Title: APEX2 proximity labeling of RNA in bacteria

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Highlights:

- APEX2 proximity labeling can be applied to RNA in bacteria
- APEX2 RNA labeling reactions occur on the sub-minute timescale.
- APEX2 workflow requires less material and time than current methods.
- Alkyne-Phenol APEX2 substrate provides flexibility with click-chemistry.

Motivation:

Studies over the past several years have shown that distinct RNAs can be targeted to subcellular locations in bacterial cells. The ability to investigate localized RNAs in bacteria is currently limited to imaging-based approaches or to laborious procedures to isolate ribonucleoprotein complexes by grad-seq, HITS-CLIP, or Rloc-seq. However, a major challenge in studying mRNA localization in bacterial cells is that bacterial mRNAs typically last for only a few minutes in the cell, while experiments to investigate their localization or interaction partners can take much longer. Therefore, rapid methods of studying RNA localization are needed to bridge this technical challenge.

Summary:

Rapid spatially controlled methods are needed to investigate RNA localization in bacterial cells. APEX2 proximity labeling was shown to be adaptable to rapid RNA labeling in eukaryotic cells, and through the fusion of APEX2 to different proteins targeted to different subcellular locations, has been useful to identify RNA localization in these cells. Therefore, we adapted APEX2 proximity labeling of RNA to bacterial cells by generating an APEX2 fusion to the RNase E gene, which is necessary and sufficient for BR-body formation. APEX2 fusion is minimally perturbative and RNA can be rapidly labeled on the sub-minute timescale with Alkyne-Phenol, outpacing the rapid speed of mRNA decay in bacteria. Alkyne-Phenol provides flexibility in the overall downstream application with copper catalyzed click-chemistry for downstream applications, such as fluorescent dye-

azides or biotin-azides for purification. Altogether, APEX2 proximity labeling of RNA provides a useful method for studying RNA localization in bacteria.

Introduction:

Bacterial mRNAs have been found to localize to distinct subcellular locations. At a global level, many mRNAs have been found to be associated with the nucleoid, membrane, or cell poles[1–4] and it has been hypothesized that mRNA localization can be important for proper gene expression. For example, Flagellin mRNA in *Campylobacter jejuni* localizes to the cell poles which may facilitate cotranslational flagellar assembly[5]. In addition, biomolecular condensates, which are non-membrane bound organelles often assembled through phase separation, have been rapidly expanding in bacterial cells, with multiple involved in RNA metabolism[4,6]. Yet the functional significance of localization of RNAs to biomolecular condensates in bacteria has just started to be explored.

GRAD-seq, HITS-CLIP, and Rloc-seq methods[1,7,8] have been successful at identifying RNAs associated with specific RNPs or localized to certain subcellular locations. However, a major difficulty is that bacterial mRNAs are very short lived, and these methods require labeling or isolation times much longer than a typical mRNAs half-life, leading to the potential for false negatives. Therefore, rapid methods are needed to isolate the associated labile bacterial mRNA before they are degraded.

APEX2 proximity labeling allows for spatially controlled and rapid RNA labeling[9,10], making it a useful method to assay for localized bacterial RNAs. In this manuscript, we show that APEX2 proximity labeling can be adapted to bacterial condensates via fusion of the core BR-body scaffold RNase E to APEX2. RNA can be labeled robustly by APEX2 with H_2O_2 and Alkyne-Phenol, allowing copper catalyzed click chemistry with a variety of azides such as Cy5-azide. This procedure can be used to rapidly label bacterial RNAs by APEX2 on the sub-minute timescale, and which can be rapidly purified via copper catalyzed click chemistry conjugation of biotin-peg3-azide followed by streptavidin purification. Through the fusion of APEX2 to different localized RNA binding proteins in bacteria, APEX2 proximity labeling has the potential to improve our knowledge of RNA localization in bacteria.

Materials and Methods:

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
DYKDDDDK Tag (D6W5B) Rabbit mAb (Binds to same epitope as Sigma-Aldrich Anti-FLAG M2 antibody) #14793	Cell Signaling Technology	Cat. No.:14793S
Goat anti-Rabbit IgG (H+L) Secondary Antibody, HRP	Invitrogen	Cat. No.: 31460

Bacterial and virus strains		
Caulobacter crescentus NA1000	Lucy Shapiro's Lab	N/A
Js767 (RNE-Apex2FlgC-2)	This study	N/A
Js768 (RNE-Apex2Flg-EGFPC-2)	This study	N/A
Js87 (RNE-msfGFPC-4)	Al-Husini et al Mol Cell 2018	N/A
LS4379 (hfq-m2)	Lucy Shapiro's Lab	N/A
Chemicals, peptides, and recombinant proteins		
Alkyne-Phenol	MedChemExpress	Cat. No.: HY-131442
HYDROGEN PEROXIDE SOLN., 50%, ACS RGT.	Sigmaaldrich	Cat. No.: 7722-84-1
Sodium ascorbate	Sigmaaldrich	Cat. No.: 134-03-2
TRIzol	Thermofisher scientific	Cat. No.: 15596018
Chloroform	Thermofisher scientific	Cat. No.: AC423550010
2-PROPANOL, ANHYDROUS	Sigmaaldrich	Cat. No.: 67-63-0
Glycogen, RNA grade	Thermofisher scientific	Cat. No.: RO551
ETHANOL-D6 (D, 99%), ANHYD.	Sigmaaldrich	Cat. No.: 1516-08-1
Sodium Acetate (3 M), pH 5.5, RNase-free	Thermofisher scientific	Cat. No.: AM9740
Biotin-peg3-Azide	Vector Laboratories	CAS Number: CCT-AZ104
Cy5 Azide	Clickchemistrytools	Cat. No.: AZ118-1
BrightStar™-Plus Positively Charged Nylon Membrane, 30 cm x 45 cm	Thermofisher scientific	Cat. No.: AM10102
UltraPure™ SSC, 20X	Thermofisher scientific	Cat. No.: 15557044
SODIUM HYDROXIDE, PELLETS	Sigmaaldrich	Cat. No.: 1310-73-2
EDTA (0.5 M), pH 8.0, RNase-free	Thermofisher scientific	Cat. No.: AM9261
Sodium Dodecyl Sulfate (98.5%)	Sigmaaldrich	Cat. No.: L3771-500G

Bromophenol Blue	sigmaaldrich	Cat. No.: B0126-25G
Glycerol	sigmaaldrich	Cat. No.: 56-81-5
THPTA	Vectorslab	SKU: CCT-1010-100
Copper(II) sulfate pentahydrate	sigmaaldrich	Cat. No.: 7758-99-8
Bovine Serum Albumin	sigmaaldrich	Cat. No.: 9048-46-8
Thermo Scientific™ Pierce™ ECL Western Blotting Substrate	fisher scientific	Cat. No.: PI32109
Heparin sodium salt from porcine intestinal mucosa	sigmaaldrich	Cat. No.: 9041-08-1
Tris (1 M), pH 7.0, RNase-free	Thermofisher scientific	Cat. No.: AM9851
Tris (Tris Base)	GoldBio	Cat. No.: T-400-500
Hydrochloric acid	Carolina	Cat. No.: 867792
SODIUM CHLORIDE, CRYSTAL	Sigmaaldrich	Cat. No.: 7647-14-5
Tween-20	sigmaaldrich	Cat. No.: 9005-64-5
PageRuler™ Plus Prestained Protein Ladder, 10 to 250 kDa	Thermofisher scientific	Cat. No.: 26620
Urea (ACS)	Fisherchemicals	Cat. No.: U15-3
PBS - Phosphate-Buffered Saline (10X) pH 7.4, RNase-free	Thermofisher scientific	Cat. No.: AM9624
Agar	Fisherchemicals	Cat. No.: DF0001-17-0
Bactopeptone	Thermofisher scientific	Cat. No.: 211677
Yeast extract	sigmaaldrich	Cat. No.: 92144-500G-F
UltraPure™ Ethidium Bromide, 10 mg/mL	Thermofisher scientific	Cat. No.: 15585011
Thermo Scientific™ TriTrack DNA Loading Dye (6X	Thermofisher scientific	Cat. No.: FERR1161
Thermo Scientific™ GeneRuler 1 kb Plus DNA Ladder, ready-to-use	Thermofisher scientific	Cat. No.: FERSM1334
Magnesium sulfate	sigmaaldrich	Cat. No.: M7506-1KG

