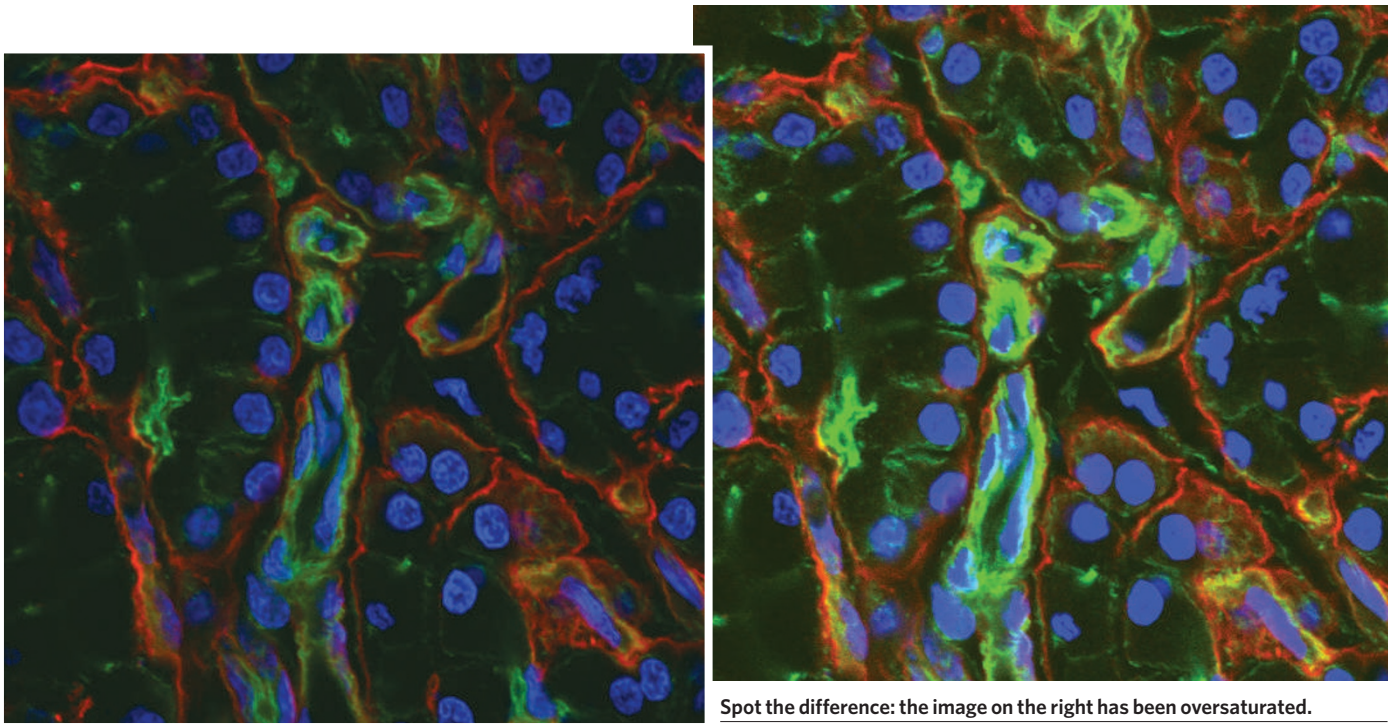


G. PAPWORTH/CENTER FOR BIOL. IMAGING, UNIV. PITTSBURGH

Spot the difference: the image on the right has been oversaturated.

THE GOOD, THE BAD AND THE UGLY

Imaging fluorescent molecules in live cells is revolutionizing cell biology. But a pretty image is not necessarily a good one, and many biologists are learning this the hard way, finds **Helen Pearson**.

The satellite imagery of Google Earth offers homeowners the chance to zoom in from outer space and hover above their rooftops. For biologists, a microscope gives a similarly exhilarating view of a cell's innards: the omnipotent eye of the nucleus, the bustling traffic of the cytoplasm and the elaborate architecture of the cytoskeleton. This is the detailed, shifting topography that cell biologists spend their lives trying to comprehend.

But looks can be deceiving, as Jack Fransen at Radboud University Nijmegen Medical Centre in the Netherlands and his colleagues found out. Using a powerful fluorescence microscope, they watched cells pulse from acid green to mellow blue when bathed in the chemical fuel ATP. The purpose of the project was to test whether ATP could cause proteins to change shape and trigger their fluorescent tags to change colour.

In the eye of the beholder

But the beauty of the imagery dimmed when the researchers checked their control protein, which had been carefully constructed to fluoresce at a constant level. This, too, pulsed prettily with ATP — as did every other control protein they could lay their hands on. "It was a complete surprise," says Fransen.

