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smFISH for plants

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Abstract: Single molecule fluorescence in situ hybridization (smFISH) is a powerful method for the visualization and quantification of individual RNA molecules within intact cells. With its ability to probe gene expression at the single cell and single-molecule level, the technique offers valuable insights into cellular processes and cell-to-cell heterogeneity. Although widely used in the animal field, its use in plants has been limited. Here, we present an experimental smFISH workflow that allows researchers to overcome hybridization and imaging challenges in plants, including sample preparation, probe hybridization, and signal detection. Overall, this protocol holds great promise for unraveling the intricacies of gene expression regulation and RNA dynamics at the single-molecule level in whole plants.

Keywords: smFISH, plant, RNA, transcription, imaging, single-molecule

1. Introduction

During the past few decades, innumerable studies have shed light on the importance of transcriptional regulation, RNA molecules and their functions. A central regulatory role was thus attributed to RNA. Indeed, apart from its primary function as a messenger for protein synthesis, the roles of RNAs over the years have been found to be more diverse and prevalent than were initially thought. RNA molecules fulfill diverse tasks in the cell, through their secondary structure and interaction with various proteins and nucleic acids, and by also being the key constituents of cellular machineries (ribosomes, tRNAs...). Tight regulations at the level of expression, post-transcriptional modifications and RNA localization have been shown to be compulsory for many biological functions of RNAs (1,2).

Intracellular transcript localization has gained a lot of interest over the years as it is a ubiquitous mechanism taking place in a wide range of organisms, from bacteria to humans. It offers local protection from degradation and restricts the transcript spatially and temporally to discrete sites within the cell, where it is of use. It is also an efficient means of mass production of localized proteins, which would facilitate protein-protein interactions, folding pathways and post-translational modifications (3,4,5).

RNA localization is a highly regulated process, indispensable to cell development, polarity and differentiation, determination of cell fate and tissue functionality, signaling and physiology. Non-uniform subcellular mRNA distribution has been described in animal oocytes (6,7) as well as specialized cells (8,9,10,11), and shown to be essential for proper embryonic patterning during development (12,13). More

recently, these discoveries extended outside of the animal kingdom, whereby specific intracellular mRNA targeting was observed also in fungi (14,15), bacteria (16) and in plants (reviewed in 4,17).

While intracellular RNA localization is a conserved process across all kingdoms, less effort has been put in its study in higher plants as compared to the animal phyla. One reason may be linked to the pecto-cellulosic cell wall, which acts as a physical barrier often reducing the efficiency of cell biology techniques. Besides, mature plant tissues are composed of differentiated cells containing a large vacuole compartment which pushes the cytoplasm to the periphery of the cell, whereas more cytoplasmically dense cells as meristematic ones are very small in size, making the observation of RNA in confined areas difficult. Despite this, a number of studies focused on the differential localization of transcripts in plants and their fundamental roles in various cellular processes.

Evidence for polar localization of mRNA in plant cells include the differential subcellular localization of expansin mRNA in xylem cells of *Zinnia elegans*, exclusively to their apical or basipetal end depending on the expansin gene and organ. Other examples include the accumulation of profilin mRNA at the tips of emerging root hair in higher plants (18), and the formation of basal/apical gradients of mRNA with development-specific patterns of distribution in the unicellular green alga *Acetabularia acetabulum* (19). Additionally, various types of mRNA have been shown to be asymmetrically distributed and concentrated in particular subcellular domains such as in developing rice endosperm (17), in ER compartments, in proximity to the mitochondria (20) and chloroplast (21).

In addition to intracellular RNA localization, RNA species can move over long distances in plants, between different tissues and organs, to ensure cell-to-cell communication and the coordination of plant growth, development, and adaptation to biotic and abiotic stresses (22, 23, 24, 25). Indeed, plants rely on systemic signals where RNAs provide an efficient and specific remote-control system to orchestrate developmental and physiological processes. The systemic migration of such a high number of RNAs between plant cells raises numerous questions regarding the pivotal roles of RNAs in distant organs, the subtle mechanisms that allow such specificity and fine-tuning of directional transport and how they may function in regulation of vital adaptational processes.

Transcript profiling and RNA analysis provide powerful tools to unravel the complexity of molecular events taking place within biological systems. While various methods have been developed and used to reliably detect genome-wide changes in gene expression (26, 27, 28, 29), they differ considerably in their abilities to detect distinct steps of gene expression, and to identify various RNA species. Additionally, they often provide collective averaged data of many RNA molecules without considering the heterogeneity in the population or the fact that a transcript's stability varies throughout its lifespan (30, 31). More importantly, these techniques lack cellular resolution and fail to measure RNAs at the level of single cells or within cellular compartments (28, 30). Hence, despite the progress in unraveling RNA functions, understanding the interplay between the different molecules and machineries remains a difficult task without the ability to visualize them in intact cells. Consequently, the implementation of techniques to visualize sequence-specific RNA is indispensable.

