CHAPTER 16

Methods for the Study of Centrosome-Independent Spindle Assembly in *Xenopus* Extracts

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I. Introduction

Microtubule organization into a bipolar spindle is essential for chromosome segregation during mitosis or meiosis. Because fidelity of this process is so important, multiple mechanisms promote proper spindle assembly. One tactic used by vertebrate somatic cells employs distinct paired structural cues, such as duplicated centrosomes that nucleate microtubules in a defined orientation, and kinetochores on chromosomes that

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provide bivalent capture sites. In addition, a set of microtubule-based motor proteins functions to cross-link microtubules and maintain spindle pole separation and organization. Motor-dependent function is even more evident in female meiotic cells that lack centrosomes (Gard, 1992; McKim and Hawley, 1995). In these systems, spindles assemble through a different pathway, termed "self-organization," in which microtubules polymerized randomly in the vicinity of mitotic chromosomes are sorted into a bipolar structure by motor proteins (Heald *et al.*, 1996). This centrosome-independent pathway can also occur in vertebrate somatic cells in which one or both centrosomes have been removed by laser ablation (Khodjakov *et al.*, 2000). Therefore, microtubule nucleation and organization can occur both in the presence and in the absence of centrosomes to form a bipolar spindle, and these two pathways of spindle assembly likely represent redundant mechanisms by which cells ensure faithful chromosome segregation.

Because most dividing somatic cells contain centrosomes, the most widely used experimental systems to explore centrosome-independent spindle assembly are *Xenopus laevis* and *Drosophila* oocytes undergoing meiosis (Endow and Komma, 1997; Gard, 1992; Theurkauf and Hawley, 1992). The *Xenopus* egg extract system has proven extremely valuable because spindle assembly reactions can be performed either in the presence or in the absence of centrosomes (Heald *et al.*, 1997). Pioneered by Lohka, Masui, and Maller (Lohka and Maller, 1985; Lohka and Masui, 1983), this system has been used to recapitulate many cellular processes, including cell cycle progression (Murray and Kirschner, 1989), chromosome condensation (Hirano and Mitchison, 1991), nuclear envelope dynamics, DNA replication (Almouzni and Wolffe, 1993), spindle assembly (Sawin and Mitchison, 1991), and anaphase chromosome segregation (Murray *et al.*, 1996; Shamu and Murray, 1992).

Xenopus eggs are arrested in metaphase of meiosis II by cytostatic factor (CSF), the product of the c-*mos* protooncogene, until fertilization triggers a calcium wave promoting entry into the first mitotic cell cycle (Sagata *et al.*, 1989). Extracts prepared from laid eggs in the presence of the calcium chelator EGTA maintain this metaphase arrest and are therefore called CSF extracts. CSF extracts can be cycled into interphase by calcium addition (Lohka and Maller, 1985) and then back into mitosis by the addition of a fresh CSF extract. Addition of demembranated *Xenopus* sperm nuclei permits the analysis of centrosome-directed spindle assembly, as the centriole-containing basal body of the flagellum remains tightly attached to the sperm and becomes competent to nucleate microtubules in the extract, to duplicate during interphase, and to define the location of spindle poles during mitosis. To study spindle assembly in the absence of centrosomes, a source of DNA lacking centrosomes can be added. Most convenient is the use of plasmid DNA immobilized on magnetic beads, which assembles into chromatin in the extract and induces microtubule polymerization and spindle self-organization (Heald *et al.*, 1996). The power of this *in vitro* system comes from the ease of biochemical manipulation. The roles of proteins involved in both types of spindle assembly can be studied by adding specific inhibitors to the extract or by immunodepletion.

This chapter discusses methods for the study of both centrosome-mediated and centrosome-independent spindle assembly in *Xenopus* egg extracts. We first describe simple assays for microtubule polymerization and organization, through the addition of dimethyl sulfoxide (DMSO) or purified centrosomes to form mitotic asters. We then provide methods for centrosome-directed and centrosome-independent spindle formation using sperm nuclei or DNA beads, respectively. Finally, we describe methods to specifically inhibit the function of extract proteins, through the addition of reagents to the extract, or by immunodepletion. In cases where protocols for particular methods have been published recently, we reference the protocols and provide our modifications when applicable.

