Molecular Basis of Spectral Diversity in Near-Infrared Phytochrome-Based Fluorescent Proteins

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SUMMARY

Near-infrared fluorescent proteins (NIR FPs) engineered from bacterial phytochromes (BphPs) are the probes of choice for deep-tissue imaging. Detection of several processes requires spectrally distinct NIR FPs. We developed an NIR FP, BphP1-FP, which has the most blue-shifted spectra and the highest fluorescence quantum yield among BphP-derived FPs. We found that these properties result from the binding of the biliverdin chromophore to a cysteine residue in the GAF domain, unlike natural BphPs and other BphP-based FPs. To elucidate the molecular basis of the spectral shift, we applied biochemical, structural and mass spectrometry analyses and revealed the formation of unique chromophore species. Mutagenesis of NIR FPs of different origins indicated that the mechanism of the spectral shift is general and can be used to design multicolor NIR FPs from other BphPs. We applied pairs of spectrally distinct point cysteine mutants to multicolor cell labeling and demonstrated that they perform well in model deep-tissue imaging.

INTRODUCTION

Bacterial phytochrome photoreceptors (BphPs) are of great interest as protein templates for the development of genetically encoded near-infrared (NIR) fluorescent probes for in vivo imaging (Piatkevich et al., 2013a; Shcherbakova et al., 2015). In an NIR optical window (650–900 nm), mammalian tissue is relatively transparent to light because the combined absorption of water, hemoglobin, and melanin is minimal, and autofluorescence is low (Weissleder, 2001). Therefore, NIR FPs are the probes of choice for truly (up to 20 mm) deep-tissue imaging in neuroscience, stem cell biology, developmental biology, and cancer research (Jiguet-Jiglaire et al., 2014; Sanders et al., 2013; Zhu et al., 2013). Non-invasive imaging of several biological processes in vivo requires spectrally distinct NIR FPs, which all have excitation and emission near or above 650 nm. Understanding of the chromophore-protein interactions responsible for spectral properties will allow us to rationally design such probes from any NIR FP type.

BphPs belongs to a family of phytochrome photoreceptors found in plants, algae, fungi, bacteria, and cyanobacteria, which use linear tetrapyrroles, also known as bilins, as a chromophore (Auldridge and Forest, 2011; Giraud and Vermeglio, 2008; Karniol et al., 2005; Rockwell and Lagarias, 2010). Importantly, BphPs incorporate the most near-infrared-absorbing bilin, biliverdin IXα (BV) (Bhoo et al., 2001), unlike plant and cyanobacterial phytochromes, which utilize phytochromobilin (PFB) and phycocyanobilin (PCB) (Rockwell and Lagarias, 2010). BV happens to be naturally present in cells and tissues of higher animals, including flies, fishes, and mammals, as a product of enzymatic heme degradation (Kapitulnik and Maines, 2012; Shu et al., 2009; Tran et al., 2014).

In all phytochromes, the bilin chromophore is positioned in a pocket of the GAF (cGMP phosphodiesterase/adenylate cyclase/FhlA) domain (Burgie et al., 2014; Essen et al., 2008; Wagner et al., 2005; Yang et al., 2009). The GAF and the N-terminal PAS (Per-ARNT-Sim) domains are minimally required for covalent BV binding (Wagner et al., 2005). Importantly, BphPs incorporate the most near-infrared-absorbing bilin, biliverdin IXα (BV) (Shoo et al., 2001), unlike plant and cyanobacterial phytochromes, which utilize phytochromobilin (PFB) and phycocyanobilin (PCB) (Rockwell and Lagarias, 2010). BV happens to be naturally present in cells and tissues of higher animals, including flies, fishes, and mammals, as a product of enzymatic heme degradation (Kapitulnik and Maines, 2012; Shu et al., 2009; Tran et al., 2014).

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The PAS and GAF domains are followed by the PHY (phytochrome-specific) domain, which is important for chromophore photoconversion and light-driven signal transduction (Takala et al., 2014; Wu and Lagarias, 2000; Yang et al., 2008). Light absorption induces a photo-isomerization of bilin at its 15/16
double bond. Two states corresponding to distinct BV conformations are Pr (red absorbing, cis 15/16 double bond) and Pfr (far-red absorbing, trans 15/16 double bond). Typical absorbance maxima of BphPs are 680–710 nm for the Pr state and 750–770 nm for the Pfr state (Giraud and Vermeglio, 2008). In addition to the main absorbance peaks of the Pr and Pfr states (Q band), phytocromes also absorb at ∼400 nm (Soret band), which does not depend on covalent binding of BV and is a common band for tetrapyrrole compounds (Franzen and Boxer, 1997).

Weak fluorescence of natural phytocromes has been known for years (quantum yields are ∼1%–4%) (Auldridge et al., 2012; Toh et al., 2011), although the utility of phytocromes as templates was first explored a decade ago by Fischer and Lagarias (2004). To engineer NIR FPs, BphPs need to be truncated to PAS-GAF domains, and mutations stabilizing the Pr state should be introduced to prevent non-radiative energy dissipation (Auldridge et al., 2012; Toh et al., 2011). In recent years, a number of NIR FPs were engineered from different BphPs. Deinococcus radiodurans DrBphP was engineered into IFP1.4 (Shu et al., 2009), IFP2.0 (Yu et al., 2014), IFP1.4rev (Bhattacharya et al., 2014), and Wi-Phy (Auldridge et al., 2012), and Bradyrhizobium BrBphP was engineered into mIFP (Yu et al., 2015). Several proteins of an iRFP series were developed based on Rhodopseudomonas palustris RpBphP2 and RpBphP6 (Filonov et al., 2011; Filonov and Verkhusha, 2013; Scherbakova and Verkhusha, 2013). The iRFP series was engineered to specifically incorporate endogenous BV, resulting in their high fluorescence in mammalian cells.

Imaging several cell populations, tissues, and organs in animals requires spectrally distinct NIR FPs. Attempts to stabilize the Pr state and make it fluorescent have been unsuccessful so far. Recently, we found that by mutating residues located close to the chromophore it was possible to shift the spectra of NIR FPs (Scherbakova and Verkhusha, 2013). Using this strategy, we developed iRFp670, iRFp682, iRFp702, iRFp713, and iRFp720, starting from two BphPs, RpBphP2 and RpBphP6. Interestingly, blue-shifted iRFp670 and iRFp682 have higher quantum yields than their respective red-shifted NIR FP variants.

