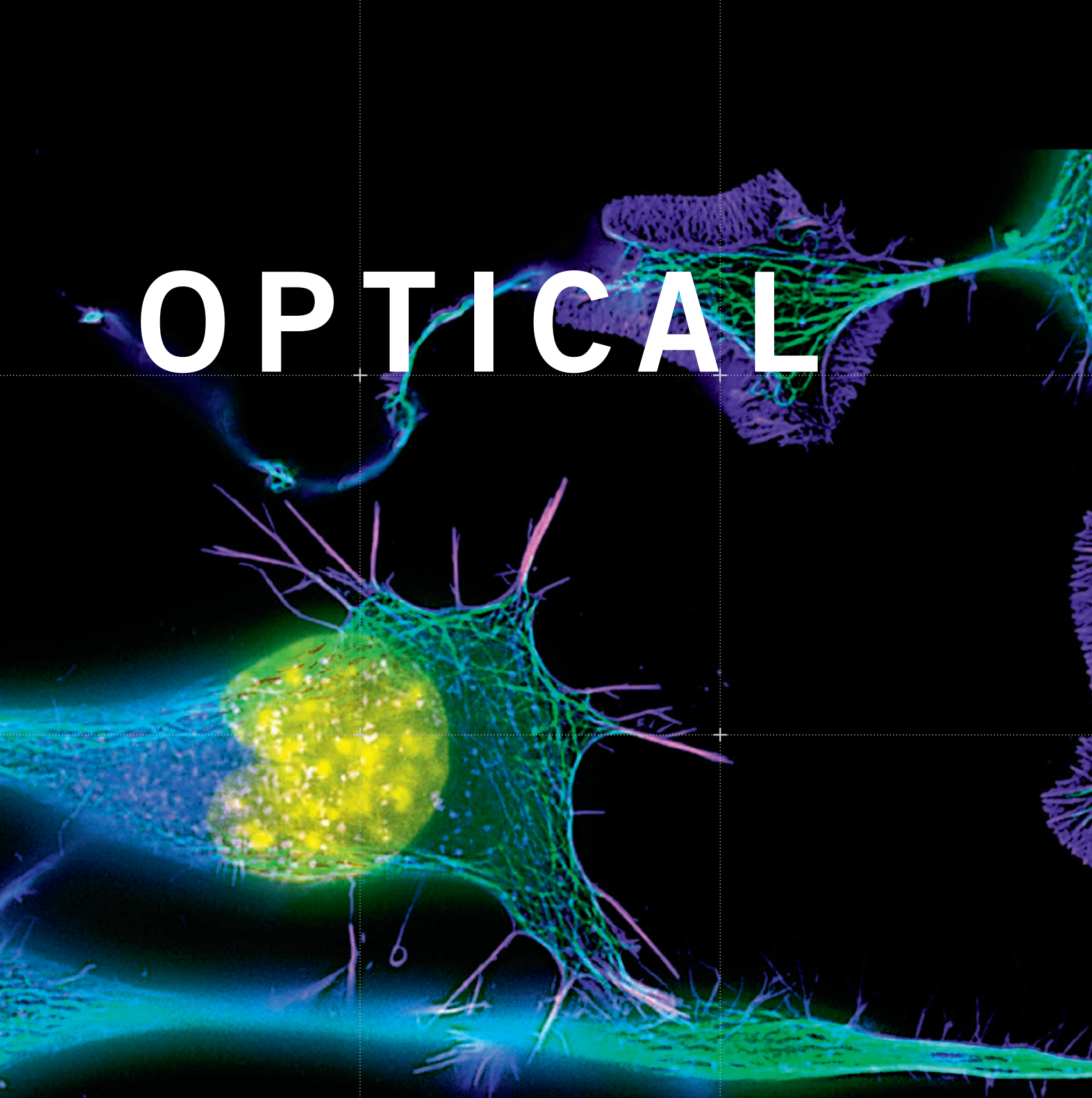




# ASPIRATIONS

At Janelia Farm, researchers will link mathematics, physics, engineering, and computing in the pursuit of better cellular pictures. **BY DAN FERBER**

# OPTICAL

A fluorescence microscopy image showing several mouse neuroblastoma cells. The cells are stained with fluorescent dyes to visualize their internal structures. The DNA is shown in yellow, the actin filaments in blue, and the microtubules in green. The cells exhibit a complex network of filaments and are surrounded by numerous fine, hair-like projections (neurites) extending outwards. The background is dark, making the brightly colored structures stand out.

