Distance segregation of sex chromosomes in crane-fly spermatocytes studied using laser microbeam irradiations.

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Abstract

Univalent sex chromosomes in crane-fly spermatocytes have kinetochore spindle fibres to each spindle pole (amphitelic orientation) from metaphase throughout anaphase. The univalents segregate in anaphase only after the autosomes approach the poles. As each univalent moves in anaphase one spindle fibre shortens and the other spindle fibre elongates. To test whether the directionality of force production is fixed at anaphase, that is, whether one spindle fibre can only elongate and the other only shorten, we cut univalents in half with a laser microbeam, to create two chromatids. In both sex-chromosome metaphase and sex-chromosome anaphase, the two chromatids that were formed moved to opposite poles (to the poles to which their fibre was attached) at speeds about the same as autosomes, much faster than the usual speeds of univalent movements. Since the chromatids moved to the pole to which they were attached, independent of the direction to which the univalent as a whole was moving, the spindle fibre that normally elongates in anaphase still is able to shorten and produce force towards the pole when allowed (or caused) to do so.

Introduction

Proper segregation of chromosomes in mitosis and meiosis occurs because metaphase chromosomes are attached to spindle fibres that extend to opposite poles: after disjunction of the chromatids (in mitosis) or half-bivalents (in meiosis), the separated chromosomes move to opposite poles, pulled by their spindle fibres. Major exceptions to this generalisation occur when individual meiotic chromosomes are unpaired, are not physically connected, yet regularly segregate to opposite poles. This was termed "distance segregation" by Hughes-Schrader (1969) who described numbers of different cell types where this takes place. In primary spermatocytes of one mantispid species, for example, the 6 sex chromosomes (3 X and 3 Y chromosomes) segregate properly without physical contact taking place (Hughes-Schrader, 1969). Autosomal univalents also can undergo distance segregation (Hughes-Schrader, 1975, 1979).

Distance segregation mechanisms are not understood. In spermatocytes of the flatworm *Mesostoma*, for example, the four univalents, of two morphological types (Göltenboth and Heitkamp, 1977; Oakley, 1985), move to the poles at the start of prometaphase and remain there

throughout division. Proper segregation is not always achieved initially, however: sometimes there are three univalents at one pole and one at the other, or there are two of one type at each pole. Distance segregation eventually occurs because the univalents move between poles to obtain proper assortment (Oakley, 1983, 1985). Nothing is known about the 'sensing' mechanisms that cause the chromosomes to switch poles or about the mechanisms of their movements.

Another example occurs in mole-cricket spermatocytes in which one bivalent is heteromorphic, composed of a large half-bivalent paired to a small half-bivalent. The univalent X chromosome always segregates to the same pole as the larger partner of the heteromorphic bivalent (Payne 1912, 1916; White, 1951). There are no physical connections between the bivalent and the single X chromosome, as determined by micromanipulation (Nicklas and Camenzind, 1968). Further micromanipulation experiments provided experimental information about mechanisms. When the X chromosome is pushed to the opposite pole, the bivalent keeps its original orientation but the X chromosome reorients and returns to its original position (Nicklas and Camenzind, 1968). When the bivalent is turned around so that the smaller half-bivalent is connected to the pole that the X chromosome is connected to, the X chromosome reorients and moves to the opposite pole (Nicklas and Camenzind, 1968). Thus the X chromosome is the active partner in ensuring that proper segregation is achieved, similar to conclusions reached from ultraviolet microbeam experiments (Wise et al., 1984). The mechanisms for this are completely unknown, however.

The present paper deals with distance segregation of sex chromosomes in crane-fly spermatocytes. The division in these cells is unusual. The three autosomal bivalents achieve bipolar orientation early in prometaphase. The two sex-chromosome univalents (X and Y), on the other hand, are at first syntelically oriented (with kinetochore spindle fibres to only one pole) and independently move back and forth along the spindle. Later, prior to anaphase, each becomes amphitelically oriented, with kinetochore fibres to both poles, and congresses to the equator (Bauer et al., 1961; Fuge, 1972; Steffen and Fuge, 1985), as seen in Figure 1A, D, E. The two sex chromosomes remain at the equator as the autosome half-bivalents disjoin at anaphase and move to the poles. The univalents move to opposite poles only after the autosomes near the poles (Fig. 2), and move with velocities considerably slower than the autosomes (e.g., Schaap

and Forer, 1979). They remain amphitelically oriented throughout: as each univalent moves, one spindle fibre shortens and the other elongates (Fuge, 1972; Fuge, 1985; Wilson and Forer, 1989), as seen in Fig. 1B,C. The univalents are not physically connected and indeed sometimes are on opposite sides of the spindle from each other as they move to the pole (Fig 1E; also Spurck et al., 1997; Sillers and Forer, 1981). As background to experiments in the present paper, we describe previous experiments that have elaborated on crane-fly spermatocyte distance segregation.

Sex chromosome behaviour in crane-fly spermatocytes is not due to inherent differences between autosomes and sex chromosomes. Rather, it depends solely on the orientation of a univalent's (or half-bivalent's) kinetochores, solely on whether the kinetochores are syntelically oriented (with fibres to one pole) or amphitelically oriented (with fibres to both poles). This is shown by several experiments. Bauer et al (1961) created spermatocytes in which an autosome fragment was translocated to the Y chromosome. When the autosome fragment paired with the corresponding portion of a bivalent its attached Y-chromosome became syntelically oriented (the spindle fibres from the two Y-chromosome kinetochores each extended to the same pole). At anaphase the syntelically-oriented Y chromosome moved to the pole together with the autosome half-bivalents, while the amphitelically oriented X chromosome remained at the equator and did not move to either pole (Bauer et al., 1961). On the other hand, when an autosomal half-bivalent was amphitelically oriented, with kinetochore fibres to both poles, it did not segregate at autosomal anaphase. Instead it underwent anaphase with the sex chromosome univalents in a distinct 'distance segregation' pattern: when there was an even number of amphitelic half-bivalents and univalents, up to 8, they segregated in equal numbers to the two poles (Dietz, 1969; Forer and Koch, 1973; Forer and Swedak, 1991). When there was an uneven number of amphitelic chromosomes, up to 7, equal numbers of amphitelic chromosomes moved to opposite poles and the odd chromosome remained at the equator (Dietz, 1969; Forer and Koch, 1973; Forer and Swedak, 1991). Thus distance segregation in crane-fly spermatocytes involves "counting" the numbers of amphitelic chromosomes. The segregation patterns of the syntelic chromosomes also influence sex chromosome movements: when four syntelically oriented half-bivalents move to one pole and two move to the other, in the subsequent sex-chromosome anaphase one of the two sex chromosomes does not move and the other moves to the pole with the two autosomes (Dietz, 1969; Forer and Koch, 1973). Autosomal spindle fibres themselves

also influence sex-chromosome movements: ultraviolet microbeam irradiation of an autosomal kinetochore fibre (in autosomal anaphase) that is adjacent to a single sex chromosome kinetochore fibre causes that sex chromosome to remain at the equator during sex-chromosome anaphase while the other sex chromosome segregates to the opposite pole (Sillers and Forer, 1981). Irradiation of an autosomal kinetochore fibre adjacent to *two* sex-chromosome kinetochore fibres causes the two sex chromosomes to move to the same pole, the pole opposite that of the fibres that were irradiated (Sillers and Forer, 1981). Thus amphitelic univalents *can* move singly, or *can* move to the same pole, but in normal circumstances they move in opposite directions. We would like to understand how the movements and directionalities of amphitelic chromosomes are influenced by "counting" each other and counting the autosomal spindle fibres, and indeed why amphitelic chromosomes move poleward after the autosomes do.

Experiments in this article use laser microbeam irradiations to examine one aspect of distance segregation mechanisms in crane-fly spermatocytes, namely whether the directions of motion of the sex chromosomes are irreversibly determined at the start of sex-chromosome anaphase or whether they are able to change if conditions change. Some previous experiments indicated that the directions of motion can change: both sex chromosomes reversed their directions of motion after micromanipulation was used to push one sex chromosome in the direction opposite to its initial movement direction, pushing it past the sex chromosome moving to the opposite pole (Forer and Koch, 1973). A recent article reached a different conclusion, that when univalents are bisected into chomatids the trailing chromatid is *not* able to reverse direction (LaFountain et al., 2012). The experiments in the present study show that the directions of sex-chromosome motion can be changed during anaphase, that the trailing chromatid can reverse direction as can a univalent itself.

Materials and Methods.

