TECHNOLOGY FEATURE

TWO MICROSCOPES ARE BETTER THAN ONE

Using two different kinds of imaging can give scientists a powerful combination of high specificity and detailed structural information.

CARL ZEISS MICROSCOPY

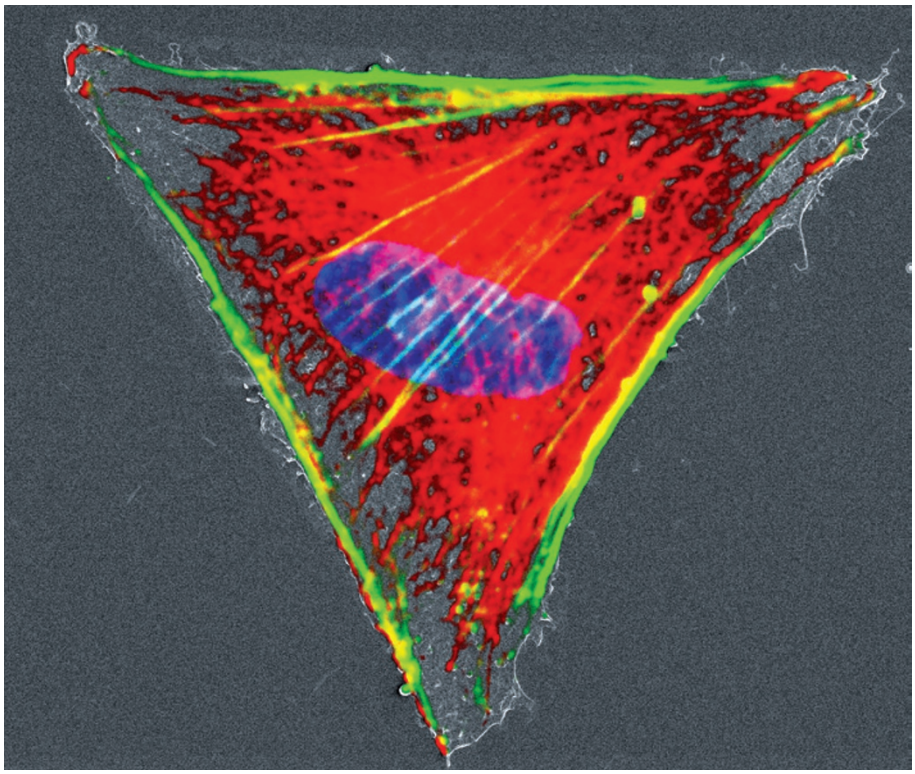
This image of a butterfly wing was constructed using two types of microscopy: a confocal image of the reflective eyespots shows scales (green) and wing (red); scanning electron microscopy reveals the different structure of non-reflective scales (upper left).

BY CAITLIN SMITH

There are many types of microscopy, each providing a unique set of benefits. Light microscopy of cells that express fluorescently labelled molecules, for example, lets scientists observe the movements of specific molecules or protein complexes in live cells in real time, or in fixed samples. Scanning electron microscopy (EM) reveals tiny details of the cell surface, and transmission EM shows the detailed cytoarchitecture of sections through fixed tissue. Other aspects of the structure of cells and tissues can be explored using techniques such as ion microscopy, total internal reflection microscopy, atomic force microscopy and super-resolution microscopy. Each type provides different information, but using two sorts of microscopy simultaneously provides even more — and molecular tools have been developed to link them together. Researchers testing the waters of this 'correlative microscopy' are beginning to discover its challenges and rewards.

Correlated light–electron microscopy, for example, provides both the specificity and real-time observation of light microscopy with fluorescent labelling and the better structural resolution of EM. But such correlative microscopy has historically been tricky to use. Scientists who tried it might have needed to switch to EM halfway through an experiment, which meant moving the sample from one ▶

M. STEVENS/IMPERIAL COLLEGE



Composite image created from correlated light–ion microscopy showing a fibroblast stained to show tubulin (red), actin (green) and the nucleus (blue).

microscope to another that may be located in a different lab, or even in another building. Not only would this journey take time, but delicate samples could be damaged by the motion, or by changes in temperature or humidity.

