Rapid separation of *Arabidopsis* male gametophyte developmental stages using a Percoll gradient

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Research investigating the dynamics of male gametophyte (MG) development has proven to be challenging for the plant science community. Here we describe our protocol for separating *Arabidopsis* MG developmental stages, which is based on the centrifugation of pollen through a discontinuous Percoll concentration gradient. This Percoll gradient can be formed using a pipette, and it does not require a gradient maker. The purity of the isolated developing spores is as high as 70%, and in most separations it is well above 80%. Using this protocol, we can separate four different stages of pollen development—uninucleate microspore (UNM), bicellular pollen (BCP), tricellular immature pollen (TCP) and mature pollen grain (MPG). The duration of the separation procedure, excluding the cutting of flower inflorescences, is 6 h. This is reduced to 4 h when using a vacuum cleaning method to remove the MPGs before the Percoll density separation.

INTRODUCTION

The angiosperm MG is a unique biological model for developmental and physiological studies, effectively allowing analysis at the single-cell level. This model can be used to investigate various processes that are involved in forming and/or establishing cellular polarity and cell cycle control, or to uncover transcriptional networks. Moreover, revealing the physiological and developmental processes of the MG itself can be biologically challenging. An understanding of these basic processes is essential to discovering more about the survival of plant species on earth.

MG development in spermatophytes (seed plants) involves the formation of a specific haploid two- or three-celled structure. The morphological and developmental differentiation of the MPG is initiated after the meiotic division of the pollen mother cell. During this process, the haploid microspore undergoes two mitotic divisions—pollen mitosis I and pollen mitosis II—resulting in one vegetative cell (VC) and two sperm cells (SCs). In this way, both cell types are clearly diversified morphologically and functionally. SCs are engulfed in the cytoplasm of the VC, which ensures their successful delivery to the female gametophyte.

The coexistence of two different cell types in the highly simplified biological structure of the MG provides an excellent opportunity to study the complex regulatory processes underlying the establishment of the generative cell lineage, pattern formation and cellular differentiation during MG development. To discover these regulatory pathways and their developmental dynamics, it is necessary to develop and use a proper methodology for separating homogeneous populations of developing spores at several stages. These stages are UNM, BCP, tricellular pollen (TCP) and MPG.

Modern technologies in pollen developmental research

More than a decade ago, high-throughput microarray technologies (e.g., the Affymetrix *Arabidopsis* ATH1 Genome Array) were applied to plants for the first time. This included analysis of the MG[1–6], which showed the dynamics of gene expression during *Arabidopsis* pollen development. New microarrays designs were introduced later and continue to be improved. New chips have been designed for a variety of biological applications, including large-scale DNA methylation mapping (i.e., Affymetrix and NimbleGen arrays)[7]. Simultaneously, the development of next-generation sequencing (NGS) techniques has increased the robustness of transcriptomic studies, resulting in their further refinement at reduced cost, increased accuracy and higher throughput[7]. Despite extensive expansion of high-throughput technologies in recent years, the vast majority of published transcriptomic studies of the MG that used NGS or advanced microarray methods were performed on mature pollen as the earliest developmental stage[8–10]. The haploid UNMs were rarely subjected to transcriptomic analyses after our initial study[2], and only one other group has analyzed microspores using NGS[11].

Comparisons with other methods

Currently, the prevailing methods for male gametophytic studies use FACS to separate SCs and vegetative nuclei in *Arabidopsis thaliana* mature pollen[6,8,12]. FACS allows for UNM or MPG separation based on their size and autofluorescence properties[3,4,12]. The disadvantage of this method is that we are unable to use it to obtain the BCP and TCP stages in their complete and fully functional form. Moreover, it is not possible to obtain the various developmental stages of the *A. thaliana* MG (UNM, BCP, TCP and MPG) in a single run. Simultaneously, the application of this method requires a highly specialized and expensive cell sorting apparatus—e.g., the Beckman Coulter MoFlo XDP[12]. For this reason, developmental studies that use *A. thaliana* BCP and TCP are stagnating while new methods for transcriptomic studies such as NGS are being introduced. Furthermore, providing additional information about the dynamics of MG development is critical to plant physiologists who are interested in more than the sexual reproduction of plants. We concluded that the unavailability of a rapid and simple protocol for the separation of individual *A. thaliana* pollen developmental stages precluded more detailed studies. To our knowledge, no alternative separation protocol has yet been published.

Development of the separation method

Here we present a protocol for our method of obtaining highly purified developmental stages of the *A. thaliana* MG using a discontinuous Percoll density gradient and rate-zonal centrifugation. The protocol describes the original method that was published by Honys and Twell in 2004[2]. Percoll has been used in biology as a separation medium for a long time, especially for the separation of animal sperm[13,14]. Several density gradient media, such as Percoll, sucrose and Ficoll (developed in the 1960s and 1970s), enabled the formation of gradient viscosity and allowed the separation of particles—e.g., cells and/or subcellular organelles from tissues and biological fluids[15]. Percoll is a modified polydisperse sodium-stabilized colloidal silica with a particle diameter between 10 and 30 nm, which is covered by a layer of polyvinylpyrrolidone[16]. This adsorbed polymer contributes to the stabilization of a colloidal gradient and diminishes the toxic effect of pure silica sol. Colloidal silica has been used since 1959 (ref. 17), when its beneficial properties for cell separation were described. The first reference to Percoll appeared in 1977 (Amersham Pharmacia Biotech, Uppsala, Sweden)[15]. Since then, it has become one of the most widely used density gradient materials; it is used predominantly for the separation of cells and/or subcellular organelles because of its close-to-ideal physical characteristics for separation[18].

Application of the Percoll separation method

The use of this protocol allows for the reproducible isolation of four pollen developmental stages—UNM, BCP, TCP and MPG—with high purity from a single plant. The purity of separated developing spores is essential for the acquisition of reliable and reproducible results. The benefits of the protocol presented here are directly applicable to further experiments designed in other laboratories—e.g., those enabling more targeted developmental transcriptomic, proteomic and metabolomic studies. The application of FACS to pollen developmental stages separated on the Percoll discontinuous gradient will boost follow-up studies of cell-specific physiological processes (e.g., DNA methylation during the MG formation and cell-specific high-throughput sequencing) of pollen developmental stages.

Advantages and disadvantages of the separation procedure

The use of Percoll as a density gradient fluid for the separation of pollen developmental stages has many advantages. First, Percoll has no effect on the biological functions of the separated pollen, as it does not penetrate pollen grains and it is not metabolized. Moreover, Percoll does not change the osmotic pressure, and it exhibits sufficient solubility. It also has a useful density range for separation, including the preparation of individual gradient layers, allowing the formation of an environment with constant osmolality in a wide range of densities that are suitable for physiological cell separation (isotonic Percoll).

The rate-zonal centrifugation technique is one type of density step gradient centrifugation that allows the complete separation of components in a mixture based on their size and weight (mass). Moreover, this type of centrifugation reduces convection, which is especially strong in cylindrical centrifuge tubes in swing-out rotors. Convection in the system is reduced to the same phase of the density medium.

This separation procedure, which is based on a discontinuous Percoll gradient, enables the purification of individual pollen

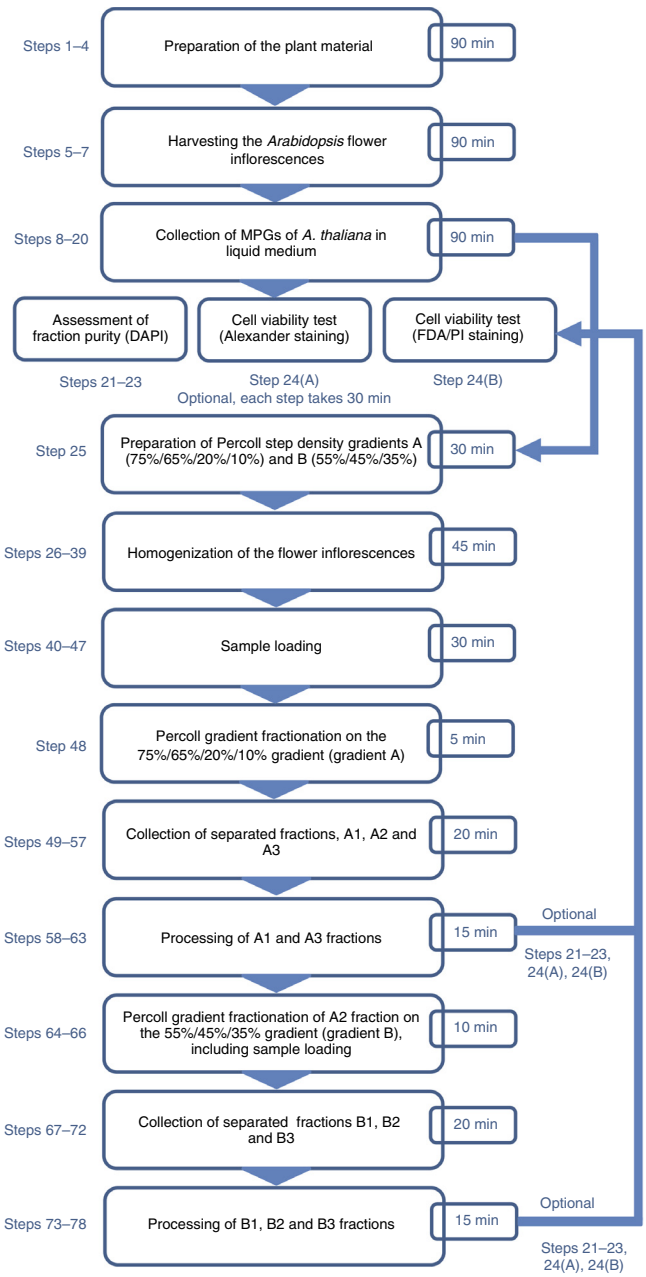

Figure 1 | Flowchart of the Percoll step gradient separation procedure. Up to 16 g of *Arabidopsis* flower inflorescences per two Percoll gradients A can be processed in this procedure. The optimal weight before homogenization was established at 4–6 g per one Percoll gradient A. The duration of Steps 5–7 (harvesting of the *Arabidopsis* flower inflorescences) is inversely proportional to the number of cooperating persons involved. The stated timing was achieved by two workers. The duration of the separation procedure, excluding cutting flower inflorescences, is ~6 h. Moreover, the total time of the procedure can be shortened by omitting protocol Steps 8–20 (collection of MPGs of *A. thaliana* in liquid medium). This part of the protocol is then replaced by the vacuum cleaning method that removes MPGs[19] and thus eliminates the risk of contaminating tricellular pollen suspension purity (A3 fraction). This reduces the total time to ~4 h. Steps 21–23 (assessment of fraction purity by DAPI), Step 24(A) (cell viability test by Alexander staining) and Step 24(B) (cell viability test by FDA/PI staining) are optional, and omitting them reduces the total time of the separation procedure. Note: it is desirable to assess the fraction purity by DAPI (Steps 21–23).

developmental stages without using a special and expensive cell sorting apparatus[12]. Furthermore, the preparation of a discontinuous gradient is not time-consuming, and it requires no special equipment for its preparation, such as a peristaltic pump. An ultracentrifuge is not required, minimum reagents are needed, and any additional steps are unnecessary, except for the removal of the Percoll medium and the collection of the plant material by centrifugation. Moreover, the method allows an effective separation of debris through the additional layer of 10% (vol/vol) Percoll, as well as recovery of a fraction formed by late UNM and early BCP.

