

Localising Total mRNA in Plant Cells.

Geraint Parry

GARNet, School of Biosciences, Cardiff University, Cardiff, CF10 3AX

geraint@garnetcommunity.org.uk

i. Summary

Visualising the location of the total cellular mRNA pool can be important to understanding how different genes effect cellular physiology. Over the past decade researchers investigating RNA processing, nuclear transport and the function of the nuclear pore complex have used this situ hybridization protocol to visualize and quantify the accumulation of the total mRNA pool within the plant cell nucleus.

ii. Keywords

In situ hybridization, nucleus, mRNA, nuclear pore complex, nuclear transport,

1. Introduction

Messenger RNA (mRNA) is the critical intermediate that transfers the genetic information encoded in DNA through to the generation of functional proteins. Transcription within the cell nucleus generates thousands of mRNAs that then travel through the nuclear pore to the cytosol where they are recognized by ribosomes, by whose action strings of amino acids are assembled into higher-order proteins. Obtaining an understanding of where mRNA is expressed and localised within a tissue and cell provide significant insights into the function of the underlining genes.

In situ hybridisation (*in situ*) is the technique that has been historically used to reveal the positioning of specific mRNAs, either through radioactive or non-radioactive labeling of a short anti-sense DNA probes. This technique can be used with both tissue sections or in whole mount samples [1,2]. *In situs* allow the identification of an expression domain, usually at the resolution of a small number of cells or to a single cell. Over recent years a refined *in situ* technique has been developed that uses multiple fluorescently labeled probes (up to 40 probes) targeted to a single mRNA. This allows the localization of single RNAs with *subcellular* resolution [3].

Each of these *in situ* techniques aim to visualize the expression of a single gene. However certain experimental circumstances require visualisation of the total cellular mRNA pool. Over the past decade these have included experiments that are investigating mechanisms of RNA processing and transport or the factors that control nuclear export, including the function of the nuclear pore complex [4-11].

Generally this technique uses a fluorescently-labeled anti-sense poly(dT) probe to evaluate the localization of all the mRNA within a plant cell. Engler *et al* [12] first published on this technique but more recently Gong *et al* (1995), who were investigating the function of the DEAD box RNA helicase LOS4, have modified this protocol (Figure 1)[4].

In comparison to wildtype cells, mutant cells show that mRNA accumulates within the nucleus. Parry (2014) showed that different NPC mutants, *nup160* and *nup62*, which have similar whole plant phenotypes, show different accumulation of total mRNA. In *nup160* mutants the total mRNA accumulates to the nucleus whilst in *nup62* this accumulation is normal [8]. Similarly when looking at the function of factors involved in nuclear export, Sørensen *et al* (2017) showed that *tex1* and *mos11* single mutants show wildtype levels of nuclear mRNA accumulation but that *tex1mos11* double mutants accumulate mRNA in their nuclei [10]. Therefore this protocol is able to define different cell biological phenotypes between seemingly similar mutants.

This protocol has only been used in Arabidopsis so might need to be adapted in order to correctly fix tissues in other organisms. The protocol has been used equally well to assess total mRNA accumulation in both root and leaf tissue.

2. Materials

- Glass vials and tissue culture dishes (see **Note 1**)
- Forceps and razor blade for tissue manipulation.
- Buffer 1: 120mM NaCl, 7mM Na₂HPO₄, 3mM Na₂PO₄, 2.7mM KCl. 80mM EGTA, 0.1% Tween, 10% dimethylsulfoxide (DMSO). (see **Note 2**).
- Paraformaldehyde (PFA)
- Xylene
- 100% Ethanol
- 100% Methanol
- 12-well plastic tissue culture dish. Other sizes of culture dishes may be used but will require more reagent.
- Perfect Hyb Plus Buffer (Sigma-Aldrich, H7033)
- Oligo(dT)₂₅ DNA primer labelled with Fluorescein (see **Note 3**)
- 20x SSC (20x SSC is 3M NaCl and 300mM sodium citrate) (see **Note 4**)
- 10% SDS (see **Note 4**)
- Glass microscope slides
- Appropriate cover slips
- VectaShield Antifade mounting material (<https://vectorlabs.com>, H-1000)
- VectaShield Antifade mounting material with DAPI (<https://vectorlabs.com>, H-1200)
- Propidium iodide (Sigma-Aldrich, P4864)

3. Methods

Carry out all procedures at room temperature unless otherwise specified.

