Supporting Information: Supporting Information for Spatially Selective Electrochemical Cleavage of a Polymerase-Nucleotide Conjugate

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1 Supplementary Figures

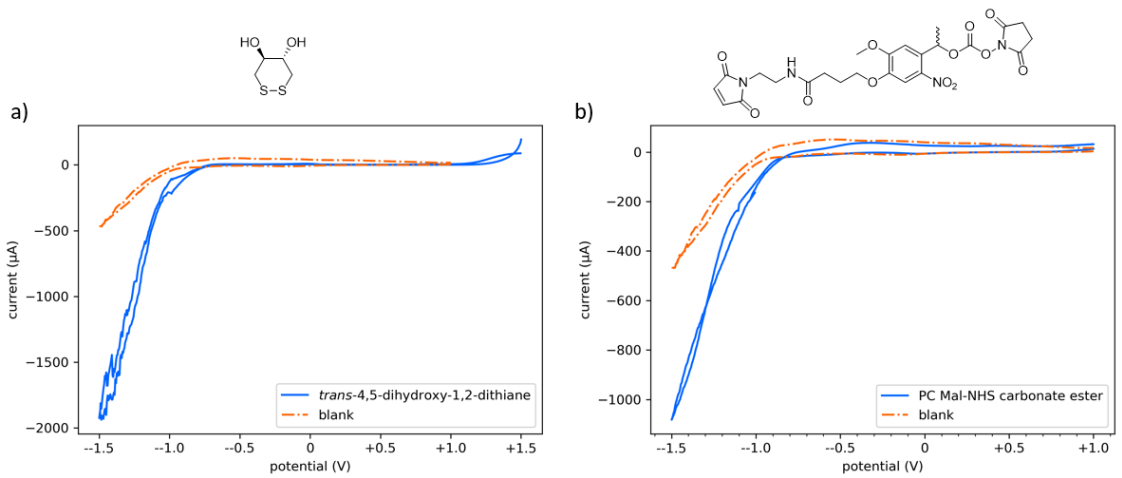


Figure S1: Cyclic voltammograms of model systems containing cleavable functionality contained in previously reported conjugates **1** and **2**. Pt disc working electrode (2 mm diameter), platinum wire counter electrode, and Ag/AgCl reference electrode. Induction period of 3 s at initial voltage prior to sweep at 10 mV/s. a) *trans*-4,5-dihydroxy-1,2-dithiane (20 mM) in 0.5 M potassium phosphate buffer, pH 7.4 b) PC Mal-NHS carbonate ester (13 mM) in 66 vol% acetonitrile, 34 vol% 0.5 M potassium phosphate buffer, pH 7.4

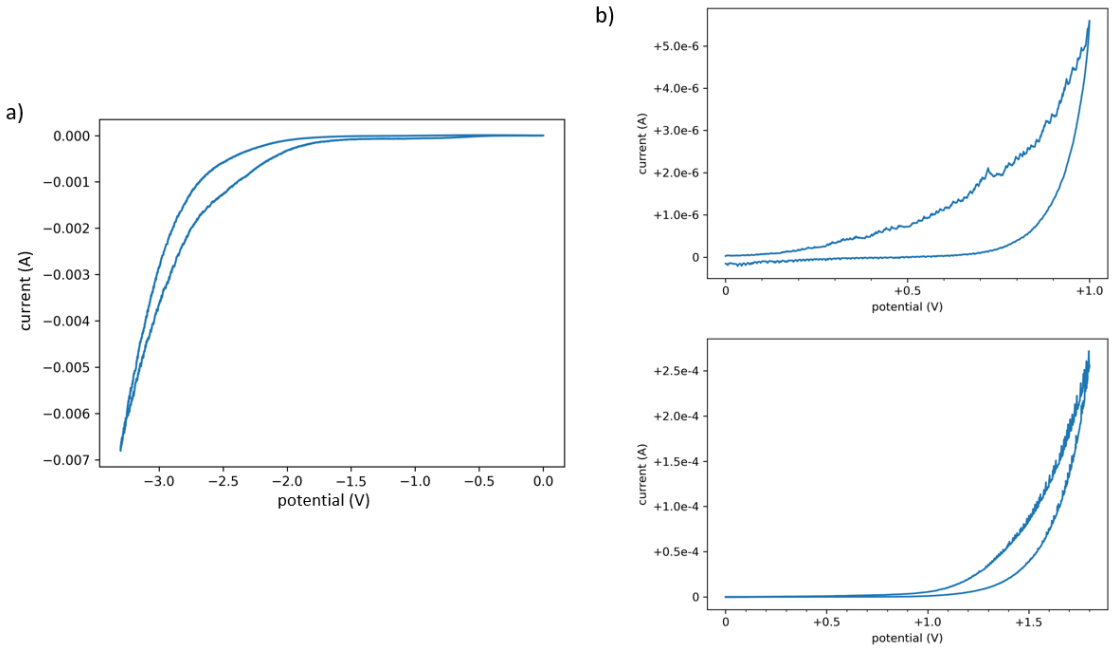


Figure S2: Observed current versus applied potential on microelectrode arrays. Arrays were covered with 50 mM potassium phosphate buffer, pH 7.4, and a potential sweep was applied between a single working electrode and the counter electrodes while measuring current flow. a) Initial negative sweep at 50 mV/s. Substantial reduction of water was not observed at cell voltages below approximated -2.0 V, at which the array is damaged by the experiment. b) Initial positive sweep at 10 mV/s.

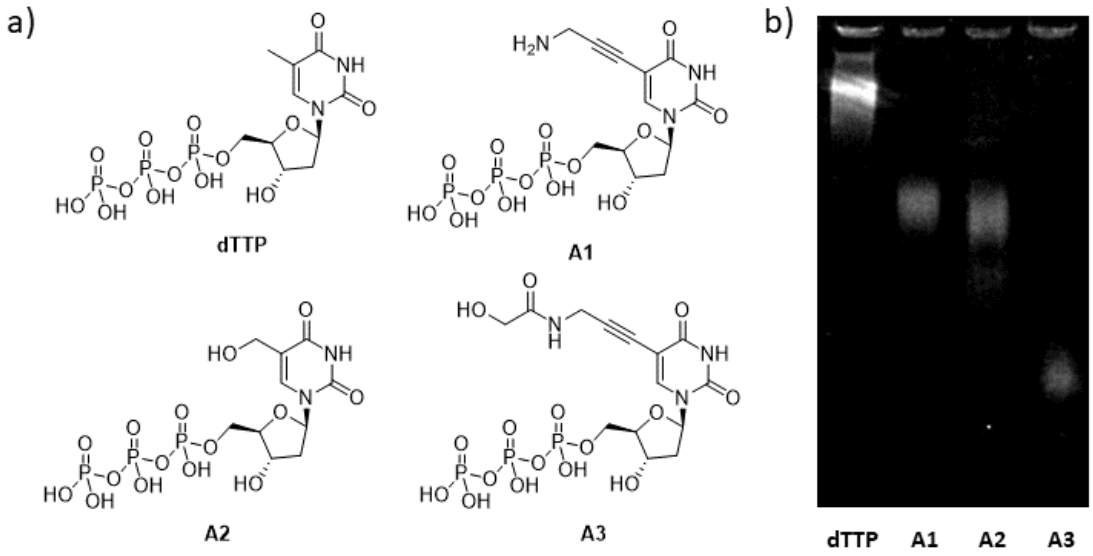


Figure S3: a) dUTP structures containing residual "scars" b) Uncontrolled TdT extension with dUTP-scar nucleotides. A 20 bp single-stranded oligonucleotide (5 pmol) was treated with TdT (1 unit) and dUTP-scar nucleotide (5 nmol) using the TdT manufacturer's recommended buffer conditions. The resulting solution was heated to 37°C for 30 min, then cooled and analyzed by PAGE.

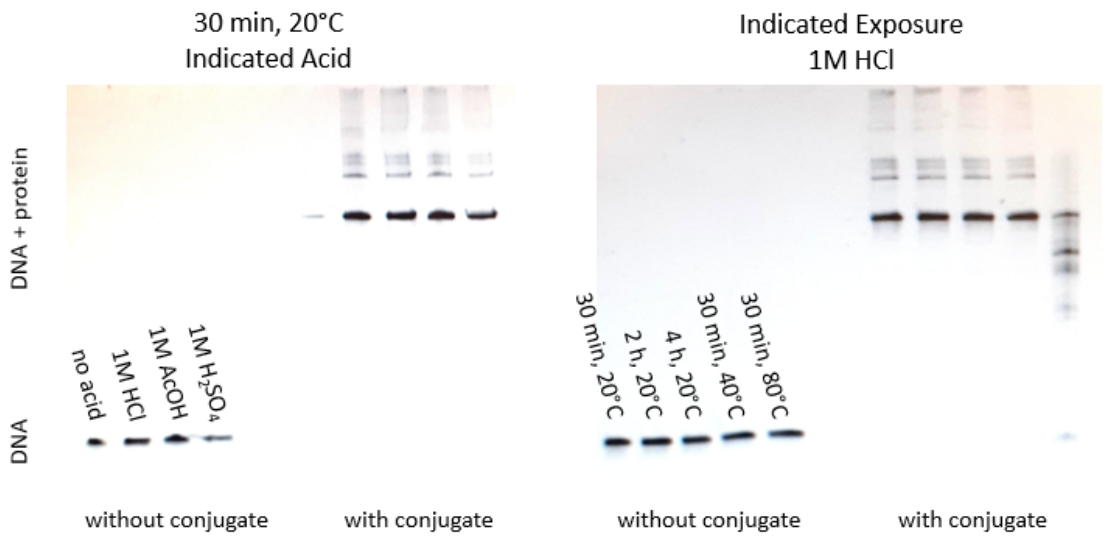


Figure S4: Stability of conjugate **5**-initiator addition products. Conjugate **5** was added to $(dT)_{60}$ in solution then treated with acid under the indicated conditions. SDS-PAGE was used to separate intact addition products (low mobility) from free oligonucleotides (high mobility).

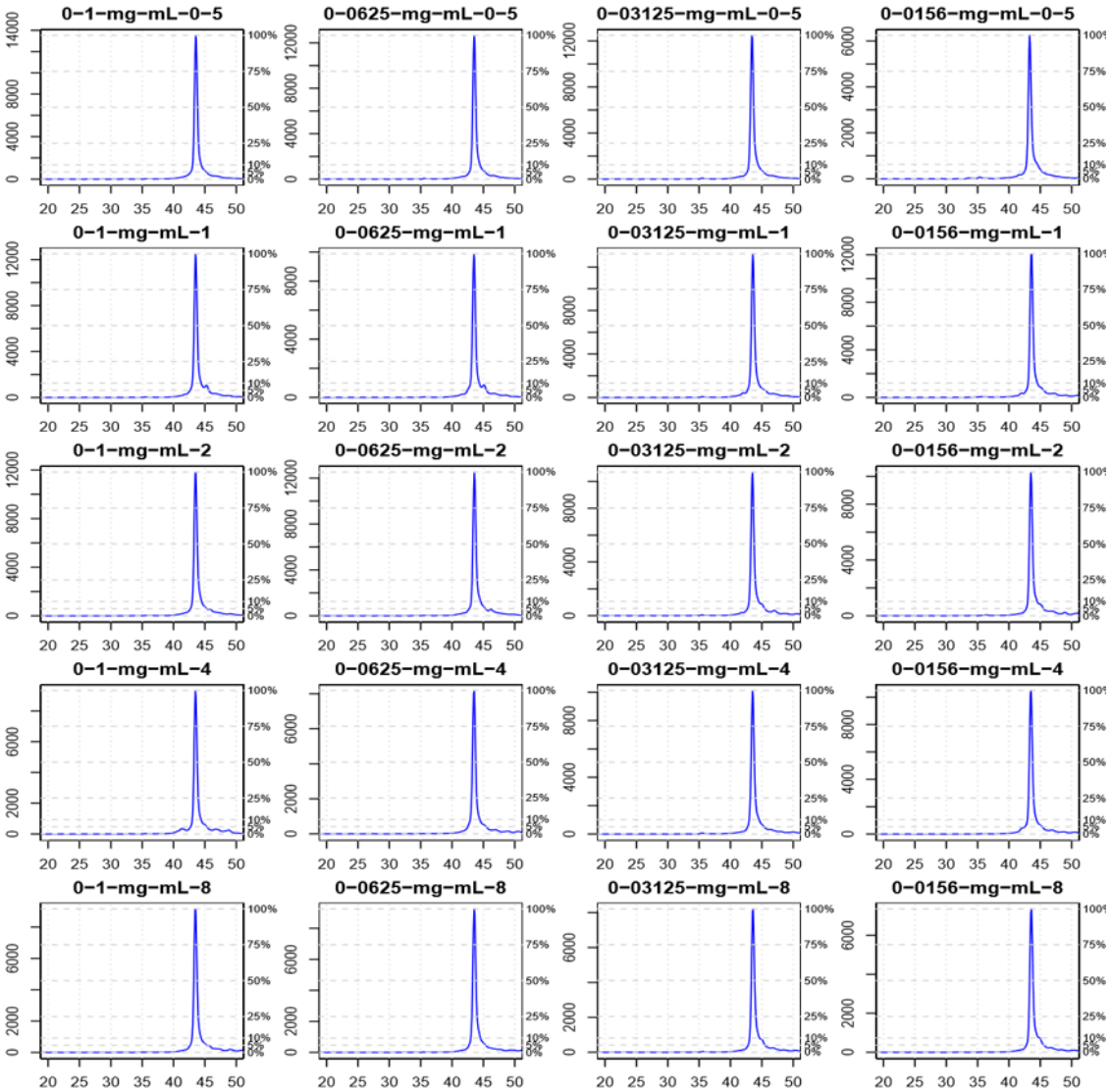


Figure S5: Complete solution-phase activity of conjugate **5**. Conjugate **5** was added to a synthetic deoxyoligonucleotide in solution. The time elapsed prior to quenching the reaction was varied per row (0.5, 1, 2, 4, 8 min). The concentration of conjugate **5** was varied per column (0.1, 0.0625, 0.03125, 0.0156 mg/mL). The disulfide bond connecting the enzyme to the linker was cleaved and the product mixture analyzed by capillary electrophoresis.

