Step-by-step user guide for the analysis of cortical microtubule response after ablation

Procedure overview

Here we describe step-by-step how to operate the image analysis workflow we developed to quantify microtubule arrays reorganization in plant cells following a mechanical stress induced by an ablation. The workflow requires several components under the form of imageJ macros and a python Jupyter notebook for the data statistical analysis. 1) First, SurfCut (Erguvan et al., 2019) is used to extract cell contours and the outer epidermal cortical microtubules (CMTs) signal. 2) Using the cell contour images extracted with SurfCut, Cell_ROIMaker.ijm (a modified version of "Segmentation4FTBatch.ijm") is then used to define each segmented cells as ROIs (Regions of interest in Fiji). 3) FibrilTool_Batch_Workflow.ijm (a modified version of FibrilTool_Batch) is then run on each microtubule image extracted with SurfCut to quantify the microtubules organization in each cell ROI previously defined. 4) Line_ROIMaker.ijm is then used to manually draw ROI lines which approximate the expected orientation of CMTs if they would circumferentially reorganize following the stress induced by the ablation. 5) Finally, the angles between the actual microtubules as quantified by FibrilTool, and their expected orientation (manually drawn line) is calculated for each cell using the Angle2Ablation.ijm macro. 6) The data is then analyzed using a python Jupyter notebook to output descriptive and inferential statistics comparing different time points, samples or conditions.

Image acquisition

Any fluorescent microtubule reporter line can be used to acquire fluorescent z-stacks images with a confocal microscope at different time points after applying a mechanical stress. In many cases, the CMT signal is sufficient to also be used as a cell contour signal, but in certain cases it can be necessary to also acquire a second channel specifically for cell contour signal. For a better quality analysis, it is recommended to use z-intervals of at least 1µm.

How to install and run the different components of the workflow

- Fiji: Most of the workflow is designed to run on Fiji. If you don't already have it, download it and install it following the procedure describe at https://fiji.sc/.

- Plugins: The macro "Cell_ROIMaker.ijm" uses the MorpholibJ (Legland et al., 2016; https://imagej.net/MorphoLibJ) plug-in. To install it, go in the "Help" menu of imageJ and click on "Update...". This will open the "ImageJ updater"; if it doesn't open, an imageJ update may be necessary so restart imageJ and do it again. In "ImageJ updater", you have to click on "Manage update sites" and in the list, find "IJPB-Plugins" and click in the square next to it to add it then close the window and apply changes on "ImageJ updater". Once this is done, imageJ needs to be restarted.

- Macros: You will need (available for download at):

- SurfCut2.ijm (https://github.com/VergerLab/SurfCut2)
- FibrilTool_Batch_Workflow.ijm (https://github.com/VergerLab/FibrilTool_Batch_Workflow)
- Cell_ROIMaker.ijm (https://github.com/VergerLab/MT_Angle2Ablation_Workflow)
- Line_ROIMaker.ijm (https://github.com/VergerLab/MT_Angle2Ablation_Workflow)
- Angle2Ablation.ijm (https://github.com/VergerLab/MT_Angle2Ablation_Workflow)
  Each of these macro can be directly downloaded in the folder of your choice. You can drag and drop the macro file into Fiji to open it, and click run in the editor window to run it.

- Python: Data analysis can be performed with any appropriate software, but here we propose a user-friendly python notebook with predefined graph output and statistical analysis to ease the analysis of the results and the comparison of the result generated in different labs using this workflow. This notebook can be run locally on your computer after installing the required software and python libraries, or with no install using binder directly from the git hub repository (https://github.com/VergerLab/MT_Angle2Ablation_Workflow). In the case of local installation, the detailed explanation for installation is described in step 6.

How to organize your folders to facilitate the data analysis/management

- From data acquisition: First, after image acquisition, keep a folder with all the raw untouched data, and create a separate folder to convert and save all individual acquired stacks in .tif format.

- Extracting cell contours and cortical microtubule signal with SurfCut: This step further described in (1.), once you have found the appropriate parameters, can be done in batch on all your images on the folder created above. If some signla extraction does not apprear correct after the batch processing, some images can be processed one by one in calibrate mode.

