Optimization and functionalization of red-shifted rhodamine dyes

Jonathan B. Grimm, Ariana N. Tkachuk, Heejun Choi, Boaz Mohar, Natalie Falco, Ronak Patel, Jennifer Lippincott-Schwartz, Timothy A. Brown, and Luke D. Lavis*

Janelia Research Campus, The Howard Hughes Medical Institute
19700 Helix Drive, Ashburn, Virginia 20147 USA

*To whom correspondence should be addressed: lavisl@janelia.hhmi.org

ABSTRACT: Expanding the palette of fluorescent dyes is vital for pushing the frontier of biological imaging. Although rhodamine dyes remain the premier type of small-molecule fluorophore due to their bioavailability and brightness, variants excited with far-red or near-infrared light suffer from poor performance due to their propensity to adopt a lipophilic, nonfluorescent form. We report a general chemical modification for rhodamines that optimizes long-wavelength variants and enables facile functionalization with different chemical groups.

The development of hybrid small-molecule:protein labeling strategies enable the use of chemical fluorophores in living cells and *in vivo*¹. Optimizing small-molecule dyes for these complex biological environments is important, as synthetic fluorophores are often brighter and more photostable than fluorescent proteins². We recently developed general methods to improve³ and fine-tune⁴ rhodamine fluorophores by incorporating four-membered azetidines into the structure, yielding the ‘Janelia Fluor’ dyes. Although our existing tuning strategies allow optimization of short-wavelength rhodamines, we discovered these methods cannot be applied to analogs excited with far-red and near-infrared (NIR) light because such dyes strongly favor formation of a colorless species. Here, we report a new complementary tuning strategy that allows rational optimization of a broader palette of fluorophores. This general method also serves as a basis for facile functionalization, enabling the synthesis of novel cell- and tissue-permeable rhodamine labels for biological imaging experiments.

The bioavailability and behavior of rhodamine dyes is dictated by a key property—the equilibrium between the nonfluorescent lactone and the fluorescent zwitterion (**Fig. 1a**). Our previous work on rhodamine dyes³⁻⁶ allows definition of a general rubric that directly correlates the lactone–zwitterion equilibrium constant (K_{L-Z}) of the free dye to the cellular performance of HaloTag⁷ ligand derivatives (**Fig. 1b**). Dyes with high K_{L-Z} exist almost exclusively in the zwitterionic form, making them useful as environmentally insensitive biomolecule labels⁸. Rhodamines with intermediate K_{L-Z} values exhibit improved cell and tissue permeability due to the modestly higher propensity of the molecule to adopt the lipophilic lactone form and rapidly traverse biological membranes^{4,6}. Dyes exhibiting even smaller K_{L-Z} values ($K_{L-Z} = 10^{-2}$ – 10^{-3}) preferentially adopt the closed lactone form, which can be exploited to create ‘fluorogenic’ ligands^{6,9,10} as binding to the HaloTag protein shifts the equilibrium to the fluorescent form. This

property also decreases *in vivo* utility, however, due to problems with solubility and sequestration in membranes. Finally, dyes with extremely small K_{L-Z} values ($< 10^{-3}$) exist completely in the nonfluorescent lactone form, rendering them effectively unusable in biological experiments.

We compared a series of Janelia Fluor rhodamine analogs with different fluorophoric systems (1–8, **Fig. 1c**). Compounds **2**, **4**, and **5** were described previously and include the azetidine-containing rhodamine (**2**), which we termed ‘Janelia Fluor 549’ (JF₅₄₉), and the carbo- and Si-rhodamine analogs **4** and **5** (JF₆₀₈ and JF₆₄₆, respectively; **Fig. 1c**)³. We expanded the wavelength range of the JF dyes by synthesizing new analogs based on known rhodamine structures containing nitrogen^{11,12} (NCH₃; **1**), sulfur^{13,14} (S; **3** and SO₂; **8**), and phosphorous^{15,16} (PO₂H; **6** and P(O)Ph; **7**) starting from *bis*-arylbromides⁵ (**Fig. 1c**, **Supplementary Note**). We evaluated the spectral and chemical properties of these dyes: absorption maximum ($\lambda_{\max}$), extinction coefficient at $\lambda_{\max}$ (ϵ), fluorescence emission maximum λ_{em} , fluorescence quantum yield (Φ), and K_{L-Z} (**Table 1**). Comparing K_{L-Z} and $\lambda_{\max}$ uncovered an inverse correlation (**Fig. 1d**), with the short wavelength NCH₃-containing JF₅₀₂ exhibiting a high $K_{L-Z} = 5.5$ and the near-infrared (NIR) dyes containing SO₂ and P(O)Ph showing a low $K_{L-Z} \approx 10^{-4}$.

