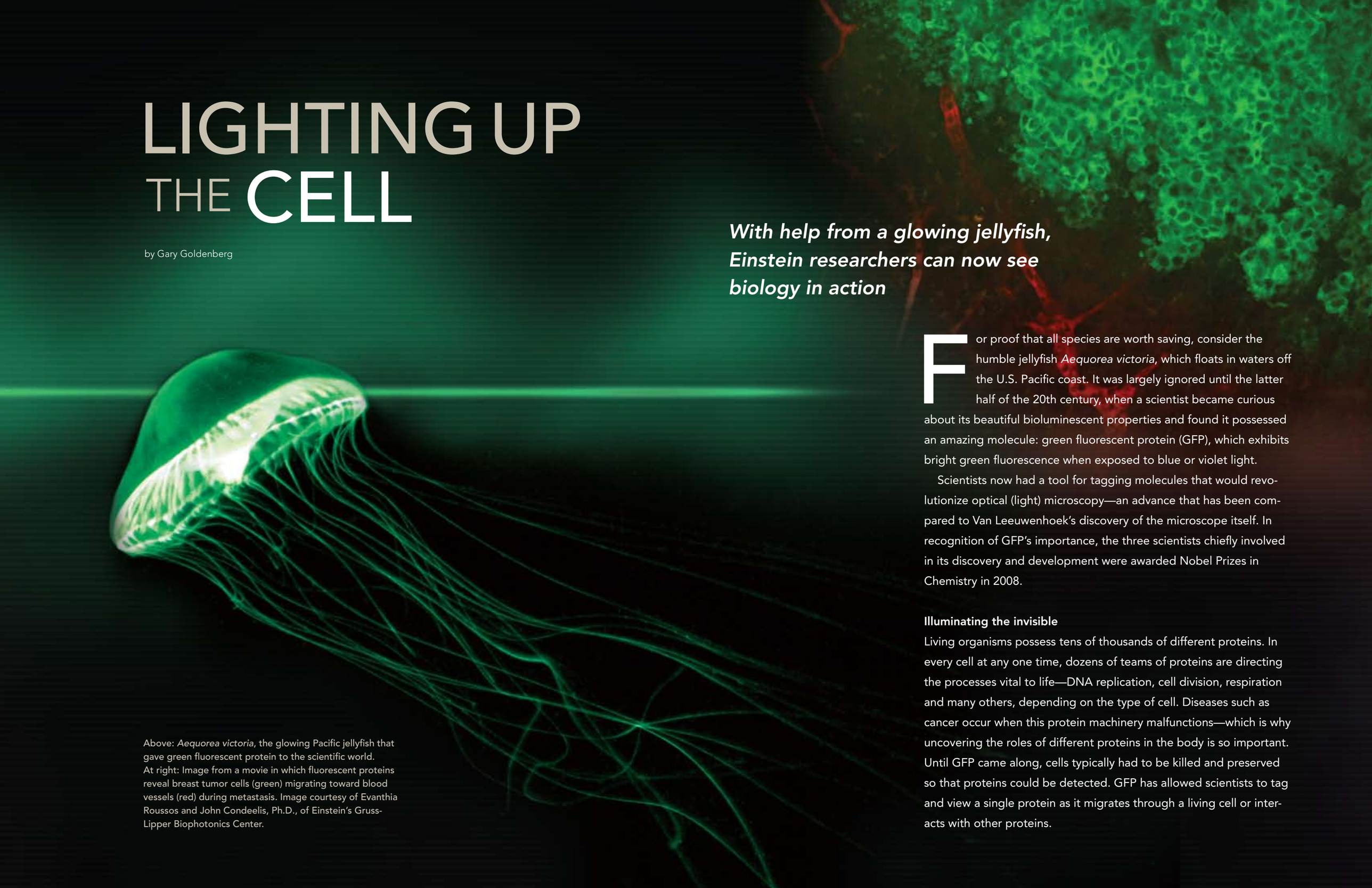
LIGHTING UP THE CELL

by Gary Goldenberg

With help from a glowing jellyfish, Einstein researchers can now see biology in action



Above: *Aequorea victoria*, the glowing Pacific jellyfish that gave green fluorescent protein to the scientific world. At right: Image from a movie in which fluorescent proteins reveal breast tumor cells (green) migrating toward blood vessels (red) during metastasis. Image courtesy of Evanthia Roussos and John Condeelis, Ph.D., of Einstein's Gruss-Lipper Biophotonics Center.

