Seeing red

The growing advantages of red to near-infrared fluorescent probes should move them beyond being a 'second color' in biological imaging.

It is impossible to overstate the profound impact that the *Aequorea victoria* GFP and its derivative, monomeric enhanced GFP (mEGFP), have had on biomedical research. Brightly fluorescent, photostable, well-behaved in fusions, and compatible with commonly available 488-nm laser lines, mEGFP is entrenched in almost every lab that performs imaging. The standard it has set for fluorescent proteins has proven surprisingly challenging to match, let alone surpass. As such, green images abound in the biological sciences.

However, treating cells with short-wavelength light can cause phototoxic effects and even cell death. Although this is most pronounced in the UV region, the blue light used to excite GFP can cause noticeable damage in cells and tissues; for extended live-cell imaging, it can cause unpredictable artifacts and affect the processes under observation.

A Commentary from Laissue and colleagues in this issue (p657) discusses the problem of phototoxicity in live imaging and champions imaging practices that prioritize sample health. The authors propose guidelines for assessing phototoxicity and reporting live imaging data transparently and promote a more systematic evaluation of sample health as a standard part of biological imaging.

Building on this theme, we encourage biologists to consider using red to near infrared (NIR) probes for live imaging applications. This idea is by no means new, but it is worth revisiting now that microscopy methods for peering into living organisms are rapidly advancing. There are two major advantages to using red probes for live imaging: longer wavelength light is compatible with imaging at depth in tissues, and longer wavelength light is less phototoxic to cells. Thus, imaging with red fluorophores could benefit sample health.

However, the dominance of GFPs is not simply because biologists have become comfortable with these tools. The road to developing the red equivalent to mEGFP has been long and bumpy, and many would argue that the ride is not yet over.

Most red fluorescent proteins (RFPs) used today are tetrameric, or they are monomers derived from naturally tetrameric proteins. This includes all of the mFruits, including the most widely used mCherry, the tandem dimer tdTomato, as well as TagRFP and derivatives. Unfortunately, the mutations that make these proteins monomeric also make most monomers dimmer and less photostable than their wild-type versions, although engineering has also improved features like chromophore formation speed and color range. In addition, many so-called monomeric RFPs still have residual tendencies to oligomerize and can cause localization defects when fused to other proteins.

Though mCherry and other RFPs have been important for biological discovery, many researchers attempting to use early versions of monomeric red fluorescent proteins may well have experienced disappointing results: aberrant tagged-protein behavior, dim signal or loss of signal over longer periods of imaging. In the late 2000s and onward, a veritable alphabet soup of 'improved' red monomers emerged, but it was not clear which of these probes were most useful. These issues have caused many biologists to turn to RFPs only as a second color, when several targets need to be labeled simultaneously.

An Analysis published in our pages in 2016 compared the behavior of over forty monomeric fluorescent proteins. This work points to mApple and mKate2 as versatile RFPs that have useful photophysical properties and should perform as true monomers in fusions. Shortly after publication of that work, the brightest monomeric RFP to date, mScarlet, was introduced. Taken together, these studies support the idea that monomeric RFPs are ready for prime time.

Proteins such as bacteriophytochromes, which bind cofactors and fluoresce in the NIR, have also been extensively engineered for use in biological imaging. These probes are particularly desirable because both their excitation and emission overlap with the 'optical window', where light can pass relatively freely through tissues. Although these probes have been plagued by low quantum yields and dependence on exogenous cofactor addition, new variants are beginning to overcome these issues, and monomeric forms are being successfully used in a variety of applications.

Beyond fluorescent proteins, dyes that are red to infrared have improved dramatically in recent years; for example, the silicon rhodamine dyes have good photophysical properties and are now widely used in super-resolution imaging; fluorogen-activating proteins bind bright NIR dyes and are compatible with live-cell imaging; and the Janelia Fluors provide bright and photostable red probes for live imaging of Halo-tagged proteins.

Much progress has been made on red to near-infrared probes. We encourage biologists, even those who may have had limited success with red probes in the past, to consider trying them. Our specimens may thank us for it.