Use of Fluorescence-Activated Nuclear Sorting (FANS) from Rare Plant Cell Types for analysis of gene expression and chromatin interactions.

Protocol adapted from:

Gutzat R, Mittelsten Scheid O (2020) **Preparing Chromatin and RNA from Rare Cell Types with Fluorescence-Activated Nuclear Sorting (FANS).** Methods Mol Biol. doi: 10.1007/978-1-0716-0179-2_7

Desvoyes B, Vergara Z, Sequeira-Mendes J, Madeira S, Gutierrez C (2018) **A Rapid and Efficient ChIP Protocol to Profile Chromatin Binding Proteins and Epigenetic Modifications in Arabidopsis.** Methods Mol Biol. doi: 10.1007/978-1-4939-7318-7_5

Slane D, Berendzen KW, Witthöft J, Jürgens G (2020) **Transcriptomic Profiling of the Arabidopsis Embryonic Epidermis Using FANS in Combination with RNAseq.** Methods Mol Biol. doi: 10.1007/978-1-0716-0342-0_12

Thibivilliers S, Anderson D, Libault M (2020) **Isolation of Plant Root Nuclei for Single Cell RNA Sequencing.** Curr Protoc Plant Biol. doi: 10.1002/cppb.20120

Weinhofer I, Köhler C (2020) **Endosperm-specific chromatin profiling by fluorescence-activated nuclei sorting and ChIP-on-chip.** Methods Mol Biol. doi: 10.1007/978-1-62703-773-0_7

Wang N, Liu C (2020) **Study of Cell-Type-Specific Chromatin Organization: In Situ Hi-C Library Preparation for Low-Input Plant Materials.** Methods Mol Biol. doi: 10.1007/978-1-0716-0179-2_9.

i. Summary

Analysis of individual cell types can add significant understanding to our knowledge the processes that drive the formation of complex tissues. However one major challenge in this area involves the difficulty in separating cells with different identifies that may be buried deep within tissues. Recently cell-type specific expression of fluorescent reporter proteins has facilitated the isolation of different cell types; initially by isolation of protoplasts and now through the use of flow cytometry and cell sorting. This latter technique allows the more rapid separation of the nuclei from specific cell files to ensure that a more realistic *in-planta* situation is revealed upon subsequent downstream analysis. This article provides a consensus methodology for the isolation of labelled nuclei and for the processing of these samples for analysis of gene expression, methylation state and of chromatin interactions.

ii. Keywords

Nucleus, Nuclei, gene expression, fluorescent reporter, chromatin, methylation, flow cytometry, cell sorting.

1. Introduction

Historically plant tissue-specific analysis of gene expression was limited by the precision of the dissection skills of the researcher, which mostly precluded the analysis of hidden or buried cell types. More recently laser dissection allowed greater precision but this technique is both time consuming and potentially damaging, as well as unable to fully solve the challenge of obtaining buried cell types (Nelson *et al*, 2006).

In the early 2000s a revolutionary manuscript from Phillip Benfey's lab analysed gene expression in protoplasts obtained from fluorescently-labelled cells in the Arabidopsis root tip, which provided a cell-type-specific expression map of this tissue (Birnbaum *et al*, 2003). Since that time the broad strategy behind this method has been built upon and improved, enabling researchers to analyse samples from smaller inputs that will have less tissue damage.

By combining advances in the use of reporter genes, analysis of cell-type specific expression patterns and cell-sorting technologies, **Fluorescence-Activated Nuclear Sorting (FANS)** has emerged as a leading technique for the analysis of any distinct group of cells. This technique is applicable to even rare cell types that may be found in the Arabidopsis embryo, shoot or root meristem. In these cases the number of cells can be vanishingly small as proportion of a total; for example use of the CLAVATA3 promotor limits reporter gene expression to only 20 cells within Arabidopsis shoot apical meristem, which in a 14-day old seedling is just 0.01% of the total (Gutzat and Mittelsten Scheid O, 2020).

FANS allows isolation of the cell nucleus so is well-designed for analysis of whole genome processes or chromatin interactions. In addition experiments have demonstrated that the nuclear RNA fraction can also be used as a proxy for total cellular mRNA levels (Slane *et al*, 2015; Gutzat *et al*, 2020). Of course FANS is not an appropriate protocol for analysis of largely cytoplasmic processes such as ribosomal-RNA interactions and/or translation rates.

Importantly FANS can be combined with a range of downstream applications such as Hi-C (Wang and Liu, 2020), RNA-Seq (Slane *et al*, 2020; Gutzat and Mittelsten Scheid, 2020), DNA methylation (Gutzat and Mittelsten Scheid, 2020), transcriptional profiling (Slane and Bayer, 2017) or Chromatin Immunoprecipitation (Desvoyes *et al*, 2018; Weinhofer and Köhler, 2020).