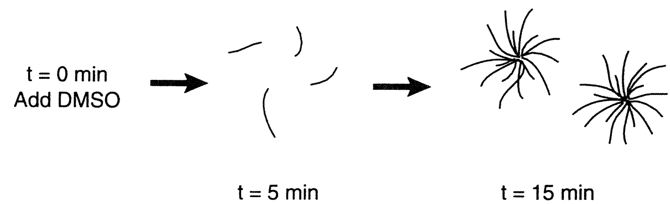
II. Preparation of *Xenopus* Egg Extract and Necessary Reagents

Methods for the preparation of *Xenopus* egg extracts are not discussed in this chapter, as good, comprehensive protocols for CSF extracts have been published elsewhere (Desai *et al.*, 1999). Preparation of *Xenopus* sperm nuclei is performed based on Murray (1991). Our stocks are diluted to a density of 2×10^6 sperm/ml in 100 mM KCl, 150 mM sucrose, 1 mM $MgCl_2$, frozen in liquid nitrogen, and stored in small aliquots at -80°C. These stocks are 20×, and upon dilution in extract, the concentration is approximately 100 sperm/μl extract. Rhodamine-labeled tubulin is prepared based on Hyman *et al.* (1991). The rhodamine tubulin stock is 200× (20–30 mg/ml) and is stored at -80°C. In order to retain maximal activity, it is best to avoid multiple rounds of freezing and thawing so rhodamine tubulin should be stored in small, single-use aliquots of 1–2 μl. Stocks can be prediluted in CSF extract at 1/10 and then added to the spindle reaction at 1/20. The preparation of mammalian centrosomes is discussed in other chapters of this volume. Our stock of KE37 centrosomes purified from lymphoid cells is at a concentration of 5×10^8 ml^{-1} and is stored in small aliquots at -80°C.

III. Microtubule Polymerization Assays in Extract

Microtubule aster reactions are useful for studying microtubule nucleation and organization. Asters form in CSF extracts through both centrosome-directed and centrosome-independent pathways, as diagrammed in Fig. 1. Purified centrosomes added to the extract nucleate microtubules with their plus ends emanating outward in a radial array, forming an aster (Fig. 3A). In the absence of centrosomes, addition of a microtubule-stabilizing agent such as DMSO or taxol induces global microtubule polymerization, and the assembled microtubules are then organized into a focused aster (Fig. 3B) by microtubule motor proteins, including cytoplasmic dynein and associated factors, dynactin (Verde *et al.*, 1991) and the spindle pole protein NuMA (Merdes *et al.*, 1996). These pathways of aster formation highlight the different mechanisms of centrosome-directed and centrosome-independent spindle assembly. Microtubule polymerization in these assays is monitored

A. DMSO aster reaction



B. Centrosomal aster reaction

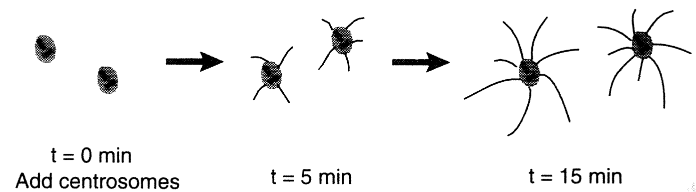


Fig. 1 Schematic drawings illustrating microtubule aster formation in *Xenopus* egg extracts. (A) DMSO aster reaction. (B) Centrosomal aster reaction.

by the addition of rhodamine-labeled tubulin, which incorporates into microtubules and allows visualization by fluorescence microscopy. Polymerization initiates within minutes after the addition of DMSO or centrosomes and incubation at 20°C. Reactions are then monitored by transferring small samples to microscope slides.

A. Materials

CSF extract
Rhodamine-labeled tubulin (20–30 mg/ml stock)
1.5-ml microcentrifuge tubes
Spindle fix: 48% glycerol, 11% formaldehyde, 1× MMR (100 m*M* NaCl, 2 m*M* KCl, 1 m*M* $MgCl_2$, 2 m*M* $CaCl_2$, 0.1 m*M* EDTA, 5 m*M* HEPES, ph 7.8) 5 μg/ml Hoechst dye
Wide-orifice or cutoff 1- to 200-μl pipette tips
20°C water bath
Anhydrous DMSO
Purified centrosomes (5×10^8 centrosomes/ml)
Microscope slides
18- × 18-mm^2 coverslips

Nail polish
Fluorescence microscope with 40× or 63× lens

B. Protocol for Centrosomal and Noncentrosomal Aster Formation

Note: For all protocols described in this chapter, it is important to mix and transfer the extract and extract reactions with wide-orifice (or cutoff) pipette tips to minimize disruption of the extract.

