# Probes: seeing in the near infrared

Vivien Marx

Peering into live cells and tissues with genetically encoded near-infrared probes requires addressing a number of hurdles.



Genetically encoded near-infrared (NIR) fluorescent proteins (FPs) offer a deeper view for optical imaging of cells and in live animals. Deep tissue imaging is his favorite application for these probes, says Loren Looger, a researcher at the Howard Hughes Medical Institute's Janelia Farm Research Campus. Katrina Forest of the University of Wisconsin–Madison agrees and says "that's the impetus for this work."

NIR FPs have not pushed the king of such tools—green fluorescent protein (GFP)—off its throne. Looger and others believe NIR FPs are neither as easy to use nor as well behaved as GFP. They are dimmer and bleach faster than GFP.

The existing NIR probes are still quite new, says Xiaokun Shu of the University of California in San Francisco. Scientists are plying their knowledge of physics, chemistry, genetics, cell biology and animal physiology as they collaborate and compete to optimize the brightness, stability and versatility of these probes.

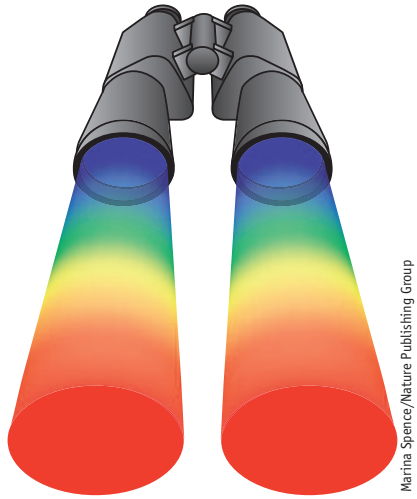
Many labs are engineering bacterial phytochromes as NIR FPs, which do not come to researchers as a "ready out-of-the-box fluorescent protein" as GFP does, says Forest. Engineered bacterial phytochromes do not have GFP's fluorescence quantum yield, which is the percentage of emitted photons per absorbed photons. Forest developed Wi-Phy, an NIR FP[1]. She is just putting the finishing touches on a new one called IFPrev, which has 9% quantum yield, an edge over Wi-Phy's 6.3%.

The quantum yield of EGFP is around 60%, says Shu. Some naturally fluorescent molecules in plants involved in photosynthesis have quantum yields that approach 100%. "That's the power of evolution," he says. None of the NIR FPs, which are mainly based on bacterial phytochromes, is in that league. "I think it's safe to say they are all currently probably around or below 10%."

If NIR FPs were sufficiently bright, they would allow microscopy at depths beyond 1 millimeter, says David Piston, who is at the Vanderbilt University School of Medicine. What limits two-photon imaging to the 1-millimeter-and-less range is the fluorescence absorption returning through the sample. But even dim dyes can shine in the NIR imaging 'window' between the cutoff for hemoglobin absorption, which is 650 nanometers (nm), and the onset of increased water absorption at 920 nm, says Piston. The field has made great strides. Not having reached the goal of bright NIR FPs does not diminish what has been achieved to date, he says. Before iRFP and mCardinal, he was beginning to think "truly useful" NIR FPs might be out of reach. NIR FPs do not have to be as good as visible ones to be better for *in vivo* or deep tissue imaging. Better NIR probes could be much, much better: several hundred-fold better, not just 20-fold, he says.

For example, Piston says, the recently developed mCardinal[2], an NIR FP from Michael Lin's lab at Stanford University, seems comparable to iRFP[3], but it has less than 20% quantum efficiency. "We need to move these out further to the red," says Piston, and also raise the quantum efficiency closer to 100%. The best emission peak for tissue transparency is 820 nm, he says. Even iRFP, which emits at 713 nm, "is not there yet."

There have been plenty of attempts to create red-shifted variants of GFP, but they have failed, says Vladislav Verkhusha of the Albert Einstein College of Medicine, who has developed iRFP and other NIR FPs. The hunt eventually led to DsRed[4,5], a red fluorescent protein cloned from coral. A decade later, researchers were stuck because they were not able to engineer NIR FPs from coral-derived proteins, he says.



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Scientists ply interdisciplinary skills to brighten NIR probes.

This first red-shifted FP began moving the quest for FPs away from its focus on bioluminescent organisms, says Konstantin Lukyanov from the Shemiakin-Ovchinnikov Institute in Moscow, and opened the door for more red and far-red GFP-like FPs. The jump to NIR, he says, became possible through work on bacteriophytochromes with excitation levels between 660 and 690 nm and emission between 680 and 720 nm, which are not achievable with GFP-like proteins.

