TITLE: *Mesostoma ehrenbergii* spermatocytes **-** a unique and advantageous cell for studying meiosis

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SUMMARY

Mesostoma ehrenbergii have a unique male meiosis: their spermatocytes have three large bivalents that oscillate for 1-2 hours before entering into anaphase without having formed a metaphase plate, have a precocious ("pre-anaphase") cleavage furrow, and have four univalents that segregate between spindle poles without physical interaction between them, i.e., via "distance segregation". These unique and unconventional features make *Mesostoma* spermatocytes an ideal organism for studying the force produced by the spindle to move chromosomes, and to study cleavage furrow control and 'distance segregation'. In the present article we review the literature on meiosis in *Mesostoma* spermatocytes and describe the current research that we are doing using *Mesostoma* spermatocytes, rearing the animals in the laboratory using methods that we describe in our companion article (Hoang et al., 2013).

Introduction

In the present article, we review the literature on male meiosis in *Mesostoma ehrenbergii* and describe features of *Mesostoma* spermatocytes that make them valuable tools for studying cell division. *Mesostoma* spermatocytes are useful for studying cell division because they have few bivalents and a large spindle. In addition, they have many unique features that are not present in conventional meiotic systems including: (1) extensive chromosome oscillations, (2) the absence of a metaphase plate, (3) distance segregation of univalents and (4) a precocious "pre-anaphase" cleavage furrow. Chromosome oscillations in mitotic and meiotic cells are fairly common but these oscillations are irregular, have low amplitudes, moderate velocities and last for a short periods of time. In *Mesostoma* spermatocytes on the other hand, oscillations are regular and coordinated, have larger amplitudes, rapid velocities and last for periods of 1-2 hours, from early prometaphase until anaphase onset, without formation of a metaphase plate. The cells contain 4 univalents, 2 each of 2 different kinds, that segregate to the two poles without physical contact between them ("distance segregation", Hughes-Schrader, 1969). Finally these cells have "precocious" cleavage furrows, cleavage furrows that form in early prometaphse, begin cleavage, arrest, and continue to cleave the cells in two only after anaphase. *Mesostoma* spermatocytes provide a single system for studying these rare aspects of meiosis that originally would have required a variety of different cell types and to study each of these phenomena separately. In the accompanying article (Hoang et al., 2013) we present our methods for how to rear *Mesostoma* and how to make preparations of living spermatocytes for those who may want to study these cells.

Previous work on* Mesostoma *spermatocytes

Mesostoma ehrenbergii, a hermaphroditic aquatic flatworm from the order Rhabdocoela and the class Turbellaria, has been well-described anatomically by ecologists for decades (Ferguson and Hayes Jr, 1941; Kolasa and Schwartz, 1988; Bedini and Lanfranchi, 1990; Kalita and Goswami, 2012). The voracious predatory nature of *Mesostoma* (Blaustein and Dumont, 1990; DeRoeck et al., 2005; Trochine et al., 2005; Trochine et al., 2006), its unique feeding behaviours (Schwartz and Herbert, 1981; Wrona and Koopowitz, 1998), its possible use in

control of mosquito larvae (Case and Washino, 1979; Kolasa and Mead, 1981; Kolasa, 1984; Kolasa et al., 1985; Blaustein, 1990; Tranchida et al. 2009), and its ability to produce both viviparous embryos and dormant eggs (Bresslau 1903; Fiore and Ioalè, 1973; Domenici and Gremigni, 1977; Heitkamp, 1977) have been well documented. Unfortunately, these worms have not received as much attention from cell biologists.

