Multiplexed No-Wash Cellular Imaging using BenzoTag, an Evolved Self-Labeling Protein

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Supplementary Information

Supplemental Discussion

On BenzoTag's decreased activity towards rhodamine substrates

The three introduced mutations that yielded BenzoTag decreased the conjugation kinetics of rhodamine-based **CA-TMR** and **CA-JF$_{635}$** by 900- and 9000-fold, respectively. HaloTag7 is the product of 25 mutations introduced into a haloalkane dehalogenase from *rhodococcus rhodochrous* (DhaA) that were introduced to improve its labeling rate with the model substrate, **CA-TMR**, and to improve its expression profile.[1, 2] Curiously, F144L, I211V, and V245A mutations are not at positions that were modified in the original HaloTag7 campaign (Table S2), posing the question why these mutations reduce activity towards rhodamine substrates. It has been computationally predicted that F144 stabilizes the *open* fluorescent isomer of a silicon rhodamine substrate,[3, 4] so the F144L mutation may account for BenzoTag's reduced activity towards rhodamine substrates. In fact, when measuring the kinetics of **CA-JF$_{635}$** conjugation to BenzoTag, high concentrations (500 nM) of dye were needed to detect turn-on fluorescence upon reacting with BenzoTag. This observation supports the critical role of F144 in the unusually rapid kinetics of HaloTag7 towards rhodamine dyes, and in promoting the isomerization to the *open* fluorescent isomer. Additionally, in work that screened site-saturation mutagenesis libraries of several positions, all mutants at F144 decreased fluorescence intensity with other fluorogenic rhodamine substrates that rely on the ground-state isomerization between a "closed" non-fluorescent spirolactone and an "open" fluorescent zwitterionic isomer.[5] This further supports a role for F144 in stabilizing the *open* fluorescent isomer for **CA-JF$_{635}$** and analogs conjugated to HaloTag7.

General Methods

All molecular biology reagents were purchased from New England Biolabs unless specified. Primers were ordered from Thermo Fisher Scientific. Primer sequences can be found in Supplemental Table 1. The HaloTag coding sequence was codon optimized for yeast expression and synthesized by Genscript. All recombinant cloning accomplished by Gibson Assembly using 2x Master Mix from New England Biolabs following manufacturer's protocols. All antibodies used were purchased from Thermo Fisher Scientific. Yeast media supplements were purchased from Sigma Aldrich.

Kinetic and fluorescence endpoint assays were performed using a Tecan Spark plate reader. Flow cytometry experiments were conducted on a Guava cell analyzer and analyzed using Guava InCyte software. Fluorescence activated cell sorting (FACS) experiments were conducted on a BioRad S3E cell sorter. All microscopy was conducted on an Olympus IX71 fluorescence microscope and image acquisition and processing was done using CellSens Dimension software. Analysis of the of kinetics of live cell imaging was done using ImageJ and the Time Series Analyzer V3 plugin.[6] Confocal microscopy was conducted on a Leica TCS SP8 microscope and image processing was done using ImageJ.

Supplemental Table 1. Primer list

Name	
P1	GGAGGCGGTAGCGGAGGCGGAGGGTCGGCTAGCTGCGGTGGCGGCGGTAT GGCTGAAATTGGTACAGGTTTTCCATTTG
P2	GTCCTCTTCAGAAATAAGCTTTTGTTCGGATCCGCCCCCAGAAATTTCTAAAG TTGACAACCATCTAGCAATTTC
P3	GTTCCAGACTACGCTCTGCAGG
P4	CTAGTGGTGGAGGAGGCTCTGGTGGAGGCGGTAGCGGAGGCGGAGGGTCG GCTAGC
P5	TATCAGATCTCGAGCTATTACAAGTCCTCTTCAGAAATAAGCTTTTGTTCGGA TCC
P6	GCTCTGCAGGCTAGTGGTGGAGGAGGCTCTGGTG
P7	CTACACTGTTGTTATCAGATCTCGAGCTATTACAAGTC
P8	ATGGCTGAAATTGGTACAGGTTTTCCATTTG
P9	AGAAATTTCTAAAGTTGACAACCATCTAGCAATTTC
P10	AGAAGGAGATATACCATGCATCACCACCATCATCACATGGCTGAAATTGGTAC AGGTTTTC
P11	AGCGGTGGCAGCAGCCTAGGTTAATTAAGAAATTTCTAAAGTTGACAACCATC
P12	GGTAGCGGGGATCCACCGGTCGCCACCATGGCTGAAATTGGTACAGGTTTTC
P13	AACGGGCCCTCTAGACTCGAGCGGTCAAGAAATTTCTAAAGTTGACAACCATC
P14	ATGGCCAGAActgGCAAGAGAAA
P15	TCATCCCATGTTGGAATTG
P16	TGAATTACCAgtgGCTGGTGAAC
P17	TTTGGAAATCTCCACAATG
P18	ACCAGGTGCTTTTATTCCACCAG
P19	CTGGTGGAATAAAAGCACCTGGT

Supplemental Table 2. Summary of HaloTag mutations reported in previous work.

More extensive HaloTag engineering, including split HaloTag and circularly permuted HaloTag, is described in ref. [7].

Los*, Encel, Wood, et. al, *ACS Chem. Biol.,* **3**, 373 (2008)	
Purpose: convert dehalogenase to self-labeling enzyme	
HT	H272F
Encel*, Wood, et. al., Curr Chem Genomics., **6**, 55 (2012)	
Purpose: improve stability, expression, and kinetics with rhodamine dyes	
HT2	K175M | C176G | H272F | Y273L
HT3	**HT2 +** S58T | D78G | A155T | A172T | A224E | F272N | P291S | A292T | plus Q294 | plus Y295
HT6	**HT3 +** L47V | Y87F | L88M | C128F | E160K | A167V | K195N | N227D | E257K | T264A
HT7	**HT6 +** Q294E | Y295I | plus S296 | plus G297
Koßmann, Niemeyer*, et. al., ChemBioChem,17,1102 (2016)	
Purpose: improve HaloTag labeling with negatively charged substrates, like oligonucleotides	
HOB	**HT7 +** E154K | E158K | A166K | D167K | E171K
Liu, Zhang*, et. al., Angew. Chemie. Int. Ed., 56, 8672 (2017)	
Purpose: proteostasis sensor; destabllized HaloTag	
HOB	**HT7 +** K73T
Kang, Rhee*, et. al., Chem. Commun., 53, 9226 (2017)	
Purpose: Improve turn-on fluorescent properties of a dansyl dye	
^{M175P}HaloTag	**HT7 +** M175P
	** also characterized M175T, N, K, C, H, Y, L, and W
Deprey & Kritzer*, *Bioconjugate Chemistry*, **32**, 964 (2021)	
Purpose: remove potential for inactivation via oxidation	
HT8	**HT7 +** C61S | C262S
Frei, Johnson*, et. al*., Nature Methods* **19**, 65 (2022)	
Purpose: modulate fluorescence lifetimes of rhodamine-based dyes	
HT9	**HT7 +** Q165H | P174R
HT10	**HT7 +** Q165H
HT11	**HT7 +** M175W
Miro-Vynyals, Liang*, et. al*., ChemBioChem*, **22**, 3398 (2021)	
Purpose: improve HaloTag labeling for a styrylpyridium dye	
HT-SP1	**HT7 +** R133C | M175Y | V245A
HT-SP2	**HT7 +** R133C | E143M | F144H | M175Y | V245A
HT-SP3	**HT7 +** E143M | F144A | L271D
HT-SP4	**HT7 +** M175Y | L271D
HT-SP5	**HT7 +** M175Y | V245A | L271D
This work	
Purpose: Improve brightness and kinetics with pro-fluorescent substrate	
Variant 1	**HT7 +** F144S | L246F
Variant 2	**HT7 +** V245A
Variant 3	**HT7 +** N195S | V245A | E251K | S291P
Variant 4	**HT7 +** L161W | V245A
Variant 5	**HT7 +** I211V | V245A
Variant 6	**HT7 +** F144L | L221S | V245A
Variant 7	**HT7 +** F144L | I211V | L221S | V245A
Variant 8	**HT7 +** F144L | L221S | V245A | L246F
Variant 9	**HT7 +** F144L | I211V | L221S | V245A | L246F
Variant 10 **BenzoTag**	**HT7 +** F144L + I211V + V245A

Yeast culture and display experiments

Synthesized DNA encoding for HaloTag7 codon-optimized for *S. cerevisiae* was cloned into the linearized pCTcon2 (Gift from Dane Wittrup,[8] Addgene plasmid # 41843) construct between restriction sites NheI and BamHI using primers P1 and P2 to introduce homologous overhang regions.[8] Successfully cloned plasmid, determined by Sanger sequencing using primer P3, was used to transform the *S. cerevisiae* strain, RJY100 (Gift from James Van Deventer[9, 10]). Transformed yeast were selected using the TRP1 auxotrophic marker and were cultured according to previously published protocols.[11] Cells were propagated in synthetic dextrose media lacking tryptophan (SD -Trp, pH = 4.5) at 30 °C and grown to stationary phase. Approximately 24 hours prior to the display experiment, the OD_{600} of the culture was measured, diluted to an OD_{600} of 0.1 (~$1x10^7$ cells/mL), and grown to mid-log phase (OD_{600} = 0.2-0.5, about 6 hours) at 30 °C. Cells were pelleted and then resuspended in synthetic galactose media with 2% dextrose and lacking tryptophan (SG -Trp, pH = 6.5) to an OD_{600} of 0.1 to induce expression of Aga2-HaloTag fusion. Cells were induced at room temperature, shaking, for 16-18 hours prior to labeling.

For yeast display experiments, the induced culture's OD_{600} was measured and $2x10^6$ cells were pelleted per sample. Cells were washed with three times with 50 µL with phosphate buffered saline (PBS) prior to labeling. To measure HaloTag activity on the surface of yeast, cells were resuspended in 50 µL of a solution of chloroalkane dye in PBS and incubated at room temperature for a set time. Cells were pelleted, dye solution was aspirated, then cells were washed three times with 50 µL with PBS. To measure HaloTag expression on the surface of yeast, primary and secondary antibody labeling of the epitope tags, HA and/or Myc, followed by a dye-labeled secondary antibody. Cells were resuspended in 50 µL of a 1:2000 diluted Rabbit anti-Myc and/or Mouse anti-HA (Invitrogen) in PBSA (PBS+0.1% w/v BSA) and tumbled at room temperature for 30 minutes. Cells were then pelleted at 4 °C and washed twice with 50 µL chilled PBSA. Cells were then resuspended in 50 µL of a 1:2000 diluted Goat anti-Rabbit labeled with AF647 and/or Goat anti-Mouse labeled with AF488 (Invitrogen) and kept on ice for 15 minutes. Cells were pelleted at 4 °C, washed once with 50 µL of chilled PBSA, resuspended in 200 mL of PBSA and transferred to a 96-well plate for flow cytometry analysis.

Yeast Codon-Optimized HaloTag DNA sequence

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ATGGCTGAAATTGGTACAGGTTTTCCATTTGATCCACATTACGTTGAAGTTTTGGGTGA
AAGAATGCATTACGTTGATGTTGGTCCAAGAGATGGTACACCAGTTTTGTTTTTACATG
GTAACCCAACTTCTTCATACGTTTGGAGAAACATCATCCCACATGTTGCACCAACTCAT
AGATGTATTGCTCCAGATTTGATTGGTATGGGTAAATCTGATAAGCCAGATTTGGGTTA
TTTCTTTGATGATCATGTTAGATTCATGGATGCTTTTATTGAAGCATTGGGTTTGGAAG
AAGTTGTTTTGGTTATTCATGATTGGGGTTCTGCATTAGGTTTTCATTGGGCTAAGAGA
AACCCAGAAAGAGTTAAGGGTATCGCTTTTATGGAGTTTATTAGACCAATTCCAACATG
GGATGAATGGCCAGAATTTGCAAGAGAAACTTTCCAAGCTTTTAGAACAACTGATGTTG
GTAGAAAGTTGATCATCGATCAAAACGTTTTTATTGAAGGTACATTGCCAATGGGTGTT
GTTAGACCATTGACTGAAGTTGAAATGGATCATTACAGAGAACCATTTTTAAACCCAGT
TGATAGAGAACCATTGTGGAGATTTCCAAATGAATTACCAATTGCTGGTGAACCAGCAA
ACATCGTTGCTTTGGTTGAAGAATACATGGATTGGTTACATCAATCTCCAGTTCCAAAG
TTGTTATTTTGGGGTACACCAGGTGTTTTAATTCCACCAGCAGAAGCTGCAAGATTGGC
TAAGTCATTGCCAAACTGTAAGGCAGTTGATATTGGTCCAGGTTTGAATTTGTTGCAAG
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TCT
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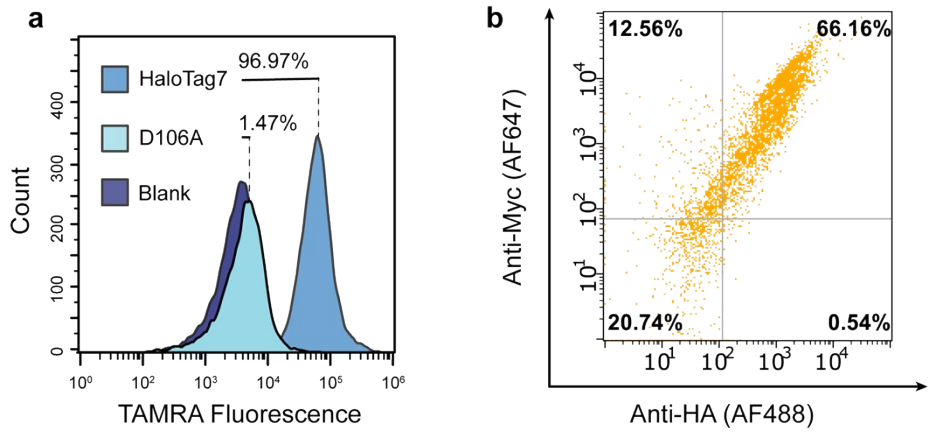


Supplemental Figure 1. HaloTag expression and activity on the surface of yeast. a) Yeast that were induced to express HaloTag fusions at the surface were labeled with 1.0 μM chloroalkane-tetramethylrhodamine (TAMRA) for 30 minutes. Gating for uniform expression, HaloTag has near quantitative labeling while the catalytically inactive enzyme (97% of HaloTag7 cells were above background), D106A, displays no labeling. Blank cells refer to unlabeled cells. B) Immunostaining of the N and C terminal epitope tags, HA and Myc, respectively, indicates robust HaloTag expression on the surface of yeast after galactose induction.

