A Perfluoroaryl-Cysteine S_NAr Chemistry Approach to Unprotected Peptide Stapling

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Supporting Information

1. General Considerations.

Hexafluorobenzene and decafluorobiphenyl were purchased from Oakwood Chemical and used as received. 2-(1H-Benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and α-Boc\Benzyl protected L-amino acids (Chem-Impex International, USA or Peptide International, USA). MBHA resin was obtained from Anaspec, USA. N,N-Dimethylformamide (DMF), dichloromethane (DCM), diethyl ether, and HPLC-grade acetonitrile were purchased from VWR International. Trifluoroacetic acid (TFA) was purchased from NuGenTec, USA or Halocarbon, USA. All other reagents were purchased from Sigma-Aldrich unless otherwise noted and used as received. HER-2 was bought from R&D Systems, Inc., Minneapolis, MN (catalog number: 1129-ER-050; lot number: FXR0711121) and reconstituted at 100 μg/mL concentration in PBS buffer.

All reactions with **1** were carried out under the atmosphere of dry argon gas. All reactions with peptides were conducted under ambient conditions. Peptides **4** and **5** were synthesized on a 0.4 mmol scale using Fmoc-Rink-MBHA resin using manual Fmoc/t-Butyl SPPS. Peptides **6**, **7** and **8** were synthesized using MBHA resin support by manual *in situ* neutralization Boc/Benzyl SPPS. Each amino acid coupling was performed in the presence of HBTU reagent (coupling time – 12-15 min). In the case of Boc chemistry, final resins were washed with DCM, dried in air and simultaneously cleaved and side-chain deprotected by treatment with 10% (v/v) *p*-thiocresol and 10% (v/v) p-cresol in anhydrous HF for 1 hr at 0 ^{o}C. Resulting crude peptide material was triturated with chilled diethyl ether, dissolved in 50:50 (MeCN:H_2O) mixture containing 0.1% TFA and subsequently lyophilized. Labeling of peptides with FITC was performed on the resin bound protected peptides by treating the protected peptide resin with the solution of fluorescein isothiocyanate (isomer I) (Sigma-Aldrich, 1.2 eq) dissolved in 2:1:1 mixture of pyridine/CH_2Cl_2/DMF, overnight. In all cases resulting crude peptide material was purified on preparative RP-HPLC (Agilent Zorbax SB C_{18} column: 21.2 x 250 mm). HPLC fractions

containing only product material (screened by MALDI) were combined and lyophilized. NMR spectra were acquired using Bruker Avance III spectrometer equipped with an autoswitchable probe and processed using TopSpin 3.1 software package. LC-MS spectra were acquired using Agilent 6520 ESI-QTOF mass-spectrometer equipped with C_3 and C_{18} Zorbax columns. Spectra were processed using Agilent Mass Hunter software package. Deconvoluted mass spectra were obtained using maximum entropy setting.

2. Synthetic Procedures.

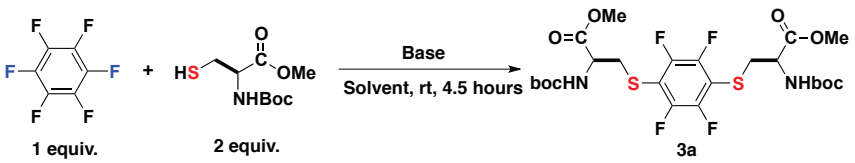
A. Representative Synthesis of **3a**.

A mixture of hexafluorobenzene (280 mg, 1.5 mmol) and *N*-(*tert*-Butoxycarbonyl)-L-cysteine methyl ester (706 mg, 3 mmol) and Na_3PO_4 (600 mg, 3.7 mmol) was magnetically stirred in 15 mL of dry acetonitrile for 4.5 h at room temperature under an atmosphere of dry argon. The resulting mixture was filtered through a pad of celite on a glass-fritted filter, evaporated *in vacuo* and subsequently purified on a silica gel column (product elutes with 4:1 hexanes/EtOAc solvent mixture). Obtained fractions containing product were combined and dried in vacuo to afford the title compound **3a** as an off-white solid (760 g, 82%). ^{1}H NMR (400.1 MHz, $CDCl_3$): δ 5.47 (m, 2H), 4.48 (m, 2H), 3.68 (s, 3H), 3.61 (s, 3H), 3.41 (m, 1), 3.24 (m, 1), 2.88 (m, 2), 1.36 (s, 6), 1.31 (s, 6); ^{13}C{^{1}H} NMR (100.6 MHz, $CDCl_3$, 24 °C): δ 170.8 (s), 170.3 (s), 155.1 (s), 154.7 (s), 147.9 (bm), 145.5 (bm), 114.1 (s), 80.1 (s), 80.0 (s), 54.9 (s), 53.7 (s), 52.6 (s), 36.3 (s), 28.2 (s), 28.0 (s), 27.1 (s); ^{19}F{^{1}H} NMR (376.4 MHz, $CDCl_3$): δ –132.6 (bs, 4F). LC-MS: m/z calcd for $[M+Na]^+$: 639.1434, found: 639.1473.

B. Representative Synthesis of **3b**.

This compound was synthesized and isolated in a procedure analogous to the one used for **3a**. (850 mg, 74%). ^{1}H NMR (400.1 MHz, $CDCl_3$): δ 5.45 (m, 2H), 4.61 (m, 2H), 3.68 (s, 6H), 3.5 (bm, 4H), 1.40 (s, 12H); ^{13}C{^{1}H} NMR (100.6 MHz, $CDCl_3$, 24 °C): δ 170.3 (s), 154.8 (s), 148.2 (d), 145.8 (d), 145.1 (d), 142.6 (d), 116.5 (m), 106.9 (m), 80.4 (s), 53.6 (s), 52.6 (s), 52.6 (s), 36.4 (s), 28.1 (s); ^{19}F{^{1}H} NMR (376.4 MHz, $CDCl_3$): δ –131.8 (bs, 4F), –137.6 (bs, 4F). LC-MS:

m/z calcd for $[M+NH_4^+]^+$: 782.1816, found: 782.1797.



Solvent	Base (2 equiv)	Yield (^{19}F NMR)
DMF	NEt_3	>95%
MeCN	NEt_3	>95%
MeOH	NEt_3	NR

Solvent	Base (2 equiv)	Yield (^{19}F NMR)
DMF	Na_3PO_4	>95%
MeCN	Na_3PO_4	>95%
DMSO	Na_3PO_4	>95%
DMF	TRIS	>95%

Table SI-1. Solvent optimization for model cysteine perfluoroarylation. Conversion and yields were estimated by *in situ* ^{19}F NMR spectroscopy.

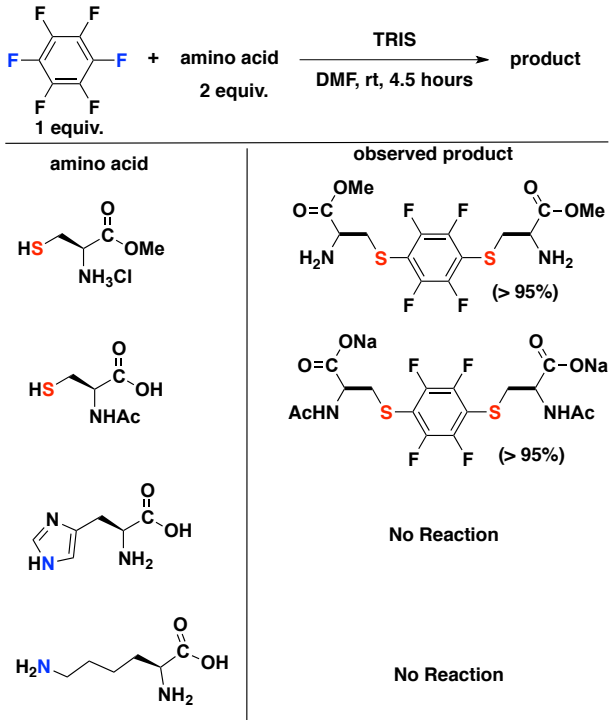


Table SI-2. Functional group tolerance screen. Conversion and yields were estimated by *in situ* ^{19}F NMR spectroscopy.

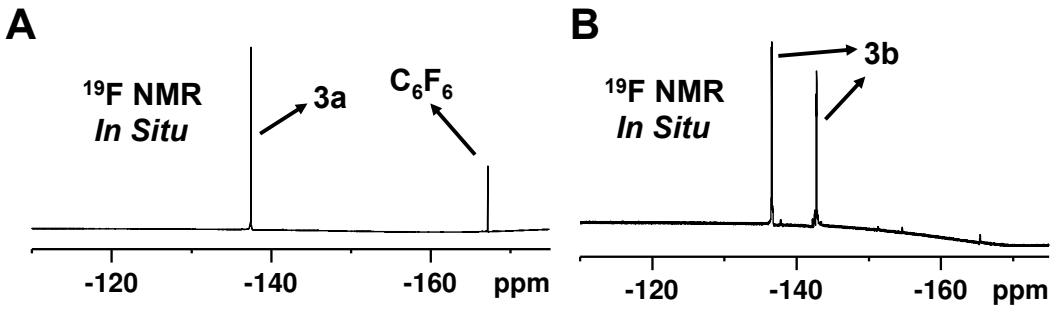


Figure SI-1. Representative *in situ* ^{19}F NMR spectra of model cysteine **1** perfluoroarylation with **2a** and **2b**.

D. Representative protocol for peptide stapling with **2a**.

To a solid sample of peptide (7.5 μmoles) in a plastic Eppendorf tube was added 1.9 mL of 100 μM solution (~ 25 equiv.) of hexafluorobenzene in DMF and 1.5 mL of 50 mM solution of TRIS base in DMF. The tube was vigorously mixed on a shaker for 30 seconds and left at room temperature for 4.5 hours. Reaction mixture with peptides **4** and **5** were characterized by LC-MS. Resulting mixture from the reactions of peptides **6** and **7** was diluted with 6 mL of 0.1% TFA solution in water and subjected to purification on HPLC. Fractions containing stapled peptide product (analyzed by LC-MS) were combined and lyophilized. **6a** and **7a** were isolated in 73% and 69% yields respectively.

E. Representative protocol for peptide stapling with **2b**.

To a solid sample of peptide (7.5 μmoles) in a plastic Eppendorf tube dissolved in 3 mL of DMF was added 0.15 mL of 100 μM solution (~ 2 equiv.) of decafluorobiphenyl **2b** dissolved in DMF and 1.5 mL of 50 mM solution of TRIS base in DMF. The tube was vigorously mixed on a shaker for 30 seconds and left at room temperature for 4.5 hours. Reaction mixture with peptides **4** and **5** were characterized by LC-MS. Resulting mixture from the reactions of peptides **6** and **7** was diluted with 6 mL of 0.1% TFA solution in water and subjected to purification on HPLC. Fractions containing stapled peptide product (analyzed by LC-MS) were combined and lyophilized. **6b** and **7b** were isolated in 68% and 65% yields respectively.

F. Syntheses of **8a** and **8b**.

8a and **8b** were synthesized from **8** similarly to the procedure used to prepare **7a** and **7b** using 10 equiv. of perfluoroaryl reagent **2a** and **2b** respectively. All starting material was consumed within 4 hours of the reaction time. Resulting products were purified via HPLC and analyzed using LC-MS (*vide supra*).

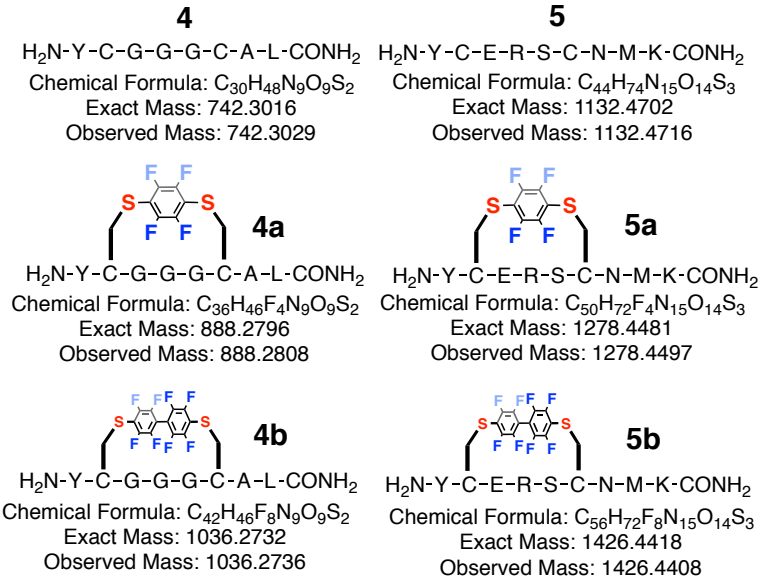


Figure SI-2. Model peptides utilized in the development of i, i+4 stapling with **2a** and **2b**. Calculated masses correspond to $[M+H]^+$ species observed directly via LC-MS.

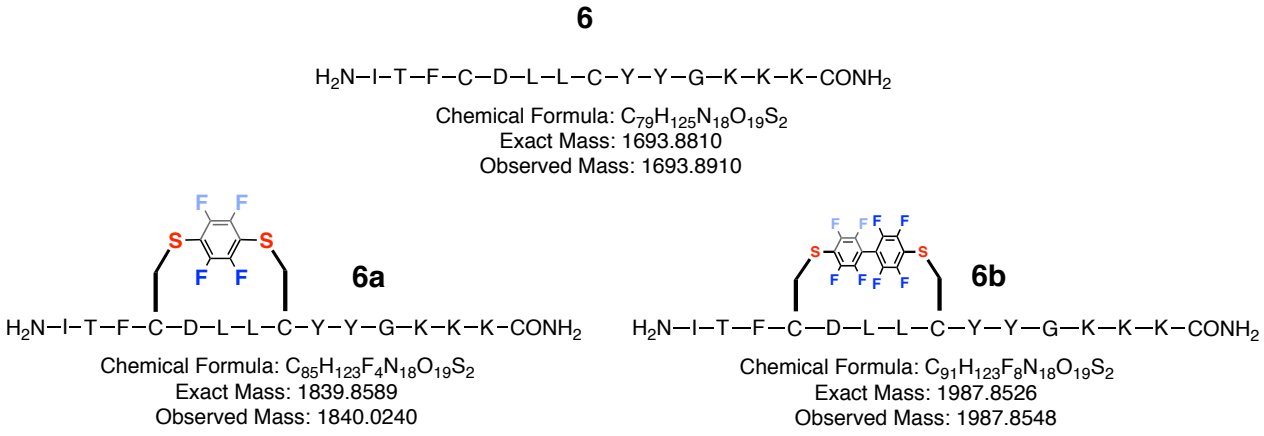


Figure SI-3. Peptides **6**, **6a**,**b**. Calculated masses correspond to $[M+H]^+$ species observed directly via LC-MS.

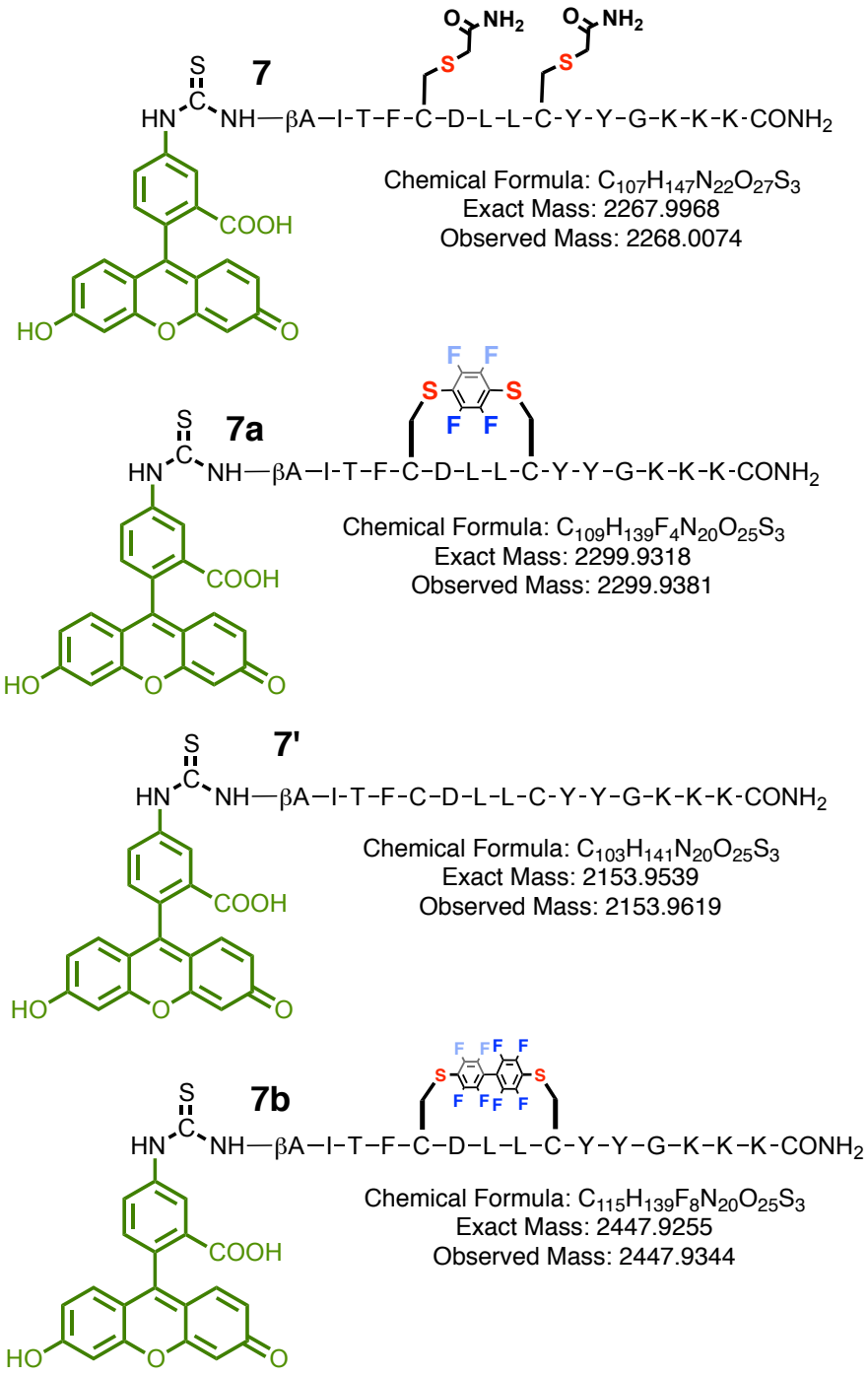


Figure SI-4. Peptides **7**, **7' 7a**,**b**. Calculated masses correspond to $[M+H]^{+}$ species extrapolated from the observed $[M+2H]^{+2}$ via LC-MS.

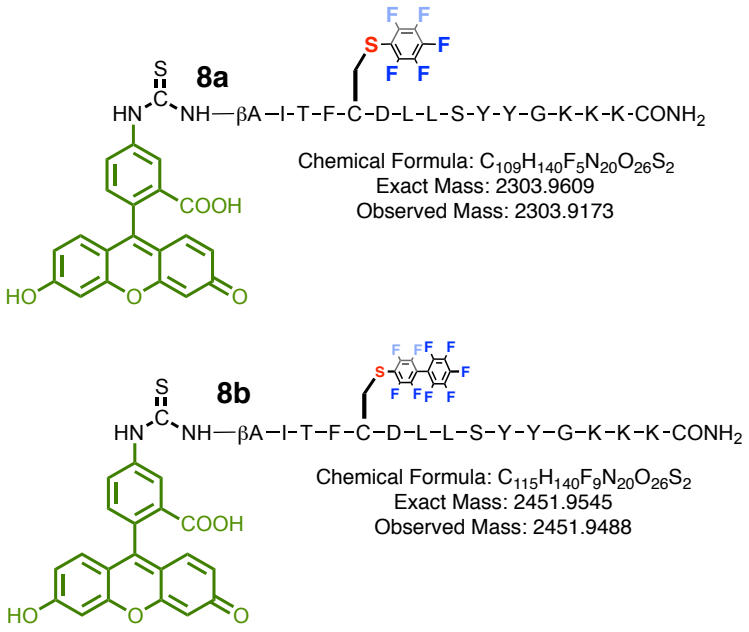


Figure SI-5. Peptides **8a**,**b**. Calculated masses correspond to $[M+2H]^+$ species observed directly via LC-MS.

D. Synthesis of the stapled affibody **10a**.

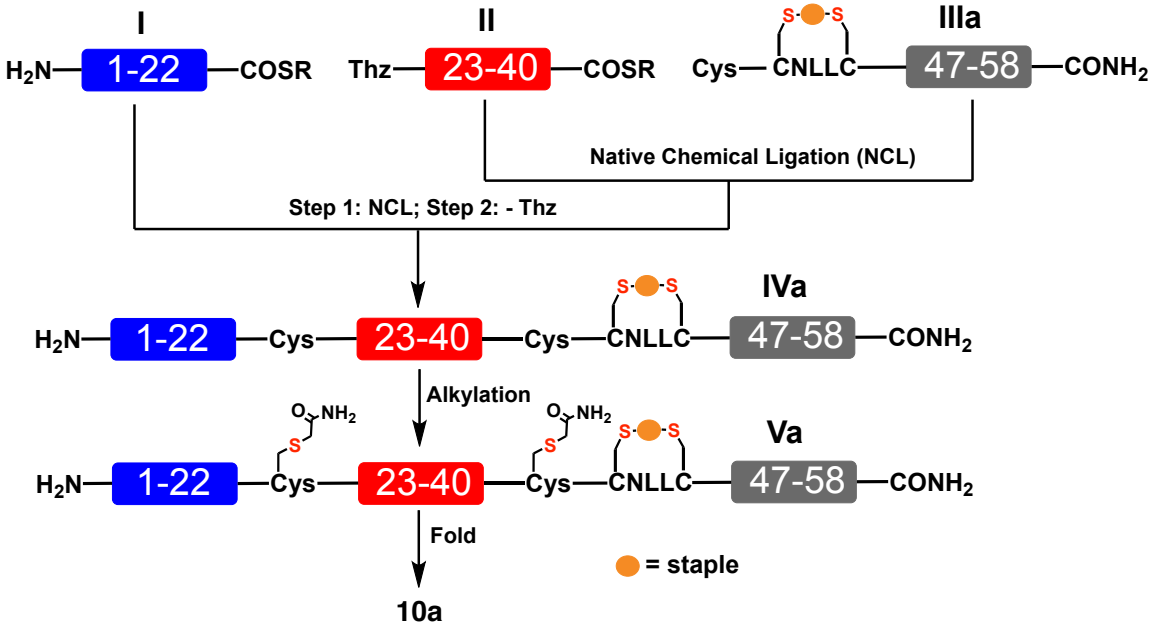


Figure SI-6. Synthetic strategy for the synthesis of stapled affibody.

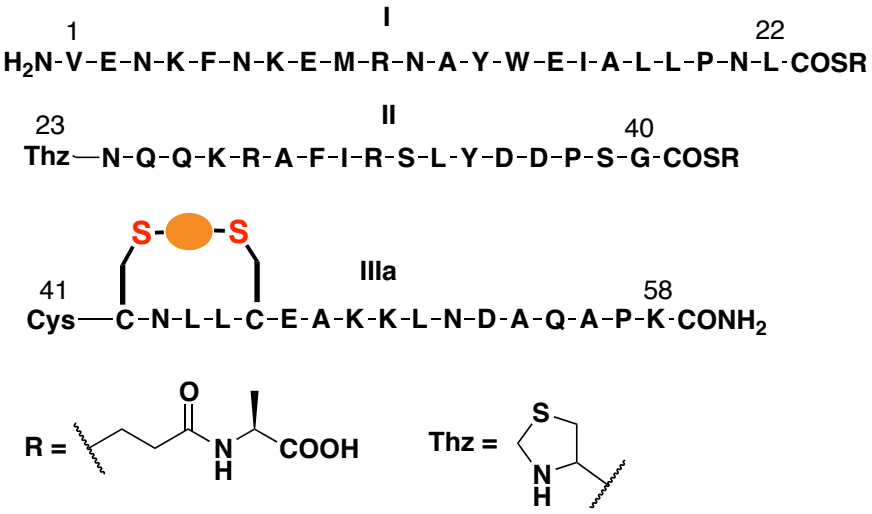


Figure SI-7. Three peptide segments used in the synthesis of stapled affibodies.

Peptide Thz^{41}-K^{58}-CONH$_2$ (1.0 mM) was treated with hexafluorobenzene (20.0 mM) or decafluorobiphenyl (5.0 mM) in the solution of TRIS base in DMF (25.0 mM) for 4 hours at room temperature to provide corresponding stapled peptide (77.3 % yield, calcd 2119.5 Da, obsd 2119.0 ± 0.1 Da). The stapled [Thz^{41}-Lys^{58}] peptide was then treated with 0.2 M MeONH$_2$·HCl at pH 4.0 at room temperature for 5 hours to give stapled [Cys^{41}-Lys^{58}] peptide (**IIIa**, 82 % yield, calcd 2107.4 Da, obs 2107.0 ± 0.1 Da) after RP-HPLC purification and lyophilization.

General strategy for one-pot native chemical ligation of peptides **I**, **II**, and **IIIa**:

[Thz^{23}-Gly^{40}]-thioester (3.2 μmol) and stapled [Cys^{41}-Lys^{58}] peptide (3.0 umol) were dissolved in a buffer (1.0 mL, pH 6.8) with guanidine·HCl (6 M), TCEP·HCl (20 mM), MPAA (40 mM) and sodium phosphate (0.2 M). The reaction mixture was incubated at room temperature for 7 hours. Without isolation, the crude reaction mixture was treated with MeONH$_2$·HCl at pH 4.0 at room temperature overnight to give stapled [Cys^{23}-Gly^{40}]-[Cys^{41}-Lys^{58}]. Then, [Val^1-Leu^{22}]-thioester (3.0 μmol) was added to the reaction mixture with the adjustment of pH 6.8 and incubated at room temperature for 5 hours to give stapled [Val^1-Leu^{22}]-[Cys^{23}-Gly^{40}]-[Cys^{41}-Lys^{58}] peptide (**IVa**, 34 % yield, calcd 6861.4 Da, obsd 6862.3 ± 0.1 Da) after purification by RP-HPLC.

Alkylation of stapled [Val^1-Leu^{22}]-[Cys^{23}-Gly^{40}]-[Cys^{41}-Lys^{58}] peptides:

Stapled [Val^{1}-Leu^{22}]-[Cys^{23}-Gly^{40}]-[Cys^{41}-Lys^{58}] peptides were alkylated at the two cysteine position with 2-bromoacetamide (50 mM) at room temperature in a buffer [pH 7.1, guanidine·HCl (6 M), TCEP·HCl (20 mM) and sodium phosphate (0.2 M)]. After 30 minutes, the reactions were quenched with MESNa (100 mM) to give stapled [Val^{1} -Lys^{58}] peptides (**Va**, 78.3 % yield, calcd 6976.9 Da, obsd 6976.4 ± 0.1 Da) after RP-HPLC purification and lyophilization.

Refolding of stapled [Val^{1}-Lys^{58}] peptide **Va**:

The stapled [Val^{1}-Lys^{58}] peptides were dissolved in a buffer (pH 7.5) with guanidine·HCl (6 M), TCEP·HCl (20 mM), Tris (20 mM) and NaCl (150 mM), and then sequentially diluted from guanidine·HCl (6 M) to guanidine·HCl (1 M). The peptide solutions were desalted into a buffer (pH 7.5) with Tris (20 mM) and NaCl (150 mM) using a HiTrap Desalting column (GE Healthcare, UK) and provided the stapled [Val^{1}-Lys^{58}] peptides (**10a**, 78 % yield, calcd 6976.9 Da, obsd 6977.0 ± 0.1 Da).

3. Cell Imaging.

293T HEK cells were cultured with DMEM with 10% FBS (*v/v*) in imaging dishes (70K cells/well) in 37°C, 5% CO_2 incubator for two days until they are about 70% confluent. Appropriate amounts of peptides **7, 7a, 7b, 8a** and **8b** dissolved in autoclaved H_2O were added to the cells to final concentrations of 5 μM. Peptide **9** was first dissolved in DMSO to make a 200 μM stock and then added to cells to a final concentration of 5 μM. The cells were incubated with the samples for 4 hours at 37°C and 5% CO_2. After incubation, cells were washed 3 times with DPBS and then fixed with 4% formaldehyde (Alfa Aesar, MA) in DPBS for 10 minutes. They are then washed 3 times with HBSS and stained with 5 μg/ml wheat germ agglutinin-tetramethylrhodamine conjugate (Invitrogen, CA) in HBSS for 20 minutes. The cells were subsequently washed with HBSS once and DPBS twice and stained with 5 μg/ml Hoechst 33342 trihydrochloride (Invitrogen, CA) in DPBS for 30 minutes. They were washed 3 times with DPBS and covered in one drop of prolong Gold antifade reagent (Invitrogen, CA) and cover slide. Images of peptide localization in cells were taken on PerkinElmer Ultraview Spinning Disk

Confocal with 30% of its maximum laser power in 488 channel with 500 ms exposure time and 0.5 μm Z-stacks. Image processing was done using Volocity software package (PerkinElmer).

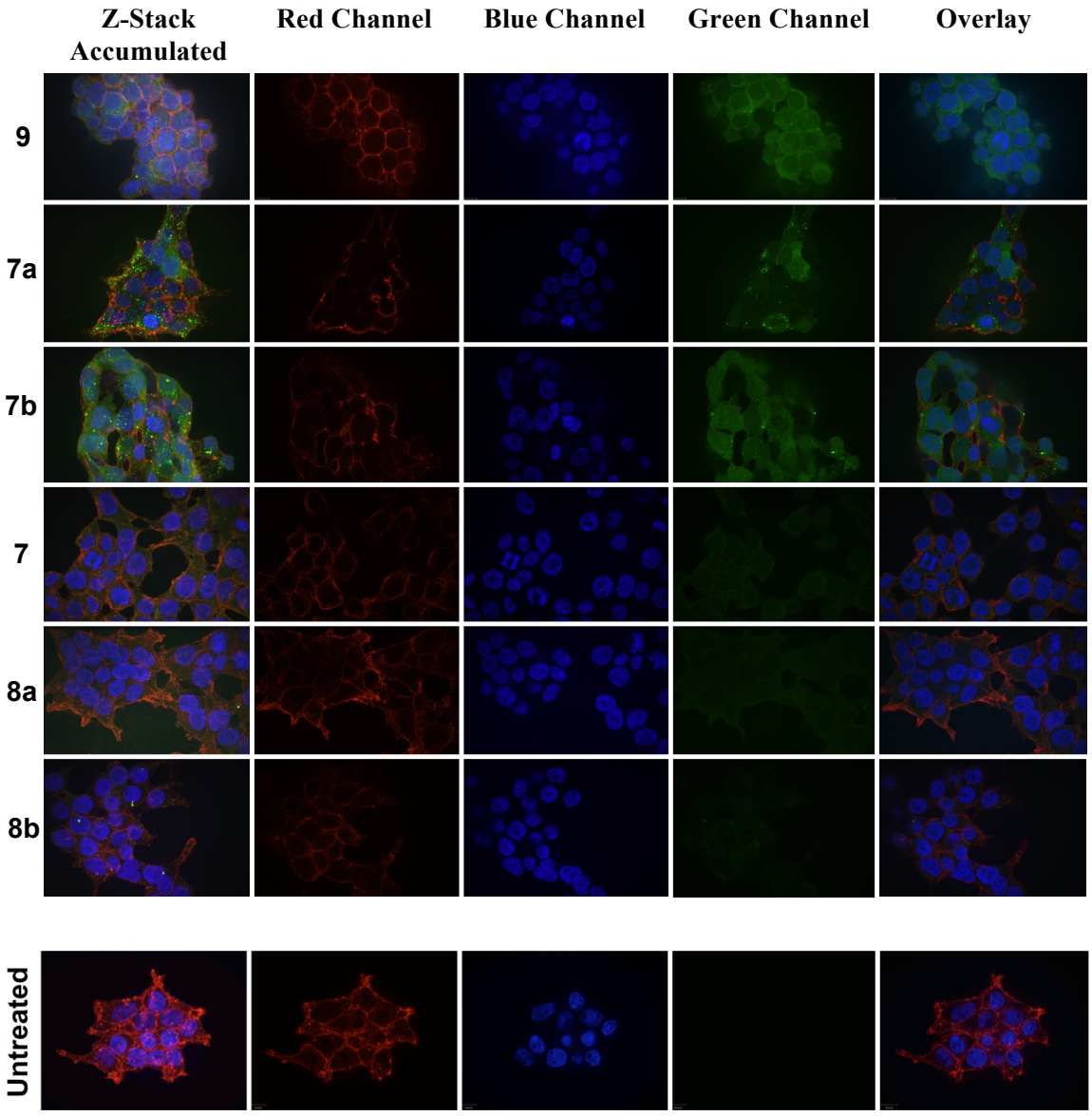


Figure SI-8. Fluorescent confocal microscopy of cells studied for peptide cell permeability. Z-stack accumulated fluorescent confocal microscopy images and representative slice overlays (DNA – blue; cell membrane – red; peptides – green (FITC)) of the HEK293T cells treated with peptides **7**, **7a**-**b** (5 μM), associated unstapled controls **8a** and **8b**, NYAD-2 – **9** and untreated control. Refer to pages S19-S50 for individual high-resolution images used in this figure.

4. Molecular cloning, protein expression and purification.

The plasmids encode Gag-derived proteins from the HIV-1 strain. The full-length gag expression vector was obtained from the Invitrogen. After PCR amplification and purification, the C-CA DNA fragment was obtained by digestion and then inserted into the pET21b vector with subsequently overnight ligation. The C-CA protein were expressed and purified as described previously[1-2].

5. Circular Dichroism Spectroscopy (CD).

CD measurements were done with Aviv 202 spectrometer using 1 mm quartz cuvette. Peptide solutions were made by dissolving solid samples in 25% acetonitrile/water mixture, CD spectra of **3a** and **3b** were measured in acetonitrile, CD spectra of affibodies was collected in 10 mM phosphate buffer. Concentration of affibodies was estimated by UV absorbance measurements at 280 nm. Contribution of the perfluorinated staple in **6a** on the absorbance value at 280 nm was taken into account (estimated molar extinction coefficient for [–S-C_6F_4-S-] ~ 5000 $cm^{-1}M^{-1}$ from UV measurements of solutions of **6** and **6a**) in these measurements. Data processing included solvent background correction (subtraction) and adjustment for pathlength and concentration (MRE = $[\theta]\lambda = \theta_{obs}$ x 1/(10 lcn); θ_{obs} = measured ellipticity, θ = mean residual ellipticity in deg x cm^2 x $dmol^{-1}$, l = pathlength (cm); c = concentration of peptide (M); n = # of amino acids). α–helicities of the peptides were estimated using previously established methods.[3]

6. Proteolysis Assays.

100 μL of peptide (100 μM) in phosphate buffer (pH 8.1) was mixed and incubated with 40 μL of the protease solution (trypsin – 70 μg/mL; chymotrypsin – 50 μg/mL) at 37 ^oC. Aliquots of 35 μL were quenched with 55 μL of 1% TFA solution in MeCN and subjected to LC-MS analysis at 10, 25 and 40 minutes respectively. Peptide concentrations at different time points were quantified by integration of the TIC trace relative to the starting peptide sample. Results of these experiments are summarized in the Figure below. Note, that in the case of experiments with unstapled peptide **7**, <1% of the intact peptide was observed after 10 minutes.

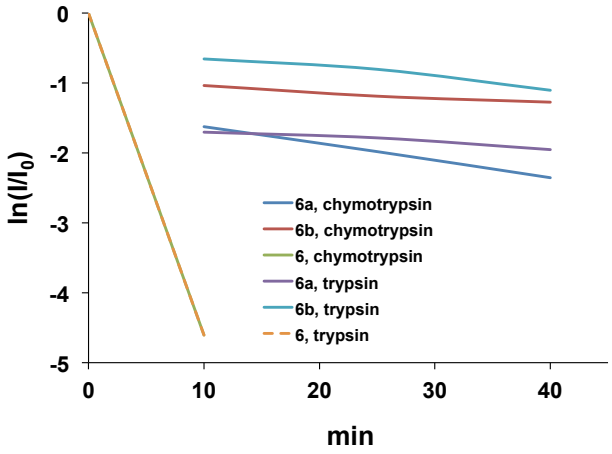


Figure SI-9. Proteolitic assays of peptide **6** series with trypsin and chymotrypsin.

For experiments with proteinase K, 100 µL of peptide (100 µM) in phosphate buffer was incubated with the 40 µL of protease solution (100 µg/mL) for 3 hours at 37 ^{o}C. 35 µL aliquot of the cleaved peptide solution was diluted with 55 µL of 1% TFA solution in MeCN and subjected to LC-MS analysis.

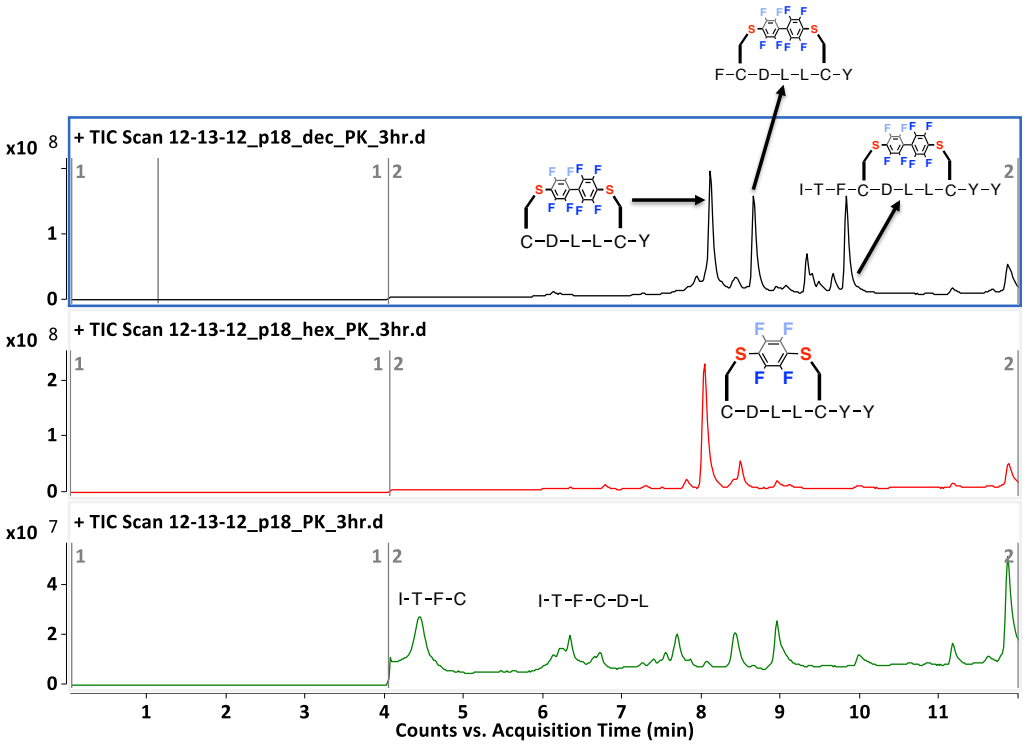


Figure SI-10. Proteolitic assays of peptide **6** series with proteinase K.

7. Flow cytometry.

293T HEK cells were cultured with DMEM with 10% FBS (*v/v*) in 24-well plates (70K cells/well) in 37°C, 5% CO_2 incubator for two days until ~ 70% confluent. Solid peptide samples were dissolved in autoclaved H_2O (except for NYAD-2, which was dissolved in DMSO) were added to the cells to final concentrations of 5 µM or 25 µM. The cells were incubated with the samples for 4 hours at 37°C and 5% CO_2. After incubation, cells were lifted by pipetting then transferred to V-bottom 96-well plates and spun at 1000 rpm for 3 min to pellet. The pellets were washed 4 times with DPBS then re-suspended in PBS with 2% FBS (*v/v*), 0.1% BSA (*w/v*) and 1% pen-strep (*v/v*) for FACS analysis on BD LSR II HTS instrument.

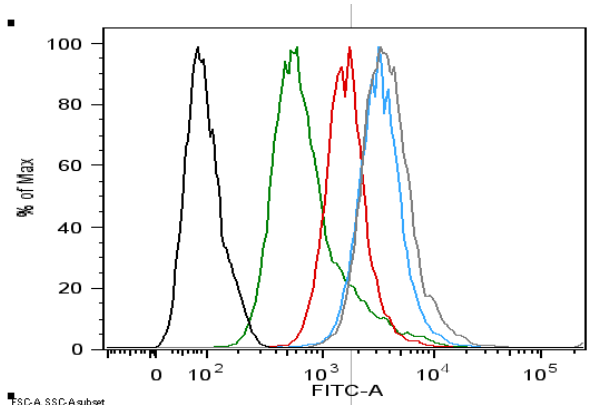


Figure SI-11. FACS data obtained from the experiments with 5 µM peptide solutions (blank – black, **7** – green, **7a** – red, **7b** – blue, NYAD-2 - grey).

