

Supplementary Material

¹⁸F-labeled Single-Stranded DNA Aptamer for PET Imaging of Protein Tyrosine Kinase-7 Expression

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Materials and Methods

General

All solvents and chemicals were purchased from Sigma-Aldrich. Tetrabutylammonium bicarbonate (TBAHCO₃) (**23**) and spirocyclic hypervalent iodine(III) precursor (**1**) (**24**) were previously reported. ¹⁸F-Fluoride was acquired from the NIH Cyclotron Facility. Sgc8-alkyne aptamer (5-Hexynyl-AT CTA ACT GCT GCG CCG CCG GGA AAA TAC TGT ACG GTT AGA, formula weight = 12775 Da) was purchased from Integrated DNA Technologies Inc. Female athymic nude mice (Harlan Laboratories) were housed in an animal facility under

pathogen-free conditions. In vivo studies were conducted under an animal protocol approved by the NIH Clinical Center Animal Care and Use Committee. LC-MS analysis was done similar to the reported procedure (25). ^{18}F -Tr-Sgc8 quality control analysis and stability studies were performed on high performance liquid chromatography (HPLC) using Phenomenex Luna C8 column (5 μm , 4.6 \times 150 mm) with a gradient system, starting from 90% of solvent A (0.1M triethylammonium acetate in water) and 10% of solvent B (CH_3CN) and changing to 70% solvent A and 30% solvent B at 35 min at flow rate of 1 mL/min. The ultraviolet (uv) absorbance was monitored at 254 nm. The retention time (rt) of ^{18}F -Tr-Sgc8 was 17.4 min and un-reacted Sgc8-alkyne was 14.7 min.

Chemistry

Synthesis of Fluoro-Tr-Sgc8 standard. 11 nmol of aptamer (150 μg) were dissolved in 15 μL of H_2O . Then 0.7 mg of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ dissolved in 20 μL of H_2O were added followed by 5.6 mg of sodium ascorbate in 200 μL of 0.1M Borate buffer pH 8.6. Next, 1.5 equivalent of fluorobenzyl azide in 50 μL of CH_3CN was added. The mixture was shortly vortexed and incubated at 37 $^\circ\text{C}$ for 15 min and then purified by HPLC with a retention time of 17.28 min for fluoro-Tr-Sgc8. The collected HPLC fraction was lyophilized. Deconvolution analysis of LC-MS (supplemental material) confirmed mass of 12925.

Radiochemistry

Labeling of ^{18}F -fluorobenzyl azide. Optimization utilized this same system with changes in temperatures, concentrations, reagents etc. until final conditions settled. The radiosynthesis of ^{18}F -fluorobenzyl azide was performed on a modular system (Eckert & Ziegler Eurotope GmbH).

^{18}F -fluoride solution was transferred directly to the reactor, without trapping on an anion exchange column, and followed by addition of TBAHCO_3 (1.52 moles equivalent amount from the precursor) in 15% water: CH_3CN . The reactor was heated to $120\text{ }^\circ\text{C}$ and under a stream of argon and vacuum; the water and CH_3CN were removed by azeotropic evaporation using additional 0.5 mL of CH_3CN . Then after spirocyclic hypervalent iodine(III) precursor (**1**) was added in 0.4 mL anhydrous dimethylformamide. The reactor was sealed and heated to $120\text{ }^\circ\text{C}$ for 10 min. subsequently the reactor was cooled to $40\text{ }^\circ\text{C}$, followed by addition of 1 mL water. The crude reaction was injected into a built-in HPLC using Agilent column [300SB-C18, 9.4X250 mm] and isocratic conditions of 32% CH_3CN :water at flow rate of 6 mL/min. Under these conditions, ^{18}F -fluorobenzyl azide had a retention time of 16 min. ^{18}F -fluorobenzyl azide was collected into a glass flask containing 20 mL of water and loaded on a C-18 Sep-pak. The Sep-pak was dried under Argon for 3 min and finally ^{18}F -fluorobenzyl azide was eluted into a glass tube using 0.8-1 mL of CH_3CN . ^{18}F -benzylazide quality control and specific activities calculation were done on a Phenomenex Luna C8 column ($5\text{ }\mu\text{m}$, $4.6\times 150\text{ mm}$) using a gradient system starting from 80% of solvent A (0.1% TFA in water) and 20% of solvent B (0.1% TFA in acetonitrile) for 2 min and increasing to 10% solvent A and 90% solvent B at 15 min, with a flow of 1 mL/min and a rt of 10.9 min. The ultraviolet absorbance was monitored at 254 and 210 nm.

