Title: Sialic acid aptamer and RNA in situ hybridization-mediated proximity ligation assay (ARPLA) for spatial imaging of glycoRNAs in single cells

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[H1] Abstract:

Glycosylated RNAs (glycoRNAs) have recently emerged as a new class of molecules of significant interest due to their potential roles in cellular processes and diseases. However, studying glycoRNAs is challenging due to the lack of effective research tools, including but not limited to imaging techniques to study the spatial distribution of glycoRNAs. Recently, we reported the development of the first glycoRNA imaging technique, called sialic acid Aptamer and RNA in situ hybridization-mediated Proximity Ligation Assay (ARPLA), to visualize sialic acid-containing glycoRNAs (sialoglycoRNAs) with high sensitivity and specificity. Here, we describe the experimental design principles and detailed step-by-step procedures for ARPLA-assisted glycoRNA imaging across multiple cell types. The procedure includes details for target selection, oligo design and preparation, optimized steps for RNA in situ hybridization (RISH), glycan recognition, proximity ligation, rolling circle amplification (RCA), and a guideline for image acquisition and analysis. With properly designed probe sets and cells prepared, ARPLA-based glycoRNA imaging can typically be completed within 1 day, by users with expertise in biochemistry and fluorescence microscopy. The ARPLA approach enables researchers to explore the spatial distribution, trafficking, and functional contributions of glycoRNAs in various cellular processes.

[H1] Introduction:

Post-transcriptional modifications of RNA molecules have been shown to significantly alter their structural and functional properties, and contribute to diverse cellular processes, including tRNA-mediated translation[1], RNA epigenetics[2], chromatin structure modulation[2], and RNA maturation[3]. The glycosylation of RNAs has recently been characterized as a new form of RNA modifications[4]. This discovery is exciting because cellular glycans play important roles by regulating many essential functions, including cell communications, homeostasis, immunomodulation, and organism development[5]. When RNA undergoes glycosylation, it becomes linked with a diverse array of glycans and is translocated to the external surface of living cells[4, 6, 7]. Interestingly, once localized on the cell membrane, glycosylated RNAs (glycoRNAs) have been demonstrated to interact with cell surface proteins, including human Siglec receptors[4, 8], P-selectin[7], and many other cell surface RNA-binding proteins[9]. These interactions between glycoRNAs and proteins suggest a potential new dimension of surface RNA-mediated signaling pathways, promising to reveal novel functional roles for RNA molecules.

Despite the growing interest and potential significance of the glycoRNAs[4, 10], the tools available for their study remain notably limited. To address the limitations, several methodologies have been developed to identify and characterize glycoRNAs *in vitro* (Table 1). For example, metabolic chemical reporters (MCRs) can be used to label and enrich glycoRNAs, followed by gel blot quantification and next-generation sequencing (NGS)[4]. Alternatively, a solid-phase chemoenzymatic method (SPCgRNA) was developed for oxidization-based native glycoRNA enrichment and sequencing[11]. Meanwhile, a periodate oxidation and aldehyde ligation method (rPAL) was developed for native glycoRNA labeling, detection, and enrichment[12]. Focusing on the glycan moieties in glycoRNAs, mass spectrometry-based technologies were widely applied. For example, high-performance liquid chromatography and mass spectrometry (PGC-LC-MS) was employed on PNGase-F (a glycosidase cleaves intact N-glycan from glycoproteins and glycoRNAs) treated glycoRNA samples, identifying 260 unique glycans from 293T, H9, and HeLa cell lines[4]. Reversed Phase Liquid Chromatography coupled with Tandem Mass Spectrometry (RPLC-MS/MS) strategy was used to investigate glycoRNAs from 12 human organs, discovering 236 unique glycans and revealing the glycan composition heterogeneity among different human organs[13]. The combination of rPAL and MS/MS has been instrumental in identifying the detailed molecular structure of glycan and RNA linkages in glycoRNAs[14, 15]. A Data-Independent Acquisition-based Glycomic Workflow (GlycanDIA) was developed for glycomic analysis and identified the abundance of more than 200 *N*-Glycans on glycoRNAs from different mouse tissues, which were different from the glycan profiles of glycoprotein samples[15].

Despite these advancements, most existing methods focus on isolated glycoRNAs or separate analyses of RNA and glycans, and lack the ability to provide spatial information within cells. The visualization of RNA modifications through imaging remains challenging, particularly for spatially resolving glycoRNAs within their native cellular environments. To address this issue, we developed ARPLA for spatial imaging of glycoRNAs in situ at single cells (Fig. 1)[6]. ARPLA enables the visualization of glycoRNAs within their native cellular contexts, providing critical spatial information and offering insights into their subcellular distributions, changes, and interactions. This method provides unique spatial information in cells and may therefore be useful for researchers who are interested in investigating glycoRNAs in their systems.

Table 1. A summary of methodologies for glycoRNA research *in vitro*.

Methodology	Sample Type	Chemical Moiety to Detect	Detection method	Acquisition Information	References
MCR-based glycoRNA sequencing	Isolated RNA	Sialic acid	NGS	RNA sequences	[4]
SPCgRNA	Isolated RNA	Galactose	NGS	RNA sequences	[11]
rPAL	Isolated RNA	Sialic acid	Gel blot	GlycoRNA quantities	[12, 14]

PGC-LC-MS	Isolated RNA	Glycans	MS/MS	Glycan profiles	[4]
RPLC-MS/MS	Isolated RNA	Glycans	MS/MS	Glycan profiles	[13]
GlycanDIA	Isolated RNA	Glycans	HCD-MS/MS	Glycan profiles	[15]
ARPLA	Fixed cells	Sialic acid and RNA sequences	Imaging	*In situ* spatial distribution	[6]
HieCo 2	Fixed or live cells	Sialic acid and RNA sequences	Imaging	*In situ* spatial distribution	[8]

[H2] Principles of ARPLA

To visualize glycoRNAs, we need to develop imaging probes that can simultaneously recognize both glycan and RNA on the glycoRNAs. To recognize the glycan, we utilize an aptamer that can bind glycans[6]. Aptamers are single-stranded nucleic acids that can fold into unique tertiary structures and bind to their targets with high selectivity and affinity[16-18]. Aptamers have been utilized for detecting various targets, including metal ions[19], small metabolites[20-22], proteins[23, 24], viruses[25], and cells[26,27]. Many aptamers have been developed for the recognition of either monosaccharides in glycans (galactose[28], glucose[28, 29], β-N-acetylglucosamine[30], sialic acid[31-33]), or certain specific forms of the glycans (glycans of RNase b[34], prostate-specific antigen[35], fibrinogen[36], and of other proteins[37-39]). To demonstrate the design of ARPLA, we chose an aptamer that is specific for sialic acid[31] because glycoRNAs have been shown to be highly sialylated, and sialic acid is the terminal sugar moiety of most glycans of glycoRNAs[4, 13, 15]. While a sialic acid-binding aptamer is currently utilized due to the terminal exposure of sialic acid in glycan structures, ARPLA is not inherently restricted to this specific aptamer. The method can be adapted for use with other glycan-binding aptamers[28-39], small molecules like phenylboronic acid[40, 41], proteins (e.g. lectins and antibodies)[42, 43], and metabolic chemical reporters[4, 44] to enable the detection of various glycoRNAs. New aptamers that are specific for different types of glycans can also be selected using Systematic evolution of ligands by exponential enrichment (SELEX)[45, 46]. These adaptations will allow researchers to explore glycoRNAs with different glycan components, providing a comprehensive understanding of glycoRNA biology.

To recognize the RNA moiety of the glycoRNAs, we used a DNA probe similar to those in RNA in situ hybridization (RISH) to specifically hybridize to the RNA component of the glycoRNAs. The dual recognition of the glycan and RNA moieties on the glycoRNAs is achieved by employing both aptamer and RISH through a proximity ligation assay (PLA) so that only specific glycoRNAs are recognized by our probes. Since the glycoRNAs are often very low in abundance, we amplified them by rolling circle amplification (RCA). The amplified glycoRNA sequences can then be imaged by a fluorophore-labeled oligonucleotide that can hybridize with the glycoRNA (Fig. 1).

[H2] Overview of the procedure

An overview of the ARPLA approach is shown (Fig. 1). The workflow involves: a) designing and preparing glycan probe and RISH probe; b) preparing cells for imaging; c) RNA in situ hybridization; d) aptamer-assisted glycan recognition and proximity ligation; e) rolling circle amplification and fluorescent probe staining; f) fluorescence images acquisition; and g) data analysis.

[H2] Applications

ARPLA has proven effective in imaging multiple glycoRNAs across diverse cell models[6] (Fig. 2a,b). To validate the specificity of ARPLA for glycoRNAs, we have carried out a number of controls, including treatments with RNases, glycosidases, and glycosylation inhibitors, which resulted in no observable fluorescent signal[6]. To rule out artifacts of detecting glycans and RNAs on a separate molecule, we designed a DNA probe (anti-Y5 probe) to cover all putative glycosylation sites of Y5 glycoRNA[47] and employed RNase H to digest the RNA section of the DNA/RNA hybrid while keeping the RISH binding sites (Fig. 2c). Additionally, we used poly T oligo as a control probe to ensure that the signals observed were due to specific interactions with glycoRNAs. We then performed RISH-RCA control to detect the RISH binding sites (see Experimental design section) and ARPLA to image Y5 glycoRNAs. As shown in Fig. 2d, after RNase H and anti-Y5 probe incubation, the RISH signals were retained, but ARPLA signals were not generated. These results confirm that the observed ARPLA signals are from intact Y5 glycoRNA and verify the sequence and glycan specificities of ARPLA for glycoRNAs.