Here we developed a novel NIR FP, named BphP1-FP, from wild-type RpBphP1. BphP1-FP exhibits the most blue-shifted absorbance and fluorescence emission and the highest quantum yield among the available BphP-derived NIR FPs. We performed biochemical, structural, and mass spectrometry analyses of BphP1-FP and its mutants to characterize the binding of BV chromophore. We found that the BphP1-FP mutant forms two unique BV adducts. Based on this novel phenomenon, we propose a mechanism for the chromophore blue shift and a rational design strategy to tune color and brightness of NIR FPs.

**RESULTS**

**Development of a Blue-Shifted Near-Infrared Protein BphP1-FP**

We chose RpBphP1 as a starting template for directed molecular evolution. The Pr state of this BphP absorbs at 680 nm, which is 20 and 30 nm blue-shifted compared with the Pr states of RpBphP6 and RpBphP2 (Giraud and Vermeglio, 2006). The crystal structure is available for this protein, although at a low resolution (2.9 Å) (Bellini and Papiz, 2012).

First, we truncated RpBphP1 to the chromophore-binding PAS-GAF domain. This protein was weakly fluorescent with a quantum yield of 3.3% (Table S1). Then we randomly mutated the key residue Asp201 and adjacent Ile202 in a conserved PXSDIP motif and screened the library of mutants for brightness in bacteria producing BV. These mutations were shown to stabilize the chromophore in the Pr state and increase the fluorescence quantum yield (Auldridge et al., 2012; Scherbakova and Verkhusha, 2013). A mixture of brightest mutants was subjected to random mutagenesis in bacteria. Using flow cytometry followed by screening of bacterial colonies on Petri dishes, we specifically selected mutants with the blue-shifted fluorescence spectra. Following three rounds of random mutagenesis, we obtained the protein named BphP1-FP. This FP had an excitation peak at 639 nm and an emission peak at 669 nm. The absorbance peak was at 643 nm, which is 36 nm shorter than the absorbance of RpBphP1 in the Pr state (Figure 1A). Interestingly, the fluorescence quantum yield of BphP1-FP was 13%, which is the highest quantum yield observed in BphP-derived FPs.

**Spectral Blue Shift is Caused by a Cysteine in the GAF Domain**

We noticed that, in addition to conserved Cys at the N-extension of the PAS domain (C20), BphP1-FP contains Cys in the conserved -SPXH- motif in the GAF domain (C253). Two Cys residues at the same positions were also observed in previously reported blue-shifted iRFp670 and iRFp682, developed from RpBphP6 and RpBphP2, respectively (Figure 1B and Supplementary Data File). Cys253 is located at the same position as a chromophore binding Cys in plant and cyanobacterial phytocromes.

We hypothesized that Cys253 in the GAF domain may covalently bind BV causing the observed spectral blue shift. To test this, we obtained mutants with a single Cys in either the PAS (BphP1-FP/C253I) or the GAF (BphP1-FP/C20S) domains, and a mutant without both cysteine residues. The proteins were expressed and purified from bacteria producing BV. We found that both Cys20 and Cys253 covalently bind BV, whereas the BphP1-FP/C20S/C253I double mutant does not bind BV covalently (Figure 1C). Next, we characterized the spectral properties of the mutants. The C253I mutation in BphP1-FP resulted in a red shift. The absorbance shifted to 677 nm and the emission to 704 nm, respectively (Figures 1D and 1E). The C20S mutation resulted in a protein with absorbance and fluorescence spectra very similar to those of BphP1-FP (Figures 1D and 1E). This suggests that in the protein with two Cys residues BV is preferably bound to Cys in the GAF domain. The double mutant BphP1-FP/C20S/C253I had red-shifted spectra similar to BphP1-FP/C253I, which is consistent with previous observations (Boruczi et al., 2009; Wagner et al., 2007) (Figures 1D and 1E; Table 1). BphP1-FP/C20S had a quantum yield of 13.9% (Table 1). Quantum yields of both red-shifted BphP1-FP/C253I and BphP1-FP/C20S/C253I were considerably lower and did not exceed 4%. Interested by the effect of Cys253, we tested the properties of the wild-type RpBphP1 (PAS-GAF domains) with C20S/I253C mutations. We found that this mutant with the chromophore-binding Cys in the GAF domain was blue-shifted relative to the
wild-type protein, with an absorbance peak at 650 nm and an emission peak at 671 nm (Table S1 and Figures S1A–S1C). The quantum yield of this mutant (4.1%) was higher than that of wild-type RpBphP1 (3.3%).

To check for possible heterogeneity of the chromophore and the presence of protoporphyrin IX (PPIX) in BphP1-FP/C20S, we recorded emission spectra at different excitation wavelengths. We found no significant heterogeneity and no presence of PPIX (manifested by emission peaks with maxima at 622–626 nm according to Wagner et al., 2008) (Figures S1D–S1G).

We concluded that covalent BV adducts bound to the Cys253 in the GAF domain are responsible for the high brightness and the blue-shifted spectra of BphP1-FP and BphP1-FP/C20S.

**Structure of BV Chromophore Bound to the Cysteine in the GAF Domain**

To study the nature of the BV adduct bound to Cys in the GAF domain, we crystallized BphP1-FP/C20S. The structure was determined at 1.64 Å resolution (Tables S2 and S3).

Overall, BphP1-FP/C20S has a fold typical of the chromophore-binding domains of BphPs (Figures 2A and S2). In contrast to other BphP structures, the N-terminal extension of BphP1-FP/C20S is mainly disordered, as there is no stabilizing covalent bond between residue 20 and BV. The figure-eight knot common to all BphPs is preserved (Figure S2C). The chromophore species in BphP1-FP/C20S are linked to Cys253 in the GAF domain (Figure 2B).