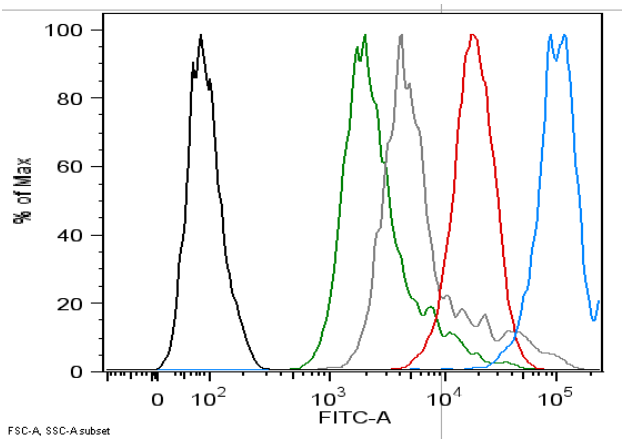


Figure SI-12. FACS data obtained from the experiments with 25 µM peptide solutions (blank – black, **7** – green, **7a** – red, **7b** – blue, NYAD-2 – grey).

8. Biacore Measurement.

Biacore 2000 and 3000 instruments (GE) were used for on-surface real-time biospecific interaction analysis between HER-2 with affibodies, and C-CA with peptides. HER-2 (~ 3000 RU) and C-CA (~ 2500 RU) were immobilized onto a CM5 sensor chip according to the normal procedures.[4] A second flow-cell surface was activated and deactivated with ethanolamine and used as a reference surface. Binding analyses were done at 25°C, and commercial HBS buffer (GE) was used as the running buffer for all of the measurements (300 sec – adsorption, 300 sec – desorption, 10 µL/min flow rate). Surface regeneration between each binding experiment was done with 10 mM glycine solution (pH 2, 5 minutes, 10 µL/min flow rate).

9. Molecular models.

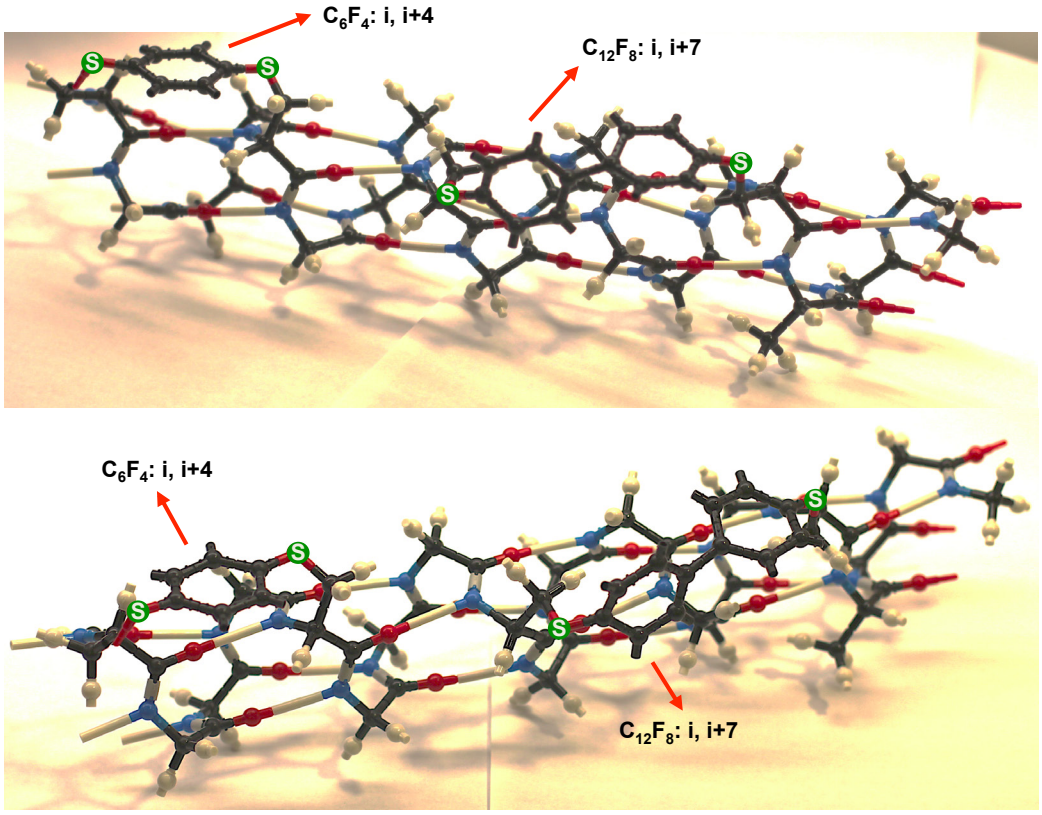


Figure SI-13. Comparison of the aryl- and biaryl-based linkers relevant to the i, i+4 and i, i+7 stapling based on the HGS Biochemistry Molecular Kit (Japan). Note, that monoaryl linker fits best (length-wise) the i, i+4 motif, and its biaryl congener – i, i+7 arrangement.

10. Molecular Dynamics Simulations

Molecular dynamics simulations were performed for the wild-type **6** and the two stapled peptides **6a**,**b** to characterize the effects of the staples on peptide structure. All simulations were performed using Gromacs 4.5.5 [5] in conjunction with the OPLS-AA force field [6] and TIP4P water model [7]. The OPLS-AA atom types for the cross-linkers as well as the sulfur atom in the CYS residue used in the simulations are given in Figure SI-14. The addition of the linkers requires 4 additional bonded parameters, which are not available in the OPLS-AA force field. These missing angle and dihedral parameters, which were determined by chemical similarity to existing parameters, are listed in Table SI-3 and SI-4.

Simulations were run in the isobaric-isothermal (NPT) ensemble at a temperature of 300 K and a pressure of 1 bar. The temperature was maintained using the v-rescale thermostat [8] with a coupling time constant τ_T = 0.1 ps. To avoid the "hot solvent-cold solute" problem [9, 10], the peptide and solvent were coupled to separate thermostats. The pressure was controlled using an isotropic Parrinello-Rahman barostat [11] with a coupling time of τ_P = 2.0 ps and a compressibility of 4.5×10^{-5} bar^{-1}. All bonds were constrained with the LINCS algorithm [12]. A 2 fs time step with the leap-frog algorithm was used to evolve the dynamics. The non-bonded interactions (Lennard-Jones and electrostatic) were truncated at 1.0 nm without shift or switch functions. Long-range electrostatic interactions beyond the cut-off distance were calculated by the Particle Mesh Ewald summation method [13] with a Fourier spacing of 0.12 nm and an interpolation order of 4. A long-range analytic dispersion correction was applied to both the energy and pressure to account for the truncation of Lennard-Jones interactions [14].

The initial structure of the wild type peptide was prepared with the Molefacture plugin in VMD [15]. The stapled peptides were constructed using the Builder module of PyMol [16]. The simulation system was set up as follows. The starting structure was solvated in a cubic periodic box of water after an energy minimization in vacuum. The dimension of the water box was chosen such that the minimum distance between any atom of the fully extended wild type peptide

and the box walls is 1.0 nm. Na^+ and Cl^- ions were added to obtain a neutral system with physiological ion concentration of 150 mM. The resulting system was further optimized by steepest descent algorithm to remove bad contacts. A 50 ps NVT (isochoric-isothermal) and a 50 ps NPT simulations with the peptide heavy atoms restrained by a harmonic potential with a force constant of 1000 $kJ \cdot mol^{-1} \cdot nm^{-2}$ were implemented sequentially to equilibrate the solvent molecules and adjust the density.

All the three peptides were started from the α-helix conformation. Each peptide was subjected to ten independent runs with different initial velocities assigned from the Maxwell-Boltzmann distribution at 300 K. Before data collection, an additional 100 ps NVT simulation followed by a 100 ps NPT simulation was also performed to equilibrate the whole system. During production, the trajectory was recorded every 10 ps. All production runs were 500 ns in length. The ten independent runs bring a total of 5 μs trajectory and 500,000 snapshots for analysis for each peptide.

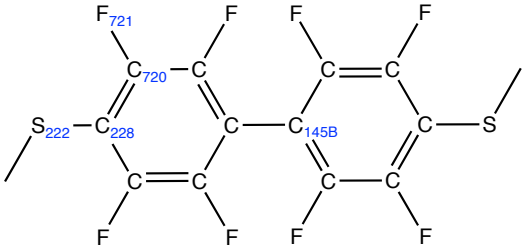


Figure SI-14. OPLS-AA atom types used for the cross-linker.

New type	OPLS-AA type	θ_{eq} (degrees)	k_θ ($kJ \cdot mol^{-1} \cdot rad^{-2}$)
C!-CA-F	CA-CA-F	120.0	669.440

Table SI-3. Angle bending parameter.

New type	OPLS-AA type	C_0	C_1	C_2	C_3
CT_2-CT-S-CA	CT-CT-S-CT	0.941	2.314	2.410	-5.665
F-CA-C!-CA	HA-CA-C!-CA	30.334	0.000	-30.334	0.000
F-CA-C!-C!	HA-CA-C!-C!	30.334	0.000	-30.334	0.000

Table SI-4. Dihedral angle parameters in Gromacs Ryckaert-Bellemans form. The unit is $kJ \cdot mol^{-1}$ for all parameters.

10. References.

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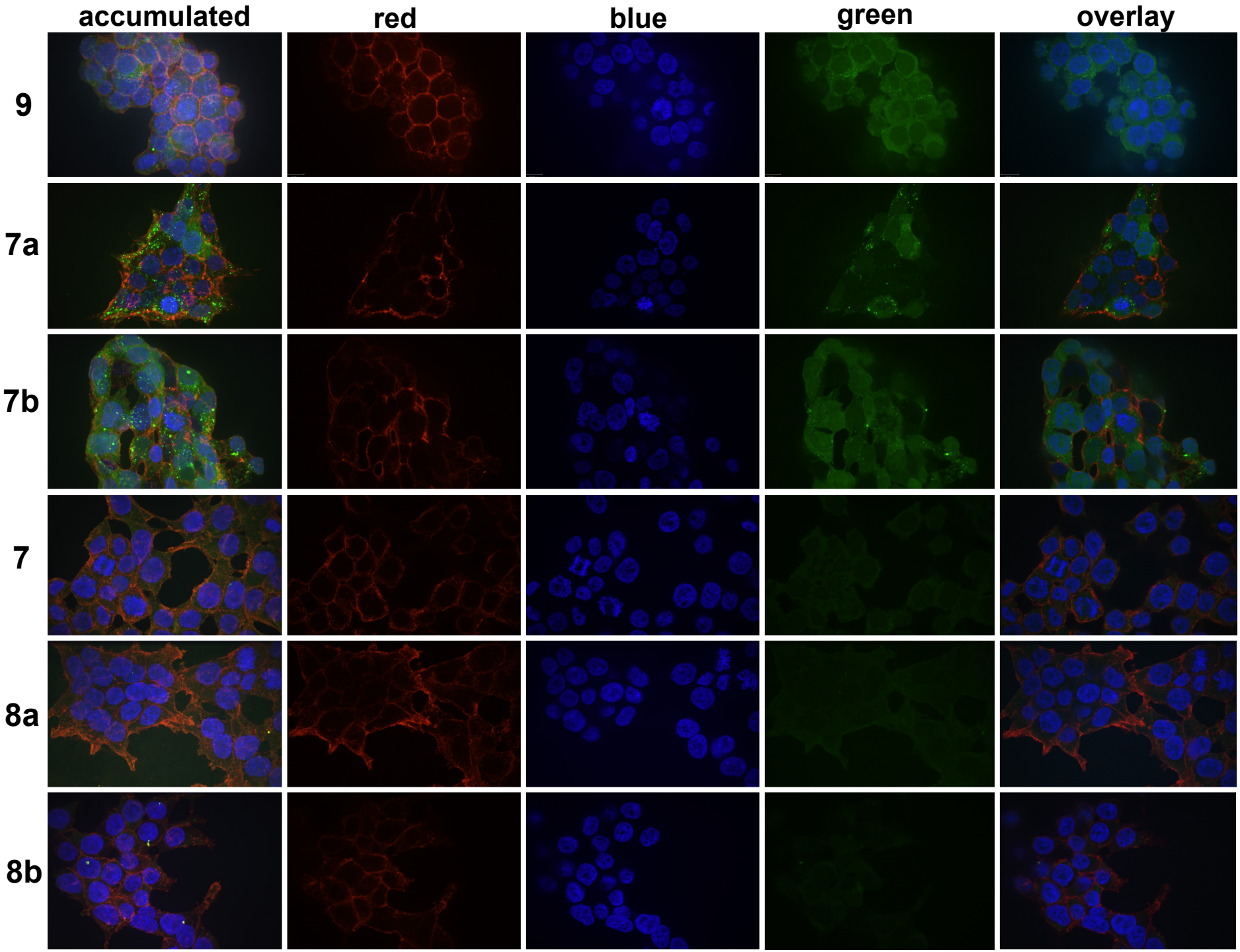
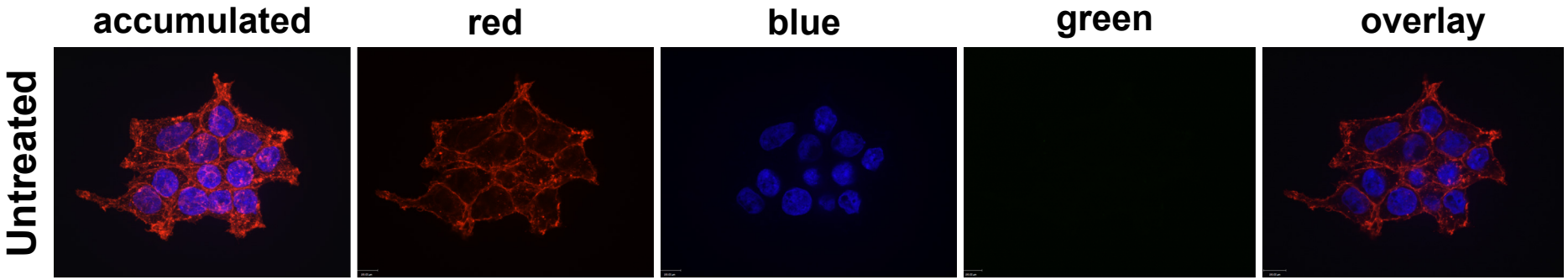
Enlarged confocal microscopy cell images. Refer to Figure SI-8 and section 3 in the SI for details.

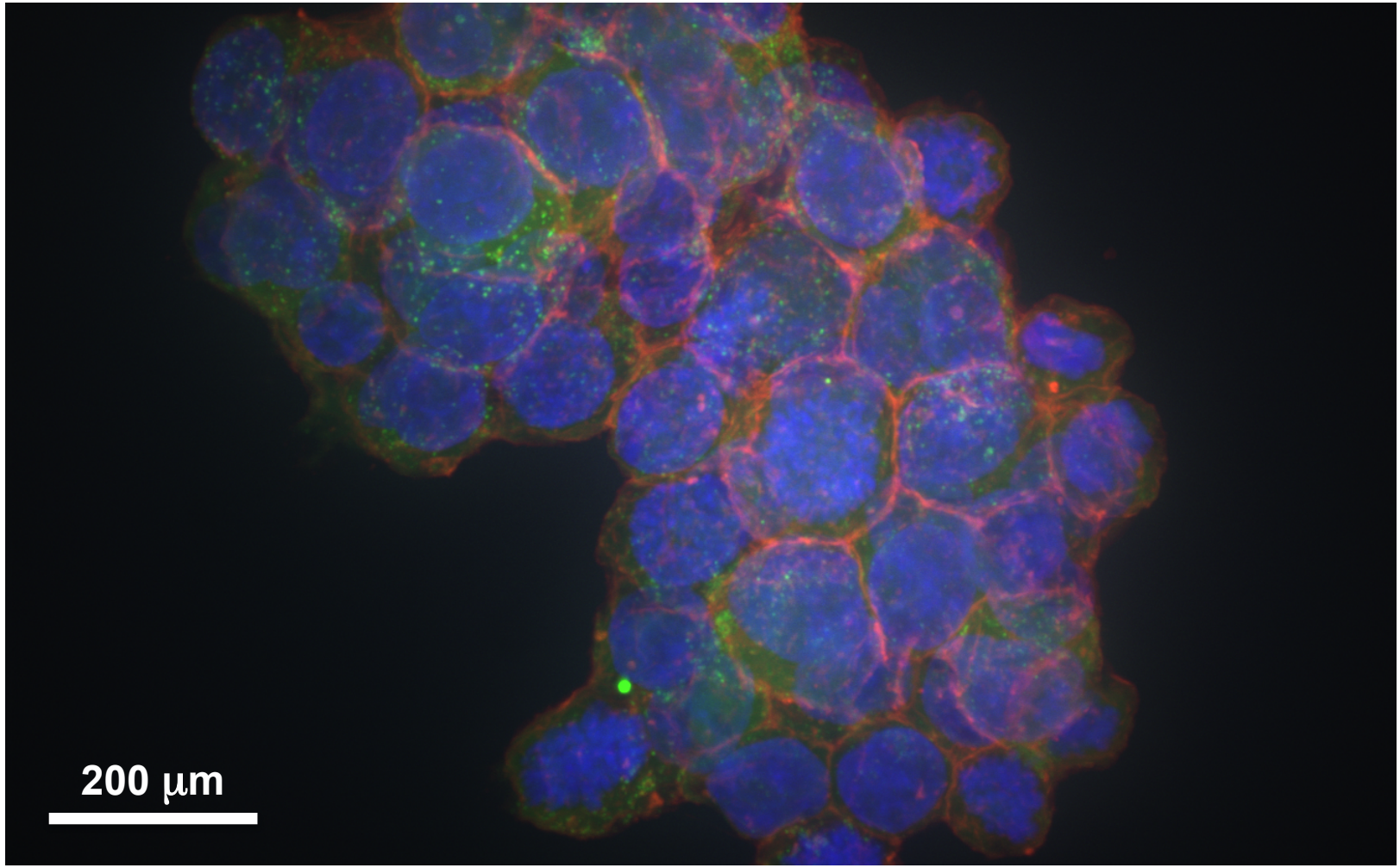




Enlarged confocal microscopy cell images. Refer to Figure SI-8 and section 3 in the SI for details.

Z-stack accumulated

9



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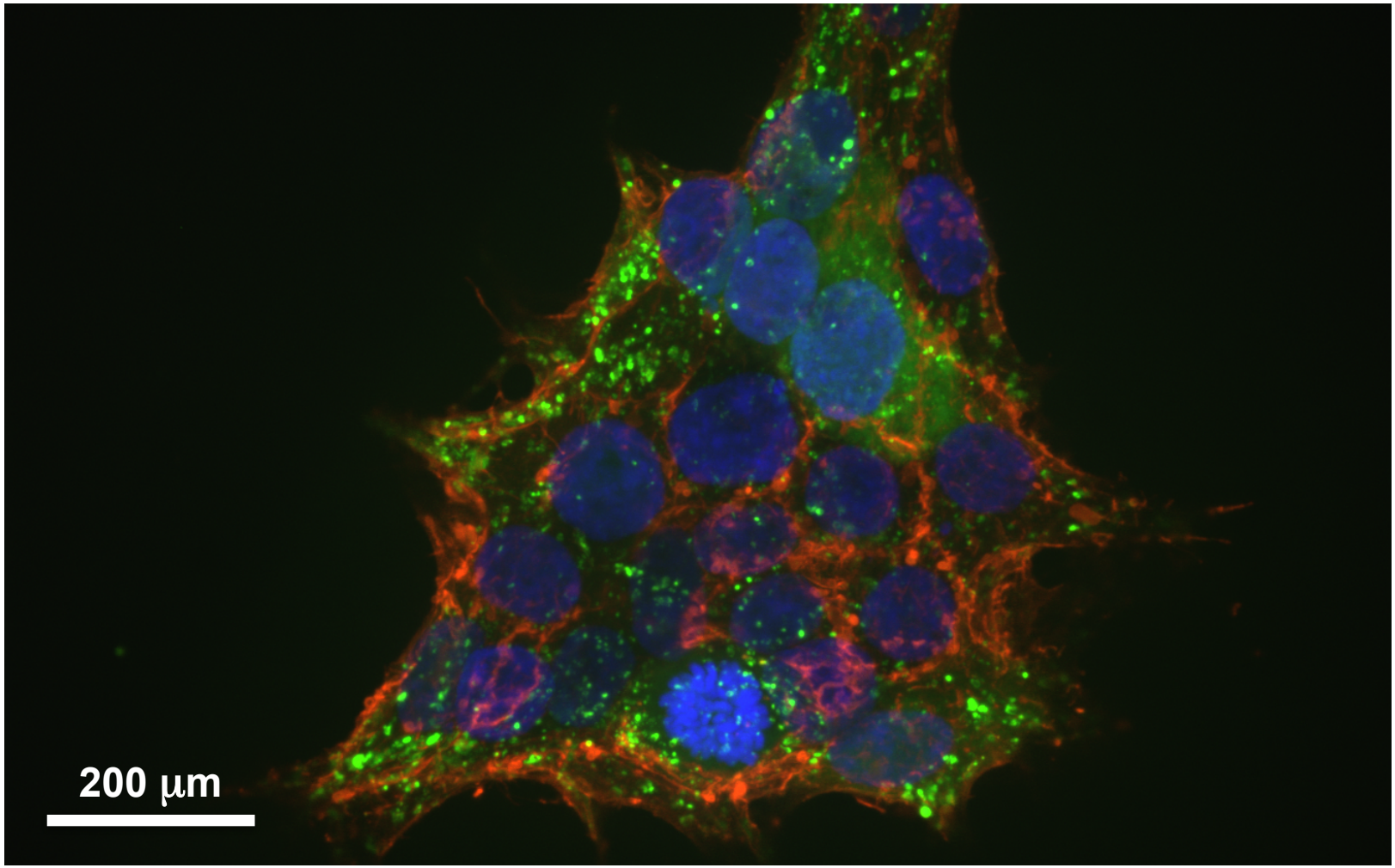

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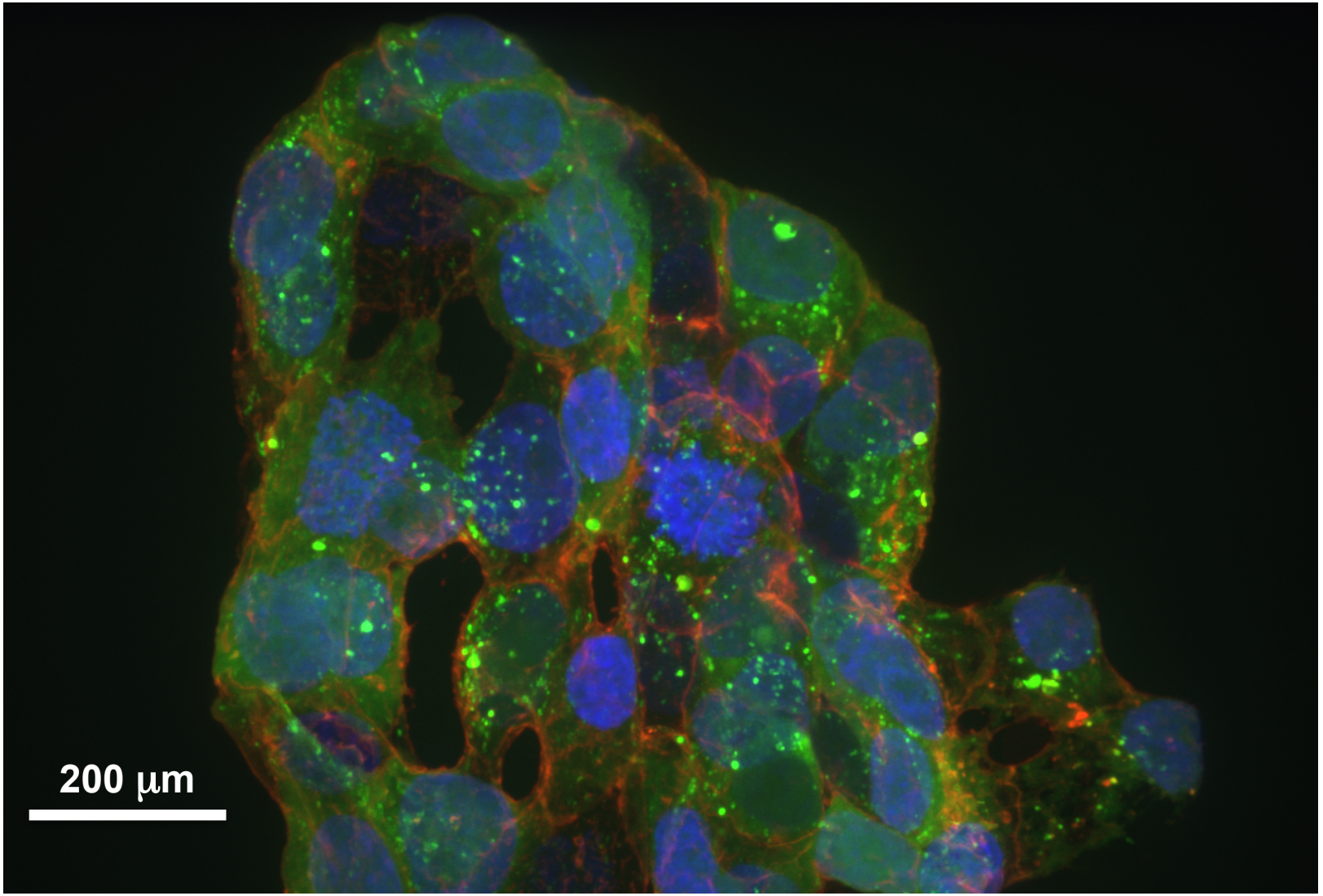

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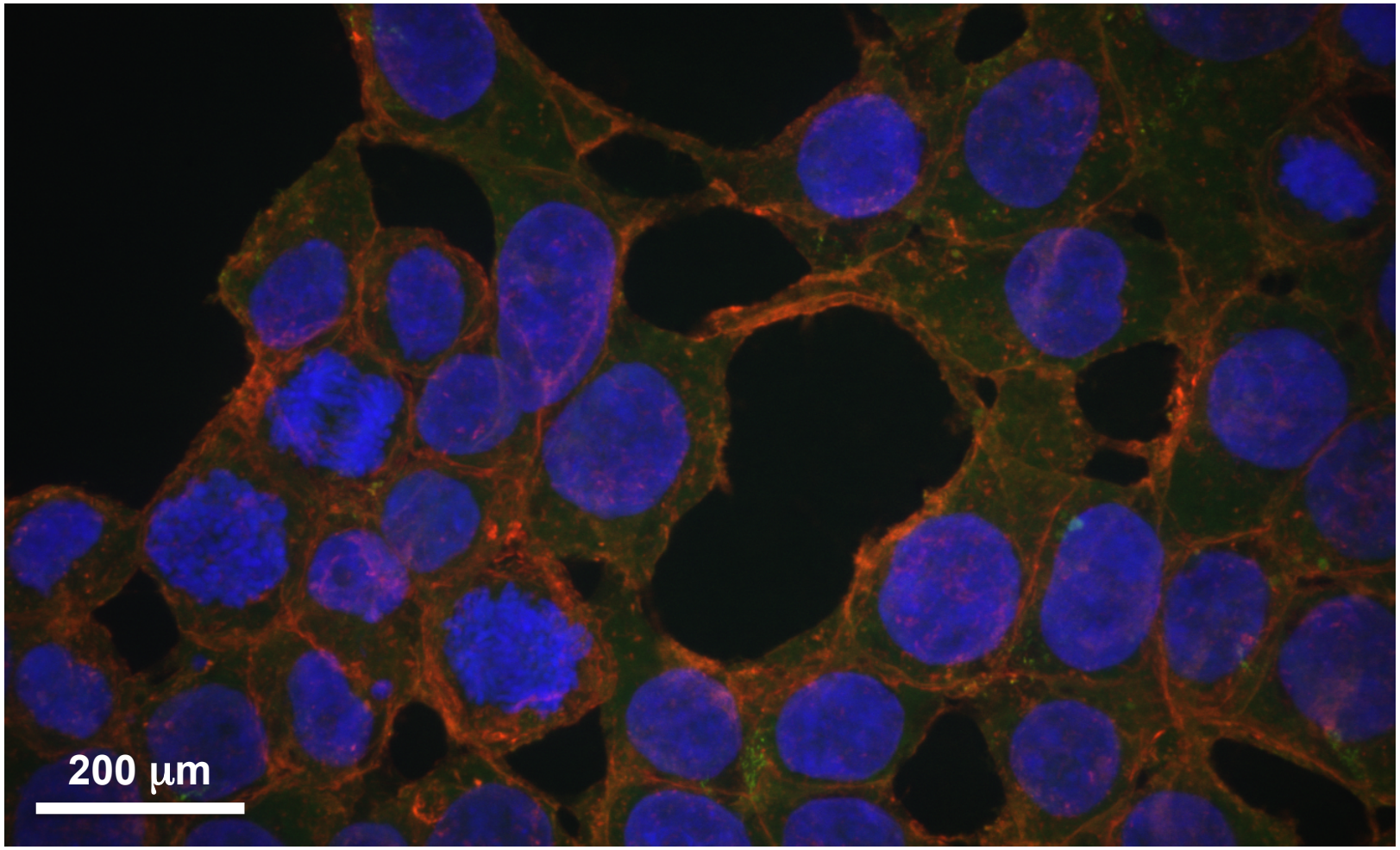

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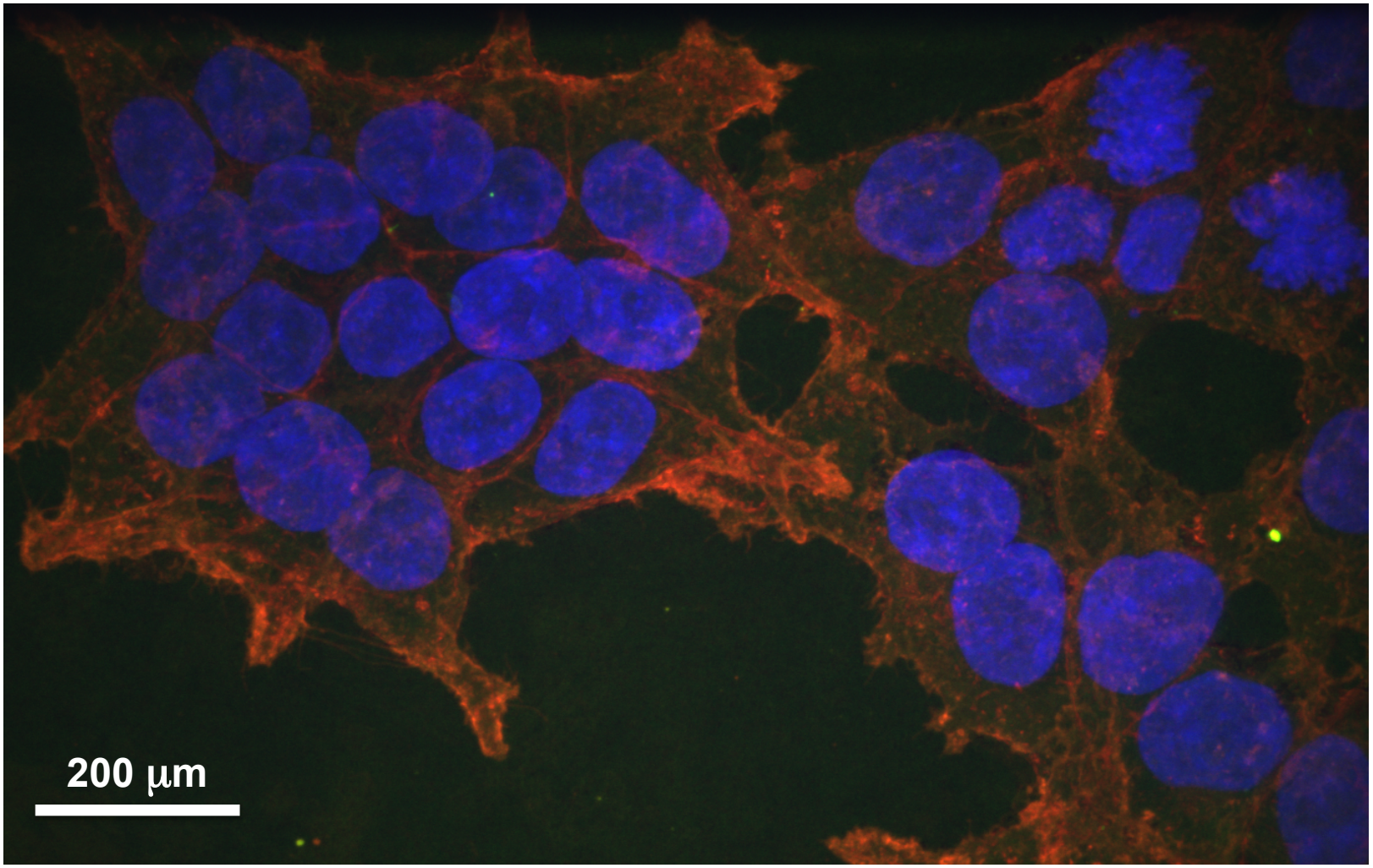

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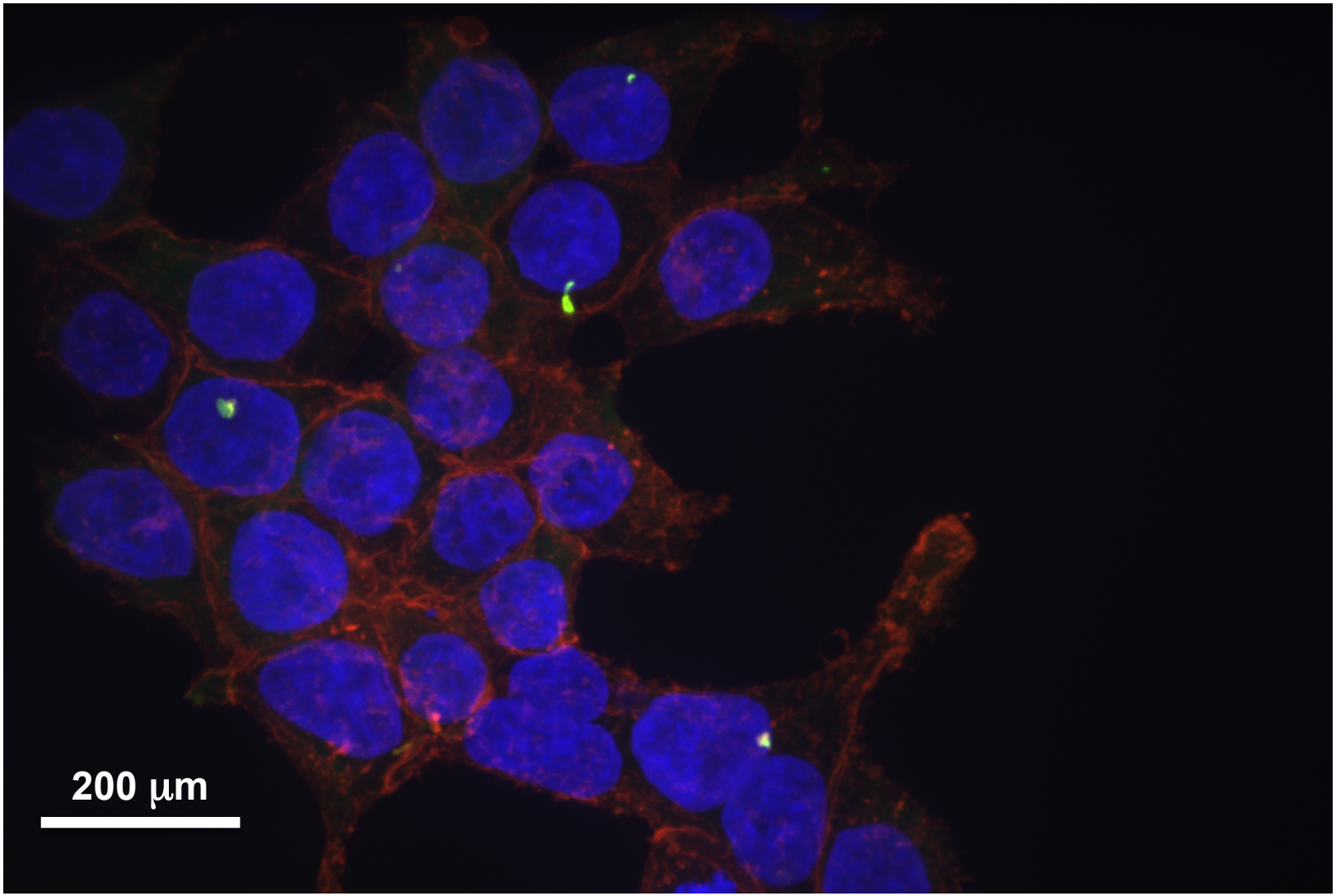

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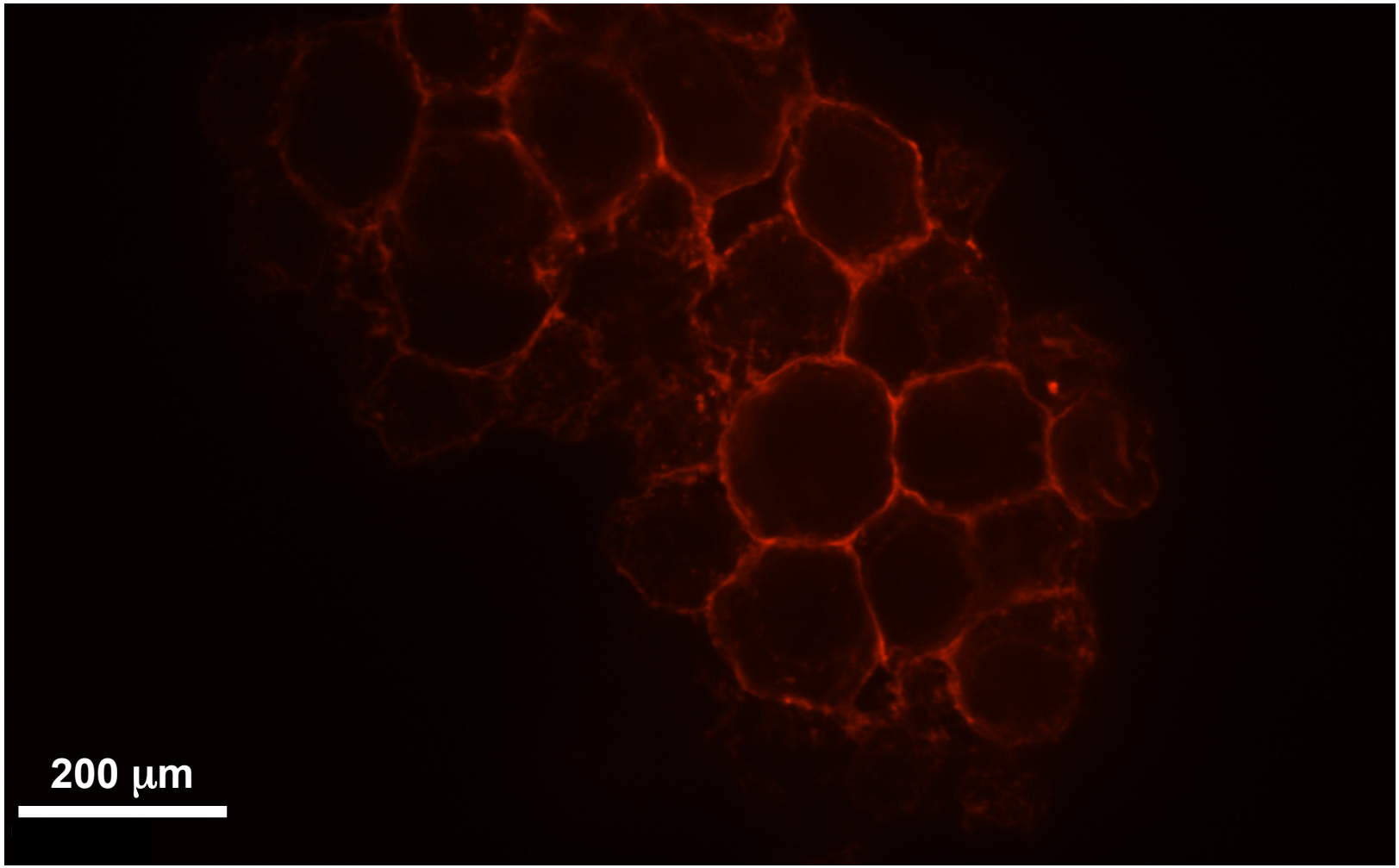