Combinatorial Library Generation

Combinatorial HaloTag libraries were generated using error-prone PCR with mutagenetic nucleotides as described previously.[11-13] Briefly, HaloTag was amplified in a 50 μL reaction of 50 ng of template vector for 10-20 rounds using 0.2 μM of primers **P4** and **P5**, 2.5 mM $MgSO_4$, 5 units of *Taq* polymerase, 0.2 mM dNTPs, 2% DMSO, and 2 or 10 μM each of mutagenic nucleotide analogs 8-oxo-2'-deoxyguanosine-5'-triphosphate (8-oxo-dGTP) and 2'-deoxy-P-nucleoside-5'-triphosphate (dPTP) (Jena Biosciences). The PCR products were gel purified and amplified again under the same PCR conditions but without 8-oxo-dGTP and dPTP for 35 rounds using primers **P6** and **P7**.

Entry	[8-oxo-dGTP], μM	[dPTP], μM	# of PCR cycles
1	2	2	10
2	2	2	20
3	10	10	10
4	10	10	20

The inserts were electroporated into freshly made electrocompetent RJY100 with NheI-BamHI digested pCTcon2 vector in a 10:1 mass ratio following established protocols.[11] The electroporated cultures were rescued in 2 mL of yeast extract peptide dextrose media (YPD) for 1 hour at 30 °C without shaking. 10 μL of the recovered culture was used to generate 100x, 1000x, 10000x, and 100000x diluted samples of which 20 μL was plated on SD-Trp plates and incubated at 30 °C. Three days later, colony forming units were counted to estimate library size

where one colony on 100x, 1000x, 10000x, and 100000x diluted plates corresponds to $1x10^4$, $1x10^5$, $1x10^6$, and $1x10^7$ transformants, respectively. Results are summarized in Table S4. The remaining recovered transformed culture was diluted in 100 mL of SD-Trp media and grown to saturation. This saturated culture was used to freeze library stocks that contained a number of cells corresponding to 10 times the library diversity.

Supplemental Table 3. HaloTag Variant Libraries.

	Number of CFUs counted on diluted plates (# of transformants)				
Sublibrary	100x[a]	1000x[a]	10000x	100000x	Average
1	N.D.	N.D.	64 ($6.4x10^7$)	8 ($8x10^7$)	$7.2x10^7$
2	N.D.	N.D.	116 ($1.16x10^8$)	15 ($1.5x10^8$)	$1.33x10^8$
3	N.D.	N.D.	24 ($2.4x10^7$)	2 ($2x10^7$)	$2.2x10^7$
4	N.D.	N.D.	19 ($1.9x10^7$)	4 ($4x10^7$)	$2.95x10^7$

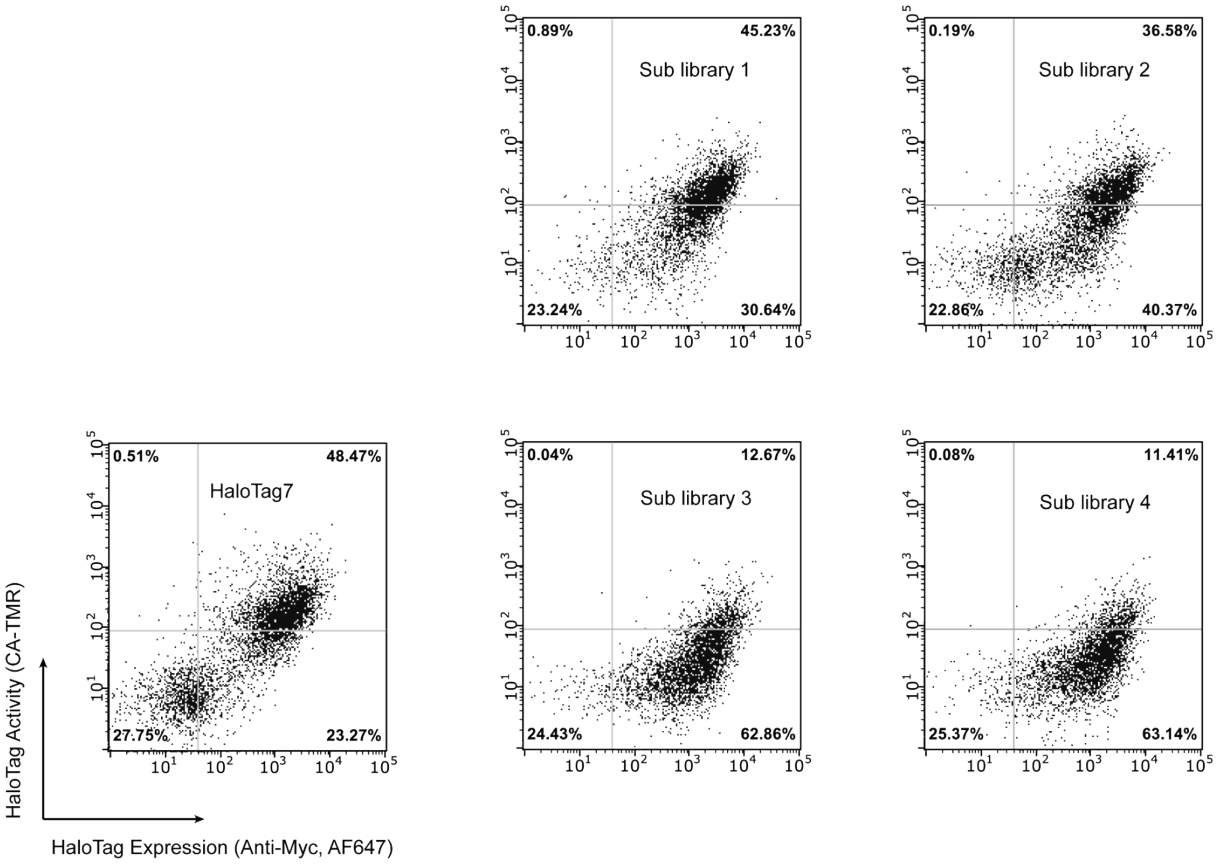
[a]*N.D* = Not determined; grown colonies were too dense to accurately count.

To evaluate amino acid mutation frequency, plasmid from each sublibrary was harvested by ZymoPrep (ZymoResearch), purified by precipitation, and re-transformed in NEB 10β *e. coli* cells by heat shock. Up to 24 random colonies from each sublibrary were inoculated into Luria-Bertani (LB) broth supplemented with 100 µg/mL ampicillin (GoldBio), plasmid was harvested by mini-prep (Invitrogen), and the insert region was by Sanger sequencing using **P3** to identify HaloTag variant sequences and mutation frequencies (Table S5).

Supplemental Table 4. Amino Acid Mutation Frequency in HaloTag Variant Sublibraries.

SubLibrary	Colonies sequenced	Average # of Mutations	Native HaloTag sequences detected
1	19	2.9	3
2	21	2.2	2
3	24	6.4	0
4	21	9.5	0

The activity of each sublibrary was determined by flow cytometry (Fig. S2). Because each sublibrary appeared to have many active mutants, we pooled the four libraries together at 10x the sublibrary diversity to yield the input HaloTag variant library that has a diversity of ~$2.6x10^8$ total HaloTag variants. We estimated that <10% of this input library contains the native HaloTag sequence.



Supplemental Figure 2. HaloTag sublibrary activity. Sublibraries were treated with 1.0 μM chloroalkane tetramethylrhodamine (CA-TMR) for 30 minutes.

Cell Sorting and Screens

Magnetic – The magnetic sort of the input library was done following a previously reported protocol.[11] Briefly, $4x10^9$ induced cells were pelleted (approximately 16x library diversity in four tubes of $1x10^9$ cells each) and each resuspended in 1 mL of PBSA. 20 μL of washed, streptavidin-coated magnetic beads were added to each tube of induced cells and tubes were tumbled at 4 °C for two hours. The tubes were placed a magnetic block, and the suspended cells were transferred to a fresh 2.0 mL tube and pelleted, thus removing cells that stuck nonspecifically to the magnetic beads. Cells were resuspended in 1.0 mL of 10 μM chloroalkane-biotin in PBS (estimated to be >10x excess of HaloTag variant copies) and tumbled at room temperature for one hour. The cells were then pelleted and washed three times with 1.0 mL PBS to remove excess ligand and resuspended in 1.0 mL of PBSA. Fresh, washed streptavidin-coated magnetic beads were added to the resuspended cells, tubes were tumbled at 4 °C for one hour, and then the tubes were place on a magnetic rack. The supernatant was carefully removed and the immobilized beads were gently washed twice with chilled PBSA, then pooled and suspended in 2.0 mL of SD-Trp. 10 μL was removed and used to make serial dilutions of beads (1:100, 1:1000, 1:10000, 1:100000) and 20 μL of each dilution was plated on a SD-Trp agar plate and incubated at 30 °C for 3 days prior to counting colonies. The remaining

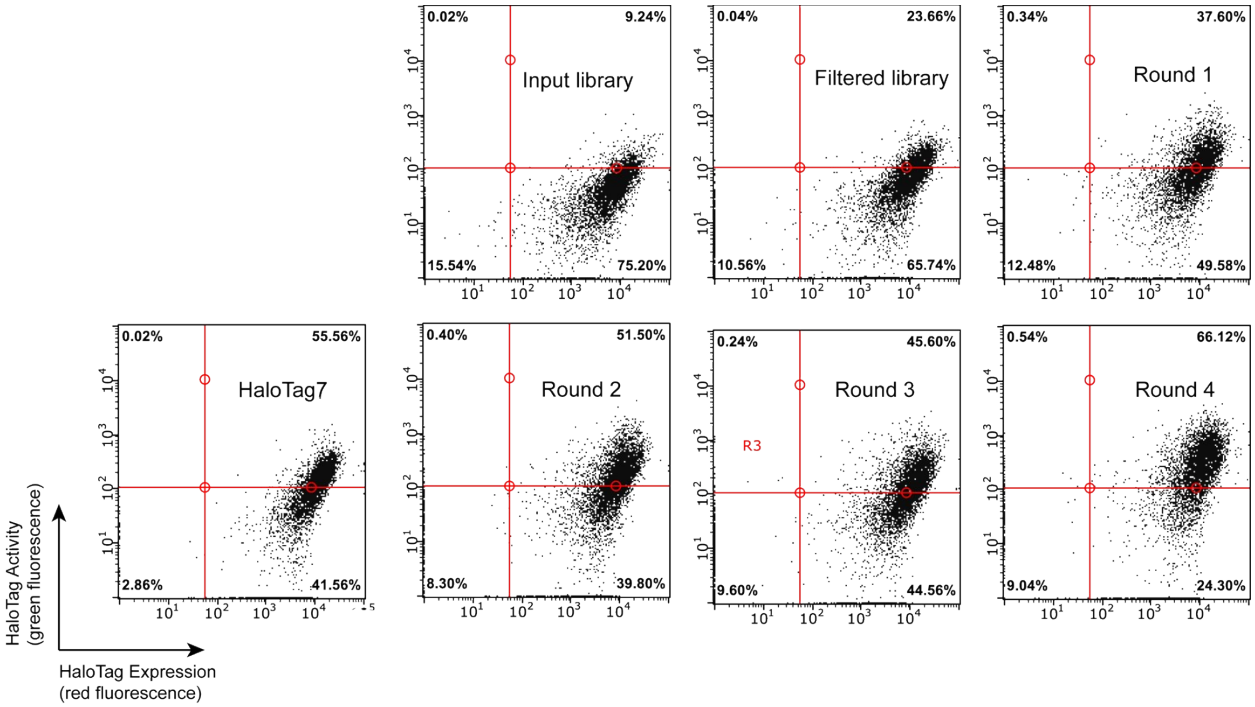
suspended beads were further diluted to 200 mL of SD-Trp media and grown to saturation. Based on colony counts, and an excess factor of 16-fold, we estimated that this magnetic sort retained ~20% of the input cells yielding a filtered library of approximately $5.3x10^7$ active HaloTag variants.

FACS –$1x10^7$ yeast cell from the filtered library (or, later, from the previous round's output pool) were grown and induced as described above. All volumes for labeling and wash steps were increased to 250 µL to accommodate the 5x increase in labeled cells per tube. The labeling conditions of dye were varied for each round of FACS as described in Table S6. For FACS, labeled batches were pooled and resuspended to a final concentration of $1x10^7$ cells/mL and subjected to sorting in which the top ~0.5% brightest green cells were isolated. Screening gates were shaped to include the brightest cells that were those well above the diagonal when green fluorescence (from the dye) was plotted against red fluorescence (from Myc immunostaining). Recovered cells were grown in SD-Trp media at 30 °C until saturation (~3 days) and the resulting stocks were then prepared for a subsequent round of sorting and frozen as glycerol stocks for later analysis.

After output pools round 3 and round 4 were isolated, $1x10^8$ cells were pelleted and plasmid was harvested by ZymoPrep (ZymoResearch). Plasmids were purified, transformed, and sequenced as described above. Twenty colonies from R3 and 75 colonies from R4 were sequenced in total. Selected hit sequences were transformed into chemically competent RJY100 *S. cerevisiae* cells as described above, and HaloTag activity was evaluated by flow cytometry (Table S7, Fig. S4, S5). Six sequences were chosen for their high activity and/or representative mutations and their HaloTag variant sequences were isolated by PCR (20 ng of template, 0.2 µM primers **P8** and **P9,** 2.0 mM $MgSO_4$, 0.2 mM of each dNTP, 1 unit of Phusion High-Fidelity polymerase, and 2% DMSO) for cloning into bacterial expression vectors.

