Supporting Information

Imaging GPCR Internalization Using Near-Infrared Nebraska Red-Based Reagents

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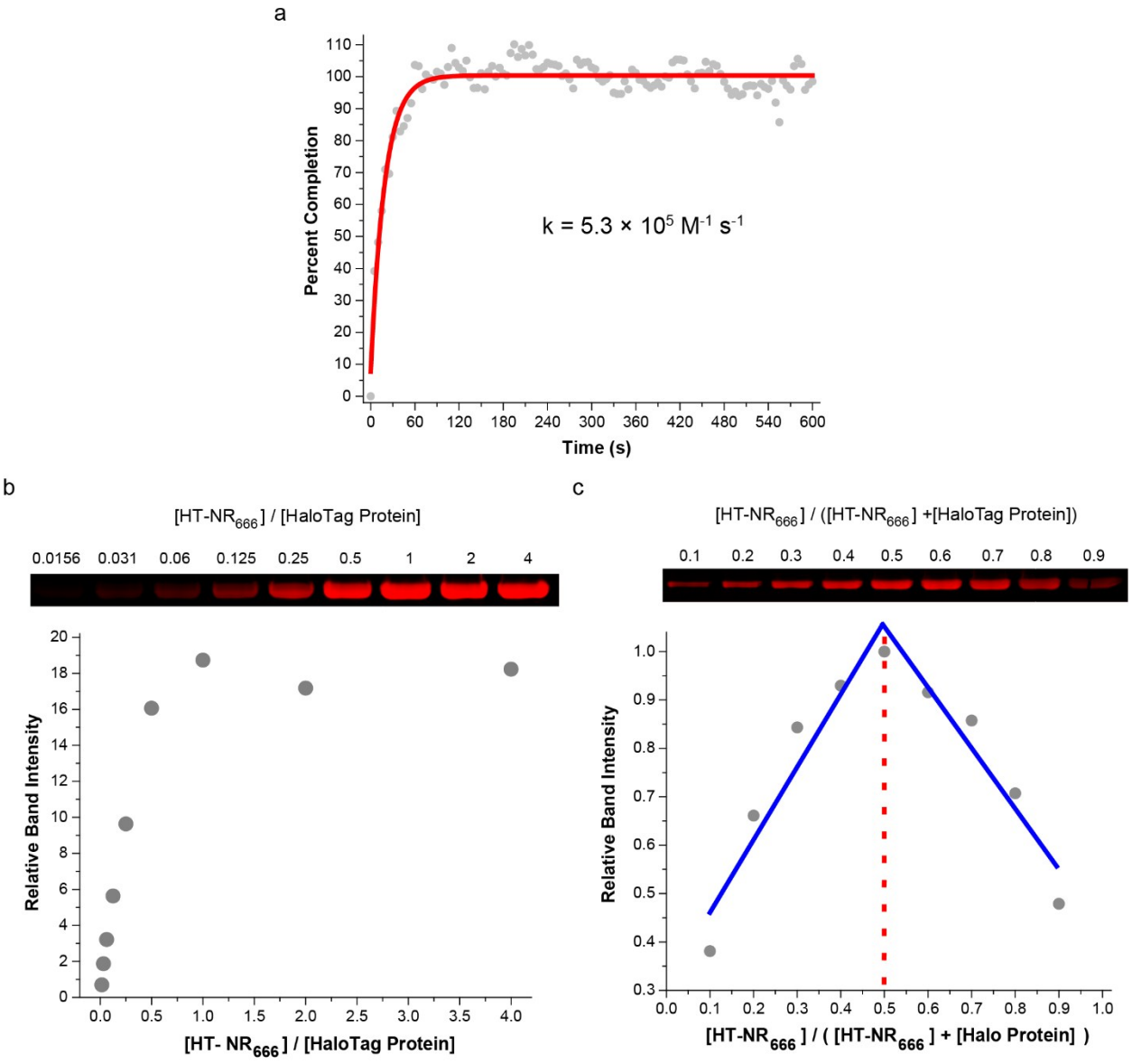
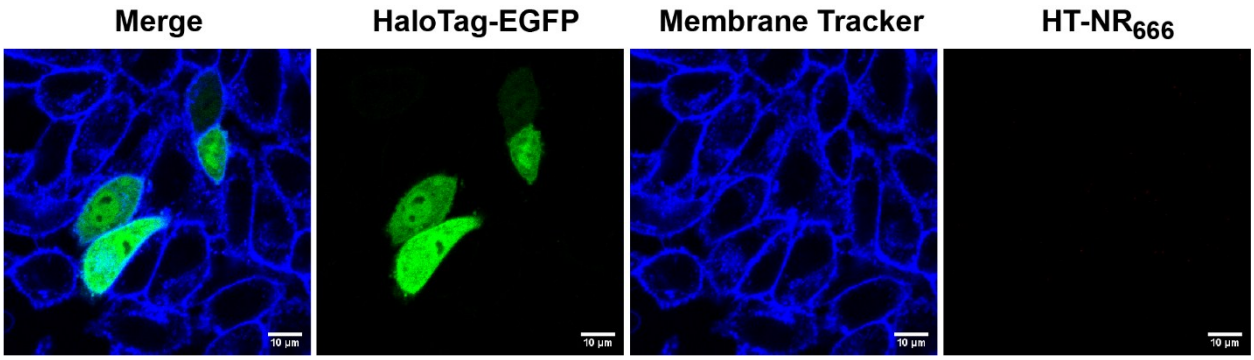
Figure S1



