Recent progress in fluorescent protein development has generated a large diversity of near-infrared fluorescent proteins (NIR FPs), which are rapidly becoming popular probes for a variety of imaging applications. However, the diversity of NIR FPs poses a challenge for end-users in choosing the optimal one for a given application. Here we conducted a systematic and quantitative assessment of intracellular brightness, photostability, oligomeric state, chemical stability and cytotoxicity of 22 NIR FPs in cultured mammalian cells and primary mouse neurons and identified a set of top-performing FPs including emiRFP670, miRFP680, miRFP713 and miRFP720, which can cover a majority of imaging applications. The top-performing proteins were further validated for in vivo imaging of neurons in Caenorhabditis elegans, zebrafish, and mice as well as in mice liver. We also assessed the applicability of the selected NIR FPs for multicolor imaging of fusions, expansion microscopy and two-photon imaging.

Fluorescent proteins (FPs) quickly became ubiquitous tools for optical imaging after the cloning of the green FP (GFP) from jellyfish Aequorea victoria in 1992 (ref. 1). Expanding spectral diversity of FPs into the near-infrared (NIR) range (~650–900 nm) of the electromagnetic spectrum enabled new imaging capabilities ranging from multiplexed super-resolution imaging of live cells to whole-body in vivo imaging. The red-shifted fluorescence of NIR FPs enables efficient excitation with standard red lasers (630–640 nm), widely used light sources in microscopy, to unlock the standard Cy5 filter set for FPs, which was previously used only for red-shifted dyes imaging. Correspondingly, NIR FPs can be readily imaged with other conventional FPs such as GFPs and red FPs (RFPs), enabling multiplexed structural and functional imaging. Further development of monomeric NIR FPs enabled their application as fluorescent tags for subcellular structures in cultured cells under conventional and super-resolution microscopy. In addition, NIR FPs are particularly beneficial for in vivo imaging of model organisms due to reduced autofluorescence, low light scattering, and minimal absorbance of tissue at longer wavelengths.

The unique photophysical properties of NIR FPs make them compatible with multiphoton microscopy and photoacoustic imaging, thus further increasing the utility of these probes for in vivo imaging of mammals. However, the majority of currently available NIR FPs are derived from bacteriophytochromes and cyanobacteriochromes, which utilize a linear tetrapyrrole biliverdin IXα as a chromophore. This feature makes the fluorescence of bacteriophytochrome photo-receptor (BphP)-based NIR FPs dependent on the heme metabolism in host cells. As a result, the performance of BphP-based NIR FPs in cultured cells and in vivo preparations cannot be predicted based on the in vitro characteristics measured on proteins purified from bacteria, which is the most common way to select FPs for an imaging experiment. Further development of NIR FPs enabled the application as fluorescent tags for subcellular structures in cultured cells under conventional and super-resolution microscopy. However, intracellular brightness of BphP-based NIR FPs in one cell type is not necessarily retained in another cell type. Altogether, this creates a need to test multiple NIR FPs in a particular preparation before selecting the one suitable for the desired application. To assist end-users to select the right NIR FPs, we performed a careful side-by-side quantitative assessment of the most popular NIR FPs.

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FPs in HEK cells and primary neurons, characterizing their intracellular brightness, photostability, oligomeric state, chemical stability and cytotoxicity. Furthermore, the top-performing NIR FPs were expressed in vivo in Caenorhabditis elegans, zebrafish and mice for a side-by-side comparison of intracellular brightness and photostability. In addition, the proteins with the highest performance were tested for multicolor imaging and validated for expansion microscopy (by quantifying fluorescence retention in cultured cells) and two-photon imaging (by measuring action cross-section spectra).

Results
Characterization of NIR FPs in cultured mammalian cells
To perform the quantitative assessment in mammalian cells, we selected 22 NIR FPs: 19 biliverdin IXα-binding FPs and three GFP-like FPs (Extended Data Table 1, see Supplementary Note 1 for the selection criteria). The selected NIR FPs span more than 70 nm of the visible spectrum, and thus they can be subdivided into two spectral groups matching the Cy5 or Cy5.5 filter set with red (630–640 nm) or NIR (680 nm) excitation lasers, respectively (see Methods for the exact specification of the Cy5 and Cy5.5 filters). In this study we also used the Cy5-LP filter (with a 664 nm long-pass emission filter that enables the collection of as much NIR fluorescence as possible) and the Cy5-BP filter (with a 679/41 nm narrow bandpass filter to improve spectral separation with the Cy5.5 filter), which are the two most common emission filters for red lasers on standard imaging set-ups. We used a wide-field microscope equipped with all three filter sets to characterize intracellular brightness and photostability of the selected NIR FPs expressed under the CAG promoter in cultured HEK cells and primary mouse neurons. Quantification of intracellular brightness was done by normalization of the NIR fluorescence by fluorescence of the GFP co-expressed via P2A self-cleavage peptide, given that this approach was characterized by superior performance for brightness normalization compared with other co-expression strategies such as IRES2 and direct fusion of two FPs (Supplementary Note 2, Supplementary Table 1).

All proteins were evenly localized both in the cytoplasm and nucleus of HEK cells except for E2-Crimson, which was excluded from the nucleus due to its tetrameric state (Supplementary Fig. 2). In live cultured neurons the fluorescence of the NIR FPs was evenly distributed in the cytosol, individual dendrites and nucleus without any noticeable aggregation or non-specific localization except for iRFP670, which showed small puncta in neuropils in both the Cy5 and green channels (Supplementary Fig. 3). Quantification of intracellular brightness and photostability identified the brightest and most photostable NIR FPs in each channel when expressed in HEK cells and neurons (Fig. 1a–f, Extended Data Fig. 1 and Supplementary Table 3 for HEK cells, and Extended Data Fig. 2 and Supplementary Table 4 for neurons, also see Supplementary Table 2 and Supplementary Dataset 1 for detailed descriptive statistics and statistical analysis). Further analysis showed low correlation in the relative brightness of NIR FPs between HEK cells and primary neurons, characterized by linear regression coefficients of 0.09, 0.27 and 0.33 for the Cy5-LP, Cy5-BP and Cy5.5 channels, respectively (Supplementary Fig. 4). The most notable was the low relative brightness of GFP-like NIR FPs in neurons compared with HEK cells. For example, while mCardinal and E2-Crimson were the brightest FPs in the Cy5-LP channel in live HEK cells, they ranked only 15th and 11th, respectively, in cultured neurons. In contrast, the relative brightness of miRFP713 and miRFP720 was 3.6- and 3.4-fold higher in neurons than in HEK cells. The emiRFP670, miRFP670-2 and miRFP680 proteins exhibited consistent brightness in both cell types and were among the top five FPs in Cy5 channels (Fig. 1a and Extended Data Fig. 1). We also noted a low correlation of intracellular brightness and effective molecular brightness (Supplementary Fig. 4). Use of exogenous biliverdin IXα at 25 μM increased intracellular brightness of all biliverdin IXα-binding NIR FPs by, on average, twofold in HEK cells and fivefold in neurons (Extended Data Fig. 1 and Supplementary Table 3 for HEK cells and Extended Data Fig. 2 and Supplementary Table 4 for neurons). Given that we encountered situations in which some NIR FPs had a high NIR-to-green fluorescence ratio but at the same time low absolute NIR fluorescence intensity (for example, compare BDPL1.6 and emiRFP670 in Supplementary Figs. 2 and 3), we conducted additional quality control experiments using western blot analysis as well as mass spectrometry for selected NIR FPs. According to western blot results, cleavage efficiency was more than 90% while the molecular ratio of NIR FP to GFP varied from 1.3 to 1.1 for most of the constructs (the mRFP713-P2A-GFP construct was an outlier with a 6.1 ratio) (Supplementary Fig. 5). Mass spectrometry analysis showed multiple post-translation modifications of the selected NIR FPs (Supplementary Fig. 6). To verify whether high intracellular brightness was associated with increased cytotoxicity, we determined the fraction of apoptotic and dead HEK cells at 48 hours after transient transfection. The cytotoxicity of the top five brightest NIR FPs from three spectral channels was comparable to the negative control (dummy plasmid transfection), while mCardinal, E2-Crimson, iRFP670 and SNIFP produced an approximately 1.5-fold larger fraction of apoptotic cells than the negative control (Supplementary Table 5 and Supplementary Fig. 7). smURFP and miRFP2 produced the largest fraction of apoptotic cells (>5-fold than the control) in the test NIR FPs.

Photobleaching rates measured under matching light powers in the Cy5 and Cy5.5 channels were consistent between HEK cells and neurons for all NIR FPs, with linear regression coefficients of >0.94 (Supplementary Fig. 4). On average, photobleaching half-times in neurons were 20–40% longer than in HEK cells except for E2-Crimson, smURFP, BDPL1.8 and miRFP713, which were slightly less photostable in neurons than in HEK cells (Fig. 1b,e, also see Extended Data Fig. 1 and Supplementary Table 3 for HEK cells and Extended Data Fig. 2 and Supplementary Table 4 for neurons). There was a more than 22-fold difference in photobleaching rates between the least photostable E2-Crimson and the most photostable miRhubarb713 both in HEK cells and neurons. Interestingly, the intracellular brightness in HEK cells had a moderately negative correlation with photostability (Pearson's coefficients, −0.46 and −0.5 in the Cy5-LP and Cy5.5 channels, respectively), while brightness and photostability in neurons were not correlated (Pearson's coefficients, 0.06 and 0.27 in the Cy5-LP and Cy5.5 channels, respectively; Supplementary Fig. 4). Given that brightness and photostability are the major considerations for choosing FPs, we compared the overall performance of NIR FPs using the product of intracellular brightness and photostability, and then selected the five variants with the highest such products for each tested channel for further validation in vivo (Fig. 1c,f, Extended Data Figs. 1 and 2 and Supplementary Table 6).

Chemical fixation with paraformaldehyde (PFA) is widely used for biological sample preservation and FPs that retain bright and photostable fluorescence after PFA fixation would be beneficial for structural fluorescence imaging. To assess the applicability of NIR FPs for imaging in chemically fixed samples, we measured their intracellular brightness, fluorescence retention and intracellular photostability in PFA-fixed HEK cells (Fig. 1g–i, Extended Data Fig. 3, Supplementary Table 7 and Supplementary Fig. 8). The top five NIR FPs by brightness in fixed HEK cells mainly overlapped with that in live HEK cells, except that mCardinal and miRFP670-2 were replaced by iRFP670 and miRFP720 (Fig. 1g). All biliverdin IXα-binding NIR FPs retained more than 60% of their fluorescence after fixation, with miRFP2 > BDPL1.5 > SNIFP > mRFP680 > smURFP being the most stable (Extended Data Fig. 3f). The GFP-like NIR FPs mNeptune2,5 and mCardinal appeared to be among the least stable, retaining only 45% and 26% of the fluorescence, respectively (Supplementary Table 7), while GFP preserved approximately 85% of its fluorescence. Photostability in fixed HEK cells was on average 30–34% higher than in live HEK cells (Fig. 1h and Supplementary Tables 3 and 7). To further validate the chemical stability of NIR FPs, we used the protein retention expansion microscopy protocol to process HEK cells.
**Fig. 1 | Quantitative characterization of NIR FPs in cultured mammalian cells.**

**a.** Normalized brightness of NIR FPs in live HEK cells imaged in the Cy5-LP channel (n > 1,945 cells for each NIR FP from three independent transfections). Brightness for each FP was normalized to the EGFP signal (used throughout the figure). Daggers (†) indicate signal-to-background ratio <2.0 throughout.

**b.** Photobleaching half-times of NIR FPs in live HEK cells under Cy5 excitation (n > 93 cells for each NIR FP from four independent transfections). Single value product of mean brightness and mean photobleaching half-time in live HEK cells. Normalized brightness of NIR FPs in live HEK cells. Excitation power for brightness imaging, 55 mW mm⁻²; for photostability measurements in live HEK cells, 55 mW mm⁻²; in live neurons and fixed HEK cells, 55 mW mm⁻². Box plots with notches: narrow part of notch, median; top and bottom of the notch, 95% confidence interval for the median; top and bottom horizontal lines, 25% and 75% percentiles for the data; whiskers extend 1.5-fold the interquartile range from the 25th and 75th percentiles; horizontal line, mean; outliers not shown but are included in all calculations and available in the source datasets. See Statistics and Reproducibility. Supplementary Table 2, Supplementary Data for detailed statistics and exact P values.
Table 1 | Quantification of NIR FP monomeric state in live HeLa cells using OSER assay

| Protein               | MFI : NE MFI (%) | Normal cells (%) | Product relative to miRFP720 (%) |
|-----------------------|------------------|------------------|----------------------------------|
| mNeptune2.5           | 4.4 ± 0.9        | 76.0 ± 5.9       | 34.1                             |
| mCardinal             | 3.3 ± 0.6        | 70.0 ± 2.4       | 41.9                             |
| E2-Crimson            | 4.6 ± 0.3        | 1.9 ± 1.3        | 0.8                              |
| smURFP                | NA               | NA               | NA                               |
| BDFP1.6               | 2.4 ± 0.5        | 74.7 ± 3.1       | 61.5                             |
| emiRFP670             | 2.6 ± 0.2        | 72.4 ± 8.3       | 55.0                             |
| miRFP670-2            | 1.9 ± 0.1        | 66.4 ± 6.2       | 69.0                             |
| irFP670               | 1.6 ± 0.1        | 68.7 ± 1.8       | 84.8                             |
| miRFP680              | 1.8 ± 0.1        | 78.7 ± 9.3       | 86.4                             |
| irFP682               | 2.3 ± 0.1        | 70.9 ± 3.5       | 60.9                             |
| emiRFP703             | 2.1 ± 0.2        | 72.8 ± 4.7       | 68.5                             |
| BDFP1.8               | 2.1 ± 0.1        | 60.8 ± 6.6       | 57.2                             |
| miRFP2                | NA               | 80.5 ± 6.7       | NA                               |
| mIFP                  | NA               | NA               | NA                               |
| IFP2                  | NA               | 86.5 ± 3.3       | NA                               |
| mRhubarb713           | 2.3 ± 1.1        | 75.6 ± 9.9       | 64.9                             |
| miRFP713              | 1.9 ± 0.1        | 80.8 ± 5.6       | 84.0                             |
| irFP713               | 2.4 ± 1.8        | 48.5 ± 4.8       | 39.9                             |
| SNIFP                 | 2.8 ± 0.4        | 69.8 ± 8.3       | 49.2                             |
| mRhubarb719           | NA               | NA               | NA                               |
| mRhubarb720           | NA               | 70.3 ± 8.1       | NA                               |
| miRFP720              | 1.6 ± 0.1        | 81.0 ± 5.6       | 100                              |

Data given as mean ± s.e.m. NA, not applicable. Calculated using <10 cells due to few bright cells with whorls. *CyERM fusion is either too dim or mislocalized. Calculated using 100–1000 cells. Calculated using <1,000 cells. Less than 10 cells. 10–100 cells. Calculated as the product of the reciprocal of the MFI : NE MFI value and the fraction of normal cells.

expressing emiRFP670, miRFP680, emiRFP682, emiRFP703, BDFP1.8, miRFP713, iRFP713 and miRFP720, which possessed the highest proportion of absolute NIR fluorescence and photo-stability in the Cy5 and/or Cy5.5 channels (Fig. 1i and Extended Data Fig. 3d). The tested NIR FPs had a >3-fold lower fluorescence retention than GFP except for BDFP1.8, which preserved ~20% of its fixed fluorescence intensity versus 33% for GFP (Extended Data Fig. 3e,g,h). These results demonstrate that the biliverdin IXα-binding NIR FPs preserve brightness and photo-stability in PFA-fixed samples, although their chemical stability was limited in the expansion microscopy preparations.

The oligomeric state of NIR FPs was evaluated based on their performance in CytéRM fusion in live HeLa cells. We calculated two parameters, the ratio of whorl structure monomeric fluorescent intensity (MFI) to nuclear envelope (NE) MFI (MFI : NE MFI), and the fraction of transfected cells without visible OSER (organized smooth endoplasmic reticulum) whorl structures, which were previously proposed for the quantification of the oligomerization tendency of FPs in live mammalian cells22,23 (see Supplementary Figs. 9 and 10 for representative images). The top five proteins in intensity assay were miRFP703, irFP670, miRFP680, miRFP670-2 and miRFP713, while in the percentage assay they were miRFP703, miRFP713, iRFP670, miNeptune2.5 and mRhubarb713 (Table 1). Several NIR FPs (smURFP, miRFP72, miFP, IFP2, mRhubarb719 and mRhubarb720) were either too dim or did not form classical whorl structures and thus were excluded from further assessment. However, our final selection of monomeric NIR FPs was not based on either assay alone. The MFI : NE MFI ratio threshold set by Costantini et al. was based on the GFP-like proteins and thus may require additional validation for biliverdin IXα-binding FPs24. For example, in the present study emiRFP703 and BDFP1.8 scored the same mean MFI : NE MFI ratio of 2.1, even though emiRFP703 was characterized as a monomer through analytical centrifugation11 and BDFP1.8 as a dimer through size-exclusion chromatography25. In another study using OSER assay for GFP-like FPs, Cranfill et al. set the threshold for the percentage of normal cells at 90%22, which was not achieved by any of the tested NIR FPs in the present study. Moreover, we noted a low positive correlation between the fraction of normal cells and the MFI : NE MFI ratio of 2.1, even though emiRFP703 was characterized as that of well-established monomeric GFP-like FPs, including mCherry, mRuby, mNeonGreen and so on22,26, suggesting that OSER assay was valid for biliverdin IXα-binding FPs.

