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Quantification of Cell-Substrate Adhesion Area and Cell Shape Distributions in MCF7 Cell Monolayers

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TITLE:

Quantification of Cell-Substrate Adhesion Area and Cell Shape Distributions in MCF7 Cell Monolayers

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KEYWORDS:

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SUMMARY:

The article describes quantification of 1) the size and number of focal adhesions and 2) cell shape index and its distribution from confocal images of the confluent monolayers of MCF7 cells.

ABSTRACT:

The methods presented here quantify some parameters of confluent adherent cell monolayers from multiple appropriately stained confocal images: adhesion to the substrate as a function of the number and size of focal adhesions, and cell shape, characterized by the cell shape index and other shape descriptors. Focal adhesions were visualized by paxillin staining and cell-cell borders were marked by junction plakoglobin and actin. The methods for cell culture and staining were standard; images represent single focal planes; image analysis was performed using publicly available image processing software. The presented protocols are used to quantify the number and size of focal adhesions and the differences in cell shape distribution in the monolayers, but they can be repurposed for the quantification of the size and shape of any other distinct cellular structure that can be stained (e.g., mitochondria or nuclei). Assessing these parameters is important in the characterization of the dynamic forces in adherent cell layer, including cell adhesion and actomyosin contractility that affects cell shape.

INTRODUCTION:

Epithelial cell monolayers act as a collective in which cell-cell and cell-substrate adhesion as well as contractile forces and tensions represent important parameters and their proper balance contributes to the overall integrity of the unit[1-3]. Thus, assessing these parameters represents a way to establish the current status of the cell layer.

The two methods described here represent a two-dimensional analysis of the confluent monolayers of adherent, epithelial cells (in this case MCF7 breast cancer cell line). The analysis is performed using confocal images (single Z-slices) from different regions on the Z-axis; basal region near a substrate for focal adhesion (FA) measurements and apical region for cell shape measurements. The presented methods are relatively simple and require standard laboratory techniques and open-source software. Confocal microscopy is sufficient for this protocol, so it can be performed without employing more specialized TIRF (Total Internal Reflection Fluorescence) microscopy. Thus, the protocol could be implemented in a relatively standard laboratory setting. Although the accuracy of the methods is limited, they can distinguish basic differences in focal adhesion and cell shape.

Both methods described here consist of the standard experimental procedures such as cell culturing, immunostaining, confocal imaging and image analysis performed using ImageJ. However, any image processing software with the appropriate functions can be used. The presented methods can track and compare changes inflicted by pharmacological treatment or minimal genetic modification. Obtaining definite values is not recommended, due to the limited precision of these methods. Two automated macros were included, to facilitate the measurements of many images.

PROTOCOL:

1. Preparatory steps

1.1. Cell seeding to obtain confluent monolayers

1.1.1. Before seeding, coat the wells of a 4-wells chamber slide with collagen I (or other ECM component of choice). For collagen I coating, follow a commercial protocol: https://www.sigmaaldrich.com/technical-documents/articles/biofiles/collagen-product-protocols.html at a concentration of 8 $\mu g/cm^2$.

1.1.2. Seed 400,000 cells to one well of a 4-well chamber slide.

1.1.3. Culture the cells for 24 h (or longer, depending on the experimental endpoints) before staining, in an incubator at 37 °C, 5% CO_2. This step allows for a maturation of cell-cell contacts and the formation of monolayers.

1.1.4. Use an optical, inverted microscope to verify confluency (about 90% is required) and a general condition of monolayers. Do not proceed if cells are floating or look stressed.

1.2. Immunofluorescent staining

NOTE: Cells can be stained with a protocol of choice. In here, immunofluorescence was performed as previously described[4].

1.2.1. For focal adhesion analysis, stain the cells with a focal adhesion protein of choice (in this protocol paxillin). For cell shape analysis stain with cell-cell junction protein of choice (in this protocol desmosomal protein plakoglobin).

1.2.2. For the procedure, use 0.5 mL of specified solutions unless specified otherwise.

1.2.3. Fix cells in 4% formaldehyde in PBS for 30 min on ice.

1.2.4. Incubate with 0.1 M ammonium chloride (in PBS) for 10 min to quench auto-fluorescence.

1.2.5. Add 0.5% Triton X-100 (in PBS) for 30 min (permeabilization).

1.2.6. Block with 5% milk (or 1% BSA) in TBS-T for 1 h.

1.2.7. Incubate with primary antibody at 4 °C overnight (anti-paxillin: rabbit, 1:250, anti-plakoglobin: rabbit, 1:400)

1.2.8. Incubate with secondary antibody for 30 min (Alexa Fluor 594 goat anti-rabbit, 1:500, 1:500)

1.2.9. Stain with 300 nM DAPI for 1-5 min, protected from light.

NOTE: Optionally, additional staining of actin with fluorescent phalloidin conjugates (conc. 1:400) may be performed; phalloidin should be added at the same step as the secondary antibody.

1.3. Confocal imaging

1.3.1. Take images of single Z-slices using a confocal microscope (e.g., Zeiss LSM800).

NOTE: Optional actin staining may help assess the proper focal plane. Cortical actin staining is present in the apical region while actin stress fibers are present in the basal region, near the substrate, as illustrated on **Figure 1**.

1.3.2. Focal adhesion imaging

1.3.2.1. Choose Z-slices for focal adhesion analysis from the basal region, close to the substrate.

1.3.2.2. Use an objective with the highest numerical aperture available (preferable 63x N.A.:

1.4).

NOTE: The shape of FA is very specific and easily recognizable. Thus, it is recommended to start the scan from a focal plane below cells and then slowly scan towards them, until FAs are clearly visible. Image analysis will be more accurate from smaller fields of vision, which tend to be more uniform, but this implies that fewer cells will be calculated in a single field of view.

1.3.3. Cell-cell contacts imaging

1.3.3.1. Use a 40x or 63x objective.

1.3.3.2. Select channels for nuclear staining and the preferred junction protein staining.

1.3.3.3. Choose Z-slices for cell shape analysis from the apical region of the monolayers.

1.3.3.4. Take pictures for at least 3 different fields of vision (200-400 cells).

2. Image analysis

NOTE: Provided macros work optimally on ImageJ version 1.50f or newer. Use for quantification only of images with a high signal-to-noise ratio and without under- or oversaturated pixels. The described methods include steps requiring manual parameter adjustment. Thus, a blind analysis/blinded experiment setup is recommended. For encrypting image file names, ImageJ plugins such as “Blind Analysis Tool” (available at: https://imagej.net/Blind_Analysis_Tools) can be used.