1. Grow Arabidopsis seedlings using your standard growth conditions.
2. Prepare required tissue samples from leaf and/or root tissue (see **Note 5**)
3. Place samples into glass container with Buffer 1 + 5% PFA (freshly made with PFA). Depending on the container use enough liquid to ensure tissue was fully submerged. Incubate at room temperature with slow shaking for >30minutes (see **Note 6**).
4. Remove liquid and wash with slow shaking for 5minutes with 100% methanol (x2) and 100% ethanol (2x) for a total of 4 washes. Completely cover tissue samples with wash solutions.
5. Remove liquid and wash for 30minutes with slow shaking with 1:1 ethanol:xylene (see **Note 7**). Always use a glass vial for this wash.
6. Remove liquid and wash for 5minutes with 100% ethanol.
7. Remove liquid and wash for 5minutes with 1:1 methanol:Buffer 1 (without PFA) mix.
8. Remove liquid and postfix for Buffer 1 with 5% PFA for >30minutes.
9. Remove liquid and wash for 5minutes with Buffer 1 (without PFA) (x2) and for 5minutes with Perfect Hyb Plus Buffer (1x) (see **Note 8**)
10. Tissue samples are pre-hybridized at 50°C in fresh Perfect Hyb Plus Buffer for >1hour (see **Note 9**).
11. Add 1pmol μl^{-1} oligo(dT) probe labeled with fluorescein (see **Note 10**) to Perfect Hyb Plus Buffer and incubate with tissue sample overnight at 50°C in the dark (see **Note 11**)
12. Remove liquid and incubate at 50°C with 2xSSC, 0.1% SDS wash solution for 1hour and then 0.2xSSC, 0.1% SDS wash solution for 20minutes.
13. Samples are transferred to fresh 0.2xSSC (no SDS) and can be held at 4°C for weeks prior to imaging (see **Note 12**)
14. Samples are processed for size in order to fit under available cover slips (see **Note 13**) and mounted in VectaShield Antifade mounting material +/- DAPI depending on the visualization requirements (see **Note 14**).
15. Samples should be evaluated using appropriate filters to visualise position of nucleus (with DAPI or PI) alongside the expression of the fluorescein-labeled probe. Both confocal and epifluorescence microscopes have been successfully used for visualising fluorescein expression.
16. If necessary, quantification of mRNA accumulation can be achieved by selecting a single point or set area in the DAPI image, one within the nucleus and another in the

cytosol adjacent to the nucleus (Figure 2). The pixel intensity is then measured at the equivalent positions in the Fluorescein-image. This will give a value that represents the amount of fluorescein expression in the nucleus compared to cytoplasm. It is important to select the location for measurement using the DAPI image to rule-out the chance of any bias that might occur if the position was selected in the fluorescein image.

17. Within a single experiment multiple nuclei should be visualized across independent tissue samples. Experiments should be repeated on at least three occasions to verify any findings.

4. Notes

NOTE 1. Plastic vials will be melted by xylene. Use of a shallow glass vial or dish allows for easier manipulation of tissue samples.

NOTE 2. Buffer 1 without DMSO and PFA can be prepared in advance. DMSO and PFA are added prior to the experiment.

NOTE 3. This type of labeled oligo(dT) can be ordered as from most oligonucleotide supplies.

NOTE 4. Dilute to working concentrations from 20XSSC and 10% SDS stocks

NOTE 5. Most studies either use small leaves (<5mm) or root tissue for this assay. Parry (2014) and Sorensen *et al* (2017) quantify nuclear mRNA accumulation in cells of the root elongation zone that have larger nuclei.

NOTE 6. Take care when working with PFA as it is a carcinogen.

NOTE 7. Xylene should be pipetted in a fume-hood with a glass pipette.

NOTE 8. Samples can be transferred to a plastic tissue culture dish for final wash with Perfect Hyb Plus Buffer.

NOTE 9. Wrap tissue culture dish (or other sample holder) with parafilm to prevent evaporation of solution. Repeat for all steps conducted at 50°C.

NOTE 10. Depending on available imaging systems other fluorophores may be used in place of fluorescein. However all recently published articles have used fluorescein or a fluorescein-derivative such as Alexa Fluor 488 [11].

NOTE 11. Usually the tissue-culture dish is wrapped in foil but this can be omitted if using a dark 50°C incubator.