Due to the inverse correlation of K_{L-Z} and $\lambda_{\max}$, short-wavelength dyes would typically require tuning K_{L-Z} *lower* to improve tissue permeability and create fluorogenic HaloTag ligands (**Fig. 1d**)^{4,6,10}. In previous work⁴, we found incorporation of 3,3-difluoroazetidines into the Janelia Fluor structure decreases K_{L-Z} and elicits a hypsochromic shift of ~25 nm. This strategy transformed JF₅₄₉ (**2**; $K_{L-Z} = 3.5$) into JF₅₂₅ (**9**; $K_{L-Z} = 0.68$, **Fig. 1e,f**, **Table 1**); the JF₅₂₅–HaloTag ligand shows improved *in vivo* bioavailability and is an excellent component of the hybrid voltage indicator Voltron². This modification also transformed JF₆₀₈ (**4**; $K_{L-Z} = 0.091$) into the highly fluorogenic JF₅₈₅ (**10**; $K_{L-Z} = 0.001$, **Fig. 1e,f**, **Table 1**). Given the relatively high $K_{L-Z} = 4.33$ observed for

JF₅₀₂ (**1**) we applied this same tuning strategy to yield the fluorinated JF₄₇₉ (**11**) that showed the expected decrease in $\lambda_{\max}$ and $K_{L-Z} = 2.88$ (**Fig. 1e,f, Table 1, Supplementary Fig. 1a–c**). The JF₄₇₉–HaloTag ligand (**12, Fig. 1g**) exhibits similar spectral properties to enhanced green fluorescent protein (GFP) when attached to the HaloTag protein (**Fig. 1h**) allowing imaging with low bleed-through compared to the similarly cell-permeable—albeit brighter—JF₅₀₃ ligand (**13**) when multiplexed with JF₅₂₅–SNAP-tag ligand (**14; Fig. 1i, Supplementary Fig. 1d–i**)^{4,7}.

For the far-red and NIR rhodamines (**5–8, Fig. 1c**) the K_{L-Z} vs. $\lambda_{\max}$ relationship reveals the need for alternative tuning strategy to *increase* K_{L-Z} (**Fig. 1d**). This would improve the *in vivo* performance of Si-rhodamine dyes such as JF₆₄₆ and rescue the NIR P(O)Ph- and SO₂-containing dyes (**7–8**). We previously showed that halogenation of the pendant phenyl ring system can substantially increase the K_{L-Z} of Si-rhodamine dyes⁵, presumably by lowering the pK_a of the benzoic acid moiety; this substitution also elicits a bathochromic shift¹⁷. For example, JF₆₄₆ (**5**; $\lambda_{\max}/\lambda_{\text{em}} = 646 \text{ nm}/664 \text{ nm}$) exhibits a $K_{L-Z} = 0.0014$ but the fluorinated analog, JF₆₆₉ (**15**; $\lambda_{\max}/\lambda_{\text{em}} = 669 \text{ nm}/682 \text{ nm}$), is higher with $K_{L-Z} = 0.262$ (**Fig. 1j,k, Supplementary Fig. 1j,k**). This manifests in a higher absorptivity in aqueous solution with **5** exhibiting $\epsilon = 5,600 \text{ M}^{-1}\text{cm}^{-1}$ but the fluorinated analog **15** showing $\epsilon = 112,000 \text{ M}^{-1}\text{cm}^{-1}$ (**Fig. 1l, Table 1**). We surmised this strategy would be general and prepared the fluorinated PO₂H-, P(O)Ph-, and SO₂-containing rhodamines (**16–18, Fig. 1j**) by replacing phthalic anhydride with tetrafluorophthalic anhydride in our synthetic scheme (**Supplementary Fig. 1j, Supplementary Note**). This modification increased K_{L-Z} and ϵ and elicited a $\sim 23 \text{ nm}$ shift in $\lambda_{\max}$ (**Fig. 1j–l, Table 1, Supplementary Fig. 1l–n**). In particular, the fluorinated P(O)Ph-derivative **17** strongly absorbs visible light in aqueous solution ($\epsilon = 87,000 \text{ M}^{-1}\text{cm}^{-1}$; $\lambda_{\max} = 722 \text{ nm}$) compared to the parent compound **7** ($\epsilon < 200 \text{ M}^{-1}\text{cm}^{-1}$; **Fig. 1l**). This trend was generalizable to oxygen- and sulfur-containing rhodamines based on **2** and **3**

