Chromosomes selectively detach at one pole and quickly move towards the opposite pole when kinetochore microtubules are depolymerized in *Mesostoma ehrenbergii* spermatocytes.

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ABSTRACT

In a typical cell division chromosomes align at the metaphase plate before anaphase commences. This is not the case in *Mesostoma* spermatocytes. Throughout prometaphase the three bivalents persistently oscillate towards and away from either pole, at average speeds of 5-6 μm/min., without ever aligning at a metaphase plate. In our experiments nocodazole (NOC) was added to prometaphase spermatocytes to depolymerize the microtubules. Traditional theories state that microtubules are the producers of force in the spindle, either by tubulin depolymerizing at the kinetochore (PacMan) or at the pole (Flux). Accordingly, if microtubules are quickly depolymerized, the chromosomes should arrest at the metaphase plate and not move. However, in 57/59 cells at least one chromosome moved to a pole after NOC treatment, and in 52 of these cells all three bivalents moved to the same pole. Thus the movements are not random to one pole or other. After treatment with NOC chromosome movement followed a consistent pattern. Bivalents stretched out towards both poles, paused, detached at one pole, and then the detached kinetochores quickly moved towards the other pole, reaching initial speeds up to more than 200 μm/min., much greater than anything previously recorded in this cell. As the NOC concentration increased the average speeds increased and the microtubules disappeared faster. As the kinetochores approached the pole they slowed down and eventually stopped. Similar results were obtained with colcemid treatment. Confocal immunofluorescence microscopy confirms that microtubules are not associated with moving chromosomes. Thus these rapid chromosome movements may be due to non-microtubule spindle components such as actin-myosin or the spindle matrix.

KEYWORDS: *meiosis, microtubules, non-random segregation, spindle matrix , nocodazole*

INTRODUCTION

The present work deals with what causes asymmetries in spindle behaviour and with what causes chromosome movements during cell division. Possible asymmetric spindle behaviour occurred after nocodazole (NOC) was added to *Mesostoma* spermatocytes during prometaphase. In general, lower concentrations of NOC cause kinetochore fibres to lose their formerly tight connection to the kinetochore and higher concentrations produce microtubule fragments and cause microtubules to detach from the centrosomes (Vasquez 1997; Yang et al. 2010). Similar effects are also seen with high concentrations of the microtubule depolymerizer colcemid (Yang et al. 2010). Therefore, after NOC is added to *Mesostoma* cells one would expect the chromosomes to stop moving and stay at the equator, as they do in other cells (Cassimeris et al. 1994). Instead, however, all the chromosomes selectively moved towards only one pole, in the one cell that was described (Forer and Pickett-Heaps 2010). One focus of this work was to see if all chromosomes consistently moved to one pole. Previous work on *Mesostoma* spermatocytes raised the possibility that the chromosomes segregate non-randomly in meiosis-I (Ferraro-Gideon et al. 2013), which might be a possible explanation for why all chromosomes move to the same pole, if indeed that occurs consistently. In non-random segregation, contrary to Mendel's law of independent assortment, partners of different bivalents in meiotic cells always go to the same pole. Non-random segregation was first observed between two chromosomes in spermatocytes of the mole cricket *Gryllotalpa borealis* by Payne in 1912, and later confirmed by Camenzind and Nicklas (1968). Since then it has been observed in a variety of species, such as liverworts (Lorbeer 1934), flea beetles (Wilson et al. 2003) and sciarid flies (Metz et al. 1926), as reviewed in Brady and Paliulis (2015). For example, in mealy bugs and *Sciara*, all male-derived autosomes go to one pole and all female-derived chromosomes go to the other pole (mealy bugs: Schrader 1921, 1923; Nur 1982. *Sciara*: Metz et al. 1926; Metz 1938; Fuge 1994, 1997).

There are two pieces of evidence that suggest that *Mesostoma* spermatocytes might divide via non-random segregation. The first is univalent excursions (Oakley 1983). In *Mesostoma* spermatocytes, bivalents are bipolarly oriented and the two univalent pairs are unipolarly oriented. By anaphase each univalent is present at a spindle pole (Figure 1), one of each kind at each pole, but throughout prometaphase univalents often move between poles, presumably to correct errors in segregation. Since there are more excursions than necessary to correct improper segregation if the two partners of each univalent pair were assorting randomly, Oakley (1985) suggested that the univalents might segregate non-randomly. Another line of evidence is from bivalent reorientations (Ferraro-Gideon et al. 2014). The bipolarly oriented bivalents in *Mesostoma* spermatocytes are clearly under tension because the bivalents elongate as the bivalents oscillate along the spindle axis. They nonetheless frequently reorient during prometaphase, and since in most reorientations the bivalent kinetochores switched poles, i.e., reversed their initial segregation, this might suggest that the bivalents, too, segregate non-randomly (Ferraro-Gideon et al. 2014; Brady and Paliulis 2015).

In this article we present a third possible piece of evidence. When we depolymerized *Mesostoma* spermatocyte spindle microtubules using nocodazole (NOC), the three bivalents

detached selectively from one pole in 52 out of 59 cells and all moved very quickly to the opposite pole. This supports the suggestion that non-random segregation occurs in these cells.

The second main issue from our work is what causes chromosome movement in the apparent absence of microtubules. Microtubules are considered the producers of forces that move chromosomes during cell division. According to the prevalent hypotheses, microtubules produce forces either because microtubules shorten at the poleward end (Cameron et al. 2006), the traction fibre or flux model, or because microtubules shorten at the kinetochore end, the PacMan model (Rieder and Salmon, 1994). However, there is growing evidence suggesting that microtubules may play a more passive role as laid out in the spindle matrix model (Johansen and Johansen 2007; 2011, Pickett-Heaps and Forer 2009, Spurck et al. 1997). In this model, microtubules impede rather than assist chromosome movement, and in their absence chromosomes are able to move quicker (Forer et al. 2015). Some of the evidence for this is as follows. When kinetochore fibres are cut during anaphase, chromosomes can continue polewards movement. In grasshopper (Gordon and Inoué 1979; Gordon 1980) and newt fibroblasts (Spurck et al. 1997, Pickett-Heaps et al. 1996) kinetochore fibres were severed with a UV microbeam and chromosomes continued moving and moved faster than previously. Crane-fly spermatocyte chromosomes do not change speed when their microtubules are severed, but do speed up when the "tether" that connects separating chromosomes is first cut with a laser (Sheykhani et al. 2017). These data suggest that microtubules limit the velocity of chromosome movements so that the chromosomes speed up when the 'impeding' microtubules are severed. There are related data from metaphase cells, also, as follows.

Chromosomes are at the equator at metaphase because forces on them to each pole are equal. Thus, when microtubules are depolymerized it is expected that chromosomes should stay in the middle of the cell and arrest at the equator, which occurs in many cells (Cassimeris et al. 1994). However, when kinetochore fibres were depolymerized during metaphase in diatoms, the bipolarly attached chromosomes detached at random and moved to either pole at random (Pickett-Heaps and Spurck 1982). Pickett-Heaps and Spurck (1982) argued that microtubules resisted force from another spindle element, the spindle matrix, and that once colcemid was added and the resistance was gone, the matrix pulled the chromosomes towards either pole at random. In our experiments, immunofluorescence images show that after NOC treatment *Mesostoma* spermatocyte microtubules are highly fragmented and are not associated with the moving kinetochores when they move. Since the selective movement of chromosomes to one pole occurs in the absence of microtubules, this raises the issue of what causes their movement.

MATERIALS AND METHODS

Living cell preparations and drug treatment

Mesostoma were reared in the lab as described in detail by Hoang et al. (2013). To study live spermatocytes, their testes are sucked up using pulled 5, 10 or 15 μL micropipettes (Fisher) and then expelled onto a cover slip with *Mesostoma* Ringers solution (61 mM NaCl, 2.3 mM KCL, 0.5 mM $CaCl_2$ and 2.3 mM phosphate buffer) that contains 0.2 mg/mL fibrinogen (Calbiochem). Thrombin is added, to cause formation of a fibrin clot, as previously described (Forer and Pickett-

Heaps 2005, 2010; Ferraro-Gideon et al. 2014), and the cells then immersed in *Mesostoma* Ringers solution in a perfusion chamber. In order to ensure the cells were healthy, they were recorded for at least 10 minutes to track several cycles of bivalent oscillations. After concluding that the cells were healthy, the cells were perfused either with *Mesostoma* Ringers, as a control, or with drugs: 5μM, 10μM, 20μM NOC in *Mesostoma* Ringers (Sigma) or 10μM, 20μM, 50μM, 100μM colcemid in *Mesostoma* Ringers (Sigma). Both drugs were diluted with Ringers from a 1000x concentrated stock solution in Dimethyl sulfoxide (DMSO). Addition of diluted DMSO by itself had no effect on oscillations (Forer and Pickett-Heaps 2010). The cells either were left in drug or rinsed out with Ringers solution 10-15 minutes later. The cells were followed on average for 45 minutes after drug addition. Live cells were viewed with phase-contrast microscopy using a Nikon 100x oil immersion lens (NA 1.3) and recorded in real-time on a DVD recorder.

Data Analyses

The free online program VirtualDub (www.virtualdub.com) was used to time-lapse the movies. We then imported the videos into our in-house program WinImage (Wong and Forer 2003). The position of each visible kinetochore in each frame was marked. The poles as seen electron microscopically are at the cell membrane at the ends of the spindle. They are not visible as discrete positions as viewed in phase-contrast microscopy, and if we were to measure kinetochore positions versus the pole, the pole positions we chose would invariably vary in different images since we cannot see discrete pole positions. The cells do not move when held in a clot so to overcome the variability in choosing pole position, in the first frame we analysed we chose a point that seemed to be 'the pole', and we used that same, fixed pixel position to measure against in all subsequent images. Thus the movement graphs are in comparison to a fixed point at or near the spindle pole (see diagram in Figure 2B); the values obtained may be slightly off in terms of actual distances from the pole, depending on how close our fixed point is to the actual pole position, but they are close, and they are accurate in terms of speeds and distances that the kinetochores travelled. Distances were calculated by the WinImage program and were converted to movement graphs using the commercial program SlideWrite. Adobe Photoshop was used to construct diagrams representing different kinds of chromosome movement and to generate montages from a series of single images. Student's t-test was done to compare the different times and velocities of the different concentrations of drugs.

Immunostaining

Cells that were destined for immunostaining were not perfused with drug. After the prep was made and the cells were deemed healthy, the coverslip was directly submersed in a Petri dish with the desired concentration of drug for varying lengths of time (less than 5 seconds, 30 seconds, 1 minute, 3 minutes, 4 minutes, 6 minutes, 10 minutes) and then added to a lysis buffer with glutaraldehyde. Control cells that were not treated with drug were immediately placed into this lysis buffer. This lysis buffer, called NTSDc, contains 3% NP40, 2% Triton X-100, 2% Saponin, 5% DMSO, 0.5% Sodium Deoxycholate and 0.25% glutaraldehyde, all in PEM. (PEM is 100mM PIPES, 10mM EGTA, 5mM MgSO4 adjusted to pH 6.9.) After 2 minutes in NTSDc the preps were then put for 1 to 2 hours into NTSDc without glutaraldehyde. Preps were then rinsed twice with phosphate-buffered saline (PBS) for 5 minutes each, then put in sodium borohydride (1 mg/mL) for up to 10 minutes in order to neutralize the free aldehyde groups, then rinsed twice

again in PBS for 5 minutes each. The preps were then stored in a solution containing equal parts PBS:Glycerol at 4°C for up to two weeks.

Both primary antibodies were added at the same time, and both secondary antibodies were added at the same time. The primary antibodies were anti-tubulin YL1/2 rat monoclonal antibody diluted 1:1000. The secondary antibodies were Alexa 568 or 594 goat anti-rat diluted 1:100. All antibodies were from Invitrogen, Burlington, ON, Canada, and all antibody dilutions were in PBS. The incubation time was 24 hours at room temperature in the dark for each double antibody stain, after which they were submersed in PBS twice for 5 minutes. Before adding the next double antibody, the preps were rinsed in PBS containing 0.1% Triton X-100 to aid in spreading the antibodies. Once the staining was complete, the preps were submersed in PBS twice for 5 minutes, then PBS: glycerol was added on top of the cells, to allow the glycerol in the Mowiol (described in the next step) to penetrate into the cell layer. Coverslips were then placed in Mowiol (Osborn and Weber 1982) which contained 0.2 g/L paraphenylene diamine (PPD) as anti-fading agent (described in Fabian and Forer 2005), put onto slides, and left to harden for 24-48 hours, after which they were stored at 4°C until viewed with the confocal microscope. The cells were viewed using an Olympus Fluoview 300 confocal microscope using an Olympus plan-apo 60x objective, NA 1.4. Images were taken using the Fluoview 300 program, and colour was added to Z-projected stacks using the free online program ImageJ. Tubulin is shown in green and DIC in black and white.

RESULTS

Control cells

Bivalent oscillations

Bivalent chromosomes in *Mesostoma* spermatocytes constantly oscillate towards and away from their respective poles, from the time the bivalents are bipolarly oriented until anaphase (Fuge 1987, 1989; Oakley 1983, 1985; Ferraro-Gideon et al. 2013, 2014). An example of oscillation in one bivalent is illustrated in Figure 2A, images A-C. The arrows indicate the position of the bottom and top kinetochores of an oscillating bivalent. Between A and B the bottom and top kinetochores of the bivalent both oscillate towards their respective poles, and from B to C they both oscillate away from their pole returning to their original positions. The oscillations of the two kinetochores of one bivalent vary during prometaphase (Ferraro-Gideon et al., 2014): sometimes they both go towards the pole at the same time as in Figure 2A, thereby stretching the chromosome, and sometimes one moves toward its pole while the other moves away, so the chromosome moves as a whole. Different bivalents oscillate independently, with different speeds and directions of their two kinetochores (Ferraro-Gideon et al., 2014). Chromosome movement is also shown graphically by plotting the relative distance of each bivalent's kinetochore to a fixed position (shown by the X) near the pole, seen in Figure 2B. The oscillation speeds towards and away from the pole differ: movement away from the pole averages 5.2 μm/min. and towards the pole 6.2 μm/min. (Ferraro-Gideon et al. 2014). Since bivalents do not stop oscillating before anaphase, they never align along a metaphase plate. There is question whether the entire period should be called prometaphase or metaphase, or whether prometaphase lasts only until the univalents are properly oriented at the

two poles after which the stage is metaphase (discussed in Fuge 1989); we will consider that the majority of meiosis-I is spent in prometaphase, which lasts up to two hours, after which the cell goes directly into anaphase-I, with continuing oscillations throughout prometaphase but no stable intervening metaphase (Figure 3 i).

Second division

Meiosis II has not been studied in these cells, and we have been able to follow only 3 cells from meiosis-I to -II. After completing anaphase-I the two resultant nuclei form two groupings of chromatids which then arrange themselves into a hollow circle in the newly reformed nucleus (Figure 3i). These observations appear similar to Steven's early drawings of spermatogenesis (1905, 1906). Although not pictured here, the nuclei and their chromatids rotate back and forth. Two or more hours after anaphase-I completion, the cells enter meiosis-II as a spindle is set up and the chromatids begin oscillating up and back in the spindle at speeds similar to those recorded in prometaphase-I.

