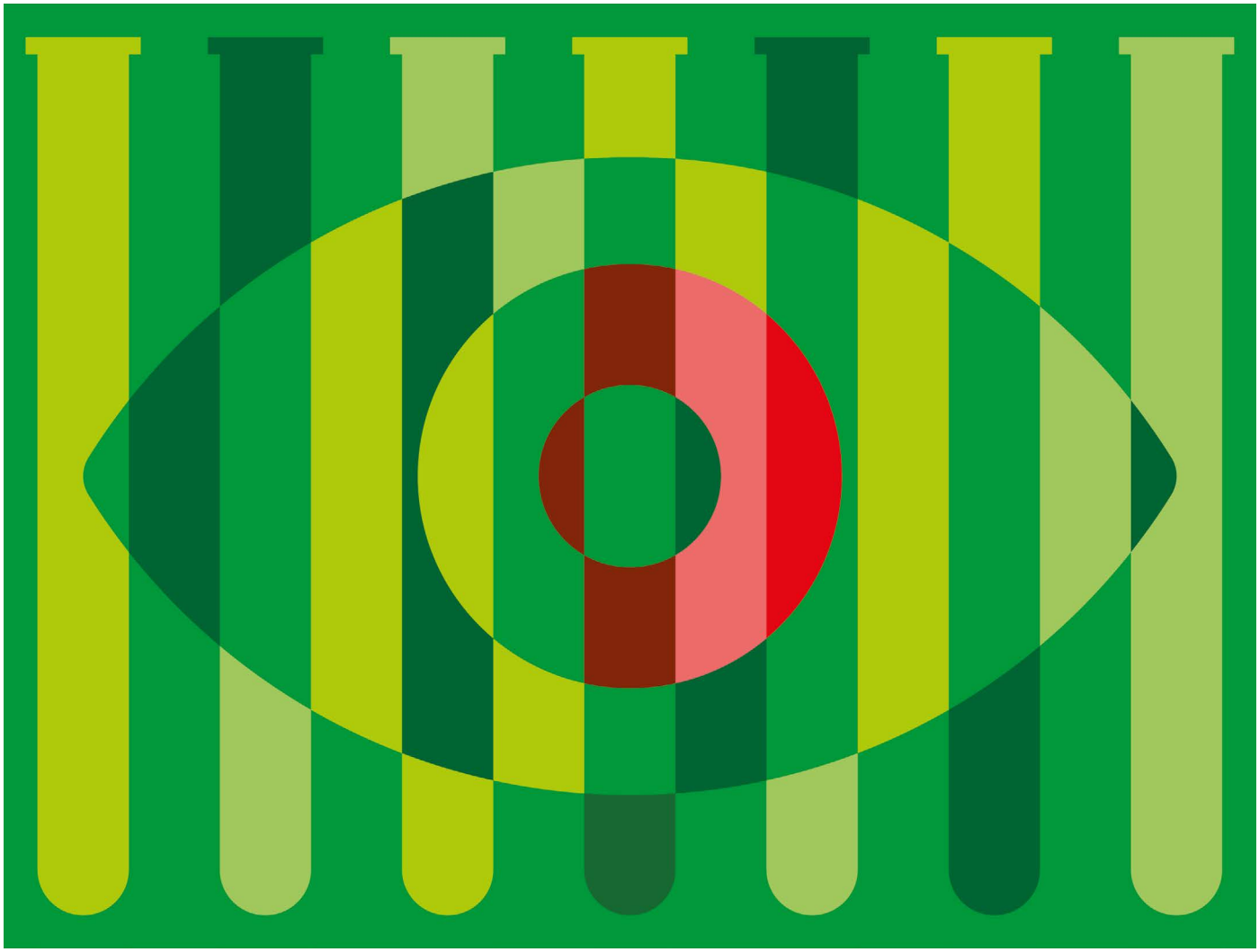

ILLUSTRATION BY THE PROJECT TWINS

THE HUNT FOR RED FLUORESCENT PROTEINS

By pushing fluorescent proteins further into the red, bioengineers are expanding the palette and penetration depth of biological imaging. **By Amber Dance**

Green fluorescent protein is one of the most popular items in the microscopist's toolbox. It is a Nobel-prizewinning innovation that brilliantly lights up molecules of interest across a diverse range of biological fields, laboratories and techniques. But it does not work for physical chemist Julie Biteen.

