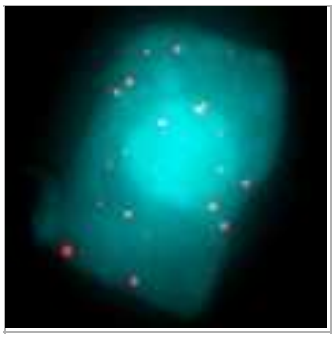

JAN 05, 2023

OPEN ACCESS

DOI:
dx.doi.org/10.17504/protocols.io.rm7vzyworlx1/v1

Protocol Citation: Susan Duncan, Hans Johansson 2023. Modified Arabidopsis Root smRNA FISH Protocol. **protocols.io** https://dx.doi.org/10.17504/protocols.io.rm7vzyworlx1/v1

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

Created: Aug 18, 2022

Last Modified: Jan 05, 2023

PROTOCOL integer ID: 68850

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

Modified Arabidopsis Root smRNA FISH Protocol

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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.

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 TEXT

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

Stellaris Hybridization Buffer (Catalog # SMF-HB1-10)
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
cgaattcggtgtcggatgat
atcggagctaggatttggat
ttgaacacgctccgaattcg
agacttcgctaggttgagat
gatgcaacctcatcatcatt
ccacaccttcacgcaataaa
cttaacaaaacctctcctcc
cttccatcatcagctttata
attagcttccctaatctcat
ctcatcaacatggcgttttc
tccatgacgtagaagcatca
tgcctgagttactcagtatg
gttcatctccatatctggaa
gctgaaaatccagcaagctt
accaccattctccacaaaag
ccttgactaagttgacacgg
aagacaacctggtgatttcc
agatatattggccccttaac
tgtttcatctgtacagctga

A
tatagaagatccaactgccc
gcaagggtgtcatatcgaac
tgagaagacagcatctgtcc
ctgggttccagctaaatgaa
ccttaacgcatatggaaggg
acttcttgcaatatggttcc
gaataagacaccagacgcgc
tgtgtctgcacattagtagt
gtagaagcaagtcatcggga
tgagcgttgtatcttggtag
agaagtggacataggcatgc
gaacgcatctgaacgtgtgg
taccctgcaatctttgagat
cgcgattgatctttgaacca
ggtacatcttcaacacgcat
tagtagatgatcgacgcctg
ggtgagtcttgttcttgatt
atgccaaagtccaaagggac
gttactgggggtgagaattc
gacttttctgttgtctgtga
agtttctggtatgctcgata
ccaatccttttacatgcatt
gtgtagttttcatctcttct
tggtgtgacccaacatagaa
gcaagtggatcaaatgctgc
cacacctgattgcatatctt
cttcattctccacatctttt
aaaggactagctccttgcaa

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 mL of TE buffer (10 mM Tris-HCl, 1mM EDTA pH 8.0). This creates a probe stock of [M] 12.5 micromolar (µM) . To minimise freeze thaw cycles make small aliquots and store them at -20 °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 (1/2 MS) near the top of a 10 cm square petri plate. 30m

2 Stratify the seeds at 4 °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 hours dark at a constant 20 °C (a plate holder can be useful to ensure stability). 5m

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 then 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 scape 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

Probe Hybridization

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 [M] 10 % (v/v) formamide Wash Buffer A. 5m

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

Note

Stellaris RNA FISH probes are shipped dry and can be stored at +2 to 8 °C in this state. Dissolved probe mix should e 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 with 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 1 µg /mL DAPI Solution (diluted in Wash Buffer A) 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 of 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

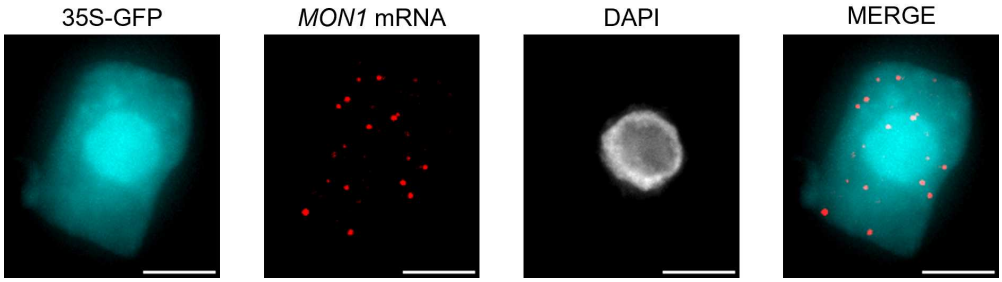
21 A wide-field fluorescence microscope is required to image single molecules of mRNA. A high numerical aperture (>1.3) and ideally 100x oil-immersion objectives are ideal. Strong light sources, such as a mercury or 2h

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 be 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 emmission bandwidth	LP655 nm
Q670 probe exposure time	1000 ms
Q670 probe EM Gain	24
DAPI detection laser	405 nm laser diode
DAPI emission bandwith	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

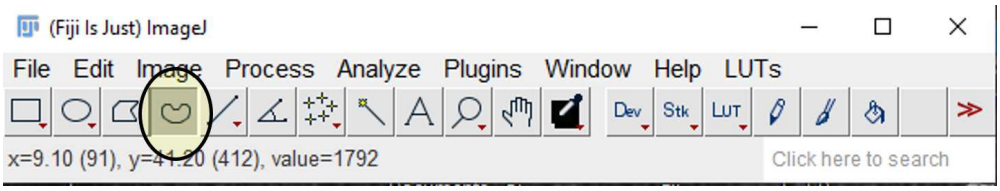
https://doi.org/10.1038/nmeth.2019

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).

