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Visualizing and Analyzing Branching Microtubule Nucleation Using Meiotic Xenopus Egg Extracts and TIRF Microscopy

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Abstract

Mitotic and meiotic spindles consist primarily of microtubules, which originate from centrosomes and within the vicinity of chromatin. Indirect evidence suggested that microtubules also originate throughout the spindle, but the high microtubule density within the spindle precludes the direct observation of this phenomenon. By using meiotic *Xenopus laevis* egg extract and employing total internal reflection (TIRF) microscopy, microtubule nucleation from preexisting microtubules could be demonstrated and analyzed. Branching microtubule nucleation is an ideal mechanism to assemble and maintain a mitotic spindle, because microtubule numbers are amplified while preserving their polarity. Here, we describe the assays that made these findings possible and the experiments that helped identify the key molecular players involved.

Keywords

Cell division; Mitotic spindle; Meiotic spindle; Cytoskeleton; Microtubule; Microtubule nucleation; *Xenopus laevis* egg extract; TIRF microscopy

1 Introduction

The mitotic spindle is assembled from several microtubule (MT) organizing centers, most prominently centrosomes and chromosomes, all of which require the universal MT nucleating molecule gamma-tubulin (γ-TB). More recently, MTs were also found to originate from within the body of the spindle [1, 2], and this was shown to be important for spindle assembly [3, 4]. Furthermore, a targeting factor for γ-TB to spindle MTs was identified in a whole-genome RNAi screen and termed augmin, as it increases the MT density within the spindle [5–8]. Yet the high MT density in the spindle precludes direct observation MT nucleation events, leaving the question of how MTs are generated within the body of the spindle open.

This roadblock could be circumvented by using meiotic *Xenopus* egg extract, which allowed resolving individual MTs with a low background by total internal reflection (TIRF) microscopy [9]. By simultaneously imaging MTs and their growing MT plus ends via fluorescently labeled tubulin and end-binding protein 1 (EB1), respectively, it was directly demonstrated that MTs originate from the wall of preexisting MTs. Addition of recombinant proteins and immunodepletion of endogenous proteins revealed the key molecular players to be augmin, γ-TB, RanGTP and its downstream factor “targeting factor of Xklp2” (TPX2)

[9]. The goal of this chapter is to describe the assay and its variations that first characterized branching MT nucleation and its molecular players in *Xenopus* egg extract.

2 Materials

2.1 Proteins and Antibodies

2.1.1 Unlabeled and Fluorescently Labeled Tubulin

1. Purified and polymerization competent tubulin [10].
2. Biotinylated tubulin [11].
3. Alexa 568-labeled tubulin.
4. Cy5-labeled tubulin.

2.1.2 Recombinant Proteins for Addition to Extract

1. GFP-labeled H.s. End-Binding protein 1 (EB1).
2. X.l. Ran^{Q69L}.
3. X.l. TPX2.

2.1.3 Rabbit Polyclonal Antibody Production and Purification for Immunodepletion

1. Polyclonal antibody serum specific against protein of interest.
2. Affigel-10 and -15 matrix (Bio-Rad).
3. Glycerol.

2.2 Branching MT Nucleation Assay in Xenopus Egg Extract

2.2.1 Preparation of Xenopus Egg Extracts

1. Freshly prepared *Xenopus laevis* CSF extract of high quality (*see* Note 1).

2.2.2 Flow Chamber Assembly

1. Frosted glass slides.
2. Double-sticky tape.
3. Razorblade.
4. No. 1.5 glass coverslips (22 × 22 mm).

2.2.3 Basic Branching Reaction

1. CSF-XB buffer (10 mM K-HEPES pH 7.7, 2 mM $MgCl_2$, 0.1 mM $CaCl_2$, 100 mM KCl, 5 mM EGTA, and 50 mM sucrose).

[1]Only freshly prepared *Xenopus laevis* CSF extract of high quality remains arrested in meiosis II and displays microtubule behavior necessary for spindle assembly.

2. Hot candle wax or nail polish.
3. Objective-based TIRF microscope equipped with a 100× 1.49 NA objective, a low noise EM-CCD camera and laser power of at least 20 mW out-of-fiber.

