Intracellular pH measurements using perfluorocarbon nanoemulsions

Supporting Information

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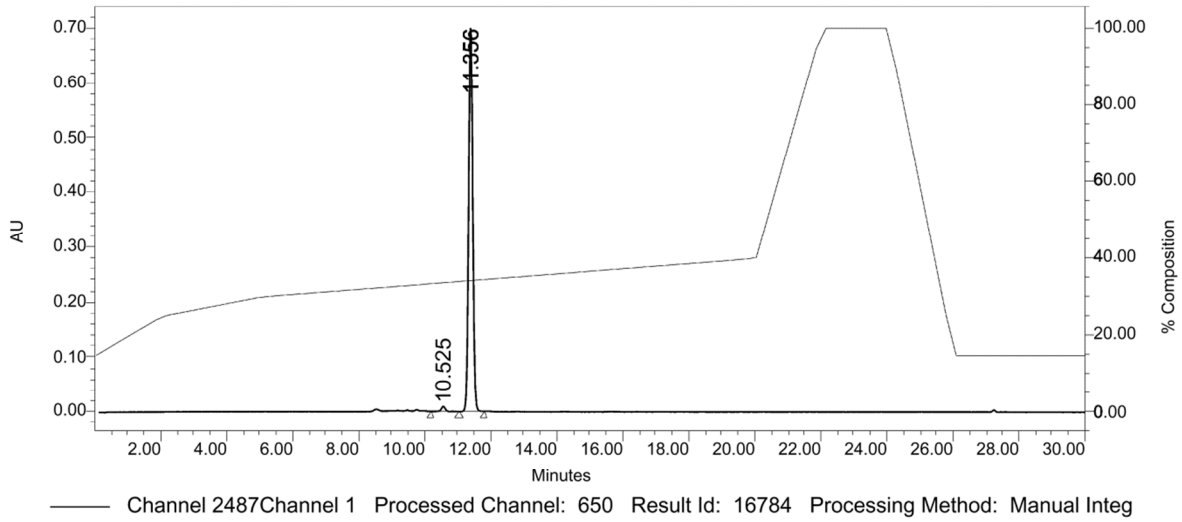
Overview

Section 1 contains: (1) Detailed synthesis of fluorescent reagents, Perfluoropolyether (PFPE) oils and nanoemulsions, (2) spectroscopy, dynamic light scattering (DLS) and nuclear magnetic resonance (NMR) data (^{1}H, ^{19}F), and (3) additional discussion. **Section 2 contains** further experimental assessment of nanoemulsions for cell labeling applications and additional discussion. **Section 3 contains** biological evaluation of nanoemulsions via ^{19}F NMR and fluorescence, flow cytometry analysis and confocal microscopy. **Section 4 contains** intracellular pH measurement by flow cytometry.

S1. Experimental methods: Synthetic procedures and analytical data

General experimental conditions

All reagents and solvents were purchased from Sigma-Aldrich, (Milwaukee, WI), Acros (Morris Plains, NJ), TCI America (Portland, OR) or Fluka (Milwaukee, WI) and used without further purification. Perfluoropolyether (PFPE) oils (PFPE ester and PFPE oxide) were purchased from Exfluor Research Corp., Roundrock, TX, and used without further purification. Dimethylformamide (DMF) was purchased anhydrous grade and used without further purification. High Performance Liquid Chromatography (HPLC) solvents were purchased HPLC grade (Fisher Scientific, Fairlawn, NJ) and used without further purification. All chemical reactions were performed under argon atmosphere. Preparative scale chromatography was performed using a Waters Prep LC 4000 System fitted with a Waters μbondapak™ C18 10 μm 125 Å, 19 x 300 mm prep column (Waters WAT025828), a Waters 2487 Dual λ Absorbance Detector and a Waters Fraction Collector II, purchased from Waters Corp., Milford, MA. Analytical chromatography was performed using a Waters 600 Controller fitted with a Waters μbondapak™ C18 10 μm 125 A, 3.9 x 300 mm analytical column (Waters WAT02734) and a Waters 2487 Dual λ Absorbance Detector. Analytes were separated using acetonitrile (AcN) gradient elution with modified aqueous mobile phases 0.1% trifluoroacetic acid (TFA) or 0.1 M triethylammonium acetate (TEAA). Reversed Phase Thin Layer Chromatography (RP-TLC) was performed using reversed phase hydrocarbon impregnated silica gel with UV254, 250 μm (Product #52521, Analtech, Inc., Newark, DE). ^{1}H and ^{13}C NMR (Bruker Instruments, Inc., Billerica, MA) spectra were obtained at 300 and 75 MHz in $CDCl_3$ unless otherwise noted. ^{19}F NMR (Bruker) was obtained at 470 MHz in water, methanol (MeOH) or water/ethanol (H_2O/EtOH) unless otherwise noted. ^{1}H and ^{13}C NMR chemical shifts were reported in parts per million (ppm) using the residual solvent signal as an internal standard. ^{19}F NMR chemical shifts were reported as ppm using TFA added at 0.1% v/v to the NMR sample, with chemical shift set at -76 ppm. ^{1}H NMR spectra were tabulated as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, qn = quintet, m = multiplet, b = broad), and number of protons and assignment(s). ^{13}C NMR spectra were acquired using a proton decoupled pulse sequence with a pulse sequence delay of 5 seconds. Low resolution mass spectra (MS) were obtained by electrospray ionization (ESI-MS) in negative ion mode using an AP 4700 MALDITOF/TOF-MS (Applied Biosystems Inc., Bedford, MA); samples were dissolved in MeOH (23μM).



	Processed Channel Descr.	RT	Area	% Area	Height
1	650	10.525	84344	1.37	9184
2	650	11.356	6085593	98.63	702278

Figure S1. Analytical HPLC of CypHer5-COOH **(3)**. UV/Vis detection was at 650 nm. Gradient elution was AcN versus 0.1% TFA at a flow rate of 1 ml/min. AcN composition is shown as a thin line.

S1.1 Synthetic and purification procedures

Cy3.29-COOH (1)

Cy3.29 free carboxylic acid **(1)** was prepared as described by Mujumdar et al.[1] and further purified using preparative HPLC using gradient elution with 15 to 35% AcN versus water (containing 0.1% TFA) over 40 minutes (min) at 10 ml/min, with detection at 550 and 280 nm. The major project eluted from 20-25 min. The purity of fractions was evaluated by RP-TLC (20% MeOH, R_f = 0.45); the final product was confirmed by ^{1}H NMR as described by Mujumdar.[1]

Cy5.29-COOH (2)

Cy5.29 free carboxylic acid **(2)** (150 mg, 0.216 mmol) was prepared as described by Mujumdar *et al.*[1] and further purified using preparative HPLC with gradient elution using 15 to 35% AcN versus water (containing 0.1% TFA) over 40 min at 10 ml/min, with detection at 650 and 280 nm. The major product eluted at 30.5-35.5 min. The purity of fractions was evaluated by analytical HPLC (20% to 40% AcN gradient versus 0.1% TFA in water over 20 min at 1 ml/min); the final product was confirmed by ^{1}H NMR.[1] The final yield was 105.1 mg at 0.151 mmol (70.0%).

CypHer5-COOH (3)

The pH-sensitive Cy5 analog **(3)** (30 mg, 0.0450 mmol) was prepared by published methods (described as "Dye IV" by Cooper *et al.*[2]) and further purified using preparative HPLC with gradient elution in 5 to 40% AcN versus water (containing 0.1% TFA) over 40 min at 10 ml/min, with detection at 650 and 490 nm. In order to achieve retention necessary for separation, the method began at 5% AcN for 1 min, then was immediately ramped up to 25% AcN, followed by a linear increase from 25% to 30% over 9 min. Separation of components was achieved using gradient elution of 30% to 40% AcN over 30 min. Flow rate was 10 ml/min throughout the process. The purity of fractions was evaluated by analytical HPLC (15% to 40% AcN non-linear gradient versus 0.1% TFA at 1 ml/min). Specifically, the AcN content began at 15%, and then was linearly increased to 25% over 2 min, and then increased to 30% over 3 min. Separation of analytes was achieved by increasing AcN to 40% over 15 min. The retention time of CypHer5-COOH was 11.4 min (98.6% purity), Figure S1. The final yield was 4.9 mg at 7.34 µmol (16.3%). The ^{1}H NMR results were (300 MHz, MeOH-d_4) δ 8.163 –8.079 (2H, t) Phe, 7.920 (2H, s) Phe, 7.891 – 7.838 (2H, m) Phe, 7.366 – 7.339 (1H, d) =CH-, 7.250 – 7.223 (1H, d) =CH-, 6.694 – 6.611 (1H, t) =CH-, 6.392 - 6.3452 (1H, d) =CH-, 6.454-6.395 (1H, d) =CH-, 5.05-4.65 (broad) H_2O, 4.168 – 4.121 (2H, t) =N-CH_2-, 3.65 (s) (*methyl ester formed* during preparation of NMR sample), 3.345 – 3.320 (broad) solvent (MeOH), 2.393 – 2.317 (2H, m) -CH_2-, 1.851 – 1.462 (12H, m), -CH_2-, 2(-CH_3) -CH_2-, 1.609 (6H, s) 2(-CH_3), 1.539 – 1.4623 (2H, m) -CH_2-. Figure S2 displays the full NMR spectrum.

Cy3.29-NBoc (5)

HPLC-purified Cy3.29-COOH **(1)** (24 mg, 0.0357 mmol) was dissolved in 1.8 ml DMF. After 5 min, 18.0 µl of diisopropylethylamine (DIPEA) (0.103 mmol) was added, followed by 1.5 molar equivalent N,N,N′,N′-Tetramethyl-O-(N-succinimidyl)uronium tetrafluoroborate (TSTU) (16.1 mg, 0.035 mmol). The reaction progress was monitored by analytical HPLC using 15-35% AcN versus water containing 0.1% TFA over 20 min at a flow rate of 1 ml/min with 550 and 280 nm detection. Retentions times (t_R) were 16.7 min (Cy3.29-COOH) and 20.7 min (Cy3.29-OSu). After 1 hour (hr), 1.5 molar equivalent NBoc ethylenediamine **(4)** was added (12.7 µl, 0.0535 mmol.) and monitored by HPLC (t_R = 19.5 min). The retention times of Cy3.29-OSu and Cy3.29-NBoc were very close (t_R=20.7 min and 19.5 min, respectively), and further HPLC analysis of NBoc derivatives was performed using AcN gradient versus TEAA buffer. Formation of NBoc de-

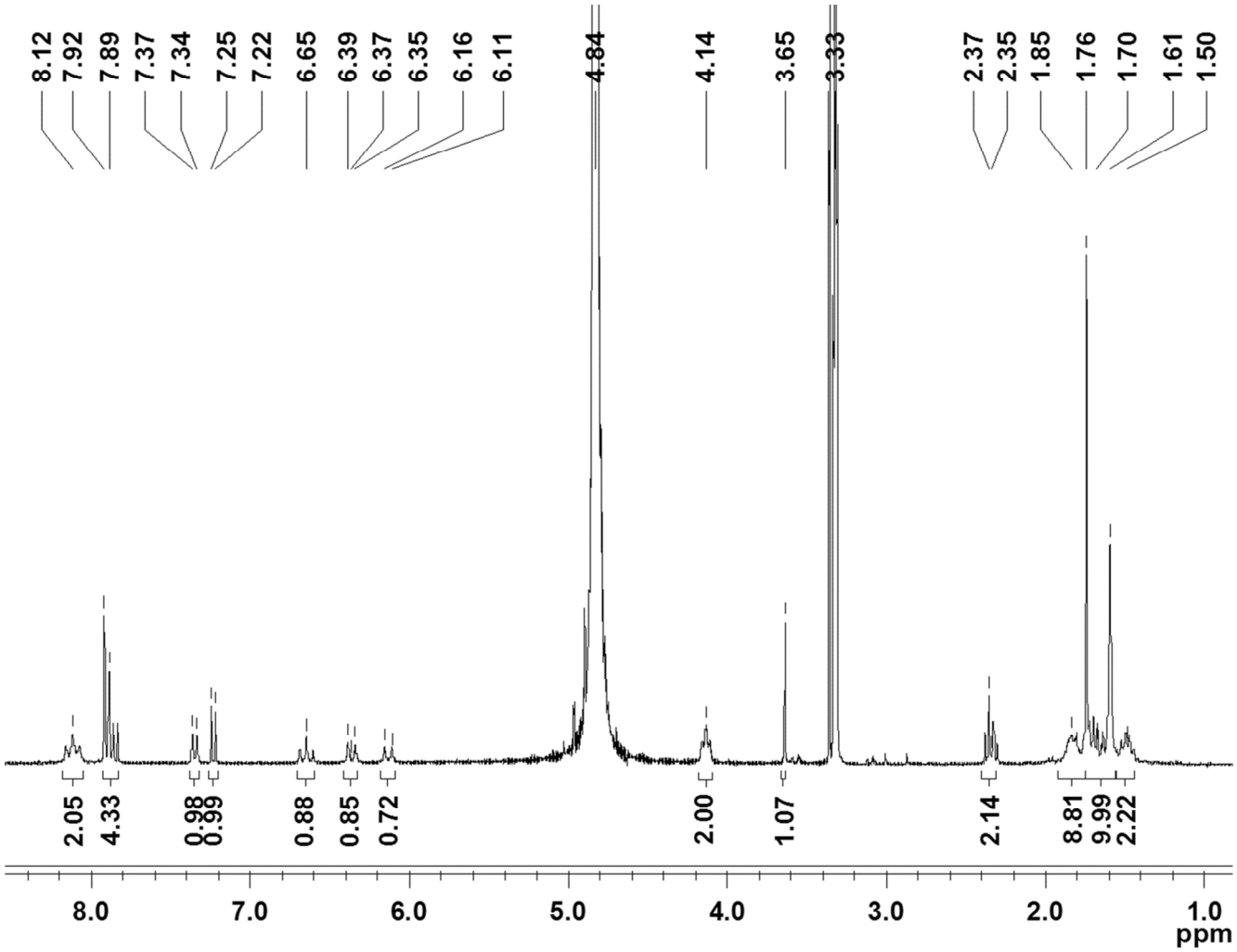


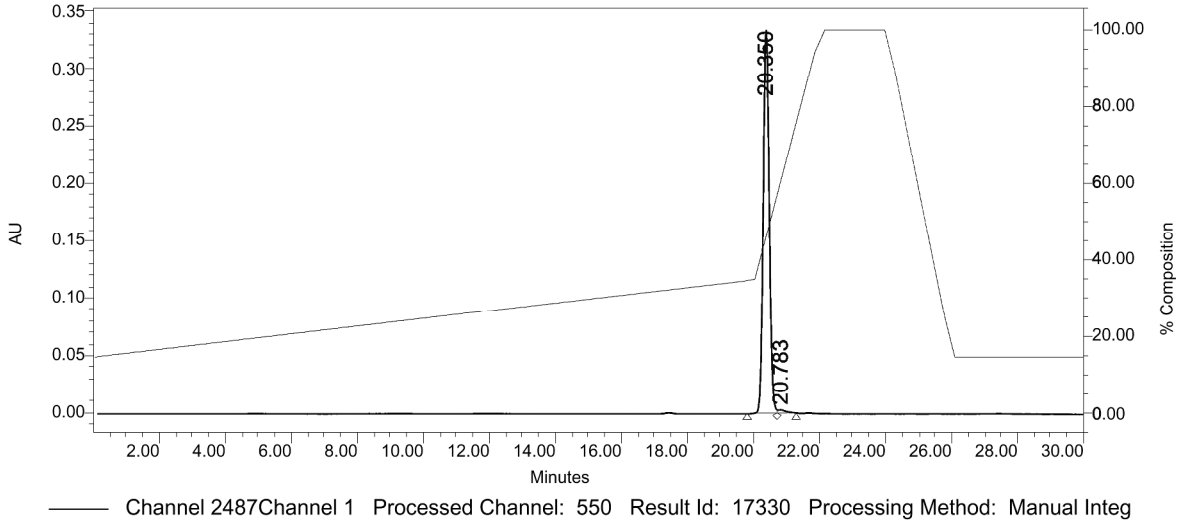
Figure S2. ^{1}H NMR (300 MHz in MeOH-d_4) of CypHer5-COOH **(3)**.

rivative was confirmed by exposure to 1% TFA and evidenced by a shift in the retention time to 15.7 min (Cy3.29-NH_2) **(8)** from 21.0 (Cy3.29-NBoc). The retention times for the other analytes were 19.1 min (Cy3.29-OSu) and 12.0 min (Cy3.29-COOH). The product was isolated by precipitation with ethyl acetate (EtOAc) (42 ml) and by centrifugation separation. The precipitate was further washed with 2x 5 ml of EtOAc and solid dried under vacuum in a desiccator for 4 hr. The pre-purification yield was 26.6 mg at 0.0345 mmol (96.8%). The bulk derivative was purified by preparative HPLC equilibrated to 20% AcN versus 80% 0.1 M TEAA with detection at 550 and 280 nm. Samples of 8 mg in 8 ml injection volume, filtered through 0.22 µm filter, were purified by gradient elution of 20% to 40% AcN over 40 min at 10.0 ml/min. The product was collected in 5 ml fractions over the range of the major peak at 23.5-28.0 min. The purity of fractions (>97.5%) was determined by analytical HPLC in TEAA buffer (Figure S3). The final purity by analytical HPLC was 98.8%. The final yield was 24.9 mg at 0.0323 mmol (90.2%). The ^{1}H NMR showed (300 MHz, D_2O) δ 8.557 (1H, t) –CH=, 7.945 (2H, s) -CH=, 7.914 – 7.870 (2H, m) Phe, 7.439 – 7.383 (2H, t) Phe, 6.454 – 6.395 (2H, d of d) =CH-, 4.790 (s) D_2O, 4.184–4.153 (4H,m) =N-CH_2-, 3.289–3.216 (12H,m) solvent (triethylammonium -CH_2-), 3.189 – 3.060 (4H, m) O=CNH-CH_2-, 2.261 – 2.215 (2H, t) -CH_2-, 1.963 (s) solvent AcN, 1.903 – 1.853 (2H, m) -CH_2-, 1.779 – 1.772 (12H, d) solvent (acetate ion),1.671 – 1.622 (2H, m), -CH_2-, 1.432 – 1.306 (32H, m), -CH_2-, -CH_3, -CH_3 (NBoc), solvent (triethylammonium –CH_3). Figure S4 displays the full NMR spectrum.

Figure S5 shows the Cy3.29-NBoc mass spectrometry results, yielding (negative ion) 771.4 (monoisotopic mass 771.31).

Cy5.29-NBoc (6)

HPLC-purified Cy5.29-COOH **(2)** (100 mg, 0.1439 mmol) was dissolved in 7.2 ml DMF. After 5 min, 144.0 µl of DIPEA (0.827 mmol) was added, followed by 1.5 equivalents of TSTU (64.9 mg, 0.216 mmol). The progress of the reaction was monitored by analytical HPLC with (20-40% AcN versus water containing 0.1% TFA, Cy5.29-COOH t_R = 14.7 min, Cy5.29-OSu t_R = 17.8 min). The next day, 1.5 equivalents of NBoc ethylenediamine **(4)** (34.2 µl, 0.216 mmol) and 72.0 µl of DIPEA (0.413 mmol) were added and monitored by analytical HPLC with 20 to 40% AcN versus 0.1 M TEAA over 20 min at 1 ml/min with 650 and 280 nm detection. The retention times were 11.1 min (Cy5.29-COOH), 17.2 min (Cy5.29-OSu) and 17.4 min (Cy5.29-NBoc). The formation of the Cy5.29-NBoc derivative was confirmed by de-protection of the amine, which was verified by the shift of retention time from 17.4 min (Cy5.29-NBoc) to 12.1 min (Cy5.29-NH_2) **(9)**. The product was isolated by precipitation with EtOAc (43 ml) and separated by centrifugation. The solid was washed with 2x 5



	Processed Channel Descr.	RT	Area	% Area	Height
1	550	20.350	4218232	98.77	334328
2	550	20.783	52602	1.23	3010

Figure S3. Analytical HPLC of Cy3.29-NBoc **(5)**. UV/Vis detection was at 550 nm. Gradient elution was AcN versus 0.1 M TEAA at a flow rate of 1 ml/min. AcN composition is shown as a thin line.

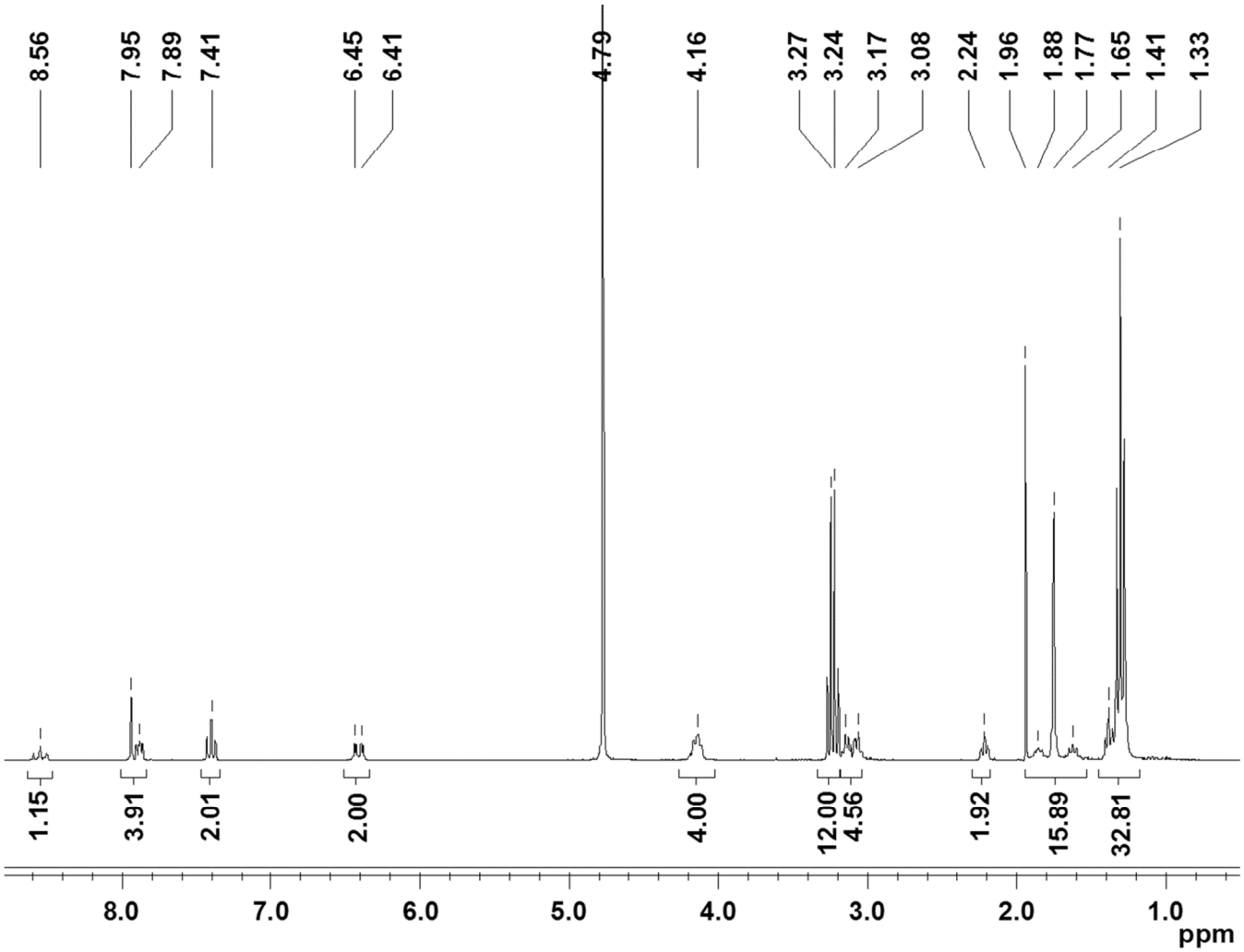


Figure S4. ^{1}H NMR (300 MHz, D_2O) of Cy3.29-NBoc **(5)**.

