Chapter 7

Encapsulation of *Xenopus* Egg and Embryo Extract Spindle Assembly Reactions in Synthetic Cell-Like Compartments with Tunable Size

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Abstract

Methods are described for preparing *Xenopus laevis* egg and embryo cytoplasm and encapsulating extract spindle assembly reactions in cell-like compartments to investigate the effects of cell size on intracellular assembly. Cytoplasm prepared from the eggs or embryos of individual frogs is screened for the ability to form interphase nuclei and metaphase spindles, and subsequently packaged, along with DNA, into droplets of varying size using microfluidics. The dimensions of these cell-like droplets are specified to match the range of cell diameters present in early embryo development. The scaling relationship between droplets and spindles is quantified using live fluorescence imaging on a spinning-disk confocal microscope. By comparing the encapsulated assembly of spindles formed from cytoplasmic extracts prepared from embryos at distinct stages of *Xenopus* early development, the influence of cell composition and cell size on spindle scaling can be evaluated. Because the extract system is biochemically tractable, the function of individual proteins in spindle scaling can be evaluated by supplementing or depleting factors in the cytoplasm.

Key words Spindle assembly, Size-scaling, *Xenopus*, Egg extract, Cytoplasm, Encapsulation, Cell-like compartment, Droplet microfluidics, Emulsion, Embryogenesis

1 Introduction

The cytoplasmic extracts of *Xenopus* eggs are an established model system for investigating the molecular mechanisms of the vertebrate cell cycle in vitro [1–6]. The extracts are capable of carrying out many cellular processes, including genome and centrosome replication, and spindle and nucleus assembly. Spindle assembly is accomplished in vitro by adding DNA, in the form of either frozen demembranated sperm nuclei or freshly prepared interphase nuclei (generated by cycling extracts into interphase with calcium), to CSF-arrested extracts. Microtubules assemble around the exogenous DNA to form spindles and are imaged using labeled α/β--tubulin [7] and DNA. The extracts are especially useful because they are amenable to biochemical manipulation. Although this

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system has proven to be quite powerful, cytoplasmic extracts lack cell boundaries and are therefore missing much of spatial information encoded in the structure of a cell.

There is now a major thrust to understand how physical boundaries, mechanical perturbations and the spatial positioning of molecules influence the behavior of cellular processes, such as cytoskeletal or organelle assembly and signaling. For example, aspects of cell division have been reconstituted on supported lipid bilayers [8], assembly of cytoskeletal proteins has been confined to microfabricated chambers [9] or water-in-oil emulsions [10, 11], and cytoplasmic extracts have been encapsulated to create cell-like compartments [12–16].

Because the tools for microfabrication and lithography are now readily available, it is feasible to use droplet microfluidics [17, 18] to encapsulate aqueous solutions in cell-size compartments whose dimensions are precisely defined. These techniques make it possible to analyze how the geometric constraints and limited volumes of a vertebrate cell impact biochemical reactions reconstituted in vitro. For example, by combining *Xenopus* extract spindle assembly with droplet microfluidics it is possible to directly test the hypothesis that cell size regulates spindle and nucleus assembly. Importantly, with the acquisition of just a few prefabricated parts, any lab can generate microfluidic devices that can be tailored to a specific biological question.

Cell size is a largely unexplored regulatory parameter in biology. There is growing interest in the observation that cell size and organelle size are coupled. For example, during the reductive divisions of early vertebrate embryogenesis, cell size is rapidly decreased. Concomitant with the reduction in cell size, intracellular structures such as the spindle, nucleus, and centrosome also decrease in size [12, 19–22]. But how do organelles know how to reach the correct size, and how do these assembly pathways adapt to changes in cell size? One approach to directly answer these questions is to build synthetic cells whose dimensions can be tuned to match the range of cell sizes found in embryo development [12]. These non-replicative cell-like compartments contain a defined boundary and are filled with cytoplasm. In addition, because cytoplasm can be isolated from various stages of development [22, 23], it is possible to utilize these extract-filled droplets to distinguish between the relative contributions of a developmental program (e.g., composition changes) and cell size (a physical change) in organelle scaling.

Here, we describe a robust protocol for generating cytoplasm-filled compartments with tunable dimensions. Using this system, it is possible to precisely specify compartment size (from 5 to 500 μm) and to use live imaging to quantify spindle and nucleus scaling as a function of droplet size. The three methodologies described include: (1) Preparation of egg and embryo cytoplasmic extracts from individual *Xenopus laevis* frogs, (2) Fabrication of T-junction

microfluidic droplet-generating devices starting from plastic replicas, and (3) Encapsulation of spindle assembly reactions inside of synthetic cell-like compartments with tunable size.

2 Materials

2.1 Egg Extract Preparation from Individual Frogs

1. Female *Xenopus laevis* frogs.
2. Pregnant mare serum gonadotropin (PMSG). 200 U/mL in sterile Milli-Q water. Store at 4 °C.
3. Human chorionic gonadotropin (HCG). 1000 U/mL in sterile Milli-Q water. Store at 4 °C.
4. 1 mL disposable syringes with 18-gauge and 27-gauge needles.
5. 4 L plastic containers with tight-fitting lids (hole-punched for air exchange).
6. 20× Marc's Modified Ringer's (MMR): 100 mM HEPES (free acid), 2 mM EDTA, 2 M NaCl, 40 mM KCl, 20 mM $MgCl_2$, 40 mM $CaCl_2$. Adjust pH to 7.8 with NaOH. Autoclave and store at room temperature.
7. 16 °C room or large incubator set to 16 °C.
8. 4 L plastic beaker and 500 mL glass beakers.
9. Plastic transfer pipettes.
10. 2 M sucrose. Filter-sterilize and store in 12.5 mL aliquots at −20 °C.
11. 1 M K-HEPES, pH 7.7, filter-sterilize and store in 5 mL aliquots at −20 °C.
12. 0.5 M K-EGTA, pH 7.7, filter-sterilize and store in 2 mL aliquots at −20 °C.
13. 2 M $MgCl_2$ solution.
14. 20× extract buffer (XB) salts: 2 M KCl, 20 mM $MgCl_2$, 2 mM $CaCl_2$. Autoclave and store at 4 °C.
15. Dejelly solution: 2 % l-cysteine base (~275 mM) in 1× XB salts, pH adjusted to 7.8 with NaOH. Prepare fresh and store at 16 °C. For 1 L solution, add 20 g l-cysteine, 50 mL 20× XB salts, and fill to 1 L. Adjust pH to 7.8 with NaOH.
16. XB: 100 mM KCl, 0.1 mM $CaCl_2$, 1 mM $MgCl_2$, 50 mM sucrose, 10 mM K-HEPES, pH 7.7. Prepare fresh and store at 16 °C. For 1 L solution, add 50 mL 20× XB salts, 25 mL 2 M sucrose, 10 mL of 1 M K-HEPES, and fill to 1 L with H_2O. Adjust pH to 7.7 with KOH.
17. CSF-XB: 1× XB + 1 mM $MgCl_2$, 5 mM K-EGTA, pH 7.7. Prepare fresh and store at 16 °C. For 400 mL solution, add 200 µL of 2 M $MgCl_2$ and 4 mL of 0.5 M K-EGTA pH 7.7 to ~400 mL of XB.

