Supporting information

Near-Infrared Fluorogenic Dimer Enables Background-Free Imaging of Endogenous GPCR in Living Mice

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1. Chemical Synthesis

1.1. General Information

Reagents were obtained from commercial sources and used without any further purification. Fmoc-NH-PEG2-COOH was obtained according to the described protocol.[1]

Solid-phase reactions were performed in polypropylene tubes equipped with polyethylene frits and polypropylene caps using an orbital agitator shaking device. Fmoc-protected Rink Amide AM resin (loading 0.7 mmol/g) was purchased from Iris Biotech. SPOrT resin was prepared as previously described.[2] The completion of couplings and Fmoc cleavages was monitored with the Kaiser test and the TNBS test.
Analytical reverse-phase high performance liquid chromatography (RP-HPLC) was performed on a C18 Sunfire column (5 μm, 4.6 mm × 150 mm) using a linear gradient (5% to 95% in 20 min, flow rate of 1 mL.min\(^{-1}\)) of solvent B (0.1% TFA in MeCN, v/v) in solvent A (0.1% TFA in H\(_2\)O, v/v). Detection was set at 220 and 254 nm. Semi-preparative RP-HPLC chromatography was performed on a SunFire C18 column (5 μm, 19 × 150 mm) using a gradient of solvent B (0.1% TFA in MeCN, v/v) in solvent A (0.1% TFA in H\(_2\)O, v/v) and a flow-rate of 20 ml.min\(^{-1}\). High resolution mass spectra (HRMS) were obtained on an Agilent Technologie 6520 Accurare-Mass Q.Tof LC/MS apparatus equipped with a Zorbax SB C18 column (1.8 μm, 2.1 × 50 mm) using electrospray ionization (ESI) and a time-of-flight analyzer (TOF). \(^1\)H and \(^{13}\)C NMR spectra were recorded on a Bruker Advance spectrometer (400 MHz for \(^1\)H spectra and 126 MHz for \(^{13}\)C) at 25 °C. Chemical shifts are reported in parts per million (ppm) relative to residual solvent and coupling constants (\(J\)) are reported in Hertz (Hz). Signals are described as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), br s (broad singlet) and br d (broad doublet).

1.2. Synthesis of Pegylated Cy5.5

![Chemical diagram](attachment:chemical_diagram.png)

25-chloro-2,5,8,11,14,17,20,23-octaoxapentacosane 4
Octaethylene glycol monomethyl ether (3.58 g, 9.32 mmol) was dissolved in DCM (20 mL) and pyridine (1.5 mL). To this solution was added thionyl chloride (1 mL) the solution was allowed to stir at 40°C overnight. The product was extracted with DCM and the organic phase was washed with HCl (1 M) then neutralised with a saturated solution of NaHCO₃ before being dried over anhydrous MgSO₄. The solution was filtered and evaporated to give 3.10 g of a yellowish oil (Yield = 83%). The TLC showed that the product was pure (Rf = 0.74, DCM/MeOH, 9/1). The product was used for the next step with no further purification.

1,1,2-trimethyl-3-(2,5,8,11,14,17,20,23-octaoxapentacosan-25-yl)-1H-benzo[e]indol-3-ium iodide 5

To a solution of 4 (3.00 g, 7.46 mmol) in ACN (20 mL) was added trimethyl-1H-benzo[e]indole (2.00, 9.57 mmol, 1.3 eq) followed by sodium iodide (5.00, 33.55 mmol, 4.5 eq). The solution was allowed to stir at 120 °C overnight. The deep blue solution was evaporated and the product was extracted with DCM and the organic phase was washed with water three times before being dried over anhydrous MgSO₄. The solution was filtered and evaporated and the product was solubilised in a minimum of acetone and was poured in ether. The precipitation step was repeated until the TLC indicated that the product was pure. Rf = 0.37 (DCM/MeOH, 96/4). 2.17 g of 5 were obtained as a blue oil (Yield = 41%). ¹H-NMR (400 MHz, CDCl₃): δ 8.10-8.01 (m, 4H, H Ar), 7.73 (t, J = 7.6 Hz, 1H, H Ar), 7.65 (t, J = 7.6 Hz, 1H, H Ar), 5.13 (t, J = 4.9 Hz, 2H, CH₂N⁺), 4.10 (t, J = 4.9 Hz, 2H, CH₂ PEG), 3.64-3.53 (m, 22H, CH₂ PEG), 3.46 (m, 6H, CH₂ PEG), 3.36 (s, 3H, OMe PEG), 3.14 (s, 3H, CH₃ indolenine), 1.86 (s, 6H, 3 CH₃). ¹³C-NMR (101 MHz, CDCl₃): δ 197.7 (CN⁺), 138.2 (C Ar), 136.7 (C Ar), 133.6 (C Ar), 131.3 (C Ar), 130.0 (C Ar), 128.4 (C Ar), 127.7 (C Ar), 127.4 (C Ar), 122.7 (C Ar), 113.2 (C...
Ar), 71.9, 70.5, 70.5, 70.5, 70.4, 70.4, 70.3, 70.2, 70.2, 67.3, 58.9, 55.9, 50.5, 30.9, 22.5, 16.5. HRMS (ESI+), calcd for C_{32}H_{50}NO_{8}^+[M+] 576.3531, found 576.3524.

