Angewandte Chemie

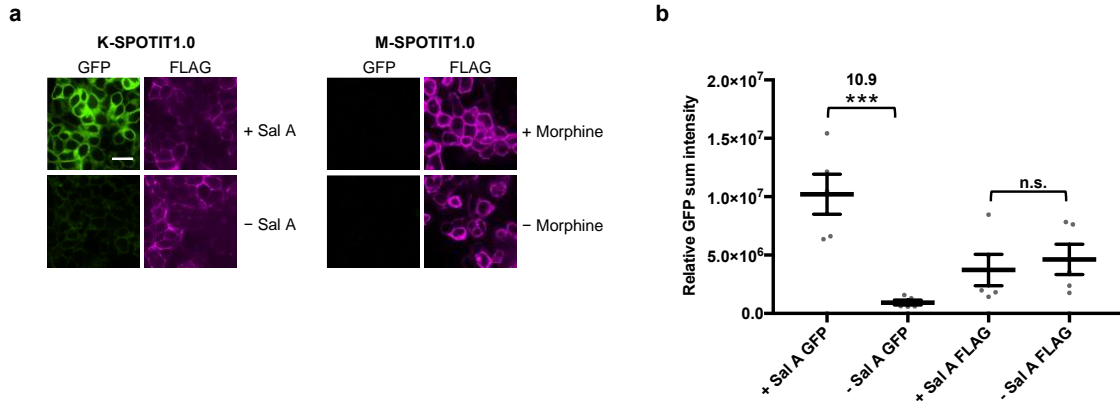*Eine Zeitschrift der Gesellschaft Deutscher Chemiker*

Supporting Information

Designing a Single Protein-Chain Reporter for Opioid Detection at Cellular Resolution

