Single Molecule Fluorescence *in situ* Hybridization in Whole Mount Tissue

Closely adapted from

- Duncan S, Olsson TSG, Hartley M, Dean C, Rosa S (2016) **A method for detecting single mRNA molecules in *Arabidopsis thaliana*.** Plant Methods. doi: 10.1186/s13007-016-0114-x.

- Rosa S, Duncan S, Dean C (2020) **Mutually exclusive sense-antisense transcription at FLC facilitates environmentally induced gene repression.** Nat Commun. doi: 10.1038/ncomms13031.

- Duncan, S., Olsson, T. S., Hartley, M., Dean, C. and Rosa, S. (2017). **Single Molecule RNA FISH in *Arabidopsis* Root Cells.** *Bio-protocol* DOI: 10.21769/BioProtoc.2240.

i. Summary

Traditional *in situ* hybridization is a powerful tool for the localization of RNA molecules within plant tissues. However the sensitivity of this technique is limited to single cell resolution and is unable to discriminate between sub-cellular expression domains. Single molecule fluorescent *in situ* hybridization (smFISH) is an evolution of this technique that allows for the resolution of expression to the level of a single messenger or non-coding RNA. This technique relies on the use of multiple fluorescent probes targeted to a single RNA sequence, which amplifies the signal such that is it visible using epi-fluorescent imaging. This allows both the localization and the quantification of single RNA molecules thus providing an increased level of precision in the study of gene expression.

ii. Keywords

In situ hybridization, nucleus, cytoplasm, non-coding RNA, mRNA, single molecule, Arabidopsis thaliana

1. Introduction

An aspirational gold standard in molecular biology is the ability to localize and quantify the activity of single RNA molecules in a cellular context. Although *in situ* hybridization has been routinely used to define the expression domain of populations of RNA molecules it lacks the sensitivity for sub-cellular localization and/or quantification. As such it can be a relatively blunt tool used for the identification of an expression domain, notably in the plant shoot or root apical meristems (Yang *et al*, 2020).

Single molecule fluorescent *in situ* hybridization (smFISH) emerged as a technique that was able to localize individual transcripts in yeast or mammalian cells (Li and Neuert, 2019; Timme and Wood, 2019). This technique inverts the principles that enable the localization of repetitive RNA sequences (such as rRNA), which rely upon a single (or small number) of probes to visualize a highly repetitive sequence. In contrast smFISH uses large number of probes targeted a single RNA to visualize the position of that molecule.

Plant smFISH was developed by members of Caroline Dean's lab at the John Innes Centre, UK. The method was used in Arabidiopsis root tips to assess the mRNA localization of the key regulatory gene Flowering Locus C (FLC) and of its regulatory non-coding RNAs (Duncan *et al*, 2016; Rosa *et al* 2016). This technique has been adapted by the Caplan group at the University of Delaware for use in maize anther tissues, showing that is can have broad applicability (Huang *et al*, 2020). However the significant difference between these uses of the protocol is that the Arabidopsis technique assesses expression in whole mount tissues whereas the maize technique uses embedded and sectioned tissues. Whereas probe design is similar in each protocol there are major differences in the preparation of tissue, which leads to changes in the required immediacy of the downstream imaging. Whole mount samples need to be imaged almost immediately whereas sectioned maize tissues can be stored prior to sectioning, pre-hybridisation, hybridisation or imaging.

In this article we will outline the steps required for whole-mount analysis of Arabidopsis roots. This method is more standarised than those that would be used for the analysis of sectioned samples, for which there is a lot of variation in their treatment.

2. Materials [NOTE 1]

- User preferred Arabidopsis growth media and dishes
- RNAse free Petri Dish (plastic or glass)
- 6-well tissue culture dish (optional)
- Razor blades
- Liquid nitrogen
- Fine forceps for dissection, larger forceps to hold slides in nitrogen
- Poly-Lysine slides, 20mmx20mm coverslips [**NOTE 2**]
- Empty 50 mL tube Falcon for 2 slides with 30 mL 70% ethanol
- Nail Varnish
- Hybridization chamber [**NOTE 3**]
- 37°C chamber

- 4% Paraformaldehyde in 1xPBS- (*Maximum required amount 20ml*)
- 1xPBS (*50ml*).
- 2xSSC (*250ul*)
- **Wash buffer**: 10% formamide, 2x SSC (*50ml*)
- **smFISH Probe Stock:** (25 µM) Stellaris 5 nmol oligo probe set, 200 µl Tris-EDTA buffer solution (See advice on Probe Design below) (*2ul*)
- **Hybridization solution**: 100 mg/ml dextran sulphate, 10% formamide, 2x SSC
- **Probe Solution**: 1µl of each required probe stock solution to 100 µl of hybridization solution (250 nM final concentration). (*100ul*)
- **DAPI solution**: 100 ng/µl DAPI, 10% formamide, 2x SSC (*200ul*)
- **Anti-fade GLOX(-) buffer** (minus enzymes): 0.4% glucose, 10 mM Tris-HCl, 2x SSC (*100ul*)
- **Anti-fade GLOX(+) buffer** (containing enzymes): For a final volume of 102 µl, mix:100 µl anti-fade GLOX(-) with 1µl glucose oxidase, 1µl bovine liver catalase suspension (mildy vortexed) (*110ul*) [**NOTE 4**]

