Detection of Individual RNA in Fixed Cells and Tissues by Chromogenic ISH

Meng Jiang, Chen Lin and Rongqin Ke*

Center for Precision Medicine, School of Biomedical Sciences and School of Medicine, Huaqiao University, Quanzhou, Fujian, 362021, China

*For correspondence: rke@hqu.edu.cn

[Abstract] Visualization of RNA molecules *in situ* helps to better understand the functions of expressed genes. Currently, most conventional *in situ* hybridization methods for visualization of individual RNAs are based on fluorescence detection. Herein we present a chromogenic *in situ* hybridization protocol for visualization of single RNA molecules in fixed cells and tissues. The protocol is based on padlock probing and rolling circle amplification to generate detectable chromogenic signal from single RNA molecules. Chromogenic signal can avoid background autofluorescence and can be preserved for a longer period than fluorescence signal.

Keywords: Chromogenic *in situ* hybridization, Gene expression, Padlock probe, Rolling circle amplification

[Background] The abundance and spatial location of expressed RNA molecules indicates the physiological and pathological status of cells and tissues. Therefore, there is an increasing interest for *in situ* RNA detection. Compared to conventional *in situ* hybridization methods, novel methods that can detect RNA at individual molecule level are more sensitive and specific (Crosetto *et al.*, 2015). These methods include single molecule fluorescence *in situ* hybridization (smFISH) (Femino *et al.*, 1998), methods based on rolling circle amplification (Larsson *et al.*, 2010), hybridization chain reaction (HCR) (Shah *et al.*, 2016), and branched DNA (bDNA) technology (Wang *et al.*, 2012; Battich *et al.*, 2013). Current methods for individual RNA *in situ* detection assays are normally readout as fluorescence signal, which offers good sensitivity and is easy to multiplex. Chromogenic readout is often used in conventional RNA ISH assays, providing stable signal that can be stored for long period of time, and is not affected by autofluorescence or photobleaching. Therefore, we introduce the single molecule chromogenic *in situ* hybridization (smCISH) assay that enables sensitive and specific detection of individual RNA in fixed cells and tissues (Jiang *et al.*, 2019). Our protocol is based on padlock probing and rolling circle amplification (Nilsson *et al.*, 1994; Banér *et al.*, 1998). First, padlock probe is designed to directly bind to target RNA specifically. After padlock probe hybridization, probe circularization is performed using SplintR DNA ligase. The closed circle is RNA-templated ligation of its two ends. Next, an RCA primer is hybridized to the circularized padlock probe to initiate rolling circle amplification reaction. The padlock probe is then amplified into its complementary concatenated form to generate RCP. HRP labeled detection probes are then hybridized to the RCP. Finally, chromogenic signal is developed using H₂O₂ and 3,3'-Diaminobenzidine (DAB) to generate brown insoluble dots from individual RCPs, which

corresponds to single RNA molecules. Our results show that single molecule chromogenic *in situ* hybridization assay can count and localize individual RNA molecules in fixed cells and tissue samples.

