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Live cell imaging of microtubule cytoskeleton and micromechanical manipulation of the Arabidopsis shoot apical meristem

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Dear editor,

Please find attached the protocol for mechanical perturbation of shoot apical meristem in plants.

Yours truly

Arun Sampathkumar

TITLE:

Live Cell Imaging of Microtubule Cytoskeleton and Micromechanical Manipulation of the *Arabidopsis* Shoot Apical Meristem

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KEYWORDS:

Arabidopsis, shoot apical meristem, mechanics, cortical microtubules, live cell imaging, physical ablation

SUMMARY:

Here we describe a protocol for live cell imaging of the cortical microtubule cytoskeleton at the shoot apical meristem and monitoring its response to changes in physical forces.

ABSTRACT:

Understanding cell and tissue level regulation of growth and morphogenesis has been at the forefront of biological research for many decades. Advances in molecular and imaging technologies allowed us to gain insights into how biochemical signals influence morphogenetic events. However, it is increasingly evident that apart from biochemical signals, mechanical cues also impact several aspects of cell and tissue growth. The *Arabidopsis* shoot apical meristem (SAM) is a dome-shaped structure responsible for the generation of all aboveground organs. The organization of the cortical microtubule cytoskeleton that mediates apoplastic cellulose deposition in plant cells is spatially distinct. Visualization and quantitative assessment of patterns of cortical microtubules are necessary for understanding the biophysical nature of cells at the SAM, as cellulose is the stiffest component of the plant cell wall. The stereotypical form of cortical microtubule organization is also a consequence of tissue-wide physical forces existing at the SAM. Perturbation of these physical forces and subsequent monitoring of cortical microtubule organization allows for the identification of candidate proteins involved in mediating mechano-perception and transduction. Here we describe a protocol that helps investigate such processes.

INTRODUCTION:

Plant cells are surrounded by an extracellular matrix of polysaccharides and glycoproteins that mechanically resembles a fiber reinforced composite material capable of dynamically changing its mechanical properties[1]. Growth in plant cells is driven by the uptake of water into the cell, which results in a concomitant buildup of tensile forces on the cell wall. In response to such forces, modifications to the physical state of the cell wall allows for cell expansion. Cells with primary walls are capable of undergoing rapid growth compared to

secondary cell wall containing cells mainly due to differences in the chemical composition of the polysaccharides within. Primary wall cells are composed of cellulose, hemicellulose, and pectin in addition to glycoproteins, and lack lignin, a component that is present in the secondary cell wall[2]. Cellulose, a glucose polymer linked via β-1,4 bonds, is the major component of the cell walls. It is organized into fibrillar structures that are capable of withstanding high tensile forces experienced during cell growth[3]. In addition to withstanding tensile forces, mechanical reinforcement along a preferential direction results in turgor-driven expansion along an axis perpendicular to the net orientation of the cellulose microfibril. The organization of the cellulose microfibrils is influenced by the cortical microtubule cytoskeleton, as they guide the directional movement of the cellulose-synthesizing complexes located at the plasma membrane[4]. Therefore, monitoring cortical microtubule organization using a microtubule-associated protein or tubulin fused with a fluorescent molecule serves as a proxy for the observation of overlying patterns of cellulose in plant cells.

The patterning of the cortical microtubule cytoskeleton is under the control of cell and tissue morphology derived mechanical forces. Cortical microtubule organization does not have any preferential organization over time in cells located at the apex of the SAM, whereas cells in the periphery and the boundary between the SAM and the emerging organ have a stable, highly organized supracellular array of cortical microtubules[5]. Several approaches have been developed to physically perturb the mechanical status of the cells. Changes to osmotic status, as well as treatment with pharmacological and enzymatic compounds that influence the stiffness of the cell wall can result in subsequent changes in the tensile forces experienced by the cell[6,7]. The use of contraptions that allow for the gradual increase in compressive forces experienced by tissues is another alternative[8]. Application of centrifugal forces has also been shown to influence the mechanical forces without any physical contact with the cells[9]. However, the most widely used means of changing directional forces in a group of cells take advantage of the fact that all epidermal cells are under tension and physical ablation of cells will eliminate turgor pressure locally as well as disrupting cell-to-cell adhesion, thereby modifying the tensile forces experienced by the neighboring cells. This is performed either by targeting a high-powered pulsed ultraviolet laser or by means of a fine needle.

