

Scanning Electron Microscopy of Motile Male Gametes of Land Plants

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[Abstract] The only motile cells produced in land plants are male gametes (spermatozoids), which are reduced to non-flagellated cells in flowering plants and most gymnosperms. Although a coiled architecture is universal, the complexity of land plant flagellated cells varies from biflagellated in bryophytes to thousands of flagella per gamete in the seed plants *Ginkgo* and cycads. This wide diversity in number of flagella is associated with vast differences in cell size and shape. Scanning electron microscopy (SEM) has played an important role in characterizing the external form, including cell shape and arrangement of flagella, across the varied motile gametes of land plants. Because of the size and scarcity of released swimming sperm, it is difficult to concentrate them and prepare them for observation in the SEM. Here we detail an SEM preparation technique that yields good preservation of sperm cells across plant groups.

Keywords: Flagella, Male gamete, Scanning electron microscopy, Spermatozoid

[Background] Motile gametes of land plants are strikingly diverse and develop through transformations that involve repositioning cellular components and the assembly of a complex locomotory apparatus (Renzaglia and Garbary, 2001). Because of constraints imposed by cell walls, elongation of the cell and flagella is around the periphery of a nearly spherical space, resulting in a coiled configuration of the mature gamete. The degree of coiling varies from just over one to as many as 10 revolutions per cell. The number of flagella per gamete is even more variable, ranging from two in bryophytes (mosses, hornworts, and liverworts) to an estimated 1,000-40,000 in *Ginkgo* and cycads. Following the diversification of *Ginkgo* and cycads, all vestiges of basal bodies and flagella were lost in the remaining seed plants that utilize pollen tubes to deliver non-motile sperm to egg cells (Southworth and Cresti, 1997). Male gametes provide a wealth of biological information, including biodiversity and cell differentiation and evolution (Garbary *et al.*, 1993; Renzaglia *et al.*, 1995; Renzaglia and Garbary, 2001; Renzaglia *et al.*, 2000; Lopez-Smith and Renzaglia, 2008; Lopez and Renzaglia, 2014). Of the range of microscopic techniques utilized to characterize plant spermatozoids, SEM provides the most direct means of elucidating cell shape, and flagella number, length and arrangement. Together with TEM observations, SEM studies lead to comparative descriptions of gamete architecture, and organellar content and arrangement across plant lineages (Renzaglia *et al.*, 2001 and 2002; Lopez-Smith and Renzaglia, 2008).

Materials and Reagents

Scanning Electron Microscopy (SEM) and the motile sperm cell architecture

1. Pipette tips 1-200 μ l (Carolina Biological Supply, catalog number: 215055)
2. Pasteur pipettes (Fisher Scientific, catalog number: 13-678-4)
Manufacturer: Corning, catalog number: C7095B5X.
3. 1.5-ml centrifuge tubes (USA Scientific, catalog number: 1615-5500)
4. Glass coverslips 22 x 22 mm (Fisher Scientific, catalog number: 12-542B)
5. Male plants with mature sperm cells
 - a. *Pellia epiphylla*
 - b. *Conocephalum conicum*
 - c. *Equisetum arvense*
 - d. *Ceratopteris richardii*
6. Sorensens phosphate buffer, 0.2 M, pH 7.2 (Electron Microscopy Sciences, catalog number: 11600-10)
7. Glutaraldehyde (Electron Microscopy Sciences, catalog number: 16120)
8. Sodium cacodylate buffer, 0.2 M, pH 7.4 (Electron Microscopy Sciences, catalog number: 11652)
9. Osmium tetroxide (Electron Microscopy Sciences, catalog number: 19150)
10. Ethanol (Decan Laboratories, catalog number: 2705HC)
11. Hexamethyl disilizane (HMDS) (Electron Microscopy Sciences, catalog number: 16700)
12. 0.01 M phosphate buffer (pH 7.2) (see Recipes)
13. 0.05 M phosphate buffer (pH 7.2) (see Recipes)
14. 0.02 M phosphate buffer (pH 7.2) (see Recipes)
15. 2.5% glutaraldehyde (see Recipes)
16. 0.05 M sodium cacodylate buffer (pH 7.2) (see Recipes)
17. 2% aqueous osmium tetroxide (see Recipes)