Electron density maps of the chromophore revealed two chromophore species that form thioether covalent bonds with Cys253 in the GAF domain. These species are linked to Cys253 via C31 or C32 carbon atoms of the A ring (Figure 2C). The presence of two chromophores was confirmed by both composite OMIT Fo-Fc and conventional 2Fo-Fc maps. We also confirmed it by calculated feature-enhanced electron density mapping (Afonine et al., 2015) (see also Experimental Procedures). Similarly to other BphPs in the Pr state (Auldridge et al., 2012; Wagner et al., 2007; Yu et al., 2014), both chromophores adopt a 5Zsyn,10Zsyn,15Zanti conformation for the methylene
linkers connecting the four pyrrole rings (Figures 2C and 2D). However, there is a remarkable difference between the A rings of chromophores in BphP1-FP/C20S and BV adducts observed in other BphP-derived proteins. In both chromophores linked to Cys253, C2, and C3, carbon atoms assume tetrahedral geometry indicative of sp³ hybridization similarly to A rings in reduced PCB and PφB bilins (Figure 2C). The methyl group of C²¹ atom points away from the GAF cysteine residue. The two chromophore species differ by the orientations of the A rings. Whereas the chromophore linked to the Cys253 via C³¹ atom has ring A in a plane with rings B and C, the chromophore linked to the Cys253 via the C³² atom has ring A rotated by 11° out of this plane (Figures 2E and 2F).

The nearest chromophore environment in BphP1-FP/C20S in general is similar to that of other BphP-based FPs with some unique characteristics (Figure 2G). In addition to a well-ordered “pyrrole water” (W₁), there is an additional water molecule W₂, which is located within hydrogen bond distance to W₁ and forms a hydrogen bond with the carbonyl oxygen of the chromophore A ring (Figures 2G and S2D). Another unique feature is the orientation of the propionate group of the C ring, which is rotated toward the side chain of Arg216 and is stabilized by a hydrogen bond with this residue.

**BV and PφB Bound to the Cysteine in the GAF Domain Exhibit Similar Spectral Properties**

As the C₃ atom in the A ring does not form a double bond, the BV adducts in BphP1-FP/C20S should have the same number of conjugated double bonds as bound PφB (Figure 3A). PφB is enzymatically produced from BV by ferredoxin-dependent bilin reductase (Rockwell and Lagarias, 2010). Importantly, in plant phytochromes, PφB is covalently bound to Cys in the GAF domain.

We tested chromophore binding and measured the spectral properties of the BphP-FP mutants expressed in bacteria. Initially, a similar maximum of 672 nm was observed for BphP1-FP/C253I assembled with BV, whereas the mass of the chromophore bound to the protein assembled with PφB is ~2 Da larger and corresponds to PφB (Figures 3E and S4; Table S3). Thus, the BV chromophore is not reduced when assembled with BphP1-FP/C20S.

**Holoprotein with BV Bound to the Cysteine in the GAF Domain Assembles Autocatalytically**

Next, we tested whether a holoprotein can be assembled without any additional enzymes and cofactors. We purified the BphP1-FP/C20S and control BphP1-FP/C253I apoproteins and assembled them with BV in vitro as described previously (Quest and Gartner, 2004).

When BphP1-FP/C20S was mixed with BV, the absorbance maximum at the Q band gradually shifted toward shorter wavelengths (Figure 3F). Starting from 672 nm, which corresponds to non-covalently bound BV (Table 1, see BphP1-FP/C20S/C253I + BV), the absorbance maximum reached 641 nm and this matches the value observed for BphP1-FP/C20S assembled in bacteria. Initially, a similar maximum of 672 nm was observed for BphP1-FP/C253I. The absorbance then shifted to 676 nm, which corresponds to the maximum observed for this protein expressed with BV in bacteria (Figures S3G and S3C, and S3D with an overlay of fluorescence spectra). Thus, the blue-shifted chromophore bound to Cys in the GAF domain forms autocatalytically.

**The Nature of the Hypsochromic Spectral Shift**

The structural and biochemical analyses indicate that both BV adducts bound to Cys253 via either C³¹ or C³² atoms have no double bonds formed by the C₃ atom in ring A, and that they are not chemically reduced and form without additional enzymes and cofactors.

These data suggest that, upon binding, BV adducts autocatalytically isomerize to change the initial position of the double bond between C₃ and C₂ or C₃ and C³ atom to the double bond between C³¹ and C³² atoms (Figure 4). The
re-arrangement of initially bound intermediates (Figure S5) with the double bond between C3 and C2 or C3 and C31 atoms should reduce the steric constraint. In contrast to the chromophore-binding Cys in the PAS domain lying almost in the same plane with BV, the Cys in the GAF domain is located above the BV chromophore plane (Figure 2B). The latter Cys location causes a steric constraint if the C3 atom forms a double bond with C2 or C31 atoms and has a planar geometry.

The proposed BV adducts explain the significant blue shift in absorbance and fluorescence observed in the NIR FPs with BV bound to Cys in the GAF domain. The nearly identical spectra of BphP1-FP/C20S assembled with BV and assembled with PFPB (Figures 3C and 3D) correlate with the similarity between the p-conjugated systems of the bound chromophores (Figures 4A and 4B).

In both BV and PFPB chromophores bound to the Cys in the GAF domain, C3 atoms in ring A do not form double bonds and have tetrahedral geometry (sp3 hybridization) that can be seen from the overlay of chromophores in BphP1-FP/C20S and plant phytochrome from Arabidopsis thaliana AtPhyB (Burgie et al., 2014) (Figure 4C). According to the chemical structures of the BV adducts (Figure 4A), the C31 atom in ring A forms a double bond with the C32 atom and thus adopts planar geometry (sp2 hybridization). This is not the case for the PFPB chromophore, in which the C31 atom is tetrahedral (sp3 hybridization) (Figure 4B).

The overlays of each of the BV chromophores with the PFPB chromophore indicate the presence of a double bond between C31 and C32 atoms in BV adducts (Figures 4D and 4E).

Figure 2. Structure of BphP1-FP/C20S
(A) Superposition of the structures of BphP1-FP/C20S (PDB: 4XTQ, in green) and Deinococcus radiodurans DrBphP PAS-GAF domains (PDB: 2O9C, in magenta).
(B) Superimposed structures as in (A) showing the chromophores and binding cysteine residues. Biliverdin adducts are green and yellow in BphP1-FP/C20S and magenta in DrBphP PAS-GAF.
(B–E) Carbon atoms of the molecules connected to C253 via C31 and C32 atoms are shown in yellow and green, respectively. Nitrogen atoms are in blue, oxygen in red, sulfur in dark yellow. (C) Biliverdin adducts in FEM electron density mapping countered at 1.5σ. (D) Top view of the two overlaid biliverdin adducts. Pyrrole rings and carbon atoms in ring A are labeled. (E and F) Side views of the two biliverdin adducts connected via C31 (E) or C32 (F) atoms.
(G) The nearest chromophore environment with a detailed view of the water molecules in the FEM electron density mapping countered at 2.5σ. See also Figure S2; Tables S2 and S3.

