CHAPTER

16 Quantifying the polymerization dynamics of plant cortical microtubules using kymograph analysis

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Chapter outline

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Abstract

The plant cortical microtubule array is a dynamic structure that confers cell shape and enables plants to alter their growth and development in response to internal and external cues. Cells use a variety of microtubule regulatory proteins to spatially and temporally modulate the intrinsic polymerization dynamics of cortical microtubules to arrange them into specific configurations and to reshape arrays to adapt to changing conditions. To obtain mechanistic insight into how particular microtubule regulatory proteins mediate the dynamic (re)structuring of cortical microtubule arrays, we need to measure their effect on the dynamics of cortical microtubules. In this chapter, we describe new ImageJ plugins to generate kymographs from time-lapse images and to analyze them to measure the parameters that quantitatively describe cortical microtubule dynamics.

1 Introduction

Microtubules are long, tube-like polymers composed of 13 protofilaments of αβ-tubulin dimers and are an essential part of the cytoskeleton of all eukaryotic cells. The αβ-tubulin dimers assemble in a head-to-tail manner leading to microtubule polarity. The β-tubulin facing end of a microtubule is more dynamic and is referred to as the plus-end, whereas the α-tubulin facing end is less dynamic and is referred to as the minus-end. A distinctive feature of microtubule dynamics is that they switch randomly between periods of growth and shortening, a behavior referred to as dynamic instability (Mitchison & Kirschner, 1984). Cells control dynamic instability through a host of microtubule-associated proteins (MAPs) to regulate the assembly, disassembly, and function of microtubule arrays.

In plants, interphase microtubules form a cortical array beneath the plasma membrane that guides the deposition of cellulose microfibrils (Crowell et al., 2009; Gutierrez, Lindeboom, Paredez, Emons, & Ehrhardt, 2009; Paredez, Somerville, & Ehrhardt, 2006) and non-cellulosic wall polysaccharides (Kong et al., 2015; McFarlane, Young, Wasteneys, & Samuels, 2008; Takenaka et al., 2018; Zhu et al., 2015). Through these activities, cortical microtubules influence cell wall mechanics, which in turn determines growth anisotropy. Different cell types assemble morphologically distinct cortical microtubule arrays as they acquire their characteristic shapes (Szymanski, 2009). Cortical microtubule arrays are also responsive to developmental and environmental signals (Chen, Wu, Liu, & Friml, 2016) as well as mechanical stress imposed by the cell wall (Hamant et al., 2008; Robinson & Kuhlemeier, 2018; Sampathkumar et al., 2014; Verger, Long, Boudaoud, & Hamant, 2018) to enable plants to alter their growth to adapt to prevailing conditions. Therefore, patterning of the cortical microtubule array is fundamentally important to plant morphogenesis.

Seed plants lack centrosomes, and cortical microtubules are nucleated in a dispersed manner primarily from the lateral walls of preexisting cortical microtubules (Chan, Sambade, Calder, & Lloyd, 2009; Murata et al., 2005; Nakamura, Ehrhardt, &

Hashimoto, 2010; Wasteneys & Williamson, 1989). Newly created cortical microtubules are released from their nucleation sites and both their plus and minus ends are dynamic (Shaw, Kamyar, & Ehrhardt, 2003). Interactions between two or more cortical microtubules and between cortical microtubules and other cellular structures such as cell edges affect their dynamics and orientation (Ambrose, Allard, Cytrynbaum, & Wasteneys, 2011; Dixit & Cyr, 2004). A quantitative understanding of the polymerization dynamics of cortical microtubules is vital to decipher how these complex activities together affect the patterning of cortical microtubules. Quantitative data allow statistical comparisons to determine whether and how microtubule dynamics differ between cell types, developmental states and experimental treatments. Combined with mutant analysis and pharmacological perturbation, this approach provides a powerful means to uncover molecular mechanisms underlying cortical microtubule organization. A quantitative description of microtubule dynamics also facilitates the use of mathematical modeling to study array organization (Deinum & Mulder, 2013; Eren, Gautam, & Dixit, 2012).

