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DOI
10.1038/s41592-022-01425-2
Publication date
2022
Document Version
Final published version
Published in
Nature Methods

SENSORS AND PROBES

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A novel bright near-infrared fluorescent protein inserted into a nanobody enables visualization of native proteins inside living cells and specific manipulation of cell function, including Boolean protein-based operators.

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The genetically encoded fluorophore GFP from *Aequorea victoria* has revolutionized cell biology. Initially, GFP color variants were limited to blue, cyan, green and yellow[1], but the structurally related red fluorescent proteins (RFPs) found in stony corals and subsequent engineering efforts yielded an extended color palette that added orange, red and far red fluorescent proteins[2]. From various efforts to further red-shift RFPs, it appears that there is a physical limit around approximately 660 nm, above which the RFPs become very dim, with quantum yields <0.1. Above 660 nm, near infrared (NIR)-fluorescing bacterial phytochromes or cyanobacteriochromes provide an attractive alternative. They can be excited above 640 nm, with much lower light scattering, absorption and autofluorescence, which is advantageous for deep tissue imaging[3]. Initially, the use of these bacterially derived near infrared fluorescent proteins (NIR-FPs) was limited by their dimerization, poor incorporation of the biliverdin chromophore and low quantum yield. Extensive engineering has solved most of these problems, miRFP670-2 being the brightest monomeric variant in this spectral class with a quantum yield of 0.14 and an extinction coefficient of 103,000 M^{-1} cm^{-1}. Using a single domain from a cyanobacteriochrome, a much smaller variant, miRFP670nano, has been generated[3]. This issue of *Nature Methods* describes[4] the engineering of a twofold brighter single-domain miRFP670nano3 variant with a quantum yield of 0.19 and extinction coefficient of 129,000 M^{-1} cm^{-1}. Introducing an additional covalent bond between the apo-protein and the biliverdin chromophore of miRFP670nano3 further enhances maturation, leading to a stunning fourfold increased brightness as detected in cells as compared to miRFP670nano. In addition, photostability and pH tolerance have been increased. As a result, miRFP670nano3 is by far the best NIR-FP available to date. Oliinyk et al.[4] show the versatility of this new probe for NIR-FP tagging of a variety of proteins and possibilities for deep tissue imaging.

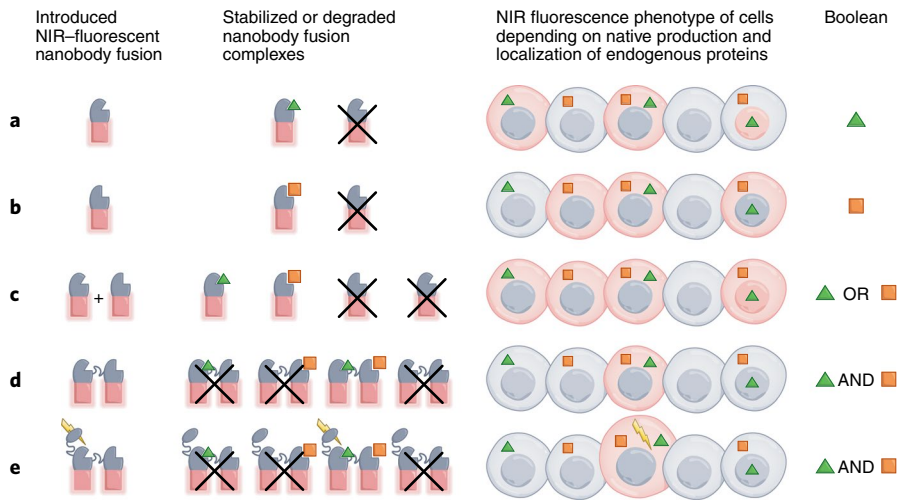

Fig. 1 | Boolean readout of protein levels in individual cells. The new NIR fluorescent protein miRFP670nano3 is indicated in red, nanobodies in blue, protein 1 as a green triangle and protein 2 as an orange square; red glow shows NIR fluorescence. Crossouts indicate nanobody–miRFP670nano3 fusion complexes that are unstable and are degraded. Situations **a**–**e** represent detection of the following: **a**, only protein 1; **b**, only protein 2; **c**, protein 1 OR protein 2; and **d**,**e**, protein 1 AND protein 2. In **e**, the nanobody–NIR-FP fusion is extended with an effector domain stimulating cell growth.

Despite the generation of many fully biofunctional fluorescent fusion proteins[2,5], there is always the possibility of a covalently attached fluorescent protein (or other tag[6]) influencing the properties of the protein, as well as the possibility that ectopic expression of fluorescent fusion proteins can alter the labeled protein's overall cellular concentration or function. Conversely, unmodified proteins at their native concentration can be studied with immunocytochemistry in fixed cells that are permeabilized and treated with fluorescently labeled antibodies, but this precludes imaging in live cells or in vivo, and even then it can be hampered by artifacts related to poor penetration depth of the labeled antibodies[7].

