

Microscopy: seeing through tissue

Vivien Marx

Tissue samples scatter light and shut out a deeper gaze. Tissue-clearing agents have changed that, but scientists must consider optimizing the optics when using these methods.

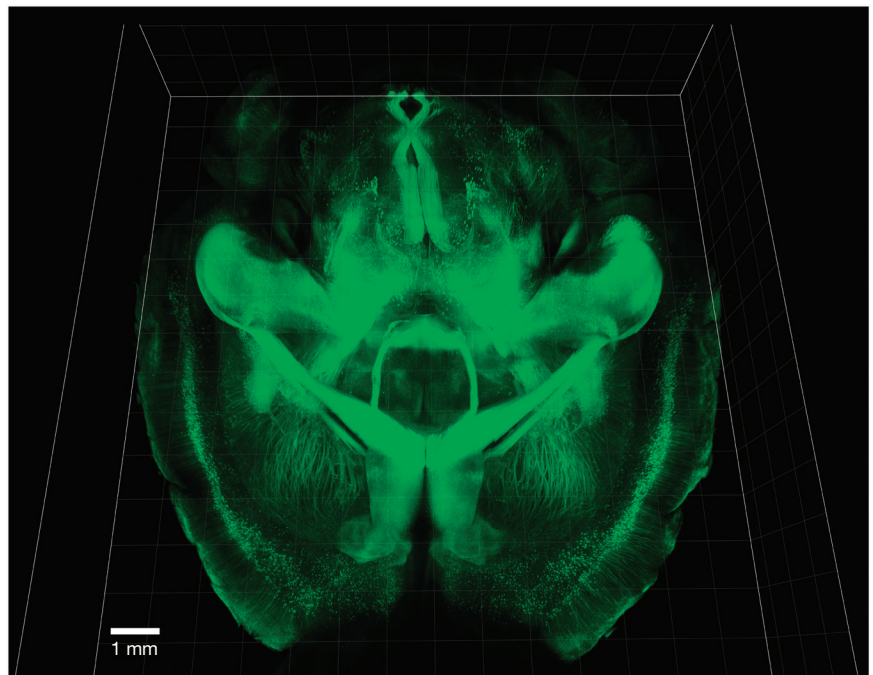
To study the causes of heart attacks, German anatomist Werner Spalteholz developed a way to render heart tissue transparent with a mixture of benzyl alcohol and methyl salicylate. He displayed 370 of his cleared specimens at an exhibition in 1911.

Spalteholz's method was fashionable at the time, point out Philipp Keller from the Janelia Farm Research Campus of the Howard Hughes Medical Institute and Hans-Ulrich Dodt of the Vienna University of Technology, who work on clearing agents and microscopy techniques¹. Yet his approach did not have a strong scientific impact, even though researchers could have applied the earliest versions of light-sheet microscopes to image these samples.

Considering the ways tissue clearing is affecting many fields, particularly neuroscience, the historic lack of scientific impact is striking, says California Institute of Technology (Caltech) researcher Viviana Gradinaru. She was a research associate in the lab of Stanford University researcher Karl Deisseroth, a Howard Hughes Medical Institute investigator who led the development of the clearing agent CLARITY².

In addition to CLARITY, there are a number of other agents, including CUBIC, LUMOS, Scale, SeeDB and 3DISCO, that render opaque tissue transparent. Their use helps to image, label and identify structures at single-cell resolution. A cleared mouse brain sample allows the imaging of structurally intact brain circuits.

The ability to see biological structures in their three-dimensionality enables new types of experiments with fluorescent probes, thereby highlighting particular features and functions. *Nature Methods* spoke with clearing agent developers and microscopy companies about optimizing clearing agents, immersion media and microscope objectives.



A mouse brain cleared with CLARITY and imaged with a 10× water-immersion objective.

Customized objectives

A number of specialty objectives customized for clearing agents either exist or are in development. For example, Leica sells an objective for CLARITY; Olympus sells objectives for CLARITY, Scale and SeeDB. Zeiss offers objectives that can be used for several clearing agents (CLARITY, Scale, CUBIC, LUMOS and glycerol-water mixture) and the company's light-sheet fluorescence microscope (**Table 1**).

