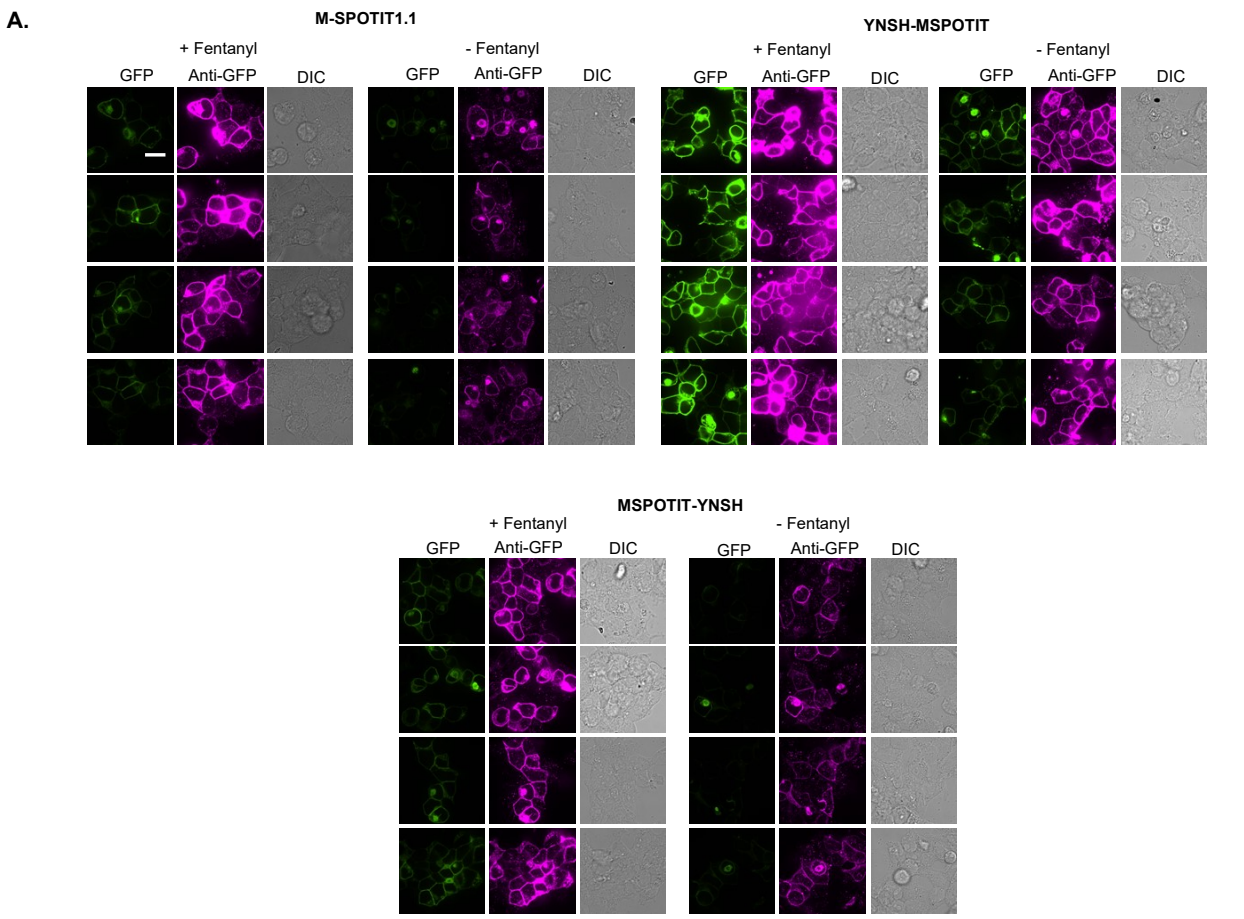
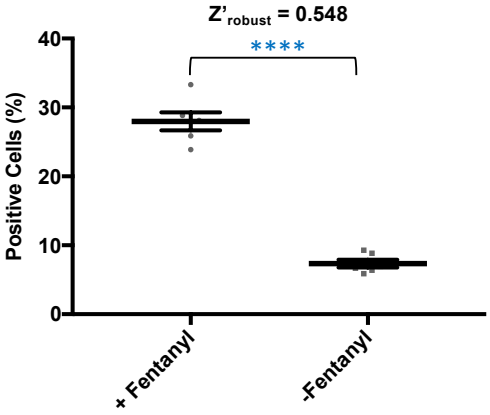
Electronic Supplementary Material (ESI) for ChemComm.

