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A Near-Infrared Photoswitchable Protein–Fluorophore Tag for No-Wash Live Cell Imaging

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Abstract

FR-1V, a fluorene-based aldehydic chromophore, binds its target protein as an imine to yield a highly bathochromic pigment, **CF-2**, a prototypic protein–dye tagging system whose NIR emission can be spatiotemporally switched ON by rapid UV-light activation. This is achieved through photoisomerization of the imine and its subsequent protonation. We demonstrate a no-wash protocol for live cell imaging of subcellular compartments in a variety of mammalian cell lines with minimal fluorescence background.

Keywords

confocal imaging; fluorene; NIR fluorescence; photoswitch; red-shifted fluorophores

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Conflict of interest

The authors declare no conflict of interest.

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Laser scan fluorescence microscopy (LSFM) is an indispensable tool for biological research,^[1] which is largely due to the availability of appropriate fluorescent proteins (FPs).^[2] FPs have an innate advantage as they can be genetically attached to proteins of interest (POIs) as fusion reporters to achieve extremely high labeling specificity. After decades of development, an arsenal of FPs is available with emissions that span almost the entire visible spectrum and possess high fluorescence brightness, giving end users a wide palette of choices. Recently, successful development of alternative phytochrome-based far-red/NIR FPs such as GAF-FP, iFPs, and iRFPs,^[3] has extended the excitation/emission wavelengths into the NIR-I regime (700–900 nm)^[4] such that deep-tissue fluorescence imaging becomes attainable. Another major advance has been the development of reversibly switchable FPs (RSFPs), such as Dronpa and Padron, with the ability to turn ON and OFF their fluorescence by light. These systems have led to applications in super-resolution microscopy (such as STORM/PALM).^[5] Despite the many advantageous features, FPs do not always exhibit large Stokes shifts, are oxygen-dependent, and need time to mature. Furthermore, FPs may induce POI mislocalization.^[6] With as many FPs developed thus far, it is difficult to integrate all these features, namely, far-red/NIR emission, large Stokes shift, photoswitchability, fast labeling kinetics, and small size, into a single variant.

In contrast to FPs, the photophysical properties of synthetic dyes are more tunable. A good example is the Janelia Fluors that have varied fluorescence lifetimes controlled as a result of changes in their twisted intramolecular charge transfer.^[7] Combination of synthetic dyes with various post-translationally modifiable protein/peptide tags have led to the development of site-selective chemical labeling systems.^[8] These include self-labeling enzymes (Halo, SNAP/ CLIP),^[8] protein affinity ligands (Y-FAST, TMP and FAP),^[8,9] bioorthogonal tags (CuAAC, SPAAC, IEDDA, and other click reactions),^[10] or mixed approaches (LpLA mediated PRIME,^[11] biarsenical FAsH/ReAsH,^[12] and dicysteine tag dC10*).^[13] These methods have greatly complemented FPs and have broadened the scope and utility of LSFM. Nonetheless, except for the biarsenics, the dC10* tags, and the PYP-tags,^[14] these approaches generally use non-fluorogenic fluorochromes (that is, always ON), hence requiring wash steps after staining to remove excess dye to increase signal-to-background ratio (SBR). Current no-wash strategies are generally based on PeT (photoinduced electron transfer) quenched fluorogens and quenched activity-based probes (qABPs).^[10,15] Successful examples include tetrazine-derived fluorogens,^[16] which require the expression of chemically modified non-canonical amino acids to facilitate the fluorescence activation. The fluorogenic qABPs are larger in size than their parent emitters, and their effectiveness relies on enzymatic activity. Recently, environment-sensitive dyeincorporated probes have been explored. Nonetheless, they mainly rely on polarity change and lack labeling ubiquity.^[17]

In this context, novel imaging tags, especially those that can incorporate multiple features into a single system, are in great demand. These features include: 1) high labeling specificity, to minimize nonspecific background; 2) far-red/ NIR emission, to obtain high tissue transparency; 3) high brightness, to increase image contrast; 4) large Stokes shift, to reduce self-quenching and further increase imaging depth; 5) high photostability, to prevent photobleaching; 6) ON/ OFF fluorogenicity, to enable spatiotemporal control; 7) fast labeling kinetics, to realize real-time imaging; 8) small size, to avoid perturbing target

functionality; and 9) minimal cytotoxicity. It is reasonable to envision that the latter goals are achievable with the use of protein/fluorophore combination that has the proper photophysical/photochemical properties.

