

VERSION 2
DEC 08, 2023

Modified Arabidopsis Root smRNA FISH Protocol V.2

Journal of Experimental Botany

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Arabidopsis Root smRNA FISH



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ABSTRACT

Single molecule RNA FISH (smRNA FISH) is an imaging method that labels individual mRNA molecules in cells to facilitate localization and quantitative studies. Here we present a modified protocol for mRNA labelling in Arabidopsis root meristem cells that retains GFP fluorescence. Although the processing steps impact GFP intensity, this protocol demonstrates visualization of RNA together with highly expressed protein. This protocol is presented together with simplified image analysis steps that aim to support inexperienced cell biologists through from experimental set up to mRNA quantification.

OPEN ACCESS



DOI:
dx.doi.org/10.17504/protocols.io.rm7vzyworlx1/v2

External link:
<https://doi.org/10.1093/jxb/erac521>

Protocol Citation: Susan Duncan, Hans Johansson 2023. Modified Arabidopsis Root smRNA FISH Protocol. [protocols.io](https://dx.doi.org/10.17504/protocols.io.rm7vzyworlx1/v2) <https://dx.doi.org/10.17504/protocols.io.rm7vzyworlx1/v2> version created by Susan Duncan

MANUSCRIPT CITATION: Duncan S, Johansson HE, Ding Y, Reference genes for quantitative Arabidopsis single molecule RNA fluorescence hybridization. Journal of Experimental Botany 74(7). doi: [10.1093/jxb/erac521](https://doi.org/10.1093/jxb/erac521)

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Protocol status: Working
We use this protocol and it's working

Created: Dec 08, 2023

Last Modified: Dec 08, 2023

PROTOCOL integer ID: 92013

Keywords: smRNA FISH, Arabidopsis root, mRNA quantification, RNA imaging

Funders
Acknowledgement:
Susan Duncan
Grant ID: Gatsby Charitable Foundation
Susan Duncan
Grant ID: John Innes Centre Strategy Funding

GUIDELINES

When performing Stellaris RNA FISH, it is imperative to limit RNA degradation. You should try to ensure that all consumables and reagents are RNase-free.

MATERIALS

TE buffer (10nM Tris-HCl, 1 mM EDTA, pH 8.0)
Liquid Nitrogen

Nuclease-free water - not DEPC treated (Qiagen, Catalog # 129225)
Methanol Free 16% formaldehyde (Sigma, Catalog # P6148)
Nuclease-free 10x Phosphate Buffered Saline (PBS) (Thermo Scientific, Catalog # AM9624)

Ethanol suitable for molecular biology.
Deionized Formamide (Sigma, Catalog # F9037)
Stellaris Wash Buffer A (Catalog # SMF-WA1-60)

- Note: Before use 10 mL Wash Buffer A must be made up as follows:
- 2 mL Wash Buffer A Stock
- 1 mL Deionised Formamide
- 7 mL Nuclease free water

Stellaris Wash Buffer B (Catalog # SMF-WB1-20)


- Note: 88 mL Nuclease free water must be added before use

Stellaris Hybridization Buffer (Catalog # SMF-HB1-10)

- Note: For effective probe hybridization, the final solution must contain 10% deionised formamide

Stellaris Custom probe set (see [Stellaris website for details](#))

Hybrislips (Grace Bio-Labs, Catalog #GBL722222)
4',6-diamindino-2-phenylindole, DAPI Solution 1mg/mL (Sigma, Catalog # MBD0015)
VECTASHIELD Antifade Mounting Media (Catalog # H-1000)
Cover slip sealant e.g. CoverGrip (Biotum, Catalog # 23005)
Razor blades

Forceps
Standard frosted ended microscope slides
3cm petri dishes (Thermo Scientific, Catalog # 121V or similar)
22 mm x 22 mm No.1 glass coverslips
Coplin jar (Sigma, Catalog # S6016 or similar)
Hybridization chamber (see protocol step 16 for more details)
Laboratory oven set to  37 °C .

