## **Supplementary information**

# The HaloTag as a general scaffold for far-red tunable chemigenetic indicators

In the format provided by the authors and unedited

## **SUPPLEMENTARY INFORMATION**

# The HaloTag as a general scaffold for far-red tunable chemigenetic indicators

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#### SUPPLEMENTARY FIGURES

gagttgtataaataa E L Y K . a – HaloCaMP1a

**Supplementary Figure 1**. DNA and amino acid sequences of HaloCaMP1a (a), HaloCaMP1b (b), HASAP1 (c), and HArcLight1 (d) annotated with sequence features.

	10	20	30	40	50	60	70	80	90	100	110	120
		nuck	ear export signal				6xHis affinity to	ag		peptide -	MLCK	
atget	gcagaacgag	cttgctctta	agttggctgga	acttgatatta	acaagact	ggaggttetea	atcatcaccaco	caccatgga	tecgecegte	gtaaatggca	gaaaacaggcc	atgcg
ML	Q N E	LAL	K L A G	LDI	Ν Κ΄Τ	GGSI	ннн	ННĞ	SAR	R K W Q	КТĞ	H A
_	poptido M		linker					an Hala Taa				
attea	tactatogat		accetaaattt	.ucaucuuau	acettecam	accttocaca	caccgacgto	адесадова	retgateateg	atcagaacgti	tttatcgagg	ataca
V R	A I G	R L S	S P E F	A A E	T F Q	A F R	гтр v	G R K	L I I	D Q N V	F I E	G T
						cpHaloTag						
ctgcc	gatgggtgtc: M G V	gtccgcccgc	tgactgaagto I T F V	gagatggaco F M D	attaccgc	gageegtteet	gaatcetgtt	Jaccgcgag	P I W	gcttcccaaa R F P N	gagetgecaa	tcgcc
		, K L				2 7 1 1		DKL				
						cpHaloTag						
ggtga	gccagcgaac	atcgtcgcgc	tggtcgaagaa	tacatggac	ggctgcac	cagteceetgt	cccgaagetg	ctgttctgg	Iggcaccccag	gcgttctgat	cccaccggccg	aagcc
G E	PAN	I V A	LVEE	Y M D	WLH	QSP	/ P K L	LFW	GTP	GVLI	PPA	ΕA
						coHeloTeg						
actea	rectagecaaa	agectgecta	actocaagoot	atagacato	acceagat	stgaatetget	gcaagaagac	accordac	ctgatcggca	acasastcac	acactaactat	cgacg
A R	L A K	S L P	N C K A	V D I	G P G	L N L I	. Q E D	N P D	L I G	S E I A	R W L	S T
0	cpHaloTag		c	rcular permutation	n linker				cpHa	aloTag		
ctcga	gatttccggc	ggaggaacag	gtggttctggt	ggaacaggg	gtagcgga	ggtacaggag	gaagtatggcg	gagategga	actggattcc	cgtttgatcc	jcattatgtgg	aagtt
		0 0 1	0 0 3 0	0 1 0	0 3 0	0 1 0 0	5 5 M A	2 1 0	107	Frur		L V
						cpHaloTag						
ctggg	agagegeatg	cattatgtgg	acgttggtcct	cgtgatggg	acaccagtg	etgtteettea	acggcaatccg	acategteg	tacgtgtggc	gtaatatcato	eccgcacgttg	ccccc
LG	ERM	ΗΥV	D V G P	R D G	TPV	LFLI	I G N P	T S S	Y V W	RNII	PHV	A P
						cpHalo lag						
T H	R C I	A P D	L I G M	G K S	D K P	D L G Y	/ F F D	D H V	R F M	D A F I	E A L	G L
						cpHaloTag						
gagga	agtagttttg	gtgatccatg	attggggtagt	gctctgggg	tccattgg	gccaagcgtaa	acccagaacgc	gtgaaagga	attgccttta	tggagttcat	cgtccgattc	caaca
EE	V V L	V I Н	D W G S	ALG	FHW	AKRI	N P E R	VKG	IAF	MEFI	RPI	РТ
cpH	HaloTag	linker 2					calmodu	lin				
taaa	cgaatggcga	gaatttgcac	gcgatcaatta	acagaggaa	cagattoco	gagtttaagg	agegttetet	ttatttgat	aaggatggcg	acggtacaat	cactactaaag	aatto
W D	EWR	EFA	R D Q L	TEE	QIA	EFKI	E A F S	LFD	κ́DĞ	DĞTI	ттк	EL
						calmodulin						
ggaac G T	V M R	S L G	aaaatccgaca Q N P T	E A E	L Q D	M I N E	iggtagacgcco E V D A	D G N	G T I	actttccggaa D F P E	F L T	ntgatg M M
						calmodulin						
gcacg	caaaatgaaa	gataccgatt	ctgaagaagag	atccgtgaa	gettteegt	gtttttgataa	agatgggaac	ggetacate	agtgetgetg	agttacgccat	gtgatgacaa	atctg
АК	кмк		SEEE	IRE	AFR	VFDI	C D G N	GYI	SAA	ELRH	VMI	NL
					calmo	dulin					linke	r
qqqqa	aaaacttacc	gacgaagaag	tagacgaaato	attcgcgag	geggatatt	gacgggggatg	acaagtaaac	tacgaggaa	itttgtgcaga	tgatgaccgc	caagetegaga	tttcc
ĞΕ	KLT	DEE	V D E M	IRE	A D I	DGD	G Q V N	Y E E	FVQ	м м т а	K L E	I S
	linker						EGFP					
ggcgg G G	Cggaagegge G S G	M V S	aggggggaggaa K G E E	L F T	grgrgrggrg G V V	P I L \	agagetegate	G D V	N G H	AATTTCCTGTC K F S V	S G E	gtgag G E
						EGFP						
ggaga	tgcaacttat	ggaaaactga	cattgaaatto	atctgcaca	acaggcaaa	ttgcctgtcco	ctggcctacc	ctggtaaco	acceteactt	atggcgttca	gtgetteteec	ggtat
GD	AIY	GKL	ILKF	ICI	IGK	LPV	, w p i	LVI	1 L 1	YGVQ	CFS	RY
						EGFP						
cctga	tcacatgaaa	cagcatgatt	tetteaaatea	gcaatgccc	aaggttat	attcaagage	gaccatetti	ttcaaggac	gatggaaact	ataaaaccag	agetgaggtta	agtte
PD	н м к	Q H D	FFKS	A M P	EGY	VQEI	ξ T I F	FKD	DGN	YKTŔ	A E V	КF
						EGFP						
gaggg	agatactttg	gtgaatcgga v N R	tcgaactgaag	gggatagaci	E K F	gatgggaacat	tttggggcata	aaactcgaa KIF	itataactaca	actcccacaa N S H N	gtgtatatca	tggct M A
_ 0			L K	5								
						EGFP						
gacaa	acagaagaac	gggatcaaag	taaattttaag	gatacgacaca	aatatagag	gacggtagtg	ccaacttgct	gatcactac	cagcagaata	cacccatcgga	agacggacccg	ttctc
D K	Q K N	GIK	VNFK	IRH	NIE	DGS	Q L A	DHY	QQN	TPIG	DGP	V L
						FOFP						
ttacc	coataateac	tatetetete	cacaateeget	ctatctasa	atocaaao	raaaagggggg	accacatanta	atacttasa	ttoattacaa	coactaat >+	cacottogoca	taa>+
L P	D N H	Y L S	T Q S A	L S K	D P N	E K R I	) H M V	L L E	F V T	A A G I	T L G	M D