Kymograph analysis is widely used to obtain quantitative data about microtubule dynamics from time-lapse image series (Zwetsloot, Tut, & Straube, 2018). Kymographs are plots of intensity values along a defined path over time. They display the life-history of selected microtubules in the form of an image that can be quantitatively analyzed. In this chapter, we present ImageJ plugins that generate kymographs to accurately capture the dynamically changing trajectories of microtubules and extract dynamic instability parameters from kymographs in a semi-automated manner.

2 Requirements

2.1 Microscopy

Kymograph analysis requires an image series that captures microtubule dynamics over time. This is commonly done using time-lapse fluorescence microscopy of cortical microtubules in living cells. Several fluorescent protein-labeled markers are available to visualize cortical microtubules in live plants (Celler et al., 2016). For technical details of fluorescence microscopy instrumentation and use, we refer the reader to chapters "Super-resolution imaging of microtubules in *Medicago sativa*" by Tichá et al. and "Live imaging of microtubules in petal conical cells" by Dang et al. in this volume.

2.2 Computer and software

A computer running ImageJ is required to use our plugins to generate and analyze kymographs of cortical microtubule dynamics. We recommend the Fiji ImageJ package (Schindelin et al., 2012), which can be downloaded from http://fiji.sc/. In the section below, we first describe the Dynamic Kymograph plugin, which uses keyframes and linear interpolation to generate kymographs from image series. We then describe the Microtubule Kymograph Analysis plugin, which extracts dynamic instability parameters from kymographs. Both of these plugins work on Windows and Macintosh operating systems.

3 Methods

3.1 Dynamic kymograph plugin

Traditionally, kymographs are generated using a static line to define the path of a dynamic object. In the case of microtubule polymerization dynamics, this type of kymograph analysis can be problematic because microtubules frequently change trajectory when they switch between phases of growth and shortening (Fig. 1A). In addition, cortical microtubules can dramatically change orientation due to interactions with other cortical microtubules or become floppy if they dissociate from the plasma membrane. In such cases, a static line will miss portions of microtubule dynamics that deviate significantly from the predefined path (Fig. 1B). This shortcoming restricts kymograph analysis to a subset of cortical microtubules that may not accurately represent the population behavior. To overcome this problem, we developed the Dynamic Kymograph plugin, which allows the line tracing the microtubule path to be modified on any frame to best match the position of the microtubule in that frame. This enables kymograph analysis of all microtubules and produces kymographs that faithfully capture all phases of microtubule dynamics (Fig. 1C).

Our plugin allows the user to define the path of a microtubule as a polyline made up of a sequence of connected line segments. In contrast to a straight line, a polyline can better characterize a curved path. To deal with changes in the shape and orientation of microtubule paths in the image sequence, a naïve approach would be asking

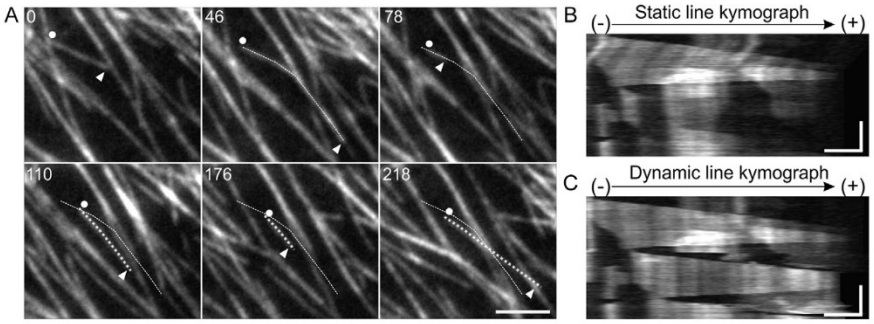

FIG. 1

Polymerization dynamics of cortical microtubules. (A) Example of a cortical microtubule exhibiting multiple phases of growth and shortening from the plus end (arrowhead). The minus end (dot) exhibits slow depolymerization. The thin dashed line marks the trajectory of the initial growth phase. The thick dashed line marks the trajectory of the microtubule in subsequent growth and shortening phases. Note that the three growth phases significantly differ in their trajectories. The numbers indicate time in seconds. Scale bar = 3 μm. (B) Kymograph of the cortical microtubule created using the thin dashed line shown in (A). X-axis scale bar = 3 μm. Y-axis scale bare = 60 s. (C) Kymograph of the same cortical microtubule created using keyframing and interpolation through the Dynamic Kymograph plugin. X-axis scale bar = 3 μm. Y-axis scale bar = 60 s.