Indeed, this method has its limits. The main one is that separation of various fractions is based on the size and mass of divided spores. For these reasons, the purity of some isolated fractions can be reduced because the size variation of developing spores may cause, for example, the contamination of the TCP fraction with late BCP. Moreover, the collection of plant material is time-consuming, but with more workers it can be reduced. The method was optimized for swing-out rotors. Up to 8 g of homogenized flower inflorescences can be layered onto one Percoll discontinuous gradient. However, the use of more starting material is not recommended, because a distortion of the Percoll interface can occur between layers. However, the procedure can be scaled down to 4 g of material per one Percoll gradient.

Experimental design

Overview of the method. The presented method consists of several successive steps, and it should be completed within one working day (~6 h; **Fig. 1**). The time can be shortened to ~4 h if wet MPGs are not required (e.g., from the PROCEDURE section 'Collection of *A. thaliana* MPGs in liquid medium'). The time required for sowing and planting is not included in this period. The first step of the protocol involves planting of the plant material and collecting inflorescences. MPGs are then isolated by shaking inflorescences in a liquid medium. A Percoll gradient is then prepared for use in separating out each developmental stage. Pollen developmental stages are released from anthers by gentle homogenization of flower buds, and they are separated by the Percoll gradient. Each separated fraction is collected and concentrated into pellets for storage. Each step of this protocol is of varied duration, and this is specified for each step separately. The duration of some steps, especially planting and harvesting of plant material, is indirectly proportional to the number of people involved (Steps 1–7). Two co-workers were engaged during the time cited, but the separation process itself (Steps 8–78) was designed for one person.

Rate-zonal centrifugation using a discontinuous gradient leads to the fractionation of individual particles (e.g., developing spores) relative to their size and mass. The larger particles (late BCP and TCP) tend to move faster than the smaller ones (UNMs and early BCP). Gradient separation itself is preceded by collecting MPGs and shaking them into a 0.1 M D-mannitol medium. The full description of this method is part of the published protocol, as the spore separation protocol itself allows the isolation of pollen-developing spores from UNM to immature tricellular pollen grain. Such plant material is suitable for Percoll fractionation because there is no contamination of the TCP fraction by MPGs. Furthermore, this approach enables the isolation of highly purified MPGs (80–90% purity). Our method is very exact, and as a result it leads to the isolation of undamaged developing spores (**Figs. 2–4**).

Plant material. The first step of the procedure is the preparation of the plant material. ~300–350 flowering plants of *A. thaliana* Col-0 are required to obtain 10–30 mg of the different pollen stages (UMN, BCP, TCP and MPG). If mutant plants with underdeveloped flowers, inflorescences and/or pollen production are used, it is necessary to plant an adequately larger population.

Seed germination and plant growth. The plants to be subjected to the separation of MG stages should be sown 6–8 weeks beforehand. If mutant plants or different accessions are used that have a delayed

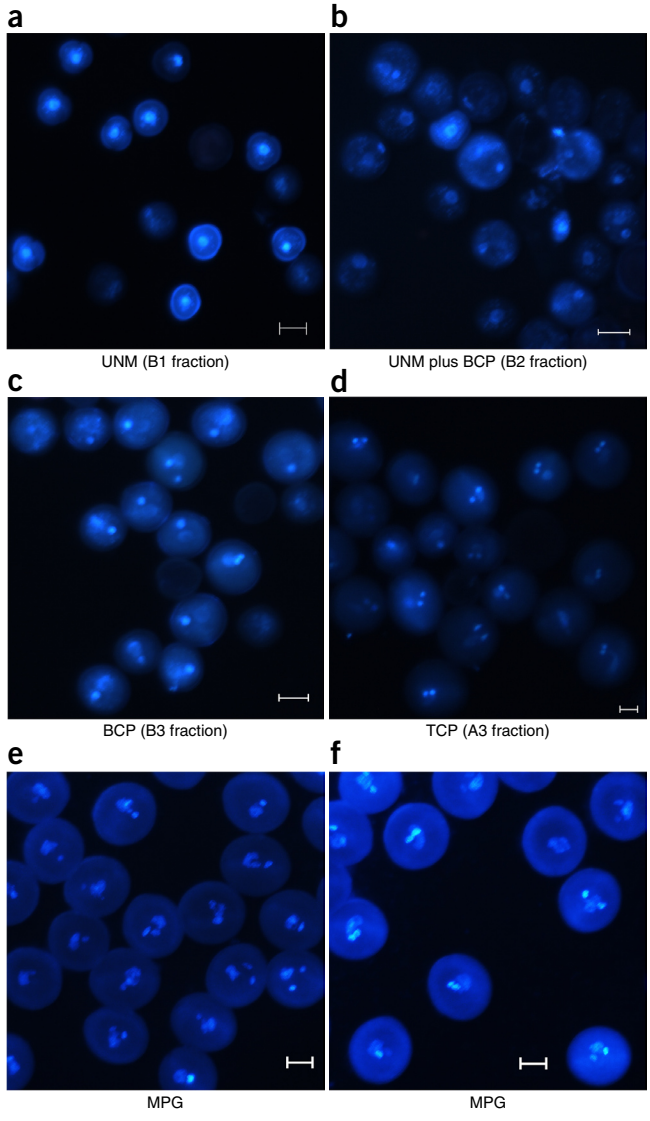

Figure 2 | DAPI staining of cell nuclei for the purity assessment of individual male gametophyte spore fractions after Percoll separation. (**a**–**e**) The samples were taken before the last concentration step and diluted in the staining solution with DAPI in a 1:1 dilution ratio (final DAPI concentration ~3 µg/ml). Blue fluorescence (DAPI) was viewed under a Nikon UV-2A filter set (excitation BP325–375, DM400LP, emission 420LP; Nikon Eclipse TE2000-U. (**a**) Early UNM from fraction B1. (**b**) Late UNM and early BCP from fraction B2. (**c**) Late BCP from fraction B3. Fractions B1, B2 and B3 were separated on a 55%/45%/35% Percoll gradient. (**d**) TCP from fraction A3 after the separation on a 75%/65%/20%/10% Percoll gradient. (**e**) MPGs from collection into liquid medium as a part of Percoll separation procedure. (**f**) MPGs obtained directly from fresh dehiscent anther show a substantially lower concentration compared with MPGs from flower shaking in 0.1 M D-mannitol. Scale bars, 10 µm.

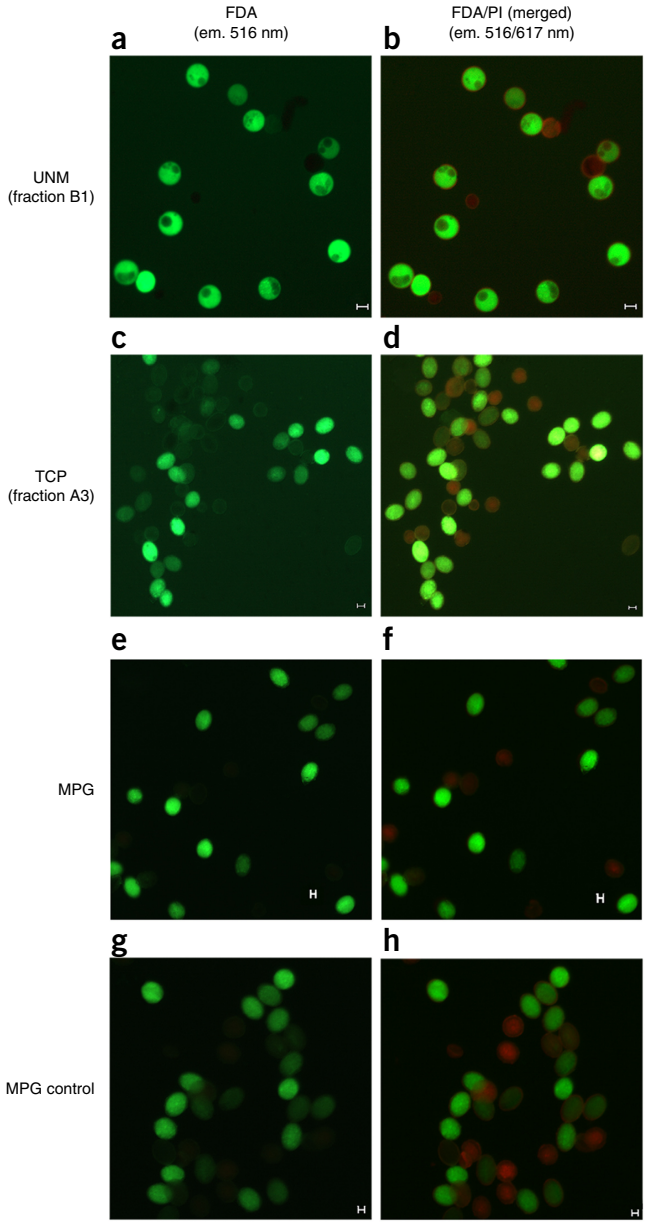

Figure 3 | Pollen viability was evaluated with cell-permeable esterase-substrate fluorescein diacetate (FDA) and the cell-impermeant nucleic acid stain propidium iodide (PI). (**a**–**h**) This test is applicable after obtaining fractions A1, A3 and B1, including MPG. Viable cells stained yellowish green and inviable cells stained red. With FDA, the fluorescence is produced by the stain's hydrolysis inside the living cells, whereas the PI fluorescence is produced by the stain's interaction with the nuclei of dead cells. Green fluorescence (FDA) was viewed under a Nikon B-2E/C FITC filter set (excitation at BP465–495, DM505, emission at BP515–555; Nikon Eclipse TE2000-U) and red fluorescence (PI) was viewed under a Nikon G-2E/C TRITC filter set (excitation at BP528–553, DM565, emission at 590–650; Nikon Eclipse TE2000-U). (**a**,**b**) UNM from fraction B1. (**c**,**d**) TCP from fraction A3. (**e**,**f**) MPGs after shaking and collecting in 0.1 M D-mannitol. (**g**,**h**) MPGs from a fresh dehiscent anther as a control. em., emission. Scale bars, 10 µm.

