Detailed Synthesis of Fluorous Hydroxamic Acid (FHOA)

Except where noted, chemicals were purchased from Sigma-Aldrich (St. Louis, MO) or TCI America (Portland, OR), and solvents were purchased from Thermo Fisher Scientific (Pittsburgh, PA) and used as received. Fluorous solvents were purchased from Exfluor Research Corporation (Round Rock, TX). Zirconium-89 (148-185 MBq in 1 M HCl or oxalic acid) was purchased from the University of Wisconsin-Madison. All handling and disposal of radioactive materials met local and Federal regulations.

All synthetic reactions were carried out under N_2 unless otherwise noted. Reactions were monitored by thin layer chromatography and LC-MS (Ion Trap XCT with 1100 LC, Agilent, Santa Clara, CA) using an analytical Luna C18(2) reverse-phase column (Phenomenex, Torrance, CA), $MeCN/H_2O$ (with 0.05% v/v CF_3CO_2H) linear gradients, 1 mL/min flow, and ESI positive or negative ion mode.

Compounds were purified by pre-packed silica gel columns on a CombiFlash automated system (Lincoln, NE, USA) equipped with a UV-Vis detector.

High-resolution mass spectrometry (HRMS) was performed by the Molecular Mass Spectrometry Facility at the University of California San Diego. UV−Vis absorption spectra were recorded on a Shimadzu UV-2700 (Kyoto, Japan) spectrophotometer. Nanoemulsion size and polydispersity was measured by dynamic light scattering (DLS) on a Malvern Zetasizer ZS (Malvern, PA). Solution NMR measurements were performed on a 9.4 Tesla spectrometer (AVANCE III HD-NanoBay, Bruker, Billerica, MA).

The starting material **FA** was prepared in ~5 g scale as colorless oil using fluorous alcohol as a starting material based on published methods *(18)* and confirmed by LC-MS (10-100% CH_3CN/H_2O for 20 min then 100% CH_3CN for 5 min, *t*=23.4 min, $[M+H]^+$ measured 522.9,

calculated 523.1). ^{1}H NMR (400 MHz, CDCl$_3$) δ 7.43 –7.36 (m, 5H), 6.70 (dd, J = 17.1, 10.4 Hz, 1H), 6.44 (dd, J = 17.2, 1.9 Hz, 1H), 5.77 (dd, J = 10.3, 1.9 Hz, 1H), 4.86 (s, 2H), 3.92 (t, J = 8.0 Hz, 2H), 2.44 (tt, J = 17.9, 7.7 Hz, 2H). ^{19}F NMR (376 MHz, CDCl$_3$) δ -80.77 (t, J = 10.62 Hz, 3F), -113.98 – -114.40 (m, 2F), -121.88 (s, 2F), -122.85 (s, 2F), -123.05 – -123.91 (m, 2F), -126.00 – -126.29 (m, 2F).
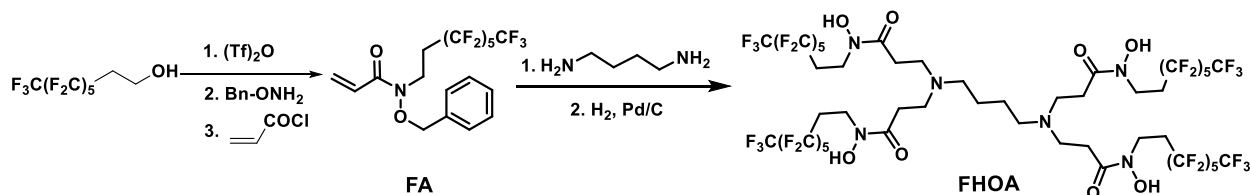
A mixture of compound **FA** (4.88 mmol, 2.55 g) and 1,4-diaminobutane (1.08 mmol, 108.8 µL, Sigma-Aldrich) in acetonitrile (50 mL) were refluxed under N$_2$ for 72 hours to obtain the benzoyl-protected precursor **FHOA-OBn**. LC-MS showed FHOA-OBn to be the major product (10-100% MeCN/H$_2$O for 20min then 100% CH$_3$CN for 5 min, t=23.7 min, [M+H]$^+$ measured 2179.8, calculated 2180.4). After the reaction cooled to room temperature, the solvent was evaporated, and the residue was re-dissolved in ethyl acetate (3 mL). The crude product was purified using a silica gel column (40 g) and eluted with CH$_3$OH/CH$_2$Cl$_2$ (0-8%, 15 min, linear gradient). A major peak centered at 11.5 min was collected and identified as **FHOA-OBn** by LC-MS. Solvents were evaporated, leaving FHOA-OBn as a colorless oil with a yield of 61±6% (1.44 g, n = 3). HRMS (m/z, ESI-TOF) for [M+2H]$^{2+}$ was calculated to be 1091.2207 and 1091.2198 was measured. ^{1}H NMR (400 MHz, CDCl$_3$) δ 7.37 (s, 20H, -C$_6$**H**$_5$), 4.82 (s, -O**CH**$_2$Ph, 8H), 3.82 (t, J = 7.6 Hz, 8H), 2.73 (t, J = 7.3 Hz, 8H), 2.52 (t, J = 7.1 Hz, 8H), 2.47 – 2.19 (m, 12H), 1.74 (s, 4H). ^{19}F NMR (376 MHz, CDCl$_3$) δ -80.83 (t, J = 8.99 Hz, -C**F**$_3$, 12F), -114.30 (p, J = 18.4 Hz, -CH$_2$C**F**$_2$-, 8F), -121.97 (q, J = 14.2 Hz, 8F), -122.95 (s, 8F), -123.50 (t, J = 13.8 Hz, 8F), -126.10 – -126.30 (m, 8F). ^{13}C NMR (101 MHz, CDCl$_3$) δ 174.71 (**C**=O), 134.16, 129.37, 129.23, 128.84,

