bio-protocol

Single Molecule RNA FISH in *Arabidopsis* Root Cells

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[Abstract] Methods that allow the study of gene expression regulation are continually advancing. Here, we present an *in situ* hybridization protocol capable of detecting individual mRNA molecules in plant root cells, thus permitting the accurate quantification and localization of mRNA within fixed samples (Duncan *et al.*, 2016; Rosa *et al.*, 2016). This single molecule RNA fluorescence *in situ* hybridization (smFISH) uses multiple single-labelled oligonucleotide probes to bind target RNAs and generate diffraction-limited signals that can be detected using a wide-field fluorescence microscope. We adapted a recent version of this method that uses 48 fluorescently labeled DNA oligonucleotides (20 mers) to hybridize to different portions of each transcript (Raj *et al.*, 2008). This approach is simple to implement and has the advantage that it can be readily applied to any genetic background.

Keywords: Single RNA molecules, Fluorescent *in situ* hybridization, Gene expression, *Arabidopsis*, Transcription

[Background] While single molecule FISH has been developed to quantitatively measure mRNAs at the single cell level for cultured cells, tissue sections and whole-mount invertebrate organisms, this method was not optimized for use in single cells in plants. Fluorescence imaging in plants is considerably challenging due to endogenous autofluorescence of plant tissues. Here, we report a method to detect single RNA molecules in plants. We describe the detection and automated counting of single transcripts within cells of fixed *Arabidopsis* root squashes. This method generates isolated cells and single-cell layers, which together with the use of red and far-red dyes maximizes signal-to-noise ratio limiting background noise.

Materials and Reagents

1. 1.5 ml microcentrifuge tube
2. Sterile Sterilin™ 10 cm square Petri dishes for plant growth media (Thermo Fisher Scientific, Thermo Scientific™, catalog number: 109)
3. 22 x 22 mm No. 1 glass coverslips (VWR, catalog number: 631-0124)
4. Poly-L-Lysine slides (Sigma-Aldrich, catalog number: P0425)
5. Razor blades (Agar Scientific, catalog number: AGT586)
6. Parafilm M® sealing film (Bemis, catalog number: PM992)

7. Hybridization chamber

    Note: Although these are available commercially (Corning, catalog number: 2551) we used 10 cm square Petri dishes covered externally with a layer of black insulation tape (RS Components, catalog number: 494-405). A double layer of tissue (KCWW, Kimberly-Clark, catalog number: 7557) 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 1).
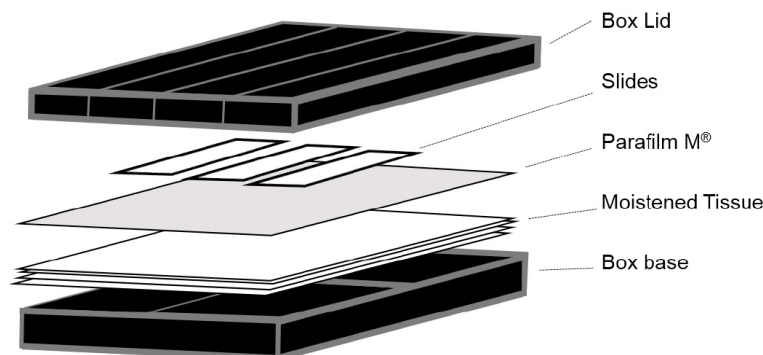
Figure 1. Illustration of the hybridization chambers used for smFISH experiments

8. Low stender-form preparation dishes (VWR, catalog number: 470144-866; or similar rimmed glass dish)

9. *Arabidopsis thaliana* roots

10. Sodium hypochlorite (NaClO) (VWR, BDH®, catalog number: CABDH7038-4L)

11. Sucrose (Sigma-Aldrich, catalog number: S9378-1KG)

12. Phytagel (Sigma-Aldrich, catalog number: P8169)

13. Paraformaldehyde (Sigma-Aldrich, catalog number: P6148)

14. Nuclease-free phosphate buffered saline solution (PBS, 10x) pH 7.4 (Thermo Fisher Scientific, Ambion™, catalog number: AM9624)