Calcium chloride (97%)	sigmaaldrich	Cat. No.: 746495-500G
Kanamycin	Thermofisher scientific	Cat. No.: 11815032
Potassium phosphate dibasic anhydrous	Fisherchemicals	Cat. No.: P288-500
Ammonium Chloride (99.5%)	sigmaaldrich	Cat. No.: A9434-1KG
Sodium phosphate dibasic	sigmaaldrich	Cat. No.: S7907-1KG
Glucose (99.5%)	sigmaaldrich	Cat. No.: G8270-1KG
Luria Broth Base	Thermofisher scientific	Cat. No.: 12795084
Iron (II) sulfate heptahydrate	sigmaaldrich	Cat. No.: F8633-250G
FD Kpn1	Thermofisher scientific	Cat. No.: FD0524
FD EcoR1	Thermofisher scientific	Cat. No.: FD0274
FD EcoRV	Thermofisher scientific	Cat. No.: FD0303
FD Dpn1	Thermofisher scientific	Cat. No.: ER1701
Hifi DNA Assembly Master Mix	NEB	Cat. No.: E2621L
T4 DNA Ligase	Thermofisher scientific	Cat. No.: EL0011
Nalidixic Acid	Fisher scientific	Cat. No.: 50488853
Gentamycin Sulfate	sigmaaldrich	Cat. No.: 345814-1GM
Agarose	Alkali	Cat. No.: A7705
RNAprotect Bacteria Reagent	Qiagen	Cat. No.: 76506
Rifampicin	Sigmaaldrich	Cat. No.: 557303-1GM
Pierce™ ECL Western Blotting Substrate	Thermofisher scientific	Cat. No.: 32106
GeneJET Gel Extraction Kit	Thermofisher scientific	Cat. No.: K0692
GeneJET Plasmid Miniprep Kit	Thermofisher scientific	Cat. No.: K0503

GeneJET PCR Purification Kit	Thermofisher scientific	Cat. No.: K0702
Glycine (99%)	sigmaaldrich	Cat. No.: G8898
Methanol (NF), Fisher Chemical™	Fisher scientific	Cat. No.: 67-56-1
PVDF Transfer Membranes, 0.2 µm	Thermofisher scientific	Cat. No.: 88520
DTT	sigmaaldrich	Cat. No.: 3483-12-3
Experimental models: Organisms/strains		
Caulobacter crescentus NA1000	T00841	N/A
Escherichia coli K-12 MG1655	T00007	N/A
Oligonucleotides		
HY1F: tgctgatgccgactacaaggatgacgatg	Invitrogen	N/A
HY1R: actttcccatacgcgtaacgttcgaattc	Invitrogen	N/A
HY2F: cgttacgcgtatgggaaagtcttacccaac	Invitrogen	N/A
HY2R: ccttgtagtcggcatcagcaaacccaag	Invitrogen	N/A
HY3F: gtacaagtgagctagctgcagcccgggg	Invitrogen	N/A
HY3R: gcagctgcagcttgtcatcgtcatccttgtagtcggc	Invitrogen	N/A
HY4F: cgatgacaagctgcagctgcctcccctg	Invitrogen	N/A
HY4R: tgcagctagctcacttgtacagctcgtccatgc	Invitrogen	N/A
CtrA forward: actgatgctgaagtctgaagg	Al-Husini,et al, Mol Cell. 2020	N/A
CtrA reverse: gattgaggtcgagcaggataag	Al-Husini,et al, Mol Cell. 2020	N/A
5s forward: ccattccgaactcggttcgttaag	Al-Husini,et al, Mol Cell. 2020	N/A
5s reverse: tggcggcgacctactct	Al-Husini,et al, Mol Cell. 2020	N/A
Recombinant DNA		
pFlgC-2	Thanbichler et al NAR 2011	N/A
pRNE-YFPC-1	Al-Husini et al Mol Cell 2018	N/A
Apex2FlgC-2	This paper	N/A
RNE-Apex2FlgC-2	This paper	N/A
RNE-Apex2Flg-EGFPC-2	This paper	N/A
pcDNA5/FRT/TO APEX2-GFP	Addgene	Cat. No.: 129640
Software and algorithms		
ImageJ	Imagej	https://imagej.net/software/fiji/downloads

Ibright analysis software	Thermofisher scientific	https://www.thermofisher.com/us/en/home/technical-resources/software-downloads/ibright-western-imager.html

60

61 ***In vivo* RNA proximity labeling**

62 <u>APEX2, Alkyne-Phenol, and H2O2 are needed for RNA proximity labeling</u>

63 NA1000 and Js767 (RNE-Apex2FlgC-2) were grown in PYE at 28°C overnight and re-
64 inoculated into 5 mL M2G the next day. Once the cultures reached an optical density (OD)
65 of 1.2 where BR-bodies were strongly induced, 2.5 mM of alkyne-phenol was added and
66 incubated for 30 minutes in a shaker incubator at 28°C at 200 rpm. A 100 mM H_2O_2 solution
67 (996 µL H_2O + 6 µL H_2O_2, 50%) was then freshly prepared. 1 mM of H_2O_2 was then added for
68 45 seconds as the cultures were still shaking. The labeling experiment was carried out
69 again with specific omissions as controls: no alkyne-phenol added, no H_2O_2 added, no
70 alkyne-phenol and H_2O_2 added, different concentrations of alkyne-phenol added (0.5 mM
71 and 1 mM), and different H_2O_2 exposures (15 seconds, 30 seconds, and 60 seconds). 4 mL
72 of cell culture were then quickly transferred into two 2 mL Eppendorf tubes and centrifuged
73 at 14,000 rpm for 10-15 seconds. The supernatant was quickly discarded, and the cell
74 pellets were resuspended in 1 mL of 65°C pre-warmed TRIzol. Because proximity labeling
75 requires the folded APEX2 protein, its denaturation in TRIzol can be used to quench the
76 proximity labeling reactions. RNA extraction followed by ethanol precipitation was then
77 carried out. 55 µL of a 10 mM Tris-HCl pH 7.0 (no EDTA) solution was added to dissolve the
78 air-dried RNA pellets. The samples were vortexed briefly to aid resuspension. 5 µL were
79 taken from the samples and placed in new tubes for Agilent Tapestation analyses and cell
80 lysate RNA sequencing.