Engineering of Spectrally Distinct NIR FPs for Multicolor Imaging
To test whether the proposed mechanism of the blue shift in BphP-based FPs is broadly applicable, we tested it on two previously reported blue-shifted NIR FPs, iRFP670 and iRFP682, which both acquired Cys in the -SPXH- motif of GAF domains in the process of molecular evolution (Shcherbakova and Verkhusha, 2013). Whereas BphP1-FP was developed from RpBphP1, iRFP670 and iRFP682 were engineered from RpBphP6 and RpBphP2, respectively. We obtained iRFP670 and iRFP682 mutants containing a single Cys either in the PAS or in the GAF domain. The spectral properties of these mutants corresponded to those of the respective mutants of BphP1-FP (Table S4). The spectra of iRFP670 and iRFP682 mutants with Cys in the PAS domain demonstrated a 35- to 40-nm red shift, while the mutants with Cys in the GAF domain had spectra similar to the original proteins with two Cys residues.

The proposed mechanism of the blue shift in BphP (Figure 4) can be applied to engineer spectrally distinct NIR FPs for multicolor in vivo imaging. We suggest that each BphP can be developed into two NIR FPs with a spectral difference of up to 40 nm, which have chromophore-binding Cys either in the PAS or in the GAF domains. First, the blue-shifted NIR FPs can be designed by introduction of chromophore-binding Cys in the GAF domain of a BphP template at the early stages of molecular evolution. Then the red-shifted NIR FPs can be obtained from the respective blue-shifted NIR FPs by removing Cys from the GAF domain, and both obtained variants can be improved by subsequent random mutagenesis.
We tested whether Cys point mutants of BphP1-FP can be directly applied for multicolor labeling in mammalian cells. The BphP1-FP/C20S- and BphP1-FP/C253I-expressing cells were successfully spectrally resolved using standard flow cytometry and epifluorescence microscopy (Figures 5A and 5B). Similar two-color detection was also possible for the Cys point mutants of iRFP670 and iRFP682 (Figure S6). All tested NIR FP variants efficiently bound endogenous biliverdin, which is abundant in eukaryotes.

The use of several different BphPs will allow engineering of more than two multicolor NIR FPs. The Pr state absorption maxima of known BphPs vary from 670 to 710 nm (Giraud and Vermeglio, 2008), providing an additional 40-nm range to the ~40-nm spectral difference between NIR FPs developed from a single BphP. Thus, it should be possible to develop several pairs of spectrally distinct NIR FPs, of which the most blue-shifted and red-shifted ones will differ by ~80 nm.

To demonstrate how the pairs of NIR FPs obtained from different BphP templates can be applied for multicolor imaging, we tested the Cys point mutants of BphP1-FP and iRFP682, which are all spectrally distinct (Figure 5C). All four NIR FPs were spectrally resolved using flow cytometry of live mammalian cells (Figure 5D). Moreover, these proteins were also spectrally separated using a commercial multispectral imaging system (Figure 5E). To test the performance of bright blue-shifted NIR FPs in deep-tissue imaging, we quantitatively compared BphP1-FP and its
mutants with a widely used red-shifted NIR FP, iRFP713 (Filonov et al., 2011), and one of the brightest far-red GFP-like FPs, mNeptune (Lin et al., 2009). Equal amounts of purified proteins were placed inside a fluorescent mouse phantom engineered to have absorbance, light scattering, and autofluorescence matching those of mouse tissues. The fluorescence signals were imaged at two depths (7.0 and 18.1 mm) in different filter channels (Figures 6A and 6C), and signal-to-autofluorescence background ratios were quantified. One can see that blue-shifted BphP1-FP and BphP1-FP/C20S exhibit signal-to-background ratios comparable with that of iRFP713 and 6–8 higher than that of mNeptune (Figures 6B and 6D).

BphP1-FP and its mutant with Cys in the GAF domain exhibit a higher quantum yield than the mutant containing Cys only in the PAS domain. Intrigued by this, we analyzed the quantum yields of all known BphP-based FPs engineered from the PAS-GAF domains (Auldridge et al., 2012; Bhattacharya et al., 2014; Filonov et al., 2011; Shcherbakova and Verkhusha, 2013; Shu et al., 2009; Yu et al., 2014). We found that all NIR FPs containing Cys in the GAF domain exhibit a higher quantum yield than the FPs with Cys in the PAS domain (Figure 6E). Possibly, the higher quantum yield in NIR FPs with Cys in the GAF domain is caused by tightening of the interaction between BV and the protein. It is likely that the bilin bound to the structurally restrained Cys in the GAF domain has a higher structural rigidity than the bilin bound to the structurally loose Cys in the PAS domain and, thus, radiationless decay processes are partially inhibited.

**DISCUSSION**

We engineered the RpBphP1 phytochrome into the near-infrared BphP1-FP, which exhibits the most blue-shifted spectra and the highest quantum yield of 13% among the BphP-derived FPs. We found that a cysteine residue introduced in the -SPXH- motif of the GAF domain is the key residue responsible for these changes. This cysteine covalently binds BV via either C31 or C32 atoms of ring A. Upon binding, both BV chromophores autocatalytically isomerize, possibly to reduce the structural steric constraints. This results in a spectral blue shift because the isomerized double bond between C31 and C32 is no longer conjugated with the π-electron system of the chromophore.

We found that spectral shifts can be engineered in NIR FPs derived from different BphP templates. Evidently, the BV isomerization phenomenon is not limited to BphP-derived NIR FPs, but can also occur in the PAS-GAF domains of BphPs as we demonstrated for the PAS-GAF domain of RpBphP1 (Table S1). Previously, it was shown that DrBphP/C24A/M259C truncated to the PAS-GAF domains binds BV via Cys259 in the GAF (Wagner et al., 2007). An absorbance spectrum of this mutant contained two peaks, a major one at 698 nm and a minor one at 673 nm. We hypothesize that the major peak corresponded to non-covalent incorporation of BV, because (1) its absorbance maximum is identical to the maximum of the mutant DrBphP/C24A with no chromophore-binding Cys residues, and (2) DrBphP/C24A/M259C lacks Cys in the PAS and is thus unable to form a
covalent bond with BV. We suggest that the minor peak corresponded to the same covalently bound BV adduct bound to the Cys in the GAF domain that is observed in BphP1-C20S. Interestingly, when the chromophore-binding Cys was introduced into the GAF domains of full-length BV-binding Calothrix CphB (Quest and Gartner, 2004) and Agrobacterium tumefaciens Agp1 (Borucki et al., 2009), these BphPs started to covalently incorporate PCB. However, BV did not bind covalently, and no spectral shift was observed. Possibly, the photosensory module (PAS-GAF-PHY domains) in full-length BphPs restricted the covalent binding of BV to Cys in the GAF domain.