the user to specify the polyline at each frame, which can be extremely time-consuming. Our solution is inspired by the "keyframing" concept from character animation in computer graphics. To create a smooth animation of a digital character spanning many frames in a movie clip, an artist would "pose" the character at a small number of time points (called *keyframes*), and the character poses in the remaining frames are automatically generated by the system, which interpolates the character poses between successive keyframes, usually by linear interpolation. In our plugin, the polyline describing the microtubule path is our "character". The user only needs to specify the location of the polyline nodes (i.e., the "pose" of the character) at a small number of keyframes, and a sequence of continuously changing polylines are then automatically generated by linearly interpolating the polylines between successive keyframes.

Initially, the user starts by creating a polyline path at any frame of the video (the first keyframe). That polyline is then duplicated to all other frames. The user can then scroll through the video, identify a frame where the microtubule deviates from the polyline, and move nodes of the polyline on that frame to better fit the microtubule (note that the user cannot add or remove vertices to the polyline). Such action will add that frame as a new keyframe, and polylines on all frames between the new keyframe and an adjacent, existing keyframe will be updated by linear interpolation. The user can add as many keyframes as needed until the sequence of the polylines satisfactorily fits the microtubule dynamics. Mathematically, let $v(i,j)$ denote the location of the i-th vertex of the polyline in the j-th frame, and j^-, j^+ be the indices of the nearest keyframe before and after the j-th frame. Note that one of (but not both) j^-, j^+ could be empty, indicating that the j-th frame is either before the first keyframe or after the last keyframe. We assign:

$$v(i,j) = \begin{cases} v(i,j^+) & \text{if } j^- \text{ is empty} \\ v(i,j^-) & \text{if } j^+ \text{ is empty} \\ \dfrac{v(i,j^-)(j^+ - j) + v(i,j^+)(j - j^-)}{j^+ - j^-} & \text{otherwise} \end{cases}$$

Given the polyline on each frame, we create a kymograph by first extracting one row of pixels from each frame along the frame's polyline and then stacking the rows together to form an image. To compute a row of pixels for a given frame, we sample uniformly along the frame's polyline to get the same number of sample points as the Euclidean length of the polyline (rounded to the nearest integer). For each sample point (with floating-point coordinates), we create a pixel whose intensity is obtained from the nearby pixel values using bilinear interpolation. To align the pixel rows computed from different frames, we ask the user to designate one of the polyline vertices as the *anchor*, and place the anchor vertex at an identifiable location along the microtubule (e.g., where it is fixed to the plasma membrane). The choice of the anchor vertex is fixed for all frames (e.g., the second vertex in all polylines). To create the kymograph, we stack up the pixel rows from all frames such that the pixels corresponding to the anchor vertex are located in the same column.

3.2 Workflow to generate kymographs using the dynamic kymograph plugin

3.2.1 Installing the dynamic kymograph plugin

1. Download the "Dynamic Kymograph-(version number).jar" file from https://imagej.net/Dynamic_Kymograph. The most recent version of the plugin can be found in the Release tab on the download page. To install a more recent version of the plugin, delete the older file and replace with the new one.
2. Save the file in the Fiji "Plugins" folder.
3. Restart Fiji. The plugin will appear in "Process" as "Dynamic Kymograph". Make sure you have updated Fiji to the latest version.

3.2.2 Using the Dynamic Kymograph plugin

1. In Fiji, open a fluorescence time-lapse image series to be analyzed. We typically use 8-bit or 16-bit Tagged Image File Format (TIFF) files, but other file formats that can be opened in Fiji are also compatible with this plugin.
2. Run the Dynamic Kymograph plugin.
3. The features of the user interfaces of this plugin are (Fig. 2):
   - The Dynamic Kymograph window consists of the following four buttons and a status message below the buttons.

     Select anchor point—Prompts the user to click on a node of a polyline region of interest (ROI) to set it as the anchor point. The status message will indicate which node was selected as the anchor point.