Now, FP developers tinker with mutations to raise the quantum yield and other qualities of NIR FPs. Verkhusha and his team have developed three types of NIR FPs, including a palette of spectrally distinct iRFPs; photoactivatable FPs called PAiRFPs; and iSplit,

an NIR reporter that detects protein-protein interactions. These probes have been used, among other applications, in mammalian cells and tissue, for stem cell biology, to study development, to characterize tumors in mice and to label organelles and cell compartments, he says[6].

When it comes to applications, Verkhusha is convinced that his and all other current NIR FPs should not be used for microscopy. "You have plenty other colors to use in microscopy," he says, including bright blue, cyan, green, yellow, orange and red variants of the GFP-like family. They allow multicolor imaging, even with a single excitation wavelength, he says.

## Brighter and brighter

As engineers tweak their NIR FPs to have both excitation and emission in the NIR, it is worth keeping in mind how they differ from GFP-like proteins, says Daria Shcherbakova, who is a postdoctoral fellow in the Verkhusha lab. All NIR FPs based on the bacterial phytochrome use biliverdin as the chromophore, the unit that fluoresces when bound to the protein. And although biliverdin is present in mammalian cells and tissues, a protein can have low affinity to it or bind other biliverdin-like molecules such as heme or protoporphyrins, she says. "We extensively screened iRFPs in mammalian cells in order to obtain proteins with high affinity and high specificity of biliverdin incorporation," she says. The resulting probes do not require adding external biliverdin to become fluorescent.

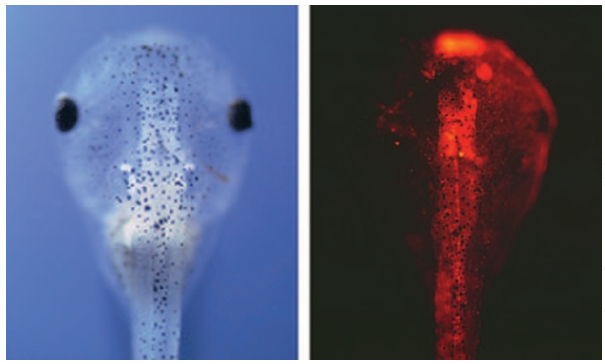
UCSF

The best NIR FP probes will be the ones that best serve biologists, says Xiaokun Shu.

Besides the dimness and low quantum yield, the need to add biliverdin to obtain fluorescence is a "serious drawback" of many NIR FPs, says Lukyanov. The fact that iRFP and its variants from the Verkhusha lab do not have this caveat lets researchers skip the extra step and not worry how the added biliverdin might, he says, "potentially lead to some alterations of biological models."

Getting enough biliverdin or bilirubin, another type of chromophore, to an NIR FP remains a big problem, says Looger. The new FPs are better at incorporating it, but, "depending on your sample, it can still be very hard," he says. As engineers make progress, Forest believes this hurdle will eventually be dismantled. "That's just my forward-looking hypothesis."

Tool developers working with the bacterial phytochromes do not use the proteins in their entirety: just the domains that bind the chromophore, absorb the photon and deliver the fluorescence, says Forest. "It's better as a tool without all that other stuff dangling off it."



Figures from ref. 4, Nature Publishing Group

This first red-shifted FP was made from coral-derived proteins. Here, a tadpole expresses the proteins in an mRNA microinjection assay.

Biliverdin is a breakdown product of heme and is available in mammalian cells. Despite this availability, the real question is whether the level is sufficient to load the NIR FPs, she says.

In an attempt to overcome some of the problems in supplying biliverdin to NIR FPs, some scientists are successfully expressing the heme oxygenase enzyme at the same time as the fluorophore in the same cell. "There's a cost to the cell to express that," Forest says. "I don't think there's a perfect solution just yet."

Coexpressing enzymes to obtain fluorescence is indeed a challenge, says Verkhusha. For example, heme oxygenase is a stress-response enzyme, which means its expression can affect cell metabolism.

For some labs, the choice of NIR FP is about what works for them. Although he likes the fact that iRFP does not need external biliverdin, Piston says that "in our hands that does not lead to usable signal." One explanation might be that he and his team are not imaging liver and tumor tissue, where biliverdin might reach higher levels than in the pancreas, where he and his team have tried to use it, he says.