Biteen studies gut bacterial communities at the University of Michigan in Ann Arbor, and was eager to use fluorescent proteins to identify individual species in complex mixtures. But gut bacteria don't like oxygen — something green fluorescent protein (GFP) absolutely requires. No oxygen, no fluorescence.

So, she turned to a label that can do without oxygen. A relatively new addition to the fluorescent-protein palette, IFP2.0 fluoresces mainly in the near-infrared — a portion of the electromagnetic spectrum that is barely visible to the human eye but readily apparent to microscope cameras[1]. "We're really excited," Biteen says. "We could see single cells and identify them."

Imaging at the red end of the spectrum offers other advantages, too: lower background fluorescence, reduced toxicity and deeper tissue penetration. "All other factors being the same, redder is better," says Robert Campbell, a protein engineer who spends half his time at the University of Tokyo and the other half at the University of Alberta in Edmonton, Canada. It also provides a way to add another hue, or two, to experiments. "The more channels we can pack into an experiment, without significant bleed-through, the more interactions we can study," says Talley Lambert, a microscopist at Harvard Medical School in Boston, Massachusetts.

Reddish fluorescent proteins have existed for decades, but they are still generally no match for GFP in terms of both brightness and

hue. Even the 'red' fluorescent protein RFP is closer to orange. Scientists are making headway in developing fluorescent proteins that are truly red — often called 'far red' to distinguish them from earlier attempts. Infrared offers similar advantages. Development is still in its infancy, but advances in bioprospecting, protein engineering and synthetic chemistry are helping to improve the labels. Most are available, in gene form, on the plasmid repository Addgene.

There's clearly a need. Scientists are desperate for tags and sensors that they can use alongside standard tools, such as GFP, blue DNA stains and channelrhodopsins, which are activated by green and blue light. Brian Almond, senior manager for product management at Thermo Fisher Scientific in Carlsbad, California, says an alternative hue is often customers' first request for new fluorescent tools. "Everything is green," they tell him. "Please, don't make it green."

Scarlet solution

A typical fluorescence-microscopy experiment can use around three colours without overlap. But picking labels that will work together is not as simple as yellow-green-blue. There are hundreds of fluorescent proteins to choose from, and they vary in factors such as hue, brightness and fluorescence longevity. Some are single-unit proteins, but others have the potential to stick to each other and perhaps even glue the protein of interest to others like it, interfering with the results. No one protein will be the best for every application.

When choosing a tag, it's best not to rely too closely on published data, warns Roberto Chica, a protein engineer at the University of Ottawa. Proteins that work well in a test tube might not shine in a model organism, and data tables are often incomplete. It's best to test a few fluorescent proteins and pick the best for your experiments.

Several free online resources can help scientists to choose candidate fluorescent proteins, including Lambert's FPbase; Thermo Fisher's Fluorescence SpectraViewer; and the Fluorescence Spectra Analyzer developed by BioLegend in San Diego, California. Users can view the excitation and emission curves for hundreds of fluorescent proteins and dyes, and by inputting their light sources, filters and detectors, they can choose accordingly.

One strategy for using multiple colours is to let computers sort out any overlapping emission spectra after the data are collected. With this technique, called 'spectral imaging and linear unmixing' or 'fluorescent unmixing', researchers can probe many more colours — up to 40 tags in the same flow-cytometry experiment, says BioLegend product manager Kenta Yamamoto. But such rainbow panels require careful planning, he warns: for example, rarer proteins might need to be paired with brighter tags if they are to stand out from the crowd.

A simpler approach is to use a colour that doesn't overlap with any of the others. That's where far-red and near-infrared labels are handy; they make it easy to get at least four non-overlapping signals from the same cells. In his lab at Westlake University in Hangzhou, China, tool developer Kiryl Piatkevich routinely records five signals from the same microscope slide by using three visible colours and two in the near-infrared range. Such experiments can often be performed without major equipment upgrades.

Where the fluorophore grows

Whereas many fluorescent proteins are found in sea creatures, far-red and near-infrared molecules tend to come from bacteria. But unlike GFP and similar proteins, bacterial light receptors lack a component to absorb light (called a chromophore) of their own; they require the addition of a pigment known as biliverdin. The good news is that biliverdin is a natural intermediate in the breakdown of haem, which binds to oxygen to transport it through the blood, so it's naturally present in mammals. The bad news is that biliverdin is also quickly degraded, so it's far from abundant.