     Make kymograph—Prompts the user for line width and then generates a kymograph using all interpolated keyframes and the selected anchor point.

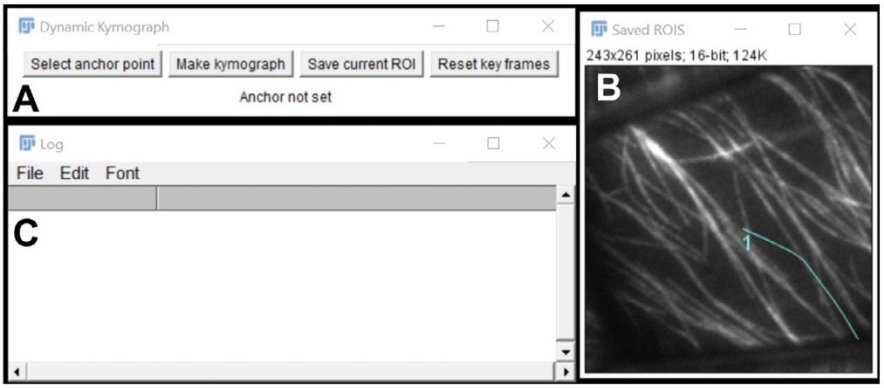

FIG. 2

User interface of the Dynamic Kymograph plugin. (A) The plugin user interface. See the text in Section 3.2.2 for details. (B) Polylines used to create kymographs are recorded in the Saved ROIs image. (C) The Log window records actions taken by the user.

Save current ROI—Records and numbers the current ROI on the "Saved ROIs" window.

Reset key frames—Clears all keyframes and anchor point.

- The Saved ROIs window displays the first frame of the image series. ROIs saved using the "Save current ROI" button will appear here. The ROIs are drawn on this image using random colors.
- The Log window records events such as when a keyframe is generated, when an anchor point is set and when the plugin is closed. It is used primarily for debugging and most users can ignore this window.

4. Survey the image series to select a microtubule to analyze. Use the segmented line tool to trace the selected microtubule. The line can be drawn in any frame of the image series. We recommend drawing the segmented line starting at the minus end so that the microtubule polarity is the same in all kymographs.

   Note: This plugin currently does not support changing the number of nodes in a segmented line. Therefore, we recommend that users include enough nodes to provide degrees of freedom necessary to track any changes in microtubule trajectory. This is particularly important for cortical microtubules because both ends are dynamic.
5. Scroll through the image series and select a node that is positioned on a region of the microtubule that remains spatially fixed over time. If needed, drag a predefined node to such a point on the microtubule. Then use the "Select anchor point" button or press "Ctrl" while the mouse is hovering over the node to set it as the anchor point. NOTE: If needed, the anchor point can be moved after setting it, but it cannot be reassigned once set.
6. Scroll through the image series to determine if the segmented line correctly traces all phases of microtubule dynamics during the observation period. If the microtubule or portion of the microtubule deviates significantly from the segmented line in any frame, edit the line to best trace the microtubule path in that frame. This can be done by changing the position of any node by dragging it or by translating the entire segmented line using the arrow keys. Each time the segmented line is modified, the plugin records a new keyframe. The plugin will automatically interpolate between all keyframes when scrolling through the image series.
7. If the modifications to the segmented line are satisfactory, press the "Make kymograph" button to generate a kymograph. If the modifications are not satisfactory, continue editing the segmented line as in step 6 and generate a new kymograph.
8. If analyzing additional microtubules in the same image series, press "Save current ROI" to make a record of which microtubule was analyzed. Then press "Reset key frames" to begin working on the next microtubule. Note: "Save current ROI" will draw the current ROI in a random color in the "Saved ROIs" image. To change the color of the ROI, keep pressing the "Save current ROI" button to cycle through colors until you find one you like.

9. To analyze a different image series, close the Dynamic Kymograph plugin and the current image series. Then, open the new image series and re-run the Dynamic Kymograph plugin as in step 4.