Here we elaborate on the process of imaging and assessing cortical microtubule behavior for mechanical perturbation at the SAM.

PROTOCOL:

1. Plant growth

1.1. Sow *Arabidopsis* seeds expressing microtubule binding domain fused with green fluorescent protein (MBD-GFP)[10] on soil and keep in long day (16 h day /8 h night), 20 °C/6 °C conditions for 1 week for germination.

1.2. After germination, transfer seedlings to new pots with sufficient growth space to allow robust vegetative growth. Keep plants in short day (8 h day /16 h night), 20 °C/16 °C conditions for 3–5 weeks.

1.3. Transfer plants to long day (16 h day /8 h night), 20 °C/16 °C conditions and keep until plants bolt (2–3 weeks). Allow the inflorescence to grow up to 2–5 cm long.

2. Medium preparation

2.1. Prepare the dissecting dishes by filling small Petri dishes (approximately 5.5 cm wide, 1.5 cm deep) to approximately half their depth with 2% agarose. The dissecting dishes could also be used for single time point imaging.

2.2. Prepare the growth medium.

2.2.1. Prepare 50 mL of a 1,000x vitamin stock solution with 5 g of myo-inositol, 0.05 g of nicotinic acid, 0.05 g of pyridoxine hydrochloride, 0.5 g of thiamine hydrochloride, and 0.1 g of glycine.

2.2.2. Prepare the growth medium, composed of ½ Murashige and Skoog medium, 1% sucrose, 1.6% agar, 1x vitamins, pH = 5.8. Autoclave and allow it to cool.

2.2.3. On a sterile, clean bench, sterilize hinged plastic boxes (approximately 5.2 cm x 5.2 cm x 3 cm) by immersing in 70% ethanol for 15 min.

2.2.4. On a sterile, clean bench, once the medium is at bearable warmth, add N6-benzyladenine to a final concentration of 200 nM, and 1/1,000 PPM (plant preservative mixture) and mix well. Fill the sterile boxes to approximately half their depth with the medium.

3. Dissection of the SAM

3.1. Cut the inflorescence and remove the older flower buds with sharp forceps by peeling the flowers at the base of the peduncles until it is difficult to see them with the naked eye.

3.2. Create a slit in the agarose in the dissecting dish with forceps and plant the inflorescence base into the thick agar. This gives good solid support for further fine dissection of younger flowers to expose the SAM.

3.3. Remove the remaining flower buds by pushing them down with the forceps starting with the oldest and sequentially progressing to the younger stages under a dissecting microscope until the SAM is visible. The SAM is usually exposed when older flowers up to stage 6 to 7 are removed. Once the SAM is exposed, avoid dehydration of the sample by moving quickly to the next step.

4. Transfer and growth of cultured SAMs

4.1. Plant the freshly dissected sample as detailed in section 1 into the growth medium in the rectangular plastic hinged culture box with the SAM just exposed above the medium surface. Add a few drops of sterile deionized water to the edges of the culture boxes and close the lid to maintain the humidity inside the box. Ensure that the added water does not cover the SAM.

4.2. Close the lid and wrap the box with micropore tape. Place the growth box in long day or continuous day conditions at 22 °C and grow for 12–24 h to allow the SAM to recover from the dissection procedure and adapt to the culture conditions.

5. Imaging of the SAM

5.1. Fill the culture box containing the SAM with sterile deionized water to cover the sample. Check under the dissecting microscope and remove any air bubbles by forcefully spraying water directed at the sample with a 1 mL pipette.

