

Destabilized near-infrared fluorescent nanobodies enable background-free targeting of GFP-based biosensors for imaging and manipulation

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Near-infrared (NIR) probes are highly sought after as fluorescent tags for multicolor cellular and in vivo imaging. Here we develop small NIR fluorescent nanobodies, termed NIR-Fb_{LAG16} and NIR-Fb_{LAG30}, enabling background-free visualization of various GFP-derived probes and biosensors. We also design a red-shifted variant, NIR-Fb₍₇₁₈₎, to simultaneously target several antigens within the NIR spectral range. Leveraging the antigen-stabilizing property of the developed NIR-Fbs, we then create two modular systems for precise control of gene expression in GFP-labeled cells. Applying the NIR-Fbs in vivo, we target cells expressing GFP and the calcium biosensor GCaMP6 in the somatosensory cortex of transgenic mice. Simultaneously tracking calcium activity and the reference signal from NIR-Fb_{LAGS} bound to GCaMP6 enables ratio-metric deep-brain in vivo imaging. Altogether, NIR-Fb_{LAGS} present a promising approach for imaging and manipulating various processes in live cells and behaving animals expressing GFP-based probes.

Near-infrared (NIR) fluorescent proteins (FPs) are useful for deep-tissue imaging. Compared with a visible spectral range (~450–650 nm), a NIR optical window (~650–900 nm) has the advantages of less phototoxicity, less absorption by tissue pigments, and less light-scattering¹. NIR FPs can also be combined with FPs, biosensors, and optogenetic tools operating in visible spectral range without spectral crosstalk in multiplexing experiments². Moreover, NIR FPs and their fusions can be used as optical guide stars for light focusing and correcting aberrations of visible-light FPs and biosensors in fluorescence^{2,3} and photoacoustic deep tissue imaging^{4,5}.

Recently, a series of small (17 kDa) NIR FPs, termed miRFPnanos^{6–9}, have been designed. Because of their small size and closely located N- and C-termini, miRFPnanos are ideal probes for insertion into proteins as internal tags. An insertion of miRFP670-nano3 into GFP-binding nanobody (Nb; 15 kDa) resulted in a

fluorescent Nb, termed NIR-Fb_{GFP}⁷. Importantly, while common Nb and their N- or C-terminal FP fusions are expressed in mammalian cells regardless of the presence of cognate antigen, NIR-Fbs are allosterically unstable and degrade fast but become highly stabilized and, consequently, brightly fluoresce when bound to their cognate antigen. This unique feature of NIR-Fb_{GFP} allows background-free visualization of GFP-tagged proteins⁷, unlike N- or C-terminal fusions of Nbs to FPs, known as chromobodies. While, similarly to NIR-Fbs, chromobodies are advantageous in the cases where a direct fusion of a protein of interest with FPs alters behavior¹⁰, the latter ones suffer from the high background due to an excess of unbound chromobodies in the cell¹¹. However, we found that NIR-Fb_{GFP} cannot bind biosensors based on GFP-derived FPs, likely because their binding epitope is hindered by analyte-binding parts^{12,13}.

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FPs, mainly GFP-derived FPs, serve as the major scaffold to design the majority of available intensimetric fluorescent biosensors that sense various ions^{14,15}, analytes^{16,17}, neurotransmitters^{18,19}, receptors¹⁸, G-proteins²⁰, and enzymes²¹. Among them, genetically encoded calcium biosensors are a remarkable example of GFP-based probes for studying brain functioning in vivo^{14,22}. The most popular series of calcium biosensors is GCaMP²³ and its improved versions^{14,24–26}.

The ability to chronically monitor calcium dynamics in specific cell types is the main advantage of calcium biosensors. To this, several *Thy1* transgenic mice with a high level of expression were generated^{25,27}, which were successfully applied for recording and mapping neuronal activity in various brain regions with high spatio-temporal resolution^{28,29}. However, transgenic mice express GCaMP with significant differences in the level and patterns of expression between lines due to stochasticity in the transgene cassette integration site. It was shown that *Thy1*-GCaMP2.2, *Thy1*-GCaMP3, *Thy1*-GCaMP6s, and *Thy1*-GCaMP6f transgenic mice express calcium biosensors in different cell fractions across different cortical regions^{25,27}. Additionally, GCaMP transgenic mice exhibit mosaic expression with uneven transgene distribution across brain regions in one line^{25,27}.

On the other hand, the basal calcium ions level may differ in various neuronal populations, and calcium biosensors differ in their affinity to calcium ions³⁰. All this complicates an interpretation of data obtained during the imaging of multiple brain areas and requires the use of several transgenic lines for comparison. In addition, in vivo imaging may suffer from animal movements, photobleaching, and autofluorescence, especially when FPs with blue-green spectra are used. Therefore, the second fluorescent dye, which recolors cell populations heterogeneously expressing GFP and GFP-based probes and thus acting as a reference, could solve the problem of discrepancies in the patterns and levels of the probe expression and imaging conditions.

In this work, we develop a series of NIR-Fbs, named NIR-Fb_{LAG16} and NIR-Fb_{LAG30}, which bind not only GFP-derived FPs but also GFP-based biosensors for calcium ions, pyruvate, and glucose. The binding of NIR-Fb_{LAGs} to biosensors has no pronounced effect on their dynamics and ability to recognize respective analytes in live cells. We show that NIR-Fb_{LAGs} effectively recognize biosensors and degrade in their absence, allowing background-free imaging in live cells and deep in the mouse brain in vivo. We also expand the spectral palette of Nbs by designing an additional NIR-Fb with emission at 718 nm, compared to 670 nm of the current NIR-Fbs, and apply it to multicolor NIR imaging of different antigens. We then design two modular NIR-Fb_{GFP}-based systems, which activate gene transcription only in the presence of GFP. Next, we validate NIR-Fb_{GFP} and both NIR-Fb_{LAGs} in the mouse brain of several transgenic mouse lines. Finally, we simultaneously monitor calcium activity and a stable reference signal of NIR-Fb_{LAGs}, bound to calcium biosensor, in GCaMP6f-expressing neurons in vivo.

Results

Design and screening of NIR Nbs for GFP-based biosensors

To determine whether previously developed NIR-Fb_{GFP} can bind GFP-based biosensors, we coexpressed it with GCaMP6s and GFP-derived blue EBFP2 targeted to the nucleus, and observed precise colocalization of NIR-Fb_{GFP} with EBFP2 but no NIR fluorescence in the cytoplasm where GCaMP6s was expressed (Supplementary Fig. 1). This indicated that NIR-Fb_{GFP} labels various GFP-derived FPs, such as EBFP2, but not GFP-based biosensors. Nevertheless, this NIR-Fb_{GFP} feature can be useful for NIR-targeting of different transgenic mouse and cell lines expressing proteins of interest fused with GFP-derived FPs, making them compatible with the co-expressed GFP-based biosensors.

Indeed, according to the crystal structure of GBP1 Nb^{12,13,31} (PDB ID: 3OGO), which was used to design NIR-Fb_{GFP}⁷, it binds GFP in proximity to the chromophore hydroxyl¹² and, therefore, does not bind

GFP-based biosensors since analyte-binding part of the sensor interferes with the Nb: GFP interaction interface (Fig. 1a, b).

Therefore, to engineer a NIR Nb for GFP-based biosensors (Fig. 1a), we first analyzed crystal structures of several high-affinity anti-GFP Nbs. For further analysis, we have chosen two Nbs, called LAG16 and LAG30, with K_d s values in the sub-nanomolar range (0.7 nM for LAG16 and 0.5 nM for LAG30³²) and two Nbs for thermostable GFP (TGFP), called Sb44 and Sb92, with slightly lower K_d s (4.4 and 3.3 nM, respectively³³). We also used GBP2, GBP6, and GBP7 anti-GFP Nbs with unknown crystal structures but which recognize different epitopes compared to GBP1¹³.

We inserted miRFP670nano3 into these seven Nbs between the residues, corresponding to Nb_{GFP} residues Ser65 and Val66 with Gly₄Ser linkers (Fig. 1a and Supplementary Fig. 2). The internally tagged Nbs were co-expressed in HeLa cells with its cognate antigen mEGFP or with non-GFP-derived blue fluorescent protein mTagBFP2³⁴, as a negative control. As expected, no NIR fluorescence was detected in mTagBFP2 co-expressed cells, whereas the highest fluorescence level was observed for LAG16 and LAG30 Nbs (Fig. 1c and Supplementary Fig. 3). We named these constructs NIR-Fb_{LAG16} and NIR-Fb_{LAG30}, respectively. Compared with parental NIR-Fb_{GFP}, the fluorescence intensity of NIR-Fb_{LAG16} and NIR-Fb_{LAG30} was 2.7- and 1.7-fold lower, respectively (Fig. 1c and Supplementary Fig. 4). LAG16 and LAG30 have 90% sequence identity (Supplementary Fig. 5) and bind GFP at closely located epitopes on the opposite to the chromophore hydroxyl side of the protein β -barrel^{35,36} (Fig. 1d). It makes NIR-Fb_{LAG16} and NIR-Fb_{LAG30} perfect candidates for binding GFP-based biosensors. A low NIR fluorescence was also observed for GBP6 with inserted miRFP670-nano3 (Fig. 1c), though the affinity of GBP6 to GFP is much lower than that of GBP1, LAG16 and LAG30^{33,32}. No fluorescence was observed for Sb44 and Sb92 with inserted miRFP670nano3 (Fig. 1c), likely because their affinity to GFP is ~10-fold lower compared to LAG16 and LAG30^{32,33}.

Characterization of NIR-Fb_{LAG} constructs

To validate that inserted miRFP670nano3 did not change its spectra, we expressed NIR-Fb_{LAG30} in bacteria and found that it preserved the parental spectral properties³², exhibiting fluorescence excitation and emission maxima at 645 nm and 670 nm, respectively (Supplementary Fig. 6a, b). When mEGFP was added to the Ni-NTA agarose preloaded with NIR-Fb_{LAG30}, the bright green and NIR fluorescence were observed only in the elution fraction (Supplementary Fig. 6c, d), confirming the ability to produce functional recombinant NIR-Fb_{LAGs} in bacteria.

To assess whether an antigen concentration affects the protein level of NIR-Fb_{LAGs}, we cotransfected cells with the fixed amount of plasmids encoding either NIR-Fb_{LAG16} or NIR-Fb_{LAG30} and decreasing amounts of a plasmid encoding EGFP antigen. Although we used the same amount of the NIR-Fb_{GFP} plasmid for cell transfection, the fluorescence intensity of NIR-Fb_{LAGs} varied and depended on the EGFP level (Supplementary Fig. 7), confirming that NIR-Fb_{LAGs} exhibit the antigen-dependence in a dose-dependent manner.

It has been shown that in the absence of EGFP antigen, NIR-Fb_{GFP} degrades by a ubiquitin-proteasome system⁷. To validate this mechanism for the NIR-Fb_{LAGs} nanobodies, we treated cells expressing NIR nanobodies in the absence of a cognate antigen with a proteasomal inhibitor bortezomib. While nontreated cells exhibited no NIR fluorescence, incubation of cells with 10 μ M bortezomib for 20 h increased the fluorescence of NIR-Fb_{LAGs} by 75% (Supplementary Fig. 8), confirming that unbound NIR-Fb_{LAGs} degrade by the ubiquitin-proteasome system.

To assess if engineered NIR-Fb_{LAG16} and NIR-Fb_{LAG30} could bind GFP-based biosensors, we cotransfected them with GCaMP6s¹⁴ into HeLa cells. Cells were incubated in calcium-saturated (4 mM CaCl₂) or calcium-depleted (2 mM EDTA) buffers to determine to which GCaMP6s conformation NIR-Fb_{LAGs} would predominantly bind.

To eliminate conformation changes in calcium-binding parts upon binding calcium ions, we constructed a truncated GCaMP6s version with deleted M13 peptide, which did not respond to elevated calcium³⁷. After 1 h incubation, no fluorescence was observed for NIR-Fb_{GFP} (Fig. 1e), indicating it predictably does not bind GCaMP6s because its epitope on GFP is structurally hidden by calcium-binding parts (Fig. 1a, b)^{38,39}. Both NIR-Fb_{LAGS} effectively bound the GCaMP6s version without M13 (Fig. 1e). However, NIR-Fb_{LAGS} exhibited ~2.3–2.8-fold lower fluorescence in the calcium-saturated conditions than in calcium-free conditions, suggesting lower binding affinity of NIR-Fb_{LAGS} to calcium-bound GCaMP6s during a prolonged time scale. At the same time, the green fluorescence of GCaMP6s was ~2.2-fold higher in calcium-saturated conditions (Fig. 1f), which is consistent with its mechanism of action¹⁴.

To estimate whether NIR-Fb_{LAGS} binding affects the fast association and dissociation calcium kinetics of GCaMP6s, we performed stopped-flow experiments with purified GCaMP6s only or with GCaMP6s in the presence of NIR-Fb_{LAG30}. The fluorescence changes during rapid mixing of GCaMP6s, either with or without NIR-Fb_{LAG30} with a series of Ca²⁺ buffers, provided observed relaxation rate constants (k_{obs}). We analyzed the dependence of k_{obs} from the free calcium ions concentration in the range of 0–350 nM (Supplementary Fig. 9a–c) and found that k_{obs} were not affected by the NIR-Fb_{LAG30} binding up to 250 nM of free calcium (Supplementary Fig. 9c). At 300 nM, GCaMP6s in the presence of NIR-Fb_{LAG30} has a 1.3-fold slower calcium binding kinetics (Supplementary Fig. 9c). At the same time, we found that dissociation rate constants (k_{off}) were rather similar for free and NIR-Fb_{LAG30}-bound GCaMP6s: 1.26 s⁻¹ and 1.36 s⁻¹, respectively.

