

Mitosis in primary cultures of *Drosophila melanogaster* larval neuroblasts

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Summary

Although *Drosophila* larval neuroblasts are routinely used to define mutations affecting mitosis, the dynamics of karyokinesis in this system remain to be described. Here we outline a simple method for the short-term culturing of neuroblasts, from *Drosophila* third instar larvae, that allows mitosis to be followed by high-resolution multi-mode light microscopy. At 24°C, spindle formation takes 7 ± 0.5 minutes. Analysis of neuroblasts containing various GFP-tagged proteins (e.g. histone, fizzy, fizzy-related and α -tubulin) reveals that attaching kinetochores exhibit sudden, rapid pole-directed motions and that congressing and metaphase chromosomes do not undergo oscillations. By metaphase, the arms of longer chromosomes can be resolved as two chromatids, and they often extend towards a pole. Anaphase A and B occur concurrently, and during anaphase A chromatids move poleward at 3.2 ± 0.1 $\mu\text{m}/\text{minute}$, whereas during anaphase B the spindle poles

separate at 1.6 ± 0.1 $\mu\text{m}/\text{minute}$. In larger neuroblasts, the spindle undergoes a sudden shift in position during mid-anaphase, after which the centrally located centrosome preferentially generates a robust aster and stops moving, even while the spindle continues to elongate. Together these two processes contribute to an asymmetric positioning of the spindle midzone, which, in turn, results in an asymmetric cytokinesis. Bipolar spindles form predominately (83%) in association with the separating centrosomes. However, in 17% of the cells, secondary spindles form around chromosomes without respect to centrosome position: in most cases these spindles coalesce with the primary spindle by anaphase, but in a few they remain separate and define additional ectopic poles.

Key words: GFP imaging, Kinetochores, Centrosome, Spindle, Cytokinesis

Introduction

There are two primary approaches for analyzing the effects of mutation on mitosis in *Drosophila*, and the one chosen depends largely on the nature of the mutation(s) being investigated (Theurkauf and Heck, 1999). Since little transcription occurs during early embryogenesis, these divisions utilize maternal stores of proteins and mRNAs stockpiled in the egg (see Ripoll et al., 1987). With time these reserves are depleted, making zygotic gene expression necessary for survival into adulthood (Gatti and Baker, 1989). Studies of mitosis involving maternal effect lethal mutations are therefore routinely performed on early embryos (Yucel et al., 2000; Wakefield et al., 2000; Wojcik et al., 2001). By contrast, phenotypic analyses of recessive zygotic gene mutations are conducted primarily on preparations obtained from larval tissues that exhibit normal diploid cell cycles; these tissues are limited to the brain, imaginal discs and histoblasts (reviewed in Theurkauf and Heck, 1999). In this regard a comparison of mitosis between neuroblasts from wild-type larvae with those from recessive zygotic mutants has been (Gatti and Baker, 1989), and continues to be, the 'method of choice' for defining genes in *Drosophila* that play a role in cell division (Sunkel et al., 1995; Wilson et al., 1997; Cullen et al., 1999; Avides and Glover, 1999; Yucel et al., 2000; Inoue et al., 2000; Wojcik et al., 2000; Giansanti et al., 2001).

Neuroblasts are progenitor cells that give rise to the central

nervous system in metazoans. In some organisms (e.g. flies and grasshoppers), these cells divide asymmetrically and, following mitosis, the larger of the two daughters retains its neuroblast identity (see Doe and Bowerman, 2001). The other, smaller ganglion mother cell (GMC), then undergoes a single symmetrical division to produce two daughters that ultimately differentiate into neurons (Poulson, 1950). In *Drosophila melanogaster*, dividing neuroblasts are first found in stage-nine embryos (Truman et al., 1993), and division continues throughout larval development (Truman and Bate, 1988).

Surprisingly, despite the fact that *Drosophila* neuroblasts can be cultured (e.g. Seecof et al., 1973; Wu et al., 1983; Furst and Mahowald, 1985; Broadus and Doe, 1997) and despite their popularity for mitotic studies, there is only one report detailing division in living neuroblasts, and it focused on the mechanism of asymmetric cytokinesis in embryos (Kaltschmidt et al., 2000). As a result, the processes of spindle formation (Wilson et al., 1997; Bonaccorsi et al., 2000; Giansanti et al., 2001) and chromosome behavior (Scaerou et al., 1999; Donaldson et al., 2001; Wojcik et al., 2001) in neuroblasts have been inferred instead from the examination of fixed, and often squashed, cells. Although these approaches have identified many proteins important to mitosis (aurora and polo-like kinases, ZW10/rod, asp, etc.), reconstructing the various dynamic and superimposed events that occur during mitosis from static images produces a limited and sometimes misleading picture

of the division process. Also, fixed material provides no data on the dynamic behavior of the proteins and organelles involved in mitosis or on how they are altered in mutants. Such behaviors, which in mutants can be manifested as obvious or even subtle changes in, for instance, the rate of chromosome motion (e.g. Savoian et al., 2000), must be defined for a complete understanding of how a particular genetic defect affects the division process.

Here we describe a rapid and simple method for generating short-term neuroblast cultures from *Drosophila* third instar larvae that allows individual cells to be followed by high-resolution time-lapse video microscopy. Using this approach we have characterized division in these cells, focusing primarily on chromosome behavior and spindle formation. Our data provide novel insights into how mitosis works in neuroblasts and also defines the control work for additional live cell studies on mutants.

Materials and Methods

Fly strains

Fly strains were maintained using standard techniques. Neuroblasts from Oregon R larvae were used for all differential interference contrast (DIC) recordings. GFP-expressing flies were the kind gift of J. Raff (Wellcome/CRC Institute, Cambridge, England). Chromosome behavior was followed in 4D using two approaches. Initially, chromosomes were directly imaged using a GFP-tagged histone variant [His2AvDGFP (Clarkson and Saint, 1999)]. In the second method, virgin *GFP-fzy* females, which display fluorescent kinetochores, were mated with *GFP-fzr* males, which have GFP-tagged centrosomes (J. W. Raff, K. Jeffers and J.-Y. Huang, personal communication). The *GFP-fzy/GFP-fzr* progeny simultaneously display fluorescent kinetochores throughout prometaphase and centrosomes throughout the cell cycle. Studies of spindle formation utilized a GFP- α -tubulin construct (Grieder et al., 2000) whose expression was driven in the larval brain using the GAL4 system (Brand and Perrimon, 1993).

Primary neuroblast cultures

Primary cultures were prepared on clean 25×25 mm glass coverslips

(#1) modified as follows. First, a drop of molten VALAP (1:1:1 Vaseline:lanolin:paraffin) was placed near the edge of each corner. Next two concentric rings of Vaseline were drawn in the center of the coverslip using a syringe and 18-gauge needle (Fig. 1A).

Drosophila third instar larvae of the appropriate genotype were removed from mating vials, bathed for 20 seconds in saline (0.7% NaCl in H₂O) to remove residual media, and then placed on #4 filter paper until dry.

All subsequent steps were performed on a dissecting microscope containing a mirror in its base and a transparent stage. This microscope allows the specimen to be illuminated from the side by a fiber optic system. By alteration of the incident light angle, the specimen can be viewed by dark field-like illumination (Figs. 1B-D).

After cleaning, a larva was transferred to the upper portion of the innermost Vaseline ring on the coverslip. Voltalef 10s oil (Elf Atochem; Paris, France) was then added until the larva was completely covered and the innermost ring filled. Brains were isolated by placing downward pressure on the black larval mouthparts with a no. 11 scalpel blade, while at the same time placing another blade one-third of the larval length back and pulling in a posterior direction. After separating the brain from the rest of the body (Fig. 1B arrow), adjacent tissues were then dissected away and discarded. During dissection the brain becomes surrounded by fluid released from the larva, which was removed by thin slivers of filter paper to ensure cell adherence to the coverslip. The intact, isolated, brain (Fig. 1C) was then moved to the center of the coverslip, which was free of debris.

