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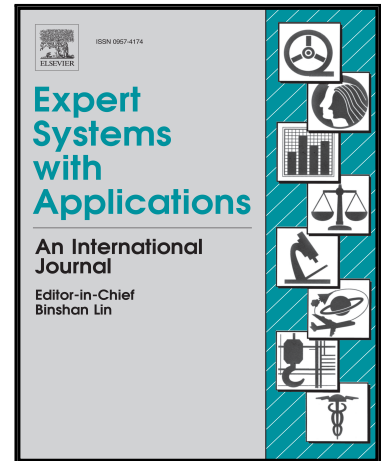
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Highlights

- A quantitative image analysis method to quantify cellular cytoskeleton changes
- The method segments cell parts using marker-controlled watershed segmentation
- Morphological operation, distance transform and post-processing play important roles
- Two texture analysis methods quantify several cytoskeletal features in tumor
- It can be used to test different biological hypothesis using cellular image sample

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A Quantitative Image Analysis for the Cellular Cytoskeleton during *In vitro* Tumor Growth

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Abstract

The cellular cytoskeleton is a dynamic subcellular structure that can be a marker of key biological phenomena including cell division, organelle movement, shape changes and locomotion during the avascular tumor phase. Little attention is paid to quantify changes in the cytoskeleton while nuclei and cytoplasmic both are present in subcellular microscopic images. In this paper, we proposed a quantitative image analysis method to analyze subcellular cytoskeletal changes using a texture analysis method preceded by segmentation of nuclei, cytoplasm and ruffling regions (area except nuclei and cytoplasm). To test and validate this model we hypothesized that Mammary Serine Protease Inhibitor (maspin) acts as cytoskeleton regulator that mediates cell-extracellular matrix (ECM) adhesion in tumor. Maspin-a tumor suppressor gene shows multiple tumor suppressive properties such as increasing tumor cell apoptosis and reducing migration, proliferation, invasion, and overall tumor metastasis. The proposed method obtained separated ruffling regions from segmentation steps and then adopted gray-level histograms (GLH) and grey-level co-occurrence matrix (GLCM) texture analysis techniques. In order to verify the reliability, the proposed texture analysis method was used to compare the control and maspin expressing cells grown on different ECM components: plastic, collagen I, fibronectin and laminin. The results show that the texture parameters extracted reflect the different cytoskeletal changes. These changes indicate that maspin acts as a regulator of the cell-ECM enhancement process, while it reduces the cell migration. Overall, this paper not only presents a quantitative image analysis approach to analyze subcellular cytoskeletal architectures but also provides a comprehensive tool for the biologist, pathologist, cancer specialist, and computer scientist to understand cellular and subcellular organization of cells. In long term, this method can be extended to be used in live cell tracking in *in vivo*, image informatics based point-of-care expert system and quantification of various complex architectures in organisms.

Keywords: Cellular cytoskeleton, *MASPIN*, Tumor growth, Quantitative image analysis, Marker-controlled watershed segmentation, and Texture analysis.

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1. Introduction

The cellular cytoskeleton is a highly dynamic structure that maintains cell shape and itself gets remodeled constantly in a living cell (Ridley, 2011). It is required during cell division, organelle movement, and cellular motion. It provides protection, shape and elasticity to the cell contents spanning the cytoplasm. In tumors, the cellular cytoskeleton aids all kinds of cellular morphogenesis including migration and adhesion required for a cell to extend, retract, and stabilize. The cytoskeleton aids cell locomotion during the avascular phase of tumor growth (Chakrabarti et al., 2015). At the leading edge of a motile cell, focal contacts hook the cytoskeleton to extracellular matrix (ECM) for providing a net force of forward traction. Then, the trailing edge disassembles its adhesive contacts and allows retraction to commence by actomyosin contraction (Sander et al., 1999). Novel imaging techniques (e.g. fluorescent microscopy, confocal and optical coherence tomography etc.) have provided a detailed view of cellular and molecular migration dynamics in tumor cells during *in vitro* and *in vivo* study (Matuszewski et al., 2011).

