

# Optimized Methods for the Isolation of *Arabidopsis* Female Central Cells and Their Nuclei

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The *Arabidopsis* female gametophyte contains seven cells with eight haploid nuclei buried within layers of sporophytic tissue. Following double fertilization, the egg and central cells of the gametophyte develop into the embryo and endosperm of the seed, respectively. The epigenetic status of the central cell has long presented an enigma due both to its inaccessibility, and the fascinating epigenome of the endosperm, thought to have been inherited from the central cell following activity of the DEMETER demethylase enzyme, prior to fertilization. Here, we present for the first time, a method to isolate pure populations of *Arabidopsis* central cell nuclei. Utilizing a protocol designed to isolate leaf mesophyll protoplasts, we systematically optimized each step in order to efficiently separate central cells from the female gametophyte. We use initial manual pistil dissection followed by the derivation of central cell protoplasts, during which process the central cell emerges from the micropylar pole of the embryo sac. Then, we use a modified version of the Isolation of Nuclei TAGged in specific Cell Types (INTACT) protocol to purify central cell nuclei, resulting in a purity of 75-90% and a yield sufficient to undertake downstream molecular analyses. We find that the process is highly dependent on the health of the original plant tissue used, and the efficiency of protoplasting solution infiltration into the gametophyte. By isolating pure central cell populations, we have enabled elucidation of the physiology of this rare cell type, which in the future will provide novel insights into *Arabidopsis* reproduction.

## INTRODUCTION

Double fertilization occurs specifically during angiosperm reproduction. Each of the two sperm cells, egg and central cells

harbor genetic and epigenetic footprints for development of the next generation. Upon fertilization, the egg cell develops into the embryo, and the central cell into the embryo-nourishing endosperm. Whilst the central cell and endosperm do not contribute genetic material directly to the embryo, the endosperm has a unique epigenetic profile, hypomethylated genome-wide, compared to the embryo. This hypomethylated state is required for gene imprinting and proper endosperm development, without which embryo development fails and the seed aborts. The DEMETER DNA glycosylase protein is expressed specifically in the central cell, and is required for endosperm hypomethylation, gene imprinting and seed development. As such, it is strongly suspected that the genome-wide hypomethylation of the endosperm is inherited from the precursor central cell. However, buried deep within the female gametophyte, central cell isolation has not previously been possible.

The presence of a cell wall renders many molecular techniques routine in other organisms highly challenging for normal plant cells. However, first reported in 1960 (Cocking, 1960) was the successful isolation of viable plant cells surrounded only by a plasma membrane, so-called protoplasts. Protoplasts behave similarly to animal cells *in vitro*, providing a versatile cell-based experimental system. As such, bacteria, cell organelles, and macromolecules such as DNA, RNA and proteins can be easily delivered to protoplasts using a variety of methods including PEG-calcium fusion, electroporation and microinjection (Chen et al., 2015; Ohyama et al., 1972; Yoo et al., 2007). Efficient systems for transient gene expression using protoplasts have been developed for a wide range of plant species including *Arabidopsis* (Im and Yoo, 2014; Schapire and Lois, 2016; Yoo et al., 2007) tobacco (Fischer and Hain, 1995), maize (Sheen, 2001), rice (Zhang et al., 2011) and even *Brachypodium* (Hong et al., 2012). However, most protoplasting techniques are based on isolation of cells from the leaf mesophyll or young seedlings (Zhai et al., 2009) and are not appropriate for isolation of inaccessible and rare cells, such as those within the female gametophyte (Chen et al., 2015; Faraco et al., 2011).

Laser capture microdissection (LCM) and fluorescence-activated cell sorting (FACS) provide alternative strategies to study specific cell types, however, both methods use harsh treatment conditions that likely alter cellular physiology during isolation, require highly complex and expensive equipment, and offer a relatively low yield and purity of target cells (Deal and Henikoff, 2011). To overcome these problems, the Isolation of Nuclei TAGged in specific Cell Types (INTACT) method has

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been developed (Deal and Henikoff, 2010; 2011). Nuclei are affinity-labeled through transgenic expression of a biotinylated nuclear envelope protein in the cell type of interest. Highly pure populations of transgenically tagged nuclei can then be isolated in large quantities using streptavidin-coated magnetic beads, allowing genomic and epigenomic profiling (Deal and Henikoff, 2011). The only limitation of INTACT, therefore, is the requirement for a known cell-type specific promoter and the time to generate transgenic plants.