The procedures for making preparations of living crane-fly spermatocytes have been described previously (Forer and Pickett-Heaps, 2005) as have procedures for irradiation (Harsono et al., 2012; Shi et al., 2012). Briefly, crane-fly larvae (*Nephrotoma suturalis* Loew) were dissected under halocarbon oil and placed in a drop of Halocarbon oil. The testes were

rinsed in insect Ringer's solution to remove the oil, were placed on a coverslip in a drop of Ringer's solution that contained fibrinogen, and then were broken open so the cells flowed out. Thrombin was added to form a clot and the cells were placed in a perfusion chamber and perfused with Ringer's solution. Cells were imaged using phase-contrast microscopy on a microscope interfaced with a laser that was used to cut parts of the cell in user-specified geometries and planes (single or multiple). Briefly, the system was an inverted microscope Axio Observer (Zeiss, Germany) coupled with a 200 femtosecond pulsed laser (Mai Tai, Newport, Co., Irvine, CA, USA) emitting 730 nm wavelength light. The laser beam was expanded to fill the back aperture of the objective (Zeiss Plan-Apochromat 63/1.40 Oil Ph3, Zeiss, Germany) and was steered by fast-scanning mirrors (FSM300, Newport Co., Irvine, CA, USA) such that the focus of the laser scissors was moved within the sample plane. Images were recorded live by an ORCA R2 camera (Hamamatsu, Japan) at variable intervals ranging from 2 seconds to 10 seconds. In these experiments we cut single lines in multiple planes (3 planes up and down from the plane of focus). Images were recorded in png and tiff formats, cropped and converted to bmp files using IrfanView (freeware), and assembled into video (avi) sequences using Virtual Dub (freeware). Graphs were obtained using an in–house program (Wong and Forer, 2003) in which observers mark on bmp files the positions to be measured. When crane-fly spermatocytes are spread on a coverslip under oil they continually change positions throughout the time series, which necessitates the constant alignment of images (La Fountain et al., 2012). When these cells are held in a clot in our preparations, on the other hand, they do not move during filming, as seen clearly when playing back time-lapsed video sequences. Thus we can mark one pixel position (for example, pole or equator) and be confident that this is a reliable, stationary coordinate from which to measure movement. For graphic analyses the resultant distance values were imported into SlideWrite, a commercially available graphics program.

Some irradiated cells were subsequently studied using confocal fluorescence microscopy using preparation and observation procedures described elsewhere (Fabian et al., 2007). Briefly, immediately after irradiation cells were lysed using cytoskeleton stabilising buffer that contained a detergent; their positions on the coverslip were marked so they could be located later. Other (lysed) cells on the same preparations also were irradiated and their positions marked. Then the cells were fixed for 5 minutes with 0.25% glutaraldehyde, treated with sodium borohydride, rinsed, reacted with antibody YL1/2 to stain tyrosylated tubulin, and then reacted with

fluorescently labelled secondary antibody. Cells were observed using an Olympus confocal microscope.

Results.

Sex chromosomes were cut into single chromatids either during sex-chromosome anaphase, or during sex-chromosome metaphase when autosomal anaphase was at least half finished but before univalents had started to move to the poles. The major result from this basic experiment is that the two chromatids moved to opposite poles, both during sex-chromosome metaphase (Figure 3) and during sex-chromosome anaphase (Figure 4). It is not surprising that the chromatids move to opposite poles during sex-chromosome metaphase, since the common assumption is that the metaphase equatorial position for amphitelic chromosomes (such as in mitotic metaphase) is determined by a balance of forces to both poles. It perhaps is more surprising that the chromatids created during sex-chromosome anaphase moved to opposite poles. When a univalent moves toward a pole the shortening fibre must be producing force. One would thus expect that the leading chromatid would move to its pole after being separated from the univalent. However, the trailing chromatid attached to the opposite pole moved *away* from its pole when it was part of the univalent, yet it moved *toward* its pole once freed from the univalent. Thus, after chromatids were separated from the univalent the former elongating fibre produced force toward the pole to which it was connected, in the direction opposite to the previous direction of motion.

Chromatids also were separated from univalents that were stationary during sex-chromosome anaphase. In previous experiments, one univalent moved to a pole and the other remained stationary at the equator after individual autosomal spindle fibres were irradiated in anaphase using an ultraviolet microbeam (Sillers and Forer, 1981). We reproduced that result by irradiating autosomal spindle fibres with the laser microbeam. Stalled univalents were cut in half in three cells: in each cell the two separated chromatids moved to opposite poles (e.g., Figure 5). Thus both spindle fibres are capable of producing force when chromatids are released from stalled anaphase univalents.

Further details of the chromatid movements help clarify some possible interpretations.

Chromatids moved poleward with constant velocities that were faster than the velocities of univalents themselves, velocities that approached or exceeded those of anaphase autosomal half-bivalents (Table 1). A small fraction of the chromatids (12/69) initially accelerated (for up to 1½ minutes) before slowing to constant velocity. Most initial accelerations (10/12) were during sex-chromosome anaphase. Our analyses were of the slower 'steady state' chromatid velocities. The average poleward velocities of chromatids separated in sex-chromosome metaphase (0.62μm/min) are not statistically significantly different from velocities of chromatids separated in sex-chromosome anaphase (0.77μm/min). Some chromatids separated in sex-chromosome anaphase moved significantly faster than autosomes in the same cells. Some also moved considerably faster than any created in sex-chromosome metaphase (Fig 6), suggesting that there might be differences between the two stages.

We compared chromatid velocities with autosomal velocities in the same cells to better ascertain whether there were differences between chromatids produced in metaphase versus anaphase. This is a more apt comparison than comparing overall averages because anaphase poleward velocities in control cells vary from cell to cell (e.g., Schaap and Forer, 1979). For those cells in which autosomal velocities could be directly compared to chromatid velocities (Table 2), there was no statistically significant difference between average ratios (chromatid velocities divided by autosome velocities) for metaphase chromatids (1.2) versus anaphase chromatids (1.5). Any difference between the two stages is due primarily to some chromatids separated in sex-chromosome anaphase that moved 3 to 5 times faster than autosomes (Figure 7). To determine whether this might be because of differences between chromatids separated from a single univalent, we compared 'partner' chromatids that moved to opposite poles.

After cutting univalents in half we sometimes could not follow both chromatids. When the cut was slightly off centre, so that the univalent was not cut exactly in the middle, the smaller piece of the univalent usually was visible long enough to measure its velocity; often, however, its phase-contrast microscope image lost contrast and faded and the chromatid became difficult to follow (e.g., Figure 4A). When our aim was even further off the laser created substantially unequal fragments; the smaller fragment very quickly lost contrast and faded, so it was not possible to measure positions. For those cells in which both partner chromatids were followed, the two chromatids sometimes moved with the same velocity (within 25%), but often did not

(Figure 8). When comparing chromatid pair ratios for sex-chromosome metaphase with those of sex-chromosome anaphase (dividing the faster velocity by the slower velocity), there was a statistically significant difference between metaphase and anaphase (Table 2). The metaphase partner chromatids were more likely to be the same speed (within 25%) than anaphase partner chromatids (Figure 8). Indeed, fewer than half the pairs in metaphase (5/12) differed in velocity by more than 25% whereas more than half (14/20) the pairs in anaphase did. When univalents were cut in half in sex-chromosome anaphase, the faster chromatid partner was generally the one that went to the near pole (Figure 9). In our sample, in 10/17 pairs the chromatid moving to the near pole was faster (by >25%) than the partner moving to the far pole, and in only 2/17 was the partner moving to the far pole the faster.

In summary, partner chromatids separated in sex-chromosome metaphase tended to move with the same velocities as each other, and to move with the same velocities as the anaphase poleward movements of autosomes in the same cell. Partner chromatids separated in sex-chromosome anaphase moved at different speeds at least half the time. The faster partner most often was the chromatid that moved to the nearer pole. About a third of the time the anaphase chromatids moved with faster velocities than anaphase movements of autosomes in the same cell.

Sex chromosome locations in the spindle sometimes influenced chromatid movements. Sex chromosomes very often are near the centre of the spindle prior to anaphase so that they are nearly superposed. Thus their four spindle fibres are in a straight line or in two lines very close to each other (Figure 1B, C, Figure 2D, E, Figure 4A). It is less common that they remain spatially separate, on opposite sides of the spindle (e.g., Figure 1D, E) with four separated spindle fibres. We released chromatids from spatially separated sex chromosomes in two anaphase cells. In each cell when the chromatid moving to the far pole reached and passed the non-cut univalent, the non-cut univalent reversed its direction of motion (Figure 10). This indicates that univalent movement directions can change even after anaphase segregation has started.

When the anaphase univalents were more-or-less in a straight line, chromatids separated from one univalent generally slowed down when they reached the non-cut univalent, as if the univalent was in the way. In a few cells, after one univalent was cut the other, non-cut univalent sped up: the non-cut univalent and the chromatid moving to the same pole both moved poleward

with velocities faster than normal univalent movement. This suggests that when the laser cut the univalent in half it also cut those microtubules that extended from the other univalent to its far pole. To test this we irradiated spindle fibres directly, in multiple Z planes to ensure that the fibre was cut, using about the same size line used to cut univalents in half. In all 4 irradiated cells, cutting the elongating fibre sped up the associated univalent. In one cell, with laterally separated univalents, the univalent's velocity increased by a factor of 3 after its elongating fibre was cut. In 3 cells, with the sex chromosomes in a straight line, we irradiated between the separating univalents and in all cells at least one univalent sped up (e.g., Fig. 11). These data suggested that the laser cut microtubules, and that in the cells in which the univalents were in a line, the laser cut univalent fibres. To confirm that the laser cut spindle microtubules we cut a line across spindle fibres, in multiple Z planes, using the same power and line length as when we cut univalents in half. The cell was lysed immediately for immunofluorescence staining. We also irradiated spindle fibres in the lysed preparation. The laser irradiations severed microtubules in the irradiated region (Figure 12). These data show that the laser cuts unlabelled spindle microtubules, and that univalents speed up after their elongating (trailing) microtubules are cut.