The problem lay with some mysterious behaviour of ATP that so far remains unexplained and hard to spot. Researchers less knowledgeable or meticulous about microscopy could easily have their results thrown off by such phenomena. This is why the team published a cautionary note about the discovery earlier this year (Willemse, M. *et al. Nature Biotechnol.* **25,** 170–172; 2007).

To correctly capture images using a modern microscope, researchers must have a good grasp of optics, an awareness of the microscope's complexity and an obsession for detail. Such skills can take months or even years to master, and yet, owing to inexperience or the rush to publish, are all too often squeezed into hours or days. Popular methods such as fluorescence microscopy are particularly fraught with dangers.

Most researchers are not intentionally cutting corners; they may simply be unaware of the possible pitfalls. And most oversights are harmless — for example, making a fluorescent protein appear dimmer or fuzzier than it is. But inept microscopy, and subsequent analysis, can easily generate results that are misleading or wrong.

It is difficult to gauge how much published microscopy is of poor quality, and it is a rare biologist, such as Fransen, who will be able to identify, let alone admit to, a specific problem. But one expert contacted by *Nature* estimated that as many as half of all experiments that report two proteins in one spot have not been performed properly. Another estimated that 5–10% of images don't match what is reported in the text. "It's easy to pick up any journal — even *Nature* — and see poor microscopy data," says Jennifer Waters, who directs the Nikon Imaging Center at Harvard Medical School. "I don't know how often the results are blatantly wrong, but I do worry about the accuracy."

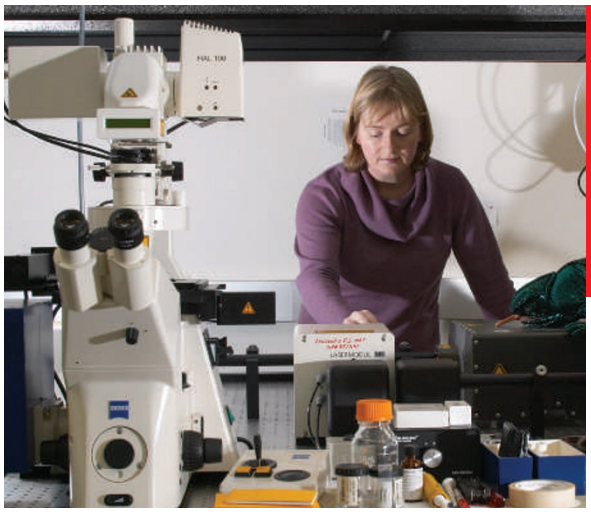
The modern light microscope comes with the accoutrements and price tag of a high-speed racing car and offers an exhilarating ride. It can boast numerous knobs, a foot pedal, winking lights and touch-control climate. Such microscopes can cost anything from US$50,000 to $1 million. But not everyone should be allowed behind the eyepiece.

"It's much more complicated than sitting down and pressing the buttons," says Simon Watkins, who runs a biological-imaging centre at the University of Pittsburgh in Pennsylvania. "If you got into a fast car but didn't know how to drive it, you'd crash very quickly."

And that's what has happened. During the past 10–15 years, these souped-up machines have become a mainstay of most cellular and

molecular biology laboratories. But many biologists' ability to handle the instruments has not kept pace with the technology, and the road to results is becoming littered with scrapes, prangs and outright wrecks.

Only 20 years ago, light microscopy was very different. Most biologists used conventional cameras to take snapshots of illuminated slices of dead tissue. That changed in the early 1990s with the discovery that proteins could be spliced onto jellyfish green fluorescent protein (GFP), allowing their location to be tracked in living cells. Since then, a rainbow of fluorescent proteins has become available, as have highly sensitive digital cameras that can detect signals invisible to the naked eye.



A. NORTH

"It's a big problem when the reviewers are more concerned with how aesthetically pleasing an image is than whether the scientific content is clear." — Alison North

Getting a look in

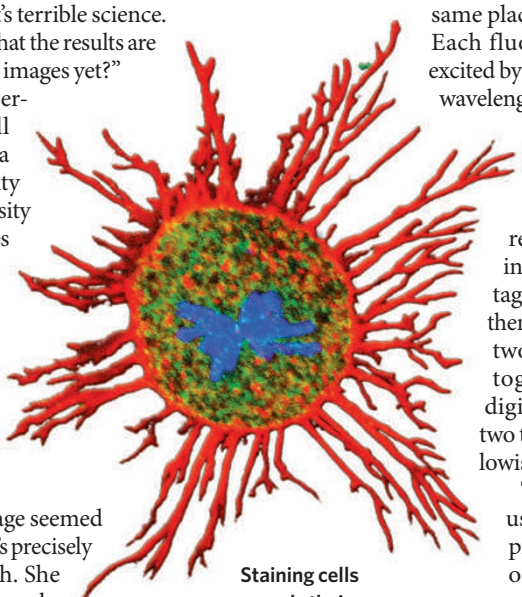
It is now a routine part of many studies to investigate, using microscopy, where in the cell a fluorescently labelled protein is concentrated and where it goes. This type of microscopy has hooked cell biologists because it allows them to gaze inside living tissues and monitor molecules in their native environment. But although most biologists graduate with some training in chopping and splicing DNA, few will have laid their hands on a pricey fluorescent microscope. "Your average molecular biologist can make all these fantastic fluorescent tools," says Kurt Anderson of the Beatson Institute for Cancer Research in Glasgow, "but then imaging is just a little bit tacked on the end."