3. Methods

3.1 Probe Design

smFISH relies on the hybridization of ~50 individual labelled probes targeted at a single RNA (mRNA or non-coding). This number of probes targeted to a single sequence allows for the visualization of individual molecules [**NOTE 5**]. Previously 48 probes have been used for a successful visualization and these can be designed using the Stellaris Probe Designer Software (https://www.biosearchtech.com/support/education/stellaris-rna-fish). This uses an oligo probe length of 20nucleotides (nt) with a minimum distance of 2nt between individual probes. A mixture of probes that target exonic and intronic allow resolution of nascent and processed transcripts [**NOTE 6**].

Prior to ordering it is advisable to perform a BLAST search against each sequence to ensure target specificity; against Arabidopsis sequences this can be performed on the TAIR website (https://www.arabidopsis.org/Blast/).

3.2 Sample Preparation

1. Grow Arabidopsis seedling on user preferred media in the growth conditions of choice.
2. After 10d use a sharp razor blade to remove ~1cm from the root tip of Arabidopsis seedlings. [**NOTE 7**].
3. Immediately place root tips into petri dish with 4% paraformaldehyde in PBS and incubate for 30 min at room temperature under a fume hood [**NOTE 8**].
4. Remove roots from the fixative and wash twice with 1x PBS in a fresh petri dish or 6-well tissue culture dish [**NOTE 9**].
5. Arrange 3 roots onto a poly-lysine microscope slide and add a coverslip. Gently squash each root onto the slide using either your thumb being careful to avoid breaking the coverslip. Aim to splay the roots sufficiently to produce multiple files and isolated cells in a single cell layer.
6. Use larger forceps to hold the squashed roots under the coverslip and immerse each slide in liquid nitrogen for ~5 sec.
7. After removal from the liquid nitrogen, carefully ease a razor blade between the coverslip and the slide and flick off the coverslip.
8. Leave samples to air dry at room temperature for a minimum of 30 min [**NOTE 10**].
9. Permeabilize the samples by immersing the slides into a 50 mL Falcon tube containing 30 mL of 70% ethanol for a minimum of one hour at RT or can be stored at 4°C until needed [**NOTE 11**].

3.3 Hybridization of probes and slide preparation

1. Remove slides from the ethanol and allow residual ethanol to evaporate at room temperature.
2. Wash twice on the slide with 100 µL of **Wash buffer** for 5 min.
3. Whilst washing prepare **Probe Solution**.
4. Add 100ul **Probe Solution** to roots, add a coverslip to prevent evaporation and incubate in a humid chamber at 37 °C in the dark for at least 4hr or overnight.
5. *Optional Overnight Break*
6. Pre-warm **Wash Buffer** and **DAPI Solution** at 37°C
7. Remove the cover slip and wash the samples in 200 µl pre warmed **Wash buffer** for 5 min on the slide.

8. Immerse the slide in a Falcon Tube containing ~30 ml of **Wash buffer** and incubate for 30 min at 37 °C, in the dark.
9. Wash the samples in 200 µl pre-warmed **DAPI solution** and incubate for 30 min at 37°C, in the dark.
10. Remove DAPI solution carefully with a pipette and rinse with ~100µl 2xSSC
11. Remove 2xSSC and add 100µl **GLOX(-) buffer** for 2 minutes.
12. Remove **GLOX(-) buffer** and add 100µl of anti **GLOX(+) buffer** to each slide.
13. Add a cover slip to the sample and remove excess **GLOX(+) buffer** [**NOTE 12**].
14. Seal the cover slip with nail varnish.
15. Begin imaging on the same day to avoid sample drying and fluorophore fading [**NOTE 13**]

3.4 Image Acquisition

An advantage of this protocol is that imaging can be performed using an epi-fluorescent microscope and therefore does not rely upon use of a con-focal microscopy. However a standard CCD camera that is optimized for low light level imaging is needed for successful image capture. The individual user should be familiar with the filters required to image the fluorophores that they have used to label their probes.

Other considerations for the imaging microscope are the requirement for a high numerical aperture (> 1.3) and 60 or 100x oil-immersion objective as well as a strong light source, such as a mercury or metal-halide lamp. Previously research has shown that Xenon or LEDs are typically not bright enough.

