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Near-infrared fluorescent proteins engineered from bacterial phytochromes

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Near-infrared fluorescent proteins (NIR FPs), photoactivatable NIR FPs and NIR reporters of protein–protein interactions developed from bacterial phytochrome photoreceptors (BphPs) have advanced non-invasive deep-tissue imaging. Here we provide a brief guide to the BphP-derived NIR probes with an emphasis on their *in vivo* applications. We describe phenotypes of NIR FPs and their photochemical and intracellular properties. We discuss NIR FP applications for imaging of various cell types, tissues and animal models in basic and translational research. In this discussion, we focus on NIR FPs that efficiently incorporate endogenous biliverdin chromophore and therefore can be used as straightforward as GFP-like proteins. We also overview a usage of NIR FPs in different imaging platforms, from planar epifluorescence to tomographic and photoacoustic technologies.

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Introduction

Near-infrared (NIR) fluorescent probes are superior for deep-tissue and whole-body imaging of small mammals because of reduced autofluorescence, low light scattering and minimal absorbance of hemoglobin, melanin and water in the NIR 'optical window' (~650–900 nm) of mammalian tissue [1]. Significant efforts to develop NIR fluorescent proteins (FPs) from the GFP-like family of proteins resulted in FPs with autocatalytically formed chromophores exhibiting maximally red-shifted absorbance of 611 nm in TagRFP657 [2] and fluorescence of 675 nm in TagRFP675 [3]. The most far-red shifted chromophore found in PSmOrange absorbs at 634 nm and fluoresces at 662 nm [4], however, its formation requires an irradiation with high-power green light. Neither of these protein have both excitation and emission maxima within the NIR optical window (Figure 1a).

To overcome this likely fundamental limit of the chromophore chemistry of the GFP-like FPs [5], recently another family of proteins was employed to engineer truly NIR FPs, namely bacterial phytochrome photoreceptors (BphPs) (Figure 1b). BphPs belong to a large family of the phytochrome photoreceptors found in plants, algae, fungi, bacteria and cyanobacteria, which use linear tetrapyrrole compounds, also known as bilins, as a chromophore [6]. The utility of phytochromes for development of fluorescent probes was first explored a decade ago by Lagarias and co-workers [7]. Among phytochromes, BphPs are the most suitable templates for engineering of NIR FPs. By contrast to plant and cyanobacterial phytochromes, BphPs utilize the most far-red absorbing bilin, biliverdin IXα (BV) [8–10]. Being an enzymatic product of heme degradation (Figure 1c), BV is ubiquitous in many eukaryotic organisms including flies, fishes and mammals, unlike tetrapyrrole chromophores of all other phytochrome types [11]. This important feature makes BphP applications in live mammalian cells, tissues and whole mammals as easy as conventional GFP-like FPs, requiring no enzymes or exogenous cofactors [12].

Recently, numerous BphP-based NIR fluorescent probes of different phenotypes have become available. They consist of permanently fluorescent NIR FPs [13••,14–16,17••,18], photoactivatable NIR FPs [19] and NIR reporters of protein–protein interaction [20•,21–23].

Here we overview available NIR FPs and their applications. We describe NIR FP phenotypes and molecular basis of their fluorescence. We discuss NIR FP characteristics including their advantages and limitations. Next we focus on NIR FP applications in basic biology and biomedicine. We overview imaging modalities beyond planar imaging that allow for higher resolution and sensitivity. Lastly, we provide a brief perspective on future NIR FPs.

Phenotypes and properties of near-infrared fluorescent proteins

In natural BphP photoreceptors, BV isomerizes at its 15/16 double bond upon light absorption [6]. This conformational

change is sensed by a photosensory module and is transmitted to an output effector domain, initiating the light-driven molecular signaling pathway. The photosensory module is formed by PAS (Per-ARNT-Sim repeats), GAF (cGMP phosphodiesterase/adenylate cyclase/FhlA transcriptional activator), and PHY (phytochrome-specific) domains (Figure 1b). BV is located in a pocket of the GAF domain and is covalently attached to a conserved Cys residue in the N-terminal extension of the PAS domain. This N-terminal extension passes through a lasso in the GAF domain, forming a figure-of-eight knot structure [24]. Although the PAS-GAF domains are minimally required for BV binding, the PHY domain is important for the chromophore photoconversion and light-driven signal transduction [25,26]. Two states corresponding to two distinct BV conformations are historically called as Pr ('pigment red absorbing'; 15/16 double bond is in a *cis* conformation) and Pfr ('pigment far-red absorbing'; 15/16 double bond in a *trans* conformation).