Until recently, there were no such objectives. Deisseroth says he and his team completed the work for the first CLARITY paper with off-the-shelf objectives. Gradinaru is happy to see that microscopy companies have responded to an emergent need over the last year or two. "We hope for more objectives to come," she says, "which

will drive better capabilities and hopefully affordable objectives, too."

Deisseroth says that researchers who want to image the brain's axons at high resolution need an appropriate objective. Without financial involvement, he and his team have advised microscope manufacturers on objective design and specifications. This type of interaction with companies is also true for the developer of Scale³, Atsushi Miyawaki of RIKEN's Brain Science Institute and for Takeshi Imai of RIKEN's Center for Developmental Biology, who developed SeeDB⁴.

When Miyawaki and his group developed Scale, they sought a clearing agent with a refractive index (RI) similar to that of water, because of the existing water-immersion objectives with long working

TECHNOLOGY FEATURE

Table 1 | Some objectives customized for clearing methods.

Model (manufacturer)	Numerical aperture	Clearing agent or immersion medium	Working distance	Refractive index range
HC FLUOTAR L 25×/1.00 IMM (n _e = 1.457) motCORR VISIR (Leica)	1.0	CLARITY	6 mm	1.45
10× UIS2- XLPLN10XSVM (Olympus)	0.6	CLARITY, glycerol, Scaleview-A2, SeeDB, water	8 mm	1.33–1.52
25× UIS2-XLSLPLN25XGMP (Olympus)	1.0	Glycerol, CLARITY, SeeDB	8 mm	1.41–1.52
LSFM Clearing 20×/1.0 (Zeiss)	1.0	CLARITY, CUBIC, LUMOS	5.6 mm	1.45 ± 0.03
LD Plan-Apochromat 20×/1.0 (Zeiss)	1.0	Scale	5.6 mm	1.38 ± 0.03
EC Plan-NEOFLUAR 5×/0.16 (Zeiss)	0.16	CLARITY, CUBIC, LUMOS	5.6 mm	1.45

distances (WD) and high numerical aperture (NA).

Miyawaki's original clearing reagents, including ScaleA2 and ScaleU2, have refractive indices of 1.34, which is close to that of water. After the Scale paper was published, new clearing methods emerged with higher RIs. These can no longer be imaged with conventional water-immersion, long-WD objectives, he says. Addressing the desire of scientists to image deep into tissue in a high-RI medium, companies have advanced objective technology, says Miyawaki, developing new models with long WDs and high NA. "And I am watching their progress with great interest," he says.

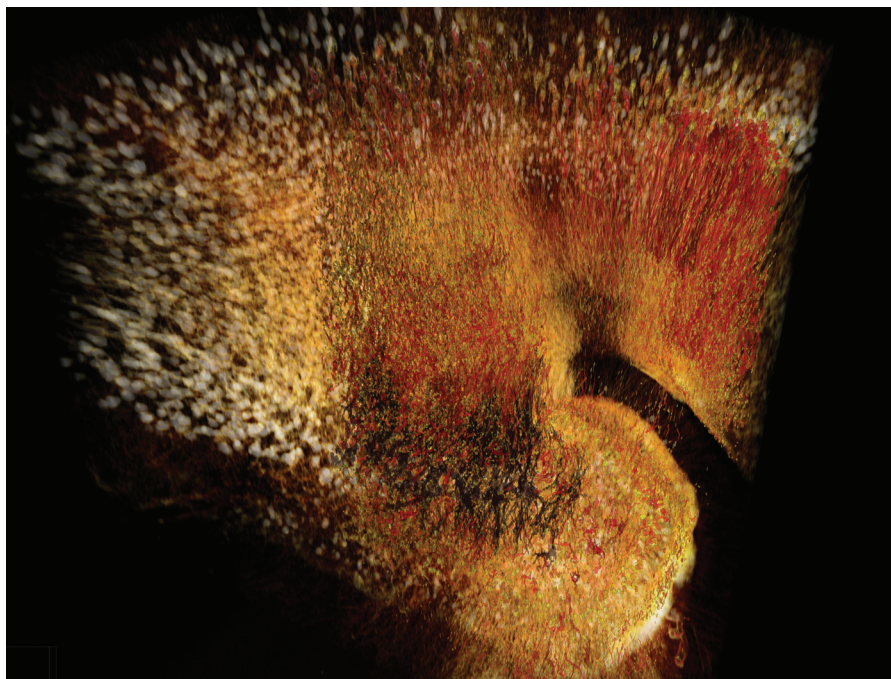
SeeDB developer Imai says that using conventional objectives is not a problem when imaging relatively thin samples. However, aberrations increase with imaging depth.

