SI Appendix for

Molecular imaging of biological systems with a Clickable dye in the broad 800- to 1,700-nm near-infrared window

Content list in SI Appendix.

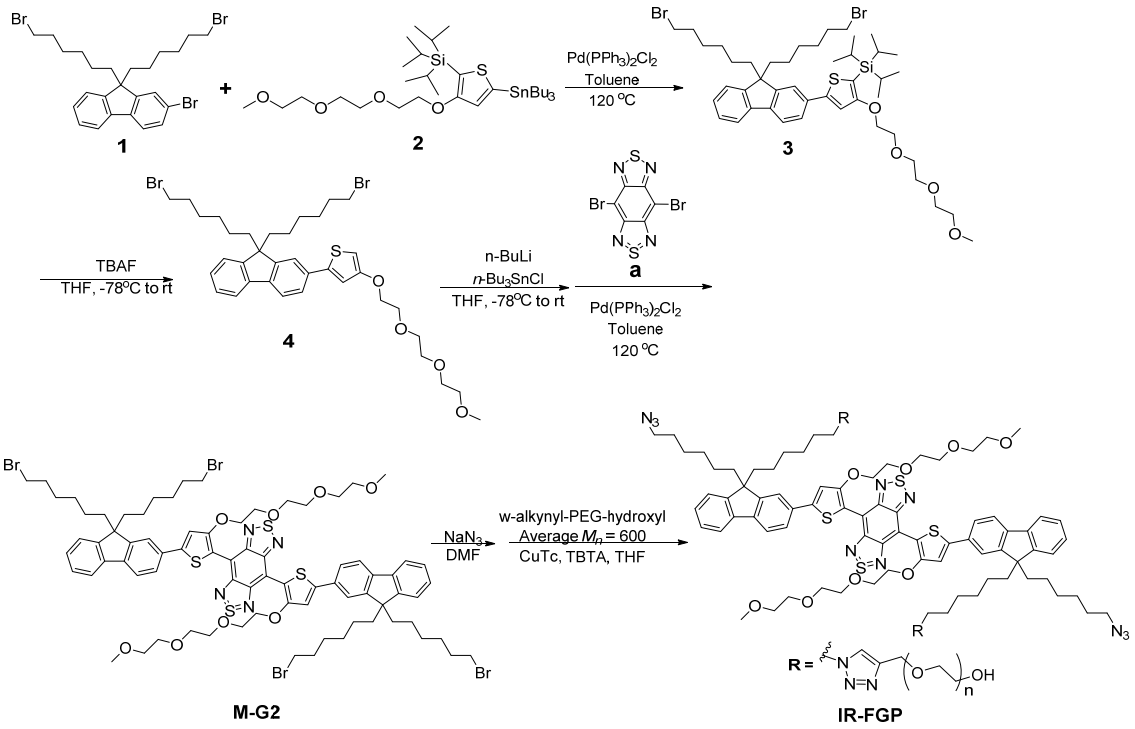
1. Materials. All air and moisture sensitive reactions were carried out in flame-dried glassware under a nitrogen atmosphere. Reactive liquid compounds were measured and transferred by gas-tight syringes and were added in the reaction flask through rubber septa. Tetrahydrofuran (THF), toluene, and dimethyl formamide (DMF) used for anhydrous reactions were purified by solvent purification system (Innovative Technology, Inc.) Unless otherwise noted, all reagents were obtained commercially and used without further purification, EZ-Link™ Sulfo-NHS-LC-Biotin, BCA protein assay kit, was purchased from thermofisher Scientific. Streptavidin (016-000-084) was purchased from Jackson Immunoresearch. PBS was purchased from HyClone. 4-(N-Maleimidomethyl) cyclohexane-1-carboxylic acid 3-sulfo-N-hydroxysuccinimide ester sodium salt (sulfo-SMCC) was obtained from Sigma-Aldrich. Erbitux (cetuximab) (2 mg/mL) was purchased from Imclone Systems Incorporated. Anti-mouse IgG was obtained from Vecton Laboratories, Inc. Bovine Serum Albumin (BSA) was purchased from Sigma-Aldrich. BCA^{TM} protein assay kit was purchased from Pierce. 1,2-Distearoyl-phosphatidylethanolamine-methyl-polyethyleneglycol (DSPE-mPEG) and DSPE-PEG-amine was purchased from Laysan Bio Inc. Purified Rat Anti-Mouse CD31 (557355)

was purchased from BD Pharmingen™. Mouse Anti-Neuronal Nuclei (NeuN) (MAB377) was purchased from Chemicon. DBCO-PEG$_4$-NHS was purchased from "Click chemistry tools" Bioconjugate technology company.

2. Synthesis and characterizations of M-G2 and IR-FGP.

General information：^{1}H and ^{13}C NMR spectra were performed on 500 MHz NMR spectrometers (Bruker AVANCE) using $CDCl_3$. Mass spectra were in general recorded on a QSTAR Elite (ABI); Column chromatography was performed with silica gel (200-300 mesh ASTM); Ultraviolet-visible near-infrared (UV-VIS-NIR) absorption spectra were recorded on Shimadzu UV-3600Plus; Size exclusion chromatography (SEC) was performed on Malvern Viscotek 270 max (10 μm PLgel 600 × 7.5 mm column, THF used as the mobile phase at a flow rate of 1.0 mL/min at 40 °C, linear polystyrene calibration, equipped with ultraviolet (UV) detector).

The synthetic routes toward **M-G2** and **IR-FGP** were shown in Scheme S1. And the detailed procedures and data were described as below:



Scheme S1. Synthesis of **M-G2** and **IR-FGP.**

(1) (5-(9,9-bis(6-bromohexyl)-9H-fluoren-2-yl)-3-(2-(2-(2-methoxyethoxy)ethoxy)ethoxy) thiophen-2-yl)triisopropylsilane **(3)** To a solution of compound 2-bromo-9,9-bis(6-bromohexyl)-9H-fluorene (1) (1.14 g, 2.0 mmol) and compound **2** (1.66 g, 2.4 mmol) in toluene (10 mL) under protection gas atmosphere, $Pd(PPh_3)_4$ (142 mg, 0.122 mol) was added. The mixture was reacted with stirring at 110 °C for 24 h. After cooling to room temperature, the mixture was poured into water and extracted twice with ethyl acetate. The organic phase was dried with $MgSO_4$ and evaporated *in vacuo.* The crude material was purified via column

chromatography on silica gel with PE/EA 2:1 to afford **3** as a light yellow oil (856 mg, 48 %). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.70 (dd, *J* = 10.3, 7.4 Hz, 2H), 7.62 (dd, *J* = 7.9, 1.6 Hz, 1H), 7.54 (d, *J* = 1.3 Hz, 1H), 7.35 (tt, *J* = 6.6, 3.6 Hz, 3H), 7.27 (s, 1H), 4.25 (t, *J* = 5.3 Hz, 2H), 3.85 (t, *J* = 5.2 Hz, 2H), 3.77 – 3.65 (m, 6H), 3.59 (dd, *J* = 5.6, 3.7 Hz, 2H), 3.41 (s, 3H), 3.29 (t, *J* = 6.8 Hz, 4H), 2.06 – 2.00 (m, 4H), 1.71 – 1.63 (m, 4H), 1.47 (dt, *J* = 14.8, 7.5 Hz, 3H), 1.25 – 1.05 (m, 26H), 0.61 – 0.68 (m, 4H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 163.66, 151.09, 150.48, 148.34, 140.79, 140.70, 133.38, 127.23, 126.99, 124.29, 122.77, 119.11, 112.59, 109.05, 71.97, 70.77, 70.64, 70.07, 70.01, 59.09, 55.10, 40.30, 34.02, 32.65, 29.04, 27.78, 23.50, 18.87, 12.14. HRMS (ESI) calcd for $C_{45}H_{69}Br_2O_4SSi$, ([M+H$^+$]) 893.2987, Found 893.3026.

(2) 2-(9,9-bis(6-bromohexyl)-9H-fluoren-2-yl)-4-(2-(2-(2-methoxyethoxy)ethoxy)ethoxy) thiophene **(4)** To a solution of compound **3 (**893 mg, 1 mmol) in THF (4 mL) at -78 ºC under protection gas atmosphere, tetrabutylammonium fluoride (1.0 M in THF, 4 mL, 4 mmol) was added. After stirring at this temperature for 1.0 h. the mixture was then slowed warmed to room temperature and stirred for another 3 h. The mixture was poured into water and extracted twice with ethyl acetate. The combined organic phase was dried with $MgSO_4$ and evaporated *in vacuo*. The crude material was purified via flash column chromatography, eluting with hexanes and ethyl acetate. The desired product was isolated as a yellow tinted oil (691 mg, 94 %). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.70 (t, *J* = 7.4 Hz, 2H), 7.56 (d, *J* = 8.0 Hz, 1H), 7.51 (s, 1H), 7.35 (d, *J* = 7.1 Hz, 3H), 7.11 (s, 1H), 6.26 (d, *J* = 1.3 Hz, 1H), 4.24 – 4.17 (m, 2H), 3.93 – 3.86 (m, 2H), 3.78 (dd, *J* = 5.8, 3.5 Hz, 2H), 3.71 (ddd, *J* = 9.3, 5.0, 2.9 Hz, 4H), 3.59 (dd, *J* = 5.6, 3.7 Hz, 2H), 3.41 (s, 3H), 3.29 (t, *J* = 6.8 Hz, 4H), 2.00 (t, *J* = 8.2 Hz, 4H), 1.73 – 1.61 (m, 4H), 1.23 – 1.05 (m, 8H), 0.76 – 0.57 (m, 4H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 157.54, 151.08, 150.54, 143.31, 140.96, 140.58, 133.17, 127.29, 127.00, 124.41, 122.79, 120.10, 119.83, 119.51, 115.53, 96.88, 71.96, 70.84, 70.70, 70.61, 69.71, 69.42, 59.09, 55.03, 40.23, 33.99, 32.64, 29.05, 27.76, 23.53. HRMS(ESI) calcd for $C_{36}H_{48}Br_2O_4S$, ([M+H$^+$]) 737.1653, Found 737.1692.