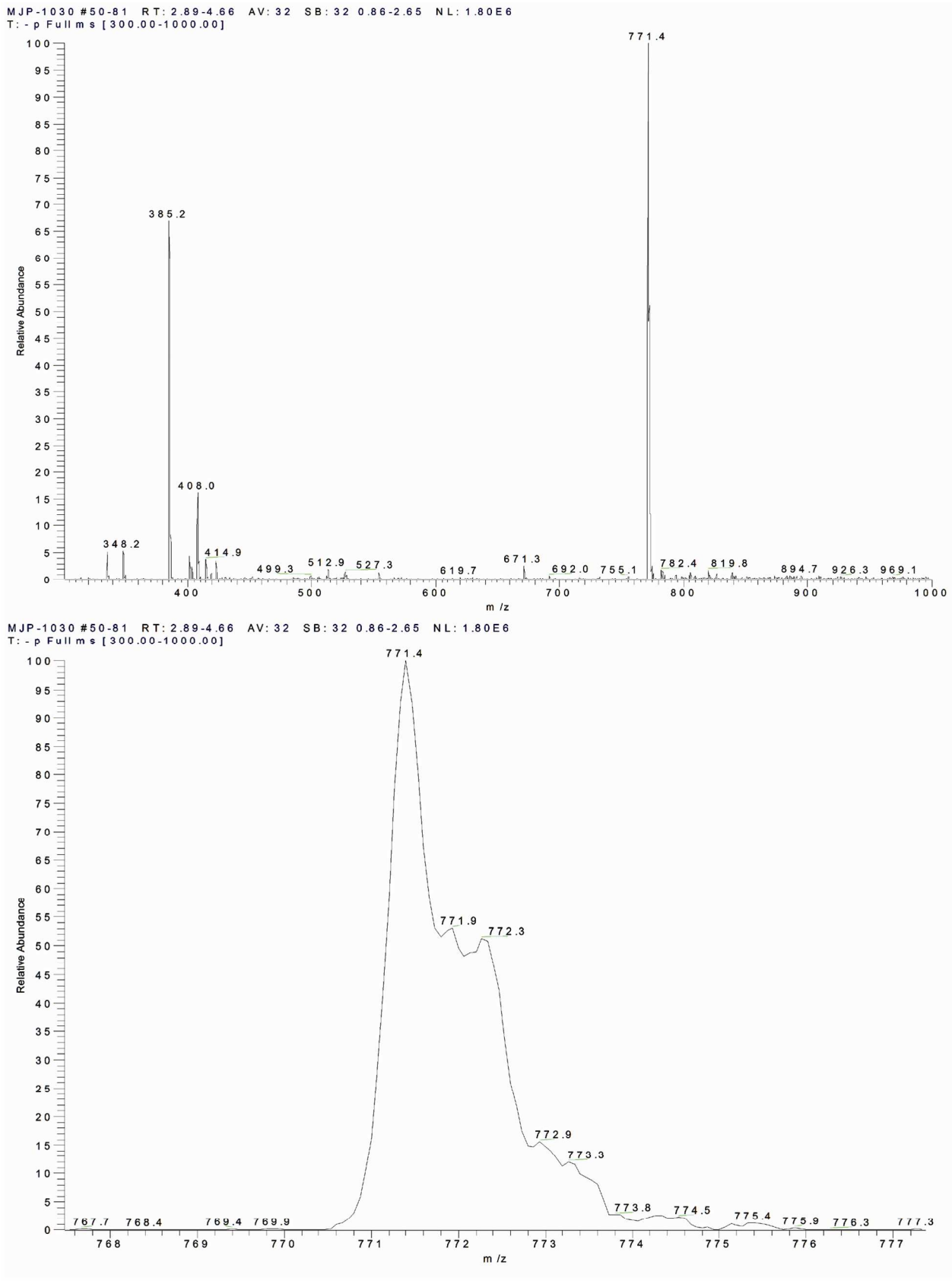
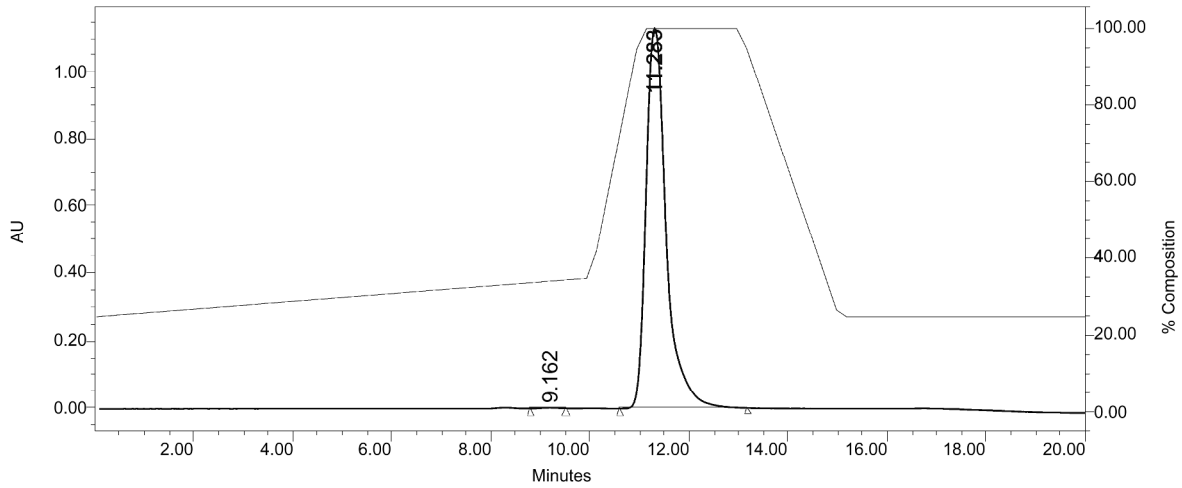


Figure S5. Mass Spectrum (negative ion) of Cy3.29-NBoc **(5)**.

ml of EtOAc and dried under vacuum for 4 hr. The pre-purification yield was ~130 mg, 0.169 mmol (86.7% by HPLC). Bulk derivative was purified by preparative HPLC equilibrated to 20% AcN versus 80% 0.1 M TEAA with detection at 650 and 280 nm. Filtered samples (0.22 μm) up to 14 mg in 14 ml injection volume were separated by gradient elu-



	Processed Channel Descr.	RT	Area	% Area	Height
1	650	9.162	45819	0.14	2083
2	650	11.283	32125184	99.86	1130118

Figure S6. Analytical HPLC of Cy5.29-NBoc **(6)**. UV/Vis detection was at 650 nm. Gradient elution was AcN versus 0.1 M TEAA at a flow rate of 1 ml/min. AcN composition is shown as a thin line.

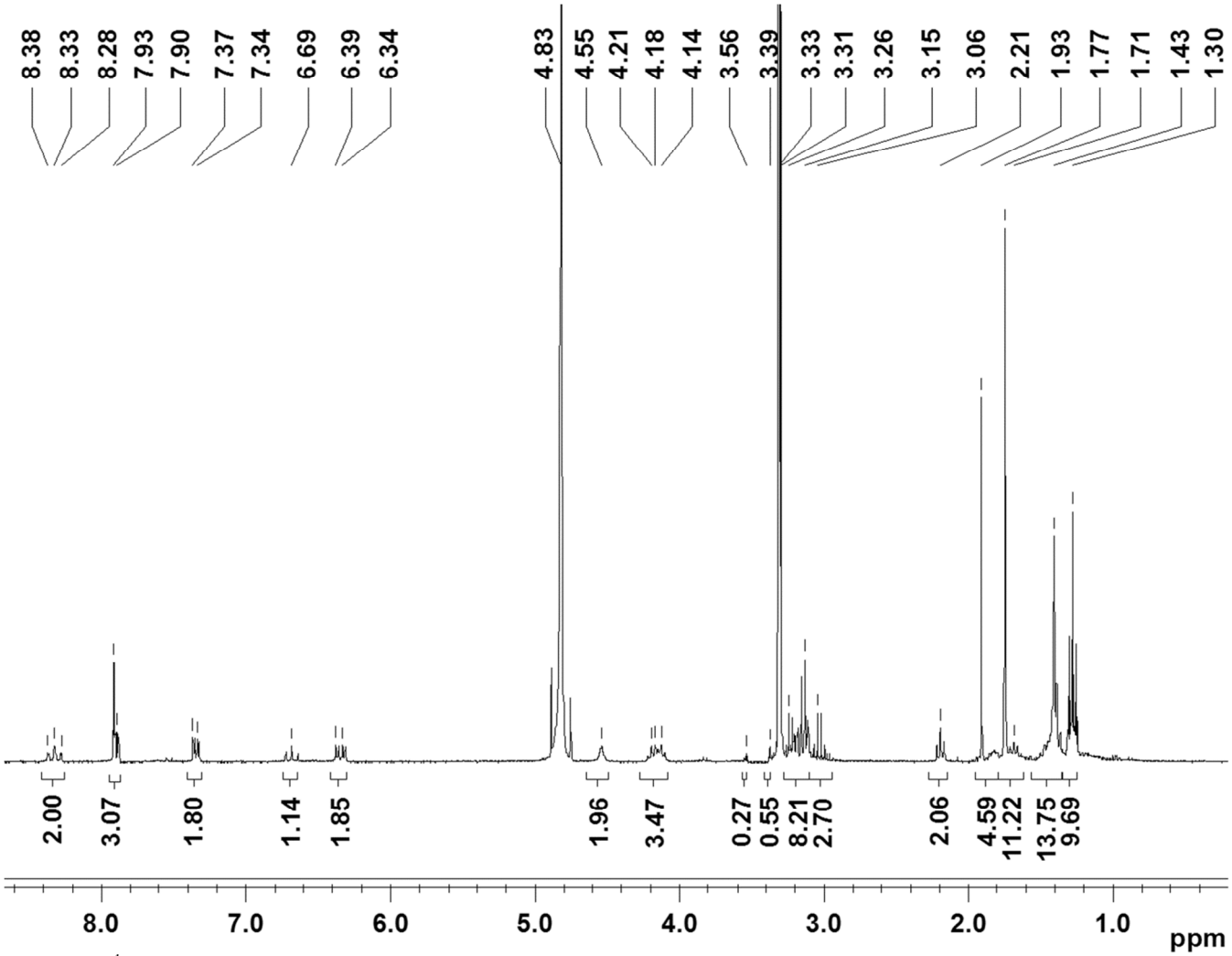


Figure S7. ^{1}H NMR (300 MHz, MeOH-d_4) of Cy5.29-NBoc **(6)**.

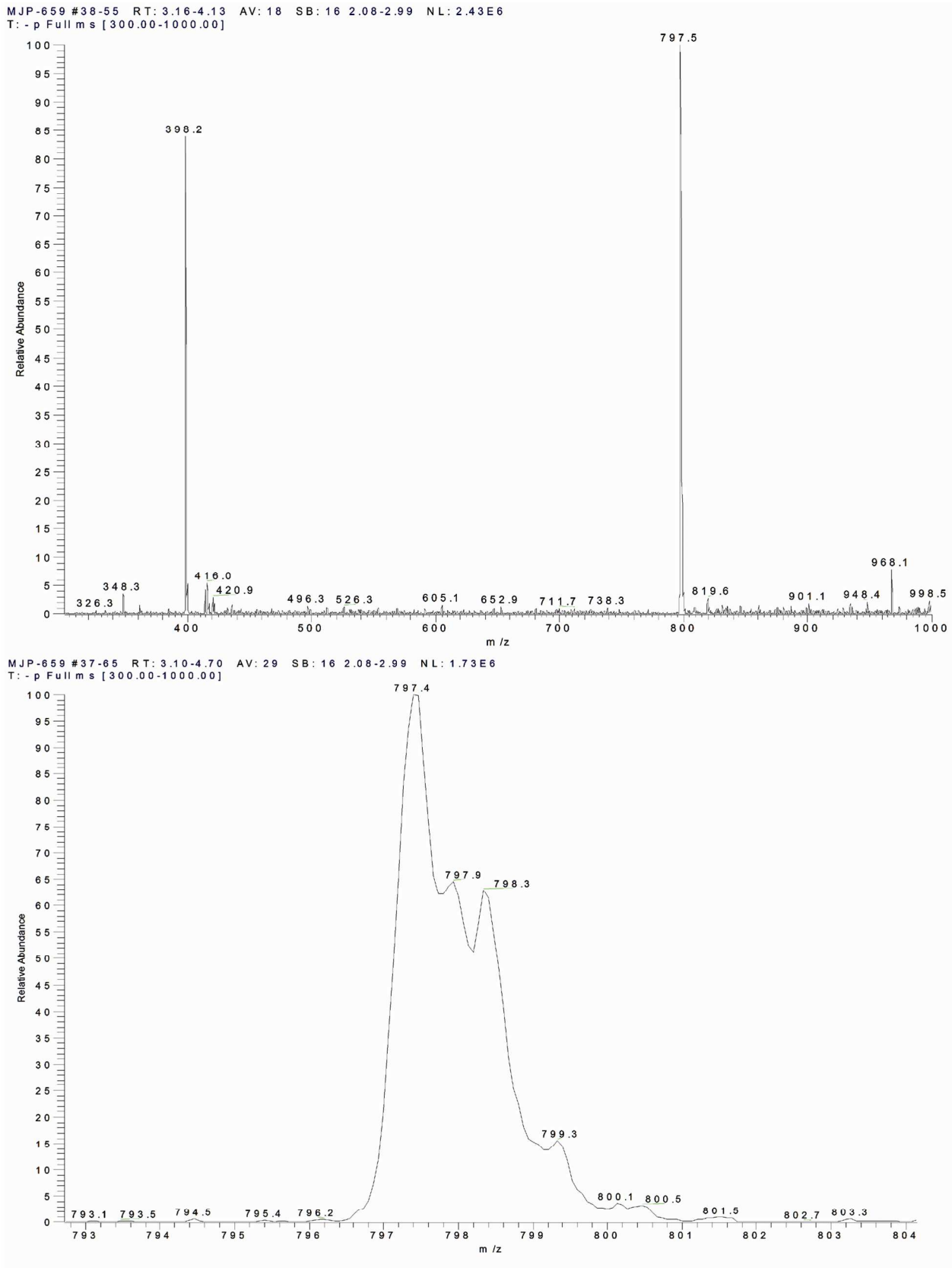
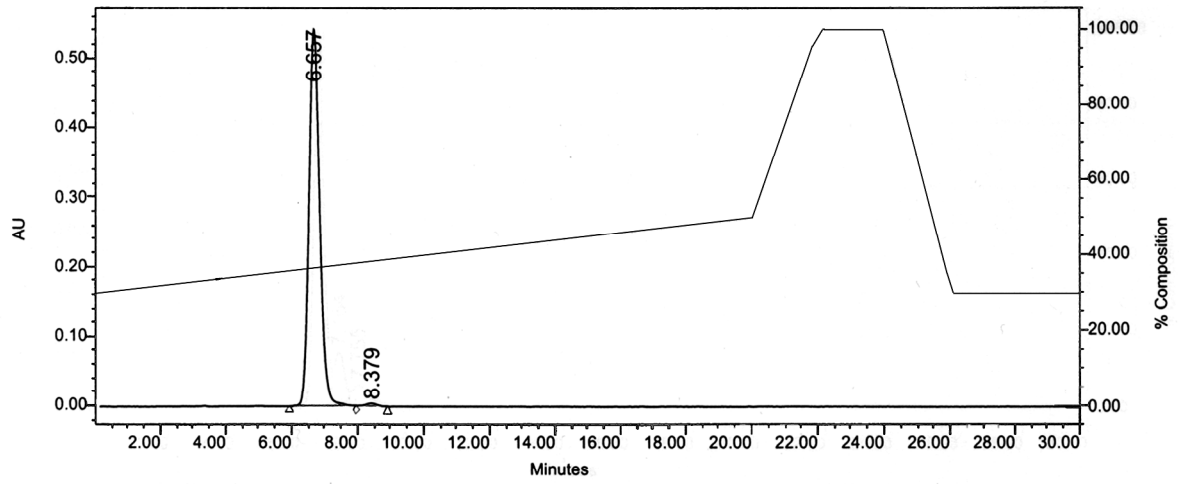


Figure S8. Mass spectrum (negative ion) of Cy5.29-NBoc **(6)**.



	Processed Channel Descr.	RT	Area	% Area	Height
1	490	6.657	12251575	99.05	542614
2	490	8.379	117016	0.95	4469

Figure S9. Analytical HPLC of CypHer5-NBoc **(7)**. UV/Vis detection was at 490 nm. Gradient elution was AcN versus 0.1 M TEAA at a flow rate of 1 ml/min. AcN composition is shown as a thin line.

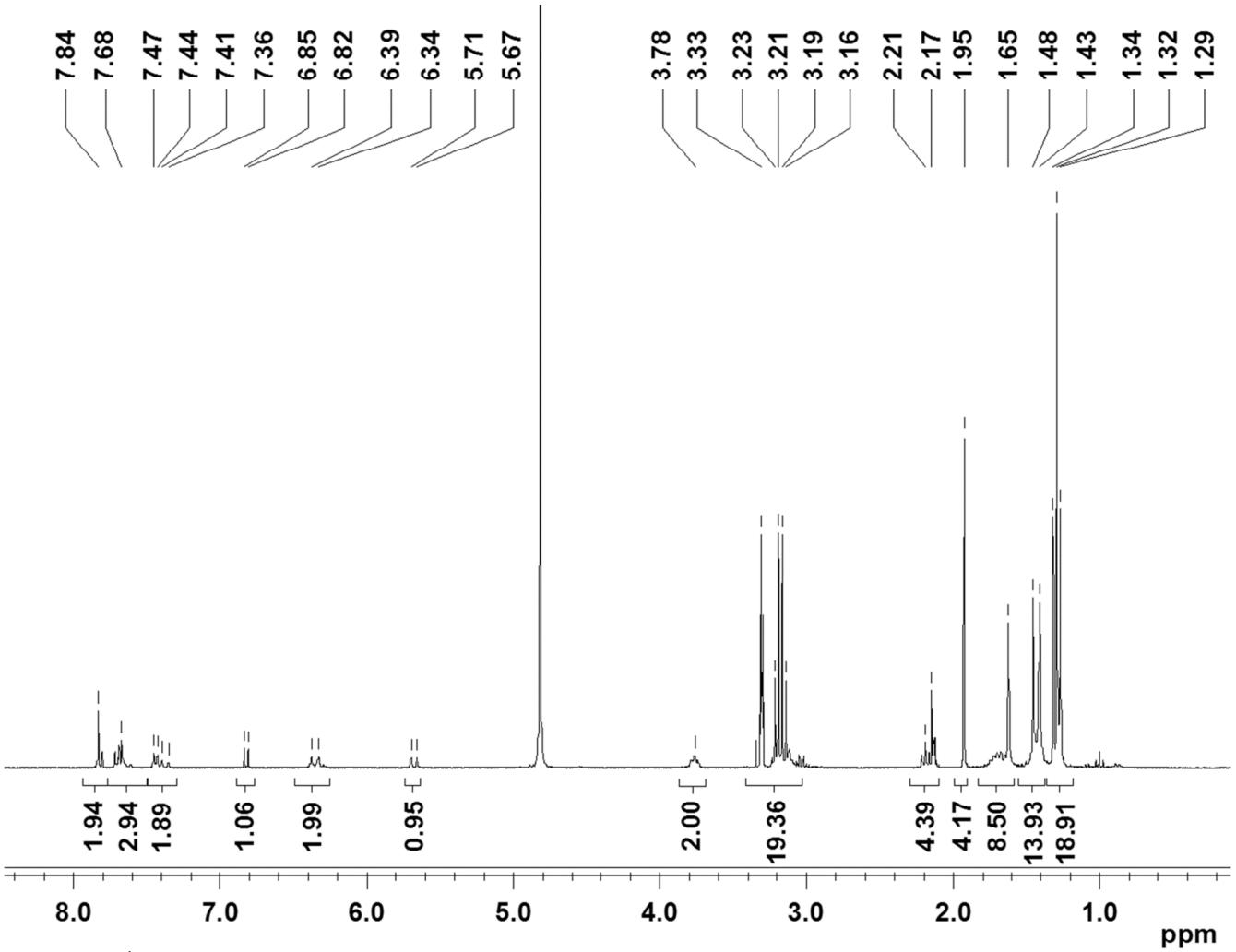


Figure S10. ^{1}H NMR (300 MHz in MeOH-d_4) CypHer5-NBoc **(7)**.

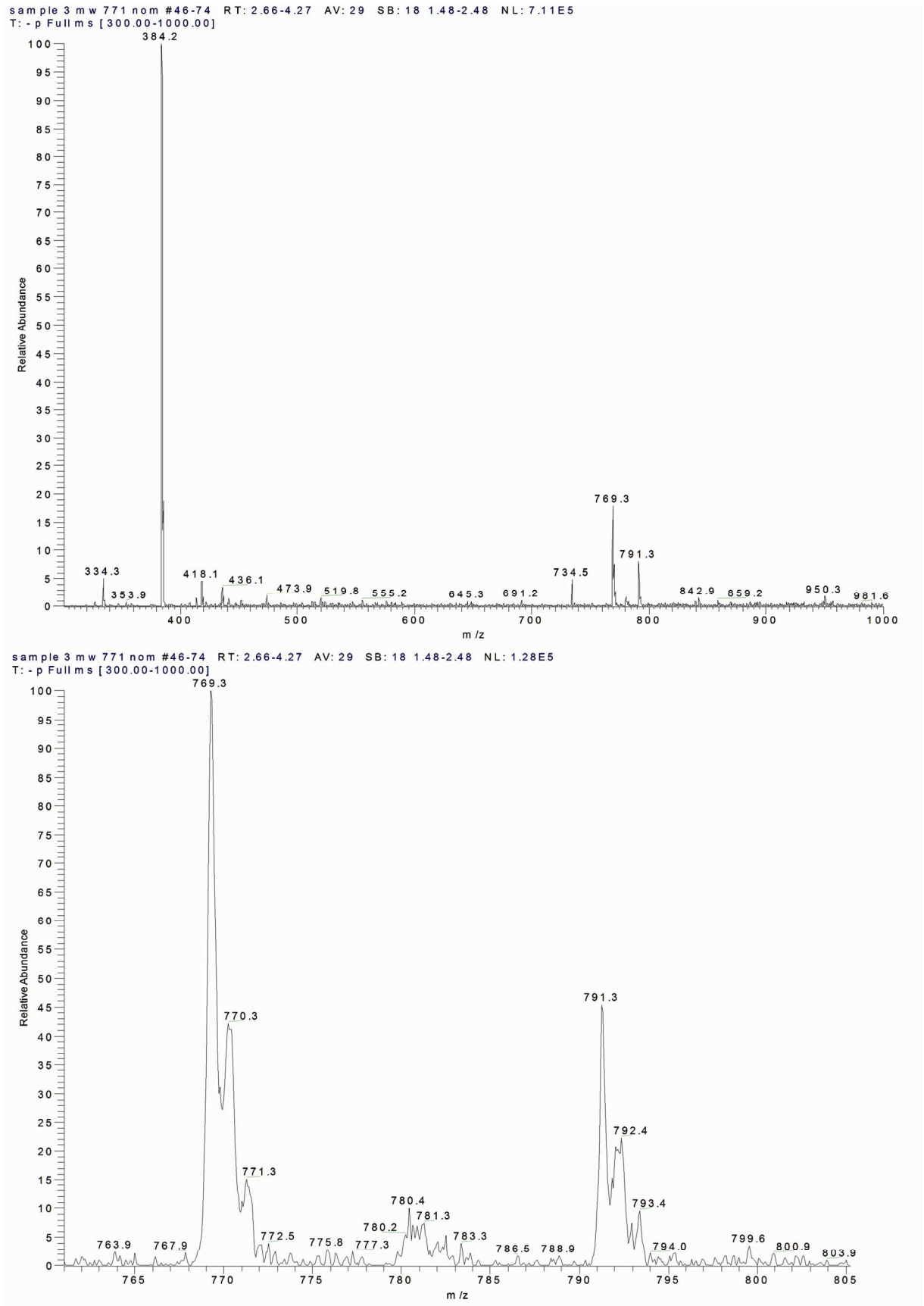


Figure S11. Mass spectrum (negative ion) of CypHer5-NBoc **(7)**.

tion with 20% to 35% AcN over 40 min at 10.0 ml/ min, with an initial hold time of 5 min, and 5 ml fractions were collected. The purity of fractions was determined by analytical HPLC (TEAA buffer 25% to 35% AcN over 10 min at 1 ml/min). The fraction yield was >97% (26.5 mg, 0.0332 mmol) via HPLC, as displayed in Figure S6, and fractions 80-97% purity (~100 mg) were saved for future re-purification. The final purity by analytical HPLC was 99.9%. The ^{1}H NMR displayed (300 MHz, MeOH-d_4) δ 8.377 – 8.290 (2H, t) *Phe*, 7.925-7.884 (3H, m) *–CH= & Phe*, 7.374 – 7.326 (2H, d of d) *Phe*, 6.735 – 6.651 (2H, t) *=CH-*, 6.389 – 6.324 (2H, s) *-CH_2=*, 4.834 (s) *solvent (water)*, 4.554 (2H, broad singlet) *–NH-*, 4.2325 – 4.119 (4H, m) *=N-CH_2-*, 3.56 (0.35H), 3.39-3.38 (0.5H), *solvent* peaks; 3.33 *solvent (MeOH-d_4)*; 3.176 (2H, m) *O=CNH-CH_2-*, 3.152 (2H, m) *O=CNH-CH_2-*, 2.238 – 2.189 (2H, t), *-CH_2-*, 1.931 (s) solvent (AcN), 1.86-1.65 (10H, m) -CH_3, -CH_3, -CH_2-, 1.522-1.193 (22H, m) -CH_2-, -CH_2-, 6(–CH_3) Figure S7 shows the full NMR spectrum.