Biology

HCT116 and U87MG Cell Culture and Flow Cytometry. HCT116 cells were grown in McCoy's 5A medium (Hyclone) and U87MG cells were grown in EMEM medium (Invitrogen). All cell culture media were supplemented with 10% fetal bovine serum (Gibco). Cells were grown at $37\text{ }^\circ\text{C}$ in a humidified atmosphere containing 5% CO_2 . PTK7 expression levels on both cell lines

were evaluated by staining the cells with Phycoerythrin-labeled anti-PTK7 antibody or isotype control (Miltenyi Biotec) and the cells were acquired using a LSRII flow cytometer (Becton-Dickinson). Data was analyzed using FlowJo (Tristar).

Cell Binding Assays. HCT116 and U87MG cells were harvested and re-suspended in 1% (w/v) bovine serum albumin/phosphate buffered saline (binding buffer). 1×10^5 cells per well were placed in a 96-well membrane plate (Corning). ^{18}F -Tr-Sgc8 (0.1 nCi, 3.7 Bq) and different concentrations of unlabeled aptamer ranging from 1 pM to 1 μM were added to the wells. After 1 h of gentle shaking at room temperature, the plate was washed six times with binding buffer and dehydrated in an oven at 60 °C. The membrane was punched out of the plate and was exposed to a phosphor-imager plate for 18-24 h. Thereafter, the plate was analyzed on a Cyclone Plus phosphor imager (PerkinElmer) using Image Gauge (Fujifilm Software) software. Binding data was calculated using GraphPad Prism software (GraphPad Software).

Western Blot. The flash-frozen tumors or tissues were thawed in T-PER Tissue Protein Extraction Reagent (Pierce Biotechnology Inc) supplemented with proteinase inhibitor cocktail (Roche Life Science) followed by cutting into small pieces and probe sonication (Ultrasonic Processor) to extract tissue proteins. Protein concentration was titrated using BCA Protein Assay Kit (Pierce Biotechnology Inc). Protein separation used the 4%-12% pre-casted Bis-Tris SDS-PAGE Protein Gel (Life Technology). An equal amount of protein (30 μg) was loaded into each well. PVDF membrane (0.45 μm pore size, Life Technology) was used for protein transfer. Immunoblotting was carried out using monoclonal anti PTK7 antibody (1:500; Millipore), and Horseradish Peroxidase conjugated donkey anti mouse secondary antibody (IgG) (1:4000, Jackson Immunoresearch Laboratories Inc.) was used. SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology Inc.) was used for signal production. The image signal was

collected from Amersham Imager 600 (GE Healthcare Life Science). Data were processed and analyzed by ImageJ software (NIH) (n=3).

Magnetic resonance imaging (MRI). MRI was conducted on a high magnetic field micro-MR scanner (7.0 T, Bruker, Pharmascan) with small animal-specific body coil. 1.5–2% Isoflurane in O₂ was employed to anesthetize the mice. During the experiment, the mice were kept warm by a circulating warm water (37 °C) and were placed in a stretched prone position with a respiratory sensor. T2-weighted images were acquired by a fast spin echo sequence and the parameters were as the following: repetition time, 2,500 ms; effective echo time, 30 ms; RareFactor = 6; Flip Angle=180; number of excitations, 1; matrix size, 256 × 256; field of view, 4 × 4 cm; slice thickness, 1 mm. The artifacts caused by respiration were controlled by a magnetic resonance-compatible small animal respiratory gating device.

