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Xenopus extract approaches to studying microtubule organization and signaling in cytokinesis

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Abstract

We report optimized methods for preparing actin-intact *Xenopus* egg extract. This extract is minimally perturbed, undiluted egg cytoplasm where the cell cycle can be experimentally controlled. It contains abundant organelles and glycogen, and supports active metabolism and cytoskeletal dynamics that closely mimic egg physiology. The concentration of the most abundant ~11,000 proteins is known from mass spectrometry. Actin-intact egg extract can be used for analysis of actin dynamics and interaction of actin with other cytoplasmic systems, as well as microtubule organization. It can be spread as thin layers, and naturally depletes oxygen through mitochondrial metabolism, which makes it ideal for fluorescence imaging. When combined with artificial lipid bilayers, it allows reconstitution and analysis of the spatially controlled signaling that positions the cleavage furrow during early cytokinesis. Actin-intact extract is generally useful for probing the biochemistry and biophysics of the large *Xenopus* egg. Protocols are provided for preparation of actin-intact egg extract, control of the cell cycle, fluorescent probes for cytoskeleton and cytoskeleton-dependent signaling, preparation of glass surfaces for imaging experiments, and immunodepletion to probe the role of specific proteins and protein complexes. We also describe methods for adding supported lipid bilayers to mimic the plasma membrane and for confining in microfluidic droplets to explore size scaling issues.

Keywords

Cytokinesis; Cell-free system; *Xenopus* egg extract; Microtubule; Actin; Cell cycle; *Xenopus*; Microfluidics

1. Introduction

Cell-free systems provide a experimental bridge between living cells and pure protein systems and allow chemical and physical manipulations that are not possible in living cells. *Xenopus* egg extracts are unique among cell-free systems in providing essentially undiluted cytoplasm with intact metabolism, organelles, and experimental control of cell cycle state. Quantitative proteomics studies in these extracts have established the identity and approximate concentration of the most abundant ~11,000 proteins in *Xenopus* egg cytoplasm (Wühr et al., 2014), which has been extremely useful in designing extract

experiments. When air is excluded, e.g., when extract is sandwiched between two glass surfaces, mitochondria in the extract remove oxygen down to very low levels within minutes, but ATP production continues by anaerobic metabolism (Niethammer, Kueh, & Mitchison, 2008). This greatly reduces photobleaching and phototoxicity of synthetic fluorophores such as Alexa dyes, making the system ideal for live fluorescence imaging. *Xenopus* eggs are naturally adapted for development in low oxygen environments, and this may be an important reason that the extract prepared from them can recapitulate normal metabolism under conditions of limited gas exchange.

Actin-intact extract is a variant of classic CSF extract, which was developed by Murray for dissecting cell cycle regulation (Murray, 1991). CSF extract recapitulates the cell cycle state of the unfertilized egg, which is arrested in metaphase of meiosis II. CSF extract is normally prepared with cytochalasin D to block actin polymerization and to prevent dramatic gelation-contraction that occurs when actin-intact CSF extract is warmed to room temperature (Field et al., 2011). To prepare actin-intact CSF extract we leave out the cytochalasin, and modify the conditions in the crushing spin to facilitate separation of cytoplasm from yolk and fat. CSF extract, whether actin-intact or cytochalasin treated, has high Cdk1 activity, reflecting the M-phase cell cycle state of the unfertilized egg. It can be easily converted to interphase (I-phase) by addition of 0.4 mM calcium to mimic fertilization (Murray, 1991). The calcium concentration rapidly returns to baseline by pumping into the endoplasmic reticulum (ER). The resulting I-phase extract is stable on ice for at least 2 hrs. Reentry into M-phase can be permanently blocked by addition of protein synthesis inhibitor to block Cyclin B synthesis. Importantly, I-phase actin-intact extract does not undergo bulk gelation-contraction, so microtubule and actin dynamics can be imaged without this complication.

Until recently, we lacked the ability to reconstitute two important aspects of egg biology in extract: actin dynamics and plasma membrane biology. Recent progress has filled these gaps. Here, we report methods for preparing extract that leaves actin dynamics intact, but still allows clean separation of cytoplasm from yolk and fat in the important crushing and stratifying spin. We also report addition of supported lipid bilayers (SLBs) to model the plasma membrane. Using these tools we recently reconstituted the spatiotemporal regulation of early cytokinesis in egg extracts, and probed the role of the chromosomal passenger complex (CPC) and several cytokinesis-associated kinesins in the signaling events contributing to this regulation (Nguyen et al., 2014).

2. Actin-intact extract preparation

2.1 Requirements

HORMONE STOCKS

- Pregnant mare serum gonadotropin (PMSG)
 - Calbiochem 367222
 - 1000 units/mL, dissolved in water
 - Store at -20°C , do not freeze/thaw

- Human Chorionic Gonadotropin (HCG)
 - Prospect HOR-250
 - 1000 units/mL, dissolved in 10 mM NaPO₄ pH 7.2
 - Store at 4°C

BUFFERS AND REAGENTS

- 25x MMR
 - 50 mM Na-HEPES, pH to 7.8 with NaOH
 - 1 mM EDTA
 - 1 M NaCl
 - 20 mM KCl
 - 10 mM MgCl₂
 - 20 mM CaCl₂
 - Autoclave, store at room temperature (RT)
- 20x XB Salts
 - 2 M KCl
 - 20 mM MgCl₂
 - 2 mM CaCl₂
 - Sterile filter, store at RT
- 2 M Sucrose
 - Sterile filter, store at 4° C
- 1 M K-HEPES
 - pH to 7.7 with KOH
 - Sterile filter, store at 4 °C
- 0.5 M K-EGTA
 - pH to 7.7 with KOH
 - Sterile filter, store at RT
- 1 M MgCl₂
 - Sterile filter, store at RT
- Protease inhibitors (PIs, aka LPC)
 - 10 mg/mL leupeptin (Sigma L2884)
 - 10 mg/mL pepstatin A (Sigma P5318)

- 10 mg/mL chymostatin (Sigma C7268)
- Dissolve in DMSO; store in 100 μ L aliquots at -20° C
- Cysteine, free base (Sigma C7352)
- 100x energy mix, to supplement extract before freezing
 - 900 mM creatine phosphate (Sigma 10621614001)
 - 100 mM ATP (Sigma A3377)
 - 100 mM MgCl_2
 - Store in 100 μ L aliquots at -20° C

EQUIPMENT

- Glass crystallizing dishes, for washing eggs (VWR 89000-294)
 - 150 mm diameter x 75 mm height or equivalent
- 13 x 51 mm UltraClear tubes (Beckman 344057)
- Clinical centrifuge, to pack eggs
 - Low speed, with a swinging bucket rotor
 - Use at $16-18^{\circ}$ C
- Vacuum
- Centrifuge and swinging bucket rotor that can go $15,000 \times g$
 - Use at 4° C
- Wide bore Pasteur pipets
 - Option 1: Polypropylene transfer pipets. Cut off tips
 - Option 2: Glass Pasteur pipets. Break tips and fire polish
 - Pipettes cut to two different diameters are needed, gardening size: 22 mm inner diameter, and transfer size: 3.5 mm inner diameter. note: A *Xenopus* egg is 1.2 mm in diameter.

2.2 Extract preparation

This protocol is for two to three frogs, the number someone familiar with the preparation can manage in parallel. Some steps (e.g., dejellying, Section 2.2.4) are time sensitive and require constant attention. Therefore, to increase the scale, one can (1) recruit others to help with time-sensitive steps, or (2) pool eggs from multiple frogs. The latter is not ideal, because different batches of eggs have different properties.

2.2.1 Several days before—At least 2 days before, prime frogs with PMSG hormone to induce maturation of oocytes. Inject 100 U (100 μ L) per frog into the dorsal lymph sac using a 27 gauge needle. Store primed frogs in dechlorinated water with 2 g/L rock salt (aka frog water) in a cool room (ambient temperature of $16-18^{\circ}$ C). We wait at least two days after

priming before inducing them to lay eggs. The priming lasts for up to two weeks. Do not feed frogs for 2–3 days before injecting them to induce ovulation (Section 2.2.2). Frogs fed recently can defecate and foul the laid eggs.

2.2.2 Day before—16–18 hours before extract preparation, two to three primed frogs are induced to ovulate by injection with 500 U (0.5 mL) of HCG. After injection, frogs are placed in separate containers with 1 L of 1x MMR for egg laying. Containers are placed in a dark and quiet 16 °C incubator overnight. Eggs sitting in MMR longer than 18 hours produce inferior extract. Egg quality must take precedence over egg quantity. A good frog usually produces eggs that yield about 0.8 mL of extract. It is best not to use any batches of eggs containing >10% of lysed eggs, activated¹ eggs, or “puffballs” (swollen eggs that are often white and puffy). Strings of eggs that do not have fully separated jelly coats are also inferior. In general, we keep each frog separate and prepare separate extracts from the eggs of each frog.

2.2.3 Hours before—BUFFERS: All buffers should be at 16–18 °C. Prepare just before use unless indicated otherwise; rinse all glassware with double distilled water prior to use.

- 1.5 L 1x MMR (can make in advance and store at 16 °C)
- 600 mL dejellying solution
 - 30 mL 20x XB salts
 - 2.61 mL 10 N KOH, gives pH 7.8
 - 12 g cysteine powder, gives 2% (w/v) cysteine
 - Add cysteine within an hour before and make sure fully dissolved
- 1.5 L CSF-XB
 - 75 mL 20x XB salts, to 100 mM KCl, 1 mM MgCl₂, 0.1 mM CaCl₂
 - 37.5 mL 2 M sucrose, to 50 mM
 - 15 mL 1 M K-HEPES, to 10 mM
 - 165 µL 10 N KOH, to maintain pH 7.7
 - 15 mL 0.5 M K-EGTA², to 5 mM
 - 1.5 mL 1 M MgCl₂, to (additional) 1 mM
- 100 mL CSF-XB + protease inhibitors (PIs)
 - 100 mL CSF-XB (from the 1.5 L above)
 - 100 µL LPC (1 aliquot) to 10 µg/mL, mix immediately

¹Activated eggs have lost their metaphase arrest. When this occurs the black pigment at the animal pole will contract. If significant numbers of activated eggs are included in the preparation, the M-phase arrest of the extract will be lost.