18. Leupeptin, pepstatin, chymostatin (LPC). 10 mg/mL each of leupeptin, pepstatin, chymostatin, in dimethylsulfoxide (DMSO). Store in ~55 µL aliquots at −20 °C.
19. Cytochalasin D: 10 mg/mL in DMSO. Store in ~55 µL aliquots at −20 °C.
20. CSF-XB+: to 100 mL of the previously prepared CSF-XB, add 100 µL of LPC. Prepare fresh and use immediately.
21. SW-55 ultra-clear thin-wall 5 mL centrifuge tubes (Beckman). 13×51 mm.
22. Sarstedt round-bottom 13 mL polypropylene tubes. 16.8×95 mm.
23. Refrigerated clinical centrifuge set to 16 °C.
24. High-speed refrigerated floor centrifuge containing Sorvall HB-6 rotor and rubber adapters for round-bottom centrifuge tubes (Kimble-Chase). Set to 16 °C.
25. Energy mix (50×): 190 mM creatine phosphate, 25 mM adenosine triphosphate, 25 mM $MgCl_2$, 2.5 mM K-EGTA pH 7.7. Store in 100 µL aliquots at −20 °C.

2.2 Embryo Extract Preparation

Subheading 2.2 requires reagents from Subheading 2.1, plus the following:

1. Testes freshly isolated from a male *Xenopus laevis* frog.
2. Plastic pestle for 1.5 mL microfuge tube (USA Scientific).
3. Glass petri dishes: 6 cm diameter.
4. 1/3× MMR stored at 16 °C.
5. Dissecting stereomicroscope.
6. Dejelly solution without XB salts: 2 % w/v cysteine (0.275 M final) in Milli-Q H_2O, pH to 7.8 with NaOH. Store at 16 °C.
7. 0.5 mg/mL (~13 µM) human Δ90-CyclinB1 in XB.
8. 15 mg/mL (~750 µM) human UbcH10-C114S in XB.

2.3 Fabrication of Microfluidic Droplet-Generating Devices

1. Plastic replicas: molds for casting microfluidic devices from PDMS.
2. Scotch tape.
3. Large plastic petri dishes: 150 mm diameter.
4. Slygard elastomer 184 kit: polydimethylsiloxane (PDMS) elastomer and curing agent.
5. Red solo cups.
6. 2 mL serological pipette.
7. Vacuum dessicator chamber.
8. Vacuum pump.

9. Oven set to 65 °C.
10. Transparencies.
11. 3 mm biopsy hole punch (e.g., Harris Uni-Core).
12. 0.5 mm biopsy hole punch.
13. Wire (<0.5 mm).
14. Razor.
15. Microscope slides.
16. Plasma cleaner (March Plasmod).

2.4 Extract Encapsulation and Spindle and Nucleus Assembly in Droplets

1. PDMS droplet-generating devices.
2. Aquapel rain repellant.
3. Gasket imaging chambers: laser cut 3 or 4.5 mm thick acrylic with 2–4 mm diameter circles (Fig. 4a). Affix to 18 mm glass circle using Norland 61 optical adhesive; expose to UV light for at least 2 h, or overnight.
4. Hamilton 1700 series glass syringes with removable needle: 25 μL (extract) and 50 μL (oil).
5. 28-Gauge needle for Hamilton syringes, 0.75 in. length.
6. Teflon (PTFE) tubing from Cole-Parmer: ID ~0.30 mm, OD ~0.76 mm.
7. Forceps with serrated tip.
8. Syringe pumps.
9. Stereomicroscope.
10. *Xenopus laevis* egg or embryo cytoplasmic extracts (from Subheadings 2.1 or 2.2).
11. Squalene (Sigma-Aldrich). Store at 4 °C.
12. Cithrol DPHS (Croda). Store at room temperature. Seal container with Parafilm.
13. 50 mg/mL solution of Cithrol DPHS in squalene: store 1 mL aliquots in glass vials with screw caps, topped with N_2 gas sealed with Parafilm, at 4 °C for up to 1 month.
14. Hoechst: 1 mg/mL Hoechst 33342 in Milli-Q H_2O. Store at 4 °C.
15. Demembranated sperm nuclei (a stock of 200,000 nuclei/μL). Store in 3 μL aliquots at −80 °C.
16. 50 mM $CaCl_2$ in Milli-Q H_2O. Filter-sterilize.
17. Rhodamine labeled porcine brain tubulin stock (20 mg/mL, or ~200 μM).
18. Spindle fix: 48 % glycerol, 11 % formaldehyde in a solution of 1× MMR. Add Hoechst to achieve a final concentration of 5 μg/mL Hoechst. Store at room temperature for up to 1 week.

3 Methods

Overview: The following sections describe procedures for the (1) Preparation of *Xenopus laevis* egg and embryo cytoplasmic extracts, (2) Fabrication of microfluidic droplet-generating devices from plastic molds, and (3) Encapsulation of cytoplasm and DNA in cell-size droplets to investigate spindle scaling as a function of compartment size.

3.1 Cytoplasmic Extract Preparation from Xenopus Eggs and Embryos

A key requirement of the extract experiments is the preparation of fresh egg or embryo cytoplasm capable of forming spindles with a high efficiency (percentage of sperm DNA around which bipolar spindles assemble). To ensure that at least one extract is functional, do not combine the eggs of each ovulated female frog. A significant hurdle in working with the extract system is that poor quality eggs from a single frog can inhibit the ability to (1) maintain extracts in CSF arrest, (2) cycle extracts into interphase and form nuclei, or (3) form bipolar spindles with high efficiency, after cycling nuclei back into mitosis. On the day of an experiment, my lab typically generates three separate extracts from individual frogs (yielding approximately 1 mL of pure cytoplasm from each clutch of eggs, or approximately 0.4 mL of cytoplasm from each dish of embryos) and screens these extracts for spindle and nucleus assembly in both a microfuge tube and inside cell-size droplets. Quality is scored and the best extracts are then systematically encapsulated and imaged in microfluidics droplets to plot the scaling relationship between spindle and compartment size (described in Subheading 3.3).

3.1.1 Egg Extract Preparation from Individual Frogs

See Fig. 1.