2.2.4 Variations of the Basic Branching Reaction

1. Sodium orthovanadate (100 mM, NEB) or purified p150-CC1 (*see* Note 2).

2.2.5 Immunodepletion of CSF Extract and Add-Back of Recombinant Protein

1. Dynabeads Protein A (Invitrogen).
2. DynaMag-2 Magnet (Invitrogen) for retrieving Dynabeads.
3. TBS-T (50 mM Tris–HCl, 150 mM NaCl, 0.05 % Tween 20).
4. Antibody of desired specificity and control total IgG fraction.
5. CSF-XB buffer (10 mM K-HEPES pH 7.7, 2 mM $MgCl_2$, 0.1 mM $CaCl_2$, 100 mM KCl, 5 mM EGTA, and 50 mM sucrose).
6. SDS-sample buffer, SDS-PAGE and Western blot equipment.
7. Recombinant protein that can replace the immunodepleted endogenous protein.

2.3 Branching MT Nucleation from Stabilized MT Seeds

1. Passivated coverslips [12].
2. GpCpp/GMPCPP (Jena Bioscience).
3. BRB80 buffer (80 mM K-PIPES, 1 mM $MgCl_2$, 1 mM EGTA, pH 6.8 with KOH).
4. Anti-biotin antibody (Invitrogen).
5. Kappa-casein (Sigma).
6. Oxygen scavenger system consisting of Trolox, protocatechuate 3,4-dioxygenase (PCD) and protocatechuic acid (PCA) according to Ref. [13] (all Sigma).

3 Methods

3.1 Proteins, Antibodies, and Xenopus Egg Extract

3.1.1 Unlabeled and Fluorescently Labeled Tubulin (See Note 3)

1. Tubulin is purified from bovine brain using two cycles of polymerization–depolymerization [10].

[2]Sodium orthovanadate and p150-CC1 are both inhibitors of the minus-end directed motor protein cytoplasmic dynein.
[3]Alternatively, unlabeled and labeled tubulin can be obtained from Cytoskeleton, Inc. if necessary.

2. Fractions of purified tubulin are then fluorescently labeled [11] with 6((biotinoyl)amino) hexanoic acid, succinimidyl ester (Invitrogen), Alexa 568 carboxylic acid, succinimidyl ester (Invitrogen) and Cy5 NHS ester (GE). Briefly, MTs are polymerized and pelleted before being incubated with the dye at 37 °C. Labeled MTs are pelleted and depolymerized on ice. Dimeric, labeled tubulin is separated from polymerized MTs through another pelleting step at 4 °C and subsequently flash-frozen in small aliquots at 200 μM.

3.1.2 Recombinant Proteins for Extract Addition

1. GFP-labeled H.s. End-Binding protein 1 (EB1), X.l. Ran^{Q69L}, and X.l. TPX2 are purified as recently described [14–16].
2. All proteins are dialyzed into CSF-XB buffer containing 10 % sucrose, which does not disturb microtubule assembly dynamics in *Xenopus laevis* egg extract. During this process, the sample is further concentrated and introduced to sucrose, which serves as a cryopreservant.
3. Immediately after purification and dialysis, all proteins are flash frozen in small aliquots at concentrations of 20 μM (TPX2), 220 μM (Ran^{Q69L}), and 10 mg/mL (GFP-EB1).

3.1.3 Rabbit Polyclonal Antibody Production and Purification for Immunodepletion

1. Polyclonal antibodies for immunodepletion are generated at a commercial offsite facility, here Covance. The antigen is ideally the complete protein or protein domains, instead of peptides, to generate high affinity antibodies, as was performed for TPX2 and the augmin subunit Dgt4. Purified antibody against gamma-tubulin's C-terminus was a gift from Christiane Weise [17].
2. Couple antigen covalently to a matrix, such as Affigel matrix (Bio-rad) used here.
3. Perform immuno-affinity purification to isolate the antibody from the serum.
4. Supplement antibodies with 20 % glycerol before flash-freezing them in small aliquots.

3.1.4 Xenopus Egg Extract

1. Preparation of Xenopus egg extracts have previously been described in great detail with complete lists of reagents required for mitotic arrested Xenopus egg extracts [18–20]. We precisely follow all steps outlined in Ref. [19]. In order to adapt the procedure to modern equipment, the packing spin during which the eggs are concentrated without lysing is no

longer conducted in a clinical centrifuge, but a table centrifuge at 150 × *g* for 60 s followed by 598 × *g* for 25 s.

3.2 Branching MT Nucleation Assay in Xenopus Egg Extract

3.2.1 Preparation of Xenopus Egg Extracts—*Xenopus laevis* CSF extract is prepared by the standard method (Murray [18]). Freshly made extract should be stored on ice and must not subjected to shearing forces during pipetting. Extract can either be directly used (*see* Subheading 3.2.2) or first subjected to immunodepletion (Subheading 3.2.5) before its test in the branching assay (*see* Note 4).

