Supporting Information

Single dish gradient screening of small molecule localization

Paolo Beuzer,[a] Joshua Axelrod,[a] Lynnie Trzoss,[b] Willam Fenical,[b] Ramesh Dasari,[c] Antonio Evidente,[d] Alexander Kornienko,[c] Hu Cang*[,a] and James J. La Clair,*[,a,e,f]

[a]The Salk Institute for Biological Sciences, 10010 North Torrey Pines Rd, La Jolla, CA 92037, United States; [b]Center for Marine Biotechnology and Biomedicine, Scripps Institution of Oceanography, University of California at San Diego, La Jolla, CA, 92093, United States; [c]Department of Chemistry and Biochemistry, Texas State University, San Marcos, TX 78666, United States; [d]Dipartimento di Scienze Chimiche, Universita' di Napoli Federico II, Complesso Universitario Monte Sant'Angelo, Via Cintia 4, 80126 Napoli, Italy; [e]Department of Chemistry and Biochemistry, University of California at San Diego, La Jolla, CA 92093, United States, and [f]Xenobe Research Institute, P. O. Box 3052, San Diego, CA 92163, United States

Correspondence should be directed to hucang@salk.edu (H. C.) or i@xenobe.org (J. J. L)

Contents **Page**

A. General Experimental Methods. Reagents were purchased from Sigma-Aldrich or Fisher Scientific unless stated otherwise. The XRI-TF35 anti-IAF mAb was prepared as previously described in reference 8. Samples of this antibody are available from the Xenobe Research Institute. Conjugation of the XRI-TF35 anti-IAF mAB was conducted by incubating 200 µg of the XRI-TF35 anti-IAF mAb with 55 µg Alexa647 succinimidylester (Life Sciences) in 225 µL of 100 mM $NaHCO_3$ at rt for 1 h. The reaction was terminated by the addition of 22 µL of 1 M glycine pH 7.5. The Alexa647-conjugated anti-IAF TF35 mAb was purified using a PD-10 desalting column (GE Healthcare) and stored at a concentration of 4.3 mg/mL in phosphate buffered saline (PBS).

B. Cell Culture. Human osteosarcoma U2OS cells were cultured on 18 mm round No. 1.5 microscope cover glass (VWR Scientific) and placed in 12-well plates (Corning) in Dulbecco Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. The cells were cultured overnight to 60% confluence in a 37 °C tissue culture incubator with 5% CO_2 atmosphere.

C. Probe Treatment. Cells growing on coverslips in 12 well plates at 60% confluence in 1 mL of supplemented DMEM media were treated with 5 µL drop of IAF probes **1c** or **2b** in DMSO using the method in Scheme 1 and incubated in DMEM for 1 h at 37 °C in an atmosphere of 5% CO_2.

D. Fixation and Permeabilization. After probe treatment was complete, the cells were washed with PBS (4 × 1 mL). The plates were then charged with 0.5 mL of 4% formaldehyde and incubated for 15 min to fix the cellular structures. After fixation, the cells were washed again in PBS (4 × 1 mL). The cells were then permeabilized by treatment with 0.5 mL of 0.2% Triton X-100 for 5 min at rt, followed by washing with PBS (4 × 1 mL).

E. Immunofluorescent Staining. After permeabilization, the cells were blocked with 0.5 mL of a blocking buffer comprised of 5% bovine serum albumin (BSA) and 0.1% Tween-20 for 1 h at rt. Samples were incubated with 1:250 diluted 4.25mg/mL primary Alexa647-conjugated anti-IAF TF35 mAb (final concentration of 80 µM) in blocking buffer for 1 h at rt. Coverslips were then washed and mounted on 35 mm Glass Bottom Microwell Dishes (MatTek Corporation) and glued with Optical Adhesive 81 (Norland).

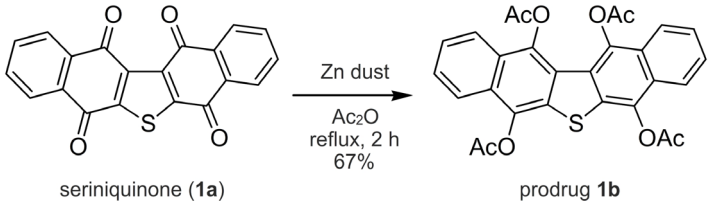
F. STORM Imaging. STORM imaging was carried out on a Ti inverted microscope (Nikon) equipped with a 60× CFI Apo TIRF objective lens, NA 1.49 (Nikon Apochromat). An ASI CRISP autofocus system (ASI Imaging) coupled with a 3D piezo stage (Physik Instruments) was used to lock in the focus during imaging. The maximum 642 nm power (Coherent OBIS) was used followed by activation with 405 nm laser (Coherent OBIS) for Alexa647 stained samples. A custom Labview (National Instruments) program was used to control the switching between the lasers. A homemade dual-viewer was placed in front of an EMCCD camera (Andor iXon3, 10242 pixels) for dual color imaging. A 1.5× tube lens was used to achieve a compound magnification of 90×. We added to the sample an imaging buffer comprised of 50 mM Tris pH 8.0, 10 mM NaCl containing 40 mM D-glucose, 0.5 mg/ml glucose oxidase (Sigma G6125), 40 µg/ml catalase (Sigma C1345), and 150 mM β-mercaptoethanol (Sigma M6250). Single molecule blinking events were collected from a FOV of 256×256 pixels with an integration time of 19 ms for a total of about 10,000 frames. STORM images were analyzed by ThunderSTORM (GitHub thunderstorm) using Wavelet filter (B-spline) for image filtering, approximate localization of molecules by Local Maximum and PSF:integrated Gaussian for subpixel localization of molecules. Lateral drift was corrected by cross-correlation using ThunderSTORM software with 5 bins and magnification 5.

G. Epifluorescence Imaging. Semrock filter sets were used to image the blue fluorescent IAF tag with excitation at 385-400 nm (bandpass, 393 CWL), dichromatic mirror at 435-470 nm (bandpass), barrier filter at 450-465 nm (bandpass, 458 CWL). Far red Alexa647 was imaged

using an excitation filter at 590-650 nm, dichromatic mirror 660 nm (longpass), and barrier filter at 663-738 nm. Epifluorescence imaging was conducted before STORM imaging.

H. General Synthetic Methods. Unless otherwise noted, all commercially available reagents were purchased and used without further purification. Dichloromethane (CH_2Cl_2) was refluxed in the presence of CaH_2 prior to use. *N*,*N*-Diisopropylethylamine (DIPEA) was distilled under normal pressure and stored in the presence of molecular sieves. Acids **5** and amine **7** were prepared using methods described in references 7 and 8, respectively. ^1H and ^{13}C NMR spectra were recorded on a Mercury Plus 500 (Varian) or VX500 equipped with Xsens cold probe (Varian) spectrometer in $CDCl_3$ unless otherwise noted. Chemical shifts are reported in parts per million (ppm) and coupling constants (*J*) are reported in Hertz (Hz). Coupling patterns are described by abbreviations: s (singlet), d (doublet), t (triplet), m (multiplet). HR-EIS-TOFMS data were obtained on an Agilent 6530 Accurate-Mas Q-TOF LMCS spectrometer.

I. Syntheses of prodrug 1b and IAF probe 1d. The syntheses of **1b** and probe **1d** were accomplished in 1 or 3 steps from **1a** and **1c**, respectively, as shown in Scheme 2, Scheme S1 (below) and Scheme S2 (below). The following sections provide an overview of the experimental procedures used for these conversions.



Scheme S1. Synthesis of prodrug **1b**.

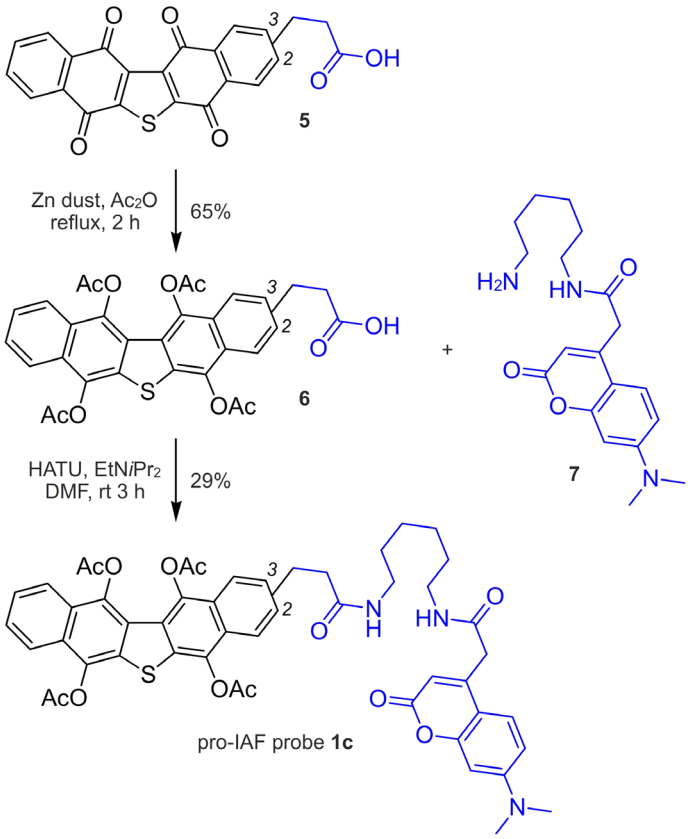
Dinaphtho[2,3-*b*:2',3'-*d*]thiophene-5,7,12,13-tetrayl tetraacetate (1b). A mixture of seriniquinone (**1a**) (50.0 mg, 0.15 mmol), Zn dust (81.0 mg, 1.23 mmol) and Ac_2O (2 mL) was brought to reflux. After 2 h, the reaction mixture was cooled to rt and the unreacted Zn dust was filtered and the filtrate was concentrated under reduced pressure. The residue was purified by silica flash chromatography eluting with a gradient of 1:1 hexanes/CH_2Cl_2 to CH_2Cl_2 to provide **1b** (51.2 mg, 67%).

