WHEN CAN WE BELIEVE WHAT WE SEE?

ABIGAIL SAWYER AND JOSEPH MARTIN INVESTIGATE HOW MICROSCOPY IS EVOLVING IN ORDER FOR CELLS AND PROCESSES TO BE BETTER VISUALIZED IN THEIR NATIVE ENVIRONMENTS

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In recent years, super-resolution microscopy has hit the headlines for surpassing the limitations of optical microscopy. Utilizing fluorescent molecules, super-resolution microscopy has pushed past Ernst Abbe's diffraction limit, enabling the visualization of molecular processes on a nanoscale and bringing microscopy into the age of 'nanoscopy'.

In 2014, Eric Betzig, Stefan Hell and William Moerner were all recognized for their efforts within this field with the award of the Nobel Prize for Chemistry [1]. However, even super-resolution microscopy has its own limitations.

As put by Betzig himself, *"it's often said that seeing is believing, but when it comes to cell biology, I think the more appropriate question is, 'When can we believe what we see?'"* [2].

Super-resolution microscopy has faced many challenges with the imaging of live samples. One such problem occurs when trying to visualize live cells in their natural environment. There are many environmental factors that play into the physiology of an organism. Therefore, interactions between components, from molecular to cellular networks, should be visualized in their native form.

However, light can be scattered and deformed by surrounding components, hence visualizing individual cells is generally easier. These microscopes also use extremely intense light, which Betzig believes *"contributes to the fear that we are not seeing cells in their natural, unstressed form."*

On top of this, images blur when a specimen moves under high resolution and the microscopes are too slow to visualize and track cells.

Chemists and physicists have been working in collaboration with biologists to develop new – and improve on current – microscope technologies to ensure that we truly can believe what we see.

Cells: the stars of their own 3D movies

Earlier this year research published in *Science* detailed how a team of scientists, led by Betzig, has created a microscope fast enough to track and record cells in living systems in 3D [3]. The research has accounted for many of the issues associated with super-resolution microscopy. The team at the Howard Hughes Medical Institute's Janelia Research Campus (VA, USA) combined two imaging technologies in order to create 3D movies of cells in their natural environment.

Scientists have been able to image living cells with microscopes for many years. However, the best images have always come from isolated cells because large groups of cells scramble light. *"This raises the nagging doubt that we are not seeing cells in their native state, happily ensconced in the organism in which they evolved,"* explained Betzig.

In this new study, Betzig's group has made use of adaptive optics (AO), which is the same technology used to provide clear views of distant objects in space from Earth. This measures sample-induced distortions to the image of a fluorescent 'guide star' and compensates for these by changing the shape of a mirror to create an equal but opposite distortion.

They combined this with lattice light sheet microscopy to sweep a thin sheet of light through the cell whilst acquiring a series of 2D images to build the high-resolution 3D movie. As the illumination is confined to a thin plane, this reduces anything outside the area being exposed to illumination. The parallel collection of fluorescence also ensures less intrusive intensities are used.

The new microscope, termed AO-LLSM, has already been used to visualize an immune cell engulfing sugar particles whilst wriggling through a zebrafish's ear and a cancer cell rolling through a blood vessel attempting to stick to the vessel wall.

Figure 1. Eric Betzig in 2015. Reproduced with permission from [4].

The next move for the researchers is to ensure the microscope is affordable and user friendly. Currently it fills a 10 ft-long table; however, the team is now working towards ensuring the microscope is affordable for research labs and can fit onto a small desk.

Pushing super-resolution to new heights

As mentioned, researchers are bringing microscopy into the age of ‘nanoscopy’. A recent note-worthy example comes from researchers at Purdue University (IN, USA), who have developed a super-resolution ‘nanoscope’ that provides a 3D view of brain molecules with much greater detail than previously possible [5].

The new technology has helped Indiana University (IN, USA) researchers better understand the structure of plaques that form

in the brain of Alzheimer’s patients, identifying the characteristics that are possibly responsible for damage.

The deposition of amyloid plaques is currently the earliest detectable evidence of pathological change leading to Alzheimer’s disease. The natural thickness of brain tissue when paired with the limited resolution associated with conventional light microscopes has prevented researchers from accurately observing the 3D morphology of amyloid plaques.

“Brain tissue is particularly challenging for single molecule super-resolution imaging because it is highly packed with extra-cellular and intracellular constituents, which distort and scatter light – our source of molecular information,” commented Fang Huang (Purdue University). “You can image deep into the tissue, but the image is blurry” [6].

AO are used by the super-resolution nanoscopes, which Huang’s research team has already developed to visualize cells, bacteria and viruses in fine detail. AO uses mirrors that change shape to compensate for light distortion, also known as aberration, which occurs when light signals from single molecules travel through different parts of cell structures at different speeds.

In order to compensate for the aberration introduced by brain tissue, Huang and his team developed new techniques that adjust the mirrors in response to sample depths. Additionally, these techniques intentionally introduce extra aberration to maintain the position information carried by a single molecule.

Mice that were genetically engineered to develop the characteristic plaques that are associated with Alzheimer’s disease were studied. Through these 3D reconstructions, amyloid plaques were found to entangle surrounding tissue via their small fibers that branch off waxy deposits. The nanoscope reconstructs

the whole tissue at a resolution up to ten-times higher than conventional microscopes, allowing a clear view through 30µm-thick brain sections of a mouse’s frontal cortex.