2.1. Focal adhesion analysis

NOTE: The recommended input files for the following methods are: images of FAs represented in 8-bit grayscale saved in .tiff format.

2.1.1 Open image using ImageJ.

2.1.2. Set the scale of an image to pixels (**Analyze | Set Scale***;* Remove Scale and check **Global** option).

2.1.3 Subtract background (**Process | Subtract Background;** set **Rolling ball radius** parameter to 50 pixels; check **Sliding paraboloid** option). In the case of pseudocolored RGB images: split RGB channels, leave the channel with FAs opened, close remaining channels (**Image | Color | Split Channels**).

2.1.4 Determine the area of the smallest region of interest (ROI). Using freehand or polygon selections outline the smallest single focal adhesion and measure its area (**Analyze | Measure**). Repeat this step for different ROIs (FAs) from a few randomly chosen images (for a total of 20

ROIs). Calculate and save the mean of obtained results.

NOTE: This step is required only when a given set of images is analyzed for a first time (specific cell line, coating slides with specific extracellular matrix components, different culture conditions).

2.1.5 Convert image to **binary** by using one of the following methods:

2.1.5.1. Set the global threshold (**Image | Adjust | Threshold;** check **Default, B&W** and **Dark Background** options, adjust threshold manually or set it automatically).

2.1.5.2. Set the local threshold (**Image | Adjust | Auto Local Threshold…;** set **Method** to Phansalkar and check **White objects on black background** option. Next, invert the image (**Edit | Invert**).

2.1.6 Include the file name and the area of ROI in measurement options (**Analyze | Set Measurements…**); check **Area** and **Display label** options.

2.1.7 Measure the number and the area of ROIs. Select **Analyze | Analyze Particles**; check **Pixel units, Display results, Clear results** and **Summarize options**, set **Size parameter**, as a lower boundary use the mean of the smallest ROIs from step 2.1.5. The upper boundary can be set to 25% of a typical cell area.

2.1.8 Transfer data (that includes image name, number of FAs, total and mean area of FAs; respectively **Slice, Count, Total Area, Average Size**) from the **Summary** window to the data managing program of choice.

2.1.9 Determine the number of cells per image by counting DAPI-stained nuclei. Counting can be done manually (**Plugins | Analyze | Cell Counter**) or as in available protocols such as: https://imagej.net/Nuclei_Watershed_Separation.

2.1.10 To facilitate FAs counting, use the attached ImageJ macro (FAs.ijm).

2.1.10.1. Move .ijm file with the macro to plugins or macros folder located in ImageJ source files folders.

2.1.10.2. Determine an area of the smallest ROI as described in step 2.1.4.

2.1.10.3. Open macro file (**Plugins | Macros | Edit...**).

2.1.10.4. Before running the macro set the value of three variables: fill value of **area_of_the_smallest_region_of_interest** with a number acquired during step 2.1.4. Set **threshold_type** value to **manual** or **auto**.

2.1.10.5. Save changes (the macro should be ready to use).

2.1.10.6. Call the macro from ImageJ panel or make a shortcut to it. The macro starts with the standard **open** dialog window. Select the image to be processed.

NOTE: In case of manual threshold adjustment, manual confirmation of threshold value will be required (avoid accepting changes using **Apply** button on **Threshold** dialog window, use **Action Required** custom dialog window instead). Results obtained by working with the macro are the same as those described in step 2.1.8 (included in **Summary** dialog window). Additionally, in the case of manual threshold adjustment **Lower Threshold Level** is displayed in a **Log** dialog window, as this value allow to reproduce obtained results in the future if needed. **Supplemental Figures S1** and **S2** were included as a training dataset for the FAs.ijm makro.

2.2. Cell shape analysis

2.2.1. Manual

2.2.1.1. Open an image in ImageJ or another image processing software with a similar set of functions (further instructions pertain to ImageJ). Choose the parameters to be measured by selecting from the menu **Analysis | Set Measurements** and ticking **Shape descriptors** in the appearing box.

2.2.1.2. Manually delineate cell borders, marked by junction protein(s) of choice, using **Freehand selections** icon. The chosen parameters are automatically calculated for each cell. Store the results after outlining each cell by clicking **Edit | Selection | Add to manager**. Only complete, entirely visible cells, with uninterrupted borders should be counted.

2.2.1.3 When all cells in the field of view are outlined, make the measurement by marking all of the numbers appearing in the left box of the ROI Manager (corresponding to cells) and clicking **Measure** The results appear in the **Results** box and can be transferred to the spreadsheet of choice.

2.2.2. Automated

NOTE: To facilitate the quantification of cell shape descriptors (CSI, aspect ratio, roundness, solidity) an ImageJ macro has been prepared and attached to this article (CSI.ijm). The macro is mainly based on ImageJ plugin called MorphoLibJ (https://imagej.net/MorphoLibJ)[5]. The macro executes the following steps: 1) Extension of each border of image by 10 black pixels [MorpholibJ]; 2) Rounds of dilations and erosions - morphological filter [MorpholibJ]; 3) Generation of binary image of cells boundaries by morphological segmentation [MorpholibJ]; 4) Dilation of cell boundaries; 5) Inversion of pixels value; 6) Generation of selections and measurement of area and perimeter of cells on the image; and 7) Saving image with outlined cells and ImageJ ROI selections to a new file.

2.2.2.1. Move the .ijm file with the macro to the plugins or macros folder located in the ImageJ source files folders. Call the macro from the ImageJ panel or make a shortcut to it.

2.2.2.2. Before the quantification of a new dataset, determine values of the smallest and the largest regions of interest. Outline (freehand or polygon selection) a few (3-10) examples of the smallest and the largest cells on the image and then measure their area (**Analyze | Measure**).

2.2.2.3. Alternatively, run the macro with default settings (lower size limit is set to 0 and upper limit is set to infinity), wait for the macro to finish and select **Set cell size boundaries** option. Measure the area of the smallest and the largest cells by clicking on their label and then press **Measure** in the ImageJ ROI Manager. Set the value of **the_smallest_cell and the_biggest_cell** variables. Save changes, close all macro dialog windows and run the macro again.

NOTE: The macro can be used without setting ROI size boundaries but it is not recommended because it significantly increases a chance of measuring inappropriate cell fragments or cell clusters.