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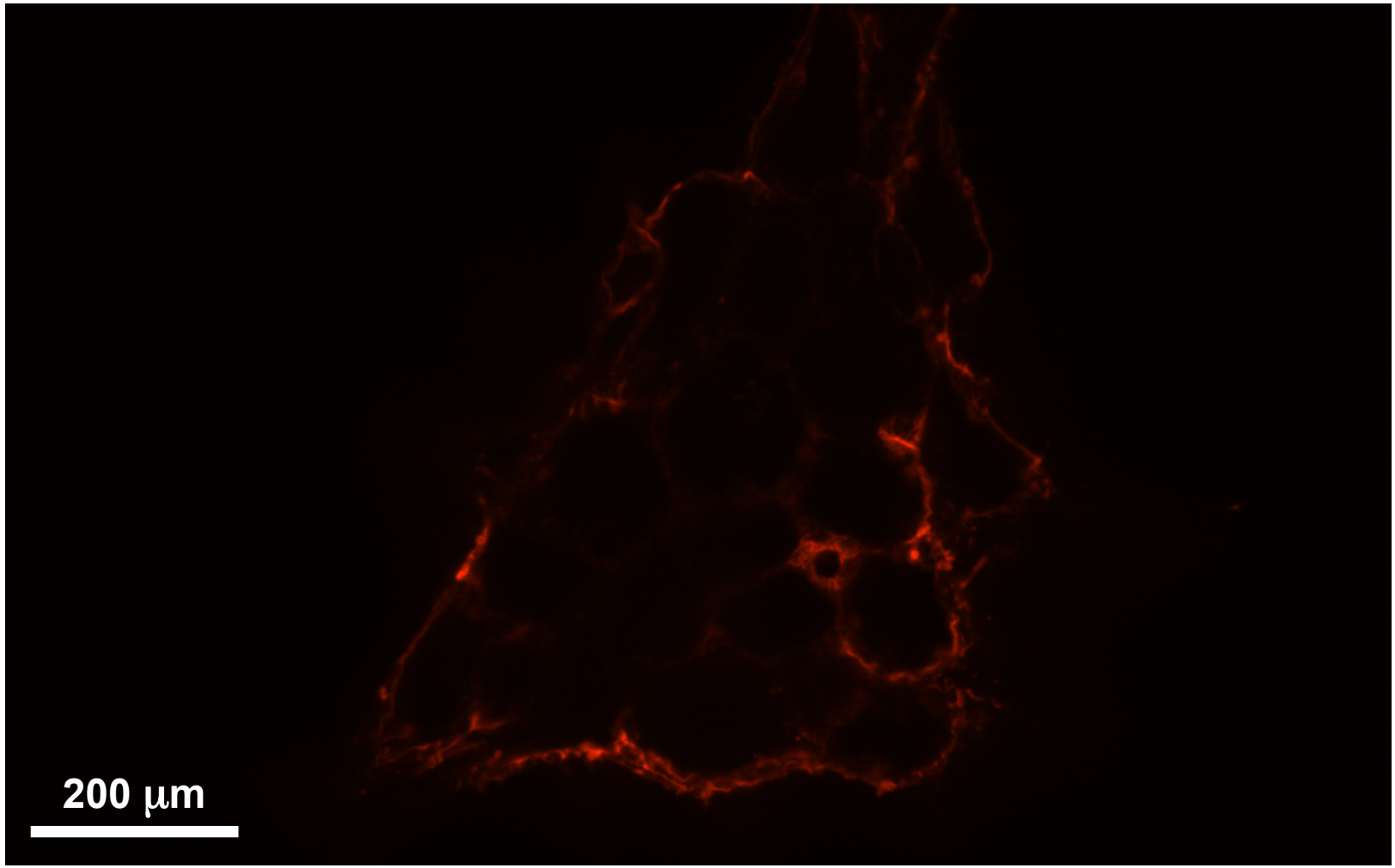

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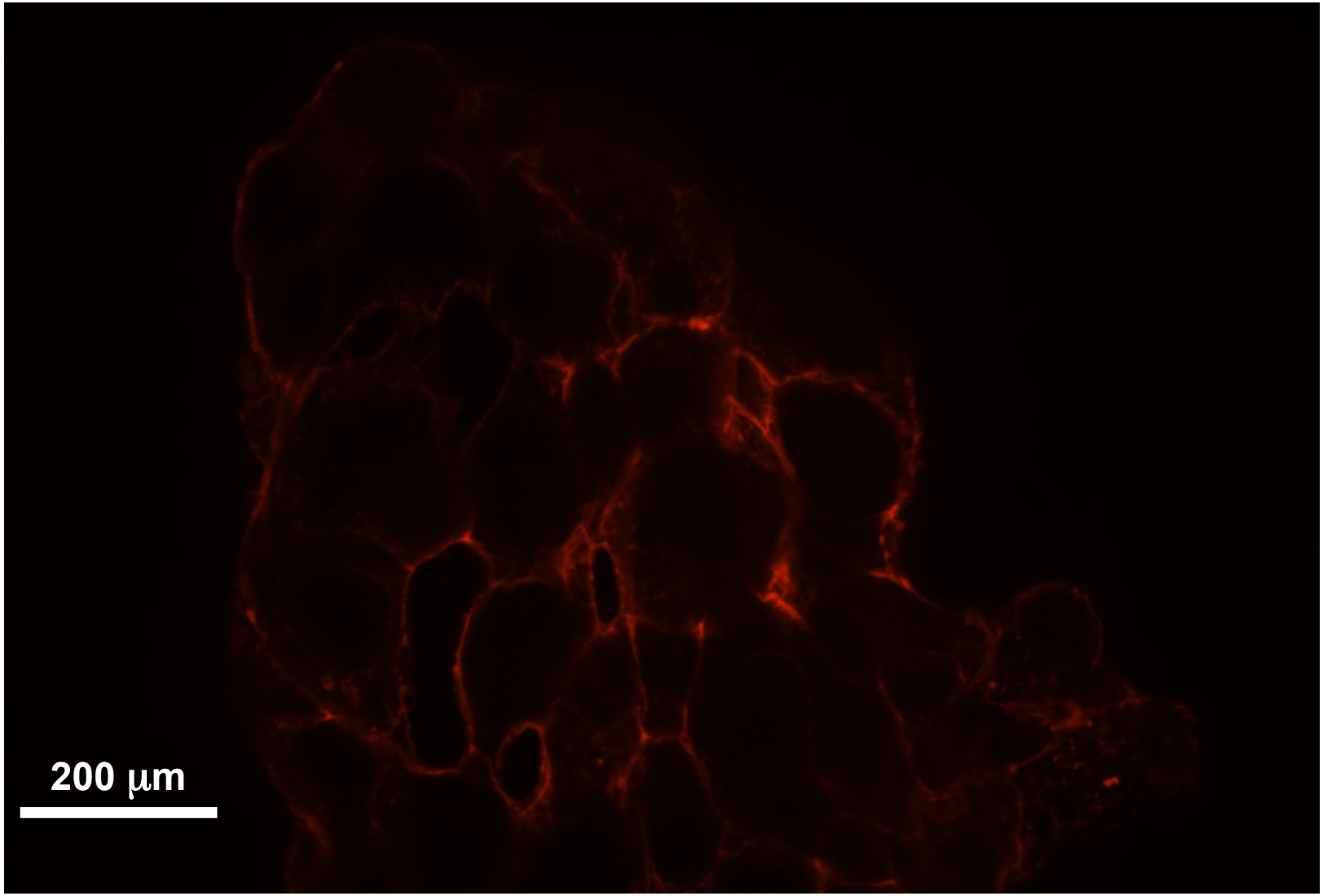

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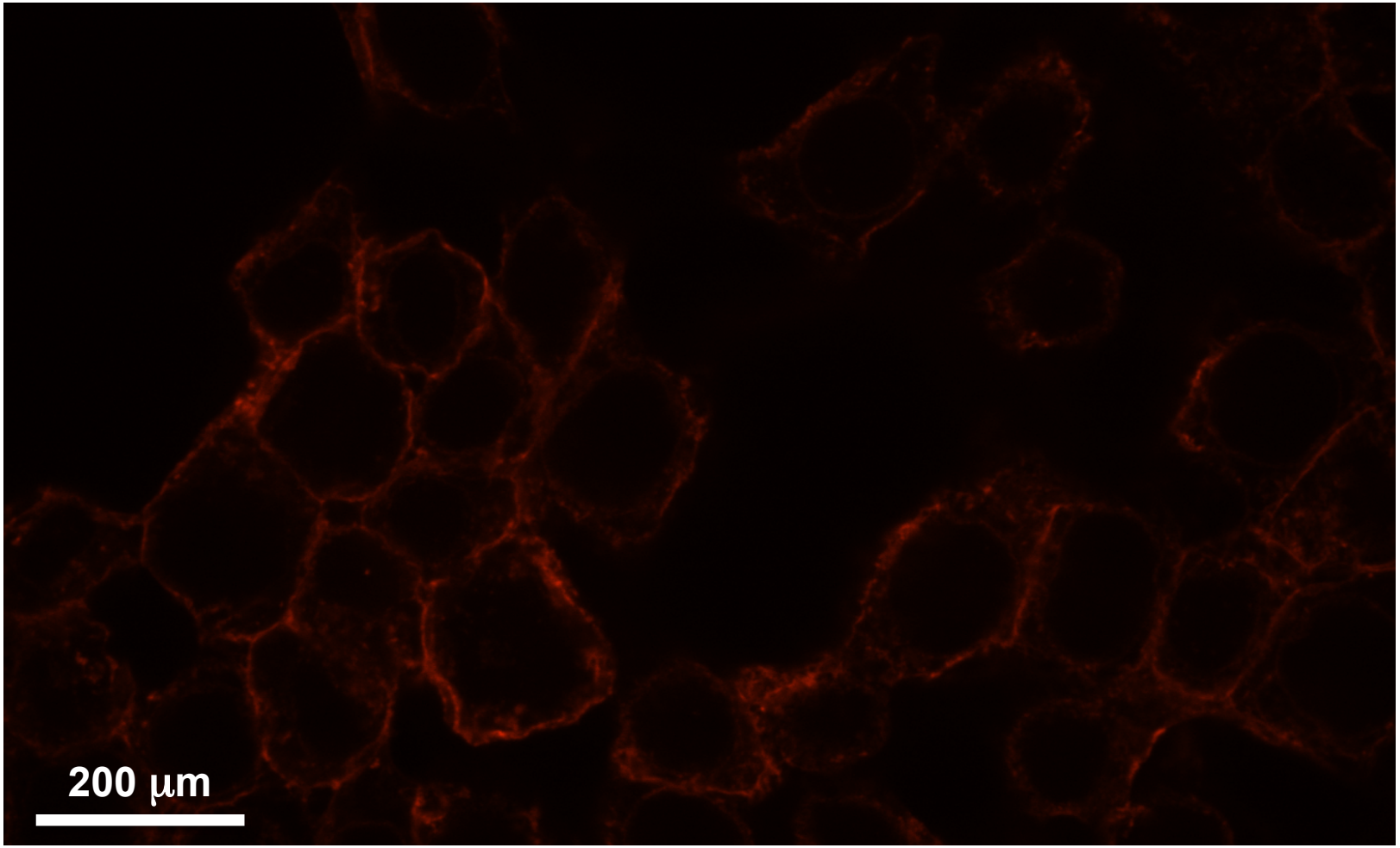

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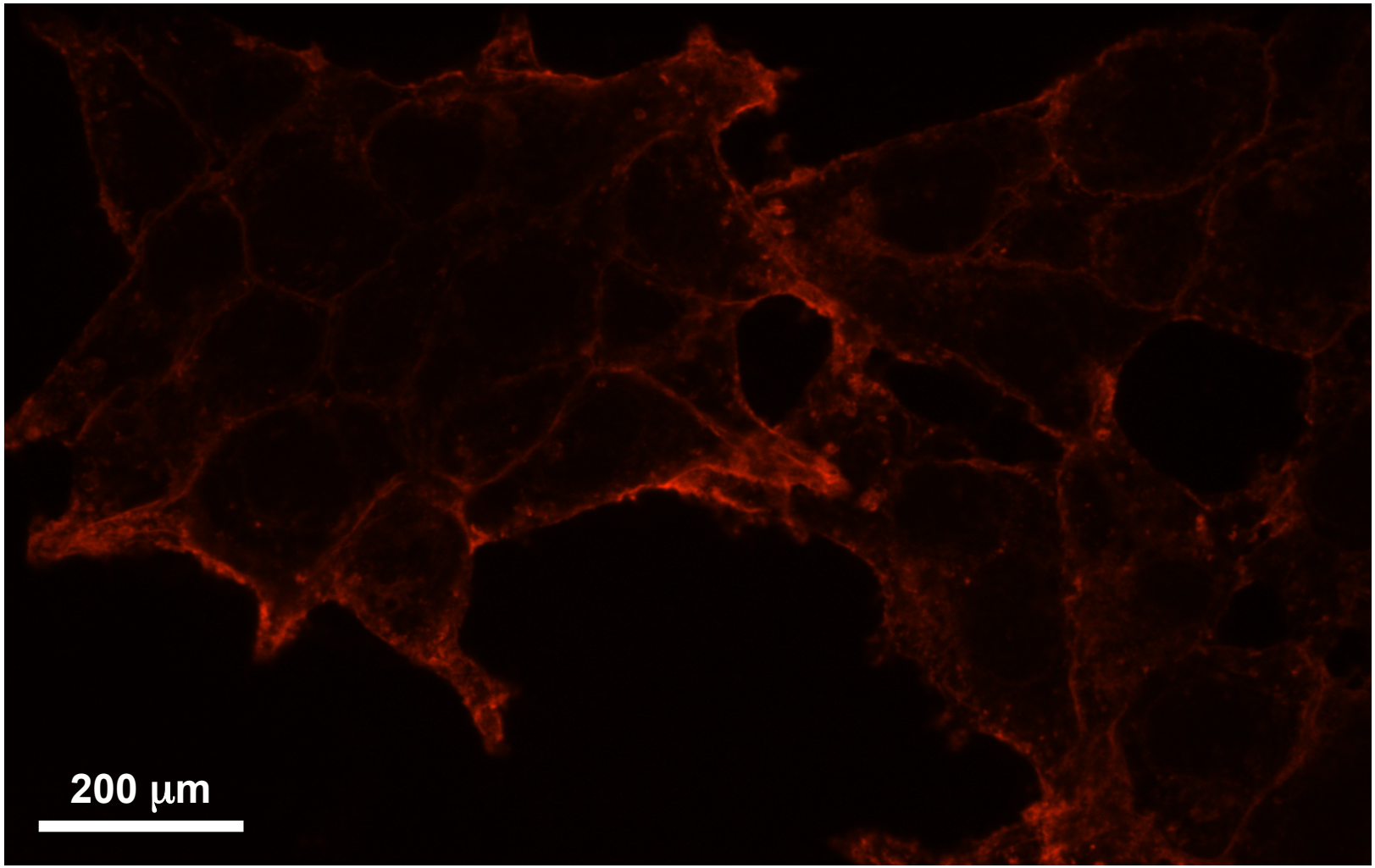

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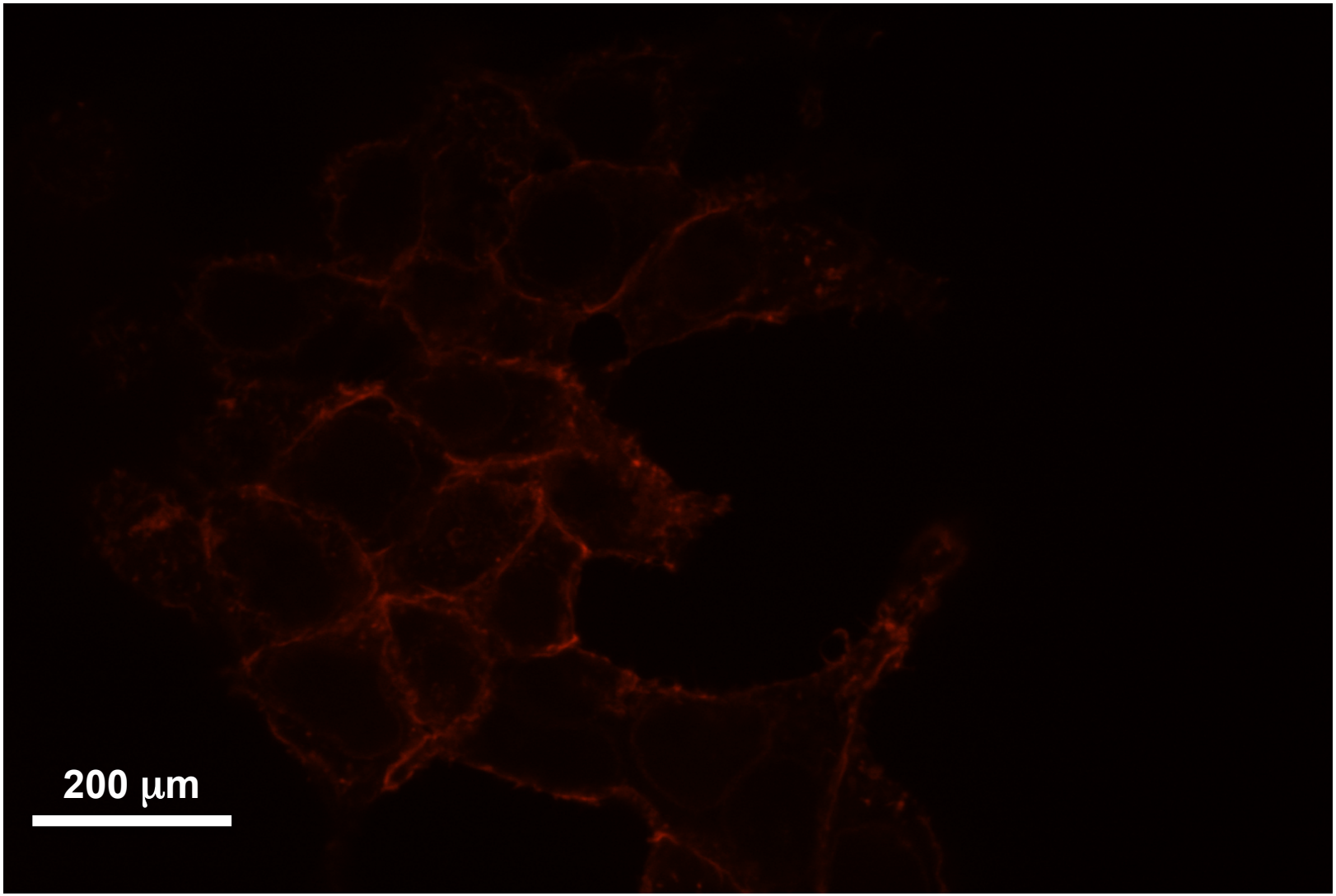

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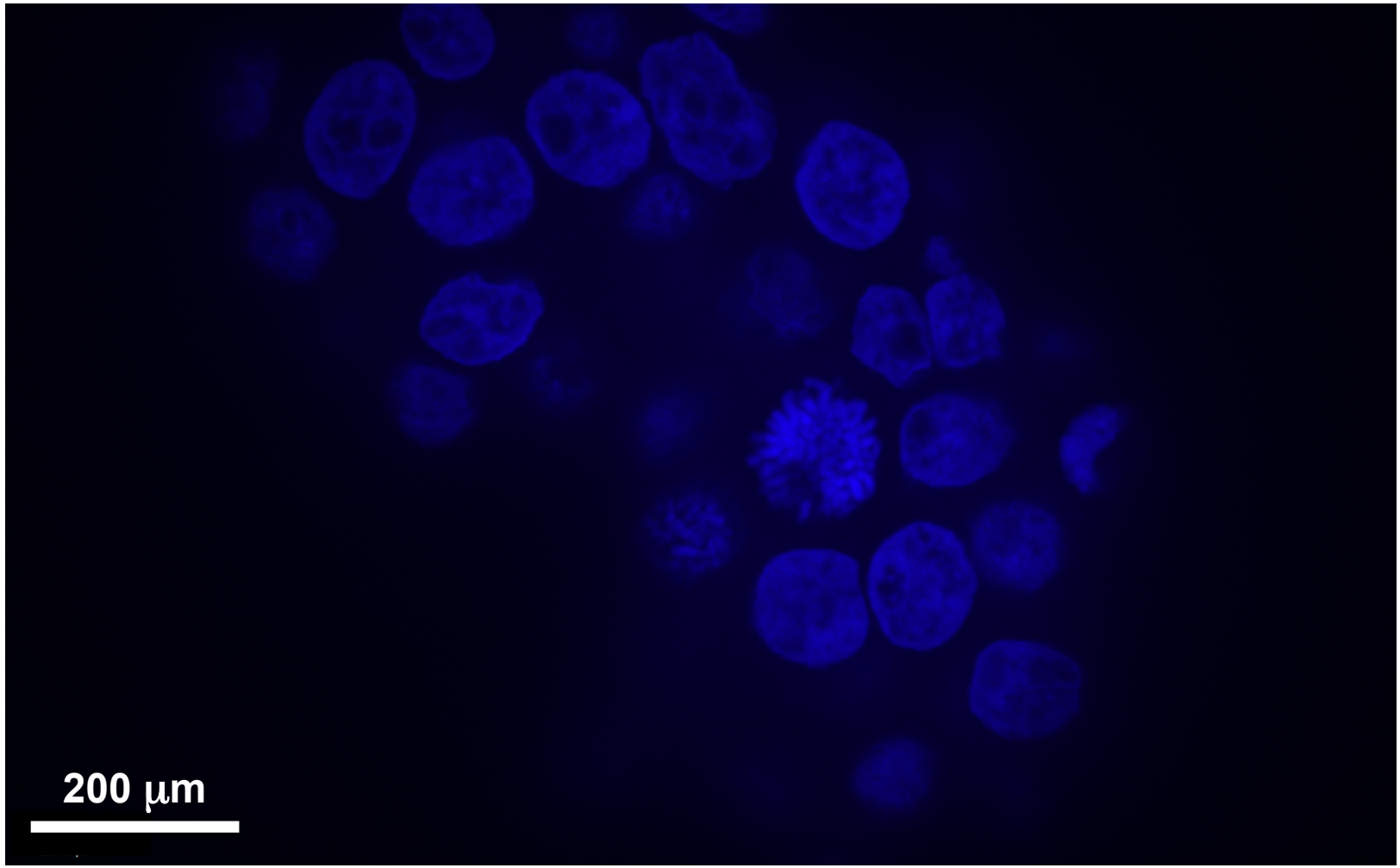

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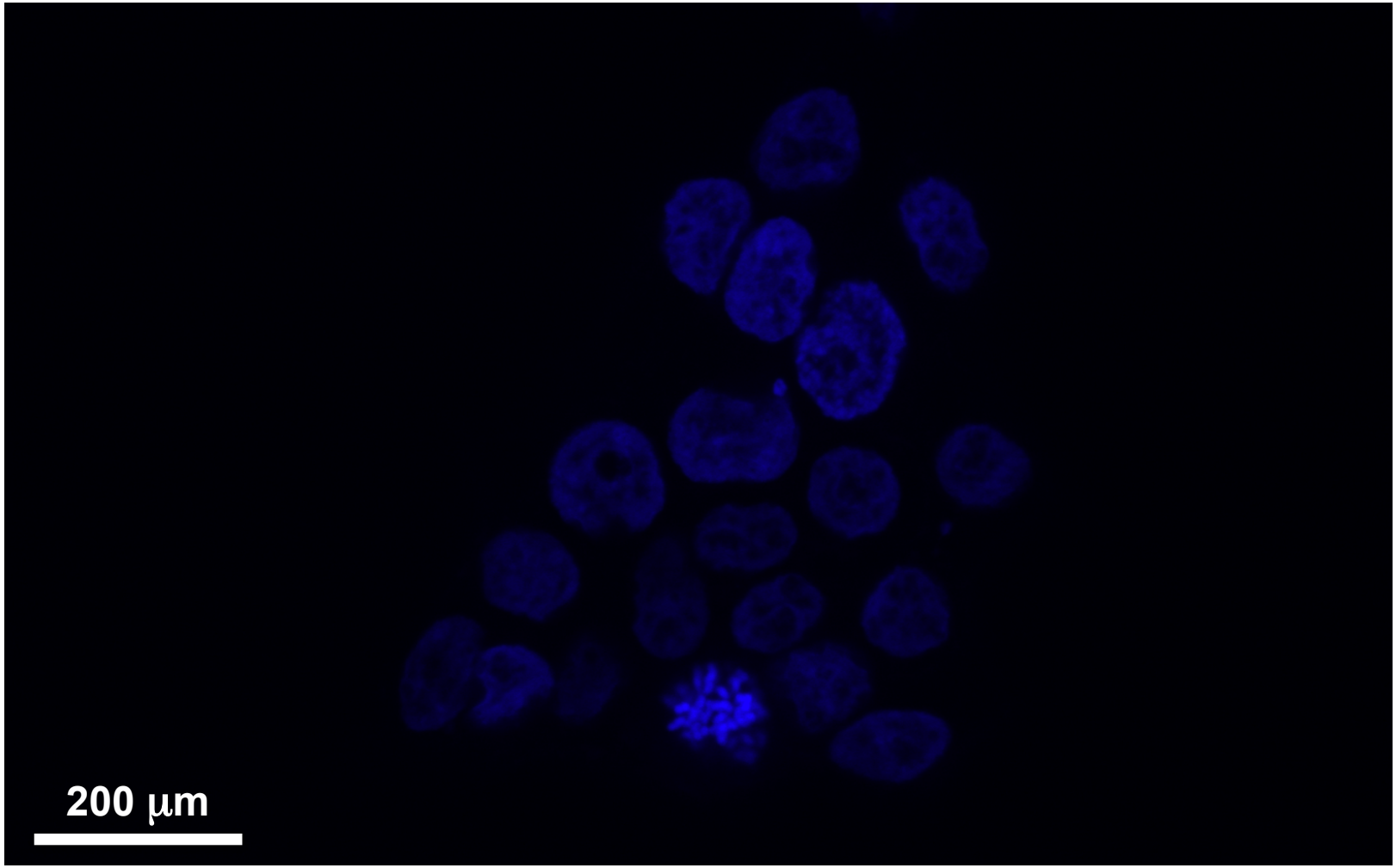

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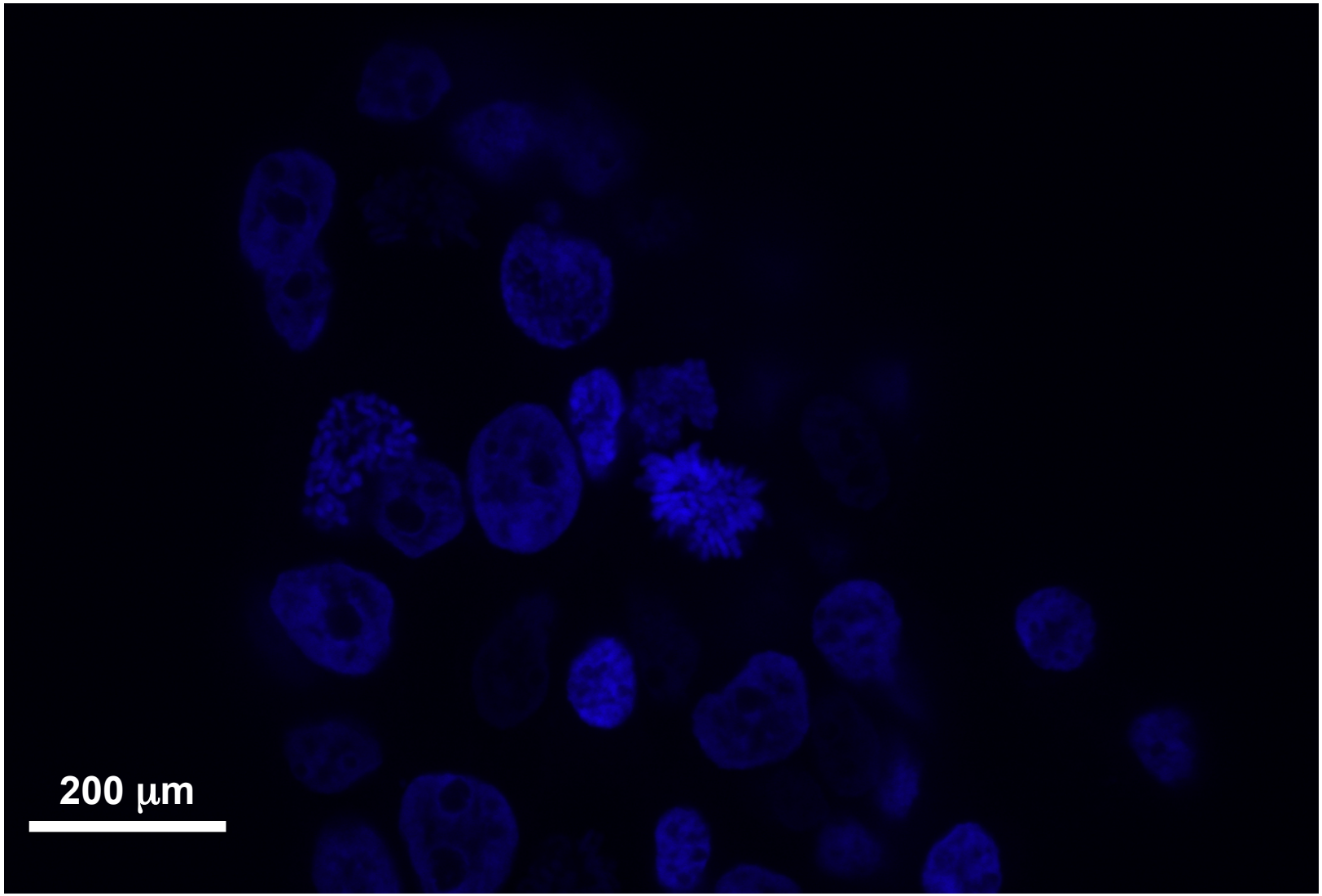

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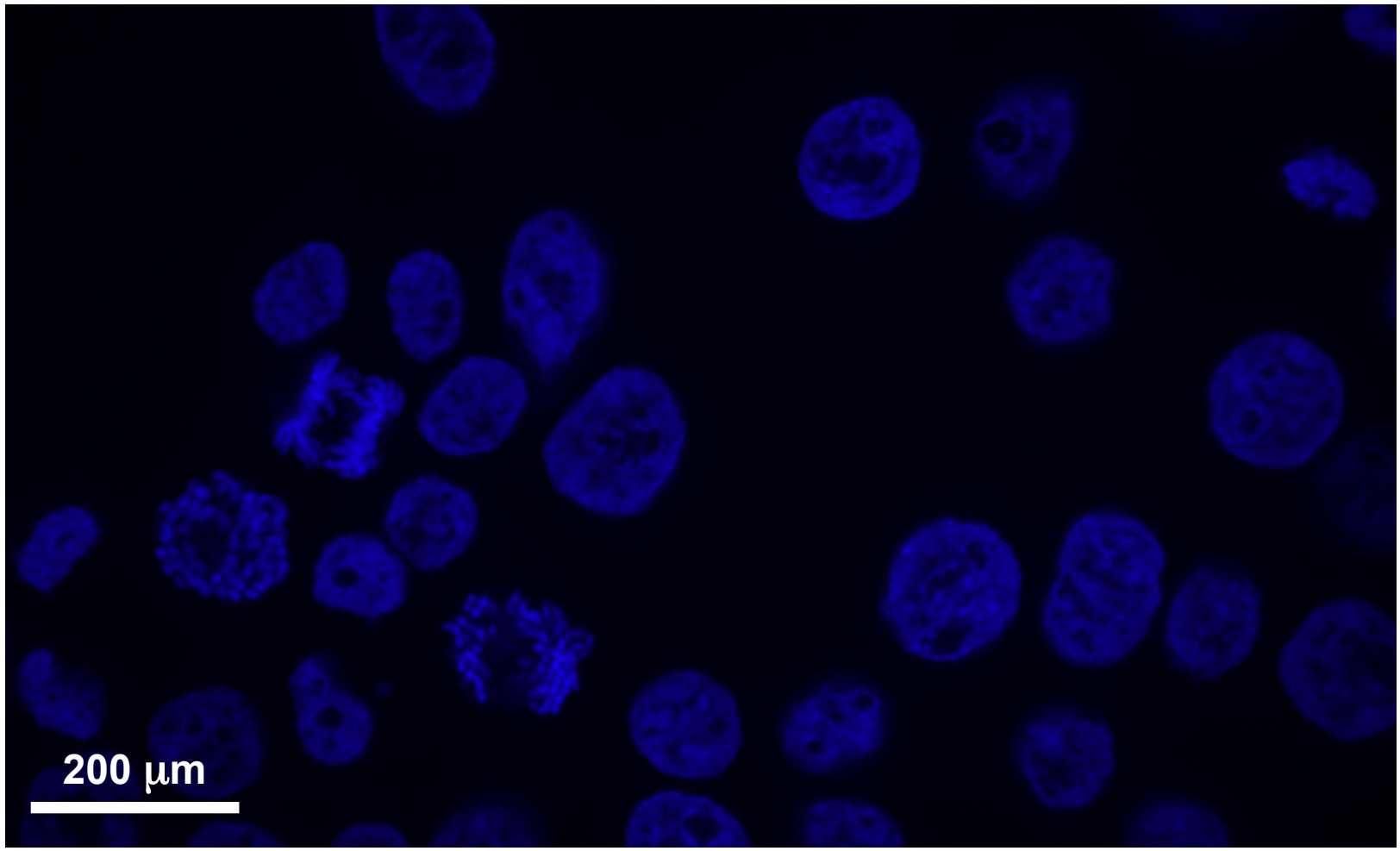

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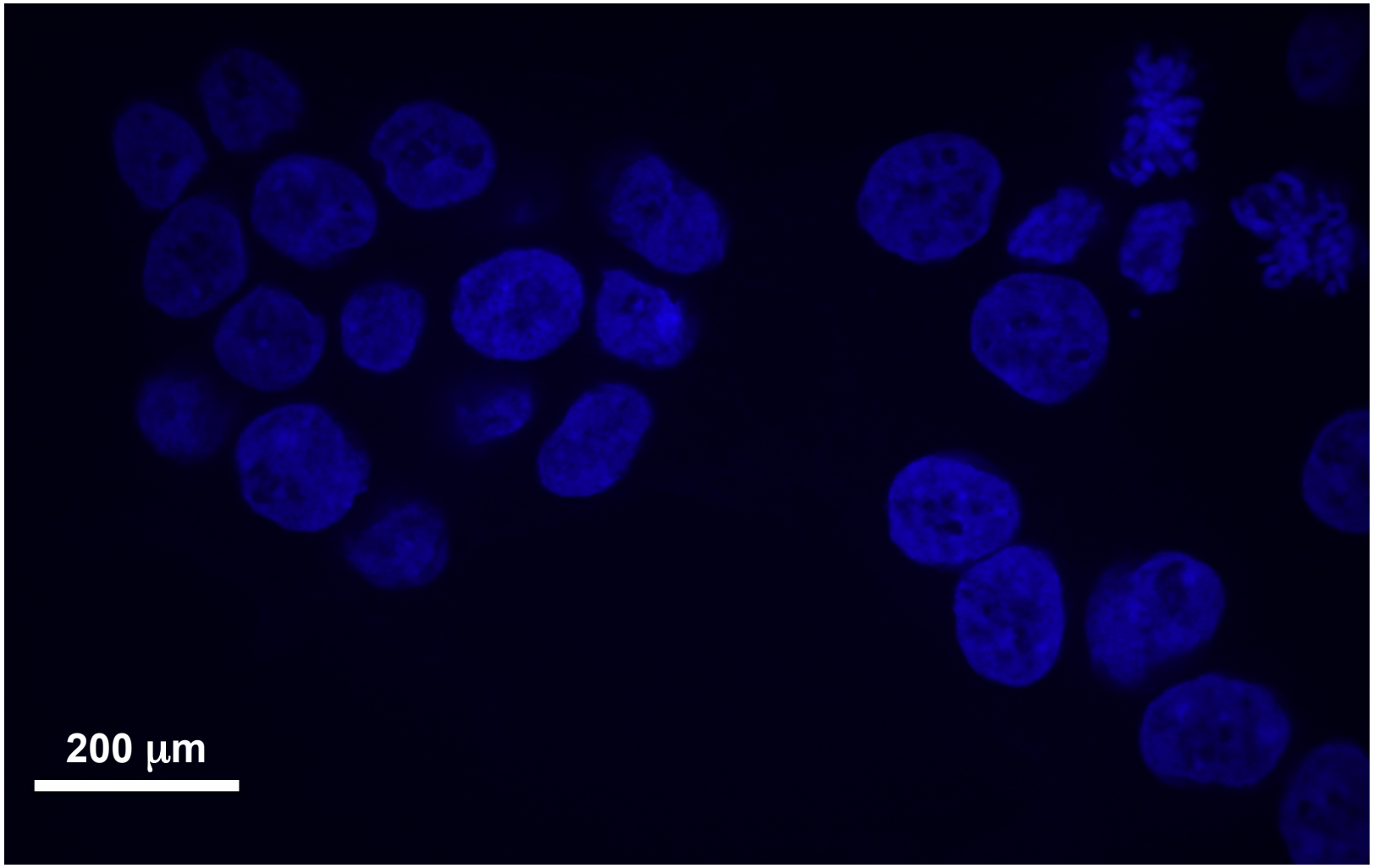

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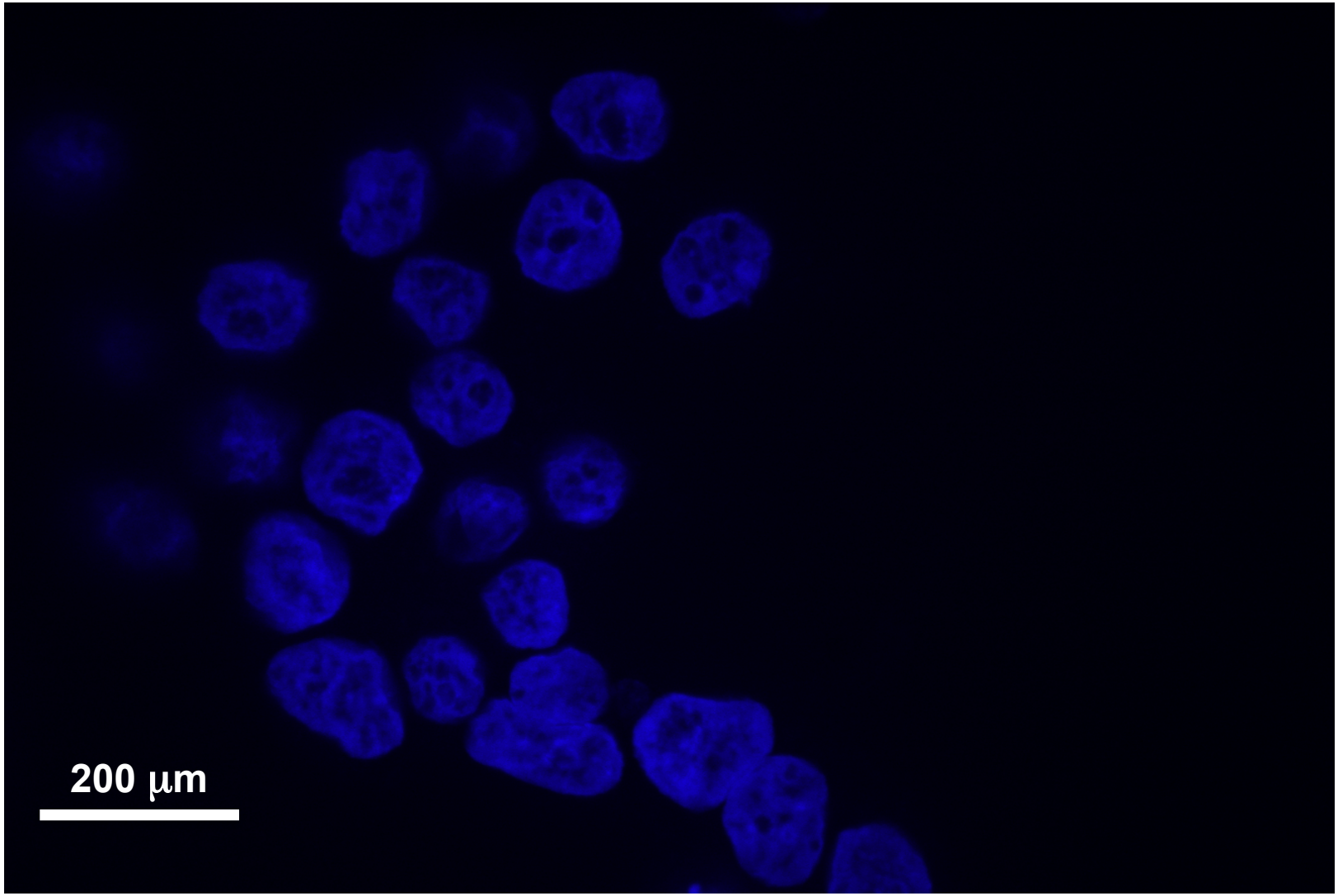