²Unfertilized *Xenopus* eggs are arrested at metaphase of meiosis II by a biochemical activity termed cytostatic factor (CSF), partly composed of the APC/C inhibitor, XErp1/Emi2. Upon fertilization, sperm entry induces a calcium rise, which results in degradation of XErp1/Emi2 and release of M-phase arrest (Rauh et al., 2005). CSF-XB buffer contains the calcium chelator EGTA and thus metaphase arrest is maintained during extract preparation.

MATERIALS

- 6 (2 per frog) UltraClear tubes (13 x 51 mm)
 - Add 1 mL CSF-XB plus PIs to each tube.
- 3 (1 per frog) glass crystallizing dishes (aka egg dishes)
 - Rinse well with ddH₂O then MMR
- Wide bore Pasteur pipette, both sizes (Section 2.1)

2.2.4 Extract preparation—Perform all steps at 16–18 °C unless noted otherwise. Higher temperatures are a common cause of bad extracts. Eggs are fragile, so handle them gently and always keep them under buffer, especially after jelly coat removal. The quality of dejellied eggs deteriorates over time, so work efficiently. The procedure from washing the eggs in MMR to crushing the eggs should take less than 1 h.

1. Garden eggs, if necessary. Use a wide bore Pasteur pipette to delicately remove sloughed skin, lysed eggs, activated eggs, and puffballs (Section 2.2.2). Gardening may continue throughout but should not delay the preparation.
2. Transfer eggs in MMR to an egg dish.
3. Wash eggs three times in ~150 mL fresh MMR until all debris removed. Pour buffer gently and not directly onto the eggs. Find a compromise between delicate handling and thorough mixing.
4. Dejelly eggs.
 - 4.1 Remove as much MMR as possible. Decant larger volumes of buffer, or use a transfer pipette to aspirate smaller volumes of buffer. When the volume of buffer is small, decanting the buffer may stretch the eggs and damage them.
 - 4.2 Add ~100 mL dejellying solution. Intermittently, swirl eggs gently in the dejellying solution. After 3 min, pour off most of the dejelly solution and add 100 mL more dejelly solution. As jelly dissociates from the egg, the jelly appears as transparent shells or schlieren streaks in the buffer.
 - 4.3 After eggs are dejellied (5–7 min total in dejelly solution), they will pack more tightly and orient with their animal poles upward. The volume of settled eggs will decrease ~5 fold.
5. Remove dejelly solution.
 - 5.1 Remove as much dejellying solution as possible (see above).
 - 5.2 Rinse eggs thoroughly, three to four times in ~150 mL fresh CSF-XB. To minimize residual dejelly solution, remove as much buffer as possible each time. Dejellied eggs are quite fragile. Tilt the egg dish to keep the eggs under buffer. Eggs exposed to air will lyse. Pour solutions down the side of the wash vessel or aspirate with transfer

10. Store M-phase extract on ice for 4–8 hours without loss of activity.
 - 10.1 Monitor the gelation-contraction activity of the extract³ (Section 2.3.2).
 - 10.2 For long term storage, see Section 2.4.4.

2.3 Cell cycle control and monitoring

Microtubules, actin, and keratin polymers, as well as endomembrane systems and the cortex, are highly regulated by the cell cycle. When working with *Xenopus* egg extract, it is important to control and monitor its cell cycle state.

2.3.1 Converting M-phase to I-phase—Sperm entry upon fertilization induces a transient rise in cytosolic calcium, triggering degradation of CSF and release of M-phase arrest. We exploit this natural response to calcium to prepare I-phase extracts. CaCl₂ from a 50x or 100x stock is added to M-phase extract to a final concentration of 0.4 mM. The ER rapidly uptakes calcium, decreasing the cytosolic concentration, so immediate and thorough mixing is crucial to release CSF arrest. We use 1.5 mL Eppendorf tubes with 25–100 µL extract per tube. Larger volumes may cause problems with mixing and gas exchange. After calcium is added, gently pipet the entire solution up and down three to five times to ensure mixing, or flick the tube about eight times. Do not vortex the extract. Incubate at 20 °C for 10 min, flicking occasionally. Then place on ice for at least 2 min. To prevent M-phase reentry, protein synthesis inhibitors (cycloheximide 100 µg/mL or puromycin 50 µM) can be added to block synthesis of cyclin B and cell cycle progression (Field, Nguyen, Ishihara, Groen, & Mitchison, 2014).

Two alternative methods for making I-phase extract have previously been described and are reviewed in (Field, Nguyen, Ishihara, Groen, & Mitchison, 2014). Both involve releasing the M-phase arrest just before or during the egg crushing spin. Even on ice, interphase extract changes over time due to cell cycle progression. Therefore, we prefer to maintain M-phase arrest during extract preparation and storage by use of EGTA in CSF-XB buffer.

2.3.2 Monitoring cell cycle state—The classic test for cell cycle state in egg extract was direct measurement of Cdk1 kinase activity using ³²P-ATP (Gautier et al., 1990). This may still be the best method, but radioactive assays are no longer routine in most laboratories. We use several methods to monitor cell cycle state:

Gelation-contraction: Actin-intact M-phase extract undergoes bulk gelation-contraction within a few minutes of warming to RT, while I-phase extract does not. This assay is fast and easy, and also tests the integrity of the actin cytoskeleton. We apply it to every extract preparation before starting experiments. After several hours storage on ice, we often repeat to check if the extract is still good. Fill a small Petri dish with mineral oil. Pipette several 1 µL droplets of extract under the mineral oil and monitor with a dissecting microscope or just by eye. In a good actin-intact M-phase extract, light scattering particulates will contract

³Inferior extract does not perform well in the gelation contraction assay (Section 2.3.2) and loses cell cycle arrest earlier. Inferior extract can be partially “rescued” by filtering it through a 0.9–1.2 µm syringe filter (at a loss of approximately 50% of the volume). Filtered extract maintains its cell cycle state.

towards the center of the droplet within 2–3 min, and form a tight ball in the center by ~10 min. I-phase extract, made by adding calcium to CSF extract and incubating at RT for 2–3 min, still forms an actin gel, but it does not contract, or contracts very slowly (Field et al., 2011). We often use this assay as a spot check for conversion to interphase.

Microtubule aster morphology: Add fluorescent tubulin probe (see Section 3.1.2) to a 20–30 μ L aliquot of extract, then bring the extract to 7.5% DMSO to induce microtubule polymerization. Incubate at 20 °C for 20 min and image. In M-phase, microtubules assemble into asters under the influence of dynein and NUMA (Verde, Berrez, Antony, & Karsenti, 1991). In I-phase they form long, interconnected bundles.

Chromatin morphology: Add demembranated *Xenopus* sperm nuclei (see Section 4.2) prelabeled with Hoechst at 1 μ g/mL (Sigma B2261) to extract at a final concentration of ~100 nuclei/ μ L. Remove a few microliters of extract over time and image. In M-phase, chromatin is condensed and slowly resolves into mitotic chromosomes. In I-phase, it gradually decondenses, acquires a nuclear envelope, and forms spherical nuclei (Murray, 1991).

Phosphoprotein analysis via Western blot: The mitotic phosphoprotein monoclonal antibody (MPM2; Millipore 05-368) recognizes many phosphorylated Cdk1 substrates. Cdc27 protein, recognized by antibody AF 3.1 (Upstate 05-906) undergoes a large mobility shift by SDS page due to loss of M-phase phosphorylation (Rankin & Kirschner, 1997; Field et al., 2011).

2.4. General considerations

2.4.1 Temperature—Prepare extract in a cool room at 16–18 °C. If the eggs or extract warm, actin may polymerize and sediment out upon centrifugation. Move packed eggs on ice to the centrifuge used for the crushing spin, and store harvested extract on ice. It is important to realize that, even on ice, biochemical reactions continue; for example, kinases still phosphorylate substrates, albeit more slowly than at 20 °C. For imaging experiments, a temperature of 18–20 °C is optimal, though a wider range is tolerated.

2.4.2. Dilution—It is important to keep the extract concentrated. The total volume of all aqueous additions should be <10%. Dilution by a larger factor can release the cell cycle arrest and damage cytoskeleton assembly reactions. The buffer used for dilution is important. High salt concentrations can damage the extract. Water, or dilute buffer, are preferred. The total volume of DMSO added must be <0.5% if microtubule dynamics are important. Higher concentrations of DMSO induce microtubule polymerization.

2.4.3. Addition of small molecules/drugs—We find that the EC₅₀ for drug action is often 10- to 1000-fold higher in extract than in tissue culture cells, although mechanism and specificity are maintained. We believe there are two reasons: (1) tissue culture cells are surrounded by a large reservoir of drug in the medium, and can accumulate drugs to a higher internal concentration and (2) hydrophobic drugs partition into lipid reservoirs and/or acidic

compartments present in extract. For these reasons, EC₅₀ for drug action in extract must be determined empirically, and we aim to use them at ~5x the EC₅₀ to maximize specificity.

If drugs are added from a DMSO stock, it is important to keep the total percent DMSO at <0.5% (see Section 2.4.2). Drugs stocks are often prepared as concentrated stocks in DMSO, e.g. 1000x. These should be diluted in small volumes of egg extract to give 10–50x final stocks. Dilution in aqueous buffer can cause hydrophobic drugs to precipitate, in which case the final concentration is unreliable.

2.4.4. Storage—It is optimal to work with freshly made extract. This can be stored on ice for up to ~8 h, with best results obtained in the first ~4 h. M-phase extract can be flash-frozen in liquid nitrogen in PCR tubes and stored at –80 °C for up to 1 month. If the extract is to be frozen, add sucrose (50 mM final concentration) and energy mix (Section 2.1) prior to freezing. Sucrose protects the organelles, and energy mix may compensate for mitochondrial damage. Upon thawing, frozen extract behaves similarly to fresh extract in many assays, e.g., gelation-contraction, but is never quite as good as fresh for reconstituting complex microtubule arrays. When working with I-phase extract, it is better to convert M-phase extract to I-phase before freezing (Section 2.3.1).