2.2.2.4. Start the macro with the standard **Open** dialog window. Select the image to be processed (grayscale).

2.2.2.5. Analyze the results. The output provided by the macro consists of: table of the results (cell label, image label, cell area [pixels^2], cell perimeter [pixels^2], circularity [CSI], aspect ratio, roundness, solidity), image with outlined cells and ROI selections list (which will be also saved in new file in the **Results** subfolder). The results table will be automatically copied to the user's clipboard.

NOTE: **Supplemental Figures S3** and **S4** were included as a training dataset for the CSI.ijm makro.

3. Quantification

3.1. Quantification of FAs

3.1.1 Calculate the mean FAs number and average FA size per cell/nuclei.

NOTE: For some cell lines it is possible to count FAs separately in distinct cells. For cell lines that have strong cell-cell contacts and grow as monolayers such as MCF7, number and size of FAs per cell can be calculated by dividing the values obtained from FAs counting by the number of nuclei in the whole image.

3.1.2 Assess statistical significance of potential differences between populations (experimental groups). Depending on the distribution and variance of the data, for the comparison of the two different groups use Student t-test (normal distribution) or non-parametric U-test (Mann-

Whitney). For comparison of multiple groups use one-way ANOVA or Kruskal–Wallis in conjunction with the appropriate post-hoc tests.

3.2. Cell shape analysis

3.2.1. Calculation of shape descriptors

3.2.1.1. Manual analysis: Calculate cell shape index (CSI, also called circularity or cell shape) in the spreadsheet of choice for each measured cell from the appropriate area and perimeter using the formula:

$$(1)\ CSI = 4\pi \times area/(perimeter)^2$$

NOTE: CSI assumes values between 1 (circular) and 0 (elongated). The examples of various cell shapes (with the same area) and their respective CSIs are presented in **Figure 2**. In automated analysis the values of shape descriptors (enlisted and defined below) are calculated automatically and appear in the result box:

(1) $CSI = 4\pi*area/(perimeter)^2$
(2) AR = major axis/minor axis
(3) $Roundness = 4*area/\pi*(major\ axis)^2$
(4) Solidity = area/convex area

3.2.2. Histograms of cell shape distribution

3.2.2.1. Plot cell shape distribution as a histogram of circularity (CSI). Classify cells according to their CSI value (calculated for the minimum of 200–400 cells), to one of the ten uniform intervals (range: 0-1, bin width: 0.1). The histogram displays the number of cells in every category.

NOTE: The histogram showing shape distribution of the typical MCF7 cell layer shows a peak of around 0.7-0.8 CSI. If the cells' shape is distorted by some factor (for example paclitaxel treatment, which causes G2/M phase arrest and in the consequence more cells are round) it should be reflected on the histogram.

3.2.3. Cumulative distribution plots

3.2.3.1. Compare cumulative CSI distributions for each cell line, because it is the best way to assess statistically important differences in cell shape changes (or any other changes in distribution. For example, it can be applied to track the changing distribution of FAs.

3.2.3.2. Calculate the Cumulative Distribution Function (CDF) to compare distributions. CDF assigns for a given CSI value (plotted on X axis) the percentage (or relative count) for which all values are less or equal to this CSI value (plotted on Y axis). Thus, as the CSI value gets higher,

the percentage of the set of values that are less or equal to this value also gets higher. CDF can be calculated by the statistical software of choice, or manually.

3.2.3.3. For statistical analysis, use Kolgomorov-Smirnov nonparametric test.

REPRESENTATIVE RESULTS:

Focal adhesion analysis

The knockdown of *HAX1* gene was previously shown to affect focal adhesions[6]. Cells were cultured on collagen I-coated surface for 48 h. Images of the MCF7 control cells and MCF7 cells with a *HAX1* knockdown (*HAX1* KD) from three independent experiments stained with focal adhesion protein paxillin were obtained using a confocal microscope (image from single focal plane/Z-slice from basal region). FAs from about 2,000-2,500 cells from each cell line were quantified using the described protocol. The mean value for the smallest focal adhesion was set to 50 (pixel^2). Representative images of FAs count with ImageJ, including the final, numbered outlines and the overlay of the FAs outlines with the original image, are shown for both cell lines on **Figure 3A**. Differences in number and size of FAs in both cell lines are presented on **Figure 3B**.

Cell shape analysis

Manual assessment: MCF7 cells were cultured for 24 h, the medium was exchanged for the same fresh medium (untreated) or the medium with 0.1 μM paclitaxel (PTX) – to induce cell rounding - and cultured for another 24 h. Images of the confluent MCF7 monolayers stained with anti-plakoglobin antibody and DAPI were obtained using a confocal microscope (single Z-slice from apical region). About 200-400 cells (2-3 fields of view) from each experiment (untreated/treated) were documented (40x objective) and the images were assessed using ImageJ image processing software (**Figure 4A**). Representative regions of each cell layer with outlined cells are shown in **Figure 4A** (untreated and PTX-treated cells). All cells were categorized according to their CSI values (10 intervals, bin width 0.1) and presented in the respective histograms (**Figure 4B**), which show an increase in the last bin (0.9-1) and the flattening of the main peaks (0.6-0.9) for the PTX-treated cells, comparing to the untreated control. Differences in the cumulative distribution of CSI were calculated for statistical significance using Kolgomorov-Smirnov test (K-S), a nonparametric test of the equality of one-dimensional probability distributions. Frequency distribution histograms and cumulative distribution plots (**Figure 4C**) were generated with software.

Automated assessment: MCF7 cells were cultured for 24 h, fixed and stained with fluorophore-conjugated phalloidin to visualize actin. Images were taken using a confocal microscope (single Z-slice from apical region). The grayscale images of the monolayers were analyzed using the attached macro file, according to the included protocol (**Figure 5A-C**). Overall, 512 cells from 12 fields of view were quantified. Results were plotted as a histogram presenting distribution of circularity (**Figure 5D**).

FIGURE AND TABLE LEGENDS:

Figure 1. Actin staining with fluorophore-conjugated phalloidin, two different Z-slices from

the same field of view. (**A**) The apical region with cortical actin. (**B**) The basal region with actin stress fibers. Bar: 10 μm.

Figure 2. Examples of different Cell Shape Index (CSI) values for the shapes with distinct perimeters, but the same area. The very elongated shape on the left has CSI close to 0, while the ideal circle on the right has CSI of 1.

Figure 3. Focal adhesion quantification. (**A**) Representative images of MCF7-based cell lines: CONTROL and *HAX1* KD stained with paxillin; from left to right: (1) paxillin and nuclei (2) outlined and numbered FAs (3) overlay of FAs outlines and the original image. Bar: 20 μm. Insets (below each picture) show zoomed boxed areas. (**B**) Plots showing the difference in FAs size and number between the two cell lines. Statistical significance was assessed using Student t-test.