Discussion

The main conclusion from our experiments is that for crane-fly sex-chromosome univalents the directionality of forces can be changed even during anaphase: the elongating fibres attached to each univalent can produce force to their poles if conditions change after anaphase has started. This conclusion is based on several experimental results. When anaphase univalents were cut in half to create chromatids, each chromatid moved to the pole to which it was connected. When stalled anaphase univalents were cut in half to create chromatids, each chromatid moved to the pole to which it was connected. When a chromatid released in anaphase overtook the non-cut univalent, the non-cut univalent reversed direction, showing that both leading and trailing fibres can change directionality in anaphase. Thus, when circumstances change, the elongating kinetochore fibre can shorten and produce force toward its pole and the shortening fibre can switch to elongation.

This conclusion is opposite to that of LaFountain et al. (2012). Working with the same species of crane-fly spermatocytes that we did, LaFountain et al. (2012) used laser irradiation to cut univalent sex chromosomes in half during sex-chromosome metaphase and sex-chromosome anaphase, to ablate trailing kinetochores in sex-chromosome anaphase, and to ablate single kinetochores in sex-chromosome metaphase. Our results agree with theirs in general in that chromatids separated from a sex-chromosome univalent in sex-chromosome metaphase both move poleward with velocities faster than normal univalent anaphase movements. Our results starkly disagree with their conclusion that when chromatids are created from sex-chromosome univalents during sex-chromosome anaphase there is "negligible movement" (LaFountain et al., 2012) of the trailing chromatid. We are puzzled by their conclusion because the results from our experiments are clear and unambiguous: the chromatids created during sex-chromosome anaphase move to opposite poles, as shown in pictures in Figure 4A, and in graphs in Figures 4B, 5 and 10. There are differences between the two sets of experiments in techniques, namely in laser pulse durations, wavelength and energy; in cell preparation methods; and in LaFountain et al. (2012) treating some cells with cytochalasin prior to cutting univalents. But since they cut univalents in half during sex-chromosome anaphase in only 3 cells, since the speed of the trailing chromatid often was much slower than the leading chromatid (our Figure 9,their Figure 4D), and since the main points addressed by their experiments concerned changes in tubulin flux in kinetochore fibres and not relative movements of chromatids during sex-chromosome anaphase, we think it likely that they too would have seen movement of the trailing chromatid had their sample size been larger.

When univalents were cut in half, a small fraction (12/69) of chromatids accelerated initially and then slowed down to a steady slower velocity. LaFountain et al. (2012) describe the same phenomenon. It is tempting to consider the acceleration a response to release from internal tension across the univalent, but it is difficult to generalize in that way since in our experiments acceleration occurred in less than 20% of the chromatids formed from cut univalents.

Sex chromosome univalents move much slower in anaphase than autosomes (in their anaphase), presumably because the trailing fibre acts as a "drag" on movement. The drag presumably occurs because the trailing fibre either produces force in the opposite direction (albeit less than the force in the forward direction) or simply slows the movement so that the

elongating microtubules actually can elongate (e.g., Fuge, 1972). The main evidence for this conjecture has been that the trailing kinetochore was greatly stretched out (Fuge, 1972; Fuge, 1985). The first direct experimental evidence that the trailing fibre slows univalent movement was presented by LaFountain et al. (2012), who showed that univalent movement sped up when the trailing kinetochore was ablated. A similar conclusion arises from our experiments in which univalents sped up after their trailing (elongating) spindle fibre was cut, both when the other univalent was cut in half and when the trailing spindle fibre was cut directly (Figure 11, 12). We do not know from any of these experiments, however, whether the elongating fibre produces force toward its pole or not. We could decide whether it did if the velocities of chromatids created in metaphase and anaphase represent the underlying forces that are present prior to cutting the univalent. In metaphase the underlying forces to the two poles are equal, and in anaphase the force from the shortening fibre is larger than the force from the elongating fibre. Our data show, however, that chromatid velocities do *not* represent the underlying forces prior to separation of the chromatids. (1) When chromatids were separated in metaphase their velocities often (5/12) differed from their partners by >25%, and sometimes differed by up to 75% (Figure 8). (2) When chromatids were separated in anaphase, those associated with the elongating spindle fibres sometimes (2/17) moved faster than their partners, and sometimes (5/17) moved at the same speeds as their partners (Figure 9). Thus the velocities of the resultant chromatids cannot be used to infer whether the elongating fibre actually produces poleward force as it elongates.

Chromatids released in anaphase on average moved poleward faster than anaphase autosomes in the same cells (Table 2). This might be because half-bivalent movements are slowed down by elastic "tethers" that connect arms of separating half-bivalents (La Fountain et al., 2002). LaFountain et al. (2011) showed that kinetochores cut away from their half-bivalent arms (and hence from tethers) moved poleward twice as fast (on average) as kinetochores connected to arms and tethers. They argued that this is because tethers slow down normal anaphase movement. Thus chromatids might move faster than the autosome half-bivalents because they have no tethers to hold them back. It is important to note, though, that the chromatids move faster than autosomes only on average. In more than half the experiments (in

both metaphase and anaphase) the autosomes move either with the same velocity, or faster than the chromatids (Figure 7). Therefore, parameters other than tethers would seem to be important.

Our experiments have elucidated some parameters of univalent motion in crane-fly spermatocytes: (a) that forces are changeable during anaphase, (b) that cutting univalents into chromatids causes changes in the forces on the chromatids, and (c) that severing the trailing fibre causes faster movement toward the pole. These conclusions are part of a larger set of puzzles: what determines which fibre elongates and which shortens? How is movement controlled so that in normal cells the two univalents move to opposite poles? Or, in other circumstances (described in the Introduction), how is movement controlled such that both move to the same pole or only one moves? That is, what is it that determines that the elongating fibre produces no force (or reduced amount of force), and determines this in such a way that the two univalents move in opposite directions? What is the "cue" that determines which 2 of the 3 amphitelic chromosomes in the cell move (when there are 3), or that determines that univalents reverse direction mid anaphase when a univalent is overtaken by the other univalent (Forer and Koch, 1973) or by a chromatid (Figure 10)? Unfortunately, we know so little about these phenomena that it is difficult even to speculate on possible mechanisms. Dietz (1969) suggested that some aspects of distance segregation were coordinated via pools of tubulin monomers, but later results from micromanipulation experiments did not fit his model (Forer and Koch, 1973). Nor did results from ultraviolet microbeam irradiations (Sillers and Forer, 1981). We think it more likely that coordination feedback for distance segregation arises through tensegral networks (Ingber, 1993) and from the spindle matrix (Johansen et al, 2011), through mechanical coupling of chromosomes and spindle fibres to an overall spindle tensegral structure (e.g., Pickett-Heaps, et al., 1997). In the absence of more concrete models to explain any examples of distance segregation we resort to continued study of more details of the overall mechanism, and hope that these details might help toward answering these bigger, more important questions.

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Figure Legends

Figure 1 illustrates various configurations of the sex chromosomes in metaphase and anaphase. A, B and C are confocal microscope images of tubulin stained cells. D and E are polarising microscope image taken using techniques described in Spurck et al., 1997; the spindle fibres appear dark, and each univalent (arrows) has a spindle fibre to each pole. **A**: autosome metaphase, sex chromosome metaphase. Each sex chromosome (arrows) has spindle fibres to both poles. **B** and **C**: sex chromosome anaphase. These are different focal planes of the same cell illustrating that each sex chromosome (arrows) has spindle fibres to both poles as they move in anaphase. **D** and **E**: autosome anaphase, sex chromosome metaphase, illustrating variable lateral separation between univalents. **D** and **E**: Bars: 10μm.

Figure 2 is a sequence of phase contrast images of one living cell from metaphase through late autosome anaphase. The sex chromosomes, indicated by arrows in A, remain at the equator (sex chromosome metaphase) until the autosomes near the poles and only then move to opposite poles (in sex chromosome anaphase). The bar is 10 μm.

Figure 3 is a graph illustrating movement of a pair of autosomal half-bivalents during their anaphase (black triangles and crosses, from 0-6 minutes) and movement of chromatids after cutting univalents during sex chromosome metaphase. The blue crosses and squares indicate the positions of the two kinetochores of one sex chromosome and the red diamonds and inverted triangles the positions of the two kinetochores of the other univalent. The vertical blue and red lines (as in all subsequent graphs) indicate the times at which the corresponding (blue or red) univalents were cut in half. We were able to plot the two chromatids of the univalent indicated in blue but only one kinetochore of the other univalent. The lines (as in all subsequent graphs) are lines of best (least mean squares) fit to the indicated points.