Kayla E. Kroning and Wenjing Wang*

anie_202101262_sm_miscellaneous_information.pdf

Supporting Figure S1: **Initial testing of SPOTIT.** (a) Confocal imaging of M-SPOTIT1.0 and K-SPOTIT1.0 in fixed HEK293T cells. Two days post infection with lentiviruses expressing SPOTIT, cells were stimulated with 10 μM of morphine and Sal A for 24 hours. Cells were then fixed, immunostained and imaged. (b) Analysis of K-SPOTIT1.0 testing from Fig. 1b. Values above the dots represent the fold-change of GFP signal compared to the no drug condition. Error bars, standard error of the mean. The mean is represented by the thicker horizontal bar. Stars indicate statistical significance analyzed using an unpaired Student's t-test. Three stars indicate a p-value of 0.0007. "n.s." indicates no significant difference between the two conditions, p-value of 0.6429. n=5 for each condition. GFP, cpGFP fluorescence; FLAG, protein expression level. Scale bar, 20 μm.
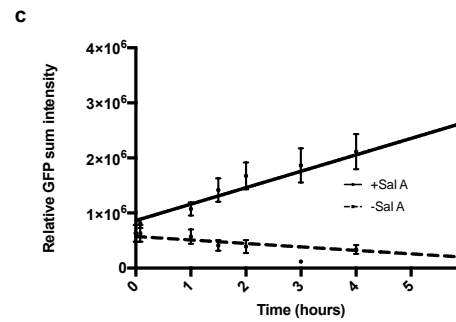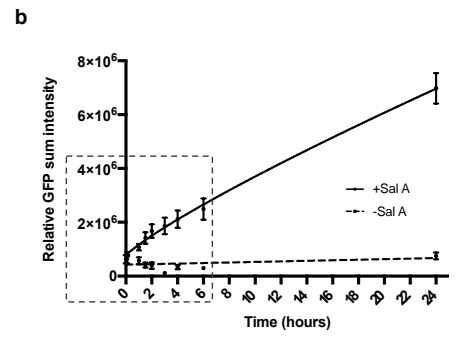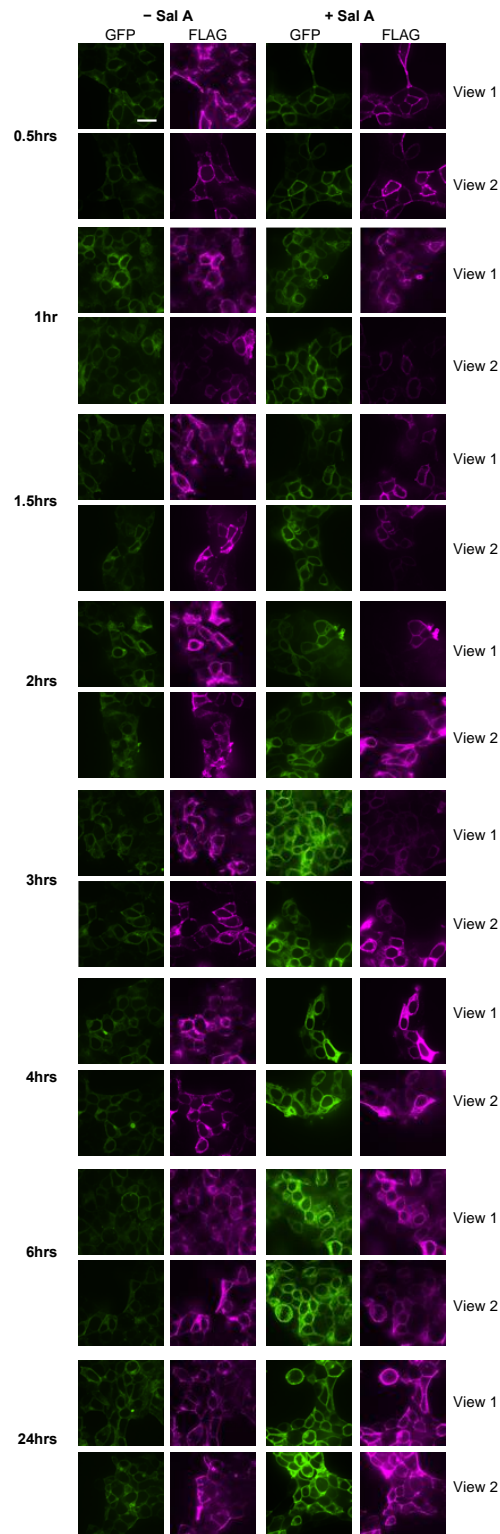
Figure S2: **Characterization of K-SPOTIT1.0's maturation time dependence.** Related to Figure 1c. (a) Two additional fields of view for Figure 1c. (b) Analysis of the imaging experiment in Figure 1c. (c) Zoomed in of b. Error bars, standard error of the mean. The mean is represented by each point in the plot. n=13-30 for each time point. GFP, cpGFP fluorescence; FLAG, sensor protein expression. Scale bar, 20 µm.
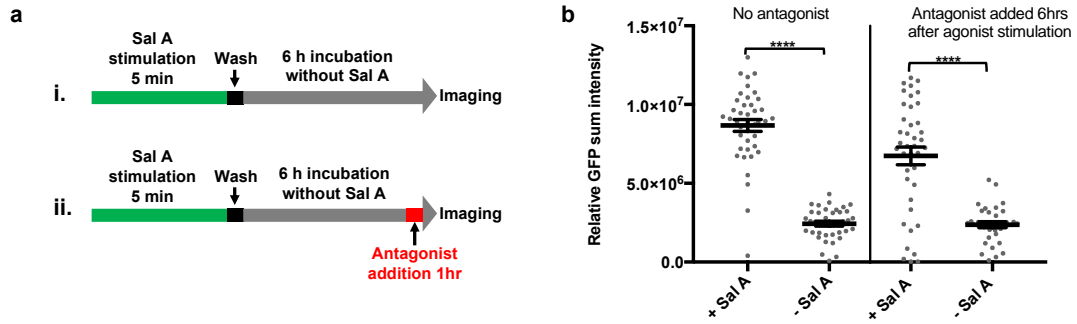
Figure S3: **Characterization of the effect of an antagonist on K-SPOTIT1.0 fluorescence post sensor-maturation.**
(a) Experimental design for testing the effect of the SPOTIT conformational state on its fluorescence post sensor maturation. 10 μM Sal A was used to stimulate HEK293T cells expressing K-SPOTIT1.0. After 5 minutes of incubation, the drug was removed, and cells were incubated for 6 hours without drug to allow the cpGFP fluorophore to mature. Then, 10 μM of Nor-BNI, a KOR antagonist, was added to the cells and incubated for 1 hour before live imaging. (b) Image analysis of the experiment described in **a**. Error bars, standard error of the mean. The mean is represented by the thicker horizontal bar. Stars indicate statistical significance analyzed using an unpaired Student's t-test. Four stars indicate a p-value <0.0001. n= 38-40 for each condition.
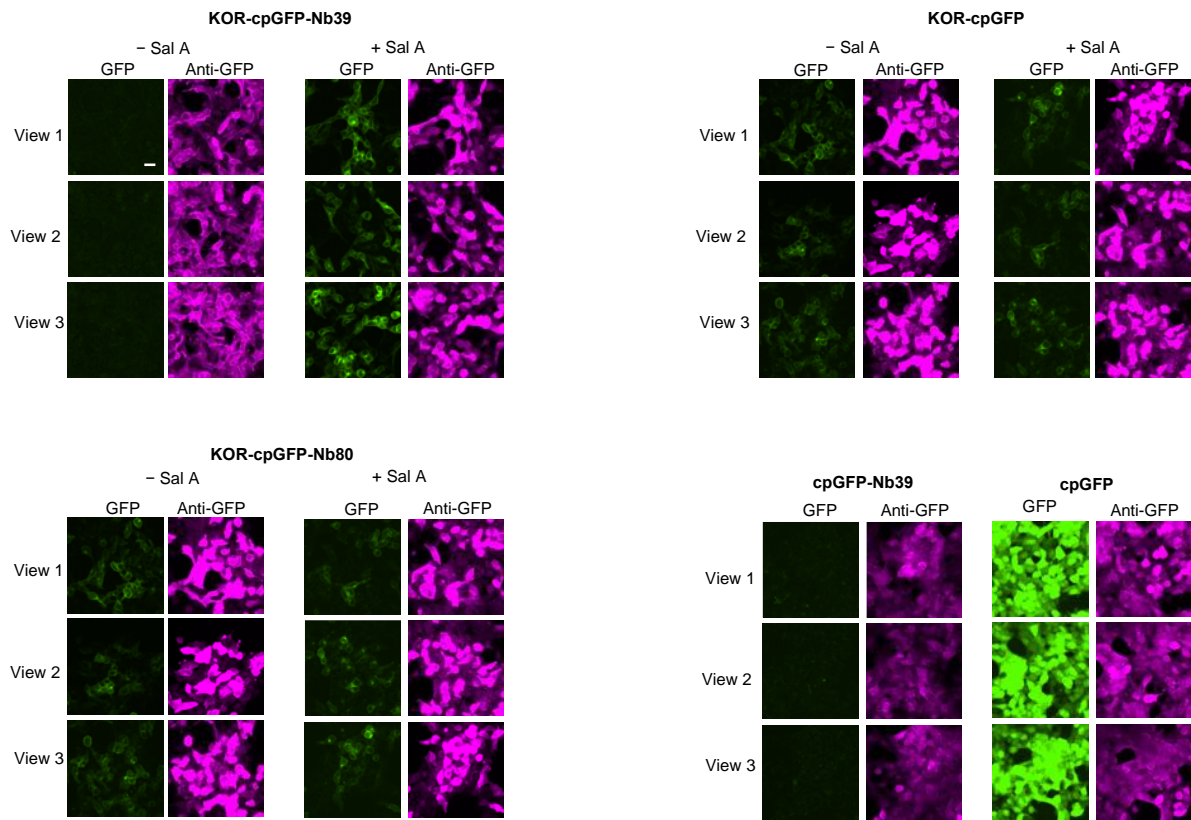
Figure S4: **Mechanistic studies of the role of Nb39 in inhibiting K-SPOTIT1.0 fluorophore maturation in the basal state.** Related to Figure 2. Three additional fields of view for the experiments shown and described in Figure 2. GFP, cpGFP fluorescence; anti-GFP, sensor expression level. Scale bar, 20 μm.
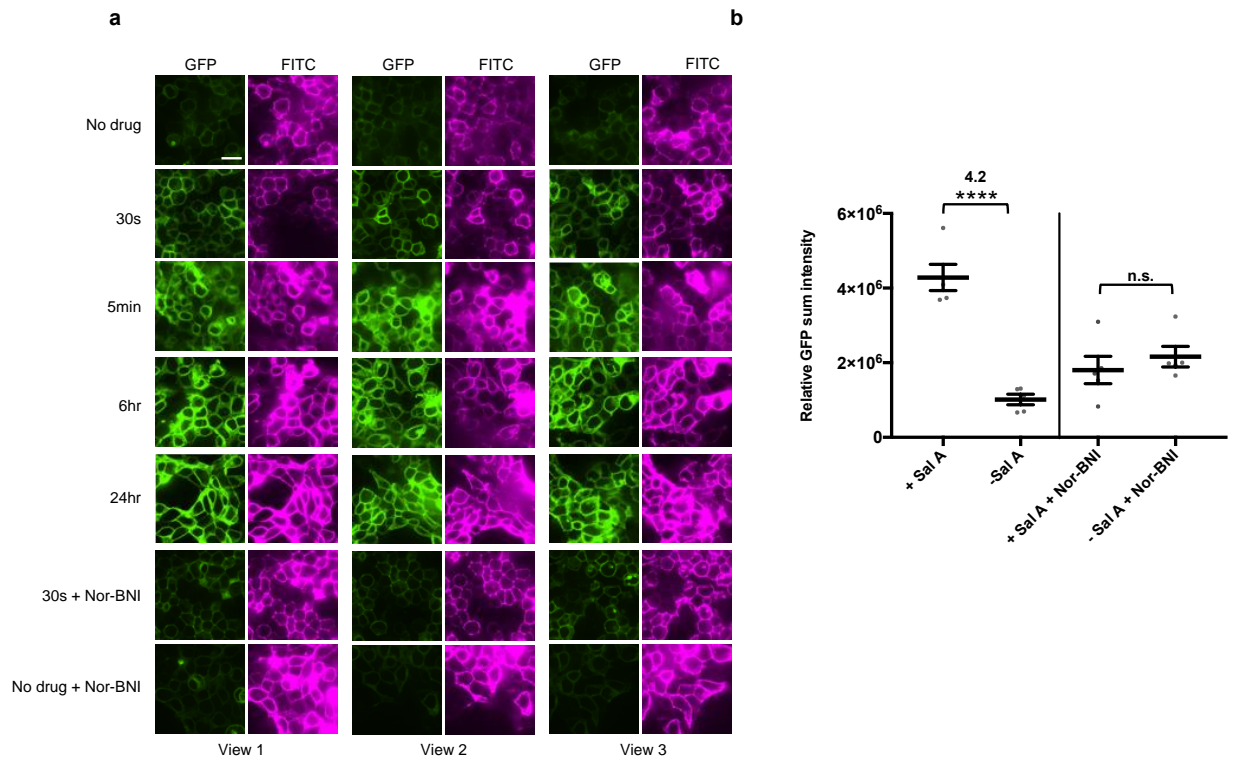
Figure S5: **Characterization of K-SPOTIT1.0's dependence on agonist exposure time.** (a) Related to Figure 3c. Three representative fields of view for the fixed images used to produce the agonist incubation time plot in Figure 3c. (b) Testing the importance of the KOR-Nb39 bound state in sensor activation. Values above the dots represent the fold-change of GFP signal compared to the no drug condition. Error bars, standard error of the mean. The mean is represented by the thicker horizontal bar. Stars indicate statistical significance analyzed using an unpaired Student's t-test. Four stars indicate a p-value <0.0001. "n.s" indicates no significant difference between the two conditions, p-value of 0.4583. n=5 for each condition. GFP, cpGFP fluorescence; FLAG, sensor protein expression. Scale bar, 20 µm.