Precocious furrow.

Another unusual feature to this cell's meiosis-I is that most *Mesostoma* spermatocytes have a precocious, pre-anaphase cleavage furrow, as shown in Figure 2A and 3 i). This furrow moves in response to chromosome imbalances in the two half spindles which can occur throughout prometaphase. When a univalent excursion or a bivalent reorientation occurs, for example, the furrow responds by shifting an average of 1-2 μm towards the half cell with fewer chromosomes (Pickett-Heaps and Forer 2010; Ferraro-Gideon et al. 2013, 2014). This creates a cell with one half cell that is smaller than the other.

NOC-treated cells

Chromosome movement

There were three different responses after addition of nocodazole (NOC). Chromosomes (1) detached from only one pole, (2) detached from both poles, or (3) poles collapsed. We describe these responses in turn.

Response (1), detached from only one pole. When chromosomes detached from only one pole (52/59 cells), they always followed a consistent 6-stage pattern of movement (Figures 2B, 2C). After addition of drug, the chromosomes stretch towards both poles, pause for some period of time, detach at only one pole, and the detached kinetochores quickly move towards the opposite pole, slow down and then stop. The 'stretch' increases the length of the bivalent by about 25-30% from its length immediately prior to NOC, and the length decreases back to its original length immediately after detachment, in the first 10-15 seconds during the fast movement, after which the movements of the kinetochore take place at constant length. In most cases, only the bivalents moved, but in 14 cells both the bivalents and univalents detached and moved to the same pole. In 7 of these 14 cells the univalents moved at the same time as the bivalents, and the rest moved from times varying from 1 to 15 minutes later (e.g., Figure 2A and supplemental video 3). The univalents

that moved at the same time as the bivalents moved with the same speed as the bivalents. The other 7 moved with average speed of 11.8 ± 2.6 μm/min (s.d), range 8.7-16.5 μm/min

In order to understand the forces acting on the chromosome kinetochores it is worth looking more carefully at the manner in which the chromosomes move, and the speeds of the movements, to see, for example, whether the movements could be due to recoil of the stretched chromosomes after release from a pole or whether other forces need be invoked. Immediately after detachment the kinetochores move extremely rapidly toward the opposite pole, usually at an angle to the direction the kinetochore partners were aligned in, and the kinetochores do not move in a straight line (Figure 4A, B). As the one kinetochore moves the bivalent bends at an angle to its original orientation, as illustrated in Figure 4 B i-iii) and supplemental video 1. Bivalents in any given cell often move at the same time, but each kinetochore and bivalent move independently, and not as a linked group: the chromosome arrangements change during movement and some move farther than others. Furthermore in some cells, bivalents detach at separate times, though all the chromosomes nonetheless move towards one pole (Figure 5). The drawings in Figure 6 illustrate the configurations at the final 'stop' positions.

Response (2), detached from both poles. In 5/59 cells one bivalent detached from either pole. [One such cell is shown in supplemental video 4.] This resulted in a mix of some bivalents moving towards one pole, and some moving to the other pole. As illustrated in Figure 6 (2), the left bivalent detached from the top pole, while the right bivalent detached from the bottom pole. Sometimes one of three bivalents did not detach and remained in the stretched configuration, such as the middle bivalent in Figure 6 (2). Univalents always remained at the poles.

Response (3), chromosomes remain in the middle. Chromosomes stretched out towards either pole as in responses (1) and (2), paused at either pole, then the bivalents shortened slowly. There was no visible, abrupt detachment from either centrosome. Later the univalents at both poles slowly drifted < 3 μm/min towards the cell equator and bivalents shortened as the univalents and bivalents moved away from the poles as a clump, for a short distance, never reaching the equator [one example is shown in supplemental video 5]. This response occurred very rarely, in only 2/59 cells.

Washout

We assume that the chromosome movements that occur after NOC treatment reflect on the functioning and organisation of the force-producing apparatus in normal cells. Because treated cells change drastically after treatment with NOC, however, and do not look 'normal', we need to be sure that chromosome movements after NOC reflect the ordinary function of the spindle and are not abnormal, pathological movements, induced by imminent apoptosis. Several lines of evidence indicate that the cells do not die after NOC treatment. For one, in the continuing presence of NOC the furrow ingresses and moves toward the pole with fewer chromosomes (Fegaras and Forer, 2017), sometimes moving off the end of the cell but often forming two different sized cells, still attached, resembling somewhat the appearance of a "shmoo" (Capp, 1948), seen in supplemetal video 3. Further, univalents left behind at a pole sometimes move across the equator

to the opposite pole, up to 15 minutes after addition of NOC. These all indicate that the cells may be wounded, but that they are alive and functioning.

To further study whether the cells are alive and functioning, we washed out the NOC after the kinetochores moved to the opposite pole, to test whether normal cell activity resumes after removal of NOC. After washout the spindle does not reform but the cells undergo changes reminiscent of the changes that occur between meiosis-I and meiosis-II in control cells, as illustrated in Figure 3 ii). In that particular cell, NOC was washed out 7.5 minutes after NOC was added, shortly after the kinetochores moved to one pole. Subsequently the cell developed morphological changes characteristic of spermatocytes between meiosis-I and meiosis-II, namely formation of an apparent nucleus, and then formation of the chromatid clump and hollow circle phases, and even developing what looks like a spindle. Although the chromosomes oscillate up and back in direction of the spindle axis, in what appears to be a meiotic-II spindle, the oscillations are irregular, are slow, the chromosomes remain in a clump, and it is hard to discern the position of the kinetochores. Figure 3 iii) and iv) provide a comparison of the NOC treated cell and a control cell about to enter second division, showing that the phases between division are very similar in both circumstances. The NOC treated cells take a prolonged period of time before attempting to enter second division, up to 7 and a half hours instead of the usual 2 hours. We have not been able to follow all of the cells for which we washed out the NOC for the >7 hours to form a meiosis-II spindle, but we have seen chromatid clump and hollow circle phases in the majority of cells after NOC washout. We have not seen meiosis-II anaphase in cells after washing out the NOC, but all the changes that occur as described above indicate that the NOC itself was not lethal to the cells – chromosomes move, the cleavage furrow functions, and the cells differentiate toward meiosis-II - and therefore that the movements that occur as a result of NOC treatment are not pathological but are due to normal forces in the living cell.

Colcemid-treated cells

To test whether the results after treatment with NOC were due to depolymerization of microtubules and not to side effects of the NOC, we treated cells with colcemid, another agent that depolymerizes microtubules. NOC suppresses microtubule dynamics by decreasing the elongation and shortening rates and thereby increases catastrophes; colcemid at high concentrations causes microtubules to fragment and depolymerize (Vasquez 1997; Jordan et al. 1992) and may also cause the centrosome to fragment (Jordan et al 1992). In comparison, colcemid at low concentrations binds to microtubule plus-ends and can stabilize microtubules, and at higher concentrations it binds to minus ends and interferes with microtubule dynamics, and may also promote microtubule detachment from the centrosome (Jordan and Wilson 2004; Yang et al. 2010).

The results after treatments with higher concentrations of colcemid are identical to those after treatments with NOC, but results with lower concentrations of colcemid are different than after NOC, perhaps due to their different mechanisms of action. At lower concentrations of colcemid (10 μM and 20 μM), bivalents continued oscillating for a short period of time with continually decreasing amplitude and period (Figure 7B, and supplemental video 2), and bivalents did or did not detach abruptly. In all cells treated with 10 μM colcemid bivalents stretched and remained stretched in the middle of the cell (response 3 of Figure 6). At 20μM colcemid the

chromosomes detached from one pole in 3 out of 6 cells, and the detached chromosomes slowly moved towards the opposite pole. The number of oscillations that occurred in the presence of colcemid depended on the colcemid concentration (Figure 7A). At the higher concentrations of 50 and 100 μM colcemid, all bivalents detached and moved to one pole (Figure 7C) (response 1 of Figure 6) similar to treatment with NOC. Thus, when depolymerisation of microtubules is rapid, the colcemid effects are the same as those after NOC, and thus the effects we observe are due to depolymerisation of microtubules.

Sidedness

What is the difference between the two poles, such that all the chromosomes detach from one and move to the other? We have not been able to tell which pole the chromosomes will detach from by scrutinising the video sequences and graphs and looking at the behaviour of the bivalents, univalents, and the furrow before addition of drug. We considered various possibilities. For example, if bivalent kinetochores at one pole oscillated at an irregular speed that may be the pole from which bivalents detach. Or, if a univalent excursion recently occurs from one pole, perhaps that is the pole the bivalents will detach from. We also analysed kinetochore oscillation directions to see if they would indicate the pole to which the chromosomes would move after addition of NOC, but they gave no indication: when a bivalent was oscillating towards a pole before addition of drug, the kinetochore did not necessarily move toward that pole after NOC. None of these parameters allowed us to predict the sidedness. We then considered whether furrow asymmetry might indicate the sidedness of the response. Asymmetric cells occur when there is an unequal distribution of chromosomes, for example three univalents at one pole and one at the other (Pickett-Heaps and Forer 2010). We treated asymmetric cells with NOC or colcemid to see if chromosomes would consistently move into either the larger or smaller half-cell (Table 1). If the response was due to the location of the furrow there would only be bivalent movement to the larger or smaller half-cell. However, chromosomes were equally likely to move into the larger half-cell or the smaller half-cell when treated with either NOC or colcemid (Table 1). Thus, the non-random movement of chromosomes, the sidedness of the spindle, is due to some factor we have not yet determined.

Chromosome speeds increase as drug concentration increases

The speeds that chromosomes move after they detach from one pole after addition of NOC or colcemid increase with increasing concentration of drug (Figure 8A). The speeds for chromosome movement are significantly different at each drug concentration, and are much larger than prometaphase oscillation speeds in non-treated *Mesostoma* spermatocytes, which average 5.1 μm/min. (Ferraro-Gideon et al. 2014). Cells treated with 10μM NOC most often had speeds in the 36-40 μm/min. range, with a maximum speed of 100 μm/min. Cells treated with 20 μM NOC had the fastest speeds; 4 cells had speeds over 100 μm/min., up to 145 μm/min. (Figure 8B). [Chromosome speeds indicated in Figure 8B and throughout are low estimates. They derive from graphs with data points 2 seconds apart. When 30 points were obtained per second, as in Figure 4

B (iv), speeds were above 200µm/min.]. In cells treated with lower concentrations of colcemid, the chromosomes did not detach and chromosome speeds were more in line with speeds in control cells, whereas in cells treated with higher concentrations of colcemid the chromosomes detached and moved with speeds in the same range as after NOC treatment (Figure 8A). We tested whether the increased speeds were related to the length of the stretch period and the length of the stretch itself before chromosomes detached.

Chromosome speeds increase as the times to detachment decrease, but are independent of stretched length.

The fast speeds of chromosome movement did not seem to be related to the length of the stretched chromosomes (Figure 8C) but did seem related to NOC concentration. As the NOC concentration increased, the stretch duration and pause duration decreased, while the fast speed of chromosome movement increased (Table 2). We considered "the time to detachment" as the stretch and pause duration lumped together. The relationship between the time to detachment and chromosome speeds is shown graphically in Figure 8D. Based on the trend of the data, chromosomes that detach and begin movement earlier are more likely to move at a quicker speed, whereas chromosomes that take a longer time to detach move at a slower speed. Chromosomes in 5µM-NOC-treated cells take the longest time to detach, and have the slowest speeds, whereas those in 20µM-NOC-treated cells generally detach quickly and move the fastest (Figure 8D). This suggests that the faster the spindle microtubules are lost the faster the chromosomes move.

But we need to test when microtubules are lost after the drugs are added.

Immunofluorescence

Control cells

Staining for tubulin in *Mesostoma* primary spermatocyte cells shows that the spindle consists of many thin non-kinetochore microtubules and a few thick kinetochore fibers (Figure 9 and 10A). There are also brightly stained centrosomes at each pole. The kinetochore fibers radiate from the centrosome and terminate at the kinetochore of a bivalent. In the cell illustrated in Figure 9, image Ai, there are two easily distinguishable kinetochore fibres at the top pole connecting to two separate bivalents near the top pole, and one very thick bundle at the bottom pole that is associated with one of the bivalents near that pole. In Figure 9, image Bi there are two kinetochore fibers at the bottom pole both of which connect to the bottom kinetochore.

NOC treated cells

The microtubules of the meiotic spindle immediately began to fragment and depolymerize after NOC addition. This is illustrated in Figure 10, a montage of single confocal microscope sections of spindles that were fixed after NOC treatment. After only 30 seconds in 10 µM NOC, the non-kinetochore microtubules have fragmented and have begun to depolymerize, and the effects are even stronger in 20 µM NOC (by studying adjacent confocal sections we confirmed that the microtubules indeed were fragmented). The kinetochore microtubules are also not as thick

or bright as the control cell, are fragmented, and appear to be disconnected and/or angled away from the centrosome at the top pole in both cells. After 6 minutes in 10 μM NOC there are barely any microtubules remaining, and ones that remain have very large breaks and any remaining kinetochore fibres are disconnected from the centrosome. The progression of microtubule depolymerisation over time, relative to the concentration of NOC, in cells fixed after different times of treatment, are illustrated in Figure 11, which is a montage of Z-stacks that include all images throughout the depth of the cell. Immediately upon addition of all concentrations of NOC non-kinetochore microtubules begin to fragment and contort. The non-kinetochore microtubules depolymerize quickest in 20 μM NOC; they are gone between 3 - 4.5 minutes, whereas some are still detectable in 5 μM NOC up to 6 minutes, albeit fragmented and with greatly reduced fluorescence. The kinetochore microtubules are more resistant to depolymerisation and although their fluorescence fades over time, some fragmented microtubules with reduced fluorescence are present in 5 μM and 10 μM NOC for up to 10 minutes. All kinetochore microtubules appear to be gone in 20 μM NOC after 4.5 minutes.

We have not seen microtubules attached to kinetochores that have moved, or to the chromosomes arms that have moved. Any microtubules present are either not in contact with the bivalents, are fragmented or bent, or are not in the same plane of focus of the bivalents, as illustrated in Figure 12, in which the Z-stacks include only those planes of focus that include chromosomes. For example, in Figure 12-Aiii and Ciii the remaining kinetochore fiber(s) do not reach the kinetochore of the bivalents that have moved to the bottom pole. In Biii the microtubules radiating from the bottom pole lead to the top kinetochore of a bivalent, but they are fragmented into several pieces. In this cell the univalents remained at the top pole and appear to be held there by the centrosome and its associated microtubules. Centrosomes either move with the chromosomes or remain at their respective poles. This occurs irrespective of whether any univalents remain behind. As seen in Biii, the centrosome stayed behind with the univalent and did not move. In Diii the centrosome moved with the bivalents and left the univalent behind at the pole.