In vitro characterization of selected NIR fluorescent proteins

The top-performing NIR FPs in neurons, namely emiRFP670, miRFP670-2, miRFP680, mRhubarb713, miRFP713, iRFP713 and miRFP720, were expressed in Escherichia coli, purified according to a standard protocol, and used for spectroscopic and biochemical characterization in PBS at pH 7.4. The mCardinal protein, which was not among the best performers in neurons, was included as an additional control because it does not require biliverdin IXα. We measured absorbance and steady-state fluorescence spectra (Supplementary Fig. II), extinction coefficients, quantum yields, fluorescence lifetime (Extended Data Table 2) and fluorescence pKₐ (the pH at which fluorescence intensity drops to 50% of its maximum value; Supplementary Fig. 12). Overall, the obtained values matched those previously reported with some minor deviations (Extended Data Table 2). Extinction coefficients calculated using the Strickler–Berg equation were on average 70% lower than those calculated based on the Soret band absorbance, although extinction coefficients for mCardinal measured using alkaline denaturation and calculation with the Strickler–Berg equation matched closely (Extended Data Table 2).

Previously we demonstrated that second-generation dimeric BphP-based NIR FPs can be used for dual-color multiphoton imaging using the standard titanium–sapphire laser27,28. To facilitate application of the top-performing NIR FPs for intravital multiphoton imaging we characterized their two-photon cross-section spectra in the range 800–1,300 nm (Extended Data Fig. 5). The recorded spectra had 7–10-fold larger absolute cross-sectional values at the Soret band (800–950 nm) than at the Q-band (1,100–1,300 nm), matching the spectral profiles of other BphP-based NIR FPs29. However, the absolute value of the cross-sections was approximately twofold smaller than our previously published data, which we attributed to the reduced value of the cross-sections was approximately twofold smaller than our previously published data, which we attributed to the reduced value of the cross-sections was approximately twofold smaller than our previously published data, which we attributed to the reduced.

Characterization in model organisms

Red-shifted spectra of biliverdin IXα-binding FPs are particularly beneficial for in vivo imaging due to low background autofluorescence,
reduced light scattering, and minimal absorbance of tissue in the NIR range of the spectrum. For quantitative assessment in vivo we expressed selected NIR FPs in neurons in mice, zebrafish and C. elegans, which are among the most popular model organisms in life science research. We also tested selected NIR FPs in liver in mice to evaluate tissue-specific variability of NIR FP performance. Given that the primary cell type for in vivo assessment was neurons, we chose to use all top five performing NIR FPs from three spectral channels (some proteins were among the best performing in more than one channel, resulting in a total of seven proteins) in neuronal culture, namely emiRFP670, miRFP670-2, miRFP680, mRhubarb713, miRFP713, iRFP713 and miRFP720 (Supplementary Table 4). Because the best-performing NIR FPs were BphP-based, we included the GFP-like NIR FP mCatalyst as a reference for biliverdin IXα availability in vivo. In addition, emiRFP2, an enhanced version of miRFP2 (ref. 30), was assessed in zebrafish.

To express selected NIR FPs in the cortex of mouse brain, we injected the rAAV2/9-CAG-NIR-FPs-P2A-GFP viruses into mouse brain at the neonatal stage and performed imaging of acute brain slices at 1 month, 2 months and 3 months after injection, and of fixed brain tissue at 1 month after injection. First, we acquired structural images of fixed brain slices from 1-month-old mice using confocal microscopy and qualitatively compared the co-localization of NIR FP and GFP fluorescence (Fig. 2a and Supplementary Fig. 13 for representative images at lower magnification). The emiRFP670 protein exhibited excellent localization in cortical neurons, enabling better visualization of small neuropil compared with GFP. The other NIR FPs provided a lower quality of neuropil visualization than GFP, with miRFP680 and miRFP713 showing significant aggregation. Minor aggregation in neuropil was observed for mCatalyst and mRhubarb713. Moreover, miRFP680, mRhubarb713, miRFP713, iRFP713 and miRFP720 exhibited noticeable nuclear accumulation, which was most prominent for miRFP713. Intra-cellular brightness measured at neuronal somas in the Cy5-LP channel achieved a maximum at the 2 month time point for all NIR FPs except for mRhubarb713 (Fig. 2b–d). The miRFP680 protein had superior brightness in both the Cy5-LP and Cy5-BP channels, outperforming other NIR FPs by ≥1.8-fold. In turn, miRFP720 was the brightest NIR FP in the Cy5.5 channel at the 1 month and 2 month time points, and miRFP713 at the 3 month time point (Extended Data Fig. 6 and Supplementary Table 8). The fluorescence of BphP-based NIR FPs was retained well in PFA-fixed brain tissue while mCatalyst fluorescence diminished by ~4-fold, reminiscent of that measured in HEK cells (Fig. 2e and Extended Data Fig. 6). miRFP713 had superior photostability in both acute and fixed brain slices, however, the photostability of the brightest NIR FP, miRFP680, was reduced by sixfold upon PFA fixation. In contrast, the photostability of emiRFP670 was ~1.8-fold higher in fixed tissue than in live tissue (Fig. 2f,g, Extended Data Fig. 6 and Supplementary Table 9).

Despite their superior brightness and photostability, miRFP680 and miRFP713 were not optimal for neuron imaging in mice due to their poor neuronal labeling in vivo. To validate their performance in other cell types and tissues, we expressed selected NIR FPs in mouse liver using the same adeno-associated viruses (AAVs) as were used for expression in the brain. Quantification of brightness in live liver tissue showed a similar ranking by brightness to that observed in the cortical neurons at the 2 month time point, although the differences in relative brightness between the NIR FPs were less significant and mCatalyst appeared to be the dimmest NIR FP (Extended Data Fig. 7 and Supplementary Table 8). Correspondingly, miRFP680 was the brightest NIR FP in the Cy5-LP and Cy5-BP channels, while miRFP720 was brightest in the Cy5.5 channel. Importantly, within the limitations of wide-field imaging in thick tissue, we did not observe accumulation of NIR FPs in the cell nucleus (Extended Data Fig. 7a).

Similar to mammals, the heme metabolism in zebrafish involves the formation of biliverdin IXa by the HMox1a and HMox1b enzymes homologous to heme oxygenase 1 (HO1)32. Furthermore, we previously demonstrated that the BphP-based NIR FPs can be visualized in zebrafish larvae without co-expression of recombinant HO1 (refs. 28, 30). However, it is likely that biliverdin IXa concentration in zebrafish is a limiting factor for the intracellular brightness of BphP-based NIR FPs, because their fluorescence can be significantly enhanced by co-expression of HO1 (ref. 18). We therefore decided to investigate the effect of heterologous HO1 on intracellular brightness and photostability of BphP-based NIR FPs in zebrafish by co-expressing either functional wild-type HO1 (wtHO1) or its enzymatically inactive variant (HO1-H25A) together with each NIR FP, while mCatalyst was used for brightness normalization. To enable co-expression of the proteins of interest across the nervous system in zebrafish larvae, we used expression constructs, which carried the genes of NIR FPs fused to P2A-mCatalyst and wtHO1 or HO1-H25A under a bidirectional promoter controlled by the neuron-specific Gal4 transcription factor encoded in the same vector (Supplementary Fig. 14). The hindbrain and spinal cord regions of live zebrafish larvae at 4 days post-fertilization (dpf) were imaged in the Cy5-LP channel under a confocal scanning microscope to assess the intracellular brightness and photostability of NIR FPs in individual neurons (Fig. 3, see Supplementary Figs. 15 and 16 for dual-color images with mCatalyst). While all BphP-based FPs could be imaged in neurons when co-expressed with the inactive form of HO1, they were ~1.7–2.9-fold dimmer than mCatalyst. Co-expression of wtHO1 enhanced the intracellular brightness of all BphP-based NIR FPs, and the most significant increase (~2.5-fold) was observed for emiRFP670 and miRFP680, which were the two brightest NIR FPs, as well as for miRFP2. By contrast, mRhubarb713, miRFP713 and iRFP713 showed the lowest intracellular brightness even when co-expressed with wtHO1 and were characterized by ~4-fold and ~8-fold lower relative brightness values in the hindbrain and spinal cord neurons, respectively, compared with mCatalyst (Supplementary Table 10). All assessed NIR FPs, except for mRhubarb713, miRFP713 and iRFP713, had sufficient brightness and cytoplasmic distribution in both hindbrain and spinal cord neurons, further enabling visualization of their neurites. Furthermore, no aggregation was observed for any of the NIR FPs in neurons. Continuous confocal imaging of spinal cord neurons showed that the photostability of the BphP-based NIR FPs was ~1.7–2.9-fold higher when co-expressed with wtHO1 except for mRhubarb713 and miRFP2, which were characterized by only an insignificant increase (~14%) in photostability with wtHO1 (Fig. 3e,f, see Supplementary Fig. 17 for representative images). The brightest NIR FP, miRFP680, exhibited limited photostability with a photobleaching half-time of ~87 s, which was almost threefold shorter than that of mCatalyst, emiRFP670 and miRFP720, which are the next brightest NIR FPs. Based on the product of intracellular brightness and photostability, emiRFP670/wtHO1 was the best-performing NIR FP, followed by mCatalyst and miRFP720/wtHO1, which were characterized by an approximately twofold lower product.

In contrast to mice and zebrafish, functional expression of BphP-based FPs in C. elegans requires a synthetic pathway for biliverdin IXa biosynthesis32,33. Therefore, we co-expressed the codon-optimized genes of the selected BphP-based NIR FPs with HO1 in neurons using extrachromosomal array expression systems. mNeon-Green, co-expressed from an independent plasmid, was used as a co-expression marker for NIR fluorescence normalization. The fluorescence of the NIR FPs was well-co-localized with mNeonGreen (Supplementary Fig. 18) and was distributed evenly in neuronal cell bodies and processes without formation of fluorescent aggregates or puncta (Fig. 4a). While all tested NIR FPs were imagable in the Cy5-LP channel, miRFP680 showed superior brightness with an approximately 3–10-fold higher relative fluorescence than most of the other NIR FPs and was approximately 1.5-fold brighter than the second brightest NIR FP, emiRFP670 (Fig. 4b and Supplementary Table 11). The miRFP713 protein was the brightest in the Cy5.5 channel, outperforming iRFP713, mRhubarb713 and miRFP680 by 13%, 50% and 9%, respectively (Fig. 4c). The miRFP720, emiRFP670 and miRFP670-2 proteins were more than 3.5-fold dimmer than miRFP713.
Fig. 2 | Quantitative characterization of the selected NIR FPs expressed in L2/3 cortical neurons in mouse brain tissue. 

a, Representative confocal fluorescence images of fixed brain slices expressing NIR-FPs-P2A-GFP (n = 2 slices from one or two mice for each protein). Imaging conditions, Cy5: excitation 639 nm, emission 655–735 nm; FITC: excitation 488 nm, emission 500–550 nm. To facilitate visual comparison of FP localization, the dynamic range was adjusted independently for each image and images were generated through maximum projection. Scale bars, 20 µm. 

b–d, Intracellular normalized brightness of NIR FPs imaged in acute brain slices from 1-month-old (b), 2-month-old (c) and 3-month old (d) mice in Cy5-LP channels (excitation power 55 mW mm⁻²; n ≥ 100 neurons from three mice for each protein at each time point). Brightness for each FP was normalized to the EGFP signal (here and in e). 

e, Intracellular normalized brightness of NIR FPs imaged in PFA-fixed brain slices from 1-month-old mice in Cy5-LP (excitation power 55 mW mm⁻²; n > 90 neurons from two mice for each protein). 

f, g, Normalized photobleaching curves of NIR FPs measured in acute brain slices (f) and PFA-fixed brain slices (g) in Cy5-LP (excitation power 55 mW mm⁻²; n ≥ 40 neurons from two mice for each protein in each channel; solid lines, experimental data; short dashed line, extrapolation; dashed line, 50% of initial intensity). Fluorescence was normalized to the intensity value of the corresponding FP at t = 0 s. Box plots with notches; narrow part of notch, median; top and bottom of the notch, 95% confidence interval for the median; top and bottom horizontal lines, 25% and 75% percentiles for the data; whiskers extend 1.5-fold the interquartile range from the 25th and 75th percentiles; horizontal line, mean; outliers not shown but are included in all calculations and available in the source datasets. See Methods (Statistics and Reproducibility section) and Supplementary Data 1 for detailed descriptive statistics and exact P values.
while mCardinal fluorescence was not detectable in the Cy5.5 channel. Under continuous wide-field illumination in the Cy5-LP and Cy5.5 channels at ~12 mW mm$^{-2}$ light power (which was ~4-fold lower than that used in cultured cells), the fluorescence of miRFP680, mRhubar713, miRFp713, iRFP713 and miRFP720 was stable for more than 20 min (less than 10% of fluorescence decline from initial values; Fig. 4d,e). mCardinal, emiRFP670 and miRFP670-2 were less photostable, losing approximately 20%, 40–50% and 45–60% of their initial fluorescence in 20 min, respectively (Fig. 4d,e and Supplementary Table 11). Based on the results, miRFP680 and miRFp713 are the proteins of choice for C. elegans imaging in the Cy5-LP and Cy5.5 channels, respectively.

**Multicolor imaging with NIR fluorescent proteins**

Analysis of intracellular brightness suggested a possibility for dual-color NIR imaging of subcellular structures using a combination of blue-shifted and red-shifted NIR FPs. In particular, NIR FPs with emission maxima below ~680 nm or beyond ~700 nm had a signal-to-background ratio of <2 in the Cy5.5 and Cy5-BP channels, respectively (Extended Data Fig. 1). To validate dual-color NIR imaging we selected two pairs
of NIR FPs, namely iRFP670–miRFP720 and miRFP670–2–miRFP720, which had the largest spectral separation among the NIR FPs characterized by high performance in OSER assay (Table 1). The H2B and keratin fusions with the selected NIR FPs were co-expressed in HeLa cells and imaged in the Cy5-LP–Cy5.5 and Cy5-BP–Cy5.5 channels (Fig. 5a,b, see Supplementary Fig. 19 for more representative images). The images from the separate channels clearly demonstrated the correct localization pattern of the target fusions, however, we also observed a minor
bleed-through of iRFP670 and miRFP670-2 fluorescence into the Cy5.5 channel, as well as miRFP720 fluorescence into the Cy5-LP and Cy5-BP channels. Application of dual-color imaging with BphP-based NIR FPs may require optimization of filter sets or further validation of the cross-talk; advanced spectral unmixing strategies can be also applied33-34.

Next, we performed quadruple-color imaging of subcellular structures and organelles in zebrafish neurons using combinations of emiRFP670 with bright cyan, green and red FPs, which can be visualized under standard imaging set-ups without spectral crosstalk. emiRFP670 was selected due to its superior performance in zebrafish larvae (Fig. 3). Co-expression of the fusions of the four FPs with subcellular localization peptides or structural proteins was achieved using a single UAS (upstream activation sequence) plasmid, either co-injected with a construct driving neuron-specific expression of Ga4 or injected into specific Ga4 transgenic zebrafish one-cell stage embryos. First, we co-expressed emiRFP670 fused to EB3, a microtubule plus-end-tracking protein32, which enables monitoring of the microtubule growth of axons in neurons, with the Lifeact-mClove3 fusion, which marks filamentous actin31. Using a conventional confocal microscope we were able to observe the real-time dynamics of actin microfilaments and comet-like EB3-emiRFP670 microtubule structures at the growing axon tip of developing primary motoneurons (Supplementary Video 1). Additional co-expression of the cyan FP mTurquoise2 fused with H2B (H2B-mTurquoise2) and the membrane-targeted red FP mScarlet-I (Fyn-mScarlet-I) enabled visualization of the nuclei of primary motoneurons as well as their highly branched axonal projections, illustrating the normal outgrowth of EB3-emiRFP670 expressing motor axons (Fig. 5c).