Figure 4. Changes in cell shape induced by paclitaxel (PTX) in MCF7 cell layer; manual count. (**A**) Representative regions of MCF7 monolayers, cells untreated and treated with PTX, stained with junction protein plakoglobin and DAPI, bar: 20 μm. The panel on the right shows the image processed in ImageJ; cell edges are outlined and all counted cells are numbered. (**B**) Histograms showing CSI distribution in untreated and PTX-treated cells, 200-400 cells in each experiment (untreated/treated), bin width: 0.1. Plots were generated using frequency distribution analysis with relative frequencies as a fraction. (**C**) The plot of cumulative distribution for the untreated and PTX-treated monolayers. Statistical difference calculated using Kolgomorov-Smirnov nonparametric test (KS).

Figure 5. Cell shape in MCF7 monolayers assessed using the automated method. (**A-C**) An example of the automated image analysis illustrating subsequent steps executed by the attached macro. (**A**) Input image; cortical actin; grayscale, scale bar 10 μm (for informative purposes; scale bars should not be embedded into analyzed images). (**B**) Cell layer after segmentation; cell borders outlined; cells without complete borders eliminated. (**C**) Overlay of the cell outlines on the original image. (**D**) A histogram showing the distribution of CSI in the analyzed dataset, 512 cells.

Supplemental Figure S1, S2: MCF7 cells, paxillin and DAPI-stained to visualize FAs and nuclei. Provided as a training dataset for the FAs.ijm macro.

Supplemental Figure S3, S4: MCF7 cells, plakoglobin and DAPI-stained to visualize cell-cell junctions and nuclei. Provided as a training dataset for the CSI.ijm macro.

DISCUSSION:

Cell-cell and cell-substrate adhesion constitute inherent attributes of the epithelial cells and play the critical role in tissue morphogenesis and embriogenesis. In adult tissues the proper regulation of mechanical properties of the cell layer is crucial in maintaining homeostasis and preventing pathological responses like tumor progression and metastasis. The size and number of focal adhesions depend on the strength of cell-substrate adhesion, while cell shape depends on contractile forces and is related to the status of cell-cell-contacts.

Here, we describe two simple methods of quantitative analysis of the area, number and shape of cellular structures stained by immunofluorescence, in this case focal adhesions and the whole cells in the cell layer. However, the proposed tools can be repurposed for the quantification of any chosen structure. The key issue for these analyses is the quality of immunofluorescent staining and confocal imaging. These methods can be implemented in any standard laboratory equipped with a cell-culture unit and a confocal microscope. They are designed to compare cell lines, especially when the differences (natural or induced by specific treatment) in the measured parameters are substantial. They are not recommended to measure minute differences or to establish absolute measurements, because they are sensitive to minimal changes in the initial arbitrary settings, especially in the case of focal adhesions. This method of FAs quantification is inferior to more advanced and specific methods like TIRF microscopy, but it has an advantage of not requiring sophisticated equipment.

Similar methods of focal adhesion measurements were described before[7-10]. Here, we specified the settings and created a free ImageJ macro, with several options, to facilitate the measurements.

Cell shape analysis was described many times, including very complex and detailed methods[11,12]. Here, we present a simple method to track the changes in epithelial cell monolayers, which could be very important for comparing cell morphology or developmental changes. The manual method of cell shape analysis in the monolayers presented in this protocol was described in a previous report[6]. The CSI formula as a way to describe and compare the shape of an object(s) is widely used in different disciplines[13,14], including geology from which it originated. Presentation of the results in a form of a histogram and/or as a cumulative distribution function is commonly used for comparisons of distributions of any kind[8,10,15].

Notably, we present here a tool for the automated cell shape analysis based on ImageJ plugin MorhpLibJ (https://imagej.net/MorphoLibJ). We provide a macro file, which can perform this analysis quickly and efficiently. This method does not always recognize the correct cell borders, but the percentage of faulty measurements (which are present in every automated analysis) is minimal and should not significantly affect the final result, especially if enough number of cells is analyzed. Cells without complete borders are eliminated. Automated cell shape analysis has undeniable advantages and we present this method so that it can be appreciated by the scientific community.

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DISCLOSURES:
The authors have nothing to disclose.