3.2.2 Flow Chamber Assembly—Assemble a flow chamber as depicted in Fig. 1 consisting of a microscope slide, two double-sticky tape strips separated by 2–4 mm, and a glass coverslip. The chamber volume should be approximately 5 μL.

3.2.3 Basic Branching Reaction (Fig. 1)

1. Pipette 7.5 μL CSF extract into a 1.5 mL tube on ice without shearing the extract via pipetting.
2. Add Alexa 568-labeled tubulin to a final concentration of 0.86 μM to visualize MTs (*see* Note 5).
3. Add EB1-GFP to a final concentration of 0.8 μM to visualize growing MT plus ends.
4. Add CSF-XB to obtain a final sample volume of 10 μL.
5. Mix the reaction mixture by gently pipetting it up and down once.
6. Pipette 5 μL of the reaction mixture into the flow cell to fill the channel without introducing bubbles.
7. Quickly seal the sample chamber with hot candle wax or nail polish.
8. Start imaging with an objective-based TIRF microscope equipped with a 100× 1. 49 NA objective, a low noise EM-CCD camera and laser power of at least 20 mW out-of-fiber.
9. Optimize imaging parameters (laser power and exposure time) to clearly observe MTs but prevent overexposure and thus photobleaching (*see* Note 6).
10. Acquire frames every 2 s for 30 min.

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[4] *Xenopus* CSF extract only remains active for short periods of time (typically 10 h for good extract). Therefore, it is critical to plan the experiments well and minimize any delays in the procedures.

[5] *Xenopus* CSF extract must not be diluted. When choosing the stock concentration of a reagent to be added, maximize the concentration and balance it with the smallest volume that can be added accurately (~0.5 μL).

[6] TIRF angle, laser power and exposure time need to be adjusted with the first sample in order to clearly observe dynamic MTs within a bright fluorescent background. Once that is achieved, laser power and exposure time should further minimized while keeping MTs visible to prevent overexposure of the sample, which may lead to photobleaching during the course of the reaction.

11. Collect a stitched slide overview of fields of view using the slide explorer function in uManager [21] at equal time points (for example after 15 or 30 min; *see* Note 7).

3.2.4 Variations of the Basic Branching Reaction (Fig. 1)

1. Replace 0.5 μL CSF-XB buffer with 0.5 μL vanadate (10 mM) for a final concentration of 0.5 mM or p150-CC1 to a final concentration of 0.125 mg/mL to inhibit dynein and prevent MT gliding on glass.
2. Replace 0.5 μL CSF-XB buffer with Ran^{Q69L} to for a final concentration of 11 μM to release endogenous spindle assembly factors from importins, such as TPX2, and induce branching MT nucleation.
3. Replace 0.5 μL CSF-XB buffer with TPX2 to a final concentration of 1 μM to induce branching MT nucleation.

3.2.5 Immunodepletion of CSF Extract and Add-Back of Recombinant Protein (Fig. 1)

1. Wash 150 μL slurry of magnetic Dynabeads 3× in 300 μL TBS-T.
2. Couple 36 μg total antibody to washed Dynabeads overnight at 4 °C. Use total IgG fraction as a control.
3. Wash off unbound antibody with 2× 150 μL TBS-T and 2× 150 μL CSF-XB.
4. Split beads into three 50 μL fractions. Keep two on ice, remove CSF-XB from one and add 50 μL of CSF extract after setting aside an extract gel sample.
5. Incubate extract with beads for 45 min on ice and gently mix every 10 min by flicking the tube or pipetting up and down with a wide bore pipette.
6. Retrieve beads completely on a magnet for 5 min and retrieve a gel sample of extract after this first round of immunodepletion.
7. Remove CSF-XB from the second bead batch and transfer extract from first to second tube for a second round of immunodepletion.
8. Repeat one more time for a third round of immunodepletion (*see* Note 8).
9. Perform imaging and observe phenotype.
10. Add-back recombinant protein to immunodepleted extract at similar concentrations as the endogenous version and take a gel sample of the final reaction mixture to verify this. Perform imaging and observe phenotype.

[7] uManager's slide explorer function is useful for quantifying the number of fan structures on a slide and to assess local variation within a flow chamber.
[8] High-affinity antibodies can deplete the protein of interest within two rounds of immunodepletion.

11. Prepare SDS-PAGE gel samples by mixing 2 μL of extract with 48 μL of 1× loading buffer. Perform Western blot for immunodepleted proteins and controls, and determine extent of depletion.