NOTE 12. When samples are not immediately imaged then ensure that expression of appropriate controls is assessed at the same time in future. This should include an unlabeled sample, wildtype sample and appropriate mutant or treated sample. Samples should only be directly compared with those that have been visualized and/or quantified at the same time.

NOTE 13. A whole leaf sample or a whole root might be too large to fit under a cover slip so these samples can be cut with a sharp razor to fit on the slide.

NOTE 14. The position of a nucleus within a cell can be visualized with DAPI or by addition and mixing of propidium iodide (1 $\mu\text{g ml}^{-1}$) to Vectashield prior to aliquoting onto microscope slide.

5. References

- 1 - Javelle M, Marco CF, Timmermans M (2011) In situ hybridization for the precise localization of transcripts in plants. *J Vis Exp.* 57:e3328. doi: 10.3791/3328
- 2 - Hejátko J, Blilou I, Brewer PB, Friml J, Scheres B, Benková E. In situ hybridization technique for mRNA detection in whole mount Arabidopsis samples (2006) *Nat Protoc.* 1(4):1939-46
- 3 - Duncan S, Olsson TSG, Hartley M, Dean C, Rosa S (2016) A method for detecting single mRNA molecules in Arabidopsis thaliana. *Plant Methods.* 12:13. doi: 10.1186/s13007-016-0114-x
- 4 - Gong Z, Dong CH, Lee H, Zhu J, Xiong L, Gong D, Stevenson B, Zhu JK (2005) A DEAD box RNA helicase is essential for mRNA export and important for development and stress responses in Arabidopsis. *Plant Cell.* 17(1):256-67
- 5 - Parry G, Ward S, Cernac A, Dharmasiri S, Estelle M (2006) The Arabidopsis SUPPRESSOR OF AUXIN RESISTANCE proteins are nucleoporins with an important role in hormone signaling and development. *Plant Cell.* 18(7):1590-603
- 6 - Lu Q, Tang X, Tian G, Wang F, Liu K, Nguyen V, Kohalmi SE, Keller WA, Tsang EW, Harada JJ, Rothstein SJ, Cui Y (2010). Arabidopsis homolog of the yeast TREX-2 mRNA export complex: components and anchoring nucleoporin. *Plant J.* 61(2):259-70. doi: 10.1111/j.1365-313X.2009.04048.x
- 7 - MacGregor DR, Gould P, Foreman J, Griffiths J, Bird S, Page R, Stewart K, Steel G, Young J, Paszkiewicz K, Millar AJ, Halliday KJ, Hall AJ, Penfield S (2013) HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENES1 is required for circadian periodicity through the promotion of nucleo-cytoplasmic mRNA export in Arabidopsis. *Plant Cell.* 25(11):4391-404. doi: 10.1105/tpc.113.114959
- 8 - Parry G (2014) Components of the Arabidopsis nuclear pore complex play multiple diverse roles in control of plant growth. *J Exp Bot.* 65(20):6057-67. doi: 10.1093/jxb/eru346
- 9 - Gallemí M, Galstyan A, Paulišić S, Then C, Ferrández-Ayela A, Lorenzo-Orts L, Roig-Villanova I, Wang X, Micol JL, Ponce MR, Devlin PF, Martínez-García JF (2016) DRACULA2 is a dynamic nucleoporin with a role in regulating the shade avoidance syndrome in Arabidopsis. *Development.* 143(9):1623-31. doi: 10.1242/dev.130211
- 10 - Sørensen BB, Ehrnsberger HF, Esposito S, Pfab A, Bruckmann A, Hauptmann J, Meister G, Merkl R, Schubert T, Längst G, Melzer M, Grasser M, Grasser KD (2017) The Arabidopsis THO/TREX component TEX1 functionally interacts with MOS11 and modulates mRNA export and alternative splicing events. *Plant Mol Biol.* 93(3):283-298. doi: 10.1007/s11103-016-0561-9
- 11 - Ehrnsberger HF, Pfaff C, Hachani I, Flores-Tornero M, Sørensen BB, Längst G, Sprunck S, Grasser M, Grasser KD (2019) The UAP56-Interacting Export Factors UIEF1 and UIEF2 Function in mRNA Export. *Plant Physiol.* 179(4):1525-1536. doi: 10.1104/pp.18.01476