#### b – HaloCaMP1b

gagttgtataaataa

20 120 10 6xHis affinity tag atgetgeagaacgagettgetettaagttggetggaettgatattaacaagaetggaggtteteateateaceaceaceatggagtgegggttatteeeagaettgataeeetgataete M L Q N E L A L K L A G L D I N K T G G S H H H H H H G V R V I P R L D T L I L linker1 cpHaloTag gtgaaagcaatgggccaccgaaaacgattcggtaacccctttaggcctaaggagaccttccaggccttccgcaccaccgacgtcggccgcaagctgatcatcgatcagaacgtttttatc ccaatcgccggtgagccagcgaacatcgtcgcgctggtcgaagaatacatggactggctgcaccagtcccctgtcccgaagctgctgttctggggcaccccaggcgttctgatcccaccg cpHaloTag gccgaagccgctcgcctggccaaaagcctgcctaactgcaaggctgtggacatcggcccgggtctgaatctgctgcaagaagacaacccggacctgatcggcagcgagatcgcgcgctgg A E A A R L A K S L P N C K A V D I G P G L N L L Q E D N P D L I G S E I A R W cpHaloTag circular permutation linker cpHaloTag ctgtcgacgctcgagatttccggcggaggaacaggtggttctggtggaacagggggtagcggaggtacaggaggaagtatggcggagatcggaactggattcccgtttgatccgcattat L S T L E I S G G G T G G S G G T G G S G G T G G S M A E I G T G F P F D P H Y cpHaloTag gtggaagttetgggagagegeatgeattatgtggaegttggteetegtgatgggaeaecagtgetgtteetteaeggeaateegaeategtegtagtggegtaatateateeegeae V E V L G E R M H Y V D V G P R D G T P V L F L H G N P T S S Y V W R N I I P H cpHaloTag gttgcccccaagcaccgctgcattgcccctgacttaattggtatggggaaaagtgataagcctgatctgggggtacttctttgacgaccacgtacgcttcatggatgcttttattgaagca V A P K H R C I A P D L I G M G K S D K P D L G Y F F D D H V R F M D A F I E A cpHaloTag cpHaloTag linker2 -atgatgggcacgcaaaatgaaagataccgattctgaagaagagatccgtgaagctttccgtgtttttgataaagatgggaacggctacatcagtgctgctgagttacgccatgtgatgaca M M A R K M K D T D S E E E I R E A F R V F D K D G N G Y I S A A E L R H V M T linker aatctgggggaaaaacttaccgacgaagaagtagacgaaatgattcgcgaggcggatattgacggggatggacaagtaaactacgaggaatttgtgcagatgatgaccgccaagatttcc N L G E K L T D E E V D E M I R E A D I D G D G Q V N Y E E F V Q M M T A K I S ggcggcggaagcggcatggtctctaagggggaggaactctttaccggtgtggtgccaatacttgtagagctcgatggggacgttaatggacacaaattttctgtctcaggtgagggtgag ggagatgcaacttatggaaaactgacattgaaattcatctgcacaacaggcaaattgcctgtcccctggcctaccctggtaaccaccctcacttatggcgttcagtgcttctcccggtat . cctgatcacatgaaacagcatgattcttcaaatcagcaatgcccgaaggttatgttcaagagcggaccatctttttcaaggacgatggaaactataaaaccagagctgaggttaagttc P D H M K Q H D F F K S A M P E G Y V Q E R T I F F K D D G N Y K T R A E V K F gagggagatactttggtgaatcggatcgaactgaaggggatagacttcaaagaggatgggaacattttgggggcataaactcgaatataactacaactccccacaacgtgtatatcatggct E G D T L V N R I E L K G I D F K E D G N I L G H K L E Y N Y N S H N V Y I M A -gacaaacagaagaacgggatcaaagtaaattttaagatacgaccaatatagaggacggtagtgtccaacttgctgatcactaccagcagaatacacccatcggagacggaccgttctc D K Q K N G I K V N F K I R H N I E D G S V Q L A D H Y Q Q N T P I G D G P V L t tgcccgataatcactatctctctacaaatccgctctgtctaaagatccaaacgaaaagcgggaccacatggtactgcttgagttcgttacagccgctggtatcaccttgggcatggat L P D N H Y L S T Q S A L S K D P N E K R D H M V L L E F V T A A G I T L G M D

<b>c – HASAP1</b> . At amino acid position 467, HASAP0.1=R, HASAP1=G.	

10	20	30	40	50	60	70	80	90	100	110 12	D
					GgVSD S1-3						
atggagacgactgtga M E T T V	ggtatgaaca R Y E Q	ggggtcagag G S E	ctcactaaa L T K	acttcgagct T S S	ctccaacagca S P T A	agatgagccc D E P	acgataaaga TIK	ittgatgatgo I D D 0	rtcgtgatgag R D E	ggtaatgaacaagac G N E Q D	120
					GgVSD S1-3						
agctgttccaatacca S C S N T	ttaggagaaa I R R K	aatttccccg ISP	tttgtgatg F V M	tcatttggat S F G	tcagagtatt F R V F	tggagttgtg G V V	cttatcatto L I I	tagacatcat V D I I	agtggtgatt VVI	gtggatctggccatc V D L A I	240
					GgVSD S1-3						
agtgagaagaaaagag S E K K R	gcattagaga G I R E	gattettgaa I L E	ggtgtttcc G V S	ctggctatag L A I	geactettette A L F F	ccttgttgat L V D	gtteteatga V L M	lgagtgtttgt RVFV	tgaaggette / E G F	aagaactatttccgg K N Y F R	360
			GgVSD S1-3	3					cpHaloTag		
tccaaactgaatactt S K L N T	tggatgcagt L D A V	catagtagtg I V V	ggcactctg G T L	ctaattaata L I N	Atgacctactco M T Y S	F S D	CttgCtgCCt L A A	F A R E	igacettecag TFQ	geetteegeaceace A F R T T	480
					cpHaloTag						
gacgtcggccgcaagc D V G R K	tgatcatcga L I I D	Cagaacgtt Q N V	tttatcgag F I E	ggtacgctgc G T L	cgatgggtgto PMGV	v R P	ctgactgaag L T E	rtcgagatgga V E M E	CCATTACCGC HYR	gagccgttcctgaat E P F L N	600
					cpHaloTag						
cctgttgaccgcgagc P V D R E	cactgtggcg P L W R	cttcccaaac F P N	gagetgeea E L P	ategeeggtg I A G	jagccagcgaad E P A N	catcgtcgcg IVA	ctggtcgaag L V E	jaatacatgga E Y M D	etggetgeae WLH	cagtcccctgtcccg Q S P V P	720
					cpHaloTag						
aagetgetgttetggg K L L F W	gcaccccagg G T P G	cgttctgatc V L I	CCACCGGCC PPA	gaagccgctc E A A	cgcctggccaaa R L A K	aagcetgeet S L P	aactgcaago N C K	ictgtggacat A V D I	G P G	ctgaatctgctgcaa LNLLQ	840
			cpHaloTag					circu	ar permutation link	ər	
gaagacaacccggacc E D N P D	tgatcggcag L I G S	cgagatcgcg E I A	cgctggctg R W L	tcgacgctcg S T L	gagatttccggd E I S G	cgagccaacc E P T	actggaggca T G G	igeggaggead S G G 1	caggaggcagc G G S	ggaggcacaggaggc G G T G G	960
cir					cpHaloTag						
agcatggcagaaatcg S M A E I	gtactggctt G T G F	P F D	CCCCATTAT	gtggaagtco V E V	ctgggcgagcgo L G E R	catgcactac M H Y	gtcgatgttg V D V	gtccgcgcga G P R D	tggcacccct G T P	gtgctgttcctgcac V L F L H	1080
					cpHaloTag						
ggtaacccgacctcct G N P T S	cctacgtgtg S Y V W	gegeaacate R N I	atcccgcat I P H	gttgcaccga V A P	acccatcgctgo T H R C	I A P	gacctgatco D L I	gtatgggcaa G M G M	atccgacaaa ( S D K	ccagacctgggttat PDLGY	1200
					cpHaloTag						
ttettegaegaeeaeg F F D D H	tccgcttcat V R F M	ggatgccttc D A F	atcgaagcc I E A	ctgggtctgg L G L	gaagaggtcgto E E V V	CCTGGTCATT L V I	cacgactggg H D W	geteegetet G S A L	gggtttccac . G F H	tgggccaagcgcaat W A K R N	1320
			cpHaloTag						GgVSD S4		
ccagagcgcgtcaaag P E R V K	gtattgcatt G I A F	tatggagttc M E F	atccgccct	atcccgacct I P T	gggacgaatg W D E W	gccagaattt P E F	R467G gccgggacag A G T	atcagatgco D Q M F	ctcaaatggtg Q M V	acacttttgcgagtt T L L R V	1440
		GgVS	SD S4								
ctgcgaatagtgatcc L R I V I	tgattcgaat L I R I	ctttcgcctt F R L	gccagccag A S Q	аадааасаас ККО	tggaggtagt L E V V	aacataa T.					1512