At 1.5 mm depth, the lateral resolution in a sample is worsened by around

twofold, and the axial resolution by around fourfold, when a water-immersion lens is used to image samples cleared with SeeDB. Fluorescence intensity also decreases, Imai says. For higher-resolution or deeper imaging, new index-optimized objective lenses are more powerful, he says.

When using a nonoptimized objective, says Imai, it is best to use the one designed for the RI closest to that of a chosen clearing agent and immersion medium. For example, glycerin with an RI of 1.47 and an oil immersion lens with an RI of 1.52 should be fine for SeeDB, which has an RI of 1.49. However, these lenses typically have short WDs. Water-immersion lenses with an RI of 1.33 work better than air-immersion objective lenses with an RI of 1.0 for SeeDB, says Imai.

Apart from the spherical aberrations that will occur with nonoptimized objective lenses, depth measurements can be



Mouse brain tissue cleared with Scale and imaged using a customized objective. Nearly 9,000 micrographs are stitched together for this image.

Olympus, data from A. Miyawaki, RIKEN

BOX 1 MAKING A CUSTOMIZED OBJECTIVE

Leica's R&D team decided to design and manufacture an objective tailored to CLARITY to help scientists image as much intact tissue as possible, at high resolution. The larger the NA, says Leica objective designer Christian Schumann, the more challenging things get for the optics.

To develop the objective, Schumann and his colleagues measured the optical characteristics of tissue cleared with CLARITY, working with samples from the labs of Kwanghun Chung, a CLARITY co-developer at the Massachusetts Institute of Technology, and Deisseroth.

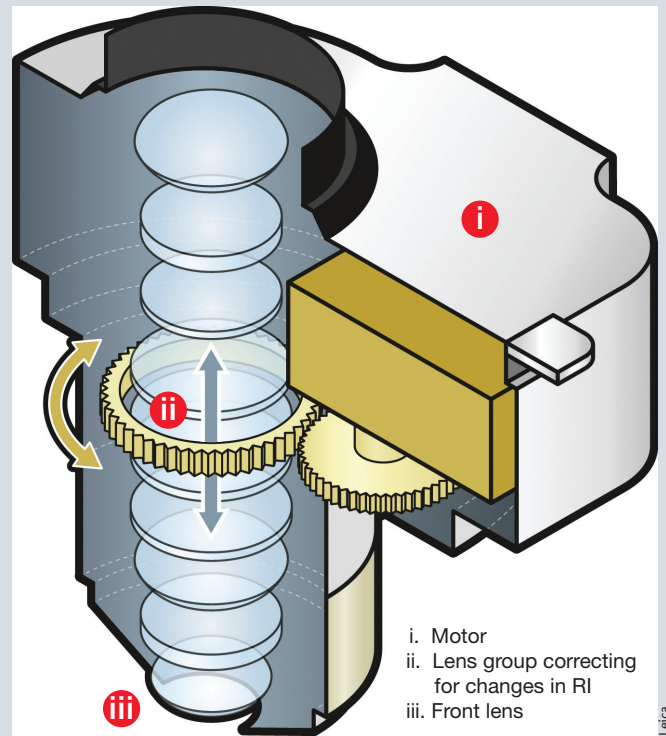
The company drew on experience with its objective for an early clearing agent, benzyl alcohol–benzyl benzoate (BABB). Because it clears samples differently from CLARITY, that objective could not be adapted for CLARITY, but the company had a design process in place, for example, for high-precision measurements of light-dispersion characteristics and refractive indices (RIs) in such samples. The design also had to include optical correctives because the RI is not constant throughout every cleared tissue sample. "The objective has to adapt and be tolerant to error," says Bernd Sägmüller, who oversees confocal microscopy at Leica.

