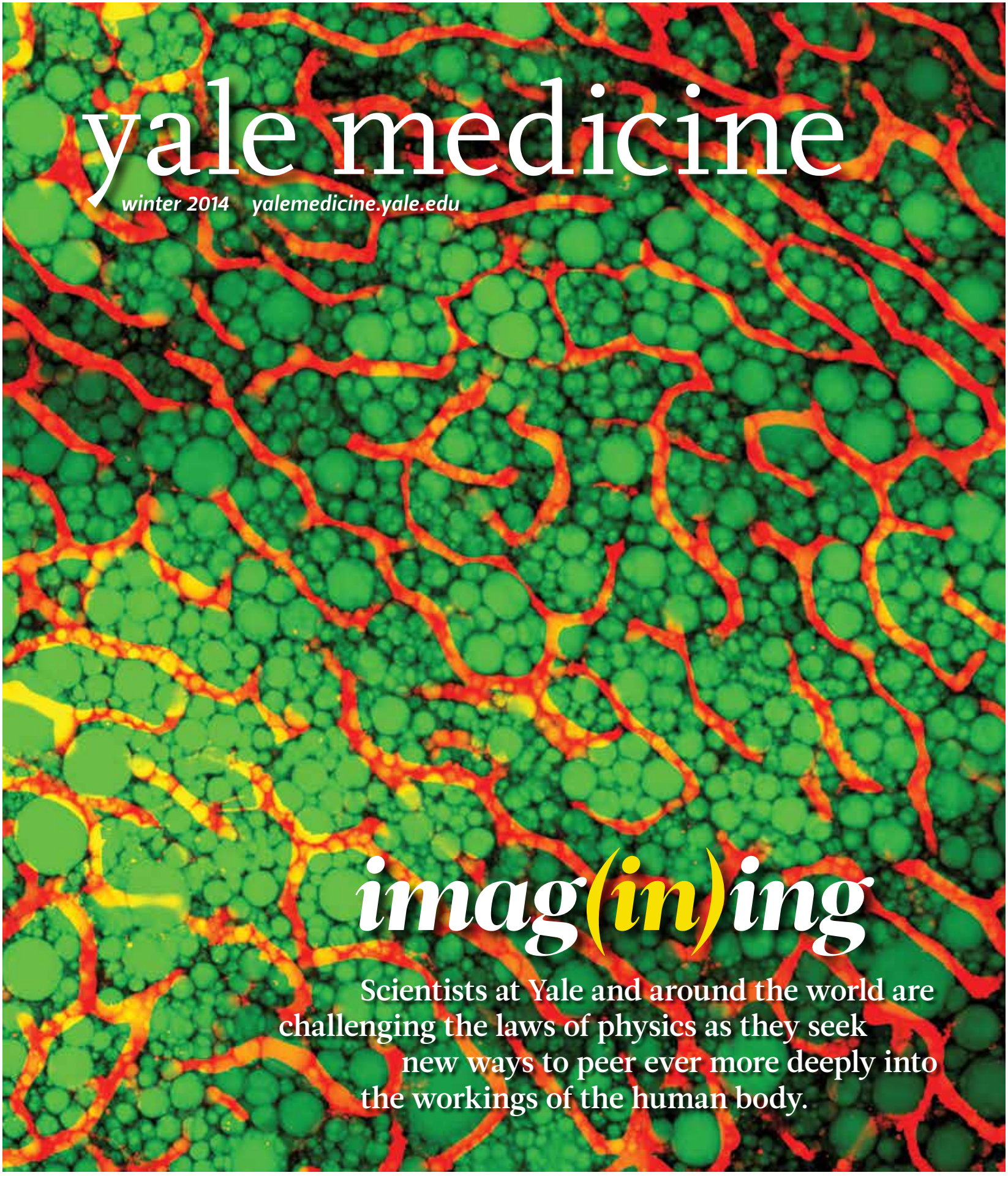

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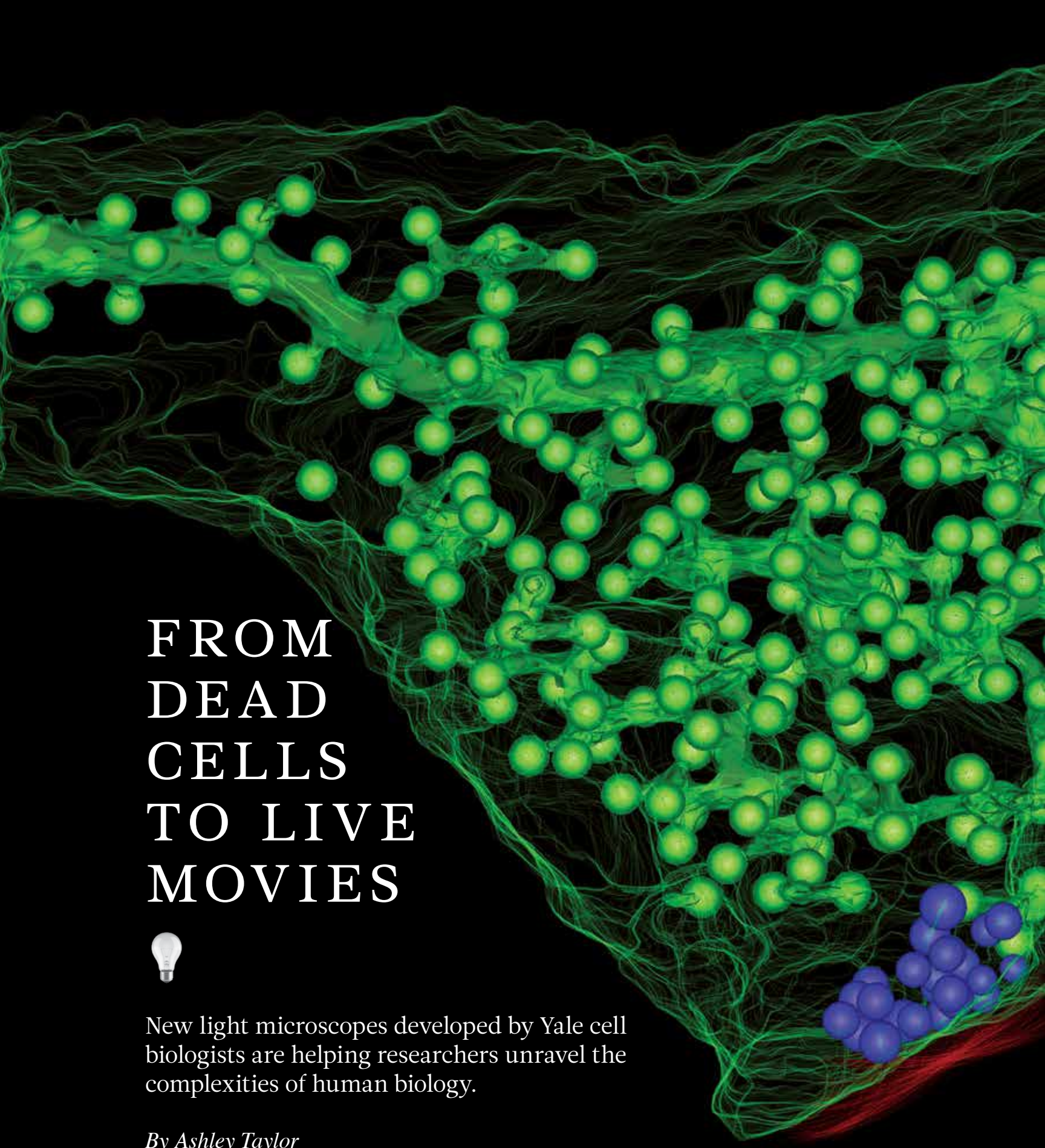
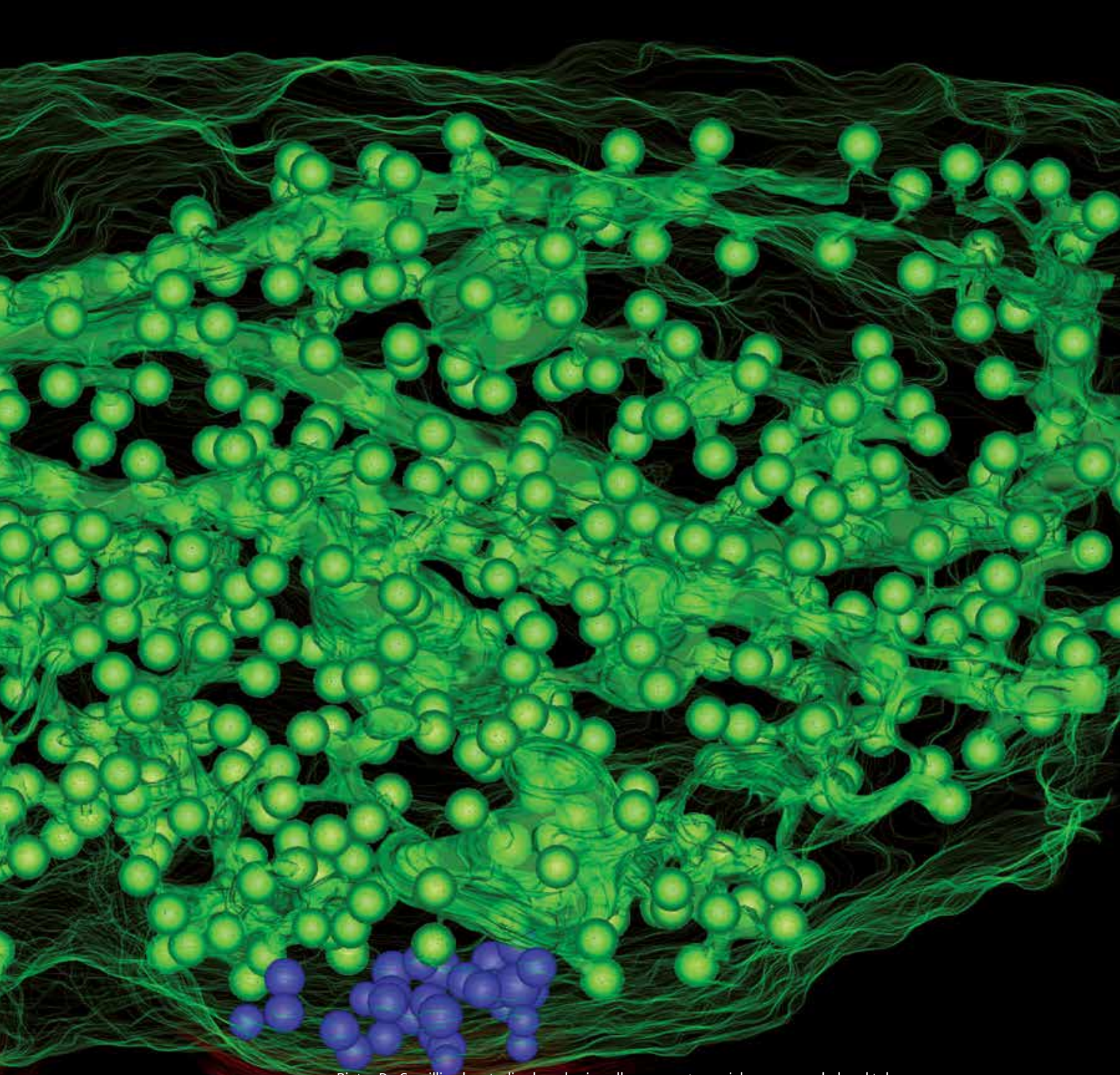
Scientists at Yale and around the world are challenging the laws of physics as they seek new ways to peer ever more deeply into the workings of the human body.