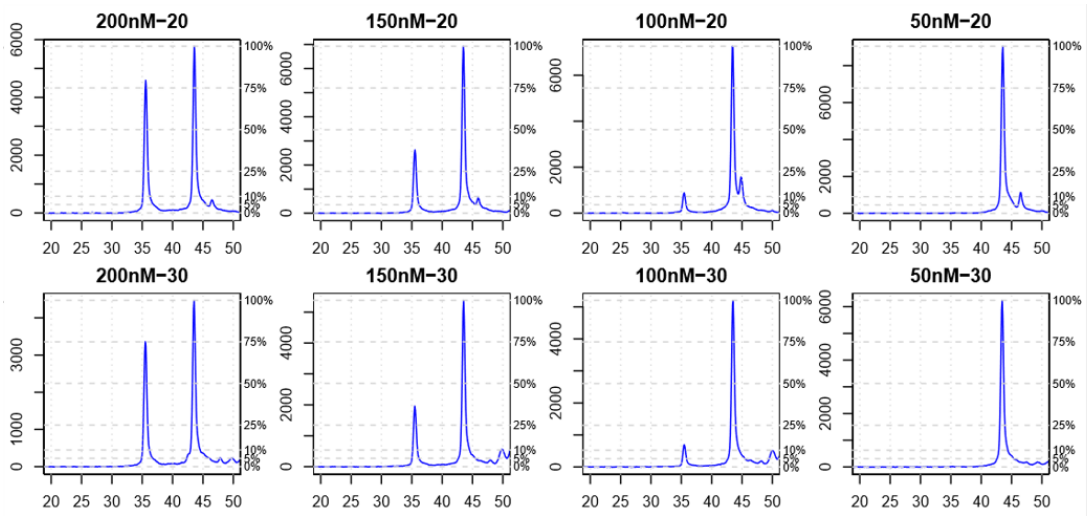
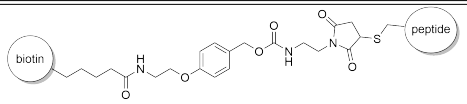
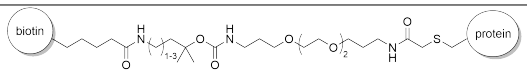
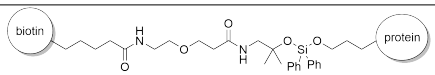
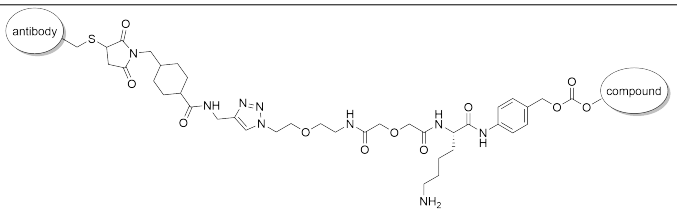
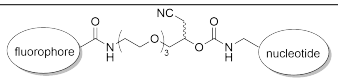
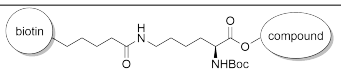
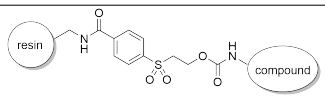
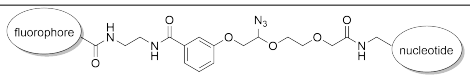
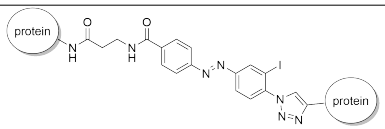
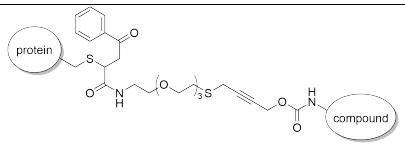
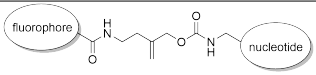
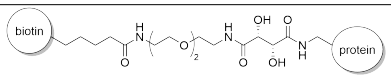


Figure S6: Active site titration of conjugate **5**. Conjugate **5** was added to a 1:1 mixture of FAM-labelled to unlabelled synthetic deoxyoligonucleotide in solution. The time elapsed prior to quenching the reaction was varied per row (20, 30 min). The total concentration of deoxyoligonucleotide was varied per column (200, 150, 100, 50 nM). The disulfide bond connecting the enzyme to the linker was cleaved and the product mixture analyzed by capillary electrophoresis.

Table S1: Previously reported biologically compatible cleavable linkers.

	Linker Structure	Cleavage	Reference
1		acid	van der Veken, 2005[S1]
S1		acid	Fauq, 2006[S2]
S2		acid	Szychowski, 2010[S3]
S3		acid proteolysis	Bargh, 2010[S4]
S4		base	Knapp, 2010[S5]
S5		base	Jahng, 2003[S6]
S6		base	Wade, 2000[S7]
S7		reduction	Bentley, 2008[S8]
S8		reduction	Denny, 1984[S9]
S9		oxidation	Stenton, 2018[S10]
S10		oxidation	Bi, 2006[S11] Guo, 2010[S12]
S11		oxidation	Yang, 2013[S13]

2 Supplementary Methods

2.1 General

Chemicals were purchased from Thermo Fisher Scientific (Waltham, MA) or Sigma Aldrich (St. Louis, MO) unless otherwise indicated. *N*-(methoxycarbonyl)maleimide was purchased from Carbosynth (United Kingdom). PC Mal-NHS carbonate ester was purchased from BroadPharm (San Diego, CA). dUTP-scar nucleotides were purchased from MyChem (San Diego, CA). Terminal transferase (M0315) and associated reagents were purchased from New England Biolabs (Ipswich, MA). Mini-PROTEAN TBE-urea and SDS-PAGE gels were purchased from Bio-Rad Laboratories (Hercules, CA). Synthetic deoxynucleotides were purchased from Integrated DNA Technologies (Coralville, IA). Phosphoramidites and related reagents were purchased from Glen Research (Sterling, VA) or BioAutomation (Irving, TX).

Cyclic voltammetry was performed using a Pine WaveNow potentiostat and analytical electrodes purchased from CH Instruments (Austin, TX). NMR spectroscopy was performed using a Bruker AVance series 300 MHz spectrometer. Gel imaging was performed using either an Invitrogen E-Gel Imager with blue light base or a General Electric Typhoon FLA 9000 fluorescent scanner. Unpatterned phosphoramidite synthesis was performed using an Applied Biosystems Expedite 8900 according to the manufacturer's recommended methods. Patterned phosphoramidite synthesis was performed as previously described.[S14] Capillary electrophoresis was performed by Genomics Services at the Fred Hutchinson Cancer Research Center (Seattle, WA) using an Applied Biosystems 3730xl DNA Analyzer. Fluorescence microscopy was performed using an Olympus BX53MTRF-S equipped with a DP74 color CMOS camera. Images were rotated, cropped, and brightness/contrast adjusted using ImageJ.[S15]

2.1.1 Protein and Deoxyoligonucleotide Sequences

TdT Mutant

MGH HHH HHH HHH SSG HID DDD KHM MKI EEG KLV IWI NGD KGY NGL AEV GKK FEK DTG IKV TVE HPD KLE EKF PQV AAT GDG PDI IFW AHD RFG GYA QSG LLA EIT PDK AFQ DKL YPF TWD AVR YNG KLI AYP IAV EAL SLI YNK DLL PNP PKT WEE IPA LDK ELK AKG KSA LMF NLQ EPY FTW PLI AAD GGY AFK YEN GKY DIK DVG VDN AGA KAG LTF LVD LIK NKH MNA DTD YSI AEA AFN KGE TAM TIN GPW AWS NID TSK VNY GVT VLP TFK GQP SKP FVG VLS AGI NAA SPN KEL AKE FLE NYL LTD EGL EAV NKD KPL GAV ALK SYE EEL VKD PRI AAT MEN AQK GEI MPN IPQ MSA FWY AVR TAV INA ASG RQT VDE ALK DAQ TNS SSN NNN NNN NNN LGI EGR ISH MSM GGR DIV DGS EFS PSP VPG SQN VPA PAV KKI SQY AVQ RRT TLN NYN QLF TDA LDI LAE NDE LRG NEG RAL AFM RAS SVL KSL PFP ITS MKD TEG IPSL GDK VKS IIE EII EDG ESS EVK AVL NDE RYK SFK LFT SVF GVG LKT AEK WFR MGF RTL SEI QSD KSL RFT QMQ KAG FLY YED LVS CVN RPE AEA VSM LVK EAV VTF LPD ALV TMT GGF RRG KMT GHD VDF LIT SPE ATE DEE QRL LHK VTD FWK QQG LLL YAD ILE STF EKF KQP SRK VDA LDH FQK AFL ILK LDH GRV HSE KSG QQE GKG WKA IRV DLV MSP YDR RAF ALL GWT GSR QFN RDL RRY ATH ERK MML DNH ALY DRT KRV FLE AES EEE IFA HLG LDY IEP WER NA

Anchor_T10_Amine

5'-CGC ATT CAT ATA CCA ATG TTG ACT TTG TCT AGC GGC CAG GCA ATT TGA AGT TTG GTG GTG TTT TTT TTT T/iSp18//3AmMC6T/-3'

FAM_Anchor*_Init

5'-/56-FAM/CAC CAC CAA ACT TCA AAT TGC CTG GCC GCT AGA CAA AGT CAA CAT TGG TAT ATG AAT GCG CCG CGA CTG GTC AGA TAC GA-3'

2.1.2 Buffers

1X KP Buffer

A solution of 1X KP buffer was prepared from Milli Q water containing 200 mM K_2HPO_3 and 150 mM NaCl, adjusted to pH 6.5 with hydrochloric acid.

1X TP6.5 Buffer

A solution of 1X TP6.5 buffer was prepared from Milli Q water containing 50 mM KOAc and 20 mM tris(hydroxymethyl)aminomethane, adjusted to pH 6.5 with acetic acid.

1X TBE Buffer

A solution of 1X TBE buffer was prepared from Milli Q water containing 130 mM tris(hydroxymethyl)amino
45 mM boric acid, and 2.5 mM ethylenediaminetetraacetic acid.

10X TP8 Buffer

A solution of 10X TP8 buffer was prepared from Milli Q water containing 500 mM KOAc, 200 mM tris(hydroxymethyl)aminomethane, and 0.1 vol% Cytiva Surfactant P20, adjusted to pH 7.9 with acetic acid.

10X Metal Mix

A solution of 10X metal mix was prepared from Milli Q water containing 100 mM $Mg(OAc)_2$ and 5 mM $Co(OAc)_2$.

1X TGS Buffer

A solution of 1X TGS Buffer was prepared from Milli Q water containing 25 mM tris(hydroxymethyl)aminon
192 mM glycine, and 0.1 wt% sodium dodecyl sulfate, adjusted to pH 8.6 with hydrochloric acid.

1X PBST Buffer

A solution of 1X PBST buffer was prepared from Milli Q water containing 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, and 0.1% w/v Tween 20. The resulting solution was adjusted to pH 7.4 with hydrochloride acid.

1X TE Buffer

A solution of 1X TE buffer was prepared from Milli Q water containing 10 mM tris(hydroxymethyl)aminome and 1 mM ethylenediaminetetraacetic acid. The resulting solution was adjusted to pH 8.0 with hydrochloric acid.

2X PBS Buffer

A solution of 2X PBS buffer was prepared from Milli Q water containing 274 mM NaCl, 5.4 mM KCl, 20 mM Na_2HPO_4, and 3.6 mM KH_2PO_4. The resulting solution was adjusted to pH 7.4 with hydrochloric acid.

2.1.3 Microelectrode Array Preparation

Prior to use, microelectrode arrays were treated with Nano-strip for 90 s, washed with DI water, and blown dry with compressed air. The Nano-strip cleaning process was repeated, then microelectrode arrays were further treated with plasma generated from air at 45 W for 30 min using a Harrick Plasma PDC-001-HP. Clean arrays could be stored in a low-dust environment for up to two weeks.

2.2 Synthesis of Alcohol Model Systems

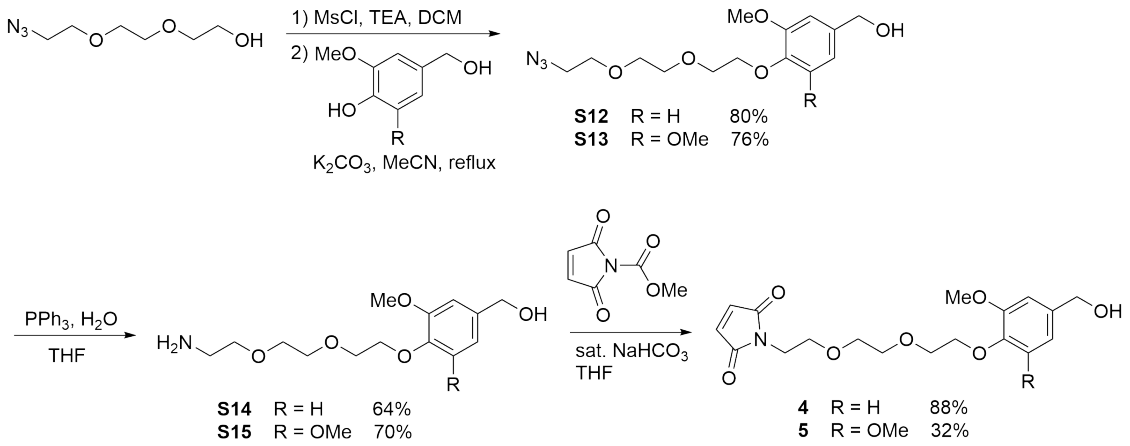


Figure S7: Synthesis of alcohol model systems.

