

Supplement
Materials and Methods

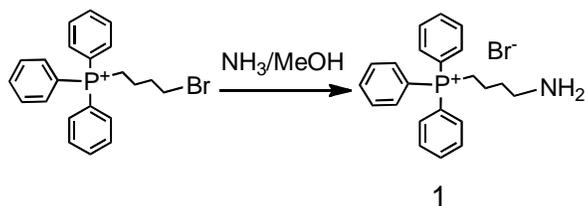
Title: Fluorescent and Radiolabeled Triphenylphosphonium Probes for Imaging Mitochondria

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Materials and Methods

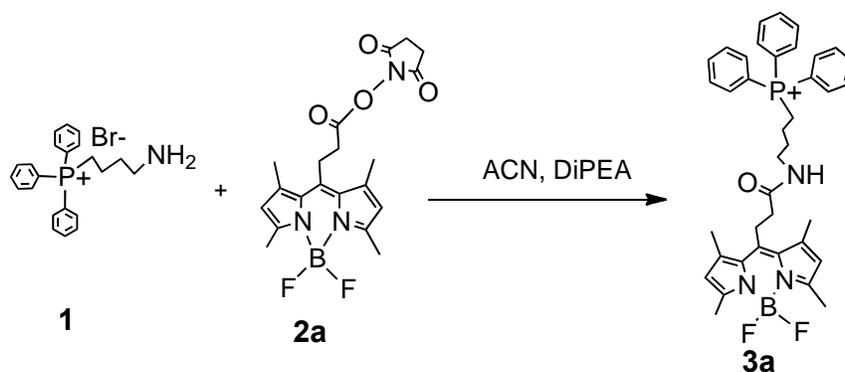
For TTP-Fluorochrome probe synthesis. (4-aminobutyl)triphenylphosphonium bromide was from Sigma-Aldrich. MBG-NHS ester **2a** [BODIPY® 493/503, SE, 4,4-Difluoro-1,3,5,7-Tetramethyl-4-Bora-3a,4a-Diaza-s-Indacene-8-Propionic Acid, Succinimidyl Ester (D-2191)] and BR-NHS ester **2b** [4,4-difluoro-5-(2-pyrrolyl)-4-bora-3a,4a-diaza-s-indacene-3-propionic acid, succinimidyl ester (D-2225)] were from Invitrogen. Cy5.5 NHS ester (47020) **2c** was from Lumiprobe. All the other solvents and chemicals were from Sigma-Aldrich and used without further purification. Compounds were characterized by mass spectra and ¹H NMR. Molecular weights were obtained by MS-ESI Micromass (Waters). NMR was recorded on a 11.7t-Vnmrs500 spectrometer in chloroform-d₃. RP-HPLC purification (Varian ProStar detector and delivery modules) employed an eluant A (0.1% TFA /water) and eluant B (0.1% TFA in 9.9% water in acetonitrile).

Synthesis of 4-aminobutyltriphenylphosphonium bromide (1):



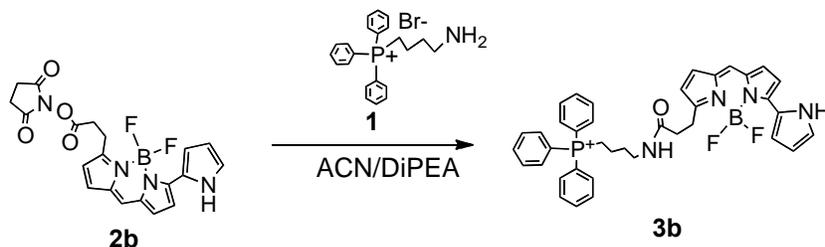
4-Aminobutyltriphenylphosphonium bromide was prepared as described ¹. Briefly, a solution of (4-bromobutyl)triphenyl-phosphonium bromide (1g, 2.512 mmol) in ammonia methanol solution (7N, 100 mL, 700 mmol) was incubated under room temperature in a sealed flask for 3 days. After evaporation of the solvent the residue was viscous liquid. After vacuum drying, a colorless foam was obtained and was used without further purification as described ¹.

