TechNews

COLORFUL ENGINEERING

Advances in fluorescent dye and protein design have led to a wider color palette for microscopy. Nathan Blow explores how this happened and what is still to come.

Credit: J. Grimm

The old saying “a picture is worth a thousand words” is certainly true when it comes to multi-color, high-resolution microscopy images. With the possibility of imaging specific cellular structures, proteins, or even nucleic acids and then following them in real time, our ability to visualize the mechanics of the cellular world through the microscope lens is more robust now than ever before.

Although advances in microscope technology have made much of this possible, microscopes by themselves do not bring cellular ecosystems into sharp focus—a lot of the credit goes to fluorophores (i.e., fluorescent dyes and proteins). These specialized tags make it possible to track molecules within cells and also enable specialized applications such as super-resolution imaging.

Fluorescent dyes have actually been around for a long time; rhodamine dyes were first described in 1887 and are still being used today. That's not say there haven't been changes over the past 130 years. Chemists have modified rhodamine by tinkering with the molecular structure to create versions of the dye with different excitation and emission properties.

Fluorescent dyes are not alone when it comes to developer tinkering—since the first reports of using green fluorescent protein (GFP) as a molecular biology tool in 1994, fluorescent protein (FP) engineering efforts have exploded as many of the key biochemical properties responsible for absorbing and emitting specific wavelengths of light have been discovered. In fact, it's this growing understanding of the chemistry and physics behind both fluorescent dyes and proteins that has produced today's numerous fluorophore options.

Fine-tuning colors

Initially, it was small-molecule fluorophores and dyes that found wide use in biological imaging applications. As fluorescence microscopy technology and dye-based lasers emerged in the middle of the 20th century, additional advances in chemical fluo-

rophores for imaging were made. Eventually, solubility and photostability improvements made through major structural changes gave rise to the CyDye and Alexa Fluor series in the 1990s.

But as far as trends go, dyes and small molecule fluorophores lost favor as the primary labeling tool for fluorescence microscopy during the mid-to-late 1990s when FPs came on the scene. With the ability to genetically tag a protein of interest for live-cell imaging and the possibility of modulating the protein's excitation and emission maxima by simply changing amino acids, a strong groundswell of interest emerged among developers in generating as many different versions of GFP as possible.

During this FP revolution, fluorescent dyes continued to find use for certain applications, typically for imaging of dead cells (immunofluorescence and FISH). Quietly, however, chemists and biochemists started to think of ways to combine the genetic specificity and live-cell compatibility of FPs with the photophysics of synthetic dyes such as rhodamine. These efforts resulted in the emergence of enzyme-based self-labeling tags (e.g., SNAP-tag, HaloTag) that allow construction of fluorescent bioconjugates inside living cells. Despite these advances, biologists were still faced with using cutting-edge protein engineering techniques with dyes from the 19th century. The reality was that for dyes to come out of the shadow of FPs, a major drawback would have to be overcome—and that had to do with the very nature of dye synthesis chemistry.

Unlike GFP, from which site-directed mutagenesis can create modified FPs, for the longest time performing rhodamine dye modification reactions required the use of harsh chemical conditions, including acid reactions, which made dye synthesis incompatible with many different functional groups that could alter excitation and emission properties. To generate new, enhanced versions of rhodamine compatible with modern in-cell labeling strategies, someone would have to fundamentally improve the synthesis chemistry.

Finding a new route

Luke Lavis is a group leader at the Janelia Research Campus in Ashburn, Virginia. Prior to coming to Janelia, Lavis spent 4 years as an organic chemist working at the companies Molecular Probes and Molecular Devices before doing his PhD work at the University of Wisconsin-Madison, where he designed novel fluorescent dye tools, work he continues in his lab at Janelia. On the Lavis lab website there is a simple quote that sums up his research interests: “We use modern organic chemistry to transform old dyes into tools for 21st century biology.”

In 2011, Lavis, along with a senior scientist in his lab named Jonathan Grimm, described a new approach for the synthesis of rhodamines from fluoresceins (1). Their method makes use of a palladium-catalyzed carbon–nitrogen cross-coupling strategy for the synthesis, avoiding the harsh acidic reaction conditions that were required in earlier synthesis techniques. While initially providing a means to generate new versions of rhodamine, Lavis’ method actually set the stage for an even bigger advance in the way fluorescent dyes are generated.

