A single *Drosophila* embryo extract for the study of mitosis *ex vivo*

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Spindle assembly and chromosome segregation rely on a complex interplay of biochemical and mechanical processes. Analysis of this interplay requires precise manipulation of endogenous cellular components and high-resolution visualization. Here we provide a protocol for generating an extract from individual *Drosophila* syncytial embryos that supports repeated mitotic nuclear divisions with native characteristics. In contrast to the large-scale, metaphase-arrested *Xenopus* egg extract system, this assay enables the serial generation of extracts from single embryos of a genetically tractable organism, and each extract contains dozens of autonomously dividing nuclei that can be prepared and imaged within 60–90 min after embryo collection. We describe the microscopy setup and micropipette production that facilitate single-embryo manipulation, the preparation of embryos and the steps for making functional extracts that allow time-lapse microscopy of mitotic divisions *ex vivo*. The assay enables a unique combination of genetic, biochemical, optical and mechanical manipulations of the mitotic machinery.

INTRODUCTION

Cell extracts are powerful tools for studying a variety of complex intracellular processes in a simplified *ex vivo* setting that permits biochemical and mechanical perturbations. Nuclear or cytoplasmic extracts from egg, embryonic or somatic cells have been used to study DNA replication and repair[1,2] transcription and splicing[3–6], translation and protein degradation[7–9], post-translational modifications[10–12], nuclear membrane formation[13], chromatin condensation and meiotic spindle assembly[14,15]. Cell extracts from amphibian eggs have been especially powerful for elucidating the biochemical reactions underlying progression through the cell cycle and spindle assembly during meiosis[16,17]. Recently, we developed a new method for the preparation of autonomously cycling *Drosophila melanogaster* embryo extract[18]. The assay enabled the first detailed visualization of dividing nuclei from early syncytial (preblastoderm) embryos, allowing us to quantify the characteristic separation distance of dividing nuclei in the absence of a cell or plasma membrane and test the effect of spatial constraints on nuclear separation. This experimental system is complementary to, and offers several advantages over, amphibian egg or embryo extracts, opening new avenues for the combined genetic, biochemical and mechanical study of the cell division machinery.

Xenopus egg and embryo extract

Cytoplasmic extract from the eggs of *Xenopus laevis* or *X. tropicalis* has become a standard for microscopy-based analysis of spindle assembly and microtubule dynamics[16]. Large volumes of extract (up to milliliters) can be prepared from the eggs of several frogs, making it ideal for biochemical studies, protein purification or protein-protein interaction studies. *Xenopus* eggs are naturally arrested in metaphase II of meiosis, and this arrest is preserved during cytoplasmic fractionation. Extracts can proceed through a limited number of cell cycles in a test tube, as indicated by oscillating kinase activities and cyclin levels[15]. However, repeated cycles of nuclear division and spindle assembly and disassembly reactions have not been reported to date, revealing a limitation of this model system. The *Xenopus* egg extract has therefore mainly been used to study the assembly and maintenance of meiotic metaphase II spindles[16]. More recently, extract from early *Xenopus* embryos that are artificially arrested in their cell cycle by inhibiting the activity of the anaphase-promoting complex (APC) have been used for the study of mitotic spindles[19]. Despite their inability to undergo repeated spindle assembly and disassembly cycles autonomously, the addition of calcium can trigger naturally arrested egg extracts to undergo a single transition to interphase, in which nuclear reassembly and DNA synthesis can be observed[15,16]. Interphasic extracts can then re-enter a mitosis-like metaphase after the addition of nondegradable cyclin B[20] or after supplementing with metaphase-arrested extract[16]; such mitotic spindles can be triggered to transition to anaphase by the addition of calcium[20–23]. Recent powerful applications of frog egg extract have included studies of mechanical properties of metaphase-arrested spindles by direct mechanical perturbation[24,25] and of the biochemical factors that control spindle size by mixing egg extracts from different species[26–28]. A major limitation of the amphibian extract system is its very limited genetic toolbox. This is due to the relatively long generation time, the polyploidy of most species and the lack of complete genomic sequence information for most frogs (except for *X. tropicalis*, see http://www.xenbase.org and ref. 29). This severely limits our ability to perform genetic manipulations in this experimental system.

Single *Drosophila* embryo extract

The *D. melanogaster* syncytial blastoderm embryo is a well-established system for the study of chromosome segregation and checkpoint control[30,31], cytoskeletal rearrangements during mitosis[32] and spindle function[33,34]. Because many nuclei are anchored at the embryo cortex at this developmental stage, the intact embryo is ideally suited for fluorescence microscopy. The first nuclear divisions, however, take place deep inside the preblastoderm embryo, making live microscopy a challenge[35,36]. Consequently, comparatively little is known about these early divisions. For example, it is unclear how the spindle apparatus generates forces that lead to fast and reliable chromosome segregations in the absence of a cell cortex in the preblastoderm embryo. Once spindles are anchored at the cortex at the

blastoderm stage, the dissection of intrinsic spindle activities from contributions of the actin-based cell cortex and of the proposed spindle matrix[37] is challenging in intact cells or embryos. Therefore, a cortex-free assay in which the remaining components are tangible and controllable by genetic or biochemical interference is needed for the analysis of these multiple contributions to spindle functioning.

Recently we developed a method for preparing the extract from the cytoplasm of individual syncytial *Drosophila* embryos at this early developmental stage[18]. This extract differs from classical *Drosophila* embryo extracts[38] that are prepared from thousands of embryos, covering a range of developmental stages and containing substantial amounts of buffer, which have been used for protein purification, the study of native protein complexes[39] and gene expression profiling[9,40]. In contrast, single *Drosophila* embryo extract is prepared from individual preblastoderm embryos at a well-defined developmental stage. It supports repeated nuclear cycling with typical *in vivo* characteristics[18], and it currently represents the only experimental *ex vivo* system with this property. This extract overcomes the difficulty of imaging early nuclear divisions, because they can be observed in volumes that are much smaller than the entire embryo. Another advantage is that genetic manipulations are easily performed using well-established methods of *Drosophila* genetics, such as classical mutagen-induced mutations, gene-targeted knockouts and knockdowns, transgenesis and homologous recombination[41,42]. These methods offer a wide variety of possibilities for achieving gain- or loss-of-function situations, as well as fluorescent labeling of proteins with genetically encoded tags[43]. An intriguing genetic feature of *Drosophila* is the existence of compound chromosomes[44], which can serve to increase chromosomal load and to test the effect of DNA mass on the nuclear division machinery. Finally, a large number of extracts can be prepared within a day, and numerous divisions can be observed, allowing good experimental throughput and robust statistical analysis based on a large data pool. A disadvantage in comparison with *Xenopus* egg extract is the much smaller volume of the single-embryo *Drosophila* extract, which therefore makes it most suitable for microscopy applications. The 30–50% smaller size of *Drosophila* embryo spindles compared with *Xenopus* egg extract spindles might be a minor disadvantage for fluorescence microscopy imaging, but it can be expected to be advantageous for correlative light or electron microscopic studies. Finally, for some applications, the natural cell cycle arrest of amphibian egg extract can be advantageous, but *Drosophila* embryo extracts can also be artificially arrested by adding nondegradable cyclins, allowing the study of long-lived mitotic metaphase spindles. We have already shown, using microchambers, that the single *Drosophila* embryo extract is amenable to mechanical perturbation[18], making it an ideal tool for studying the mechanical properties of the native or genetically modified spindle apparatus.

Proven applications

The single *Drosophila* embryo extract has been demonstrated to be a powerful tool for studying the transitions of the cell division machinery in the absence of a cell membrane. The demonstrated applications[18] are as follows.

- *The production of functional extracts* with endogenous fluorescently labeled DNA and microtubule markers, providing an advantage over the *Xenopus* egg extract system.
- *Confocal fluorescence microscopy imaging* of repeated nuclear divisions and of the distribution of nuclei within the cytoplasmic space, allowing the detailed study of the dynamics of these synchronous divisions driven by the early developmental program.
- *Encapsulation of dividing nuclei in microchambers* to probe spatial constraints ("artificial cell").
- *Laser ablation of cytoskeletal components* to probe force-generating elements in the spindle.
- *Addition of small molecules* at defined concentrations.

Additional potential applications

We believe that the single *Drosophila* embryo extract is also suitable for the following as yet unpublished applications that take advantage of the genetic toolbox available for *Drosophila* and the properties of a cell-free system.