127.15-99.89 (m, -**CF$_2$**-, weak), 54.03, 48.38, 38.48, 29.97, 29.73, 27.87 (t, -**CH$_2$CF$_2$**-, J =22.03 Hz), 24.96.

The **FHOA-OBn** (1.44 g) obtained was dissolved in ethanol (60 mL), then Pd/C (10%, 120 mg) was added. The mixture was stirred at room temperature under 1 atm H$_2$ for 16 h. LC-MS confirmed the disappearance of FHOA-OBn and formation of FHOA (10-100% CH$_3$CN/H$_2$O for 20 min then 100% CH$_3$CN for 5 min, t=22.6 min, [M+H]$^+$ measured 1819.5, calculated 1820.2). The mixture was first filtered through Celite, then through a 0.22 μm membrane to remove insoluble components. Residual solvents were removed by evaporation under vacuum, leaving FHOA as yellow semisolid with a yield of 89±4 % (1.06 g, n=3). HRMS (m/z, ESI-TOF) for [M+2H]$^{2+}$ was calculated to be 911.1268 and 911.1267 was measured. ^{1}H NMR (400 MHz, CDCl$_3$) δ 3.93 (t, J = 8.38 Hz, 8H), 2.93 – 2.61 (m, 12H), 2.56 – 2.30 (m, 20H). ^{19}F NMR (376 MHz, CDCl$_3$) δ -80.81 – 81.56 (m, -**CF$_3$**, 12F), -114.40 – -114.86 (m, -**CH$_2$CF$_2$**-, 8F), -122.11 – -122.91 (m, 8F), -122.91 – -123.35 (m, 8F), -123.38 – -123.95 (m, 8F), -125.52 – -127.00 (m, 8F). ^{13}C NMR (101 MHz, CDCl$_3$) δ 173.26 (**C**=O), 124.12 – 100.03 (m, -**CF$_2$**-, weak), 53.32, 49.90, 40.48, 29.73, 27.97 (m, -**CH$_2$CF$_2$**-), 24.27.