Supplemental Table 5. FACS labeling conditions

	[**Bz-1**], µM	Time (min.)
Round 1	1.0	15
Round 2	1.0	1
Round 3	0.2	1
Round 4	0.04	1

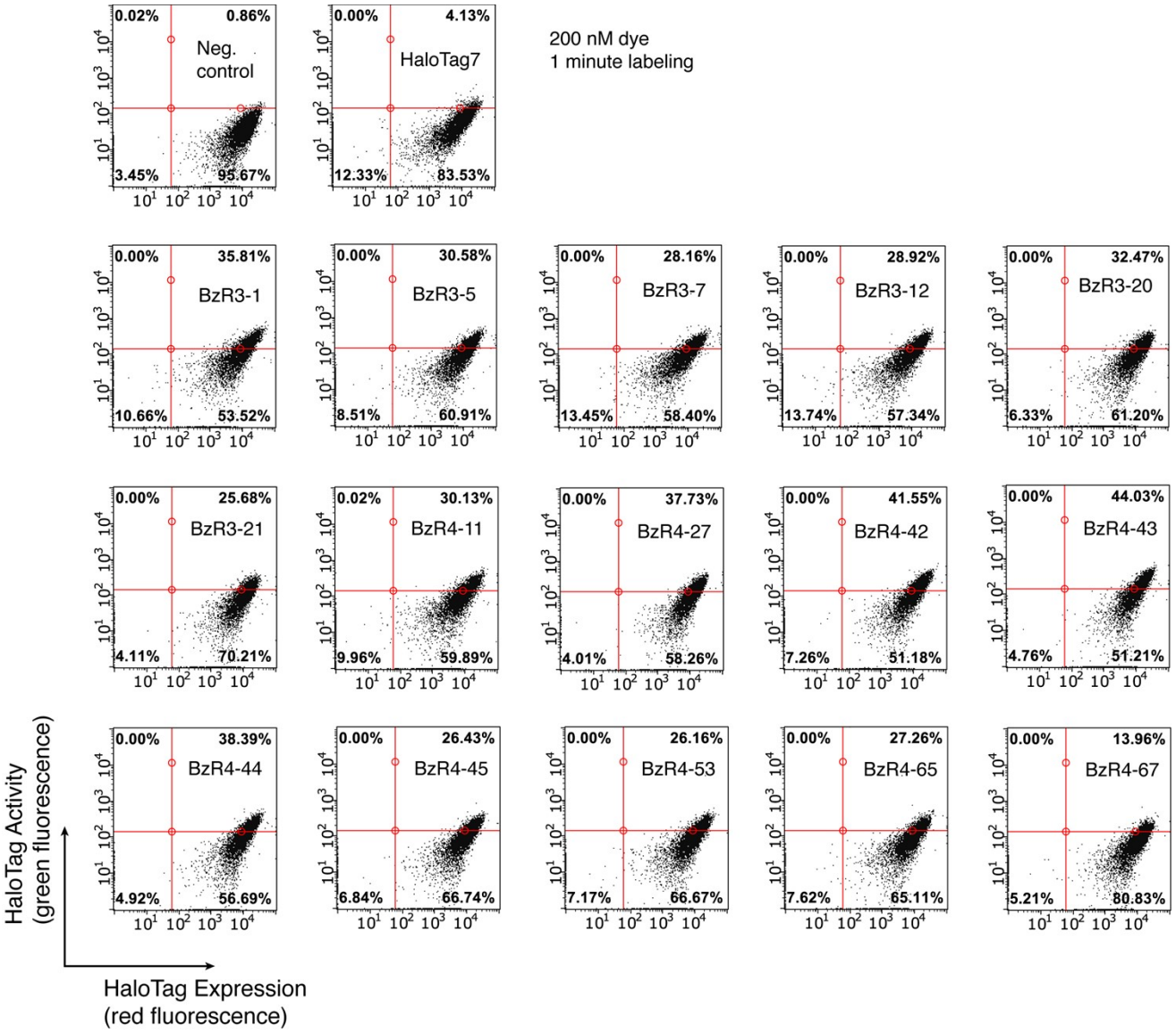


Supplemental Figure 3. Evaluating activity of the output pools from each round of screening. HaloTag activity was measured by labeling with 1.0 μM **Bz-1** for 15 minutes. 10,000 cells are reported in each plot. HaloTag expression is measured by immunostaining against Myc using an AlexaFluor647-conjugated antibody.

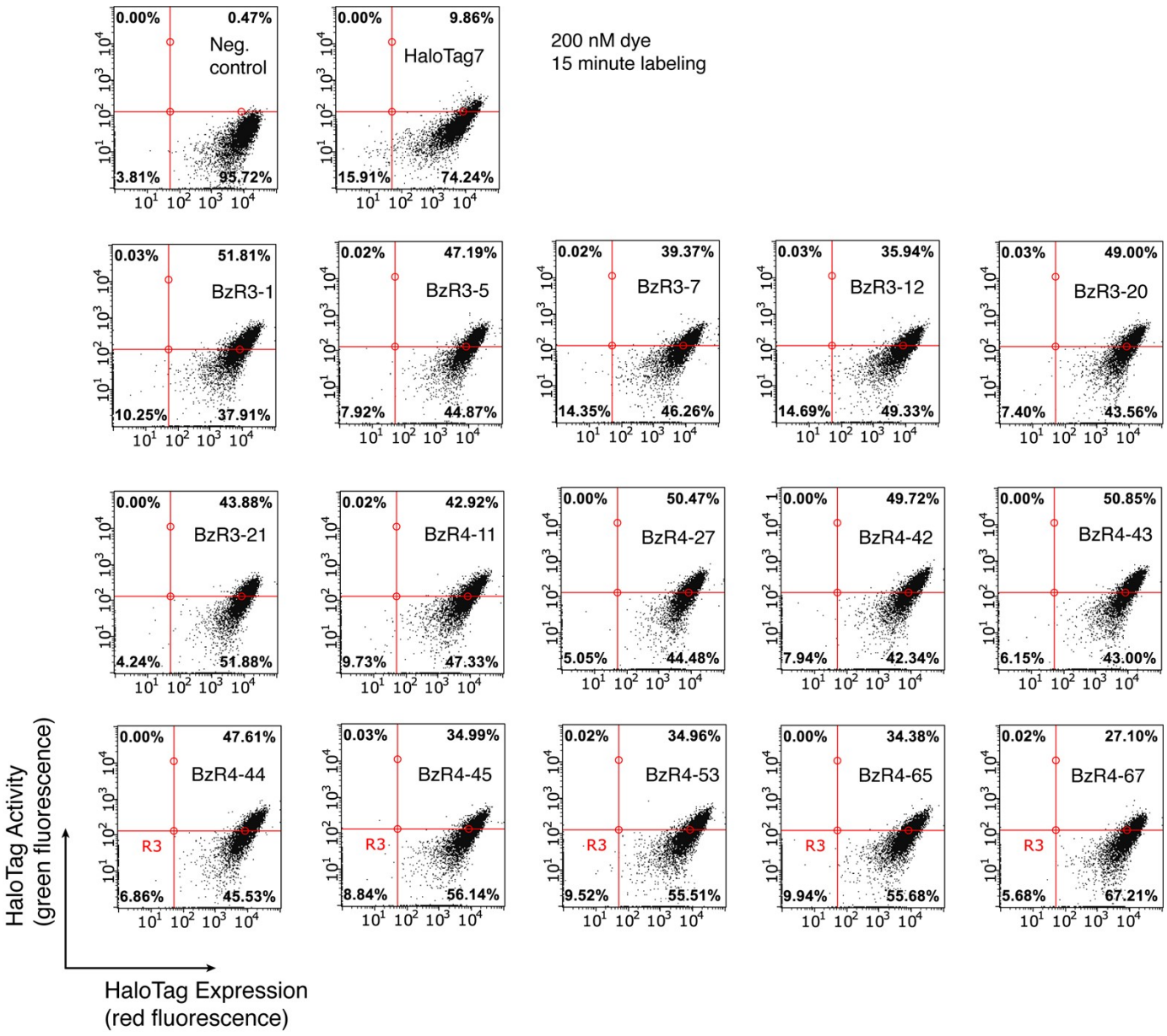
After output pools round 3 and round 4 were isolated, $1x10^8$ cells were pelleted and plasmid was harvested by ZymoPrep (ZymoResearch). Plasmids were purified, transformed, and sequenced as described above. Twenty colonies from R3 and 75 colonies from R4 were sequenced in total. Selected hit sequences were transformed into chemically competent RJY100 *S. cerevisiae* cells as described above, and HaloTag activity was evaluated by flow cytometry (Table S7, Fig. S4, S5). Six sequences were chosen for their high activity and/or representative mutations and their HaloTag variant sequences were isolated by PCR (20 ng of template, 0.2 μM primers **P8** and **P9,** 2.0 mM $MgSO_4$, 0.2 mM of each dNTP, 1 unit of Phusion High-Fidelity polymerase, and 2% DMSO) for cloning into bacterial expression vectors.

Supplemental Table 6. Hit HaloTag Variants with Identified Mutations

Variant	Alternate Name	Mutations
BzR3-1	Variant 1	F144S | L246F
BzR3-5		D156N | E191K | V245A
BzR3-7		H13R
BzR3-12		E224K | V245A
BzR3-20	Variant 3	N195S | V245A | E251K | S291P
BzR3-21		L246F | L259W
BzR4-11		F144L | I211V | V245A | L271V
BzR4-27	Variant 4	L161W | V245A
BzR4-42	Variant 6	F144L | L221S | V245A
BzR4-43	Variant 5	I211V | V245A
BzR4-44	Variant 2	V245A
BzR4-45		D78G | E98G | E121G, V245A
BzR4-53		V245A | A250V
BzR4-65		Q231R | V245A
BzR4-67		A212V | L246F



Supplemental Figure 4. **Activity of screening hits, expressed on the surface of yeast analysis.** Single HaloTag variant clones were analyzed as described above. Cells were treated with 0.2 μM **Bz-1** for 1 minute.



Supplemental Figure 5. **Activity of screening hits, expressed on the surface of yeast analysis.** Single HaloTag variant clones were analyzed as described above. Cells were treated with 0.2 μM **Bz-1** for 15 minutes.

Supplemental Table 7. DNA sequences of selected HaloTag variants produced from yeast display experiments.

Name	Sequence
BzR3-1 (Variant 1)	ATGGCTGAAATTGGTACAGGTTTTCCATTTGATCCACATTACGTCGAAGTTTTGGGTGAAAGAA TGCATTACGTTGATGTTGGTCCAAGAGATGGTACACCAGTTTTGTTTTTACATGGTAACCCAAC TTCTTCATACGTTTGGAGAAACATCATCCCACATGTTGCGCCAACTCATAGATGTATTGCTCCA GATTTGATTGGTATGGGTAAATCTGATAAGCCAGATTTGGGTTATTTCTTTGATGATCATGTTA GATTCATGGATGCTTTTATTGAAGCATTGGGTTTGGAAGAAGTTGTTTTGGTTATTCATGATTG GGGTTCTGCATTAGGTTTTCATTGGGCTAAGAGAAACCCAGAAAGAGTTAAGGGTATCGCTTTT ATGGAGTTTATTAGACCAATTCCAACATGGGATGAATGGCCAGAATCTGCAAGAGAAACTTTCC AAGCTTTTAGAACAACTGATGTTGGTAGAAAGTTGATCATCGATCAAAACGTTTTTATTGAAGG TACATTGCCAATGGGTGTTGTCAGACCATTGACTGAAGTTGAAATGGATCATTACAGAGAACCA TTTTTAAACCCAGTTGATAGAGAACCATTGTGGAGATTTCCAAATGAATTACCAATTGCTGGTG AACCAGCAAACATCGTTGCTTTGGTTGAAGAATACATGGATTGGCTACATCAATCTCCAGTTCC AAAGTTGTTATTTTGGGGTACACCAGGTGTTTTCATTCCACCAGCAGAAGCTGCAAGACTGGCT AAGTCATTGCCAAACTGTAAGGCAGTTGATATTGGTCCAGGTTTGAATTTGTTGCAAGAAGATA ACCCAGATTTGATTGGTTCTGAAATTGCTAGATGGTTGTCAACTTTA
BzR3-20 (Variant 3)	ATGGCTGAAATTGGTACAGGTTTTCCATTTGATCCACATTACGTTGAAGTTTTGGGTGAAAGAA TGCATTACGTTGATGTTGGTCCAAGAGATGGTACACCAGTTTTGTTTTTACATGGTAACCCAAC TTCTTCATACGTTTGGAGAAACATCATCCCACATGTTGCACCAACTCATAGATGTATTGCTCCA GATTTGATTGGTATGGGTAAATCTGATAAGCCAGATTTGGGTTATTTCTTTGATGATCATGTTA GATTCATGGATGCTTTTATTGAAGCATTGGGTTTGGAAGAAGTTGTTTTGGTTATTCATGATTG GGGTTCTGCATTAGGTTTTCATTGGGCTAAGAGAAACCCAGAAAGAGTTAAGGGTATCGCTTTT ATGGAGTTTATTAGACCAATTCCAACATGGGATGAATGGCCAGAATTTGCAAGAGAAACTTTCC AAGCTTTTAGAACAACTGATGTTGGTAGAAAGTTGATCATCGATCAAAACGTTTTTATTGAAGG TACATTGCCAATGGGTGTTGTCAGACCATTGACTGAAGTTGAGATGGATCATTACAGAGAACCA TTTTTAAGCCCAGTTGATAGAGAACCATTGTGGAGATTTCCAAATGAATTACCAATTGCTGGTG AACCAGCAAACATCGTTGCTTTGGTTGAAGAATACATGGATTGGTTACATCAATCTCCAGTTCC AAAGTTGTTATTTTGGGGTACACCAGGTGCTTTAATTCCACCAGCAAAAGCTGCAAGATTGGCT AAGTCATTGCCAAACTGTAAGGCAGTTGATATTGGTCCAGGTTTGAATTTGTTGCAAGAAGATA ACCCAGATTTGATTGGTTCTGAAATTGCTAGATGGTTGTCAACTTTA
BzR4-27 (Variant 4)	ATGGCTGAAATTGGTACAGGTTTTCCATTTGATCCACATTACGTTGAAGTTTTGGGTGAAAGAA TGCATTACGTTGATGTTGGTCCAAGAGATGGTACACCAGTTTTGTTTTTACATGGTAACCCAAC TTCTTCATACGTTTGGAGAAACATCATCCCACATGTTGCACCAACTCATAGATGTATTGCTCCA GATCTGATTGGTATGGGTAAATCTGATAAGCCAGATTTGGGTTATTTCTTTGATGATCATGTTA GATTCATGGATGCTTTTATTGAAGCATTGGGTTTGGAAGAAGTTGTTTTGGTTATTCATGATTG GGGTTCTGCATTAGGTTTTCATTGGGCTAAGAGAAACCCAGAAAGAGTTAAGGGTATCGCTTTT ATGGAGTTTATTAGACCAATTCCAACATGGGATGAATGGCCAGAATTTGCAAGAGAAACTTTCC AAGCTTTTAGAACAACTGATGTTGGTAGAAAGTGGATCATCGATCAAAACGTTTTTATTGAAGG TACATTGCCAATGGGTGTTGTCAGACCATTGACTGAAGTTGAAATGGATCATTACAGAGAACCA TTTTTAAACCCAGTTGATAGAGAACCATTGTGGAGATTTCCAAATGAATTACCAATTGCTGGTG AACCAGCAAACATCGTTGCTTTGGTTGAAGAATACATGGATTGGTTACATCAATCTCCAGTTCC AAAGTTGTTATTTTGGGGTACACCAGGTGCTTTAATTCCACCAGCAGAAGCTGCAAGATTGGCT AAGTCATTGCCAAACTGTAAGGCAGTTGATATTGGTCCAGGTTTGAATTTGTTGCAAGAAGATA ACCCAGATTTGGTTGGTTCTGAAATTGCTAGATGGTTGTCAACTTTA
BzR4-42 (Variant 6)	ATGGCTGAAATTGGTACAGGTTTTCCATTTGATCCACATTACGTTGAAGTTTTGGGTGAAAGAA TGCATTACGTTGATGTTGGTCCAAGAGATGGTACACCAGTTTTGTTTTTACATGGTAACCCAAC TTCTTCATACGTTTGGAGAAACATCATCCCACATGTTGCACCAACTCATAGATGTATTGCTCCA GATTTGATTGGTATGGGTAAATCTGATAAGCCAGATTTGGGTTATTTCTTTGATGATCATGTTA GATTCATGGATGCTTTTATTGAAGCATTGGGTTTGGAAGAAGTTGTTTTGGTTATTCATGATTG GGGTTCTGCATTAGGTTTTCATTGGGCTAAGAGAAACCCAGAAAGAGTTAAGGGTATCGCTTTT ATGGAGTTTATTAGACCAATTCCAACATGGGATGAATGGCCAGAACTTGCAAGAGAAACTTTCC AAGCTTTTAGAACAACTGATGTTGGTAGAAAGTTGATCATCGATCAAAACGTTTTTATTGAAGG TACATTGCCAATGGGTGTTGTCAGACCATTGACTGAAGTTGAAATGGATCATTACAGAGAACCA TTTTTAAACCCAGTTGATAGAGAACCATTGTGGAGATTTCCAAATGAATTACCAATTGCTGGTG AACCAGCAAACATCGTTGCTTCGGTTGAAGAATACATGGATTGGTTACATCAATCTCCAGTTCC