M. ehrenbergii was first reported in Europe by Focke in 1836 and later in different localities around the world (Woodworth 1897; Graff 1913). Early researchers who looked at their cells were primarily interested in determining the number and morphology of meiotic chromosomes in the European (Luther, 1904; Bresslau, 1904; Voss, 1914) and North American (Husted et al., 1939; Husted and Ruebush, 1940) variants of *M. ehrenbergii*. Husted and Ruebush (1940) were amongst the first researchers to illustrate the karyotype of *Mesostoma* spermatocytes (as seen in Figure 1) and to document the length of each chromosome and the differences in centromere positions in both the European and North American worms. Husted and Ruebush (1940) determined that the European *M. ehrenbergii* (subspecies: *ehrenbergii*) has spermatocytes with 3 bivalents and 4 univalents (n=10) (Figure 1A), whereas the North American *M. ehrenbergii* (subspecies: *wardii*) has spermatocytes with 3 bivalents and 2 univalents (n=8) (Figure 1B). This observation was later contradicted by Hebert and Beaton (1990) who found that the North American subspecies of *M. ehrenbergii* have spermatocytes with the same number of chromosomes (n=10) as the European worms. Husted and Ruebush (1940) were also amongst the first researchers to describe that each of the three bivalents have a single distally located chiasma. This was later confirmed by electron microscopy studies performed by Oakley and Jones (1982) and Croft and Jones (1989). Oakley and Jones (1982) identified that chromosome pairing in *M. ehrenbergii* is incomplete, as synaptonemal complex (SC) formation is restricted to the lobed region of the nucleus which limits each of the three bivalents to short SC sequences. Jones and Croft (1989) further examined this phenomenon and determined that chromosome pairing is indeed incomplete, synaptonemal complexes are in fact only short segments on each of the bivalents. But unlike Oakley and Jones (1982), they determined that each synaptonemal complex contains one recombination nodule.

After the work of Husted and Ruebush in 1940 on fixed preparations, there were no further studies of chromosomes during meiosis I in *Mesostoma* spermatocytes until almost 40 years later (Oakley and Jones, 1982; Oakley, 1983; Oakley, 1985; Fuge, 1987; Croft and Jones, 1989; Fuge, 1989 and Fuge and Falke 1991).

Non-Random Distance Segregation of Univalents

Oakley and Fuge studied chromosome movements in living spermatocytes. Oakley focused her research on the pole-to-pole univalent movements during prometaphase I in *Mesostoma* spermatocytes (Oakley, 1983; 1985). In early prometaphase, univalents are present at the spindle poles. They remain there until anaphase, but sometimes individual univalents move from one pole to the opposite pole. From squash preparations, Oakley determined that the four univalents are actually two pairs of two and that members of each pair are morphologically identical to each other but morphologically different from the other pair. Prior to anaphase, there can be different numbers of univalents at each pole or there can be two of the same kind at each pole; but by anaphase I there is one of each kind at each pole. So, if the two univalent pairs in *Mesostoma* spermatocytes consist of X1,X2 and Y1,Y2 chromosomes, the end result by the start of anaphase is one X and one Y chromosome at each pole. The univalents seem to segregate

properly by anaphase, an example of 'distance segregation', as described by Hughes-Schrader (1969), in which partners segregate to opposite poles without having first been conjoined.

From observations of living cells Oakley described univalents moving from pole to pole, and she presumed that the movements were necessary to obtain one X and one Y univalent at each pole. Her observations suggested to her that there was non-random assortment of the univalents. If assortment were random, then the two poles in any given cell would have X1, Y2 and X2, Y1 or would have X1,Y1 and X2,Y2 chromosomes. She noticed, however, that the univalents moved from pole to pole more often than needed in order to obtain one random X and one random Y at each pole, and she noticed that members of one univalent pair often changed poles (e.g., X1 and X2 changing poles); neither of these would occur if the required end point was only that there be one of each kind of univalent at each pole. Thus she suggested that there is non-random assortment of univalents in these cells, resulting, e.g., in the two poles having only X1, Y1 and X2,Y2 chromosomes.

In addition to her discovery that univalents undergo distance segregation and possible non-random assortment, Oakley characterized the pole-to-pole movements of these univalents (Oakley, 1983, 1985). The univalents move rapidly, moving from one spindle pole to the other in about 1-2 minutes (Oakley, 1983), as seen in Figure 2A-F, with the kinetochores always leading the way (Oakley, 1985). Univalents moved one at a time, with intervals as short as 5-10 minutes before the next univalent excursion (Oakley, 1983), as illustrated graphically in Figure 2G. The mechanism of how univalent chromosomes achieve distance segregation is completely unknown, and it is not known whether there actually is non-random assortment of univalent chromosomes as suggested by Oakley (1985).

