An Ever-Brighter Future for Fluorescence

New, less toxic fluorescent proteins and tags, and strategies for delivering them, are allowing researchers to watch processes within cells never before observed. Industry and academia are now working to not only detect these processes, but also localize and quantify them. By Anne Harding

Fluorescent dyes and tags have come a long way since the cloning of green fluorescent protein (GFP) nearly two decades ago. Researchers now have a literal rainbow of products to choose from, in an array of forms from nanoparticles to proteins, and can increasingly multiplex their experiments by using several different colors. “Twenty years ago, people were lucky to be able to detect green fluorescence,” says Kathy Free, senior product manager of Invitrogen, part of Life Technologies. “With the advances in instrumentation, they actually now are comfortable with four or five colors at once.”

“Probably since about 2007 there has been a real explosion of new fluorochromes,” says Richard Eglen, president of biodiscovery at PerkinElmer. “It really is starting to open up the field dramatically.”

Roger Tsien, who shared the 2008 Nobel Prize in Chemistry for his work in developing GFP as a tagging tool, developed a series of recombinant fluorescent proteins, including the far-red mPlum and mRaspberry, all derived by directed mutagenesis from a monomeric mutant of DsRed, which itself was derived from a Discosoma reef coral fluorescent protein. Clontech, a part of the Takara Bio Group, which first commercialized GFP technology as a cell biology research tool, back in 1994, also has commercialized these tags as Fruit Fluorescent Proteins.

As always, the makers of fluorescent dyes and tags are aiming for ever-improving brightness and photostability, while the increasingly widespread use of live cells has pushed companies to come up with new ways to illuminate activities within cells without harming them. Progress remains to be made in the development of probes that are spectrally distinct enough in fluorescence and activation to increase multicolor experiment capabilities; meantime, researchers are using photoactivatable and photoswitching proteins to observe biological processes at the molecular scale.

GETTING COLOR INTO THE CELL
Fluorescence is not just about color, of course; it’s also about finding new ways to get those colors into the cell. The click chemistry concept introduced by K. Barry Sharpless of the Scripps Research Institute (who shared the 2001 Nobel Prize in Chemistry for this work) is allowing investigators to tag proteins using a much simpler, less toxic process. Invitrogen offers a number of Click-iT labeling kits and reagents based on click chemistry, with reactive probes incorporating the company’s Alexa Fluor dyes.

Invitrogen’s Click-iT tools use a copper-catalyzed alkyne and azide reaction to join biomolecules. Because the alkyne-azide links use functional groups not found in biological systems, they help investigators avoid nonspecificity and background noise.

Fredika Robertson, a professor at The University of Texas M.D. Anderson Cancer Center, uses Invitrogen’s Click-iT EdU assay to study the behavior of the tumor-initiating, putative cancer stem cells that give rise to inflammatory breast cancer, a rare and lethal form of the disease that does not respond to chemotherapy or radiation. “The way to enrich for these cells and to study them is to develop three dimensional models in culture,” Robertson explains. She and her colleagues culture the cells under low adherence conditions so they spontaneously form tumor spheroids. Robertson has been able to label and track the cells from which these breast cancers emerge using this model.

Initially, Robertson used bromodeoxyuridine (BrdU) to observe replication of these cells. Unlike BrdU, the Click-iT EdU assay does not require extensive pretreatment to visualize the nucleoside label that defines them as “label-retaining cells.”

“This is an improvement over BrdU since the quality of the images of the 3D spheroids is improved, allowing quantitation of the label-retaining cells and providing an assay to examine the effect of agents that may target these cancer stem cells,” Robertson says.

One limit to the click chemistry approach is the toxicity of copper, Robertson says, although Carolyn Bartozzi, director of the Molecular Foundry at the Lawrence Berkeley National Laboratory, and her colleagues are working on a copper-free version. In continued *
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February, Bartozzi and her team reported on their use of the copper-free technique to monitor the dynamics of glycans in mice, the first time that click chemistry has been safely used in a living organism.

"I think click chemistry has given us the means to view these dynamics in a way that we couldn’t do before," Robertson says. "But there are innovations coming soon that will make it even better."