	AAAGTTGTTATTTTGGGGTACACCAGGTGCTTTAATTCCACCAGCAGAAGCTGCAAGATTGGCT AAGTCATTGCCAAACTGTAAGGCAGTTGATATTGGTCCAGGTTTGAATTTGTTGCAAGAAGATA ACCCAGATTTGATTGGTTCTGAAATTGCTAGATGGTTGTCAACTTTA
BzR4-43 (Variant 5)	ATGGCTGAAATTGGTACAGGTTTTCCATTTGATCCACATTACGTTGAAGTTTTGGGTGAAAGAA TGCATTACGTTGATGTTGGTCCAAGAGATGGTACACCAGTTTTGTTTTTACATGGTAACCCAAC TTCTTCATACGTTTGGAGAAACATCATCCCACATGTTGCACCAACTCATAGATGTATTGCTCCA GATTTGATTGGTATGGGTAAATCTGATAAGCCAGATTTGGGTTATTTCTTTGATGATCATGTTA GATTCATGGATGCTTTTATTGAAGCATTGGGTTTGGAAGAAGTTGTTTTGGTTATTCATGATTG GGGTTCTGCATTAGGTTTTCATTGGGCTAAGAGAAACCCAGAAAGAGTTAAGGGTATCGCTTTT ATGGAGTTTATCAGACCAATTCCAACATGGGATGAATGGCCAGAATTTGCAAGAGAAACTTTCC AAGCTTTTAGAACAACTGATGTTGGTAGAAAGTTGATCATCGATCAAAACGTTTTTATTGAAGG TACATTGCCAATGGGTGTTGTCAGACCATTGACTGAAGTTGAAATGGATCATTACAGAGAACCA TTTTTAAACCCAGTTGATAGAGAACCATTGTGGAGATTTCCAAATGAATTACCAGTTGCTGGTG AACCAGCAAACATCGTTGCTTTGGTTGAAGAATACATGGATTGGTTACATCAATCTCCAGTTCC AAAGTTGTTATTTTGGGGTACACCAGGTGCTTTAATTCCACCAGCAGAAGCTGCAAGATTGGCT AAGTCATTGCCAAACTGTAAGGCAGTTGATATTGGTCCAGGTTTGAATTTGTTGCAAGAAGATA ACCCAGATTTGATTGGTTCTGAAATTGCTAGATGGTTGTCAACTTTA
BzR4-44 (Variant 2)	ATGGCTGAAATTGGTACAGGTTTTCCATTTGATCCACATTACGTTGAAGTTTTGGGTGAAAGAA TGCATTACGTTGATGTTGGTCCAAGAGACGGTACACCAGTTTTGTTTTTACATGGTAACCCAAC TTCTTCATACGTTTGGAGAAACATCATCCCACATGTTGCACCAACTCATAGATGTATTGCTCCA GATTTGATTGGTATGGGTAAATCTGATAAGCCAGATTTGGGTTATTTCTTTGATGATCATGTTA GATTCATGGATGCTTTTATTGAAGCATTGGGTTTGGAAGAAGTTGTTTTGGTTATTCATGATTG GGGTTCTGCATTAGGTTTTCATTGGGCTAAGAGAAACCCAGAAAGAGTTAAGGGTATCGCTTTT ATGGAGTTTATTAGACCAATTCCAACATGGGATGAATGGCCAGAATTTGCAAGAGAAACTTTCC AAGCTTTTAGAACAACTGATGTTGGTAGAAAGTTGATCATCGATCAAAACGTCTTTATTGAAGG TACATTGCCAATGGGTGTTGTCAGACCATTGACTGAAGTTGAAATGGATCATTACAGAGAACCA TTTTTAAACCCAGTTGATAGAGAACCATTGTGGAGATTTCCAAATGAATTACCAATTGCTGGTG AACCAGCAAACATCGTTGCTTTGGTTGAAGAATACATGGATTGGTTACATCAATCTCCAGTTCC AAAGTTGTTATTTTGGGGTACACCAGGTGCTTTAATTCCACCAGCAGAAGCTGCAAGATTGGCT AAGTCATTGCCAAACTGTAAGGCAGTTGATATTGGTCCAGGTTTGAATTTGTTGCAAGAAGATA ACCCAGATTTGATTGGTTCTGAAATTGCTAGATGGTTGTCAACTTTA
Variant 7	ATGGCTGAAATTGGTACAGGTTTTCCATTTGATCCACATTACGTTGAAGTTTTGGGTGAAAGAA TGCATTACGTTGATGTTGGTCCAAGAGATGGTACACCAGTTTTGTTTTTACATGGTAACCCAAC TTCTTCATACGTTTGGAGAAACATCATCCCACATGTTGCACCAACTCATAGATGTATTGCTCCA GATTTGATTGGTATGGGTAAATCTGATAAGCCAGATTTGGGTTATTTCTTTGATGATCATGTTA GATTCATGGATGCTTTTATTGAAGCATTGGGTTTGGAAGAAGTTGTTTTGGTTATTCATGATTG GGGTTCTGCATTAGGTTTTCATTGGGCTAAGAGAAACCCAGAAAGAGTTAAGGGTATCGCTTTT ATGGAGTTTATTAGACCAATTCCAACATGGGATGAATGGCCAGAACTTGCAAGAGAAACTTTCC AAGCTTTTAGAACAACTGATGTTGGTAGAAAGTTGATCATCGATCAAAACGTTTTTATTGAAGG TACATTGCCAATGGGTGTTGTCAGACCATTGACTGAAGTTGAAATGGATCATTACAGAGAACCA TTTTTAAACCCAGTTGATAGAGAACCATTGTGGAGATTTCCAAATGAATTACCAATTGCTGGTG AACCAGCAAACATCGTTGCTTCGGTTGAAGAATACATGGATTGGTTACATCAATCTCCAGTTCC AAAGTTGTTATTTTGGGGTACACCAGGTGCTTTTATTCCACCAGCAGAAGCTGCAAGATTGGCT AAGTCATTGCCAAACTGTAAGGCAGTTGATATTGGTCCAGGTTTGAATTTGTTGCAAGAAGATA ACCCAGATTTGATTGGTTCTGAAATTGCTAGATGGTTGTCAACTTTA
Variant 8	ATGGCTGAAATTGGTACAGGTTTTCCATTTGATCCACATTACGTTGAAGTTTTGGGTGAAAGAA TGCATTACGTTGATGTTGGTCCAAGAGATGGTACACCAGTTTTGTTTTTACATGGTAACCCAAC TTCTTCATACGTTTGGAGAAACATCATCCCACATGTTGCACCAACTCATAGATGTATTGCTCCA GATTTGATTGGTATGGGTAAATCTGATAAGCCAGATTTGGGTTATTTCTTTGATGATCATGTTA GATTCATGGATGCTTTTATTGAAGCATTGGGTTTGGAAGAAGTTGTTTTGGTTATTCATGATTG GGGTTCTGCATTAGGTTTTCATTGGGCTAAGAGAAACCCAGAAAGAGTTAAGGGTATCGCTTTT ATGGAGTTTATTAGACCAATTCCAACATGGGATGAATGGCCAGAACTGGCAAGAGAAACTTTCC AAGCTTTTAGAACAACTGATGTTGGTAGAAAGTTGATCATCGATCAAAACGTTTTTATTGAAGG TACATTGCCAATGGGTGTTGTCAGACCATTGACTGAAGTTGAAATGGATCATTACAGAGAACCA TTTTTAAACCCAGTTGATAGAGAACCATTGTGGAGATTTCCAAATGAATTACCAATTGCTGGTG AACCAGCAAACATCGTTGCTTCGGTTGAAGAATACATGGATTGGTTACATCAATCTCCAGTTCC

	AAAGTTGTTATTTTGGGGTACACCAGGTGCTTTAATTCCACCAGCAGAAGCTGCAAGATTGGCT AAGTCATTGCCAAACTGTAAGGCAGTTGATATTGGTCCAGGTTTGAATTTGTTGCAAGAAGATA ACCCAGATTTGATTGGTTCTGAAATTGCTAGATGGTTGTCAACTTTA
Variant 9	ATGGCTGAAATTGGTACAGGTTTTCCATTTGATCCACATTACGTTGAAGTTTTGGGTGAAAGAA TGCATTACGTTGATGTTGGTCCAAGAGATGGTACACCAGTTTTGTTTTTACATGGTAACCCAAC TTCTTCATACGTTTGGAGAAACATCATCCCACATGTTGCACCAACTCATAGATGTATTGCTCCA GATTTGATTGGTATGGGTAAATCTGATAAGCCAGATTTGGGTTATTTCTTTGATGATCATGTTA GATTCATGGATGCTTTTATTGAAGCATTGGGTTTGGAAGAAGTTGTTTTGGTTATTCATGATTG GGGTTCTGCATTAGGTTTTCATTGGGCTAAGAGAAACCCAGAAAGAGTTAAGGGTATCGCTTTT ATGGAGTTTATTAGACCAATTCCAACATGGGATGAATGGCCAGAACTGGCAAGAGAAACTTTCC AAGCTTTTAGAACAACTGATGTTGGTAGAAAGTTGATCATCGATCAAAACGTTTTTATTGAAGG TACATTGCCAATGGGTGTTGTCAGACCATTGACTGAAGTTGAAATGGATCATTACAGAGAACCA TTTTTAAACCCAGTTGATAGAGAACCATTGTGGAGATTTCCAAATGAATTACCAATTGCTGGTG AACCAGCAAACATCGTTGCTTCGGTTGAAGAATACATGGATTGGTTACATCAATCTCCAGTTCC AAAGTTGTTATTTTGGGGTACACCAGGTGCTTTTATTCCACCAGCAGAAGCTGCAAGATTGGCT AAGTCATTGCCAAACTGTAAGGCAGTTGATATTGGTCCAGGTTTGAATTTGTTGCAAGAAGATA ACCCAGATTTGATTGGTTCTGAAATTGCTAGATGGTTGTCAACTTTA
Variant 10 (BenzoTag)	ATGGCTGAAATTGGTACAGGTTTTCCATTTGATCCACATTACGTTGAAGTTTTGGGTGAAAGAA TGCATTACGTTGATGTTGGTCCAAGAGATGGTACACCAGTTTTGTTTTTACATGGTAACCCAAC TTCTTCATACGTTTGGAGAAACATCATCCCACATGTTGCACCAACTCATAGATGTATTGCTCCA GATTTGATTGGTATGGGTAAATCTGATAAGCCAGATTTGGGTTATTTCTTTGATGATCATGTTA GATTCATGGATGCTTTTATTGAAGCATTGGGTTTGGAAGAAGTTGTTTTGGTTATTCATGATTG GGGTTCTGCATTAGGTTTTCATTGGGCTAAGAGAAACCCAGAAAGAGTTAAGGGTATCGCTTTT ATGGAGTTTATCAGACCAATTCCAACATGGGATGAATGGCCAGAATTTGCAAGAGAAACTTTCC AAGCTTTTAGAACAACTGATGTTGGTAGAAAGTTGATCATCGATCAAAACGTTTTTATTGAAGG TACATTGCCAATGGGTGTTGTCAGACCATTGACTGAAGTTGAAATGGATCATTACAGAGAACCA TTTTTAAACCCAGTTGATAGAGAACCATTGTGGAGATTTCCAAATGAATTACCAGTGGCTGGTG AACCAGCAAACATCGTTGCTTTGGTTGAAGAATACATGGATTGGTTACATCAATCTCCAGTTCC AAAGTTGTTATTTTGGGGTACACCAGGTGCTTTAATTCCACCAGCAGAAGCTGCAAGATTGGCT AAGTCATTGCCAAACTGTAAGGCAGTTGATATTGGTCCAGGTTTGAATTTGTTGCAAGAAGATA ACCCAGATTTGATTGGTTCTGAAATTGCTAGATGGTTGTCAACTTTA

Supplemental Table 8. Protein sequences of selected HaloTag variants produced from yeast display experiments.