Lavis and his team started to play around a little more with their new rhodamine synthesis strategy, eventually developing a way to incorporate 3-substituted azetidine rings into rhodamine (2). The impact of being able to incorporate these azetidine rings into the structure of rhodamine was profound. Suddenly, true fine-tuning of the spectral properties of rhodamine dyes was possible. Lavis’ team generated 8 different versions of rhodamine possessing excitation wavelengths in the 525–550 nm range. Prior to this, there was only one rhodamine dye known to be excitable in this range of the light spectrum. Perhaps more importantly, using these azetidine rings, Lavis uncovered a way to make predictions about the spectral properties of rhodamine-based dyes using chemical structure alone.

Cells in metaphase stained with fluorescent dye JF646, which was developed by Luke Lavis at the Janelia Research Campus. Credit: L. Lavis.

Luke Lavis, a group leader at the Janelia Research Campus, develops novel fluorescent dye tools for imaging applications. Credit: L. Lavis.

“We learned some general tuning rules for rhodamine dyes where we can predict the changes in spectral properties with high precision,” explains Lavis. Having tuning rules fundamentally changes the way in which a developer approaches fluorophore design. Without any guidance, dye developers must employ brute-force strategies, often resorting to screening large numbers of chemical derivatives in order to identify a molecule with the desired properties. But Lavis had found a cheat sheet when it came to making rhodamine variants.

“Experimentally, we did not have to make dozens of molecules to find ones that match common laser lines—we knew precisely what to make from the start,” he says. And it’s not just the spectral properties that Lavis and his team found they could predict and tune; it’s possible to alter a dye’s fluorescence level in a rational way as well.

Rhodamine dyes exist in two states: an open, zwitterionic fluorescent form and a closed, colorless lactone form. Lavis’ team showed that they could use substituted azetidine to modulate this equilibrium and tune a dye’s fluorogenicity and cell permeability, which opened the possibility for use in more complex biological environments such as tissue or even intact animals. “We could even take dye scaffolds that were not fluorogenic and make them fluorogenic through simple and rationale substitutions.”

Beyond rhodamine

But rhodamines are not the only fluorescent dyes used by researchers—several other dye classes are currently available. As it turns out, the rules Lavis developed for tuning rhodamine dyes can also be applied to other dye chemistries.

“The azetidine substitution works for essentially all the dye classes that we previously reported, and our latest work demonstrates tuning for rhodols, rhodamines, carbhodamines, and Si-rhodamines,” Lavis says. Based on work currently in

The near-infrared (NIR) fluorescent protein iRFP713, engineered from bacterial phytochrome, brightly fluoresces in cultured mouse hippocampal neurons without a supply of any exogenous compounds. Credit: V. Verkhusha.

progress in his lab, he suggests that these azetidine tuning rules could extend to even more dye classes. So far, Lavis and his team have created a series of tuned rhodamines with excitation wavelengths ranging 500–650 nm, matching many of the common excitation sources. These dyes are also cell-permeable, and according to Lavis, they can even be introduced to flies, fish, and mice through feeding, incubation, or IV injection.

So does this mean that anyone can go out and create a version of rhodamine or another fluorescent dye specifically tailored to their spectral and fluorogenicity needs? While more possible now than before, unfortunately it's still not that simple when it comes to working with fluorescent dyes. Even with this newfound set of rules for dye design, Lavis says that generating a new dye takes considerable time and effort. "The challenge is to get in front of the hood and making the stuff. It can take a minute to draw a new fluorophore but months to actually build it in the lab."

Sensitive to fluorescent proteins

Vladislav Verkhusha, a professor in the Department of Anatomy and Structural Biology at Albert Einstein College of Medicine in New York, has spent the majority of his research career engineering novel FPs and biosensors for use in a wide variety of microscopy applications.

"Initially, we started working with GFP, trying to make different spectral variants of the protein. But around 2010, we turned our attention to another group of weakly fluorescent proteins known as bacterial phytochrome photoreceptors," recalls Verkhusha. It was this move that led Verkhusha and his lab on a quest to push the spectral boundaries of FP design into the near-infrared (NIR) range. In the process, they would learn more about how FPs function.

NIR FPs hold much promise for in vivo imaging applications, especially deep imaging in tissues. Mammalian tissues have minimal absorption in what is known as the NIR tissue transparency window, a spectral region ranging from 650 nm to 900 nm, and there is less autofluorescence in the NIR spectral band, a significant advantage when imaging tissues. There is also the

fact that when using shorter wavelengths of light for FP excitation, the high-intensity excitation light can damage cells. But excitation in the NIR range is less invasive, resulting in lower levels of phototoxicity and cell damage. All of this means that NIR probes are great for in vivo imaging, but the challenge for developers has been finding FPs or FP variants that have both excitation and emission wavelengths in that part of the spectrum.