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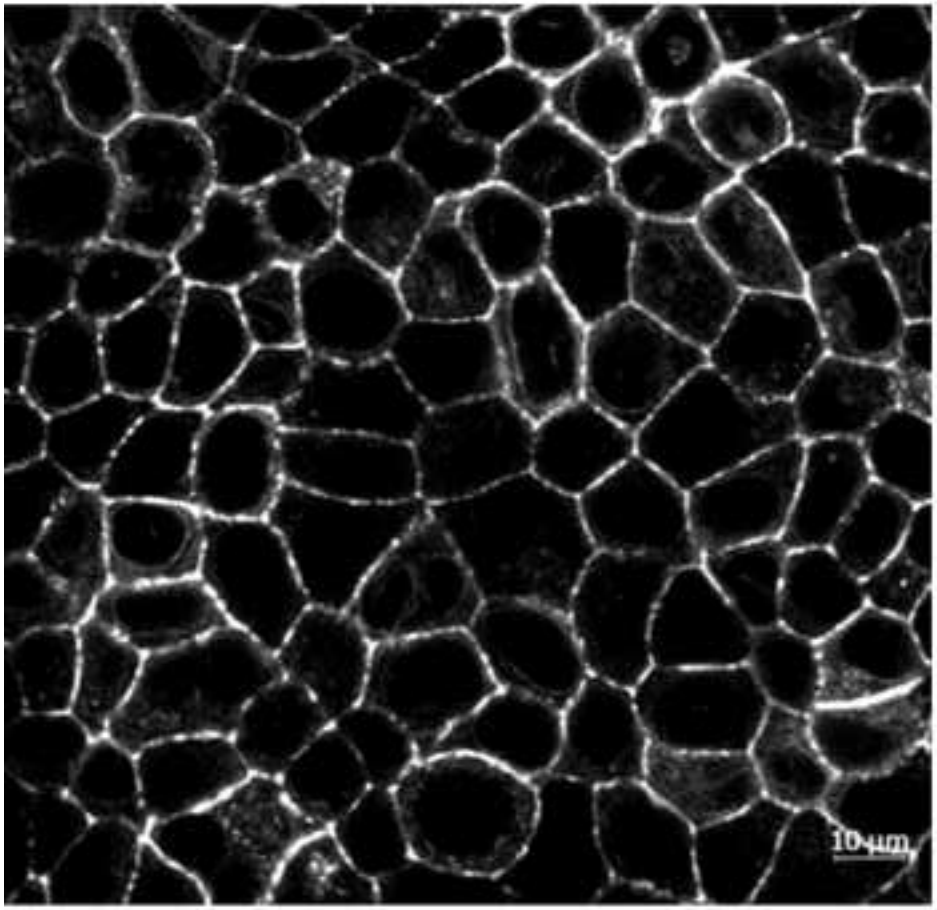
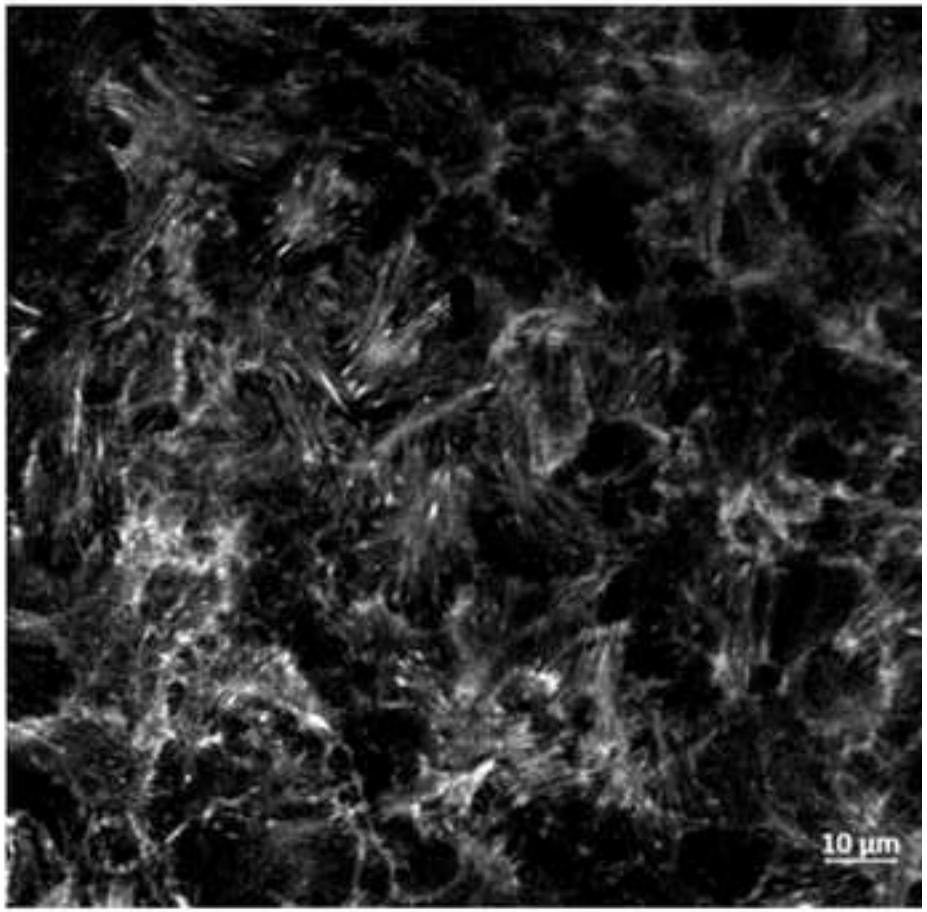
Figure 1