5.2. Place the culture box on an upright confocal microscope stage. Take care that the culture box does not contact the microscope objectives. Use a long distance 40x or 60x water dipping lens of numerical aperture 0.8–1 that is optimal for imaging without a cover glass.

5.3. Lower the objective into the water and check for air bubbles formed on the objective's front lens. Remove any bubbles by lowering the stage and gently wiping the lens with an optical tissue and adding a small amount of water to the front lens of the objective with a Pasteur pipette before reimmersion into the water.

5.4. Using the GFP filter and epi-illumination module of the confocal microscope, adjust the XY controller to locate the sample. Adjusting the position of the oculars, put the SAM directly under the light source and focus along the Z axis until the apex is located.

NOTE: Do not look directly at the ultraviolet light.

5.5. Once the sample is found, illuminate it using a laser capable of exciting GFP (i.e., a 488 nm or 496 nm laser source). Adjust the optical zoom of the microscope so that the entire SAM and the stage 1 floral primordia are in the field of view. Further adjust the power of the laser output and gain settings to ensure optimal signal-to-noise ratio.

NOTE: The high intensity of the laser will result in photo bleaching of the sample. The under- and overexposure palette help ensure better adjustment of these settings.

5.6. Allow the sample to settle down for 2–5 min. Acquire confocal Z stacks of the sample at 0.25 µm–0.5 µm Z slice intervals at a resolution of approximately 0.3 µm pixel size. Ensure that the total imaging time required for acquiring Z stacks for one sample does not exceed 10 min from the time the sample is immersed into water until the completion of the acquisition.

5.7. Immediately remove the water and transfer the culture box back to the growth chamber. Keeping the samples for a long time under water will influence the osmotic status of the cells.

6. Micromechanical perturbation of the SAM

6.1. Set up the imaging conditions as described in section 5 and acquire preablation image stacks of the cortical microtubule organization.

6.2. Decant the water in the culture dish. Perform the ablation with a clean syringe needle (0.4 mm x 20 mm).

6.2.1. Using a dissecting microscope, carefully hold the needle and slowly approach the SAM.

NOTE: Breath holding and handling the needle with a relaxed grip helps avoid shaking.

6.2.2. Briefly contact the SAM dome with the needle tip to confirm that the ablation is done. Preferably perform the ablation on the periphery of the SAM, which allows visualization of the behavior and transitioning of unorganized cortical microtubules in the central region of the dome.

6.3. Refill the culture dish with sterile deionized water and add propidium idodide (10 µg/mL). In addition to monitoring the GFP cortical microtubule signal, visualizing the propidium iodide using a separate channel can clearly mark regions of ablated cells. Propidium iodide is illuminated using the 561 nm or any other suitable laser with emission recorded between 600–650 nm.

6.4. Acquire image stacks right after ablation (see section 6) and repeat the acquisition process every 2 h for a period of 6 h. Return the culture dish along with the sample into the incubation chamber after every time point. Ensure there is some water left on the culture media and wrap the culture dish with micropore tape to prevent desiccation.

7. Data visualization and quantification

7.1. Generate surface projections using freely available software such as MORPHOGRAPHX[11], FIJI[12], or macro SurfCut[13].

7.2. Perform extraction of cortical microtubule anisotropy using FibrilTool[14] macro in FIJI.

NOTE: Detailed protocols for software use can be obtained from the respective citations.