Synthesis of TPP-Green (3a): 4-Aminobutyltriphenyl-phosphonium bromide was reacted with Green-NHS ester [BODIPY® 493/503, SE, 4,4-Difluoro-1,3,5,7-Tetramethyl-4-Bora-3a,4a-Diaza-s-Indacene-8-Propionic Acid, Succinimidyl Ester] **2a**.



To a solution of Green-NHS ester **2a** (5mg, 0.012 mmol) and TPP-Amine **1** (5.9mg, 0.012 mmol, 1 eq.) in anhydrous acetonitrile (1 ml), was added DIPEA (2.0 μ l, 0.012 mmol). The mixture was incubated under RT for 2hr. HPLC was employed for purification with a condition of: gradients: 20% B to 100%B in 15min, back to 20%B in 2min, and isocratic for 3min; column: Higgins Analytical Inc. Clipeus C18 10um, 250x20mm, P/N: CS-2520-C181, S/N: 186532; UV: 500nm; flow: 15ml/min. The major peak around 15min was found to be the right product. After lyophilization, **3a** was obtained as a brown red powder. Yield: 8mg, 89%. MS: $C_{38}H_{42}BF_2N_3OP^+$: Cal. 636, found 636 (M^+). 1H NMR ($CDCl_3$, ppm): 8.36 (br, 1H), 7.65-7.82 (m, 15H), 6.03 (s, 2H), 3.41-3.44 (m, 2H), 3.34-3.40 (m, 2H), 3.23-3.27 (m, 2H), 2.50 (s, 6H), 2.45-2.48 (m, 2H), 2.43 (s, 6H), 1.82-1.85 (m, 2H), 1.68-1.73 (m, 2H).

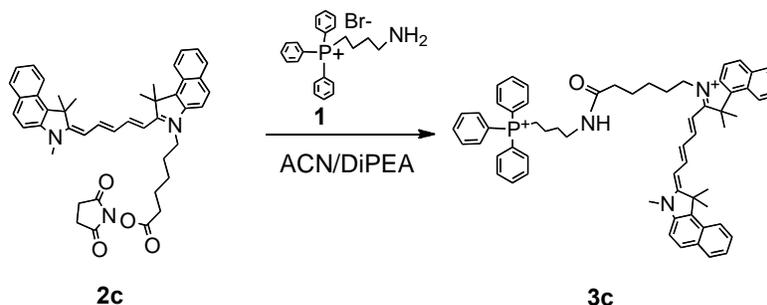
Synthesis of TPP-Red 3b: 4-Aminobutyltriphenyl-phosphonium bromide **1** was reacted with Red-NHS ester [BODIPY® 576/589, 4,4-difluoro-5-(2-pyrrolyl)-4-bora-3a,4a-diaza-s-indacene-3-propionic acid, succinimidyl ester] **2b**.



To a solution of Red-NHS ester **2b** (Bodipy 576/589, 5mg, 0.01173mmol) and TPP-Amine **1** (5.8mg, 0.01173 mmol, 1 eq.) in anhydrous acetonitrile (1 ml), was added DIPEA (2.0 μ l, 0.012mmol). The mixture was incubated under RT for 2hr. HPLC was