С

gagategegegetggetggetggegetggegetggegetggetggetggetggetggetggetggetggetggetggetggegetggegetggegetggegegetg

	10	20	30	40	50	60	70	80	90	100	110	120
						CiVSD						
atgga M E	gggattcgac G F D	ggttcagatt G S D	ttagtcctcc F S P P	agctgattta A D L	gttggcgttg V G V	gcggtgcagt G G A \	catgcggaac M R N	gtcgttgacg V V D	tcacgataaa V T I N	tggtgacgtc G D V	actgctccgc T A P	cgaaa P K
						CiVSD						
gcagc A A	gccaagaaaa P R K	agtgaatcgg S E S	taaagaaagt v к к v	tcattggaat H W N	gatgtagacc D V D	aaggaccgag Q G P S	tgaaaaacca E K P	igagacaagac E T R	aggaggaacg Q E E R	aatagatata I D I	cccgagattt P E I	caggt s G
						CiVSD						
ctatg L W	gtggggcgag W G E	aatgaacatg N E H	gagtgggcgg G V G G	tgggagaatg G R M	gagataccta E I P	ictactggtgt T T G \	aggtcgcgtc G R V	cagtttcgtg Q F R	tccgagcagt V R A V	gattgatcat I D H	ctagggatgc L G M	gagcc R A
						CiVSD						
tttgg F G	agtetteeta V F L	attctcttgg I L L	acatcatcct D I I L	Catgatcatto M I I	gateteagte DLS	ttccaggaaa LPGP	aagtgaatct S E S	tcacaatcct SQS	tttatgacgg F Y D G	gttggctttg L A L	gctctttctt A L S	gttat C Y
						CiVSD						
F M	gctggattta L D L	ggattaagga G L R	tatttgccta I F A Y	CGGGCCCAAG G P K	aatttettea NFF	T N P V	ggaggttgct 'EVA	.gatggcttga D G L	ttatcgttgt I I V V	cacattcgtc T F V	gtcacgatat V T I	tttac F Y
						CiVSD						H
actgto T V	gttagatgaa L D E	tactttcaag Y F Q	aaacaggagc E T G A	cgatggtttg D G L	gggcagttgg G Q L	yttgtgttggd VVL4	CCGTTTGCTG R L L	R V V	gattagcaag R L A R	aatatttat IFY	tcccatcaac S H Q	aaatc Q I
						HaloTag						
ggtac G T	tggctttcca G F P	ttegaceece F D P	attatgtgga H Y V E	agteetggge V L G	gagegeatge E R M	actacgtcga H Y V [	tgttggtccg V G P	gegegatggea R D G	cccctgtgct T P V L	gttcctgcac F L H	ggtaacccga G N P	cctcc T S
						HaloTag						
tccta S Y	cgtgtggcgc V W R	aacatcatcc NII	cgcatgttgc P H V A	accgacccat P T H	cgctgcattg R C I	JCTCCAGACCT A P D L	gatcggtatg I G M	jggcaaatccg G K S	acaaaccaga D K P D	cctgggttat L G Y	ttettegaeg F F D	accac D H
						HaloTag						
gtccg V R	cttcatggat F M D	gccttcatcg A F I	aagccctggg E A L G	L E E	gtegteetgg VVL	ytcattcacga V I H [	ctggggctcc W G S	gctctgggtt A L G	tccactgggc F H W A	caagcgcaat K R N	ccagagcgcg P E R	tcaaa V К
						HaloTag						
ggtat G I	tgcatttatg A F M	gagttcatcc E F I	gecetatece R P I P	gacctgggac T W D	gaatggccag E W P	gaatttgccco E F A F	cgagacette E T F	Caggeettee Q A F	gcaccaccga R T T D	cgtcggccgc V G R	aagctgatca K L I	tcgat I D
						HaloTag						
cagaa Q N	CGTTTTTTTT V F I	gagggtacgc E G T	tgccgatggg L P M G	tgtcgtccgc V V R	ccgctgactg P L T	jaagtcgagat EVEM	ggaccattac D H Y	cgcgagccgt R E P	teetgaatee F L N P	tgttgaccgc V D R	gagccactgt E P L	ggcgc W R
						HaloTag						
F P	aaacgagctg N E L	ccaatcgccg PIA	gtgagccagc G E P A	gaacatcgtc N I V	gegetggteg A L V	jaagaatacat E E Y M	ggactggctg DWL	JCACCAGTCCC H Q S	ctgtcccgaa PVPK	gctgctgttc L L F	tggggcaccc W G T	caggc P G
						HaloTag						
gttct V L	gateccaceg I P P	gccgaagccg A E A	ctcgcctggc A R L A	caaaagcctg K S L	cctaactgca PNC	aggctgtgga K A V [	catcggcccg I G P	ggtctgaatc G L N	tgctgcaaga L L Q E	agacaacccg D N P	gacctgatcg D L I	gcagc G S
		ł	laloTag									



a<sub>0</sub> was constrained to 1.0.

**Supplementary Figure 2.** Kinetics of calcium unbinding from HaloCaMP1a or HaloCaMP1b bound to JF<sub>635</sub>-HTL. A stopped flow instrument was used follow the decrease in fluorescence emission from recombinant calcium-saturated HaloCaMP<sub>635</sub> following rapid mixing with excess calcium chelator (EGTA, 10 mM). HaloCaMP1a was fit to a two-phase exponential model and HaloCaMP1b was fit to a one-phase exponential model. Mean and s.d. for 27 trials over 3 independent days, normalized to the initial fluorescence intensity at time 0.



**Supplementary Figure 3.** Fluorescence response of HASAP1<sub>635</sub> in response to a 100 mV potential step. Insets: Zoom in on fluorescence response to membrane depolarization (from -70 mV to +30 mV), and repolarization (from +30 mV to -70 mV). Solid black line is fit of rise and decay kinetics to a double exponential function. Image acquisition rate 1200 Hz. See Table S4 for full kinetic data.



**Supplementary Figure 4.** Fluorescence response of HArclight1<sub>635</sub> in response to a 100 mV potential step. Insets: Zoom in on fluorescence response to membrane depolarization (from -70 mV to +30 mV), and repolarization (from +30 mV to -70 mV). Solid black line is fit of rise and decay kinetics to a double exponential function. Image acquisition rate 1200 Hz. See Table S4 for full kinetic data.