¹⁸F-Tr-Sgc8 Stability in Mouse Serum. ¹⁸F-Tr-Sgc8 (50 µL) was incubated in 500 µL of mouse serum. At 30, 1 and 2 h an aliquot of 100 µL was taken, diluted with 100 µL of saline and heated at 95 °C for 5 to denature the large proteins. Then after, it was centrifuged at 13,000 g for 10 min. At 1 h an additional aliquot was taken, diluted with 150 µL of saline and loaded on NAP5 column. The supernatants and NAP5 fraction 4 were loaded on 2% agarose gel (Fisher Scientific). The Gel was imaged after staining with GelStar (Lonza) using a Fluorchem camera (Alpha Innotech).

Figure Legend

Figure S1. Representative HPLC chromatogram (uv absorbance) of “cold” reaction between Sgc8-alkyne (11 nmol) and 1.5 eq of fluorobenzylazide in the presence of sodium ascorbate, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ and 0.1M borate buffer.

Figure S2. LC-MS results of Sgc8-alkyne (A) and the plus 151 molecular weight of the conjugated unlabeled fluoro-Tr-Sgc8 (B) after deconvolution.

Figure S3. HPLC chromatogram of (A) ^{18}F -Tr-Sgc8 crude reaction – before NAP5 purification and (B) ^{18}F -fluorobenzylazide – before conjugation with Sgc8-alkyne.

Figure S4. Representative PET images of mouse bearing tumor in the peritoneum, injected with 100 μCi (3.7 MBq) of ^{18}F -Tr-Sgc8. The PET images are 1 h pi. Left is photograph of the tumor on the peritoneal wall; three PET images of different coronal slices.

Figure S5. (A) Representative HPLC chromatogram of ^{18}F -Tr-Sgc8 in mouse serum after 30 min incubation and extraction. (B) *In vivo* blood stability analysis of ^{18}F -Tr-Sgc8 by HPLC. (C) 1, 2 – Radioactive and mass HPLC of ^{18}F -Tr-Sgc8 standard; 3, 4 - Radioactive and mass HPLC of urine sample, 30 min after injection of ^{18}F -Tr-Sgc8.

Figure S6. Representative PET images of mice injected with 100 μCi (3.7 MBq) ^{18}F -Tr-Sgc8 containing low amount of Sgc8 (1-2 μg) at 30 min and 1 h p.i.

Figure S7. Biodistribution data of Sgc8 aptamer conjugated to ^{18}F -SFB. The results are presented as average of 4 mice \pm SD.

Figure S8. Metabolite analysis of ^{18}F -Tr-Sgc8 in the urine, 30 min pi. (A) UV chromatogram. (B) m/z data of peak extracted at 4.8 min. (C) molecular weight of the peak at 4.8 min after deconvolution. (D) m/z spectrum of urine from mouse that was not injected with ^{18}F -Tr-Sgc8.

Supplemental Movie

Movie 1. A 3D movie of PET imaging of mice bearing HCT116 tumors. Left mouse – mouse from Figure 4; Right mouse – mouse from supplemental Figure 4.

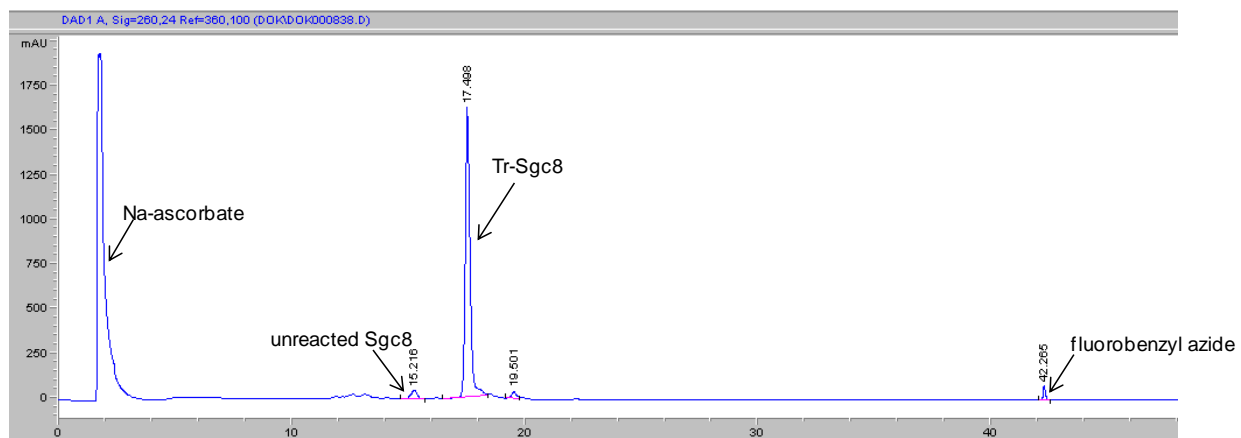
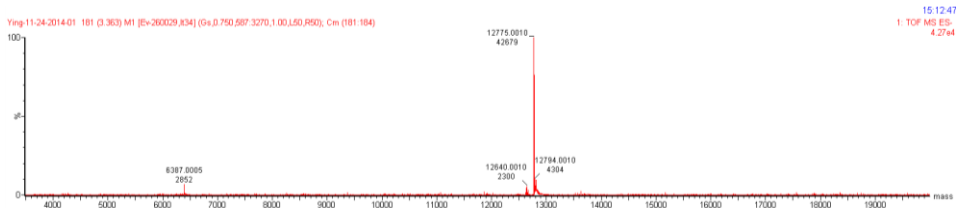