The same field of vision

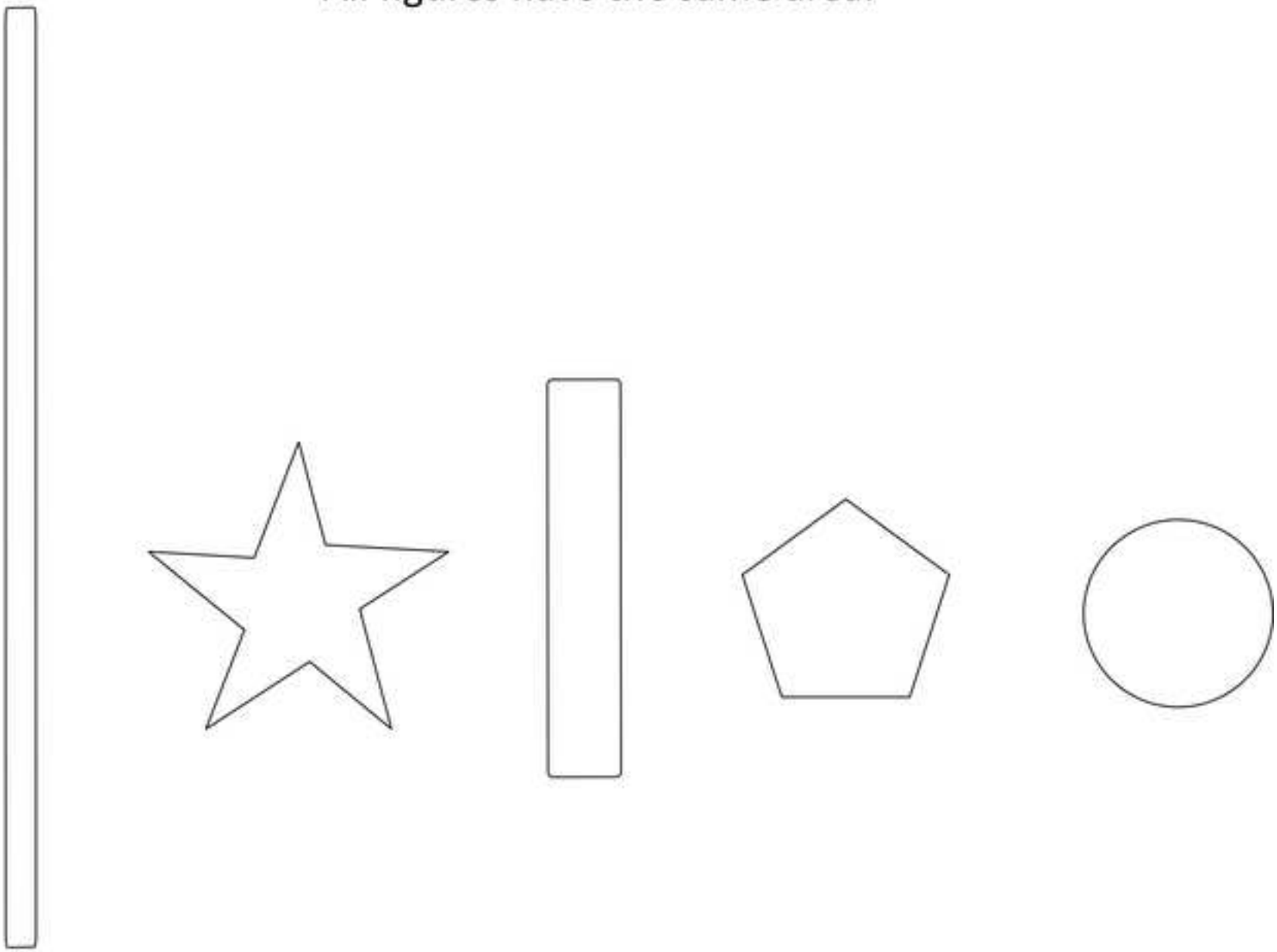


Cortical actin; apical region



Actin stress fibers; basal region

Examples of different Cell Shape Index (CSI) values for distinct cell shapes.
All figures have the same area.