Next, to monitor neuronal autophagy in differentiating Purkinje cells in larval zebrafish cerebellum we generated a UAS construct carrying LAMP1-emiRFP670, which labels lysosomal membranes, with a derivative of the tandem fluorescent pH-sensitive autophagic flux sensor33, TagRFP-T-mClove3-1LC3, and cytoplasmic mTurquoise2. Purkinje cell-specific Ga4 transgenic embryos were injected with the construct, and subsequently autophagy was induced in 4 dpf larvae by inhibiting mTOR using bath incubations with the compound Rapalink-1 (ref. 39). Using confocal microscopy imaging with an Airyscan detector, three different vesicles associated with the autophagic fusion process were easily distinguished in the cytoplasm of Purkinje cells: autophagosomes, autolysosomes and lysosomes (Fig. 5c, see Supplementary Fig. 20 for negative control experiment).

Finally, synaptic labeling of Purkinje cells in larval zebrafish was achieved with a UAS reporter plasmid co-expressing the presynaptic and excitatory post-synaptic markers35,36, synaptophysin b-emIRFP670 and PSD95-mScarlet-I, respectively. Expression of synaptic proteins including synaptophysin and PSD95 was reported to be reduced by HO1 overexpression in mice neurons, causing synaptic alterations36. Thus, the reporter construct without the HO1 gene was injected into Purkinje cell-specific Ga4 transgenic embryos37, which resulted in detectable expression of both synaptic markers in Purkinje cells with appropriate localization, together with Fyn-mClove3 and H2B-mTurquoise2 (Supplementary Fig. 21). This showed that emiRFP670 is sufficiently bright and can be used to label synaptic structures together with additional FPs, even without boosting its NIR fluorescence by HO1 expression. Taken together, emiRFP670 is a superior NIR FP for the use of multicolor cellular compartment labeling in zebrafish and is useful for multiplex imaging of in vivo cell biological studies.

**Discussion**

A systematic characterization of NIR FPs enabled us to identify the best-performing FPs for applications in cultured cells and in vivo in model organisms. Although there was no single best FP for every tested condition, a small set of NIR FPs can cover the majority of potential applications. For example, for imaging of non-neuronal cells in culture and in vivo, miRFP680 has proven to be reliable due to its superior brightness and high photostability both in live and fixed samples. In addition, its monomeric behavior in cells is advantageous for the most demanding applications. For neuroimaging in mice, particularly if the goal is to image neuronal morphology, emiRFP670 is the only suitable choice due to its exceptional neuronal labeling and high intracellular brightness in acute brain slices (it ranked second in brightness following miRFP680). The photostability of emiRFP670 in PFA-fixed brain tissue appeared to be almost 20-fold higher than in live tissue, making it more suitable for imaging in preserved samples. emiRFP670 can be used as a fluorescent tag for structural proteins and organelle labeling suitable for real-time imaging in zebrafish larvae. However, in *C. elegans* neurons, miRFP680 significantly outperformed emiRFP670 in both brightness and photostability while demonstrating high-quality neuronal labeling. As a result, miRFP680 and emiRFP670 can perhaps cover the majority of applications unless imaging in the Cy5.5 channel is required. In that case, miRFP720, miRFP713 or IRFP713 are the potential candidates. miRFP720 is a protein of choice for structural imaging in preserved samples given that it appeared to be the most monomeric FP by the OSER assay and demonstrated high performance in fixed cells. For live sample imaging, miRFP720 is optimal for neuronal labeling in zebrafish. miRFP720 might also be useful when fast cellular labeling is needed given that it demonstrated superior brightness during short-term expression in the brain and liver. In turn, miRFP713 is beneficial for long-term imaging (more than 2–3 months) in mammalian neurons if neuropil labeling is not required (note that miRFP720 demonstrated better neuronal labeling than miRFP713). miRFP713 should also be used for *C. elegans* imaging in the Cy5.5 channel. IRFP713 can be applied for cytoplasmic labeling when live imaging of non-neuronal cells in Cy5.5 channels is needed. However, if downstream sample processing requires PFA fixation, miRFP713 would be a better choice due to its higher performance in fixed samples and only slightly lower brightness in live cells. In short, miRFP680 and emiRFP670 are the most optimal for imaging in the Cy5-LP and Cy5-BP channels, while miRFP720, miRFP713 and IRFP713 (to a lower degree than the former two NIR FPs) should be selected for imaging in the Cy5.5 channel based on the particular cell type or model organism requirements, as well as the sample preparation method. The use of BphP-based NIR FPs in small model organisms, including zebrafish, flies and worms, requires an additional experimental consideration such as co-expression of HO1. First, heme metabolism is tightly controlled, and expression of recombinant HO1 may interfere with cellular physiology. Second, expression cassettes need to include an addition of ~1 kbp (size of the HO1 gene), which further increases the size of the DNA cargo for intracellular delivery (BphP-based genes are 1.5-fold bigger than GFP-like FPs). These factors should be taken into account during experimental design.

Besides characterization in cultured cells and in vivo, the selected NIR FPs were assessed under different imaging modalities. We demonstrated the potential applicability of blue- and red-shifted NIR FPs for dual-color imaging of fusion proteins, however, minor cross-bleed was observed between the Cy5-LP and Cy5.5 channels. Optimization of filter sets or selection of NIR FPs with a larger spectral separation are potential options for improving dual-color NIR imaging. Alternatively, advanced spectral unmixing algorithms can be applied at the image processing step33-34. Based on the measured two-photon absorption spectra, the NIR FPs are suitable for dual-color imaging with GFPs using the standard titanium–sapphire laser. However, two-photon photostability cannot be reliably predicted based on one-photon photobleaching data, and therefore end-users may need to estimate two-photon photostability to choose an optimal NIR FP. Despite high performance in PFA-fixed samples, the chemical stability of biliverdin IXα-binding FPs is insufficient for super-resolution imaging with expansion microscopy. Further development of NIR FPs might be required to extend their applicability for imaging techniques that involve harsh chemical treatment of samples. We suggest emiRFP670 and BDFP1L8 as promising starting templates for the development of chemically stable NIR FPs.
Fig. 5 | Multicolor imaging with NIR FPs. a, Representative dual-color NIR imaging of live HeLa cells co-expressing H2B-iRFP670 and keratin-miRFP720 acquired in the Cy5-BP and Cy5.5 channels (n = 30 cells from two independent transfections). b, Representative dual-color NIR imaging of live HeLa cells co-expressing H2B-miRFP670-2 and keratin-miRFP720 acquired in the Cy5-BP and Cy5.5 channels (n = 30 cells from two independent transfections). c, Schematic representation (top) of the expression cassette used for co-expression of quadruple reporters with HO1, and (bottom) representative pseudocolor fluorescence images from 33 hours post-fertilization (hpf) zebrafish larva in which the primary spinal motor neurons co-express EB3-emiRFP670 (red) and three additional fluorescent markers labeling nucleus (H2B-mTurquoise2, yellow), plasma membrane (Fyn-mScarlet-I, blue) and F-actin (LifeAct-mClover3, green; n = 8 fish). Right, higher magnification image of the boxed region showing that signals for EB3-emiRFP670 marking the microtubule plus-end (red) are distributed along the axons (blue) where F-actin was visualized with LifeAct-mClover3 (green) and concentrated highly at the axon terminals. Imaging conditions: see Methods section for the exact optical set-up. Z-stack projected images were generated, followed by adjustment of the dynamic ranges independently for each image from four different channels. The images are further processed to facilitate visualization in different colors in four different channels (Fiji software). 
d, Schematic representation (top) of the expression cassette used for co-expression of triple reporters with HO1, and (bottom) representative pseudocolor fluorescence images of a Purkinje cell in 4 dpf zebrafish exposed to Rapalink-1, showing co-expression of cytoplasmic mTurquoise2 (yellow), autophagy flux sensor TagRFP-T-mClover3-LC3 (blue and green) and LAMP1-emiRFP670 (red), which enables identification of autophagosomes (white arrows) and autolysosomes (red arrows; n = 8 fish). Each image was processed directly using the Zeiss Airyscan image processing algorithm in the Zen black software, allowing for linear deconvolution with the Wiener filter and alignment of each offset detector to the central element. Z-stack projected images were generated, followed by adjustment of the dynamic range independently. To facilitate visualization, images are further processed in four different channels to discriminate each signal in the merged images (Fiji software). Scale bars: a, b, 20 µm; c, 30 µm (merge), 10 µm (inset); d, 1 µm.
We are confident that protein engineers will continue to develop improved NIR FPs for both general and specialized applications. This study may provide certain guidelines for future protein engineering efforts. First, the molecular brightness of NIR FPs expressed by E. coli should not be considered as a screening parameter during protein evolution because it has low correlation with intracellular brightness (Supplementary Fig. 4). Second, relative intracellular brightness in one cell type does not necessarily translate to another cell type. Therefore, expression host systems for NIR FP screening should be selected based on the intended applications. Co-expression of a reference FP via P2A peptide is an acceptable approach for intracellular brightness quantification both in cell culture and in vivo. Brightness in cultured neurons can be used as a selection criterion for imaging neurons in vivo in mammals, however, the quality of neuronal labeling in vivo is not possible to predict from neuronal culture results. Third, relative photostability measured under matching illumination is highly correlated between different cell types, however, PFA fixation may alter photobleaching rates considerably. Fourth, the OSC assay is valid for BphP-based NIR FPs, and we suggest using miRFP720 as a control; however, the values reported in Table 1 should not be interpreted as absolute values. Finally, we suggest that newly developed NIR FPs are benchmarked against miRFP680 and emiRFP670 in Cy5 channels and against miRFP720 and miRFP713 in the Cy5.5 channel.

Protocol registration
The study was conducted according to the registered peer-reviewed protocol available at https://springernature.figshare.com/articles/journal_contribution/Piatkevich_AIP_pdf/17213501. Other than pre-registered and approved pilot data, all data reported in the paper were acquired after the date of the registered protocol publication.

Online content
Any methods, additional references, Nature Portfolio reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at https://doi.org/10.1038/s41592-023-01975-z.

References
1. Prascher, D. C., Eckenrode, V. K., Ward, W. W., Prendergast, F. G. & Cormier, M. J. Primary structure of the Aequorea victoria green-fluorescent protein. Gene 111, 229–233 (1992).
2. Shcherbakova, D. M., Stepanenko, O. V., Turoverov, K. K. & Verkhusha, V. V. Near-infrared fluorescent proteins: multiplexing and optogenetics across scales. Trends Biotechnol. 36, 1230–1243 (2018).
3. Filonov, G. S. et al. Bright and stable near-infrared fluorescent protein for in vivo imaging. Nat. Biotechnol. 29, 757–761 (2011).
4. Chu, J. et al. Non-invasive intravitral imaging of cellular differentiation with a bright red-excitable fluorescent protein. Nat. Methods 11, 572–578 (2014).
5. Shcherbakova, D. M. & Verkhusha, V. V. Near-infrared fluorescent proteins for multicolor in vivo imaging. Nat. Methods 10, 751–754 (2013).
6. Babakhanova, S. et al. Rapid directed molecular evolution of fluorescent proteins in mammalian cells. Protein Sci. 31, 728–751 (2022).
7. Qian, Y. et al. A genetically encoded near-infrared fluorescent calcium ion indicator. Nat. Methods 16, 171–174 (2019).
8. Qian, Y. et al. Improved genetically encoded near-infrared fluorescent calcium ion indicators for in vivo imaging. PLoS Biol. 18, e3000965 (2020).
9. Shcherbakova, D. M., Cox Cammer, N., Huisman, T. M., Verkhusha, V. V. & Hodgson, L. Direct multiplex imaging and optogenetics of Rho GTPases enabled by near-infrared FRET. Nat. Chem. Biol. 14, 591–600 (2018).
10. Shcherbakova, D. M. et al. Bright monomeric near-infrared fluorescent proteins as tags and biosensors for multiscale imaging. Nat. Commun. 7, 12405 (2016).
11. Kamper, M., Ta, H., Jensen, N. A., Hell, S. W. & Jakobs, S. Near-infrared STED nanoscopy with an engineered bacterial phytochrome. Nat. Commun. 9, 4762 (2018).
12. Wegner, W. et al. In vivo mouse and live cell STED microscopy of neuronal actin plasticity using far-red emitting fluorescent proteins. Sci. Rep. 7, 11781 (2017).
13. Matlashov, M. E. et al. A set of monomeric near-infrared fluorescent proteins for multicolor imaging across scales. Nat. Commun. 11, 239 (2020).
14. Piatkevich, K. D., Subach, F. V. & Verkhusha, V. V. Engineering of bacterial phytochromes for near-infrared imaging, sensing, and light-control in mammals. Chem. Soc. Rev. 42, 3441–3452 (2013).
15. Shcherbakova, D. M., Baloban, M. & Verkhusha, V. V. Near-infrared fluorescent proteins engineered from bacterial phytochromes. Curr. Opin. Chem. Biol. 27, 52–63 (2015).
16. Piatkevich, K. D. et al. Near-infrared fluorescent proteins engineered from bacterial phytochromes in neuroimaging. Biophys. J. 113, 2299–2309 (2017).
17. Shemetov, A. A. et al. A near-infrared genetically encoded calcium indicator for in vivo imaging. Nat. Biotechnol. 39, 368–377 (2021).
18. Yu, D. et al. A naturally monomeric infrared fluorescent protein for protein labeling in vivo. Nat. Methods 12, 763–765 (2015).
19. Paez-Segala, M. G. et al. Fixation-resistant photoactivatable fluorescent proteins for CLEM. Nat. Methods 12, 215–218 (2015).
20. Campbell, B. C., Paez-Segala, M. G., Looger, L. L., Petsko, G. A. & Liu, C. F. Chemically stable fluorescent proteins for advanced microscopy. Nat. Methods 19, 1612–1621 (2022).
21. Tillberg, P. W. et al. Protein-retention expansion microscopy of cells and tissues labeled using standard fluorescent proteins and antibodies. Nat. Biotechnol. 34, 987–992 (2016).
22. Cranfill, P. J. et al. Quantitative assessment of fluorescent proteins. Nat. Methods 13, 557–562 (2016).
23. Costantini, L. M., Fossati, M., Francolini, M. & Snapp, E. L. Assessing the tendency of fluorescent proteins to oligomerize under physiologic conditions. Traffic 13, 643–649 (2012).
24. Li, X. D. et al. Design of small monomeric and highly bright near-infrared fluorescent proteins. Biochim. Biophys. Acta Mol. Cell Res. 1866, 1608–1617 (2019).
25. Shaner, N. C. et al. A bright monomeric green fluorescent protein derived from Branchiostoma lanceolatum. Nat. Methods 10, 407–409 (2013).
26. Day, R. N. & Davidson, M. W. The fluorescent protein palette: tools for cellular imaging. Chem. Soc. Rev. 38, 2887–2921 (2009).
27. Dobrzhev, M., Makarov, N. S., Tillo, S. E., Hughes, T. E. & Rebane, A. Two-photon absorption properties of fluorescent proteins. Nat. Methods 8, 393–399 (2011).
28. Piatkevich, K. D. et al. A robotic multidimensional directed evolution approach applied to fluorescent voltage reporters. Nat. Chem. Biol. 14, 352–360 (2018).
29. Mikhailov, A. et al. Hot-band absorption can mimic entangled two-photon absorption. J. Phys. Chem. Lett. 13, 1489–1493 (2022).
30. Babakhanova, S. et al. Rapid directed molecular evolution of fluorescent proteins in mammalian cells. Protein Sci. 31, 728–751 (2022).
31. Holowiecki, A., O’Sheils, B. & Jenny, M. J. Characterization of heme oxygenase and biliverdin reductase gene expression in zebrafish (Danio rerio): basal expression and response to pro-oxidant exposures. Toxicol. Appl. Pharmacol. 311, 74–87 (2016).
32. Qian, Y. et al. Improved genetically encoded near-infrared fluorescent calcium ion indicators for in vivo imaging. PLOS Biol. 18, e3000965 (2020).
33. Seo, J. et al. PICASSO allows ultra-multiplexed fluorescence imaging of spatially overlapping proteins without reference spectra measurements. Nat. Commun. 13, 2475 (2022).
34. Chiang, H. J. et al. HyU: Hybrid Unmixing for longitudinal in vivo imaging of low signal-to-noise fluorescence. Nat. Methods 20, 248–258 (2023).
35. Stepanova, T. et al. Visualization of microtubule growth in cultured neurons via the use of EB3-GFP (end-binding protein 3-green fluorescent protein). J. Neurosci. 23, 2655–2664 (2003).
36. Riedl, J. et al. Lifeact: a versatile marker to visualize F-actin. Nat. Methods 5, 605–607 (2008).
37. Kimura, S., Noda, T. & Yoshimori, T. Dissection of the autophagosome maturation process by a novel reporter protein, tandem fluorescent-tagged LC3. Autophagy 3, 452–460 (2007).
38. Namikawa, K. et al. Modeling neurodegenerative spinocerebellar ataxia type 13 in zebrafish using a Purkinje neuron specific tunable coexpression system. J. Neurosci. 39, 3948–3969 (2019).
39. Rodrik-Outmezguine, V. S. et al. Overcoming mTOR resistance mutations with a new-generation mTOR inhibitor. Nature 534, 272–276 (2016).
40. Meyer, M. P. & Smith, S. J. Evidence from in vivo imaging that synaptogenesis guides the growth and branching of axonal arbors by two distinct mechanisms. J. Neurosci. 26, 3604–3614 (2006).
41. Niel, C. M., Meyer, M. P. & Smith, S. J. In vivo imaging of synapse formation on a growing dendritic arbor. Nat. Neurosci. 7, 254–260 (2004).
42. Li, L. et al. Overexpression of heme oxygenase 1 impairs cognitive ability and changes the plasticity of the synapse. J. Alzheimers Dis. 47, 595–608 (2015).

