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METHODS PAPER

A simple and robust protocol for immunostaining *Arabidopsis* pollen nuclei

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Abstract

Pollen represents the male sexual lineage in flowering plants. At maturity, pollen grains are composed of a companion vegetative cell with embedded sperm. During pollen development, these two cell types acquire vastly differing cell fates. Underlying this differential fate acquisition is dramatic reconfiguration of pollen chromatin that is highly evident at a cytological level. The precise link between histone mark deposition and fate acquisition remains largely unexplored, which in part has been hindered by the difficulty in working with pollen in model plant species like *Arabidopsis*. Here, we describe a simple and robust protocol to isolate *Arabidopsis* pollen nuclei and immunostain for histone marks. Plant growth aside, the protocol can be performed over 2 days with few *Arabidopsis* plants, thus allowing multiple genotypes to be analysed in parallel. We also describe a method to de-mask epitopes through antigen retrieval, which vastly improves the signal for antibodies that target heterochromatic histone marks.

Keywords Pollen · Chromatin · Immunostaining · Cytology

Introduction and scope

Pollen represents the haploid male gametophyte generation in flowering plants and has evolved to nurture, protect and deliver the male gametes. Pollen development begins after male meiosis with the production of four haploid microspores. Each microspore undergoes a highly asymmetric cell division, producing a generative (or germ) cell that becomes engulfed in the cytoplasm of a larger companion vegetative cell (Borg et al. 2009). The germ cell will go on to divide once more to produce two sperm, which can occur before or after pollen shed (Williams et al. 2014). During pollination, pollen is deposited on the female stigma and results in hydration of the vegetative cell, which will grow a pollen tube by rapid directional tip growth to the female embryo sac (Higashiyama and Takeuchi 2015). A complex series of signalling events triggers the release of sperm to the two female gametes, the egg and central cell, leading to double fertilisation and subsequent seed production (Lafon-Placette and Köhler 2014).

Aside from its obvious importance for plant fertility, pollen is an attractive developmental system to explore the molecular basis of cell fate determination (Borg et al. 2009). Despite only being separated by two cell divisions, the sperm and vegetative cell acquire vastly differing fate, which is reflected in their unique gene expression profiles (Borges et al. 2008; Rutley and Twell 2015). This differential transcriptional rewiring is underpinned by dramatic epigenetic reprogramming that is evident at the chromatin level (Borg and Berger 2015). Sperm nuclei (SN) are relatively condensed compared to the diffuse chromatin of the vegetative cell nucleus (VN) (Fig. 1a). Chromatin decondensation in the VN is partly explained by the loss of pericentromeric identity and associated H3K9me2 marks (Fig. 2a) (Schoft et al. 2009; Mérai et al. 2014). In contrast, SN remain relatively compact but retain nucleosome-based chromatin (Fig. 1b), unlike basal land plants and most animal species, where global histone-to-protamine exchange causes extreme chromatin condensation (Reynolds and Wolfe 1984; Braun

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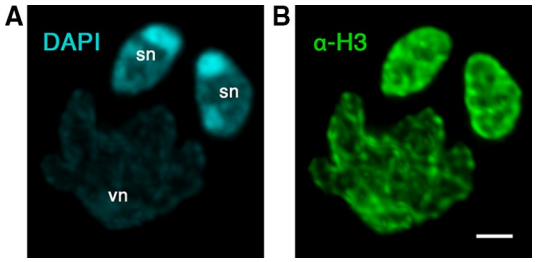

Fig. 1 Paternal chromatin is protamine-free and histone-based in flowering plants. **a** Maximum intensity projection images of 4′,6-diamidino-2-phenylindole (DAPI) stained vegetative cell nuclei (VN) and sperm nuclei (SN) isolated from *Arabidopsis* pollen. The two pollen cell types acquire different cellular fate that is highly evident at the chromatin level. The VN undergoes extensive chromatin decondensation while SN chromatin remains relatively compact. Scale, 2 µm. **b** Corresponding H3 antibody (α-H3—Abcam, #ab1791) immunostaining of the nuclei depicted in **a**. Unlike animals, sperm chromatin is not reprogrammed by global replacement of histones with protamine, as illustrated by appreciable levels of histone H3 in both SN and VN

