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***In situ* hybridization for detecting mature microRNAs *in vivo* at the single cell resolution**

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Abstract

microRNAs (miRNAs) are key regulators of cell and tissue development. Yet, spatial resolution of miRNA heterogeneity and accumulation patterns *in vivo* remains uncharted. Next-generation sequencing methods assay miRNA abundance in tissues. These analyses however do not provide spatial resolution. A method to assay miRNA expression at the single cell resolution *in vivo* should clarify the cell-autonomous functions of miRNAs, their roles in influencing the cellular microenvironment, and analysis of miRNA perdurance and turnover rate. We present an *in situ* hybridization protocol to map miRNA subcellular expression in single cells *in vivo* in four days. Using this protocol, we mapped distinct miRNAs that accumulate in the cytoplasm of one sibling oocyte but not another, dependent on the oocyte developmental stage. Thus, this method provides spatial and temporal resolution of the heterogeneity in expression of miRNAs during *C. elegans* oogenesis. This protocol can be generally adapted to any tissue amenable to dissection and fixation.

Keywords

microRNA; *in situ* hybridization; Locked Nucleic Acid probe; oogenesis; *C. elegans*

INTRODUCTION

To study microRNA (miRNA) function *in vivo*, we designed an *in situ* hybridization method to map the spatial expression of miRNAs at the single cell level in a whole tissue (Figure 1). To visualize the spatial expression pattern of oocyte-expressed miRNAs in *C. elegans* at the single cell resolution, we used Locked Nucleic Acid (LNA) probes labeled with Digoxigenin (DIG) on tissue (gonad) obtained through dissection and extrusion. Dissection and extrusion of the gonad followed rapidly by fixation in a paraformaldehyde and glutaraldehyde solution led to maintenance of tissue integrity and spatial context so as to increase the cellular resolution for each germ cell and somatic gonadal cell (Figure 2). Fixation of the gonad also aids with permeabilization of the tissue which is essential for later steps in the protocol that require the use of an anti-DIG antibody to gain access to the DIG labeled LNA probes (Figure 3A). While the LNA probes can be directly conjugated to fluorophores for detection,

our experience suggests that low copy number miRNAs generate low signal-to-noise ratio, and the DIG based chemical development with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) allows for controlled development and amplification of miRNA signal to provide high signal-to-noise ratio irrespective of miRNA abundance per each cell type. As mentioned, we utilize LNA-based probes for detection. LNA probes are generated using modified RNA nucleotides (Figure 3B) (Kaur et al., 2006, Owczarzy et al., 2011). The ribose is modified such that an extra bridge connects the 2' oxygen with the 4' carbon (Figure 3B). This modification allows for greater structural stability of the nucleotides requiring a higher temperature for hybridization with the target RNA species (Kaur et al., 2006, Owczarzy et al., 2011). This higher temperature by definition results in increased specificity and a higher signal-to-noise ratio (Kloosterman et al., 2006, Nuovo et al., 2009, Obernosterer et al., 2007). Utilizing this protocol, we successfully mapped distinct oocyte-expressed miRNAs in sibling oocytes. Because the method is performed on whole gonads, it allows us to also assess the expression of the miRNA in the somatic gonadal cells in conjunction with the germ cells.

Materials

M9 buffer (see recipe)

3% Paraformaldehyde / 0.25% glutaraldehyde / 0.1 M K₂HPO₄ (pH 7.2) (see recipe)

CAUTION: Paraformaldehyde and glutaraldehyde is a hazardous chemical. Always handle under a chemical fume hood.

1x PBS (SIGMA-ALDRICH, cat. no. P2194)

1x TBS (SIGMA-ALDRICH, cat. no. T6664)

0.1% Tween 20 (Fischer Scientific, cat. no. BP337-500)

Methanol (Electron Microscopy Sciences, cat. no. 18510)

CAUTION: Methanol is a hazardous chemical. Always handle under a chemical fume hood.

Proteinase K (10 mg/mL) (SIGMA-ALDRICH, cat. no. RPROTK-RO)

0.2% Glycine in 1x TBS (see recipe)

0.13 M 1-Methylimidazole, 300 mM NaCl (pH 8.0) (see recipe)

N-(3-Dimethylaminopropyl)-*N*'-ethylcarbodiimide hydrochloride (EDC) (SIGMA-ALDRICH, cat. no. 03449)

Hybridization buffer (see recipe)

50% 1x TBS-T / 50% hybridization buffer (see recipe)

1x TBS-T / 0.5 mg/mL BSA (see recipe)

Staining Solution (see recipe)

0.1 M Levamisole hydrochloride (SIGMA-ALDRICH, cat. no. L9756)

SIGMAFAST™ BCIP®/NBT (SIGMA-ALDRICH, cat. no. B5655)

cel-miR-39-3p miRCURY LNA Detection probe (Qiagen, cat. no. YD00619874)

cel-miR-35-3p miRCURY LNA Detection probe (Qiagen, cat. no. YD00613449)

U6, hsa/mmu/rno miRCURY LNA Detection probe, positive control (Qiagen, cat. no. YD00699002). The probe works in *C. elegans* due to 100% sequence complementarity.