Our findings provide a rational design strategy to engineer spectrally distinct NIR FPs. To demonstrate this, we applied the Cys point mutants in multicolor imaging in cells (Figure 5). We found that the blue-shifted NIR FPs with Cys in the GAF domain have higher fluorescence quantum yields than NIR FPs with Cys in the PAS domain. We also showed that bright blue-shifted NIR FPs perform as well as the red-shifted NIR FPs, and considerably better than far-red GFP-like FPs in deep-tissue imaging (Figure 6).

NIR FPs engineered from bacterial phytochromes enable non-invasive in vivo imaging. The lack of understanding of NIR FPs with substantially higher quantum yield than currently observed in BphP-derived FPs. Twice higher fluorescence brightness will allow non-invasive tracking of four-fold smaller objects and cell populations, imaging of four-fold deeper organs and tissues, and detection of developmental, cancer, and infection processes at substantially earlier stages.

The possibility to engineer spectrally shifted NIR FPs based on any BphP template demonstrated here can be applied to NIR FPs of other phenotypes. For example, by introducing Cys point mutations, it will be possible to create spectrally distinct bimolecular fluorescence complementation reporters based on split NIR FPs, such as iSplit (Filonov and Verkhusha, 2013), and spectrally resolvable monomeric NIR FP for protein tagging. The multicolor NIR FPs will enable simultaneous labeling and tracking of two and more cellular proteins, protein-protein interactions, cell populations, tissues, and organs in live animals in normal and pathogenic conditions.

SIGNIFICANCE

NIR FPs engineered from bacterial phytochromes enable non-invasive in vivo imaging. The lack of understanding of
chromophore-protein interactions that influence the spectral properties of these FPs has hindered their development. We engineered the most blue-shifted NIR FP with the highest fluorescence quantum yield, and uncovered the molecular basis of its properties. Biochemical, structural, and mass spectrometry analyses revealed unique BV chromophore adducts. We showed that the formation of these BV adducts is generally applicable to NIR FPs of different origins and that they can be used for the rational design of spectrally distinct NIR FPs and biosensors for multicolor imaging in whole animals. We demonstrated that pairs of cysteine mutants allow multicolor imaging in live cells and that they perform well in deep-tissue imaging modeling.

EXPERIMENTAL PROCEDURES

Mutagenesis and Directed Molecular Evolution

The DNA fragment corresponding to PAS-GAF domains (first 315 amino acids) of RpBphP1 was amplified by PCR and inserted as the BglII/EcoRI fragment into a pBAD/His-B vector (Invitrogen). Site-specific mutagenesis was performed using a QuickChange mutagenesis kit (Stratagene). Random mutagenesis was performed with a GeneMorph II random mutagenesis kit (Stratagene), using conditions that resulted in a mutation frequency of up to 16 mutations per 10³ base pairs. After mutagenesis, a mixture of mutated genes was electroporated into LMG194 bacterial cells (Invitrogen) containing the pWA23h plasmid encoding heme oxygenase (hmuO) from Bradyrhizobium ORS278 under the rhamnose promoter (Piatkevich et al., 2013b; Shcherbakova and Verkhusha, 2013). Typical mutant libraries consisted of more than 10⁶ independent clones. The LMG194 cells were grown overnight at 37°C in RM minimal medium supplemented with ampicillin and kanamycin. Protein expression in the libraries was induced with 0.002% arabinose and 0.02% rhamnose. The cells were grown for 6–8 hr at 37°C and then at 18°C for 24 hr. For flow cytometry screening, bacterial cells were washed with PBS and diluted with PBS to an optical density of 0.03 nm at 600 nm. The libraries were screened using a MoFlo XDP (Beckman Coulter) fluorescence-activated cell sorter using a 592-nm laser for excitation and a 680-nm LP emission filter for positive selection. The 200–1,000 brightest infrared bacterial cells collected were rescued in SOC medium at 37°C for 1 hr, grown on LB/ampicillin/kanamycin Petri dishes supplemented with 0.02% arabinose and 0.2% rhamnose overnight at 37°C, and then incubated at 18°C. Screening for spectrally blue-shifted mutants on Petri dishes was performed with a Leica MZ16F fluorescence stereomicroscope equipped with 605/40 and 650/45 nm excitation filters and 640 and 690 nm LP emission filters (Chroma). Spectral properties of 10–20 selected mutants were tested, and their DNA was sequenced. A mixture of several selected mutants was then used as a template for the next round of mutagenesis.

Protein Expression and Purification

For expression of apoproteins, the genes encoding the proteins in the pBAD/His-B vector (Invitrogen) were expressed in TOP10 bacterial cells (Invitrogen). Bacterial cells were grown in RM medium supplemented with ampicillin. To
initiate protein expression, 0.002% arabinose was added and bacterial culture was incubated for 12 hr at 37 °C, followed by 24 hr at 18 °C. Proteins were purified using Ni-NTA agarose (Qiagen).

For expression of proteins in bacteria in the presence of BV, the genes coding for the proteins in pBAD/His-B vector (Invitrogen) were expressed in TOP10 bacterial cells (Invitrogen) bearing the pLPl-PB8 (Fischer et al., 2005; Gambetta and Lagarias, 2001) according to the protocol described by Burgie et al. (2014). In brief, cells were grown in termic broth containing 0.4% glycerol and 1 mM MgCl2 at 37 °C until the cells reach an optical density of 0.4–0.6 at 600 nm. The temperature was then decreased to 18 °C, and 100 mM l-α-aminolevulinic acid was added. After 1 hr, isopropyl β-D-1-thiogalactopyranoside was added to 0.4 mM, followed by addition of 0.02% arabinose after an additional 1 hr. The cells were then cultured for 16 hr. Proteins were purified using Ni-NTA agarose as described earlier.