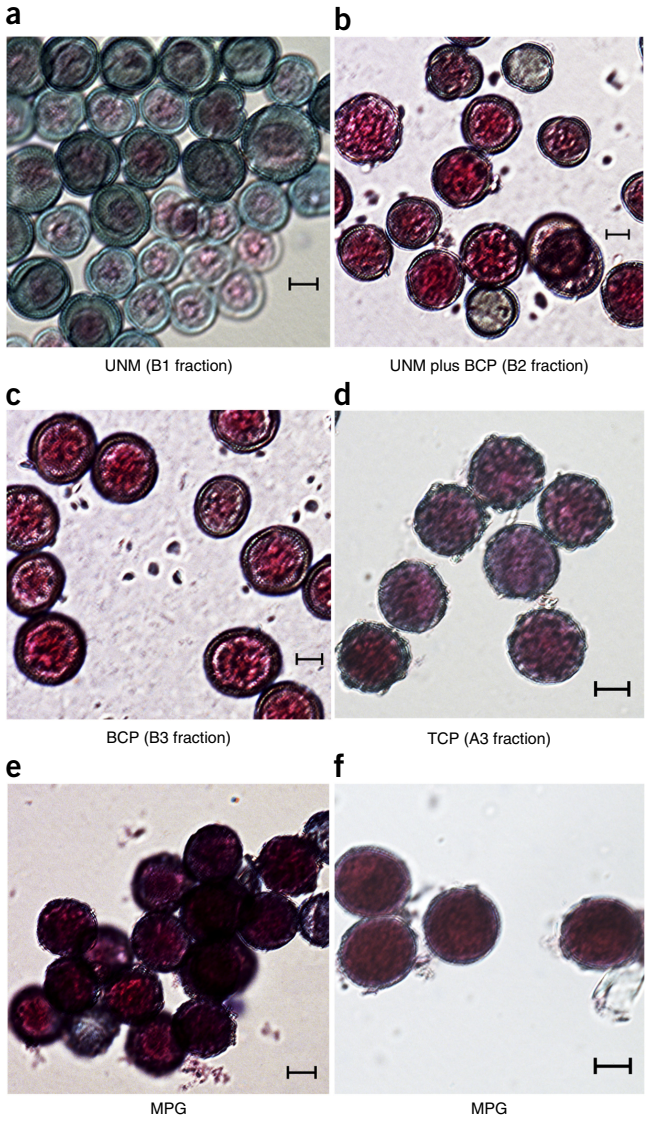

Figure 4 | Pollen viability test by Alexander staining is applicable for each fraction that was obtained during Percoll separation procedures (A1, A2, A3, B1, B2 and B3), including MPG. (**a**–**f**) Distinction between viable and nonviable grains is based on the differential staining of the protoplasm and the cellulose-containing wall. Viable spores are stained on a pink-to-purple color scale, and dead cells are stained blue. Analyzed samples were stored for 6 months at –20 °C after the separation procedure and before staining. Stained samples were visualized by bright-field microscopy. (**a**) Early UNM from fraction B1. (**b**) Late UNM and early BCP from fraction B2. (**c**) Late BCP from fraction B3. (**d**) TCP from fraction A3. (**e**) MPGs from collection into liquid medium as a part of Percoll separation procedure. (**f**) MPGs obtained directly from a fresh dehiscent anther. Scale bars, 10 µm.

or expanded flowering time, it is necessary to take this into consideration. The use of older plants with fewer or smaller flower buds is generally not recommended. For seed germination and plant growth, Jiffy-7 tablets (pellets) are used. They are manufactured from sphagnum peat and coir fibers in a dried compressed form. The pellets are easily rehydrated, and they can be planted in a few minutes. Jiffy plugs are packed in a soft biodegradable net covering that holds the substrate in a compact form, enabling space-friendly, long-term storage. Seed sowing and subsequent plant cultivation is carried out on the non-netted side. The advantages of Jiffy tablets include economical storage; reduced time requirements for sowing compared with conventional soil substrate preparation; an absence of pests, which eliminates the requirement for sterilization; and optimal conditions for plant rooting; in addition, the tablets conduct water with a high efficiency.

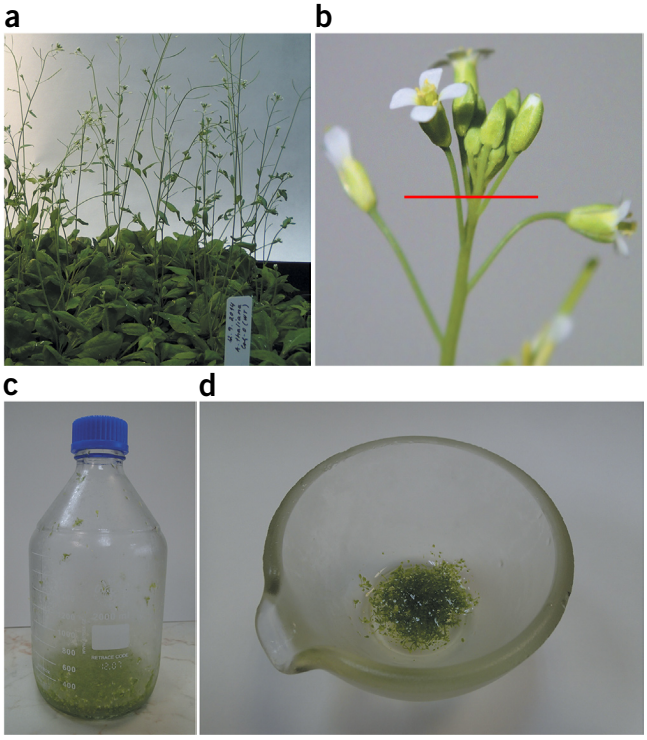

Figure 5 | Harvesting and homogenization of *A. thaliana* Col-0 (wild-type) flower inflorescences. (**a**) Plants were grown in plastic trays. Those shown were prepared for flower inflorescence clipping. (**b**) Only flowers grown on the main stem were collected; those in which the length of the pistil exceeded the length of the stamens and young siliques (stage 15 and later, according to Smyth *et al.*[22]) were not collected. The red line indicates the cutting site. (**c**) The flowers were collected into 2-l glass bottles. (**d**) Flowers were put into a cooled glass mortar and gently disturbed with a pestle during the homogenization step. Limiting homogenization to only ~1–1.2 g of plant material at a time is important.

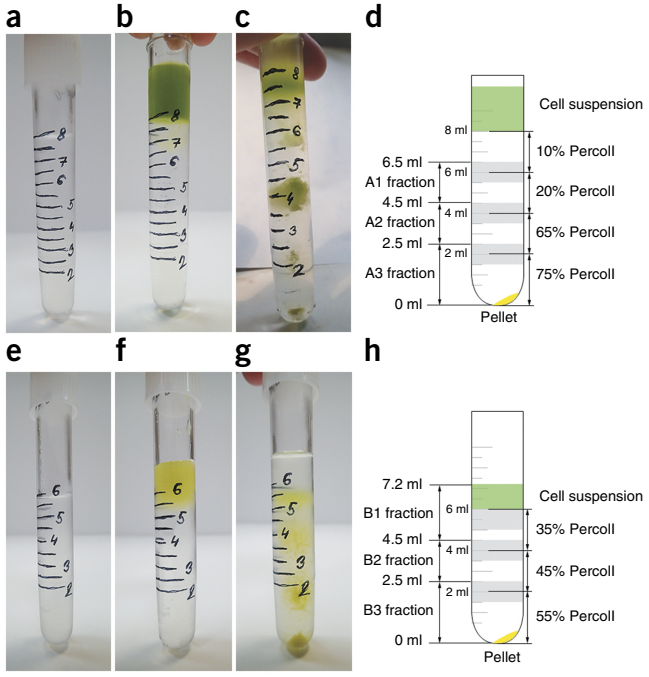

Figure 6 | Percoll step gradients. (**a**,**e**) Both gradients (75%/65%/20%/10% and 55%/45%/35%) must be prepared at least for 1 h but not more than 12 h before the separation. Marking the line with permanent pen is essential after adding each 500-µl aliquot of Percoll layer, as it aids the separation of obtained fractions. (**a**) Gradient A (75%/65%/20%/10%) without plant material and (**b**) after the loading of homogenized plant material. (**c**,**d**) Three fractions of developing spores formed after the centrifugation in gradient A—fractions A1, A2 and A3. All fractions were collected with a syringe and a needle. Fractions A1 and A3 were subsequently pelleted. Fraction A2 was loaded onto gradient B. (**d**) Schematic figure of individual fractions in gradient A containing specific bands formed by different developmental stages of spores. The fractions were taken as the band (shown in gray) and the area beneath the band in a total volume of 2 ml or 2.5 ml, depending on the fraction (see below). Fraction A1 is specifically contained in the band between 5.5 and 6 ml (6.5 ml), where uninucleate microspores (UNMs) were present. Then, the band in fraction A2 ranged between 3.5 and 4 ml (4.5 ml), and it was a mixture of UNMs and bicellular pollen (BCP). The last band in fraction A3, ranging between 1.5 and 2 ml (2.5 ml), contained TCP together with the pellet. The top layer at the 6.5-ml line contained debris and was discarded. (**e**) Gradient B (55%/45%/35%) without suspension and (**f**) the gradient after loading of cell suspension. After loading the homogenized plant material (**b**) or cell suspension (**f**) onto the gradient, we observed that it overflowed immediately into the top layer of the gradient. (**g**,**h**) Three fractions were obtained after the centrifugation of the fraction A2 pellet (resuspended in 0.1 M D-mannitol) in gradient B, fractions B1, B2 and B3. All fractions were collected with a syringe and a needle. Fractions B1, B2 and B3 were subsequently pelleted. (**h**) Schematic figure of individual fractions in refining gradient B, containing a specific band formed by a different developmental stage of spores. The separated bands are highlighted in gray. The fractions were taken again as the gray band plus the area beneath the band in a total volume of 2 or 2.5 ml, depending on the fraction. Fraction B1 contained the band between 5 and 5.5 ml (6 ml) with UNMs. Fraction B2, with the band between 3.5 and 4 ml (4.5 ml), contained late UNM together with early BCP. The band in fraction B3, ranging between 1.5 and 2 ml (2.5 ml), together with the pellet, contained BCP.

Harvesting the inflorescences. The next step in the separation procedure is the collection of *Arabidopsis* inflorescences (**Fig. 5a**). All flower buds collected should be up to stage 13, including fully opened flowers (stage 14) containing MPGs (**Fig. 5b**). Flowers with a pistil length exceeding the length of the stamens (stage 15) contain almost no pollen grains, as with subsequent flower developmental stages (fruit formation). A higher proportion of these latter harvested materials increases the amount of undesirable material (such as debris, other cells and so on) on the Percoll gradient, and the yield and purity of the spore fractions will be substantially reduced as a result.

Collection of MPGs. Collection of MPGs precedes Percoll gradient separation; MPGs are harvested by shaking into the liquid medium (0.1 M D-mannitol; **Fig. 5c**). This removal of MPGs substantially reduces the possible contamination of the TCP fraction (fraction A3). Moreover, the resulting MPG fraction is of a reasonably high purity (80–90%).

