Fluorescent capable microscope equipped with DAPI filter and GFP or other capable fluorescent filter dependent upon staining.
Wilton Toolmaker Vise, Cat #: 11715 Penn Tools, Maplewood, NJ, USA.
Torque Wrench fitted with a hex nut attachment with a visible readout that can apply 15N of force.

Using scanning electron microscope for documenting Drosophilid sperm head morphology.

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Introduction

Morphological features of sperm heads can be used to hypothesize evolutionary relationships among species (Talarico *et al*., 2008; Meisner *et al*., 2005; Jamieson *et al*., 1999). We expect to find variability in drosophilid sperm head morphology, because there is already known variability in the sperm length. For example, *D. bifurca* has the longest sperm, approximately 6 cm (Pitnick *et al*., 1995; Joly *et al*., 1995). In addition the external and internal genitalic structures themselves vary and have traditionally been used for taxonomic and phylogenetic purposes (Patterson and Stone, 1952).

Drosophilid sperm have been investigated using bright field, fluorescence, and/or confocal microscopy (Desai *et al*., 2009; Snook *et al*., 2000; Joly *et al*., 1991; Shoup 1967). The mature sperm head has been reported as long and cylindrical, similar to a needle (Tokuyasu, 1974). The mature sperm head is approximately 0.5 - 0.3 microns, and the tail one fourth of this width (Shoup, 1967). These dimensions are actually the limits of bright field microscopy; therefore, it lacks the ability to resolve and identify fine structural differences in the surface morphology of sperm heads among species.

Transmission electron microscopy (TEM) has yielded images demonstrating difference in sperm maturation and cellular structure (Shoup, 1967). Sperm head surface structure can only be indirectly inferred using the TEM and would be better understood using the scanning electron microscope (SEM), which provides the required resolution along with surface imaging capabilities. The SEM has been used to image the large sperm balls of *D. bifurca*; however, the sperm head is not visible these images (Bjork *et al*., 2007; Bjork and Pitnick, 2006). This paper is the first to report on efforts to employ scanning electron microscopy (SEM) to document the morphology of drosophilid sperm heads.

Methods

The scanning electron microscope (SEM) sample preparation procedures are based upon the methods of Meisner *et al*. (2005), Michalik and Huber (2006), and Bjork and Pitnick (2006). Mature

sperm was isolated from male seminal vesicles of *D. simulans* obtained from the UC San Diego *Drosophila* Stock Center.

The freshly dissected seminal vesicle was placed in a drop of 0.1 M sodium phosphate buffer pH 7.2 (PB), on a cover glass coated with 0.1% poly L-lysine. The sperm were released from the seminal vesicle using homemade minuten pin probes. This is similar to procedures employed for SEM preparation of spiders (Michalik and Huber, 2006), *D. bifurca* (Bjork and Pitnick, 2006), and numerous light microscopy studies measuring sperm head and tail length (*e.g*., Joly *et al*., 1989; Joly and Lachaise, 1994). The sperm sample was allowed to incubate on the cover glass for 30 minutes in a humidified chamber, then rinsed by dipping in PB. The sample was then fixed in glutaraldehyde by placing a drop of 2.5% glutarldehyde in PB on the cover glass for one hour in a humidified chamber. The samples were rinsed three times in PB and subsequently dried in a series of ethanol washes (20%, 50%, 70%, 80%, 95%, 100%, 100%). The cover glass containing the sperm was critical-point dried in a Bal Tec CPD (model 030). The cover glass was mounted on an SEM stub and sputter coated with gold/palladium. Specimen was viewed and images were taken using an Hitachi S-4700 cold field-emission scanning electron microscope (SEM) at the Microscopy and Imaging Facility of the American Museum of Natural History.

Results

To identify sperm, we were looking for a string-like structure with a visible constriction indicating the juncture of the head and tail regions (Figure 1). This putative sperm with an intact head (Figure 1) has dimensions that correspond with those reported in the literature (Shoup, 1967; Tokuyasu, 1974).