Even with a technique such as INTACT, the isolation of angiosperm reproductive cells is not trivial, since they are embedded deep inside the gametophytes, which are additionally contained within sporophytic tissues. Enzymatic procedures for the isolation of female gametes or embryo sacs have been described for several plant species including *Zea mays*, *Lilium longiflorum*, petunia and *Alstroemeria* (Hoshino et al., 2006). Whilst *Arabidopsis* is a powerful model for flowering angiosperms, the microscopic size and delicacy of its reproductive tissues has meant that the preparation of protoplasts from ovules, and further isolation of the central cell and their nuclei has not yet been reported.

Central cell molecular biology is instructive for both egg cell physiology and development of the endosperm, and as such its analysis is key to understanding flowering plant reproduction. Here, we report an optimized and detailed protocol for the isolation of highly enriched *Arabidopsis* central cell protoplasts, as well as subsequent central cell nuclei, using INTACT assays, thus facilitating future genomic and epigenomic profiling of this highly important cell type.

## MATERIALS AND METHODS

### Growth of plants

*Arabidopsis* plants were grown on soil in either a greenhouse or in an environmentally-controlled room at 22-25°C with a long photoperiod (16 h of light / 8 h of dark). Plants were carefully monitored and grown across several greenhouse chambers, in order to minimize the effect of pest infections, which were highly detrimental to this experiment. Having ascertained the specificity of the DD7 promoter to central cells, we generated *DD7::NTF* and *DD7::BirA* plants based on Deal and Henikoff (2011) and made double homozygous plants for both constructs.

### Generation of transgenic plants

To specifically isolate central cell nuclei, we used a modified version of INTACT (Deal and Henikoff, 2010). This method needs Nuclear Targeting Fusion protein (NTF) and BirA under the same specific promoter. The NTF consists of three parts: 1) the WPP domain of *Arabidopsis* RAN GTPase activating protein 1 (RanGAP1, locus At3g63130; amino acids 1-111, inclusive), which is necessary and sufficient for the association with the nuclear envelope in plants (Rose and Meier, 2001), 2) green fluorescent protein (GFP) for visualization, and 3) the biotin ligase recognition peptide (BLRP), which serves as a substrate for the *E. coli* biotin ligase BirA. Thus, the expression of BirA and NTF in the same cell type produces biotin-labeled nuclei exclusively in those cells. We chose the DD7 promoter for central cell-specific expression (Steffen et al., 2007) and generated *DD7::NTF* and *DD7::BirA* plants. These two transgenic plants were then crossed to produce double homozygous plants.

### Protoplasting solution

Composition and proprietary product information for the protoplasting enzyme solution is as described by Yoo et al. (2007),



**Fig. 1.** Manual pistil dissection. Dissecting the pistils, whilst maintaining individual ovule integrity is important to allow sufficient exposure of intact ovules to the enzyme solution (A) Using the sharp tip of the forceps, pistils are opened along the red line under a stereoscopic microscope. (B) An open pistil with intact ovules. In this state, the pistil is placed into the enzyme solution. It is not necessary to separate each ovule from the pistil at this stage since other parts of the pistil will be filtered out by the 70  $\mu$ m mesh during protoplast isolation.

where leaf mesophyll cells were isolated, except for the following modifications; we tested the concentration of each protoplasting solution component to identify the optimal solution for central cells, which was 20 mM MES (4-morpholineethanesulfonic acid), pH 5.7, 1.25% cellulase R10, 0.1% pectolyase, 1% hemicellulase, 2.3% pectinase, 0.4 M mannitol and 20 mM KCl. Before the addition of enzymes and  $\text{CaCl}_2$ , the MES solution containing mannitol and KCl was preheated at 70°C for 5 min to resolubilize any crystals. The solution was then allowed to cool to room temperature, and enzymes added followed by incubation of the solution at 55°C for 10 min, enhancing enzyme solubility and to inactivate DNases and proteases. 10 mM  $\text{CaCl}_2$  was then added after the solution reached room temperature. The final enzyme solution was filter sterilized by syringing through a 0.45  $\mu$ m membrane. 6 ml of the enzyme solution was then added to each well of a 6-well plate, with each well already containing a 70  $\mu$ m cell strainer (CELLSTAR EASYstrainer, Greiner Bio-One, cat. no. 542070) to filter out the larger pistil debris following dissection.