Figure S8 shows the Cy5.29-NBoc mass spectrometry results, yielding (negative ion) 797.4 (monoisotopic mass 797.33).

CypHer5-NBoc (7)

HPLC-purified CypHer5-COOH **(3)**, (16.3mg, 0.0244 mmol) was dissolved in 2.4 ml DMF. After 5 min, 5.4 µl of DIPEA (0.031 mmol) was added, followed by 1.5 equivalents of TSTU (11.1 mg, 0.0369 mmol). The progress of the reaction was monitored by analytical HPLC (equilibrated at 15% AcN versus water containing 0.1% TFA, gradient elution starting after 2 min from 30% to 40% over 18 min at 1 ml/min with 650 and 280 nm detection). The retention times were 11.4 min (CypHer5-COOH) and 14.4 min (CypHer5-OSu). Two hr later, 1.5 equivalents of NBoc ethylenediamine **(4)** (5.76 µl, 0.036 mmol) were added, and after 1 hr, analytical HPLC was used to determine reaction completion. To preserve NBoc derivative in the TFA mobile phase, the sample was diluted in 1% acetic acid, prior to injection, with t_R = 14.1 min. The TEAA mobile phase was used in later analyses using 30 to 50% AcN versus 0.1 M TEAA over 20 min at 1 ml/min and monitoring at 490 nm. The retention times were 2.5 min (CypHer5-COOH), 6.2 min (CypHer5-OSu) and 6.8 min (CypHer5-NBoc). Dye product was semi-purified by precipitation with EtOAc (30 ml) and separated by centrifugation. The solid was washed with 2x 2.5 ml of EtOAc and solid dried under vacuum at ambient temperature for 2 hr (pre-purification yield was 16.2 mg, 0.0210 mmol, 86.1%). Bulk derivative was purified by preparative HPLC equilibrated to 30% AcN versus 70% 0.1 M TEAA with detection at 650 and 490 nm. Product was dissolved in HPLC mobile phase to make 1 mg/ml concentration and passed through a 0.22 µm filter. Injection volume was up to 10 ml per run, using gradient elution 30% to 90% AcN over 40 min at 10.0 ml/min, with an initial hold time of 5 min; 5 ml fractions were collected. The purity of fractions was determined by analytical HPLC (TEAA buffer 30 to 50% AcN over 20 min at 1 ml/min) with detection at 490 nm (Figure S9). Final purity analyzed by analytical HPLC was 99.1%. The final yield was 6.2 mg at 8.0 µmol (32.9%). The ^{1}H NMR showed (300 MHz, MeOH-d_4) δ 7.840 –7.809 (2H, m) Phe, 7.722 – 7.676 (3H, m) Phe, 7.465 – 7.362 (2H, m) Phe, =CH- 6.847 – 6.820 (1H, d) =CH-, 6.390 – 6.339 (2H, m) =CH-, 5.711 – 5.670 (1H, d) =CH-, 4.833 (broad singlet) H_2O, 3.804 – 3.757 (2H, t) =N-CH_2-, 3.362 – 3.314 (m) solvent (MeOH), overlapping multiplet (16H, m) solvent (triethylammonium -CH_2-) 2 (O=CNH-CH_2-), 2.233 – 2.1837 (2H, t) -CH_2-, 2.1695 – 2.136 (3.5H, m), 1.948 solvent (AcN), 1.766-1.645 (10H m) 2(-CH_2-) 2(-CH_3), 1.477 – 1.433 (18H, m) solvent (acetate ion), 2(-CH_3); 1.339 -1.272 (27H, m) -NBoc-CH_3, solvent (triethylammonium –CH_3,). The full NMR spectrum is shown in Figure S10.

The ^{13}C NMR results in MeOH-d_4 are: δ 191.747, 178.942, 176.783, 176.102, 155.862, 145.371, 144.892, 143.664, 143.318, 142.394, 141.550, 128.121, 125.995, 121.817, 121.411, 113.300, 112.353, 112.176, 111.664, 104.944, 49.511-48.059, 45.097, 44.824, 43.523, 40.999, 40.615, 38.011, 36.720, 36.315, 30.705, 28.775, 28.206, 28.056, 27.999, 27.337, 27.166, 26.686, 26.422, 26.141, 24.194, 11.506, 9.776, 9.231, 8.829. (Spectrum not shown).

CypHer5-NBoc mass spectrometry (negative ion) results are 769.3 (Monoisotopic mass 769.29) (Figure S11), where the compound is unprotonated.

Cy3.29-PFPE-oil (12, 15 and 18)

Purified Cy3.29-NBoc **(5)** (3.0 mg, 3.89 µmol) was dissolved in 1% TFA in MeOH (2.0 ml) and mixed under Ar for 1 hr. Solvents were removed by rotary evaporation at 30 ^{o}C. Residue (**8**) was used immediately. PFPE-methyl ester **(11)**, (408 µl, 0.388 mmol, Avg. MW = 1,750), was diluted into 366 µl of ethanol (EtOH) (90% volume of PFPE ester), 217 µl triethylamine (TEA) was added (1.555 mmol, 4 molar excess to PFPE) and mixed with a magnetic stirrer. After 15 min, separately, Cy3.29-NH_2 **(8)** (3.89 µmol, prepared *in situ*) was dissolved in EtOH (854 µl, 210% volume of PFPE ester) and added to the mixture. MeOH (~400 µl) was used to rinse undissolved dye residue into the vial. Mixture was stirred at ambient temperature in the dark for 48 hr. To block the unreacted ester, 121 µl of diethylamine (DEA) (1.17 mmol, 3 molar excess to PFPE) was added to the mixture and stirred for 72 hr. Afterwards, the solvent was removed by rotary evaporation for 1 hr at 35 ^{o}C, and the residue dried under vacuum in a desiccator overnight. The yield was 0.732 g of polymer mixture. Material was used for formulation following spectral analysis (presented in Section S2.2).

In preparation of conjugated oils for ratiometric nanoemulsions **(24-29)**, an alternative NBoc removal method was used, where Cy3.29-NBoc **(5)** (4.64 mg, 6.01 µmol) was dissolved in 3 M hydrochloric acid (HCl) in MeOH (1.395 ml), to provide 700 molar excess H+, and mixed under Ar for 3 hr. Formation of Cy3.29-NH_2 (98.0%) was confirmed by analytical HPLC (described in Cy3.29-NBoc synthesis). AcN (3 ml) was added to mixture, and solvents were removed by rotary evaporation at 35 ^{o}C for 20 min. Residue was re-dissolved in MeOH (1 ml) and AcN (3 ml), rotary evaporated further for 15 min and dried in a vacuum desiccator for 2 hr. PFPE-conjugation was continued as a TFA-deprotected product, but scaled to batch size. The yield was 1.11 g of polymer mixture.

The ^{19}F NMR showed (0.1% TFA in H_2O/EtOH) δ -56.9553 (0.3 F), -58.2192 (0.2 F), -72.9373 (0.3 F), -73.8704 (0.3 F), -75.9991 (TFA Standard 1 F), -78.2388-78.6800 (1.9 F), -80.0654 (0.2 F), -88.3546-91.7116 (28.5 F), -92.9808 (0.2 F). The full spectrum is given in Figure S12.

Cy5.29-PFPE-oil (13, 16 and 18)

Purified Cy5.29-NBoc **(6)** (5.3 mg, 6.63 µmol) was dissolved in 1% TFA in MeOH (4.0 ml) and mixed under Ar for 1.5 hr.

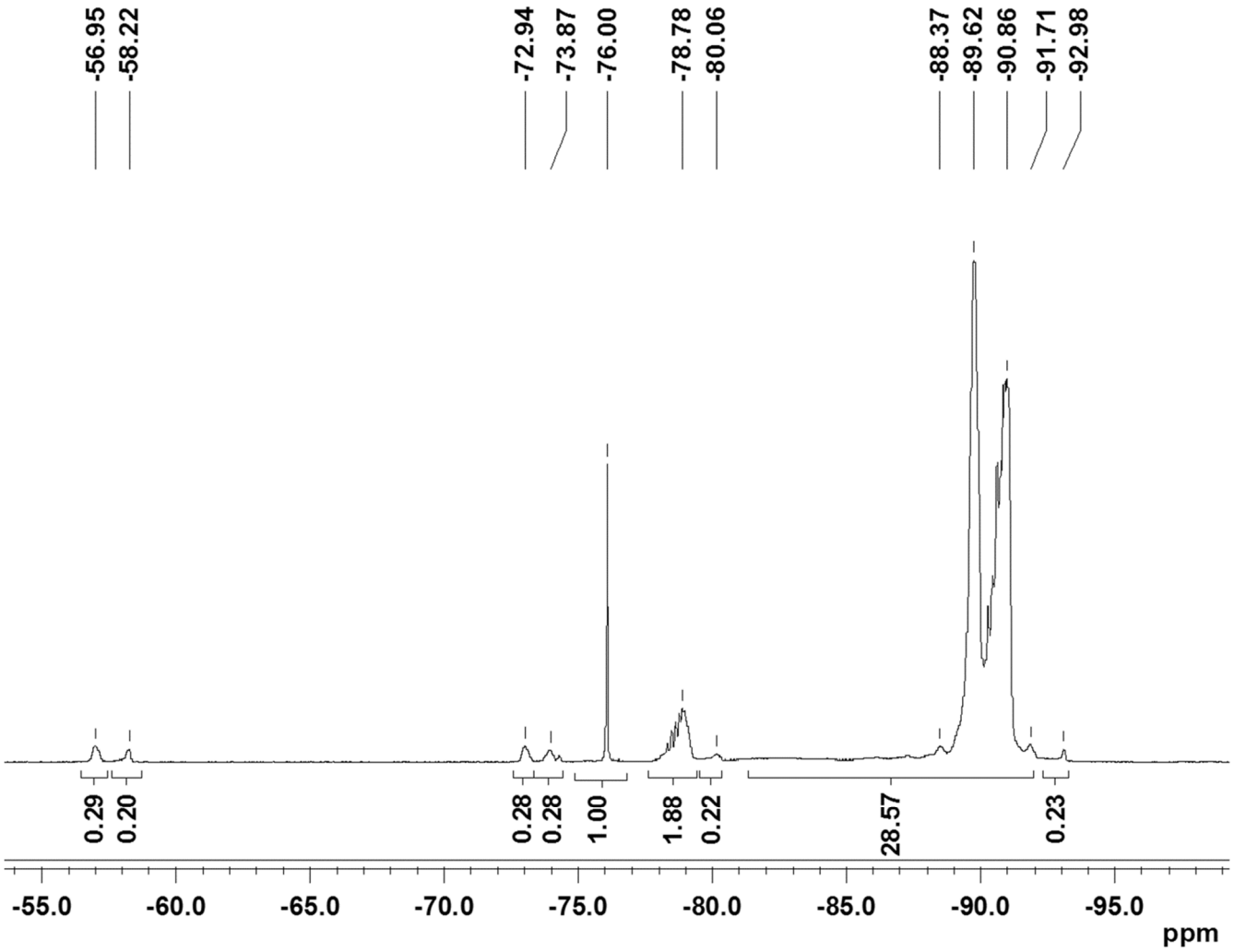


Figure S12. ^{19}F NMR of Cy3-PFPE-oil **(12, 15, 18)** in H_2O/EtOH containing 0.1% TFA.

Solvents were removed by rotary evaporation at 30 ^oC. Residue **(9)** was used immediately. PFPE-methyl ester **(11)**, (688 µl, 0.663 mmol, Avg. MW = 1,750) was diluted in 619 µl of EtOH (90% volume of PFPE ester), 370 µl TEA was added (2.65 mmol, 4 molar excess to PFPE) and mixed with a magnetic stirrer. After 15 min, separately, Cy5.29-NH_2 **(9)** (6.63 µmol, prepared *in situ*) was dissolved in EtOH (1.44 ml, 210% volume of PFPE ester), with the aid of bath sonication, and added to the mixture. MeOH (700 µl) was used to rinse undissolved dye residue into the vial. Mixture was stirred at ambient temperature in the dark for 48 hr. To block the unreacted ester, 206.2 µl of DEA (1.99 mmol, 3 molar excess to PFPE) was added to the mixture and stirred for 72 hr. Afterwards, the solvent was removed by rotary evaporation for 1 hr at 30^oC and the residue dried under vacuum in a desiccator overnight. The yield was 1.22 g of polymer mixture. Material was used for formulation following spectral analysis (presented in Section S2.2).

The ^{19}F NMR showed (0.1% TFA in H_2O/EtOH): δ -58.2952 (1 F), -74.0978 (0.3 F), -75.9991 (TFA Standard 1 F), -78.5603-79.0201 (3 F), -80.4000-92.4365 (44.5 F), -93.0538 (1 F). The full spectrum is displayed in Figure S13.

CypHer5-PFPE-oil (14, 17 and 18)

Purified CypHer5-NBoc **(7)** (3.0 mg, 3.89 µmol) was dissolved in 1% TFA in MeOH (2.0 ml) and mixed under Ar for 1.5 hr. Solvents were removed by rotary evaporation at 30 ^oC. Residue **(10)** was used immediately. PFPE-methyl ester **(11)**, (408 µl, 0.389 mmol, Avg. MW = 1,750), was diluted into 366 µl of EtOH (90% volume of PFPE ester), 217 µl TEA was added (1.555 mmol, 4 molar excess to PFPE) and mixed with a magnetic stirrer. After 15 min, separately, CypHer5-NH_2 **(10)** (3.89 µmol, prepared *in situ*) was dissolved in EtOH (854 µl, 210% volume of PFPE ester), with the aid of bath sonication, and added to the mixture. MeOH (400 µl) was used to rinse undissolved dye residue into the vial. Mixture was stirred at ambient temperature in the dark for 48 hr. To block the unreacted ester, 120.8 µl of DEA (1.166 mmol, 3 molar excess to PFPE) was added to the mixture and stirred for 72 hr. Afterwards, the solvent was removed by rotary evaporation for 1 hr at 30 ^oC, and the residue was dried under vacuum in a desiccator overnight. The yield was 0.743 g of polymer mixture. Material was used for formulation following spectral analysis (see Section S2.2).

In preparation of conjugated oils for ratiometric nanoemulsions **(24-29)**, an alternative NBoc removal method

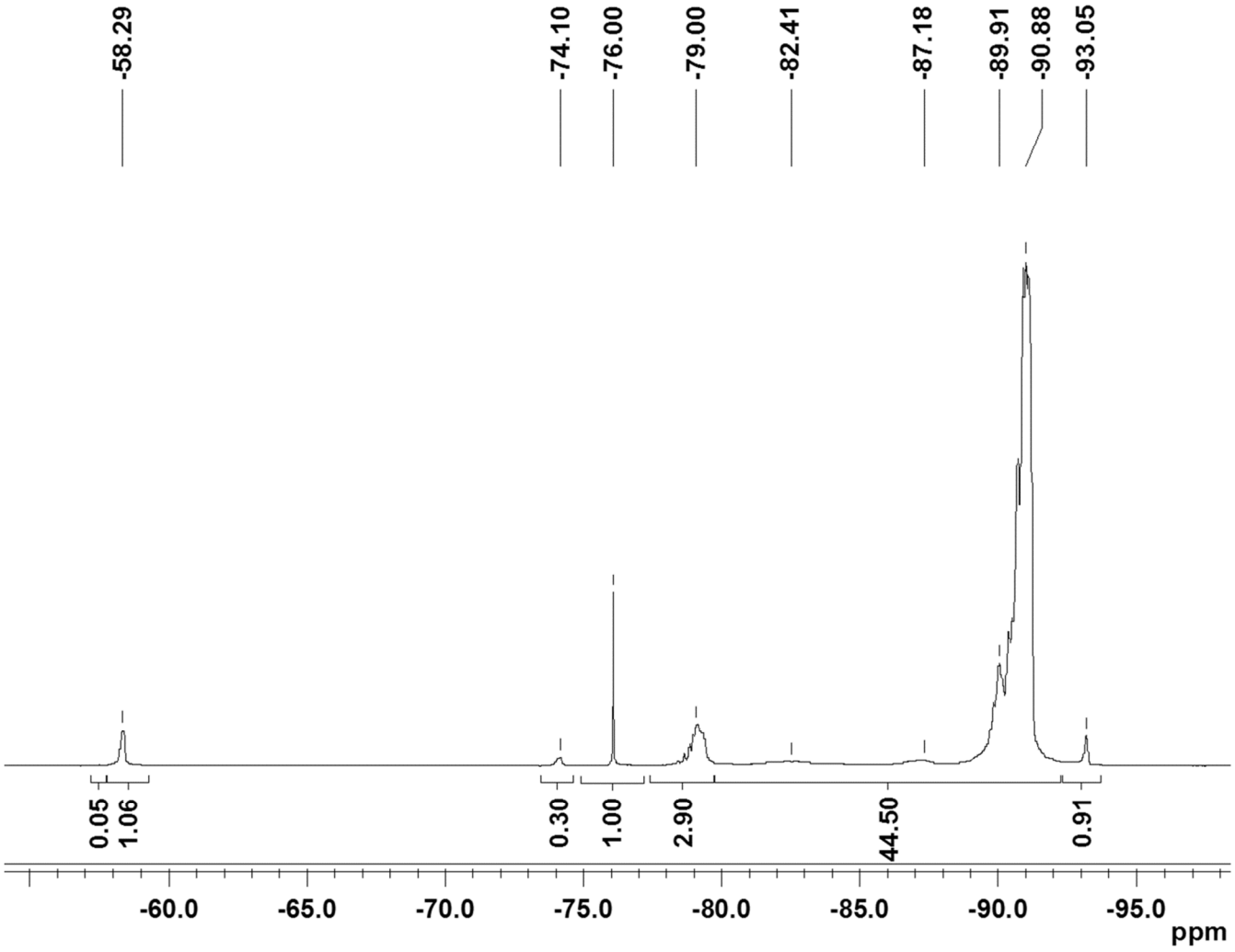


Figure S13. ^{19}F NMR of Cy5-PFPE-oil **(14, 17, 18)** in H_2O/EtOH containing 0.1% TFA.

was used, where CypHer5-NBoc **(7)** (5.82 mg, 7.55 μmol) was dissolved in 3 M HCl in MeOH (1.75 ml), to provide 700 molar excess H+, and mixed under Ar for 3 hr. Formation of CypHer5-NH_2 (98.9%) was confirmed by analytical HPLC (as described in CypHer5-NBoc synthesis). AcN (3 ml) was added to mixture and solvents removed by rotary evaporation at 35 ^oC for 20 min. Residue was re-dissolved in MeOH (1 ml) and AcN (3 ml), rotary evaporated further for 15 min, and dried under vacuum in a desiccator for 2 hr. PFPE-conjugation was continued as the TFA-deprotected product, but scaled to batch size. Additional MeOH (approximately 1 ml/5 mg CypHer5-NH_2) was needed to wet-transfer the material to the PFPE reaction vial. The yield was 1.38 g of polymer mixture.

The ^{19}F NMR displayed (0.1% TFA in H_2O/EtOH): δ -57.0440 (0.3 F), -58.2572 (0.5 F), -72.9660 (0.2 F), -73.9668 (0.4 F), -75.9999 (TFA Standard 1 F), -78.8366 (2.7 F), -80.0055 (0.3F), -80.8067-92.4490 (40F), -93.0117 (0.7 F). Figure S14 shows the full NMR spectrum.

S1.2 Blending of cyanine-PFPEs

S1.2.1 Stoichiometry of cyanine-PFPEs

For ratiometric nanoemulsions, the concentrations of Cy3-PFPE **(12)** and CypHer5-PFPE **(14)** conjugates were estimated in triplicate via UV/Vis spectroscopy measurements. Samples were dispersed in pH 5.8 phosphate buffer (described in Section S2.2.1). Extinction coefficients 150,000 and 250,000 $M^{-1}cm^{-1}$ were used for Cy3-PFPE and CypHer5 chromophores.[1-3] Single-color nanoemulsions were prepared from the corresponding PFPE-conjugates without further analysis. Afterward, the concentrations of **13** and **14** were measured to be 16.9 mM and 5.37 mM, respectively; the concentration of **12** was not determined, but was estimated to be 10-20 mM.

S1.2.2 Blending of cyanine-PFPE oils for ratiometric formulations (24-29)

Cy3-PFPE **(12)** and CypHer5-PFPE oils **(14)** were blended in volume ratios based on known concentrations by first warming oils to ambient temperature, and then combining the two oils in various proportions, referred to as "Blended Ratiometric Oils" (Table S1). For single component ratiometric control formulations **(28-29)** PFPE-Amide **(19)** was substituted for either **12** or **14**. EtOH (200 μl) was added and vortex mixed on high speed for 5 min to ensure adequate blending. The spectral contribution of each fluorescent conjugate to the blended product was determined by fluorescence synchronous scan (see Section S2.3). Fluorescence intensities at EX 649 and EX 548 were used to calculate the pre-formulation ratio of CypHer5/Cy3 nanoemulsion (Table S1).