DISCUSSION

Our experiments deal with two issues, (1) why the ‘sidedness’ of the spindle such that detached chromosomes all move to the same pole, and (2) what causes movement of chromosomes (kinetochores) at very high speeds after treatment with drugs that remove microtubules.

Non-random movement to one pole

After the addition of NOC, chromosomes detach and move towards one pole. It is highly unlikely that the movement to only one pole was due to chance since this was observed in 52 out of 59 cells. The bivalent kinetochores move independently, in that they take different paths, change arrangements, move to the pole to different extents, and while all move at roughly the same time, often they move at separate times (e.g., Figure 5, and supplementary video 3). We think this is

because their kinetochore microtubules attached at that pole all are detached at near the same time. This possibility could be tested using UV or laser microbeams to cut the kinetochore microtubules at only one pole.

We do not know what the difference is between the two poles, but we think that there is a difference, and that it may be related to possible non-random segregation in these cells suggested by several lines of evidence reviewed in the Introduction and in Brady and Paliulis (2015). It may be that the 'sidedness' of the spindle is a marker for which pole the paternal-derived or maternal-derived chromosomes segregate to. We recognise that this is speculative on our part, but it seems a distinct possibility when considered together with the other evidence for non-random segregation in these cells. While difficult to prove, we could negate it if we could find some predictor of the sidedness prior to addition of NOC. Nothing we looked at in chromosome behaviour enabled us to predict which direction the detached chromosomes would move, however. The position of asymmetric furrows can vary from one side of the spindle to the other, several times in the course of division, and seems to be due to chromosome 'bulk' imbalance (e.g., Forer and Pickett-Heaps, 2010). If the 'sidedness' of chromosomes moving to a pole was associated with the asymmetry in numbers of univalent at a pole – e.g., the chromosomes always move to the pole with fewer univalents – then that would negate the hypothesis and mean that there is no inherent sidedness to the spindle itself. But asymmetry of the cleavage furrow also was not a predictor of sidedness of chromosome movement (Table 1). So far we have not been able to negate the possibility that the movements to one pole of all bivalent kinetochores (and univalents, when they move) are because of non-random segregation, so we speculate that the sidedness may be related to non-random chromosome segregation.

We might be able to test some of the speculations by staining the centrioles. The mother centriole is implicated in asymmetric cell division in both *Drosophila* and budding yeast (Yamashita 2009) and may also be implicated in symmetrically dividing cells. In HeLa cells treated with low concentrations of NOC, it was found that mis-segregated chromosomes moved towards the pole with the older, mother centriole (Gasic et al. 2015). Perhaps in *Mesostoma,* bivalents detach and move towards (or away from) the pole containing the mother centriole.

Chromosome movement after addition of NOC or colcemid

After the addition of NOC, chromosomes stretched, paused, detached from one pole, and then the detached kinetochores moved toward the other pole. The stretch elongates bivalents by about 25-30% and after detachment bivalent length returns almost immediately to the pre-stretch value and it remains at that length during subsequent fast movements. Immunofluorescence observations indicated that during the stretch and pause stages non-kinetochore microtubules depolymerised and fragmented and kinetochore microtubules fragmented, separated from the centrosome, changed angle and began to depolymerise (Figures 10, 11, 12). By the time of the detachment and movement phases most microtubules had depolymerised. An explanation for some of these events might be as follows. During the stretch phase bivalents move towards both poles

at a rate similar to normal prometaphase poleward oscillations, 6.2 ± 1.8 μm/min. (Ferraro-Gideon et al. 2014). If poleward oscillations of bivalents are caused by (or accompanied by) the disassembly of microtubules as suggested by Fuge (1989) and Ferraro-Gideon et al. (2013), movement of bivalents towards the two poles during the stretch phase may be caused by microtubules depolymerising in response to NOC. Microtubules and components of the brightly stained centrosome may be responsible for anchoring the chromosomes at the poles during the pause stage. Detachment occurs when there appear to be no kinetochore microtubules remaining. The detached kinetochores move extremely fast away from the pole of detachment towards the opposite pole, in an arc, not a straight line, with velocities up to 200μm/min. or more, at constant bivalent length. The moving kinetochores swing to one side and as they move they cause the chromosomes to bend (Figure 4 A,B).

What is responsible for this very rapid movement? We think it highly unlikely that the detached kinetochores are pushed by kinetochore microtubules, because those movements would be linear, not in an arc, and the microtubules would have to elongate at speeds over 200μm/min. while causing the kinetochore to swing out and then move toward the opposite pole. In any event, we saw no indication of kinetochore microtubule attachment to the detached kinetochores or chromosomes.

Chromosomes move faster when sliding along microtubules than when attached head on to them. Could the very rapid movement be due to the kinetochores sliding along non-kinetochore microtubules? There are non-kinetochore microtubules near kinetochores, as seen in electron micrographs of *Mesostoma* spermatocytes by Fuge (1987) and Fuge and Falke (1991), so sliding might be a possible mechanism. However, when anaphase chromosomes are attached to microtubules at kinetochores the highest velocities in anaphase for chromosome movements are up to 6μm/min. (Wang et al. 2010), and in prometaphase are up to 10μm/min. (Ferraro-Gideon et al. 2013). When not-attached chromosomes slide along microtubules (Rieder and Alexander, 1990) average speeds are up to 40μm/min., though instantaneous speeds can be up to 50μm/min. (Alexander and Rieder, 1991). The *in vitro* movement of microtubules along kinesin support speeds up to 30 μm/min. (DeLuca et al. 2001). We know of no reports of sliding at the highest speeds at which the kinetochores move after NOC treatment. Our immunofluorescence data indicates that the non-kinetochore microtubules are fragmented very soon after addition of NOC, within 30 seconds, earlier than the movements of the detached kinetochores, and none are present during the movements of the univalent chromosomes which may occur up to 15 minutes later. We see no indication in the immunofluorescence images that even fragments of microtubules are close to each detached kinetochore or moving chromosome arms, so we think it is highly unlikely that the detached kinetochores move by sliding against microtubules.

In sum, there are several reasons why we think it unlikely that microtubules produce the forces that move the detached kinetochores. One is that the kinetochores do not travel in a straight path but curl to one side; thus the force has to change directions rather than act in a straight line in the direction of a linear microtubule. The force must allow for high speeds, and we know of no

reports that microtubules and microtubule motors are able to produce speeds near or above 200 μm/min. And lastly the force must act on all kinetochores at the same time to produce the co-ordinated movement to only one pole, yet in the immunofluorescence images kinetochore microtubules are highly fragmented and any microtubules that do remain are not associated with the kinetochores (Figures 10 and 12). Even if one microtubule-kinetochore association remained, that still could not be responsible for the co-ordinated movement of all three bivalents that was consistently observed in 52 out of the 59 cells. Since we have not seen attachment of microtubules to moving chromosomes, either direct or along the side of the chromosome, we conclude that the rapid kinetochore movements do not appear to be due to microtubules. There are other sources of forces, however, forces that do not arise from microtubules.

Could the movements be due to "recoil", after tension is released on stretched chromosomes? Release in tension due to stretching could cause the shortening of the detached chromosome, and this undoubtedly occurs, release of tension causes some of the movement, but the fact that kinetochores swing to one side early in their movement indicates that the movement we observe is not due solely to a release in tension along the length of the chromosome. Recoil forces along the length of the chromosome would result in travel in a straight path to the opposite kinetochore but not at an angle to it as occurs in these cells (Figure 4B). Further, were the movements arising solely from recoil from the stretch, the speeds would not vary with concentration of NOC or colcemid and would not vary depending on how long it took for the movements to occur after adding the drug (Figure 8A). Nor do the speeds of movement seem to vary with length of stretched bivalent: chromosomes that are stretched to a longer inter kinetochore distance do not move any faster than shorter chromosomes (Figure 8C). Finally, when kinetochore microtubules in untreated cells are severed using a laser, the associated kinetochores move quickly towards the other pole, likely from 'recoil' after being stretched; these movements are at speeds an order of magnitude slower than those seen after NOC treatment, maxima of 20μm/min (Ferraro-Gideon, 2013; Hoang, 2013), instead of the 200μm/min seen after NOC. Thus we think that most of the movement we see is not due to 'recoil' from elastic stretch and we therefore must look for outside forces acting on the chromosomes. Consistent with this proposition is that univalent chromosomes move toward the equator at high speeds, averaging 11.8 μm/min (n=7), up to 16.5 μm/min, often after the bivalents have moved, at times when there are no spindle microtubules remaining in the cells.

One potential force away from the pole is the elastic 'tethers' that connect chromosome arms. When arms of anaphase chromosomes are cut, the arm fragments move rapidly toward the partner chromosomes because of elastic tethers that extend between the arms (LaFountain et al. 2002). Tethers are present in most (or all) animal cell spindles, including *Mesostoma* (Forer et al. 2017), and probably are universally present (Paliulis and Forer, 2017). In *Mesostoma* spermatocytes tethers also extend between the free arms of the prometaphase/metaphase bivalents so that when an arm is cut, the arm fragment moves rapidly to the other free arm (Forer et al., 2017). One could imagine that when a kinetochore detaches from the pole the tether would pull

the attached telomere toward the telomere of the other arm to which it is attached. Forces from tethers may contribute to the force for the movement; they would only act between telomeres and depending on the geometry of the arms they might cause the twisting, but there would not be differences in speed depending on NOC or colcemid concentration or on how long the bivalents remain stretched (Figure 8D). Thus other forces must contribute, perhaps those from the spindle matrix or from actin-myosin.

The spindle matrix model, as discussed in Pickett-Heaps and Forer (2009), wherein a spindle matrix propels the chromosomes towards the poles, is consistent with the movements we have described. In the original descriptions of spindle matrix in diatoms, in which the matrix is identifiable using the electron microscope, the matrix is connected to kinetochores and after depolymerisation of microtubules using colchicine the chromosomes detached and moved at random to either pole (Pickett-Heaps and Spurck, 1982). Our findings are the same, except that in *Meosostoma* spermatocytes the chromosomes move towards only one pole, and a matrix is not visible using electron microscopy (e.g., Fuge and Falke, 1991; Forer and Pickett-Heaps, 2010). Data from other cells show that the spindle matrix remains after microtubules are depolymerized, but that the matrix is compressed and gradually loses shape after the microtubules are gone (Yao et al. 2012). This suggests that in our experiments the higher speeds for kinetochore movements might be due to the complete matrix remaining, because they occur when kinetochore movements are initiated at shortest times after drug treatment (Figure 8); slower speeds, at later times after drug treatment, to matrix that is partially gone or destabilized; and when microtubules are removed gradually (as with low concentrations of colcemid), the matrix is completely gone and the chromosomes are not able to move. We can extend this interpretation to explain the spindle sidedness: we suggest that the matrix may be polarised toward one pole. This could be tested by looking at the arrangements of spindle matrix components.

Another possibility for producing force in the absence of microtubules is actin and myosin. Actin and myosin have been identified as being present in large numbers of spindles (Forer et al. 2003, Table 1) and physiological data support actin and myosin being involved in spindle function (e.g., Mogessie and Schuh, 2017; Sheykhani et al. 2013a, 2013b). Another possibility is the ‘spindle matrix’ (e.g., Johansen and Johansen, 2007; Pickett-Heaps and Forer, 2009). Consideration of actin-myosin and spindle matrix are not mutually exclusive since, as discussed in detail in Johansen et al. (2011), the spindle matrix includes proteins such as the nuclear derived proteins Skeletor, Megator, Chromator and EAST and muscle proteins such as actin, myosin, and titin, that colocalize or align with the microtubule spindle in a variety of different cells types (Johansen and Johansen, 2007; Johansen et al. 2011). In crane-fly spermatocytes, for example, actin, phosphorylated myosin, and titin are aligned along kinetochore microtubules (Fabian et al, 2007; Sheykhani et al. 2013b), in close proximity to spindle matrix proteins (Fabian et al. 2007). Just as the spindle matrix ‘framework’ of a spindle shape remains after removal of spindle microtubules (Johansen and Johansen, 2007; Yao et al. 2012), and slowly disappears, the actin ‘spindle’ that remains after microtubules in mouse meiotic spindles are depolymerised with NOC

also seems to slowly disappear (Mogessi and Schuh, 2017, Figure S2B). The slow disappearance of the putative force production mechanism that acts on *Mesostoma* chromosomes after microtubules are gone (after NOC treatment) could explain why chromosome movements are slower when time to detachment is longer. Thus actin/myosin too might be possibilities for causing the rapid movements of chromosomes/kinetochores.

One issue that arises from these considerations is the directionality of the forces produced by the underlying spindle-matrix/actin-myosin: if, as argued elsewhere, these are the forces that propel chromosome poleward when kinetochore microtubules are severed during anaphase, or when microtubules are completely gone (Pickett-Heaps and Forer, 2009; Forer et al. 2015), how might the same components act to propel *Mesostoma* bivalents and univalents in the opposite direction, from pole to equator, when the kinetochore microtubules are depolymerised? This is not necessarily a contradiction, however. As seen in *Drosophila* cells, the spindle matrix is not static: it changes organisation with time and depending on interactions with microtubules (e.g., Yao et al. 2012). Chromosome movements in the *Mesostoma* spermatocytes we have described herein are in cells prior to anaphase, in which the chromosomes move both toward and away from the poles. It is conceivable that the underlying matrix-actin-myosin network prior to anaphase is bidirectional and it reorganizes so that by anaphase it is unidirectional. It also is conceivable that the matrix components are unidirectional toward one pole prior to anaphase, toward the pole which the chromosomes all move after NOC treatment. Experimental analysis of the presence and organization of the matrix and actin-myosin components of *Mesostoma* spermatocytes can test for the presence of the components and for whether its organization changes at anaphase, and could help decide whether our interpretations and speculations have merit.

In conclusion, studies investigating possible differences between the two poles, and what is responsible for chromosomes movement in the absence of microtubules, in particular investigating possible involvement of the spindle matrix and actin-myosin, might provide insight into the normal functioning of these meiotic cells.

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CONFLICT OF INTEREST

Neither author has any conflict of interest.

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FIGURE LEGENDS

Figure 1. Illustration of a *Mesostoma* spermatocyte modified from Husted and Ruebush, (1940) and Ferraro-Gideon et al. (2013). The large bracket indicates the position of the three metacentric bivalents which are shown in black; the small brackets indicate the position of the univalents, with the two kinds indicated by blue and green. The arrow with a C points to a chiasma, and KT points to a kinetochore.