(3) 4,8-(di-5-(9,9-bis(6-bromohexyl)-9H-fluoren-2-yl)-3-(2-(2-(2-methoxyethoxy)ethoxy) ethoxy)thiophene)-1H,5H-benzo[1,2-c:4,5-c']bis([1,2,5]thiadiazole) **(M-G2)** To a solution of compound **4 (**1.47 g, 2 mmol) in THF (15 mL) at -78 ºC under protection gas atmosphere, *n*-BuLi (1.6 M in Hexane, 1.5 mL, 2.4 mmol) was added dropwise. After the mixture was stirred at this temperature for another 2.0 h, tributyltinchloride (0.812 mg, 2.5 mmol) was added to the solution. The mixture was slowly warmed to room temperature and stirred for 8 h. After that, it was poured into water and extracted twice with ethyl acetate. The combined organic phase was dried with $MgSO_4$ and evaporated *in vacuo* without further purification. To a solution of 4,8-dibromo-1H,5H-benzo[1,2-c:4,5-c']bis([1,2,5]thiadiazole) **(a)** (234 mg, 0.67 mmol) and the crude product (2 mmol) in toluene (15 mL) under protection gas atmosphere, $Pd(PPh_3)_2Cl_2$ (150 mg) was added. The mixture was stirred at 110 ºC for 12 h. After cooling to room temperature, the mixture was poured into water and extracted twice with ethyl acetate. The organic phase was dried with $MgSO_4$ and evaporated *in vacuo*. The crude product was subjected to column chromatography on silica gel with DCM/EA 5:1 to afford **M-G2** as a dark green solid (565 mg, 52 %). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.75 (m, 3H), 7.67 (s, 1H), 7.49 (s, 1H), 7.38 (m, 3H), 4.49 (t, *J* = 5.1 Hz, 2H), 3.76 (t, *J* = 5.1 Hz, 2H), 3.66 – 3.56 (m, 6H), 3.54 (dd, *J* = 5.8, 3.5 Hz, 2H), 3.38 (s, 3H), 3.31 (t, *J* = 6.8 Hz, 4H), 2.07 (t, *J* = 8.1 Hz, 4H), 1.69 (dd, *J* = 14.6, 7.0 Hz, 4H), 1.28

– 1.20 (m, 4H), 1.16 – 1.09 (m, 4H), 0.75 – 0.63 (m, 4H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 156.62, 152.79, 151.23, 150.60, 146.70, 141.55, 140.56, 133.06, 127.49, 127.08, 124.60, 122.82, 120.24, 119.94, 119.66, 113.98, 113.77, 71.91, 71.15, 70.72, 70.60, 70.55, 70.06, 59.05, 55.18, 40.33, 34.03, 32.65, 29.06, 27.81, 23.56. HRMS(ESI) calcd for $C_{78}H_{94}O_8N_4Br_4NaS_4^+$, ([M+$Na^+$]) 1681.2537, Found 1681.2580.

(4) Synthesis of **IR-FGP.** Compound **M-G2** (100 mg, 0.06 mmol) was dissolved in DMF (10 mL) and then sodium azide (47 mg, 0.72 mmol) was added. The mixture was heated for 3 h at 70 ºC. After that, substantial water was added and the mixture was stirred until all solids were dissolved. Then it was extracted twice with ethyl acetate, and the combined organic phase was dried with $MgSO_4$ and evaporated *in vacuo*. The crude product was subjected to flash column chromatography on silica gel to afford dark green solid 96 mg. The dark green solid was dissolved in THF (5 mL) and copper(I) thiophene-2-carboxylate (CuTc) (10 mg), w-alkynyl-PEG-hydroxyl (M_n = 600, 72 mg), and tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine (TBTA) (5 mg) was added. The system was stirred at RT for 0.5 h and then filtered with diatomite. The solution was evaporated *in vacuo*. The crude product was purified by thin layer chromatography eluting with DCM/MeOH 10:1. **IR-FGP** (130 mg) was afforded as a green oil. The number-average molecular weight was measured to be 2800 by SEC, suggesting that two PEG chains were connected to the dye molecule by click reaction.

3. Bio-conjugation between IR-FGP and proteins (streptavidin, Erbitux) based on click chemistry.

Take streptavidin as an example, the following is the bio-conjugation protocol.

Linker: SA was reacted with DBCO-PEG_4-NHS cross-linker.

Reaction buffer: All the reactions were carried in the 1*PBS. The optimal ratio of SA:Dye:linker is 1:10:20.

1. First step: For a typical reaction: SA (5 uL, 90.9 uM) and DBCO-PEG_4-NHS (2.95 uL, 3.08 mM in DMSO) were added in PBS (50 uL) sequentially. The mixture was vortexed a little bit, and reacted at shaker or stirring for 2 hours.
2. Washing: After the reaction, the product was washed by 30 k filter for 4 times, the final volume is 100 uL.
3. Second step: Add IR-FGP (29.7 uL, 152.9 uM) to SA-DBCO (100 uL) (the concentration of the dye can be tuned). The mixture was vortexed a little bit, and reacted at stirring for 3 hours.
4. The product (the output can be enlarged based on the same ratio) was subjected to DGU purification. Sucrose gradient column for 4 mL DGU tube was made by interval adding sucrose solutions: 15, 20, 25, 30, 35, 40, 45, 50, 55 % (400 μL of each and then tilt-permeate); after adding the sample, the column was ultracentrifuged for 18 hours at 50000 rpm and 4 ºC. Sucrose gradient for 50 mL DGU tube was made by auto-gradient maker: 5 mL 60 % sucrose was firstly added at the bottom of the tube, then sucrose gradient column was made from 20% to 50 % (16 mL), at last the column was ultracentrifuged for 48 hours at 31000 rpm and 4 ºC
5. The DGU sample was collected by auto-fractionating machine and stored in -4 ºC for further using.

6. The bio-conjugation between IR-FGP and Erbitux/anti mouse IgG followed the same protocol.

Note 1: Density gradient ultracentrifugation, a technique whereby a buoyant density or sedimentation coefficient difference drives biomolecule separation during ultracentrifugation after establishing a linearly changing density gradient along the length of a centrifuge tube, has demonstrated excellent separation of various species within the reaction mixture post-bioconjugation (1-3).

Note 2: Growing interest in NIR-II imaging mandates the production of high quality molecular probes, yet most NIR-II fluorophore conjugates do not allow purification through standard techniques.

4. Calculation of dye/protein ratio and protein concentration.

1). Measure the protein concentration of the conjugation by Pierce™ BCA Protein Assay Kit.

2). Using the free protein to get the absorbance at 808 nm ($A_{protein,808}$) and 280 nm ($A_{protein,280}$) by UV-vis spectra (nanodrop one).

3). Measure the absorbance of the conjugate at 808 nm ($A_{conjugation,808}$) by nanodrop one.

4). Calculate the absorbance of the dye: $A_{dye,808} = A_{conjugation,808} - A_{protein,808}$

5). Using the following formula to determine the ratio and protein concentration:

$D/P = (A_{dye,808}/\varepsilon_{dye}) : (A_{protein,280}/\varepsilon_{protein})$

$\varepsilon_{dye,808}$=2.83 $L*g^{-1}cm^{-1}$ (808 nm) (If the concentration of dye is too low, use $A_{dye,375}$ and $\varepsilon_{dye,375}$ to determine the concentration of dye) ($\varepsilon_{Erbitux,280}$= 1.45 L/g/cm; $\varepsilon_{SA,280}$= 0.617 L/g/cm; $\varepsilon_{IgG,280}$= 1.36 L/g/cm)

6). The ratio of protein: IR-FGP is ca. 3.1-4.4.