REPRESENTATIVE RESULTS:

Figure 1 shows typical projection images obtained from MBD-GFP lines with cells at the center of the dome containing disorganized cortical microtubules, and cells at the periphery having a circumferential distribution (**Figure 1A**,**B**), whereas the boundary domain cells contain cortical microtubules aligned parallel to the cell's long axis. These observations show differences in the spatial distribution of cortical microtubules in the different domains of the SAM. Time lapse imaging showed cortical microtubule alignment changing from a highly disordered array to a more organized array within 6 h of ablation (**Figure 1C**,**D**). The tensors generated using FibrilTool could be superimposed on the cortical microtubule image. A longer tensor represented a higher the degree of anisotropy. The tensors also showed the major axis of alignment of the cortical microtubules. The extracted information can be represented by potting the mean anisotropy over time (**Figure 1E**)[15]. A sample size of four to five is recommended per treatment or genotype that must be tested. The results showed a gradual increase in cortical microtubule anisotropy within a period of 6 h and allowed us to conclude

that modulation to mechanics of the SAM results in concurrent changes in cortical microtubule organization.

FIGURE LEGENDS:

Figure 1: Example result of cortical microtubule anisotropy quantification and mechanical ablation in *Arabidopsis* SAM. (**A**) Surface projection of 35S: MBD-GFP SAM Z stacks. (**B**) Nematic tensors in red showing cortical microtubule anisotropy of manually segmented cells in the central and boundary domain. (**C, D**). Surface projections of cortical microtubule time lapse data from an ablation experiment. (**D**) Magnified images from (**C**) showing cortical microtubule realignment near the site of ablation (red asterisk) overlaid with nematic tensor information of individual cells. (**E**) Cortical microtubule anisotropy changed after ablation from 0–6 h, quantified from the nematic tensor labeled region in **C** with the circle representing the mean value and the bars indicating the 95% confidence interval. Scale bars = 25 µm.

DISCUSSION:

The assessment of mechanical signal transduction events is crucial to identify molecular regulators involved in the mechano-perception and transduction pathways. The protocol described here provides a quantitative view of such events by using the cortical microtubule response as a readout for such a process in *Arabidopsis* SAMs. The procedure described here is routinely used in several studies in various tissue types[16-19]. A significant increase in microtubule anisotropy is observed in the range of 4 h in all tissue types.

The most critical step in the protocol is ensuring that alterations to the microtubules do not occur due to prolonged submergence in water, because water on its own results in an increase of the cells' turgor pressure. Therefore, the imaging time should be minimized. This can compromise the image resolution, but it provides a more accurate readout of the perturbation results. The second most crucial step is careful dissection of the SAM. Due to its very small size, it is very likely to be damaged during the procedure. The organs become more difficult to remove if the peduncles of older organs are not entirely removed. Finally, during dissection the sample should not be allowed to dry out due to prolonged air exposure of the SAM. This is especially common when handling mutant SAMs that are hard to access or that are smaller than 50 µm in diameter. Frequent application of water drops on the SAM between the dissection steps prevent this issue.

A limitation of this procedure is that there is no real control over the ablated area. This is an issue in comparing changes occurring in different conditions and genotypes. Therefore, it is necessary to use control SAMs perturbed in a similar manner for comparison. Another alternative is performing a more controlled ablation using a pulsed ultraviolet laser[20]. In addition, while ablation is widely known to create changes in mechanical properties, it is also associated to a certain degree with wound-induced responses. For this reason, other approaches, such as mechanical compression and turgor-induced perturbations need to be performed to confirm the observations[6,9].

A significant advantage of this method compared to other ways of manipulating mechanical forces is that it uses very basic tools yet provides a robust readout of microtubule response.

ACKNOWLEDGMENTS:

None.

DISCLOSURES:

The authors have nothing to disclose.