A second obstacle is the need to 'find back', which means searching the thin section prepared for EM to relocate the region observed under the light microscope. To help them find the sample, scientists could create fiducial markers, which act as landmarks to pinpoint the area of interest — they could be marks etched onto the sample dish, for example. But even then, the process is not straightforward, and correlative microscopy has faced a slow uptake.

BRUKER

"There is no need to switch between microscopes if one instrument integrates both forms of microscopy."

Instrument manufacturers have developed several solutions to address these problems. In the summer of 2010, Carl Zeiss, based in Jena, Germany, released the Shuttle & Find. This consists of a sample holder, enabling tissue sections to be transported safely, and a software module that connects to both microscopes and uses a coordinate system to find the region of interest. According to Kirk Czymmek, director of North American labs at Carl Zeiss Microscopy in Thornwood, New York, this approach offers a way of "relocating the region of interest in different microscope systems within a matter of minutes — a task that until now had taken hours, and sometimes even days".

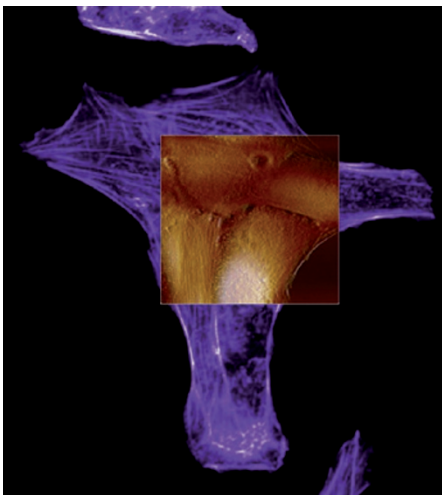
Another way to make finding back easier is to use thicker tissue sections in EM. Cell biologist Judith Klumperman, director of the Cell Microscopy Center at the Utrecht University Medical Center in the Netherlands, says that she uses correlative light–electron microscopy to characterize "distinct endosome populations by their dynamics, interactions, subcellular localization, cargo, protein composition and ultrastructural morphology". After observing the dynamics of fluorescently labelled lysosomal proteins with a light microscope, for example, her group uses EM to study their structural details. "When using thin 80–100-nanometre sections, these structures will appear in only one or two EM sections of the 20 to 40 that are generally obtained from one cell," says Klumperman. "With three-dimensional electron tomography, we can use 300–400-nanometre sections, which increases the chance of finding back the region of interest."

There is no need to switch between microscopes if one instrument integrates both fluorescence and electron microscopy. Furthermore, using the same stage reduces the chance of damaging the specimen and makes it easier to find back the region of interest. Zeiss's Merlin, for example, has all these features and also offers the option of an integrated atomic force microscope (see 'Multimodal microscopy') in the vacuum chamber of its scanning EM. A range of similar correlative microscopes is produced by FEI based in Hillsboro, Oregon.

The ClairScope, produced by Japanese company JEOL, based in Tokyo, has a different solution to the same problems. It has an inverted scanning EM below the culture dish, which can be viewed at atmospheric pressure, and an optical microscope above it. A window coated with a silicon nitride film allows electrons to be projected from underneath while maintaining a vacuum between the EM and the sample dish. This set-up allows researchers to perform concurrent imaging of a sample in solution by both microscopes, says Donna Guarrera, assistant director in the SEM Division at JEOL USA. "There is no running from the optical microscope lab, then preparing the sample to be vacuum-compatible for SEM imaging."