### Emasculatation

The developmental stage of the inflorescences used to generate protoplasts is key to the success of this technique. Mature siliques and open-flowers with bursting pollen were removed from 6 to 8-week-old plants using fine scissors or forceps (Fine Science Tools, Inox Fine Forceps, Dumont #5). Inflorescences were then immobilized under a stereomicroscope with 10 to 20  $\times$  magnification. Healthy, unopened floral buds specifically at flower stage 12, which have yellow but non-bursting pollen, were chosen for emasculatation (Boyes et al., 2001). Single healthy inflorescences usually have 2-3 buds at the ideal stage. Using forceps, we removed all of the floral organs except the pistil from each bud. The number of emasculatations to be per-

formed in total for each experiment was calculated based on the ability of the researcher to perform the following rate-limiting pistil dissection step, with a given number of buds, within the period available to complete the experiment. Typically for us this meant 100 emasculations per experiment, with two experiments possible per day, however, with additional researchers this number could be increased. Emasculated plants were then incubated in an environmentally-controlled room or growth chamber at 22°C with 60% humidity under long-day photoperiods (16 h light, 8 h dark) for 24 hours to allow them undergoing the final stages of gametophyte development.

### Pistil dissection and protoplast isolation

Emasculated pistils were cut from the plants and each pistil was dissected by hand using forceps under the stereomicroscope (Fig.1), to allow access of the enzyme solution to the ovules. Each dissected pistil was transferred gently into the enzyme solution and completely submerged with the forceps. Vacuum infiltration was used to maximize uptake of the enzyme solution by the ovules. The samples were placed in a vacuum desiccator chamber at 40-50 kPa for 150 min in the dark. Following this, samples were incubated in the enzyme solution for three hours. Shaking was not used since we found that shaking the sample, and incubations of more than three hours, gave rise to an increase in non-target protoplasts without concomitant increases in central cell yield. After diluting the enzyme/protoplast solution with an equal volume (6 ml) of 0.45  $\mu$ m syringe filter-sterilized W5 solution (2 mM MES, pH 5.7, 154 mM NaCl, 125 mM CaCl<sub>2</sub> and 5 mM KCl) (Yoo et al., 2007), the 70  $\mu$ m cell strainer was removed and the solution containing the protoplasts transferred into a 50 ml tube. Protoplasts were gently pelleted by centrifugation for five minutes at 200 g and the supernatant was removed.

### Nuclei purification and INTACT

Composition of the protoplast lysis buffer and concentration of each reagent were based on a previously published protocol (Sheen, 1993), which was originally designed for obtaining the nuclei fraction from maize mesophyll protoplasts. We optimized the protoplast lysis buffer for isolating central cell nuclei from central cell protoplasts as we had for the protoplast enzyme solution, testing the optimal concentration of each component. Among the tests, lysis buffer with 1 mM MgCl<sub>2</sub> concentration gave us more intact nuclei than that with 2.5 mM MgCl<sub>2</sub> used in the original paper. The addition of 30 mM beta-mercaptoethanol in the lysis buffer did not alter the nuclei purity and yield in our hands, so we omitted this. Therefore, our optimized protoplast lysis buffer was: 20 mM Tris-HCl, pH 7, 250 mM sucrose, 25% glycerol, 20 mM KCl, 2 mM EDTA, 1 mM MgCl<sub>2</sub>, 1X protease inhibitor cocktail (Roche: 11873580001), 1% Triton X-100.

During optimization of the nuclei resuspension buffer, we found that adding Triton X-100 to a final concentration of 0.1%, which was not in the original paper, significantly removed off-target binding in following the INTACT procedure, thereby increasing the purity of central cell nuclei in the final product. Therefore, our optimized nuclei resuspension buffer was: 20 mM Tris-HCl, pH 7, 25% glycerol, 1 mM MgCl<sub>2</sub>, 1X protease inhibitor cocktail (Roche: 11873580001), 0.1% Triton X-100.

One milliliter of the protoplast lysis buffer was added to the prepared pellet of central cell protoplasts, breaking the protoplasts and allowing simultaneous nuclei purification. After incubating the samples on ice for 8 min, the following steps were performed in a 4°C cold room: the sample was centrifuged at

200 g for 5 min and the supernatant was discarded, the pellet was resuspended by gentle inverting with 1 ml of the nuclei resuspension buffer.

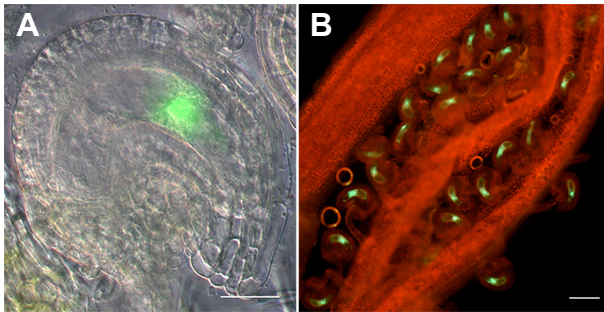
Following nuclei resuspension, clumps of non-target cells were present in the solution, as identified by their lack of GFP fluorescence and non-central cell nucleus appearance. We found that these were still present following antibody binding of the sample and subsequent purification with Dynal Protein G beads (Invitrogen: 100-03D). Since these cell clumps did not express GFP, and following close observation of them under the microscope, we hypothesized that their irregular structure may allow their non-specific attachment to the beads. We therefore incorporated a step to add magnetic Dynal Protein-G beads to sequester and remove these non-specific cell-clumps, prior to incubation of the sample with the GFP antibody.