where the fluorine substitution on the pendant phenyl ring also increased K_{L-Z} and $\lambda_{\max}$ (**19–20**, **Fig. 1m,n**, **Table 1**, **Supplementary Fig. 1o,p**).

We then explored conjugatable versions of these new far-red and NIR dyes. In addition to increasing $\lambda_{\max}$ and K_{L-Z} , the halogenated phenyl ring motif can also serve as an electrophile for attack by thiols through a nucleophilic aromatic substitution reaction (S_NAr), an established strategy for preparing xanthene dye derivatives^{8,18}. The reactivity of other nucleophiles was unknown, however; we discovered that N_3^- , CN^- , NH_3 , and NH_2OH could react with JF₆₆₉ (**15**) to provide derivatives **21–24** (**Fig. 2a**). This reaction type was generalizable to other fluorinated rhodamines and regioselective at the 6-position (**Supplementary Note**). Although beyond the scope of this report, we briefly investigated some of these derivatives, finding azide **21** was an excellent reactant in strain-promoted ‘click chemistry’ with cyclic alkynes¹⁹ **25** and **26** to form triazole adducts **27** and **28** (**Supplementary Fig. 2a**), validating the regiochemistry of the amine addition to form **22** using intermediates **21** and **29** (**Supplementary Fig. 2b**), and testing the reactivity of amine-containing ion-chelating groups **30** and **31** which generated novel prototype far-red indicators for K^+ and Zn^{2+} (**32–33**, **Supplementary Fig. 2c–e**).

We then sought to create carboxy derivatives compatible with different labeling strategies such as the HaloTag. We explored the reactivity of malonates and related carbon nucleophiles, all of which showed reaction with fluorinated rhodamines with regioselectivity at the 6-position (**Supplementary Note**). In particular, the addition of masked acyl cyanide²⁰ reagent **34**, an *umpolung*-type acyl anion equivalent, to JF₆₆₉ resulted in intermediate **35**, which could be deprotected to yield a reactive acyl cyanide suitable for conjugation with the HaloTag ligand amine (**36**) to form JF₆₆₉–HaloTag ligand **37** (**Fig. 2a**). This late-stage, regioselective introduction of the carboxy handle has distinct advantages over previous rhodamine syntheses that generate isomeric

mixtures²¹. Compound **37** was an excellent label for cell biological experiments (**Fig. 2b,c**) and was also blood–brain-barrier permeable, labeling HaloTag-expressing neurons throughout the mouse brain after *intravenous* injection (**Fig. 2d, Supplementary Fig. 3a**). This chemistry was generalizable across fluorinated rhodamines, allowing facile synthesis of HaloTag ligands **37–42** from dyes **15–20** with λ_{max} ranging from the green to NIR (**Supplementary Fig. 3b,c, Supplementary Note**). These compounds selectively labeled HaloTag fusions in cells, demonstrating that fluorination on the pendant phenyl ring does not prohibit HaloTag labeling (**Fig. 2e**).

