PUSHING THE FRONTIER OF FLUORESCENCE MICROSCOPY REQUIRES THE DESIGN OF ENHANCED FLUOROPHORES WITH FINELY TUNED PROPERTIES. WE RECENTLY DISCOVERED THAT INCORPORATION OF FOUR-MEMBERED AZETIDINE RINGS INTO CLASSIC FLUOROPHORE STRUCTURES EILTS SUBSTANTIAL INCREASES IN BRrightNESS AND PHOTOSTABILITY, RESULTING IN THE JANELIA FLUOR (JF) SERIES OF DYES. WE REFINED AND EXTENDED THIS STRATEGY, FINDING THAT INCORPORATION OF 3-SUBSTITUTED AZETIDINE GROUPS ALLOWS RATIONAL TUNING OF THE SPECTRAL AND CHEMICAL PROPERTIES OF RHODAMINE DYES WITH UNPRECEDENTED PRECISION. THIS STRATEGY ALLOWED US TO ESTABLISH PRINCIPLES FOR TUNING THE PROPERTIES OF FLUOROPHORES AND TO DEVELOP A PALETTE OF NEW FLUORESCENT AND FLUOROGENIC LABELS WITH EXCITATION RANGING FROM BLUE TO THE FAR-RED. OUR RESULTS DEMONSTRATE THE VERSATILITY OF THESE NEW DYES IN CELLS, TISSUES AND ANIMALS.

Small molecule fluorophores are essential tools for biochemical and biological imaging \(^1,2\). The development of new labeling strategies \(^3\) and innovative microscopy techniques \(^4\) is driving the need for new fluorophores with specific properties. A particularly useful class of dyes is the rhodamines, first reported in 1887 (ref. 5) and now used extensively because of the superb brightness and excellent photostability of this fluorophore scaffold \(^1,2,6\). The photophysical and chemical properties of rhodamines can be modified through chemical substitution \(^6–14\), allowing the creation of fluorescent and fluorogenic labels, indicators and stains in different colors \(^7–11,20\).

Despite a century of work on this dye class, the design and synthesis of new rhodamines remains severely limited by chemistry. The classic method of rhodamine synthesis—acid-catalyzed condensation \(^2,5,6\)—is incompatible with all but the simplest functional groups. To remedy this long-standing problem, our laboratory developed a method to synthesize rhodamine dyes using a Pd-catalyzed cross-coupling strategy starting from simple fluorescein derivatives \(^21\). This approach facilitated the discovery of a previously unknown class of dyes containing four-membered azetidine rings, which exhibit substantial increases in the quantum yield relative to classic rhodamines containing \(N,N\)-dimethylamino groups \(^13\). The flagship member of this new dye class is Janelia Fluor 549 (JF549, Fig. 1a). The enhanced brightness and photostability of this rhodamine dye has made it an exceptionally useful label for single-molecule experiments in living cells \(^13,22–25\), and the recent development of a photoactivatable derivative has further extended its utility in advanced imaging experiments \(^26\).

An important feature of rhodamine dyes is the ability to tune the spectral and chemical properties using chemistry \(^5\). JF549 (Fig. 1a and Table 1) absorbs green light \((\lambda_{\text{abs}}/\lambda_{\text{em}} = 549 \text{ nm}/571 \text{ nm})\), absolute fluorescence quantum yield value \((\Phi = 0.88)\), making it an excellent match for light sources centered near 550 nm. Replacing the xanthene oxygen in JF549 (1) with a quaternary carbon yields carborhodamine Janelia Fluor 608 (JF608, 2), with an expected 59-nm shift in spectral properties \((\lambda_{\text{abs}}/\lambda_{\text{em}} = 608 \text{ nm}/631 \text{ nm}, \Phi = 0.67)\). A larger red-shift can be achieved using the established Si-rhodamine strategy \(^10,12\) to yield Janelia Fluor 646 (JF646, 3, \(\lambda_{\text{abs}}/\lambda_{\text{em}} = 646 \text{ nm}/664 \text{ nm}, \Phi = 0.54\)). Finally, a shift to shorter wavelengths can be imposed by replacing one azetidine group in JF549 (1) with an oxygen atom to yield rhodol \(^27\) Janelia Fluor 519 (JF519-4, \(\lambda_{\text{abs}}/\lambda_{\text{em}} = 519 \text{ nm}/546 \text{ nm}, \Phi = 0.85\)).

In addition to large shifts in \(\lambda_{\text{abs}}\) and \(\lambda_{\text{em}}\), these modifications modulate the equilibrium between the colorless, nonfluorescent, ‘closed’ lactone (L) form and the colored, fluorescent, ‘open’ zwitterionic (Z) form. Rhodamine JF549 (1, \(\varepsilon = 1.01 \times 10^{5} \text{ M}^{-1} \text{ cm}^{-1}\)), carborhodamine JF608 (2, \(\varepsilon = 9.9 \times 10^{4} \text{ M}^{-1} \text{ cm}^{-1}\)) and rhodol JF519 (4, \(\varepsilon = 5.9 \times 10^{4} \text{ M}^{-1} \text{ cm}^{-1}\)) primarily adopt the open, zwitterionic form in water, as evidenced by their large extinction coefficients \((\varepsilon; \text{Table 1})\). In contrast, JF646 (3) predominantly adopts the colorless, nonfluorescent and lipophilic lactone form in aqueous solution \((\varepsilon = 5.0 \times 10^{3} \text{ M}^{-1} \text{ cm}^{-1})\). The shifted L-Z equilibrium of JF646 and other Si-rhodamines renders these dyes highly cell permeable as well as chromogenic and fluorogenic, where the change in chemical environment after binding of dye ligands to a variety of biomolecular targets can shift the equilibrium to the fluorescent zwitterionic form \(^12–14,18\).

The strategies described above allow only ‘coarse’ tuning of spectral and chemical attributes: spectral shifts >30 nm and substantial changes in L-Z equilibrium and absorptivity. We developed...
a general method to finely tune the spectral and chemical properties of rhodamine dyes with unprecedented precision. The use of 3-substituted azetidines on the Janelia Fluor 549 (1) scaffold allows modulation of λ_{abs}, λ_{em} and the L-Z equilibrium without affecting fluorescence quantum yield. The shifts in chemical and spectral properties can be explained using physical organic chemistry principles and computational chemistry. The structure-activity relationships that we determined for rhodamine dyes were generalizable to related structures, allowing the rational design of four new fluorophores aligned with standard laser excitation sources across the visible spectrum: rhodol JF$_{503}$-rhodamine JF$_{525}$-carborhodamine JF$_{585}$ and Si-rhodamine JF$_{635}$. These dyes exhibited improved properties for cellular imaging and could be extended to applications in tissue or in vivo.