Supplementary Information



Supplementary Figure 1. (A) Four representative fields of view for Figure 2, Testing of M-SPOTIT1.1, YNSH-MSPOTIT, and MSPOTIT-YNSH in HEK293T cells. Cells were imaged 24-hours post-stimulation of 10 μM of fentanyl or complete media alone and imaged at pH 11. (B) Figure 2 with lower contrast. GFP, cpGFP signal. Anti-GFP, protein expression level. DIC, differential interference contrast. Scale bars, 20 μm.



Supplementary Figure 2. Z-factor calculation for M-SPOTIT2. Cells were imaged 24-hours post stimulation with 10 μM of fentanyl or DMSO. A high throughput fluorescent imager was used for imaging, and cells with GFP signal above a set threshold were counted as "positive cells." Percent positive cells are plotted on the y-axis.

Materials and methods

Plasmids and cloning

Constructs for HEK293T cell expression were cloned in an ampicillin-resistant lentiviral vector with a cytomegalovirus (CMV) promoter. Constructs for neuron expression were cloned in an ampicillin-resistant Adeno-associated virus (AAV) vector with a CAG promoter. Standard cloning procedures were used (Q5 polymerase PCR amplification, NEB restriction enzyme digest, and T4 ligation) and ligated plasmids were transformed into XL1-blue competent cells using heat shock transformation.

HEK293T cell culture

HEK293T cells were cultured in complete growth media at 37 °C under 5% CO_2. Complete growth media consists of: 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% penstrep (Gibco). The cells are passaged (plated in a new flask) once 80% confluence is reached.

HEK cell transfection with PEI MAX

PEI MAX (polyethyleneimine, Polysciences) was used for all transfection experiments.

Before plating the HEK293T cells, we incubated all flasks/plates with 20 μg/mL human fibronectin (Milipore Sigma) for 10 minutes at 37 °C. After removal of the fibronectin, HEK293T cells were plated so they would be 90% confluent after settling. Immediately after plating, HEK293T cells were transfected with a homemade PEI max solution mixed with DNA. For transfection of a 6-well plate, 1 μg of SPOTIT DNA and 10 μL of PEI max solution (1mg/mL in H_2O) in 100 μL DMEM were incubated for 10 minutes at room temperature (RT). After 10 minutes of incubation, the DNA-PEI mixture was added to the cell suspension that was just plated. For transfection of a T25 flask, multiply all amounts by 2.5. For

transfection of a T75 flask, multiple all amounts by 7.5. Cells were incubated at 37 °C with 5% CO_2 until re-plating and stimulation 24 hours later.

Lentivirus production

HEK293T cells were plated in a T75 flask pre-coated with human fibronectin following the steps outlined in "HEK cell transfection with PEI MAX." 7.5 μg sensor DNA, 0.75 μg pVSVG, and 6.75 μg Δ8.9 lentiviral helper plasmid were mixed in 600 μL of DMEM without FBS. Then, 75 μL of PEI max solution was added to the DNA mixture and the whole solution was mixed. After 10 minutes of incubation at room temperature, the solution was pipetted on top of the HEK293T cells that were just plated. After two days of incubation at 37 °C with 5% CO_2, the virus-containing 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 were plated at 40-60% confluence in plates/flasks pretreated with 20 μg/mL human fibronectin (Millipore Sigma). Virus quantities were added based on the volume of virus that would give a 100% infection efficiency. 40-48 hours after infection, HEK293T cells were re-plated and stimulated.

HEK cell stimulation

20-24 hours after transfection and 40-48 hours after infection, HEK293T cells were re-plated and stimulated. HEK293T cells were plated at 90-100% confluence in 24-well glass bottom plates (Corning) pre-treated with human fibronectin. Drugs were diluted in pre-warmed complete media to the desired concentration. 100 μL of the diluted drug was dropped on top of the cells immediately after plating. For stimulation with peptides, 2 μL of a protease inhibitor cocktail (Millipore Sigma) was added to the 24-well

plates prior to peptide stimulation. After stimulation, the cells were incubated at 37 °C with 5% CO_2 until 20-24 hours later.