Finally, we sought to create a bright, fluorogenic NIR HaloTag ligand suitable for biological imaging. The SO₂-containing rhodamine JF₇₂₄ (**18**) possessed a promising $K_{L-Z} = 10^{-3}$ for creating fluorogenic compounds; the JF₇₂₄–HaloTag ligand (**42**) showed a 15-fold increase upon reaction with HaloTag protein *in vitro* (**Supplementary Fig. 3d**). Nevertheless, this dye was plagued with a low $\Phi = 0.05$ (**Table 1**), making it suboptimal for imaging experiments. In contrast, the P(O)Ph-containing fluorophore JF₇₂₂ (**17**) exhibits a larger $\Phi = 0.11$ but also a relatively high $K_{L-Z} = 0.026$; the JF₇₂₂–HaloTag ligand (**41**) was not fluorogenic (**Supplementary Fig. 3e**). We investigated whether our two tuning strategies could work synergistically, using JF₅₇₁ (**19, Fig. 1m**) as a proof-of-concept. We introduced a fluorine substituent on each azetidine ring to create JF₅₅₉ (**43; Supplementary Fig. 3f**) and found this dye exhibits $K_{L-Z} = 6.22$, intermediate between JF₅₄₉ (**2; $K_{L-Z} = 3.5$**) and JF₅₇₁ (**19; $K_{L-Z} = 7.93$** ; **Table 1, Supplementary Fig. 3g–i**), demonstrating the compatibility of these strategies. The JF₅₅₉–HaloTag ligand (**44**) could be used in live cell labeling (**Supplementary Fig. 3j,k**). We then applied this modification to JF₇₂₂ by synthesizing derivatives with fluorine substituents on the azetidine ring, yielding JF₇₁₁ (**45, Fig. 2f, Table 1, Supplementary Fig. 3l**). Compound **45** exhibited a further improvement in $\Phi = 0.17$ (**Table 1**)

and was predicted to yield fluorogenic ligands based on its $K_{L-Z} = 10^{-3}$ (**Fig. 2g**). The JF₇₁₁–HaloTag ligand **46** (**Fig. 2h**) showed a 5-fold increase upon binding HaloTag (**Fig. 2i**) with excellent performance in live-cell imaging experiments (**Fig. 2j**).

In summary, we expanded the palette of Janelia Fluor dyes by replacing the central oxygen in JF₅₄₉ (**2**) with nitrogen (NCH₃, **1**), sulfur (S and SO₂, **3,8**), and phosphorous (PO₂H and P(O)Ph, **6–7**; **Fig. 1c**). As the nitrogen-containing dye JF₅₀₂ (**1**) exhibited relatively high K_{L-Z} and long $\lambda_{\max}$, we applied our established tuning strategy⁴ to transform this dye into the GFP-like JF₄₇₉ (**11**; **Fig. 1e–i**). The NIR-excited dyes **7** and **8** exhibited the opposite problem with low K_{L-Z} values that rendered the compounds unusable in biological environments (**Fig. 1d**). We therefore established a complementary general method to increase both $\lambda_{\max}$ and K_{L-Z} by incorporating fluorines on the pendant phenyl ring of rhodamine dyes (**Fig. 1j,k**) followed by facile, generalizable S_NAr chemistry to install groups for bioconjugation (**Fig. 2a**). This strategy yielded the bioavailable JF₆₆₉–HaloTag ligand (**37**, **Fig. 2b–e**) along with other new fluorophores (**38–42**, **Fig. 2e**) and could be combined with our previous tuning method to create the fluorogenic NIR-excited JF₇₁₁ HaloTag ligand (**46**, **Fig. 2f–j**). Although we have focused here on HaloTag ligands and mammalian cells, we expect this general rubric relating K_{L-Z} to cellular performance (**Fig. 1b**) to be applicable to other ligands and biological systems⁶. We also anticipate this expanded fluorophore palette to enable the rational design of finely tuned labels for biological imaging experiments in cells or animals and the new derivatization chemistry to facilitate the synthesis of novel ligands, labels, stains, and indicators, particularly those excited by far-red or NIR light.

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AUTHOR CONTRIBUTIONS

L.D.L. and J.B.G. conceived the project. J.B.G. contributed organic synthesis and 1-photon spectroscopy measurements. A.N.T. and H.C. contributed cultured cell imaging experiments. B.M. contributed *in vivo* labeling and tissue imaging experiments. N.F. contributed organic synthesis. R.P. contributed 2-photon spectroscopy measurements. J.L-S and T.A.B. directed the project. L.D.L. directed the project and wrote the paper with input from the other authors.

COMPETING FINANCIAL INTERESTS STATEMENT

The authors declare competing interests: J.B.G. and L.D.L. have filed patent applications whose value may be affected by this publication.