Essentially, changes in the cellular cytoskeleton can be crucial markers of cell migration and adhesion when Mammary Serine Protease Inhibitor (maspin), a tumor suppressor gene, is present in the tumor cells. Previously, maspin was discovered in an *in vivo* study where it was silenced in breast cancer (Zou et al., 1994). After this, maspin was characterized as a tumor suppressor because of its involvement in three key areas of tumor development: metastasis, apoptosis, and angiogenesis. In particular, researchers studied its localization (Zou et al., 1994), regulation of expression (Cella et al., 2006), and more recently protein interactions of maspin (Endsley et al., 2011). Many studies suggest that maspin is present at the cell surface (Sheng et al., 1996; Pemberton, et al., 1997; SefTOR et al., 1998; Ngamkitidechakul, et al., 2001; 2003; Law et al., 2005; Cella et al., 2006; Khalkhali-Ellis and Hendrix, 2007; Bass et al., 2009; Ravenhill et al., 2010; Endsley et al., 2011). But, exogenous activities of maspin are still debatable (Teoh et al., 2010; 2014). To date, few computational attempts have been taken to investigate the fact that maspin resides exogenously and acts as cytoskeleton regulator by reducing cell migration and increasing cell-ECM adhesion (Al-Mamun et al., 2013; 2016a). Recently, Al-Mamun et al., (2016b) presented a multidisciplinary quantitative image model where reduction of cell migration phenomena was investigated through both *in vitro* and computational study. This study measured the nuclei, cytoplasmic and ruffling regions using different quantitative descriptors like area, perimeter, and circularity of the cells, fractal dimension, and integrated optical density. Recently, few studies attempted quantitative analysis of subcellular structures of a cell such as grayscale analysis of microtubules of cardiac myocytes (Dang et al., 2015), combined method of gray-level-histogram (GLHs), a gray-level co-occurrence matrix (GLCM) (Lu et

al., 2014) and wavelet packet energy analysis (WPEA) to analyze different facets of microtubules morphology (Lan et al., 2015). These motivated us to hypothesize that cellular cytoskeletal changes due to maspin can be detectable by quantifying the textures of ruffling regions at the surface of cell membrane.

Identifying cellular changes from confocal microscopic images is a challenging task and usually, it requires experienced assessors to be interpreted. Practically, most subcellular microscopic images do not just contain single cells, but also clumped and layered cells. For analyzing a complex subcellular microscopic image we need to define areas containing the nuclei, cytoplasm, and peripheral substances. Generally, different image segmentation methods help us to differentiate these regions. Many researchers have developed different image segmentation methods to quantify the endo- and exogenous structures of a cell. Usually, segmentation of nuclei and/or cytoplasm is performed as the first step and then other quantification techniques are implemented on those segmented pieces. Some popular segmentation techniques include Otsu's method (Otsu, 1979), edge detection (Wählby et al., 2004), watershed (Mukherjee et al., 2004), mean shift method (Debeir et al., 2005), level set algorithms (Nath et al., 2006), active contour models (Kayser et al., 2009), sliding band filter (Quelhas et al., 2010), and iterative thresholding (Cai et al., 2014). To overcome the limitation of over-segmentation of the watershed method, marker-controlled watershed techniques have been developed to improve the performance (Yang et al., 2006; Fenistein et al., 2008; Plissiti et al., 2011, Koyuncu et al., 2012; Arco et al., 2015). Before applying a segmentation method, morphological analysis is helpful to extract useful information from the cellular images (Amini et al., 2010; Plissiti et al., 2011). A two-step binarization method is proposed to split the clumped nuclei (LaTorre et al., 2013).

Extracting quantitative texture features from microscopic images of the cellular cytoskeleton warrants proper segmentation of cell parts - nuclei, cytoplasm and other regions. Though selecting an appropriate segmentation method depends on three considerations: a) whether the method will be able to separate the features of interest when there is great variability in background patterns and intensity in images, b). whether it will be able to resolve the overlapping nuclei or cell separation problem and c) whether it will require human intervention and supervision to solve the problem. Sometimes, segmentation becomes more difficult because of different heterogeneous complexity in the image samples. Firstly, the inherent image acquisition processes may generate noisy image samples because of the dust on the confocal slits in the microscope. These noisy image samples requires a pre-processing step prior supplying to the segmentation algorithm, otherwise, these may create a problem during binarization process (LaTorre et al., 2013). Secondly, overlapped nuclei may generate over-segmentation, because the clumped nuclei or cytoplasm may belong to different cells. Thirdly, even though the nuclei splitting algorithm successfully segment the nuclei, but the division of clumped cytoplasm area may arise the under or over-segmentation problem (Lindblad et al., 2004). Finally,