The structurally much simpler and smaller nanobodies originating from camelid mammals provide a possible solution. Because nanobodies consist of a single polypeptide chain and do not require post-translational modifications, they can be easily fused to a fluorescent protein, yielding a so called chromobody. Chromobodies can be expressed in live cells, which enables specific in situ labeling of native proteins[8]. A limitation is that unbound overexpressed chromobodies yield non-specific background fluorescence. By inserting the newly developed miRFP670nano3 tag in a loop of a nanobody not involved in epitope binding, Oliinyk et al. solve this background problem. Surprisingly, the nanobody with the inserted NIR-FP is unstable and prone to ubiquitination and degradation in cells unless it is stabilized by binding to its target protein. Together, this technology allows in situ visualization of proteins in their native (unmodified) state and at their native concentration under native regulation inside live cells and organisms, a breakthrough for cell and developmental biology. There are limitations. The technique does require the generation of a specific

nanobody and subsequent cloning of an inserted miRFP670nano3 tag. In addition, the binding of the modified nanobody may interfere with protein function, and it may titrate away free protein. The maturation of NIR-FPs is also relatively slow (>24 hours), but nevertheless, the property of built-in degradation of superfluous unbound nanobody–NIR-FP tags is a unique asset that will undoubtedly enable novel applications.

Not only does this property allow visualization of native proteins, it also can be used to manipulate cells in a manner dependent on the presence of a specific protein. The engineered nanobody (and everything fused or interacting with it) is constantly degraded unless an epitope is present. If a transcriptional activator is fused to this NIR–fluorescent nanobody, transcription will become dependent on the presence of a specific protein. This is nicely shown for a GAL4 promotor[4]. One can also fuse an inhibitory protein domain to the NIR–fluorescent nanobody — for instance, to modulate cAMP-dependent kinase signaling[4].

The most intriguing application of the new methodology is the fusion of two different nanobodies (both with an inserted nano-FP tag) into one polypeptide chain. The fusion protein will be degraded if one or both of the nanobodies are not bound to their epitope. This allows specific labeling of cells producing two different proteins of interest. Only if both proteins are present will the cell or compartment become NIR fluorescent. By contrast, introducing both nanobodies separately (for example, separated by a 2A peptide) will yield cells that fluoresce in the NIR when they produce either one or both of the proteins. Together this sets the stage for Boolean NIR-fluorescence cell labeling (Fig. 1). One can label cells with only protein 1 (Fig. 1a) or only protein 2 (Fig. 1b), cells with protein 1 or protein 2 (Fig. 1c), or cells simultaneously producing proteins 1 and 2 (Fig. 1d). In addition to fluorescence, all these situations can be combined with specific cellular manipulation by attaching an effector protein to the nanobody fusion: a theoretical example is shown for an effector that stimulates cell growth only if two proteins of interest are both produced (Fig. 1e). The technique can also be used to remove a protein of interest in a manner dependent on the absence of, for instance, GFP by fusing an anti-GFP nanobody to a nanobody against a protein of interest. Subsequently, the latter protein will be degraded in cells that do not ectopically express GFP[4]. Using cell-type-specific promotors, this allows cell-type-specific protein depletion, with many applications in developmental biology or medical biology. Importantly, after NIR fluorescence labeling, cells can be isolated from tissues by fluorescence-activated cell sorting and subjected to all sorts of detailed molecular analyses, such as sequencing or mass spectrometry. This new NIR–fluorescent nanobody toolkit has tremendous potential in synthetic biology for engineering artificial protein networks with Boolean operators[9], for developmental biology, and for screening or identifying specific cellular states. ❐

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Published online: 23 May 2022

https://doi.org/10.1038/s41592-022-01425-2

References

1. Tsien, R. Y. *Annu. Rev. Biochem.* **67**, 509–544 (1998).
2. Chudakov, D. M., Matz, M. V., Lukyanov, S. & Lukyanov, K. A. *Physiol. Rev.* **90**, 1103–1163 (2010).
3. Shcherbakova, D. M., Stepanenko, O. V., Turoverov, K. K. & Verkhusha, V. V. *Trends Biotechnol.* **36**, 1230–1243 (2018).
4. Oliinyk, O. S. et al. *Nat. Methods* https://doi.org/10.1038/s41592-022-01467-6 (2022).
5. Enterina, J. R., Wu, L. & Campbell, R. E. *Curr. Opin. Chem. Biol.* **27**, 10–17 (2015).
6. Crivat, G. & Taraska, J. W. *Trends Biotechnol.* **30**, 8–16 (2012).
7. Schnell, U., Dijk, F., Sjollema, K. A. & Giepmans, B. N. G. *Nat. Methods* **9**, 152–158 (2012).
8. Rothbauer, U. et al. *Nat. Methods* **3**, 887–889 (2006).
9. Miyamoto, T., Razavi, S., DeRose, R. & Inoue, T. *ACS Synth. Biol.* **2**, 72–82 (2013).

Competing interests

The author declares no competing interests.