Prodrug **1b**: ^1H NMR ($CDCl_3$, 500 MHz) δ 8.12 (td, J = 1.2, 8.2 Hz, 2H), 7.80 (td, J = 0.9, 8.2 Hz, 2H), 7.64 (ddd, J = 1.3, 6.7, 8.3 Hz, 2H), 7.59 (ddd, J = 1.3, 6.8, 8.1 Hz, 2H), 2.61 (s, 6H), 2.58 (s, 6H); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 168.8, 168.7, 140.2, 137.7, 128.7, 127.2, 126.6, 126.2, 125.6, 122.9, 120.6, 20.8, 20.5; HR-ESI-MS *m/z* calcd. for $C_{28}H_{20}O_8SNa$ $[M+Na]^+$: 539.0879, found 539.0775.

3-(5,7,12,13-Tetraacetoxydinaphtho[2,3-*b*:2',3'-*d*]thiophen-2-yl)propanoic acid and 3-(5,7,12,13-tetraacetoxydinaphtho[2,3-*b*:2',3'-*d*]thiophen-3-yl)propanoic acid (6). A mixture of acid **5** (80.0 mg, 0.19 mmol), Zn dust (97.0 mg, 1.48 mmol) and Ac_2O (2 mL) was brought to reflux for 2 h. Upon completion, the reaction mixture was cooled to rt and the unreacted Zn dust was filtered and the filtrate was concentrated under reduced pressure. Pure acid **6** (70.6 mg, 65%) was obtained by flash chromatography eluting with a gradient of 1:1 hexanes/CH_2Cl_2 to 100:1 CH_2Cl_2/MeOH.

Acids **6**: ^1H NMR ($CDCl_3$, 500 MHz) δ 8.12 (m, 1H), 8.05 (d, J = 8.7 Hz, 1H), 7.91 (m, 1H), 7.80 (d, J = 8.4 Hz, 1H, major isomer), 7.74 (d, J = 8.6 Hz, 1H, minor isomer), 7.63 (m, 1H), 7.60 (m, 1H), 7.49 (dd, J = 1.7, 8.7 Hz, 1H, minor isomer), 7.44 (dd, J = 1.7, 8.8 Hz, 1H), 3.17 (m, 2H), 2.78 (m, 2H), 2.60 (s, 3H, minor isomer), 2.60 (s, 3H, major isomer), 2.58 (s, 6H, minor isomer), 2.58 (s, 9H, major isomer), 2.57 (s, 3H, minor isomer); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 170.8, 177.9, 168.5, 168.4, 168.4, 168.3, 168.2, 168.2, 152.7, 140.7, 140.7, 140.6, 140.4, 138.6, 138.2,

138.2, 137.9, 130.7, 130.3, 130.2, 129.7, 129.2, 128.1, 127.6, 127.4, 127.2, 127.1, 127.0, 126.8, 126.8, 126.5, 126.5, 126.4, 126.4, 126.0, 125.9, 125.6, 123.2, 122.8, 122.7, 121.3, 121.0, 120.5, 120.5, 119.1, 35.2, 35.2, 31.1, 31.1, 21.1, 21.1, 21.1, 20.8; HR-ESI-MS *m/z* calcd. for $C_{31}H_{25}O_{10}S$ $[M+H]^+$: 611.1021, found 611.1003.



Scheme S2. Synthesis of pro-IAF probe **1c**.

2-(3-((6-(2-(7-(Dimethylamino)-2-oxo-2*H*-chromen-4-yl)acetamido)hexyl)amino)-3-oxopropyl)-dinaphtho[2,3-*b*:2',3'-*d*]thiophene-5,7,12,13-tetrayl tetraacetate and 3-(3-((6-(2-(7-(dimethyl-amino)-2-oxo-2*H*-chromen-4-yl)acetamido)hexyl)amino)-3-oxopropyl)dinaphtho[2,3-*b*:2',3'-*d*]thiophene-5,7,12,13-tetrayl tetraacetate (1c). Acids **6** (74.0 mg, 0.12 mmol) were dissolved in anhydrous DMF (12 mL). Amine **7** (65.0 mg, 0.15 mmol) was added followed by EtN*i*Pr$_2$ (0.064 mL, 0.37 mmol) and HATU (61.0 mg, 0.16 mmol) at 0 °C. The reaction was then warmed to rt. After 3 h, the DMF was removed under reduced pressure. Pure probe **1c** (32.0 mg, 29%) was obtained by silica gel flash chromatography eluting with a gradient of 100:1 CH_2Cl_2/MeOH to 20:1 CH_2Cl_2/MeOH).

Probe **1c**: ^{1}H NMR ($CDCl_3$, 500 MHz) δ 8.10 (d, J = 9.1 Hz, 1H), 8.03 (d, J = 8.7 Hz, 1H), 7.78 (d, J = 10.1 Hz, 1H), 7.60 (m, 3H), 7.56 (d, J = 7.0 Hz, 1H), 7.33 (d, J = 8.9 Hz, 1H), 6.51 (dd, J = 9.0, 2.6 Hz, 1H), 6.44 (d, J = 2.4 Hz, 1H), 5.83 (m, 1H), 5.79 (s, 1H), 5.45 (m, 1H, 3.21 (s, 2H), 3.16 (m, 2H), 3.07 (m, 2H), 2.98 (m, 6H), 2.93 (m, 2H), 2.59 (m, 12H), 1.06 (m, 10H); ^{13}C NMR (DMSO-d_6, 125MHz) δ 171.5, 169.4, 169.3, 169.2, 168.2, 161.4, 156.0, 153.5, 152.1, 143.3, 140.8, 140.6, 138.3, 137.9, 129.4, 129.3, 127.7, 127.5, 127.1, 126.9, 126.6, 126.3, 125.5, 125.4, 123.5, 121.2, 119.2, 110.0, 109.6, 108.8, 98.1, 49.2, 39.3, 39.0, 29.7, 29.5, 26.7, 26.6, 21.3, 21.2, 21.1; HR-ESI-MS *m/z* calcd. for $C_{50}H_{50}N_3O_{12}S$ $[M+H]^+$: 916.3070, found 916.3146.

J. Synthesis of probe 2b. The synthesis of **2b** was accomplished in 2 steps from ophiobolin A (**2a**), as shown in Scheme 3. The following sections provide an overview of the experimental procedures used for these conversions.

(3*S*,3a*R*,3'*S*,5'*R*,6a*S*,9*R*,9a*S*,10a*R*,*E*)-5'-((*R*)-1-bromo-2-methyl-2-(prop-2-yn-1-yloxy)propyl)-9-hydroxy-3',9,10a-trimethyl-7-oxo-1,3a,4,4',5',6a,7,8,9,9a,10,10a-dodecahydro-2*H*,3'*H*-spiro[dicyclopenta[*a*,*d*][8]annulene-3,2'-furan]-6-carbaldehyde (3). *N*-bromosuccinimide (2.4 mg, 0.013 mmol) and NaOAc (6.0 mg, 0.07 mmol) were added to a solution of ophiobolin A (**2a**) (5.0 mg, 0.012 mmol) in propargyl alcohol (0.3 mL). After stirring at rt for 20 h, the reaction mixture was diluted with EtOAc and washed with satd. $NaHCO_3$. The resulting organic layer was dried over Na_2SO_4 and concentrated. Pure alkyne **3** (1.5 mg, 23 %) was obtained by pTLC using a chamber charged with 3:10 EtOAc/hexanes.

Alkyne **3**: ^{1}H NMR (400 MHz, C_6D_6) δ 9.00 (s, 1H), 6.04 (m, 1H), 4.24 (m, 1H), 4.14 (d, J = 3.8 Hz, 1H), 3.83 (d, J = 2.4 Hz, 1H), 3.81 (d, J = 2.4 Hz, 1H), 3.43 (d, J = 10.1 Hz, 1H), 3.10 (d, J = 16.5 Hz, 1H), 2.74 (dd, J = 14.7, 4.3 Hz, 1H), 2.50 (m, 1H), 2.20 (m, 2H), 2.07 (t, J = 2.4 Hz, 1H), 2.01 (m, 2H), 1.81-1.66 (m, 4H), 1.55 (m, 2H), 1.25 (m, 2H), 1.19 (s, 3H), 1.18 (s, 3H), 0.94 (s, 3H), 0.74 (d, J = 6.9 Hz, 3H), 0.60 (s, 3H); ^{13}C NMR (100 MHz, C_6D_6) δ 213.8, 193.7, 156.0, 143.3, 96.4, 81.5, 77.7, 76.0, 74.4, 73.7, 68.1, 55.2, 52.7, 50.9, 49.9, 48.8, 42.8, 42.6, 42.4, 40.3, 35.6, 30.1, 30.0, 25.4, 24.9, 24.1, 22.4, 15.6; HR-ESI-MS *m/z* calcd. for $C_{28}H_{39}BrNaO_5$ $[M+Na]^+$: 557.1879, found 557.1882.

***N*-(2-(4-((((*R*)-1-bromo-1-((3*S*,3a*R*,3'*S*,5'*R*,6a*S*,9*R*,9a*S*,10a*R*,*E*)-6-formyl-9-hydroxy-3',9,10a-trimethyl-7-oxo-1,3a,4,4',5',6a,7,8,9,9a,10,10a-dodecahydro-2*H*,3'*H*-spiro[dicyclopenta-[*a*,*d*][8]annulene-3,2'-furan]-5'-yl)-2-methylpropan-2-yl)oxy)methyl)-1*H*-1,2,3-triazol-1-yl)ethyl)-2-(7-(dimethylamino)-2-oxo-2*H*-chromen-4-yl)acetamide (2b).** A freshly prepared 1M aqueous solution of $CuSO_4$•$5H_2O$ (1.0 mL) was added to a 10 M aqueous suspension of sodium ascorbate (1.0 mL) and the mixture was stirred at rt until the black solution turned light yellow-brown. A 10 µL aliquot of this solution was then added to a solution of alkyne **3** (3.4 mg, 0.006 mmol) and azide **4** (2.0 mg, 0.006 mmol) in *t*-BuOH (0.3 mL) and H_2O (0.5 mL). The resulting mixture was stirred at rt for overnight. The crude product was obtained via rotary evaporation. Pure probe **2b** (5.6 mg, 88 %) was obtained by pTLC in a chamber containing 5% MeOH/$CHCl_3$.