The time taken for purification of individual fractions of developing spores can be reduced by using the vacuum cleaning method, which removes MPGs[19]. Therefore, the protocol section, 'Collection of MPGs of *A. thaliana* in a liquid medium,' can be omitted. The final shaking of flower inflorescences in water should be performed within 1 min, and the suspension acquired by filtration through a 53-µM nylon mesh will be discarded. Plant material is collected on a nylon mesh. After collection, floral inflorescences are subjected to homogenization. This procedure ensures that there will be no contamination of the fractions.

As we did not wet the MPGs in this procedure, the time for Percoll separation was shortened to ~4 h.

Flower homogenization and filtration. The collected inflorescences are homogenized to release the developing spores from the flower buds. The obtained suspension contains particles with different sedimentation rates. The flower homogenization is carried out in a chilled glass mortar with a pestle (inner diameter = 8 cm; **Fig. 5d**). The mortar with the pestle must be stored in a refrigerator or in a cold room 1 d before homogenization. For homogenization, two sets of mortar and pestle per sample are always used. While one set is in use for homogenization, the second one is chilled on ice. The mortar and pestle sets are exchanged after completing each homogenization step to keep them as cold as possible. Before cooling, it is important to wipe off the inside of the mortar and clean the pestle. The use of two nylon meshes with different pore sizes (53 µm, 100 µm) during the filtration step removes an enormous amount of debris created by plant homogenization.

Percoll step-density gradient. The density of the stock isotonic Percoll (SIP) solution is 1.123 g/ml, and further dilutions to lower the density values with 0.1 M D-mannitol showed a linear correlation between the concentration and density. A Percoll step gradient is prepared by layering several Percoll gradient solutions of varied concentrations into the centrifuge tube. All gradient layers are formed manually using a pipette. Discontinuous Percoll gradients can also be made by a peristaltic pump. The separation of MG spore fractions is achieved by centrifugation of a mixed spore suspension through two different Percoll gradients (a two-step gradient separation; **Fig. 6**). In the first run, plant material is applied to a 75%/65%/20%/10% Percoll gradient (hereafter designated as gradient A, **Fig. 6a**,**b**). This leads to the separation of three fractions: A1, A2 and A3 (**Figs. 6c**,**d** and **7a**). Subsequently, the collected fraction A2, containing UNM and BCP, is separated by the discontinuous gradient B (55%/45%/35% Percoll, **Fig. 6e**,**f**) to separate three A2 subfractions, B1, B2 and B3 (**Figs. 6g**,**h** and **7b**). The method of preparation for the two gradients is identical. The top 10% (vol/vol) Percoll layer reduces the contamination of fraction A1 by cell debris.

Weight of the plant material for Percoll separation. One tube of Percoll gradient A can be used for the separation of up to 4–8 g of collected inflorescences. If the weight of collected plant material reaches 8–16 g, two gradient tubes must be used. The obtained A2 fraction(s) are then separated with one tube of Percoll gradient B.

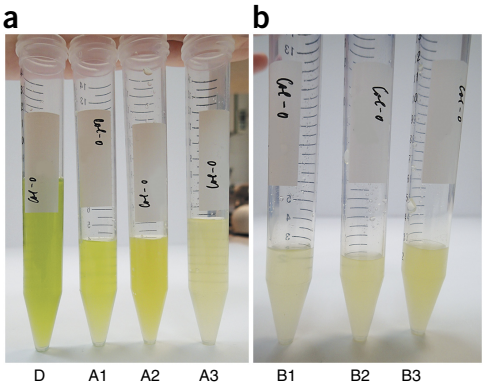

Figure 7 | Individual collected fractions from the Percoll separation procedure (**a**) A1 (UNM), A2 (UNM plus BCP), A3 (TCP) and cellular debris (D) were markedly different in color from each other. (**b**) A similar situation occured with fractions B1 (early UNM), B2 (late UNM plus early BCP) and B3 (late BCP; after the separation in Percoll gradient B). However, the differences were not as apparent.

Percoll step-density gradient after centrifugation. After separation by centrifugation, discrete zones should be visible to the naked eye within the individual fractions A1, A2 and A3/B1, B2 and B3 (**Fig. 6c**,**g**). These bands contain the respective developmental stages of MGs. Their size is proportional to the amount of separated pollen. The A1 zone usually appears in the range of 5.5 to 6–6.5 ml. The A2 zone is expected to be between 3.5 and 4–4.5 ml. Finally, the A3 zone appears in the range of 1.5 to 2–2.5 ml (**Fig. 6d**). The B1 zone usually appears in the range of 5–5.5 to 6 ml, whereas the B2 zone is expected between 3.5 and 4–4.5 ml. Finally, the B3 zone appears in the range from 1.5 to 2–2.5 ml (**Fig. 6h**).

Cell viability. Cell viability was assessed using a fluorescein diacetate (FDA)/propidium iodide (PI) pollen viability assay[20] (**Fig. 3a–f**) and Alexander staining[21] (**Fig. 4a–e**). It was clearly demonstrated by FDA/PI staining that the individual developmental fractions of *A. thaliana* MG (B1 and A3) remained viable after the Percoll separation (**Fig. 3a–d**). Almost all MPGs remained viable after they were collected in a liquid medium (**Fig. 3e–f**). Because the FDA dye reveals the viability of only the following developmental stages—UNM (B1 fraction; **Fig. 6a**,**b**), TCP (A3 fraction; **Fig. 3c**,**d**) and MPG (**Fig. 3e**,**f**)—we used another pollen viability test: Alexander staining. The viability of all separated fractions (B1, B2, B3 and A3) and MPGs was clearly shown (**Fig. 4a–e**).

MATERIALS

REAGENTS

- Plant material (Reagent Setup)
- Percoll (GE Healthcare, cat. no. 17-0891-01) ▲ **CRITICAL** Percoll is available only from this manufacturer.
- D-mannitol ($C_6H_{14}O_6$, molecular weight (Mw) 182.17; Sigma-Aldrich, cat. no. M4125)
- EDTA–disodium salt ($C_{10}H_{14}N_2O_8Na_2.2H_2O$, Mw 372.24; Sigma-Aldrich, cat. no. E0399)
- DAPI for nucleic acid staining ($C_{16}H_{15}N_5.2HCl$, Mw 350.25; Sigma Aldrich, cat. no. D9542) ! **CAUTION** DAPI is a suspected mutagen; wear gloves and mask while preparing the stock solution.
- *N*,*N*-dimethylformamide (DMF, $HCON(CH_3)_2$, Mw 73.09; Sigma-Aldrich, cat. no. D4551) ! **CAUTION** DMF is potentially toxic; wear protective gloves under a chemical hood while preparing the solution.
- Sodium phosphate monobasic monohydrate($NaH_2PO_4{\cdot}H_2O$, Mw 137.99; Sigma-Aldrich, cat. no. 71507)
- Sodium phosphate dibasic heptahydrate ($Na_2HPO_4{\cdot}7H_2O$, Mw 268.07; Sigma-Aldrich, cat. no. S9390)
- Fluorescein diacetate (FDA, $C_{24}H_{16}O_7$, Mw 416.38; Sigma-Aldrich, cat. no. F7378) ! **CAUTION** FDA is a suspected mutagen; wear gloves and a mask while preparing the stock solution.
- Propidium iodide (PI, $C_{27}H_{34}I_2N_4$, Mw 668.39; Sigma-Aldrich, cat. no. P4170) ! **CAUTION** PI is suspected as a mutagen; wear gloves and mask while preparing the stock solution.
- Acetone (C_3H_6O, Mw 58.08; Sigma-Aldrich, cat. no. 179124)

! CAUTION Acetone is toxic; wear protective gloves under a chemical hood while preparing the solution.
- Liquid nitrogen (N_2, Mw 28.01) **! CAUTION** Wear safety glasses or a face shield when you are transferring liquid nitrogen; wear gloves when you are touching any object cooled by liquid nitrogen.
- Malachite Green oxalate salt ($C_{25}H_{26}N_2O_4$, Mw 418.48; Sigma-Aldrich, cat. no. M9015) **! CAUTION** Malachite Green oxalate salt is a toxic, mutagenic and carcinogenic substance; wear gloves and mask while preparing the stock solution.
- Ethanol (C_2H_6O, Mw 46.07; Sigma-Aldrich, cat. no. 34923)
- Acid Fuchsin calcium salt ($C_{20}H_{17}N_3O_9S_3Ca$, Mw 579.64; Sigma-Aldrich, cat. no. 857408)
- Orange G ($C_{16}H_{10}N_2Na_2O_7S_2$, Mw 452.37; Sigma-Aldrich, cat. no. 861286)
- Phenol (C_6H_6O, Mw 94.11; Sigma-Aldrich, cat. no. P1037)
! CAUTION Phenol is a toxic and mutagenic substance; wear protective gloves under a chemical hood while preparing the solution.
- Chloral hydrate ($C_2H_3Cl_3O_2$, Mw 165.40; Sigma-Aldrich, cat. no. C8383)
! CAUTION Chloral hydrate is a potent neurotoxin; wear gloves and a mask while preparing the stock solution.
- Acetic acid, glacial ($C_2H_4O_2$, Mw 60.05; Sigma-Aldrich, cat. no. ARK2183)
! CAUTION Always pour acid slowly into the solution.
- Glycerol ($C_3H_8O_3$, Mw 92.09; Sigma-Aldrich, cat. no. G5516)
- Triton X-100 ($(C_2H_4O)_nC_{14}H_{22}O$; Sigma-Aldrich, cat. no. T8787)
▲ CRITICAL The product specification and Material Safety Data Sheet for each of the reagents listed here should be obtained where available and read before use. Personal safety protection clothing (laboratory coat, gloves and shoes) should be worn at all times. These reagents can be purchased by alternative chemical companies if they meet the parameters specified in this protocol, with the exception of chemicals for which the protocol prohibits replacement with an alternative.

