Method Article

A quantitative method to analyse F-actin distribution in cells

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A B S T R A C T

Changes in actin structure and distribution are involved in many cellular processes, such as differentiation, proliferation and migration. Differences in cell shape and size make the analysis of actin distribution difficult. Here, we have developed a Fiji macro that analyzes the distribution of actin within the cell, regardless of cell size or shape. The staining intensity is measured along an automatically drawn line over the cell. The intensity data is divided in equal bins, making the analysis insensitive to changes in cell size or shape. We have also created an R script that further processes the acquired data. Together, final data can be acquired within minutes from a set of images, with freely available software. We demonstrate our method with F-actin staining of cytochalasin D treated cells. The advantages of our methods are:

- The analysis is not influenced by cell shape or size
- All steps in the analysis are shown, and can therefore easily be verified for each image
- All software required for the analysis is freely available

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A R T I C L E  I N F O

Method name: Fiji macro for staining distribution quantification
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**Specification Table**

| Subject Area: | Biochemistry, Genetics and Molecular Biology |
|--------------|---------------------------------------------|
| More specific subject area: | Automated image quantification |
| Method name: | Fiji macro for staining distribution quantification |
| Name and reference of original method: | Not applicable |
| Resource availability: | Fiji software: https://fiji.sc |
|                      | R software: https://www.r-project.org |

**Method details**

We have analyzed the F-actin distribution in human mesenchymal stromal cells (hMSCs) using a Fiji macro that we have developed. The macro could be used to test any staining intensity distribution within any cell type. Images were acquired using a confocal microscope with a 25x objective at 512 × 512 pixels and 8-bit. Depending on the desired resolution, other imaging parameters can be used. To ensure fair quantification among conditions, all images should be taken with the same objective and microscope settings. For optimal post-processing, cells should not be in contact with each other. Z-stacks were taken to ensure the whole cell was in focus. The image analysis was then done in Fiji.

To install the Fiji macro, install Fiji (see useful links), and drag the Fiji macro file (see supplementary data) into the Fiji window, save it and press run.

When the Fiji macro is started, it first asks to choose a folder to save the output data file. After this is selected, a window with several options is opened. There is the option for fully automated cell selection, manual cell selection, or to manually draw a line. In addition, there is an option to perform a maximum intensity projection of a Z-stack, as the macro does not work with Z-stacks. The maximum intensity images are automatically saved in a separate folder in the folder of the original files.

The second set of options is about the line creation. A line can be drawn perpendicular or parallel with the long axis of the cell or the nucleus. Select the option that is most relevant to your research question. In this paper, we were interested in the actin distribution of hMSCs. In our experiments with hMSCs presented here, most actin fibers that formed were in line with the major axis of the cells. To best analyze the actin distribution, we therefore analyzed the cells with lines drawn perpendicular to the major (long) axis of the cell. The reader is kindly referred to the “additional information” section for a more detailed discussion on the different line options.

In the window, the slice (channel) number that contains the nuclear or actin staining (or other staining of interest) and the name of the data group should be filled in.

Lastly, there is the option to analyze all files in a folder for rapid image processing, or to analyze an image that is already opened. The last option runs the macro on the last active image.

After selecting the desired options, the macro will run as follows:

1. Clearing background around cell of interest.
2. A threshold is made using the ‘Default’ threshold method. (See tips and tricks on how to adjust and optimize this process)
   - The ‘Fill Holes’ function is run.
   - The ‘Analyze particles’ function is run on the actin image slice, minimal size 200 μm². (See tips and tricks on how to adjust the minimal cell size)
3. If manual selection was selected, the ROI’s are displayed and the cell of interest can be selected manually, if there is more than one cell present in the image. If automated selection is selected, the largest ROI in the image is considered the cell of interest.
   - The ‘Clear Outside’ function clears everything outside of the cell of interest.
4. Selecting the nucleus
   - The ‘Analyze particles’ function is run on the nucleus image slice, minimal size 25 μm². (See tips and tricks on how to adjust the minimal nuclear size)
5. If manual selection was selected, the ROI’s are shown and the nucleus of interest can be selected manually, if there are multiple nuclei in the image. For automated selection, the largest ROI within the cell of interest is considered the nucleus.
Step 3. Measuring average actin intensity over the nucleus and in the whole cell

For the actin intensity over the nucleus, the nucleus of interest is selected and the mean gray value (mean pixel intensity) in this ROI is measured in the actin slice (channel).

For the average actin intensity in the cell, the cell of interest is selected and the mean gray value (mean pixel intensity) in the actin slice (channel) is measured.

Step 4. Measuring actin intensity over the line

A line can be drawn through the cell or through the nucleus, depending on what was selected in the main window.