1. At 3–7 days prior to extract preparation, prime females with a subcutaneous injection of 100 U of PMSG using a 27-gauge needle and 1 mL syringe. These frogs should be used within 1 week. Numbers vary, but a typical week requiring three extract preparations necessitates the priming of 12 females.
2. At 16–18 h prior to egg collection subcutaneously inject four females with 500 U of HCG and store them separately in individual plastic containers filled with 2 L of 1× MMR (prepared with deionized water) at 16 °C. Assume that three out of four females will lay high-quality eggs, allowing the preparation of three separate single-frog extracts.
3. Analyze egg quality to determine how many extracts can be prepared. Good eggs should have clearly delineated animal (dark) and vegetal (light) poles. Clutches of eggs that are activated or lysed (white and puffy appearance) or stringy should not be used. A few lysed eggs (up to 10 in a container of ~2500 eggs) can be tolerated. These lysed eggs should be removed using a transfer pipette (*see* **Note 1**).

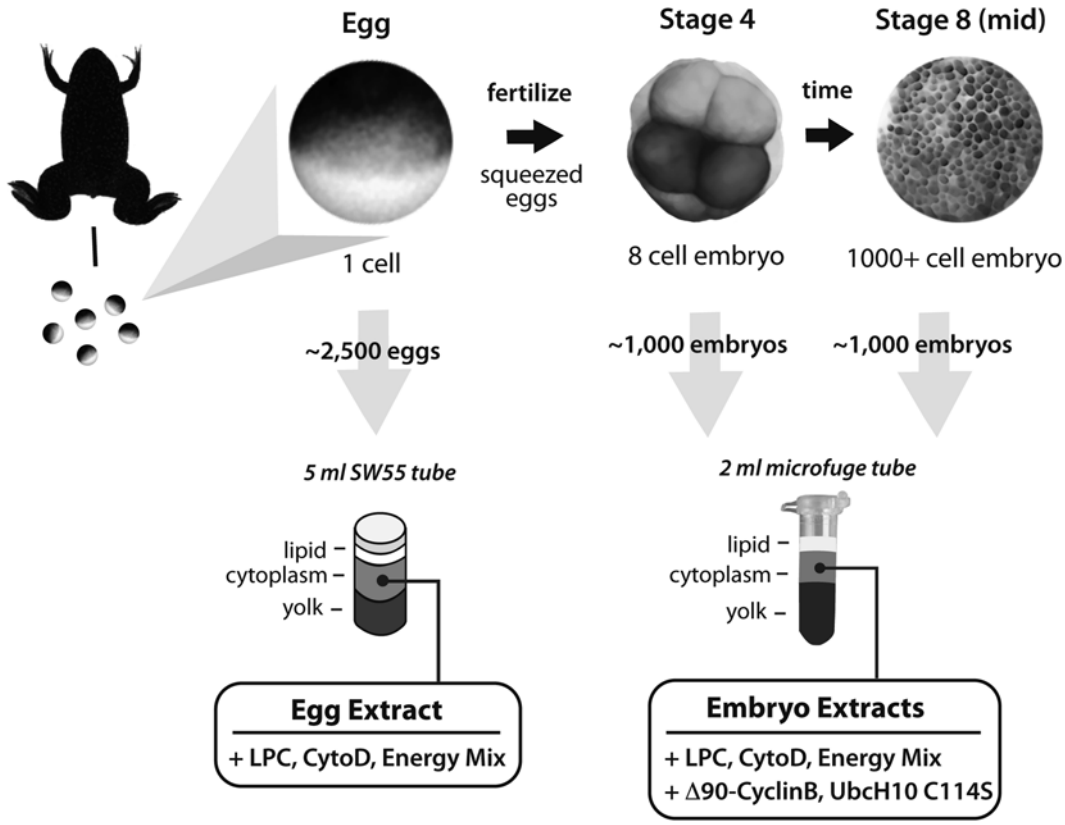

Fig. 1 Schematic of *Xenopus laevis* egg and embryo extract preparation. Starting from ovulating females, laid eggs can be collected and used to generate an "egg extract," or freshly squeezed eggs can be fertilized and synchronized embryos can be used to generate "embryo extracts" at various stages of early development

4. Separate frogs from eggs, keeping eggs in their containers at 16 °C. Frogs should be placed in a bucket of deionized water and returned to the colony by the end of the day.
5. At 2 h prior to extract preparation, begin preparing buffers, supplies and equipment (*see* **Note 2**). For three single-frog extracts: Prepare 1 L dejelly, 1 L XB, and 0.4 L CSF-XB. Ten minutes prior to the start of experiments, prepare 0.1 L CSF-XB+, and fill each of 4 Beckman SW55 tubes with 1 mL CSF-XB+ and 15 μL of 10 mg/mL cytochalasin D (final concentration of 150 μg/mL). For supplies: cut tips from 4 transfer pipettes, and leave 4 uncut. Collect one 4 L plastic beaker, 3 × 500 mL glass beakers and all reagents and supplies and use a cart to transport into the 16 °C room. Make sure that all centrifuges are set to 16 °C.

   In the next steps [6–8], gently pour buffers down the side of the beaker, and lightly swirl eggs. Between steps, pour off as much buffer as possible without exposing the eggs to air,

which will shear them. Manipulate eggs carefully to prevent activation and lysing. Additionally, once eggs are dejellied, make sure to carry out subsequent washing and centrifugation steps in rapid succession.

6. Clean off the eggs: Pour off additional water inside plastic container to remove, urine, feces and other particulate matter. For containers that are especially dirty, wash the eggs once with 500 mL of 1× MMR.
7. Dejelly eggs: Pour off remaining MMR and add approximately 150 mL of dejelly solution to each of three beakers, each containing eggs from a separate frog. The egg jelly coat can be visualized by eye: the jelly coat will prevent eggs from packing directly on top of one another (there will be a clear gap between the eggs, which are dark). Swirl the eggs gently, and after 1–2 min pour off and replenish dejelly solution. Total time of dejelly will vary depending on eggs. Swirl the eggs every 20 s until they pack together tightly (up to ~5 total minutes), without gaps, when beaker it tipped to the side. Take care to not let the eggs dejelly for too long. Once ready, pour off the dejelly solution and immediately add 150 mL of XB. Begin staggering the timing of exchanging buffers in each of the 3 beakers. Keep the order the same throughout the remaining steps.
8. Wash eggs: Pour off the first addition of XB and add an additional 150 mL to each beaker. Pour off the XB and add 125 mL of CSF-XB. Pour off the CSF-XB and add 30 mL of CSF-XB+. At this point the eggs should be clean and largely pole-rotated (dark animal pole will be facing up). Do one final quick-sort to remove any lysed or misshapen eggs.
9. Transfer eggs very carefully to SW55 tube (prefilled with 1 mL of CSF-XB+ and 15 μL of 10 mg/mL cytochalasin D). Use trimmed transfer pipette, and avoid exposing the eggs to air by first drawing up 0.5 mL of CSF-XB+ buffer and then gently drawing up the eggs (Note: each transfer pipette holds a max of about 2 mL of eggs). Typically 2–3 fillings of the transfer pipette are required to deposit all the eggs from beaker containing CSF-XB+ into SW55 tube. Do not overfill the tube, exposing eggs to air. The eggs from a female which ovulates robustly will fill ~75–100 % of the volume of the SW55 tube (*see* **Note 3**).
10. Packing spin: Place SW55 tube inside 13 mL Sarstedt tube using a forceps. Transfer tubes to a refrigerated clinical centrifuge, set to 16 °C. Spin at 250×*g* for 1 min, and then at 500×*g* for an additional 30 s. After spinning, aspirate excess buffer from the top of the eggs using a transfer pipette. This step prevents buffer from diluting the cytoplasm.

