Quantitative evaluation of cytoskeletal organizations by microscopic image analysis

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Summary: Visualization of cytoskeletal organizations is a fundamental research method for understanding plant cell activities. Conventionally, immunostaining methods have been used to visualize cytoskeletons, but some technical difficulties make it hard to obtain a large number of reliable microscopic images. The introduction of fluorescent protein tagging technology and the development of high-throughput method for microscopic image acquisition have made it easier and quicker to obtain many reliable microscopic digital images of cytoskeletons. Based on these technical improvements, a method for quantitative evaluation of cytoskeletal organizations by image analysis has been developed. This method has become an indispensable research approach in state-of-the-art plant cell biology. In this minireview, I outline a practical method to measure image features to quantitatively evaluate the orientation, parallelness, bundling, and density of cytoskeletons using the ImageJ image analysis software.

Key words: actin filament, cytoskeleton, image processing, microtubule, quantitative evaluation

INTRODUCTION

Organizations of cytoskeletons are tightly related to various plant cell activities. For example, the orientation of actin filaments is deeply involved in the direction of cytoplasmic streaming (Kachar and Reese 1988, Shimmen and Yokota 2004) and the structures of transvacuolar strands (Shimmen et al. 1995, Higaki et al. 2006). The orientation of cortical microtubules determines the cellulose deposition direction, which regulates cell growth orientation (Hasezawa et al. 1988, 1989, Hasezawa and Nozaki 1999, Baskin et al. 2004). Therefore, to understand plant cell activity, visualization of cytoskeletons is an important research approach.

Although fluorescent protein (FP) tagging techniques with cytoskeleton-related proteins have already become the main solution for visualizing cytoskeletal organizations (Kost et al. 1998, Marc et al. 1998, Ueda et al. 1999, Hasezawa et al. 2000, Kumagai et al. 2001), before the introduction of FP technology, immunostaining was commonly used to observe cytoskeletons (Hasezawa et al. 1988, 1989, 1991, 1994, 1997, 1998, Hasezawa and Nagata 1991, 1993, Eun and Lee 1997, Miyake et al. 1997, Fukuda et al. 1998, Hasezawa and Nozaki 1999). Of course, immunostaining of cytoskeletons is still an important technique that does not require transformation. A serious problem with immunostaining method is that the cytoskeletal structures will usually be broken during the chemical fixation or staining process, even if they are carried out by technically skilled researchers. Therefore, it is very difficult to obtain a large number of reliable microscopic images of cytoskeletons by immunostaining methods. As a result, it was a laborious task to observe many samples, classify the cells based on the cytoskeletal organizations, and examine the time-course of their appearance frequencies by using immunostaining methods (Eun and Lee 1997, Miyake et al. 1997, Fukuda et al. 1998, Hasezawa et al. 1998, Hwang and Lee 2001). With FP technology, although it is obviously necessary to pay attention to the risk of artifacts caused by transformation and overexpression of FP probes, it is relatively easy to obtain many reliable microscopic images of cytoskeletons in living cells to be analyzed. Specifically, the FP technology makes it easy to classify the state of cytoskeletal organizations into several groups and to plot the frequency of occurrence of each group over time (Higaki et al. 2007a, 2008, Gao et al. 2008, Ma et al. 2016). In addition, because of recent advances in microscope technology, the image acquisition efficiency has also been improved (Abu-Abied et al. 2006, Salomon et al. 2010), and it is now easy to obtain a large number of digital microscopic images in a short time. With this technical background, a research method for the quantitative evaluation and classification of cytoskeleton organizations by microscopic image analysis has been developed (Higaki et al. 2010a). Quantitative analysis of cytoskeletal organizations has already contributed to the elucidation of various plant cell activities, including stomatal movement (Higaki et al. 2010a, 2012, 2013, Zhao et al. 2011), pollen tube growth (Su et al. 2012, Qu et al. 2013, Zheng et al. 2013, Zhu et al. 2013, Qin et al. 2014, Madison et al. 2015, Zhou et al. 2015), leaf pavement cell morphogenesis (Akita et al. 2015, Higaki et al. 2016, 2017), zygote polarization (Kimata et al. 2016), response to plant hormones (Lanza et al. 2012, Wu et al. 2015, Ma et al. 2016, Scheuring et al. 2016, Takahashi et al. 2016), defense response (Henty-Ridilla et al. 2013, 2014, Li et al. 2015, Inada et al. 2016a, 2016b, Shimono et al. 2016), and response to mechanical stress (Louveau et al. 2016). This quantitative evaluation technique has now become a basic research method that is indispensable for state-of-the-art plant cell biology. In this review, I outline the quantitative evaluation methodology of cytoskeletal organizations and share practical ways of using the ImageJ image analysis software (Abramoff et al. 2004).