## SUPPLEMENTARY TABLES

	HaloTag-TMR	Ca <sup>2+</sup> -HaloCaMP1b-
Data collection	(FDB 0032)	
Space group	P42212	P2
Cell dimensions		1 2
a h c (Å)	62 53 62 53 164 17	92 56 60 66 122 60
$\alpha, \beta, \gamma(\circ)$	90 90 90	90 91 0 90
Resolution (Å)	62,53 = 1,80	9254 - 200
	$(1.90 - 1.80)^*$	(2 11 - 2 00)
Rev.m (%)	10.3 (54.5)	9.6 (69.6)
	11 2 (2 4)	65(11)
Completeness (%)	97.0 (97.6)	98.6 (97.6)
Redundancy	5 8 (5 9)	4 6 (4 4)
Rodandanoy	0.0 (0.0)	
Refinement		
Resolution (Å)	58.43 – 1.80	122.58 – 2.00
No. reflections	30,022	90,798
R <sub>work</sub> / R <sub>free</sub>	15.7/19.3	18.8/22.6
No. atoms		
Protein	2350	7420
Dye-HaloTag ligand	76	100
Chloride ions	1	2
Calcium ions	-	8
Water	177	203
<i>B</i> -factors		
Protein	28.8	53.1
Dye-HaloTag ligand	37.9	87.9
Chloride ions	20.8	38.9
Calcium ions	-	54.0
Water	36.4	47.8
R.m.s. deviations		
Bond lengths (Å)	0.030	0.026
Bond angles (°)	2.54	2.44

**Supplementary Table 1.** X-ray diffraction data collection and model refinement statistics. \*Values in parentheses are for highest-resolution shell. One crystal was used for each structure.

HaloCaMP variant	Peptide	L1	L2	€ <sub>sat</sub> (M⁻¹.cm⁻¹)	$\Phi_{sat}$	Brightness (mM <sup>-1</sup> .cm <sup>-1</sup> )	∆F/F₀	K₀ (nM)
1a	MLCK	PEFAA	REFAR	96,000	0.78	74.9	5.0	190
1b	СКК	PK	PFAR	60,000	0.75	45.0	9.2	43

**Supplementary Table 2**. Properties of HaloCaMP variants 1a and 1b labeled with JF<sub>635</sub>-HaloTag ligand.

Dye	λ <sub>ex</sub> (nm)	λ <sub>em</sub> (nm)	ε (M <sup>-1</sup> .cm <sup>-1</sup> )	Φ
<b>JF</b> 635	635	652	~400	0.56
<b>JF</b> 646	646	664	5000	0.54
<b>JF</b> 639	639	656	5000	0.62
<b>JF</b> 630	630	649	~700	NM
<b>JF</b> 629	629	648	<200	NM
<b>JF</b> 626	626	638	<200	NM
<b>JF</b> 614	614	631	<200	NM

**Supplementary Table 3.** Photophysical properties of azetidine-substituted Si-rhodamines in 10 mM HEPES, pH = 7.4. NM: not measured.

Ligand		λ <sub>ex</sub> (nm)	λ <sub>em</sub> (nm)	ε (M <sup>-1</sup> .cm <sup>-1</sup> )	Φ
1 / IE. HoloTog ligond \9	– HaloTag	635	652	~400	NM
I (JF635-Halo ray ligariu)	+ HaloTag	640	656	81000	0.75
<b>5</b> (IFace HoleTeg ligend) <sup>9</sup>	– HaloTag	649	666	6000	0.52
<b>5</b> (JF646-Halo ray ligarid)*	+ HaloTag	652	666	95000	0.64
6 (IE., HoloTog ligond)	– HaloTag	645	658	5300	0.63
<b>6</b> (JF639-Halo I ag ligand)	+ HaloTag	647	663	120000	0.71
7 (IEase HoloTog ligond)	– HaloTag	633	657	1200	NM
(JF630-Halo Tag liganu)	+ HaloTag	639	656	32000	0.70
9 (IE., HoloTog ligond)	– HaloTag	638	655	<200	NM
<b>8</b> (JF629-Hai0Tag ligand)	+ HaloTag	638	656	29000	0.81
<b>0</b> (IE HoloTog ligond)	– HaloTag	634	647	<200	NM
<b>9</b> (JF626-Hai0Tag ligand)	+ HaloTag	639	654	57000	0.73
10 (IE., HeleTeg ligand)	- HaloTag	622	640	<200	NM
i (JF614-Haio l'ag ligand)	+ HaloTag	628	646	7000	0.74

**Supplementary Table 4.** Photophysical properties of Si-rhodamines HaloTag ligands in the presence or absence of HaloTag protein in 10 mM HEPES pH = 7.4 containing 0.1 mg·mL<sup>-1</sup> CHAPS. NM: not measured.

-	HaloC	aMP1a	HaloC	aMP1b
Ligand	∆F/F₀	K <sub>d</sub> (nM)	∆F/F₀	K <sub>d</sub> (nM)
<b>1</b> (JF <sub>635</sub> -HaloTag ligand) <sup>9</sup>	5.0	190	9.2	43
5 (JF <sub>646</sub> -HaloTag ligand) <sup>9</sup>	0.6	65	0.9	19
<b>6</b> (JF <sub>639</sub> -HaloTag ligand)	1.1	128	2.3	32
7 (JF <sub>630</sub> -HaloTag ligand)	7.8	340	8.7	67
<b>8</b> (JF <sub>629</sub> -HaloTag ligand)	13.8	118	20.9	44
<b>9</b> (JF <sub>626</sub> -HaloTag ligand)	11.8	391	7.4	42
<b>10</b> (JF <sub>614</sub> -HaloTag ligand)	29.5	892	10.8	61

**Supplementary Table 5.** Ca<sup>2+</sup> binding properties of HaloCaMP1a and 1b bound to Si-rhodamine ligands measured in solution.

	Activation (-70 mV to 30 mV	) )		Deactivation (30 mV to -70 mV)				
	τ <sub>fast</sub> (ms)	$ au_{slow}$ (ms)	% fast	$ au_{fast}\left(ms\right)$	$ au_{slow}$ (ms)	% fast		
HASAP1- JF <sub>635</sub>	2.1 ± 0.2	5.2 ± 0.6	96 ± 3	1.1 ± 0.1	3.7 ± 0.3	50 ± 8		
HArclight1- JF <sub>635</sub>	2.2 ± 0.2	8.5 ± 0.3	54 ± 5	1.6 ± 0.2	8.1 ± 0.6	37 ± 3		

**Supplementary Table 6**. HASAP1 and HArclight1 kinetics in primary rat neuron cultures. Neurons expressing HASAP1 and Harclight1 were imaged at 1 kHz during whole cell voltage clamp. Fluorescence traces were fit using a double exponential function (Supplementary Figs. 12,14). % fast is the percentage of fluorescence change attributed to the fast-changing component of the bi-exponential fit to the fluorescence change. The remainder is attributed to the slow-changing component. Errors are s.e.m. N = 8 cells for HASAP1 and N = 6 cells for HArclight1.

#### SUPPLEMENTARY NOTE

#### SYNTHETIC PROCEDURES

**Procedure A:** Synthesis of Si-rhodamines by Pd-catalyzed cross-coupling. The following procedure for (**12**; **JF**<sub>639</sub>) is representative. A vial was charged with silafluorescein ditriflate **11**<sup>10</sup> (50 mg, 78 µmol), 3-methoxyazetidine hydrochloride (39 mg, 312 µmol, 4 eq), Pd<sub>2</sub>dba<sub>3</sub> (7.1 mg, 7.8 µmol, 0.1 eq), XPhos (11.2 mg, 23.4 µmol, 0.3 eq), and Cs<sub>2</sub>CO<sub>3</sub> (204 mg, 625 mmol, 8 eq). The vial was sealed and evacuated/backfilled with nitrogen (3x). Dioxane (2 mL) was added, and the reaction was flushed again with nitrogen (3x). The reaction was then stirred at 100 °C overnight. It was subsequently cooled to room temperature, diluted with MeOH, deposited onto Celite, and concentrated to dryness. The residue was purified as described.