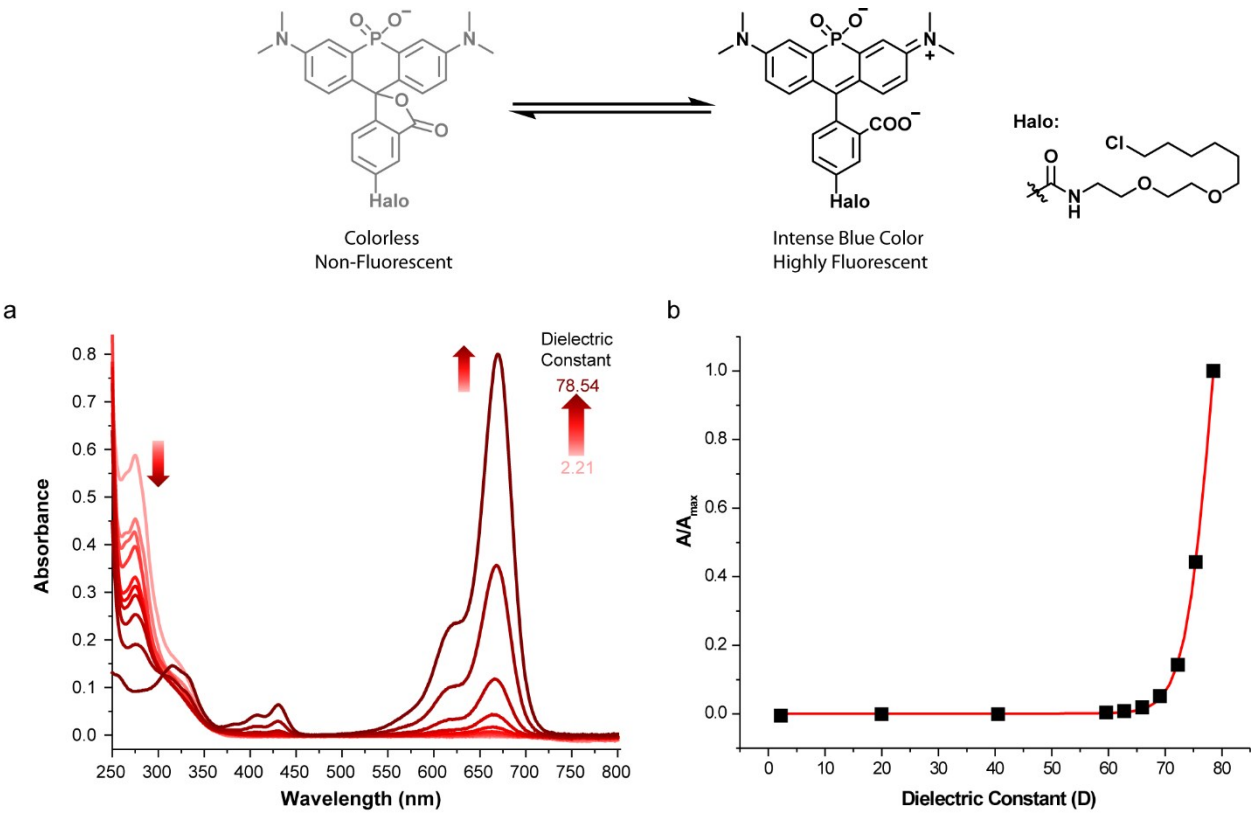
Evaluating HT-NR_{666} labeling of recombinant His_6-HaloTag protein *in vitro.* (a) Labeling kinetics measured by fluorescence polarization. SDS-PAGE gels imaged by in-gel fluorescence corresponding to labeling of His_6-HaloTag with increasing concentrations of HT-NR_{666} (b) as well as a Job's plot assay (c) demonstrate a 1:1 binding stoichiometry.

Figure S2



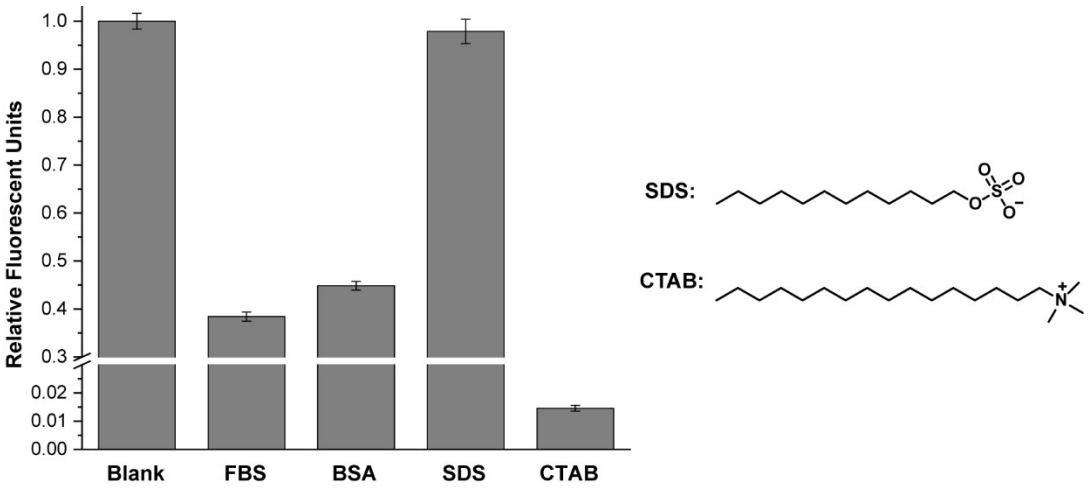
$HT\text{-}NR_{666}$ is not cell permeable. CHO cells were transfected with a HaloTag-EGFP construct and labeled with membrane tracker. After washing 3 times with DPBS, cells were loaded with 10 µM $HT\text{-}NR_{666}$ in HEPES buffered DMEM for 20 min. Images were taken in HEPES buffered DMEM after washing 3 times with DPBS.