Spectral and Biochemical Characterization

Fluorescence spectra were recorded using a FluoroMax-3 spectrofluorometer (Jobin Yvon). A Hitachi U-2000 spectrophotometer was used for absorbance measurements, unless specified in the figure legends, excitation spectra were recorded using 750 nm emission; and emission spectra were recorded using 590 nm excitation. The extinction coefficient was calculated from a comparison of absorbance values at the main peak at the Q band with the absorbance value at Soret band, assuming that the latter had an extinction coefficient of free BV of 39,900 M⁻¹ cm⁻¹ (Filonov et al., 2011; Shu et al., 2009). To determine quantum yield, we compared the fluorescence of a purified FP with that of an equally absorbing Nile blue dye (quantum yield is 0.27 in acidic ethanol (Gens and Drexhage, 1981)) using several dilutions.

For studies of BV assembly, purified apo-proteins (15 μM) were mixed with 10 μM BV in PBS in the presence of 1 mM DTT. Absorbance spectra were monitored immediately after mixing (in about 1 min) and then at the indicated time points until the absorbance reached a steady state.

For multicolor spectral imaging of bacterial cells, Petri dishes with bacterial streaks of LMG194 strain expressing each of four NIR FPs were prepared. For linear spectral unmixing, a set of images in 19 filter channels was collected on the IVIS Spectrum. Pure fluorescence signals of each NIR FP were identified by shortening the N-terminal polyhistidine tag to the MGSHHHHHHGRS amino acid sequence. Expressed and purified as above, the protein was transferred to a buffer containing 20 mM Tris-HCl, 200 mM NaCl (pH 8.0), and concentrated to 20 mg/ml using an Amicon Ultra centrifugal filter (Millipore). An initial search for crystallization conditions was carried out with Mosquito Robotic Crystallization System (TTP LabTech). The successful hits were further optimized manually. Large-scale crystallization was set up using the hanging drop vapor diffusion method. Typically, 2 μl of protein stock solution was mixed with 2 μl of the well solution and incubated against 500 μl of the same reservoir at 20 °C for 2 weeks. The best crystals of BphP1-FP/C20S were obtained from 3.2 M NaCl, 0.08 M Tris-HCl (pH 8.0) buffer.

X-Ray diffraction data were collected at the Advanced Photon Source, SERCAT beamline 22-BM (Argonne National Laboratory). Diffraction intensities were registered on a MAR225 CCD detector (Rayonix). Prior to data acquisition, a single crystal was dipped into cryoprotecting solution comprised of 20% (v/v) glycerol and 80% (v/v) of the corresponding well solution, and flashed cooled in a 100-K nitrogen stream. Cryogenic temperature was maintained throughout the diffraction experiment with an Oxford Cryostream cooling device (Oxford Cryosystems). Diffraction images were indexed, integrated, and scaled with HKL2000 (Otwinowski and Minor, 1997). The statistics of data processing are shown in Table S2.

The structure of BphP1-FP/C20S was solved by a molecular replacement method with MOLREP (Vagin and Teplyakov, 2010), using the PAS and GAF domains of RpBphP1 (PDB: 4GW9 (Bellini and Papiz, 2012)) as a search model. Maximum likelihood refinement and real space model correction were performed with REFMAC5 (Murshudov et al., 2011), PHENIX.REFINE (Adams et al., 2002) and COOT (Emsley and Cowtan, 2004). Ordered water molecules were added to the appropriate difference electron density peaks with COOT and PHENIX.REFINE. Improved noiseless feature-enhanced maps (FEMs) were calculated with PHENIX.FEM (Afonine et al., 2015). Structure quality was validated with PROCHECK (Laskowski et al., 1993) and COOT. The refinement statistics are summarized in Table S3. Structure figures were generated with PyMOL (Delano Scientific).

The absence of unusual chromophore species observed in the structure was confirmed by feature-enhanced electron density mapping (Afonine et al., 2015). The FEM procedure modifies the 2Fo − Fc and Fc − Fo maps to reduce noise and model bias, retaining and enhancing existing features. The FEM algorithm computes a large ensemble of slightly perturbed
maps and combines them into one map, exploiting the fact that map artifacts are more sensitive to randomization than the signal. It also performs signal equalization, making strong and weak signals similar in strength, and applies a OMIT map filter to reduce model bias.

Expression in Live Mammalian Cells

To construct mammalian expression plasmids, the respective genes of FP s were PCR-amplified as AgeI-NotI fragments and swapped with a gene encoding EGFP in the pEGFP-N1 plasmid (Clontech). HeLa cells were grown in DMEM medium supplemented with 10% FBS, 0.5% penicillin-streptomycin and 2 mM glutamine (Invitrogen). For microscopy, cells were cultured in 35-mm glass-bottom Petri dishes with no. 1 coverglass (MatTek). Plasmid transfections were performed using an Effectene reagent (Qiagen).

Flow Cytometry and Fluorescence Microscopy

Flow cytometry analysis was performed using a BD LSRII flow cytometer equipped with 488 and 640 nm lasers and 530/40, 670/30, and 710/20 nm emission filters. A total of 20,000 events for each cell type were analyzed. The obtained dot plots were superimposed.

Epifluorescence microscopy of live HeLa cells was performed 48 hr after the transfection. HeLa cells were imaged using a Nikon Eclipse Ti inverted epifluorescence microscope equipped with a 60×/1.4 NA oil immersion objective lens (UPlanSApo, Olympus), and two filter sets (605/40 nm exciter and 667/30 nm emitter, and 682/10 nm exciter and 721/42 nm emitter) (Chroma). SlideBook v.4.1 software (Intelligent Imaging Innovations) was used to operate the microscope.

ACCESSION NUMBERS

The coordinates and structure factors for BphP1-FP/C20S have been deposited in the PDB under the accession code PDB: 4XTQ.

SUPPLEMENTAL INFORMATION

Supplemental Information includes a Supplemental Data File, six figures, and five tables and can be found with this article online at http://dx.doi.org/10.1016/j.chembiol.2015.10.007.

AUTHOR CONTRIBUTIONS

D.M.S. and M.B. developed the protein and its mutants and characterized them in vitro. M.B. characterized the protein variants in mammalian cells. S.P., V.N.M., and Z.D. crystallized the protein, and S.P. performed its structural analysis. H.X. performed the mass spectrometry analysis. V.V.V. planned and directed the project and together with D.M.S. designed the experiments, analyzed the data, and wrote the manuscript.