11. Crushing spin: Transfer tubes with packed eggs to Sorvall HB-6 rotor containing adapters, and centrifuge at 18,000 × *g* for 15 min at 16 °C.
12. Collecting cytoplasm: Immediately after crushing the eggs remove SW55 tubes from Sarstedt tubes and place them on ice. The visibly colored layers correspond to components of the egg, top: lipid (white-yellow), middle: cytoplasm (gold), bottom: yolk granules and pigment (black/brown). Using a 18-gauge needle attached to a 1 mL syringe, puncture the thin-wall tube at the bottom of the cytoplasmic layer. Carefully remove the cytoplasmic layer without taking up any of the surrounding yolk or lipid layers. Be conservative. Expect about 0.9–1.2 mL of pure cytoplasm from a SW55 tube that was completely filled with eggs. Remove the needle and transfer the cytoplasm to a prechilled, labeled 1.5 mL microfuge tube on ice (*see* **Note 4**).
13. Supplement the extract with a final concentration of: 10 μg/mL or ~16.67 μM LPC (1:1000 of 10 mg/mL stock), 10 μg/mL or ~20 μM cytochalasin D (1:1000 of 10 mg/mL stock). Add 1:50 of energy mix to achieve a final concentration of ~38 mM creatine phosphate, 0.5 mM ATP, 0.5 mM $MgCl_2$, and 0.05 mM EGTA. Mix by inverting the microfuge tube. The extract can be used for the next 6–8 h.

3.1.2 Embryo Extract Preparation

Note: We have had the best results preparing high-quality Stage 4 (8 cell) and Stage 8 (1000s of cells) embryo extracts by fertilizing and incubating freshly squeezed eggs at 16 °C (Fig. 1). The extracts are less functional when the steps are carried out at 22 °C. Freshly squeezed eggs are necessary to achieve >90 % fertilization efficiency. Do not collect loose eggs in the container that houses the ovulating female. The methods here have been revised from the procedure described by Wilbur and Heald [23].

1. As in Subheading 3.1.1, prime females with a subcutaneous injection of 100 U of PMSG 3–7 days prior to in vitro fertilization (IVF).
2. At 14–15 h prior to squeezing, subcutaneously inject four females with 500 U of HCG and store them separately in individual plastic containers filled with 1× MMR (prepared with deionized water) at 16 °C. Assume that three out of four females will lay high-quality eggs, allowing the preparation of three separate single-frog embryo extracts.
3. 14 h after HCG injection, analyze egg quality. Avoid using females that have already laid lysed or stringy eggs, or that have laid no eggs at all.
4. Fresh egg collection: For selected frogs, promote egg-laying by squeezing females in a manner that mimics amplexus, and

collect freshly laid eggs from single females in individual 6 cm glass dishes. Repeat every 15 min (up to 4 total squeezes) or until the dish is full. Place females in deionized water in between rounds of egg collection.

5. Prepare sperm slurry: For each IVF reaction, use 1/8 of a testis recently isolated from a *Xenopus* male. Crush a piece of gonad in 1 mL of Milli-Q H_2O inside a 1.5 mL microfuge tube, using an appropriate plastic pestle, for 1–2 min.
6. Fertilize eggs: For each IVF reaction (each dish), pipette 1 mL of sperm slurry onto the eggs and incubate for 5 min at 16 °C. Wash once and replace the solution with 1/3× MMR. Let embryo development progress at 16 °C for either 4.5 h to reach mid-Stage 4, or 13.5 h to reach mid-Stage 8. To confirm fertilization, wait for 10–20 min and observe whether embryos pole-rotate such that the animal cap (dark) is facing up (*see* **Note 5**).
7. Prepare 100 mL of XB-free dejelly solution from 3 g cysteine in Milli-Q H_2O, and add 600 μL of 10 N NaOH to reach an approximate pH of 7.8.
8. Dejelly eggs at 2–3 h post-fertilization, after the first cleavage has occurred (at 16 °C). Pour off 1/3× MMR and add 20 mL of XB-free dejelly solution to each dish. Swirl gently for 2–3 min, until embryos pack tightly together when dish is tilted. Pour off dejelly and wash 3 times with 1/3× MMR. Add 15 mL of 1/3× MMR and continue incubation at 16 °C.
9. Begin sorting embryos: Using a dissecting stereomicroscope, remove embryos that have not divided, that have enlarged, or that appear misshapen. Continue to monitor developmental progression by recording the timing of the second and third divisions. Aspirate and remove embryos that are lagging or beginning to lyse.
10. Prepare remaining buffers and carry out final sort 30 min prior to embryo collection (4 h for Stage 4 and 13 h for Stage 8). Following the materials in Subheading 3.1.1 and instructions in Subheading 3.1.1, **step 5**, prepare 0.2 L of XB solution, and 100 mL of CSF-XB+ (plus 100 μL of 10 mg/mL cytochalasin D). Perform final sort to ensure that the remaining ~1000 embryos in each dish are homogeneous.
11. Wash embryos: Pour off 1/3× MMR and add in succession to each dish: 2 × 25 mL of XB, 2 × 15 mL of CSF-XB+ (plus cytochalasin D). Gently swirl and pour off buffers between each addition. Tilt dish so that surface tension helps to carefully pull the solution over the lip of the dish. This ensures that embryos will not be poured off during buffer exchanges.
12. Pack embryos: After pouring off remaining buffer, and without exposing embryos to air, carefully transfer the embryos

from a single dish into 2 mL microfuge tube using a cut transfer pipette. A full dish of embryos, unpacked, will fill the tube to a level of ~1.7 mL. Centrifuge at 200 × *g* for 1 min and then at 500 × *g* for 30 s in a benchtop microcentrifuge. Aspirate remaining buffer: first using P1000 and finally using a P200.