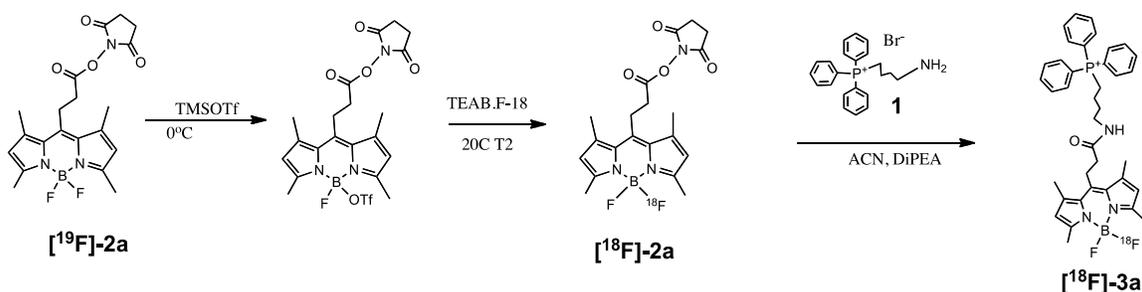
employed for purification with a condition of: column: Higgins Analytical Inc. Clipeus C18 10um, 250x20mm, P/N: CS-2520-C181, S/N: 186532; Gradients: 20% B to 100%B in 15min, back to 20%B in 2min, and isocratic for 3min; UV: 570nm; flow: 15ml/min. Major PK around 14-15min was found to be the right product. **3b** was obtained as a dark red powder after lyophilization. Yield: 5.5 mg, 73%. MS: $C_{38}H_{37}BF_2N_4OP^+$, cal. 645.28, found 645.10 (M^+). 1H NMR ($CDCl_3$, ppm): 10.42 (s, 1H), 7.62-7.75 (m, 15H), 7.10 (m, 1H), 6.99 (d, 1H, $J = 4.5Hz$), 6.94 (s, 1H), 6.92 (m, 1H), 6.81 (d, 1H, $J = 4.5Hz$), 6.78 (d, 1H, $J = 4Hz$), 6.31 (m, 1H), 6.30 (d, 1H, $J = 4Hz$), 3.33-3.40 (m, 4H), 3.23 (t, 2H, $J = 7.8Hz$), 3.0 (b, 1H), 2.63 (t, 2H, $J = 8Hz$), 1.80 (m, 2H), 1.68 (m, 2H).

Synthesis of TPP-NIR (3c): a-Aminobutyltriphenyl-phosphonium bromide **1** was reacted with Cy5.5-NHS ester [Lumiprobe] **2c**. The Lumiprobe Cy5.5 lacks sulfate groups.



To a solution of NIR-NHS ester **2c** (5.42mg, 0.008 mmol, Lumiprobe) and TPP-Amine **1** (5.7 mg, 0.0115 mmol, 1.4 eq.) in anhydrous acetonitrile (1 ml), was added DiPEA (2 μ l, 0.012mmol). The mixture was incubated under RT for 3hr. HPLC was employed for purification with a condition of: column: Higgins Analytical Inc. Clipeus C18 10um, 250x20mm, P/N: CS-2520-C181, S/N: 186532; Gradients: 30% B to 100%B in 15min, back to 30%B in 3min, and isocratic for 3min. UV: 674nm, flow: 15ml/min. The peak around 15min was found to be the right product. After lyophilization, **3c** was obtained a blue powder. Yield: 5.5 mg, 76%. MS: $C_{62}H_{66}N_3OP^{2+}$: cal. 899.49, found: 449.90 (M^{2+}) (double charges). 1H NMR ($CDCl_3$, ppm): 8.04-8.14 (m, 5H), 7.91 (d, 4H, $J=8.5Hz$), 7.75 (b, 3H), 7.63-7.66 (m, 12H), 7.60 (t, 2H, $J=8Hz$), 7.45 (t, 2H, $J=7.5Hz$), 7.35 (dd, 2H, $J_1=13Hz$, $J_2=9Hz$), 6.31 (t, 1H, $J=12Hz$), 6.18 (d, 2H, $J=13Hz$), 4.06 (t, 2H, $J=8Hz$), 3.69 (s, 3H), 3.30 (b, 4H), 2.22 (t, 4H, $J=7.5Hz$), 1.98 (s, 6H), 1.96 (s, 6H), 1.78-1.81 (m, 4H), 1.66 (m, 4H), 1.46 (m, 2H).

Synthesis of [^{18}F] TPP-Green [^{18}F]-3a:



The synthesis of $[^{18}\text{F}]\text{-2a}$ was as described² with minor modifications consisting of replacing the phase transfer agent with tetraethylammonium hydrogen carbonate, increasing the amounts of $[^{18}\text{F}]$ (15mCi) and $[^{19}\text{F}]\text{-2a}$ (1.2 μmol).