3.3 Microtubule Kymograph Analysis plugin

Once kymographs are generated, they need to be analyzed to measure the parameters that quantitatively describe the dynamic instability of microtubules. These parameters are microtubule growth and shortening rates, time spent in growth, pause and shortening phases, and the frequency of transition between these phases. Because both ends of cortical microtubules are dynamic, we developed a tool called Microtubule Kymograph Analysis that can separately analyze each end. By convention, the more dynamic end is the microtubule plus end and the less dynamic end is the minus end. Another way to distinguish between the two microtubule ends is to use protein markers that label either the plus-ends or minus-ends of cortical microtubules (Chan, Calder, Doonan, & Lloyd, 2003; Dixit, Chang, & Cyr, 2006; Fan, Burkart, & Dixit, 2018; Leong, Yamada, Yanagisawa, & Goshima, 2018; Nakamura, Lindeboom, Saltini, Mulder, & Ehrhardt, 2018).

3.4 Workflow to extract dynamic instability parameters from kymographs using the Microtubule Kymograph Analysis plugin

3.4.1 Installing the Microtubule Kymograph Analysis plugin

1. Download the "Microtubule_Kymograph_Analysis-(version number).jar" file from https://github.com/mrsata/Microtubule_Kymograph_Analysis. The most recent version of the plugin can be found in the Release tab on this page. To install a more recent version of the plugin, delete the older file and replace with the new one.
2. Save the file in the Fiji "Plugins" folder.
3. Restart Fiji. The plugin will appear in "Process" as "Microtubule Kymograph Analysis"

3.4.2 Using the Microtubule Kymograph Analysis plugin

1. In Fiji, open a kymograph to be analyzed.
2. Run the Microtubule Kymograph Analysis plugin. NOTE: the plugin assumes that distance is represented along the X-axis and time is represented along the Y-axis of the kymograph image.
3. The Microtubule Kymograph Analysis window (Fig. 3A) has the following buttons. Note: Sometimes when the plugin is run for the first time, it shows a blank window or a window with missing components. To solve this, change the size of the window by dragging the corner or maximizing it.

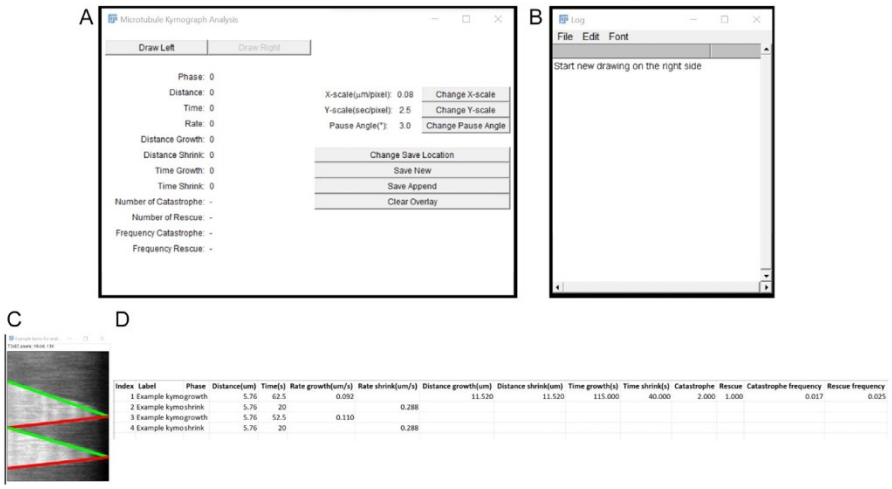

FIG. 3

User interface of the Microtubule Kymograph Analysis plugin. (A) The plugin user interface. See the text in Section 3.4.2 for details. (B) The Log window records actions taken by the user. (C) Example output kymograph image with colored segmented line overlay. (D) Example csv file with calculated parameters from the kymograph in (C).