For proof that all species are worth saving, consider the humble jellyfish *Aequorea victoria*, which floats in waters off the U.S. Pacific coast. It was largely ignored until the latter half of the 20th century, when a scientist became curious about its beautiful bioluminescent properties and found it possessed an amazing molecule: green fluorescent protein (GFP), which exhibits bright green fluorescence when exposed to blue or violet light.

Scientists now had a tool for tagging molecules that would revolutionize optical (light) microscopy—an advance that has been compared to Van Leeuwenhoek's discovery of the microscope itself. In recognition of GFP's importance, the three scientists chiefly involved in its discovery and development were awarded Nobel Prizes in Chemistry in 2008.

Illuminating the invisible

Living organisms possess tens of thousands of different proteins. In every cell at any one time, dozens of teams of proteins are directing the processes vital to life—DNA replication, cell division, respiration and many others, depending on the type of cell. Diseases such as cancer occur when this protein machinery malfunctions—which is why uncovering the roles of different proteins in the body is so important. Until GFP came along, cells typically had to be killed and preserved so that proteins could be detected. GFP has allowed scientists to tag and view a single protein as it migrates through a living cell or interacts with other proteins.

FPs Against Disease

Serious diseases affect the entire body. But they arise at the cellular level, due to proteins or other molecules that behave abnormally. FPs allow scientists to observe, in real time and in living cells, the molecular glitches that underlie diseases—knowledge vital for developing therapies against health problems that so far resist effective treatment. In this table, we list several of the faulty molecular and cellular processes that FPs have illuminated and diseases associated with those aberrations.

Abnormality Detected	Disease
Proteins travel to wrong cellular compartment	cystic fibrosis
Protein clumping creates "traffic jams" in nerve cells	Parkinson's disease
Impaired intracellular "garbage removal"	Huntington's disease
Movement of cancer cells into blood vessels	metastatic cancer
Altered intracellular structures	Parkinson's, Progeria

Image courtesy of Nobel Foundation

Right, structure of green fluorescent protein, containing 238 amino acids. White region is the chromophore, the group of three amino acids that absorbs blue or violet light and fluoresces green.

Researchers can also follow cells as they move through living tissues. So thanks to GFP and other fluorescent proteins (FPs), scientists can observe both normal and pathological biological processes that had previously been invisible to them.

Fluorescent microscopy is flourishing at Einstein, where scientists are devising new types of FPs and using them in novel ways. Einstein researchers are employing FPs to learn how cancer spreads, how cells carry out their internal housekeeping, how misfolded proteins trigger neurological diseases and more.

From jellyfish to coral

Until about a decade ago, there were fewer than a dozen FPs—most of them variations on green, yellow and blue. So as a practical matter, researchers studying living cells could follow just two or three different molecules at a time. In 1999 came the next great advance in fluorescent microscopy, when Russian scientists studying coral isolated the first red FP. The next year Vladislav Verkhusha, Ph.D., working in Kyoto, Japan, published a paper describing the biochemical and photochemical properties of this new FP. Dr. Verkhusha is now associate professor of anatomy and structural biology at Einstein, where he remains in the vanguard of FP research.

> By creating FPs in different hues, that switch from one color to another and even change color over time, Dr. Verkhusha has helped revolutionize optical microscopy.

In a paper published earlier this year in *Chemistry & Biology*, Dr. Verkhusha reported discovering the crystal structure of two key fluorescent proteins—one red and one blue. That crucial knowledge has enabled him to design new and differently colored FPs in a rational way.

"Creating novel FPs had been a hit-or-miss affair," says Dr. Verkhusha. "Scientists had to fuse the genes of already-discovered FPs to the genomes of bacteria and then expose millions of those bacteria to radiation, in the hope that random genetic mutations would lead to new FPs. Now that we know those two crystal structures, we have a road map for designing new and differently colored FPs."

By greatly expanding the FP palette in recent years—creating FPs in different hues of red, blue and orange, FPs that switch from one color to another and even FPs that change color over time—Dr. Verkhusha has helped revolutionize optical microscopy.