Kinetochore Oscillations of Autosomal Bivalents

Descriptions of bivalent chromosome movements in living *Mesostoma* spermatoctyes followed the work of Oakley. Fuge (1987, 1989) described the unique kinetochore oscillations of the three bipolarly oriented bivalents that occur during prometaphase/metaphase in *Mesostoma* spermatocytes. In his first article on the oscillatory movements of bivalent kinetochores in *Mesostoma* spermatoctyes, Fuge (1987) was able to analyze only short sequences (up to 8 minutes) of movement due to the short length of film that he had available. These short sequences of movement, however, were the first to illustrate the regular, rapid, and coordinated kinetochore oscillations that the bivalents in *Mesostoma* spermatocytes exhibit throughout prometaphase/metaphase (Figure 2A-C and Figure 2G). There is no defined 'metaphase' as commonly described, however, because the bivalents continually oscillate and do not align at the equator. In a later article, Fuge (1989) analysed longer sequences and was able to provide a more descriptive analysis of these kinetochore oscillations. In the cells he studied, kinetochores oscillated to and away from the spindle poles for the entire observation period, up to approximately one hour, with average kinetochore velocities of 8-10 μm/min and maximum velocities of up to 17 μm/min. Fuge determined that kinetochores move 5-7 μm away from the pole and then back to the pole, and repeat this every 100s with the two kinetochores of any given bivalent moving either in phase or out of phase (Fuge, 1989, 1991). (He classified kinetochores as moving in-phase when partner kinetochores moved to the pole at the same time and away from the pole at the same time; partner kinetochores were classified as moving out-of-phase when one kinetochore moved to the pole and the other kinetochore moved away from pole.) In order to better understand how kinetochores move to and from the pole, electron microscopy

studies were undertaken (Fuge, 1987, 1989; Fuge and Falke, 1991). The electron microscope images showed that *Mesostoma* kinetochores have a cup-like invagination that allow the deep insertion of kinetochore microtubules (Fuge 1987), and that chromosomal fibres are several µm in length, well-developed, and contain kinetochore microtubules that insert into the kinetochore and contained as well as non-kinetochore microtubules that surround the kinetochores and bivalents (Fuge 1989). These results could explain kinetochore movement to the pole by shortening of kinetochore microtubules but not the backward movement of the entire chromosome and the away from pole movement of kinetochores. In a subsequent article Fuge and Falke (1991) suggested that chromosome spindle fibres resemble a "microtubular fir-tree", composed of kinetochore microtubules and non-kinetochore microtubules that associate with both bivalents and kinetochores. Although Fuge and Falke (1991) were able to provide a more intricate picture of the spindle of *Mesostoma* spermatocytes, they still could only speculate as to how kinetochores oscillate to and away from the pole. And neither Fuge nor Oakley described anaphase in these spermatocytes.

The research conducted by Husted and Ruebush (1940), Oakley (1983, 1985) and Fuge (1987, 1989, 1991) laid the groundwork for further study of chromosome movements in *Mesostoma* spermatocytes, which was then hindered for decades by the absence of laboratory stocks of these animals.

Precocious "pre-anaphase" Cleavage Furrow

Approximately 20 years after the last article published on *Mesostoma* spermatocytes, Forer and Pickett-Heaps (2010) published an article on yet another unique feature of *Mesostoma* spermatocytes: the presence of a precocious ("pre-anaphase") cleavage furrow. Cleavage furrows in a multitude of cell types begin ingression after the onset of anaphase (Burgess and Chang, 2005; Barr and Gruneberg, 2007). Forer and Pickett-Heaps (2010), however, described a different phenomenon. In *Mesotoma* spermatocytes, the precocious cleavage furrow begins ingression during prometaphase when the bivalents achieve bipolar orientation; the furrow then arrests until the start of anaphase, almost 1-2 hours later, when it cleaves the cell into two equal daughter cells (Forer and Pickett-Heaps, 2010). A well–developed precocious furrow can be seen in the cell shown in Figure 2A. Surprisingly, precocious furrows shift their positions along the length of the cell in response to imbalances in chromosome numbers associated with the two poles, which occur when univalents move between spindle poles trying to achieve proper segregation (Forer and Pickett-Heaps, 2010). The furrow compensates for these imbalances by shifting from its primary position at the equator toward the spindle pole associated with the fewer number of chromosomes. Changes in the position of the furrow can occur in the absence of microtubules but not in the absence of actin and myosin as the latter are required to maintain the shape of the furrow (Forer and Pickett-Heaps, 2010).

