In just 20 hours, a zebrafish embryo advances from a single cell to a 20,000-cell organism capable of twitching its tail. Philipp Keller, as he began his Ph.D. in 2005, wanted to watch how that complexity is built up in such a short time. With embryonic cells migrating as fast as several microns per second, he needed high-speed video that could capture the process.

The answer was light-sheet microscopy, says Keller, now a biologist and physicist at Janelia Research Campus near Ashburn, Virginia. The concept is simple, even if the internal optics are not: Illuminate an entire plane of the sample at one time, take a widefield image, then move the plane. This method obtains 3D, live volumes much faster than the point-by-point scanning of a typical confocal microscope. It also exposes individual cells to less toxic light than other techniques do. Thus, light-sheet systems have become the next wave in live-cell imaging for many scientists interested in high-speed cellular activities, such as the firing of neurons or the flowing of blood cells. Other techniques advancing in the live-imaging space include multiphoton microscopy and label-free processes such as Raman microscopy and third harmonic generation imaging.

“Live-cell imaging is—and has always been—the ultimate thing you can do with a microscope,” says Christian Hellriegel, an applications specialist at ZEISS Microscopy in Jena, Germany. It goes beyond the structure of life and gets to biological processes, including how cells or molecules move, how cells respond to their environment or neighbors, and how the brain works or how injuries heal, he says.

Companies and scientists are developing an array of options, all of which boil down to the following trifecta, says Chris Xu, an applied physicist at Cornell University in Ithaca, New York: “Everybody wants to go deeper, everybody wants to go faster, everybody wants to go wider.”

Deeper: Multiphoton imaging

To achieve greater depth in microscopy, one solution is multiphoton imaging, says Julian Burke, chief scientific officer of Leica Microsystems, in Cambridge, United Kingdom. Leica just launched SP8 DIVE, a multicolor two-photon system that looks deeply into live tissues. The trick is that instead of one photon, it uses two longer-wavelength photons to excite the fluorophore. Those redder, longer light waves can penetrate deeper into tissues—up to 500 microns at optimal conditions, says Burke—because they’re less likely to be scattered by the tissue itself. They’re also less toxic and less likely to bleach fluorophores, he adds.

David Monchaud, a chemist at the University of Burgundy in France, was drawn to multiphoton imaging for its precision, sensitivity, and ability to image living cells. He studies DNA and RNA quadruplexes, nucleic acid structures that were first visualized via confocal microscopy in fixed, dead cells. Using multiphoton microscopy, he could confirm that quadruplexes exist in living cells, indicating they likely have some function there. “This microscopy was invaluable,” says Monchaud.

If two photons are good, might three be better? That’s what Xu is investigating. By requiring three photons—of very long wavelength—to activate a fluorophore, he can peer even deeper into a living sample. In 2017, he reported visualization of mouse neurons firing at a depth of 1 millimeter. The microscope could image right through the dense, light-scattering white matter, a feat no prior microscope could achieve, says Xu. Several labs are using the technology to study topics such as cell migration and neuroscience, he says.
Faster: Sheets of light

Multiphoton imaging still relies on slow point-scanning, though. “Speed and depth is always the trade-off,” says Xu. And Keller wanted his entire zebrafish embryo scanned in 60–90 seconds, so light-sheet microscopy was the answer.

While light-sheet microscopy is an old idea—scientists at ZEISS Microscopy and collaborators first came up with it in 1903—only in this century has the convergence of fluorescent labels that work to process image volumes combined to make light-sheet mainstream. The ZEISS Lightsheet Z.1 microscope includes a life-support chamber that can hold entire organisms—a fruit fly or even a small octopus, for example—dangling in front of the objective, and permits easy rotation for a better point of view.

A 2008 version of Keller’s light-sheet microscope, called digitally scanned laser light-sheet fluorescence microscopy (DSLM), gave him the speed he craved: 1.5 billion voxels per minute. The design differs from that of a conventional commercial microscope: Keller placed the laser and objective lens at right angles to each other atop a table; then he embedded zebrafish embryos in agarose in a sample tube between the two. He translocated the sample back and forth through the light sheet to hit all planes. He could observe never-before-seen instances of zebrafish embryo development, and the formation of germ layers.

Since then, Keller has updated the technology. One issue was that if a sample is large or opaque, the light sheet might not reach all the way through it. To deal with this, Keller developed SiMView: He doubled the light sheets and cameras to collect four images within 20 milliseconds or less, because each camera can focus on the two sheets in rapid succession.

Another issue he tackled was anisotropy, that is, the fact that a single objective will typically give better resolution in the lateral xy direction than in the axial z direction. To create a 3D image with equal resolution at any viewing angle—a technology called IsoView—Keller doubled the number of cameras and sheets again to four, and digitally combined those images.

At the National Institute of Biomedical Imaging and Bioengineering in Bethesda, Maryland, microscopist Hari Shroff is also interested in developmental neuroscience. In collaboration with scientists at Yale University in New Haven, Connecticut, and the Sloan-Kettering Institute in New York, he’s imaging how the brain of a nematode develops.

