Supplementary Information for

Click-ready perfluorocarbon nanoemulsions for $^{19}$F MRI and multimodal cellular detection

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**DBCO-lipid synthesis.** The preparation of DBCO-lipid with a 4 carbon linker was modified from a literature procedure with changes to the stoichiometry (3:2) and base (Na₂CO₃ instead of Et₃N)¹². Dioctadecylamine (0.186 mmol) and Na₂CO₃ were dissolved in 3 ml anhydrous tetrahydrofuran (THF). The mixture was stirred under Ar for 30 min. DBCO-NHS (0.124 mmol) was dissolved in 1.5 ml anhydrous THF and then added in the previous mixture. The reaction was stirred at 50 °C for 5 hours under argon gas. The product was purified by chromatography (silica gel, hexane: ethyl acetate= 3:2) and dried by rotary vaporizer. The DBCO-lipid with 4 carbon linker was obtained as pale-yellow solid with a 90% yield and characterized by ¹H NMR and electronic spray anionization mass spectrometry. ¹H NMR (800 MHz, Chloroform-d) δ 7.69 (dd, J = 7.7, 1.2 Hz, 1H), 7.41 – 7.38 (m, 1H), 7.38 (d, J = 1.9 Hz, 1H), 7.37 – 7.35 (m, 1H), 7.33 – 7.31 (m, 1H), 7.31 – 7.28 (m, 1H), 7.25 – 7.24 (m, 1H), 5.15 (d, J = 13.9 Hz, 2H), 3.65 (d, J = 14.0 Hz, 2H), 3.23 – 3.18 (m, 2H), 3.08 – 3.03 (m, 2H), 2.26 – 2.20 (m, 2H), 2.12 – 2.07 (m, 2H), 1.91 (ddd, J = 15.1, 8.6, 5.8 Hz, 2H), 1.59 – 1.18 (m, 64H), 0.88 (td, J = 7.1, 1.4 Hz, 6H). MS (m/z, ESI-TOF) for [M+H]⁺ calculated 837.35, found 837.72.
Figure S1. Characterization of DBCO-lipid. (A) 1H NMR characterization of DBCO-lipid. (B) MS characterization of DBCO-Lipid (target mass = 836.5)
**Nanoemulsion preparation and characterization.** 95 mg egg yolk phospholipids (EYP, Sigma-Aldrich, St Louis, MO), 20 mg cholesterol (Avanti Polar Lipids, Alabaster, AL), 2.4 mg 1,2 dipalmitoyl-sn-glycero-3-phosphoethanolamine (DPPE, Avanti), and 5.45 mg DBCO-lipid were dissolved in 2 ml chloroform and dried as a thin film in a 20 ml glass vial under argon. The sample was dried overnight under high vacuum. The next day, the lipids were rehydrated in 122 mg D-mannitol (Sigma-Aldrich) and 4.5 ml of miliQ water by vortexing for 30 s and then ultrasonication for 2 mins (Omni Ruptor 250 W, 30% power, 2 min Omni international, Kennesaw, GA). Then 2.136 g perfluoropolyether (PFPE, Exflour, Round Rock, TX) and 180 mg Cremophor (Sigma-Aldrich) were added to the solution, followed by a brief vortex and sonication for 2 mins. The crude solution was passed 5 times through a microfluidizer (LV1, Microfluidics, Newton, MA) operating at 20,000 psi. The emulsion was filtered through a 0.22 µm Supor membrane (no. 4187, Pall, Port Washington, NY) into sterile glass vials. Size characterizations were performed with dynamic light scattering (DLS, Malvern Zetasizer ZS, Malvern, PA). Fluorine concentration was determined by $^{19}$F NMR spectroscopy (9.4 T, Bruker Biospin, Billerica, MA). Nanoemulsion morphology was assessed by cryoEM (Thermo Fisher Glacios) under 11,000x and 45,000x magnification.

**Fluorescence quantification assay.** Perfluorocarbon concentration was normalized to 200 mg/mL before aliquoting DBCO-NE to react with Alexa Fluor 488 (AF488, Click Chemistry, San Diego, CA) at the desired concentration. For quantification of surface available DBCO, DBCO-NE was reacted with Alexa Fluor 488 azide (AF488) in various conditions to produce its fluorescently labeled form (AF488-DBCO-NE). Fluorescence measurements were collected by Tecan reader (Infinite M200PRO, Morrisville, NC). Conditions tested included the following: (i) DBCO mol% ranges (0.2, 0.5, 1 and 5, $n = 4$), (ii) reaction time at 2, 24 and 48 hr timepoints, (iii) AF488 concentration range ($n = 3$). Saturated fractions (maximum bound) were collected and content of AF488 determined by absorbance for each of the selected DBCO mol% ranges. The amount of AF488 clicked to the surface was calculated from a standard curve. The ratio of reactive DBCO
compared to total DBCO added was determined by manuscript Eq. (1). Since AF488 and DBCO are equimolar based on the click reaction, the concentration of AF488 \([AF488]\) was equated to the reactive DBCO concentration \([DBCO]_r\). Lastly, the DBCO starting material \([DBCO]_T\) is compared to its \([DBCO]_r\) value using Eq. 1 to yield the percentage of clicked ratio of reactive DBCO to total available \(E_{DBCO}\). Differences in clicking efficiency can also be identified by comparing \(E_{DBCO}\) values across DBCO mol%.