12 - Engler JA, Van Montagu M, and Engler, G. (1994). Hybridization *in situ* of whole-mount messenger RNA in plants. *Plant Mol. Biol. Rep.* 12, 321–331

6. Figure Legends

Figure 1: Poly(A) RNA export is blocked in *los4-2* cells at warm and high temperatures. Wild-type and *los4-2* mutant plants were grown at 4°C for 2 months or 22°C for 2 weeks. *In situ* hybridization with fluorescein-labeled oligo(dT) probe was performed with seedlings growing at 4°C (a) or 22°C (b). Reproduced from Gong *et al* (2005) [4]

Figure 2: Epifluorescence microscope images (63x) of cells in the root elongation zone from 7do seedlings treated with an oligo(dT)-Fluorescein (FLO) probe (**b, d**) and post-stained with DAPI (**a, c**). Nuclei in *nup85* mutant roots show an increase in FLO accumulation when compared to wildtype. FLO accumulation is quantified by setting single-point measurement positions in images of DAPI-stained nuclei (**a, #1, #2; c, #3, #4**) and then measuring pixel intensity in an identically positioned FLO-image (**b, #1a, #2a; d, #3a, #4a**). The pixel intensity is compared between **#1a and #2a** (or **#3a and #4a**) to give a value for FLO accumulation in the nucleus. Scale bar- 10µm. Figure amended from Parry (2014).

Figure 1

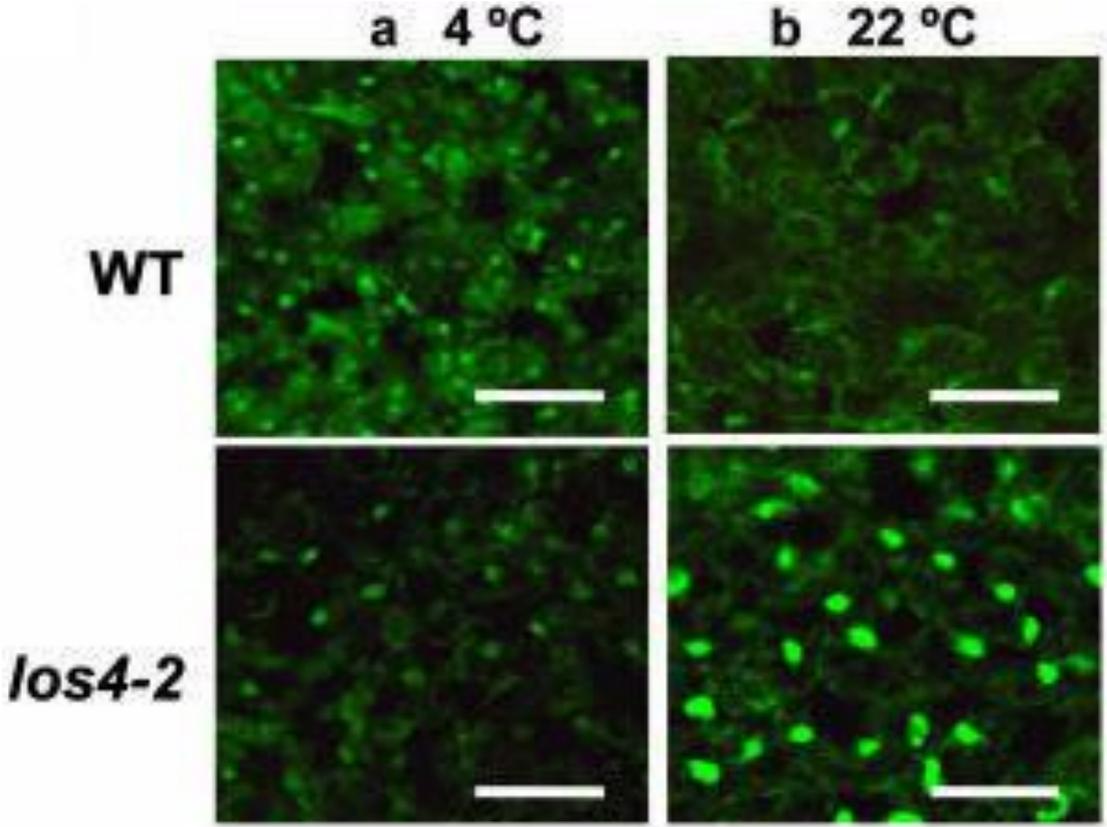


Figure 2