Here we present a consensus methodology for using FANS to isolate nuclei from rare tissue types as well as for three subsequent downstream applications; namely RNA-based techniques, genomic DNA-based techniques and chromatin immunoprecipitation. The type of available flow cytometer will determine the specific methodology for cell sorting and as such that portion of the protocol should be developed with input from an expert user.

2. Materials

If working with RNA, it is recommended to use all RNase-free disposable items such as pipette tips and eppendorf tubes. Where possible work in an RNAse-free environment that has been treated with an appropriate RNAse decontamination solution.

2.1 Nuclei Isolation

- Single use plastic round petri dishes
- Sharp razor blades. We find that double-sided blades are sharper than single-sided blades.
    - Pestle and Mortar (*optional*)
    - Dounce homogeniser (*optional*)
- 30µm cell strainer or Miracloth (typical pore size: 22-25 µm)
- Nuclei isolation buffer (NIB): 500 mM sucrose, 100 mM KCl, 10 mM Tris–HCl pH 9.5, 10 mM EDTA, 4 mM spermidine, 1 mM spermine. Filter-sterilize and keep at 4°C. Alternatively, NIB can be stored at -20°C for several months.
  Add 2-ME (mercaptoethanol) (1% v/v) just before use.
- Galbraith buffer (GB): 45 mM $MgCl_2$-$6H_2O$, 30 mM sodium citrate (trisodium), 20 mM MOPS (3-(N-mor- pholino)propanesulfonic acid), pH 7.0. Autoclave or filter- sterilize and store at 4°C. Add 5 µL 2-ME/ per ml GB before use.
- Modified Galbraith buffer (Mod-GB) as GB with 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?)
- Staining Buffer (SB): GB plus 5ug/ml DAPI (from 5mg/ml DAPI stock solution)
- Triton X-100
- CelLyticTM Plant Nuclear kit (*optional*)
  https://www.sigmaaldrich.com/catalog/product/sigma/cellytpn1?
- Flow sorting tubes (specific to the brand of available flow cytometer)

2.2 RNA extraction

- TRIzol LS. https://www.thermofisher.com/order/catalog/product/10296028#/10296028
- Chloroform
- Isopropanol
- RNA-grade glycogen
- Qiagen RNeasy Micro Kit. https://www.qiagen.com/us/products/discovery-and-translational-research/dna-rna-purification/rna-purification/total-rna/rneasy-micro-kit

2.3 DNA Extraction

- Zymo Quick-gDNA MicroPrep Kit. https://www.zymoresearch.com/collections/quick-dna-kits
- Quant-iT™ PicoGreen™ dsDNA Assay Kit
https://www.thermofisher.com/order/catalog/product/P7589#/P7589
- Pico Methyl-Seq Library Prep Kit (*optional*)
  https://www.zymoresearch.com/products/pico-methyl-seq-library-prep-kit

2.4 Chromatin Immunoprecipitation

- Phosphate buffer saline (PBS)
- Paraformaldehyde powder
- RNaseA
- 2x ChIP Lysis buffer: 100 mM Tris–HCl pH 8.0, 20 mM EDTA, 2% SDS (from 10% SDS stock), 2 mM PMSF and appropriate amount of cOmplete™ Protease Inhibitor

- ChIP Dilution buffer: 16.7 mM Tris–HCl pH 8.0, 167 mM NaCl, 1.2 mM EDTA and 1.1% Triton X-100. Add before use 1 mM PMSF and appropriate amount of cOmplete™ Protease Inhibitor.
- Protein A or Protein G agarose beads (available from many suppliers).
- ChIP grade antibodies appropriate for the particular experiment.
- Low Salt Wash Buffer (make 100ml stock): 20 mM Tris–HCl pH 8.0, 2 mM EDTA, 150 mM NaCl, 0.1% SDS, and 1% Triton X-100. Add before use 1 mM PMSF and appropriate amount of cOmplete™ Protease Inhibitor
- High Salt Wash Buffer (make 100ml stock): 20 mM Tris–HCl pH 8.0, 2 mM EDTA, 500 mM NaCl, 0.1% SDS, and 1% Triton X-100. Add before use 1 mM PMSF and appropriate amount of cOmplete™ Protease Inhibitor
- LiCl Wash Buffer (make 100ml stock): 10 mM Tris–HCl pH 8.0, 1 mM EDTA, 0.25 M LiCl, 1% Igepal CA-630, and 1% sodium deoxycholate. Add before use 1 mM PMSF and appropriate amount of cOmplete™ Protease Inhibitor
- TE Buffer ((make 100ml stock): 10 mM Tris–HCl pH 8.0, and 1 mM EDTA. Add before use 1 mM PMSF and appropriate amount of cOmplete™ Protease Inhibitor.
- ChIP Elution Buffer (freshly prepared): 1% SDS, 0.1 M $NaHCO_3$
- MinElute PCR Purification Kit, https://www.qiagen.com/us/products/discovery-and-translational-research/dna-rna-purification/dna-purification/dna-clean-up/minelute-pcr-purification-kit/