3. Fluorescent probes

3.1 Fusion proteins and fluorescently-tagged proteins

Proteins can be expressed as fusions with fluorescent proteins or expressed unlabeled and tagged with reactive dyes. All probes have the potential to perturb and should be added at the minimum concentrations needed for imaging. Reactions with and without a given probe should be carefully compared to ensure minimal perturbation. We dilute probes as 10x to 100x stocks in actin-intact extract, and often freeze in liquid nitrogen in small aliquots. Adding 10% (v/v) of frozen extract to fresh has no discernable negative effect. We focus on a few of our key protein probes in later sections.

3.1.1 F-actin—We have mainly used Utrophin-GFP (Burkel et al., 2007, Field et al., 2011) and Lifeact-GFP (Riedl et al., 2008; Field et al., 2011; Nguyen et al., 2014). Directly labeled G-actin gives very low contrast images, presumably due to the large monomer pool (Rosenblatt, Agnew, Abe, Bamberg, & Mitchison, 1997). Labeled phalloidin gives high contrast images and is convenient, but it perturbs actin dynamics. Care is required when using Utrophin-GFP. It can be perturbing, as it tends to induce bundles in a concentration-dependent manner. We prefer Lifeact-GFP, expressed in bacteria as a GFP fusion with a 6xHis tag for purification. Use at a final concentration of 100–200 nM.

3.1.2 Microtubules—We standardly use labeled bovine brain tubulin as a probe, adding it at approximately 200–250 nM final concentration. We prepare it as described (Hyman et al., 1991) using the NHS esters of Alexa 488, 568, and 647 (Life Technologies). See Figure 2A. High quality labeled tubulin can also be purchased from Cytoskeleton Inc. Labeled *Xenopus* egg tubulin probes (Groen & Mitchison, 2016) improve signal/noise for imaging, presumably because 20 °C is too cold for optimal polymerization of a mammalian tubulin

probe. These probes are superior when the quality of the microtubule image is important, or for quantification of polymer levels.

3.1.3 EB1—We use human EB1-GFP in egg extract to quantify the direction, rate, and persistence of microtubule polymerization (Figure 2A). Human EB1-GFP tagged with 6xHis construct was obtained from Kevin Slep (UNC Chapel Hill), expressed in *E. coli*, and is typically added at 40 to 60 nM. Endogenous EB1 is ~1.6 μ M (Wühr et al., 2014). *Xenopus* EB1-GFP provided inferior images, perhaps due to less efficient partitioning onto microtubules. We used the plusTipTracker MATLAB software module (Applegate et al., 2011) to perform automated detection and frame-to-frame linking of EB1 to obtain growth tracks (Figure 2B). (Nguyen et al., 2014; Ishihara, Nguyen, Groen, Field, & Mitchison, 2014)

3.1.4 RhoA-GTP reporter—RhoA-GTP at the inner surface of the plasma membrane is a central positive regulator of actin and myosin II during cytokinesis (Piekny, Werner, & Glotzer, 2005). To report on its localization during cytokinesis signaling we adapted a reporter based on the RhoA-binding domain of Rhotekin that was developed for imaging cleavage furrow induction in *Xenopus* embryos (Bement, Benink, & von Dassow, 2005). The rGBD of mouse Rhotekin fused to mCherry (mCherry-rGBD) tagged with 6xHis was expressed in *E. coli* and added to extract at a final concentration of 140 nM. This probe accumulates on supported lipid bilayers (SLBs) at the interface between I-phase microtubule asters (Nguyen et al., 2014). RhoA-GTP localization was dependent on activity of the AurkB subunit of the CPC, but was not dependent on the presence of Kif23, the kinesin subunit of Centralspindlin. This unexpected finding suggests the existence of a signaling pathway from the CPC to RhoA that does not depend on Centralspindlin (Nguyen et al., 2014).

3.2 Antibodies

We often use directly labeled antibody probes. They must be used with caution since antibody binding can activate or inactivate protein targets. With this caveat they provide a quick readout of endogenous protein localization. We typically affinity purify IgG from rabbit serum by binding to antigen and eluting with low pH. Many of our antibodies are generated by immunizing with ~20 amino acid peptides from the C-terminus of the protein (Field, Oegema, Zheng, Mitchison, & Walczak, 1998). We currently use two micro-scale methods for labeling IgG:

3.2.1. IgG labeling with anti-IgG Fab fragments—Rabbit IgG is mixed with Fab fragments of goat anti-rabbit IgG labeled with a fluorophore, such as DyeLight 647 (Jackson ImmunoResearch 111-607-003). Jackson sells Fab fragments coupled to a wide range of other fluorophores. We typically mix equal mass of IgG and Fab, corresponding to a molar excess of Fab. After 30 min at RT, the mixture is diluted into extract for imaging. Typically, we aim for a final concentration of labeled IgG of ~1 μ M. The IgG–Fab mixture can be stored for at least a month at 4 °C.

3.2.2. On-resin IgG labeling—This method is very useful for direct labeling of small amounts of antibody and has given reliable results for many antibodies and several fluorophores. The labeling works well using Protein G UltraLink resin (Thermo Scientific 53125) for rabbit and mouse IgG and Affi-Prep Protein A resin (Biorad 156-0006) for rabbit IgG.

1. To hold the resin, a 200 μ L polypropylene pipette tip is sealed at the pointed end by brief passage through the flame of a Bunsen burner. To allow liquid egress while retaining resin, a 1 to 2 mm slit is made parallel to the long axis of the sealed tip by cutting with a thin razor blade. All procedures are performed at room temp (20–25 $^{\circ}$ C).
2. 40 μ L of 50% resin slurry is loaded into the tip and washed 3x with 100 μ L of 0.15 M NaCl, 10 mM K-HEPES (pH 7.7) to give a packed resin bed of $\sim$ 20 μ L. Use a pipetman to force buffer through the packed resin bed, here and in all subsequent steps.
3. Purified antibody against a protein of interest, typically an affinity-purified rabbit IgG or a commercial monoclonal antibody, is passed through the resin bed three to five times sequentially to bind. For 20 μ L of packed resin we typically label 20–100 μ g IgG per tip. Alternatively, IgG can be incubated with resin in a tube for $\sim$ 1 h with gentle rotation, then packed and washed.
4. The resin is washed three times with 100 μ L of 200 mM K-HEPES pH 7.7 to remove unbound contaminants.
5. The resin-bound IgG is then labeled with an amine-reactive fluorescent dye. We always use Alexa Fluor NHS esters (Life Technologies), but similar dyes and reactive esters should work. Dye is dissolved in DMSO at $\sim$ 50 mM and stored as frozen aliquots at -20° C. 0.5 μ L of dye stock is diluted into 25 μ L of 200 mM K-HEPES pH 7.7 and immediately loaded onto the resin bed. Make sure the resin is saturated with dye (i.e. a little comes out the bottom) and leave a small amount of dye solution on top of the resin bed so that it does not dry out. NHS esters hydrolyze with a half-life of a few minutes in aqueous solution above pH 7, so it is important to load the diluted dye onto the resin rapidly after dilution. We use the same amount of NHS ester independent of the amount of labeled IgG on the resin. It is a large molar excess over the IgG, but most reacts with the resin or gets hydrolyzed. The tip is then incubated at room temperature for 10–60 min.
6. A second aliquot of dye is diluted, added to the resin bed, and incubated in the same manner.
7. After the second labeling reaction, the bed is washed five times with 0.15 M NaCl, 10 mM K-HEPES pH 7.7 to remove unbound dye. The wash buffer should be free of color by the third wash.
8. Labeled IgG is then eluted with five times 20 μ L aliquots of 200 mM acetic acid. Each aliquot of eluate is immediately mixed with 5 μ L of 1 M Tris-Cl pH 9 to neutralize and cooled to 4 $^{\circ}$ C. The colored fractions are pooled. We store

antibody in the resulting Tris-acetate buffer for months at 4 °C. If necessary, antibody can be exchanged into other buffers by two to three rounds of dilution and reconcentration using a 10 kDa centrifugal concentration device (Sigma Z677108), or by desalting using a small G25 column. For longer term storage of labeled IgGs we add glycerol to 50% (v/v) and store at –20 °C.

4. Microtubule organizing centers (MTOCs) and MT nucleation

To nucleate asters we have used four different microtubule organizing center (MTOC) types: anti-AurkA IgG on beads, centrosomes attached to sperm nuclei, isolated centrosomes, and *Tetrahymena* pellicles. During aster growth in I-phase extract, microtubules from adjacent asters do not interpenetrate due to mutual inhibition of microtubule growth. Instead, they form aster-aster interaction zones (AAIZs) where Aurora B kinase (AurkB) localizes. Aster-aster interactions are important for cytokinesis signaling (Nguyen et al., 2014). Due to AAIZ formation, higher MTOC densities result in smaller asters. In practice, we dilute MTOCs in extract so the final concentration is 1–20 per 20x imaging field. To some extent, adding more nucleating sites mimics decreasing blastomere size during embryo development (Field, Groen, Nguyen, & Mitchison, 2015).

4.1 Anti-AurkA IgG coated beads

These were developed by Tsai and Zheng (2005). They are very reliable and easy to store, but require IgG to AurkA. Asters nucleated from AurkA beads are symmetric. We have used them to explore how a single MTOC can nucleate a microtubule array that fills a millimeter sized egg (Ishihara, Nguyen, Groen, Field, & Mitchison, 2014) and to reconstitute cytokinesis signaling (Nguyen et al., 2014). See Figure 2A.