DAPI (SIGMA-ALDRICH, cat. no. D9542)

Vectashield (Vector Laboratories, cat. no. H-1000)

Equipment

Flat bottom glass watch dish (Agar Scientific, cat. no. AGL4467)

5 mL glass conical tube (Corning, cat. no. 8060-5)

25 gauge needles (Becton Dickinson, cat. no. 305122)

9" disposable glass Pasteur pipette (Fisher Scientific, cat. no. 13-678-20D)

Amber glass vial, 8MM x 40MM (SIGMA-ALDRICH, cat. no. 29434-U) (Figure 4)

Clinical benchtop centrifuge, accommodating 5mL glass conical tubes

Hybridization oven, accommodating temperatures from 40°C – 65°C

25°C incubator

4°C refrigerator

Dissection microscope (Leica stereo-microscope used for worm breeding, dissection and extrusion of the tissue)

Compound microscope (Zeiss Apotome microscope designed for high resolution imaging)

Dissection and Extrusion of Gonadal Tissue

1. Pick 100 –150 wild type (N2) or selected genotype of *C. elegans* worms at the chosen developmental stage into 1000 µL of M9 buffer in a flat bottom glass watch dish.
2. Add 1 –3 µL of 0.1 M levamisole to the flat bottom glass watch dish containing the worms and M9 buffer. Levamisole is used to temporarily immobilize the worms for dissection.
3. Gently swirl the levamisole and M9 to enable even mixing.

CRITICAL STEP: Wait no longer than 2 minutes before moving to next step. Worms may still be mobile, but dissections need to be completed in under 5 minutes for reproducible miRNA detection.

4. Attach two 25 G needles to two 1 mL syringes. Position one syringe in each hand.
5. Under a dissecting microscope, position each worm between the needle points and place a fine cut near the second pharyngeal bulb of the worm (Figure 5). Use the needles as a pair of scissors by placing one needle above the other and sliding them across each other. After a single cut in the worm has been made, move to the next animal and repeat until all the worms in the dish have been dissected.

CRITICAL STEP: Dissections should be completed in under 5 minutes for reproducible miRNA detection. If all 150 dissections cannot be completed in under 5 minutes, use smaller batches of worms.

CAUTION: Once one cut has been made in the worm, do not make a second cut in the same worm. Instead move to the next worm. A second cut in the same worm does not enable better gonad extrusion, instead it often leads to fragmentation of the extruded tissue.

FIXATION (Time: 3 Hours)

6. In a chemical fume hood, add 2 mL of 3% paraformaldehyde / 0.25% glutaraldehyde / 0.1M K_2HPO_4 solution to the dissected gonads in the flat bottom glass watch dish and cover with parafilm.

CAUTION: Paraformaldehyde and glutaraldehyde are hazardous chemicals. Always handle under a chemical fume hood.

7. Incubate the dissected gonads for 2 hours at room temperature ($\sim 24^\circ C$) in the flat bottom glass watch dish covered with parafilm.
8. After the completion of the 2 hours, add 3 mL of 1x TBS-T buffer to the flat bottom glass watch dish containing the dissected gonads.
9. Using a 9" glass Pasteur pipette, draw the liquid from the flat bottom glass watch dish into the Pasteur pipette and transfer the contents into a fresh 5 mL glass conical tube. The flat bottom glass watch dish and 5 mL glass conical tubes can be washed with soap and water and re-used.

CRITICAL STEP: Use glass Pasteur pipettes and glass conical tubes as dissected gonads stick to plastic, which causes loss or breakage of the tissue.

10. Centrifuge the 5 mL glass conical tubes for 30 seconds in a clinical benchtop centrifuge at 1,000 x g.
11. Remove and discard the supernatant using a 9" glass Pasteur pipette taking care so as not to disturb the dissected gonads.

CRITICAL STEP: Removal of the liquid after each wash should be completed under a dissection microscope so as not to affect the dissected gonads, and to minimize loss of the tissue.

12. Add 5 mL of 1x TBS-T buffer to the glass conical tubes.
13. Gently mix the gonads with the 1x TBS-T buffer by drawing up into the 9" glass Pasteur pipette and releasing the contents
14. Centrifuge the glass conical tubes in a clinical benchtop centrifuge for 30 seconds at 1,000x g.
15. Remove and discard the supernatant using a 9" glass Pasteur pipette taking care as to not disturb the dissected gonads.
16. Repeat steps 12 –15 for a total of three times.
17. Pool ~150 dissected and washed gonads into a single 5 mL conical tube.

CRITICAL STEP: Each individual in situ probe hybridization will need approximately 100–150 dissected animals, so pooling dissected batches together is required in the event that one set of dissections does not yield 150 extruded gonads. The large number of starting material is needed because gonads will be lost during the various wash steps throughout the protocol.

18. Add 2 mL of ice cold methanol (100% concentration) to the tubes, and gently mix the gonads with the methanol by drawing up into the 9" glass Pasteur pipette and releasing the contents.
19. Incubate the tube at –20°C for a minimum of 1 hour.

PAUSE POINT: Dissected gonads can be stored in methanol for up to two days for miRNA in situ hybridizations. Storage for more than two days in methanol results in a reduction in miRNA signal and is not recommended.

PERMEABILIZATION and HYBRIDIZATION (Time: ~21 Hours)

20. After the desired incubation time, repeat steps 12 –15 for a total of three times in order to wash the gonads, and remove the methanol.
21. After the final wash, remove and discard the supernatant using a 9" glass Pasteur pipette taking care as to not disturb the dissected gonads.
22. Add 2 mL of Proteinase K / TBS solution to the 5 mL conical tube.
23. Gently mix the gonads with the Proteinase K solution by drawing up into the 9" glass Pasteur pipette and releasing the contents.

