Isolation of the Plant Nucleolus

Protocol adapted from

Pontvianne F, Boyer-Clavel M, Sáez-Vásquez J (2016) **Fluorescence-Activated Nucleolus Sorting in Arabidopsis.** Methods Mol Biol. doi: 10.1007/978-1-4939-3792-9_15.

Pendle AF, Shaw PJ (2017) **Isolation of Nuclei and Nucleoli.** Methods Mol Biol. doi: 10.1007/978-1-4939-6533-5_3

Montacié C, Durut N, Opsomer A, Palm D, Comella P, Picart C, Carpentier MC, Pontvianne F, Carapito C, Schleiff E, Sáez-Vásquez J (2017) **Nucleolar Proteome Analysis and Proteasomal Activity Assays Reveal a Link between Nucleolus and 26S Proteasome in *A. thaliana*.** Front Plant Sci. doi: 10.3389/fpls.2017.01815.

i. Summary

This article describes a protocol for Fluorescence-Activated Nucleolar Sorting (FANoS), which allows for clean extraction of the nucleolus from a range of plant tissues. This generates a nucleolar extract that is appropriate for use for downstream multi-'omic characterisation of this important nuclear sub-domain.

ii. Keywords

Nucleolus, nucleus, chromatin, proteome, proteomics, genomics

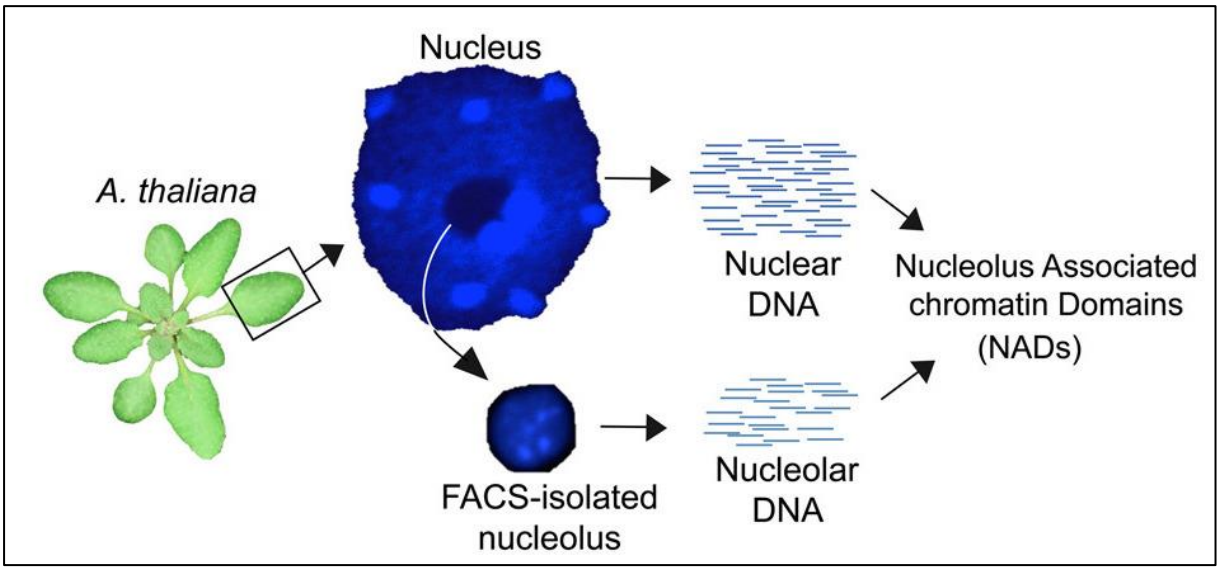

Figure 1: Schematic of nuclear and nucleolar extraction for identification of nucleolar associated chromatin domains (adapted from Pontvianne *et al*, 2016)

1. Introduction

The nucleus lies at the heart of every eukaryotic cell, where it sequesters nucleic acid and is the starting point for cellular processes that rely on gene expression. However the nucleus is far from a single homogenous unit as it contains a large number of sub-domains that carry out discrete functions (Santos *et al*, 2020).