a

| Signal Peptide | FLAG | KOR transmembrane domain | Receptor C-terminal cytosolic domain | cpGFP | Nb39 |

K-SPOTIT 1.0, full length: CFPLKMRMERQSTSRVRNTVQDPAYLRDIDGMNKPV
K-SPOTIT 1.1, 10 AA: CFPLKMRMER
K-SPOTIT 1.2, 6 AA: CFPLKM

b

K-SPOTIT1.0   K-SPOTIT1.1   K-SPOTIT1.2

c

d cpGFP fluorescence

e

f

	LOD (logM)	Sensitivity (intensity/logM)	Linear dynamic range (logM)
1.0	-8.5	2.7×10^5	-6.9 to -5.5
1.1	-7.7	5.0×10^5	-6.6 to -5.4
1.2	NA*	1.5×10^5	-6.9 to -5.5

Figure S6: **Characterization of K-SPOTIT variants.** (a) DNA constructs of K-SPOTIT with C-terminal truncations. A signal peptide was used to aid membrane trafficking of the sensor. (b) Confocal imaging of the K-SPOTIT variants. Cells were simulated with 10 μM Sal A for 24 hours, and then fixed, immunostained, and imaged. (c) Analysis of variants using live-cell imaging. Error bars, standard error of the mean. The mean is represented by the thicker horizontal bar. Stars indicate statistical significance analyzed using an unpaired Student's t-test. Four stars indicate a p-value <0.0001. n=30 for each condition. (d) Confocal imaging of a titration with K-SPOTIT variants. Cells were simulated with 100-0.001 μM Sal A for 24 hours, and then fixed and imaged. (e) Analysis of titration curves. Error bars, standard error of the mean. The mean is represented by each point in the plot. n=7-10 for each time point. (f) Calculations of limit of detection (LOD), sensitivity, and linear dynamic range for all three sensors using the titration curve plotted in **e**. LOD was calculated by taking the mean of the blank intensity value plus three times its standard deviation and then extrapolating the x-value for this calculation from the titration curve. *For SPOTIT1.2, LOD could not be extrapolated due to its low background in the no agonist state. Sensitivity was determined by calculating the slope of the linear region of the titration curve. The linear dynamic range was determined by estimating the upper and lower limits of linearity from the titration curve. GFP, cpGFP fluorescence; FLAG, sensor protein expression. Scale bar, 20 μm in **b** and 50 μm in **d**.

! max = 395 nm   ! max = 470 nm

Figure S7. Protonated and deprotonated states of the cpGFP fluorophore.
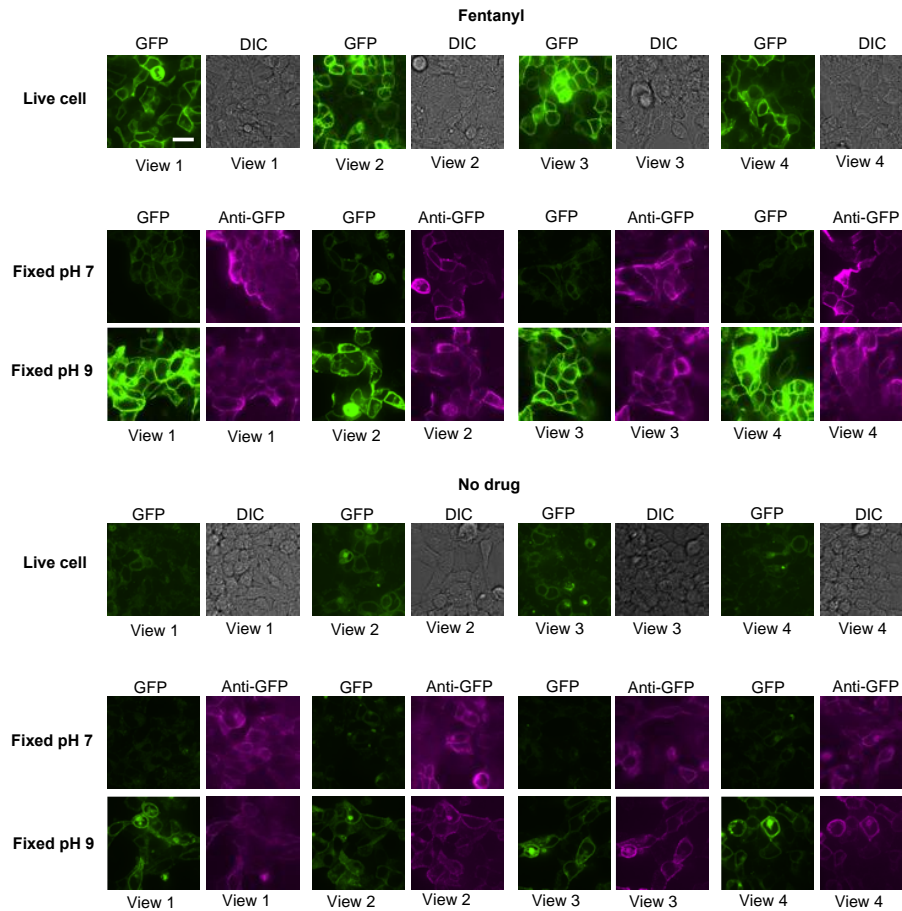
Figure S8: Confocal imaging characterization of M-SPOTIT1.1 in live and fixed cells. Related to Figure 5a. Four representative fields of view for pH testing. GFP, cpGFP fluorescence; anti-GFP, protein expression level. Scale bar, 20 μm.