2.2.1 4-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)-3-methoxybenzyl alcohol (S12)

Methanesulfonyl chloride (1.04 mL, 1.1 eq) was added dropwise to a solution of 2-(2-(2-azidoethoxy)ethoxy)ethanol[S16] (2.16 g, 1 eq) and triethylamine (5.14 mL, 3 eq) in dichloromethane (45 mL). The resulting mixture was stirred at room temperature for 2 h then diluted with additional dichloromethane. The mixture was washed sequentially with 1 M HCl (aq) and sat. $NaHCO_3$ (aq), dried over Na_2SO_4, and concentrated *in vacuo* to yield a colorless oil that was used directly without further purification.

A solution of the crude mesylate prepared above (3.12 g, 1 eq), 4-hydroxy-3-methoxybenzyl alcohol (2.28 g, 1.2 eq), and K_2CO_3 (2.55 g, 1.5 eq) in acetonitrile (50 mL) was heated to reflux overnight. The reaction mixture was cooled to room temperature and filtered, washing with ethanol, then the filtrate was concentrated *in vacuo*. The crude residue was purified by flash column chromatography on silica gel, eluting with a gradient from 0-6 vol% methanol in dichloromethane to give 3.07 g viscous yellow oil (80%). **^{1}H NMR**: 6.95-6.80 (m, 3H), 4.63 (d, 6 Hz, 2H), 4.18 (t, 6 Hz, 2H), 3.92-3.87 (m, 2H), 3.87 (s, 3H), 3.77-3.72 (m, 2H), 3.70-3.65 (m, 4H), 3.38 (t, 6 Hz, 2H).

2.2.2 4-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)-3,5-dimethoxybenzyl alcohol (S13)

Prepared as described above from 2-(2-(2-azidoethoxy)ethoxy)ethanol (0.93 g, 1 eq) substituting 4-hydroxy-3,5-dimethoxybenzyl alcohol (1.17 g, 1.2 eq) for 4-hydroxy-3-methoxybenzyl alcohol to yield 1.38 g viscous yellow oil (76%). Product contaminated with 4-((2-(2-(2-azidoethoxy)ethoxy)ethoxy)methyl)-2,6-dimethoxyphenol. **^{1}H NMR**: 6.59 (s, 6 Hz, 2H)), 4.62 (d, 6 Hz, 2H), 4.13 (t, 6 Hz, 2H), 3.83 (s, 6H), 3.83-3.78 (m, 2H), 3.76-3.71 (m, 2H), 3.70-3.66 (m, 4H), 3.38 (t, 6 Hz, 2H).

2.2.3 4-(2-(2-(2-aminoethoxy)ethoxy)ethoxy)-3-methoxybenzyl alcohol (S14)

Triphenylphosphine (1.08 g, 1.1 eq) was added to a solution of 4-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)-3-methoxybenzyl alcohol (1.15 g, 1 eq) in water (1.2 mL) and tetrahydrofuran (11 mL). The resulting mixture was stirred overnight, then the volatiles were removed *in vacuo*. The residue was purified by flash column chromatography on silica gel, eluting sequentially with dichloromethane, 10 vol% methanol in dichloromethane, and 10 vol% methanol in dichloromethane with 1 vol% triethylamine. Isolated 0.88 g yellow semi-solid (64%) eluting in the final solvent system. Product contaminated with unknown material. **^{1}H NMR**: 7.00-6.80 (m, 3H), 4.55 (s, 2H), 4.15 (t, 6 Hz, 2H), 3.90-3.80 (m, 2H), 3.85 (s, 3H), 3.70-3.65 (m, 2H), 3.60-3.55 (m, 2H), 3.40 (t, 3 Hz, 2H), 2.80-2.75 (m, 2H).

2.2.4 4-(2-(2-(2-aminoethoxy)ethoxy)ethoxy)-3,5-dimethoxybenzyl alcohol (S15)

Prepared as described above substituting 4-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)-3-methoxybenzyl alcohol (1.38 g, 1 eq) for 4-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)-3-methoxybenzyl alcohol to yield 0.88 g viscous yellow oil (70%). Product contaminated with triethylamine hydrochloride. **^{1}H NMR**: 6.58 (s, 6H), 4.60 (s, 2H), 4.16 (t, 6 Hz, 2H), 3.84 (s, 6H), 3.80 (t, 6 Hz, 2H), 3.65 (t, 3 Hz, 2H), 3.57 (t, 3 Hz, 2H), 3.45 (t, 6 Hz, 4H), 2.80 (t, 6 Hz, 2H).

2.2.5 1-(2-(2-(4-(hydroxymethyl)-2-methoxyphenoxy)ethoxy)ethoxy)ethyl)-1*H*-pyrrole-2,5-dione (2)

(*N*-methoxycarbonyl)maleimide (0.31 g, 1 eq) was added portionwise to a solution of 4-(2-(2-(2-aminoethoxy)ethoxy)ethoxy)-3-methoxybenzyl alcohol (0.57 g, 1 eq) in sat. $NaHCO_3$ (aq) (10 mL) and tetrahydrofuran (10 mL) cooled in an ice/water bath. The resulting mixture was stirred for 30 min, the cooling bath removed, and stirring continued for an additional 30 min. The reaction mixture was diluted with additional sat. $NaHCO_3$ (aq) and extracted with

three portions of dichloromethane. The combined organic layers were dried over Na_2SO_4 and concentrated *in vacuo*. The residue was purified by flash column chromatography on silica gel, eluting with a gradient from 50-80 vol% ethyl acetate in dichloromethane to give 0.112 g pale yellow oil (88%). **^{1}H NMR**: 6.92-6.82 (m, 3H), 6.68 (s, 2H), 4.63 (s, 2H), 4.16 (s, 6 Hz, 2H), 3.87 (s, 3H), 3.85 (t, 6 Hz, 2H), 3.72 (t, 6 Hz, 2H), 3.70-3.61 (m, 6H).

2.2.6 1-(2-(2-(4-(hydroxymethyl)-2,6-dimethoxyphenoxy) ethoxy)ethoxy)ethyl)-1*H*-pyrrole-2,5-dione (3)

Prepared as described above substituting 4-(2-(2-(2-aminoethoxy)ethoxy)ethoxy)-3,5-dimethoxybenzyl alcohol (0.88 g, 1 eq) for 4-(2-(2-(2-aminoethoxy)ethoxy)ethoxy)-3-methoxybenzyl alcohol to yield 0.38 g pale yellow oil (32%). **^{1}H NMR**: 6.68 (s, 2H), 6.59 (s, 2H), 4.62 (d, 3 Hz, 2H), 4.10 (t, 6 Hz, 2H), 3.84 (s, 6H), 3.76 (t, 5 Hz, 2H), 3.71 (t, 5 Hz, 2H), 3.68-3.59 (m, 6H).

2.3 Linker Preparation and Modification of Nucleotide

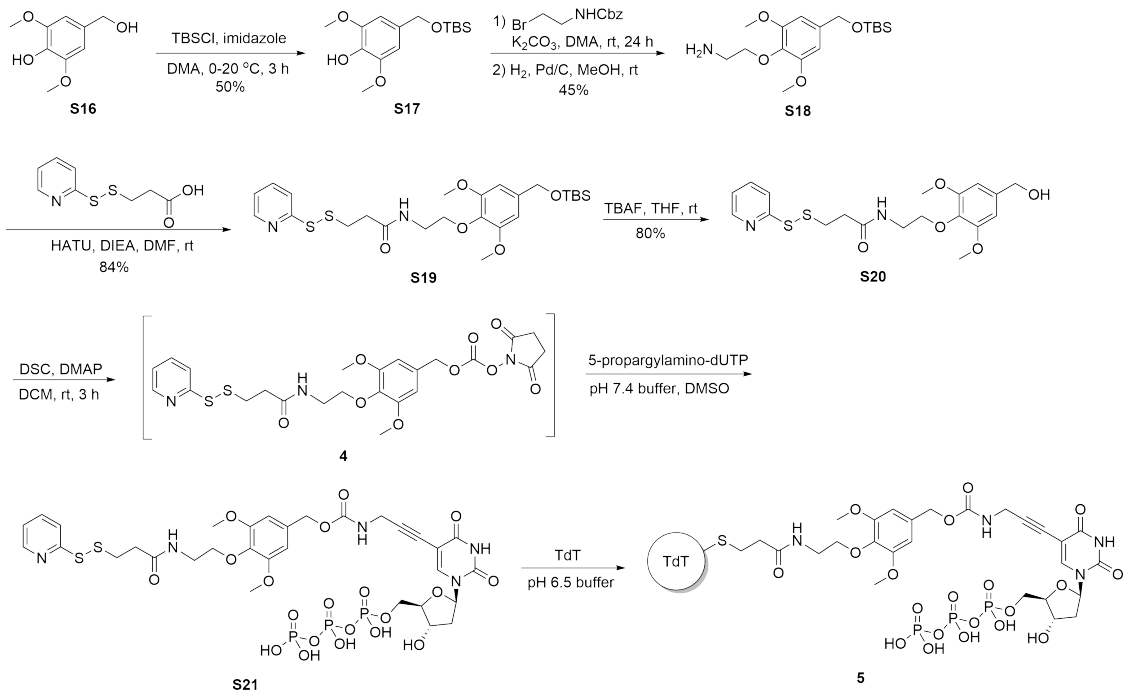
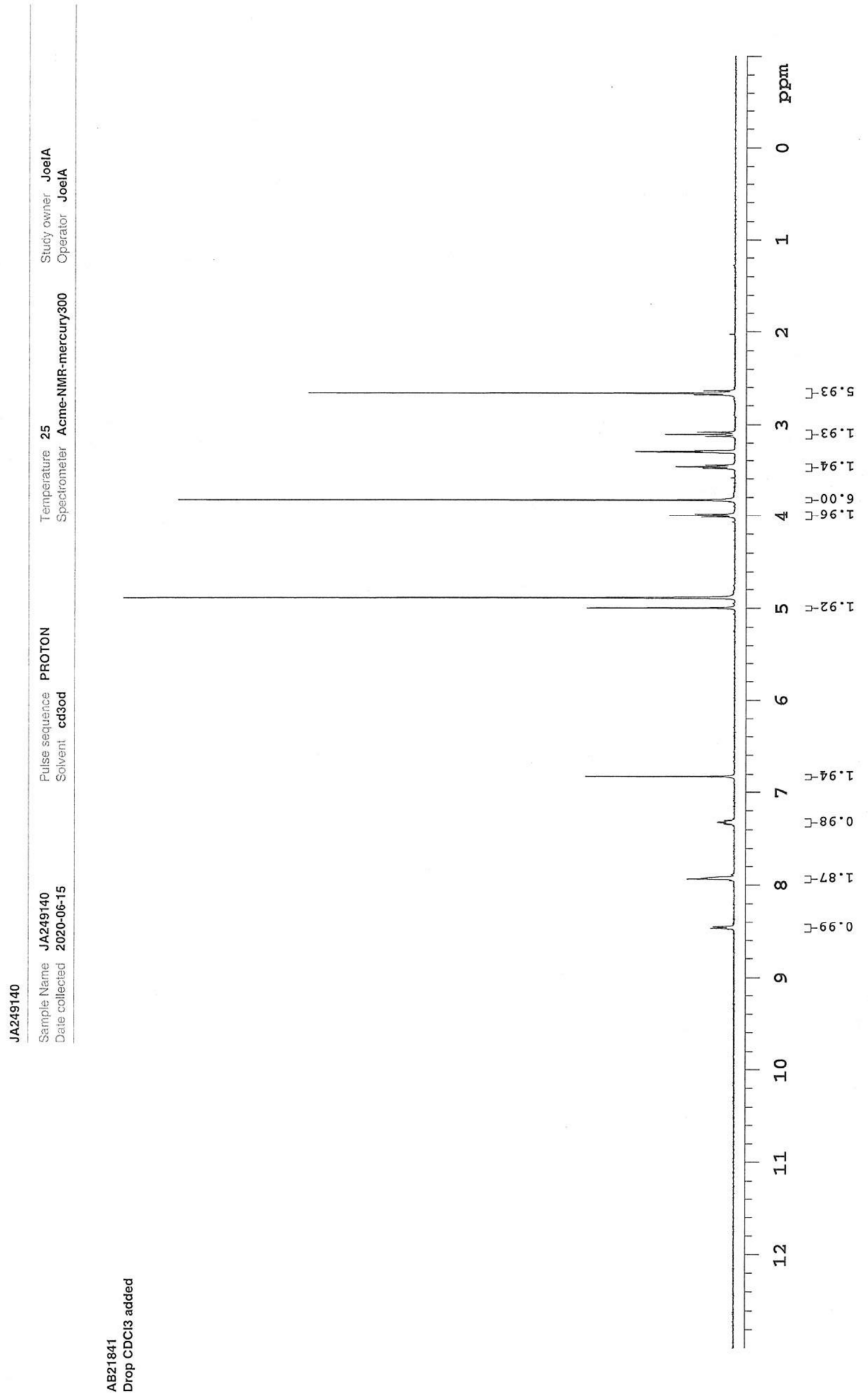