WHAT WE SHOULD MEASURE FROM OUR CYTOSKELETAL IMAGES

Using image analysis techniques, we can obtain a vast variety of information from a single microscopic image. In other words, one microscopic image can be regarded as one plot in a multidimensional feature space. Some image features may
be biologically very meaningful, and some features may have no value in biological research. Therefore, when working on biological image analysis, it is necessary first to decide what kind of biological features should be measured from the image. Deciding what features to measure depends on the biological problem being addressed; therefore, what should be measured from a microscopic image should be decided by experimental researchers, the so-called ‘wet’ researchers. On the other hand, developing a computer program to measure those features from images may require the help of image analysis experts, so-called ‘dry’ researchers. Fortunately, I have supported many image analysis studies in collaborative research projects in parallel with conducting my own experimental research on plant cytoskeletons. Based on my experiences, I consider the four features, orientation, parallelness, bundling, and density, are important to grasp the cytoskeletal organizations in plant cells. However, what kind of features should be measured should be carefully considered and customized according to the objective of the research. The following is a sample set of highly versatile cytoskeletal features in the field of plant cell biology.

(1) Orientation
The direction that a cytoskeleton is arranged is a fundamental feature because the cytoskeletal direction is closely related to the plant cell activities, as mentioned above. Because it is unlikely that all the cytoskeletons will be oriented completely in the same direction, all angles of the cytoskeleton per unit length should be measured, and the average value should be calculated from the obtained angle distribution. The cytoskeletal orientation with respect to the biological reference direction can be evaluated by differences between the average angle of cytoskeletons and the cell morphological reference angle. The reference direction will vary according to the research purpose, but in many cases, direction is derived from the cell shape, including the frequently used cell long axis (Ueda et al. 2010, Kimata et al. 2016, Takahashi et al. 2016, Higaki et al. 2017), branching cell growth axis of jigsaw puzzle-like shaped leaf epidermal cells (Akita et al. 2015), and edge of the stomatal pore (Higaki et al. 2010a, Shimono et al. 2016). Also, measuring the difference in the average angles is also informative to examine the parallel running property between two kinds of filamentous structures (e.g. cortical microtubule and cellulose microfibril) (Yoneda et al. 2007, 2010).

(2) Parallelness
Although parallelness has a close relationship with the orientation, it is an index that shows the variations in the angle distribution of the cytoskeleton per unit length. For example, parallelness is used as an index to quantitatively evaluate the phenotype of the myosin mutants, in which most of actin filaments are randomly oriented, while wild-type show longitudinally oriented actin filaments (Ueda et al. 2010, Madison et al. 2015). Parallelness measurements have also been used to study reorganization of actin filaments during zygote polarization (Kimata et al. 2016) and in response to auxin (Takahashi et al. 2016). Unlike the angle mentioned above, a reference direction is not needed to measure parallelness. Measurement of parallelness is not only useful for knowing the variation of the cytoskeleton orientation, but also for correctly interpreting the mean angle data. No matter the value the average angle, if the value of parallelness is sufficiently low, it indicates randomness of cytoskeletal orientation thus it will be difficult to find a biologically significant meaning from the average value. Therefore, when discussing a biological feature based on the average angle of the cytoskeleton, measurement of parallelness should also be considered.