Figure 4A is a series of phase contrast images of a cell that is graphed in **Figure 4B**. In Figure **4A** The two sex chromosomes (in their anaphase) are indicated by arrows in A; when cut into chromatids the two chromatids are indicated by arrows (in E, F, H, I , J, K and L) in the same directions as the arrows of the original not-cut univalents. The bottom chromatid in H is smaller than the upper chromatid, and faded as it moved to the pole so we could not follow it as well as the partner chromatid. The green lines in B and G indicate where the laser cut the chromosomes. The bar is 10μm. In Figure **4B** the upper univalent (and chromatids) seen in Fig. 4A are uppermost (in red crosses and squares) and the lower univalent is bottom-most (in blue crosses and triangles). The two symbols in each colour indicate the two kinetochores of that particular univalent (and then of the chromatids). The red and blue vertical bars are placed at the times that the red and blue univalents were cut in half.

Figure 5 graphically illustrates the result of cutting a univalent that is stalled at the equator. The two kinetochores of the stalled univalent are indicated by the blue crosses and triangles; the green dashed lines indicate the lines of best fit for the one kinetochore before the univalent was cut, and then of both kinetochores of the chromatids. The vertical blue and red lines indicate the times at which the corresponding (blue or red) univalents were cut. The black dashed lines associated with the red crosses (the moving univalent) are lines of best fit for one kinetochore before the univalent was cut. The dashed black lines associated with the red crosses and squares are the lines of best fit for the kinetochores of the chromatids after the univalent was cut.

Figure 6 is a bar graph showing the distribution of chromatid velocities for chromatids created in sex-chromosome metaphase (solid red bars) and in sex-chromosome anaphase (hollow green bars).

Figure 7 is a bar graph comparing chromatid velocities with autosome velocities in the same cells: the distribution is of ratios of chromatid velocities divided by autosome velocities for chromatids separated from univalents during sex-chromosome metaphase (solid red bars) and for chromatids separated from univalents during sex-chromosome anaphase (hollow green bars).

Figure 8 is a bar graph comparing the velocities of partner chromatids (moving to opposite poles) separated from univalents during sex-chromosome metaphase (solid red bars) or during sex-chromosome anaphase (hollow green bars), presented as ratios of the larger velocity of the two partners divided by the smaller velocity.

Figure 9 is a bar graph comparing the velocities of partner chromatids (moving to opposite poles) separated from univalents during sex-chromosome metaphase (solid red bars) or during sex-chromosome anaphase (hollow green bars). This is different from the previous figure in that in this graph the ratio for chromatids separated in sex-chromosome anaphase is of velocity of the chromatid moving to the *nearer* pole (to which the univalent itself was moving) divided by the velocity of the chromatid moving to the *farther* pole.

Figure 10 graphically illustrates that the non-cut univalent reverses its direction of motion when a chromatid created from the other univalent passes its trailing kinetochore. The two kinetochores of each univalent are indicated by blue or red symbols. Prior to the first cut (of the univalent whose kinetochores are indicated by blue crosses and triangles), the blue univalent moved upwards (indicated by the green line of best fit for the leading kinetochore), and the red univalent moved downwards (indicated by the solid black lines of best fit for each of the kinetochores). The vertical blue line indicates when the blue univalent was cut; after the lower blue chromatid (triangles) passed the upmost kinetochore of the red univalent (crosses), the red univalent reversed its direction of motion (as indicated by the dashed black lines of best fit for each of the kinetochores). The vertical red line indicates when the red univalent was cut into chromatids, after which the two chromatids moved toward their respective poles.

Figure 11 illustrates altered univalent anaphase movement when the laser (green line in the inset) cut the spindle fibres (green vertical line in the graph): the lower univalent accelerated initially and then slowed down, to a much faster speed than usual univalent anaphase movement. The plot is of only the leading kinetochore of each univalent (the kinetochore closest to the pole toward which the univalent was moving). The solid lines are the best-fit lines to the kinetochores prior to the cut, and the dashed line is the best-fit line to the bottom kinetochore after the initial acceleration after the laser cut.

Figure 12 illustrates a lysed anaphase cell in which the laser cut the spindle fibres in two places (green lines in **A**). **B** and **C** are two confocal images of the same cell after staining for tubulin: B is the merged images of those sections closest to the plane of the cut, and C is the merged images of most of the planes throughout the cell. The arrows indicate where the microtubules are cut, seen most prominently in B, but even seen in C. The scale bar represents 10μm.