Probe **2b**: ^{1}H NMR (400 MHz, CD_3OD) δ 9.17 (s, 1H), 7.77 (s, 1H), 7.47 (d, J = 9.1 Hz, 1H), 7.00 (m, 1H), 6.76 (dd, J = 9.1, 2.6 Hz, 1H), 6.57 (d, J = 2.6 Hz, 1H), 5.99 (s, 1H), 4.53 (m, 6H), 4.32 (d, J = 4.1 Hz, 1H), 4.20 (m, 1H), 3.69 (m, 2H), 3.63 (m, 2H), 3.09 (s, 6H), 2.99 (d, J = 16.6 Hz, 1H), 2.87 (m, 1H), 2.69 (m, 1H), 2.38-2.26 (m, 3H), 2.11 (m, 2H), 1.78 (m, 4H), 1.54 (m, 2H), 1.43 (s, 3H), 1.39 (s, 3H), 1.38 (s, 3H), 1.18 (m, 1H), 1.03 (d, J = 6.5 Hz, 3H), 0.88 (s, 3H); ^{13}C NMR (100 MHz, CD_3OD) δ 219.0, 194.5, 174.3, 170.0, 162.8, 158.5, 155.8, 153.4, 151.0, 145.5, 142.3, 125.6, 123.7, 109.3, 109.2, 97.4, 96.7, 78.1, 76.9, 75.5, 74.3, 70.1, 67.5, 55.4, 54.5, 52.5, 50.0, 48.9, 48.5, 42.22, 42.16, 42.06, 39.7, 39.2, 38.9, 38.6, 35.4, 29.8, 24.1, 23.5, 23.3, 21.4, 14.6; HR-ESI-MS *m/z* calcd. for $C_{43}H_{56}BrN_5NaO_8$ $[M+Na]^+$:872.3210, found 872.3223.

