Supporting Information

Turn-on Near-Infrared Fluorescence/Positron Emission Tomography Dual-modal Probe for Intracranial Hemorrhage Diagnosis

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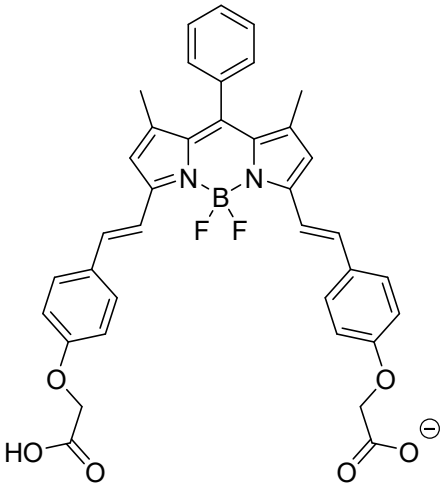
Contents

Chemical Synthesis:

Synthesis of Bodipy (II):



(II)

Chemical Formula: $C_{37}H_{31}BF_2N_2O_6$

Exact Mass: 648.2243

Molecular Weight: 648.4596

^{1}H NMR (500 MHz, d6-DMSO, 21°C, TMS) δ 13.09 (s, 2H), 7.58 - 7.52 (m, 9H), 7.45 - 7.40 (m, 4H), 7.02 (d, *J* = 8.9 Hz, 4H), 6.95 (s, 2H), 4.75 (s, 4H), 1.41 (s, 6H); ^{13}C NMR (125 MHz, d6-DMSO, 21°C, TMS) δ 170.43, 159.31, 152.62, 142.00, 138.75, 136.99, 134.71, 132.95, 129.76, 129.73, 129.68, 129.24, 128.80, 118.63, 116.63, 115.68, 65.01, 14.69; ^{19}F NMR (500 MHz, d6-DMSO, 21°C, $CFCl_3$) [^{10}B]-BF_2: δ -138.293, [^{11}B]-BF_2: δ -138.433; MS calcd for $[M-H]^-$ =$[C_{37}H_{30}BF_2N_2O_6]^-$: 647.2170, found: $[M-H]^-$= 647.2158.

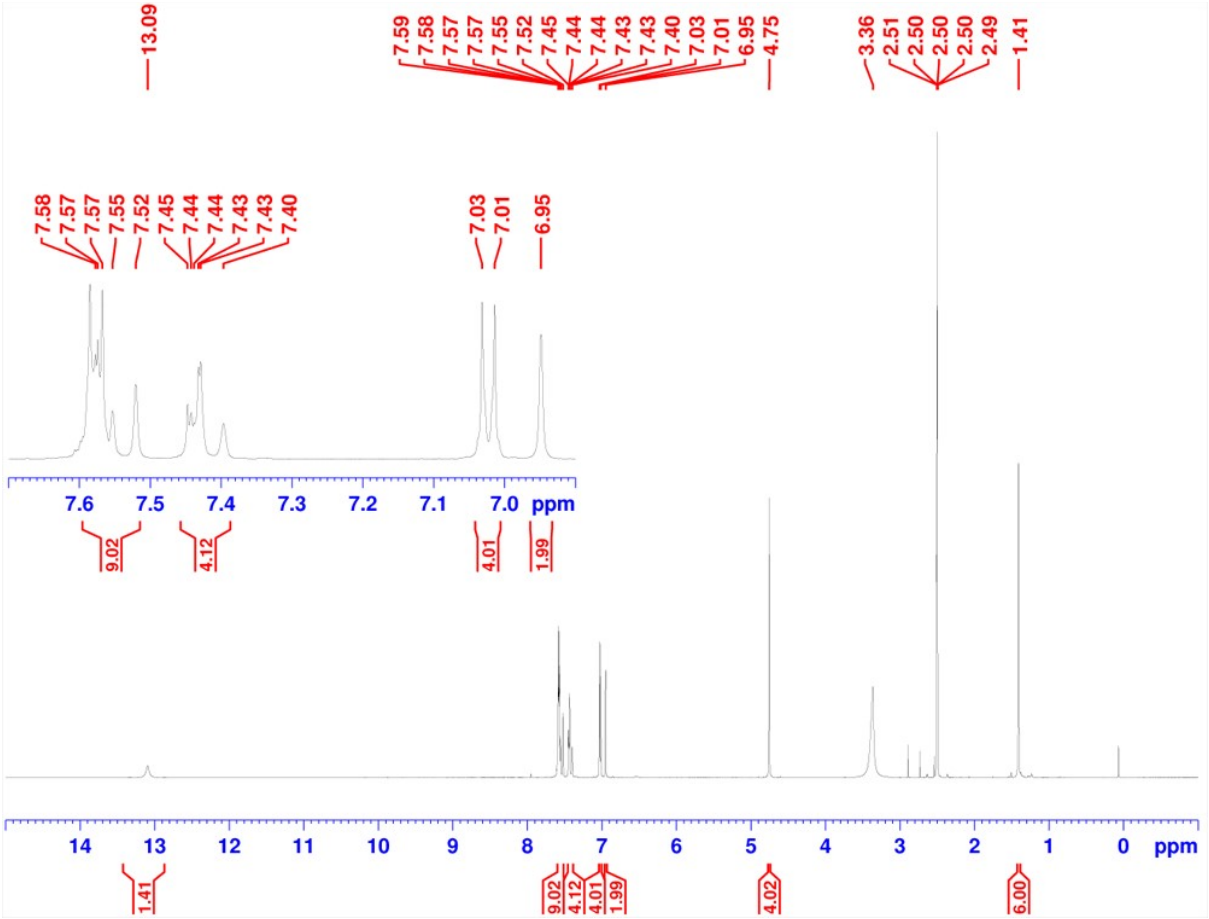


Figure S1: ^{1}H NMR (500 MHz, d6-DMSO, 21°C, TMS) for Bodipy (II).

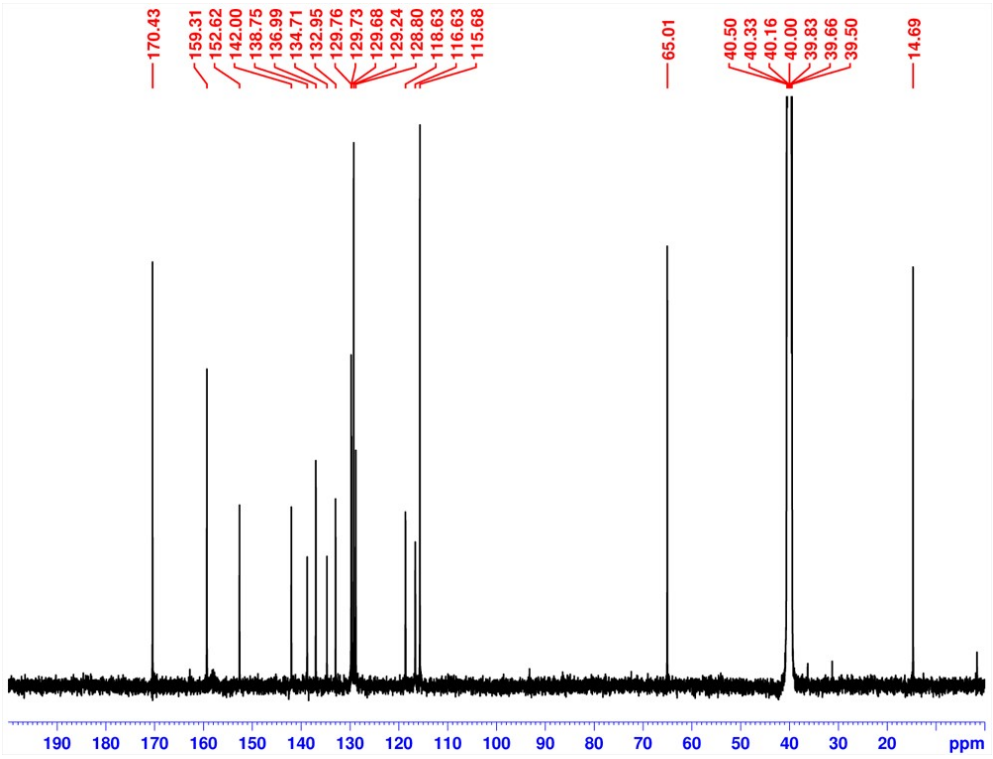


Figure S2: ^{13}C NMR (125 MHz, d6-DMSO, 21°C, TMS) for Bodipy (II).