Figure S3



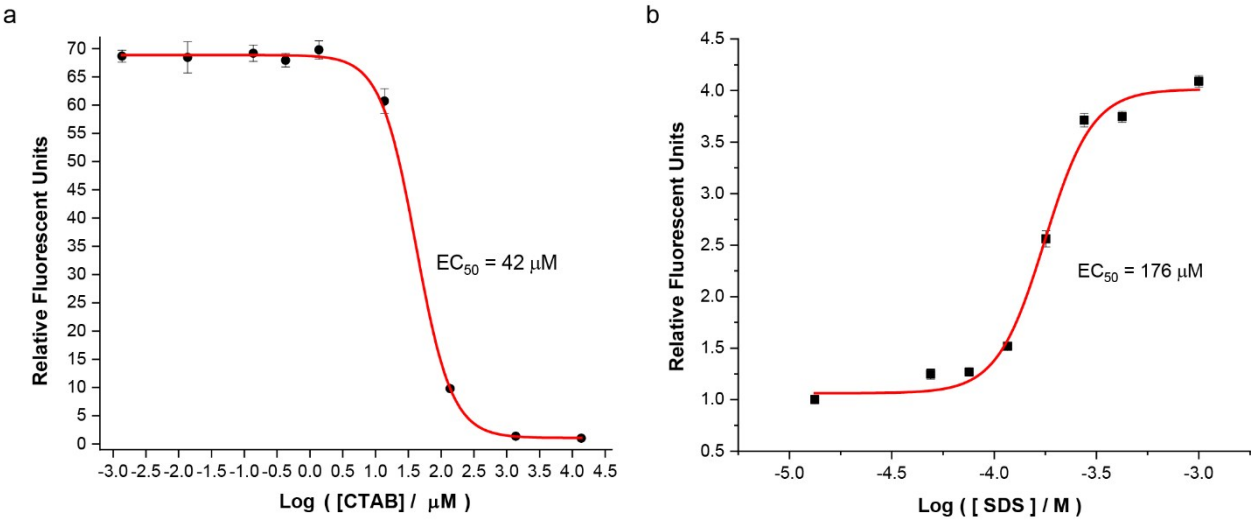
In vitro evaluation of the fluorogenic behavior for mHT-spiroNR$_{666}$. (a) Absorbance spectrum of 10 μM mHT-spiroNR$_{666}$ in water/dioxane mixtures with different dielectric constants. (b) The normalized absorption at 670 nm for mHT-spiroNR$_{666}$ across the indicated dioxane-water gradient.

Figure S4



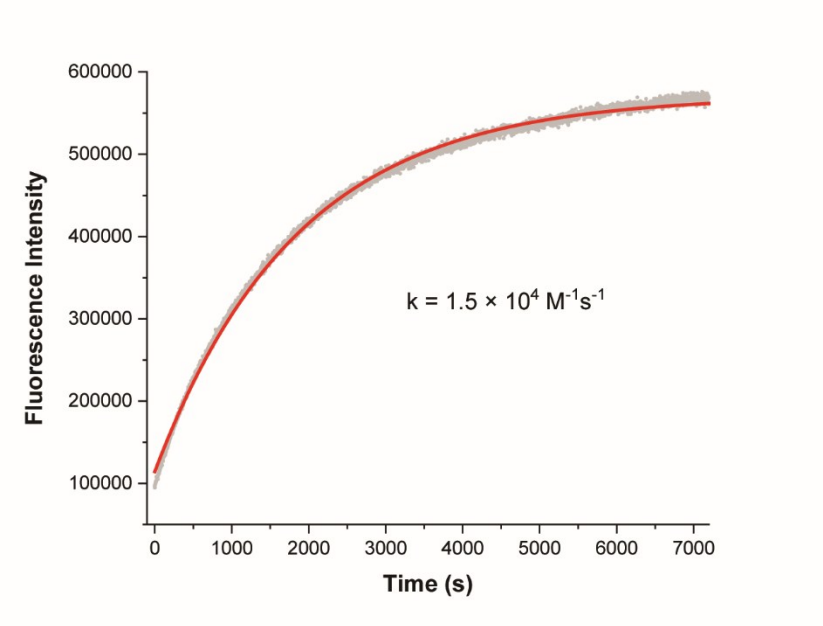
Normalized fluorescence intensity of mHT-spiroNR$_{666}$ (490 nM) in DPBS containing 10% FBS, BSA (2.3 mg/mL), SDS (1 mM), or CTAB (1 mM). Error bars represent the standard deviation of triplicate experiments.

Figure S5



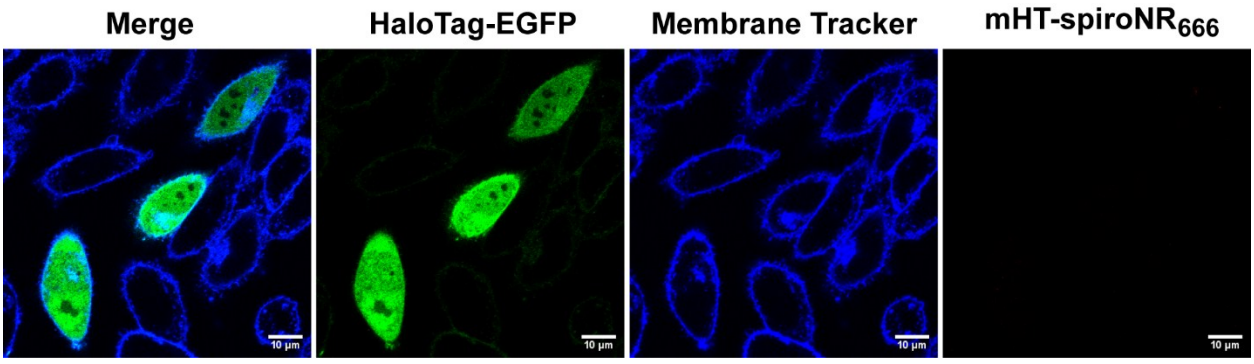
(a) The fluorescence of mHT-spiroNR$_{666}$ (490 nM) in the presence of increasing concentrations of CTAB. (b) The fluorescence of solutions containing BSA (2.3 mg/mL) and mHT-spiroNR$_{666}$ (490 nM) incubated with increasing concentrations of SDS. Error bars represent the standard deviation of triplicate experiments.