13. Crush embryos: Transfer 2 mL tubes containing packed eggs to a Sorvall HB-6 rotor containing microfuge tube adapters, in a superspeed centrifuge set to 16 °C. Spin at 18,000 × *g* for 12 min.
14. Collect embryo cytoplasm (*see* **Note 6**): After crushing the embryos into stratified layers, place the 2 mL microfuge on ice. The layers correspond to, top: lipid (white-yellow), middle: cytoplasm (gold), bottom: yolk granules and pigment (black/brown). Using a bent P200 pipette tip, insert the tip from above, through the lipid layer and begin carefully withdrawing cytoplasm, making sure to avoid surrounding layers. Dispense the cytoplasm into a prechilled 1.5 mL microfuge tube. Expect a yield of ~350–400 μL of pure cytoplasm for each full plate of embryos.
15. Supplement the embryo extract with to reach a final concentration of: 10 μg/mL or ~16.67 μM LPC (1:1000 of 10 mg/mL stock), 10 μg/mL or ~20 μM cytochalasin D (1:1000 of 10 mg/mL stock). Add 1:50 of energy mix to achieve a final concentration of ~38 mM creatine phosphate, 0.5 mM ATP, 0.5 mM $MgCl_2$, and 0.05 mM EGTA. Mix by inverting the microfuge tube. Additionally, to ensure that the extracts are synchronized and arrested in mitosis add a nondegradable form of cyclin B along with a dominant-negative form of UbcH10. Achieve a final concentration of: ~0.35 μM Δ90-CyclinB1 (1:40 v:v of ~13 μM stock), and ~18 μM UbcH10-C114S (1:30 v:v of ~750 μM stock). Mix by inverting tube. Embryo extracts are functional for up to 6 h (*see* **Note 7**).

3.2 Fabrication of Microfluidic Droplet-Generating Devices

Using the procedures described below, droplet-generating devices can be fabricated in a standard molecular biology lab, without the need for a clean room (Fig. 2). The key requirement is a plastic mold from which a microfluidic device can be cast using PDMS. For an overview on the process of creating a plastic mold, *see* **Note 8**. Alternatively, a number of universities and private companies have microfluidic foundries that will fabricate devices or molds on a fee-for-service basis (*see* **Note 9**).

1. Wash plastic replicas with deionized water and blow dry with pressurized air.
2. Attach clear tape around the perimeter of plastic replica to hold in elastomer poured onto the mold. About 2 mm of tape should be affixed to the wall (depth) of plastic replica and another 10 mm of tape should be visible above the feature-side

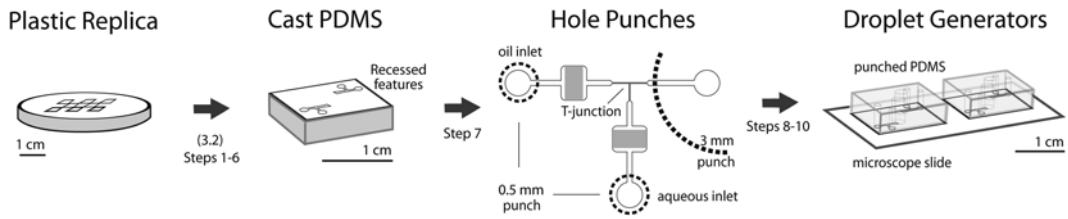

Fig. 2 Schematic of PDMS droplet-generator fabrication from a plastic mold. Starting from a plastic replica of multiple T-junction microfluidic droplet-generating device, PDMS is cast, degassed, and cured. Holes are punched in the PDMS to generate a collection chamber (3 mm hole) at the outlet, and to later connect tubing to 0.5 mm holes over inlets. Plasma-cleaned PDMS devices are then bonded to glass microscope slides. Each slide contains a total of four T-junction droplet generators. Scale bar: 1 cm

of the replica. Place 4–5 taped replicas in a large 15 cm petri dish. Features should face up.

3. Mix 10:1 (by weight) elastomer and curing agent, in a red solo cup. Stir with 2 mL pipette for 1–2 min to ensure thorough mixing. Assume approximately 20 g is required per plastic replica. Because it takes >30 min for PDMS to begin to harden, this can be done at a modest pace.
4. Slowly pour a layer of approximately 4 mm in thickness of PDMS onto taped plastic replica. Pouring a thicker layer of PDMS will make it more challenging to punch holes and connect tubing in later steps. Transfer everything to a dessicator chamber.
5. Degas the uncured PDMS. First use a house vacuum for 30 min to remove most of the bubbles, and follow with suction from a vacuum pump (~5 min). Monitor the dessicator to ensure that PDMS does not boil over under the pressure of the vacuum pump. If it starts bubbling, close down the valve on the dessicator chamber lid.
6. Cure PDMS overnight in a 65 °C oven. Allow a minimum of 2 h to thoroughly set.
7. Punch holes: Peel off tape and remove the PDMS cast carefully from the plastic replica. Place on a transparency with feature side facing down. Punch a large 3 mm hole over the outlet, just beyond the T-junction, and punch two small (0.5 mm) holes over the oil and aqueous inlets of the device. Use <0.5 mm diameter wire to remove any small plugs left in the smaller of the punched holes.
8. Cut the larger PDMS cast into individual droplet-generating devices (~1–1.5 cm rectangles) using a flat razor. Place feature side down on a second, unused transparency. The procedure can be paused here and devices can be sealed with clear tape and stored at room temperature.

9. Wash devices with deionized water to clean off and remove any residual polymer or dust in the punched holes. Dry with pressurized air.
10. Use oxygen plasma to generate hydrophilic surfaces on the PDMS casts and glass microscope slides. Place two PDMS casts and one microscope slide in Plasmod and expose to oxygen plasma for approximately 20 s. Immediately attach two PDMS casts to the microscope slide, feature side down. Using medium pressure by hand, ensure complete bonding of the PDMS and glass.
11. Place completed devices in a large 15 cm petri dish. Tape over the punched holes to ensure the devices remain dust-free. Cover and store at room temperature.

3.3 Extract Encapsulation and Spindle and Nucleus Assembly in Droplets

The successful encapsulation of *Xenopus* cytoplasmic egg and embryo extracts requires the experimenter to have prepared droplet-generating devices in Subheading 3.2, prepared cytoplasm in Subheadings 3.1.1 or 3.1.2, and to screen single-frog extracts for spindle and nucleus assembly activities. The following section describes procedures for: (1) Preparing the required equipment, supplies and reagents, (2) Evaluating extract quality, and (3, 4) Generating and imaging extract-filled droplets. All equipment, reagents and extracts should be placed in a 4 °C cold room. This strategy ensures that that microtubule polymerization is restricted, thereby preventing spindle assembly until the droplets are shifted to 22 °C (a temperature at which spindles are fully formed in 40 min) (*see* **Note 10**).

3.3.1 Preparation

Day Before Experiment

1. Treat microfluidic channels with Aquapel to ensure that they are hydrophobic. This will prevent droplets from wetting onto PDMS after being formed. Pipette 2 μL of Aquapel rain repellant into the oil inlet. The channels will lose contrast as the Aquapel is wicked into them. Blow out excess Aquapel using a N_2 stream. Cure and dry devices at 50 °C overnight (*see* **Note 11**).
2. Either place prepared imaging chambers at 4 °C, or laser cut new chambers from 3 to 4.5 mm thick acrylic and glue to 18 or 22 mm glass circle cover slips using Norland 61 optical adhesive (Expose to UV light overnight at room temperature)
3. Submerge teflon tubing in Milli-Q H_2O in a large petri dish overnight, at 4 °C. Additionally, place large items in the cold room, including syringe pumps and a stereomicroscope. Also store forceps, Hamilton syringes, pipettes and tips, and a microfuge tube rack in cold room.