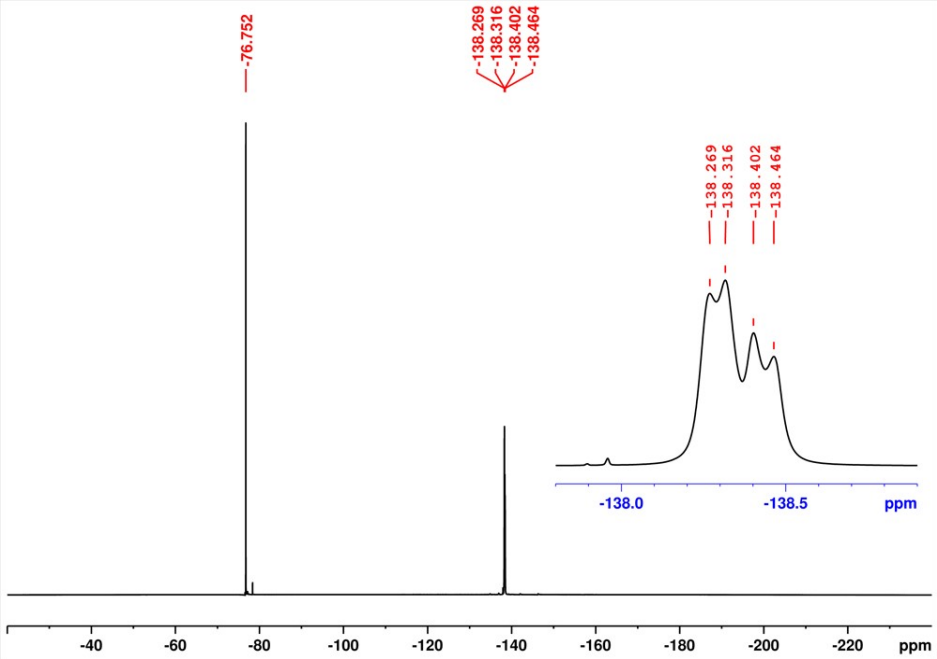


Figure S3: ^{19}F NMR (500 MHz, d6-DMSO, 21°C, $CFCl_3$) for Bodipy (II).

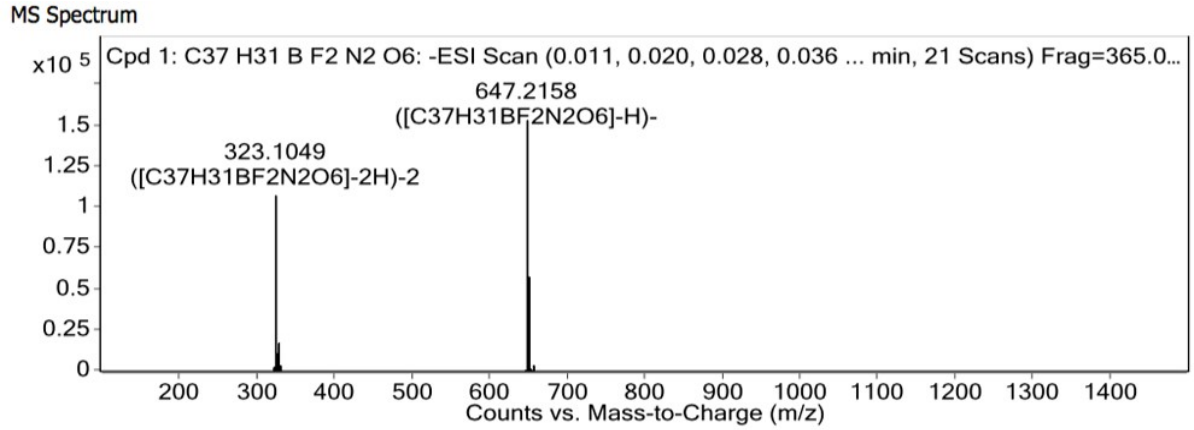


Figure S4: HRMS ES^- for Bodipy (II).

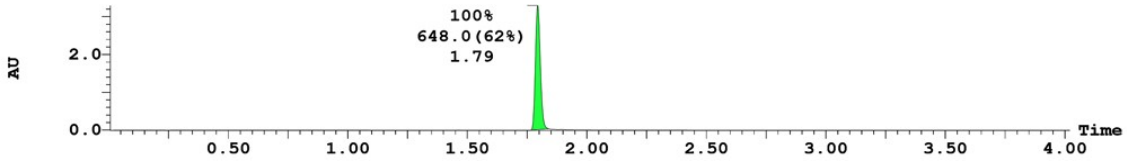
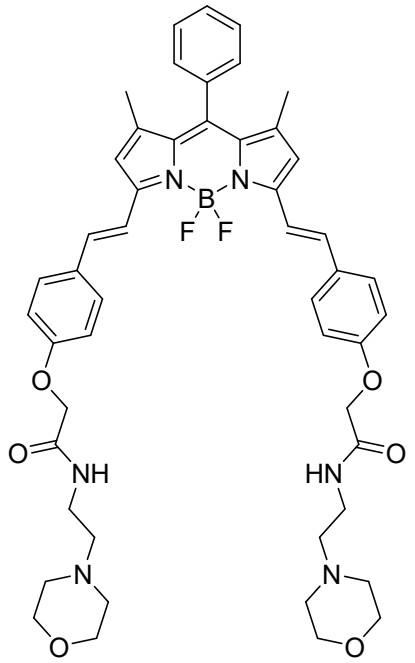


Figure S5: C18 Reverse phase UPLC elution profile of Bodipy (II).
a10-90 1.5 min, Absorbance monitoring at 636 nm.

Synthesis of Bodipy (III):



(III)
Chemical Formula: $C_{49}H_{55}BF_2N_6O_6$
Exact Mass: 872.4244
Molecular Weight: 872.8054

^{1}H NMR (500 MHz, d6-DMSO, 21°C, TMS) δ 8.45 (s, 2H), 7.62 - 7.54 (m, 9H), 7.44 - 7.40 (m, 4H), 7.09 (d, *J* = 8.9 Hz, 4H), 6.95 (s, 2H), 4.60 (s, 4H), 3.97 (s, 4H), 3.66-3.11 (m, 20H), 1.41 (s, 6H); ^{13}C NMR (125 MHz, d6-DMSO, 21°C, TMS) δ 168.31, 158.50, 152.11, 141.60, 138.40, 136.48, 134.15, 132.50, 129.58, 129.28, 129.22, 128.81, 128.27, 118.21, 116.25, 115.44, 66.95, 63.32, 55.21, 33.10, 14.20; ^{19}F NMR (500 MHz, d6-DMSO, 21°C, $CFCl_3$) δ [^{10}B]-BF_2: δ -138.307, [^{11}B]-BF_2: δ -138.449; MS calcd for $[M]^+$=$[C_{49}H_{55}BF_2N_6O_6]^+$: 872.4244, found: $[M]^+$=872.4255.

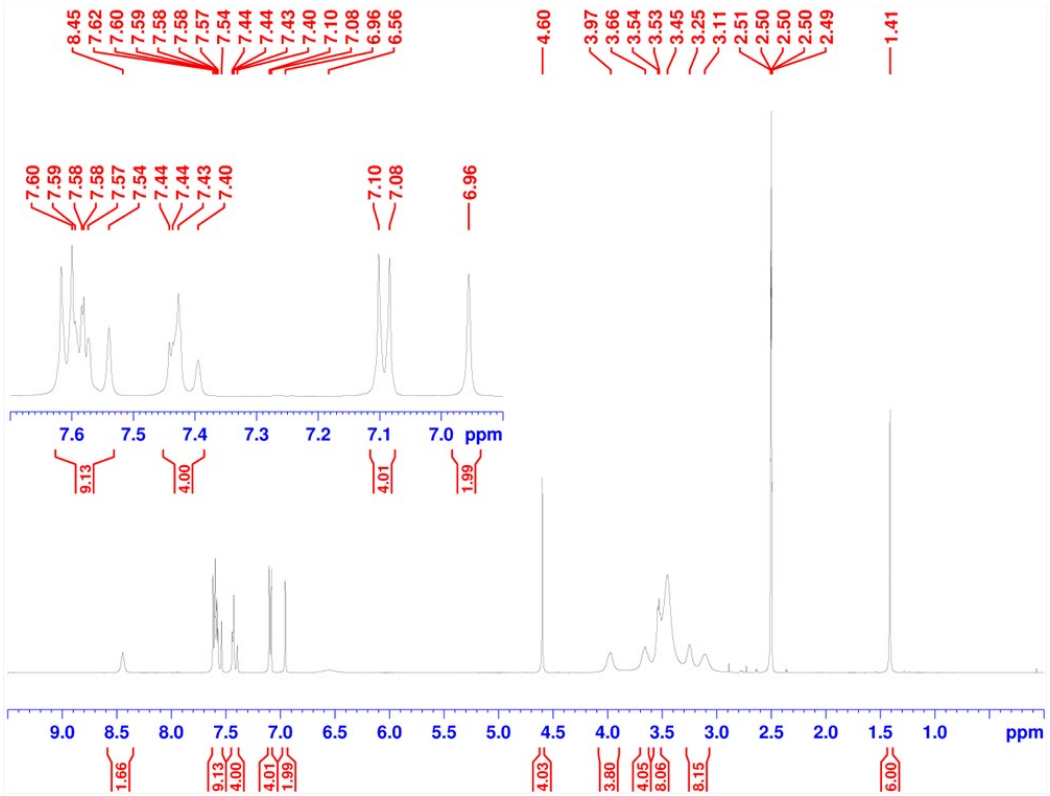


Figure S6: [1]H NMR (500 MHz, d6-DMSO, 21°C, TMS) for Bodipy (III).