MON1 mRNA Probe set sequences:

A
ggagaagacctcgaatctga

A
cgaattcgggtgctcgatgat
atcggagctaggatttgat
ttgaacacgctccgaattcg
agacttcgctaggttgagat
gatgcaacctcatcatcatt
ccacaccttcacgcaataaa
cttaacaaaacctctcctcc
cttccatcatcagctttata
attagcttcctaattctcat
ctcatcaacatggcgtttc
tccatgacgtagaagcatca
tgctgagttactcagtatg
gttcatctccatatctggaa
gctgaaaatccagcaagctt
accaccattctccacaaaag
ccttgactaagttgacacgg
aagacaacctggtgatttcc
agatatattggcccctaac
tgtttcatctgtacagctga
tatagaagatccaactgccc
gcaaggggtgcatacgaac
tgagaagacagcatctgtcc
ctgggttccagctaaatgaa
ccttaacgcatatggaaggg
acttcttgcaatatggttcc
gaataagacaccagacgcgc
tgtgtctgcacattagtagt
gtagaagcaagtcacgga
tgagcgttgatcttggtag

A
agaagtggacataggcacgc
gaacgcacatctgaacgtgtgg
taccctgcaatctttgagat
cgcgattgatctttgaacca
ggtacatcttcaacacgcac
tagtagatgatcgacgcctg
ggtgagtcttgttcttgatt
atgccaaagtccaaagggac
gttactgggggtgagaattc
gacttttctgtgtctgtga
agtttctggtatgctcgata
ccaatcctttacatgcatt
gtgtagtttcatctcttct
tggtgtgaccaacatagaa
gcaagtggatcaaagtctgc
cacacctgattgcatactt
cttcattctccacatctttt
aaaggactagctccttgcac

SAFETY WARNINGS







WARNING: Formaldehyde is a carcinogen and should be used in a chemical fume hood.

WARNING: Formamide is a teratogen that is easily absorbed through the skin and should be used in a chemical fume hood.







WARNING: Formamide should be warmed to room temperature before opening the bottle.

BEFORE START INSTRUCTIONS

1. Before starting the fixation step, clean bench surfaces and equipment with RNase Away
2. Ensure your 5 nmol dried oligonucleotide probe blend has been suspended in  400 μL of TE buffer (10 mM Tris-HCl, 1mM EDTA pH 8.0). This creates a probe stock of  12.5 micromolar (μM). To minimise freeze thaw cycles make small aliquots and store them at  $-20\text{ }^{\circ}\text{C}$
3. Prepare  50 mL 1xPBS using nuclease free 10x PBS and nuclease free water.





Plant Growth

1w 0d 0h 30m

- 1 Sterilize then sow a row of Col-0 Arabidopsis seeds onto half strength Murashige and Skoog Medium  30m (1/2 MS) near the top of a 10 cm square petri plate.
- 2 Stratify the seeds at  $4\text{ }^{\circ}\text{C}$ in a cold room for two days.  2d
- 3 Take the plate out of the cold and place it vertically in a growth cabinet set for 16 hours light and 8 h  5m dark at a constant  $20\text{ }^{\circ}\text{C}$ (a plate holder can be useful to ensure stability).
- 4 Leave seeds to germinate and grow for 5 days  5d


Root Fixation

40m

- 5 Prepare  4 mL 4% fixative in a 3 cm petri dish by diluting  1 mL of 16% methanol free formaldehyde in  3 mL of 1x PBS.  5m

Safety information

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- 6 Use forceps to remove whole seedlings from the plate and submerge them in the 4% formaldehyde to leave to fix for  00:30:00 . 30m





Safety information

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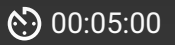

- 7 Move seedlings into a fresh petri dish and carry out three washes with 1 x PBS. 5m



Sample Preparation

40m

- 8 Arrange 3 plants on a slide, place a No 1 coverslip on top. Then, starting at the root tip, squash each seedling flat using a pencil eraser. 5m
- 9 Whilst holding the seedlings under the coverslip with forceps, carefully immerse in liquid nitrogen for  00:00:10 . 10s
- 10 Immediately after the slide is removed from the liquid nitrogen, flip off the coverslip using a razor blade and carefully scrape away the frozen leaves. Leave the slide to air dry for  01:00:00 at  Room temperature . 1h
- 11 Immerse the slide in 70% EtOH in a coplin jar for  01:00:00 . 1h

16h 15m





12 Remove the slide from the ethanol, tip off residual liquid and leave for  00:05:00 at  Room temperature . 5m

13 Carry out 1 x 5 min  200 μL wash on the slide with freshly prepared  10 % (v/v) formamide 5m
Wash Buffer A.




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WARNING: Formamide should be warmed to room temperature before opening the bottle.

14 Thoroughly mix  100 μL of freshly prepared  10 % (v/v) formamide Hybridization Buffer with 5m
 1 μL of each probe type of interest (dissolved to  250 nanomolar (nM) concentration in TE buffer).

Note

Stellaris RNA FISH probes are shipped dry and can be stored at +2 to  8 °C in this state. Dissolved probe mix should be subjected to a minimum number of freeze-thaw cycles. For daily and short-term use of dissolved probe mix, storage at +2 to  8 °C in the dark up to a month is recommended. For storage longer than a month, Stellaris recommend aliquoting and freezing probes in the dark at -15 to  -30 °C .