Nanoemulsion Formulation

A lipid film was prepared by dissolving 115.5 mg egg lecithin (60%, Alfa Aesar, Haverhill, MA), 9.3 mg cholesterol (Anatrace, Maumee, OH) and 16.8 mg DSPE-mPEG(2000) (Avanti Polar Lipids, Alabaster, AL) in chloroform (1.5 mL), followed by rotatory evaporation under N$_2$ flow, and drying under high vacuum for 24 h. The lipid film was hydrated with purified water (4.8 mL), vortexed at high for 2 min and probe sonicated for 2 mins (Omni Ruptor 250 W, 30% power, Omni International, Tusla, OK). FHOA (1 mM, 2.2 mg) was fully dissolved in PFOB or PFCE (1.2 mL)

oil and was added, and the final mixture was vortexed and sonicated sequentially for 2 min each. The pre-emulsion obtained was passed five times through a microfluidizer (LV1, Microfluidics, Newton, MA) operating at 20,000 psi and filtered through a 0.8/0.2 μm Supor membrane (Port Washington, NY) into sterile glass vials. The vials were sealed and stored at 4 °C before use. Nanoemulsion containing a higher concentration of FHOA (10 mM, 22 mg) was prepared in the same way to study the binding behavior by NMR and UV-Vis. Blank PFOB nanoemulsion was formulated in a similar way, except that no FHOA was added. FERM nanoemulsion using a polymeric surfactant (Pluronic F68) was prepared by mixing an aqueous solution of Pluronic F68 (4.8 mL, 31 g/L) with PFCE (1 mM FHOA, 1.2 mL) directly, followed by sonication and microfluidization as stated above. No phase separation was observed for >2 months of storage at 4 °C for all nanoemulsions prepared.

Metal Binding

Direct binding of $ZrCl_4$ to FHOA in solution was characterized by NMR spectroscopy. 0-2 equivalents of 40 mM $ZrCl_4$ in CD_3OD were titrated into 0.4 mL of 0.2 mM FHOA in CD_3OD in 5 mm NMR tubes. Samples were incubated at room temperature for 1 hour prior to acquisition of ^1H and ^{19}F NMR spectra. To determine binding kinetics, ^{19}F NMR spectra were recorded at 10, 20 and 30 min timepoints after addition of 1 equivalent of $ZrCl_4$.

Binding and transmetallation of Zr^{4+} and Fe^{3+} to FHOA was observed by UV-vis spectroscopy and ^{19}F T_1 relaxation time measurements. 0.16 µmol of $FeCl_3$ or $ZrCl_4$ in 90% H_2O/10% D_2O was added to 40 µL of FERM nanoemulsion in 260 µL of H_2O/10% D_2O. After incubation for 3 hours, ^{19}F T_1's were determined using inversion recovery experiments, and UV-vis spectra recorded. To investigate transmetallation, an equal amount of $ZrCl_4$ or $FeCl_3$ was added

to Fe and Zr bound emulsions, respectively, and T_1 and UV-vis measurements were repeated 16 h after addition. To determine transmetallation rate, UV-Vis spectra were also recorded every 5 min automatically in the 16 h period following addition of $ZrCl_4$ to Fe-saturated nanoemulsion.

Radiolabeling of FERM Nanoemulsion

For radiolabeling, 29.6 MBq of $^{89}ZrCl_4$ (~5 µL in 1M HCl) was added to 1 mL of FERM nanoemulsion and gently mixed in a vial. After incubation at room temperature for 3 h, the mixture was desalted into 10 mM Tris-HCl buffer (pH=8.0) containing 2% (v/v) of propylene glycol using a Sephadex G-25 gel filtration column (NAP-10, GE Healthcare, Chicago, IL) and collected in a glass vial. The nanoemulsion was typically adjusted to a concentration of 3.7 MBq per 0.2 mL with Tris buffer for *in vivo* injection. The radiolabeling yield of FERM was calculated from the ratio of activity in the collected eluate to total activity. A prolonged reaction time (16 h) did not significantly increase the yield. Quantitative ^{19}F NMR from decayed samples showed a typical recovery of $61.2\pm8.0\%$ (0.8 mL, n=3) of total fluorine atoms loaded into the column.