REQUIREMENTS

- 2.8 µm magnetic Dynabeads coated with Protein A (Thermo 10002D)
- Rack to hold tubes with magnet to pull beads
 - For 1.5 mL Eppendorf tubes (Bangs Laboratories LS001)
- Wash buffer
 - 50 mM KCl
 - 10 mM K-HEPES pH 7.7
 - 1 mM MgCl₂
 - 1 mM EGTA
- Storage buffer
 - Wash buffer plus
 - 0.05% sodium azide
 - 100 µg/mL bovine serum albumin (BSA)
- Rotating wheel for incubation

PREPARATION OF MTOCS

1. Rabbits were immunized with full length *Xenopus* AurkA fused to GFP and expressed in bacteria.
2. Antisera were first passed over a column of GFP, to remove antibodies against the fusion protein partner.
3. The antisera were then passed over an AurkB column, to remove Aurora B antibodies before the final purification. This ensures the purified antibody is specific for Aurora A.
4. Dynabeads Protein A are washed 3x with Wash Buffer and then coated with saturating amounts of anti-AurkA IgG (30 min, room temperature).
5. Beads with antibody attached are then washed twice with Storage Buffer and stored at 4 °C. Beads are stable for months. The BSA prevents bead aggregation and nonspecific adsorption, however it should not be added prior to antibody binding to the beads.

4.2 Demembranated *Xenopus* sperm with centrosomes attached

Centrosomes attached to sperm nuclei are the natural MTOC that is introduced to eggs during fertilization. However, asymmetric signals from the chromatin make their nucleation activity complex. Chromatin can nucleate microtubules (Heald et al., 1996), and it also sends an asymmetric signal via AurkB activity. As a result, sperm centrosomes nucleate asters that are polarized in their structure and activity early in the cell cycle (Field, Groen, Nguyen, & Mitchison, 2015). See Figure 3A. Later sperm form nuclei and centrosomes split and migrate apart (Figure 3B, C). The following protocol is adapted from (Murray, 1991).

BUFFERS AND REAGENTS: All buffers should be prepared and placed on ice before starting the preparation.

- MMR (Section 2.1)
- 2x NPB
 - 500 mM sucrose
 - 30 mM K-HEPES pH 7.4 with KOH
 - 2 mM EDTA pH 8.0
 - 1 mM spermidine trihydrochloride (Sigma S2501)
 - 0.4 mM spermine (Sigma S1141)
 - 2 mM DTT (Sigma D9779)
- Protease inhibitors (LPC) 1/1000 of 10 mg/mL stock (see Section 2.1)
- Lysolecithin (L- α -lysophosphatidylcholine Type 1) (Sigma L4129)
 - 10 mg/mL, made fresh the morning of the prep

- 10% BSA
- 10 mg/ml Hoechst 33342 stock (Sigma B-2261)
 - Stored in water in a light-tight vessel at -20°C
- Sperm dilution buffer (SDB)
 - 100 mM KCl
 - 10 mM K-HEPES pH 7.7
 - 1 mM MgCl_2
 - 150 mM sucrose
 - Can prepare as a 5x stock and store at -20°C
- Sperm Storage Buffer
 - 1x NPB
 - 30 % glycerol
 - 30 μL 10% BSA for 1 mL
- Hoechst stock solution, 10 mg/mL in water

EQUIPMENT

- Small Petri dish
- Razor blade or scalpel
- Cheesecloth
- Funnel
- Centrifuge and Beckman JS 13.1 swinging bucket rotor or equivalent
- 14 mL round bottom polypropylene tubes (Falcon 352059)
- Hemocytometer (or another method to quantitate sperm yield)

4.2.1 Isolation of sperm

1. Remove testes from one to two male *Xenopus laevis*.
2. Place in small Petri dish and remove as much fat and blood vessels as possible without causing damage.
3. Rinse 3 times in 1x MMR and 2 times in 1x NPB.
4. Transfer testes to a dry Petri dish and macerate thoroughly with a razor blade until they look like a slurry.
5. Add 2 mL NPB + LPC to the macerate. Gently pipette up and down through a fire-polished truncated Pasteur pipette with an opening of ~ 3 mm in diameter.

6. Pass macerate through about 4 thicknesses of cheesecloth on a funnel, collecting into a 14 mL round bottom Falcon tube. Rinse dish with 8 mL of NPB + LPC. Pass this rinse through the cheesecloth, collecting it in the tube.
7. Centrifuge at 3000 rpm for 10 min at 4 °C in a Beckman JS-13.1 or equivalent swinging bucket rotor.
8. Discard supernatant. Resuspend the pellet in 8 mL NPB + LPC.
9. Centrifuge again, as in step 7.
10. Resuspend pellet in 1 mL NPB + LPC, using a clipped (larger opening) P1000 pipette tip to minimize damage to the sperm due to shear forces.

4.2.2 Demembration and concentration of sperm

1. Reserve 1 μ L sperm in NPB on ice to use as a control later when monitoring the progress of demembration. Warm the rest of the sample to room temperature, and add 50 μ L of 10 mg/mL lysolecithin. Mix by gentle flicking and incubate for 5 min at room temperature.
2. Monitor demembration.
 - 2.1 To the control sample from Step 1 and an equivalent sample after lysolecithin treatment, add 9 μ L of NPB and 1/100 Hoechst stock. Image 5 μ L of each. After demembration, the Hoechst signal should be much brighter and sperm should look more stringy. It is typical for 1–5% of nuclei to be partially membranated, with part or all of the nuclei remaining globular, circular, or rounded.
3. Add 10 mL cold NPB + 3% BSA + LPC to the tube of demembrated sperm. Mix gently by pipetting up and down and spin at 3000 rpm for 10 min at 4 °C.
4. Decant supernatant. The lysolecithin-treated pellet should look slightly more translucent than it did prior to treatment.
5. Resuspend in 5 mL NPB + 0.3% BSA without LPC. Mix gently by pipetting, then centrifuge at 3000 rpm for 10 min at 4 °C.
6. Decant supernatant and resuspend pellet in 500 μ L Storage Buffer. This is now your demembrated sperm stock. Keep on ice, and determine yield (Section 4.2.3). Aliquot in 20 μ L volumes, flash freeze in liquid nitrogen, and store at –80 °C.

4.2.3 Yield determination

1. Add 1 μ L of the demembrated sperm stock and 1 μ L of 1/100 dilution of Hoechst stock to 98 μ L of SDB. Mix the sperm stock very well using a clipped (larger opening) pipette tip just before removing the 1 μ L sample.
2. Mix the diluted SDB/Hoechst/sperm very well and allow a small amount to flow into the chamber of a hemocytometer by capillary action.

3. Count nuclei in a square of the hemocytometer under a compound microscope (we usually visualize with Hoechst and bright field combined). From two males, you should obtain counts of at least $200\text{--}250 \times 10^4$ cells/mL for a 1:100 dilution of the 0.5 mL suspension.

4.3 Free centrosomes

Centrosomes can be purified from tissue culture cells (Reber, 2011). For us it has been easier to prepare them from *Xenopus* sperm, using M-phase extract to naturally separate the centrosome from the nuclear envelope. Free centrosomes nucleate symmetrical asters, unlike sperm, where the chromatin causes strong asymmetry in microtubule organization (Field, Groen, Nguyen, & Mitchison, 2015).

REQUIREMENTS

- *Xenopus* sperm (Section 4.2)
 - M-phase extract (Section 2)
 - Cytochalasin D, 10 mg/mL in DMSO (Sigma C8273)
 - 1 M CaCl_2
 - Ficoll 400 (Sigma F9378)
 - 1x XB salts (Section 2.1)
 - 1x CSF-XB Buffer (Section 2.1)
 - Fluorescent tubulin (Section 3.1.2)
 - 14 mL round bottom polypropylene tubes (Falcon 352059)
 - Microcentrifuge
 - Centrifuge and swinging bucket rotor that can run at $25,000 \times g$
1. Add cytochalasin D (0.2 μL of 10 mg/mL stock) to 100 μL of M-phase extract, or prepare M-phase extract in the presence of cytochalasin. Add sperm nuclei (up to 5,000 per μL) and incubate for 10–20 min at 20°C . This incubation naturally dissociates centrosomes from chromatin. Addition of cytochalasin prevents gelation-contraction of the extract.
 2. Add calcium to the extract to a final concentration of 0.4 mM. This cycles the extract into interphase (Section 2.3.1). Incubate for 1 h and 20 min at room temperature. During this time the DNA decondenses and forms nuclei (Section 2.3.2). Additionally, the centrioles replicate and centrosomes assemble.
 3. The remaining steps of the protocol should be performed at 4°C , using buffers chilled to 4°C .
 4. Dilute the extract 5x in cold 1x XB and centrifuge in a microfuge at $3000 \times g$ for 5 min.

5. Keep the supernatant. The pellet is very soft and should contain most of the nuclei.
6. Dilute the supernatant 5x in cold CSF-XB.
7. Prepare a 3 mL 20% Ficoll cushion in CSF-XB in a 14 mL Falcon tube.
8. To concentrate centrosomes, pellet the supernatant onto the Ficoll cushion. The cushion is important, since pelleting them in a tube leads to irreversible aggregation (Mitchison & Kirschner, 1986). Carefully layer the diluted centrosomes atop the Ficoll cushion, and spin in a swinging bucket rotor at 25,000 x g for 15 min at 4 °C.
9. Discard most of the diluted extract far above the cushion, then carefully collect fractions nearer the cushion. Most of the centrosomes will be in the Ficoll cushion, close to the interface with the diluted extract.
10. Assay by adding 1–2 µL of centrosome preparation to 20 µL of M-phase extract and fluorescent tubulin (Section 3.1.2). One should see one aster form per centrosome. Centrosomes can be stored at –80 °C for prolonged periods.
11. Modifications
 - 11.1 To increase the purity of centrosomes, dilute the most concentrated fraction(s) again and centrifuge through an additional cushion. Alternatively, centrosomes can be purified on a sucrose gradient, then stored in high sucrose (Mitchison & Kirschner, 1986).
 - 11.2 To scale up, start with a larger volume of extract.

4.4 Tetrahymena Pellicles

Tetrahymena pellicles are detergent-extracted cortices of deciliated *Tetrahymena* cells. They contain a dense array of basal bodies that nucleate a dense array of microtubules. They provide a convenient local source of polymerized microtubules that is not centrosomal in origin (Lombillo, Stewart, & McIntosh, 1995). We used them to compare microtubule stability inside and outside a mitotic spindle (Needleman et al., 2010), and as a source of microtubules nucleated independently of centrosomes or chromatin (Ishihara, Nguyen, Groen, Field, & Mitchison, 2014). See (Coue, Lombillo, & McIntosh, 1991) for pellicle isolation.