Morning of Experiment

4. Remove Aquapel-treated PDMS devices from the oven, tape over the holes with Scotch tape, and move them to 4 °C. Place gasket imaging chambers in the cold room.

Immediately Before Experiment

5. Clean out Hamilton syringes and tips with appropriate reagent. Wash extract syringe with water, blow out, add 50 μL of extract (any extract), blow out, and then let it incubate with extract (any extract) until the start of experiments. Pipette 100 μL of 50 mg/mL mixture of oil and surfactant (squalene and Cithrol DPHS) into the oil syringe, blow out, add then let it incubate with the oil/surfactant mix.
6. Assemble PTFE tubing onto syringe needles using forceps. Cut tubing to an appropriate length (~6 in.).
7. Fill oil syringe and tubing with 100 μL oil/surfactant mix by pipetting into the plunger end of the syringe. Fill the aqueous syringe with 25 μL of extract. Withdraw it from a microfuge tube using the tubing.

3.3.2 Evaluate the Quality of Egg or Embryo Extracts

The goal is to identify the single-frog extract that is most capable of forming nuclei and spindles at high efficiency, both in microfuge tubes and inside microfluidic droplets. The following extract qualities are scored to identify the best extract.

1. *Evaluate mitotic character*: If demembranated sperm nuclei are added to CSF-arrested egg extracts, do spindles form with high efficiency in a 25 μL reaction (~24 μL egg extract + 0.25 μL of 50,000 sperm/μL stock + 0.25 μL of 20 mg/mL rhodamine-labeled tubulin + 0.25 μL of 20 μg/mL Hoechst) in a microfuge tube? Flick tube every 5–10 min to ensure that spindles do not clump. After 40 min at room temperature, mix 1 μL of extract reaction with 4 μL of spindle fix and image (*see* **Note 12**).
2. *Evaluate interphase character*: If 0.5 mM $CaCl_2$ and 1000/μL demembranated sperm nuclei are added to egg extracts, does the reaction (~24 μL egg extract + 0.25 μL of 200,000 sperm/μL stock + 0.25 μL of 50 mM $CaCl_2$ + 0.25 μL of 20 mg/mL rhodamine-labeled tubulin) generate large, round nuclei within 60 min at room temperate? Flick tube every 5–10 min to ensure that nuclei do not clump. Monitor progression at 45 and 60 min after calcium addition by imaging a mixture of 1 μL of extract reaction with 4 μL of spindle fix (also *see* **Note 12**).
3. *Evaluate formation of cycled spindles* from interphase nuclei + fresh CSF extract (50:50 mix). The ideal extract will form spindles at high efficiency within 40 min at 22 °C. A typical reaction is made from ~12.5 μL interphase extract containing 1000/μL fully formed sperm nuclei + 12.5 μL of CSF extract containing

0.4 mg/mL rhodamine-labeled tubulin and 0.4 μg/mL Hoechst. The 25 μL spindle assembly reaction has a final concentration of 500 nuclei/μL along with 0.2 mg/mL rhodamine-labeled tubulin and 0.2 μg/mL Hoechst. Flick tube every 5–10 min to ensure that spindles do not clump. After 40 min, mix 1 μL of extract reaction with 4 μL of spindle fix and image.

4. *Evaluate cycled spindle assembly inside hand-formed extract-in-oil emulsions*. Add 2 μL of freshly prepared cycled spindle reaction (from Subheading 3.3.2, **step 3**) to 100 μL of 50 mg/mL mixture of Cithrol DPHS in squalene in a 1.5 mL microfuge tube. Using a standard P200 tip and pipette set at 50 μL, pipette up and down 5–10 times, keeping tip pressed against the bottom of the tube. The shear forces will break the extract apart into droplets with a diameter of 20–200 μm. Transfer the droplets to a gasket imaging chamber using a pipette, and incubate for 40 min at 22 °C. Image structures formed in droplets using an inverted fluorescence microscope. This assay determines whether extract functionality is altered by the oil/surfactant mixture or the encapsulation process, and is the preferred test to determine which extract will perform at the highest level when encapsulated in microfluidic droplets.
5. *Evaluate embryo extracts*: The evaluations in the previous steps (Subheading 3.3.2, **steps 1–4**) are critical for optimizing embryo extract spindle assembly reactions. A typical embryo reaction is comprised of 85 % embryo cytoplasm, 10 % CSF-arrested egg cytoplasm, and 5 % cycled interphase nuclei (from egg extracts), along with rhodamine-tubulin and Hoechst to monitor microtubules and DNA. Choose the most mitotic egg extract for the 10 % addition (to promote mitotis), and choose the egg extract best able to make large round interphase nuclei as the source of DNA. Additionally, if time permits, test the ability of each extract to form spindles from interphase nuclei, prior to picking one embryo extract for encapsulation. The following is a typical embryo extract reaction: ~21 μL embryo extract + 1.25 μL of 6000 sperm/μL ("12×") freshly prepared nuclei in interphase egg extracts, 2.5 μL of CSF-arrested egg extract, and includes a final concentration of 0.2 mg/mL rhodamine-labeled tubulin and 0.2 μg/mL Hoechst. Monitor the reactions both in a test tube, and inside of droplet emulsions formed by hand (*see* Subheading 3.3.2, **step 4**).

Moving forward, for egg extract spindle assembly reactions, use a combination of the best CSF extract and best cycled nuclei, combining them at a 50:50 volume ratio. Generate interphase nuclei reactions at concentrations that are appropriate for the droplet sizes being generated (*see* Table 1). Additionally, it is possible to characterize the effect of the addition or depletion of factors from the extracts.

Table 1
List of common droplet diameter and volumes, and sperm concentrations necessary to generate droplets containing a single sperm nucleus

Droplet diameter (μm)	Droplet volume (L)	Ideal extract sperm conc. (number/μL)	Conventional sperm conc. (stock: 1× = 500/μL)	% Droplets loaded (assuming max of 10× sperm/reaction) (%)
1000	5.2E-07	2	0.004×	100
100	5.2E-10	1910	4×	100
80	2.7E-10	3730	7×	100
70	1.8E-10	5568	11×	91
60	1.1E-10	8842	18×	57
50	6.5E-11	15,279	31×	33
40	3.4E-11	29,842	60×	17
30	1.4E-11	70,736	141×	7
25	8.2E-12	122,231	244×	4

Spindle reactions containing the sperm concentrations highlighted in *gray* are not feasible without encapsulation. At very high sperm concentrations, spindles assembling in bulk extracts will fuse