Figure S6



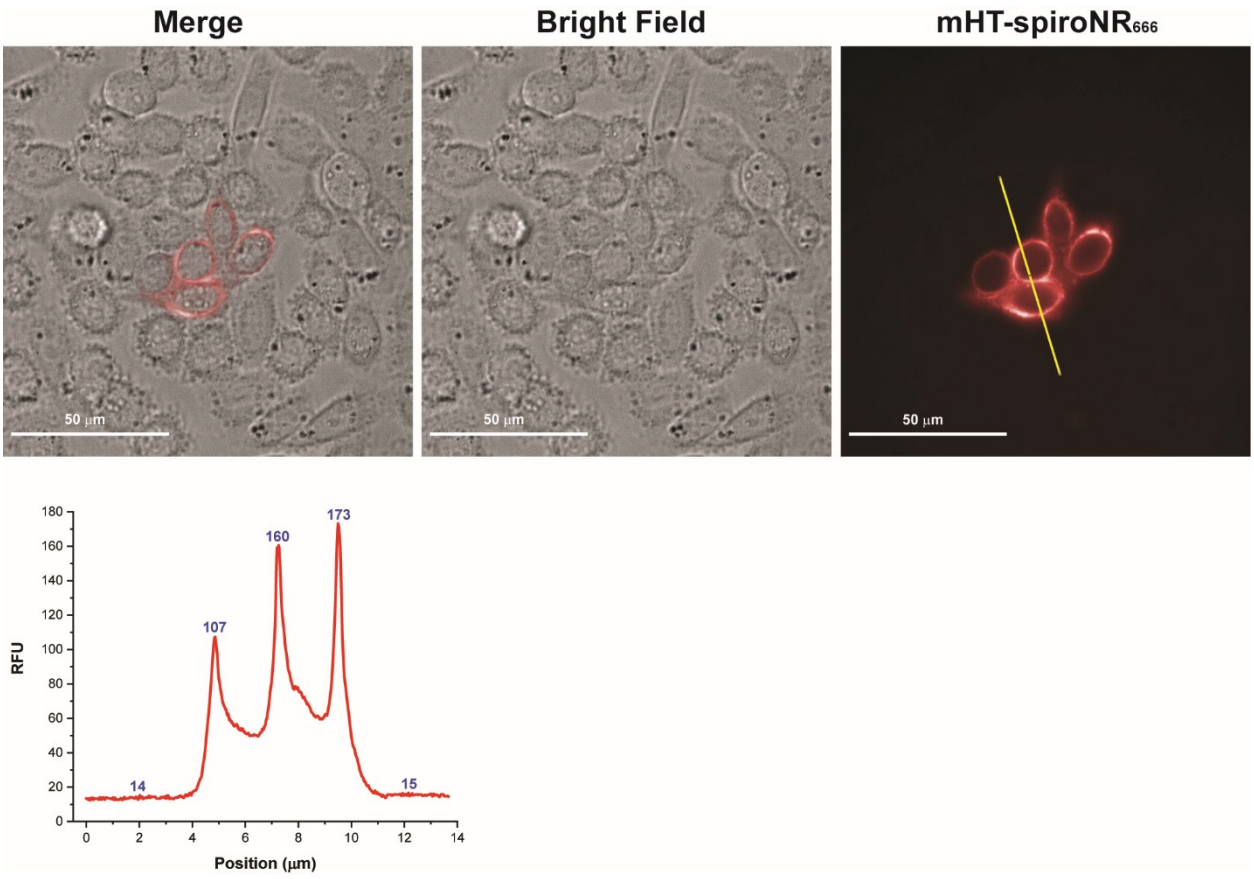
Labeling kinetics of mHT-spiroNR$_{666}$ (25 nM) with HaloTag-GST fusion protein (100 nM, Promega G4491) in PBS containing 10% FBS, measure by fluorescence turn-on (ex. = 669 nm and em. = 685 nm).