Shu says that the level of biliverdin indeed varies. In neurons, for example, it is "relatively low" compared with the level in human embryonic kidney cells. He took this variation into account on his latest engineering route to create his newest NIR FP, called IFP2.0: he mitigated biliverdin variation by overexpressing heme oxygenase in neurons. To address the stress-response aspect, he and his team compared neurons expressing the NIR FP with or without coexpression of this enzyme to completely unengineered neurons. They found that the neurons expressing IFP2.0 and the engineered enzyme formed regular neural networks in culture and showed regular electrophysiological properties, he says. And yet the system may still be perturbed.

Even GFP has the potential to perturb a system under study, says Shu, because it crowds the cells with foreign proteins. Two decades of work with GFP have led researchers to worry less about unwanted GFP effects. Now NIR probes will get the same attention. "We should not be scared of this potential perturbation," he says, but rather characterize it, take it into account and control for it.

## Monomeric parents

Shu believes that an NIR FP is best monomeric so that it can be used to tag other proteins and to study protein-protein interaction in live animals. And, he says, "it's time to make sensors": for cell signaling, for example, or functional reporters for kinases, proteases, voltage or calcium. Shu engineered IFP1.4 (ref. 7), a monomeric version of IFP1.2, and further improved it, creating IFP2.0 (ref. 8). And he wants to keep engineering it. Although it is a monomer, its parent is a dimer, he says. "It's better if we started with a parent that is a monomer."

Residues are his main concern when engineering a dimer into a monomer. Mutating residues in the dimeric parent will keep the proteins from interacting. But a large number of residues are buried in the hydrophobic interface of a dimer, and their disruption exposes them to a new, aqueous environment in the cell. Of the resulting NIR FPs, Shu says, "I suspect they can become not that stable or soluble." Although IFP2.0 has been used to label the cell membrane and has behaved well in the experiment, he has started engineering a "naturally monomeric"

NIR FP that is made from a monomeric parent.

When an NIR FP is a dimer, as is the case with iRFP, it is inappropriate for labeling proteins, but it is useful as is and can be used to label cells, says Shu. His IFP1.4 was much dimmer than iRFP. "This one is as bright as iRFP," he says of IFP2.0, and it has the monomeric properties of IFP1.4.

### Lights, action

Part of the NIR FP development process is about choosing imaging instruments, filters and light sources. NIR FPs are still emerging, which may explain, says Forest, why "most companies are not really positioned with their lasers and detectors to really think about these longer-wavelength fluorophores," she says. They are optimized to work well for slightly shorter-wavelength fluorescence emissions and, as a consequence, are less efficient at longer wavelengths. As the probes mature, so will the filters, detectors and lamps so that they are better at these longer wavelengths, she says.

Tess Forest

The field's impetus is to gain a deeper view for optical imaging of cells and live animals, says Katrina Forest.

The efficiency of too many fluorescence detectors falls off at longer wavelengths, says Forest. Scientists need to not use just "any old fluorometer off the shelf." For example, typical plate readers do not deliver efficient measurements at these wavelengths.

These equipment needs apply to developers who want precise, comparative data about their NIR FPs. Users looking at total fluorescence readouts for one fluorophore will not have the same demands.

Piston says he finds "superb" NIR detectors, but they tend not to be used in fluorescence microscopy because they do not perform well in the visible range of the electromagnetic spectrum. Charge-coupled device (CCD) cameras can perform well in the NIR but are usually blocked at those wavelengths to reduce background noise from residual heat. "If we had a good probe, it would be short order before appropriate detection systems were available," says Piston.

In multicolor cellular imaging, an NIR FP needs to be excitable in the NIR, and it also needs low absorption in the 400- to 600-nm range, which makes it insensitive to the wavelengths already being used for imaging visible fluorescent proteins and for photoactivation, says Jeff Carmichael, technical product manager at Chroma Technology, a company that makes filters.

If NIR FPs are dim, scientists run risks as they image, particularly when looking at a live animal and exploring open questions as opposed to working in cell culture, where they might know exactly what they are tracking. "You don't want to photobleach it before you've properly imaged it," says Carmichael. One shortcoming he sees in some NIR FPs is that their broad excitation spectra extend well into the visible range.

If a sample contains no other NIR FPs, he recommends using emission filters with wide passbands or a long-pass filter. "That way, you can acquire as much fluorescence emission bandwidth as possible, which will reduce exposure times," says Carmichael. This approach leverages the lack of autofluorescence and spectral overlap from neighboring fluorochromes. But the situation can be trickier when a sample contains more than one NIR FP.

Optimizing an imaging application using NIR FPs can mean trying a few different combinations of excitation and emission filters. It can be helpful, he says, to determine the best excitation range and bandwidth first.