Name	Sequence
BzR3-1 (Variant 1)	MAEIGTGFPFDPHYVEVLGERMHYVDVGPRDGTPVLFLHGNPTSSYVWRNIIPHVAPTHRCIAP DLIGMGKSDKPDLGYFFDDHVRFMDAFIEALGLEEVVLVIHDWGSALGFHWAKRNPERVKGIAF MEFIRPIPTWDEWPESARETFQAFRTTDVGRKLIIDQNVFIEGTLPMGVVRPLTEVEMDHYREP FLNPVDREPLWRFPNELPIAGEPANIVALVEEYMDWLHQSPVPKLLFWGTPGVFIPPAEAARLA KSLPNCKAVDIGPGLNLLQEDNPDLIGSEIARWLSTL
BzR4-44 (Variant 2)	MAEIGTGFPFDPHYVEVLGERMHYVDVGPRDGTPVLFLHGNPTSSYVWRNIIPHVAPTHRCIAP DLIGMGKSDKPDLGYFFDDHVRFMDAFIEALGLEEVVLVIHDWGSALGFHWAKRNPERVKGIAF MEFIRPIPTWDEWPEFARETFQAFRTTDVGRKLIIDQNVFIEGTLPMGVVRPLTEVEMDHYREP FLNPVDREPLWRFPNELPIAGEPANIVALVEEYMDWLHQSPVPKLLFWGTPGALIPPAEAARLA KSLPNCKAVDIGPGLNLLQEDNPDLIGSEIARWLSTL
BzR3-20 (Variant 3)	MAEIGTGFPFDPHYVEVLGERMHYVDVGPRDGTPVLFLHGNPTSSYVWRNIIPHVAPTHRCIAP DLIGMGKSDKPDLGYFFDDHVRFMDAFIEALGLEEVVLVIHDWGSALGFHWAKRNPERVKGIAF MEFIRPIPTWDEWPEFARETFQAFRTTDVGRKLIIDQNVFIEGTLPMGVVRPLTEVEMDHYREP FLSPVDREPLWRFPNELPIAGEPANIVALVEEYMDWLHQSPVPKLLFWGTPGALIPPAKAARLA KSLPNCKAVDIGPGLNLLQEDNPDLIGSEIARWLSTL
BzR4-27 (Variant 4)	MAEIGTGFPFDPHYVEVLGERMHYVDVGPRDGTPVLFLHGNPTSSYVWRNIIPHVAPTHRCIAP DLIGMGKSDKPDLGYFFDDHVRFMDAFIEALGLEEVVLVIHDWGSALGFHWAKRNPERVKGIAF MEFIRPIPTWDEWPEFARETFQAFRTTDVGRKWIIDQNVFIEGTLPMGVVRPLTEVEMDHYREP FLNPVDREPLWRFPNELPIAGEPANIVALVEEYMDWLHQSPVPKLLFWGTPGALIPPAEAARLA KSLPNCKAVDIGPGLNLLQEDNPDLVGSEIARWLSTL
BzR4-43 (Variant 5)	MAEIGTGFPFDPHYVEVLGERMHYVDVGPRDGTPVLFLHGNPTSSYVWRNIIPHVAPTHRCIAP DLIGMGKSDKPDLGYFFDDHVRFMDAFIEALGLEEVVLVIHDWGSALGFHWAKRNPERVKGIAF MEFIRPIPTWDEWPEFARETFQAFRTTDVGRKLIIDQNVFIEGTLPMGVVRPLTEVEMDHYREP FLNPVDREPLWRFPNELPVAGEPANIVALVEEYMDWLHQSPVPKLLFWGTPGALIPPAEAARLA KSLPNCKAVDIGPGLNLLQEDNPDLIGSEIARWLSTL
BzR4-42 (Variant 6)	MAEIGTGFPFDPHYVEVLGERMHYVDVGPRDGTPVLFLHGNPTSSYVWRNIIPHVAPTHRCIAP DLIGMGKSDKPDLGYFFDDHVRFMDAFIEALGLEEVVLVIHDWGSALGFHWAKRNPERVKGIAF MEFIRPIPTWDEWPELARETFQAFRTTDVGRKLIIDQNVFIEGTLPMGVVRPLTEVEMDHYREP FLNPVDREPLWRFPNELPIAGEPANIVASVEEYMDWLHQSPVPKLLFWGTPGALIPPAEAARLA KSLPNCKAVDIGPGLNLLQEDNPDLIGSEIARWLSTL
(Variant 7)	MAEIGTGFPFDPHYVEVLGERMHYVDVGPRDGTPVLFLHGNPTSSYVWRNIIPHVAPTHRCIAP DLIGMGKSDKPDLGYFFDDHVRFMDAFIEALGLEEVVLVIHDWGSALGFHWAKRNPERVKGIAF MEFIRPIPTWDEWPELARETFQAFRTTDVGRKLIIDQNVFIEGTLPMGVVRPLTEVEMDHYREP FLNPVDREPLWRFPNELPIAGEPANIVASVEEYMDWLHQSPVPKLLFWGTPGAFIPPAEAARLA KSLPNCKAVDIGPGLNLLQEDNPDLIGSEIARWLSTL
(Variant 8)	MAEIGTGFPFDPHYVEVLGERMHYVDVGPRDGTPVLFLHGNPTSSYVWRNIIPHVAPTHRCIAP DLIGMGKSDKPDLGYFFDDHVRFMDAFIEALGLEEVVLVIHDWGSALGFHWAKRNPERVKGIAF MEFIRPIPTWDEWPELARETFQAFRTTDVGRKLIIDQNVFIEGTLPMGVVRPLTEVEMDHYREP FLNPVDREPLWRFPNELPVAGEPANIVASVEEYMDWLHQSPVPKLLFWGTPGALIPPAEAARLA KSLPNCKAVDIGPGLNLLQEDNPDLIGSEIARWLSTL
(Variant 9)	MAEIGTGFPFDPHYVEVLGERMHYVDVGPRDGTPVLFLHGNPTSSYVWRNIIPHVAPTHRCIAP DLIGMGKSDKPDLGYFFDDHVRFMDAFIEALGLEEVVLVIHDWGSALGFHWAKRNPERVKGIAF MEFIRPIPTWDEWPELARETFQAFRTTDVGRKLIIDQNVFIEGTLPMGVVRPLTEVEMDHYREP FLNPVDREPLWRFPNELPVAGEPANIVASVEEYMDWLHQSPVPKLLFWGTPGAFIPPAEAARLA KSLPNCKAVDIGPGLNLLQEDNPDLIGSEIARWLSTL
(Variant 10) **BenzoTag**	MAEIGTGFPFDPHYVEVLGERMHYVDVGPRDGTPVLFLHGNPTSSYVWRNIIPHVAPTHRCIAP DLIGMGKSDKPDLGYFFDDHVRFMDAFIEALGLEEVVLVIHDWGSALGFHWAKRNPERVKGIAF MEFIRPIPTWDEWPELARETFQAFRTTDVGRKLIIDQNVFIEGTLPMGVVRPLTEVEMDHYREP FLNPVDREPLWRFPNELPVAGEPANIVALVEEYMDWLHQSPVPKLLFWGTPGALIPPAEAARLA KSLPNCKAVDIGPGLNLLQEDNPDLIGSEIARWLSTL

Recombinant Protein Expression

The six isolated DNA fragments and the yeast codon-optimized HaloTag7 sequence were amplified by PCR using primers **P10** and **P11** to introduce homologous overhangs and an N-terminal 6xHis sequence for cloning into the pET51(+)b plasmid between the NsiI and AvrII restriction sites. Cloned sequences were transformed into NEB BL21 competent *E. coli* by heat shock for protein expression and purification, as previously reported.[14] Recovered cells were grown on LB agar plates with ampicillin (100 µg/mL) overnight at 37 °C. Individual colonies were inoculated in 10 mL of LB broth with ampicillin and grown overnight at 37 °C. Overnight cultures were then diluted into 1 L of LB broth with ampicillin and grown for 2-4 hours at 37 °C until reaching an optical density at 600 nm of 0.6. Protein expression was induced with 0.5 mL IPTG (1 mM) for 4 hours at 37 °C or overnight at 20 °C before pelleting at 5,000 rpm for 10 min at 4 °C. Pellets were stored at -80 °C before lysis in sterile filtered His-binding buffer (50 mM Tris-Cl, 100 mM NaCl, 5 mM imidazole, 0.1 mM EDTA, 5 mM 2-mercaptoethanol, pH 8.0) with 30 mg lysozyme (Thermo Fisher Scientific) and ½ tablet cOmplete EDTA-free protease inhibitor (Roche). Lysed pellets were sonicated on ice for 10 minutes (10 seconds on/off, amplitude: 60%). Lysate was then incubated with Pierce Universal Nuclease for Cell Lysis (Thermo Fisher Scientific) for 5 minutes on ice and clarified by centrifugation for 30 minutes at 10,000 rpm at 4 °C before decanting and centrifuging again. The clarified lysate was purified by batch affinity purification with 3 mL HisPur™ Ni-NTA resin (Thermo Scientific). Before purification, Ni-NTA resin was washed and then equilibrated with His-binding buffer for >45 min on a rocking platform at 4 °C. After equilibration, clarified lysate was incubated with the Ni-NTA resin in His-binding buffer for 1 hour on a rocking platform at 4 °C. After draining the flowthrough, the Ni-NTA resin was washed 10 times: 3 times with His-binding buffer and 7 times with His-wash buffer (50 mM Tris-Cl, 500 mM NaCl, 10 mM imidazole, 0.1 mM EDTA, 5 mM 2-mercaptoethanol, pH 6.8). We found that washing with a higher salt concentration (500 mM NaCl rather than to 100 mM) and a lower pH (pH 6.8 rather than pH = 8.0) yielded purer HaloTag. Purified HaloTag was eluted from the resin with His-elution buffer (50 mM Tris-Cl, 50 mM NaCl, 300 mM imidazole, 0.1 mM EDTA, 5 mM 2-mercaptoethanol). Finally, eluted fractions were pooled and buffer exchanged into PBS using 7k MWCO Zeba Spin Desalting Columns (Thermo Fisher Scientific) before aliquoting and snap freezing in liquid nitrogen. Frozen protein samples were stored at -80 °C. This protocol routinely produced purified protein yields of ~20 mg/L with the yeast codon-optimized HaloTag sequences. By contrast, a bacteria codon optimized sequence produced ~90 mg/L in our hands.[14]

Variants 7-10 were generated using site-directed mutagenesis using Variant 5 and Variant 6 constructs as templates. Mutagenesis was done using the Q5® Site-Directed Mutagenesis Kit from NEB while following manufacturers procedures. Primers P14 and P15 were used to introduce F144L, P16 and P17 were used to introduce I211V, and P18 and P19 were used to introduce L246F. Successful mutagenesis was verified by Sangar sequencing of ten random colonies and variants were expressed and purified following the same protocol from above.

Kinetics

Conjugation rates of **Bz-1** and related substrates to HaloTag7 and identified variants were measured as previously described using a fluorescence plate reader.[15] Briefly, 0.5 µM substrate in PBS + 0.1% Tween was added to varying concentrations of 2x protein (4, 3, 2, 1.5, 1, 0.5 µM) in PBS + 0.1% Tween in a 1:1 ratio. Final dye concentration was 0.25 µM and final protein concentrations were 2, 1.5, 1, 0.75, 0.5, and 0.25 µM. The growth of fluorescence (excitation

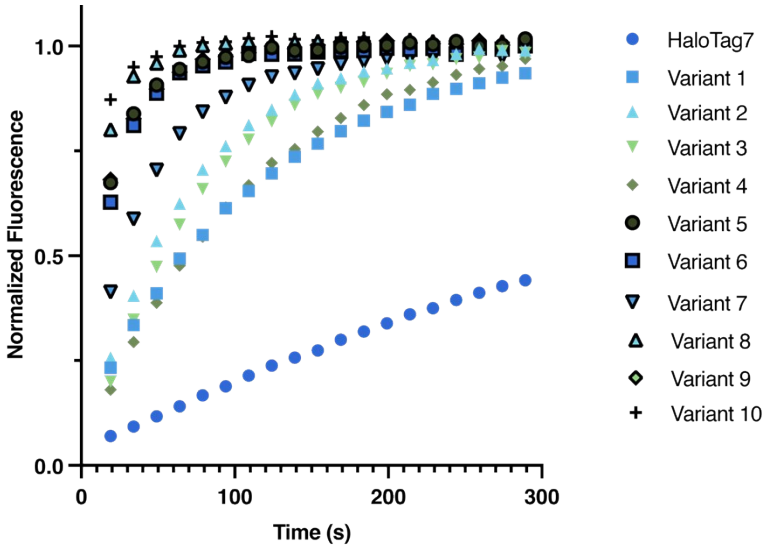
450 nm, emission 535 nm) was measured as a function of time accounting for the gap between addition of protein and the first measurement (<20 seconds). Fluorescence was measured every 15 seconds for 30 minutes. Kinetic traces were normalized and fit to a one-phase association exponential curve (Equation 1) where Y_0 was set to 0. All results reported are the average of at least three independent biological replicates and errors are reported as the standard error of the mean (Table S10, Fig. S6 & S7). We note that the larger error associated with the faster measured Variant 7 and 10 are a result of these rates approaching the limit of detection using traditional plate reader fluorescence measurements. Stopped-flow kinetics will be more suitable for rates any faster than 10^5 $M^{-1}s^{-1}$.

$$(1)\quad Y = Y_0 + (Plateau - Y_0)\left(1 - e^{-k_{obs}X}\right)$$

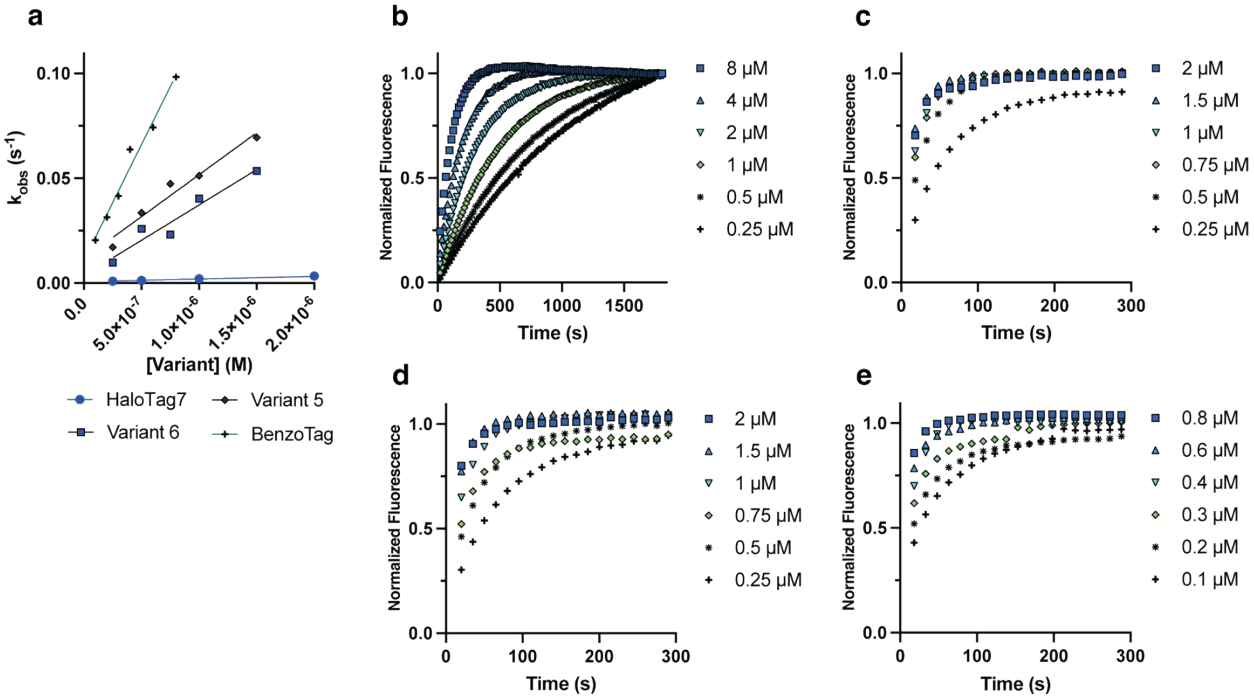
Conjugation rates of CA-TMR with BenzoTag and select variants were measured by fluorescent plate reader and fluorescence polarization as previously reported.[14] Rates between 0.05 μM substrate and identical protein concentrations as above were used. Conjugation rates of CA-JF_{635} were measured by monitoring the growth of fluorescence emission at 665 nm. As noted in the main text, larger concentration of dye (500 μM), and thus higher protein concentrations (100, 50, 25, 12.5, 6.25, and 3.125 μM) were used to detect a large enough change in fluorescence upon reacting with BenzoTag.