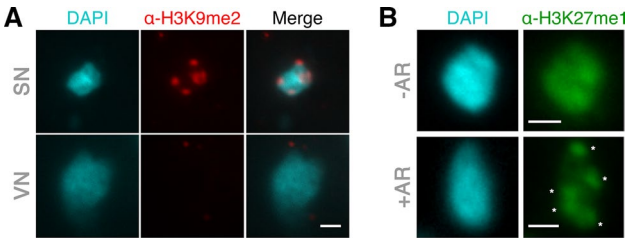

Fig. 2 Antigen retrieval improves detection of heterochromatic marks. **a** Standard fluorescence images of H3K9me2 antibody (α-H3K9me2—Abcam, #ab1220) immunostained vegetative cell nuclei (VN) and sperm nuclei (SN) isolated from *Arabidopsis* pollen. Diffuse chromatin in the VN is caused by decondensation of pericentromeric heterochromatin and loss of associated H3K9me2 marks. In contrast, SN retain pericentromeric identity as illustrated by the detection of five distinct chromocenters—one for each *Arabidopsis* chromosome. The samples were processed using antigen retrieval and results are consistent with that previously published in Schoft et al. (2009). Scale, 2 µm. **b** Standard fluorescence images of H3K27me1 antibody (α-H3K27me1—Millipore, #17-643) immunostained sperm nuclei isolated from *Arabidopsis* pollen. Depicted are examples of staining without (−AR) and with (+AR) antigen retrieval. H3K27me1 is a typical heterochromatic mark that is enriched at pericentromeric regions (Jacob et al. 2009). However, this only becomes evident after antigen retrieval with an enriched signal at the five chromocenters (shown with an asterisk). Scale, 1 µm

2001; Boskovic and Torres-Padilla 2013). A distinguishing feature of both VN and SN chromatin is the incorporation of distinct classes of atypical, pollen-specific histone variants (reviewed in Borg and Berger 2015). It is expected that these histone variants possess properties that participate in reprogramming of specific histone marks in pollen, although a biological demonstration of this has remained elusive.

The precise molecular mechanisms that underlie chromatin reorganisation in pollen remain an exciting yet challenging avenue of research. This in part has been hindered by the difficulty in collecting and working with pollen in model plant species like *Arabidopsis*. Moreover, the tough pollen exine wall makes the efficient release of intact pollen nuclei problematic. To this end, we have optimised a simple and robust method to immunostain *Arabidopsis* pollen nuclei. We have used this method to study the localisation of histone marks in SN and VN chromatin while reproducing previously published findings (Fig. 2a).

The protocol does not require large-scale collection of pollen and can be performed with 3–4 large and healthy *Arabidopsis* plants, thus allowing multiple genotypes to be processed on the same day. We find that the lysis buffers used in classical plant immunostaining protocols (Lysak et al. 2006) do not efficiently disrupt and dissolve the plasma membranes that form the male germ unit (McCue et al. 2011), often causing high background and inefficient staining. We recommend using an isolation buffer with a high concentration of detergent that is often used for nuclear flow cytometry (Galbraith et al. 1983; Borges et al. 2012). We also describe a method to de-mask epitopes by antigen retrieval (Taylor et al. 1996), which vastly improves the immunostain signal with certain antibodies, particularly those targeting heterochromatic histone marks (Fig. 2b). The immunostaining protocol should be performed with and without antigen retrieval when testing out new antibodies.

Materials

Slide preparation

- Galbraith buffer: 45 mM $MgCl_2$, 30 mM Tri-Sodium acetate, 1% Triton X-100, 20 mM MOPS pH 7.0. Filter-sterilise and store at 4 °C. Prepare at least 2.5 ml for each genotype being processed. Freshly add 0.7 µl 14.3 M ß-mercaptoethanol per 1 ml buffer (10 mM final concentration).
- 50 × Protease Inhibitor Cocktail: dissolve one tablet of Complete Protease Inhibitor Cocktail (Roche, # 000000011697498001) in 1 ml of water. Store at − 20 °C.
- 16% paraformaldehyde (Alfa Aeser, # 43368.9L): replace within 3 months once opened.
- 2 M glycine: prepare fresh on the day by dissolving 0.751 g glycine with 5 ml of sterile distilled water.
- 1× phosphate-buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4, adjust to pH 7.3.
- 0.5-mm glass beads (Scientific Industries, #SI-BG05): glass beads of similar diameter should also work well.
- Miracloth (Merck-Millipore, # 475855): cut into 4 cm × 4 cm pieces.