First, a number of experiments could be undertaken that address the understanding of nuclear division as such, including the following:

- *Confocal and/or super-resolution imaging* of the assembly and disassembly dynamics of the spindle apparatus in a wild-type background and after genetic perturbation, and testing the spatial control of spindle size and inter-spindle distance.
- *Correlative fluorescence/electron microscopy imaging* of natural and genetically modified spindle architecture without or after arrest in metaphase; the small volume of extract in comparison with an intact embryo provides an advantage especially for electron microscopy (complete vitrification of water, simpler data collection).
- *Fluorescence imaging of cell cycle regulation and checkpoint control* of the first rapid divisions.
- *Probing the mechanism of synchronization* of early embryonic divisions.
- *Investigating the biochemical versus mechanical triggers of developmental transitions* (e.g., from preblastoderm to blastoderm embryo).
- *Probing spatial scaling of the nuclear division machinery* by introducing compound chromosomes[44] to increase or decrease chromosome mass.

Second, this cell-free experimental system has great potential for probing the mechanical properties of the mitotic spindle machinery; here, an extract is essential for the free design of the mechanical contact without being limited by the presence of a cell cortex. To our knowledge, it is the first open system that allows mechanical perturbations throughout mitotic phase transitions. These perturbations can be implemented using microneedles, micropipettes or nanofabricated cantilevers, and using encapsulation in microchambers, droplets in oil or liposomes. Probing the mechanical integrity of centrosomal versus acentrosomal spindles is another direct application.

Third, this single-embryo extract is an ideal tool for studying nuclear function by mixing different extracts, followed by imaging of chromosome condensation and histone exchange kinetics. Possibly, transcription can be visualized as well.

Finally, it will help investigate the regulation of filament dynamics (microtubules and actin filaments) and of motor protein transport in a close-to-physiological environment.

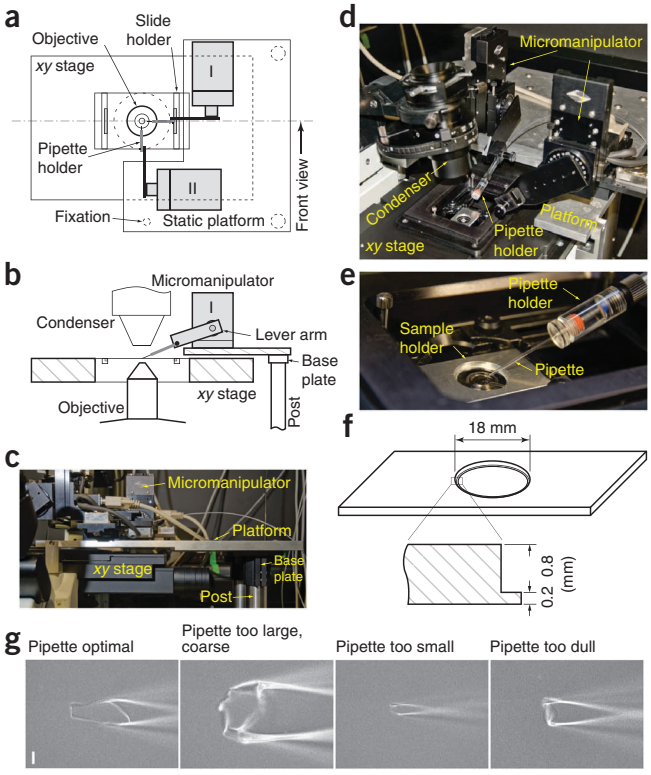

Figure 1 | Pipette-based extraction system on an inverted light microscope. (**a**) Schematic of the top view of the extraction system. Two micromanipulators are arranged perpendicularly on a static platform. They hold a pair of micropipettes, which in the manipulator's work position are close to the optical axis of the objective. The static platform is mounted directly on the microscope frame and the optical table. The dash-dotted line marks the front-view plane. (**b**) Front view of the system. Note that the static platform is independent of the *xy* stage. The manipulator arm and pipette holder have to fit between the objective and condenser lenses. (**c**) Photograph of the front view of the microscope stage and the static platform that is mounted rigidly on the table with posts and a base plate. (**d**) Photograph of an implemented system. (**e**) Photograph of a zoomed view of the sample holder and the pipette holder with a pipette being positioned at the focus point of the objective. (**f**) Schematic of the sample holder fitting between the two movable levers of the microscope slide holder. The round opening with a tiny bottom rim will hold a round coverslip. (**g**) Images of different pipette apertures, showing an optimal pipette for extraction with a sharp edge and a diameter of ~40 µm and three suboptimal pipettes, either too large, too small or at the right size but with a blunt aperture (from left to right). Vertical scale bar, 20 µm.

As a further outlook, we propose that this cell-free assay may be very suitable for performing time-lapse confocal fluorescence imaging of pronuclear migration and karyogamy in *Drosophila ex vivo*. Well-fed female flies in isolation lay unfertilized eggs that are arrested after meiosis. In principle, the extraction protocol can be easily adapted for unfertilized eggs. After extraction, once the female pronucleus is located in an extract droplet, purified sperm can be added to the cytoplasm, and the migration and fusion could be observed in unprecedented quality. Moreover, the mechanical characteristics of the male pronuclear migration in the cytoplasm could be tested for the first time by applying space constraints and direct mechanical perturbation.

Experimental design

Animal model. In the following protocol, we describe the preparation of single-embryo extract from transgenic *D. melanogaster* expressing the histone variant H2Av-RFP as a chromosomal marker and Jupiter-GFP as a microtubule marker in a wild-type (w^{1118}) background. It is critical to note that a fly strain that overexpresses a fluorescently tagged tubulin may not give sufficient contrast to detect the mitotic state of the embryo, a critical step in the PROCEDURE. If a mutant strain is being tested, the fluorescent microtubule marker Jupiter will need to be introduced by a genetic cross to facilitate extraction with the right timing. Moreover, in parallel, it is advisable to raise a batch of flies expressing only Jupiter-GFP (possibly also histone-RFP) in a wild-type background as a control to distinguish between a normal phenotype and unsuccessful extraction procedure.

Microscope-based extraction setup. In contrast with large-volume cell extracts prepared from hundreds of eggs or embryos (*Xenopus*[16], *Drosophila*[38]) by lysis and centrifugation, the single-embryo extract described here is prepared with pipette-based fluidic devices mounted directly on a microscope that permits confocal fluorescence imaging. We show schematically (**Fig. 1a**,**b**) and in photographs (**Fig. 1c**–**e**) how an inverted microscope frame is equipped with a static platform that can host one or two mechanical micromanipulators. This platform is independent of the microscope's motorized stage, which allows translation of the sample holder (**Fig. 1f**) while the manipulator handle remains steady relative to the objective. A pipette holder is mounted on the manipulator and connected with tubing to a foot pedal–operated bidirectional pump. Importantly, all manipulators are motorized and connected to a micro-controller, which facilitates fast, programmable and reproducible positioning of the pipettes in the field of view of the microscope. All manipulation is performed under polarized bright-field illumination and monitored through the oculars after passing a polarizer in 90° (crossed) configuration. A motorized filter turret hosting the polarizing optics, a motorized port changer and illumination shutters allows fast switching between polarized transmission and confocal fluorescence mode. Highly efficient (i.e., fast and precise) instrument control is needed for the success of the experiment. For confocal fluorescence imaging, a spinning-disk confocal scanner and a camera (e.g., electron-multiplying charge-coupled device (EM-CCD)) are mounted on the side port of the microscope frame, and two laser lines are coupled into the light path of the scanner for selective GFP or RFP excitation.

Micropipettes. The extraction pipette is the core instrument of the extract assay. We use a capillary puller and a heating filament to produce small pipettes, and we cut their tip and enlarge the aperture with a scalpel. Such a pipette is rather coarse (with respect to embryo size), has sharp edges and serves as a means for puncturing the vitelline membrane of the embryo and sucking out cytoplasm with nuclei and cytoskeletal components. Interestingly, the size of the tip aperture is an important determinant of extract functionality. A small tip creates large shear forces and possibly higher flow rates. Shear forces could disrupt organelles or the cytoskeletal features of the cytosol that are vital for nuclear cycling and for extract function in general. Once pipettes have been used, they often become narrower because the cytoplasm sticks to the tip and dries out when it comes in contact with air. Therefore, it is crucial

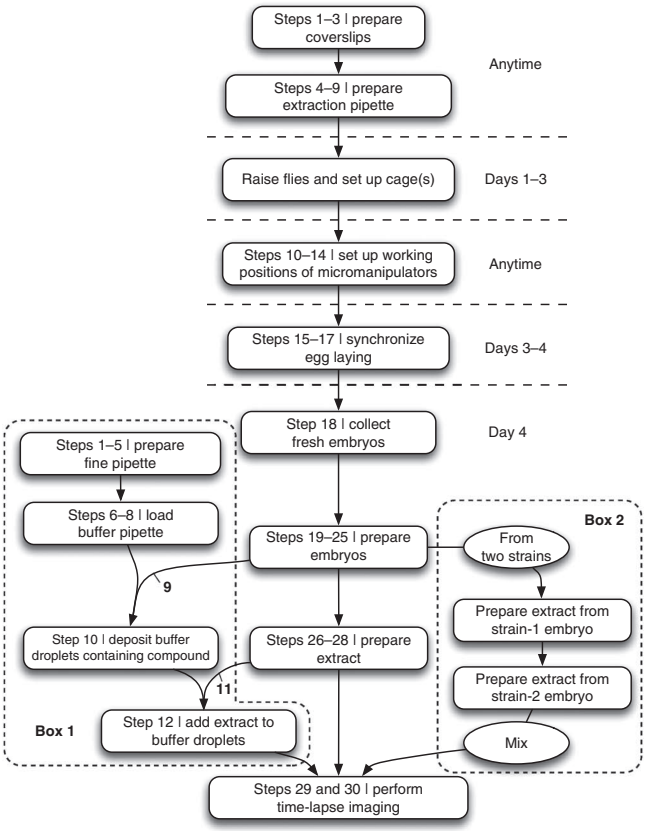

Figure 2 | Flowchart of working steps for embryo extract preparation. An approximate time scale is given for planning, together with two alternative procedures for the addition of chemicals or proteins (**Box 1**) and for mixing two extracts from different embryo strains (**Box 2**).

to the experiment to have an extraction pipette with the correct tip size and shape installed (**Fig. 1g**), and to replace this pipette frequently. A two-way syringe pump hosting a gas-tight syringe facilitates both suction and ejection of the cytoplasm into and out of the pipette, respectively, as well as rapid switching between the two modes.