- Draw Left—Resets statistics (if any) and prepares the user to draw a segmented line to track microtubule dynamics on the left side of the kymograph image.
- Draw Right—Resets statistics (if any) and prepares the user to draw a segmented line to track microtubule dynamics on the right side of the kymograph image. Note: We generate kymographs such that the cortical microtubule plus-end is on the right side and the minus-end is on the left side. We analyze plus-end dynamics first and hence this button is selected by default when the Microtubule Kymograph Analysis plugin is first opened.
- Change X-scale—Allows user to input a value which corresponds to the distance (in microns) represented by one image pixel in the X-axis. The default value is 0.08 μm/pixel based on our imaging system.
- Change Y-scale—Allows user to input a value which corresponds to the time (in seconds) represented by one image pixel in the Y-axis, which corresponds to the imaging time interval. The default value is 2.5 s/pixel based on our imaging system.
- Change Pause Angle—Allows user to input a value to define the extent of angular deviation (in degrees) from a vertical line to be considered as a pause phase (i.e., phase when a microtubule is neither growing nor shortening). The default value is 3 degrees.
- Change Save Location—Allows user to specify the directory of the output files.

- Save New—Saves output files in the same directory as the kymograph image.
- Save Append—Allows user to append newly calculated parameters to a previous csv and image overlay file.
- Clear Overlay—Toggles overlay of growth, pause and shortening phases on kymograph. Note: Hotkey for Clear Overlay is "Ctrl".
- The Log window (Fig. 3B) records events and prompt the user for the next step.

4. Draw a segmented line to track the path of the plus end (or minus end) of a microtubule in the kymograph image. The plugin will automatically color the line segments green, blue or red to represent the growth, pause and shortening phases, respectively. Note: Opening the plugin automatically selects the segmented line tool.
5. The plugin will automatically calculate the parameters listed in the Microtubule Kymograph Analysis window. The user can change the segmented line by dragging any of the nodes and the calculated numbers will update automatically.
6. When satisfied with the line, select "Save New" or "Save Append" to save the calculated parameters. The plugin will output an image of the kymograph with the segmented line overlay for your record (Fig. 3C) and a csv file with all the measurements (Fig. 3D). "Save New" outputs these files in the same directory as the image. "Save Append" will prompt the user to select a csv file to append the new data and a kymograph image to save the segmented line overlay. The names of the csv and image overlay files contain date and time information in the "month.day.hour.minute" format.
7. The output csv file contains the following columns:
   Index: numbers each phase of microtubule dynamics.
   Label: name of kymograph image file.
   Phase: growth, pause or shrink phase.
   Distance: distance microtubule grew, paused or shortened in μm.
   Time: time microtubule grew, paused or shortened in seconds.
   Growth rate: rate of microtubule polymerization in μm/s.
   Shrink rate: rate of microtubule depolymerization in μm/s.
   Distance grown: the amount of microtubule growth in μm.
   Distance shrink: the amount of microtubule shortening in μm.
   Time growth: the duration of microtubule growth in seconds.
   Time shrink: the duration of microtubule shortening in seconds.
   Catastrophe: a catastrophe event (switch from growth to shortening) is denoted as 1. No catastrophe is denoted as 0.
   Rescue: a rescue event (switch from shortening to growth) is denoted as 1. No rescue is denoted as 0.
   Catastrophe frequency: calculated as the number of catastrophe events divided by the total growth time. Units are sec^{-1}.
   Rescue frequency: calculated as the number of rescue events divided by the total shortening time. Units are sec^{-1}.
8. Once the calculations have been saved, click anywhere on the kymograph image to clear the segmented line and floating calculations in the Microtubule

Kymograph Analysis window. To analyze another microtubule in the same kymograph image, draw a new segmented line and continue from step 4.

9. When done analyzing a kymograph, close the image and open another one without reopening the plugin.

4 Concluding remarks

In this chapter, we have provided the rationale and procedures to use the Dynamic Kymograph and Microtubule Kymograph Analysis plugins to generate kymographs of microtubules and to measure the parameters describing their polymerization dynamics. While we have focused on plant cortical microtubules here, both of these ImageJ plugins can be used to analyze microtubule dynamics in any experimental system (in vivo or in vitro) and during any phase of the cell cycle. The Dynamic Kymograph plugin should be particularly useful to generate kymographs of animal interphase microtubules since they are generally quite floppy and hence difficult to track using traditional kymograph analysis. In addition, while the Microtubule Kymograph Analysis plugin is specifically designed to quantitatively analyze kymographs of microtubules, the Dynamic Kymograph plugin can be used to generate kymographs of any motile structure.

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