FROM DEAD CELLS TO LIVE MOVIES

New light microscopes developed by Yale cell biologists are helping researchers unravel the complexities of human biology.

By Ashley Taylor



SCIENTIFIC IMAGE PROVIDED BY PIETRO DE CAMILLI

Pietro De Camilli, who studies how brain cells package neurotransmitters, used electron tomography to capture this 3-D image that shows what happens when the process goes awry. Vesicles about 40 nanometers in diameter (blue) deliver neurotransmitters between cells by fusing with the outer membranes of neurons. Normally, after they release their cargo, vesicles are recycled and take on more neurotransmitters for another shipment. When the protein dynamin is mutated, this recycling does not occur and vesicle buds (green) that cannot be released to generate new vesicles accumulate at the cell surface and its infoldings.

IN 1974, THE LATE GEORGE E. PALADE, PH.D., chair of Yale's newly formed cell biology department, shared a Nobel Prize in physiology or medicine for using electron microscopy to elucidate the inner workings of cells—groundbreaking findings that some say ushered in the modern field of cell biology. But although the electron microscope opened new avenues of research, it had a huge drawback as a tool for studying life: it can observe cells only after they are dead, treated with special fixatives, and sliced into thin sections or coated in a layer of metal. The grayscale world pictured in such detail in electron micrographs, while powerful, is "a cellular cemetery," in the words of Pietro De Camilli, M.D., FW '79, the Eugene Higgins Professor of Cell Biology, professor of neurobiology, and director of the Yale Program in Cellular Neuroscience, Neurodegeneration and Repair.

Because electron microscopy's vision is limited to dead cells, it provides just a snapshot of a cell's inner workings. Derek K. Toomre, Ph.D., associate professor of cell biology, likes to compare an electron micrograph of a cell to a still photograph taken during a football game. If you are trying to learn the rules of the game, Toomre said, a snapshot doesn't get you very far. The same is true in biology. "There are a lot of biological problems that—if you could see them in living cells in action—we would be able to unravel."

To observe live cells, scientists use light microscopy, which includes the dissecting microscopes familiar from high school biology and extends to high-tech microscopes whose images brighten the pages of scientific journals.

But standard light microscopy too has a major limitation in resolution: scientists have known since the 19th century that it cannot resolve, or distinguish between, structures smaller than about the size of organelles. Smaller structures—the vesicles carrying cellular messages and the protein scaffolding that gives cells their heft and shape—blur together because of what is called the diffraction limit, described in 1873 by Ernst K. Abbe, a contemporary of the microscope manufacturer Carl Zeiss.

A light microscope, even with an excellent lens, cannot resolve structures smaller than about half the wavelength of the light used to illuminate them. That works out to a resolution of about 250 nanometers, around the size of the measles virus and about 400 times smaller than the width of a strand of human hair.

In trying to learn the rules of cell biology's game, scientists had at their disposal detailed still images from the electron microscope and views of the cell in action from the light microscope, with some of the most interesting players too small to see. Although each type of microscope had its uses, between them lay a large gap.

In the last 20 years, however, scientists have found ways to overcome the diffraction limit and close that gap through what is called super-resolution light microscopy. Using custom-made fluorescence microscopes, some designed by Yale scientists, researchers at Yale are observing the live-cell dynamics of structures that they could previously see only in snapshots. With these new data, they are beginning to answer scientific questions nearly as old as the limit that once held them back.

"This is the direction in which we have to go," said De Camilli, who studies how brain cells package neurotransmitters, the chemicals that pass along neuronal signals. "Super-resolution microscopy is really the next critical step." Gesturing toward a photo on his wall of a smiling Palade, who looks as though he's listening in on our conversation, De Camilli continued, "Palade was a pioneer in the use of electron microscopy. We feel like super-resolution microscopy is the next frontier in microscopy, and we think it's appropriate that it happen here, in the heritage of George Palade."