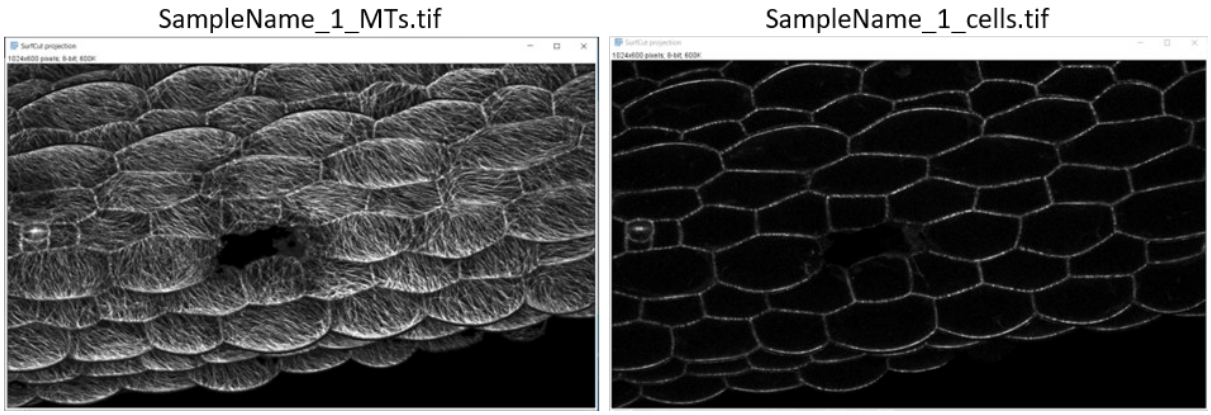
- Angle2ablation analysis: For the following steps, the images need to be separated in different sub-folders according to conditions/mutants/time points. This is because all the data present in a single such folder will be ultimately pooled to correspond to one “population” for later statistical comparisons (e.g. WildType_T0, WildType_T8, Mutant1_T0, Mutant1_T8,...). The following macros in the workflow (“Cell_ROIMaker.ijm”, “FibrilTool_Batch_Workflow.ijm”, “Line_ROIMaker.ijm”, “Angle2Ablation.ijm”) are meant to process images in batch in a single folder. Ultimately the macro “Angle2Ablation.ijm” generate a .txt file containing all the quantification for one such population.

Step-by-step procedure

1. **Cell contour and Microtubule signal extraction with “SurfCut.ijm”**

a. Run “SurfCut.ijm” macro on imageJ to extract the cell contours of the tissue and the outer epidermal CMT signal on all images. First, you have to determine the right parameters for the cell contours and CMTs with the “Calibrate” mode of SurfCut or you can use the “Batch” mode to analyze your images semi-automatically. For more details, see the SurfCut Step-by-step user guide (https://github.com/sverger/surfcut).

b. At the end of the macro, it is important to add specific suffix to the cell contour images “_cells” and “_MTs” to the CMTs images when saving them. These suffixes will be recognized by the next macros used later on. Note that there is no need to save the Original Projection or the SurfCut Stack, but only the SurfCut Projection.



c. The SurfCut macro automatically creates a folder. This folder contains the extracted 2D projections of cell contours with the “_cells.tif”, CMTs with the “_MTs.tif” suffix and the parameter files for each signal extraction.

