

# Fluorescence in situ Localization of Pri-miRNAs in Isolated *Arabidopsis thaliana* Nuclei

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## Abstract

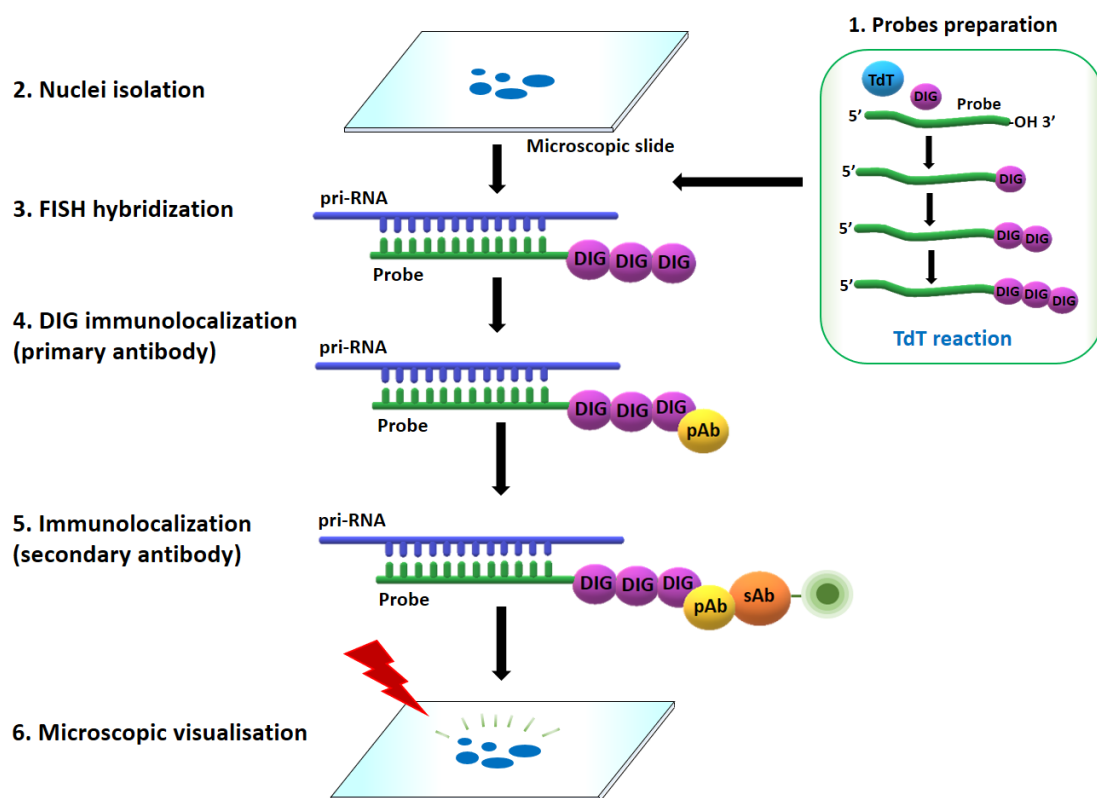
Here, we present an approach combining fluorescence in situ hybridization (FISH) and immunolabeling for localization of pri-miRNAs in isolated nuclei of *A. thaliana*. The presented method utilizes specific DNA oligonucleotide probes, modified by addition of digoxigenin-labeled deoxynucleotides to its 3' hydroxyl terminus by terminal deoxynucleotidyl transferase (TdT). The probes are then detected by immunolabeling of digoxigenin (DIG) using specific fluorescent-labeled antibodies to visualize hybridized probes. Recently, we have applied this method to localize pri-miRNA156a, pri-miRNA163, pri-miRNA393a, and pri-miRNA414 in the nuclei isolated from leaves of 4-week-old *A. thaliana*. The present approach can be easily implemented to analyze nuclear distribution of diverse RNA classes, including mRNAs and pri-miRNAs in isolated fixed cells or nuclei from plant.

**Keywords:** Fluorescent in situ hybridization, Immunolabeling, Digoxigenin, Pri-miRNA, Confocal microscopy

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## Background

Microscopic imaging allows to observe and analyze the localization of diverse RNA molecules in their native cellular environment. It was found to be essential for understanding their turnover and function in different types of cells. Conventional fluorescence in situ hybridization (FISH) techniques have been designed for visualization of RNAs and rely on using fluorescently labeled DNA oligonucleotides (Hu et al., 2014). However, FISH methods are often challenging to detect RNAs expressed at low levels in the cells, such as pri-miRNAs, mainly due to low efficiency of resulting fluorescence signal. Here, we describe a method to detect pri-miRNA molecules in isolated nuclei of *A. thaliana* by combining FISH together with immunolocalization of digoxigenin (Figure 1). This method has been shown to be more sensitive and effective than conventional FISH (Bhat et al., 2020; Gonzalo et al., 2022; Stepień et al., 2022), and can successfully be applied to detect pri-miRNAs in plant and animal cells.