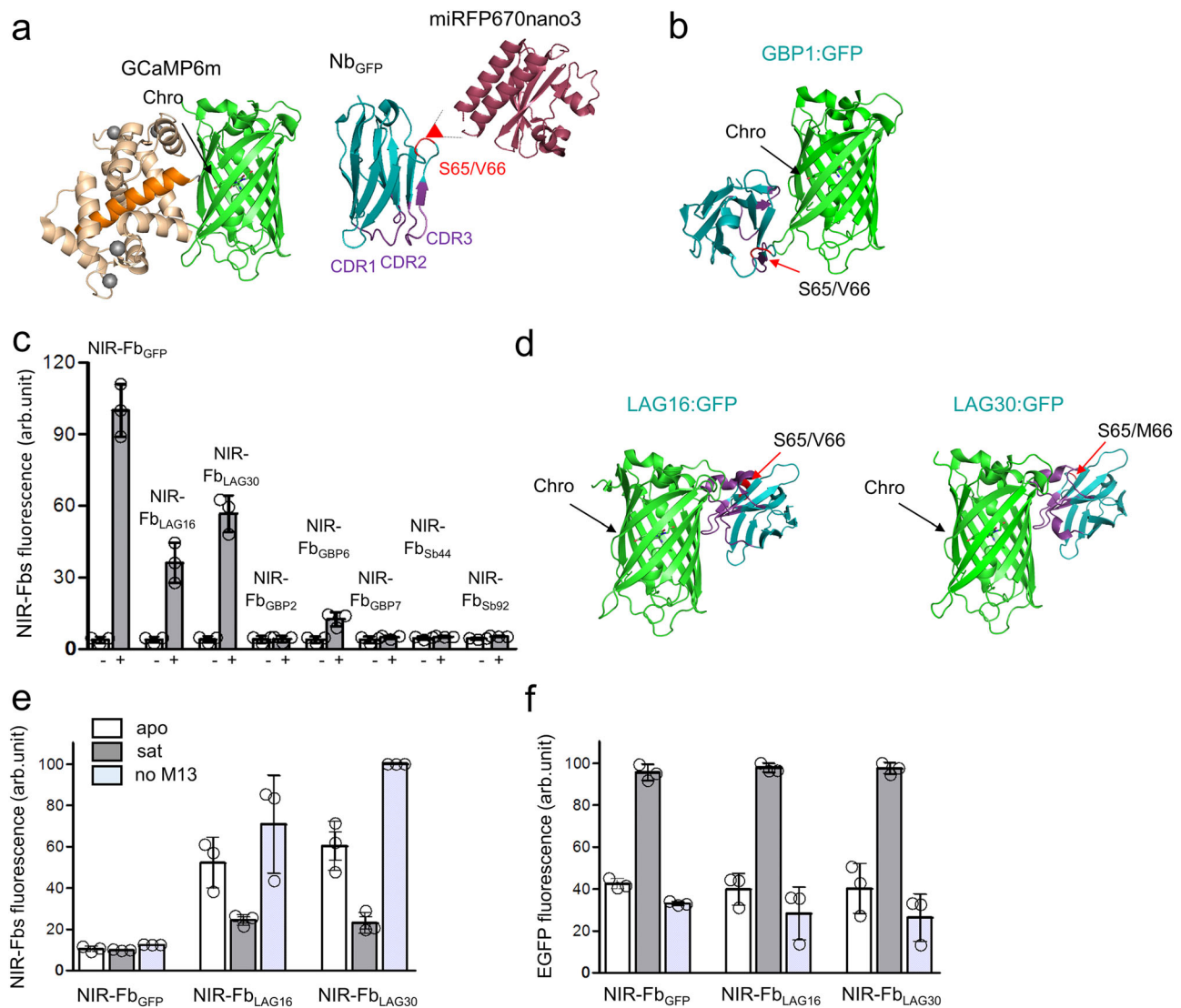


Fig. 1 | Design and screening of nanobodies internally fused with miRFP670-nano3 (NIR-Fbs). **a** Crystal structure of a Nb (PDB ID: 3OGO) with internally fused miRFP670nano3 (PDB ID: 7LSC) (NIR-Fb) bound to GFP-based biosensor GCaMP6m (PDB ID: 3WLD). Complementarity-determining regions (CDRs) are highlighted in violet. The position of miRFP670nano3 insertion to the anti-GFP Nb is indicated with a red arrow. **b** Crystal structure of GBP1 (PDB ID: 3OGO) Nbs to GFP. **c** Fluorescence intensity of cells transfected with eight different NIR-Fbs to GFP co-expressed with mEGFP (+) or mTagBFP2 (-). **d** Crystal structures of LAG16 (PDB ID: 6LR7) and LAG30 (PDB ID: 7SAI) Nbs to GFP. In **a**, **b** and **d** black arrows indicate chromophore. Red arrows indicate the position where miRFP670nano3 was inserted. **e** Far-red fluorescence intensity of cells transfected with NIR-Fb_{LAG16}, NIR-

Fb_{LAG30} or NIR-Fb_{GFP} and coexpressed with GCaMP6s biosensor in its apo or saturated states or with GCaMP6s with truncated M13 peptide. **f** Green fluorescence intensity of cells transfected with NIR-Fb_{LAG16}, NIR-Fb_{LAG30} or NIR-Fb_{GFP} and co-expressed with GCaMP6s in its apo or saturated states or with GCaMP6s with truncated M13 peptide. In **c**, **e** and **f** fluorescence intensity was analyzed by flow cytometry using a 640 nm excitation laser and a 660/20 nm emission filter for mEGFP, a 405 nm excitation laser and a 525/50 nm emission filter for mEGFP, a 405 nm excitation laser and a 450/50 nm emission filter for mTagBFP2. **c**, **e**, **f** Data are presented as mean values $\pm$ s.d. for $n = 3$ transfection experiments. Source data are provided as a Source Data file.

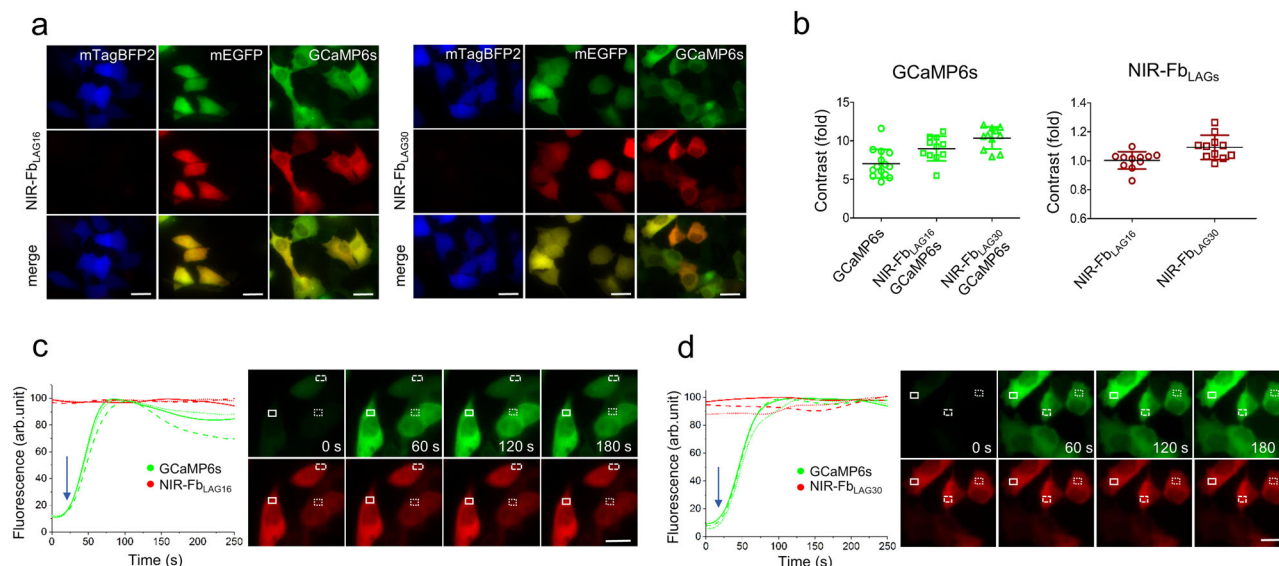


Fig. 2 | Performance of NIR-Fb_{LAGS} nanobodies co-expressed with GCaMP6s in HeLa cells. **a** Fluorescence images of HeLa cells coexpressing NIR-Fb_{LAG16} or NIR-Fb_{LAG30} anti-GFP Nb together with mTagBFP2 (negative control), mEGFP (positive control), and GCaMP6s. **b** Left, contrast of GCaMP6s only ($n = 14$) and GCaMP6s coexpressed with either NIR-Fb_{LAG16} ($n = 11$) or NIR-Fb_{LAG30} ($n = 11$) after the addition of 5 μM ionomycin. Right, the contrast of NIR-Fb_{LAG16} and NIR-Fb_{LAG30} for the data presented in the left graph. Data are presented as mean values ± s.e.m. for $n = 3$ transfection experiments. **c** Change in fluorescence intensity of the cells co-

expressing GCaMP6s (green) and NIR-Fb_{LAG16} (red) in response to 5 μM ionomycin. **d** Change in fluorescence intensity of the cells co-expressing GCaMP6s (green) and NIR-Fb_{LAG30} (red) in response to 5 μM ionomycin. In **c** and **d**, data are shown for 3 cells, and the arrow indicates the time point when 5 μM ionomycin was added. For imaging of mTagBFP2, mEGFP or GCaMP6s and NIR-Fb_{LAGS}, 390/40 nm excitation and 460/40 nm emission, 480/40 nm excitation and 530/40 nm emission, and 605/40 nm excitation and 640LP nm emission filters were used, respectively. Scale bar, 40 μm. Source data are provided as a Source Data file.

(Supplementary Fig. 9d). We then assessed the effect of NIR-Fb_{LAG30} binding to the GCaMP6s on the affinity of the indicator for calcium ions. According to the calcium titration experiment, GCaMP6s demonstrated comparable K_d in the presence and absence of NIR-Fb_{LAG30}: 148 ± 14 nM and 144 ± 4 nM, respectively (Supplementary Fig. 9e), which is consistent with the kinetics data. Because GCaMP6s is $\geq 90\%$ saturated by bound calcium at 300 nM, differences in binding kinetics at calcium concentration above 300 nM should not contribute much to the fluorescence changes of NIR-Fb_{LAG30}-bound GCaMP6s. Overall, these data indicate that NIR-Fb_{LAG30} does not interfere with the calcium binding and release and does not block GCaMP6s in “on” or “off” fluorescent states during fast changes of calcium concentration, which might be particularly valuable for in vivo brain imaging.

Performance of NIR-Fb_{LAGS} co-expressed with GCaMP6s

To study NIR-Fb_{LAGS}’ performance in live mammalian cells, they were co-expressed with mEGFP, GCaMP6s, or mTagBFP2 negative control and imaged with an epifluorescence microscope. NIR fluorescence was observed in the cells co-expressing mEGFP or GCaMP6s but not mTagBFP2 (Fig. 2a), suggesting that NIR-Fb_{LAGS} are stabilized only in the presence of its cognate antigen. The intracellular patterns of expression for NIR-Fb_{LAGS} coincided with those of mEGFP and GCaMP6s expression, confirming efficient NIR-Fb_{LAG16} and NIR-Fb_{LAG30} binding to their cognate antigen in live cells (Fig. 2a).

To test whether NIR-Fb_{LAG16} or NIR-Fb_{LAG30} fluorescence will change upon calcium increase and whether NIR-Fb_{LAGS} interaction with GCaMP6s will change its ability to bind calcium ions, we added 5 μM ionomycin ionophore to the HeLa cell cultures co-expressing NIR-Fb_{LAGS} and GCaMP6s and time-lapsed imaged them. We observed that the maximal contrast of GCaMP6s was not substantially changed in cells co-expressing NIR-Fb_{LAGS} (Fig. 2b). Fluorescence intensity for both NIR-Fb_{LAG16} and NIR-Fb_{LAG30} did not change after ionomycin addition too (Fig. 2b–d), suggesting that NIR-Fb_{LAGS} do not influence GCaMP6s performance, and calcium binding does not affect NIR-

Fb_{LAGS} interaction with GCaMP6s, reflected by the unchanged NIR fluorescence after calcium increase (Fig. 2c, d).

Targeting various types of GFP-biosensors with NIR-Fb_{LAGS}

To study whether NIR-Fb_{LAGS} can bind other GFP-based biosensors, we have chosen biosensors that detect chemical analytes other than calcium, such as Green Pegassos¹⁶, PyronicSF⁴⁰, and iGlucoSnFR^{17,41}. Green Pegassos and PyronicSF are pyruvate biosensors based on pyruvate dehydrogenase transcriptional regulator (PdhR) from *E. coli* inserted into GFP or linked to a circularly permuted version of GFP (cpGFP), respectively^{16,40}. iGlucoSnFR is a glucose biosensor based on the insertion of cpGFP into a glucose-binding protein from *Thermus thermophilus*¹⁷.