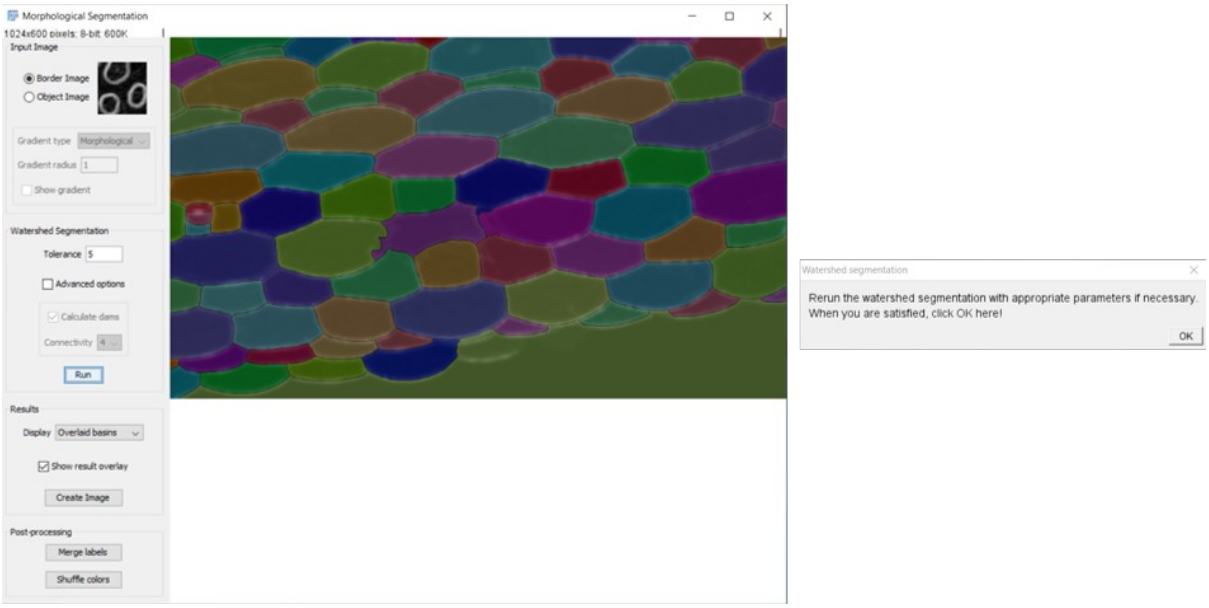
2. Define cells as region of interest with “Cell_ROIMaker.ijm”

Before running “Cell_ROIMaker” you need to install or make sure that you have installed the MorpholibJ plugin in your Fiji.

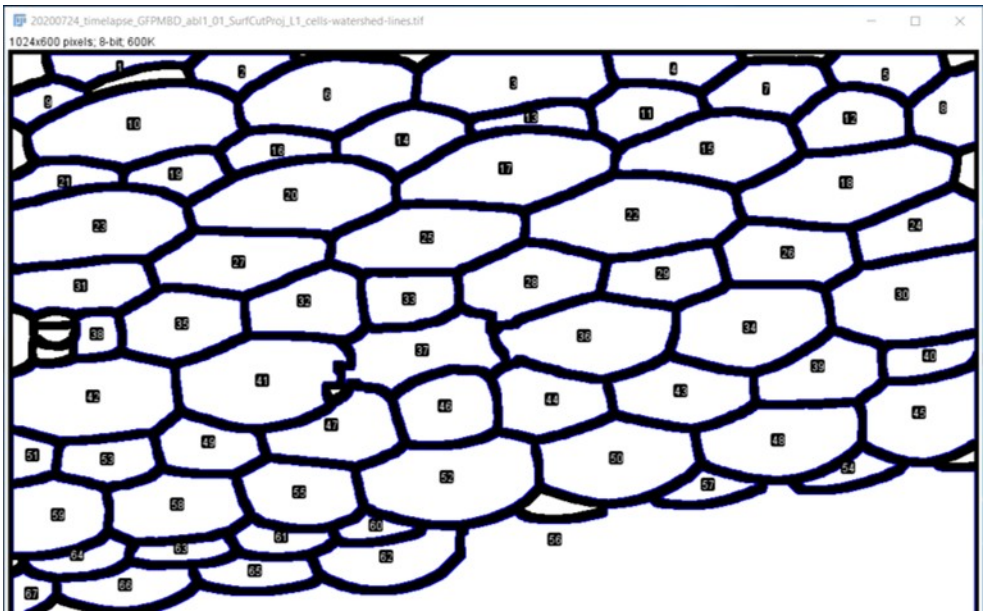
a. Drag and drop the Cell_ROIMaker.ijm file on your Fiji window.

b. Run the “Cell_ROIMaker.ijm” macro. Here you will be asked to choose the subfolder where your “_cells.tif” and “_MTs.tif” images are located. This macro, as well as the next ones will open the images one by one and process them in a batch mode to save automatically the results and ROISet folders in that same subfolder. If the experiment includes different mutants/conditions/time points, the macro has to be run for each subfolder.