Equipment

1. Scanning electron microscope (SEM) (FEI, model: QuantaTM 450 FEG)
2. Shaker (Thermolyne, model: M-16715)
3. Centrifuge (Thermo Fisher Scientific, Thermo ScientificTM, model: mySPINTM 6, catalog number: 75004061)
4. Oven (General Signal, model: Gravity convection)
5. SEM Specimen Mount Stubs, Aluminum Slotted Head (Electron Microscopy Sciences, catalog number: 75220)
6. Samdri 790 Critical Point Dryer (Tousumis)
7. Sputter coater (Denton Vacuum, model: Desk V)

Procedure

Scanning Electron Microscopy (SEM) and the motile sperm cell architecture:

SEM examination of mature sperm cells has enabled rapid visualization and comparisons of whole gamete architecture compared with laborious TEM observations and cell reconstructions. The diversity of gamete architecture and organization of flagella in mature cells is readily seen in the SEM (Figure 1).

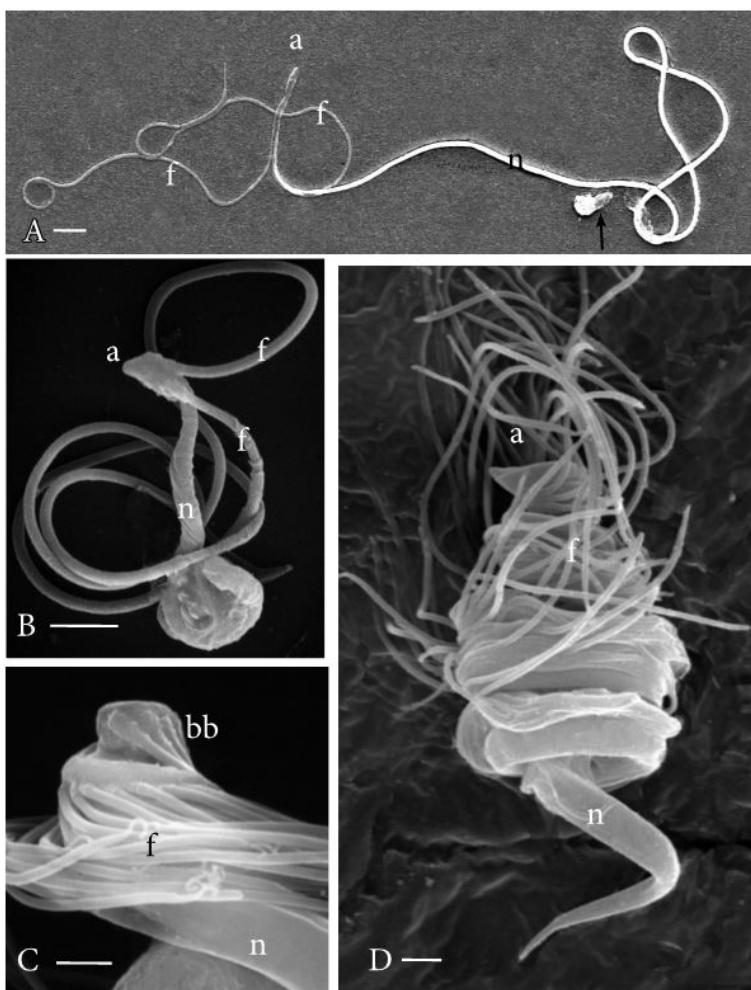


Figure 1. Scanning electron micrographs of plant sperm cells. A. *Pellia epiphylla*, a simple thalloid liverwort. This is one of the longest sperm cells produced by bryophytes. The two flagella (f) are staggered in their insertion near the cell anterior (a) and the cell body consists of a long cylindrical nucleus (n) with a plastid and mitochondrion at the posterior (arrow). B. *Conocephalum conicum*, a complex thalloid liverwort. This sperm cell is much smaller than that of *Pellia*. The two flagella (f) are inserted anteriorly (a) and are much longer than the cell body, which consists of a cylindrical nucleus (n) and posterior cytoplasm that in this case is not fully extended. C. *Equisetum arvense*, a eusporangiate fern. The approximately 36 basal bodies (bb) attach flagella (f) along the anterior coils of the cell. The nucleus (n) extends to the cell posterior

(not visible). D. *Ceratopteris richardii*, a leptosporangiate fern. This cell coils four times and has about 80 flagella (f) inserted anteriorly (a). The cylindrical nucleus (n) extends over three coils and tapers at the cell posterior. Bars = 5.0 μm (A), 1.0 μm (B, C, D).