15. Liquid nitrogen

16. Ethanol suitable for molecular biology

17. Nuclease-free 20x saline-sodium citrate (20x SSC) (Thermo Fisher Scientific, Ambion™, catalog number: AM9763)

18. Clear nail varnish (Electron Microscopy Sciences, catalog number: 72180; or similar)

19. Dextran sulphate (Sigma-Aldrich, catalog number: RES2029D)

20. Custom RNA FISH Stellaris® probe sets

    We used the online Stellaris Probe Designer to design our smFISH probe sets: https://www.biosearchtech.com/support/education/stellaris-rna-fish. We used a default masking level 2 to avoid general problematic RNA sequences and selected the maximum number of 48 probes, an oligo length of 20 nt and 2 nt minimum spacing between probes.

    We also performed TAIR BLAST searches (https://www.arabidopsis.org/Blast/index.jsp) for each probe sequence and considered the results collectively to ensure target specificity. We

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found Quasar 570 and Quasar 670 dyes equally suitable for imaging RNA in *Arabidopsis* root cells, however we were unable to detect RNA labelled with Fluorescein modified probes.

21. Murashige & Skoog basal medium with vitamins (any equivalent source would be suitable) (PhytoTechnology Laboratories®, catalog number: M519)

22. Nuclease-free Tris-EDTA buffer solution (10 mM Tris-HCl, 1 mM EDTA pH 8) (Sigma-Aldrich, catalog number: 93283)

23. DAPI (Thermo Fisher Scientific, Molecular Probes™, catalog number: D1306)

24. Glucose (Sigma-Aldrich, catalog number: 158968-500G)

25. Tris HCl buffer 1 M pH 8, nuclease-free (Thermo Fisher Scientific, Ambion™, catalog number: AM9855G)

26. Glucose oxidase (Sigma-Aldrich, catalog number: G0543)

27. Bovine liver catalase (Sigma-Aldrich, catalog number: C3155)

28. Deionized formamide (Sigma-Aldrich, catalog number: F9037)

29. 1 N HCl (Sigma-Aldrich, catalog number: 71763)

30. 10% (v/v) bleach (Vortex 5-10% sodium hypochlorite, Procter & Gamble, UK) diluted in dH_2O

31. TE buffer (10 nM Tris-HCl, 1 mM EDTA, pH 8.0)

32. Nuclease-free water–not DEPC treated (QIAGEN, catalog number: 129117)

33. Murashige and Skoog medium (see Recipes)

34. Wash buffer (see Recipes)

35. Hybridization solution (see Recipes)

36. DAPI solution (see Recipes)

37. Anti-fade GLOX buffer (minus enzymes) (see Recipes)

38. Anti-fade GLOX buffer (containing enzymes) (see Recipes)

Equipment

1. Forceps (Fisher Scientific, S Murray™, catalog number: E017/01; or similar)

2. Coplin jar (Sigma-Aldrich, catalog number: S6016; or similar)

3. Plant growth chamber (Panasonic Healthcare, Sanyo, catalog number: MLR-352H-PE; or similar)

4. Fume cupboard (Labconco, catalog number: 2247300; or similar)

5. Laminar flow cabinet (Thermo Fisher Scientific, Thermo Scientific™, model: Heraguard™ ECO Clean Bench Floor Stands, catalog number: 50109313; or similar)

6. Laboratory oven (Cole-Palmer, catalog number: WZ-52412-83; or similar)

7. Orbital shaker (Cole-Palmer, catalog number: WZ-51700-13; or similar)

8. Wide-field fluorescence microscope
   Note: We used an Andor iXon EM-CCD camera (Andor, model: iXon EMCCD Camera) fitted to a Zeiss, Elyra PS-1 (Zeiss, model: Elyra PS.1), however similar images have also been obtained using a standard CCD camera optimized for low light level imaging.