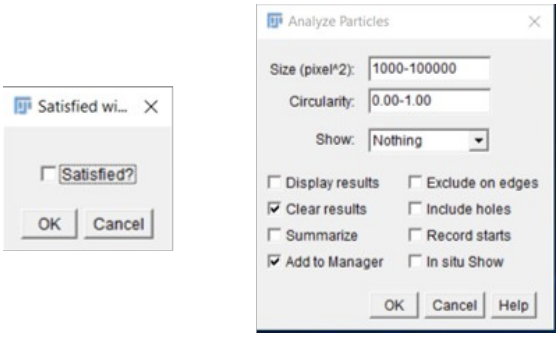
c. The “Morphological Segmentation” tool (MorphoLibJ library; Legland et al., 2016) opens with an image. The segmentation runs automatically by using default parameters. The Watershed Segmentation has a tolerance of 10 but this can be modified until the segmentation fits your needs. When you are satisfied, you can tick the box and click “OK” on the window (see below) to pursue the segmentation. Note that if some of the cells are not segmented properly they can be corrected, removed or added manually during the next steps.



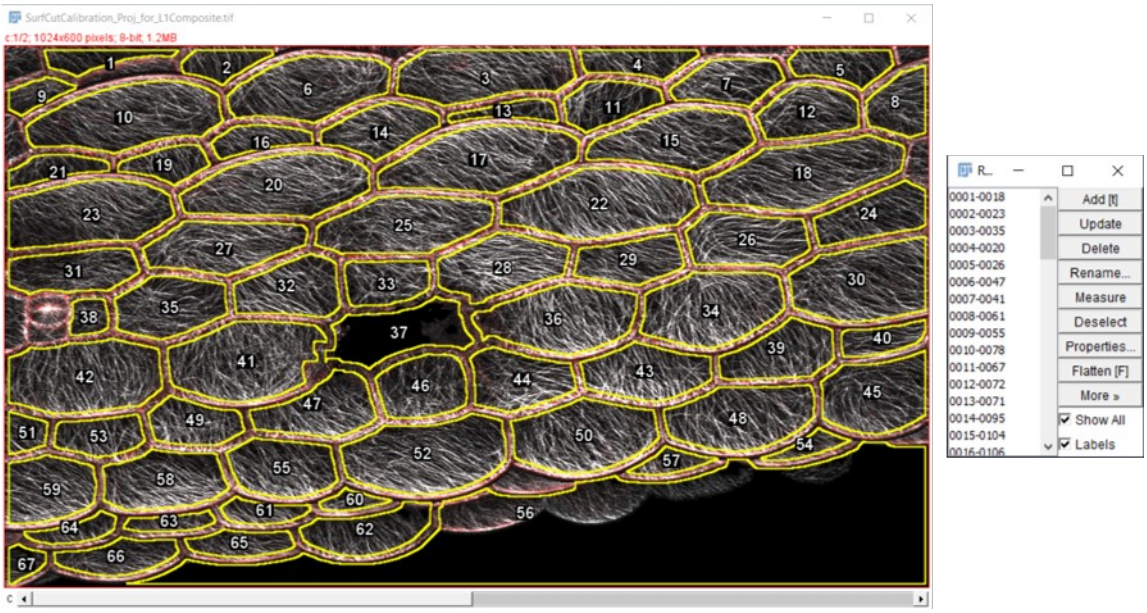
d. An outline of the segmentation with watershed lines is automatically generated. These lines are automatically dilated four times in order to exclude the signal of cell contours and to avoid any bias in the later CMTs array analysis. The Analyze particles tool is automatically run to show the ROI of the segmented cells as an overlay and to save them in the ROI manager.



e. Note that the parameters of the Analyze particles tool can be modified by unclicking the “satisfied” window and clicking “OK”. Otherwise, keep the box ticked and click “OK”.