(3) Bundling
Cytoskeleton bundling is appropriately regulated by various cytoskeletal binding proteins and is deeply involved in various cellular phenomena (Higaki et al. 2010b). For example, bundling of actin filaments has been observed in various physiological events such as the formation of transvacuolar strands (Higaki et al. 2006) and stomatal opening (Higaki et al. 2010a). To quantitatively evaluate the bundling level of actin filaments, we reported that skewness of the intensity distribution derived from FP probes is useful (Higaki et al. 2010a, 2010b). Skewness is a statistic parameter that shows the asymmetry of distribution. We assumed that actin filaments would have a normal distribution of FP fluorescent intensities, and an increase in brighter pixels of the actin bundles would shift and skew the FP intensity distribution to the left in the horizontal axis that indicates fluorescent intensities, resulting in higher skewness values. This method has been widely accepted and is now recognized and used as a fundamental method to evaluate the bundling of actin filaments (Zhao et al. 2011, Lanza et al. 2012, Su et al. 2012, Qu et al. 2013, Zheng et al. 2013, Zhu et al. 2013, Henty-Ridilla et al. 2013, 2014, Qin et al. 2014, Li et al. 2015, Wu et al. 2015, Zhou et al. 2015, Inada et al. 2016a, 2016b, Kimata et al. 2016, Scheuring et al. 2016, Shimono et al. 2016, Takahashi et al. 2016). It has also been experimentally shown that this skewness method is useful for microtubules (Louveaux et al 2016, Ma et al. 2016).

(4) Density
Density is an indicator of the amount of cytoskeleton per unit area of target cell regions. Degree of cytoskeletal disruption by inhibition of gene function or external stimuli can be evaluated quantitatively by this indicator (Higaki et al. 2010a, Su et al. 2012, Fujita et al. 2013, Henty-Ridilla et al. 2013, 2014, Qin et al. 2014, Li et al. 2015, Inada et al. 2016a, 2016b, Kimata et al. 2016, Scheuring et al. 2016, Shimono et al. 2016, Takahashi et al. 2016).

HOW WE CAN MEASURE CYTOSKELETON FEATURES
For an example of practical image analysis, how to quantitatively evaluate (1) orientation, (2) parallelness, (3) bundling, and (4) density of cortical actin filaments in tobacco BY-2 cells stably expressing GFP-ABD2 (Sano et al. 2005, Higaki et al. 2007b) is shown as Figure 1. Orientation is evaluated by differences
between average cytoskeletal angle and angle of cell long axis (Kimata et al. 2016). Bundling level is evaluated by skewness of fluorescent intensity distribution (Higaki et al. 2010a). Please also refer to the papers for detailed definitions of parallelness (Ueda et al. 2010) and density (Higaki et al. 2010a).

0. Installation of ImageJ software and plug-ins
0-1. Download and install ImageJ software (Abramoff et al. 2004). Please refer to the official ImageJ website for details (URL: https://imagej.nih.gov/ij/download.html).
0-2. Download and install LPX ImageJ plug-ins. Please refer to the official website for details (URL: https://lpixel.net/services/research/lpixel-imagej-plugins/). LPX plug-ins were developed by Dr. Natsumaro Kutsuna (The University of Tokyo, LPixel Inc.). They were formerly known as KBI ImageJ plug-ins and were distributed on the website of Professor Seiichiro Hasezawa laboratory in The University of Tokyo.

Step 1. Segmentation of target cell regions
1-1. Open the target image using the ImageJ menu “File-Open”. In this example, the target image file was the multi-tiff file containing a cell to analyze in each slice (Figure 1).
1-2. Draw the target cell regions (region of interest: ROI) using the ImageJ toolbar menu “Freehand selections” (Figure 1, white closed curve).
1-3. Add the drawn cell regions to the ROI Manager using the ImageJ menu “Edit-Selection-Add to Manager”.
1-4. Repeat steps 1-2 and 1-3 until all slices have been segmented.
1-5. If a ROI is selected in the ROI Manager, deselect the ROI using the ImageJ ROI Manager menu “Deselect”.
1-6. Save the ROIs as a zip file using the ImageJ menu “More-Save”. The zip file can be opened in ImageJ.

