Visualization and functional analysis of spindle actin and chromosome segregation in mammalian oocytes

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Running head: Studying spindle actin function in mammalian oocytes

Abstract

Chromosome segregation is conserved throughout eukaryotes. In most systems, it is solely driven by a spindle machinery that is assembled from microtubules. We have recently discovered that actin filaments that are embedded inside meiotic spindles (spindle actin) are needed for accurate chromosome segregation in mammalian oocytes. To understand the function of spindle actin in oocyte meiosis, we have developed high-resolution and super-resolution live and immunofluorescence microscopy assays that are described in this chapter.

Key words: Actin, microtubules, chromosomes, meiosis, oocytes, eggs, spindle, fertility, high-resolution live microscopy

1. Introduction

Every mammalian life begins with the fertilization of an egg by a sperm cell. For the resulting genetically unique zygote to grow into a healthy offspring, both the egg and sperm should first contain the correct number of chromosomes. However, for reasons we are only starting to understand, eggs are often likely to have additional or missing copies of certain chromosomes before fertilisation – they are aneuploid [1]. Embryos formed from fertilisation of aneuploid eggs frequently die, leading to pregnancy failures, or result in offspring with genetic disorders such as Down's syndrome. Importantly, the rate of egg aneuploidy increases dramatically with advancing maternal age [2]. This phenomenon, often referred to as 'the maternal age effect', is highly attributed to errors in meiosis, the specialised form of cell division that generates eggs from oocytes [3]. Indeed, a number of factors including oocyte chromosome cohesion [4] and microtubule dynamics [5] are known to deteriorate with increasing maternal age.

Meiotic chromosome segregation is driven by a spindle machinery that is assembled from microtubules and separates the chromosomes in two rounds of cell division [3]. Interestingly, we recently discovered that the actin cytoskeleton plays a vital role inside the meiotic spindle – actin filaments embedded inside the spindle help to organise microtubules into functional bundles that can accurately separate the chromosomes [6]. This finding constitutes an important safety mechanism in mammalian meiosis that prevents aneuploidy in oocytes and eggs. The association of actin filaments with meiotic spindles of mouse oocytes has long been known [7,8], but its functional analyses had been thwarted due to lack of high spatial and temporal resolution microscopy assays. This chapter discusses in detail the live and immunofluorescence microscopy assays that have for the first time enabled highly resolved visualisation and functional analysis of spindle actin in mammalian oocyte meiosis.

2. Materials

2.1 Ovary dissection and oocyte isolation

1. N6,2′-O-Dibutyryladenosine 3′,5′-cyclic monophosphate sodium salt (dbcAMP)
2. 35 mm tissue culture dishes
3. Pastettes (e.g. Alpha Laboratories, LW4206)
4. Paraffin (mineral oil)
5. 37ºC incubator
6. 1.5 ml microcentrifuge tubes
7. Mouth aspirator tube assemblies for calibrated microcapillary pipettes (e.g. Sigma, A5177-5EA)
8. 100 µl micropipettes (e.g. PIP3022, Scientific Laboratory Supplies)
9. 0.22 µm filters
10. 2x Dumont Tweezers #2, 12 cm, Straight, 0.34x0.14 mm Tips (e.g. Electron Microscopy Sciences, 0103-2-PO)
11. 2x Dumont Tweezers #2, 8.3 cm, Straight, 0.06x0.1 mm Tips (e.g. Electron Microscopy Sciences, 0302-M5S-PS)
12. 1 ml BD Plastipak precision syringe (e.g. VWR, 613-3908)
13. 50 ml BD Plastipak Luer lock concentric syringe (e.g. VWR, 613-3925)
14. 13 mm gauge needle
15. Mouse ovaries from 8-12 weeks old CD1, 129s, FVBN or C57BL/6 mice

16. Zeiss Discovery V8 stereomicroscope

2.2 Stock solutions for oocyte culturing and *in vitro* maturation M2 medium

1. BSA stock: 10 mg/ml BSA in embryo transfer water (Sigma, W1503)
2. Stock A: 55.5 mg/ml NaCl, 3.8 mg/ml KCl, 1.74 mg/ml KH_2PO_4, 3.14 mg/ml $MgSO_4$, 10.76 mg/ml Glucose, 0.67 mg/ml Penicillin sodium salt, 0.54 mg/ml Streptomycin sulfate, 2.1% w/w Sodium Lactate in embryo transfer water
3. Stock B: 3.4 mg/ml $NaHCO_3$ in embryo transfer water
4. Stock C: 3.36 mM Sodium Pyruvate in embryo transfer water
5. Stock D: 2.6 mg/ml $CaCl_2$ in embryo transfer water
6. Stock E: 0.02 mg/ml Phenol red in embryo transfer water
7. Stock F: 175.2 mM HEPES
8. Store all stock solutions at -20ºC until use.
9. Thoroughly defrost aliquots of 10 mg/ml BSA and M2 stock solutions in a beaker filled with double-distilled or MilliQ water or by placing on a rotator at room temperature. Make sure there are no precipitates in any of the stock solutions before proceeding to the step 2.
10. Constitute M2 medium by adding 5 ml each of stock solutions A, B,C, D, E and F to 20 ml of 10 mg/ml BSA solution in a 50 ml tube and mixing by inversion. Refreeze unused stocks for future preparation of M2 medium.
11. Add 20 µl of 1M NaOH to the M2 medium and transfer 2 ml of it into a 14 ml tube for pH measurement.
12. Measure the pH and adjust to pH 7.2-7.4 by adding 1M NaOH in 2 µl increments to the 50 ml tube of M2.
13. Once the desired pH range is reached, load M2 medium into a 50 ml syringe and filter it through a 0.22 µm filter into a new 50 ml tube.
14. Divide filtered M2 medium into 8 ml fractions in 15 ml tubes and store at 4ºC for a maximum of two weeks.

2.3 mRNA synthesis and preparation for microinjection

1. mRNA synthesis kit (e.g. ThermoFisher, AM1344)
2. Nuclease free water
3. Sterile Microtubes

4. Special glass capillary tube (100 mm x 0.8 mm outer diameter) (e.g. Jaytec Glass, CAP-SPEC-100-8)
5. Silicone grease, medium viscosity, Bayer (e.g. VWR, ACRO386110010)
6. Microscope slide
7. 15 cm petri dish
8. Dimethylpolysiloxane (capillary oil)

2.4 Microinjection

1. 22x22 mm coverslips
2. Hydrochloric acid solution
3. Microwave
4. Absolute Ethanol
5. Tungsten carbide scriber (glass cutter) (e.g. Micro-Mark, 50299)
6. Microinjection chamber constructed from plastic according to dimensions specified in section 3.2.2.
7. Scotch double-sided tape, 12.7x7.9 mm
8. P-1000 micropipette puller fitted with a box filament (World Precision Instruments)
9. Zeiss AxioVert A1 microscope equipped with Narishige MN-4, MO-202U and NZ-19-2 micromanipulators and a CellTram 4r Oil hydraulic manual microinjector (Eppendorf)
10. Mercury

2.5 High-resolution and super-resolution live imaging, and drug addition experiments

1. For high-resolution live imaging, a Zeiss LSM 710, LSM 780, LSM 800, LSM 880, or Leica SP8 microscope equipped with sensitive Gallium Arsenide Phosphide (GaAsP) detectors and a piezo stage.
2. For super-resolution live imaging, a Zeiss LSM 800 or LSM 880 microscope equipped with Airyscan technology
3. Glass bottom imaging dishes (e.g. MatTek Corporation, P35G-0-14-C)
4. Dimethyl sulfoxide (DMSO)
5. 0.6 mM stock SNAP-Cell-647-SiR solution by dissolving compound in DMSO (NEB, S9102S)
6. 1 mg/ml Cytochalasin D stock solution prepared from powder

7. 5 mM Nocodazole stock solution prepared from powder
8. 1 mM SiR-Actin stock solution prepared by dissolving compound in DMSO (Cytoskeleton Inc., CY-SC001)