K. Selected NMR spectra

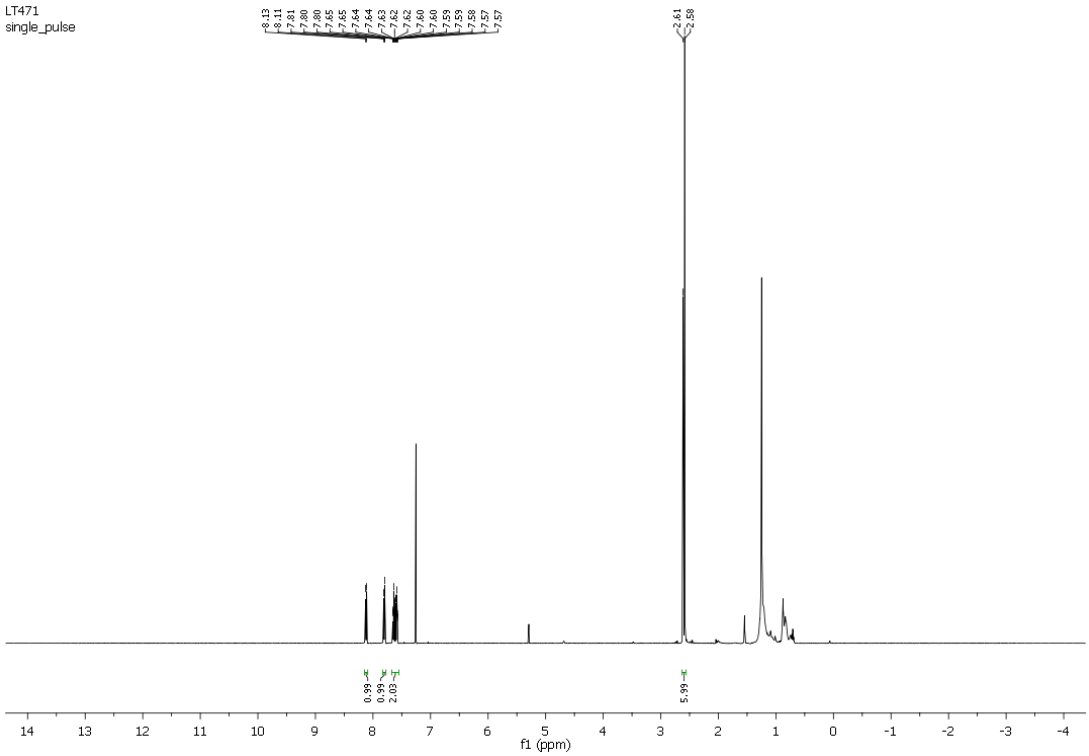


Figure S1. ^{1}H-NMR (500 MHz) spectra of prodrug **1b** in $CDCl_3$

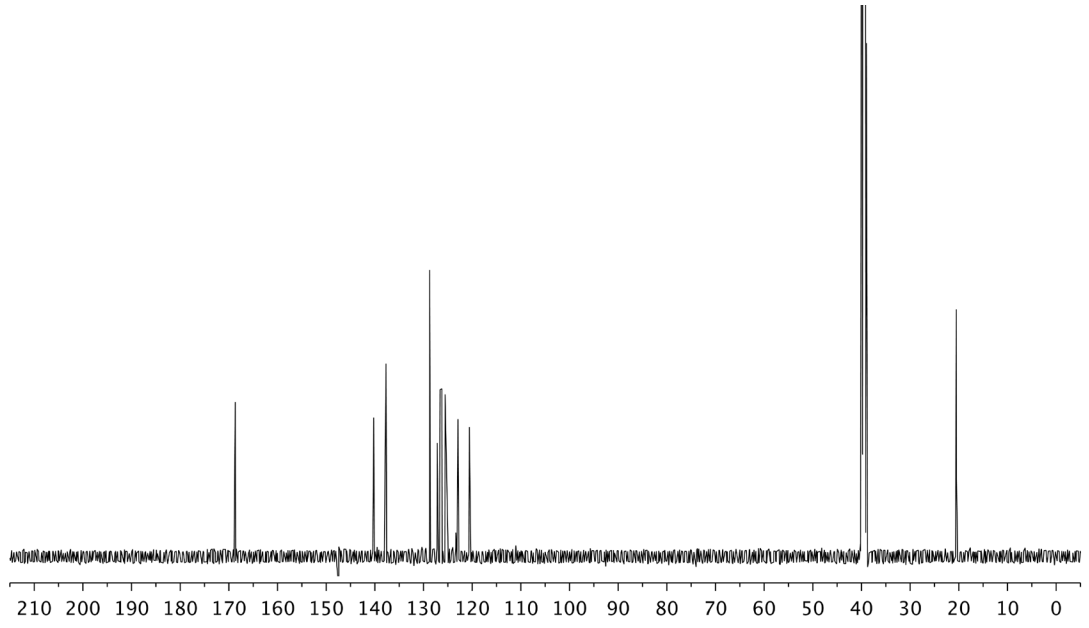


Figure S2. ^{13}C-NMR (125 MHz) spectra of prodrug **1b** in DMSO-d_6

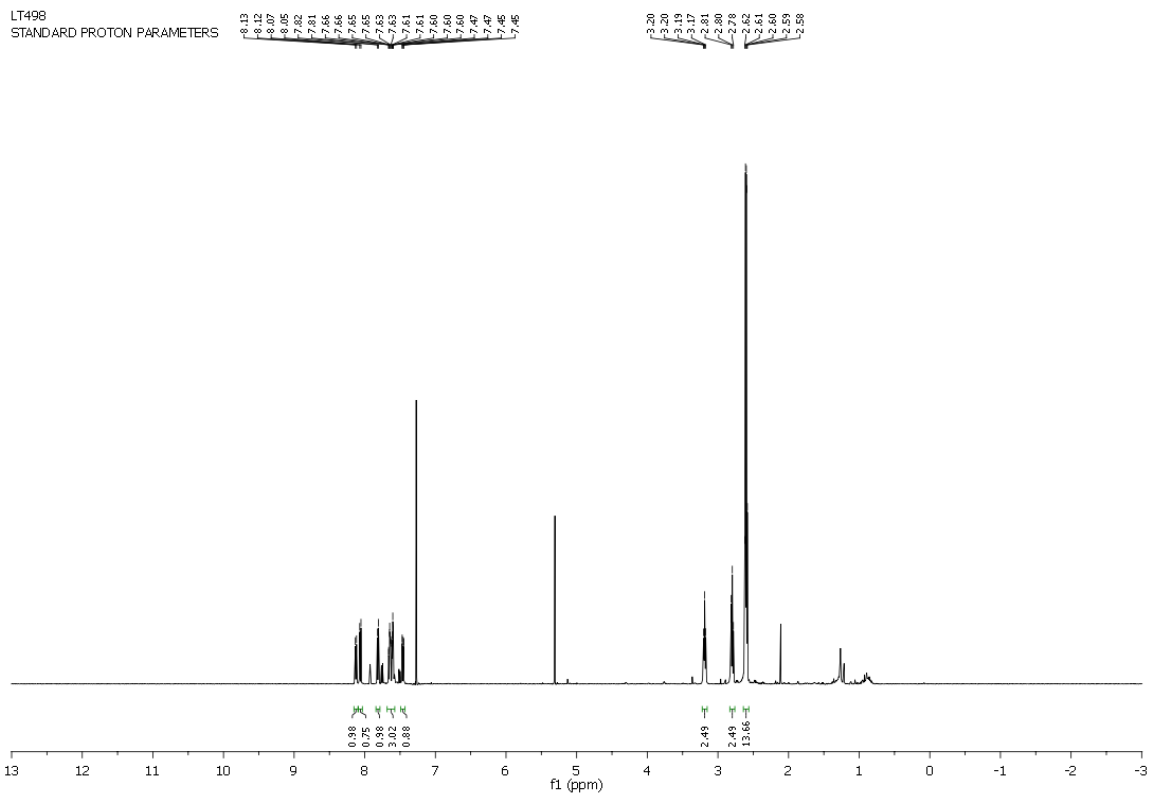


Figure S3. ^{1}H-NMR (500 MHz) spectra of acid **6** in $CDCl_3$

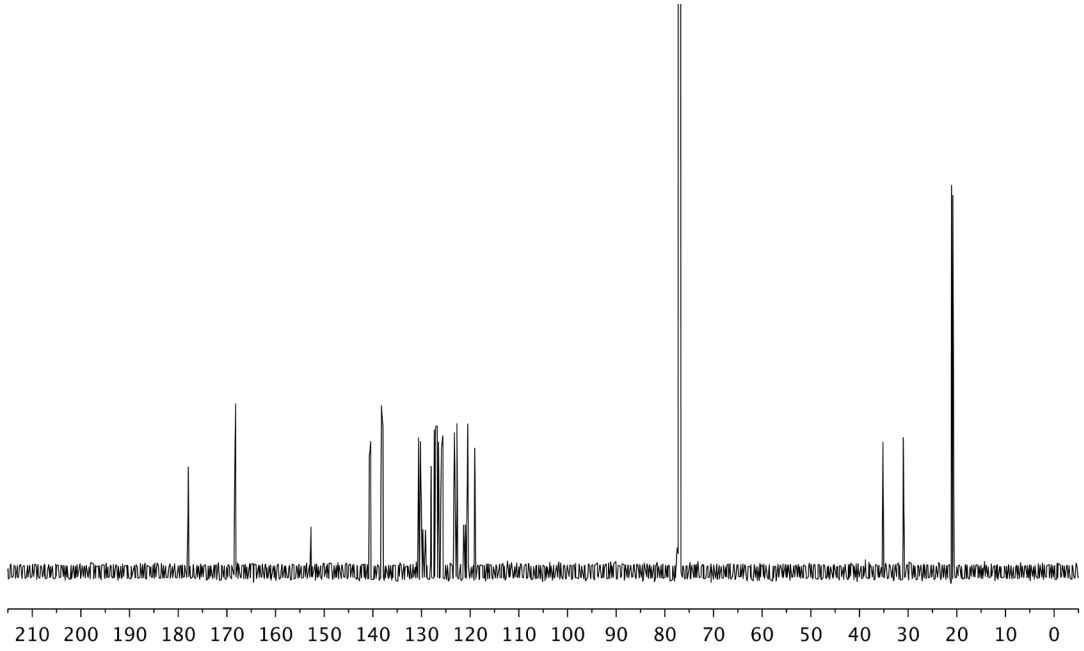


Figure S4. ^{13}C-NMR (125 MHz) spectra of acid **6** in $CDCl_3$

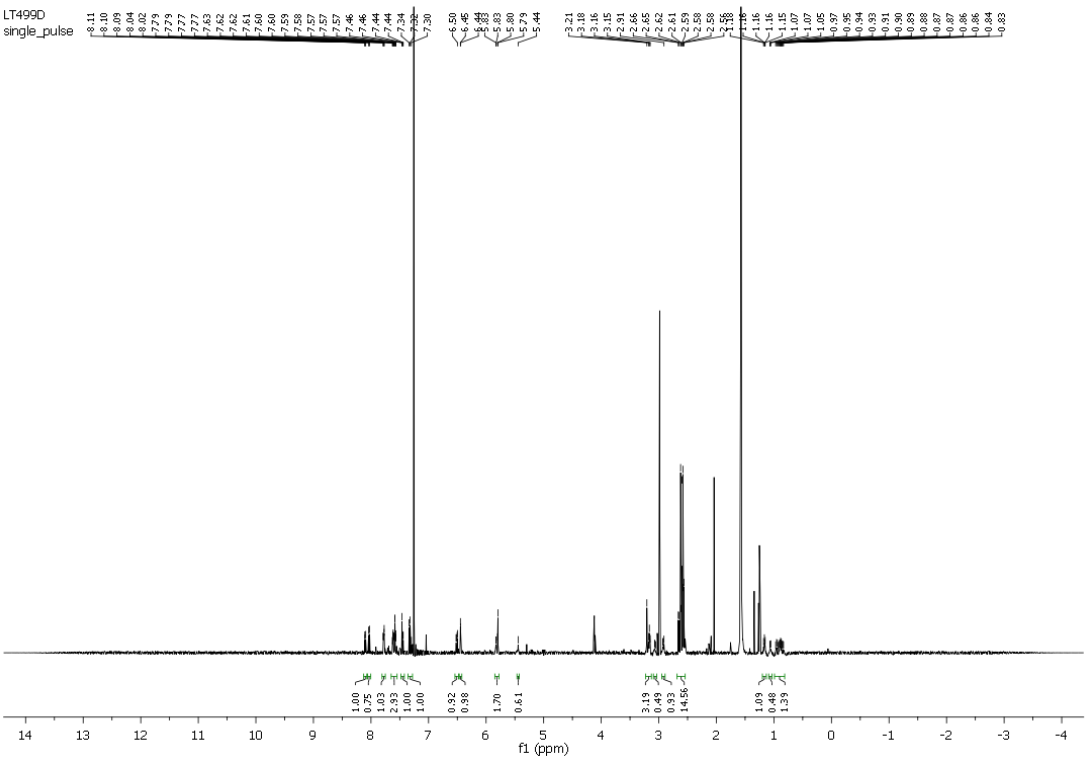


Figure S5. ^{13}C-NMR (125 MHz) spectra of pro-IAF probe **1c** in $CDCl_3$

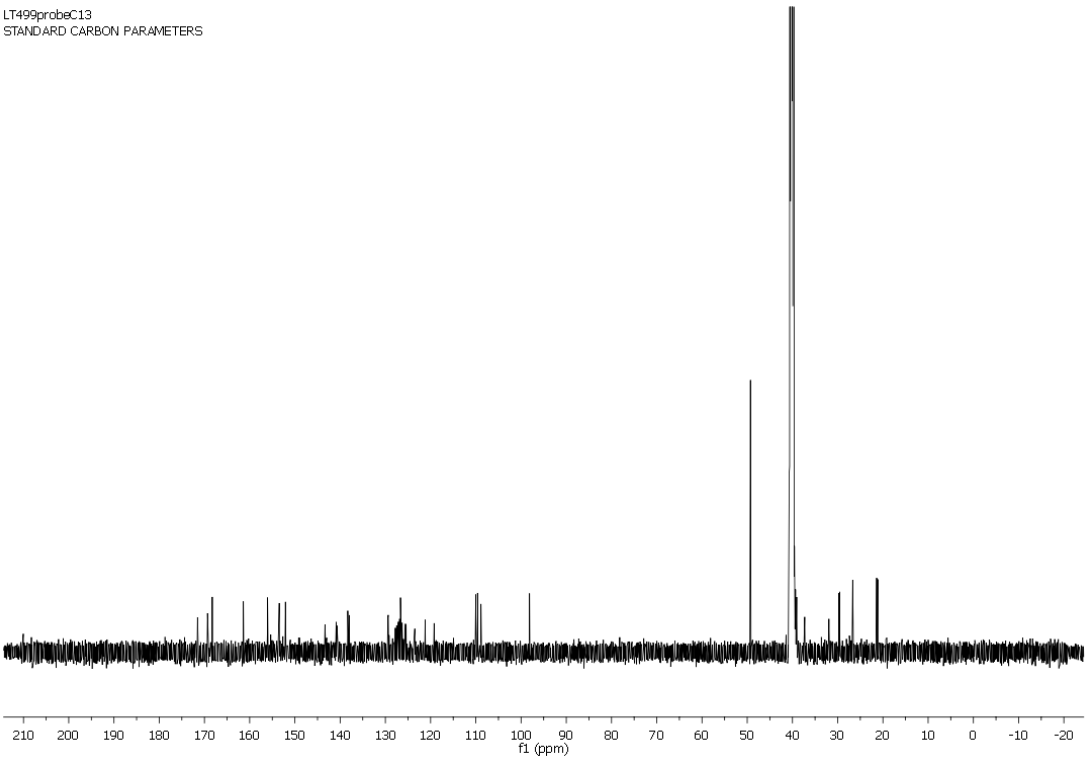