**RAW 264.7 cells.** Frozen RAW 264.7 cells (ATCC), were thawed and maintained in DMEM media containing 10% FBS, 100 U/ml penicillin, and 100 lg/ml streptomycin in T75 flasks (Sigma). Cells were incubated at 37 °C in 5% CO₂.

**In vitro cell cytotoxicity assay.** RAW 264.7 cells were plated at a density of 100,000 cells per well (36 wells total) in 6-well tissue culture plates (Corning, Inc., Corning, NY) and were allowed to adhere. Wells \((n = 3, \text{ per condition})\) received: (i) NE dose range (2-10 mg/ml, control), (ii) DBCO-NE dose range (2-10 mg/mL), or (iii) AF488-DBCO-NE dose range (2-10 mg/mL). After 16 hr incubation, cells were washed twice with 1X sterile PBS and incubated with 0.25% trypsin resuspension buffer (2 mL per well) for 5 min. Then DMEM media was added (8 mL per well) and extracted into a 10-mL sterile Falcon tube. An aliquot of suspended cells (10 µL) was mixed with trypan blue stain (10 µL) in a 96-well plate and assayed with a cell counter (Countess II FL, Life Technologies, Carlsbad, CA). The relative cell cytotoxicity was obtained by normalization to the non-dosed control.

**Acute Inflammation Model.** All animal work was approved through the University of California, San Diego, Institutional Animal Care and Use Committee (IACUC). In vivo studies were performed to confirm colocalization of AF488-DBCO-NE \(^{19}\)F and fluorescence signals at site of inflammation.
Female mice (n = 3) mice 8-9 weeks of age (ICR CD-1, Envigo) received footpad injections in the right paw, each containing 1% carrageenan solution (CAS# 9064-57-7, Sigma) in 0.9% saline. After 1 hour, mice received 112 mg/mL AF488-DBCO-NE in TRIS-HCl buffer (2% propylene glycol) by intravenous injection via tail vein. After 24 hours, fluorescence and $^{19}$F signals were measured.

**Fluorescence imaging.** All mice underwent serial fluorescence imaging (IVIS, PerkinElmer, Waltham, MA) one hour after receiving AF488-DBCO-NE. Prior to imaging, hair was removed using depilatory cream. Mice were anesthetized with 1-2% Isoflurane in air. A white-light body surface image was collected, followed by a fluorescent image using the GFP filter set rendered in pseudo-color, which was overlaid onto the surface image. Quantitative analysis of the total radiance (photons/s) was performed with the Living Image Software (Perkin Elmer) by defining identical regions of interest covering the left (control) and right paws.

**Magnetic Resonance Imaging (MRI).** Mice were anesthetized with 1–2% isoflurane in O$_2$ and positioned an 11.7 T Bruker BioSpec preclinical scanner with a dual-tuned $^1$H/$^{19}$F birdcage volume coil. Animal temperature was regulated, and respiration was monitored during scans. A reference capillary with AF488-DBCO-NE was positioned in the image field of view (FOV). Lower body $^1$H anatomical images were acquired using the (rapid acquisition with relaxation enhancement) RARE sequence with TR/TE = 1250/15 ms, RARE factor 6, matrix 256 × 192, FOV 40 × 30 mm, slice thickness 1 mm, 20 slices, and 6 averages. $^{19}$F images were also acquired using a RARE sequence with parameters TR/TE = 1000/20 ms, RARE factor 8, matrix 48 × 320, FOV 40 × 30 mm, slice thickness 2 mm, 10 slices, and 300 averages. The total number of fluorine atoms per voxel in hind paw were measured directly in regions of interest (ROIs) by segmenting around relevant $^{19}$F signals (right paw, left paw), and ROI voxel
intensities were displayed as histograms. For display, $^{19}$F images were thresholded remove background noise, and $^1$H/$^{19}$F renderings were constructed in VivoQuant™ (Invicro, Boston, MA) by overlaying $^1$H (grayscale) and $^{19}$F (hot-iron scale) slices.

Immunohistochemical analysis. After imaging, the right and left paws were embedded in optimal cutting temperature compound (OCT, Sakura Finetek USA, Inc., Torrance, CA) and stored at −80 °C. All tissues were cryosectioned (CM1950, Leica Microsystems Inc., Buffalo Grove, IL) at 10 μm thickness. Sections were fixed with 4% paraformaldehyde and stained for macrophage using rabbit anti-mouse mannose receptor (ab64693, 1:500 dilution, Abcam), followed by donkey anti rabbit Alexa fluor 647 (A-31573, 1:1000 dilution, ThermoFisher). Hoechst 33342 (1:500, ThermoFisher) was used to stain nuclei. Confocal images were acquired on a Leica SP5 2 confocal system with a Leica DM 6000 CFS microscope and a ×63 immersion objective.

Statistical analysis. Measurements are presented as mean ± SD. For stability assays, a linear mixed model from lme4 package was used and followed by a z test of the model estimated change over time with the emmeans package in R software, the differences were considered significant with p values < 0.01. For the cell viability assays, a generalized linear model ANOVA from lme package was used and P values were corrected for multiple comparisons within a given outcome with the Bonferroni-Holm correction for 30 tests in R software. For optical and MR imaging in vivo, an unpaired t-tests were used with unequal variances to compare between groups. Two tailed p values <0.05 were considered statistically significant.

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