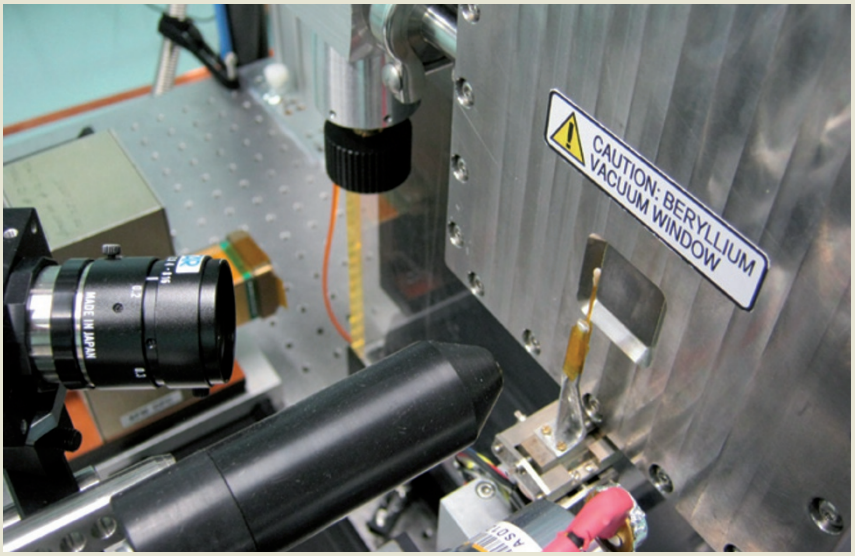
Despite recent improvements, technical difficulties still limit what scientists can accomplish with correlated light and electron microscopy. Jeffrey Caplan, associate director of the University of Delaware's bioimaging centre, finds that the speed of fixation is holding him back — he would like to study live-cell dynamics and then immobilize the cells rapidly for EM imaging. "Current cryo-fixation methods take about 10 seconds to a couple of minutes," he says, "but we would like a tool that can stop movement of dynamic structures in less than one second if possible." This would make it easier to study dynamic events such as vesicle docking and trafficking, cytoskeletal remodelling and calcium signalling[1].

Immunogold labelling has historically been the gold standard for identifying subcellular structures in EM. However, the sheer bulk of gold-labelled antibodies can interfere with the identification of targets with single-molecule precision. Caplan's group is now developing



Correlated epifluorescence (purple) and atomic force microscopy (brown) images of live osteocarcinoma cells stained for actin.

Multimodal microscopy



DARYL HOWARD, AUSTRALIAN SYNCHROTRON

The Australian Synchrotron's beamline allows fine measurements in multimodal microscopy.

Correlative microscopy is becoming more widespread as fresh opportunities to correlate different types of imaging data arise. "Rather than just correlative imaging, the goal is to combine a whole suite of characterization methods into something that might better be described as multimodal microscopy," says Andrew Peele, director of science at the Australian Synchrotron in Clayton, a suburb of Melbourne, Australia. The synchrotron is a circular particle accelerator, about the size of a football field, where strong magnetic fields force high-energy electrons to travel round its tunnels. The intense beams of light it generates, usually optimized in the X-ray region, can be used for imaging experiments that may include X-ray fluorescence, X-ray diffraction, light microscopy, electron microscopy, tomography or infrared microscopy, says Peele.

Despite all these options, multimodal imaging is not straightforward. "One of the challenges we face is comparing information across widely different techniques," says Peele, "especially those with quite different resolutions", such as fluorescence data and high-resolution diffraction data.

Other labs are using multimodal imaging, too. Molly Stevens, a biomedical materials professor at Imperial College London, hopes her research using correlative microscopy will advance our understanding of cells and their environments, perhaps leading to applications such as the design of biomaterials and scaffolds for tissue engineering. Her group is currently developing a protocol for correlating images from electron and ion microscopy, and following it with fluorescence imaging. "Then we will be able to correlate topographical information, ultrastructures of cells and specific biochemical changes using fluorescently tagged proteins," she says. C.S.

an alternative to immunogold labelling for electron and super-resolution microscopy "to ensure that single-molecule localizations are accurately mapped onto the EM image", he says.