Ergonomic optimization. Quick sequential operation of the microscope's light sources, the stage controller, the micromanipulator control handle and any additional microscrew-driven injector is of critical importance for maintaining the functional state of the extract. Optimize the entire experimental setup such that the various control handles and buttons are easily and efficiently reachable while using the microscope and looking through the ocular of the microscope.

Extract preparation. An overview of the protocol and a rough time frame is presented in **Figure 2**. We first provide a description of initial cleaning and silanization (passivation) of coverslips, as we have found that the glass surface must be free of contamination for preserved extract functionality. Washing with NaOH renders the surface hydrophilic, which in turn causes the cytoplasm to spread and the proteins to stick to the surface. Thus, surface passivation after cleaning is essential for extract functionality, and we have used both silane and PEG treatment successfully[45]. Fly husbandry, synchronized egg laying, removal of the chorion and embryo immobilization are essentially identical as for DNA injection, which is routinely done in many laboratories, however, with slightly different timing of embryo collection. Fresh embryos are immobilized in a densely packed row on the coverslip and covered with halocarbon oil. The entire procedure of extracting the cytoplasm followed by placing small droplets of cytoplasm on the glass surface next to the embryos all occurs under oil. The glass surface for droplet deposition must remain free of any contamination during the course of sample preparation. Preserved function of the nucleo-cytoplasm can be achieved by choosing the right moment of extraction with respect to the mitotic cycle of the embryo: mechanical manipulation of the embryo cytoplasm is only tolerated during late telophase and interphase. This could be attributable to a higher susceptibility to damage of prophase and metaphase spindles upon mechanical manipulation.

Alternative procedures. In addition to the standard extract preparation, two alternative procedures are described, thereby offering the possibility to add drugs, purified proteins or RNA dissolved in buffer to the extract (**Box 1**) or to mix different extracts (**Box 2**). For the addition of compounds in buffer, a second pipette with

Box 1 | Extract dilution and addition of compounds ● TIMING ~45 min + imaging time

A key feature of every cell-free system is the ability to add compounds, such as drugs, proteins, RNA or latex beads in a solution. Here, because of the small volumes of cytoplasm generated by the extraction procedure, additional compounds must be deposited with a micropipette by means of micromanipulation, analogous to the droplet formation of cytoplasm. However, a pipette with an aperture in the submicrometer range in combination with a hydraulic injection system guarantees a much higher precision in volume control. Although fine pipettes for cell injection are commercially available (see MATERIALS), they can be produced with the available equipment by pulling thicker glass capillaries and beveling the pipette tip[49]. Here we provide a short protocol for the production of such fine pipettes, and we describe in detail additional steps to the main PROCEDURE that will facilitate biochemical perturbations of the cytoplasm after extraction.

Preparation of fine pipettes for buffer deposition ● TIMING 30 min

1. Clamp a capillary with a thick wall (inner diameter 0.50 mm) to the holding device of the pipette puller. Adjust the position of the capillary relative to the heating filament such that the resulting pipette is about 20–30 mm long.
2. Adjust heat and pulling strength to produce a medium long taper (Narishige PC-10: two-step pulling, drop length: 10 mm, heater slider: 2 mm, 4 weights, heating power step 1: 65%, step 2: 60%). Remove the pipette carefully without damaging the tip.

(continued)

Box 1 | Extract dilution and addition of compounds ● TIMING ~45 min + imaging time (continued)

3. Use a pipette beveler to open the tip of the pipette; bevel at an angle of ~20° and achieve a tip aperture of 0.5–1 µm. The aperture can be observed under a high-magnification microscope.
4. Fill the hydraulic microinjector with low-viscosity mineral oil. Remove all air bubbles in the cylinder, the tubing and the pipette holder.
5. Seal-mount the pipette to the holder; fill the pipette with oil by carefully turning the knob of the injection cylinder.

Adding compounds to the extract ● TIMING 10–15 min + imaging time

6. Before embryo preparation (Step of the main PROCEDURE), take a clean empty coverslip and place 1 µl of buffer (containing any reagent or protein) as a drop on the glass surface. Cover the drop with a larger drop of halocarbon oil such that it completely seals the buffer, which should stay in contact with the surface. Place it in the sample holder and install it on the microscope.

▲ CRITICAL STEP Adjust the pH to ≥7.8 with a molarity of ~20 mM to preserve extract cycling.

7. Set the focus slightly above the glass, and adjust the field of view at the interface between the buffer and oil. Maneuver the fine pipette to the field of view using the saved work position, and penetrate the oil-buffer interface. Suck some buffer into the pipette by monitoring the fluid interface receding toward the back of the pipette.
8. Exit the buffer by moving the stage, and monitor the buffer-oil interface moving into the pipette tip. The aspirated oil will seal the pipette and prevent drying. Quickly adjust the cylinder (pressure) to stop any further flow. Move the pipette out of the field of view (home button) and remove the coverslip.

▲ CRITICAL STEP By filling the pipette through the tip and not through the large opening before mounting it, any air bubbles and any resulting high compliance are prevented.

9. Perform Steps 19–25 of the main PROCEDURE to prepare embryos.
10. Adjust the focus level slightly above the glass surface, and set the field of view to the clean side of the coverslip. Bring the buffer-loaded pipette into the field of view and produce an array of small buffer droplets on the clean area beside the row of embryos (**Fig. 3b**, diameter: 10–20 µm, 15–20 droplets) by operating the microscrew-driven cylinder of the injector, the microscope's *xy* stage and the *z* axis movement of the micromanipulator. Finally, remove the pipette (home button).

? TROUBLESHOOTING

11. Perform Steps 26 and 27 of the main PROCEDURE to find an embryo in the correct mitotic state and perform the extraction.
12. With the pipette kept at a safe z-level in the field of view, move with the stage to the buffer droplets previously made. Adjust the focus. Slowly eject the cytoplasm into several buffer droplets by operating the foot pedals and the *z*-rotational handle of the micromanipulator (**Fig. 3e**). Monitor the size of the droplet before and after addition of extract using the micrometer scale installed in the ocular.

▲ CRITICAL STEP The volume ratio can be estimated from the increase in droplet size. Ideally, adjust the concentration of any compound in the buffer such that a dilution of one volume of buffer to three volumes of extract will result in the final concentration of the compound in the extract. Because the droplets are flat, adding three volumes of extract translates to approximately a doubling in the diameter of the final droplet.

13. Perform Steps 29 and 30 of the main PROCEDURE to acquire multicolor time-lapse images of the nuclear cycling.

? TROUBLESHOOTING

a tip in the sub-micrometer range is implemented on a second micromanipulator perpendicular to the first. This pipette is connected to a manual, oil-driven injector, which offers better volume control than an air-based injection system. As such, this system and its associated micromanipulator are not absolutely required, as they are not used for the actual extraction procedure. However, this system enables biochemical manipulation of the cytoplasm. In contrast, mixing two extracts can be done without any additional equipment, and it enables several types of experiments including mutant rescue and dosage (**Box 2**).

Box 2 | Mixing of extracts from different embryos ● TIMING 15–20 min + imaging time

Another important feature of this cell-free assay is the possibility to mix the cytoplasm from two embryos that have, for example, different genetic backgrounds. These mixing experiments enable the user to perform rescue experiments with loss-of-function or null mutants, allowing good control of the doses of the added proteins. Furthermore, with the use of different fluorescent tags expressed in transgenic lines, it is possible to perform pulse-chase experiments and measure kinetic parameters during the nuclear cycle. For example, mixing cytoplasms with differently tagged histone variants enables the measurement of histone exchange in the time course of nuclear cycling. Finally, the extract-mixing assay enables the study of cycle regulation[30], checkpoint control[55,56]

(continued)

Box 2 | Mixing of extracts from different embryos ● TIMING 15–20 min + imaging time (continued)

and synchronization, because two extracts to be mixed will typically not cycle in a synchronous manner. Here we describe the modifications required to the main PROCEDURE to perform these extract-mixing experiments.