MOUSE NEUROBLASTOMA CELLS WERE STIMULATED TO DIFFERENTIATE IN VITRO, AND THEN LABELED FLUORESCENTLY. THE IMAGE, CAPTURED WITH AN EPIFLUORESCENCE MICROSCOPE, SHOWS ACTIN (BLUE) AND MICROTUBULES (GREEN) IN TWO SUCH CELLS DURING THE EARLY STAGES OF NEURITE OUTGROWTH (DNA IS YELLOW). INDIVIDUAL CHANNELS WERE SUBSEQUENTLY OVERLAID AND COLORIZED. PHOTOGRAPH COURTESY OF TÖRSTEN WITTMANN

# IN THE WORLD OF BIOMEDICAL RESEARCH,

there are big-idea guys and detail guys, and Nikolaus Grigorieff is a detail guy. He doesn't just sweat the details—he thrives on them. For the problems he addresses, details are everything. Grigorieff, an HHMI investigator at Brandeis University, has been appointed one of the first group leaders at HHMI's new Janelia Farm research campus, which opens later this year. He uses electron microscopy to visualize tiny three-dimensional protein structures inside cells. That means grappling with a sea of details. A single molecular machine may contain dozens of proteins, each with hundreds of amino acids. To understand how it works, you need to “open it up, see what's inside, and see how those bits and pieces fit together,” Grigorieff says. And that's where microscopy comes in.

Since Theodor Schwann first peered inside animal cells in the 1830s, curious biologists have sought to identify cellular components and comprehend how they work together. But until recently, light microscopes could not distinguish objects much smaller than a mitochondrion—an organelle about one-fiftieth the diameter of a typical cell. Electron microscopes have long helped scientists like Grigorieff make out smaller objects, such as molecular machines and small organelles—but only in dead, chemically fixed cells. To better understand such objects, biologists tried to obtain sharper images of them and see them in action. But until the last decade, they had little luck.

Now that's changing. “Every 20 years or so there's a big technical advance that really changes the way biomedical research is done,” says Gerald M. Rubin, vice president of HHMI and director of Janelia Farm. In the late 1950s, x-ray crystallography allowed biologists to see the atomic structure of proteins, which carry out most of the work of the cell; in the 1970s, cloning and DNA sequencing led to new insights into evolution, gene regulation, and the biochemical workings of individual proteins.

At Janelia Farm, HHMI has focused a good deal of its efforts on developing new

microscopy methods and new computing methods to analyze images. Nationally, the organization is investing tens of millions of dollars each year in microscopy. That's because imaging, Rubin says, “is the most important technology that we need now.”

## Limited Vision

### THE HUMAN EYE

can readily distinguish objects as small as 100 micrometers across, about the width of a human hair. A typical human cell is about 10 micrometers in diameter and therefore invisible to the naked eye. As of the early 1990s, even state-of-the-art light microscopes could distinguish only objects larger than 0.2 micrometer in diameter—half the wavelength of blue light—which meant that smaller but important structures like ribosomes and spliceosomes were impossible to see in living cells. Biologists' vision was restricted by the light microscope's resolution—its ability to create a sharp image by distinguishing between two adjacent points. Optics dogma dictated that resolution was limited to about half the shortest wavelength of light used, so many scientists thought it could get no better.

Cellular structures were also difficult to view because they're usually transparent, making it hard to distinguish them from the watery cytoplasm in which they sit. Phase-contrast microscopes made that easier by using interfering light waves to distinguish cellular structures from background. And biologists developed a plethora of chemical stains and fluorescent antibodies that bound to specific cellular structures, making them visible. But cells usually had to be killed first, and scientists then had to surmise what the structures did when the cells were alive.