Supplemental Table 9. Second-order rate constants for Bz-1 Conjugation to HaloTag7, and Variants 1-6.

	k_{app} ($M^{-1}s^{-1}$)					
	Trial 1	Trial 2	Trial 3	Trial 4	Trial 5	Average
HaloTag7	1229	1944	1541	1194	-	1477 ± 174
Variant 1	6264	5501	9910	8152	-	9329 ± 2023
Variant 2	13088	9708	11804	9430	-	11008 ± 873
Variant 3	5335	8117	16818	11482	7648	8317 ± 997
Variant 4	13849	10632	9803	7098	-	10346 ± 1390
Variant 5	41090	43023	36382	39734	46113	41268 ± 1625
Variant 6	33688	31757	28303	40014	-	33441 ± 2458
Variant 7	56574	14313	77788	36241	59796	68844 ± 8161
	62359	78259	104179	131318	96792	
	40027	50245	95765	35912	93088	
Variant 8	12232	12721	16085	19501	17559	15620 ± 1395
Variant 9	34860	19916	51566	46157	48808	44601 ± 4149
	48551	53187	36241	62127		
Variant 10	61758	147628	58690	102928	75640	95366 ± 9249
BenzoTag	89957	110609	109900	101182		

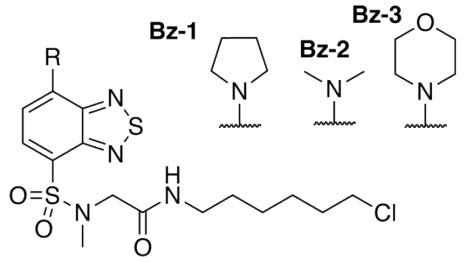


Supplemental Figure 6. Representative kinetic traces Bz-1 conjugation to HaloTag7, BenzoTag, and Variants 1-9. Example kinetic traces of 0.25 μM **Bz-1** reacting with 1.0 μM of protein for the first five minutes of measurement.



Supplemental Figure 7. Representative kinetic traces of Bz-1 conjugation used to determine second-order rate constants. (a) First-order rate constants (k_{obs}) of **Bz-1** conjugation to HaloTag7, Variant 5, Variant 6, and BenzoTag plotted as a function of protein concentration. The slope of the linear fit yielded the second-order rate constant. Representative kinetic traces of **Bz-1** are shown with varying concentrations of (b) HaloTag7, (c) Variant 5, (d) Variant 6 and (e) BenzoTag; these are the primary data used to determine the first-order rate constants shown in (a).

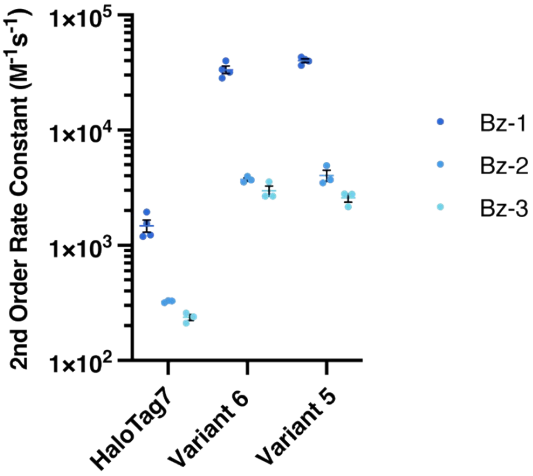
a



b

	k_{app} ($M^{-1}s^{-1}$) (x10^3)		
	HaloTag7	Variant 6	Variant 5
Bz-1	1.47 ± 0.17	33.44 ± 2.4	40.06 ± 1.40
Bz-2	0.32 ± 0.00	3.72 ± 0.12	4.04 ± 0.44
Bz-3	0.24 ± 0.01	2.97 ± 0.30	2.57 ± 0.21

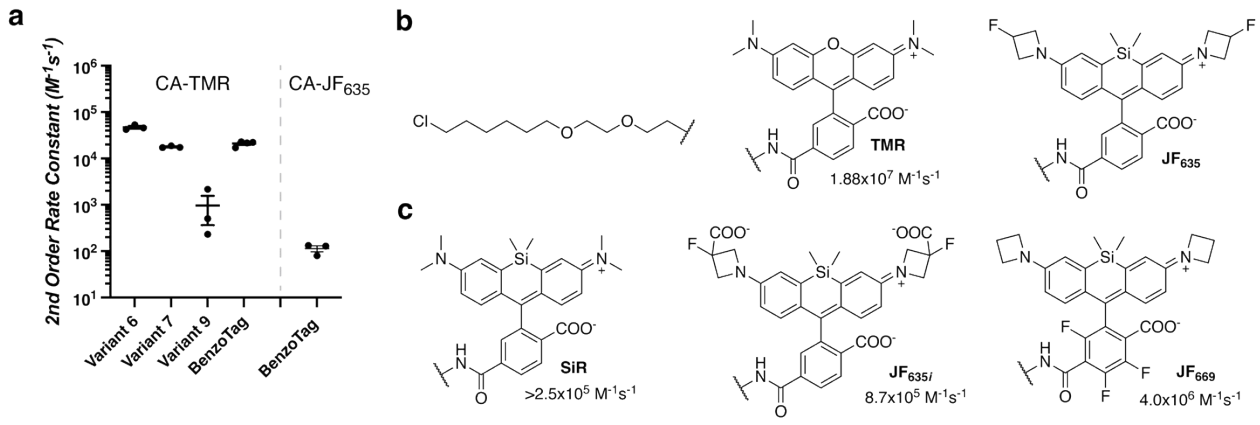
c



Supplemental Figure 8. Reaction rates of other benzothiadiazole substrates with HaloTag7, BenzoTag, and Variant 5. (a) Structures of **Bz-1, Bz-2,** and **Bz-3**. (b,c) Summary of second-order rate constants of **Bz-1, Bz-2,** and **Bz-3** with HaloTag7, BenzoTag, and Variant 5. All results reported are the average of at least three independent biological replicates and errors are reported as the standard error of the mean.

Supplemental Table 10. Second-order rate constants for CA-TMR and CA-JF_{635} Conjugation to BenzoTag and select variants.

	k_{app} ($M^{-1}s^{-1}$)					
	Trial 1	Trial 2	Trial 3	Trial 4	Trial 5	
	CA-TMR					Average
BenzoTag	16975	22330	21751	22986	16975	21011 ± 1369
Variant 6	42499	45729	53562	-	-	47263 ± 3284
Variant 8	17186	17390	18765	-	-	17780 ± 495.8
Variant 9	232	2160	506	-	-	966.0 ± 602.2
	CA-JF_{635}					Average
BenzoTag	79.54	132.4	129	-	-	113.6 ± 17.08



Supplemental Figure 9. Reaction rates of rhodamine chloroalkane substrates with HaloTag7, BenzoTag, and other variants. (a) Plot of apparent second order rate constants of **CA-TMR** and **CA-JF$_{635}$** with BenzoTag and selected variants. (b) Structures of **CA-TMR** and **CA-JF$_{635}$**. (c) Structures of analogous silicon rhodamine dyes, **SiR**[16]; **JF$_{635i}$**[17]; **JF$_{669}$**[18, 19], and their apparent second order rate constants used to estimate the conjugation rate of **CA-JF$_{635}$** to HaloTag7.

Photophysical Measurements

Steady state photophysical measurements were conducted in aerated solvents at approximately 10 μM of dye such that the absorbance did not exceed 0.1. Quantum yield of fluorescence was calculated using eq. 2, where F is the integrated intensities, *f* is the overlap absorbance value between the dye and standard, and η is the refractive index of the solvent; *i* and s stand for sample and standard, respectively. Coumarin153 in ethanol was used as the standard ($\varphi_{Fl,\ standard}$ = 0.53). Properties were measured in duplicate.

$$\Phi_{fl}^{\ i} = \left(\frac{F^i f_s \eta_\iota^2}{F^s f_i \eta_s^2}\right) \Phi_{fl}^{\ s} \quad (1)$$

Supplemental Table 11. Photophysical measurements of the BenzoTag system

	Φ_{fl}	ε ($M^{-1}cm^{-1}$)	$\lambda_{abs,\ max}$ (nm)	$\lambda_{em,\ max}$ (nm)
BenzoTag•**Bz-1**	0.58	8100	446	530

Cell Culture, Flow Cytometry, and Confocal Fluorescence Microscopy

General – HaloTag7 and BenzoTag were subjected to PCR using primers **P12** and **P13** to introduce homologous overhangs for cloning into pCDNA/FRT/TO_TOMM20 plasmid (Gift from Kai Johnsson,[5] pCDNA5/FRT/TO_TOMM20_HaloTag9 was a gift from Kai Johnsson (Addgene plasmid # 169335) between the AgeI and XhoI restriction sites.[5] After cloning, these plasmids were transformed into NEB 5α competent *E. coli* and plasmid isolated by maxi-prep (Invitrogen).

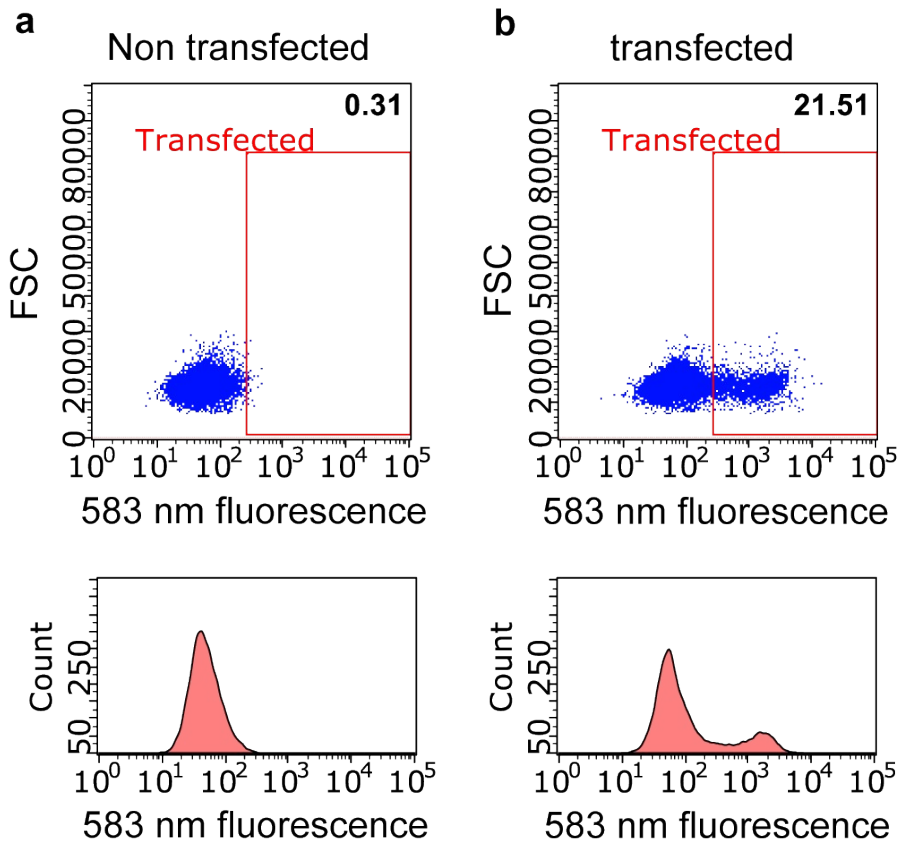
U-2 OS cells (ATCC) were cultured in high glucose MyCoy's 5A media (Thermo Fisher Scientific) supplemented with 10% fetal bovine serum and grown at 37 °C in a humidified

environment of 5% CO_2. Two days prior to an experiment, 10,000 cells were seeded per well in a 96-well plate and grown for 24 hours. Cells were then transiently transfected with expression plasmid using X-tremeGene 9 transfection reagent (Roche) according to manufacturer protocols. Cells were incubated with transfection mix for 18-24 hours.

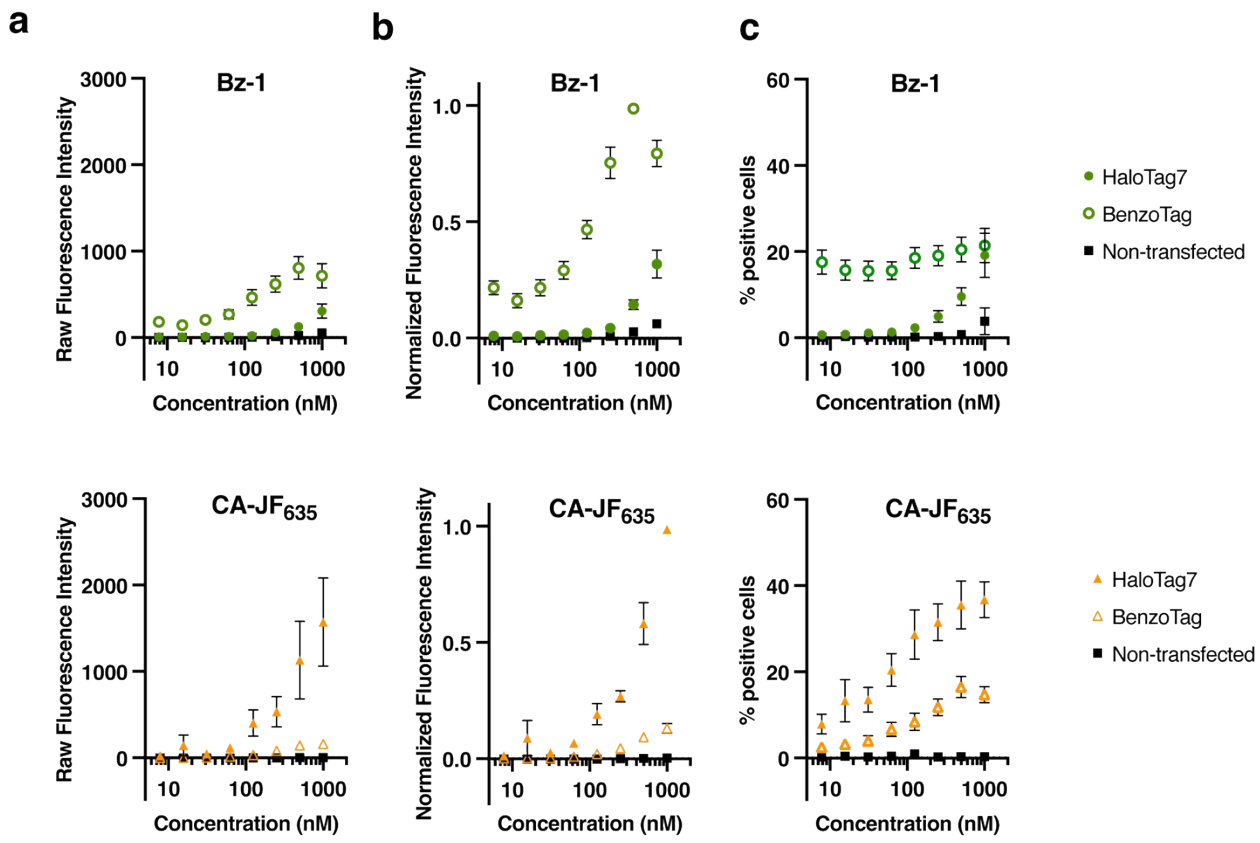
Flow Cytometry – Transfection media was aspirated, and cells were washed with PBS. Non-transfected cells and cells transfected with either HaloTag7 or BenzoTag, and non-transfected cells were then treated with varying concentrations of **Bz-1** or **Bz-3** 100 µL in Opti-MEM reduced serum media (Thermo Fisher Scientific) for 10 minutes. Media was then aspirated, and cells were trypsinized without wash steps from the well using 0.05% Trypsin in PBS, diluted to 200 µL. 10,000 live, single cells were analyzed by flow cytometry. The **Bz-1** fluorescence (green) of each cell was measured using a 488 nm laser and a 525 nm emission filter, and the **CA-JF$_{635}$** fluorescence (red) was measured using a 642 nm laser and a 661 nm emission filter. The mean green fluorescence of 10,000 cells was calculated and the background due to cell autofluorescence was subtracted using measurement from non-transfected, non-dye treated cells. Signal-over-background was calculated by taking the ratio of green fluorescence between dye-treated, transfected cells and dye-treated, non-transfected of cells. The reported mean green fluorescence values are for the entire analyzed population and are thus likely and underestimation of the true enhancement of green fluorescence in live cells as it includes ~80% of cells that were not transfected.