CRITICAL STEP: Make a fresh Proteinase K solution each time to achieve maximum effective permeabilization.

24. Incubate the dissected gonads in the Proteinase K solution at room temperature for 20 minutes.

25. After the 20 minutes of incubation, remove as much of the Proteinase K solution as possible taking care as to not disturb the dissected gonads. Centrifugation is not needed as dissected gonads will settle at the bottom of the 5 mL conical tube by gravity.
26. Add 1x TBS buffer to the 5 mL conical tube, and gently mix the gonads with the 1x TBS buffer by drawing up into the 9" glass Pasteur pipette and releasing the contents.
27. Centrifuge the glass conical tubes for 30 seconds at 1,000 x g.
28. Remove and discard the supernatant taking care as to not disturb the dissected gonads.
29. Repeat steps 26 –28 for a total of two times.
30. Add 2 mL of 3% paraformaldehyde / 0.25% glutaraldehyde / 0.1M K_2HPO_4 to the 5 mL conical tube.

CRITICAL STEP: A second fixation step is required to maintain the tissue integrity of the dissected gonads. Without this additional fixation, dissected gonads break and lose tissue integrity during the many harsh washes and treatments that follow in the protocol.
31. Gently mix the gonads with the paraformaldehyde and glutaraldehyde solution by drawing up into the 9" glass Pasteur pipette and releasing the contents, then cover with parafilm.

CAUTION: Paraformaldehyde and glutaraldehyde are hazardous chemicals. Always handle under a chemical fume hood.
32. Incubate the gonads in the paraformaldehyde and glutaraldehyde solution for 10 minutes at room temperature in the 5 mL conical tube covered with parafilm.
33. After incubation, add 3 mL 1x TBS-T buffer to the 5 mL conical tube and centrifuge for 30 seconds at 1,000 x g.
34. Remove and discard the supernatant taking care as to not disturb the dissected gonads.
35. Repeat steps 33 –34 for a total of two times.
36. Add 2 mL of the 0.2% glycine solution to the glass conical tube. This wash step is necessary to stop the second fixation reaction and to ensure the removal of any residual paraformaldehyde and glutaraldehyde solution.
37. Gently mix the gonads and the solution by drawing up into the 9" glass Pasteur pipette and releasing the contents.
38. Incubate the gonads in the glycine solution for 10 minutes at room temperature.
39. After the incubation, centrifuge the 5 mL conical tube for 30 seconds at 1,000 x g.

40. Remove as much of the supernatant as possible without disturbing the dissected gonads.
41. Repeat step 36 –40 for a total of two times.
42. Add 2 mL of the 0.13M 1-methylimidazole, 300mM NaCl solution to the glass conical tube. This solution is utilized to remove any extraneous phosphates. This is critical since extraneous phosphates could interfere with step 48 (to follow) where the 5' mono-phosphate of the miRNA is linked to the protein matrix in the tissue to block the small RNAs from escaping the cells / tissue.
43. Gently mix the gonads and the solution by drawing up into the 9" glass Pasteur pipette and releasing the contents.
44. Incubate the dissected gonads in the solution for 10 minutes at room temperature.
45. After incubation, centrifuge the 5 mL conical tube for 30 seconds at 1,000 x g.
46. Remove as much of the supernatant as possible without disturbing the dissected gonads.
47. Repeat step 42 –46 for a total of two times.
48. Add 1 mL of EDC solution to the 5 mL conical tube. The EDC solution is utilized to crosslink the 5' mono-phosphate of the miRNA to the protein matrix to prevent loss of the miRNA to other cells within the tissue.
49. Gently mix the gonads and the solution by drawing up into the 9" glass Pasteur pipette and releasing the contents.
50. Incubate the gonads and the solution for 2 hours at 25°C.
CRITICAL STEP: Make a fresh EDC solution each time you complete this step for best results.
51. After the two hours, remove as much of the EDC solution as possible without disturbing the dissected gonads.
52. Add 1 mL of the 0.2% glycine solution to the glass conical tube.
53. Gently mix in the solution with the gonads by drawing up the mix into the 9" glass Pasteur pipette and releasing the contents.
54. Centrifuge the glass conical tubes for 30 seconds at 1,000 x g.
55. Remove as much of the supernatant as possible without disturbing the dissected gonads.
56. Repeat step 52 –55 for a total of two times.
57. Add 2 mL 1x TBS buffer to the glass conical tube.
58. Gently mix the gonads and the solution by drawing up into the 9" glass Pasteur pipette and releasing the contents.
59. Centrifuge the glass conical tubes for 30 seconds at 1,000 x g.