Figure S6. ^{13}C-NMR (125 MHz) spectra of pro-IAF probe **1c** in $CDCl_3$

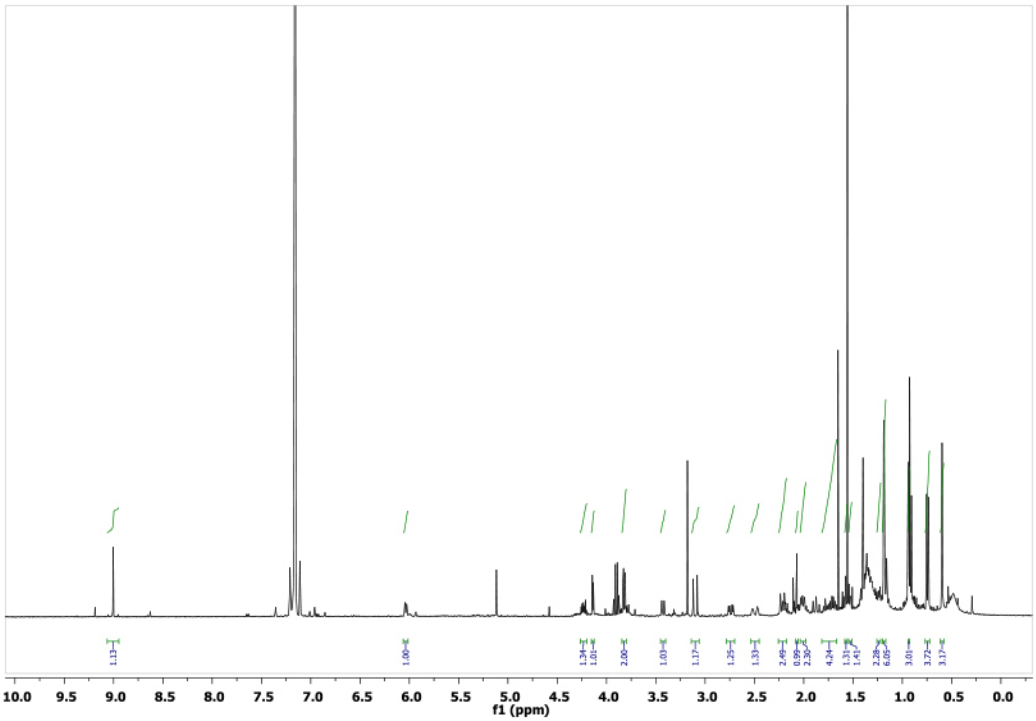


Figure S7. ^{1}H-NMR (400 MHz) spectra of alkyne **3** in benzene-d_6

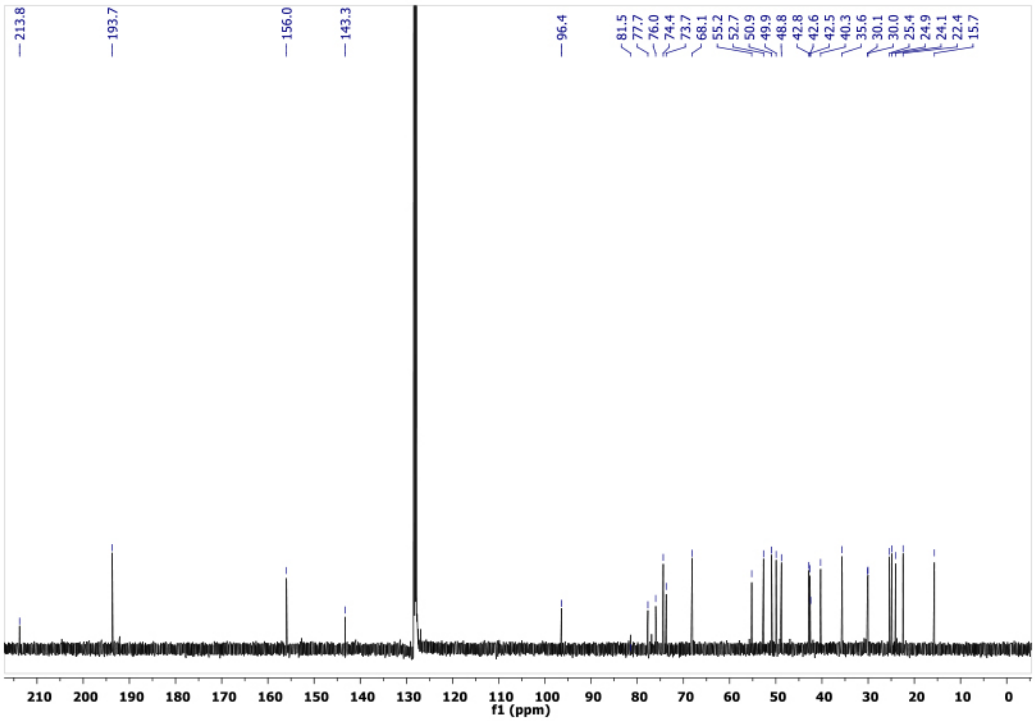


Figure S8. ^{13}C-NMR (100 MHz) spectra of alkyne **3** in benzene-d_6

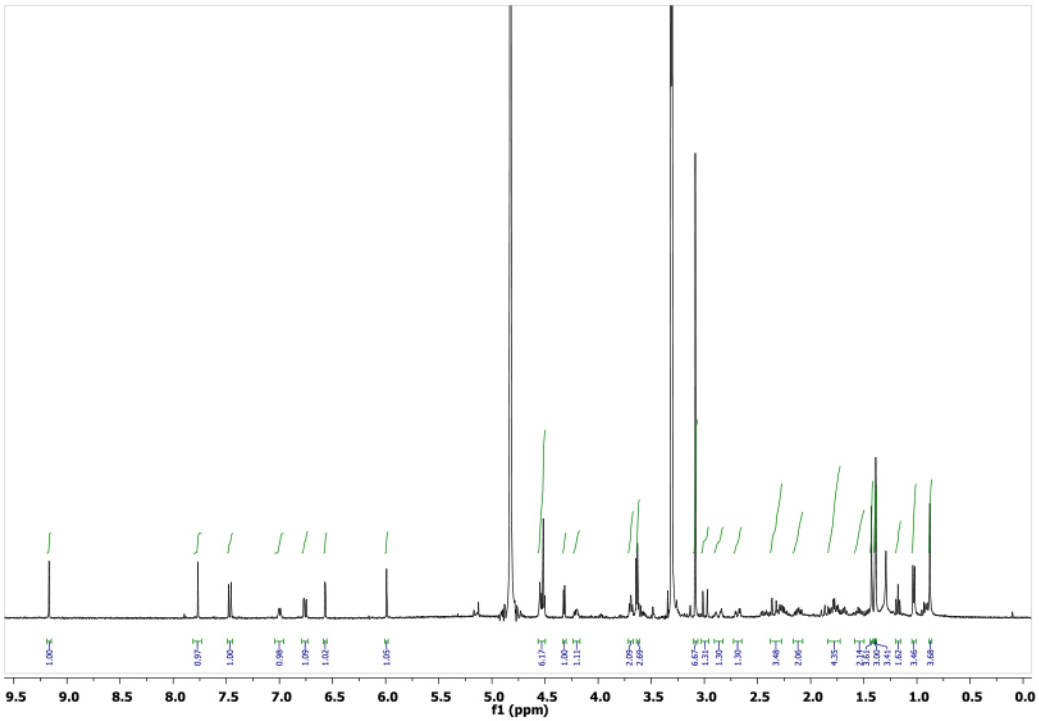


Figure S9. ^{1}H-NMR (400 MHz) spectra of IAF probe **2b** in CD_3OD

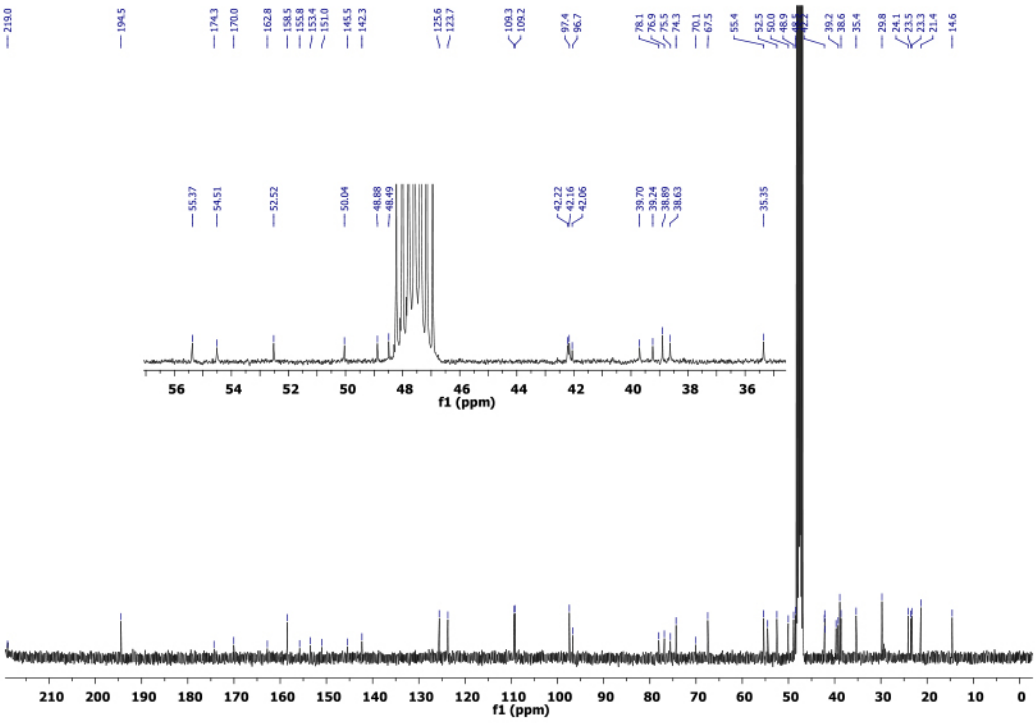


Figure S10. ^{13}C-NMR (100 MHz) spectra of IAF probe **2b** in CD_3OD

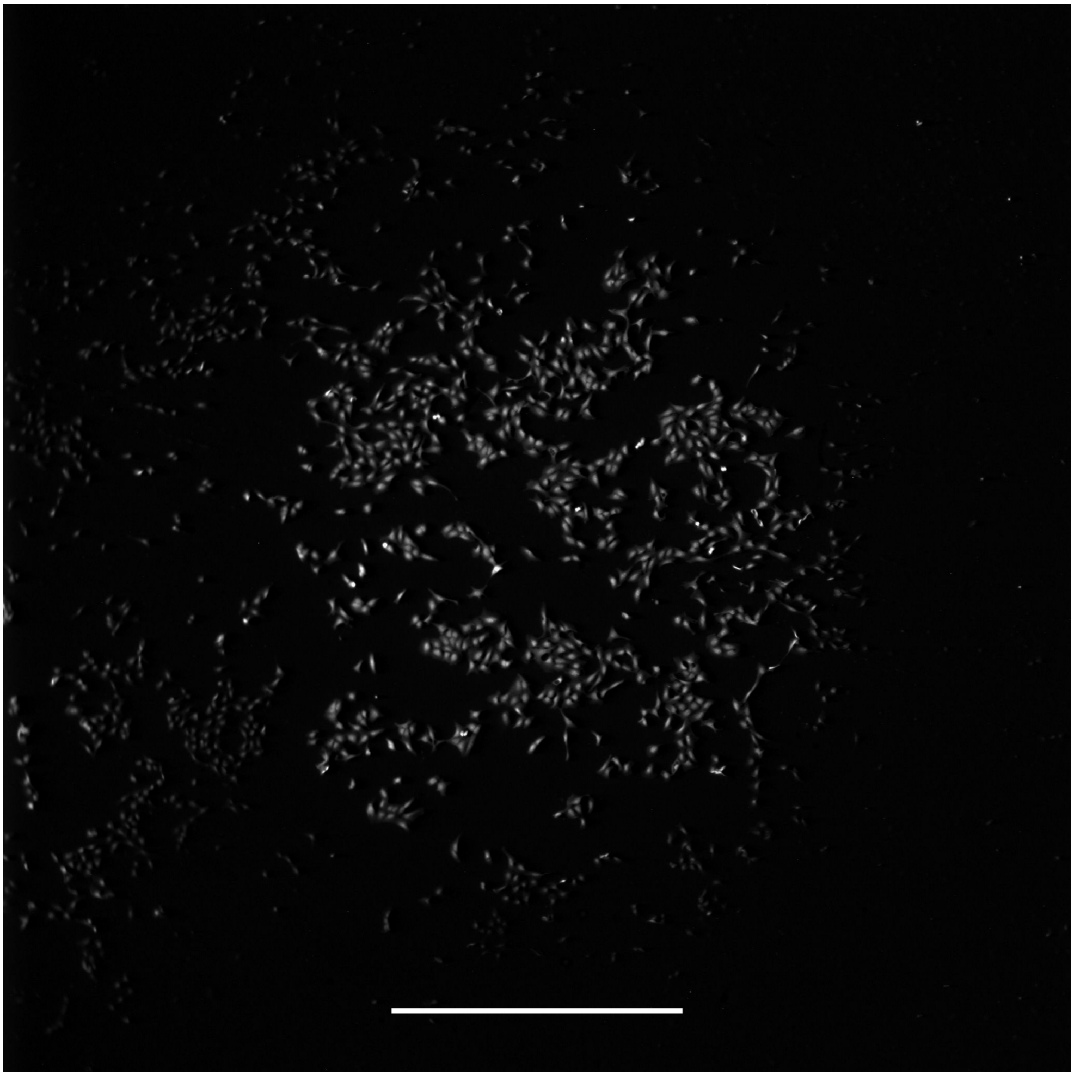


Figure S11. Full size image of the panel in Fig. 1b.