even both nuclei and cytoplasm segmentation algorithms may successfully segment the major cell parts; but they may not distinguish the outer cellular parts (cytoskeleton). In these cases, co-staining of nuclei and cytoplasm during *in vitro* experiment setup may resolve some problems by providing separate nuclei and cytoplasm image samples for analysis (Lindblad et al., 2004). In this paper, we used co-stained image samples of nuclei and cytoplasm for four ECM substrates, fibronectin, laminin, collagen I and plastic. A marker-controlled watershed segmentation method was used to segment two channel image samples into three cellular parts: nuclei, cytoplasm and ruffling area. Then we used several texture analysis methods to analyze the texture of ruffling regions. Here, we propose two quantitative texture analysis methods to analyze the cellular cytoskeletal morphology for two groups of image samples: control and maspin for four ECM components. We also perform comparison and validation of the method with an existing method using domain expert's knowledge.

The rest of the paper is organized as follows. Section 2 presents the details of the *in vitro*, biochemical fractionation, western blotting, image acquisition technique, segmentation model, and texture analysis methods. Section 3 presents the segmentation and texture analysis results. It also provides the *in vitro* supports to the texture analysis finding using biochemical fractionation of F and G-actin. Section 4 discusses the relevance of the results with the biological hypothesis and contribution and implications. Section 5 summarizes the paper and states future direction of the proposed method.

2. Materials and Methods

2.1. *In vitro* methods

MCF-7 cell lines were obtained from American Type Culture Collection (ATCC) and grown with differential maspin expression and maintained (described previously in Ravenhill *et al.* 2010); “control cells” are maspin null MCF-7-pcDNA3.2, “maspin cells” are MCF-7-pcDNA3.2-maspin transfected cells expressing maspin. The cell lines were authenticated by the DNA Diagnostics Centre (London, UK). For actin staining, 0.2 unit of Alexa Fluor 568 labelled phalloidin was used. The secondary antibodies and Control IgG were brought from Life Technologies (Paisley, UK) and Dako (Ely, UK), respectively. The mouse monoclonal antibodies were used to detect maspin (commercially available, BD Biosciences, Oxford, UK).

2.2. Image acquisition

The coverslips were coated with isolated ECM components at 5µg/ml for 15 hours at 4°C. Cells were plated and incubated for 17 hours, then fixed as subconfluent monolayers with 4% formaldehyde and washed with PBS (phosphate buffered saline). For actin staining, Fluor 568 labelled phalloidin was added to each sample and incubated in PBS for 40 minutes at room temperature. This was followed by two PBS washes. Slides were mounted with hydromount (National Diagnostics, GA, USA). A

Charge Coupled Device (CCD) upright microscope or an LSM confocal microscope (Carl Zeiss Ltd, Hertfordshire, UK) was used to visualize the cells. Two capturing software kits: Axiovision 4.7.1 software and Zeiss LSM Examiner 4.0 were used. Figure 1 displays the raw image samples extracted from confocal microscope.

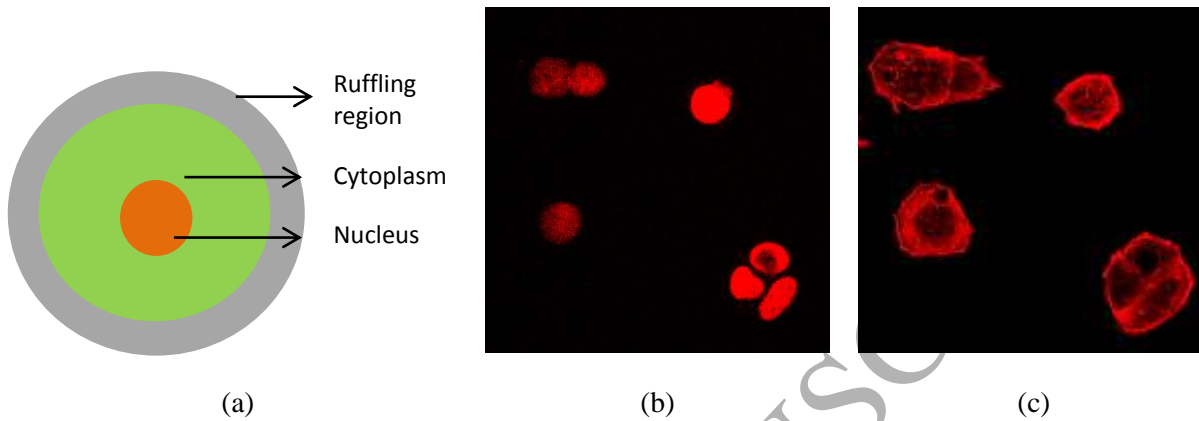


Figure 1. The grayscale two channel image samples extracted from LSM confocal microscope: a) three parts of a cell in a sketch b) channel 1: nuclei, and c) channel 2: cytoplasm with outer periphery.