Figure 1 (a) The lactone-zwitterion equilibrium of Janelia Fluor rhodamine dyes and the equilibrium constant: K_{L-Z} . (b) Phenomenological plot detailing the properties of different dyes based on K_{L-Z} . (c) Synthesis, structures, and λ_{max} of dyes 1-8. (d) Plot of K_{L-Z} vs. λ_{max} for dyes 1-8 and general tuning strategies for dyes with short or long λ_{max} . (e) Structures of dyes 9-11. (f) Plot of K_{L-Z} vs. λ_{max} showing decreased K_{L-Z} for dyes 9-11. (g) Structure of JF479-HaloTag ligand (12). (h) Fluorescence excitation (ex) and emission (em) spectra of 12-HaloTag conjugate and GFP. (i) Two-color confocal imaging experiment of U2OS cells expressing histone H2B-HaloTag fusion protein labeled with either 12 or JF503-HaloTag ligand (13) excited with 488 nm and TOMM20-SNAP-tag fusion protein labeled with JF525-SNAP-tag ligand (14) excited with 532 nm; scale bars: 10 μ m. (j) Structures of dyes 15-18. (k) Plot of K_{L-Z} vs. λ_{max} showing increased K_{L-Z} for dyes 15-18. (l) Absorption spectra of 5-8 and fluorinated analogs 15-18. (m) Structures of dyes 19-20. (n) Plot of K_{L-Z} vs. λ_{max} showing increased K_{L-Z} for dyes 19-20.

Figure 2 (a) Nucleophilic aromatic substitution (S_NAr) of JF₆₆₉ (15) to form derivatives 21–24 and 35; subsequent synthesis of JF₆₆₉–HaloTag ligand (37). (b) Airyscan image of U2OS cell expressing endoplasmic reticulum-localized Sec61β–HaloTag labeled with 37. (c) Airyscan image of U2OS cell expressing mitochondria-localized TOMM20–HaloTag labeled with 37; color scale in b and c indicates z-depth; scale bars: 5 μm. (d) Image of fixed coronal mouse brain slice from animal expressing GFP–HaloTag fusion protein in neurons after *intravenous* injection of 37; scale bar = 500 μm. (e) Structures of HaloTag ligands 37–42 and high-magnification images of U2OS cell nuclei expressing histone H2B–HaloTag fusion protein and labeled 37–42; scale bar = 10 μm. (f) Structures of dyes 17 and 45. (g) Plot of K_{L-Z} vs. λ_{max} showing decreased K_{L-Z} for dye 45. (h) Structure of JF₇₁₁–HaloTag ligand (46). (i) Absorption spectra of 46 in the absence (–HT) or presence (+HT) of excess HaloTag protein. (j) Confocal imaging experiment of U2OS cells expressing TOMM20–HaloTag fusion protein labeled with 46; scale bar = 20 μm.

Compound	Name	X	Y	NR ₂	λ_{max} (nm)	ϵ_w (M ⁻¹ cm ⁻¹)	λ_{em} (nm)	Φ	K_{L-z}
1	JF ₅₀₂		H		502	57,800	533	0.71	4.33
2	JF ₅₄₉		H		549	101,000	571	0.88	3.47
3	JF ₅₇₀		H		570	83,600	593	0.63	2.24
4	JF ₆₀₈		H		608	99,000	631	0.67	0.091
5	JF ₆₄₆		H		646	5,600	664	0.54	0.0014
6	JF ₆₆₈		H		668	26,700	687	0.34	0.0093
7	-		H		~704	<200	~723	-	<0.0001
8	-		H		-	<100	-	-	<0.0001
9	JF ₅₂₅		H		525	94,000	549	0.91	0.068
10	JF ₅₈₅		H		585	1,500	609	0.78	~0.001
11	JF ₄₇₉		H		479	47,900	517	0.62	2.88
15	JF ₆₆₉		F		669	112,000	682	0.37	0.262
16	JF ₆₉₀		F		690	150,000	707	0.24	2.90
17	JF ₇₂₂		F		722	87,200	743	0.11	0.026
18	JF ₇₂₄		F		724	6,600	748	0.05	~0.001
19	JF ₅₇₁		F		571	101,000	590	0.78	7.93
20	JF ₅₉₃		F		593	90,300	612	0.55	6.06
43	JF ₅₅₉		F		559	106,000	579	0.85	6.22
45	JF ₇₁₁		F		711	12,400	732	0.17	~0.001

Table 1 Properties of Janelia Fluor dyes **1–11**, **15–20**, and **43**, and **45**.