- 60.** After the 1x TBS buffer wash, leave ~500 μ L of 1x TBS buffer in the 5 mL conical tube.
- 61.** Using a fresh 9" glass Pasteur pipette transfer the remaining contents of the conical tube into a fresh 1 mL amber glass vial (8 mm X 40 mm) (Figure 4).
- 62.** Allow the gonads to settle by gravity for 5–10 minutes.
- CRITICAL STEP: From this step onwards, do not centrifuge / vortex the gonads when in the amber glass vials. We previously found that the force of vortex or centrifugation damages the gonads and results in their loss. Allow gonads to completely settle by gravity to prevent any loss.
- 63.** Remove as much of the supernatant as possible without disturbing the dissected gonads.
- 64.** Add 400 μ L of 50% 1x TBS-T / 50% hybridization buffer to the amber glass vials.
- 65.** Incubate for 5 minutes at the miRNA probe specific hybridization temperature (Table 1).
- CRITICAL STEP: The hybridization temperature should be ~30°C below the melting temperature of the LNA probe, and should be standardized for each probe prior to experimental testing. If 30°C below the melting temperature of the LNA probe does not produce any visible signal, various temperatures above and below the initially tested temperature should be tested for optimization of probe binding.
- CAUTION: This protocol is designed for LNA probes labeled with DIG for detection. However, probes can be labeled with other methods as long as detection methods are compatible with the probe label.
- 66.** Remove as much of the supernatant as possible without disturbing the dissected gonads.
- 67.** Add 400 μ L of hybridization buffer to the amber glass vials.
- 68.** Incubate for 1 hour at the appropriate hybridization temperature for the miRNA being tested.
- 69.** After the 1 hour incubation, dilute the miRNA probe in hybridization buffer to a final concentration of 100 nM.
- CRITICAL STEP: Dilutions of the miRNA probe can be kept at –80°C for reuse. Aliquot the probes so as to decrease the amount of freeze thaw cycles for each probe. Standardize the final concentration of the miRNA probe for accurate detection of your miRNA of interest (Table 2).
- 70.** Remove as much of the supernatant as possible from the amber glass vial without disturbing the dissected gonads.
- 71.** Add 100 μ L of the diluted miRNA probe, at a final concentration of 100 nM, to each amber glass vial.

72. Allow the miRNA probe to hybridize for 12–16 hours, without any shaking, at the optimized hybridization temperature for the miRNA being tested.

Probe Detection (Time: 16 –18 Hours)

73. Pre-warm the hybridization buffer to the optimized hybridization temperature (for the miRNA being tested).
74. Add 400 μ L of the pre-warmed hybridization buffer to the amber glass vials containing the dissected gonads.
75. Incubate for 15 minutes at the optimized hybridization temperature.
76. Remove as much of the supernatant as possible without disturbing the dissected gonads.
77. Repeat steps 74 –76 for a total of four times.
78. Add 400 μ L of 50% 1x TBS-T / 50% hybridization buffer to the amber glass vials containing the dissected gonads.
79. Incubate for 10 minutes at the optimized hybridization temperature.
80. Remove as much of the supernatant as possible without disturbing the dissected gonads.
81. Add 400 μ L of 1x TBS-T buffer to the amber glass vials containing the dissected gonads.
82. Incubate for 10 minutes at the optimized hybridization temperature for the miRNA being tested.
83. Remove as much of the supernatant as possible without disturbing the dissected gonads.
84. Repeat steps 81 –83 for a total of two times.
85. Add 1x TBS-T buffer to the amber glass vials containing the dissected gonads.
86. Incubate at room temperature for 5 minutes.
87. Remove as much of the supernatant as possible without disturbing the dissected gonads.
88. Add 400 μ L of 1x TBS-T / 0.5 mg/mL BSA to the amber glass vials containing the dissected gonads. This step is the “blocking step” before application of the primary anti-DIG antibody.
89. Incubate at room temperature for 15 minutes.
90. Dilute the alkaline-phosphatase-conjugated anti-DIG antibody at 1:1000 in 1x TBS-T buffer.

CRITICAL STEP: Keep diluted antibody at 4°C until ready to use.
91. After incubation with 1x TBS-T / 0.5 mg/mL BSA, remove as much of the supernatant as possible without disturbing the dissected gonads.

92. Add 500 μ L of the diluted alkaline-phosphatase-conjugated anti-DIG antibody to the amber glass vials.
93. Incubate at room temperature for 2 hours or overnight at 4°C.

PAUSE POINT: Incubation for 2 hours at room temperature may be used, but overnight incubation at 4°C generates lower background signal.

Signal Development (Time: 2–5 Hours), Mounting for Visualization (Time: 30 Minutes)

94. After incubation, remove as much of the supernatant as possible without disturbing the dissected gonads.
95. Add 500 μ L of 1x TBS-T buffer to the amber glass vials.
96. Allow gonads to settle by gravity for 5–10 minutes.
97. Remove as much of the supernatant as possible without disturbing the dissected gonads.
98. Repeat steps 95 –97 for a total of two times.
99. Add 500 μ L of 1x TBS-T / 0.5 mg/mL BSA to the amber glass vials.
100. Incubate at room temperature for 20 minutes.
101. Remove as much of the supernatant as possible without disturbing the dissected gonads.
102. Repeat steps 99 –101 for a total of three times.
103. Add 500 μ L of staining solution to the amber glass vials.
104. Allow gonads to settle by gravity for 5–10 minutes.
105. Remove as much of the supernatant as possible without disturbing the dissected gonads.
106. Add 1 mL of the SIGMAFAST™ BCIP®/NBT solution to the amber glass vials.