Using a wider-band emission filter might allow the use of a narrower-band excitation filter, if the excitation needs to be as specific as possible. If an FP is photostable and the emission range is problematic, a wider passband for an excitation filter might provide a high enough signal for a specific emission passband. What turns out to be right will be "completely application dependent," says Carmichael.

### Deeper, deeper

"I'd love to see LEDs become more widely available for fluorescence imaging," Carmichael says about light-emitting diodes. He recommends light "with decent output" in the NIR. Traditional xenon lamps are becoming obsolete, and mercury sources have much lower NIR output, he says. Lasers can be good sources for cellular imaging but more difficult for whole-animal imaging unless used in scanning mode.

Piston believes that scientists "should only be using LEDs and lasers for excitation," but he points to the large installed base of other illuminators and companies that are invested in continuing to sell those systems. LED systems are still more expensive on the front end, and most scientists are facing a tight funding climate, he says. A xenon lamp gives a researcher every wavelength. For experiments with a new dye or FP, the system just needs a filter as opposed to a new laser or LED.

For confocal imaging, Shu uses lasers, and for epifluorescence imaging, he uses a white-light LED. It is an LED that covers all the spectra, he says. Xenon lamps and LEDs offer low excitation in the NIR, but, he says, it is high enough. When using xenon lamps, his team needed neutral density filters, which can be avoided with LEDs. "We can use much less energy," he says, and LEDs can be readily dimmed.

Verkhusha says he uses PerkinElmer's IVIS and FMT imaging platforms with standard filters. He highlights the wealth of software packages, from both established companies and amateur "enthusiasts."

When he hears scientists using the term two-photon microscopy to describe 'deep' tissue imaging, he says with a laugh, it is time for "deep-deep imaging" or "double-deep" tissue macroscopy. In his view, this field of NIR FP imaging awaits new hardware breakthroughs, not unlike the situation prior to the advent of super-resolution microscopy.

### Better, brighter

No NIR FP is a clear winner yet: "none is perfect," says Looger, and it remains to be seen how much "headroom" there is to brighten these FPs. It is equally unclear how easy it will be to do other things with these FPs as has been possible with GFP, such as engineering sensors or creating photoswitchable NIR FPs versions for photoactivated localization microscopy and other applications, he says.

Verkhusha continues to work on his NIR FP family and is eager to develop biosensors based on NIR FPs, such as calcium sensors that fluoresce only if there is protein-protein interaction in the presence of calcium. He bristles at NIR FP comparisons in which

Linda Tammisto

It's time for "deep-deep imaging" or "double-deep" tissue macroscopy, says Vladislav Verkhusha.

Vanderbilt University School of Medicine

NIR FPs could be much, much better: several hundred-fold better, not just 20-fold, says David Piston. Here, he aligns a laser to a microscope.

proteins are optimally expressed in bacteria, purified, and tested at temperatures just right for protein folding, all of which are "nonmammalian conditions." Instead he wants brightness and measurements to be made in mammalian cells. "That's our target," he says.

Forest's new NIR FP, IFPrev, will not yet be "big in animals" because it is "just" IFP1.4, meaning it is comparable in terms of stability and monomer status. But she calls it "an interesting proof of principle" in terms of quantum yield.

Shu, who is also working on sensors and reporters, says that the probes considered to be the most talented will not be determined as such by developers' comparisons. Rather, they will be the probes that best serve biologists. "We need to measure those specs," he says, just as tablet computer manufacturers give users device specs. Users will decide which one is best for them, and so it will be for NIR FPs, which must get into biologists' hands. "They are the real users," he says. As he engineers FPs, he collaborates with biologists who test them. "Of course, we cannot say it will work for everyone; at least we know it works in these tested systems."

Shu likes helping out with his colleagues' biological questions. Most recently, when testing IFP2.0 in fruit flies, he enjoyed imaging neurons as well as seeing the colorful movement of all the cells during development. When the project began, he assumed the larval brain was transparent. Then he realized it can be imaged around only 100 micrometers deep using green light. Imaging deeper in the larval brain using single-photon and green fluorescence results in the signal growing fuzzy because the photons are scattered and some green light is absorbed by the tissue. That is why researchers also use two-photon microscopy to obtain excitation in a much smaller area.

Many of the issues that NIR FP developers face did not happen with GFP and GFP-like proteins, which are expressed equally well in bacteria and mammalian cells. "It's luck," Verkhusha says. With NIR FPs, luck is partnering with engineering to get the probes to a brighter place.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