The sample was rotated for 2 min to allow binding of the non-target cell clumps to the Protein-G beads, then placed on a magnet for 7-9 min to remove beads from the supernatant fraction. The supernatant (containing central cell nuclei) was then transferred to a 1.5 ml tube containing 1  $\mu$ g GFP antibody (Invitrogen: G10362) in 0.1 ml nuclei resuspension buffer. The sample was incubated for 10 min with rotation to bind the antibody to the nuclei. Following this, Protein G beads were added to the sample in order to capture the GFP-labeled central cell nuclei, and the sample was then rotated for another 10 min to allow the beads to bind the antibody. The solution was then diluted by gentle inversion in 9 ml nuclei resuspension buffer in a 15 ml tube. In order to pellet the beads, the 15 ml tube containing the sample was placed on a magnet for 8 min. Keeping the tube in place on the magnet, the supernatant was removed slowly and discarded. The sample was then removed from the magnet and washed by adding 10 ml nuclei resuspension buffer, and the beads were resuspended by shaking gently. Again, the tube was placed on a magnet for 8 min before the supernatant was removed and discarded. The beads were washed a second time by adding 1.5 ml nuclei resuspension buffer, resuspending by gentle shaking. The sample and beads were then transferred to a 1.5 ml tube and rotated for 1 min. To check the yield and the purity of the sample, 300  $\mu$ l (1/5 of total volume) of this sample was placed into a new 1.5 ml for further analysis as follows: The tube was placed on a magnet for 5 min and excess supernatant was removed from the pelleted beads. The remaining sample was pipetted onto a glass slide and a cover slip laid gently on top. Under a fluorescence microscope, the total number of nuclei and the proportion of GFP-positive nuclei were counted, thereby assessing the purity of the sample. The tube containing the remaining 1.2 ml sample was placed on a magnet for 5 min to pellet the beads. After removing and discarding the supernatant, the bead pellet was finally resuspended in 4  $\mu$ l of nuclei resuspension buffer, and stored at -80°C.

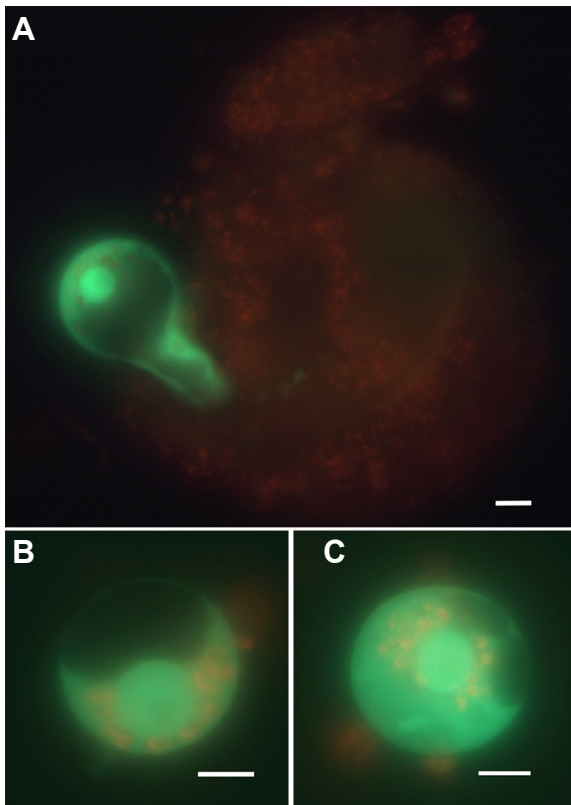
## RESULTS

### The DD7 promoter is active in the central cell specifically

We chose the DD7 promoter (Steffen et al., 2007) and verified its specific activity in central cells by visualizing GFP expression. Figure 2 shows the specific expression of GFP in the central cells of developing ovules, but not in any surrounding tissue. Since our isolation involved manual dissection, and therefore unavoidable contamination with surrounding pistil tissues, the lack of GFP expression in these tissues meant that we could be confident that GFP expressing cells were our target cell type, and that the DD7 promoter is appropriate to use for this procedure.