RESULTS

Fine-tuning rhodamines: Janelia Fluor 525

We reasoned that we could finely tune the physiochemical properties of JF$_{549}$ (1) by exploring different substitution patterns on the azetidine ring. Indeed, the azetidinyl-rhodamine system provides an ideal test case for N-substituent effects because of the high-yielding Pd-catalyzed cross-coupling synthesis$^{21}$; the commercial availability of assorted 3-substituted azetidines; the short, three-bond separation between the substituent and the rhodamine aniline nitrogen; and the symmetry of the system. We hypothesized that electron-withdrawing groups would decrease the λ$_{abs}$ of the fluorophore and shift the L-Z equilibrium toward the closed, colorless lactone form based on initial computational chemistry experiments (Fig. 1b and Online Methods) and reports of fluoroalkane-substituted rhodamine dyes$^{19,28}$.

To test these predictions, we synthesized compounds 5–12 (Fig. 1a and Table 1) using our Pd-catalyzed cross-coupling approach$^{13,21}$. We then evaluated the photophysical properties of compounds 5–12 in aqueous solution, comparing them with JF$_{549}$ (1; Table 1). All of the substituted azetidinyl dyes showed high ε values above 1 × 10^5 M$^{-1}$ cm$^{-1}$ except for the 3,3-difluoroazetidinyl compound 12, which exhibited a slightly lower absorptivity (ε = 9.4 × 10^4 M$^{-1}$ cm$^{-1}$). Similarly, the quantum yield values of the azetidine dyes 5–12 were all >0.80, with the exception of the N,N-dimethyl-azetidin-3-amine compound 8, which showed Φ = 0.57 at pH 7.4. The quantum yield value for 8 was rescued at pH 5.0 (Φ = 0.89; Table 1), which is suggestive of photoinduced electron transfer (PeT) quenching by the unprotonated dimethylamino groups$^{29}$.

Although the ε and Φ of the different azetidinyl-rhodamine dyes was largely immune to substitution at the 3-position, the λ$_{abs}$ and λ$_{em}$ values were strongly affected by the nature of the substituent (Table 1 and Supplementary Fig. 1). Groups with greater electron-withdrawing character elicit larger hypsochromic shifts in λ$_{abs}$. This effect was additive, for example, the 3-fluorooazetidinyl compound 10 showed a 13-nm blue shift (λ$_{abs}$ = 536 nm) relative to the parent dye (1), and the 3,3-difluoroazetidinyl-rhodamine (12) showed a further hypsochromic shift of 11 nm (λ$_{abs}$ = 525 nm). We plotted λ$_{abs}$ against the available Hammet inductive substituent constants (σ$_J$)$^{30}$ for the azetidine substituents in dyes 1, 5 and 8–12 and observed an excellent correlation (Fig. 1c), suggesting that the inductive effect of the substituents was primarily responsible for the decrease in absorption and emission maxima. The experimental λ$_{abs}$ values also showed excellent agreement with calculated λ$_{abs}$ values (Fig. 1b).

We then analyzed how the azetidine substitutions can tune the L-Z equilibrium (Fig. 1d), first examining the absorbance of fluorophores 1, 5 and 9–12 as a function of dielectric constant using dioxane-water titrations$^{11,16}$ (Fig. 1e); compounds 6–8 were not examined as a result of the ionizable substituents on the azetidine ring. On the basis of these data, we determined the equilibrium constant (K$_{L-Z}$)$^{31}$ in 1:1 dioxane-water, which gave the largest distribution of absorbance measurements (Fig. 1f), and therefore K$_{L-Z}$ values (Fig. 1d and Table 1). We determined these equilibrium values from the maximal extinction coefficients (ε$_{max}$) measured in acidic alcohol solutions (Table 1 and Online Methods). JF$_{549}$ (1) and the 3,3-dimethyazetidinyl-rhodamine (5) showed K$_{L-Z}$ values >3, indicating that these dyes exist primarily in the open form. In contrast, the equilibrium constant of the 3,3-difluoroazetidinyl-rhodamine (12) was substantially smaller (K$_{L-Z}$ = 0.068), suggesting that the electron-withdrawing fluorine substituents can shift the equilibrium toward the closed lactone form. The remainder of the dyes exhibited K$_{L-Z}$ values that were intermediate and correlated with σ$_J$ (Fig. 1g). Collectively, these results yield rational and general rules for tuning both λ$_{abs}$ and the L-Z equilibrium using different 3-substituted azetidines without compromising fluorophore brightness.

Rhodamine 12 exhibited λ$_{abs}$ at 525 nm and a high quantum yield (Φ = 0.91), making it a useful label for imaging with blue-green excitation (514–532 nm). On the basis of the λ$_{abs}$, we named this fluorophore Janelia Fluor 525 (JF$_{525}$) and prepared the JF$_{525}$-HaloTag$^{12}$ ligand (13; Fig. 1h), which showed excellent labeling in live cells expressing histone H2B-HaloTag fusions (Fig. 1i). We posited that the JF$_{525}$-HaloTag ligand (13) would show improved cell permeability relative to the parent JF$_{549}$-HaloTag ligand (14; Fig. 1h) based on its higher propensity to adopt the lactone form (Fig. 1d–f and Table 1). We compared the labeling efficiency of 13 or JF$_{549}$-HaloTag ligand 14 in live cells expressing HaloTag-histone H2B fusions (Online Methods). Compound 13 labeled intracellular proteins faster than JF$_{549}$ ligand 14 (Fig. 1j). These results support the hypothesis that shifting the L-Z equilibrium toward the lactone form can improve cell permeability. We also synthesized the JF$_{525}$-SNAP-tag ligand 15, which was useful for intracellular labeling (Supplementary Fig. 2a,b), validating JF$_{525}$ as the first cell-permeable self-labeling tag ligand with an excitation maximum near 532 nm. None of the reported HaloTag or SNAP-tag ligands showed acute cellular toxicity at standard labeling concentrations and incubation times (Supplementary Fig. 2c).

Fine-tuning rhodols: Janelia Fluor 503

Since fluorine is the most electronegative atom, the difluoroazetidinyl-containing JF$_{525}$ (12) represents the tuning limit of the azetidinyl-rhodamines toward the blue region of the spectrum. To access shorter wavelength dyes, we turned to the rhodol Janelia Fluor 519 (4; Figs. 1a and 2a). On the basis of the tuning rules that we determined for the rhodamine dyes (Fig. 1a), we surmised that replacement of the single azetidine substituent with a 3,3-difluoroazetidine could elicit a desirable ~15-nm blue-shift to yield a dye with maximal absorption closer to 488 nm. To test this hypothesis, we synthesized the 3,3-difluoroazetidinyl-rhodol 16, which showed the expected blue-shifted spectra with λ$_{abs}$/λ$_{em}$ = 503 nm/529 nm, ε = 8.3 × 10^4 M$^{-1}$ cm$^{-1}$ and Φ = 0.87 (Fig. 2a, Table 1 and Supplementary Fig. 1); we named this compound Janelia Fluor 503 (JF$_{503}$).
We then synthesized the JF503-HaloTag ligand (17; Fig. 2b), which was an excellent label for histone H2B-HaloTag fusions in live cells (Fig. 2c). We compared this label to two other 488-nm-excited HaloTag ligands based on the classic rhodamine 110 ($\lambda_{\text{abs}}/\lambda_{\text{em}m} = 497\text{ nm}/520\text{ nm}$, 18) and the recently described N,N'-bis(2,2,2-trifluoroethyl)rhodamine ($\lambda_{\text{abs}}/\lambda_{\text{em}m} = 501\text{ nm}/525\text{ nm}$, 19; Supplementary Fig. 2d)19. The cell-loading time course for these structurally distinct and relatively polar dyes was similar (Supplementary Fig. 2e), but the JF503 ligand showed higher photostability than the other two dyes in live cells (Fig. 2d), consistent with previous reports comparing the photostability of rhodols to rhodamines27. JF503 could be extended to the SNAP-tag labeling system with JF503-SNAP-tag ligand (20; Supplementary Fig. 2f,g).