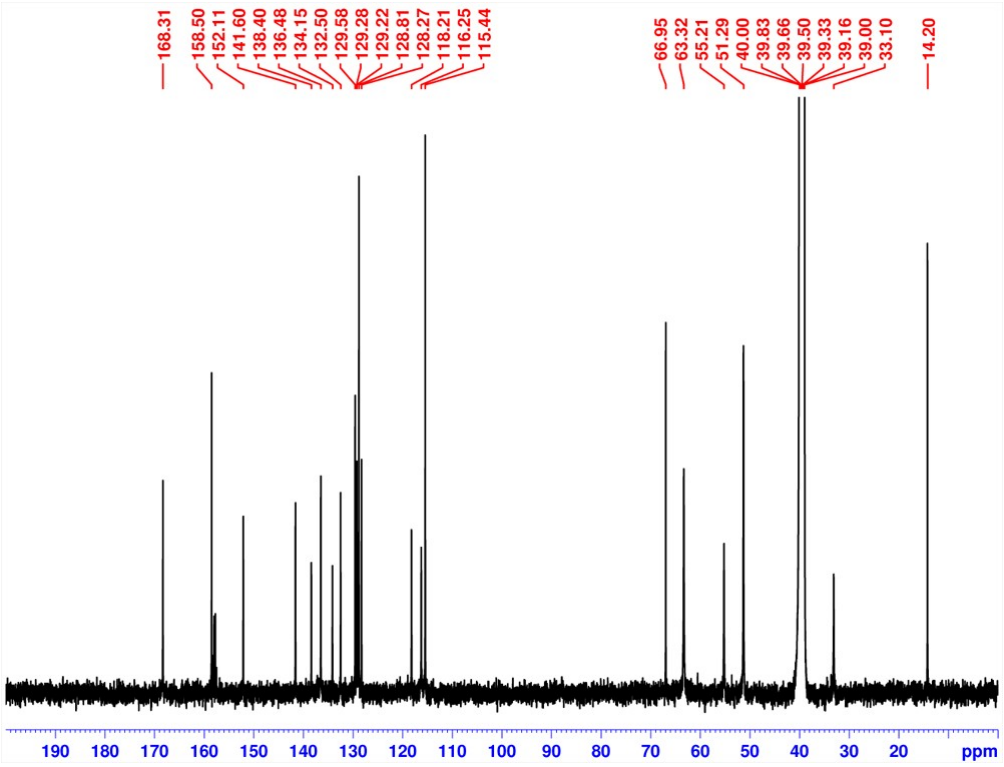


Figure S7: [13]C NMR (125 MHz, d6-DMSO, 21°C, TMS) for Bodipy (III).

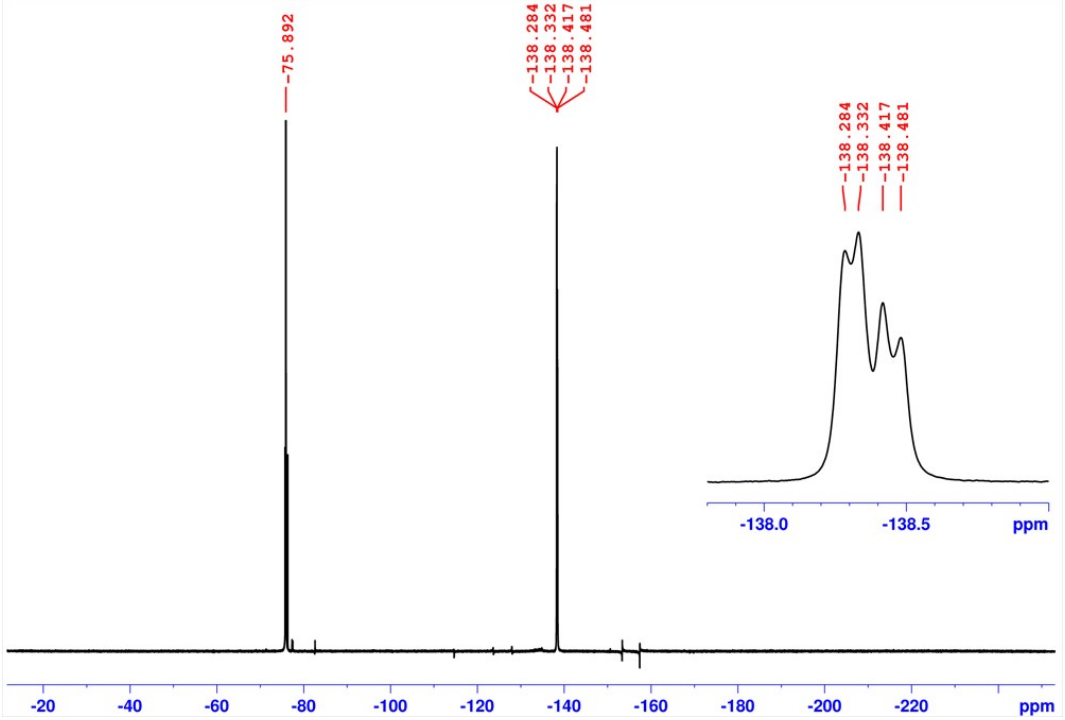


Figure S8: ^{19}F NMR (500 MHz, d6-DMSO, 21°C, $CFCl_3$) for Bodipy (III).

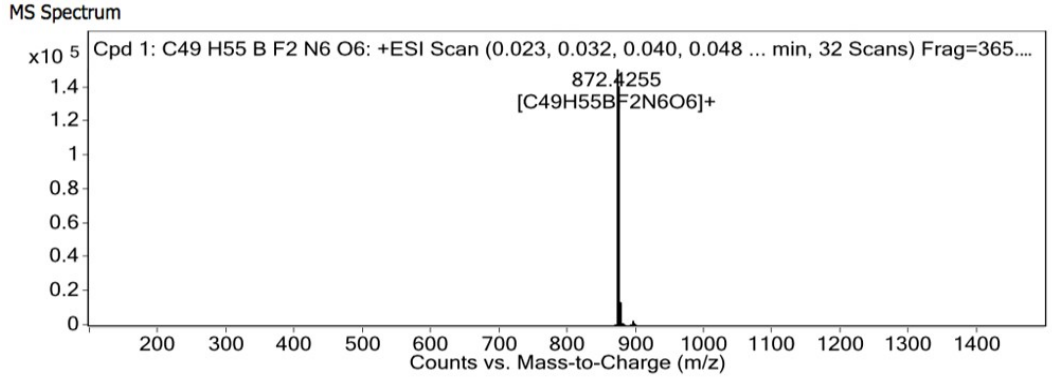


Figure S9: HRMS ES report for Bodipy (III).

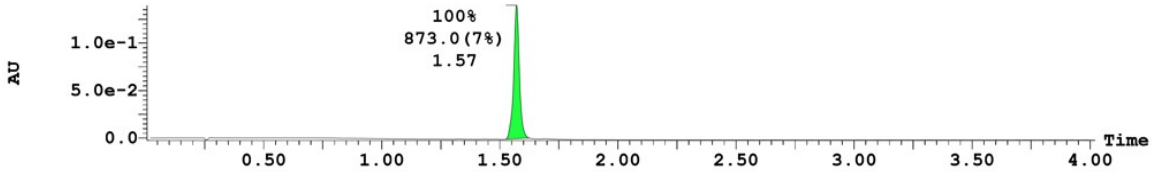


Figure S10: C18 Reverse phase UPLC elution profile of Bodipy (III). ACN10-90 1.5 min, absorbance monitoring at 636 nm.

Additional Methodology:

Radiochemistry and Imaging:

PET/CT Imaging:

All procedures conducted in mice were approved by the Weill Cornell Medicine Institutional Animal Care and Use Committee (#2014-0030) and are consistent with the recommendations of the American Veterinary Medical Association and the National Institutes of Health Guide for the Care and Use of Laboratory Animals. This work is compliant with ARRIVE guidelines for how to report animal experiments. Mice were imaged with an Inveon PET/CT (Siemens) 2 h post injection. Mice were sacrificed and tissues were collect 1 h post injection in preparation for fluorescence imaging and scintillated biodistribution studies. Tissue was imagined with Bruker Xtreme Imaging System with a 5 second exposure with an excitation and emission filters set at 650 nm and 700 nm respectively. After fluorescence imaging, collected organs were weighed and scintillated with a Wallac Wizard gamma counter. Fluorescent images were adjusted and analyzed using ImageJ. PET/CT dynamic reconstructions were performed on Siemen's Inveon Acquisition Workplace software. Reconstructed .hdr files were exported as DICOM files for PET/CT/MR alignment using open source Amide v1.0.4 software.