**Fig. 2.** DD7::GFP expression is found in the central cell nucleus only. Thus we are able to use the whole pistil as an input tissue and all of the GFP fluorescent nuclei can be considered as central cell nuclei. (A) An individual ovule, showing the location of GFP fluorescence in the central cell within the embryo sac, and an absence of staining of other surrounding cell types (GFP and bright field merged) (B) Fluorescent image of part of a pistil prior to dissection from a plant homozygous for the DD7::NTF transgene showing again that GFP fluorescence is confined to the central cells only, and is not present in any other tissue. Scale bars = 25  $\mu$ m (A), 100  $\mu$ m (B).



**Fig. 3.** Emergence of GFP-positive central cell protoplasts from within the embryo sac. (A) Central cell protoplasts tend to be released from the ovule through the micropylar pole of the embryo sac. (B, C) Two isolated central cell protoplasts. Central cell protoplasts are perfectly round and contain the characteristically large central cell nucleus. Scale bars = 10  $\mu$ m.

### Manual and enzymatic ovule degradation do not allow isolation of central cells containing nucleus

Extracting an intact central cell from the ovule was the first challenging step during the isolation procedure. During female gametophyte development, haploid female gamete cells are buried deep inside the embryo sac, as they become surrounded by multiple layers of integument (Truernit and Haseloff, 2007; Yadegari and Drews, 2004). Following as much as 24 hours incubation with our degradation enzyme solution, the structure of the ovule remained almost completely intact. Thus, integument cell layers cause considerable resistance to enzyme solution penetration. To disrupt the integuments, we tried manual grinding of the ovules using a pestle and mortar, both with and without liquid nitrogen, as per previous reports (Deal and Henikoff, 2010; 2011). However, this technique resulted in degradation of central cell nuclei and loss of GFP expression. The susceptibility of central cell nuclei to disruption by pestle may be due to its relatively large size (8-15  $\mu$ m) compared to the root cell nuclei (3-5  $\mu$ m) that had been successfully isolated using the original INTACT technique (Deal and Henikoff, 2011). In addition, it may be also more fragile.

### Protoplasting enables isolation of GFP positive central cell protoplast

In order to isolate intact GFP positive central cell nuclei, we opted to introduce an intermediate protoplasting step. Ovule integuments and leaves exhibit similarities and share a number of features at the morphological, molecular and structural levels, such as determinant growth, bilateral symmetry, organ initiation and overlapping transcriptional profiles (Kelley and Gasser, 2009; Lenhard et al., 2001; Long et al., 1996; Pillitteri et al., 2007). Therefore, we used an enzymatic solution as applied previously to isolate leaf mesophyll protoplasts (Yoo et al., 2007). This way, we sought to sufficiently break down the ovule structure to allow access to the central cell, whilst preserving individual nuclei in the form of protoplasts. Subsequently, our GFP labeling of central cell nuclei would allow us to specifically isolate these nuclei using INTACT.

During protoplasting, GFP fluorescence was visible throughout when we observed the sample. We tracked the location of the fluorescence, and were able to observe emergence of central cells from the micropylar end of the ovule (Fig. 3). Thus, while the overall structure of the ovule is maintained during enzymatic treatment, thanks to the micropylar pore, the micropylar end is more susceptible to degradation, allowing release of the central cell. Despite obtaining some complete central cells, however, we also observed many central cells that appeared to have been broken into non-specific small vesicles that did not contain distinct fluorescent nuclei. This suggested that either the hole made by the enzymes in the micropylar end was often too narrow to maintain central cell integrity, or, that the enzymes did not sufficiently infiltrate into the embryo sac to break the cell adhesions holding the central cell in place, resulting in partial-central cell vesicles without a nucleus.

### Pectinase and mannitol concentrations, as well as vacuum infiltration time, are key for the isolation of complete central cell protoplasts

Although we had successfully isolated central cell protoplasts, we next sought to improve our yield by optimizing their intact emergence from the embryo sac. A large number of central cells is required for successful downstream applications such as INTACT (Deal and Henikoff, 2010) or FACS (Bimbaum et al., 2005; Gronlund et al., 2012) in order to allow for losses that

**Table 1.** The yield of isolated central cell protoplasts increases with increasing vacuum infiltration time until reaching between 150 to 180 minutes, at which point yields begin to decrease.