In Situ Hybridization (ISH) has evolved significantly over the years, transitioning from a time-consuming month-long assay to a much faster technique capable of detecting single transcripts in just a couple of

days (32, 33). This transformation was facilitated by the development of various enhancements, such as the use of radioactively labeled probes initially (33), followed by histochemical detection methods (34) and, notably, the introduction of fluorescent oligonucleotides (32). Single-molecule fluorescent in situ hybridization (smFISH) became a powerful application of ISH that employs multiple fluorescently labeled DNA probes to visualize individual mRNA molecules as diffraction-limited fluorescent spots, enabling the study of gene expression patterns, RNA distribution, and transcription kinetics with cellular resolution.

SmFISH has been used in various organisms and different cell types with variable success. As opposed to older methods, it provides cellular resolution and allows the study of gene expression patterns, asymmetric RNA distribution within cells and revealed cell-to-cell variability existing in tissues. Multiple technological advancements made it the method of choice for quantifying low-abundance mRNAs and providing insights on transcription kinetics and the bursty nature of gene expression (35, 36), mRNA export (37), translation and even decay (38, 39). Various improvements also enabled high quality three-dimensional imaging, multiplexing different RNA species, co-visualization of RNA with proteins (40, 41) and paved the way for image-based transcriptomics (42, 43).

Although well documented, the smFISH workflow in general holds some drawbacks. A first inconvenience is the cost of synthesis of fluorescent oligonucleotide probes. Another major impediment is that smFISH always suffers from background and non-specific binding of stray probes which generates false-positive signals and affects the ability to differentiate true targets from background noise. Minimizing these artifacts necessitates the use of a larger number of probes. To achieve this and reduce costs, a single molecule inexpensive FISH (smiFISH), was developed (44). The approach is based on the use of an increased number of unlabelled primary probes specific to the targeted RNA, which carry an extra readout sequence that can be detected by fluorescently labeled secondary probes. This in general yields higher signal-to-noise ratio and enhanced signal quality at low costs (43; 44).

In plants, the permeability of cell walls creates difficulties and restricts efficient probe penetration. Hence, hybridization outcomes were limited. Classically, mRNA tissue hybridization used sections of biological samples to allow better probe accessibility to deeply embedded cell types (45). This provides access to the localization of mRNA in various plant tissues (46, 47, 48), and organs using Dig-labeled probes. It was shown to be reliable for the analysis of transcript localization in different developmental processes (49) but could not reach single molecule resolution. Multi-color whole-mount ISH was also successfully implemented in plants (50).

To overcome the limited sensitivity provided by these techniques, more direct labeling of transcripts with fluorescently labeled probes has been employed. However, the optical properties of plant cells and tissues present considerable challenges for fluorescence microscopy. Many endogenous molecules emit high levels of background and autofluorescence adversely affects detection efficiency. Nevertheless, recent advances done in that regard allowed to circumvent these problems and use smFISH in *Arabidopsis* roots (51). This also enabled the quantification of mRNAs per cell and exploration of cell-to-cell variations to study RNA polymerase II transcription, gene bursting (36) and to explore different steps associated with the RNA regulation (Figure1).

In the present work, we describe smFISH protocols that are successfully used in plants. We focus on *Arabidopsis* root since our genes of interest were highly expressed in that organ and rapidly altered due to nutritional stress (36). However, the protocol also works well for leaves and stems.

Overall, the technique provides a resource for plant researchers investigating transcription dynamics, RNA metabolism and single-molecule studies. Adequate computational tools and algorithms must be combined for image analysis, molecule counting and spatial organization characterization. Consequently, smFISH holds great promise for visualizing transcripts from birth-to-death and unraveling the intricacies of gene expression regulation in plants.