DLS, TEM, and photophysical characterization:

Characterization of the nano-aggregates of Bodipy (III) in deionized water:
A 10 μL DMSO solution (1 mM) of non-radioactive Bodipy (III) was mixed with 1 mL deionized water at room temperature for 10 min. A few drops of solution were added via a dropper onto copper mesh and allowed to dry. The resulting copper mesh was stained with uranyl acetate for 30 s and imaged with a JEOL 1400 Transmission Electron Microscope (TEM).
Another fraction of the aqueous Bodipy solution was transferred into a cuvette for dynamic light scattering (DLS) (Zetasizer Nano ZS, Malvern) and scanned at 25 °C in order to determine size distribution and nano-aggregate zeta potential.

The photophysical characterizations:
Bodipy (II), (III), and (IV) were dissolved in 100% DMSO and analyzed on a Cary 60 UV-Vis Spectrophotometer and a Cary Eclipse Fluorescence Spectrophotometer to determine their UV-Vis absorption spectra and photoluminescence spectra, respectively. Photo-luminescent quantum yields were determined using methylene blue (MB) as a standard (quantum yield = 0.04 in ethanol [1]).

In vitro cell experiments:

Biocompatibility experiments:

Bodipy (II), (III), and (IV) biocompatibility was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. A549 human lung cells were pre-seeded in 96-well plates at a concentration of 5000 cell/well. After 24 h of incubation at 37°C in RPMI media (1640), media was replaced with 100 μL volumes of RPMI-Bodipy mixtures containing different concentrations of Bodipy. Cells were incubated with Bodipy (III) for 48 h more hours at 37°C before 25 μL of MTT solution (5 mg/mL) was added. After 4 h of incubation, MTT containing culture media was replaced with warm 1X PBS buffer (37°C pH 7.4). 1X PBS was used to wash each well 3 times. 150 μL DMSO was added into each well. 96-well plates were read with a Tecan Infinite M1000 Pro Microplate Reader at 570 nm. A control group of cells that received media without Bodipy (III) was used with a cytoviability that set as 1.0, to which other wells were normalized.

Confirmation of quick labeling of suspended A549 cells by Bodipy (III):

Suspended A549 cells (in 1X PBS) were incubated with 1 μM Bodipy (III) for 15 min before they were transferred onto 24-well plates. Cells were promptly imaged on an epifluorescence microscope (EVOS FL Auto Cell Imaging System. Ex = 628/40; Em = 692/40).

In vitro cell imaging and localization:

A549 cells (in RPMI Medium 1640) were pre-seeded in a 24-well plate at 37°C. After 24 h, Bodipy (II), (III), and (IV) were mixed with RPMI 1640 medium and added into the 24-well plates at final concentrations of 1 μM. After 15 min, RPMI Medium 1640 containing Bodipy (III) were decanted and replaced with 37 °C with 1 μM fresh solutions of LysoTracker Green (Thermo Scientific). Cells were incubated at 37°C for another 30 min before the cells were washed with 37 °C RPMI Medium 1640 and imaged by fluorescence microscopy (EVOS FL Auto Cell Imaging System. Ex = 628/40; Em = 692/40).

Quantifying and comparing A549 probe uptake by FACS:

A549 cells were incubated with 0.1 μM Bodipy (III) for 10 min, and then washed with 150 μL 1X PBS buffer (pH 7.4). Suspended A549 cells were analyzed by Fluorescence-activated cell sorting (FACS, Gallios), 10000 cells of each sample were counted. A549 cells incubated with 1x PBS were used as a FACS control.

No-wash Bodipy cell labeling and imaging:

A549 cells were pre-seeded in two wells of a 24-well plate for 24 h and then incubated with 1 μM of Bodipy (III) for 15 min. One well was washed with 100 μL warm (37 °C) cell culture solution (RPMI) 3 times and the other one was not washed. The two samples were imaged using the same imaging conditions by fluorescence microscopy (EVOS FL Auto Cell Imaging System. Ex = 628/40; Em = 692/40). To quantitate the difference in sample preparation, Image J was used to draw lines across cells in fluorescent tiff format images. Fluorescence intensities along the lines were plotted and analyzed.

Long-term retention of Bodipy by A549 cells:

Pre-seeded A549 cells were incubated with 1 μM of Bodipy (III) for 15 min, washed with 1X PBS buffer (pH 7.4), and then transferred to fresh culture media at 37°C. Cells were digested, diluted and seeded in new 24-well plates every other day for 6 passages (12 days in total.).

Confirmation that Bodipy is retained by cells and does not leak or transfer Bodipy from A549 post-labeling:

A549 stained with Bodipy (III) were mixed and co-incubated with GFP expressing Hela cells for 24 h. The cells were then imaged by fluorescence microscopy in the different channels (EVOS FL Auto Cell Imaging System. Ex = 628/40; Em = 692/40 for Bodipy (III) in A549. Ex = 470/22; Em = 510/42 for GFP in Hela cells.). Brightfield, GFP, Cy5/Bodipy (III) fluorescent figures were overlaid to show that Bodipy does not transfer from A549 (No GFP) to Hela (GFP) cells.

The intracellular photostability study:

A549 cells were labeled with Bodipy (III) (1 μM) or Lysotracker (1 μM) for 2 h, then subjected to continuous irradiation with a 650 nm light (10 mW/cm^2, for Bodipy (III)) or 488 nm light (10 mW/cm^2, for Lysotracker). Fluorescence images were captured at defined time intervals (0–10 min for Bodipy (III); 0–100 s for Lysotracker), and the relative fluorescence intensity was quantified (normalized to the initial signal).

pH stability:

1 μL DMSO solutions (1 mM) of Bodipy was added to 10 mL solutions of PBS/DMSO (1/2, v/v) at pH 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, and 9.0, pHs were confirmed by pH meter before and after dissolving Bodipy in PBS/DMSO solutions. The fluorescence intensity of each sample was determined on a fluorescence spectrophotometer (Cary Eclipse) with an excitation wavelength of 610 nm. Fluorescence measurements were repeated three times. Data is normalized to the most intense fluorescence intensity.

PET imaging of A549 cellular pellets:

Approximately one million A549 cells in 1 mL 1X PBS buffer were mixed with 10 μL DMSO solution of ^{18}F fluoride labeled Bodipy (IV) (50 μCi) at room temperature. After 15 min incubation at room temperature, 1.5 mL Eppendorf tubes were centrifuged at 1000 rpm for 3 min to pellet cells. The tube was placed in an Inveon PET/CT (Siemens) for a 15 min PET scan.

In vivo/ex vivo experiments:

Ex vivo imaging of A549 cancer cell metastasis:

Mice were intravenously injected (tail-vein) with two million A549/Luciferase expressing cancer cells. After 105 days, mice were injected intraperitoneally (i.p.) with D-Luciferin firefly potassium salt (15 mg/mL, 200 μL). Mice were sacrificed 15 min later and organs were harvested for bioluminescence imaging. Bioluminescence imaging of the collected organs were performed immediately after tissue collection on a Pre-Clinical, IVIS Optical Imaging System (5 min collection time).

Fluorescence histology:

Collected brain specimens were fixed with formalin. A cryotome was used to generate 20 μm sections. Individual, adjacent sections were DAPI stained (nuclear dye), analyzed for Bodipy fluorescence (Cy5 filters), or subject to TER-119 RBC-specific horseradish peroxidase/ diaminobenzidine immunostaining.

Supporting Figures for *in vitro* and *in vivo* experiments:

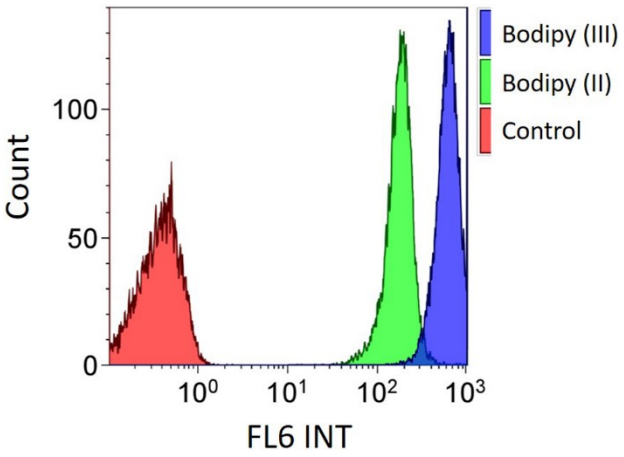


Figure S11. FACS fluorescence characterization of A549 cells labeled with equimolar amounts of Bodipy (II) and Bodipy (III) (10 min).
Bodipy (III) labels cells to a superior degree than Bodipy (II).