Figure S1

A



B

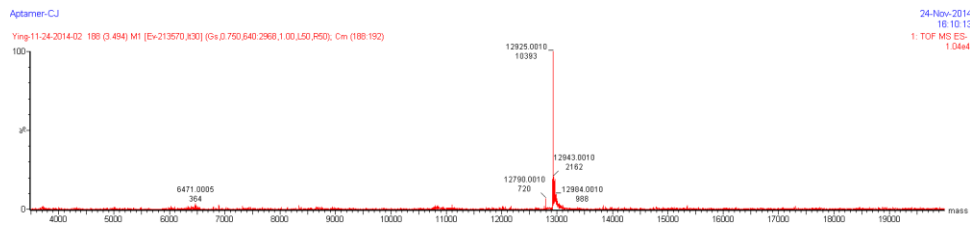
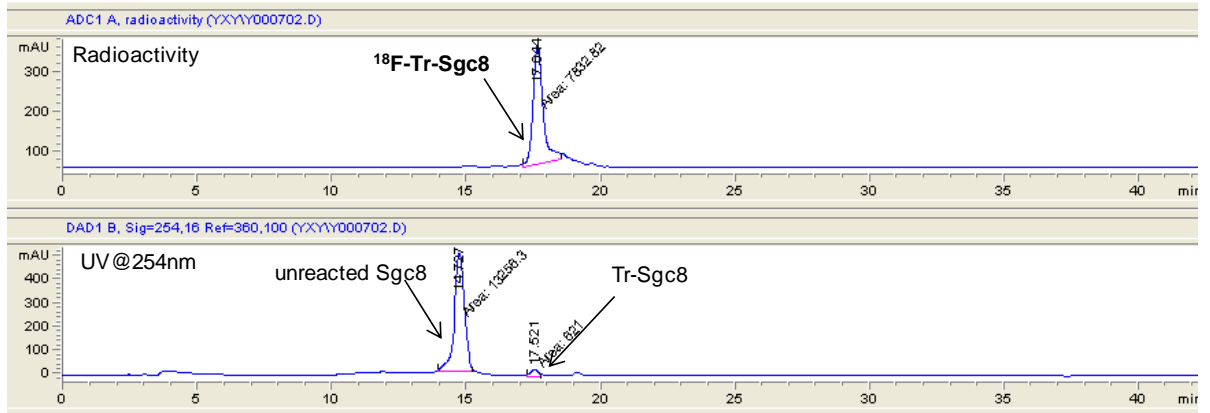