Supplementary Figure 1 (a–b) Fluorescence excitation (ex) and emission (em) spectra of **1** (a) and **11** (b). (c) Two-photon absorption spectra of **1**, **11**, and fluorescein. (d) Structures of JF₅₀₃-HaloTag ligand (**13**) and JF₅₂₅-SNAP-tag ligand (**14**). (e–h) Widefield imaging experiment of U2OS cells expressing either histone H2B-HaloTag fusion protein (**e,g**; scale bars: 51 μm) and confocal imaging of PDGFR transmembrane domain (TMD)-HaloTag fusion protein (**f,h**; scale bars: 21 μm) labeled with JF₄₇₉-HaloTag ligand (**12**; **e,f**) or JF₅₀₃-HaloTag ligand (**13**; **g,h**) (i) Nuclear fluorescence vs. time upon addition of ligands **12** or **13** to cells expressing histone H2B-HaloTag fusion protein. (j) Synthesis of Janelia Fluor dyes **15–20**. (k–p) Fluorescence excitation (ex) and emission (em) spectra of **15** (k), **16** (l), **17** (m), **18** (n), **19** (o), and **20** (p).

Supplementary Figure 2 (a) Reaction of azide **21** with strained alkynes **25** or **26** to form triazole adducts **27** or **28**. (b) Synthesis of amine **22** via reaction of JF₆₆₉ (**15**) with NH₃, reduction of azide **21**, or Curtius rearrangement starting from ester **29** showing consistent regioselectivity of S_NAr. (c) Reaction of **15** with amine-containing chelator groups **30** and **31** to form far-red K⁺ indicator **32** and far-red Zn²⁺ indicator **33**. (d) Fluorescence emission spectra of **32** in the absence or presence of 100 mM K⁺. (e) Fluorescence emission spectra of **30** in the absence or presence of 10 μM Zn²⁺.

Supplementary Figure 3 (a) Two-color mosaic image of fixed coronal slices from a mouse expressing HaloTag-GFP (green) in neurons transduced by *intravenous* injection of the viral vector PHP-eB-Syn-HaloTag-GFP followed by *intravenous* injection of **37** (magenta), perfusion, and slicing; scale bar = 3 mm. (b) Expanded synthetic scheme of HaloTag ligands **37-42**, **44** and **46** starting with nucleophilic aromatic substitution (S_NAr) with **34**. (c) Two-photon absorption spectra of the HaloTag conjugates of HaloTag ligands **37-42**, **44** and **46**. (d) Absorption spectra of **42** in the absence (-HT) or presence (+HT) of excess HaloTag protein. (e) Absorption spectra of **41** in the absence (-HT) or presence (+HT) of excess HaloTag protein. (f) Structure of JF₅₇₁ (**19**) and JF₅₅₉ (**43**). (g-h) Full plot of K_{L-Z} vs. λ_{max} (g) and zoom (h) showing decreased K_{L-Z} for dye **43**. (i) Fluorescence excitation (ex) and emission (em) spectra of **43**. (j) Structure of JF₅₅₉-HaloTag ligand (**44**). (k) Widefield imaging experiment of U2OS cells expressing histone H2B-HaloTag fusion protein labeled with **44**; scale bar: 51 μm . (l) Fluorescence excitation (ex) and emission (em) spectra of JF₇₁₁ (**45**).

ONLINE METHODS

Chemical synthesis. Methods for chemical synthesis, full characterization of all novel compounds, and crystallographic confirmation of regioselective S_NAr 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% v/v. 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_{abs}), extinction coefficient (ϵ), and maximum emission wavelength (λ_{em}) were taken in 10 mM HEPES, pH 7.3 buffer unless otherwise noted; reported values for ϵ are averages ($n = 3$). Normalized spectra are shown for clarity. For prototype ion indicators **32** and **33** (Supplementary Fig. 2c) the compounds were dissolved in 10 mM HEPES, pH 7.3 buffer alone or with either 100 mM KCl or 10 μ M $ZnCl_2$; the fluorescence emission spectra of these solutions were recorded using $\lambda_{ex} = 575$ nm and $\lambda_{em} = 625$ –825 nm.