Materials and Reagents

1. Secure-Seal hybridization chamber, 9 mm diameter, 0.8 mm deep (Containing seal tabs, Thermo Scientific, catalog number: S24732)
2. EasYFlask 25 cm² (Thermo Scientific Nunc, catalog number: 156340-ROS)
3. Serological Pipette (Thermo Scientific Nunc, 2 ml, 5 ml, 10 ml, 50 ml)
4. Cell culture Dish, 150 mm x 20 mm (Thermo Scientific Nunc, catalog number: 168381-ROS)
5. Adhesion Microscope Slides (Citotest, catalog number: 188105)
6. 15 ml Conical Centrifuge Tubes (Thermo Scientific Nunc, catalog number: 339650-SPL)
7. 50 ml Conical Centrifuge Tubes (Thermo Scientific Nunc, catalog number: 339652-SPL)
8. Filtered Pipette Tip (QSP, 0.1-10 µl, 2-20 µl, 10-100 µl, 20-200 µl, 100-1,000 µl)
9. 0.2 ml PCR Tubes (AXYGEN, catalog number: PCR-02-C)
10. 1.5 ml Microtubes (AXYGEN, catalog number: MCT-150-C)
11. 0.22 µm membrane filter (Millipore, catalog number: SLGP033RS)
12. HER2 padlock probe (sequence: 5'-ATTACTTGCAGGTTCTTTCTTTTACGACCTCAATGCTGCTGCTGTACTACTCTTCAGAATT
CGTCCCCGG, underline: target hybridization sequences and detection probe complementary part, ***RCA primer binding part***) (Synbio Tech, China)
13. ACTB padlock probe (sequence: 5'-CTGTGCTCGCGGGGCGTTCCTTTTACGACCTCAAT
GCTGCTGCTGTACTACTCTTGGCAAAGGCGAGGCT, underline: target hybridization sequences and detection probe complementary part, ***RCA primer binding part***) (Synbio Tech, China)
14. RCA primer (sequence: 5'-ACAGCAGCAGCATTGAGGTC) (Synbio Tech, China)
15. HRP labeled detection probe (sequence: 5' HRP-CCTCAATGCTGCTGCTGTACTAC) (TaKaRa)
16. Pepsin (Sigma-Aldrich, catalog number: P7012-1G), store at -20 °C
17. Fetal Bovine Serum (Hyclone, catalog number: 10270-106), store at -20 °C
18. Trypsin (Hyclone, catalog number: SH30042.01), store at -20 °C
19. ATP, 100 mM Solution (Thermo Scientific, catalog number: R0441), store at -20 °C
20. T4 Polynucleotide Kinase, 10 U/µl (Thermo Scientific, catalog number: EK0032, containing PNK buffer A), store at -20 °C
21. dNTP Set, 100 mM Solutions (Thermo Scientific, catalog number: R0182), store at -20 °C
22. SplintR Ligase, 25 U/µl (NEB, catalog number: M0375L), store at -20 °C
23. phi29 DNA Polymerase, 10 U/µl (Thermo Scientific, catalog number: EP0094), store at -20 °C
24. BSA, 20 mg/ml (NEB, catalog number: B9000S), store at -20 °C
25. RiboLock RNase Inhibitor, 40 U/µl (Thermo Scientific, catalog number: EO0384), store at -20 °C
26. Tween-20 (Sigma-Aldrich, catalog number: P9416-100ML), store at room temperature (RT)

27. Triton X-100 (Sigma-Aldrich, catalog number: T8787-100ML), store at RT
28. NaOH (Sigma-Aldrich, catalog number: 795429-500G), store at RT
29. KCl (Sigma-Aldrich, catalog number: P9541-500G), store at RT
30. NaCl (Sigma-Aldrich, catalog number: S3014-1kG), store at RT
31. Na₂HPO₄ (BBI LIFE SCIENCES, catalog number: A610404-0500), store at RT
32. KH₂PO₄ (Sangon Biotech, catalog number: A100781-0500), store at RT
33. Sodium iodate (BBI LIFE SCIENCES, catalog number: A600859), store at RT
34. Aluminum potassium sulfate dodecahydrate (Sangon Biotech, catalog number: A500755), store at RT
35. Glycerol (Sigma-Aldrich, catalog number: G9012-100ML), store at RT
36. Hematoxylin (Sigma-Aldrich, catalog number: H3136-25G), store at RT
37. Neutral balsam mounting medium (BBI LIFE SCIENCES, catalog number: E675007), store at RT
38. Xylene (XILONG SCIENTIFIC, catalog number: 33535-500ML), store at RT
39. Ethanol (Macklin, catalog number: E809061-500 ML), store at RT
40. Acetate (Macklin, catalog number: A801295-500 ML), store at RT
41. Hydrochloric acid (SCR, catalog number: 10011018), store at RT
42. DEPC (Sigma-Aldrich, catalog number: D5758-25ML), store at 4 °C
43. Paraformaldehyde (Sigma-Aldrich, catalog number: 16005-1KG-R), store at 4 °C
44. Formamide (Sigma-Aldrich, catalog number: F9037-100ML), store at 4 °C
45. 20x SSC buffer (Sigma-Aldrich, catalog number: S6639), store at 4 °C
46. 1x Phosphate Buffered Saline (Hyclone, catalog number: SH30256.01), store at 4 °C
47. DNase/RNase-Free Water (Solarbio, catalog number: R1600), store at 4 °C
48. Hydrogen peroxide solution, 30 wt.% in H₂O (Macklin, catalog number: H811240-500 ml), store at 4 °C
49. DAB Immunohistochemistry Color Development Kit (BBI LIFE SCIENCES, catalog number: EP670033), store at -20 °C
50. EDTA (Sigma-Aldrich, catalog number: RDD017-500G), store at RT
51. Serum-free medium, DMEM/HIGH GLUCOSE (Hyclone, catalog number: SH30022.01), store at 4 °C
52. Penicillin-Streptomycin (Hyclone, catalog number: SV30010), store at -20 °C
53. Culture media (50 ml) (see Recipes)
54. 10x PBS (500 ml), pH 6.8 (see Recipes)
55. 1x DEPC-PBS (1 L), pH 7.4 (see Recipes)
56. 1x DEPC-PBST (1 L) (see Recipes)
57. 4% (w/v) PFA (500 ml) (see Recipes)
58. 1x TE buffer (100 ml), pH 8.0 (see Recipes)
59. 0.5%(v/v) Triton X-100 (5 ml) (see Recipes)
60. 3% (w/v) H₂O₂ solution (50 µl) (see Recipes)