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Methods

Molecular cloning

Synthetic DNA oligonucleotides used for cloning were synthesized by Tsingke Biotechnology. PrimeStar Max master mix (Takara) or 2×HiTff PCR Master Mix (Yeasen) was used for high-fidelity polymerase chain reaction (PCR) amplifications. Restriction endonucleases were purchased from New England Biolabs and used according to the manufacturer’s protocols. DNA ligations were performed using T4 DNA ligase (New England Biolabs) or the NovoRecom plus one step cloning kit (Novoprotein). The ligation products were chemically transformed into the TOP10 E. coli strain (Biomed) and cultured according to the standard protocols. Sequencing of bacterial colonies and purified plasmids was performed using Sanger sequencing (Zhejiang Youkang Biological Technology). Small-scale isolation of plasmid DNA was performed with commercially available Mini Prep kits (Qiagen or Tiangeng); large-scale DNA plasmid purification was done with Midi Prep kits (Qiagen).

The mammalian codon-optimized genes of NIR FPs were de novo synthesized by Tsingke Biotechnology or Shanghai Generay Biotech based on the amino acid sequences reported in the original publications. Oligonucleotides for cloning were purchased from Tsingke Biotechnology or Zhejiang Youkang Biological Technology (see the list of all primers used in this study in Supplementary Dataset 2). To clone pAAV-CAG-NIR-FPs-P2A-EGFP plasmids, the complementary DNA of the NIR FPs was PCR amplified with KpnI/NotI flanking sites and swapped with the TagRF638 gene in the pAAV-CAG-TagRF638-P2A-EGFP plasmid (Addgene 178971). To clone pAAV-CAG-NIR-FP plasmids, the cDNA of the NIR FPs was PCR amplified with KpnI/EcoRI flanking sites and swapped with the GFP gene in the pAAV-CAG-GFP (Addgene 37823). To construct Cy5ERM (cytoplasmic end of an endoplasmic reticulum signal anchor membrane protein) fusions, the NIR FPs were PCR amplified with AgeI/Nool flanking sites and swapped with the mScarlet gene in the pCy5ERM-mScarlet-N1 (Addgene plasmid 85066). To construct plasmids for expression of structural protein fusions, the cDNA of the selected NIR FPs was PCR amplified and swapped with the corresponding FP genes in the mRuby-Cx-43-7 (Addgene 55856), pkeratin-miRFP670nano (Addgene 127437), pActin-Electra1 (Addgene 184941), pH2B-Electra1 (Addgene 179478) and pTubulin-Electra1 (Addgene 184929) plasmids. All plasmids used in this study are available from Addgene and WeKwikGene (https://wekwikgene.wllsb.edu.cn).

Characterization in cultured mammalian cells

All animal maintenance and experimental procedures were conducted according to the Westlake University Animal care guidelines, and all animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) of Westlake University, Hangzhou, China under animal protocol 19-044-KP. For all experiments involving mice throughout, the C57BL/6J strain (supplied by the animal facility of Westlake University) was used regardless of sex. HEK293FT (Invitrogen) and HeLa (ATCC CCL-2) cells were authenticated by the manufacturer using STR (short tandem repeat) profiling, re-authenticated in our lab by inspecting the stereotypical morphological features under wide-field microscope, and tested to ensure negativity for mycoplasma contamination to their standard levels of stringency. Authentication by morphology was performed every time before transient transfection.

The intracellular brightness and photostability were measured in HEK cells and in primary hippocampal mouse neurons using a Nikon TiE wide-field fluorescence microscope equipped with a Spectra III Light Engine (LumenCore), 680 nm CNL laser, and an ORCA-Flash 4.0 V3 sCMOS camera (Hamamatsu) with ×10.45 numerical aperture (NA) and ×20.75NA objective lenses (Nikon) controlled by NIS Elements AR 5.2.1.00 (Nikon). To facilitate unbiased comparison of NIR FPs in cell culture, we used blinding and randomization. The experimenters were blinded to protein identity; all vials with plasmids were barcoded, and the identity of the proteins was shown only after data collection and analysis were finished. The transfection and imaging of the samples were performed in random order; all vials with plasmids were pooled together and randomly picked one by one during transfection. Independent transfections were randomized independently.

HEK293FT cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin, and seeded in a 24-well glass-bottom plate (P24-O N Cellvis) after Matrigel (356235, BD Biosciences) coating. Cells were transfected at 80–90% confluency using Hieff Trans Liposomal Transfection Reagent (Yeasen Biotechnology, 40802ES02) according to the manufacturer’s protocol, and imaged using a fluorescence wide-field microscope at 36–48 h after transfection. First, transfected cells were imaged without exogenous biliverdin IXα, then 25 µM biliverdin IXα was added to the cell medium and incubated for 3 h at 37°C in a cell incubator. Before imaging, cells were washed once with PBS to remove excess biliverdin IXα and the same fields of view were imaged again in fresh culture media under the same imaging settings. Cells were imaged in the FITC (excitation 475/28 nm at 5.30 mW mm⁻² from Spectral LumenCor; emission 535/46 nm), Cy5-BP (excitation: 635/22 nm at 56–58 mW mm⁻² from Spectral LumenCor; emission 679/41 nm), Cy5-LP (excitation: 635/22 nm at 56–58 mW mm⁻² from Spectral LumenCor; emission 664LP), and Cy5.5 (excitation: 680/13 nm at 56–58 mW mm⁻² from 680 nm CNI laser; emission 710LP) channels. To obtain statistically significant datasets, we performed three independent transfections and analyzed more than 100 regions of interest (ROIs) from each transfection (at least 300 ROIs for analysis in total per construct). ROIs were determined using the auto-detect function of NIS Elements software and limiting the smallest ROI to 50 µm² (the size of an HEK cell). The mean fluorescence intensity in the green, Cy5-BP, Cy5-LP and Cy5.5 channels for ROIs was extracted and the Cy5-BP-to-green, Cy5-LP-to-green and Cy5.5-to-green fluorescence ratios were calculated for each construct after background subtraction for each channel, and used for further comparison of intracellular brightness under corresponding imaging conditions. The data were excluded from analysis if the cells died after transfection or if the cell culture was contaminated with bacteria or yeast.

To measure photostability in live HEK cells, HEK cells were transfected as described above and imaged under continuous Cy5 and Cy5.5 wide-field excitation. The photobleaching curves were calculated for each cell individually, and average photobleaching half-time with standard deviation was reported for each protein. Photobleaching was performed for at least a 50% fluorescence drop, when possible, and reported as the mean photobleaching curve for each protein (averaged from all individual curves). Cells that detached or died during photobleaching experiments were excluded from data analysis.

To quantify intracellular brightness in live primary mouse hippocampal neurons, cultured neurons were prepared as previously described and transfected with pAAV-CAG-NIR-FPs-P2A-EGFP plasmids using the calcium phosphate transfection method or transduced using rAAV2/9-CAG-NIR-FPs-P2A-EGFP viruses (Shanghai Sunbio Medical Biotechnology). In brief, hippocampal neurons were transfected at DIV (days in vitro) 4–5 using the calcium phosphate transfection kit (K278001, Invitrogen) according to the previously described protocol. To obtain a high density of neurons expressing NIR FPs, transduction was performed with ~1 µl rAAV2/9 viruses (>10¹² vector genome (v.g.) ml⁻¹) per well, in 1 ml culture medium at DIV 4–5 (this procedure was not proposed during stage 1 but was included in the final version to compensate for the low density of calcium phosphate transfection). Neurons expressing NIR FPs were imaged at DIV 12–18 using the same imaging set-up as described above for HEK cells. Mean fluorescence intensity was calculated for neuronal soma only. Intracellular brightness with and without biliverdin IXα, and photostability were measured and quantified using the same procedure as described for HEK cells above. The culture was excluded from the analysis if cells died after transfection or if the culture was contaminated with bacteria or yeast.
Bottom-up proteomic analysis was performed using liquid chromatography with tandem mass spectrometry (LC-MS/MS). In brief, 10^6 HEK cells transiently transfected with pAAV-CAG-NIR-FPs-P2A-GFP plasmids as described above were lysed using RIPA buffer (89900, Thermo Fisher) and the extracted peptides were analyzed using an Orbitrap Fusion Lumos mass spectrometer (Thermo Fisher) in data-independent acquisition (DIA) mode. The precolumn (75 μm inner diameter (i.d.), 360 μm outer diameter (o.d.), 2 cm long) was packed with 3 μm C18 packing material (100 Å pore size, 164946, Thermo Scientific) and liquid chromatograph columns (75 μm i.d., 360 μm o.d., 25 cm long) were packed with 1.9 μm C18 particles (164941, Thermo Scientific). The flow rate of the liquid chromatography separation was 300 nL/min using a nanoUPLC pump (UltiMate 3000 RSLC nano, Thermo Scientific). A linear 60 min gradient of 5–98% buffer B (0.1% formic acid in 80% acetonitrile) was used for the liquid chromatography separation. All samples were analyzed using an Orbitrap Fusion Lumos mass spectrometer (Thermo Fisher Scientific, tune version 3.4 and Xcalibur version 4.4) with high-field asymmetric waveform ion mobility spectrometry pro (FAIMS pro). The peptide precursors were first isolated by Orbitrap (60,000 resolution) and interrogated by MS2 in the Orbitrap (15,000 resolution) using high energy collisional dissociation at a collision energy of 30. The MS/MS spectra were recorded at a target value of 5×10^4 with a 30 ms maximum ion injection time. Acquired data were processed with DIA-NN software (https://github.com/vdemichev/diann) for protein identification and quantification, and PEAKS Studio Xpro software (Bioinformatics Solutions) was used to identify post-translational modifications. Here, we report protein identity for each analyzed sample as well as identified post-translational modifications.

For western blot analysis (which was not proposed during stage 1 but was included in the final version to complement the mass spectrometry results), HEK cells transfected with pAAV-CAG-NIR-FPs-P2A-GFP were washed twice with ice-cold PBS and then lysed for 10 min in 200 μL RIPA Lysis Buffer 1 (lot no. CS00005-0010, Sangon Biotech) containing protease inhibitor cocktail (buffer B) and phosphatase inhibitor cocktail (buffer C). The cell lysates were sonicated with an ultrasonic processor (VCX150, Sonics) and the protein concentration was determined using a BCA Protein Quantification Kit (20201ES76, Yeasen Biotechnology). Proteins were detected using an enhanced chemiluminescence detection kit (36222ES60, Thermo Fisher) and the extracted peptides were analyzed using an Amersham Imager 680 Analysis software.

To perform the cytotoxicity assay of the NIR FPs, HEK cells were transiently transfected with pAAV-CAG-NIR-FP plasmids and dummy DNA (pUC19) as a negative control, using HiEff Trans Liposomal Transfection Reagent (40802ES02, Yeasen Biotechnology), and assessed at 36–48 h after transfection using Annexin V, Alexa Fluor 687 conjugate (A13202, ThermoFisher) and Live/Dead reagent (L34985, Viability/Vitality Kit, ThermoFisher), according to the manufacturer’s protocol with minor adjustments. Annexin V conjugates are designed to detect the externalization of phosphatidylserine, one of the earliest indicators of apoptosis. In brief, we prepared annexin-binding buffer: 10 mMHEPES, 140 mM NaCl and 2.5 mM CaCl2, pH 7.4, and added dyes indicating live and dead cells into the buffer at working concentrations. The HEK cells were collected at a concentration of 1×10^6 cells/ml in each 1.5 ml tube using EDTA-free Trypsin (BL527A, Biosharp) and washed in cold PBS. The washed cells were centrifuged, and supernatant was removed. One tube of non-transfected cell pellet was heated at 50 °C for 10 min to obtain the heat-killed cells as a positive control for apoptotic and dead cells. The HEK cells including the positive controls were resuspended in 100 μL annexin-binding buffer containing Live/Dead dyes. After incubating the cells on ice for 15 min, 5 μL annexin V conjugate were added into each 100 μL cell suspension and the cells were further incubated at room temperature for 15 min. A total of 400 μL annexin-binding buffer without Live/Dead dyes was added, mixed gently, and cells were briefly kept on ice. The stained cells were analyzed using a flow cytometer (Cytomics LX, Beckman Coulter) equipped with a 405 nm laser and 450/43 bandpass for calcein violet-labeled live cells (Live V450-PB-A), and 525/40 bandpass for the aqua fluorescent reactive dye-labeled dead cells (Dead V525-KrO-A); a 561 nm laser and 585/42 bandpass (Apoptosis Y585-PE-A) were used for Annexin V-labeled live cells. The channels Cy5-BP R660-APC-A and Cy5-LP R712-APC700-A were used to analyze the NIR FP-positive cells with a 638 nm laser. Cytotoxicity was quantified as the fraction of dead cells and cells in the early stage of apoptosis in the total transfected cells. Given that the NIR FP signal exhibited crosstalk into the channels used for detection of the dye signals, the signals were compensated correspondingly.

To quantify fluorescence retention upon PFA fixation, HEK cells were transfected as described above, and imaged using a fluorescence wide-field microscope at 36–48 h after transfection without exogenous biliverdin IXa. After imaging in the Cy5-LP and Cy5.5 channels, cells were washed with PBS twice and fixed with 4% PFA (15714, Electron Microscopy Sciences) in PBS at room temperature for 15 min. Fixed cells were gently washed with PBS twice and the same fields of view were imaged under identical imaging settings. Image analysis was performed as described above for live cells. To obtain statistically significant datasets, we performed two independent transfections and analyzed more than 1,000 ROIs for each transfection.

The protein retention expansion microscopy (proExM) experiments were conducted according to a previously described protocol. HEK cells, seeded and transfected in glass-bottom dishes (MatTek) as described above, were treated with 4% PFA in PBS for 15 min, washed three times for 5 min with PBS, and imaged in FITC and Cy5-LP channels with a ×20 0.75NA objective lens under a Nikon wide-field microscope. After imaging, fixed cells were incubated with 0.1 mg/ml αCyt (A20770, Life Technologies) for at least 6 h at 23 °C. After washing with PBS, cells were incubated with activated monomer solution (8.625% (w/w) sodium acrylate, 2.5% (w/w) acrylamide, 0.15% (w/w) N,N’-methylenebisacrylamide, PBS, 2 M NaCl, 0.2% (w/w) APS and 0.2% (v/v) TEMED) and gelled for 2 h in a humidified incubator at 37 °C in a nitrogen atmosphere. Formed sample–hydrogel composites were digested with proteinase K (New England Biolabs) in digestion buffer (50 mM Tris, 1 mM EDTA, 0.5% Triton X-100, 1 M NaCl, pH 8.0) overnight at 23 °C. Following digestion, the samples were processed by extensively washing with PBS, and then shrunk in 1 M NaCl and 60 mM MgCl2. Digested samples were imaged using the same setting as fixed cells. The same cells before and after proExM treatment were identified manually and analyzed using NIS Elements software.