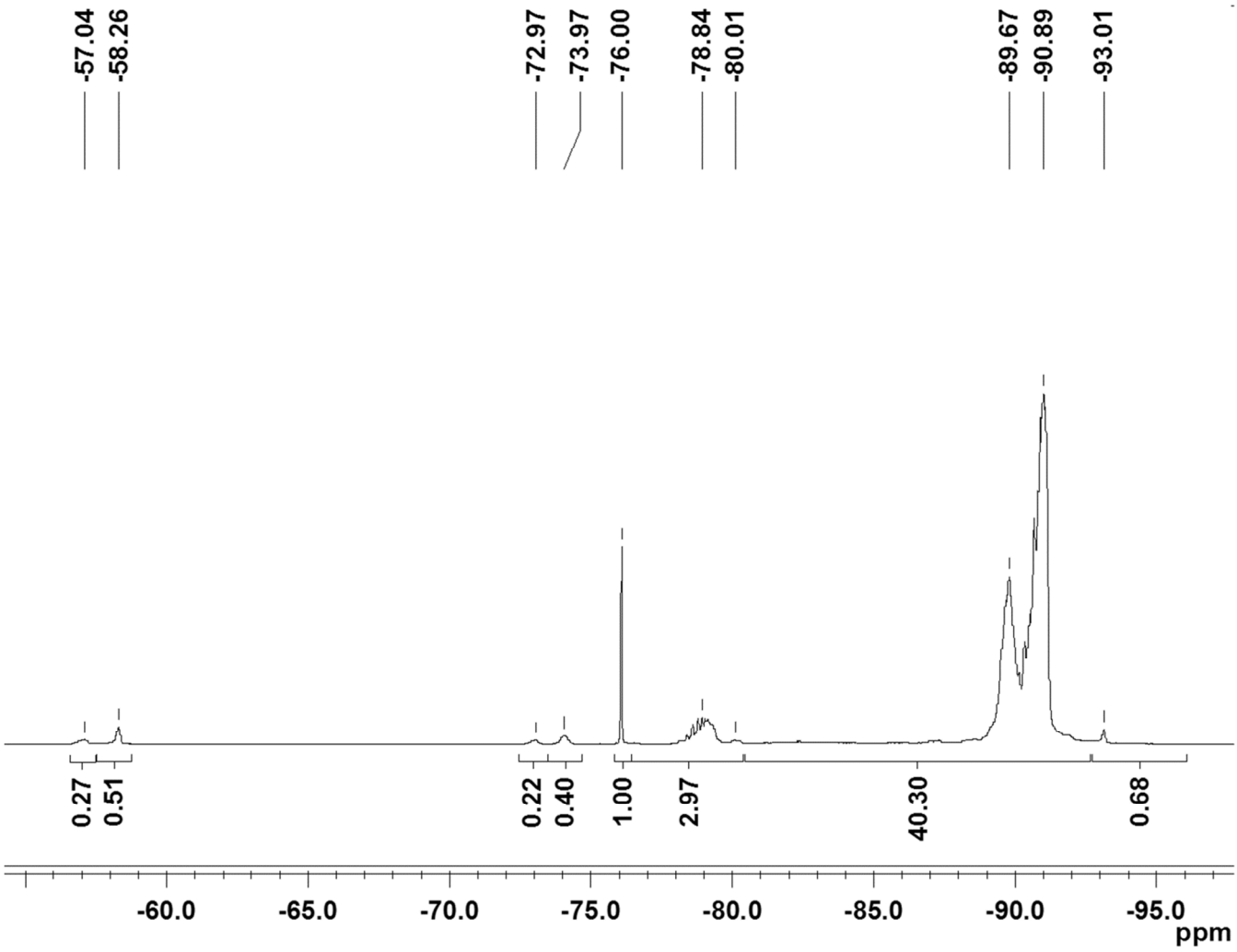


Figure S14. ^{19}F NMR of CypHer5-PFPE-oil **(14, 17, 18)** in H_2O/EtOH containing 0.1% TFA.

S1.2.3 Blending of cyanine-PFPE with PFPE-oxide

Our procedures were modified from Janjic et al.[4] For single-color nanoemulsions **(20-23)**, 100 µl cyanine-PFPE-oils **(12, 13,** or **14)** or PFPE-Amide **(19)** were mixed with 100 µl of EtOH for 15 min, and then added to 1.9 ml of PFPE-oxide (Fluoromed AFP-500HP-0711E, Fluoromed, L.P., Round Rock, TX), mixed with vortex on high speed, followed by a magnetic stirrer for 10 min. Nanoemulsion formulation (S1.3) proceeded immediately afterwards. For ratiometric nanoemulsions **(24-29)**, 200 µl of blended ratiometric oils (Section S1.2.2) were combined with PFPE-oxide (2.0 ml) and mixed with vortex on high speed, followed by a magnetic stirrer for 10 min. Nanoemulsion formulation (S1.3) proceeded immediately afterwards. Table S1 provides further details.

S1.3 Nanoemulsion preparation by microfluidization

A Microfluidizer® M110S (Microfluidics, Inc., Newton, MA), operating at a liquid pressure of approximately 15,000-20,000 psi, was used for all nanoemulsion preparations. The chamber and coil tubing were cooled with an ice bath for at least 30 min prior to using. Cyanine-PFPE nanoemulsions were prepared as Janjic *et al.*[4] with modifications. For single-color nanoemulsions **(20-22)**, 2.00 ml of blended PFPE oil (Section S1.2.3) was combined with 1.36 ml of Pluronic F68 in water (100 mg/ml) and mixed with vortex for 2.5 min. Polyethyleneimine (PEI) solution (1.15 ml of 100 mg/ml in water) was added and mixed with vortex for an additional 2.5 min, then 25 ml of water was added and mixed for 2 min. Nanoemulsions were prepared using microfluidization, with 10-15 cycles at a dynamic pressure of 70 psi. Ratiometric nanoemulsions **24-29** and non-fluorescent **23**, were prepared likewise, but included chamber rinses with water (Table S1).

The final nanoemulsion product (25-45 ml) was drained into a collection container and sat at ambient temperature for 20 min, followed by sterilization by filtration though a 0.2 µm Supor membrane filter (PALL #4652, Pall Corp., Port Washington, NY). The prepared nanoemulsion was stored at 4 °C until use. Further formulation details are shown in Table S1. Stability was tested at two temperatures (4 and 37 °C) by monitoring nanoemulsion droplet diameter (Z average) and polydispersity (PDI) over time by DLS measurements (Section S1.4). Concentration of nanoemulsion was determined by ^{19}F NMR as described in the Supplemental Methods of Janjic *et al.*[4] The ^{19}F NMR spectra for the nanoemulsion were very similar to the neat oil; selected full spectra are shown in Figure S15.

The chemical shifts for each nanoemulsion product are presented here:

Table S1. Nanoemulsion formulation components.

Nanoemulsion Type	Single Color				Ratiometric				Single Component Ratiometric Controls	
Nanoemulsion number	**20**	**21**	**22**	**23**	**24**	**25**	**27**	**28**	**29**	**30**
Cy3-PFPE (12) volume (µl)*	100.0	-	-	-	100	73.5	20.8	8.25	-	45.9
Cy5-PFPE (13) volume (µl)*	-	100.0	-	-	-	-	-	-	-	-
CypHer5-PFPE (14) volume (µl)*	-	-	100.0	-	100	126.5	179.2	141.8	79.1	-
PFPE-amide (19) volume (µl)	-	-	-	100.0	-	-	-	-	45.9	79.1
Ethanol (µl)	100.0	100.0	100.0	100.0	200.0	200.0	200.0	150.0	125.0	125.0
Pre-formulation ratio, pH 5.8 (synchronous scan EX649/EX548)	-	-	-	-	1.10	2.39	12.0	16.9	-	-
Blended EtOH & PPFE-conjugates (µl)	200.0	200.0	200.0	200.0	200.0	200.0	200.0	200.0	200.0	200.0
PFPE-oxide (ml)	1.900	1.900	1.900	1.900	2.000	2.000	2.000	2.000	2.000	2.000
F68 (100 mg/ml) volume (ml)	1.360	1.360	1.360	1.360	1.360	1.360	1.360	1.360	1.360	1.360
PEI (100 mg/ml) volume (ml)	1.150	1.150	1.150	1.150	1.150	1.150	1.150	1.150	1.150	1.150
Water (ml)	25	25	25	15	15	15	15	15	15	15
Volume expelled from microfluidizer (ml)	25	25	25	15	20	30	33	32	45	30
Number and volume of microfluidizer water rinses (ml)	-	-	-	10	2x 10	3x 5	1x 10	1x 10	-	3x 5
Final yield (ml)	25	25	25	25	40	45	43	42	45	45
Final ^{19}F concentration (mg/ml)	114.3	101.2	110.0	111.6	81.4	83.1	90.4	89.5	66.2	72.2

*For single color nanoemulsions, concentrations of **13** and **14** were 16.9 mM and 5.37 mM respectively; concentration of **12** was not determined, but is assumed to be 10-20 mM. For ratiometric nanoemulsions, concentrations of **12** and **14** were 4.37 mM and 2.56 mM respectively.

Cy3-PFPE nanoemulsion (20):

^{19}F NMR (0.1% TFA in water) δ -58.9351 (4 F), -75.9999 (TFA Standard 1 F), -91.5644 (32 F), -93.6363 (3 F). Figure S15 displays the full spectrum.

Cy5-PFPE nanoemulsion (21):

^{19}F NMR (0.1% TFA in water) δ -58.9354 (4 F), -76.0004 (TFA Standard 1 F), -91.5663 (28.6 F), -93.6381 (2.6 F). Figure S15 displays the full spectrum.

CypHer5-PFPE nanoemulsion (22):

^{19}F NMR (0.1% TFA in water) δ -58.9412 (4 F), -76.0000 (TFA Standard 1 F), -91.5672 (31 F), -93.6394 (3 F). Figure S15 displays the full spectrum.

PFPE-Amide nanoemulsion (23):

^{19}F NMR (0.1% TFA in water) δ -58.9358 (4 F), -75.9996 (TFA Standard 1 F), -91.5620 (30 F), 93.6333 (3 F).

0.6:1 Ratiometric nanoemulsion (24):

^{19}F NMR (0.1% TFA in water) δ -58.9509 (3 F), -75.9999 (TFA Standard 1 F), -91.5661 (22 F), -93.6383 (2 F). Figure S15 displays the full spectrum.

1:1 Ratiometric nanoemulsion (25):

^{19}F NMR (0.1% TFA in water) δ -58.9512 (3 F), -75.9997 (TFA Standard 1 F), -91.5643 (22.5 F), -93.6365 (2.1 F).

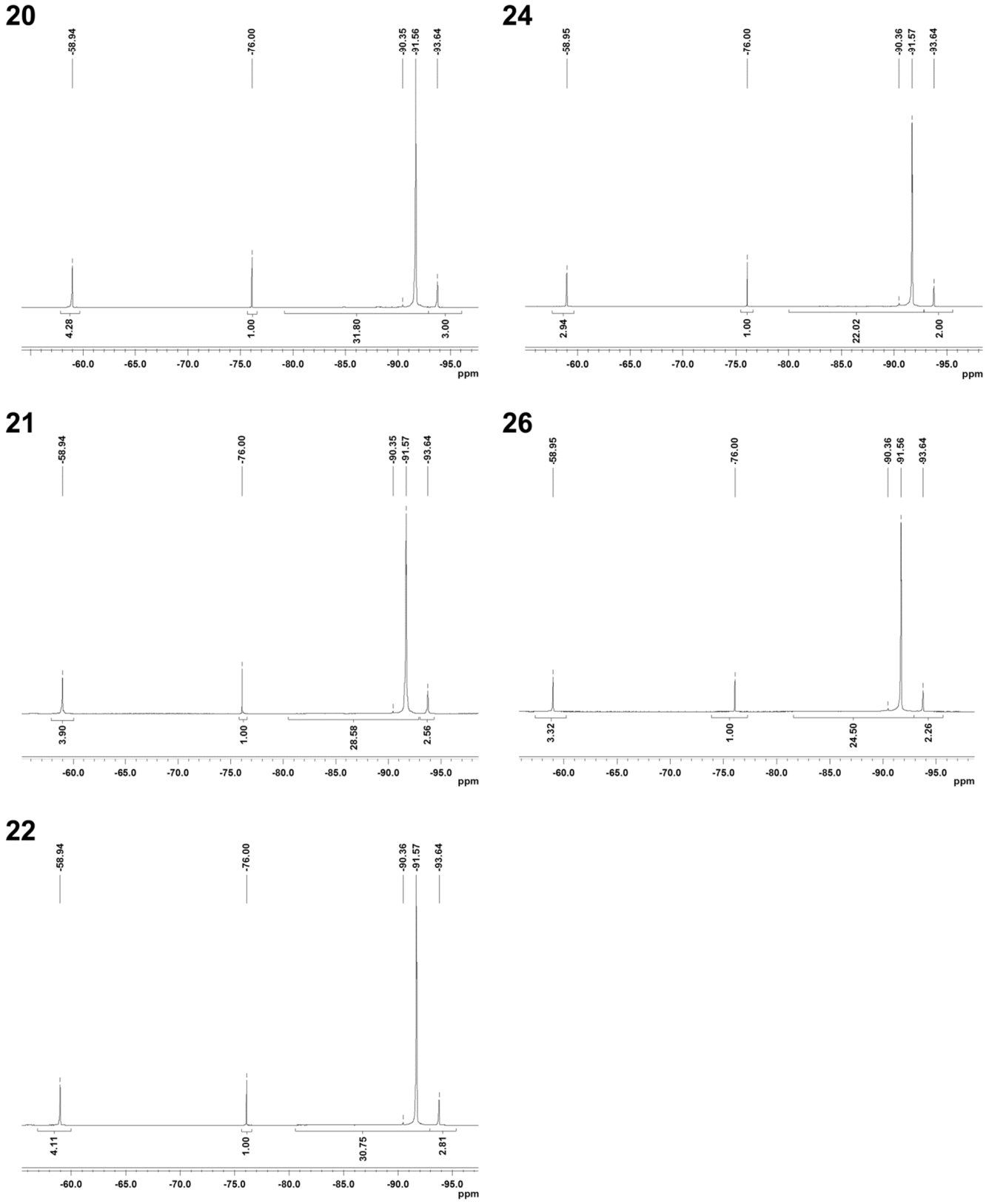


Figure S15. ^{19}F NMR of nanoemulsions **20-22, 24** and **26** in 0.1% TFA.

5:1 Ratiometric nanoemulsion (26):

^{19}F NMR (0.1% TFA in water) δ -58.9512 (3 F), -75.9997 (TFA Standard 1 F), -91.5643 (24.5 F), -93.6365 (2.3 F). Figure S15 displays the full spectrum.

8:1 Ratiometric nanoemulsion (27):

^{19}F NMR (0.1% TFA in water) δ -58.9502 (3.3 F), -75.9999 (TFA Standard 1 F), -91.5659 (24.2 F), -93.6378 (2.2 F).

CypHer5 single component ratiometric control nanoemulsion (28):

^{19}F NMR (0.1% TFA in water) δ -58.9520 (2.4 F), -75.9991 (TFA Standard 1 F), -91.5664 (18 F), -93.6397 (1.6 F).

Cy3 single component ratiometric control nanoemulsion (29):

^{19}F NMR (0.1% TFA in water) δ -58.9515 (2.6 F), -76.0007 (TFA Standard 1 F), -91.5666 (19.6 F), -93.6398 (1.8 F).

S1.4 Nanoemulsions size and stability

The size distribution of nanoemulsion droplets was determined in aqueous media by DLS using Zetasizer Nano ZS instrument (Malvern Instruments, Inc., United Kingdom). Samples were equilibrated to room temperature for at least 20 min and diluted 50:950 (1:20 v/v) into deionized water. Nanoemulsions were stable at 4 °C and 37 °C for at least 10 months. Variations were within experimental error of DLS. Plots for nanoemulsions **20-29** are shown in Figure S16.

S1.5 Additional discussion of synthetic and formulation procedures

S1.5.1 Synthetic rationale

Ratiometric nanoemulsion measurement accuracy relies on detection of the reference and reporter fluorophores at the same exact physical location. Cyanine dyes are directly conjugated to PFPE and stay with the fluorous oil inside the cell. In earlier reported synthetic and formulation studies,[5-7] a fluorescent lipophilic dye was added to the surfactant mixture in the perfluorocarbon (PFC) nanoemulsion to facilitate the fluorescence detection. However, this approach is less robust than the direct conjugation used in our study because lipophilic dyes have a tendency to dissolve and distribute in the cell membranes that they come into contact with, potentially leading to non-specific fluorescent cell labeling *in vivo*. Therefore, fluorescence signals should be from PFPE-conjugates in the fluorous phase and not fluorescent precursors carried along in the aqueous phase or with surfactants.

S1.5.2 Synthetic strategies

It has been well documented that the longer the polymethine bridge within cyanine dyes, the less stable the chromophore.[8] Because of the environmental sensitivities of the CypHer5 chromophore, such as chemical reactivity or susceptibility to decomposition, care was taken to preserve the compound stability during synthesis and purification by HPLC. The direct addition of ethylenediamine to the succinimidyl ester (OSu) was attempted, but was abandoned because of instabilities caused by the methyl ester and the free amino group. It was anticipated that the sensitive CypHer5 chromophore would not withstand these conditions. Therefore, an NBoc-protected ethylenediamine was used to limit reactivity to the one amine on the linker.

Cy3.29 (Cy3) and Cy5.29 (Cy5) chromophores were used as controls to evaluate synthetic and application procedures, particularly exposure to alkaline conditions, heat and storage conditions of intermediates and final products. Cy3.29 was chosen as a robust cyanine of similar structure to CypHer5 to aid in the evaluation of synthetic and formulation processes, where Cy5.29 serves as a same-wavelength, non-environmentally sensitive control. During the HPLC purification of CypHer5-COOH **(3)** and CypHer5-NBoc **(7)**, the stability of CypHer5 derivatives was dependent upon handling conditions. If acidity/alkalinity, temperature and physical state were not carefully controlled, the fluorogen degraded. While in solution, CypHer5 derivatives had to be kept cold (4 °C) and acidic; however, for long term storage, cold (-20 °C), solid and dry was best.

S1.5.3 Preparative HPLC

Reversed phase preparative HPLC was used to purify all of the cyanine free acid starting materials and the NBoc conjugates, yielding good separation of the reaction by-products. Typically, Cy3.29 or Cy5.29 analogs could be purified using AcN/water gradients without the aid of mobile phase modifiers. However, because of the removable proton of CypHer5 analogs **(3, 7)**, care had to be taken to keep the molecule protonated at each stage of the purification process. CypHer5-COOH **(3)** was purified by preparative HPLC in AcN/H_2O gradients containing 0.1% TFA to keep the CypHer5 molecules protonated. Fractions where concentrated to dryness at < 30 °C immediately following purification, and unpurified bulk solution was stored at 2-8 °C to prevent decomposition. NBoc conjugates **(5-7)** were also purified by preparative HPLC. Analytical and preparative HPLC methods required the use of 0.1 M TEAA instead of TFA to preserve the NBoc groups. TFA could prematurely remove the NBoc protecting group, rendering the amine reactive, and thereby producing unwanted by-products or lower yields. To protonate NBoc conjugates in solution, 1% acetic acid was used to dilute the samples. For a chromophore with a removable proton such as CypHer5, ion exchange chromatography would seem to be a likely purification method; however, since the final product and reaction by-products all contain the CypHer5 chromophore (and thus a removable proton), this method would do little to separate CypHer5 by-products from the compound of interest.

S1.5.4 Analytical HPLC

Cyanine-NBoc derivatives were characterized by ^{1}H NMR. HPLC was used to indicate purity of the NBoc conjugate and document the functionality of the NBoc group. Functionality was demonstrated by a shift in retention time due to the conversion of the –NBoc group to –NH_2 upon exposure to TFA. The progress of the cyanine-NBoc **(5, 6, 7)** conjugation was monitored by analytical HPLC; however, the retention times of the cyanine-OSu intermediate and cyanine-NBoc product **(5, 6, 7)** were very close. To confirm the conjugation of the NBoc group to the dyes, a sample was exposed to 1% TFA and then analyzed by an HPLC method using AcN/TEAA mobile phase. For CypHer5-NBoc, the retention time shifted from 13.95 min to 10.48 min when the free amino group was liberated **(10)**. The hydrolysis of the NHS ester would result in the re-formation of CypHer5 free acid **(3)** at a retention time of 12.2 min. When purifying CypHer5 **(7)** with TEAA mobile phases (pH 8.5), the detector was set at 650 and 490 nm, because CypHer5 has two absorption maxima at this pH.

S1.5.5 NMR spectra

HPLC purification of NBoc derivatives using aqueous solvents resulted in a large signal from water, despite extended drying under vacuum. In an attempt to remove residual water molecules during NMR analysis of CypHer5-COOH **(3)**, the sample was dissolved in MeOH-d_4 and rotary evaporated to dryness at 30 °C; however, this did little to remove the water and instead resulted in formation of methyl ester in the NMR sample. The use of higher temperature (37 °C) was considered

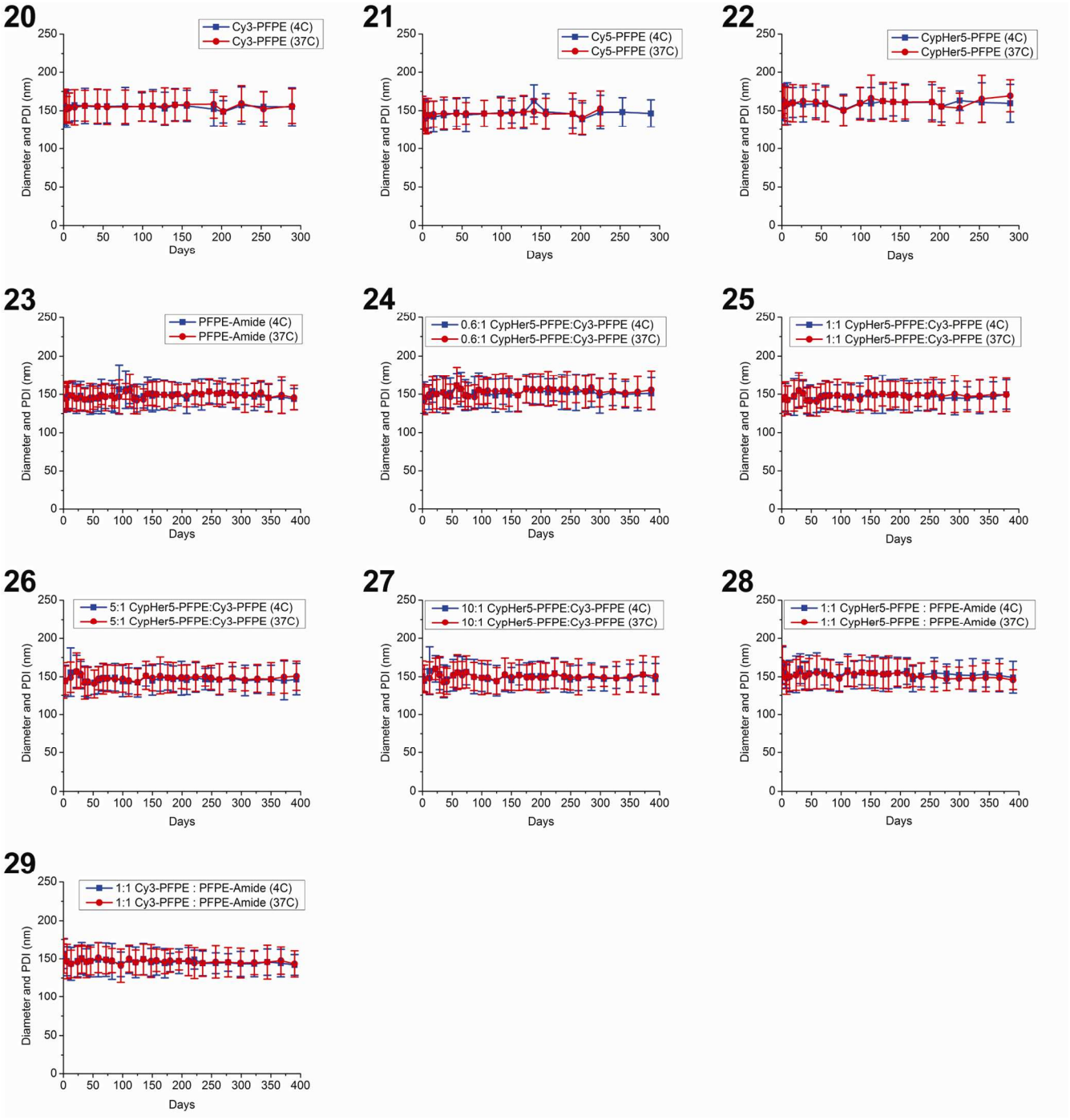


Figure S16. Droplet size of nanoemulsion products shown by compound number **(#)**. The diameters were followed over time at 4 ^{o}C and 37 ^{o}C and measured by DLS.

to aid dehydration, but the stability of CypHer5-NBoc **(7)** was a concern. The affinity of the derivative for water was not a problem in later synthetic steps because during the conjugation to PFPE oil, alcohols were used to mix the hydrophilic and hydrophobic reagents. Cy3.29-NBoc **(5)** and Cy5.29-NBoc **(6)** NMR spectra showed triethylammonium and acetate ions due to the HPLC mobile phase. CypHer5-NBoc may have a higher affinity for water over triethylammonium and acetate ions because it is in the unprotonated form when in TEAA mobile phase (pH ~8.5), causing it to have one less positive charge than its Cy3.29 and Cy5.29 counterparts, and thus less affinity for ion-pairing.