Extract-mixing assay ● TIMING 15–20 min + imaging time

1. Perform Steps 19–25 (embryo preparation) of the main PROCEDURE with the following modification: collect embryos from two different fly strains (after synchronized laying) and dechorionate them. Transfer the embryos from both collections onto the same agar block and keep them spatially separate; remember their origin. Align the embryos from strain 1 in one half of the row and embryos from the other strain in the second half, with a clear gap to distinguish between the two groups (see Step 23 of the main PROCEDURE).

2. Perform Steps 26–29 (extraction procedure) of the main PROCEDURE first for strain 1 and subsequently for strain 2 embryos with the following modifications: Produce smaller droplets of cytoplasm during the first extraction. After the first extraction, check the mitotic state of nuclei in the droplets—which should ideally be interphase or prophase—and start a timer. Add roughly the same volume of extract from a strain 2 embryo to the droplets previously made from a strain-1 embryo (similar to step 12 of **Box 1**) within 5–7 min; adding an equal volume translates to an ~40% increase in droplet diameter.

▲ CRITICAL STEP Keep in mind that during extraction of the strain 2 embryo, the nuclei in the droplets of the first extraction undergo mitotic division. A complete nuclear cycle takes ~9 min, during which time the second extraction should be performed.

3. Perform multicolor time-lapse imaging of the nuclear cycles in a droplet of mixed cytoplasm (Steps 29 and 30 of the main PROCEDURE). For control purposes, it is recommended to acquire in parallel and to compare time-lapse movies of a droplet produced by mixing of two extracts and a droplet of single-embryo extract. Simply generate two or three droplets of the control strain without any mixing; choose one of each (mixed and control droplet) and adjust the microscope software to move the *xy* stage between the two different positions within each frame of the acquisitions.

? TROUBLESHOOTING

MATERIALS

REAGENTS

- Active dry yeast (http://www.Flystuff.com, "Redstar", cat. no. 62-103)
- Chlorotrimethylsilane (Sigma, cat. no. 386529) ! CAUTION Chlorotrimethylsilane is hazardous and reacts violently with water. Handle it under a hood while wearing protective gloves and goggles.
- Halocarbon oil, Voltalef 10S (VWR International, cat. no. 24627.188)
- Heptane (Sigma, cat. no. 49629) ! CAUTION Heptane is highly flammable. Handle it under a hood.
- Mineral oil (Sigma, cat. no. M5904)
- Pressurized nitrogen (10-liter bottle with pressure reducer)
- Pressurized CO_2 (fixed gas line or a 25-liter bottle with pressure reducer)
- Sodium hypochlorite (Merck, cat. no. 1.05614.2500) stored at 4 °C
  ! CAUTION Sodium hypochlorite causes severe skin burn and eye damage. Wear protective gloves and goggles when handling it.
  ▲ CRITICAL Sodium hypochlorite has a limited shelf life; it loses 10% active chlorine within 3 months.

Reagents for buffers

- DMSO (Sigma, cat. no. D8418) ! CAUTION DMSO is an irritant. Wear protective gloves when handling it.
- HEPES (Sigma, cat. no. 43375)
- Imidazole (Sigma, cat. no. A6558) ! CAUTION Imidazole is hazardous and corrosive. Avoid inhalation and handle it under a fume hood.
- Magnesium chloride ($MgCl_2$; Sigma, cat. no. M8266)
- Potassium aspartate (K-aspartate; Sigma, cat. no. I5513)
- Potassium chloride (KCl; Sigma, cat. no. P9541)
- Potassium hydroxide (KOH; Merck, cat. no. 1.05012.1000) ! CAUTION KOH is corrosive, and highly exothermic when dissolved in water. Wear protective gloves and goggles when handling it.
- Potassium phosphate (K-phosphate; Sigma, cat. no. P5655)
- Sodium hydroxide (NaOH; Merck, cat. no. 1.06462.1000) ! CAUTION NaOH is corrosive and highly exothermic when dissolved in water. Wear protective gloves and goggles when handling it.
- Millipore (deionized) water

Reagents for apple juice agar plates

- Agar (Sigma, cat. no. A1296)
- Apple juice, 100% juice
- Ethanol (Sigma, cat. no. 459844)
- Methyl paraben (cat. no. H3647)
- Sucrose (Sigma, cat. no. S0389)

Animals

- w^{1118} ; ; *P{PTT-GA} Jupiter*^{G00147} fly line (no. 6836, Bloomington *Drosophila* Stock Center)
- w^{1118} ; ; *P{His2Av-mRFP1}* fly line (no. 23650, Bloomington *Drosophila* Stock Center)

EQUIPMENT

Equipment for pipette production

- Stereomicroscope, ×0.8–5.6 magnification (Olympus, model SZX7 or comparable)
- LED ring illumination (Olympus, cat. no. RL-S80-55-2 and MC-1500 or comparable)
- Glass capillary puller (Narishige, model PC-10 or Sutter Instruments, model no. P1000)
- Black plastic board (circa 100 × 100 mm)
- Borosilicate capillaries, 0.75-mm inner diameter (Sutter Instruments, cat. no. B100-75-10)
- Pipette storage char (WPI, cat. no. E210)
- Scalpel handle (WPI, cat. no. 500236-G)
- Scalpel blades, no. 11 (WPI, cat. no. 500240) ! CAUTION Handle scalpel blades cautiously and dispose of used blades in a special container for sharp objects.

Additional pipette equipment (for experiments described in Box 1)

- Pipette beveler (Sutter Instruments, model no. BV-10)
- Borosilicate capillaries, 0.50-mm inner diameter (Sutter Instruments, cat. no. B100-50-10)
- Alternatively, use premade pipettes, µTip (WPI, cat. no. TIP01TW1F)
  ▲ CRITICAL Handle fine pipettes carefully and protect the tip from damage.

Equipment for embryo preparation
- Air pistol (http://www.Flystuff.com, cat. no. 54-104 or comparable)
- Artist's brush (fine, no. 0)
- Acrylic glass cylinder (diameter 50 mm, wall thickness: 2–5 mm, length: 100 mm)
- Bath sonicator, 1.5-liter volume
- Cell culture dishes, 35 × 10 mm (BD Bioscience, cat. no. 351008)
- Cell culture dishes, 60 × 15 mm (BD Bioscience, cat. no. 351007)
- Conical flasks, 1,000 ml (Schott Duran)
- Coverslips, 18 mm round, no. 1 (Menzel, cat. no. CB00180RA1)
- Coverslip rack, ceramic (×2, Thomas Scientific, cat. no. 8542E40)
- Double-sided adhesive tape (TESA or Scotch 3M)
- Fly pad (http://www.Flystuff.com, cat. no. 59-114)
- Forceps, bent, Dumont no. 7 (WPI, cat. no. 14097)
- Forceps, fine, Dumont no. 5 (WPI, cat. no. 500342)
- Glass bottle, sealable, 50 ml (Schott Duran)
- Microwave oven (Panasonic, or comparable)
- Scalpel handle (WPI, cat. no. 500236-G)
- Scalpel blades no. 10 (WPI, cat. no. 500239) ! **CAUTION** Handle scalpel blades cautiously and dispose of used blades in a special container for sharp objects.
- Rocker (Benchmark Scientific, model no. BR2000)
- Stainless steel mesh (TWP, 150–200 mesh/inch or 150-µm mesh)
- Tissue paper (Kimberly-Clark, Kimtech Science, cat. no. 7102)

Equipment for building an extraction setup
- Optical table with vibration isolation (Thorlabs, cat. no. PBH51513 and PFA51501)
- Self-assembled motorized micromanipulation system: motorized three-axis micromanipulator (Sutter Instruments, model no. MP-285); manipulator control unit (Sutter Instruments, model MPC-200 and ROE-200); pipette holder connected to Teflon tubing (Sutter Instruments, cat. no. MI-10010) aluminum breadboard, circa 300 × 300 × 10 mm (Thorlabs, cat. no. MB2025/M); large mechanical post with a base plate (×2, ~300 mm long, Thorlabs, cat. no. P200/M and PB2/M); mounting post bracket (×2, Thorlabs, cat. no. C1505/M); small mechanical post (~30 mm long, Thorlabs, cat. no. TR30/M) ▲ **CRITICAL** The micromanipulators should be programmable with 'home' and 'work' positions, and ideally they should offer several levels of coarse and fine movement. A second motorized three-axis micromanipulator is needed for experiments involving the addition of buffer and chemical compounds (**Box 1**).
- Alternatively, use the mechanical part of an IVF system for an inverted light microscope, motorized, programmable, without pumps (e.g., Eppendorf, model PatchMan NP 2)
- Foot pedal for syringe pump (×2, Marquardt, cat. no. 2410.0401)
- Gas-tight syringe (Hamilton, cat. no. 1750TLL-SAL)
- Motorized syringe pump, bidirectional (WPI, model no. SP210CZ)
- Tube connectors (GE Healthcare, cat. no. GE 18-1112-55, GE 18-1027-12, GE 18-1112-57)
- Oil-driven manual injection system (Eppendorf, model CellTram Oil), for experiments described in **Box 1**