The largest of these sub-domains is the nucleolus, whose function is defined by the production of ribosomal RNA through the grouping of rRNA genes. Recent work has revealed that the nucleolus has other functions and it certainly plays a role in shaping the organisation of nuclear chromatin domains as well as contacting multiple loci and associated proteins whose role is not involved with ribosome biogenesis (Picart-Picolo *et al*, 2019; Pontvianne and Liu, 2020).

Julio Sáez-Vásquez, Frederic Pontvianne and colleagues have been in the vanguard of the study of the plant nucleolus. They have contributed to an analysis of its proteomic composition (Montacie *et al*, 2017) as well as characterisation of its interaction with chromatin domains, in which they show that the NUCLEOLIN1 protein is required for correct nuclear organisation (Pontvianne *et al*, 2016). They have developed a novel methodology in which they use Fluorescence-Activated Nucleolus sorting (FANoS) to isolate nucleoli extracts (Pontvianne *et al* 2013; Pontvianne *et al* 2017; Carpentier *et al*, 2018, Figure 1). FANoS improves upon previous methods of nucleolus extraction that do not use cell sorting (Pendle *et al*, 2005; McKeown *et al*, 2008; Pendle and Shaw, 2017).

Although the Sáez-Vásquez lab well characterised the nucleolar proteome that was extracted from 3-week old leaves (Montacie *et al*, 2017). However the opportunity remains to understand the role of the nucleolar proteins during the processes of cell division and differentiation. It is reasonable to assume that the composition of the nucleolus will change depending on developmental context. Improvements in Fluorescent Activated Nucleus Sorting (FANS) now allow extraction and analysis of samples from specific-tissues and therefore the opportunity exists to analyse cell type specific activities of the nucleolus (Gutzat and Mittelsten Scheid, 2020; INDEPTH Academy, Oxford Brookes RADAR https://doi.org/10.24384/fvmf-km57)

Nucleoli can be simply isolated through successive homogenization of tissues to generate nuclear and then nucleolar fractions. However despite best efforts this will inevitably generate accompanying cell debris that may impact the downstream processing of samples. In recent times fluorescent activated nuclear sorting (FANS) or more specifically FANoS (fluorescent activated nucleolar sorting) has been used to more cleanly isolate samples. This method of course relies upon availability of a reliable fluorescently labelled nucleolar-localised protein. This rules out a range of experimental organisms that do not have the same access to molecular tools as are available in Arabidopsis.

Nucleolar samples have also been successfully isolated from protoplasts generated from Arabidopsis suspension cell cultures. This article does not refer to this method but for more information please consult Pendle *et al* (2005) and Palm *et al* (2016). This articles presents a consensus FANoS methodology for nucleolar isolation, after which samples can be used for downstream applications to analyse the DNA, RNA or protein content. However this simple protocol can be used for isolation of nucleoli from tissues that are not available for fluorescence sorting.

2. Materials

2.1 Reagents

- Cooled Falcon tubes (15ml or 50ml depending on the amount of starting material)
- 37% Paraformaldehyde stock solution
- Wash solution: 10 mM Tris–HCl pH 7.5, 10 mM Na EDTA pH 8, 100 mM NaCl. Prepare fresh from individual stock solutions.
- Galbraith's buffer (GB): 45 mM $MgCl_2$, 20 mM MOPS, 30 mM sodium citrate, 0.1 % Triton X-100, adjusted to pH 7 with NaOH. Can be prepared in advance and stored at −20 °C for several months. Before use add 5 µL 2-ME/ per ml GB.
  For downstream analysis of protein samples then also add 100 µg/mL PMSF, 1 µg/mL Pepstatin A and a broad action protease inhibitor (such as cOmplete™ Protease Inhibitor, https://www.sigmaaldrich.com/catalog/product/roche/04693159001?)
  Downstream analysis of RNA samples requires additional of a RNAse inhibitor (available from many suppliers).
- Razor blades, in our hands double-sided blades are sharper.
- 30ml plastic Petri-dish
- 30um non-sterile filter (available from CellTrics or similar https://us.sysmex-flowcytometry.com/consumables/celltrics-filters/non-sterile-celltrics-filters/1444/non-sterile-celltrics-filters-250/box?c=12
- DAPI stock solution: 1 mg/mL of stock solution. Dilute 1000× for DAPI staining.