81 Copper-catalyzed click chemistry reactions [12] were then conducted on the labeled RNA.
82 The reactions have a 300 µL total volume and contained: 50 µL of 100 ng/uL RNA (5 µg) +
83 175 µL H_2O + 12 µL of 125 mM sodium ascorbate + 3 µL of 10 mM Cy5-Azide + 60 µL of a
84 mix of 2.5 mM Cu(II)SO_4/12.5 mM THPTA (tris-hydroxypropyltriazolylmethylamine). The
85 reagents were added in the order they are listed. The click reaction was vortexed for 5
86 seconds and incubated for 10 minutes at room temperature away from light exposure.
87 Afterward, RNA precipitation was conducted and the RNA pellets were resuspended in 50

88 µL of 10 mM Tris-HCl pH 7.0 and 0.1 mM EDTA. The samples were then subjected to dot
89 blot filtration on a nylon membrane following the manual's instructions of BIO RAD's Bio-
90 Dot® and Bio-Dot SF Microfiltration Apparatus. A nylon membrane was pre-wetted in a 6X
91 SSC solution for at least 10 minutes. The pre-wetted nylon membrane was then assembled
92 into the dot blot apparatus. Vacuuming was applied to the apparatus and tighter sealing
93 was implemented. The wells were then washed 3 times with 1 mL of an ice-cold mixture of
94 10 mM NaOH/1 mM EDTA. Afterward, the 5 µg RNA samples were directly applied to the
95 wells (no RNA dissolution was performed). The wells were washed 3 times with 1 mL of an
96 ice-cold mixture of 10 mM NaOH/1 mM EDTA. The blotted membrane was washed 3 times
97 for 5 minutes with a 30 mL solution containing 2X SSC and 0.1% SDS. Fluorescent blot
98 imaging was then visualized using an ibright imaging system at a fluorescent light exposure
99 of 50 ms under the Cy5 channel.

100 RNase A & DNase I treatments

101 50 µL of 100 ng/µL of RNA collected from a proximity labeling reaction (4 mL of *Js767* cells
102 grown in M2G media with 2.5 mM alkyne phenol, 45 seconds H_2O_2) were subjected to
103 either an RNase A (5 µL of 10 mg/mL RNase A) or DNase I (5 µL of 1 U/µL DNase I)
104 treatment. The treated and mock samples were incubated for 2 hours at 37°C. Following
105 the enzyme or mock treatments, the RNA was precipitated and resuspended in 50 µL of 10
106 mM Tris-HCl pH 7.0 and then subjected to the Copper catalyzed click-chemistry reaction
107 with Cy5-azide. Following the 10-minute click reaction incubation, the RNA was then
108 precipitated and subjected to dot blot filtration as previously described.

109 Streptavidin capture of biotin labeled RNA

110 To enrich the transcriptome of BR-bodies, RNA labeled with alkyne-phenol was subjected
111 to the following click chemistry reaction: 50 µL of RNA (total RNA volume) + 175 µL H2O +
112 12 µL of 125 mM sodium ascorbate + 3 µL of 10 mM Biotin-peg3-azide + 60 µL of a mix of
113 2.5 mM Cu(II)SO_4/12.5 mM THPTA . The reagents were added in order as they are listed. The
114 click reaction was vortexed briefly and incubated for 10 minutes at room temperature away
115 from light exposure. RNA precipitation was then conducted and the air-dried RNA pellets
116 were resuspended in 100 µL B&W buffer (5 mM Tris-HCl, pH 7.5, 1 M NaCl, 0.5 mM EDTA,
117 0.1% v/v tween-20). The RNA samples were then immediately used for biotinylated RNA
118 enrichment.

119 High-capacity magnetic streptavidin beads (Vectorslab) were washed three times with 1
120 mL B&W buffer, twice with 1 mL of a mix of 0.1 M NaOH/0.05 M NaCl, and once with 1 mL
121 0.1 M NaCl at room temperature. The beads were then blocked using a blocking buffer (1
122 mg/mL BSA + 1 mg/ml heparin salt, dissolved in B&W buffer) at room temperature for 2

123 hours. The beads were then washed three times with 1 mL of B&W buffer under gently
124 vortexing. Afterward, the 100 µL RNA solution was added to the beads along with an extra
125 100 µL of B&W buffer. The RNA and beads were allowed to mix at room temperature for
126 one hour. The RNA-loaded beads were washed three times with 1 mL of B&W buffer, twice
127 with 1 mL of a PBS solution containing 4 M urea + 0.1% SDS, and twice with 1 mL of just
128 PBS at room temperature. To elute the enriched RNA, 900 uL of 65°C pre-warmed TRIzol
129 was added to the beads and the samples were incubated at 65°C for 10 minutes.
130 Afterward, 200 µL of chloroform was added and the samples were inverted 6-7 times and
131 then were incubated for 5 minutes at room temperature. The samples were centrifuged at
132 14,000 rpm for 10 minutes at 4°C. The upper aqueous layer was transferred to a new
133 Eppendorf tube and the enriched RNA was precipitated as previously described. The air-
134 dried RNA pellets were resuspended in 10 µL of 10 mM Tris-HCl pH 7.0 and 0.1 mM EDTA. 4
135 µL were then used for Agilent Tapestation analysis. The remaining volume could be utilized
136 for the follow-up RNA-seq experiments.

137 <u>RNA quality assessment</u>

138 *Js767 (RNE-Apex2FlgC-2)* was grown in PYE at 28°C overnight and re-inoculated into 5 mL
139 M2G the next day. Once the cultures reached an OD of 1.2, 2.5 mM of alkyne-phenol was
140 added and incubated for 30 minutes in a shaker incubator at 28°C at 200 rpm. 1 mM of
141 H_2O_2 was added to the culture for 45 seconds. Total RNA was then collected and
142 precipitated from 4 mL of culture as previously described using TRIzol. Afterward, 50 µL of
143 100 ng/µL of alkylated RNA were subjected to the following copper catalyzed click-
144 chemistry reaction: 50 µL of 100 ng/µL RNA (5 µg) + 175 µL H_2O + 12 µL of 125 mM sodium
145 ascorbate + 3 µL of 10 mM Cy5-Azide + 15 µL of 100 mM aminoguanidine hydrochloric acid
146 + 60 µL of a mix of 2.5 mM Cu(II)SO_4/12.5 mM THPTA. The click reaction was incubated for
147 10 minutes at room temperature away from light exposure. The RNA from the click reaction
148 was then precipitated and the air-dried RNA pellet was resuspended in 50 µL of 10 mM
149 Tris-HCl pH 7.0 and 0.1 mM EDTA. 250 ng of RNA from the *Js767* RNA lysate and the click
150 reaction RNA were sent for Agilent Tapestation analysis.

151 **RNA precipitation**

152 RNA precipitation was carried out by adding, in order, 2 µL of 1 mg/mL glycogen, 1/10
153 sample volume of 3 M sodium acetate pH 5.5, and 1 sample volume of isopropanol. The
154 sample mixtures were vigorously vortexed and stored at -80°C overnight. The samples were
155 spun for 1 hour at 14,000 rpm. The supernatant was then decanted and 1 mL of ice-cold
156 80% ethanol was added. The samples were spun for 15 minutes at 14,000 rpm. The
157 ethanol was pipetted out and the samples were then spun for 1 minute at 14,000 rpm. Any

158 remaining ethanol was pipetted out and the pellets were allowed to air-dry until they
159 became translucent. The RNA was then resuspended in the buffer of choice.

160 **Western Blot**

161 The following buffers were initially prepared:

162 1) 10X transfering buffer: 144 grams of glycine and 30.2 grams of Tris-Base were
163 dissolved in 900 mL of ddH2O. The buffer volume was adjusted to 1 L of ddH2O and
164 then filtered utilizing a Nalgene Rapid-flow sterile disposable filter unit.
165 2) 10X TBS: 24 grams of Tris-Base and 88 grams of NaCl were dissolved in 900 mL of
166 ddH2O. The pH of the buffer was adjusted to 7.6 utilizing hydrochloric acid. The
167 buffer volume was adjusted to 1 L of ddH2O and then sterilized utilizing a Nalgene
168 Rapid-flow sterile disposable filter unit.
169 3) 1X TBST: 10X TBS was diluted 10-fold with ddH2O to make 1X TBS. 0.1% Tween-20
170 was then added to the 1X TBS solution to make 1X TBST.