9. A high numerical aperture (> 1.3) and 60 or 100x oil-immersion objective
10. Strong light source, such as a mercury or metal-halide lamp (Xenon or LEDs are typically not bright enough)
11. Filter sets appropriate for the fluorophores

Software

1. ImageJ software: http://rsbweb.nih.gov/ij/index.html (Schindelin *et al.*, 2015)
2. FISHcount: https://github.com/JIC-CSB/FISHcount
3. GraphPad Prism Software or MS Excel

Procedure

A. Plant growth and microscope slide preparation
1. Surface sterilize seeds in 5% (v/v) sodium hypochlorite for 5 min in a 1.5 ml microcentrifuge tube (inverting ~10 times) and then rinse three times in sterile distilled water.
2. Plate the seeds on to Murashige and Skoog medium (pH 5.8) supplemented with 1% (w/v) sucrose and 0.5% (w/v) phytagel.
3. Stratify the seeds by incubating for 2 days at 4 °C in darkness. Then, seedlings were grown at 22 ± 1 °C, in vertically oriented Petri dishes for at least 5 days (16 h light and 8 h dark).

B. Fixation and preparation of *Arabidopsis* roots (see Figure 2 for an overview)
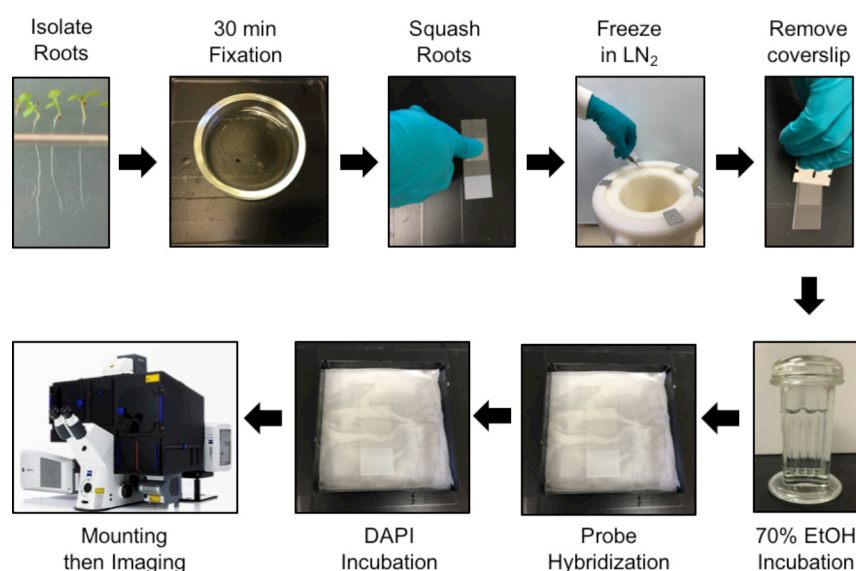
Figure 2. Protocol steps for smFISH in *Arabidopsis* root cells. See main text for details.

1. Cut root tips from *Arabidopsis* seedlings (approx. 1 cm) whilst still on plates and place into a small glass dish containing freshly prepared 4% paraformaldehyde and incubate for 30 min at room temperature under a fume hood.

2. Remove the roots from the fixative and wash twice with 1x PBS.

3. Arrange 3-4 roots onto a slide and cover with a coverslip. Gently squash each root onto the slide using either your thumb and be careful to avoid breaking the coverslip. Aim to splay the roots sufficiently to produce multiple files and isolated cells in a single cell layer (see Figure 3).
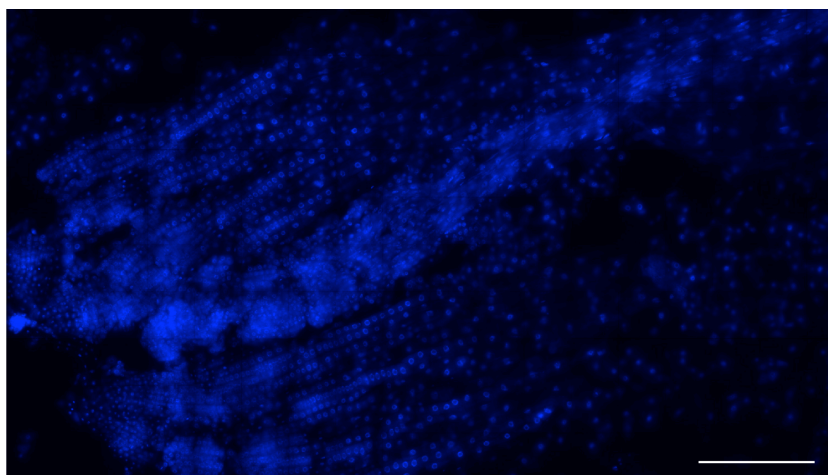
Figure 3. Low magnification image a root meristem squash. Nuclei stained with DAPI (blue). This method yields many single-layer cells that are either isolated or within files. Scale bar = 200 μm.