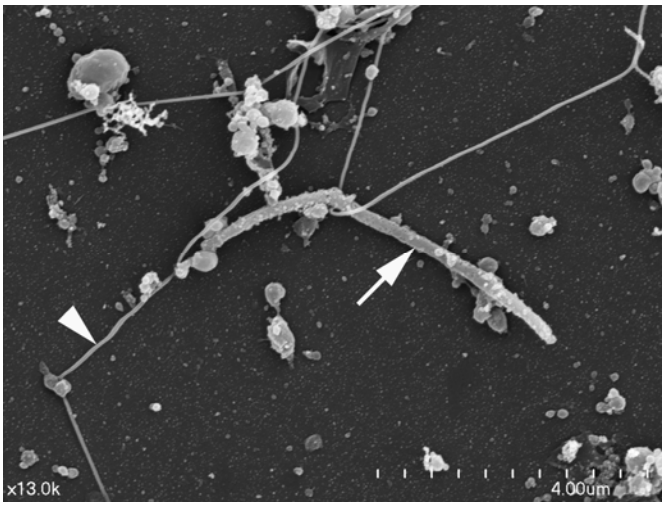

Figure 1. Mature sperm with head. Sperm from *D. simulans* seminal vesicle preserved with gluteraldehyde. The head width is approximately 0.3 micron, and the tail width is slightly less than 0.1 micron. Arrow indicates sperm head; arrowhead indicates sperm tail.

Conclusions and Future Directions

This paper demonstrates the ability to examine drosophilid sperm head morphology using the SEM. Ultrastructural details in sperm head morphology can be useful to various fields of biology. For example, the cell biologist might gain insight regarding the molecular interactions required by DNA compaction and sperm formation (*e.g*., Jayaramaiah Raja and Renkawitz-Pohl 2005), and characters for systematic analysis (Talarico *et al*., 2008; Meisner *et al*., 2005; Jamieson *et al*., 1999).

Although this procedure is useful, there are areas for improvement. Sperm within insects, but especially within drosophilids, are extremely long in relation to the size of the male (Joly *et al*., 1995; Bjork and Pitnick, 2006). The main obstacle for visualizing sperm heads is the ability to relax the sperm enough to expose the sperm head properly. Many times it seemed that the sperm heads were blocked by the high concentration of sperm tails. We might try increasing the length of time for the

sperm to unwind (Joly *et al*., 1989; Joly and Lachaise, 1994). Future experiments might want to test the use of poly-L-lysine treated cover glass. Poly-L-lysine's positive charge attracts the negative sperm to the cover glass and might indirectly promote clumping. Additionally, we will try immobilizing the sperm on polycarbonate filters instead of cover glass to eliminate the sample loss and facilitate dissociation of the sperm (Meisner *et al*., 2005).

Perhaps methods for sperm uncoiling should be examined and compared using the fluorescence microscopy prior to preparation for SEM. Since 4'-6-Diamidino-2-phenylindole (DAPI) stained sperm heads can be seen separate from the sperm tails using fluorescence microscopy, one could flip between bright field and fluorescence settings to determine the optimal method(s) for preventing tails from occluding the sperm heads.

Another future option is to collect sperm from the female rather than, or in addition to, the male. Sperm are stored individually with the tails coiled around the head within the male seminal vesicle; whereas, sperm from multiple mating are stored within the female's spheroid spermatheca as one large mass (Miller and Pitnick, 2002; Pitnick *et al*., 1999). Dissociating sperm stored in the spermatheca may be easier than uncoiling the sperm stored in the male seminal vesicle. Within the female, sperm stored in the linearized narrow seminal receptacle is believed to remain elongated (Miller and Pitnick, 2002; Pitnick *et al*., 1999). One drawback to obtaining sperm from the female is that the ejaculatory fluid might coat the sperm surface and obscure fine morphological details. This problem might be remedied by buffer washes.

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