When examining the subcellular distributions of glycoRNA, ARPLA showed that glycoRNAs are colocalized with lipid rafts on the cell membrane and are present within lipid vesicles during intracellular trafficking[6] (Fig. 3). This technique has also been applied to investigate glycoRNA abundance variations among different biological models. In a breast cancer model, sialoglycoRNA intensities decreased in the breast cancer cell line (MCF-7) and further in the metastatic cancer cells (MDA-MB-231) compared to healthy breast cells (MCF-10A) (Fig. 4). This result highlighted a unique glycosylation regulatory process of glycoRNA, distinct from other glycoconjugates, where hyper sialylation is known as a hallmark of cancer progression[48]. In an immune cell model, ARPLA revealed reduced surficial sialoglycoRNA intensities during monocyte THP-1 differentiation into macrophages. Conversely, *Escherichia coli*-derived lipopolysaccharide (LPS) stimulation significantly increased glycoRNA signals. In summary, ARPLA is adaptable for a diverse glycoRNA imaging application across various cell models.

[H2] Limitations of ARPLA

The current version of ARPLA has some limitations that can be improved through further optimization. 1) As glycoRNA is typically present in low abundance, the RCA is required to amplify the signal, but the RCA may potentially lead to false positive signals. Carefully design and optimization of the ARPLA probes, including probes for control experiments, as outlined in our experimental design, should be implemented to avoid the false positive signals. 2) While RCA enables signal amplification, it sacrifices resolution. The estimated resolution of ARPLA is ~300 nm using a Zeiss 710 confocal microscopy[6]. When quantifying the RCA amplicon numbers in relatively small cells (such as THP-1 and HL-60), it is difficult to separate individual particles due to the resolution limitation (Fig. 3). 3) ARPLA can provide only a semi-quantitative analysis of glycoRNAs abundance. To improve the resolution and obtain a more quantitative analysis, super-resolution imaging techniques like super-resolution microscopy[49, 50], DNA-based points accumulation for imaging in nanoscale topography (DNA-PAINT)[51], and expansion microscopy[52] can be employed in conjunction with ARPLA staining. 4) ARPLA relies on the sequence information of glycoRNAs, limiting its application for investigating glycoRNAs with unknown sequences. To address this issue, general RNA labeling strategies like anti-RNA antibodies[4] or metabolic chemical reporters[7] for RNA can be introduced to ARPLA for sequence-independent glycoRNA detection. 5) Fixed Cell Limitation: ARPLA is currently optimized for fixed cells to ensure high sensitivity and specificity. The buffer conditions required for RNA in situ hybridization and removal of unbound probes are not suitable for live cell imaging. This limits the ability to study dynamic processes involving glycoRNAs in live cells. Additionally, the fixation process itself may potentially alter the native distribution of glycoRNAs, introducing artifacts or causing the redistribution of molecules. Future work could focus on adapting ARPLA for live cell applications, potentially through the development of milder buffer conditions and real-time imaging techniques. 6) Proximity Limitation: One concern with ARPLA is the potential for false positives arising from the binding of aptamers to glycoproteins in close proximity to glycoRNAs. To minimize the influence of false-positive induced misinterpretation of ARPLA, control experiments (see Experimental Design section) are necessary. Future improvements in glycoRNA-specific recognition strategy could enhance the specificity and overcome the proximity limitations.

[H2] Comparison of ARPLA with other glycoRNA imaging methods

To image glycoRNAs on the surface of cells, several methods have been reported. Initially, a double-stranded RNA (dsRNA) antibody was used to verify the existence of membrane-localized RNAs[53]. Later, a 5′-bromouridine (BrU) was then employed as an MCR for RNAs and achieved cell surface RNA imaging with an anti-BrU antibody[7]. However, neither method can detect glycans. As a result, they cannot differentiate glycoRNAs from other RNAs on the cell surface. In addition, both methods lack sequence specificity and thus cannot distinguish one glycoRNA from another that has a different sequence. To address these issues, we reported ARPLA that provides dual recognition of glycan and RNA as well as glycoRNA with different sequences[6]. Since our report, HieCo 2[8] was also developed to address the same issue (Table 1). Both methods employ RISH to identify glycoRNA sequences, but they differ in their approaches to glycan recognition and signal amplification. ARPLA leverages a sialic acid-specific aptamer to bind glycans, followed by proximity ligation and RCA for signal enhancement. In comparison, HieCo 2 employs metabolic labeling with azido sugars to tag the sialic acids of glycoRNAs, followed by click chemistry to attach DNA probes that can trigger a hybridization chain reaction (HCR) for signal amplification, together with the RISH probes. ARPLA allows for higher spatial resolution in glycoRNA imaging and is applicable to native samples without the need for MCR incubation. On the other hand, HieCo 2 is capable of live-cell imaging without the requirement for cell fixation. Overall, while both methods provide specificity and sensitivity in glycoRNA detection, the choice between ARPLA and HieCo 2 depends on the specific requirements of the study.

[H2] Experimental design

[H3] Oligo design

ARPLA for a specific glycoRNA employs the following three probes and two connectors (Fig. 1, Fig. 5):

1) The glycan probe comprises an aptamer that binds sialic acid specifically, a DNA linker that is complementary to Connectors 1 and 2 as shown in Fig. 1 and a spacer to prevent steric hindrance during hybridization. The spacer length needs to be optimized due to the differences of the space between glycan modification and RNA hybridization site. Generally speaking, a spacer ranging from 8-15 nucleotides allows for flexibility.

The DNA linker should have a melting temperature (Tm) below 28 ºC before ligation and over 50 ºC after ligation. Adding 3 rU bases at the end of the glycan probe can prevent RCA from this strand ensuring that amplification occurs only from the intended RNA strand.

2) The RISH probe includes an antisense region to the targeted glycoRNA, a DNA linker for connectors 1 and 2 that is complementary to Connectors 1 and 2 as shown in Fig. 1, and another spacer to prevent steric hindrance during hybridization. The binding properties, especially the specificity and stability of the antisense probe, should be verified via software, such as NUPACK[54], UNAfold[55], and BLAST[56, 57]. In the current protocol, we use a hybridization buffer containing 250 mM NaCl and 50 mM $MgCl_2$ (see Reagent setup). So, when using the software to calculate the binding properties like the melting temperature, the parameter should be set with the ionic strength as mentioned. The probes should be designed following general RISH probe designing principles[58]: a) 18–25 nucleotide length; b) melting temperature 55–75 °C; c) minimal self-complementarity; d) 40-60 % GC content; e) (optional) modifications that stabilize hybridization and lessen degradation (e.g., locked nucleic acid (LNA) and 2'-O-methyl RNA). The spacer and linker designs should follow the guidelines described in 1) above.
3) Two connectors enable circular DNA formation upon proximity ligation. The ligated product should include two hybridization regions complementary with the two linkers of the glycan probe and the RISH probe. The suggested length for each hybridization region in the circular DNA is 22-28 nucleotides to ensure minimal binding before ligation and strong binding after ligation, while other regions can be customizable with complementary regions for the imager probe.
4) The imager probe, a short oligonucleotide with a fluorophore at either end, should be complementary to a region in the proximity ligation product and not bind to other cell surface RNAs. This design can be verified by performing BLAST with GSE150237[53].

[H3] RNA hybridization

Given that we designed our RISH probes targeting glycoRNAs at their loop or terminal single-stranded regions, we conducted RNA hybridization at 37 ºC. To increase the specificity against RNA, hybridization conditions can be optimized by adding formamide (to ~10%) and raising the hybridization melting temperature to 42-50 ºC, guided by the melting temperature of potential bindings. The use of modified nucleotides like LNAs or 2'-O-methyl RNAs in the antisense region of the RISH probe can enhance stability and specificity, especially for complex RNA structures, reducing the risk of off-target bindings.

[H3] Aptamer-assisted glycan recognition

The aptamer in glycan probe was initially selected for N-acetylneuraminic acid (Neu5Ac)[31]. The binding affinity (K_d) is 91 nM based on our measurement using isothermal titration calorimetry (ITC)[6]. The aptamer binding is buffer-sensitive; thus, the glycan recognition should occur under the same buffer conditions as their Systematic Evolution of Ligands by Exponential Enrichment (SELEX) (50 mM Tris-HCl, 5 mM KCl, 100 mM NaCl, and 1 mM $MgCl_2$ at pH 7.4)[31] as it was characterized. Substituting the sialic acid aptamer with other aptamers that target different sugars or glycoforms is possible, and it requires thorough validation to ensure the aptamer maintains the necessary affinity and specificity for the target glycan after completing the glycan probe through ITC or other biophysical approaches.

[H3] Proximity ligation and RCA

The proximity ligation and RCA are two critical steps in ARPLA for specific and sensitive glycoRNA detection. The simultaneous binding of the glycan and RNA hybridization promotes the connector hybridization, enabling DNA ligation and circular DNA creation. A successful ligation is crucial for amplification and signal enhancement through RCA. RCA uses circular DNA products as templates for DNA polymerase (Phi 29 polymerase), producing long single-stranded DNA with target sequence repeats. Phi 29 polymerase was chosen for the RCA step due to its high processivity, strong strand displacement activity, and ability to amplify circular DNA templates with high fidelity, generating long concatemeric products. An amplified signal is then generated using a fluorophore-conjugated imager probe.