171 *NA1000, LS4379 (hfq-m2), and Js767(RNE-Apex2FlgC-2)* were inoculated in PYE and
172 incubated overnight at 28°C until they reached log phase (OD ~0.3-0.6). 1 mL was taken
173 from each culture, placed in 1.5 mL Eppendorf tubes, and centrifuged for 2 minutes at
174 6,000 rpm. The liquid cultures were discarded, and the cells were resuspended in 4X SDS
175 loading dye. 125 µL of 4X SDS loading dye was added to cultures with an OD~0.5. The
176 samples were boiled at 95 °C for 5 minutes then vortexed vigorously. The vortexed samples
177 were quickly spun down and placed on ice. 5 µL of a pre-stained PageRuler marker and 20
178 µL of lysate from each sample were loaded onto an SDS-PAGE gel. 1X transferring buffer
179 was freshly prepared (20 mL of 10X transfer buffer + 20 mL of methanol in 160 mL ddH2O)
180 while the samples were running on the gel. A PVDF transferring membrane was wetted in
181 methanol and 6 blotting papers were wetted using 1X transferring buffer for at least 10
182 minutes. Once the lysate samples on the SDS-PAGE were properly resolved, the gel was
183 placed on top of 3 pre-wetted blotting papers, and 2 mL of 1X transferring buffer was
184 poured on the gel. The PVDF membrane was briefly plunged into the 1X transferring buffer
185 and then placed on top of the gel. The remaining 3 blotting papers were placed on top of
186 the PVDF membrane. The transferring of the lysates from the SDS-PAGE gel to the PVDF
187 membrane occurred by utilizing the BIO-RAD Trans-Blot Turbo Transfer system (1 amps,
188 2.5 volts, 15 minutes). Once the transfer was completed, the PVDF membrane was placed
189 in a new container containing 5% BSA (blocking solution). The container was nutated for 1
190 hour at room temperature. Afterward, the 5% BSA blocking solution was discarded and the
191 PVDF membrane was washed 5 times with ddH2O for 5 minutes per wash. The primary
192 antibody (DYKDDDDK anti-flag tag) was then diluted (1:10000) into a fresh 5% BSA
193 solution. Once the last ddH2O wash was completed, the primary antibody solution was

194 poured on the PVDF membrane, and the container was nutated at 4°C overnight. The
195 following day, the primary antibody solution was discarded and the PVDF membrane was
196 washed 5 times with TBST for 5 minutes per wash. The secondary antibody (goat anti-rabbit
197 secondary antibody, HRP conjugated) was then diluted (1:10000) into a fresh 5% BSA
198 solution. Once the last TBST wash was completed, the secondary antibody solution was
199 poured on the PVDF membrane, and the container was nutated at room temperature for 1
200 hour avoiding light exposure. The secondary antibody solution was then discarded and the
201 PVDF membrane was washed 5 times with TBST for 5 minutes per wash. Following the final
202 washing procedure, a pierce ECL western blotting substrate solution was prepared (1 mL
203 of reagent 1 + 1 mL of reagent 2 were mixed and vigorously vortexed). The PVDF membrane
204 was placed on Seram wrap and the 2 mL substrate solution was poured on top of the
205 membrane. The substrate was incubated on the PVDF membrane at room temperature for
206 5 minutes avoiding light exposure. The membrane was then placed into an iBright imaging
207 system and the signal was detected under the Chemi-blot filter.

208 **Dilution Plates**

209 *NA1000 & Js767 (RNE-Apex2FlgC-2)* were grown in PYE overnight at 28°C. The overnight
210 cultures were diluted into fresh media and incubated at 28°C until the cells reached an OD
211 of ~0.3-0.6. Cells were then diluted to an OD=0.05 in PYE and 4 10-fold serial dilutions
212 were made. 5 µL were spotted from each dilution onto PYE plates. The plates were
213 incubated at 28°C for two days, and images were taken using an ibright imaging system.

214 **Fluorescent Cell Imaging**

215 *Js87 (RNE-msfGFP) & Js768 (RNE-Apex2FlgC-EGFPC-2)* were grown in PYE + gent (0.5
216 µg/mL) and PYE + Kan, respectively, overnight at 28°C. Cells were then fixed on M2G +
217 1.5% agarose pads placed on microscope slides (3051, Thermofisher scientific). Nikon
218 elements software was used to control a Nikon Eclipse NI-E equipped with a CoolSNAP
219 MYO-CCD camera and a 100x Oil CFI Plan Fluor (Nikon) objective to capture the images.
220

221 **mRNA half-lives measurement**

222 *NA1000* and *Js767 (RNE-Apex2FlgC-2)* were grown in liquid PYE at 28°C overnight. Cells
223 were then serially re-inoculated into liquid M2G and incubated until overnight log phase
224 (OD ~0.3-0.6) was reached. Log phase cultures were then re-inoculated to an OD = 0.05 in
225 25 mL liquid M2G. Before adding rifampicin, at time point 0, 1 mL of cells were added to 2
226 mL of RNAprotect Bacterial reagent (Qiagen) and vortexed for 5 seconds. 200 µg/mL of
227 Rifampicin was administered to the cultures and 1 mL of cells were extracted and added to
228 2 mL of RNAprotect Bacterial reagent at each of the following time points (followed by 5
229 seconds vortexing): 1, 2, 4, and 8 minutes. Cells were incubated at room temperature in

230 the RNAprotect Bacterial reagent for 5 minutes before being spun at 5000 rpm for 5
231 minutes. The bacterial pellets were resuspended in 1 mL of 65°C pre-heated TRizol
232 (Ambion) and incubated at 65°C for 10 minutes. 200 µL of chloroform was then added and
233 the samples were incubated at room temperature for 5 minutes. Subsequently, the
234 samples were centrifuged at 14,000 rpm for 10 minutes at 4°C. The aqueous layer was
235 removed and placed in a new 1.5 mL Eppendorf tube. The RNA was then precipitated and
236 the pellets were resuspended in 50 µL elution buffer (10 mM Tris-HCl, pH=7.0, 0.1 mM
237 EDTA). PCR tubes were filled with a master mix that contained 0.4 µM of *ctrA* forward
238 primer and 0.4 µM of *ctrA* reverse primer, 1X Luna Universal One-Step Reaction Mix, 1X
239 Luna WarmStart RT Enzyme Mix, and water. 100 ng/µL of RNA template was additionally
240 aliquoted into each of the PCR tubes. The samples were mixed well and quickly spun
241 down. A QuantStudio Real-Time PCR apparatus was utilized to conduct and examine the
242 qRT-PCR experiments. The same qRT-PCR experiment was done using instead the 5S
243 forward and reverse primers. To determine the mRNA-decay rates, we fitted a linear curve
244 to the ln (fraction RNA remaining) at each time point. Using a standard curve, the Ct was
245 converted into the quantity of RNA. Each time point's 5S rRNA amount was divided by the
246 amount of 5S rRNA at time point 0. The number obtained at each time point was then used
247 to normalize the amount of *ctrA* at each time point. The natural log of % RNA remaining
248 found in each sample was divided by the natural log of RNA at time point 0. The slopes of
249 the linear curve fit were then converted into mRNA half-life using the following equation:
250 mRNA half-life=−ln(2)/slope.