CRITICAL STEP: Make a fresh SIGMAFAST™ BCIP®/NBT solution each time you complete this step. SIGMAFAST™ BCIP®/NBT is light sensitive so it should be stored in a dark place.
107. Using a fresh 9" glass Pasteur pipette, remove the contents of the amber glass vials and place in a fresh flat bottom glass watch dish.
108. Cover the flat bottom glass watch dish with aluminum foil and protect from light.
109. The reaction can be monitored by visualizing the contents of the flat bottom glass watch dish periodically under a dissecting microscope.

CRITICAL STEP: Start with a one hour development time, and monitor every hour for detectable purple signal under the dissecting microscope. miRNA expression should be detectable between 1 and 5 hours of development time.

110. Once the signal is visible under the dissecting microscope, transfer the contents using a fresh 9" glass Pasteur pipette into a fresh amber glass vial.
111. Allow the gonads to settle by gravity for 5–10 minutes.
112. Remove as much of the supernatant as possible without disturbing the dissected gonads.
113. Add 1 mL of 1x TBS-T buffer to the amber glass vials.
114. Allow gonads to settle by gravity for 5–10 minutes.
115. Remove as much of the supernatant as possible without disturbing the dissected gonads.
116. Repeat steps 113 –115 for a total of three times.
117. Add 2 μ L of 100 ng/mL DAPI to the amber glass vials and fill the rest of the vial with 1x TBS buffer.
118. Incubate at room temperature for 20 minutes in the dark.
119. Remove as much of the supernatant as possible without disturbing the dissected gonads.
120. Add 1 drop (~35 μ L) of Vectashield solution.
121. Mount and arrange gonads on a 2% agarose pad on a slide and cover with a large coverslip (Figure 6).
122. Store the slides flat and in the dark at 4°C for 24 hours to allow the germlines to settle and flatten before imaging.
123. After the 24 hour period and the residual liquid has evaporated, seal the edges of the coverslip with clear nail polish.
124. Image the slides.

CRITICAL STEP: For best resolution use high magnification (we use 63X) to image the dissected gonads. The entire gonad cannot be imaged in one frame, so montages of images are acquired along the length of the germline. The images are then assembled in any available image software. Image the dissected gonads using DIC to visualize the miRNA accumulation and 405 nm laser to visualize the DAPI signal.

REAGENTS AND SOLUTIONS

M9 Buffer (Stiernagle, 2006)

For 1 L: Add 3 g KH_2PO_4 , 6 g Na_2HPO_4 , 5 g NaCl, 1 ml 1 M MgSO_4 , H_2O to 1 liter.
Sterilize by autoclaving.

3% paraformaldehyde / 0.25% glutaraldehyde / 0.1 M K₂HPO₄ (pH 7.2)

For 100 mL: mix 18.75 mL of 16% paraformaldehyde, 12.5 mL 2% glutaraldehyde, 10 mL 1 M K₂HPO₄ (pH 7.2), 58.75 mL PBS. Dispense in 10 mL aliquots and store at −20°C.

CAUTION: Paraformaldehyde and glutaraldehyde are hazardous chemicals. Always handle under a chemical fume hood.

1x TBS Buffer

Dissolve 150 mM NaCl and 50 mM Tris-Cl (pH 7.5) into 1 L H₂O. Store at room temperature.

1x TBS-T Buffer

Make 1x TBS buffer as described above. Add 1 mL of 0.1% Tween 20 to the 1x TBS buffer and mix well. Store at room temperature. If solution becomes cloudy make a fresh solution.

Proteinase K in 1x TBS (5 µg/mL)

Weigh 100 mg of Proteinase K and dissolve in 10 mL of H₂O. Aliquot in 50 µL aliquots and store at −20°C.

For 100 mL: Add 50 µL of Proteinase K (10 mg/mL) to 100 mL of 1x TBS buffer. Prepare fresh before each use.

0.2% glycine in 1x TBS

For 100 mL: Add 0.2 g of glycine to 100 mL of 1x TBS buffer. Dissolve with a stir bar gently on a magnetic stirrer. Filter sterilize the solution before use. Store at room temperature.

0.13 M 1-methylimidazole, 300 mM NaCl (pH 8.0)

For 160 mL: Add 1.6 mL of 1-methylimidazole to 130 mL of H₂O. Add 20 µL of 12 M HCL to this solution to obtain a pH of 8.0. Add 16 mL of 3 M NaCl (final concentration) to this solution, and make up the final volume to 160 mL with H₂O. Filter sterilize the solution before use. Store at room temperature.

CAUTION: HCL is a hazardous chemical. Always handle under a chemical fume hood.

EDC solution

Add 176 µL of EDC solution (*N*-(3-Dimethylaminopropyl)-*N*'-ethylcarbodiimide hydrochloride) to 10 mL of 0.13 M 1-methylimidazole, 300 mM NaCl solution (above). Prepare fresh before each use.

Hybridization buffer

For 100 mL: Add 50 mL formamide to 25 mL of 20x SSC (3M NaCl and 300 mM sodium citrate, pH 7.0). To this solution, add 1 mL Salmon sperm DNA (100 µg/mL), 10 mL of Heparin (50 µg/mL) and 0.1 mL Tween 20. Make up the final volume to 100 mL with H₂O.

Dissolve with a stir bar gently on a magnetic stirrer. Dispense in 10 mL aliquots and store at -20°C .

CAUTION: Formamide is a hazardous chemical. Always handle under a chemical fume hood.