5. Bovine Serum Albumin conjugated with biotin (BSA-biotin).

BSA (6 mg) was dissolved in 1*PBS (1 mL), and EZ-Link™ Sulfo-NHS-LC-Biotin (1 mg) was dissolved in 1*PBS (180 μL). the EZ-Link™ Sulfo-NHS-LC-Biotin was added to BSA and it was reacted at room temperature for 1.5 hour (shaker). After reaction, the volume was concentrated to 100 μL by 30 k Amicon centrifugal filter. At last, the BSA-biotin was purified by illustra NAP-5 columns (GE Healthcare, 17-0853-01). The final concentration of BSA is 4 mg/mL.

6. Lysate preparation.

Lysate was extracted from SCC, U87 MG cells as following.

- 1 million cell of certain type in PBS (1 mL) was obtained from fresh cultured cells.
- Centrifuged the solution at 1500 g for 2 min.
- Removed supernatant and leave cell pellet in the tube.
- Added 50 uL cell lysis solution CeLytic M (Sigma, Cat# C3228-50ML) to the cell pellet and resuspend the cell.
- Incubated the solution for 15 min on a shaker.
- Centrifuged the lysed cells for 10 min at 15000 g to pellet the cellular debris.
- Transfered the protein-containing supernatant to a chilled test tube.

The concentration of lysate was determined by BCA protein assay kit.

7. SA@IR-FGP detection.

(1) Printed BSA-biotin (0.5 mg/mL) on plasmonic Au slide (4-7).

(2) Blocked by BSA + 1 mM Tris in PBS (50 mg/mL), add 100 μL in each reaction well for 1 hour.

(3) Added SA@IR-FGP (pre- and post-DGU samples, normalized as 20 nM) 100 μL in each reaction well for 1 hour.

Note: after step 2 and 3, PBST was used to wash the slide five times and dry for testing.

8. Erb@IR-FGP detection.

(1) Printed SCC cell lysate and U87 cell lysate (2 mg/mL) on plasmonic Au slide.

(2) Blocked by BSA + 1mM Tris in PBS (50 mg/mL), add 100 μL in each reaction well for 1 hour.

(3) Added Erb@IR-FGP (pre- and post-DGU samples, normalized as 100 nM for Erb) 100 μL in each reaction well for 1 hour.

Note: after step 2 and 3, PBST was used to wash the slide five times and dry for testing.

9. SWCNT conjugated with SA or other proteins.

1) Making sodium cholate (SC) coating Laser vaporization (LV) SWCNT (8). DI water (10 mL) was added to a flask with SWCNT powder (4 mg) and sodium cholate (0.1 g). The system was sonicated for 4-6 hours and then centrifuged overnight at 15000 g for 8 hrs. The semiconductor SWCNT was purified by column method as reported by us previously (8). The supernatant was mixed with 1 wt.% sodium dodecyl sulfate (SDS) with a volume ratio of 2:3. The resulting solution was then added to a filtration column filled with ~20 mL of ally dextran-based size-exclusion gel (Sephacryl S-200, GE Healthcare). A mixture surfactant solution of 0.6 wt.% SDS /0.4 wt.% SC was used to wash the column to remove the metallic nanotubes, leaving mainly semiconducting LV SWNTs in the column. The semiconducting LV SWCNT were collected by eluting the column with a solution of 1 wt.% SC.

2) The semiconducting LV SWCNT was exchanged into DSPE-mPEG5k and DSPE-PEG5k-amine with a 2.5:1 weight ratio (1 mg/mL in total), and then dialyzed for 7 days, 1*PBS was changed every 8 hours. The SWCNT solution was stored at 4 ºC for further using.

3) The aggregation of the exchanged SWCNT was removed by ultracentrifuge at 50 k rpm for half an hour. The SWCNT was washed for 6 times before conjugation (30 kDa) to remove excess surfactants. The remaining volume in the filter should be <0.5 mL.

4) After the final wash step (concentrate the SWCNT solution down to as much as possible), and 10X PBS was added to make the SWCNT in 1x PBS. Tiny sample was diluted to test the absorption value at 280 nm by UV-vis. Then divided by 3.5 to obtain the absorption at 808 nm (OD at 808 nm is 3.5 lower than that at 280 nm) (9). The concentration of SWCNT was determined by using extinction coefficient 7900000 cm*L/mol (9-11).

5) Calculate amount of needed reagents. The mole ratio of SWCNT:SA:Traut's was 1:20:200. SWCNT and SA were dissolved in 1*PBS. Traut's reagent was dissolved in 1*PBS with 5 mM EDTA.

6) Traut's solution (8.3 μL, 10 mM) was added to SA (92 μL, 90.9 μM). Then EDTA (0.92 μL, 0.5 M) was added to adjust the final concentration of EDTA to 5 mM. The system was shaken at room temperature for 1.5 hour.

7) At the same time, Sulfo-SMCC (0.5-1 mg) was dissolved in DMSO (50-100 μL), and SWCNT (200 uL, 2.08 uM) was added. The reaction was left at room temperature for 1-1.5 h by shaker.

8) Prepared SA-Traut's washing solution. TCEP (3 mg), NaOH (35 μL, 1M) (since TCEP is acidic) and EDTA/1x PBS (65 μL, 5 mM) (can scale appropriately if more is needed) were mixed. The solution was verified to have pH ~7.4. 10x dilution was performed using 5 mM EDTA/1x PBS to make it 1 mL.

9) Once incubation of antibody was complete, excess Traut's reagent was removed by filtration using 500 μL 30 kDa filters. The prepared SA washing solution was added to reach the total volume of the filter device and centrifuge was conducted for 5 times for 5 min at 10,000 g and at room temperature. The SA washing solution was replenished after each wash. The final volume was 50-100 μL.

10) Removed excess Sulfo-SMCC using an Amicon centrifugal filter device (MWCO = 30 kDa). The solution was washed 6 times with DIUF. After the final step, 10*PBS was used to make the final solution 1*PBS, and the final volume was ca. 200 uL.

11) Mixed the Sulfo-SMCC-modified SWCNT with the thiolated antibody. The reaction solution was incubated for 48 h at 4 ºC.

12) The product (the output can be enlarged based on the ratio) was subjected to DGU purification. Sucrose column gradient for 4 mL DGU tube was made by interval adding sucrose solutions: 5, 10, 15, 20, 25, 30, 40 % (400 μL of each and then tilt-permeate), ultracentrifuged for 2 hours at 50000 rpm and 4 ºC.

13) The DGU sample was collected by fractionating and then left for testing or using.

10. Anti CD 31 conjugated with biotin (anti CD 31-biotin).

EZ-Link™ Sulfo-NHS-LC-Biotin (1 mg) was dissolved in 1*PBS (180 μL, 10 mM). EZ-Link™ Sulfo-NHS-LC-Biotin (2 μL, 10 mM) was added to anti CD31 (100 μL, 10 μM) to react at room temperature for 1.5 hour (shaker). After reaction, the anti CD 31-biotin was purified by illustra NAP-5 columns (GE Healthcare, 17-0853-01). The final concentration of anti CD 31-biotin was 2 μM.

11. Cell staining.

SCC/U87 cells were cultured in DMEM medium plus 10% FBS. The cell line was maintained in a 37 °C incubator with 5% (v/v) CO_2 for 24 h firstly. Cells were then incubated with 20-200 nM Erbitux@IR-FGP for 1 h. At last, the cells were washed with PBS for 3 times and scanned by NIR confocal.

12. Tissue staining.

12.1. Brain tissue three color staining

1.1 Brought the tissue slides to room temperature and let sit for at least 30 min.

1.2 Fixed in 4% PFA (4g PFA in 100 mL PBS) for 20 min. Washed sections in TBST 3 times for 5 min shaker each time.

1.3 Pretreated for 20 min with 0.1% Triton and 0.3% H_2O_2 in TBST.

1.4 Washed with TBST 3 times (5 min for every time).

1.5 Blocked with 10% goat serum for 1 hour.

1.6 Applied anti CD31-biotin and mouse anti Neuron primary antibody (20-500 nM of each in 10% goat serum), 500 uL for each slice. Incubated overnight at 4 °C refrigerator.

1.7 Washed tissue using TBST 3 times (5 min for every time).

1.8 Applied SA@SWCNT and anti mouse IgG@IR-FGP (20-500 nM of each in 10% goat serum), 500 uL for each slice. 3 hours at room temperature.
1.9 Added Deep Red (250 times dilute) for each slice for 45 min.
1.10 Washed tissue 3 times with TBST (5 min for every time) and scanned by home-built NIR confocal set-up.
1.11 For 3D staining, hydration/dehydration process was added between 1.2 and 1.3.

12.2. SCC tumor three color staining
1.1 Brought the tissue slides to room temperature and let sit for at least 30 min.
1.2 Fixed in 4% PFA (4g PFA in 100 ml PBS) for 20 min. Washed sections in TBST 3 times for 5 min shaker each time.
1.3 Permeated for 20 min with 0.1% Triton and 0.3% H_2O_2 in TBST.
1.4 Washed with TBST 3 times (5 min for every time)
1.5 Blocked with 10% goat serum for 1 hour.
1.6 Applied anti CD 31-biotin (20-500 nM in 10% goat serum), 500 uL for each slice. Incubated overnight at 4 °C refrigerator.
1.7 Washed tissue using TBST 3 times (5 min for every time).
1.8 Applied SA@SWCNT and Erbitux@IR-FGP (20-500 nM of each), 500 uL for each slice. 3 hours at room temperature.
1.9 Added Deep Red (250 times dilute) for each slice for 45 min.
1.10 Washed tissue 3 times with TBST (5 min for every time) and scanned by home-built NIR confocal set-up.