FOCUSING ON THE RIGHT END OF THE SPECTRUM

Far-red is the hottest end of the spectrum right now for fluorescent dye development, for both microscopy and in vivo imaging, many in the industry say. The instant signal-to-noise ratio advantage that near-infrared fluorescence offers in imaging biological tissues, which do not autofluoresce in this wavelength, allows for seeing more deeply and clearly into samples and animals.

Steve Shifflet, technical product manager for Thermo Scientific Pierce Protein Detection Products, says that far-red and near-infrared dyes will continue to be a significant part of his company’s efforts.

“This will continue to be an area of increasing focus in the life science industry,” he said. “This is still a relatively new area that presents lots of opportunities.”

In February of this year, Thermo released its DyLight 680B Dye, a brighter version of its DyLight 680. The new dye is significantly brighter than Alexa Fluor 680 Dye, Shifflet says.

Moscow-based Evrogen has also made red and far-red fluorescent proteins a focus, says head of product development Ilya Kelmanson.

“Now our product line includes four extremely bright red and far-red fluorescent proteins that can be used in different kinds of in vivo imaging applications, including two-photon microscopy and whole body imaging,” Kelmanson says.

Evrogen just released a second-generation version of its far-red monomeric fluorescent protein mKate, mKate2, which is three times brighter than its predecessor and 10 times brighter than mPlum, and has a maturation half-time of less than 20 minutes, compared to mCherry’s 40 minutes, according to the company.

Clontech also boasts several far-red products designed for in vivo use. “DsRed-Express2 and E2-Crimson were developed and validated specifically for high solubility and use in stem cells and other sensitive cells,” says Suvarna Gandlur, product manager at Clontech.

“Unlike most far-red fluorescent proteins, E2-Crimson combines a favorable emission wavelength with a bright signal, for easy detection in vivo. Another ideal choice for in vivo imaging is tdTomato, which has been imaged as deep as 1 cm below the surface in SCID [severe combined immunodeficiency] mice.” Gandlur said the company is working on developing additional, application-specific fluorescent proteins for in vivo and other uses.

Carestream Health stakes the claim for near-infrared dyes with the biggest Stokes shift. The company’s Kodak X-Sight Large Stokes Shift Dyes and LSS Dye Conjugates offer 85 to 98 nanometer separation of excitation and emission peaks within the near-infrared range, the only products now available with such a large, naturally occurring Stokes shift in the near-infrared range, says Seth Gammon, in vivo product manager at Carestream Molecular Imaging.

TARGETING STRUCTURES AND PROCESSES

Fluorescent labels that target specific cell structures and systems for quickly identifying particular processes in cellular metabolism and division are other important areas of growth in the field. Evrogen offers expression vectors for labeling of six subcellular structures and nine key proteins, and a choice of colors is available for most of them. Active Motif sells two self-staining kits, one for staining mitochondria, another for staining the cytoskeleton in fixed cells. And Invitrogen’s Cellular Lights target six key proteins, while its Organelle Lights target 11 subcellular structures.

Limitations of organelle-specific probes include photobleaching and quenching upon concentration in the target organelle, or only transient association with that organelle, Enzo Life Sciences researchers noted in a 2009 report in FASEB Journal. In that report, they describe a new “family” of red-emitting, organelle-specific probes—part of their CELLestial product line—that they say are “highly resistant” to these problems.

Cellular processes that can now be studied with “ready to go” fluorescence kits include cellular division, autophagy, apoptosis, and more. Probes produced by Invitrogen and MBL International that license work done in the lab of Atsushi Miyawaki at the Brain Science Institute for Physical and Chemical Research (RIKEN) in Tokyo use the fluorescent ubiquitination-based cell cycle (Fucci) to visualize the cell cycle in real time.

Clontech’s Lenti-X Actin Dynamics Monitoring Kit allows users to monitor actin filament behavior in live cells. The company also offers Precloned CRE and NFκB DD Reporter Systems, which use Clontech’s brightest red, green, and cyan reporters to monitor commonly studied signal transduction pathways.

MAKING SENSE OF SENSORS

One goal companies are reaching for is to make sensors that not only indicate that something is happening, but show where it’s happening, quantitatively. “The two strategies are going to be fluorophore/quencher pairs, as well as probes that are actually modified by the chemical they’re trying to detect,” predicts Carestream’s Gammon.