2.6 Immunofluorescence microscopy of fixed oocytes

1. 60-well Terasaki plates (Greiner Bio-One, 653180)
2. Soft tissue paper such as Kimwipes
3. BSA
4. HEPES
5. EGTA
6. $MgSO_4.7H_2O$
7. Formaldehyde, 10%, methanol free, ultra-pure
8. Triton X-100 solution
9. A 37ºC incubator designated for storage of fixative solution containing plates
10. Fixative stock solutions dissolved or diluted in double-distilled or Milli-Q water (1 M HEPES, 0.25 M EGTA, 1 M $MgSO_4.7H_2O$, 10% v/v TritonX-100)
11. Fixative solution in double-distilled or Milli-Q water (0.1 M HEPES, 0.05 M EGTA, 0.01 M $MgSO_4$, 2% Formaldehyde, 0.2 % TritonX-100)
12. Extraction (PBT) buffer (PBS+0.1% v/v Triton X-100)
13. Wash buffer (3% v/v PBT + BSA)
14. Anti-tubulin primary antibody
15. Stock labelling solutions from ThermoFisher prepared according to manufacturer's instructions (Phalloidin A488, A12379; Rhodamine Phalloidin, R415; Hoechst, 62249)

3 Methods

3.1 Oocyte isolation, culturing and *in vitro* maturation

Mouse ovaries are typically collected from 6-12 weeks old females (e.g. 129s, CD1, C57BL/6). Depending on the strain used, typically 30-50 oocytes can be obtained from each animal. This is normally sufficient for one live imaging or immunofluorescence experiment. Ovaries should be dissected and processed with as little delay as possible to maximise the number of meiosis-competent oocytes that can be isolated. All culturing medium and dishes should be prepared and stored at 37°C for prompt transfer of dissected ovaries. Oocytes should be cultured in

dbcAMP (a non-hydrolysable analogue of cAMP) to keep them arrested in prophase during micromanipulation.

3.1.1 Preparation of oocyte transfer and washing dishes

1. Defrost an aliquot of 100 mM stock dbcAMP by briefly shaking at room temperature or 37ºC.
2. Dilute dbcAMP to 250 µM in M2 medium. For 129s strains, we find three-times higher concentration of dbcAMP is necessary to maintain prophase arrest (see Note 1).
3. Prepare two 35 mm culture dishes with 1ml and 2ml of M2+dbcAMP and two 35 mm culture dishes each with 9 droplets of M2+dbcAMP (Fig. 1).
4. Overlay the droplets with mineral oil using a pastette as in Fig. 1 and prewarm dishes at 37ºC for a minimum of 5 minutes.
5. Prepare a sterile microcentrifuge tube with 500 µl of M2+dbcAMP for ovary collection and prewarm at 37ºC for 5 minutes (see Note 2).

3.1.2 Isolation and preparation of oocytes for microinjection

1. Transfer collected ovaries to the 1 ml M2+dbcAMP culture dish above (Fig. 1).
2. Separate ovaries from associated fat and other tissues using fine tweezers while taking extra care not to tear them (Fig. 2A and B).
3. Transfer ovaries to the 2 ml dbcAMP culture dish above (Fig. 1).
4. Puncture one ovary at a time with a 13 mm gauge needle fitted to a 1 ml syringe to expel oocytes from follicles (Fig. 2C). For efficient puncturing, hold the ovary down with one needle and puncture with the other (see Note 3).
5. When finished puncturing one side of the ovary, flip it over and continue to puncture until it appears flat (Fig. 2C).
6. Exchange syringes between hands and puncture the second ovary similarly with the unused needle (see Note 4).
7. Transfer oocytes from the puncture dish into a dish with M2+dbcAMP droplets (Fig. 1) using a mouth pipette assembly (Fig. 3A) and micropipettes produced as described in section 3.1.4 (see Note 5).
8. Remove debris and sort meiosis-competent oocytes by washing oocytes that have centrally-positioned nuclei through M2+dbcAMP droplets prepared (Fig. 3B).

9. Leave cleaned oocytes in the last droplet and keep at 37ºC while preparing mRNAs for microinjection.

3.1.3 Production of capillaries for mouth pipetting

1. Using both hands, hold a micropipette over a Bunsen burner so that the middle of the glass is exposed to the flame (Fig. 3C).
2. When the glass is red hot, pull both ends of the micropipette apart with force. This will produce two pieces of micropipettes with bent ends where the glass surface was heated (Fig. 3C).
3. While still holding non-heated ends of the two pieces of micropipettes, gently caress the bent end of one piece with that of the other until it breaks and repeat the same for the second piece (Fig. 3C).
4. Observe pulled micropipettes under a stereomicroscope and discard those with sharp or broken edges (Fig. 3C) by observing the glass pipettes under a stereomicroscope.
5. Ensure that produced mouth pipettes are at least 80 µm in diameter before using them routinely by pipetting some oocytes first. Discard pipettes where oocytes appear to be squeezed into during aspiration.

3.2 Studying spindle actin function using high-resolution and super-resolution live microscopy

Microinjection of oocytes is a principal method for studying mammalian meiosis by high-resolution live imaging. We routinely perform quantitative microinjection of oocytes using a modification of a method that was described in great detail by Jaffe and Terasaki [10]. In this section, technical details in the context of mouse oocyte microinjection are provided along with visual aids.

3.2.1 Preparation of mRNAs for microinjection

Visualisation of spindle actin, microtubules and chromosomes in live oocytes is achieved by microinjection of *in vitro* transcribed mRNAs that encode their fluorescently labelled markers. We routinely use the calponin-homology domain of Utrophin (UtrCH) to mark actin filaments, the microtubule binding domain of MAP4 (MAP4-MTBD) to mark meiotic spindles and histone H2B to mark chromosomes. To avoid degradation of mRNAs, they should be prepared for microinjection as follows

only after isolation of oocytes from ovaries. In addition, all mRNA samples should be handled on RNAse free bench with gloved hands and using pipettes thoroughly cleaned with 70% ethanol.

1. Defrost mRNAs quickly under RNAse free conditions and store on ice.
2. If two separate mRNAs are to be microinjected, prepare a new RNAse free tube and mix them to the desired dilution (see Note 6).
3. Fashion a small apparatus for holding mRNA capillaries from a small piece of cardboard, a glass slide and a scotch tape (Fig.4A-C) (see Note 7).
4. Place a dollop of grease in the centre of the capillary holder fold (Fig. 4C) (see Note 8).
5. Prepare a special glass capillary tube by breaking it in half and placing it on the capillary holder grease dollop (Fig. 4E).
6. mRNA containing capillaries need to be stored on ice. To prepare the capillary storage container, line the inside of a 15 cm petri dish with folded tissue paper (Fig. 4F).
7. Place a glass slide on the tissue paper and apply a dollop of grease on the slide to serve as a capillary holder (Fig. 4F).
8. Place the capillary storage petri dish on ice (see Note 9).
9. Load 1 µl of capillary oil into the non-sharp end of the half-broken glass capillary from step 5 and place it back on the holder.
10. Load 0.5 µl of mRNA into the capillary and place it back on the holder.
11. Load 0.5 µl of capillary oil into the capillary and place it on ice inside the capillary storage dish by gently laying it over the grease dollop (Fig. 4D).

3.2.2 Preparation of microinjection chamber

A U-shaped microinjection chamber can be constructed from clear plastic with little effort by most workshops according to the dimensions in Fig. 5A. In this chamber, oocytes are lined up for microinjection inside a small glass shelf created by spacing two specially cleaned coverslips with a double-sided tape. The cleanliness of coverslips used for microinjection strongly affects oocyte health. We use the following steps to thoroughly clean and store coverslips:

1. Fill a coverslip washing plastic box (maximum volume 1L) with 500 ml of Milli-Q water and bring to a boil in a microwave.

2. Dilute a dash of washing up liquid detergent in the heated water.
3. Using fine tweezers, drop 22x22 mm coverslips one by one into the detergent solution.
4. Swirl container box gently to mix and let it sit for 15-20 minutes.
5. Pour out the detergent solution taking care not to lose coverslips.
6. Wash three times with Milli-Q water while thoroughly but gently shaking at every wash.
7. Bring to a boil in microwave.
8. Wash three times with Milli-Q water while thoroughly but gently shaking at every wash.
9. Lay out paper towels on a clean bench and transfer coverslips from wash box to tissues using fine tweezers.
10. Prepare a 50% v/v HCl solution by adding 200 ml of 10 M HCl to 200 ml of Milli-Q water inside the wash box (see Note 10).
11. Using fine tweezers, transfer coverslips individually into the wash box and leave in 50% v/v HCl overnight.
12. Drain the acid solution into a glass bottle for reuse.
13. Wash five times with Milli-Q water while thoroughly but gently shaking at every wash and let it sit for 10 minutes.
14. Wash again five times with Milli-Q water while thoroughly but gently shaking at every wash.
15. Using fine tweezers, transfer coverslips individually into 50 ml Falcon tubes containing 80% v/v EtOH and 20% Milli-Q water for storage (see Note 11).