2.5 Equipment

- Flow Cytometer such as BD Biosciences FACS Aria II, BD FACSMelody or Beckman Coulter MoFlo Astrios.
- Sonicator such as BioruptorTM200
- Nanodrop
- BioAnalyser
- Rotating shaker

3. Methods

Within the source protocols there are important differences for the fixation of tissues prior to nuclei isolation. The protocols that focus on isolated cells from early embryonic tissue fix tissue with paraformaldehyde prior to nuclei isolation (Slane *et al*, 2020)[**NOTE 1**].

However the methods that intend to analyse cells derived from root or shoot meristem do not require a fixation step (Gutzat and Mittelsten Scheid, 2020; Thibivilliers *et al*, 2020).

The method below omits the fixation step but if this is needed see Slane *et al* (2020) [**NOTE 3**]. Researchers developing a new protocol are advised to use both methods to understand which provides the best results in their hands. Steps highlighted in Red represent overnight stopping points.

3.1 Plant Growth and Isolation of Nuclei

1. Plants should be grown according to user preference depending on tissue type of interest [**NOTE 4**]. Researchers should be confident of the tissue-specificity and strength of the nuclear-localised fluorophore used to sort fluorescent nuclei [**NOTE 5**].

2. All steps of the workflow should be conducted at 4°C. Whilst speed of activity is important, for optimal reproducibility it is critical that where possible samples are treated identically [**NOTE 6**]

3. Dissect tissue and place in Nuclei Isolation Buffer (NIB) prior to downstream processing **[NOTE 7; NOTE 8**].

4. Tissue samples are placed a plastic petri dish in Galbraith buffer (GB) and chopped with a sharp razor blade for up to 10 minutes/ sample. Ensure samples are coated with GB but there is not excess liquid as this will decrease the effectiveness of the chopping. After chopping rinse petri dish with 500ul GB to ensure all nuclei have been isolated [**NOTE 9**].

5. Remove isolated nuclei in GB and strain through a pre-wet 30um sieve into a 1.5ml Eppendorf.

6. Add Triton X-100 to 0.3% [**NOTE 10**].

7. Centrifuge for 15minutes at 1000x g (always in a pre-cooled centrifuge)

8. Remove supernatant and carefully resuspend nuclei in 1.5ml SB [**NOTE 11**].

9. Incubate for 15minutes on ice.

10. Filter sample again through 30um filter into flow sorting tubes.

3.2 Fluorescence Activated Nuclei Sorting (FANS)

11. Sort nuclei using the available cell sorter. As mentioned above an expert user should be consulted before using the machine..

12. The technical parameters used on each machine and with various fluorophores will be different and will need to be empirically tested. However the following guidelines can aid these 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. A usual nozzle size of 70um and default sheath pressure of 70psi is used but if whole intact nuclei are required for downstream analysis then a 100um nozzle size with 20psi is used.
    c. With both positive and negative samples begin by using the flow cytometer to analyse DAPI-labelled nuclei to test the quality of the nuclei extraction. As a minimum record 100K total events (corresponding to 10K DAPI events) in order to check nuclei quality and set gates. Cell sorting can then proceed.
    d. Prior to sorting, set the correct gates for collection of fluorophore +/- nuclei.
    e. The volume of 1000 sorted-nuclei with a 70µm nozzle and 70psi will be approximately 1µl; with 100µm nozzle and 20 PSI this will be 3.5µl. To have an estimate of the required sorting time Gutzat and Mittelsten Scheid (2020) estimate that dissected SAMs from 25 14-day-old seedlings/replicate and using 70psi obtained ~100K–150K DAPI-labeled nuclei in 90 minutes of sorting. Furthermore Slane *et al* (2020) indicate that roughly 1ng of total RNA can be extracted for every 1000–2000 collected nuclei.
    f. An example of flow sorted nuclei extraction is shown in **Figure 1**.

13. Samples are sorted into buffers for appropriate downstream applications. This might be TRIzol LS for RNA applications (**see 3.3**), into DNA lysis buffer for analysis of genomic DNA (**see 3.4**) or into 2xChIP lysis buffer for ChIP (**see 3.5**)[**NOTE 12; NOTE 13**].

3.3. RNA Extraction

14. Prepare RNase-free eppendorf tubes to receive sorted nuclei by adding 375ul of TRIzol LS.

15. Sort up to 125ul of nuclei into TRIzol tubes, incubate for at least 5 minutes at room temperature (RT) (see **Step 12.e** above).