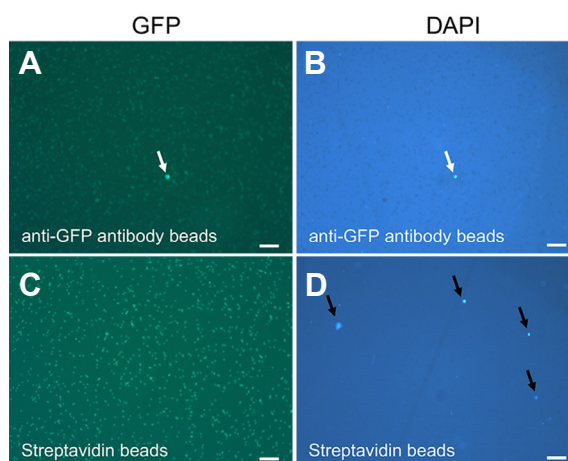
Vacuum time (min)	30	60	120	150	180
Yield* (%) of CC1	7.5	12	18.5		
Yield (%) of CC2			8.6	9.5	6.5

Yield = GFP protoplasts in the solution / input central cell number

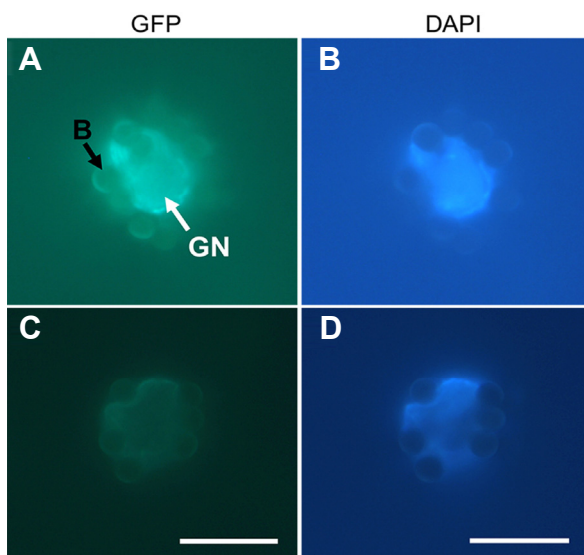
occur during each process. Our previous experiments had found that central cells were not completely released from the embryo sac, often leaving their nuclei behind in the ovule, likely due to incomplete infiltration of the enzyme solution to disrupt cell-to-cell adhesions. The diameter of the central cell is 35-50  $\mu\text{m}$  and its location reaches the chalazal end of the embryo sac. To obtain intact central cells, it was therefore key for the enzyme solution to sufficiently infiltrate the embryo sac as far as possible. In order to achieve this, we used vacuum infiltration (40-50 kPa), which has been used previously to infiltrate enzyme solutions to make leaf mesophyll protoplasts (Bechtold and Pelletier, 1998; Yoo et al., 2007). The use of this method was successful, and in fact we were unable to achieve a central cell yield of more than 2% without vacuum infiltration. We assessed the effect of vacuum infiltration time on the yield of isolated central cells (Table 1). Initially testing 30 min, 60 min and 120 min vacuum infiltration time with healthy plant tissue, we obtained the highest yield of central cell protoplasts at 120 min. For the second trial, the plant conditions were not as good, and we found the optimal time from 120 min, 150 min and 180 min to be 150 min. We therefore used the 150 min incubation time for our experiments.

Next, we systematically altered the buffer components of the protocol from Yoo et al. (2007) to optimize the derivation of central cells, notably finding that BSA slightly reduced central cell protoplast yield, so we omitted BSA in the enzyme solution. The variation in enzyme concentrations did not significantly increase the yield of central cell protoplasts, except for when we varied the concentration of pectinase. The main role of pectin is to provide a tensile strength to plant cell walls, providing a barrier against the outside environment (Harholt et al., 2010). When we compared 1.25-1.5% and 2.3% pectinase concentrations (for a fixed 30 min vacuum infiltration treatment), the isolated central cell yield increased with the pectinase concentration, being highest in the 2.3% concentration condition (data not shown). Hereafter, we used a 2.3% pectinase concentration.

The addition of osmotic pressure protective agents, such as mannitol, is also essential in maximizing protoplast release from plant tissues. Many studies suggest that the optimum concentration of osmotic pressure protective agents can vary according to species and even cultivar (Wu et al., 2009; Zhang et al., 2011). The effective osmotic concentration is also dependent on the endogenous cell osmotic pressure at the time of isolation, which are markedly influenced by environmental growth conditions and the age of donor tissues (Shepard and Totten, 1977). As such, we thoroughly examined the effect of varying mannitol concentrations. 0.7 M mannitol in the enzyme solution increased the central cell protoplast yield from ovules in comparison to 0.4-0.6 M mannitol concentrations. However, central cell protoplasts obtained using 0.7 M mannitol were shrunken and more fragile. We found that these shrunken protoplasts