4. 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.

5. Leave samples to air dry at room temperature for a minimum of 30 min.
    Note: To avoid increased levels of autofluorescence do not leave to dry for longer than 2 h.

6. Permeabilize the samples by immersing the slides into a Coplin jar containing 70% ethanol for a minimum of one hour at room temperature. Fixed roots can be stored at 2 to 8 °C in 70% ethanol up to a week prior to hybridization.

C. Probe hybridization
    1. Remove slides from the ethanol and allow residual ethanol to evaporate at room temperature.
    2. Wash 2 times on the slide with wash buffer for 5 min.
    3. Prepare a probe solution by adding 1 μl of each required probe stock solution to 100 μl of hybridization solution (250 nM final concentration).
    4. Add 100 μl probe solution to each slide, cover with a coverslip to prevent evaporation and incubate in a humid chamber at 37 °C for a minimum of 4 h (or overnight) in the dark.

D. Sample mounting

1. Remove the cover slip and wash the samples 2 times in 200 µl wash buffer for 5 min on the slide.

2. Immerse the slide in a Coplin jar containing ~30 ml of wash buffer and incubate for 30 min at 37 °C, in the dark.

3. Remove the slide from the jar and add 100 µl of DAPI solution to the slide then incubate at 37 °C for 30 min in the dark.

4. Aspirate the DAPI solution carefully with a pipette and rinse with ~100 µl 2x SSC.

5. Remove 2x SSC and add 100 µl anti-fade GLOX buffer (minus enzymes). Leave it to equilibrate for 1-2 min.

6. Remove anti-fade GLOX buffer (minus enzymes) and add 100 µl of anti-fade GLOX buffer (containing enzymes) to each slide.

7. Cover the samples with a coverslip, remove excess anti-fade GLOX buffer (plus enzymes) and seal with nail varnish.

8. Image the slides the same day to avoid sample drying and fluorophore fading.

E. Imaging

1. Images are acquired on a wide-field epifluorescence microscope, with an optical sectioning size of 0.2 µm per z-plane and spanning the entire volume of the cell (around 5 µm, depending on cell size). Exposure times will vary depending on your microscope setup (light source, objective, *etc.*). We acquired our images using a Zeiss Elyra PS with a 100x oil-immersion objective (1.46 NA) and a cooled electron multiplying-CCD (charge-coupled device) Andor iXon 897 camera (512 x 512, QE > 90%). For probes labeled with Quasar 570 an excitation line of 561 nm was used and signals were detected at 570-640 nm; for probes labelled with Quasar 670 an excitation line of 642 nm and signals were detected at 655-710 nm; for DAPI an excitation line of 405 nm and signals were detected at wavelengths of 420-480 nm. Typically, we used 30 msec exposure time for DAPI, and 200-300 msec for Quasar dyes.