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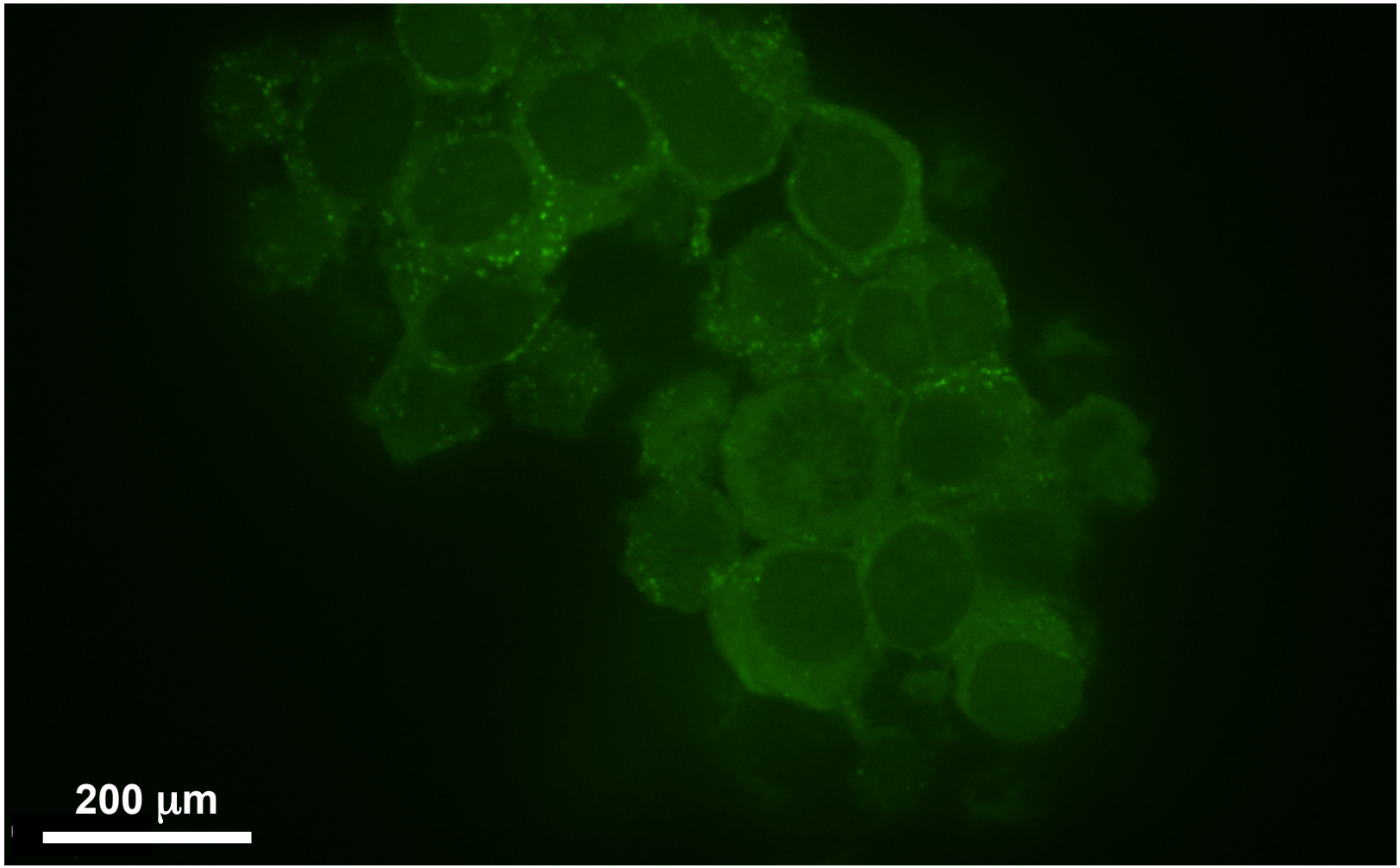

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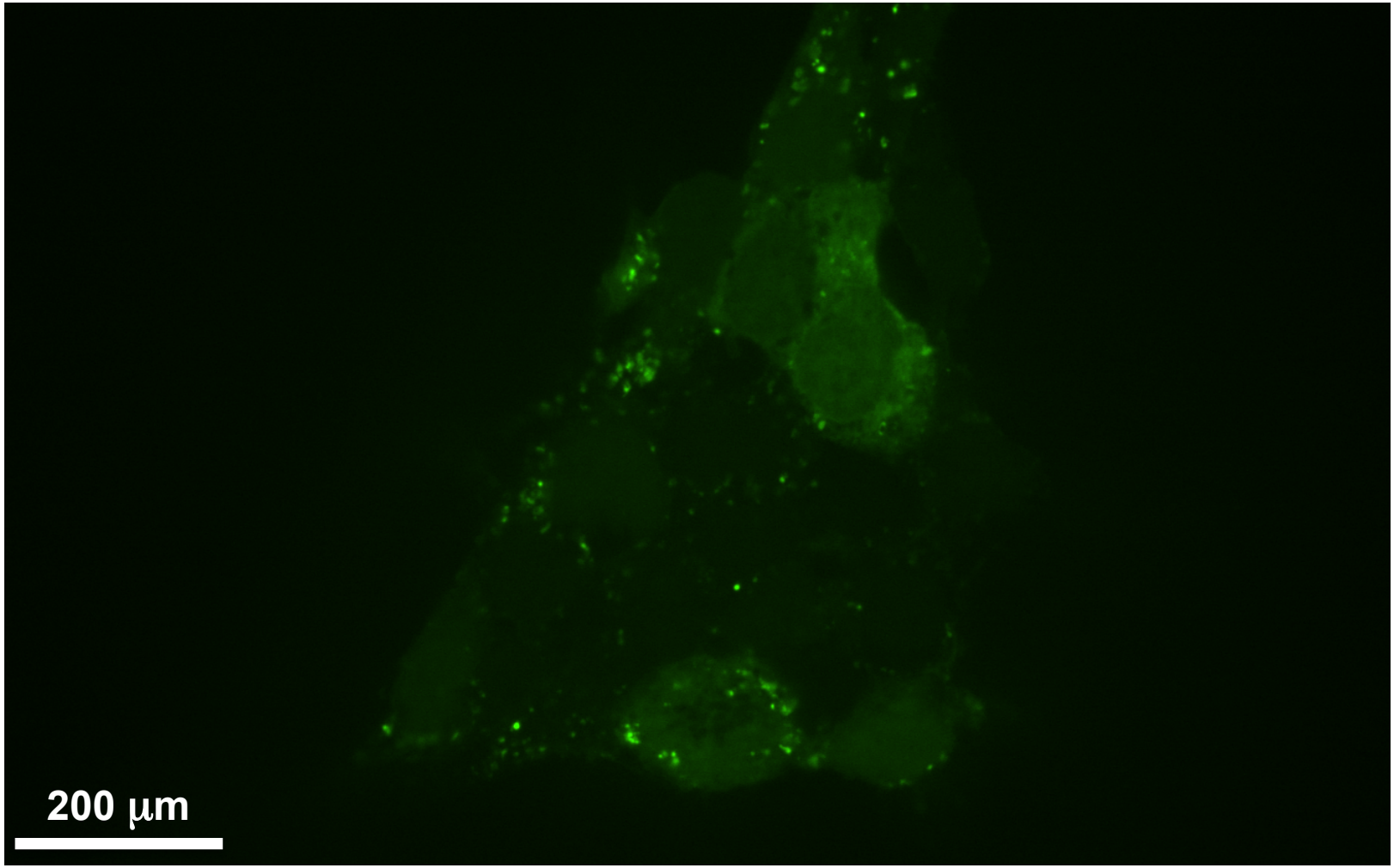

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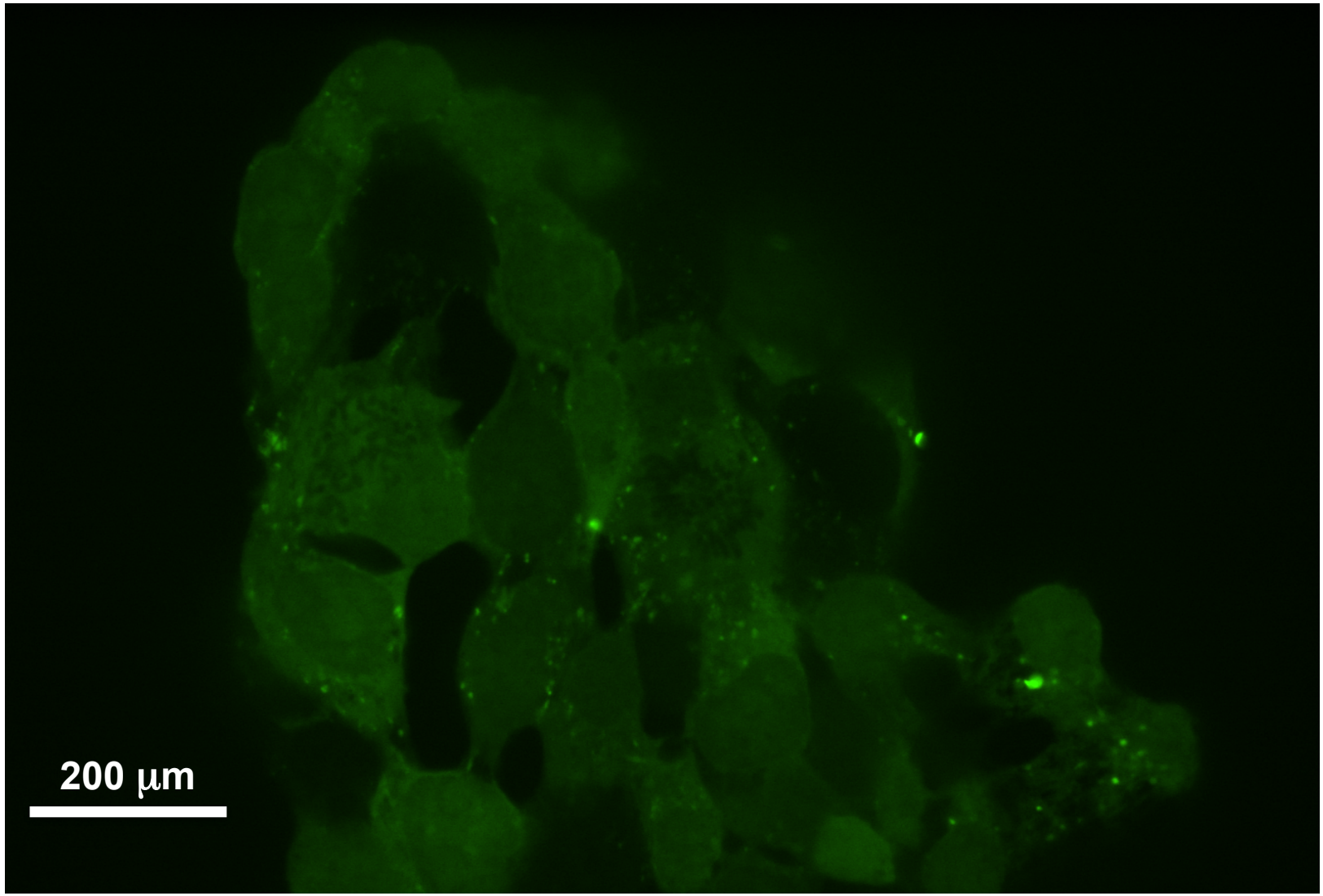

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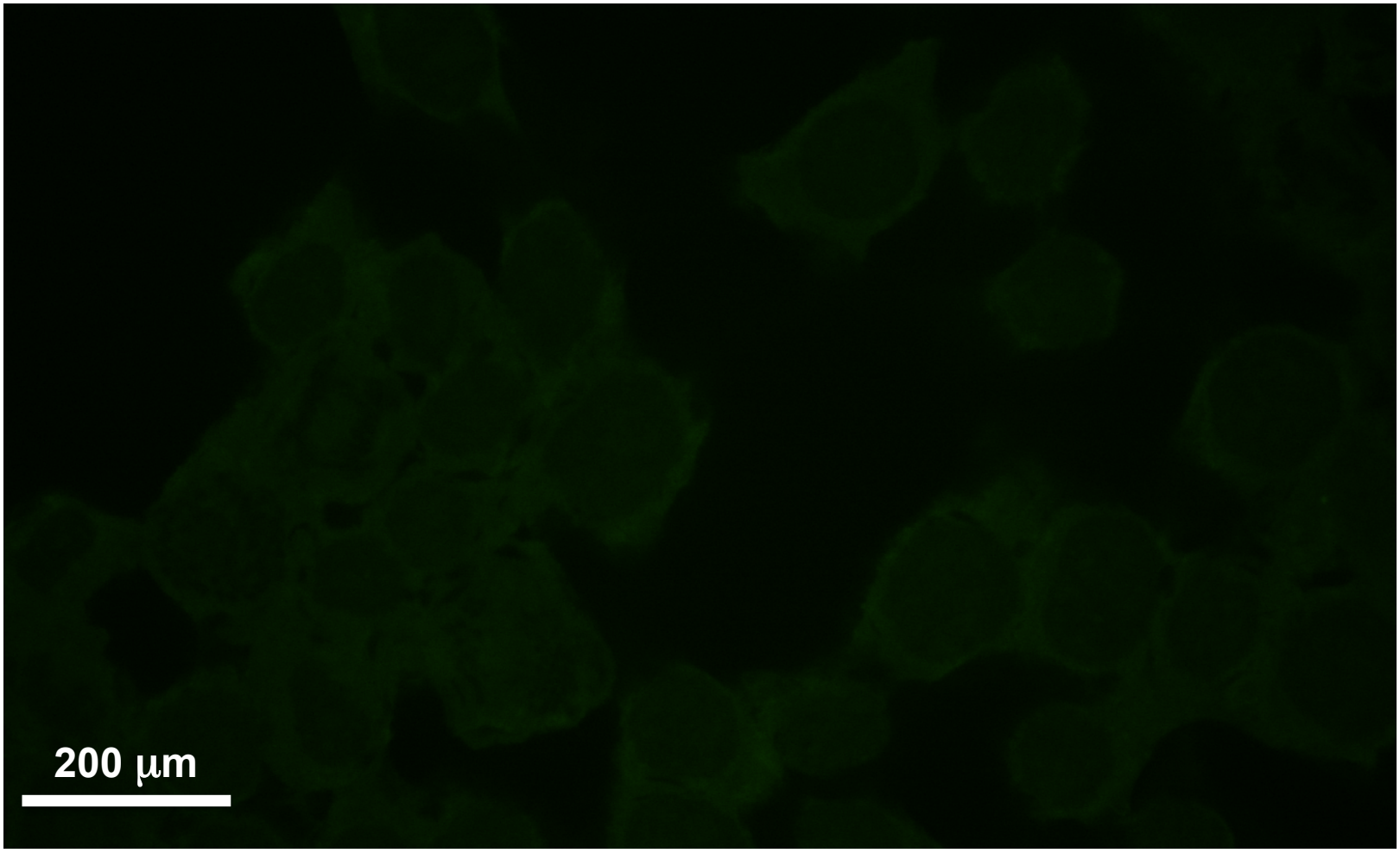

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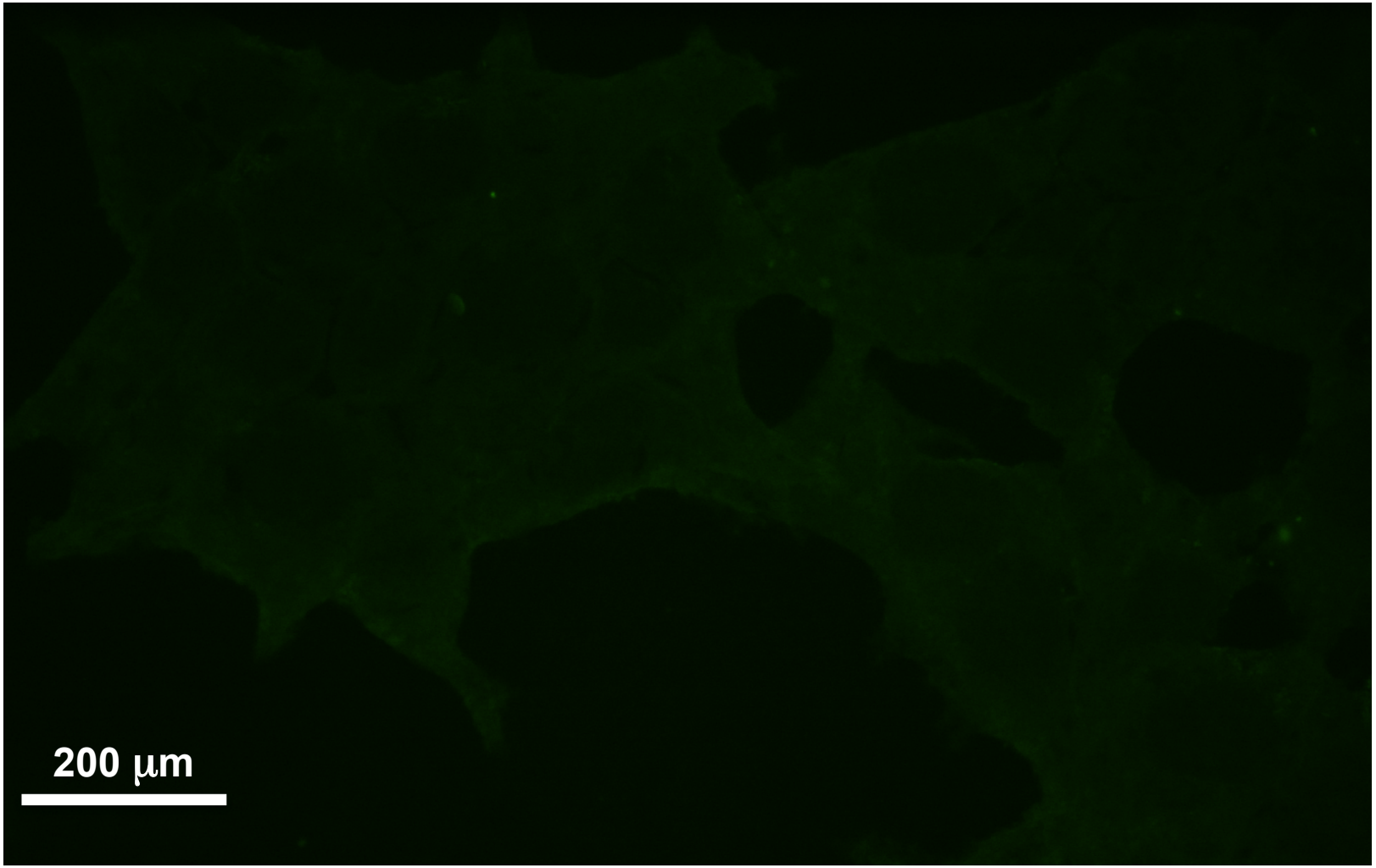

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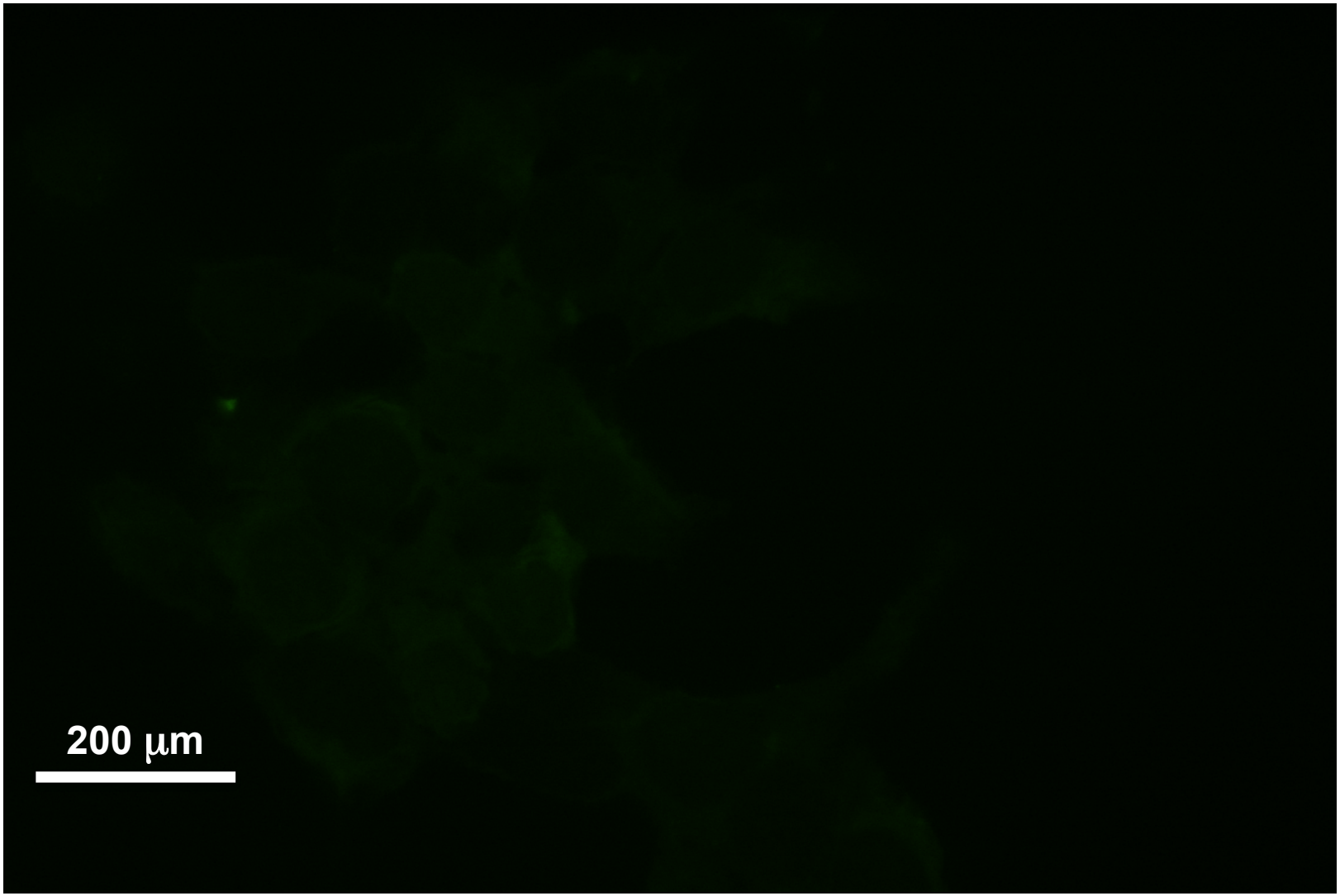

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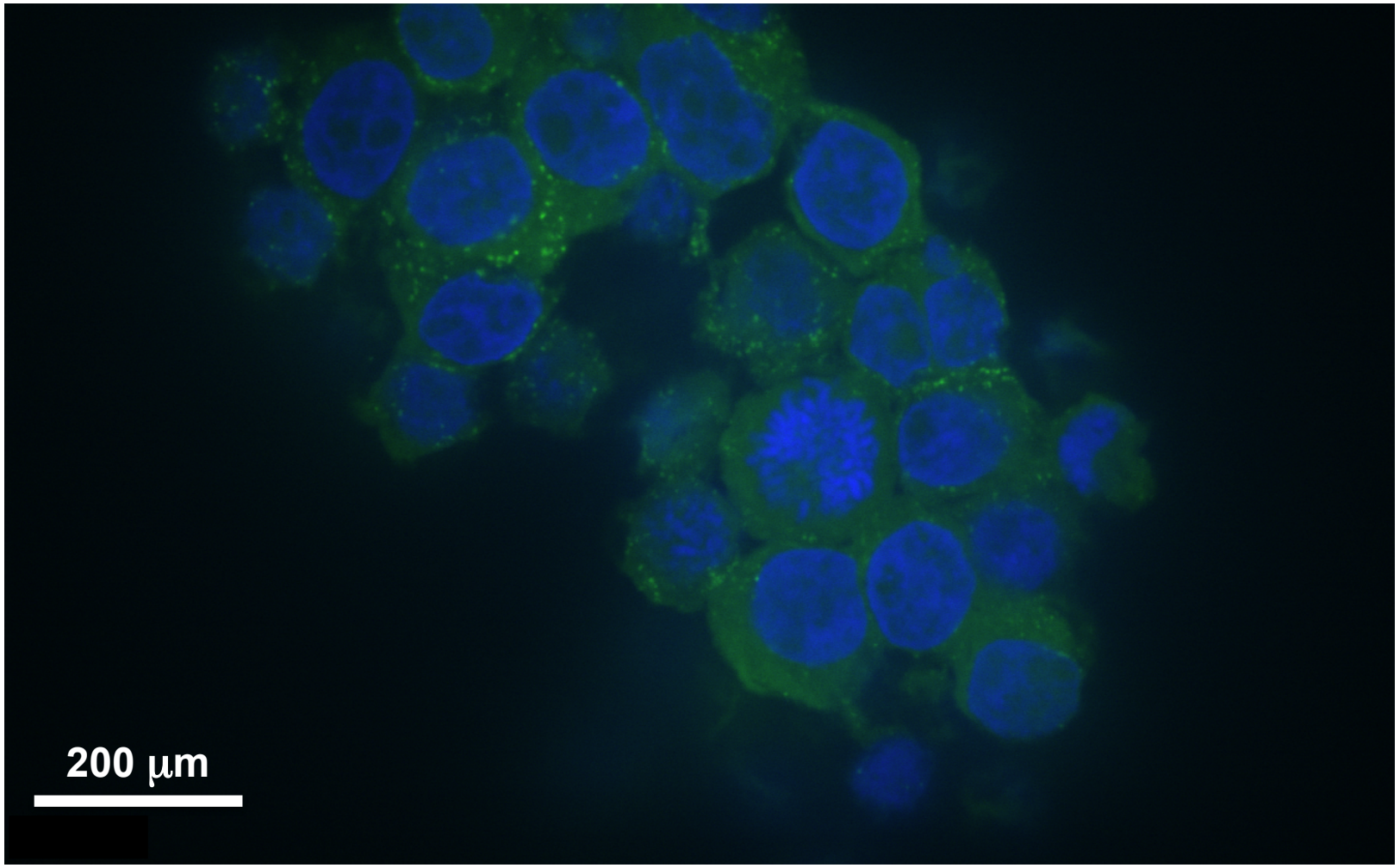

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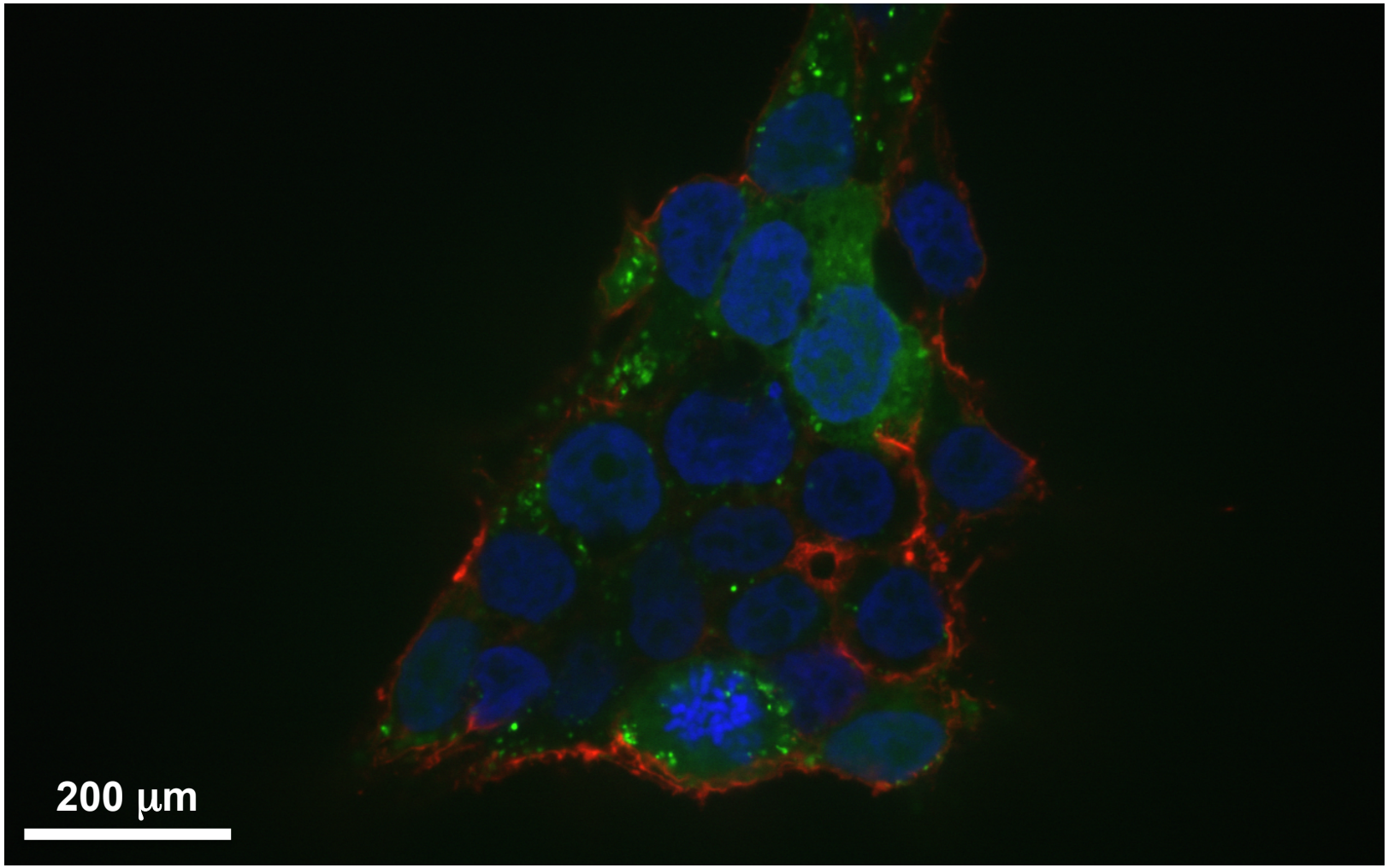

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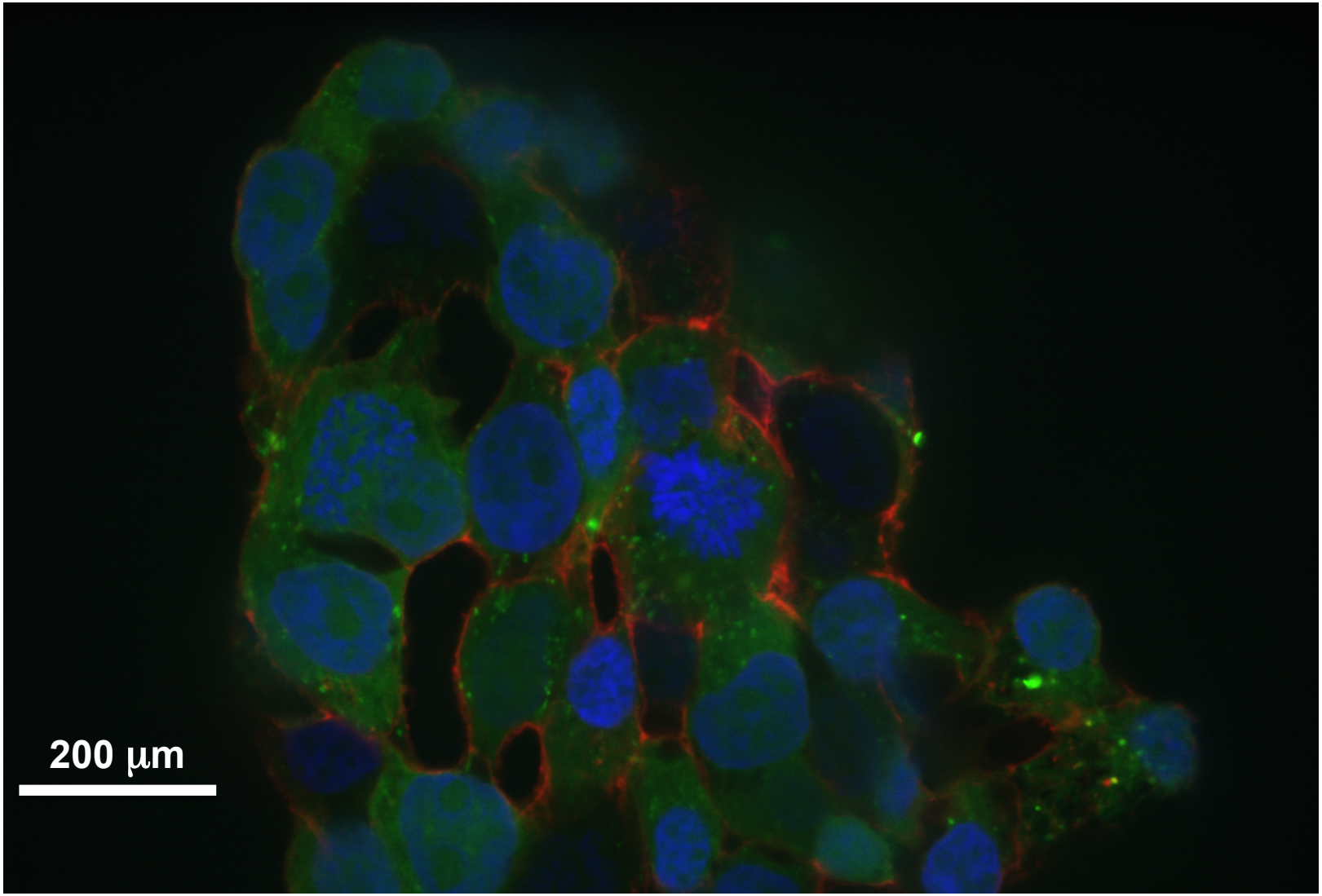

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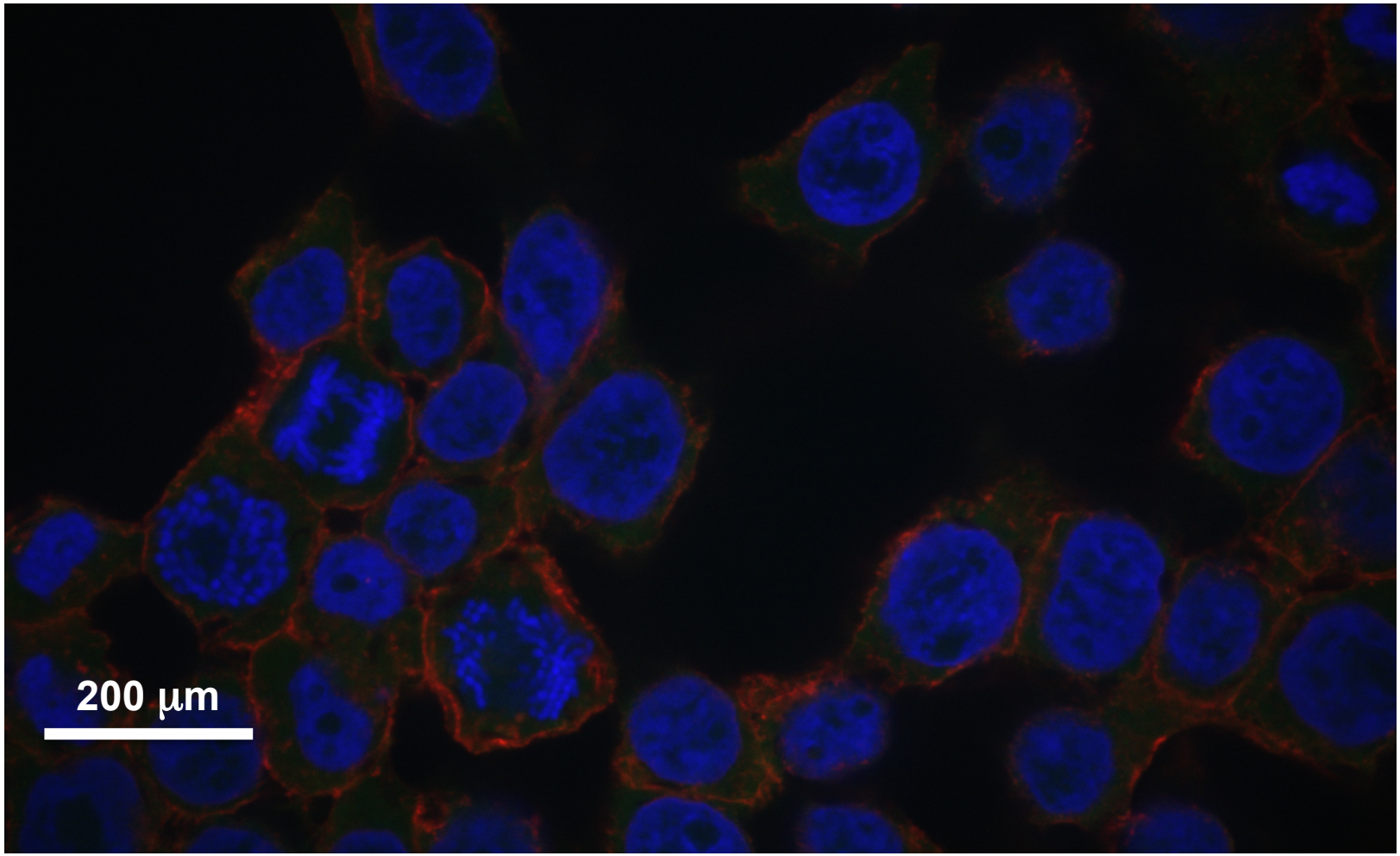

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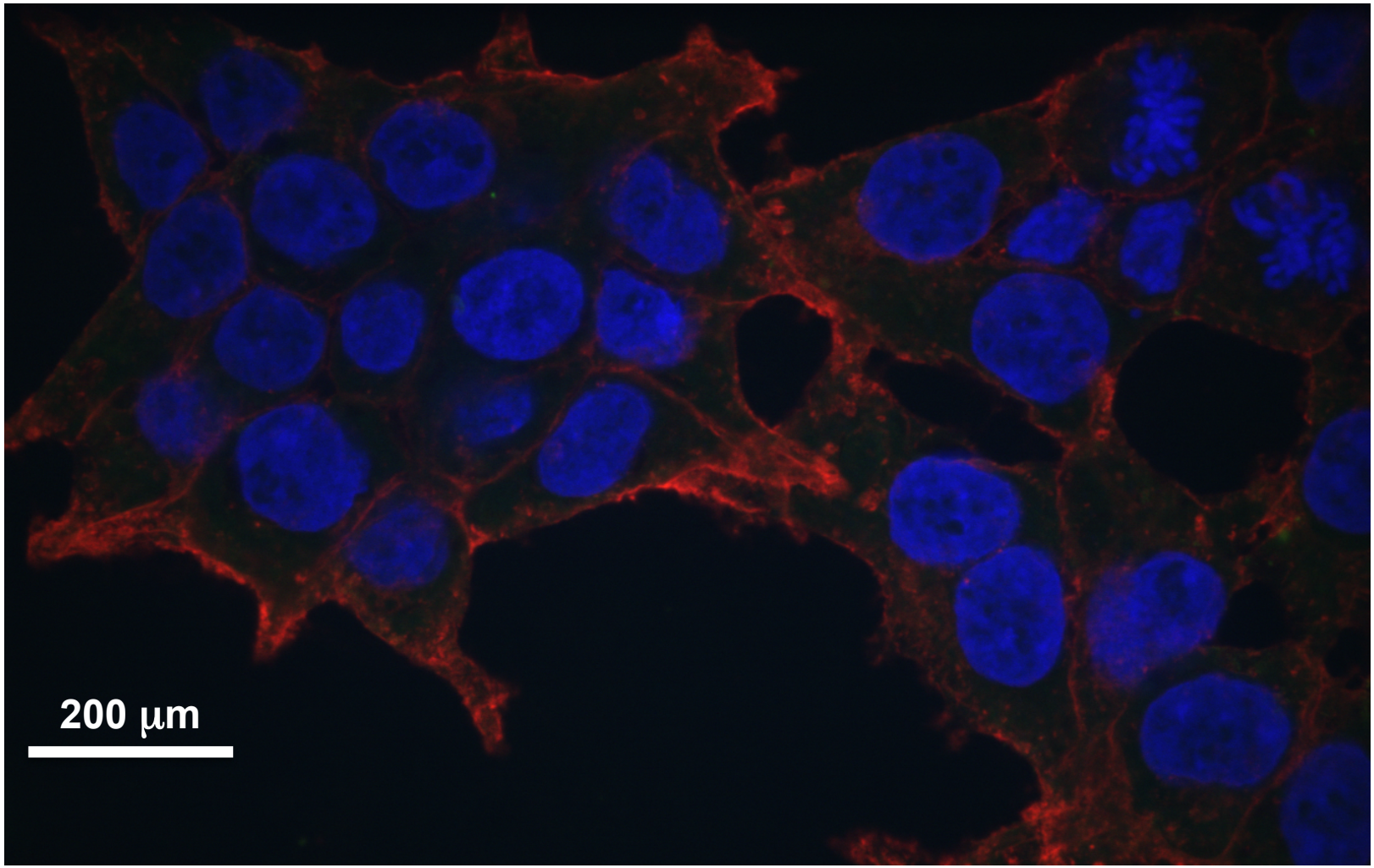