16. Add 100ul chloroform and vortex vigorously for 20seconds.

17. Incubates for 10minutes at RT.

18. Centrifuge at full speed in a microcentrifuge at 4°C for 15minutes.

19. Remove aqueous phase and transfer to new RNAse-free 1.5ml tube.

20. Add 250ul isopropanol and 1.5ul RNA-grade glycogen.

21. Incubate at -20C overnight.

22. Centrifuge at full speed for 20minutes at 4°C.

23. Wash pellet in 750ul 80% ethanol and centrifuge at full speed for 5minutes at 4°C.

24. Dry pellet and resuspend in 100ul* nuclease-free water [**NOTE 14**].

25. Use Qiagen RNeasy Micro Kit to perform RNA cleanup and concentration protocol with addition of on-tube DNase digest step [**NOTE 15**].

26. Take 1.5ul to quantify RNA concentration using a Bioanalyzer according to manufacturer protocols.

27. If sufficient quality and quantity of RNA are extracted, proceed to either generate sequencing libraries for the appropriate NGS platform or generate cDNA for downstream qPCR analysis to evaluate expression of specific genes.

3.4 DNA extraction

28. Prepare collection tubes for DNA extraction. This can proceed using a favoured genomic DNA extraction kit, such as a Zymo Quick-gDNA MicroPrep Kit.

29. These kit-based protocols feature column-based isolation and should proceed as per user manual.

30. Elute genomic DNA into 16.5ul of nuclease-free water. Take 1.5ul for DNA quantification and flash freeze remaining sample for future analysis.

31. Quantify genomic dsDNA using a Nanodrop with Pico-green fluorescent label [**NOTE 16**].

32. The methylation status of genomic DNA can be analysed through an experimental pipeline for bisulfite genomic sequencing [**NOTE 17**].

3.5 Chromatin Immunoprecipitation (ChIP, ChIP-ChIP or ChIP-Seq)

Nuclei isolated by FANS can be used for downstream analysis by chromatin immunoprecipitation. This requires that chromatin proteins are not denatured so Modified-GB solution is used when chopping samples [**NOTE 18**]. Furthermore after **Step 7** samples for ChIP will need to be crosslinked to form the DNA-protein complex.

At this stage insert the following:

Step 7a*: Remove the supernatant and gently resuspend the pellet in 1 mL 1× PBS containing 1% paraformaldehyde. Mix gently and incubate for 8 min on ice [**NOTE 19**]*

Step 7b*. Stop crosslinking reaction by addition of a final concentration of glycine of 125mM and incubate for 5 min on ice.*

Step 7c*. Centrifuge at 1500×g for 5 min at 4℃. Remove the supernatant and wash the pellet with 1 ml ice-cold 1× PBS containing protease inhibitors [**NOTE 20**]. Repeat this step.*

Return to **Step 8** above where pelleted nuclei are resuspended in 1.5ml SB and proceed with final filtering and cell sorting.

To accompany the ChIP extraction an RNA extraction is required in order to assess gene expression in the same tissues. RNA extractions should be conducted using **Steps 14-27**. This may require using a larger amount of starting material than needed to perform a single RNA or DNA extraction.

3.5.1 Sonication

33. Proceed from **Step 13** by sorting 100K nuclei into an equal volume of 2x ChIP lysis buffer. Transfer samples into a 1.5ml Eppendorf, incubate on ice for 10minutes.

34. Sonicate samples on ice at high power for 30s ON, 1min OFF for 10minutes [**NOTE 21]**.

35. Centrifuge at full speed for 10minutes at 4°C. Transfer supernatant to a fresh tube. Remove 50ul for quantification in **Step 36**. If needed remaining samples can be flash-frozen at this time.

3.5.2 Quantification of Chromatin

36. To quantify chromatin: 50ul sample is added to 450ul nuclease-free water, 50ul 5M NaCl. Boil for 15minutes and once cooled add 5ul DNase-free RNaseA. Incubate at 37C for 15minutes then add 5ul Proteinase L and incubate at 67°C for 15minutes. Use a QIAquick Min Elute column (or similar) to purify the DNA fraction and quantify on a Nanodrop (or similar). Remove known quantity of chromatin and run on a 1.5% agarose gel to check that sonication has successfully generated the required 300bp fragments [**NOTE 22**].

37. If the sonication and subsequent quantification of the chromatin has been

successful (**Step 36**) then proceed with the immunoprecipitation (IP) [**NOTE 23**].

3.5.3 Chromatin Immunoprecipitation

38. Take remaining chromatin from **Step 35** and dilute with 10x ChIP dilution buffer to decrease SDS concentration to a minimum of 0.1% [**NOTE 24**]. Use up to 1ml of diluted chromatin in each ChIP.