Previously, we have disclosed the use of engineered human cellular retinol binding protein II (hCRBP_{II}) variants containing an active site lysine residue (Q108K) that reacts with synthetic aldehydic chromophores to form protonated (PSB) or neutral (SB) Schiff bases. hCRBP_{II} is a small (15 kDa) cytosolic protein, belonging to the intracellular lipid binding protein family, and has high tolerance to mutations without affecting its structure.^[18] These studies have led to a robust platform to design fluorescent complexes that have controlled emission profiles based on fluorophore-protein interactions. Similar to *cis-trans* isomerization observed in some RSFPs,^[5] engineered CRABP_{II} (cellular retinoic acid binding protein II), having a similar structure to hCRBP_{II} but a deeper binding cavity, exhibited *cis-trans* imine isomerization of bound all *trans*-retinal in both solution and crystals.^[19] As shown crystallographically, the *cis*-iminium (PSB) is first formed, followed by thermal isomerization to the *trans*-imine (SB). It was further demonstrated in single crystals that the same cycle could be driven photochemically, with green light irradiation driving the *cis*-iminium to the *trans*-imine, and UV irradiation driving the photocycle in the opposite direction (Figure 1a, retinylidene chromophore). These observations inspired a search for a fluorophore-hCRBP_{II} complex capable of recapitulating the imine/iminium photoisomerization, such that SB and PSB would have spectrally resolved absorption and emission peaks. We further speculated that the photochemical activation required for the PSB emission would suffice to make the complex fluorogenic in the confocal long-wavelength channel. Although other cellular proteins have the potential to bind the fluorophore as a SB, the photoisomerization event is specific to unique hCRBP_{II} mutants, and thus non-specific SB formation with non-targeted proteins is of no concern with regards to generating undesired background emission. This should constitute a no-wash protocol for live cell imaging.

To obtain large Stokes shift and long-wavelength emission, we synthesized dyes characterized by strong intramolecular charge transfer (ICT). In principle, ICT dyes are hyper-sensitive to changes in the local environment, owing to the highly polar nature of their excited states. The net result is that environmental polarity induces a wavelength shift in emission rather than absorption, leading to a large Stokes shift. The FR0 dye was first selected due to its strong ICT nature and exceptionally high fluorescence quantum yield.^[20] FR0 derivatives with extended conjugation between the fluorene and the aldehyde were synthesized to shift the emission wavelengths further to the red end. Prior to in vitro studies, we used n-butylamine as a surrogate for lysine to form SB with the derivatives and screen for the fluorophore with proper spectroscopic features. FR-1V (1V denoting one vinyl group) was selected because of its strong environmental response and high brightness (Supporting Information, Figure S1 and Table S1). To shift the emission wavelength even redder, the SB was acidified to obtain the PSB, further minimizing the HOMO-LUMO energy gap. Gratifyingly, the PSB emission maximum was in the NIR-I region (Supporting Information, Figure S2).

In search of an optimal protein for imaging purposes, we carried out site-directed mutagenesis of residues within 4 Å of the chromophore, based on a retinal-bound hCRBP II crystal structure (PDB ID: 4EXZ). Addition of **FR-1V** to psHex (wt-Q108K/K40L/T51V/T53S/Y19W/R58Y; Supporting Information, Figure S3) led initially to the formation of a mixture of SB ($\lambda_{\text{abs}} = 378$ nm) and PSB ($\lambda_{\text{abs}} = 600$ nm), which equilibrated to a final ratio of the two species, as seen in Figure 1b. Conversion to the SB under in vitro conditions exhibits a second-order rate constant of $264 \text{ m}^{-1} \text{ s}^{-1}$ at 23°C (Supporting Information, Figure S4). A short UV exposure (5 sec, ca. 365 nm) of this psHex-**FR-1V** complex (designated CrimFluor-1, **CF-1**) converts the thermodynamically equilibrated OFF state ($\lambda_{\text{abs/em}} = 378/445$ nm, Figure 1b) to a photoactivated ON state ($\lambda_{\text{abs/em}} = 600/686$ nm, Figure 1c). The two states can be interconverted repeatedly by UV (BP 300–400) and yellow light (LP 500) irradiation with good fatigue resistance (Figure 1d; Supporting Information, Figure S5). In contrast to the thermal conversion of the PSB (ON) to the SB (OFF), the light-assisted isomerization is about 50% faster (Figure 1e; Supporting Information, Figures S6–S8). This light-triggered OFF-to-ON switchability enables spatiotemporal control of activation for the NIR fluorescence of **CF-1**.