61. 2x hybridization buffer (5 ml) (see Recipes)
62. Washing buffer (15 ml) (see Recipes)
63. 2x detection buffer (5 ml) (see Recipes)
64. Staining buffer (50 μ l) (see Recipes)
65. 0.1% hydrochloric acid-ethanol (5 ml) (see Recipes)
66. Hematoxylin Staining Solution (50 ml) (see Recipes)
67. 25 mM dNTPs (100 μ l) (see Recipes)

Equipment

1. Vortex Mixers (MIULAB, model: MIX-25P)
2. Microcentrifuge (MIULAB, model: Mini-6K)
3. Magnetic stirrer (IKA, RCT basic)
4. pH meter (OHAUS, model: starter3100)
5. PCR machine (BIOER, model: TC-XP)
6. Analytical balance (OHAUS, model: AS220.R2)
7. Pipette (Eppendorf AG, Research plus)
8. Cell culture incubator (Esco, model: CCL-170B-8)
9. Biosafety cabinet (Esco, model: AC2-4S1)
10. Ultra-low temperature freezer (Thermo, model: DW-86L338)
11. Freezer (Siemens, model: KG32EV2S0C)
12. Electro-heating standing-temperature cultivator (ENXIN, model: DRP-9052)
13. Microscope (Leica, model: DM6B, equipped with a DFC7000T camera)

Software

1. CellProfiler
2. GraphPad Prism

Procedure

A. Cell culture and sample preparation

1. Pre-warm 2 ml 1x DEPC-PBS, 23 ml culture media and 0.8 ml 0.25% (w/v) trypsin-EDTA to 37 °C.
2. Remove the used culture media in the cell culture flask (take 25 cm² flask for example).
3. Wash briefly with 2 ml 1x DEPC-PBS. Aspirate PBS.
4. Add 0.8 ml 0.25% (w/v) trypsin-EDTA into flask and cover the cells.
5. Check the cells with inverted microscope to be sure that most of the cells are rounded up, but still attached to the flask. Carefully remove trypsin.

6. Add 3 ml culture media to the flask, suspend the cells from attached cell layer and mix them well by pipetting.
7. Put 5 slides into a Petri dish and add 20 ml culture media into it to cover the slides.
8. Seed the resuspended cells on slides.
9. Rest for at least 10 min until resuspended cells subside on the slides.
10. Transfer the Petri dish into the cell culture incubator (37 °C, 5% CO₂).
11. Culture the cells until they reach 80% confluence (12-48 h).
12. After 12-48 h incubation, remove and discard the culture media.
13. Wash 3 times with 25 ml 1x DEPC-PBS for 3 min each.
14. Add 25 ml 4% (w/v) PFA into the Petri dish to cover the slides for 30 min at RT.
15. Wash twice with 25 ml 1x DEPC-PBS for 3 min each.
16. Perform dehydration using an ethanol series of 70%, 85%, and ethanol absolute for 5 min each.
Air dry.