For radiolabeling with ^{89}Zr-oxalate (in 1 M oxalic acid), 22.2 MBq (~10 µL) of ^{89}Zr-oxalate in 30 µL 1 M $NaHCO_3$, and 10 µL 1 M Tris buffer (pH=8.0) was added to 1 mL of lipid FERM nanoemulsion, which resulted in a final pH between 6-6.5 as tested by pH paper. After incubation at room temperature for 3 h, the mixture was desalted using the above method.

MTT Assay

The murine macrophage cell line RAW264.7 (ATCC, Manassas, VA) was maintained in DMEM containing 10% fetal bovine serum (FBS), 10 mM HEPES, 1 mM sodium pyruvate, and 1.5 g/L sodium bicarbonate at 37 °C in 5% CO_2 atmosphere. Cells were grown in 10 mL cell culture tubes.

Nanoemulsion with FHOA (1 mM in oil) or without chelate were added at a fluorine concentration of 5 mg/mL overnight. Cells without nanoemulsion labeling were used as control groups. After incubation overnight at 37 °C, cells were washed three times in PBS and counted using a Countess II FL Automated Cell Counter (Fisher Scientific, Waltham, WA). The labeled and unlabeled cells were seeded in 96-well plate with 10^5/well in cell culture medium. An MTT assay kit (ab211091, Abcam, Cambridge, UK) was used following the manufacturer's protocol at 0, 18, 24, and 48 hours. The absorbance at OD=590 nm was measured using an Infinite 200 plate reader (Tecan, Männedorf, Switzerland).
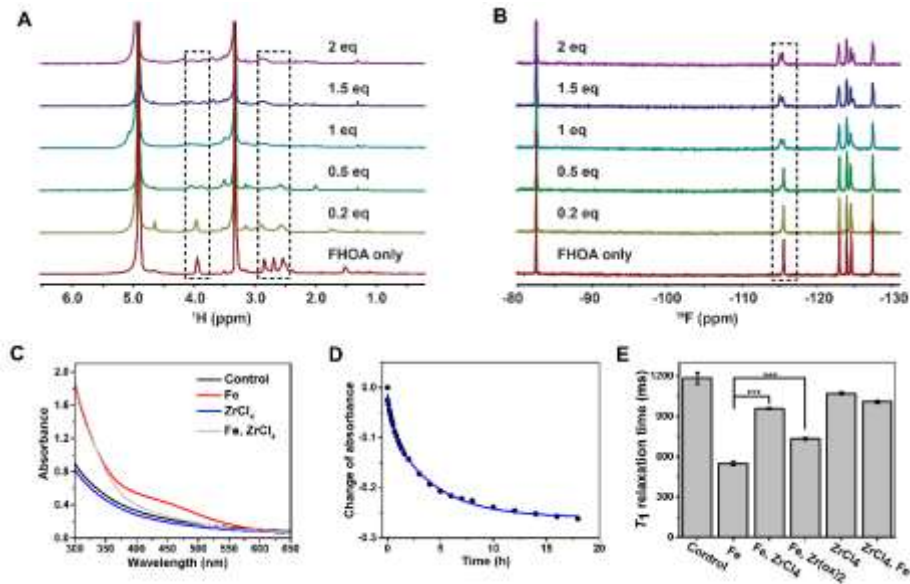
Supplemental Table

Oil	Surfactant	Size (nm)	PDI	^{19}F (mg/mL)
PFOB Blank	Lipids	162.0 ± 7.7	0.045 ± 0.031	179.9 ± 6.6
PFOB-FHOA	Lipids	158.0 ± 1.9	0.085 ± 0.006	168.5 ± 5.8
PFCE-FHOA	Lipids	151.8 ± 4.7	0.073 ± 0.030	154.1 ± 9.0
PFCE-FHOA	Pluronic F68	137.3 ± 6.6	0.084 ± 0.052	161.2 ± 6.1