Figure S7



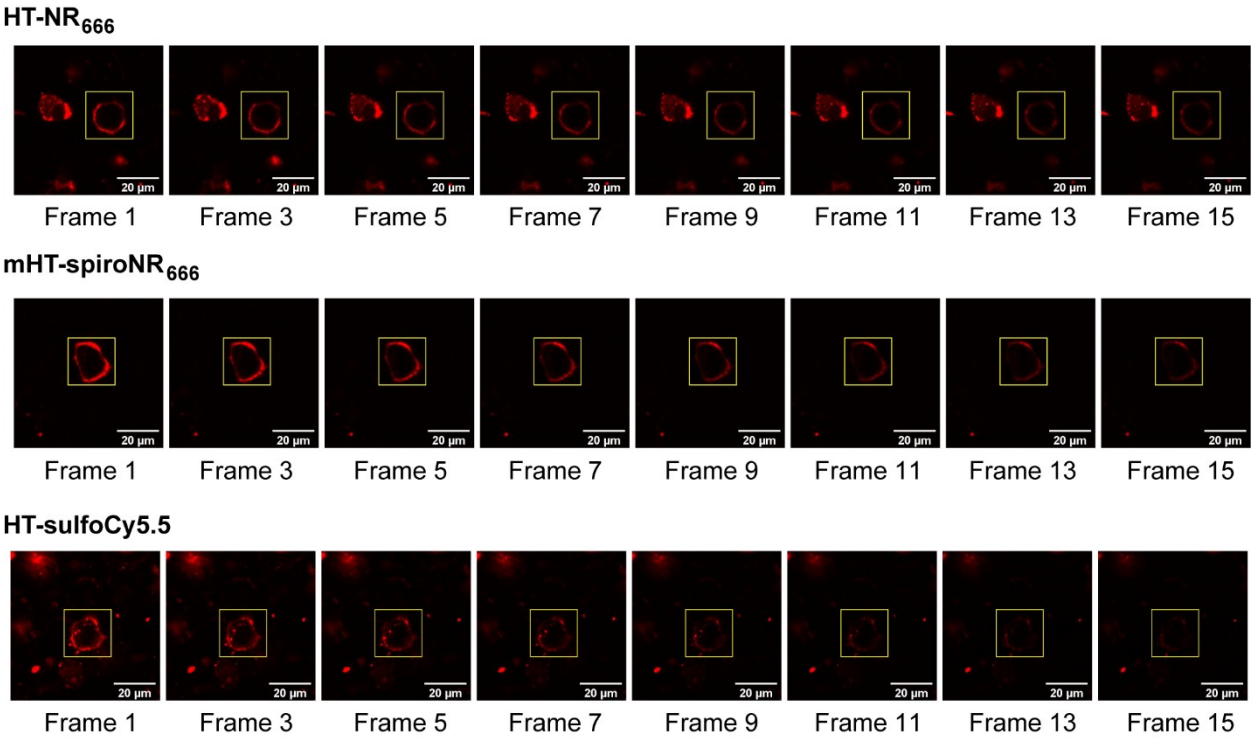
mHT-spiroNR$_{666}$ is not cell permeable. CHO cells were transfected with a HaloTag-EGFP construct and labeled with membrane tracker. After washing 3 times with DPBS, cells were loaded with 10 µM mHT-spiroNR$_{666}$ in HEPES buffered DMEM for 20 min. Images were taken in HEPES buffered DMEM after washing 3 times with DPBS.

Figure S8



mHT-spiroNR$_{666}$ produces a clear fluorogenic signal using no-wash epifluorescence imaging. Epifluorescence images of CHO cells transiently transfected with HaloTag-hOX2R and labeled with 100 nM mHT-spiroNR$_{666}$. Cells were grown on a 24-well, glass-bottom plate (MatTek, P24G-1.5-13-F) and imaged using the Alexa 633 channel of an Operetta CLS High-Content Imaging System (top). Fluorescence was quantified across the indicated region of interest (bottom). Scale bar: 50 μm.

Figure S9


HT-NR666
Frame 1
Frame 3
Frame 5
Frame 7
Frame 9
Frame 11
Frame 13
Frame 15
mHT-spiroNR666
HT-sulfoCy5.5
20 µm
HT-sulfoCy5.5
HT-NR666
mHT-spiroNR666
Relative Florescent Units
Frame
k = 0.203 frame-1
k = 0.217 frame-1
k = 0.298 frame-1

Comparison of photobleaching rates for HT-NR_{666}, mHT-spiroNR_{666}, and HT-sulfoCy5.5. CHO cells transiently transfected with HaloTag-hOX2R were labeled with the indicated probe (10 μM). Cells were washed and imaged using laser scanning confocal microscopy at 5 seconds per frame using a 640 nm laser (2 mW) until fluorescence signal was lost. Regions of interest (ROI) are indicated by yellow squares. These ROIs were analyzed in ImageJ and fluorescence intensities were normalized to time zero. Changes in fluorescence over time were then fit to a first-order decay curve (see methods) yielding the bleaching rate constants (k, $r^2 > 0.999$ for each fit).

Table S1

Photophysical properties of probes.

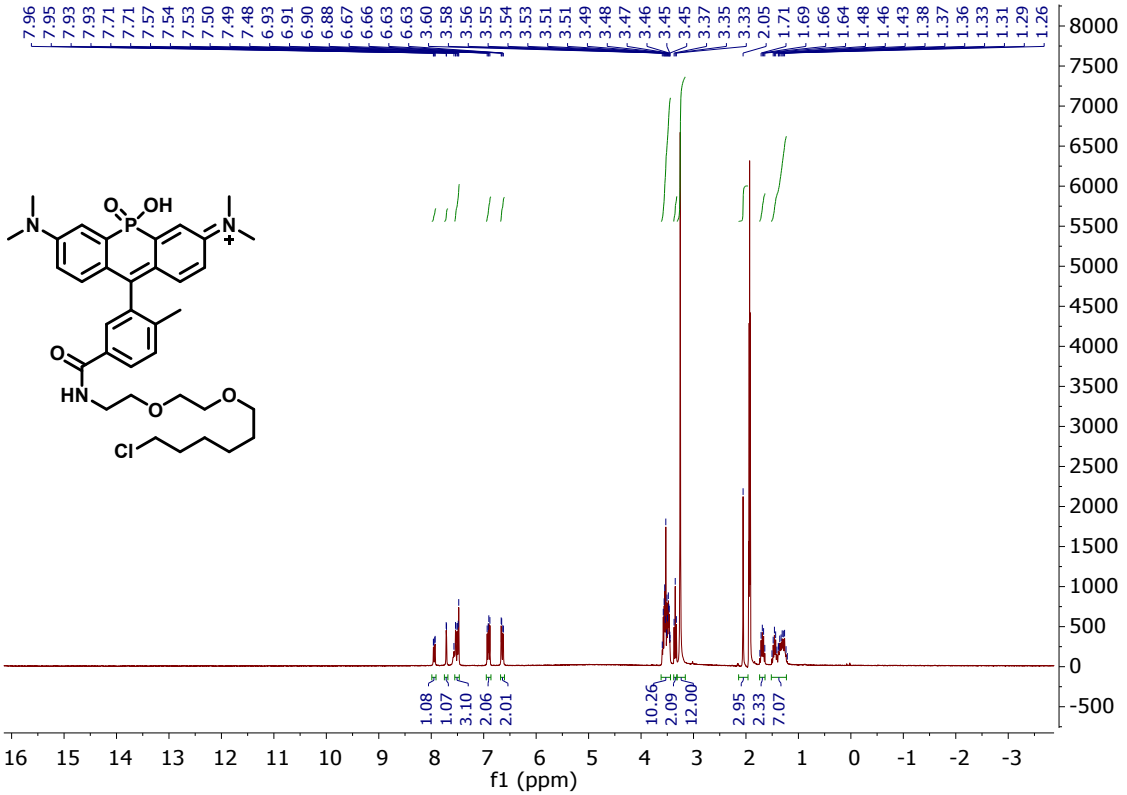
Dye	λ_{ab} (nm)	λ_{em} (nm)	ε ($M^{-1} \cdot cm^{-1}$)	φ
HT-NR_{666}	671	685	80,367	0.37
mHT-spiroNR_{666}	669	685	62,200	0.11