Successful proximity ligation and RCA depend on the efficiency of DNA connector hybridization, accurate DNA ligation, and the enzymatic activity of DNA polymerase. Optimizing these steps, along with control experiments, is critical for reliable and specific glycoRNA detection with ARPLA.

[H3] Controls

Whenever applicable, use the following general controls:

1) A negative control with an inactivated aptamer sequence, achieved with DNA oligo having a scrambled sequence matching the aptamer's composition.
2) A negative control involving a mutated RNA antisense region of RISH probe that will not bind to the target.
   Both controls 1) and 2) are crucial for assessing background signals from either autofluorescence or nonspecific binding-induced proximity ligation and RCA amplification.
3) A positive control featuring a linear proximity connector with a nick at the RISH probe linker region. This serves as an RCA-RISH to evaluate the presence of surface RNAs with the target sequence and the enzymatic activities of proximity ligation and RCA enzymes (Fig. 2 c,d).

[H3] Imaging

For imaging, various microscopes such as wide-field epi-fluorescent microscopes (e.g., Zeiss Observer 7) or confocal microscopes are suitable. For glycoRNAs with unknown or low abundances, start with wide-field epi-fluorescent microscopy with high-sensitivity cameras or confocal microscopes.

Capture 2D images at the focus of the strongest fluorescence or clearest nuclei signal, taking at least 5, usually 10 frames per sample, either randomly or at pre-set x, y positions. Acquire 3D image stacks with z-slices at 0.3 or 0.5 μm steps, then use maximum-intensity projection for 2D images or orthographic projection to display z-scale spatial glycoRNA distributions (Fig. 3).

[H3] Image analysis

To analyze images, we usually perform cell segmentation by using CellPose 2.0 with bright field images to generate ROIs for each individual cell[59, 60]. An enhanced cell segmentation can be achieved by staining the cells with Hoechst and cell indicators such as CellMask. The raw images are then imported into ImageJ (FIJI) to measure mean fluorescence intensity per cell, RCA amplicon quantity and size, and fluorescence intensity of each RCA amplicon using ROIs and particle measurement functions (Fig. 4). Descriptive statistics are obtained using Origin or GraphPad Prism.

[H1] Materials:

[H2] Cell lines

ARPLA is applicable to a variety of cell lines. Cell types we used to produce the data in the current protocol include:

- HeLa (ATCC, Cat. CCL-2)
- HL-60 (ATCC, Cat. CCL-240)
- MCF-7 (ATCC, Cat. HTB-22)
- HEK-293T (ATCC, Cat. CRL-3216)
- THP-1 (obtained from Cancer Center at Illinois; identical cell line can also be purchased from ATCC, Cat. TIB-202)

  CAUTION Perform STR analysis to prevent misidentification and cross-contamination of cell lines.

  CAUTION Additionally, routinely use a mycoplasma testing kit for potential issues arising from bacterial infection.

[H2] Reagents

- Deionized water
- Nuclease-free water (Invitrogen, Cat. AM9932 or similar)
- Sodium chloride (NaCl) (Fisher Scientific, Cat. S271-1)
- Tris base (Millipore Sigma, Cat. 64-831-0500)
- Hydrochloric acid (HCl) (Millipore Sigma, Cat. HX060375)
- Magnesium chloride hexahydrate, $MgCl_2 \cdot 6H_2O$ (Fisher Scientific, Cat. M35-212)
- Potassium chloride, KCl (Fisher Scientific, Cat. P330-3)
- Tri-sodium citrate dihydrate (Fisher Scientific, Cat. S466-3)

[H2] Oligonucleotides

All the oligonucleotide sequences were purchased from Integrated DNA Technologies (IDT) and purified by high-performance liquid chromatography (HPLC). The sequences can be found in Supplementary Table 1.

[H2] Cell culture

- RPMI-1640 cell culture medium (Cytiva HyClone, Cat. SH30255.FS)

- IMDM cell culture medium (Cytiva HyClone, Cat. SH30228.02)
- DMEM cell culture medium (Corning, Cat. 10-013-CM)
- Phorbol 12-myristate 13-acetate (PMA) (CAS: 16561-29-8; Cayman Chem, Cat. 10008014 or similar)
- Fetal bovine serum (FBS; GeminiBio, Cat. 100-106)
- Penicillin-Streptomycin (5,000 U ml^{-1}; Gibco, Cat. 15070063 or similar)
- 100x non-essential amino acids (NEAA; Gibco, Cat. 11140050)
- Trypsin-EDTA (0.05%) (Gibco, Cat. 25300062)
- TrypLE Express Enzyme (Gibco, Cat. 12605010)
- L-Glutamine (200 mM) (Gibco, Cat. A2916801)
- Lipopolysaccharide (LPS) Solution (500X) (eBioscience, Cat. 00-4976-03)

[H2] ARPLA materials

- 35-mm glass bottom dishes, poly-D-Lysine coated (MatTek, Cat. P35GC-1.5-14-C)
- 4% paraformaldehyde (PFA) solution (ThermoScientific, Cat. AAJ19943K2 or similar)
  CAUTION PFA is toxic through skin contact and inhalation. Handle the PFA solution carefully in a chemical hood.
  CAUTION The shelf life of PFA solution is less than 1 month in the fridge once opened.
- 10x Phosphate-Buffered Saline (PBS), pH 7.4, RNase-free (Invitrogen, Cat. AM9624 or similar)
- RNase-free BSA (50 mg ml^{-1}, Invitrogen, Cat. AM2616 or similar)
- ATP solution (10 mM) (NEB, Cat. P0756S)
- T4 DNA ligase (400 U μl^{-1}) (NEB, Cat. M0202S)
- 10x T4 ligation buffer (NEB, Cat. B0202S)
- Phi 29 polymerase (10 U ul^{-1}) (NEB, Cat. M0269S)
- dNTP solution (10 mM) (NEB, Cat. N0447L)
- 10x phi29 DNA polymerase buffer (NEB, Cat. B0269S)
- Poly T oligonucleotides (d(T)$_{20}$) (IDT)
- T-25 cell culture flask (Thermo Fisher, Cat. 130189)
- RNase H (NEB, Cat. M0297S)
- Formamide (Fisher Scientific, Cat. 014835.D6)
  CAUTION Handle formamide solution in a chemical hood.

[H2] Equipment

- Fridge and freezers (4°C, -20°C, and -80 °C)
- Nanodrop (Fisher Scientific, Cat. 13-400-518 or similar)
- 1.5 ml microcentrifuge tube (Fisher Scientific, Cat. 05-408-129)
- 15 ml centrifuge tube, RNase free (Corning, Cat. 430790)
- 50 ml centrifuge tube, RNase free (Corning, Cat. 352070)
- 5 ml serological pipette, RNase free (Fisher Scientific, Cat. 02-923-203)
- 10 ml serological pipette, RNase free (Fisher Scientific, Cat. 02-923-204)
- 10 ul filter tips, RNase free (FroggaBio, Cat. L10F)
- 20 ul filter tips, RNase free (FroggaBio, Cat. L20F)
- 200 ul filter tips, RNase free (FroggaBio, Cat. L200F)

- 1000 ul filter tips, RNase free (Corning, Cat. MRF-1000XT-L-R-S)
- Parafilm (Fisher Scientific, Cat. 1337416)
- Cover glass (Cardinal Health, Cat. M6045-1A)
- Kimwipe (Millipore sigma, Cat. Z671584)
- Benchtop centrifuge (Thermo Fisher Scientific, Cat. 75007200 or similar)
- Tissue culture flask (VWR, Cat. 10062-872)
- Cell culture humidified incubator, 37 ºC and 5% CO_2 (Fisher Scientific, Cat. 51030414 or similar)
- Cell counter (Thermo Fisher Scientific, Cat. C10283)
- Basic Inverted Microscope (VWR, Cat. 76317-470 or similar)
- Confocal microscope (Nikon W1 spinning disk confocal microscope and ZEISS 710 laser scanning microscope, or similar)
- 0.22-micron acetate filter, 47 mm (Millipore sigma, Cat. GPWP04700)
- 0.22-micron acetate filter, 25 mm (Millipore sigma, Cat. SLMP025SS)
- pH meter (e.g., Fisher Scientific, Cat. AB315ACERT)

[H2] Software

- UNAfold: http://www.unafold.org/
- NUPACK: https://nupack.org/
- IDT oligo analyzer: https://www.idtdna.com/pages/tools/oligoanalyzer
- BLAST: https://blast.ncbi.nlm.nih.gov/blast/Blast.cgi
- CellPose 2.0: https://cellpose.readthedocs.io/en/latest/#
- Image J (FIJI): https://imagej.net/software/fiji/
- ZEN lite 3.8: https://www.zeiss.com/microscopy/en/products/software/zeiss-zen-lite.html
- Nikon NIS Element Viewer: https://www.microscope.healthcare.nikon.com/products/software/nis-elements/viewer
- OriginLab: https://www.originlab.com/Origin
- GraphPad Prism: https://www.graphpad.com/
- ox DNA: https://dna.physics.ox.ac.uk/index.php/Main_Page

[H2] Reagent setup (buffer preparation)

- **1M Tris-HCl solution (pH 7.5)**
  Weight 121.14 g of Tris base and add approximately 800 ml nuclease free water. Carefully add concentrated HCl to adjust pH to 7.4 by monitoring with a pH meter. Adjust the volume to 1 L with nuclease free water and mix thoroughly. Filter-sterilize the solution using a 0.22 µm filter. Store the 1M Tris-HCl solution at room temperature for up to one year. For longer storage, keep it at 4°C.
- **0.5M $MgCl_2$ solution**
  Dissolve 10.165 g of magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$) with 100 ml nuclease free water. Filter-sterilize the solution using a 0.22 µm filter. Store the 0.5 M $MgCl_2$ solution at room temperature for less than 6 months. For longer storage, keep it at 4°C.
- **1M NaCl solution**
  Dissolve 58.44 g of sodium chloride (NaCl) in 1 L of deionized water. Filter-sterilize the solution using a 0.22 µm filter. Store the 1 M NaCl solution at room temperature for up to one year.
- **3M KCl solution**

Dissolve 223.8 g of potassium chloride (KCl) in 1 L of nuclease free water. Filter-sterilize the solution using a 0.22 μm filter. Store the 3 M KCl solution at room temperature for up to one year.