1. On ice, add the rhodamine tubulin stock at a 1:200 dilution to 25 μl of CSF extract in a 1.5-ml tube. It is important to use 1.5-ml tubes and not let reaction volumes exceed 100 μl to permit gas exchange.

2. Add 1.25 μl of anhydrous DMSO or 1.25 μl of purified centrosomes to stimulate microtubule polymerization and transfer the tubes to a 20°C water bath. The bath can be prepared by adding ice to a room temperature bath to adjust to 20°C. Additional ice should be added as needed to maintain temperature. Once reactions are initiated at 20°C, samples should be maintained at that temperature. Placing reactions back on ice before taking samples will result in rapid microtubule depolymerization.

3a. For centrosome reactions, incubate at 20°C and take "squash samples" at time points ranging from 5 to 15 min by transferring 1 μl of the reaction to a microscope slide. Carefully overlay the drop of extract with 5 μl of spindle fix and squash by gently lowering an 18 × 18-mm coverslip on top. For best results, lower the coverslip as carefully as possible. Because squashes can be somewhat variable, it is best to take duplicate samples. Two or three coverslips fit on each slide. In order to minimize the variability of samples, reactions should be mixed with a cutoff pipette tip before taking the squash sample. Samples can be analyzed on a fluorescence microscope using the rhodamine channel. Red asters of microtubules emanating from single focal points should be apparent at early time points, and aster size will increase with longer incubation time.

3b. To monitor DMSO reactions, incubate for 5–30 min and remove a 1-μl sample of the reaction to fix and squash as described earlier. At early time points, extensive microtubule polymerization is apparent. By 20 min, asters have organized that contain many more microtubules than centrosome asters.

4. Squash samples can be stored in the dark at 4°C after sealing the edges of the coverslips with nail polish. Alternatively, the entire reaction mixtures can be spun onto a coverslip, fixed, and mounted as described in Section VI.

IV. Spindle Assembly Assays

Spindle assembly with and without centrosomes can be recapitulated in *Xenopus* egg extracts through the addition of sperm nuclei or DNA-coated beads, respectively (Figs. 3C and 3D). Each *Xenopus* sperm nucleus has an associated centrosome, which

becomes competent to nucleate microtubules upon incubation in extract. Sperm spindles can be formed in extract by two different methods (Sawin and Mitchison, 1991). Addition of sperm nuclei to CSF extracts results in microtubule nucleation at each centrosome, forming monopolar or "half" spindles. These half spindles then fuse pairwise to yield bipolar spindles. Although this form of spindle assembly does not mimic *in vivo* spindle assembly, the half-spindle reaction is a simple and quick assay. Sperm spindles can also be formed by adding sperm nuclei to the extract and then cycling the extract through interphase and back into mitosis. This cycling step allows centrosome and DNA replication, and therefore spindles are assembled in a more physiological manner. Microtubules nucleated from the centrosomes can attach to duplicated kinetochores on the replicated chromosomes, mimicking what occurs *in vivo*. Protocols for these pathways of centrosome-directed spindle assembly will not be given here, as a comprehensive protocol has been published in Desai *et al.* (1999).

Spindle assembly in the absence of centrosomes can be performed in *Xenopus* egg extracts using DNA-coated beads (Heald *et al.*, 1996). In this protocol, biotinylated plasmid DNA is coupled to magnetic beads, taking advantage of a biotin–streptavidin linkage. These beads are added to the CSF extract that is then cycled into interphase to allow DNA replication and chromatin assembly on the beads. Upon cycling of the extract back into mitosis, microtubules are nucleated around the chromatin and then organized into a bipolar spindle by microtubule-based motors. Beads can also be retrieved on a magnet after incubation in the extract to allow biochemical characterization of proteins bound to the DNA.

As before, microtubule formation and spindle assembly in these assays are monitored by the addition of rhodamine-labeled tubulin to the extract. Squashes of reactions can be taken at various time points, and the reactions can also be spun down onto coverslips for long-term storage.