"The numerous functions going on in living cells occur mainly through molecular interactions—especially proteins interacting with other proteins," says Steven C. Almo, Ph.D., professor of biochemistry and of physiology & biophysics at Einstein and Dr. Verkhusha's coauthor on the *Chemistry & Biology* paper. "To understand what is happening, you ideally want to follow a number of different protein-protein interactions simultaneously—which means the more colors you have for tagging those proteins, the better. That's why the FPs that Vlad is developing are so exciting—they allow you to actually see the machinery of life as it comes together."

An FP primer

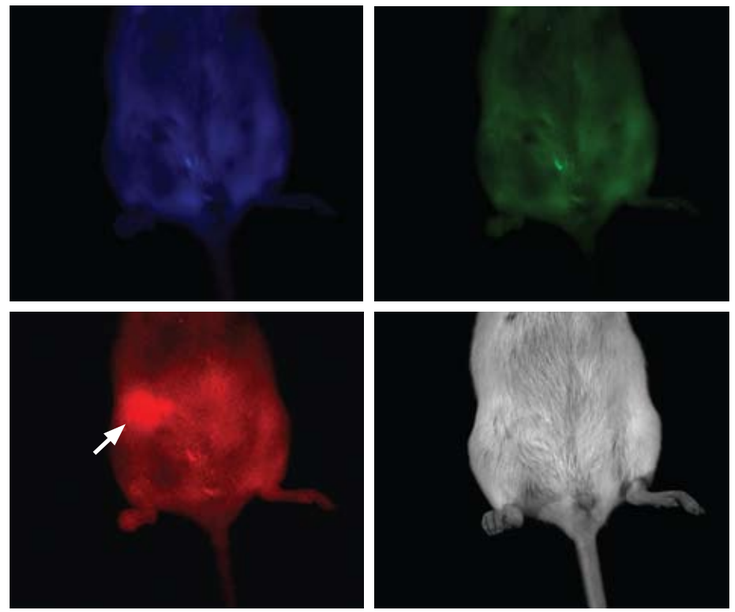
Dr. Verkhusha's colorful creations belong to several categories of FPs, including the following:

Far-red FPs: Until recently, FPs couldn't be used to peer inside a live animal. The reason: hemoglobin in an animal's blood effectively absorbs the blue, green, red and other wavelengths used to stimulate standard FPs and that are emitted by FPs when they fluoresce. Now, in an exciting development described earlier this year in *Biophysical Journal*, Dr. Verkhusha has created an FP that can be stimulated by wavelengths that pass right through hemoglobin—wavelengths at the far-red end (611 nm) of the visible spectrum. This far-red FP, called TagRFP657, fluoresces at the far-red end as well, and this emitted light (657 nm) can be seen with the use of special emission filters. (See images at right.)

Far-red FPs will allow scientists noninvasively to track molecules and cells that participate in biological processes occurring deep within tissues, organs or even entire animals. In addition, far-red FPs can reveal tumors—an important medical advance.

Recently, in collaboration with the laboratory of John Condeelis, Ph.D., Dr. Verkhusha developed three populations of mouse breast cancer cells, each of which expressed a different FP: CyanFP (blue), GFP (green) and TagRFP657 (far red). A mixture of the three cell populations was injected into a mouse. Two weeks later, the mouse had developed a mammary-gland tumor that was composed of cells expressing the three FPs.

The images below show what happened when the researchers tried to visualize the tumor by stimulating each of its three FPs to fluoresce. As expected, efforts to cause fluorescence of tumor cells expressing the blue and green FPs failed to reveal the tumor. By contrast, tumor cells expressing the far-red TagRFP657 fluoresced brightly, clearly revealing—noninvasively—the tumor deep within the mouse.



Inside a living mouse, a breast tumor containing three types of FPs has been stimulated to emit blue, green and far-red light. The tumor is clearly visible in the lower left image due to fluorescence from the far-red TagRFP657 (see arrow). Animal in the first three images appears tinted due to autofluorescence of its hair. The black-and-white image shows the animal in ordinary light.