To see molecular machines and small organelles, scientists used transmission electron microscopes, which utilize electromagnetic coils to focus electron beams instead of glass lenses to focus light. But electron beams destroy biological

tissue, so researchers could only see into dead, chemically fixed cells. And electron microscopists like Grigorieff who wanted to determine the atomic structures of protein complexes could not do it. So biologists filled textbooks with descriptions of what they could see, saying little about what they couldn't.

## Freeze Frame

### IN A DARK,

high-ceilinged room near Grigorieff's laboratory, Carsten Sachse pours liquid nitrogen into a stainless steel sample holder on the side of an electron microscope, a foot-thick gray column taller than an NBA star. It hisses and boils off an icy steam.

He points to a computer monitor next to the microscope, to an image of gray fibers packed tightly side by side. They're lab-grown fibrils of amyloid beta peptide, the molecule in the brain suspected of causing Alzheimer's disease, magnified 59,000 times. The regular packing of the fibrils means that Sachse, a visiting graduate student who works with Marcus Fändrich at the Leibniz Institute for Age Research, in Jena, Germany, may well be able to use electron microscope images of similar preparations to determine the three-dimensional atomic structure of amyloid peptide packed into fibrils. Understanding that could be key to blocking tissue damage in Alzheimer's patients. “It looks very promising,” he says.

Electron microscopy, Grigorieff says, can help elucidate the molecular structures of complexes too big to analyze by crystallography or nuclear magnetic resonance yet too small to see with a light microscope. “EM is a good technique to bridge the gap to high resolution,” Grigorieff says.

But not just any electron microscope. Grigorieff's \$2 million microscope contains a 300,000-volt field-emission electron gun to accelerate electrons through relatively thick samples, while ensuring they march in lockstep—a property needed to enhance contrast. It

has specialized CCD cameras—tricked-out large-format digital cameras, essentially—to capture electron images and diffraction patterns. The researchers run recently developed algorithms on high-powered clusters of computers to turn huge amounts of electron microscopy data into three-dimensional images. And the darkened room where Sachse works contains a \$400,000 climate-control system that prevents even the smallest drafts and shifts in temperature—all to keep their samples extraordinarily steady, which they must do to obtain good data.

Such attention to technology has paid dividends for Grigorieff. Working in collaboration with HHMI investigator Melissa J. Moore of Brandeis University and former postdoc Melissa Jurica, the group got the first-ever glimpse of the three-dimensional structure of the spliceosome, a molecular machine that splices newly formed RNA to form messenger RNA, which in turn encodes the correct amino acid sequence of

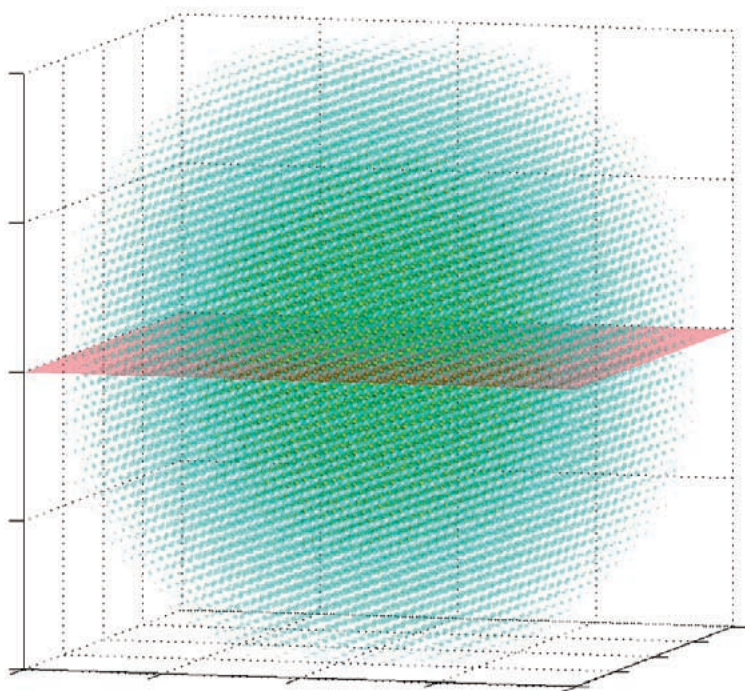
the protein. The researchers obtained thousands of electron microscope images of single frozen spliceosomes and then reconstructed the complex's three-dimensional shape on a computer. The result: a cylinder on a hollow ovoid domain with an armlike extension that seems optimized to perform the contortions necessary to cut and splice a threadlike RNA molecule.