50% 1x TBS-T / 50% hybridization buffer

For 10 mL: Add 5 mL of 1x TBS-T buffer to 5 mL hybridization buffer. Store at room temperature.

1x TBS-T / 0.5 mg/mL BSA

For 10 mL: Dissolve 5 mg of BSA in 10 mL of 1x TBS-T buffer. Store at room temperature.

Staining solution

For 100 mL: Add 10 mL of 1 M Tris-HCL (pH 9.5), 2 mL of 5 M NaCl, 5 mL of 1M MgCl_2 , 100 μl of 0.1% Tween 20, 10 mL of 0.1 M Levamisole, and make up the volume with H_2O to 100 mL. Filter sterilize the solution before use. Store at room temperature.

SIGMAFAST™ BCIP®/NBT solution

Thaw one tablet of SIGMAFAST™ BCIP®/NBT at room temperature (The BCIP®/NBT tablets are stored at -20°C). Add the tablet to 10 mL staining solution and cover with foil. Place on rocker and gently rock until tablet has completely dissolved. Prepare fresh before each use and store in the dark during the duration of the experiment.

COMMENTARY

Background Information

miRNAs are small noncoding RNAs that target downstream mRNAs for gene regulation (Hammond, 2015). miRNAs are 21–22 nucleotides in length, and bind to the 3'UTR of a target mRNA and either degrade (Behm-Ansmant et al., 2006), protect (Dallaire et al., 2018), or inhibit its translation (Kiriakidou et al., 2007). miRNAs are generated through a canonical biogenesis pathway (Figure 7) comprised of Drosha and Dicer, RNase IIIb enzymes ((Bortolin-Cavaille et al., 2009, Hammond et al., 2001, Lee et al., 2004). Primary transcripts are folded to form primary miRNAs, which have a hairpin structure due to internal sequence complementarity (Hammond, 2015, Bortolin-Cavaille et al., 2009, Lee et al., 2004). The primary miRNAs undergo two consecutive rounds of processing in order to become a functionally mature miRNA. The first processing step is completed by the microprocessor. The microprocessor is a complex between Drosha and Pasha. Together, this complex cleaves the single stranded overhangs of the primary miRNA thereby generating a pre-miRNA in the nucleus (Hammond, 2015, Denli et al., 2004, Gregory et al., 2004, Han et al., 2004, Lee et al., 2003). The pre-miRNAs are then transported out of the nucleus into the cytoplasm where Dicer processes the pre-miRNA into a miRNA duplex. One strand of the miRNA duplex is loaded into the RNA Induced Silencing Complex (RISC) comprised of Argonaute proteins (Hammond et al., 2001, Tabara et al., 1999). The RISC complex guides the miRNAs to their target mRNAs, where Argonaute proteins either degrade the target

mRNA or inhibit its translation (Hammond, 2015, Grishok et al., 2001, Hutvagner et al., 2001, Ketting et al., 2001).

miRNAs were first discovered in 1993 to regulate the developmental timing in *C. elegans* (Lee et al., 1993, Wightman et al., 1993). Since then, miRNAs have been shown to regulate multiple cellular processes. For example, in *Drosophila* the miRNA bantam controls cell proliferation and apoptosis during tissue growth (Brennecke et al., 2003, Thompson and Cohen, 2006). miR-1, which is conserved from worms to mammals, regulates growth and maintenance of muscle tissue (Aboobaker et al., 2005, Sokol and Ambros, 2005, Zhao et al., 2005). In addition, miRNAs have been found to play a role in the vertebrate brain, specifically in regulating neuronal morphogenesis (Schratt et al., 2006, Vo et al., 2005). Although miRNAs have been studied for nearly 25 years, the *in vivo* function of miRNAs are recently beginning to become clear. With *in vivo* functional analyses of miRNAs, questions then arise on spatial expression and accumulation of mature miRNAs in individual cell types of a tissue.

Recent models propose that miRNAs are expressed in a tissue specific manner instead of being expressed globally within an organism (Lagos-Quintana et al., 2002, Ludwig et al., 2016). Moreover, miRNA expression seems restricted to unique cell types within a given tissue. For example, in *C. elegans*, 29 oocyte-expressed miRNAs are localized in four different spatial patterns that are restricted to the developing and maturing oocytes in the germline (Minogue et al., 2018). These analyses allowed spatial resolution of each miRNA relative to another in a tissue comprised of multiple cell types. This spatial information together with genetic analysis of miRNA function within the tissue is a powerful method for understanding the role of miRNAs in diverse cell and tissue types.

Next generation sequencing (NGS) methods are useful in identifying novel miRNAs and determining the relative abundance of each miRNA in a given tissue or cell type. However, NGS quantitation's do not provide spatial information on the heterogeneity i.e. the number and types of cells that the miRNAs are expressed in within the tissue. To map the miRNA expression at a cellular level, *in situ* hybridization technologies need to be used. *In situ* hybridization for miRNAs require ensuring that the small miRNAs do not escape out of the tissue, and due to the labile nature of RNA, the tissue requires careful handling to prevent contamination by RNases. Careful coordination of the multiple steps that are required for *in situ* hybridization, designed with appropriate positive and negative controls, are also necessary to determining miRNA expression. Overall, *in situ* hybridization methods for detecting miRNAs in tissues have proven to be challenging and are not commonly used. We present a protocol that allows for easier and broader applications of the technology for the detection of miRNAs *in vivo*.