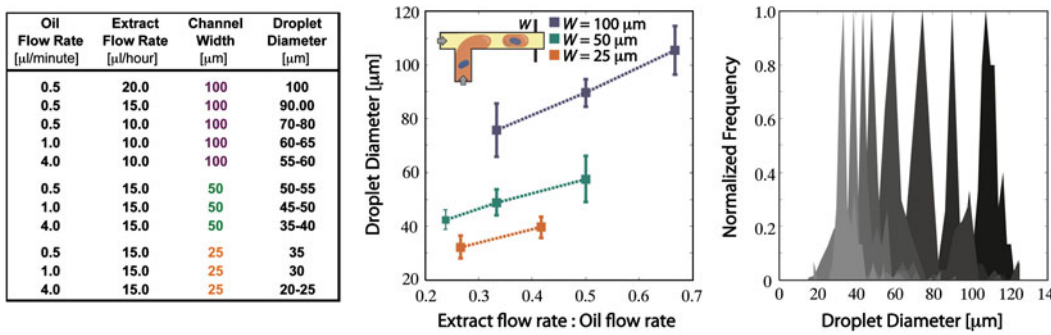

Fig. 3 Parameters for controlling droplet size in microfluidic devices. Table shows flow rates programmed for a 50 μL syringe containing a mix of oil and surfactant and 25 μL syringe filled with extracts, in devices with various channel widths. Values for generating droplets whose diameters range from 20 to 100 μm are shown. A plot of droplet diameter as a function of relative flow rate is also shown. Each peak in the histogram corresponds to a distribution of droplet sizes generated from a single experiment

3.3.3 Generate Extract-Filled Microfluidic Droplets of Specific Sizes

The channel widths and syringe flow rates described in Fig. 3 should be used as a guide to specify droplet size.

1. Switch on the syringe pump driving the oil syringe and set to rate of 0.5–1 μL/min. Insert the PTFE tubing attached to oil syringe into the punched hole over the oil inlet. Use forceps to pull the tubing near to the bottom (glass-side) of the PDMS device, as observed from the side. Place the droplet-generating

device onto the stage of stereomicroscope and watch for oil entering the channels. A reduction in contrast signals that the oil has filled the channels and the device is ready to be hooked up to the extract syringe tubing.

2. Generate spindle assembly reactions: Mix 10 μL interphase extract containing an appropriate number of sperm nuclei + 10 μL of CSF extract containing 0.2 mg/mL rhodamine-labeled tubulin and 0.2 μg/mL Hoechst in 1.5 mL microfuge tube on ice. Use immediately by transferring to the extract syringe.
3. After cleaning out the extract syringe using compressed air, fill the extract syringe by pulling back on the plunger to withdraw at least 12 μL of spindle assembly reaction through the attached PTFE tubing. Mount the syringe on the extract syringe pump. Push the plunger forward to remove any bubbles and to create a small bolus of extract. Set the appropriate flow rate (e.g., 10–15 μL/h). Use forceps to connect the tubing to the 0.5 mm punched hole over the extract inlet. Pull the tubing near to the bottom (glass-side) of the PDMS device.
4. Wait for 2 min for droplets to begin forming. Occasionally observe the device under a stereomicroscope until extract enters the T-junction. Take care not to overexpose the sample as this may increase temperature and reduce functionality.
5. As droplets begin to form, allow them to collect in the larger punched hole (collection chamber) for up to 5 min. As they accumulate, use a P20 pipette to transfer them to form a monolayer of droplets in a gasket imaging chamber.
6. Increase the oil syringe flow rate to produce slightly smaller droplets, using the same device and extract (*see* **Note 13**). Wait for 2 min, clearing out any new droplets which are likely incorrect in size. Wait for another 5 min and transfer new droplets to a second gasket imaging chamber. Allow these droplets to sit for 3 min before taking them out of cold room.
7. Repeat **steps 5–6**. Select different channel widths and flow rates to create a series of droplets that cover a range of diameters (e.g., 80/70, 60/50, 40/35, 25/20 μm). In the best-case scenario, the control and an experimental spindle assembly reaction can each be encapsulated in 8 sizes of droplets (16 total sets of droplets, each requiring separate imaging) (*see* **Notes 14** and **15**).

3.3.4 Imaging Spindle Assembly Inside of Droplets

See Fig. 4.

1. Remove droplet-filled gasket imaging chambers from the cold room and incubate at 22 °C for 40 min. Three minutes prior to the 40 min timepoint, place the imaging chamber on the stage of a spinning disk confocal microscope.
2. Identify focus using transmitted light and a 20× NA = 0.5 air objective. Set up the software to Acquire a multi-wavelength

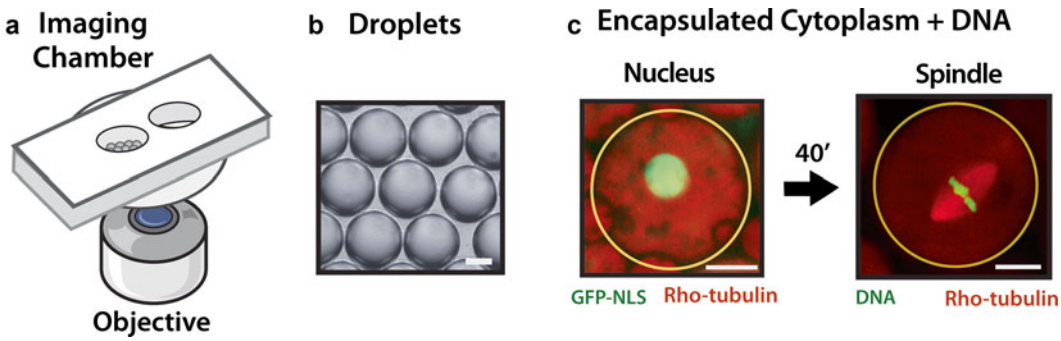

Fig. 4 Images of spindle and nucleus assembly inside of cell-size droplets. (**a**) Schematic of a monolayer of droplets being imaged inside of a gasket imaging chamber on an inverted microscope. (**b**) Representative image showing the homogeneity of 50 μm droplets generated using a T-junction microfluidic device. (**c**) Encapsulated spindle assembly reaction (a 50:50 mix of interphase nuclei : CSF-arrested cytoplasm), at time = 0. Nuclei are visualized using a GFP construct fused to a nuclear localization signal. (**d**) Encapsulated spindle assembly reaction in (**c**), after incubation at 22 °C for 40 min. Microtubules are visualized by the presence of 0.2 mg/mL rhodamine-labeled tubulin, and DNA is stained with 0.2 μg/mL Hoechst. Droplet boundaries are indicated in *yellow*. Scale bar 20 μm

(405 and 561 nm excitation) set of images, for an 8 × 8 area in XY (total of ~2 × 2 mm region of interest), across multiple slices in Z (*see* **Note 16**). Acquisition time is typically 5–7 min, depending on exposure times.

3. Repeat **steps 1–2** for each new set of droplets. If new droplets are generated every 10 min, there should be no delays in the imaging pipeline. Spindles and nuclei should form with similar efficiency inside of droplets and in unencapsulated bulk extracts (*see* **Note 17**). If spindles are not assembling properly or form with low efficiency, *see* **Note 18**.