Step 2. Measurements of the cell area and reference angles
2-1. Select the ROI measurements items using the ImageJ menu “Analyze-Set Measurements” and check “Area” and “Fit ellipse”.
2-2. Open the ROI zip file using the ImageJ menu “File-Open”.
2-3. Measure the cell region area and angle of cell long axis obtained by ellipse fitting using the ImageJ ROI Manager menu “Measure”. A results table will appear in which ‘Area’ and ‘Angle’ indicate area of the cell region and angle of cell long axis, respectively.
2-4. Save the result table as a csv file using the result table menu “File-Save As”. The saved csv file can be opened as a spreadsheet.

Step 3. Skeletonization of cytoskeletons
3-1. Select the target image and generate the skeletonized image using the ImageJ menu “Plugins-LPX-Lpx Filter2d (filter = lineFilters, linemode = lineExtract)”. In this example, the parameters were fixed as: giwsiter = 5, mdnmsLen = 15, pickup = otsu, shaveLen = 5, delLen = 5 (Figure 1).
3-2. Save the skeletonized image as a multi-tiff file using the ImageJ menu “File-Save As-Tiff...”.

Figure 1 Schematic illustration of image processing for quantitative evaluation of cytoskeleton structures. As an example, confocal images of fluorescently labeled actin filaments in tobacco BY-2 cells (Sano et al. 2005, Higaki et al. 2007b) are shown. Using the original confocal images, target cell regions (white closed curve) are manually segmented (Step 1), and the cell region area and angle of the cell long axis are measured (Step 2). Skeletonized images are obtained from the original confocal images (Step 3), and masked with the manually segmented cell region images (Step 4). Using the masked skeleton images, cytoskeleton features are measured (Step 5). N is the number of target cells.
Step 4. Masking of the skeletonized image
4-1. Select the skeletonized image and pick the background (Black: Intensity value is zero) in the skeletonized image using the ImageJ toolbar menu “Color picker”.
4-2. Open the ROI zip file using the ImageJ menu “File-Open”.
4-3. Select a ROI using the ImageJ ROI Manager.
4-4. Inverse the ROI on the skeletonized image using the ImageJ menu “Edit-Selection-Make Inverse”.
4-5. Mask the skeletonized image using the ImageJ menu “Edit-Fill”.
4-6. Repeat steps 4-4 to 4-6 until all slices have been painted.
4-7. Save the masked image as a multi-tiff file using the ImageJ menu “File-Save As-Tiff…”.

Step 5. Measurements of cytoskeletal features
5-1. Select the masked image and measure the features using the ImageJ menu “Plugins-LPX-Lpx Filter2d (filter: lineFilters, lineMode: lineFeature)”. A LineFeature table of the results will appear.
5-2. Save the results table using the LineFeature table menu “File-Save As”. The saved file can be opened as a spreadsheet.

The ‘a_avgTheta’ value is the mean angle of the cytoskeleton, and the angle against the cell long axis is calculated as the difference between this value and the angle of cell long axis that was obtained in step 2-3. The ‘a_normAvgRad’ value indicates parallelness. The ‘i_skewness’ value indicates skewness of intensity distribution, which is an indicator for bundling. The ‘i_nPix’ value indicates the skeletonized pixel number, and density is calculated as this value divided by the cell area that was obtained in step 2-3.

CONCLUSIONS
In this minireview, I outlined the method of quantitative evaluation of cytoskeleton organizations using ImageJ plugins. Although I did not discuss here, using the ImageJ macro can make the image processing work more efficient (Abramoff et al. 2004), so it may be worth trying when handling many images. Other analysis tools for quantifying the cytoskeleton organizations have been published (Jacques et al. 2013, Boudaoud et al. 2014). In the field of cell biology, not only in plant cytoskeleton research, obtaining quantitative data from microscopic images is now an indispensable and fundamental research method. I encourage so-called ‘wet’ biologists who may not be fully familiar with computers to consider image analysis as an extension of their microscope observations because ImageJ can be used without hesitation. I am sure that the inclusion of image analysis in the wider field of cell biology will naturally lead to the development of new research areas.

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