To quantify the monomeric state of NIR FPs in mammalian cells, the OSER assay was used as previously described. In brief, HeLa (ATCC CCL-2) cells were cultured and transfected with the pcYER-MIR-NIR-FPs-NI plasmids as described for HEK cells and imaged 12–18 h after transfection using the Cy5-LP channel. For each NIR FP we performed at least two independent transfections and analyzed at least 250 individual cells for each transfection. Following the protocol described by Cranfill et al., positive cells selected for analysis had overall similar fluorescence brightness, and cells that were significantly brighter were excluded (indications of unhealthy or highly stressed cells). Cells with non-spherical nuclei, endoplasmic reticulum sheet architectures, or condensed nuclei were also excluded from the assay.
(see Supplementary Fig. 10 for representative images of cells excluded from the analysis). Image analysis was performed by three blinded independent researchers, using NIS Elements software, and mean values with standard errors were calculated. Moreover, each NIR FP was further assessed by calculating the ratio of structure MFI (mean fluorescence intensity) to NE (nuclear envelope) MFI as proposed by Costantini et al. Structures visibly distinct from the endoplasmic reticular networks were manually traced using the free cycle selection tool in ImageJ, and the MFI for the selected area was measured. Using the freehand line selection tool in ImageJ, three separate regions of the nuclear envelope were manually traced avoiding karnemellae, and the NE MFI was measured and averaged. Live HeLa cells expressing NIR FP fusions with structural proteins were imaged at 24–36 h after transfection using an Olympus FY3000 inverted confocal microscope (except mIRFP660, which was additionally imaged in fixed cells using a Nikon CSU-W1 SoRa), acquiring on average four fields of view in the Cy5 channel (excitation 640 nm; emission 660–735 nm). Dual-color NIR imaging was performed in the Cy5-LP–Cy5.5 and Cy5-BP–Cy5.5 channels using the Nikon wide-field microscope described above.

In vitro characterization

For protein purification, the NIR FPs genes were inserted between the Xhol and PstI sites of the pBAD vector that encodes HO1 from the cyanobacteria "Synechocystis sp. CACIAM 05 to convert an endogenous heme in bacteria into biliverdin IXα, as previously described. The plasmids were transformed into the TOP10 E. coli strain (Biomed) and grown in RM medium supplemented with ampicillin and 0.06% arabinose for 18–20 h at 37 °C followed by incubation for 24 h at 18 °C. Proteins were purified with metal affinity chromatography using Ni-NTA resin (20S035ES50, Yeasen Biotechnology) according to the manufacturer’s protocol. Proteins were eluted with PBS containing either 400 mM imidazole or 100 mM EDTA (EDTA was used for mRubarb713 elution given that 400 mM imidazole resulted in protein discoloration, perhaps due to the chromophore substitution). The purified proteins were dialyzed against PBS at pH 7.4 and adjusted to the required concentration for spectroscopy. The UV–visible absorption spectra were measured in the range 250–800 nm using the UV-VIS-NIR UV3600Plus spectrophotometer (Shimadzu). The steady-state fluorescence spectra were measured using the Photoluminescence Spectrometer FS5 (Edinburgh Instruments). Absolute fluorescence quantum yields (QYs) were measured with an integrating sphere fluorometer (Quantaurus-QY, Hamamatsu) using 1 cm quartz cuvettes at a set of excitation wavelengths from 530 nm to 700 nm with 10 nm steps. Quantum yields were plotted against excitation wavelength for each protein and the region where the dependence was flat (QY did not depend on wavelength) was selected. QYs are presented as an average of the data obtained in these regions. The peak optical density of the samples was <0.1. The reference (1x PBS buffer only) measurements were done in the same cuvette as was used for the sample measurements.

Fluorescence lifetimes were measured with a digital frequency domain system, ChronosSDFD (ISS), as previously described. In brief, fluorescence was excited with a 635 nm laser diode (ISS), modulated at a number of harmonics. LDS798 dye (Exciton) in ethanol (τ = 0.15 ns) was used as a reference standard to obtain the instrumental response function. Fluorescence was collected at 90° to the excitation beam using a Nikon CSU-W1 SoRa. The same measurement protocol was used for a whole series of the selected NIR FPs to enable a robust direct comparison of their two-photon absorption properties. To measure the two-photon excitation spectrum, the laser wavelength is automatically stepped and the corresponding total fluorescence signal is recorded at each wavelength using our customized LabView program. Well-characterized two-photon standard dye solutions of Coumarin 540A in dimethylsulfoxide and LDS798 in chloroform were used to correct for the two-photon spectral profile of the sample for wavelength-to-wavelength variations of laser parameters (spatial intensity distribution and pulse duration).

We used a modification of the previously described method of measurement of the two-photon absorption cross-section (σ2). In particular, we collected a total (without monochromator) fluorescence signal of the sample and reference, first upon two-photon excitation at a certain wavelength, and then upon one-photon excitation with a continuous-wave laser with a visible wavelength matching the absorption spectra of the sample and reference. In these measurements, the sample and the reference solutions are contained in 3 × 3 mm SOG (special optical glass) cuvettes (Starna) and have a maximum optical density of <0.1. The fluorescence is collected at 90° to the excitation laser beam through a set of filters that block both the femtosecond NIR and the visible laser wavelengths, typically including FF01-770/SP, or FF01-842/SP in combination with 561 nm EdgeBasic best-value long-pass filters (all Semrock) using the left emission channel of the spectrophotofluorometer. We first collected the two-photon excited fluorescence signal I as a function of laser power P for both the sample and reference solutions and fitted them to a quadratic function $I = aP^2$. From these fits, the coefficients $a_s$ and $a_r$ are obtained for the sample (index S) and reference (index R) solutions, respectively. Second, we measured the one-photon excited fluorescence signals as a function of laser power for the same solutions and in the same registration conditions as in the two-photon experiment, and fitted them to a linear function: $I = bP$. The best fits provide coefficients $b_s$ and $b_r$. The two-photon absorption cross-section was then calculated as follows:

$$\sigma_{2,s}(\lambda_2) = \frac{a_s}{2b_s} \frac{\varepsilon_2(\lambda_2)}{\varepsilon_1(\lambda_1)} \sigma_{2,r}(\lambda_1)$$

Here, $\lambda_2$ is the wavelength used for one-photon excitation (561 nm), $\lambda_1$ is the wavelength used for two-photon excitation (1,060 or 1,064 nm), and $\varepsilon_2(\lambda_1)$ are the corresponding extinction coefficients, measured at $\lambda_1$. This approach enables us to automatically correct for the laser beam properties (pulse duration and spatial intensity distribution), fluorescence collection efficiencies for one- and two-photon modes, photomultiplier tube spectral sensitivity, and differences in quantum yield and concentration between the sample and reference solutions.

The two-photon absorption cross-sections were measured at 1,060 nm relative to LDS798 in deuterated chloroform, the value of which is 220 GM.

Characterization in mice

All animal maintenance and experimental procedures for mice were conducted according to the Westlake University Animal care...
were excluded from the study. We calculated for individual cells. Animals that show no GFP fluorescence were tested at least three livers. The mean fluorescence intensity was measured at postnatal days 20–30.

We perfused mice using 4% PFA according to the protocol described in the methods. For assessment of intracellular brightness and photostability in fixed brain tissue, we used the neonatal intraventricular injections of custom-made recombinant AAVs, serotype 2/9 (rAAV2/9-CAG-NIR-FPs-P2A-GFP, titer: >10^12 v.g. ml^-1; Shanghai Sunbio Medical Biotechnology) as described previously⁴⁹. In brief, viruses were injected pan-cortically into pups at postnatal day 0 regardless of sex with a Hamilton microliter syringe. To induce hypothermic anesthesia, the pups were put on soft tissue with ice underneath under close monitoring until they stopped responding to gentle squeezing on the limbs. For each hemisphere, 0.5 µl virus solution (titer: >10^12 v.g. ml^-1) supplemented with 0.1% FastGreen dye (Sigma-Aldrich) was injected under the skull manually. After injections in both hemispheres, the pups were moved onto a heating pad maintained at 37°C for a 5 min recovery. After the pups regained response to gentle squeezing, they were returned to the home cages. The whole injection process for each pup was controlled within a few minutes to prevent cannibalism. At least 11 postnatal day 0 mice were injected for each NIR FP. Acute brain slices were obtained from mice injected with AAVs at postnatal days 20–30 as well as at 2 months and 3 months of age using standard techniques as described previously⁴⁹.

To assess the intracellular brightness of each protein in the Cy5 and Cy5.5 channels, at least nine live brain slices from three mice were imaged for each time point. To quantify the photostability of each protein, at least three live brain slices were obtained from three 1-month-old mice for each protein. In brief, mice were deeply anesthetized with 1% pentobarbital sodium (8 µg g^-1) and perfused transcardially using cold saline (oxygenated with 95% O_2 and 5% CO_2) containing 145 mM NaCl, 3.5 mM KCl, 1.2 mM NaHPO_4, 0.2 mM CaCl_2, 2 mM MgCl_2, 26 mM NaHCO_3, 10 mM HEPES (pH 7.4), adjusted with NaOH, 320–340 mMosm l^-1. Coronal slices (300–500 µm thick) were cut using a slicer (VT1200 S, Leica Microsystems) and then incubated for 10–15 min in a holding chamber (BSK4, Scientific System) at 37°C for a 5 min recovery. After the pups regained response to gentle squeezing, they were returned to the home cages. The whole injection process for each pup was controlled within a few minutes to prevent cannibalism. At least 11 postnatal day 0 mice were injected for each NIR FP. Acute brain slices were obtained from mice injected with AAVs at postnatal days 20–30 as well as at 2 months and 3 months of age using standard techniques as described previously⁴⁹.

Characterization of NIR FPs in neurons of living zebrafish larvae

All experiments involving zebrafish were performed according to European Union guidelines and German legislation (EU Directive 2010.63 and license AZ 325.L53/561-TU-BS). Zebrafish husbandry and breeding were performed according to the standard procedures. The NIR FPs that showed high performance in cultured neurons were expressed transiently in neurons of zebrafish larvae and assessed for their brightness and photostability using zebrafish-specific expression vectors. The neuronal expression vector for these experiments carries two expression components flanked by Tol2 recognition sites. The neuron-specific Gal4 expression unit includes a Gal4 variant KalNFB, the expression of which is regulated by a compact, 377 bp-long promoter (nbt<sup>3002n</sup>): a 300-bp-long Xenopus neuron-specific β-tubulin (nbt) gene core promoter (gene bank accession; EF989124, 3182–3481), followed by a 2x neuron restrictive silencer element (2x NRSE: 2n) in the 5′ untranslated region (5′UTR). The KalNFB gene was linked to three copies of the target site of microRNA1 (3×mir1T) to suppress ectopic expression in muscles, followed by SV40 (simian virus 40) polyA. Furthermore, the predicted 3′ UTR of zebrafish synaptotagmin 2a was inserted between 3xmir1T and SV40 polyA to enhance the level of expression in neurons (C. Riegler, personal communication).

The final construct, named pTol2-nbt<sup>3002n</sup>:KalNFB-3xmir1T-syt2a (Addgene 196990) successfully enhanced the neuron specificity of Gal4-UCAS-mediated reporter expression by eliminating muscle expression while maintaining high levels of neuronal expression. The resultant construct, designated pTol2-nbt<sup>3002n</sup>:KalNFB-3xmir1T-syt2a, was used to insert the following reporter genes as below. The second expression module carries a regulatory element composed of the 10×UAS flank by two Elb minimal promoters (10xUAS), inducing bidirectional co-expression of transgenes in a Gal4-dependent, cell type-specific manner. To validate neuron-specific co-expression of two reporters inserted into pTol2-nbt<sup>3002n</sup>:KalNFB-3xmir1T-syt2a, TagRFP-T and EGFP were cloned into the left hand and right hand of 10×UAS, respectively (Addgene 196161) (Supplementary Fig. 14). For expression of NIR FPs, the mammalian codon-optimized NIR FPs linked to P2A-mClover3 (NIR-FPs-P2A-mClover3) were cloned as one cistron into the right hand of bidirectional 10×UAS (Addgene 196118-196133). As the other cistron, the zebrafish codon-optimized open reading frame (ORF) of wild-type human heme oxygenase 1 (hOh1), or its catalytically inactive form (H01-H25A)⁵⁸ was inserted into the left hand of 10×UAS (see Supplementary Fig. 14 for the schematics of the expression cassette). The obtained Gal4/UAS construct drove multicistronic transgene expression, enabling the assessment of NIR FP fluorescence in Gal4-expressing neurons with or without H01 activity. As a control, a construct carrying mCardinal-P2A-mClover3 with neither H01 nor H01-H25A was cloned, the expression of which is under the control of 6×UAS (Addgene 19617) (Supplementary Fig. 14).

The generated expression constructs were co-injected with Tol2 transposase mRNA (1.5 nl injection mix containing 25 ng µl^-1 of both Tol2 plasmid and pTol2 mRNA) into pigmentation-compromised brass mutant embryos at the single-cell stage. Injection of this construct together with Tol2 mRNA into zebrafish embryos led to Tol2 transposon-mediated early genomic integration of the transgene, followed by broad expression in neuronal cells of larval zebrafish in a semi-mosaic manner. Injected larvae were screened for green fluorescence throughout the nervous system at 2–3 dpf using a stereomicroscope (Leica Microsystems). Subsequently, larval zebrafish at 4 dpf were sedated with 0.02% Tricain (E10521, Sigma-Aldrich) and embedded in 1.0% low-melting agarose (A9414, Sigma-Aldrich) for imaging. Fluorescence signals of TagRFP-T and EYFP were captured with a fluorescence stereomicroscope (MZ220FA, Leica Microsystems), whereas those for NIR FPs and mClover3 were imaged using a confocal microscope (Leica SP8, Leica Microsystems) with a ×40 1.3NA water immersion objective in the green channel (excitation, 488 nm from
an argon laser; emission, 496–530 nm) and cfCy5-LP channel (excitation, 633 nm from an HeNe laser; emission, 645–780 nm). To quantify the intracellular brightness of NIR FPs in neurons, reconstructions and projections from z-stacks of images were generated, and further processed with Fiji to measure the mean fluorescence intensity in the green and cfCy5-LP channels and calculate the mean NIR FP/mClover3 ratio for individual neurons in the hindbrain and spinal cord. Four individual larval fish were imaged to collect the data from 120 neurons for analysis in total per construct. The mean fluorescence intensity of each NIR FP was calculated, followed by the evaluation of statistically significant differences in brightness and compared with their behavior in cultured cells.

To quantify the photostability of NIR FP fluorescence in zebrafish neurons, four independent 4 dpf larvae co-expressing HO1 and the respective NIR FP underwent confocal microscopy imaging using a ×40 NA1.1 water immersion objective in the cfCy5-LP channel; an ROI was selected (96.88 × 96.88 µm) encompassing 5–10 positive neurons. To collect photobleaching data from 40 neurons (10 cells per larvae), a single plan image (optical section, 1.271 µm) was recorded continuously for 3 min with 70% laser power. Acquired images were processed with Fiji to measure the fluorescence intensity in individual neurons for each time point. The data were excluded from the analysis if zebrafish moved during image recording. Statistically significant differences in brightness were evaluated as described in the Data Analysis and Statistics section.

Characterization of NIR FPs in C. elegans neurons

The selected NIR FPs were expressed using extrachromosomal arrays in C. elegans and assessed for intracellular localization, brightness, and photostability in the Cy5-LP and Cy5.5 channels. For expression in C. elegans, the target genes were codon-optimized using the C. elegans Codon Adapter application (https://worm.mpi-cbg.de/codons/cgi-bin/optimize.py) with insertion of two introns and synthesized de novo (Tsingke Biotechnology). The optimized genes of NIR FPs were cloned into pSF11 vector (Addgene plasmid 179485) under the pan-neuronal tag-168 promoter in frame with the T2A-HO1 gene (HO1 from Homo sapiens). In addition, we cloned the mcCardinal gene into the pSF11 vector without the T2A-HO1 gene. The codon-optimized gene of mNeonGreen cloned into the pSF11 vector was used as a reporter marker for screening positive worms and for normalizing NIR fluorescence (cloned by GenScript USA). The establishment of transgenic lines was done by SunyBiotech according to standard protocols. In brief, wild-type N2 worms were co-injected with two plasmids encoding wNIR-FPs-T2A-HO1::wmNeonGreen with a 10 ng µl⁻¹ final concentration each. Transgenic lines were selected by green fluorescence and confirmed with sequencing of the target. Selected worms were maintained and grown on nematode growth medium (NGM) plates seeded with E. coli OP50-1 at 20 °C following standard protocols. Positive worms with green fluorescence (used without regard to sex) at the L4 stage of development were selected for further imaging. Worms were mounted on immunofluorescence assay slides (Jiangsu Shitai Zhenduan Jishu, 80383-0209-01), immobilized with 25 mM levamisole and imaged using a wide-field Nikon microscope with a ×100 0.45NA objective lens for quantification (exact microscope configuration described above; for Fig. 4b–d and Supplementary Fig. 18) and a Zeiss 980 confocal microscope for structural imaging (×100 0.45NA, ×40 0.95NA, 639 nm excitation laser for NIR, 635–757 nm emission range; for Fig. 4a) controlled by ZEN blue v3.5. Individual neurons were selected manually, and mean fluorescence intensity was calculated using Nikon Elements software.