EQUIPMENT
- Jiffy-7 tablets (seed pellets, peat pellets; Jiffy Products International; http://www.Jiffygroup.com), diameter 41 mm
- Square plastic plant pot (3.2 × 3.2 cm; e.g., from http://www.hummert.com)
- Appropriate flat plastic tray for water retention; e.g., from http://www.hummert.com)
- Plastic plug tray with cells measuring 4.5 × 4 cm (54 holes) for Jiffy-7 tablets; e.g., from http://www.hummert.com)
- Tweezers (e.g., Sigma-Aldrich, cat. no. T4162)
- Plastic bucket (e.g., Brand, cat. no. BR71772)
- 2 l glass bottles with screw caps (e.g., DURAN)
- Scissors (e.g., Sigma-Aldrich, cat. no. Z265977)
- 100-ml graduated cylinder (e.g., Brand, cat. no. BR41638)
- 500- and 250-ml Erlenmayer flasks
- 53- and 100-µm SEFAR nylon mesh (ELKO Filtering; 53-µm mesh membrane, mesh opening 53 µm, open area 30%, cat. no. 03-53/30; 100-µm mesh membrane, mesh opening 100 µm, open area 32%, cat. no. 03-100/32)
- 50- and 15-ml polypropylene conical centrifuge tubes (e.g. Eppendorf, cat. nos. 0030122151 and 0030122178)
- 5.0-ml polypropylene conical centrifuge tubes (e.g., Eppendorf, cat. no. 0030119401) **▲ CRITICAL** Using 5-ml tubes is preferrable for ease of handling and storage.
- Polystyrene round-bottom centrifuge tubes, capacity 10 ml, 110 × 16 mm (e.g., Nunc, cat. no. 348224)
- Eppendorf 5810R centrifuge with swing-bucket rotor or similar alternative, max. RCF 4,500*g*
- Eppendorf swing-bucket rotor A-4-62, max. RCF 3,220*g*
- Eppendorf swing-bucket rotor S-4-104, max. speed 3,220*g*
- Eppendorf adaptor for 50-ml conical centrifuge tubes for A-4-62 rotor, flat with rubber mat, centrifugation radius 173 mm, max. RCF 3,050*g* (Eppendorf, cat. no. 5810 757.002)
- Eppendorf adaptor for 15-ml conical centrifuge tubes for A-4-62, rotor, conical bottom shape, centrifugation radius 178 mm, max. RCF 3,150*g* (Eppendorf, cat. no. 5810 755.000)
- Eppendorf adaptor for 5.0-ml conical centrifuge tubes and S-4-104 rotor, centrifugation radius 189 mm, max. RCF 3,214*g* (cat. no. 5825 739.000)
- Automatic pipette (e.g., Eppendorf Xplorer)
- Manual pipette (e.g., Gilson)
- 0.80 × 120 mm needle (e.g., B/Braun, cat. no. 466 5643)
- 2-ml syringe (e.g., BD Discardit II, cat. no. 3009 28)
- 1.5-ml nontransparent or transparent capped microtube (e.g., Eppendorf, cat. no. 0030120086)
- Microscope slides (e.g., Knittel Gläser, 76 × 26 mm, cat. no. VA31100 001FKB)
- Coverslips of 0.13–0.16 mm thickness; 18 × 18 mm (e.g., Marienfeld Superior, cat. no. 0101030)
- Plastic Dewar flask (e.g., Nalgene)
- Plastic and glass beakers (e.g., Brand)
- Laminar flow box
- Magnetic stirrer (e.g., IKA)
- Funnel (e.g., Brand, cat. no. BR148040)
- Water vacuum pump (e.g., Dynalon, cat. no. 312635)
- Glass mortar and pestle (inner diameter = 8 cm; e.g., Cole-Parmer, cat. no. EW-34500-55) **▲ CRITICAL** Store these in a refrigerator or in a cold room (4 °C) 1 d before homogenization.
- Growth room
- Fluorescence microscope (e.g., Nikon Eclipse TE2000-U)

▲ CRITICAL All laboratory equipment coming in contact with Percoll should be washed immediately with a strong stream of water. Its sediments (especially on the walls and the bottoms of the tubes) are difficult to remove when dry. Percoll is not toxic, and washing with tap water is sufficient.

REAGENT SETUP

Plant material This protocol has been optimized for *A. thaliana* ecotype Col-0 (wild type, WT). In principle, it can be used with other accessions, such as Landsberg erecta (Ler), Wasillewskija (Ws) and so on. *A. thaliana* Col-0 (WT) plants were grown in a growth room under controlled conditions (21 °C, illumination of 150 µmol/m²/s, 16-h photoperiod, 50% relative humidity). **! CAUTION** When working with mutant lines, the conditions for cultivating genetically modified (GMO) plants have to be in full compliance with the laws of the respective country.

Stock isotonic Percoll Percoll is supplied as a 23% (wt/wt) colloidal water solution with a density of 1.130 ± 0.005 g/ml and osmolality <25 mOs/kg H_2O. Before preparing the Percoll gradient with various densities, it is necessary to make a prediluted isotonic Percoll stock solution (stock isotonic Percoll, SIP). Diluted SIP solution has adjusted osmolality, in contrast to nondiluted Percoll. Stock SIP is 90% (vol/vol) Percoll solution osmotically adjusted by the addition of 10% (vol/vol) 1 M D-mannitol. Mix nine parts of nondiluted Percoll (63 ml) with one part of 1 M D-mannitol (7 ml). Use an automatic or manual pipette. Keep the SIP in a glass bottle with a screw cap. The SIP density in D-mannitol is 1.123 g/ml, the density of Percoll is 1.130 g/ml and the density of 1 M D-mannitol is 1.0604 g/ml. The SIP density in 1 M D-mannitol is equal to the density of SIP in 1.5 M NaCl. As compared with sucrose, D-mannitol has higher viscosity and lower maximal density. **▲ CRITICAL** Percoll is supplied as a sterile solution, and it can be autoclaved, but only under specific conditions (see the manufacturer's manual for Percoll from GE Healthcare). For this reason, prepare SIP and Percoll gradients under sterile conditions (under a flow hood). Originally packed Percoll can be stored at room temperature (24 °C) for 2–5 years. Upon opening, it can be stored at 2–8 °C for 2–5 years. Store SIP at 2–8 °C, and do not autoclave. This solution can be used for at least 6 months. **▲ CRITICAL** Long-term storage of Percoll may result in the formation of aggregates. However, they do not affect the separation of most biological particles. For specific experiments, however, it is important to eliminate aggregates by filtration using, e.g., A15 Millipore prefilters.

1 M D-Mannitol Weigh out 18.22 g of D-mannitol and dissolve it in 80 ml of water with a magnetic stirrer. Add water to a final volume of 100 ml. Transfer the solution to a 250-ml glass bottle and sterilize it by autoclaving (121 °C, 30 min). Store it at 2–8 °C. This solution can be used for at least 1 year under sterile conditions. **▲ CRITICAL** Open the bottle containing D-mannitol solution in a sterile flow hood to avoid repeated autoclaving. The stir bar should be kept in the prepared solution so that it dissolves any possible D-mannitol precipitate, as it is saturated in a 1 M solution. **▲ CRITICAL** During long-term storage, D-mannitol crystals form and are dissolved by stirring on a combined hot plate and magnetic stirrer device (stirring rate 6 × 100 per min, heating to 40 °C).

▲ CRITICAL Use sterilized 1 M and 0.1 M D-mannitol whenever called for. Use double-distilled or double-deionized water whenever water is called for.

0.1 M D-Mannitol Weigh out 5.46 g of D-mannitol and dissolve it in 240 ml of water with a magnetic stirrer. Add water to a final volume of 300 ml. Transfer the solution to a 500-ml glass bottle and sterilize it by autoclaving (121 °C, 30 min). Store it at 2–8 °C. This solution can be used for at least 1 year under sterile conditions. **▲ CRITICAL** 1 M D-mannitol and 0.1 M

D-mannitol can be autoclaved repeatedly after being used in a nonsterile area.0.2 M $NaH_2PO_4 \cdot H_2O$.

Dissolve 2.76 g of $NaH_2PO_4 \cdot 1H_2O$ in 50 ml of water and adjust the final volume to 100 ml. Store it in a glass bottle at 2–8 °C. This solution can be used for at least 6 months.

0.2 M $Na_2HPO_4 \cdot 7H_2O$ Dissolve 5.362 g of $Na_2HPO_4 \cdot 7H_2O$ in 50 ml of water and adjust the final volume to 100 ml. Store the solution in a glass bottle at 2–8 °C. This solution can be used for at least 6 months.

100 mM Sodium phosphate buffer The 100 mM Sodium phosphate buffer is 0.2 M $NaH_2PO_4 \cdot 1H_2O$ and 0.2 M $Na_2HPO_4 \cdot 7H_2O$, pH 7.0. Mix 19.5 ml of 0.2 M $NaH_2PO_4 \cdot H_2O$ with 30.5 ml of 0.2 M $Na_2HPO_4 \cdot 7H_2O$. Add water to a final volume of 100 ml. Store the buffer at 2–8 °C in a glass bottle. The buffer can be used for at least 6 months.

Extraction buffer The extraction buffer is 100 mM sodium phosphate buffer, 10 mM EDTA–disodium salt and 0.1% Triton X-100, pH 7.0. Add 372.3 mg of EDTA–disodium salt and 100 µl of Triton X-100 to 75 ml of 100 mM phosphate buffer. Adjust the pH to 7.0 with 10 M NaOH while stirring with a magnetic stirrer, and then dilute the solution to 100 ml and store it at 2–8 °C in a glass bottle. This buffer can be used for at least 6 months.

DAPI stock solution (DAPI and DMF) Dissolve 0.5 mg of DAPI in 1 ml of DMF, and store the solution in a 1.5-ml nontransparent capped microtube at −20 °C. This solution can be used for at least 1 year.

Nuclei staining solution (DAPI stock solution and extraction buffer) Mix 120 ìl of DAPI stock solution in 10 ml of extraction buffer in a 15-ml screw-cap tube. The final concentration is 6 µg of DAPI in 1 ml of buffer. Store the solution at 2–8 °C, protected from light. This solution can be used for at least 6 months.

0.2% (wt/vol) FDA stock solution Dissolve 2 mg of FDA in 1 ml of acetone, and store the solution in a 1.5-ml nontransparent capped microtube at −20 °C. This solution can be used for at least 6 months. **! CAUTION** Store the FDA powder in a jar with silica desiccant at −20 °C in darkness indefinitely.

FDA working solution Mix 1.5 µl of FDA stock solution in 125 µl of 0.1 M D-mannitol in a 1.5-ml nontransparent capped microtube. The final concentration is 24 µg of FDA in 1 ml of 0.1 M D-mannitol. **▲ CRITICAL** Always use freshly prepared working solution (same day as sample staining).

1.5 mM PI stock solution Dissolve 1 mg of PI in 1 ml of water, and store it in a 1.5-ml nontransparent capped microtube at 2–8 °C. This solution can be used for at least 6 months.

1% (wt/vol) Malachite Green solution Dissolve 0.1 g of Malachite Green in 10 ml of ethanol. Store it in a 15-ml Falcon tube at 2–8 °C. This solution can be used for at least 1 year.

1% (wt/vol) acid fuchsin solution Dissolve 0.1 g of acid fuchsin in 10 ml of water. Store the solution in a 15-ml screw-cap tube at room temperature. This solution can be used for at least 2 years.

1% (wt/vol) Orange G solution Dissolve 0.1 g of Orange G in 10 ml of water. Store the solution in a 15-ml screw-cap tube at room temperature. This solution can be used for at least 1 year. The solution may partially crystallize on storage; it can be re-dissolved by heating it slightly.