**Fine-tuning carborhodamines: Janelia Fluor 585**

Previously, we extended our Pd-catalyzed cross-coupling approach to carborhodamines11, resulting in the synthesis of JF608 (2; Figs. 1a and 2e, and Table 1)13. We also discovered that carborhodamines...
generally exhibit a higher propensity to adopt the colorless lactone form compared with rhodamines ($K_{L-Z}$ for 2 = 0.091). Nevertheless, this shift in the L-Z equilibrium is not sufficient to achieve fluorogenic ligands and its relatively long $\lambda_{abs}$ value makes JF508 suboptimal for multicolor cellular imaging experiments using orange (589 nm) and red (640 nm) excitation. Based on the rhodamine tuning (Fig. 1a), we expected that incorporation of a 3,3-difluoroazetidine would elicit a blue-shift of approximately 24 nm, bringing the $\lambda_{abs}$ closer to the desired excitation wavelengths. We were also curious whether this modification would create a fluorogenic ligand given that, in the rhodamine series, the 3,3-difluoroazetidine motif decreased the $K_{L-Z}$ by nearly two log units (Fig. 1d). Given the linear relationship between log $K_{L-Z}$ and $\sigma_1$ (Fig. 1g) we reasoned that this substitution should tune the equilibrium of JF508 (2, $K_{L-Z}$ = 0.091) closer to the fluorogenic JF646 (3, $K_{L-Z}$ = 0.0012; Table 1). We note previous efforts to shift the L-Z equilibrium of carborhodamines using direct fluorination produced a HaloTag ligand with modest, nine-fold fluorogenicity, but also severely decreased quantum yield. On the basis of the general trend to higher $\Phi$ values following incorporation of electron-withdrawing substituents in both rhodamines and rhodols (Table 1), we expected that substitution with fluorine atoms on the 3-position of azetidine would also increase quantum yield of carborhodamines.

To test these predictions, we synthesized the 3,3-difluoroazetidinyl-carborhodamine (21), which showed the expected blue-shift in spectra ($\lambda_{abs}/\lambda_{em} = 585 \text{ nm}/609 \text{ nm}$) and increase in quantum yield ($\Phi = 0.78$; Fig. 2e, Table 1 and Supplementary Fig. 1). Based on these properties the dye was named ‘Janelia Fluor 585‘ (JF585). As predicted, JF585 (21) also exhibited low visible absorption in water ($\varepsilon = 1.5 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$) and a $K_{L-Z}$ near zero (Table 1). We then evaluated these carborhodamines as biomolecule labels, preparing JF608–HaloTag ligand (22) and JF585–HaloTag ligand (23; Fig. 2f)11,13,21. We first determined the absorbance of these ligands.

Figure 2 | Rational fine-tuning of other dyes. (a) Tuning of JF519 (4) to yield JF503 (16). (b) Structure of JF503–HaloTag ligand 17. (c) Image of live, washed COS7 cells expressing histone H2B-HaloTag fusions and labeled with ligand 17. Scale bar represents 35 µm. (d) Comparison of the photostability of cells labeled with 17 and cells labeled with 488-nm-excited dyes 18 and 19 (Supplementary Fig. 2d); the initial photobleaching measurements are fitted to a linear regression. (e) Tuning of JF608 (2) to yield JF605 (21). (f) Structure of HaloTag ligands derived from JF608 (22) and JF585 (23). (g) Absorbance of HaloTag ligands 22 and 23 in the presence (+HT) or absence (−HT) of excess HaloTag protein; n = 2. (h,i) Representative images of COS7 cells expressing HaloTag-histone H2B fusion, labeled with 250 nM of HaloTag ligands 22 and 23 for 1 h and imaged directly without washing. The image for each dye pair was taken with identical microscope settings, $\lambda_{ex} = 594 \text{ nm}$. Numbers indicate mean signal (nuclear) to background (cytosol) ratio (S/B) in three fields of view. (h) JF608 ligand 22 (S/B from n = 224 areas). (i) JF585 ligand 23 (S/B from n = 235 areas). (j) Tuning of JF564 (3) to yield JF565 (25). (k) Structure of HaloTag ligands derived from JF564 (26) and JF635 (27). (l) Absorbance of HaloTag ligands 26 and 27 in the presence (+HT) or absence (−HT) of excess HaloTag protein (n = 2). (m,n) Representative images of COS7 cells expressing HaloTag-histone H2B fusion, labeled with 250 nM of HaloTag ligands 26 and 27 for 1 h and imaged directly without washing. The image for each dye pair was taken with identical microscope settings, $\lambda_{ex} = 647 \text{ nm}$. Numbers indicate mean signal (nuclear) to background (cytosol) ratio in three fields of view. (m) JF564 ligand 26 (S/B from n = 175 areas). (n) JF635 ligand 27 (S/B from n = 278 areas). Scale bars in h, i, m and n represent 15 µm.
ligands in the absence and presence of excess HaloTag protein. JF608-HaloTag ligand (22) showed only an 11% increase in absorption following reaction with the HaloTag protein, but JF585 ligand 23 showed a substantially higher absorbance increase of 80-fold (Fig. 2g). We then evaluated these dyes in ‘no wash’ cellular imaging experiments. Incubation of JF608–HaloTag ligand (22, 250 nM) with cells expressing histone H2B–HaloTag showed excellent nuclear labeling, but high background, as a result of the free ligand staining internal membrane structures (Fig. 2h). In contrast, cells that were incubated with 250 nM of JF585–HaloTag ligand 23 and imaged directly showed bright nuclei with low fluorescence background (Fig. 2i). The JF585–SNAP-tag ligand (24) also functioned as a cellular label (Supplementary Fig. 2h,i), and the orange JF585–HaloTag ligand (23) could be used in three-color experiments with the green JF503–SNAP-tag ligand (20) and our previously described red JF646–Hoechst stain33 (Supplementary Fig. 2j).