2. Materials

2.1 reagents

1. Sterile Petri dishes
2. Autoclaved plant growth medium: Murashige and Skoog medium diluted tenfold (MS/10), 5 g/L sucrose and 8 g/L agar, pH 5.7 (described in 52), with 0.5 mM KH₂PO₄ or without phosphate (0.013 mM KH₂PO₄).
3. Sterilized Arabidopsis seeds
4. Carbonate buffer: 0.1 M Na HCO₃ pH 8.8
5. Micropore tape.
6. x50, 0,7M, pH 5.8 (or 7 if we want observe GFP signal) stock solution of 2-(N-morpholino) ethane sulfonic acid (MES) buffer solution.
7. 4% PFA/MES: 4% paraformaldehyde (PFA) diluted in x1 (or 14 mM) MES buffered at pH 5.8 or 7 if we want observe GFP.
8. Liquid nitrogen
9. Forceps
10. Glass microscopy slides (0.17 mm thick) and coverslips
11. Razor blade
12. 100%, 80%, 70% Ethanol
13. 20x Saline-Sodium citrate buffer (SSC)
14. 15% Formamide solution: 15% formamide, 1x SSC in DDW
15. Cy3 or Cy5 labeled oligonucleotide probes (amino-modified - C6 dT and labeled in vitro, see section 3.2 'probe labeling')
16. 20 mg/ml E. Coli tRNA
17. Autoclaved water
18. 20 mg/mL RNase-free BSA
19. 200 mM Vanadyl ribonucleoside complex (VRC) (non-mandatory)
20. 40% Dextran sulfate (100 mg/mL)
21. Anti-fading mounting medium containing DAPI (Prolong Diamond Gold, Invitrogen)
22. 3M Sodium acetate pH 5.2
23. DMSO
24. Tris-EDTA buffer
25. Probe mix 1: 1xSSC, 360 ng/μL *E. coli* tRNA, 15% formamide, 0.4 ng/μL probe mix (see Note 3,4).
26. Probe mix 2: 200 μg/μL RNase-free BSA, 2mM VRC (Vanadylribonucleoside), 10,8% dextran sulfate

2.2 Equipment

1. Plant growth chamber for *Arabidopsis* plants under a 16 h light/8 h dark regime with 25°C/22°C respectively.
2. Heating block
3. Horizontal shaker

4. Zeiss Axioimager Z1 wide-field upright microscope equipped with a camera sCMOS ZYLA 4.2 MP (Andor), using a 100x, NA 1.4 Plan Apochromat oil objective.
5. Dragonfly (Oxford instrument) equipped with four laser lines and an ultrasensitive EMCCD camera (iXon Life 888, Andor) mounted on a Nikon Eclipse Ti2 microscope body, a 40x, NA 1.3 Plan Fluor oil objective or a 60x, NA 1.4 Plan Apochromat oil objective coupled with a supplementary lens of 2x.

2.3 Software

Microscope image acquisition and analysis software (e.g. ImageJ, HotSpot (53), FISHquant (54)).

3 Methods

3.1 Probe design (Figure 2)

For DNA oligonucleotide design, aim for about 40 oligonucleotides of ~45 bases with 65-70 °C T_m and a GC content below 60%. We use <http://biotools.nubic.northwestern.edu/OligoCalc.html>, set with 50mM salt, nearest neighbor, 10 nM primers, ssRNA. Use the reverse complementary strand for the mRNA of interest. Search for an area with a convenient T_m and where you can locate two internal T (on the probe strand) separated from each other and from extremity by at least 10 bases. Adjust the length of the oligos to reach a proper T_m. Label the two internal T which will be modified into C6dT. Then add a 5'T X at the beginning of the oligo and X T at the 3' end. Both X will also correspond to C6dT modified base. Order the oligonucleotide with the C6dT modified bases.

3.2 Probe labeling Cy3 or Cy5:

1. 1 vial of Cy3 or Cy5 (Cytiva PA23001 or PA25001, Amersham) allows the preparation of 15µg oligonucleotides.
2. For 1 vial, add 30µl DMSO and vortex twice for 30s.
3. To label 3 probes: take 5 µg of each unprotected oligonucleotide, add 23 µl of 0.1M carbonate buffer pH 8.8 and 10 µl of DMSO/Cy3 solution per probe.
4. Vortex twice for 30s and leave over-night (O/N) at room temperature in the dark.
5. Add 10 µg of *E. coli* tRNA and precipitate with 3.0 M sodium acetate, pH 5.2 (1/10 volume) and 3 vol 100% ethanol . Leave O/N at -20°C and wash with 80% ethanol. Supernatant and pellet should be red or green according to the fluorochrome used.
6. Resuspend oligo in 100 µl H₂O/0.3M Sodium Acetate. Precipitate again with 3 vol 100% ethanol and wash with 300 µl 80% ethanol. Repeat this procedure until the supernatant becomes transparent and only the pellet is colored.
7. Resuspend in 250 µl TE (final concentration 20 ng/ µl).

Adapt volumes to optimize the number of probes to label (5 maximum), for example with 5 probes: 3 µg oligonucleotide, 13.8 µl buffer and 6µl DMSO/Cy3 per probe. **We do not advise to label reduced amount of oligonucleotides to avoid loss of material during precipitation procedure.**

3.3 Sample preparation and fixation (Figure 3)

1. Prepare petri dishes with *in vitro* plant growth medium, suitable for the experimental requirements. Once set, sow sterilized *Arabidopsis* seeds.
2. Seal the petri dish with two rounds of micropore tape and store overnight at 4°C for stratification.
3. Transfer the plate to a growth cabinet with 16h light/8h dark regime with 25°C/22°C respectively.
4. Grow the seedlings for the appropriate duration and conditions in which the gene of interest is induced. In parallel, grow negative control seedlings in which the gene of interest is repressed or deleted.