4 Notes

1. When generating *Xenopus* cytoplasmic extracts, the most critical parameter is egg quality. Egg yield and quality are largely dependent on the age and health of the frog. It is important to allow females to rest for at least 4–6 months between ovulations and to remove frogs which repeatedly produce poor quality (lysed, stringy, or too few) eggs. Additionally, younger frogs tend to have the best quality eggs.
2. When handling frogs, eggs and embryos, be sure to use the appropriate source of water. Frogs should only be kept in deionized water or solutions made from deionized water. Industrial tap water often contains contaminants that are harmful to aquatic species. All buffers used for making extracts

should be formulated using Milli-Q H_2O, and the buffers, dejelly, XB, CSF-XB, and CSF-XB+, should always be made fresh, the day of extract preparation.

3. When working with extracts it is important to work quickly, but also carefully, to ensure that manipulations do not physically damage the eggs. For example, the smaller the window between dejellying and crushing the eggs with centrifugation, the better the extract quality. However, rapid pipetting of eggs (e.g., from beaker to SW55 tubes) will cause them to shear and lyse and can reduce overall extract activity.
4. To prevent cytoplasm from warming up when withdrawing it using a needle, only hold the SW55 tube at the very top and keep the bottom of the tube in contact with ice. It is also helpful to prechill the 1 mL syringe. In general, it is important to work quickly and keep the cytoplasm cool.
5. If fertilization efficiency is low, consider freshly preparing another clutch of eggs, dissecting a fresh set of testes and possibly using a larger portion of the testes for fertilization.
6. Prechill a box of P200 tips and make sure not to handle the 2 mL tube for an extended period of time, which will warm up the extracts.
7. Embryo extract spindle assembly reactions are much more efficient at forming spindle upon addition of 5–10 % freshly prepared CSF egg extracts. Also, the best way to coordinate timing is to prepare the egg extracts about an hour prior to generating embryo extracts (while embryos are developing).
8. To create plastic replicas for generating PDMS microfluidic devices, the following steps were performed: (I) An SU-8 mold was generated using standard soft-lithography techniques [18]. First, a 25 μm thick film of SU-8 2025 was deposited on a silicon substrate, soft-baked, and UV exposed through a custom chrome mask using a contact aligner. After a second bake, the wafers were developed in SU-8 developer and silanized under vacuum. (II) 1–2 mm of PDMS was cast on the developed and silanized wafer (from step I), degassed, and cured at 65 °C overnight. (III) A 50:50 mix of Smooth-cast 310 plastic (total 15 mL) was cast onto the PDMS and air-cured to generate the final "plastic replica" mold. From this plastic replica, PDMS microfluidic devices can be generated in any research lab (*see* Fig. 2). Because of its size, each 6 cm plastic replica contains the features of ~20–24 individual T-junction droplet-generating devices.
9. There are a number of commercial options for prototyping microfluidic devices from PDMS. Companies including Flowjem(http://www.flowjem.com/) and uFluidics (http://ufluidix.com/) will fabricate devices starting from a user-

submitted mask created in Autocad. Additionally, it is worth investigating whether a microfluidic foundry is available on your campus. Many universities have invested in microfabrication facilities for soft-lithography, and some offer custom fee-based fabrications services.

10. For those without convenient access to a cold room, or in cases in which it is preferable to keep extracts at room temperature (e.g., *Xenopus tropicalis* egg extracts), microfluidic droplets can be generated at temperatures above 4 °C. However, in these cases the process becomes time-sensitive—microtubules begin to polymerize before and during encapsulation—and therefore the experimenter must closely monitor and account for the time elapsed.
11. Residual Aquapel will inhibit spindle assembly. Make sure that it is fully removed by drying in a 50 °C oven overnight.
12. Strongly mitotic extracts are useful because they can be used to make CSF spindles in droplets or to add as a supplement to embryo extracts to improve spindle assembly efficiency. However, strongly mitotic extracts are less useful if the goal is to generate interphase nuclei from sperm DNA. Weakly mitotic or interphasic extracts are characterized by the transition into interphase even in the absence of calcium addition. Strongly interphasic extracts are useful for generating cycled nuclei although they may make it difficult to generate cycled spindles from the mixture of 50 % interphase extract + 50 % CSF-arrested extracts.
13. By staggering the removal of consecutive sets of droplets formed from the same PDMS device, a 10 min interval is created which is necessary to perform live imaging exactly 40 min after initiation of spindle assembly (via temperature shift from cold room to 22 °C).
14. Experiments in Subheading 3.3.3 are easiest to perform with two people. Ideally one person manages the droplet-generators in the cold room, and one person assembles the spindle reactions and runs the microscope. Once the best extract is selected (Subheading 3.3.2), it takes approximately 2.5–3 h to generate and image a full range of droplets sizes.
15. The major benefits of making uniformly sized microfluidic droplets rather than hand emulsions are: (1) spindles inside homogenously sized droplets are sitting in roughly the same imaging plane, making it much easier to acquire images of spindles across a large region of droplets in XY, and (2) better control over exact number droplets generated for a particular size. Although it is easy to make droplets by hand, it is difficult to generate a specified number of droplets of a given size (e.g., 1000 each for 70, 60, 50 μm droplets).

16. Because the acrylic used in making the imaging chambers is not perfectly flat, there can be 10–20 μm of tilt across the diameter of the droplet chamber. This will alter the focus from one side of the well to the other, so choose an "average" *z*-plane by finding the mean of the ideal focus in Z for spindles in frames in the upper left and lower right of the region of interest. This will ensure that the maximum number of droplets are in focus during the XY scan.
17. Extracts of the highest quality exhibit the following features: An ideal CSF extract will generate sharp-looking spindles around exogenously added sperm DNA at high efficiency, within 45 min at room temperature. Spindles should form in the absence half-spindles, or asters and spindle microtubules should look sharp, not "hairy." An ideal interphase nuclei preparation forms large, round nuclei at high efficiency within 60 min hour after addition of 0.5 mM $CaCl_2$ and sperm DNA. The best cycled spindle reactions (from 50:50 mix of interphase extracts + CSF-arrested extracts) generate bipolar spindles at extremely high efficiency. In all cases, the spindles should form easily both in bulk reactions in a test tube and within hand emulsions or 70–80 μm diameter microfluidic droplets.
18. When extracts are not performing optimally, the following issues will arise: (1) CSF extract: forms asters and nuclei rather than spindles. The extract may be interphasic and unable to hold mitotic arrest. (2) Cycled interphase extract: does not form large swollen, round nuclei within 45–60 min, but instead forms nonspherical, small nuclei. The extract may be too mitotic. (3) Encapsulated reactions: the ability to form spindles in a test tube but not inside 70–80 μm diameter droplets signals an issue with the oil/surfactant mixture. If spindles are able to form in hand emulsions but not microfluidic droplets, PDMS devices (and residual solvent) or tainted PTFE tubing are likely to blame. In this case, you often observe nuclei and asters inside microfluidic droplets (as if extracts and nuclei were stuck in interphase).

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