(12; JF<sub>639</sub>): Purification by silica gel chromatography (0–35% EtOAc/toluene, linear gradient) afforded 12 (78%) as a light blue solid. <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400 MHz)  $\delta$  7.96 (d, *J* = 7.6 Hz, 1H), 7.64 (td, *J* = 7.5, 1.2 Hz, 1H), 7.54 (td, *J* = 7.5, 1.0 Hz, 1H), 7.32 – 7.27 (m, 1H), 6.77 (d, *J* = 8.7 Hz, 2H), 6.69 (d, *J* = 2.7 Hz, 2H), 6.28 (dd, *J* = 8.7, 2.7 Hz, 2H), 4.38 – 4.27 (m, 2H), 4.10 (d, *J* = 7.3 Hz, 4H), 3.73 (dt, *J* = 7.7, 4.0 Hz, 4H), 3.32 (s, 6H), 0.61 (s, 3H), 0.58 (s, 3H); <sup>13</sup>C NMR (CDCl<sub>3</sub>, 101 MHz)  $\delta$  170.7 (C), 154.3 (C), 150.4 (C), 137.1 (C), 133.8 (CH), 133.3 (C), 128.9 (CH), 128.1 (CH), 127.0 (C), 125.9 (CH), 124.7 (CH), 116.1 (CH), 112.7 (CH), 91.9 (C), 70.1 (CH<sub>3</sub>), 58.9 (CH<sub>2</sub>), 56.2 (CH), 0.5 (CH<sub>3</sub>), -1.4 (CH<sub>3</sub>); HRMS (ESI) calcd for C<sub>30</sub>H<sub>33</sub>N<sub>2</sub>O<sub>4</sub>Si [M+H]<sup>+</sup> 513.2210, found 513.2202.



(13; JF<sub>630</sub>): Synthesized following procedure A from silafluorescein ditriflate and 3-methylsulfonyl-azetidine hydrochloride. Purification by silica gel chromatography (50–100% EtOAc/hexane, linear gradient) afforded 13 (80%) as a light blue solid. <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400 MHz)  $\delta$  7.96 (dt, *J* = 7.6, 1.0 Hz, 1H), 7.65 (td, *J* = 7.5, 1.2 Hz, 1H), 7.55 (td, *J* = 7.5, 1.0 Hz, 1H), 7.28 – 7.25 (m, 1H), 6.83 (d, *J* = 8.7 Hz, 2H), 6.70 (d, *J* = 2.7 Hz, 2H), 6.31 (dd, *J* = 8.7, 2.7 Hz, 2H), 4.29 – 4.15 (m, 8H), 4.07 (tt, *J* = 7.5, 5.7 Hz, 2H), 2.96 (s, 6H), 0.62 (s, 3H), 0.59 (s, 3H); <sup>13</sup>C NMR (CDCl<sub>3</sub>, 101 MHz)  $\delta$  170.6 (C), 154.2 (C), 149.2 (C), 137.1 (C), 134.8 (C), 134.1 (CH), 129.1 (CH), 128.2 (CH), 126.6 (C), 126.0 (CH), 124.6 (CH), 116.2 (CH), 113.0 (CH), 91.2 (C), 52.5 (CH<sub>2</sub>), 52.4 (CH<sub>2</sub>), 51.7 (CH), 38.3 (CH<sub>3</sub>), 0.4 (CH<sub>3</sub>), -1.3 (CH<sub>3</sub>); HRMS (ESI) calcd for C<sub>30</sub>H<sub>33</sub>N<sub>2</sub>O<sub>6</sub>SiS<sub>2</sub> [M+H]<sup>+</sup> 609.1549, found 609.1548.



(14; JF<sub>629</sub>): Synthesized following procedure A from silafluorescein ditriflate and 3-azetidinecarbonitrile hydrochloride. Purification by HPLC (35-95% MeCN/H<sub>2</sub>O + 0.1% TFA additive) afforded 14 (42%) as a light blue solid. <sup>1</sup>H NMR (CD<sub>2</sub>Cl<sub>2</sub>, 400 MHz)  $\delta$  7.93 (dt, *J* = 7.6, 1.0 Hz, 1H), 7.66 (td, *J* = 7.5, 1.2 Hz, 1H), 7.57 (td, *J* = 7.5, 1.0 Hz, 1H), 7.24 (dd, *J* = 7.7, 1.0 Hz, 1H), 6.84 (d, *J* = 8.7 Hz, 2H), 6.72 (d, *J* = 2.7 Hz, 2H), 6.33 (dd, *J* = 8.7, 2.7 Hz, 2H), 4.20 (ddd, *J* = 8.6, 7.1, 1.8 Hz, 4H), 4.13 – 4.03 (m, 4H), 3.60 (tt, *J* = 8.4, 6.1 Hz, 2H), 0.63 (s, 3H), 0.57 (s, 3H); <sup>13</sup>C NMR (CD<sub>2</sub>Cl<sub>2</sub>, 101 MHz)  $\delta$  <sup>13</sup>C NMR (101 MHz, CD<sub>2</sub>Cl<sub>2</sub>)  $\delta$  170.7 (C), 154.8 (C), 150.1 (C), 137.3 (C), 135.0 (C), 134.6 (CH), 129.6 (CH), 128.5 (CH), 126.8 (C), 126.3 (CH), 124.8 (CH), 120.5 (C), 116.6 (CH), 113.4 (CH), 91.4 (C), 55.9 (CH<sub>2</sub>), 19.1 (CH), 0.4 (CH<sub>3</sub>), -1.1 (CH<sub>3</sub>); HRMS (ESI) calcd for C<sub>30</sub>H<sub>27</sub>N<sub>4</sub>O<sub>2</sub>Si [M+H]<sup>+</sup> 503.1903, found 503.1899.



(15; JF<sub>626</sub>): Synthesized following procedure A from silafluorescein ditriflate and 3-(trifluoromethyl)azetidine hydrochloride. Purification by silica gel chromatography (0–100% EtOAc/hexane, linear gradient), followed by purification by silica gel chromatography (0–35% EtOAc/toluene) afforded 15 (76%) as a light blue solid. <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400 MHz)  $\delta$  7.97 (dt, *J* = 7.7, 1.0 Hz, 1H), 7.66 (td, *J* = 7.5, 1.2 Hz, 1H), 7.56 (td, *J* = 7.5, 1.0 Hz, 1H), 7.29 (dd, *J* = 7.7, 0.9 Hz, 1H), 6.82 (d, *J* = 8.7 Hz, 2H), 6.70 (d, *J* = 2.7 Hz, 2H), 6.29 (dd, *J* = 8.7, 2.7 Hz, 2H), 4.14 – 4.04 (m, 4H), 4.01 – 3.91 (m, 4H), 3.49 – 3.29 (m, 2H), 0.62 (s, 3H), 0.60 (s, 3H); <sup>19</sup>F NMR (CDCl<sub>3</sub>, 376 MHz) = -73.4 (d, <sup>3</sup><sub>JHF</sub> = 8.8 Hz); <sup>13</sup>C NMR (CDCl<sub>3</sub>, 101 MHz)  $\delta$  170.5 (C), 154.1 (C), 149.5 (C), 137.2 (C), 134.1 (CH), 133.9 (C), 129.1 (CH), 128.2 (CH), 126.5 (q, <sup>2</sup><sub>JCF</sub> = 81.5Hz, CF<sub>3</sub>), 126.0 (CH), 124.7 (CH), 115.8 (CH), 112.5 (CH), 91.5 (C), 51.4 (CH<sub>2</sub>), 51.3 (CH<sub>2</sub>), 32.8 (q, <sup>3</sup><sub>JCF</sub> = 32.3 Hz, C), 0.5 (CH<sub>3</sub>), -1.5 (CH<sub>3</sub>); HRMS (ESI) calcd for C<sub>30</sub>H<sub>27</sub>N<sub>2</sub>O<sub>2</sub>SiF<sub>6</sub> [M+H]<sup>+</sup> 589.1746, found 589.1751.