We then prepare a microinjection shelf and chamber using the following steps:

1. Using fine tweezers, place a double-sided tape roughly 5 mm away from either side of a 22x22 mm coverslip (Fig. 5B) and firmly stick it down by pressing on it using the non-sharp end of the tweezers.
2. Using a ruler and a glass cutting knife, cut another coverslip into smaller pieces as shown in Fig. 5B. Keep four of the small pieces for building shelves.
3. Using fine tweezers, place a shelf piece coverslip from step 2 onto the double-sided tape affixed to the coverslip in step 1. The smaller coverslip piece should have its cut end resting on the tape while its non-cut end should visibly hang

over the tape (Fig. 5B), thus creating a space (the ‘shelf’) between the two coverslips where oocytes will be loaded for microinjection.

4. Press firmly on the small piece coverslip while taking care not to crack it (see Note 12 and Note 13).
5. Position the plastic microinjection chamber upside down and apply a thin layer of grease around its U-shape (Fig. 5C).
6. Using fine tweezers, place a clean and uncut coverslip on the grease and press down on it using the non-sharp end of the tweezers to remove air bubbles from between the grease and coverslip (Fig. 5C).
7. Flip the chamber over and apply a thin layer of grease around its U-shape (Fig. 5D).
8. Using fine tweezers, place a coverslip containing the shelf, with the shelf facing inside (Fig. 5D), and press down on it using the non-sharp end of the tweezers to remove air bubbles. This concludes assembly of a microinjection chamber consisting of a shelf and a medium dam.

3.2.3 Microinjection of oocytes

We microinject oocytes with mercury-filled needles produced using a horizontal micropipette puller (see Note 14). Microinjection needles are connected to oil-filled hydraulic pumps through a micropipette holder. Due to high surface tension of mercury, pressure displacement of oil within the hydraulic pump translates picolitre scale displacement of mRNA samples within the micropipettes. When performed on calibrated microscopes fitted with binoculars bearing measurement rulers, this allows quantitative microinjection of mRNA into oocytes. We produce microinjection needles using a P-1000 micropipette puller (Sutter Instruments) equipped with a box filament (FB255B). The parameters provided in Table 1 yield microinjection needles that in our hands allow smooth microinjection and do not affect oocyte health and maturation. We find that adjustment of the parameters according to the manufacturer’s instruction is necessary from time to time (see Note 15). For oocyte loading, capillary fitting and oocyte microinjection, we use the following steps:

1. Add 800 µl of M2+dbcAMP into the medium dam of the microinjection chamber (Fig. 5E).
2. Load oocytes into microinjection shelf by mouth pipetting (Fig. 5F, 6A).

3. Place the microinjection chamber down with the shelf side away from the bench surface and apply two minimal amounts of grease onto the front and back of the capillary groove (Fig. 5G).
4. Using fine tweezers, place the mRNA capillary inside the capillary groove, supported by the grease (Fig. 5G).
5. Transfer the microinjection chamber-mRNA capillary assembly to a microinjection microscope and place it with the shelf side facing away from the stage (Fig. 6C).
6. Attach a mercury-filled needle to the micropipette holder of a hydraulic pump (Fig. 6B) and place on a micromanipulator.
7. Bring microinjection needle and mRNA capillary into focus (Fig. 7A).
8. Use the micromanipulator to slowly move the needle to one edge the mRNA capillary (Fig. 7B) and break its tip by gently tapping on the capillary surface.
9. Push oil outward through the micropipette holder using the hydraulic pump until the mercury moves to the front of the needle (Fig. 7C). This should normally happen after a few rotations of the hydraulic pump dial. If not, it is an indication that the needle is not sufficiently broken. Repeat the tip breaking step and try to move the mercury to the front of the needle again.
10. Using the micromanipulator, move the needle into the front oil portion of the capillary and bring it into focus (Fig. 7D).
11. Move the needle into the capillary and take up 3 units of oil by applying negative pressure through the hydraulic pump (Fig. 7E and F).
12. Move the needle farther inside the capillary into the mRNA portion and bring it into focus (Fig. 7G).
13. Take up 6-7 picolitre unit equivalents of mRNA (Fig. 7H).
14. Move the needle back into the first oil portion of the capillary and bring it into focus (Fig. 7I).
15. Take up 2.5 units of oil (Fig. 7J).
16. Move the needle out of the capillary and bring it into focus (Fig. 7H).
17. Using the stage and the micromanipulator, move the needle to the front of the microinjection shelf (Fig. 8A). Take extra care not to hit the needle against the chamber coverslip when entering into the chamber.
18. Move the needle into the shelf and slightly push the oocyte to be injected to determine if it is in the ideal microinjection plane (Fig. 8B and C) (see Note 16).

19. Using the micromanipulator to pierce through the zona pellucida and move the needle into the oocyte cytoplasm.
20. Apply positive pressure inside the needle through the hydraulic pump until the front oil portion of the needle is expelled into the oocyte cytoplasm. This is quickly followed by the mRNA portion, which may not be readily visible. Once the mercury has moved back to the front of the needle and an oil droplet is inside the oocyte (Fig. 8D), it can be assumed the mRNA has been successfully introduced into the oocyte.
21. As soon as oil expulsion is seen, quickly manoeuvre the stage to withdraw it away from the needle holder until the needle comes out of the chamber (see Note 17).
22. Reposition the microinjection needle to the front of the mRNA capillary. If they are not both in focus, bring the capillary into focus by gently pressing down or lifting up its front end using fine tweezers.
23. Once needle and mRNA capillary are in focus, repeat microinjection steps for each oocyte. As long as the capillary is kept in the adjusted position, it should not be necessary to move the needle in the Z-axis throughout the rest of the microinjection experiment.
24. After microinjection, transfer oocytes to a prewarmed dish of M2+dbcAMP droplets prepared earlier for mRNA expression.

3.2.4 High-resolution live imaging of spindle actin assembly and chromosome segregation during meiosis I and II

For live imaging of meiosis in mammalian oocytes, we have routinely used confocal microscopes from Zeiss (LSM 710, LSM 780, LSM 800, LSM 880) and Leica (SP8). To achieve maximum resolution during imaging, we use 40x and 63x water immersion objectives (1.1 – 1.2 NA). In addition, we are able to achieve high signal-to-noise ratio using the Airyscan super-resolution module on LSM 800 and LSM 880 microscopes. Combined, these setups allow us to perform four-dimensional (x,y,z,t) live cell imaging of meiosis without compromising oocyte viability.

To perform live imaging of microtubules, chromosomes and spindle actin, oocytes must first be released from prophase arrest by washing out dbcAMP. Depending on the mouse strain used, the release from prophase arrest can take 30 minutes to 1 hour. We find that oocytes from 129s mouse strains release from prophase arrest

faster than oocytes from CD1, C57BL/6 and FVBN strains. Achieving a temperature of 37ºC inside the imaging dish, close to the objective lens where oocytes eventually settle is absolutely critical for oocyte development (see Note 18). To visualise the entire process of meiosis starting from nuclear envelope breakdown, oocytes should be transferred to the microscope immediately after washing out dbcAMP.

1. Prepare a 35 mm culture dish containing 9 droplets of M2 and a glass bottom imaging dish containing a droplet of M2 covered with paraffin oil 30-45 minutes before starting a live imaging experiment and store at 37ºC.
2. If using SNAP-MAP4-MTBD to simultaneously image actin, microtubules and chromosomes, prepare an additional dish with droplets of SNAP-Cell-647-SiR substrate diluted 1:200 in M2+dbcAMP.
3. After 2-3 hours of mRNA expression, remove dbcAMP by mouth pipetting oocytes through the 9 droplets of M2 prepared in step 1. If labelling microtubules with SNAP-MAP4-MTBD, wash oocytes through droplets of M2+dbcAMP+SNAP-Cell-647-SiR prepared in step 2 (see Note 19).
4. Move washed oocytes into the imaging dish containing only M2 medium by mouth pipetting and transfer the dish to a confocal microscope (see Note 20).
5. Locate oocytes through the binoculars using only transmission light (see Note 21).
6. Switch to confocal mode and using coarse imaging settings (high scan speed, no line averaging, 512x512 pixels or less, low zoom) mark the positions of oocytes after centring them in the field of view.
7. Adjust the laser settings as necessary to determine the lowest laser intensities that give good signal-to-noise ratio for microtubules and chromosomes in prophase-arrested oocytes (Fig. 9B) or for spindle actin at the start of meiotic spindle bipolarisation (Fig. 9D) (See Note 22).
8. For the actual imaging experiment, increase the image size to 512x512 pixels, set the pinhole size to 3 μm and adjust zoom (to cover the entire oocyte), scan speed and directionality (uni-directional or bi-directional scan) to achieve a time-lapse of 3-6 minutes while acquiring a z-stack thickness of at least 20 μm at confocal sections of 1.5 μm. The identified imaging conditions should allow continuous live imaging of oocytes from nuclear envelope breakdown through

meiosis I to anaphase I and metaphase II spindle assembly without compromising oocyte health (Fig. 10) (see Note 23).