Figure S2

A



B

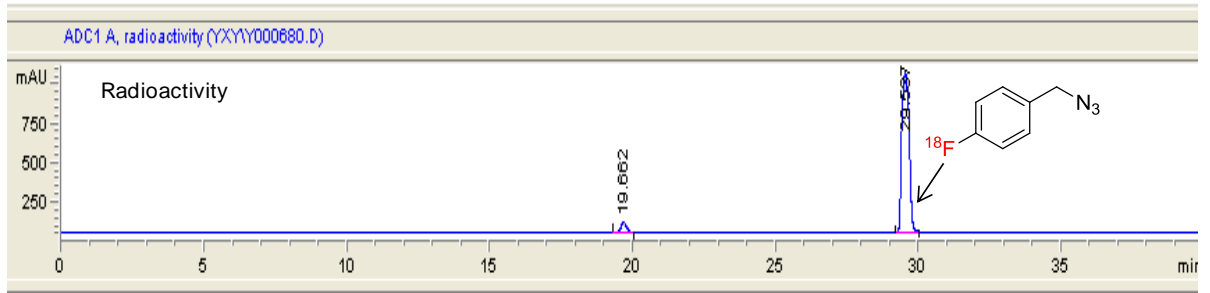


Figure S3

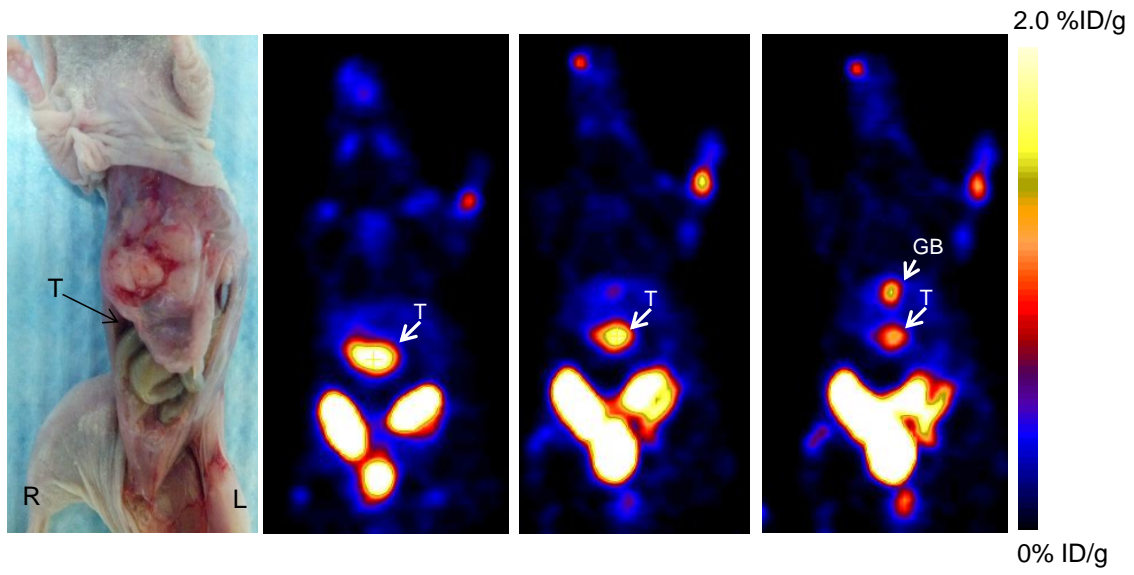
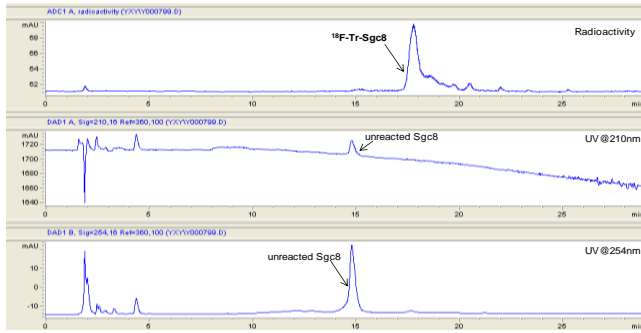
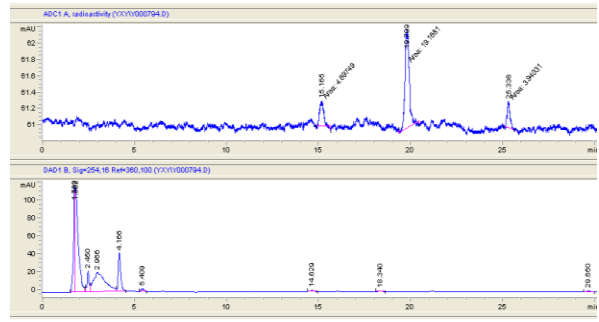
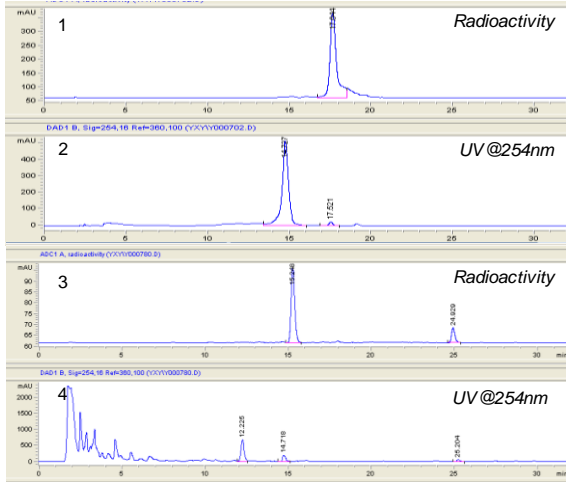