**Figure 1. Overview of combined fluorescence in situ hybridization (FISH) and immunolabeling approach to detect pri-miRNAs in situ.** Prior to the FISH, DNA oligonucleotides were labeled with digoxigenin at their 3'-ends by terminal transferase (TdT) (1), and the nuclei were isolated following the procedure described in (2). Pri-miRNAs were next localized by applying FISH (3) combined with the immunolocalization of digoxigenin attached to 3'-ends of the probes (4 and 5). The results were registered with confocal microscope (6). DIG: digoxigenin, TdT: terminal transferase, pAb: primary antibody, sAb: secondary antibody

## Materials and reagents

### Reagents

1. Roche Terminal Transferase 8000 U (Merck, catalog number: 3333566001)
2. ChromaTide™ Alexa Fluor™ 488-5-dUTP (Thermo Fisher, catalog number: C11397)

3. Roche DIUTPS-RO (DIG-11-dUTP) (Digoxigenin-11-dUTP, alkali-stable) (Merck, catalog number: 11093088910)
4. Hoechst 33342, trihydrochloride, trihydrate, 100 mg (Thermo Fisher, catalog number: H1399)
5. ProLong™ Gold Antifade Mountant (Thermo Fisher, catalog number: P36930)
6. Paraformaldehyde, 16% w/v aq. soln., methanol free (Thermo Fisher, catalog number: 043368.9M)
7. Formamide (Merck, catalog number: FX0420)
8. Sodium chloride (Merck, catalog number: 1.16224)
9. Potassium chloride (Merck, catalog number: 7447-40-7)
10. Magnesium chloride (Merck, catalog number: 7786-30-3)
11. Sodium citrate (Merck, catalog number: 1613859)
12. Sodium phosphate, 0.5 M, pH 7.0 buffer (Thermo Fisher, catalog number: J63791.AK)
13. Saline sodium citrate (SSC) 20× concentrate (Merck, catalog number: S6639)
14. Ethylenediaminetetraacetic acid (EDTA) (Merck, catalog number: E9884-100G)
15. Bovine serum albumin (BSA) (Merck, catalog number: A9418-5G)
16. Ficoll® 400 (Merck, catalog number: F2637-5G)
17. Polyvinylpyrrolidone (Merck, catalog number: P0930-50G)
18. BSA, acetylated (Thermo Fisher, catalog number: AM2614)
19. Spermine (Merck, catalog number: S3256-1G)
20. Anti-digoxigenin antibody (Merck, 11333062910)
21. Goat anti-mouse secondary antibody, Alexa Fluor Plus 488 (Thermo Fisher, catalog number: A55058)
22. Goat anti-mouse secondary antibody, Alexa Fluor Plus 555 (Thermo Fisher, catalog number: A32727)
23. RNase A (Thermo Fisher, catalog number: EN0531)
24. Tris-HCl at pH 7.5 (Thermo Fisher, catalog number: 15567027)
25. Tween 20 (Merck, catalog number: P1379)
26. Sucrose (Merck, catalog number: S0389)
27. Phosphate buffered saline (PBS) (Merck, catalog number: P4417)
28. Triton X-100 (Merck, catalog number: 9036-19-5)

## Solutions

1. Hybridization buffer (see Recipes)
2. Denhardt's solution 100× (50 mL) (see Recipes)
3. LB01 buffer (10 mL) (see Recipes)
4. Sorting buffer (10 mL) (see Recipes)

## Equipment

1. Humid chamber (e.g., StainTray Slide Staining, Polysciences, catalog number: 25502-1)
2. Polylysine adhesion slides (e.g., Roth, catalog number: ET10.1)
3. Cover glasses for microscope slides (e.g., Merck, Brand cover glasses, catalog number: BR470045)
4. CellTrics 30 µm (Sysmex, catalog number: CTS220112)
5. Confocal microscope, Leica DMI 6000B, TCS TrinoTCS SP8
6. Plan Apochromat DIC H oil immersion lens (63×/1.4)

## Software

1. ImageJ, Fiji (<https://imagej.net/software/fiji/>)

## Procedure

### A. Nuclei isolation

*Note: Nuclei isolation was performed according to the method described in Pontvianne et al. (2007 and 2012).*

1. Collect 2–3 leaves from 4-week-old *A. thaliana* plants and fix them for 20 min at room temperature in 4% formaldehyde solution in Tris buffer (10 mM Tris-HCl at pH 7.5, 10 mM EDTA, 100 mM NaCl). Leaves should be completely immersed.