Confocal Fluorescence Microscopy – Transfection media was aspirated, and cells were treated with Hoechst stain (1 µg/mL) for 30 minutes in Opti-MEM. Cells were washed twice with PBS and then treated with Opti-MEM for an extended 30-minute wash step. 50 µL of HEPES-buffered saline (HBS) were added to the cells. Prior to imaging, cells were then treated with 50 µL of 250 nM or 20 nM of **Bz-1** in HBS (final concentration 125 nM or 10 nM) and imaged directly without removing excess dye. Images were acquired on a Leica TCS SP8 microscope and processed using ImageJ software.

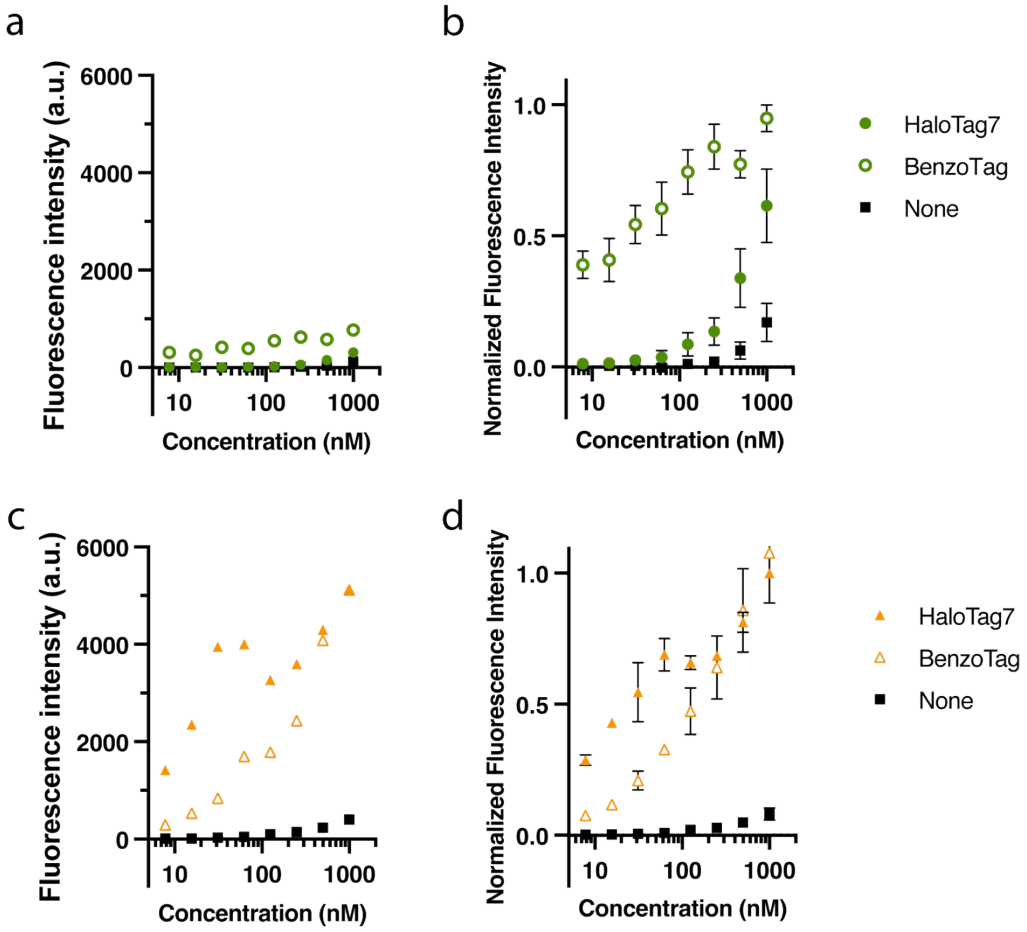
Live cell labeling kinetics – Transfected cells were labeled with Hoechst stain as described above. To each well, 50 µL HBS was added. Shortly after the start of video recording, 50 µL of **Bz-1** in HBS was added to a final concentration of 125 or 10 nM. The appearance green fluorescence of transfected cells was recorded in real-time. Videos were recorded at 6 frames/second for five minutes. Videos were analyzed using ImageJ with the Time Series Analyzer V3 plugin.[6] The fluorescence intensity of at least 10 transfected cells (as determined by the last frame) was measured every 25 frames (~4 seconds) and plotted as a function of time. The average background fluorescence of 10 non-transfected cells was also measured every 25 frames and was subtracted from the fluorescence intensity of the transfected cells. Averages of the ≥10 transfected cells are shown in Fig. 4c in the main text.



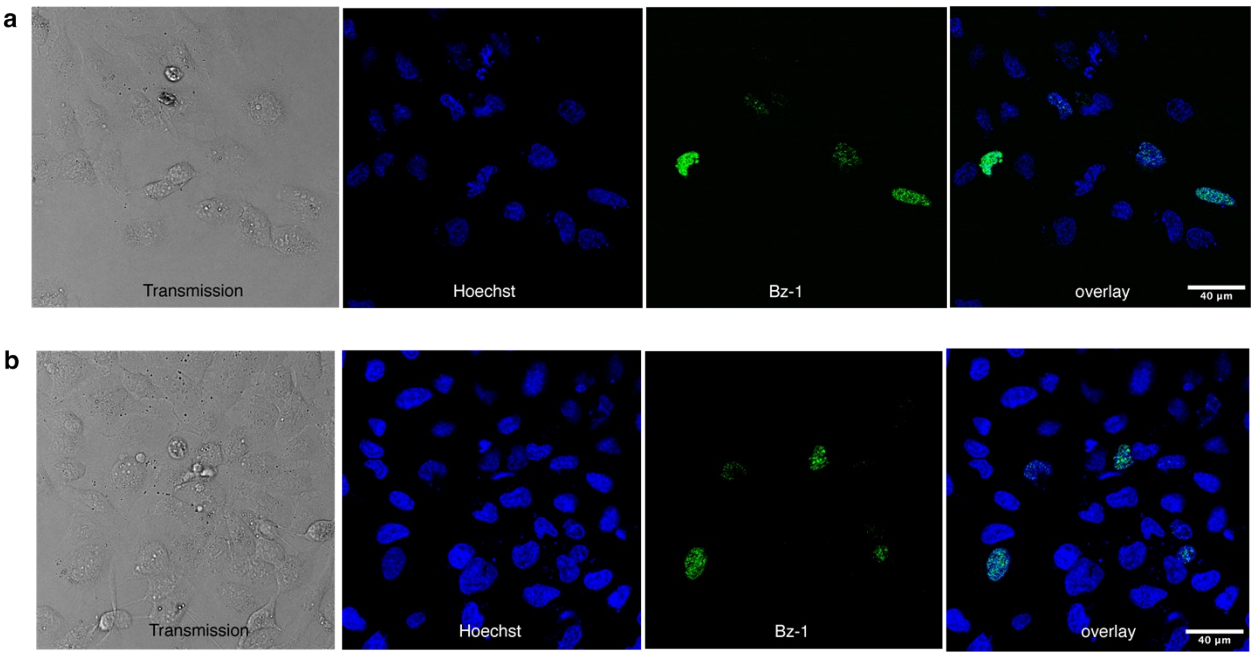
Supplemental Figure 10. Transient transfection efficiencies for live cell experiments. Representative examples of raw flow cytometry data indicating transfection efficiencies. To verify and quantitate transfection, cells were treated with excess (1.0 μM) CA-TMR for 15 minutes, followed by a wash-out step and then analyzed by flow cytometry using the Blue (488 nm) excitation source and Yellow (583 nm) emission filter. The gate was made relative to cells that were no transfected but treated with dye.



Supplemental Figure 11. Flow cytometry data for cells expressing BenzoTag or HaloTag7 after 10 minutes incubation with Bz-1 or CA-JF$_{635}$. (a) Dose-response raw fluorescence intensity of **Bz-1** or **CA-JF$_{635}$** treated U-2 OS cells or U-2 OS cells transiently expressing H2B fusions fo HaloTag7 or BenzoTag. Cells were treated for 10 minutes prior to analysis. (b) Normalized fluorescence intensity. (c) Dose-response of the percentage of **Bz-1** or **CA-JF$_{635}$** labeled U-2 OS cells, or U-2 OS cells expressing HaloTag7 or BenzoTag above background autofluorescence of untreated control cells. All data are shown as the mean and standard error of six biological replicates with 10,000 cells analyzed in each replicate.

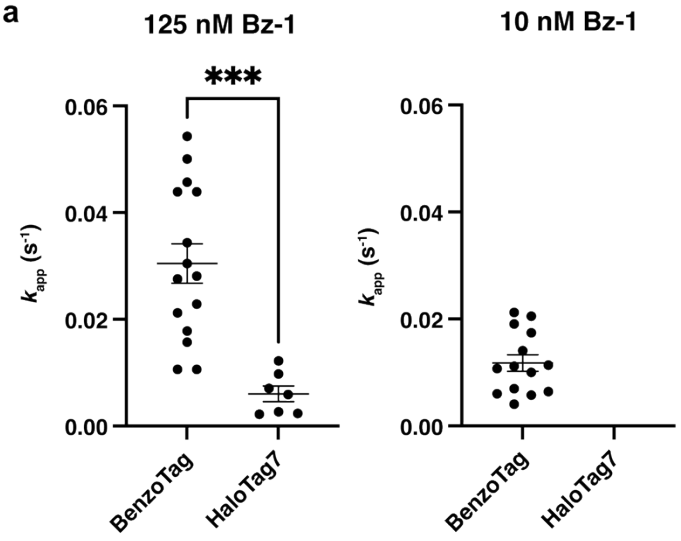


Supplemental Figure S12. Flow cytometry data for cells expressing BenzoTag or HaloTag7 after 60 minutes incubation with Bz-1 or CA-JF$_{635}$. (a,c) Representative dose-response raw fluorescence intensity of **Bz-1** (a) or **CA-JF$_{635}$** (c) treated U-2 OS cells or U-2 OS cells transiently expressing H2B fusions of HaloTag7 or BenzoTag. Cells were treated for 60 minutes prior to analysis. (b,d) Normalized fluorescence intensity. Data are shown as the mean and standard error of three biological replicates with 10,000 cells analyzed in each replicate.



Supplemental Figure 13. No-wash, turn-on fluorescence in BenzoTag-expressing cells. Confocal microscopy images of **Bz-1** with live U-2 OS cells that were transiently transfected with a H2B-BenzoTag fusion, treated with (a) 10 nM of **Bz-1** or (b) 125 nM of **Bz-1** and imaged directly without washing. Blue is Hoechst staining, and green fluorescence is **Bz-1**.