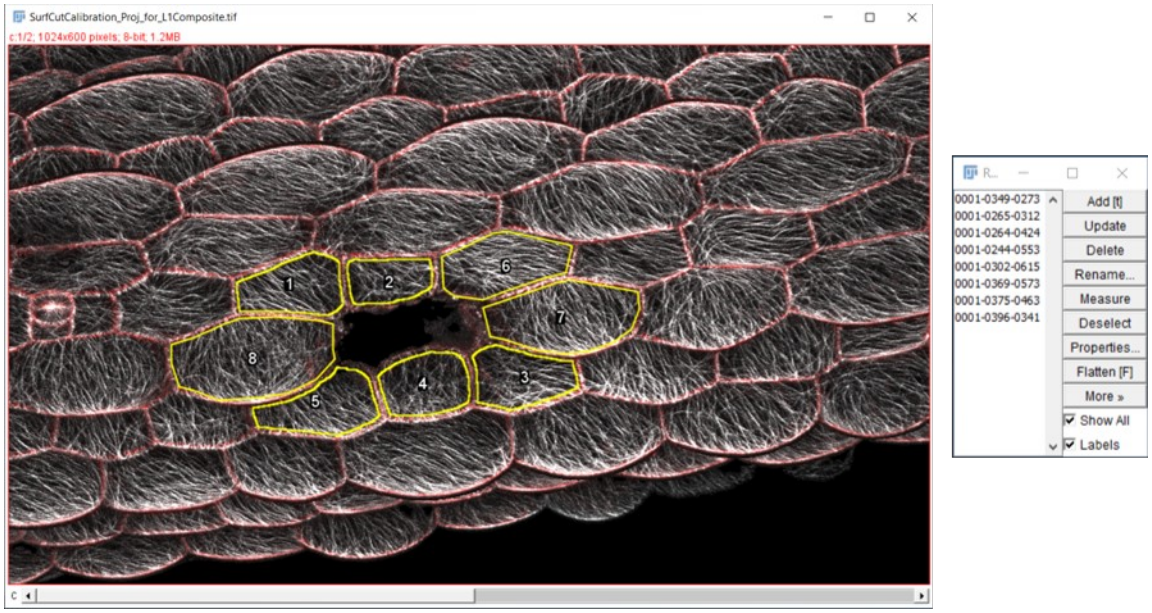
f. A composite image of the “_MTs” and “_cells” with the overlay of all the Cell ROIs will then appear. On this overlay image you can select the Cell ROI that you don’t want to analyze and click on delete of the ROI Manager. We recommend to only keep the cells directly around the ablation site because the CMTs of these cells are the most relevant to analyze for the response to the mechanical stress induced by the ablation.



g. If a ROI doesn’t fit a cell compared to the cell contour, you can move that ROI to fit and update the new ROI position on the ROI Manager.

h. If some cells are missing, you can also add a ROI of a cell by using the polygon selection (see below) and by adding the ROI on the ROI Manager (hit ctrl+T to add a newly drawn ROI to the ROI manager). This is possible because of the composite image of _MTs and _cells; it makes it easier to follow the cell contours of the missing cell.

i. Once the selection of ROIs to analyze is done, you can click on “OK” (see below). The ROIset file will be saved automatically, the curent image closed and the next open. The steps above are repeated for each image until all of the images in the folder are processed.