Creating new objectives involves selecting from hundreds of types of glass for the dozen or so lenses used in a high-performance objective. Lenses have different radii and thickness. Tiny variations make a big difference, says Schumann.

Glass affects fluorescence detection. Chromatic aberration, in which light of different wavelengths is focused differently, needs to be corrected so the light comes into focus in the same focal plane.

Light passes not only through the cleared sample but also through immersion medium, which has its own RI. Testing and adjusting the performance of an objective's lenses is always tricky, but especially so with immersion liquids used with cleared samples. These media can contain bubbles and temperature gradients, which change the RI and affect imaging quality.

At first these factors led to oscillating, unacceptable fluctuations on Schumann's interferometer. But he and his colleagues in the manufacturing and application departments adjusted all of the optical components. The objective is



The objective is equipped with a motorized correction collar for researchers to adapt the optics remotely.

equipped with a motorized correction collar for researchers to adapt the optics to different experiments. The Leica objective optimized for CLARITY can be used for multiphoton or single-photon experiments with a variety of fluorophores. "There is a bouquet of parameters that cannot all be optimized," says Sägmüller. "There is no objective that will be perfect in all respects."

These optics challenges explain why scientists often visit microscope manufacturers with samples in hand. Microscopy companies tend to have open-door policies about such visits.

imprecise, so scientists will want to perform axial calibration.

The high RI itself is the key when clearing biological samples, Imai says, which is why most new clearing techniques have high RIs and the future ones will probably be similar. As such, long-WD, high index–optimized objective lenses will probably become more popular in bioimaging, says Imai.

Given the price of specialty objectives, Caltech's Gradinaru hopes it will not be necessary to have an objective for each clearing agent. It also takes time for manufacturers to design and make objectives.

Scientists will pick and optimize the clearing approach most suited to their experiment, says Annette Bergter, who is

product manager for the Zeiss Z.1 light-sheet microscope. Not every tissue is suited for the methods that work well on brain tissue.

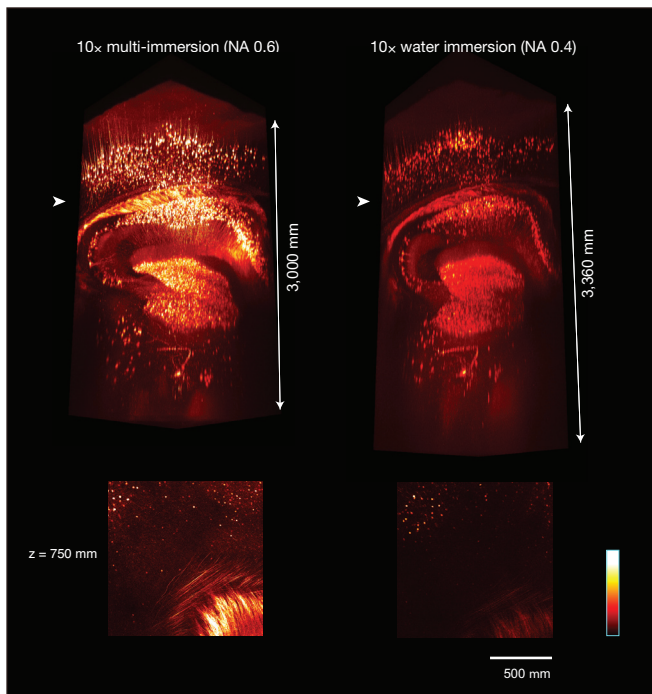
Deisseroth and his team developed CLARITY-optimized light-sheet microscopy (COLM), which allows for high-speed data collection. It is up to 1,000 times faster than conventional scanning methods and offers less photobleaching, he says. They use Olympus objectives optimized for CLARITY with this imaging method. When CLARITY and light-sheet microscopy are used together in this way, "it becomes increasingly plausible to have an objective working to support many projects or even labs at once, so the cost should not be limiting," says Deisseroth.