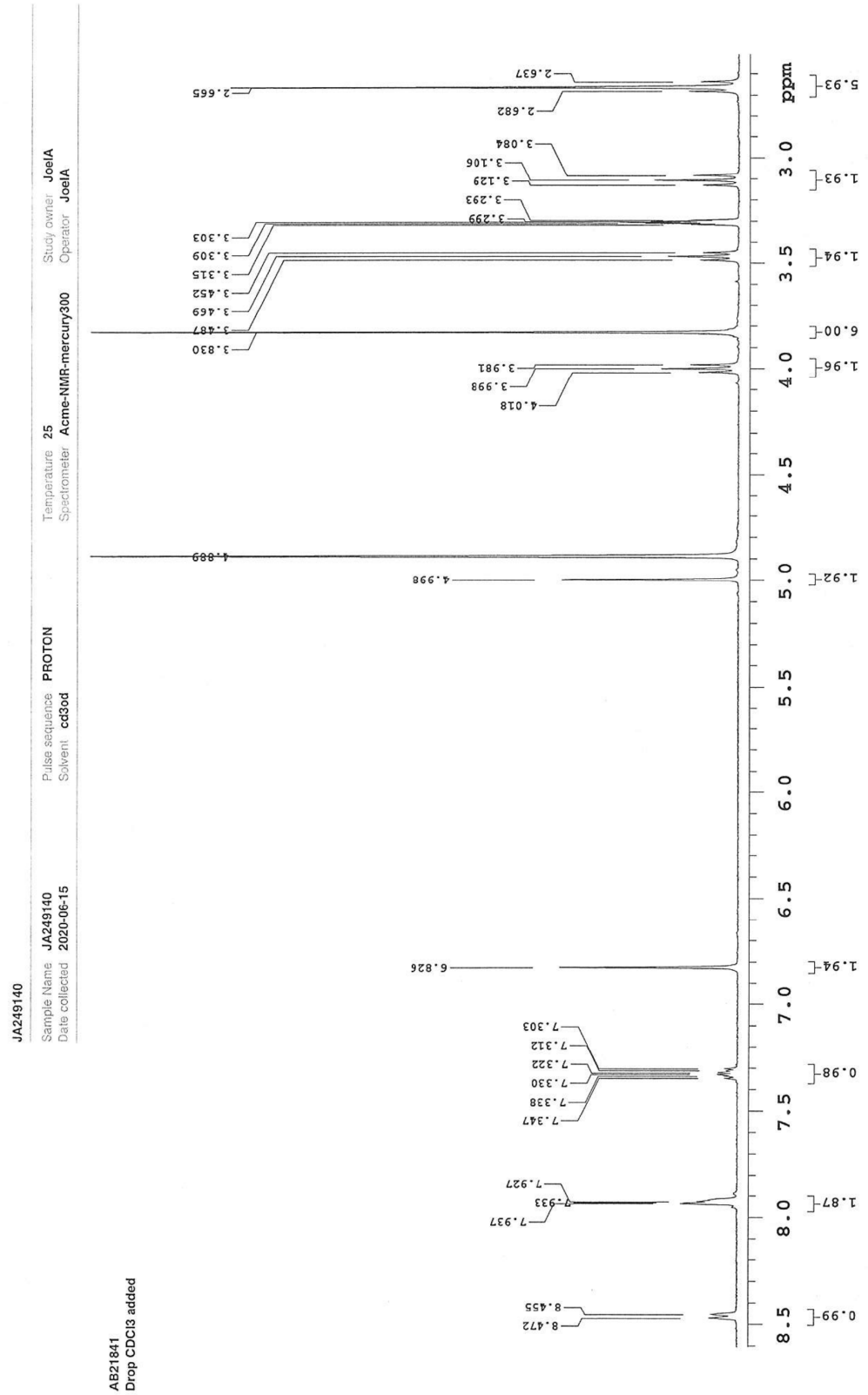
Figure S8: Synthesis of conjugate **5**.

Synthesis of cleavable linker **4** was performed by Acme Bioscience (Palo Alto, CA) as depicted in Figure S8. Briefly, 3,5-Dimethoxy-4-hydroxybenzyl alcohol (**S16**) was mono-protected by treatment with *tert*-butyldimethylsilyl chloride. The resulting phenol **S17** was alkylated with benzyl 2-bromoethylcarbamate and the carbamate protecting-group removed by catalytic hydrogenation. The resulting amine **S18** was coupled to 3-(2-pyridinyldithio)propanoic acid and the *tert*-butyldimethylsilyl protecting group removed with tetrabutylammonium fluoride in tetrahydrofuran to yield alcohol precursor **S20**. Cleavable linker **4** was found to be unstable to prolonged isolation; therefore, it was generated *in situ* by treatment of **S20** with *N*,*N*'-disuccinimidyl carbonate and conjugated directly to 5-propargylamino-2'-deoxyuridine-5'-triphosphate by MyChem (San Diego, CA) to yield linker-nucleotide complex **S21**.

3 Characterization Data

3.1 Cleavable Linker 4

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Date collected 2020-06-15
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Study owner JoelA
Operator JoelA
AB21841
Drop CDCl3 added
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8.455
7.937
7.933
7.927
7.347
7.338
7.330
7.322
7.312
7.303
6.826
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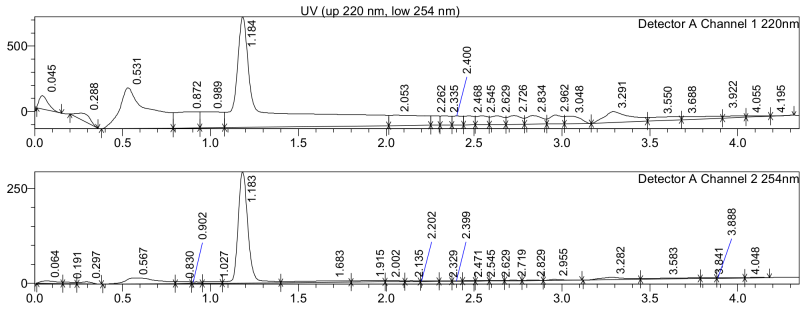
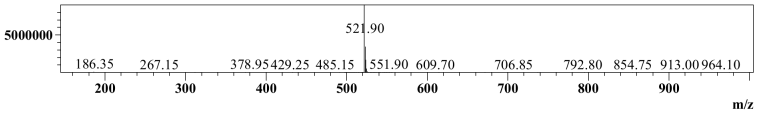
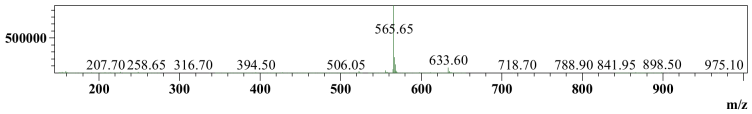
3.303
3.299
3.293
3.129
3.106
3.084
2.682
2.665
2.637
ppm

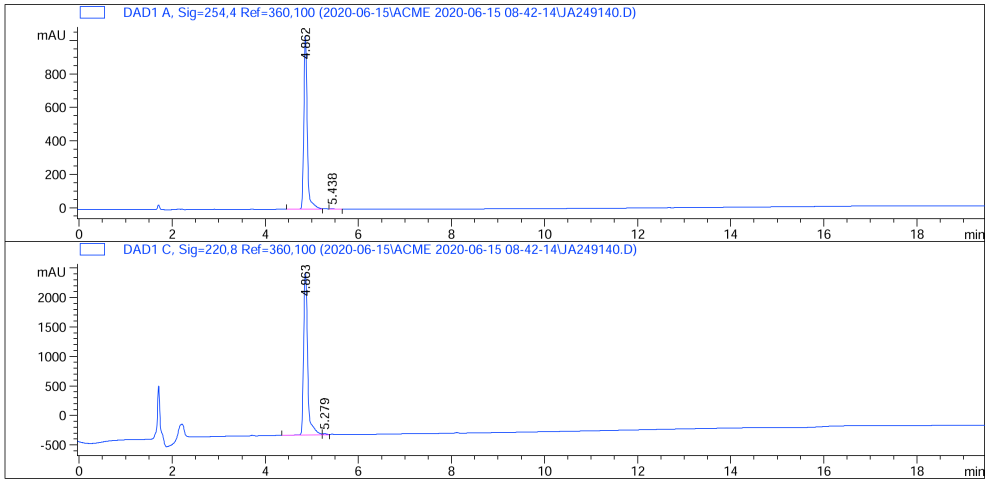


Line#:1 R.Time:1.187(Scan#:357)
MassPeaks:943
Spectrum Mode:Single 1.187(357) BasePeak:521.90(8976308)
BG Mode:None Segment 1 - Event 1
ESI Positive

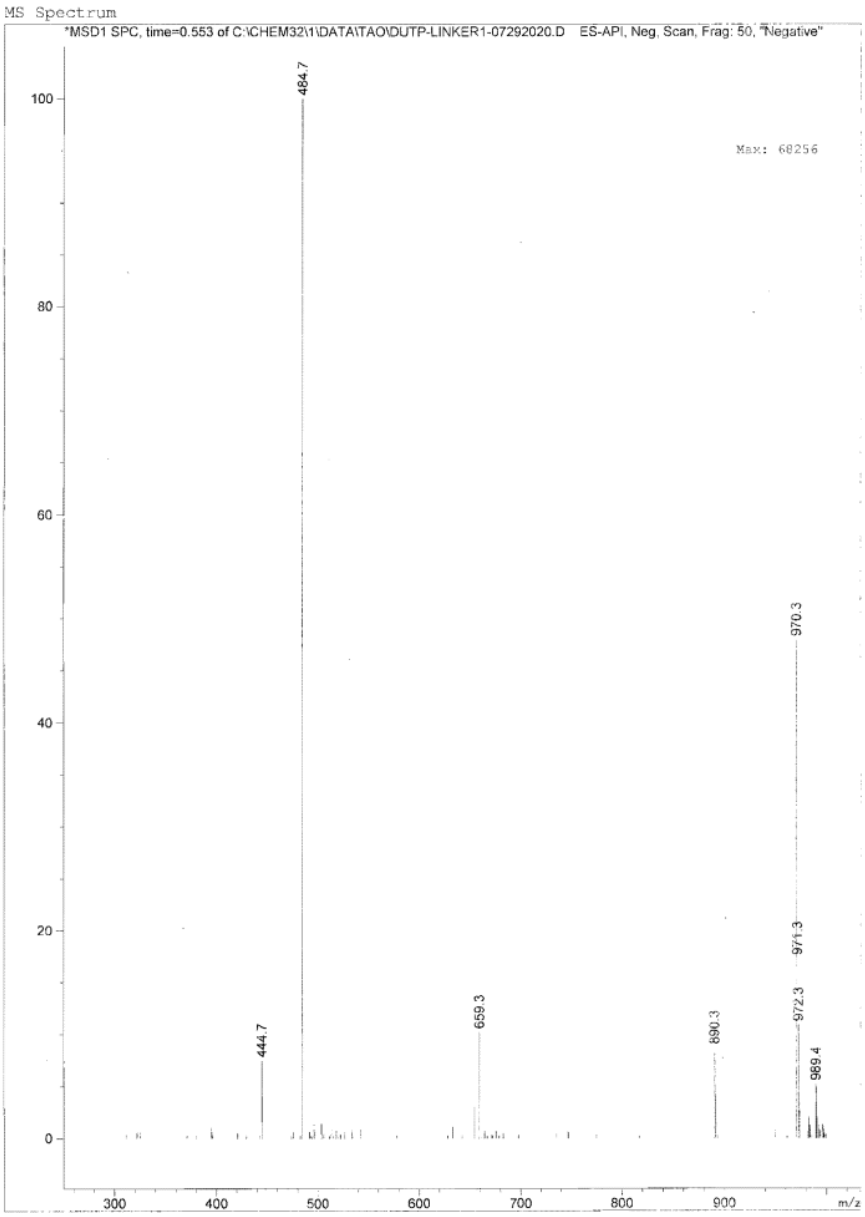


Line#:2 R.Time:1.190(Scan#:358)
MassPeaks:891
Spectrum Mode:Single 1.190(358) BasePeak:565.65(965605)
BG Mode:None Segment 1 - Event 2
ESI Negative