We co-expressed Green Pegassos, PyronicSF, and iGlucoSnFR with either NIR-Fb_{LAG16} or NIR-Fb_{LAG30} and analyzed their fluorescence in live cells (Fig. 3a, b). We observed bright fluorescence of NIR-Fb_{LAGS} in cells co-expressing GFP-based biosensors or mEGFP but not mTagBFP2 negative control, suggesting their efficient antigen-dependent stabilization. Moreover, NIR-Fb_{LAGS} fully colocalized with the biosensors and exhibited no background fluorescence (Fig. 3a, b), confirming the NIR-Fb_{LAGS} ability to bind various GFP-based biosensors.

To test if NIR-Fb_{LAGS} affect the ability of the biosensors to detect their analytes, we treated the co-expressing cells with 1 mM or 10 mM pyruvate, or 50 mM glucose, for Green Pegassos, PyronicSF, or iGlucoSnFR, respectively, and time-lapsed imaged them (Fig. 3c–e and Supplementary Figs. 10–12). As Green Pegassos and PyronicSF have different affinities for pyruvate (70 μM for Green Pegassos and 480 μM for PyronicSF)^{16,40}, we used different pyruvate concentrations for these biosensors. No difference in the contrasts of biosensors’ fluorescence was observed without or with co-expressed NIR-Fb_{LAG16} or NIR-Fb_{LAG30} (Fig. 3c–e), suggesting that binding of NIR-Fb_{LAGS} may have a limited effect on the ability of GFP-based biosensors to detect their analytes.

Adding the second color to the NIR-Fb family

NIR-Fb_{GFP}, NIR-Fb_{LAG16}, and NIR-Fb_{LAG30} have the inserted mRFP670-nano3 (ex. 645 nm, em. 670 nm). For simultaneous labeling of several antigens, it would be beneficial to have distinct Nb colors.

Recently, cyanobacteriochrome-based NIR FP mRFP718nano (ex. 690 nm, em. 718 nm), spectrally resolvable from mRFP670nano3, was reported⁸. Since mRFP670nano3 and mRFP718nano have the same predecessor⁶, we have hypothesized that we could swap mRFP670-nano3 for mRFP718nano in NIR-Fbs, including NIR-Fb_{GFP} (Fig. 4a and Supplementary Fig. 13), thus targeting two different antigens simultaneously. This red-shifted construct was named NIR-Fb₍₇₁₈₎. In cells co-expressing mEGFP, NIR-Fb₍₇₁₈₎ exhibited evenly distributed fluorescence whose pattern coincided with that of mEGFP expression (Fig. 4b), confirming that NIR-Fb₍₇₁₈₎ was dependent on the presence of the antigen.

For targeting two antigens in a cell, we used a previously reported NIR-Fb_{59H10}⁷, which recognizes the p24 antigen of HIV (human immunodeficiency virus) and has internally fused mRFP670nano3. We fused p24 with mTagBFP2 and NES (nuclear exclusion signal) to direct it to cytosol and mEGFP with histone 2B (H2B) to localize it in the nucleus (Fig. 4c). When NIR-Fb₍₇₁₈₎ and NIR-Fb_{59H10} were co-expressed with these antigens, the NIR-Fb_{59H10} colocalized with p24-mTagBFP2 in the cytosol and NIR-Fb₍₇₁₈₎ colocalized with mEGFP in the nucleus (Fig. 4c), demonstrating the multicolor NIR imaging in the same cell.

Multicolor targeting GFP probes with NIR-Fbs

The developed NIR-Fbs of two distinct colors specific to GFP or GFP-based biosensors should be useful in designing various molecular tools. We have analyzed various GFP mutants and found that a pH-stable tandem dimer GFP (pH-tGFP)⁴² could serve as the second antigen for the NIR-Fb₍₇₁₈₎ - NIR-Fb_{LAG} pair. Analyzing the dimerization interface of pH-tGFP using EGFP crystal structure⁴³, we hypothesized

that only NIR-Fb_{LAG30} would bind pH-tGFP, whereas the epitope for NIR-Fb₍₇₁₈₎ would be buried in the dimerization interface and, thus, NIR-Fb₍₇₁₈₎ should be destabilized in the pH-tGFP presence.

To check the possibility of multicolor imaging of two NIR-Fbs to GFP-like antigens, we transfected HeLa cells with NIR-Fb_{LAG30} and NIR-Fb₍₇₁₈₎ and two antigens, pH-tGFP with an NES signal and blue EBFP2 with a nuclear localization signal (NLS) in various combinations (Fig. 5). When either NIR-Fb₍₇₁₈₎ or NIR-Fb_{LAG30} was co-expressed with NLS-EBFP2, both NIR-Fbs predictably exhibited NIR fluorescence in the nucleus (Fig. 5a, b). In the presence of NES-pH-tGFP antigen, there was no NIR fluorescence when NIR-Fb₍₇₁₈₎ was cotransfected (Fig. 5c), whereas NIR-Fb_{LAG30} exhibited bright NIR fluorescence in the cytosol (Fig. 5d), confirming our initial hypothesis. When two NIR-Fbs were cotransfected with both NES-pH-tGFP and NLS-EBFP2 antigens, we observed cytosolic and nuclear localization of NIR-Fb_{LAG30} and only nuclear localization of NIR-Fb₍₇₁₈₎ (Fig. 5e). These results demonstrated the ability of simultaneous tracking several GFP-based probes in the cell by combining different NIR Nbs.

NIR-Fb-based systems for transcription control

A unique feature of NIR-Fbs is their stabilization upon antigen binding and degradation in the absence of a cognate antigen, allowing the development of systems to control gene expression in an antigen-dependent manner. Since LAG16 (or LAG30) and GBP1 Nbs bind different GFP epitopes, it was shown that they can co-occupy GFP, forming complexes^{35,36}. We decided to utilize this property and use superfolder GFP (sfGFP) as a dimerizer for two NIR-Fbs. To this, we first constructed a sfGFP version split at position 157⁴⁴, in which each split fragment was fused to mTagBFP2 (Supplementary Fig. 14a). HeLa cells transfected with one sfGFP split fragment exhibited no green fluorescence, whereas two complementing sfGFP fragments fused to mTagBFP2 restored its green fluorescence (Supplementary

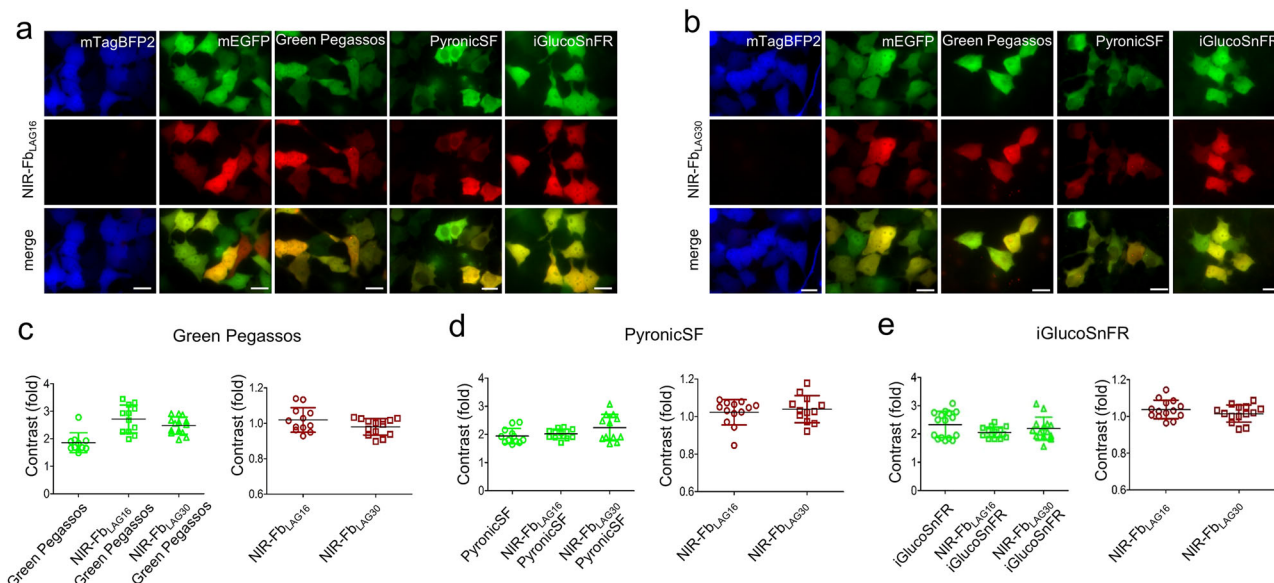


Fig. 3 | Performance of NIR-Fb_{LAGs} co-expressed with GFP-based pyruvate and glucose biosensors. **a** Fluorescence images of HeLa cells co-expressing NIR-Fb_{LAG16} anti-GFP Nb together with mTagBFP2 (negative control), mEGFP (positive control), pyruvate sensors Green Pegassos and PyronicSF and glucose sensor iGlucoSnFR. **b** Fluorescence images of HeLa cells co-expressing NIR-Fb_{LAG30} anti-GFP Nb together with mTagBFP2 (negative control), mEGFP (positive control), pyruvate sensors Green Pegassos and PyronicSF and glucose sensor iGlucoSnFR. **c** Left, contrast of Green Pegassos only ($n = 10$) and Green Pegassos coexpressed with either NIR-Fb_{LAG16} ($n = 12$) or NIR-Fb_{LAG30} ($n = 14$) after the addition of 1 mM pyruvate. Right, the contrast of NIR-Fb_{LAG16} and NIR-Fb_{LAG30} for the data presented in the left graph. **d** Left, contrast of PyronicSF only ($n = 12$) and PyronicSF co-

expressed with either NIR-Fb_{LAG16} ($n = 13$) or NIR-Fb_{LAG30} ($n = 12$) after addition of 10 mM pyruvate. Right, the contrast of NIR-Fb_{LAG16} and NIR-Fb_{LAG30} for the data presented in the left graph. **e** Left, contrast of iGlucoSnFR only ($n = 16$) and iGlucoSnFR co-expressed with either NIR-Fb_{LAG16} ($n = 14$) or NIR-Fb_{LAG30} ($n = 14$) after addition of 50 mM glucose. Right, the contrast of NIR-Fb_{LAG16} and NIR-Fb_{LAG30} for the data presented in the left graph. For imaging of mTagBFP2, mEGFP or Green Pegassos, PyronicSF and iGlucoSnFR, 390/40 nm excitation and 460/40 nm emission, 480/40 nm excitation and 530/40 nm emission, and 605/40 nm excitation and 640LP nm emission filters were used, respectively. Scale bar, 40 μ m. **c–e** Data are presented as mean values $\pm$ s.e.m. for $n = 3$ transfection experiments. Source data are provided as a Source Data file.

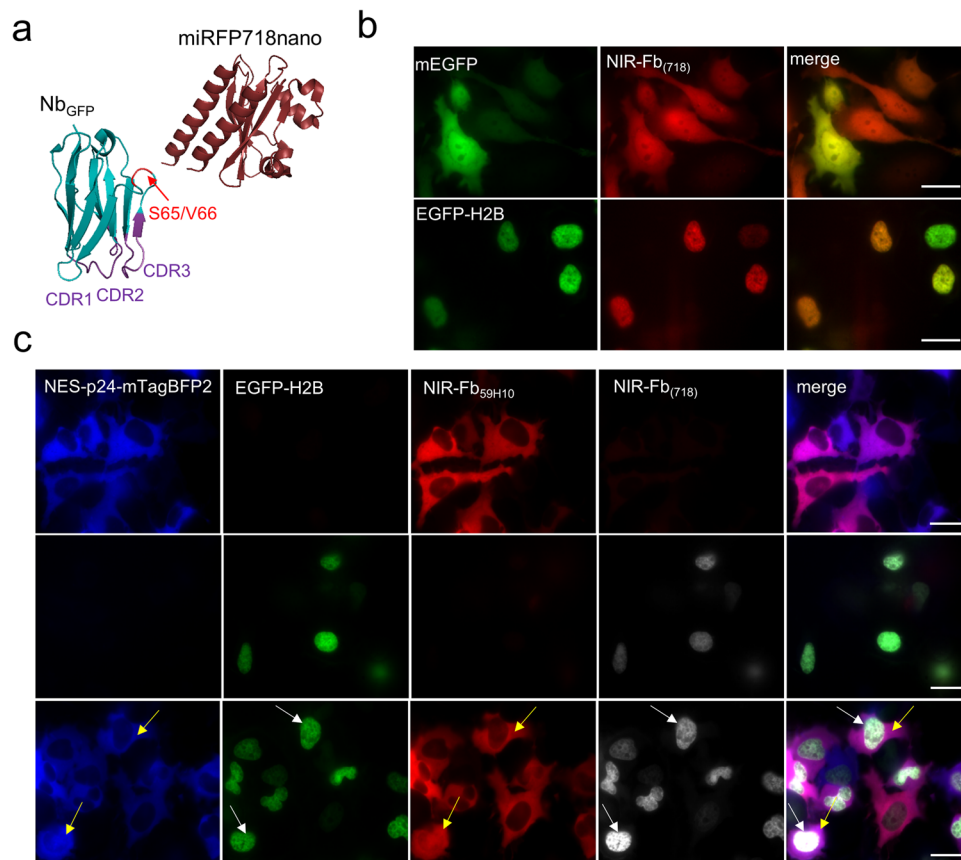


Fig. 4 | Multicolor imaging of two different NIR-Fbs. **a** Design of NIR Nb for GFP (Nb_{GFP}) (PDB ID 3OGO) with indicated position for insertion of miRFP718nano (PDB ID 7LSD). CDRs of Nb_{GFP} are highlighted with violet and the position of miRFP718nano insertion is indicated in red. **b** Co-expression of NIR Nb_{GFP} containing inserted miRFP718nano (NIR-Fb₍₇₁₈₎) with cognate mEGFP antigen in the cytoplasm (upper panels) or in the nucleus (bottom panels) of HeLa cells. **c** Upper panels, fluorescent images of HeLa cells co-expressing near-infrared Nb for p24 HIV protein with inserted miRFP670nano3 fluorescent protein (NIR-Fb_{59H10}) and cognate antigen, NES-p24-mTagBFP2. Middle panels, fluorescent images of HeLa cells co-expressing NIR Nb for GFP with inserted miRFP718 (NIR-Fb₍₇₁₈₎) and EGFP-

H2B. Bottom panels, fluorescent images of HeLa cells co-expressing NIR-Fb_{59H10} and NIR-Fb₍₇₁₈₎ and two respective antigens, NES-p24-mTagBFP2 and EGFP-H2B. Patterns of co-expression are indicated with yellow and white arrows, respectively. For imaging of mTagBFP2 and mEGFP, 390/40 nm excitation and 460/40 nm emission and 480/40 nm excitation and 530/40 nm emission filters we used, respectively. For imaging of NIR-Fb_{59H10} and NIR-Fb₍₇₁₈₎, 605/40 excitation and 667/30 emission, and 682/12 excitation and 721/42 emission filters were used, respectively. Scale bar, 40 μ m. For more details, see “Statistics and reproducibility” section of the Methods.