Figure 1

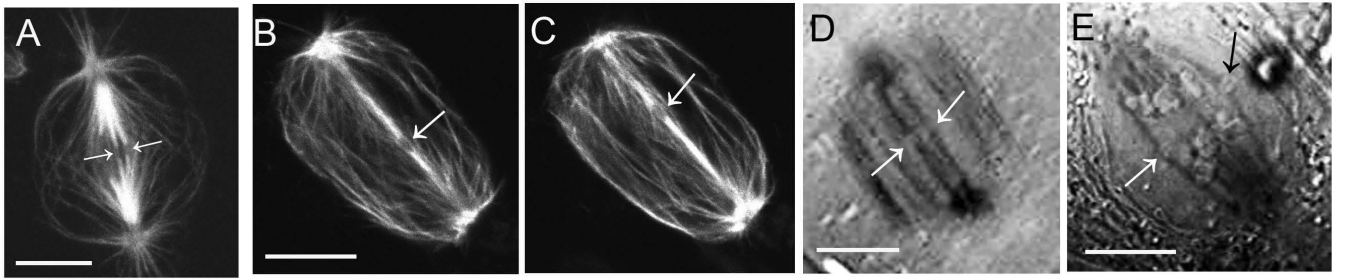

Figure 2

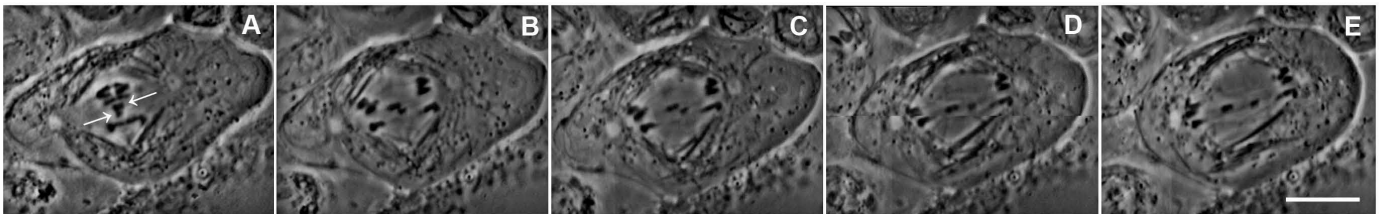

Figure 3

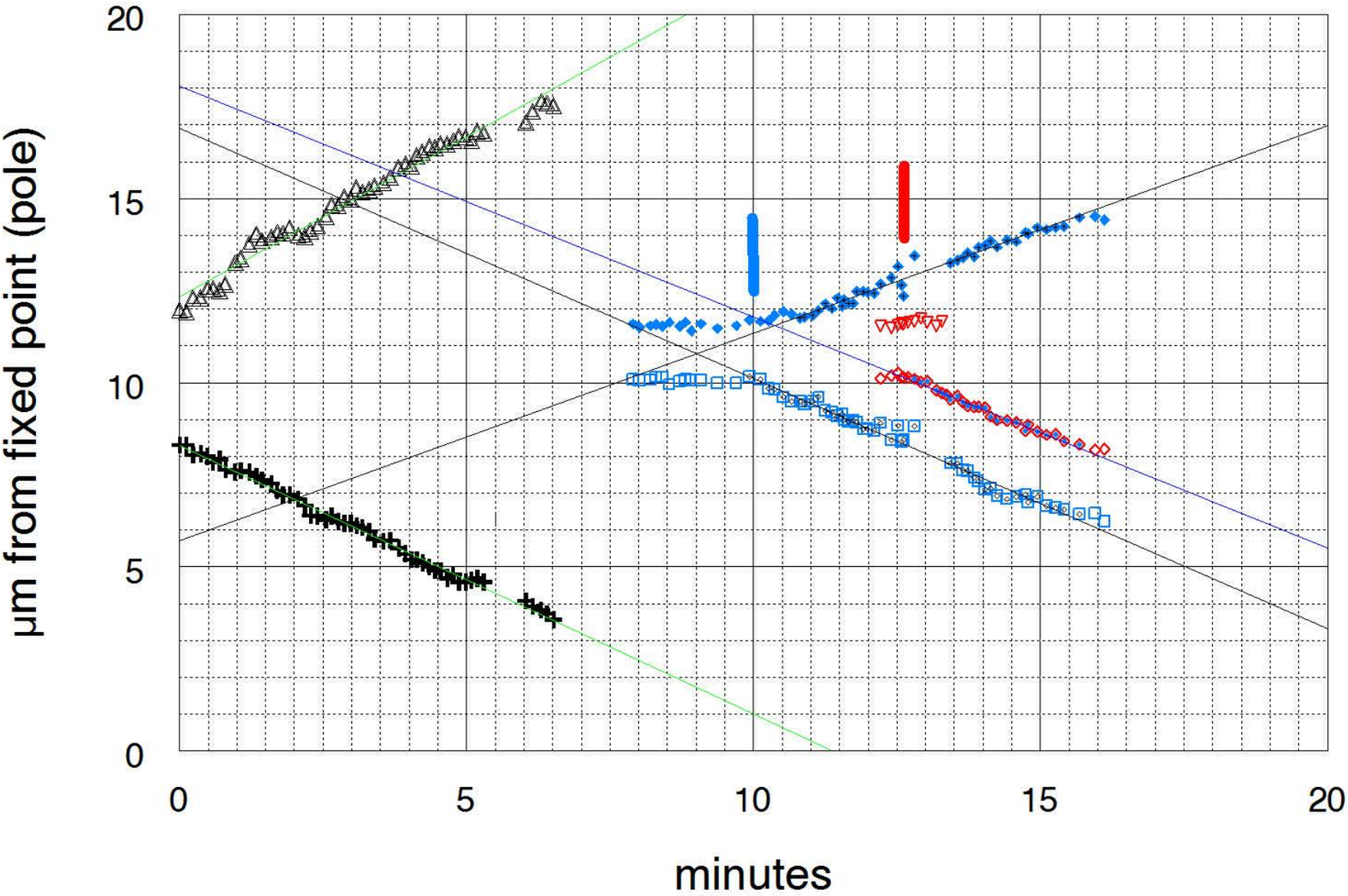

Figure 4A

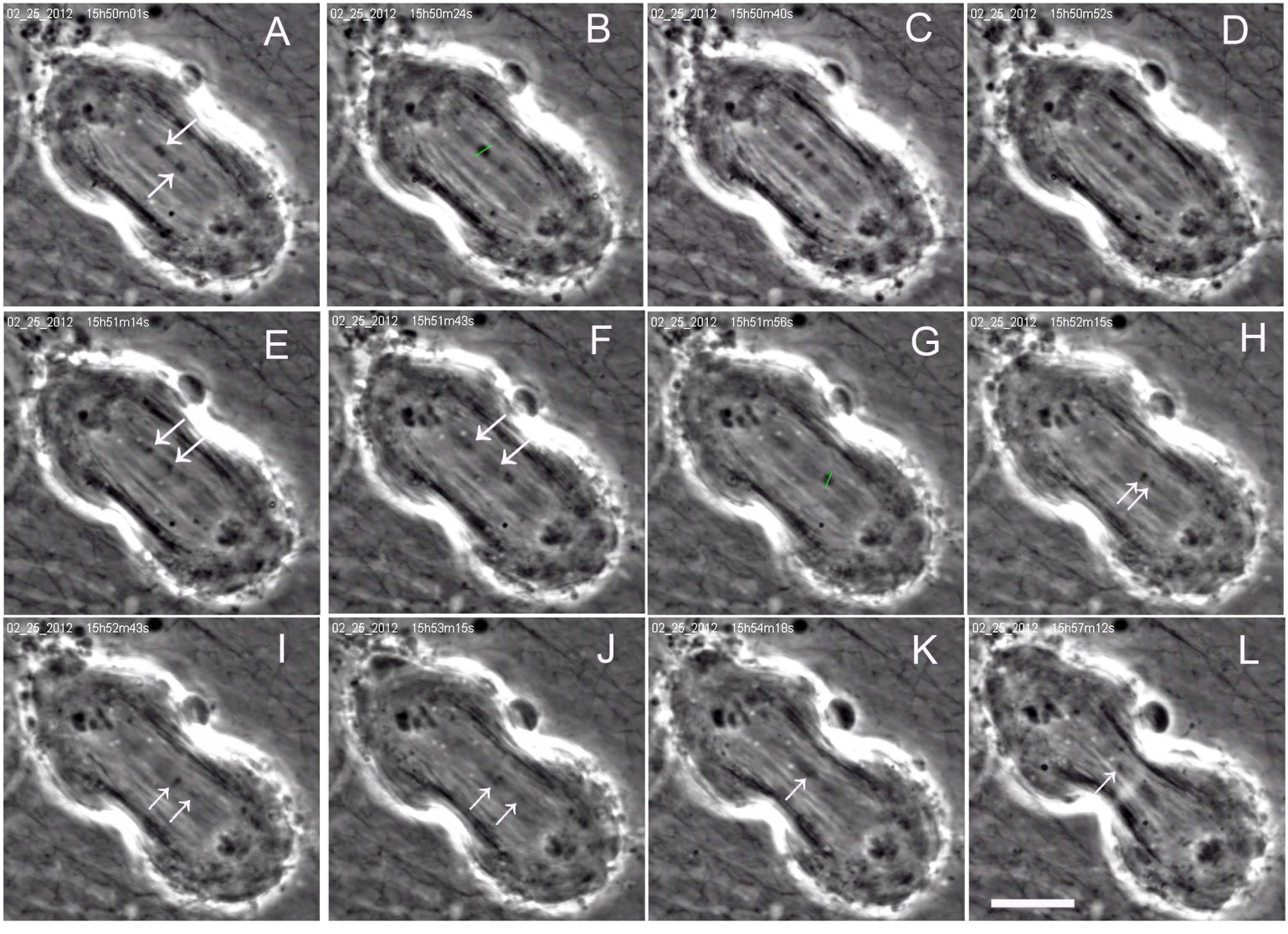
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Figure 4B