LIGHT–ION MICROSCOPY

One drawback of correlative light–electron microscopy is the fixed order of tasks: fluorescence microscopy must be carried out before EM, rather than after it, because the electron beam can destroy the fluorescence signal. It is therefore not possible to verify fluorescence microscopy results after EM. Scanning ion microscopy may provide the answer.

Biomedical materials professor Molly Stevens and her colleagues at Imperial College London recently described correlated light–ion microscopy (CLIM), in which fluorescence microscopy is correlated with ion microscopy[2]. Ion microscopy is similar to EM except that a beam of ions is used to scan the sample, instead of electrons. After imaging the fluorescent signal, Stevens and her colleagues fixed the sample and performed ion microscopy by scanning it with a beam of gallium ions. "I believe that helium ions might also work," says Sergio Bertazzo, a postdoctoral researcher in Stevens' lab. "One concern we have is that helium ions might penetrate much more into the sample, damaging the fluorescence signal." Because the gallium ions did not damage it, the scientists could go back and forth between the two microscopy techniques. They could revisit the fluorescence in the sample after assessing its three-dimensional structure with scanning ion microscopy — several times for the same sample, in fact.

"We have used this to study how cells interact with environments such as biomaterials, scaffolds for tissue engineering, and 2D micropatterned surfaces", which guide cell growth in specified geometries, Stevens says.

Her group has also combined ion microscopy with total internal reflection fluorescence microscopy, a technique that can boost the resolving power to single-molecule resolution near the edges of the cell. The resulting sub-micrometre scale resolution allowed Stevens' group to see a migrating fibroblast in an intermediate step of the migration process, with one edge mobilized for travel and the opposite edge still firmly adhering to the substrate[2].

FLUORESCENCE–ATOMIC FORCE MICROSCOPY

In another approach called atomic force microscopy (AFM), the sample is scanned by the tip of a sensitive cantilever probe, providing high-resolution, three-dimensional, structural information. Unlike electron microscopy, AFM can deliver three-dimensional images of live cells without requiring imaging agents such as fluorophores, and it can detect single molecules with nanometre resolution.

There is a practical reason why microscopy companies sometimes correlate AFM and light microscopy. "AFM can only visualize a very small area at a time, so you are essentially hunting around blind without correlation with light microscopy," says Ben Ohler, product manager for research atomic force microscopes at Bruker, based in Billerica, Massachusetts. "Some basic correlation is simply a requirement to participate in the market."

Nicholas Geisse, a bio-applications scientist at Asylum Research in Santa Barbara, California, which specializes in AFM, thinks that AFM needs to have faster imaging to keep up with millisecond-scale fluorescence microscopy measurements. Asylum's Cypher AFM was designed for faster scanning and data acquisition, he says. "Many of Cypher's technical advancements, including the use of small cantilevers, have enabled high-speed scanning."

Bruker's latest instruments are also designed with speed in mind, says Ohler. "Where AFM images have typically taken minutes per image, Dimension FastScan Bio now acquires images in seconds, or even several images per second," he says. He believes that such technology can lead to AFM measurements of dynamic biological events.

JOE BELCOVSON

A research group in Italy is also heading in that direction. Physicist Alberto Diaspro, director of the nanophysics department at the Italian Institute of Technology in Genoa, is correlating AFM with super-resolution stimulated emission depletion (STED) microscopy to develop tools for topographical imaging, nanomechanical imaging, and measurements of cell stiffness[3]. In STED, researchers intentionally deactivate some fluorophores in part of the sample, which enhances the resolution in that area. Diaspro is optimistic that combining correlative-microscopy techniques with STED's nanoscale resolution will reveal valuable information about cellular nanostructures. The rapid image acquisition possible with STED also holds promise for measuring fast events, such as intracellular vesicle dynamics during secretion or neurotransmitter release, or cytoskeletal remodelling during cell motility.

Clodagh O'Shea and her team use a variety of techniques to investigate viral oncoproteins.