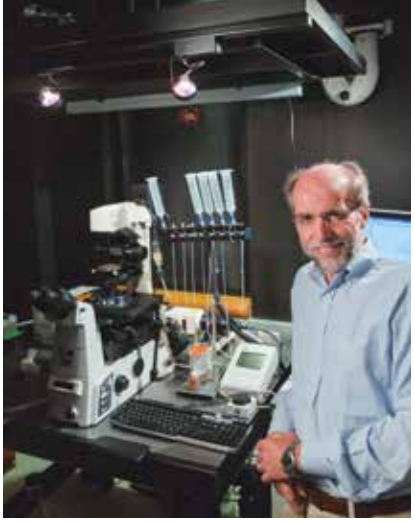
CELLULAR FIREWORKS

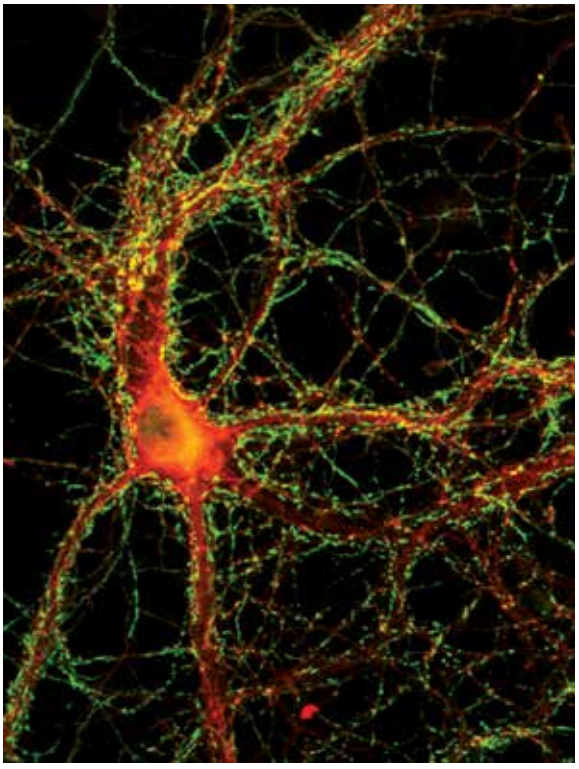
Joerg Bewersdorf, Ph.D., assistant professor of cell biology and of biomedical engineering, "stumbled into microscopy" in 1996. Then an undergraduate studying physics at the University of Heidelberg, Bewersdorf wanted to develop technologies that would help scientists in other fields. After taking an optics class with Stefan W. Hell, Ph.D., who, Bewersdorf said, was then "a junior professor, not really known, just a very dynamic person," Bewersdorf joined Hell's new lab at the Max Planck Institute for Biophysical Chemistry in Göttingen, which was quite small at the time. "Eight years later, when I left," Bewersdorf remembers, "the lab had like 35 people, Hell was famous and had won a lot of awards, and this whole field of super-resolution microscopy had taken off."

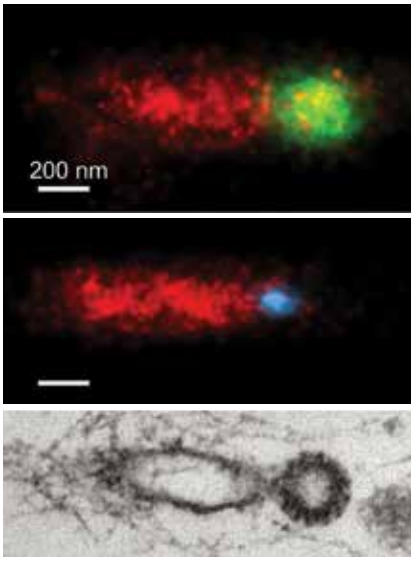
Hell, director of the institute since 2002, had broken the diffraction limit, building a microscope that allowed scientists to see tiny biological structures in a way not thought possible. For years Abbe's diffraction limit had been considered dogma, with scientists skeptical of attempts to bypass it, Bewersdorf said. Breaking the limit required a change in the way scientists thought about microscopy.

"To really break the diffraction limit, you can't think of the microscope as just optics," said Bewersdorf. "And this is what people had done for 150 years—it was always about lenses or it was always about light." Instead, said Bewersdorf, Hell was thinking about the interaction of the microscope light with the cells or tissue being examined. Hell believed that resolution could be improved, not by modifying the light used to make a sample fluoresce but by altering the fluorescent light as it is emitted. Using this approach, Hell theorized in a visionary 1994 paper in the journal *Optics Letters* that he would be able to achieve a resolution of 35 nanometers—small enough to see not just organelles but structures within them, like the involutions of the mitochondria, the cell's power plants, or the many layers of the Golgi apparatus, the cell's protein-processing pipeline.

By 1999, Hell had built a super-resolution microscope. His new technique was called stimulated emission depletion (STED) microscopy. A year later his lab showed that STED could work with biological material. In 2006, other research groups independently published papers