Critical Parameters

In order to maximize the effectiveness of this protocol, several considerations need to be kept in mind. First, because of the various wash steps that occur in this protocol, dissected and fixed gonads are lost at each step. This will result in very few gonads remaining at the end of the protocol for imaging and processing. Thus, it is critical to start with at least 100 dissected gonads per treatment in order to obtain 20–30 full gonads to image. At each step of

the protocol that requires aspiration of the supernatant after washing, it is best to remove the wash buffer by visually inspecting the tube under a dissection microscope; this ensures that dissected gonads that may be floating are not accidentally aspirated. The use of positive and negative controls throughout the experiment will aid in enhanced specificity and sensitivity in the assay (Figure 8). We recommend the U6 small nucleolar RNA (U6 snRNA) as a positive control (Figure 9), and a deletion mutant for the miRNA of interest as a negative control to test the probe specificity (Figure 8). U6 snRNA is a very abundant RNA that is uniformly expressed in all cells of the tissue. Being an abundant RNA, the time taken for development of this probe is under 1 hour. The timed development in each experiment allows one to calibrate the time that may be needed for miRNAs that are less abundant in the same experimental set up. Finally, the concentration of the probe (for optimal signal-to-noise ratio), temperature of hybridization of the probe to its target, and development of the probe signal will need to be optimized for each miRNA based on its abundance in the cell or tissue type (Figure 9).

miRNAs are often expressed at very low levels. Consequently, detection of low abundant miRNA species may not always be possible as they may be below the detection limit of this protocol. Other more sensitive methods such as qRT-PCR with Taqman™ probes (Tian et al., 2015), which assay whole germlines or tissues, can be used to confirm the tissue specific identity of the miRNA of interest.

Troubleshooting

Please see Table 3 for troubleshooting considerations.

Understanding Results

In wild type hermaphroditic animals, the LNA miRNA probe should detect signal in the cells where the miRNA is expressed. For example, miR-35-3p localizes to developing oocytes in wild type germlines and is not detected in the deletion mutant (*mir-35-41(nDf50)*) (Figure 8). The U6 snRNA positive control is expressed in all germ cell nuclei as well as the somatic cell nuclei of the gonad (Figure 9A). Optimization of probe concentration and development time for each miRNA is important. miR-39-3p localization, from the late pachytene region and into the developing oocytes, is visible only at 100 nM probe concentration with five hours of development time (Figure 9F). The miRNA localization patterns are reproducibly visualized at the single cell resolution in hermaphroditic gonads.

Time Considerations

This protocol can be completed in four day's time. Please see each section's time considerations throughout the protocol for timing on individual parts.

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Figure 1.
Protocol flowchart

Figure 2. Cartoon of a hermaphroditic gonad.

(A) *C. elegans* carries two U-shaped gonad arms connected at the uterus. These gonad arms take up most of the internal cavity of the worm in adulthood. (B) Dissection at the pharyngeal bulb results in extrusion of an entire gonad arm that unfolds as a linear structure. Each gonad arm consists of the germ cells that are organized in a spatial and temporal manner from germline stem cells to mature oocytes, as well as somatic cells. The cartoon depicts one gonad arm with mitotic germline stem cells on the anterior side (left) and growing oocytes on the posterior side (right). The somatic cells of the gonad are the distal tip cell and sheath cells (arrows) which encase the germ cells. Mitotic germline stem cells transition into pachytene of meiosis I as the germline develops from anterior to posterior. Oocytes arrest at diakinetic stages. In the hermaphroditic gonad, sperm are housed in the spermatheca (a somatic structure). Oocytes ovulate into the spermatheca where fertilization occurs.

Locked Nucleic Acid (LNA) Structure

Figure 3. LNA probe structure.

(A) LNA probes are 21 nucleotides in length that correspond to the complementary sequence of a mature miRNA. The probes used in this protocol are dual labeled on the 3' and 5' end with DIG. DIG is detected by an alkaline-phosphate-conjugated anti-DIG antibody catalyzed by alkaline phosphatase which results in the colorimetric reaction and produces the purple color. (B) LNA probes utilize a modified ribose that contains an extra bridge between the 2' oxygen with the 4' carbon giving increased stability to the

nucleotides. This modification results in higher hybridization temperatures, which increases specificity in the reaction and allows for a lower signal-to-noise ratio.

Figure 4. The amber glass vial.

The amber glass vial is used for all hybridization steps. It provides ideal conditions for the light sensitive reagents. The vial is used without a lid and without inversion or shaking to prevent unnecessary damage or loss to gonads. The vial can be stabilized during hybridization steps by using a microtube rack. If concerned about evaporation, a small piece of parafilm can be used to seal the opening of the vials during hybridization.

Figure 5. Cartoon visualization of a *C. elegans* gonad dissection.

To extrude the gonad arms, the animal is immobilized in levamisole and cut behind the second pharyngeal bulb (red dashed line). Loss of turgor pressure inside the animal because of the cut results in extrusion of one of the two gonads; rarely both gonad arms will extrude.

Figure 6. Cartoon visualization of slide preparation to visualize the dissected gonads.

2% agarose is flattened between two glass slides to generate an “agarose pad”. The dissected gonads are placed onto the pad using a 9” Pasteur pipette. The gonad arms sometimes fall underneath the body carcass or intestine (also extruded in the dissection process). To move the gonad arm out from under the body, a fine deer hair brush is used to position the gonad arm away from the intestine and body carcass, under a dissecting stereo microscope. A coverslip is then gently placed on top of the gonads for visualization.