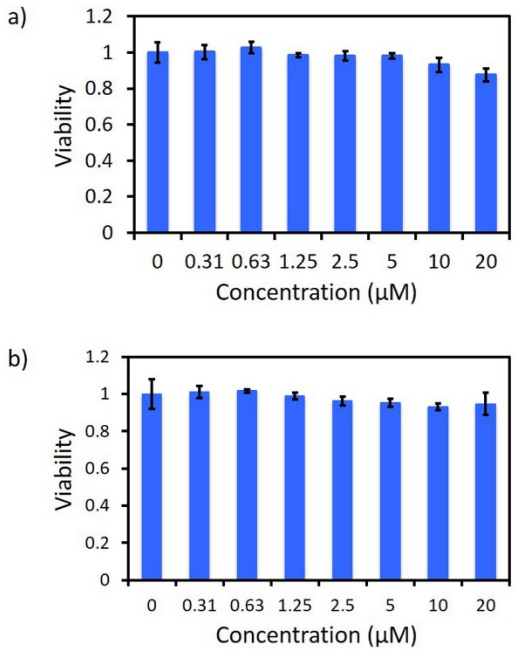


Figure S12. A549 cell viability results (MTT) following 24 h (a) and 48 h (b) incubation with various concentrations of Bodipy (III).
Bodipy (III)- A549 toxicity is not observed.

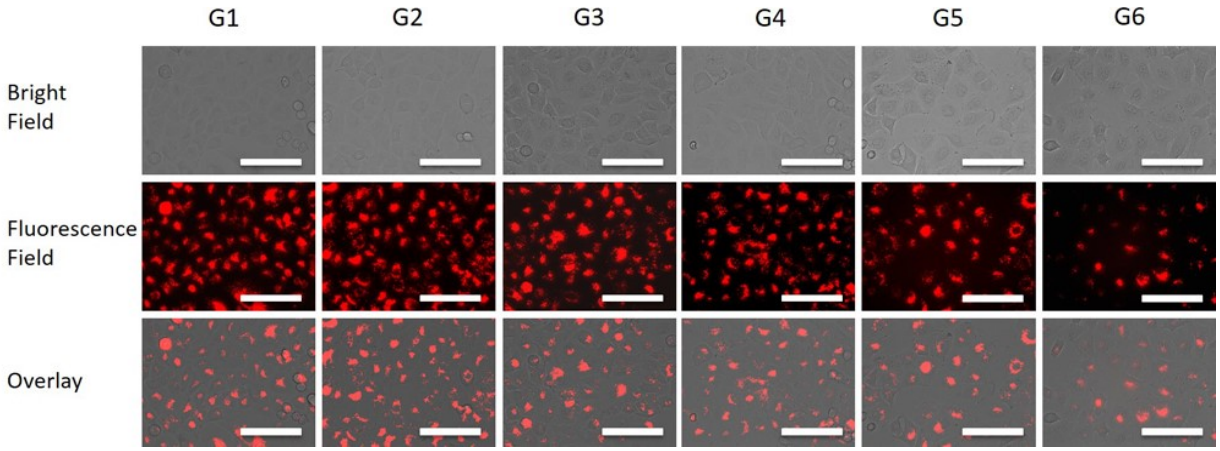


Figure S13. Epifluorescent confirmation of long-term Bodipy (III) retention by A549 cells after 12 days of division. Bodipy (III) stained A549 cells were digested, diluted and seeded in new plates every other day. 6 passages were performed over 12 days. Scale bars = 100 µm.

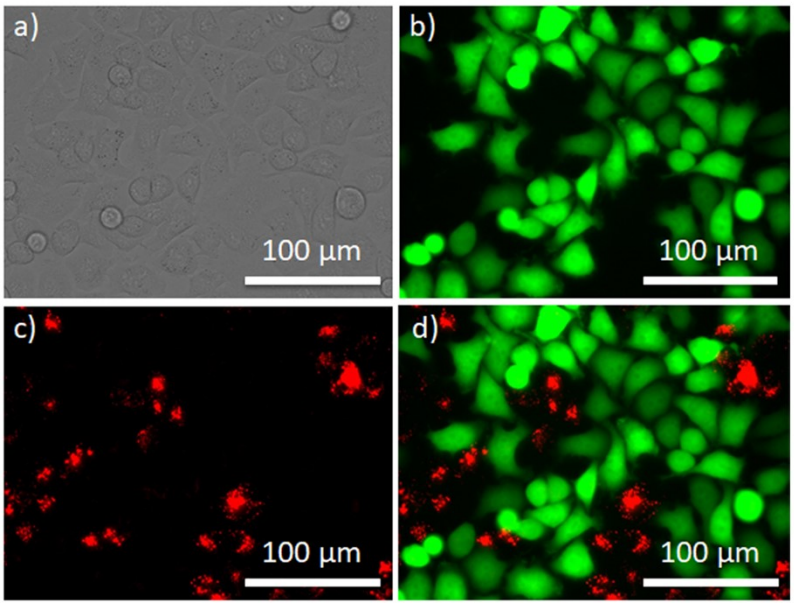


Figure S14. Epifluorescent confirmation of no dye transfer following initial A549 labeling. Bodipy (III) stained A549 cancer cells were co-incubated with GFP expressing Hela cells (no Bodipy labeling). a) Bright field image; b) GFP fluorescence channel (Ex = 470/22 nm, Em = 510/42 nm, GFP expressing Hela cells). c) Cy5 fluorescence channel (Ex = 628/40 nm, Em = 692/40 nm) for Bodipy (III) stained A549 cancer cells. d) The overlay of b) and c). Bodipy (III) transfer from A549 cells into Hela-GFP cells is not observed after 24 hours of co-incubation.

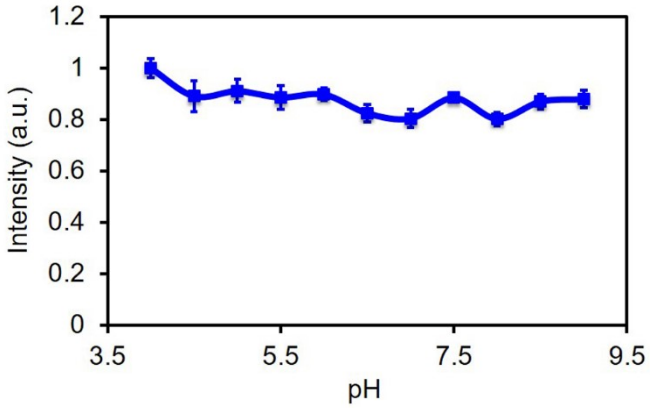


Figure S15. Bodipy (III) fluorescence intensity does not vary with pH.
Sodium hydroxide or hydrochloric acid was used to adjust the pH of phosphate buffers that were used to solubilize (III). Bodipy (III) is fully soluble in 1x PBS/DMSO (1/2, v/v) solutions.