ACKNOWLEDGMENTS

We thank Eric Giraud (Institute for Research and Development, Montpellier, France) for the pSpBphP1 gene, Clark Lagarias (University of California at Davis) and Richard Vierstra (University of Wisconsin at Madison) for the plasmids for production of BV and P48 in bacteria. We are grateful to Clark Lagarias for useful comments on the manuscript. We thank Jinhang Zhang for assistance with flow cytometry. Use of the Advanced Photon Source was supported by the US Department of Energy, Office of Science, Office of Basic Energy Sciences under Contract No. W-31-109-Eng-38. This work was supported in part with US Federal funds from the National Cancer Institute of the NIH under contract HHSN261200800001E, the Intramural Research Program of the NIH, by the NIH grants CA164468, GM073913 and GM108579 (all to V.V.V.) and ERC-2013-ADG-340233 (to V.V.V.) grant from the EU FP7 program.

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Supplemental Information

Molecular Basis of Spectral Diversity in Near-Infrared Phytochrome-Based Fluorescent Proteins

Daria M. Shcherbakova, Mikhail Baloban, Sergei Pletnev, Vladimir N. Malashkevich, Hui Xiao, Zbigniew Dauter, and Vladislav V. Verkhusha
Figure S1, related to Figure 1. (A-C) Spectral properties of the PAS-GAF domains of wild-type RpBphP1 and its C20S/I253C mutant. Overlays of (A) absorbance, (B) excitation and (C) emission spectra of these two proteins are shown. (D-G) Fluorescence emission spectra recorded at different excitation wavelengths for (D, F) BphP1-FP and (E, G) BphP1-FP/C20S. The fluorescence excitation wavelengths are indicated in the panels.
Figure S2, related to Figure 2. (A) Superposition of 3D structures of BphP1-FP/C20S (blue), PAS and GAF domains of RpBphP1 (PDB: 4GW9; in green), Wi-Phy (PDB: 3S7Q; in red), and IFP2.0 (PDB: 4CQH; in yellow). (B) Positions of amino acid residues mutated in BphP1-FP/C20S (blue) relative to PAS and GAF domains of parental RpBphP1 (PDB: 4GW9; pink). Non-identical residues are shown as sticks. Out of twenty-four amino acid residues, which differ in BphP1-FP/C20S compared to PAS-GAF of RpBphP1, five residues (201, 202, 253, 286 and 282) are positioned in the vicinity of the chromophore, and the other nineteen reside on the protein surface. The chromophores of BphP1-FP/C20S connected to Cys253 via C3¹ and C3² atoms are shown in yellow and green, respectively. (C) Superimposed structures of BphP1-FP/C20S (PDB: 4XTQ; in green) and Deinococcus radiodurans DrBphP PAS-GAF domains (PDB: 2O9C; in magenta. N-terminal extension and the knot lasso in both structures are highlighted with brighter color than the rest of the protein. (D) The immediate chromophore environment. The system of hydrogen bonds is shown as red dashed lines. Water molecules are shown as red spheres.
Figure S3, related to Figure 3. (A, B) Spectral properties of BphP1 variants assembled with BV and PΦB. (A) Overlay of absorbance spectra of BphP1-FP/C253I assembled with BV and PΦB. (B) Overlay of excitation and emission spectra of BphP1-FP/C253I assembled with BV and PΦB. (C, D) Fluorescence of BphP1 variants assembled with BV in bacteria and in vitro. (C) Overlay of excitation and emission spectra of BphP1-FP/C20S with BV in bacteria and in vitro. (D) Same as in (C) for BphP1-FP/C253I mutant.
Figure S4, related to Figure 3. The MS/MS spectra of the S249 – K259 peptide isolated from the trypsinized samples of the purified BphP1-FP/C20S expressed with BV and BphP1-FP/C20S expressed with PΦB. The BV-bound and PΦB-bound peptides elute at the similar retention time, one at 48.48 min and another at 47.20 min.
Figure S5, related to Figure 4. Proposed formation of the blue-shifted BV chromophores in BphP1-FP/C20S. The hypothetical intermediates are shown in parentheses. To release steric constraint in these intermediates caused by location of the Cys in the GAF domain above the BV chromophore plane, an autocatalytic chromophore isomerization occurs. This results in the spectral blue-shift. Pyrrole rings and carbon atoms in A ring are labeled in unbound BV.
Figure S6, related to Figure 5. Two-color cell labeling using single Cys point mutants of iRFP670 and iRFP682 proteins. **(A)** Flow cytometry analysis of live HeLa cells expressing iRFP670/C10A and iRFP670/C247S. **(B)** Representative images of live HeLa cells expressing iRFP670/C10A and iRFP670/C247S. **(C)** Flow cytometry analysis of live HeLa cells expressing iRFP682/C15S and iRFP682/C254S. A 640 nm laser and a combination of two indicated emission filters were used for analysis of 20,000 events in each sample (A, C). **(D)** Representative images of live HeLa cells expressing iRFP682/C15S and iRFP682/C254S. The images in the 605/40 nm excitation and 667/30 nm emission channel are presented in a green pseudocolor, and images in the 682/12 nm excitation and 721/42 nm emission channel are presented in a red pseudocolor. Scale bar is 10 µm (B, D).
Table S1, related to Figure 1. Properties of the PAS-GAF domains of wild-type \( R\)pBphP1 and its C20S/I253C mutant.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Absorbance maximum, nm</th>
<th>Excitation maximum, nm</th>
<th>Emission maximum, nm</th>
<th>Quantum yield, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>( R)pBphP1 PAS-GAF</td>
<td>684</td>
<td>684</td>
<td>704</td>
<td>3.5</td>
</tr>
<tr>
<td>( R)pBphP1 PAS-GAF C20S/I253C</td>
<td>650</td>
<td>647</td>
<td>671</td>
<td>4.1</td>
</tr>
</tbody>
</table>
Table S2, related to Figure 2. BphP1-FP/C20S data collection statistics.