13. Instruments and characterization.

Tissue slide was cut on Microw HM550. A plasmonic gold films composed of tortuous gold nano-islands was fabricated as previous report (4). Assay spots was printed by GeSiM Nano-Plotter 2.1. UV-Vis-NIR spectrophotometer (Cary 6000i) with background correction was employed to measure the optical absorption spectrum of IR-FGP in water in the range of 300-1200 nm. A home build confocal setup was used to scan cell or tissue samples with three color channels. A home build setup was used to measure the fluorescence spectra of IR-FGP and SWCNT in the region of 900-1800 nm using an array detector (Princeton OMA-V) and a spectrometer (Acton SP2300i) under an 808-nm diode laser (RMPC lasers) excitation (160 mW). During emission measurements, two 850-nm (Thorlabs), 900 nm (Thorlabs), 1,000-nm (Thorlabs), 1,100-nm (Omega), 1,200-nm (Omega), 1,300-nm and 1,400-nm short-pass filter (Omega) were used as excitation filters and one 900-nm long-pass filter (Thorlabs) was used as emission filter. The obtained emission spectra were further corrected by the detector sensitivity profile and the absorbance features of the filter. All the staining images were plotted by Matlab and ImageJ.

14. Animals.

The experiments were approved by the Institutional Animal Care and Use Committees of VA Palo Alto Health Care System and of Stanford University. All experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Nude or Balb/C mice were purchased from The Jackson's Laboratory (Bar Harbor, ME). Bedding, nesting material, food and water were provided ad libitum. Ambient temperature was controlled at 20 to 22°C with 12-hour light/12-hour dark cycles. SCC tumor was formed by injecting 1.5 million SCC cells under subcutaneous in nude mice. Brain tissue was obtained from heathy Balb/C mice.

15. Figure S1-S10.

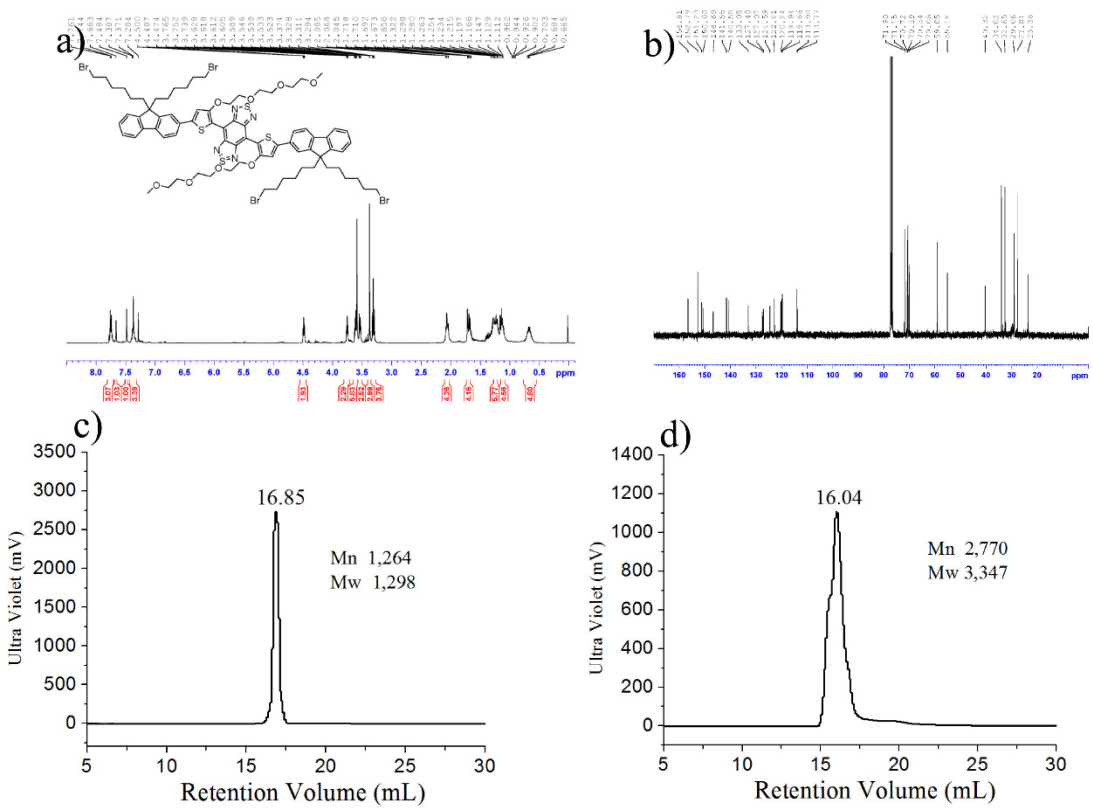


Figure S1. Structure characterizations: a) ^{1}H NMR and b) ^{13}C NMR spectrum of **M-G2**. c) SEC trace and molecule weight of **M-G2**. d) SEC trace and molecular weight of **IR-FGP**.

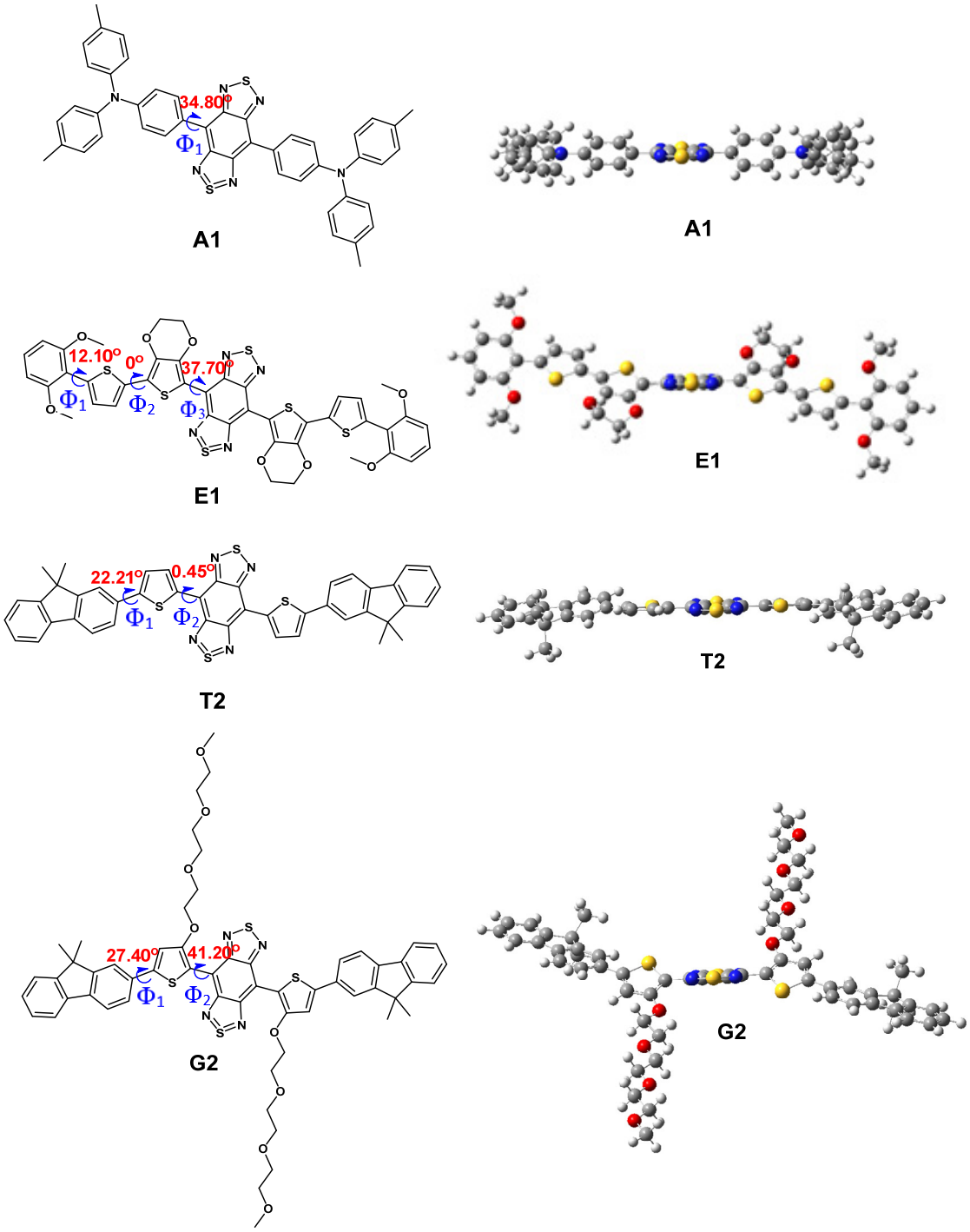


Figure S2. Dihedral angles and molecular shapes from the simulated results of the NIR-II molecular fluorophores analogues: **A1** (CH1055) (12), **E1** (IR-E1) (13), **T2** (IR-FTP) (14) and **G2** (IR-FGP). The calculations were performed using the Gaussian 09 with the B3LYP, 6-31g(d) functional. The geometric structures of all species were optimized in the gas phase. The fluorescence quantum yields for these PEGylated fluorophores in aqueous solution under 808 nm excitation are as following: CH1055 ~ 0.30%, IR-E1 ~ 0.70%, IR-FTP ~ 0.020%, and IR-FGP ~ 1.9%. The enhanced QY of IR-FGP in water results from the synergistic effect of both TEG substituted thiophene donor and fluorene shielding groups in the dye structure.