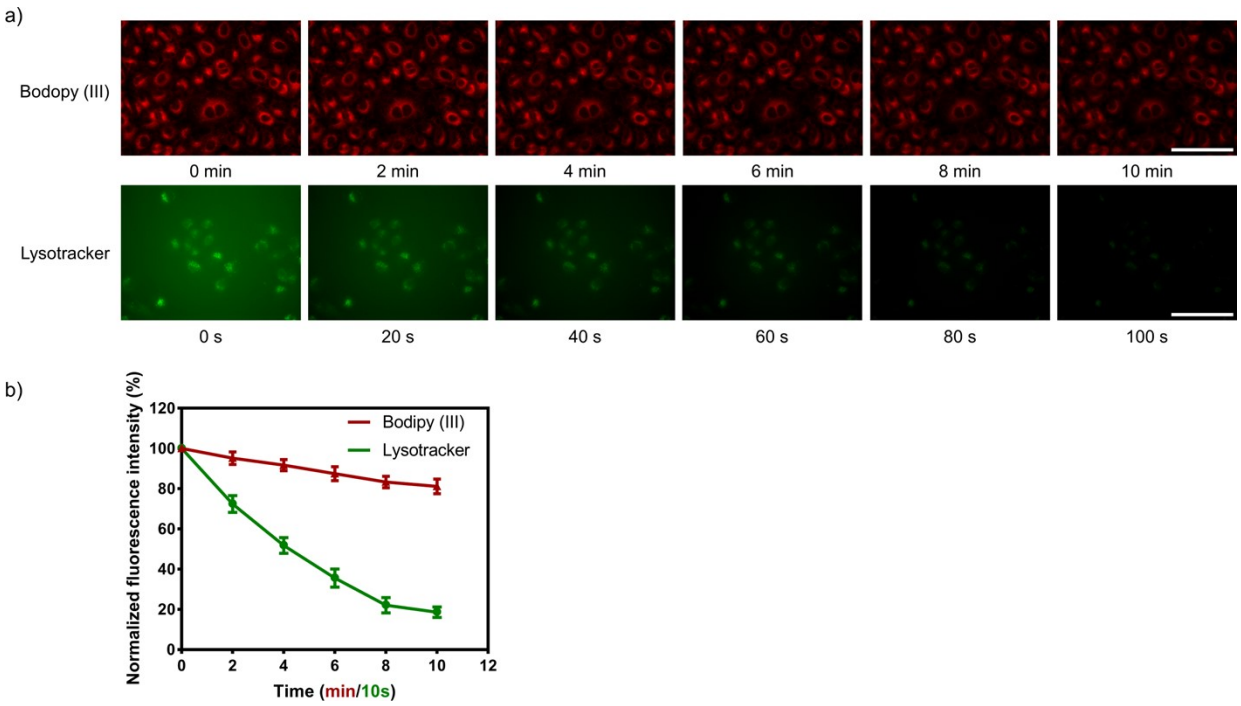


Figure S16. Intracellular photostability comparison between Bodipy (III) and commercial Lysotracker probe. a) Top row: Fluorescence images of A549 cells labeled with Bodipy (III) (Cy5 fluorescence channel, Ex = 628/40 nm, Em = 692/40 nm) under continuous 650 nm light irradiation (10 mW/cm^2) at 0, 2, 4, 6, 8, and 10 min. Bottom row: Fluorescence images of A549 cells labeled with Lysotracker (GFP fluorescence channel, Ex = 470/22 nm, Em = 510/42 nm) under continuous 488 nm light irradiation (10 mW/cm^2) at 0, 20, 40, 60, 80, and 100 s. Scale bars = 100 μm. b) Quantitative analysis (not shown) revealed that Bodipy (III) retained 81.2% ± 3.7% of its initial fluorescence intensity after 10 min of irradiation, whereas Lysotracker retained < 20% of its initial intensity after 100 s, demonstrating the superior photostability of Bodipy (III) for long-term live-cell imaging.

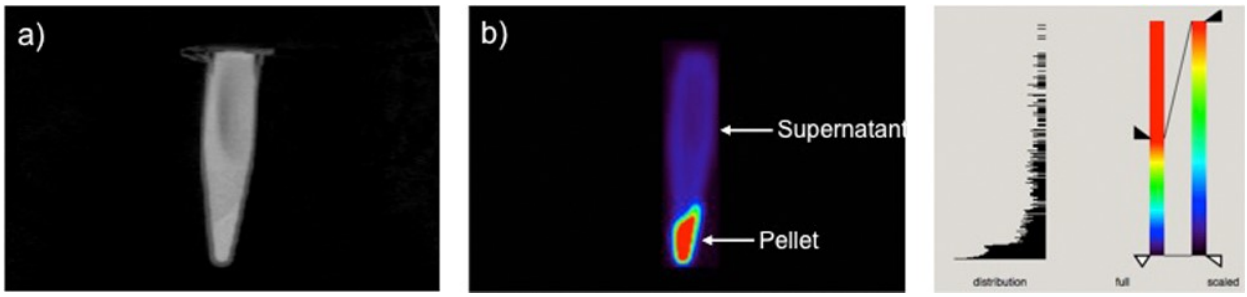


Figure S17. No wash labeling of cells shows cell-specific labeling by 18F-PET. A549 cells were labeled with radioactive Bodipy (IV) for 15 min at room temperature in a 1.5 ml Eppendorf tube. Labeled A549 cells were centrifuged at 1000 rpm (94 g) for 3 min to form a pellet. The pellet was imaged on an Inveon PET/CT. a) CT imaging. b) PET imaging.

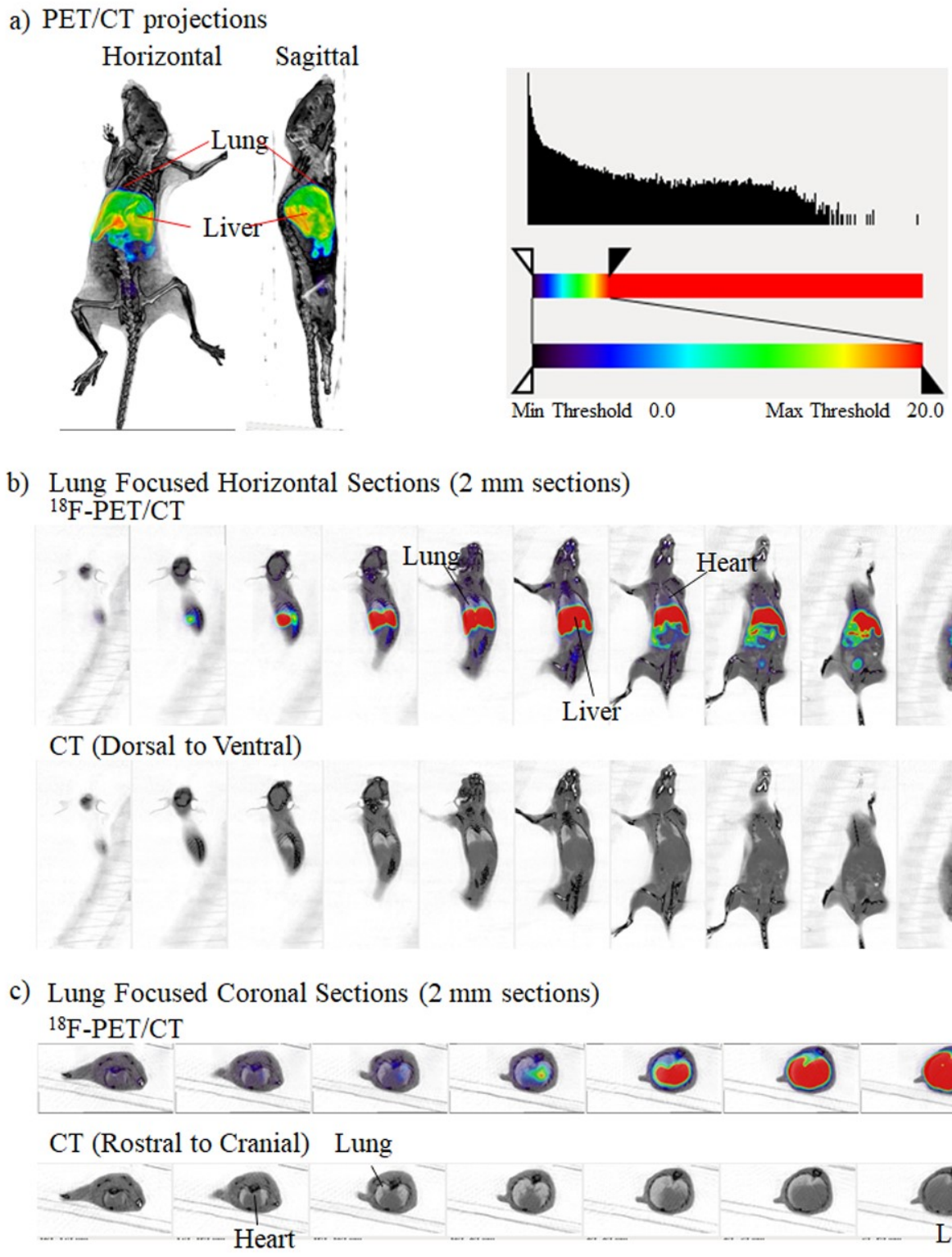


Figure S18. Control PET imaging 2h post i.v. injection of Bodipy (IV) solution (30 μCi of injected activity per mouse) alone. Bodipy (IV) is not present in the lungs.