5. Transfer the plants into a small glass dish containing freshly prepared 4% PFA/ MES buffer (pH ~5.8 or 7 depending if GFP signal should be conserved), and incubate for 20 min at room temperature in a fume hood (see Note 1).
6. Isolate material to label (here roots but can be other tissue) and remove from the fixative solution. Wash twice with 1x MES buffer.
7. Arrange 3-4 roots onto a microscopic slide and cover with a coverslip. Gently squash each root onto the slide using your thumb and be careful to avoid breaking the coverslip. Aim to splay the roots sufficiently to produce multiple files of isolated cells in a single cell layer.
8. Use tweezers to hold the squashed roots under the coverslip and immerse each slide in liquid nitrogen for ~5 sec. After removal from the nitrogen, ease a razor blade between the coverslip and the slide and flick the coverslip off.
9. Leave samples to air dry at room temperature for a minimum of 30 min. To avoid increased levels of autofluorescence do not leave to dry for longer than 2 h.
10. Permeabilize the samples by immersing the slides into a Coplin jar containing 70% ethanol overnight at 4°C. Fixed roots can be stored at 2 to 8°C in 70% ethanol up to a week prior to hybridization.

3.4 *In situ* hybridization (Figure 3)

1. Rinse plants once with MES then aspirate the liquid.
2. Incubate the slides in 15% formamide solution (see Notes 1-2) for 15 min at room temperature:
3. During the incubation time, prepare Mixes 1 and 2 separately on ice according Section 2.1.
4. A volume of 100µL (Mix 1 + Mix 2) is sufficient for one slide (22 x 50 mm).
5. Vortex thoroughly Mix 2. Heat Mix 1 at 85°C for 3 mins in order to denature secondary structures and then place on ice (see Note 5).
6. Then add Mix 1 to Mix 2 and vortex again.
7. Add 100 µL of the hybridization mix on top of the fixed plants on the slide, then lay a coverslip on top to prevent evaporation. Be careful to avoid air bubbles.
8. Arrange the slides in a square petri dish. Put a cap of a falcon tube containing some water inside the Petri dish to create humidity and close the Petri dish.
9. Wrap a Parafilm sheet around it, and incubate at 37°C overnight.

3.5 Washing and mounting (Figure 3)

1. Remove the coverslip and place the slides in a coplin jar containing freshly prepared 15% formamide (same as in section 3.4). Put the jars on a horizontal shaker for 45 min at 37°C. Repeat this step twice.
2. Rinse the slides twice in 1xMES buffer before mounting.
3. Drop 20 µL of an anti-fading mounting medium containing DAPI on the slide and lay the coverslip on top (see Note 6). Observations can be performed immediately.

3.6 Imaging and image analysis

1. Observe and image plants on a microscope. We use either a spinning disk confocal or a wide field microscope. For spinning disk microscopy, we use a Dragonfly (Oxford instrument) equipped with four laser lines and an ultrasensitive EMCCD camera (iXon Life 888, Andor) mounted on a Nikon Eclipse Ti2 microscope body, using a 40x, NA 1.3 Plan Fluor oil objective or a 60x, NA 1.4 Plan Apochromat oil objective coupled with a supplementary lens of 2x, using z-stacks of about 50-80 slices with a 0.5 μm or 0.4 μm step. For wide field imaging, we use a Zeiss Axioimager Z1 wide-field upright microscope equipped with a camera sCMOS ZYLA 4.2 MP (Andor), using a 100x, NA 1.4 Plan Apochromat oil objective. For these z stacks, a step of 0.3 or 0.4 μm is used.
2. Images properties (e.g. brightness, contrast, colors) can be adjusted using ImageJ (Figure 4).
3. Quantification of smFISH spots and transcription sites is performed using FISH-quant. Follow the detailed instructions of the developers. Use the negative control plants as basis to determine the background of FISH spots.

4. Notes

1. If the plants used are transgenic and fluorescent, we recommend fixing in 2% paraformaldehyde for 12 min to maintain the fluorophore, or replacing formamide with 2.4 M Urea (55).
2. In case of high background, the formamide concentration can be increased from 15 to 50% for both the washes and hybridization buffer (or use 8 M urea).
3. We also obtained good results with an alternative hybridization buffer (100 mg/mL dextran sulfate and 10% formamide in 2X SSC).
4. The amount of probes can be increased if the signal is too low, or decreased if the background is too high.
5. In our hands, the protocol described here works also for smiFISH in plants (but turns out to be not as good as smFISH). For smiFISH, step 5 in section 3.4 for denaturation of secondary probes must be omitted, otherwise the secondary probes would detach.
6. For long-term storage, the slides can be kept at -20°C for future imaging.

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