Alexander's stain solution Mix 10 ml of ethanol, 1 ml of 1% (wt/vol) Malachite Green solution, 5 ml of 1% (wt/vol) acid fuchsin solution, 0.5 ml of 1% (wt/vol) Orange G solution, 5 g of phenol, 5 g of chloral hydrate, 2 ml of glacial acetic acid and 25 ml of glycerol in a glass beaker with a magnetic stirrer. Adjust the total volume to 50 ml with double-dH_2O in a measurement cylinder. Store the solution in a 50-ml screw-cap tube at 2–8 °C. This solution can be used for at least 1 year.

Percoll gradient solutions (Percoll plus 0.1 M D-mannitol) Prepare the following Percoll gradient solutions under sterile conditions (under a flow hood). Prepared individual Percoll gradient solutions can be stored at 4 °C for a few weeks.

Stock isotonic Percoll (SIP) (ml)	0.1 M D-Mannitol (ml)	Final layer concentration[a] (vol/vol)	Total volume (ml)	Density (g/ml)
15	5	75%	20	1,093
13	7	65%	20	1,082
11	9	55%	20	1,070
9	11	45%	20	1,058
7	13	35%	20	1,046
4	16	20%	20	1,028
2	18	10%	20	0,916

[a]For simplified calculation of final layer concentration, SIP is designated as 100% Percoll.

▲ CRITICAL Check for possible contamination of Percoll gradient solutions before use.

▲ CRITICAL As Percoll sediments by gravity during long-term storage, shake the solution vigorously by hand to homogenize the solution before the preparation of Percoll step-density gradients.

EQUIPMENT SETUP

Centrifugation The Percoll gradient protocol was established empirically and optimized for the particular types of centrifuge, rotor and tubes stated above. Other types of general-purpose centrifuges, such as Beckman (Allegra 6 series) or Sorvall, can be used, but centrifugation conditions must conform exactly to those described in this protocol. 5-ml Eppendorf tubes with the corresponding swing-out rotor adaptor were used for the final fraction collection. If the 5-ml Eppendorf tube adaptors cannot be used (e.g., if using an alternative brand of centrifuge), the fractions can be deposited into the 15-ml Falcon tubes. Gradient Nunc 10-ml polystyrene centrifuge tubes with a round-bottom shape are preferred. It is not recommended to use larger centrifuge tubes to separate a larger amount of biological material at once. Additional tubes of aforementioned volume could be used instead. The tubes must be properly balanced before the centrifugation.

Preparation of gradient layers manually using a pipette Conventional manual pipettes with suitable disposable tips were used to prepare the Percoll gradient in Nunc 10-ml polystyrene centrifuge tubes. The most important criteria for the selection of pipettes are (i) ease of handling and (ii) the option of pipetting the 500-µl volume (e.g., Gilson, Eppendorf and so on). An easy-dispensing pushbutton is an important feature of the pipette used. The necessity to apply higher pressure to deliver a given volume of gradient solution may cause an increased flow, resulting in mixing together of the Percoll layers.

Percoll layers are formed by adding 500 µl of Percoll solution to the previous layer or the part created before. The total volume of each layer required for a given density is 2 ml—i.e., four subsequent 500-µl pipetting steps for each density layer.

Sample visualization and analysis A fluorescence microscope with an appropriate source of light (e.g., xenon lamp in the Nikon Eclipse TE2000-U) and filters are used for detection and visualization of counterstaining fluorescent dyes (DAPI, FDA and PI). Samples stained by Alexander staining were visualized by bright-field microscopy. The microscopic samples were kept at 4 °C in darkness until they were observed under a microscope.

PROCEDURE

▲ CRITICAL All flasks and tubes containing medium with/without MG, including those used for harvesting plant material, must be kept on ice or in a refrigerator during the whole procedure. All centrifugation steps cited below were performed with the Eppendorf 5810R centrifuge.

Preparation of the plant material ● TIMING 90 min

1| *Rehydration of Jiffy tablets*. Rehydrate Jiffy tablets with water in an appropriate container (e.g., a beaker). After rehydration, transfer the soaked tablets to pots and/or trays for subsequent sowing and/or planting.

▲ CRITICAL STEP When the tablets are rehydrated, the volume increases fivefold. This fact should be taken into account when planning the container size. The diameter of a fully swollen tablet is ca. 3.5 cm.

2| *Seed germination*. Place four fully swollen Jiffy tablets into each pot (size 3.2 × 3.2 cm). Push the tablets well to the bottom of the pot to ensure optimal water transport from the tray. Sow the seeds onto the soil surface of the tablets.

3| Leave the pots in a cold room (7 °C) for 2 d (seed stratification). Next, transfer them to the growth room for seed germination (see plant material in the Reagent Setup).

4| *Seedling planting*. Fill six plastic trays with fully swollen tablets (Steps 1 and 2). Using tweezers, make one hole in each tablet. Transfer the seedlings into these holes (one seedling per Jiffy tablet) once they have reached a diameter of ~0.5–0.7 cm (ca. 12 d after sowing).
▲ **CRITICAL STEP** To ensure an efficient plant transfer, pull the Jiffy tablet with the seedlings out of the pot and stir up the sphagnum peat coat with your fingers. Remove one seedling at a time and transfer it with tweezers to the new Jiffy tablet with a hole. The seedling roots should be covered with peat. When transporting the seedlings with the tweezers, hold them under the cotyledons and be attentive to the fragility of the plant material.
■ **PAUSE POINT** Seedlings should be grown for 6–weeks in the growth conditions described in the Reagent Setup (**Fig. 5a**).

Harvesting of the *Arabidopsis* flower inflorescences ● TIMING 90 min
5| If two people will be involved in harvesting the inflorescences, cool two laboratory glass beakers on ice and add 50 ml of ice-cold 0.1 M D-mannitol to each. After the inflorescences are harvested, put the collections together in one 2-l glass bottle. If only one worker is harvesting, collect the inflorescences directly into one 2-l bottle containing 100 ml of 0.1 M D-mannitol (**Fig. 5c**). The stated timing was achieved by two workers.

6| Cut the stem of each plant with scissors so that the whole plant can be easily manipulated.

7| Cut the flowers just below the last fully open flower with a pistil length not exceeding the length of the stamens (stage 14)[22] (**Fig. 5b**). Hold the inflorescences above the opening of the bottle or beaker, and cut so that they fall inside. All flower buds that are collected should be up to stage 14.
▲ **CRITICAL STEP** The duration of this protocol is inversely proportional to the number of people involved in collecting the plant material. The subsequent part of the protocol (from the collection step onward) was carried out by one person.

Collection of MPGs of *A. thaliana* in a liquid medium ● TIMING 90 min
▲ **CRITICAL** The successive volume reduction of the suspension increases the yield of MPGs in the final fraction.
8| Prepare a 500-ml Erlenmayer flask with a funnel fitted with a 100-µm nylon mesh and filter.

9| Hold the bottle containing the inflorescences tightly, and shake it vigorously up and down for 1 min to release the pollen grains into the medium.

10| Pour the medium containing MPGs through the 100-µm nylon mesh. Squeeze the residual MPG suspension from the mesh into a flask. Put the remaining plant material back into the original glass bottle using tweezers.

11| Again add 100 ml of ice-cold 0.1 M D-mannitol to the bottle with the plant material, and repeat the shaking and filtration procedure as in Steps 9 and 10. The procedure should be repeated four times in total.

12| After the last round, put the plant material into the bottle, add 200 ml of double-dH_2O and store it on ice in a cold room (7°C) until you perform the steps for homogenization of the floral inflorescences (Steps 26–39).

13| Filter the harvested mature pollen suspension (total volume 400 ml) through a 53-µm nylon mesh into a new 500-ml Erlenmeyer flask.

14| Divide the MPG suspension into eight 50-ml Falcon tubes (50 ml of MPG suspension in each tube) and centrifuge them in a swing-bucket rotor A-4-62 using the Eppendorf adaptor for 50-ml Falcon tubes at 900*g* (speed acceleration 5, speed braking 9) at 4 °C for 10 min.

15| Decant half of each supernatant without disturbing the MPG pellet, and then resuspend the pellet in each 50-ml Falcon tube by vigorous shaking.

16| Reduce the total number of 50-ml Falcon tubes by half by pooling the MPG suspensions from two Falcon tubes in one. Spin the tubes again at 900*g* (speed acceleration 5, speed braking 9) at 4 °C for 10 min. Repeat Steps 15 and 16 until the volume of the MPG suspension is reduced to only 25 ml in one 50-ml tube.

17| Change the rotor adaptor for the 15-ml Falcon tubes. Divide the resuspended MPG suspension into two 15-ml Falcon tubes and spin them (each 15-ml tube contains ca. 12.5 ml of MPG suspension). Spin the tubes at 900*g* (speed acceleration 5, speed braking 9) at 4 °C for 10 min.

18| Use an automatic pipette to discard 7.5 ml of the supernatant from each 15-ml Falcon tube. Resuspend the pellet, by shaking it by hand and transferring the concentrated MPG suspension to two new 5-ml Eppendorf tubes.
▲ **CRITICAL STEP** If it is necessary to determine the isolation yield, weigh the empty tube in which the pollen material will be stored for further applications.

19| Spin the tubes at 900*g* (speed acceleration 5, speed braking 9) at 4 °C for 10 min. Discard half of the supernatant using an automatic pipette. Resuspend the pellet and centrifuge it again.

20| Decant as much as possible of the clear supernatant. Resuspend the pellet with a pipette in the remaining buffer.

Assessment of MPG fraction purity by fluorescence microscopy (DAPI) ● **TIMING 30 min**
21| Put 5 µl of concentrated MPG suspension into a 1.5-ml tube containing 5 µl of nuclei staining solution. Leave the remaining MPG suspension on ice for cell viability assessment in Step 24. If you do not wish to perform assessment of MPG fraction purity or viability, instead proceed to Step 24A(iv).

22| Incubate the MPG samples in a nuclei-staining solution in darkness at room temperature for at least 30 min to achieve an efficient staining of the nuclei for the microscopic evaluation. The final DAPI concentration in the MPG suspension should be ~3 µg/ml.