We used brain hemispheres and the ventral ganglion. These components were severed from one another and placed in adjacent regions of the coverslip center. Cell monolayers were then produced by inserting both scalpel blades into a single piece of tissue and drawing them in opposite directions using smooth sweeping or arching motions. This process was repeated until most of the tissues were spread (Fig. 1D).

Once the cells were spread, a clean glass slide was pressed against the coverslip. As force is applied, the VALAP in each corner begins to compress and act as a spacer. Excessive compression does not generally affect karyokinesis, but in overly flattened cells cleavage furrows often regress before forming mid-bodies. Under these culture conditions, cells continue to enter and complete mitosis for over 1 hour, after which they stop dividing and ultimately become refractive under DIC optics.

Neuroblasts could be easily distinguished from other cells, including GMCs, because they are the largest cells in the preparation

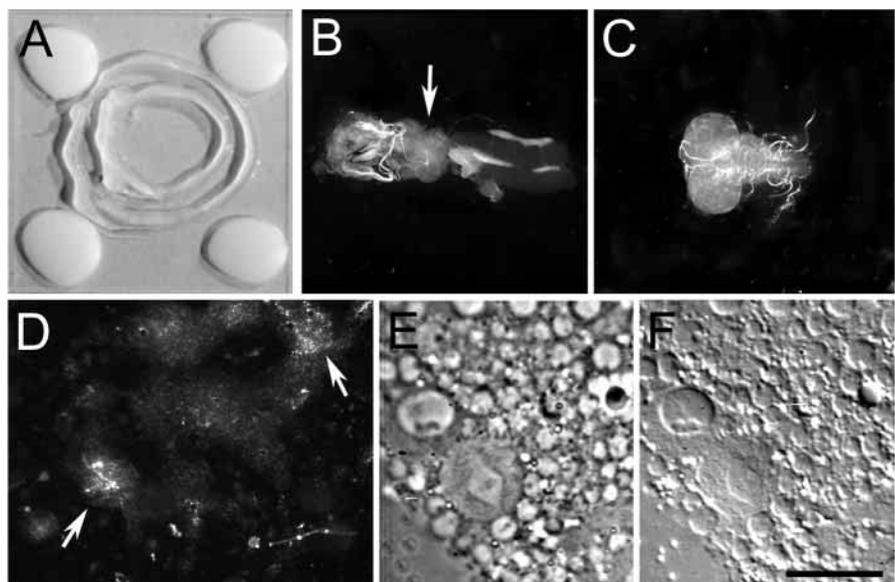


Fig. 1. Larval neuroblast culture. The chamber (A) is constructed from a coverslip by placing VALAP on each corner and two concentric Vaseline rings in the center. A larva is placed in the centermost ring and covered with Voltalef oil. After dissection, the brain (arrow in B) usually remains associated with the mouthparts and salivary glands, which can then be removed (C). After spreading, most cells form a monolayer (D), although some groups remain (arrows). (E) Phase-contrast image of a culture. Two neuroblasts, one in metaphase, are visible and are recognized by their large size relative to other cells. (F) Same as in E but viewed under DIC optics. See Materials and Methods for details. Bar, 20 μ m (for E and F).

(Fig. 1E,F) (Wu et al., 1983). That we were indeed following neuroblasts could also be verified, at the end of the division, by the unique asymmetric cytokinesis that these cells undergo. Thus, even though GMCs could be followed throughout mitosis in the same preparations, we focused our study on the neuroblast.

DIC microscopy and analysis

DIC imaging was conducted on a Nikon Diaphot 200 light microscope (LM) equipped with de Senarmont compensation and DIC optics using a 60 \times 1.4 Planapo objective lens and matching condenser. Cells were illuminated with shuttered and filtered green (546 nm) light obtained from a tungsten filament. Images were acquired at 4 second intervals with a Micromax 5 MHz cooled CCD camera (Roper Scientific, Trenton, NJ) and captured to a PC with Image Pro Plus (Media Cybernetics, Silver Springs, MD).

Measurements from sequential images were made as described previously (Savoian et al., 2000). Briefly, cursors were manually placed on the leading edge of anaphase chromosomes and the centrosomes using Image J (Public domain software; NIH, Bethesda, MD). After program calibration with a micrometer the distance between the cursors was determined and exported into Excel (Microsoft, Redmond, WA) for plotting. Anaphase chromosome velocities were determined from the slope of the steepest 10 sequential points.

4D fluorescence microscopy and analysis

All fluorescence imaging was conducted on a Deltavision Restoration Microscopy System, centered on an Olympus IX70 DIC inverted LM, running the included SoftWORx software (v2.5; Applied Precision Inc., Issaquah, WA). Cells were illuminated using the FITC filter set (excitation 490 \pm 10 nm; emission 528 \pm 19 nm) with shuttered light generated by a Hg-arc lamp and scrambled through a fiber optic cable. They were viewed with a 100 \times (NA 1.35) objective lens and the images were recorded with a Roper CM350 camera using a 2 \times bin.

Live cell 4D (3D over time) fluorescence studies are subject to two constraints. First, the fluorescence intensity often varies among different GFP-expressing strains. As a result, to record 4D sequences with useful temporal resolution, the exposure times were kept to a minimum, and even with binning some images had a sub-optimal signal-to-noise ratio. Second, despite minimal exposure times, photobleaching still occurs, and the severity of its impact varies with the strains and recording conditions used. We therefore used different parameters to study each of the GFP-expressing strains. However, regardless of the strain used, the radiation levels required for time-lapse 4D imaging did not harm the cells, as revealed by the fact that they entered and completed karyokinesis, and often cytokinesis, with the same kinetics as those recorded by DIC LM.

During our studies we used 4D LM to follow the behavior either of the chromosomes, the centrosomes and kinetochores or Mts. Chromosomes were directly labeled using His2AvDGFP. For this strain, a z-series composed of eight optical sections, recorded with a step size of 0.6 μ m, was collected at 7 second intervals. Because the two spindle poles were often in different planes, z-series of kinetochore and centrosome interactions in *GFP-fzy/GFP-fzr* expressing cells consisted of 10 optical sections, each of which had a step size of 0.6 μ m. These were collected every 8 seconds. To minimize photobleaching during studies on spindle formation, z-series in *GFP- α -tubulin*-expressing cells were collected every 15 seconds, and each series consisted of eight optical sections captured at 0.5 μ m step intervals. 4D data sets were then iteratively deconvoluted using the SoftWORx program.

Measurements on neuroblasts expressing *GFP-fzy/GFP-fzr* were made with the measure/distance function in the SoftWORx software package. The distances between the two centrosomes, or between the centrosome and kinetochores, were calculated by two methods. First

we measured the distance in the maximum intensity projection for each time point by manually placing the cursors on the brightest central portion of each object of interest. For the second method, the same objects were followed in 4D space, which allowed the contribution of z-axis displacement to be included in distance calculations. Optical sections were selected in which the objects of interest displayed their maximum brightness. Cursors were then placed on the central portions of each object, and the resulting distances exported to Excel. These two techniques could give slightly differing results that were significant only in cells where the spindles rotated, so that the centrosomes became non-coplanar. When analyzed in maximum intensity projections, such spindles appeared to shorten as they rotated out of the z-plane. By contrast, because attached kinetochores tend to be in the central portion of the cell volume, both measuring methods gave similar results for kinetochore-to-centrosome distances.

Figures for illustration were compiled from selected recordings and contrast enhanced using Adobe Photoshop (Adobe Systems, Mountain View, CA). Each GFP panel is the z-series maximum intensity projection.