The center of mass and fit ellipse functions are used on the ROI of the selected cell to determine the middle and the shape of the ROI, respectively.

A line is then drawn through the middle of the ROI, parallel, or perpendicular to the long axis of the fitted ellipse of the ROI.

The line is drawn through the whole image.

When the manually drawn line option is selected, the macro skips the previous steps and starts here.

The pixel intensity along the line is measured using the getProfile() function.

The line is trimmed on both ends until the first value higher than 0, to measure only the cell of interest.

As cells are not equally sized, the pixel intensity data from the line is split up into equally sized bins. The average is taken of all data points within each bin. Because pixel intensity is only measured where the line goes over the cell, the cell shape and size determines the size of each bin, and will be different between different cells. The standard bin size is set to 10, so the first bin always contains the outer 10% of the cells, and the last bin the outer 10% of the other side of the cell. See tips and tricks on how to change the bin size.

Step 5. Exporting data to csv

The actin intensity over the nucleus, within the whole cell and within each bin is exported into a single.csv file. When the macro is started, a folder is selected where the data file will be stored. If a data file already exists in the folder, the new data will be added to that file. If the added data has the same data group name, the R script (see step 6) will treat it all as one data group. This allows for measurements to be done at different times and all data can be selected in a single file.

Step 1–5 are done automatically by the macro in Fiji. To automatically analyze the data, we have written a script in R.

Step 6. Data analysis with R

To run the code, install R (see useful links), File -> New document; copy the code in this window, save as Unicode(UTF-8) file. Follow the instructions in the code to set the right working folder. Copy the code in the R console window and press enter.

The data is separated per data group and the values for each bin are averaged using the aggregate function.

The standard deviation and the n are also determined using the aggregate function.

These values, along with the actin intensity over the nucleus and within the whole cell, are exported to a new.csv file.

This file gives a clear overview of the different measurements per data group. See tips and tricks on how to calculate the 95% confidence interval, as is used in Fig. 1, from these values.

To validate our method, we have treated bone-marrow derived human mesenchymal stromal cells (hMSCs) with cytochalasin D (cytoD) (Sigma-Aldrich), an inhibitor of actin polymerization [1] (Fig. 1a).

hMSCs were isolated from the bone-marrow by Texas A&M Health Science Center [2]. hMSCs were seeded on glass coverslips at passage 5 at 1000 cells/cm² in growth medium (αMEM + Glutamax+10% FBS). 1 μM cytoD was added to the medium for 24 h and cells were fixed the following day in 3,6% (v/v) paraformaldehyde. Permeabilization and blocking was done using 2% (w/v) bovine serum albumin in PBS + 0.1% Triton-X for 1 h at room temperature. After one PBS + 005% Tween–20 wash, actin and nuclei were visualized using 66 nM phallolidin–488 (ThermoFisher Scientific) and 014 μg/ml DAPI (Sigma-Aldrich) in PBS + 005% Tween–20 (Sigma-Aldrich), for 20 min at room temperature, followed by three PBS + 005% Tween–20 washes. The unmounted cells on coverslips were imaged using a confocal microscope with a 25x objective (Numerical aperture: 0.95; working distance: 2.4 mm) at 512 × 512
Fig. 1. **F-Actin distribution in hMSCs treated with Cytochalasin D.** hMSCs were cultured for 24 h on coverslips and then incubated with 1 μM cytochalasin D for 24 h. (a) Cells were stained for F-actin (phalloidin, green) and nuclei (DAPI, blue). Scale bars 50 μm. (b–d) Graphs show mean ± 95% CI. Statistical differences were calculated using Graphpad Prism 8. (b) A two-way ANOVA with Sidak’s multiple comparisons for each bin was performed. Stars indicate statistically significant difference between the two groups within the same bin. (c, d) An unpaired t-test was performed. * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001. N = 15 for Untreated, N = 13 for CytoD.
pixels and 8-bit, pinhole 1 Airy unit (5587 µm). Z-stacks were made with a z step size of 1 µm between images and typically 5 to 10 images, depending on cell shape, to image the cell from top to bottom. Using the Fiji macro and R script we analyzed the F-actin distribution in the cell. The line over which the actin intensity was measured was perpendicular to the major (long) axis of the cell and through the cell center. As expected, the distribution of F-actin changed upon treatment with cytoD (Fig. 1b). In the untreated hMSCs, actin was distributed equally over the cell. In the cytoD treated hMSCs, F-actin intensity was higher on the cell border than in the cell center due to a lack of stress fibers. Less F-actin was found over the nucleus (Fig. 1c). Interestingly, on average more F-actin per cell was observed in the CytoD treated hMSCs, probably due to strong decrease in cell size (Fig. 1d).