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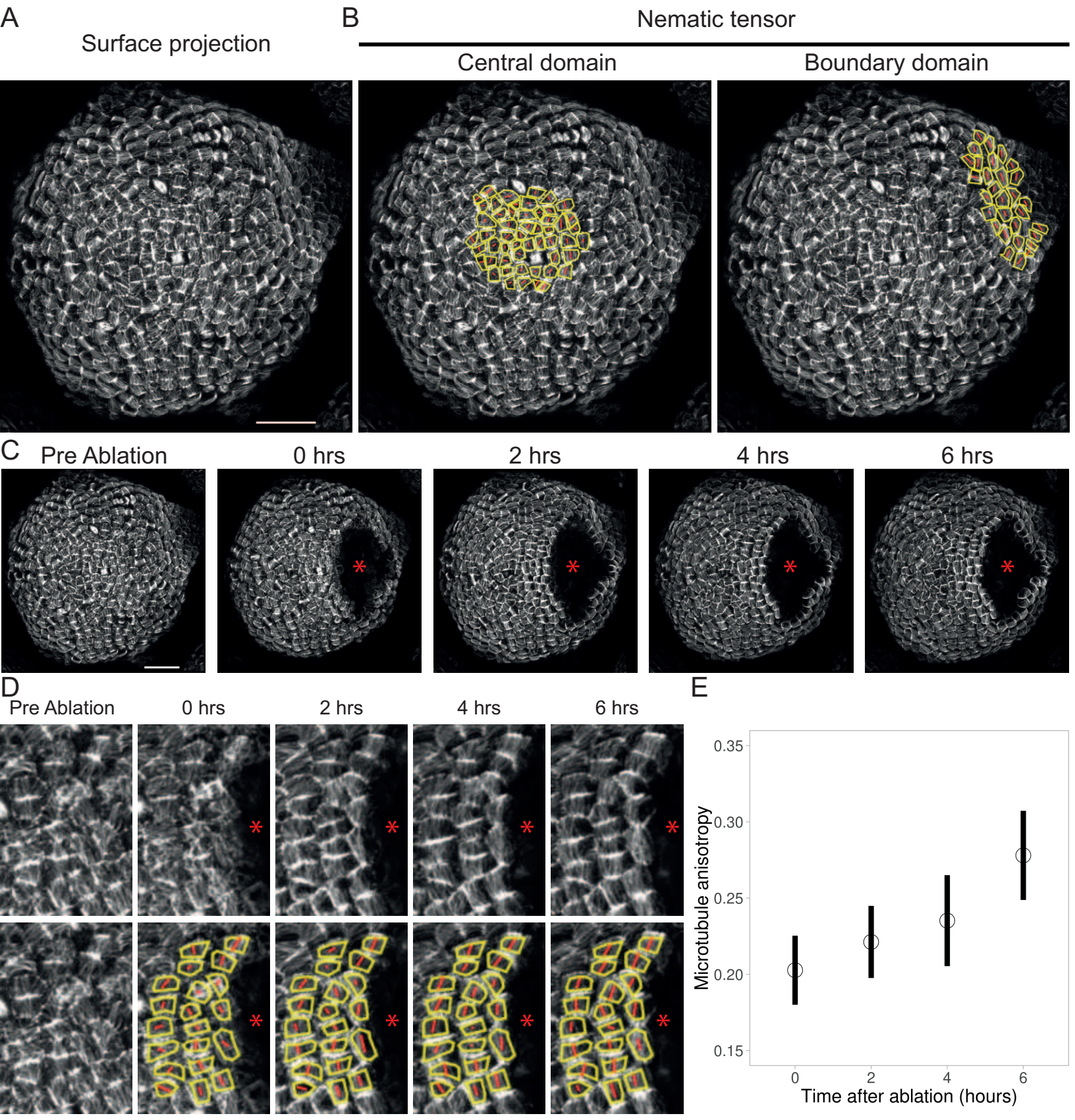

Name of Material/ Equipment	Company	Catalog Number
FibrilTool		
FIJI		
glycine	Merck	1.04201.1000
Leica SP8 confocal microscope	Leica	DM6000 CS
MAP4-GFP		
micropore tape	Leukopor	02482-00
MorphographX		
myo-inositol	Sigma	I5125
N6-benzyladenine	Sigma	B3408
nicotinic acid	Sigma	N4126
plastic hinged box	Electron microscopy sciences	64312
PPM (Plant Preservative Mixture)	Plant Cell Technology	PPM
Propidium iodide	Sigma	P4864
pyridoxine hydrochloride	Sigma	P9755
SURFCUT		
thiamine hydrochloride	Sigma	T4625