1. Using excised male tissue (antheridia of male gametophytes), place several antheridia in each droplet (ca. 100 μl) of 0.01 M phosphate buffer, pH 7.2 (see Recipes), on a dental wax plate, cover to retard evaporation, and leave overnight at room temperature to facilitate release of sperm. Alternatively, excised antheridia can be placed in 1.5 ml Eppendorf centrifuge tubes adding 1 ml of 0.01 M phosphate buffer, pH 7.2 to completely cover tissue and left overnight. Antheridial tissue can be removed and discarded the next day. Swimming sperm will be in the buffer.
2. Remove tissue and place the droplet in a 1.5 ml Eppendorf centrifuge tube containing 1 ml 2.5% glutaraldehyde (see Recipes) in 0.05 M sodium cacodylate buffer, pH 7.2 (see Recipes).
3. Shake tube gently and fix the spermatozoid suspension for 1 h at room temperature.

Note: From this point on, spermatozoids are concentrated by centrifugation at 100 $\times g$ for 5 min between each solution change. Because the sperm are colorless, you will most likely not see a pellet after centrifugation.

4. Remove the glutaraldehyde supernatant down to 0.1 ml leaving the pellet at bottom of tube. Rinse fixed sperm cells three times (10 min each) in 1 ml 0.05 M sodium cacodylate buffer.
5. Post-fix tissue for 20 min in 1 ml 2% aqueous osmium tetroxide (see Recipes).
6. Rinse specimens twice in 1 ml double distilled autoclaved water (10 min), once each in 25%, 50% and in 75% ethanol (10 min), twice in 100% ethanol (10 min), and then twice in 100% hexamethyl disilizane (HMDS; 10 min).
7. Pipet sperm cells in 1 ml HMDS on to a glass coverslip affixed to a standard aluminum pin-type mounting stub and dry for 20 min in a 60 °C oven.
8. Specimens on mounting stubs are sputter-coated with an ultra-thin layer (ca. 390 nm) of a conducting metal (palladium–gold) that prevents charging from non-conducting biological specimens, increases the number of secondary electrons from the specimen surface that improves topographical contrast, and minimizes damage to the specimen.

Basic steps:

- a. Vacuum down sputter coater
- b. Out-gas with Argon gas
- c. Apply voltage
- d. Open gas to the specific emissions
- e. Coat with ca. 390 nm palladium–gold

9. View on a high-resolution SEM.

Note: Often cells aggregate around the edges of the coverslip, so it is better to put a very small drop of sperm cell suspension in HMDS in the middle of the coverslip to dry.

Recipes

1. 0.01 M phosphate buffer (pH 7.2)
Add 5 parts 0.20 M Sorenson's phosphate buffer (pH 7.2) to 95 parts double distilled autoclaved water
2. 0.05 M phosphate buffer (pH 7.2)
Add 25 parts 0.20 M Sorenson's phosphate buffer (pH 7.2) to 75 parts double distilled autoclaved water
3. 0.02 M phosphate buffer (pH 7.2)
Add 1 part 0.20 M Sorenson's phosphate buffer (pH 7.2) to 9 parts double distilled autoclaved water
4. 2.5% glutaraldehyde
 - a. Add 1 part 10% glutaraldehyde to 1 part double distilled autoclaved water
 - b. Add 1 part 0.20 M Sorenson's phosphate buffer (pH 7.2) to 1 part double distilled autoclaved water
 - c. Add 1 part 5% glutaraldehyde to 1 part 0.10 M Sorenson's phosphate buffer (pH 7.2)
5. 0.05 M sodium cacodylate buffer (pH 7.2)
Add 1 part 0.30 M sodium cacodylate buffer (pH 7.2) to 5 parts double distilled autoclaved water
6. 2% aqueous osmium tetroxide
Add 1 part 4% aqueous osmium tetroxide to 1 part double distilled autoclaved water

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