A. Materials

DNA bead preparation:

- Plasmid DNA (> 5 kb)
- Appropriate restriction enzymes (see protocol)
- TE: 10 m*M* Tris, 1 m*M* EDTA, pH 8
- Biotin-dATP, biotin-dUTP, thio-dCTP, thio-dGTP
- Klenow fragment of DNA polymerase
- Pharmacia nick columns
- Washing and binding solutions from Dynal Kilobase BINDER kit
- Bead buffer: 2 *M* NaCl, 10 m*M* Tris, 1 m*M* EDTA, pH 7.6
- Streptavidan dynabeads from Dynal Kilobase BINDER kit
- Dynal MPC (magnetic particle concentrator, Dynal)
- Rotator at 16°C.
- UV spectrophotometer

Spindle assembly:

Materials used for aster reactions (Section III,A)

10X calcium solution: 4 mM $CaCl_2$, 100 mM KCl, 1 mM $MgCl_2$

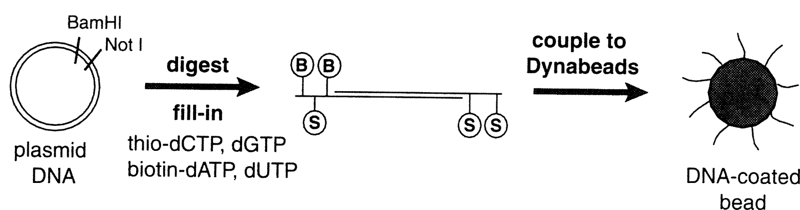
B. Centrosome-Independent Spindle Assembly

1. DNA Bead Preparation

An overview of the bead preparation protocol is shown in Fig. 2A. Plasmid DNA is first linearized using restriction enzymes that produce sticky ends, and then the ends are filled in using the Klenow fragment of DNA polymerase. One end is filled in with thio nucleotides to prevent exonuclease digestion, and the other is filled in with biotin-conjugated nucleotides, which allows coupling to streptavidin-coated dynabeads. After an overnight binding reaction, beads are washed and resuspended in bead buffer and can be stored at 4°C indefinitely.

1. Purify plasmid DNA using Qiagen column purification or a similar method. In theory, any plasmid can be used for coupling to the beads because the sequence is not important, although the length of the linearized DNA should be greater than 5 kb to ensure efficient chromatin assembly on the beads. In addition, a plasmid should be

A. DNA bead preparation



B. Bead spindle reaction

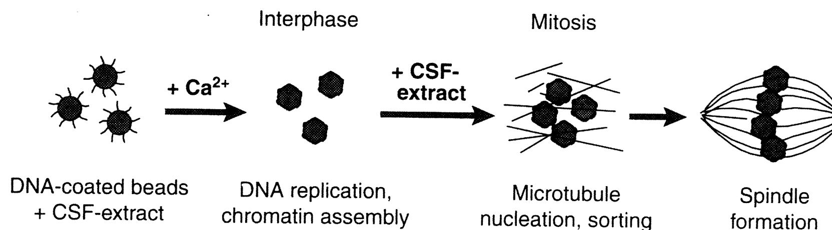


Fig. 2 Steps involved in noncentrosomal spindle assembly. (A) DNA bead preparation. (B) Bead spindle assembly.

chosen that has unique restriction enzyme sites in the poly linker. One enzyme should leave an overhang containing only Gs and Cs, while the other should also contain As and Ts. *Not*I and *Bam*HI are good choices.

2. Digest 50 μg of plasmid DNA to produce one short and one long (>5 kb) DNA fragment. Ethanol precipitate the DNA, resuspend in 25 μl TE, and quantify recovery by measuring the OD_{260}.

3. Set up the fill-in reaction in a total volume of 70 μl. This reaction contains 30 μg DNA, 1X Klenow buffer, 50 μM each nucleotide (biotin-dATP, biotin-dUTP, thio-dCTP and thio-dGTP), and 20 units Klenow fragment of DNA polymerase. Incubate at 37°C for 2 h.

4. Use Pharmacia nick columns to remove unincorporated nucleotides, following the supplied instructions. These columns allow better recovery than spin columns. Elute the DNA in 400 μl TE.