Grigorieff's team has also determined the structures of other cellular machines, including, in collaboration with HHMI investigator Axel T. Brünger of Stanford University, a molecular machine called NSF that helps nerve cells export packets of molecules that enable them to signal

their neighbors, and, with Thomas Walz of Harvard Medical School, a soccer-ball-shaped delivery structure called a clathrin coat. "We need to know what these molecules look like," Grigorieff says.

For particularly complex molecular machines, it's often necessary to combine methods. David A. Agard, an HHMI investigator at the University of California, San Francisco, uses a technique he's optimized called cryoelectron tomography that takes images of particles from different angles and then assembles those images into a three-dimensional model. He's used the method to visualize the centrosome, an organelle that manages the cell's internal skeleton. To show how the centrosome

**AT JANELIA FARM, Nikolaus Grigorieff says, he'll try to push single-particle cryoelectron microscopy methods to "routinely get to such high resolution that you can build an atomic model."**



**A SPHERICAL REGION OF ILLUMINATION WITHIN AN OPTICAL LATTICE MICROSCOPE MAY CONTAIN THOUSANDS OF POINTS OF LIGHT FOR MASSIVELY PARALLEL, RAPID IMAGING OVER A LARGE VOLUME WITHIN A CELL.**

carries out that task, he uses single-particle electron microscopy and x-ray crystallography to view its components at atomic resolution.

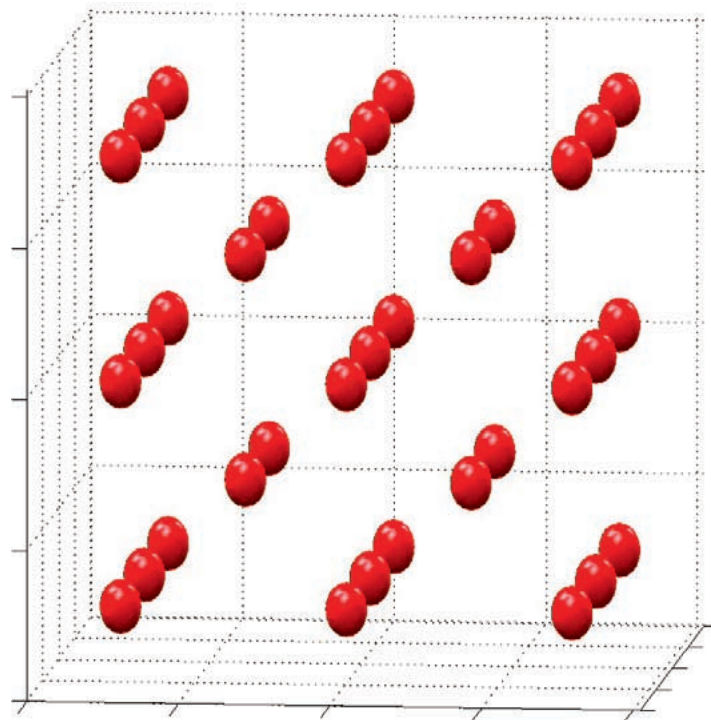
Grigorieff and others also use electron microscopy to visualize two-dimensional crystals of membrane proteins in a lipid bilayer, much like that in the cell. He and others have even used electron microscopy to visualize membrane proteins in atomic detail. In December, Walz reported using this technique, called electron crystallography, to determine the three-dimensional structure of a cellular water-pore protein called aquaporin; the resolution was high enough to spot discrete lipid molecules clinging to the side of the protein. Electron microscope images of single protein complexes (as opposed to crystals) have lower resolution, but they can distinguish parts of proteins, such as helices and loops. At Janelia Farm, Grigorieff says, he'll try to push single-

particle cryoelectron microscopy methods to “routinely get to such high resolution that you can build an atomic model.”