HEK cell fixation and immunostaining

For fixation, media was removed from HEK293T cells and 200 μL of 4% formaldehyde in PBS (phosphate buffered saline) was added to the wells. After 20 minutes of fixative incubation, the fixative was removed, and the wells were washed. To permeabilize the cells, 200μL of -20 °C methanol was added. The cells were incubated at -20 °C for 5 minutes then washed. For immunostaining, chicken anti-EGFP (abcam, ab13970) was diluted in a 1% BSA in PBS to a concentration of 1:200 anti-GFP: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 and 200 μL of a 1:200 concentration of anti-chicken 647 (abcam, A21449) was added to each well. Again, the cells rocked for 30 minutes at RT with 5% CO_2 and were washed. 200 μL pH 11 CAPS buffer were added back to the cells, and the cells were imaged.

Confocal microscopy of HEK cells and Analysis of HEK293T cell images

For detailed information of the confocal microscope and analysis used in this paper, see Kroning and Wang, *Angew. Chemie Int. Ed.*, 2021. method section. Confocal microscopy was performed on a Nikon inverted confocal microscope and 20x air objectives and 60x oil immersion objectives were used for imaging. The mean FITC intensities and object areas (determined by an intensity threshold) were recorded. These two values were multiplied together to achieve the sum intensity value. The sum was subtracted by the object area multiplied by the mean intensity of a field of view with no cells. Only intensities above a set threshold were analyzed. This threshold was set a few intensity units above the autofluorescence of the cells. Fields of view with no cells (determined by object areas of "0") were omitted from analysis. 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.

M-SPOTIT initial testing

HEK293T cells were cultured and transfected with M-SPOTIT1.1 and M-SPOTIT2 DNA following the protocol outlined in "HEK cell transfection with PEI MAX." Following 24-hours of transfection, cells were plated in 24-well glass imaging plates and stimulated with 10 μM fentanyl or HEK cell media alone. 24-hours post plating and stimulation, cells were fixed, imaged, and immunostained following the "HEK cell fixation and immunostaining" protocol. Then, 10 images were taken per well and analysis was performed following "Confocal microscopy of HEK cells and Analysis of HEK293T cell images" protocol.

M-SPOTIT pH titration

HEK293T cells were cultured and infected with M-SPOTIT1.1 and M-SPOTIT2 lentiviruses following the **"**HEK293T cell infection**"** protocol**.** 24 hours post stimulation with fentanyl or a no drug control (HEK cell media alone), all cells were fixed and imaged. One field of view was imaged at different pH values. This was repeated 2 additional times for a total of 3 biological replicates. For M-SPOTIT1.1, the following pH values were used: 6, 7, 7.5, 8, 8.5, 9, and 10. For M-SPOTIT2, the following pH values were used: 6, 7, 8, 8.5, 9, 10, and 11. The following buffers were used pH 6: 1x PBS, pH 7: 1x PBS, 7.5 1x PBS, pH 8: 100 mM Tris-HCl, pH 8.5: 100mM 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. Analysis was performed following "Confocal microscopy of HEK cells and Analysis of HEK293T cell images" protocol. Curve was fitted using an agonist vs. response curve, four parameters with variable slope. pKa value was determined by the Prism software.

M-SPOTIT agonist titration

After plating M-SPOTIT2 infected cells in 24-glass bottom plates following the **"**HEK293T cell infection**"** protocol, cells were stimulated with 0.0001, 0.001, 0.01, 0.02, 0.1, 1, 10, and 50 μM of fentanyl. Three technical replicates were performed for each condition. Following 24-hours of incubation at 37 °C with 5% CO_2, HEK293T cells were fixed and imaged at pH 11 with a CAPS buffer. Five images were taken per technical replicate to give 15 images in total for each condition. Analysis was performed following "Confocal microscopy of HEK cells and Analysis of HEK293T cell images" protocol. Curve was fitted using an agonist vs. response curve, four parameters with variable slope. EC_{50} value was determined by the Prism software. Sensitivity was calculated as the slope of the linear region of the titration curve. Limit of detection was calculated by taking the mean of the lowest concentration (0.0001 μM) intensity value plus three times its standard deviation and then extrapolating the x-value for this calculation from the titration curve. Dynamic range was determined as the range of concentrations where the titration curve gave a linear response.