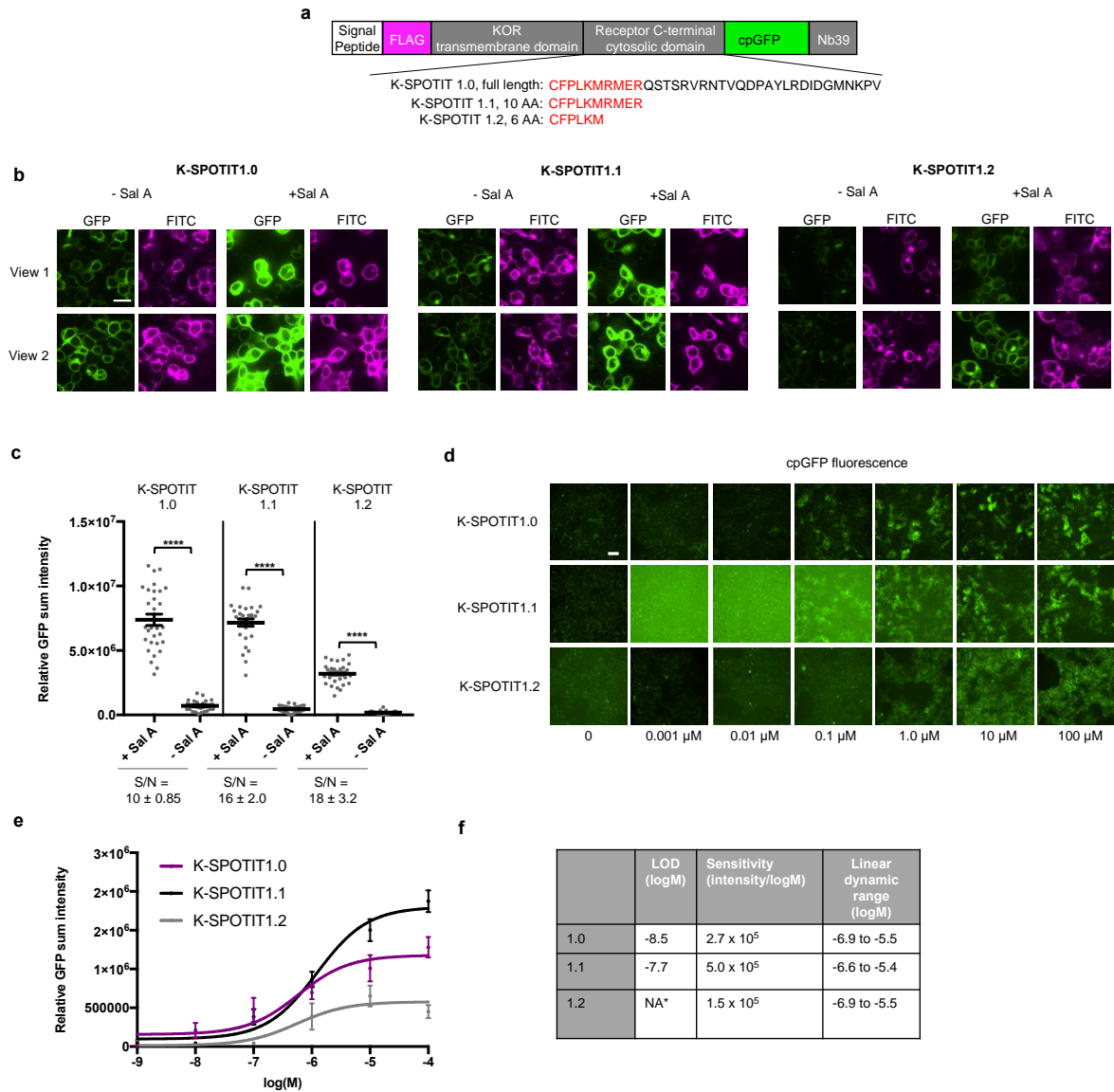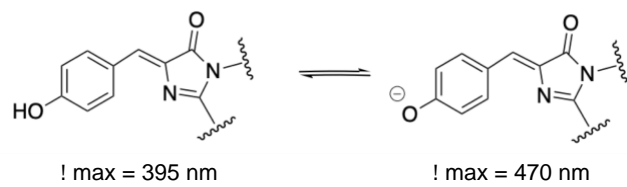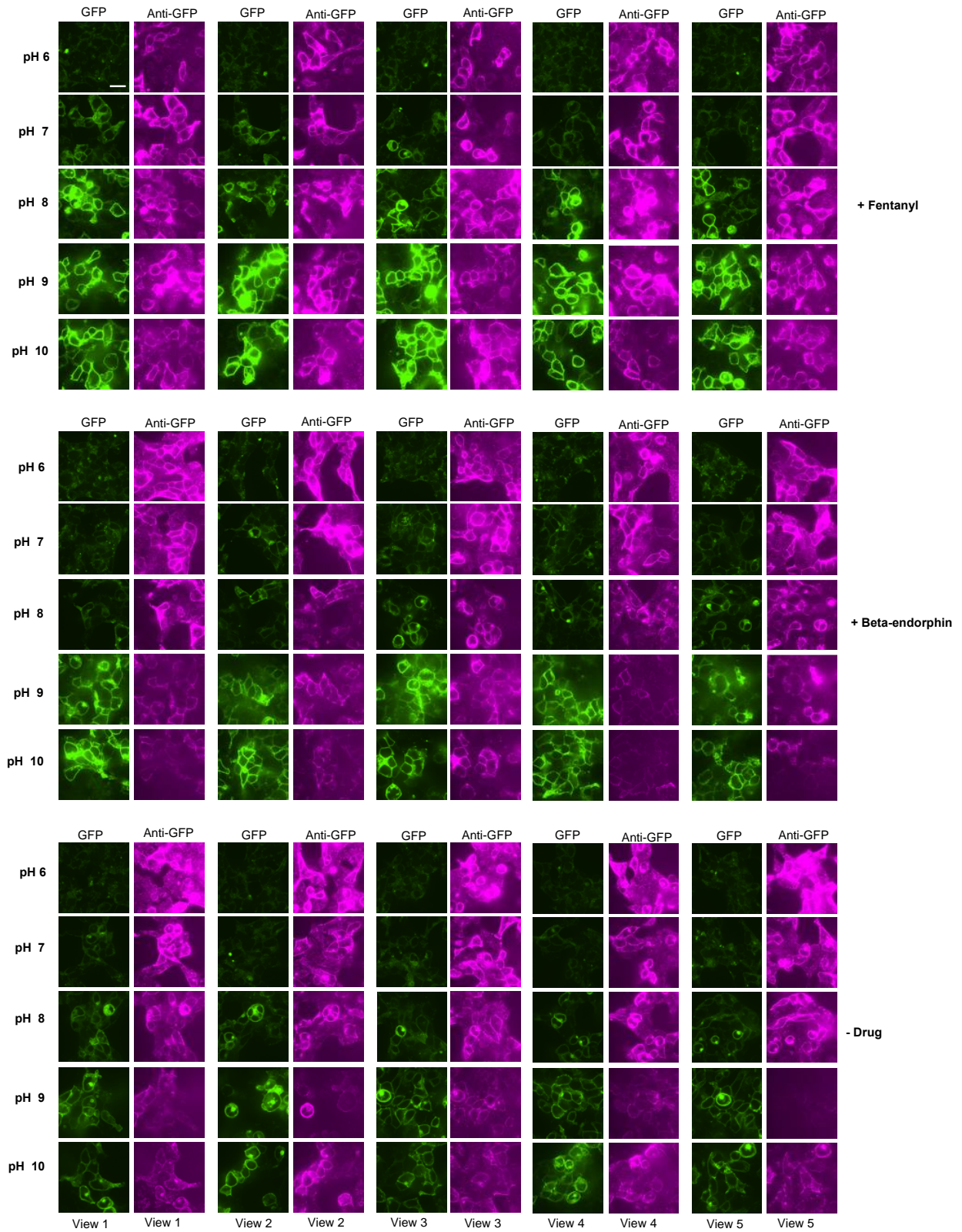**Figure S9:** **pH titration of M-SPOTIT1.1 in fixed HEK cells.** Related to Figure 5c. Five representative fields of view for pH titration. GFP, cpGFP fluorescence; anti-GFP, protein expression level. Scale bar, 20 μm.