2.3. Biochemical fractionation

The method for fractionating actin was based on that described elsewhere (Zou et al., 2007). Cells were plated onto the substrate at 2×10^5 /ml in serum free medium and incubated for 17 hours. After 24 hours cells were washed twice in Stabilization Buffer (50mM PIPES pH 6.9, 50mM sodium chloride, 5mM EGTA, 5mM magnesium chloride, 1mM adenosine triphosphate, 1mM Dithiothreitol, 5% (v/v) glycerol, 0.1% (v/v) β -mercaptoethanol). Extraction Buffer (Stabilization Buffer supplemented with 0.5% (v/v) Triton-X100 and Protease Inhibitor Cocktail (Roche, West Sussex, UK) was added for 10 minutes at 37°C and soluble proteins removed. The culture plate was then washed in Extraction Buffer and insoluble proteins scraped into protein sample buffer warmed to 37°C.

2.4. Cell lysates, SDS-PAGE and western blotting

Cell lysates were prepared by the method reported previously (Ravenhill et al., 2010). For Western blotting, samples separated by SDS-PAGE on a 10% resolving gel were transferred to polyvinylidene difluoride membranes (Bio-Rad). Protein bands were detected by incubation with the appropriate antibody followed by HRP-conjugated secondary antibodies (0.65 g/ml). For western blotting the concentration of primary antibodies is indicated in the figure legends, secondary antibodies were used at 1:1000.

2.5. Segmentation model

The segmentation model took two channel image samples (nuclei and whole cell) as input and then segmented the three cellular parts: nuclei, cytoplasm and ruffling region (the area without nuclei and cytoplasm). The proposed model is divided into three blocks: nuclei segmentation, cytoplasm segmentation and texture analysis. Figure 2 describes all the three blocks. Firstly, nuclei image was fed into the nuclei segmentation block where single and clumped nuclei were separated and segmented using watershed segmentation. Secondly, the separated seeded nuclei were used as marker for cytoplasm segmentation followed by a post-processing algorithm. Thirdly, bordered nuclei and cytoplasm were supplied to texture analysis block where ruffling area was extracted. Fourthly, ruffled regions were analyzed using GLHs and GLCM methods. Finally, the texture parameters were analyzed using statistical analysis.