251 **Plasmid Construction**

252 <u>pAPEX2-FlgC-2 *Kan*^{R}</u>

253 The pFlgC-2 vector was PCR amplified using primers HY1F & HY1R. APEX2 was PCR
254 amplified using primers HY2F & HY2R from the addgene template #129640. The amplicons
255 were ran on a 1% agarose gel and gel extracted using a GeneJET Gel Extraction Kit. The
256 purified vector was Dpn1 treated then column purified using the GeneJET PCR Purification
257 Kit. The Apex2FlgC-2 *Kan*^{R} plasmid was assembled via Gibson assembly (NEB) and
258 transformed into chemically competent DHbeta10 *E. coli* and plated on LB + Kan (50
259 µg/mL) plates. The resulting KanR colonies were minipreped using the GeneJET Plasmid
260 Miniprep Kit and screened via restriction digestion (EcoR1) and the insert sequence was
261 verified by Sanger sequencing (Genewiz).

262 <u>pRNE-Apex2-FlgC-2 *Kan*^{R}</u>

263 The last 534 RNase E (RNE) base pairs were obtained by digesting pRNE-YFPC-1 [11] with
264 Nde1 and Kpn1. APEX2FlgC-2 was digested with Nde1 and Kpn1. The digestion reactions
265 were ran on a 1% agarose gel and the RNE fragment and Apex2-FlgC-2 vector were gel
266 extracted using a GeneJET Gel Extraction Kit. The RNE fragment and Apex2FlgC-2 vector
267 were ligated using T4 ligase, transformed into chemically competent DHbeta10 *E. coli*, and
268 selected on LB + Kan plates. The resulting KanR colonies were minipreped using the
269 GeneJET Plasmid Miniprep Kit and screened via restriction digestion (EcoRV) and the insert
270 sequence was verified by Sanger sequencing (Genewiz).

271 pRNE-Apex2FlgC-EGFPC-2 *Kan*^{R}

272 The RNE-Apex2FlgC-2 vector was PCR amplified using primers HY3F & HY3R. The EGFP
273 insert was PCR amplified using primers HY4F & HY4R from the addgene template #129640.
274 The amplicons were ran on a 1% agarose gel and gel extracted using a GeneJET Gel
275 Extraction Kit. The purified vector was Dpn1 treated then column purified using the
276 GeneJET PCR Purification Kit. The RNE-Apex2FlgC-EGFPC-2 plasmid was assembled via
277 Gibson assembly (NEB) and transformed into chemically competent DHbeta10 E. coli and
278 selected on LB + Kan plates. The resulting KanR colonies were minipreped using the
279 GeneJET Plasmid Miniprep Kit and screened via restriction digestion (EcoRV) and the insert
280 sequence was verified by Sanger sequencing (Genewiz).

281 **Strain construction**

282 *Js767: NA1000 rne::rne-apex2-flg Kan*^{R}

283 The RNE-Apex2FlgC2 plasmid was recombined into the *rne* locus in NA1000 via mating and
284 the selection was carried out on PYE + Nal (20 µg/mL) + Kan (25 µg/ mL) plates. The
285 resulting KanR colonies were first grown in PYE + Kan (5 µg/mL) cultures and then screened
286 by PCR.

287 *Js768: NA1000 rne::rne-apex2-flg-egfp Kan*^{R}

288 The RNE-Apex2FlgC-EGFPC-2 plasmid was recombined into the *rne* locus in NA1000 via
289 mating and the selection was carried out on PYE + Kan plates. The resulting KanR colonies
290 were first grown in PYE + Kan cultures and then screened by PCR.

291 ## Results:

292 ### RNaseE-APEX2 proximity labeling of RNA requires Alkyne-Phenol, H_20_2, and
293 APEX2

294 APEX2 is an engineered ascorbate peroxidase which can catalyze the creation of a
295 free radical on the oxygen of alkyne-phenol, and this highly reactive species can react with
296 molecules within a 10-20 nm radius [13,14] (Fig 1A). It was shown that APEX2 can label RNA in
297 eukaryotic cells, making this a useful experimental system for identifying localized RNAs
298 [10,15,16]. In order to perform RNA proximity labeling in bacteria, we first generated a gene
299 fusion between RNase E and APEX2. RNase E is known to phase-separate into bacterial
300 ribonucleoprotein bodies (BR-bodies), biomolecular condensates containing mRNA and
301 promoting the mRNA decay process[17]. To determine whether APEX2 fusions are tolerated
302 in bacteria, we examined the subcellular localization, cell fitness, and mRNA decay activity
303 of RNase E-APEX2 fusions in the bacterium *Caulobacter crescentus* (Fig 1B). We observed
304 that fusing APEX2 to RNase E led to proper expression of RNase E-APEX2 (Fig S1) and did
305 not alter its ability to phase separate into BR-bodies (Fig 1B). In addition, since RNase E's
306 ability to degrade mRNAs is essential for cell growth [11,18], we also examined the cellular
307 fitness of the RNase E-APEX2 fusion and found that both CFUs and colony size were
308 indistinguishable from wild-type (Fig 1B). Finally, we compared the mRNA decay activity of
309 the RNase E-APEX2 fusion and found that it has similar rates of mRNA decay as compared
310 to wild-type (Fig 1B), while 5S rRNA remained stable. Altogether, we could not detect any
311 measurable differences in RNase E function when fused to APEX2. To examine whether
312 the APEX2 fusion can be used to label RNA we added all combinations of proximity labeling
313 reactants (H_2O_2 and Alkyne-Phenol) in combination with or without the RNase E-APEX2
314 fusion (Fig 1C). Each of the proximity labeling conditions were performed, and then we
315 extracted the RNA and used copper click chemistry to conjugate a Cy5-azide to the RNA.
316 The RNA was then deposited on a positively charged nylon membrane using a dot blot
317 apparatus, and the Cy5 signal was measured in a fluorescent gel imager. We find that only
318 in the presence of APEX2, H_2O_2, and alkyne-phenol do we observe RNA labeling (Fig 1C),
319 suggesting that APEX2 proximity labeling reactions occurred. To determine whether the
320 Cy5 signal was a result of RNA, or contaminating DNA we performed RNase A or DNase I
321 digestions on our samples before spotting them on the dot blot (Fig 1C). Here we see that
322 RNase A treatment leads to a complete loss of Cy5-fluorescence, while DNase I treatment
323 led to no difference in fluorescence signal, suggesting that our Cy5 fluorescence is from
324 RNA.

325 Rapid labeling of cellular RNA with Alkyne-Phenol

326 One of the major challenges of studying bacterial mRNA decay is the very short
327 lifetimes of cellular mRNAs. Most bacterial mRNAs in rapidly growing species have half-
328 lives between 1-4 minutes [19,20], making it hard to harvest the RNA before they are degraded.
329 To address this technical limitation, we first optimized the Alkyne-phenol concentration
330 used for labeling cells and found that 2.5 mM Alkyne-Phenol robustly labels cellular RNA

331 (Fig 2). Next, we performed a time-course of H_2O_2 exposure to identify the timescale of
332 RNA labeling, ranging from 15 seconds reaction times to 1 minute reaction times. We
333 found that robust labeling could be achieved in as little as 15 seconds, while labeling
334 increased upon longer proximity labeling reaction times (Fig 2). Importantly, the labeling
335 reaction occurs on the sub-minutes timescale, making this method well-suited to the use
336 in exponentially growing bacterial cells.