DAPI is imaged with an excitation line of 405nm and signal was detected at wavelengths of 420–480 nm. In our experience exposure times between 200–250 ms were used and if appropriate a series of optical sections with z-steps of 0.2 µm were collected.

Depending on available hardware, users will need to establish their own parameters for imaging. Importantly we recommend that as users establish these parameters the following controls are carried out:
A, No probe (where probes are omitted from the hybridization solution)
B, RNase A treatment to confirm RNA specificity. Samples were incubated with RNase for 1 h at 37 °C in a humid chamber after the ethanol permeabilization in **Step 3.2.9**, rinsed in 10 mM HCl for 5 min, washed twice with 2× SSC for 5 min before the protocol is continued in **Step 3.3.1**.

The method will typically allow >100 cells to be imaged from 3 roots. These images are then suitable for further analysis using the automated mRNA counting programme.

Open source analysis software requires images to be in TIFF format so these can be collected at time of imaging or converted using Bio-Formats software, which is available as an ImageJ plug-in [**NOTE 14**]

3.5 Image Analysis

smFISH image analysis consists of two components—cell segmentation and mRNA counting. These have been incorporated into open access software available for

download for use on a Mac OS at https://github.com/JIC-CSB/FISHcount. This software is run on Python so depending on their level of ability in coding the user may need to take the advice from a more experienced bioinformatician.

For users working on a Windows machine software has been developed by Mueller *et al* (2013) and can be downloaded here https://code.google.com/archive/p/fish-quant/

Mac or Windows users should make themselves familiar with the software that they will use. A detailed explanation of the rationale behind use of the software can be found at Duncan *et al* (2016) [**NOTE 15**].

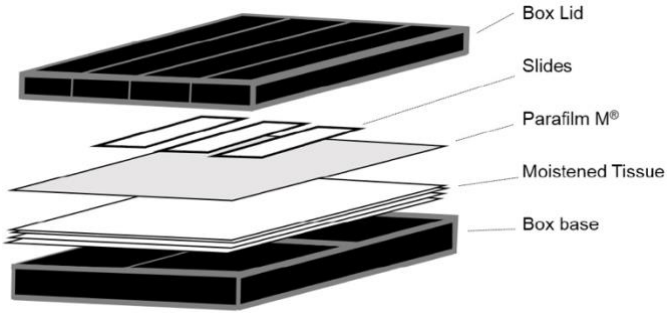
After performing analysis to a whole dataset, we recommend that users manually inspecting each ‘mRNA Per Cell’ output image to ensure that the image analysis workflow has not generated inaccurate results through incorrect segmentation. Previous experience places the majority of output numbers in double-digits so datasets that deviate from this expectation may indicate that an error has occurred during experimentation or image analysis [**NOTE 16**].

4. Notes

NOTE 1: All procedures should be prepared to remove RNase contamination. This includes use of gloves, barrier tips and RNAse free plastic wear. Use nuclease free water and NOT DEPC treated water.

NOTE 2: These slides are not essential but the samples adhere better to these than to untreated slides

NOTE 3: Although hybridization chambers are available commercially a home-made version can be made using a 10 cm square Petri dishes covered externally with a layer of black insulation tape. A double layer of tissue was then placed in the base and saturated with sterile water before slides were placed on top of a single layer of Parafilm (see figure taken from Duncan *et al*, 2017)



NOTE 4: Oxygen-scavenging GLOX buffer maximised the stability of our smFISH fluorophores and rapid bleaching was observed when substituted with the commercial anti- fade mounting media Vectorshield.

NOTE 5: Previously we have found that Quasar 570 and Quasar 670 dyes are equally suitable for imaging RNA in *Arabidopsis* root cells but crucially we were unable to detect RNA labelled with Fluorescein modified probes. Depending on their needs users can experiment with different probes but this might be a costly and time consuming undertaking.

NOTE 6: The resolution of nascent versus processed transcripts can be achieved by double labelling of samples. This requires two set of 48-probes targeted at specific sequences. An example of this dual labelling is taken from Duncan *et al* (2017), Figure 4.