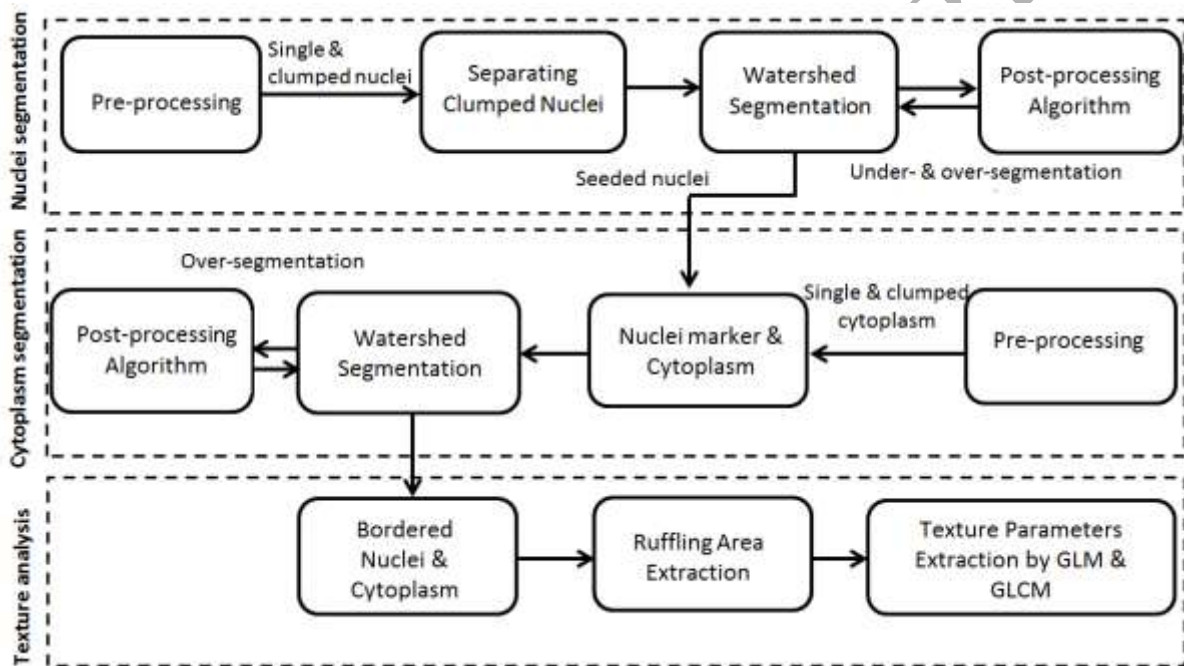


Figure 2. A general schematic of proposed quantitative image analysis method. Abbreviations: GLM: gray-level histogram, GLCM: gray-level co-occurrence matrix.

2.6. Nuclei segmentation algorithm

Each image sample contained single and/or combined nuclei, cytoplasm and scattered ruffling area around the cytoplasm. The algorithm first segmented the nuclei and then segmented the cytoplasm using the nuclei as a marker. Normally, the watershed transform works better if it can mark or identify the foreground objects and background location, in other words this is called marker-controlled watershed segmentation technique (MCWS). The preprocessing step identified the cells from different background contrast. This preprocessing step is essential because some image samples had background contrast problem. It might occur because of uneven illumination and striped pattern problems in the microscope. We used median filtering iteratively with contrast limited adaptive

histogram Equalization (CLAHE) until the background problem was resolved (Al-Mamun et al., 2016b). Normally, CLAHE is useful when both the background and foreground object are bright at the same time or else both are dark at the same time. During median filtering 3×3 neighbors and for CLAHE 8×8 tiles have chosen. Once we got the preprocessed image, we used the following procedure to segment the nuclei.

Procedure 1: Nuclei Segmentation

- Step 1:* Filtering with sobel horizontal edge-emphasizing filter with gradient magnitude calculation to produce a variable contrast between the foreground object bordered with the background (shown in Figure 3b)
- Step 2:* Using morphological opening (object size- 50 pixels) to make a good nuclei seed (shown in Figure 3c)
- Step 3:* Using two times dilation used to reconstruct the original nuclei size (shown in Figure 3d)
- Step 4:* Euclidian distance transform (EDT) was used to create the separate line between clumped nuclei (Breu et al., 1995). During this process, h-minima level was set to 2 (shown in Figure 3e)
- Step 5:* Then clumped nuclei was separated by applying watershed (shown in Figure 3f)
- Step 6:* If under- or over-segmentation occurred for any nuclei then it was sent to post-processing algorithm to solve the splitting problem
- Step 7:* The segmented nuclei was delineated with a border using 4 connecting neighborhood points (Srisukkhram et al., 2013)

The details of the morphological operation and EDT are given in the supplementary file, section S1 and S2, respectively.