In sum, *Mesostoma* spermatocytes present opportunities for studying several unique occurrences, namely distance segregation, possible non-random chromosome assortment, kinetochore oscillations, absence of a true metaphase, and precocious ("pre-anaphase") cleavage furrows. Although meiosis in this organism would be defined as "unconventional" according to textbook descriptions of meiosis, these 'unusual' phenomena all exist in this one cell, and need to be understood if we are to really understand cell division. We have tried to expand our understanding of these cells in recent experiments.

Our Current Studies using Mesostoma Spermatocytes

We have been able to expand on the previous work on *Mesostoma* spermatocytes because we are able to rear the animals in the laboratory, as described in our companion article (Hoang et al. 2013). The *Mesostoma* that we rear in the laboratory however, are different from the *Mesostoma* that Oakley and Fuge originally used. In the cells we study, the bivalents are metacentric and have only one chiasma, so each bivalent has one free arm, whereas in the cells that Oakley and Fuge studied two of the bivalents are acrocentric (and have no free arms) and one bivalent is metacentric. Although some variation exists in the morphology of the bivalents in the North American subspecies we study versus the European subspecies that Oakley and Fuge studied, the same phenomena of kinetochore oscillations, distance segregation and non-random assortment of univalents and presence of a precocious cleavage furrow exist in our cells. Therefore, we have been able to expand on the previous literature on kinetochore oscillations and univalent movements, as well as providing a detailed description of anaphase chromosome movements, bivalent reorientations, and shifts in the position of the precocious furrow in response to alterations in spindle components. We primarily focused on the kinetochore oscillations that occur during prometaphase/metaphase.

Oscillatory kinetochore movements are rapid, regular, coordinated and last for periods of 1 to 2 hours which is uncommon when compared to oscillatory kinetochore movements in other cells types that are usually slower, irregular, uncoordinated and last only for a short period of time (Bajer, 1982; Ault *et al*. 1991; Skibbens *et al.* 1993, 1995; Khodjakov *et al*. 1997; Jaqaman *et al*. 2010). In the cells we studied kinetochores oscillated to and from the pole with average excursions of 4.0µm and velocities of 6.2µm/min and 5.2µm/min, respectively, as based on analysis of approximately 1700 kinetochores. The velocities of these movements are much faster than the prometaphase oscillatory kinetochore movements in most cells, which are around 1.0µm/min to 3.0µm/min (Bajer, 1982; Ault *et al*. 1991; Skibbens *et al.* 1993, 1995; Khodjakov *et al*. 1997; Jaqaman *et al*. 2010) but they are similar to the rapid oscillatory movements in early *Drosophila* embryo cells which have average velocities of 3.6µm/min and 6.6µm/min, depending on temperature (Maddox *et al*. 2002), but which, however, last for only 50-100s and have excursions that are only 0.5-2µm away from the pole (Civelekoglu-Scholey *et al*. 2006). The velocities of kinetochore movements in *Mesostoma* spermatocytes are comparable to the velocities of chromosome movement in early *Drosophila* embryo cells but the length of time oscillatory movements take place and the distance of each excursion are much greater than those in early *Drosophila* embryos.

Kinetochore oscillations in *Mesostoma* spermatocytes are unique and therefore allow us to use a variety of tools to manipulate different components of the spindle so we can better understand how chromosomes move. Through the use of an optical trapping laser (1064nm), an ultraviolet microbeam (290nm) and an optical cutting laser (730nm), we determined that kinetochore movement to the pole is different from kinetochore movement away from the pole. We measured the force required to stop chromosome movement by holding oscillating kinetochores with an optical trap (Ferraro-Gideon et al., 2013). The lengthy oscillation periods allowed us to perform multiple trapping experiments in the same spermatocyte and to collect a lot of data in a short period of time. We determined the power that would stop kinetochore movement in the presence of the trap but would allow kinetochore movement to resume when the trap was released, indicating that though motion was stopped, the laser did not damage the

cell (Ferraro-Gideon et al., 2013). UV microbeam irradiations of kinetochore fibres suggested that movement mechanisms are different for oscillations to and from the pole (unpublished data): After irradiation of a kinetochore fibre, kinetochore movement continued until the kinetochore reached the pole (or the movement reversed direction so the kinetochore could move to the pole). Movement stopped when the kinetochore was at the pole, but movement resumed only for chromosomes whose fibres were irradiated as the kinetochore moved to the pole. We still are investigating differences in the components and mechanisms between movement to the pole versus away from the pole.