Objectives and RIs

There are trade-offs between WD, NA and correction of various imaging errors, which all must be integrated in a microscope objective and also matched to the RI range of both the immersion liquid and the cleared tissue sample.

WD is the distance between the objective's front edge and the focal plane at which an area of the sample to be studied is in focus; it roughly describes the depth to which a researcher can image into a sample. NA determines how much light enters the objective and the objective's ability to capture a sample's detail.

As Leica microscope objective designer Christian Schumann explains, a traditional objective does not have the longer WD



A mouse brain cleared with SeeDB and imaged with two objectives shows differing brightness and spatial resolution. The water-immersion lens (right) has a lower NA than the multi-immersion lens (left).

Mt. Ke. I. Linai, RIKEN CDB

needed to deliver crisp images from cleared samples. Schumann was part of the team that developed Leica's CLARITY objective (Box 1).

Clearing agents enable deep 3D imaging and capture images of neuronal circuits that must be assembled. But, says Bernd Sägmüller, who oversees confocal microscopy at Leica, blurring can hinder assembly into a full 3D rendering of the cleared tissue. The deeper the microscopist's gaze, the weaker the fluorescent readout can be, if the fluorophore's signal does not make it to the objective, explains Sägmüller's colleague Andrea Mülter, who directs Leica's activities related to confocal microscopy applications, including clearing agents. When that happens, researchers tend to then turn up the laser intensity, says Sägmüller. But the lots-of-light approach will not help much, he says. It tends to bleach fluorophores sooner and can accelerate phototoxicity.

Zeiss's Bergter explains that clearing solutions have RIs ranging from 1.38 to 1.55 (the RI of air is 1; water's is 1.33; oil's is 1.52). The wealth of clearing methods and immersion media challenge objective designers, but Zeiss researchers took note that many of the new clearing agents have an RI of around 1.45.

At first glance, says Bergter, objective design appears straightforward: maximize

an objective's WD, NA, magnification and color stability. But there is a trade-off between these parameters. Maximizing NA can reduce the possibilities of aberration-free imaging, for instance. "It is still an open question which clearing agents will establish themselves in research, which means we have to practice flexible optical systems design," says Bergter.

Zeiss developed objectives optimized for use with clearing agents and its Z.1 light-sheet microscope. Light-sheet microscopes image samples with much less photobleaching and at higher speeds than scanning microscopes. The company developed two objectives for light-sheet microscopy on cleared samples: a 5 \times objective for an RI of 1.45 to image larger sample areas and a 20 \times objective for an RI of 1.0 to obtain high-resolution images of the same sample.

Olympus's first customized objective for clearing agents was for Scale, which Olympus sells as Scaleview. The team

studied how a cleared sample scatters light and how to match the RI of tissue and the clearing agent, and designs that capture as many photons as possible from the sample, says Brendan Brinkman, who is in Olympus's product-planning department. On the basis of the physics of lenses, there are trade-offs when adjusting for a wide range of RIs while offering high resolution and long WD.

The first Olympus objectives for cleared samples were 25 \times with 4-mm and 8-mm WDs. Longer WDs tend to mean lower resolution. The engineers improved the lens design to increase the resolution of the 8-mm objectives, Brinkman says. The Scaleview objectives were originally designed for work with cleared, fixed tissue, but they have also been used in *in vivo* experiments. The company now sells a 10 \times lens with a WD of 8 mm that can be used for Scale, CLARITY and SeeDB for cleared fixed tissue and, separately, for *in vivo* experiments. The Scaleview objectives can match the RIs of various aqueous immersion solutions.