Figs. 14a and 15a, b), confirming a weak dimerization ability of mTagBFP2. Analyzing the mEGFP crystal structure and published data^{12,35,36}, we hypothesized that NIR-Fb_{LAG30} would bind the N-split fragment (sfGFP(1-157)) (Supplementary Fig. 14b, c) while NIR-Fb₍₇₁₈₎ would bind only restored sfGFP, allowing two NIR-Fbs to bind the same sfGFP molecule simultaneously. When we co-expressed N- or C-end split fragments of sfGFP with NIR-Fb_{LAG30} or NIR-Fb₍₇₁₈₎, respectively, NIR-fluorescence was observed only in the cells transfected with N-end split sfGFP and NIR-Fb_{LAG30} (Supplementary Figs. 14d and 15c, d). When both split fragments were co-expressed, NIR fluorescence was observed for both NIR-Fb_{LAG30} and NIR-Fb₍₇₁₈₎ (Supplementary Figs. 14e and 15c, d). This confirms the possibility of using GFP as a dimerizer for two NIR-Fbs binding distinct epitopes. Notably, since NIR-Fb₍₇₁₈₎ binds only restored sfGFP, it can be used as a NIR reporter of the split GFP complementation in the protein-protein interaction assays.

To study whether NIR-Fb could be used for gene regulation, we have chosen the GAL4-UAS system⁴⁵. In the first approach, we fused the GAL4 DNA-binding domain to each of the split sfGFP fragments and NIR-Fb₍₇₁₈₎ to the VP16 activation domain. As a reporter, we used a pG12-mTagBFP2 with 12xUAS (upstream activating sequence; termed G12 here) repeats upstream of the reporter gene (Fig. 6a). In this

design, binding of UAS sites by two GAL4 domains restores sfGFP molecule, then NIR-Fb₍₇₁₈₎ binds its sfGFP epitope, resulting in the formation of GAL4-VP16 complex and, consequently, activation of a mTagBFP2 reporter transcription (Fig. 6a). We observed blue reporter fluorescence only when cells were co-transfected with sfGFP(1-157)-GAL4, sfGFP(158-238)-GAL4, VP16-NIR-Fb₍₇₁₈₎, and pG12-mTagBFP2. The dynamic range of the system is -78.8 ± 7.1 (Fig. 6c and Supplementary Fig. 16a). In the absence of one of the split sfGFP fragments, NIR-Fb₍₇₁₈₎ was destabilized, and no mTagBFP2 reporter fluorescence was observed (Fig. 6c).

In the second approach, we fused GAL4 to the NIR-Fb_{LAG30} and VP16 to NIR-Fb₍₇₁₈₎. In this system, mEGFP molecule acts as a dimerizer for two NIR-Fbs, stabilizing their fusions, resulting in the formation of GAL4-VP16 complex and, consequent, activation of mTagBFP2 reporter transcription with a dynamic range of -100.0 ± 0.5 (Fig. 6b, d and Supplementary Fig. 16b). We also tested if this NIR-Fb-based system for transcription control could be triggered by *Discosoma*-derived FPs, such as mCherry⁴⁶ and mOrange⁴⁶. As expected, none of them induced gene transcription (Fig. 6d and Supplementary Fig. 16b). Both NIR-Fbs-based gene transcription systems can be further used for manipulation of GFP-expressing cell populations in numerous GFP transgenic organisms generated to date.

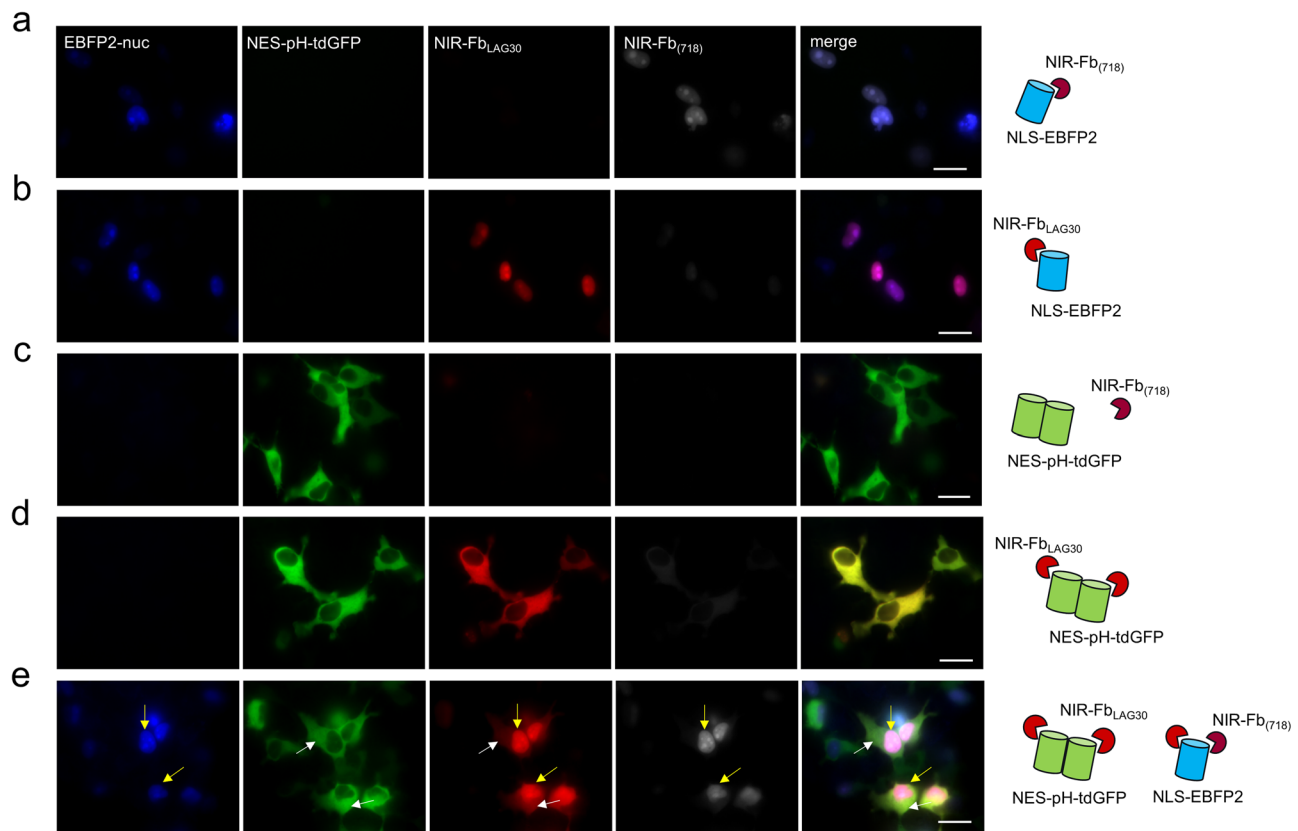


Fig. 5 | Multicolor imaging of two NIR-Fbs for GFP. Fluorescence images of HeLa cells co-expressing EBFP2-nuc with either (a) NIR-Fb for GFP with inserted miRFP718nano fluorescent protein (NIR-Fb₍₇₁₈₎) or (b) with NIR-Fb for GFP with inserted miRFP670nano3 (NIR-Fb_{LAG30}). Fluorescence images of HeLa cells co-expressing pH-tdGFP with either (c) NIR-Fb₍₇₁₈₎ or (d) with NIR-Fb_{LAG30}. **e** Fluorescence images of cells co-expressing EBFP2-nuc, pH-tdGFP, NIR-Fb_{LAG30}, and NIR-Fb₍₇₁₈₎. Yellow arrows indicate patterns of co-expression for EBFP2-nuc

with NIR-Fb_{LAG30} and NIR-Fb₍₇₁₈₎ in the nucleus, white arrows indicate patterns of co-expression for pH-tdGFP with NIR-Fb_{LAG30} in the cytosol. For imaging of EBFP2 and pH-tdGFP, 390/40 nm excitation and 460/40 nm emission and 480/40 nm excitation and 530/40 nm emission filters we used, respectively. For imaging of NIR-Fb_{LAG30} and NIR-Fb₍₇₁₈₎, 605/40 excitation and 667/30 emission and 682/12 excitation and 721/42 emission filters were used, respectively. Scale bar, 40 μ m. For more details, see “Statistics and reproducibility” section of the Methods.

Targeting GFP-expressing cells with NIR-Fb_{GFP} in vivo

To demonstrate multiplex imaging of GFP-expressing cells targeted in vivo with NIR-Fb_{GFP}, we constructed a CAG promoter carrying AAV9 expression vector. The vector was injected into the somatosensory cortex of *Thy1*-GFP-M and *Aldh1l1*-GFP mice with fluorescently labeled neurons and astrocytes, respectively. 3–5 weeks after the injections, animals were prepared for in vivo two-photon imaging to determine how accurately NIR-Fb_{GFP} targets GFP-expressing neurons and astrocytes in the brain of transgenic mice. We found that NIR-Fb_{GFP} brightly fluoresced both in the transduced neurons and astrocytes (Fig. 7a, b and Supplementary Movies 1–4). NIR-Fb_{GFP}/GFP-positive neurons were visualized through the entire depth of the cortex (approximately 800 μ m; Fig. 7c); and NIR-Fb_{GFP}/GFP-positive astrocytes were routinely visualized at the depth of 450 μ m (Fig. 7d and Supplementary Movies 1–4).

After two-photon imaging, animals were perfused and brain tissue was sectioned to 40 μ m slices. Slices analysis demonstrated very good (>94%) correspondence between NIR-Fb_{GFP} and GFP-expressing cell bodies both for neurons and astrocytes near the injection sites (Fig. 7e, f), indicating that NIR-Fb_{GFP} targeted various cell types expressing GFP in the mouse brain with precise accuracy.

Targeting GCaMP6-expressing neurons with NIR-Fb_{LAGS} in vivo

To evaluate the utility of the developed NIR-Fb_{LAGS} for targeting neurons expressing GFP-based genetically encoded biosensors, we constructed CAG promoter-carrying AAV9 vectors with either NIR-Fb_{LAG16} or NIR-Fb_{LAG30}. The AAVs were then injected into the somatosensory

cortex of *Thy1*-GCaMP6f mice with neurons expressing GCaMP6f. 3–4.5 weeks after the cortical injection, we performed two-photon imaging in awake mice on a spherical treadmill. Fluorescence of NIR-Fb_{LAGS} was detectable up to ~600 μ m cortical depth and was co-localized with GCaMP6f expression (Supplementary Movies 5–8). NIR+/GCaMP6f+ neurons exhibited spontaneous and running-evoked calcium spiking with NIR fluorescence showing few signs of photobleaching during the extended (>10 min continuous) recordings (Fig. 8a, b, e, f and Supplementary Movies 9, 10). Vigorous mouse running led to motion-induced NIR+ and GCaMP6f+ fluorescence fluctuations (Fig. 8b, c, f, g). The stability of NIR fluorescence allowed us to use it as a ratiometric reference for the GCaMP6f signal. Calculating the ratio (R) of GCaMP6f and NIR-Fb_{LAG} fluorescence resulted in activity traces with noticeably reduced motion artifacts (Fig. 8a, d, e h). As shown in Fig. 8d h, normalizing the GCaMP6f trace to the NIR-Fb_{LAG16} (or NIR-Fb_{LAG30}) trace from the same cell corrects the fluorescence baseline fluctuations, which often occur during animal locomotor activity (Fig. 8d, h). This, in turn, substantially improves the neuronal calcium spike estimation, for example, by helping to identify false positives and false negatives.