23| Resuspend the MPG suspension by pipetting, and transfer 10 µl onto the microscope slide and cover it with a cover glass. Visualize with an appropriate filter—e.g., a UV-2A filter set (420 LP nm, Nikon).
? TROUBLESHOOTING

Cell viability test
24| Viability of pollen developmental stages can be assessed either by Alexander staining (option A) or by FDA and PI staining (option B).
(A) Alexander staining ● **TIMING 30 min**
▲ **CRITICAL STEP** This is applicable to fractions A1, A2, A3, B1, B2, B3 and MPG.
(i) Put 5 µl of concentrated MPG suspension (Step 20) into a 1.5-ml tube containing 5 µl of Alexander staining solution.
(ii) Incubate the MPG samples in the staining solution at room temperature for a few minutes.
(iii) Resuspend the MPG suspension by pipetting. Transfer 10 µl onto a microscope slide and cover it with a cover glass. Visualize by bright-field microscopy. Viable spores are stained on a pink-to-purple color scale; dead cells are stained blue (**Fig. 4a–e**).
(iv) Spin the remaining MPG suspension from Step 20 at 900*g* (speed acceleration 5, speed braking 9) at 4 °C for 10 min, and remove the supernatant using a long needle attached to a vacuum pump.
■ **PAUSE POINT** Freeze the MPG pellet in liquid nitrogen, and store it at –80 °C until subsequent analysis (e.g., hormone extraction, total RNA extraction and so on). If needed, the pellet can be weighed before freezing.
(B) FDA and PI staining ● **TIMING 30 min**
▲ **CRITICAL STEP** This is applicable to fractions A1, A3, B1 and MPG.
(i) Spin the tubes again with the remaining MPG suspension, and remove the supernatant using a long needle attached to a vacuum pump.
(ii) Add 500 µl of 0.1 M D-mannitol to the pellet, and resuspend the cells with a pipette.
(iii) Put 125 µl of concentrated MPG suspension into a new 1.5-ml tube, and add 125 µl of FDA working solution. Vortex the sample vigorously. The final FDA concentration in the MPG suspension should be ~12 µg/ml. Incubate the sample in darkness at room temperature for 5 min.
(iv) Add 5 µl of PI stock solution to the tube after incubation with FDA. The final PI concentration in the MPG suspension should be 20 µg/ml. Incubate the sample in darkness at room temperature for 5 more min.

(v) Briefly spin the tube at 14 000*g* at room temperature for 30 sec and then transfer 10 µl of MPG suspension from the bottom of the tube onto the microscope slide and cover it with a cover glass. Visualize it under appropriate filters—e.g., for PI use the TRITC HYQ filter set (590–650 nm, Nikon) and for FDA use B2-E/C FITC (515–555 nm, Nikon). Viable cells are stained yellowish green and inviable cells are stained red (**Fig. 3a–f**).

(vi) Spin the remaining MPG suspension (from Step 24B(ii)) at 900*g* (speed acceleration 5, speed braking 9) at 4 °C for 10 min, and remove the supernatant using a long needle attached to a vacuum pump.
■ **PAUSE POINT** Freeze the MPG pellet in liquid nitrogen, and store it at –80 °C until subsequent analysis (e.g., hormone extraction, total RNA extraction and so on). If needed, the pellet can be weighed before freezing.

Preparation of Percoll step-density gradients A (75%/65%/20%/10%) and B (55%/45%/35%) ● **TIMING 30 min**

25| Prepare the required number of Percoll gradients in Nunc 10-ml polystyrene centrifuge tubes with a round bottom. Normally, two tubes of Percoll gradient A (75%/65%/20%/10%) and one tube of Percoll gradient B (55%/45%/35%) are required because the weight of the starting plant material collected from six trays usually does not exceed 16 g. Add 500-µl aliquots of Percoll solutions of decreasing concentration at the bottom of the tube (e.g., in the 75%/65%/20%/10% gradient, lay the 75% layer first). Add the next 500 µl of the Percoll solution onto the previous layer until the total volume of 2 ml is reached. This means that four subsequent pipetting steps for each density layer (e.g., 75%) are performed. Keep the tube sloped at a 45° angle when pipetting the layers. The pipette tip should touch the tube wall just above the top of the last created layer. The solution from the pipette tip should be discharged slowly (the flow rate should be under 0.5 ml of 15 per s) to prevent mixing it with the previous solution. As the Percoll solution is being pushed out from the pipette tip, the pipette must be lifted up to prevent its immersion in the previously formed layer. Mark the level of each Percoll layer every 500 µl with a water-resistant marker for optimal removal of Percoll layers with individual spore fractions after the centrifuge separation.
▲ **CRITICAL STEP** It is essential to prevent air bubbles when preparing a density gradient.
▲ **CRITICAL STEP** Discontinuous Percoll gradients must be prepared at least 1 h before the separation so that a uniform diffusion of particles is provided in each interphase. Formed Percoll gradients should not be stored longer than 12 h.
▲ **CRITICAL STEP** Check for contamination of Percoll gradient solutions before making each gradient.

Homogenization of the flower inflorescences ● **TIMING 45 min**

26| Take the bottle with plant material from Step 12 and two mortar and pestle sets from a cold room (7°C).

27| Put both mortar and pestle sets on ice.

28| Perform the final shaking of flower inflorescences in water for 1 min as in Step 9.

29| Filter the suspension through a 53-µM nylon mesh, and discard this last suspension.

30| Squeeze out excess liquid and weigh the remaining plant material that was retained in the nylon mesh using a balance. Keep the plant material on ice.
▲ **CRITICAL STEP** The weight of the homogenized material applied on one Percoll gradient A should not exceed 8 g. The optimal weight before homogenization was established at 4–6 g.

31| Take one mortar and pestle set. Wipe off the inside of the mortar, and clean the pestle.

32| Transfer the inflorescences with tweezers into the prechilled mortar, add 5 ml of 0.1 M D-mannitol and disturb the buds by gently tapping with a prechilled pestle for 1 min.
▲ **CRITICAL STEP** The amount of homogenized material for one homogenization step should not exceed 1–1.2 g (**Fig. 5d**). Homogenizing a higher amount of plant material at once notably decreases the yield of isolated spores.

33| Transfer the mixed spore suspension to a 50-ml Falcon conical centrifuge tube, and keep it on ice.

34| Rinse the mortar and the pestle with 5 ml of 0.1 M D-mannitol, and transfer the liquid containing washed spores to the same 50-ml centrifugation tube. Repeat this procedure once again.

35| Vortex the 50-ml centrifuge tube containing the spore suspension (total volume ~15 ml) intermittently for 30 s.

36| Pour the spore suspension through a 100-µm nylon membrane into a prechilled 250-ml Erlenmeyer flask. Squeeze the residual suspension into the flask by hand.

37| Discard the debris retained on the mesh.

38| Repeat this procedure (Steps 31–37) until all the plant material is depleted.
▲ **CRITICAL STEP** After completing each homogenization step, change the mortar and pestle set for a new one that was kept on ice. Before use, wipe off the inner space of the mortar and pestle.

39| Filter the clean mixed spore suspension through a 53-µm nylon mesh into a new prechilled 250-ml Erlenmeyer flask and keep it on ice.

Sample loading ● TIMING 30 min
40| Divide the mixed spore suspension from Step 39 into an appropriate number of 50-ml Falcon tubes, and centrifuge them on a swing-out rotor A-4-62 with an adaptor at 900*g* (speed acceleration 5, speed braking 9) at 4 °C for 10 min.

41| Discard half of the supernatant by decanting, resuspend the pellet by shaking and pool the suspension from two Falcon tubes into one. Repeat the centrifugation.

42| Repeat Step 41 until only two Falcon tubes with pelleted MGs are left.

43| Decant half of the supernatant, resuspend the pellet by shaking it, and pool the suspension from two Falcon tubes into one. Divide the mixed spore suspension into two 50-ml Falcon tubes and centrifuge again.

44| Pour away the supernatant without disturbing the pellet.

45| Resuspend the pellets in the first 50-ml tube in 1 ml of ice-cold 0.1 M D-mannitol, and, using a manual pipette, transfer the suspension to the top of the Percoll gradient A (contained in Nunc 10-ml polystyrene centrifuge tubes prepared in Step 25). Layer the sample as a narrow zone at the top of the Percoll density gradient.

46| Rinse the 50-ml tube with an additional 1 ml of D-mannitol, and transfer again to the same Percoll gradient A. The total volume of the transferred suspension should be 2 ml plus the volume of the pellet with the remaining solution (**Fig. 6b**).

47| Repeat the procedure from Steps 45 and 46 with the pellets in the second 50-ml tube containing the mixed pellet of the spore population. Use the other Nunc 10-ml polystyrene centrifuge tubes containing Percoll gradient A.

Percoll gradient fractionation on the 75%/65%/20%/10% gradient (gradient A) ● TIMING 5 min
48| Centrifuge the Nunc 10-ml centrifuge tubes with the Percoll step gradient topped with a mixed spore sample at 450 g for 5 min at 4°C, with acceleration 0 and braking 0, on the swing bucket rotor A-4-62 with adaptor.
▲ **CRITICAL STEP** Maintaining the recommended centrifugation time and speed is necessary. The value of acceleration and braking must be set at 0 so that the gradient will not be disturbed. The density of the particles is higher than the density of the bottom gradient, so all particles will eventually form a pellet if they are centrifuged long enough.

Collection of separated fractions A1, A2 and A3 ● TIMING 20 min
49| Take the top layer, between 10.4–10.5 and 6.5 ml, and discard it.
▲ **CRITICAL STEP** The upper limit (10.4 or 10.5 ml) depends on the volume of the spore suspension applied on preformed Percoll gradient A.
? TROUBLESHOOTING

50| Take the layer between 6.5 and 4.5 ml (fraction A1, volume 2 ml) and place it into a prechilled 15-ml centrifuge tube using a long needle and a syringe (**Figs. 6c–d** and **7a**). This fraction contains UNM.
? TROUBLESHOOTING

51| Take the layer between 4.5 and 2.5 ml (fraction A2, volume 2 ml) and place it into prechilled 15-ml centrifuge tubes using a long needle and a syringe (**Figs. 6c–d** and **7a**). This fraction contains a mixture of UNM and BCP and will be further separated by Percoll gradient B.
? TROUBLESHOOTING

52| Take the layer between 2.5 ml and 0 ml, including the pellet (fraction A3, volume 2.5 ml), and place it into respective prechilled 15-ml centrifuge tubes (one tube per fraction) using a long needle and a syringe (**Figs. 6c**,**d** and **7a**). This fraction contains TCP.
? TROUBLESHOOTING

53| Pool together the corresponding fractions from individual Percoll gradients. The total volume of fractions A1 and A2 after separation by two identical gradients should be 4 ml, whereas the volume of fraction A3 should be 5 ml (**Fig. 7a**).

54| Dilute each spore suspension containing a fraction with two volumes of 0.1 M D-mannitol (e.g., add 8 ml of 0.1 M D-mannitol to 4 ml of spore suspension).

55| Mix the diluted suspension thoroughly by inverting the tube at least five times.
▲ **CRITICAL STEP** Proper dilution and mixing of suspension ensures proper sedimentation of spores from suspension and pelleting.

56| Centrifuge the tubes on a swing-out rotor A-4-62 with an adaptor at 900*g* (speed acceleration 5, speed braking 9) at 4 °C for 10 min.