M-SPOTIT selectivity

Immediately after re-plating in 24-well glass imaging plates, cells were stimulated with 10 μM of different agonists diluted in complete HEK cell media or a DMSO and media control. 24 hours post stimulation, HEK cells were fixed, and pH 11 CAPS buffer was added to the wells. The cells were then imaged and analyzed following the "Confocal microscopy of HEK cells and Analysis of HEK293 cell images" protocol. 9-10 images were taken per condition.

AAV supernatant production for neuronal infection

A CAG promoter was used to express M-SPOTIT1.1 and M-SPOTIT2 in an AAV viral vector. AAV virus supernatant was prepared by PEI transfection, following the "HEK cell transfection with PEI MAX"

protocol. For each well of a 6-well plate, 0.35 μg AAV-M-SPOTIT2 and AAV-M-SPOTIT1.1, 0.29 μg AAV1 serotype, 0.29 μg AAV2 serotype plasmid, and 0.7 μg helper plasmid pDF6 was combined in 80 μL serum-free DMEM. Then, 10μL of PEI max was added to the DNA-DMEM mixture and mixed. After 10 minutes of incubation at room temperature, the mixture was pipetted onto 2mL of HEK293T cells plated in the fibronectin-pretreated 6-well plate. After 48 hours, the virus-containing supernatant was collected and spun down at 4000 rpm for 5 minutes to remove cell debris. Then, the supernatant was used for infection.

Neuronal culture experiments.

Half area 96-well glass plates (Corning, CLS4580-10EA) were coated with 50μl Poly-D-lysine (Gibco, 0.1mg/mL in water) for 2 hours. Then, the plates were washed twice with water. A tube of frozen rat cortical neurons (Thermo Fisher Scientific, Cat# A1084001) was partially thawed in a 37 °C water bath by swirling until there was only a small piece of ice. Then, the cells were transferred to a 50mL conical tube and 1mL of a pre-warmed 3:1 complete neurobasal media (NM) and glial enriching medium (GEM) mix was gently added to the cells while swirling the conical tube of neurons. Exact compositions of NM and GEM can be found in the method section of Kroning and Wang, *Angew. Chemie Int. Ed.*, 2021. An additional 4 ml of NM:GM (3:1) mix media was then added to the cells. 100 μl of neurons were plated. The next day, 50 μl of neuronal media was removed and 70 μl of fresh media was added back in. Five days after plating, neurons were infected. For infection, 20 μl of each virus was mixed with 20 μl of NGM and the full mixture was added to the well. Seven days after infection, neurons were stimulated with fentanyl or a media blank for 24 hours. Then, the neurons were fixed on ice and imaged at pH 11 with a CAPS buffer.

Analysis of Neuron images

Mean and object area FITC values were taken for 9 fields of view per condition at 20x magnification. Only intensities above a set threshold were analyzed. This threshold was 3 x the mean intensity of the uninfected condition. NIS-Elements General Analysis 3 software was used to analyze the images. The mean and object areas were multiplied together to get the sum intensity. This value was subtracted by the mean of an uninfected field of view. The mean and 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.

Z-factor calculation

HEK293T cells transfected with M-SPOTIT2 DNA were plated in a 384-well plate using a multi-channel automatic pipette. Six replicate wells were used for the 10μM fentanyl stimulated and DMSO alone conditions. One day after stimulation, cells were imaged using an MDC Image Express Micro imager. Total HEK293T cell quantities were counted during analysis. HEK293T cells with GFP fluorescence intensity above a set threshold were also counted; these cells were considered "positive cells." The number of positive cells were divided by the total number of cells and multiplied by 100 to obtain the "percent positive cells." The percent positive cells in both the fentanyl and DMSO conditions were used to calculate the Z'_{robust}.