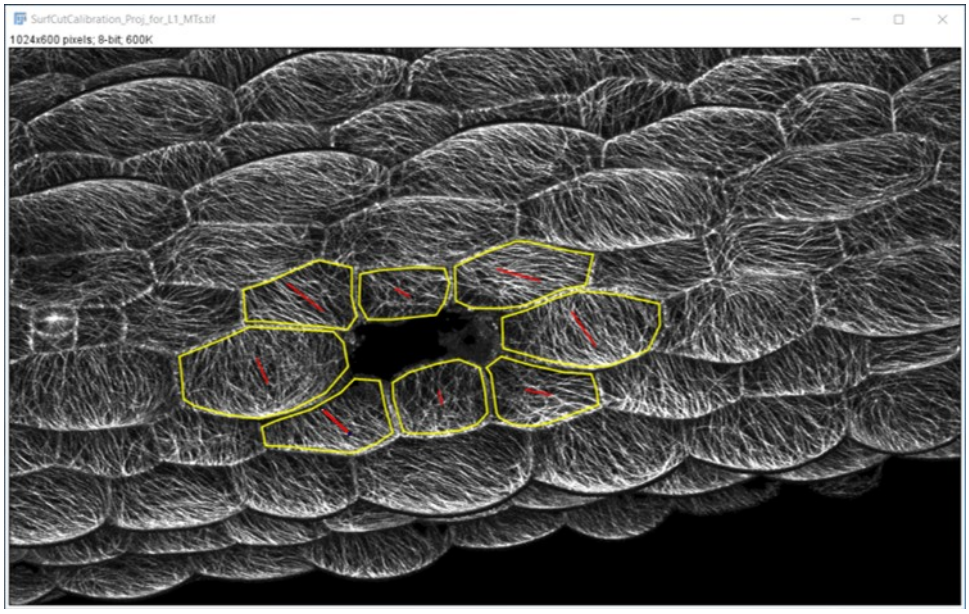


3. **Microtubule quantification with “FibrilTool_Batch_Workflow.ijm”**

a. To analyze the CMTs arrays, run “FibrilTool_Batch_Workflow.ijm” macro (adapted from Boudaoud et al., 2014; Louveaux et al., 2018) on Fiji.

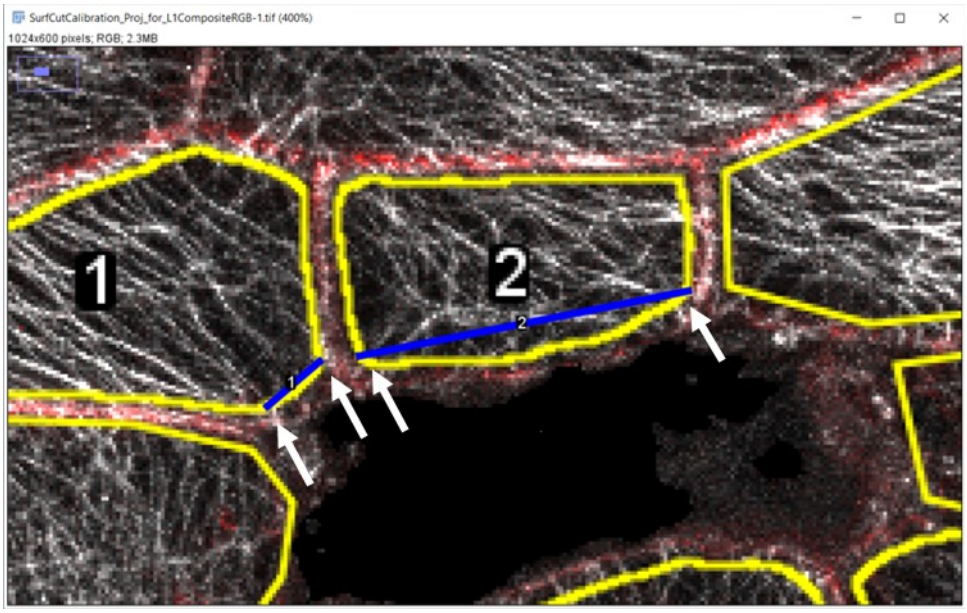
b. A FibrilTool window opens to allow you to choose some parameters. These are mostly to change the result display on the output image and do not affect the analysis (lines color, numbering...).

c. Once you have defined what you need, you can click “OK” to start the analysis. The analysis will run in loop for all the images in the folder and the image output and quantification results will be saved automatically.



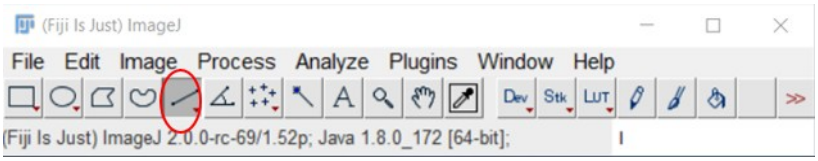
4. **Line representing the ablation stress for each cell with “Line_ROIMaker.ijm”**

To analyze the changes in the CMT arrays orientation in response to mechanical stress, we need to take into account how each cell is positioned toward the ablation site. An ablation in a (ideally isotropically) tensed tissue is expected to generate a circumferential tension around the ablation site. Here, to approximate this expected stress pattern, our strategy is to trace a line along the side of the cell facing the ablation. To limit user induced bias this line should be drawn between the two edges of the cell facing the ablation site (see figure below).