a

Fentanyl | No drug

0.5hrs
1hr
1.5hr
2hr
3hr
4hr
6hr

View 1 | View 2 | View 1 | View 2

c

Morphine
Fentanyl
Buprenorphine
Loperamide
Oxycodone
DAMGO
Beta-endorphin
Leu-enkephalin
Endomorphin-1

Endomorphin-2
DADLE
Spiradoline
Sal. A
BRL52537
Isoproterenol
Naloxone
No drug

b

30s fentanyl
5min fentanyl
6hr fentanyl
30s fentanyl then 24 hour naloxone
No drug

30s Morphine
5min Morphine
6hr Morphine
30s morphine then 24 hour naloxone
24hr naloxone
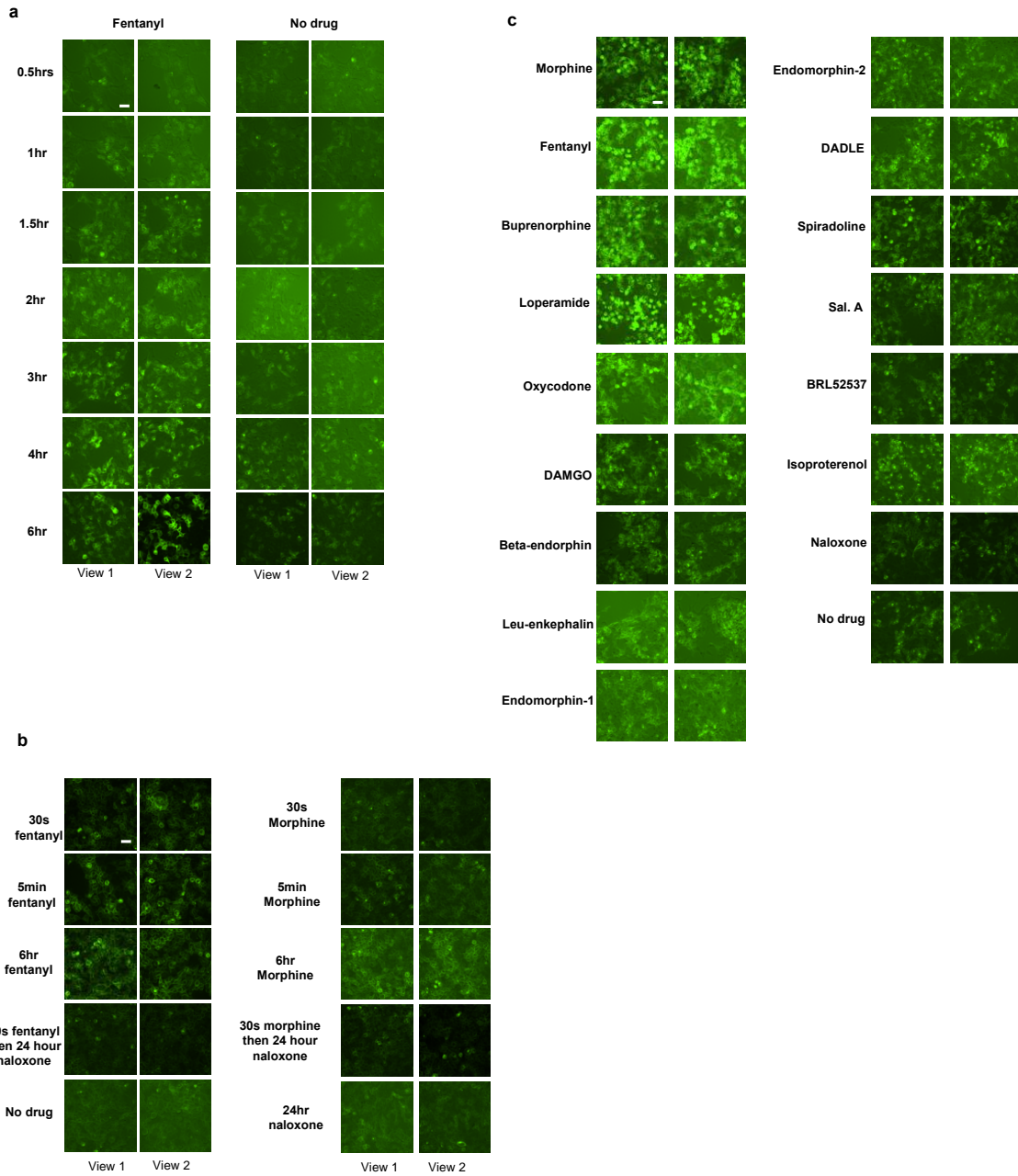
View 1 | View 2 | View 1 | View 2

Figure S10: **M-SPOTIT characterization at pH 9.** Related to Figure 5. Two representative fields of view for (a) M-SPOTIT1.1 maturation assay, (b) agonist exposure assay, and (c) selectivity testing. All images shown are of cpGFP fluorescence. Scale bar, 50 µm

No drug

	View 1	View 2	View 3
DIC			
mCherry			
GFP			

10 µM Fentanyl

	View 1	View 2	View 3
DIC			
mCherry			
GFP			

50 µM Beta-endorphin

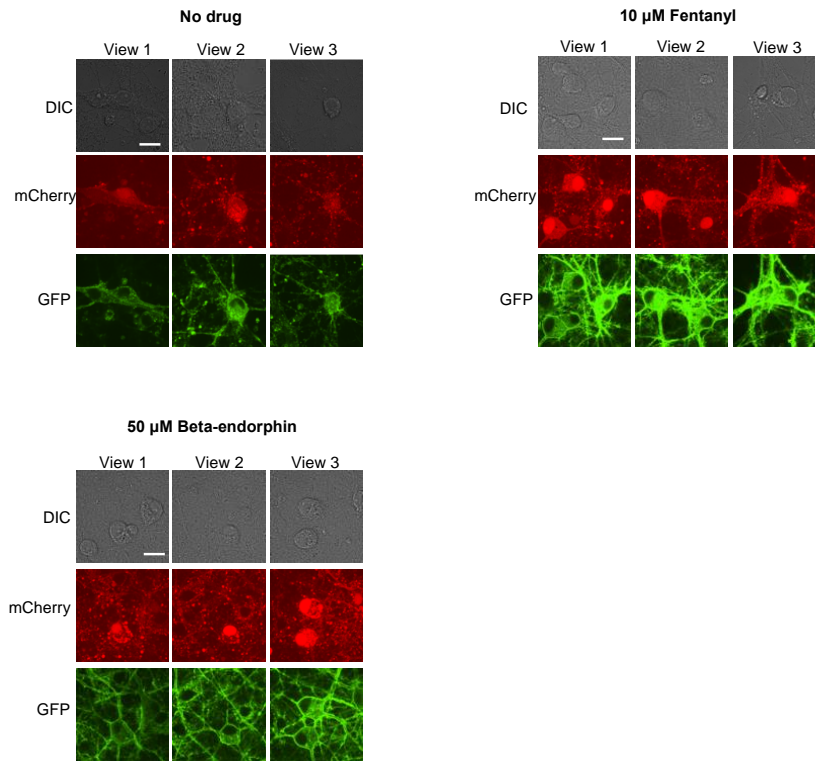|	View 1	View 2	View 3
DIC			
mCherry			
GFP			

Figure S11: Initial testing of M-SPOTIT1.1 in cultured neurons. Related to Figure 6. Three representative fields of view of the fixed-cell images used to characterize M-SPOTIT1.1's performance in cultured neurons. GFP, cpGFP fluorescence; mCherry, protein expression level. Scale bar, 20 µm.