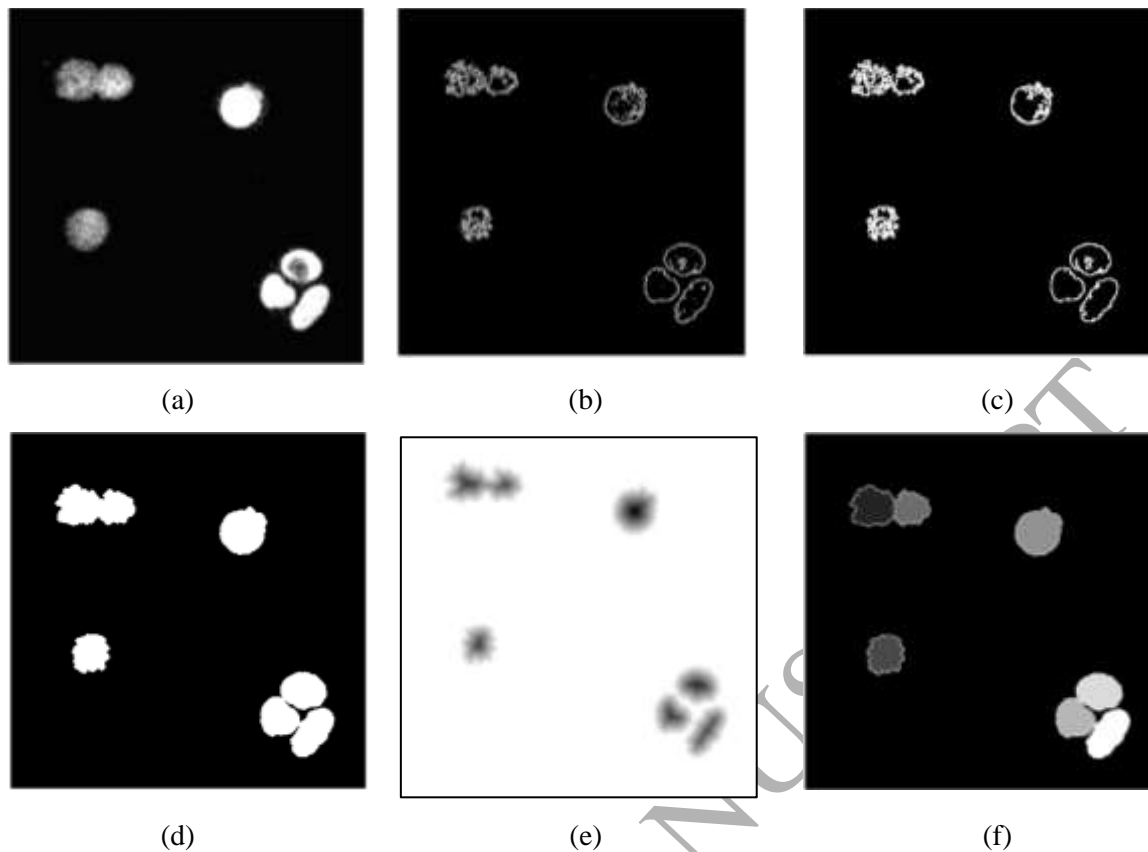


Figure 3. The steps of nuclei segmentation: (a) The preprocessing step, (b) the gradient magnitude variation of background and border, (c) output of the morphological opening, (d) removing small objects size after 2 times dilation, (e) distance transform map of nuclei, (f) separated nuclei after applying watershed algorithms.

2.7. Cytoplasm Segmentation

The separated nuclei were used as seeds of cytoplasm segmentation. Before implementing the cytoplasm segmentation process, it was needed to run the preprocessing routine (described in section 2.6) for cytoplasm image samples. Once we obtained the preprocessed cytoplasm images, then we undertook the following procedure to segment the cytoplasm

Procedure 2: Cytoplasm Segmentation

Step 1: Filtering with sobel horizontal edge-emphasizing filter with gradient magnitude calculation to produce a variable contrast between the foreground object bordered with the background (shown in Figure 4a)

Step 2: Using morphological opening (object size-500pixels and line shaped-2pixels)

Step 3: Filling the holes of whole cytoplasmic area (shown in Figure 4b)

Step 4: Using extended- h -minima transform (regional minima of the h minima transform level) to impose the nuclei seed as a mask (shown in Figure 4c), The larger the h -value is, the fewer the numbers of the segmented regions. This step was essential because it creates a good

marker for the GWDT (shown in Figure 4d)

Step 5: Applying gray-weighted distance transform (GWDT) to the grayscale image and applied into $-\infty$ background (shown in Figure 4e)

Step 6: Applying watershed to make the labelled cytoplasmic areas for single and combined cells (shown in Figure 4f)

Step 7: The segmented nuclei was delineated with a border using 4 connecting neighborhood points (Srisukkhom et al., 2013)

The details of GWDT method is discussed in supplementary file in section S3.