Determination K_{L-Z} and ϵ_{max} . We calculated K_{L-Z} using the following equation²²: $K_{L-Z} = (\epsilon_{dw}/\epsilon_{max})/(1 - \epsilon_{dw}/\epsilon_{max})$. ϵ_{dw} is the extinction coefficient of the dyes in a 1:1 v/v dioxane:water solvent mixture; this dioxane–water mixture was chosen to give the maximum spread of K_{L-Z} values⁴. ϵ_{max} refers to the maximal extinction coefficients measured in different solvent mixtures empirically determined depending on dye type: 0.1% v/v TFA in ethanol for the Si-rhodamines (**5**

and **15**); 0.1% v/v trifluoroacetic acid (TFA) in 2,2,2-trifluoroethanol (TFE) for all the other rhodamines. We note that accurate determination of low K_{L-Z} values ($\leq 10^{-3}$) is complicated by the relatively poor sensitivity of absorbance measurements. We estimated $K_{L-Z} = 10^{-3}$ when we observed a small but significant absorbance signal in 1:1 v/v dioxane:water solvent mixture over the dye-free control, and $K_{L-Z} \approx 10^{-4}$ when we observed no significant absorbance of the dye solution.

Quantum yield determination. All reported absolute fluorescence quantum yield values (Φ) were measured in our laboratory under identical conditions using a Quantaurus-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 corrections²³ were performed using the instrument software. Reported values are averages ($n = 3$).

1-Photon spectroscopy of HaloTag conjugates. HaloTag protein was used as a 100 μ M 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-mL quartz cuvettes. HaloTag ligands **12**, **41–42**, **46** (5 μ M) were dissolved in 10 mM HEPES, pH 7.3 containing 0.1 mg·mL⁻¹ CHAPS. An aliquot of HaloTag protein (1.5 equiv) was added and the resulting mixture was incubated until consistent absorbance signal was observed (60–120 min). To measure the fold-increase of absorbance upon HaloTag binding (**41**, **42**, **46**) an equivalent volume of TBS–glycerol blank was added in place of enzyme to record the ‘–HT’ absorbance. Absorbance scans are averages ($n = 2$).

Multiphoton spectroscopy. For the environmentally insensitive compounds **1**, **11**, and a fluorescein control (**Supplementary Fig. 1c**) we prepared 5 μM solutions of the free dyes in 10 mM HEPES buffer, pH 7.3. For other rhodamines (**Supplementary Fig. 3c**), we measured spectra of the HaloTag conjugates: compounds **37–42**, **44**, and **46** (5 μM) were incubated with excess purified HaloTag protein (1.5 equiv) in 10 mM HEPES, pH 7.3 containing 0.1 $\text{mg}\cdot\text{mL}^{-1}$ CHAPS as above 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^{48,49}. Briefly, measurements were taken on an inverted microscope (IX81, Olympus) equipped with a 60 $\times$, 1.2NA 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 1000–1300 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, Perkin Elmer). 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. U2OS cells (ATCC) were cultured in Dulbecco's modified Eagle medium (DMEM, phenol red-free; Life Technologies) supplemented with 10% (v/v) fetal bovine serum (Life Technologies), 1 mM GlutaMAX (Life Technologies) and maintained at 37 °C in a humidified 5% (v/v) CO₂ environment. For confocal imaging of cell nuclei (**Fig. 1i**, **Fig. 2e**, **Supplementary Fig. 1e,g**, **Supplementary Fig. 3k**), we used U2OS cells