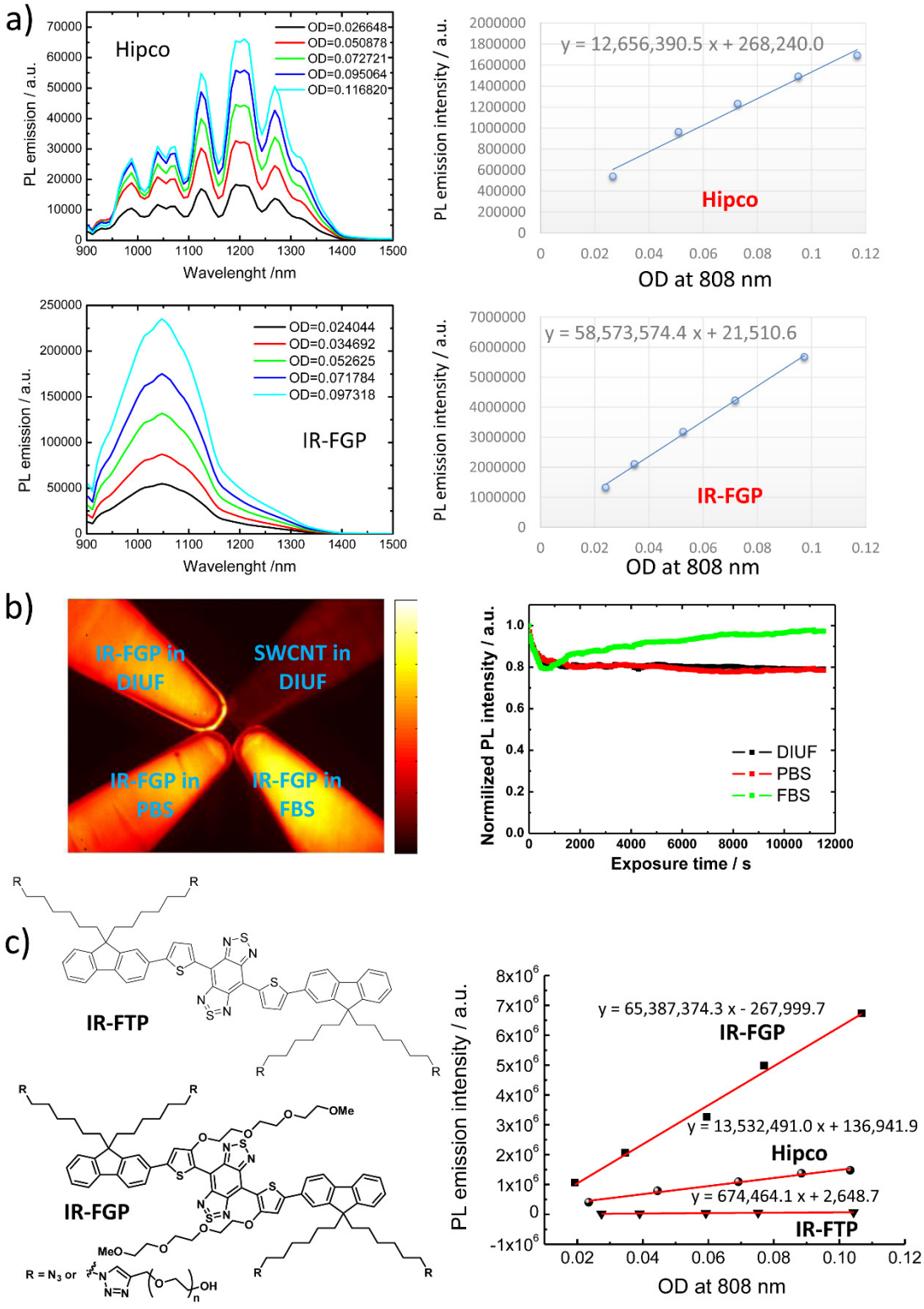


Figure S3. Quantum yield and photo-stability of IR-FGP. a) Quantum yield measurement of IR-FGP by Hipco CNT as reference. The quantum yield of Hipco CNT is 0.40%, so the QY of IR-FGP=0.40%*58573574.4/12656390.5=1.9% (significant digits are different for OD, PL intensity and QY to precisely express the value). b) Photo-stability of IR-FGP in water, PBS, and FBS under continuous 808-nm radiation for up to 3 h. IR-FGP in DIUF and PBS exhibited considerable photostability with less than 20% decay for continuous excitation of over 3h, while it

shows much better photo-stability in FBS compared with in DIUF and PBS. c) Chemical structure of IR-FGP and IR-FTP (left) and the measurements of their fluorescence quantum yield in aqueous solution (right). The QY of IR-FTP is only 0.020%, which is much lower than IR-FGP (It should be noted that the data of Fig. S3a and c came from two times of measurement with home-built spectrophotometer).

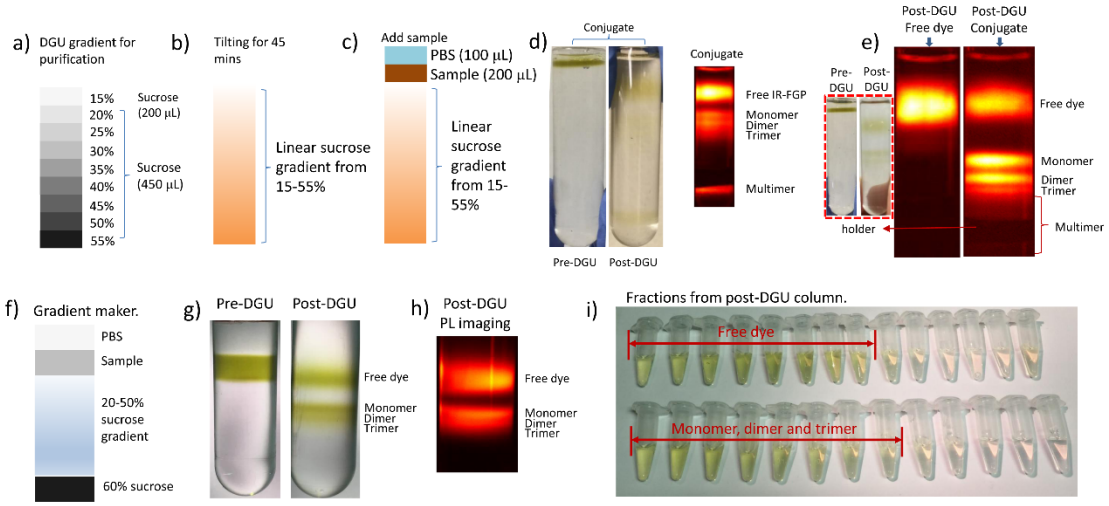


Figure S4. The used DGU gradient for purifying proteins@IR-FGP, the volume of DGU tube is 4 mL. a) The added sucrose for preparing DGU gradient. b) After tilting for 45 min at 20° angle, the linear gradient formed. c) And the sample and protecting PBS were added in the column, respectively. After that, the column was subjected to ultracentrifugation at 50 k RPM for 18 hours. d) Pictures of pre- and post-DGU tubes of SA@IR-FGP (the bottom part of DGU tube is not included in fluorescent picture). e) Day light and PL (1100 nm LP) pictures of the DGU tubes of Erbitux@IR-FGP (the bottom part of DGU tube is not included in fluorescent picture). f-i) Big DGU purification of IR-FGP and Erbitux conjugates for scaling up. f) The used DGU gradient for purifying Erbitux@IR-FGP which was made by gradient maker. g) Picture of pre- and post-DGU samples (50 mL DGU tube), and the monomer, dimer and trimer can be observed by naked eyes. h) PL (1100 nm long pass filter) imaging of post-DGU sample. i) Fractions from post-DGU column, the monomer can be extracted without any free dye or dimer/trimer using auto-fraction machine.