Comments/Description

Boudaoud, A. *et al., Nat Protoc. 2014*
Schindelin, J. *et al., Nat Methods. 2012*

Marc, J. *et al., Plant Cell* 1998

Strauss, S. *et al., Methods Mol Biol.* 2019

Erguvan, O. *et al., BMC Biol.* 2019

We would like to thank the reviewers for their comments. Specific responses below. We have submitted a version with changes indicated in blue in addition to the final manuscript.

Editorial comments:
Changes to be made by the Author(s):
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

Checked.

2. Please include at least 6 keywords or phrases.

This is done.

3. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: names of the software IMARIS, Fibriltool, etc.

We have done this for IMARIS. Fibriltool is not a commercial software just a macro that is available for FIJI.

4. Please revise the Introduction to include all of the following with citations:
a) A clear statement of the overall goal of this method
b) The rationale behind the development and/or use of this technique
c) The advantages over alternative techniques with applicable references to previous studies
d) A description of the context of the technique in the wider body of literature
e) Information to help readers to determine whether the method is appropriate for their application

This is addressed.

5. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.”

We have checked this.

6. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections.

We have checked this.

7. Please use complete sentences throughout.

We checked the sentences.

8. Please ensure you answer the “how” question, i.e., how is the step performed?

Checked and more details added.

9. Step 5, 6, 7: Please describe all the button clicks, the knob turns, etc to show how the step is being performed. Click “Open” to open the software, then click “Analyze”.

Such steps are not present.

10. There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

The protocol does not exceed three pages.

11. Please describe the result with respect to your experiment, you performed an experiment, how did it help you to conclude what you wanted to and how is it in line with the title.

We have made this change.

12. Please include a scale bar for all images taken with a microscope to provide context to the magnification used. Define the scale in the appropriate Figure Legend.

We have included this now.

13. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your

Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

No permissions required.

14. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

We have included this.

15. Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials in separate columns in an .xls/.xlsx file. Please sort the materials table in alphabetical order.

We revised the table and several changes were made.

Reviewers' comments:
Reviewer #1:

Manuscript Summary:
Wang and Sampathkumar provided a microscopy-based analysis protocol for tissue mechanics in the Arabidopsis shoot apical meristem. With the increasing focus on biomechanics, the protocol is timely. The protocol covered sample preparation, imaging, micromechanical preturbation and data analysis. Whereas all previous aspects are well detailed, the authors desperately need to expand on the data analysis section.

Major Concerns:

The authors largely used the general term "microtubule" throughout the protocol, whereas the real focus is more cortical microtubule. The authors should clearly define and specify what is being observed and measured.

We have include the word cortical.

Step 2.2: The composition of apex growth medium is slightly different from other published protocols (e.g. Stanislas et al., 2017, https://smex-ctp.trendmicro.com:443/wis/clicktime/v1/query?url=http%3a%2f%2fdx.doi.org%2f10.1016%2fbs.mcb.2016.11.008&umid=5d971ce9-db66-4058-8b62-ac6df1926d51&auth=59ab812f8dc1d135cfb4a1fdb744694347dd7c19-

1d7d8a464803538e084ba9e4bd80ff65b7b167de). Besides using a different medium name, the authors should also consider to point out the differences for clarificaion.

We have now changed the name from apex growth medium to just growth medium. We do not use the media composition described in Stanislas et al so we do not mention or discuss this here.

Line 154: I have no experience with exciting GFP at 514nm, but this is a rather unconventional wavelength. The authors should consider to justify this alternative choice or consider removing it.

We agree and have now removed 514nm.