“We can see now that this is where the damage to the brain occurs. The mouse gives us validation that we can apply this imaging technique to human tissue,” commented Gary Landreth (Indiana University).

Work has already begun using the nanoscope to observe amyloid plaques in samples of human brains, as well as providing a closer look at how the plaques interact with other cells and get remodelled over time.

“This development is particularly important for us as it had been quite challenging to achieve high-resolution in tissues. We hope this technique will help further our understanding of other disease-related questions, such as those for Parkinson’s disease, multiple sclerosis and other neurological diseases,” concluded Huang.

Figure 2. (A) A β plaque at a depth of ~ 24 μm with ~ 3.6 μm thickness. (B) Magnified view of i from A. (C) yz cross-section of B. (D) Equivalent confocal image of the region shown in C. (E) xz cross-section of ii from A. (F) Equivalent confocal image of the region shown in E. (G) yz cross-section of ii from A. (H) Equivalent confocal image of the region shown in G. (J) Magnified view of iii from A. (K) yz cross-section of iii from A. (L) Equivalent confocal image of the region shown in K. The scale bar in A is 5 μm and in B–L is 500 nm. The colored bar in A indicates the depth of the molecules from the surface of the coverslip. This figure represents 37 different imaged plaques. Reproduced with permission from [5].

Figure 3. An optical section of a 12.5 day old mouse embryo, imaged with the mesolens. It is possible to see individual cell nuclei in the inset eye region. It is also possible to see developing heart muscle fibers in the main image. Reproduced with permission from [9].

Adapting super-resolution

Researchers at the National Institute of Biomedical Imaging and Bioengineering (MD, USA) have combined two different microscope technologies to create sharper images of rapidly moving processes inside a cell [7].

The recent research describes new improvements to traditional total internal reflection fluorescence (TIRF) microscopy. TIRF microscopy works by illuminating a sample at a sharp angle so that the light reflects back, illuminating only a thin section of the sample that is exceptionally close to the coverslip. By eliminating much of the background light that conventional microscopes pick up, a much higher contrast image is produced.

One drawback of TIRF microscopy concerns its ability to capture small features within cells. Previously, super-resolution microscopy techniques applied to TIRF microscopes have been able to improve the resolution; however, such attempts have always compromised speed, thus making the imaging of rapidly moving objects extremely difficult. Consequently, a vast majority of cellular processes remain too small or fast to observe.

Hari Shroff, chief of the National Institute of Biomedical Imaging and Bioengineering lab section on High Resolution Optical Imaging noticed that if they modify a high-speed, super-resolution microscope to act like a TIRF microscope, they could obtain the benefits of both technologies.

Developed by the Shroff lab in 2013, instant structured illumination microscopy (iSIM) can capture video at 100 frames per second. However, iSIM falls are inferior to TIRF microscopes in terms of contrast. In order to combine the strengths of both types of microscopy, the team blocked most of the illumination from the iSIM, using a simple 'mask'. This combination of both iSIM and TIRF allows for observation of rapidly moving objects about ten-times faster than other microscopes at a similar resolution.

"TIRF microscopy has been around for more than 30 years and it is so useful that it will likely be around for at least the next 30," commented Shroff. "Our method improves the spatial resolution of TIRF microscopy without compromising speed – something that no other microscope can do. We hope

it helps us clarify high-speed biology that might otherwise be hidden or blurred by other microscopes so that we can better understand how biological processes work" [8].

Interestingly, this new technology has promising applications and was used to follow the rapidly moving Rab11 particles near the plasma membrane of human cells, which are usually blurred when imaged by other microscopes. With the new microscope, Shroff and his team also revealed the dynamics and spatial distribution of HRas, a protein that has been implicated in facilitating the growth of cancerous tumors.

Optical microscopy: teaching an old dog new tricks

In order to study intact specimens without compromising detail, a team from the University of Strathclyde (Glasgow, Scotland), led by Brad Amos, has developed a new microscope lens for optical microscopy – the Mesolens [9]. It was named as one of Physics World's Top Ten Breakthroughs in 2016 [10].

"It has the large coverage of a photographic camera lens and the fine resolution of the microscope objective, so it has the advantages of the two approaches," explains Amos. "The images are extremely useful."

The Mesolens has a lower magnification of only four-times, but it has a wider field of view, which enables it to capture up to 5 mm. It can image live specimens and the team has already utilized it to watch a mouse embryo develop in real time.

"It's almost unheard of for anyone to design a new objective lens for light microscopy and we have done this to try to accommodate the new types of specimens that biologists want to study," commented Gail McConnell (University of Strathclyde).

"If you develop an objective different than anything that has been made for the past 100 years, it opens up all kinds of unknown possibilities," concludes Amos. "We are just beginning to get at what those possibilities are."

So, when can we believe what we see?

The field of microscopy is continuously evolving and doing so at an incredible pace. These recent innovations are just more proof of scientists' ability to recognize a problem, confront it and find a solution.

Whilst it seems this is an incredibly philosophical question to warrant a simple answer, these new technologies are already being utilized to visualize cells and processes in an environment that is as close to native as has ever been before. It does appear that if not now, in the near-future, we can confidently say that we can believe what we see – most of the time.

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BioTechniques 65: 245–249 (November 2018)
10.2144/btn-2018-0151

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