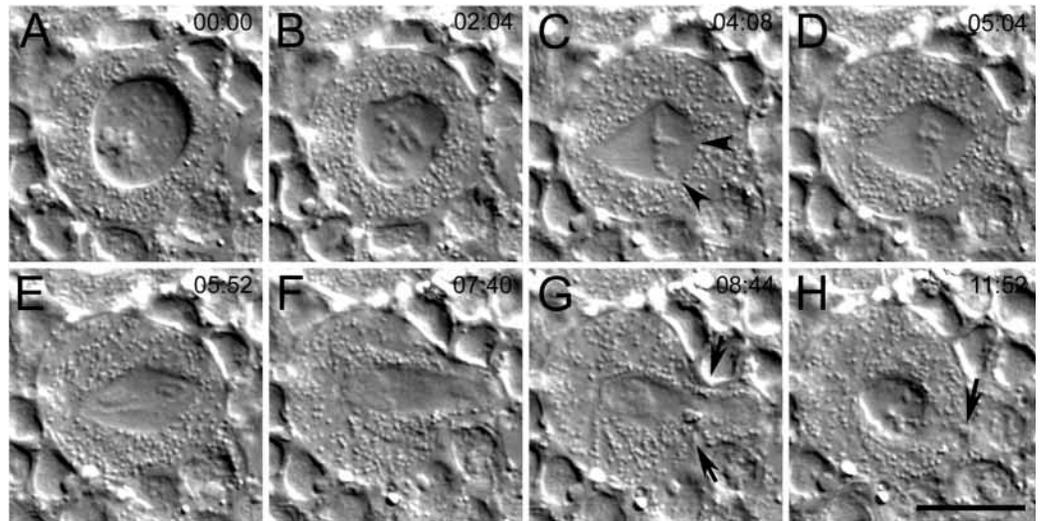
Results

General features of mitosis in neuroblasts

We initially characterized karyokinesis in neuroblasts using video-enhanced DIC LM, which provides the highest spatial resolution of all transmitted LM modes (Salmon and Tran, 1998). During late G2, the nucleus appears relatively homogenous with the exception of a single prominent nucleolus. The first signs of prophase are marked by the sudden appearance of numerous granules (Fig. 2A), which then rapidly (within \sim 2 minutes) coalesce to form the chromosomes (Fig. 2B, Fig. 3A). At nuclear envelope breakdown (NEB), the chromosomes begin to move (Fig. 2B, Fig. 3B), and some exhibit a sudden and rapid displacement towards one of the spindle poles. Prometaphase is short, and all of the chromosomes congress to the spindle equator within 3-4 minutes of NEB (Fig. 2C, Fig. 3C). Time-lapse records reveal that congressed chromosomes do not exhibit oscillatory motions around the spindle equator and also that throughout metaphase the arms of one or more long chromosomes are aligned parallel to the spindle long axis in \sim 50% of the cells (Fig. 2C, Fig. 3C, arrowheads). The pre-anaphase spindle usually rocks within the cell and, as a result, in DIC images the spindles poles are seldom co-planar for extended periods (compare Fig. 2C with Fig. 3C).

At anaphase onset (Fig. 2D, Fig. 3D), spindle rocking decreases, the chromosomes disjoin synchronously and move poleward (Fig. 2D-E, Fig. 3D-E) at 3.2 \pm 0.1 μ m/minute ($n=10$ in four cells; range 2.9-3.7 μ m/minute). Spindle elongation (anaphase B) starts concurrently with this chromosome-to-pole (anaphase A) motion (see below). Chromosomes decondense and form karyomeres near the spindle poles 2.5 \pm 0.1 minutes ($n=10$; range 120-208 seconds) after anaphase onset (Fig. 2F, Fig. 3F). Near the onset of telophase, the elongating spindle undergoes a sudden, but slight, positional shift, which moves it closer to that region of the cortex destined to become incorporated into the GMC (see below). As anaphase B continues, the future GMC becomes visible as a deformation and protrusion of the cell membrane (Fig. 2F, Fig. 3F). The cleavage furrow then begins to constrict the cell at the junction between the protrusion and major cell body, and continues to ingress until a midbody forms between

Fig. 2. Mitosis in a neuroblast. During prophase (A) the nucleoplasm becomes granulated as the chromosomes condense. After NEB (B) the chromosomes attach to the forming spindle and quickly congress to a metaphase configuration (C). Metaphase chromosomes show little motion, and long chromosome arms are often aligned parallel to the spindle long axis (arrowheads). Sister chromatids disjoin synchronously at anaphase (D), and at this time the spindle also begins to elongate. Restitution nuclei form after the chromosomes reach the poles (F), and the spindle becomes



asymmetrically positioned within the cell near the onset of telophase. Once this occurs the centrally located centrosome stops moving but spindle elongation continues by movement of the cortical centrosome (F-H). Cytokinesis is initiated during telophase (arrows in G) and progresses to form a midbody that separates the GMC from the neuroblast (arrow in H). Time is in minutes and seconds. Bar, 10 μ m.

the small GMC and the larger neuroblast (Fig. 2G-H, arrows, Fig. 3G-H, arrows).

In culture, cytokinesis can either go to completion or the furrow can relax before bisecting the cell. Since binucleated cells are rare in neuroblast cultures, this variation is caused by the culture conditions and appears related to the degree of cell flattening.

Chromosome behavior and kinetochore/centrosome interactions

The continuous changes in spindle positioning made it difficult to follow selected components and their interaction, over an extended period by DIC. To more thoroughly characterize these interactions we therefore followed cells expressing various GFP-tagged proteins using 3D fluorescence LM. We began our analysis using a GFP-tagged histone variant, which revealed that the chromosomes were paired by late prophase,

when they were well condensed (Fig. 4A). With 4D imaging, we could readily define when the chromatids disjoined to initiate anaphase, which occurred relatively synchronously. In 40% of the cells, the small #4 chromosomes lead the motion during anaphase (insets in Fig. 4D-H), and chromosome decondensation occurred 2.7 ± 0.1 minutes ($n=11$) after anaphase onset (essentially the same timing as obtained from DIC observations; see above).

To follow kinetochore behavior relative to the centrosomes, we used larvae expressing two different GFP tags (Fig. 5). Kinetochores were labeled with GFP-fizzy (GFP-fzy), the *Drosophila* homologue of Cdc20 (Dawson et al., 1995; Lorca et al., 1998). Similarly, centrosomes were labeled with a GFP-fizzy-related fusion (GFP-fzr) (Sigrist and Lehner, 1997). During prophase, GFP-fzy is excluded from the nucleus (data not shown) until it first becomes permeable, after which it rapidly localizes to kinetochores. It then remains on kinetochores throughout spindle formation, but is only weakly

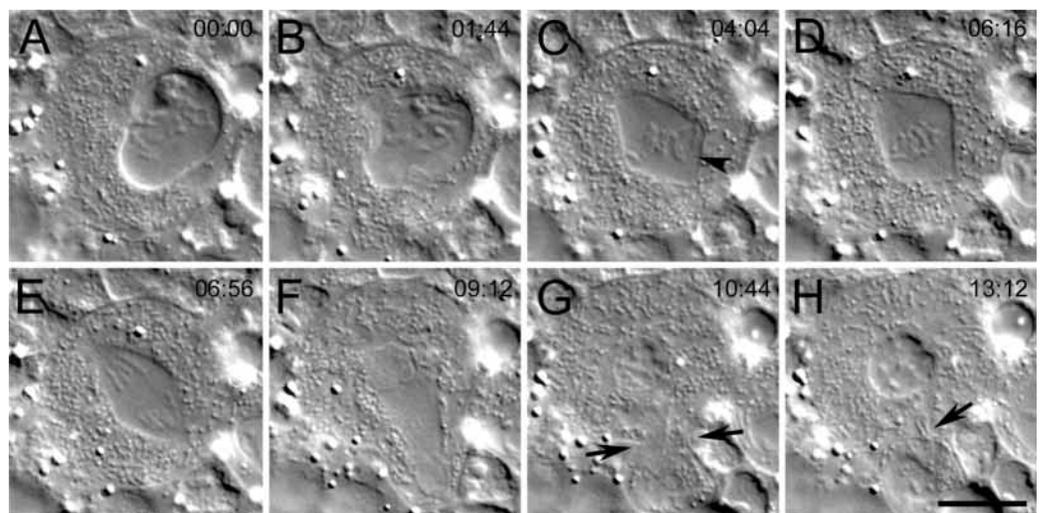


Fig. 3. Mitosis in a cultured neuroblast. Similar to Fig. 2, except the spindle poles remain co-planar during prometaphase and metaphase (C). (D) Anaphase onset. (E) Late anaphase prior to spindle repositioning. (F) Telophase, just after spindle repositioning. (G,H) Cytokinesis (arrows note the furrow and midbody). Time is in minutes and seconds. Bar, 10 μ m.