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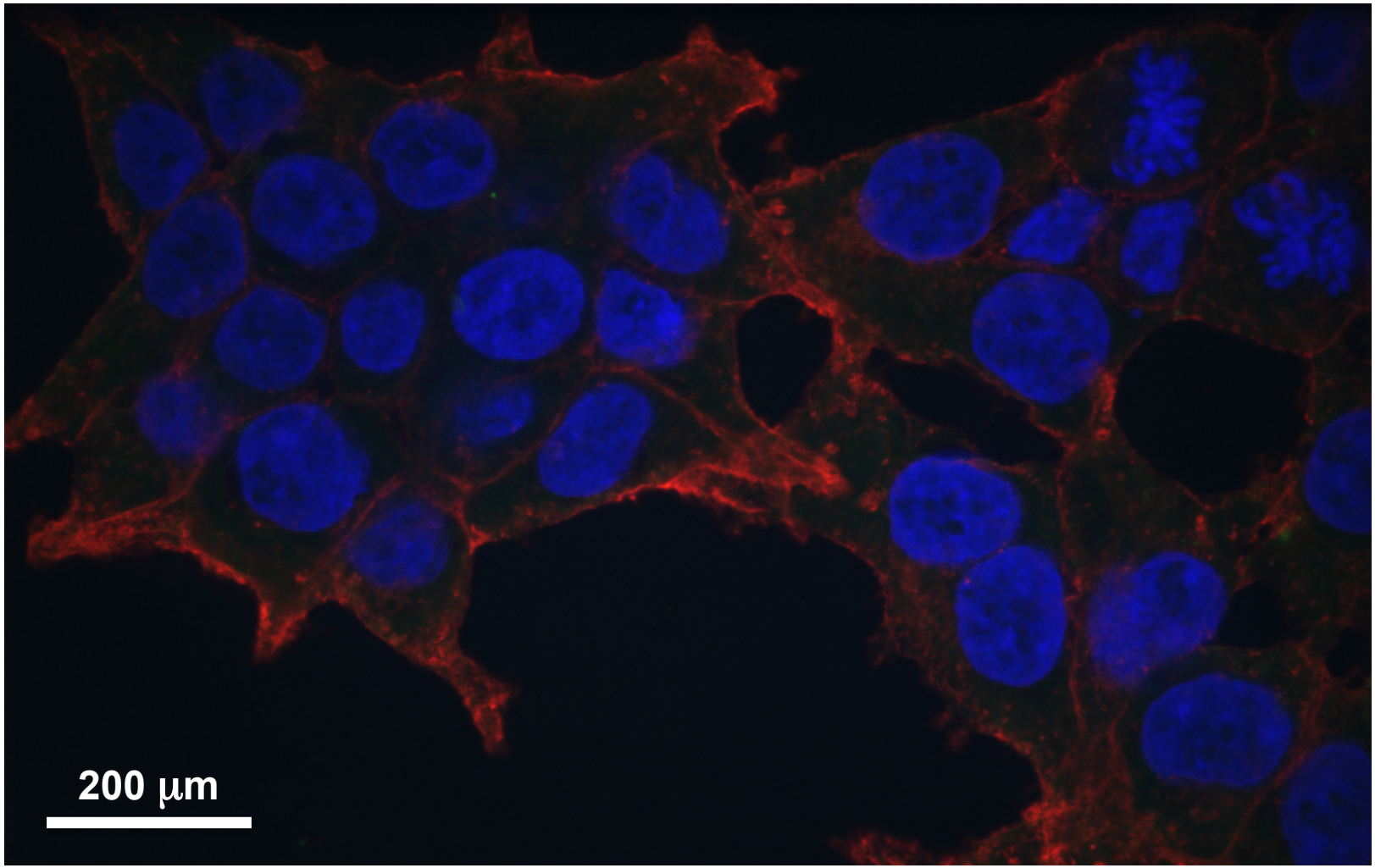

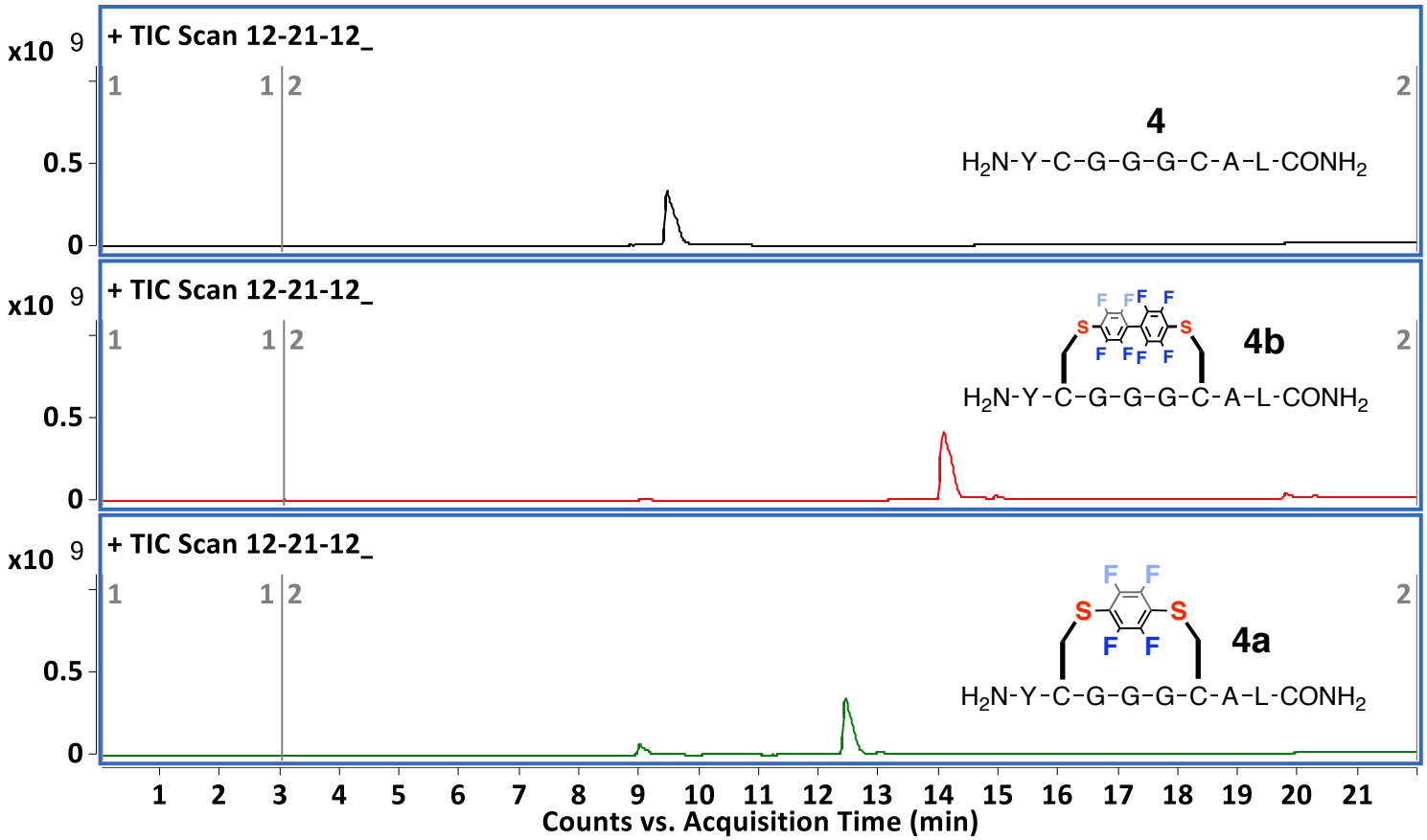
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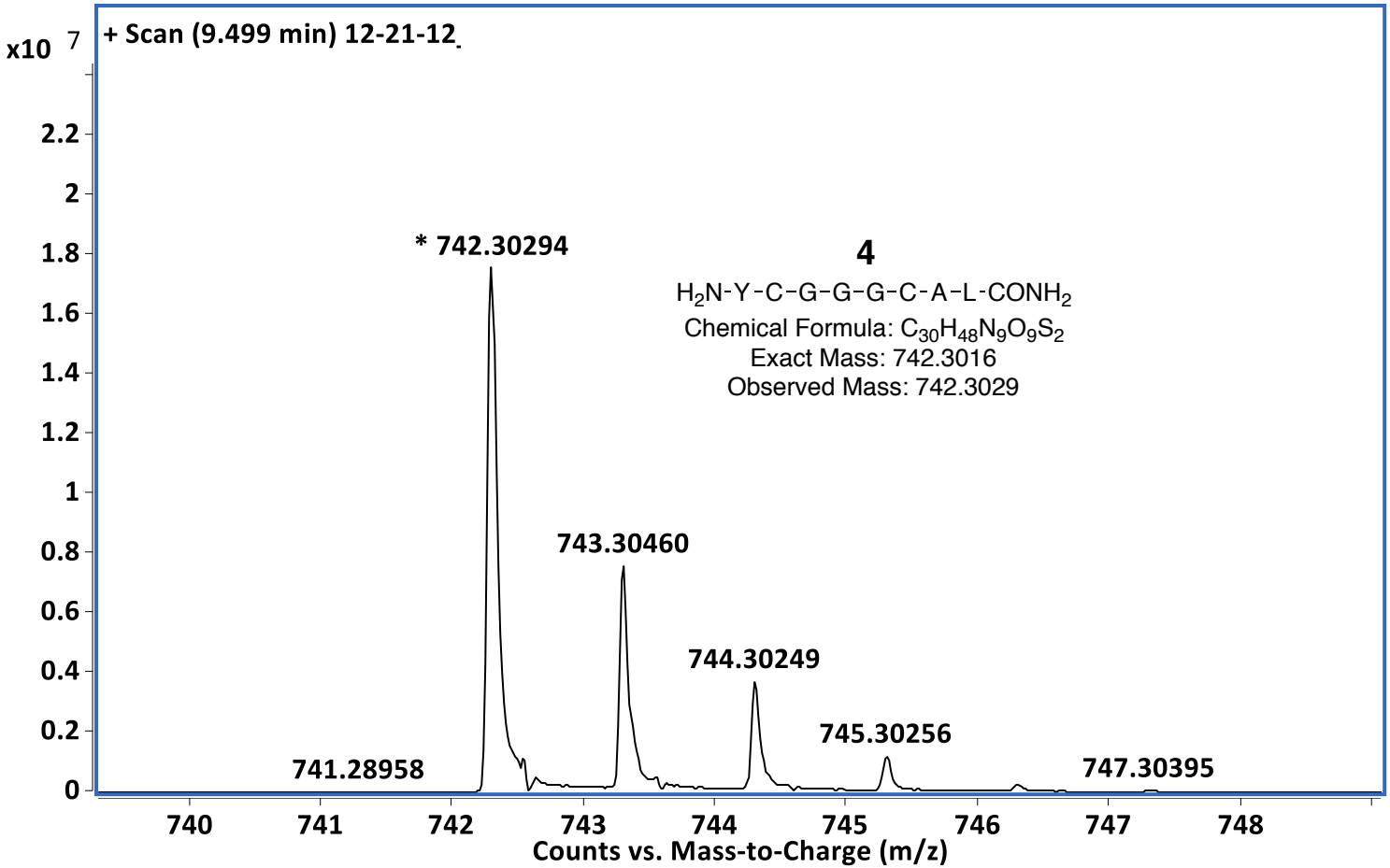
2
0.5
0
4
H2N-Y-C-G-G-G-C-A-L-CONH2
4b
4a
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
Counts vs. Acquisition Time (min)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2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
* 742.30294
743.30460
744.30249
745.30256
741.28958
747.30395
4
H2N-Y-C-G-G-G-C-A-L-CONH2
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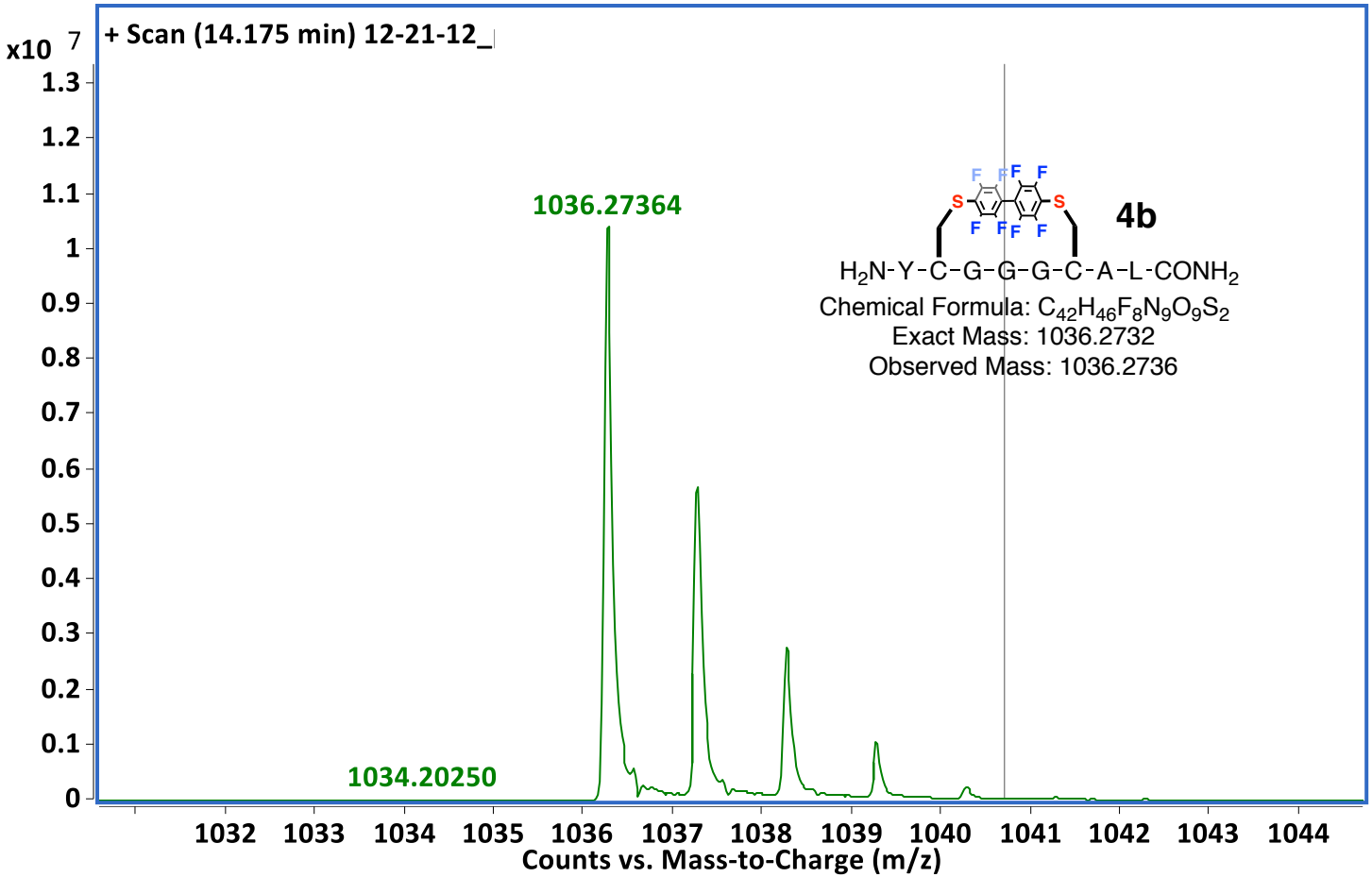
Exact Mass: 742.3016
Observed Mass: 742.3029
740
741
742
743
744
745
746
747
748
Counts vs. Mass-to-Charge (m/z)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1.1
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0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
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1034.20250
4b
H2N-Y-C-G-G-G-C-A-L-CONH2
Chemical Formula: C42H46F8N9O9S2
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Observed Mass: 1036.2736
1032
1033
1034
1035
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1037
1038
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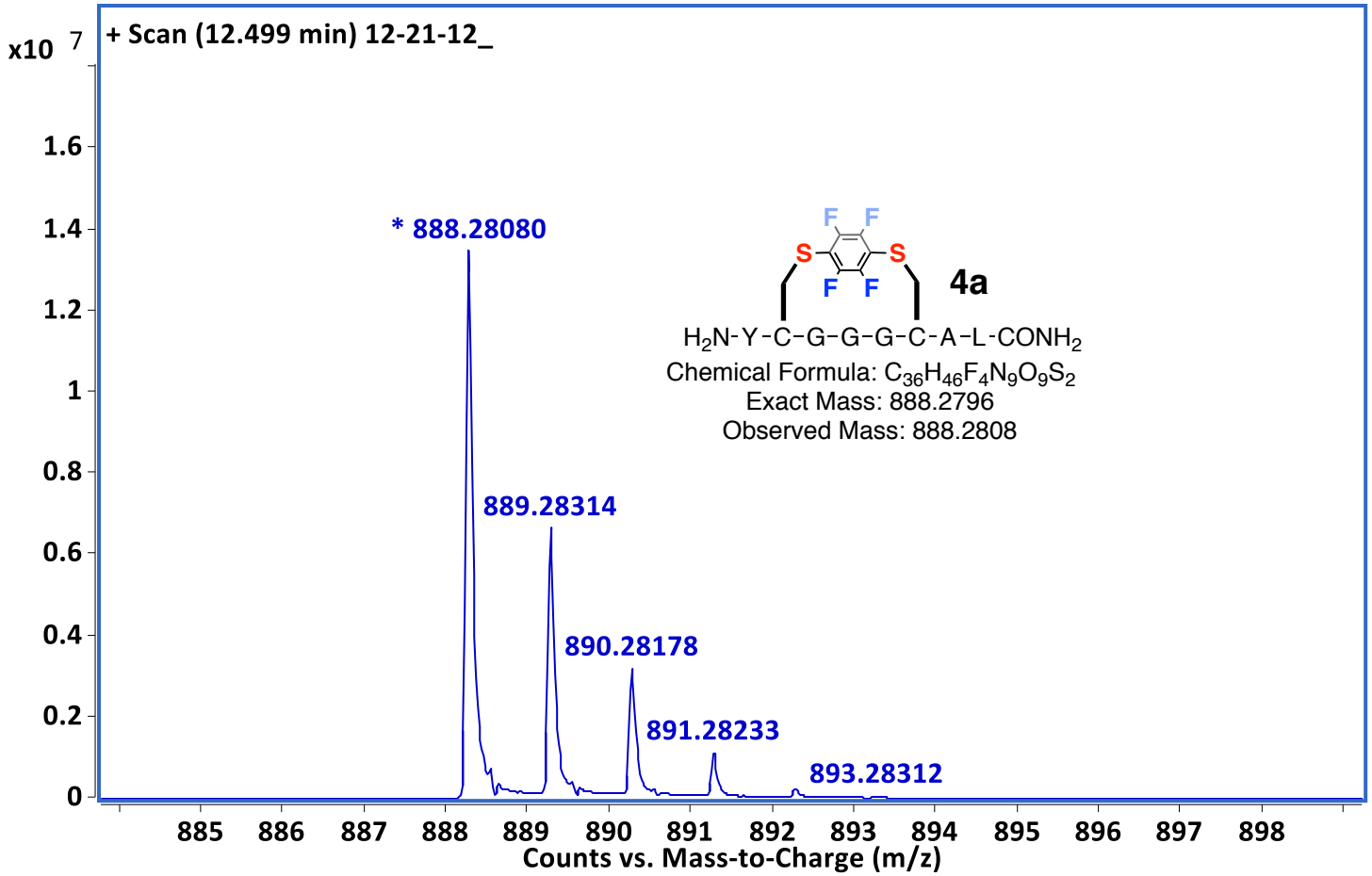
1040
1041
1042
1043
1044
Counts vs. Mass-to-Charge (m/z)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+ Scan (12.499 min) 12-21-12_
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890.28178
891.28233
893.28312
F F
S S
F F
4a
H2N-Y-C-G-G-G-C-A-L-CONH2
Chemical Formula: C36H46F4N9O9S2
Exact Mass: 888.2796
Observed Mass: 888.2808
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
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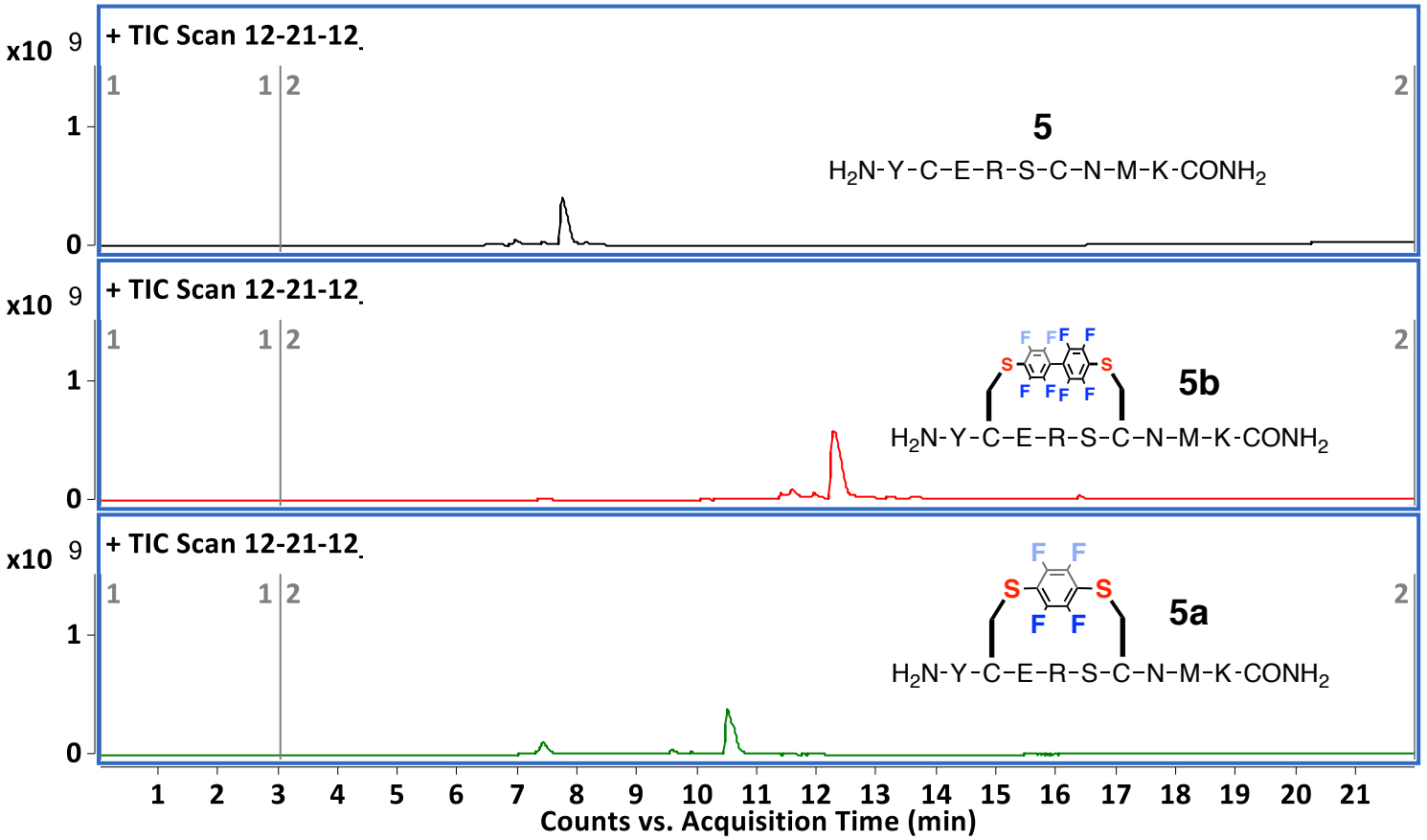
Counts vs. Mass-to-Charge (m/z)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1 2
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0
5
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x10 9
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1
1 2
2
0
5b
H2N-Y-C-E-R-S-C-N-M-K-CONH2
x10 9
+ TIC Scan 12-21-12
1
1 2
2
0
5a
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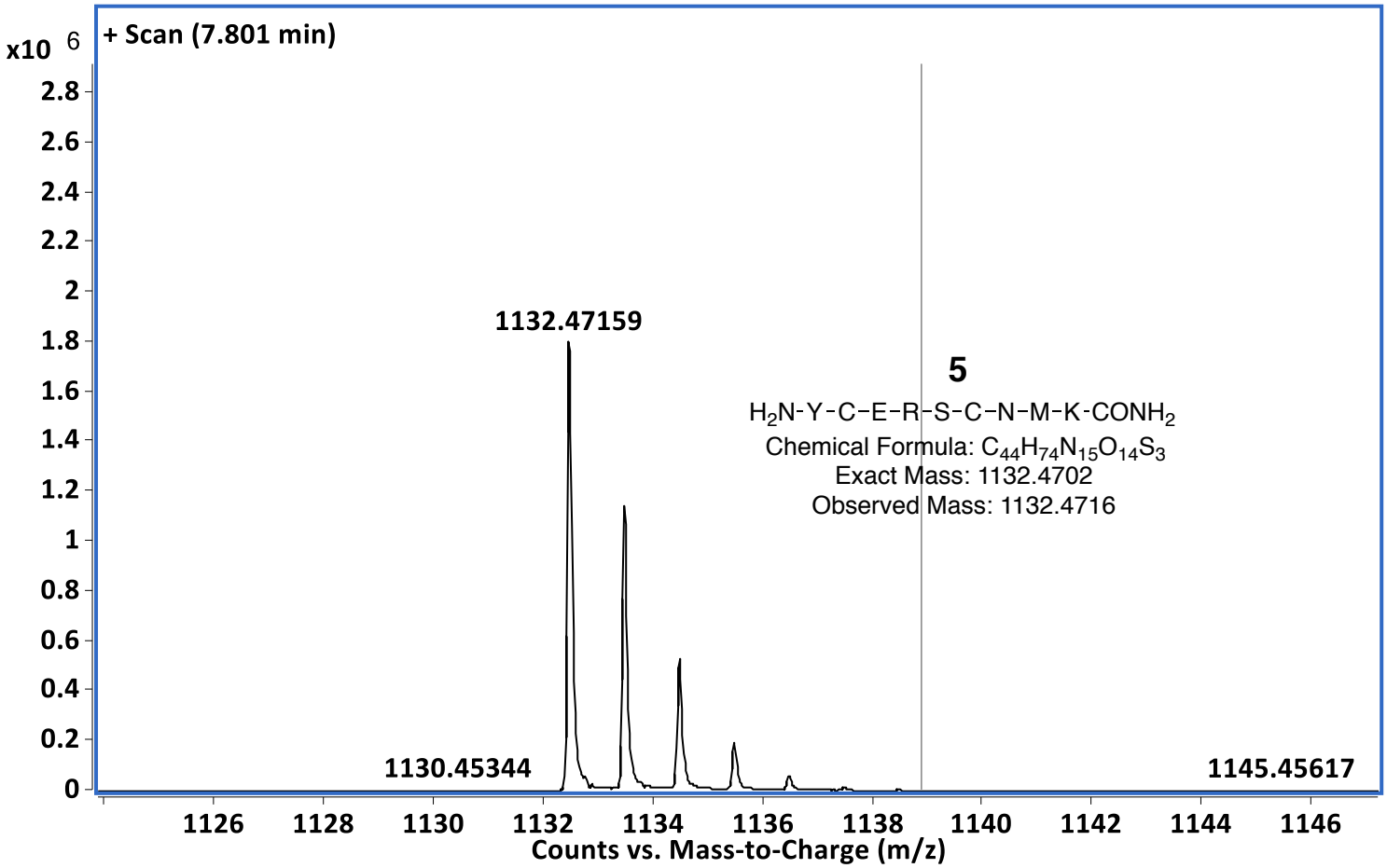
Counts vs. Acquisition Time (min)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2.4
2.2
2
1.8
1.6
1.4
1.2
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0.2
0
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1132
1134
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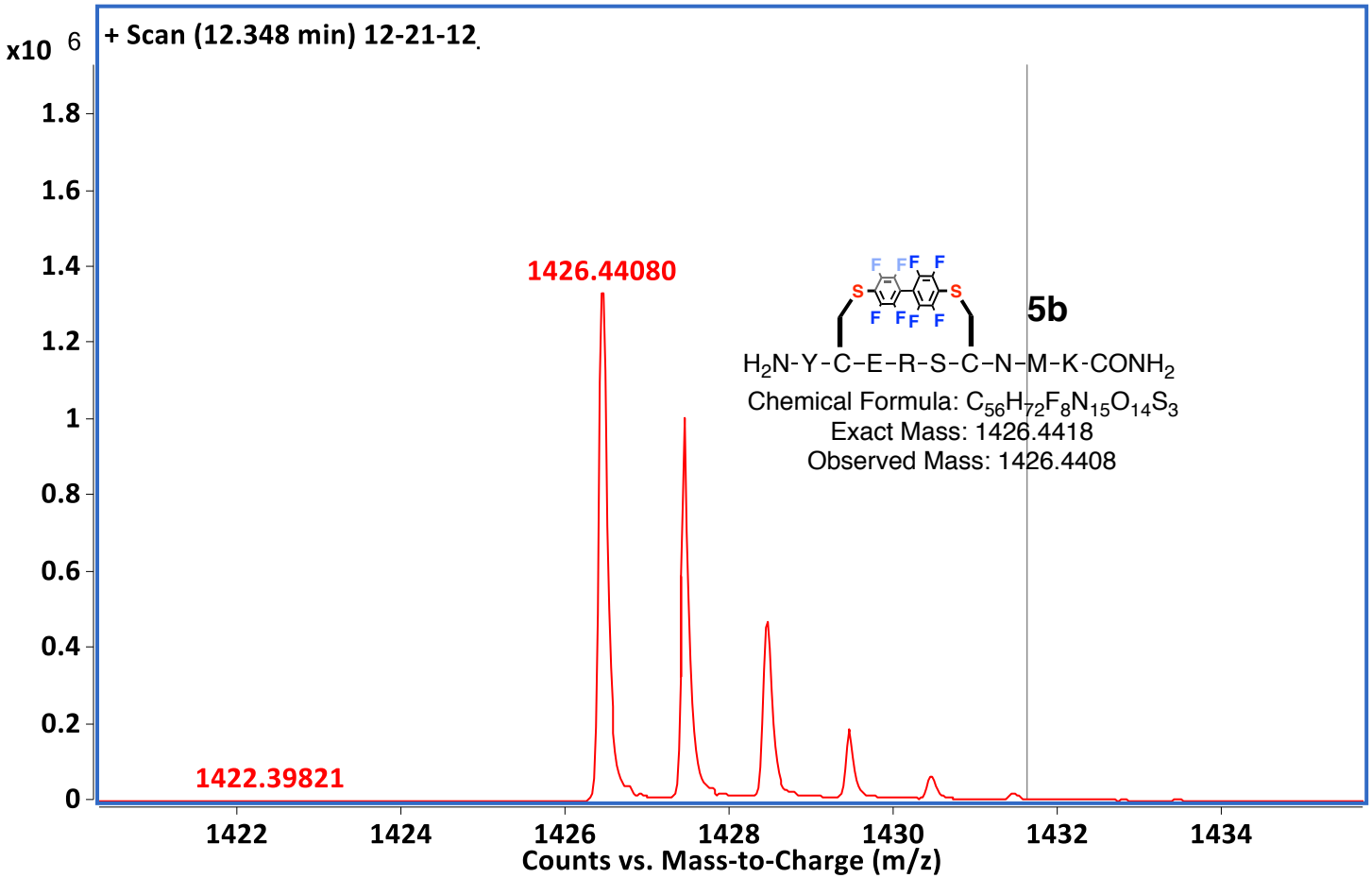
1144
1146
Counts vs. Mass-to-Charge (m/z)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1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
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1422.39821
5b
H2N-Y-C-E-R-S-C-N-M-K-CONH2
Chemical Formula: C56H72F8N15O14S3
Exact Mass: 1426.4418
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1426
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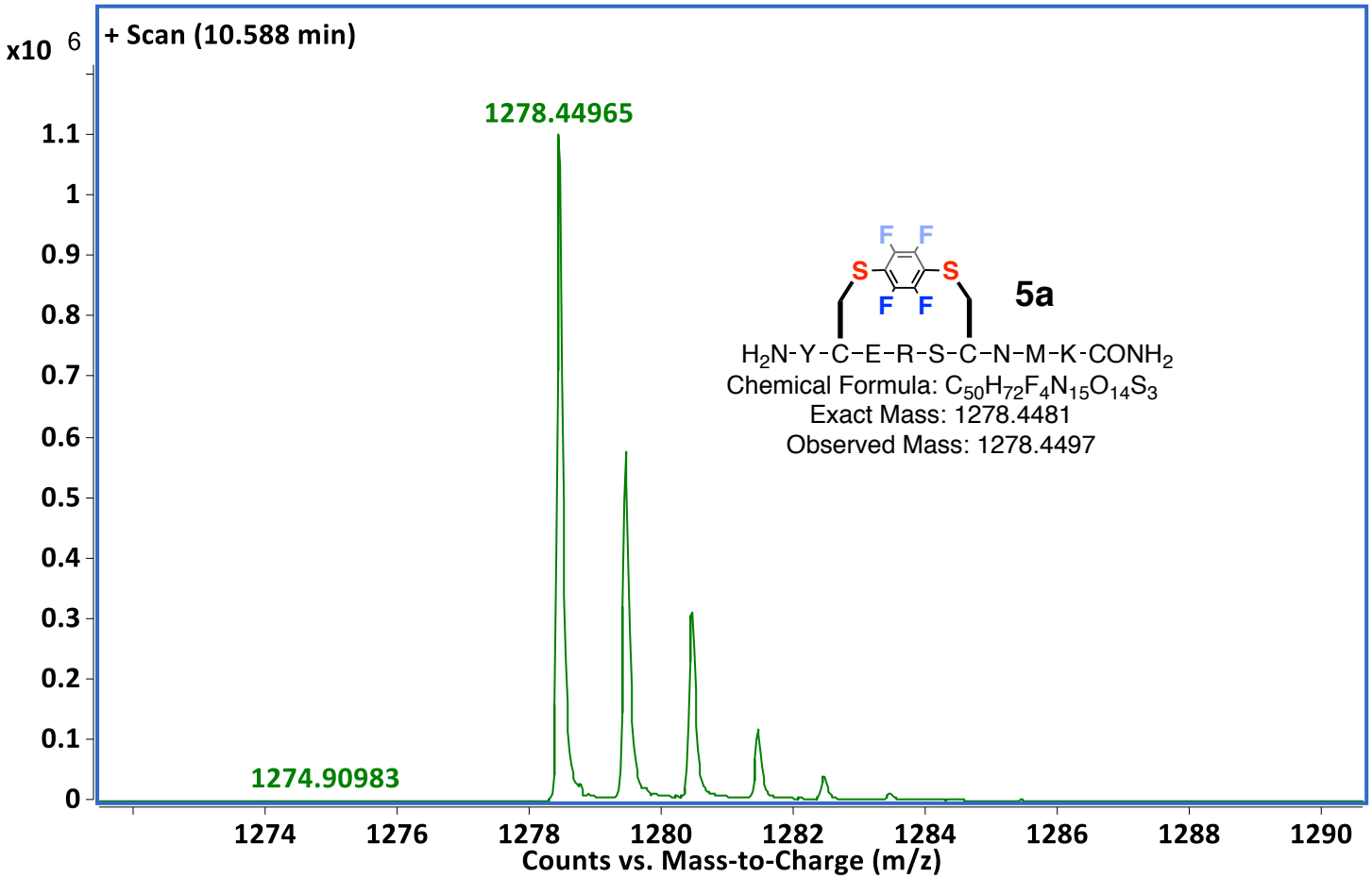
1432
1434
Counts vs. Mass-to-Charge (m/z)