3.2.5 Pharmacological drug addition experiments

Cytoskeletal loss-of-function assays can be readily performed in oocytes by addition of widely-used cytoskeletal inhibitors to the culture medium. Cytoskeletal disruption is reversible when oocytes are treated with drug concentrations we routinely use. We typically treat oocytes with 5 µg/ml Cytochalasin D to disrupt the actin cytoskeleton, with 5 µM Nocodazole to disrupt microtubules and 5 µM SiR-Actin to stabilise actin. In addition, Cytochalasin D and Nocodazole can be combined to simultaneously disrupt actin filaments and microtubules in loss-of-function assays. Drugs can be added before releasing oocytes from prophase arrest. Alternatively, acute cytoskeletal disruption or stabilisation can be achieved by addition of drugs at any desired stage of meiosis (e.g. spindle relocation, immediately before anaphase I or II, or before and after metaphase II spindle assembly). For analysis of spindle actin function in meiosis II chromosome alignment and segregation, Cytochalasin D or SiR-Actin should be acutely added at least four hours after metaphase II spindle assembly to ensure that all chromosomes are fully aligned first.

1. For drug addition experiments, dilute a stock of the drug of choice in M2 medium without dbcAMP. Make a similar dilution of DMSO for use as control (see Note 24).
2. Prepare control and experimental 35 mm culture dishes by placing 9 droplets of diluted DMSO and drug respectively and overlay with paraffin oil (Fig. 11).
3. Prepare one glass-bottom imaging dish with one droplet of diluted DMSO and one droplet of diluted drug sufficiently spaced apart to avoid contamination of the control droplet by the drug. Overlay these carefully by gently applying paraffin oil between the two droplets so that it spreads out to the droplets (Fig. 11). This minimises any chance of carryover of the drug to the control group by the spreading paraffin oil.
4. Prewarm culture and imaging dishes at 37ºC.
5. Divide oocytes into two groups and wash them through the droplets of control and drug dishes.

6. Transfer oocytes to the imaging dish containing DMSO and drug droplets accordingly and proceed with microscopy.
7. If the experiment involves acute perturbation of spindle actin in meiosis II, perform the steps above with oocytes that have matured into eggs and have been arrested at metaphase II for at least four hours (see Note 25).

3.2.6 Parthenogenetic activation of mouse eggs to visualise spindle actin and chromosome segregation during anaphase II

After release from prophase arrest, mouse oocytes progress through meiosis I, segregate the homologous chromosomes in anaphase I and become arrested in metaphase II until fertilised, when the sister chromatids are segregated in anaphase II. Importantly, fertilisation can be chemically mimicked *in vitro* to induce anaphase II and observe sister chromatid separation. We follow these steps to efficiently achieve release from metaphase II arrest in mouse eggs:

1. Immediately before activation experiment, prepare a 1M $SrCl_2$ stock solution in embryo transfer water.
2. Dilute $SrCl_2$ stock solution to 10 mM in calcium-free M2 medium (prepared by replacing stock D with equal volume of embryo transfer water; see Materials section).
3. If anaphase II is to be observed in control versus drug-treated oocytes, $SrCl_2$ should be diluted to 10 mM in calcium-free M2 containing DMSO or the cytoskeletal drug of choice.
4. Prepare a 35 mm culture dish with 9 droplets of 10 mM $SrCl_2$ alone or with DMSO and cytoskeletal drugs. Overlay droplets with paraffin oil and store at 37ºC.
5. Prepare a glass bottom imaging dish with corresponding 10 mM $SrCl_2$ droplets, overlay with paraffin oil and store at 37ºC.
6. Wash oocytes through $SrCl_2$ droplets and place in $SrCl_2$ droplet inside imaging dish.
7. Transfer immediately to confocal microscope and acquire time-lapse images as described earlier.

3.2.7 Quantification of chromosome alignment and segregation errors from live imaging datasets

Drug-mediated disruption of spindle actin or its genetic disruption in Formin-2 knockout oocytes leads to chromosome alignment and segregation errors that lead to oocyte aneuploidy [6]. High temporal resolution is critical for quantification of chromosome misalignment and segregation. 3-6 minutes time-lapse live imaging datasets allow us to capture oocyte chromosome alignment and segregation in detail and to reproducibly quantify errors. These chromosomal defects can be quantified in two ways – firstly through manual quantification using well-defined criteria and secondly through automatic detection of chromosomes in live imaging datasets using Imaris software (Bitplane).

The first method takes into account the meiotic spindle length in maximum intensity projection confocal images to define outliers of chromosome alignment. As such, it should be used for quantification only in oocytes where the meiotic spindle is positioned parallel to the imaging plane (Fig. 12A). Z-projection of meiotic spindles that are oriented at various angles relative to the imaging plane will lead to inaccurate measurement of spindle length and the perception of chromosome misalignment where there is none (Fig. 12A). Alignment should be quantified in the metaphase frame immediately before anaphase onset. Anaphase onset is defined as the first frame where homologous chromosomes (anaphase I) or sister chromatids (anaphase II) noticeably start to move apart (Fig.12B).

1. Using ImageJ/Fiji or a suitable image analysis software such as Zen (Zeiss), perform maximum intensity projection of the acquired confocal sections.
2. Select time-lapse movies where the meiotic spindle is parallel to the imaging plane.
3. Measure the spindle length and determine the spindle equator position.
4. Draw a rectangle from the spindle equator to either spindle pole spanning one-third of the half spindle.
5. Score as misaligned any chromosome that clearly lies outside this rectangle region in the last metaphase frame before anaphase onset (Fig. 12C).
6. Starting from the next frame, score as lagging or severely lagging those chromosomes that fail to clear the central spindle region within 12 or 18 minutes of anaphase I onset (Fig. 12C).

The second method, uses the three-dimensional surface reconstruction module of Imaris software (Bitplane) to automate the detection of misaligned and lagging chromosomes from high-resolution live imaging datasets. This approach can be used to independently confirm results from manual analyses. We have confirmed the steps described here can be performed using Imaris versions 7.0 – 9.2.

1. Navigate to the ‘Surpass’ menu of Imaris and open a time-lapse imaging dataset (without maximum intensity projection) where the meiotic spindle is oriented parallel to the imaging plane at anaphase onset. Imaris can open a wide range of raw data file types including ZEN (Zeiss) and LAS X (Leica) files.
2. If acquired, switch of the transmission light channel in the ‘Display adjustment’ pane for easier visualisation of the dataset.
3. Under the volume properties submenu, select ‘add new surface’ to reconstruct the chromosomes.
4. In the automatic creation menu that shows up, untick ‘Track Surfaces (over time)’ and press next (blue and white arrow button).
5. In the next window, select the chromosome channel as the source channel for reconstruction and press next without changing default settings (Thresholding based on absolute intensity is the default setting for surface creation). If the signal-to-noise ratio for H2B-mRFP in the live imaging dataset is optimal, chromosomes will likely appear as a single large mass before anaphase onset (see Note 26).
6. In the thresholding adjustment window, make sure the ‘Region Growing’ box is unticked and press the finish button (green) to complete the creation process.
7. For ease of visualisation, switch off the chromosomes channel in the ‘Display adjustment’ pane.
8. Navigate to the frame immediately before anaphase onset and score the number of misaligned chromosomes that appear separated from the main mass of chromosomes (Fig. 12D).
9. Navigate through the next frames and score lagging chromosomes that are disconnected from the main separating chromosome mass (Fig. 12E) as lagging and severely lagging using the same criteria applied in manual quantifications.

3.3 Studying spindle actin function in fixed mouse oocytes

3.3.1 Fixation and preparation of mouse oocytes for immunofluorescence

For best results, mouse oocyte fixative solution should be prepared and prewarmed at 37ºC prior to fixation. In addition, the extraction and washing buffers should be prepared beforehand and stored at 4ºC when not being used.