Photoactivatable FPs: More versatile than ordinary FPs, photoactivatable FPs can be turned on or off with a pulse of light. Dr. Verkhusha has created several novel photoactivatable FPs, including PAmCherry, which goes from dark to red when exposed to a particular wavelength of light. Photoactivatable FPs are used mainly to mark a select population of protein molecules within a cell or to highlight whole cells of interest. Once their FPs are activated, these tagged proteins or cells can be followed in real time. For proteins in particular, photoactivatable FPs provide unsurpassed resolution for revealing their fate in single cells over both time and space. (See image on page 30, upper left.)

Photoswitchable FPs: These FPs change from one color to another after activation by a particular wavelength of light. There are two classes: reversible photoswitchable FPs, which can repeatedly be turned off (dark) and on (e.g., green) with pulses of light, and

irreversible photoswitchable FPs, which change color only once. In practice, researchers use photoswitchable and photoactivatable FPs for similar purposes. Dr. Verkhusha developed the first irreversible photoswitchable FP, called PSCFP, which changes from blue to green. More recently he developed Dendra, an irreversible photoswitchable FP that changes from green to red. (See image on page 30, upper right.)

Fluorescent timer FPs: These are FPs that change color over time. Until recently, fluorescent timer molecules were so big that they were useful only for tagging entire cells. In a major advance, Dr. Verkhusha's lab has developed a new class of fluorescent timers, called monomeric fluorescent timers, which are small enough to tag individual proteins and which change color (blue to red) at different speeds: "Slow" fluorescent timers take nearly a day to change color, "medium" timers change color in about 12 hours and "fast" timers change from blue to red in a few hours. The ratio of blue to red timers can indicate, for example, whether most of the molecules of a tagged cellular protein are old or newly synthesized or whether new and old proteins have localized to the same or different regions of a cell. (See images on page 31.)

The FP clearinghouse

To help researchers who want to use these imaging tools, Dr. Verkhusha and Erik Snapp, Ph.D., assistant professor of anatomy and structural biology, have established the Fluorescent Protein Resource Center at Einstein. The center serves as an information clearinghouse for FPs and assists researchers in choosing the best FPs for their experiments. The advice is crucial, since FPs can be tricky to use—failing to fluoresce in some cellular environments and creating unwanted artifacts in others.

FRET, PALM and Intracellular Intimacy

For today's optical microscopists, observing the proximity of different molecules within a cell can provide crucially important information. Proteins that bind together, for example, may be involved in an enzymatic reaction, while proteins that migrate to the same organelle may be participating in the same biochemical pathway. Dr. Verkhusha's FPs are used in two techniques that allow researchers to visualize two different molecules that are much less than a hair's breadth from each other.

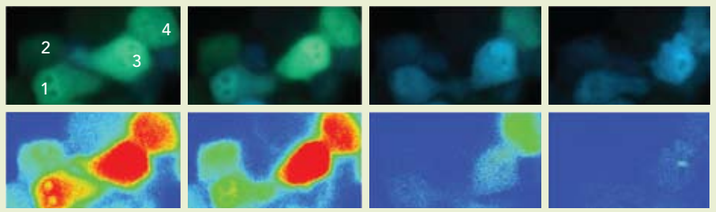
FRET (fluorescence resonance energy transfer) imaging can resolve two molecules that are bound together in a cell or otherwise extremely close together—from zero to five nanometers (five billionths of a meter) apart. The "ET" in FRET refers to the maximal "energy transfer" that occurs when molecules containing blue and green FPs are close together: The blue FP transfers its energy to the green FP—dimming the blue, brightening the green and confirming a close encounter. Conversely, as shown in the image below, FRET signaling disappears—and the color shifts from green to blue—when two once-close entities separate.

In 2008, Dr. Verkhusha and colleagues published a paper in *Chemistry & Biology* showing that FRET imaging can be used to detect apoptosis—the orderly (genetically programmed) cell death that can occur in response to viruses, toxins or other stressors. The enzyme caspase-3, activated during apoptosis, carries out the death sentence by degrading cellular proteins.

In this experiment, human cancer cells have been manipulated into expressing a "sensor" protein consisting of a blue FP on one end, a green FP on the other and a peptide sequence in the middle that caspase-3 targets when it degrades proteins. At the start of the experiment, the four cells shown in the images below were exposed to a drug that triggers apoptosis. The cells were then followed for 150 minutes.