Synthesis of $[^{18}\text{F}]\text{-3a}$ was carried out by incubating the solution of $[^{18}\text{F}]\text{-2a}$ under room temperature for 5min after the acetonitrile solutions of *t*-BuOH (750mM, 18 μl , 13.19 μmol) and DIPEA (250mM, 100 μl , 25 μmol) were added. Then the solution of TPP-NH₂ (**1**) in acetonitrile (56.46mM, 335 μl , 19 μmol) was added and the final mixture was incubated at room temperature for 15 min. A HPLC purification was employed with the condition of: gradient: of 25% buffer B to 100%B in 10min, back to 25%B in 5min and isocratic for 5min; 496nm; flow: 5ml/min; on a semi-preparative C18-column (Higgins Analytical Inc. Proto 300 C18 5 μm , 250X10mm, P/N: CS-2520-C185). The peak between 10.5 min was the right compound calibrated with the $[^{19}\text{F}]\text{-3a}$ internal standard. The fraction was diluted with water (0.1% HOAc) with a ratio of 1:4 (v/v) (10ml to 40ml) and loaded onto a C18 cartridge (Waters, preconditioned by rinsing with ethanol (0.1% HOAc) (2x1ml) and water (0.1% HOAc) 3x1ml)). The cartridge was washed by water (0.1% HOAc) 5ml and purged by air. The compound was eluted off the cartridge by ethanol (0.1% HOAc) (2x1ml). The product was dried by Ar flow under 70°C with total activity of 1.753mCi (specific activity around 0.37mCi/ μmol). The final residue was reconstituted with 1xDPBS (0.7ml, pH 7.4) and EtOH (0.06ml). Three doses were aliquoted for injections and one for analysis. The final compound was analyzed by HPLC with the same condition as preparation, see Figure S1.

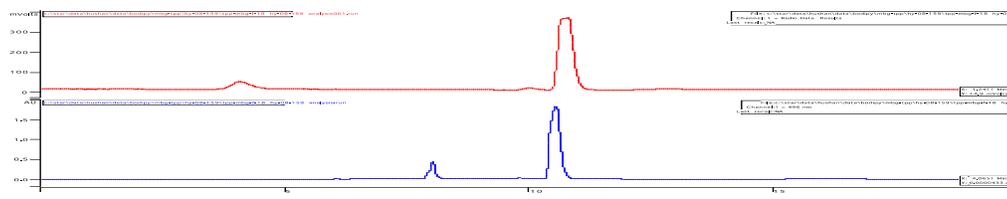


Figure S1. Radiochemical purity of $[^{18}\text{F}]\text{-2a}$ by RP-HPLC. Top trace is radioactivity. Bottom trace is absorbance at 496 nm.

For FACS and microscopy, tetramethylrhodamine, ethyl ester (TMRE), Rho123, and Hoechst 33342 were purchased from Invitrogen. Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) was from Sigma. A549 cells were from ATCC.

For mitochondria staining of TPPs by confocal microscopy A549 cells were cultured and plated in a 6-well glass bottom plate. Cells were incubated for one day before treating TPP-fluorochromes and stained with 300 nM of TPP-fluorochromes for 30min. Mitochondrial stains TMRE (549/574) and Rh123 (507/530) were used. TMRE was incubated with TPP-Green (500/510) and Rho123 with TPP-Red (577/590) and TPP-NIR (673/707). Hoechst 33342 (2mg/mL) was used for counterstaining of nuclei. After incubation, cells were washed with D becco's phosphate buffered saline (DPBS) and then, resuspended in DPBS with Ca²⁺, Mg²⁺ and 1% fetal calf serum. LSM 510 confocal microscope equipped with a stage incubator was used for performing live cell imaging.