Figure S12: **Fentanyl titration in cultured neurons.** Related to Figure 6. Three representative views of the fixed-cell images used to characterize M-SPOTIT1.1's performance in cultured neurons. GFP, cpGFP fluorescence; mCherry, protein expression level. Scale bar, 50 μm.
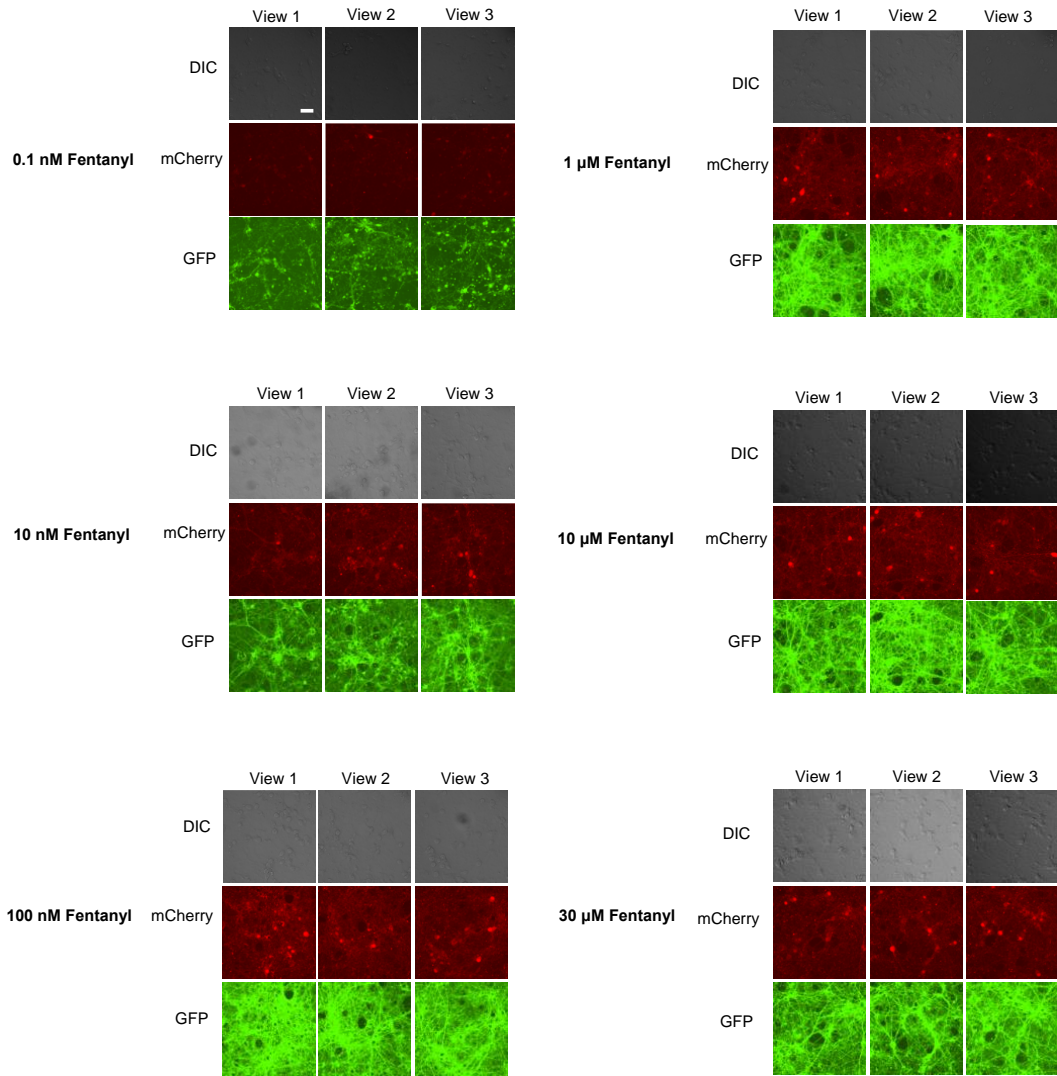
Plasmids and cloning

Constructs for HEK293T cell expression were cloned in an ampicillin-resistant lentiviral vector using a cytomegalovirus (CMV) promoter. cpGFP was amplified from AAV-hSyn1-GCaMP6s-P2A-nls-dTomato (addgene plasmid #51084, Jonathan Ting laboratory.) MOR and KOR sequences were gifts from Bryan Roth[24] (Addgene plasmid #66464 and #66462). Nb39 and Nb80 were synthesized as a gene block from IDT. Standard cloning procedures, such as Q5 polymerase PCR amplification, NEB restriction enzyme digest, and T4 ligation or Gibson assembly were used. Ligated plasmids were transformed into XL1-blue competent cells using heat shock transformation. After full sequencing of all constructs, we found a point mutation in MSPOTIT1.0 and MSPOTIT1.1 sequences, leading to a isoleucine to threonine mutation at MOR amino acid position 140. This is in the extracellular portion of the third loop and, therefore, does not affect the receptor functionality which depends on an intracellular conformational change. As a result, we went ahead with this construct.

HEK293T cell culture

HEK293T cells were cultured in complete growth media: 1:1 DMEM (Dublecco's Modified Eagle medium, GIBCO): MEM (Modified Eagle medium, GIBCO), 10% FBS (Fetal Bovine Serum, Sigma), 1% (v/v) penicillin (Gibco), and 1% penstrap (Gibco). We pretreated 24-well glass bottom plates (Corning) with 200 μL 20 μg/mL human fibronectin (Milipore Sigma) for 10 min at 37 $^\circ$C under 5% CO_2. Cells were plated at a density so that they would reach 90% confluence on the day of stimulation, for transfection this was next day and for viral infection this was in two days.

HEK cell transfection with PEI MAX

PEI MAX (polyethyleneimine, Polysciences) was used for all transfection experiments. 1-3 h after plating, cells were transfected with a homemade PEI max solution. For transfection per well of a 24-well plate, 200 ng SPOTIT DNA and 2 μL PEI max solution (1mg/mL in H_2O) in 20 μL DMEM was incubated for 10 min at room temperature (RT). After 10 min, 200 μL of complete media was added, and 220 μL of this solution was gently pipetted on top of the plated cells. Cells were incubated at 37 °C with 5% CO_2 until stimulation 24 h later.

Lentivirus production

90% confluent HEK293T cells in a T25 flask were transfected with 2.5 μg sensor DNA, 0.25 μg pVSVG, and 2.25 μg Δ8.9 lentiviral helper plasmid mixed in 200 μL of DMEM without FBS. After 10 min of incubation at room temperature, the DMEM, PEI, and DNA solution was pipetted gently on top of the HEK293T cells in the T25 flask. After two days of incubation at 37 °C with 5% CO_2, the virus-containing T25 supernatant was collected, aliquoted into 500 μL volumes, flash-frozen using liquid nitrogen, and stored in -80 °C for future use.

HEK293T cell infection

HEK293T cells (less than 20 passages) were plated in 24-well glass bottom plates (Cellvis) pretreated with 350 μL 20 µg/mL human fibronectin (Millipore Sigma) for 10 min at 37 °C. HEK293T cells were plated at 40%-60% confluence. For infection of a single well in a 24-well plate, 25-100 μL of each supernatant virus was added gently to the top of the media and incubated for 48 h before stimulation

HEK cell stimulation

20-24 h after transfection and 40-48 h after infection, HEK293T cells were stimulated. Drugs were diluted in pre-warmed complete media to the desired concentration. 100 μL of the diluted

drug was gently dropped on top of the plated cells. For stimulation with beta-endorphin and leu-enkephalin, 2 μL of a protease inhibitor cocktail (Millipore Sigma) was added to the 24-well plates prior to peptide stimulation. After the desired drug incubation time, all the media in the well was removed and the well was washed three times with complete media. 400 μL of complete media was added back to the well, and the cells were kept at 37 °C with 5% CO_2 prior to imaging. We noticed that SPOTIT is sensitive to temperature. Incubation at temperatures lower than 37 °C will increase the background fluorescence. Presumably the cpGFP can be better folded at lower temperate to allow the fluorophore to mature. For all experiments other than chromophore maturation experiments, the cells were imaged 24 h after stimulation.