### Going Live AT JANELIA FARM,

other scientists will press light-microscopy methods to observe cells or even entire brains at high resolution. Janelia Farm group leader Eugene W. Meyers, who wrote programs that dramatically sped the sequencing of the human genome, will design new computing methods to assemble three-dimensional reconstructions of working brains from two-dimensional microscope images. Group leader Karel Svoboda, currently an HHMI investigator at Cold Spring Harbor Laboratory, will use two-photon excitation microscopy, a form of fluorescence microscopy developed in the 1990s, to observe living neurons in mouse brains. “We need new imaging

A CLOSER VIEW WITHIN AN OPTICAL LATTICE MICROSCOPE REVEALS INDIVIDUAL, TIGHTLY FOCUSED EXCITATION POINTS (RED) ARRANGED IN A PERIODIC ARRAY.



## KAREL SVOBODA AND OTHER researchers use two-photon excitation microscopy to peer into opaque tissues like the brain—an ability akin to Superman’s x-ray vision.

methods to figure out how the brain works,” Rubin says.

Janelia Farm biologists will make use of more than a decade of extraordinary advances in light microscopy. Until the early 1990s, some biologists examined cell shape and behavior by using light microscopy, while others tried to understand their protein of interest by localizing it in cells that had been killed and fixed, says Doug Murphy, a cell biologist at Johns Hopkins School of Medicine in Baltimore who will move to Janelia Farm to direct its shared light microscopy facility. “Now we don’t just want to see where it is, but how it’s behaving.”

New fluorescent probes make that possible. In the mid-1990s, HHMI

investigator Roger Y. Tsien of the University of California, San Diego, began working on a naturally fluorescent jellyfish molecule called the green fluorescent protein (GFP). Since then, he and others have developed GFP variants that glow red, yellow, and many other colors. Biologists quickly learned to tag proteins by fusing them with GFP or its cousins. Today biologists can follow several differently colored proteins simultaneously in live cells and in real time. “To see molecules zip around inside living cells and tissues—that’s been huge,” Svoboda says.

Xiaowei Zhuang, an HHMI investigator at Harvard University, makes movies of single fluorescently tagged influenza viruses

invading cells. Others had used electron microscopes to spot influenza virus in membrane-bound compartments called coated vesicles and endosomes, indicating that cells engulfed the viruses in a process called receptor-mediated endocytosis. But no one knew whether the virus homed in on existing pits in the membrane or induced the cell to create new ones.

Zhuang’s movies showed a red virus surrounded by a green coat of clathrin molecules, which coat membrane pits. The pits then bud off to become coated vesicles. “We actually saw pits grow right outside a virus,” she says. That means the virus most likely persuades the cell to take it up by receptor-mediated endocytosis—a result she confirmed by statistical analyses. Until Zhuang made her movies, no one knew influenza viruses used this trick to invade cells. Drugs that target key parts of the process could one day help block viral infection.

Svoboda and other researchers use two-photon excitation microscopy to peer into

opaque tissues like the brain—an ability akin to Superman’s x-ray vision. Optically, brain tissue resembles milk, he says. The interface between fat and water “acts like little mirrors” that scatter light and make the substance appear white, which makes it impossibly murky under older fluorescent scopes.

In two-photon excitation microscopy, however, a new type of laser emits bright, synchronized pulses of infrared light that are focused on tiny volumes in the cell. Tagged molecules fluoresce only when they simultaneously absorb two such photons. Outside of the zone of focus, that’s rare, so the method dramatically

reduces background. Recently, Svoboda’s team watched single nerve endings fire by combining the method with a second fluorescence method called fluorescence resonance energy transfer (FRET).

FRET works like this: One fluorescent dye emits light of a specific color that excites a nearby dye to glow a different color. By tagging one protein with the donor dye and a second protein with the acceptor dye, scientists can see when and where the two proteins interact. Svoboda chose proteins that would come together only when an enzyme called Ras was activated. It turned out that activation of single synapses in the hippocampus activates Ras, which then

stimulates the synapse to reshape itself—a phenomenon that underlies learning.