- **BSA solution** (10 μg $μl^{-1}$)
  Dilute RNase-free BSA stock solution (50 mg ml^{-1}) with nuclease free water to make it to 10 ug ul^{-1} by mixing 1 volume of BSA stock solution with 4 volumes of nuclease free water. Aliquot and store it in the freezer (-20 ℃) for 1 year. Avoid repeated freeze-thaw cycles.
- **20x saline-sodium citrate (SSC) buffer**
  Dissolve 175.3 g NaCl and 88.3 g tri-sodium citrate to a final volume of 1 L with nuclease free water. Filter-sterilize the solution using a 0.22 μm filter. It can be stored at room temperature for up to 1 year.
- **2x SSC buffer**
  Dilute 20x SSC buffer to 2x SSC buffer with nuclease free water, 1 volume 20x SSC with 9 volume nuclease free water. Filter-sterilize the solution using a 0.22 μm filter.
- **1x PBS**
  Dilute 1 volume of RNase-free 10x PBS with 9 volumes of nuclease free water to make 1x PBS solution.
- **10x hybridization buffer**
  Add 0.4 ml $MgCl_2$ solution (0.5 M) and 0.6 ml nuclease free water to 1 ml Tris-HCl solution (1M, pH 7.5). The final concentrations are 500 mM Tris-HCl and 100 mM $MgCl_2$. Store in aliquots at −20 °C; the shelf life is around 3 months. Avoid repeated freeze–thaw cycles.
- **2x glycan binding buffer**
  Add 5 ml Tris-HCl solution (1 M, pH 7.5), 0.2 ml $MgCl_2$ solution (0.5 M), 10 ml NaCl solution (1 M), and 0.167 ml KCl solution (3 M) to 34.633 ml nuclease free water to make 50 ml 2x glycan binding buffer. The final concentrations are 100 mM Tris-HCl, 10 mM KCl, 200 mM NaCl, and 2 mM $MgCl_2$ at pH 7.4. Store in the fridge (4-8 °C) for up to 6 months.
- **Blocking buffer**
  For each imaging dish, prepare 100 μl blocking buffer, which contains 10 μl 10x hybridization buffer, 2.5 μl BSA solution (10 μg $μl^{-1}$), 2 μl poly T oligo (stock in 20 μM) into 85.5 μl nuclease free water. Prepare the blocking buffer freshly every time before the experiment.
- **1x RNA hybridization buffer**
  RNA hybridization buffer contains 1.5 μM RISH probe, 0.25 μg $μl^{-1}$ BSA, and 250 mM NaCl in 1x hybridization buffer. For each imaging dish, prepare 100 μl 1x RNA hybridization buffer by adding 2.5 μl BSA solution (10 μg $μl^{-1}$), 25 μl NaCl solution (1 M), 10 μl 10x hybridization buffer, and 3 μl RISH probe (50 μM) to 59.5 μl nuclease free water. Prepare freshly every time before use.
- **Hybridization washing buffer.**
  Mix 10% (v/v) formamide solution in 2X SSC. Exercise caution when handling formamide. Prepare it fresh before use.
- **1x glycan and connector solution**
  Glycan and connector solution containing 100 nM glycan probe, 0.25 μg $μl^{-1}$ BSA, 100 nM poly T oligo, 125 nM connector 1, 125 nM connector 2 in 1x glycan binding buffer. For each imaging dish, prepare 100 μl 1x glycan and connector solution by adding 50 μl 2x glycan binding buffer, 1 μl glycan probe, 1.25 μl Connector1, 1.25 μl Connector2, 2.5 μl BSA solution (10 μg $μl^{-1}$), and 2 μl poly T oligo (stock in 20 μM) to 42 μl nuclease free water. Prepare fresh every time before use.

- **T4 ligase solution**
  For each imaging dish, prepare T4 ligation solution by adding 0.25 μl T4 DNA ligase (400 U μl^{-1}), 1 μl ATP solution (100 mM), and 10 μl 10x T4 ligation buffer into 90 μl 1x glycan binding buffer. Prepare fresh on ice every time before use.
- **RCA working solution**
  RCA working solution contains 2.5 U μl^{-1} phi29 DNA polymerase, 0.25 mM dNTP, 0.2 $\mu g\ \mu l^{-1}$ BSA, 5% (vol/vol) glycerol, and 1× phi29 DNA polymerase reaction buffer. For each imaging dish, prepare 100 μl RCA working solution by mixing 2.5 μl Phi 29 polymerase (10 U ul^{-1}), 2.5 μl dNTP solution (10 mM), 2.5 μl BSA solution (10 $\mu g\ \mu l^{-1}$), 10 μl Glycerol (50%, vol/vol), 10 μl 10x RCA buffer, and 73 μl nuclease free water.
- **Probe hybridization buffer**
  The probe hybridization buffer contains 100 nM of imager probe, 0.2 $\mu g\ \mu l^{-1}$ BSA, 100 nM poly T oligo in 2x SSC buffer. For each imaging dish, prepare 100 μl to use. Add 1 μl imager probe (10 μM), 10 μl 20x SSC buffer, 2 μl 10x blocking reagent, and 84.5 μl nuclease free water.

[H1] Procedure:

[H2] Target selection and oligo preparation Timing: several days to a few weeks, depending on the experimental design.

1. Verify the target glycoRNA by referring to the glycoRNA sequencing database (e.g., GSE136967 and SPCgRNA-seq[11]) to confirm the exact sequences.
2. Use UNAFold or NUPACK or find literatures to understand the secondary structures of the RNA target. Design a RISH probe with an antisense sequence that targets regions of the RNA expected to be single-stranded or become single-stranded under mild denaturing conditions.
3. Analyze hybridization properties of the designed probe with glycoRNA using UNAfold, NUPACK, or IDT oligo analyzer.
4. Design the RISH probe for ARPLA by combing the antisense probe designed in steps 1-3, a spacer DNA (see Supplementary Table 1), and a linker DNA to bridge connector gaps between connector 1 and connector 2 (see Supplement Table 1). Check the full RISH probe with UNAfold or NUPACK to avoid self-hybridization.
5. Design the glycan probe, including the sialic acid aptamer, a spacer, and a complementary DNA linker to bridge connector gaps (see Supplement Table 1).
6. Design connector 1 and connector 2 to hybridize with linkers in the RISH probe and glycan probe, enabling in situ ligation for circular DNA generation.
7. Design a reporter probe complementary to the RCA product, conjugated with a fluorophore (e.g., Alexa 647) for glycoRNA visualization.
8. Order the DNA oligos designed in steps 1-7 from IDT with HPLC purification.
9. Dissolve the DNA oligos in nuclease free water at 150 μM (per the manufacturer's information), check concentration with nanodrop, and adjust to 100 μM with nuclease free water.
   PAUSE POINT Oligo orders may take several days to weeks for synthesis and can be stored at -20 °C for years.
   ? Troubleshooting

[H2] Cell culture, plating, and fixation Timing: ~ 1 to 3 d, depending on the cell types and experiment aims.

CRITICAL This protocol focuses on performing ARPLA on fixed cells.

CRITICAL To avoid RNase contamination, after cell fixation, all the materials should be prepared with RNase-free water.

10. Cells are cultured at 37 °C in a humidified incubator with 5% CO_2. HeLa and HEK293T are cultured in DMEM cell culture medium supplemented with 10% FBS and 100 U ml^{-1} penicillin-streptomycin. MCF-7 is cultured in DMEM with 10% FBS, 100 U ml^{-1} penicillin-streptomycin, and 1x NEAA. HL-60 is cultured in IMDM medium supplemented with 20% FBS, 1× GlutaMAX, and 1× MEM NEAA. THP-1 is cultured in RPMI-1640 medium supplemented with 2.5 mM glutamine, 1× MEM NEAA, and 10% heat-inactivated FBS. To differentiate THP-1 cells into macrophage-like cells (M0), THP-1 is treated with 250 nM PMA in its culture medium for 24-48 h until its attachment to the dish and then rest in RPMI-1640 medium containing 5% FBS for another 2 d. To activate M0 macrophages, macrophages were incubated in the serum-free RPMI-1640 medium supplemented with 12.5 μg ml^{-1} LPS overnight.
11. For imaging, we recommend using glass bottom imaging dishes or plates to have a better imaging quality. Here, we use poly-D-lysine coated 35-mm glass-bottom imaging dishes (MatTek) as an example. For attached growing cells, such as HeLa, MCF-7, and HEK293T, use 0.05% trypsin or TrypLE to detach the cells. Then, seed 0.4 x 10^6 cells per dish in 2 ml complete culture medium (around 30% confluent).