**Fine-tuning Si-rhodamines: Janelia Fluor 635**

Having success in applying these tuning rules to rhodol and carbodamine dyes, we then turned to the Si-rhodamine JF646 (3; Figs. 1a and 2j)13. We anticipated that addition of a single fluorine atom on each azetidine ring would elicit a ~13-nm hypsochromic shift and further decrease $K_{L-Z}$, yielding a dye with a $\lambda_{abs}$ near 633 nm and higher degree of fluorogenicity. True to this prediction, the synthesis of the desired fluorinated derivative 25 (Fig. 2j) afforded a dye that showed $\lambda_{abs}/\lambda_{em} = 635$ nm/652 nm and a slightly higher $\Phi = 0.56$ relative to JF646 (3). Compound 25 also exhibited an extremely low absorbance in water with

| Table 1 | Properties of azetidine-containing fluorophores 1–12, 16, 21 and 25 |
|---|---|
| Dye | X | R<sup>1</sup> | R<sup>2</sup> | $\lambda_{abs}$ (nm) | $\epsilon$ (M<sup>–1</sup> cm<sup>–1</sup>) | $\lambda_{em}$ (nm) | $\Phi$ | $K_{L-Z}$<sup>b</sup> |
| 1 (JF<sub>549</sub>) | O | | | 549 | 101,000 | 134,000 | 571 | 0.88 | 3.5 |
| 5 | O | | | 550 | 110,000 | 143,000 | 572 | 0.83 | 3.2 |
| 6 | O | | | 549 | 111,000 | 138,000 | 572 | 0.87 | — |
| 7 | O | | | 545 | 108,000 | 130,000 | 568 | 0.87 | — |
| 8 | O | | | 542 | 111,000 | 127,000 | 565 | 0.57<sup>c</sup> | — |
| 9 | O | | | 541 | 109,000 | 137,000 | 564 | 0.88 | 2.5 |
| 10 | O | | | 536 | 113,000 | 141,000 | 560 | 0.87 | 1.0 |
| 11 | O | | | 533 | 108,000 | 133,000 | 557 | 0.89 | 0.24 |
| 12 (JF<sub>525</sub>) | O | | | 525 | 94,000 | 122,000 | 549 | 0.91 | 0.068 |
| 13 (JF<sub>533</sub>) | O | | | 519 | 59,000 | 69,000 | 546 | 0.85 | — |
| 16 (JF<sub>503</sub>) | O | | | 503 | 83,000 | 95,000 | 529 | 0.87 | — |
| 2 (JF<sub>568</sub>) | N | | | 608 | 99,000 | 121,000 | 631 | 0.67 | 0.091 |
| 21 (JF<sub>509</sub>) | O | | | 585 | 1,500 | 156,000 | 609 | 0.78 | <0.0001 |
| 3 (JF<sub>646</sub>) | O | | | 646 | 5,000 | 152,000 | 664 | 0.54 | 0.0012 |
| 25 (JF<sub>593</sub>) | O | | | 635 | 400 | 167,000 | 652 | 0.56 | <0.0001 |

<sup>a</sup>Maximal extinction coefficient measured in ethanol or TFE with 0.1% TFA (rhodamines) or 0.01% Et<sub>3</sub>N (rhodols). <sup>b</sup>Equilibrium constant measured in 1:1 (vol/vol) dioxane:water. <sup>c</sup> $\Phi = 0.89$ in pH 5.0 buffer.

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an extinction coefficient value of approximately 400 M$^{-1}$ cm$^{-1}$, which gave a $K_{\text{L-Z}}$ near zero (Table 1 and Supplementary Fig. 1).

We call this dye Janelia Fluor 635 (JF$_{635}$).

Analogous to the experiments with JF$_{608}$ and JF$_{585}$, we synthesized the JF$_{635}$-HaloTag ligand (27) and compared it with the JF$_{666}$ ligand 26 (Fig. 2k). As reported previously$^{13}$, ligand 26 showed a 21-fold increase in absorbance following binding to the HaloTag protein (Fig. 2i). The shifted L-Z equilibrium of JF$_{635}$ caused HaloTag ligand 27 to show exceptionally low background and a 113-fold increase in absorbance following conjugation (Fig. 2i). Both of these absorbance increases were substantially larger than the previously published SiTMR ligand 28 (Supplementary Fig. 2k), which showed a 6.7-fold increase in absorption following reaction with the HaloTag protein$^{12,13}$. These in vitro results were mirrored in no-wash cellular imaging experiments, in which we incubated cells expressing histone H2B-HaloTag fusions with 250 nM ligands 26-28. JF$_{608}$ ligand 26 (Fig. 2m) and JF$_{635}$ ligand 27 (Fig. 2n) exhibited substantially lower nonspecific extranuclear fluorescence than the SiTMR compound 28 (Supplementary Fig. 2i), with JF$_{635}$ showing the highest contrast. The SNAP-tag ligand of JF$_{635}$ (29) effectively labeled SNAP-tag fusions in cells (Supplementary Fig. 2m,n) and the JF$_{635}$-HaloTag ligand (27) could be used in a two-color experiment with JF$_{525}$-SNAP-tag ligand (15; Supplementary Fig. 2o).

**Applications in tissue and in vivo**

The HaloTag ligands of tunable fluorophores Janelia Fluor 585 (23) and Janelia Fluor 635 (27) are small, cell permeable and exhibit high fluorogenicity following reaction with the HaloTag protein. We were curious whether these properties would make them useful for labeling in more complex biological environments such as tissue or whole animals. We first attempted labeling in living brain tissue from Drosophila larvae using the JF$_{635}$-HaloTag ligand (27; Fig. 2k) because of its far-red excitation (Fig. 2j) and high on/off ratio (Fig. 2l,n). We used a Drosophila GAL4 line expressing myristoylated HaloTag protein in ‘Basin’ neurons, which project basin-shaped arbors into the ventral nerve cord (VNC) of the larval fly$^{34}$. Explants from Drosophila third instar larvae were dissected, incubated briefly with 27 (1 µM, 10 min) and imaged using the SiMView light-sheet microscope$^{35}$. As shown in the projection of the SiMView three-dimensional reconstruction, the JF$_{635}$ label exhibited consistent labeling throughout...
the living tissue and low nonspecific background staining (Fig. 3a,b and Supplementary Fig. 3a–c), demonstrating its utility beyond simple cell culture.