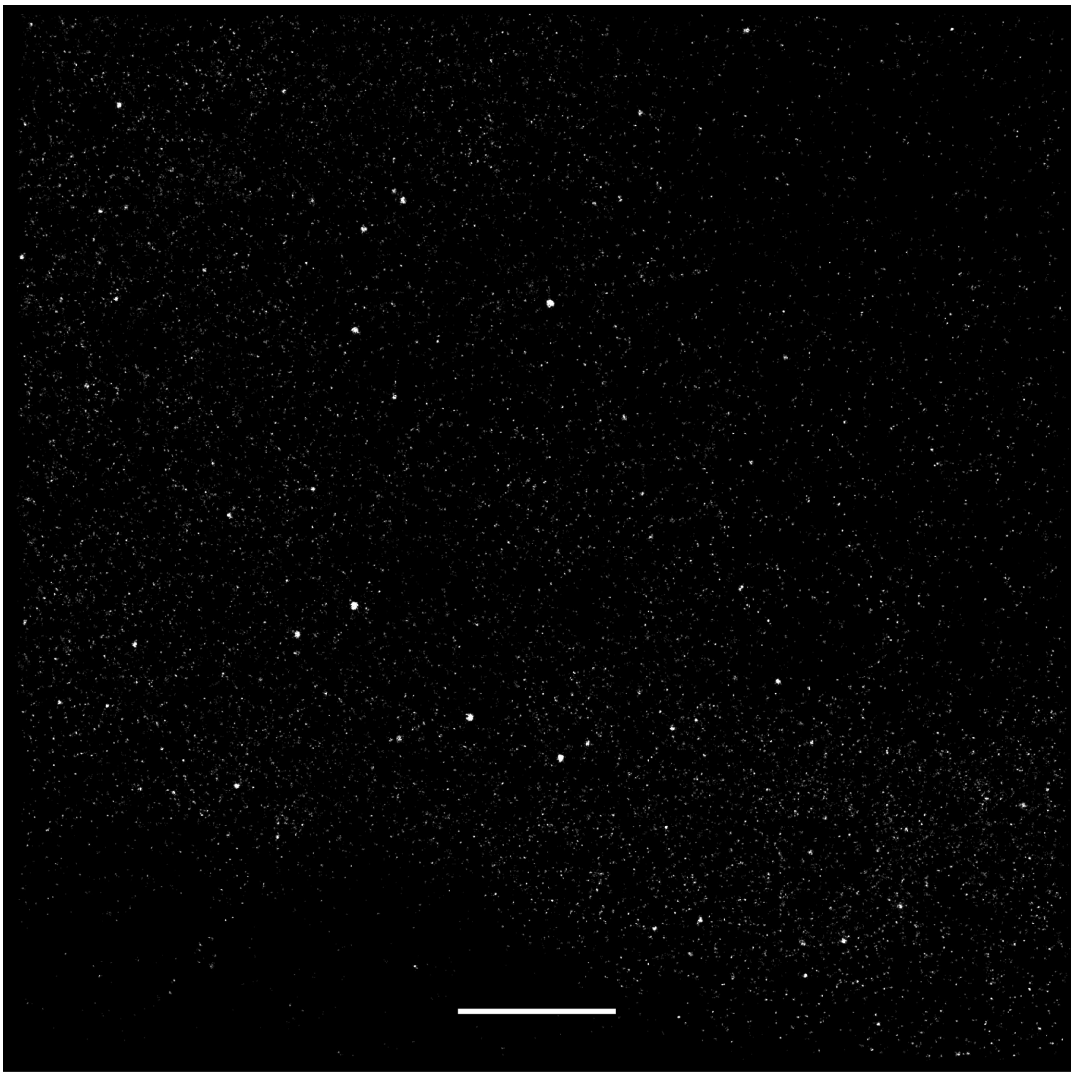


Figure S12. Full size image of the STORM image from the right column of Fig. 2a.

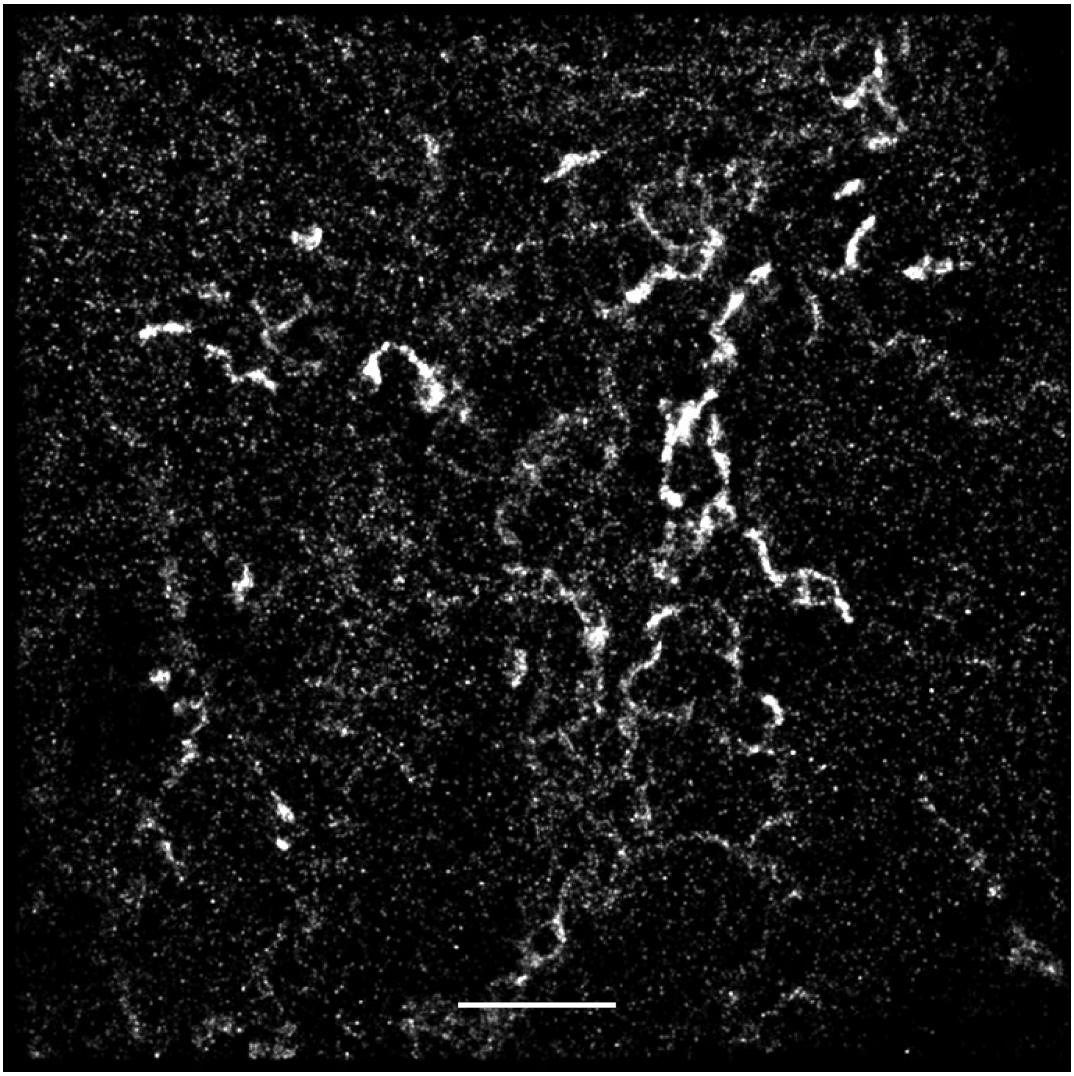


Figure S13. Full size image of the STORM image from the right column of Fig. 2b.

