**Evaluating pavement cell size and circularity (Cifrová and Šliková, unpublished; modif. from Rosero et al 2015)**

1. Use images from cotyledons stained with 1μM FM4-64 (Sigma) for 1 hour in the dark. Three single-plane images should be taken from nonoverlapping regions of the adaxial epidermis in the apical third of the cotyledons using confocal laser-scanning microscope with a 20 x/1.2 water-immersion objective and 515 nm excitation. Alternatively, in plants carrying fluorescent cytoskeletal markers, the FM4-64 staining can be omitted and marker fluorescence (or autofluorescence after contrast enhancement in case of cells which have silenced marker expression) can be instead used to detect cell to cell boundaries. Measure at least 100 cells total per variant.
2. Open each image by dragging the microscope-generated file onto the Fiji window. In the Bio-Formats dialog go to import Options- Hyperstack , Default.
3. Image-stack-Zprojection-max.intesity
4. Proces-smooth
5. Image-adjust-tresholds-set Huang and BaW, optimize cell border width by cursor, set Dark background and Apply)
6. Proces-binary-make Binary
7. Proces-binary-skeletonize
8. Connect gaps in cell contures using the Pencil and erase garbage using the Eraser (for Pencil edit-option-line weight-1), Use More tools – Drawing tools to see the Eraser. Use magnifier – left and right mouse is mínus and plus – to see enough details. Proces-binary-make Binary again when done.
9. Analyze-set scale- unit length – micron (není nutné)
10. Analyze-set measurements - set area, perimeter, shape description, display label, uncheck the rest.
11. Select 8-10 cells per sample within a field were selected for evaluation. To avoid bias, cells that made contact with a diagonal line were chosen in each measured field. Mark each cell of interest (Wand –tracing tool) -Analyse-measure (or Ctrl+M)
12. Transfer results to Excel for further analysis.
13. Test the hypothesis that samples E17 and E18 differ in either cell size or shape (circularity). Use ANOVA at <http://www.astatsa.com/OneWay_Anova_with_TukeyHSD/> to check for significance. Then do the same combining results from all participants.

**Quantifying actin dynamics using QuACK - Quantitative Analysis of Cytoskeletal Kymograms (Cvrčková and Oulehlová 2017)**

For serious results, aim towards 10 movies per sample, recorded from at least 5 plants, with no more than 2 recordings from the same plant. Only 4 movies per sample will be done in the course for time reasons.

**Conventions:**

Instructions in plain text, file/folder names and variable names in Courier, program and menu names in ***bold italics***, commands in **bold**, notes, remarks and comments in *italics*.

**Before you begin:**

* Copy the macros MovieProcess.ijm and LineToSkeleton.ijm to a local drive into a location where you can easily browse to them from within ***Fiji*** (either the Fiji.app/macros directory or a folder that also contains your data).
* Open the folder containing your data.

**Step 1: processing the movies**

* Select the files to be analyzed and open them by dragging onto the ***Fiji*** icon. In the ***Bio-Formats import Options*** dialog, select **View stack with Hyperstack**, **Stack order XYZT**, **Open files individually**, **Autoscale**, **Split channels**. Make sure no other boxes are checked and click **OK**. Open all the movies you are considering for analysis, including those where you are in doubt. You should now have a stack of windows, each containing a movie, on your desktop.
* Set the parameters to be measured: in the ***Fiji*** menu, go to ***Analyze*** – ***Set Measurements***, check **Shape Descriptors** (and nothing else), and click **OK**.
* If working with anisotropic organs such as roots or hypocotyls, rotate all the movies to align organ axis or another well-defined structural landmark to the horizontal or the vertical (the same for all movies). We shall not do it here.
* Activate the MovieProcess.ijm macro: go to ***Plugins*** – ***Macros*** – ***Install***, browse to the location of the macro, and click **Open**. You will now have a new command ***MovieProcess*** in the ***Plugins*** – ***Macros*** menu.
* Apply this macro to each of the open movies. To do so, select a movie by clicking its top frame and run ***Plugins*** – ***Macros*** – ***MovieProcess*** This (1) converts the movie to grayscale, (2) stretches the contrast in a predefined manner, and (3) overlays a randomly positioned 5 m x 5 m (or 2 m x 2 m) grid over the movie. You will thus now have a pile of grayscale movies with a grid overlay. Discard (close without saving changes) any obviously bad, especially very noisy, movies and select the required number of movies for further analysis, starting with those of the best technical quality. *The random grid positioning ensures that you can in case of need re-measure the same movie as a technical replicate without actually duplicating the data obtained.*