2. After image acquisition, Z-stacks can be reduced to a 2D dataset using the maximum projection command in ImageJ (Image/Stack/Z Project). For a detailed description of this step please refer to ImageJ User Guide available at: https://imagej.nih.gov/ij/docs/guide/user-guide.pdf. The maximum projection command will result in an image with diffraction-limited spots corresponding to single RNAs. When exonic probes are used in conjunction with intronic probes for the same gene, some spots will co-localize within nuclei, corresponding to nascent RNA molecules that were being transcribed at the time of fixation (Figure 4).
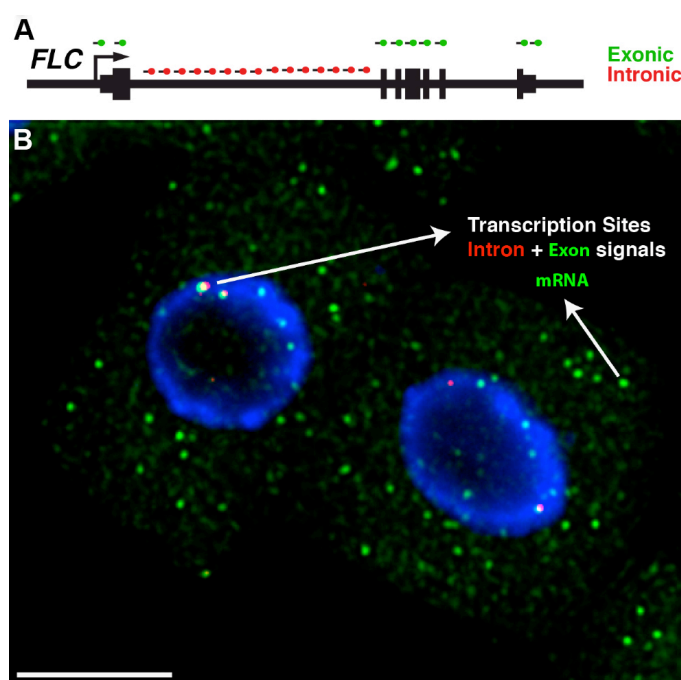
Figure 4. Detection of *FLC* transcripts in single cells. A. Schematic of the probes used to detect *FLC* transcripts: intronic (red) and exonic (green). *FLC* exons are represented as thick lines and introns shown as thin lines. B. Representative image showing *FLC* mRNA (green) and *FLC* nascent RNA (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.

Data analysis

1. For our image analysis we used a publically available mRNA counting programme that had been optimised for our experimental setup. Details about how to install this programme on a Mac computer can be found at https://github.com/JIC-CSB/FISHcount.
   Note: We recommend FISH-quant for researchers wishing to quantify FISH data on Windows machines (Mueller et al., 2013).

2. Figure 6 shows examples of 'RNA identification', 'Cell Segmentation' and 'mRNA Per Cell' output images that are generated by FISHcount. After performing analysis to a whole dataset, we recommend manually inspecting each 'mRNA Per Cell' output image (Figure 6D) to ensure that the image analysis workflow has not generated inaccurate results through incorrect segmentation. A detailed explanation of the image analysis workflow can also be found the Methods section in Duncan *et al.*, 2016 (Figure 5).