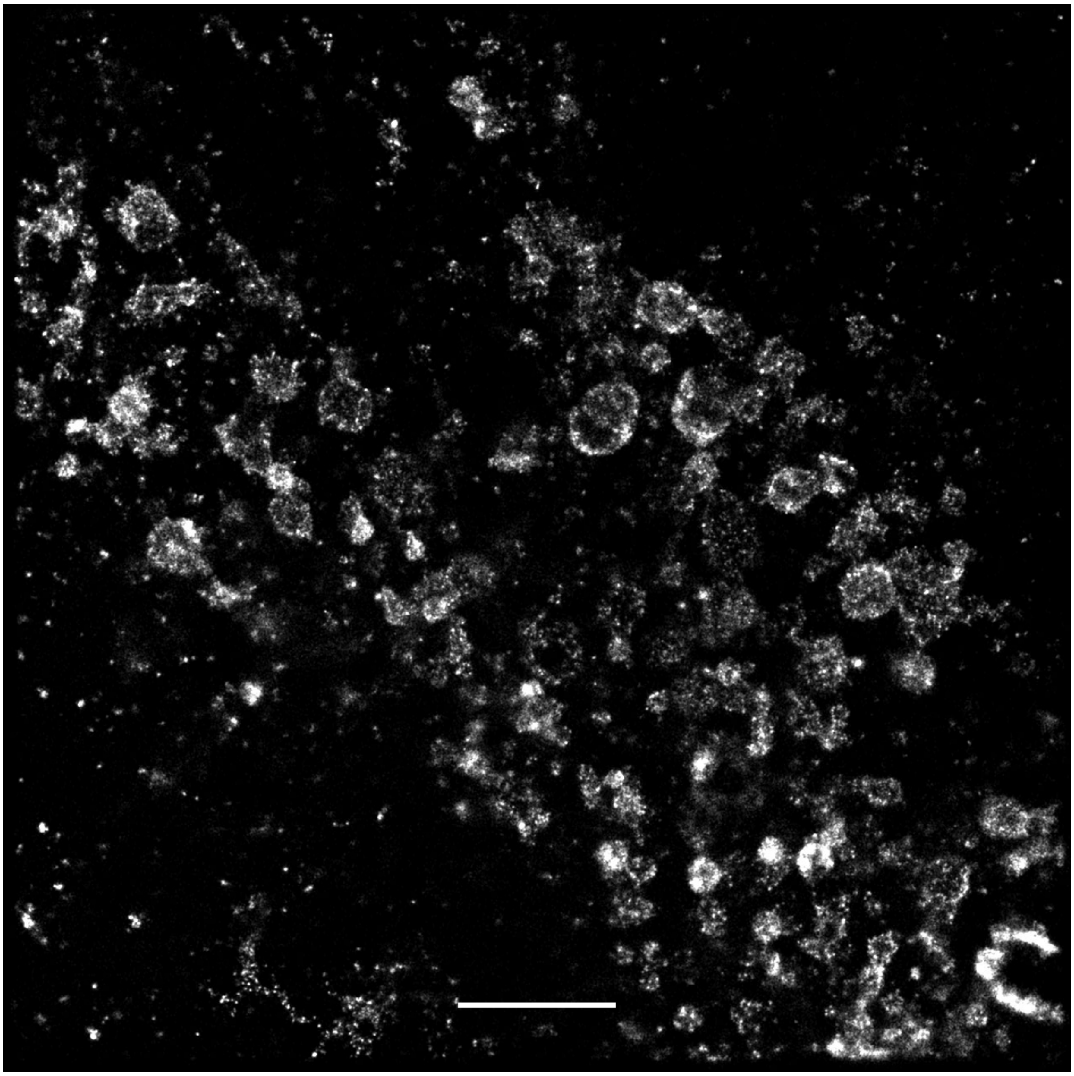


Figure S14. Full size image of the STORM image from the right column of Fig. 2c.

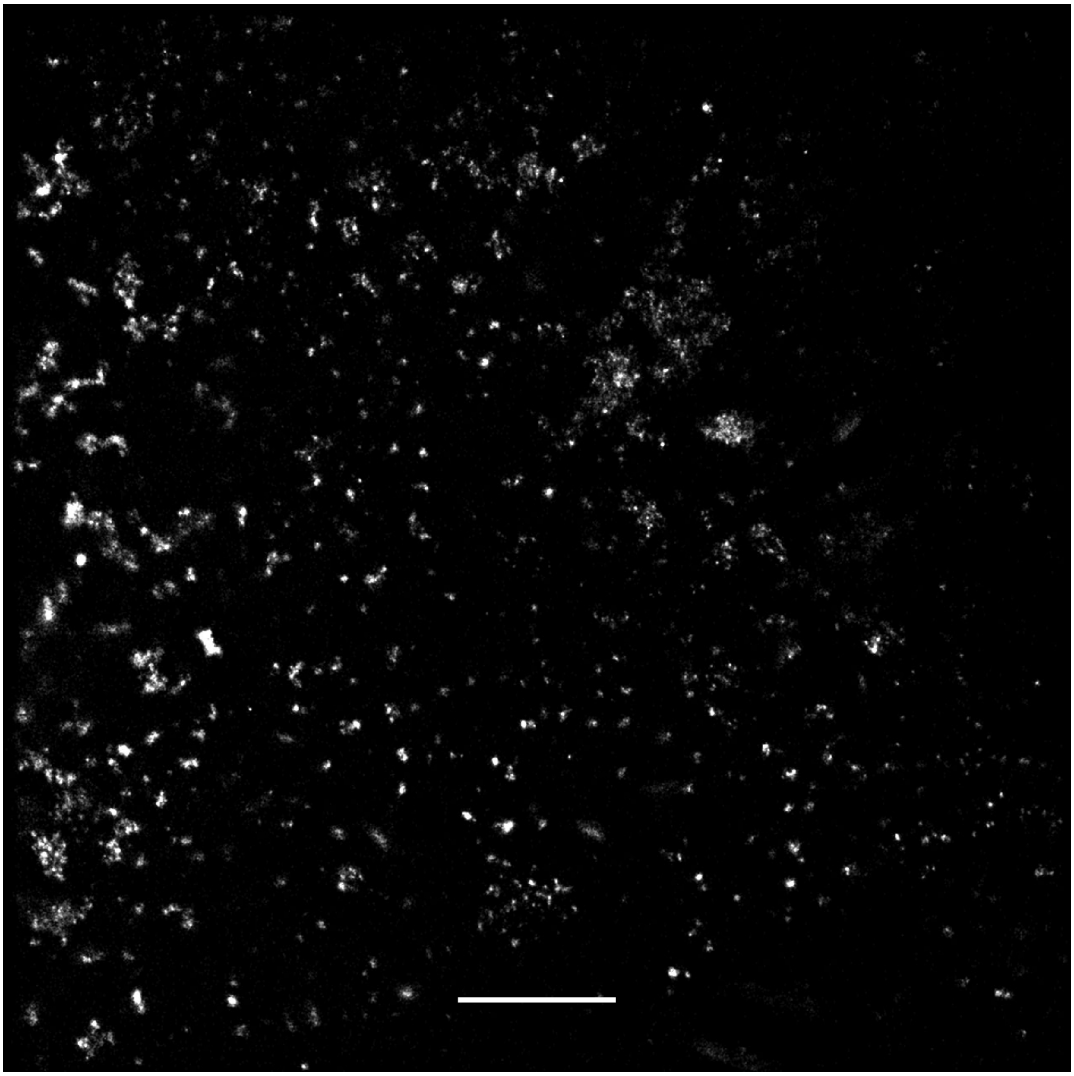


Figure S15. Full size image of the STORM image from the right column of Fig. 2d.

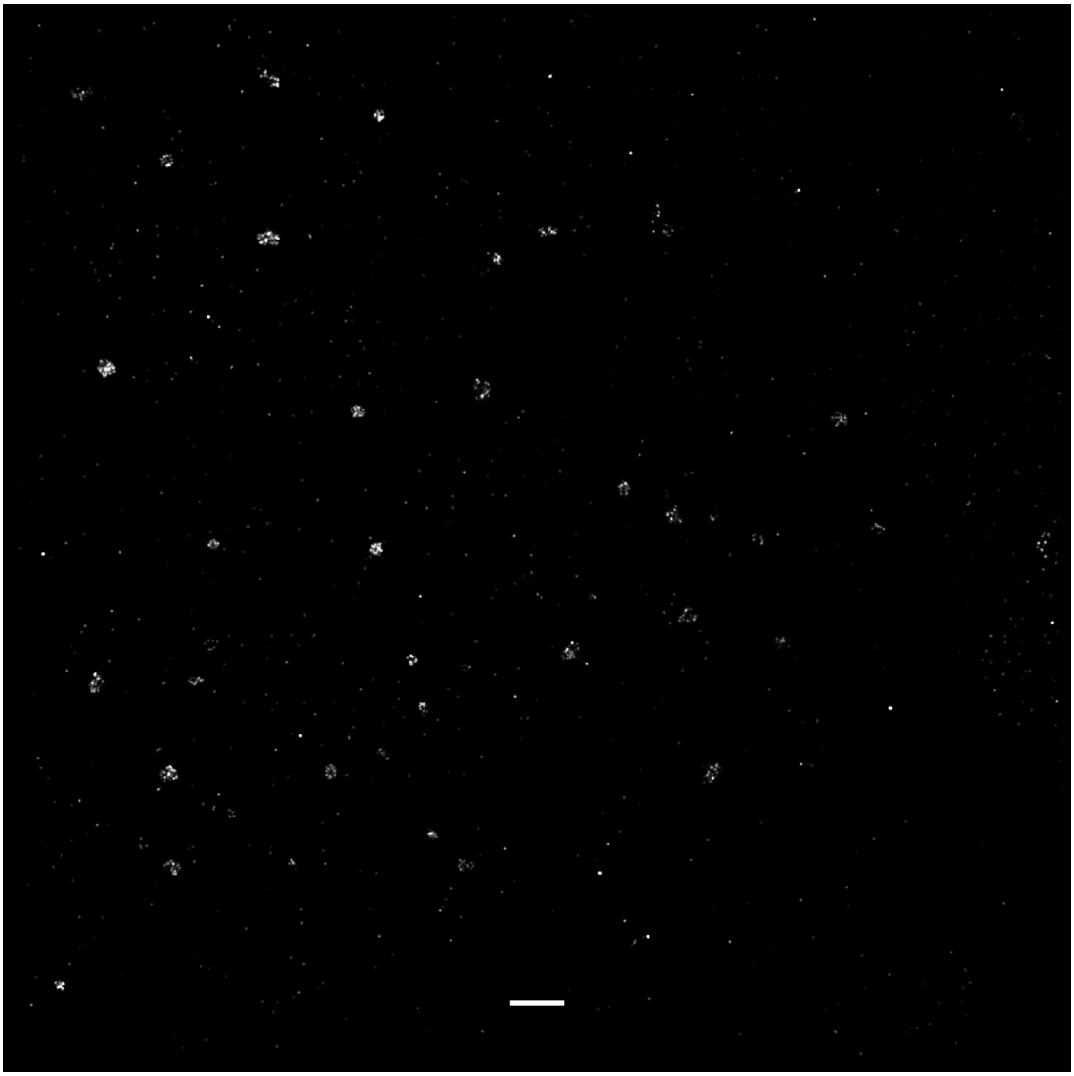


Figure S16. Full size image of the STORM image from the right column of Fig. 3a.