The upper panels consist of overlaid images of the blue and green FPs at different times in the four cells. The transition in color indicates apoptosis in action: as increasing numbers of sensor proteins are cleaved, their blue and green FPs drift apart, causing the color to shift from green to blue.

In the lower panels, the four cells have been analyzed to detect the FRET signaling produced by the proximity of the green and blue FPs. In these pseudocolored images, red indicates maximal FRET signaling (i.e., the green and blue FPs are practically touching), while blue indicates minimal signaling (i.e., the FPs have drifted apart). The change from red to blue over the 150 minute period depicts the destruction wrought by caspase-3 during apoptosis.



PALM (photoactivated localization microscopy) uses photoactivatable FPs to resolve images 10 to 200 nanometers apart. This makes PALM ideal for distinguishing molecules that "colocalize" in a living cell, meaning they gather within the same organelle, for example, or in a large protein complex.

At Einstein, PALM allows researchers to study the clustering of proteins, including one that plays a crucially important role in cancer: epidermal growth factor receptor (EGFR).

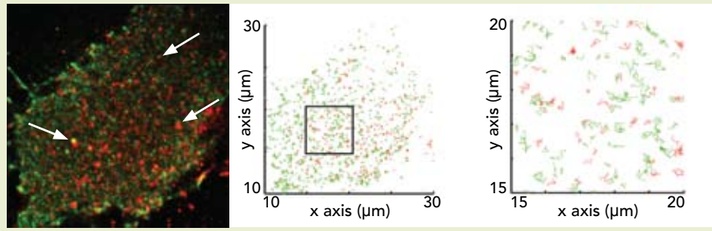
After being synthesized in the cytoplasm, EGFR migrates to the cell membrane. It sends a signal to the cell when a protein called epidermal growth factor binds to it. "Normal" EGFR signaling may tell the cell to divide. However, aberrant EGFR signaling is associated with many types of solid tumors: abnormally high levels of EGFR can often be found on the surface of tumor cells. A focus of cancer chemotherapy is to develop drugs that muffle EGFR signaling and prevent the uncontrolled cell division associated with cancer.

In a paper published earlier this year in the *Journal of the American Chemical Society*, Dr. Verkhusha and colleagues used PALM to answer this question: Do EGFR proteins insert themselves randomly into the cell membrane or instead localize to particular regions? To follow EGFR in its travels, the researchers tagged it with a photoactivatable red fluorescent protein called PATagRFP; then the green photoactivatable fluorescent protein PAGFP was used to mark a small protein called VSVG—a short sequence of amino acids that is thought to attach uniformly to the cell membrane everywhere throughout its surface. If EGFR colocalized with VSVG, it would indicate that EGFR molecules, as is the case with VSVG molecules, arrive randomly at their positions on the cell membrane.

The PALM image, below left, is an overlay of EGFR (red) and VSVG (green) in a living human cell. It shows the relative distribution of molecules of the two proteins following their synthesis and arrival at the cell membrane. The arrows indicate some of the few places where the two types of protein molecules have colocalized.

The middle image is a graph in which "tracks" (movements) made by EGFR and VSVG molecules lasting longer than a second have been plotted according to distance and direction traveled. EGFR molecules are represented in red and VSVG molecules are in green.

The right-hand image is a close-up of the region indicated by the square in the middle image. In this particular region of the cell membrane, EGFR and VSVG appear to be localizing differently. Results indicate that EGFR does not insert randomly in the cell membrane but instead chooses specific membrane regions.



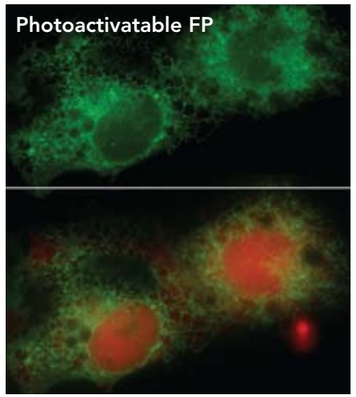
"FPs are large compared to the proteins to which they are attached, which means that an FP can sometimes block a protein from binding to its intended target," says Dr. Snapp. "You still see the protein fluoresce, so you assume everything is fine, but the FP that the protein is lugging around has actually rendered the protein inactive. That is why it's essential, whenever we fuse an FP to a protein, to have assays that can tell us whether the FP is interfering with the protein's known function."