Figure 2 A B). Image sequences and movement graphs of chromosome movement in a *Mesostoma* spermatocyte before and during treatment with 20 μM NOC. **(A) A-L.** In images A-C the cell is immersed in Ringers solution. The white arrows point to the bottom and top kinetochores of one bivalent. They both oscillate towards their respective poles in A to B, then away from the poles in B to C. Image D is during the perfusion of 20 μM NOC. In images E-L the cell is immersed in 20 μM NOC. Bivalents begin stretching towards either pole in E, and once they reach both poles they pause for a period of time as shown in F. The open arrow in G shows the initial detachment of only the bivalents from the bottom pole. Images H and I are during the fast and slow movement of the bivalents towards the top pole. The kinetochores swing out towards the left side of the cell. Both univalents remain at the bottom pole during bivalent movement, but one univalent detaches in J, moves towards the top pole in K, and stops in L. A video sequence of this cell is presented as supplemental video 3. In this and all other video figures the times are given in hours:minutes:seconds. **(B)** Graphical representation of chromosome movement in the same cell before and during 20 μM NOC addition. Bivalent oscillations during prometaphase appear as sine or saw tooth waves. NOC addition is indicated by the solid black line. Chromosome speeds are determined from the lines of best fit, as indicated in grey. The diagram to the right of the graph shows how kinetochore positions were determined. Kinetochore positions, as indicated by the pink and green arrowheads, were plotted relative to their distance from a fixed point at the bottom edge of the cell membrane near the centrosome, as shown by the red X. **(C)** Close up of the movement of the bottom kinetochore (which is the green triangle in B). This pattern of movement is consistent among all the 52 cells in which detachment occurred at only one cell pole.

Figure 3 i-iv). Cells treated with NOC appear similar to control cells in-between meiosis I and II, and attempt to go into second division. **(i)** A control cell completes anaphase I and the two resultant nuclei enter into meiosis II. In C and D the bivalents have formed into two distinct groupings of chromatids and in E the chromosomes are arranged at the periphery of the nucleus, appearing as a hollow circle. In F-H the left nucleus enters second division and the chromatids begin oscillating along the spindle axis. Although not shown, the second nucleus enters second division sometime later and both cells complete anaphase II. **(ii)** After the addition of 10 μM NOC in image J, the bivalents stretch out, detach and move to the bottom pole. NOC is washed out with Ringers in figure M, after which the chromosomes in the

cell move, a nucleus forms, and the chromosomes become arranged as in control cells between meiosis-I and meiosis-II. This is followed by an abortive attempt at second division (figures O-S). In O the bivalents have moved down in the cell and formed into one distinct grouping of chromatids. In P the nucleus reforms and the chromatids arrange themselves in a hollow circle. In Q-S the cleavage furrow reforms and the chromosomes begin to oscillate but do so irregularly and the cell does not enter anaphase. The **(iii)** 'chromatid clump' and **(iv)** 'hollow circle' phases appear almost identical when comparing the NOC treated cell to the control cell.

Figure 4 A, B i-iv. Image sequence of a *Mesostoma* spermatocyte treated with 20 μM NOC. **(A) A-L.** In images A-C the cell is immersed in Ringers solution. In A, the triangles indicate the positions of the univalents at the two poles. The white arrow points to the bottom kinetochore which oscillates away from the pole in A to B, then towards the pole in B to C. Image D is during the perfusion of 20 μM NOC. In images E-L the cell is immersed in 20 μM NOC. Image E-F shows bivalents stretching towards their poles, G shows they pause at both poles. H shows the initial detachment of only the bivalents from the top pole, as indicated by the open arrow. Images I and J are during the fast movement of the kinetochores towards the bottom pole, and the triangle indicates the univalents that stay behind. Image K is during the slow movement, and L is after bivalents stop moving. **(B).** The same cell as illustrated in Figure 4A. Chromosomes do not move in a straight line during the addition of NOC. The image sequences in i-iii) all use the same image frames. These images were originally at an angle but were tilted in order to line the cell axis vertically. This cell is shown in supplemental video 1, and is the same cell shown in Figure 4A. **(i)** The positions of the two kinetochores are shown by the two white dots in each frame. Each image frame is one second apart. An overlay of the kinetochore positions in images A-J shows that the kinetochores do not move in a straight line, but change orientation and move towards the right. [There appears to be one dot fewer associated with the right kinetochore than the left kinetochore because two of the dots superposed.] **(ii)** A vertical line that includes the centrioles at both poles (as indicated by the directions the kinetochores were moving in) is drawn through the midline of the cell to show bivalents bend to the left side as the kinetochores move towards the right. **(iii)** A tracing of the bivalents was drawn on top of frames A-J, and the background was then subtracted. As the kinetochores move towards the bottom pole and to the right, the body of each bivalent bends towards the left side. The length of the bivalents also decreases (the length is the distance between the sister kinetochores of one bivalent). For stretched bivalents we measured the straight-line lengths from kinetochore to kinetochore but as the bivalents started to bend, we estimated straight-line lengths from each kinetochore to the middle of the bend and then summed the two. The left bivalent decreased in length (from its maximum length during stretch) from 20.5 μm to 16.4 m, i.e., to about 80% of its stretched length. The actual length of the right bivalent may be longer than we measured because the bivalent appears to bend backwards out of the plane of focus. **(iv)** Graphical representation of the fast and slow movement of the right kinetochore. The kinetochore position was plotted 30 times per second over a course of 12 seconds. Each second from 2-11 seconds lines up with the corresponding images in i-iii), as labelled. As seen by comparing the images in i-iii to the speeds in the graph, kinetochore movement to the right and bivalent bending towards the left occur during both the fast and slow movements.

Figure 5 A-E. Bivalents detach at separate times within the same cell, during the addition of NOC. As seen in image A, the position of two visible kinetochores of two separate bivalents that are in the process of stretching are indicated by white arrows. In B the left kinetochore begins to detach

before the right one, and in C the left kinetochore is seen moving towards the top pole. In D the right kinetochore has detached and begins moving towards the top pole, and both bivalents reach the top pole in E.

Figure 6. The 6 observed configurations of chromosomes in *Mesostoma* spermatocytes in response to the addition of NOC and the numbers of each that were seen. The univalents are seen at either pole, and the bivalents stretch across the middle of the cell. When chromosomes move, they curl and continue moving, kinetochore first, either past or not past the equator. The equator of the cell is the site where the precocious cleavage furrow pinches in, creating the dumbbell shape. The univalents may either travel with the bivalents or stay behind at their respective poles. Numbers of cells seen in each category are included below each diagram, arranged by drug concentration.

Figure 7 A-C. *Mesostoma* spermatocytes treated with varying concentrations of colcemid. At low concentrations of colcemid the bivalents continue to oscillate; there are fewer and fewer oscillations as the colcemid concentration is increased. At 50 and 100μM colcemid there are no oscillations and the response is the same as in NOC treated cells. **(A)** The average number of bivalent oscillations in each cell during the addition of colcemid. One oscillation is when a kinetochore completes an entire wavelength in the graph. N= number of cells. The capped bars indicate the standard deviations. **(B)** Movement graph of a cell treated with 10 μM colcemid. The vertical black line indicates addition of colcemid, and the black box indicates the oscillations which continue in the presence of drug. As visualized by the green triangles, the kinetochore moves towards the pole while oscillating but eventually stops oscillating and stays at that pole. The other kinetochore on the same bivalent, pink circles, also continues to oscillate, stops, then both kinetochores move towards the same pole. **(C)** Cell treated with 100 μM colcemid. The vertical black line indicates addition of colcemid. The chromosome responds in the same pattern as cells treated with NOC. In this cell the detached kinetochores moved at around 91 μm/min. towards the opposite pole.

Figure 8 A-C. Speeds of chromosome movement in control cells or after addition of NOC or colcemid. **(A)** Speeds of chromosomes after detachment with NOC or colcemid increase with increasing concentration of drug, and are much larger than speeds in non-treated cells. [The data for non-treated cells is from Ferraro-Gideon et al. 2014]. Standard deviations are represented by the half-lengths of each bar. At the top of each bar is the average speed for that condition and above that is N, the number of cells. These values are also shown in Table 2. Using Student's t-test, the speeds at the different concentrations of NOC are significantly different ($p<0.05$), as are those for the different concentrations of colcemid ($p<0.05$). **(B)** Distribution of fast chromosome movement speeds after detachment with respect to concentration of NOC. **(C)** Length of stretched bivalents (at their maximum stretch before NOC was added) versus the fast speed of the kinetochore movement, at different concentrations of NOC. There doesn't seem to be any relatioin between speed and length. **(D)** Time until chromosomes detach in the presence of NOC vs. fast chromosome speed. Chromosomes that detach earlier are more likely to move at a faster speed, whereas chromosomes that take a longer time to detach are more likely to move at a slower speed. These data include only cells in which kinetochores detached from one pole and moved across the cell equator.

Figure 9 A-B. Tubulin immunofluorescence staining in control cells, showing the presence of non-kinetochore microtubules (some are indicated by white arrows), kinetochore fibres (enclosed in yellow boxes) and centrosomes at either pole. DIC image of the chromosomes are in grey. Kinetochores marked with the yellow asterisk have visible kinetochore fibre attachments as seen in the merged images. The tubulin images are of a Z-stack; the DIC images and tubulin:DIC images are of only one image in the stack.

Figure 10 A-D. Tubulin immunofluorescence staining shows both non-kinetochore and kinetochore microtubules fragment upon the immediate addition of NOC. Each image is one layer taken from the Z-stack. The asterisk indicates the position of the centrosomes, arrowheads point to regions in the process of fragmenting, and brackets span across larger regions where microtubules have depolymerised. (**A)** A control cell showing a long non-kinetochore microtubule spanning across the middle of the cell, as well as brightly stained kinetochore microtubule oriented towards the bottom kinetochore of a bivalent. **(B)** and **(C)** After the addition of either 10 or 20 μM NOC, there are no continuous non-kinetochore microtubules and the kinetochore microtubules have broken away from the centrosome. As seen in B) the kinetochore microtubules have also angled away from the centrosome. **(D)** After the addition of 10 μM NOC for 6 minutes barely any microtubules remain, and both kinetochore and non-kinetochore microtubules are highly fragmented.

Figure 11. Tubulin immunofluorescence staining at various time intervals after the addition of 5, 10 or 20 μM NOC, showing the progression of microtubule depolymerisation. All images are oriented so that chromosome movement (based on the DIC images of the same cells) was towards the bottom pole. 15 individual cells are illustrated. Images are of the entire Z-stack. In some images there is some background fluorescence of the chromosomes (e.g., the cell at 10:00 minutes treated with 20 μM NOC).

Figure 12 A-D. Tubulin immunofluorescence staining during or immediately after chromosomes have moved after the addition of NOC, as seen in 4 separate cells. White arrows point to univalents which remain at their respective poles. Although some microtubules have remained, many of the remaining microtubules are fragments and few of the remaining microtubules contact the kinetochores of the chromosomes that have moved, and at most are near only one of the moving kinetochores, or a chromosome itself. All images are oriented so that chromosome movement is towards the bottom pole. Tubulin images are of a Z-stack that includes only the 2 or 3 slices that include chromosomes, whereas the DIC images are of only one slice in the stack.

SUPPLEMENTARY VIDEO 1: A *Mesostoma* spermatocyte treated with 20μm NOC. The NOC was added between 15:21:33 and 15:21:38. This is the same cell as illustrated in the montage of Figure 4 A and B i-iv.

SUPPLEMENTARY VIDEO 2: A *Mesostoma* spermatocyte treated with 20μm colcemid. The colcemid was added between 10:15:57 and 10:16:21.

SUPPLEMENTARY VIDEO 3: A *Mesostoma* spermatocyte treated with 20µm NOC. The NOC was added between 13:46:01 to 13:46:07 (frames 88-91) and again at 13:46:31, indicated in both instances as where the focus changes. Bivalent kinetochores detached and moved towards the top pole (starting frame 143, at 13:37:51). One of the two univalents detached (starting at frame 190) and moved towards the top pole. The second univalent begins to move toward the upper pole considerably later, in frame 491, sometime after 13:59:xx. This is the same cell illustrated in the montage of Figure 2A and the graphs in 2B and C.

SUPPLEMENTARY VIDEO 4: A *Mesostoma* spermatocyte treated with 20µm NOC. The NOC was added 17:21:46 to 17:22:59 and again from 17:22:33 to 17:22:45. A kinetochore detached from the top pole at 17:25:15 and moved toward the bottom pole. A different kinetochore detached from the bottom pole at 17:27:01 and moved toward the top pole.

SUPPLEMENTARY VIDEO 5: Two *Mesostoma* spermatocytes treated with 20µm NOC. The NOC was added from 13:26:13 to 13:26:17, after which the bivalents in both cells stretched out. **Left cell**: All kinetochores detached from the bottom pole at 13:27:09 and then moved toward the top pole. **Right cell**: All bivalents remained stretched until .13:28:11, at which time they slowly shortened. At 13:28:34 the univalent began to move toward the equator and moved a short distance, taking the bivalents with them.

TABLES.

Table 1. Comparison of whether bivalents move into the larger or smaller half-cell, when the cell was asymmetric prior to addition of NOC or colcemid. All illustrations are of an asymmetric cell prior to drug addition in which there are three univalents at the top pole, and only one at the bottom. The imbalance in the number of univalents causes the furrow to shift towards the pole with fewer univalents, the bottom pole. **(A)** The cell prior to addition of drugs. **(B)** and **(C)** Chromosome movement after addition of drug. In (B) movement is towards the large half-cell, and in (C) movement is towards the smaller half-cell. The number totals indicate that there was no preference for movement to either the larger or the smaller side.

Drug	Conc. (μM)	Prior to drug, furrow was asymmetric (Response A)	During drug, bivalents moved into: larger half cell (Response B)	smaller half cell (Response C)
Col-cemid	10	1	0	1
	20	1	1	0
	50	0	0	0
	100	4	2	2
Noco-dazole	5	4	2	2
	10	6	3	3
	20	2	1	1
Totals:		18	9	9

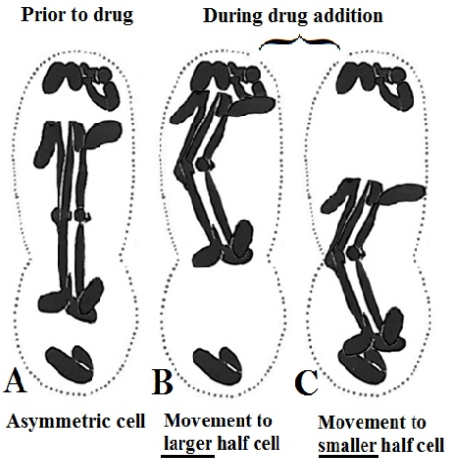


Table 2. Average times and speeds for each stage of the chromosome response for the different concentrations of NOC. The fast chromosome movement speeds also are shown in Figure 5. The ±standard deviations are included for each value, and the cell sample size is indicated by the n=value.
* indicates values that are significantly different using Student's t-test at $p<0.05$.