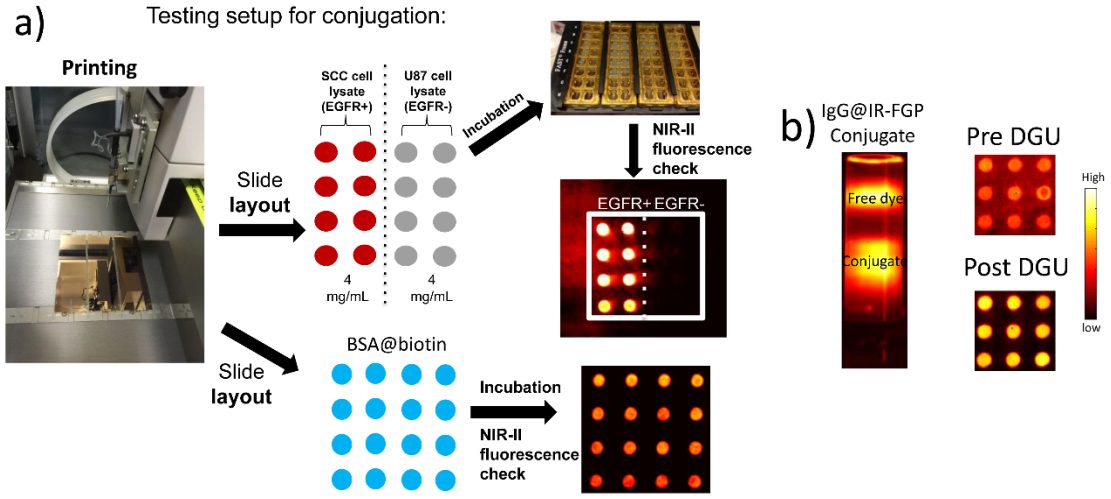


Figure S5. a) Assay testing setup for bio-conjugation. 1. The BSA-biotin, "catching" primary antibody or cell lysate were printed on the plasmonic fluorescence-enhancing gold slide. 2. The testing conjugates (pre/post DGU conjugates) were incubated on the top of the printed spots. 3. Check the PL intensity by 10* magnification NIR II set-up with 808 nm excitation and 1100/1500 nm long-pass emission filter. b) PL pictures of the DGU tubes of anti mouse IgG@IR-FGP; Assay testing the conjugation of anti-mouse IgG@IR-FGP (the printed spot is mouse anti her2 antibody). Anti her2 was printed on the plasmonic fluorescence-enhancing gold slide; then the pre/post DGU conjugates were incubated on the slide; at last the slide was scanned by NIR II set-up with 808 nm excitation and 1100 nm long pass filter. The enhanced SBR or P/N ratio is due to the removal of free IR-FGP, which could cause non-specific binding on the slide.

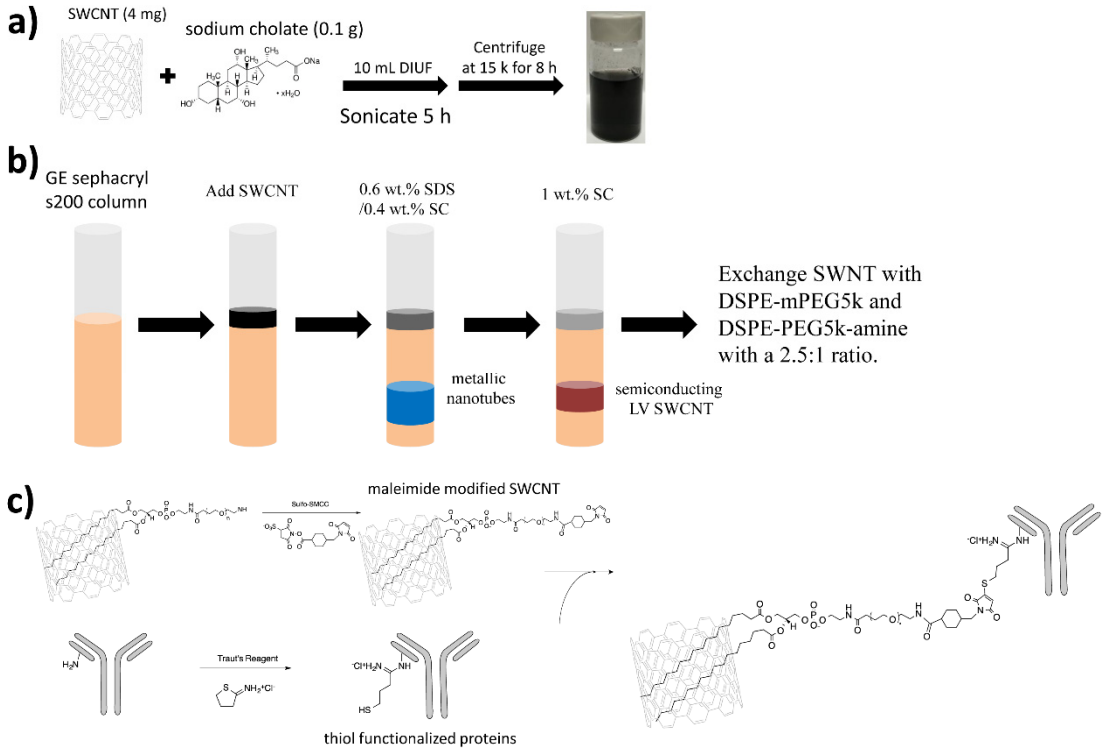


Figure S6. Conjugation between SWCNT and protein. a) Laser-vaporization SWCNTs with

fluorescence emission past 1500 nm were dispersed with an ionic surfactant to preserve quantum yield. b) Semiconducting SWCNT was separated with higher QY by column method, following by coating with amine terminated polymers through a surfactant exchange method with DSPE-mPEG/DSPE-PEG-amine. c) Conjugation route between SWCNT and protein. The amino groups were reacted with a sulfosuccinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (sulfo-SMCC) linker, resulting in maleimide modified SWCNT capable of reacting with thiol functionalized proteins with high reactivity and selectivity.

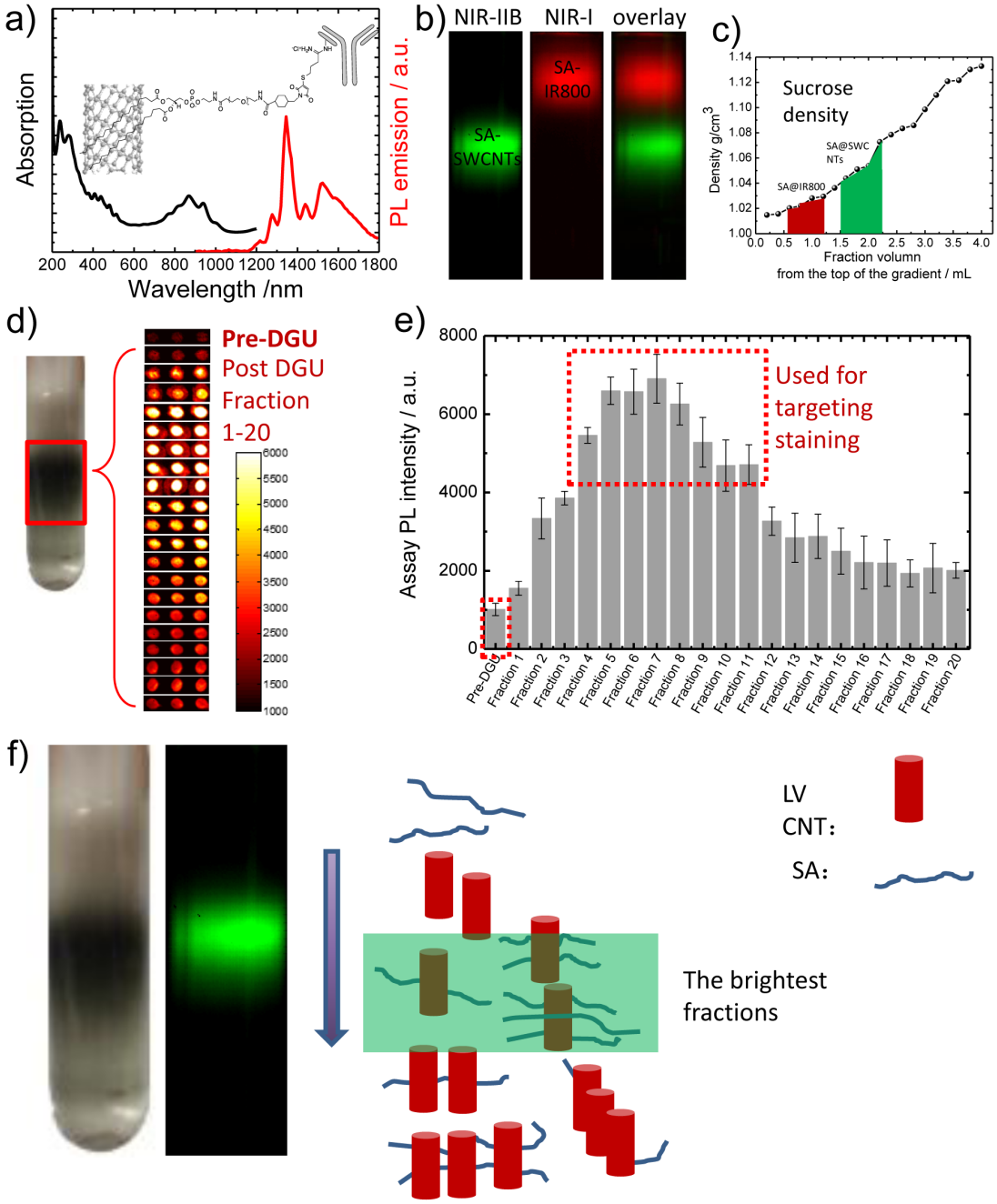


Figure S7. a) Absorption and PL (808 nm excitation) spectra of SWCNT (inset is the chemical structure of SA@SWCNT conjugate). b) The DGU purification of the SA@SWCNT: Fluorescent images of post-DGU sample under NIR IIb (1500 nm long pass filter) and NIR I (850 nm long pass plus 900 nm short pass filters) windows: a little SA-IR800 was added for visualizing free SA