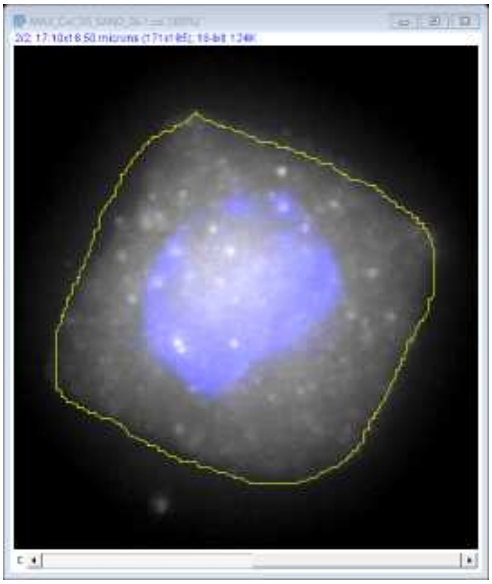
For automated image analysis it is essential that acquisition is completed carefully so that stacks do not include images above or below the cells. This is because if full stack projection is completed blindly it can allow high intensity out-of-focus light to impair smFISH spot detection. If max projection 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 interest (ROI) by pressing t. This will add the area to the ROI manager.

2m



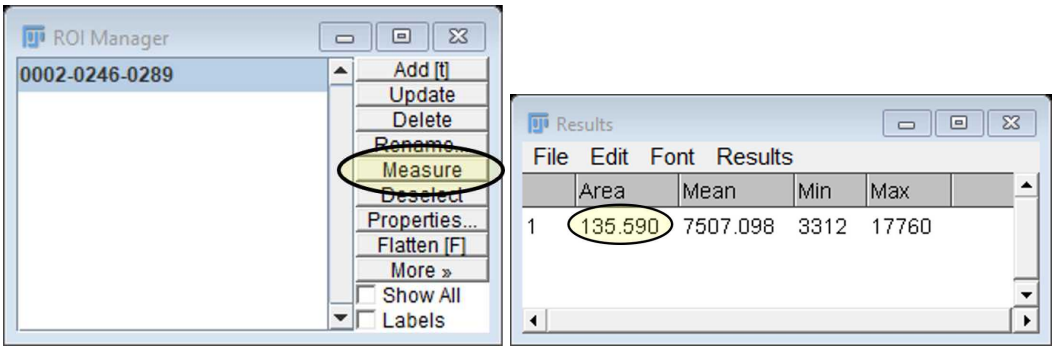
The freehand selection tool

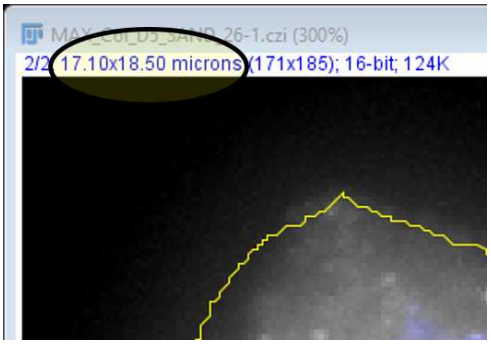


This example image demonstrates how heterogeneous background labelling makes thresholding essential for determining quantitative mRNA data. (The mRNA probe channel is presented in a grey scale and blue indicates the nucleus stained with DAPI)

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.

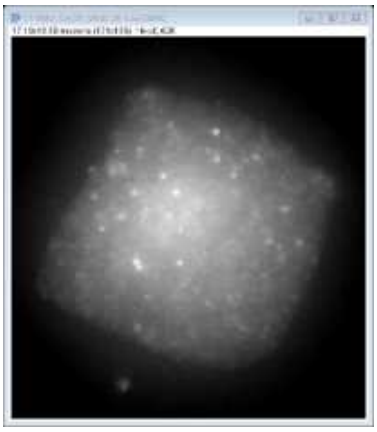
Note: Be sure to check that the image file has provided FIJI with calibrated data before trusting these values!





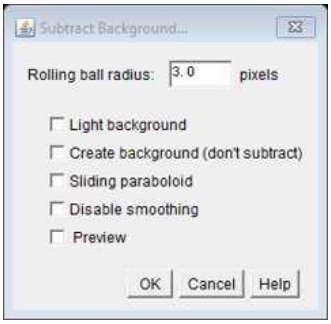
A quick check at the top of the image should confirm whether it is calibrated correctly.

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.

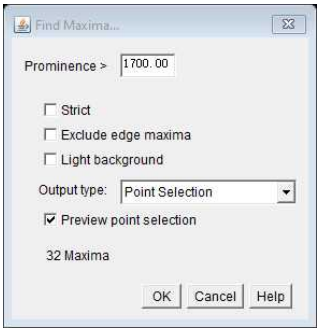
27 We apply the Subtract Background command with a 3 pixel rolling ball radius. This radius setting will depend on you camera set up. We acquire images with 100 x 100 nm pixels so a 3 pixel radius is required to highlight the ~300 nm diameter smFISH spots. 1m

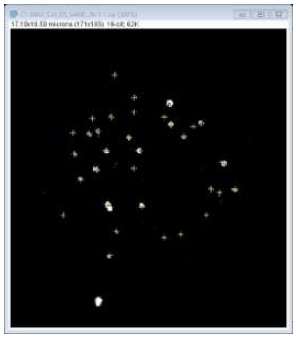


This window can be opened by selecting Process -> Subtract Background

28 Highlight the cell again 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. 1m





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 µm). 2m