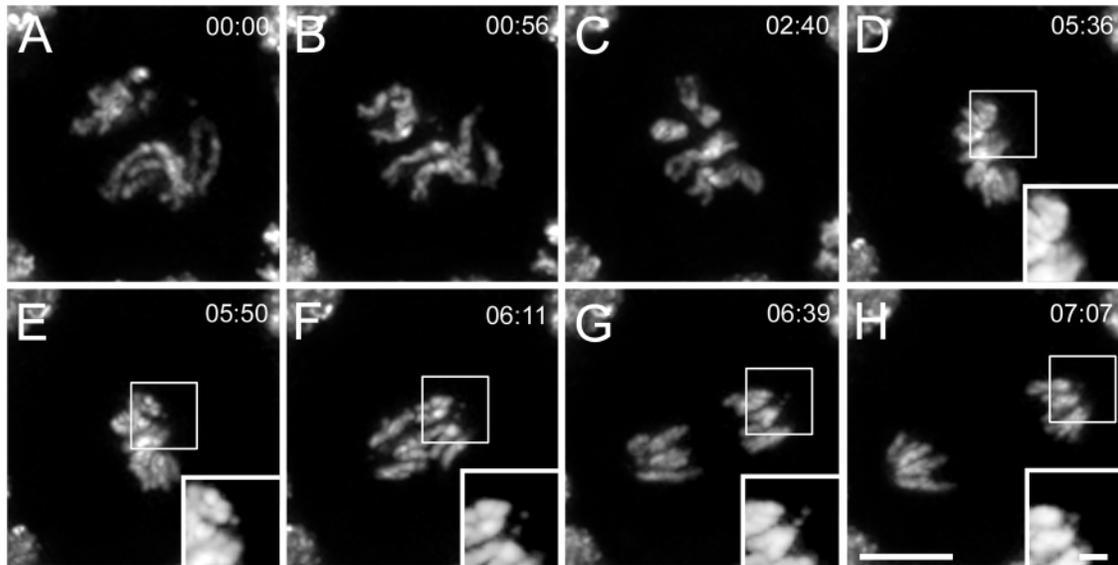


Fig. 4. Maximum intensity projections from a 4D time-lapse recording of mitosis in a neuroblast tagged with GFP-histone. By late prophase (A) homologous chromosomes appear paired, and after NEB sister chromatids can be resolved as individual units often separated in the telomere region (B,C). Chromosomes congress rapidly (B-D), and remain in metaphase for several minutes prior to anaphase (E). During anaphase the small fourth chromosomes often lead the way poleward (inset in D-H). Time is in minutes and seconds. Bar, 5 μm ; inset is 1 μm .

visible after anaphase. The nuclear infusion of GFP-fzy provides a reliable marker for NEB, whereas the separation of GFP-fzy-tagged kinetochores provides a similar marker for anaphase onset. Using these criteria the duration of prometaphase, at 24°C, is 7.0 ± 0.5 minutes ($n=13$; range 282-578 seconds).

The timing of centrosome separation, relative to NEB, is highly variable: in some neuroblasts the centrosomes are already positioned on opposite sides of the nucleus at NEB, whereas in others they separate after NEB as the kinetochores attach to the asters (see, however, exceptions in next section). During prometaphase, sister kinetochores exhibit various

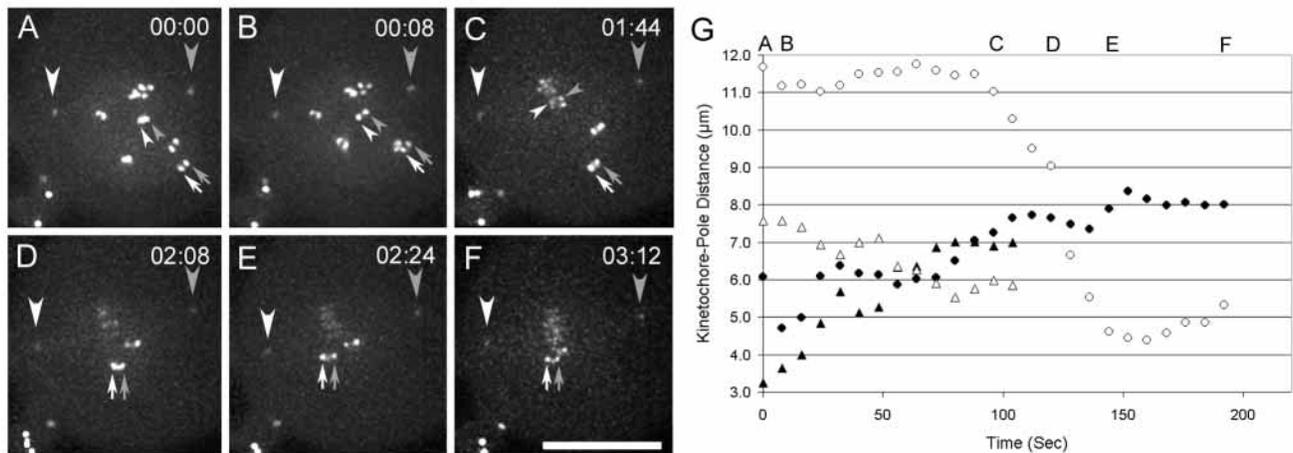


Fig. 5. Maximum intensity projections from a 4D sequence of a dividing neuroblast containing GFP-tagged kinetochores and centrosomes. Kinetochores are labeled with GFP-fzy and centrosomes with GFP-fzr. GFP-fzy appears on kinetochores (A, arrows and small arrowheads) at NEB, while GFP-fzr is present on centrosomes (A, large arrowheads) as cells enter mitosis. After attaching to the spindle, sister kinetochores (e.g. gray and white arrows and arrowheads in A-F; filled and open circles and triangles in G) rapidly achieve a stable equatorial position. During early prometaphase some attaching kinetochores (A,B, gray arrows) exhibit a sudden rapid motion towards the proximal pole (A,B, large gray arrowhead). In the example here, the chromosome then moves away from the pole along a vector that does not intersect the distal centrosome (small white and gray arrows in B,C). These sister kinetochores then exhibited an arc-like motion that positioned them on the metaphase plate (C,D), after which they remained stationary (E,F). By comparison, sister kinetochores on chromosomes more centrally located between the centrosomes at NEB quickly become bioriented, after which they congress in one relatively smooth motion (small arrowheads in A-C; open and filled triangles in G). Time is in minutes and seconds. Bar, 10 μm . (G) Plot showing the behavior of sister kinetochores marked by the gray and white arrows/arrowheads in A-F. The positions of sister kinetochores denoted by the gray and white small arrows/arrowheads, relative to their respective poles, are plotted on the graph as filled and open circles and triangles. Letters at the top of the graph note the time points corresponding to panels (A-F).

behaviors and sometimes they undergo a sudden displacement towards one pole (Fig. 5A,B, gray arrows and large arrowheads; Fig. 5G, closed circles). We interpret this as a monopolar attachment (mono-orientation). Unfortunately, the temporal resolution of our data (z-series every 8 seconds) was insufficient to determine the duration, and thus the absolute magnitude, of these rapid motions. In other cases the sister kinetochores appear to attach simultaneously (Fig. 5A-C, small white and gray arrowheads; Fig. 5G, open and filled triangles) and rapidly establish a stable metaphase position with very little motion.

Sister kinetochores that undergo a sudden poleward motion during attachment can subsequently make several motions towards the spindle equator during congression, after which they become stably positioned (data not shown). However, they may also move away from the proximal pole along a vector that does not intercept the opposing centrosome (Fig. 5B,C white and gray arrows; Fig. 5G open and filled circles). Sister kinetochores positioned in this manner usually exhibit a subsequent lateral motion towards the interpolar axis, which brings them into alignment with the other metaphase kinetochores (Fig. 5C,D, white and gray arrows; Fig. 5G open and filled circles). After reaching the spindle equator, the kinetochores do not exhibit notable oscillations (Fig. 5A-F, arrows and small arrowheads; Fig. 5G, open and closed triangles and circles).