"The only time I end up in stand-up fights with users," says microscope specialist Alison North, "is when they say, 'I need to get a picture, I've never used that microscope before but I'm sending out the paper tomorrow.' Then I scream at them, because that's terrible science. How do they know what the results are if they haven't got the images yet?"

North knows the perils of microscopy all too well. She runs a 12-microscope facility at Rockefeller University in New York and gives a two-hour lecture on general microscopy and its pitfalls. After I sat through an abbreviated version of the talk, my brain felt heavy and my palms damp, and the chance of capturing a good image seemed near impossible. That's precisely the point, says North. She aims to scare users enough that they will consult her before embarking on a doomed microscopy project: "It's quite cruel of me isn't it?"

The list of potential mistakes in fluorescence microscopy is long and complex (see 'Top tips for taking images', overleaf). Seemingly small steps, such as using the correct thickness of glass coverslip, are crucial to obtaining a good image. Even the intermittent cooling of an air conditioner can cause a microscope to drift in and out of focus. "There are an infinite number of settings that a poor microscopist can make mistakes on," says microscope expert Michael Davidson at the Florida State University in Tallahassee.

One of the most common uses of fluorescence microscopy — and therefore the source of many problems — involves looking for two proteins labelled with different coloured tags in order to determine whether they sit in the same place in a tissue or cell. Each fluorescent protein is excited by a particular range of wavelengths and emits at different wavelengths that are collected through microscope filters. If a researcher uses GFP in combination with a tag that emits red light then, in places where the two proteins are close together, combining digital images of these two tags will create a yellowish signal.



D. STOLZ/CENTER FOR BIOL. IMAGING, UNIV. PITTSBURGH

Staining cells reveals their inner workings.

Things go wrong if users select an inappropriate pair of tags or incorrect microscope settings with which to detect them. Problems can arise if the light used to excite one tag also partially excites the second and a poorly chosen filter lets some of that unwanted light in. This phenomenon, known as bleed-through, can wrongly suggest that two proteins are located together, because one of the tags will fluoresce under both excitation conditions and thus appear to be two tags in precisely the same spot. Such a mistake can be avoided by choosing tags with non-overlapping emission and excitation spectra, and by a control experiment in which only one tag is used, to see if it fluoresces under both conditions.

Not for the faint-hearted

Microscopists save most of their expletives for more sophisticated techniques such as FRET (fluorescence resonance energy transfer), one of several four-letter acronyms for methods that are both popular and treacherous. FRET is the technique that Fransen and his colleagues stumbled over. It's so temperamental that Waters says she advises new graduate students "to turn around and run away" if a prospective supervisor suggests FRET for their thesis work.

FRET is highly susceptible to both false-positive and false-negative results because it is used to detect very close interactions between two proteins or parts of the same protein and users are not always aware of its limitations.

So who is responsible for ensuring that microscope users are competent, and what can be done to help those who are not? Most scientists are willing to admit their inexperience and accept that it is their responsibility to operate microscopes correctly. They can turn to information-packed websites and books for help. But it is hard to beat hands-on experience from the many highly regarded — and oversubscribed — crash courses in microscopy. A handful of universities are starting to offer graduate courses in biological imaging, but more are needed. And microscope manufacturers such as Olympus and Nikon provide training and troubleshooting.

A growing number of institutions also host a central imaging facility similar to that run by North, with a suite of machines and one or more dedicated specialists. These experts urge

researchers to consult them at the planning stages of an experiment to ensure that their imaging will be successful.

But even with all this advice on offer, biologists can remain oblivious to the mistakes they are making. In such cases, some believe that journals and reviewers could do more to police poor-quality microscopy. One improvement journals could make would be to require more details about microscopy techniques in methods sections or supplementary information. Experts say that few journals require enough detail to properly judge the quality of data or to reproduce them. For example, including the type of filters used would help others judge whether a result could be due to bleed-through. Several experts also support the idea of asking a microscopist to review imaging data in papers that rely strongly on imaging to support their conclusions. Editors at the *Journal of Cell Biology* and *Nature Cell Biology* say they already consult reviewers with microscopy expertise when necessary.