Step 7: With current descriptions in "data visualization and quantification", the readers will not be able to recreate the analysis. Detailed descriptions are needed here. At least, the authors need to suggest how deep the signals should be projected, how to judge projection quality, how ROI should be selected, etc. (or clearly state that the choices of these parameters can be found in specific references, if that fits journal requirement).

The process of quantification and data visualization in 7 is described in a step by step manner in Boudaoud et al Nature Protocol. We feel that this will be a reiteration of the same process and that is not necessary. We have added a statement telling about the availability of a detailed protocol in addition to the citation that are included.

Minor Concerns:

Depending on journal requirement, the authors may consider to add a "Here, we..." sentence in the abstract.
We have now included such a statement
Line 41: Since SAM cells have no central vacuole, and the "fragmented" vacuoles do not take up the majority of cellular space, would it be easier to simply say "water uptake into the cell"?
We have made this change
Line 41, 48, 49, etc.: "tensional force" is not a correct term. It should be "tensile force", if at all.
We have made this change
Line 50: "cellulose micro fibrils determine the directional expansion of the cell". How?
We have modified the sentence “In addition to withstanding tensile forces mechanical reinforcement along a preferential direction results in turgor expansion along an axis that is perpendicular to the net orientation of the cellulose microfibril”
Line 70: Physical ablation does not only "elimitates turgor pressure", but also disrupts cell-cell adhesion.
We agree and include this “as well as disrupting cell to cell adhesion”
Line 212: "avoid dehydration". How?
We have included the statement “by quickly moving to the next step”
Step 5.2: Depending on journal requirement, the authors may suggest an example lens model.
Since this is a commercial product we follow the guidelines suggested by the journal and have not included this.
Line 149: After a GFP filter, the light is not UV. The warning is nevertheless sincerely

appreciated.
Thank you
Lines 156-160: Sentences may be swapped around to clarify what "proper visualization" means (e.g. optimal SNR, reduced bleaching, as authors already stated).
We have now modified this.
Line 162: "Allow the sample to settle down". Why and how?
Hydrogels (solid media) absorb water this would result in movement of the sample.
Line 164: The authors may take the chance to provide a guideline for resolution choices based on e.g. pixel size, pixel dwell time, MT density, MT dynamics, etc.
We have provided the pixel size here that should be sufficient to resolve MT bundles in cells of the SAM. The dwell time could vary based on individual microscope capabilities and we know that MT dynamics is stable in the recommended imaging time suggested.
Line 210: Fig. 1E is neither a histogram nor a line chart.
Yes, we agree and modified this sentence. "The extracted information can be represented by potting the mean anisotropy over time (Figure 1E)"

Reviewer #2:

Manuscript Summary:
In this manuscript Wang and Sampathkumar describe a protocol for imaging the cortical microtubule cytoskeleton (CMT) at the shoot apical meristem (SAM) of Arabidopsis. In addition, they provide a protocol to ablate cells and to quantify the effects on CMT organisation over time after ablation.

Major Concerns:
Since this is a protocol paper, I think it is important to better describe the confocal setup including the types of objectives used (N.A.) and typical laser power for acquisition.

This can be any conventional confocal microscope with an upright stage. The N.A. of the objective is now provided. The laser power is however different based on the power of the actual laser supplied with the company and it varies. In addition to this, the laser output at the objective is also different based on the setup. The imaging conditions provided is described in Step 5.5.

Also it would be good if the authors could show the effects of using lower magnification or lower NA objectives to demonstrate how these affect the image quality and the ability to quantify the CMT. It is important to show the readers where potential artefacts may come from.

We agree this is good but unfortunately, we do not have lower NA objectives or objectives of different magnification.

In addition, I Ould like to argue that the authors are not ablating but rather massively wound the SAM. The described protocol for ablation is rather vague and the results will very much depend on the person who holds the needle. I do not think that this will give reproducible results. Maybe the authors could show 10 independently "ablate" SAMs and show the variation they get. I would argue that a ablation laser is much more precise. This is important since the authors want to quantify the changes of CMT organisation in response to the mechanical stimulation. I think

that also the image analysis should be more detailed and maybe show the results obtained with different software to show that they in principle give the same results?