One explanation for why chromosomes do not oscillate during congression and metaphase in neuroblasts, as they do in vertebrate somatic cells (Skibbens et al., 1993; Khodjakov and Rieder, 1996), is that opposing poleward forces are constantly acting on the sister kinetochores. We attempted to evaluate this by measuring the distance between sister kinetochores before attachment, as well as during congression and metaphase. Our rationale was that, if sister kinetochores behave independently, then the inter-kinetochore distance should vary with time. However, although our impression from time-lapse sequences is that the inter-kinetochore distance increased during chromosome attachment, and became maximal at metaphase, the temporal (8 second) resolution and noise in our data made it impossible to demonstrate this with certainty.

Once a chromosome achieves a position on the spindle equator, the intensity of the GFP-fzy tag on its sister kinetochores is attenuated (Fig. 5). This is not caused by global

changes in the cell, because the GFP-fzy label does not decrease simultaneously on all kinetochores, and kinetochores on late congressing chromosomes consistently display a brighter signal (Fig. 5A-F). As a result, with this marker it was not possible to reliably track kinetochores beyond mid-metaphase. By contrast, GFP-fzr remains at spindle poles throughout mitosis, which allowed us to continuously monitor centrosome behavior during spindle formation (e.g., Fig. 5 large arrowheads) and anaphase. During prometaphase, after the centrosomes have achieved a maximum separation distance, this distance is either maintained or, in some cases, the spindle shortens during metaphase. In all cases, anaphase B spindle elongation begins immediately following chromatid disjunction at anaphase onset (data not shown). During this time the rate that centrosomes move apart is relatively constant at $1.6 \pm 0.1 \mu\text{m}/\text{minute}$ ($n=4$; range 1.3-1.9 $\mu\text{m}/\text{minutes}$).

Spindle formation and microtubule distribution during anaphase and telophase

We used 4D LM to study spindle formation and maturation in neuroblasts cultured from GFP- α -tubulin-expressing larvae. Our goal was to detail these processes *in vitro* as a prelude to future mutational analyses.

In GFP- α -tubulin-expressing neuroblasts, the centrosomes appear during prophase as two similarly sized intense spots from which Mts radiate (Fig. 6, Fig. 8A, Fig. 9A). In cells where the centrosomes are well separated by late prophase (e.g. Fig. 6) numerous centrosomal Mts are seen to invade the area of the former nucleus during NEB (Fig. 6B, Fig. 8A). These Mts appear to arise from that portion of the centrosome facing the chromosomes, and at this time their associated 'astral' Mt arrays become greatly attenuated (Fig. 6B, Fig. 8B). As prometaphase progresses, many of the Mts between the two centrosomes become organized into discreet bundles that terminate in a non-fluorescing band at the spindle equator (Fig. 6C, arrowheads). Co-labeling with Hoechst 33342 confirms that this region corresponds to the chromosomes (data not shown) and that these bundles are therefore kinetochore fibers (k-fibers). By metaphase, k-fibers are the most conspicuous components of the spindle (Fig. 6C).

At anaphase onset, the k-fibers begin to shorten (Fig. 6D, arrowheads), and the spindle begins to disassemble. Spindle elongation starts as soon as the chromatids disjoin, and during

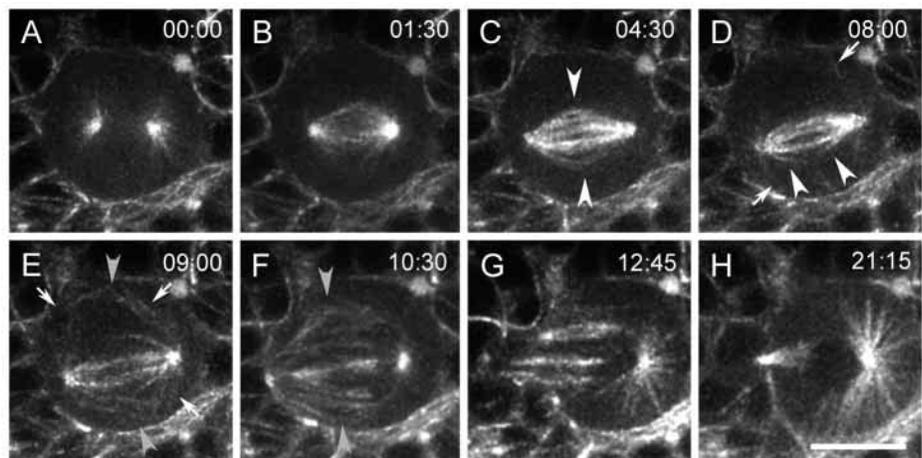


Fig. 6. Maximum intensity projections from a 4D sequence of a neuroblast expressing GFP- α -tubulin, as it progresses from late-prophase through cytokinesis. (A) Late prophase. (B) Early prometaphase. (C) Metaphase. (D) Anaphase. (E) Late anaphase. (F) Telophase. (G-H) Late telophase/cytokinesis. See text for details. Time is in minutes and seconds. Bar, 10 μm .

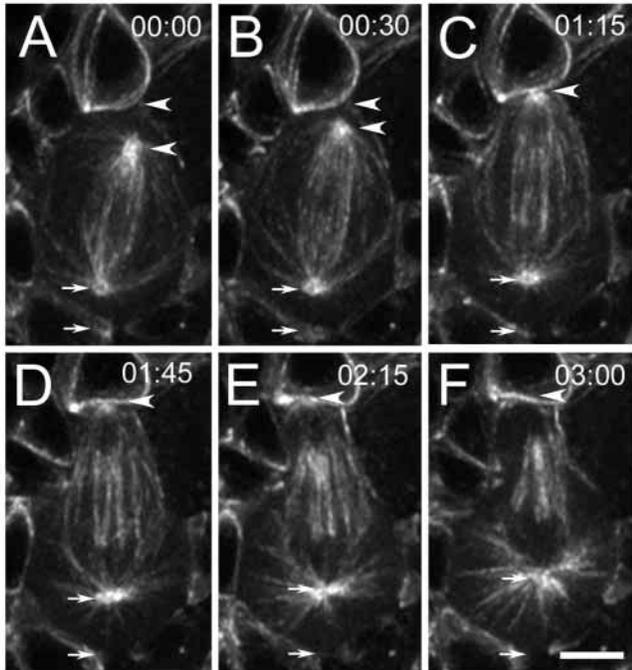


Fig. 7. Spindle behavior during anaphase and telophase in a neuroblast expressing GFP- α -tubulin. The elongating spindle remains centrally positioned until mid-anaphase (A), at which time it undergoes a sudden shift towards the cell cortex (B,C), with the future GMC cell centrosome leading (white arrowheads in A-F). After this shift, the centrally located centrosome (white arrows) stops moving (C-F) and begins to generate a more robust aster (D-F), while the spindle continues to elongate. In time-lapse recordings this elongation appears to ‘push’ against the cortex to form a cytoplasmic protrusion into which the GMC centrosome (and its associated nucleus) move. Time is in minutes and seconds. Bar, 5 μ m.

this time astral Mts again begin to grow from each centrosome (Fig. 6D arrows). These rapidly elongate until reaching the cell cortex and often continue to grow along this boundary (Fig. 6D,E, arrows). As anaphase B progresses, the opposing arrays of centrosome-derived overlapping Mts (Fig. 6E,F gray arrowheads) form a ‘cage’ around the elongating spindle (Fig. 6E,F, Fig. 7A,B).

Shortly after this cage forms, one of the centrosomes suddenly moves towards the cell cortex, which induces the whole spindle to shift position (Fig. 6E,F; Fig. 7A-C). This shift occurs quickly (over \sim 30 seconds), while the spindle is elongating. It is readily evident in larger cells where it covers several μ m (see Fig. 7B,C), but less so in smaller cells in which spindle elongation has already positioned the centrosomes adjacent to the cortex by mid-anaphase.