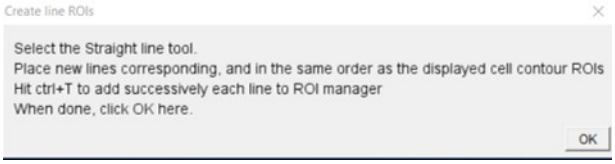
a. Run the “Line_ROIMaker.ijm” macro.

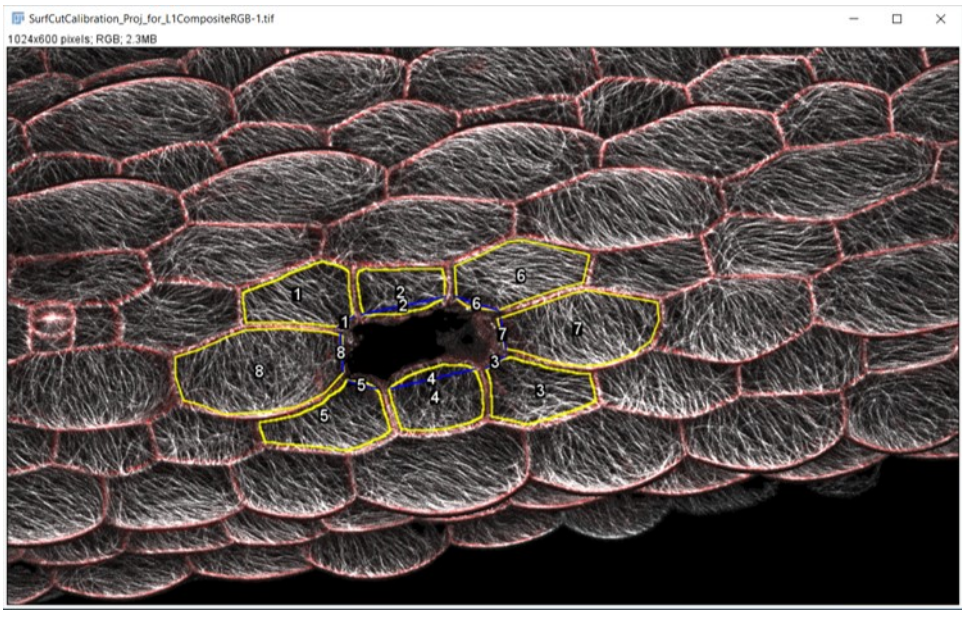
b. Select the Straight line tool in imageJ (see below).



c. Draw manually each lines corresponding to each cell on the image. Here it is important to note that each line ROI number must correspond to the cell ROI number. In the image, the cell ROI numbers are displayed. Start by drawing the line that correspond to the cell (ROI) number 1 and add it to the ROI manager (Click “add” or hit ctrl+T). Then draw the line corresponding to the cell number 2, and so on.

d. Check that each Line ROI number fits to the Cell ROI number and then click “OK”.