S1.5.6 pH-sensitive color changes during synthesis and ratiometric nanoemulsion preparation.

The pH-sensing ability of CypHer5 analogs was evident during preparation and formulation. During the synthesis of CypHer5-NBoc **(7)** and CypHer5-PFPE oils **(14, 17)**, the color changed from blue to deep orange upon the addition of DIPEA, an organic amine used to de-protonate the free amino group on the linker. After amines were diluted by acidic solution for preparative HPLC purification or removed by vacuum prior to blending, the blue color of CypHer5 conjugates returned. During nanoemulsion formulation, the color of

Table S2. Spectral properties of cyanine-NBoc conjugates. Samples were analyzed in 50 mM potassium phosphate buffers ranging from pH 5.8-8.0 unless specified.

Compound name	Cy3-NBoc	Cy5-NBoc	CypHer5-NBoc
Compound number	**5**	**6**	**7**
Absorption max (nm)	550*	647*	646†, 494‡
Isobestic point (nm)	N/A	N/A	562
Emission λmax (nm)	N/A	N/A	664†
Fractional fluorescence (I/I_0) at pH 8.0/pH 5.8	N/A	N/A	0.08
pK_a (I/I_0 = 0.5)	N/A	N/A	6.75

* Water † Phosphate buffer, pH 5.8 ‡ Phosphate buffer, pH 8.0

Cy5.29-PFPE **(21)** was light blue, whereas the color of CypHer5-PFPE **(22)** was light pink. The color difference is due to the alkalinity of PEI used in the formulation, which results in a nanoemulsion pH of 8-9. Color of blended ratiometric oils used to prepare **24-29** ranged from blue to purple to red depending on the content of CypHer5 and Cy3. During preformulation, the mixture changed color with the addition of PEI because CypHer5 responded to pH, turning from deep blue to orange in color. Because of the reddish color of Cy3, pre-formulated nanoemulsions ranged from pale orange to pink in color, depending upon the content of CypHer5 and Cy3.

S2. Experimental analysis of spectral properties

Absorption and fluorescence measurements for all products were acquired using a Tecan Safire2 Fluorescence Plate Reader (Tecan Group Ltd., Switzerland).

S2.1 Spectroscopic evaluation of cyanine-NBoc derivatives (5-7)

S2.1.1 Absorption spectra

Cy3.29-NBoc **(5)** and Cy5.29-NBoc **(6)** were dissolved in water to make solutions with an optical density of 0.5-1.0 absorbance units; 150 µl was used for measurement in a 96-well flat bottom transparent plate. CypHer5-NBoc **(7)** was dissolved in MeOH (13.2 µM), and 10 µl diluted into 150 µl of phosphate buffers of equal ionic strength ranging from pH 5.8 to 8.0 (in 0.2 pH-unit increments)[9] in a 96-well plate. Absorbance measurements were taken from 350 to 750 nm at 2 nm intervals. See Table S2 for results.

S2.1.2 Fluorescence emission spectra

Emission scans (650-750 nm) with excitation at 630 nm and 10 nm bandwidth were obtained using CypHer5-NBoc **(7)** using samples prepared in S2.1.1. Detector gain was set from the most fluorescent sample (pH 5.8). See Table S2 for results. Section S2.4.3 describes the pK_a determination.

S2.2 Spectroscopic evaluation of PFPE-conjugates (12-18)

S2.2.1 Absorption spectra

Absorption spectra of Cy3.29-PFPE **(12, 15, 18)**, Cy5.29-PFPE **(13,16,18)** and CypHer5-PFPE **(14,17,18)** oils (1% in EtOH) were measured in a series of phosphate buffers of equal ionic strength ranging from pH 5.8 to 8.0.[9] Cyanine-PFPE oils (nominally **12**, **13** and **14**), 7.5 µl each, were dissolved in 750 µl of EtOH. 60 µl of this solution was then added to 90 µl of 50 mM phosphate buffer. Absorbance measurements were taken from 350 nm to 800 nm (Figure 2 and Table S3).

S2.2.2 Fluorescence emission spectra

Fluorescence emission spectra were obtained for PFPE conjugated to Cy3.29, Cy5.29 and CypHer5 **(**nominally **12, 13** and **14)**. Dye-PFPE conjugate was dissolved in EtOH at 1% (v/v), and then diluted with an equal volume of water. The solvated oil was further diluted (5% v/v) into phosphate buffers ranging from pH 5.8 to 8.0.[9] More specifically, 1.0 µl of Cy3.29-PFPE-oil **(12)**, Cy5.29-PFPE-oil **(13)** or CypHer5-PFPE-oil **(14)** were dissolved in 100 µl of EtOH, and then mixed with 100 µl of water. 10 µl of this solution was diluted into 200 µl of 50 mM phosphate buffer, and then 150 µl was transferred to 96-well plate. The fluorescence emission spectra of Cy5.29-PFPE **(13, 16, 18)** and CypHer5- PFPE oil **(14, 17, 18)** were measured from 650 to 750 nm using an excitation wavelength of 630 nm. The gain setting was automatically calculated from the most fluorescent sample for each dye set (for CypHer5-PFPE this was at pH 5.8). Cy3-PFPE-oil **(12)** was evaluated likewise, but with excitation at 530 nm and emission at 550-650 nm. Results are presented in Table S3 and in Figures 2 and S17 (panels A-B). Cy3-PFPE scans were similar to Cy5-PFPE scans (Figure S17B), except blue-shifted by 100 nm (spectra not shown). See Section S2.4.3 for pK_a determination.

Follow-up studies of the fluorescence stability of the conjugates were performed using the same sample preparation, emission scan parameters and detector gain settings used for the initial analyses.

S2.2.3 Fluorescence excitation spectra

The samples prepared for emission scans in S2.2.2 were used for excitation scans. The Cy5-PFPE-oil **(13)** and CypHer5-PFPE-oil **(14)** excitation range was 400 to 660 nm, using a 690 nm emission wavelength. Excitation spectra were measured from 400-670 nm at 690 nm emission for Cy5-PFPE-oil **(13)** and CypHer5-PFPE-oil **(14)**; Cy3-PFPE **(12)** employed 400-570 nm excitation and 590 nm emission. Excitation and emission bandwidths were 5 and 10 nm respectively. Detector gain was set from the most acidic sample. The results are summarized in Table S3.

S2.2.4 Fluorescence synchronous scan spectra

The samples prepared for emission scans were used for synchronous scans. For all samples, scanning parameters were 500-750 nm, 20 nm offset, step size 4 nm and 10 nm band-

Table S3. Spectral properties of cyanine-PFPE Conjugates (12-18). Samples were analyzed in 50 mM potassium phosphate buffers ranging from pH 5.8-8.0 unless specified.

Compound name	Cy3-PFPE	Cy5-PFPE	CypHer5-PFPE
Compound number	**12, 15, 18**	**13, 16, 18**	**14, 17, 18**
Absorption max (nm)	550†	648†, 648‡	648†, 488‡
Isobestic point (nm)	N/A	N/A	560
Excitation λmax (nm)	550†	647†	644†
Emission λmax (nm)	562†	662†	662†
Synchronous scan maximum (20 nm offset)	548†	649†	649†
Fractional fluorescence (I/I_o) at pH 8.0/pH 5.8	N/A	0.83	0.39
pK_a (I/I_0 = 0.7)	N/A	N/A	6.81

† Phosphate buffer, pH 5.8 ‡ Phosphate buffer, pH 8.0

of excitation and emission. The detector gain was set by automatic optimization, using the most acidic sample. The PFPE-conjugates yielded a single peak, summarized in Table S3.

S2.3 Spectroscopic evaluation of blended ratiometric oils

Blended ratiometric oil samples prepared in EtOH (Section S1.2.2) were diluted 1:200 in water (5 µl into 995 µl), which is comparable to final nanoemulsion concentrations, and then 10 µl was further diluted into 490 µl of pH 5.8 phosphate buffer.[9] A 150 µl aliquot was used for fluorescence synchronous scans (20 nm offset, 4 nm step, 500-750 nm range), using a Tecan Safire2 fluorescent plate reader. Detector gain was adjusted automatically for each sample. Fluorescence ratios were calculated (Section S2.4.3) and are summarized in Table S1 (preformulation ratio).

S2.4 Spectroscopic evaluation of nanoemulsions

S2.4.1 Fluorescence spectroscopy of single-color nanoemulsions (20-22)

Single-color nanoemulsions **20-22** were analyzed in the same manner as the PFPE-oil conjugates, but with simplified sample preparation; Cy3.29-PFPE **(20)** was diluted 10 µl into 490 µl (2% v/v) into water, and Cy5.29-PFPE **(21)** or CypHer5-PFPE **(22)** were diluted (2% v/v) into 50 mM phosphate buffer.[9] Then 150 µl of these dilutions were used for fluorescence measurements. Emission spectra of samples were obtained with an excitation wavelength of 530 nm for Cy3.29, and 630 nm for CypHer5 and Cy5.29. The emission wavelengths were scanned from 550 nm to 650 nm for **20** and 650 nm to 750 nm for **21-22**. All detector gain settings were automatically calculated from the most acidic sample. For follow-up studies, the original gain settings were used. Excitation spectra were obtained with an emission wavelength of 590 nm for Cy3.29, and 690nm for CypHer5 and Cy5.29. The excitation wavelengths were scanned from 400 nm to 560 nm for **20** and 400 nm to 660 nm for **21-22**. Data are summarized in Table S4. Excitation spectra for nanoemulsions **21-22** are shown in Figure S17 (panels E-F). Cy3-PFPE spectra were similar to Cy5-PFPE scans, only blue-shifted ~100 nm (data not shown).

S2.4.2 Fluorescence spectroscopy of ratiometric nanoemulsions (24-29)

Ratiometric nanoemulsions **(24-29)** samples were prepared as single-color nanoemulsion samples (1:50) in 50 mM phosphate buffer with pH 5.8-8.0.[9] Nanoemulsions **23, 24, 26** were further evaluated in high potassium phosphate buffer, pH range 4.0-8.0 (see Section S2.6), in 1:50 dilution. 150 µl of diluted sample was used for full spectral characterization. The plate reader was used at ambient temperature. For synchronous scans, a range of 500-750 nm was used, with 20 nm offset, a step size 4 nm, and a 10 nm bandwidth for excitation/emission. The detector gain was set for each nanoemulsion by automatic optimization using the most acidic sample, and the same gain values were used for subsequent samples and follow-up studies. Emission scans were done likewise, with excitation of 630 nm and emission of 650-750 nm for CypHer5, and excitation of 530 nm and emission of 550-750 nm for Cy3. Excitation and emission bandwidths were 10 nm each; emission wavelength step size was 2 nm. Excitation scans were similar to emission scans, with excitation of 400-660 nm and emission of 690 nm for CypHer5, and excitation of 400-560 nm and emission of 590 nm for Cy3. Excitation and emission bandwidths were 10 nm each; excitation wavelength step size was 4 nm. Fixed Wavelength Scans were set either for the optimal excitation/emission of each fluorescent dye (544/564 nm for Cy3 and 644/664 nm for CypHer5) or at wavelengths of synchronous scan measurements (548/568 nm and 649/669 nm for Cy3 and CypHer5, respectively). For these types of measurements, the sample was optimized for each excitation/emission pair separately using the most acidic sample and the same gain values were used for subsequent sample measurements. See Figures 3-4 and Table S4 for summary. Excitation spectra for nanoemulsions **24** and **26** are shown in Figure S18.

S2.4.3 Fluorescence ratios and pK_a calculations

Synchronous scan spectra were normalized to excitation 548 nm (Cy3 signal). Ratios were calculated from CypHer5 signal to Cy3 signal from emission scans (i.e., EM_{max} CypHer5/Cy3), fixed wavelength scans (emission 664/564 nm or emission 669/568 nm as specified), or synchronous scans (excitation 649/548 nm). A normalization calculation to generate the fractional fluorescence (I/I_0), which is described further in S2.8.1, was performed using the most acidic sample, with I_0 set at pH

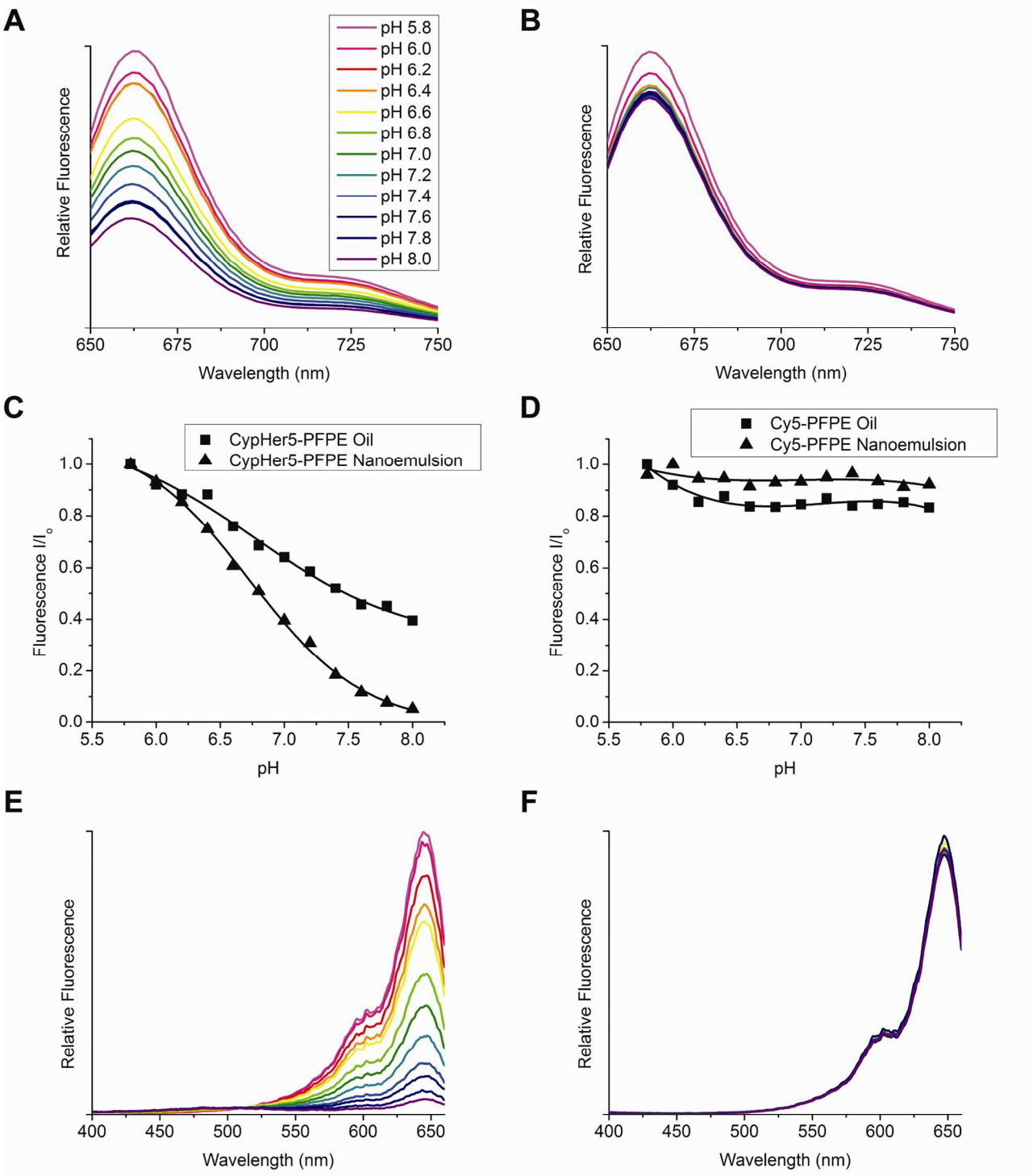


Figure S17. Fluorescence emission spectra of CypHer5-PFPE-oil **(14, 17 and 18)** and Cy5-PFPE-oil **(13, 16** and **18)** in phosphate buffers using 630 nm excitation **(A, B)**. Inset **(A)** shows pH range for series. Panel **(C)** shows a comparison of pK_a of CypHer5-PFPE oils **(14, 17** and **18)** and CypHer5-PFPE nanoemulsion **(22),** and **(D)** displays Cy5.29-PFPE oils **(13, 16** and **18)** and Cy5-PFPE nanoemulsion **(21)** using 630 nm excitation. Panels **(E-F)** show excitation spectra of CypHer5-PFPE **(22)** and Cy5-PFPE **(21)** nanoemulsions using 690 nm emissions.

5.8 in phosphate buffer,[9] at pH 4.4 in MES/HEPES {2-(N-morpholino)ethanesulfonic acid}/{4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid} buffers[10] or at pH 4.0 in high potassium phosphate buffer (see Section 2.6). Data plots were fitted to a Boltzmann sigmoidal curve using Origin 8.5 software (OriginLab Corp., Northampton, MA). For CypHer5-NBoc **(7)** and nanoemulsions **22-28**, the pK_a was determined from the plot of I/I_0 versus pH, at the pH where $I/I_0 = 0.5$ (Figure S17C, S17D). For CypHer5-PFPE conjugate oil **(14, 17, 18)**, pK_a was determined from the plot of I/I_0 versus pH, at the pH where $I/I_0 = 0.7$, which is the midpoint between the start and end of the sigmoidal curve (Figure S17C, S17D). To compare pH-sensitivity of the products, I/I_0 was calculated using I at pH 8.0, and I_0 at the most acidic value. Data is summarized

Table S4. Spectral properties of nanoemulsions 20-29. Samples were dissolved in 50 mM potassium phosphate buffers ranging from pH 5.8-8.0 unless specified.

Nanoemulsion number	20	21	22	23	24	25	26	27	28	29
Excitation λmax (nm)	550*	646	644	N/A	544,644	544,644	544,644	544,644	644	544
Emission λmax (nm)	562*	664	662	N/A	564,664	564,664	564,664	564,664	664	564
Synchronous scan absolute ratio 649/548 (I_o = pH 5.8)	N/A	N/A	N/A	N/A	0.570	1.07	5.39	7.79	> 90	< 0.005
Synchronous scan normalized ratio 649/548 (I/I_o = pH 8.0/pH 5.8)	N/A	N/A	N/A	N/A	0.12	0.12	0.12	0.12	0.11	N/A

* Water

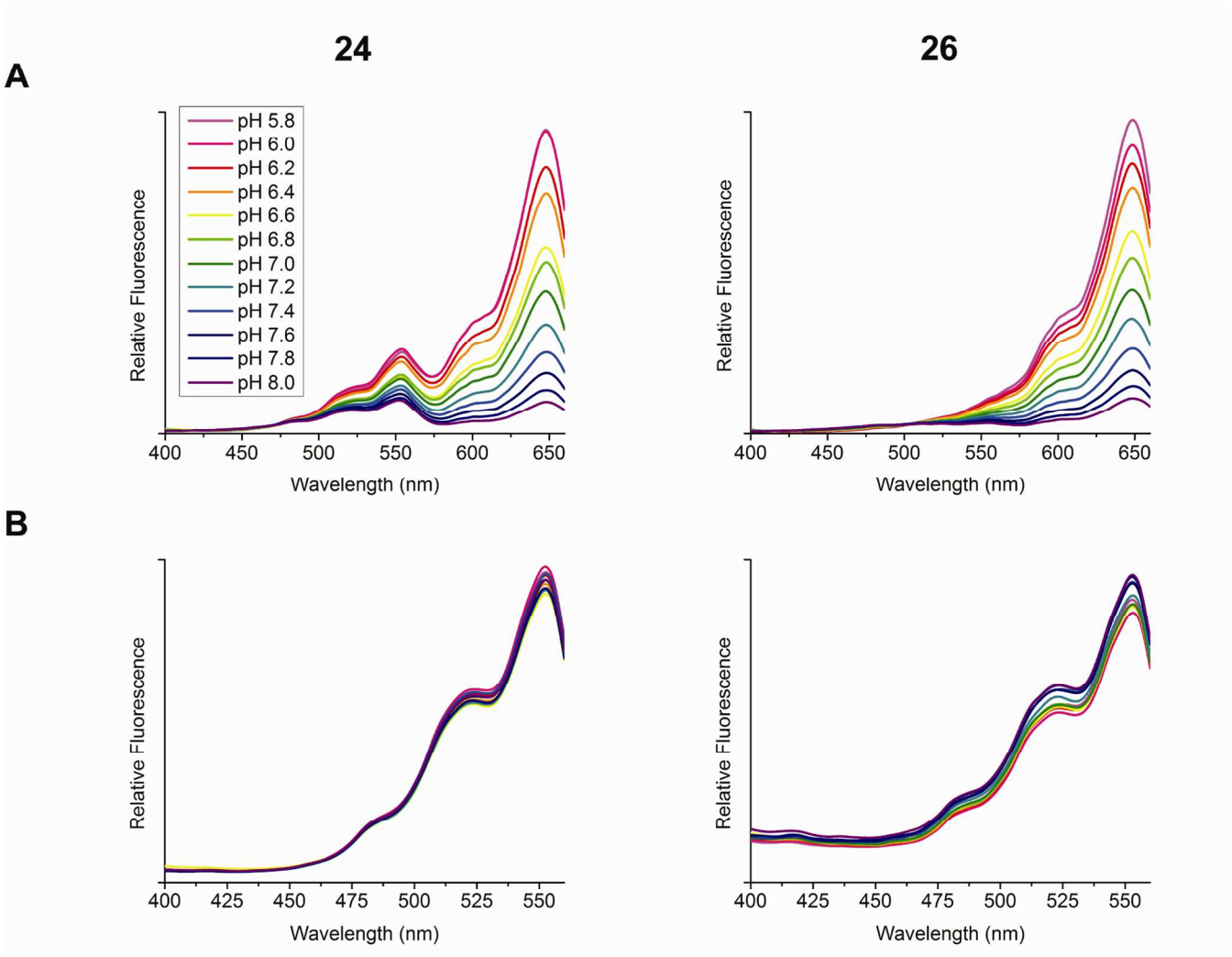


Figure S18. Fluorescence excitation spectra of ratiometric nanoemulsions **24** and **26**. Here, **(A)** is CypHer5 (EM = 690 nm) and **(B)** is Cy3 (EM = 590 nm). Inset **(A)** shows pH range for series.

in Tables S2-S4 and Figures S19-S20.