We next evaluated the JF dyes in the brains of living mice. The JF_{585}-HaloTag ligand 23 was chosen on the basis of its high fluorogenicity (Fig. 2g,i) and superior two-photon fluorescence at 1,100-nm excitation (Fig. 3c), which is sufficiently separated from GFP-based indicators such as GCaMP6 (2-photon \( \lambda_{ex} = 940 \) nm)\(^{34} \) to allow multicolor imaging. Cytosolic HaloTag protein was coexpressed in layer 4 or layer 5 visual cortex (V1) neurons with GCaMP6s via viral transduction and the mice were fitted with a chronic cranial window (Online Methods). Injection of 100 nmol of HaloTag ligand 23 into the tail vein (intravenous, IV) revealed that the JF_{585} ligand was blood–brain-barrier-permeable and gave measureable labeling in the brain within 5 min, peaking around 6 h and lasting for nearly 2 weeks as measured by epifluorescence (Fig. 3d and Supplementary Fig. 3d). Subsequent intraperitoneal (IP) injection into the same set of mice also showed effective delivery to the brain, although with different pharmacokinetics in the early time points (<7 d; Fig. 3d). Under two-photon imaging, we observed that the GCaMP6s and JF_{585} signals colocalized (Fig. 3e, Supplementary Fig. 3e and Supplementary Video 1), and the labeling showed no significant effect on spontaneous neuronal activity (Fig. 3f,g), establishing the utility of this fluorophore and labeling strategy in vivo.

**DISCUSSION**

Despite the broad utility of rhodamines, methods to modulate the physicochemical properties of this dye class are relatively coarse and empirical. We developed a method that allows rational fine-tuning of fluorophore properties for specific biological applications. We first determined the tuning rules in the rhodamine system by synthesizing a panel of rhodamine variants using the bright and photostable JF_{549} scaffold (Fig. 1a). This resulted in the development of JF_{525} (12) and its derivatives 13 and 15, which constitute the first ligands for self-labeling tags with absorption maxima near 532 nm. The tuning rules that we discovered are generalizable to other fluorophore classes—rhodols, carboxorhodamines and Si-rhodamines—allowing the rational design of finely tuned fluorophores such as JF_{503} (16; Fig. 2a), JF_{585} (21; Fig. 2e) and JF_{635} (25; Fig. 2f). Together with JF_{549} (1) and JF_{666} (3; Fig. 1a)\(^{13} \), we have now described six dyes that span the visible region of the spectrum and match common excitation wavelengths for fluorescence microscopy. These bright fluorophores can be used immediately for structured illumination and stimulated emission depletion imaging and could be converted to photoactivatable derivatives\(^{26} \) for single-molecule localization microscopy experiments. Our general rules should allow fine-tuning of a variety of fluorescent reagents, including classic fluorophores\(^{13} \), emerging red-shifted rhodamine variants\(^{37} \) and fluorogenic ligands\(^{12,14,18,19,33} \), to further extend the range of bright fluorophores useful for fluorescence microscopy. Notably, the new HaloTag ligands derived from JF_{585} and JF_{535} showed a high degree of chromogenicity and fluorogenicity (Fig. 2g,i,l,n), a critical parameter in advanced imaging experiments\(^{38} \). Of particular interest is the ability to deliver these dyes to neural tissue in explants (Fig. 3a,b) or whole animals (Fig. 3d–g), which could allow the imaging of deeper structures in the brain or the in situ assembly of semisynthetic indicators for monitoring cellular activity\(^3 \).

Although we focused on the fluorine-substituted azetidines, the other substitutions (Fig. 1a) could be exploited to prepare fluorophores for specific applications. For example, the carboxy groups in compounds 6 and 7 could serve as attachment sites for a variety of chemical modifiers to improve solubility\(^7 \), quench unwanted triplet states\(^{39} \) or to allow the molecule to serve as a multivalent fluorescent cross-linker. The modest pH sensitivity and presence of the basic amine in compound 8 could allow it to function as a pH sensor or stain for lysosomes. The methoxy group on compound 9 could be elaborated to a polyethylene glycol (PEG) or another solubilizing group. Finally, the cyano group in compound 11 could be used in multimodal regimes in which both fluorescence and Raman\(^{40} \) modalities are used for imaging. In all, this general method to rationally tune photophysical and chemical properties against a backdrop of high quantum yield will allow the precise design of many new fluorophores for specific, sophisticated biological imaging experiments in increasingly complex systems.

**METHODS**

Methods, including statements of data availability and any associated accession codes and references, are available in the online version of the paper.

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**AUTHOR CONTRIBUTIONS**

L.D.L. and J.B.G. conceived the project. J.B.G. contributed organic synthesis and one-photon spectroscopy measurements. A.K.M. contributed organic synthesis and computational chemistry experiments. Y.L., R.L. and N.J. contributed mouse imaging experiments. T.A.B. contributed cultured cell imaging experiments. W.C.L. and F.J.K. contributed larval explant imaging experiments. R.P. and J.J.M. contributed two-photon spectroscopy measurements. L.D.L. contributed one-photon spectroscopy measurements and wrote the manuscript with input from the other authors.

**COMPETING FINANCIAL INTERESTS**

The authors declare competing financial interests: details are available in the online version of the paper.

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ONLINE METHODS

**Chemical synthesis.** Methods for chemical synthesis and full characterization of all novel compounds can be found in the Supplementary Note.

**UV-vis and fluorescence spectroscopy.** Fluorescent and fluorogenic molecules for spectroscopy were prepared as stock solutions in DMSO and diluted such that the DMSO concentration did not exceed 1% (vol/vol). Spectroscopy was performed using 1-cm path length, 3.5-ml quartz cuvettes or 1-cm path length, 1.0-ml quartz microcuvettes from Starna Cells. All measurements were taken at ambient temperature (22 ± 2 °C). Absorption spectra were recorded on a Cary Model 100 spectrometer (Agilent). Fluorescence spectra were recorded on a Cary Eclipse fluorometer (Varian). Maximum absorption wavelength (λ<sub>abs</sub>), extinction coefficient (ε), and maximum emission wavelength (λ<sub>em</sub>) were taken in 10 mM HEPES, pH 7.3 unless otherwise noted; reported values for ε are averages (n = 3). Normalized spectra are shown for clarity.

**Determination K<sub>L-Z</sub> and ε<sub>max</sub>.** To determine K<sub>L-Z</sub> we first performed dioxane-H<sub>2</sub>O titrations in spectral grade dioxane (Aldrich) and milliQ H<sub>2</sub>O (Fig. 1e). The solvent mixtures contained 0.01% (vol/vol) triethylamine to ensure the rhodamine dyes were in the zwitterionic form. The absorbance values at λ<sub>abs</sub> were measured on 5 µM samples (n = 4) using a quartz 96-well microplate (Hellma) and a FlexStation3 microplate reader ( Molecular Devices). Values of dielectric constant (ε<sub>r</sub>) were as previously reported<sup>43</sup>. We then calculated K<sub>L-Z</sub> using the following equation:<sup>31</sup> K<sub>L-Z</sub> = (ε<sub>dw</sub>/ε<sub>max</sub>)/(1 − ε<sub>dw</sub>/ε<sub>max</sub>). ε<sub>dw</sub> is the extinction coefficient of the dyes in a 1:1 (vol/vol) dioxane:water solvent mixture (Fig. 1f); this dioxane-water mixture was chosen to give the maximum spread of K<sub>L-Z</sub> values (Fig. 1e). ε<sub>max</sub> is the maximal extinction coefficients determined in different solvent mixtures depending on dye type: 0.1% (vol/vol) trifluoroacetic acid (TFA) in 2,2,2-trifluoroethanol (TFE) for the rhodamines (1, 5–12) and carboxohromaines (2, 21); 0.1% (vol/vol) TFA in ethanol for the Si-rhodamines (3, 25); 0.01% (vol/vol) Et<sub>3</sub>N in TFE for the rhodols (4, 16).