To more quantitatively assess NIR-Fb_{LAGS} targeting of GCaMP6f-expressing neurons, we prepared coronal slices from AAV9-CAG-NIR-Fb_{LAG16} and AAV9-CAG-NIR-Fb_{LAG30} injected *Thy1*-GCaMP6f mice. Near the injection sites, NIR fluorescence was seen in cell bodies and processes of GCaMP6f+ neurons (Fig. 9a, b) with ~99% overlap (Fig. 9c, d). Therefore, our CAG promoter-carrying AAV9 vectors with NIR-Fb_{LAG16} and NIR-Fb_{LAG30} enabled the targeting of GCaMP6f-expressing

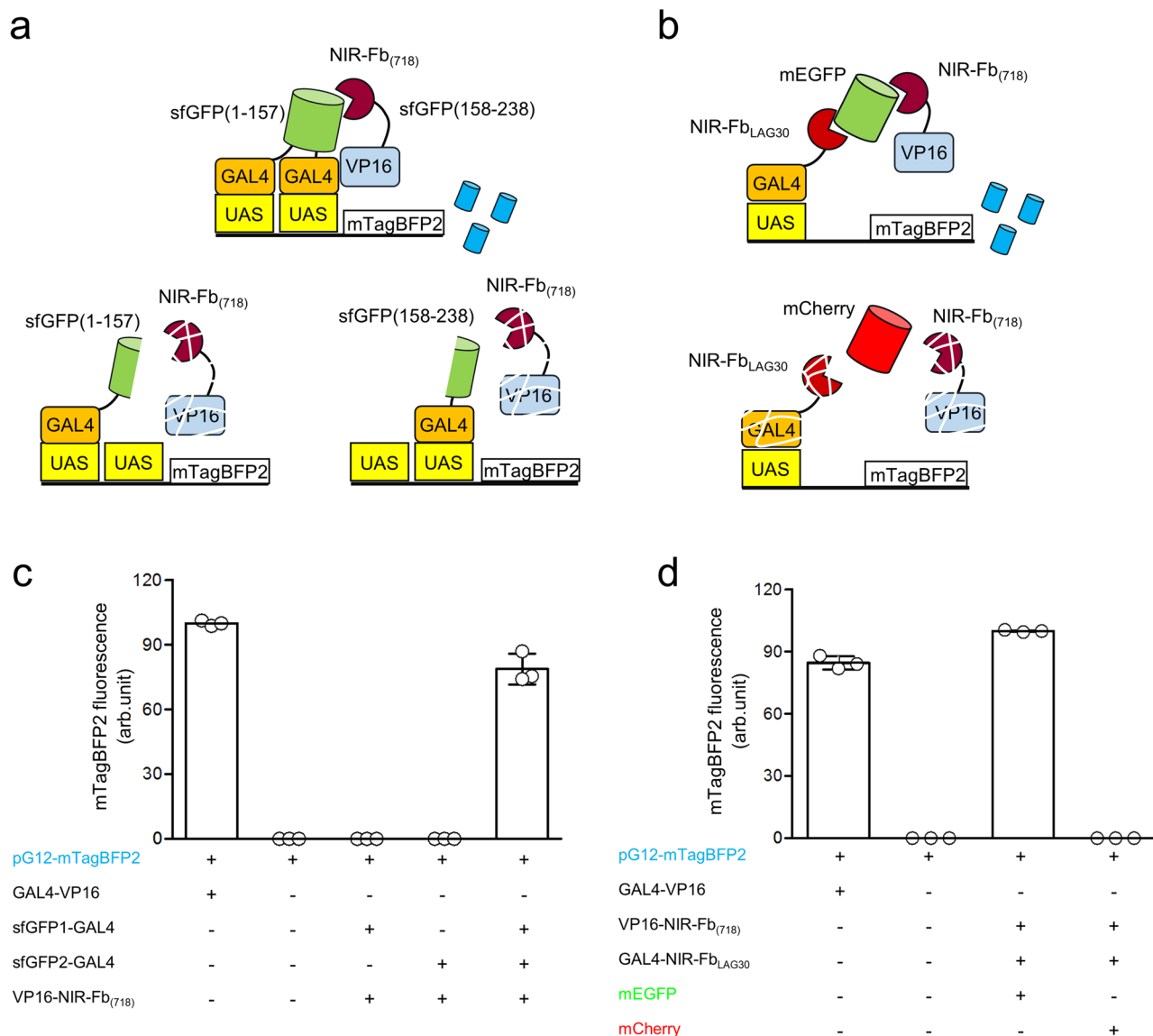


Fig. 6 | NIR-Fb_{GFP}-based systems for transcription activation. **a** Scheme of NIR-Fb₍₇₁₈₎-based system for activation of mTagBFP2 reporter transcription. Two fragments of split-sfGFP are fused to GAL4 DNA-binding domains, while NIR-Fb for GFP with inserted miRFP718nano (NIR-Fb₍₇₁₈₎) is fused to VP16 transcriptional activation domain. In the presence of the fusions of GAL4 to the split sfGFP fragments and a VP16-NIR-Fb₍₇₁₈₎ fusion, sfGFP reconstitutes and can bind NIR-Fb₍₇₁₈₎. In turn, transcriptional activation domain VP16 forms a complex with GAL4 DNA-binding domain which binds to the UAS regulatory sequence and activates transcription of the mTagBFP2 reporter gene. **b** Scheme of NIR-Fb_{LAG30} and NIR-Fb₍₇₁₈₎-based system for activation of mTagBFP2 reporter transcription. Here GAL4 DNA-binding domain is fused to the NIR-Fb_{LAG30}. In the presence of mEGFP, both GAL4-NIR-

Fb_{LAG30} and VP16-NIR-Fb₍₇₁₈₎ bind different epitopes of mEGFP molecule and activate mTagBFP2 reporter transcription. **c** Fluorescence intensity of mTagBFP2 in HeLa cells transfected with a plasmid from the scheme (a). As a control, GAL4-VP16 plasmid with pG12-mTagBFP2 reporter plasmid or pG12-mTagBFP2 reporter plasmid only were used. **d** Fluorescence intensity of mTagBFP2 in HeLa cells transfected with plasmids from the scheme (b). Controls are the same as in c. Fluorescence intensity was analyzed by flow cytometry using a 640 nm excitation laser and a 660/20 nm emission filter for NIR-Fbs, a 488 nm excitation laser and a 525/50 nm emission filter for mEGFP, a 405 nm excitation laser and a 450/50 nm emission filter for mCherry. Data are presented as mean values $\pm$ s.d. for $n = 3$ transfection experiments. Source data are provided as a Source Data file.

neurons with high specificity and penetrance (Supplementary Movies 5–10), permitting ratiometric functional imaging for improved spike estimation in behaving animals.

Discussion

By using the bright small cyanobacteriochrome-derived miRFP670-nano3 as an internal tag and structural and experimental screening of anti-GFP Nbs, we developed a series of NIR-Fb_{LAG} constructs that recognize GFP-based biosensors (Figs. 1–3). NIR-Fb_{LAG}s preserve spectral properties of parental miRFP670nano3 and can be produced recombinantly in bacteria (Supplementary Fig. 6). Stabilization of NIR-Fb_{LAG}s by antigen binding in mammalian cells enables visualization of

cell populations heterogeneously expressing GFP or GFP-based probes in a background-free manner.

We showed that NIR-Fb_{LAG}s bind a broad range of GFP-based biosensors, irrespective of the analyte they recognize, while having limited effect on the functionality of biosensors (Figs. 2b–d and 3c–e). At the same time, the bound NIR-Fb_{LAG}s provide stable longitudinal fluorescence. Although NIR-Fb_{LAG}s exhibit about twice higher affinity to the calcium-free conformation of GCaMP6s according to the flow cytometry data, we observed no change in NIR fluorescence after the increase in calcium level, suggesting the NIR-Fb_{LAG}s dissociation from calcium-bound GCaMP6s is quite slow at least for minutes after an increase in calcium concentration (Fig. 2c, d). This discrepancy may be

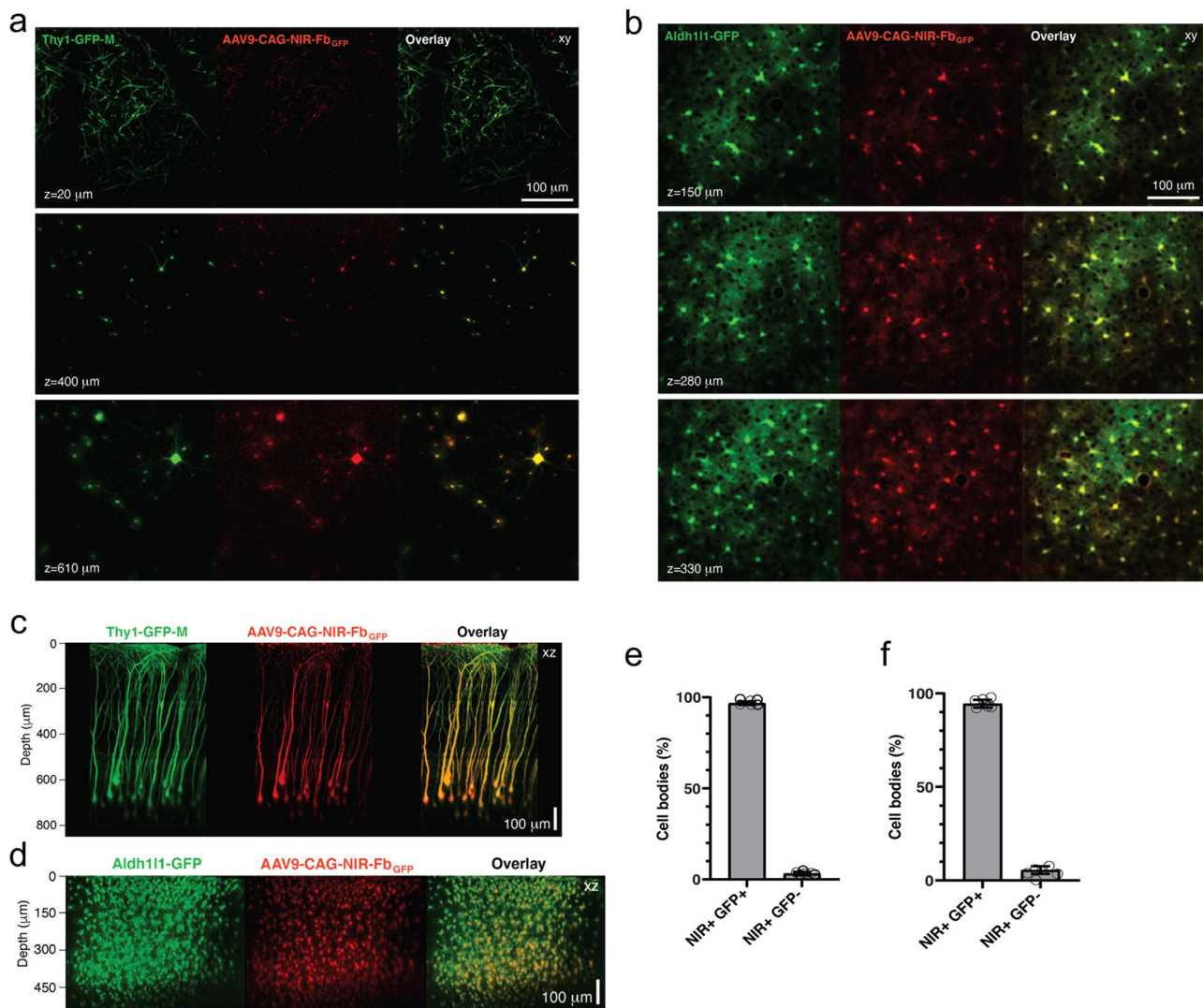


Fig. 7 | Targeting GFP-expressing neurons and astrocytes in the brain of live mice with NIR-Fb_{GFP}. **a** Left, example images from a xy fluorescence image stack showing GFP-expressing neurons and their processes (green) in the somatosensory cortex of an anesthetized 9.5-week-old *Thy1*-GFP-M mouse at the indicated depths (z) from the pial surface. Center, the same neurons show miRFP670nano3 expression three weeks after stereotactic AAV9-CAG-NIR-Fb_{GFP} vector delivery. Right, overlay of the simultaneously acquired two-photon images. Scale bar, 100 μm. **b** Left, example images from a xy fluorescence image stack showing GFP-expressing astrocytes and their processes (green) in the somatosensory cortex of an anesthetized 16-week-old *Aldh1l1*-GFP mouse at the indicated depths (z) from the pial surface. Center, the same astrocytes show miRFP670nano3 expression 4.5 weeks after stereotactic AAV9-CAG-NIR-Fb_{GFP} vector delivery. Right, overlay of the simultaneously acquired two-photon images. Scale bar, 100 μm. **c** Example

maximum-intensity side projection (xz) from the image stack in panel **a** showing the GFP (left) and miRFP670nano3 (center) expression pattern across depth. An overlay image is shown on the right. Scale bar, 100 μm. **d** Example maximum-intensity side projection (xz) from the image stack in panel **b** showing the GFP (left) and miRFP670nano3 (center) expression pattern across depth. An overlay image is shown on the right. Scale bar, 100 μm. **e** Population analysis showing the percent overlap between miRFP670nano3 and GFP-positive cell bodies (NIR + GFP+: 96.8% ± 1.1%; NIR + GFP-: 3.2% ± 1.1%; *n* = 6 tissue sections from two mice). **f** Population analysis showing the percent overlap between miRFP670nano3 and GFP-positive cell bodies (NIR + GFP+: 94.4% ± 2.6%; NIR + GFP-: 5.6% ± 2.6%; *n* = 6 tissue sections from two mice). The entire xy fluorescence image stacks and xz side projections are shown in Supplementary Movies 1–4. **e**, **f** Data are presented as mean values ± s.d. Source data are provided as a Source Data file.

due to the prolonged, 40–60 min, incubation of cellular cultures in calcium-saturated conditions before detection by flow cytometer, which may fasten dissociation of the NIR nanobody from GCaMP6s. At the same time, the fast calcium association and dissociation kinetics, as well as affinity to calcium ions of GCaMP6s, were not affected by NIR-Fb_{LAG30} binding (Supplementary Fig. 9). This makes NIR-Fb_{LAG30} beneficial for ratiometric deep-brain mapping of fast neuronal activity in vivo by simply comparing green and NIR fluorescence intensities.