1,1-dimethyl-3-(2,5,8,11,14,17,20,23-octaoxapentacosan-25-yl)-2-((1E,3E)-4-(N-phenylacetamido)buta-1,3-dien-1-yl)-1H-benzo[e]indol-3-ium iodide 6

To a solution of 5 (1.00 g, 1.42 mmol) and malonaldehyde dianilide hydrochloride (0.40 g, 1.55 mmol, 1.1 eq) in acetic anhydride (10 mL) was added 1 mL of acyl chloride. The solution was allowed to stir at 100 °C before being evaporated. The crude was purified by column chromatography on silica gel (DCM/MeOH, 9/1) to obtain 1.00 g of 6 as a dark syrup (Yield = 80%). Rf = 0.62 (DCM/MeOH, 9/1). The product was involved in the next step without further characterization.

2-((1E,3E,5E)-5-(3-(5-carboxypentyl)-1,1-dimethyl-1,3-dihydro-2H-benzo[e]indol-2-ylidene)penta-1,3-dien-1-yl)-1,1-dimethyl-3-(2,5,8,11,14,17,20,23-octaoxapentacosan-25-yl)-1H-benzo[e]indol-3-ium iodide 2

6 (1.00 g, 1.14 mmol) and C6-Indo[3] (500 mg, 1.23 mmol, 1.1 eq) were dissolved in pyridine (15 mL) and the solution was allowed to stir at 60 °C for 1 h. The solvents were evaporated and the product was extracted with DCM and the organic phase was washed with HCl (1 M) before
being dried over anhydrous MgSO₄. The solution was filtered and the crude was purified by column chromatography on silica gel (DCM/MeOH, 99/1 to 85/15) to obtain 490 mg of 2 as a dark blue syrup (Yield = 40%). Rf = 0.57 (DCM/MeOH, 9/1). ¹H-NMR (400 MHz, CDCl₃): δ 8.42-8.33 (m, 2H, H Ar), 8.19-8.17 (m, 2H, H Ar), 7.95-7.89 (m, 4H, H Ar), 7.60 (dd, J = 9.9, 5.4 Hz, 2H, H Ar), 7.48 (dt, J = 16.8, 8.4 Hz, 3H, H Ar), 7.39-7.37 (m, 1H, H Ar), 6.96-6.90 (m, 1H, H Ar), 6.55-6.52 (m, 1H, H Ar), 6.33 (d, J = 13.6 Hz, 1H, H Ar), 4.51-4.48 (m, 2H, CH₂), 4.20-4.16 (m, 2H, CH₂), 4.01-3.99 (m, 2H, CH₂), 3.65-3.51 (m, 32H, CH₂ PEG), 3.37 (s, 3H, OMe PEG), 2.45-2.42 (m, 2H, CH₂), 2.10 (s, 12H, 4 CH₃), 1.92-1.87 (m, 2H, CH₂), 1.78-1.74 (m, 2H, CH₂), 1.62-1.58 (m, 2H, CH₂). ¹³C-NMR (126 MHz, CDCl₃): δ 174.8 (CN), 174.1 (CN), 152.8 (CO), 140.0 (C Ar), 139.2 (C Ar), 134.0 (C Ar), 133.7 (C Ar), 131.7 (C Ar), 131.7 (C Ar), 130.5 (C Ar), 130.1 (C Ar), 129.9 (C Ar), 129.9 (C Ar), 128.2 (C Ar), 128.0 (C Ar), 127.7 (C Ar), 127.5 (C Ar), 126.5 (C Ar), 126.5 (C Ar), 125.0 (C Ar), 124.9 (C Ar), 122.3 (C Ar), 111.5 (C Ar), 110.4 (C Ar), 104.0 (C Ar), 103.2 (C Ar), 71.7, 71.0, 70.4, 70.3, 70.3, 70.3, 70.2, 68.3, 59.0, 51.2, 51.2, 51.2, 45.0, 45.0, 45.0, 44.3, 33.9, 27.8 (2 CH₃), 27.8 (2 CH₃), 27.1 (CH₂), 26.2 (CH₂), 24.3 (CH₂), 24.3 (CH₂), 24.3 (CH₂), 24.3 (CH₂). HRMS (ESI) calcd for C₅₆H₇₅N₂O₁₀ ([M-\text{I}⁺]: 935.5422; found: 935.5393.