Immersion media

Immersion media have to match clearing agent and budget. The protocol for CLARITY includes the immersion medium FocusClear, which is expensive, acknowledges Deisseroth. But FocusClear is not absolutely obligatory, he says, as there are several home-cooked alternatives (Box 2). One of them is RI Matching Solution (RIMS), developed in Gradinaru's lab⁵.

Adapting the immersion medium is a way to work with the optical requirements

BOX 2 HOME-COOKED IMMERSION MEDIA

In addition to using commercial immersion media, many labs develop their own to cut down on costs. Viviana Gradinaru and her colleagues at the California Institute of Technology developed recipes for immersion media that can be mixed in the lab⁵.

Refractive index matching solution (RIMS):

Dissolve 40 g of Histodenz in 30 ml of 0.02 M phosphate buffer with 0.01% sodium azide and bring pH to 7.5 with NaOH. This leads to a final concentration of 88% (weight by volume) Histodenz with a refractive index (RI) of 1.46. Estimated cost: \$3 per milliliter.

sRIMS:

Combine 70% sorbitol (weight by volume) in 0.02 M phosphate buffer with 0.01% sodium azide, bring pH to 7.5 with NaOH. Cost: \$0.2 per milliliter.

In Gradinaru's lab, RIMS outperformed sRIMS in terms of resolvable imaging depth. Sorbitol is commonly available in labs and is thus a convenient and cost-effective alternative to glycerol-based mounting solutions.

of cleared samples without resorting to different objectives, says Gradinaru. Many labs trying CLARITY expressed concern about the lack of availability of FocusClear and its cost. This concern led her to develop RIMS so that work with CLARITY and other clearing approaches is easier and costs less. RIMS can be made in the lab, and samples can be stored in it for months without damaging fluorescence, Gradinaru says. Her lab has also obtained good results with an affordable sorbitol solution called sRIMS. When it comes to immersion media, Gradinaru points out that no single solution fits all. Labs will be interested in tissues with different properties. Tissues might be more or less dense, myelinated or not or more or less fatty, collagen-rich or protein-rich.

These variables and the fact that only certain microscope objectives might be available in a lab mean that researchers will look for a solution with a particular RI. For their paper⁵, Gradinaru and her colleagues used mainly one RIMS formulation, but the team shows how to make RIMS with RIs ranging from 1.36 to 1.50.

Many clearing agents are mixtures of several chemicals, which leads to inhomogeneous optical characteristics that detract from image quality, says Zeiss's Bergter. Scientists can troubleshoot these issues by keeping the temperatures of the clearing agent constant and handling the solutions with care to avoid turbulences in the liquid, for example.

SeeDB developer Imai also points out issues relating to RIs when imaging cleared samples. Biological samples contain 'high-index' components, such as lipids, which calls for media with RIs of around 1.5 (CLARITY removes lipids from samples; SeeDB does not). Imai and his team originally used a conventional confocal microscope and air-immersion dry objective lenses. They imaged 500–1,000 μm deep, which sufficed for their studies of the mouse olfactory bulb. But Imai wanted to try other imaging techniques, and his experience has heightened the importance of immersion media to him.

Battling blur

Imai says that Miyawaki's *Scale* paper³ is what encouraged him to publish his fructose-based clearing agent, SeeDB. It also encouraged him and his group to try two-photon imaging of a SeeDB-cleared sample. They found that they could image

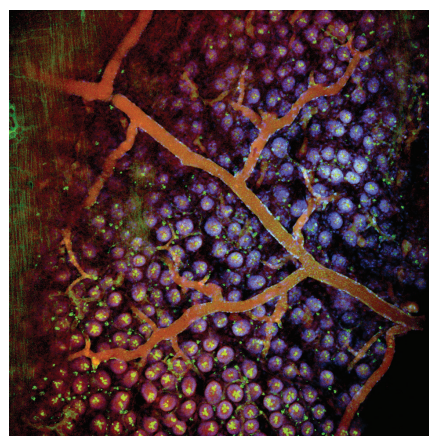
as far as the objective's WD of 2 mm allowed.