**Step 2: generating skeletonized kymograms**

* Activate the LineToSkeleton.ijm macro: go to ***Plugins*** – ***Macros*** – ***Install***, browse to the location of the macro, and click **Open**. You will now have a ***LineToSkeleton*** instead of ***MovieProcess*** in the ***Plugins*** – ***Macros*** menu.
* Decide on the length of transect you want to analyze, which will affect sensitivity of the assay. For maximum sensitivity with highly dynamic structures (actin), aim to the longest line that fits into most cells in both horizontal and vertical direction. 20 um is a good start for actin in pavement cells. In the course, generate 4 kymograms per movie. For isotropic samples, horizontal and vertical transects can be mixed.
* For each movie, generate a set of skeletonized kymograms (“skeletons”) over the given transect length. To produce a skeleton:
  + Draw a horizontal or vertical transect across a well-focused portion of the movie. In the ***Fiji*** menu, select the ***straight line*** tool and draw a line segment of the selected length along the grid while holding **Shift** (depress the **left mouse** button at line beginning and release it at the end). Aim for equally spacing the transects across the focused part of the image.
  + Run ***Plugins*** – ***Macros*** – ***LineToSkeleton***. A skeletonized kymogram will pop out in a separate window.
* Proceed until you have processed all the movies and accumulated a full set of kymograms for the given parameter/data point. Keep the movie windows open unless you are sure that you will not be returning to them. *It is now safe to close any unused movies. If you are generating the last set of kymograms (last transect length or direction) in an assay, you may now close the movies already processed (without saving any changes). In a typical case, you now have 40 skeletons and possibly 10 movies on the desktop.*

**Step 3: measuring the skeletons**

* To measure structure lifetime, determine the length of the longest trace in the time (vertical) dimension of each skeletonized kymogram.
  + Select a kymogram window by clicking its top. In the ***Fiji*** toolbar, select the ***magnifying glass*** tool and click the kymogram as needed to see details well.
  + In the ***Fiji*** toolbar, select the ***straight line*** tool and draw a vertical line segment along the vertical length of the tallest contiguous trace while holding **Shift** (depress the **left mouse** at line beginning and release it at the end; you can go either top-down or bottom-up, this does not matter). Press **Ctrl M**. A new row of values will appear in the ***Results*** window.
  + If you are not sure which is the tallest trace, measure several (2-3) candidates and delete those which are not tallest (in the ***Results*** window, select the row to be deleted by clicking it, then go ***Edit*** – ***Clear***).
  + If you do not plan measuring other parameters, close the kymogram without saving.
* Proceed until all kymograms have been measured. *Typically, you will now have 40 measurements per sample.* Open the ***spreadsheet program*** of your choice and create a new spreadsheet, or open an existing spreadsheet you will add your data to. In the ***Fiji***´s ***Results*** window, go ***Edit*** – ***Select All***, then ***Edit*** – ***Copy***. Paste the copied data into the spreadsheet. Clean up and annotate the data in the spreadsheet – you will only be interested in the last column (Length). Save the spreadsheet.
* To measure lateral mobility, determine the width of the widest trace in the space (horizontal) dimension of each skeletonized kymogram in a manner analogous to that described above.
* When done, close all ***Fiji*** windows without saving (***File*** – ***Close all***) and exit ***Fiji***.

**Step 4: data interpretation and presentation**

Once you have processed all your samples using steps 1-3, you will have a set of numeric measurements for each condition (or set of experimental parameters) examined. While it is tempting to use standard statistical approaches such as the t-test or ANOVA to assess significance of between-group differences, this is very likely a wrong approach, because long-living events (or, possibly, large movements) are not recorded whole and thus the data may not fulfill the condition of normal distribution. Therefore other methods are needed.

* To compare two groups, use the Wilcoxon-Mann-Whitney test (also known as WMW, Wilcoxon, Mann-Whitney, or U-test). A good calculator can be found e.g. at <http://www.socscistatistics.com/tests/mannwhitney/Default2.aspx>
* To compare more than two groups, use the Kruskal-Wallis test. Calculator e.g. at <http://www.mathcracker.com/kruskal-wallis.php>.