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A novel immunofluorescence method to visualize microtubules in the antiparallel overlaps of microtubule-plus ends in the anaphase and telophase midzone

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ABSTRACT

Cell division, in which duplicated chromosomes are separated into two daughter cells, is the most dynamic event during cell proliferation. Chromosome movement is powered mainly by microtubules, which vary in morphology and are organized into characteristic structures according to mitotic progression. During the later stages of mitosis, antiparallel microtubules form the spindle midzone, and the irregular formation of the midzone often leads to failure of cytokinesis, giving rise to the unequal segregation of chromosomes. However, it is difficult to analyze the morphology of these microtubules because microtubules in the antiparallel overlaps of microtubule-plus ends in the midzone are embedded in highly electron-dense matrices, impeding the access of anti-tubulin antibodies to their epitopes during immunofluorescence staining. Here, we developed a novel method to visualize selectively antiparallel microtubule overlaps in the midzone. When cells are air-dried before fixation, aligned α-tubulin staining is observed and colocalized with PRC1 in the center of the midzone of anaphase and telophase cells, suggesting that antiparallel microtubule overlaps can be visualized by this method. In air-dried cells, mCherry-α-tubulin fluorescence and β-tubulin staining show almost the same pattern as α-tubulin staining in the midzone, suggesting that the selective visualization of antiparallel microtubule overlaps in air-dried cells is not attributed to an alteration of the antigenicity of α-tubulin. Taxol treatment extends the microtubule filaments of the midzone in air-dried cells, and nocodazole treatment conversely decreases the number of microtubules, suggesting that unstable microtubules are depolymerized during the air-drying method. It is of note that the air-drying method enables the detection of the disruption of the midzone and premature midzone formation upon Aurora B and Plk1 inhibition, respectively. These results suggest that the air-drying method is suitable for visualizing microtubules in the antiparallel overlaps of microtubule-plus ends of the midzone and for detecting their effects on midzone formation.

1. Introduction

Microtubules are a major cytoskeleton filament in the cell and play essential roles in various intracellular processes, including the transport of membrane vesicles in the cytoplasm, cell motility, and chromosome movement during cell division [1–3]. Microtubules are polymers of a heterodimer of α- and β-tubulin, and have polarity; the end where β-tubulin is exposed is referred to as the plus end, and the opposite end, where α-tubulin is exposed, is referred to as the minus end [4]. The polymerization rate is higher at the plus end than at the minus end [5]. However, polymerization and depolymerization dynamics can be modified by microtubule-associated proteins to accomplish the biological functions of microtubules, and the regulation of these dynamics is pivotal to microtubule function [6–10].

Cell division, in which duplicated DNA is separated into two daughter cells, is the most dynamic event during cell proliferation. The mitotic spindle is the apparatus by which chromosomes are segregated and is composed of chromosomes and microtubules, by which chromosome movement is mainly powered. Microtubules vary in their morphology and are organized into characteristic structures according to mitotic progression. From the entry into mitosis to just before the onset of anaphase, microtubules are organized into three different types: kinetochore, astral, and interpolar microtubules. During anaphase and telophase, the spindle midzone, which consists of antiparallel microtubule bundles, forms and plays crucial roles in positioning and signaling for the assembly of the contractile ring. At the last step of cell

Abbreviations: CE, contrast-enhanced; DIC, differential interference contrast

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division, cytokinesis, the midbody forms and regulates the timing of abscission. A variety of microtubule-bound proteins organize microtubule structures by regulating the polymerization, depolymerization, and bundling of microtubules [11]. PRC1 [12], centralspindlin [13–15], CLASP [16–18], MKLP2 [19,20], and KIF4 [21–24] are core organizers of the spindle midzone and midbody, and are regulated by upstream kinases [25,26].

During mitosis, different populations of microtubules have distinct polymerization and depolymerization dynamics and stabilities. When centrosomes are positioned closely at the beginning of cell division, the kinesin Eg5 tethers the antiparallel microtubules and a pushing force generated by the slide of polymerizing antiparallel microtubules separates centrosomes [27–29]. Together with chromosome condensation and breakdown of the nuclear envelope, microtubules nucleated from centrosomes radiate microtubule-plus ends to capture chromosomes. By repeatedly extending and shrinking, namely, switching between polymerization and depolymerization, respectively, microtubules search for chromosomes. Once microtubules capture chromosomes, microtubules are stabilized [30]. Compared to astral microtubules, kinetochore microtubules are relatively stable against stimuli promoting depolymerization, such as low concentrations of nocodazole, a microtubule-depolymerizing agent [31], and low temperatures [32–34]. In anaphase B, the slide of elongating antiparallel microtubules of the midzone in opposite directions makes centrosomes move apart, resulting in spindle elongation [35–37]. At the final phase of cell division, microtubules of the central spindle and midbody are stabilized by their rigid bundling [12].

The microtubules in the spindle midzone play prerequisite roles in cytokinesis, and the midbody regulates abscission timing. Irregular formation of the spindle midzone and midbody during late mitosis often leads to a failure in cytokinesis, giving rise to the unequal segregation of chromosomes through the following cell cycle [38–42]. Thus, close analysis of these characteristic structures will give important insights into the regulation of cell division, especially cytokinesis and abscission. However, the microtubules in the antiparallel overlaps of microtubule-plus ends (sometimes referred to as "stem bodies") and Flemming body in the midbody are embedded in highly electron-dense matrices [43,44], impeding anti-tubulin antibodies from accessing their epitopes on microtubules during immunofluorescence staining and making it difficult to analyze the morphology of the overlapping antiparallel microtubules [44]. In the present study, we developed a novel immunofluorescence method to visualize selectively microtubules in the antiparallel overlaps of microtubule-plus ends in order to analyze the regulation of the spindle midzone.