337 Copper click-chemistry of Azide-Biotin and streptavidin purification of labeled 338 RNA.

339 APEX2 proximity labeling of RNA is rather flexible due to the diversity of azides
340 commercially available for copper catalyzed click chemistry. To isolate cellular RNA for
341 RNA-sequencing, we altered the azide from azide-Cy5 dye to azide-biotin to allow for
342 streptavidin-mediated RNA purification from the cell (Fig 3A). In this approach, labeled
343 RNA is extracted from cells and conjugated to azide-biotin using copper catalyzed click
344 chemistry, which allows for the subsequent purification of the labeled RNA using
345 streptavidin-resin. While copper can cleave RNA, using a short incubation time for the
346 click reaction minimizes RNA cleavage by copper (Fig S2). After click chemistry, the RNA
347 can be purified under stringent conditions and eluted from the resin under denaturing
348 conditions. When applied to the RNase E-APEX2 fusion presented earlier, we found that
349 the eluted RNA profile indeed matched that of BR-body RNA isolated via density
350 centrifugation (Fig 3)[11,21]. In particular, the rRNA and tRNAs were depleted from the RNA
351 samples, showing a similar profile in RNA size abundance to density centrifugation
352 isolated BR-body RNA. Importantly though, while centrifugation-based isolation of BR-
353 bodies required 200 mL of cell culture and 5 days of hands-on work to isolate the BR-body
354 enriched RNAs, APEX2 proximity labeling required only 4 mL of cell culture and 3 days of
355 hands-on work, making it higher throughput.

356 Discussion:

357 RNP complexes are important regulators of RNA biology, including transcription, RNA
358 processing, transport, translation, and decay [4,22–24]. In addition to RNP complex formation,
359 these complexes have been increasingly found to localize into biomolecular condensate
360 structures, which help to facilitate the spatial organization of the stages in the mRNA life
361 cycle via phase-separation [11,17,25–27]. As realizations that RNA localization has become
362 increasingly important in bacteria, methods to identify the population of localized RNAs
363 have been developed, yet these methods have been limited due to the long timescale of
364 the procedures compared to the short mRNA lifetimes in bacteria. APEX2 proximity
365 labeling can rapidly label RNA and can be easily genetically fused to genes whose proteins
366 have known patterns of subcellular localization. APEX2 proximity labeling of RNA requires

367 small amounts of cells, can be completed in a few hours by a single scientist, and yields
368 sufficient RNA for downstream analysis. The reactivity radius of APEX2 is estimated to be
369 10-20nm[28], suggesting it gives high spatial precision. Genetic fusion of APEX2 is easy to
370 generate, and APEX2 is similar in size to a fluorescent protein and has no observable
371 functional impacts when fused to RNase E, so we anticipate that this is unlikely to disrupt
372 target protein function. Altogether, APEX2 proximity labeling of RNA will help to accelerate
373 the discovery of localized RNAs in bacteria.

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376 to HY.

377

378 Figure legends:

379 **Figure 1. APEX2 proximity labeling of RNA in bacterial cells with minimal perturbation**.
380 A) Schematic of APEX2 labeling of RNA protocol. The APEX2 protein was fused to RNase E,
381 the major protein that scaffolds BR-bodies. Cells are incubated in media containing
382 alkyne-phenol, and labeled was initiated with H_2O_2. After a brief labeling reaction, RNA was
383 extracted from the cells, and Cy5-azide was conjugated to the RNA by copper catalyzed
384 click chemistry. B) APEX2 fusion does not dramatically impact localization or function
385 (growth rate). Left: *In vivo* localization of RNase E-msfGFP vs RNase E APEX2-msfGFP
386 shows that fusion does not impact the formation of BR-bodies. Scale bar is 1µm. Middle:
387 Growth of RNase E-APEX2 fusion is similar to wild type *Caulobacter* cells. Right: mRNA
388 half-life measurements by qRT-PCR show that RNase E-APEX2 degrades mRNAs with a
389 similar half-life to wild-type. Data are from three biological and technical replicates. C)
390 APEX2 labeling requires H_2O_2 and alkyne-phenol to label RNAs. RNA labeling reactions
391 were placed on a nylon membrane to bind to the RNA in a dot blot apparatus and scanned
392 for Cy5 fluorescence in a gel imager. As a control, the RNA samples were incubated for 2
393 hours with DNase I and RNase A. The RNA was then precipitated before being subjected to
394 the azide-Cy5 click chemistry reaction and was re-precipitated before being filtered on the
395 nylon membrane in the dot blot apparatus.

396 **Figure 2. APEX2 proximity labeling of RNA works rapidly.** Optimization of APEX2 labeling
397 of RNA with Alkyne-Phenol. Top: Alkyne-phenol titration reveals that peak labeling occurs
398 with 2.5 mM Alkyne-Phenol. RNA was labeled in the scheme shown in Fig 1A, and the Cy5
399 intensity was measured in a gel imager. Bottom: Time course of APEX2 labeling. RNA was
400 labeled in the scheme shown in Fig 1A, and RNA labeling is apparent in as short as 15

401 seconds of H_2O_2 incubation, while peak labeling efficiency is observed at 45 seconds of
402 H_2O_2 incubation.

403 **Figure 3. APEX2 proximity labeled RNA can be isolated by streptavidin purification**. A)
404 Schematic of the conjugation of biotin-azide to clicked alkyne-phenol and the resulting
405 streptavidin purification. B) Streptavidin purification of biotinylated RNAs. Tapestation
406 RNA profiles of the lysates and elution fractions of biotinylated proximity labeled RNAs.
407 The bottom two bands are tRNAs, which are known to be highly depleted in BR-bodies from
408 differential centrifugation-based isolation of BR-bodies[11,21].

409 **Figure S1. RNase E-APEX2-FLAG Western Blot**. Protein lysates generated from *NA1000,*
410 *Hfq-FLAG (LS4379)* and *RNase E-APEX2-FLAG (JS767)* were run on an SDS PAGE gel,
411 transferred to PVDF, and probed with an anti-FLAG antibody.

412 **Figure S2. Effects of copper catalyzed click reaction on RNA integrity**. RNA extracted
413 from APEX2 proximity labeling reactions were ran on an Agilent Tapestation from cells
414 harboring RNase E-APEX2 fusion. The APEX2 labeled RNA was also incubated with Copper
415 Sulfate under similar conditions for click chemistry reactions where we observe minimal
416 RNA degradation from the copper.