x10 6
+ Scan (10.588 min)
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1274.90983
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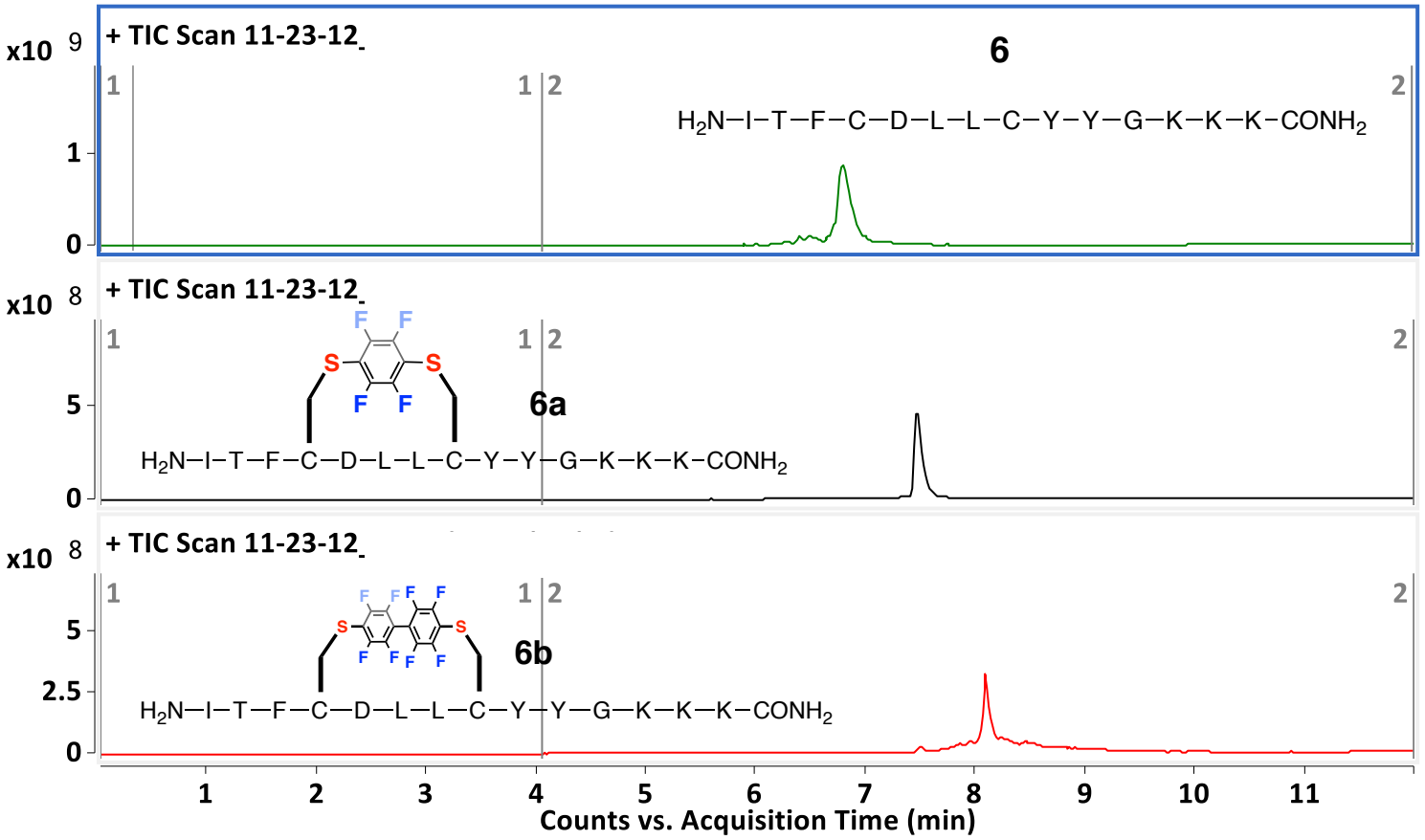
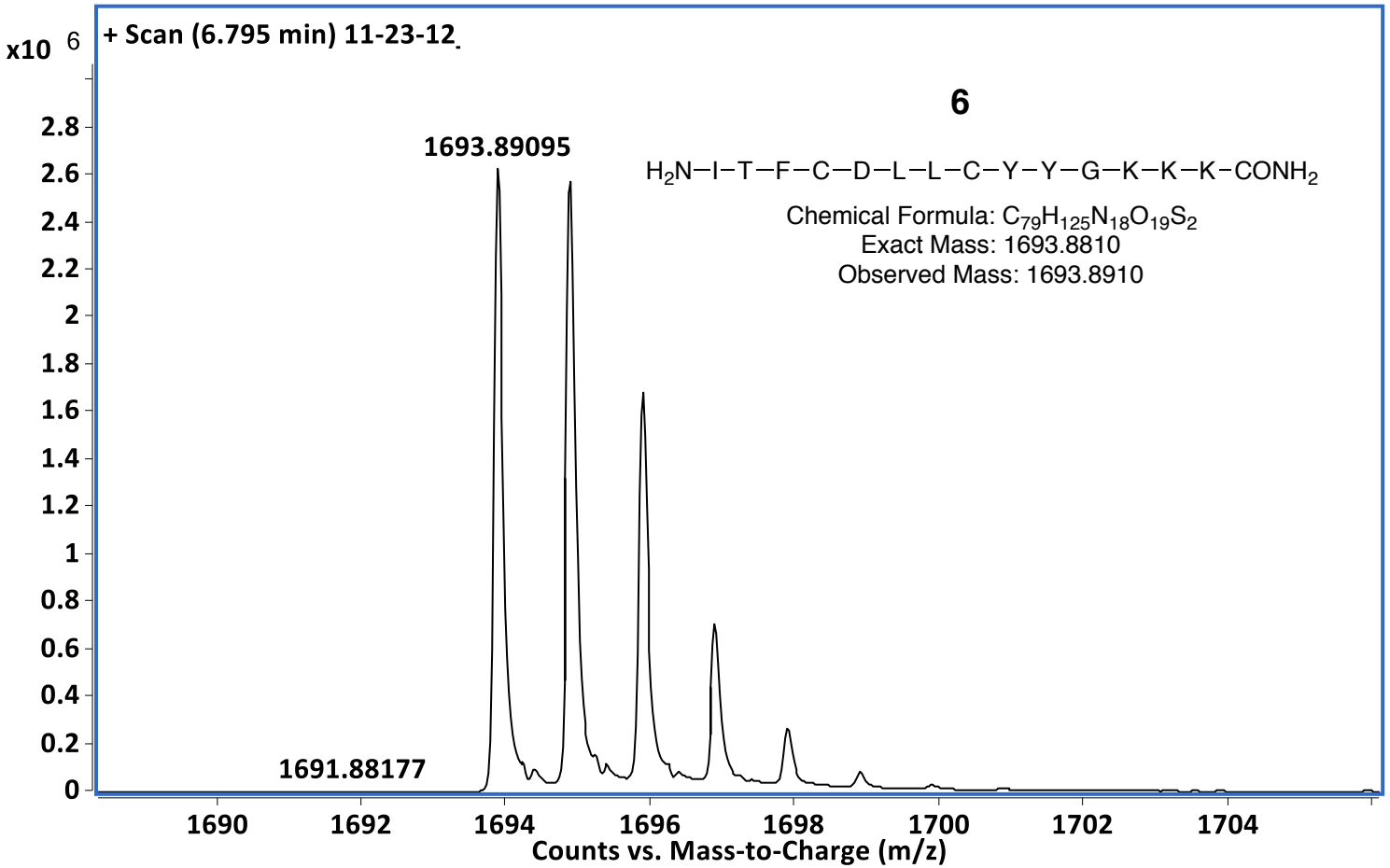
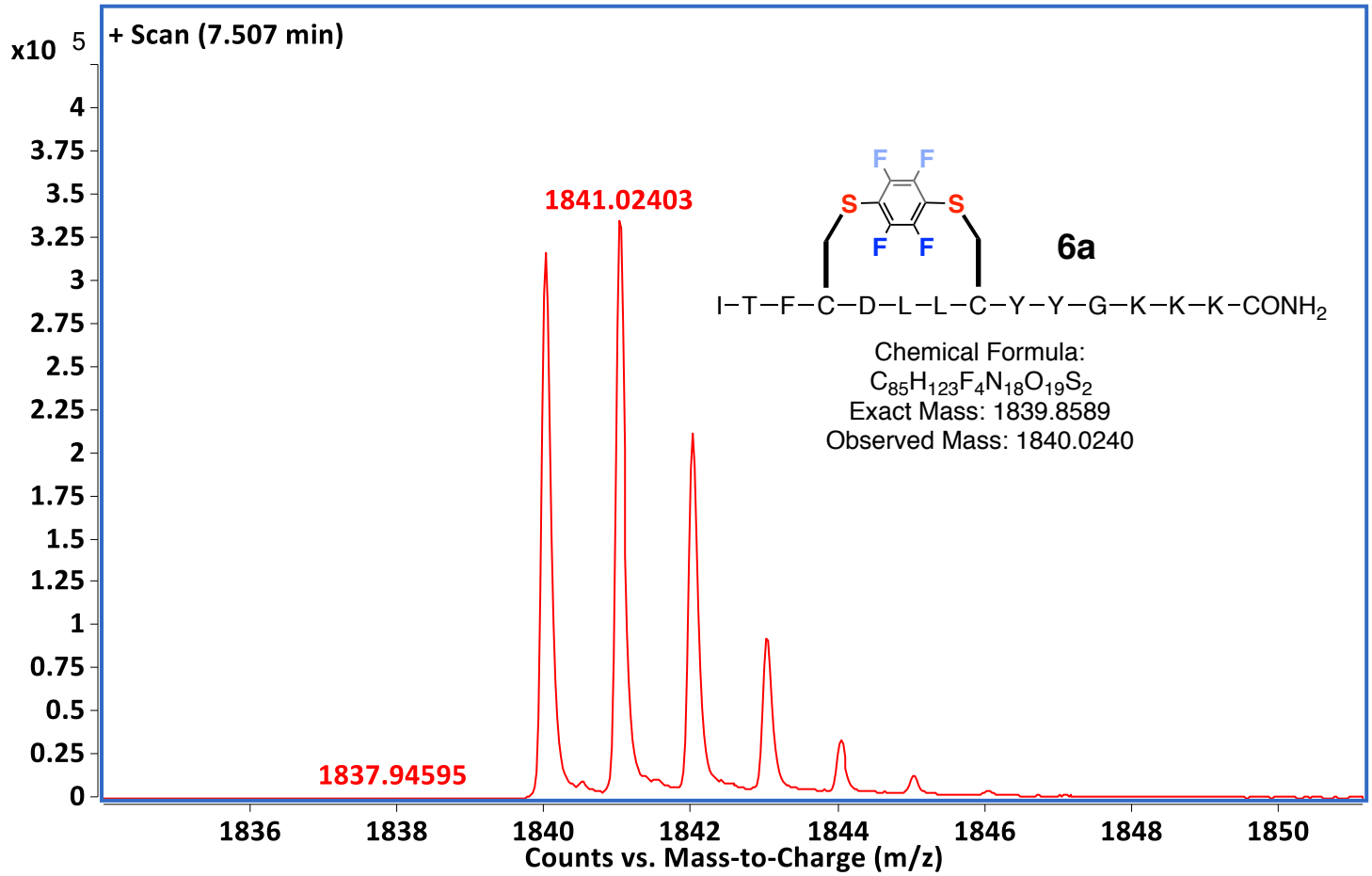
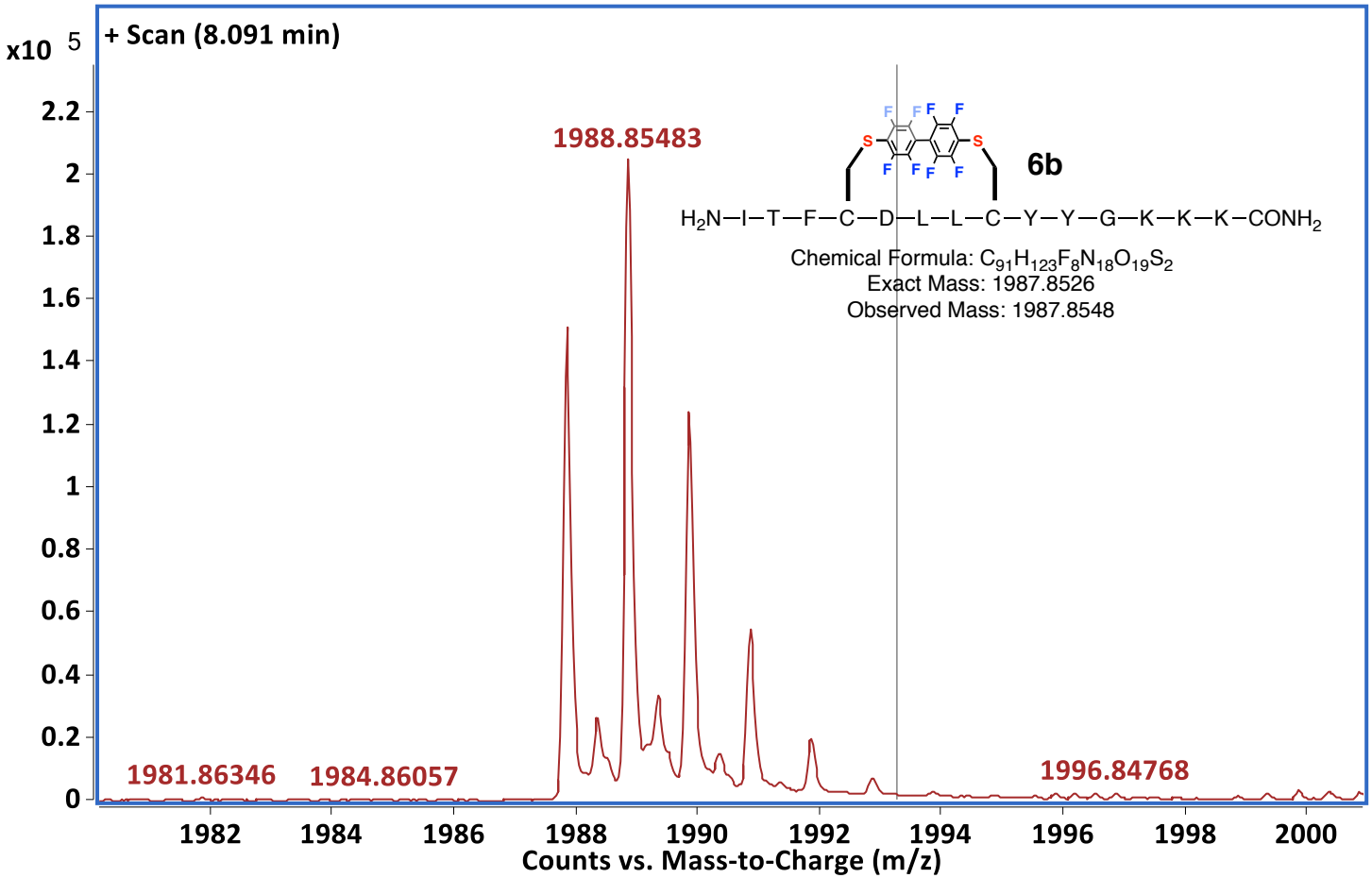
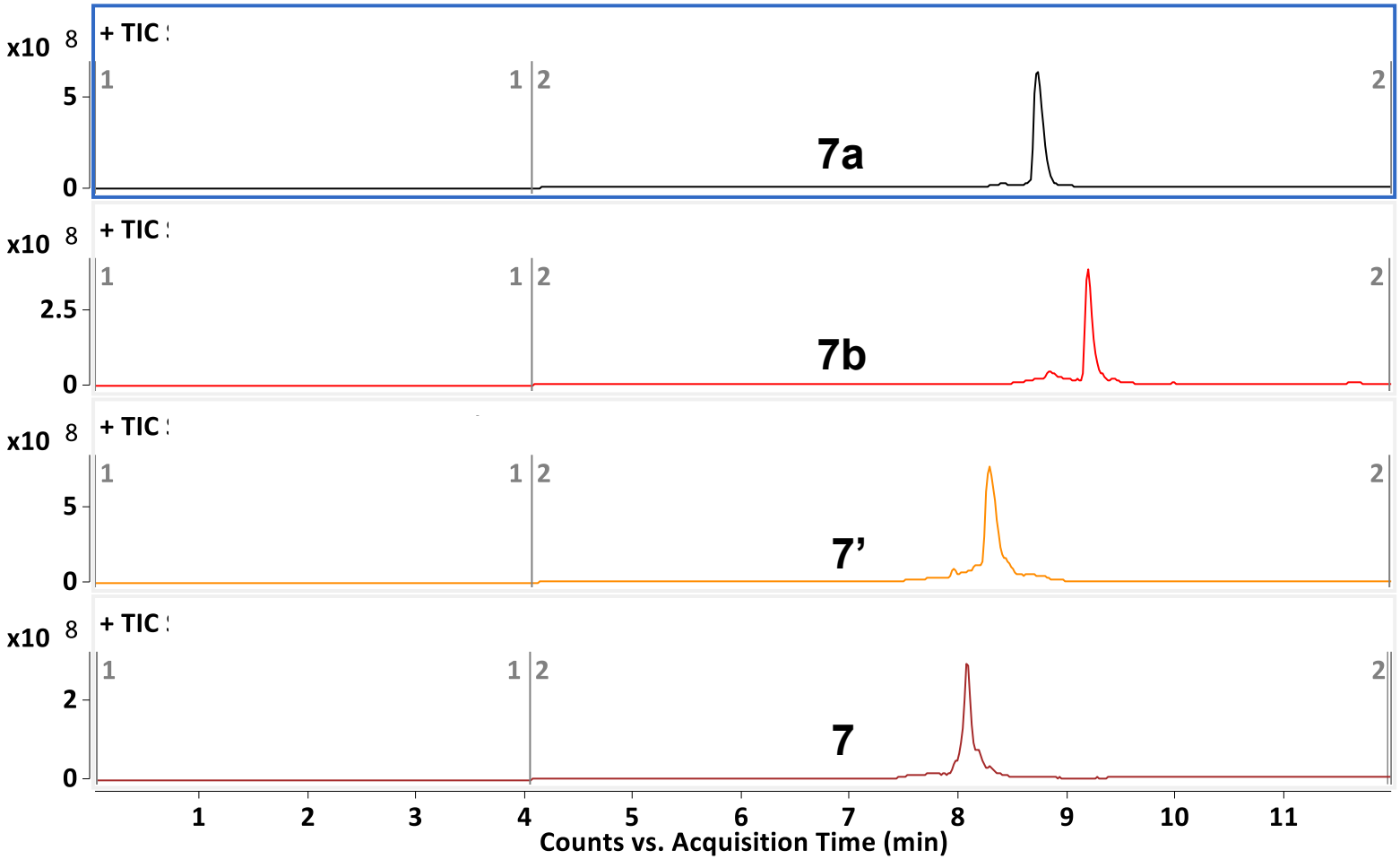
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
1274
1276
1278
1280
1282
1284
1286
1288
1290
Counts vs. Mass-to-Charge (m/z)
F F
S S
F F
5a
H2N-Y-C-E-R-S-C-N-M-K-CONH2
Chemical Formula: C50H72F4N15O14S3
Exact Mass: 1278.4481
Observed Mass: 1278.4497

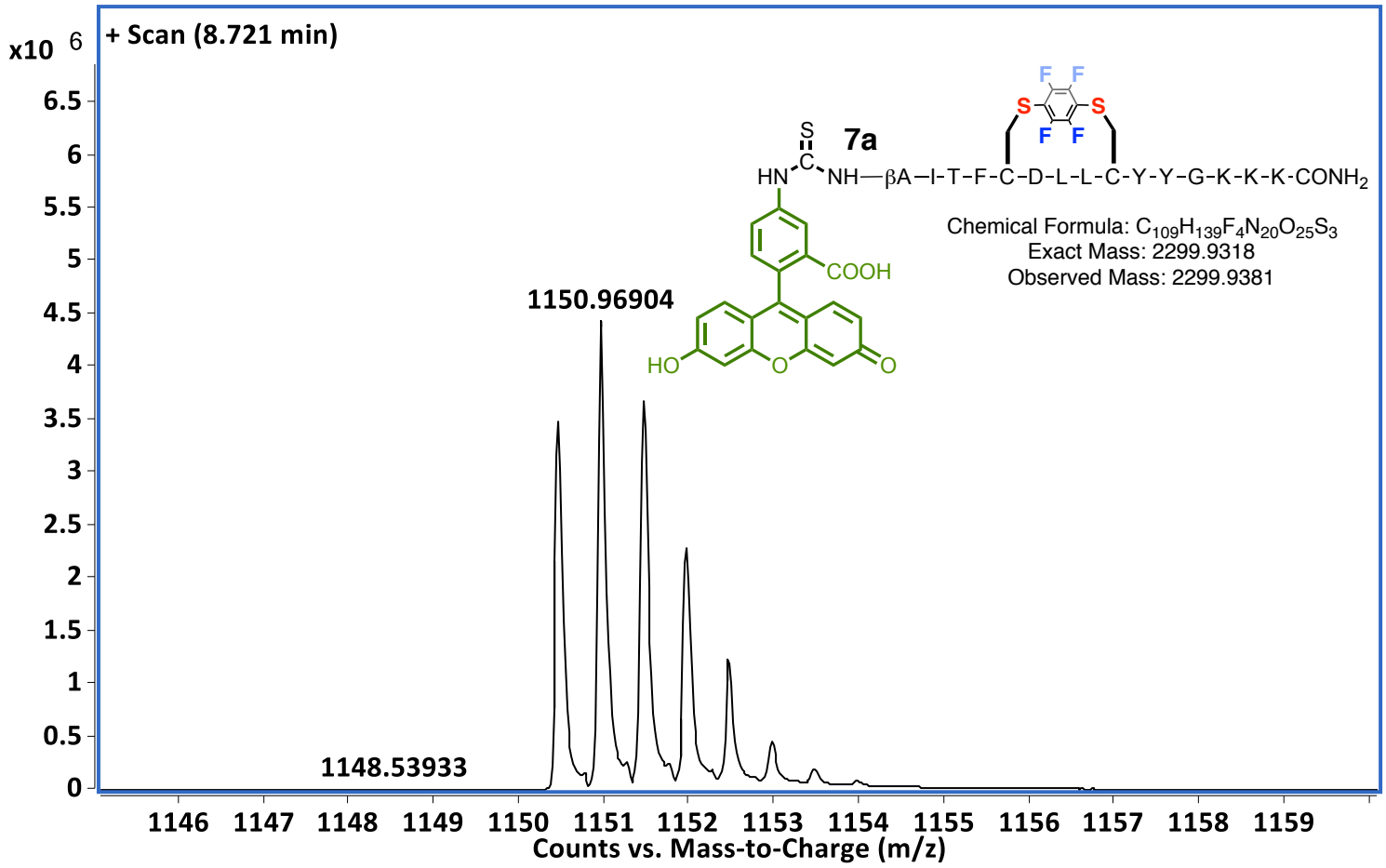

+ TIC Scan 11-23-12_
x10 9
6
1
2
$H_2N-I-T-F-C-D-L-L-C-Y-Y-G-K-K-K-CONH_2$
+ TIC Scan 11-23-12_
x10 8
6a
$H_2N-I-T-F-C-D-L-L-C-Y-Y-G-K-K-K-CONH_2$
+ TIC Scan 11-23-12_
x10 8
6b
$H_2N-I-T-F-C-D-L-L-C-Y-Y-G-K-K-K-CONH_2$
Counts vs. Acquisition Time (min)

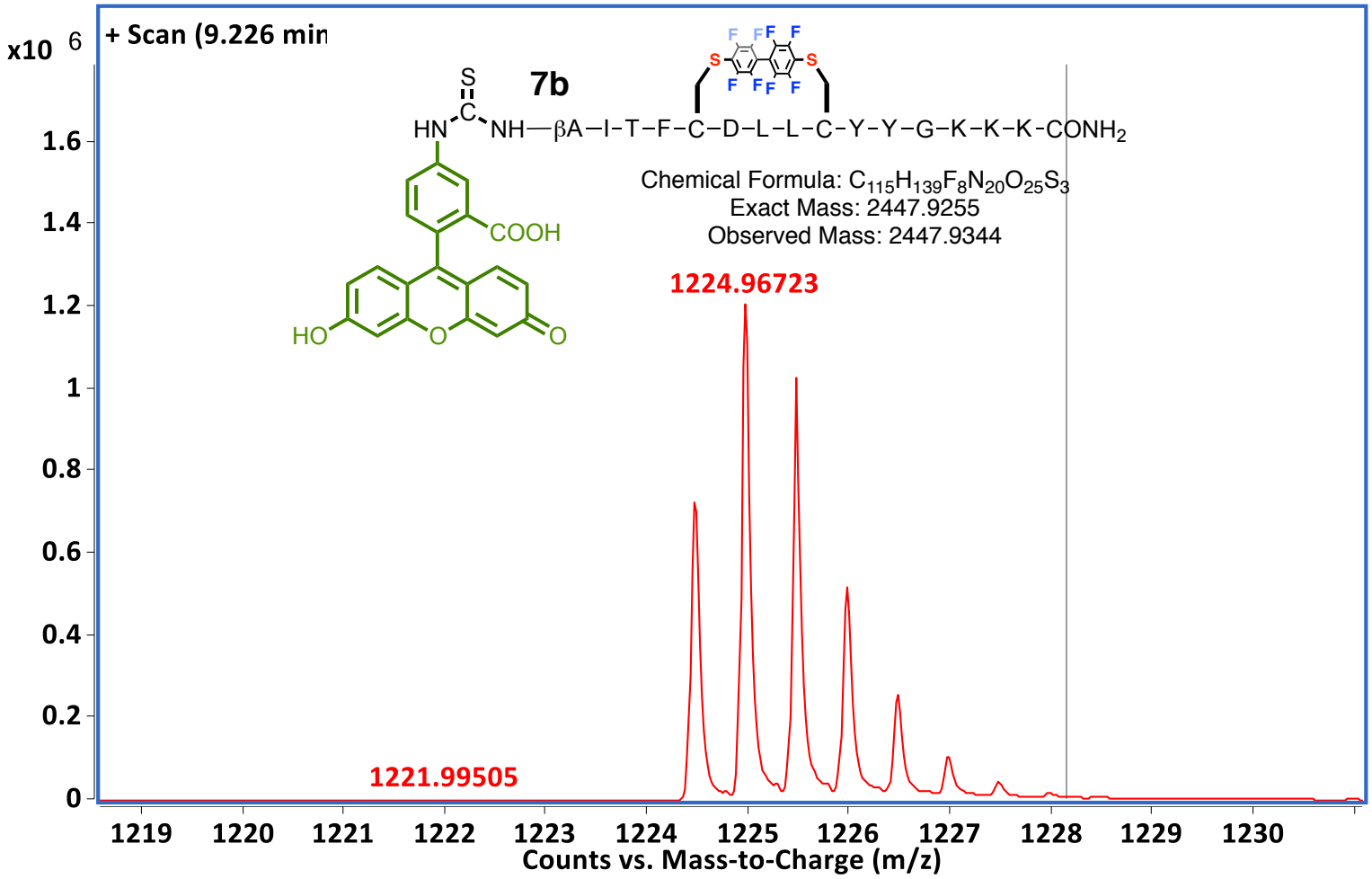

+ Scan (6.795 min) 11-23-12
x10 6
2.8
2.6
2.4
2.2
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
1693.89095
1691.88177
6
H2N–I–T–F–C–D–L–L–C–Y–Y–G–K–K–K–CONH2
Chemical Formula: C79H125N18O19S2
Exact Mass: 1693.8810
Observed Mass: 1693.8910
1690
1692
1694
1696
1698
1700
1702
1704
Counts vs. Mass-to-Charge (m/z)

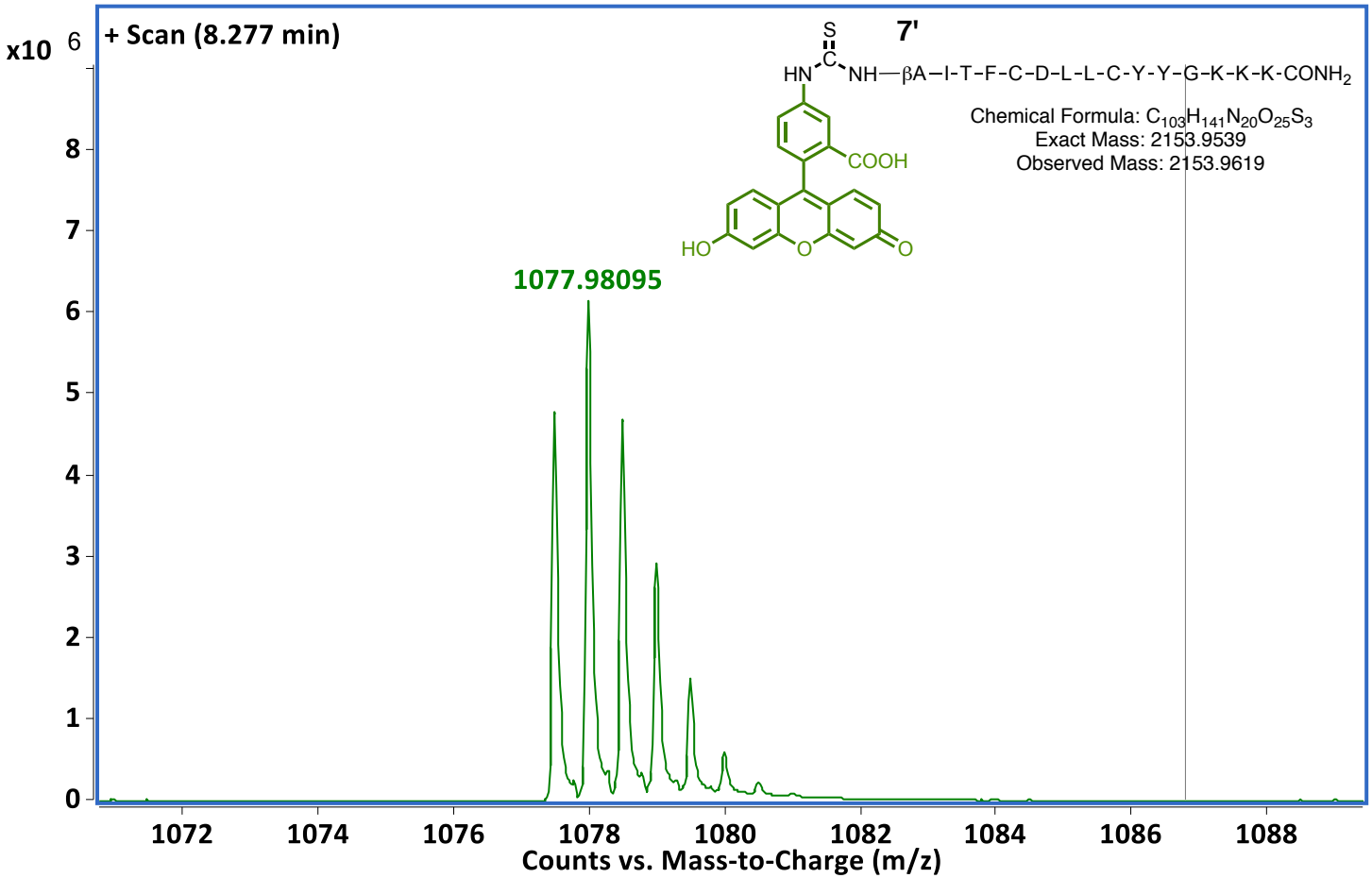

+ Scan (7.507 min)
x10 5
4
3.75
3.5
3.25
3
2.75
2.5
2.25
2
1.75
1.5
1.25
1
0.75
0.5
0.25
0
1841.02403
1837.94595
F F
S S
F F
6a
I–T–F–C–D–L–L–C–Y–Y–G–K–K–K–CONH2
Chemical Formula:
C85H123F4N18O19S2
Exact Mass: 1839.8589
Observed Mass: 1840.0240
1836
1838
1840
1842
1844
1846
1848
1850
Counts vs. Mass-to-Charge (m/z)

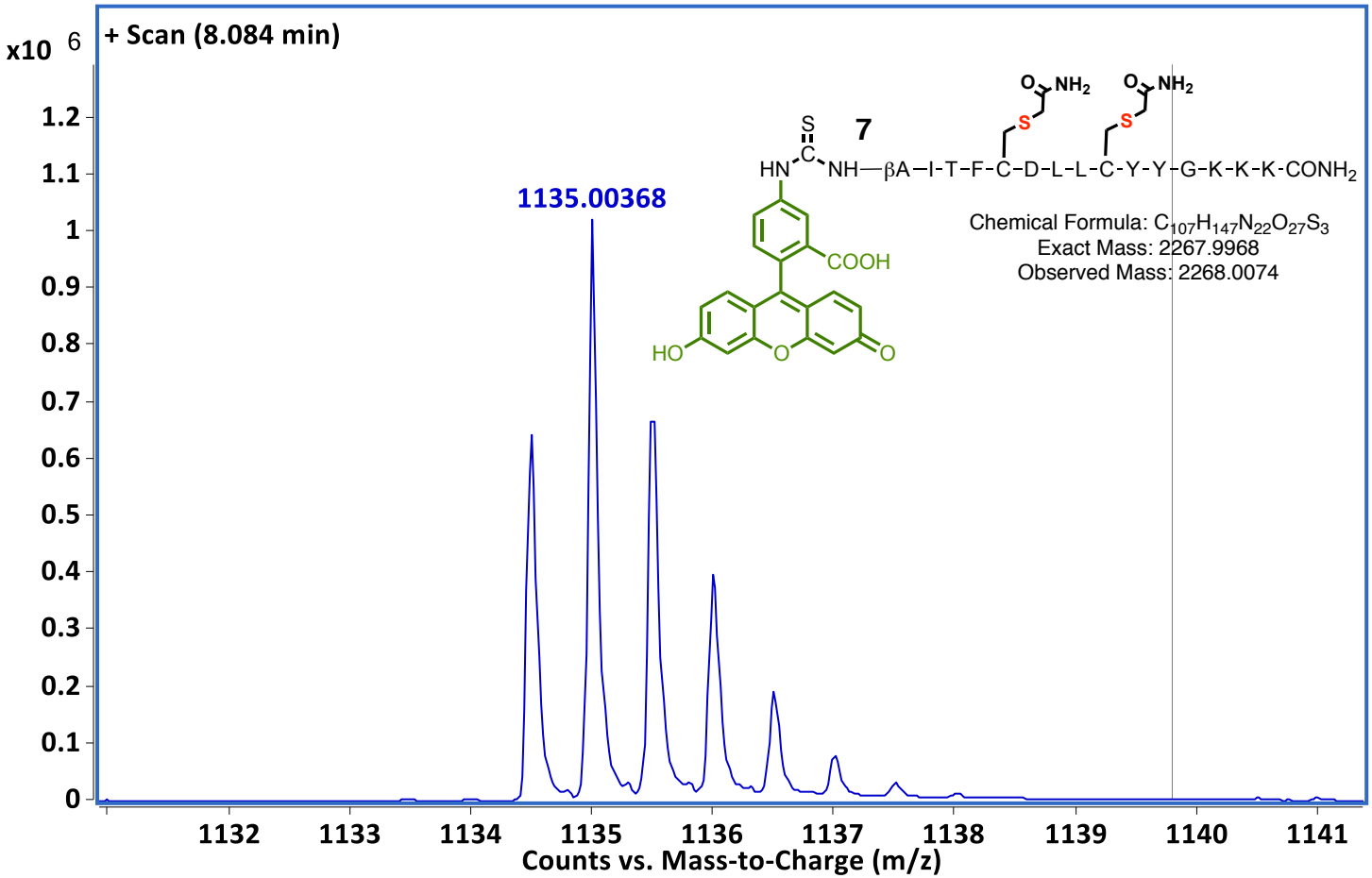

+ Scan (8.091 min)
x10 5
2.2
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
1988.85483
1981.86346
1984.86057
1996.84768
6b
H2N—I—T—F—C—D—L—L—C—Y—Y—G—K—K—K—CONH2
Chemical Formula: C91H123F8N18O19S2
Exact Mass: 1987.8526
Observed Mass: 1987.8548
1982
1984
1986
1988
1990
1992
1994
1996
1998
2000
Counts vs. Mass-to-Charge (m/z)

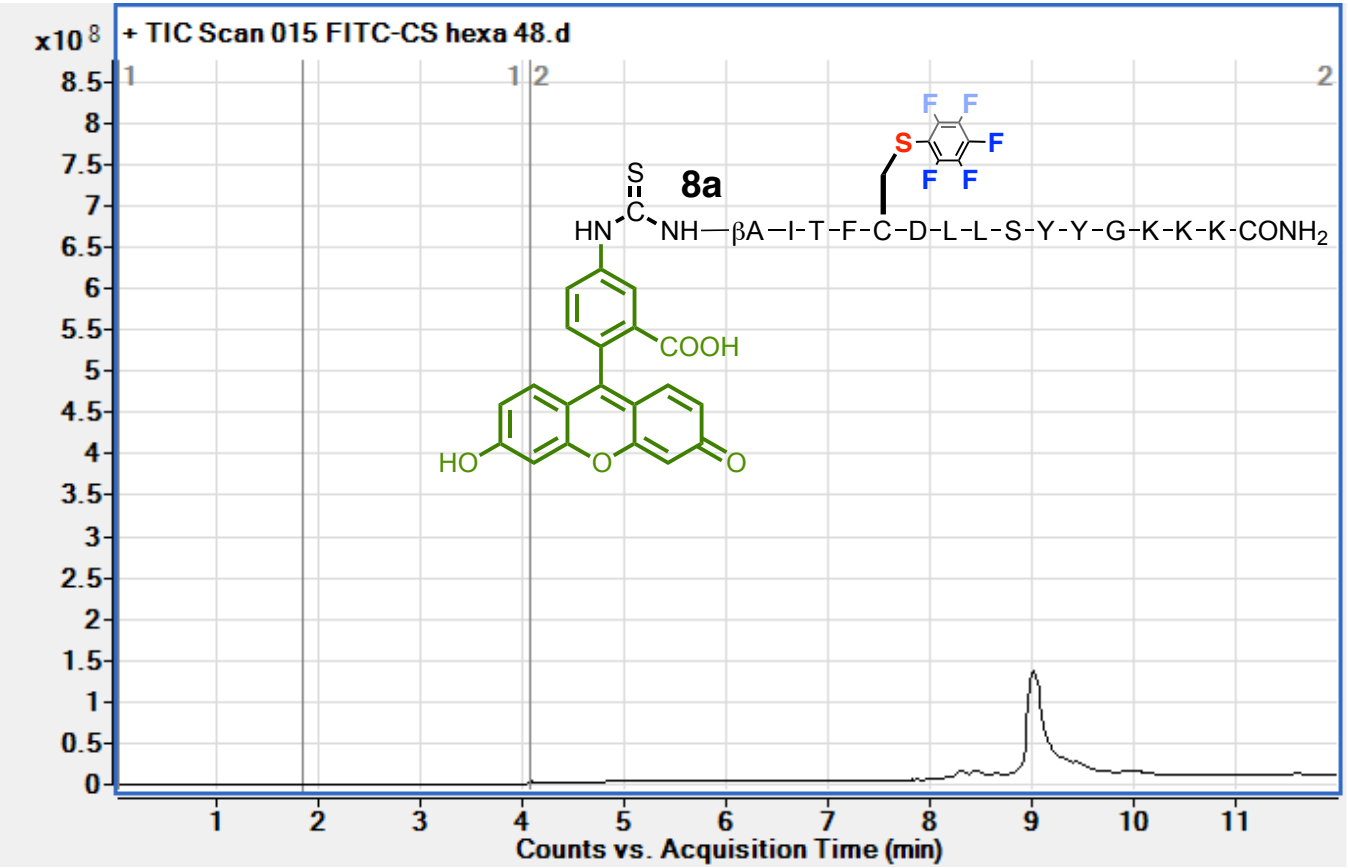

x10 8
+ TIC
5
0
7a
x10 8
+ TIC
2.5
0
7b
x10 8
+ TIC
5
0
7’
x10 8
+ TIC
2
0
7
1 2 3 4 5 6 7 8 9 10 11
Counts vs. Acquisition Time (min)

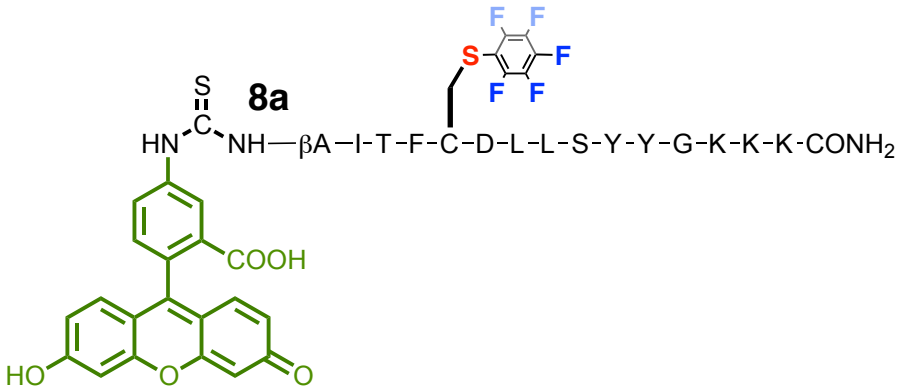

+ Scan (8.721 min)
x10 6
6.5
6
5.5
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
1150.96904
1148.53933
7a
HN
S
C
NH—βA—I-T-F-C-D-L-L-C-Y-Y-G-K-K-K-CONH2
F F
S S
F F
COOH
HO
O
O
Chemical Formula: C109H139F4N20O25S3
Exact Mass: 2299.9318
Observed Mass: 2299.9381
1146 1147 1148 1149 1150 1151 1152 1153 1154 1155 1156 1157 1158 1159
Counts vs. Mass-to-Charge (m/z)

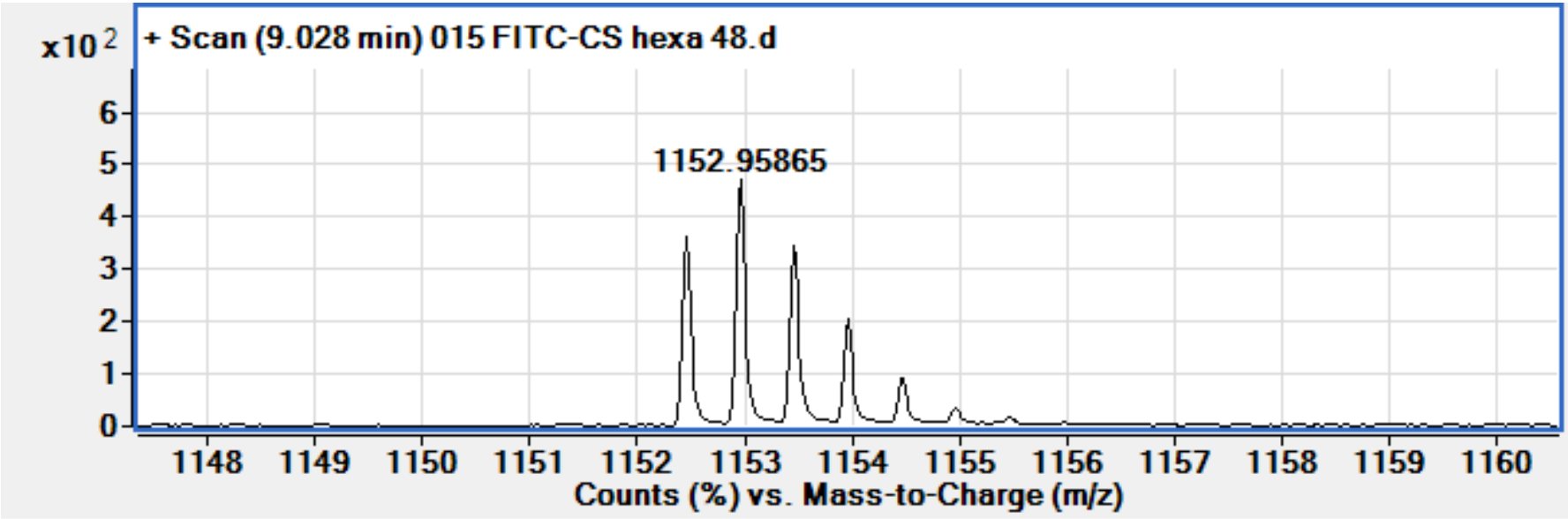

+ Scan (9.226 min
x10 6
7b
HN
S
C
NH—βA–I–T–F–C–D–L–L–C–Y–Y–G–K–K–K-CONH2
COOH
HO
O
O
Chemical Formula: C115H139F8N20O25S3
Exact Mass: 2447.9255
Observed Mass: 2447.9344
1224.96723
1221.99505
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
1219
1220
1221
1222
1223
1224
1225
1226
1227
1228
1229
1230
Counts vs. Mass-to-Charge (m/z)

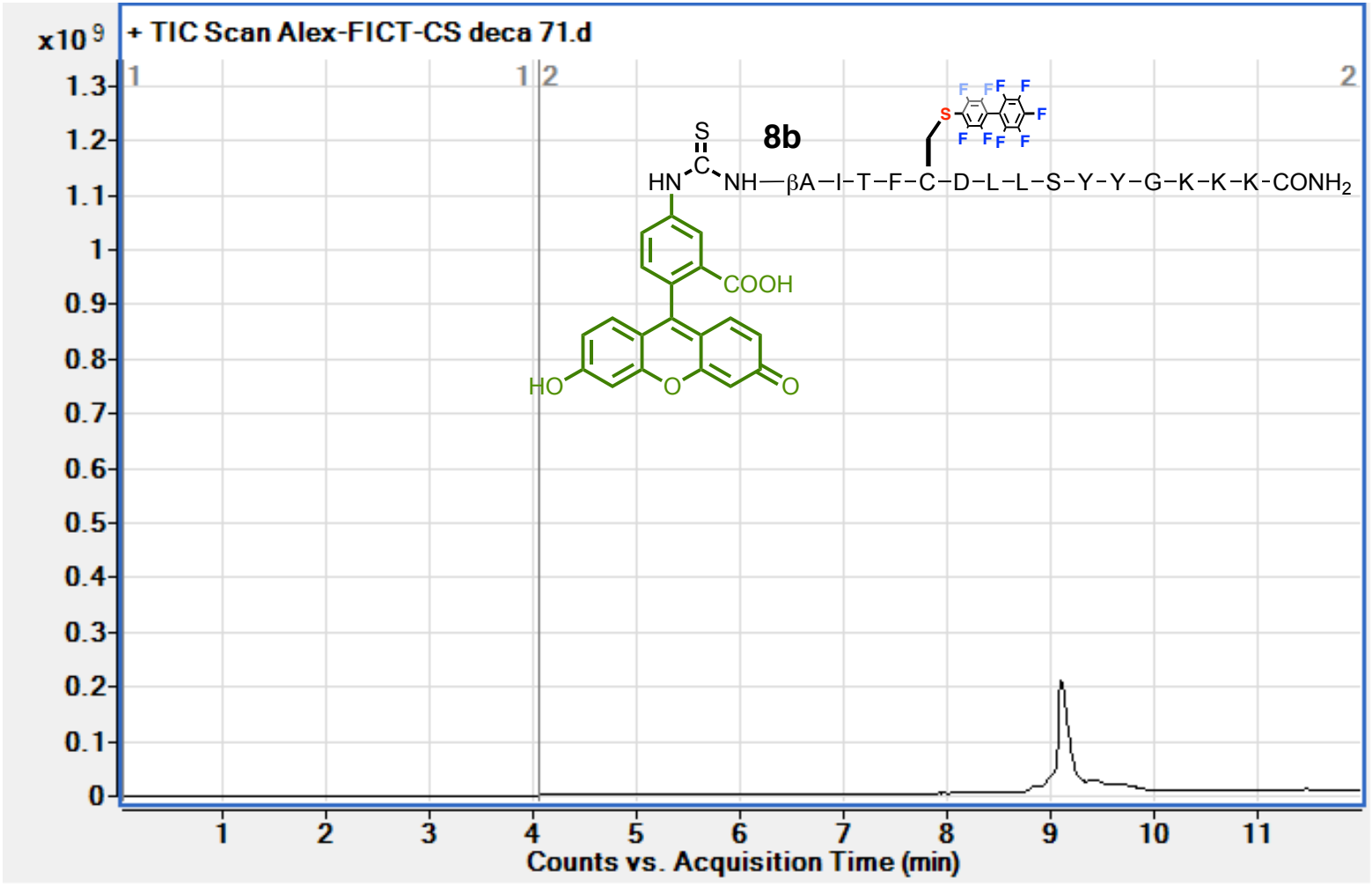

x10 6
+ Scan (8.277 min)
7'
HN
S
C
NH—βA–I-T-F-C-D-L-L-C-Y-Y-G-K-K-K-CONH2
COOH
HO
O
O
Chemical Formula: C103H141N20O25S3
Exact Mass: 2153.9539
Observed Mass: 2153.9619
1077.98095
8
7
6
5
4
3
2
1
0
1072
1074
1076
1078
1080
1082
1084
1086
1088
Counts vs. Mass-to-Charge (m/z)