2.2 Equipment

- Flow Cytometer such as BD Biosciences FACS Aria II, BD FACSMelody or Beckman Coulter MoFlo Astrios.
- Sonicator, such as BioruptorTM200
- Epifluorescent microscope with appropriate filters
- Haemocytometer
- Rotating wheel/shaker

2.3 Challenges of reproducibility

As with any experiment it is important to consider the correct controls so as to gain maximum information from the data generated by FANoS. These controls should include a non-fluorescent control (wildtype) and a nuclear-extraction control. A reproducible picture of the nucleolar content can be understood by comparing the outputs of the FANoS extraction to these appropriate controls.

Previous experiments that have isolated and analysed the nucleolar proteome have characterised a various number of proteins. Both Pendle *et al* (2005) and Palm *et al* (2016) used traditional nucleolar extraction methods from Arabidopsis cell cultures to identify 217 and 1602 proteins respectively. Montacie *et al* (2017) performed two replicate nucleolar extracts using FANoS from Arabidopsis leaves and identified 1001 and 778 proteins. Importantly 562 proteins were shared between these extractions, meaning that 44% and 28% of initially identified proteins were not considered subsequent downstream analysis. Finally they showed that 17% (99) or 73% (409) of these 562 nucleolar proteins had been previously identified in Pendle *et al* (2005) or Palm *et al* (2016) respectively. This highlights that; *1*. Extraction methods may have improved over the past decade; *2.* There is actually

(perhaps surprisingly) good similarity between samples extracted from suspension cell cultures and *in planta* samples.

Ultimately the variation in nucleolar extracts necessitates that all appropriate controls should be taken from within a single experiment, as drawing conclusions by comparing nuclear and nucleolar extracts made at different times is not a fair comparison. Montacie *et al* (2017) show that increasing the experimental replicates can improve confidence in the identity of the nucleolar proteins, which will ultimately improve the strength of the conclusions drawn from the downstream analysis.

2.4 Growth of Plant Material

To perform downstream immunohistochemistry Pendle and Shaw (2016) use a non-FANoS method to isolate nucleoli from the root tips of Arabidopsis, Wheat or Pea plants with no change in each methodology (aside from the number of root samples). They suggest collecting 10mm samples from 100 Arabidopsis root tips or 5mm of emerging roots from 15 wheat/pea samples. For their FANoS method Pontvianne *et al* (2016) uses 1gram of leaves from 3-week old Arabidopsis plants. As expected this demonstrates that downstream 'omic analysis requires a much larger amount of starting tissue. As such use of FANoS to extract nucleoli from root tip tissue will require >> 100 Arabidopsis seedlings.

Leaf tissue from 3week old plants contains large cells with nuclei that individually may have ploidy levels over 32C [**NOTE 1**]. This in turn increases the size of the nucleolus and the absolute amount of nucleolar tissue that can be isolated. In Arabidopsis root tips the meristematic zone contains numerous small cells with concomitantly small nuclei/nucleoli; whilst further back in the root elongation zone the cells and nuclei/nucleoli will be larger [**NOTE 2**].

Ultimately the type and amount of tissue relies upon the questions that an experiment is aiming to answer. Although nucleoli extracted from a root or shoot meristem will provide different biological insights to those gained from nucleoli extracted from 3-week old leaf tissue, it will be more challenging to obtain the amount of tissue required for these experiments.