HEK cell fixation and immunostaining

For fixation, media was removed from HEK293T cells and 200 μL of fixative (4% formaldehyde in PBS) was added to the wells. After 15 min of fixative incubation, the fixative was removed, and the wells were washed three times with PBS. To permeabilize the cells, 200μL of cold methanol (~20 °C) was added to each well. The cells were incubated in -20 °C for 5 min then washed 3x with PBS. For immunostaining, mouse anti-flag (Sigma, F3165) or chicken anti-EGFP (abcam, ab13970) was diluted in a 1% BSA in PBS (phosphate buffered saline) solution to a concentration of 1:1000 antibody:solution. 200 μL of the antibody solution was added to each well, and the cells were incubated with the primary antibody for 30 minutes at RT on a rocker. Then, the cells were washed 3x with PBS and the same volume and concentration of anti-mouse 647 (Life Technologies, A21235) or anti-chicken 647 (abcam, A21449) was added to each well. Again, the cells rocked for 30 min at RT with 5% CO_2 and were washed 3x with PBS. 200 μL of PBS or pH 9 CAPS buffer were added back to the cells, and the cells were imaged.

Confocal microscopy of HEK cells

Confocal imaging was performed on a Nikon inverted confocal microscope with 20× air objective and 60x oil immersion objective, outfitted with a Yokogawa CSU-X1 5000RPM spinning disk confocal head, and Ti2-ND-P perfect focus system 4, a compact 4-line laser source: 405 nm (100 mW) 488 nm (100 mW), 561 nm (100 mW) and 640-nm (75 mW) lasers. The following combinations of laser excitation and emission filters were used for various fluorophores: EGFP/Alexa Fluor 488 (488 nm excitation; 525/36 emission), mCherry (568 nm excitation; 605/52 emission), Alexa Fluor 647 (647 nm excitation; 705/72 emission), and differential interference contrast (DIC). Acquisition times ranged from 1 to 2 s and 50% laser intensity was used for all excitation filters. ORCA-Flash 4.0 LT+sCMOS camera. 1-1.5x magnification was used for the 20x objective and 1x magnification was used for 60x. All images were collected using Nikon NIS-Elements hardware control and processed using NIS-Elements General Analysis 3 software.

Analysis of HEK293T cell images

For live cell 30x magnification images, 10-15 fields of view per well were taken and three technical replicates were performed. For 60x and 20x magnification fixed images, 5-10 images were taken per well. NIS-Elements General Analysis 3 software was used to analyze the images. Data for both the mean FITC (or Cy5) intensity and object areas were taken for all fields of view and all technical replicates. These values were multiplied together to collect the sum FITC (or Cy5) intensity per field of view. This value was subtracted by the mean intensity value of a position with no cells multiplied by the object area taken for that specific field of view. This accounts for the background of the laser against the plate. For glass plates, a representative mean intensity value can be used for the background subtraction of all fields of view and wells. For plastic plates, the laser background significantly differs per field of view, so it is important to take the mean intensity value of an area with no cells for each field of view to perform

background subtraction. The mean and standard error of the mean (SEM) were calculated for each condition. Two-sided Student's t-tests were used to evaluate the significance between data points. Plots were made using Prism GraphPad software. Fields of view with no cells (due to low confluence or cells lifting during washing steps) were omitted from analysis. These fields of view were identified by a "0" object area.

K-SPOTIT and M-SPOTIT initial testing

HEK293T cells were cultured in 24-well imaging plates and transfected with K-SPOTIT1.0 and M-SPOTIT1.0 lentiviruses following the above protocol. 20-24 h post transfection, the cells were stimulated with 10 μM Salvinorin A for K-SPOTIT and 10 μM morphine for M-SPOTIT. The cells were incubated with drug for 24 h before fixation, FLAG immunostaining, imaging, and data analysis following the previously stated protocols.

K-SPOTIT addition of antagonist

To test the persistence of the SPOTIT signal, HEK293T cells were cultured in 24-well imaging plates and infected with K-SPOTIT1.0 lentivirus following the above protocol. 40-48 h after infection, cells were stimulated for 5 min with 10 μM of Salvinorin A. After 5 min, the cells were washed 3x with complete media and allowed to incubate for 6 h at 37 $°$C without agonist . After 6 h, 10 μM Nor-BNI was added and incubated for 1 h. Then, the cells were fixed and immunostained for FLAG tag expression. The cells were imaged and analyzed using the protocol stated above.

K-SPOTIT and M-SPOTIT linker optimization

HEK293T cells were cultured in 24-well imaging plates and transfected with SPOTIT DNA coding for the different linker lengths and types following the above protocol. 20-24 h post

transfection, the cells were stimulated with 10 µM Salvinorin A for K-SPOTIT and 10 µM morphine for M-SPOTIT. The cells were incubated with drug for 24 h before fixation, FLAG immunostaining, imaging, and data analysis following the previously stated protocols.

K-SPOTIT and M-SPOTIT maturation assays

For the K-SPOTIT maturation assay, HEK293T cells were cultured in 24-well imaging plates and infected with K-SPOTIT1.0 lentiviruses following the above protocol. For the M-SPOTIT1.1 maturation assay, HEK293T cells were cultured in 48-well plates and infected with M-SPOTIT1.1 lentiviruses following the above protocol. Wells were plated and infected with K-SPOTIT1.0 lentivirus for the following conditions: 0 h, 0.5 h, 1 h, 1.5 h, 2 h, 3 h, 4 h, 6 h, and 24 h. 0.5 h means the cells were imaged 30 min post stimulation; 1 h means the cells were imaged 1 h post stimulation, and so on. The same conditions were plated for M-SPOTIT1.1, except for 24 h. 40-48 h post infection, the cells were stimulated at different time points with 10 µM Salvinorin A for K-SPOTIT and 10 µM of fentanyl for M-SPOTIT1.1. K-SPOTIT cells were fixed and immunostained with 1:1000 mouse anti-flag antibody and 1:1000 anti-mouse 647 antibody. M-SPOTIT1.1 was fixed and imaged at pH 9. All wells were imaged at the same time following the imaging and data analysis technique stated above.