When they tried a *Scaleview* objective (the Olympus lens customized for *Scale*), Imai invited the company's objective developers to the lab. "The results were surprising to us," says Imai, because of how well the objective worked. They imaged up to the objective's 4-mm WD. But as they imaged deeper, both the resolution and brightness decreased, and it was difficult to see fine structures beyond 3 mm.

A point-spread function analysis revealed that the result was not due to insufficient transparency, but rather to aberrations. The RI of *Scale* did not match SeeDB. Imai and Olympus began talking about a customized objective for SeeDB, which Olympus eventually made. Imai says the company used its experience designing *Scaleview* to work on an objective for SeeDB that would deliver crisp, bright images. But the customized lens delivered blurry images that were much worse than those captured with other objectives, says Imai.

The problem lay not with the objective but the immersion medium. SeeDB had been used as a clearing and immersion medium, but during long-term imaging, the water gradually evaporated from the surface of the immersion medium, creating a subtle gradient in its RI. "Because of its longer working distance, the new objective lens was more sensitive to the subtle inhomogeneity of the refractive index," Imai says.

The researchers replaced the immersion medium with a less viscous liquid of the same RI, namely 90% 2,2'-thiodiethanol (TDE). And the problem was solved. The



Clearing methods keep evolving. Here, CLARITY that is distributed via the vasculature which shows mouse intestinal tissue.

solution maintained a uniform RI throughout and enabled the researchers to image a cleared mouse brain from top to bottom using the customized objective, says Imai. The scientists could trace individual callosal axons across brain hemispheres, an imaging task not easily accomplished with existing objectives. On the basis of this experience, the team built a two-part imaging chamber; the bottom part contains the sample mounted in SeeDB and is sealed to avoid evaporation⁶. The upper chamber is capped with a coverslip and contains the TDE immersion medium.

Looking wide

It is too time consuming to image a large area using a 25 \times objective with a WD of 8 mm, says Imai. "Stitching of multiple tiles of images was also tricky," he says. He points out Olympus has a 10 \times objective with a WD of 8 mm, which made it easier to image over larger areas. The Olympus objectives all have correction collars and cover the RIs of various clearing agents. Deisseroth and his group recently used the Olympus 10 \times lens to image CLARITY-cleared samples with COLM.

In Imai's view, combining a long-WD objective and light-sheet microscopy may be the most efficient path to plan how to image all structures in cleared brains from mice. Given that NA worsens as WD increases and NA determines image resolution, there are necessary trade-offs when setting out to image the whole body of a model organism such as a mouse, or a larger brain such as that of a rat or a marmoset, says Imai. Or it means facing the challenge of designing an objective with a larger diameter lens, he says.

Another aspect to keep in mind, he says is the possibility of morphological changes caused by a clearing agent, although such changes might not be a problem in low-resolution imaging. SeeDB does not cause such changes, he says, but CLARITY and CUBIC involve some tissue swelling. "Even if you use correct optics, you will not get correct images from deformed samples," says Imai.

Deisseroth highlights that CLARITY-based tissue swelling is transient and less extensive than the swelling that occurs with other clearing methods. The sample returns to its original dimensions in the final RI-matching step. Deisseroth also points out that CLARITY has changed⁷. Originally, it involved an electrophoresis step. But in the next-generation version of CLARITY, that

step can be eliminated and replaced with a passive clearing step. Over the course of two weeks, an adult mouse brain can be cleared and readied for deep macromolecular labeling, says Deisseroth.

What comes next

Clearing agents make up a dynamic research area with which objective design will be keeping pace. As with any new method, the research community is now “at version 1,” similarly to the situation with optogenetics between 2005 and 2007,

says Gradinaru. She and her colleagues addressed and solved the optogenetics challenges one by one. In the same vein, developers are still troubleshooting clearing agents and applying them in new ways, such as to bones, work that is ongoing in her lab.

With such developments, “it takes many a village,” she says. She hopes that as scientists continue to work with clearing agents and immersion media they will keep communicating successes and challenges to allow the field to thrive.

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