- 10-µm nylon mesh (Mercateo, # 2843-9068213): cut into 4 cm × 4 cm pieces.
- Glass slides: the precise model or poly-L-lysine-coated slides are not critical.
- Glass slide holder: a solid glass holder that can withstand microwave heating is critical.
- Pre-cooled microcentrifuge at 4 °C.

Antigen retrieval

- Microwave with adjustable power settings.
- 1× DAKO target retrieval solution: dilute 10× DAKO Target Retrieval pH 6.0 solution concentrate (DAKO, #S1699). Around 200 ml should be sufficient for one slide holder although the exact volume should be determined for own apparatus.
- 1× phosphate-buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4, adjust to pH 7.3.
- Glass slide holder: a solid glass holder that can withstand microwave heating is critical.

Blocking, immunostaining and washes

- Blocking solution: 2% BSA, 1% 1× PBS, 0.1% Tween-20.
- 1× PBS-T: 1× PBS, 0.1% Tween-20.
- Primary antibody: use desired primary antibody. Exact dilution depends on the antibody being used, but we typically start at a 1:100 dilution.
- Secondary antibody: we have good experience using goat antirabbit or antimouse Alexa Fluor®-conjugated secondary antibodies from Thermo Fisher Scientific. Use at a 1:500 dilution.

DAPI staining and mounting

- DAPI staining solution: dissolve 1 mg of 4′,6-diamidino-2-phenylindole (DAPI, Sigma #D9542) in 1 ml of water, filter-sterilise and store 100 µl aliquots at − 20 °C. Be sure to protect from direct light. Make DAPI staining solution fresh on the day by adding 1.5 µl of 1 mg/ml DAPI stock solution to 1 ml of sterile distilled water.
- Vectashield antifade mounting medium with DAPI (Vector Laboratories, #H-1200).
- Coverslips: 20 mm × 20 mm coverslips are preferred although the precise size or model is not critical.
- Nail varnish: a cheap transparent varnish is sufficient for sealing the edges of the coverslip.

Methods

Slide preparation

1. Grow healthy *Arabidopsis* plants in deep pots for 5 to 6 weeks until they start to produce plenty of flowers.
2. Pick open flowers and fill a 5-ml microfuge tube to the ~ 1.5 to 2.0 ml mark.
3. Add 2 ml of ice-cold *Galbraith buffer* and vortex for 3 min at full speed to wash out the pollen grains.
4. Filter 750 µl of the pollen suspension through a Miracloth filter[1] and pellet the pollen grains (13,000 *g*, 30 s, 4 °C).
5. Discard supernatant and repeat filtration of the remaining 750 µl of pollen suspension.
6. Discard supernatant and resuspend the pollen pellet in 250 µl of ice-cold *Galbraith buffer* containing 1× protease inhibitor cocktail.[2]
7. Add approximately 50 µl of glass beads and vortex at full speed for 3 min to disrupt the pollen wall and release nuclei.[3]
8. Remove excess pollen wall debris by filtering[4] 200 µl of the nuclear suspension through a 10-µm mesh (1000 *g*, 1 min, 4 °C).
9. Disassemble the mesh column and pellet the nuclei (3000 *g*, 10 min, 4 °C).
10. Discard supernatant and add 200 µl of ice-cold *Galbraith buffer* containing 1× protease inhibitor cocktail. Gently resuspend the pellet until precipitates disappear.
11. Immediately add 65 µl of 16% *paraformaldehyde* (to a final concentration of 4%) and incubate on ice for 20 min to fix the nuclei.
12. Quench the fixation by adding 18 µl of *2 M Glycine* (to a final concentration of 125 mM) and mix well.

[1] To assemble filters, we make use of spent miniprep columns. Flame a razor blade and cut off the end of the column containing the embedded silica matrix and wash the plastic column thoroughly. The resulting column can then be used to trap a single layer of miracloth into a 1.5 ml microfuge tube to allow quick and efficient filtering of the pollen suspension.