NOC conc.	Stretch duration (min:sec)	Pause duration (min:sec)	Time from drug addition to detachment (min:sec)	Fast and slow movement duration (min:sec)	Fast speed chromosome movement (μm/min)
5μM (n=17)	01:11* ±00:26	01:29* ±00:54	02:40 ± 01:20	01:21 ± 00:26	15.5* ± 9.07
10μM (n=21)	01:00* ±00:23	01:06* ±00:27	02:06 ± 00:50	01:20 ± 00:51	35.1* ± 24.2
20μM (n=21)	00:46* ±00.18	00:58* ±00:36	01:44 ± 00:54	00:43 ± 00:17	57.8* ± 35.1

Fig 1

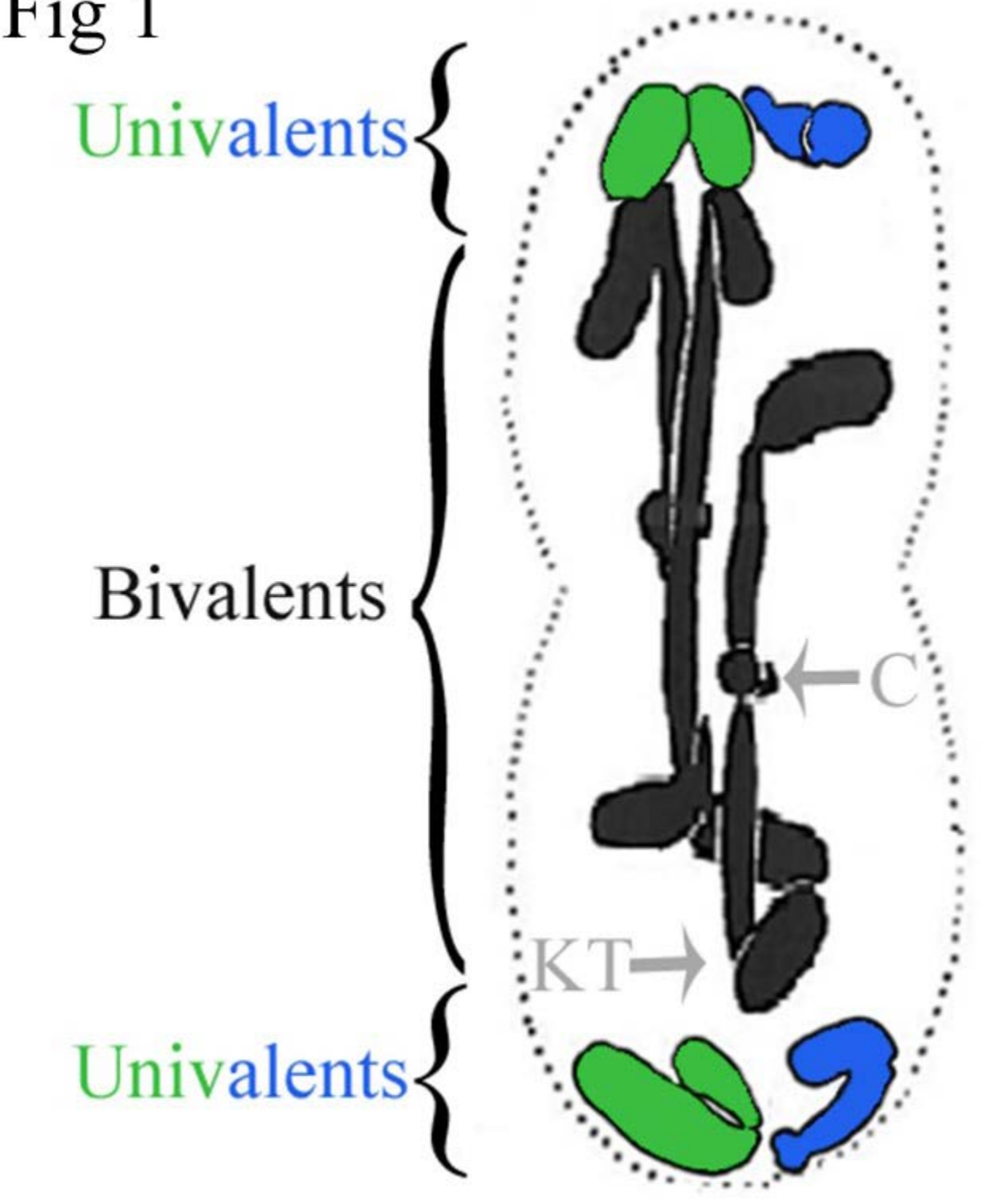


Fig 2A

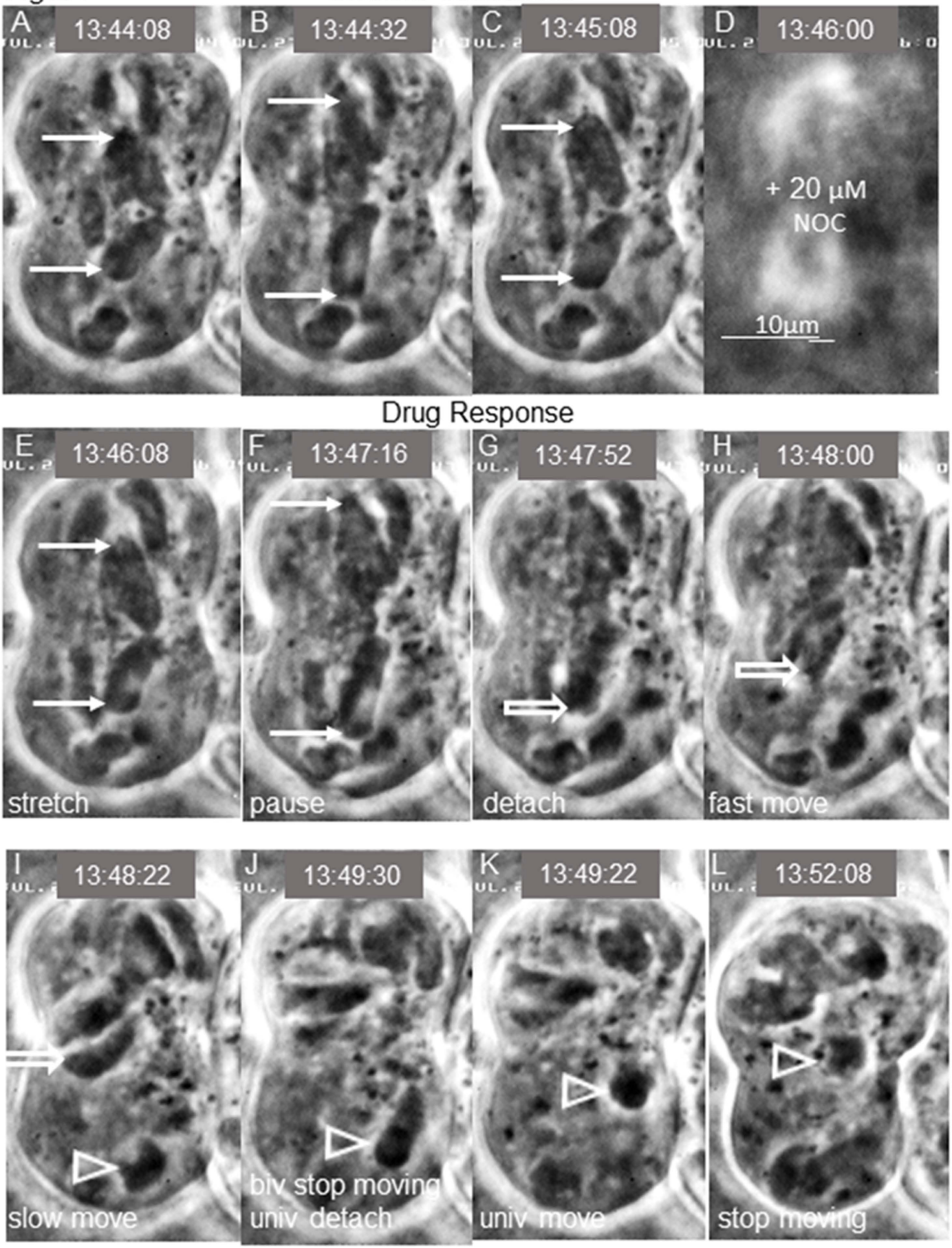

Bivalent Oscillations
A
13:44:08
B
13:44:32
C
13:45:08
D
13:46:00
+ 20 µM
NOC
10µm
Drug Response
E
13:46:08
stretch
F
13:47:16
pause
G
13:47:52
detach
H
13:48:00
fast move
I
13:48:22
slow move
J
13:49:30
biv stop moving
univ detach
K
13:49:22
univ move
L
13:52:08
stop moving

Fig 2B

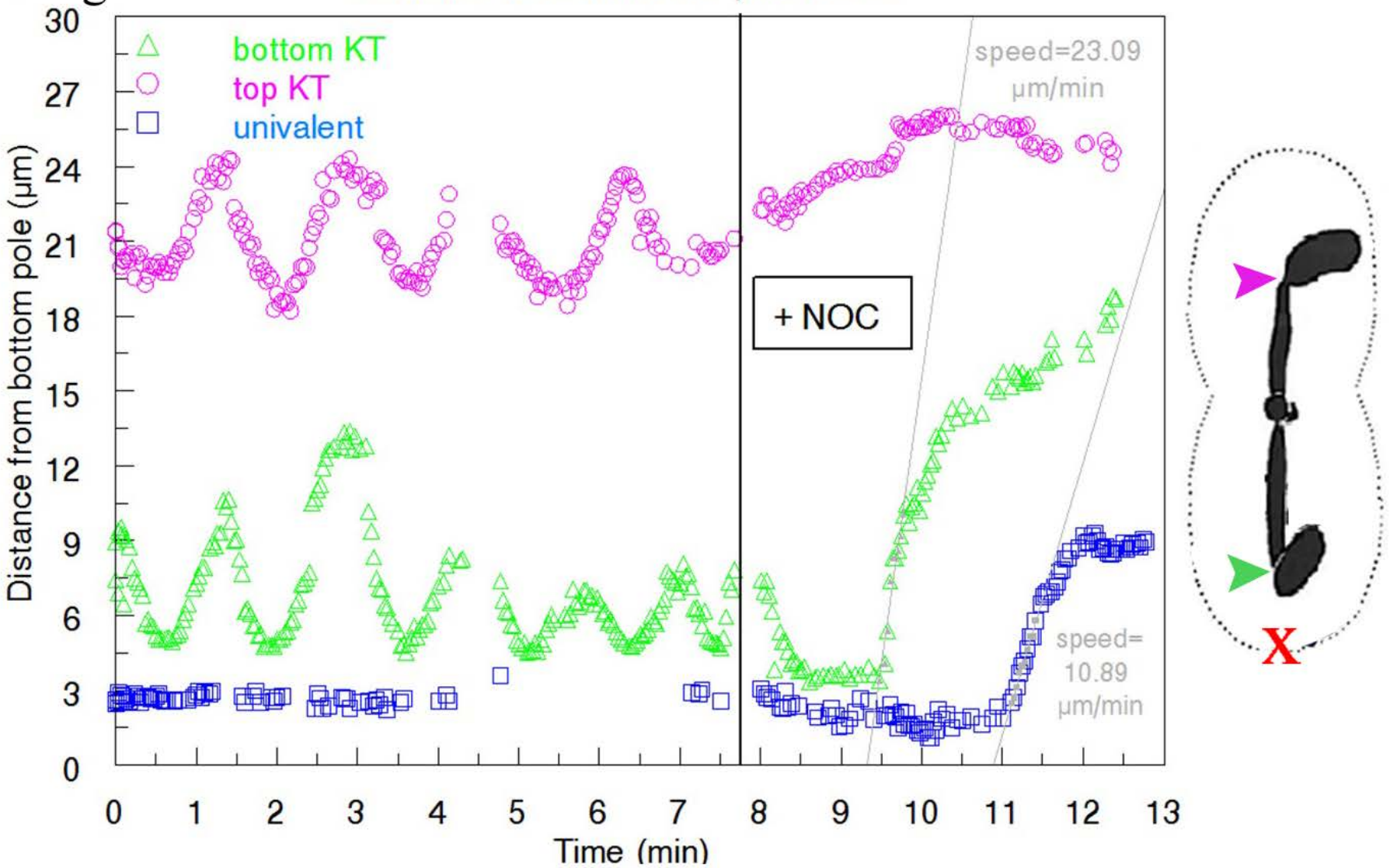

Cell treated with 20 µM NOC
bottom KT
top KT
univalent
speed=23.09
µm/min
+ NOC
speed=
10.89
µm/min
X
Distance from bottom pole (µm)
Time (min)