K-SPOTIT and M-SPOTIT agonist incubation time assays

For the K-SPOTIT agonist incubation time assay, HEK293T cells were cultured in 24-well imaging plates and infected with K-SPOTIT1.0 lentiviruses following the above protocol. For the M-SPOTIT1.1 agonist incubation assay, HEK293T cells were cultured in 48-well plates and infected with M-SPOTIT1.1 lentiviruses following the above protocol. HEK293T cells were cultured in 24-well imaging plates and infected with K-SPOTIT1.0 and M-SPOTIT1.1 lentiviruses following the above protocol. Wells were plated and infected with K-SPOTIT1.0 lentivirus for the

following conditions: No agonist, 30 s agonist, 5 min agonist, 6 h agonist, 24 h agonist, and 30 s agonist followed by antagonist. K-SPOTIT1.0 was stimulated with Salvinorin A for the time points indicated, and M-SPOTIT1.1 was stimulated with both fentanyl and morphine for the time points indicated. For example, for the K-SPOTIT1.0 30 s agonist time point, 10 μM Salvinorin A was added to the well for 30 s. After 30 s, the well was washed 3x with complete HEK cell media. 400 μL of fresh complete media was then added back to the well. The same procedure was followed for the other time points of 5 min, 6 h, and 24-26 h. The same procedure was also followed for M-SPOTIT1.1 with fentanyl and morphine. For the time points with antagonist added, post-stimulation with agonist, the agonist was washed out 3x and 10 μM of Nor-BNI for K-SPOTIT1.0 and naloxone for M-SPOTIT1.1 was added into the well. This was not washed out. 24-26 h post stimulation, the cells were imaged. K-SPOTIT1.0 was fixed and imaged at pH 7 and M-SPOTIT1.1 was fixed and imaged at pH 9.

Mechanism testing

HEK293T cells were cultured in 24-well imaging plates and infected with lentivirus DNA constructs used to interrogate the mechanism of SPOTIT (as seen in **Fig. 2**). 40-48 h post transfection, the cells infected with constructs containing K-SPOTIT were stimulated with 10 μM Salvinorin A. The cells were incubated with Salvinorin A for 24 h before fixation, EGFP immunostaining, and imaging following the protocols described above. For all mechanism studies, GFP and cy5 intensities and object areas were collected as described before. Final figures were made by normalizing the GFP intensity to the protein expression level by dividing the GFP sum intensity values with the cy5 sum intensity values.

M-SPOTIT initial pH testing

HEK293T cells were cultured in 24-well imaging plates and infected with M-SPOTIT1.1 lentiviruses following the above protocol. The following conditions were plated: live cell, fixed pH 7, and fixed pH 9. Live cell conditions were plated on a separate plate from the fixed conditions. 40-48 h post infection, cells were stimulated with fentanyl. 24 h post fentanyl stimulation, live-cell images were taken and the fixed conditions were fixed and immunostained with anti EGFP chicken antibody and anti-chicken 647. After immunostaining, a pH 7 PBS solution or pH 9 CAPS buffer was added to the cells. The fixed cells were then imaged and analyzed using the protocol stated above.

M-SPOTIT pH titration

HEK293T cells were cultured in 24-well imaging plates and infected with M-SPOTIT1.1 lentiviruses following the above protocol. The following conditions were plated for fentanyl, beta-endorphin, and no drug stimulations: pH 6, pH 7, pH 8, pH 9, and pH 10. 40-48 h post infection, cells were stimulated with 10 μM fentanyl or 10 μM beta endorphin. 24 h post stimulation, all cells were fixed and immunostained with anti EGFP chicken antibody and anti-chicken 647. After immunostaining, solutions with different pHs were added to the appropriate wells. The following buffers were used pH 6: 1x PBS, pH 7: 1x PBS, pH 8: 100 mM Tris-HCl, pH 9: 100 mM Tris-HCl, pH 10: 100 mM Tris-HCl, pH 11: 100 mM CAPS buffer. All buffers were adjusted with 1 M NaOH and HCl to achieve the correct pH. The cells were then imaged and analyzed using the protocol stated above.

M-SPOTIT selectivity

HEK293T cells were cultured in 48-well plates and infected with M-SPOTIT1.1 lentiviruses following the above protocol. 40-48 h post infection, cells were stimulated with 10 μM of different agonists. 24 h post stimulation, cells were fixed and immunostained with anti EGFP chicken

antibody and anti-chicken 647. After immunostaining, a pH 9 CAPS buffer was added to the cells. The fixed cells were then imaged and analyzed using the protocol described above.

AAV supernatant production for neuronal infection

AAV virus supernatant was used for neuronal culture experiments. 6-well plates were pretreated with human fibronectin for 10 min at 37 °C. HEK293T cells were plated on the fibronectin-treated plates, so they were 60-90% confluent. For each well, 0.35 µg AAV expression DNA, 0.29 µg AAV1 serotype, 0.29 µg AAV2 serotype plasmid, and 0.7 µg helper plasmid pDF6 with 80 µL serum-free DMEM and 10 µL PEI max were mixed and incubated for 10 min at room temperature, and then 2 mL complete growth media was added and mixed. The DNA mix was added gently on the top of the cells. HEK293T cells were incubated for 40-48 h at 37 °C and then the virus supernatant was collected. The virus supernatant was stored in sterile Eppendorf tubes (0.5 mL/tube), flash frozen by liquid nitrogen and stored at −80 °C.

Neuronal culture experiments.

Frozen rat cortical neurons (Thermo Fisher Scientific, Cat# A1084001) were plated according to the user protocol. The half area 96-well glass plates (Corning, CLS4580-10EA) were coated with 50 µl poly-D-lysine (Gibco, 0.1 mg/ml in water) for 1 h, and then washed twice with ultrapure water. The frozen rat cortical neurons were quickly removed from liquid nitrogen and thawed in the 37 °C water bath by swirling until a small piece of ice was present. The cells were gently transferred to a 50 ml conical tube. To the cells, 1 ml pre-warmed 3:1 ratio of complete neurobasal media (NM) and glial enriching medium (GEM) mix was very slowly dropped in at one drop per second with gentle swirling. NM is composed of neurobasal (Thermo Fisher Scientific) supplemented 2% B27 (Thermo Fisher Scientific), 50 mM HEPES (Thermo Fisher Scientific), 1% Penicillin-Streptomycin (50 units/mL penicillin and 50 µg/mL streptomycin,

Thermo Fisher Scientific), and 1% GlutaMAX (Thermo Fisher Scientific). GEM is composed of DMEM (Gibco) supplemented with 10% FBS (Fetal Bovine Serum, Sigma), 2% B27 (Thermo Fisher Scientific), 50 mM HEPES (Thermo Fisher Scientific), 1% Penicillin-Streptomycin (50 units/mL penicillin and 50 µg/mL streptomycin, Thermo Fisher Scientific), and 1% GlutaMAX (Thermo Fisher Scientific). Additional 4 ml of NM:GM (3:1) mix media was added to the cells. Viable cell density was determined by mixing 10 µl of the cell suspension with 10 µl 0.4% Trypan blue and cell counting was performed using hemocytometer. Around 0.25×10^5 viable cells were plated on each well, and cells were incubated at 37 °C with 5% CO_2. Half of the media was replaced with fresh 3:1 NM:GEM mix media within 4-24 h after plating.

For neuronal infection, supernatant AAV virus mix encoding TREp-M-SPOTIT1.1-IRES-mCherry and Synapsin-tTA (20 µl of each virus) were added the neurons at DIV4-DIV8 (days in vitro). Seven days after infection, neurons were treated with fentanyl and endorphin at the concentrations indicated in Fig. 6 for 24 h and then fixed on ice and imaged at pH 9.

Analysis of Neuron images

For initial neuron testing, 5 fields of view of 60x magnification images were taken. For the titration curve, 5 fields of view of 20x magnification images were taken. This experiment was performed twice with similar results. NIS-Elements General Analysis 3 software was used to analyze the images. Data for the mean FITC intensity of the entire image was taken for each field of view. This value was subtracted by the mean FITC intensity of an empty well to adjust for the background of the laser. The mean and standard error of the mean (SEM) were calculated for each condition. Two-sided Student's t-tests were used to evaluate the significance between data points. Plots were made using Prism GraphPad software.