57| Decant the supernatant from the pelleted fraction A2, and store the pellet on ice until Step 64.
? TROUBLESHOOTING

Processing of A1 and A3 fractions ● TIMING 15 min
58| Discard the supernatant of the A1 and A3 fractions (from top to bottom) with an automatic pipette. Leave 5 ml of supernatant with the undisturbed pellet still in the centrifuge tube.
? TROUBLESHOOTING

59| Resuspend the pellet in a residual solution by shaking, and transfer the suspension to a new 5-ml centrifuge tube. Centrifuge the mixture at 900*g* (speed acceleration 5, speed braking 9) at 4 °C for 10 min.

60| Discard half of the supernatant with an automatic pipette. Resuspend the pellet again and repeat the centrifugation step.

61| Decant as much as possible of the clear supernatant. Resuspend the pellet in a residual solution by pipetting. See Steps 21–23 if you need to assess the purity of the fraction by DAPI staining, or Step 24 to test cell viability. If not, proceed to the next step.
? TROUBLESHOOTING

62| Spin the tubes at 900*g* at 4 °C for 10 min. Remove the supernatant completely with a long needle attached to a vacuum pump.

63| Freeze the pellet in liquid nitrogen and store it at –80 °C for further application(s) (e.g., hormone extraction, total RNA extraction and so on). If needed, the pellet can be weighed before freezing.

Percoll gradient fractionation of A2 fraction on the 55%/45%/35% gradient (gradient B), including sample loading ● TIMING 10 min
64| Resuspend the A2 pellet from Step 57 in 1 ml of ice-cold 0.1 M D-mannitol by pipetting.

65| Transfer the spore suspension to the top of the Percoll gradient B, contained in Nunc 10-ml polystyrene centrifuge tubes (Step 25), using a manual pipette (**Fig. 6f**). Layer the sample as a narrow zone at the top of the Percoll density gradient. The total volume of the transferred suspension should be 1 ml plus the spore pellet volume.

66| Centrifuge the Percoll step gradient topped with a mixed spore sample at 450*g* for 5 min at 4°C, with acceleration 0 and braking 0, on the swing bucket rotor A-4-62 with adaptor.
▲ **CRITICAL STEP** It is essential that the acceleration and deceleration value be set at 0 so that the gradient is not disturbed.

Collection of separated fractions B1, B2 and B3 ● TIMING 20 min
67| Take the top layer, between 7.3–7.2 and 4.5 ml (fraction B1, volume ca. 2.8 ml). The upper limit (7.3 or 7.2 ml) depends on the volume of the spore suspension applied on Percoll gradient B. This fraction contains UNM. Transfer the collected fraction B1 (ca. 2.8 ml) to the new prechilled 15-ml Falcon tubes (**Figs. 6g**,**h** and **7b**).
? TROUBLESHOOTING

68| Take the layer between 4.5 and 2.5 ml (fraction B2, volume 2 ml). This fraction contains the mixture of late UNM and early BCP. Transfer the collected fraction B2 (2 ml) to new prechilled 15-ml Falcon tubes (**Figs. 6g**,**h** and **7b**).
? TROUBLESHOOTING

69| Take the bottom layer, between 2.5 ml and 0 ml, including the pellet (fraction B3, volume 2.5 ml). This fraction contains late BCP. Transfer the collected fractions B3 (2.5 ml) to new prechilled 15-ml Falcon tubes (**Figs. 6g**,**h** and **7b**).
? TROUBLESHOOTING

70| Dilute each spore suspension–containing fraction by two volumes of 0.1 M D-mannitol (e.g., add 4 ml of 0.1 M D-mannitol to 2 ml of spore suspension).

71| Mix the diluted suspension thoroughly by inverting the tube at least five times.
▲ **CRITICAL STEP** Proper dilution and mixing of suspension ensures proper sedimentation of spores from their suspension and their pelleting.

72| Centrifuge the tubes on a swing-out rotor A-4-62 with an adaptor at 900*g* (speed acceleration 5, speed braking 9) at 4 °C for 10 min.

Processing of B1, B2 and B3 fractions ● TIMING 15 min
73| Leave 5 ml of supernatant with the undisturbed pellet in the centrifugation tube. Discard the remaining supernatant (from the top to the bottom) with an automatic pipette.
? TROUBLESHOOTING

74| Resuspend the pellet, shake the residual 0.1 M D-mannitol and transfer the suspension to a new 5-ml centrifuge tube. Centrifuge the mixture again.

75| Discard half of the supernatant with an automatic pipette. Resuspend the pellet again and repeat the centrifugation step.

76| Pour out as much as possible of the clear supernatant. Resuspend the pellet with a pipette.
See Steps 21–23 if you need to assess the purity of the fraction by DAPI staining, or Step 24 to test cell viability. If not, proceed to the next step.
? TROUBLESHOOTING

77| Spin the tubes at 900*g* at 4 °C for 10 min. Remove the supernatant completely with a long needle attached to a vacuum pump.

78| Freeze the pellet in liquid nitrogen and store it at –80 °C for further application(s) (e.g., hormone extraction, total RNA extraction and so on). If needed, the pellet can be weighed before freezing.

? TROUBLESHOOTING
Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Step	Problem	Possible reason	Solution
23, 61, 76	Low yield of pollen	The floral inflorescences contained a low number of developing spores	It is necessary to collect enough plant material before the separation
		An incorrect concentration was used in the preparation of the Percoll density solutions	Check the proper preparation of all Percoll solutions used for gradient formation
		Pollen grains were damaged during homogenization	Reduce the force applied to the pestle during homogenization

(continued)

TABLE 1 | Troubleshooting table (continued).

Step	Problem	Possible reason	Solution
49, 50, 51, 52, 67, 68, 69	The obtained fractions are indistinct or fused together after separation	Too much plant material was loaded onto the gradient	Decrease the amount of collected plant material to below 16 g. Loading 4–6 g or less onto one Percoll gradient improves the separation
		The Percoll layers of different density are mixed together	Take more care during the Percoll gradient formation. The pressure on the pipette dispense button should be as low as possible (the flow rate should be <0.5 ml per 30 s)
		The braking speed is too fast	Reduce the braking centrifugation speed to value 0
57, 58, 73	The purified material does not pellet well	The Percoll was not sufficiently dissolved in cell suspension	The isolated spore fraction must be diluted with the 0.1 M D-mannitol in a ratio of at least 1:2 (one part of the removed Percoll layer after separation:two parts of D-mannitol)

● TIMING

Steps 1–4, preparation of the plant material: 90 min
Steps 5–7, harvesting of the *Arabidopsis* flower inflorescences: 90 min
Steps 8–20, collection of MPGs of *A. thaliana* in a liquid medium: 90 min
Steps 21–23, assessment of MPG fraction purity by fluorescence microscopy (DAPI): 30 min
Step 24(A), cell viability test (Alexander staining): 30 min
Step 24(B), cell viability test (FDA/PI staining): 30 min
Step 25, preparation of Percoll step-density gradients A (75%/65%/20%/10%) and B (55%/45%/35%): 30 min
Steps 26–39, homogenization of the flower inflorescences: 45 min
Steps 40–47, sample loading: 30 min
Step 48, Percoll gradient fractionation on the 75%/65%/20%/10% gradient (gradient A): 5 min
Steps 49–57, collection of separated fractions A1, A2 and A3: 20 min
Steps 58–63, processing of A1 and A3 fractions: 15 min
Steps 64–66, Percoll gradient fractionation of A2 fraction on the 55%/45%/35% gradient (gradient B) including sample loading: 10 min
Steps 67–72, collection of separated fractions B1, B2 and B3: 20 min
Steps 73–78, processing of B1, B2 and B3 fractions: 15 min

ANTICIPATED RESULTS

To evaluate the purity of individual separated fractions, nuclei of isloated spore populations were stained by DAPI (**Fig. 2a**–**e**)[23]. After separation on a Percoll gradient 75%/65%/20%/10% (gradient A, **Fig. 6a**–**d**), three different fractions were obtained—A1, A2 and A3 (**Fig. 6c**,**d**). Fraction A1 (**Fig. 7a**) is composed of UNM. The purity of this fraction is often lower than that of fraction B1 (**Figs. 2a** and **7b**), which also contains microspores. Therefore, it is advisable to process these fractions separately. Fraction B1 is obtained after the separation of diluted fraction A2 on Percoll step gradient B (55%/45%/35%) (**Fig. 6e**–**h**). Fraction B2 contains the mixture of late UNM and early BCP (**Figs. 2b** and **7b**). The centrifuge-based separation of these stages is not feasible because of their very similar sedimentation characteristics. On the other hand, fraction B3 contains mostly BCP (**Figs. 2c** and **7b**). TCP forms fraction A3, the lowest band after the separation on gradient A (**Figs. 2d** and **7a**). The purity of all fractions was at least 70%. However, the purity of most fractions was higher, ranging between 75 and 85%. It is possible (but highly unlikely) to obtain highly pure fractions (i.e., >90%) pertaining to the method principle (rate zonal centrifugation). The purity of the MPG suspension after its collection in liquid medium was also assessed by DAPI staining (**Fig. 2e**).

The yield of separated spores ranged from 10 mg to 30 mg in each fraction. This amount of plant material was obtained after using 4 g of flower inflorescences per Percoll gradient, which substantially reduced the total protocol time.

During our project, dedicated hormonal profiling was carried out and analyzed for each stage of MG development (UNM, BCP, TCP and MPG) by high-performance liquid chromatography coupled with mass spectrometry (HPLC/MS). All of these materials were obtained by the Percoll separation protocol, and for each analyzed developmental stage, a different level of biologically active substances connected to the auxin metabolism was detected. In pursuance of these results, it can be stated that this separation protocol yields a sufficient amount of viable cells to be used in a downstream biological application (**Table 2**). The advantage of this protocol is that it can be scaled up or down should a larger or lower number of fractions be required.

TABLE 2 | The levels of phytohormones in isolated fractions enriched for different male gametophyte developmental stages compared with a diploid sporophytic tissue (from seedlings) in *Arabidopsis thaliana* Col-0.

Fraction	IAA (pmol/gFW)	IAA-Asp (pmol/gFW)	OxIAA (pmol/gFW)	OxIAA-GE (pmol/gFW)	IAN (pmol/gFW)
B1 (UNM)	26.72	1.46	26.80	7.19	11.21
B2 (late UNM and early BCP)	12.91	1.81	20.87	3.84	1.92
B3 (late BCP)	12.30	0.54	13.48	9.24	4.45
A3 (TCP)	14.40	0.23	11.95	23.99	5.12
MPG	10.47	0.39	22.44	5.79	24.32
18-day-old seedlings	67.07	13.78	125.23	1652.47	709.70

BCP, bicellular pollen; IAA, indole-3-acetic acid; IAA-Asp, indole-3-acetyl-aspartate; IAN, indole-3-acetonitrile; OxIAA, oxindole-3-acetic acid; OxIAA-GE, oxindole-3-acetyl-glucose ester; TCP, tricellular pollen; MPG, mature pollen grains; pmol/gFW, pmol per gram of fresh weight; UNM, uninucleate microspore.

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