S2.5 Fluorescent signal and ratio stability of ratiometric nanoemulsions

The fluorescence stability of ratiometric nanoemulsions **24-29** was followed at two temperatures, 4 °C and 37 °C. Samples were equilibrated to room temperature for at least 20 min before dilution. Ratiometric nanoemulsions **24-29** were diluted (2% v/v) in duplicate into 50 mM phosphate buffer,[9] pH 5.8, and prepared as described in Section S2.4.2. The initial data point was taken singly, as part of the formulation process analysis, and then all subsequent samples were done in duplicate. Synchronous scan settings and detector gain values determined during the formulation process analysis were kept for subsequent measurements. Synchronous scan parameters were

500-750 nm, 20 nm offset and 4 nm step. To accelerate measurements, abbreviated synchronous scans were performed that captured the fluorescence maxima of CypHer5 (645-653 nm) and Cy3 (544-552 nm) in 3 data points each using the established detector gain settings. CypHer5 (excitation 649 nm) and Cy3 signals (excitation 548 nm) were plotted against time. The ratio of CypHer5/Cy3 fluorescence signals (excitation 649 nm / excitation 548 nm) were calculated separately for each replicate and plotted against time. Data plots were fitted to the best curve (linear, polynomial, sigmoidal or exponential decay) using Origin 8.5 software and are shown in Figure S21.

S2.6 High potassium phosphate buffer

Buffers ranging from pH 4.0 to 8.0 were prepared at ambient temperature by combining stock solutions of potassium dihydrogen phosphate (pH ~4) and dipotassium hydrogen phosphate (pH ~9) in appropriate proportions to make the desired pH solutions, which were verified by a pH meter. Acidic phosphate stock (pH ~4) contained 20 mM potassium dihydrogen phosphate, 115 mM potassium chloride and 15 mM sodium chloride. Alkaline phosphate stock (pH ~9) contained 20 mM dipotassium hydrogen phosphate, 95 mM potassium chloride and 25 mM sodium chloride. Both stock solutions were prepared to have an ionic strength of 300 mM. The pH 4.0 buffer was adjusted to pH with the addition of a few microliters of 0.1 M HCl. Buffers and stock solutions were stored 4 ^{o}C; warmed to room temperature before use, and pH verified by a pH meter. Adjustments were made by addition of acidic or alkaline stock as needed.

S2.7 Stability of ratiometric nanoemulsions in media at 37˚C

Nanoemulsion droplet stability at 37 ^{o}C, nanoemulsion fluorescence stability and pH-sensitivity were analyzed following incubation in typical cell-labeling culture conditions and compared against incubation in water. Nanoemulsions **24**, **25** and **26** were diluted in duplicate to make 1 mg/ml in both water and labeling Dubelco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% penicillin streptomycin (PS), and 25 mM HEPES (no Phenol Red indicator), each component was obtained from Gibco (Life Technologies, Grand Island, NY). One vial of each solution was incubated at 4 and 37 ^{o}C. Samples were taken in triplicate at 0, 3, 24 and 48 hr and diluted 10:490 (2% v/v) into pH 5.5, 6.5 and 7.5 high potassium HEPES buffer, prepared as described by Barriere.[10] 150 µl of diluted sample was added to a 96-well flat bottom clear plate. Fluorescence fixed wavelength scans were taken with excitation/emission at 548/568 nm (Cy3 signal) and excitation/emission 648/668 nm (CypHer5 signal), each with 10 nm bandwidth. Detector gain was set for each excitation wavelength separately, using the settings from the 0 hr pH 5.5 samples that produced the largest signal for each nanoemulsion. Fluorescence signals at emission 568 nm and emission 668 nm were plotted against time. For each replicate, the ratio of 668/568 nm emission was calculated and the plotted against time. For 0 and 48 hr data, the same fluorescence ratio was calculated for pH 5.5, 6.5 and 7.5, normalized to the pH 5.5 ratios for the data set, and plotted against pH. Plots were similar for all three nanoemulsions; representative plots are shown in Figure S22.

S2.8 Additional discussion of spectroscopy and analytical testing

S2.8.1 Spectral analyses

The fractional fluorescence (I/I_0) was used to quantify pH sensitivity of CypHer5 as described by Briggs and Cooper,[2,3] which is defined as the emission of the probe at pH n, where n is a number between 5.8 and 8.0, divided by the emission where the probe is 100% protonated.[2] This concept is used with our ratiometric reagents; however, in this case, I/I_0 refers to the ratio of reporter/reference emission of the probe at pH n divided by ratio of reporter/reference emission where the probe is 100% protonated. In this manuscript, the fluorescence of ratiometric reagents (I) is referred to as "absolute ratio," while the fractional fluorescence of ratiometric reagents (I/I_0) is referred to as "normalized ratio." This extension of fractional fluorescence to ratiometric reagents allows for comparison of pH sensitivity between the ratiometric products and the original reagents. Additionally, fractional fluorescence (I/I_0) at the alkaline extreme (pH 8.0) provides an effective measure of a fluorophore's responsiveness to pH changes, and thus its effectiveness as a pH sensor. The lower I/I_0 is at alkaline pH, the better the pH/fluorescence response is.

Sample preparation methods were different for spectral analyses of cyanine derivatives **(5-7)**, cyanine-PFPE conjugates **(12-18)** and cyanine-PFPE nanoemulsions **(20-29),** due to differences in miscibility of each product with aqueous buffers. Free acids and -NBoc conjugates were isolated in solid form and dissolved directly into aqueous solutions for spectral measurements. However, once the cyanines were conjugated to PFPE oils, the water solubility of the cyanine dyes was diminished by the hydrophobicity of the PFPE oils. To obtain fluorescence emission spectra of the PFPE conjugate oils in aqueous solutions, EtOH (2.5%) was required. Once the cyanine-PFPE oils were microfluidized into nanoemulsions, the hydrophobic character of the PFPE oil no longer dominated the aqueous solubility characteristics, and nanoemulsions could be diluted directly into aqueous solutions.

Absorption maxima of the cyanines changed very little (± 1 nm) between free acids, NBoc-derivatives and PFPE-conjugates; likewise, excitation and emission maxima of PFPE-conjugates and nanoemulsions agreed (± 2 nm), as shown in Tables S2-S4. Deviation can be attributed to subtle differences between spectrometer instrumentation and acquisition parameters (such as wavelength interval being even or odd numbered). The emission maxima of the PFPE conjugates and nanoemulsions were very close to the cyanine free acids, which were 565 nm for Cy3.29 **(1)**,[1] 666 nm for Cy5.29 **(2)**,[1] and 660 nm of the equivalent pH-sensitive Cy5.29 free acid **(3).**[2]

CypHer5-PFPE-oil **(14)** and CypHer5-PFPE nanoemulsion **(22)** both exhibit fluorescence spectral differences in acidic versus alkaline solution; however, the CypHer5-PFPE-oil alone shows less difference in the change in fractional fluorescence (I/I_0) between acidic and alkaline solutions. I/I_0 at pH 8.0 was 10 times higher for CypHer5-PFPE-oil (~0.5) than for CypHer5-PFPE nanoemulsion (~0.05), as shown in Figure S17C. As a control, Cy5.29-PFPE oil **(13)** and nanoemulsion **(21)** were tested similarly and, as expected, did not show pH-sensing behavior (Figure S17D). However, CypHer5-PFPE **(14)** and Cy5.29-PFPE **(13)** oils exhibited different fluorescence intensity ratios at various pH values than their nanoemulsion counterparts (**22** and **21)**, and the pK_a curve of

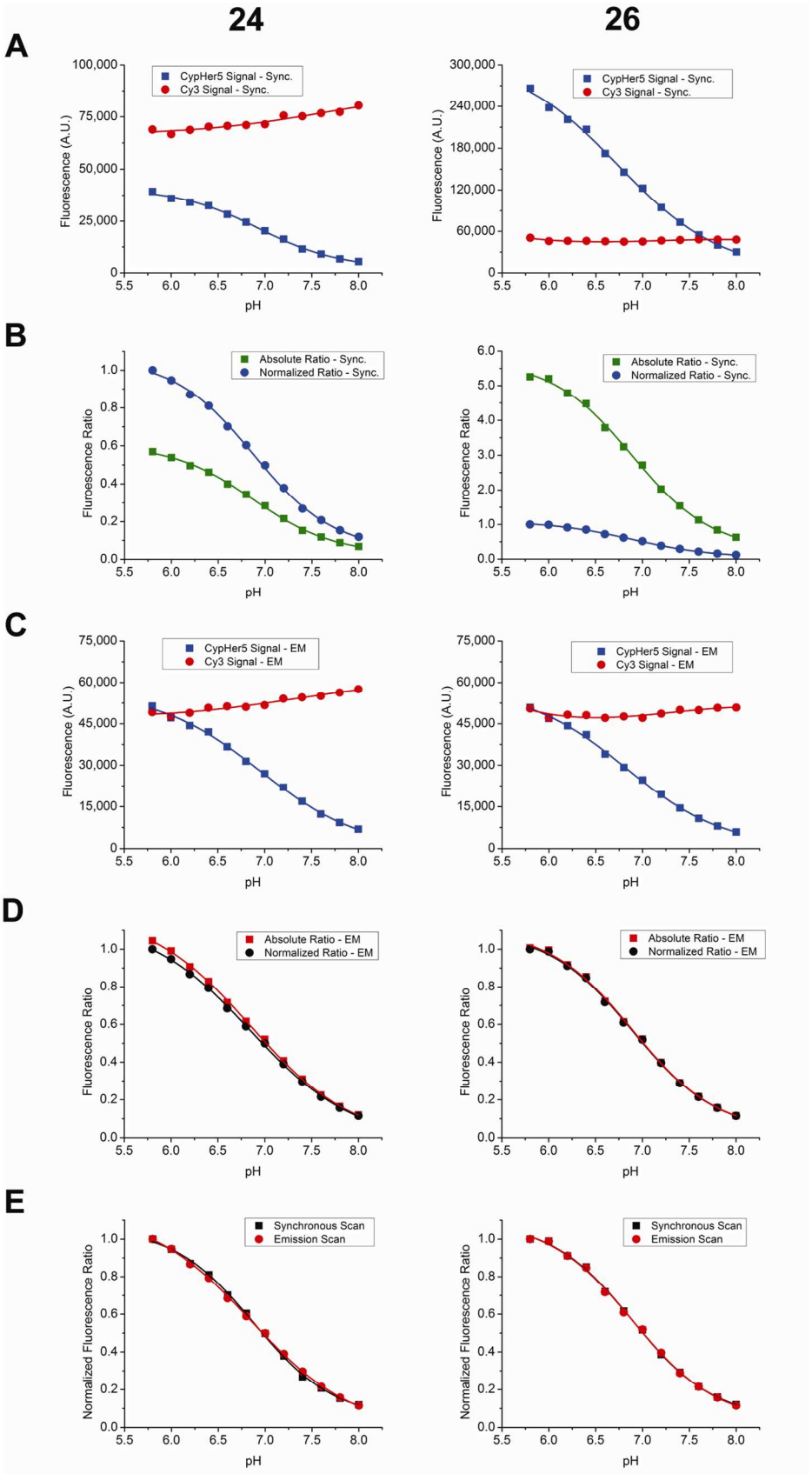


Figure S19. Comparison of synchronous and emission scan data for nanoemulsions **24** and **26**. Displayed are synchronous scan signals versus pH **(A)** and CypHer5/Cy3 ratio-pH curves **(B),** shown as absolute ratio and normalized to pH 5.0 value. Emission scan signals versus pH are shown **(C)** and corresponding ratio-pH curves **(D)**. Comparison of normalized ratio-pH curves obtained by synchronous and emission scans are also displayed **(E)**.

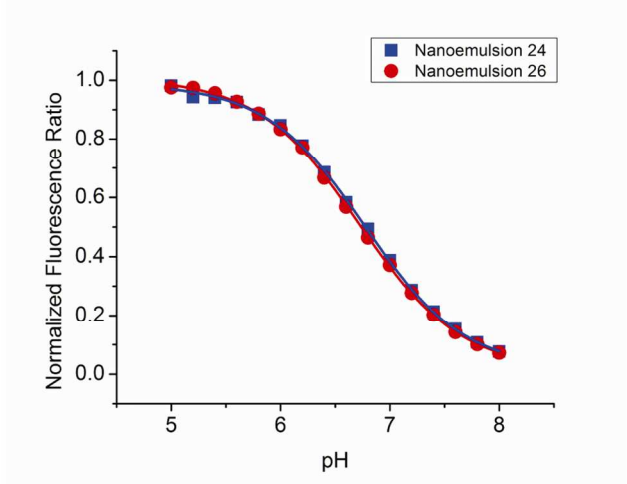


Figure S20. Ratio-pH curves of nanoemulsions **24** and **26.**

nanoemulsion **(22)** was less steep than those presented by Cooper[2] for the original free acids. This is due to the microenvironment that the PFPE-conjugate oil occupies during measurement. When the PFPE-conjugate oil is solvated with ethanol and diluted into aqueous solution for spectral measurements unformulated, it most likely forms a hydrophobic micelle; however, once formulated into a nanoemulsion through microfluidization, the droplets are much smaller and less hydrophobic. Additionally, light scatter from the colloidal dispersions may also affect the spectra, exhibiting larger effects with larger droplet size (formulated vs. unformulated). Differences in pK_a curve shape of nanoemulsion **22** and its corresponding free acid may also be attributed to the PFPE chain hindering access to the removable proton on the conjugate, compared to the unencumbered access of the free acid. Spectral quantification of the unformulated CypHer5-PFPE oil **(14)** is not particularly reliable as a pH sensor because of the hydrophobicity of the PFPE-derivatives, demonstrated by the larger I/I_0 value at pH 8.0, when compared to that of the nanoemulsion (discussed further in Section S2.8.2). However, fluorescence synchronous scans of the cyanine-PFPE oils were sufficient for providing an estimate of the fluorescence contribution of each dye to ratiometric nanoemulsions during preformulation analysis.

S2.8.2 Effect of residual HCl on pH sensitivity of CypHer5-PFPE oil

When Cy3-PFPE **(12)** and CypHer5-PFPE **(14)** were resynthesized for use in ratiometric nanoemulsions, the removal of the -NBoc group was performed using 3 M HCl instead of 1% TFA (Section S1.1). For the conjugate products, it was observed that I/I_0 at pH 8 was ~0.8, compared to ~0.5 when TFA was used as the deprotecting reagent. It was suspected that residual HCl was protonating the molecule and developing an affinity for the dye molecule due to the non-aqueous environment of the PFPE oil, which comprised 99% of the molecular content.

To investigate this further, HCl-deprotected PFPE-conjugate **(14)** was titrated with 3 M excess of either TEA or DIPEA in EtOH, by diluting into amine-EtOH solution 1:100 and then further in water (1:1). Then, the titrated product was diluted 1:200 into four phosphate buffers spanning pH 5.8-8.0. Fluorescence emission scans were taken as described in Section S2.2.2. Emission intensity maximum (EM_{max}) was plotted against pH for each condition (data not shown). The addition of 3 M TEA or DIPEA prior to dissolving in phosphate buffers resulted in a decrease in I/I_0 to 0.1 at pH 8, indicating that at least 10% of residual HCl was titrated using this method. However, the hydrophobic CypHer5-PFPE molecule may be buried in the PFPE core, unable to reach the surrounding aqueous solution at the perimeter of the oil droplet. If the dye molecule is trapped in the protonated form, it will contribute higher fluorescent signal in alkaline solutions than if free, thus contributing to the observed higher I/I_0 at pH 8, instead of ~0.1 as with the –NBoc or nanoemulsion.

Ultimately, residual HCl proved to be a stabilizing factor for the CypHer5 molecule, which was protonated and had a chloride counterion. Spectrally, the residual HCl protonation maximized the fluorescent signal in the analysis of the oil since CypHer5 molecules were protonated. During the analysis of the CypHer5-PFPE **(14)** oil, residual HCl was not titrated fully by pH 8 buffer due to hydrophobicity of PFPE. However, during the formulation process, the residual HCl was titrated by alkaline PEI, and I/I_0 was ~0.1 at pH 8. The effect of the protonated PEI was seen later as enhanced cellular uptake of nanoemulsions **24-27**, evidenced by saturation of the dose curve at 1 mg/ml (HCl-deprotected) versus 5 mg/ml using TFA-deprotected reagents (Section S3.1).

S2.8.3 Fluorescence stability of PFPE-conjugates and nanoemulsions

For PFPE-conjugates prepared using TFA as the deprotecting reagent, Cy3-PFPE **(12)** fluorescence intensity decreased an average of 15.2% per month, while Cy5-PFPE **(13)** and CypHer5-PFPE **(14)** decreased by 5.0% and 2.3% per month. However, when HCl was used as the deprotecting reagent, fluorescence intensity decreases were an average of 1.4% and 1.9% per month for Cy3-PFPE **(12)** and CypHer5-PFPE **(14)**. Residual HCl preserved Cy3 fluorescence better than TFA, while little difference was seen in CypHer5 with either deprotecting agent. This may have been due to more efficient removal of residual TFA during Cy3-PFPE preparation, thus removing any residual acid for stabilization. Once formulated, single color nanoemulsions (**20-22)** showed 11.8%, 5.6% and 3.6% average loss of fluorescence intensity per month for Cy3-PFPE **(20)**, Cy5-PFPE **(21)** and CypHer5-PFPE **(22)** nanoemulsions, respectively, which were similar to the stability of the conjugates used to prepare them.

For single component ratiometric control formulation **28**, the 649 / 548 nm excitation ratio dropped 25% in 6 months at 4 ^{o}C. The presence of PFPE-amide (or its decomposition products) in the single component ratiometric control formulation may hasten loss of the dye, which is absent in the complete ratiometric formulations (**24-27**). In the absence of residual acid, and/or in the presence of increased alkalinity via PFPE-amide, Cy3-PFPE fluorophore **(29)** degraded faster than CypHer5-PFPE **(28)**, which is the opposite behavior of the corresponding water-soluble free acids and NBoc derivatives. This is likely due to charge and hydrophobicity effects of PFPE-conjugates and nanoemulsion formulations that make them much different from their precursors. Ultimately, in ratiometric nanoemulsions, it is CypHer5 decomposition due to heat that makes absolute fluorescence ratios decrease over time. Practical considerations of time-related decrease in absolute ratios are discussed further in Section S2.8.7.

S2.8.4 Buffer considerations

The choice of buffer composition can also affect ratio-pH calibration curves. High potassium MES and HEPES buffers[10]

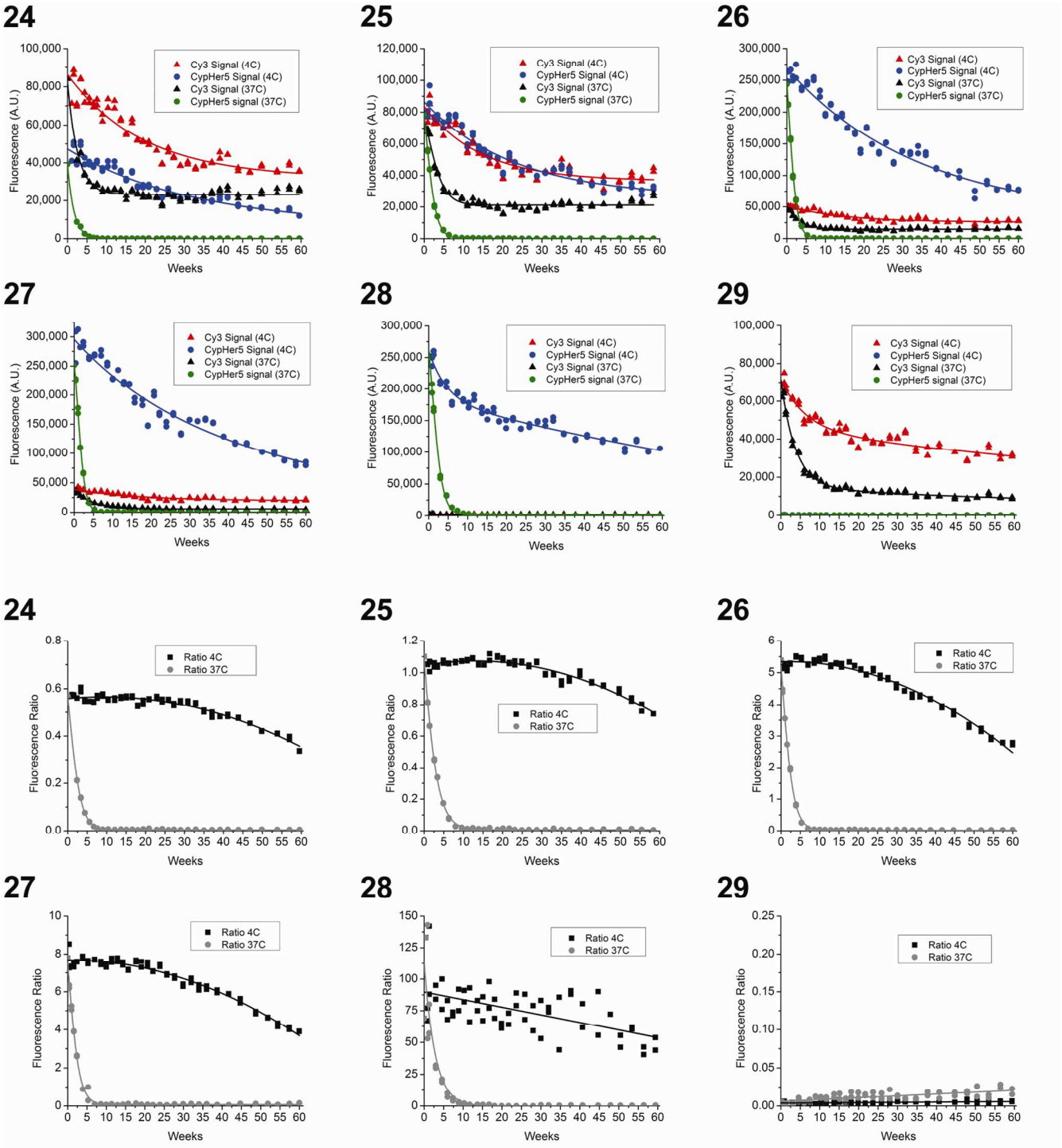


Figure S21. Long-term fluorescence of bulk nanoemulsions **24-29** stored at 4 °C and 37 °C, measured in pH 5.8 phosphate buffers. Top panel shows fluorescence signals of Cy3 (EX 548 nm) and CypHer5 (EX 649 nm). Bottom panel shows fluorescence ratios (EX 649 nm/EX 548 nm).

were used in some tests (MES for pH < 5.5, HEPES for pH>5.5), but it was discovered that at pH values below 5.5 the fluorescence signal of ratiometric nanoemulsions **(24-27)** at the Cy3 wavelength was *decreased*, which resulted in an increased 649 / 548 nm excitation ratio over the pH range. This jump in ratio caused the pK_a to be erroneously measured as 6.5. This discrepancy was traced to MES buffer, which decreased the fluorescence of Cy3. When the system was switched to a high potassium phosphate buffer (see Section S2.6), the Cy3 reference signal remained constant across the pH range 4-8, and thus produced a pK_a value of 6.8 for **24** and **26**.