Figure S4

A**B****C****Figure S5**

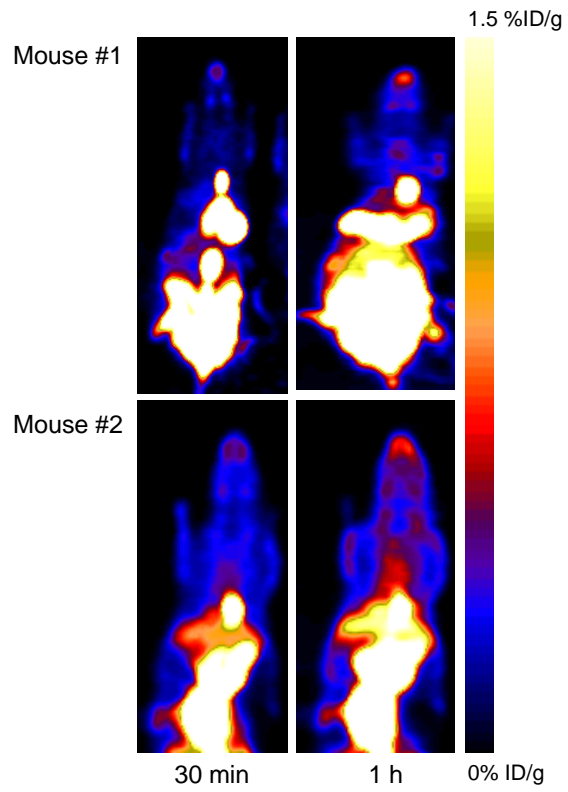


Figure S6

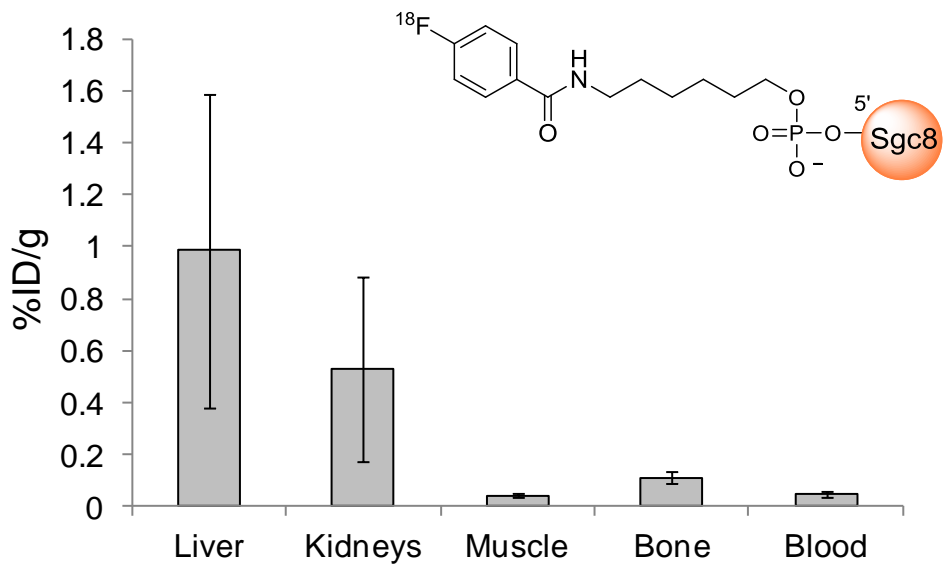


Figure S7

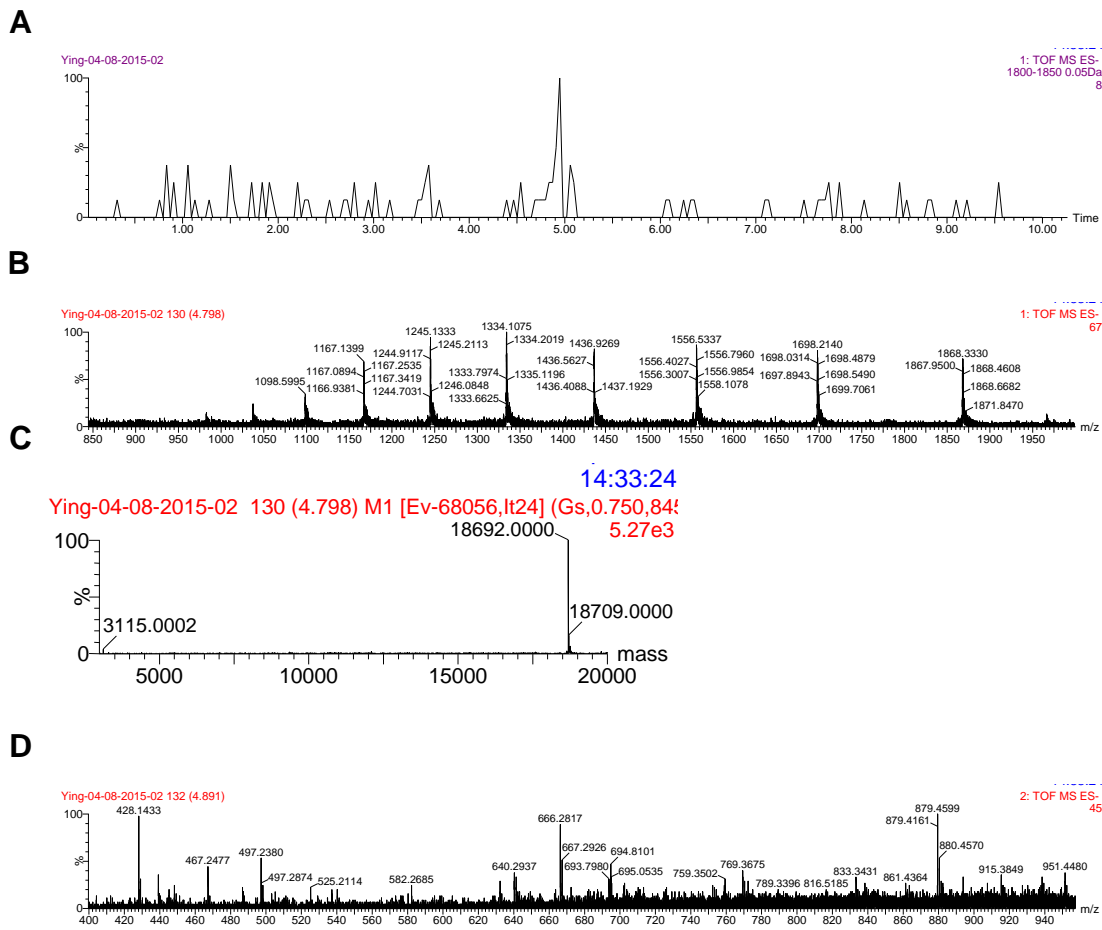


Figure S8

Table S1. Click chemistry reaction conditions between ^{18}F -fluorobenzyl azide and Sgc8-alkyne. 3-5 mCi and same volume concentration was used for all studies. Percentage of conversion was determined by HPLC.

^{18}F -fluorobenzyl azide (μL)	Sgc8-alkyne μg (nmol)	% conversion
50	150 (11)	>90
100	150 (11)	60
50	50 (3.8)	>90
50	13 (1.0)	48