**Quantum yield determination.** All reported absolute fluorescence quantum yield values (Φ) were measured in our laboratory under identical conditions using a QuantaSUry-QY spectrometer (model C11374, Hamamatsu). This instrument uses an integrating sphere to determine photons absorbed and emitted by a sample. Measurements were carried out using dilute samples (A < 0.1) and self-absorption corrections<sup>42</sup> were performed using the instrument software. Reported values are averages (n = 3). The quantum yield for compound 8 at pH 5.0 was taken in 10 mM sodium citrate buffer containing 150 mM NaCl.

**Computational chemistry.** Computational experiments were performed using Gaussian 09 (ref. 43). DFT and TD-DFT methods were used to calculate the spectral properties of the azetidinyl-rhodamine compounds (Fig. 1b). Calculations were performed at the B3LYP/6-31+G(d,p)/IEFPCM and TD-B3LYP/6-31+G(d,p)/IEFPCM theory levels for the ground states and excited states respectively. Frequency calculations confirmed that an energy minimum was found in geometry optimizations. Linear response solvation with the IEPFCM model was sufficient to study the excited state energies. Evaluations of TD-DFT theory have discussed the overestimation of excitation energies<sup>44,45</sup>, and previous studies of rhodamine excited states have reported using ~0.4-eV correction to account for this overestimation<sup>46,47</sup>. We applied a consistent ~0.4-eV correction to the calculated excited state energies, which gave good agreement with spectroscopy experiments (Fig. 1b).

**Measurement of increase in absorbance of HaloTag ligands 22, 23, 26–28 following attachment with HaloTag protein.** HaloTag protein used as a 100 µM solution in 75 mM NaCl, 50 mM TRIS-HCl, pH 7.4 with 50% v/v glycerol (TBS–glycerol). Absorbance measurements were performed in 1.0-ml quartz cuvettes. HaloTag ligands 22, 23, 26–28 (5 µM) were dissolved in 10 mM HEPES, pH 7.3 containing 0.1 mg·ml<sup>−1</sup> CHAPS. An aliquot of HaloTag protein (1.5 equiv) or an equivalent volume of TBS–glycerol blank was added and the resulting mixture was incubated until consistent absorbance signal was observed (~60 min). Absorbance scans are averages (n = 2).

**Multiphoton spectroscopy.** HaloTag ligands 13, 14, 17, 23, 26 and 27 (5 µM) were incubated with excess purified HaloTag protein (1.5 equiv) in 10 mM HEPES, pH 7.3 containing 0.1 mg·ml<sup>−1</sup> CHAPS as above and incubated for 24 h at 4 °C. These solutions were then diluted to 1 µM in 10 mM HEPES buffer, pH 7.3 and the two-photon excitation spectra were measured as previously described<sup>48,49</sup>. Briefly, measurements were taken on an inverted microscope (IX81, Olympus) equipped with a 60×, 1.2 NA water objective (Olympus). Dye–protein samples were excited with pulses from an 80 MHz Ti-Sapphire laser (Chameleon Ultra II, Coherent) for 710–1080 nm and with an OPO (Chameleon Compact OPO, Coherent) for 1,000–1,300 nm. Fluorescence collected by the objective was passed through a dichroic filter (675DCSXR, Omega) and a short pass filter (720SP, Semrock) and detected by a fiber-coupled Avalanche Photodiode (SPCM_ AQRH-14, PerkinElmer). For reference, a two-photon excitation spectrum was also obtained for the red fluorescent protein mCherry (1 µM), in the same HEPES buffer. All excitation spectra are corrected for the wavelength-dependent transmission of the dichroic and band-pass filters, and quantum efficiency of the detector.

**General cell culture and fluorescence microscopy.** COS7 and U2OS cells (ATCC) were cultured in Dulbecco’s modified Eagle medium (DMEM, phenol red-free; Life Technologies) supplemented with 10% (vol/vol) FBS (Life Technologies), 1 mM GlutaMAX (Life Technologies) and maintained at 37 °C in a humidified 5% (vol/vol) CO<sub>2</sub> environment. The COS7 cells have integrated a histone H2B-HaloTag expressing plasmid via the piggyback transposase (that is, H2B–HaloTag), and the U2OS cells have integrated a Sec61<sup>ΔH</sup>-HaloTag expressing plasmid via the piggyback transposase. Both cells were kept under the selection of 500 µg/ml Geneticin (Life Technologies). Cell lines undergo regular mycoplasma testing by the Janelia Cell Culture Facility. Cells were imagined on confocal microscopes in the Janelia Imaging Facility (Zeiss LSM 710, W Plan APO 20×/1.8 D –or- Zeiss LSM 880, C-APO 40 ×/1.2 W Corr FCS M27) using the indicated filter sets.
Comparison of JF549 and JF525. For the dye loading comparison (Fig. 1j), H2B-Halo COS7 cells were stained for varying amounts of time with 100 nM of either JF525-HaloTag ligand 13 or JF549-HaloTag ligand 14. The dye was washed from the cells and subsequently labeled with JF646-HaloTag ligand 26 at 1 µM for 30 min. Fluorescence of JF549-HaloTag ligand was quantified from the nuclear signals in summed confocal image stacks collected with 633 nm Ex/638–759 nm Em and analyzed using Fiji. The integrated density of the nuclear signal was corrected by subtracting the integrated density of adjacent background regions. Labeling is expressed as the percent of the JF549-HaloTag fluorescence displaced by the JF525- and JF549-HaloTag ligands. The nuclear staining of these cells by the JF525-HaloTag ligand (Fig. 1i) is displayed as a maximum intensity projection of confocal image stacks, 514 nm Ex/530–657 Em.

Comparison of JF583 and other 488-nm-excited dyes. H2B-Halo COS7 cells were labeled with 200 nM of JF603-HaloTag ligand 17. HaloTag R110Direct ligand (18, Promega), or HaloTag ligand 19 (ref. 28), over a time course of 0–2 h. Cells were washed 2× with phosphate-buffered saline (PBS) and fixed with 4% (wt/vol) paraformaldehyde in 0.1 M phosphate for 30 min, followed by two more washes with PBS. Cells were imaged using confocal microscopy with 488 nm Ex/515–565 nm Em. The nuclear staining of these cells by the JF583-HaloTag ligand (Fig. 2c) is displayed as a maximum intensity projection of confocal image stacks. Corrected nuclear fluorescence was calculated as above to determine the cell loading profile (Supplementary Fig. 2e). To test the relative bleaching rates of these three dyes under imaging conditions (Fig. 2d), cells were stained and fixed as previously described for 2 h and then bleached with 488 nm at twice the typical excitation power and imaged after each of 70 cycles. Bleached fluorescence data are normalized to the initial fluorescence levels.