5. Set up the coupling mixture by combining the 400 μl of eluted biotinylated DNA and 400 μl binding solution (included in the Dynal Kilobase BINDER kit). Save 25 μl of this mixture for later determination of coupling efficiency.

6. Determine the volume of streptavidin dynabeads to use in the coupling reaction. Four microliters of dynabeads should be used for each microgram of DNA (120 μl beads for 30 μg DNA). Use the magnetic particle concentrator (MPC) to retrieve the beads, wash once with 5 volumes of binding solution, retrieve the beads again, and then resuspend them in the coupling mixture prepared in step 5.

7. Couple the DNA by incubating the bead/DNA mixture on a rotator at 16°C for several hours (the reaction can also be left overnight).

8. Retrieve the beads using the MPC and save the supernatant. Measure the OD_{260} of a 1:40 dilution of the supernatant and compare to the precoupling mixture to determine coupling efficiency. Two-thirds of the DNA is usually coupled.

9. Wash the beads twice with washing solution (Dynal kit) and twice with bead buffer (2 M NaCl, 10 mM Tris, 1 mM EDTA, pH 7.6). Resuspend the beads in bead buffer to a final concentration of 1 μg immobilized DNA for every 5 μl of beads. Some batches of beads may be clumpy. If so, pass the beads through a 27-gauge needle before use.

2. Spindle Assembly Reaction

A schematic of this protocol is shown in Fig. 2B. After resuspending DNA-coupled dynabeads in fresh CSF extract, the extract is cycled into interphase so that chromatin assembles on the beads, and then into mitosis to allow mitotic chromatin assembly. Resuspension of the chromatin beads in fresh CSF extract then supports efficient bead spindle assembly. The sticky nature of the DNA beads causes them to aggregate into differently sized clumps. Clumps of 10–20 beads are optimal, as this corresponds to the amount of DNA in a single *Xenopus* sperm nucleus (Heald *et al.,* 1996). Although aggregate size cannot be controlled precisely, excessive clumping can be prevented by frequent mixing.

1. Pipette 3 μl of DNA beads (approximately 0.5 μg DNA) into a 0.5-ml microcentrifuge tube and place on ice. Collect the beads on a MPC magnet, remove the supernatant, and wash the beads by resuspending them in 20 μl of CSF extract. Retrieve the beads on the magnet, remove the supernatant again, and resuspend in 100 μl fresh CSF extract.

2. Transfer the reaction to a 1.5-ml microcentrifuge tube and incubate at 20°C.

3. After 10 min of incubation, add 10 μl of 10× calcium solution (4 m*M* $CaCl_2$, 100 m*M* KCl, 1 m*M* $MgCl_2$) and mix with a cutoff pipette tip to release the extract into interphase. Incubate for 2 h at 20°C, mixing every 20–30 min to reduce clumping.

4. Add 50 μl of fresh CSF extract to return the extract reaction to mitosis. Incubate for an additional 30 min at 20°C.

5. Incubate the extract reaction on ice for a few minutes and then place the mixture on a magnet for 10–15 min to retrieve the chromatin beads. This retrieval step is slow because the extract is viscous. Pipetting the extract every few minutes while keeping the tube on a magnet accelerates bead retrieval.

6. Keeping the tube on the magnet, remove the extract and verify that the beads have been retrieved. Resuspend the beads in 100 μl of fresh CSF extract containing rhodamine-labeled tubulin (1/200 dilution of stock).

7. Incubate the reaction at 20°C and monitor spindle assembly by transferring 1-μl samples to a microscope slide. Overlay with 5 μl spindle fix and squash with a coverslip as described previously. Spindles can sometimes be seen after 30 min, although in some extracts the reaction takes as long as 90 min.

8. Squash samples can be stored at 4°C after sealing the coverslips with nail polish or the reactions can be spun down onto coverslips as described in Section VI.

V. Studying Proteins Involved in Spindle Assembly

The roles of proteins involved in spindle assembly can be evaluated easily using *Xenopus* egg extracts because the extracts are open to manipulation. Four approaches to inhibit the function of a protein in the extract are dominant-negative protein addition, antibody addition, immunodepletion, and drug addition. A good example of the first approach is the addition of a subunit of the dynactin complex, p50/dynamitin, to inhibit dynein function (Echeverri *et al.*, 1996; Wittmann *et al.*, 1998). A protocol for this method is provided in Wittmann and Hyman (1999). Second, the addition of antibodies to extract has been used successfully to inhibit a wide variety of proteins, including many microtubule-based motors (Boleti *et al.*, 1996; Heald *et al.*, 1996; Vernos *et al.*, 1995). Methods for this approach are discussed in Desai *et al.* (1999).