Measurements were conducted in PBS (pH = 7.4) with 0.5% DMSO. Sulfo-cyanine5.5 NHS ester (Lumiprobe) was used as the fluorescence reference standard (ex. = 670 nm, em. = 690 nm), which has a quantum yield of 0.237 in PBS at pH = 7.4.

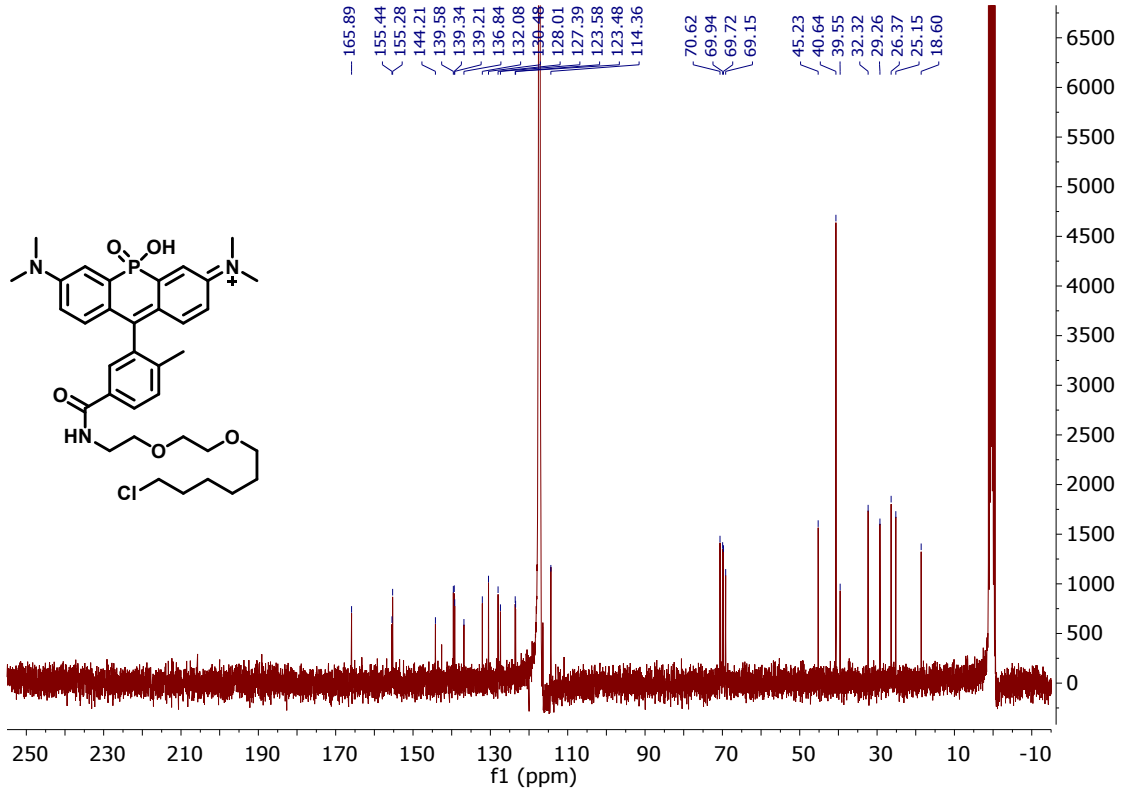
Movie Captions

Time-lapse live-cell imaging of CHO cells expressing HaloTag-hOX2R and labeled with HT-NR_{666} (10 μM). Cells were treated with orexin A (1 μM, Movie S1) to stimulate receptor internalization or with DMSO (control, Movie S2). Cells were imaged every 30 seconds for 32.5 minutes. Scale bar: 10 μm.