Comparison of HaloTag ligands 22, 23 and 26–28 in cells. H2B-Halo COS7 cells were labeled with 250 nM of JF608-HaloTag ligand (22), JF585-HaloTag ligand (23), JF646-HaloTag ligand (26), JF635-HaloTag ligand (27) or SiTMR-HaloTag ligand (28; Supplementary Fig. 2k) and imaged by confocal microscopy using 594 nm Ex/599–734 nm Em (JF608 and JF585) or 633 nm Ex/638–759 nm Em (JF646, JF635, or SiTMR). All five samples were imaged via confocal microscopy without washing out the dyes. Signal to background (S/B) ratios were determined using the mean fluorescence of the nuclei relative to a region adjacent to each nuclei using Fiji (n = 152–275 areas as noted; Fig. 2h,i,m,n and Supplementary Fig. 2i).

Staining with SNAP-tag ligands. COS7 cells were transfected with histone H2B–pSNAP-tag (New England Biolabs) and stable integration of this plasmid was selected for using 600 µg/ml Geneticin (Life Technologies). This cell line expresses the histone H2B protein fused to the 26th version of the SNAP-tag protein. Cells were stained with four different dyes as follows; JF503-SNAP-tag ligand (20, 2 µM for 90 min), JF525-SNAP-tag ligand (15, 3 µM for 30 min), JF585-SNAP-tag ligand (24, 2 µM for 3 h with 0.2% (wt/vol) Pluronic F-127), JF635-SNAP-tag ligand (29, 2 µM for 2 h with 0.2% (wt/vol) Pluronic F-127). After staining, cells were washed three times with complete media, followed by a 20-min incubation in a 37 °C, 5% CO2, humidified incubator. The media was replaced again immediately before imaging.

Multiplexed imaging using HaloTag and SNAP-tag. U2OS cells expressing Sec61β-HaloTag fusion were transfected with either histone H2B-SNAP-tag piggybac or TOMM20-pSNAPf plasmids using Lipofectamine 2000 (ThermoFisher). Sec61β encodes an endoplasmic reticulum membrane protein translocator protein, and TOMM20 encodes an outer mitochondrial membrane protein as part of a protein translocase complex. Live cells were simultaneously stained with combinations of JF635-SNAP-tag ligand (20, 1 µM) and JF585-HaloTag ligand (23, 100 nM; Supplementary Fig. 2j) or JF535-SNAP-tag ligand (15, 1 µM) and JF635-HaloTag ligand (27, 100 nM, Supplementary Fig. 2o) for 60 min. Cells were fixed with 4% paraformaldehyde (20 min), and simultaneously stained with JF646-Hoechst (5 µM, 30 min), as indicated, before imaging.

Cell viability assays. The effects of various Janelia Fluor ligand compounds on cell viability were tested using the tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Supplementary Fig. 2c). This colorimetric cell viability assay relies on NAD(P)H-dependent oxidoreductase enzymes that reduce the dye to an insoluble and highly absorbing formazan. COS7 cells were plated at 1.5 × 104 cells per well of a 96-well plate 24 h before the assay. Dyes were applied to cells at different concentrations to span standard labeling conditions in live cells (n = 3). HaloTag ligands 13, 14, 17, 23, 26 and 27 were applied for 1 h and SNAP-tag ligands 15, 20, 24 and 29 were applied for 3 h before addition of MTT to reflect typical maximum staining times for these dyes.

Staining of Drosophila larvae. The CNS of third instar Drosophila melanogaster larvae were dissected in physiological saline. For the JF635-HaloTag ligand staining (Fig. 3a,b and Supplementary Fig. 3a,c), we used larva expressing a previously described HaloTag containing a myristoylation sequence in the ‘Basin’ neurons under control of the enhancer fragment R72F11. The isolated nervous system was incubated in physiological saline containing 1 µM JF635-HaloTag ligand (27) for 10 min at ambient temperature (22 ± 2 °C). For the pan-neuronal comparison (Supplementary Fig. 3b), this animal expressed GCaMP6s via the Gal4/UAS system using a 57C10-Gal4 driver line. The specimens were then embedded in agarose and imaged with the SiMView light-sheet microscope. The VNC zoomed images for direct comparison (Supplementary Fig. 3a,b) show the raw image data after fusion and deconvolution, whereas the full image of JF635-HaloTag ligand labeling (Fig. 3a,b, Supplementary Fig. 3c) additionally uses filtering and gamma correction to show neuronal morphologies more clearly.

General information for mouse in vivo experiments. Male mice, 3–8 months old, were used for viral infection, dye injection and in vivo imaging of neurons in the visual cortex (V1): the Scnn1a-Tg3-Cre (Jax no. 009613) line was used for imaging in layer 4 cortical neurons (L4); and Rbp4-Cre mice (MMRRC no. 031125-UCD) were used for imaging in layer 4 cortical neurons (L5) neurons. All experimental protocols were conducted according to the National Institutes of Health guidelines for animal research.
Cranial window implant and virus injection. A craniotomy was carried out at the same time as the virus injection to provide optical access for in vivo imaging experiments. Mice were anesthetized with isoflurane (1–2% (vol/vol) in O2) and given the analgesic buprenorphine (SC, 0.3 mg/kg). Using aseptic technique, a 3.5 mm-diameter craniotomy was made over the left V1 region of the brain of anaesthetized mouse (center: 3.4 mm posterior to Bregma; 2.7 mm lateral from midline). The dura was left intact. HaloTag and GCaMP6s was cotransduced using the viral vector: AAV2/1.synapsin.FLEX.GCaMP6s.P2A.HaloTag.WPRE (~5 × 1012 infectious units per ml, 30 nl per site). The virus was injected using a glass pipette beveled at 45° with a 15–20-µm opening and back-filled with mineral oil. A fitted plunger controlled by a hydraulic manipulator (Narashige, MO10) was inserted into the pipette and used to load and inject the solution into six sites of left V1 (3.4–4.4 mm posterior to Bregma; 2.2–2.8 mm lateral from midline; ~0.5 mm distance between each injection site, 0.5 mm below pia). A cranial window made of a single glass coverslip (Fisher Scientific no. 1.5) was embedded in the craniotomy and sealed in place with dental acrylic. A titanium head-post was attached to the skull with cyanoacrylate glue and dental acrylic.