<table>
<thead>
<tr>
<th>Protein</th>
<th>BphP1-FP/C20S</th>
</tr>
</thead>
<tbody>
<tr>
<td>Space group</td>
<td>$P2_12_12_1$</td>
</tr>
<tr>
<td>Unit cell parameters (Å)</td>
<td>a = 52.7, b = 53.1, c = 107.1</td>
</tr>
<tr>
<td>Temperature (K)</td>
<td>100</td>
</tr>
<tr>
<td>Wavelength (Å)</td>
<td>1.00</td>
</tr>
<tr>
<td>Resolution (Å)</td>
<td>50.0 - 1.64</td>
</tr>
<tr>
<td>Total reflections</td>
<td>267,748</td>
</tr>
<tr>
<td>Unique reflections</td>
<td>37,535</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>99.9 (99.9)</td>
</tr>
<tr>
<td>$I/\sigma&lt;I&gt;$</td>
<td>32.1 (2.2)</td>
</tr>
<tr>
<td>R-merge</td>
<td>0.058 (0.76)</td>
</tr>
<tr>
<td>Multiplicity</td>
<td>7.1 (6.5)</td>
</tr>
</tbody>
</table>

Data in parentheses are given for the outermost resolution shell, 1.70 – 1.64 Å.
Table S3, related to Figure 2. BphP1-FP/C20S refinement statistics.

<table>
<thead>
<tr>
<th>Protein</th>
<th>BphP1-FP/C20S</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of protein atoms</td>
<td>2,552</td>
</tr>
<tr>
<td>No. of solvent atoms</td>
<td>398</td>
</tr>
<tr>
<td>Resolution range (Å)</td>
<td>50.0 - 1.64</td>
</tr>
<tr>
<td>R-work</td>
<td>0.175</td>
</tr>
<tr>
<td>R-free</td>
<td>0.208</td>
</tr>
<tr>
<td>R.m.s.d. bond lengths (Å)</td>
<td>0.021</td>
</tr>
<tr>
<td>R.m.s.d. angles (°)</td>
<td>2.57</td>
</tr>
<tr>
<td>R.m.s.d. chirality (°)</td>
<td>0.16</td>
</tr>
<tr>
<td>R.m.s.d. planarity (°)</td>
<td>0.012</td>
</tr>
<tr>
<td>R.m.s.d. dihedral (°)</td>
<td>17.5</td>
</tr>
<tr>
<td>Mean B factors (Å²)</td>
<td></td>
</tr>
<tr>
<td>Protein atoms</td>
<td></td>
</tr>
<tr>
<td>overall</td>
<td>17.7</td>
</tr>
<tr>
<td>main chain</td>
<td>16.0</td>
</tr>
<tr>
<td>side chain</td>
<td>19.4</td>
</tr>
<tr>
<td>chromophore</td>
<td>16.6</td>
</tr>
<tr>
<td>Ramachandran statistics (%) (for non-Gly/Pro residues)</td>
<td></td>
</tr>
<tr>
<td>most favorable</td>
<td>94.5</td>
</tr>
<tr>
<td>additional allowed</td>
<td>5.1</td>
</tr>
<tr>
<td>generously allowed</td>
<td>0.4</td>
</tr>
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**Table S4, related to Figure 3.** Masses of bound chromophores in BphP1-FP/C20S expressed with BV and with PΦB obtained by mass-spectrometry analysis.

<table>
<thead>
<tr>
<th>Peptide fragments</th>
<th>m/z in unmodified peptide (ms/ms of 654.12^{2+}, Da)</th>
<th>m/z in modified peptide from BphP1-FP/C20S BV (ms/ms of 944.9^{2+}, Da)</th>
<th>Chromophore mass in BphP1-FP/C20S BV, Da</th>
<th>m/z in modified peptide from BphP1-FP/C20S PΦB (ms/ms of 946.6^{2+}, Da)</th>
<th>Chromophore mass in BphP1-FP/C20S PΦB, Da</th>
</tr>
</thead>
<tbody>
<tr>
<td>b7</td>
<td>756.1483</td>
<td>1338.3887</td>
<td>582.2404</td>
<td>1340.3516</td>
<td>584.2033</td>
</tr>
<tr>
<td>b8</td>
<td>884.2838</td>
<td>1466.3969</td>
<td>582.1131</td>
<td>1468.3889</td>
<td>584.1051</td>
</tr>
<tr>
<td>b9</td>
<td>1047.306</td>
<td>1629.646</td>
<td>582.3397</td>
<td>1631.4281</td>
<td>584.1218</td>
</tr>
<tr>
<td>b10</td>
<td>1160.455</td>
<td>1742.6017</td>
<td>582.1472</td>
<td>1744.4568</td>
<td>584.0023</td>
</tr>
<tr>
<td>y8^{2+}</td>
<td>501.4435</td>
<td>792.6021</td>
<td>582.3172</td>
<td>793.3693</td>
<td>583.8516</td>
</tr>
<tr>
<td>y9^{2+}</td>
<td>544.8135</td>
<td>836.0978</td>
<td>582.5686</td>
<td>837.1528</td>
<td>584.6786</td>
</tr>
<tr>
<td>Average chromophore mass Δm</td>
<td></td>
<td></td>
<td>582.2877±0.164281</td>
<td></td>
<td>584.2033±0.281166</td>
</tr>
</tbody>
</table>

Peptide fragments are extracted from MS/MS spectra corresponding to unmodified chromophore-binding peptide S249 – K259 and to the same peptides with bound chromophores (Figure S4). Chromophore masses were calculated as differences between mass-to-charge ratios (m/z) of peptide fragments with and without modification, multiplied by charge (z).
Table S5, related to Table 1. Spectral properties of iRFP670, iRFP682 and their mutants.

<table>
<thead>
<tr>
<th>NIR FP</th>
<th>Natural BphP template</th>
<th>Absorbance maximum, nm</th>
<th>Excitation maximum, nm</th>
<th>Emission maximum, nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>iRFP670</td>
<td>RpBphP6</td>
<td>643</td>
<td>642</td>
<td>670</td>
</tr>
<tr>
<td>iRFP670/C10A</td>
<td>RpBphP6</td>
<td>641</td>
<td>639</td>
<td>669</td>
</tr>
<tr>
<td>iRFP670/C247S</td>
<td>RpBphP6</td>
<td>675</td>
<td>673</td>
<td>704</td>
</tr>
<tr>
<td>iRFP682</td>
<td>RpBphP2</td>
<td>663</td>
<td>660</td>
<td>682</td>
</tr>
<tr>
<td>iRFP682/C15S</td>
<td>RpBphP2</td>
<td>659</td>
<td>657</td>
<td>683</td>
</tr>
<tr>
<td>iRFP682/C254S</td>
<td>RpBphP2</td>
<td>694</td>
<td>692</td>
<td>714</td>
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</table>