Supplemental Table 1. FERM nanoemulsion size and polydispersity index (PDI), measured by DLS, for various formulations with different fluorocarbons and surfactants types. The fluorine content of prepared nanoemulsions were assayed using ^{19}F NMR. Data are shown as mean ± standard error (n=3).
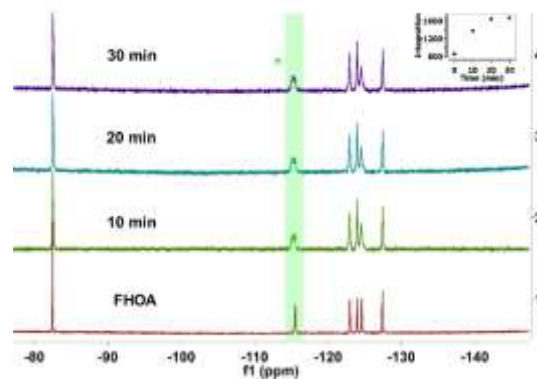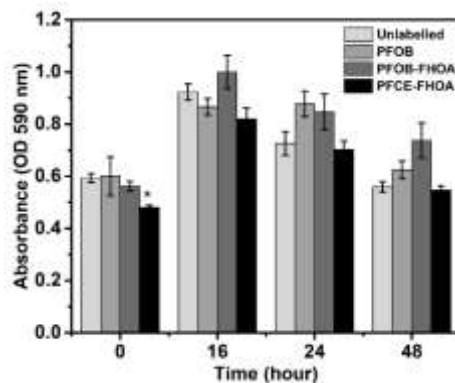
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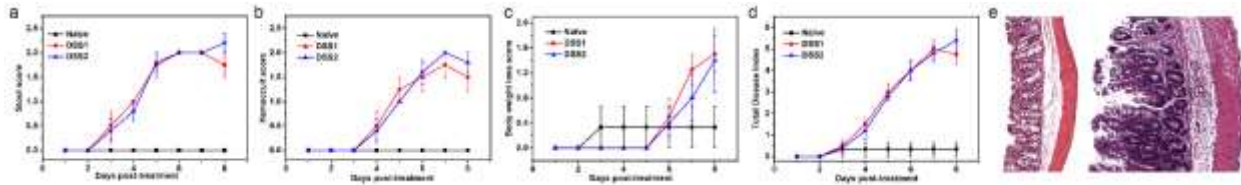
Supplemental Fig. 1. Characterization of dextran sulfate sodium (DSS) treated IBD C57BL/6 mouse model. Control mice (naïve, n=3) were fed with regular water, while IBD mice were fed with water containing 3% DSS (groups DSS1, n=4; DSS2, n=5) for 8 days before imaging. Panel (a) shows stool consistency score (0=normal, 1=soft but still formed, 3=very soft, 4=diarrhea). In (b), hemoccult scores are shown (0=negative, 1=positive, 2=visible traces of blood, 3=gross rectal bleeding). (c) Displays body weight loss score (0 for 0-1% body weight loss, 1 for 1-5% loss, 2 for 5-10% loss, 3 for 10-20% loss and 4 for >20% loss). (d) Represents total disease index, defined as the summation of scores from (a)-(c). Two mice from DSS1 group and three mice from DSS2 group were used for imaging studies. Panel (e) displays representative H&E-stained sections from Swiss rolls made from colon of an untreated mouse (left) and a mouse treated with DSS (right). The DSS colon (right) displays pronounced inflammation characterized by mucosal hyperplasia, goblet cell depletion and mononuclear cell infiltration The fixed segments (2% PFA) were embedded in paraffin, cut into 5 µm-thick transverse sections, and stained with hematoxylin and eosin (H&E) and imaged (200×).