1. Roll a Kimwipe tissue and moisten with water. Squeeze out excess water and place moistened tissue on the side of a multi-well Terasaki plate to maintain humidity during fixation and immunostaining.
2. Add 18 µl of fixative solution to three wells of a Terasaki plate for each group of oocytes (e.g. DMSO-treated controls) that is to be fixed.
3. Collect oocytes in as little medium as possible and transfer to fixing well using a mouth pipette (see Note 27).
4. Fix oocytes at 37°C in a designated immunofluorescence incubator for 30 minutes. If spindle actin is to be labelled, reduce this to 25 minutes.
5. Wash oocytes three times with 18 µl PBT extraction buffer to replace fixative solution.
6. Extract by incubation in PBT at 4°C overnight. For urgent analyses, 1hr extraction may give sufficient results.

3.3.2 Labelling of spindle actin, chromosomes and microtubules in fixed oocytes

1. Wash oocytes three times with PBT-BSA and block for 30-60 minutes in PBT-BSA at room temperature.
2. Dilute primary antibodies (e.g. anti-tubulin) in PBT-BSA.
3. Wash oocytes three times with 18 µl primary antibody solution and incubate for 1-3 hours at room temperature or 4°C overnight (see Note 28).
4. After incubation with primary antibodies, wash oocytes three times with 18 µl volumes of PBT-BSA.
5. Dilute secondary antibodies and other dyes (e.g. Hoechst to label chromosomes, Phalloidin-Alexa-488 or Rhodamine-Phalloidin to label actin) in PBT-BSA.
6. Wash oocytes three times with 18 µl volumes of secondary antibody or dye solution and incubate for 1-2 hours at room temperature.

7. After incubation, wash oocytes three times with 18 µl volumes of PBT (see Note 29).
8. Place droplets of PBT-BSA on glass-bottom imaging dishes and cover with paraffin oil.
9. Transfer oocytes to droplets using mouth pipettes and proceed with imaging.

4. Notes

1. 6 ml of M2+dbcAMP per mouse (two ovaries) is usually sufficient for isolation, preparation and microinjection of oocytes.
2. Dissected ovaries should be kept in M2+dbcAMP during transfer from animal facility to the lab.
3. Make sure to move ovaries around the entire culture dish surface during puncturing and avoid keeping them in a small area throughout to avoid excessive cell death.
4. During puncturing, needles often become bent and efficiency of oocyte expulsion from ovaries eventually drops. It is important to use fresh needles for each ovary to maximise the number of oocytes obtained.
5. Glass pipettes should always be wider than the diameter of mouse oocytes (about 80 µm) to prevent cell death due to squeezing.
6. For EGFP/SNAP-MAP4-MTBD, EGFP-UtrCH and H2B-RFP, we find that the *in vitro* mRNA transcription kit from ThermoFisher (AM1344) consistently produces similar mRNA yields from 10 µg DNA templates. We therefore routinely use these at final dilutions of 1:16 for EGFP/SNAP-MAP4-MTBD, 1:100 for H2B and 1:8 for EGFP-UtrCH.
7. This apparatus can be reused for holding capillaries during mRNA loading and does not need to be fashioned for each experiment.
8. Due to reuse, the amount of grease on the capillary holder will eventually lower and will need to be topped up from time to time.
9. This capillary storage dish can be reused and should be kept, while topping up the grease and cleaning or replacing the glass slide from time to time.
10. Concentrated HCl is highly corrosive and the appropriate personal protective equipment should be worn when handling it.

11. Before use, coverslips from the 80% v/v EtOH solution should be wiped off on tissue until dry and gently cleaned by scrubbing off with fine tissue such as Kimwipes.
12. It's essential to make sure this coverslip is fully affixed on the double-sided tape. Improperly fixed coverslips will eventually float away into the M2+dbcAMP medium during microinjection and lead to oocyte loss. In addition, oocytes will not be supported by the double-sided tape during microinjection if the shelf coverslip is not fully affixed on the tape. This will make microinjection a very cumbersome and frustrating task. Taking the time to assemble a very good shelf is key to a smooth microinjection experiment and maximises post-injection oocyte survival rate.
13. The remaining small coverslip pieces can be used to construct shelves for future experiments. Preparing several shelves at once and storing them saves time during microinjection experiments.
14. Mercury is highly toxic and appropriate personal protective equipment should be worn when handling it. We typically backfill microinjection needles with 3-5 µl of mercury in a fume cupboard using Hamilton syringes.
15. It is important to calibrate the volume of needles produced to maintain the quantitative element of this microinjection setup. This is achieved by dispensing capillary oil into a microinjection chamber filled with water. By measuring the diameter of the oil droplet via the binocular ruler and assuming that it is a sphere, a ruler unit versus volume table can be constructed. Manufacturers provide the value of ruler units in the form 1St unit/µm or similar. This can be used to translate binocular ruler units into SI units for volume calculations. We routinely microinject 6-7 picolitres of mRNA to simultaneously label actin and chromosomes or microtubules and chromosomes. Mouse oocytes are about 200 picolitres in volume. The final concentration of the introduced mRNA inside an oocyte can therefore be calculated using this quantitative microinjection setup.
16. The ideal microinjection plane is the oocyte hemisphere as oocytes often stay stationary and do not roll when being pushed at this position. Post-injection, oocytes are also more likely to stay inside the shelf when injected at their hemisphere, which simplifies their retrieval after microinjection. Using the membrane curvature as a readout, move the needle (using the Z-axis component of the micromanipulator) to the oocyte's hemispheric region.

17. Oocytes may stick to the needle and come out of the shelf during withdrawal. However, they will detach at the medium-air interface and remain inside the chamber. It is important to periodically top up the M2+dbcAMP medium during microinjection by mouth pipetting more medium into the chamber under a stereomicroscope.
18. Very often, temperatures displayed on microscope environmental chamber controllers vary considerably from the actual temperature inside the imaging dish. It is therefore important to first measure the temperature inside a glass-bottom imaging dish containing M2 medium covered with paraffin oil using a thermocouple thermometer. Once temperature controller settings that yield 37ºC inside the M2 medium are determined, the microscope should be equilibrated at this temperature for at least one hour before each live imaging experiment.
19. Oocytes should be incubated in SNAP-tag substrates for 30 minutes at 37ºC and washed out and stored in M2+dbcAMP without the substrate for an additional 30 minutes before imaging. The time needed for labelling should be taken into consideration when planning an experiment to avoid prolonged expression of microinjected mRNAs.
20. Mammalian oocytes are non-adherent cells. Extra care should be taken when transferring the imaging dish to a microscope to avoid that they move randomly within the M2 droplet, which would make locating them all on the microscope very difficult. We bunch oocytes together by mouth pipetting them and they typically remain together as long as the imaging dish is moved gently during transfer to microscope. It is also critical to determine a microscope stage speed and acceleration that does not cause oocytes to float away during multi-position imaging.
21. To easily locate oocytes, align the centre of the objective lens with the edge of the M2 droplet through visual inspection. This greatly simplifies finding the oocytes in transmission light as they will be close to the focal plane where the edge of the droplet is in best focus.
22. For live imaging experiments, we perform simultaneous imaging of actin, microtubules and chromosomes on a single track as sequential imaging of channels will produce time delays between imaging each colour. Before nuclear envelope breakdown, only some acentriolar microtubule organising centres (aMTOC) can be seen with MAP4-MTBD. UtrCH will produce a clear labelling of

cortical actin and fainter labelling of cytoplasmic actin filaments, which will allow for later detection of spindle actin without stabilising actin filaments (Fig. 9C). Upon release from prophase arrest, chromosomes labelled with H2B-mRFP will adopt a surrounded nucleolar (SN) configuration (Fig.9A). However, if the release from prophase takes long in a particular strain, they may not yet have this configuration while the live imaging experiment is being setup. Instead, a non-surrounded nucleolar (NSN) configuration will be seen (Fig. 9A). Oocytes that achieve the surrounded nucleolar configuration before nuclear envelope breakdown are most likely to progress through meiosis. Since computing storage space is often a limitation with long-term live imaging of meiosis, it might be beneficial to only select those oocytes that have SN configuration for the overnight live imaging experiment. Although the microinjection techniques described here are highly quantitative and reproducible, some oocytes will often express more fluorescently-labelled proteins than others. It is important to avoid imaging oocytes with much brighter fluorescence of actin, microtubules and chromosomes as overexpression of each marker by itself will significantly perturb meiosis. In particular, overexpression of EGFP-UtrCH will stabilise actin filaments and prevent spindle relocation while overexpressed EGFP-MAP4-MTBD and SNAP-MAP4-MTBD will stabilise microtubules and lead to chromosome segregation errors. Similarly, overexpression of H2B-mRFP will interfere with chromosome individualisation and segregation.