**(16; JF**<sub>614</sub>**):** Synthesized following procedure A from silafluorescein ditriflate and 3,3-difluoroazetidine hydrochloride. Purification by silica gel chromatography (0–100% EtOAc/hexane, linear gradient), followed by purification by silica gel chromatography (0–35% EtOAc/toluene) afforded **16** (24%) as a light blue solid. <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400 MHz)  $\delta$  7.98 (dt, *J* = 7.6, 1.0 Hz, 1H), 7.66 (td, *J* = 7.5, 1.2 Hz, 1H), 7.56 (td, *J* = 7.5, 1.0 Hz, 1H), 7.30 – 7.28 (m, 1H), 6.85 (d, *J* = 8.7 Hz, 2H), 6.73 (d, *J* = 2.7 Hz, 2H), 6.34 (dd, *J* = 8.7, 2.8 Hz, 2H), 4.23 (t, <sup>3</sup>*J*<sub>HF</sub> = 11.8 Hz, 8H), 0.64 (s, 3H), 0.61 (s, 3H); <sup>19</sup>F NMR (CDCl<sub>3</sub>, 376 MHz) = -99.9 (p, <sup>3</sup>*J*<sub>HF</sub> = 11.7 Hz); <sup>13</sup>C NMR (CDCl<sub>3</sub>, 101 MHz)  $\delta$  170.5 (C), 154.0 (C), 148.7 (t, <sup>4</sup>*J*<sub>CF</sub> = 2.6 Hz, C), 137.3 (C), 134.8 (C), 134.0 (CH), 129.2 (CH), 128.2 (CH), 126.8 (C), 126.1 (CH), 124.6 (CH), 116.8 (CH), 115.9 (t, <sup>1</sup>*J*<sub>CF</sub> = 276 Hz, CF<sub>2</sub>), 113.6 (CH), 91.2 (C), 63.4 (t, <sup>2</sup>*J*<sub>HF</sub> = 25.9,CH<sub>2</sub>), 0.4 (CH<sub>3</sub>), -1.4 (CH<sub>3</sub>); HRMS (ESI) calcd for C<sub>28</sub>H<sub>25</sub>N<sub>2</sub>O<sub>2</sub>SiF<sub>4</sub> [M+H]<sup>+</sup> 525.1621, found 525.1629.



(17): Synthesized following procedure A from 6-*tert*-butoxycarbonylsilafluorescein ditriflate  $4^{10}$  and 3-methoxyazetidine hydrochloride. Purification by silica gel chromatography (0–30% EtOAc/hexane, linear gradient), afforded **17** (94%) as an off-white solid. <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400 MHz)  $\delta$  8.11 (dd, *J* = 8.1, 1.3 Hz, 1H), 7.96 (d, *J* = 8.0 Hz, 1H), 7.81 (d, *J* = 1.2 Hz, 1H), 6.85 (d, *J* = 8.7 Hz, 2H), 6.68 (d, *J* = 2.7 Hz, 2H), 6.32 (dd, *J* = 8.7, 2.7 Hz, 2H), 4.38 – 4.27 (m, 2H), 4.16 – 4.04 (m, 4H), 3.78 – 3.68 (m, 4H), 3.32 (s, 6H), 1.55 (s, 9H), 0.65 (s, 3H), 0.58 (s, 3H); <sup>13</sup>C NMR (CDCl<sub>3</sub>, 101 MHz)  $\delta$  170.3 (C), 164.4 (C), 155.4 (C), 150.4 (C), 137.3 (C), 136.2 (C), 132.8 (C), 130.0 (CH), 129.1 (C), 127.7 (CH), 125.7 (CH), 125.1 (CH), 116.1 (CH), 113.1 (CH), 91.7 (C), 82.4 (C), 70.1 (CH<sub>3</sub>), 58.9 (CH<sub>2</sub>), 56.2 (CH), 28.2 (CH<sub>3</sub>), 0.2 (CH<sub>3</sub>), -0.7 (CH<sub>3</sub>); HRMS (ESI) calcd for C<sub>35</sub>H<sub>41</sub>N<sub>2</sub>O<sub>6</sub>Si [M+H]<sup>+</sup> 613.2734, found 613.2726.



(18): Synthesized following procedure A from 6-*tert*-butoxycarbonylsilafluorescein ditriflate 4 and 3-methylsulfonyl-azetidine hydrochloride. Purification by silica gel chromatography (50–100% EtOAc/hexane, linear gradient), afforded 18 (87%) as a light blue solid. <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400 MHz)  $\delta$  8.12 (dd, *J* = 8.0, 1.3 Hz, 1H), 7.97 (dd, *J* = 8.0, 0.7 Hz, 1H), 7.79 (t, *J* = 1.0 Hz, 1H), 6.93 (d, *J* = 8.7 Hz, 2H), 6.72 (d, *J* = 2.7 Hz, 2H), 6.38 (dd, *J* = 8.8, 2.7 Hz, 2H), 4.31 – 4.19 (m, 8H), 4.15 – 4.03 (m, 2H), 2.97 (s, 6H), 1.55 (s, 9H), 0.67 (s, 3H), 0.59 (s, 3H); <sup>13</sup>C NMR (CDCl<sub>3</sub>, 101 MHz)  $\delta$  170.2 (C), 164.3 (C), 155.2 (C), 149.2 (C), 137.5 (C), 136.2 (C), 134.3 (C), 130.2 (CH), 128.7 (C), 127.8 (CH), 125.9 (CH), 124.9 (CH), 116.2 (CH), 113.3 (CH), 90.9 (C), 82.6 (C), 52.5 (CH<sub>2</sub>), 51.7 (CH), 38.3 (CH<sub>3</sub>), 28.2 (CH<sub>3</sub>), 0.1 (CH<sub>3</sub>), -0.6 (CH<sub>3</sub>); HRMS (ESI) calcd for C<sub>35</sub>H<sub>41</sub>N<sub>2</sub>O<sub>8</sub>SiS<sub>2</sub> [M+H]<sup>+</sup> 709.2073, found 709.2074.