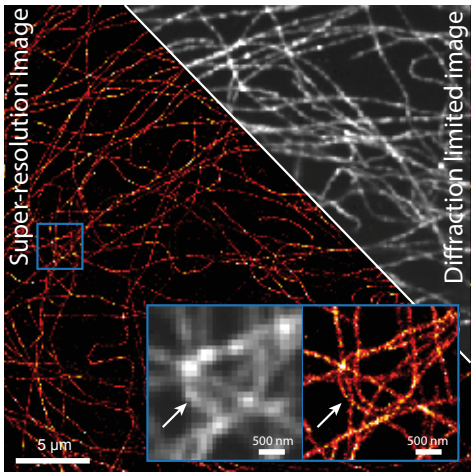


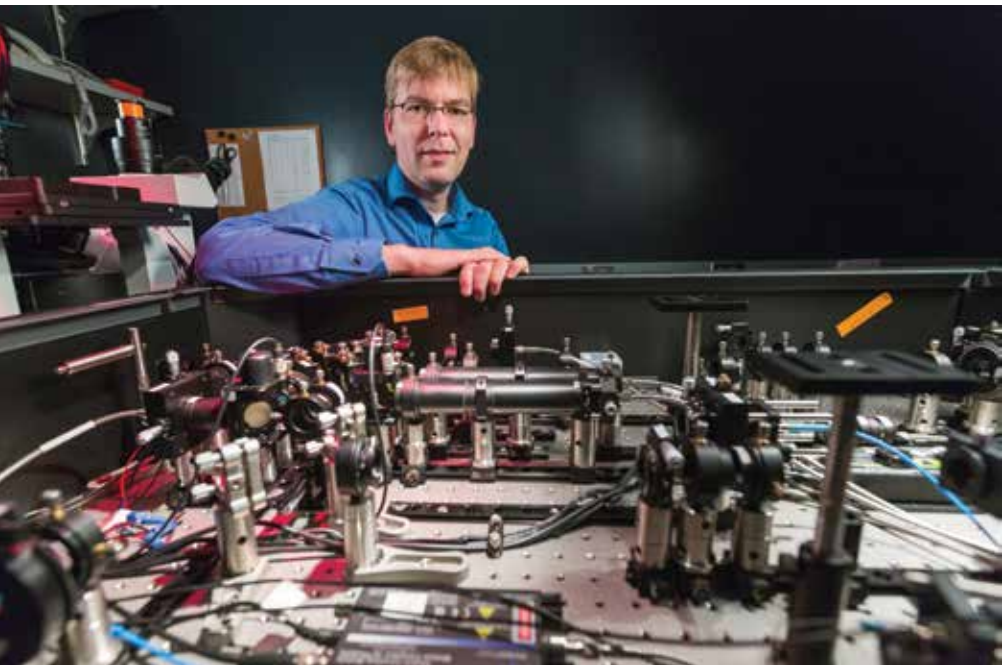
TOP "This is the direction in which we have to go," said Pietro De Camilli. High-resolution imaging technologies can overcome the diffraction limit that held back advances in cell biology for many years. De Camilli, shown here with a TIRF microscope, uses the new imaging modalities to study how brain cells package neurotransmitters.

MIDDLE Epifluorescence microscopy produced this image of synapsin (green spots on the surface of two neurons) and adaptin (in red). These two proteins play a role in forming and storing neurotransmitter-filled synaptic vesicles at sites where neurons communicate. Comparing the staining patterns of synapsin and alpha-adaptin—under different conditions of neuronal activity and/or after pharmacological and genetic perturbations—allows researchers to assess the status of synaptic vesicles and their recycling. Shawn Ferguson, who produced this image, noted that it "does not boast a high degree of resolution—hundreds of nanometers."

BOTTOM De Camilli and his collaborators used super-resolution microscopy to create these images of vesicles being reformed. The color images show the location of two proteins, dynamin and clathrin, involved in that process. "We are zooming in at incredible levels of resolution," he said. The color images were taken with fluorescence microscopy and the other image was taken with electron microscopy.

ROBERT LISAK PHOTO, SCIENTIFIC IMAGES PROVIDED BY PIETRO DE CAMILLI





TOP A split image of microtubule structures shows the advantage of a super-resolution pointillism technique called fPALM/STORM over traditional imaging methods. Thousands of images of fluorescent molecules are taken and reassembled by computers. Microtubule structures are important in processes ranging from maintaining cell structure to providing platforms for intracellular transport.

ABOVE This is not your grandparent's microscope. In their efforts to obtain images of smaller and smaller structures, scientists have moved away from lenses and light to such complex and sophisticated devices as this STED microscope, which relies on fluorescence and laser beams. Joerg Bewersdorf trained in physics, but now works with cell biologists looking for images of ever-smaller cellular structures.

describing a different approach to light microscopy that achieved even higher resolution than Hell's technique. Each group gave its version of the technique a different name: photoactivated localization microscopy (PALM); fluorescence photoactivated localization microscopy (fPALM); and stochastic optical reconstruction microscopy (STORM). All three versions, however, rely on the same principle—imaging a fluorescently labeled sample a few scattered points at a time. By 2012, scientists were using the term "diffraction-unlimited microscopy," or even "nanoscopy," to reflect the fact that these new microscopes work on the scale of nanometers. The paradigm shift was complete.

At about the time that Hell was developing his new microscope, from 1997 to 2001, Toomre was also in Germany (though he did not meet Bewersdorf until they both came to Yale) as a postdoctoral fellow at Heidelberg's European Molecular Biology Laboratory. Toomre was trying to learn about vesicles, the bubble-like structures that cells use to shuttle materials in and out and from one cellular location to another. Vesicles are ubiquitous in biology—they transport everything from hormones to neurotransmitters. In the brains of animals, signals pass from one neuron to another thanks to a process called exocytosis, in which vesicles packed with neurotransmitters fuse with the cell membrane of one neuron and empty the chemicals into the synapse—the space between it and the next neuron—to transmit the message. (James E. Rothman, Ph.D., the Fergus F. Wallace Professor of Biomedical Sciences and professor and chair of cell biology, shared in the 2013 Nobel Prize for physiology or medicine for his studies of vesicles.)

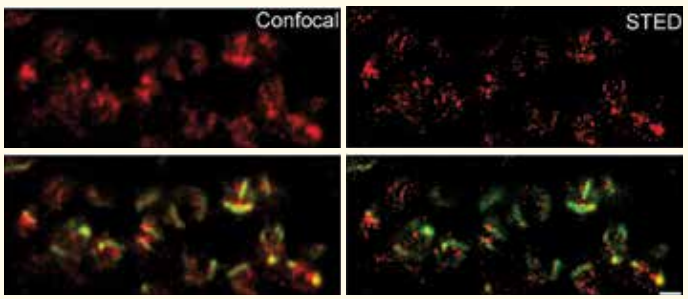
"I was frustrated," said Toomre, "because we knew biochemically that these things had to go out to the surface, and we could see these little vesicles moving, but we really didn't see them fuse." He had tried to simulate vesicle fusion in a test tube, an effort that failed after a year. But he had heard about a microscope called a total internal reflection fluorescence (TIRF) microscope that might help. The TIRF microscope could selectively illuminate objects in a thin 80-nanometer optical section and achieve much higher resolving power than traditional confocal microscopes—but only near the surface of the cell. The technology was perfect for observing the fusion of a vesicle with the cell membrane. Toomre "begged and pleaded" to

ROBERT LISAK PHOTO, SCIENTIFIC IMAGES PROVIDED BY JOERG BEWERSDORF (THIS PAGE AND OPPOSITE)

Breaking the diffraction limit

The period at the end of this sentence is 1 million nanometers wide. With super-resolution microscopy, scientists can see synaptic vesicles as small as 30 nanometers wide. Imagine taking a picture of the continental United States from the stratosphere and being able to distinguish a single strand of hair.