39. Wash 30ul of protein A or Protein G coated agarose beads with ChIP dilution buffer **[NOTE 25**]. Centrifuge at 1000x g at RT for 2minutes. Discard supernatant and repeat wash.

40. Preclear chromatin by adding 30ul washed agarose beads (from **Step 39**) with diluted chromatin (from **Step 38**).

41. Incubate with shaking at 4°C for 1hour.

42. Centrifuge at 1000x g at 4°C for 5minutes [**NOTE 26**]. Collect supernatant.

43. Take 10% of this supernatant (the diluted and precleared chromatin) and freeze at -20C for later analysis.

44. Add appropriate antibodies to tubes of diluted and precleared chromatin. This will require negative (IgG) and positive (specific to the experiment) controls as well as specific 'experimental' antibody [**NOTE 27**]. Incubate overnight at 4°C on a rotating shaker.

3.5.4 Recovery of Immune complexes

45. Pulldown ChIP reaction with 50ul of pre-washed ProteinA/G. Incubate 2 hours at 4°C on a rotary shaker.

46. Prepare ChIP Elution buffer and keep at 65°C.

46. Wash experimental ProteinA/G beads twice with 1ml of the following buffers:
    - Low Salt wash
    - High Salt wash
    - LiCL wash
    - TE buffer

For each wash buffer add 1ml for a few seconds, centrifuge 2 min at 1000 x g at 4°C, remove supernatant and replace with another 1ml for 5minutes at 4°C with rotation.

47. To elute DNA-chromatin-antibody complexes add 200ul preheated elution buffer to Protein A/G beads. Vortex briefly and incubate at 65°C with agitation for 15minutes.

48. Centrifuge beads for 1minute at 1000x g. Transfer supernatant to a new tube.

49. Repeat **Steps 47 and 48**.

50. Adjust volume of sample to 400ul with elution buffer.

3.5.5 Purification of ChIPed DNA.

51. Add 16ul of 5M NaCl to each sample. These are the eluted samples from **Step 50** and the input sample from **Step 43**.

52. Incubate at 65°C overnight with agitation.

53. Digest any residual RNA by addition of 8ul RNaseA, 16ul 1M Tris-HCl pH 6.8. Incubate for 60minutes at 37C with agitation.

54. Digest proteins add 8ul of 0.5M EDTA and 2ul 10mg/ml proteinase K. Incubate at 2hours at 37°C with agitation.

55. Isolate DNA with QIAquick MinElute columns (or similar)[**NOTE 29**]

56. At this stage you will have output DNA samples from:
    1. *Input;*
    2. *Negative Ab;*
    3. *Positive Ab;*
    4. *Experimental Ab*

These samples can then be analysed depending on the researcher plans. This might involve analysis of specific DNA by qPCR or global analysis by ChIP-CHIP or ChIP-Seq. Users could consult the specific directions of their selected protocols.

4. Notes

NOTE 1: A mild fixation is needed as isolation of deeply buried tissues will apply greater stresses to tissues and possibly increase sample damage. Fixation better ensures that the native state of the tissue is maintained during extraction. According to Slane *et al*, (2020) higher PFA concentrations can provide a better yield but with a potential loss of quality. The possible use of fixation might also be influenced by the lab-specific challenges of being able to treat samples identically [**NOTE 2**].

NOTE 2: Isolated nuclei left at 4°C will inevitably shows cold-increased gene expression changes. Therefore experiments should be planned so that samples are treated in as best an identical manner as possible. In this method it is advisable to treat samples individually and perform nuclei-sorting on individual samples before preparing subsequent samples. This will require a cold workspace close to the cell-sorter. If this is not possible then researchers should make their best precautions to treat tissues identically.

NOTE 3: A later fixation step is needed during the protocol if the researcher will be analysing their samples using ChIP.

NOTE 4: A trade-off must be made between the required amount of tissue and the time of processing, particularly when tissues are not fixed prior to nuclei isolation. Dissection of the required tissues will extend the process such that samples obtained at the start and end will show different expression patterns. Root tips can be analysed from 7do Arabidopsis root tips, shoot apical meristems can be dissected from 14do seedlings and embryo samples should be taken from siliques matured for an appropriate time.

NOTE 5: Gutzat and Mittelsten Scheid (2020) recommend use of a Histone2B-FP tag as they find no phenotypic changes when the FP tag is attached to this protein. In general they have found that use of a fluorescently labelled nuclear protein whose nuclear import is driven by a simple NLS does not provide a strong enough signal to be effectively sorted. Researchers should empirically test the constructs that will work best their hands.

NOTE 6: If possible using pre-cooled equipment an available 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 nucleus.