### Optical Advances RECENT ADVANCES IN

fluorescence microscopy occurred only because microscope designers focused on developing and adapting new technologies. To see GFP and its cousins, for example, they built new lenses from materials that allow for brighter samples and greater contrast, created thin-film interference filters that transmit only a specific color of light, and replaced film cameras with low-noise CCD cameras to record very dim fluorescence. Two-photon excitation microscopy required new microscope objectives that were transparent to infrared light, and mode-locked pulsed lasers, developed in the 1980s, to create very short, very bright pulses of photons.

The past few years have seen an explosion of new microscopy methods, which sometimes read like alphabet soup: two-photon fluorescence correlation microscopy (TPFCM), which helped scientists trace drug transport in tumors; three-dimensional live-cell microscopy, which helped identify never-before-seen threadlike transport lines between live cells; and the GRIN lens, a needle-shaped, insertable lens that can create microscopic images several centimeters deep in the brain.

But microscope designers are far from done. At Janelia Farm, group leader Eric Betzig, a physicist, will see if he can build microscopes that use optical tricks to see objects far smaller than previous light microscopes. Betzig is developing what he calls the optical lattice microscope, in which multiple beams of light interfere with each other to create a three-dimensional lattice that will fill a sample with spots of light. The result, according to his theories, would be a microscope that can image objects three times smaller than today’s best light microscopes and thousands of times faster. Others in the small field of ultra-high-resolution microscopy are pursuing the same goals using different designs.

“The holy grail is to see dynamically imaged living cells noninvasively, to see at the level of an individual protein molecule, and to see how the molecules interact in a cell,” Betzig says. “It’s many years away, but you can dream about it.” ■

## THE CHALLENGES OF IMAGING

**TO BRING CELLS INTO SHARPER FOCUS**, biologists need a world-class imaging facility, and that’s exactly what HHMI plans at Janelia Farm. But building such a facility offers challenges of its own. **FOR STARTERS, DEVELOPING NEW MICROSCOPES** takes a broad range of expertise. For example, Eric Betzig, a physicist and Janelia Farm group leader, has developed a blueprint for a new type of ultra-high-resolution light microscope (see main story) by drawing on mathematics, theoretical and experimental physics, and engineering. To make his blueprint a reality, he’ll work with experimental physicists; computer scientists; and electrical, optical, and mechanical engineers to build prototypes and to develop them into reliable instruments. His long-term goal is “to make instruments that are widely used by biologists.” **TO DEVELOP BETTER ELECTRON MICROSCOPES**, Chen Xu, a physicist in HHMI investigator and Janelia Farm group leader Nikolaus Grigorieff’s lab who will manage Janelia Farm’s shared electron microscope facility, will collaborate with microscope manufacturers like FEI and JEOL to obtain the best electron beams, the best phase plates, and the most sensitive CCD detectors—and customize them. Grigorieff’s team will develop fast new computer programs that choose particles to analyze, align them, and piece together a three-dimensional structure. **DEVELOPING NEW MICROSCOPES IS** only part of the challenge. “You also need a facility for [biologists] to do high-end microscopy with established techniques,” says Winfried Denk, a leading microscopist who directs the department of biomedical optics at the Max Planck Institute in Heidelberg, Germany. At Janelia Farm, HHMI plans to create several core facilities, including Xu’s shared electron microscope facility, to provide expert technical support to the biologists who use them. **RESEARCH INSTITUTES LIKE THE MAX PLANCK** Institute and Janelia Farm are great places to develop new technologies, says Denk. “Developing new technology involves taking risks,” he says. Academic scientists, with their eye on tenure and their next grant, can’t always do that. Research institutes also foster cross-disciplinary collaboration, which is critical for developing new microscopes. At Janelia Farm, physicists will rub elbows with biologists, chemists will talk with computer scientists, and molecular biologists will mingle with mathematicians, says Gerald Rubin, director of Janelia Farm. “We’re going to have all those kinds of people working side by side,” he says.