This chapter concentrates on the other two methods to inhibit protein function, immunodepletion, and drug addition. Both are powerful ways to analyze the function of

proteins in the extract. We first provide modifications to published protocols that we use for immunodepletion in order to minimize the loss of extract activity. We then discuss the use of chemical compounds to inhibit extract proteins and provide a protocol for screening drugs to identify inhibitors of spindle assembly. Using chemical compounds to disrupt the function of proteins has proven to be an effective way of studying cell division processes (Gray *et al.*, 1998; Mayer *et al.*, 1999; Rosania *et al.*, 2000). In addition, the ability to couple drugs to affinity matrices and identify their targets biochemically represents a powerful new approach to identify proteins involved in mitosis (Rosania *et al.*, 1999).

A. Materials

20X demembranated sperm nuclei, 2 × 10^6/ml stock

Materials used for aster reactions (Section III,A)

Immunodepletion:

- Protein A-coated dynabeads
- TBS/0.1% Triton X-100
- CSF-XB: 10 m*M* HEPES, pH 7.7, 2 m*M* $MgCl_2$, 0.1 m*M* $CaCl_2$, 100 m*M* KCl, 5 m*M* EGTA, 50 m*M* sucrose
- 1000X protease inhibitors: 10 mg/ml leupeptin, pepstatin, and chymostatin dissolved in DMSO and stored at −20°C

Drug addition:

- Compounds in 1–10 m*M* stocks, dissolved in DMSO

B. Immunodepletion of Extracts

Immunodepletion has been used to inhibit the function of many extract proteins, including Eg5, a tetrameric kinesin-like protein involved in cross-linking and organizing microtubules in the spindle (Sawin *et al.*, 1992; Walczak *et al.*, 1998). Following Eg5 depletion from *Xenopus* egg extracts, spindles are unable to form around DNA-coated beads and instead form monopolar astral arrays (Walczak *et al.*, 1998), as shown in Fig. 3F.

Good protocols for immunodepletion have been published elsewhere (Desai *et al.*, 1999). We use a modification of this protocol, designed to minimize disruption of the extract. While most immunodepletion protocols utilize protein A-coated Affiprep beads as a solid support, protein A-coated dynabeads offer the advantage that they are small and remain resuspended in the extract without constant rotating, thereby causing minimal disruption of the extract. After the addition of antibody-coated dynabeads to the extract, beads are mixed by flicking the tube gently and then the reaction is incubated on ice. Dynabeads have been used successfully for immunodepletion by other groups (Funabiki and Murray 2000; Wittmann *et al.*, 2000) and our protocol is similar to those published.

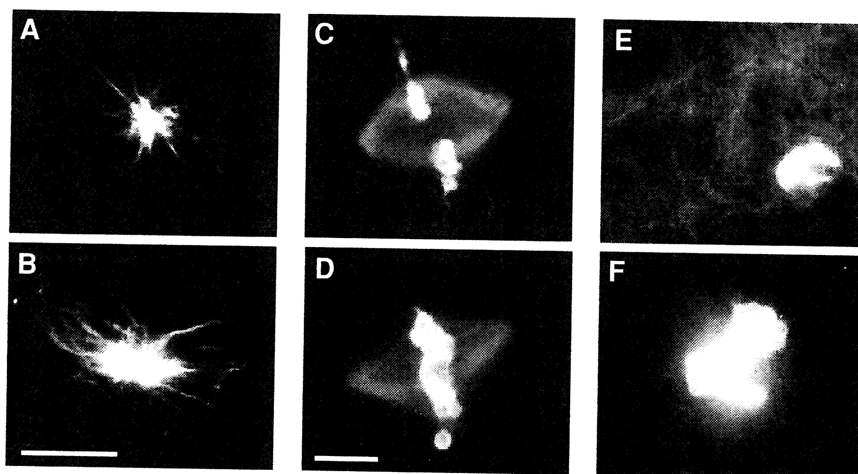


Fig. 3 Examples of microtubule aster and spindle reactions visualized by fluorescence microscopy. (A) Centrosomal aster. (B) DMSO aster. (C) Sperm spindle. (D) DNA bead spindle. (E) Sperm spindle reaction performed in the presence of 200 μM NG97, which inhibits cdk1. (F) DNA bead spindle assembled in *Xenopus* egg extract depleted of Eg5. Scale bar in A and B is 5 μm, bar in C–F is 10 μm. Images in C–F are overlays of microtubule staining (gray) and DNA staining (bright).