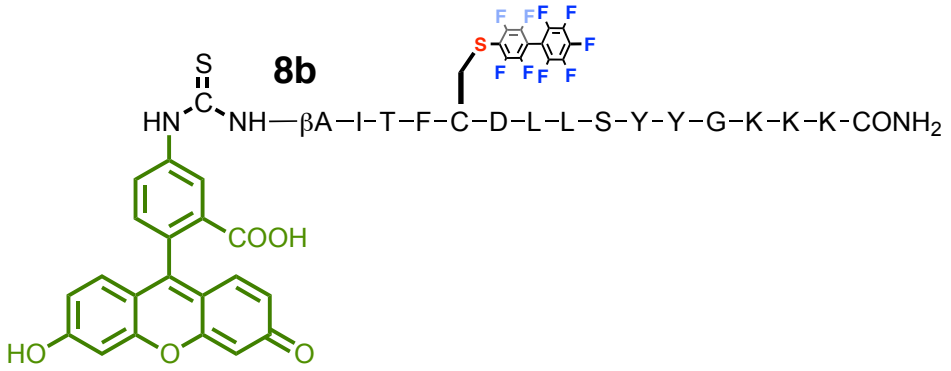

x10 6
+ Scan (8.084 min)
1.2
1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
1135.00368
7
HN
S
C
NH—βA–I-T-F-C-D-L-L-C-Y-Y-G-K-K-K-CONH2
NH2
NH2
COOH
HO
O
O
Chemical Formula: C107H147N22O27S3
Exact Mass: 2267.9968
Observed Mass: 2268.0074
1132
1133
1134
1135
1136
1137
1138
1139
1140
1141
Counts vs. Mass-to-Charge (m/z)

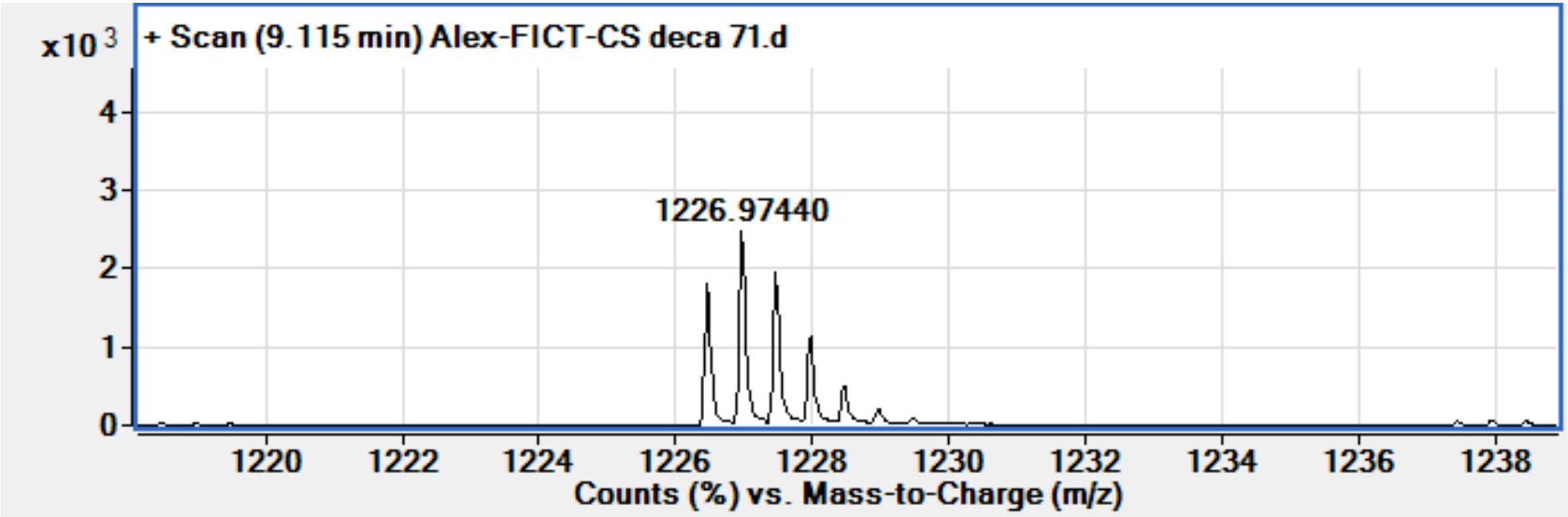

x10 8
+ TIC Scan 015 FITC-CS hexa 48.d
8a
HN
S
C
NH—βA—I-T-F-C-D-L-L-S-Y-Y-G-K-K-K-CONH2
F
F
S
F
F
F
COOH
HO
O
O
Counts vs. Acquisition Time (min)

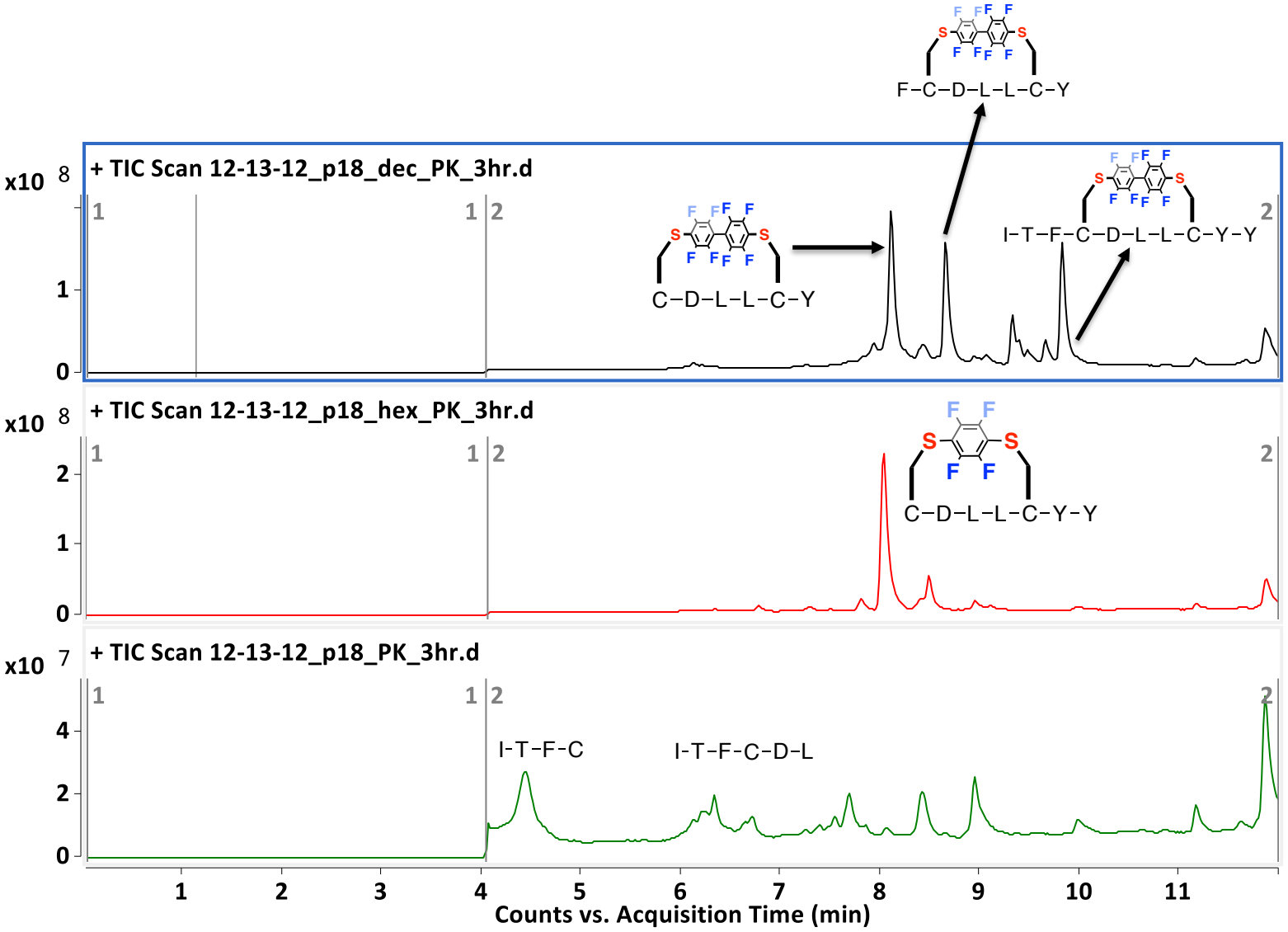

8a
HN
S
C
NH—βA—I-T-F-C-D-L-L-S-Y-Y-G-K-K-K-CONH2
F
F
S
F
F
F
COOH
HO
O
O

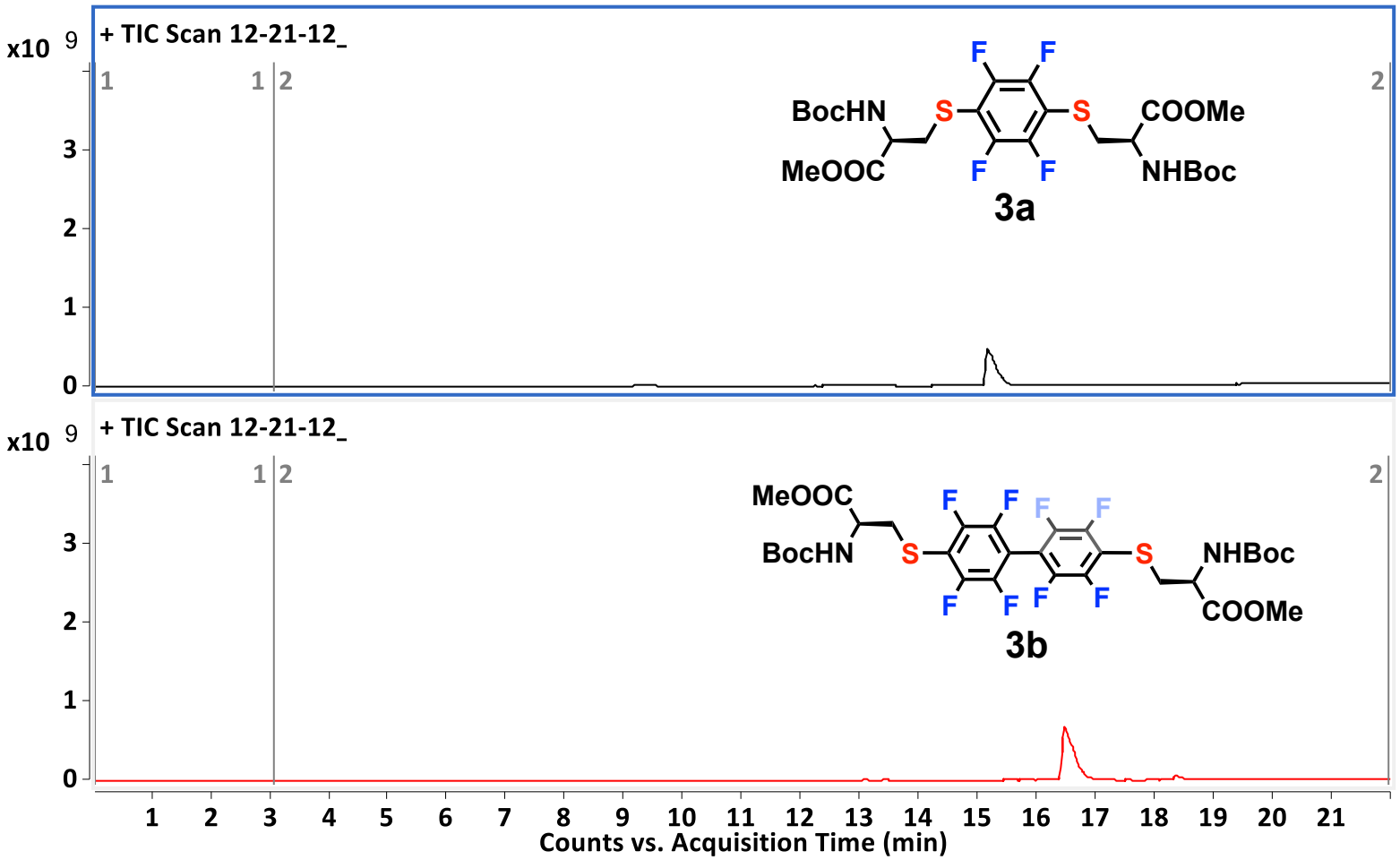

x10 2
+ Scan (9.028 min) 015 FITC-CS hexa 48.d
1152.95865
6
5
4
3
2
1
0
1148
1149
1150
1151
1152
1153
1154
1155
1156
1157
1158
1159
1160
Counts (%) vs. Mass-to-Charge (m/z)

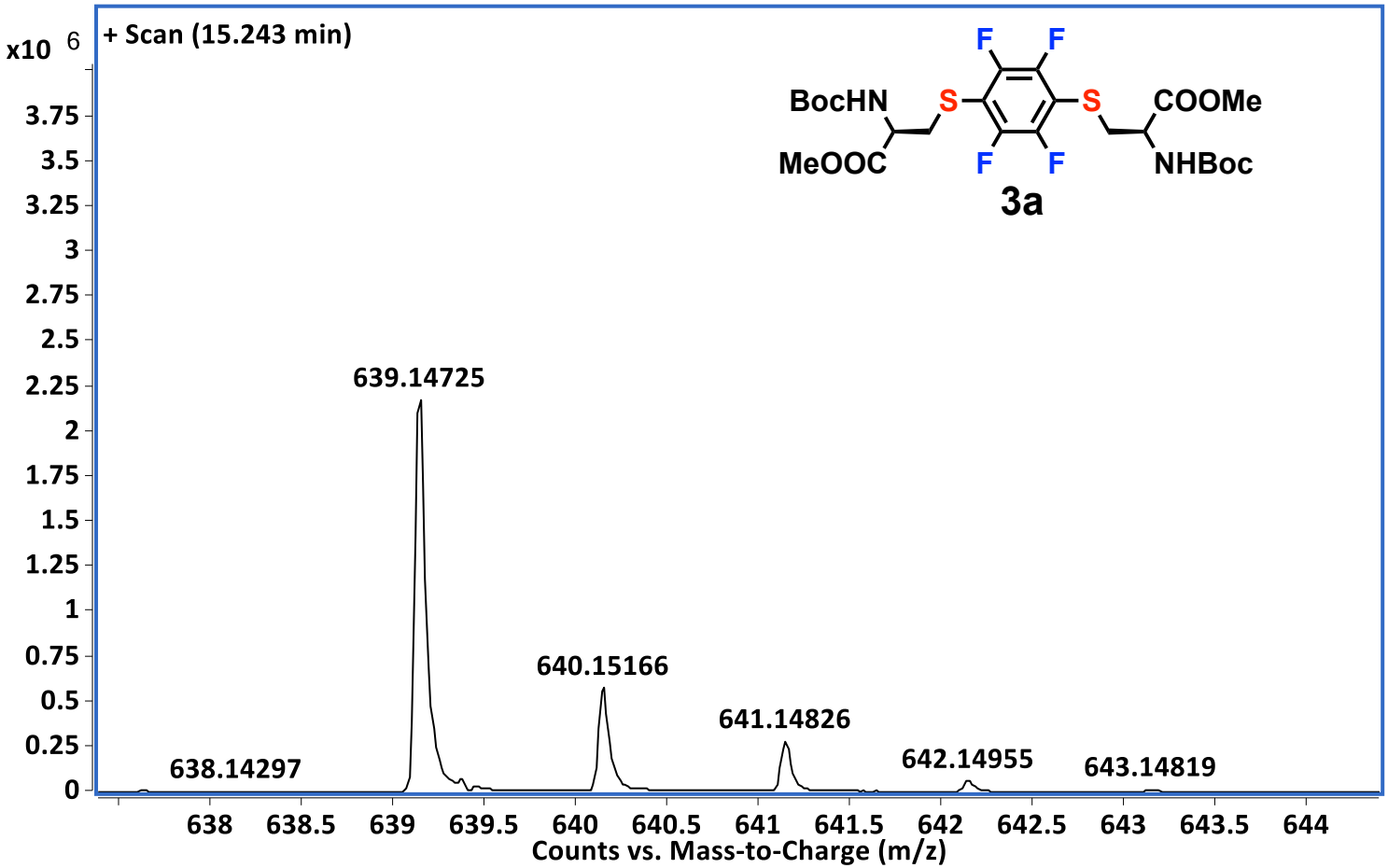

x10 9
+ TIC Scan Alex-FICT-CS deca 71.d
1
2
8b
HN
S
C
NH—βA–I–T–F–C–D–L–L–S–Y–Y–G–K–K–K-CONH2
COOH
HO
O
O
1.3
1.2
1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
1
2
3
4
5
6
7
8
9
10
11
Counts vs. Acquisition Time (min)

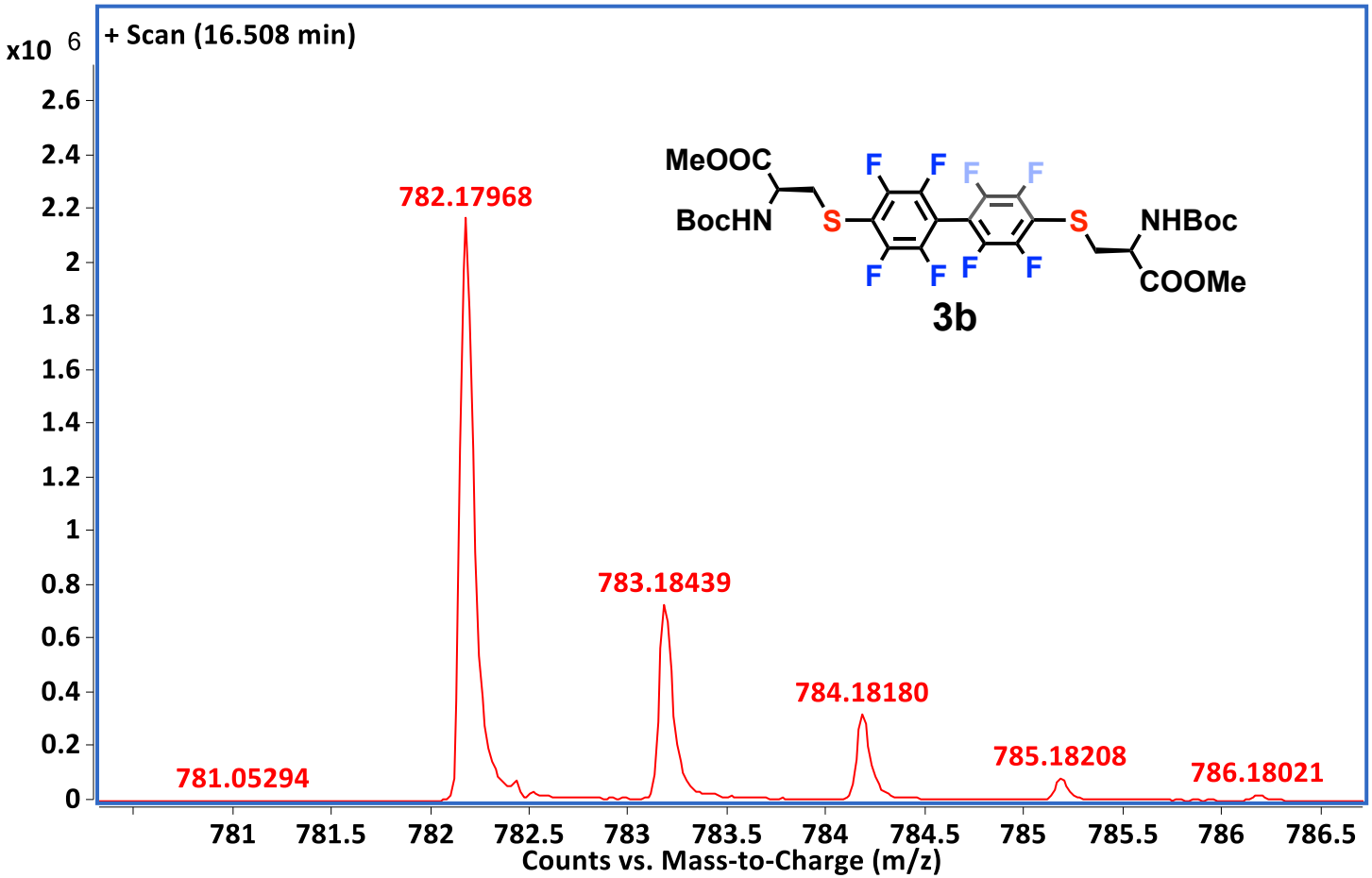

8b
HN
S
C
NH—βA–I–T–F–C–D–L–L–S–Y–Y–G–K–K–K-CONH2
COOH
HO
O
O
F
F
F
F
F
F
F
F
S

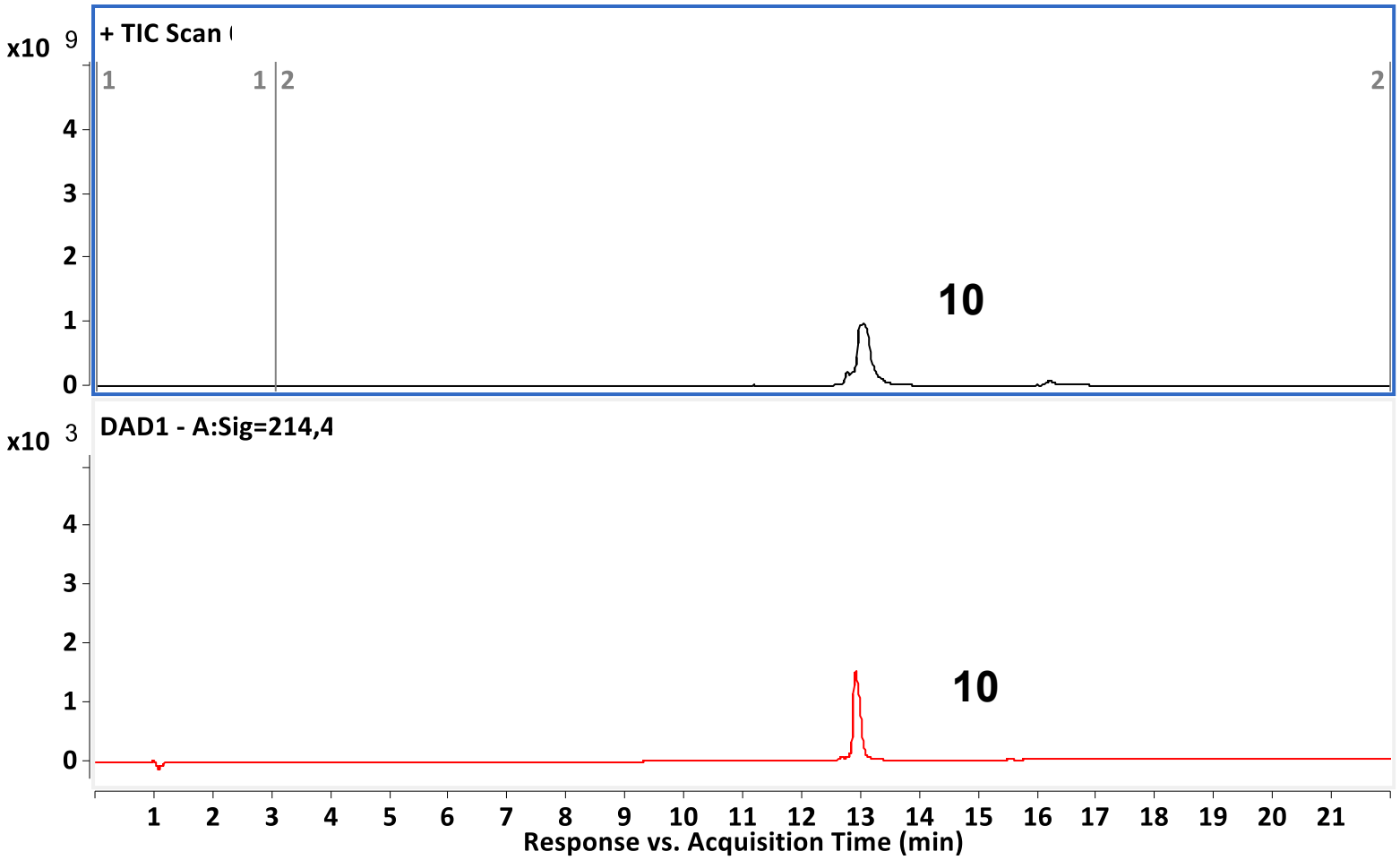

x10 3
+ Scan (9.115 min) Alex-FICT-CS deca 71.d
4
3
2
1
0
1226.97440
1220
1222
1224
1226
1228
1230
1232
1234
1236
1238
Counts (%) vs. Mass-to-Charge (m/z)

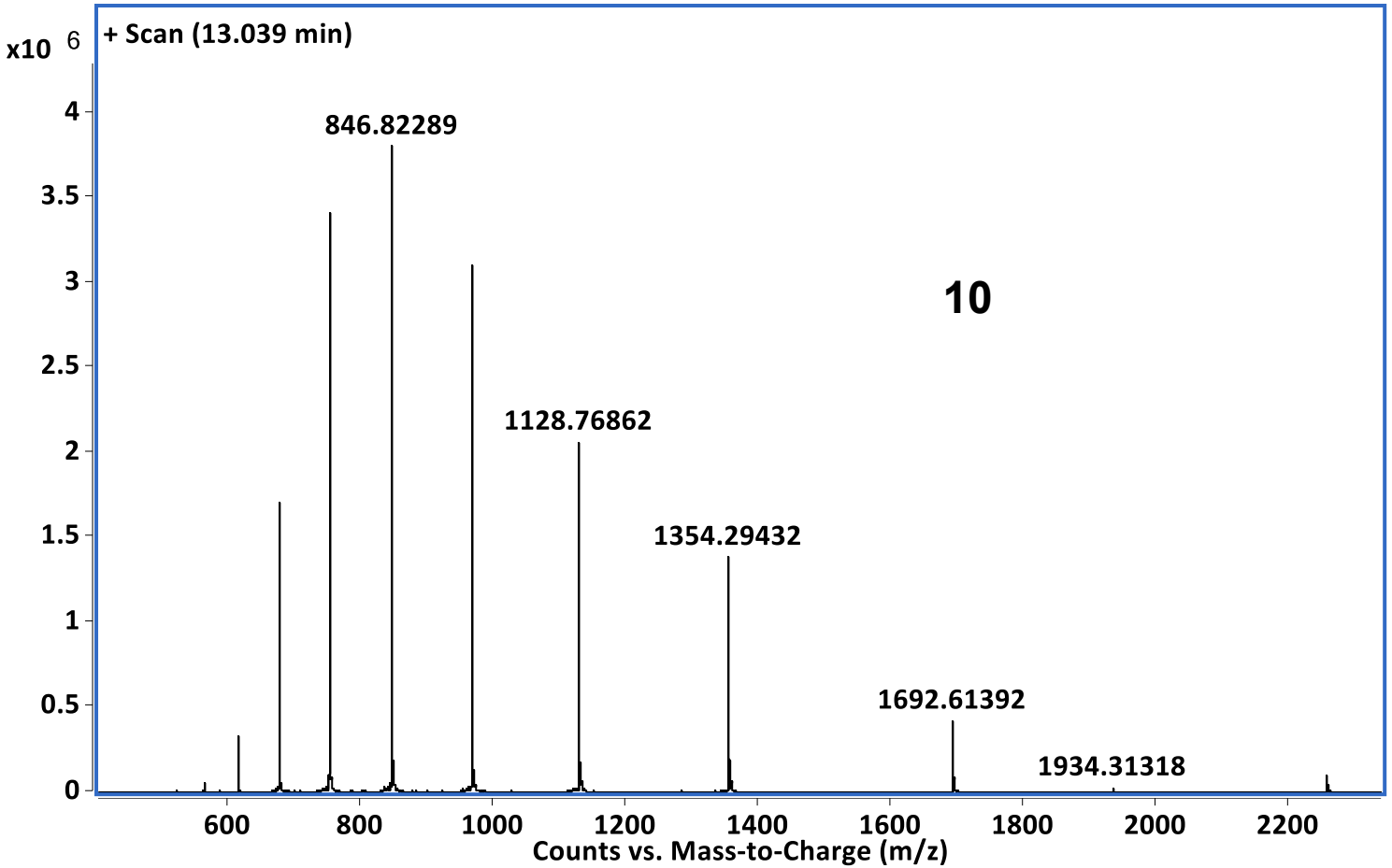

+ TIC Scan 12-13-12_p18_dec_PK_3hr.d
x10 8
F–C–D–L–L–C–Y
C–D–L–L–C–Y
I–T–F–C–D–L–L–C–Y–Y
+ TIC Scan 12-13-12_p18_hex_PK_3hr.d
x10 8
C–D–L–L–C–Y–Y
+ TIC Scan 12-13-12_p18_PK_3hr.d
x10 7
I-T-F-C
I-T-F-C-D-L
Counts vs. Acquisition Time (min)

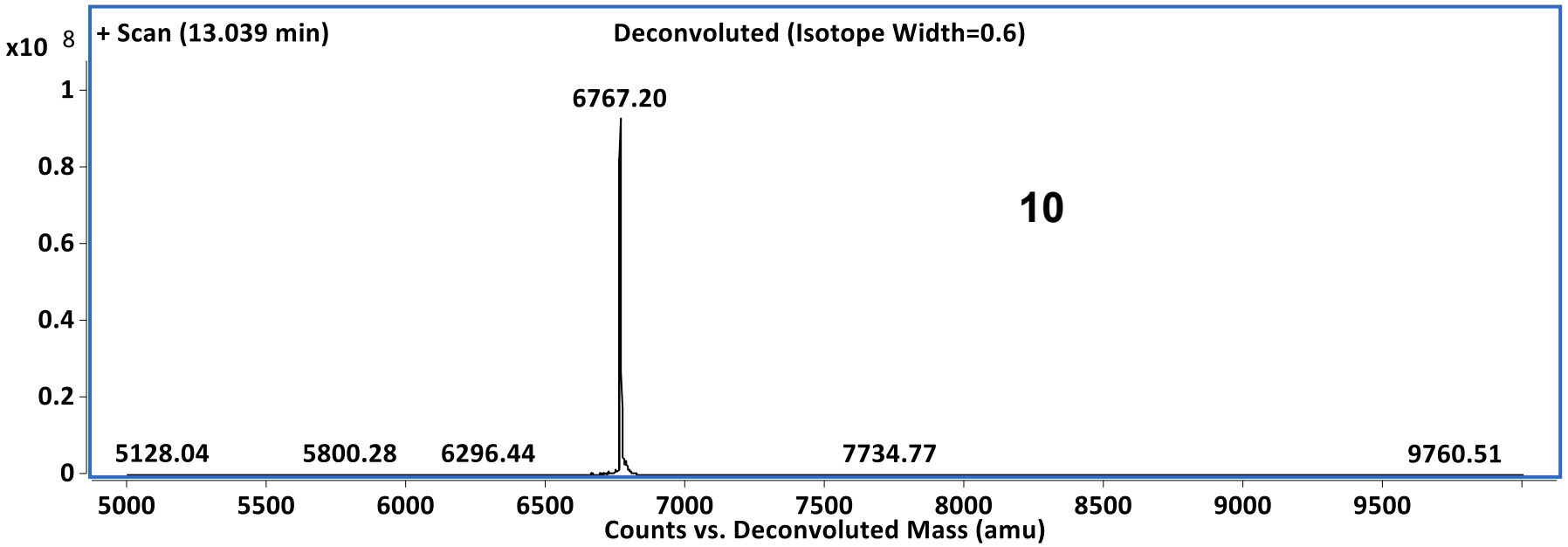

x10 9
+ TIC Scan 12-21-12_
1
1 2
2
3
2
1
0
BocHN
MeOOC
S
F
F
F
F
S
COOMe
NHBoc
3a
x10 9
+ TIC Scan 12-21-12_
1
1 2
2
3
2
1
0
MeOOC
BocHN
S
F
F
F
F
F
F
F
F
S
NHBoc
COOMe
3b
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
Counts vs. Acquisition Time (min)

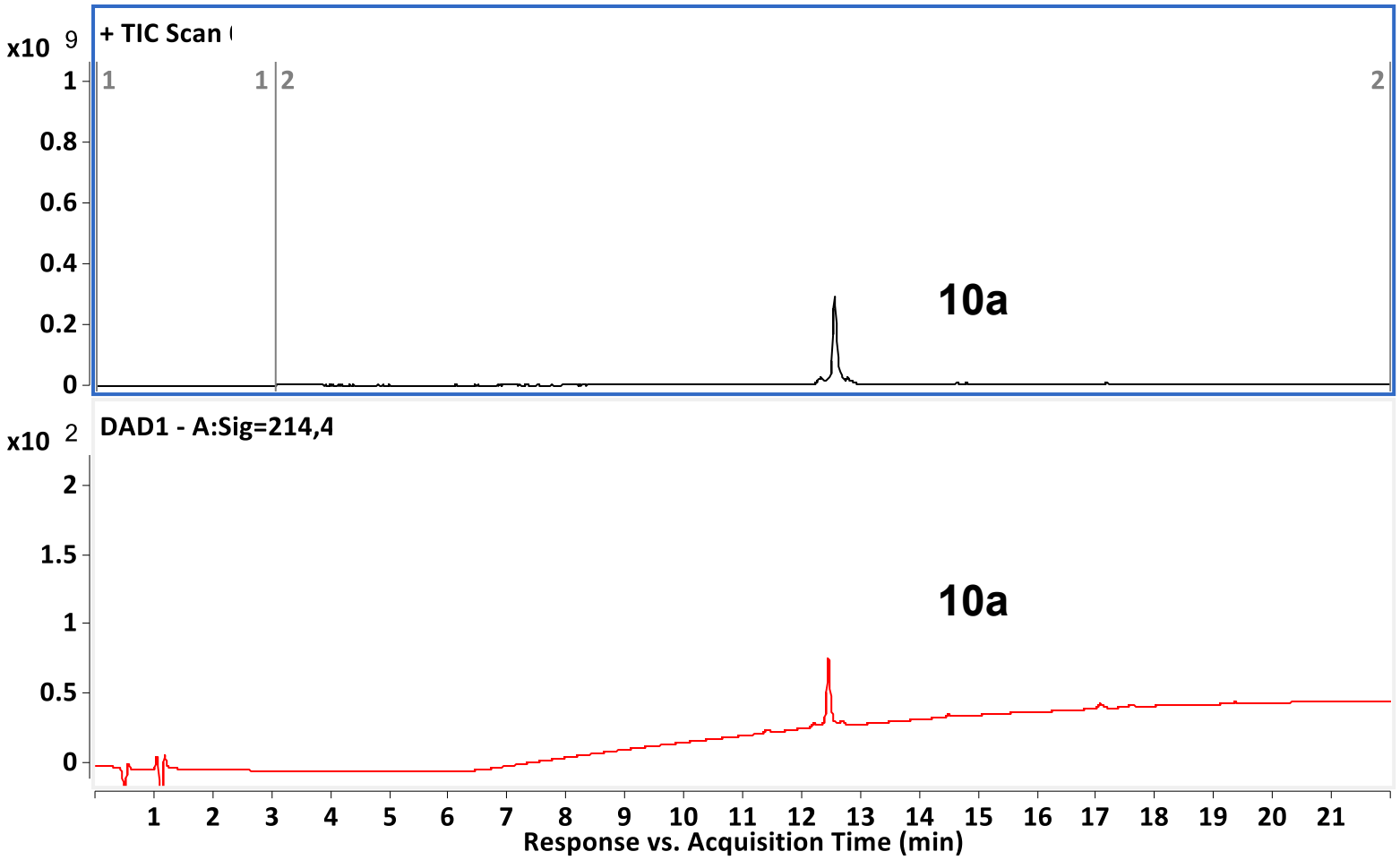

+ Scan (15.243 min)
x10 6
3.75
3.5
3.25
3
2.75
2.5
2.25
2
1.75
1.5
1.25
1
0.75
0.5
0.25
0
639.14725
640.15166
641.14826
642.14955
643.14819
638.14297
638
638.5
639
639.5
640
640.5
641
641.5
642
642.5
643
643.5
644
Counts vs. Mass-to-Charge (m/z)
F
F
BocHN
S
S
COOMe
MeOOC
F
F
NHBoc
3a

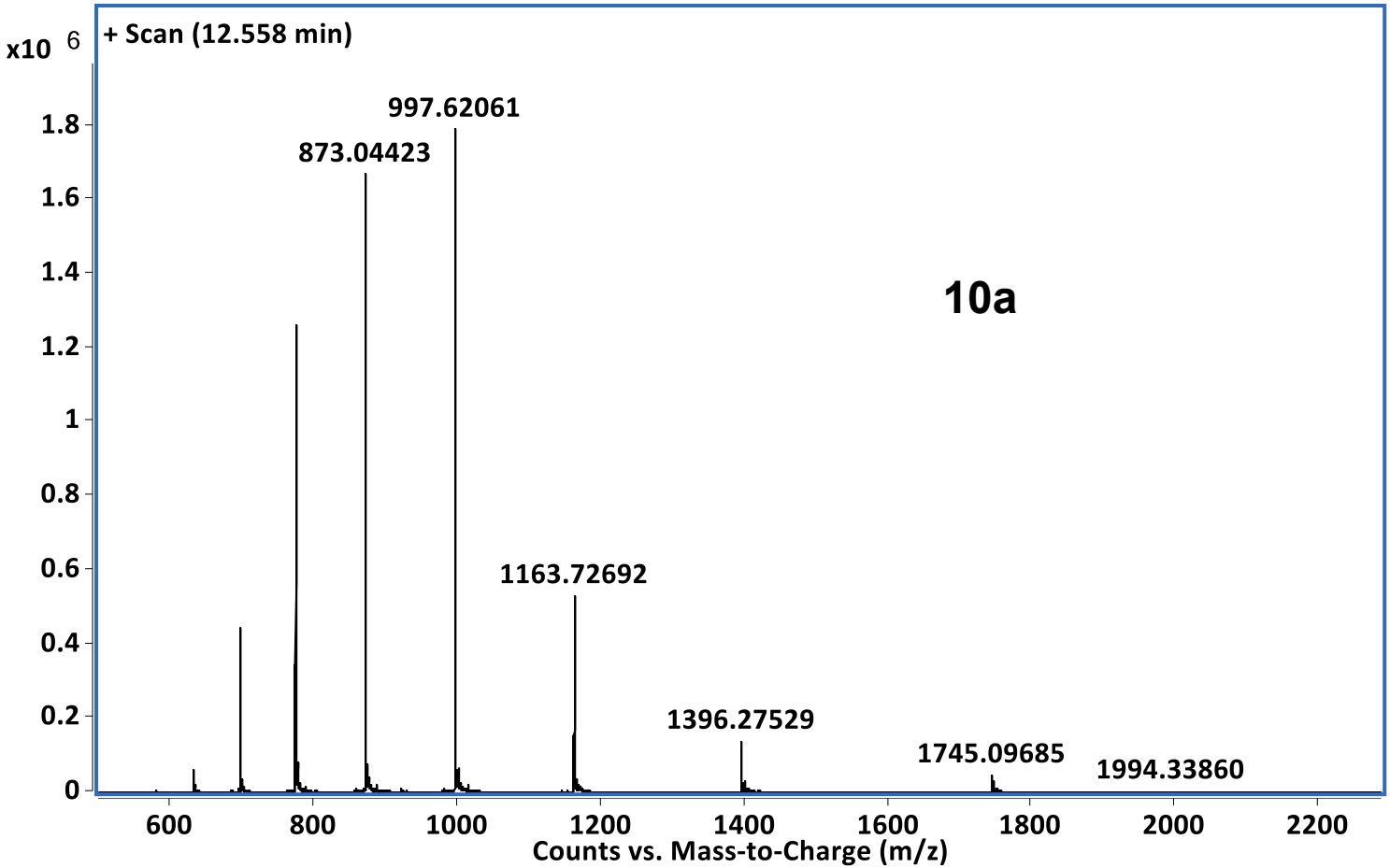

+ Scan (16.508 min)
x10 6
2.6
2.4
2.2
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
782.17968
783.18439
784.18180
785.18208
786.18021
781.05294
MeOOC
BocHN
S
F
F
F
F
F
F
F
F
S
NHBoc
COOMe
3b
781
781.5
782
782.5
783
783.5
784
784.5
785
785.5
786
786.5
Counts vs. Mass-to-Charge (m/z)