4.5 Spontaneous nucleation

Microtubules nucleate spontaneously in egg extract. The timing varies from 15 to 45 min post calcium addition, depending on the extract and its age (older extracts have more spontaneous microtubules). Once initiated, nucleation tends to amplify rapidly, perhaps due to positive feedback (Ishihara, Nguyen, Groen, Field, & Mitchison, 2014). Activated *Xenopus* eggs, those released from CSF arrest by electrical stimulation, for example, also exhibit spontaneous microtubule assembly. Motors in the extract and egg can organize these microtubules into aster-like structures (Ishihara, Nguyen, Groen, Field, & Mitchison, 2014).

5. Preparation of glass surfaces for imaging

For imaging experiments, we typically squash small volumes of extract between two coverslips to provide a 5- to 25- μ m thick preparation. Cleaning and preparation of the glass surface is important. Untreated glass tends to bind motor proteins such as dynein that influence microtubule organization, and it also prevents Chromosomal Passenger Complex (CPC) recruitment to zones between asters. For cytokinesis signaling experiments we routinely passivate the glass using polyethylene glycol (PEG). Many PEG passivation schemes have been described in the literature. We discuss several here, starting with the most simple (Section 5.1), which is our current favorite. Passivation with poly-L-lysine-g-PEG was reported in (Lee & Vörös, 2005), now commercially available from SuSoS Chemicals. See Figure 4 for examples of asters growing on different surfaces.

5.1 Passivation with poly-L-lysine-g-polyethylene glycol (PLL-g-PEG)

5.1.1 Requirements

- Standard glass coverslips, No. 1.5 thickness, various sizes
- Parafilm
- Teflon rack for coverslips (Life Technologies C14784)
- Poly-L-lysine-g-polyethylene glycol (PLL-g-PEG) (SuSoS PLL(20)-g[3.5]-PEG(2)).
- VALAP, 1:1:1 mixture of wax, Vaseline, and lanolin
- Nitrogen or house air

5.1.2 Flamed coverslips—To flame clean the coverslip surface, coverslips are individually dipped in 70% ethanol for a few seconds, pulled out, flamed with a Bunsen burner, cooled in air for a few seconds, then placed onto a droplet of 0.1 mg/mL PLL-g-PEG in 10 μ M HEPES pH 7.4 on a piece of Parafilm. Coverslips 22 x 22 mm in size require 110 μ L, and 18 x 18 mm coverslips require 90 μ L. Allow coverslips to incubate at room temperature for 15–30 min. They are then washed 3x in distilled water. The first two washes involve placing each coverslip PEG side down in a droplet of distilled water and allowing them to sit for 5 min. The last wash involves dipping each coverslip into a beaker of distilled water. Coverslips are then placed in slots in a Teflon rack so they do not contact one another. They are dried with a jet of nitrogen, to prevent residue from water droplets. They are generally used the same day, but can be stored for up to a week at room temperature in the dark (Field, Groen, Nguyen, & Mitchison, 2015).

5.1.3 Chemically cleaned coverslips—Deposit many (>50) coverslips in a beaker and clean by sonicating in 3 M KOH, rinsing in distilled water, then sonicating in 100% ethanol. Coverslips can be stored in 100% ethanol before use. Coverslips are removed from ethanol and incubated in drops of 0.1 mg/mL PLL-g-PEG solution in 10 mM K-HEPES pH 7.4 buffer for 15–30 min. After incubation, coverslips are placed in a Teflon drying rack and dried using house air (Ishihara, Nguyen, Groen, Field, & Mitchison, 2014).

5.2. Chemical coupling of silane-PEG to glass surfaces

This method was described in (Bieling, Telley, Hentrich, Piehler, & Surrey, 2010). We obtained similar or better results with the following simplifications: (1) Instead of cleaning coverslips with Piranha solution, we use oxygen plasma (Technics plasma etcher 500-II, oxygen gas at 100 mTorr, glow discharge at 200 W for 4 min). (2) Instead of a two-step silanization followed by PEG coupling procedure, we directly couple to the glass a silane-PEG (methoxy PEG silane, MW 5000, JenKem Technology USA # A3037). Silane-PEG is dissolved in 100% ethanol to 10% (w/v). Fully dissolving the silane-PEG requires heating. Coverslip sandwiches are made with this solution in between, and incubated for 2 hr at 75 °C. These coverslips can be stored with a desiccant at -20 °C for up to one year. We used these in (Nguyen et al., 2014).

5.3 Passivation with casein

An advantage of casein passivation over PEG passivation is that growing microtubules track within a TIRF illumination field along the coverslip surface. This presumably reflects some adsorption of microtubule-associated proteins (MAPs) and motors. We made extensive use of kappa-casein coating in recent work, where we tracked single microtubules by TIRF (Ishihara, Nguyen, Groen, Field, & Mitchison, 2014; Nguyen et al., 2014). To clean the coverslips, use protocol in Section 5.1.3. Then, instead of PLL-g-PEG solution, coverslips are incubated in droplets of 1 mg/mL casein in 10 mM HEPES pH 7.7 for 15–30 min. Coverslips are passed through two droplets of distilled water (no incubation), placed in a Teflon rack, and dried with a stream of house air.

As assayed by aster morphology and microtubule sliding, coverslips passivated with whole casein (Sigma C3400) bind less dynein but enrich for plus end directed motors (Maloney, Herskowitz, & Koch, 2011). Coverslips passivated with kappa-casein (Sigma C0406) bind much less kinesin but more dynein, resulting in significant dynein activity on the glass surface. We obtained good TIRF images of dynamic astral microtubules while minimizing motor-generated forces from the substrate by coating the imaging coverslip with kappa-casein, coating the other coverslip with PEG, and adding a dynein inhibitor to the extract. For dynein inhibition we routinely use 0.04 mg/mL CC1, an easily expressed fragment of p150Glued that blocks dynein function (Quintyne et al., 1999). Vanadate usually has similar effects in extract systems, but may interfere with phosphatase activity.

5.4 Metal support slides

To hold the passivated coverslips we machined stainless steel or aluminum slides ~2 mm thick with 20 mm circular holes in the center. 22 x 22 mm square coverslips are attached to top of the metal slide using VALAP, with the passivated surface facing away from the slide. 4–12 µL of extract is pipetted to the center of the coverslip, then an 18 x 18 mm coverslip is placed on top with passivated side towards the extract. After the droplet spreads out fully (~5 s), we seal with VALAP so the sample does not dry out. 7.5 µL extract gives a squash nominally 23 µm in thickness, but this may vary across the coverslip. These chambers can be viewed using both upright and inverted microscopes. We make metal slides with one, two, and four holes (Fig. 5A and B). With the four-hole slides, we can image four different reactions in parallel using an automated stage, visiting each reaction every 1–3 min.

6. Protein immunodepletion

Proteins can be specifically removed from egg extract by immunodepletion. This method has advantages when compared to gene knockdown in cultured cells, where expression of other proteins may change in response to target protein depletion over time. The degree of depletion can be monitored via Western blots and also by adding fluorescent antibody probes and imaging. Rescue experiments can be performed by adding back active fusion proteins. The most important requirement is high-quality IgG.

6.1 Requirements

- 2.8 μ m magnetic Dynabeads coated with Protein A (Thermo 10002D)
- IgG antibody, raised against protein of interest
- Random rabbit IgG for control (Jackson ImmunoResearch 011-000-003)
- Rack to hold tubes with magnet to pull beads
 - For 1.5 mL Eppendorf tubes (Bangs Laboratories LS001)
- Wash Buffer, see Section 4.1
- Storage Buffer, see Section 4.1
- Rotating wheel for incubation

6.2 Bead preparation

1. Dynabeads are washed three times in Wash Buffer.
2. Saturate the beads with an affinity-purified polyclonal antibody raised against the protein of interest. In a typical preparation, we use 20 μ g IgG per 50 μ L of a 50% bead slurry in a 1.5 mL microfuge tube. Random rabbit IgG at an equivalent antibody concentration is used as a control.
3. Incubate the bead/antibody mixture while gently rotating overnight at 4 °C or at room temperature for 30–60 min.
4. Wash three times with 1 mL Storage Buffer, and resuspend in 50 μ L Storage Buffer. Beads with antibody attached can be stored at 4 °C for 1–2 months. BSA reduces clumping and nonspecific adsorption of beads, and azide prevents bacterial growth.

6.3 Protein depletion

We optimize the immunodepletion protocol for particular target proteins. Typically, two to three rounds of immunodepletion are required to remove proteins to <5%, but more IgG may be required to deplete abundant proteins or in cases where the IgG binds less efficiently. In some cases we have obtained more effective immunodepletion by adding IgG directly to extract, incubating 15 min at 0 °C, then adding washed protein A (or G) beads, incubating 30 min, and removing beads.

1. Add *Xenopus* extract to prepared beads in a 1.5 mL tube at approximately a 1:20 dilution (typical would be 5 μ L of beads and 100 μ L of extract).
2. Incubate at 4 °C for 45–60 min. Occasionally (every 5–10 min) flick the bead/extract mixture to disperse the beads and return to 4 °C.
3. Pull beads to the side of the tube with a magnet, then move the extract to a second tube of prepared beads. Repeat the depletion step 2.
4. After the second depletion, separate the extract from the beads and assay the extract for activity.
5. Run an SDS PAGE gel and Western blot to determine extent of depletion.

7. Supported lipid bilayers (SLBs)

The plasma membrane plays a central role in cell division. It defines the cell boundary, is shaped by the cortical actin cytoskeleton, interacts with microtubules, and ingresses during cytokinesis. There has been huge interest in reconstituting plasma membrane biology in the biophysics community, which led to several approaches that might be extended to egg extracts, notably giant unilamellar vesicles (GUVs) and supported lipid bilayers (SLBs). So far, we have focused on use of SLBs to model the plasma membrane in egg extract experiments, because these are technically easier than GUVs, and better suited for microscopy experiments. In particular, SLBs are an excellent match to TIRF imaging, because they confine plasma membrane biochemistry to a thin layer near the coverslip which is ideally placed for TIRF imaging. By combining actin-intact extract, artificial MTOCs, and SLBs we were able to reconstitute spatially organized cytokinesis signaling in a cell-free system for the first time (Nguyen et al., 2014).