After the spindle changes its position, a robust Mt array forms in association with the more centrally located centrosome (Fig. 6F-H, Fig. 7C-F), which will become incorporated into the new neuroblast. By contrast, the Mt-nucleating ability of the cortical centrosome, which is incorporated into the GMC, is attenuated. At this time the centrally located centrosome stops moving (Fig. 7C-F), and the spindle continues to elongate primarily by movement of the cortical (GMC) centrosome away from the more stationary, centrally positioned neuroblast centrosome (arrows, Fig. 7).

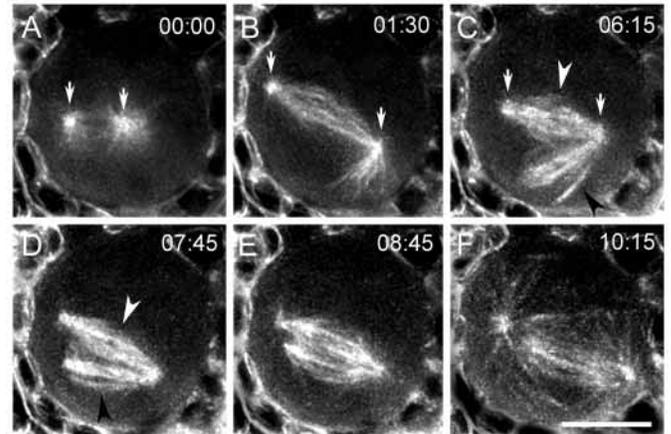


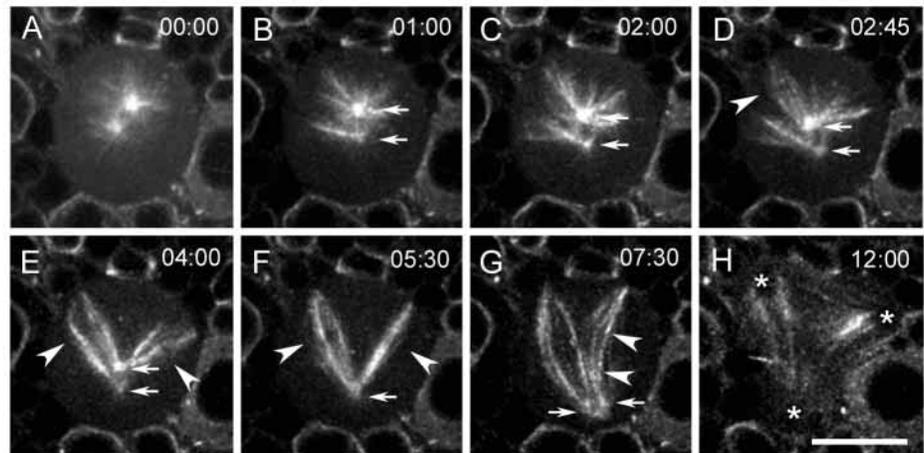
Fig. 8. Secondary spindles can form in association with chromosomes attached to the centrosome-containing spindle. In this GFP- α -tubulin-expressing neuroblast a half-spindle is generated in association with one of the centrosomes (right-hand arrow in B). This half-spindle then grows to form a fusiform-shaped structure containing a blunt acentrosomal pole (C,D). This ‘secondary spindle’ forms around one or more chromosomes, as shown by the fact that it contains a central band of reduced fluorescence (black arrowheads in C,D) as seen on the major spindle (white arrowheads in C,D). It then coalesces with the centrosome-containing bipolar spindle (D,E), and the cell undergoes a normal anaphase and telophase (F). Time is in minutes and seconds. Bar, 10 μ m.

During the elongation process, the cortical centrosome impacts and appears to ‘push’ against the cell membrane. This, in turn, correlates with the formation of a progressive bulge in the membrane into which the centrosome and its associated nucleus continue to move (Fig. 2E,F, Fig. 6F,G, Fig. 7). Concurrently, the Mt cage surrounding the spindle aggregates into multiple bundles (Fig. 6F,G; Fig. 7A-D) that coalesce during cytokinesis into one large bundle (mid-body) between the centrosomes (Fig. 6G,H; Fig. 7E-F).

The formation of acentrosomal spindle poles

As described above, in 83% of the GFP- α -tubulin-labeled neuroblasts, bipolar spindles formed between two separating centrosomes. However, in 17% (12/70) of these cells the spindles formed via a different pathway that could be further sub-divided into two distinct routes. In the majority (8/12), an additional half-spindle-shaped Mt array suddenly formed in association with one of the centrosomes (Fig. 8). This additional half-spindle was first detected as Mts began to emanate from one of the centrosomes towards a defined region of the cell (Fig. 8B), after which they became organized into bundles (Fig. 8C). Over time, these Mt bundles elongated and interacted along their length to form a spindle-shaped structure, one end of which contained a blunt acentrosomal polar region (Fig. 8C,D). Like the primary spindle (Fig. 8C,D white arrowheads), these spindles displayed a central band of decreased fluorescence (black arrowheads in Fig. 8C,D), which indicates the presence of one or more chromosomes. In all of these cells, the secondary spindle ultimately became incorporated into the primary spindle prior to anaphase (Fig. 8D,E), which then proceeded normally (Fig. 8E,F).

Fig. 9. Similar to Fig. 8, except that in this example a tri-polar spindle is organized in the absence of centrosome separation. At NEB (B) the two centrosomes are closely associated and remain so throughout mitosis (arrows C-G). Regardless, two bipolar spindles ultimately form (D-F) that share a common polar region defined by the two non-separated centrosomes. Each of these spindles contains chromosomes (white arrowheads in D-F) that, during anaphase (G), segregate into three nuclei (asterisks in H). Time is in minutes and seconds. Bar, 10 μ m.



The second unusual route of spindle formation was less common (4/12 cells) and occurred after a failure in centrosome separation. In two of these cells, a bipolar spindle was formed using the replicated centrosome as one of the spindle poles. In the other two, Mts emanating from the non-separated asters became bundled over time into two half-spindles, joined at one apex, that were directed to different regions of the cell (Fig. 9A-F). Over time these half-spindles elongated into normal fusiform-shaped structures, even while the two centrosomes remained adjacent to one another (Fig. 9E,F). The net result was the formation of a 'V'-shaped metaphase spindle that contained three poles, two of which were acentrosomal (Fig. 9). Both of these 'joined' spindles contained chromosomes, as revealed by the attenuated fluorescence at the spindle equators (arrowheads in Fig. 9E,F). In these cases the spindle remained tri-polar throughout the ensuing anaphase (Fig. 9G,H).

Discussion

Here we describe a rapid and simple method for generating short-term cultures of neuroblasts from *Drosophila* third instar larvae, which allows the neuroblasts to be followed by high-resolution video-enhanced 4D LM. Using this method we have detailed, for the first time, spindle formation and chromosome behavior in vitro during karyokinesis in these cells. Since *Drosophila* is a powerful system for studying zygotic gene mutations affecting mitosis and since larval neuroblasts are routinely used in such screens, our approach will allow a more accurate description of how mutant phenotypes arise - thereby refining the roles that various proteins play in the division process.

At 24°C, mitosis in wild-type neuroblasts takes ~15 minutes. During this time the condensing chromosomes are visible ~2 minutes before NEB; spindle formation (prometaphase to anaphase onset) requires ~7 minutes; anaphase (chromatid disjunction until chromosome fusion) requires ~3 minutes; and telophase/cytokinesis requires ~3 minutes. Therefore, of the mitotic cells in a fixed population of wild-type neuroblasts, ~15% should be in prophase, ~45% in prometaphase/metaphase, ~20% in anaphase and the remainder (~20%) in various stages of telophase/cytokinesis. Indeed, in fixed wild-type populations, the percentage of neuroblasts in anaphase, relative to all mitotic cells, is ~20% (e.g. Wilson et al., 1997;

Cullen et al., 1999; Lemos et al., 2000; Giansanti et al., 2001). Although it is sometimes reported that 75-80% of the dividing cells in such preparations are in 'metaphase' (e.g. Sunkel et al., 1995; Inoue et al., 2000), our analyses suggest that this proportion is really only 45-50%. This discrepancy probably arises from the fact that after fixation and squashing, prophase cells are difficult to distinguish from those in prometaphase/metaphase. Also, telophase cells, in which the chromosomes have reformed nuclei, may not be scored as mitotic (as we do), which would lead to an enhanced percentage of prometaphase/metaphase cells in the mitotic population.