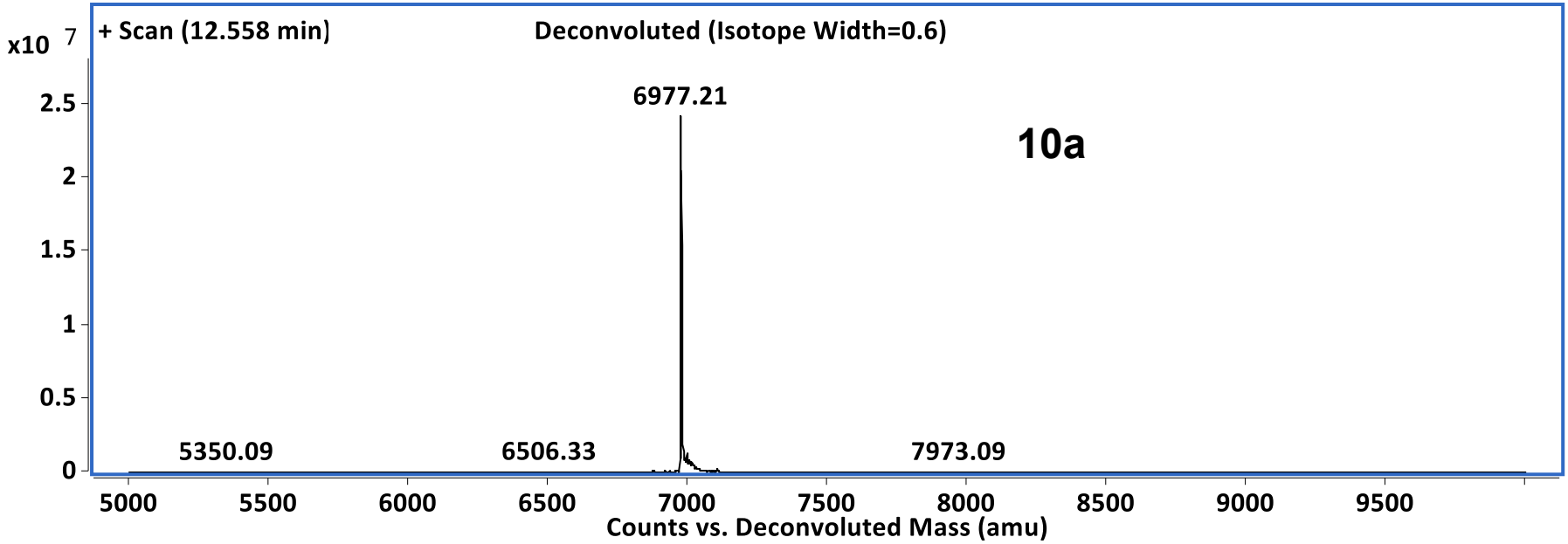

x10 9
+ TIC Scan (
1
1 2
2
4
3
2
1
0
10
x10 3
DAD1 - A:Sig=214,4
4
3
2
1
0
10
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
Response vs. Acquisition Time (min)

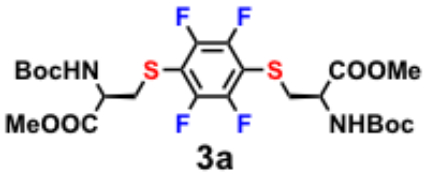

+ Scan (13.039 min)
x10 6
4
3.5
3
2.5
2
1.5
1
0.5
0
846.82289
1128.76862
1354.29432
1692.61392
1934.31318
10
600
800
1000
1200
1400
1600
1800
2000
2200
Counts vs. Mass-to-Charge (m/z)


+ Scan (13.039 min)
Deconvoluted (Isotope Width=0.6)
x10 8
1
0.8
0.6
0.4
0.2
0
6767.20
10
5128.04
5800.28
6296.44
7734.77
9760.51
5000
5500
6000
6500
7000
7500
8000
8500
9000
9500
Counts vs. Deconvoluted Mass (amu)


x10 9
+ TIC Scan (
1
1 2
2
1
0.8
0.6
0.4
0.2
0
10a
x10 2
DAD1 - A:Sig=214,4
2
1.5
1
0.5
0
10a
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
Response vs. Acquisition Time (min)

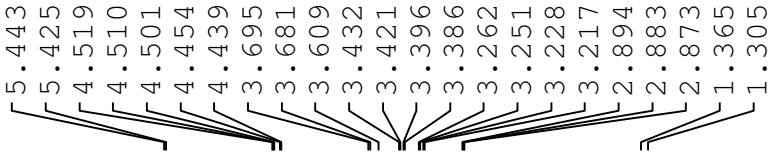

+ Scan (12.558 min)
x10 6
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
997.62061
873.04423
1163.72692
1396.27529
1745.09685
1994.33860
10a
600
800
1000
1200
1400
1600
1800
2000
2200
Counts vs. Mass-to-Charge (m/z)

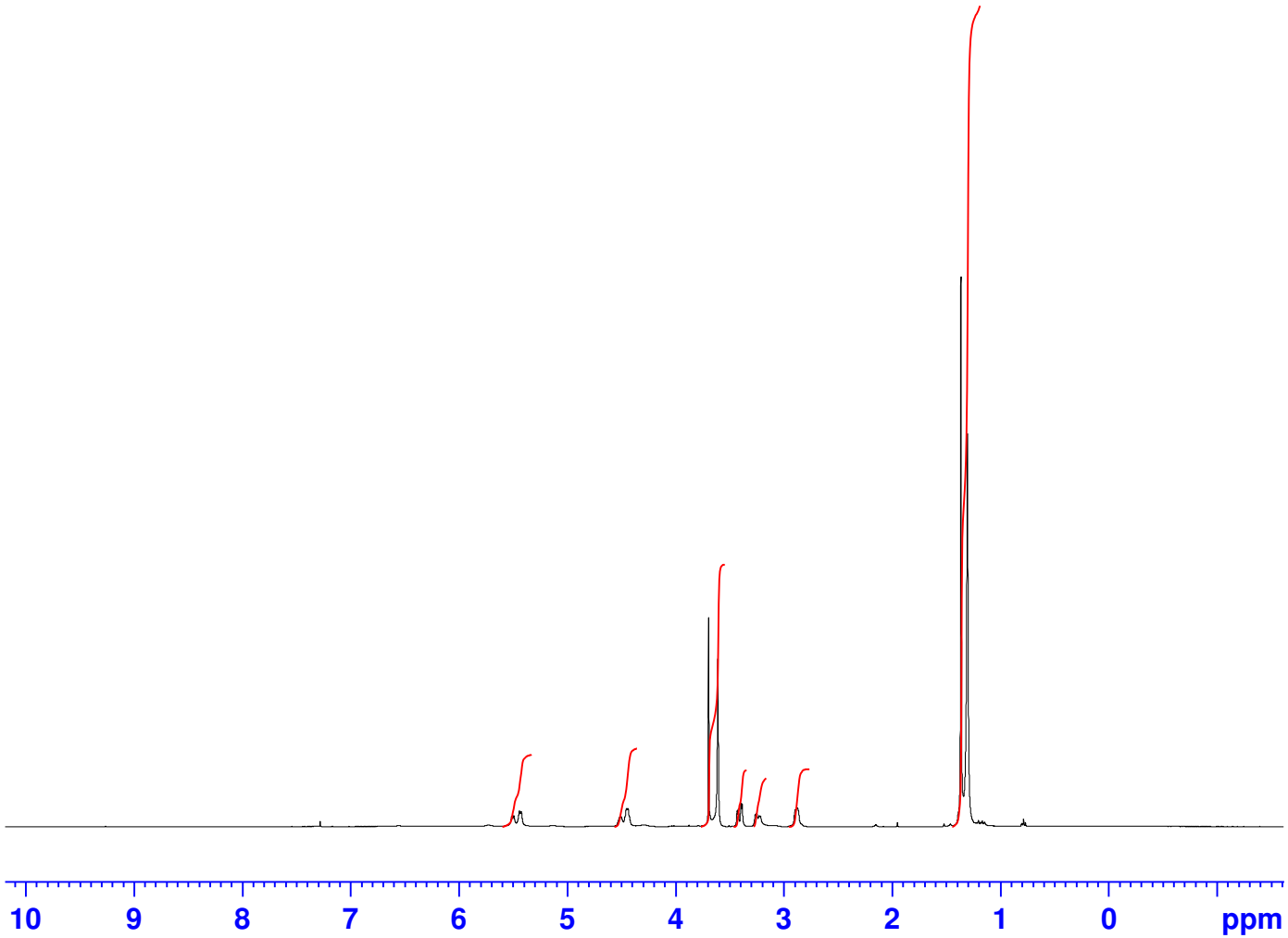

+ Scan (12.558 min)
Deconvoluted (Isotope Width=0.6)
x10 7
2.5
2
1.5
1
0.5
0
6977.21
10a
5350.09
6506.33
7973.09
5000
5500
6000
6500
7000
7500
8000
8500
9000
9500
Counts vs. Deconvoluted Mass (amu)



3a

cleaned, H1



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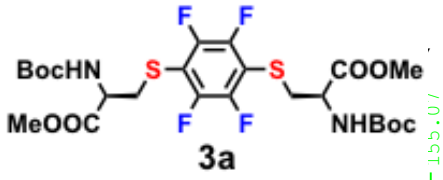
Current Data Parameters
NAME          Nov28_2012
EXPNO                 16
PROCNO                 1

F2 - Acquisition Parameters
Date_           20121204
Time               15.25
INSTRUM            spect
PROBHD   5 mm PABBO BB/
PULPROG             zg30
TD                 65536
SOLVENT            CDCl3
NS                     4
DS                     2
SWH             8012.820 Hz
FIDRES          0.122266 Hz
AQ             4.0894465 sec
RG                    32
DW                62.400 usec
DE                 6.50 usec
TE                294.2 K
D1           1.00000000 sec
TD0                    1

======== CHANNEL f1 ========
SFO1         400.1324710 MHz
NUC1                  1H
P1                14.50 usec
PLW1        10.00000000 W

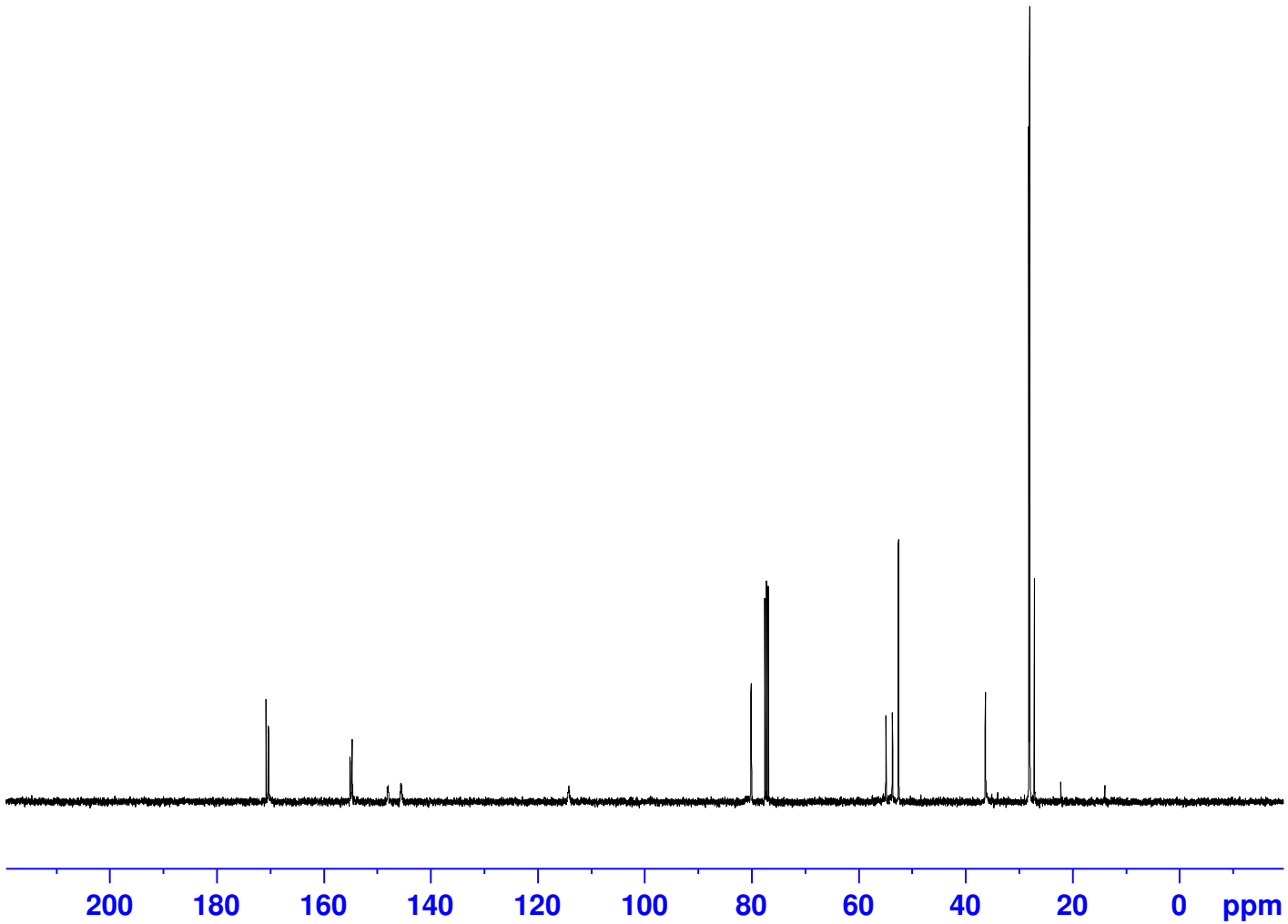
F2 - Processing parameters
SI                 65536
SF          400.1300000 MHz
WDW                   EM
SSB       0
LB                 0.30 Hz
GB        0
PC                 1.00
```



3a

cleaned

155.07
154.68
147.96
145.58
114.18
80.10
80.03
77.51
77.19
76.87
54.88
53.66
52.54
36.29
34.00
28.17
28.03
27.11
22.21
13.95



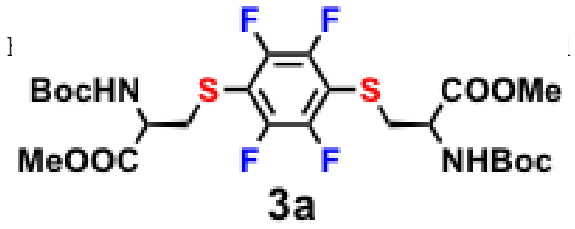

```
Current Data Parameters
NAME          Nov28_2012
EXPNO                 15
PROCNO                 1

F2 - Acquisition Parameters
Date_           20121204
Time               15.24
INSTRUM            spect
PROBHD   5 mm PABBO BB/
PULPROG          zgpg30
TD                65536
SOLVENT           CDCl3
NS                   48
DS                    4
SWH           24038.461 Hz
FIDRES         0.366798 Hz
AQ            1.3631488 sec
RG                  203
DW                20.800 usec
DE                 6.50 usec
TE                294.8 K
D1           2.00000000 sec
D11          0.03000000 sec
TD0                   1

======== CHANNEL f1 ========
SFO1        100.6228293 MHz
NUC1                13C
P1                10.00 usec
PLW1        44.00000000 W

======== CHANNEL f2 ========
SFO2        400.1316005 MHz
NUC2                1H
CPDPRG[2         waltz16
PCPD2             90.00 usec
PLW2        10.00000000 W
PLW12        0.25957000 W
PLW13        0.21025001 W

F2 - Processing parameters
SI                 32768
SF          100.6127690 MHz
WDW                  EM
SSB       0
LB                1.00 Hz
GB        0
PC                1.40
```



3a


```
Current Data Parameters
NAME          Nov28_2012
EXPNO                  4
PROCNO                 1

F2 - Acquisition Parameters
Date_           20121128
Time               18.45
INSTRUM            spect
PROBHD   5 mm PABBO BB/
PULPROG          zgflqn
TD              319980
SOLVENT           None
NS                  23
DS                   4
SWH          200000.000 Hz
FIDRES         0.625039 Hz
AQ            0.7999500 sec
RG                 128
DW               2.500 usec
DE               6.50 usec
TE               300.0 K
D1           1.00000000 sec
TD0                  1

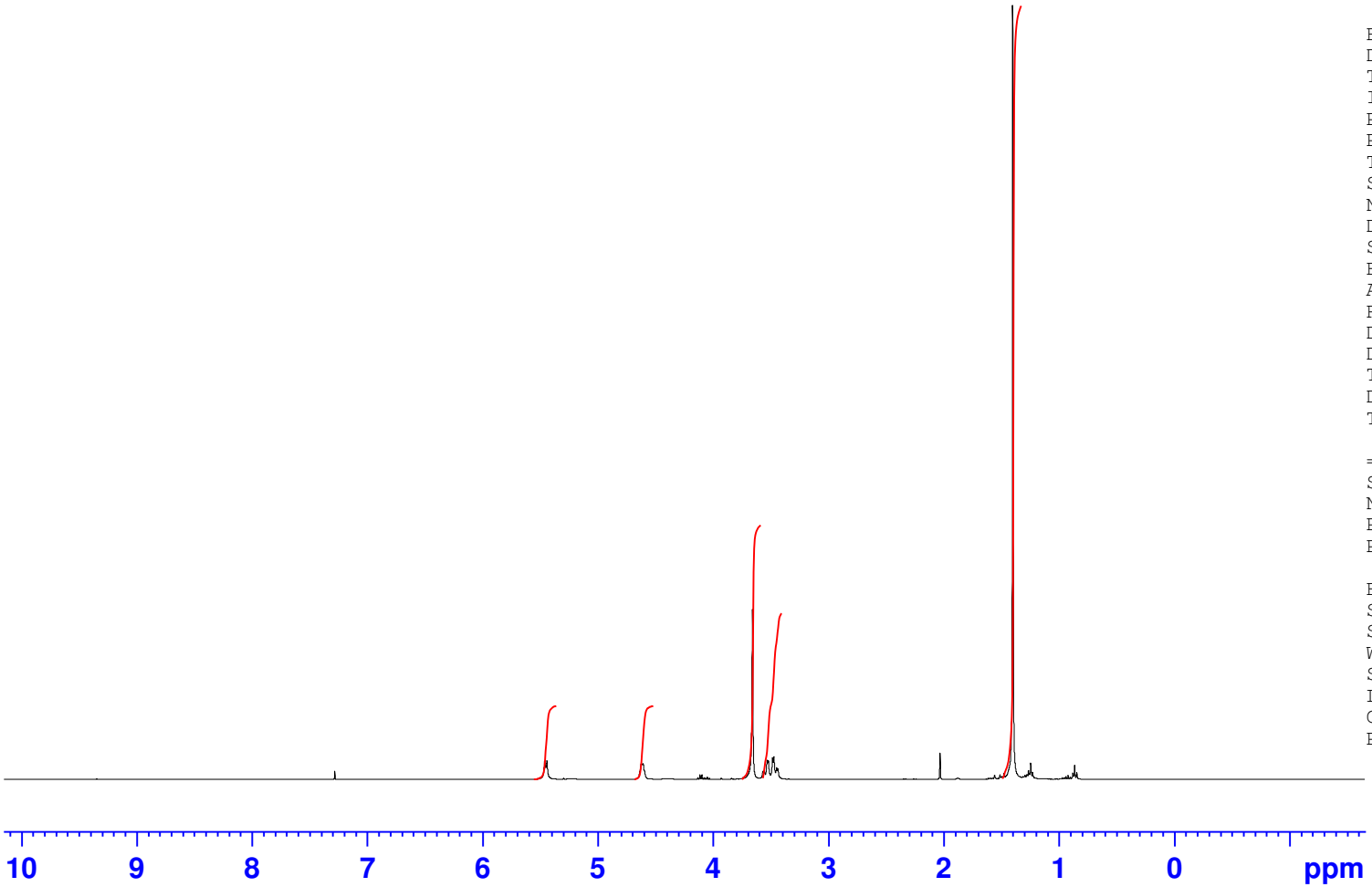
======== CHANNEL f1 ========
SFO1        376.4607164 MHz
NUC1               19F
P1               14.25 usec
PLW1        17.00000000 W

F2 - Processing parameters
SI                65536
SF          376.4983660 MHz
WDW                EM
SSB       0
LB               0.30 Hz
GB        0
PC               1.00
```

3b

C13

5.443
4.624
4.615
4.607
3.659
3.532
3.521
3.484
3.473
3.449
3.438
1.401

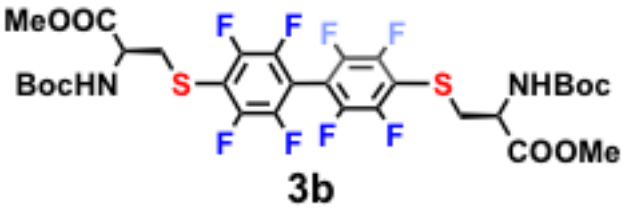



```
Current Data Parameters
NAME          Nov28_2012
EXPNO                 14
PROCNO                 1

F2 - Acquisition Parameters
Date_           20121204
Time               15.15
INSTRUM            spect
PROBHD   5 mm PABBO BB/
PULPROG             zg30
TD                 65536
SOLVENT            CDCl3
NS                     5
DS                     2
SWH             8012.820 Hz
FIDRES          0.122266 Hz
AQ             4.0894465 sec
RG                    32
DW                62.400 usec
DE                 6.50 usec
TE                294.2 K
D1            1.00000000 sec
TD0                    1

======== CHANNEL f1 ========
SFO1         400.1324710 MHz
NUC1                   1H
P1                14.50 usec
PLW1         10.00000000 W

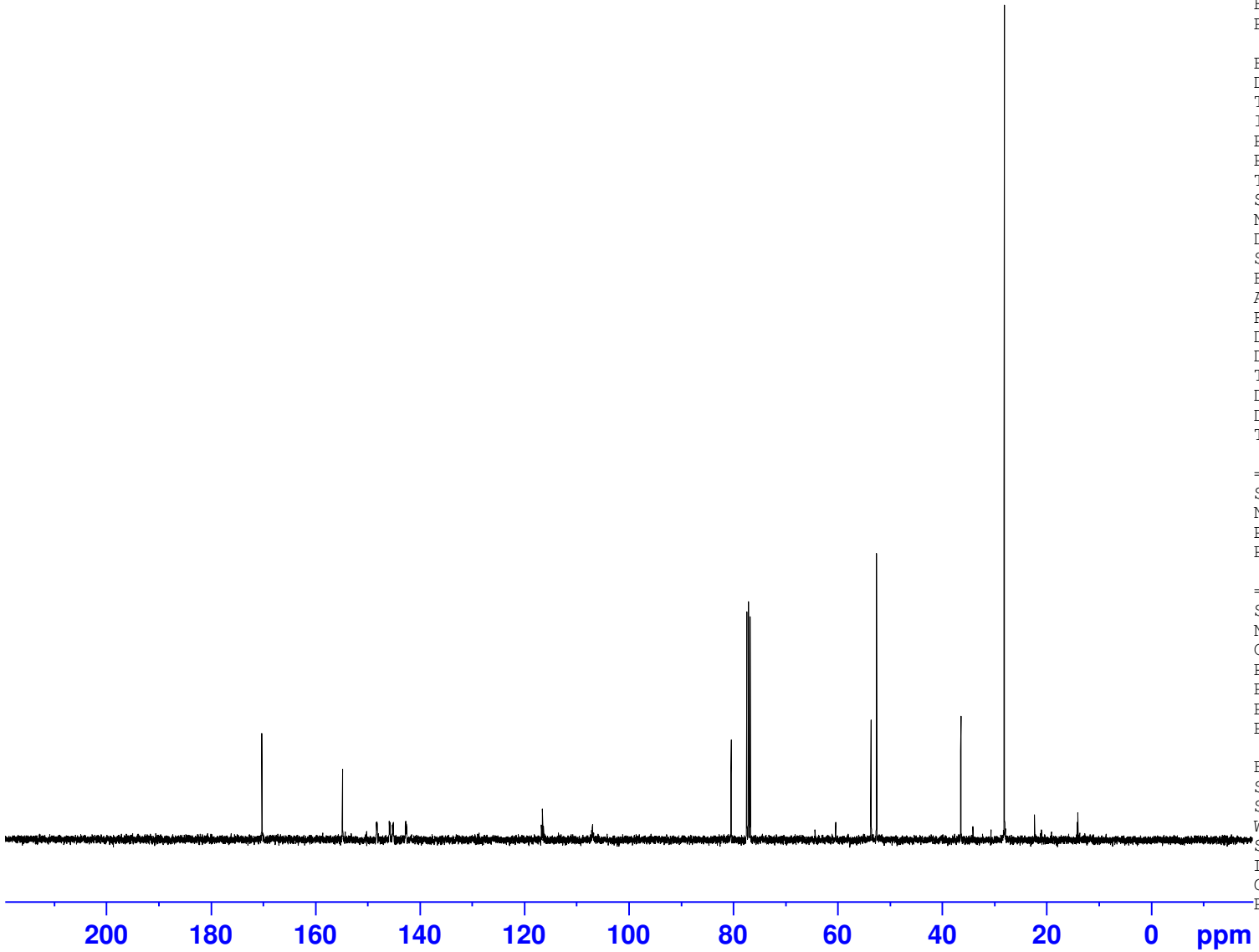
F2 - Processing parameters
SI                 65536
SF           400.1300000 MHz
WDW                   EM
SSB       0
LB                 0.30 Hz
GB        0
PC                 1.00
```



3b

C13

116.71
116.51
116.32
106.93
80.39
77.38
77.06
76.74
53.61
52.56
36.40
28.09




```
Current Data Parameters
NAME          Nov28_2012
EXPNO                 13
PROCNO                 1

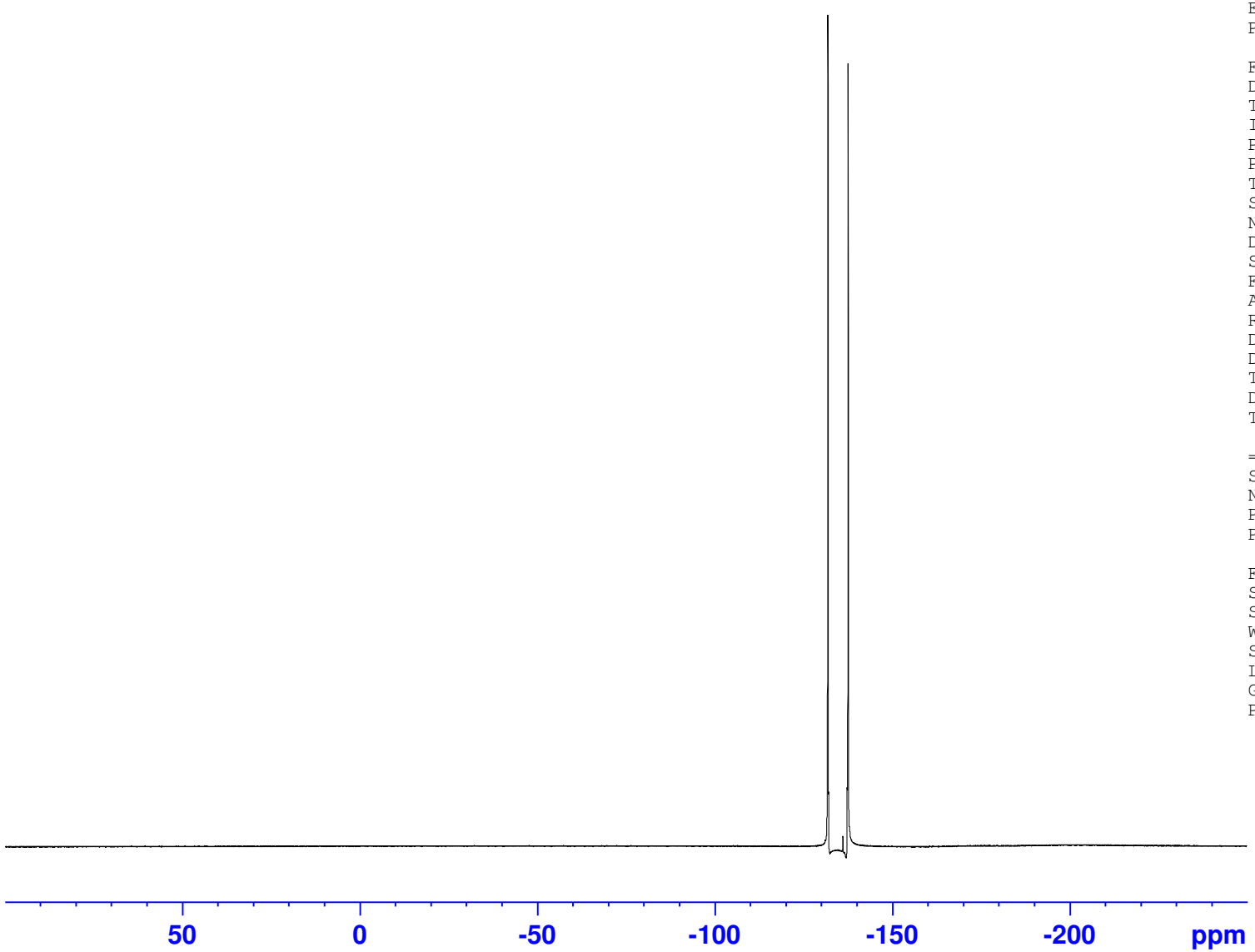
F2 - Acquisition Parameters
Date_           20121204
Time               15.13
INSTRUM            spect
PROBHD   5 mm PABBO BB/
PULPROG          zgpg30
TD                65536
SOLVENT           CDCl3
NS                   62
DS                    4
SWH           24038.461 Hz
FIDRES         0.366798 Hz
AQ            1.3631488 sec
RG                  203
DW                20.800 usec
DE                 6.50 usec
TE                294.8 K
D1           2.00000000 sec
D11          0.03000000 sec
TD0                   1

======== CHANNEL f1 ========
SFO1        100.6228293 MHz
NUC1                13C
P1                10.00 usec
PLW1        44.00000000 W

======== CHANNEL f2 ========
SFO2        400.1316005 MHz
NUC2                1H
CPDPRG[2         waltz16
PCPD2             90.00 usec
PLW2        10.00000000 W
PLW12        0.25957000 W
PLW13        0.21025001 W

F2 - Processing parameters
SI                 32768
SF          100.6127690 MHz
WDW                EM
SSB       0
LB                1.00 Hz
GB        0
PC                1.40
```

3b

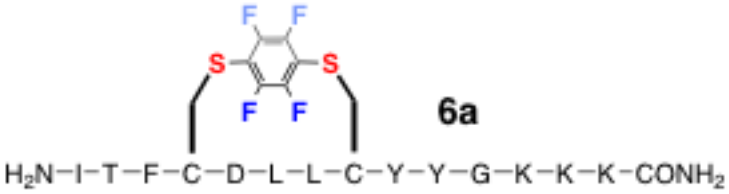


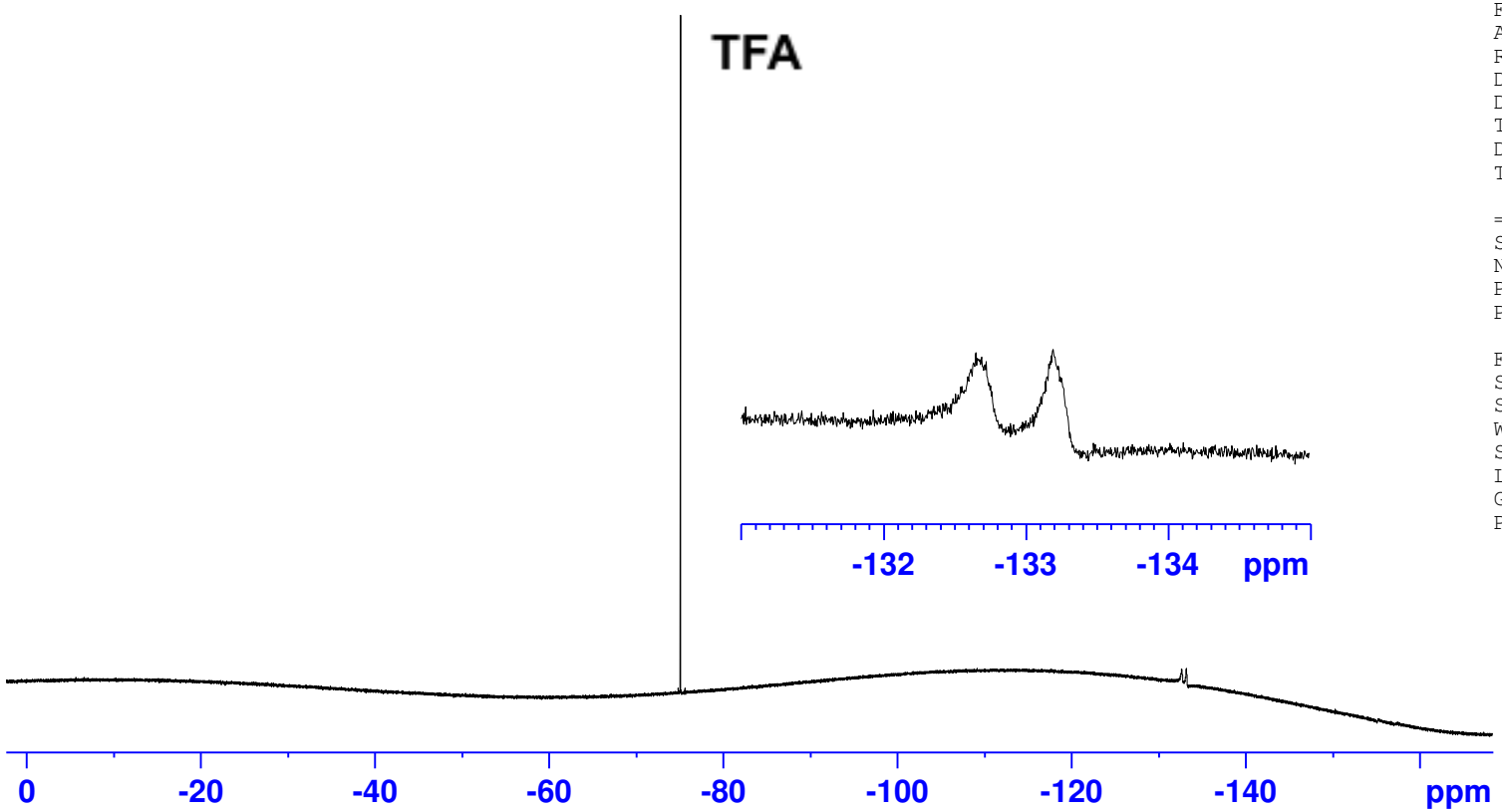

```
Current Data Parameters
NAME          Nov28_2012
EXPNO                  3
PROCNO                 1

F2 - Acquisition Parameters
Date_           20121128
Time               18.42
INSTRUM            spect
PROBHD   5 mm PABBO BB/
PULPROG          zgflqn
TD                319980
SOLVENT             None
NS                    28
DS                     4
SWH           200000.000 Hz
FIDRES          0.625039 Hz
AQ             0.7999500 sec
RG                   128
DW                 2.500 usec
DE                 6.50 usec
TE                 300.0 K
D1            1.00000000 sec
TD0                    1

======== CHANNEL f1 ========
SFO1         376.4607164 MHz
NUC1                19F
P1                14.25 usec
PLW1         17.00000000 W

F2 - Processing parameters
SI                 65536
SF           376.4983660 MHz
WDW                   EM
SSB       0
LB                 0.30 Hz
GB        0
PC                 1.00
```



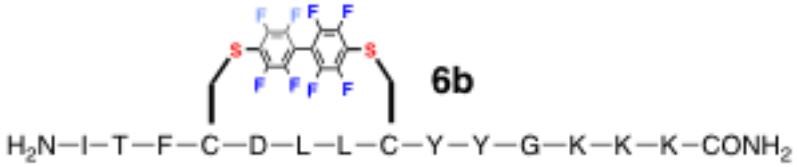


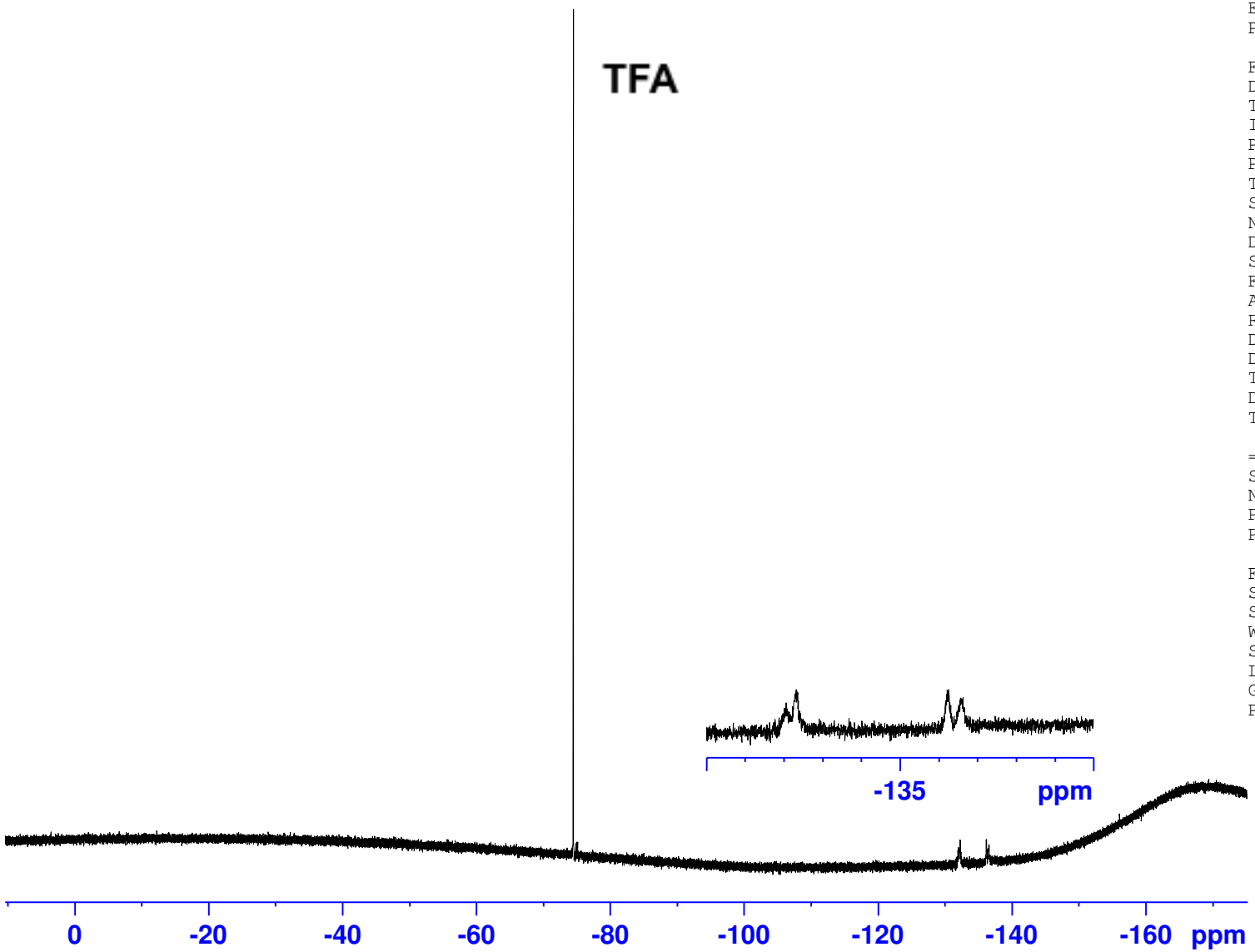

```
Current Data Parameters
NAME          Dec23_2012
EXPNO                  5
PROCNO                 1

F2 - Acquisition Parameters
Date_           20121223
Time               12.32
INSTRUM            spect
PROBHD   5 mm PABBO BB/
PULPROG          zgflqn
TD                199988
SOLVENT             None
NS                   255
DS                     4
SWH          100000.000 Hz
FIDRES         0.500030 Hz
AQ             0.9999400 sec
RG                   128
DW                 5.000 usec
DE                 6.50 usec
TE                 300.0 K
D1           1.00000000 sec
TD0                    1

======== CHANNEL f1 ========
SFO1         376.4607164 MHz
NUC1                19F
P1                14.25 usec
PLW1         17.00000000 W

F2 - Processing parameters
SI                 65536
SF           376.4983660 MHz
WDW                   EM
SSB       0
LB                 0.30 Hz
GB        0
PC                 1.00
```






```
Current Data Parameters
NAME          Dec21_2012
EXPNO                  8
PROCNO                 1

F2 - Acquisition Parameters
Date_           20121221
Time               11.39
INSTRUM            spect
PROBHD   5 mm PABBO BB/
PULPROG          zgflqn
TD                131072
SOLVENT              DMF
NS                    99
DS                     4
SWH            89285.711 Hz
FIDRES          0.681196 Hz
AQ             0.7340032 sec
RG                   128
DW                 5.600 usec
DE                 6.50 usec
TE                 298.1 K
D1            1.00000000 sec
TD0                    1

======== CHANNEL f1 ========
SFO1         376.4607164 MHz
NUC1                19F
P1                14.25 usec
PLW1         17.00000000 W

F2 - Processing parameters
SI                 65536
SF           376.4983660 MHz
WDW                   EM
SSB       0
LB                 0.30 Hz
GB        0
PC                 1.00
```