7.1 Requirements

LIPIDS (all from Avanti Polar Lipids)

- L- α -phosphatidylcholine (PC) (Brain, Porcine) (840053)
- L- α -phosphatidylserine (PS) (Brain, Porcine) (840032)
- L- α -phosphatidylinositol (PI) (Liver, Bovine) (840042)
- L- α -phosphatidylinositol-4,5-bisphosphate (PI(4,5)P₂) (Brain, Porcine) (ammonium salt) (840046)

EQUIPMENT TO CREATE SMALL UNILAMELLAR VESICLES (SUVS)

- 10 μ L Hamilton syringe, 1700 series, point style 2 (Sigma 20972)
- 100 μ L Hamilton syringe, 1700 series, point style 2 (Sigma 20688)
- Glass vials (Borosilicate glass, phenolic screw cap) (VWR 66011-020)
- Nitrogen gas
- Argon gas

- Branson CPX1800 ultrasonic cleaner, or equivalent
- 2 Teflon racks (Life Technologies C14784), to clean coverslips
- VALAP, 1:1:1 mixture of wax, Vaseline, and lanolin

EQUIPMENT FOR CLEANING GLASS

- Glass coverslips 1.5 mm thickness
 - Option 1: 22 x 22 mm (VWR 48404-133)
 - Option 2: 22 x 60 mm (VWR 48367-059)
- Racks to wash 22 x 22 mm coverslips and keep them separate
 - Option 1: Polypropylene rack (Sigma-Aldrich Z688568)
 - Option 2: Teflon rack, (Life Technologies C14784)
- Rack to wash 60 x 22 mm coverslips
 - Coplin staining jar (Thomas Scientific 8541K92)
- Glass beakers
 - 100 mL
 - 500 mL
- Plasma cleaning device
 - Option 1: Glow discharge unit (Electron Microscopy Sciences)
 - Option 2: Chemical vapor deposition system (Denton Vacuum)
 - Option 3: Other plasma cleaning device (e.g., Harrick Plasma Inc.)
 - Option 4: Handheld corona treater (Electro-Technic Products BD20AC). This option is less reproducible but less expensive and more portable than the other options.
- UV light source
 - Compact UV lamp (UVP 95-0019-16)
 - Benchtop transilluminator (UVP 95-0449-01)
- Thin-walled PCR tubes, 0.2 mL (VWR 20170-012)
- Custom metal sample chambers (Section 5.4)
- Hot plate or temperature block

BUFFERS AND REAGENTS

- Extract buffer (XB) (Section 2.1)
- High-salt extract buffer (HS-XB): XB with 200 mM KCl
- VALAP (Section 5.1)

- Potassium hydroxide
- 100% ethanol (VWR 89125-186)
- Acetone
- Chloroform
- Norland Optical Adhesive 63, ultraviolet curing glue (Norland Products 6301)

7.2 Preparation of small unilamellar vesicles (SUVs)

GENERAL INFORMATION: Prepare SUVs at least 1 day prior to the experiment. Lipids are usually dissolved in organic solvents such as chloroform. To handle and store chloroform solutions, avoid plastic pipette tips and containers. Instead use glass or polytetrafluoroethylene (PTFE, e.g., Teflon). We use Hamilton syringes and glass vials with PTFE screw cap lids.

To store organic solutions of phospholipids: prepare aliquots, cover the solution with a layer of argon to prevent oxidation of the lipids, close the cap tightly, and seal with Parafilm. Store at -20°C . More polar lipids, such as PI(4,5)P₂, are dissolved in more polar solvent mixtures, such as chloroform/methanol/water 60/33/7% (v/v). Furthermore, PI(4,5)P₂ is fairly unstable and volatile in organic solution at -20°C (lasting no longer than 1 week), thus it should be stored as SUVs in aqueous solution under argon at -80°C , as described in the following.

To mimic the inner leaflet of the plasma membrane of animal cells, we mixed two different mammalian lipids stocks

- Stock A: 3 lipids in the molar ratio PC 0.6/PS 0.3/PI 0.1
- Stock B aka 5% PI(4,5)P₂: 3 lipids in Stock A plus PI(4,5)P₂ in the molar ratio of PC 0.6/PS 0.3/PI 0.05/ PI(4,5)P₂ 0.05

Depending on the quality of the extract, we occasionally added small amounts of PI(4,5)P₂ by combining the two stocks (Section 7.5.3).

1. In 4 mL clean glass vials, add lipid solutions in desired amounts and ratios, so the total concentration of lipids is 1–2 mM. An optimal volume of lipid per vial is 100–300 μL . Dry down lipids in a nitrogen gas stream, to obtain a translucent lipid film on the bottom of the glass vial.
2. Vacuum desiccate for 1–2 h to remove residual organic solvent.
3. Rehydrate lipid film with appropriate amount of HS-XB buffer to obtain a final 5 mM lipid suspension. Incubate buffer with lipid film for 30 min at 37°C , then vortex rigorously to obtain large multilamellar vesicles (MLVs). The result should be a milky solution.
4. To create SUVs freeze-thaw the MLVs five times using a dry ice/isopropanol mix. Vortex after each thaw step.

5. Sonicate suspension in bath sonicator for 2 min at a time until the solution is clear. Keep at 37 °C when not sonicating. Take care not to overheat the lipid mixture during sonication. Some lipid suspensions, especially those made from mammalian lipids or with a high PE content, can take two to three rounds of 2-min sonication or longer sonication times. We found that freeze-thaw followed by sonication for each round is the fastest and most convenient method to break up larger MLVs into SUVs. This method produces SUVs with diameters in the range of 15–50 nm.
6. Aliquot SUVs from Stock A into desired volumes, typically 24 µL or larger. Store at –20 °C. Aliquot SUVs from Stock B in smaller aliquots 5–10 µL and store at –80 °C under argon, as PI(4,5)P₂ is more volatile.
7. Bilayers are assembled by mixing the appropriate amounts of Stocks A and B and adding the SUVs to small chambers affixed to coverslips (Section 7.4). For a larger reaction chamber prepared using a 0.5 mL microfuge tube, 10 µL is required to make one bilayer. For a smaller reaction chamber made from a PCR tube, 6 µL is sufficient for one bilayer.

7.3 Cleaning glass and preparing chambers

We typically clean coverslips in alkaline solutions and organic solvents using sonication in a bath sonicator. These should be prepared the day before the experiment and can be stored for several days in ethanol at 4 °C. Then we use air plasma for final cleaning and assemble the imaging chambers ~2 h prior to the experiment.

7.3.1 Chemical cleaning

1. Place coverslips into slots in a Coplin jar (for 22 x 60 mm coverslips) or a polypropylene rack (for 22 x 22 mm coverslips). It is important to keep each coverslip separate. Immerse coverslips into a beaker of 3 M KOH and sonicate for 20 min.
2. Discard cleaning solution, and rinse five times with ddH₂O.
3. Immerse coverslips in ddH₂O, and sonicate for 20 min.
4. Discard ddH₂O and rinse with 100% ethanol.
5. Immerse coverslips into 100% ethanol and sonicate for 20 min.
6. Immerse into fresh ethanol. Proceed to plasma cleaning (Section 7.3.2), or store the coverslips in a sealed container for several days at 4 °C.

Note: With certain batches of coverslips⁴, we found that a more rigorous pre-cleaning procedure was helpful:

1. Sonicate in 100% ethanol for 20 min, then rinse three times with ddH₂O.
2. Sonicate in 100% acetone for 20 min, then rinse three times with ddH₂O.

⁴We have successfully used coverslips from various vendors. Most commonly we used #1.5 thickness, 24 x 60 mm or 22 x 22 mm square coverslips made of standard glass.

3. Incubate in 100% nitric acid (HNO_3) for 20 min, then rinse three times with ddH₂O.
4. Proceed to Step 1 in the cleaning procedure above.

7.3.2 Plasma Cleaning

1. Collect items required for chamber assembly before plasma cleaning. See Section 7.3.3. Everything must be ready before plasma cleaning begins so chambers may be assembled as soon as cleaning is complete.
2. Use forceps to remove the rack with chemically cleaned coverslips from ethanol. Keep coverslips in the rack and blow dry using compressed nitrogen gas. Make sure each coverslip is dry.
3. We routinely used an EM Sciences Glow Discharge Unit. Place individual coverslips flat inside a Petri dish, then place the Petri dish into the chamber. Glow discharge at 25 mA for 8 min.
4. Immediately prepare the reaction chamber (Section 7.3.3) after plasma cleaning. Freshly cleaned and dried glass surfaces can adsorb dirt particles from unfiltered air, so we add the SUV solution within 30 min after plasma.

7.3.3 Preparation of reaction chambers

1. Cut either PCR tubes or 0.5 mL microfuges tubes to make chambers. Remove the lid and the lower conical part of the tube. See Figures 5C and 6B.
2. Use metal slides (stainless steel or aluminum) with circular holes 20 mm in diameter. Attach a 22 x 22 mm plasma cleaned coverslip to bottom of the slide using VALAP. The plasma-cleaned side should face up through the hole. Or use 22 x 60 mm coverslip as a platform, again plasma-cleaned side up (Fig. 6C). The longer coverslips can be used in place of a slide in a microscope slide holder and can accommodate more chambers; however, they are less robust.
3. Cover the rim of a cut PCR tube with a thin layer of UV glue. Insert a pipette tip into the rim opening to insure it is not occluded by glue.
4. Place tube with glue-covered rim down flat onto the surface of the freshly cleaned glass coverslip (Figure 6B).
5. Expose to 365 nm UV light for 5–10 min to allow glue to polymerize⁵. It is crucial to load SUVs into the reaction chambers as soon as possible, so while the glue is polymerizing, thaw and mix lipid stocks, to form supported bilayers (Section 7.4).

⁵To prevent loss of valuable lipids, one should practice gluing chambers to uncleaned coverslips, then adding water to check for leakage.