Microscope equipment
- Halogen lamp (Olympus, cat. no. U-LH100-3)
- Long-distance bright-field condenser (Olympus, cat. no. IX2-LWUCD)
- Motorized inverted light microscope (Olympus, model IX-81 or comparable)
- Motorized filter turret (Olympus, cat. no. IX-RFACA)
- Motorized *xy* stage (Prior Scientific, model H117 and ProScanIII control unit)
- Ocular with micrometer scale (Olympus, cat. no. WHN10X3-H)
- Polarizer (Olympus, cat. no. IX-LWPO) and analyzer (Olympus, cat. no. IX2-MDICT)
- Laser-based spinning-disk confocal scanner (Andor, REVOLUTION) with: laser combiner with 488-nm and 561-nm lines (50–100 mW); Yokogawa CSU-X1 scan unit; fast filter wheel (Prior Scientific, model HF110); EM-CCD or sCMOS camera (Andor, model iXon or comparable)
- UPlanApo 0.8 numerical aperture (NA) oil-immersion objective, ×20 (Olympus), working distance: 0.19 mm
- UPlanFL 1.3 NA oil-immersion objective, ×40 (Olympus), working distance: 0.20 mm ▲ **CRITICAL** For optimal imaging and repeated extraction, we recommend using oil-immersion objectives for both manipulation and imaging. Avoid the use of low-magnification air objectives throughout the experiments. Water immersion could cause problems when using halocarbon oil to cover embryos; the two fluids might come in contact, form unwanted interfaces and cause optical distortion.
- (Optional) ×60 PlanSApoN 1.35 NA oil-immersion objective (Olympus)
- Microscope control software (Andor IQ2, Metamorph, or comparable)

REAGENT SETUP

Yeast paste In a cell culture dish, mix 5 g of instant yeast granules with 10 ml of dH_2O in a 60-mm Petri dish; stir the mixture manually using a spatula and produce a smooth yeast paste. Cover the dish with the lid and keep it at 4 °C for up to 5 d.

Embryo glue Prepare embryo glue by soaking 2–3 m of double-stick adhesive tape, unrolled and cut into small pieces, in 20 ml of heptane in a 50-ml sealable glass bottle on a rocker for at least 48 h. Before use, remove the pieces of tape. During use, add a small amount of heptane if the glue is too viscous. ▲ **CRITICAL** Preparation of the glue takes a long time and needs to be done in advance.

Animals Follow established procedures for fly husbandry, initiating egg laying and collecting synchronously developing embryos[46]. In addition, introduce the microtubule-binding protein Jupiter-GFP (and, ideally, an RFP-tagged histone as a chromosomal marker) into the fly strain of interest (typically w^{1118}) by genetic crosses[18]. Keep several batches of 6–7 fly vials per generation, each containing 15–20 flies, in order to set up a fly cage for egg laying. ▲ **CRITICAL** Keep in mind that the generation of a particular fly line of interest may take up to several weeks.

NaOH, 3 M Dissolve 30 g of NaOH pellets in 250 ml of dH_2O. Freshly prepare the solution; use the solution only once for cleaning purposes and discard it afterward.

KOH, 1 M Dissolve 5.61 g of KOH pellets in 100 ml of Millipore water. Store the solution at room temperature (20–25 °C) for several months. This solution is to be used for pH adjustments.

Dilution buffer For the addition of small-molecule drugs or purified proteins to the extract (**Box 1**), prepare either a standard injection buffer (150 mM potassium aspartate, 10 mM potassium phosphate, 20 mM imidazole)[47] or a low- to medium-ionic-strength buffer designed for the particular protein. The pH should be ~7.8, as we found that mitotic divisions fail in diluted extract with a pH of ≤7.6. The following buffer has been tested for extract dilution: 20 mM potassium HEPES, pH 7.8, up to 100 mM KCl, 1 mM $MgCl_2$ and up to 1% (vol/vol) DMSO. Adjust the pH with KOH pellets and fine-adjust it with 1 M KOH solution. ▲ **CRITICAL** Freshly prepare the buffer just before setting up the microscope and the pipettes, but at the earliest 1 d before the experiment. Keep the buffer at 4 °C. We advise thorough testing of a buffer before its use as a compound solvent for biochemical perturbations.

EQUIPMENT SETUP

Fly cage As a fly cage for egg laying, use an acrylic glass cylinder with a 50-mm outer diameter, which fits into a standard 60-mm cell culture dish (actual size of dish: 54.8-mm outer diameter, ~51-mm inner diameter). Cover the top of the cylinder with a fine metal mesh (attach using heat) or a commercially available fly screen (using a rubber band).

Apple juice agar plates Follow an established recipe from ref.48 for making apple juice agar plates. In brief, mix separately 18 g of agar in 600 ml of water and 20 g of sucrose in 200 ml of apple juice. Boil both in a microwave until the agar and sucrose are dissolved; next, carefully add the agar solution to the juice and let it cool down. Add 20 ml of 20% (wt/vol) methyl paraben in ethanol and stir the mixture for a few minutes. Pour the fluid into 60-mm cell culture dishes (the smaller dish, not the lid); fill the dishes halfway and let the agar solidify at room temperature. Keep the plates refrigerated at 4 °C for 4 weeks.

Embryo basket For embryo collection, assemble a small mesh basket by removing the lid of an Eppendorf tube; slice away the bottom two thirds and fix a fine stainless steel mesh to the original opening by using a heat plate. ▲ **CRITICAL** Choose fine stainless steel meshes over galvanized or zinc-plated products.

Microscopy system Mount the frame of an inverted, motorized light microscope rigidly on an optical table that offers vibration isolation. Attach the ×20 and ×40 oil-immersion objectives to the microscope revolver, install bright-field optics and add a polarizer and analyzer set in a 'crossed' configuration. Ensure that the analyzer is quickly removable, for example, by installing the optics in a motorized filter turret. Adjust the angle of the polarizer, which is mounted on top of the condenser, to match a 90° angle to the analyzer located in the filter turret with respect to their

polarization properties. An optimal angle can be achieved by illuminating the field of view at a high intensity and by turning the angle of the polarizer until the field of view is almost dark. Finally, set up the spinning-disk confocal scanning unit for excitation and detection of GFP and RFP. Ideally, the stereomicroscope for embryo preparation is located close to or in the same room as the confocal microscope in order to avoid any delays between embryo preparation and the extraction procedure.

Micromanipulation In a mechanical workshop, cut a standard aluminum breadboard (300 × 300 × 10 mm) into an L-shaped board (**Fig. 1a**); the final dimensions of this platform will depend on the particular microscope stage in use. Leave the microscope's slide holder accessible and ensure that the *xy* stage does not hit the platform during calibration and operation. Position the breadboard as an independent, static platform on top of the microscope's *xy* stage (**Fig. 1b**) by mounting it on three mechanical posts, two bolted on the optical table and one attached to the microscope frame (anterior attachment site). If possible, fix the platform also at the back side of the microscope frame. Adjust the height of the post's base plate such that the gap between the *xy* stage and the static platform is minimal (<10 mm; **Fig. 1b–c**). Mount a three-axis motorized micromanipulator onto the static platform sideward (**Fig. 1a**, labeled I) and as close to the optical axis of the objective as possible while leaving space for the upright condenser. Attach the supplied lever arm to each micromanipulator. Clamp the pipette holder for extraction on the arm of the right manipulator I (**Fig. 1b**). Adjust the angle of the pipette holder to 15–20° relative to the horizontal plane (the stage); this can be achieved by mounting the lever arm as low as possible to the micromanipulator. Ensure that the arm, holder and pipette do not touch any edges of the stage, sample holder and condenser during operation (**Fig. 1d–e**).

Sample holder Fabricate a small aluminum plate with similar dimensions to a standard microscopy slide (25 × 75 × 1 mm) and with a central round hole (diameter 18 mm) containing a 0.2-mm-thick and ~0.5-mm-wide rim at its bottom side to hold a coverslip (**Fig. 1f**, bottom). Ideally, the plate should be black anodized to prevent reflections and light scattering.