S2.8.5 Fluorescent scan mode and ratiometric measurements

For a given ratiometric nanoemulsion, fluorescent ratios can be calculated from emission signals of CypHer5 / Cy3 generated by either synchronous fluorescence scans, emission scans or fixed wavelength scans. However, based on the acquisition

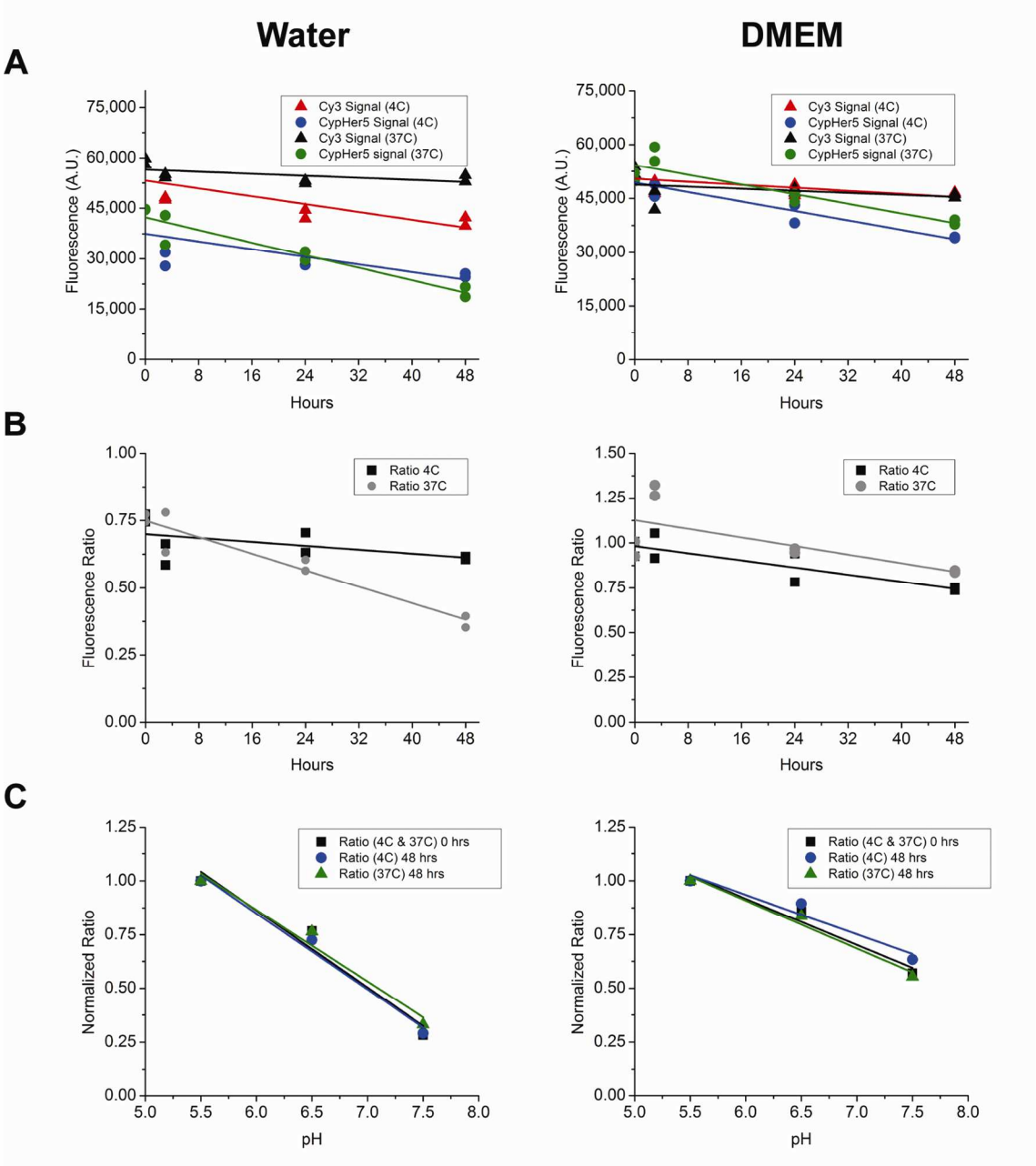


Figure S22. Short-term incubation of nanoemulsion **26** in water and DMEM. Panel **(A)** shows fluorescence signals at EM 664 (CypHer5) and EM 564 (Cy3). Panel **(B)** displays the ratio of CypHer5/Cy3 (EM 664 nm/EM 564 nm) in pH 5.5 high K+ HEPES buffer. **(C)** Shows **s**tability of ratio-pH curves over 48 hours, where data is normalized to the pH 5.5 values for each time-temperature measurement set.

method and detector gain settings, the absolute ratios calculated may differ significantly (Figures S19B and S19D). For pH measurement of any sample, the same conditions must be used to measure samples as were used to generate the ratio-pH calibration curve. When the data is normalized to I_0 for each scanning mode, the I/I_0 versus pH curves align (Figure S19E) and the pK_a values agree within 0.05 pH units between the scanning modes. Thus, the scanning mode can be selected to fit the experimental design parameters to yield accurate ratiometric measurement.

S2.8.6 Stability of ratiometric nanoemulsion signals *in situ*

The reliability of fluorescent signals for plate reader experiments was examined. Nanoemulsion **26** was diluted to 2% in phosphate buffers (pH 5.8-8.0), and read immediately using synchronous scans as described in Section S2.4.2. The same bulk samples were incubated at 37 °C for 4 hr, and at ambient temperature (25 °C) for 5 hr, and then re-measured. In both cases, the CypHer5 signals were 25 percent lower than initial readings (data not shown). When CypHer5/Cy3 ratios were normalized to I_0 at pH 5.8 for each sample set, the standard deviation of the normalized fluorescence ratios between all three conditions was 0.03, roughly corresponding to a 0.1 pH unit difference near the pK_a of 6.8. These observations provide a practical indication of the variation of ratiometric measurements using a fluorescent plate reader; they indicate signal robustness under typical experimental conditions and show the significance of using normalized data (discussed further in S2.8.7).

S2.8.7 Discussion of absolute and normalized fluorescence ratios in experiment design

As seen in the long-term and short-term fluorescence stability experiments, individual dye fluorescence and absolute fluorescence ratios decreased over time at 37 °C (Figures S21 and S22). However, when absolute ratios were normalized to the most acidic pH, the differences between the normalized ratio values 0 and 48 hr later were small (Figure S22C). This result demonstrates that normalization of ratio-pH calibration curves by I_0 can compensate for inherent changes in absolute ratios due to time and temperature, which is an important factor in ratiometric experiment design.

In ratiometric microscopy experiments designed by Barriere *et al.*[10], a normalized ratio-pH curve was generated once initially (normalized by I_0), and subsequent experimental data was normalized by a new I_0 value generated during the current experiment; then pH values were calculated from the original curve. Normalization of the signals keeps dye decomposition effects equal, as well as other factors such as instrument intensity variation. Alternatively, a full calibration curve could be generated during the current experiment. This would be useful if conditions (cell types, light sources, detectors, etc.) changed significantly since the original curve was developed. Generation of calibration curves can be costly in both time and resources. However, for simple experiments where dye stability is not deemed a significant factor (no extended incubation), absolute ratios can be used without normalization, should all other parameters remain the same, and pH values calculated directly from a calibration curve made from the absolute ratios. This offers a more direct processing method for simple experiments.

Ultimately, the stability of both dye signals is necessary to achieve accurate ratiometric measurements. For this reason, normalized data is used to compare measurements and performance under conditions where absolute ratios are likely change significantly, i.e. days. If in doubt, ratio-pH curves can be generated at the time of measurement, which will further ensure the reliability of pH values obtained using ratiometric nanoemulsion reagents.

S3 Biological evaluation

Four of the ratiometric nanoemulsions prepared in this study **(24-27)** were used to label 9L gliomal cells, which were then analyzed with three commonly used fluorescence platforms: fluorescent plate reader, fluorescence microscope and flow cytometer. Optimal CypHer5-Cy3 stoichiometries were determined for each platform.

S3.1 Labeling of 9L cells and determination of nanoemulsion uptake via ^{19}F NMR and fluorescence plate reader

Nanoemulsion uptake per cell was determined using ^{19}F NMR as described in Janjic.[4] 9L glioma cells (rat) were plated in 6-well plates, 2 x 10^6/well, and allowed to attach overnight. Immediately before cell labeling, nanoemulsion was diluted at concentrations ranging from 0 to 10 mg/ml in DMEM supplemented with 10% (v/v) FBS and 1% PS. Labeling medium was added to cells at 2 ml/well at an approximate density of $2x10^6$ cells/well. After 3 hr incubation at 37 ºC, cell labeling medium was removed, and cells were washed three times with 2 ml of phosphate buffered saline (PBS), detached by trypsinization, washed and resuspended in 1 ml of DMEM. A portion of the cell suspension (1/10) was used for cell number estimates by Cell Titer Glo (Promega, Madison, WI). Cell viability[4] was determined as the percentage of labeled cells versus unlabeled control cells (Figure S23A). The cells were pelleted and resuspended in 180 µl of deionized water, incubated at r.t. for 20 min, mixed with 200 µL 0.2 % (v/v) TFA solution in water to make lysate in 0.1% TFA. Fluorine content per cell was measured using ^{19}F NMR (Figure S23B). The optimal labeling dose for nanoemulsions **24-27** was 80% lower than that of nanoemulsions **20-22**. During the preparation of the PFPE-conjugate precursors used to make **24-27,** the deprotection of the NBoc groups was done using 3 M HCl instead of TFA. As a result of residual HCl in the PFPE conjugates, these nanoemulsions contained a higher level of protonated PEI upon formulation (Section S2.8.2). A higher level of protonated PEI resulted in greater nanoemulsion uptake during the 3 hr incubation period than nanoemulsions **20-22**.

For ratiometric nanoemulsions **24, 25, 26, 27,** 10% volume of cell lysate isolated above (40 µl) was transferred to a 96-well flat bottom transparent plate and further diluted with 0.1% TFA (60 µl). For a fluorescence calibration curve, standards were prepared by serial dilution of each nanoemulsion stock into 0.1% TFA ranging from 300.0 to 9.4 µg/ml. 100 µl of diluted standard was plated in duplicate. Plates were read using a Tecan Safire2 fluorescent plate reader using fixed wavelength scans (excitation/emission 530/564 nm and 630/664 nm for Cy3 and CypHer5, respectively) with 10 nm bandwidth. The detector gain was set for each wavelength separately using the labeled cell sample with the highest dose of nanoemulsion for each nanoemulsion evaluated. For each nanoemulsion, the same gain value was used for the cellular measurement and fluorescence standards. Unlabeled cells were treated in the same way and used as controls. The amount of nanoemulsion (ng/cell) taken up by the cells was calculated for each wavelength from the linear fluorescence calibration based on the fluorescence of labeled cell samples, divided by cell count. Unlabeled cells were treated likewise, and the ng/cell value subtracted from the labeled cells as background.

^{19}F NMR and fluorescence uptake data were compared following background subtraction and normalization to maximum dose. First, the value of ^{19}F/cell of the unlabeled cells was subtracted from the value of ^{19}F signal/cell of labeled cells as background (attributed to signal noise). Then, for each nanoemulsion, the ^{19}F/cell (as determined by NMR) and the ng/cell (as determined by fluorescence) were normalized to the corresponding maximum dose value of each and plotted for comparison (Figures 6 and S24).

S3.2 Fluorescence Microscopy Evaluation

S3.2.1 Labeling of 9L cells with CypHer5-PFPE nanoemulsion for fluorescence microscopic imaging

To evaluate long-term detection of our nanoemulsions and determine the intracellular location of the cyanine-PFPE during labeling, rat 9L glioma cells with green fluorescent protein (GFP)-expressing cytoplasm were co-incubated attached with CypHer5-PFPE nanoemulsion **(22)** at 5 mg/ml for 3 hr. Cells were washed with PBS obtained from Gibco, detached with trypsin, washed with DMEM and incubated attached to poly-

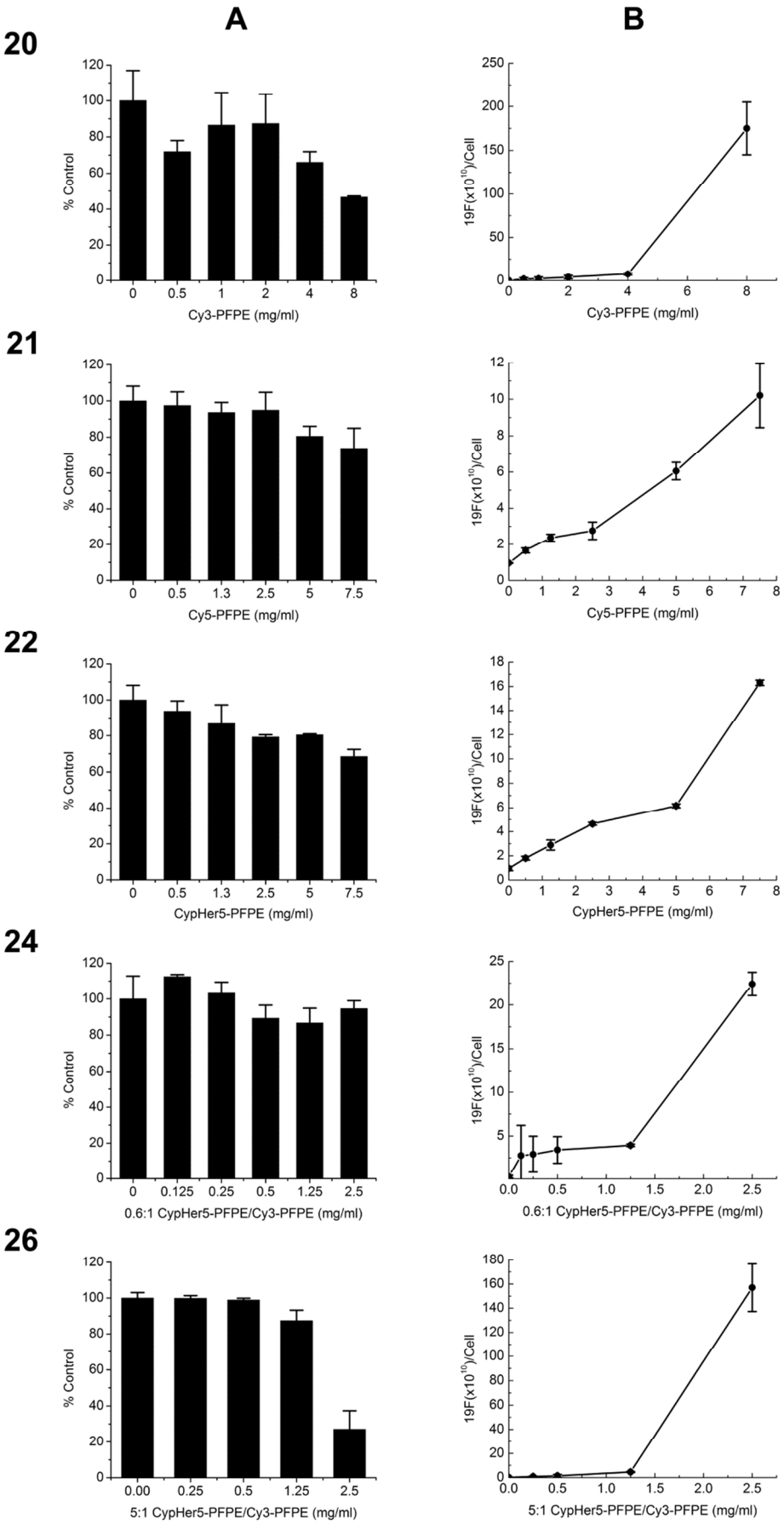


Figure S23. Cell viability **(A)** and ^{19}F uptake per cell **(B)** for nanoemulsions **20-22, 24** and **26.**

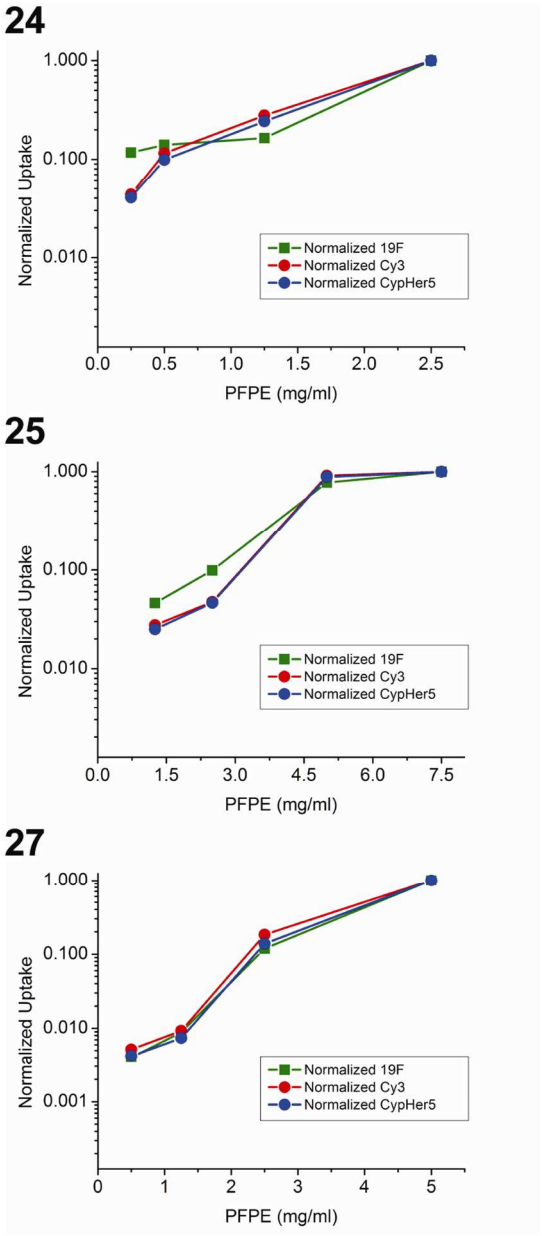


Figure S24. Nanoemulsion uptake as measured by ^{19}F and fluorescence. 9L cells were labeled with ratiometric nanoemulsions **24, 25** and **27.** Uptake values were normalized to the highest dose. Cells were lysed for measurements in 0.1% TFA.

D-lysine confocal microscopy culture dishes (MatTek P35GC-1.0-14-C, MatTek Corp., Ashland, MA). Confocal microscopy imaging was performed 25 hr after labeling. Confocal microscopy images (512x512 pixels) were acquired on a Zeiss LSM 510 Meta UV DuoScan inverted spectral confocal microscope using a 40x oil immersion objective (Carl Zeiss Microscopy, LLC., Thornwood, NY). A single scan of 1.27 µs per pixel was employed. GFP-expressing cytoplasm was excited at 488 nm with an argon laser at 4% power (1.2 mW) and emission monitored in a 505-550 nm window; CypHer5 was excited at 633 nm at 7% power (2.1 mW) and emission monitored in a 657-711 nm window. As shown in Figure S25 and in Movie S1, cyanine-PFPE nanoemulsions are clearly localized in the cytoplasm of cells labeled with CypHer5-PFPE nanoemulsion.

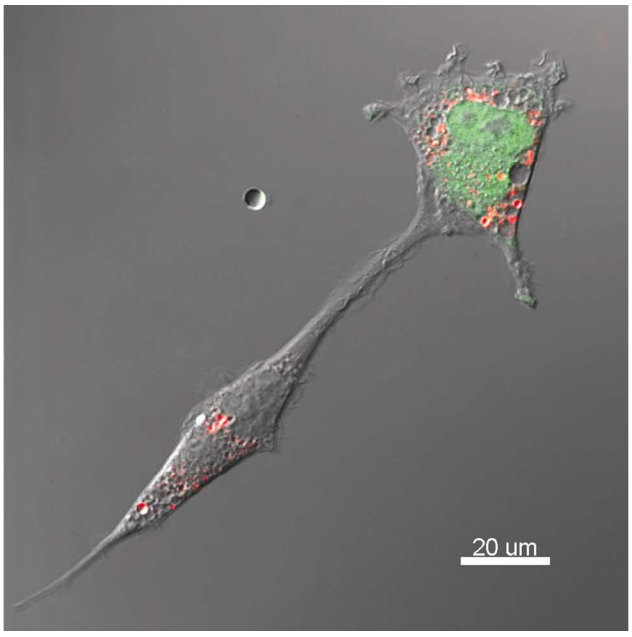


Figure S25. Orientation image corresponding to Movie S1. 9L cells expressing GFP show uptake of CypHer5-PFPE nanoemulsion **(22)** 25 hours after labeling. Three hours after the addition of nanoemulsion, cells were detached and re-plated onto poly-D-lysine coated dishes and incubated 24 hrs prior to imaging. The detection channels where GFP (505-550 nm) **(green)**, CypHer5 (657-711 nm) **(red)**, and DIC **(gray).** See Movie S1 for three-dimensional rendering of z-stack images.

S3.2.2 Labeling of 9L cells with ratiometric nanoemulsions for microscopic ratiometric imaging

Adherent 9L cells were co-incubated with ratiometric nanoemulsions **(24-27)** at 1 mg/ml for 3 hr as described in S3.1. Cells were washed with PBS to remove free nanoemulsion, and DMEM (without Phenol Red indicator) added for imaging. An Andor Revolution XD spinning disk confocal microscope (Andor Technology USA, South Windsor, CT) was used for preliminary evaluation of ratiometric quantification. Confocal images were obtained (512x512) using a 60x oil objective using sequential scanning with 560 and 640 nm laser excitation and emission filters 607±36 nm and 685±70nm for Cy3 and CypHer5, respectively. For further discussion, please see Section S3.4.

S3.3 Flow cytometry evaluation

9L cells were plated in 6-well plates, $5x10^5$/well and incubated. Immediately before cell labeling, nanoemulsions were diluted in DMEM supplemented with 10% (v/v) FBS and 1% PS at 5 mg/ml. Labeling medium was added to cells (~$2x10^6$ cells) at 2 ml/well at the following concentrations: ratiometric nanoemulsions **(24-27)** at 1 mg/ml each, single component ratiometric control nanoemulsions **(28, 29)** at 2.5 mg/ml and non-fluorescent control **(23)** and single color nanoemulsions **(20-22)** at 5 mg/ml. After 3 hr, cells were washed with DMEM, detached with trypsin, followed by centrifugation. The pellet was re-suspended in PBS to approximately 500,000 cells/ml. Fluorescence Activated Cell Sorting (FACS) analysis was performed immediately using a BD FACSVantage SE (BD Biosciences, San Jose, CA, USA) flow cytometer. Primary excitation laser was 488 nm (60 mW), Cy3 excitation 532

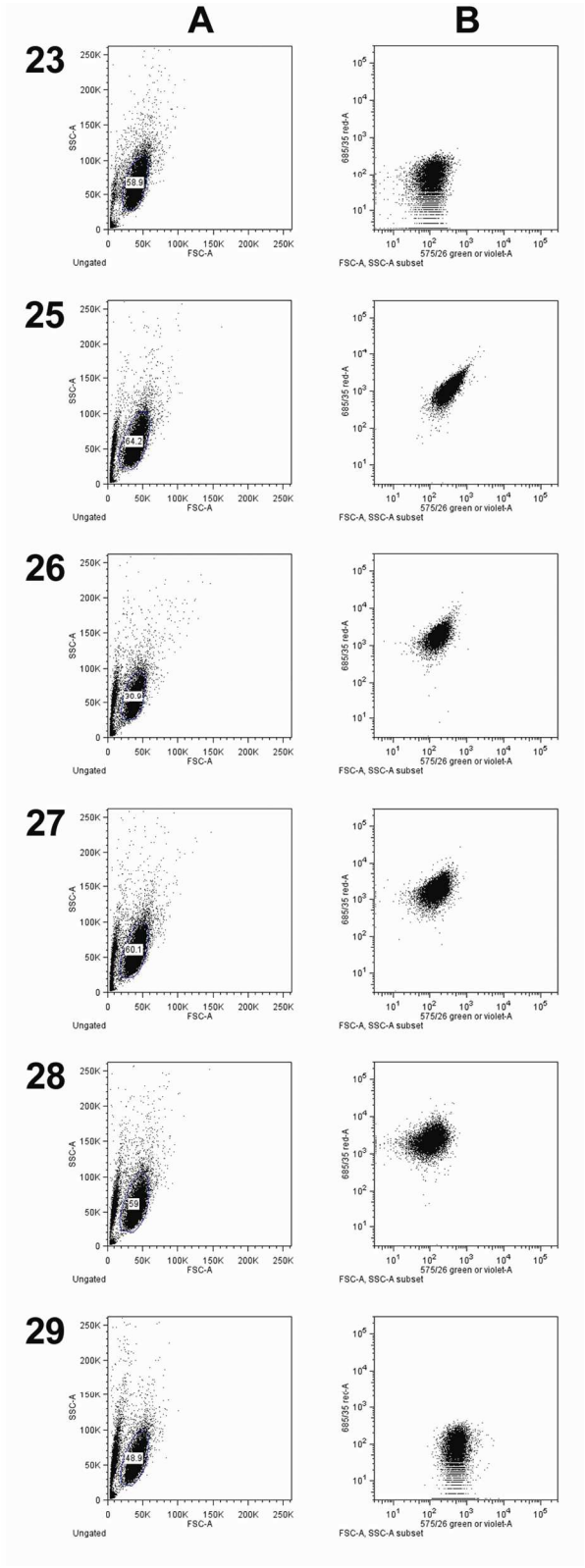


Figure S26. Flow cytometry of 9L cells labeled with nanoemulsions. FSC/SSC plots **(A)** and dot plots at 575 nm versus 685 nm **(B)** of 9L cells labeled with nanoemulsions **23** and **25**-**29**; gated regions are marked by thin blue lines in (**A).** Plots are of live, gated cells. This is the complete data used to generate Figure 9.

nm (100 mW) and Cy5/CypHer5 excitation 635 nm (30 mW). Emission detection was performed with a Cy3 filter (575/26 nm) and a Cy5/CypHer5 filter (685/35 nm). Processing was performed using FlowJo analysis software (Treestar, Inc. Ashland, OR).