due to the same isopycnic of SA and SA-IR800 in DGU tube. For DGU purification, unlike the NIR-II dye labeled proteins where minute buoyant density differences of the labeled versus unconjugated targeting proteins did not allow removal of unconjugated proteins, substantial differences in the sedimentation coefficients of unbound proteins versus protein-conjugated nanotubes enabled facile elimination of all unconjugated proteins to afford highly pure protein-nanotube conjugates. c) The tested position of free SA (SA@IR800) and SA@SWCNT in post-DGU sucrose gradient. d) Binding efficiency and PL intensity of SA@SWCNT DGU fractions were tested by assay method. The conjugate band was firstly extracted with 20 fractions; then the fractions were normalized by UV-vis at 808 nm; following by incubating on plasmonic fluorescence-enhancing gold slide with BSA-biotin spots; at last the slide was scanned by 10* magnification NIR II set-up with 808 nm laser excitation and 1500 nm long-pass emission filter. e) The statistics PL intensity of fraction assay spots. f) The different schemes of the conjugates distributed from the top to bottom in the DGU column. The observed brighter fractions near the top of the nanotube band could result from highly protein-decorated SWCNTs migrating through the gradient at a slower sedimentation velocity, while carbon nanotubes with less bound streptavidin progressively appeared at increasing densities. Another possible reason for brightness fraction of SWCNT in DGU comes from length distribution and aromatic stacking effects (15). Further, the low NIR-II tissue autofluorescence that eventually could reach near-zero levels in the NIR-IIB (1500-1700 nm) window could facilitate a marked increase in fluorescent probe signal-to-background ratios and imaging specificity (8, 16, 17).

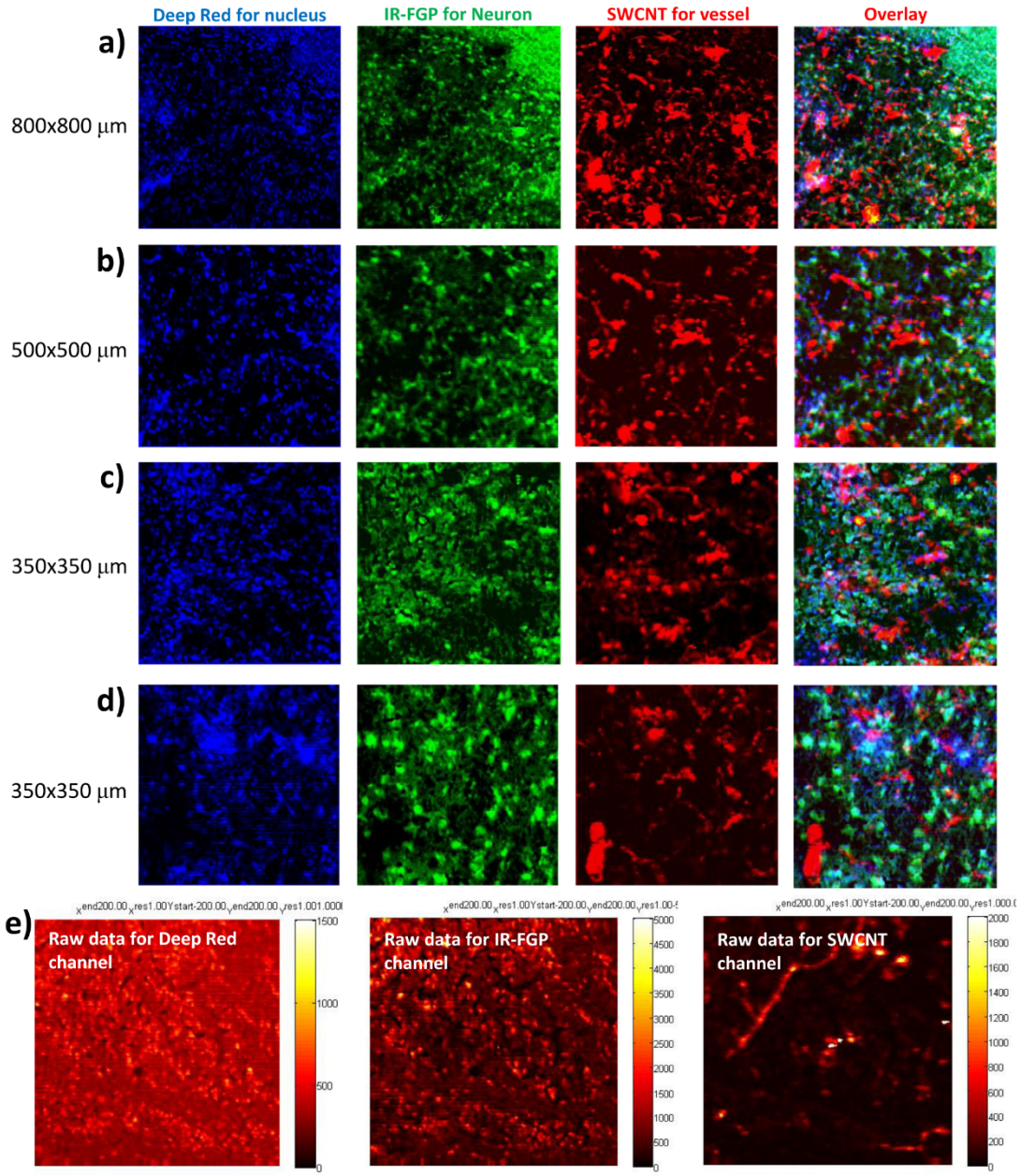


Figure S8. Several 2D scanning images (a-d) of multi-color brain tissue labelling: Deep Red with NIR I emission for staining the nucleus (blue) **(658 nm excitation with 850 nm long pass and 900 nm short pass emission filters)**; Anti-neuron and anti mouse IgG@IR-FGP with NIR IIa' emission for staining the NeuN nucleus (green) **(785 nm excitation with 1050 nm long pass and 1300 nm short pass emission filters)**; Anti CD 31 and SA@SWCNT with NIR IIb emission for staining vessel (red) **(785 nm excitation with 1500 nm long pass emission filter)**. (800-900 nm labels for cell nuclei; 1050-1300 nm for neurons (18); 1500-1700 nm for blood vessels). e) Raw data of three channels without tuning "low Z" scale: Deep red channel shows much higher autofluorescence and non-specific binding signal of background than IR-FGP and SWCNT channels (although there is little emission overlay between IR-FGP and SWCNT, the lower QY of SWCNT didn't affect IR-FGP channel).