2. Materials and methods

2.1. Cells

Human cervix adenocarcinoma HeLa S3 cells (Japanese Collection of Research Bioresources, Osaka, Japan) and pig kidney epithelial LLC-PK1 cells expressing both mCherry-tubulin and GFP-MKLP1 (provided by K. Kamijo) [45,46] were cultured in Dulbecco's modified Eagle's medium containing 5% fetal bovine serum with 20 mM HEPES-NaOH (pH 7.4) under an atmosphere of 5% CO_2 at 37 °C.

2.2. Chemicals

To modulate microtubule dynamics, Taxol (Wako, Kyoto, Japan), a microtubule-stabilizing agent, and nocodazole (Wako, Osaka, Japan), a microtubule depolymerizing agent, were used at 30 ng/mL for 10 min and 0.1 μg/mL for 10 min, respectively. To inhibit mitotic kinases, the Aurora B inhibitor ZM447439 (JS Research Chemical Trading, Wedel, Germany), the Plk1 inhibitor BI2536 (AdooQ BioScience, Irvine, CA, USA), and the Cdk1 inhibitor RO-3306 (Calbiochem, San Diego, CA, USA) were used at 10 μM for 10 min, 0.1 μM for 10 min, and 8 μM for 20 h, respectively.

2.3. Antibodies

The following antibodies were used for immunofluorescence analysis: rat monoclonal anti-α-tubulin (1:1000–1:2000; MCA78G, Bio-Rad Laboratories, Hercules, CA, USA), rabbit monoclonal anti-β-tubulin (1:200; 9F3, Cell Signaling Technology, Danvers, MA, USA), rabbit polyclonal anti-PRC1 (1:300–1:500; H-70, Santa Cruz Biotechnology, Dallas, TX, USA), anti-γ-tubulin (1:200; Poly6209, BioLegend, San Diego, CA, USA), CREST, a human anti-centromere (1:400; HCT-0100, Immunovision, Springdale, AR, USA), and mouse monoclonal anti-AIM-1 (Aurora B) (1:300; 6/AIM-1, BD Biosciences, San Jose, CA, USA) antibodies. Alexa Fluor 488-, Alexa Fluor 555-, or Alexa Fluor 647-labeled donkey anti-mouse IgG, donkey anti-rabbit IgG, donkey anti-rat IgG, and FITC-anti-human secondary antibodies were used.

2.4. Immunofluorescence microscopy

The cells were fixed in phosphate-buffered saline (PBS) containing 4% formaldehyde for 20 min at room temperature, as described previously [47–49]. Alternatively, the culture medium was completely removed from the culture dish, and the cells were air-dried for 5 min, followed by fixation with PTEMF buffer (2 mM PIPES [pH 6.8], 0.2% Triton X-100, 10 mM EGTA, 1 mM $MgCl_2$, 4% formaldehyde) for 20 min at 30 °C. After fixation, the cells were permeabilized and blocked with PBS containing 0.1% saponin and 3% bovine serum albumin, and then incubated with primary and secondary antibodies. DNA was stained with 1 μM Hoechst 33342 for 1 h together with a secondary antibody. Fluorescence images were observed under an IX-83 fluorescence microscope (Olympus, Tokyo, Japan) equipped with a 60 × 1.42 NA oil-immersion objective (Olympus). The optical system for fluorescence observations included a U-FUNA cube (360–370 nm excitation, 420–460 nm emission) for observing Hoechst 33342 fluorescence, U-FBNA cube (470–495 nm excitation, 510–550 nm emission) for Alexa Fluor 488 fluorescence, U-FRFP cube (535–555 nm excitation, 570–625 nm emission) for Alexa Fluor 555 and mCherry fluorescence, and U-DM3-CY5 cube (600–650 nm excitation, 670–740 nm emission) for Alexa Fluor 647 fluorescence.

Confocal images were obtained using an LSM800 laser scanning microscope equipped with a 63 × 1.40 NA oil-immersion objective (Carl Zeiss, Jena, Germany). Hoechst 33342, Alexa Fluor 488, and Alexa Fluor 555 were excited with the 405-, 488-, and 561-nm line, and detected with 400–465 nm, 510–550 nm, and 570–620 nm emission filters, respectively.

Signal intensities of microtubule staining vary significantly according to mitotic sub-phase; therefore, imaging conditions were kept constant in each mitotic sub-phase but set independently of other subphases to avoid fluorescence overexposure. In addition, images in air-dried cells were contrast-enhanced (CE) to allow comparison of microtubles' morphology. Composite microscopic images were edited using ImageJ software (National Institutes of Health, Bethesda, MD, USA), GIMP software (https://www.gimp.org), and Illustrator CC software (Adobe, San Jose, CA, USA).

2.5. Cell synchronization

To synchronize the cells at the M phase, they were pre-arrested at the G2/M border by treatment with 8 μM RO-3306 for 20 h, washed with pre-warmed PBS (37 °C) supplemented with Ca^{2+} and Mg^{2+} (PBS [+]), and incubated in pre-warmed medium for 60 min, as described previously [49–51].

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