Although most of our research has focused on using kinetochore oscillations in *Mesostoma* spermatocytes to better understand how chromosomes move, the same experiments showed that the position of the precocious cleavage furrow changes after various components of the spindle are altered. Following UV irradiation of a kinetochore fibre, the furrow shifts approximately 1µm, usually away from the site of irradiation; following UV irradiation of a kinetochore, the furrow immediately shifts its position either towards or away from the site of irradiation, but the furrow then completely loses its shape and the cells become rounded (unpublished data). The furrow also shifts its position in response to bivalent reorientation, when both kinetochores are temporarily associated with one spindle pole, the furrow shifts towards the pole with the fewer number of attached kinetochores (unpublished data).

Conclusion

Mesostoma spermatocytes offer a unique system for studying and better understanding why and how chromosome oscillations take place; the mechanisms of distance segregation (and possibly non-random chromosome assortment); and they provide new insight into cleavage furrow formation since the furrows form and contract in early prometaphase, one or more hours prior to anaphase. Not only are these cells large, with few chromosomes, in conventional meiotic systems a variety of different cell types would have been required to study each of these phenomena separately, whereas each of these phenomena can be studied using a single cell type, spermatocytes from *Mesostoma.* This not only allows for the study of each of these phenomena individually but it also allows for the study of these phenomena as a whole. Although some of the features of *Mesostoma* spermatocytes are unconventional to most meiotic systems, these unconventional features can allow us to shed light on important features of cell division that often are ignored.

There are other advantages to using these animals. For example, the hermaphroditic *Mesostoma* will self-fertilize if reared in isolation, which could be quite useful for genetic studies. The animals are transparent (see illustrations and videos in the accompanying article) and can easily be injected through the body wall with a variety of labeled markers. Although much still is unknown about this organism, and molecular tools have not yet been developed, the unusual kinetochore oscillations, the presence of a precocious ("pre-anaphase") cleavage furrow, the distance segregation of univalents, the non-random assortment of univalents and bivalents and the lack of metaphase make the meiotic division in *Mesostoma* spermatocytes so different from the standard paradigm of cell division, that it really should be investigated more fully.

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FIGURES:

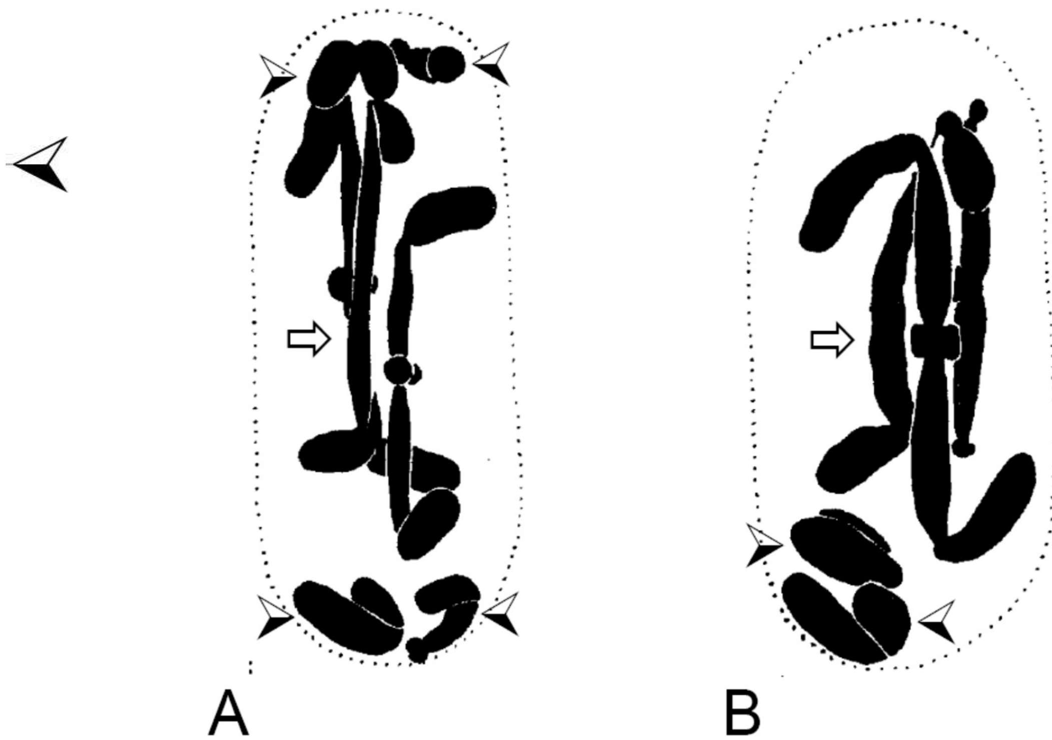
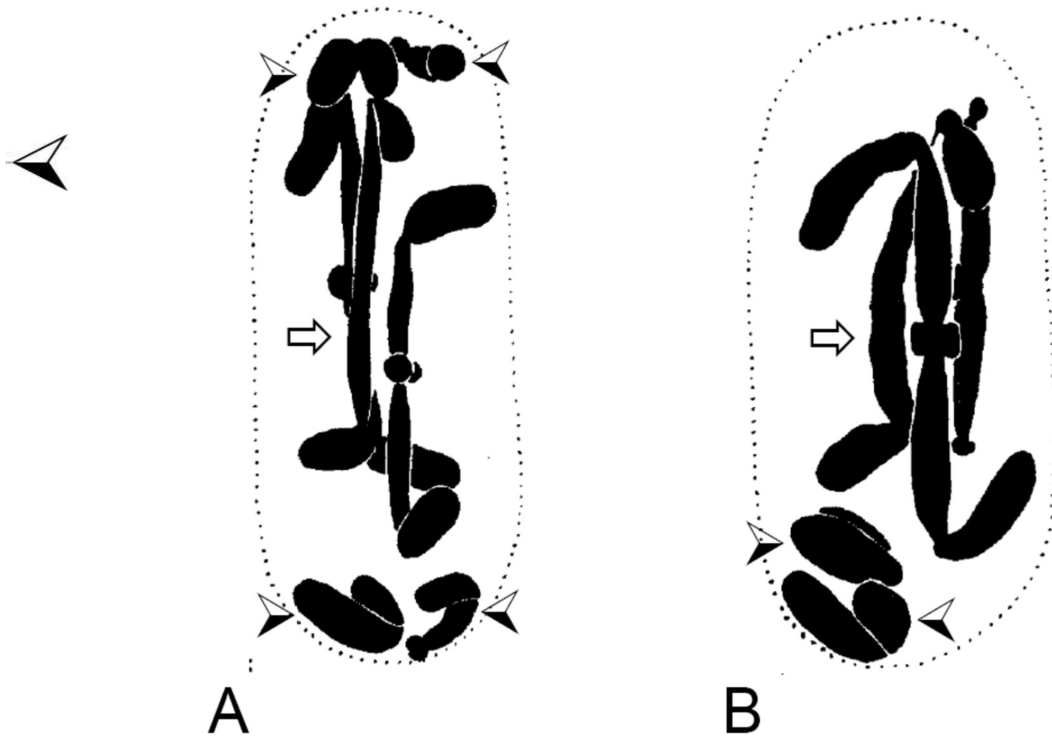


Figure 1 Picture of fixed and sectioned *M. ehrenbergii* spermatocytes modified from Husted and Ruebush (1940). (A) European form of *M. ehrenbergii* (*ehrenbergii*) showing 3 bivalents and 4 univalents, n=10. (B) North American form of *M. ehrenbergii* (*wardii*) showing 3 bivalents and 2 univalents, n=8. The open white arrows (⇨) illustrate the positions of one bivalent in each spermatocyte and the arrowheads (➤) illustrate the positions of each of the univalents at the poles in each spermatocyte.