Multicolor imaging in live zebrafish larvae

To examine the use of NIR FPs for multicolor imaging in vivo, emiRFP670 was chosen based on the result of the intracellular brightness and photostability analysis in zebrafish neurons, and its fusion proteins were co-expressed together with cyan, green and red FPs, each of which labels a different neuronal compartment. To further enhance the expression level, the zebrafish codon-optimized genes of the selected FPs were used. Zebrafish codon-optimized cDNAs of emiRFP670, mc Clover3 (green FP) and mScarlet-I (red FP) were synthesized de novo by Tsingke Biotechnology. A zebrafish codon-optimized mTurquoise2 (cyan FP) was generated by site-directed mutagenesis of the zebrafish codon-optimized Cerulean gene (Addgene 61389). A zebrafish codon-optimized TagRFP-T (red FP) was kindly provided by H. Burgess (Eunice Kennedy Shriver National Institute of Child Health and Human Development).

The expression cassette encoding dual subcellular reporters labeling plasma membrane and nucleus were composed of an amino-terminal 15-amino acid sequence of Fyn kinase (fused to mScarlet-I or mClover3) and H2B-mTurquoise2, which were linked via a T2A self-cleaving peptide. The resultant cassette was followed by the ocean pout antifreeze protein 3’UTR containing predicted polyadenylation signal (AFP polyA), and further assembled with HO1-SV40 polyA and Elb-10xUAS-Elb into the pTol2-MCS vector backbone. The inserted sequences were flanked by Tol2 transposase recognition sequence (pTol2-HO1-10U-Fyn-mScarlet-I/mC Lov3-H2B-mTurquoise2; see Fig. 5c for the expression cassette schematics). Similarly, pTol2-HO1-10U-mTurquoise2 and pTol2-10U-Fyn-mClover3-H2B-mTurquoise2 were generated for the cloning of autophagosome–lysosome (Fig. 5d) and synapse (Supplementary Fig. 21) reporters, respectively.

To monitor actin–microtubule cytoskeletal dynamics in growing axons of zebrafish neurons, LifeAct-mClover3–emiRFP670 fusions and microtubule plus-end binding protein 3 (EB3)–emiRFP670 fusions were generated for the labeling of F-actin and microtubule plus-ends, respectively.

Lysosomal-associated membrane protein 1 (LAMP1) was fused to the N-terminus of emiRFP670 to generate a reporter labeling the outer lysosomal membrane. A tandem fluorescent autophagic flux sensor, TagRFP-T-mClover3-zebrafish microtubule-associated protein 1-light chain 3 (LC3)-beta (TagRFP-T-mClover3-LC3) mimics mRFP1-EF-GFP-LC3 (ref. 37) but showed much brighter fluorescence than mRFP1-EF-GFP-LC3. This sensor visualizes autophagosomes marked with both green and red fluorescence, whereas acidic autolysosomes formed by autophagosome and lysosome fusion, are detected with only red fluorescence based on the different pH stability of these proteins (pKₐ = 6.5 and 4.5, for mClover3 and TagRFP-T, respectively, according to the data in FPbase (https://www.fpbase.org)). To visualize presynaptic vesicles, emiRFP670 was fused to the carboxy terminus of zebrafish synaptophysin b (Addgene 74316), followed by 3’UTR of zebrafish tubulinβ5 microtubule subunit gene (tubb5) (kindly provided by F. Giudicelli), enhancing the axonal transport of mRNA. Excitatory post-synapse reporter was generated by mScarlet-I fused to zebrafish post-synaptic density protein 95 (PSD95). Each reporter attached to the SV40 polyA sequence was assembled into pTol2-HO1-10U-Fyn-mScarlet-I-H2B-mTurquoise2, pTol2-HO1-10U-mTurquoise2 or pTol2-10U-Fyn-mClover3-H2B-mTurquoise2 to generate pTol2-F-actin–microtubule plus-ends (1), autophagosome–lysosome (2) or synapse reporters (3), respectively, as below. These constructs carry an insert composed of two bidirectional expression constructs (Fig. 21) reporters, respectively.

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1. pTol2-F-actin–microtubule plus-ends reporters, mediating co-expression of Fyn-mScarlet-I, H2B-mTurquoise2, EB3-emiRFP670 and LifeAct-mClover3 together with HO1 (Addgene 196134).
2. pTol2-autophagosome–lysosome reporters, mediating co-expression of cytoplasmic mTurquoise2,
TagRFP-T-mClover3-LC3 and Lamp1-emIRFP670 together with H01 (Addgene 196135).

3. pTol2-Synapse reporters, mediating co-expression of Fyn-mClover3, H2B-mTurquoise2, synaptophysin b-emIRFP670 and mScarlet-I-PSD95 (Addgene 196899).

pTol2-F-actin–microtubule reporters and pTol2-nbt3002x:KaNF- syt2a (Addgene 196901) were co-injected into brass embryos with Tol2 mRNA (1.5 nl injection mix containing 25 ng µl⁻¹ for each pTol2 plasmid and Tol2 mRNA). At thirty-three hours post-fertilization (33 hpf), larval fish expressing these reporters were sorted, followed by confocal microscopy image recording of primary motoneurons and their axons using a ×63 1.2NA water immersion objective (Leica SP8). For synapse imaging, pTol2-synapse reporters together with Tol2 mRNA (1.5 nl injection mix containing 25 ng µl⁻¹ for each pTol2 plasmid and Tol2 mRNA) were injected into Tg(2xen.cpc-E1B:KaLFNB,he1.1:MtagBP7F2) embryos in which KaNFb induces reporter expression in Purkinje cells restrictively. Injected larval fish showing green fluorescence in the cerebellum were sorted at 4 dpf, followed by confocal microscopy image recording of Purkinje cells at 5 dpf using a ×63.1 2NA water immersion objective (Leica SP8). Green and NIR fluorescence emission signals were recorded simultaneously (mClover3: excitation, 488 nm using an argon laser; emission, 490–550 nm; emIRFP670: excitation, 633 nm using a HeNe laser; emission, 641–765 nm), followed by image recording in the cyan (mTurquoise2: excitation, 405 nm using a UV laser; emission, 421–482 nm) and red (mScarlet-I: DPSS 561 nm laser, emission, 570–616 nm) emission ranges. Reconstructions and projections from z-stacks of images were generated by Fiji. For time-lapse imaging of a growing axon tip of a primary motoneuron, images were recorded every 1.29 s in a single focal plane. Acquired images were processed with Fiji, followed by their animation (25 frames per s) using the QuickTime player (Apple). For autophagosome–lysosome imaging, pTol2-autophagosome–lysosome reporters were injected into Tg(2xen.cpc-E1B:KaLFNB,he1.1:MtagBP7F2) embryos together with Tol2 mRNA (1.5 nl injection mix containing 25 ng µl⁻¹ for each pTol2 plasmid and Tol2 mRNA). At 4 dpf, larval fish expressing each reporter were sorted, sedimented with 0.02% Tricain, and embedded in 1.5% low-melting agarose. The larvae were treated with 1.5 µM Rapalpin1-1 (Ambeam) in 30% Danieau containing 0.02% Tricain for 4 h, followed by imaging of cerebellar Purkinje cells using the Airyscan mode of a ZEISS LSM 880 microscope with an LD C-Apochromat ×63 1.15NA water immersion objective (Zeiss). Each emission signal of the green (mClover3: excitation, 488 nm using an argon laser), cyan (mTurquoise2: excitation, 405 nm using a UV laser), red (TagRFP-T: excitation, 561 nm using a DPSS laser) and NIR (emIRFP670: excitation, 633 nm using a HeNe laser) FP was recorded separately. For the adjustable light path in the Airyscan system, the main beam splitter for the wavelength of 488/561/633 nm was chosen in the visible light path and 405 nm for the invisible light path. To narrow down the detectable fluorescence spectrum, different emission dual filters were used for the detection of blue (BP 465–505 nm and LP 525 nm) and green (BP 495–550 nm and LP 570 nm) fluorophores, while the same filters could be used to detect the emission of both red and NIR (BP 570–620 nm and LP 645 nm) fluorescence, respectively. For image acquisition, the system-defined optimal settings for pixel resolution and Z-stack slice thickness (0.23 µm) were used. The acquired images were processed with the ZEN 2.3 black software and the Zeiss Airyscan processing option. The brightness and contrast were later evenly adjusted across the images in ImageJ.

Data analysis and statistics
Data were analyzed offline using NIS Elements Advance Research software (versions 5.21.00 and 5.30.00), Excel (Microsoft), OriginPro (2019b, OriginLab), GraphPad Prism 8, Fiji ImageJ 2.9.01/1.31t, CytExpert 2.4, Amersham Imager 680 analysis software and the Microscope online application (https://www.fphbase.org/microscope). For fluorescence signal intensity analysis, an ROI including cells, or cell bodies in the case of neurons, and a neighboring cell-free region (as a background) were selected either manually or automatically using NIS Elements Advance Research software. Mean intensity was extracted for each ROI in the Cy5 LP, Cy5-BP, Cy5.5 and FITC channels, and the corresponding background was subtracted. The means of ratios (NIR to green) indicating the normalized intracellular brightness of the fluorescent proteins are reported in all experiments. For OSER assay analysis we counted the total number of cells and the number of cells that contain whorl fluorescent structures, which are visibly distinct from endoplasmic reticular networks. For photostability in C. elegans the data were corrected for movement using the Image plugin Time Series Analyzer V3 (https://imagej.nih.gov/ij/plugins/time-series.html). For photostability in cultured neurons and live brain slices, the data were corrected for movement using the Image plugin Template matching (https://imagej.nih.gov/ij/plugins/template-matching.html). All statistics were performed in JMP (SAS), OriginPro or R (the R Foundation).

The Shapiro–Wilks test was used for the normal distribution test for datasets with N < 5,000. For datasets where sample size exceeded 5,000, the Kolmogorov–Smirnov test for normality was used instead of the Shapiro–Wilks test. The Kolmogorov–Smirnov and Dunn’s tests were used for pairwise comparison instead of the Nemenyi test, given that the datasets under comparison did not have equal size. Moreover, we performed a power analysis using 5 out of 22 proteins spanning from mNeptune2.5 to miRFP720 to determine the minimum sample size needed to produce a statistically significant experiment (Supplementary Table 1). P < 0.05 was considered statistically significant (see Supplementary Data I for detailed statistics).

Statistics and reproducibility
Due to limited space in the figure legends, we present in detail in this section the sample size, number of independent experiments, statistical tests used with their parameters and exact P values. For Fig. 1a three independent transfections were performed for each protein and a total of n = 1,945 cells for mNeptune2.5, n = 8,481 cells for mCardinal, n = 3,562 cells for E2-Crimson, n = 5,930 cells for smURFP, n = 6,230 cells for BDFP1.6, n = 6,658 cells for emIRFP670, n = 6,035 cells for miRFP670-2, n = 8,911 cells for iRFP670, n = 4,470 cells for miRFP680, n = 5,118 cells for iRFP682, n = 3,325 cells for emIRFP703, n = 5,970 cells for BDFP1.8, n = 4,417 cells for miRFP2, n = 5,439 cells for miFF, n = 6,060 cells for IF2, n = 4,831 cells for mRhubarb713, n = 4,819 cells for miRFP713, n = 5,771 cells for IRFP713, n = 5,965 cells for SNIFP, n = 6,192 cells for mRhubarb719, n = 4,619 cells for mRhubarb720, and n = 4,116 cells for miRFP720 were analyzed. Kruskal–Wallis ANOVA was performed with 95% confidence intervals (alpha = 5%, one-sided), d.f. = 21, chi-squared = 74,637.94216, P = 0. For pairwise comparison the Kolmogorov–Smirnov test was performed with 95% confidence intervals, two-sided. Exact D, Z and P values are provided in Supplementary Dataset 1 for each pair.

For Fig. 1b three independent transfections were performed for each protein and a total of n = 99 cells for mNeptune2.5, n = 100 cells for mCardinal, n = 94 cells for E2-Crimson, n = 100 cells for smURFP, n = 117 cells for BDFP1.6, n = 111 cells for emIRFP670, n = 111 cells for miRFP670-2, n = 110 cells for iRFP670, n = 98 cells for miRFP680, n = 113 cells for IFP682, n = 98 cells for emIRFP703, n = 98 cells for BDFP1.8, n = 110 cells for miRFP2, n = 101 cells for miFF, n = 110 cells for IF2, n = 97 cells for mRhubarb713, n = 103 cells for miRFP713, n = 96 cells for iRFP713, n = 110 cells for SNIFP, n = 107 cells for mRhubarb719, n = 114 cells for mRhubarb720 and n = 108 cells for miRFP720 were analyzed. Kruskal–Wallis ANOVA was performed with 95% confidence intervals (alpha = 5%, one-sided), d.f. = 21, chi-squared = 2,154.8492, P = 0. For pairwise comparison the Kolmogorov–Smirnov test was performed with 95% confidence intervals, two-sided. Exact D, Z and P values are provided in Supplementary Dataset 1 for each pair.
For Fig. 1d two independent transductions were performed for each protein and a total of n = 44 neurons for mNeptune2.5, n = 48 neurons for mCardinal, n = 45 neurons for E2-Crimson, n = 50 neurons for smURFP, n = 50 neurons for BDFP1.6, n = 51 neurons for emiRFP670, n = 57 neurons for miRFP670-2, n = 53 neurons for iRFP713, n = 51 neurons for mRhubarb713, n = 50 neurons for SNIFP, n = 45 neurons for miRFP680, n = 54 neurons for miRFP703, n = 54 neurons for BDFP1.8, n = 53 neurons for miRFP682, n = 52 neurons for miRFP670, n = 52 neurons for miRFP680, n = 44 neurons for miRFP670-2, n = 53 neurons for miRFP680, n = 43 neurons for miRFP703, n = 47 neurons for miRFP720 and n = 51 neurons for miRFP720 were analyzed. Kruskal–Wallis ANOVA was performed with 95% confidence intervals (alpha = 5%, one-sided), d.f. = 21, chi-squared = 1,386.5542, P = 0.0. For pairwise comparison Dunn’s test was used. Exact Z and P values are provided in Supplementary Dataset 1 for each pair.

For Fig. 1e two independent transductions were performed for each protein and a total of n = 45 neurons for mNeptune2.5, n = 46 neurons for mCardinal, n = 50 neurons for E2-Crimson, n = 50 neurons for smURFP, n = 53 neurons for BDFP1.6, n = 54 neurons for emiRFP670, n = 51 neurons for miRFP670-2, n = 56 for miRFP670, n = 52 neurons for miRFP680, n = 44 neurons for miRFP682, n = 53 neurons for emiRFP703, n = 54 neurons for BDFP1.8, n = 53 neurons for miRFP720, n = 46 neurons for miRFP, n = 43 neurons for IFP2, n = 43 neurons for mRhubarb713, n = 49 neurons for miRFP713, n = 54 neurons for SNIFP, n = 50 for mRhubarb719, n = 41 neurons for mRhubarb720 and n = 49 neurons for miRFP720 were analyzed. Kruskal–Wallis ANOVA was performed with 95% confidence intervals (alpha = 5%, one-sided), d.f. = 21, chi-squared = 1,056.94323, P = 2.04 × 10−21. For pairwise comparison Dunn’s test was used. Exact Z and P values are provided in Supplementary Dataset 1 for each pair.

For Fig. 2c three mice were used for each protein and a total of n = 100 neurons for mCardinal, n = 237 neurons for emiRFP670, n = 349 neurons for miRFP670-2, n = 213 neurons for miRFP680, n = 310 neurons for mRhubarb713, n = 204 neurons for miRFP713, n = 182 neurons for IFP713 and n = 386 neurons for miRFP720 were analyzed. Kruskal–Wallis ANOVA was performed with 95% confidence intervals (alpha = 5%, one-sided), d.f. = 7, chi-squared = 1,454.85847, P = 0. For pairwise comparison Dunn’s test was used. Exact Z and P values are provided in Supplementary Dataset 1 for each pair.

For For Fig. 2e two mice were used for each protein and a total of n = 213 neurons for mCardinal, n = 98 neurons for emiRFP670, n = 252 neurons for miRFP670-2, n = 146 neurons for miRFP680, n = 190 neurons for mRhubarb713, n = 124 neurons for miRFP713, n = 149 neurons for IFP713 and n = 92 neurons for miRFP720 were analyzed. Kruskal–Wallis ANOVA was performed with 95% confidence intervals (alpha = 5%, one-sided), d.f. = 7, chi-squared = 1,019.60205, P = 7.0 × 10−21. For pairwise comparison Dunn’s test was used. Exact Z and P values are provided in Supplementary Dataset 1 for each pair.