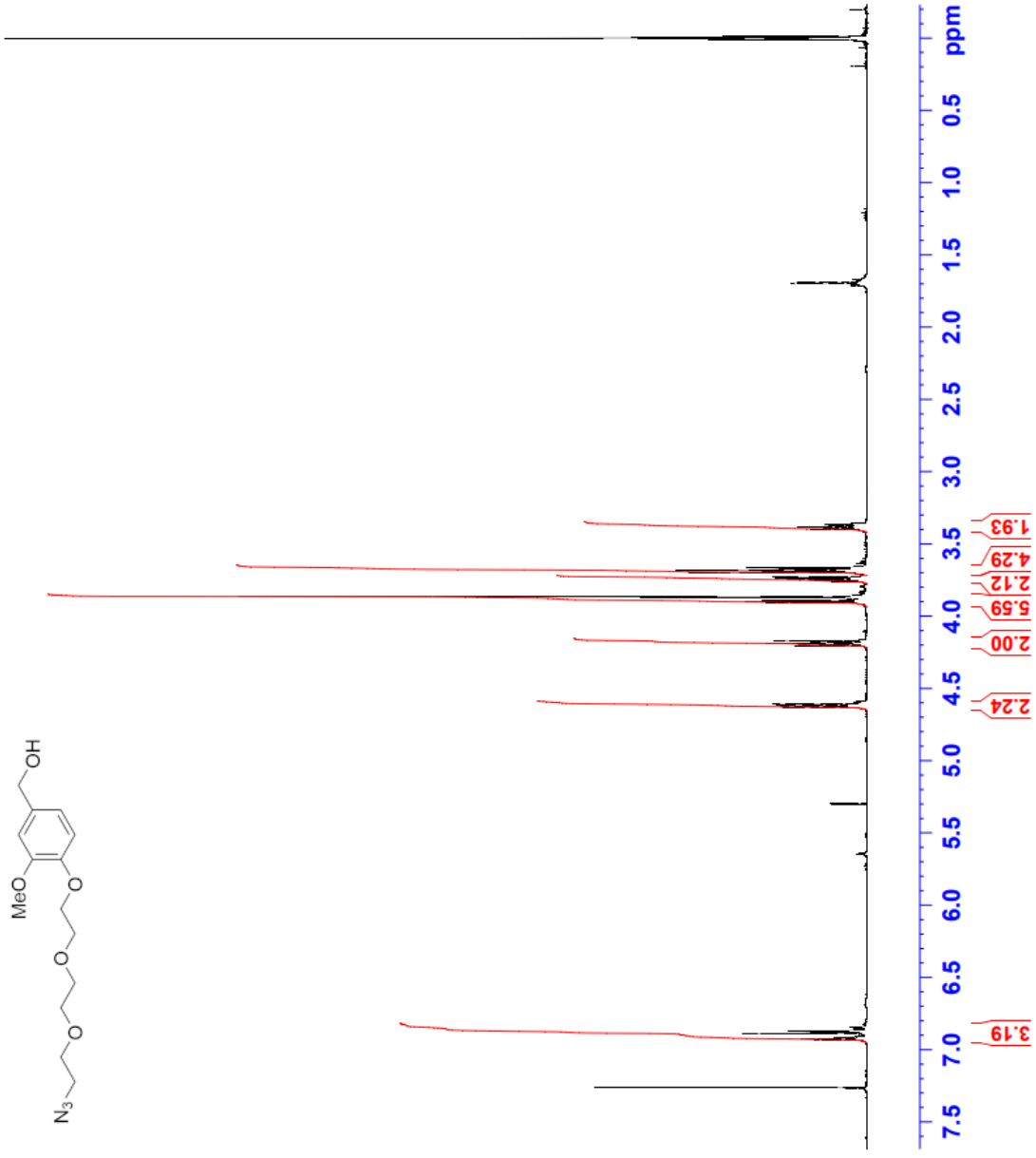


3.2 Linker-Nucleotide Complex S21

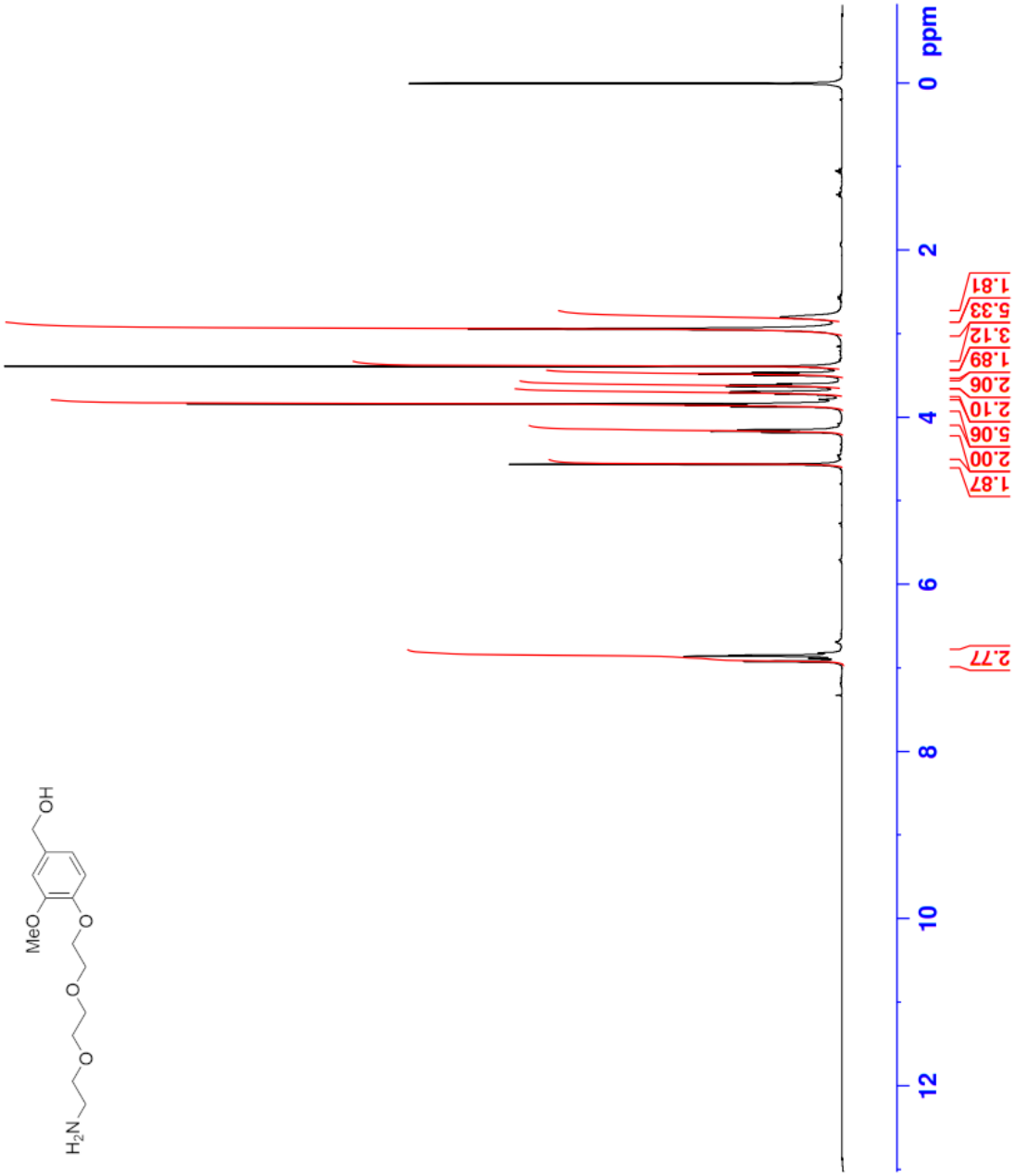


3.3 Alcohol 2

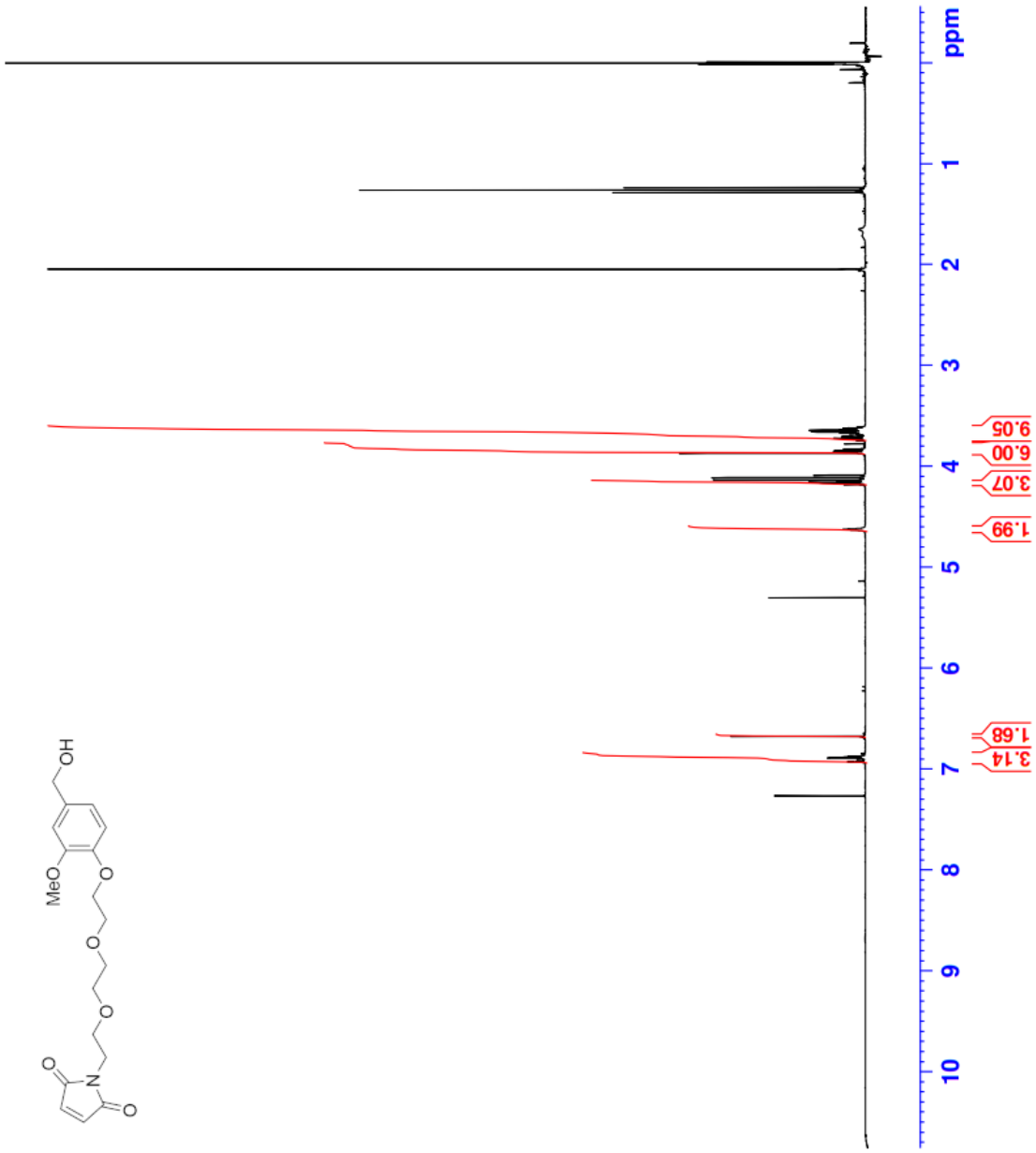
3.3.1 4-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)-3-methoxybenzyl alco- hol (S12)



3.3.2 4-(2-(2-(2-aminoethoxy)ethoxy)ethoxy)-3-methoxybenzyl alco- hol (S13)

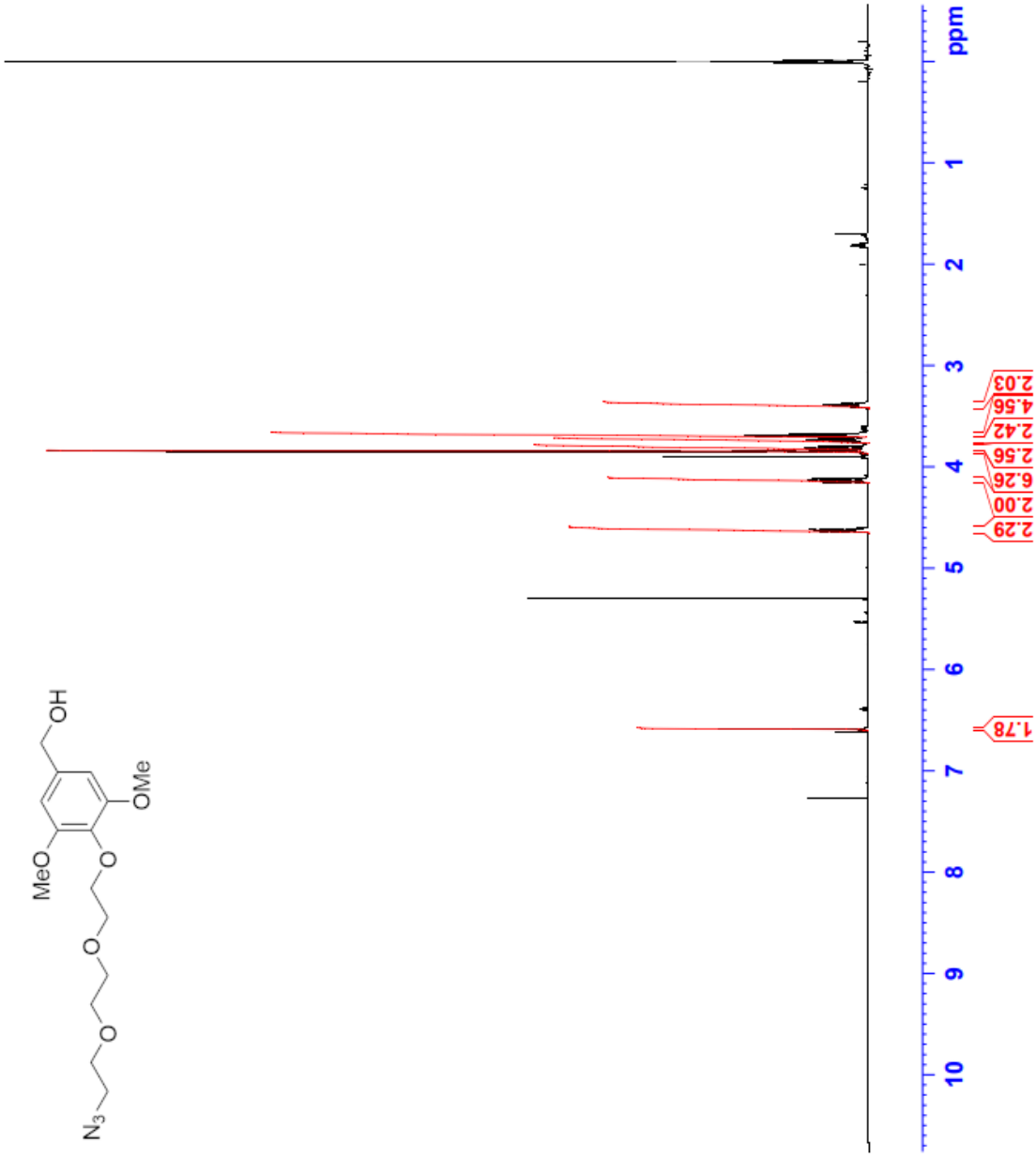


3.3.3 1-(2-(2-(4-(hydroxymethyl)-2-methoxyphenoxy) ethoxy)ethoxy)ethyl)-1*H*-pyrrole-2,5-dione (2)