2.5 Choice of fluorescent markers

To obtain a clean nucleolar extract a fluorescent marker that is exclusively expressed in the nucleolus should be used for cell-sorting. Fibrillarin2 (FIB2) is a nucleolar protein implicated in ribosomal RNA maturation and the Arabidopsis FIB2:YFP has been extensively used as an *in planta* nucleolar marker (Pontvianne *et al*, 2016; Palm *et al*, Montacie *et al*, 2017, Figure 2, **NOTE 3**). If a different nucleolar marker is used then this would need to be empirically tested. Additional candidates for *in planta* nucleolar markers have been identified by Palm *et al* (2016) and Montacie *et al* (2017).

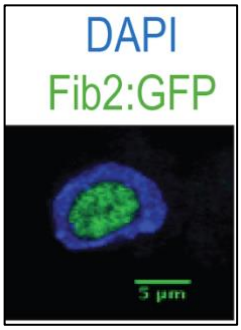

Figure 2: Nucleolar FIB2:GFP expression within a DAPI-stained nucleus from an Arabidopsis root tip (adapted from Montacie *et al*, 2017).

Pontvianne *et al* (2016) used the viral 35S promotor sequence to ubiquitously drive FIB2:YFP expression, which allows the isolation of a high number of nucleoli during nucleolar-sorting of tissues from 3-week old leaves.

If an experiment aims to investigate the activity of the nucleolus in a particular developmental content then an appropriate tissue-specific promotor should be used to drive FIB2:YFP expression. However a consideration must be made as to the amount of tissue that will be required for experiments that extract nucleoli from a small expression domain.

3. Methods

For all steps of the protocol samples should be kept on ice and also preferably conducted in a cold room. Whilst speed of activity is important, for optimal reproducibility it is critical that where possible, samples are treated identically [**NOTE 4**].

3.1 Nuclei Extraction

1. Grow plants as required for particular experimental design

2. Harvest tissue (from leaves or root tips, etc) and place into 15ml/*50ml* Falcon tube for 20min in 10/*30ml* mL of 4% formaldehyde in Wash buffer. Rotate continuously.

3. Remove buffer and wash 2x10minutes in cold Wash buffer.

4. Blot dry tissue and add into plastic petri dish with amount of Galbraith's buffer (GB) that will wet but not flood samples [**NOTE 5**].

5. Chop samples for at least 1minute with a fresh razor blade. Use a maximum of 0.5g tissue when chopping tissue.

6. Filter debris through a pre-wet 30um cell sieve on ice. Add additional liquid to the petri dish to recover all released nuclei. Repeat until all samples have been chopped. The total volume of nuclei should be minimised but will vary depending on amount of starting tissue [**NOTE 6**].

7. Centrifuge sample at 200x g and carefully resuspend in 1ml GB with 1ug/ml DAPI.

8. The amount of extracted nuclei can be assessed at this stage by loading a small sample (1-5ul) onto a haemocytometer and quantified under an epifluorescence microscope with a DAPI/UV filter.

3.2 Nucleolar extraction

9. Set aside 1/3 of the total sample as the nuclear extract control.

10. To release nucleoli from the nuclear extract use an available sonicator [**NOTE 7**]. Pontvianne *et al* (2016) use a Bioruptor and sonicate three times for 5 min (30 s ON/30 s OFF) at medium power **[NOTE 8**, **NOTE 9**]. Sonicate no more than 700ul of sample in a cooled 1.5ml eppendorf tube.

11. After sufficient sonication [**NOTE 9**] remove remaining cell and nuclear debris by centrifuging briefly at 200xg. The nucleolar sample is in the pellet and should be resuspended in 1ml GB.