CSI:	0.097	0.276	0.408	0.862	1.000

Figure 3

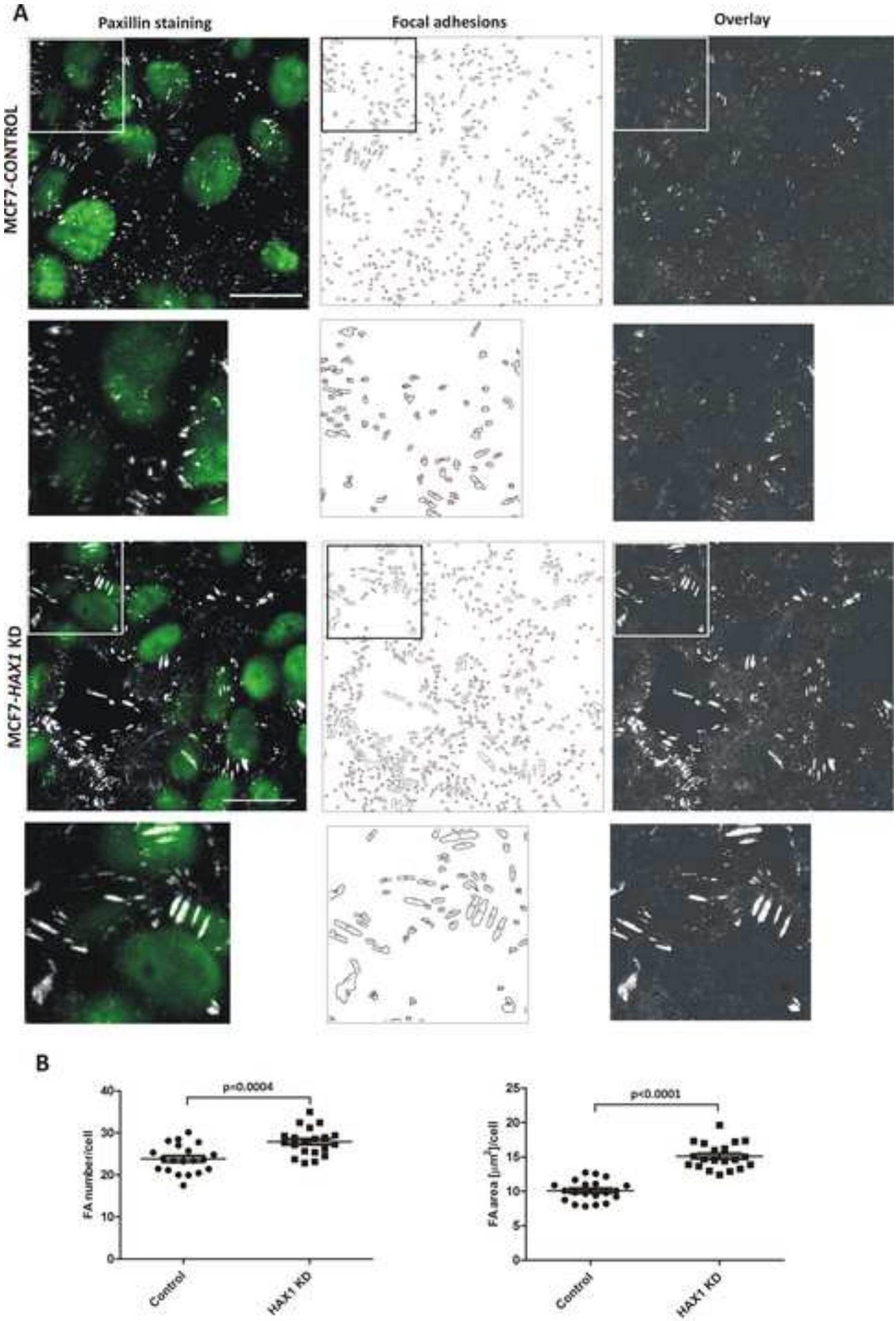


Figure 4

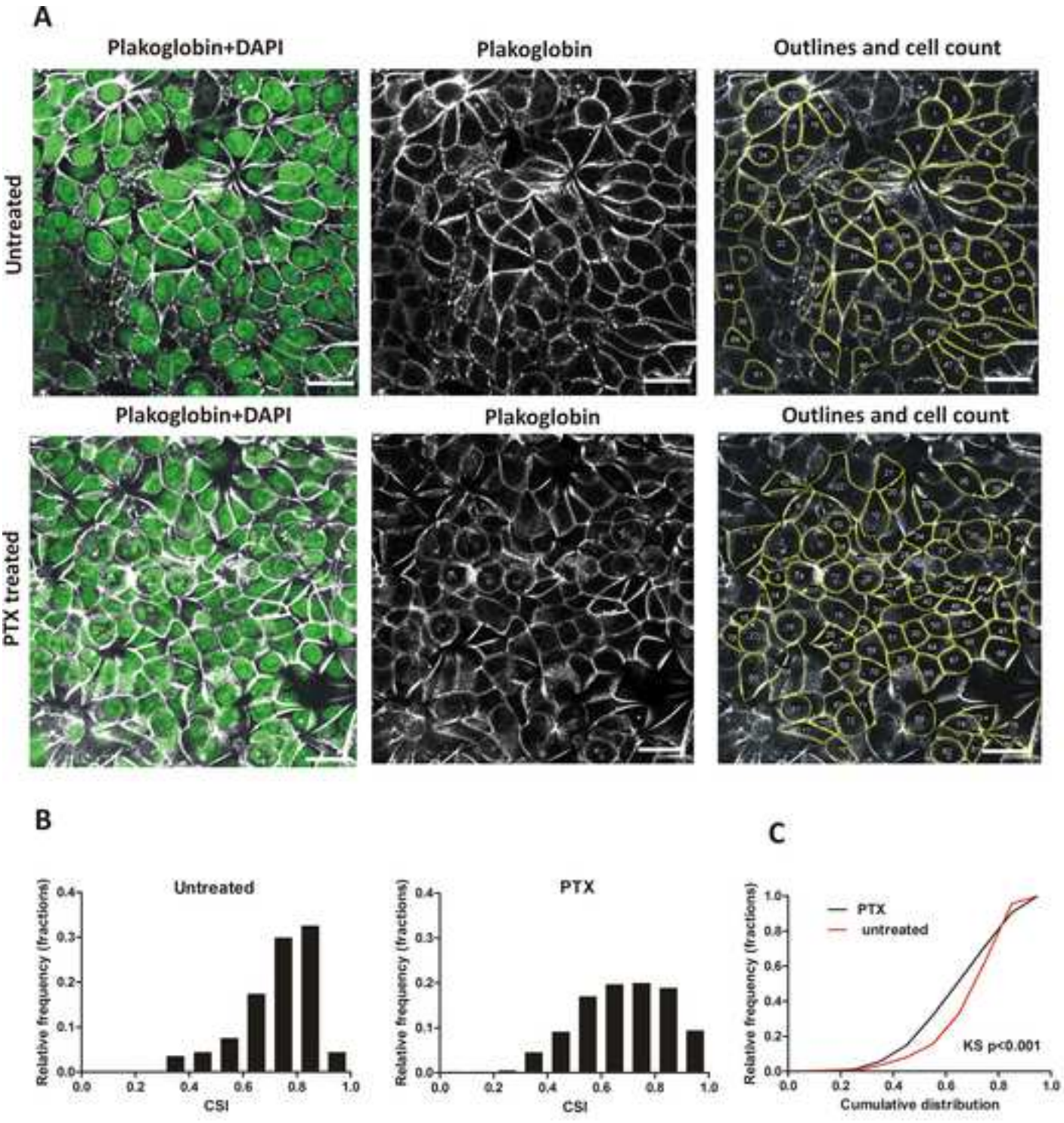
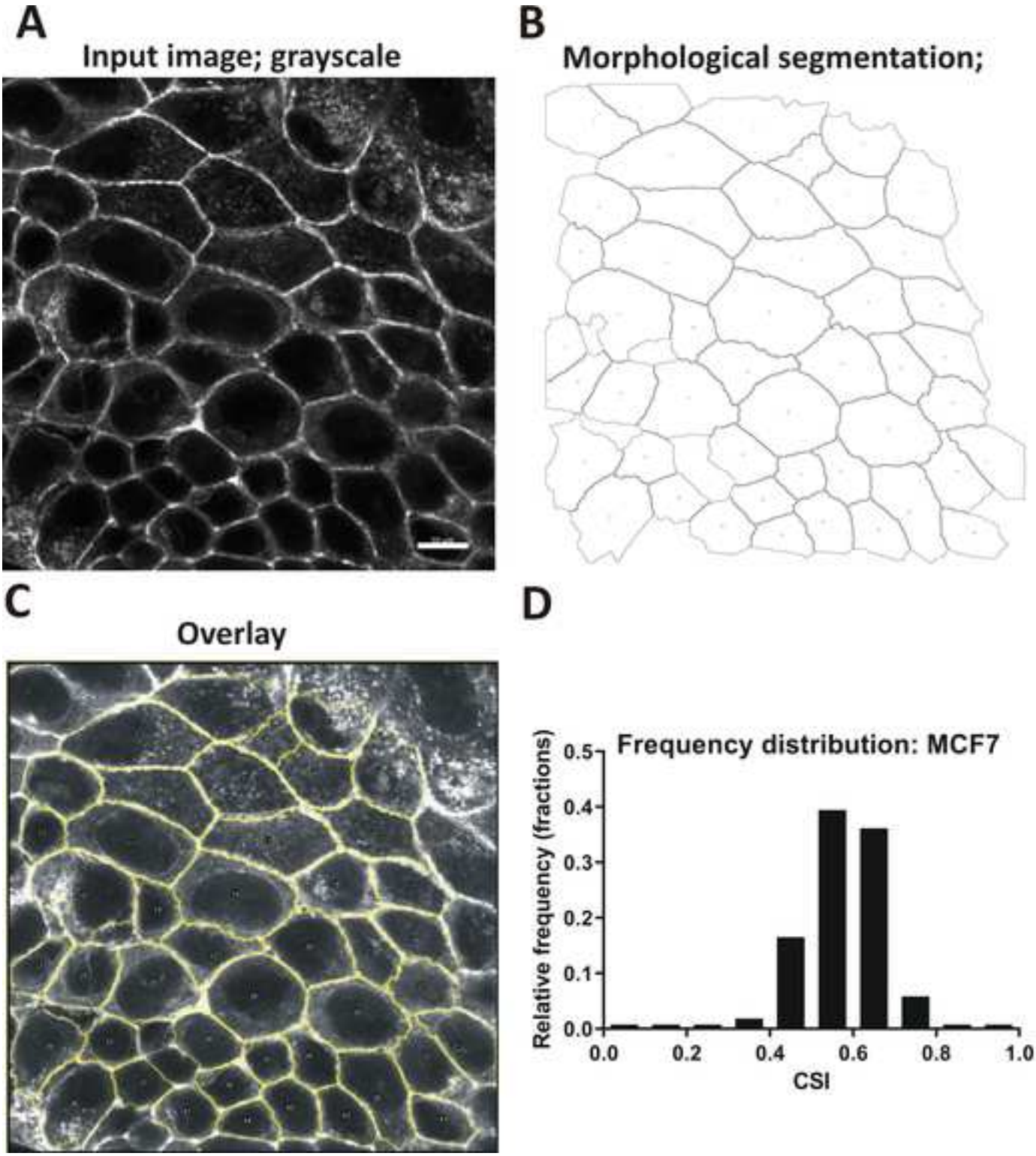