NOTE 7: The composition of Nuclei Isolation Buffer varies between methods. Thibivilliers *et al* (2020), and Slane *et al* (2020) recommend use of the CelLyticTM Plant Nuclear kit (Sigma Aldrich) but this is not essential, especially if consumable budget is tight. Gutzat and Mittelsten Scheid (2020) recommend use of Galbraith Buffer (GB).

NOTE 8: If tissue harvesting does not take a significant time then samples can be immediately placed into GB. This may be the case for harvesting from leaf or root tissue but for tissues that requires a longer dissection NIB should be used to hold samples prior to nuclei extraction. Ensure majority of NIB is removed before tissues are then chopped in GB.

NOTE 9: Other common methods can be used to disrupt tissues but the best quality nuclei are isolated using simple chopping; although there may be a yield penalty when using this method. Other options include:

1. Grind samples with a pestle and mortar in liquid nitrogen. Although this will generate a fine powder it can also produce more tissue debris that will increase the noise of the outputs from the cell sorter.
2. Use of a TissueRuptor (or similar automatic device, https://www.qiagen.com/us/shop/automated-solutions/sequencers/tissueruptor-ii/).

Be sure to treat the samples gently as vigourous disruption can destroy nuclear integrity.

3. Use a dounce homogenizer for larger amounts of tissue.

NOTE 10: This is a critical step. Amount of Triton X-100 in GB should be empirically tested and can be used up to a concentration of 1%. As Triton X-100 facilitates cell membrane lysis and dissociates nuclei from one another, nuclear extract purity increases with longer treatment times or higher Triton X-100 concentrations. However increased purity will proportionally reduce the number of fluorophore positive nuclei. Quality and number of nuclei can be empirically tested by using an epifluorescent microscope with a UV/DAPI filter. Measurement of nuclei can be performed using a haemocytometer with standard protocols.

NOTE 11: Use a cut off pipette to resuspend so as not to damage nuclei.

NOTE 12: Be aware that some flow cytometry facilities will not allow use of hazardous solution (e.g phenol-based) and therefore alternatives should be used such as RNAlater or similar products (https://www.thermofisher.com/uk/en/home/brands/product-brand/rnalater.html).

NOTE 13: During sorting, drops of liquid containing nuclei will often collect on the walls of the flow tubes. Immediately gently roll the closed tube to transfer nuclei suspension to the bottom of the tube, preventing nuclei drying.

NOTE 14: If the user is confident that RNA sample obtained at **Step 24** will have good purity it can be resuspended in 14ul nuclear-free water and **Step 25** can be omitted.

NOTE 15: Add DTT to RLT buffer instead of 2-ME prior to RNA cleanup. Continue with protocol as per manual instructions. Finally elute RNA in 14µl nuclease-free water.

NOTE 16: Pico-green will quantify dsDNA in the presence of ssDNA, RNA and free nucleotides

NOTE 17: Gutzat and Mittelsten Scheid (2020) recommend use of the Pico Methyl-Seq Library Prep Kit for Whole Genome Bisulfite Sequencing (WGBS) library preparation. This protocol can be used with as little 10pg of input DNA so is ideally suited to samples from rare cell types. The protocol prepares samples for bisulfite sequencing by Illumina NGS. https://www.zymoresearch.com/products/pico-methyl-seq-library-prep-kit

NOTE 18: Modified-GB includes 100 µg/mL PMSF, 1 µg/mL Pepstatin A and a broad action protease inhibitor such as cOmplete™ Protease Inhibitor.

NOTE 19: Prepare 1% paraformaldehyde from a 37% stock.

NOTE 20: Gently wash the pellet so as not to introduce air bubbles or shear nuclei sample.

NOTE 21: Sonicate with a BioruptorTM200 or similar equipment. Ensure that the sonicator is well-cooled with ice prior to use. Eppendorf tube should include up to 300ul for sonication. After sonication samples can be pooled into a single eppendorf.

NOTE 22: An example of the required chromatin after sonication is reproduced from Figure 1 from Desvoyes *et al*, 2018.

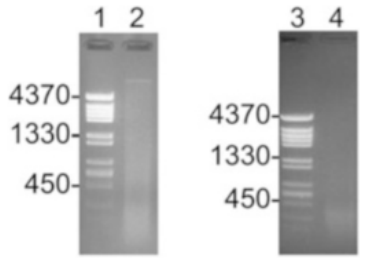

Fig. 1 Size analysis of the sonicated chromatin. The sonicated DNA is expected to have a size of 100–500 bp and no RNA contamination Samples correspond approximately to 50 mg of seedlings. *Lanes 1* and *3*: DNA marker (Φ29 DNA digested with Hind III); *Lane 2*: chromatin prior to sonication; *Lane 4*: chromatin after sonication

NOTE 23: The IP purifies the DNA that is bound to a specific protein and/or proteins with a particular modification. The specific protein(s) will be isolated using an appropriate antibody.