23. It is critical to determine imaging conditions that do not cause phototoxicity to oocytes for each microscope since lasers can vary in power from one system to another, including identical systems from a single manufacturer. It is advisable to measure the laser power using an external meter such as PM160/PM160T/PM160T-HP from ThorLabs.
24. We use stock concentrations of 1 mg/ml Cytochalasin D, 5 mM Nocodazole and 1 mM SiR-Actin. Stocks of drugs are prepared by dissolving powders in DMSO. High concentrations of DMSO alone can perturb meiosis and oocyte development. Therefore, control oocytes should be treated with DMSO diluted similarly to drug stocks in M2 medium.
25. During the wash, always clean out the glass pipette by repeatedly flushing it with water to avoid contamination of control groups with the drug.

26. In sub-optimal resolution imaging datasets where the signal-to-noise ratio is low, surfaces will be created outside chromosome regions due to detection of noise. These have to be manually deleted (please refer to Imaris user manual on how to do this.
27. Fixative solution will exclude water-based medium so mix it by briefly stirring with the mouth pipette while oocytes are being pipetted into the fixative. After each fixation step, dispose of the glass pipette. Re-using formaldehyde contaminated pipettes to transfer oocytes from culture medium into the fixative solution will lead to inconsistent fixation and is a health and safety hazard. Fixing oocytes in multiple wells will minimise the amount of M2 transferred into the fixative solution along with oocytes. Attempting to place too many oocytes in one well will inevitably dilute the fixative too much and lead to non-uniform and inefficient fixation.
28. Diluted primary antibody solutions can also be pipetted into new wells of a Terasaki plate using manual pipettes and oocytes can be transferred into antibody wells by mouth pipetting. This is particularly useful when multiple antibodies have to be used and fixed oocytes need to be separated into groups. Here, mouth pipetting has strong advantage over manual pipetting because the number of oocytes being pipetted per well can be precisely controlled.
29. Oocytes can be stored at 4°C in PBT for several days. However, wells should be topped up with PBT regularly to prevent them from drying out.

Acknowledgments

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Table 1. Micropipette pulling settings for production of microinjection needles for box filament FB255B.

Heat	Pull	Velocity	Time	Pressure	Ramp
525	30	40	250	200	515

Fig. 1

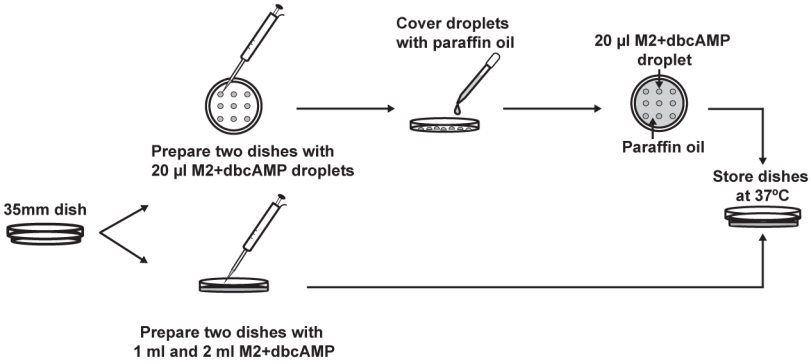

Cover droplets
with paraffin oil
20 µl M2+dbcAMP
droplet
Paraffin oil
Prepare two dishes with
20 µl M2+dbcAMP droplets
Store dishes
at 37°C
35mm dish
Prepare two dishes with
1 ml and 2 ml M2+dbcAMP

Fig. 2

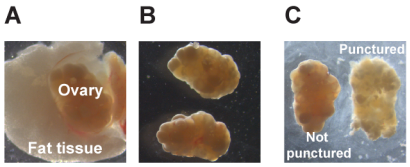


Fig. 3

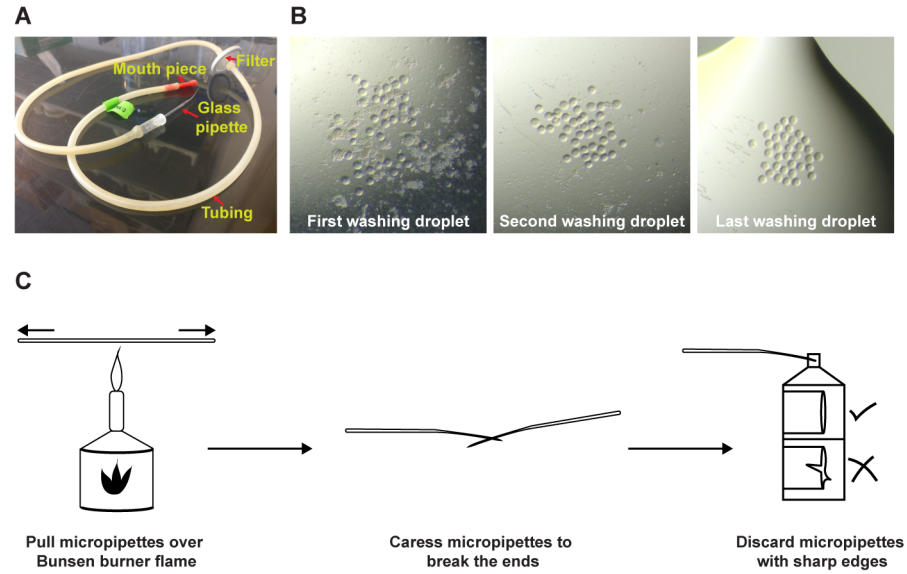

A
B
Mouth piece
Filter
Glass pipette
Tubing
First washing droplet
Second washing droplet
Last washing droplet
C
Pull micropipettes over Bunsen burner flame
Caress micropipettes to break the ends
Discard micropipettes with sharp edges

Fig. 4

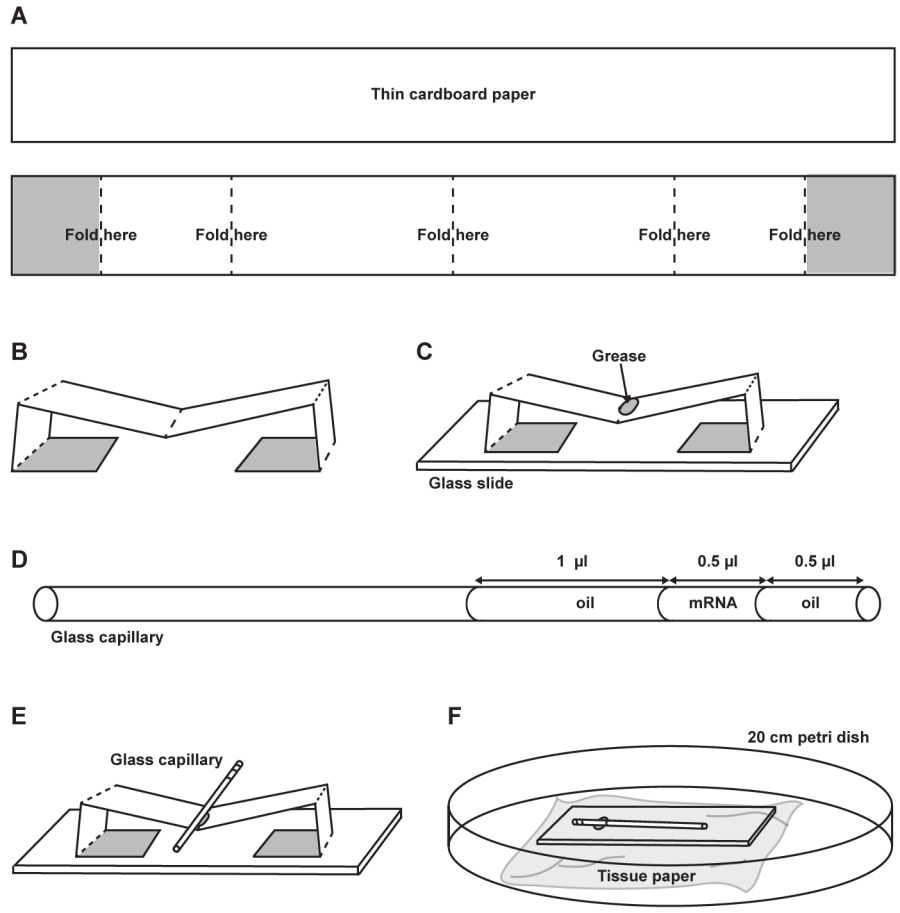

A
Thin cardboard paper
Fold here
Fold here
Fold here
Fold here
Fold here
B
C
Grease
Glass slide
D
1 µl
0.5 µl
0.5 µl
oil
mRNA
oil
Glass capillary
E
Glass capillary
F
20 cm petri dish
Tissue paper