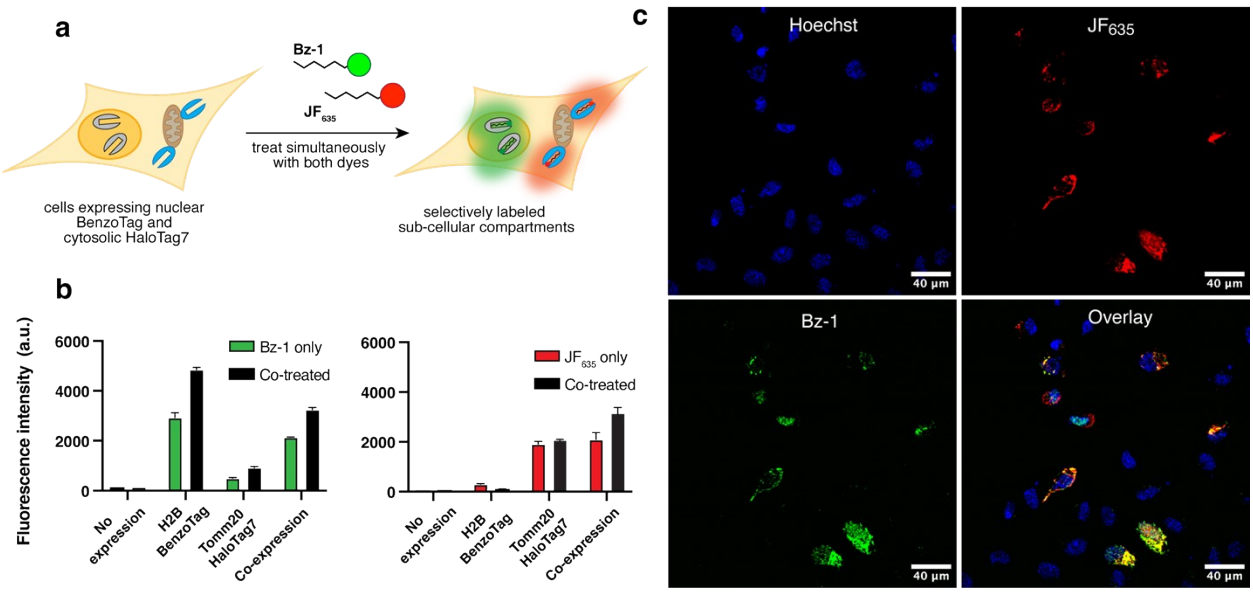
a



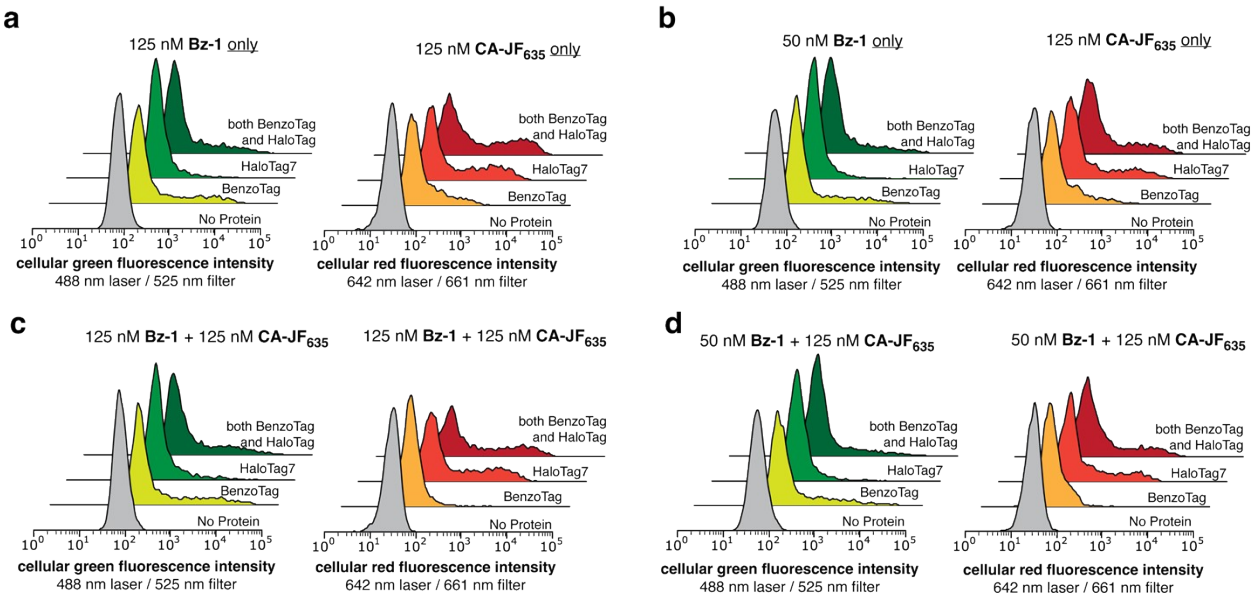
b

	k_{obs} (s^{-1})	
	125 nM	10 nM
BenzoTag	0.03 ± 0.003	0.012 ± 0.001
HaloTag7	0.006 ± 0.001	ND

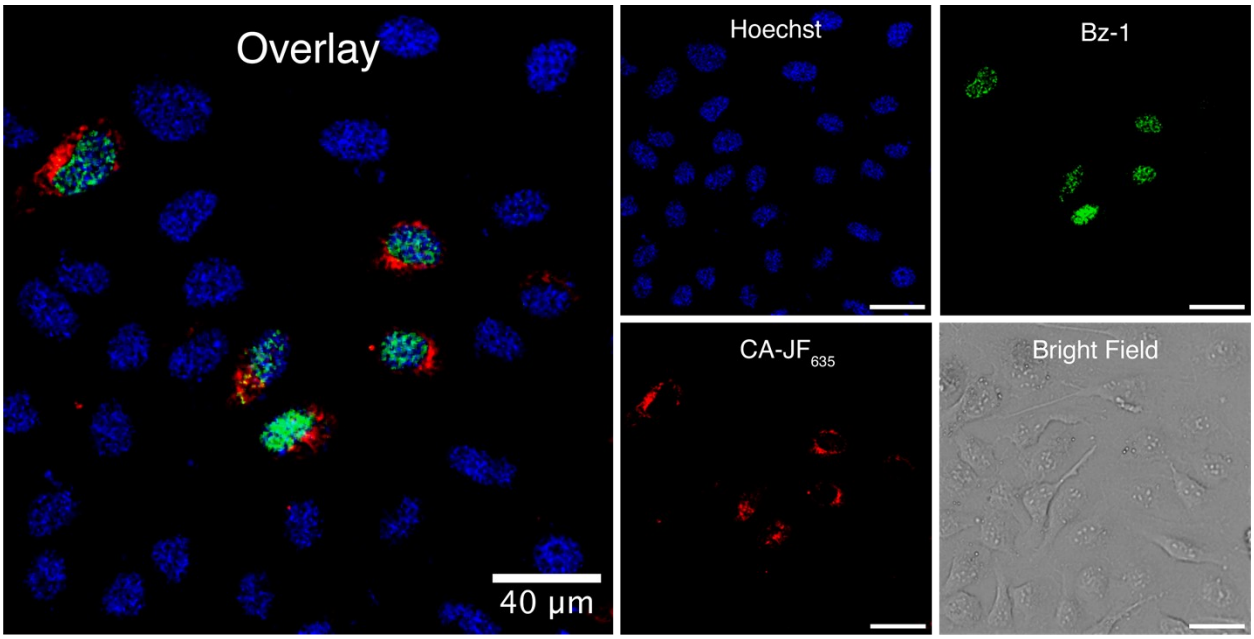
Supplemental Figure 14. Summary of live-cell labeling kinetics of U-2 OS cells transiently transfected with BenzoTag or HaloTag and treated with Bz-1. Rates were calculated by fitting curves to each of the ≥10 transfected cells analyzed. Significance was determined by an unpaired Student's t-test; p=0.0003. (a) Labeling rates for cells treated with 125 nM (left) or 10 nM (right) **Bz-1**. (b) Summary of average k_{obs} values (s^{-1}). Errors are standard error of the mean. ND represents no signal detected sufficient to fit a rate equation.



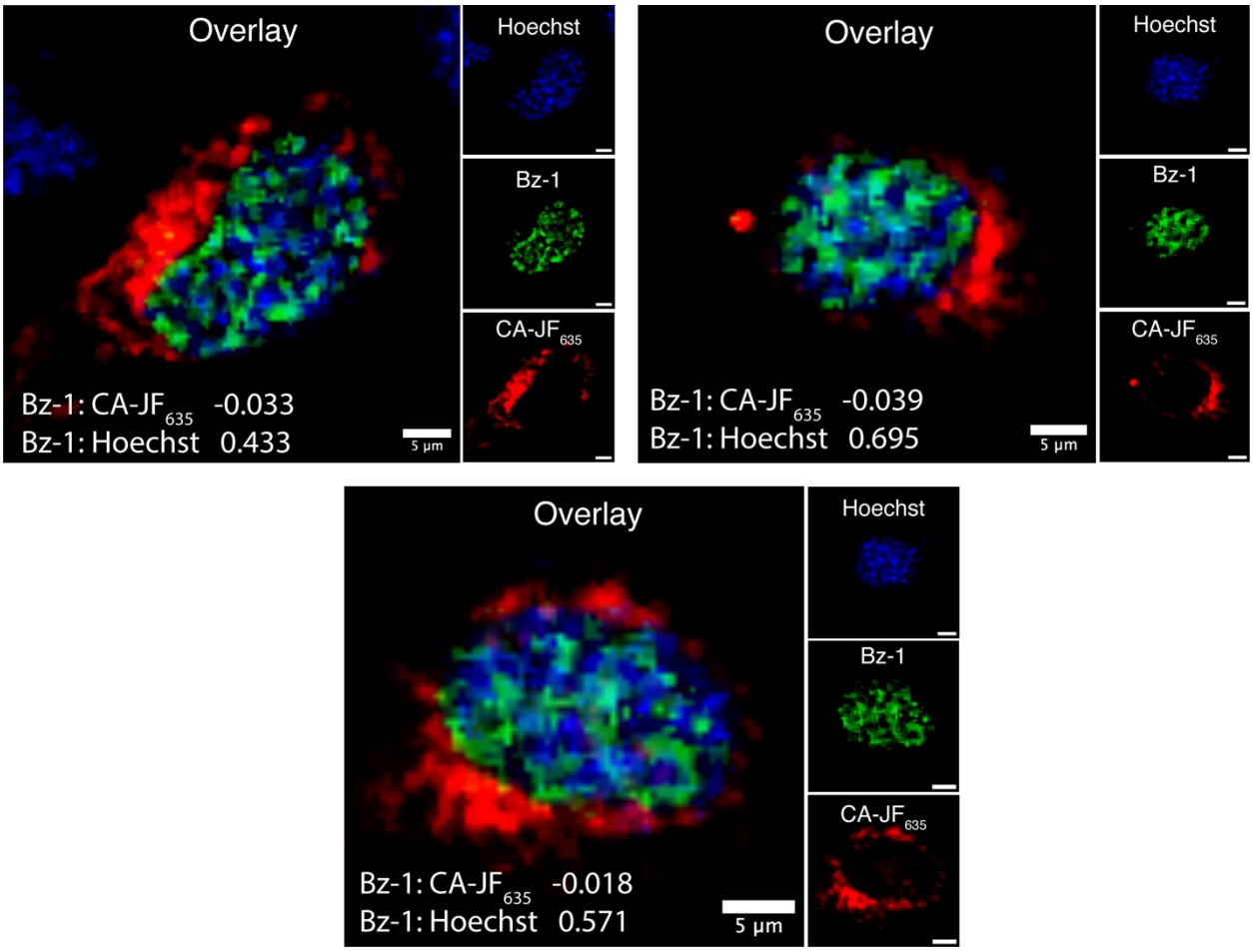
Supplemental Figure 15. Simultaneous multiplexed, no-wash labeling with Bz-1 and CA-JF$_{635}$. (a) U-2 OS cells co-expressing H2B-BenzoTag and Tomm20-HaloTag7 were labeled simultaneously with a solution of 125 nM of each **Bz-1** and **CA-JF$_{635}$** and analyzed without washing out excess dye. (b) Flow cytometry data of the brightest 15% of cells in orthogonal labeling experiments with 125 nM **Bz-1** and 125 nM of **CA- JF$_{635}$** for 10 minutes. Raw flow cytometry histograms can be found in Fig. S14. U-2 OS cells expressing H2B-BenzoTag, Tomm20-HaloTag7, or with side-by-side comparison of cells treated with only dye or a co-treated with both dyes. Data are shown as the mean and standard error of three biological replicates with 10,000 cells analyzed in each replicate. (c) Confocal microscopy images of U-2 OS cells transiently transfected with both H2B-BenzoTag and Tomm20-HaloTag7. Cells were treated with 125 nM **Bz-1** and 125 nM **CA- JF$_{635}$** for 60 minutes and are co-stained with nuclear Hoechst dye.



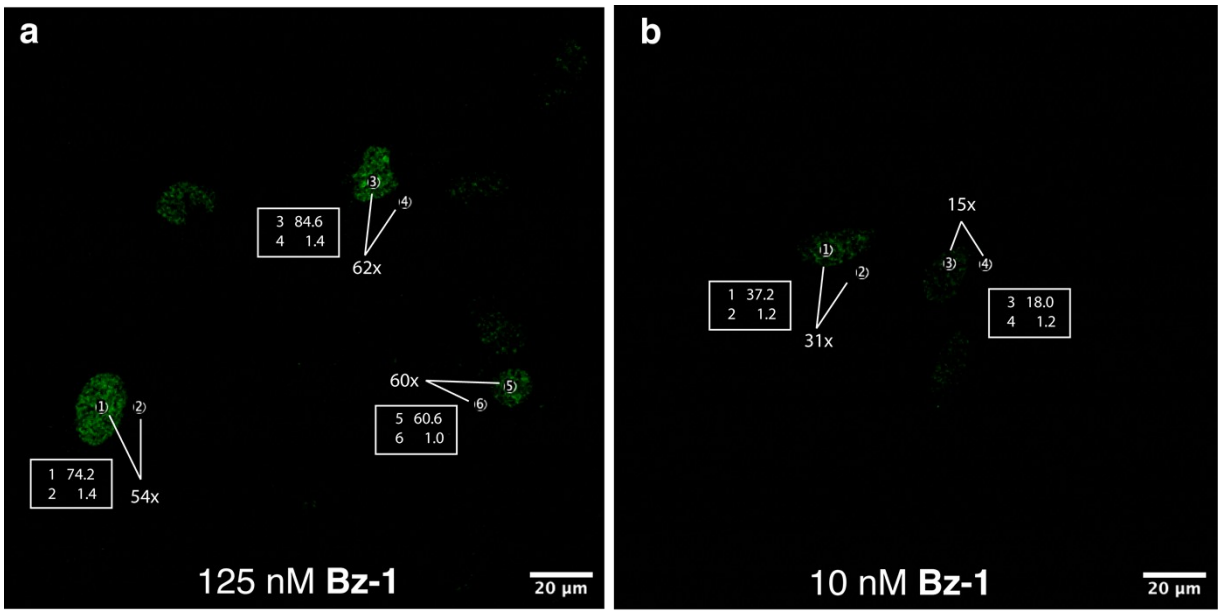
Supplemental Figure 16. Histogram of simultaneous multiplexed labeling of cells co-expressing HaloTag7 and BenzoTag. U-2 OS that were co-transfected with Tomm20-HaloTag7 and H2B-BenzoTag constructs were treated either with a solution of only **Bz-1**, **CA-JF$_{635}$,** or a mixture of both dyes, and analyzed by flow cytometry. (a, c) Cells treated with 125 nM of **Bz-1,** 125 nM of **CA-JF$_{635}$**, or a solution of both dyes for 10 minutes. (b,d) Cells treated with 50 nM of **Bz-1,** 125 nM of **CA-JF$_{635}$**, or a solution both dyes for 60 minutes. The fluorescence intensity of the top 15% of cells were extracted for analysis.



Supplemental Figure 17. Orthogonal labeling between Bz-1•BenzoTag and CA-JF$_{635}$•HaloTag7. U-2 OS that were co-transfected with Tomm20-HaloTag7 and H2B-BenzoTag constructs were treated with a mixture of 50 nM **Bz-1** and 125 nm **CA-JF$_{635}$** and imaged by confocal microscopy. This image is identical to the confocal image shown in Fig. 5 but includes the corresponding brightfield image.



Supplemental Figure 18. Confocal Images of Individual Co-labeled cells. Zoomed-in images of individual cells from Figure 5 and Supplemental Figure 17. Colocalization analysis verifies orthogonal labeling between nuclear H2B-BenzoTag labeled with **Bz-1** and cytosolic Tomm20-HaloTag7 labeled with **CA-JF$_{635}$.** Pearson correlation coefficients were determined using the ImageJ plugin, JACoP.[20]



Supplemental Figure 19. Signal over background of Bz1•BenzoTag in live cell imaging. Signal over background in live U2OS cells expressing BenzoTag as a Tomm20 fusion treated with (a) 125nM or (b) 10 nM of **Bz1** for ~60 minutes and imaged without washing or replacing the media. Mean fluorescence intensity of signal (nuclear fluorescence) over background (cytosolic fluorescence) are indicated. Analysis was conducted using ImageJ on raw image files obtained from a confocal fluorescence microscope.

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