    CRITICAL Trypsin may contain RNases. After trypsin digestion, it is advisable to culture the cells for longer than 12 h to allow for recovery of surface glycoRNA.
12. Allow the cells to grow to the desired imaging confluency in the incubator, normally around 60%. This may take 1-2 days.

    CRITICAL STEP Avoid seeding the cells and growing the cells into a high confluent (>70%). High density of cells would make it hard to image the cell edges.
13. For floating cells like THP-1 and HL-60:
    a. plate the cells on the imaging dishes:
        i) Pellet the cells by centrifuge at 500 g for 5 min, wash with 1x PBS twice, and spin at 500 g for 5 min again. Resuspend the cells in serum-free RPMI at the density of 0.4 x 106 cells per ml.
        ii) Add 2 ml (0.8 x 10^6 cells) THP-1 or HL-60 cell suspension to each imaging dish.
        iii) Allow the cells to attach for 1-2 h in the incubator.
        iv) Wash with 1x PBS once to remove unbounded cells.
14. (optional) RNase treatment: incubate live cells with 0.02 μg μl^{-1} RNase A or 1 U μl^{-1} RNase T_1, in 100 μl of HBSS at 37 °C for 20 min. Wash twice with PBS.
15. Prepare a 4% PFA solution (vol/vol). Commercial PFA solution can be stored in the fridge (4 °C) for more than 1 year before opening. However, after opening, its shelf life would be around 1 month when stored in the fridge.

    CAUTION Handle PFA solution carefully, wear appropriate personal protective equipment (PPE), and work under a hood.

    CRITICAL STEP The shelf life of the PFA solution is short (~1 month at 4°C) after opening. We recommend checking the pH of the PFA solution before use, if the pH is out of the range of 6.9-7.4, a new PFA solution should be used.
16. For all the cells, wash the cells with 2 ml 1x PBS once and replace PBS with 2 ml 4% PFA solution.
17. Incubate cells with 4% PFA solution for 15 min at room temperature.

CRITICAL STEP Inadequate incubation of the PFA solution can result in insufficient fixation and RNase inactivation. Conversely, extensive incubation can lead to membrane permeabilization.

18. Aspirate PFA solution and wash the cells 3 times with 2 ml 1x PBS.

    CRITICAL STEP Starting from this step, make sure to use RNase-free 1x PBS to avoid RNA degradation or add SUPERase inhibitor at 0.1 U μl^{-1}.

? Troubleshooting

[H2] In situ hybridization Timing: ~3 h

CRITICAL When using 35-mm glass bottom dishes, apply 100 µl of the solution for each incubation to cover the inner well only.
CRITICAL Assigning specific dishes as negative and positive controls is essential, and the recommended control types can be found in the experimental design section.
CRITICAL The design of RISH probe would significantly influence the in-situ hybridization conditions, for those antisense probes that have other potential unwanted targets, the optimization of hybridization conditions is needed.

19. Prepare 100 µl blocking buffer for each imaging dish. (see Reagent setup)
20. Add 100 µl blocking buffer to each well and incubate at 37 °C for 30-60 min.
21. Aspirate the blocking buffer and wash once with 1 ml 1x PBS.
22. Prepare 100 µl per dish RNA hybridization buffer which contains RISH probe. (see Reagent setup)
23. Add 100 µl RNA hybridization buffer to each well and incubate at 37 °C for 60 min.

    CRITICAL RNA hybridization conditions differ depending on the target transcript secondary structures and antisense region of RISH probe designs. Optimizations of hybridization temperatures ranging from 37-50 ºC and formamide concentrations ranging from 0-40 % are recommended.

24. Wash the cells with 2 ml hybridization washing buffer, which contains 2x SSC solution and 10 % formamide, by incubating the cells with the washing buffer for 20 min with slow shaking. Repeat this washing step 3 times.

    CRITICAL STEP Avoid drying the cells during buffer exchanges, which will cause false positive signals.

    CAUTION Formamide is dangerous. Handle and prepare formamide solutions under a chemical hood.

25. Aspirate the hybridization washing buffer and wash quickly with 1x PBS 3 times to remove residual formamide.

[H2] Glycan recognition and proximity-assisted *in situ* ligation Timing: ~1.5 h.

CRITICAL It is important to check the quality of the glycan probe after receiving it from IDT. We recommend running a denaturing PAGE gel to check the purity and synthesized length. If the purity of the glycan probe is a concern, we recommend doing PAGE purification.
CRITICAL The secondary structure of aptamer in the glycan probe is essential. To avoid unwanted structures, it is ideal to incubate the glycan probe at 85 °C for 5 min and transfer it to ice immediately.

26. Prepare 100 µl 1x glycan and connector solution for each imaging dish, which contains 100 nM glycan probe. (see Reagent setup)

27. Incubate the cells with 100 µl per well 1x glycan and connector solution for 30 min at 30 °C.
28. Aspirate the glycan and connector solution and add in 2 ml 1x glycan binding buffer for washing once to remove unbound glycan probes and connectors.
    CRITICAL STEP The binding between the glycan probe and glycan is dynamic. To avoid disrupting this equilibrium, we recommend limiting washing to no more than once.
29. Add 100 µl T4 ligase solution to each well and incubate at 30 °C for 30 min.
    CRITICAL STEP Check all the components of the T4 ligation buffers. Make the solution fresh before use. Pipette carefully and change tips when adding the buffer to different wells.
30. Wash twice with 1x PBS after incubation.

? Troubleshooting

[H2] Rolling Circle Amplification and labeling Timing: ~ 3.5 h.

31. Prepare 100 µl RCA working solution, which contains 2.5 U μl^{-1} Phi 29 DNA polymerase for each well. (See Reagent setup)
32. Incubate the cells with 100 µl per well RCA working solution at 37 °C for 90 min.
    CRITICAL STEP For semi-quantitative comparison among groups, ensure that the RCA reaction time is consistent across all groups.
    CRITICAL STEP RCA reaction time could be extended to overnight incubation.
    CRITICAL STEP For long-time RCA reaction, to avoid volume loss, wrap the dishes with parafilm.
33. Wash the wells with 2 ml PBS 3 times.

? Troubleshooting

34. Prepare probe hybridization buffer, which contains 100 nM imager probes, 100 µl for each well.
35. Add probe hybridization buffer to each well and incubate at 37 °C for 30 min.
36. Aspirate probe hybridization buffer and wash with 2 ml 2x SSC buffer twice, 5 min each time, at room temperature with slow shaking.
37. Wash once with 2 ml 1x PBS for 5 min, at room temperature with slow shaking.
38. (optional) Stain the cells with Hoechst solution for 10 min at room temperature, and then wash twice with 2ml 1x PBS.
39. Replace the PBS with the mounting medium progressively.
40. Store the dishes at 4 °C and keep the dishes away from the light.
    PAUSE POINT The sample is stable for several days at 4 °C in a mounting medium.

[H2] Imaging Timing: ~2 to 6 h, depending on the experiment designs

CRITICAL The image details are highly dependent on the microscope such as the sensitivity, the image quality, and the resolution. Therefore, choose a microscope based on your experimental aims. We recommend using a laser-scanning confocal microscope or a spinning disk confocal microscope for both highly sensitive and high-resolution imaging.

41. Use a confocal microscope (e.g., Nikon W1 spinning-disk microscope or ZEISS 710 laser-scanning microscope) with a 60x water immersion objective or a 63x oil immersion

objective for imaging cells to ensure a high resolution of subcellular distributions of glycoRNAs on the membrane.

42. Image the glycoRNAs with the filter sets correlated with the fluorophore conjugated to the probe (e.g., 640-nm laser and Cy5 filter for Alexas 647-conjugated probe) and acquire bright field image with DIC. Take single-layer images with a 1024 x 1024-pixel size or 512 x 512 region-of-interest (ROI) for data analysis.

? Troubleshooting

43. Take at least 5 frames randomly in each dish.
44. (optional) Acquire nuclei image with a 408 nm laser and BFP filter or similar.
45. (optional) Acquire z-stack images for single cells with a 512 x 512-pixel size area (x, y) and 0.3 μm z-axis steps for around 35 slices.

    PAUSE POINT After imaging acquisition, the time is flexible for data analysis.

[H2] Image analysis Timing: ~6 h to 1 d, depending on the sample sizes.

CRITICAL There are many analysis pipelines available for detecting and quantifying RCA amplicons in various samples. Here, we introduced a straightforward and user-friendly analysis pipeline suitable for those with limited bioinformatics skills.

46. Import all the images to the software that can segment cells into single-cell ROIs (e.g., CellPose 2.0[59]) to segment the images and generate a mask containing ROIs for each cell.
47. Import the cell masks to FIJI and record the ROI information to the ROI manager.
48. Import the raw images to FIJI. Measure the mean fluorescent intensity of individual cells using the segmented mask image-generated ROIs.
49. Change the image to 8-bit, adjust the threshold, and measure particles in FIJI to record the RCA particle counts and sizes.
50. Import the particle measurement data into the ROI manager. Measure the raw image to determine the fluorescent intensities of each RCA amplicon.
51. (optional) Import the z-stack image into ZEN lite or NIS Element Viewer, then create a maximum intensity projection.