Supplemental Fig. 2. Metal-binding properties of FERM nanoemulsion. (A, B) Display changes in ^1H and ^{19}F NMR spectra with binding between FHOA and Zr^{4+} (non-radioactive) in CD_3OD at varying doses. Panel (C) shows UV-Vis spectra of nanoemulsion with addition of Zr^{4+} (2eq) or Fe^{3+} (2eq). Adding Zr^{4+} to the Fe-bound FERM nanoemulsion (red line) causes a decrease in absorbance at ~450 nm (grey line). "Control" is FERM nanoemulsion without metal (black line). Panel (D) displays UV-Vis absorbance at 450 nm over time after adding Zr^{4+} (2 eq) to Fe-bound FERM nanoemulsion; a bi-exponential fit yields $t_{1/2}$ values of 0.32 ± 0.07 h and 2.91 ± 0.26 h, with $R^2 = 0.9971$. In (E), we show the ^{19}F T_1 of PFOB (-CF_2Br peak) nanoemulsion upon metal binding. Data are mean $\pm$ s.e.m. (n=3), where *** denotes P<0.001 for unpaired t-tests.

Supplemental Fig. 3. Time-lapse ^{19}F NMR spectra of FHOA upon addition of ZrCl$_4$ (1 eq, non-radioactive). The insert (upper right) shows the integration values of peaks centered at -115.8 ppm at each time point.