We expanded the spectral range of NIR-Fbs by developing the NIR-Fb₍₇₁₈₎ having ~50 nm red-shifted fluorescence as compared to the original NIR-Fbs⁷ (Fig. 4a, b) and demonstrated efficient multi-color targeting of two different proteins in live cells (Fig. 4c). Also,

using the distinct-colors NIR-Fbs for GFP, we visualized two GFP-derived proteins in the cells, altogether demonstrating the wide possibilities of the NIR-Fbs with shifted emission for multiplexed imaging (Fig. 5).

Recently, adaptable turn-on maturation tools (ATOMs) based on circularly permuted monobodies (Mb) or Nbs, inserted into GFP-like FPs, were developed and tested in cells, but not in vivo⁴⁷. Similarly to NIR-Fbs, the fluorescence of ATOMs increases upon binding a cognate antigen. However, ATOMs do not degrade in the absence of antigen and, therefore, though non-fluorescent, accumulate in the cells. Also, depending on Mb stability, ATOM can be always in the “on” or “off” mode, thus, not reflecting a real increase in the antigen concentration.

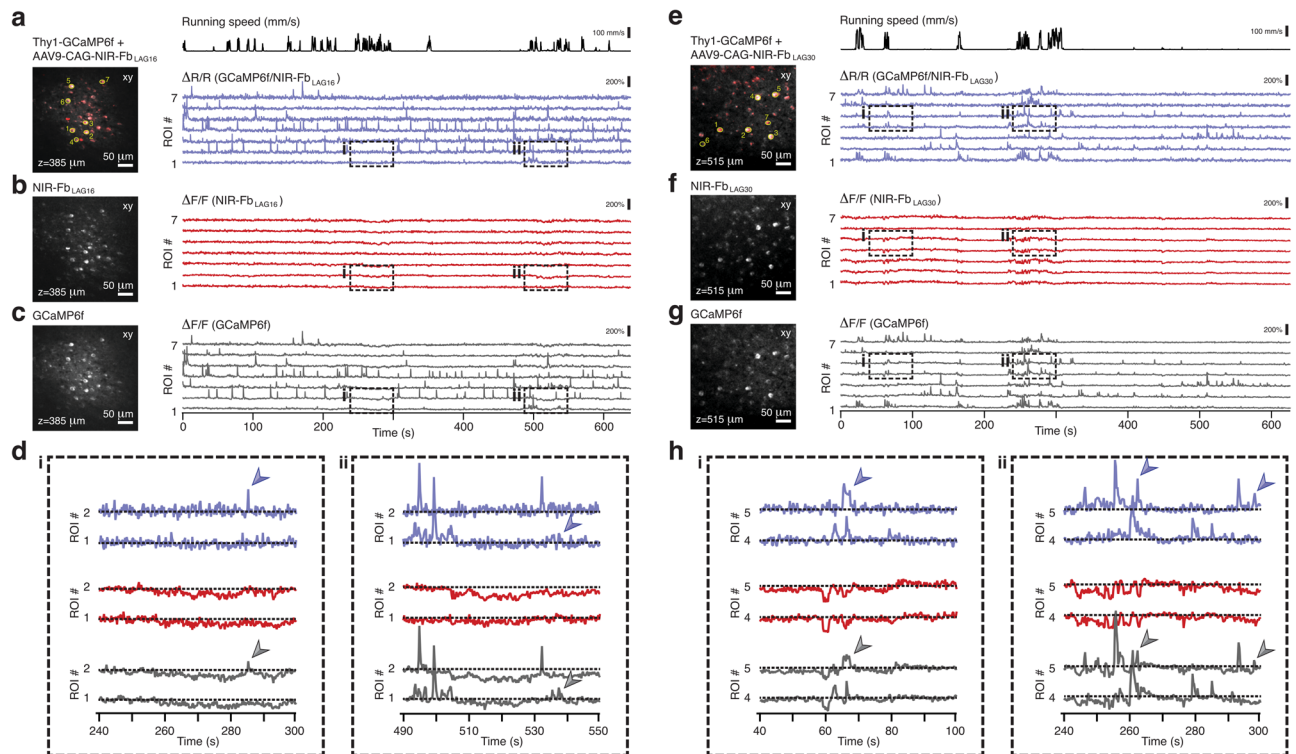


Fig. 8 | In vivo two-photon imaging of NIR-Fb_{LAG16} or NIR-Fb_{LAG30} in the somatosensory cortex of *Thy1-GCaMP6f* mice. **a** Example two-photon fluorescence image from a dual-color time-lapse recording showing GCaMP6f- and mRFP670nano3-expressing neurons in the somatosensory cortex of a behaving *Thy1-GCaMP6f* mouse. Imaging was performed ~4.5 weeks after stereotactic AAV9-CAG-NIR-Fb_{LAG16} injection. Recording depth (z) from the pial surface and seven somatic regions of interest (ROIs) are indicated. **b** Color-separated image for the NIR-Fb_{LAG16} channel. **c** Color-separated image for the GCaMP6f channel. In **a–c**, right, fluorescence transients in the indicated ROIs are shown as $\Delta R/R$ (blue) and $\Delta F/F$ (red and gray) for the combined and individual channels, respectively. The simultaneously recorded mouse's locomotor activity on a spherical treadmill is shown above the fluorescence traces. Scale bars, 50 μm (left), 100 mm/s, and 200% (right). **d** Zoom-ins of the two periods indicated in **a–c** show how the $\Delta R/R$

calculation corrects for fluorescence baseline variations caused by mouse locomotor activity and improves calcium spike estimation by identifying potential false positives and false negatives (arrowheads). **e** Example two-photon fluorescence image from a dual-color time-lapse recording for a *Thy1-GCaMP6f* mouse injected with AAV9-CAG-NIR-Fb_{LAG30}. **f** Color-separated image for the NIR-Fb_{LAG30} channel. **g** Color-separated image for the GCaMP6f channel. In **e–g**, right, fluorescent transients in the indicated ROIs are shown as in **a–c**. The simultaneously recorded mouse's locomotor activity on the spherical treadmill is shown atop. **h** Zoom-ins of the two periods indicated in **e–g** highlight the advantages of ratiometric imaging as in **d**. The corresponding time-lapse recordings are shown in Supplementary Movies 9, 10. xy fluorescence image stacks and xz side projections, including the recording sites, are shown in Supplementary Movies 5–8.

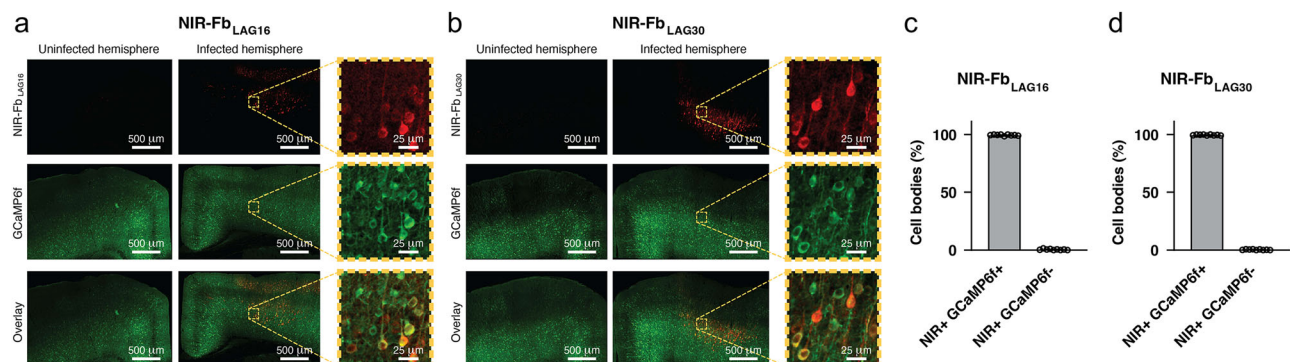


Fig. 9 | Targeting GCaMP6f-expressing neurons in the brain of mice with NIR-Fb_{LAG16} or NIR-Fb_{LAG30}. **a** Example confocal fluorescence images showing GCaMP6f- and mRFP670nano3-expressing neurons (center) in the cortex of an AAV9-CAG-NIR-Fb_{LAG16}-injected *Thy1-GCaMP6f* mouse. The uninfected control hemisphere of the same mouse is shown on the left. Right, zoom-ins of the indicated regions. Scale bars, 500 μm (center, left) and 25 μm (right). **b** Same as in panel **a** but for an AAV9-CAG-NIR-Fb_{LAG30}-injected *Thy1-GCaMP6f* mouse. **c** Population

analysis showing the percent overlap between NIR-Fb_{LAG16} and GCaMP6f-positive cell bodies (NIR+/GCaMP6f+: 99.4% $\pm$ 0.6%; NIR+/GCaMP6f-: 0.6% $\pm$ 0.6%; $n = 9$ tissue sections from three mice). **d** Population analysis showing the percent overlap between NIR-Fb_{LAG30} and GCaMP6f-positive cell bodies (NIR+/GCaMP6f+: 99.5% $\pm$ 0.4%; NIR+/GCaMP6f-: 0.5% $\pm$ 0.4%; $n = 9$ tissue sections from three mice). **c, d** Data are presented as mean values $\pm$ s.d. Source data are provided as a Source Data file.

NIR-Fbs, on the other side, simply degrade in the absence of a cognate antigen.

The antigen-stabilization of NIR-Fbs can be used to control cellular processes in an antigen-dependent manner. Coupling our engineered NIR-Fbs with GFP-labeling, we developed two systems, in which gene transcription starts upon GFP expression (Fig. 6a, b). Unlike the previously reported approach⁴⁸, the NIR-Fbs-fused fragments in our systems are rapidly degraded if unbound to GFP, allowing very tight control over gene transcription (Fig. 6c, d). These systems can be further applied to numerous available GFP-expressing transgenic animals for selective manipulations of GFP-labeled cells. Also, using NIR-Fb_{LAGS} as a destabilizing fusion partner it will be possible to target protein degradation in specific cell types expressing either GFP or GFP-based biosensors, unlike GFP only as with original NIR-Fb_{GFP}⁷.

NIR-Fb_{LAGS} offer advantages in *in vivo* imaging of transgenic animals expressing GFP or GFP-based biosensors (Figs. 7–9 and Supplementary Movies 1–10), addressing several challenges. First, transgenic mice express GFP-based probes heterogeneously in different cell populations due to random transgene integration into the genome^{27,28,49,50}. Additionally, discrepancies in GFP-based biosensor expression levels, illumination conditions, and variations in analyte concentrations complicate data interpretation. NIR-Fb_{LAGS} could serve as orthogonal reference probes, enabling the normalization of functional fluorescence changes, including in situations where correction of image motion is infeasible or suboptimal (e.g., variable within-frame distortions during low frame-rate image acquisitions, or low signal-to-noise recordings from deep tissue regions; Fig. 8e–h and Supplementary Movie 10). Second, small bright NIR-Fbs could be ideal optical guide stars for deep-tissue light focusing *in vivo*, especially in scattering tissues such as the brain. Third, targeting NIR-Fb_{LAGS} to various intracellular compartments could provide precise localization for GFP-derived probes.

These and other advances of the engineered NIR-Fb_{LAGS}, NIR-Fb₍₇₁₈₎, and NIR-Fbs-based gene transcription systems highlight their great potential to enhance the accuracy and reliability of *in vivo* studies, marking a valuable step forward in functional imaging and control of cellular processes.

Methods

Ethical statement

All live animal procedures were performed following the National Institutes of Health (NIH) guidelines and were approved by the Institutional Animal Care and Use Committee (IACUC) of the Salk Institute under the protocol number 13-00022.

Recombinant protein production in bacteria

The gene encoding the NIR-Fb_{LAG30} protein was amplified by polymerase chain reaction (PCR) and inserted into the pET22 vector. All oligonucleotide PCR primers were purchased from Thermo Fisher Scientific. NIR Nb was expressed in Rosetta(DE3)pLysS bacterial cells, cotransformed with pWA23h plasmid, encoding heme oxygenase. Bacterial cells were grown to an optical density of 0.5–0.7 at 600 nm in LB/ampicillin/kanamycin/chloramphenicol medium supplemented with 0.02% rhamnose and, then, to induce NIR-Fb_{LAG30} expression, 0.5 mM IPTG was added. Bacteria were cultured for 2–3 h at 37 °C and then for 36 h at 18 °C. Protein purification was performed with Ni-NTA agarose (Qiagen). Proteins were washed with wash buffer containing 20 mM PBS, 0.1 M NaCl, 1 mM β -mercaptoethanol and 20 mM imidazole and eluted with elution buffer containing 20 mM PBS, 0.1 M NaCl, 1 mM β -mercaptoethanol and 250 mM imidazole. For purification of NIR-Fb_{LAG30} complex with mEGFP, the same protocol was used, except for Rosetta(DE3)pLysS cells were cotransformed with pET22-NIR-Fb_{LAG30}, pWA23h, and pWA23-mEGFP.

GCaMP6s calcium biosensor in the pBAD/HisB vector was transformed into an LMG194 bacterial host (Invitrogen/Thermo Fisher

Scientific). The bacterial cells were grown to an optical density of 0.5–0.7 at 600 nm in LB/ampicillin medium, and then 0.02% arabinose was added to induce GCaMP6s expression. After 48 h culture at 30 °C, bacteria were centrifuged at 4650 $\times g$ for 10 min, and cell pellets were resuspended in PBS at pH 7.4 with 300 mM NaCl and lysed by French press. The recombinant protein was purified using Ni-NTA resin (Qiagen), followed by dialysis for 16–18 h against buffer solutions (50 mM MOPS, 100 mM KCl, pH 7.2 with either 1 mM EDTA or 1 μ M CaCl₂).