**Tips and tricks**

**Image type requirements**

Image files should be. tiff or. tif files, the macro does not work with other image files. The images should contain a µm scale. If images do not contain a scale, then a scale should be set (Analyze->Set Scale). There should be at least a channel with a nuclear staining and a channel with actin or another staining. Image files can contain more than these two channels, but the macro can only analyze two channels. In case of Z-stacks, use the macro to create maximum projection images before proceeding.

**Cell-cell contact**

As will all image processing, it’s difficult to distinguish between different cells if they are in contact. The macro can also not distinguish between different cells if there is too much cell contact. See Fig. 2 for examples of images with multiple cells that the macro is still able to handle properly. Avoid taking images of cells that are in contact with other cells. If this cannot be avoided, the macro can be run as normal, but afterwards the line can then be adjusted manually to the region of interest in the cell of interest, and the line measured manually by the macro. In case of a manually drawn line, the macro will not have the information on average cellular actin or average actin over the nucleus.

**Cell shape**

The macro shortens the drawn line to when it first reaches the border of the cell. If a cell is a particular shape so that the line goes through a cell multiple times, it can happen that the inter-cell space is being measured in part of the line. In this case, the measurement will not be reliable. A line can be drawn manually through the cell and this line can be measured by the macro. In case of a manually drawn line, the macro will not have the information on average cellular actin or average actin over the nucleus.

**Threshold**

The thresholding method should be optimized if the ‘Default’ method does not work well. Use the ‘Auto Threshold’ function with ‘Method: Try all’, to try all automatic thresholding settings and determine which works best for your image acquisition. Ideally, the thresholding is optimized before taking all images for quantification to ensure that a good automatic threshold can be made with the researcher’s image set. To change the method the macro uses, look for: ‘method = Default white’ and change ‘Default’ into the name of the preferred method.

**Adjust minimal cell or nucleus size**

The minimal surface area of what the macro will detect as a cell is 200 µm², the minimal size of a nucleus is 25 µm². If cells or objects smaller than 200 µm² are to be analyzed, the minimal detection size can easily be adjusted. Search for ‘200’ in the code, there are two locations where 200 is used. Change this number to the desired cell size in both locations. To change the minimal size of the nuclei: search for ‘25’, there are three locations where 25 is used. Change all three into the desired minimal nuclear size.
Fig. 2. Images of hMSCs pre- and post-processing by the macro. The macro was run on the images in the top panel with automatic cell selection and line perpendicular to the major (long) axis of the cell. When multiple cells are present in the image, but they are not touching, the macro is able to select the cell of interest and remove the others (bottom panel). hMSCs were untreated (left and middle panel) or treated for 24 h with 1 μM cytoD (right panel) and stained for phalloidin (green) and DAPI (blue). Scalebars 30 μm.
Changing bin number and maximum number of bins

The standard number of bins is 10. To change this, search for: ‘bin = 10’, and change 10 into the desired number. There is no upper limit for the number of bins in the macro, but keep in mind that the number of pixels in the cell where the line passes is the theoretical maximum number of bins.

Changing data in the. csv file

In case measurements need to be deleted or edited, open the. csv file in a TextEdit (mac) or Notepad (windows), and not in excel. Changing the file in excel will damage the file beyond repair and all measurements will have to be done again, before the R script can process the file.

Calculate the confidence interval

The R code gives the average and standard deviation of the measurements. To calculate the confidence interval, as was done for the results in Fig. 1, the following formula can be used: \( Z^* \sqrt{(s^2/n)} \). Z is the confidence interval factor, which is 196 for 95% confidence interval. s is the standard deviation. n is the number of samples.

Additional information

To our knowledge, only one other method has been described to quantify actin distribution within cells, regardless of cell shape [3]. Elosegui-Artola et al. use a Matlab script to transform cells into circles and measure the actin intensity radially. The difference with our method is that we measure the intensity over a line, and not radially over the whole cell. This can be an advantage or a disadvantage, depending on the use. It can be advantageous to measure over a line rather than the whole radius in a number of different situations: 1. If there is interest in a particular distribution of the staining in the cell. An example of this is front to end polarization of actin of migrating cells. 2. The line over which staining intensity is analyzed can be drawn perpendicular to, or in line with the cell. Analyzing cells using both options can give more information of the distribution of the staining through the cell. 3. When analyzing actin distribution in cells with many long protrusions, analyzing staining distribution over a line can be advantageus over measuring radially over the whole. The line could be placed over the exact region of interest, while a radial distribution could give unreliable results because of the protrusions. Lastly, our method can be done with freely available software and we have developed a fully automated pipeline, from image to final analysis. Other researchers have looked at characterizing individual actin stress fibers, or different actin structures within cell, but not distribution [4–7].

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.mex.2019.10.018.

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