Supplemental Fig. 4. Cytotoxicity in RAW264.7 cells labeled overnight with FERM nanoemulsion (PFOB or PFCE), measured by the MTT assay over 48 hours. Data are shown as mean ± standard error (n=3). Unpaired t-test analyses show insignificant differences between nanoemulsion labeled groups and unlabeled groups (p>0.05), except for PFCE-FHOA at 0 h where p<0.05 (*).
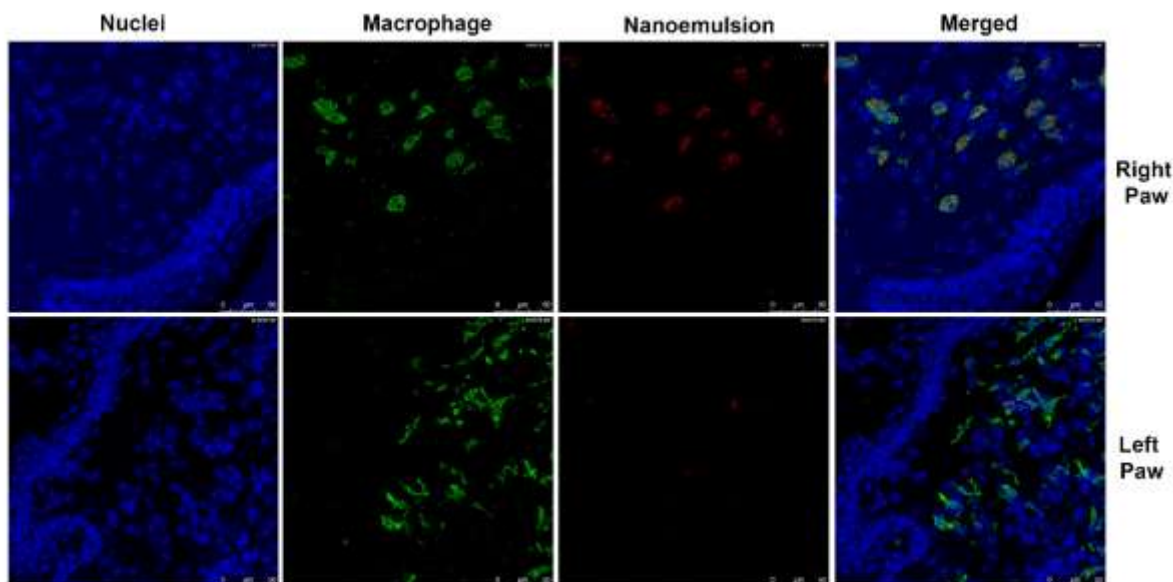
Supplemental Fig. 5. Representative immunofluorescence confocal images of mouse paw sections from λ-carrageenan inflammation model. Data show the colocalization of fluorescent fluorocarbon nanoemulsion (red) with macrophages (green) in the inflamed, right paw and absence of nanoemulsion in the (control) left paw. We employed a dual-mode fluorocarbon nanoemulsion containing a red-fluorescent dye (VS-1000H DM Red, 150 nm droplet size, Celsense, Pittsburgh, PA). The nanoemulsion was injected through tail vein of mice bearing inflammation on the right paw (n=3). After 24 h, mice were sacrificed and paws were harvested, weighted, cryo-freezed in optimal cutting compound and kept frozen at -80 °C. Nuclei are stained with Hoechst (blue, 62249, Thermo Fisher). The sections were cut along the palm direction at 10 μm thickness in a cryotome and fixed with 4% paraformaldehyde, followed by permeabilization and Fc blocking. Slices were stained with rabbit anti-mannose receptor (ab64693, Abcam, Cambridge, UK) as primary antibody and Alexa Fluor 488 goat anti-rabbit (A11008, Thermo Fisher) as the secondary antibody. Confocal images (CTR 6500, Leica Microsystems, Buffalo Grove, IL) were acquired of sections at 63× magnification. Scale bar = 50 μm.
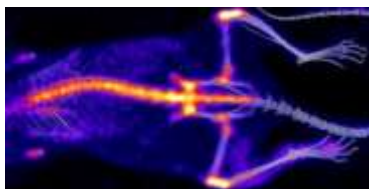
Supplemental Fig. 6. Representative PET/CT image of mouse 24 h after injected with free ^{89}ZrCl$_4$ (3.7 MBq in 0.2 mL Tris buffer). The image data is displayed as a maximum-intensity projection.
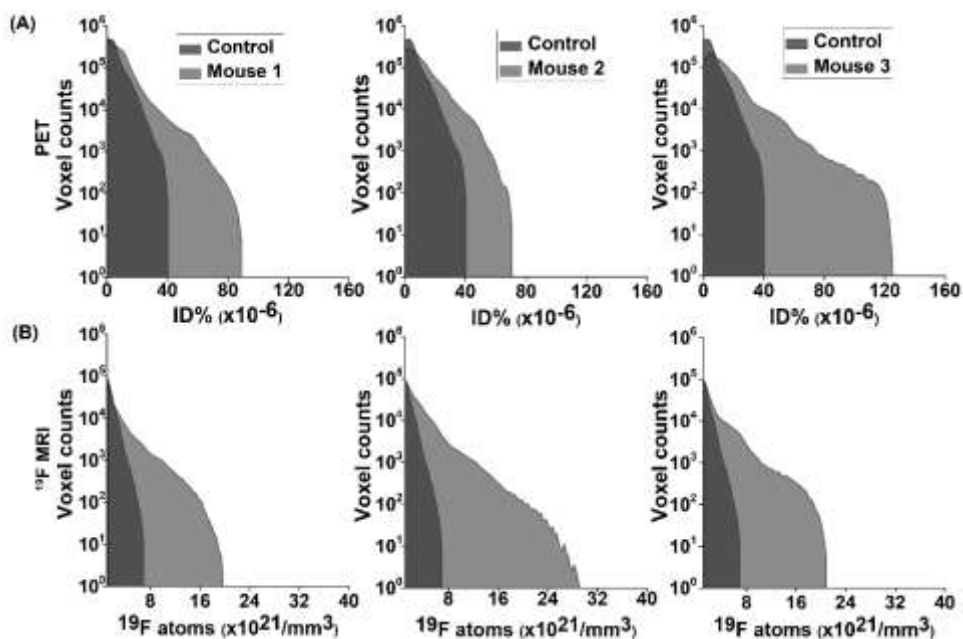
Supplemental Fig. 7. PET (A) and ^{19}F MRI (B) histograms of nanoemulsion quantification per voxel are shown for abdominal ROI encompassing entire peritoneum of IBD mice or control mice. Images for these mice are displayed in manuscript Fig. 3.