FLUORESCENT BRIDGES

Researchers have long searched for a molecule that can bridge fluorescence and electron microscopy — a fluorescent molecule that also stains in EM images, for example. The ideal tool would be a genetically encodable molecular tag, small enough to penetrate fixed tissues better than awkward gold-labelled antibodies, but fluorescent and capable of delivering good contrast in EM. Two groups at the National Center for Microscopy and Imaging Research at the University of California, San Diego, are investigating a variety of molecules for use in more than one type of microscopy. Mark Ellisman, the centre's director, and biochemist Roger Tsien, who shared the 2008 Nobel Prize in Chemistry for developing the widely used green fluorescent protein (GFP) tag, often collaborate in what Ellisman calls "molecular painting" in the search for a reliable, genetically encodable tag to label proteins in EM. "We were looking for the GFP of electron microscopy," says Ellisman.

Last year, researchers from both groups revealed a molecular tool they had engineered called mini singlet oxygen generator (miniSOG), which is derived from a plant photoreceptor[4]. Singlet oxygen generator molecules are easy to see because they fluoresce in light microscopy and can be stained by diaminobenzidine for EM[5]. The group expressed proteins labelled with miniSOG to demonstrate its utility as a genetically encoded tag for protein targets in EM. "MiniSOG is an extremely good singlet oxygen generator, so we believe this one will get to single-molecule sensitivity," says Ellisman. "We're working on that now."

Meanwhile, miniSOG is already proving useful. Using a combination of microscopy techniques, molecular biologist Clodagh O'Shea and her colleagues at the Salk Institute for Biological Studies in La Jolla, California, are studying the puzzling observation that small viral oncoproteins seem to hijack cellular machinery to stimulate both viral and pathological cellular replication. According to O'Shea, her group wondered: "how do small viral oncoproteins win?" The group imaged infected cells by using miniSOG to label the adenovirus oncoprotein E4-ORF3 (ref. 6). Serial block-face scanning EM gave them reconstructed, three-dimensional views of infected cells. "The scanning electron microscope slices an infected cell from top to bottom in tiny 60-nm blocks," says O'Shea. The group also used electron tomography to make hundreds of 0.5-nm-thick computational slices through cells, and used specialized software to recreate cells from the computed slices. "Three-dimensional reconstructions show that E4-ORF3 assembles into a remarkable network of cables that weaves through the nucleus," she says. The images showed that the weave physically separates viral DNA replication domains from cellular nucleoli. In other words, she says, the viral oncoprotein self-assembles into a trap for tumour suppressors.

"We have been looking for the green fluorescent protein of electron microscopy."

A cousin of miniSOG is also on the horizon. Ellisman and Alice Ting, now at the Massachusetts Institute of Technology in Cambridge, collaborated to create a peroxidase enzyme called APEX, which allows EM staining in relatively thick tissues[7]. Ellisman stresses the importance of genetically encoded tags for miniSOG and APEX. "Both are molecules we can introduce genetically, and both will result in contrast in EM," he says.

Researchers continue to push the limits of correlative microscopy. "We are currently developing a methodology that combines CLIM with super-high-resolution fluorescence microscopy," says Stevens. "If successful, it will offer a new way of studying molecular interactions." Meanwhile, Ellisman and his colleagues are working on expressing two genetically encoded miniSOGs that have different colours. "This is what we call multicolour EM," he says. "The idea is that you would be able to do your dynamic light microscopy, then correlate a high-resolution subvolume down to the molecular scale. It's a hard project, but we know we'll succeed."

Bridging light and electron microscopy with genetically encodable fluorescent tags may one day be as routine as labelling with GFP is today. High-throughput methods applied to tissue sections may make super-resolution microscopy faster and easier. When that happens, two — or perhaps more — microscopes will definitely be better than one. ■

Caitlin Smith *is a freelance science writer based in Portland, Oregon.*

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CORRECTION

The Technology Feature 'Reading the second genomic code' (*Nature* **491,** 143–147; 2012) wrongly stated that Constellation and Genentech are collaborating to develop inhibitors of BET proteins and EZH2 chromatin-writers. Although they are working together on inhibitor development, the targets are not those mentioned.