(19): Synthesized following procedure A from 6-*tert*-butoxycarbonylsilafluorescein ditriflate 4 and 3-azetidinecarbonitrile hydrochloride. Purification by silica gel chromatography (0–20% EtOAc/hexane, linear gradient) afforded 19 (88%) as a light blue solid. <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400 MHz)  $\delta$  8.13 (dd, *J* = 8.0, 1.3 Hz, 1H), 7.97 (dd, *J* = 8.0, 0.7 Hz, 1H), 7.81 (t, *J* = 1.0 Hz, 1H), 6.91 (d, *J* = 8.7 Hz, 2H), 6.68 (d, *J* = 2.7 Hz, 2H), 6.33 (dd, *J* = 8.7, 2.7 Hz, 2H), 4.25 – 4.17 (m, 4H), 4.14 – 4.02 (m, 4H), 3.59 (tt, *J* = 8.4, 6.2 Hz, 2H), 1.55 (s, 9H), 0.68 (s, 3H), 0.59 (s, 3H); <sup>13</sup>C NMR (CDCl<sub>3</sub>, 101 MHz) 170.0 (C), 164.3 (C), 154.9 (C), 149.4 (C), 137.5 (C), 136.3 (C), 134.4 (C), 130.2 (CH), 128.8 (C), 127.8 (CH), 125.9 (CH), 124.9 (CH), 119.7 (C), 116.1 (CH), 113.3 (CH), 90.9 (C), 82.6 (C), 55.3 (CH<sub>2</sub>), 28.2 (CH<sub>3</sub>), 18.5 (CH), 0.1 (CH<sub>3</sub>), -0.7 (CH<sub>3</sub>); HRMS (ESI) calcd for C<sub>35</sub>H<sub>35</sub>N<sub>4</sub>O<sub>4</sub>Si [M+H]<sup>+</sup> 603.2428, found 603.2425.



(20): Synthesized following procedure A from 6-*tert*-butoxycarbonylsilafluorescein ditriflate **4** and 3-(trifluoromethyl)azetidine hydrochloride. Purification by silica gel chromatography (0–20% EtOAc/hexane, linear gradient) afforded **20** (54%) as a light blue solid. <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400 MHz)  $\delta$  8.12 (dd, *J* = 8.1, 1.3 Hz, 1H), 7.97 (d, *J* = 8.0 Hz, 1H), 7.82 (s, 1H), 6.88 (d, *J* = 8.7 Hz, 2H), 6.68 (d, *J* = 2.6 Hz, 2H), 6.33 (dd, *J* = 8.7, 2.7 Hz, 2H), 4.08 (t, *J* = 8.1 Hz, 4H), 3.98 (dt, *J* = 7.8, 5.6 Hz, 4H), 3.39 (qt, *J* = 8.5, 5.8 Hz, 2H), 1.55 (s, 9H), 0.66 (s, 3H), 0.59 (s, 3H); <sup>13</sup>C NMR (CDCl<sub>3</sub>, 101 MHz)  $\delta$  170.2 (C), 164.4 (C), 155.0 (C), 149.5 (C), 137.4 (C), 136.4 (C), 133.6 (C), 130.1 (CH), 129.0 (C), 127.8 (CH), 125.8 (CH), 125.1 (CH), 125.0 (C), 115.8 (CH), 112.8 (CH), 91.3 (C), 82.5 (C), 51.33 (CH<sub>2</sub>), 51.30 (CH<sub>2</sub>), 33.2 (q, <sup>3</sup><sub>JCF</sub> = 32.1 Hz, C), 28.2 (CH<sub>3</sub>), 0.2 (CH<sub>3</sub>), -0.7 (CH<sub>3</sub>); HRMS (ESI) calcd for C<sub>35</sub>H<sub>35</sub>N<sub>2</sub>O<sub>4</sub>SiF<sub>6</sub> [M+H]<sup>+</sup> 689.2270, found 689.2282.



(21): Synthesized following procedure A from 6-*tert*-butoxycarbonylsilafluorescein ditriflate **4** and 3,3-difluoroazetidine hydrochloride. Purification by silica gel chromatography (0–30% EtOAc/hexane, linear gradient), followed by purification by silica gel chromatography (0–20% EtOAc/hexanes) afforded **21** (71%) as an off-white solid. <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400 MHz)  $\delta$  8.13 (dd, *J* = 8.0, 1.3 Hz, 1H), 7.98 (dd, *J* = 8.1, 0.8 Hz, 1H), 7.82 (t, *J* = 1.0 Hz, 1H), 6.93 (d, *J* = 8.7 Hz, 2H), 6.73 (d, *J* = 2.7 Hz, 2H), 6.38 (dd, *J* = 8.7, 2.7 Hz, 2H), 4.24 (t, *J* = 11.7 Hz, 8H), 1.55 (s, 9H), 0.68 (s, 3H), 0.61 (s, 3H); <sup>19</sup>F NMR (CDCl<sub>3</sub>, 376 MHz) = -99.3 (p, <sup>3</sup>*J*<sub>HF</sub> = 11.9 Hz); <sup>13</sup>C NMR (CDCl<sub>3</sub>, 101 MHz)  $\delta$  170.0 (C), 164.3 (C), 155.0 (C), 148.7 (t, <sup>4</sup>*J*<sub>HF</sub> = 2.8 Hz, C), 137.5 (C), 136.5 (C), 134.3 (C), 130.2 (CH), 128.9 (C), 127.9 (CH), 125.9 (CH), 125.0 (C), 116.8 (CH), 115.9 (t, <sup>1</sup>*J*<sub>CF</sub> = 276 Hz, CF<sub>2</sub>), 113.9 (CH), 91.0 (C), 82.5 (C), 63.4 (t, <sup>2</sup>*J*<sub>HF</sub> = 26.0 Hz, CH<sub>2</sub>), 28.2 (CH<sub>3</sub>), 0.2 (CH<sub>3</sub>), -0.7 (CH<sub>3</sub>); HRMS (ESI) calcd for C<sub>33</sub>H<sub>33</sub>N<sub>2</sub>O<sub>4</sub>SiF<sub>4</sub> [M+H]<sup>+</sup> 625.2146, found 625.2145.

**Procedure B:** Synthesis of HaloTag ligands. The following procedure for **6** is representative. **17** (36 mg, 59 µmol) was taken up in CH<sub>2</sub>Cl<sub>2</sub> (2 mL) and trifluoroacetic acid (0.25 mL) was added. The reaction was stirred at room temperature overnight. Toluene (3 mL) was added, the reaction mixture was concentrated to dryness and then azeotroped with MeOH three times. The residue was combined with HaloTag(O<sub>2</sub>)amine (TFA salt, 30 mg, 89 µmol, 1.5 eq), HATU (34 mg, 89 µmol, 1.5 eq) in DMF (1.5 mL). DIEA (52 µL, 295 µmol, 5.0 eq) was added and the mixture was stirred at room temperature for 4 h. It was subsequently evaporated to dryness and purified as described.



(6; JF<sub>639</sub>-HaloTag ligand): Purification by silica gel chromatography (30–100% EtOAc/hexanes, linear gradient) provided 6 (60%) as a light-blue solid. <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400 MHz)  $\delta$  7.98 (dd, *J* = 8.0, 0.7 Hz, 1H), 7.91 (dd, *J* = 8.0, 1.4 Hz, 1H), 7.68 (t, *J* = 1.0 Hz, 1H), 6.81 (br s, 1H), 6.76 (d, *J* = 8.6 Hz, 2H), 6.68 (d, *J* = 2.7 Hz, 2H), 6.29 (dd, *J* = 8.7, 2.7 Hz, 2H), 4.37 – 4.29 (m, 2H), 4.13 – 4.07 (m, 4H), 3.76 – 3.70 (m, 4H), 3.66 – 3.60 (m, 6H), 3.56 – 3.52 (m, 2H), 3.50 (t, *J* = 6.7 Hz, 2H), 3.39 (t, *J* = 6.7 Hz, 2H), 3.32 (s, 6H), 1.78 – 1.69 (m, 2H), 1.51 (p, *J* = 6.9 Hz, 2H), 1.44 – 1.35 (m, 2H), 1.34 – 1.23 (m, 2H), 0.64 (s, 3H), 0.57 (s, 3H); Analytical HPLC: t<sub>R</sub> = 13.0 min, 99% purity (10–95% MeCN/H<sub>2</sub>O, linear gradient, with constant 0.1% v/v

TFA additive, 20 min run, 1 mL/min flow, detection at 254 nm); HRMS (ESI) calculated for C<sub>41</sub>H<sub>53</sub>CIN<sub>3</sub>O<sub>7</sub>Si [M+H]<sup>+</sup> 762.3341, found 762.3352.