Fluorescence imaging of purified NIR-Fb_{LAG30} fractions was performed on the IVIS Spectrum instrument (PerkinElmer). For mEGFP imaging, 465/20 nm excitation and 520/30 nm emission filters were used. For NIR-Fb_{LAG30} imaging, 640/20 nm excitation and 720/30 nm emission filters were used.

Fluorescence and absorbance spectra of NIR-Fb_{LAG30} were recorded with a FluoroMax-3 fluorimeter (Jobin Yvon) and a Hitachi U-3010 spectrophotometer, respectively. All measurements were performed in PBS (pH 7.4).

To determine the K_d s of GCaMP6s and GCaMP6s with NIR-Fb_{LAG30} for calcium ions, a series of buffers (100 mM KCl, 50 mM MOPS, pH 7.2), supplemented with either 10 mM EGTA or 10 mM CaEGTA, containing different amount of Ca²⁺ were prepared following the instructions provided in the Calcium Calibration Buffer Kit (Invitrogen). Purified proteins were then diluted to 5 μ g/mL in the series of buffers with different concentrations of free Ca²⁺, and their fluorescence (excitation 480 nm; emission 490–650 nm) was measured with a FluoroMax-3 fluorimeter (Jobin Yvon). Binding data was fit with a Hill equation: $y = F_{max} \times [Ca^{2+}]^n / (K_d^n + [Ca^{2+}]^n)$. The K_d s were calculated as the concentration of Ca²⁺ when the fluorescence change for GCaMP6s or GCaMP6s in the presence of NIR-Fb_{LAG30} was half its maximum value.

Stopped-flow spectroscopy of Ca²⁺-binding kinetics

A SX20 stopped-flow spectrofluorometer (Applied Photophysics) equipped with a 150 W xenon lamp and a photomultiplier was used for kinetic fluorescence measurements. The excitation wavelength was set to 490 nm, and a 495 nm cut-off filter was used. All measurements were done at 22 °C. Five replicates were averaged for analysis. Kinetics records were fitted to exponential using Origin 2017 SR1 software. To measure association kinetics, the GCaMP6s indicator (0.5 μ M) or GCaMP6s with NIR-Fb_{LAG30} (in molar concentration ratio of 1:2 for GCaMP6s: NIR-Fb_{LAG30}, incubated at 4 °C overnight before kinetic measurements) in 50 mM MOPS, 100 mM KCl, pH 7.2 and 1 mM EGTA were rapidly mixed (1:1) with a series of Ca²⁺ buffers. The change in the fluorescence signal during rapid mixing provided the relaxation rate constants (k_{obs}) for the Ca²⁺ association reaction at various Ca²⁺ concentrations (from 0 nM to 350 nM).

To measure dissociation kinetics, protein solutions (0.5 μ M; molar concentration ratio 1:2 for GCaMP6s: NIR-Fb_{LAG30}) in 50 mM MOPS, 100 mM KCl, pH 7.2 and 1 μ M CaCl₂ were rapidly mixed (1:1) with 50 mM MOPS, 100 mM KCl, pH 7.2 and 10 mM EGTA. Exponential fitting of the fluorescence signal changes over time provided the dissociation rate constants (k_{off}).

Construction of mammalian plasmids

Anti-GFP Nbs internally fused with mRFP670nano3 or mRFP718nano were generated by overlap PCR, and the resulting constructs were inserted into the pcDNA plasmid at the KpnI/EcoRI sites. All oligonucleotide primers for PCR amplification were purchased from Thermo Fisher Scientific (Supplementary Table 1). LAG30 Nb to GFP was amplified from a pET21-pelB-LAG-30 plasmid (Addgene no.172754). LAG16 Nb to GFP was amplified from a pET21-pelB-LAG-16 plasmid (Addgene no.172746). GBP2, GBP6, and GBP7 Nbs to GFP were amplified from pCAG-Gal4DBD-GBP2 (Addgene no.49439), pCAG-p65AD-GBP6 (Addgene no.49440), and pCAG-GBP7-p65AD (Addgene no.49441) plasmids, respectively. Sb44 and Sb92 Nbs to TGFP were

amplified from pSb-init-Sb44 (Addgene no.159421) and pSb-init-Sb92 (Addgene no.159424) plasmids, respectively.

GCaMP6s biosensor and its truncated version were amplified from a pAAV.CaMKII.GCaMP6s.WPRE.SV40 (Addgene no.107790) plasmid and inserted into the pcDNA plasmid at the HindIII/EcoRI sites. Glucose biosensor iGlucoSnFR was amplified from a pAAV.hSynap.(cyto)iGlucoSnFR-mRuby2 (Addgene no.164510) and inserted into the pcDNA plasmid at the KpnI/EcoRI sites.

To generate the NES-p24-mTagBFP2 plasmid, the mTagBFP2 gene was swapped with mEGFP in the NES-p24-mEGFP plasmid by the NEBuilder HiFi DNA Assembly. To generate NES-p24-mEGFP plasmid, a fusion of HIV p24 with mEGFP was amplified and inserted into the pcDNA plasmid with an NES signal (Addgene no.164052) at the BamHI/XhoI sites. To construct an NES-pH-tGFP plasmid, pH-tGFP was amplified from a pH-tGFP plasmid (Addgene no.74322) and inserted into the pcDNA plasmid with an NES signal (Addgene no.164052) at the BamHI/XhoI sites.

To generate mTagBFP2-sfGFP fusions, mTagBFP2 was fused to the N-end part of the sfGFP (nucleotides 1–157) via 8 a.a. long linker (Gly₂SerGlySerGly₂Ser) by overlap PCR and C-end part of the sfGFP (nucleotides 158–238) was fused to mTagBFP2 via 8 a.a. long linker (Gly₂SerGlySerGly₂Ser) by overlap PCR. Amplified fusions were then inserted into the pcDNA plasmids by the KpnI/NotI sites.

To generate GAL4 fusions with sfGFP and NIR-Fb fusions with GAL4 and VP16, a 15-residue linker (Gly₃SerGly₄SerGly₃SerGly₂) was inserted into a pcDNA plasmid by the BamHI/NotI sites. For the construction of sfGFP(1–157) or sfGFP(158–238) fused to GAL4, GAL4 was amplified from a pQP-T2A plasmid (Addgene no.102583) and inserted into a pcDNA3.1 plasmid, containing the 15 aa-long linker at the KpnI/BamHI sites, and sfGFP(1–157) or sfGFP(158–238) was inserted at the NotI/XbaI sites. To generate NIR-Fb_{LAG30} fused to GAL4, GAL4 was amplified from a pQP-T2A plasmid (Addgene no.102583) and inserted into a pcDNA3.1 plasmid containing the 15 a.a. long linker at the KpnI/BamHI sites, and NIR-Fb_{LAG30} was inserted at the NotI/XbaI sites. To engineer NIR-Fb₍₇₁₈₎ fused to VP16, VP16 was amplified from a pQP-T2A plasmid (Addgene no.102583) and inserted into a pcDNA3.1 plasmid containing the 15 a.a. long linker at the KpnI/BamHI sites, and NIR-Fb₍₇₁₈₎ was inserted at the NotI/XbaI sites. A nuclear localization signal (NLS) was added to VP16-NIR-Fb₍₇₁₈₎ fusion by PCR amplification. For the construction of a pG12-mTagBFP2 reporter plasmid, mTagBFP2 was PCR-amplified and inserted into pG12-SEAP plasmid (Addgene no.170271) by BamHI/NotI sites.

Mammalian cell culture and transfection

HeLa (CCL-2) cells were obtained from the ATCC. Cells were cultured in a DMEM medium (Corning) supplemented with 10% FBS (Gembio), 0.5% penicillin-streptomycin (HyClone) and 2 mM glutamine at 37 °C. For live-cell fluorescence microscopy, cells were plated in 35 mm glass-bottom Petri dishes (Greiner Bio-One). Transient transfections were performed using polyethylenimine⁵¹ or Effectene reagent (Qiagen).

Green Pegassos (Addgene no.163114) and PyronicSF (Addgene no.124812) plasmids were used for transfection in GFP-based biosensors targeting experiments.

For multi-color imaging of two different NIR-Fbs, HeLa cells were cotransfected with plasmids encoding NIR-Fb_{59H10}, NIR-Fb₍₇₁₈₎, and cognate antigens, NES-p24-mTagBFP2 and H2B-EGFP, in a 1:5:1:1 ratio. For multi-color imaging of two NIR-Fbs for GFP, HeLa cells were cotransfected with plasmids encoding NIR-Fb_{LAG30}, NIR-Fb₍₇₁₈₎, NES-pH-tGFP and EBFP2-nuc, in a 1:5:10:1 ratio. For imaging and flow cytometry of NIR-Fb-based systems for transcription activation, HeLa cells were cotransfected with plasmids encoding GAL4-sfGFP(1–157), GAL4-sfGFP(158–238), VP16-NIR-Fb₍₇₁₈₎, and reporter plasmid pUAS-mTagBFP2 (12xUAS) in the 1:1:5:10 ratio or with plasmids encoding VP16-NIR-Fb₍₇₁₈₎, GAL4-NIR-Fb_{LAG30}, pUAS-mTagBFP2 (12xUAS) and mEGFP in the 1:1:1:10 ratio. As controls, cells transfected with GAL-VP16

(Addgene no.71728) and a reporter plasmid pUAS-mTagBFP2 or with pUAS-mTagBFP2 reporter plasmid only were used.

To study protein stability in mammalian cells, HeLa cells were incubated for 20 h with 10 μ M bortezomib (Millipore).

No biliverdin was exogenously supplied in all mammalian cell experiments.

Epifluorescence live-cell microscopy

Live mammalian cells were imaged using an Olympus IX81 inverted epifluorescence microscope equipped with a Lambda LS Xenon light source (Sutter). An ORCA-Flash4.0 V3 camera (Hamamatsu) was used for image acquisition. Cells were imaged using a 60 $\times$ 1.35 NA oil immersion objective lens (UPlanSApo, Olympus) and 390/40 nm, 480/40 nm, 570/30 nm, 605/40 nm, or 682/12 nm excitation filters and 460/40 nm, 530/40 nm, 615/40 nm, 667/30 nm, or 721/42 nm emission filters, respectively. Light power densities measured at the rear aperture of the objective lens were 0.76 mW cm⁻² for 390/40 nm, 3.73 mW cm⁻² for 480/40 nm, 2.1 mW cm⁻² for 570/30 nm, 3.41 mW cm⁻² for 605/40 nm, and 0.67 mW cm⁻² for 682/12 nm excitation filters, which gives an estimate (FN is 26.5 for the objective lens) for the light power densities at the front focal objective plane of 0.47 W cm⁻², 2.32 W cm⁻², 1.3 W cm⁻², 2.12 W cm⁻², and 0.42 W cm⁻², respectively. During imaging, HeLa cells were incubated in a DMEM medium and kept at 37 °C. The microscope was operated with a SlideBook v.6.0.8 software (Intelligent Imaging Innovations). For imaging performance of NIR-Fbs coexpressed with GCaMP6s biosensor, HeLa cells were washed twice with pre-warmed 20 mM HEPES-buffered Hanks' Balanced Salt Solution (HHBSS) (Corning) before imaging and incubated in HHBSS supplemented with 4 mM CaCl₂. After the first frame was imaged, 5 μ M ionomycin (Cayman Chemical) was added. Cells were maintained at 35 °C using the environmental chamber.

For imaging pyruvate biosensors, Green Pegassos and PyronicSF, HeLa cells were incubated in HHBSS 30 min before imaging. After the first frame was imaged, a 1 mM or 10 mM solution of sodium pyruvate (Gibco) was added, respectively. For imaging glucose biosensor, iGlucoSnFr, HeLa cells were incubated in a DMEM medium without glucose and pyruvate (Gibco). After the first frame was imaged, a 50 mM glucose solution was added.

For imaging of NIR-Fb_{LAG16}, NIR-Fb_{LAG30}, and NIR-Fb_{59H10}, 605/40 nm excitation and 667/30 nm or 640LP emission filters (Chroma) were used. For imaging NIR-Fb₍₇₁₈₎, 682/12 nm excitation and 721/42 nm emission filters (Chroma) were used. For imaging mEGFP, GCaMP6s, Green Pegassos, PyronicSF, iGlucoSnFr and pH-tGFP, 480/40 nm excitation and 530/40 nm emission filters (Chroma) were used. For imaging mTagBFP2 and EBFP2, 390/40 nm excitation and 460/40 nm emission filters (Chroma) were used. The data were analyzed using SlideBook v. 6.0.8 (Intelligent Imaging Innovations), GraphPad Prism v.5.04, and Origin 2017 SR1 software.

Flow cytometry

Flow cytometry analysis was performed using an LSR II (BD Biosciences) flow cytometer. Before analysis, live cells were washed with cold PBS, trypsinized for 5 min at 37 °C and diluted in cold PBS containing 4% FBS and 2 mM EDTA to a density of 500,000 cells per ml.