Fluorescent tags make this possible. Molecules and structures of interest are given a fluorescent



tag—either a dye or a genetically engineered tag like green fluorescent protein (GFP). A laser beam is directed at the target sample, which makes the tag emit light, and the resulting fluorescence is recorded to create an image.

The first fluorescence microscopes scanned an entire sample at once, which produced a fair amount of out-of-focus fluorescence. Today's standard fluorescence microscope—the confocal microscope—scans a sample one point at a time, pixel by pixel, and assembles the pixels to create an image. The size of each fluorescent point in these standard microscopes is determined by how much the laser light diffracts and is limited by the diffraction limit to between 200 and 250 nanometers.

Stefan Hell's innovation, STED, improves resolution by reducing the size of each fluorescent spot. STED targets the light returning from the sample and a second laser blocks out the fluorescence in a donut shape around the center of each fluorescent spot. Each fluorescent point is reduced to the size of the donut hole. These smaller points of light yield a higher-resolution image. STED microscopy can achieve a resolution of 25 to 80 nanometers, small enough to distinguish cellular vesicles and the folds within organelles.

PALM/fPALM/STORM capture just a few scattered molecules at a time so that they are unlikely to overlap and blur together. Using labels that turn on and off, scientists arrange to have only a few molecules fluoresce at one time; then they take a picture. A computer finds the center of each spot, representing a single fluorescent molecule, on the individual photo. This process is repeated thousands of times, and the photos are then combined. The approach is sometimes called pointillist microscopy, after Impressionist Georges Seurat's painting technique. Pointillist techniques achieve extremely high resolution, about 25 nanometers. However, the technique can also be slow—it requires many photos to generate one image, and it is dependent on high-powered computers to process the data.

TIRF microscopy, developed in the early 1980s, excites fluorescence in a thin layer near the cell surface, which reduces background fluorescence and improves resolution to between 40 and 100 nanometers. TIRF microscopy is faster than pointillist techniques but has lower resolution and can record only the cell surface.

These are only a few of the high-resolution microscopy techniques available today, and Yale is unusual in that it has all these microscopes—STED, PALM/fPALM/STORM, the electron microscope, and others—in one place, said Derek Toomre. Each has its strengths and weaknesses. "If we knew that there was one type that could do everything, we wouldn't be investing in all of them. … There's no clear winner. We'll see; maybe there will be."

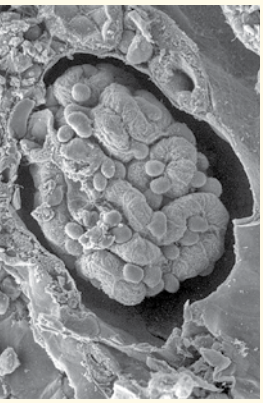
"Cavorting wee beasties"

The magnitude of advances in microscopy becomes clear in the context of its four-century history.

Seeing the unseeable

Starting around the turn of the 17th century, natural philosophers using the light microscope saw things where, to the naked eye, there was nothing to see. The Englishman Robert Hooke observed pockets of air within cork, which he called cells; the Dutch scientist Anton van Leeuwenhoek saw living bacteria in pond water and cells within blood and even found "wee beasties," as he sometimes called his "cavorting" specimens, in his own semen.

Electron microscope



In the 1930s, the German physicist Ernst Ruska developed a microscope with ultra-high resolution by using electrons, which have a smaller wavelength than light and can distinguish tinier features. Using Ruska's new electron microscope, scientists could view structures within an individual cell, with the downside that they could not look at live cells.

borrow the TIRF microscope in the lab of cell physiologist Wolf Almers, Ph.D., who was then at the Max Planck Institute. "Within a few hours of imaging," said Toomre, "we had an amazing result. We could see these vesicles arrive and explode during fusion." In 2000, Toomre, Almers, and others published their observations in *The Journal of Cell Biology*. This was Toomre's entrée into super-resolution microscopy, which he would pursue at Yale beginning in 2001.

In his office, Toomre shows a more recent video generated by a TIRF microscope of fluorescently labeled vesicles fusing with the cell membrane. Fluorescent green dots—the vesicles—move around on the screen, then flash brightly as they fuse with the cell membrane. "It's fireworks," Toomre said. "Cellular fireworks."

NEW FRONTIERS … IN BIOLOGY

Bewersdorf, one of the first physicists recruited to the highly interdisciplinary Department of Cell Biology at Yale, joined the faculty in 2009 because he wanted to collaborate with biologists who were using these new microscopes to answer important questions in biology.