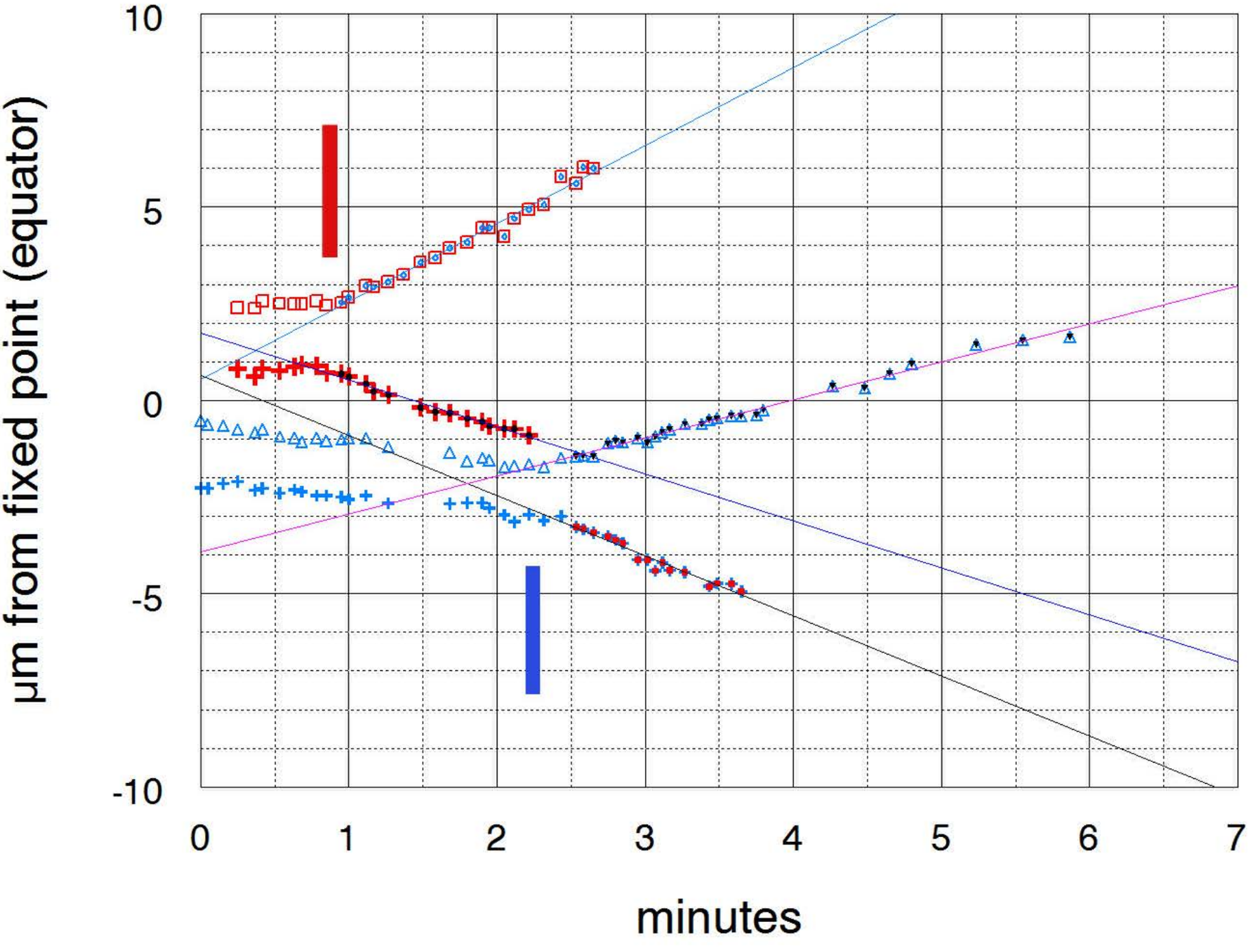

Figure 5

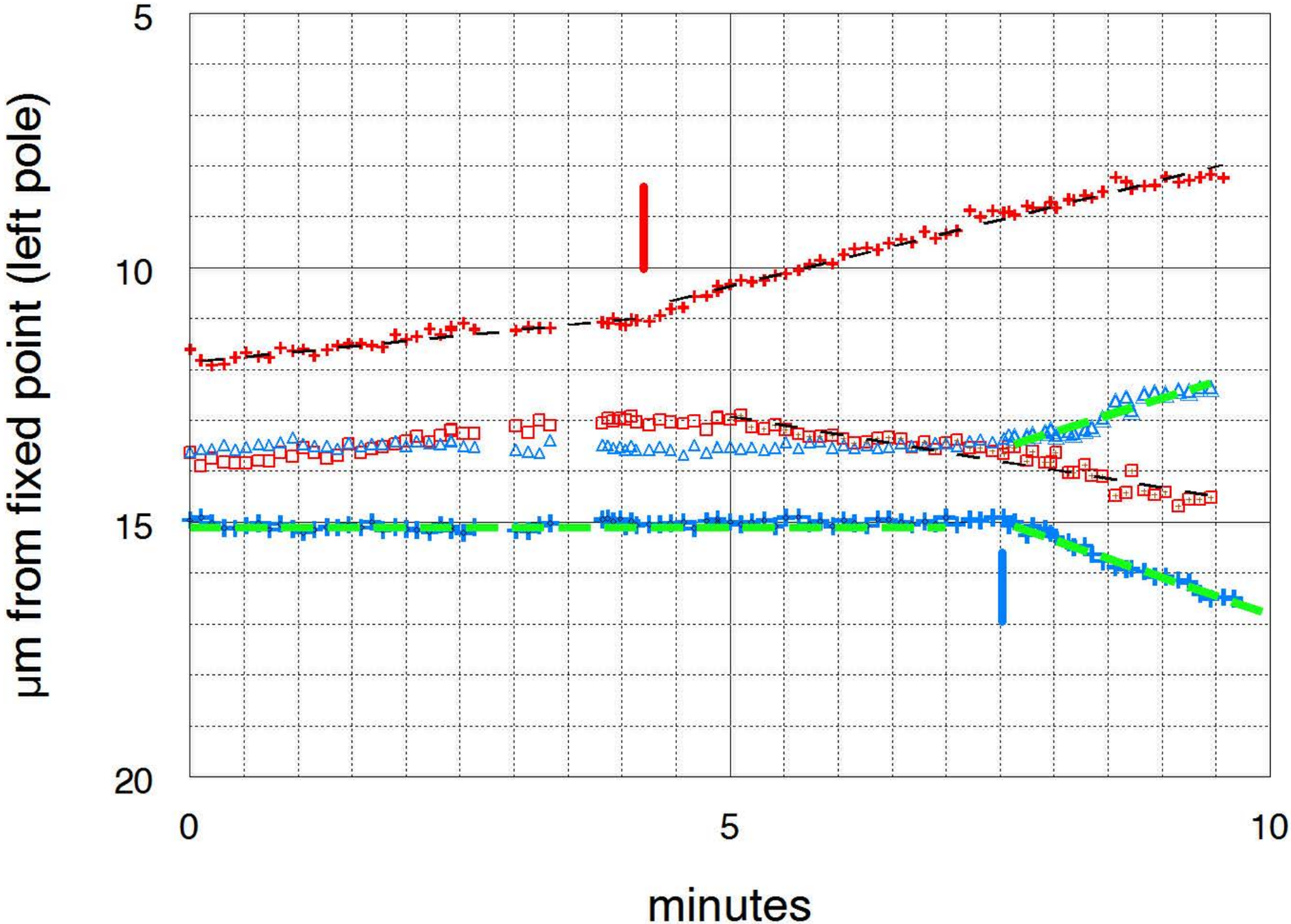

Figure 6: chromatid velocities

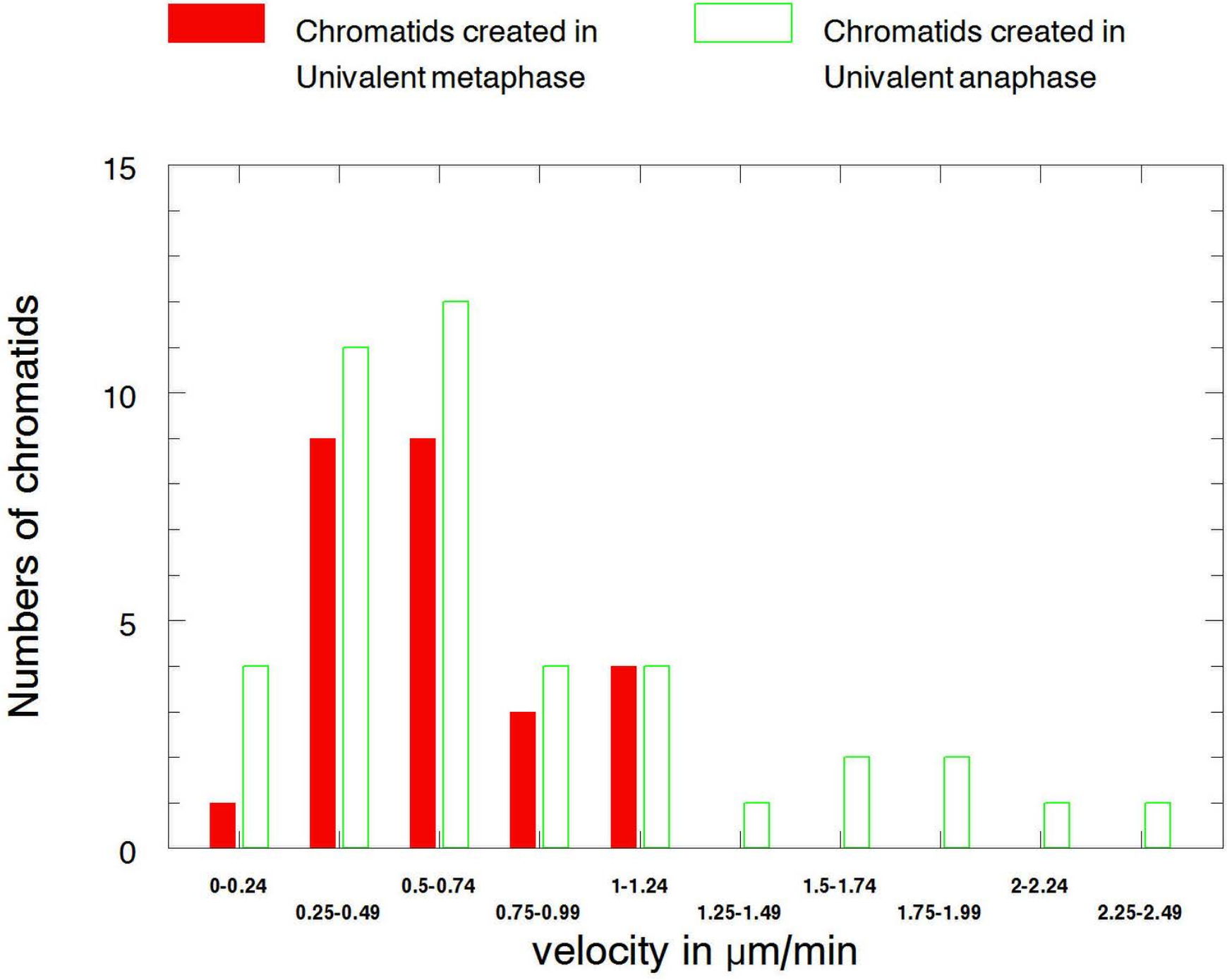