For analysis of cells co-expressing GCaMP6s in their Ca²⁺-saturated or Ca²⁺-depleted states and NIR-Fb_{LAGs}, samples were washed with cold HHBSS and trypsinized in 0.1% Soybean Trypsin Inhibitor (Thermo Fisher) for 5 min at 37 °C. Cells then were centrifuged at 4 °C, and pellets were washed with cold HHBSS, diluted in cold HHBSS containing 4 mM CaCl₂ or 2 mM EDTA to a density of 500,000 cells per ml and incubated for 40–60 min. At least 30,000 cells per sample were recorded. Data were collected using BD FACSDiva v.8.0.1 (BD Biosciences) software. The fluorescence intensity of cells expressing Nbs to GFP with internally fused miRFP670nano3 was analyzed using the 640 nm excitation laser

and 660/20 nm emission filter. The fluorescence intensity of cells expressing Nb to GFP with internally fused mRFP718nano was analyzed using the 640 nm excitation laser and 780/60 nm emission filter. The fluorescence intensity of mEGFP was analyzed using a 488 nm laser for excitation, and its fluorescence was detected with a 525/50 nm emission filter. The fluorescence intensity of mTagBFP2 was analyzed using a 405 nm excitation laser and a 450/50 nm emission filter. The fluorescence intensity of mCherry was analyzed using a 561 nm excitation laser and a 610/20 nm emission filter. The data were analyzed using FlowJo v.7.6.2 software.

Animal subjects

Live animal procedures were performed following the NIH guidelines and approved by the IACUC of the Salk Institute. We used two male *Thy1*-GFP-M (Jackson Laboratories; RRID: IMSR_JAX:007788), two male *Aldh1l1*-EGFP (GENSAT; RRID: MMRRC_011015-UCD), and eight female *Thy1*-GCaMP6f mice (Jackson Laboratories; RRID: IMSR_JAX:025393) with a typical age of ~10–17 weeks at the time of imaging (~3–4.5 weeks after stereotactic injection; two injections per hemisphere). All mice were on a C57BL/6J background. Mice were group-housed, provided with bedding and nesting material, and maintained on a 12-h light-dark cycle in a temperature ($22 \pm 1^\circ\text{C}$) and humidity controlled (45–65%) environment.

Virus production and stereotactic injection

The *NIR-Fb_{GFP}*, *NIR-Fb_{LAG16}*, and *NIR-Fb_{LAG30}* genes were PCR amplified and subcloned into an AAV transfer vector downstream of the CAG promoter. These vectors were cotransfected into HEK293-AAV cells (Vector Biolabs) along with a pAdeno-helper vector and a pRC-AAV9 rep-cap plasmid. Recombinant AAV9 production was then carried out either in-house or by the Viral Vector Core at the Salk Institute. The recombinant AAV9-CAG-NIR-Fb_{GFP}, AAV9-CAG-NIR-Fb_{LAG16}, and AAV9-CAG-NIR-Fb_{LAG30} viruses had a titer of $2.4\text{E} + 13\text{ GC ml}^{-1}$, $2.4\text{E} + 14\text{ GC ml}^{-1}$, and $2.6\text{E} + 14\text{ GC ml}^{-1}$, respectively.

0.4 μl of diluted AAV (1:10 and 1:50 for NIR-Fb_{GFP} and NIR-Fb_{LAG30}, respectively) was injected into the somatosensory cortex (coordinates: AP -0.5 – (-1.85) mm; ML 1.4 – 1.65 mm; DV 0.3 – 0.95 mm). Surgical procedures closely followed previously established protocols^{7,52}. Briefly, thin-wall glass pipettes were pulled on a Sutter Flaming/Brown micropipette puller (model P-97). Pipette tips were cut at an acute angle under $10\times$ magnification using a sterile technique. Tip diameters were typically 15 – $20\text{ }\mu\text{m}$. Pipettes that did not result in sharp bevels nor had larger tip diameters were discarded. Millimeter tick marks were made on each pulled needle to measure the virus volume injected into the brain.

Mice were anesthetized with isoflurane (4–5% for induction; 1–1.5% for maintenance) and positioned in a computer-assisted stereotactic system with digital coordinate readout and atlas targeting (Leica Angle Two). Body temperature was maintained at 36 – 37°C with a direct current (DC) temperature controller and ophthalmic ointment was used to prevent eyes from drying. A small amount of depilator cream (Nair) was used to remove hair over the dorsal areas of the injection site. The skin was cleaned and sterilized with a two-stage scrub of betadine and 70% ethanol.

A midline incision was made beginning just posterior to the eyes and ending just past the lambda suture. The scalp was pulled open and the periosteum-cleaned using a scalpel and forceps to expose the desired hemisphere for calibrating the digital atlas and coordinate marking. Once reference points (bregma and lambda) were positioned using the pipet needle and entered into the program, the desired target was set on the digital atlas. The injection pipette was carefully moved to the target site (using AP and ML coordinates). Next, the craniotomy site was marked and an electrical micro-drill with a fluted bit (0.5 mm tip diameter) was used to thin a 0.5 – 1 mm diameter part of the bone over the target injection site. Once the bone was thin enough

to flex gently, a 30 G needle with an attached syringe was used to carefully cut and lift a small (0.3 – 0.4 mm) segment of bone.

For injection, a drop of AAV was carefully pipetted onto parafilm (2 – $3\text{ }\mu\text{l}$) to fill the pulled injection needle with the desired volume. Once loaded with sufficient volume, the injection needle was slowly lowered into the brain until the target depth was reached. Manual pressure was applied using a 30-ml syringe connected by shrink tubing and $0.4\text{ }\mu\text{l}$ of the virus was slowly injected over 5 – 10 min. Once the virus was injected, the syringe's pressure valve was locked. The position was maintained for approximately 10 min to allow the virus to spread and avoid backflow upon needle retraction. Each animal received two injections (~ 1 mm apart) in one hemisphere. Following the injections, head clamps were removed, muscles were approximated, and the skin was sutured along the incision. Mice were given subcutaneous Buprenorphine SR (0.5 mg per kg) and allowed to recover before placement in their cage.

Animal preparation for in vivo two-photon imaging

Surgical procedures closely followed established protocols^{7,52,53}. Briefly, mice were anesthetized with isoflurane (4–5% for induction; 1–1.5% for maintenance) on a custom surgical bed (Thorlabs). Body temperature was maintained at 36 – 37°C with a DC temperature control system and ophthalmic ointment was used to prevent eyes from drying. Depilator cream (Nair) was used to remove hair above the imaging site. The skin was thoroughly cleansed and disinfected with a two-stage scrub of betadine and 70% ethanol.

A scalp portion was surgically removed to expose frontal, parietal, and interparietal skull segments. Scalp edges were attached to the lateral sides of the skull using a tissue-compatible adhesive (3M Vet-bond). A custom-machined metal plate was affixed to the skull with dental cement (Coltene Whaledent, cat. no. H00335), allowing the head to be stabilized with a custom holder. Animals intended for awake imaging were allowed to recover for at least three days and then habituated to head fixation on a spherical treadmill (typically three sessions over three consecutive days). On the day of imaging, an approximately 3 mm diameter craniotomy was made over the AAV injection sites. A $\sim 1.5\%$ agarose solution and coverslip were applied to the exposed tissue. The coverslip was affixed to the skull with dental cement to control tissue motion. Mice imaged under awake conditions were allowed to recover from isoflurane anesthesia for at least 1 – 1.5 h before recordings commenced.

In vivo two-photon microscopy

Live animal imaging was performed 3–4.5 weeks after AAV injection, as previously described^{7,52,53}. Briefly, a Sutter Movable Objective Microscope equipped with a pulsed femtosecond Ti:Sapphire laser (Chameleon Ultra II, Coherent) and two fluorescence detection channels were used for imaging (green emission filter: ET-525/70M (Chroma); near-infrared emission filter: ET645/75M (Chroma) supplemented with a FF01-720/SP (Semrock); dichroic beamsplitter: 565DCXR (Chroma); photomultiplier tubes: H7422-40 GaAsP (Hamamatsu)). The laser excitation wavelength was set between 880 and 920 nm. The average laser power was <10 – 15 mW at the tissue surface and adjusted with depth as needed to compensate for signal loss due to scattering and absorption. At the excitation intensities and durations used in this study, no signs of phototoxicity, such as a gradual increase in baseline fluorescence, lasting changes in activity rate, or blebbing of labeled cells, were apparent in our recordings. An Olympus $20\times$ 1.0 NA water immersion objective was used for light delivery and collection. Z-stacks included up to 900 images, acquired at $1\text{ }\mu\text{m}$ axial step size, using a two- to four-frame average, 512×512 -pixel resolution, and 1.0 – $2.5\times$ zoom (corresponding to 701 – $282\text{ }\mu\text{m}$ fields of view). Time-lapse recordings used a 256×256 -pixel resolution, $1.0\times$ zoom (corresponding to a $348\text{ }\mu\text{m}$ field of view), and 8.14 Hz frame rate. Awake recordings were performed in head-fixed mice on a spherical treadmill

equipped with an optical encoder (E7PD-720-118; US Digital). Analog and imaging data were acquired synchronously, allowing the animal's running speed to be related to cellular fluorescence changes (Fig. 8 and Supplemental Movies 9, 10).

Brain tissue fixation, slicing and immunostaining

Around 3–4.5 weeks after AAV injection, mice were euthanized in their home cage following American Veterinary Medical Association (AVMA) guidelines. Transcardial perfusion was performed using 10% sucrose followed by 4% paraformaldehyde (PFA). Brain tissue was carefully extracted and incubated in 4% PFA overnight at 4 °C. The tissue was then washed on a shaker with 1× PBS three times (15 min per wash). A Leica VT1000S vibratome was used to prepare 40-μm-thick coronal tissue sections. Immunostaining was performed on floating sections using standard techniques. Primary and secondary antibodies included GFP (1:250 dilution; Thermo Fisher Scientific; RRID: AB_2534023) and Alexa Fluor 488 goat anti-chicken (1:100 dilution; Thermo Fisher Scientific; RRID: AB_2534096), respectively.

Confocal microscopy

Confocal imaging of brain sections was performed on a Zeiss LSM 710 with ZEN Black software (v2011) and an Olympus 20×0.8 NA air-matched objective. Two-channel, 4–6×4–6 tiled z-stacks (~15 images at 1 μm axial step size) were acquired using 488 nm and 633 nm laser lines. Each z-stack image had a 1024×1024-pixel resolution.

Image and analog data processing

Data were processed, analyzed, and plotted in Fiji (v2.0.0c; SciJava), Imaris (v9.2; Oxford Instruments), Igor Pro (v9.04; WaveMetrics), and Prism software (v8.4.3; GraphPad). xz projections were created from xy fluorescence image stacks using the 'Reslice' and '3D Project' functions in Fiji. Motion artifacts in time-lapse recordings were reduced using the 'Image Stabilizer' and 'TurboReg' plugins. Analog data from the spherical treadmill encoder were processed using Igor Pro. Supplemental Movies 1–10 were created using Igor Pro. Immunostained tissue was quantified by generating digital representations of labeled cells with Imaris' spot creation wizard. To quantify the overlap of NIR+ and GFP+ or GCaMP6f+ cell bodies, cells were classified as "Spots." Spots were created on background-subtracted images. A 10 μm "XY Diameter" was used as the spot size filter. The 'Quality' filter was set to include the top 2% and 6% of spots for GFP+ and GCaMP6f+ cells, respectively, accounting for differences in labeling intensity and uniformity.

Statistics and reproducibility

No statistical method was used to predetermine sample size. No data were excluded from the analyses for all experiments, except for histology. For histology, slices with weak or uneven/irregular staining were excluded from data analysis. The experiments were not randomized. The investigators were not blinded to allocation during the experiments and outcome assessment. No sample-size estimation was performed to ensure adequate power to detect a pre-specified effect size. For flow cytometry, at least 30,000 cells per sample were recorded. For animal experiments, the sample size included 2–6 mice for each experiment. Sex as a biological variable was not considered in the research design and analyses, as the study's primary goal was to demonstrate the approach's technical capabilities.

Standard $n > 3$ independent experiments were performed for all presented nanobodies, cognate antigens, and genetically encoded sensors, unless noted in the figure legend. All attempts at replication were successful. For all experiments involving live animals, the results were replicated successfully within and across animals. For histology experiments, 2–3 biological replicates were used with 3 or 6 slices per animal quantified. Fluorescence imaging of NIR-Fb₍₇₁₈₎ (Fig. 4b), multicolor imaging of two different NIR-Fbs

(Fig. 4c) and multicolor imaging of two NIR-Fbs to GFP (Fig. 5a–e) show typical data of $n = 3$ independent experiments. Fluorescence imaging of NIR-Fbs binding to split-sfGFP (Supplementary Fig. 14a, d, e) and fluorescence imaging of NIR-Fb_{GFP}-based systems for gene transcription activation (Supplementary Fig. 16a, b) show typical data of $n = 2$ independent experiments. For other experiments n is provided in the Figure legends.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

All data supporting the findings of this study are available within the article, its Supplementary Information and Source Data file. The major plasmids constructed in this study, their maps and sequences are deposited at Addgene (#220739 - #220750). Protein structures used in this work include PDB IDs 3OGO, 7LSC, 3WLD, 6LR7, 7SAI, 7LSD. Source data are provided with this paper.

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Author contributions

N.V.B. together with O.S.O. developed the NIR-Fbs, N.V.B. characterized NIR-Fbs in cells. E.C. performed the AAV injections and mouse surgeries. A.N. planned and conducted mouse surgeries and two-photon in vivo imaging. V.V.V. planned and supervised the whole project, and together with N.V.B. and A.N. designed the experiments, analyzed the data, and wrote the manuscript. All authors reviewed the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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