*Notes:*

- a. For 4% fixative preparation, dilute 10 mL of 16% methanol-free formaldehyde in 30 mL of Tris buffer.
- b. Nuclei can also be isolated from seedlings, following the same protocol.

**WARNING:** Formaldehyde is a volatile and toxic carcinogen and should only be used in a fume hood. Gloves, safety glasses, and protective clothing should also be worn when working with it.

2. Wash the leaves twice for 10 min in Tris buffer.
3. Chop the leaves with a razor blade in 0.5 mL of LB01 buffer to obtain a soup in a Petri dish.
4. Filter the resulting lysate through a 30 µm cell strainer.
5. Add 12 µL of sorting buffer per 3 µL of filtered nuclei suspension obtained in step 3.
6. Spread the mixture on adhesion slides covered with Polylysine using a pipette and leave to dry at room temperature (can be kept overnight).

### B. Probe preparation

1. Dissolve DNA oligonucleotides with double-distilled H<sub>2</sub>O to a final concentration of 100 µM.
2. Prepare terminal transferase (TdT) working solution: 5 mM CoCl<sub>2</sub>, 1× TdT Reaction buffer, 0.1 mM DIG-11-dUTP, 0.1 mM dATP, and 400 U of TdT per reaction in PCR tube.

*Notes:*

- a. Optionally, you can supply a reaction buffer additionally with Alexa Fluor 5-dUTP (final concentration: 0.2 mM).
  - b. Instead of DIG-11-dUTP, Biotin-11-dUTP could be used.
3. Combine 1 µL of DNA oligonucleotide with 9 µL of TdT working solution and incubate it for 40 min at 37 °C in a PCR tube.

*Notes:*

- a. Incubation is performed without shaking. After incubation, the reaction mixture could be stored at -20 °C. In our studies, probes were working after three months of storage.
- b. For our studies, the probes were provided by Metabion company (Planegg, Germany).

### C. Fluorescence in situ hybridization (FISH)

1. Prepare permeabilization buffer: 0.1% Triton X-100 in 1× PBS.
2. Incubate the sample in permeabilization buffer for 10 min at room temperature. The buffer should cover the whole sample area.
3. Wash the sample twice with 1× PBS.
4. Prepare hybridization buffer (Niedojadlo et al., 2012).
5. For FISH, dilute 1 µL of the probe mixture (section B) in 100 µL of hybridization buffer (final probe concentration: 100 pM/µL).

*Note: The final probe concentration in hybridization buffer may vary for different RNAs and should be adjusted experimentally.*

6. Add 100 µL of hybridization buffer to the sample on the slide and cover with a slide cover.
7. Place the slide in a humid hybridization chamber in the 28 °C incubator and leave overnight. The slides should be prevented from drying during hybridization. To obtain this, a small volume of PBS or water should be placed inside the chamber to keep a stable humidity.

*Note: Incubation is performed with a gentle shaking (0.6 rpm)*

8. After incubation, wash the slide using the following order: 4× SSC for 5 min, 2× SSC for 5 min, 1× SSC for 5 min, and 1× PBS for 10 min.

#### D. Immunolabeling

1. Dilute primary anti-digoxigenin antibody in 1× PBS containing 0.05% acetylated BSA diluted 1:100.
2. Cover the sample with 100 µL of antibody diluent (containing anti-digoxigenin antibody from step 1) and place the slide cover. Incubate the sample in a humid chamber overnight at 10 °C.
3. Remove primary antibody from the sample and wash with 1× PBS for 5 min.
4. Dilute the secondary antibody labeled with fluorescence dye in 1× PBS containing 0.05% acetylated BSA (diluted 1:1,000).
5. Place 100 µL of secondary antibody solution on the sample, cover it with a slide cover glass, and incubate in a humid chamber for 3 h at 37 °C.
6. Remove the secondary antibody from the sample and wash it two times with 1× PBS for 5 min.