Two of Dr. Snapp's graduate students are working on strategies for surmounting the limitations of FPs. They recently discovered a technique for ensuring that FPs assume their characteristic barrel shape (required for fluorescence to occur) when they're deployed to the periplasm of bacteria. This space between the inside and outside membranes of gram-negative bacteria is often hostile to FPs, preventing them from fluorescing.

Everything is illuminated

Today, Dr. Verkusha's FPs are glowing in laboratories all over the Einstein campus. Dr. Snapp uses them in his own research to study the endoplasmic reticulum (ER), a membranous cytoplasmic network where secretory proteins (e.g., hormones and signaling molecules) are folded into their proper shape—an essential task carried out by molecules known as chaperone proteins.

Protein folding appears to go awry in Huntington's disease, the fatal inherited neurological disorder caused by a gene mutation that creates a defective form of the huntingtin protein. In the cytoplasm, defective huntingtin protein and other misfolded proteins destined for degradation are nudged into large protein complexes called proteasomes. But huntingtin appears to cripple the function of proteasomes, with potentially serious consequences for the cell.



By imparing proteasome activity in the cytoplasm, could defective huntingtin cause proteins that need degrading to back up inside the ER and damage cells? Efforts to address that question could lead to new treatments for Huntington's disease by identifying new therapeutic targets.

The **photoactivatable FP** images above are from a study in which postdoc Patrick Lajoie of Dr. Snapp's lab used two FPs to see if he could simultaneously monitor, in real time, two possibly related cellular activities: the turnover of a toxic cytoplasmic protein such as huntingtin and the buildup of misfolded proteins in the ER. The red FP is PAmCherry, the photoactivatable FP developed by Dr. Verkhusha's lab; the green FP is GFP. (These images also appear on the front cover and contents page of the magazine.)

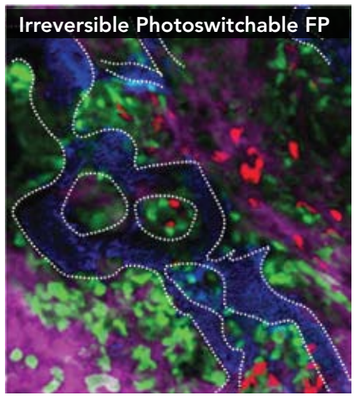
In the top image above, exposing two live human cells to blue light has caused GFP to fluoresce bright green. GFP is tagged to BiP, an important chaperone protein that binds to misfolded proteins and is used here to detect misfolded-protein buildup in the ER. Since BiP resides in the ER, its fluorescence reveals the cells' extensive ER network. The lower image, taken later, shows the same two cells after illumination for one minute with near-ultraviolet light of 420 nm wavelength. The photoactivatable FP PAmCherry, which can be attached to a toxic protein such as huntingtin, was readily activated, changing from dark to bright fluorescent red and becoming visible throughout the cells' nuclei (large red areas) as well as the cytoplasm. These studies have revealed that synthesis of defective huntingtin protein increases the misfolded-protein burden in the ER.

In the Gruss Lipper Biophotonics Center, Dr. Condeelis uses a custom-built microscope in combination with FPs to study metastasis—the migration of malignant cells into the bloodstream and then to the lungs, liver or other vital organs. Cancer patients don't usually die from their original (primary) tumors but rather from tumors "seeded" by metastatic cells. Dr. Condeelis has done pioneering research in breast

Using FPs that switch from green to red, Einstein researchers tracked the movement of single tumor cells over several days.

cancer metastasis, deciphering signaling pathways within the tumor microenvironment that guide cancer cells on their deadly journey.

In a paper published in *Nature Methods* in 2008, Dr. Condeelis and colleagues observed the movement of breast-tumor cells in living mice. Tumors were tagged with Dendra, an irreversible photoswitchable FP developed by Dr. Verkhusha that changes from green to red following exposure to blue light. Then, when the researchers photoswitched certain regions of the tumor, they were able to track single tumor cells over the course of several days.



The **irreversible photoswitchable FP** image above shows the position of breast-tumor cells 24 hours after parts of the tumor were photoswitched. Nonphotoswitched tumor cells have remained green. (Blood vessels are stained blue [outlined by white dotted lines] and the extracellular matrix is stained purple.) The photoswitched (red) cells can clearly be seen to have invaded the surrounding microenvironment—including nearby blood vessels—during that 24-hour period.