In wildtype, the mitotic index in fixed/squashed brains can vary two-fold from larvae-to-larvae (Gatti and Baker, 1989). By contrast, the duration of mitosis is considerably less variable, and as noted above, anaphase cells constitute ~20% of the mitotic figures. This being the case, the percentage of mitotic cells in anaphase, or the ratio of prometaphase/metaphase to anaphase cells (see Wojcik et al., 2000), provides a more accurate description of mitotic progression than the total mitotic index. In some cases the mitotic index may vary only by a factor of two between wild-type and mutants, whereas the number of anaphase cells varies ten-fold (e.g. Cullen et al., 1999).

In the appropriate media, neuroblasts isolated from gastrula-stage embryos (e.g. Seecof et al., 1973; Furst and Mahowald, 1985; Broadus and Doe, 1997) and even third instar larvae (Wu et al., 1983) can be cultured for several days during which they undergo multiple mitoses. By contrast, our larval neuroblast cultures begin to deteriorate after ~1 hour. Our intent, however, was not to maintain the cells for extended periods but rather to establish a rapid and simple procedure that allows for short-term high-resolution LM studies. We used halocarbon oil because it produces a much crisper DIC image than media does, and replacing this oil with growth medium does not substantially prolong the life span of our cultures. This is because high-resolution transmitted LM requires that the viewing chamber be extremely thin, which severely limits the amount of fluid bathing the cells (Rieder and Cole, 1998). We designed our approach around that used by many to study meiosis by high-resolution LM in insect spermatocytes (e.g. Nicklas and Staehly, 1967; LaFountain, 1982; Savoian et al., 2000), where long-term viability is also sacrificed for resolution. Regardless, considering that mitosis requires only

15 minutes, and that neuroblasts remain viable for >1 hour, our approach provides ample time to locate and follow one or two divisions in each culture.

Ninety-seven percent (68/70) of the cells we followed through mitosis formed bipolar spindles, entered anaphase, completed telophase and initiated (if not completed) cytokinesis. However, we did observe abnormally high numbers of tri-polar spindles (2/70 cells or 3%) and cells containing spindle poles lacking centrosomes (4/70 cells or 6% versus <1% in controls) (see Wilson et al., 1997). Although it is formally possible that these represent sick cells, strong arguments can be made that this is not the case. All of these cells entered anaphase, completed karyokinesis and at least attempted cytokinesis. Two of the most sensitive stages of the cell cycle include entry into and exit from, mitosis (Mazia, 1961). These transitions are both guarded by pathways that rapidly arrest mitotic progression when triggered in response to various stresses (reviewed in Pearce and Humphrey, 2002). For example, in addition to DNA damage, the G2/M transition is (reversibly) inhibited by anoxia, hypothermia/hypothermia, elevated CO₂, changes in pH and changes in tonicity, etc. (Rieder and Khodjakov, 1997; Mikhailov and Rieder, 2002). As a result, sick or stressed cells do not enter mitosis, yet alone form spindles, disjoin chromosomes, exit mitosis or initiate cytokinesis.

Spindle formation

Neuroblasts in *urchin*-mutant larvae, in which the kinesin-like protein KLP61F is non-functional, often (60%) form spindles in which one of the poles lacks a centrosome. Although the route by which these 'monastral bipolar' spindles are generated remains to be defined (Wilson et al., 1997), at least some are likely to arise from the organization of an acentrosomal half-spindle in the presence of non-separated centrosomes. [In mammals, inhibiting Eg5, the homologue of KLP61F, prevents centrosome separation but produces monopolar spindles (Kapoor et al., 2000).] Although rare (<1%), bipolar spindles lacking centrosomes at both poles (i.e. anastral spindles) are also observed during mitosis in wild-type and *urchin* mutant *Drosophila* neuroblasts as well as in gonial cells (Wilson et al., 1997). Finally, bipolar spindles are also reported to be organized in neuroblasts when the formation of astral (centrosomal) Mt arrays is compromised, as in *asterless* (Bonaccorsi et al., 2000; Giansanti et al., 2001) and *centrosomin* (Megraw et al., 2001) mutants. Together these data from fixed cells imply that neuroblasts (and probably other cells in *Drosophila*) normally have a route for organizing half-spindles independently from the centrosome and its astral Mt array. Our live cell data directly confirm this conclusion: in 17% of our cells a bipolar spindle formed in association with one or more chromosomes when they were attached to only a single centrosome (Figs 8 and 9).

We observed a higher incidence of cells containing both centrosomes in one spindle pole (6%) than reported for wild-type neuroblasts fixed in situ (<1%) (Wilson et al., 1997). We attribute this to excessive cell flattening during culture preparation which, relative the more rounded condition seen in situ, would be expected to enhance the frequency with which chromosomes are not readily positioned to interact with both asters at NEB. Indeed, these are the chromosomes that organize

a half-spindle lacking a centrosome. However, regardless of the extent that this acentrosomal pathway normally contributes to spindle formation in neuroblasts, our data not only demonstrate that it exists, but also that it can lead to the formation of functional bipolar spindles that are not retarded from entering anaphase. The ability of *Drosophila* neuroblasts to organize a functional bipolar spindle when the centrosomes fail to separate offers an explanation for why monopolar spindles are seldom seen in wild-type cells (e.g. Heck et al., 1993; Wilson et al., 1997; Inoue et al., 2000), and it provides a ready (although yet to be proven) mechanism for forming monastral bipolar spindles in mutants lacking a functional KLP61F (and even wild-type neuroblasts) (Wilson et al., 1997). Together our data and those of others demonstrate that neuroblasts contain a constitutive pathway for forming an acentrosomal half-spindle that is manifested when one or more chromosomes are delayed in becoming bioriented between two separated centrosomes.

Given what we know about spindle formation, it is doubtful that the secondary spindles that we observed are an artifact of α -tubulin-GFP expression. These cells were obtained from a strain of flies that constitutively express the probe without any apparent detrimental effects. We also saw what we interpret to be a similar pattern in our DIC recordings of wild-type cells and those expressing *GFP-fzy* and *GFP-fzr* (Fig. 5): sister kinetochores positioned off the axis between the two centrosomes move laterally into the metaphase plate, as occurs when a secondary spindle fuses with the major centrosomal spindle (Fig. 5). Furthermore, although the presence of secondary spindles has not been described previously in wild-type *Drosophila* neuroblasts fixed in situ, a low frequency of monastral bipolar structures (and even anastral spindles) are seen in larval brains of wild-type animals (Wilson et al., 1997). As argued above, it is likely that the formation of secondary spindles via the acentrosomal pathway is manifested only when the formation of a bipolar attachment between two asters is delayed, as occurs more frequently when the cells are flattened.

When present during mitosis or meiosis, centrosomes are thought to suppress the formation and organization of Mts around chromosomes and thus dominate as sites of Mt nucleation (Heald et al., 1997; Hyman and Karsenti, 1998). However, we find that the presence of two functional centrosomes in *Drosophila* neuroblasts does not preclude the formation of Mts near the chromosomes (Figs 8 and 9). Although the mechanism for forming these Mts is unknown, it is interesting that relatively normal looking bipolar spindles are formed during mitosis in *Drosophila* neuroblasts lacking functional γ -tubulin (Sunkel et al., 1995). As in *Xenopus* oocyte extracts (Walczak et al., 1998), once generated near the chromosomes (perhaps by the Ran/GTP pathway) (see Dasso, 2001), Mts in neuroblasts are presumably organized into stable and functional half-spindles via the action of Mt motors and other chromatin-associated proteins.