Safety information

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15 Add to the sample and cover with a Hybrislip , or other suitable plastic cut to size (good results have been achieved using the inside surface of an unused autoclave bag). **5m**

16 Place the slide in a humid, hybridization chamber to the **37 °C** incubator and leave overnight. **16h**
Note: You can make your own hybridization chamber by covering a 10 cm tissue culture plate with black insulation tape. Then line the base evenly with a flat water-saturated paper towels and apply a single layer of Parafilm on top to provide a dry flat surface for the slides. For more details see:

CITATION

Duncan S, Olsson TSG, Hartley M, Dean C, Rosa S (2017). Single Molecule RNA FISH in Arabidopsis Root Cells.. Bio-protocol.

LINK

<https://doi.org/10.21769/BioProtoc.2240>

Sample Mounting

1h 20m

17 Wash slide with **200 µL** Wash Buffer A, then add another **200 µL** Wash Buffer A and cover a fresh Hybrislip (or plastic alternative). Return to **37 °C** incubator for 30 mins. **35m**

18 Tip off Wash Buffer A and add **100 µL** of **1 µg** /mL DAPI Solution (diluted in Wash Buffer) to each slide. Cover again, place back in the hybridization chamber and return to **37 °C** incubator for 30 mins. **35m**

19 Tip the DAPI Solution from the slide, then carry out a **200 µL** Wash Buffer B wash for a minimum 5 mins. **5m**

20 Tip Wash Buffer B off the slide, then add **20 µL** of Vectashield mounting media directly onto the root tip area. Carefully cover with a No.1 coverslip and press down firmly with forceps. **5m**

Imaging

3h

21

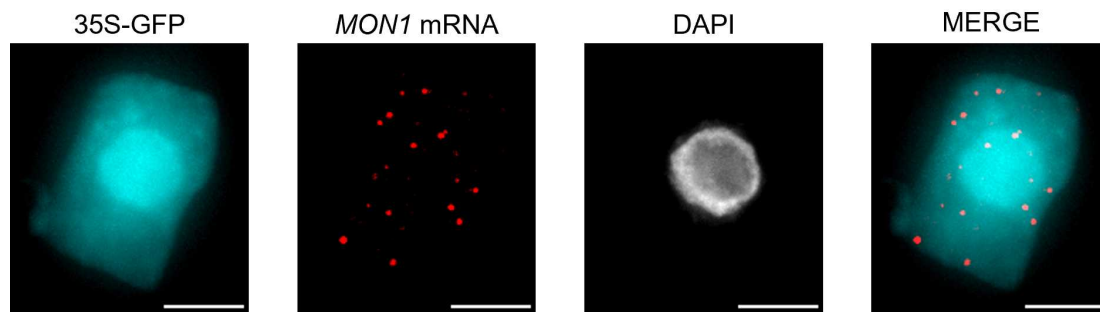
A wide-field fluorescence microscope is required to image single molecules of mRNA. A high numerical aperture (>1.3) and ideally 100x oil-immersion objectives are ideal. Strong light sources, such as a mercury or metal-halide lamp are ideal, but successful imaging has also been achieved with LEDs. Filter sets must be appropriate for the fluorophores. An EM-CCD camera is ideal, but images can also be obtained using a standard cooled CCD camera that is optimized for low-light level imaging, rather than speed. Z stacks should be set up to fully encompass cell depth and 200 nm steps should be used to provide adequate resolution for analysis.

A	B
Microscope Model and Manufacturer	Zeiss Elyra PS1 inverted wide-field microscope
Acquisition software	Zen 2.3 (Black)
Objective	Plan-Apochromat 100x/NA 1.46 DIC
Immersion	Oil
Camera	Andor iXon 897 (512x512, QE>90%)
Voxel Size	x/y 100 nm, z 200 nm
Frame Averaging	1
Acquisition	Complete z-stacks were acquired for Q670 probes then DAPI, sequentially.
Q670 probe detection laser	642 nm laser diode
Q670 probe emission bandwidth	LP655 nm
Q670 probe exposure time	1000 ms
Q670 probe EM Gain	24
DAPI detection laser	405 nm laser diode
DAPI emission bandwidth	420-480 nm
DAPI exposure time	300 ms
DAPI EM Gain	25

Details of microscope set up used to acquire the images in this study

22

When imaging suitable transgenic lines it is worth checking whether GFP signal has been retained. Below is an example image showing *MON1* mRNA in a cell expressing GFP under a 35S promoter.