Fig 2C

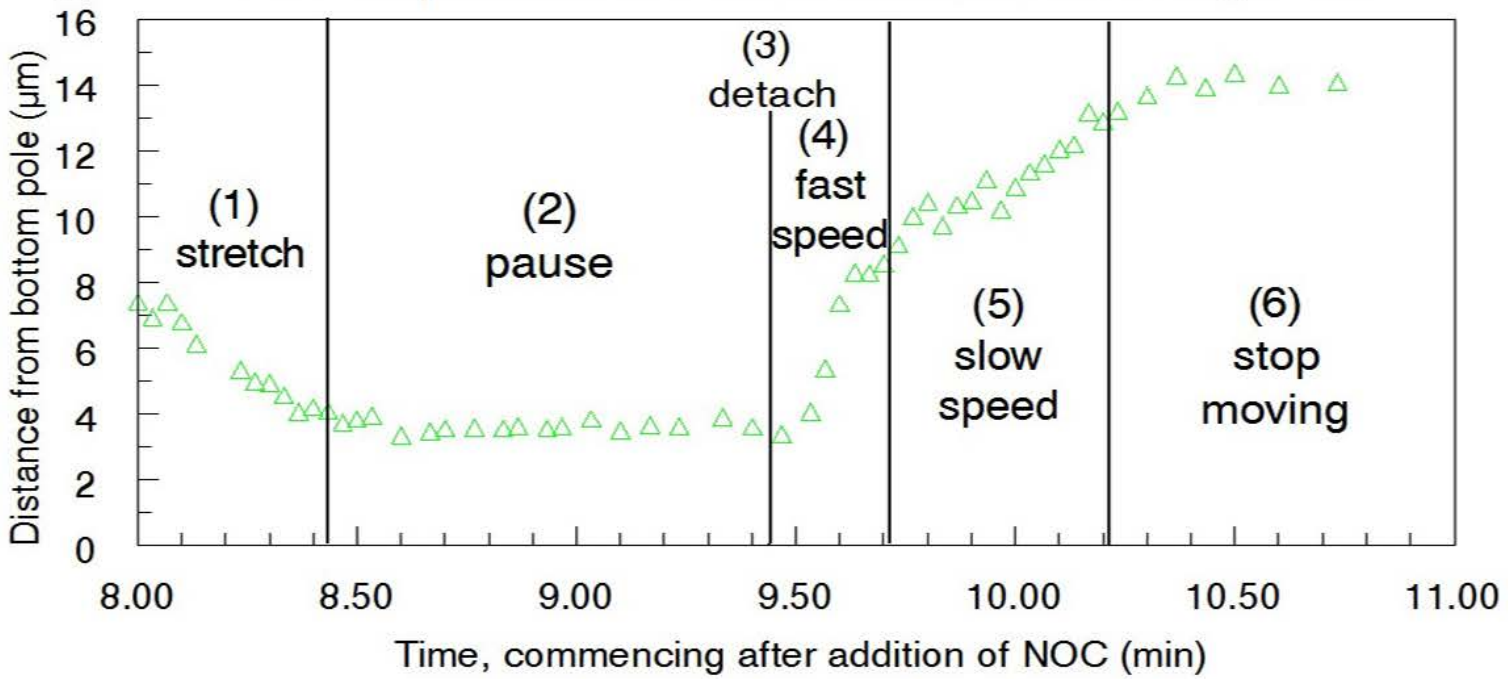
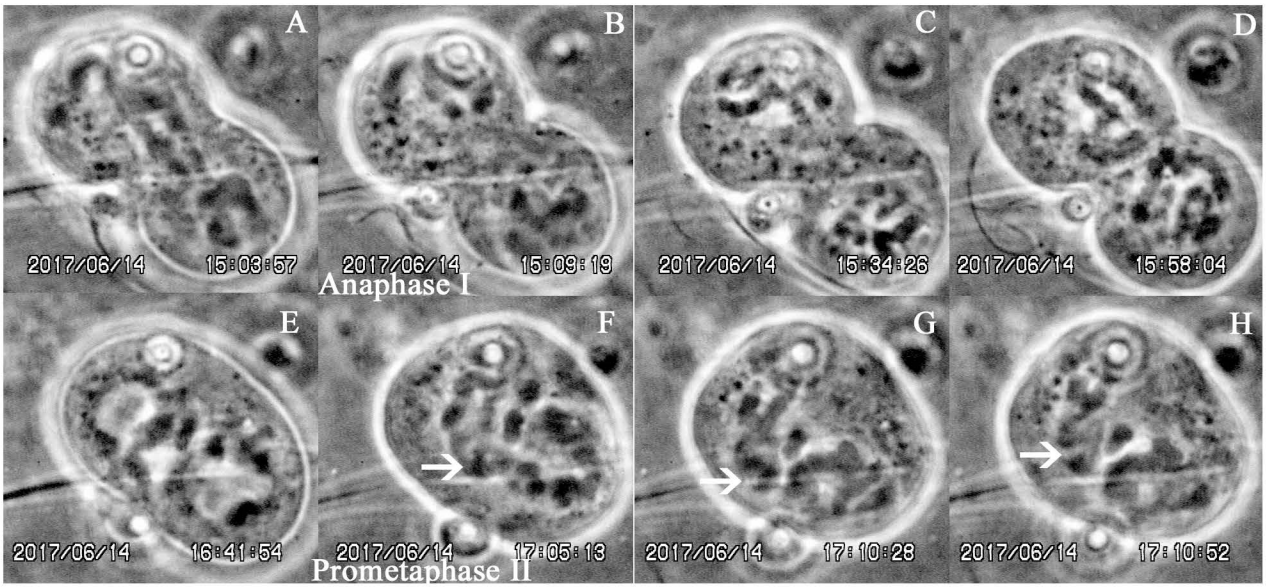
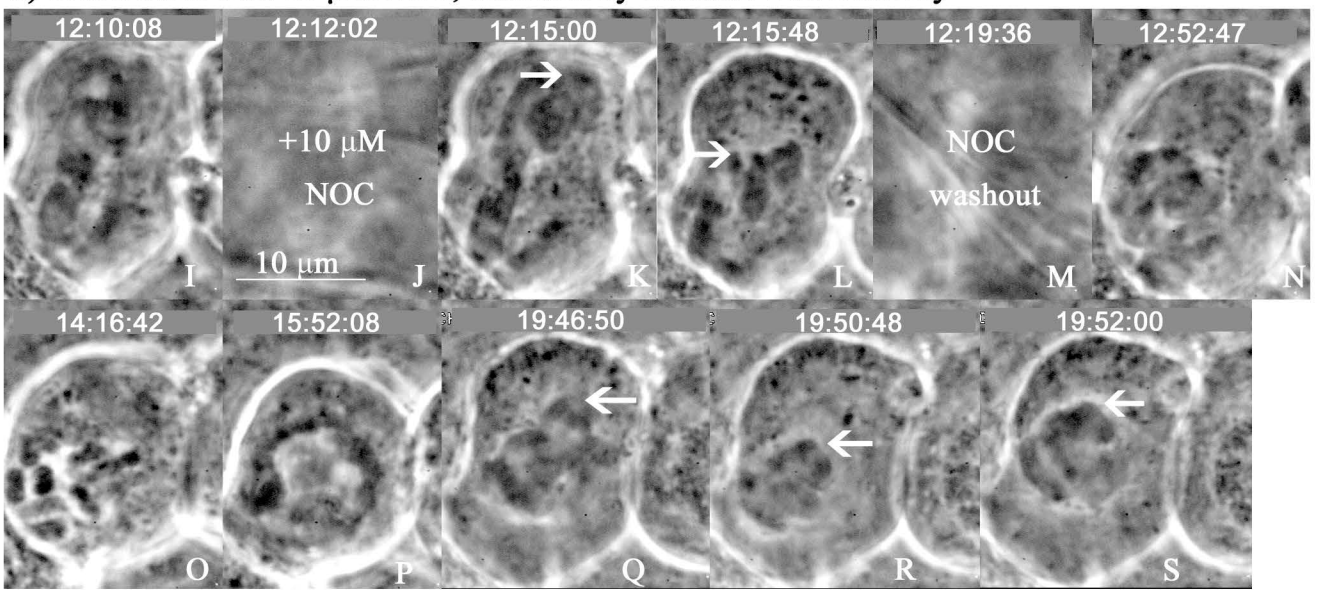

Close-up of chromosome movement during NOC
(1)
stretch
(2)
pause
(3)
detach
(4)
fast
speed
(5)
slow
speed
(6)
stop
moving
Distance from bottom pole (µm)
Time, commencing after addition of NOC (min)

Fig 3

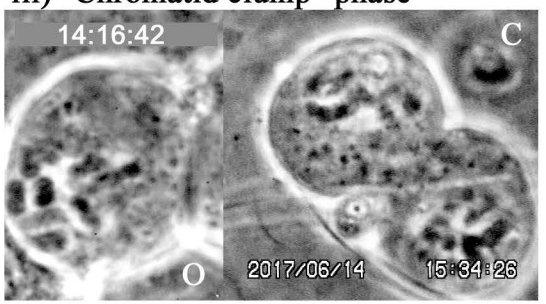
i) Control cell completing anaphase I and entering prometaphase II



ii) Cell treated with 10 μM NOC, followed by washout with recovery



iii) "Chromatid clump" phase



iv) "Hollow circle" phase

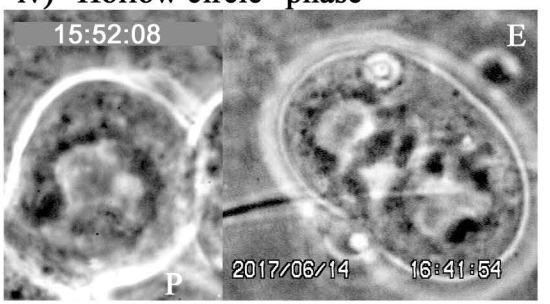
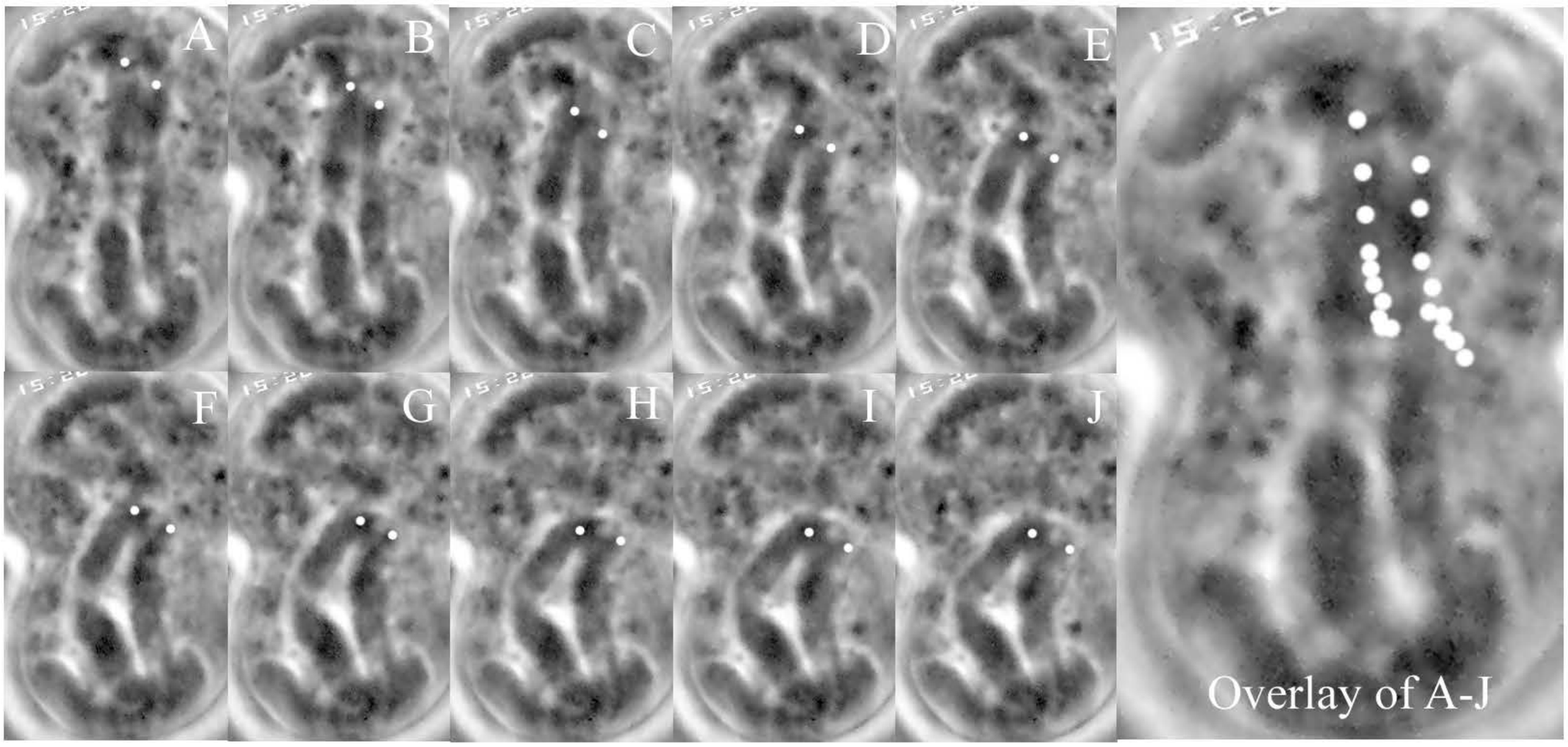
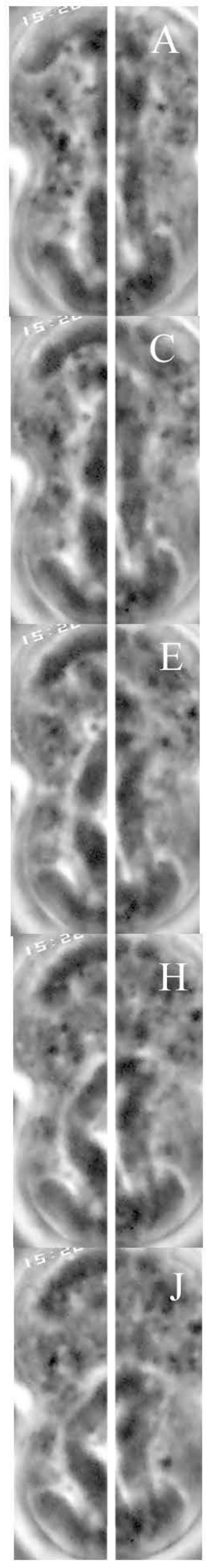


Fig 4.B Chromosomes do not move in a straight line:

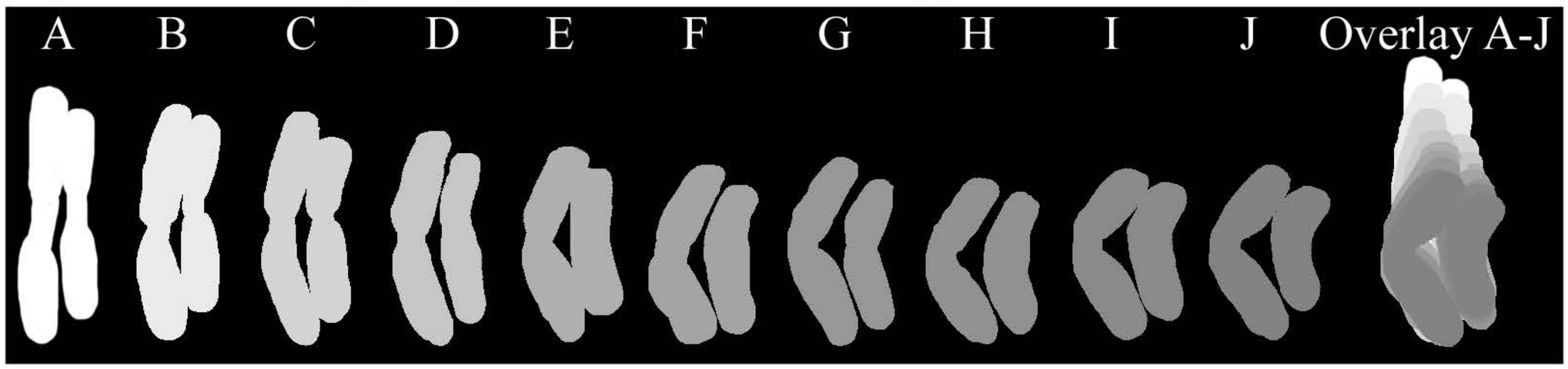
i) As indicated by the position of the kinetochore(s)