1. Couple 2–10 μg of affinity-purified antibody or control IgG to 25 μl protein A-coated dynabeads. The amount of antibody needed will depend on the nature of the antibody and epitope. Many antibodies are effective at 2-μg levels, whereas others may require multiple rounds of immunodepletion and higher amounts. The amount of antibody used should be optimized for each depletion. On ice, set up the coupling mixture in 200 μl total volume, containing affinity-purified antibody or control IgG, diluted in TBS/0.1% Triton X-100. Transfer 25 μl of protein A-coated dynabeads to a 0.6-ml low-retention microcentrifuge tube, wash twice with TBS/0.1% Triton X-100, and then add the coupling mixture. Rotate the mixture at 4°C for 2 h or overnight.

2. Retrieve the beads on a magnet (MPC) and remove the supernatant. Wash once with TBS/0.1% Triton X-100 and then four times with CSF-XB containing 1X protease inhibitors. For each wash, resuspend the beads in the wash buffer and then retrieve on the MPC.

3. After the last wash, remove as much of the wash buffer as possible using a thin gel-loading pipette tip. Resuspend the beads in 75 μl CSF extract by gently pipetting with a wide-orifice pipette tip.

4. Incubate on ice for 90–120 min. Mix the reaction every 15 min by flicking gently.

5. Retrieve the beads using the MPC for 10–15 min as described in the bead spindle protocol. Transfer the supernatant to a fresh tube, taking care to avoid the transfer of beads. Use this extract as the depleted extract, and set up aster reactions or spindle assembly reactions as described previously.

Note: This protocol can be used to generate enough depleted extract for aster or half-spindle reactions. If cycling reactions are to be used, the volumes should be scaled up accordingly.

C. Screening of Chemical Compounds in *Xenopus* Egg Extracts

Chemical compounds that can be used to inhibit specific protein function are powerful tools to study complex processes in extracts and in cells. This approach has been used successfully by many groups, including Mayer and colleagues (1999), who identified a specific inhibitor of Eg5, monastrol, using a cell-based screen. Inhibition of Eg5 using monastrol induces a phenotype similar to immunodepletion. Many chemical inhibitors are available commercially, including antimitotic drugs such as colchicine, nocodazole, and taxol. Methods for the use of these drugs are provided in Jordan and Wilson (1999). In addition, many laboratories are currently synthesizing libraries of diverse chemical compounds for use in biological screens, and many potent inhibitors have been identified (Chang *et al.,* 1999; Gray *et al.,* 1998; Haggarty *et al.,* 2000). This section describes methods to screen for inhibitors of spindle assembly in *Xenopus* egg extracts.

Libraries of chemical compounds can be screened using any of the assays discussed in this chapter. Addition of compounds to aster reactions, sperm spindle reactions, or bead spindle reactions is a good way to identify inhibitors of microtubule nucleation, growth, and organization. To simplify the screening process, we use either a simple aster reaction or a half-spindle reaction (described later) as a primary screen, as these protocols are simpler and quicker than cycled spindle assembly. Interesting compounds can then be tested in other assays.

Compounds can be added to the extract at a range of concentrations, and different compounds will have different potencies. When screening compounds, one concentration should be chosen for all compounds so that comparisons between them can be made easily. In determining a concentration, it is important to consider the goal of the screen. A low concentration (1–10 μM) will yield few hits, but the compounds identified will be potent inhibitors. Screening at higher concentrations (100 μM–1 mM) will yield more hits, but the compounds pulled out of the screen will be less potent. We typically screen compounds at a final concentration of 100 μM in the extract and, after interesting compounds are identified, titrations are performed to determine compound efficacy. Compounds can be diluted and stored in a variety of solvents. We use anhydrous DMSO because many compounds are stable in this solvent, and the addition of DMSO to the extract at low dilutions results in minimal disruption of microtubule morphology.