NOTE 24: ChIP will not work in a buffer with more than 0.1% SDS.

NOTE 25: Proteins A or G have different affinities for antibodies depending on the species and the immunoglobulin subclass. Therefore choose to use the most appropriate samples for each individual experiment. If you are using magnetic beads (rather than agarose beads) then preclearing **Steps 40-42** are not necessary.

NOTE 26: If magnetic beads are used they should be collected in a magnetic rack.

NOTE 27: The positive control is selected as appropriate for the experiment. If the experiment aims to identify chromatin containing epigenetically-modified histones (such as H3K27me3) then an anti-H3 antibody could be used as positive control. In experiments where a specific protein is targeted for ChIP then the researcher will need to consider an available appropriate positive control.

NOTE 28: Cross-linking will be affected by many factors including the type of buffer that is used. Samples are removed from GB and resuspended in PBS as this is a good option for crosslinking. The extent of cross-linking can be varied to suit the needs of each experiment and should be empirically tested.

NOTE 29: Select a product that will allow the elution in small volumes as it is likely that there will be a small amount of DNA in the final sample.

5. References

Birnbaum K, Shasha DE, Wang JY, Jung JW, Lambert GM, Galbraith DW, Benfey PN (2003) **A gene expression map of the Arabidopsis root.** Science doi: 10.1126/science.1090022

Desvoyes B, Vergara Z, Sequeira-Mendes J, Madeira S, Gutierrez C (2018) **A Rapid and Efficient ChIP Protocol to Profile Chromatin Binding Proteins and Epigenetic Modifications in Arabidopsis.** Methods Mol Biol. doi: 10.1007/978-1-4939-7318-7_5

Gutzat R, Mittelsten Scheid O (2020) **Preparing Chromatin and RNA from Rare Cell Types with Fluorescence-Activated Nuclear Sorting (FANS).** Methods Mol Biol. doi: 10.1007/978-1-0716-0179-2_7

Gutzat R, Rembart K, Nussbaumer T, Hofmann F, Pisupati R, Bradamante G, Daubel N, Gaidora A, Lettner N, Donà M, Nordborg M, Nodine M, Mittelsten Scheid O (2018) **Arabidopsis shoot stem cells display dynamic transcription and DNA methylation patterns**. EMBO J. doi: 10.15252/embj.2019103667

Nelson T, Tausta SL, Gandotra N, Liu T (2006) **Laser microdissection of plant tissue: what you see is what you get.** Annu Rev Plant Biol. doi: 10.1146/annurev.arplant.56.032604.144138

Slane D, Kong J, Schmid M, Jürgens G, Bayer M (2015) **Profiling of embryonic nuclear vs. cellular RNA in *Arabidopsis thaliana*.** Genom Data. doi: 10.1016/j.gdata.2015.03.015

Slane D, Berendzen KW, Witthöft J, Jürgens G (2020) **Transcriptomic Profiling of the Arabidopsis Embryonic Epidermis Using FANS in Combination with RNAseq.** Methods Mol Biol. doi: 10.1007/978-1-0716-0342-0_12

Thibivilliers S, Anderson D, Libault M (2020) **Isolation of Plant Root Nuclei for Single Cell RNA Sequencing.** Curr Protoc Plant Biol. doi: 10.1002/cppb.20120

Weinhofer I, Köhler C (2020) **Endosperm-specific chromatin profiling by fluorescence-activated nuclei sorting and ChIP-on-chip.** Methods Mol Biol. doi: 10.1007/978-1-62703-773-0_7

Wang N, Liu C (2020) **Study of Cell-Type-Specific Chromatin Organization: In Situ Hi-C Library Preparation for Low-Input Plant Materials.** Methods Mol Biol. doi: 10.1007/978-1-0716-0179-2_9.

6. Figure Legends

Figure 1: Output from Flow Cytometry of Nuclei Extract

Experiment 1 and Experiment 2 show flow analysis of nuclei from either wildtype plants (**Col-0, right**) or plants expressing the ***pCLV3::H2B-mCherry*** transgene **(left)**. This transgene restricts the mCherry expression to the CLAVATA3 domain in the shoot apical meristem (SAM). Nuclei samples were extracted from dissected SAMs.
DAPI plots shows ploidy levels of extracted nuclei. Experiment 2 demonstrates better quality nuclei extraction as the peaks of nuclei of different ploidy are much cleaner.
mCherry +/- plots show nuclei expressing mCherry. Nuclei have been separated for sorting through **mCherry- (#)** and **mCherry+ (*)** gates.
The **Events** table shows the number of nuclei collected in each gate **(# or *)**. In Experiment 1 >4600 nuclei have been collected in mCherry- gate **(#**). In contrast 37 nuclei have been collected in mCherry+ **(*)** gate from the *pCLV3::H2B-mCherry* extract and only 1 nucleus from the Col-0 extract. In Experiment 2 ~6000 nuclei have been collected in each mCherry- gate **(#**) and 9 nuclei have been collected in mCherry+ **(*)** gate from the pCLV3::H2B-mCherry extract. This demonstrates the small number of nuclei that can be sorted from an extract in which expression is restricted to a rare cell type. In addition it provides an insight into the time that may be required to sort enough nuclei for downstream applications. Despite the relatively poor quality of nuclei extracted in Experiment 1 (based on the DAPI ploidy distribution), it is possible to isolate mCherry+ nuclei from this sample.
Data kindly supplied by Ruben Gutzat. For more information on this experiment see Gutzat *et al* (2020).