417

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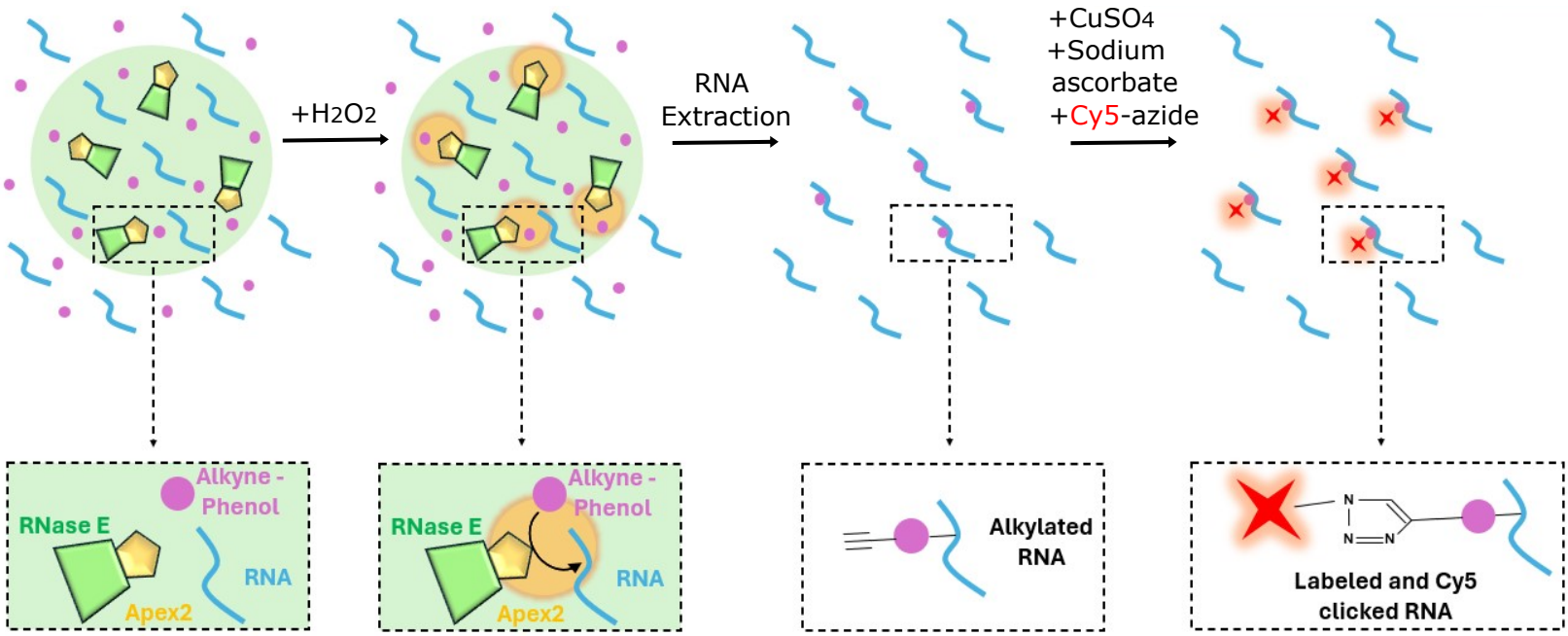
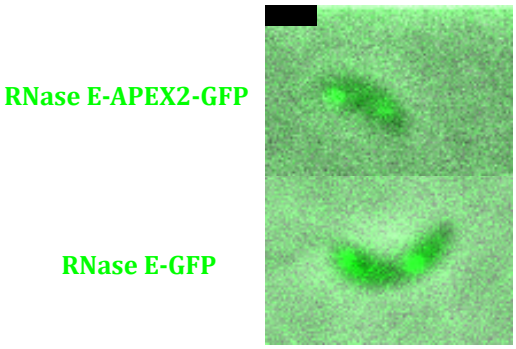
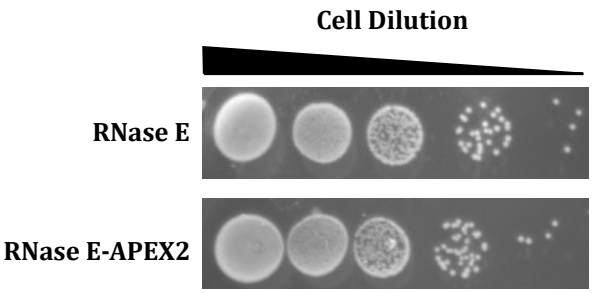
Fig 1

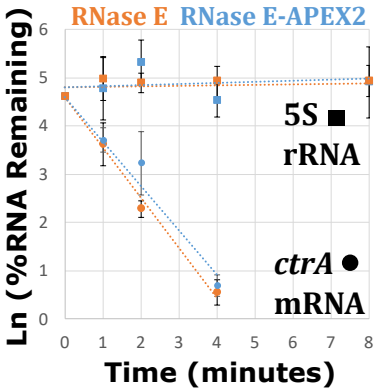
A. Flow Chart of APEX2 Proximity Labeling of RNA