Next, the performance of **CF-1** as a no-wash live cell imaging system was investigated. psHex was expressed in HeLa, HEK293, and U2OS cell lines (Supporting Information, Figures S9, S10). Cells were stained with **FR-1V** for 5 min at 37°C and were imaged without any wash step. **CF-1** retained its photoswitching ability in cellulo. A 1 s 405 nm laser excitation led to a 10-fold increase of the PSB fluorescence in the confocal NIR channel (LP650). The NIR signal specificity was measured by fusing EGFP on the N-terminus of psHex and examining the colocalization of green and NIR fluorescence. This was demonstrated with constructs localized in nucleus, cytosol, and plasma membrane, via C-terminal fused signal peptides.^[11] In every triple-fused construct, the NIR emission demonstrated the same pixel specificity as that of EGFP (Supporting Information, Figure S11), confirming the purity of NIR fluorescence that is emitted from the activated **CF-1** without any signal contamination from non-specifically labeled **FR-1V**. Furthermore, **FR-1V** elicits minimal cytotoxicity at the working concentration ($2 \mu\text{M}$; Supporting Information, Figure S12). Assessed by size exclusion chromatography, psHex is monomeric in solution up to millimolar concentration (20 mg mL^{-1} ; Supporting Information, Figure S13). This is advantageous, assuming the monomeric state dominates in cellular environments as well. Furthermore, **CF-1** operates with less than 20% intensity loss across a range of physiologically relevant pHs (6 to 7.6; Supporting Information, Figure S14).

Having demonstrated proof of concept, we next examined the possibility to further engineer **CF-1** as a fluorogenic tag without any residual PSB in the OFF state, so that a true dark NIR channel would be available before photoswitching. This would greatly increase the contrast for spatiotemporal imaging. We hypothesized that installation of more hydrophobic residues in the vicinity of the embedded **FR-1V** would destabilize the iminium (cationic species). This would have two potential consequences: 1) lessening or complete suppression of the residual PSB absorption in the OFF state, and 2) an accelerated ON-to-OFF thermal switching rate, by increasing the electrostatic penalty for maintaining a charged species. Several rounds of altering the psHex sequence led to psNona (psHex-Y58W/T29L/Q38L/

Q128L; Supporting Information, Figure S3). As illustrated in Figures 1 f,g, the psNona-FR-1V complex (designated **CF-2**) showed negligible PSB absorption in its OFF state, while still displaying comparable absorption/emission maxima, QY, fatigue resistance, and the same labeling specificity to those of **CF-1** (Figure 1, 2b,c; Supporting Information, Figure S5d; Table 1). A welcomed difference was noticed in a 6-fold increase in the in vitro ON-to-OFF thermal switching rate, and a 3-fold increased rate of complex formation for **CF-2** in comparison to **CF-1** (Table 1; Supporting Information, Figure S15). As shown (Figure 2a,d; Supporting Information, Figure S16), a completely dark NIR channel is observed before photoswitching (achieving a mean SBR of 46, a maximum SBR of 94, and 35-fold increase in fluorescence contrast) along with spatiotemporal control of the ON state demonstrated at single cell resolution (see Figure 1a, **FR-1V** chromophore for its photocycle summary). **CF-2**'s faster in cellulo ON-to-OFF thermal switching rate also makes multiple rounds of ON/OFF NIR fluorescence practical (Supporting Information, Figures S17 and S18). Finally, it should be noted that the NIR brightness of both **CF-1** and **CF-2** far exceeds that reported for other far-red/NIR FPs (Table 2),^[3,21] with significantly improved Stokes shift.