"Everything is green. Please, don't make it green."

One solution to that problem is to add more biliverdin, either from standard chemical suppliers, or by altering organisms to make more of it. Another is to engineer natural far-red and near-infrared proteins so that they work better outside their usual host, for example by boosting the efficiency of the binding between the protein and the pigment, says Vladislav Verkhusha, a molecular bioengineer at Albert Einstein College of Medicine in New York City. His group induces random mutations in the relevant genes *in vitro*, then expresses those genes in the bacterium *Escherichia coli* and selects for the reddest or brightest products. In one example[2], the team used 17 rounds of this molecular-evolution approach to obtain a near-infrared protein tag called miRFP670nano. The tag is about 60% of the size of GFP, binds to biliverdin efficiently and fluoresces brightly in mammalian cells.

Piatkevich uses molecular evolution, too, but in mammalian cells, which fold up the proteins such that they match those of the target cells more closely than those evolved in bacteria. His team has used this approach to brighten a near-infrared fluorescent voltage reporter (useful for tracking nerve-cell firing), creating a sensor called Archon1 (ref. 3).

It's also possible to engineer proteins directly. Timothy Wannier, a synthetic biologist at Harvard Medical School, used both molecular evolution and computer-based protein analysis and design on GFP relatives during his PhD studies at the California Institute of Technology in Pasadena. His goal was to turn dimeric far-red fluorescent proteins into monomers, which would help to prevent undesirable interactions. But he also had to engineer mutations to stabilize the lone monomers[4].

One of the resulting tags, mKelly1, caught the eye of Yi Shen, a protein engineer at the University of Alberta, who used it to build far-red calcium sensors named FR-GECOs (ref. 5).

Redder all the time

Despite these advances, far-red and near-infrared fluorescent proteins remain dim bulbs. Whereas some green proteins push the limits of brightness, the best near-infrared tags hover at around 10–20% of the maximum.

One solution is a workaround based on far-red and near-infrared chemical dyes, which are also becoming available more widely. Organic chemist Luke Lavis at the Howard Hughes Medical Institute Janelia Research Campus in Ashburn, Virginia, developed bright, non-toxic dyes that can switch between colourless and fluorescent forms according to their surroundings[6]. Lavis has since teamed up with protein-engineer colleague Eric Schreiter to turn these dyes into 'chemigenetic' cellular sensors that couple a chemical dye with a protein partner.

The pair use the genetically encoded 'HaloTag' as a dock for the synthetic dyes, and hook it up to sensor proteins that change shape in the presence of calcium or electrical voltage[7]. The shape change alters the local environment of the dye such that it fluoresces — and is about ten times brighter than previous red sensors, says Schreiter. Lavis gives the dyes to other scientists free of charge. He is now testing next-generation dyes that he expects will penetrate deeper into tissue for *in vivo* applications.

"The future is bright for this class of sensors," says Schreiter. He adds that it should be possible to replace GFP in any pre-existing sensor with the HaloTag to create a new, red, chemigenetic sensor. But for the rest of the rainbow, Lavis says, standard fluorescent proteins should suffice "because they're awesome".

Amber Dance is a freelance science journalist in Los Angeles, California.

1. Yu, D. *et al. Nature Commun.* **5**, 3626 (2014).
2. Oliinyk, O. S., Shemetov, A. A., Pletnev, S., Shcherbakova, D. M. & Verkhusha, V. V. *Nature Commun.* **10**, 279 (2019).
3. Piatkevich, K. D. *et al. Nature Chem. Biol.* **14**, 352–360 (2018).
4. Wannier, T. M. *et al. Proc. Natl Acad. Sci. USA* **115**, E11294–E11301 (2018).
5. Dalangin, R. *et al.* Preprint at bioRxiv https://doi.org/10.1101/2020.11.12.380089 (2020).
6. Grimm, J. B. *et al. Nature Meth.* **17**, 815–821 (2020).
7. Deo, C. *et al. Nature Chem. Biol.* **17**, 718–723 (2021).

Correction

This Technology feature misnamed the protein miRFP670nano as miR670nano.

Corrected 5 August 2021