B. APEX2 doesn't impact localization or protein function

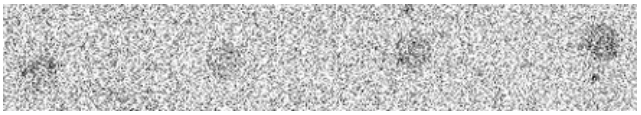


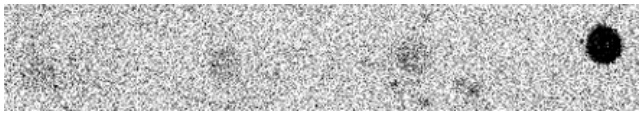




C. APEX2 Proximity Labeling of RNA

APEX2:	–	–	–	–	+	+	+	+
Alk-Ph:	–	+	–	+	–	+	–	+
H_2O_2:	–	–	+	+	–	–	+	+





Mock +RNase A +DNase I

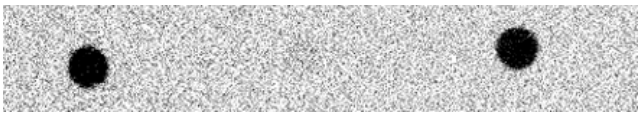


Fig 2

Optimized APEX2 RNA labeling occurs rapidly

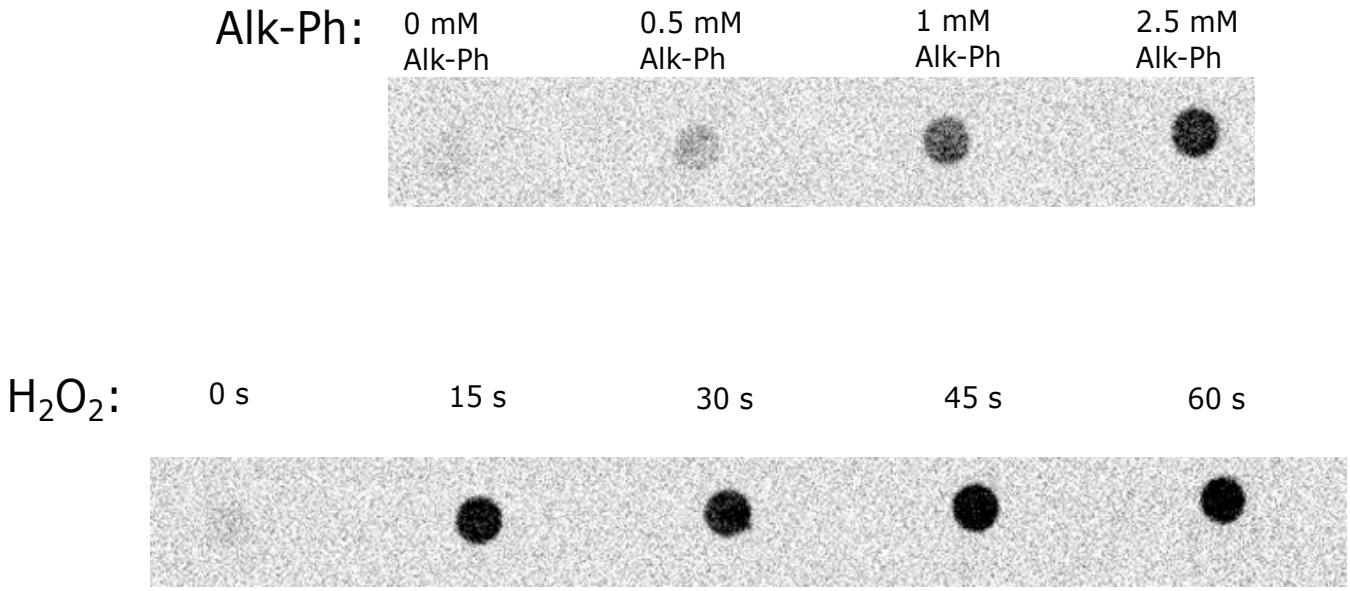
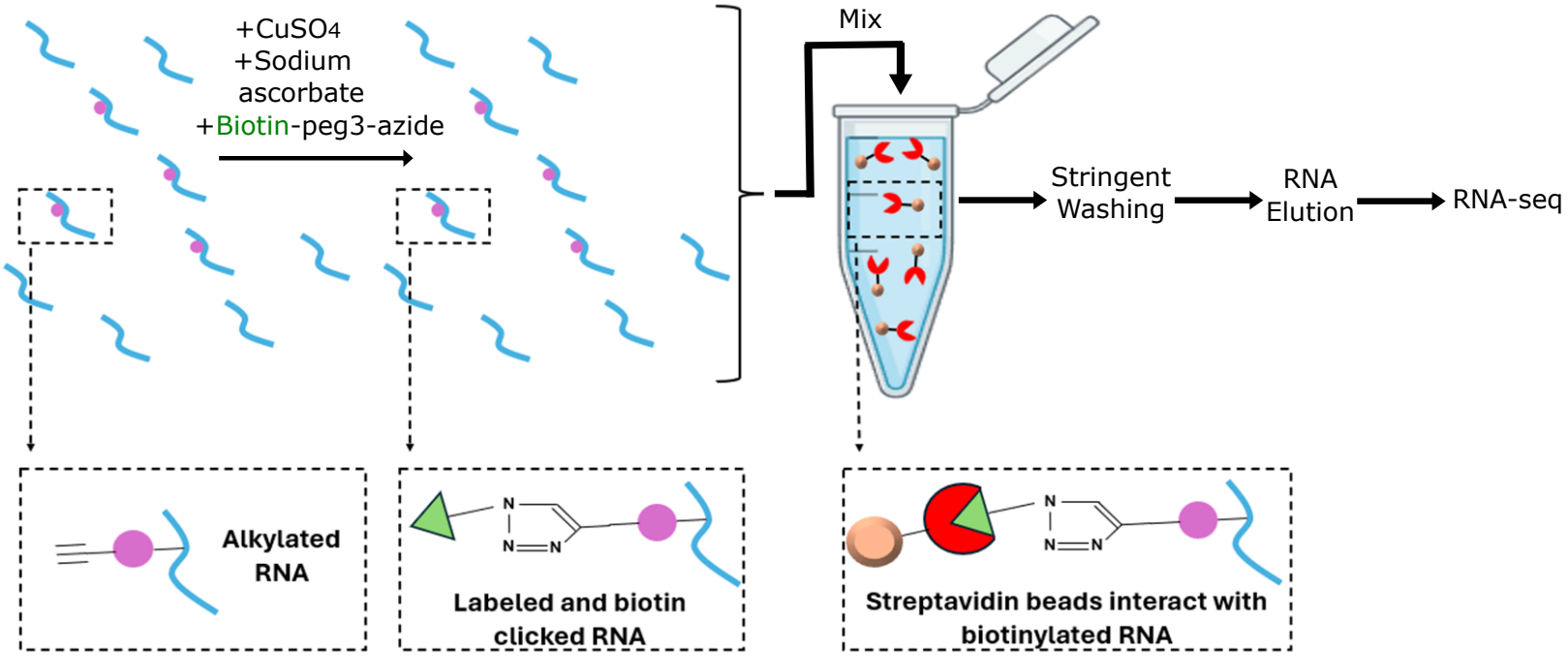


Fig 3

A. APEX2 Proximity Labeling of RNA, clicked with biotin-azide for streptavidin purification



B. Streptavidin purification

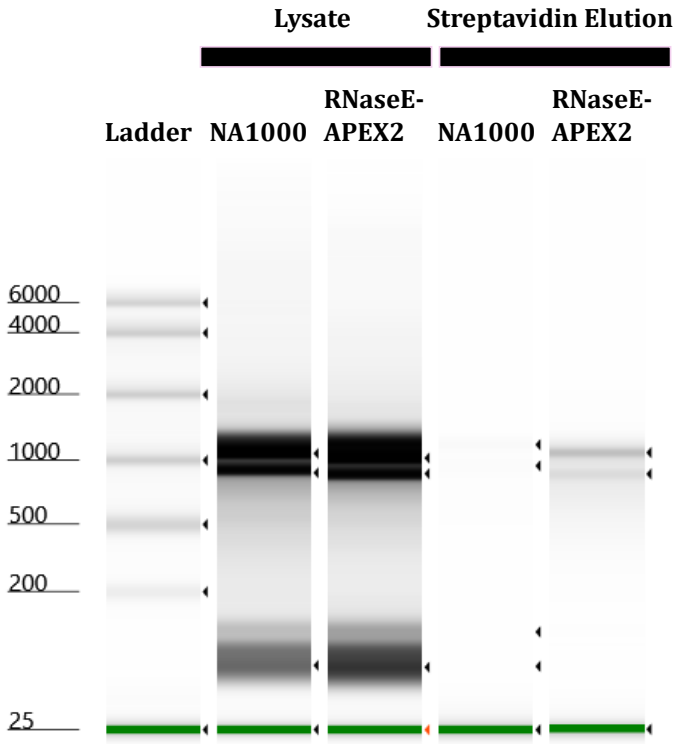


Fig S1

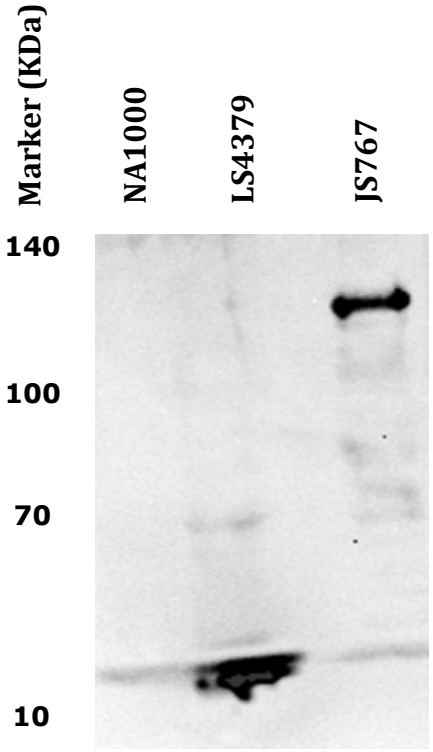
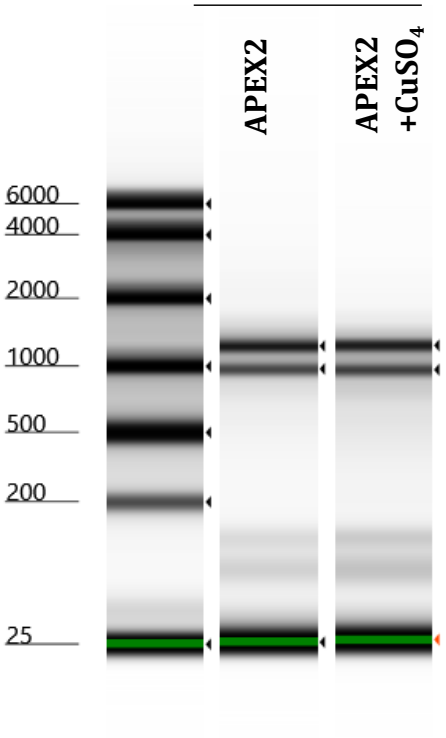


Fig S2

APEX2 Labeled RNA
Click chemistry reactions