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Step-by-step quantitative analysis of focal adhesions

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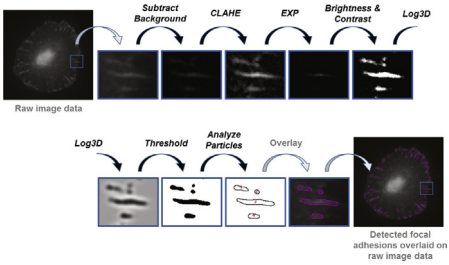
GRAPHICAL ABSTRACT



ABSTRACT

Focal adhesions (FAs) are specialized adhesive structures which serve as cellular communication units between cells and the surrounding extracellular matrix. FAs are involved in signal transduction and actin cytoskeleton organization. FAs mediate cell adhesion, which is a critical phenomenon in cancer research. Since cells can form many and micrometer scale FAs, their quantitative analysis demands well-optimized image analysis approaches [1–3]. Here, we have optimized the analysis of FAs of MDA-MB-231 breast cancer cells. The optimization is based on proper processing of immunofluorescence images of vinculin, which is one of the markers of FAs. All image processing steps are carried out using the ImageJ software, which is freely available and in the public domain. The advantages of our method are:

- The analysis steps are simplified by combining different plugins of the ImageJ program.
- FAs are better detected with minimal false negatives due to optimized processing of fluorescent images.
- This approach can be applied to quantify a variety of fluorescent images comprising focal and/or localized signals within a high background such as FAs, one of the many complex signaling structures in a cell.

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Materials

- Fluorescence images
- ImageJ software

Critical points

- Sample should be properly immunostained so that good quality fluorescence images can be captured.
- The images should be of high quality; unfocused images and inadequate resolution hinder the quality of the analysis.
- Fluorescence images should ideally be captured at 100× magnification.
- If color images are captured, single channel images should first be created with the ImageJ SPLIT CHANNEL command.
- Use raw image data which are greyscale images without any special preprocessing.

Method details

Here, we have analyzed focal adhesions of MDA-MB-231 cells. However, our method can be applied to not only any cell type such as fibroblasts, endothelial cells or leucocytes but also to any structure in the cell with a localized signal such as focal adhesions, endosomes or invadopodia. Focal adhesions were immunostained for vinculin using a vinculin specific primary antibody (Sigma Cat no: V9131), followed by a fluorophore conjugated secondary antibody. Other markers of focal adhesions such as paxillin or focal adhesion kinase could also be easily used for analysis. 8-bit images were captured using an Olympus epi-fluorescence microscope with an Olympus infinity corrected 100× oil UPlanSApo objective with a numerical aperture of 1.4 and an Olympus SLR E-330 camera such that each image was 3136 pixels by 2352 pixels and 37 pixels corresponded to 2 μm. All steps of image processing were carried out using ImageJ which is freely available and in the public domain. ImageJ provides a wide range of processing and analysis approaches possible via not only built-in functions but also numerous plugins. ImageJ has been in use for some time by researchers. However, choosing the right combination and order of processing steps is crucial for obtaining meaningful quantitative results.

The image processing optimized here aims to identify FAs immunostained for vinculin. The raw fluorescent images are processed in steps as follows:

- Step 1: Apply SUBTRACT BACKGROUND. Selection of sliding paraboloid option changes the rolling ball to a parabolic with the same ball radius in pixels but with sharper curvature. A parabola slides in different directions over the image and calculates and subtracts local background from the original image. We chose the SLIDING PARABOLOID option with the ROLLING BALL radius set to 50 pixels.
- Step 2: Enhance the local contrast of the image by running CLAHE (Contrast Limited Adaptive Histogram Equalization). The plugin CLAHE offers three critical parameters which are block size, histogram bins and maximum slope to clarify desired objects. We used the following values: block size=19, histogram bins=256, maximum slope=6, no mask and fast [4].
- Step 3: Apply mathematical exponential (EXP) to further minimize the background.
- Step 4: Adjust BRIGHTNESS & CONTRAST automatically. The BRIGHTNESS & CONTRAST tool updates the lookup table of the image based on an analysis of the histogram of the image.