Dye administration in vivo. JF585-HaloTag ligand (23) was first administered to mice 3–4 weeks after the cranial window installation and viral injection. Dye solution was prepared by first dissolving 100 nmol (76 µg) of 23 in 20 µl DMSO. After vortexing, 20 µl of a Pluronic F-127 solution (20% (wt/wt) in DMSO) was added and this stock solution was diluted into 100 µl or 200 µl sterile saline for IV (tail vein) or IP injection, respectively.

In vivo wide-field imaging and analysis. Mice were head-fixed and awake during the imaging period and were therefor habituated to experimental handling and head fixation starting 1-week post-surgery. During each habituation session, mice were head-fixed onto the sample stage with body restrained under a half-cylindrical cover. The habituation procedure was repeated 3–4 times for each animal for a duration of 15–60 min. For in vivo wide-field imaging, an external fluorescence light source (Leica EL6000, Leica) was used for excitation of GCaMP6s (green channel) and JF585-HaloTag ligand (red channel). Images were acquired via Leica Application Suite 4.5 (Leica). Wide-field images in green (1-s exposure) and red (4-s exposure) channels were acquired at multiple time intervals over 2 weeks under the same imaging conditions and the images were aligned with the Stackreg plugin in ImageJ. The mean values in the same area of red and green channels were plotted to track the labeling kinetics and turnover of JF585-HaloTag in vivo.

In vivo two-photon imaging and analysis. For in vivo two-photon imaging, GCaMP6s and JF585-HaloTag were excited at 940 nm and 1,100 nm, respectively, using a femtosecond laser source (InSight DeepSee, Spectra-Physics), and imaged using an Olympus 25×, 1.05 NA objective and a homebuilt two-photon microscope. Images were acquired from 200 to 550 µm below the pia with post-objective power ranging between 20 and 60 mW. No photobleaching or photodamage of tissue was observed.

Typical imaging settings were composed of 256 × 256 pixels, with 1.2 µm per pixel, and a ~3-Hz frame rate. The time-lapse calcium images of spontaneous neuronal activity in awake, head fixed mice were recorded and analyzed with custom programs written in MATLAB (Mathworks). Lateral motion present in head-fixed awake mice was corrected using a cross-correlation-based registration algorithm, where cross-correlation was calculated to determine frame shift in x and y directions. Cortical neurons were outlined by hand as regions of interest (ROIs). The fluorescence time course of each ROI was used to calculate its calcium transient as ΔF/F (%) = (F - F0)/F0 × 100, with the baseline fluorescence being the mode of the fluorescence intensity histogram of this ROI. For the Pearson correlation coefficient calculation, the JF585 (red channel) and GCaMP6s (green channel) fluorescence signals in each ROI were averages from 1,000 imaging frames (3 Hz).

Statistics. For spectroscopy measurements (Figs. 1e and 2g, Table 1, and Supplementary Fig. 1) reported n values for absorption spectra, extinction coefficient (ε) and quantum yield (Φ) represent measurements of different samples prepared from the same dye DMSO stock solution. For the cell loading experiment (Fig. 1j) the following reported n values represent the number of intensity values measured from three fields of view for the time points at 30 s, 1 min, 2 min, 3 min and 4 min, respectively: JF525-HaloTag ligand 13: n = 112, 120, 130, 114, 128; JF549-HaloTag ligand 14: n = 135, 129, 135, 158, 161. For the reference stain using JF646-HaloTag ligand n = 248. For cellular toxicity (Supplementary Fig. 2c) assays, reported n values represent different cell culture samples in separate microplate wells. For the contrast measurements (Fig. 2h, i, m, n and Supplementary Fig. 2l) reported n values represent intensity values measured from three fields of view for each dye type. For the IV and IP experiments (Fig. 3d), reported n = 3 values represent different fields of view taken via wide-field imaging. The one-way ANOVA analysis of spontaneous neuronal activity before and after dye administration (Fig. 3g) gave F(3, 236) = 0.1204.

Data availability. The data that support the findings of this study are provided in the Source Data files and are available from the corresponding author upon request.

A Life Science Reporting Summary is available online.
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## Experimental design

1. **Sample size**
   
   Describe how sample size was determined.
   
   All quantitative imaging experiments were performed on replicate samples or fields of view as reported in the Methods and figure legends.

2. **Data exclusions**
   
   Describe any data exclusions.
   
   None

3. **Replication**

   Describe whether the experimental findings were reliably reproduced.

   N/A

4. **Randomization**

   Describe how samples/organisms/participants were allocated into experimental groups.

   N/A

5. **Blinding**

   Describe whether the investigators were blinded to group allocation during data collection and/or analysis.

   N/A

   Note: all studies involving animals and/or human research participants must disclose whether blinding and randomization were used.

6. **Statistical parameters**

   For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or in the Methods section if additional space is needed).

   □ Confirmed

   - The exact sample size \((n)\) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.)
   - A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
   - A statement indicating how many times each experiment was replicated
   - The statistical test(s) used and whether they are one- or two-sided (note: only common tests should be described solely by name; more complex techniques should be described in the Methods section)
   - A description of any assumptions or corrections, such as an adjustment for multiple comparisons
   - The test results (e.g. \(P\) values) given as exact values whenever possible and with confidence intervals noted
   - A clear description of statistics including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range)
   - Clearly defined error bars

   See the web collection on statistics for biologists for further resources and guidance.
7. Software

Describe the software used to analyze the data in this study.

We used published or commercial software to perform the image analysis as stated in the Methods.

For manuscripts utilizing custom algorithms or software that are central to the paper but not yet described in the published literature, software must be made available to editors and reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). Nature Methods guidance for providing algorithms and software for publication provides further information on this topic.

8. Materials availability

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a for-profit company.

We will deposit all plasmids in AddGene. The Janelia Fluor dyes will be distributed freely to all nonprofit researchers until commercialization.

9. Antibodies

Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).

N/A

10. Eukaryotic cell lines

a. State the source of each eukaryotic cell line used.

All Cell Lines were from ATCC as stated in the Methods.

b. Describe the method of cell line authentication used.

N/A
c. Report whether the cell lines were tested for mycoplasma contamination.

The Janelia Cell Culture Facility regularly tests for mycoplasma contamination. This is stated in the Methods.
d. If any of the cell lines used are listed in the database of commonly misidentified cell lines maintained by ICLAC, provide a scientific rationale for their use.

N/A

11. Description of research animals

Provide details on animals and/or animal-derived materials used in the study.

Male mice, 3–8 months old, were used for viral infection, dye injection, and in vivo imaging of neurons in the visual cortex (V1): The Scnn1a-Tg3-Cre (Jax no. 009613) line was used for imaging in layer 4 cortical neurons (L4); and Rbp4-Cre mice (MMRRC no. 031125-UCD) were used for imaging in layer 4 cortical neurons (L5) neurons. All experimental protocols were conducted according to the National Institutes of Health guidelines for animal research and were approved by the Institutional Animal Care and Use Committee at the Janelia Research Campus, HHMI.

12. Description of human research participants

Describe the covariate-relevant population characteristics of the human research participants.

N/A