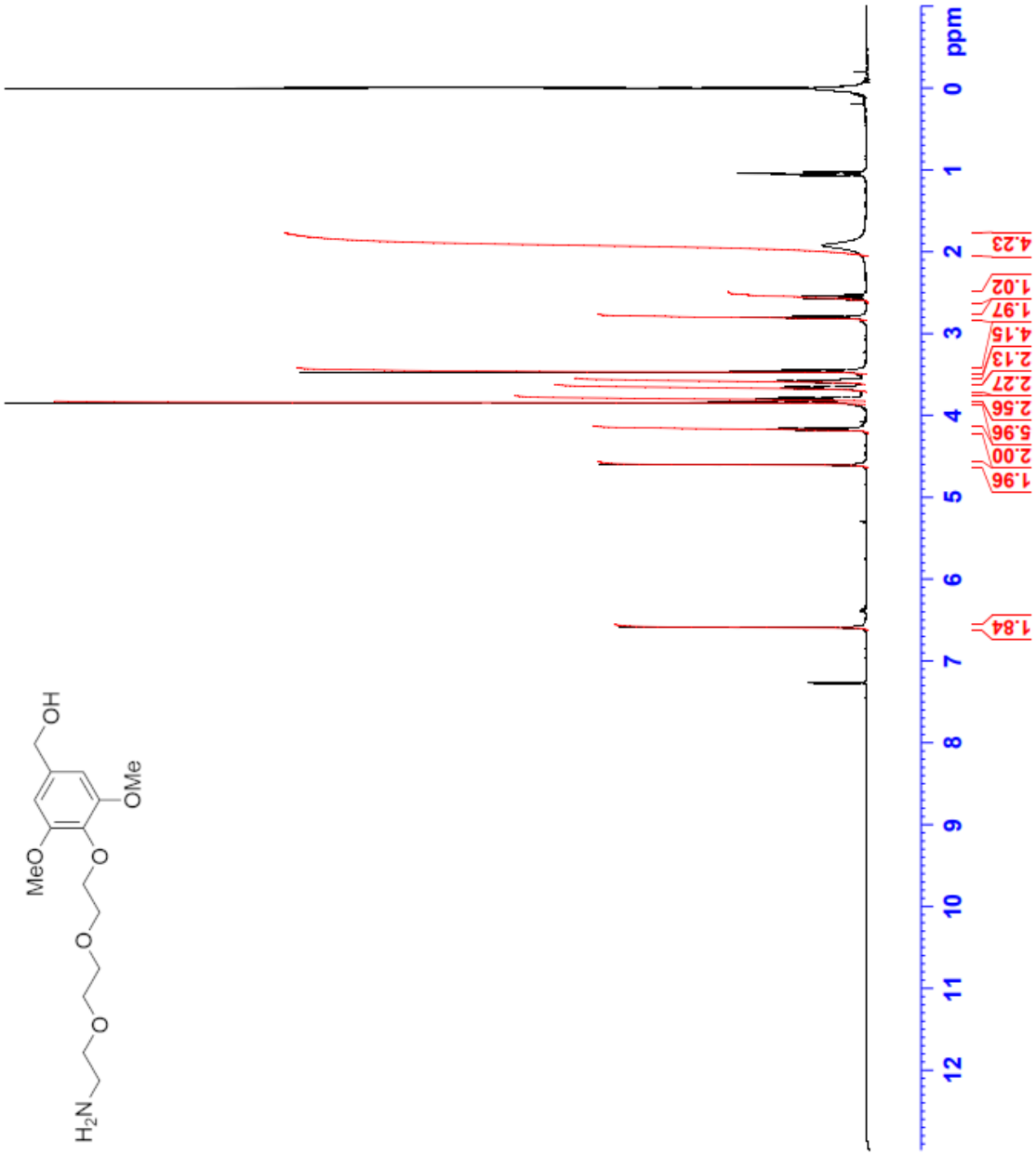


3.4 Alcohol 3

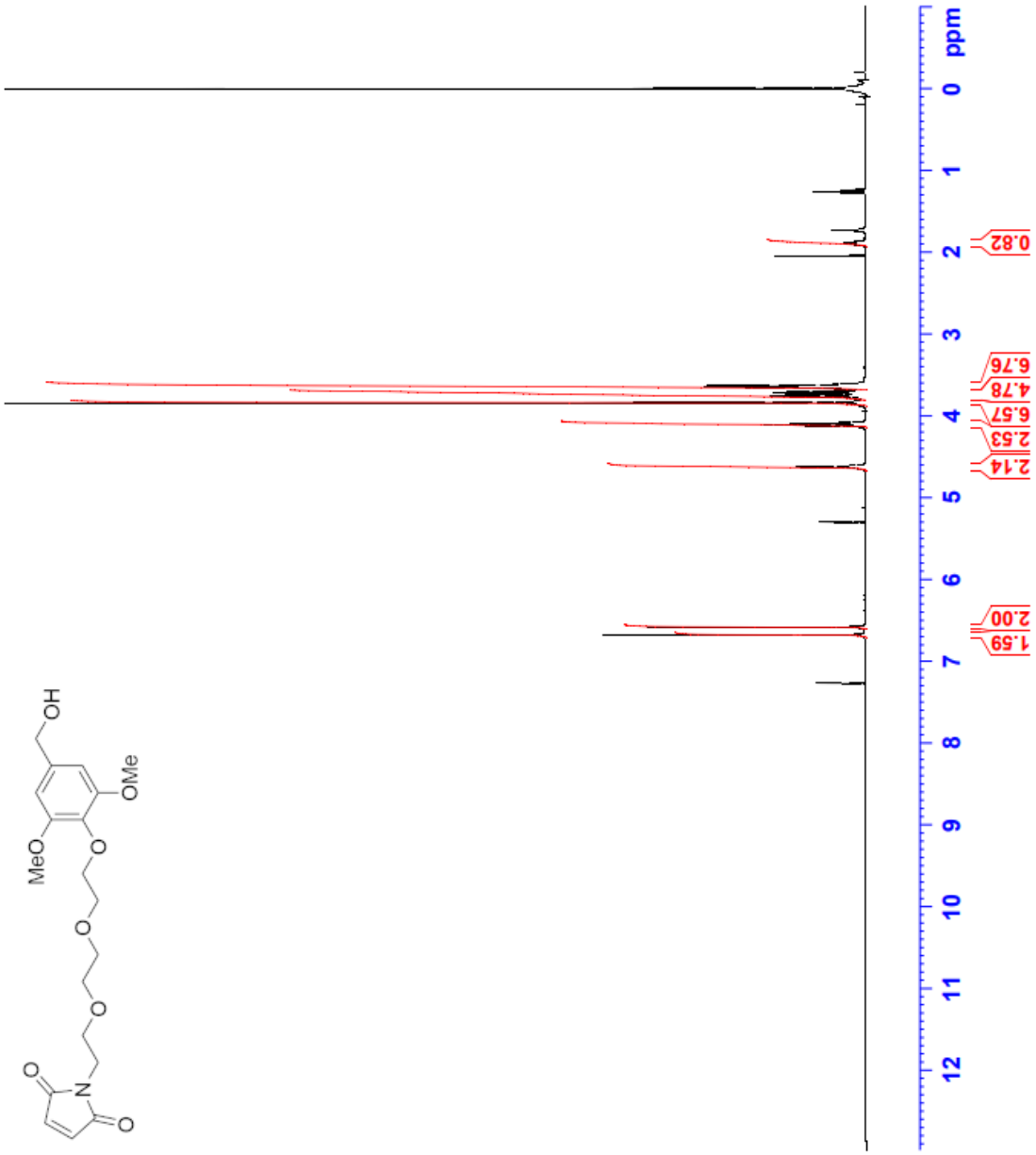
3.4.1 4-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)-3,5-dimethoxybenzyl alco- hol (S14)



3.4.2 4-(2-(2-(2-aminoethoxy)ethoxy)ethoxy)-3,5-dimethoxybenzyl alco- hol (S15)

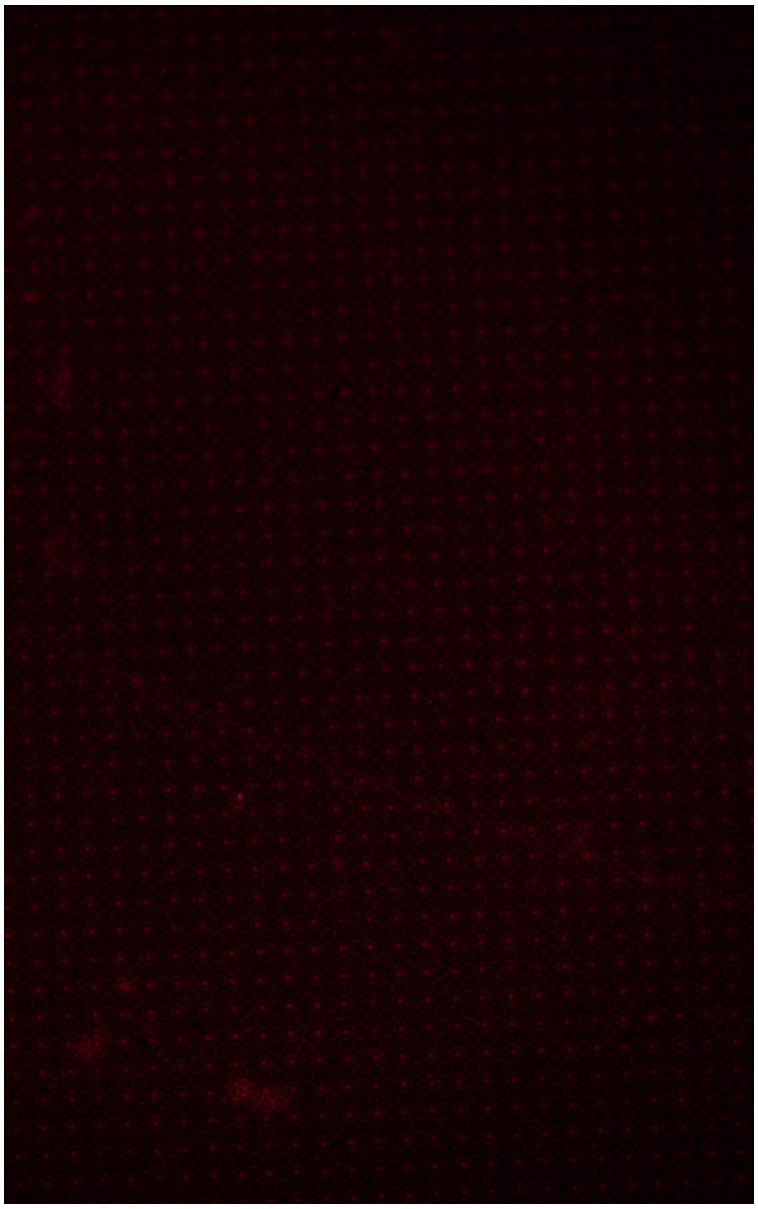


3.4.3 1-(2-(2-(4-(hydroxymethyl)-2,6-dimethoxyphenoxy) ethoxy)ethoxy)ethyl)-1*H*-pyrrole-2,5-dione (3)