Pump systems Hardwire two independent foot pedals with the transistor-transistor logic (TTL) interface of the bidirectional syringe pump to control stepwise movement and change in directionality (injection or suction). Connect a gas-tight syringe with a pipette holder by means of Teflon tubing (~0.5 m in length) and dedicated tube connectors. Insert the syringe into the holding device of the pump. The digital interface of the syringe pump will need some information regarding syringe type and volume. Set the pump to the smallest possible volume per step movement. Position the syringe pump close to the confocal microscope and arrange both foot pedals under the microscope table; this pump system is pneumatic and serves for embryo extraction.

Alternative procedures demanding additional equipment If the experiment requires the addition of chemical compounds such as small molecules or purified proteins (**Box 1**), position a commercially available, manual hydraulic (oil-driven) injection system next to the microscope's *xy* stage controller ('joystick'). Mount a second three-axis motorized micromanipulator onto the static platform in perpendicular arrangement to the first (**Fig. 1a**, labeled II). Attach the pipette holder of this hydraulic injection system on the arm of this micromanipulator. This system will be used for fast buffer deposition at precise locations. Check that all arms, holders and pipettes do not touch any edges of the stage, sample holder and condenser during operation (**Fig. 1d–e**).

PROCEDURE

Preparation of glass coverslips ● TIMING 1 h

1| Align 10–15 round coverslips in a ceramic rack using forceps. If needed, remove obvious contamination with lint-free tissue paper beforehand. Immerse the rack in 3 M NaOH and sonicate the coverslips for 10 min.

2| By using forceps, rinse each coverslip extensively with Millipore water, dry them with pressurized nitrogen and put them back in a dry, clean rack.

3| Transfer the rack into a sealable plastic container and fill the container with nitrogen. Add a drop of chlorotrimethylsilane using a syringe and quickly seal the container; the silane will vaporize. Incubate the coverslips for ~2 min. Once the silane reacts with glass, a biocompatible surface coating is generated. Silanization renders the glass surface more hydrophobic and prevents proteins from sticking.

! CAUTION Chlorotrimethylsilane is hazardous and reacts violently with water. Avoid contact with air (humidity). The reaction with the glass surface should ideally occur under nitrogen atmosphere. Wear gloves and goggles and work under a hood.

■ PAUSE POINT Store coverslips (with the rack) in a clean, sealable glass beaker at room temperature. Repeat the silane treatment if coverslips are stored for longer than 1 week.

Preparation of extraction pipettes ● TIMING 30 min

4| Clamp a capillary with a thin wall (inner diameter 0.75 mm) to the holding device of the pipette puller. Adjust the position of the capillary relative to the heating filament such that the resulting pipette is about 20–30 mm long.

5| Adjust heat and pulling strength to produce a short taper (Narishige PC-10: one-step pulling, four weights, heating power: 65%). Remove the pipette carefully without damaging the tip.

6| Place a black plastic board under a stereomicroscope to increase contrast, and switch to maximum magnification. Cut the tip of the pipette with a scalpel (straight blade); position the blade such that it is slightly angled toward the tapered end, and produce a tip aperture of 40–50 µm and preferably a sharp edge (**Fig. 1g**).

▲ CRITICAL STEP The size and shape of the tip aperture are crucial. Too large tips are disadvantageous in terms of volume control, and may not break up the vitelline membrane of the embryo. Refer to an earlier work[49] for general advice on how to produce glass pipettes from capillaries.

7| Repeat Steps 4–6 to produce several pipettes.

8| Treat the pipettes with chlorotrimethylsilane before use to prevent sticking. Follow the same procedure to expose capillaries to silane vapor as for coverslips (Step 3). Use a sealable storage jar and a pipette holder made from foam to prevent damage to the tips.
! CAUTION Chlorotrimethylsilane is hazardous and reacts violently with water. The reaction with the glass surface should ideally occur under nitrogen atmosphere. Wear gloves and goggles and work under a fume hood.

9| Carefully insert one of the pipettes into the holder of the pneumatic system (connected to the syringe pump, see Equipment Setup) and hand-tighten the fixation screw at the front of the holder for proper sealing.
■ **PAUSE POINT** Store the remaining pipettes in a dust-free storage jar at room temperature. Repeat the silane treatment if pipettes are stored for longer than 1 week.

Setting up working positions and maneuvers ● TIMING 15 min
10| Insert an extraction pipette into the pipette holder of the pneumatic system that is mounted to manipulator I (**Fig. 1a**, Equipment Setup). If needed, mount the holder of the fine pipette of the hydraulic system to micromanipulator II (**Box 1**). Adjust the mounting position of the holder(s) such that collisions cannot occur during diagonal maneuvers from the home position to the focal point of the objective.

11| Add an empty coverslip to the sample holder and place it on the confocal microscope using immersion oil. Add a drop of halocarbon oil on the coverslip and set the focus level of the objective slightly above the top glass surface. Take advantage of any dust particles on the glass surface, which are easy to find and focus on. Perform Köhler illumination adjustments to the condenser to optimize the field illumination; refer to the microscope manual for details.

12| Manually move each pipette tip carefully into the field of view by using the manipulator's control handles, by direct visual inspection and by looking through the ocular (polarized transmission mode). Finally, save the work position for the pipette(s).

13| As the surface tension pulls halocarbon oil into the air-filled extraction pipette, adjust the syringe pump such that only the tip of the pipette is filled with oil. This will later protect the extract from contact with air; it will also enhance the mobility in the pipette by reducing viscosity effects.

14| Automatically move the pipette(s) back to the home position. The extraction setup is now ready; experiments can be conducted at any time.

Preparation of the fly cage and synchronization ● TIMING 0.5 d
15| Start in the evening with parent flies of roughly the same age, raised under uncrowded conditions and fed with yeast for 3 d after eclosion. Remove half of the male flies (resulting in a 2:1 ratio of female to male flies), and transfer ~50 adults to a cage standing on an apple juice agar plate containing fresh yeast paste at 25 °C and 60% humidity ('fly room'). The female flies will lay fertilized eggs onto the agar.

16| The following morning, take 10–15 apple juice agar plates, add some fresh yeast paste to each with a spatula, and then store them next to the cage ready for several (pre) collections. Treat the flies briefly (3–5 s) with CO_2 until they are immobile; the females will expel overaged embryos retracted in their oviduct. Transfer the flies back to the cage and cover the cage with a new plate containing yeast.

17| Perform two 30-min precollections and discard the plates.

18| Collect embryos for 70 min on a fresh plate; the majority of embryos laid in this 70-min period will be in developmental stage 2 (preblastoderm). If you are performing experiments that require the addition of compounds to the extract, perform steps 1–8 of **Box 1** before proceeding to Step 19 below. If you are performing extract-mixing experiments, proceed to **Box 2** for required modifications to the main PROCEDURE.

Embryo preparation ● TIMING 5 min
19| Prepare two small cell culture dishes (35 mm), one filled with dH_2O (label with 'W') the other with 5% "bleach" (label with 'B'), a 1:1 dilution of sodium hypochlorite stock solution. Add a small embryo basket to dish W. Place everything close to the stereomicroscope for sample preparation.