Note: The sample slides can be stored at -80 °C until used.

B. Pretreatments

1. Phosphorylate padlock probes before use. Prepare 50 µl mix containing 38 µl DEPC-H₂O, 1 µl padlock probes (100 µM), 5 µl PNK buffer A (10x), 5 µl ATP (10 mM), 1 µl T4 polynucleotide kinase (10 U/µl) in the 0.2 ml PCR Tubes.
2. Incubate the above mixture at 37 °C for 30 min and 65 °C for 10 min in the PCR machine.

Note: For formalin-fixed, paraffin-embedded pathological tissue sections, perform pretreatments procedures 3 to 20, except 17 and 18. For cell samples, perform pretreatments procedures 14 to 20.

3. Bake the slide at 60 °C for 30 min.
4. Put the slide into the xylene for 15 min and another fresh xylene for 10 min, respectively.
5. Put the slide into ethanol absolute twice, 95% ethanol twice, and 70% ethanol twice for 2 min each time.
6. Treat the slide with DEPC-H₂O for 5 min to complete rehydration.
7. Wash with DEPC-PBS for 2 min.
8. Perform the fixation procedure with 4% (w/v) PFA in DEPC-PBS for 10 min.
9. Wash with DEPC-PBS for 2 min.
10. Put the slide into the solution of 0.1 mg/ml pepsin in 0.1 M HCl for 30 min at 37 °C.
11. Wash with DEPC-H₂O for 5 min.
12. Wash with DEPC-PBS for 2 min.
13. Dehydrate the slide by an ethanol series of 70%, 85%, and ethanol absolute for 1 min each.
14. Air dry.
15. Assemble the 9 mm diameter and 0.8 mm depth Secure-Seal hybridization chamber to the surface of the slide on the side where the samples are (Figure 1). All the following incubation step take place in the chamber with a volume of 50 µl.

Figure 1. Assemble hybridization chamber to the surface of slide where the samples are. Place a seal-tab over each filling port to prevent evaporation of the solutions and the drying out of the specimen.

16. Rinse the cell sample with DEPC-PBS with 0.05% Tween-20 (DEPC-PBST).
17. Add 50 μ l 0.5%(v/v) Triton X-100 (in DEPC-PBS) into the chamber to permeabilize the cells for 10 min at RT.
18. Wash 3 times with 1x DEPC-PBST.
19. Apply 50 μ l 3% (w/v) H_2O_2 solution to the sample for 15 min at RT to block endogenous peroxidases.
20. Wash 3 times with DEPC-PBST.

C. Padlock probe hybridization

1. Prepare 50 μ l padlock probe hybridization mix containing 22.5 μ l DEPC- H_2O , 25 μ l 2x hybridization buffer and 2.5 μ l phosphorylated padlock probe (2 μ M, from B2).
2. Add the mix into the chamber and incubate for 2 h at 37 $^{\circ}$ C.
3. Wash 3 times with washing buffer for 5 min each.
4. Wash 3 times with DEPC-PBST.

D. Padlock probe ligation

1. Prepare 50 μ l padlock probe ligation mix containing 6.5 μ l DEPC- H_2O , 31.25 μ l 80% (v/v) glycerol, 5 μ l BSA (2 mg/ml), 1.25 μ l RiboLock RNase Inhibitor (40 U/ μ l), 5 μ l SplintR ligase reaction buffer (10x) and 1 μ l SplintR ligase (25 U/ μ l).

2. Add the mix into the chamber and incubate for 1 h at 37 °C.
3. Wash 3 times with washing buffer for 5 min each.

E. RCA primer hybridization

1. Prepare 50 µl RCA primer hybridization mix containing 20 µl DEPC-H₂O, 25 µl 2x hybridization buffer and 5 µl RCA primer (10 µM).
2. Add the mix into the chamber and incubate for 30 min at 37 °C
3. Wash 3 times with washing buffer for 5 min each.
4. Wash 3 times with DEPC-PBST.