To our knowledge, CrimFluors are the first reported photoswitchable NIR tags that employ both a synthetic dye and a fusion protein. The design of CrimFluors accomplishes three complimentary goals towards a desirable imaging platform, namely: 1) the use of ICT capable fluorophores, 2) generation of a bathochromically-shifted PSB, and 3) the ability to photoactivatably turn ON and OFF the fluorescence signal. The use of ICT capable fluorophores leads to back-ground-free no-wash NIR fluorescence as a result of the large bathochromic shift of the fluorophore upon protonation of the imine, which happens only when bound to the engineered proteins. Tested in different cell lines, **CF-1** and **CF-2** can be turned to the ON state by a rapid 405-nm laser irradiation. Both have good photostability and a large Stoke shift, enabling data collection with a 650 nm long pass filter. If optimal NIR brightness is required, then **CF-1** is a better choice. On the other hand, **CF-2** offers a complete dark state with a faster ON-to-OFF thermal switching rate. CrimFluors can complement current systems for live cell imaging and have potential utility for bright deep-tissue imaging. With the CrimFluor platform as a starting point, further optimization of the complex (both fluorophore and protein) are underway to extend fluorescence lifetime of the emissive PSB species (to increase quantum yield) and enhance the photoswitching rates in both directions to better suit super-resolution microscopy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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References

- [1]. Kusumi A, Tsunoyama TA, Hirose KM, Kasai RS, Fujiwara TK, Nat. Chem. Biol. 2014, 10, 524–532. [PubMed: 24937070]