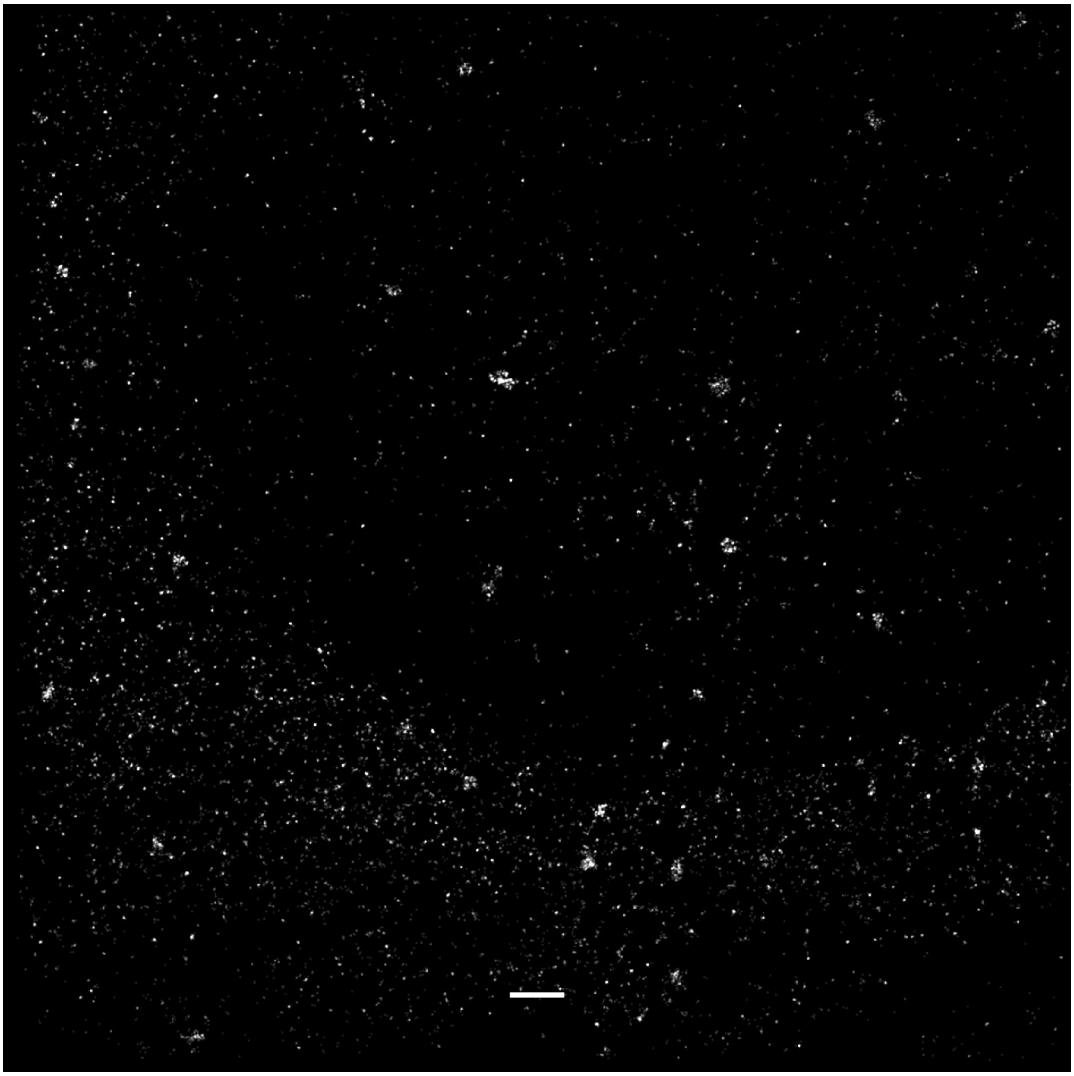


Figure S17. Full size image of the STORM image from the right column of Fig. 3b.

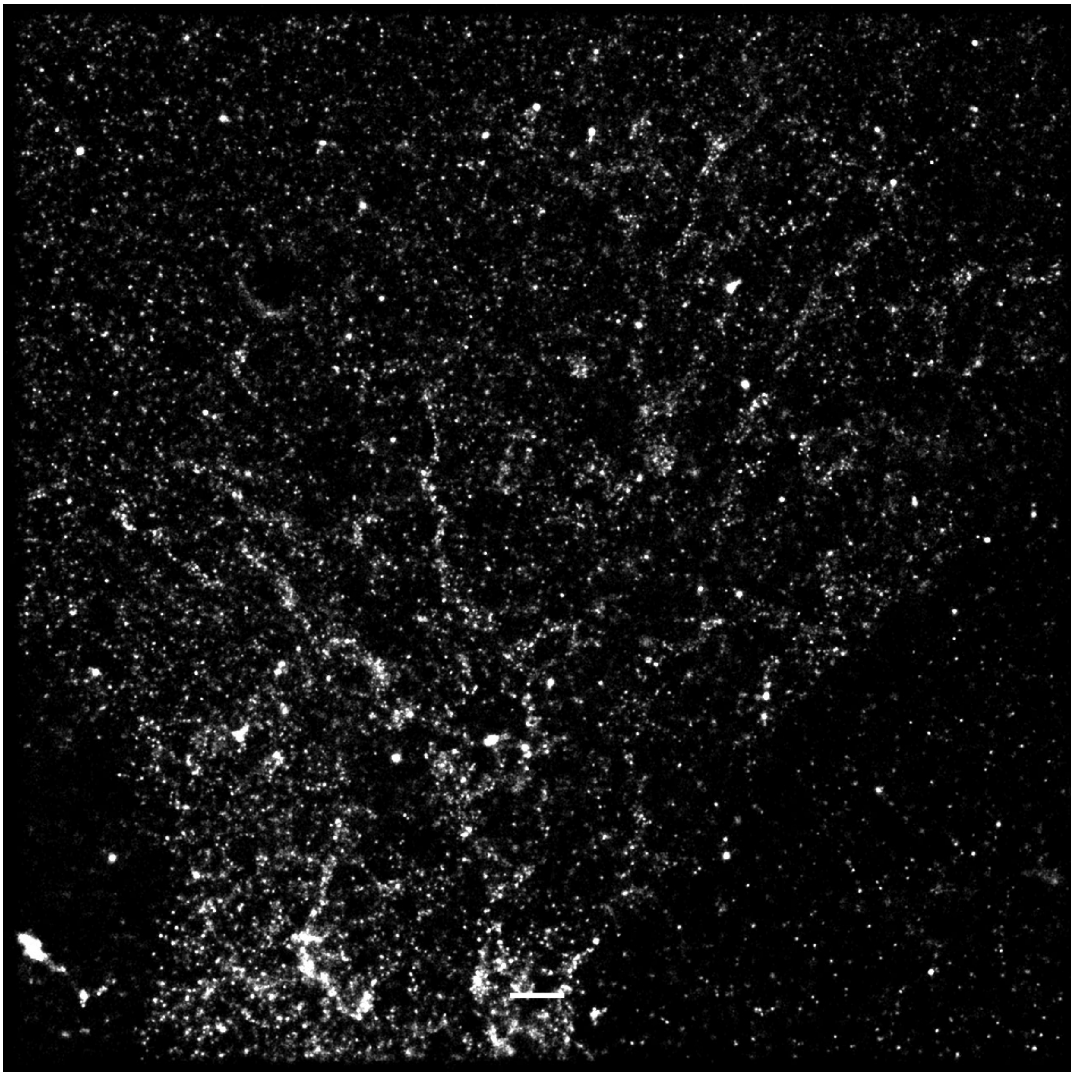


Figure S18. Full size image of the STORM image from the right column of Fig. 3c.

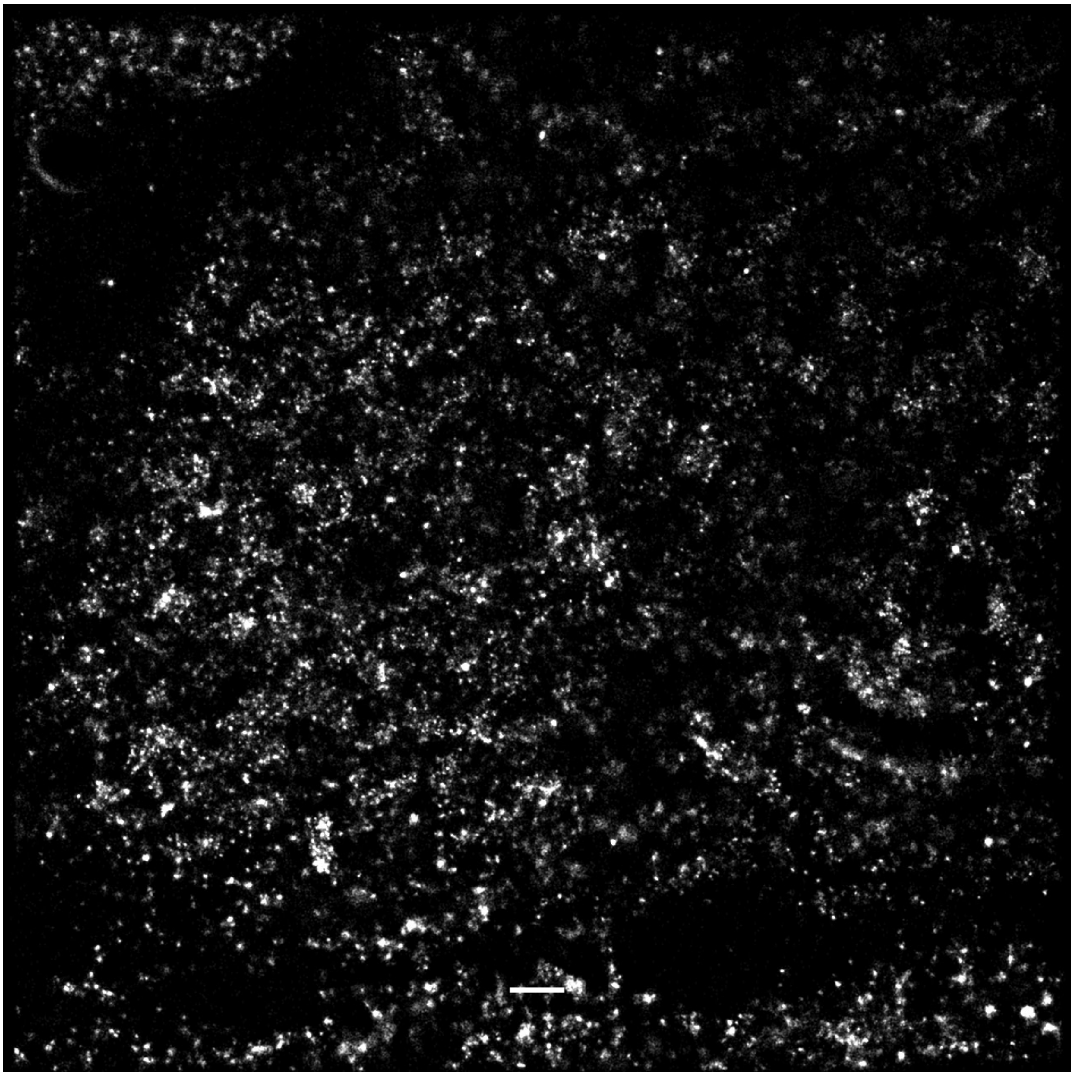


Figure S19. Full size image of the STORM image from the right column of Fig. 3d.