(7; JF<sub>630</sub>-HaloTag ligand): Synthesized following procedure B from 18. Purification by silica gel chromatography (0–4% MeOH/CH<sub>2</sub>Cl<sub>2</sub>, linear gradient), followed by purification by silica gel chromatography (50–100% EtOAc/hexanes, linear gradient) afforded 7 (65%) as a light blue solid. <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400 MHz)  $\delta$ .97 (d, *J* = 7.9 Hz, 1H), 7.89 (dd, *J* = 8.0, 1.4 Hz, 1H), 7.68 (t, *J* = 1.0 Hz, 1H), 6.91 – 6.87 (m, 1H), 6.84 (d, *J* = 8.7 Hz, 2H), 6.70 (d, *J* = 2.7 Hz, 2H), 6.32 (dd, *J* = 8.7, 2.7 Hz, 2H), 4.28 – 4.17 (m, 8H), 4.13 – 4.03 (m, 2H), 3.67 – 3.59 (m, 6H), 3.57 – 3.54 (m, 2H), 3.50 (t, *J* = 6.7 Hz, 2H), 3.40 (t, *J* = 6.7 Hz, 2H), 2.96 (s, 6H), 1.78 – 1.67 (m, 2H), 1.51 (p, *J* = 6.8 Hz, 2H), 1.44 – 1.35 (m, 2H), 1.35 – 1.26 (m, 2H), 0.65 (s, 3H), 0.57 (s, 3H); Analytical HPLC: t<sub>R</sub> = 13.0 min, 98% purity (10–95% MeCN/H<sub>2</sub>O, linear gradient, with constant 0.1% v/v TFA additive, 20 min run, 1 mL/min flow, detection at 254 nm); HRMS (ESI) calculated for C<sub>41</sub>H<sub>53</sub>CIN<sub>3</sub>O<sub>9</sub>S<sub>2</sub>Si [M+H]<sup>+</sup> 858.2681, found 858.2690.



**(8;** JF<sub>629</sub>-HaloTag ligand): Synthesized following procedure B from 19. Purification by silica gel chromatography (20–100% EtOAc/hexanes, linear gradient) afforded **8** (73%) as a light blue solid. <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400 MHz)  $\delta$  7.98 (dd, *J* = 8.0, 0.7 Hz, 1H), 7.88 (dd, *J* = 8.0, 1.4 Hz, 1H), 7.70 (t, *J* = 1.0 Hz, 1H), 6.89 – 6.82 (m, 3H), 6.67 (d, *J* = 2.6 Hz, 2H), 6.30 (dd, *J* = 8.7, 2.7 Hz, 2H), 4.20 (dd, *J* = 8.5, 7.0 Hz, 4H), 4.09 (q, *J* = 6.7 Hz, 4H), 3.65 – 3.54 (m, 10H), 3.50 (t, *J* = 6.6 Hz, 2H), 3.41 (t, *J* = 6.7 Hz, 2H), 1.77 – 1.69 (m, 4H), 1.52 (p, *J* = 6.9 Hz, 2H), 1.44 – 1.36 (m, 2H), 1.35 – 1.28 (m, 2H), 0.66 (s, 3H), 0.58 (s, 3H); Analytical HPLC: t<sub>R</sub> = 14.4 min, 97% purity (10–95% MeCN/H<sub>2</sub>O, linear gradient, with constant 0.1% v/v TFA additive, 20 min run, 1 mL/min flow, detection at 254 nm); HRMS (ESI) calculated for C<sub>41</sub>H<sub>47</sub>ClN<sub>5</sub>O<sub>5</sub>Si [M+H]<sup>+</sup> 752.3035, found 752.3044.



(9; JF<sub>626</sub>-HaloTag ligand): Synthesized following procedure B from 20. Purification by silica gel chromatography (30–100% EtOAc/hexanes, linear gradient) afforded 9 (83%) as a light blue solid. <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400 MHz)  $\delta$  <sup>1</sup>H 7.99 (d, *J* = 7.8 Hz, 1H), 7.89 (dd, *J* = 8.0, 1.4 Hz, 1H), 7.73 – 7.68 (m, 1H), 6.84 –

6.78 (m, 3H), 6.67 (d, J = 2.7 Hz, 2H), 6.29 (dd, J = 8.7, 2.7 Hz, 2H), 4.07 (t, J = 8.1 Hz, 4H), 4.01 – 3.92 (m, 4H), 3.68 – 3.60 (m, 6H), 3.58 – 3.54 (m, 2H), 3.50 (t, J = 6.6 Hz, 2H), 3.45 – 3.34 (m, 4H), 1.77 – 1.68 (m, 2H), 1.56 – 1.46 (m, 2H), 1.44 – 1.35 (m, 2H), 1.34 – 1.26 (m, 2H), 0.65 (s, 3H), 0.58 (s, 3H); <sup>19</sup>F NMR (CDCl<sub>3</sub>, 376 MHz) = -73.5 (d, <sup>3</sup> $_{JHF}$  = 8.7 Hz); Analytical HPLC: t<sub>R</sub> = 16.4 min, 98% purity (10–95% MeCN/H<sub>2</sub>O, linear gradient, with constant 0.1% v/v TFA additive, 20 min run, 1 mL/min flow, detection at 254 nm); HRMS (ESI) calculated for C<sub>41</sub>H<sub>47</sub>ClF<sub>6</sub>N<sub>3</sub>O<sub>5</sub>Si [M+H]<sup>+</sup> 838.2878, found 838.2891.



(10; JF<sub>614</sub>-HaloTag ligand): Synthesized following procedure B from 21. Purification by silica gel chromatography (0–3% MeOH/CH<sub>2</sub>Cl<sub>2</sub>, linear gradient) afforded 10 (75%) as an off-white solid. <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400 MHz)  $\delta$  8.00 (dd, *J* = 7.9, 0.7 Hz, 1H), 7.88 (dd, *J* = 8.0, 1.4 Hz, 1H), 7.70 (t, *J* = 1.0 Hz, 1H), 6.87 (d, *J* = 8.7 Hz, 2H), 6.77 – 6.70 (m, 3H), 6.36 (dd, *J* = 8.7, 2.7 Hz, 2H), 4.24 (t, *J* = 11.7 Hz, 8H), 3.67 – 3.59 (m, 6H), 3.58 – 3.53 (m, 2H), 3.50 (t, *J* = 6.6 Hz, 2H), 3.41 (t, *J* = 6.7 Hz, 2H), 1.78 – 1.69 (m, 2H), 1.57 – 1.49 (m, 2H), 1.44 – 1.36 (m, 2H), 1.35 – 1.28 (m, 2H), 0.67 (s, 3H), 0.60 (s, 3H); <sup>19</sup>F NMR (CDCl<sub>3</sub>, 376 MHz) = - 99.9 (p, <sup>3</sup><sub>JHF</sub> = 11.6 Hz); Analytical HPLC: t<sub>R</sub> = 16.3 min, 95% purity (10–95% MeCN/H<sub>2</sub>O, linear gradient, with constant 0.1% v/v TFA additive, 20 min run, 1 mL/min flow, detection at 254 nm); HRMS (ESI) calculated for C<sub>39</sub>H<sub>45</sub>CIF<sub>4</sub>N<sub>3</sub>O<sub>5</sub>Si [M+H]<sup>+</sup> 774.2753, found 774.2759.

#### NMR SPECTRA AND HPLC TRACES







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