*Note: The time and temperature of incubation, as well as antibody concentration, should be defined experimentally for different antibodies used.*

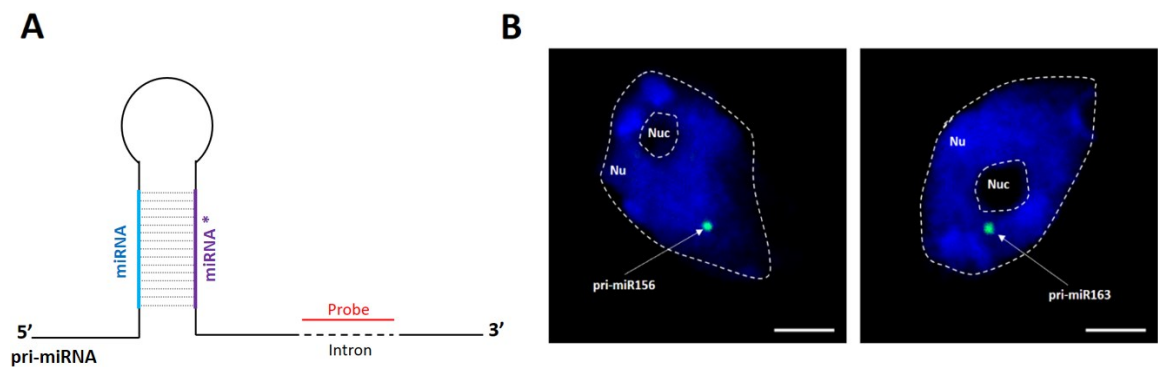
#### E. Nuclei labeling and sample mounting

1. Dilute Hoechst 33342 at 1:1,000 in 1× PBS and add 100–200 µL to the sample. Incubate for 10 min at room temperature.  
*Note: For nuclei labeling, DAPI (4',6-diamidino-2-phenylindole) diluted 1:1,000 in 1× PBS buffer can also be used.*
2. Remove the Hoechst 33342 solution from the slide and wash it once with 1× PBS for 5 min.
3. Cover the sample with 20 µL of ProLong Gold Antifade reagent, carefully place the coverslip, and press down firmly with the finger to remove air bubbles and uniformly distribute the antifade reagent over the samples.
4. Leave the sample to dry at room temperature, protecting it from the light.

## Data analysis

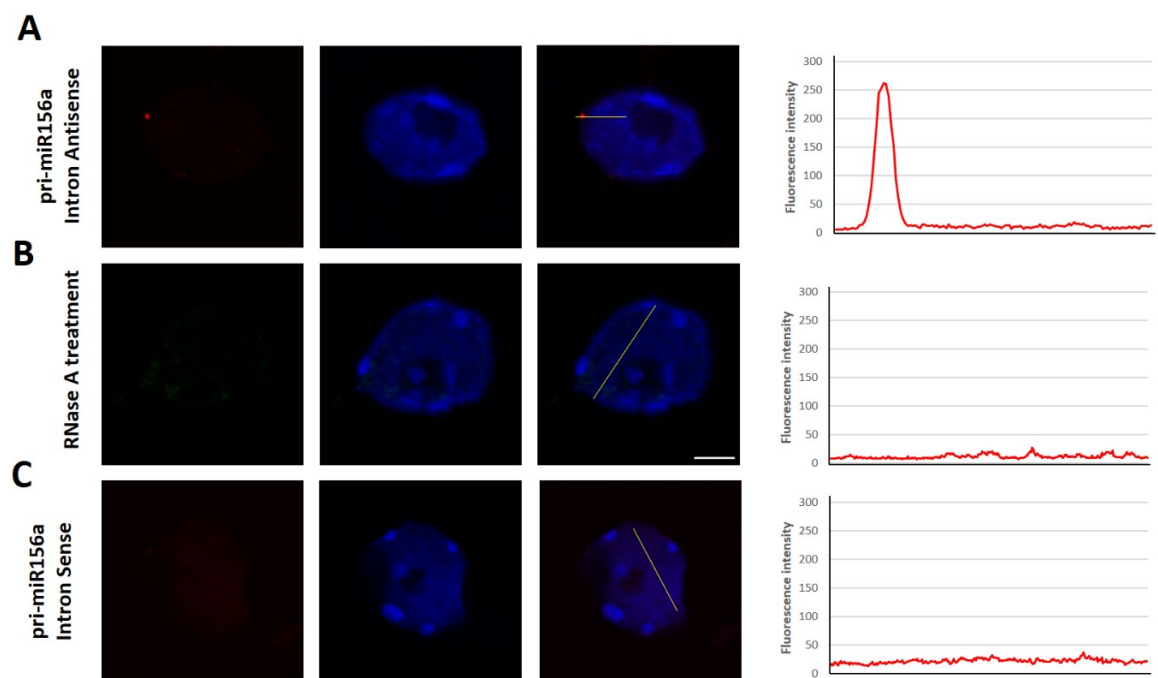
In our studies, the results were registered with the Leica SP8 confocal microscope using a diode 405 laser, an argon/ion laser with a wavelength of 488 nm, and a diode laser DPSS 561 that emitted light with a wavelength of 561 nm with an optimized pinhole, long exposure time (200 kHz) and 63× (numerical aperture, 1.4). Images were collected sequentially in blue (Hoechst 33342), green (Alexa 488 fluorescence), and red (Alexa 555) channels. The low laser power (0.4%–5% of maximum power) and a single-channel collection were set to minimize bleed-through between fluorescence channels. For bleed-through analysis, Leica SP8 software was used.