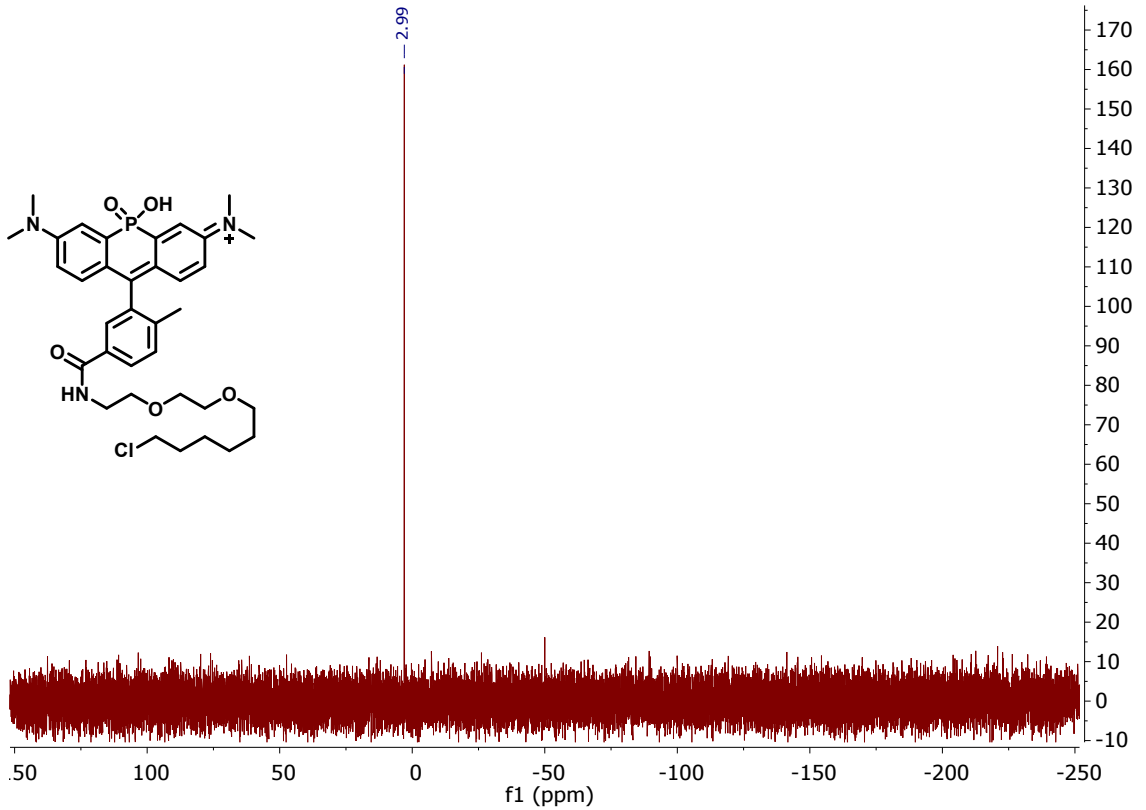
Copies of ^{1}H, ^{13}C, and ^{31}P NMR spectra.



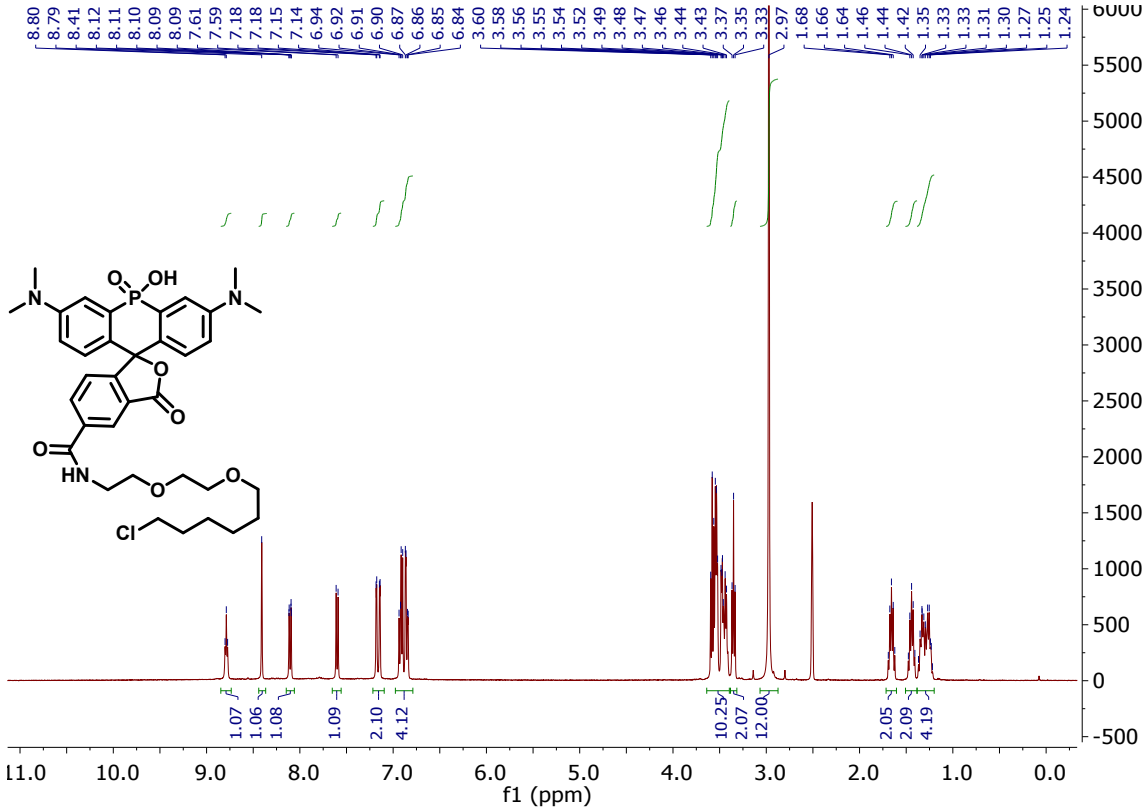
^{1}H NMR chart of HT-NR_{666} in CD_3CN (300 MHz).