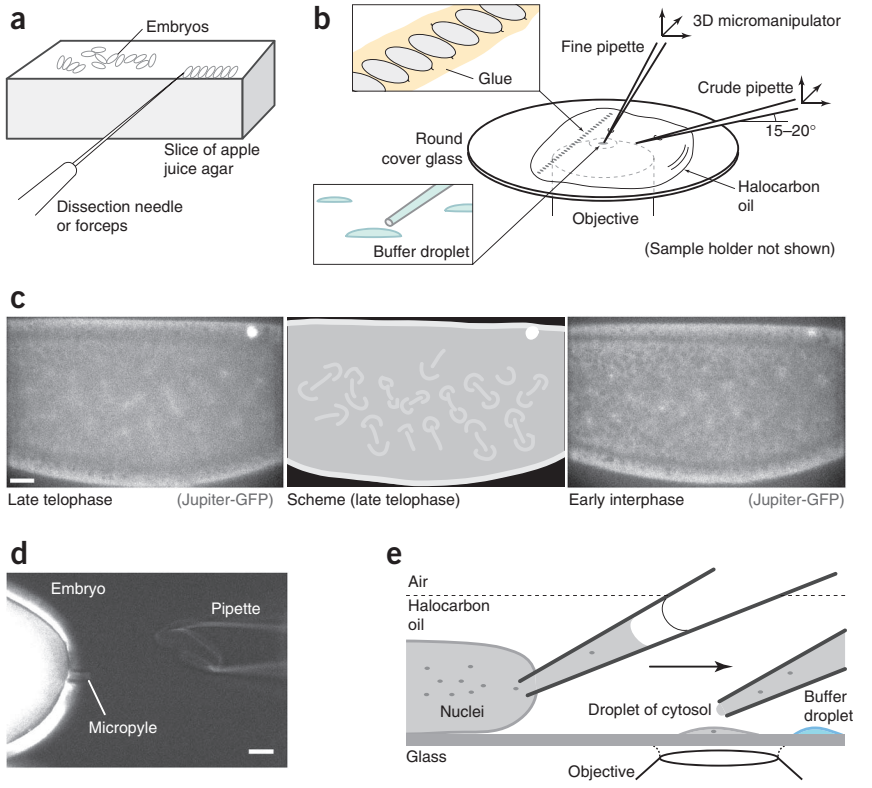

Figure 3 | Embryo preparation and extraction procedure. (**a**) Illustration of the agar block method. After removal of the chorion, embryos are placed on a slice of agar and arranged along the edge. (**b**) Schematic view of a coverslip installed on the microscope, with two pipettes arranged perpendicular to each other. Embryos are immobilized on the round coverslip that bears a strip of glue (top inset, orange) and is covered with halocarbon oil. After installation of the sample on the microscope, several buffer droplets can be placed on a clean area of the glass surface (bottom inset) before embryo extraction (**Box 1**). Note that the sample holder is omitted in this schematic. (**c**) Low-magnification view of an intact embryo showing the typical patches of Jupiter-GFP fluorescence in telophase (left), represented in a scheme (middle). The fluorescence pattern will change to smaller round patches (right) within less than 1 min. The extraction procedure should start between these two time points. Scale bar, 20 µm. (**d**) Microscope image of the top view of an extraction pipette facing the anterior end of the embryo. The pipette tip has a spiky edge that can break the vitelline membrane. Scale bar, 20 µm. (**e**) Schematic representation of the extraction procedure. After breaking up the membrane and entering the embryo, a fraction of the cytoplasm and nuclei is sucked into the pipette and ejected as droplets on the glass surface or added to previously made buffer droplets. Note that an oil phase in the pipette prevents the cytoplasm from contacting air.

20| Pipette some embryo glue onto a clean coverslip held with forceps. Produce a strip of glue—slightly off center and across the whole diameter of the coverslip—and let it dry in a dust-free unsealed container.

21| Remove the collection plate (with embryos up to 70 min old) from the fly cage and replace it with a fresh one for subsequent collection. Proceed to the stereomicroscope and carefully brush embryos from the agar surface into the water-filled basket. Wash them twice by moving the basket up and down and replacing the water.

22| Dechorionate embryos in bleach by transferring the basket to cell culture dish B; repeatedly move the basket up and down for 10–20 s until the first embryo appears shiny and floats on the fluid surface. Dry the basket quickly on a tissue and transfer it back to dish W.

▲ **CRITICAL STEP** Although the chorion is not immediately removed after brief immersion in bleach, it can be peeled off by surface tension. Simply immerse the basket briefly in water and then remove the water by placing the basket on a tissue; repeat this step several times until the majority of embryos finally float on the water surface.

? TROUBLESHOOTING

23| Cut a small block of agar from a plate with a scalpel and transfer it to the black plastic board under the stereomicroscope. Remove excess water from the basket and carefully transfer dechorionated embryos onto the agar block using a wet brush. With a needle or one prong of a blunt forceps, slide individual embryos to the edge of the block and align each one perpendicularly to the edge (**Fig. 3a**). The (anatomical) orientation does not matter. Align 20–30 embryos in a single row and tightly pack them so that they touch each other.

24| Pick up the glue-treated coverslip with forceps, turn it upside down and touch the top of the embryos with the strip of glue. They should adhere to the glue and keep their arrangement.

▲ **CRITICAL STEP** With the glue strip being slightly acentric on the coverslip, the larger side is reserved for subsequent extraction and imaging and should remain clean.

25| Quickly cover the row of embryos with halocarbon oil to avoid drying. Cover the larger glass surface with a thin layer of oil; be conservative with the amount of oil, as it should not reach the glass edge at any time and mix with the objective immersion oil (**Fig. 3b**). Place the coverslip into the dedicated sample holder (**Fig. 1f**) with the row of embryos perpendicular to the long side, and place the sample holder in the slide holder of the microscope *xy* stage (**Fig. 1d–e**). If you are performing experiments that involve the addition of compounds to the extract, carry out step 10 of **Box 1** before proceeding to Step 26 of the main PROCEDURE.

Embryo extraction procedure ● TIMING 5–10 min + imaging time

26| Switch to confocal fluorescence mode (detection of GFP) for the observation of microtubules. Use the ×20 objective and scan through the whole row of embryos while screening for a preblastoderm embryo in transition from telophase to interphase. Our experience is that embryos can be reliably characterized from the telophase of division cycle 6 onward. Look for elongated signal patches that will split to round patches within a minute (**Fig. 3c**). These signal patches are generated by the large astral microtubule arrays.

▲ **CRITICAL STEP** Do not choose an embryo that has entered mitosis or is in metaphase.

? TROUBLESHOOTING

27| Quickly switch to polarized transmission mode (via oculars), maneuver the extraction pipette to the programmed work position, approach the embryo with the sharp tip (**Fig. 3d**) and puncture it with a fast movement through the vitelline membrane. Immediately start suction by operating the foot pedals of the syringe pump. Suck in about half of the embryo's cytoplasm while monitoring the flow rate, and then stop the flow and withdraw the pipette from the embryo. If you are performing experiments that involve the addition of compounds to the extract, omit Step 28 of the main PROCEDURE. Instead, carry out step 12 of **Box 1** before proceeding to Step 29 of the main PROCEDURE.

28| With the pipette kept at a safe *z*-level in the field of view, move with the stage to a clean surface area that is still covered with halocarbon oil. Adjust the focus to the glass surface. Eject a small volume of cytoplasm and deposit it as a droplet on the glass surface by operating the *z*-handle of the micromanipulator and touching the surface (**Fig. 3e**). Produce droplets of 50–100 µm in diameter, and ensure that they firmly adhere to the glass surface. Repeat this step until the desired number of droplets is deposited (or until all of the cytoplasm is ejected). Our suggestion is 10–15 droplets per extract.

▲ **CRITICAL STEP** Work quickly during the extraction procedure. However, keep the flow in the pipette at a flow velocity of ≤200 µm s^{-1}, as observed from yolk movement. The total duration of the extraction from the embryo and production of droplets should not be more than 2 min; ideally, finish before the next mitosis starts.

? TROUBLESHOOTING

29| If necessary, eject all remaining cytoplasm as bulk and clear the pipette for the next extraction. Press the home button to remove the pipette from the field of view and switch to confocal fluorescence mode (detection of RFP) for the observation of histones in the nucleus or chromosomes.

30| Choose a droplet containing only one or two nuclei. Ideally, the extract will be entering prophase or metaphase. Switch to the ×40 or ×60 objective and perform multicolor time-lapse imaging of the nuclear cycles in one droplet or in several droplets in parallel by repositioning the *xy* stage between frame acquisitions.

? TROUBLESHOOTING

Follow-up extraction ● TIMING 5–10 min + imaging time

31| When imaging is complete, and if the age of the embryos allows it, repeat Steps 26–30 and produce another extract. Otherwise, return to Step 18, collect fresh embryos and restart embryo preparation.

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1**. An example image of an unsuccessful extraction as observed in Step 30 is presented in **Figure 4a**.

TABLE 1 | Troubleshooting table.

Step	Problem	Possible reason	Solution
22	Chorion is not removed even by long exposure to bleach	Upon exposure to air, bleach loses free radicals responsible for breaking up of the chorion membrane	Always prepare fresh bleach on the day of the experiment
	Embryos are malformed from dehydration, abnormal cytoskeletal morphology, development ceases, extensive mitotic errors	Treatment with sodium hypochlorite too long; concentration of sodium hypochlorite too high	Change the bleach solution in the dish more frequently; immerse embryos only for 5–10 s; reduce the concentration of sodium hypochlorite

(continued)

TABLE 1 | Troubleshooting table (continued).