F. RCA

1. Prepare 50 µl RCA mix containing 26.75 µl DEPC-H₂O, 5 µl 50% (v/v) glycerol, 2 µl dNTP (25 mM), 5 µl BSA (2 mg/ml), 1.25 µl RiboLock RNase Inhibitor (40 U/µl), 5 µl phi29 DNA polymerase buffer (10x) and 5 µl phi29 polymerase (10 U/µl).
2. Add the mix into the chamber and incubate for 16-18 h at RT.
3. Wash 3 times with washing buffer for 5 min each.
4. Wash 3 times with DEPC-PBST.

G. RCA products detection

1. Prepare 50 µl RCA products detection mix containing 10 µl DEPC-H₂O, 25 µl 2x detection buffer, 12.5 µl 80% (v/v) glycerol and 2.5 µl HRP-labeled detection probe (2 µM).
2. Add the mix into the chamber and incubate for 30 min at RT.
3. Wash 3 times with DEPC-PBST.

H. Staining and mounting

1. Incubate the sample with staining solution for 3 to 8 min depending on the color development.
2. Wash 3 times with DEPC-H₂O.
3. Stain the nuclei in Hematoxylin staining solution for 1 min.
4. Wash 3 times with DEPC-H₂O.
5. Differentiate with 0.1% hydrochloric acid-ethanol for 1 min.
6. Wash 3 times with DEPC-H₂O.
7. Place slide in 1x DEPC-PBS for 1 min.
8. Wash 3 times with DEPC-H₂O.
9. Remove the chamber and rinse in running tap water for 5 min.
10. Dehydrate the slide by an ethanol series of 70%, 85%, and ethanol absolute for 1 min each.
11. Mount the slide with 15-20 µl neutral balsam mounting medium.

I. Image acquisition

1. Acquire images with bright-field microscope using the 40x objective.

2. In images, brown dots are detected individual mRNA molecules and nuclei are shown in blue (Figures 2 and 3).

Figure 2. Detection of ACTB mRNA molecules in MCF-7 cells. Scale bar = 40 μ m.

Figure 3. Detection of HER2 mRNA molecules in HER2-positive and HER2-negative breast cancer FFPE tissue sections. Scale bars = 40 μ m.

Data analysis

Quantitative analysis of detected RNA molecules is achieved by using CellProfiler image analysis software. The images are processed with “Unmixcolors”, “EnhanceOrSuppressFeatures”, “IdentifyPrimaryObjects”, “IdentifySecondaryObjects”, “IdentifyPrimaryObjects” and “RelateObjects” modules in turn. To remove the background, the “Unmixcolors” module is used to

split into hematoxylin and DAB two channels. The “EnhanceOrSuppressFeatures” module is used to highlight DAB staining spots. Finally, the relation of signals with their host cells is identified with the “RelateObjects” module. Then the number of RCPs per cell was listed. Statistical plotting was performed using GraphPad Prism.

Notes

1. For FFPE tissue sections and fresh frozen tissue sections, the pretreatment is different due to different kinds of tissue, including permeabilization and fixation.
2. Oligonucleotides were dissolved and diluted with 1x TE buffer. The stock concentration of the padlock probe, RCA primer and detection probe were 100 μ M, 10 μ M and 2 μ M respectively.
3. According to the cell type, adjust the experimental conditions of nucleic counterstaining, such as staining time, concentration of stain fluid.
4. DEPC is heat inactivated before using the solution.
5. Aspirate the solutions from the previous steps before adding a new solution into the chamber.

Troubleshooting: A list of possible reasons for experiment failures and solutions are listed in Table 1.