The bacterial phytochrome photoreceptors that Verkhusha started to explore in 2010 had several properties that made them attractive alternatives to the more common GFP variants in use at the time. Bacterial phytochromes use a low-molecular-weight compound called biliverdin as a chromophore (i.e., the actual light-absorbing molecule). Biliverdin is actually an enzymatic by-product of heme and is therefore ubiquitous in mammalian cells, which means researchers using FPs based on bacterial phytochromes do not have to supply the chromophore exogenously during experiments. In addition, Verkhusha suspected that it would be possible to engineer different NIR versions of bacterial phytochromes more easily than generating GFP NIR variants.

In 2011, Verkhusha and his team published an article describing their first NIR FP, named iRFP, which was based on bacterial phytochromes (3). iRFP had an excitation maximum at 690 nm and was brighter and more photostable than previous phytochrome-based FPs.

Since the initial iRFP report, Verkhusha's lab has worked on improving iRFP to enable new applications. In 2016, they described three monomeric versions of iRFP, enabling the incorporation of iRFPs into protein fusions for tracking experiments and extending their possible use for biosensor applications (4). Currently, Verkhusha's efforts are aimed at better understanding the biochemical properties of iRFP, along with those of other FPs, to facilitate FP design.

"Our work has been guided by both developing fluorescent proteins for specific applications as well as by exploring their

Vladislav Verkhusha from the Albert Einstein College of Medicine has been instrumental in developing novel fluorescent proteins in the near-infrared (NIR) range of the spectrum. Credit: V. Verkhusha.

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chemistry,” explains Verkhusha. When designing a unique FP, his team may have a specific application in mind. For example, they are currently developing a set of monomeric NIR FRET sensors for use in vivo experiments. Alternatively, they might notice something in the data or results from an experiment or when developing a new FP variant that is just a little strange, which will lead the group to try to understand more about the basic chemistry and physics of protein fluorescence.

Verkhusha is keenly interested in moving his FP designs even further toward the red end of the spectrum—hopefully out to 800 nm or beyond. And as he is quick to note, having a large range of different FPs allows researchers to perform multiplexing experiments using different FPs together to study multiple proteins or even multiple cellular pathways at the same time.

Similarly to Lavis’ fluorescent dye work, Verkhusha and other FP designers have been able to establish some general guidelines and rules when it comes to engineering different FPs with distinct spectral properties.

Fitting it all together

Fortunately for cell biologists, the work of these developers means that new fluorophore options are regularly emerging. Much of this diversity can be traced back to the growing understanding of the chemistry and biology of these molecules, information that gives rise to tuning rules that can make designing new FPs and dyes easier and faster. But there is still work to be done, including finding better ways to target fluorescent reagents and figuring out how to produce brighter fluorophores.

“Fluorescent proteins are genetically encoded, which means it’s possible to target specific subcellular locations,” notes Verkhusha. It’s this level of specificity that continues to make FPs a go-to tool for many microscopy applications where tracking is needed. Dyes, on the other hand, have proven more challenging to target, although chemistry improvements could make targeting more effective in the future. And chemistry could also prove the quickest route to making brighter fluorophores.

“With fluorescent proteins, nature did not have much to work with, but she was able to squeeze out some fluorophores from the 20 protogenic amino acids,” Lavis says in regard to FP diversity. This, he suggests, is where synthetic dyes have an edge—there is an entire field of organic chemistry at a developer’s disposal for making brighter fluorophores. And brighter fluorophores are critical for some of today’s more sophisticated microscopy experiments. Lavis thinks dyes can have the largest impact in experiments requiring a lot of photons. As examples of this, some of the dyes Lavis has been developing work very well in single-particle tracking experiments or when performing multi-color deep imaging in the far-red channel. Yet, he concedes that, at the moment, dyes do add an additional layer of complexity to biological experiments requiring targeted delivery.

The fluorescent dye and protein landscape continues to evolve as developers such as Lavis and Verkhusha push the envelope of fluorophore capabilities. From expanding spectral ranges, to enhancing chemical properties, to allowing rational design of fluorophores for specific applications, this latest generation of fluorophores is opening new and intriguing opportunities for researchers at the bench.

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