Fig. 5

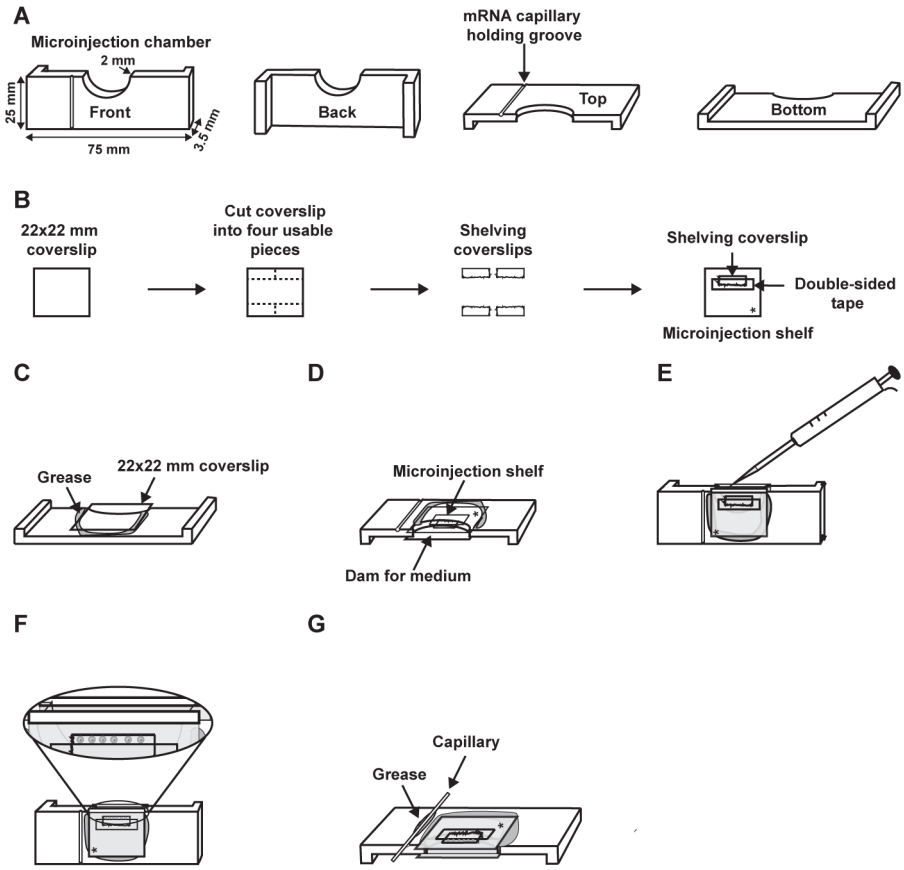

A
Microinjection chamber
2 mm
25 mm
Front
75 mm
3.5 mm
Back
mRNA capillary holding groove
Top
Bottom
B
22x22 mm coverslip
Cut coverslip into four usable pieces
Shelving coverslips
Shelving coverslip
Double-sided tape
Microinjection shelf
C
Grease
22x22 mm coverslip
D
Microinjection shelf
Dam for medium
E
F
G
Capillary
Grease

Fig. 6

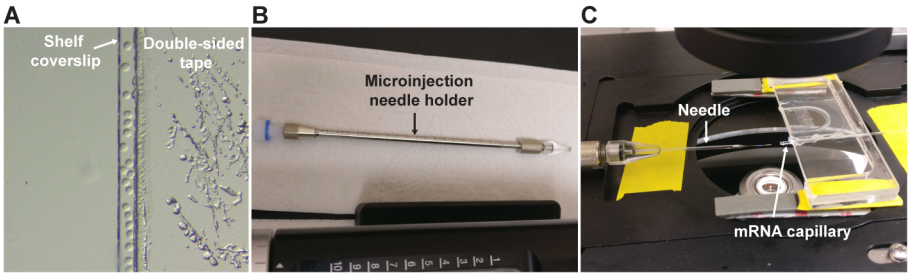

A
Shelf coverslip
Double-sided tape
B
Microinjection needle holder
C
Needle
mRNA capillary

Fig. 7

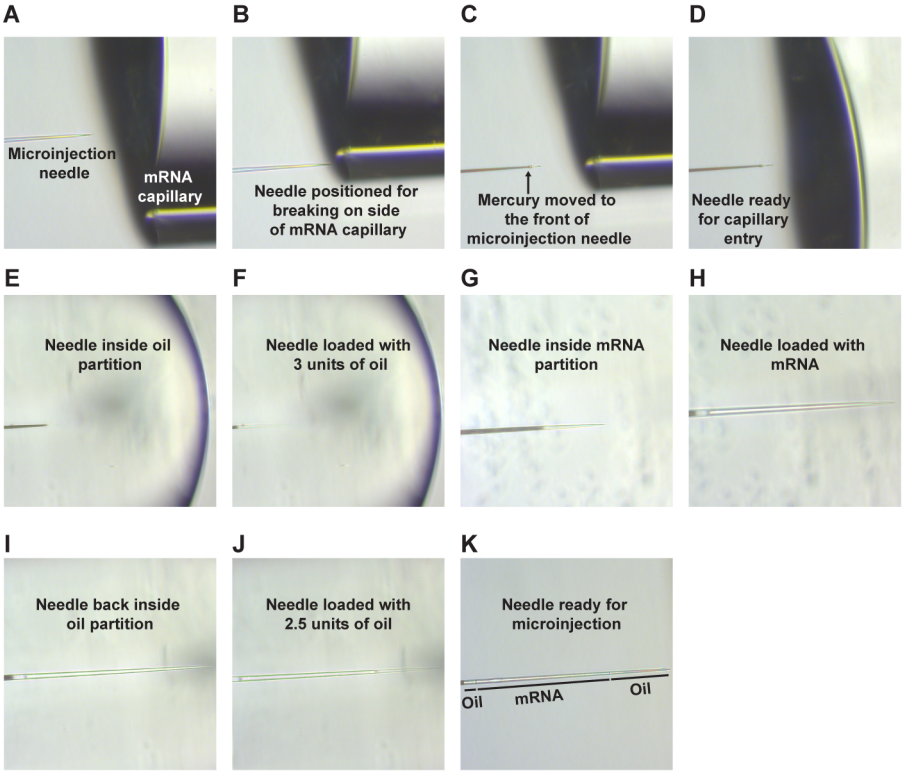

A
Microinjection needle
mRNA capillary
B
Needle positioned for breaking on side of mRNA capillary
C
Mercury moved to the front of microinjection needle
D
Needle ready for capillary entry
E
Needle inside oil partition
F
Needle loaded with 3 units of oil
G
Needle inside mRNA partition
H
Needle loaded with mRNA
I
Needle back inside oil partition
J
Needle loaded with 2.5 units of oil
K
Needle ready for microinjection
Oil
mRNA
Oil

Fig. 8

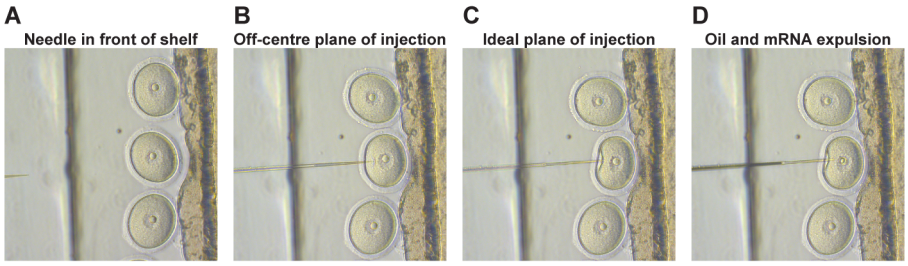

A
Needle in front of shelf
B
Off-centre plane of injection
C
Ideal plane of injection
D
Oil and mRNA expulsion