For Supplementary Fig. IgPmiRFP680-IRES-EGFP = 0.006645, PmiRFP680-IRES-EGFP-P2A-FusionRed = 4.04404 × 10−21, PmiRFP680-IRES-EGFP-P2A-FusionRed = 1.69504 × 10−14. For pairwise comparison Dunn’s test was used. Exact Z and P values are provided in Supplementary Dataset 1 for each pair.

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References

43. Papadaki, S. et al. Dual-expression system for blue fluorescent protein optimization. Sci. Rep. 12, 10190 (2022).

44. Drobizhev, M. et al. Local electric field controls fluorescence quantum yield of red and far-red fluorescent proteins. Front. Mol. Biosci. 8, 633217 (2021).

45. Luchowski, R. et al. Instrument response standard in far-red, GFP-like fluorescent proteins. PLoS One 13, e0208075 (2018).

46. Drobizhev, M., Molina, R. S. & Hughes, T. E. Characterizing the two-photon absorption properties of fluorescent molecules in the 680–1300 nm spectral range. Bio Protoc. 10(2), e3498 (2020).

47. Kim, J.-Y., Grunke, S. D., Levites, Y., Golde, T. E. & Jankowsky, J. L. Intracerebroventricular viral injection of the neonatal mouse brain for persistent and widespread neural transduction. J. Vis. Exp. 15(91), 51863 (2014).

Data availability

The mass spectrometry proteomics data generated in this study have been deposited in the iProX database with the dataset identifier IPX000576700 (https://www.iprox.cn/page/SCV017.html?query=IPX000576700). The total size of the files acquired for this study was about 2 TB, which exceeds the limit of the FigShare repository, therefore only the most essential raw datasets, that is, the raw images with metadata supporting the results in Figs. 1–5, Extended Data Figs. 1–7 and Supplementary Figs. 2, 3, 5, 7, 9, 11 are available at FigShare (https://figshare.com/authors/Hanbin_Zhang/14524646 and repository, therefore only the most essential raw datasets, that is, the raw images with metadata supporting the results in Figs. 1–5, Extended Data Figs. 1–7 and Supplementary Figs. 2, 3, 5, 7, 9, 11 are available at FigShare (https://figshare.com/authors/Hanbin_Zhang/14524646 and https://doi.org/10.6084/m9.figshare.2197570). The rest of the files are available from the corresponding author upon request. Source data files are provided with this paper. All plasmids used in this study are available from Addgene and WeKwikGene (https://wekwikgene.wlslb.edu.cn/). Source data are provided with this paper.

Code availability

The custom MatLab code for analysis photostability data is available at Zenodo (https://doi.org/10.5281/zenodo.7992722).
49. Shu, X. et al. Mammalian expression of infrared fluorescent proteins engineered from a bacterial phytochrome. Science 324, 804–807 (2009).
50. Westerfield, M. The Zebrafish Book. A Guide for the Laboratory Use of Zebrafish (Danio rerio). 5th edn (Univ. Oregon Press, 2007).
51. Urasaki, A., Morvan, G. & Kawakami, K. Functional dissection of the Tol2 transposable element identified the minimal cis-sequence and a highly repetitive sequence in the subterminal region essential for transposition. Genetics 174, 639–649 (2006).
52. Mishima, Y. et al. Zebrafish miR-1 and miR-133 shape muscle gene expression and regulate sarcomeric actin organization. Genes Dev. 23, 619–632 (2009).
53. Ishikawa, K., Sato, M., Ito, M. & Yoshida, T. Importance of histidine residue 25 of rat heme oxygenase for its catalytic activity. Biochem. Biophys. Res. Commun. 182, 981–986 (1992).
54. Green, R. A. et al. Expression and imaging of fluorescent proteins in the C. elegans gonad and early embryo. Methods Cell Biol. 85, 179–218 (2008).
55. Baraban, M., Anselme, I., Schneider-Maunoury, S. & Giudicelli, F. Zebrafish embryonic neurons transport messenger RNA to axons and growth cones in vivo. J. Neurosci. 33, 15726–15734 (2013).
56. Strack, R. L. et al. A rapidly maturing far-red derivative of DsRed-Express2 for whole-cell labeling. Biochemistry 48, 8279–8281 (2009).
57. Rodriguez, E. A. et al. A far-red fluorescent protein evolved from a cyanobacterial phycobiliprotein. Nat. Methods 13, 763–769 (2016).
58. Ding, W. L. et al. Far-red acclimating cyanobacterium as versatile source for bright fluorescent biomarkers. Biochim. Biophys. Acta Mol. Cell Res. 1865, 1649–1656 (2018).
59. Yu, D. et al. An improved monomeric infrared fluorescent protein for neuronal and tumour brain imaging. Nat. Commun. 5, 3626 (2014).
60. Rogers, O. C., Johnson, D. M. & Firnberg, E. mRhubarb: engineering of monomeric, red-shifted, and brighter variants of IRFP using structure-guided multi-site mutagenesis. Sci. Rep. 9, 15653 (2019).

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Author contributions
H.Z. and S.P. performed characterization in cultured mammalian cells. X.S. characterized proteins in the expansion microscopy protocols. M.D. and H.Z. characterized proteins in vitro. H.Z. and X.S. performed the mouse experiments, X.W., M.R., R.W.K. and K.N. performed the zebrafish experiments, S.P., L.Y. and L.W. performed the C. elegans experiments. H.Z., S.P. and K.D.P. analyzed the data and wrote the manuscript with input from all of the authors. K.D.P. designed and oversaw all aspects of the project.

Competing interests
K.D.P. is the co-founder of a company that pursues commercial applications of expansion microscopy and is listed as an inventor on several patent applications concerning development of new expansion microscopy methods. All other authors have no competing interests.

Additional information
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Extended Data Fig. 1 | See next page for caption.
Extended Data Fig. 1 | Quantitative assessment of intracellular brightness and photostability of NIR FPs in live HEK cells. (a) Intracellular normalized brightness of NIR FPs imaged in three channels: (i) Cy5-LP, (ii) Cy5-BP and (iii) Cy5.5 under 55 mW/mm² excitation power (n > 2000 cells for each NIR-FP from three independent transfections in each channel). Brightness for each FP was normalized to the EGFP signal (here and in panel e). Asterisks (*) indicate signal-to-background ratio <2.0 throughout; in panels a–c box plots highlighted with brown correspond to top 5 NIR FPs for each panel. (b) Photobleaching half-times of NIR-FPs under Cy5 (i and ii, excitation power 58 mW/mm²) and Cy5.5 (iii, excitation power 56–58 mW/mm²; n > 93 cells for each NIR-FP from four independent transfections). (c) Product of mean brightness and mean half-time fluorescence presented as bar graph (mean ± SEM) (due to small value of SEM the error bars are not visible for some bars). (d) Normalized photobleaching curves of NIR FPs under (i) Cy5 (excitation power 58 mW/mm²) and (ii) Cy5.5 (excitation power 56–58 mW/mm²) illumination for the results shown in panel b (n > 93 cells for each NIR-FP from four independent transfections in each channel). Fluorescence was normalized to the intensity value of corresponding FP at t = 0 s. NIR FPs that exhibited signal-to-background ratio lower than 2.0 in Cy5.5 channel are not shown in the graph, however the corresponding curves are available in the source datasets except for mNeptune2.5, which was not visible in Cy5.5 channel at all and thus was not measured. (e) Side-by-side normalized brightness comparison without and with BV administration imaged in (i) Cy5-LP, (ii) Cy5-BP and (iii) Cy5.5 under 55 mW/mm² excitation power (n > 2000 cells for each NIR-FP from three independent transfections in each channel). Outliers not shown but included in all calculations and available in the source datasets. See Supplementary Table 2 and Supplementary Dataset 1 for the detailed descriptive statistics and exact p-values.
Extended Data Fig. 2 | See next page for caption.
Extended Data Fig. 2 | Quantitative assessment of intracellular brightness and photostability NIR FPs in live primary cultured mouse neurons. (a, b) Intracellular normalized brightness of NIR FPs imaged after (a) Calcium Phosphate transfection and (b) viral transduction in Cy5-LP, (ii) Cy5-BP and (iii) Cy5.5 under 55 mW/mm² excitation power (n > 40 neurons for each NIR FP from two independent cultures). Brightness for each FP was normalized to the EGFP signal (here and in panel f). In panels a–d box plots highlighted with brown correspond to top 5 NIR FPs for each panel. (c) Photobleaching half-times of NIR FPs under Cy5 (i and ii, excitation power 55 mW/mm²) and Cy5.5 (iii, excitation power 55 mW/mm²; n > 42 neurons for each NIR FP from two independent cultures). NA – not applicable due to low fluorescence signal. (d) Product of mean brightness and mean half-time fluorescence presented in bar graph (mean ± SEM) (due to small value of SEM the error bars are not visible for some bars). (e) Normalized photobleaching curves of NIR FPs under (i) Cy5 (excitation power 55 mW/mm²) and (ii) Cy5.5 (excitation power 55 mW/mm²) illumination for the results shown in c. Fluorescence was normalized to the intensity value of corresponding FP at t = 0 s. (f) Side-by-side normalized brightness comparison before and after BV administration imaged in (i) Cy5-LP, (ii) Cy5-BP and (iii) Cy5.5 under 55 mW/mm² power (n > 20 neurons for each NIR FP from two independent cultures). see Supplementary Dataset 1 for the detailed descriptive statistics and exact p-values.
Extended Data Fig. 3 | See next page for caption.
Extended Data Fig. 3 | Quantitative characterization of NIR FPs in fixed HEK cells. (a) NIR-to-green fluorescence ratio in fixed HEK cells imaged in (i) Cy5-LP, (ii) Cy5-BP and (iii) Cy5.5 under 55 μW/mm² excitation power (n > 1976 cells for each NIR FP from two independent transfections in each channel). Brightness for each FP was normalized to the EGFP signal. Asterisks (*) indicate signal-to-background ratio <2.0 throughout the figure based on brightness of fixed HEK cells; in panels a, c, d box plots highlighted with brown correspond to top 5 NIR FPs for each panel. (b) Normalized photobleaching curves of NIR FPs under (i) Cy5 (excitation power 55 μW/mm²) and (ii) Cy5.5 (excitation power 55 μW/mm²) illumination in fixed HEK cells (n = 40 cells for each NIR FP from two independent transfections in each channel). Fluorescence was normalized to the intensity value of corresponding FP at t = 0 s. NIR FPs that exhibited lower than 2.0 signal-to-background ratio in Cy5.5 channel are not shown in the graph; however, the corresponding curves are available in the source datasets. (c) The products of mean NIR to-green fluorescence ratio and mean fluorescence half-time in (i) Cy5-LP and (ii) Cy5.5 are presented in the form of bar graph (mean ± SE). (d) The products of mean absolute NIR brightness to mean fluorescence half-time in (i) Cy5-LP and (ii) Cy5.5 are presented in the form of bar graph (mean ± SE). (e) Comparison of selected NIR FPs in proExM. (f) Representative images of NIR FPs in PFA fixed and proExM treated HEK cells acquired in Cy5-LP (NIR FP) and FITC (GFP) channels (n > 61 cells for each NIR FP from one independent transfection). The dynamic brightness range was adjusted independently to facilitate visualization of transfected cells in fixed and proExM processes for each NIR FPs. Images were obtained through single-plane scanning and without projection processing. (g) Fluorescence retention of NIR FPs in HEK cells upon PFA fixation presented as bar graph (mean of NIR retention for each protein) (Cy5-LP, n > 1974 cells for each NIR FP from two independent transfections). Black dots indicate ratio of mean brightness before and after fixation for two transfections. (h) Absolute NIR fluorescence of fixed (brown box plots with notches) and proExM treated (orange box plots with notches) HEK cells (n > 61 cells for each NIR FP from one independent transfection). See Supplementary Table 2 and Supplementary Dataset 1 for the detailed descriptive statistics and exact p-values.
Extended Data Fig. 4 | Fluorescence imaging of NIR FPs fusions in live HeLa cells. Representative images of (from top left) iRFP670-actin, miRFP670-2-actin, miRFP680-actin, miRFP713-actin, miRFP720-actin, H2B-iRFP670, H2B-miRFP670-2, H2B-miRFP680, H2B-miRFP713, H2B-miRFP720, Cx43-iRFP670, Cx43-miRFP670-2, Cx43-miRFP680, Cx43-miRFP713, Cx43-miRFP720, keratin-iRFP670, keratin-miRFP670-2, keratin-miRFP680, keratin-miRFP713, keratin-miRFP720, iRFP670-tubulin, miRFP670-2-tubulin, miRFP680-tubulin, miRFP713-tubulin, miRFP720-tubulin (n > 15 cells for each construct from two independent transfection). For each image the dynamic range was adjusted independently to facilitate visualization and images were generated through maximum projection.
Extended Data Fig. 5 | Two-photon cross-section spectra of selected NIR-FPs. Two-photon cross-section spectra of mCardinal, emiRFP670, miRFP670-2, miRFP680, mRhubarb713, miRFP713, iRFP713, and miRFP720 presented versus laser wavelength used for excitation (GM, Goeppert-Mayer units).
Extended Data Fig. 6 | See next page for caption.
Extended Data Fig. 6 | Quantitative characterization of the selected NIR FPs expressed in L2/3 cortical neurons in mouse brain tissue. (a, b, c) Intracellular normalized brightness of NIR FPs imaged in acute brain slices from (a) one-month, (b) two-month, (c) three-month old mice in (i) Cy5-LP, (ii) Cy5-BP, (iii) Cy5.5 channels (excitation power 55 mW/mm²; n ≥ 100 neurons from 3 mice for each protein in each channel). Brightness for each FP was normalized to the EGFP signal (here and in panel d). (d) Intracellular normalized brightness of NIR-FPs imaged in PFA-fixed brain slices from one-month old mice in (i) Cy5-LP, (ii) Cy5-BP, (iii) Cy5.5 (excitation power 55 mW/mm²; n > 90 neurons from 2 mice for each protein in each channel). (e, f) Normalized photobleaching curves of NIR-FPs measured in (e) acute brain slices and (f) PFA-fixed brain slices in (i) Cy5-LP and (ii) Cy5.5 channels (excitation power 55 mW/mm²; n > 40 neurons from 2 mice for each protein for each channel). Fluorescence was normalized to the intensity value of corresponding FP at t = 0 s. See Supplementary Dataset 1 for the detailed descriptive statistics and exact p-values.
Extended Data Fig. 7 | Quantitative characterization of the selected NIR FPs expressed in mouse liver in vivo. (a) Representative wide-field fluorescence images of NIR FPs expressed in mouse liver acquired in Cy5-LP, Cy5-BP, Cy5.5, and FITC channels (excitation power 55 mW/mm² for Cy5 and Cy5.5 channels; n = 3 liver slices from 3-4 mice for each protein). (b,c,d) Intracellular normalized brightness of NIR FPs imaged in fresh liver tissue from three-month-old mice in (b) Cy5-LP, (c) Cy5-BP, (d) Cy5.5 channels (excitation power 55 mW/mm²; n > 154 cells from 3-4 mice for each protein in each channel). Brightness for each FP was normalized to the EGFP signal. See Supplementary Dataset 1 for the detailed descriptive statistics and exact p-values.
## Extended Data Table 1 | List of the NIR FPs selected for quantitative assessment in cultured cells

| Protein        | Excitation maximum (nm) | Emission maximum (nm) | Extinction coefficient (M⁻¹cm⁻¹) | Quantum yield | Molecular brightness | Ref. |
|----------------|-------------------------|-----------------------|----------------------------------|---------------|---------------------|------|
| mNeptune2.5    | 599                     | 643                   | 95,000                           | 0.24          | 22.8                | 4    |
| mCardinal      | 604                     | 659                   | 87,000                           | 0.19          | 16.53               | 4    |
| E2-Crimson     | 611                     | 646                   | 126,000                          | 0.23          | 28.98               | 50   |
| smURFP         | 642                     | 670                   | 180,000                          | 0.18          | 32.4                | 60   |
| BDFP1.6        | 642                     | 666                   | 100,000                          | 0.197         | 19.7                | 61   |
| emiRFP670      | 642                     | 670                   | 87,400                           | 0.14          | 12.24               | 62   |
| miRFP670-2     | 643                     | 670                   | 103,000                          | 0.136         | 14.01               | 62   |
| iRFP670        | 643                     | 670                   | 114,000                          | 0.11          | 12.54               | 5    |
| miRFP680       | 661                     | 680                   | 94,000                           | 0.145         | 13.63               | 62   |
| iRFP682        | 663                     | 682                   | 90,000                           | 0.11          | 9.9                 | 5    |
| emiRFP703      | 674                     | 703                   | 90,900                           | 0.086         | 7.82                | 62   |
| BDFP1.8        | 678                     | 702                   | 77,027                           | 0.061         | 4.7                 | 24   |
| miRFP2         | 676                     | 706                   | 55,600                           | 0.043         | 2.39                | 6    |
| mIFP           | 683                     | 704                   | 82,000                           | 0.08          | 6.56                | 18   |
| IFP2.0         | 690                     | 711                   | 86,000                           | 0.08          | 6.88                | 63   |
| mRhubard713    | 690                     | 713                   | 113,457                          | 0.0763        | 8.66                | 64   |
| miRFP713       | 690                     | 713                   | 99,000                           | 0.07          | 6.93                | 62   |
| iRFP713        | 690                     | 713                   | 105,000                          | 0.06          | 6.3                 | 3    |
| SNIFP          | 697                     | 720                   | 149,200                          | 0.022         | 3.28                | 11   |
| mRhubarb719    | 700                     | 719                   | 83,769                           | 0.068         | 5.7                 | 64   |
| mRhubarb720    | 701                     | 720                   | 94,941                           | 0.0646        | 6.13                | 64   |
| miRFP720       | 702                     | 720                   | 98,000                           | 0.061         | 5.98                | 9    |
Extended Data Table 2 | Spectroscopic and biochemical properties of the selected NIR FPs measured in this study