[H1] Troubleshooting:

Troubleshooting information can be found in Table 2.

Table 2: Troubleshooting table

Step	Problem	Possible reason	Solution
9	The nanodrop curve is inaccurate.	Low quality of oligos	Check the MS spectrum from IDT and purchase a new batch of oligos or perform a PAGE purification in the lab as the protocol described elsewhere[61].

18	Cells are washed away.	Fixation failed.	Check the pH of the 4% PFA solution, if the pH is out of the range of 6.9 – 7.4, buy a new batch of 4% PFA solution. Always keep 4% PFA solution at 4 ºC and can be used for up to 1 month.
30	Ligation fails	The glycan probe is washed away at step 28.	Try without washing; directly add T4 ligase and T4 enzyme buffer to the glycan and connector solution
		High DNA concentration impairs DNA circulation	Optimize the connectors and probe concentrations.
		Low quality of T4 ligation buffer.	Check the ligation buffer to ensure the ATP and Mg^{2+} are included in the buffer. The ATP in the buffer is easily degraded after long time storage or freeze-thaw cycles. Order new ligation buffer or supplement with ATP.
		High salt or EDTA may interfere with T4 DNA ligase.	The DNA should be cleaned up with PAGE purification and dissolved in nuclease free water.
		Connectors lack phosphates	Order DNA with phosphates or phosphorylate connectors with a PNK kit.
		The temperature is too high at 30°C.	Do ligation at 16 ºC overnight.
		Ligase is inactive	Purchase a new ligase kit. Always keep the enzyme in -20 ºC freezer and avoid freeze-thaw cycles.
33	RCA fails	The RCA reaction is not highly efficient.	Replace the current vial of Phi 29 DNA polymerase with a new one.
			Change a new vial of DNA polymerase buffer, the DTT may become bad after storage.
			The optimal reaction temperature range is 37 to 42 °C. Check the reaction temperature and run RCA at a higher temperature.
42	No or low fluorescence signals	Identification of the cause of no or low fluorescence signals.	1. Test RCA steps by generating circular DNA with connectors and probes in a test tube. Apply circular DNA directly at step 26 without the addition of the glycan probe and skip to step 31 for RCA. If the RCA amplicons are not obtained, refer to the solution for RCA Fails above. 2. If RCA amplicons are detectable, then test proximity ligation by

			performing control 3), a positive control with only one nick site at the RNA hybridization probe linker region (see Experimental design section). If no fluorescence signal is observed, refer to the solution for Ligation Fails above. If the fluorescence signal is detectable, focus on the troubleshooting steps below.
		The Glycan probe is not pure.	The purity of aptamer affects the binding performance. Do a PAGE purification of the glycan probe to remove unpurified fragments.
		RNase contamination	Prepare new buffers with nuclease-free water and purchase new SUPERasein.
		Trypsin may be contaminated with RNase.	After trypsin treatment, recover the cells for longer than 24h to ensure the recovery of surface glycoRNAs. Or use TrypLE, which is a recombinant protein, to seed the cells.
		Not enough fixation	Check the pH of the 4% PFA solution, if the pH is out of the range of 6.9 – 7.4, buy a new batch of 4% PFA solution. Always keep 4% PFA solution at 4 ºC and can be used for up to 1 month.
		RNA hybridization efficiency is low	Optimize RNA hybridization conditions, including temperature (37-50 ºC), formamide concentration in the buffer (0-50 %), and incubation time (can be extended to overnight).
		Ligation fails	See the solution in Troubleshooting for step 30.
		Phi 29 DNA polymerase has lost activity	Purchase a new enzyme. Always keep the enzyme in -20 ºC freezer and avoid freeze-thaw cycles.
42	High background	Samples have dried out during incubation	During long incubation steps, the reaction volume can decrease. Always perform the incubation steps in a humidified incubator, keep the dish caps closed, or use parafilm to seal the dish.
		Low blocking efficiency	Optimize the blocking conditions (e.g., longer blocking time, higher concentration of BSA and poly T oligo).
		Auto-fluorescent objectives in the sample	Check with other filter sets to see if there are also strong backgrounds, if

			yes, the sample may have strong autofluorescence. Add autofluorescence eliminator reagent (Millipore Sigma, Cat. 2160 or similar).
		Glycan probe concentration too high	Titrate the glycan probe concentration.

Timing:

Step 1-9, target selection and oligo preparation: several days to weeks, depending on the experimental design.

Step 10-14, cell preparation: ~ 1d to 3d, depending on the cell types and experiment aims.

Step 15-18, cell fixation: ~30 min.

Step 19-25, in situ hybridization: ~3 h.

Step 26-30, glycan recognition and proximity ligation: ~1.5 h.

Step 31-40, rolling circle amplification and labeling: ~3.5 h.

Step 41-45, imaging: ~2 h to 6 h, depending on the experiment designs.

Step 46-51, image analysis: ~6h to 1d, depending on the sample sizes.

Anticipated results:

This protocol provides detailed steps for glycoRNA imaging, covering probe design and purification, cell preparation, cell fixation, RNA targeting, proximity ligation, and rolling circle amplification. Fluorescent signals are then observed in 2D or 3D images (Figure. 2,3,4). These images enable analysis of glycoRNA subcellular localization, semi-quantitative assessment of glycoRNA expression in single cells, exploration of glycoRNA heterogeneity during cellular processes (e.g., THP-1 differentiation and activation), and comparison of glycoRNA abundance across cell types.

ARPLA is compatible with imaging methods for other biomolecules using chemical stains or antibody-based immunofluorescence. For example, we co-stained lipid raft with cholera toxin subunit B (CT-B), demonstrating colocalization with U1 glycoRNA with lipid rafts on the cell membrane[6] (Fig. 3). Intracellular proteins can also be stained with antibodies alongside ARPLA. We stained soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNARE) proteins with antibodies and showed the distribution of glycoRNA intracellularly within SNARE protein-wrapped lipid vesicles[6]. Combining ARPLA with other labeling methods expands its utility for studying glycoRNA interactions with other biomolecules.

Reporting summary:

Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability:

The data discussed in the present proposal was generated as part of the original research article. More data can be accessed from the original research article. All the raw data and other example images are available at:

https://figshare.com/projects/Spatial_Imaging_of_GlycoRNA_in_single_Cells_with_ARPLA/164113.

Code availability:

The code generated and used for data analysis during the current study are available at https://figshare.com/projects/Spatial_Imaging_of_GlycoRNA_in_single_Cells_with_ARPLA/164113.

References:

1. Holoch, D.; Moazed, D., RNA-mediated epigenetic regulation of gene expression. *Nat. Rev. Genet.* **2015,** *16* (2), 71-84.
2. Li, Y.; Xia, L.; Tan, K.; Ye, X.; Zuo, Z.; Li, M.; Xiao, R.; Wang, Z.; Liu, X.; Deng, M.; Cui, J.; Yang, M.; Luo, Q.; Liu, S.; Cao, X.; Zhu, H.; Liu, T.; Hu, J.; Shi, J.; Xiao, S.; Xia, L., N(6)-Methyladenosine co-transcriptionally directs the demethylation of histone H3K9me2. *Nat. Genet.* **2020,** *52* (9), 870-877.
3. Yu, S.; Kim, V. N., A tale of non-canonical tails: gene regulation by post-transcriptional RNA tailing. *Nat. Rev. Mol. Cell Biol.* **2020,** *21* (9), 542-556.
4. Flynn, R. A.; Pedram, K.; Malaker, S. A.; Batista, P. J.; Smith, B. A. H.; Johnson, A. G.; George, B. M.; Majzoub, K.; Villalta, P. W.; Carette, J. E.; Bertozzi, C. R., Small RNAs are modified with N-glycans and displayed on the surface of living cells. *Cell* **2021,** *184* (12), 3109-3124.e22.
5. Reily, C.; Stewart, T. J.; Renfrow, M. B.; Novak, J., Glycosylation in health and disease. *Nat Rev Nephrol* **2019,** *15* (6), 346-366.
6. Ma, Y.; Guo, W.; Mou, Q.; Shao, X.; Lyu, M.; Garcia, V.; Kong, L.; Lewis, W.; Ward, C.; Yang, Z.; Pan, X.; Yi, S. S.; Lu, Y., Spatial imaging of glycoRNA in single cells with ARPLA. *Nat. Biotechnol.* **2024,** *42* (4), 608-616.
7. Zhang, N.; Tang, W.; Torres, L.; Wang, X.; Ajaj, Y.; Zhu, L.; Luan, Y.; Zhou, H.; Wang, Y.; Zhang, D.; Kurbatov, V.; Khan, S. A.; Kumar, P.; Hidalgo, A.; Wu, D.; Lu, J., Cell surface RNAs control neutrophil recruitment. *Cell* **2024,** *187* (4), 846-860 e17.
8. Liu, H.; Li, X.; Ren, Y.; Yang, Y.; Chen, Y.; Ju, H., In Situ Visualization of RNA-Specific Sialylation on Living Cell Membranes to Explore N-Glycosylation Sites. *Journal of the American Chemical Society* **2024,** *146* (12), 8780-8786.
9. Perr, J.; Langen, A.; Almahayni, K.; Nestola, G.; Chai, P.; Lebedenko, C.; Volk, R.; Caldwell, R.; Spiekermann, M.; Hemberger, H.; Bisaria, N.; Tzelepis, K.; Calo, E.; Mockl, L.; Zaro, B.; Flynn, R. A., RNA binding proteins and glycoRNAs form domains on the cell surface for cell penetrating peptide entry. *bioRxiv* **2023**, 2023.09.04.556039.
10. Quinn, J. J.; Chang, H. Y., Unique features of long non-coding RNA biogenesis and function. *Nat. Rev. Genet.* **2016,** *17* (1), 47-62.
11. Li, J.; Yue, S.; Gao, Z.; Hu, W.; Liu, Z.; Xu, G.; Wu, Z.; Zhang, X.; Zhang, G.; Qian, F.; Jiang, J.; Yang, S., Novel Approach to Enriching Glycosylated RNAs: Specific Capture of GlycoRNAs via Solid-Phase Chemistry. *Anal. Chem.* **2023,** *95* (32), 11969-11977.
12. Hemberger, H.; Chai, P.; Lebedenko, C. G.; Caldwell, R. M.; George, B. M.; Flynn, R. A., Rapid and sensitive detection of native glycoRNAs. *bioRxiv* **2023**, 2023.02.26.530106.
13. Ming, B.; Zirui, Z.; Tao, W.; Hongwei, L.; Zhixin, T., A draft of human N-glycans of glycoRNA. *bioRxiv* **2023**, 2023.09.18.558371.
14. Yixuan, X.; Helena, H.; Nicholas, A. T.; Peiyuan, C.; Christopher, W.; Charlotta, G. L.; Reese, M. C.; Benson, G.; Carolyn, R. B.; Benjamin, A. G.; Ryan, A. F., The modified RNA base acp3U is an attachment site for N-glycans in glycoRNA. *bioRxiv* **2023**, 2023.11.06.565735.
15. Yixuan, X.; Xingyu, L.; Chenfeng, Z.; Siyu, C.; Shunyang, W.; Zongtao, L.; Faith, M. R.; Benson, M. G.; Ryan, A. F.; Carlito, B. L.; Benjamin, A. G., Development and application of GlycanDIA workflow for glycomic analysis. *bioRxiv* **2024**, 2024.03.12.584702.
16. Ding, Y.; Liu, J., Pushing Adenosine and ATP SELEX for DNA Aptamers with Nanomolar Affinity. *Journal of the American Chemical Society* **2023,** *145* (13), 7540-7547.
17. Keefe, A. D.; Pai, S.; Ellington, A., Aptamers as therapeutics. *Nature Reviews Drug Discovery* **2010,** *9* (7), 537-550.
18. Liu, J.; Cao, Z.; Lu, Y., Functional Nucleic Acid Sensors. *Chem. Rev.* **2009,** *109* (5), 1948-1998.
19. Zhou, W.; Saran, R.; Liu, J., Metal Sensing by DNA. *Chem. Rev.* **2017,** *117* (12), 8272-8325.
20. Harada, K.; Frankel, A. D., Identification of two novel arginine binding DNAs. *The EMBO Journal* **1995,** *14* (23), 5798-5811.

21. Huizenga, D. E.; Szostak, J. W., A DNA Aptamer That Binds Adenosine and ATP. *Biochemistry* **1995,** *34* (2), 656-665.
22. Hong, S.; Zhang, X.; Lake, R. J.; Pawel, G. T.; Guo, Z.; Pei, R.; Lu, Y., A photo-regulated aptamer sensor for spatiotemporally controlled monitoring of ATP in the mitochondria of living cells. *Chemical Science* **2020,** *11* (3), 713-720.
23. Bock, L. C.; Griffin, L. C.; Latham, J. A.; Vermaas, E. H.; Toole, J. J., Selection of single-stranded DNA molecules that bind and inhibit human thrombin. *Nature* **1992,** *355* (6360), 564-566.
24. Lin, Y.; Padmapriya, A.; Morden, K. M.; Jayasena, S. D., Peptide conjugation to an in vitro-selected DNA ligand improves enzyme inhibition. *Proceedings of the National Academy of Sciences* **1995,** *92* (24), 11044-11048.
25. Peinetti Ana, S.; Lake Ryan, J.; Cong, W.; Cooper, L.; Wu, Y.; Ma, Y.; Pawel Gregory, T.; Toimil-Molares María, E.; Trautmann, C.; Rong, L.; Mariñas, B.; Azzaroni, O.; Lu, Y., Direct detection of human adenovirus or SARS-CoV-2 with ability to inform infectivity using DNA aptamer-nanopore sensors. *Science Advances* **2021,** *7* (39), eabh2848.
26. Sefah, K.; Shangguan, D.; Xiong, X.; O'Donoghue, M. B.; Tan, W., Development of DNA aptamers using Cell-SELEX. *Nat. Protoc.* **2010,** *5* (6), 1169-1185.
27. Xue, C.; Zhang, S.; Yu, X.; Hu, S.; Lu, Y.; Wu, Z.-S., Periodically Ordered, Nuclease-Resistant DNA Nanowires Decorated with Cell-Specific Aptamers as Selective Theranostic Agents. *Angew. Chem. Int. Ed.* **2020,** *59* (40), 17540-17547.
28. Yang, K.-A.; Barbu, M.; Halim, M.; Pallavi, P.; Kim, B.; Kolpashchikov, D. M.; Pecic, S.; Taylor, S.; Worgall, T. S.; Stojanovic, M. N., Recognition and sensing of low-epitope targets via ternary complexes with oligonucleotides and synthetic receptors. *Nat. Chem.* **2014,** *6* (11), 1003-1008.
29. Nakatsuka, N.; Yang, K.-A.; Abendroth, J. M.; Cheung, K. M.; Xu, X.; Yang, H.; Zhao, C.; Zhu, B.; Rim, Y. S.; Yang, Y.; Weiss, P. S.; Stojanović, M. N.; Andrews, A. M., Aptamer–field-effect transistors overcome Debye length limitations for small-molecule sensing. *Science* **2018,** *362* (6412), 319-324.
30. Zhu, Y.; Hart, G. W., Dual-specificity RNA aptamers enable manipulation of target-specific O-GlcNAcylation and unveil functions of O-GlcNAc on beta-catenin. *Cell* **2023,** *186* (2), 428-445 e27.
31. Yue, H.; Chen, J.; Chen, X.; Wang, X.; Zhang, Y.; Zhou, N., Systematic screening and optimization of single-stranded DNA aptamer specific for N-acetylneuraminic acid: A comparative study. *Sensors Actuators B: Chem.* **2021,** *344*, 130270.
32. Gong, S.; Ren, H.-L.; Tian, R.-Y.; Lin, C.; Hu, P.; Li, Y.-S.; Liu, Z.-S.; Song, J.; Tang, F.; Zhou, Y.; Li, Z.-H.; Zhang, Y.-Y.; Lu, S.-Y., A novel analytical probe binding to a potential carcinogenic factor of N-glycolylneuraminic acid by SELEX. *Biosens. Bioelectron.* **2013,** *49*, 547-554.
33. Cho, S.; Lee, B.-R.; Cho, B.-K.; Kim, J.-H.; Kim, B.-G., In vitro selection of sialic acid specific RNA aptamer and its application to the rapid sensing of sialic acid modified sugars. *Biotechnol. Bioeng.* **2013,** *110* (3), 905-913.
34. Yoshikawa, A. M.; Rangel, A.; Feagin, T.; Chun, E. M.; Wan, L.; Li, A.; Moeckl, L.; Wu, D.; Eisenstein, M.; Pitteri, S.; Soh, H. T., Discovery of indole-modified aptamers for highly specific recognition of protein glycoforms. *Nature Communications* **2021,** *12* (1), 7106.
35. Díaz-Fernández, A.; Miranda-Castro, R.; de-los-Santos-Álvarez, N.; Rodríguez, E. F.; Lobo-Castañón, M. J., Focusing aptamer selection on the glycan structure of prostate-specific antigen: Toward more specific detection of prostate cancer. *Biosens. Bioelectron.* **2019,** *128*, 83-90.
36. Li, M.; Lin, N.; Huang, Z.; Du, L.; Altier, C.; Fang, H.; Wang, B., Selecting Aptamers for a Glycoprotein through the Incorporation of the Boronic Acid Moiety. *J. Am. Chem. Soc.* **2008,** *130* (38), 12636-12638.
37. Sun, X.; Pan, Q.; Yuan, C.; Wang, Q.; Tang, X.-L.; Ding, K.; Zhou, X.; Zhang, X.-L., A Single ssDNA Aptamer Binding to Mannose-Capped Lipoarabinomannan of Bacillus Calmette–Guérin Enhances Immunoprotective Effect against Tuberculosis. *J. Am. Chem. Soc.* **2016,** *138* (36), 11680-11689.
38. Li, W.; Xu, S.; Li, Y.; Chen, J.; Ma, Y.; Liu, Z., High Mannose-Specific Aptamers for Broad-Spectrum Virus Inhibition and Cancer Targeting. *CCS Chemistry* **2023,** *5* (2), 497-509.