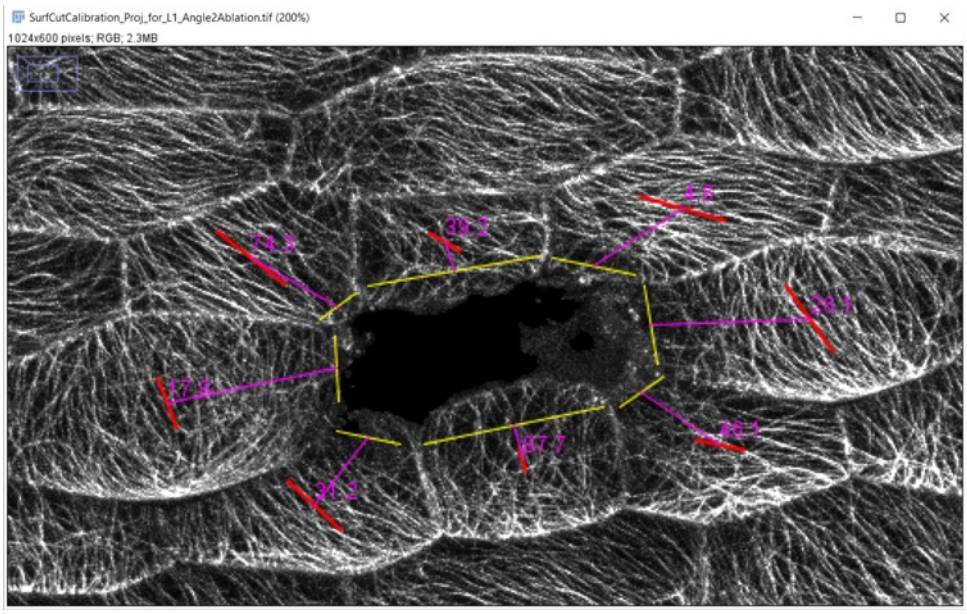




5. Angle measurements of microtubules toward the ablation site with “Angle2Ablation.ijm”

a. Run the “Angle2Ablation.ijm” macro.

b. This macro calculates the acute absolute angle (between 0 and 90 degrees) between the actual microtubule array main orientation and the manually drawn line representing the expected stress pattern. It generates an image output and two files, one for each image from the subfolder containing the measurements (FibrilTool Angle; Drawn Line Angle; Raw angle; Absolute angle; Acute absolute angle to ablation; FibrilTool Anisotropy) and a file containing all the measurements of all the images in the subfolder. The image output (see below) with the angles written on the image combined with FibrilTool output and the lines representing the ablation. This image is useful to visually confirm that there is no apparent mistake in the angle quantification.



6. Data analysis with Jupyter Notebook

Here you have two possibilities to use the data analysis script.

Access the Jupyter notebook version through Binder:

a. A temporary version of the Jupyter notebook can be used on your browser using binder. To start it go to https://github.com/VergerLab/MT_Angle2Ablation_Workflow and click on “Launch binder” (it can take a few minutes to start it).

b. Then, upload your data in the “Data” folder on the web page. Only the file called All_….txt

c. Once this is done, click on “Stats_Angle2Ablation.ipynb” this will start a new tab on your browser with the Jupyter notebook named “Statistics for Angle to ablation Analysis”.

d. Start the analysis process at the first line (“Load required packages”), click on it to select (Blue on the left side) and then use “shift + enter” to run the line. Proceed as for the first line with the rest. For the line, “single_file_path” put the path leading to your own data. To finish run the rest of the lines.

Download and Installation of Miniconda.

The miniconda installer can be found on the official website: https://repo.anaconda.com/. From this website, you can download the Miniconda2 installer for your system.
LINUX: https://repo.continuum.io/miniconda/Miniconda2-latest-Linux-x86_64.sh
MAC: https://repo.continuum.io/miniconda/Miniconda2-latest-MacOSX-x86_64.sh
Windows: https://repo.continuum.io/miniconda/Miniconda2-latest-Windows-x86_64.exe

- Miniconda2 install for Windows:

Click on “Execute” to start the installer and follow the instructions. During the installation, you can choose the “Destination folder”. In “Advanced options” make sure to select “Add Miniconda2 to my PATH environment variable”. Then, follow the instructions to finish the install.

- Commands for Anaconda prompt for Windows:

To open the Jupyter notebook for the statistical analysis of the angles to ablation. You have to open first the command on the “Start” menu. Then you have to create a new environment. To do so you enter the command:

conda env create

Following that command, before clicking on enter; write the name of the environment you wish to create (wait a few minutes until the installation of the packages in the environment is done) :

Stats_Angle2Ablation_V2

In order to activate this new environment enter the following command:

conda activate Stats_Angle2Ablation

Note that you can find the path of all the environments that you created by using the command:

conda info --envs

Once Stats_Angle2Ablation is activated type as command:

jupyter lab

A web page should open automatically if not you can find the web link to the Jupyter notebook on the command window.

- “Statistics for Angle to ablation Analysis” Jupyter notebook:

Here, start at the first line (“Load required packages”), click on it to select (Blue on the left side) and then use “shift + enter” to run the line. Proceed as for the first line with the rest. For the line, “single_file_path” put the path leading to your own data. To finish run the rest of the lines.