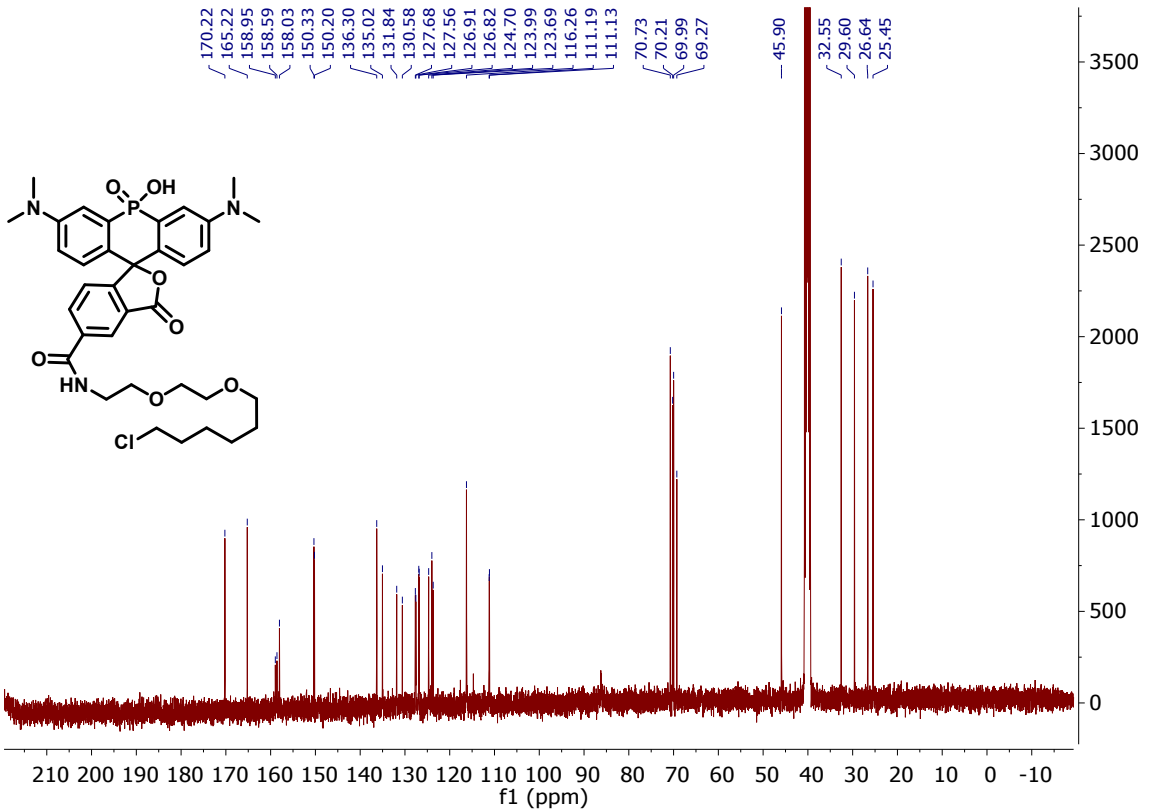
^{13}C NMR chart of HT-NR_{666} in CD_3CN (75 MHz).



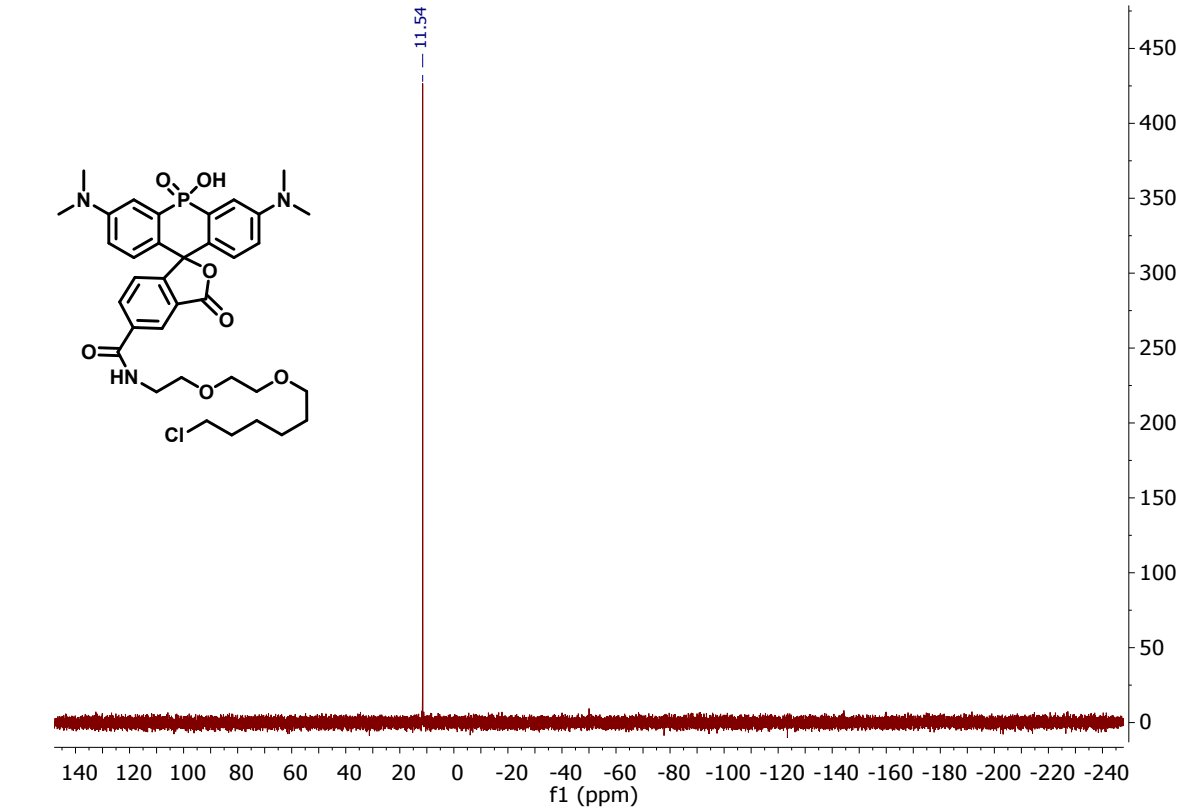
^{31}P NMR chart of HT-NR$_{666}$ in CD_3CN (121 MHz).



^{1}H NMR chart of mHT-spiroNR$_{666}$ in DMSO-d_6 (400 MHz).



^{13}C NMR chart of mHT-spiroNR$_{666}$ in DMSO-d$_6$ (101 MHz).



^{31}P NMR chart of mHT-spiroNR$_{666}$ in DMSO-d$_6$ (162 MHz).