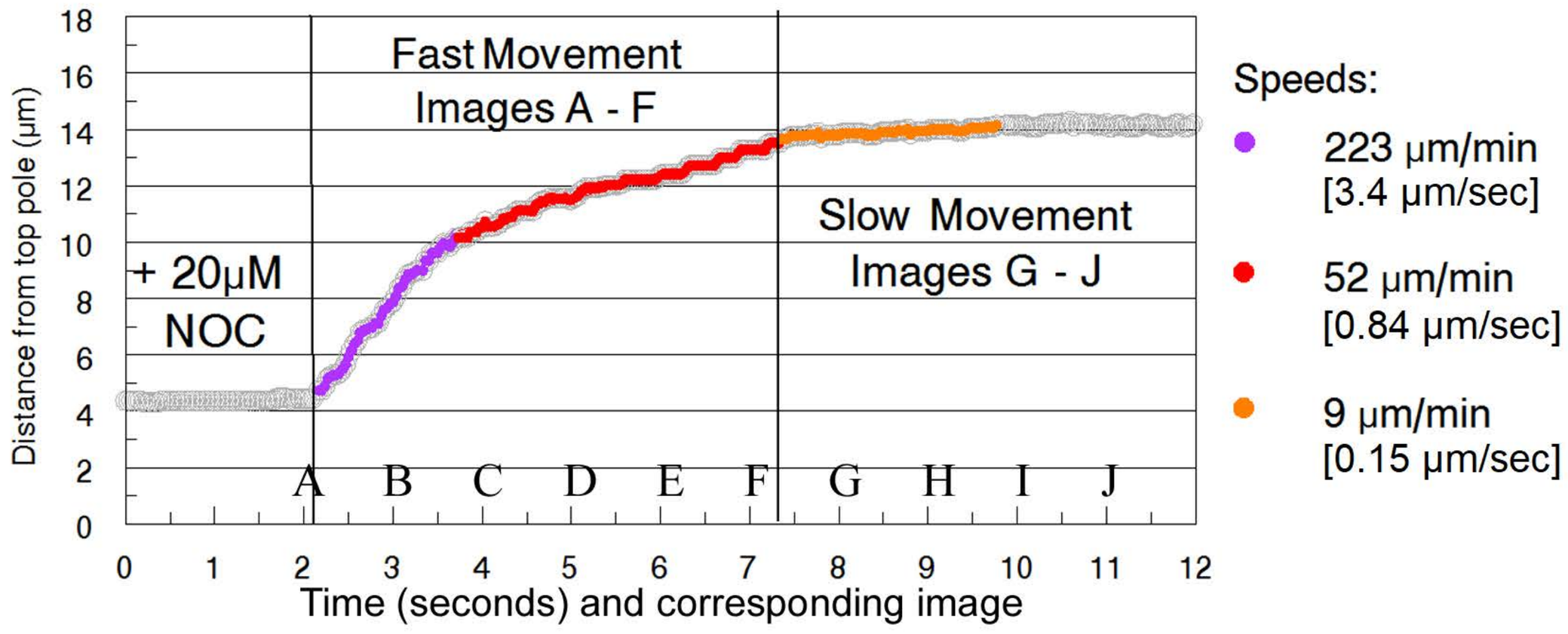
ii) In relation to the midline of the cell



iii) As indicated by the position of the bivalent(s)



iv) Close-up of of fast and slow movement, cell treated with 20μM NOC



Speeds:

- 223 μm/min [3.4 μm/sec]
- 52 μm/min [0.84 μm/sec]
- 9 μm/min [0.15 μm/sec]

Fig 4A

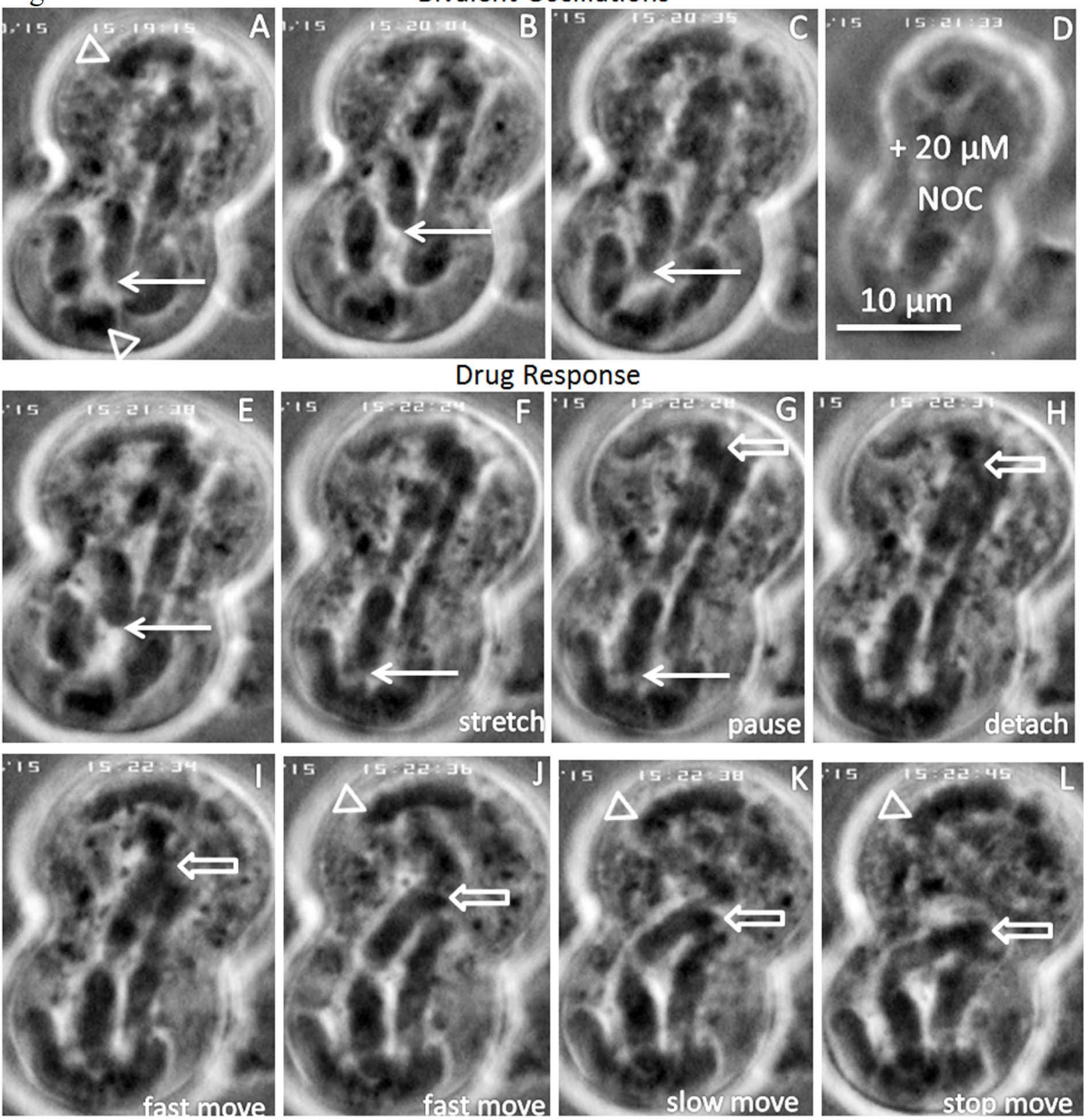

Bivalent Oscillations
A
B
C
D
+ 20 µM
NOC
10 µm
Drug Response
E
F
stretch
G
pause
H
detach
I
fast move
J
fast move
K
slow move
L
stop move

Fig 5. Bivalents detach independently, cell treated with 10 μM NOC

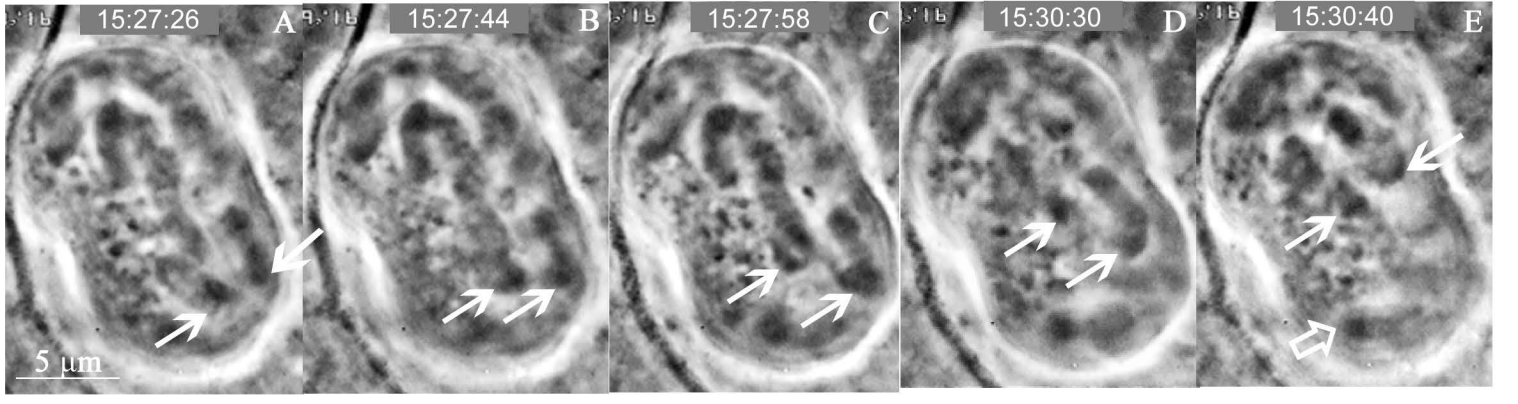


Fig 6

	Movement type:					
	(1) Chromosomes detach from only 1 pole				(2) Chromosomes detach from 2 poles	(3) Chromosomes remain in the middle
	A)Univalents don't move, bivalents move to 1 pole		B) Bivalents and univalents move to 1 pole			
	Past equator	Not past equator	Past equator	Not past equator		
						
5μM NOC	10	2	2	0	2	1
10μM NOC	5	8	5	2	1	0
20μM NOC	11	2	2	3	2	1
Cell Totals:				**=52**	**=5**	**=2**