The concept of large scale ablation to test mechanical response is widely accepted and has resulted in very similar results even though performed by different people in different labs (Uyttewaal et al 2012 Cell, Sampathkumar et al 2019 Development). Laser ablation is preferred but not all labs including ours have access to such instrumentation. We have included the statement of sample size "A sample size of four to five is recommended per treatment or genotype that has to be tested."

Minor Concerns:

The protocol for dissecting the plants in order to image the SAM will benefit from a figure showing the different stages of preparation. I know there will be a video, but it would benefit the reader if there would also be a figure summarising all the steps.

There is already a detailed JOVE article just on this process Geng and Zhou issue 145

- Please read again carefully the manuscript there are several typos and words missing in some sentences.
We have now checked this carefully and made corrections

Reviewer #3:

Manuscript Summary:
The paper quite nicely summarizes the technique of in situ live imaging of the cytoskeleton within the Arabidopsis meristem and techniques used to examine the cytoskeleton´s response to mechanical stress. However, in its present form, the manuscript does not cover a very important part of the technique, namely data processing, and looks as a very rough preliminary draft, rather than a paper ready for submission. In my opinion, it may become suitable for publication only if the following issues are addressed.

Major Concerns:

Unfortunately, the paper feels VERY unfinished, with text quality varying across the manuscript. Some parts of the protocol lack sufficient documentation. I have several substantial concerns here:

1) There is no "Discussion" section, although the jounal´s template requires it and I feel that there really should be one, if for nothing else, then at least to compare the strenghts and weaknesses of the author´s approach vs techniques used by others for related purposes (look up, e.g., studies from the Olivier Hamant lab).

This was completely overlooked we have now included all this in the discussion section

2) Part 7 of the Protocol (Data visualization and quantification) lacks sufficient detail required to make the method work. The visualisation and quantification step is essential to produce results, yet the authors merely list the software used, without any further details.

3) Related to this, insufficient information is provided concerning the data processing methods used to produce the representative results shown in Fig. 1.

The process of quantification and data visualization in 7 is described in a step by step manner in Boudaoud et al Nature Protocol and Erguvan et al BMC Biology 2019. We feel that this will be a reiteration of the same process and that is not necessary. We have added a statement telling about the availability of a detailed protocol in addition to the citation that are included.

4) The Materials and equipment list only lists common chemicals and consumables but the more important/critical materials (such as genetically defined plant lines), software and equipment (such as a sample microscope configuration) are not mentioned. This information must be provided.

We have now included this.

Minor Concerns:

1) Although I am not a native speaker of English, I think that the manuscript needs to undergo a thorough language check. Among other things, I noticed multiple sentences with subject in singular and a verb in plural (or vice versa), sentences with multiple subjects or multiple verbs (e.g. lines 47-49), words broken in two (" extra cellular" instead of "extracellular"), nonsensical terminology ("cells containing primary walls" - how can a cel contain its own wall?) etc.

We have carefully checked and made these and other changes.

2) Why are some words on line 54 in capitals?

Protein products of genes are written in Capital letters without italics.

3) While in most cases microtubules indeed can be taken as a proxy for cellulose microfibril localisation, the relationship is not that of absolute causality. Namely, the tight relationship holds only for the nascent (growing) microfibrils. Once micrtofibrils are established, microtubules can freely move away. See e.g. Whiitington et al 2001 (DOI: 10.1038/35079128). Consider therefore rephrasing the sentemce on l. 53-55.

This discussion is more relevant for a research paper and not for this method paper that realizes completely on the assumption that microtubules act as proxy for cellulose microfibrils. This issue has been address in several research papers.

3) I cannot understand the sentence on l. 175-176.

We have now modified this.