Figure 7. Cartoon of the *C. elegans* miRNA biogenesis pathway.

miRNAs are generated in a step-wise manner, starting with the transcription of the primary miRNA in the nucleus. The primary transcript is then folded into a primary miRNA based on internal sequence complementarity. The primary miRNA is then processed by Drosha and Pasha to produce the pre-miRNA. The pre-miRNA is exported out of the nucleus and processed by Dicer into a mature 21 nucleotide miRNA. Mature miRNAs together with the RISC complex bind to the target mRNA where the RISC complex either degrades the mRNA or results in its translational inhibition.

Figure 8. miR-35-3p is detected in wild type gonads and not in *mir-35* family mutant gonads. Wild type gonads were dissected at 24 hours past the L4 stage of development and oriented left to right, with the germline stem cells on the left and developing oocytes on the right. DAPI labels DNA and marks chromosomal morphologies in each of the different developing germ cells for each gonad (right panels). Bright field denotes DIC images (left panels). **(A)** 100 nM probe concentration of miR-35-3p results in no signal after 5 hours of development time in a *mir-35-41* depletion allele, *nDf50*. **(B)** 100 nM probe concentration of miR-35-3p reveals strong signal in wild type germlines and localizes to late pachytene germ cells and growing and developing oocytes after 5 hours of development time. Blue line indicates positive signal. Cartoon depicts miRNA expression pattern in orange. Scale bar = 20 μ M.

Figure 9. miR-39-3p detection using optimized probe concentration and colorimetric development time.

Wild type gonads were dissected at 24 hours past the L4 stage of development and oriented left to right, with the germline stem cells on the left and developing oocytes on the right. DAPI labels DNA and marks chromosomal morphologies in each of the different developing germ cells for each gonad (right panels). Bright field denotes DIC images (left panels). **(A)** 1 nM U6 snRNA probe concentration was used and developed for 2 hours. U6 localizes to all germ cell and somatic gonad nuclei. Cartoon depicts U6 expression pattern in orange. **(B)** 1

nM probe concentration of miR-39-3p results in no signal after 5 hours of development time. **(C)** 10 nM probe concentration of miR-39-3p results in no signal after 5 hours of development time. **(D)** 100 nM probe concentration of miR-39-3p results in no signal after 1 hours of development time. **(E)** 100 nM probe concentration of miR-39-3p results in no signal after 3 hours of development time. **(F)** 100 nM probe concentration of miR-39-3p produces strong signal after 5 hours of development time and reveals miRNA localization in late pachytene stage germ cells and oocytes. Blue line indicates positive signal. Cartoon depicts miRNA expression pattern in orange. Scale bar = 20 μ M.

Table 1:
LNA probes and target hybridization temperatures.

The LNA probes should be hybridized at 30°C below the probe melting temperature. Each hybridization reaction needs to be carefully optimized for each probe, starting from 30°C below the melting temperature until a reproducible signal is detected. Each miRNA probe, probe sequence, and hybridization temperature used in the protocol is shown here.

miRNA	LNA Probe Sequence	LNA Probe Melting Temperature	Hybridization Temperature
U6 snRNA	5'-CACGAATTTGCGTGTCATCCTT-3'	86°C	56°C
cel-miR-39-3p	5'-CAAGCTGATTACACCCGGTGA-3'	86°C	56°C
cel-miR-35-3p	5'-CAAGCTGATTACACCCGGTGA-3'	91°C	61°C

Table 2:
LNA probe concentration and development time for each miRNA.

The colorimetric reaction can extend anywhere between one to five hours depending on the abundance level of each miRNA. Development time for each reaction should be monitored and continued until a strong purple signal is detectable. Shown here is a table for the LNA probe U6 snRNA, miR-39-3p and miR-35-3p with different probe concentrations and development times that were used. Each miRNA probe should be optimized independently for probe concentration and development time before use.

miRNA	Concentration	Development Time	Figure
U6 snRNA	1 nM	2 hours	8a
cel-miR-39-3p	1 nM	5 hours	8b
cel-miR-39-3p	10 nM	5 hours	8c
cel-miR-39-3p	100 nM	1 hour	8d
cel-miR-39-3p	100 nM	3 hours	8e
cel-miR-39-3p	100 nM	5 hours	8f
cel-miR-35-3p	100 nM	5 hours	9b

Table 3:

Troubleshooting Considerations

Steps	Problem	Possible Reason	Possible Solution
22–24	Tissue is not permeabilized efficiently to provide access to the probe.	Incorrect concentration or time of Proteinase K was used.	Optimize the concentration and time of Proteinase K treatment using a positive control.
69	Probe concentration does not produce detectable miRNA signal.	The probe concentration is too low.	Optimize the concentration of the probe up to 100 nM.
109	The miRNA signal is not detected or is weak after the colorimetric reaction.	The colorimetric reaction time was too short.	Increase reaction time and monitor periodically. Do not exceed 5 hours. Or if longer development times are used, appropriate negative controls should be processed simultaneously to control for non-specific signal development.
	High background or no signal.	Reagents weren't made fresh and RNase free.	Remake all solutions fresh and repeat the experiment.