7.4 Supported lipid bilayers (SLBs) from mammalian lipids

1. Thaw stocks of SUVs A and B prepared in Section 7.2. Seal tubes with Parafilm and sonicate 1–2 min until solution is clear. We found immersing the tubes in the center of the sonicator bath such that the liquid inside the tube is just below the surface of bath water works well. Make sure that lipids do not heat up.
2. Using the SUV stocks A and B make mixtures of the desired volumes and ratios of lipids. 6 μL of SUV is required for one PCR tube chamber. For our cytokinesis signaling experiments we typically made mixtures of 0%, 0.1%, and 0.5% PI(4,5)P₂ lipid mixtures in 60% PC, 30% PS, 10% PI and tested the extract for actin assembly properties see Section 7.5.
3. Sonicate the mixtures for 1–2 min as described in Step 1.
4. Dilute SUV mixtures 5x in HS-XB buffer to 1 mM final lipid concentration.
5. Put 30 μL of diluted SUVs into each PCR tube well.
6. Add CaCl₂ to 4 μM final concentration (1.2 μL of 100 mM CaCl₂ per well).
7. Incubate on 42 °C heated temperature block for 30–60 min. This incubation temperature approximates porcine body temperature (lipids are porcine derived).
8. Wash bilayers at room temperature:
 - 8.1 Add 170 μL of regular XB buffer into each well.
 - 8.2 Pipet up and down five times, then remove 150 μL buffer from each well.
 - 8.3 Add 150 μL XB buffer into each well, pipet up and down five times, then remove 150 μL of the buffer from each well. Repeat this until they have been rinsed five times. Do not touch bilayers. Make sure a layer of ~20 μL of buffer remains above the bilayer.
9. Keep slides with bilayers in a covered Petri dish at 4 °C until use. They should last for several hours.

7.5 Evaluation of bilayer integrity and extract quality

Before the experiment begins, optimize every aspect of the protocol, such as bilayer continuity, fluidity, and composition; extract quality; and concentrations of fluorescent probes and microtubule organizing centers (MTOCs).

7.5.1 Requirements

For bilayer integrity

- Fluorescent lipids
 - Option 1: 0.5 mol % Lissamine Rhodamine PE. 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (Avanti Polar Lipids 810158C).

- Option 2: 0.5 mol % Texas Red DHPE. Texas Red-1,2-dihexanoyl-sn-glycero-3-phosphoethanolamine (Biotium 60027)
- Option 3: DiIC18 (Invitrogen D282) 10 mg/mL stock in DMSO (1000x).

For extract quality

- Small Petri dish
- Mineral oil (Sigma M5904)
- 1 M CaCl₂
- 20 °C water bath
- Fluorescent probes (Section 3). In this protocol, labeled tubulin and Alexa 647-labeled anti-AurkB IgG.
- Microtubule organizing center (Section 4). In this protocol, anti-AurkA beads.
- PEG passivated coverslips, 22 mm and 18 mm (Section 5.1)
- Metal imaging slides (Section 5.4)
- VALAP (Section 5.1.1)

7.5.2 Bilayer integrity—When first assembling supported lipid bilayers, one should verify bilayer continuity and lateral fluidity by adding a low concentration of fluorescent lipids (Section 7.5.1). One can add Rhodamine PE, Texas Red DHPE, or the lipophilic dye DiIC18 (DiI) to the lipid mixture during the vesicle preparation. To confirm continuity, one can image the fluorescent bilayer, which should appear homogeneous. To confirm fluidity, one can perform fluorescence recovery after photobleaching (FRAP): using the field aperture, photobleach a small region, then monitor diffusion of unbleached lipids into the region, which should take 1–5 min depending on the size of the bleached spot.

If slow fluorescence recovery reveals poor continuity or fluidity, one possible reason is aging of the lipids. For example, oxidized lipids do not form a fluid membrane. In this case, remake SUVs with fresh lipids. Another possible reason is adsorbed vesicles did not coalesce to form a continuous bilayer, with various causes such as insufficient cleaning of the glass coverslips. Alternatively, one may increase the incubation time, the buffer ionic strength, or the divalent cations concentration to improve results (Nair, Salaita, Petit, & Groves, 2011).

After we tested a batch of lipids, we often omitted fluorescent lipids, to enable use of all fluorescence channels for experimental probes. Still, one can estimate bilayer integrity based on the actin probe Lifeact-GFP. Actin should form a uniform dynamic layer atop the bilayer, and gaps indicate holes in the bilayer.

7.5.3 Extract quality

Actin contractility in droplets: As soon as the extract has been harvested (Section 2.2.4), assay each batch for actin contractility in metaphase. Each batch of extract should not combine eggs from multiple frogs. As described in Section 2.3.2, pipette a droplet of extract under mineral oil, then observe the speed and extent of actin contraction. If no extract contracts well, abort the experiment.

Formation of aster-aster interaction zones (AAIZs): Convert each extract into I-phase (Section 2.3.1), and test for assembly of large microtubule asters that form aster-aster interaction zones (AAIZs) between glass coverslips (Nguyen et al., 2014). In addition to testing extract quality, this assay also verifies concentrations of fluorescent probes and microtubule organizing centers (MTOCs). One should add an optimal number of MTOCs to allow formation of aster-aster interaction zones. If asters are too far from one another, they cannot interact. If asters are too close to one another, they cannot recruit AurkB or form interaction zones (Field et al., 2015).

1. To 30 μ L of extract, add tubulin and anti-AurkB IgG fluorescent probes, and anti-AurkA bead MTOCs.
2. Convert extract to I-phase with addition of calcium to 0.4 mM (Section 2.3.1).
3. Squash 7.5 μ L of extract between two PEG passivated coverslips (Section 5.1) on a metal support slide, and seal with VALAP (Section 5.4).
4. Image using a wide field fluorescent microscope at 10x or 20x.
5. Robust microtubule asters should grow from each AurkA bead. When two asters are close enough to interact, a zone of low microtubule density should form between them. AurkB should localize to this zone within 15–20 min, depending upon the temperature (Figure 6A).

PI(4,5)P₂ requirement: Egg extracts prepared from different frogs vary in their ability to promote actin polymerization at the cortical layer near the lipid membrane. To obtain the desired F-actin morphology at the cortical layer, we can vary the amount of negatively charged lipids in the supported bilayer. In general, more negatively charged lipid bilayers support more actin polymerization (Lee, Gallop, Rambani, & Kirschner, 2010). Routinely, we mix lipid stocks with (Stock B) and without (Stock A) PI(4,5)P₂ (Section 7.2) to prepare SLBs containing 0%, 0.1%, and 0.5% PI(4,5)P₂. Then, before each experiment, we assay for cortical actin morphology, such as alignment along microtubule asters (Figure 6D).

7.6 Imaging cytokinesis signaling

During cytokinesis in animal cells, the cleavage furrow is positioned by signals to the cell cortex that emanate from microtubule arrays interacting at the cell equator. An important protein complex that sends signals from microtubules is the Chromosomal Passenger Complex (CPC). Signals from the CPC help recruit additional proteins to the equatorial cortex resulting in cortical contraction and membrane ingression, separating the cell into two daughters. The key cortical signaling molecule is the active form of the small GTPase RhoA-GTP.

To reconstitute cytokinesis signaling, one requires high quality extract and bilayers, MTOCs, and an appropriate collection of fluorescent probes. To visualize tubulin we use 250 nM tubulin-Alexa Fluor 488 or 568. To visualize RhoA-GTP we use 140 nM mCherry-rGBD. To visualize filamentous actin we use 140 nM Lifeact-GFP. To visualize the CPC we use 0.1–1 µg/mL anti-AurkB IgG labeled with Alexa Fluor 647 or a GFP-tagged version of the DasraA protein, another member of the CPC complex (Nguyen et al., 2014, Field et al 2015). We have also imaged two cortical cleavage furrow proteins anillin and septin, using fluorescently labeled antibodies (Nguyen et al., 2014).

TIMING AND TEAMWORK: Due to the simultaneous, time sensitive tasks, we generally work in pairs. One or both people make the extract (approximately 1–2 h). Then one person plasma cleans the glass and assembles chambers, then prepares the supported lipid bilayers (SLBs). Meanwhile, the second person tests extract quality and probe concentrations and selects the best extract for experiments (Fig. 6). Each process takes about 2 h with experienced hands. It is crucial to coordinate the two processes and to perform the experiments in a timely manner, as both the SLBs and the extracts last about 6 h at 4 °C.

Final sample preparation

1. Prepare SLBs from mammalian lipids as described previously.
2. Convert extract to I-phase with addition of calcium to 0.4 mM (Section 2.3.1). Incubate at 20 °C for 10 min, then return to ice for at least 2 min to depolymerize microtubules and filamentous actin.
3. To exchange out buffer, wash bilayers three times with 20 µL I-phase extract. Extract used for washing bilayers can be prepared earlier and frozen (Section 2.4.4). If frozen extract is used, wash two times times with frozen extract, then a third time with fresh extract.
4. Add desired probes and AurkA beads to 20 µL of I-phase extract and gently mix. As described in Section 7.5.3, use the beads concentration optimal for formation of aster-aster interaction zones.
5. Remove the most of the last 20 µL I-phase extract used to wash the bilayer, and add the 20 µL extract containing probes and beads. At this point, we start a timer.
6. Monitor aster assembly reactions with an inverted TIRF or spinning disc confocal microscope. The temperature of the microscope room should be 18–22 °C, though up to 25 °C is tolerable. As the cell cycle progresses, microtubule asters will form and F-actin cables align with the tubulin polymer. Formation of aster-aster interaction zones (AAIZs) that recruit cytokinesis factors such as the CPC are observed starting at around 20 min. At the same time, activation of RhoA, as indicated by binding of the mCherry-rGBD probe to the bilayer, is also initiated. By 50–60 min, a stable active RhoA zone can be seen above CPC-positive zones (Figure 7). By 90 min, most microtubules usually have disappeared, and we do not image beyond this point.