Molecular Devices’ FLIPR calcium flux assays, introduced in 1999, employ a calcium-sensing fluorescent dye along with quenching technology, and eliminate the need for cell washing following dye-loading. The company, now a part of Danaher, released...
its FLIPR Calcium 5 Kit in June of last year, but expects to keep the original FLIPR Calcium Kit, as well as versions 3 and 4, on the market for as long as its customers need them. “Certain targets respond better to certain versions of the systems,” notes applications scientist Carole Crittenden.

Companies are also introducing sensors that use fluorescence resonance energy transfer, or FRET, in which a conformational change brings together two fluorophores attached to the sensor; the excited fluorophore then transfers energy to its unexcited neighbor.

Invitrogen’s Molecular Probes Premo Cameleon Calcium sensor uses genetically encoded sensors for FRET-based ratiometric detection for quantitative results, delivered by the company’s BacMam system.

Evrogen has two genetically encoded fluorescent sensors for detecting caspase-3 mediated apoptosis in living cells, both FRET-based. The company also sells non-FRET-based sensors, including its HyPer line, the first and only genetically encoded sensor capable of detecting intracellular hydrogen peroxide, and Case12, for detecting intracellular calcium ion changes.

ON AGAIN, OFF AGAIN FLUORESCENCE
Photoactivatable and photoswitchable fluorescent proteins are another key area of innovation, useful for observing dynamic processes within cells and in single molecule–based superresolution protein localization. Photoactivatable fluorescent proteins go from “off” to “on” with exposure to light of a certain wavelength, and will continue to fluoresce until they bleach out. Photoswitchable proteins can be switched from dark to bright or red to green; using light of a different wavelength, they can then be switched back to their original state.

Jennifer Lippincott-Schwartz, who started working with GFP in 1994 and has made major advances in the use of fluorescence in imaging—both at the conventional and molecular levels—has developed a way to use photoswitching and photoactivatable proteins (and even light-activated conventional fluorophores) to watch trafficking within cells at the molecular level. She and her colleagues together with physicists Eric Betzig and Harald Hess first reported on the technique, known as PALM (photoactivated localization microscopy). Xiaowei Zhuang and her colleagues at Harvard University have developed a similar technique, STORM (stochastic optical reconstruction microscopy), that uses photoswitchable dyes.

Rather than lighting up ten thousand molecules at once—producing a bunch of overlapping blurry spots—a low amount of activating energy is used to light up just a few at once; this handful of molecules will be far enough apart to be imaged as a spot shape. “That shape, because it has a Gaussian profile, can be fit into an algorithm that allows you to define with very high precision the shape’s center, used as the molecule’s position,” Lippincott-Schwartz explains. Then, thousands of these images are combined to map the total population of molecules. “It’s similar to pointillism,” she adds. “You’re building an image by combining small, distinct spots.”

There are over 20 different varieties of photoswitching or photoactivatable proteins available, says Lippincott-Schwartz. “The proliferation of these photoactivatable or photoconvertible fluorescent proteins permits a variety of novel imaging schemes for obtaining new biological insights,” she says. “That said, the fluorescent proteins don’t give off a huge amount of photons, only about a tenth of the photons that would be produced by a photoswitchable dye.”

There has been major interest in trying to develop these sorts of dyes, she adds, with one approach involving caging a dye with a protective group that is destroyed when it’s illuminated, permitting the dye to fluoresce. But dyes have their own challenges in that they need to be targeted somehow, for example with antibodies, which carry the risk of nonspecificity and also limit resolution due to their relatively large size. Click chemistry can also be useful, she adds: “Because the protein has been only slightly modified with a cysteine tag or something similar for recruitment of a dye, the protein stays close to its original size and so is more likely to target correctly.”

Lippincott-Schwartz and her team are now working on double-labeling two different proteins, and are also collaborating with Hess at Howard Hughes Medical Institute’s Janelia Farm to look at the three-dimensional distribution of molecules within cells.

According to Lippincott-Schwartz, companies that make imaging equipment have worked closely with researchers in this area to make sure their confocal microscopes and other tools keep up with these types of experimental innovations.

“It’s an absolutely amazing synergy between very different groups of people: the physicists who are building the microscopes, the chemists who are working on the probes, and then the biologists who put all of this together to answer biomedically related questions,” she says. “All three groups seem to be moving forward. It’s pretty neat to see this. It’s the right time for all these disparate groups coming together.”

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