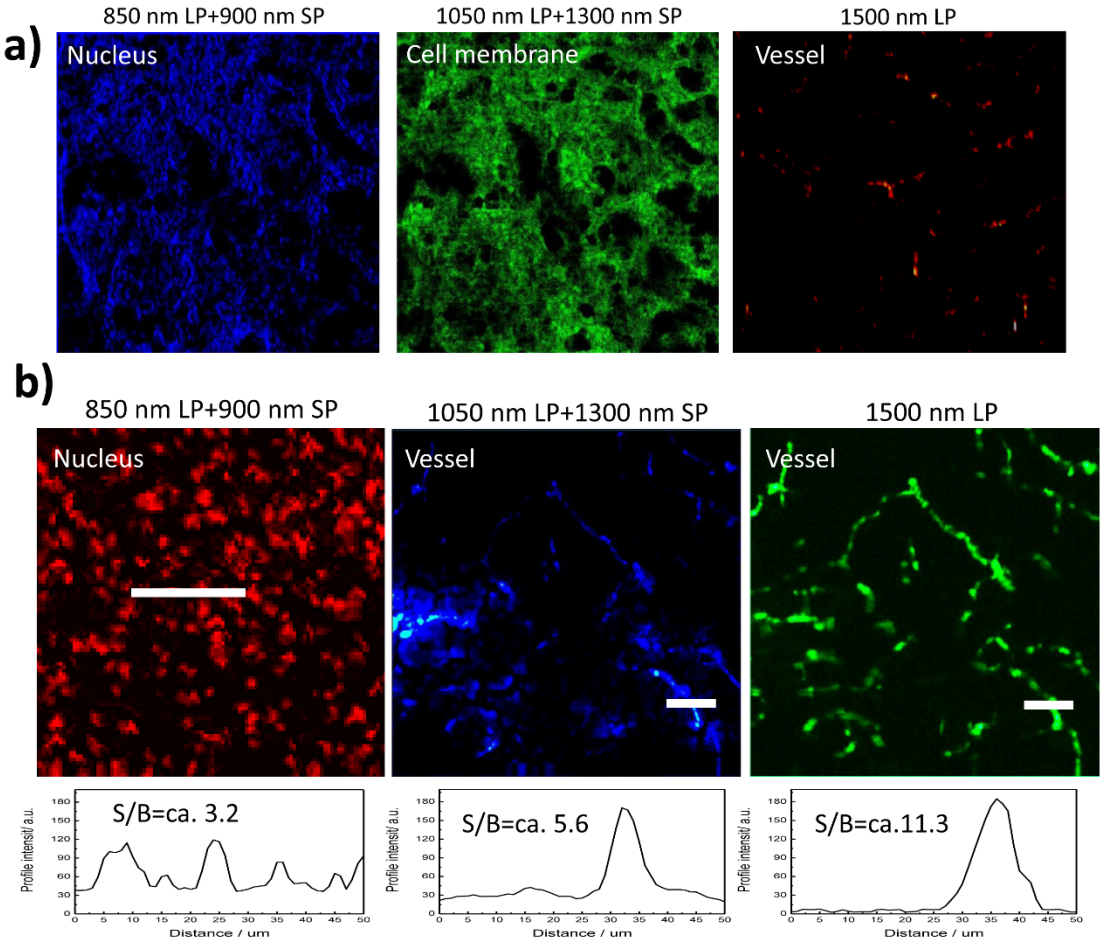


Figure S9. a) Three colors staining of SCC tumor slide (500 μm*500 μm area). Deep Red with NIR I emission for staining the nucleus (blue); Erbitux@IR-FGP with NIR IIa' emission for staining cell EGFR (green); SA@SWCNT with NIR IIb emission for staining vessel (red). b) Comparison (calculation from raw data without changing Z intensity scale) of the ratio of signal to background at NIR I and IIa'/IIb windows. The brain tissue was stained by Deep red for nucleus, anti CD 31-biotin and SA@IR-FGP / SA@SWCNT for Vessel staining (250 μm*250 μm).

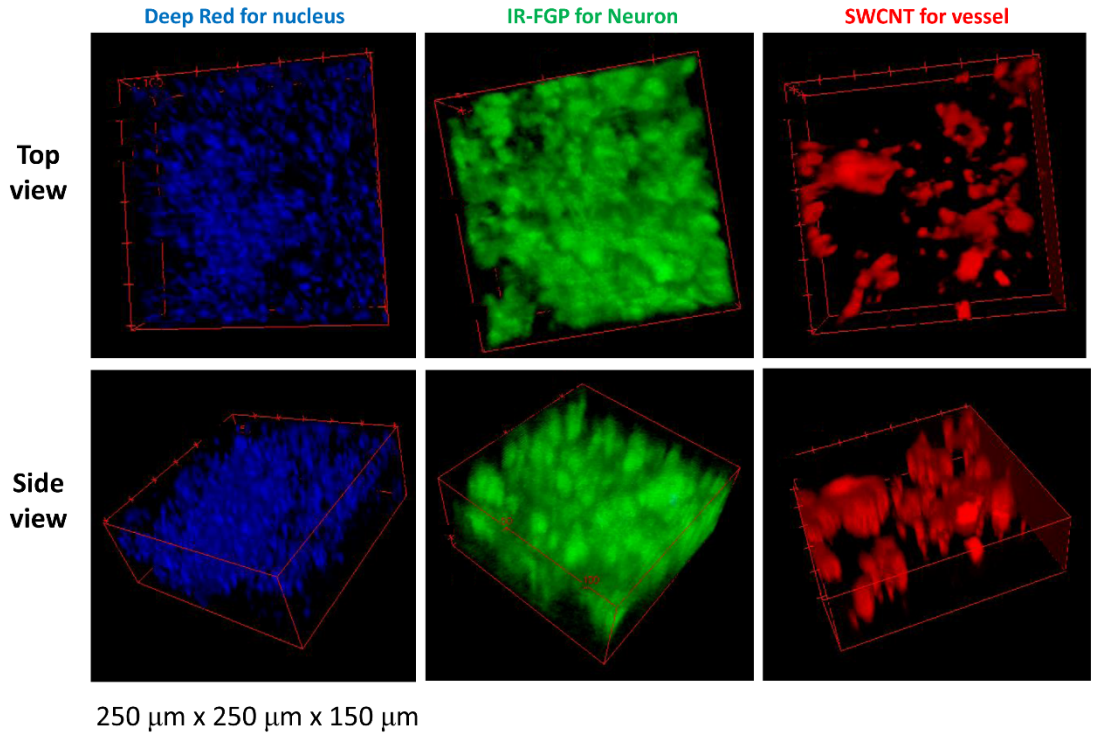


Figure S10. Separated 3D scanning of multi-color brain tissue labelling: Deep Red with NIR I emission for staining the nucleus (blue); Anti mouse IgG@IR-FGP with NIR IIa' emission for staining the NeuN nuclues (green); SA@SWCNT with NIR IIb emission for staining vessel (red). The development of a panel of NIR-II molecular probes will allow the fluorescence imaging community to truly benefit from the improved optical imaging metrics garnered by long-wavelength NIR-II imaging.

References

1. Green AA & Hersam MC (2007) Ultracentrifugation of single-walled nanotubes. *Materials Today* 10(12):59-60.
2. Ghosh S, Bachilo SM, & Weisman RB (2010) Advanced sorting of single-walled carbon nanotubes by nonlinear density-gradient ultracentrifugation. *Nature Nanotechnology* 5(6):443-450.
3. Antaris AL, *et al.* (2015) Single Chirality (6,4) Single-Walled Carbon Nanotubes for Fluorescence Imaging with Silicon Detectors. *Small* 11(47):6325-6330.
4. Zhang B, *et al.* (2012) Multiplexed cytokine detection on plasmonic gold substrates with enhanced near-infrared fluorescence. *Nano Research* 6(2):113-120.
5. Zhang B, *et al.* (2013) An integrated peptide-antigen microarray on plasmonic gold films for sensitive human antibody profiling. *PloS One* 8(7):e71043.
6. Zhang B, Kumar RB, Dai H, & Feldman BJ (2014) A plasmonic chip for biomarker discovery and diagnosis of type 1 diabetes. *Nature Medicine* 20(8):948-953.
7. Li X, *et al.* (2016) Multiplexed Anti-Toxoplasma IgG, IgM, and IgA Assay on Plasmonic Gold Chips: towards Making Mass Screening Possible with Dye Test Precision. *Journal of Clinical Microbiology* 54(7):1726-1733.
8. Diao S, *et al.* (2015) Fluorescence Imaging In Vivo at Wavelengths beyond 1500 nm. *Angewandte Chemie* 54(49):14758-14762.

9. Kam NW, O'Connell M, Wisdom JA, & Dai H (2005) Carbon nanotubes as multifunctional biological transporters and near-infrared agents for selective cancer cell destruction. *Proceedings of the National Academy of Sciences of the United States of America* 102(33):11600-11605.
10. Welsher K, Sherlock SP, & Dai H (2011) Deep-tissue anatomical imaging of mice using carbon nanotube fluorophores in the second near-infrared window. *Proceedings of the National Academy of Sciences of the United States of America* 108(22):8943-8948.
11. Liu Z, Tabakman SM, Chen Z, & Dai H (2009) Preparation of carbon nanotube bioconjugates for biomedical applications. *Nature Protocols* 4(9):1372-1382.
12. Antaris AL*, et al.* (2016) A small-molecule dye for NIR-II imaging. *Nature Materials* 15(2):235-242.
13. Zhang XD*, et al.* (2016) Traumatic Brain Injury Imaging in the Second Near-Infrared Window with a Molecular Fluorophore. *Advanced Materials* 28(32):6872-6879.
14. Woo SJ*, et al.* (2016) Synthesis and Characterization of Water-Soluble Conjugated Oligoelectrolytes for Near-Infrared Fluorescence Biological Imaging. *ACS Applied Materials & Interfaces* 8(25):15937-15947.
15. Tabakman SM, Welsher K, Hong G, & Dai H (2010) Optical Properties of Single-Walled Carbon Nanotubes Separated in a Density Gradient; Length, Bundling, and Aromatic Stacking Effects. *The journal of physical chemistry. C, Nanomaterials and Interfaces* 114(46):19569-19575.
16. Du Y*, et al.* (2010) Near-infrared photoluminescent Ag2S quantum dots from a single source precursor. *Journal of the American Chemical Society* 132(5):1470-1471.
17. Liang C*, et al.* (2014) Tumor metastasis inhibition by imaging-guided photothermal therapy with single-walled carbon nanotubes. *Advanced Materials* 26(32):5646-5652.
18. Kozai TD*, et al.* (2012) Ultrasmall implantable composite microelectrodes with bioactive surfaces for chronic neural interfaces. *Nature Materials* 11(12):1065-1073.