39. Wang, C.-Y.; Wu, C.-Y.; Hung, T.-C.; Wong, C.-H.; Chen, C.-H., Sequence-constructive SELEX: A new strategy for screening DNA aptamer binding to Globo H. *Biochem. Biophys. Res. Commun.* **2014,** *452* (3), 484-489.
40. Melavanki, R.; Kusanur, R.; Sadasivuni, K. K.; Singh, D.; Patil, N. R., Investigation of interaction between boronic acids and sugar: effect of structural change of sugars on binding affinity using steady state and time resolved fluorescence spectroscopy and molecular docking. *Heliyon* **2020,** *6* (10), e05081.
41. Brooks, W. L. A.; Deng, C. C.; Sumerlin, B. S., Structure–Reactivity Relationships in Boronic Acid–Diol Complexation. *ACS Omega* **2018,** *3* (12), 17863-17870.
42. Cummings, R. D.; Etzler, M.; Hahn, M. G.; Darvill, A.; Godula, K.; Woods, R. J.; Mahal, L. K., Glycan-recognizing probes as tools. In *Essentials of Glycobiology, 4th edition*, Cold Spring Harbor (NY), 2022.
43. Lundquist, J. J.; Toone, E. J., The Cluster Glycoside Effect. *Chem. Rev.* **2002,** *102* (2), 555-578.
44. Palaniappan, K. K.; Bertozzi, C. R., Chemical Glycoproteomics. *Chem. Rev.* **2016,** *116* (23), 14277-14306.
45. Ellington, A. D.; Szostak, J. W., In vitro selection of RNA molecules that bind specific ligands. *Nature* **1990,** *346* (6287), 818-822.
46. Tuerk, C.; Gold, L., Systematic Evolution of Ligands by Exponential Enrichment: RNA Ligands to Bacteriophage T4 DNA Polymerase. *Science* **1990,** *249* (4968), 505-510.
47. Flynn, R. A.; Smith, B. A. H.; Johnson, A. G.; Pedram, K.; George, B. M.; Malaker, S. A.; Majzoub, K.; Carette, J. E.; Bertozzi, C. R., Mammalian Y RNAs are modified at discrete guanosine residues with N-glycans. *bioRxiv* **2019**, 787614.
48. Munkley, J.; Elliott, D. J., Hallmarks of glycosylation in cancer. *Oncotarget* **2016,** *7* (23), 35478-89.
49. Rust, M. J.; Bates, M.; Zhuang, X., Sub-diffraction-limit imaging by stochastic optical reconstruction microscopy (STORM). *Nat. Methods* **2006,** *3* (10), 793-796.
50. Schermelleh, L.; Ferrand, A.; Huser, T.; Eggeling, C.; Sauer, M.; Biehlmaier, O.; Drummen, G. P. C., Super-resolution microscopy demystified. *Nat. Cell Biol.* **2019,** *21* (1), 72-84.
51. Jungmann, R.; Avendaño, M. S.; Woehrstein, J. B.; Dai, M.; Shih, W. M.; Yin, P., Multiplexed 3D cellular super-resolution imaging with DNA-PAINT and Exchange-PAINT. *Nat. Methods* **2014,** *11* (3), 313-318.
52. Wassie, A. T.; Zhao, Y.; Boyden, E. S., Expansion microscopy: principles and uses in biological research. *Nat. Methods* **2019,** *16* (1), 33-41.
53. Huang, N.; Fan, X.; Zaleta-Rivera, K.; Nguyen, T. C.; Zhou, J.; Luo, Y.; Gao, J.; Fang, R. H.; Yan, Z.; Chen, Z. B.; Zhang, L.; Zhong, S., Natural display of nuclear-encoded RNA on the cell surface and its impact on cell interaction. *Genome Biol.* **2020,** *21* (1), 225.
54. Zadeh, J. N.; Steenberg, C. D.; Bois, J. S.; Wolfe, B. R.; Pierce, M. B.; Khan, A. R.; Dirks, R. M.; Pierce, N. A., NUPACK: Analysis and design of nucleic acid systems. *J. Comput. Chem.* **2011,** *32* (1), 170-173.
55. Zuker, M., Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Res.* **2003,** *31* (13), 3406-3415.
56. Camacho, C.; Coulouris, G.; Avagyan, V.; Ma, N.; Papadopoulos, J.; Bealer, K.; Madden, T. L., BLAST+: architecture and applications. *BMC Bioinformatics* **2009,** *10* (1), 421.
57. Altschul, S. F.; Madden, T. L.; Schäffer, A. A.; Zhang, J.; Zhang, Z.; Miller, W.; Lipman, D. J., Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* **1997,** *25* (17), 3389-3402.
58. Raj, A.; van den Bogaard, P.; Rifkin, S. A.; van Oudenaarden, A.; Tyagi, S., Imaging individual mRNA molecules using multiple singly labeled probes. *Nat. Methods* **2008,** *5* (10), 877-879.
59. Pachitariu, M.; Stringer, C., Cellpose 2.0: how to train your own model. *Nat. Methods* **2022,** *19* (12), 1634-1641.
60. Stringer, C.; Wang, T.; Michaelos, M.; Pachitariu, M., Cellpose: a generalist algorithm for cellular segmentation. *Nat. Methods* **2021,** *18* (1), 100-106.

61. Lopez-Gomollon, S.; Nicolas, F. E., Chapter Six - Purification of DNA Oligos by Denaturing Polyacrylamide Gel Electrophoresis (PAGE). In *Methods Enzymol.*, Lorsch, J., Ed. Academic Press: 2013; Vol. 529, pp 65-83.

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Contributions:

W.G., Y.M. and Y.L. conceived and designed the study. W.G., Y.M. and Q.M. designed the method. L.K. performed the MD simulation of ARPLA. W.G. and Y.M. performed the experiments and analyzed the data. X.S., V.G., W.L., and Z.Y. assisted in cell experiments. X.S. and M.L. assisted in data analysis. S.L. assisted in manuscript preparation. The manuscript was written by W.G., Y.M. and Y.L.. Y.L. supervised the project.

Ethics declarations:

Competing interests

The authors declare no competing interests.

Additional information:

Figure legends:

Fig. 1: ARPLA Procedure Overview. The workflow begins with probe design and preparation for the target glycoRNA. Subsequent steps include cell culture and preparation, RNA hybridization, aptamer-assisted glycan recognition, proximity ligation, RCA, RCA labeling with an imager probe, image acquisition, and data analysis. This depiction is a simplified schematic representation for illustrating the ARPLA protocol and does not represent the real structure of glycoRNA. The figure was created with BioRender.com.

Fig. 2: GlycoRNA imaging in a variety of cell lines. a) A schematic representation of the U1 RNA structure (predicted by UNAfold) and the design of the RISH binding site for ARPLA (shown in blue). b) Imaging of U1 glycoRNA with ARPLA, illustrating the subcellular localization of U1 glycoRNA in HeLa, SH-SY5Y, PANC-1, HEK293T, HL-60, and THP-1 cell lines. c) A scheme to show the Y5 RNA structure (predicated by UNAfold) and the design of RNase H targeting site (shown in red) and RISH binding site (shown in blue); d) cell surface Y5 RNA imaging with RISH-RCA and Y5 glycoRNA imaging with ARPLA with the treatment of RNase H to remove glycosylation sites, scale bar: 20 μm. Panel b adapted with permission from ref.[6].

Fig. 3: ARPLA images highlighting the spatial subcellular locations of U1 glycoRNA in single HL-60 cells. Z-stack images were collected with the staining of U1 glycoRNA by ARPLA (green) and lipid raft (stained by CT-B, red). The spatial distributions of both U1 glycoRNA and lipid rafts were shown in z-slices format (a), orthographic projection (b), and maximum intensity projection (c). The Pearson's coefficient for the colocalization images shown in Fig. 3 is 0.74 ± 0.11, indicating the colocalization between glycoRNA and lipid rafts. Scale bar: 2 μm. 3D images were processed by using ZEN 3.8. The figures adapted with permission from ref.[6].

Fig. 4: Image analysis of ARPLA in breast cell lines. (a) representative ARPLA images of U1 glycoRNA in MCF-10A, MCF-7, and MDA-MB-231 cells. In the analysis of ARPLA images, the following features can be obtained: Mean fluorescence intensity of individual cells (b) (each signal dot indicates the average mean intensity in each frame, n = 6 frames); RCA amplicon amounts in each cell (n = 20 cells) (c); the sizes of each RCA amplicons (n = 2000 amplicons) (d); and mean fluorescent intensity of each RCA amplicons (n = 2000 amplicons) (e). Bar graph (b) and dot plot (c) are presented as mean values ±SD. The statistical significance are determined by unpaired two-tailed t test (b) as * p = 0.0344, *** p < 0.001, and by one-way ANOVA (c) as n.s. p=0.8913. Violin plots lines at the median (dotted line) and quartiles (dashed lines). Statistic assays are performed with the two-tailed unpaired t-test. In (b), * p=0.0106, *** p<0.0001; In (c), both p<0.0001.

Fig. 5 Molecular dynamic (MD) simulation of the ARPLA design. a) The general design of ARPLA, with different sites (sites 1-6), was chosen to analyze the distances; b) MD simulation of the structure of ARPLA with oxDNA, including 4 oligos: the glycan probe, the RISH probe, the connector 1, and the connector 2. c) the distances between different sites indicated in a) and b). The figures are from ref.[6] adapted with permission.