2. Absorption and Fluorescence Spectroscopy

2.1. General information

Absorption spectra were recorded on a Cary 4000 spectrophotometer (Varian) and fluorescence spectra on a Fluoromax 3 (Jobin Yvon, Horiba) spectrofluorometer. Fluorescence emission spectra were systematically recorded at 630 nm excitation wavelength at 20 °C. All fluorescence spectra were corrected for instrumental effects. Fluorescence quantum yields (QY) were measured using Rhodamine 800 in EtOH as a reference (QY = 25%)."[^4]
2.2. Results

**Figure S1.** Absorption (left) and fluorescence (right) spectra of dCy5.5-PEG (200 nM) in different solvents.

**Figure S2.** Absorption (left) and fluorescence (right) spectra of mCy5.5-PEG (400 nM) in different solvents.

**Figure S3.** Normalized absorption spectra of the cyanine 2 and dCy5.5-PEG in MeOH.
3. Fluorescence Confocal Microscopy

3.1. Cell Lines, Culture Conditions and Treatment

HEK293 cells expressing the GFP-fused oxytocin receptor (GFP-OTR) and wild-type HEK293 cells were cultured in Eagle’s minimal essential medium (MEM, Invitrogen 21090) with 10% of heat-inactivated fetal bovine serum, 100 U/mL of penicillin, 100 μg/mL of streptomycin, 2 mM of glutamine and 50 μg/mL of hygromycin B for GFP-OTR cells at 37 °C in a humidified 5% CO₂ atmosphere. 70-80% cell confluence was maintained by removal of a portion of the culture and replacement with fresh medium twice a week. For confocal microscopy studies, cells were seeded onto 35 mm ibiTreat Ibidi Polymer Coverslip at a density of 100 000 cells/Ibidi 24 h before microscopy.

3.2. Confocal Microscopy Experiments

Cells were washed two times by gentle rinsing with Hank's Balanced Salt Solution (HBSS, no phenol red), then solutions of fluorescent ligands at 10 nM in HBSS (1 mL) were added and the cells were incubated for 5 min at room temperature. For competition experiments, a mixture of 10 nM of fluorescent ligands and 2 μM of carbetocin was used. Fluorescence confocal microscopy experiments were performed on a Leica TCS SPE-II microscope with a HXC PL APO 63x/1.40 OIL CS objective. The excitation of Hoechst 33342 was performed with a 405 nm 10 mW laser and the emission was detected around 450-500 nm. The excitation of GFP was performed with a 488 nm 10 mW laser and the emission was detected around 500-550 nm. The excitation of Cy5.5 was performed with a 635 nm 18 mW laser and the emission was detected around 670-750 nm. Image treatment was proceeded using ImageJ (Wayne Rasband, National Institute of Mental Health, Bethesda).

![Figure S4.](image-url) Confocal microscopy study of dCy5.5-PEG-CBT on living HEK 293 cells expressing OTR-GFP fusion (A: GFP channel, B: Cy5.5 channel) and unmodified HEK 293 cells (C: Cy5.5 channel).
channel) under no-wash conditions. Cells were incubated with the fluorescent ligand (10 nM) for 5 min at room temperature prior to the imaging. The membrane GFP fluorescence is shown in green, the fluorescence of dCy5.5-PEG-CBT is shown in magenta, the nucleus is shown in blue (Hoechst 33342 staining, 5 µg/mL). Excitation/emission wavelengths: 405 nm / 450-500 nm for Hoechst 33342; 488 nm / 500-550 nm for GFP and 635 nm / 680-750 nm for Cyanines 5.5.

4. Small Animal Fluorescence Imaging

4.1. Animals

Twelve-week-old pregnant female Swiss mice were purchased from Janvier Laboratories (France). Animals were maintained under controlled environmental conditions (20 ± 2 °C) with a relative humidity (50 ± 10%) and a 12 hour light/dark cycle in Individually ventilated cages (GM500, Techniplast) with bedding made from spruce wood chips (Safe, villeAugy, France) and enriched with nestlets. Food (autoclavable diet, D04, Safe, France) and tap water were available ad libitum. Animal experimentation was conducted with the approval of the French ministry of agriculture and the Ethics local committee for animal experimentation of the Strasbourg University (CREMEAS) under the authorization number #11974-2017103010101372.

4.2. In Vivo Fluorescence Biodistribution Study

Animal fluorescence imaging was performed using a luminograph (NightOwl, Berthold Technologies). Lactating mice 11 days after delivery were anesthetized intraperitoneally (Ketamine 150 mg/kg, xylazine 10 mg/kg). Fluorescent compounds (100 µL containing 7.5 nmol in 0.9 % NaCl) with or without non-fluorescent carbetocin (100 µL containing 450 nmol in 0.9 % NaCl) were administered intravenously (tail vein). Mice were placed in the luminograph (30 min after intravenous administration of the probes), and positioned in decubitus dorsal. Mice were imaged using a halogen lamp (75 W, 340–750 nm) and emission of the dyes was recorded using a 630/700 nm filter. The experiments were repeated on three mice.
**Figure S5.** Ex vivo images of the organs of the mice, injected i.v. with 7.5 nmol of dCy5.5-PEG-CBT (A) or 7.5 nmol of dCy5.5-PEG-CBT and 450 nmol of CBT (B): mammary gland (1), liver (2), lungs (3), breast (4), kidney (5), spleen (6), brain (7) and uterus (8).

5. References

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6. NMR spectra

$^1$H NMR spectrum of 5

$^{13}$C NMR spectrum of 5
$^1$H NMR spectrum of 2

$^{13}$C NMR spectrum of 2