- [2]. Chudakov DM, Matz MV, Lukyanov S, Lukyanov KA, *Physiol. Rev.* 2010, 90, 1103–1163. [PubMed: 20664080]
3. Chernov KG, Redchuk TA, Omelina ES, Verkhusha VV, *Chem. Rev.* 2017, 117, 6423–6446. [PubMed: 28401765]
4. a) Weissleder R, *Nat. Biotechnol.* 2001, 19, 316–317; [PubMed: 11283581] b) Hong GS, Antaris AL, Dai HJ, *Nat. Biomed. Eng.* 2017, 1, 0010.
5. Zhou XX, Lin MZ, *Curr. Opin. Chem. Biol.* 2013, 17, 682–690. [PubMed: 23876529]
6. Landgraf D, Okumus B, Chien P, Baker TA, Paulsson J, *Nat. Methods* 2012, 9, 480–482. [PubMed: 22484850]
7. a) Grimm JB, English BP, Chen J, Slaughter JP, Zhang Z, Revyakin A, Patel R, Macklin JJ, Normanno D, Singer RH, Lionnet T, Lavis LD, *Nat. Methods* 2015, 12, 244–250; [PubMed: 25599551] b) Grimm JB, Muthusamy AK, Liang Y, Brown TA, Lemon WC, Patel R, Lu R, Macklin JJ, Keller PJ, Ji N, Lavis LD, *Nat. Methods* 2017, 14, 987–994. [PubMed: 28869757]
8. Dean KM, Palmer AE, *Nat. Chem. Biol.* 2014, 10, 512–523. [PubMed: 24937069]
9. a) Plamont M-A, Billon-Denis E, Maurin S, Gauron C, Pimenta FM, Specht CG, Shi J, Quérard J, Pan B, Rossignol J, Moncoq K, Morellet N, Volovitch M, Lescop E, Chen Y, Triller A, Vríz S, Le Saux T, Jullien L, Gautier A, *Proc. Natl. Acad. Sci. USA* 2016, 113, 497–502; [PubMed: 26711992] b) Szent-Gyorgyi C, Schmidt BF, Creeger Y, Fisher GW, Zakel KL, Adler S, Fitzpatrick JAJ, Woolford CA, Yan Q, Vasilev KV, Berget PB, Bruchez MP, Jarvik JW, Waggoner A, *Nat. Biotechnol.* 2008, 26, 235–240. [PubMed: 18157118]
10. Lang K, Chin JW, *Chem. Rev.* 2014, 114, 4764–4806. [PubMed: 24655057]
11. Uttamapinant C, White KA, Baruah H, Thompson S, Fernández-Suárez M, Puthenveetil S, Ting AY, *Proc. Natl. Acad. Sci. USA* 2010, 107, 10914–10919. [PubMed: 20534555]
12. a) Adams SR, Tsien RY, *Nat. Protoc.* 2008, 3, 1527; [PubMed: 18772880] b) Griffin BA, Adams SR, Tsien RY, *Science* 1998, 281, 269–272; [PubMed: 9657724] c) Hoffmann C, Gaietta G, Zgrn A, Adams SR, Terrillon S, Ellisman MH, Tsien RY, Lohse MJ, *Nat. Protoc.* 2010, 5, 1666. [PubMed: 20885379]
13. a) Chen Y, Clouthier CM, Tsao K, Strmiskova M, Lachance H, Keillor JW, *Angew. Chem. Int. Ed.* 2014, 53, 13785–13788; *Angew. Chem.* 2014, 126, 14005–14008; b) Chen Y, Tsao K, Acton SL, Keillor JW, *Angew. Chem. Int. Ed.* 2018, 57, 12390–12394; *Angew. Chem.* 2018, 130, 12570–12574.
14. Mizukami S, Hori Y, Kikuchi K, *Acc. Chem. Res.* 2014, 47, 247–256. [PubMed: 23927788]
15. a) Blum G, Mullins SR, Keren K, Fonvi M, Jedszko C, Rice MJ, Sloane BF, Bogoy M, *Nat. Chem. Biol.* 2005, 1, 203–209; [PubMed: 16408036] b) Nadler A, Schultz C, *Angew. Chem. Int. Ed.* 2013, 52, 2408–2410; *Angew. Chem.* 2013, 125, 2466–2469.
16. a) Devaraj NK, Weissleder R, *Acc. Chem. Res.* 2011, 44, 816–827; [PubMed: 21627112] b) Kormos A, Koehler C, Fodor EA, Rutkai ZR, Martin ME, Mezo G, E. A. Lemke, P. Kele, *Chem. Eur. J.* 2018, 24, 8841–8847. [PubMed: 29676491]
17. Klymchenko AS, *Acc. Chem. Res.* 2017, 50, 366–375. [PubMed: 28067047]
18. Wang W, Nossoni Z, Berbasova T, Watson CT, Yapici I, Lee KSS, Vasileiou C, Geiger JH, Borhan B, *Science* 2012, 338, 1340–1343. [PubMed: 23224553]
19. a) Berbasova T, Santos EM, Nosrati M, Vasileiou C, Geiger JH, Borhan B, *ChemBioChem* 2016, 17, 407–414; [PubMed: 26684483] b) Nosrati M, Berbasova T, Vasileiou C, Borhan B, Geiger JH, *J. Am. Chem. Soc.* 2016, 138, 8802–8808. [PubMed: 27310917]
20. Kucharak OA, Didier P, Y. M8ly, A. S. Klymchenko, *J. Phys. Chem. Lett.* 2010, 1, 616–620.
21. a) Bindels DS, Haarbosch L, van Weeren L, Postma M, Wieser KE, Mastop M, Aumonier S, Gotthard G, Royant A, Hink MA, Gadella TWJ, *Nat. Methods* 2017, 14, 53–56; [PubMed: 27869816] b) Shcherbo D, Murphy CS, Ermakova GV, Solovieva EA, Chepurnykh TV, Shcheglov AS, Verkhusha VV, Pletnev VZ, Hazelwood KL, Roche PM, Lukyanov S, Zarsky AG, Davidson MW, Chudakov DM, *Biochem. J.* 2009, 418, 567–574; [PubMed: 19143658] c) Shcherbo D, Shemiakina II, Ryabova AV, Luker KE, Schmidt BT, Souslova EA, Gorodnicheva TV, Strukova L, Shidlovskiy KM, Britanova OV, Zarsky AG, Lukyanov KA, Loschenov VB, Luker GD, Chudakov DM, *Nat. Methods* 2010, 7, 827–829. [PubMed: 20818379]

Figure 1.

a) Representation of chromophore-hCRBP/CRABP photoisomerization cycle. The imines in the photocycle are the product of retinal or **FR-1V**, bound to their respective protein hosts, via imine formation with an active Lys residue (Lys111 of hCRBP for retinal, and Lys108 of hCRBP for **FR-1V**). Absorbance and emission spectra of **CF-1** (complex of **FR-1V** with hCRBP-Q108K/K40L/T51V/T53S/Y19W/R58Y hexamutant, psHex), b) OFF and c) ON states (at pH 7.3 in PBS buffer, excited at absorption maxima). d) Photoswitching fatigue resistance of **CF-1** monitored at OFF and ON emission maxima. Alternate irradiation steps were illuminated using an Arc lamp (Hg(Xe), Oriol), with UV light (U-360 UV Bandpass filter) for 20 s, and yellow light (Y-50 500 nm Longpass filter) for 15 min, in non-degassed solutions. 0.6% absorption and 1% emission intensities were observed decreasing per cycle. e) Thermal (22°C) vs. yellow light-assisted (Y-50 filter) switching of **CF-1** ON-to-OFF conversion, monitored at ON-state absorption maximum. Data points collected at 3 min interval. Absorbance and emission spectra of **CF-2** (complex of **FR-1V** with psHex-Y58W/T29L/Q38L/Q128L, psNona), f) OFF and g) ON states (at pH 7.3 in PBS buffer, excited at absorption maxima).