Figure 7: chromatid velocity/autosome velocity

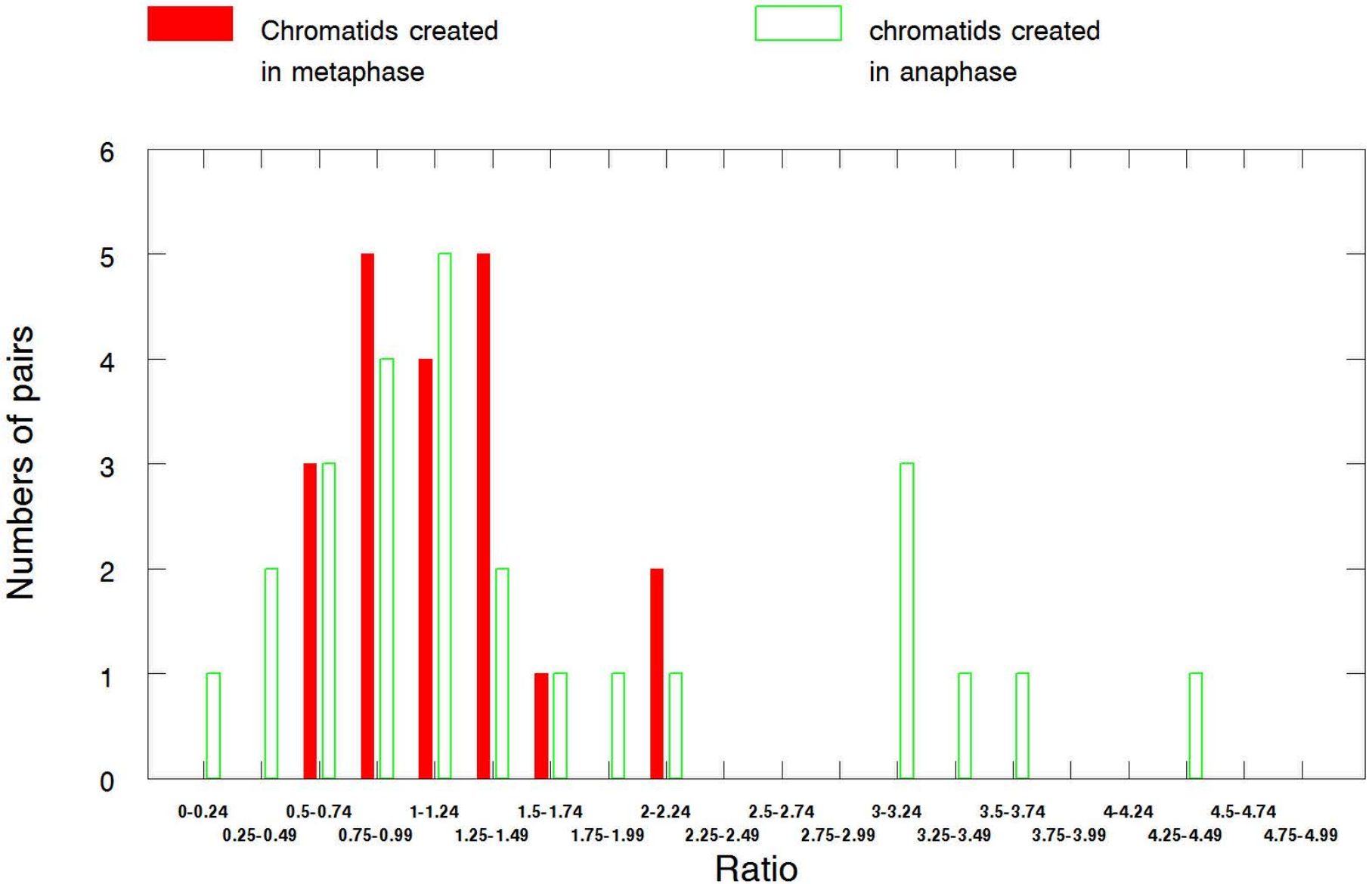

Figure 8 : Chromatid-pair velocity ratios

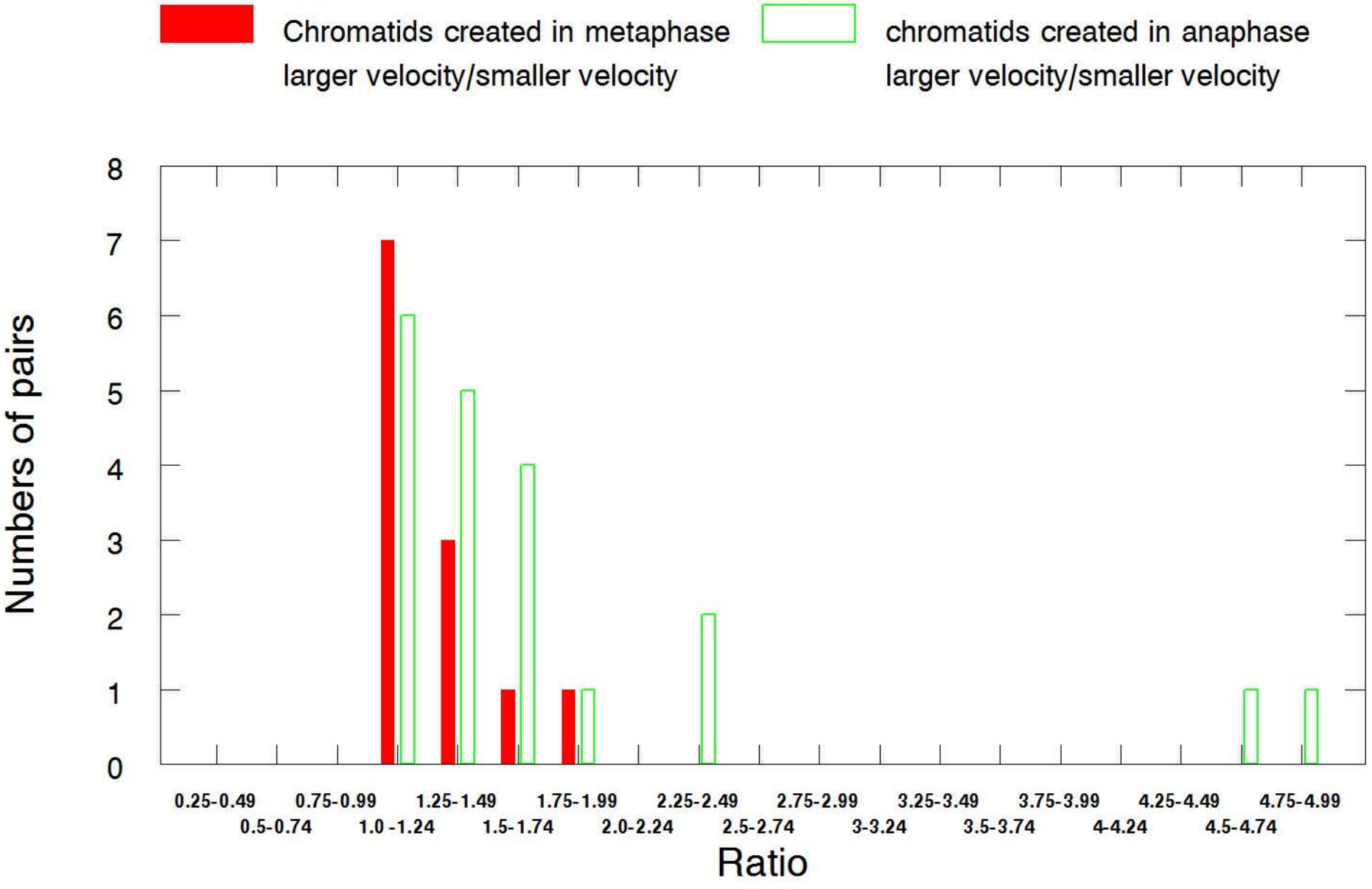