Table 1. Troubleshooting table

Problem	Possible reason	Solution
Little or no signal	Padlock probes are not 5'-phosphorylated	Phosphorylate padlock probes with PNK
	The abundance of RNA is low in cells of interest	Increase the number of padlock probes that target different hybridization sites on the same RNA
Weak staining	DAB staining solution is not fresh	Use appropriate positive-control cell line
	DAB staining time is too long	Prepare DAB staining solution fresh
High background	DAB staining time is too long	Shorten staining time
	Endogenous peroxidases are not blocked	Block endogenous peroxidases with 3% H ₂ O ₂ solution

Recipes

1. Culture media (50 ml)
 - 45 ml serum-free medium
 - 5 ml fetal bovine serum
 - 0.5 ml Penicillin-Streptomycin Solution
 - Store at 4 °C

2. 10x PBS (500 ml), pH 6.8
40 g NaCl
1 g KCl
7.2 g Na₂HPO₄
1.2 g KH₂PO₄
500 ml ultrapure water
Filter the solution using 0.22 µm membrane filter
Prepare fresh
3. 1x DEPC-PBS (1 L), pH 7.4
100 ml 10x PBS
900 ml ultrapure water
1 ml DEPC
Store at 4 °C
4. 1x DEPC-PBST (1 L)
1 L DEPC-PBS
0.5 ml Tween-20
Store at 4 °C
5. 4% (w/v) PFA (500 ml)
For a 4% paraformaldehyde solution:
 - a. Add 20 g of EM grade paraformaldehyde to 480 ml of 1x DEPC-PBS
 - b. Add 5 ml of 5 M NaOH and stir gently on a heating block at ~30 °C until the paraformaldehyde is dissolved
 - c. Allow the mixture to cool to room temperature and adjust the pH to 7.4 with HCl
 - d. Then adjust the final volume to 500 ml with 1x DEPC-PBS
 - e. Filter the solution through a 0.22 µm membrane filter to remove any particulate matter
 - f. Make the paraformaldehyde solution fresh prior to use, or store in aliquots at -20 °C for several months. Avoid repeated freeze/thawing
6. 1x TE buffer (100 ml), pH 8.0
1 ml 1 M Tris-HCl (pH 8.0)
0.2 ml 0.5 M EDTA (pH 8.0)
98.8 ml DEPC-H₂O
Filter the solution using 0.22 µm membrane filter
Store at 4 °C
7. 0.5%(v/v) Triton X-100 (5 ml)
25 µl Triton X-100
4.975 ml DEPC-H₂O
Store at RT
8. 3% (w/v) H₂O₂ solution (50 µl)
5 µl 30% (w/v) H₂O₂

- 45 µl DEPC-H₂O
Prepare fresh
9. 2x hybridization buffer (5 ml)
3 ml 20x SSC
1 ml Formamide
1 ml DEPC-H₂O
Store at RT
10. Washing buffer (15 ml)
1.5 ml 20x SSC
3 ml Formamide
10.5 ml DEPC-H₂O
Store at RT
11. 2x detection buffer (5 ml)
1 ml 20x SSC
5 µl Tween-20
0.2 ml 20 mg/ml BSA
3.975 ml DEPC-H₂O
Store at 4 °C
12. Staining buffer (50 µl)
45 µl DAB buffer
2.5 µl 20x DAB substrate solution
2.5 µl 20x DAB chromogen solution
Prepare fresh
13. 0.1% hydrochloric acid-ethanol (5 ml)
50 µl hydrochloric acid
3.713 ml ethanol
1.237 ml DEPC-H₂O
Store at RT
14. Hematoxylin Staining Solution (50 ml)
a. Dissolve 0.1 g hematoxylin with 12.5 ml ethanol
b. Dissolve 0.85 g aluminum potassium sulfate dodecahydrate with 37.5 ml DEPC-H₂O
c. Then mix the two solutions
d. After hematoxylin is oxidized to a purplish red by adding 10 mg sodium iodate, add 0.5 ml acetate
e. Filter the solution using 0.22 µm membrane filter. Store at RT
f. Concentration of hematoxylin staining solution can be diluted with DEPC-H₂O
15. 25 mM dNTP (100 µl)
25 µl 100 mM ATP
25 µl 100 mM GTP

25 µl 100 mM CTP

25 µl 100 mM TTP

Store at -20 °C

Competing interests

Authors declare no conflicts of interest or competing interests.

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