Fig 7A

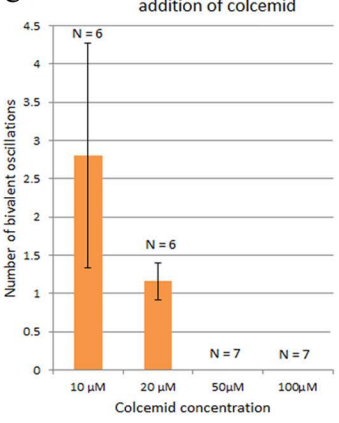


Fig 7B

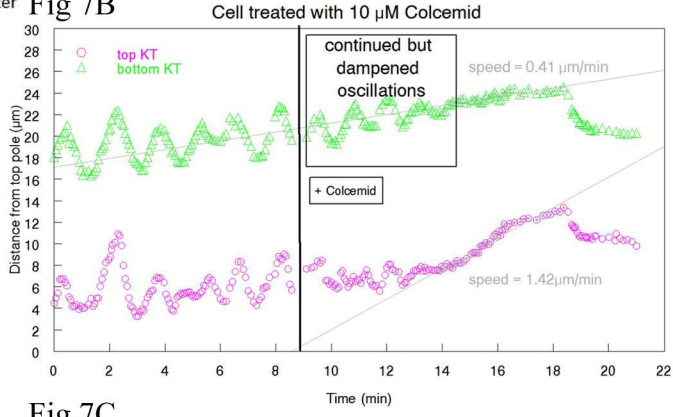


Fig 7C

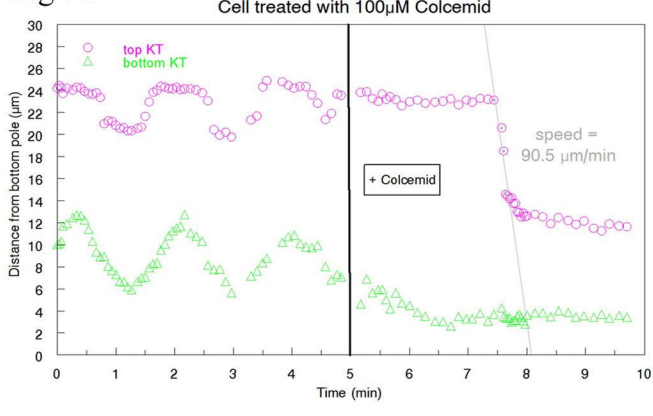


Fig 8A

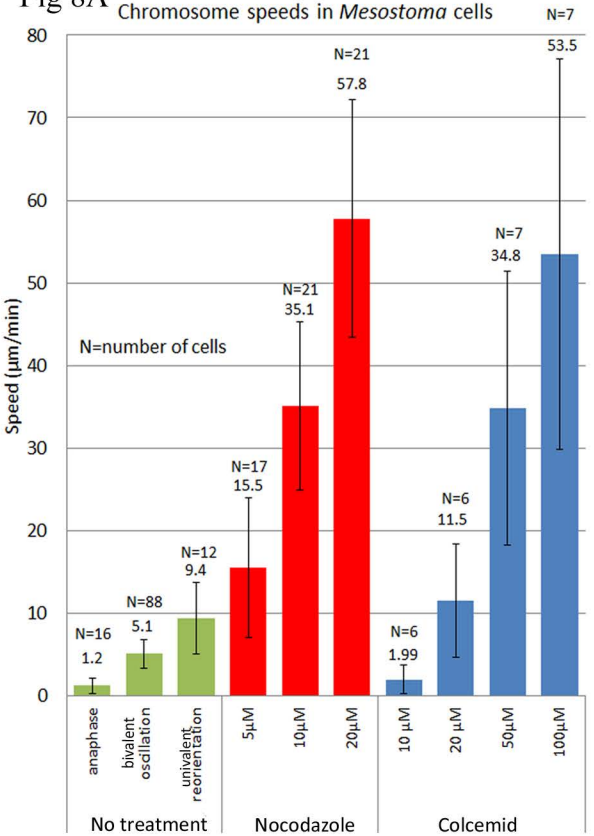


Fig 8B

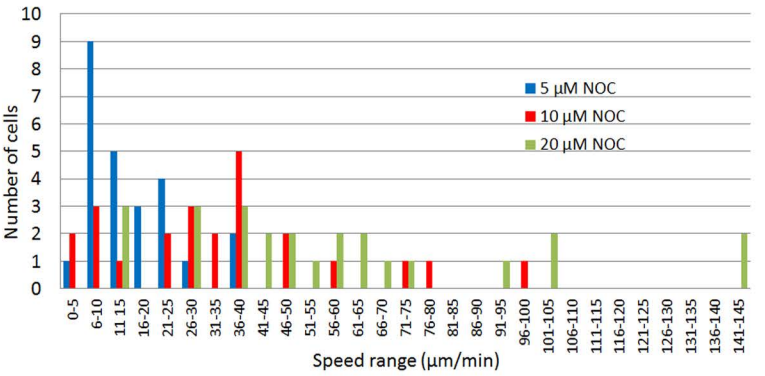


Fig 8C

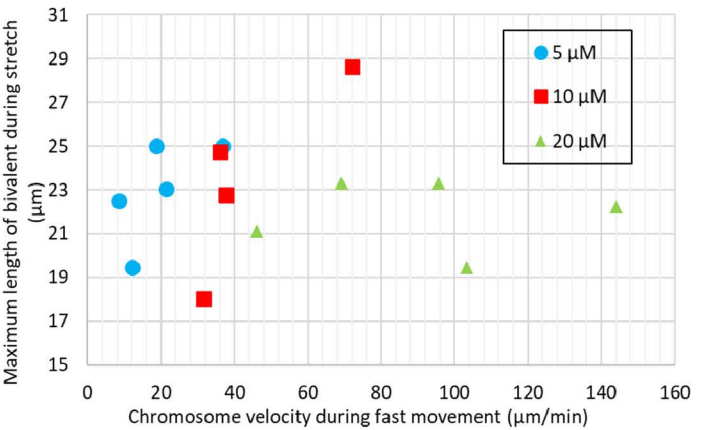


Fig 8D

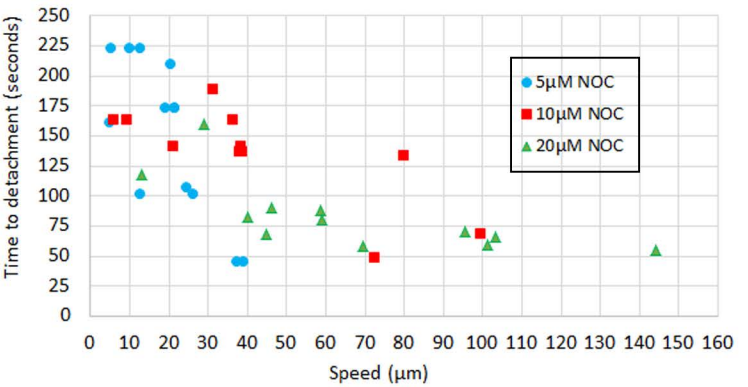
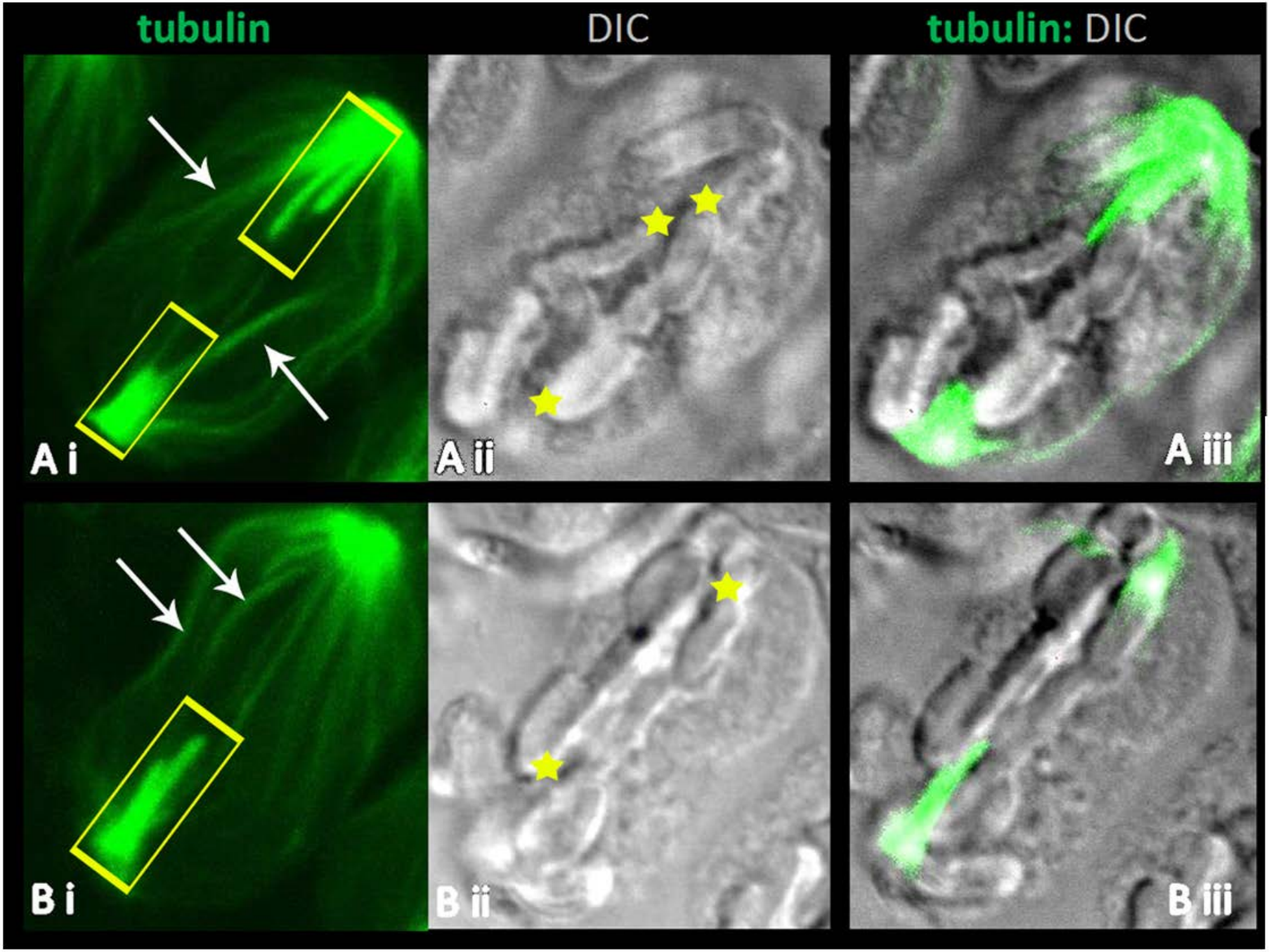


Fig 9



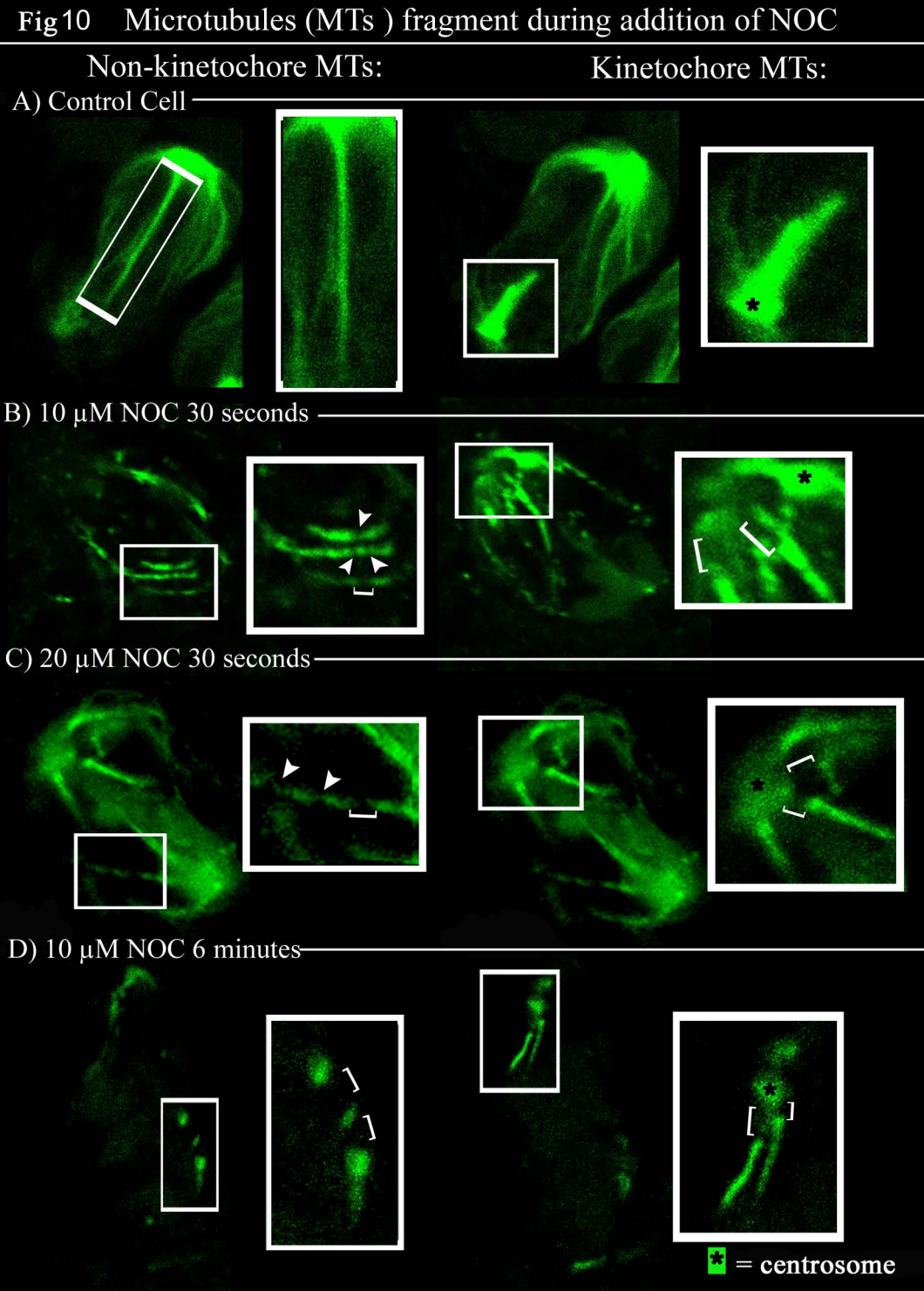


Fig 10 Microtubules (MTs) fragment during addition of NOC
Non-kinetochore MTs:
Kinetochore MTs:
A) Control Cell
B) 10 μM NOC 30 seconds
C) 20 μM NOC 30 seconds
D) 10 μM NOC 6 minutes
* = centrosome

Fig 11

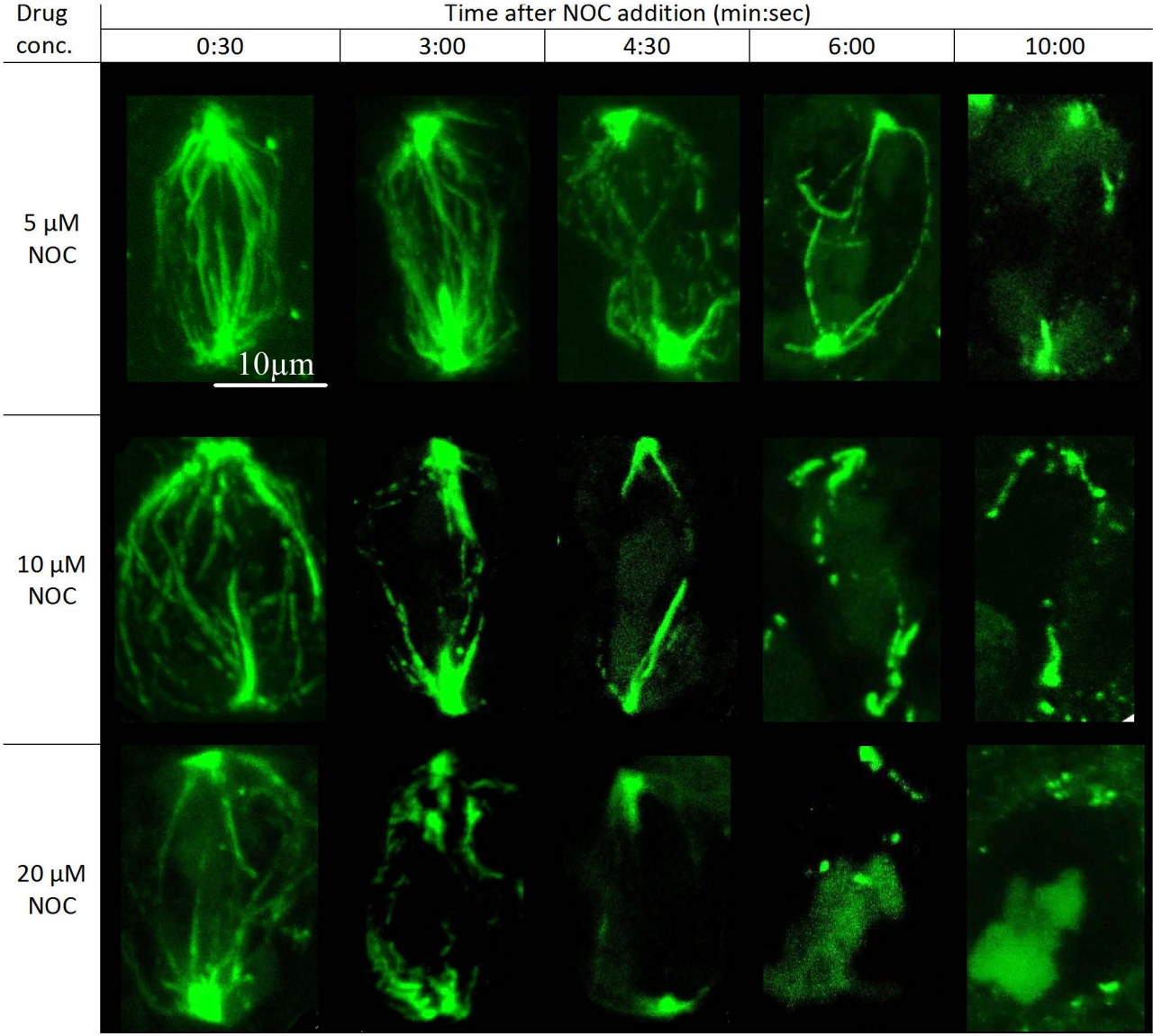
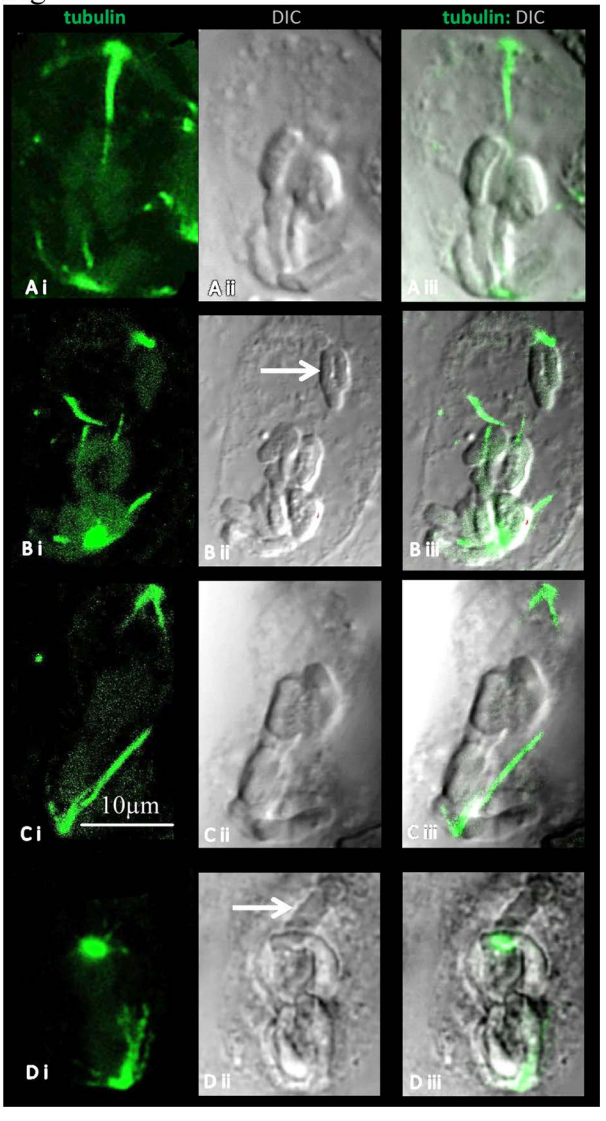

Drug conc.
Time after NOC addition (min:sec)
0:30
3:00
4:30
6:00
10:00
5 µM NOC
10 µM NOC
20 µM NOC
10µm

Fig 12


tubulin
DIC
tubulin: DIC
A i
A ii
A iii
B i
B ii
B iii
C i
10μm
C ii
C iii
D i
D ii
D iii