Fig. 9

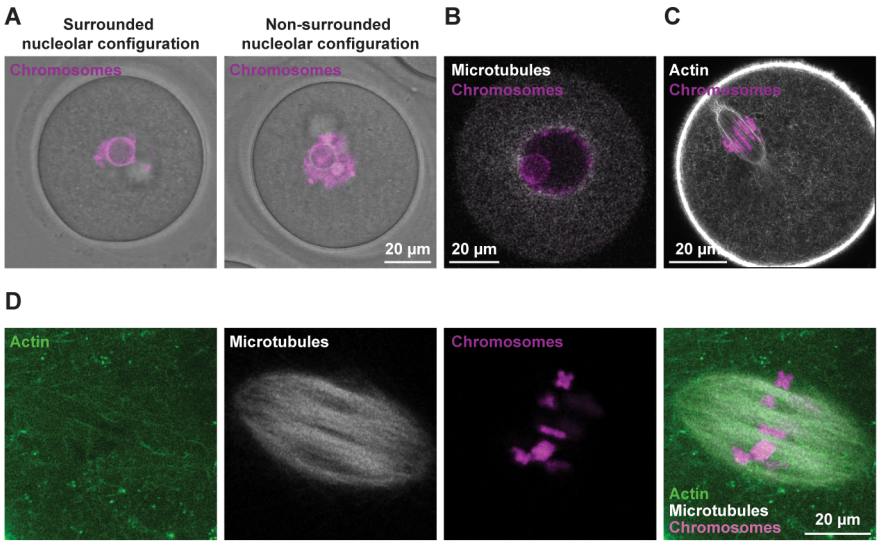

A
Surrounded nucleolar configuration
Non-surrounded nucleolar configuration
B
C
Chromosomes
Chromosomes
Microtubules
Chromosomes
Actin
Chromosomes
20 µm
20 µm
20 µm
D
Actin
Microtubules
Chromosomes
Actin
Microtubules
Chromosomes
20 µm

Fig. 10

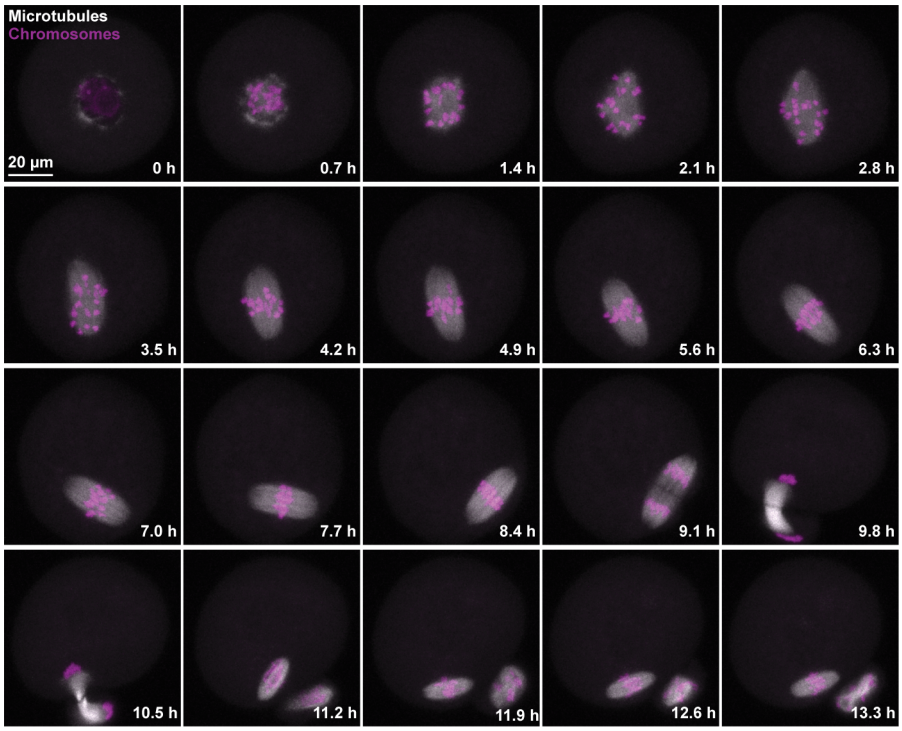

Microtubules
Chromosomes
20 µm
0 h
0.7 h
1.4 h
2.1 h
2.8 h
3.5 h
4.2 h
4.9 h
5.6 h
6.3 h
7.0 h
7.7 h
8.4 h
9.1 h
9.8 h
10.5 h
11.2 h
11.9 h
12.6 h
13.3 h

Fig. 11

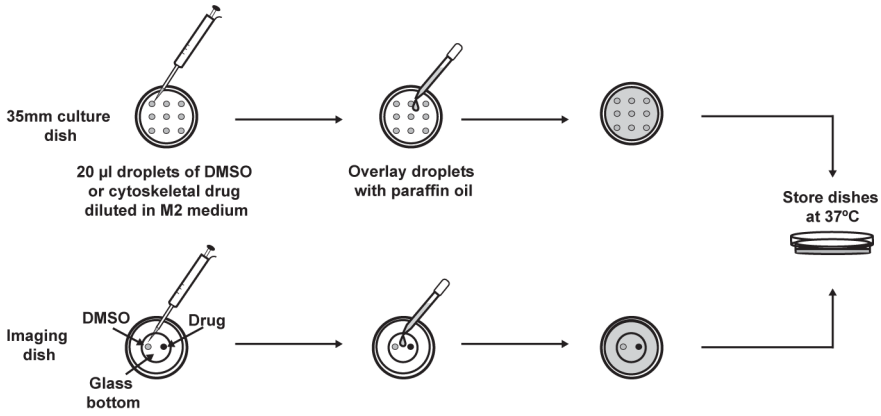
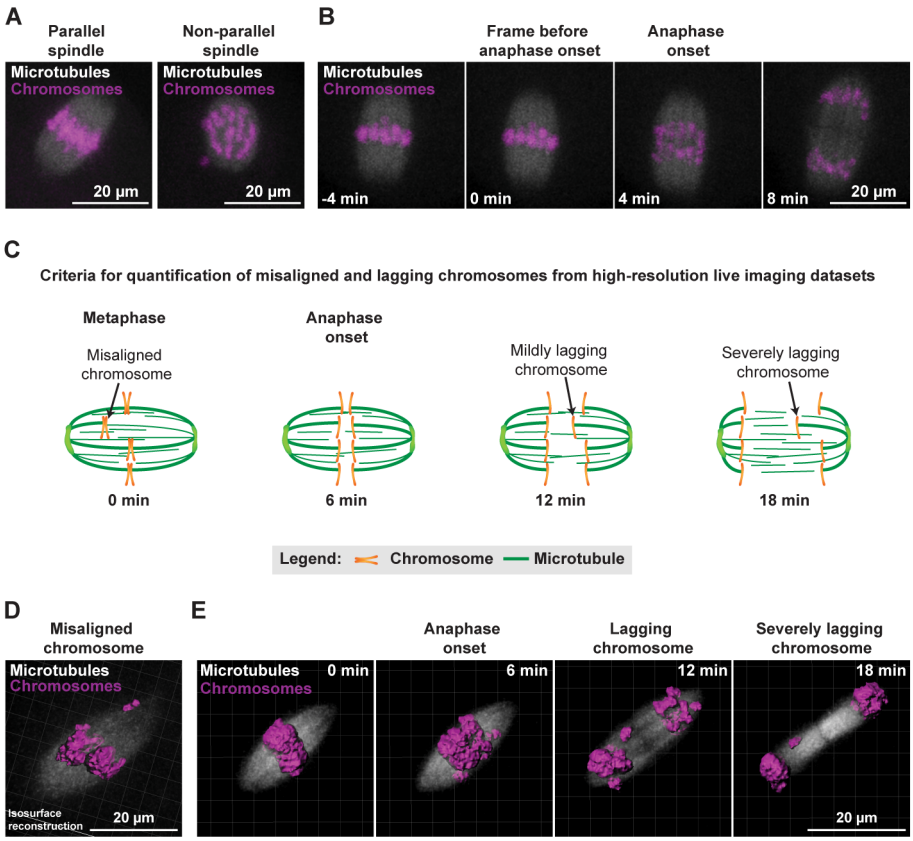

35mm culture dish
20 µl droplets of DMSO or cytoskeletal drug diluted in M2 medium
Overlay droplets with paraffin oil
Store dishes at 37ºC
Imaging dish
DMSO
Drug
Glass bottom

Fig. 12


A
Parallel spindle
Non-parallel spindle
Microtubules
Chromosomes
20 µm
B
Frame before anaphase onset
Anaphase onset
-4 min
0 min
4 min
8 min
20 µm
C
Criteria for quantification of misaligned and lagging chromosomes from high-resolution live imaging datasets
Metaphase
Misaligned chromosome
Anaphase onset
Mildly lagging chromosome
Severely lagging chromosome
0 min
6 min
12 min
18 min
Legend: Chromosome Microtubule
D
Misaligned chromosome
Isosurface reconstruction
E
Anaphase onset
Lagging chromosome
Severely lagging chromosome