Step	Problem	Possible reason	Solution
26	Difficulties in determining mitotic state; large variation in developmental stages	Embryos too young, low synchronization; female flies too old	At Step 18, perform more precollections to synchronize better; change batch of flies
28	Cytoplasm spreads on the surface and forms a thin layer	Coverslip surface is too hydrophilic	At Steps 1 and 2, reduce the duration of NaOH treatment to 5–8 min, or leave treated and dried coverslips at air overnight
	Cytoplasm does not adhere to the coverslip surface but rather flows along the pipette tip	Pipette surface is too hydrophilic	Repeat silane treatment of the pipette (Step 8) shortly before installation and use
30	Nuclei in extract without centrosomes, and difficulty in controlling volume during droplet deposition	Small pipette tips create large shear forces; large tips allow more flow	In Step 6, keep tip aperture at 40–50 µm (**Fig. 1g**); use thin-walled capillaries only
	Nuclei in extract without centrosomes	Large flow causes large shear forces in the pipette; this can break up protein complexes	At Step 27, reduce the flow rate in the pipette during extraction, or increase the diameter of pipette aperture
	Early cycle arrest; chromosome bridges at anaphase, abnormal spindle morphology	Extract droplet is deposited on agar-contaminated surface, which is toxic for the extract	At Steps 24 and 28, remember which side of the coverslip has touched the agar block; deposit extract droplets on clean glass surface
	Early cycle arrest; chromosome bridges at anaphase; abnormal spindle morphology	Extract droplet is deposited on glue-covered surface, which is toxic to the extract	At Step 28, deposit extract on clean glass surface
	Many spindles with abnormal morphology immediately after extraction, fused spindles, no cycling, arrest in all droplets	Extraction during metaphase; misjudged mitotic state; yolk spheres can operate as lenses and generate locally increased intensity, producing static signal patches	At Step 26, carefully monitor the mitotic state of the embryo, look for telophase (**Fig. 3c**) and monitor live a change in the shape of the patches; then perform the extraction
	Spindles with abnormal morphology during the cycle in many droplets, fused spindles, nuclear cycling fails	Time needed for the extraction procedure was too long; nucleo-cytoplasm entered mitosis in the pipette	At Steps 27 and 28, perform the extract procedure within 2 min, essentially before the next mitosis starts
	Chromosome bridges during anaphase	Light intensity too high	At Step 30, reduce the intensity of the excitation laser
Box 1, step 10	Difficulties in producing small buffer droplets	Air bubbles in the oil-based injection system	In step 5 of **Box 1**, remove air bubbles in the cylinder, tubing, holder and pipette
	No flow from the pipette tip although excessive pressure is applied	The tip is clogged (dried) from prolonged exposure to air, or it got clogged by dirt in the buffer or oil	Replace the pipette and start over with step 6 of **Box 1**. Before placing solution onto the cover glass, centrifuge the solution at 16,000*g* for at least 1 min to remove any debris
Box 1, step 13	Diluted extract does not cycle or arrests early	Dilution too high; wrong pH; salt too high	In step 12 of **Box 1**, reduce the volume ratio of buffer to extract; check the pH of the buffer; reduce ionic strength
Box 2, step 3	Early arrest after mixing cytoplasm of two embryos; abnormal morphology of microtubules, spindles	Addition of cytoplasm while droplets of preceding extraction are in metaphase; possibly stirring with pipette	Monitor time and consider cycle progression; the mixing of the two extracts should occur ideally during interphase, but not during metaphase or anaphase; add cytoplasm with minimal mechanical disturbance

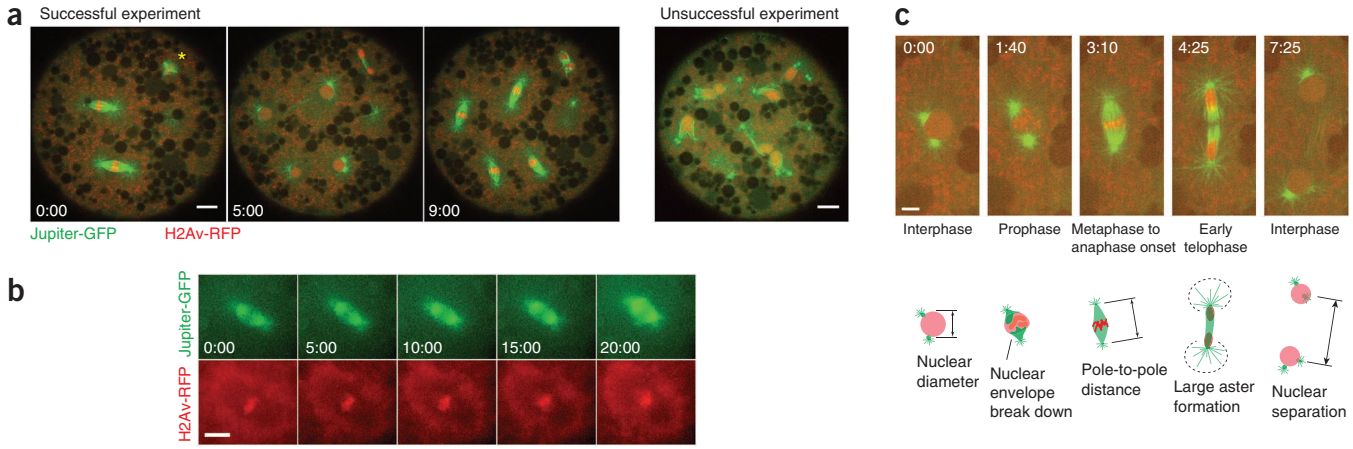

Figure 4 | Example of nuclear divisions in extracts. (**a**) Sequence of time-lapse confocal fluorescence images of a droplet of cytoplasm from a cycle 6 embryo, undergoing the seventh and eighth division, and containing two centrosomal spindles with large asters and one acentrosomal spindle (left frame, asterisk). In all cases, the chromosomes are divided and daughter nuclei are formed (middle frame). However, while four new spindles assemble, the spatial separation of the acentrosomal nuclei fails and spindles fuse (right frame). On the far right is an overview image of an unsuccessful extraction, showing many nuclei with dissociated centrosomes, deformed nuclei and multipolar microtubule structures. Green indicates microtubules, and red indicates histone marking DNA. Scale bars, 10 μm; time is in min:sec. (**b**) Sequence of time-lapse epifluorescence images of a spindle in extract during metaphase arrest following supplementation with nondegradable cyclin A. In this example, the metaphase arrest is maintained over an interval corresponding to two full cycles. Scale bar, 10 μm; time is in min:sec. (**c**) Sequence of time-lapse microscopy images and corresponding schemes illustrating the prominent phases of mitosis in the *Drosophila* syncytial embryo. The cycle starts in interphase, during which the nucleus grows because of DNA duplication, and the centrosomes migrate around the nucleus to opposite sides. In prophase, the chromosomes condense and the nuclear envelope partially breaks down. In metaphase, the spindle machinery waits for the spindle checkpoint to turn off. In telophase, the nuclear envelope reassembles and the large centrosome-nucleated microtubule aster transports the daughter nuclei apart while the central spindle microtubule bundle disintegrates. A new cycle begins with the next interphase. Arrows and circles denote measurable quantities—nuclear diameter, centrosome migration, chromosome condensation, spindle length, aster size, nuclear separation and central spindle disassembly—to characterize the mitotic division. Scale bar, 5 μm; time is in min:sec.

● TIMING

Steps 1–3, preparation of glass coverslips: 1 h
Steps 4–9, preparation of extraction pipettes: 30 min
Steps 10–14, setting up working positions and maneuvers: 15 min
Steps 15–18, preparation of the fly cage and synchronization: 0.5 d
Steps 19–25, embryo preparation: 5 min
Steps 26–30, embryo extraction procedure: 5–10 min + imaging time
Step 31, follow-up extraction on the same coverslip: 5–10 min + imaging time
Box 1, extract dilution and addition of compounds: ~45 min + imaging time
Box 2, mixing extracts from different embryos: 15–20 min + imaging time

ANTICIPATED RESULTS

Figure 4 shows examples of time-lapse imaging experiments that were performed using the protocol detailed here. Cycle time, spindle orientation and nuclear positioning can be studied in droplets of cytoplasm after ordinary extract preparations (**Fig. 4a**). The features of a successful and unsuccessful extraction with a wild-type fly strain expressing the microtubule and the chromosomal marker are presented. Multiple rapid and synchronous nuclear divisions can be observed after successful extraction, in contrast to an unsuccessful experiment in which many nuclei are deformed and lack their centrosomes, leading to various spindle defects and no further divisions. The cycle time should be 8–9 min for early embryonic divisions[50]. Moreover, nuclear cycling can be checkpoint-arrested in extract by adding a purified, nondegradable *Drosophila* cyclin A construct, lacking the first 170 nt that contain the recognition site for ubiquitin-mediated protein degradation[51]. The construct was amplified from the *Drosophila* cDNA as described earlier[52], cloned into a pET-ZZ vector for expression in *Escherichia coli*[53] and purified and dialyzed in dilution buffer according to a protocol described earlier[54]. The addition of the nondegradable cyclin A (Δ170) protein to the extract at a final concentration of 5–10 μM caused an arrest in metaphase for longer than the time two consecutive cycles would normally take (**Fig. 4b**). In nonarrested extract during progressing nuclear cycling, the measurement of nuclear diameter, chromosome condensation, spindle length, aster size and nuclear separation are spatial measures whose time course allows a detailed characterization of the dynamics of DNA duplication, packing, separation and positioning during mitosis (**Fig. 4c**), as presented in an earlier study[18].

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AUTHOR CONTRIBUTIONS I.A.T. and I.G. determined the correct timing of extraction. I.A.T. designed the instrument, drew up the protocol and performed the experiments. I.A.T. and T.S. conceived the cycle arrest experiment. I.A.T. prepared the figures. A.E. and T.S. supervised the work. All authors discussed the work and contributed to the writing of the manuscript.

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