The presented approach was previously applied to localize pri-miRNA156a and pri-miRNA163 in the nuclei isolated from leaves of 4-week-old *A. thaliana* (Figure 2).



**Figure 2. Detection of pri-miR156a and pri-miR163 using digoxigenin-labeled probes and antibodies targeting digoxigenin.** The probes hybridizing to intron sequence located downstream of stem-loop structure were applied. (A) Schematic illustration of stem-loop structure of pri-miRNA; probe is depicted in red. (B) Detection of pri-miRNA156a (left image, green) and pri-miRNA163 (right image, green). DNA was stained with Hoechst (blue). Scale bar = 2.5  $\mu$ m.

To confirm that the observed nuclear spots were due to hybridization of probes to pri-miRNA, we treated the isolated nuclei with RNase A (100  $\mu$ g/mL in PBS for 30 min) and then applied the probe recognizing the intronic sequence of pri-miR156a (Figure 3B). We have not observed hybridization signals in nuclei treated with RNase A. Similarly, we have not observed fluorescence signal if a sense probe matching the pri-miR156a intron was applied (Figure 3C). Additionally, we have calculated a fluorescence intensity by Fiji software to distinguish the positive fluorescence signal of hybridization from the background.



**Figure 3. Detection (fluorescence in situ hybridization, FISH) of pri-miR156a (red) using the intron-recognizing probe (Intron antisense probe), in the nuclei treated with RNase A and using the probe with the sequence identical with a fragment of intron (Intron sense probe).** The fluorescence intensity is plotted along the yellow line shown on each merged image. Scale bar = 2.5  $\mu$ m.

## Recipes

### 1. Hybridization buffer

Reagent	Final concentration	Quantity
Formamide	30% (v/v)	600 µL
20× SSC	4×	400 µL
100× Denhardt's solution	5× Denhardt's buffer	100 µL
Sodium phosphate buffer (0.5 M)	50 mM	200 µL
EDTA (0.5 M)	1 mM	4 µL
H <sub>2</sub> O	n/a	696 µL
Total	n/a	2 mL

### 2. Denhardt's solution 100× (50 mL)

Reagent	Final concentration	Quantity
Bovine serum albumin (BSA)	2% (w/v)	1 g
Ficoll 400	2% (w/v)	1 g
Polyvinylpyrrolidone	2% (w/v)	1 g
H <sub>2</sub> O	n/a	50 mL
Total	n/a	50 mL

### 3. LB01 buffer (10 mL)

Reagent	Final concentration	Quantity
Tris-HCl (1M)	15 mM	150 µL
EDTA	2 mM	20 µL
Spermine	0.5 mM	5 µL
Potassium chloride (KCl) (1M)	80 mM	800 µL
Sodium chloride (NaCl) (1M)	20 mM	200 µL
Triton (10%)	0.1%	1 mL

### 4. Sorting buffer (10 mL)

Reagent	Final concentration	Quantity
Tris-HCl (1 M)	100 mM	1 mL
Potassium chloride (KCl) (1 M)	50 mM	500 µL
Magnesium chloride (MgCl <sub>2</sub> ) (1 M)	2 mM	20 µL
Tween 20 (10%)	0.05 mM	50 µL
Sucrose	5%	0.5 g
Bovine serum albumin (BSA)	3%	0.3 g

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## Competing interests

There are no conflicts of interest or competing interests.

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