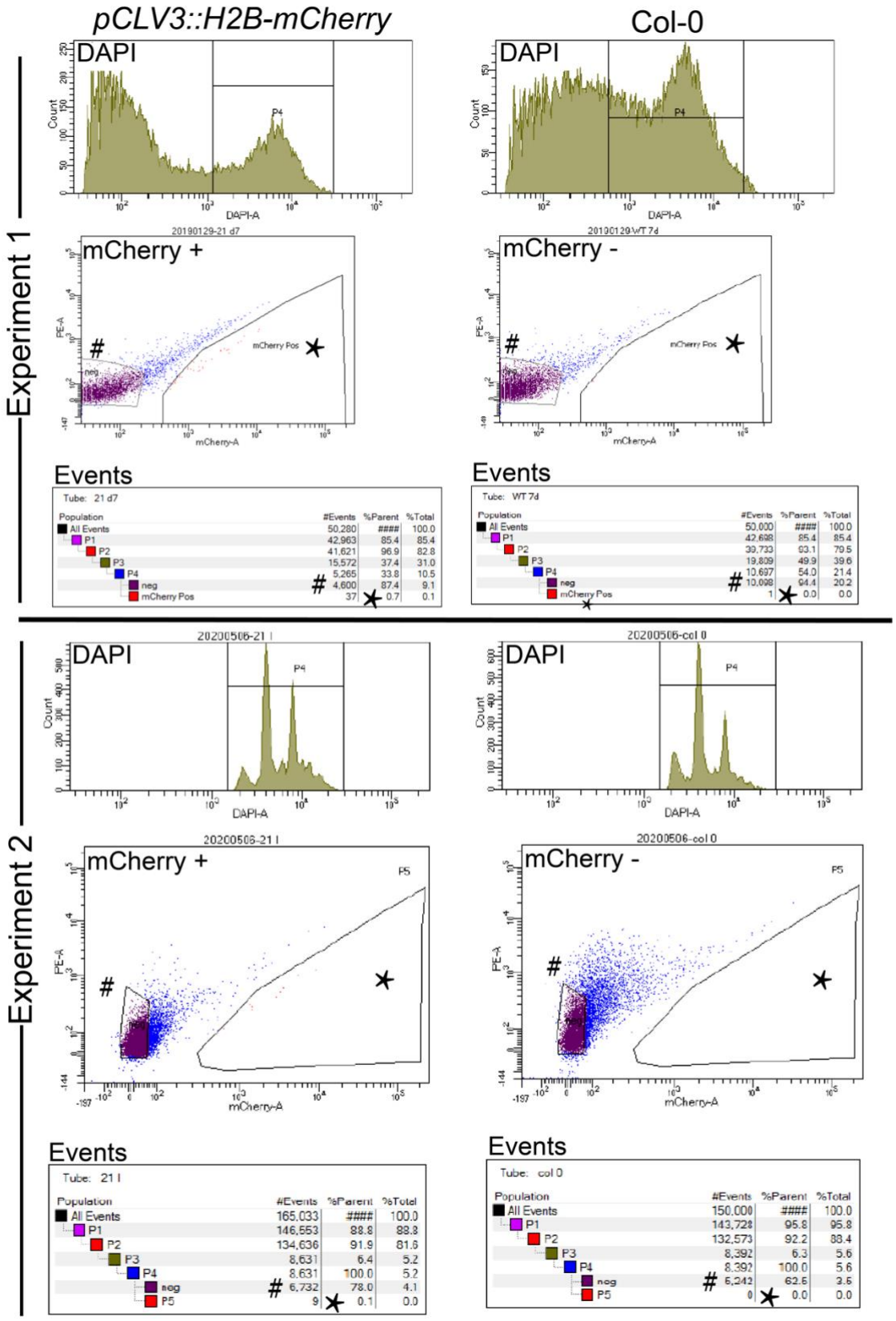

Figure 1: Output from Flow Cytometry of Nuclei Extract