3.2.6 Determining Branch Angle and Measuring MT Nucleation Kinetics (See Note 9)

1. Use ImageJ [22] for quantitating branch angle between the daughter and mother microtubules. A branch angle of 0° corresponds to parallel growth.
2. Use ImageJ [22] to duplicate the EB1 channel and apply a Gaussian Blur function with a low [1] and a high [5] radius of decay for each one.
3. Subtract the latter image sequence from the former.
4. Set a threshold to eliminate background noise before counting particles for each time frame with the Analyze Particles function in ImageJ (*see* Note 10).

3.3 Branching MT Nucleation from Stabilized MT Seeds

1. Coverslips are cleaned and passivated with dichlorodimethylsilane as described [12].
2. Prepare GMPCPP-stabilized MT seeds, which are biotinylated and fluorescently labeled by mixing porcine brain tubulin (1.7 mg/mL final concentration), Cy5-labeled tubulin (0.19 mg/mL final concentration, ~30 % labeled), biotin-tubulin (0.19 mg/mL final concentration), and GMPCPP (1 mM final concentration) in BRB80 buffer and incubate at 37 °C for 1 h. Store at room temperature and in the dark until use.
3. Prepare a flow channel with passivated coverslips.
4. Flow in 50 μL of 0.1 mg/mL anti-biotin antibody in BRB80 and incubate for 5 min.
5. Flow in 50 μL of BRB80 to wash the chamber.
6. Flow in 50 μL kappa-casein (1 mg/mL) and incubate for 5 min.
7. Wash flow chamber with 50 μL of BRB80.
8. Flow in 50 μL MT seeds diluted 1/500 in BRB80 and incubate for 5 min.
9. Wash in 50 μL BRB80 with an oxygen scavenger system.
10. Check MT seeds for desired density with TIRF microscopy.
11. Flow in CSF extract with desired factors to induce branching (*see* Note 11).

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[9] For this analysis, it is critical that the background signal is as low as possible. Usually this is only achieved with extract of highest quality.

[10] Make sure that each particle detected corresponds to the EB1 signal at the growing MT ends. Each MT, even when branched, only has one EB1 comet and therefore this method directly counts MT number over time.

[11] An additional step is to wash out the extract and flow in tubulin, which can branch from the attached seeds.

12. Image as detailed above.

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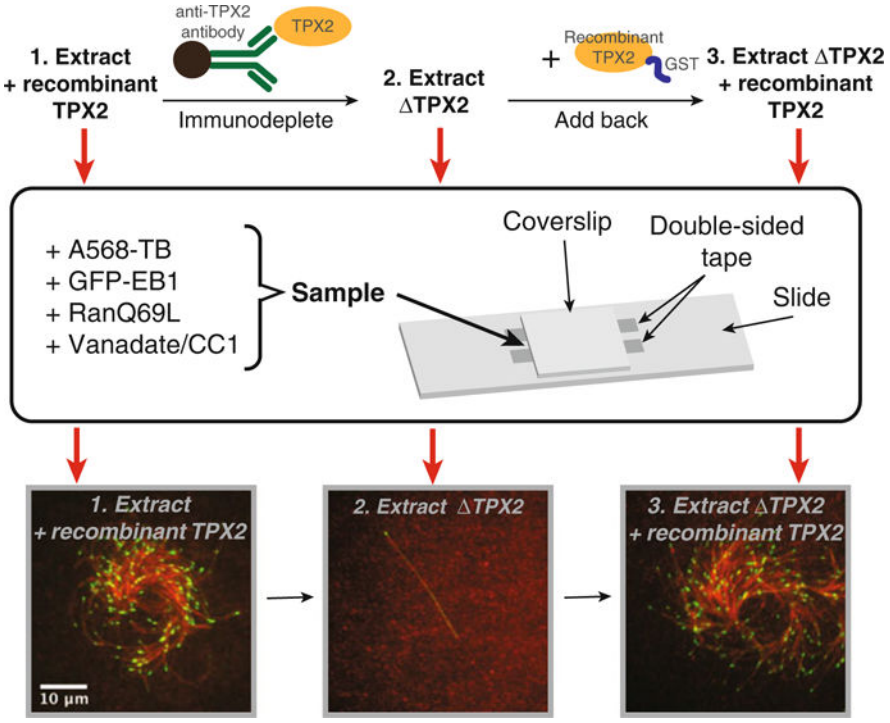

Fig. 1.

Overview of the basic branching MT nucleation assay, which is performed with Xenopus egg extract. Proteins of interest, such as TPX2, can be immunodepleted to assess their phenotype and role. Recombinantly expressed and purified proteins can then be added back to the depleted extract to test whether they complement the depleted function