The interaction of the BV chromophore with the apoprotein and its photochemistry defines NIR FP characteristics and results in several NIR FP phenotypes. Engineering of BphPs into permanently fluorescent NIR FPs (Figure 1d) requires a stabilization of the Pr state of the BV chromophore to decrease a probability of non-radiative energy dissipation that is achieved by truncation to the PAS-GAF domains and introduction of amino acid substitutions into the chromophore immediate environment [16,17••,18]. In recent years, a number of permanently fluorescent NIR FPs were engineered from different BphPs (Table 1). Majority of these proteins are dimers. Although some NIR FPs behave as monomers in *in vitro* assays, their proper localization in fusion constructs in mammalian cells was not shown. The common feature of all NIR FPs is their relatively low quantum yield comparing to GFP-like FPs. However, the high extinction coefficients and NIR-shifted spectra made them superior probes for *in vivo* imaging.

Fluorescent brightness in mammalian cells (also called effective brightness) is the important parameter for NIR FP applications (Table 1). The effective brightness depends on molecular brightness, intracellular folding and stability, affinity and specificity to BV chromophore, intracellular BV concentration and protein expression level. Low efficiency and specificity of BV binding to the apoprotein substantially decreases the cellular fluorescence because of the competition from other heme-related compounds including protoporphyrins [27,28]. Among NIR FPs, proteins of an infra-Red Fluorescent Protein (iRFP) series have been developed using extensive screening of mutants for fluorescence in mammalian cells [13••,17••]. As a result, iRFPs do not require supply of exogenous BV and can be used by delivering of a single gene to cells. By contrast, NIR FPs of an Infrared Fluorescent Protein (IFP) series require the BV supply [18] or co-expression of heme oxygenase [14], which may affect cell metabolism and proliferation as heme oxygenase is the oxidative stress-inducible enzyme [29,30].

Imaging of several biological processes *in vivo* requires spectrally distinct NIR FPs. By mutating residues in the BV-binding pocket a set of multicolor permanently fluorescent iRFPs has been developed [13••]. Recently, we uncovered a mechanism of the spectral blue-shift in BphP-derived NIR FPs (unpublished data). It was found that the chromophores in blue-shifted NIR FPs differ from the chromophores in red-shifted NIR FPs developed from the same BphP template. In natural BphPs and in red-shifted NIR FPs, the BV chromophore covalently binds to the Cys residue in the PAS domain via $C3^2$ carbon of the side chain of pyrrole ring A (Figure 1d). In blue-shifted NIR FPs, however, BV binds to the Cys residue in the GAF domain, resulting in blue-shifted chromophores linked via either $C3^2$ or $C3^1$ carbon atoms of the side chain of pyrrole ring A (Figure 1d).

By engineering of a whole photosensory module consisting of the PAS-GAF-PHY domains, two photoactivatable NIR FPs (PAiRFPs) were developed from a subtype of BphPs, called bathy BphPs, whose ground state is Pfr [19]. Initially in the dark (non-fluorescent) Pfr state, PAiRFPs photoconvert into the fluorescent Pr state upon illumination with NIR light in the range of ~650–800 nm (Figure 1e). The light-induced conversion from Pr to initial Pfr state is disabled in PAiRFPs, however, a slow thermal conversion results in relaxation back to the dark Pfr state, enabling photoactivation-relaxation cycles.

Domain organization of BphPs is favorable for design of split reporters of protein–protein interactions. Several such reporters have been developed using reconstitution of the PAS and the GAF domains into a fluorescent NIR FP via bimolecular fluorescence complementation [20•,21–23] (Figure 1f, Table 1). Among them, iSplit engineered from iRFP713 exhibits the highest cellular brightness and does not require supply of exogenous BV to mammalian cells and tissues [20•]. The complementation of iSplit reporter is irreversible. Recently, fluorescence of an IFP Protein-fragment Complementation Assay (IFP PCA) reporter was shown to be reversible [21]. Reversibility of IFP PCA complementation is not clearly understood. The knot structure between the N-terminus of the PAS domain with covalently bound BV and the lasso loop of the GAF domain where BV is positioned may inhibit dissociation of the reconstituted NIR FP [24]. Likely, the observed IFP PCA reversibility results from a non-covalently incorporated BV.

Biological applications of near-infrared fluorescent proteins

Advanced NIR FPs are superior probes for non-invasive *in vivo* imaging over GFP-like FPs. Deeper tissue penetration