"Without FPs, you wouldn't have this level of resolution," says Dr. Condeelis, cochair and professor of anatomy and structural biology, codirector of the Biophotonics Center and holder of the Judith and Burton P. Resnick Chair in Translational Research. "All of the other clinical imaging methods—MRI, PET, CT, ultrasound—provide information only about gross anatomy, not about the cellular microenvironment. With our setup, it's crystal clear."

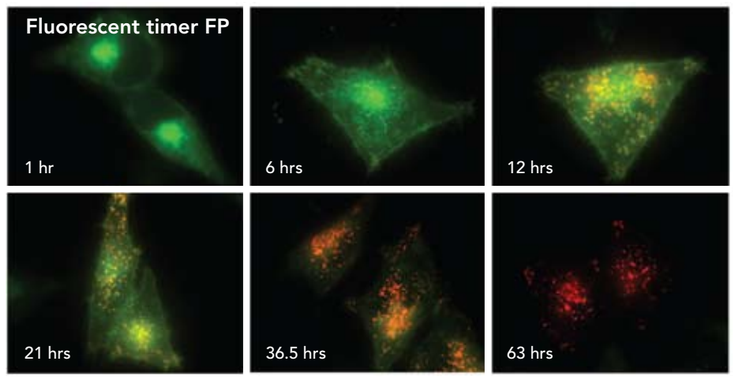
Another Einstein scientist, Ana Maria Cuervo, M.D., Ph.D., professor of developmental and molecular biology, of anatomy and structural biology and of medicine, is a leading authority on autophagy—the cellular "housekeeping" process in which worn-out or defective proteins are digested in sac-like, enzyme-filled structures called lysosomes; the proteins' amino acids are then recycled. Evidence increasingly suggests that glitches in autophagy underlie the degenerative changes associated with aging as well as diseases such as Huntington's and Parkinson's. Dr. Cuervo is researching strategies for revving up autophagy when its efficiency declines.

In one type of autophagy, molecules bearing proteins that are destined for recycling dock with LAMP-2A, a protein receptor on the lysosomal membrane. This crucial step allows the protein to pass into the lysosome for digestion. In a *Nature Chemical Biology* paper published in 2009, Dr. Cuervo and other Einstein researchers used a fluorescent timer FP to address a long-standing mystery: the sequence of events between LAMP-2A's synthesis and its arrival at lysosomes.

The gene for LAMP-2A was fused with the gene for Dr. Verkhusha's medium-speed blue-to-red fluorescent timer FP, and the fused gene was inserted into human cancer cells. This gene was allowed to synthesize fusion protein (consisting of LAMP-2A and the fluorescent timer FP) for seven hours.

The **fluorescent timer FP** images, above right, show how LAMP-2A localized in the cell at various times (from one to 63 hours) after protein synthesis was halted. The newly synthesized fusion protein changed gradually from blue to red over time. Based on the red-to-blue ratios observed for fusion protein at different times in different parts of the cell, the researchers concluded that LAMP-2A prefers a roundabout route to lysosomes.

Following LAMP-2A's synthesis, its traffic pattern takes it to the Golgi (organelles that add carbohydrates to proteins), the plasma membrane (the cell's outer membrane), early endosomes (membranous structures that funnel molecules from the plasma membrane



to internal parts of the cell), late endosomes and, finally, lysosomes. The blue and red forms of LAMP-2A/fluorescent timer FP are shown here as green and red pseudocolors, respectively.

To attach an FP to the protein they want to "follow," researchers must fuse the FP's gene to the gene for that protein. Expression of this fused gene forms the protein of interest with its attached FP tag. Vladislav Verkhusha, Ph.D., left, and Erik Snapp, Ph.D., center, have formed the Fluorescent Protein Research Center to help Einstein scientists choose the optimal FPs for their research and create successful fusion proteins.

Thankfully, *A. victoria*, the glowing Pacific jellyfish that sparked the GFP revolution, was in ample supply when it was studied 50 years ago. Unfortunately, thousands of other species have become extinct since then. Who knows what medical science has lost in its effort to cure disease? E