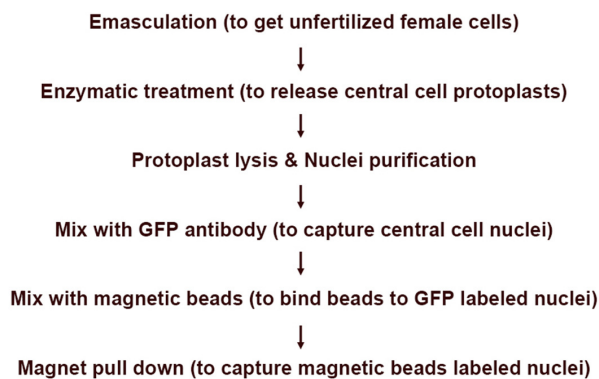
**Fig. 4.** The purity of central cell nuclei obtained using anti-GFP antibody beads to purify the sample is higher compared to when using streptavidin beads. All four panels show example fluorescent microscope images of the final product following isolation of central cell nuclei using magnetic beads. (A) and (B) show the purity when using anti-GFP antibody beads after magnetic purification using GFP and DAPI filters, respectively. In (A) one GFP positive spot (white arrow) can be seen amongst background non-stained beads, shown to be a nucleus by the corresponding spot in B (white arrow), the DAPI filter. In contrast, (C) and (D) show the purity when using streptavidin-bound magnetic beads to isolate the central cell nuclei. Whilst four nuclei are visible in the DAPI pane (D) (black arrows), there are no corresponding GFP positive nuclei in (C), with only non-stained beads being visible (with high exposure), showing that non-GFP positive nuclei have been isolated using the streptavidin beads. Scale bars = 50  $\mu\text{m}$



**Fig. 5.** GFP positive central cell nuclei are efficiently bound by anti-GFP antibodies, which in turn successfully binds magnetic beads. (A) and (C) show two GFP fluorescent central cell nuclei labelled with beads, and their DAPI images, (B) and (D), respectively. Only the nuclei labelled with anti-GFP antibody beads can be captured with the magnet. As the central cell nuclei are the only GFP fluorescent nuclei in the input tissues, all of the nuclei that are GFP fluorescent are central cell nuclei. GN, GFP fluorescent central cell nuclei; B, beads. Scale bars = 10  $\mu\text{m}$ .

**Table 2.** An example set of central cell nuclei collections, listing the total number of central cell nuclei potentially isolated over five experiments for batch 1 and six experiments for batch 2, with corresponding purity levels. The number of GFP fluorescent nuclei, which represent the central cell nuclei, were counted amongst the total nuclei.

Nucleus type	Total central cell nuclei	Total nuclei	Purity
Central cell nuclei 1	163	187	87%
Central cell nuclei 2	155	191	81%



**Fig. 6.** An overview flowchart describing isolation of central cells and their nuclei.

pared much less well in subsequent nuclei isolation step compared to those protoplasts obtained using the 0.4 M mannitol solution. Therefore, we utilized 0.4 M mannitol concentration in order to preserve protoplast integrity. This way, we were able to obtain central cell nucleus yields of up to 25%, with total input calculated using the original number of ovules harvested.

#### GFP antibodies were superior to streptavidin for pure central cell nuclei isolation

Having successfully isolated central cell protoplasts, we first tried to carry out FACS in order to purify the central cell population away from the other contaminating cells of the ovule. However, we failed to purify GFP fluorescent central cells using FACS, even using a larger FACS nozzle to account for the large size of the central cell nucleus. Therefore, we decided to use INTACT to isolate central cell nuclei. When we tried the original INTACT method [as described in detail (Deal and Henikoff, 2011)], either the unique physiology of the central cell, or its low ratio (0.02%) amongst the other cells in the tissues we were dissecting, meant we failed to isolate sufficient central cell nuclei for analysis. As described in “Materials and Methods”, we optimized each buffer component, manual step and procedure of the process to maximize central cell nuclei yield, resulting in a successful protocol for isolating central cell nuclei at a reasonable rate. Following this, however, we found that the use of streptavidin beads caused many other non-targeted nuclei to be captured along with the central cell nuclei, greatly reducing the purity of the sample (Fig. 4). Since each INTACT transgene includes a GFP tag, it is alternately possible to pull-down labeled cells using anti-GFP-antibody-bound beads (Fig. 5)

(Henry et al., 2012). The use of this alternative pull-down strategy, although it modestly reduced our yield compared to using streptavidin, enabled us, along with our systematically optimized protocol, to routinely obtain central cell nuclei isolates at 70-90% purity (Fig. 4 and Table 2). In our hands, each central cell nuclei isolation experiment yielded between 4 to 85 central cell nuclei in the final isolate, although the number of central cell nuclei we could obtain was heavily dependent on the health of the *Arabidopsis* plants. We found that the yield of central cell nuclei obtained using this method was very sensitive to the infection status of the plants. Many insect pests (notably aphids, thrips and spider mites) gather around flower buds, and even low levels of infection reduced the central cell nuclei yield significantly.