3. Data can then recorded and plotted as shown in Figure 7 using GraphPad Prism or MS Excel.
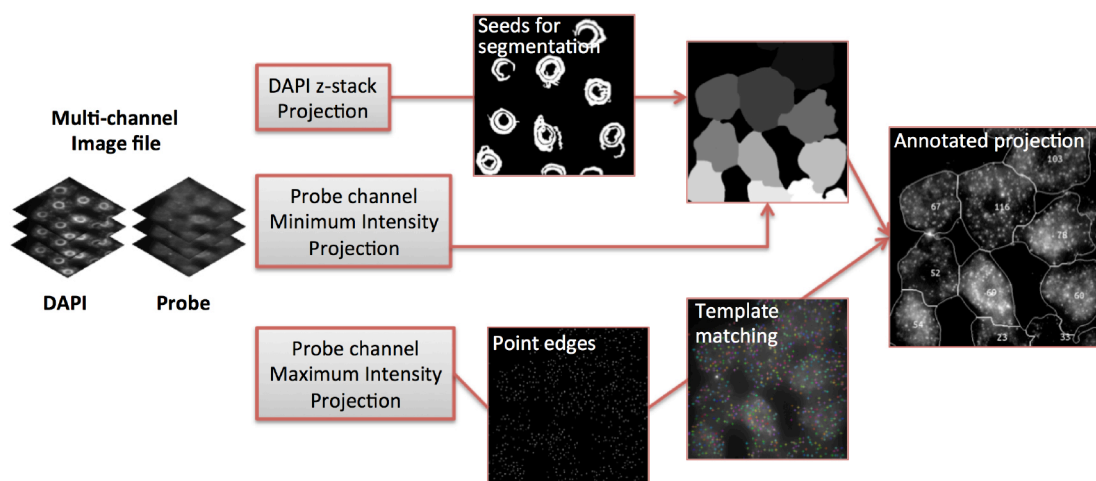
Figure 5. Image analysis workflow (adapted from Duncan *et al*., 2016)
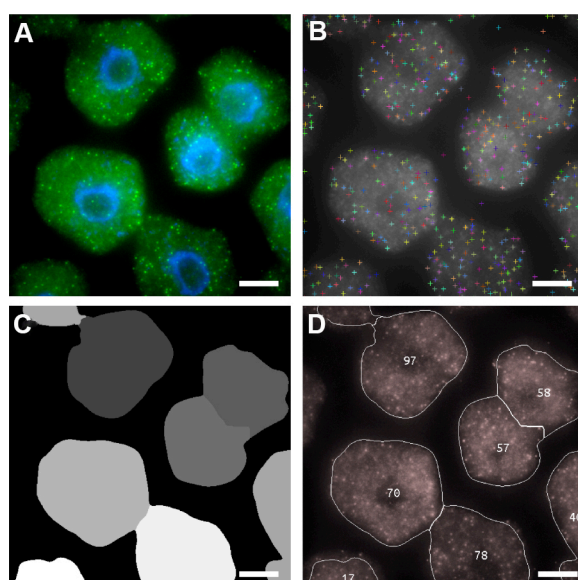
Figure 6. Automated image analysis of *FLC* mRNA. A. Representative maximum projection image of cells labeled with *FLC* mRNA probes (green). DNA labeled with DAPI (blue). B-D. Screen shots showing sequential detection steps used to determine positive mRNA signals. B. *FLC* mRNA spot locations; C. Cell Segmentation; D. Output image with number of mRNAs per cell. Scale bars = 5 μm.
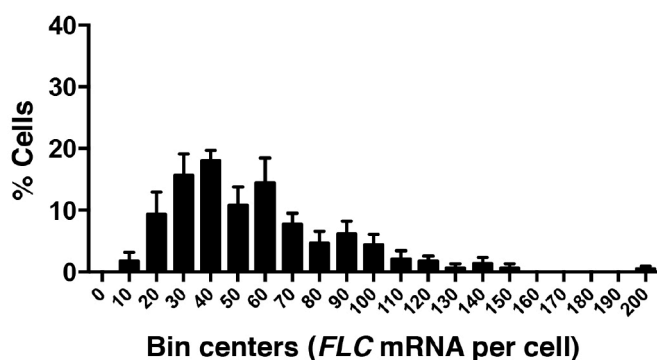
Figure 7. Frequency distribution of *FLC* mRNA molecules per cell. A total of 520 cells were analyzed from three experiments. Error bars are ± SEM.

Recipes

1. Murashige and Skoog medium
   0.025 mg/L $CoCl_2 \cdot 6H_2O$
   0.025 mg/L $CuSO_4 \cdot 5H_2O$
   36.7 mg/L $Na \cdot Fe\text{-}EDTA$
   6.2 mg/L H_3BO_3
   0.83 mg/L KI
   16.9 mg/L $MnSO_4 \cdot 2H_2O$
   0.25 mg/L $Na_2MoO_4 \cdot 2H_2O$
   8.6 mg/L $ZnSO_4 \cdot 7H_2O$
   332.02 mg/L $CaCl_2 \cdot 2H_2O$
   170 mg/L KH_2PO_4
   1,900 mg/L KNO_3
   180.5 mg/L $MgSO_4 \cdot 7H_2O$
   1,650 mg/L NH_4NO_3 (pH 5.8)
   1% sucrose
   0.5% phytagel
2. Wash buffer
   10% formamide
   2x SSC
3. Hybridization solution
   100 mg/ml dextran sulphate
   10% formamide
   2x SSC
4. smFISH probe stock (25 µM)
   Stellaris 5 nmol oligo probe set
   200 µl Tris-EDTA buffer solution

5. DAPI solution

   100 ng/µl DAPI

   10% formamide

   2x SSC

6. Anti-fade GLOX buffer (minus enzymes)

   0.4% glucose

   10 nM Tris-HCl

   2x SSC

7. Anti-fade GLOX buffer (containing enzymes)

   For a final volume of 102 µl, mix:

   100 µl anti-fade GLOX minus enzyme solution

   1 µl glucose oxidase

   1 µl bovine liver catalase suspension (mildy vortexed)

Acknowledgments

References

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