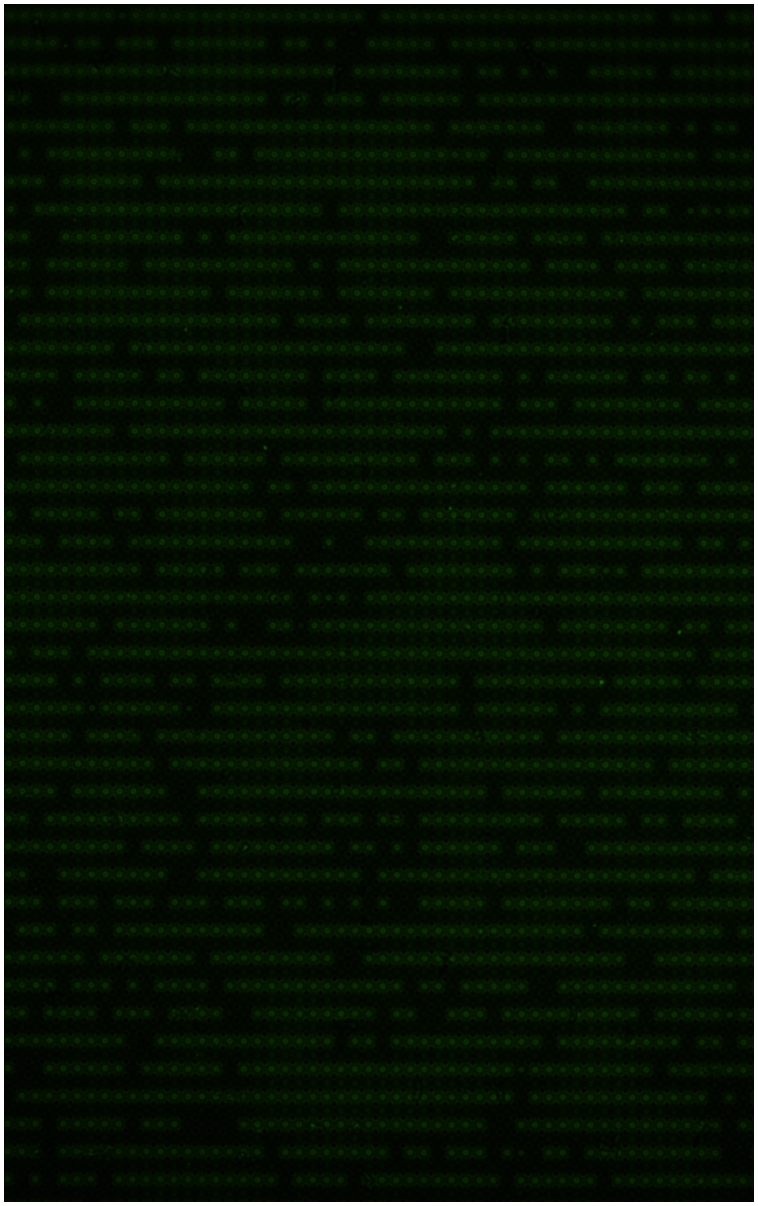


4 Unprocessed Images

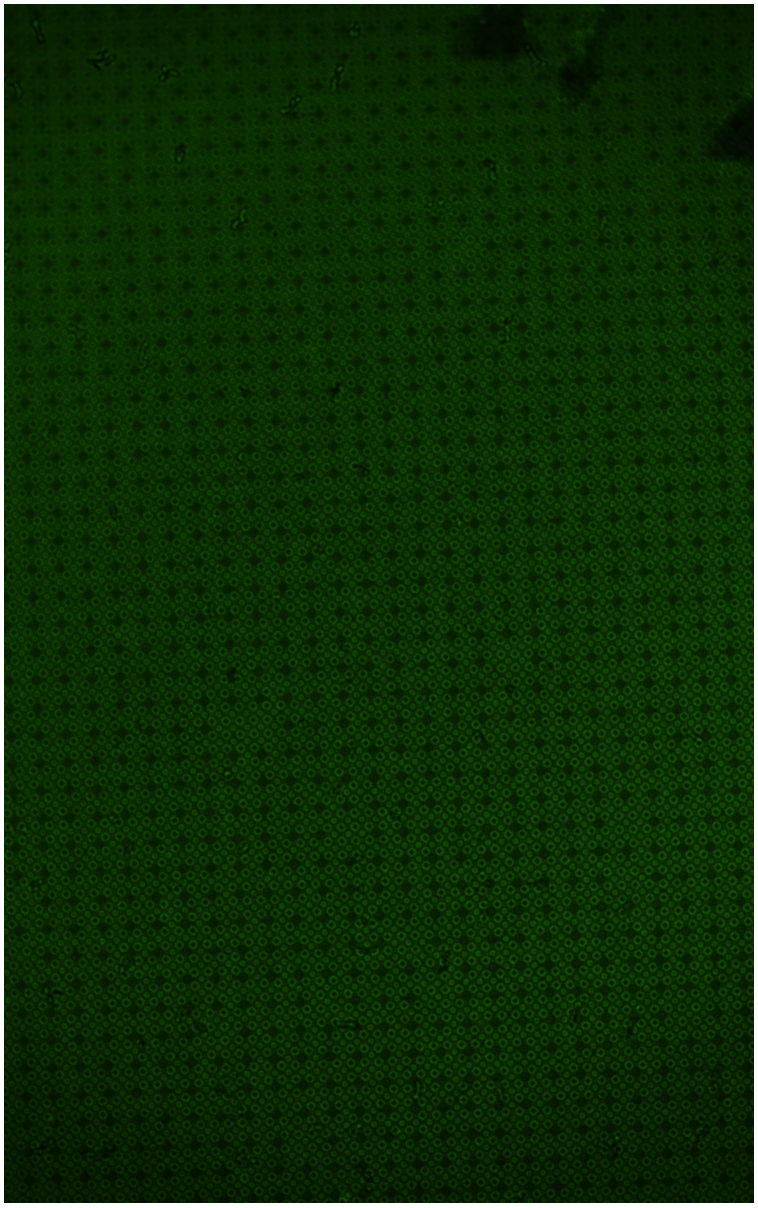
4.1 Figure 4a



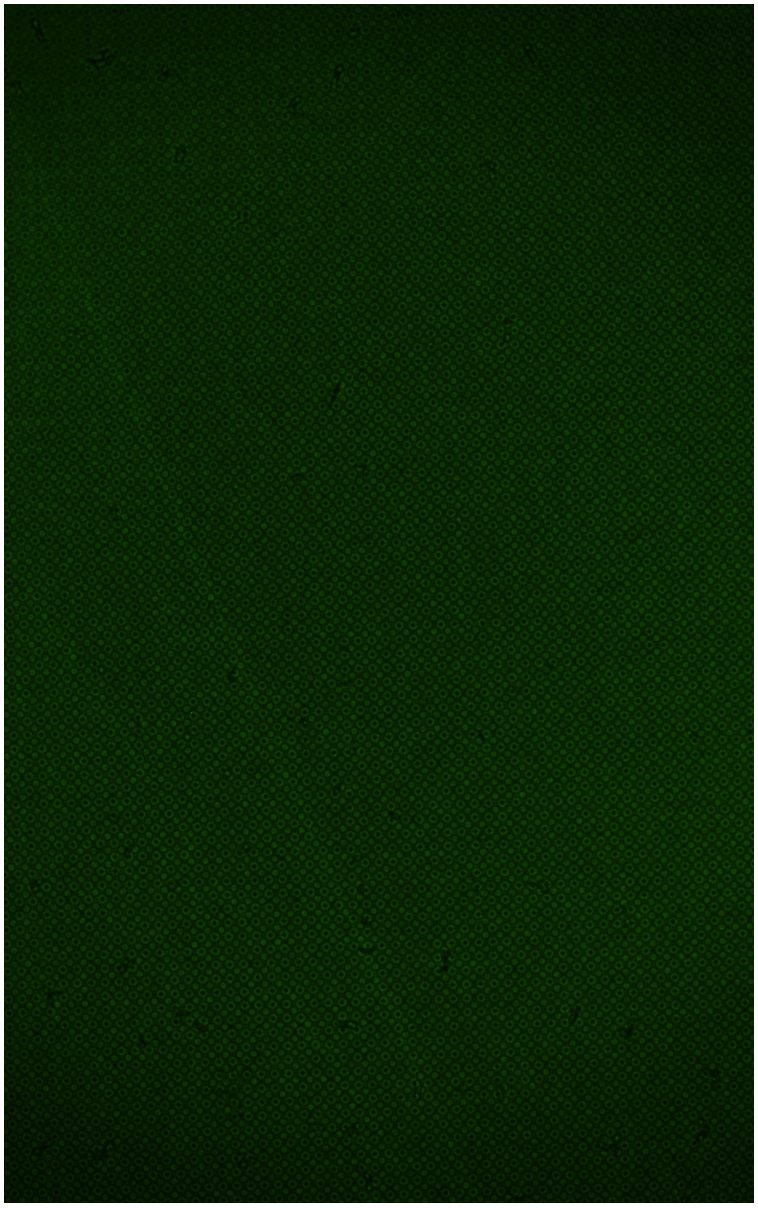
4.2 Figure 4b



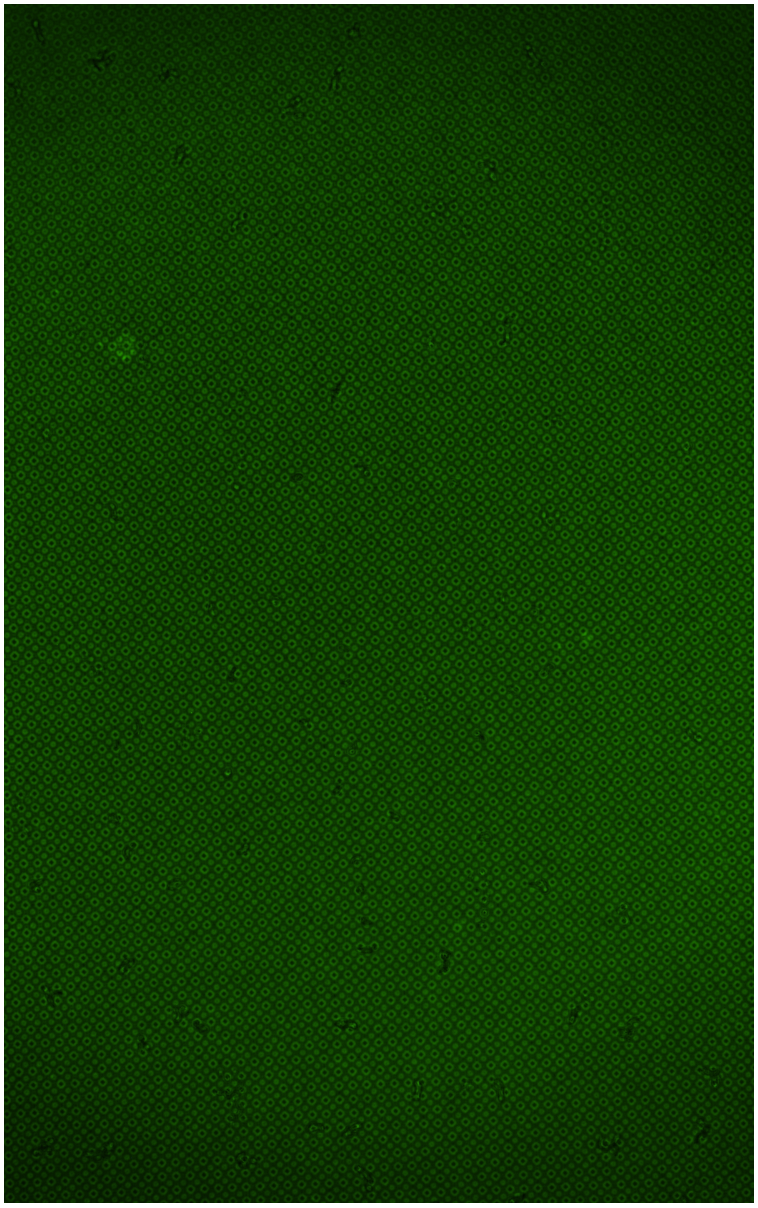
4.3 Figure 5a



4.4 Figure 5b

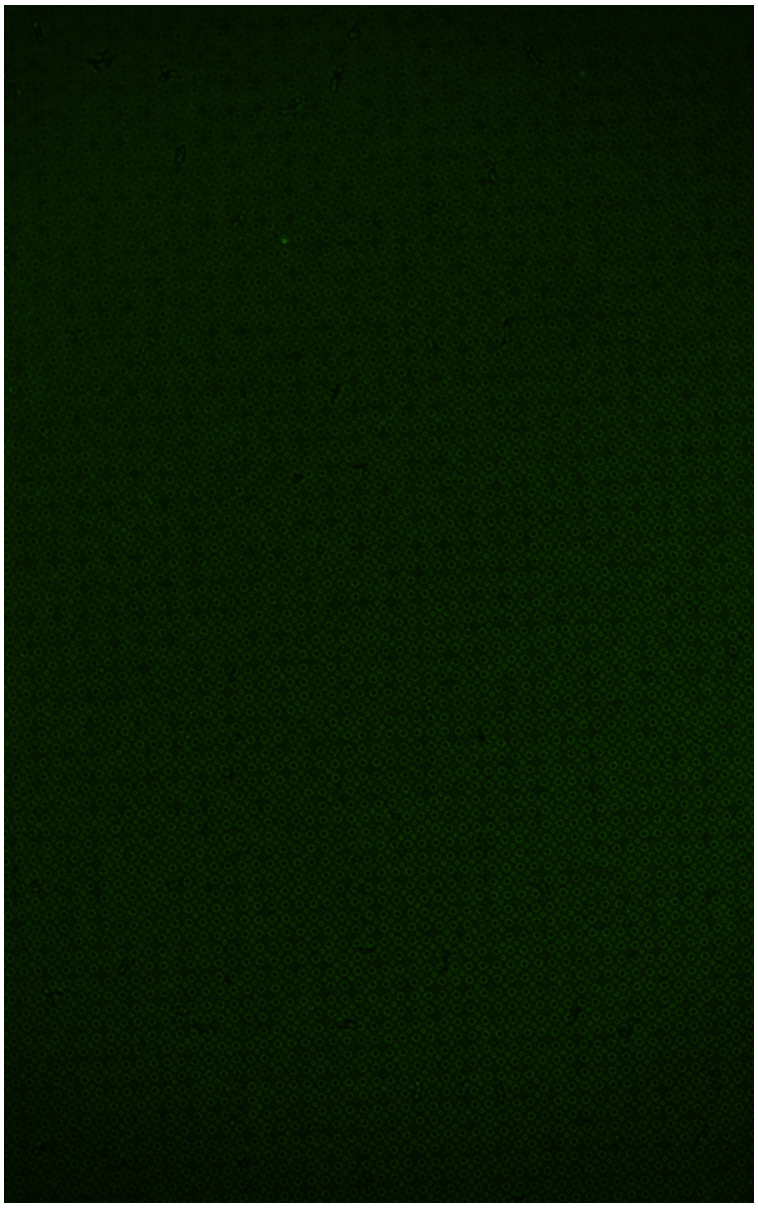


4.5 Figure 5c

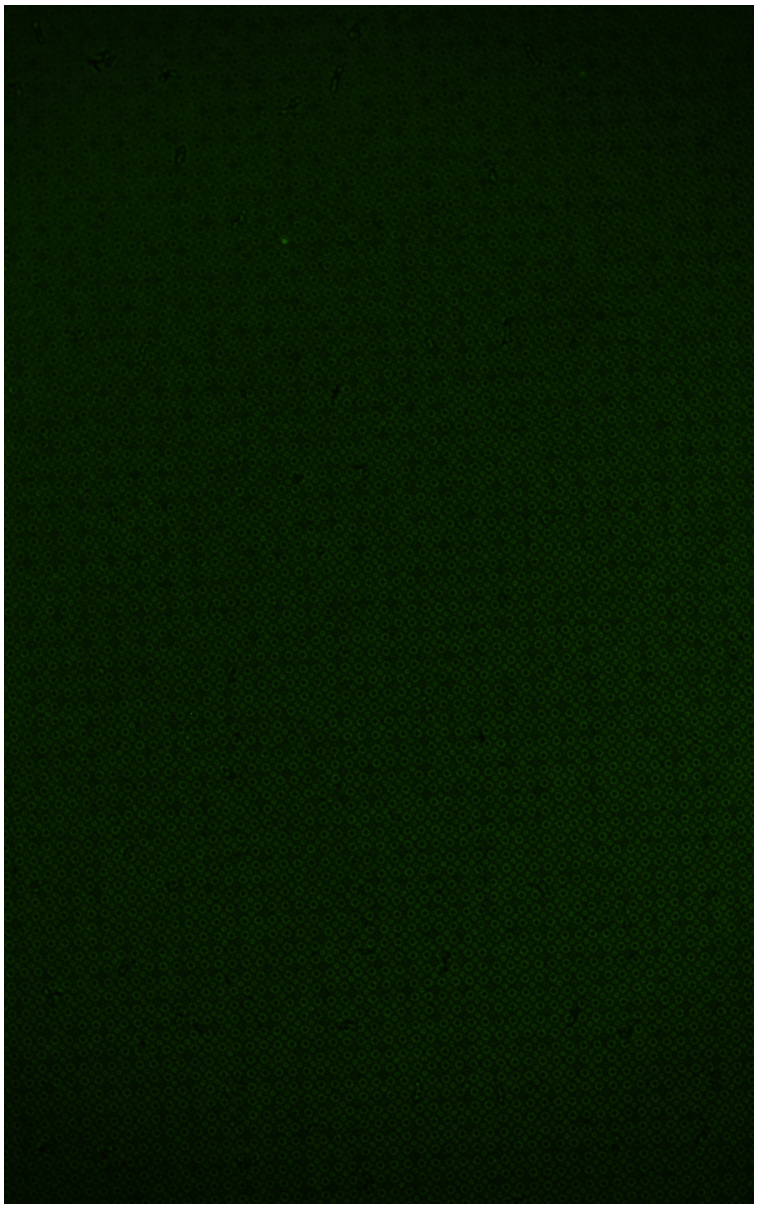


4.6 Figure 5d