## DISCUSSION

Angiosperm reproduction is a complex process involving unique epigenetic changes that dictate not only the subsequent development of the seed, but also likely transgenerational protection of the genome from harmful transposon activation. The glycosylase protein DEMETER (DME) is central to this process, and acts to demethylate DNA genome-wide, without which seed abortion occurs due to a loss of gene imprinting (Choi et al., 2002; Ibarra et al., 2012; Kim et al., 2008). The role of DME-mediated demethylation of the companion cells of the gametes; the central cell of the ovule and vegetative cell of pollen, in this process has been demonstrated in the male gametophyte. In the vegetative cell, transposable elements demethylated by DME are expressed, which in turn generates a signal, likely via RNA-directed DNA methylation, resulting in methylation of those same transposons in sperm (Ibarra et al., 2012; Slotkin et al., 2009). Male gametophytes comprise pollen grains, which are abundant and accessible, enabling its relatively straightforward access for experimentation and analysis.

In contrast, the female gametophyte is embedded deeply within sporophytic tissues. As such, the comparable analysis of female gamete epigenetic regulation has so far been impossible, and our knowledge of egg and central cell epigenetic status inferred from their downstream differentiated tissues, the embryo and endosperm. To date, genome-wide DNA methylation levels of the embryo and the endosperm (Gehring et al., 2009; Hsieh et al., 2009; Ibarra et al., 2012) and genome-wide H3K27 trimethylation levels of the embryo and the endosperm (Moreno-Romero et al., 2016; Weinhofer et al., 2010) have all been published. Genome-wide transcriptional profiles of the female gamete cells have been generated by isolating these tissues using Laser-Capture Microdissection techniques (LCM) (Schmidt et al., 2012; Wuest et al., 2010). However, LCM tissues have been fixed, a treatment which is likely to disrupt epigenetic processes within these cells, and in addition, provide a very low yield and limited purity, which is key when analysing a single cell type such as the central or egg cells (Deal and Henikoff, 2011).

When undertaking analysis of such a unique cell population as *Arabidopsis* gametes, it is important not only to consider the possible yield that can reasonably be obtained, where you have one cell buried within hundreds or thousands of non-target cells, but also the purity with which they can be obtained. The procedure for optimizing our central cell isolation focused on optimizing the maximum possible yield of central cell protoplasts, to increase the chances of meaningful downstream applications, but was constantly limited by our need for the highest possible purity. Several steps found ways to modestly increase our yield,

such as using alternate protoplasting solutions, the use of streptavidin beads, or reducing the number of magnetic bead wash steps, for example. However, in each of these cases a concurrent sacrifice in the purity of central cell nuclei in the final isolate meant that we had to change these steps, trading a slightly lower yield for a more pure product.

The technique we have pioneered to isolate high central cell populations and pure central cell nuclei from live plants will allow future genomics studies to delineate the epigenetic and transcriptional profiles of *Arabidopsis* central cells. This will allow us to understand the biology of mature female gamete cells before fertilization. By comparing future data generated from analysis of the gamete cells obtained by our method, to published data from embryo and endosperm, we may be able to elucidate the genetic and epigenetic changes that occur throughout fertilization (Hsieh et al., 2009; 2011; Ibarra et al., 2012). In addition, comparison of the central cell to the male companion cell, the vegetative cell, will allow us to draw parallels, or distinctions, between these two specific populations of DME expressing cells, helping to delineate the function of this protein in *Arabidopsis* reproduction.

This protocol is highly optimized specifically for central cells, and in our hands does not successfully allow isolation of egg cells. This may be due to the different size or the different biology that exists between central and egg cells. However, our optimization of this protocol for central cells and their nuclei has provided knowledge of the steps in these experiments at which variation provides changes in yield or efficacy, according to specific attributes of the target cell population. This facilitates not only the design of protocols for possible protoplasting and derivation of egg cells, but also the derivation of protoplasts from other rare cell types. In the future, we will optimize this technique for the novel isolation of egg cells and very early endosperm and embryo, in order to provide a comprehensive overview of the molecular biology throughout *Arabidopsis* fertilization and early embryogenesis.

Here, we have extensively modified existing leaf protoplasting methods and the root INTACT method to derive a successful method for obtaining pure populations of central cell nuclei, for the first time allowing access to the genome of the female gamete companion cell.

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