with an integrated a histone H2B–HaloTag expressing plasmid via the piggyback transposase. For confocal imaging of the cell surface (**Supplementary Fig. 1f,h**), we used U2OS cells transiently transfected with a plasmid expressing a C-terminal transmembrane anchoring domain from platelet-derived growth factor receptor (PDGFR) fused to the HaloTag protein (PDGFR–HaloTag); nucleofection (Lonza) was used for transfection. For the Airyscan imaging experiments (**Fig. 2b,c**) we used U2OS cells transiently transfected with Sec61 β –HaloTag expressing plasmid or TOMM20–HaloTag expressing plasmid using FuGENE HD (Promega). Sec61 β is an endoplasmic reticulum membrane protein translocator protein and TOMM20 is an outer mitochondrial membrane protein as part of a protein translocase complex. For confocal imaging of mitochondria (**Fig. 2j**), we used U2OS cells with an integrated a TOMM20–HaloTag expressing plasmid via the piggyback transposase. These cell lines were kept under the selection of 500 μ g/mL Geneticin (Life Technologies). Cell lines undergo regular mycoplasma testing by the Janelia Cell Culture Facility. Unless otherwise noted, cells were imaged on a Leica SP8 Falcon confocal microscope with an HC PL-APO 86 $\times$ /1.20 water objective, a Zeiss LSM 800 confocal microscope with a Plan APO 20 $\times$ /0.8 air M27 objective or Plan APO 63 $\times$ /1.4 oil DIC M27 objective, a Zeiss LSM 880 with a C-APO 40 $\times$ /1.2 W Corr FCS M27, or a Zeiss LSM 880 with Airyscan and a plan-apochromatic 63 $\times$ oil objective (NA=1.4). The airyscan images were processed using the Zen software from Zeiss, the Leica and Zeiss LSM 800 confocal images were processed using FIJI.

Dye loading kinetics. For the dye loading comparison (**Supplementary Fig. 1i**), U2OS cells stably expressing histone H2B–HaloTag were stained over a time course of 0–4 h with 200 nM of either JF₄₇₉–HaloTag ligand **12** or JF₅₀₃–HaloTag ligand **13**. Cells were washed 2 $\times$ with dye-free media and imaged live using widefield microscopy (Nikon Eclipse Ti, Plan APO λ 20 $\times$ /0.75; 470

nm Ex/FITC (515/30) Em. Fluorescence was quantified from the average of the summed intensity of nuclear signals in single plane widefield analyzed using Nikon NIS-Elements AR software (**Supplementary Fig. 1e,g**).

Multiplexed imaging comparison JF₅₀₃ and JF₄₇₉. U2OS cells stably expressing histone H2B–HaloTag fusion protein were transiently transfected with TOMM20–pSNAPf plasmids using nucleofection (Lonza). Live cells were simultaneously incubated with JF₄₇₉–HaloTag ligand (**12**, 500 nM) or JF₅₀₃–HaloTag ligand (**13**, 500 nM) for 3 h, adding JF₅₂₅–SNAP-tag ligand (**14**, 1 μ M, **Supplementary Fig. 2d**) for 60 min. These cells were then washed and imaged (**Fig. 1i**) using tunable white light laser excitation at 488 nm and 532 nm on a Leica SP8 Falcon confocal microscope with an HC PL-APO 86 $\times$ /1.20 water objective. The images are displayed as maximum intensity projections of confocal image stacks using FIJI.

Mouse *in vivo* labeling experiments. Adult C57/BL6 male mice were used to express a GFP HaloTag fusion protein throughout the brain by systemic injection using the viral vector: PHP-eB-Syn-HaloTag-GFP ($\sim 5 \times 10^{11}$ infectious units per ml, 100 μ l). The virus was injected using a 0.5ml 27G syringe to the retro-orbital sinus. JF₆₆₉–HaloTag ligand (**37**) was administered to mice 3–4 weeks after the viral injection. Dye solution was prepared by first dissolving 100 nmol (76 μ g) of **37** in 20 μ L DMSO. After vortexing, 20 μ L of a Pluronic F-127 solution (20% w/w in DMSO) was added and this stock solution was diluted into 200 μ L sterile saline for IV (retro-orbital) injection. 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.

Statistics. For spectroscopy measurements (**Fig. 1l, Table 1, Supplementary Fig. 1a,b,k-p, and Supplementary Fig. 1i,l**) 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 (**Supplementary Fig. 1i**) 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 minutes, respectively: JF₄₇₉–HaloTag ligand **12**: *n* = 112, 120, 130, 114, 128; JF₅₀₃–HaloTag ligand **13**: *n* = 135, 129, 135, 158, 161.

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

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