8. Droplet microfluidics

Microfluidic droplets are a versatile platform to study the effects of confinement and component limitation on the organization, size scaling, and dynamics of cytoskeletal structures. Extract without intact actin has been encapsulated in microfluidic droplets to investigate the organization of microtubule asters (Pinot et al., 2009) and size scaling of spindles (Good, Vahey, Skandarajah, Fletcher, & Heald, 2013; Hazel et al., 2013). Actin-intact extract in droplets has reconstituted dynamic actin networks in bulk (Pinot et al., 2012) and at the cortex (Abu Shah & Keren, 2014). This section assumes fully assembled microfluidic devices as a starting material, described in the above mentioned references. We present special considerations for encapsulating actin-intact extract in droplets, to study actin dynamics in confinement and interactions between actin and microtubules. Examples are shown in Fig. 8.

8.1 Requirements

We most often use these materials, though substitutions can be made.

- Polydimethylsiloxane (PDMS) microfluidic devices, with inlet and outlet holes cored, bonded to glass coverslips (Fig. 8A)
- Oil/surfactant mixture: 2% Pico-Surf 2 surfactant in HFE7500 fluoruous oil (Dolomite 3200281)
- Mineral oil (Sigma M5904)
- Syringes. Norm-Ject 1 mL (VWR 53548-001)
- Blunt needles. 30 g x 1/2" (Brico BN3005)
- Long needles. 16 g x 1 1/2"
- Tubing. Tygon 0.010" x 0.030" ND-100-80 formulation (Cole-Parmer EW-06419-00)
- Two syringe pumps which can be mounted vertically

8.2 Droplet generation

Droplets must be formed at 4 °C. At room temperature, gelation and contraction of the extract can clog the tubing. Furthermore, polymerization of microtubules before encapsulation in droplets can skew the concentrations of microtubule associated proteins (MAPs): at room temperature, droplets containing microtubule nucleating centers contained significantly more MAPs than droplets without nucleating centers. If you have access to a cold room, omit the ice bath from the protocol.

1. Prepare microfluidic devices and materials.
 - 1.1 Soak devices in CSF-XB buffer (Section 2.2.3) to limit evaporation through the PDMS. Prevent buffer from entering the device, for example, by blocking the holes with tubing.

- 1.2 Load 400 μ L mineral oil into a 1 mL Norm-Ject syringe and chill on ice.
 - 1.3 Cut two lengths of tubing, long enough to reach from the syringe pump to the device. Press a blunt needle into one end, and cut the other end with a taper, which makes it easier to press tubing into PDMS holes.
 - 1.4 Prepare an ice bath in a small water basin.
 - 1.5 Load 300 μ L oil/surfactant into a 1 mL Norm-Ject syringe.
 2. Fill the microfluidic device with oil/surfactant at $\sim$ 30 μ L/min. Avoid air bubbles in the device, tubing, or syringe. Oil settles in the device, so tilting the device with the outlet uphill helps avoid air bubbles.
 3. Submerge the device and tubing in the ice bath.
 4. Finish preparation of the extract. For example, add fluorescent probes, MTOCs, or cycle to I-phase. Prepare at least 50 μ L extract per device.
 5. Using a long needle, aspirate extract into the syringe containing mineral oil. Avoid air bubbles. Keep the extract as cold as possible, so quickly connect the second tubing length and submerge in the ice bath.
 6. Mount the extract syringe on the syringe pump, so the extract is in the ice bath.
 7. Purge air from the extract tubing, then reduce the flow rate to 2 μ L/min. At the same time, start the flow of oil at 2 μ L/min, so the PDMS extract inlet is full of oil, and a droplet of oil forms atop the inlet. To prevent water from entering into the device, attach the extract with both extract and oil flowing.
 8. Stop the oil flow, then flow extract at several microliters per minute. Monitor the device. When extract first enters the device, restart the flow of oil at several microliters per minute. Avoid filling the device with extract.
 9. Form droplets. Start with 5 μ L/min oil and 1 μ L/min extract then adjust; the optimal extract to oil flow ratio depends on the geometry of the T-junction generator and the desired droplet size.
 - 9.1 If the droplets are too small, or the oil altogether outcompetes the extract at the T-junction, then decrease the oil flow rate or increase the extract flow rate.
 - 9.2 Conversely, if the droplets are too large, or the extract altogether outcompetes the oil at the T-junction, then increase the oil flow rate or decrease the extract flow rate.
 10. After you make enough droplets, stop the extract flow but continue to flow oil to clear extract from the T-junction.
 11. Stop the oil flow and remove the device from the ice bath.
 12. Cut the tubing at the level of the PDMS and seal the holes with VALAP. Do not pull the tubes out of the PDMS, to avoid pressure fluctuations.

13. Mount on the microscope and image. If imaging for tens of minutes, place a Kimwipe wet with CSF-XB above the device to limit evaporation.

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Abbreviations

AurkA	Aurora kinase A
AurkB	Aurora kinase B
CPC	Chromosomal passenger complex
ER	Endoplasmic reticulum
TIRF	Total internal reflection fluorescence microscopy

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Figure 1. Preparation of actin-intact extract

A) *Xenopus* eggs deposited in a centrifuge tube. (B) After the packing spin, eggs appear tightly packed, with the animal pole facing upward. They are distended but remain intact. Excess buffer is aspirated from the top. (C) After the crushing spin, egg components stratify. The top, yellow (light gray in print versions) layer contains lipid droplets. The second, black layer contains pigment. The third, straw colored layer is the cytoplasmic extract (green bracket (gray in print versions)). The bottom, dark layer contains yolk, pigment granules and egg cortex. Lower panels are 3x magnified. Note the distended eggs in panel B.

Figure 2. Quantitative analysis of interphase aster growth using EB1 tracking

(A) Two frames from a 20x widefield movie of an aster growing from an anti-AurkA bead in I-phase extract. The extract contains 0.04 mg/mL CC1, a dynein inhibitor which blocks microtubule sliding. The coverslips are passivated with PEG. Note individual EB1 comets can be clearly visualized and counted (see 3x insert). (B) Top panel: maximum intensity projection of a short movie to illustrate EB1 comet velocity. (B) Bottom panel: automated EB1 tracking (plusTipTracker, Applegate et al., 2011) was used to count the number of microtubules crossing a given radius (red circle (light gray in print versions)). This analysis showed that most microtubules are nucleated away from the bead once the aster gets large (Ishihara, Nguyen, Groen, Field, & Mitchison, 2014).

Figure 3. Microtubule nucleation by sperm plus centrosomes

Permeabilized sperm nuclei with attached centrosomes were added to actin-intact interphase egg extract. 20x widefield imaging. (A) Example of asymmetric aster formation. Note that (A') the microtubules on the side of the aster close to the sperm chromatin are shorter and more bundled and (A'') recruit chromosomal passenger complex (CPC) to their plus ends (Dasra-GFP). For CPC imaging it is essential to use PEG-passivated glass surfaces (Field et al., 2015). (B and C) Centrosome duplication and separation. (B and B') and (C and C') are the same field in different channels taken 18 min apart. Yellow arrows (light gray in print versions) indicate a centrosome shown at 3x magnification. Note centrosome duplication and separation visualized using fluorescent tubulin and anti-Centrin IgG probes. The extent of centrosome separation was sensitive to the coverslip coating, with more separation on plain glass coverslips, which bind more dynein. This example uses partially passivated glass.

Figure 4. Surface passivation influences aster morphology

These examples are nucleated from AurkA beads (Section 4.1) in I-phase extract. (A) Uncoated coverslip. Motor activity and nonspecific adsorption of microtubules generate disorganized asters. (B) Uncoated coverslip with 40 µg/mL CC1 added to inhibit dynein (Section 5.3). Dynein inhibition increases radial ordering of microtubules. (C) PLL-g-PEG coated coverslips (Section 5.1). PEG treatment reduced nonspecific adsorption of motors and increased radial order.

Figure 5. Metal support slides

(A) Support slides made from aluminum or stainless steel. Single hole slide is 24 x 75 x 2 mm, four hole slide is 50 x 75 x 2 mm. Holes are 22 mm in diameter. (B) Coverslip mount for widefield and TIRF imaging of extract squash preparations. The same mount works on upright and inverted microscopes. (C) Coverslip and open well mount for supported lipid bilayer experiments. This mount is imaged from below by TIRF. See Figures 6 and 7 for images.

Figure 6. Evaluating extract quality and mounting wells for bilayers

(A) Squash preparation containing AurkA beads (yellow (white in print versions)), tubulin probe (green (gray in print versions)) and anti-AurkB IgG [marker for the chromosomal passenger complex (CPC)] (red (light gray in print versions)). Widefield imaging. In a good extract, microtubule asters form zones of interacting microtubules termed aster-aster interaction zones (AAIZs). AAIZs recruit CPC. (B) Metal slide with chambers for preparation of supported lipid bilayers. (C) Bilayer chambers attached to 22 x 60 mm coverslip. (D) TIRF imaging of tubulin and actin on a bilayer to test actin morphology. Actin is aligned along the microtubule bundles in the aster. When the actin had this morphology, cytokinesis signaling was successful (Nguyen et al., 2014). If there is too much PI(4,5)P₂ for a given extract we observed very dense F-actin not aligned with microtubules (not shown). If not enough, there was little F-actin on the coverslip (not shown).

Figure 7. Reconstitution of cytokinesis signaling

(A) Schematic of a bilayer experiment viewed from the side. (B) Representative experiment viewed via TIRF microscopy. Aster-aster interaction zones form where asters interact. The chromosomal passenger complex (CPC) is recruited and active RhoA is recruited to the bilayer (Nguyen et al., 2014).

Figure 8. Cytoplasmic extract confined in microfluidic droplets

(A) Each microfluidic device has an oil inlet, an extract inlet, and an outlet. Droplets are formed at the T junction where the inlets meet. (B) I-phase extract encapsulated in microfluidic droplets. Green (white in print versions) is actin (Lifeact-GFP), and magenta (light gray in print versions) is tubulin (Alexa Fluor 647). An aster nucleated from an AurkA bead spans the droplet, and actin filaments align with microtubules near the aster center. (C) M-phase extract encapsulated in microfluidic droplets, packed into a hexagonal array. Fluorescence corresponds to Lifeact-GFP. (D) Kymographs of actin bulk contraction. The arrows in panel (C) correspond to the three kymographs in panel (D).