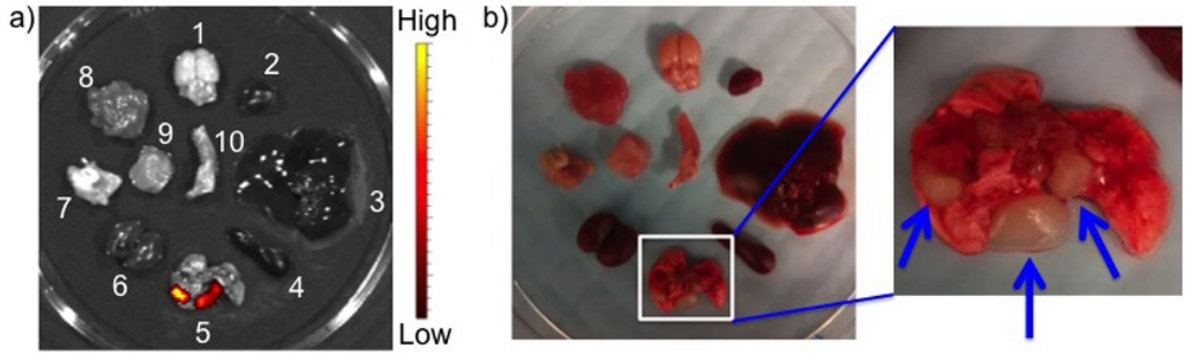


Figure S19. Ex vivo confirmation that circulating A549 cells metastasize to lung tissue. a) Bioluminescence imaging of freshly collected organs from a mouse, 105 days following a single intravenous (i.v., tail vein) injection of two million A549/Luc cells, the mouse was intraperitoneally (i.p.) injected with D-Luciferin Firefly potassium salt (15mg/mL, 200 µL). 15 min was allowed to pass before the mouse was sacrificed and organs were harvested. Bioluminescence imaging on a Pre-Clinical IVIS Optical Imaging System (5 min exposure time) was performed. 1 - Brain, 2 - Heart, 3 - Liver, 4 - Spleen, 5 - Lung, 6 - Kidney, 7 - Stomach, 8 - Intestine, 9 - Muscle. 10 - Bone. b) Bright field image of the collected organs, with an inset showing clear metastasis on/in lung tissue on visual inspection. Blue arrows indicate the tumor metastases in the lung.

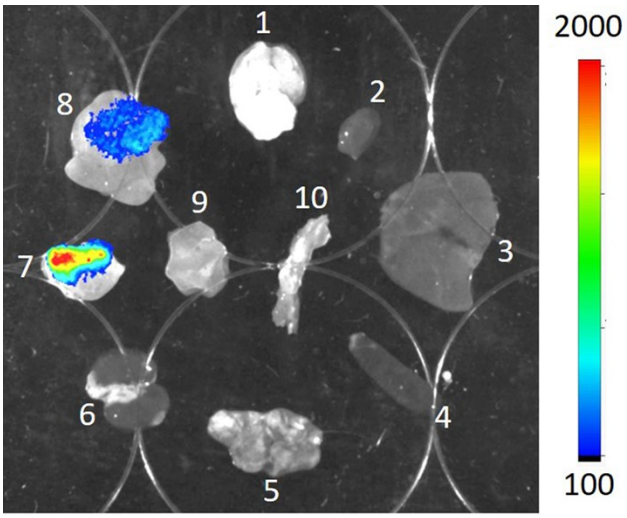


Figure S20. Ex vivo confirmation of stomach and intestinal autofluorescence. A healthy and untreated mouse was sacrificed and imaged with the Bruker Xtreme Imaging System under the same imaging conditions in Figure 7. 1 - Brain, 2 - Heart, 3 - Liver, 4 - Spleen, 5 - Lung, 6 - Kidney, 7 - Stomach, 8 - Intestine, 9 - Muscle. 10 - Bone.

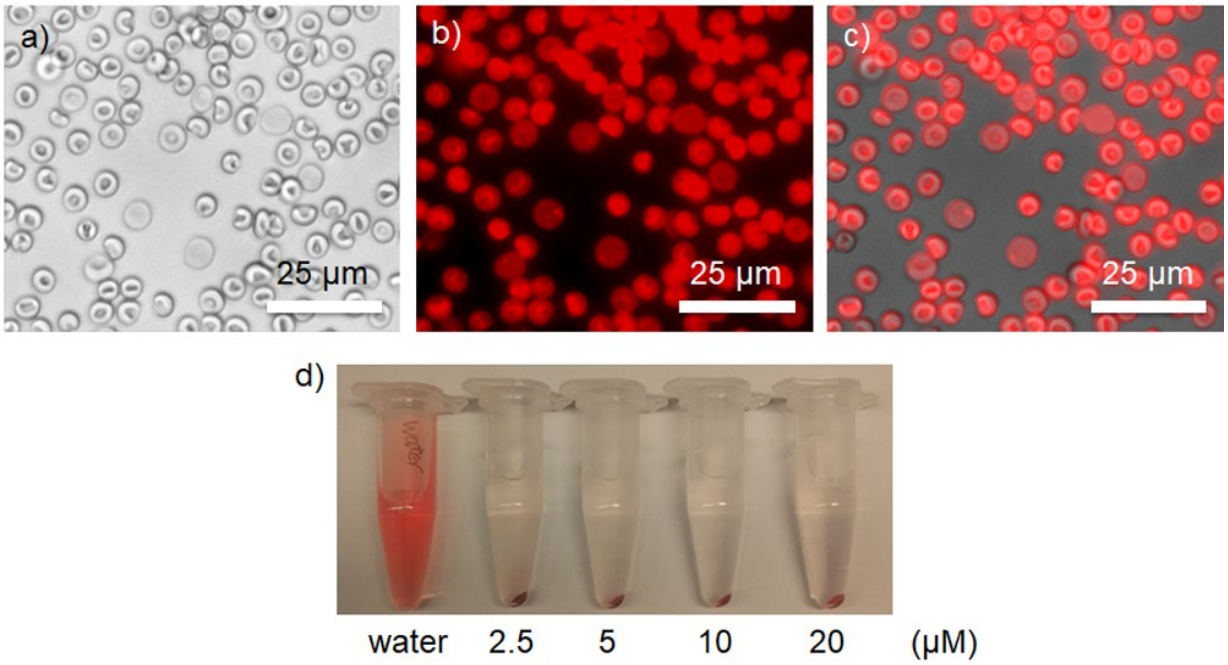


Figure S21. Confirmation of Bodipy (III) RBC labeling and lack of Bodipy (III) induced hemolysis. Microscopic analysis of RBCs labeled with 1 µM Bodipy (III) for 15 min at room temperature (no wash). a) Bright field image, b) fluorescence image (Ex = 628/40 nm, Em = 692/40 nm), c) overlay of bright field image and fluorescent images. d) Hemolysis assay of Bodipy (III) after 4 h of incubation with RBCs. Complete RBC hemolysis is observed in deionized water (control, left, shows lack of pelleting and red colored supernatant). Hemolysis is not observed with 20, 10, 5 or 2.5 µM Bodipy (III) in isotonic phosphate-buffered saline (pH 7.4, 1X PBS).

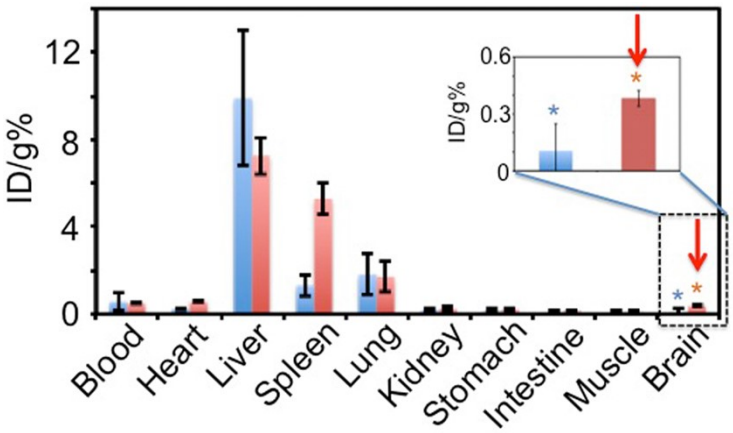


Figure S22. Scintillated biodistribution (gamma scintillation, 2h post injection) of tissues from mice injected with ~ 100 µCi Bodipy (IV) labeled RBCs (red, 4 mice) and free Bodipy (IV) solution (blue, 4 mice), respectively. Data were presented as mean ± standard deviation (SD).