Figure 5



Name of Material/Equipment	Company
Alexa Fluor 594	ThermoFisher Scientific
Ammonium chloride	Sigma
BSA	BioShop
Collagen from calf skin	Sigma
DAPI	Sigma
DMEM + GlutaMAX, 1 g/L D-Glucose, Pyruvate	ThermoFisher Scientific
FBS	ThermoFisher Scientific
Junction plakoglobin	Cell Signaling
Laminar-flow cabinet class 2	Alpina
MCF7-based HAX1KD cell line	Cell line established in the National Institute of Oncology, Warsaw, described in Balcerak et al., 2019
MCF7 cell line (CONTROL)	ATCC
Olympus CK2 light microscope	Olympus
Paxillin	Abcam
PBS	ThermoFisher Scientific
Phalloidin-TRITC conjugate	Sigma
PTX	Sigma
TBST – NaCl	Sigma
TBST – Trizma base	Sigma
Triton X-100	Sigma
Zeiss LSM800 Confocal microscope	Zeiss

Catalog Number	Comments/Description
A32740	goat anti-rabbit, 1:500
A9434	
ALB001.500	
C9791-10MG	
D9542	1:10000 (stock 1 mg/mL in H_2O), nucleic acid staining
21885-025	
10270-136	
2309S	rabbit, 1:400
	standard equipment
	MCF7 cell line with HAX1knockdown
ATCC HTB-22	epithelial, adherent breast cancer cell line
ab32084	rabbit, 1:250, Y113
10010023	
P1951	1:400 (stock 5 mg/mL in DMSO), actin labeling
T7402-1MG	
S9888	
T1503	
9002-93-11	

Authors response to the editorial comments:

1. Please copy-edit the manuscript as there remains some wordy sections in the manuscript.

The manuscript has been edited, according to the editorial comments.

2. Additional details are needed in the protocol. Please see the comments in the attached manuscript.

The protocol was corrected and completed according to the attached comments.

3. Please include scale bars for Figure 5.

The scale bar was included, but only for the informative purposes on the original image – for the analysis scale bars or any other annotations would hinder calculations, thus the other images are without it and the appropriate warning was added to the manuscript..

4. Please provide a caption for the supplemental figures. What are they to be used for? They are not mentioned in the manuscript.

The captions were provided along with the explanation of the purpose of these supplemental data (as a training datasets, according to the reviewer requirements).

Supplemental Figure S1

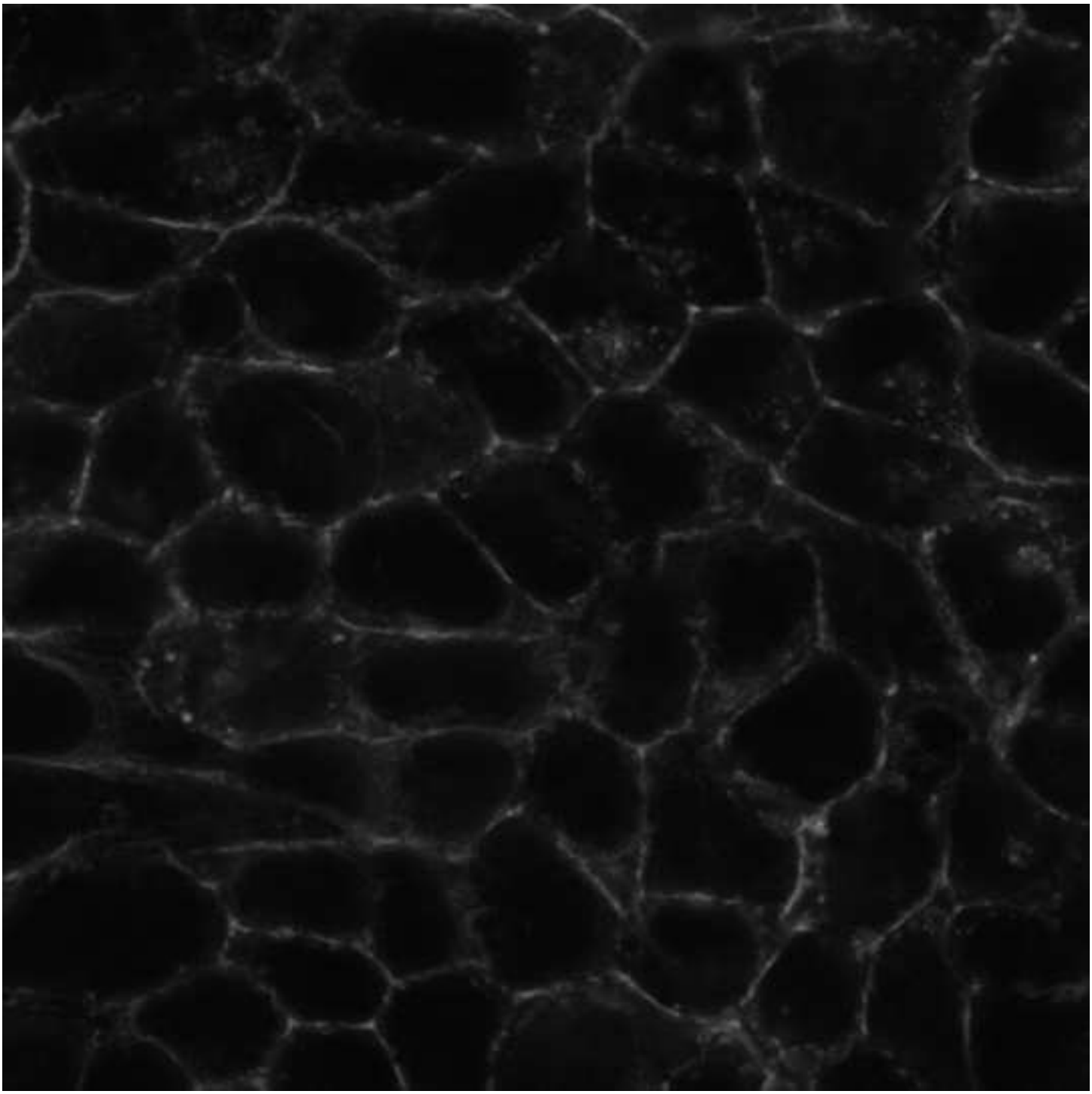
Click here to access/download;Supplemental File (Figures, Permissions, etc.);Figure S1.tif

Supplemental Figure S2

Click here to access/download;Supplemental File (Figures, Permissions, etc.);Figure S2.tif

Supplemental Figure S3

Click here to access/download;Supplemental File (Figures, Permissions, etc.);Figure S3.tif



Supplemental Figure S4

Click here to access/download;Supplemental File (Figures, Permissions, etc.);Figure S4.tif