Basic Image Analysis Steps

5m

23 Per cell mRNA counts can be achieved by basic FIJI commands

1m

CITATION

Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, Preibisch S, Rueden C, Saalfeld S, Schmid B, Tinevez JY, White DJ, Hartenstein V, Eliceiri K, Tomancak P, Cardona A (2012). Fiji: an open-source platform for biological-image analysis.. Nature methods.

LINK

<https://doi.org/10.1038/nmeth.2019>

First carry out a Maximum Z projection of the stack: Image -> Stacks -> Z project (include all slices and select maximum intensity from the drop down menu).

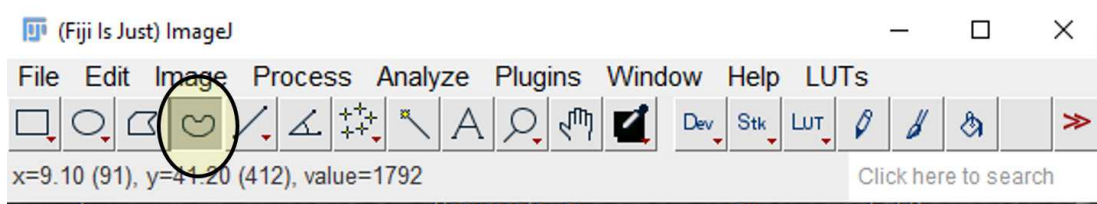
For automated image analysis it is essential that acquisition is completed carefully so that stacks do not include images above or below the cells. This is because if full stack projection is completed blindly it can allow high intensity out-of-focus light to impair smFISH spot detection. If max projection is being completed manually, it is worth specifying the exact top and bottom slices to ensure optimal downstream analysis.



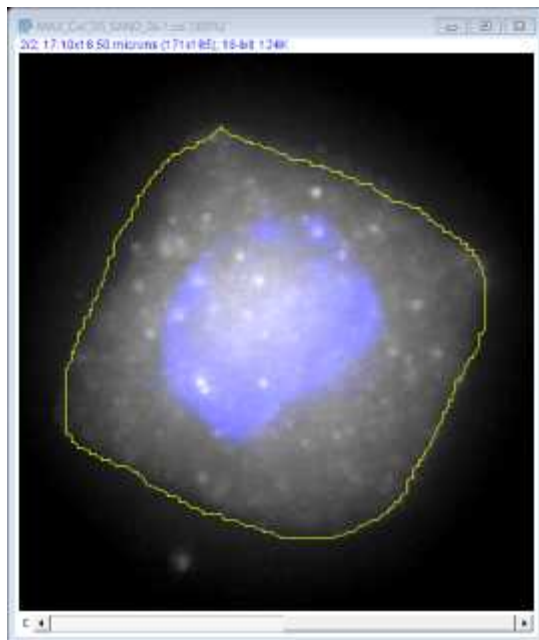
24 Use the freehand selection tool to draw around the perimeter of the cell. Select this as a region of

2m

interest (ROI) by pressing t. This will add the area to the ROI manager.



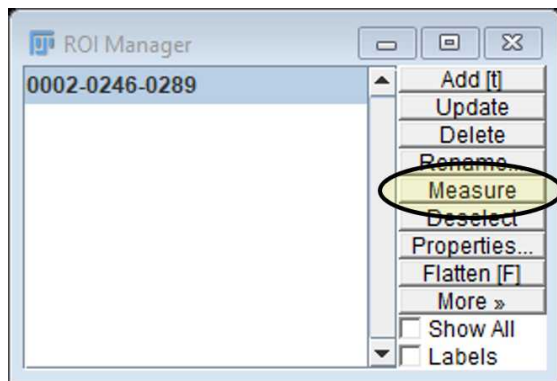
The freehand selection tool



This example image demonstrates how heterogeneous background labelling makes thresholding essential for determining quantitative mRNA data. (The mRNA probe channel is presented in a grey scale and blue indicates the nucleus stained with DAPI)

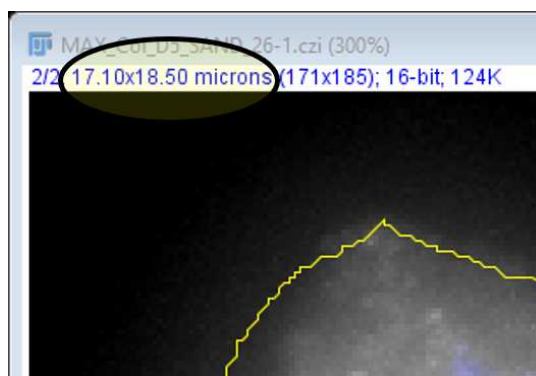
- 25** Select the ROI on the manager window and click on measure to open up a results window showing the area (μm^2). The mean, min and max fluorescence intensity values of this region are also provided for the channel selected.