But journals and reviewers are too often impressed by pretty images. North tells the story of one postdoc who slaved to capture images of her small cells only to have the paper turned down because the reviewer said the images were not good enough. "I've seen

"It's much more complicated than sitting down and pressing the buttons." — Simon Watkins

how long she spent getting the highest quality images she could possibly achieve," says North. "I think it's a big problem when the reviewers are more concerned with how aesthetically pleasing an image is than whether the scientific content is clear."

Looking ahead, the situation could get both better and worse. For those who just want to point-and-shoot with their microscope, manufacturers are building machines with less room for mistakes. Last year, Nikon launched a microscope called BioStation IM — effectively a foolproof microscope for imaging live cells with camera, software and incubator all in one box. Joseph LoBiondo, an expert in bioscience microscopes at Nikon in Melville, New York, predicts that microscopes will become even more automated in future, and says, "it takes the mystique out of it." But others worry that such automation will encourage sloppy experimentation.

At the same time, microscopy is becoming still more complex. Even those who run imaging facilities say that they struggle to keep up with the latest technology as new imaging techniques are introduced. Many potential problems, such as that encountered by Fransen, are only now being discovered.

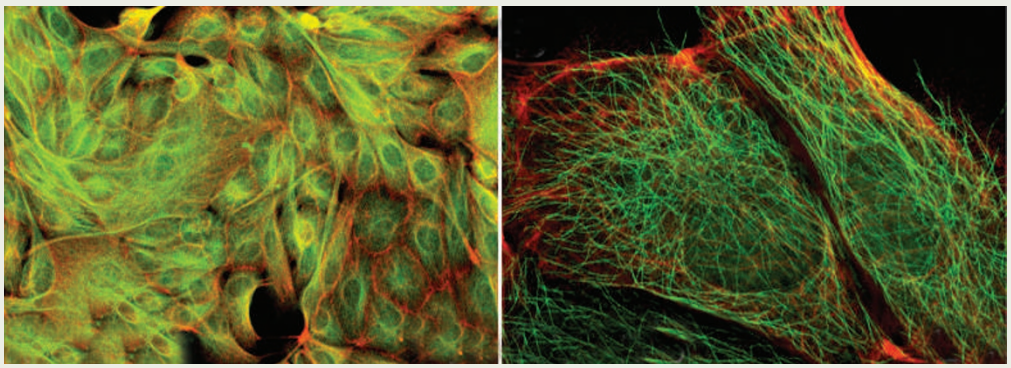
It could be argued that biologists should focus on generating hypotheses and analysing results, rather than mastering sophisticated machinery. So microscopy could become a specialized service that is outsourced to technical experts, collaborators or even companies, says John Runions, who specializes in bioimaging at Oxford Brookes University, UK.

Others disagree, saying that many biological questions simply cannot be answered without a working knowledge of microscopy. "In competitive biology, you don't necessarily need to be a mechanic but you need to be able to operate the machine," Davidson says. "If you don't know how it works you'll get creamed in the race." ■

Helen Pearson is a reporter for *Nature* based in New York.

H. GIGLIO/PITT MAGAZINE

Top tips for taking images



A. NORTH/JOURNAL OF CELL BIOLOGY

A low-resolution objective (left) suggests that two proteins are in one place; a high-resolution one shows otherwise.

Choose the right method
For different samples there are various microscopy techniques, each with strengths and weaknesses. The most expensive option is not necessarily the best.

Prepare the sample carefully
Many labs use one protocol for all samples, but preparation needs to be optimized for every protein and cell type. The wrong method can warp cells or change a protein's distribution.

Choose the right mountant
Some mounting media contain 'anti-fade' chemicals. These prevent some fluorescent tags from fading, but quench the signal from others.

Select the objective lens with care
The resolution of a microscope lens, or its ability to make out fine detail, is largely determined by its numerical aperture, not to be confused with its stated magnification. Choose carefully: a low-resolution objective may suggest two proteins are located together whereas a high-resolution one can separate them.

Choose the right tags and filters
Don't just use the fluorophores in the fridge or those that are pretty. And choose filter sets that only pick up emission from your chosen tags to avoid 'bleed-through', which can make one tag appear to be two.

Avoid aberration
Check and compensate for chromatic and spherical aberrations. These cause light to focus at slightly different points because of its different wavelengths, or because it passes through a different part of the lens. Both can smear out signals.

Don't saturate the image
To get a bright, beautiful image, some users 'saturate' regions, meaning that the pixel value is maximized but the true amount of light hitting the pixel is lost. The full range of pixel values should be used to capture the most information.

Don't always select the ideal cell
It's tempting to choose a cell in which a protein behaves as your hypothesis suggests. It is much more objective to blind the samples or randomly select cells.

Keep your cells happy
When doing live cell imaging, the main priority is to keep cells alive long enough to get images of them; everything else is a compromise. (For more details, see: North, A. J. *J. Cell Biol.* **172,** 9–18; 2006.) **H.P.**