Figure 2.

a) Compartmentalized **CF-2** labeling in HeLa cells, with N-terminal fused EGFP and C-terminal signal peptides. EGFP channel: BP 505–530. **CF-2** channel: LP650. NLS=nuclear localization sequence. NES=nuclear export sequence. CAAX=prenylation tag. **FR-1V**=2 μm. Scale bars: 20 μm. b),c) Labeling specificity of nuclei-localized CF-2 in HeLa cells. b) Top left: EGFP channel; top right: **CF-2**_ON channel, circles and number indicating mean SBR (n=440); bottom: merged channels+DIC. c) Line profiles of fluorescence intensities in two channels along the blue arrow in (b). Scale bars: 20 μm. d) Confocal imaging of HeLa cells, expressing nuclei-localized hCRBP II fused with EGFP, stained with FR-1V (2 μm) for 5 min, no wash. Red channel: **CF-2**_ON. Following the arrows: before switch-on; 1st cell switch-on; 2nd cell switch-on; 3rd cell switch-on; whole field switch-on. Compare with green channel: reference EGFP. Scale bars: 10 μm. For channels in all images: EGFP, λ_{ex} =488 nm (2.80 μW), BP 505–530; **CF-2**_ON, λ_{switch} =405 nm (4.91 μW), λ_{ex} =594 nm (1.63 μW), LP 650; DIC, λ_{ex} =488 nm (2.80 μW).

Table 1:Characteristics of **CF-1** (psHex) and **CF-2** (psNona).

Mutant	SB $\lambda_{\text{abs}}/\lambda_{\text{em}}$	PSB $\lambda_{\text{abs}}/\lambda_{\text{em}}$	QY ^[a] _{SB}	QY ^[a] _{PSB}	Binding k [M ⁻¹ S ⁻¹] ^[b]	ON→OFF $t_{1/2}$ [min] ^[c]
psHex	378/445	600/686	0.46	0.16	264	7.4
psNona	382/440	604/684	0.52	0.13	759	1.2

^[a] Absolute quantum yields measured at pH 7.3.^[b] Second-order rate constants of **FR-1V** and hCRBP_{II} complexation.^[c] Half-times were measured in dark (thermal conversion) at 23°C.

Table 2:

Comparison of *CrimFluors* with other far-red/NIR FPs.

	mCherry	mKate2	mNeptune	IFP1.4 ^[a]	iRFP682 ^[a]	mIFP	CF-1 (ON)	CF-2 (ON)
Excitation peak [nm]	587	587	599	684	663	683	600	604
Emission peak [nm]	610	631	649	708	682	704	686	684
Fluorescence QY	0.22	0.39	0.18	0.07	0.11	0.08	0.16 ^[b]	0.13 ^[b]
ϵ_{max} [M ⁻¹ cm ⁻¹]	72000	63000	57500	92000	90000	82000	50500	35700
Brightness (a.u.) ^[c]	15840	24570	10350	6440	9900	6560	8080	4641
ϵ_{635} [M ⁻¹ cm ⁻¹]	1000	–	7900	–	–	–	37550	27940
$\Phi(700-900 \text{ nm})$ ^[d]	0.04	–	0.05	–	–	–	0.07	0.05
Brightness in NIR [a.u.] ^[e]	40	87 ^[f]	395	–	–	–	2630	1400
Reference	[21a]	[21b,c]	[21a]	[3]	[3]	[3]	this work	this work

^[a]Dimer.^[b]Absolute quantum yield measured in PBS (pH 7.3).^[c]Calculated as the product of molar extinction coefficient and quantum yield.^[d]Calculated from the emission fraction between 700 nm and 900 nm.^[e]Calculated as the product of molar extinction coefficient at 635 nm and quantum yield in NIR-I (700–900 nm). Data of mCherry and mNeptune are reported in Ref. [21c].^[f]Calculated from the brightness ratio (beyond 650 nm) to mCherry, as reported in Ref. [21b].