| Protein   | Abs (nm) | Em (nm) | ε<sub>max</sub> (mM<sup>-1</sup> cm<sup>-1</sup>)<sup>a</sup> | ε<sub>max</sub> (mM<sup>-1</sup> cm<sup>-1</sup>)<sup>b</sup> | φ<sup>b</sup> | τ (ns) | τ<sub>R = τ/φ</sub> (ns) | τ<sub>0→S<sub>1</sub></sub> σ<sub>2</sub> (GM) (λ<sub>0</sub>, nm) | τ<sub>0→S<sub>2</sub></sub> σ<sub>2</sub> (GM) (λ<sub>0</sub>, nm) | pK<sub>a</sub> |
|-----------|---------|-------|-----------------------------|-----------------------------|------|------|----------------|----------------|----------------|------|
| mCardinal | 603     | 645   | 79<sup>c</sup> [87<sup>d</sup>] | 75.4 | 0.21 [0.19<sup>d</sup>] | 1.38 [1.3<sup>e</sup>] | 6.6 | 88 (800 nm) | 48 (1200 nm) | 5.4 [5.3<sup>e</sup>] |
| emiRFP670 | 385,643 | 667   | 81 [87.4<sup>f</sup>] | 46 | 0.16 [0.14<sup>e</sup>] | 1.53 | 9.6 | 200 (832 nm) | 132 (880 nm) | 17 (1196 nm) | 3.4, 10.3 [4.5<sup>f</sup>] |
| miRFP670-2 | 387,645 | 669   | 82 [103<sup>f</sup>] | 49 | 0.16 [0.136<sup>e</sup>] | 1.42 | 8.9 | 188 (832 nm) | 109 (880 nm) | 18 (1196 nm) | 3.7 [4.5<sup>f</sup>] |
| miRFP680  | 381,663 | 677   | 88 [94.0<sup>f</sup>] | 48 | 0.16 [0.145<sup>f</sup>] | 1.67 | 10.4 | 158 (860 nm) | 116 (890 nm) | 23 (1220 nm) | 3.9 [4.5<sup>f</sup>] |
| mRhubarb713 | 389,690 | 710   | 77 [113.5<sup>f</sup>] | 53 | 0.069 [0.0763<sup>f</sup>] | 0.78 | 11.3 | 142 (890 nm) | 22 (1280 nm) | 4.2 |
| miRFP713  | 391,690 | 709   | 93 [99.0<sup>f</sup>] | 52 | 0.079 [0.077<sup>f</sup>] | 0.89 | 11.3 | 154 (890 nm) | 25 (1284 nm) | 4.3, 10.9 [3.5<sup>f</sup>] |
| iRFP713   | 390,690 | 710   | 92 [98.0<sup>f</sup>] | 56 | 0.073 [0.063<sup>f</sup>] | 0.78 [0.63<sup>f</sup>] | 10.7 | 113 (890 nm) [446 (890 nm)] | 19 (1276 nm) [74 (1280 nm)] | 4.3 [4.5<sup>b</sup>] |
| miRFP720  | 393,703 | 718   | 95 [98.0<sup>f</sup>] | 52 | 0.064 [0.061<sup>f</sup>] | 0.81 | 12.7 | 166 (888 nm) | 132 (900 nm) | 26 (1300 nm) | 4.3, 10.7 [4.5<sup>i</sup>] |

ε<sub>max</sub>, extinction coefficient; φ, quantum yield; τ, fluorescence lifetime; σ<sub>2</sub>, two-photon cross-section; λ, wavelength. <sup>a</sup>Calculated based on the Soret band peak; <sup>b</sup>Calculated based on the Strickler–Berg equation. <sup>c</sup>Measured using alkaline denaturation. <sup>d</sup>Data from ref. 4. <sup>e</sup>Data from ref. 46. <sup>f</sup>Data from ref. 13. <sup>g</sup>Data from ref. 60. <sup>h</sup>Data from ref. 10. <sup>i</sup>Data from ref. 16. <sup>j</sup>Data from ref. 9.
**Reporting Summary**

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**Statistics**

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

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- The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
- A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
- The statistical test(s) used AND whether they are one- or two-sided
  - Only common tests should be described solely by name; describe more complex techniques in the Methods section.
- A description of all covariates tested
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- Estimates of effect sizes (e.g. Cohen's d, Pearson's r), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

**Software and code**

Policy information about availability of computer code

**Data collection**

Data was collected using NIS-Elements Advance Research software (version 5.21.00), Leica Application Suite X Office v3.5.5, Olympus FV3000-IX83, ZEN blue v3.5, ZEN black v2.3, CytExpert v2.4, Amersham Imager 680. Data collection for fluorescence spectra of NIR FPs were done using https://www.fpbase.org.

**Data analysis**

Data was analyzed offline using NIS-Elements Advance Research software (version 5.21.00 and 5.30.00), Excel (Microsoft), OriginPro (2019b, OriginLab), GraphPad Prism v8, Fiji 2.9.01/1.53t ImageJ, the Microscope online application (https://www.fpbase.org/microscope), CytExpert v2.4, Amersham Imager 680 analysis software.

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

**Data**

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

The mass spectrometry proteomics data generated in this study have been deposited to the iProX database with the dataset identifier IPX0005767000. The total size of the files acquired for this study was about 2 TB, which exceeds the limit of the fileshare repository, therefore only the most essential raw datasets including raw images supporting results in Fig. 1-5, Extended Data Fig. 1-7, Supplementary Fig. 2, 3, 5, 7, 9, 11 are available at FigShare [https://figshare.com/authors/].
Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

**Sample size**

For cell culture experiments we performed power based on the pilot data reported in the Stage 1 manuscript to identify the lowest number of cells needed for the experiments. For in vivo experiments we did not perform a power analysis because prior information on mean and standard deviation of brightness for tested NIR FPs in neurons in model organism is lacking, as noted in Dell et al., Sample Size Determination, 2003: "It is not possible to compute a sample size for certain types of experiments because prior information is lacking or because the success of the experiment is highly variable, such as in producing a transgenic animal* for C. elegans and zebrafish in vivo experiments we produced transgenic animals. Therefore the sample size for in vivo experiments was based on our past experience in characterizing neurotechnologies. The suggested number of samples for in vivo assessment was approved during Stage 1 of the manuscript preparation. Full statistical analysis for the performed experiments are summarized in Supplementary Table 1, 2 and Supplementary Dataset 1.

**Data exclusions**

P7. Several NIR FPs (smURFP, miRFP2, mIFP, IFP2, mRhubarb719, and mRhubarb720) were either too dim or did not form classical whorl structures and thus were excluded from further assessment.

P22. According to Cranfill et al. recommendations, positive cells selected for analysis will have overall similar fluorescence brightness, and cells that are significantly brighter will be excluded (indications of unhealthy or highly stressed cells). Cells with non-spherical nuclei, ER sheet architectures, or condensed nuclei will be excluded from the assay.

P18. The data were excluded from the analysis if cells died after transfection or cell culture was contaminated with bacteria or yeast.

P18 and P19. Cells that detached or died during photobleaching experiments were excluded from data analysis.

P26. Animals that show no GFP fluorescence were excluded from the study, the exclusion criterion was pre-established.

P28. The data were excluded from the analysis if zebrafish moved during image recording.

**Replication**

All attempts at replication were successful. For live HEK imaging, all experiments were performed independently three times except for photostability experiments, which were reproduced four times independently. For fixed HEK cells, all experiments were reproduced twice independently, except for the ExM measurements, which were performed only once. For OSER assay, all experiments performed twice independently. For fusion imaging in HeLa cells including multicolor imaging, the experiments were performed once with three replicates for each tested fusion. For live neuronal culture imaging, all experiments were reproduced twice independently with 20 replicates for each experiment. AAV injections for brain tissue in mice were performed once with 3-4 replicates for each protein. C. elegans experiments were performed once with 30 replicates for each protein. Zebrafish experiments were performed once with 4 replicates for each protein, except for multicolor imaging in zebrafish, which was performed once with 10 replicates.

**Randomization**

All cell culture experiments were performed in random order. AAV injections, animal dissections, and tissue imaging were preformed in random order.

**Blinding**

The researchers were blinded to the sample identities during data collection and analysis

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

**Materials & experimental systems**

| n/a | Involved in the study |
|-----|-----------------------|
| ☒ Antibodies |
| ☒ Eukaryotic cell lines |
| ☒ Palaeontology and archaeology |
| ☒ Animals and other organisms |
| ☒ Human research participants |
| ☒ Clinical data |
| ☒ Dual use research of concern |

**Methods**

| n/a | Involved in the study |
|-----|-----------------------|
| ☒ ChIP-seq |
| ☒ Flow cytometry |
| ☒ MRI-based neuroimaging |
Antibodies

**Antibodies used**

Primary antibodies: rabbit anti-GFP (clone D5.1, dilution 1:1000, 2956T, Cell Signaling Technology, USA), mouse anti-P2A (clone 3H4, dilution 1:1000, NBP2-59627SS, Novus Biologicals, USA).

**Validation**

All antibodies in the study were used according to the user manuals and validation statements can be found on the respective manufacture website (https://www.cellsignal.com/products/primary-antibodies/gfp-d5-1-rabbit-mab/2956 and https://www.novusbio.com/products/2a-peptide-antibody-3h4_nbp2-59627). In the current study the antibodies performed as expected (Supplementary Figure 5).

Eukaryotic cell lines

**Policy information about cell lines**

**Cell line source(s)**

HEK293FT (Invitrogen) and HeLa (ATCC CCL-2)

**Authentication**

Cells were authenticated by the manufacturer using STR profiling, and reauthenticated in our lab by inspecting stereotypical morphological features under widefield microscope and tested negative for mycoplasma contamination to their standard levels of stringency and were here used because they are common cell lines for testing new tools.

**Mycoplasma contamination**

Cells were authenticated by and tested negative for mycoplasma contamination using KIT NAME VENDOR NUMBER to their standard levels of stringency and were here used because they are common cell lines for testing new tools.

**Commonly misidentified lines (See ICLAC register)**

No commonly misidentified lines were used in the study.

Animals and other organisms

**Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research**

**Laboratory animals**

For neuronal culture, about 25 P0 pups were sacrificed to get the dissociated neurons regardless of gender. For brain slice imaging, at least 15 pups from two mother mice were injected with AAVs at P0 for each protein regardless of gender. For the brightness obtained from live brain slice, total 9 mice were sacrificed for each protein at P20-30; 2 months and 3 months (3 mice at each time point). For the photostability obtained from live brain slice, 3 mice were sacrificed for each protein at each time point. For the brightness and photostability obtained from fixed brain slice, 2 mice were sacrificed for each protein at P20-30. For the brightness obtained from live liver slice, 3 three-month-old male mice were sacrificed for each protein. 3-4 three-month-old male mice were injected with AAVs through tail vein injection for each protein. All the mice in the experiment were C57BL/6J. Mice were maintained at strict barrier facilities with macroenvironmental temperature and humidity ranges of 20-26°C and 40-70%, respectively. Food and water were provided ad libitum. The rooms had a 12 h light/12 h dark cycle. The housing conditions were closely monitored and controlled.

The pigmentation-compromised zebrafish brass strain and homozygous nacre embryos of the pan-neuronal expressing Gal4 line (tg(evf03::GAL4-VP16)mnns6) at 33 hours post fertilization and 4-5 days post fertilization regardless of sex, since sex cannot be specified at these stages, were used for proteins expression in zebrafish.

Transgenic [tag-168::wNIR-FPs-T2A-HO1, tag-168::wmNeonGreen] C. elegans worms on wild-type N2 background were used at the L4 developmental stage disregarding sex.

**Wild animals**

No wild animals were used in the study

**Field-collected samples**

No field-collected samples were used in the study

**Ethics oversight**

All animal maintenance and experimental procedures for mice were conducted according to the Westlake University Animal care guidelines, and all animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) of Westlake University, Hangzhou, China under animal protocol #19-044-KP. All experiments involving zebrafish at Technische Universität Braunschweig were conducted in accordance with protocols approved by German legislation following European Union guidelines (EU Directive 2010_63).

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Flow Cytometry

**Plots**

Confirm that:

- The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
- The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a ‘group’ is an analysis of identical markers).
- All plots are contour plots with outliers or pseudocolor plots.
- A numerical value for number of cells or percentage (with statistics) is provided.
Methodology

Sample preparation

To perform cytotoxicity assay of the NIR FPs, HEK293FT (Invitrogen) cells were transiently transfected with pAAV-CAG-NIR-FPs plasmids and dummy DNA (pUC19) as negative control using Hieff Trans Liposomal Transfection Reagent (40802ES02, Yeasen Biotechnology, China) and assessed at 36-48 h post transfection using Annexin V, Alexa Fluor™ 568 conjugate (A13202, ThermoFisher, USA) and Live/Dead reagent (L34958, Viability/Vitality Kit, ThermoFisher) according to the manufacture protocol with minor changes. Annexin V conjugates are designed to detect the externalization of phosphatidylserine, one of the earliest indicators of apoptosis. Briefly, we prepared annexin-binding buffer: 10 mM HEPES, 140 mM NaCl, and 2.5 mM CaCl2, pH 7.4, and added dyes indicating live and dead into the buffer making the dyes at working concentration. The HEK cells were harvested at a concentration of 1x10^6 cells/mL in each 1.5 ml tube using EDTA-free Trypsin (BL527A, Biosharp) and washed in cold phosphate-buffered saline (PBS). The washed cells were recentrifuged and removed the supernatants. One tube of the untransfected cell pellets was heated at 50°C for 10 minutes to obtain the heat-killed cells as positive control for apoptotic cells and dead cells. The HEK cells including heat-killed cells were resuspended in 100 ul annexin-binding buffer containing Live/Dead reagent. After incubating the cells on ice for 15 minutes, 5 µl of the annexin V conjugate were added into each 100 µl of cell suspension. Then incubated the cells at room temperature for 15 minutes. After the incubation period, added 400 µl of annexin-binding buffer without Live/Dead reagent, mixed gently, then kept the samples on ice. As soon as possible, the stained cells were analyzed using a flow cytometer (CytoFLEX LX, Beckman Coulter) equipped with a 405 nm laser and 450/45 bandpass for calcein violet-labeled live cells (Live V450-PB-A) and 525/40 bandpass for the aqua-fluorescent reactive dye-labeled dead cells (Dead V525-KrO-A); a 661 nm laser and 585/42 bandpass (Apoptosis Y585-PE-A) for Annexin V-labeled live cells. The channels of Cy5-BP R660-APC-A and Cy5-LP R712-APC700-A with a 638 nm laser of the flow cytometer were used to analyze the NIR-FPs positive cells. The cytotoxicity was quantified as fraction of dead cells and cells in early stage of apoptosis from the total transfected cells. Since the signal of NIR FPs exhibited crosstalk into the channels used for detection of dyes signals, their signals were compensated correspondingly.

Instrument

CytoFLEX LX flow cytometer, Beckman Coulter

Software

CytExpert v2.4 software was used data collection and analysis

Cell population abundance

No cell collection was performed, only cell population analysis, see Supplementary Table 5 for abundance of relevant cell populations.

Gating strategy

First, cells were gated out using forward and side scatter area (FCS-A and SSC-A), and then cell aggregates were gated out using forward scatter area and height (FSC-A, FSC-H) before desired fluorescence channels were used to analyze cells (lower left FACS dot-plot). See Supplementary Figure 7 for plots reflecting gating strategy.

Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.