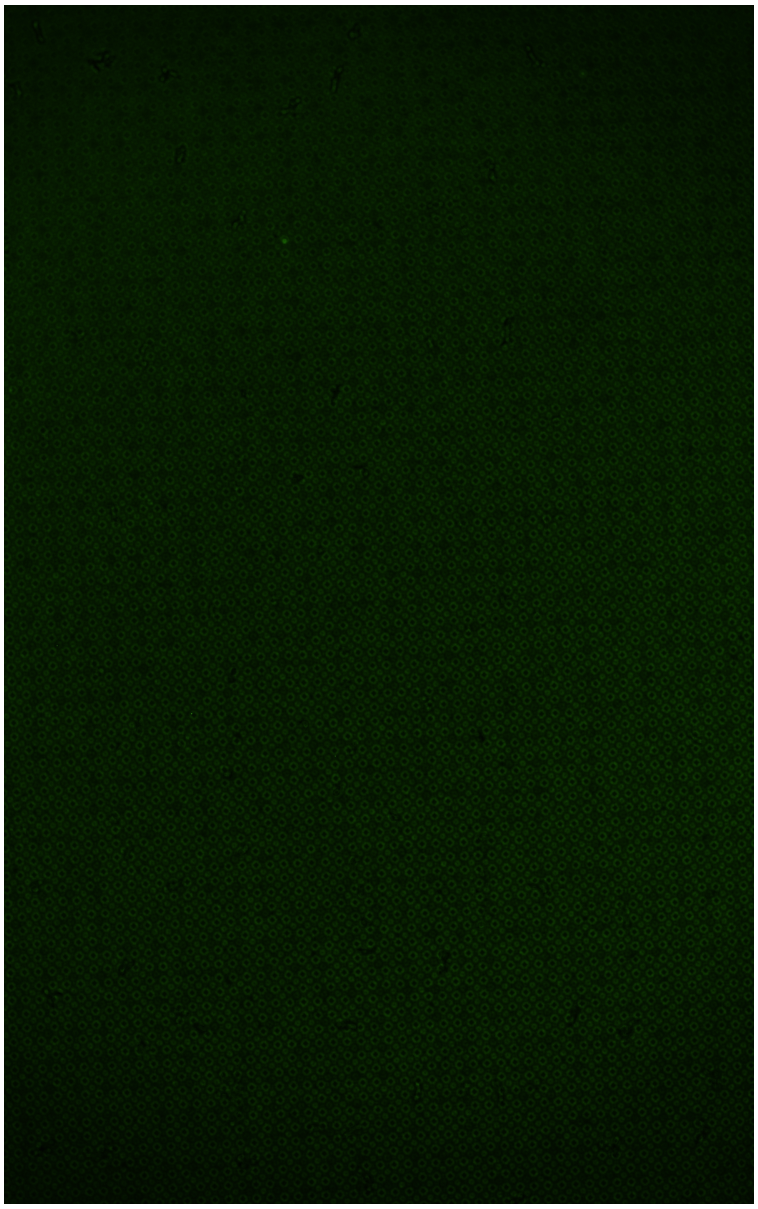
4.6.1 90 s



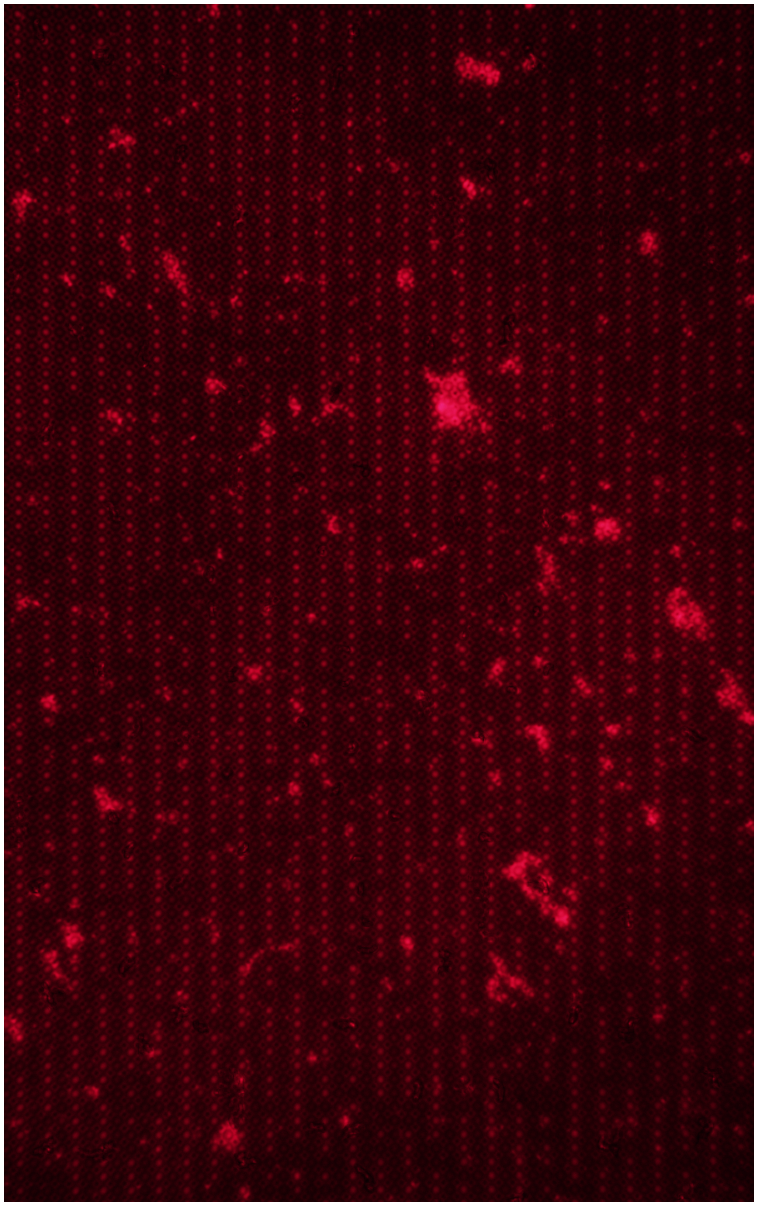
4.6.2 180 s



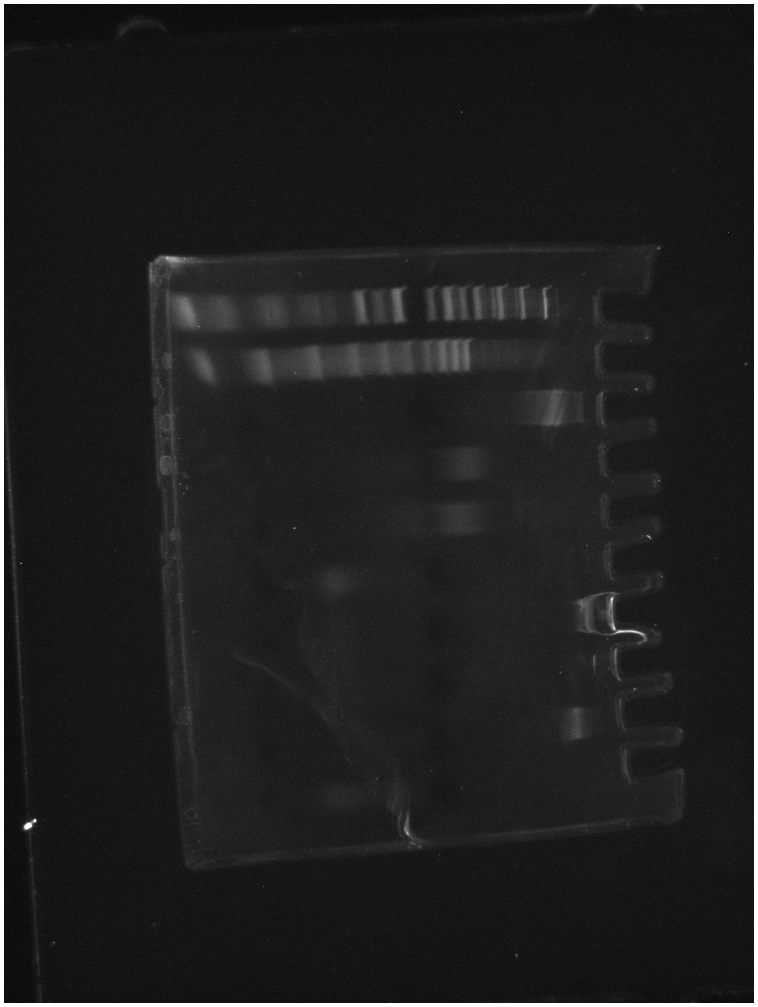
4.6.3 360 s



4.7 Figure 6

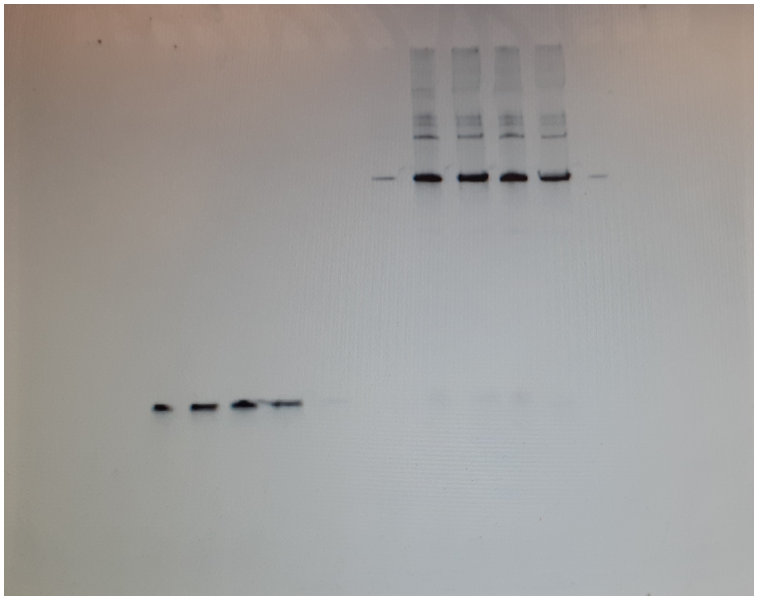


4.8 Figure S3

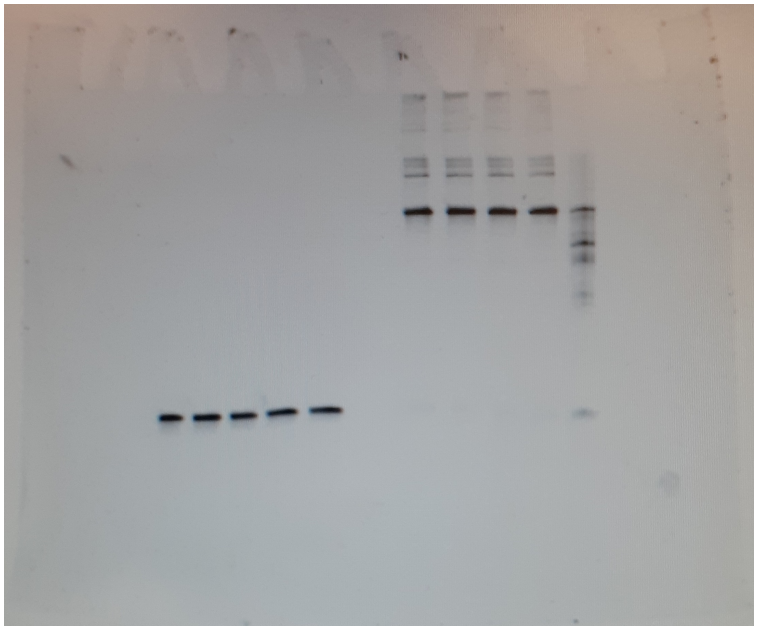


4.9 Figure S4

4.9.1 Left



4.9.2 Right



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