Figure 2 Montage of phase contrast microscope images of a *Mesostoma* spermatocyte illustrating a univalent as it moves between spindle poles during prometaphase/metaphase and bivalent kinetochore oscillations. (A-C) The univalent moves from the lower pole to the upper pole. The lower half-bivalent kinetochore as depicted by the white arrow moves towards its pole. (D-F) Approximately 10 minutes after the first univalent excursion, the same univalent moves from the upper pole to the lower pole. The black arrows in A point to the precocious cleavage furrow and the thick black arrows in A-E point to the positions of the univalent. (G) Graph of distance of the kinetochores of partner half bivalents (**O** and Δ) and of the kinetochores of a univalent (◊) from the bottom pole of the cell in μm, versus time in minutes in a *M. ehrenbergii* spermatocyte.

FIGURES:



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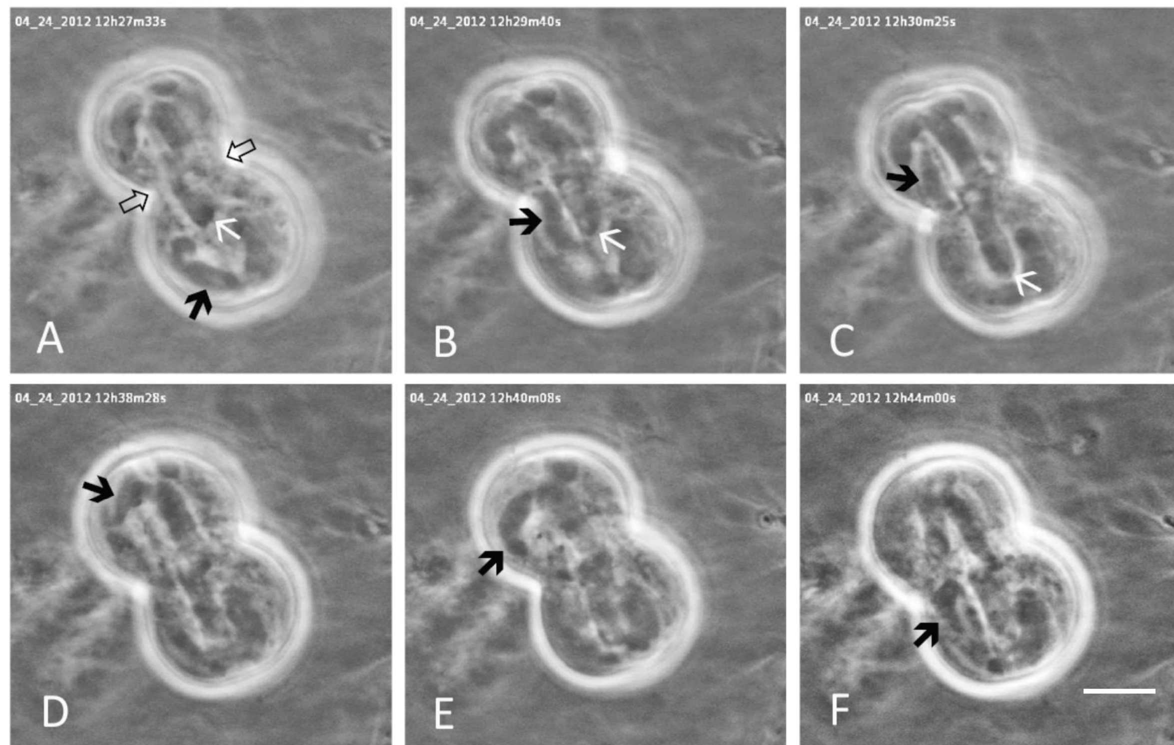

Figure 2 Montage of phase contrast microscope images of a *Mesostoma* spermatocyte illustrating a univalent as it moves between spindle poles during prometaphase/metaphase and illustrating bivalent kinetochore oscillations. (A-C) The univalent moves from the lower pole to the upper pole. The lower half-bivalent kinetochore as depicted by the white arrow moves towards its pole. (D-F) Approximately 10 minutes after the first univalent excursion, the same univalent moves from the upper pole to the lower pole. The block arrows in A point to the precocious cleavage furrow and the thick black arrows in A-E point to the positions of the univalent. (G) Graph of distance of the kinetochores of partner half bivalents (**O** and Δ) and of the kinetochores of a univalent (◊) from the bottom pole of the cell in μm, versus time in minutes in an *M. ehrenbergii* spermatocyte.