One compound that has a strong effect on spindle assembly in *Xenopus* egg extracts is NG97, which alters the cell cycle state of the extract. NG97 is an inhibitor of cdk1 and therefore causes CSF extracts to enter interphase, resulting in chromosome decondensation, increased microtubule stability, and reduction of histone H1 kinase activity (Rosania *et al.,* 1999), as shown in Fig. 3E. The following protocol is used to test the effects of NG97 and other inhibitors.

1. Compounds are typically added to the extract at a 1:100 dilution. Predilute compounds in DMSO to an appropriate concentration (for an extract concentration of 100 μM, compound stocks should be 10 mM). If a range of concentrations is being used, it is important to predilute the compound stocks in DMSO so that the volume of DMSO added to each extract reaction is the same for all reactions.

2. Aliquot 0.25 μl of each compound into a prelabeled 1.5-ml microcentrifuge tube. Set up a DMSO-only control also. Do not place these tubes on ice, as the DMSO will solidify.

3. On ice, combine the CSF extract, rhodamine tubulin (1:200 dilution), and sperm nuclei (1:20 dilution). Allow 25 μl of extract mixture for each compound, and overestimate slightly to compensate for losses that occur when aliqouting.

4. Using a cutoff pipette, transfer 25 μl of the extract mixture to each tube and mix by pipetting. Immediately place the tubes at 20°C.

5. Take squash samples at a defined time point between 45 and 60 min as described previously. At these time points, bipolar spindles should have formed. It does not matter which time point is used as long as it is consistent between screens. Due to variability in squash samples, two squashes should be done for each reaction. Mix each reaction with a cutoff pipette tip before squashing.

6. Visually screen through the squash samples to identify potential inhibitors of spindle assembly. Compounds of interest should be reassayed with a second extract to confirm results.

7. For a further characterization of compounds, the assays and compound concentrations can be varied. In addition, compounds can be added to the reactions after incubation at 20°C to determine effects on preformed spindles.

VI. Methods to Observe Spindle Assembly

The simplest way to monitor spindle assembly reactions is to take small squash samples as described in previous protocols. However, entire reactions can also be transferred onto glass coverslips, fixed, and mounted on microscope slides for long-term storage. This procedure also allows the samples to be subjected to immunostaining techniques. Protocols for this method are given in Desai *et al.* (1999), and because our procedure is similar, this section only provides a brief overview.

First, reactions are diluted into 1 ml of spin down dilution buffer [30% glycerol, 1% Triton X-100, 1X BRB80 (80 mM PIPES, 1 mM MgCl2, 1 mM EGTA, pH 6.8) for spindle reactions; 15% glycerol, 1% Triton X-100, 1X BRB80 for aster reactions]. This mixture is then layered gently onto a 5-ml spin-down cushion (40% glycerol, 1X BRB80 for spindle reactions; 25% glycerol, 1X BRB80 for aster reactions) in a modified Corex tube containing a 12-mm round coverslip. Centrifugation is performed in a swinging bucket rotor at 16°C, which pellets insoluble structures such as microtubule asters and spindles, but removes soluble material, including unincorporated rhodamine tubulin subunits. For this reason, background fluorescence is reduced significantly. Coverslips

are then fixed in methanol at −20°C, washed in PBS/0.1% Nonidet P-40, subjected to immunofluorescence if desired, and stained with Hoechst dye. The coverslips are then mounted on microscope slides using mounting media (90% glycerol, 10% 0.2 *M* Tris–HCl, pH 8) and sealed with nail polish.

VII. Conclusions

This chapter presented methods for studying centrosome-independent spindle assembly using *Xenopus* egg extracts. The protocols described include microtubule aster reactions, bead spindle reactions, and inhibition of extract proteins. This system is ideal for studying mechanisms of self-organization, including microtubule nucleation around chromatin and motor-dependent function. The value of these methods extends beyond the study of microtubule self-organization, however. Both centrosome-mediated and centrosome-independent spindle assembly share mechanisms in common. Although centrosomes constitute visible and dominant organizing centers when they are present, motor functions are superimposed and are crucial for spindle organization, as they are when centrosomes are absent. Therefore, the study of both types of spindle assembly is important to understand the principles of microtubule organization in mitosis and meiosis.

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