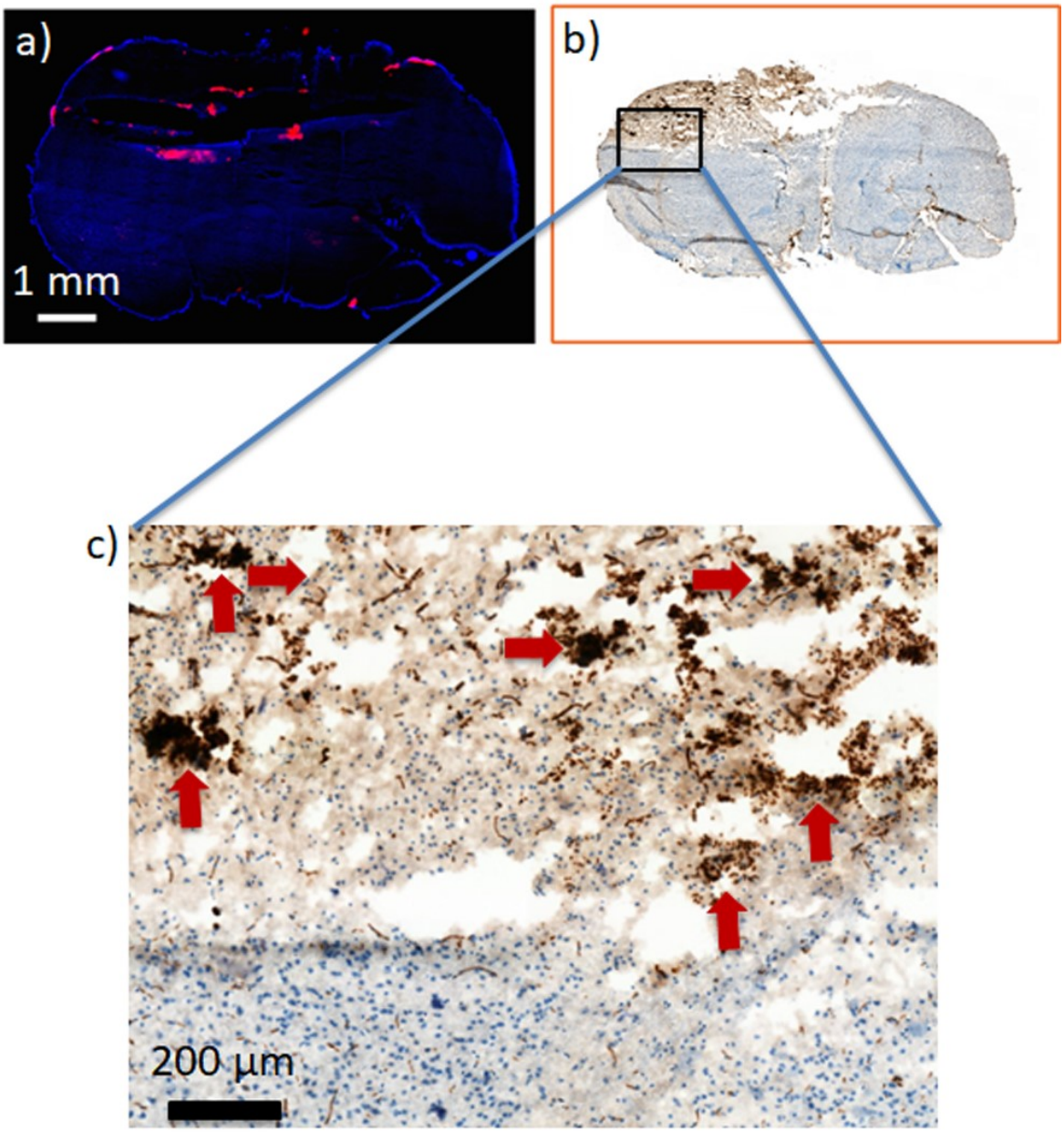


Figure S23. Bodipy (III) labeled RBC Fluorescence and TER0119 immunohistochemical analysis of brain specimens. Mice were injected with 50 µCi of Bodipy (IV) labeled RBCs 2h following the creation of a hemorrhagic lesion. Formalin-fixed paraffin, 20 µm coronal sections of brain were generated on a cryotome. The presence of fluorescent Bodipy (IV) labeled RBCs located at the hemorrhage site is verified by histology. a) Fluorescence image of Bodipy in the hemorrhagic region is generated in DAPI stained tissue. Fluorescence is shown in red (Cy5 channel) and DAPI stained nuclei are shown in blue. Fluorescence at site of hemorrhage indicates the presence of Bodipy (IV) labeled RBCs. b) A neighboring slice was stained with RBC-specific TER-119 antibody, hematoxylin, and eosin. The site of intracranial hemorrhage appears in brown and corroborates the fluorescent data in a). c) Inset showing magnified transition area between normal and brain tissue subject to hemorrhagic. RBC staining is shown in brown (red arrows) due to the oxidation of diaminobenzidine by secondary Ab labeled with horseradish peroxidase.

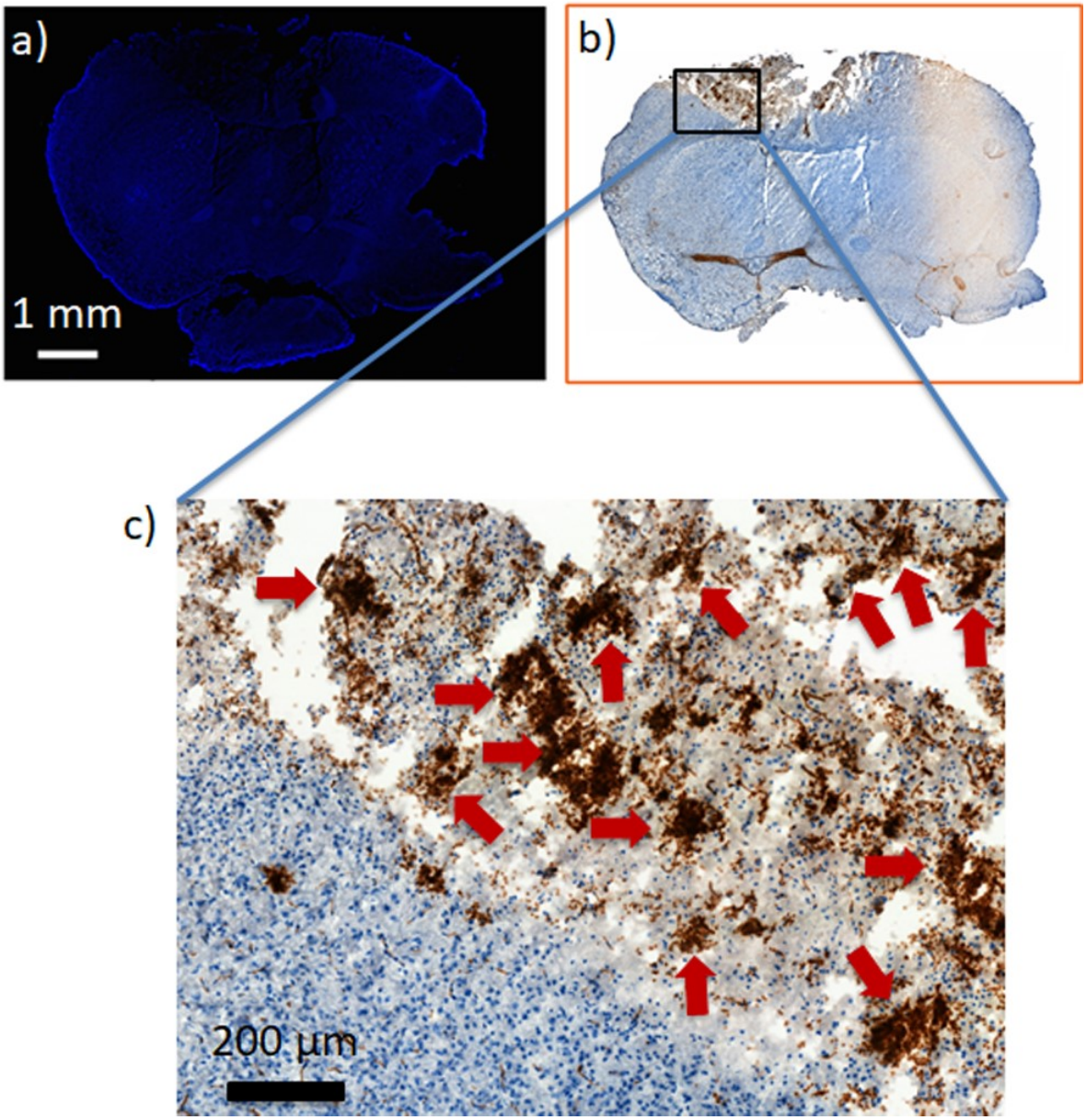


Figure S24. Bodipy (III) only (Control) Fluorescence and TER0119 immunohistochemical analysis of brain specimens. Mice were injected with 50 µCi of Bodipy (IV) 2h following the creation of a hemorrhagic lesion. Formalin-fixed paraffin, 20 µm coronal sections of brain were generated on a cryotome. The presence of fluorescent Bodipy (IV) is not present in at the hemorrhage site. a) Bodipy Fluorescence is not visible in hemorrhagic, DAPI stained tissue. Fluorescence is shown in red (Cy5 channel, not visible) and DAPI stained nuclei are shown in blue. b) A neighboring slice was stained with RBC-specific TER-119 antibody, hematoxylin, and eosin. The site of intracranial hemorrhage appears in brown and corroborates the fluorescent data in a). c) Inset showing magnified transition area between normal and brain tissue subject to hemorrhage. RBC staining is shown in brown (red arrows) due to the oxidation of diaminobenzidine by secondary Ab labeled with horseradish peroxidase.

1. Kelly, J. M.; Putten, W. J.; Mcconnell, D. J., Laser flash spectroscopy of methylene blue with nucleic acids. *Photochemistry and photobiology* **1987,** *45* (2), 167-175.