A burning question both within and outside the department: How does the Golgi apparatus, the cell's protein processing plant, work? A stack of membrane-bound disks, the Golgi processes proteins into their final forms, adding sugar and phosphate molecules as they pass from one end of the stack to the other and are sorted to other areas of the cell. If necessary, the Golgi packages them into vesicles to be released from the cell. For 100 years, Toomre said, scientists have debated whether the Golgi is a stable structure that moves vesicles around or a dynamic structure that transforms itself into the vesicles it releases. The debate continues, as a major roadblock is the inability to see small vesicles trafficking within the highly convoluted Golgi "pancake" in live cells. Now, by labeling both the Golgi and the proteins moving through it, then watching the labeled cells at super-resolution, an international consortium of researchers at Yale, Oxford, and Cambridge are hoping to find the answer.

Vesicles are also a focus of De Camilli's lab, which is studying the way they are made. Vesicles are formed by pinching off from a larger membrane, like the cell membrane or the membrane of an organelle. De Camilli wanted to know which proteins are responsible for cutting the new vesicle off from its parent membrane. Two proteins might be involved, he thought: clathrin and dynamin. He wanted to see where the two proteins are located on the vesicle. In his office, De Camilli draws furiously on a scrap of paper: green for clathrin,

TERRY DAGRADI PHOTO, SCIENTIFIC IMAGE PROVIDED BY DANIEL BIEMESDERFER

gray for dynamin. Under regular light microscopy, clathrin and dynamin seem to overlap. To demonstrate this overlap, De Camilli draws green and gray swirls, one atop the other. But using super-resolution microscopy, he shows me that dynamin is clearly distinguishable from clathrin.

… IN MEDICINE …

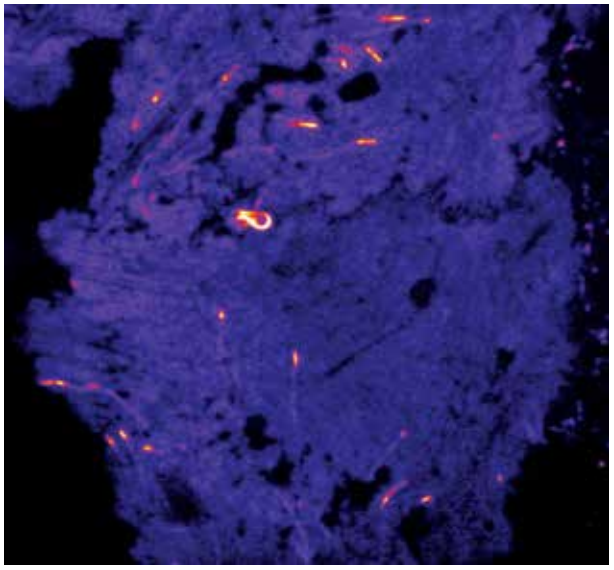
The new information about cell structure revealed by super-resolution microscopy is helping scientists to understand the mechanisms of diseases that affect humans, De Camilli said—in particular, diseases rooted in genetic mutations. Many genetic disorders, he said, result from changes in the distribution or localization of proteins in cells. "In order to understand in which way the mutation affects cell function, it is very useful to be able to localize either the mutant protein itself or organelles and proteins with which it interacts or the organelle on which it is localized," De Camilli said.

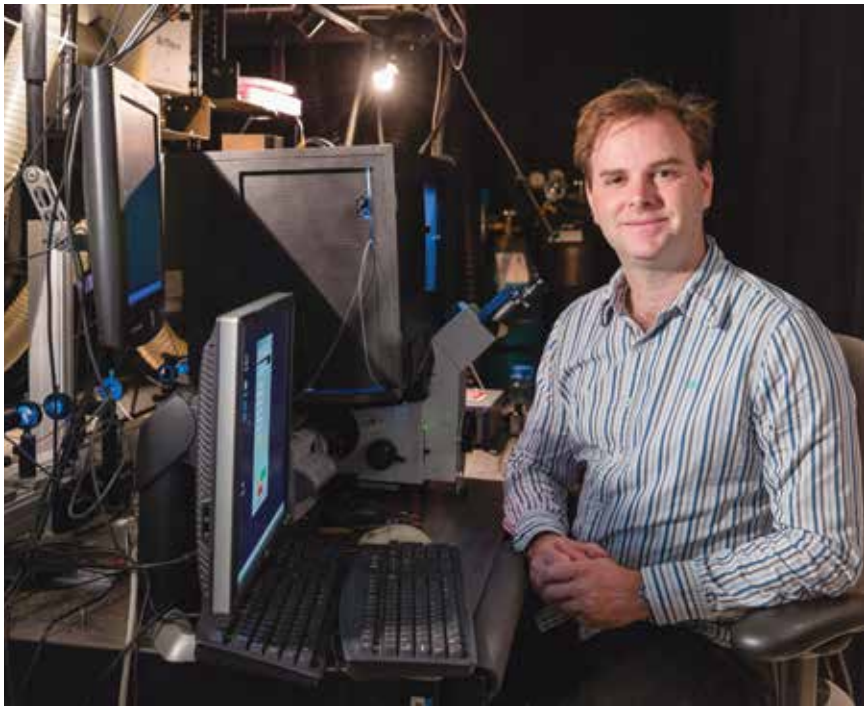
For example, De Camilli is studying Lowe syndrome, a rare disorder that almost exclusively affects males and causes intellectual disability, congenital cataracts, and kidney problems. His previous research had revealed that, on the molecular level, Lowe syndrome causes problems with endocytosis, the process by which a vesicle empties its contents into a cell. Using super-resolution microscopy to monitor the distribution of the normal and mutant Lowe syndrome proteins on endocytic vesicles, De Camilli hopes to better understand the mechanisms of the disease, with implications for therapy.