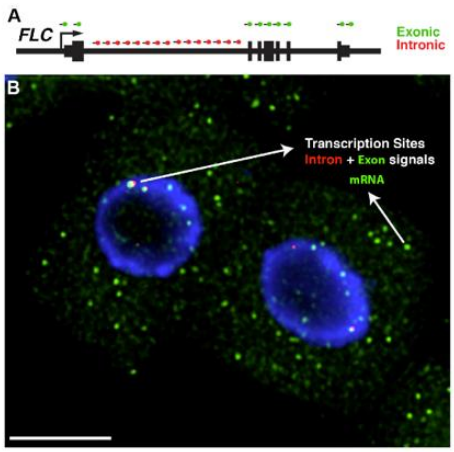


Figure 4. Detection of *FLC* transcripts in single cells. A. Schematic of the probes used to detect *FLC* transcripts: intronic (red) and exonic (green). *FLC* exons are represented as thick lines and introns shown as thin lines. B. Representative image showing *FLC* mRNA (green) and *FLC* nascent RNA (red) expression in *Arabidopsis thaliana* root cells. The diffraction-limited spots in the cytoplasm correspond to single *FLC* mRNA molecules (green), and the intense spots co-localizing with intronic signals (red) correspond to transcription sites. Depending on the cell cycle stage one can detect up to 2 dots (G1 phase) or 4 dots (G2 phase) for the intronic signals. The nucleus is stained with DAPI and shown in blue. Scale bar = 5 µm.

It is important to note that each transcript will behave differently so users should amend their probe sets according to their own targeted gene. Experience indicates that using more probes will not have a negative effect on the ability to visualise transcripts. To date the literature reports successfully use of smFISH in whole mount Arabidopsis roots targeted at the FLC locus or at the housekeeping gene Protein Phosphate2A (PP2A). It is unlikely that these loci are uniquely suited to this technique but new users will need to empirically test their probe sets against their gene of interest.

NOTE 7: A shorter piece of root tip could be removed but this makes manipulation of the samples more challenging and will also decreases the amount of tissue available for analysis. Moreover this method is most effective when looking at the larger cells of the root elongation zone.

NOTE 8: Care must be taken when using paraformaldehyde and where possible should be used under a fume hood.

NOTE 9: Roots can either be moved between filled wells of a tissue culture dish or the liquid can be replaced from a single petri dish. The former strategy can risk damaging fragile root tips but can be done safely if care is taken.

NOTE 10: To avoid increased levels of auto-fluorescence do not leave to dry for longer than 2hr.

NOTE 11: Fixed roots can be stored at 2 to 8 °C in 70% ethanol up to a week prior to hybridization

NOTE 12: 100ul of GLOX(+) buffer should be sufficient to correctly mount the cover-slip on the samples.

NOTE 13: Immediate imaging is recommended as a noticeable reduction in image quality was observed around 4 h after mounting samples.

NOTE 14: Bio-Formats is available from the Open Microscopy Environment (OME) https://www.openmicroscopy.org/bio-formats/ and is available as an ImageJ plug-in.

NOTE 15: An example output from the mRNA Counting software is taken from Duncan *et al* (2017), Figure 6.

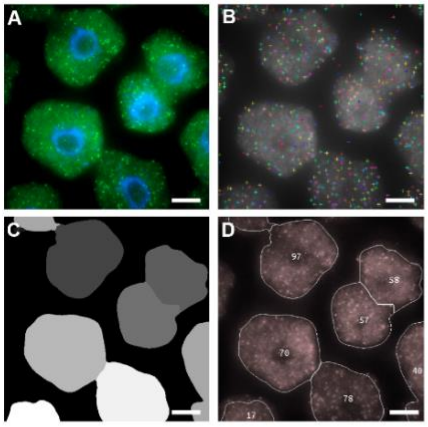


Figure 6. Automated image analysis of *FLC* mRNA. A. Representative maximum projection image of cells labeled with *FLC* mRNA probes (green). DNA labeled with DAPI (blue). B-D. Screen shots showing sequential detection steps used to determine positive mRNA signals. B. *FLC* mRNA spot locations; C. Cell Segmentation; D. Output image with number of mRNAs per cell. Scale bars = 5 µm.

NOTE 16: Distributions from FLC 'mRNA per cell' counts from a variety of conditions can be can be found in Rosa *et al* (2016) and Duncan *et al* (2017). The example below is taken from Duncan *et al* (2017), Figure 7.

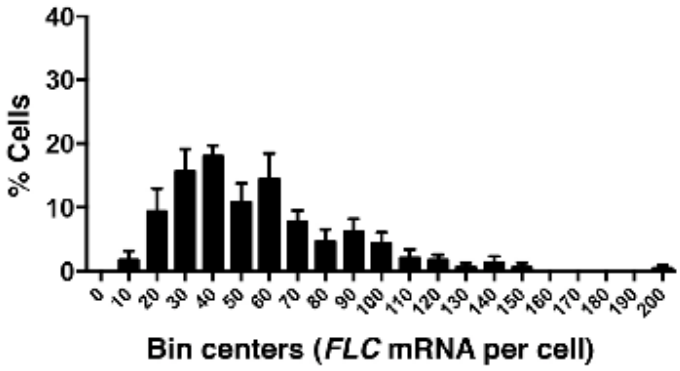


Figure 7. Frequency distribution of *FLC* mRNA molecules per cell. A total of 520 cells were analyzed from three experiments. Error bars are ± SEM.

5. References

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