In *orbit/mast* (Inoue et al., 2000; Lemos et al., 2000) and *aurora* (Glover et al., 1995) mutants, the centrosomes generate asters during prometaphase that fail to separate. As a result, monopolar spindles are formed at NEB in which the chromosomes are grouped around a single pole containing multiple asters. How does the monopolar mutant phenotypes arise given the above evidence that, in neuroblasts, the chromosomes can direct the organization of a functional half-

spindle in the absence of a second centrosomal region? One interesting possibility is that the monopolar phenotype (see also *mgr*) (Gonzalez et al., 1988) is not caused simply by a defect in centrosome separation, but that it arises instead because the mutant protein normally participates, for example, in generating or stabilizing Mts in the vicinity of the chromosomes. Indeed, orbit/mast is a Mt-associated protein, which is proposed to play an important role in regulating spindle Mt behavior (Inoue et al., 2000; Lemos et al., 2000), and the kinase activity of aurora A is similarly required for spindle Mt stabilization (Giet et al., 2002) as well as in activating KLP61F (Eg5) (reviewed in Giet and Prigent, 1999).

Spindles formed around individual chromosomes in *Drosophila* neuroblasts usually associate laterally into a common bipolar array by anaphase onset (Fig. 8). Although the mechanisms responsible for this fusion remain unclear, it appears to be retarded in *mini-spindle* (Cullen et al., 1999) and *asp* (Avides and Glover, 1999; Wakefield et al., 2000) mutants, both of which encode for Mt-associated proteins. The progressive coalescence of multiple spindles into a single functional bipolar unit may explain why neuroblasts entering mitosis in the presence of multiple (separated) centrosomes form mostly bipolar spindles, as for example, in *Slimb* mutants (Wojcik et al., 2000).

The only metazoan cell line lacking centrioles/centrosomes is derived from *Drosophila*, and in fixed preparations 40% of the mitotic cells in this line are reported to contain multipolar 'metaphase' spindles (Debec et al., 1995). Our data suggest that these figures are generated as spindles form around individual chromosomes or groups of chromosomes. It also suggests that if given time (i.e. if not fixed) most or all of these individual spindles would ultimately fuse into a common bipolar array. However, neuroblasts can clearly enter anaphase before this fusion process is complete (Fig. 9). Thus, although centrosomes are not needed to form spindles, the presence of two separated centrosomes appears to ensure (and enhance) the fidelity of bipolar spindle formation.

Chromosome behavior

During early prometaphase, chromosomes in *Drosophila* neuroblasts can exhibit a rapid motion towards one of the centrosomes. This probably reflects the sudden attachment of one sister kinetochore to an aster, which induces the chromosome to mono-orient and move towards the now forming spindle pole. The rapid poleward motion of a mono-orienting chromosome is a common feature of mitosis in a wide variety of higher eukaryotes (Rieder and Salmon, 1998), and in *Drosophila* spermatocytes it appears to be mediated by kinetochore-associated cytoplasmic dynein (Savoian et al., 2000).

We find that the metaphase plate in *Drosophila* neuroblasts can be generated via two routes. In most cells it forms through the traditional pathway, in which the sister kinetochores on each chromosome become attached to the opposing astral Mt arrays as they separate. After bi-orientation, the chromosome then quickly moves in one or several smooth motions to the metaphase plate where it remains static until anaphase. In the other route, which is seen only after a chromosome has already become attached to one of the centrosomes, a second opposing half-spindle and pole is organized around the chromosome

without reference to an existing astral Mt array. This results in the transient formation of two or more spindles that share a single common pole (e.g. Fig. 8). In these cells all of the chromosome(s) become aligned on a common metaphase plate as these secondary spindles fuse laterally with the primary spindle.

Unlike in many animal cells (Rieder and Salmon, 1994), in neuroblasts the centromere region on congressed chromosomes does not move continuously back and forth, or oscillate, across the metaphase plate, that is, the sister kinetochores do not exhibit directional instability. The stationary behavior resembles that seen in plants (Khodjakov et al., 1996), insect spermatocytes [grasshopper (Hays and Salmon, 1990); *Drosophila* (Savoian et al., 2000); crane fly, (LaFountain et al., 2001) and *Xenopus* oocytes (Funabiki and Murray, 2000)].

We found that the arms of the larger chromosomes were often directed towards a spindle pole prior to anaphase. This behavior is also seen in plants (Khodjakov et al., 1996) and some insect spermatocytes containing large chromosomes (Adames and Forer, 1996), where it has been shown by microsurgery to be mediated by pole-directed forces that act continuously along the chromosome. It is also seen in *Xenopus* oocyte extracts (Funabiki and Murray, 2000) and in vertebrate somatic cells (Levesque and Compton, 2001), after depleting the chromosome-associated kinesin-like protein kid. In insect spermatocytes, this poleward force is produced within each half-spindle by the continuous flux of tubulin subunits from the spindle equator towards the pole. During anaphase in crane fly spermatocytes (LaFountain et al., 2001) and *Xenopus* spindles (Murray et al., 1996), the flux rate is equal to the rate of poleward chromosome motion, suggesting that flux drives chromosome movement (Waters et al., 1996; Desai et al., 1998). Flux also appears to be involved in anaphase chromosome movement in *Drosophila* embryos, although a consensus has yet to be reached regarding its total contribution. The fact that the arms of long chromosomes point poleward during spindle formation in neuroblasts suggests that flux is a component of these spindles as well.

Asymmetric cytokinesis

Asymmetric cytokinesis in *Drosophila* neuroblasts has been detailed by indirect immunofluorescence LM (Bonaccorsi et al., 2000; Giansanti et al., 2001) and also by vital fluorescence imaging of embryos expressing GFP-tau (which labels Mts) (Kaltschmidt et al., 2000). The former studies provided no data on the dynamics of this process, whereas the latter focused on spindle rotation and positioning. All report that the astral Mt array associated with the centrosome that is destined to be incorporated into the GMC is greatly attenuated during anaphase, whereas the aster associated with the more centrally located neuroblast centrosome grows.

Our work provides some novel insight into the process of cytokinesis in larval neuroblasts. First, we find that both asters grow to an equal extent during early anaphase and that during this growth the opposing astral Mt arrays overlap to form a 'cage' around the spindle (Figs 6 and 7). As anaphase continues, the spindle suddenly moves closer to one side of the cell, with the centrosome destined to be incorporated into the GMC leading. This shift, which has not been reported previously for *Drosophila* neuroblasts, asymmetrically

positions the elongating spindle within the cell. It is distinct from the spindle rotations described in embryo neuroblasts (Kaltschmidt et al., 2000), which occur during metaphase and define the future orientation of the cleavage plane. The positional shift of the mid-anaphase spindle in *Drosophila* neuroblasts is similar to that described during anaphase in *Caenorhabditis elegans* embryos (Doe and Bowerman, 2001), which also leads to an asymmetric cytokinesis.

Spindle displacement in the worm is mediated by an imbalance of pulling forces acting on the Mts of each aster (Grill et al., 2001), and *Drosophila* neuroblasts probably use this same mechanism to reposition the spindle during late anaphase. As a result of this positional change, the central spindle mid-zone, which ultimately defines the sight through which the furrow will pass, becomes positioned off-center within the cell. In the *asterless* mutant, neuroblasts still divide to produce different-sized daughter cells (Giansanti et al., 2001), which implies that asters are not required for asymmetric cytokinesis in this system. However, it is possible that these mutants possess enough astral Mts to effect spindle repositioning. Alternatively, spindle repositioning may not be an absolute requisite for asymmetric cytokinesis in *Drosophila* neuroblasts, which are extremely small relative to the large *C. elegans* embryo. Indeed, we found that small neuroblasts also undergo an asymmetric cytokinesis even when there is little room in the cell for the spindle to shift position. In this context, it is noteworthy that, in contrast to what has been reported in embryonic cells (Kaltschmidt et al., 2000), we find that in larval neuroblasts the more centrally located centrosome (which is destined to be incorporated into the new neuroblast) generates a robust aster and preferentially stops moving during the early stages of spindle elongation (Figs 6 and 7). As a result, as the spindle continues to elongate, the centrosome destined for the GMC impacts the cell cortex where it appears to induce a bulge in the membrane. Thus, the asymmetric cytokinesis in larval neuroblasts arises from two sequential processes, including a sudden shift in spindle position towards a point on the cell cortex followed by a growth and immobilization of the centrally located aster, whereas the spindle continues to elongate. Both of these pathways lead to the required off-center positioning of the spindle midbody.

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