Note: Be sure to check that the image file has provided FIJI with calibrated data before trusting these values!



Results

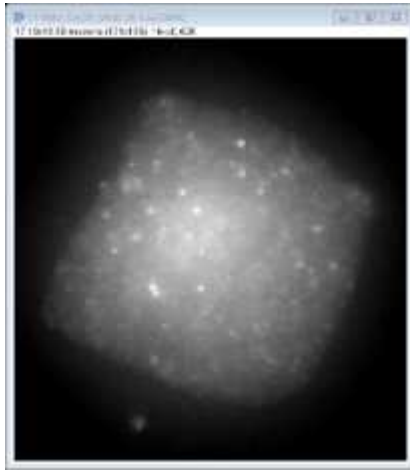
	Area	Mean	Min	Max
1	135.590	7507.098	3312	17760



A quick check at the top of the image should confirm whether it is calibrated correctly.

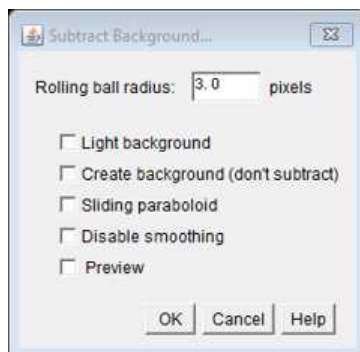
- 26** Split the DAPI and mRNA probe channels: Image -> Colour -> Split Channels.
Then select the probe channel to work on next.

1m



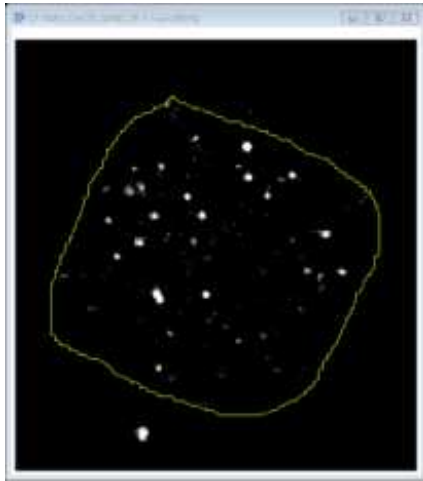
Maximum intensity Z projection of the probe channel.

- 27 We apply the Subtract Background command with a 3 pixel rolling ball radius. This radius setting will depend on your camera set up. We acquire images with 100 x 100 nm pixels so a 3 pixel radius is required to highlight the ~300 nm diameter smFISH spots. 1m

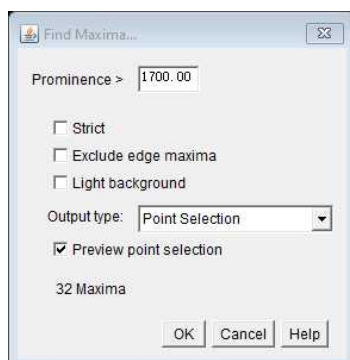


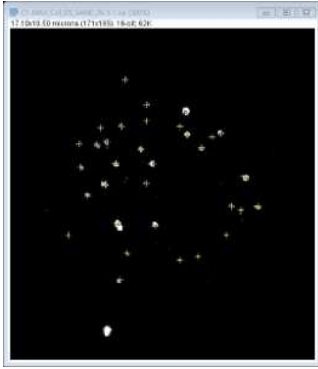
This window can be opened by selecting Process -> Subtract Background

- 28 Highlight the cell again by selecting it in the ROI manager. 1m



- 29 Use the Find Maxima command with a 1700 prominence setting (with preview selected) to highlight and quantify all mRNA signals in this cell. This setting should be considered as a starting point and will differ depending on the microscope set up and probe set. To determine the appropriate setting, compare no probe control and probe images (these must have been acquired using the same microscope settings.) Determine the lowest prominence level that retains spot detection in your probe images, but ignores almost all non-specific spots in the no probe controls. As a guide, for all our reference probe sets, we quantified mRNA labels using 1700 prominence setting and this detected an average of one non-specific signal every 3-4 cells.





By preselecting the cell area, maxima are only counted in this area.

30

Cell volumes can also be estimated for mRNA concentration measurements (mRNA per μm^3):
Take the area measurement calculated in step 25 and multiply this by the Z depth
(i.e. the number of Z steps used during image acquisition multiplied by $0.2 \mu\text{m}$).

2m