[2] Always add the Protease Inhibitor Cocktail fresh before use. Simply add 20 µl of 50× Protease Inhibitor Cocktail to every 1 ml of Galbraith Buffer.

[3] It is advisable to monitor the status of pollen wall disruption by observing a small aliquot under a fluorescence microscope. Take a 3 µl aliquot of the disrupted pollen suspension, mix with 3 µl of 1.5 mg/ml DAPI stock solution and mount on a slide. Most pollen grains should be disrupted while the suspension should be enriched for DAPI-stained sperm and vegetative nuclei. It is not necessary that all pollen grains are broken as the final concentration of nuclei will be more than sufficient for immunostain purposes, meanwhile ensuring that released nuclei remain intact and undamaged.

[4] The nylon mesh filter is assembled in a similar manner to miracloth filters. See note 1.

13. Spread 10 µl of the fixed nuclear suspension onto a glass slide and allow to dry at room temperature for about 20 min.
14. Immobilise the slides in a slide holder and submerge with *1× PBS* until other samples that require antigen retrieval are processed. Otherwise, proceed to step 19.

Antigen retrieval

15. De-mask epitopes using freshly prepared *1× DAKO target retrieval solution*.
16. Immobilise slides in a slide holder and completely submerge in *1× DAKO target retrieval solution*.
17. Microwave the slides at 500 W twice for 5 min—be sure to cool the slide for 5 min in between the treatments.[5]
18. Cool down the slides by slowly adding sterile distilled water to the slide holder. Remove the slides and store in *1× PBS*.

Blocking, immunostaining and washes

19. *Wash* the samples by incubating with 200 µl of *1× PBS-T* for 10 min. Repeat for a total of three washes.
20. *Block* the samples by incubating with 200 µl of *blocking solution* and incubate in a humid chamber[6] for 30 min at 37 °C. In the meantime, prepare adequate amounts of primary antibody (typically 1:100) by diluting in blocking solution.
21. Add 200 µl of *primary antibody* and incubate in a humid chamber for 2 h at 37 °C.
22. *Wash* the samples by incubating with 200 µl of *1× PBS-T* for 10 min. Repeat for a total of three washes.
23. Add 200 µl of *secondary antibody* (diluted in blocking solution, typically 1:500) and incubate in a humid chamber *in the dark* for 2 h at 37 °C.
24. *Wash* the samples by incubating with 200 µl of *1× PBS-T* for 10 min. Repeat for a total of three washes.

DAPI staining and mounting

25. Remove 1× PBS-T from last wash and soak off excess by gently wiping around the sample with a tissue paper.
26. Add 200 µl of *DAPI staining solution* to each slide directly over the sample and incubate *in the dark* for about 15 min.
27. Remove the DAPI solution and wash with 200 µl of sterile water.
28. Remove as much of the water as possible with a pipette tip and tissue paper.
29. Add 10 µl of Vectashield Antifade Mounting Medium with DAPI and carefully place on a coverslip. Press the slide between some filter paper to remove excess liquid. Seal the coverslip with nail varnish.
30. Allow the mounting medium to set for a few hours protected from direct light. Store in the dark at 4 °C.[7]

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[5] When using your own microwave, optimise the power setting to a point where the DAKO solution is at a gentle rolling boil. It is imperative that the DAKO solution does not boil over during this heating step, which will expose the slides and dry the samples. During heating, never leave the microwave unattended and regularly top up with fresh DAKO solution.

[6] Create a humid chamber to ensure that the samples remain hydrated during incubation steps. Place some tissue paper inside a large Petri dish, moisten with sterile distilled water and squeeze out excess water. Cover the chamber with aluminium foil for steps that require incubation in the dark.

[7] It is advisable to analyse and image the slides within 1 week to ensure a sufficient DAPI signal for counterstaining and nuclear detection. We often perform counter immunostains with a second mouse anti-H3 antibody (most histone mark antibodies are raised in rabbit). Use appropriate secondary antibodies with distinct and non-overlapping Alexa Fluor® dyes (for example, Alexa Fluor-488 with Alexa Fluor-555).

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