Figure 9: Chromatid-pair velocity ratios

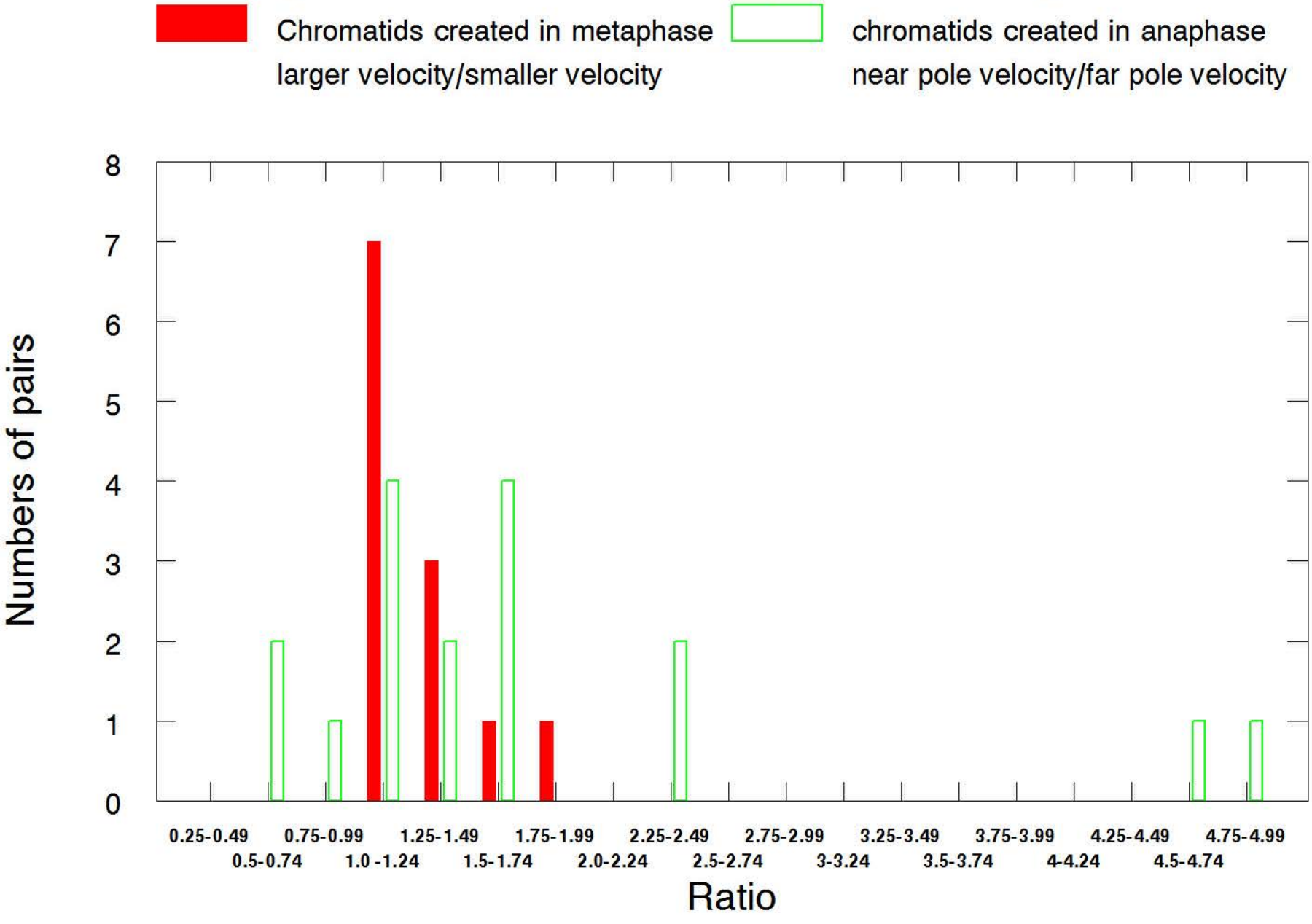

Figure 10

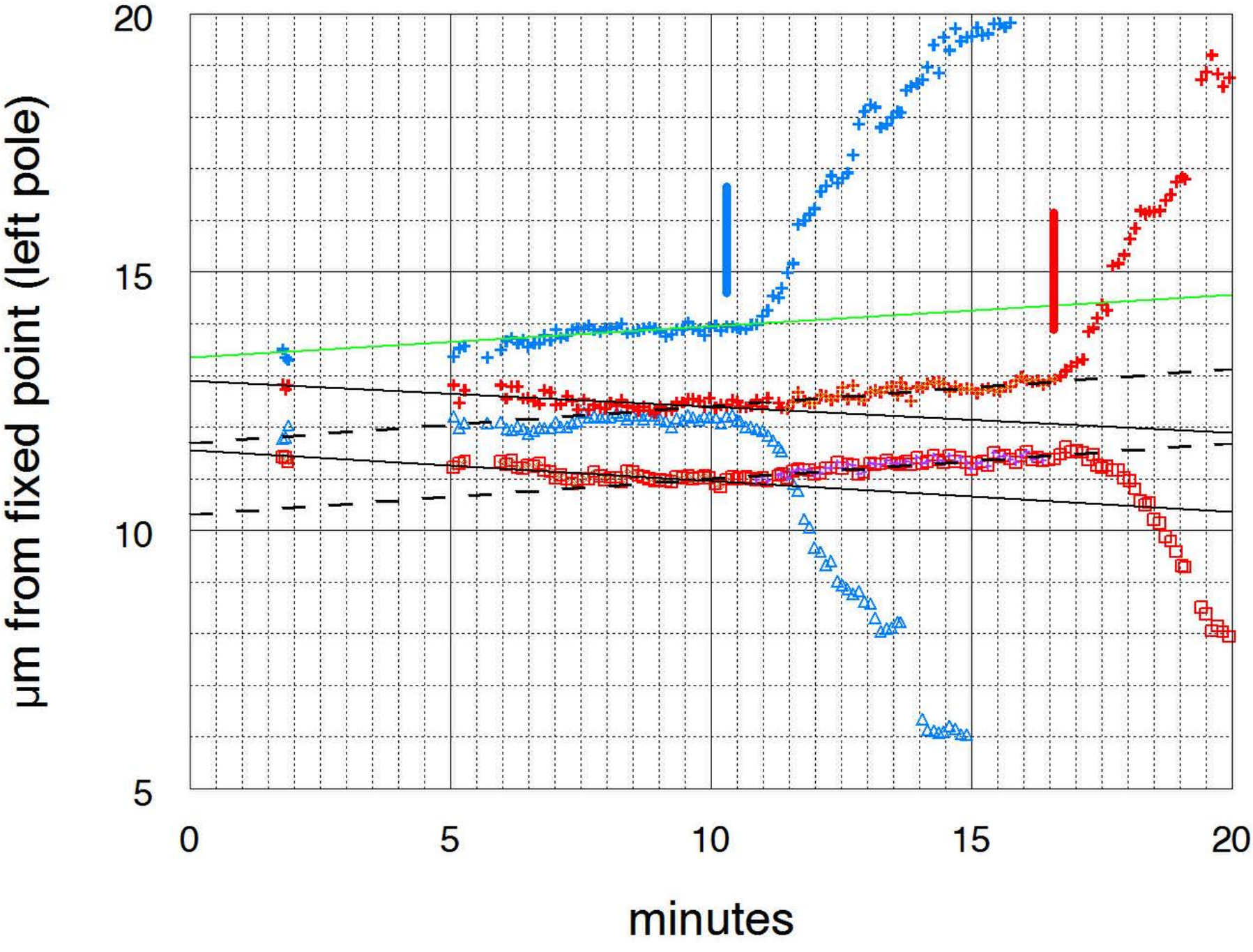

Figure 11

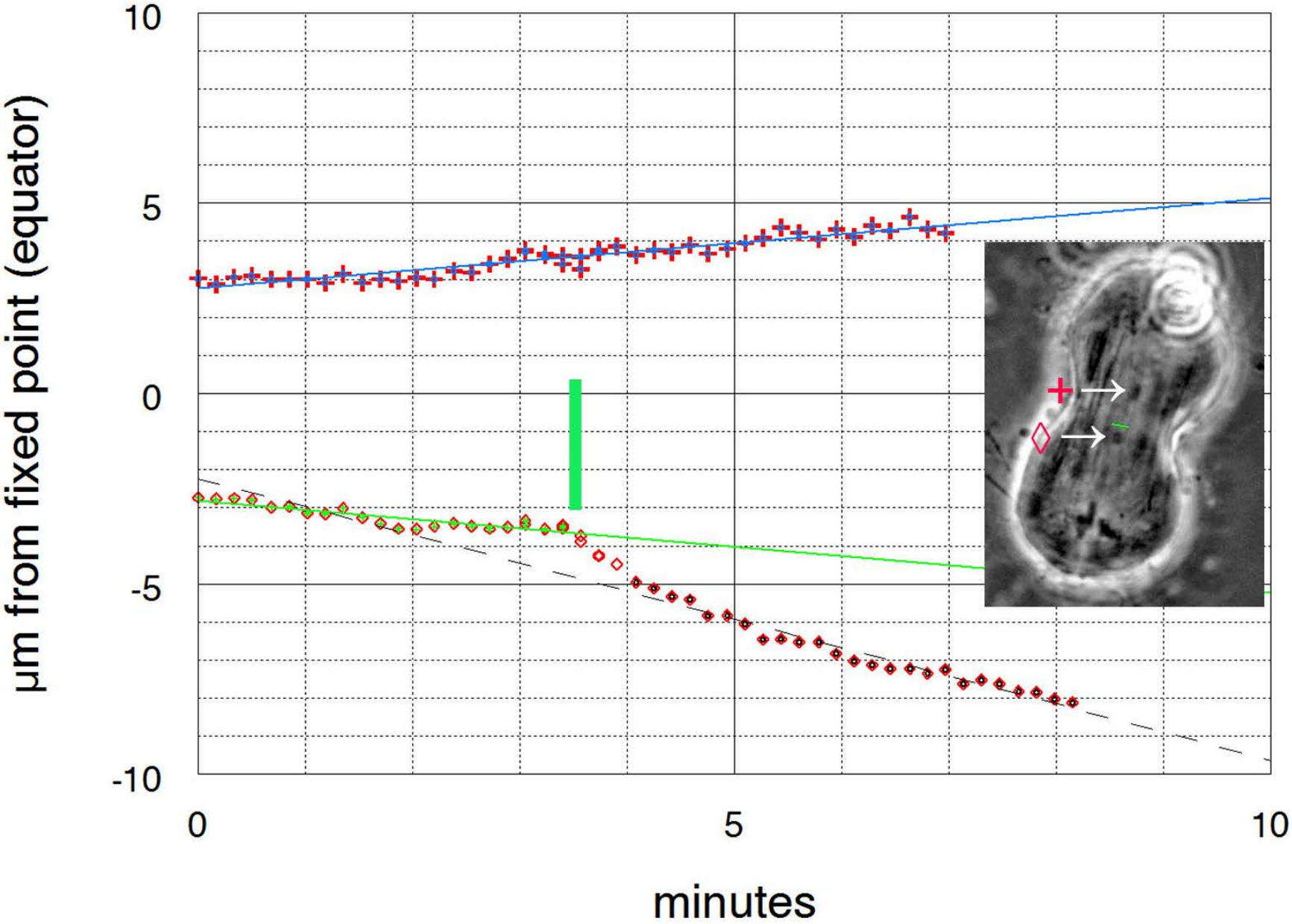

Figure 12