Toomre is using TIRF microscopy to study diabetes by watching the way fat cells respond to insulin. When fat cells are stimulated with insulin, he has found, vesicles whose membranes contain sugar transport proteins rush to the cell membrane and fuse with it, adding the transporter proteins to the cell membrane and allowing the fat cells to take up more glucose. In diabetes, this process is somehow disrupted, and Toomre hopes to find out how. So far, he said, "Using this TIRF microscopy, we discovered that there were two different types of vesicles arriving at the surface, and until we could see it, we didn't realize that."

… AND IN TECHNOLOGY

On the cellular level, the Golgi apparatus is the new frontier. In the lab, this new frontier is studded with giant microscopes enclosed in black boxes to keep out light and prevent temperature fluctuations. Bewersdorf walks me through his lab. His custom-made STED microscope looks in part like other confocal microscopes I've seen,





TOP Throughout his career Derek Toomre has tried to learn more about vesicles, structures that move proteins, neurotransmitters, and other cargo in and out of cells and from one cellular location to another. This composite image, taken with dual-color live-cell epifluorescence microscopy, shows vesicles moving along microtubule "highways."

ABOVE Toomre believes that seeing cells in real time is crucial to understanding human biology. "There are a lot of biological problems that—if you could see them in living cells in action—we would be able to unravel."

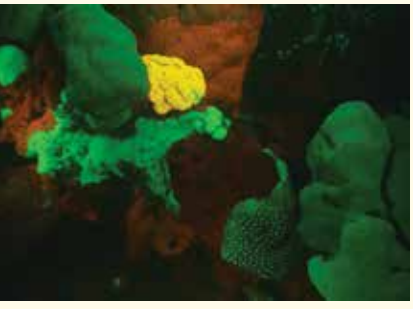
SCIENTIFIC IMAGE PROVIDED BY DEREK TOOMRE, ROBERT LISAK PHOTO

Dyes and stains

In 1873, the Italian physician and scientist Camillo Golgi stained neurons using a silver compound that turned the cells black. The Spanish neuroanatomist Santiago Ramón y Cajal put Golgi's method to fruitful use, making observations that led to the neuron doctrine, the now-accepted idea that the nervous system is composed of discrete cells. In 1886, Paul Mayer invented the hematoxylin and eosin staining procedure. Hematoxylin stains cell nuclei blue; eosin is nonspecifically attracted to proteins and gives the rest of the cell a contrasting reddish hue. The most important dyes used in light microscopy today, however, are fluorescent.

Fluorescence microscope

Though the fluorescence microscope was invented around 1910, fluorescence microscopy did not really take off until the end of the century, spurred by the development of fluorescent labels for specific biological structures. The most famous of these fluorescent tags is called green fluorescent protein, or GFP, a protein derived from jellyfish that emits green light when stimulated by blue light. [For more on the use of marine life as a source of fluorescent tags, see "In coral reefs, a treasure trove of tools" on next page.] In the 1990s, scientists isolated the gene encoding GFP, which allowed them to engineer cells genetically so that GFP could be fused to a protein of interest for visualization with the fluorescence microscope. Microscopy's palette expanded as scientists developed variations of GFP that fluoresce in different colors; and by labeling different structures with different fluorescent molecules that can be visualized at the same time, scientists can determine whether those structures are colocalized and potentially interacting. Fluorescent labels are not limited to proteins: they can also label DNA, lipid molecules, and carbohydrates. And efforts to break the diffraction limit would increasingly rely on these fluorescent proteins.



with eyepieces and a computer screen displaying an image. But nearby is a table with black sides reminiscent of a filled casket. Inside are black tubes with white labels, lined-up lenses of different tints, and blue and silver cables. The laser beam, he said, travels through the blue cables; the fluorescence travels to the detector through the silver ones. What's good about these custom-built microscopes, Bewersdorf said, is that he can easily adjust them for different samples.

"A lot of the things at the edge are not commercial. A lot of the microscopes that you'll see in Joerg's lab are custom-made," said Toomre, "and they're custom-made because that's the only way you can do it."

Bewersdorf has achieved his goal of working with scientists in other fields, and the number of scientists who can thank him is likely to grow. "Super-resolution is something that just about everyone is trying to jump into," said Michael W. Davidson, Ph.D., a Florida State University scientist who is collaborating with Bewersdorf and Toomre by providing fluorescent proteins from his large collection. "It's had a huge impact, but I think the impact is just starting. I think almost everybody's going to be doing it within 10 years."

Bewersdorf and Toomre are working with microscope companies to commercialize the instruments that they have custom-built in their labs. For now, though, this is what Toomre calls "the edge," the frontier of science. I asked Bewersdorf if he thought the resolution of light microscopy would continue to improve. "No," he said. The goals are no longer about resolution. Now the challenges are finding compatible fluorescent labels in order to watch multiple structures simultaneously and developing cameras that can capture the images faster and faster to create videos of cellular structures in motion. Perhaps the most important challenge is to apply this technological tool kit to questions of neuroscience, metabolism, and cancers whose answers may be central to human health. Bewersdorf shows me a pointillist microscope, also custom-built in his lab. That microscope, armed with a digital camera, can take photos so fast that they can be used to create high-resolution movies of fluorescently labeled cells, as Bewersdorf and colleagues reported in a *Nature Methods* paper published online in May. I think back to Toomre's analogy about trying to learn the rules of football from a snapshot. At last, a high-resolution movie of cells at play. Now scientists can really learn the rules of the game. */yale medicine*

Ashley Taylor is a freelance writer based in New York City.

CARMEN JANE BOOTH PHOTO (TOP), VINCENT PIERIBONE PHOTO (BOTTOM)