“The worm embryo: It’s hard to image, and it’s very sensitive to light,” says Shroff. “You really have to be fast.”

He and his colleagues wanted to avoid the classic light-sheet arrangement in which the sample is embedded in a tube of agarose and surrounded by lasers and cameras. Instead, they preferred to put the worm embryos on standard glass coverslips.

To get the incoming light sheet and observing objectives at right angles to each other, Shroff’s solution was to tilt each by 45 degrees from vertical. The apparatus looks much like a standard microscope, except for the two askew objectives aimed at one stage. The sample stays stationary, and the light sheet from one objective and the other detection objective move in tandem. The system is called inverted selective plane illumination microscopy (iSPIM).

Like Keller, Shroff also dealt with the anisotropy issue, but he didn’t add any more objectives to do it. He simply set each to either produce a light sheet or collect an image, and to alternate between the two. First, objective A produces the sheet and objective B the image, then they swap. The researchers called this arrangement symmetrical dual-view iSPIM (diSPIM).

But Shroff and colleagues weren’t finished. In another version they call triple-view SPIM, they added an extra objective below the coverslip, to collect extra output and improve spatial resolution at no cost to speed, and with no added dose of light. Finally, the researchers experimented with performing diSPIM with a mirrored coverslip. This creates a “virtual image” beyond the looking glass, as if the sample and the light sheets were doubled. With the right algorithms to make sense of it all, Shroff and his team can pull information from those reflections. The result is more sensitive imaging at twice the speed, all for the cost of an aluminum-coated coverslip.

Wider: Living, moving landSCAPEs

Elizabeth Hillman, a biomedical engineer at Columbia University in New York, has developed a light-sheet technique that uses only a single objective to both produce the light sheet and to collect all the signals from the sample—which could be an entire, freely moving organism. The system uses a mirror to sweep the light—and the focal point of the camera—through a sample. She refers to her system as “swept confocally-aligned planar excitation” (SCAPE) microscopy.

As with other new light-sheet techniques, SCAPE has the advantage of tremendous speed. With newer cameras, Hillman is imaging more than 100 volumes of sample per second. That allows her to image the cells of awake, moving creatures, such as the flashing neurons of crawling fly larvae or the beating hearts of twitching zebrafish, without the problem of blurring when the animal moves. “We’re going so fast that we can see timing information no one’s ever seen before.” cont.>
Labels and the lack thereof

Sometimes, scientists don’t want to use fluorescent tags. For one thing, it can be tricky to introduce them into samples. It’s also hard to be certain that the fluorophore accurately reflects the location and activity of the target protein. The label might not find the protein efficiently, or might photobleach over time.

Biophysicist Hyungsik Lim at Hunter College, City University of New York, uses third harmonic generation (THG) microscopy to image myelin—the “insulation” around nerve “wires”—in live cultures and tissues without adding any labels. THG generates a signal when the energy from three incoming photons is combined into one outgoing photon. The technique is particularly sensitive to boundaries where the refractive index of a tissue changes, such as those between aqueous solutions and lipid- or protein-rich structures, such as myelin.

Another option is Raman microscopy, a scanning version of Raman spectroscopy, says Katsumasa Fujita, an applied scientist at Osaka University in Japan, who is introducing Raman to the microscopy world. Raman spectroscopy relies on the incoming light of a single wavelength to excite the molecules in a sample. The photons that make up this light bounce, or scatter, off the molecules in the sample, mostly at the same wavelength they had coming in. But every so often (about one in 100 million photons), a photon will bounce off with a different wavelength, shifted to a lower, more reddish frequency. The frequency of the shifted light depends on the molecule it scattered from. In this way, Raman spectroscopy can identify the components of a sample.

Under the life-science microscope, Raman scattering works in a similar way, providing a profile of the component molecules in a specimen—whether it is DNA, protein, or lipid. However, it can’t distinguish much beyond that; for example, it can’t tell one kinase from another.

Meanwhile, researchers at Columbia University in New York are working on a way to add dozens of color labels to Raman-imaged cells. Traditional fluorescence imaging maxes out at around five colored labels, because the broad ranges of wavelengths emitted by the fluorophores overlap, points out biophysical chemist Wei Min. But the wavelengths emitted in Raman spectroscopy fall into a much tighter range, so it ought to be possible to use many more colors without that overlap.

A former postdoc of Min’s, Lu Wei, now a chemistry professor at the California Institute of Technology in Pasadena, took on the challenge. She and her colleagues designed 24 different fluorescent dyes by varying the triple carbon–carbon bonds, triple carbon–nitrogen bonds, and isotope content to create different colors.

Now, Wei and Min are working on more colors and methods to link the dyes to specific biomolecules or organelles of interest—Min thinks 50 or more colors should be possible.

It’s easy to adapt the SCAPE objective to a variety of sample types, says Hillman. The cells or organisms could be in a plate or dish, or in the head of a mouse; it doesn’t matter.

Burke predicts more fluorescent proteins and dyes that indicate cellular events (e.g., neurotransmission) will make real-time, rapid imaging appealing to many scientists. He also anticipates that faster, more sensitive cameras will be needed—for speedy imaging.

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