12. If samples will not be sorted then they can be processed for downstream analysis (**Step 15)**.

3.3 Nucleolar Sorting

13. Sorting is carried on using an available FACS machine. The specifics of the sorting will need to be finalised with input from an expert user. The following guidelines can aid user decisions:
    a. For DAPI excitation, a 375 nm laser is ideal (although a 405 nm laser can be used) with appropriate detection filters, e.g., 442/46 nm or 450/40 nm. For GFP use a 488 nm laser (and 530/30 nm detection) and for mCherry, a 561 nm laser with 610/20 nm detection is required.

    b. For nucleolar sorting a nozzle size of 70um and sheath pressure of 37psi is recommended.

    c. With both negative control and nuclear-sample the DAPI-labelled DNA can be assessed to test the quality of the extraction. However this is not possible (or needed) for the nucleolar sample. However extracts with poor resolution between the ploidy peaks can still be used for successful sorting.

    d. Prior to sorting set the appropriate gates for fluorophore +/- nuclear/nucleolar samples by analysing the fluorescently labelled samples. Gates are then set for sorting in all samples.

    e. The volume of nuclear and nucleolar extracts will vary depending on the tissue from which they were extracted and the sheath pressure that is used when sorting. Pontvianne *et al* (2016) suggest that nucleoli from a leaf cell will be held in a 3.88nl drop of sorted liquid (nozzle size 70um, sheath pressure 37psi). Therefore 1ml of GB from **Step 11** will yield ~250,000 nucleoli. In a separate study that isolated nuclei from the cells of a dissected shoot apical meristem, Gutzat and Mittelsten Scheid (2020) estimate the volume of 1 nuclei was equal to 1nl (70um, 70psi).

14. Samples should be sorted directly into GB solution or a buffer appropriate for subsequent downstream analysis. For RNA extraction this can TRIZOL LS, for DNA extraction it will a DNA lysis buffer or for protein analysis into an appropriate protein extraction buffer. If appropriate, use a 2x concentration of appropriate extraction buffer due to the diluting effect of the liquid within the sorted samples.

15. Downstream analysis can proceed according to the specific aims of the experiment [**NOTE 10**].

4. Notes

NOTE 1: Ploidy does not affect the number of nucleoli, but the size of the nucleolus is proportional to the level of ploidy of the cell.

NOTE 2: Some root cell-types possess a large nucleolus that can represent up to 25% of the nuclear volume, albeit in a small total volume. In larger leaf cells the nucleolus can be 5–10% of the nuclear volume although in absolute terms this will be larger than a root tip nucleolus.

NOTE 3: Although a nucleolar-localised fluorescent signal represents a small proportion of total nuclear volume it is sufficient to sort whole nuclei.

NOTE 4: If possible use pre-cooled equipment in a cold room. If one item has been forgotten then the temptation might be to use a 'warm-item' for a short step in the protocol. This should be avoided as the heat-cool cycle could damage the nuclear extract.

NOTE 5: Application of too much liquid at this stage will reduce the efficiency of chopping and nuclei release.

NOTE 6: Pontvianne *et al* (2016) add a total of 2ml GB for nuclei extraction from 1g of leaf tissue.

NOTE 7: Sonication will shear nuclear DNA and break the nuclear membrane. The tightly packed nucleoli should be retained intact but this step will need to be empirically by varying the settings on the available sonicator.

NOTE 8: It is important to keep samples cool during and between sonications. If there is no cooling system on the sonicator then place samples on ice for at least 2minutes between sonications.

NOTE 9: If needed between rounds of sonication the DAPI-stained samples can be removed and quantified with a haemocytometer. This will allow assessment of the number and quality of nuclei and nucleoli present after each round of sonication and whether extra rounds of sonication are necessary.

NOTE 10: Advice on downstream procedures can be found in INDEPTH Academy protocol on 'Fluorescent Activated Nuclear Sorting' hosted by Oxford Brookes University RADAR repository https://doi.org/10.24384/fvmf-km57.

5. References

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