Cell labeling was compared for ratiometric **(24-27)** and single-component ratiometric control nanoemulsions **(28-29)** against cells labeled with non-fluorescent nanoemulsion **23** (Figure 8). Fluorescence ratios (emission 685 nm / 575 nm) were calculated using mean fluorescence obtained from the histograms. FACS images are of live, gated cells. Cell labeling with nanoemulsion is 100%. Forward Scatter / Side Scatter (FSC/SSC), and dot plots of the data that were used to make Figures 8C-D and S27 are shown in Figure S26A. Dot plots were generated using auto-gating function on live cells (Figure 26B). The horizontal axis shows distribution of cells by Cy3 orange fluorescence; and the vertical axis depicts distribution of cells by red fluorescence (CypHer5). Histograms (Figure S27) were generated from dot-plots using gating on live cells.

S3.4 Discussion of GFP modification and ratiometric imaging.

9L cells with cytoplasmic GFP were used to show cellular location of PFPE labeling (Movie S1); however, for evaluation of ratiometric reagents, images shown in Figure 7 were obtained using 9L cells without GFP modification. GFP signals may interfere with Cy3 signal detection by making the cellular auto-fluorescence background substantially higher.

Initial evaluation of ratiometric nanoemulsions **(24-27)** was performed using a Zeiss DuoScan confocal microscope, but due to delays in sequential acquisition of the two channels, labeled cellular compartments did not co-localize completely during analysis due to nanometer scale intracellular motions within the acquisition time period. Co-localization of labeled compartments in both channels is a fundamental requirement of ratiometric imaging. The spinning disk confocal system had much faster acquisition rates (Figure 7), thus allowing ratiometric comparison of intracellular compartments to be made with sufficient co-localization of signals.

S4 Measurement of Intracellular pH by flow cytometry

S4.1 Cell labeling procedures for ratiometric pH calibration curve

9L cells (~1x10^6 cells) were incubated at 37 ^{o}C with nanoemulsion **24** at 1 mg/ml, and control cells with non-fluorescent nanoemulsion **23** at 5 mg/ml. After 3 hr, cells were washed with PBS to remove free nanoemulsion, detached with trypsin and pelleted by centrifugation. The pellets were re-suspended to make cell density ~1x10^6 cells/ ml in PBS. Cells were then resuspended in high potassium phosphate buffers (Section S2.6) containing 10 μM pH clamp reagents nigericin and monensin (Sigma-Alrich). FACS was performed within 5-30 min of exposure to pH clamp reagents[10] using BD FACSVantage SE FACS flow cytometer. The primary excitation laser was 488 nm (60 mW), Cy3 was excited at 532 nm (100 mW) and Cy5/CypHer5 was excited at 635 nm (30 mW). Emission detection was performed with a Cy3 filter (575/26 nm) and Cy5/CypHer5 filter (685/35 nm). FACS analysis was also performed on cells labeled with nanoemulsion **23**, resus-

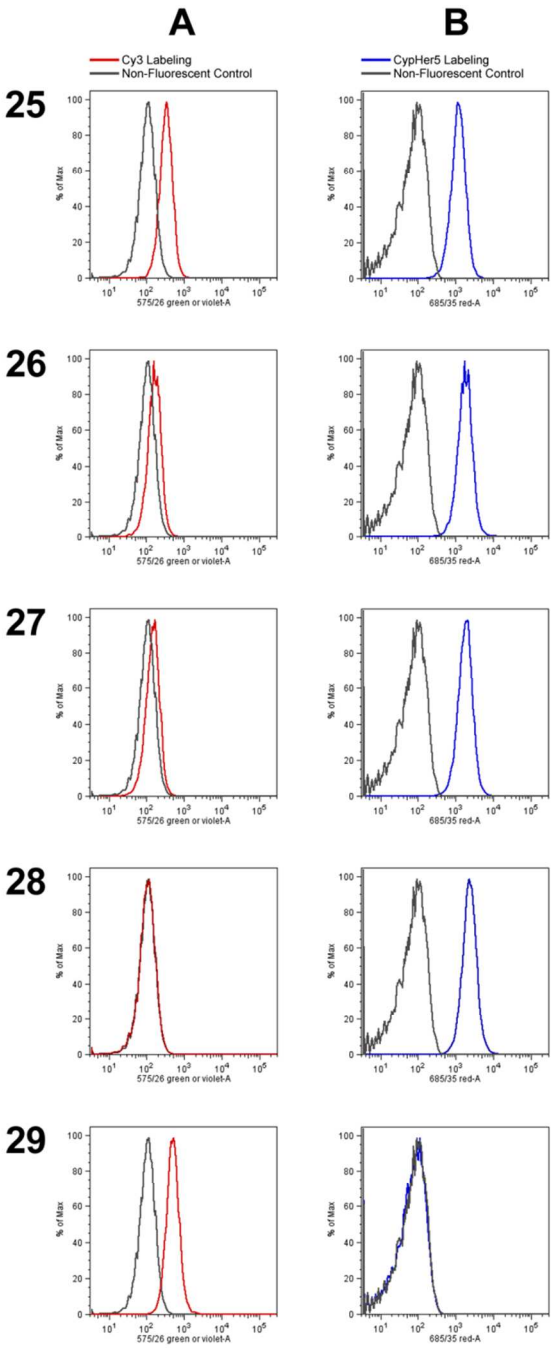


Figure S27. Flow cytometry histograms of 9L cells labeled with ratiometric nanoemulsions **25-29**. Here, Cy3 at 575 nm is shown in panel **(A)** and CypHer5 at 685 nm is shown in **(B).** Results are of live, gated cells, overlaid onto control cells labeled with non-fluorescent nanoemulsion **23**. This is the complete data used to generate Figure 9.

pended in PBS without ionophores (see Section S4.2). The FlowJo analysis software was used for data analysis. Dot plots and mean fluorescence histograms of 575/26 nm and 685/35 nm filters were generated using gating on live cells for all samples (Figure S28). Figure S30A shows the mean fluorescence of Cy3 and CypHer5 flow cytometry channels after labeled cells were exposed to pH clamp reagents and adjusted to extracellular pH. A ratio-pH calibration curve was generated (see main article, Section 4.2) and raw data was normalized to the fluorescence ratio (685nm/575nm) of the mean histograms at pH 5.0. For auto-fluorescence corrected curves, mean fluorescence values of histograms from cells labeled with **23** were subtracted from corresponding mean fluorescence values of histograms from cells labeled with **24,** and corrected the 685 nm/575 nm ratios were calculated. A ratio-pH calibration curve was generated and, for corrected data, was normalized by the fluorescence ratio of corrected mean histograms at pH 5.0 (Figure 10A).

S4.2 Cell labeling procedures for ratiometric nanoemulsion uptake study

To monitor the pH of Cy3/CypHer5-labeled cells during nanoemulsion uptake, 9L cells were labeled with nanoemulsion **24** from 0.5 to 3 hr. Cells were washed with PBS to remove free nanoemulsion and then cells were detached with trypsin and pelleted by centrifugation. The pellets were resuspended in PBS to a cell density of ~$1x10^6$ cells/ml. Flow cytometry was performed immediately using the same acquisition parameters as the pH calibration curve and within 1-2 hr of that experiment (Section S4.1).

Data processing was performed as described in Section S4.1. Dot plots and histograms of data acquired using 575/26 nm and 685/35 nm filters were generated using gating on live cells for all samples (Figure S29). Figure S30B shows the mean fluorescence of Cy3 and CypHer5 flow cytometry channels during nanoemulsion uptake. Fluorescence ratios (685 / 575 nm) were calculated from mean fluorescence value of histograms. For raw data, ratios were normalized by 0.791, the same value used to normalize the calibration curve. For autofluorescence corrected curves, mean fluorescence values of histograms from cells labeled with **23** were subtracted from corresponding mean fluorescence values of cells labeled with **24.** Corrected ratios (685 nm / 575 nm) were calculated and normalized by 1.19, the normalization factor used with the corrected calibration curve in Section S4.1.

Intracellular pH was quantified using the calculated fluorescence ratios and comparing the value to the calibration curve. If ratios were calculated after correction for cell autofluorescence, the calibration curve obtained using autofluorescence-corrected data was used (Figure 10B).

S4.3 Further discussion of intracellular pH measurement

Absolute ratio values (non-normalized) can be used to generate the ratio-pH calibration curves and calculate pH measurements from corresponding non-normalized data, but like-data must be used. The exact same pH values are obtained when the absolute and normalized data are fitted to a Boltzmann sigmoidal curve, $y = A2 + (A1-A2)/(1 + \exp((x-x_0)/dx))$. This is because the inflection point (center x_0) and the rate (dx width) are identical for absolute and normalized curves. Normalization merely changes the initial (A1) and final (A2) y values of the curve, which are exactly proportional to the normalization factor.

The intracellular pH changed from ~6.7 to ~5.5 during the 3 hr labeling experiment (Figure 10B) whether raw or corrected data was used, but in the interim, corrected pH values were 0.4 pH units lower (more acidic) than raw data. The differences in values between methods were most significant between pH 5.5-6.5 which is most likely due to the differences in pK_a values of the corresponding curves and the contribution of autofluorescence. Since autofluorescence is primarily in the

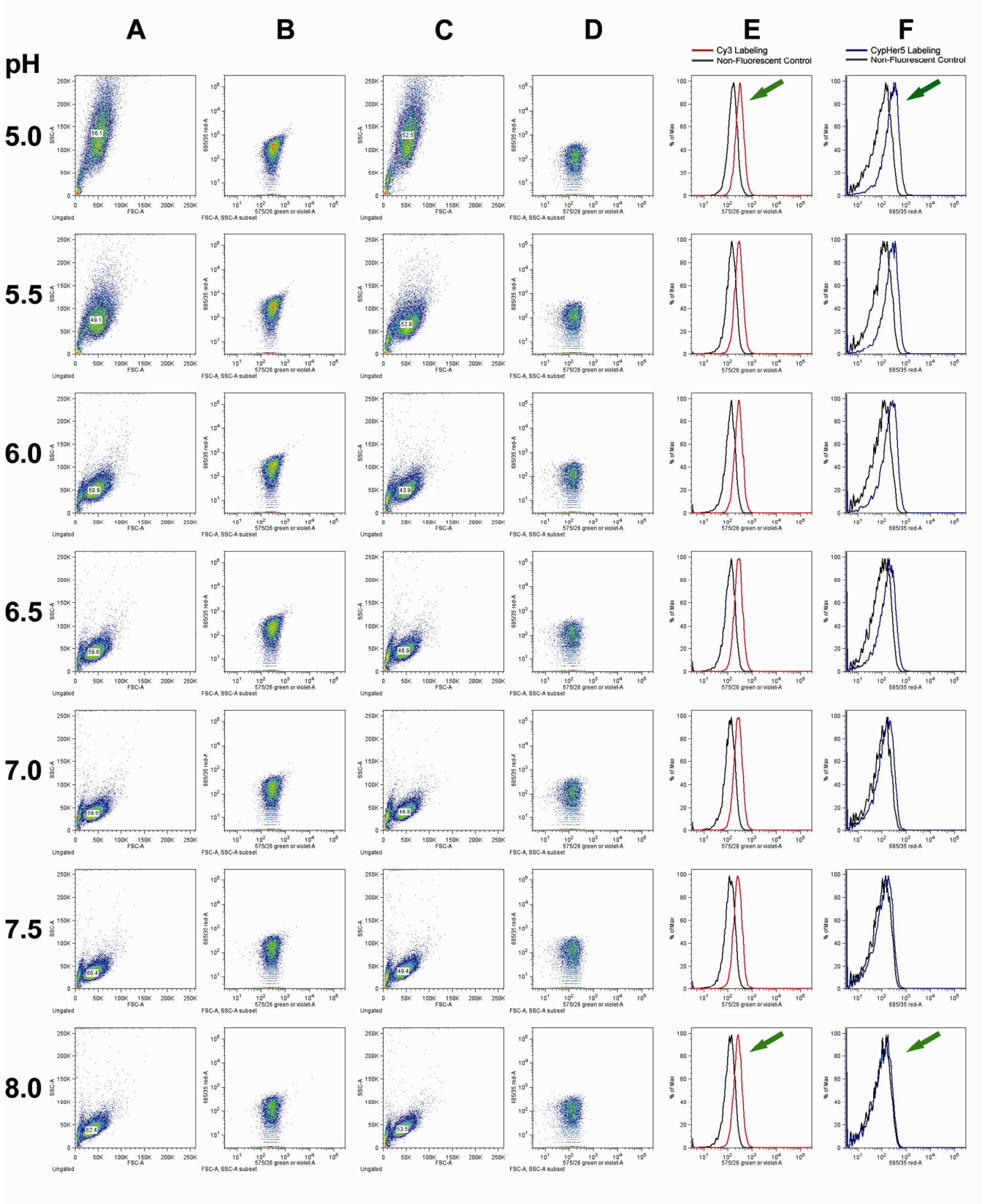


Figure 28. Complete flow cytometry data used to generate Figure 10A, the ratio-pH calibration curve. Here, cells exposed to pH clamp reagents and adjusted to extracellular pH are shown. FSC/SSC plots of 9L cells labeled with ratiometric nanoemulsion **24** are shown in **(A),** and non-fluorescent control **23** is shown in **(C)**. Dot plots (575 nm versus 685 nm) of cells labeled with **24** and **23** are shown in **(C)** and **(D)**, respectively. Histograms of live gated 9L cells labeled with **24** showing Cy3 at 575 nm is displayed in panel **(E)** and CypHer5 at 685 nm is shown in **(F)** and control cells labeled with **23**. Green arrows indicate key distinctions in data.

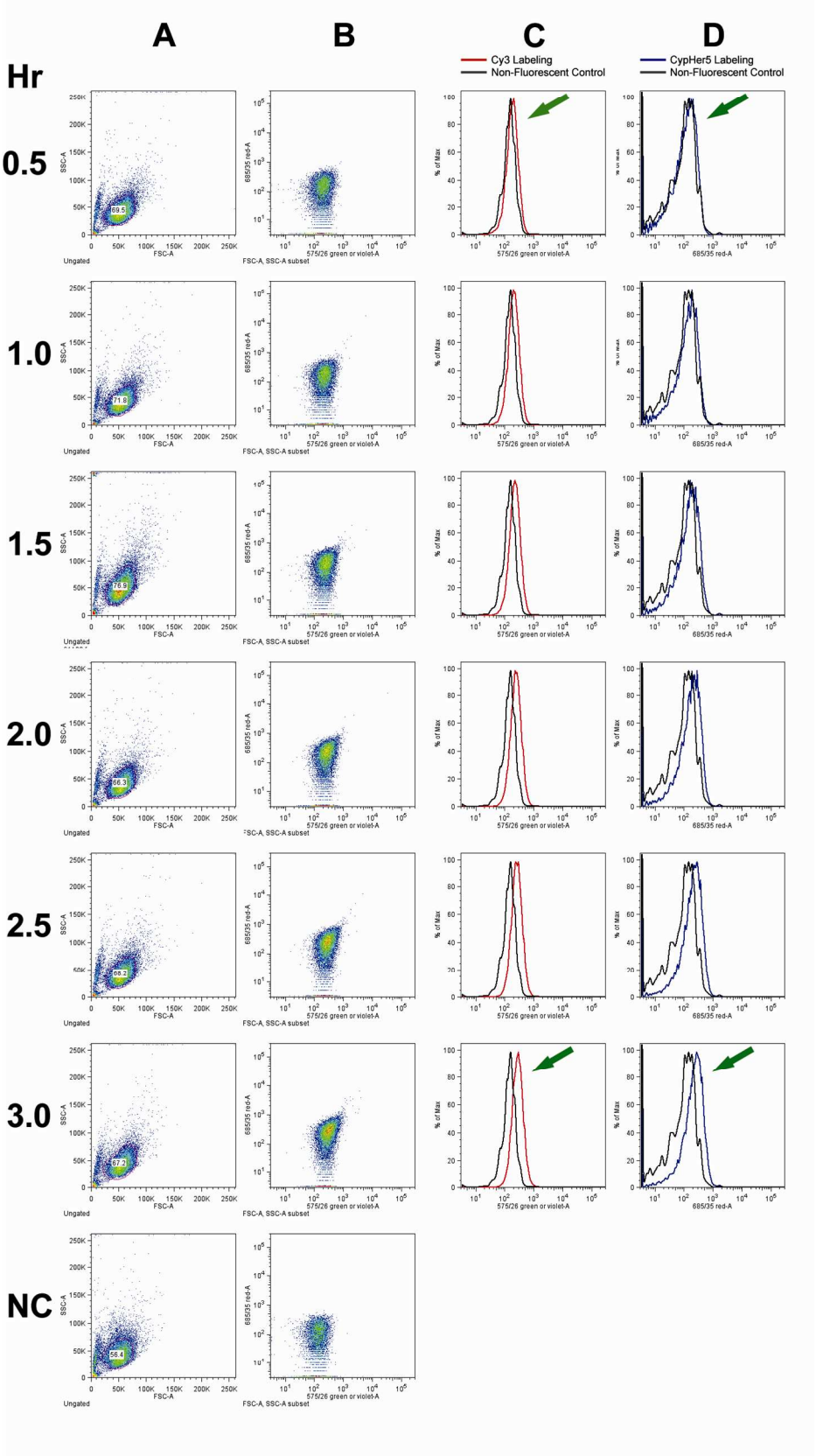


Figure S29. Complete flow cytometry data used to generate Figure 10B, which shows intracellular pH during nanoemulsion uptake. Shown are FSC/SSC **(A)** and dot plots (575 nm versus 685 nm) **(B)** of 9L cells labeled with ratiometric nanoemulsion **24** and non-fluorescent control **23** (identified as **NC**). Corresponding histograms of live gated 9L cells labeled with **24** showing Cy3 at 575 nm **(C)** and CypHer5 at 685 nm **(D)** and control cells labeled with **23**. Green arrows indicate key distinctions in data.

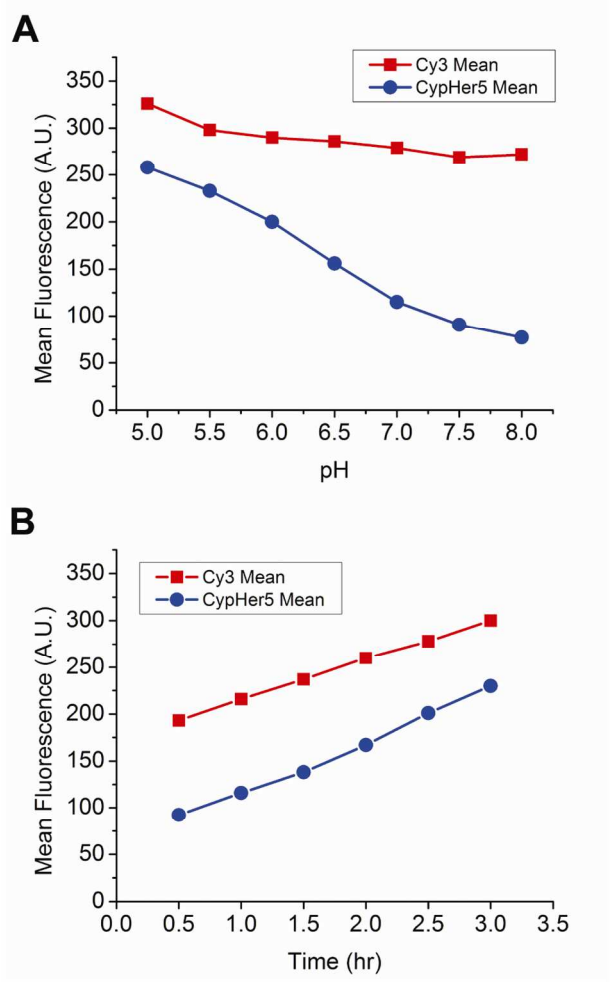


Figure S30. Mean fluorescence of Cy3 (575 nm) and CypHer5 (685 nm) flow cytometry channels using 9L cells labeled with ratiometric nanoemulsion **25**. In Panel **A**, cells were exposed to pH clamp reagents and adjusted to extracellular pH. In panel **B**, fluorescence was monitored during nanoemulsion uptake. Results are of live, gated cells. The mean fluorescence values were used to calculate the results shown in Figure 10.

Cy3 channel, uncorrected data would have a higher fluorescence ratio than corrected data, which ultimately would result in a higher calculated pH value, even when using a ratio-pH curve generated from uncorrected raw data. This is because autofluorescence varies with pH.

ASSOCIATED CONTENT

Movie S1, showing that cyanine-PFPE nanoemulsions are localized in the cytoplasm of cells labeled with CypHer5-PFPE nanoemulsion, is available as a media file.

MOVIE S1. 3D reconstruction of confocal microscopy images showing CypHer5-PFPE **(22)** labeled vesicles.

ABBREVIATIONS

AcN, acetonitrile; DEA, diethylamine; DIPEA, diisopropylethylamine; DMEM, Dubelco's modified Eagle medium; DMF, dimethylformamide; EtOAc, ethyl acetate; EtOH, ethanol; FACS, fluorescence activated cell sorting; FSC/SSC, forward scatter / side scatter; FBS, fetal bovine serum; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high performance liquid chromatography; I/I_0, fractional fluorescence; MeOH, methanol; MES, 2-(N-morpholino)ethanesulfonic acid; OSu, succinimidyl ester; PBS, phosphate buffered saline; PEI, polyethyleneimine; PFC, perfluorocarbon; PFPE, perfluoropolyether; PS, penicillin streptomycin; TEA, triethylamine; TFA, trifluoroacetic acid; TSTU, N,N,N′,N′-Tetramethyl-O-(N-succinimidyl)uronium tetrafluoroborate.

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