For FACS analysis of TPP-fluorochromes and rhodamine references, A549 cells were plated to 2x10⁵ cells/well in a 24-well plate. Cells were incubated for one day before staining mitochondria with TPPs. For co-staining of mitochondria, A549 cells were incubated with 300nM of TPP-Fluorochromes and 50nM of reference rhodamine for 30 min. After treatment, cells were washed with Dulbecco's phosphate buffered saline (DPBS) and then, detached (200 µl of 0.05% Trypsin with 0.53 mM EDTA, 10 min @ 37°C), pelleted (1,500 rpm, 5 min) and resuspended in 300 µl of DPBS with Ca²⁺, Mg²⁺ and 1% fetal calf serum and analyzed with a FACSCalibur flow cytometer.

To determine the effect of mitochondrial membrane potential on TPP-fluorochrome accumulation, mitochondrial uncoupler FCCP (5 µM) was added to the cell and incubated for 30min. After inhibition, cells were stained with TPPs, TMRE and Rhod123 (final concentration 10nM) for 30min. Cells were washed with DPBS and collected as before for the flow cytometry.

For Ex-vivo Fluorescence and PET imaging, all procedures were approved by the institutional animal care committee.

Myocardial infarction (MI) was surgically induced in female 12 week C57Bl6 mice by occluding the left coronary artery (LCA) as described³. After 1 week, the mice were injected with 100 μ L of PBS containing 10 nmol of the TPP-NIR via tail vein injection and euthanized 2 hours later.

For ex-vivo surface fluorescence images of the heart, mice (one week post infarction) were also injected immediately prior to sacrifice with fluorescent red microspheres (50 μ L, Life Technologies, Cat#: F-8834). Injection was into the left ventricle via cardiac puncture. These are polystyrene spherical particles average 10-15 μ m in size, loaded with red fluorochrome (with excitation maximum at 580 nm and emission max at 605 nm). When injected in the heart, fluorescent microspheres lodge in the capillaries and small blood vessels, thus demarcating the ischemic area (lack of microsphere accumulation) from the perfused area with healthy blood flow (highly fluorescent). Hearts were sectioned along the short axis and the mid-myocardium was imaged. Images were acquired in the fluorescence reflectance mode on the IVIS Spectrum (PerkinElmer) imaging system. TPP-NIR accumulation was detected with an excitation wavelength of 640 nm and emission wavelength of 700 nm with an auto exposure setting. Microsphere fluorescence was detected with 550 nm excitation, 600 nm emission filter setting and auto exposure. Fluorescence images were acquired with a spatial resolution of 135 μ m.

The PET/CT imaging study was conducted using Triumph, a micro PET/SPECT/CT tri-modality imaging system (Triumph, Gamma Medica, Northridge, CA). Myocardial infarction was performed as described in the fluorescence imaging section. At 1 week post-procedure, mice were injected with 100 μ L of PBS containing 1 mCi of [¹⁸F]-TPP-green conjugate (synthesized in house) via tail vein injection and dynamic PET imaging was performed.

For PET, this system provided a 0.9 mm axial spatial resolution while the axial field of view was 8 cm. PET images were reconstructed with maximum likelihood expectation maximization algorithm (30 iterations) in high resolution mode. No contrast was used for CT imaging. The voltage of X-ray tube was 70 kVp and the anode current was 0.16 mA. The axial field of view was set to 91.07 mm, while 512 projections were acquired in fly gantry motion mode. The CT acquisition time was 6.40 minutes. The

co-registration of PET and CT images was performed using PMOD software v3.2. The heart was manually segmented from the co-registered contrast-enhanced CT images based on the signal-intensity difference between the region of interest (ROI) and background.

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2. J. A. Hendricks, E. J. Keliher, D. Wan, S. A. Hilderbrand, R. Weissleder and R. Mazitschek, *Angewandte Chemie (International ed. in English)*, 2012, **51**, 4603-4606.
3. S. Huang, H. H. Chen, H. Yuan, G. Dai, D. T. Schuhle, C. Mekkaoui, S. Ngoy, R. Liao, P. Caravan, L. Josephson and D. E. Sosnovik, *Circulation. Cardiovascular imaging*, 2011, **4**, 729-737.