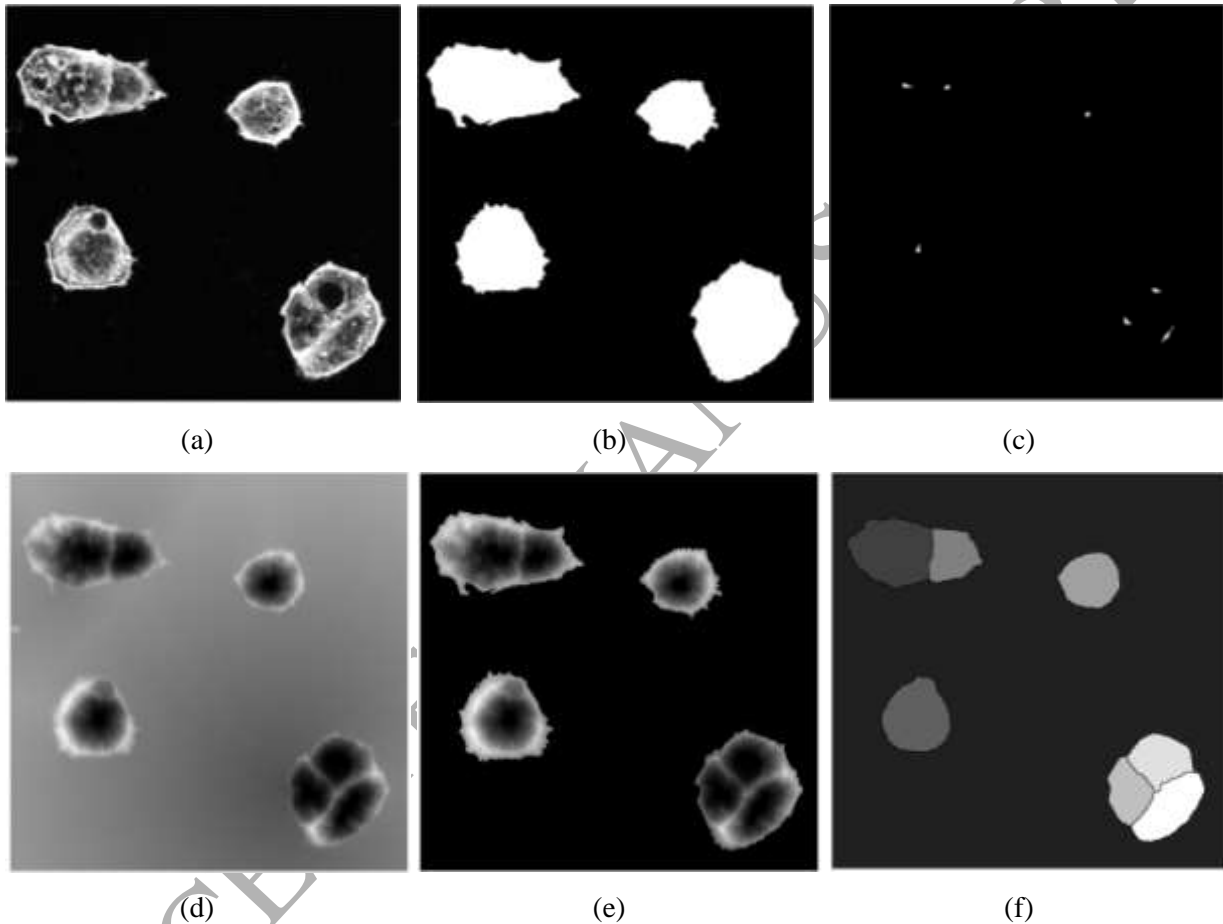


Figure 4. The steps of cytoplasm segmentation. (a) cytoplasm image after preprocessing and median filtering, (b) filling the whole cytoplasm, (c) imposing the nuclei seed as a mask using extended h-minima transform, (d) gray-weighted distance transform (GWDT) of the grayscale cytoplasm, (e) the GWDT applied into $-\infty$ (infinity) background, (f) output after applying watershed segmentation.

2.8. Post-processing and ruffling area extraction

For avoiding over- and under-segmentation, we used region merging algorithm as a post processing method. First, it took the labeled image after watershed segmentation and calculated the number of watershed flooded areas. Second, it retrieved the labeled areas as sub-images with original intensities

and created an intensity pixel list. Third, it searched for small objects; if it found one, the intensity value of the small object was compared with neighboring regions. Fourth, if it found any single touching object then the label was put into a merging list. In the case of several touching objects, it took the highest summed intensity of touching objects to the merging list. Fifth, the objects were merged based on the final merging list. In the merging list, zero represented those objects that were originally noise. The flowchart of the post-processing is shown in Figure 5.

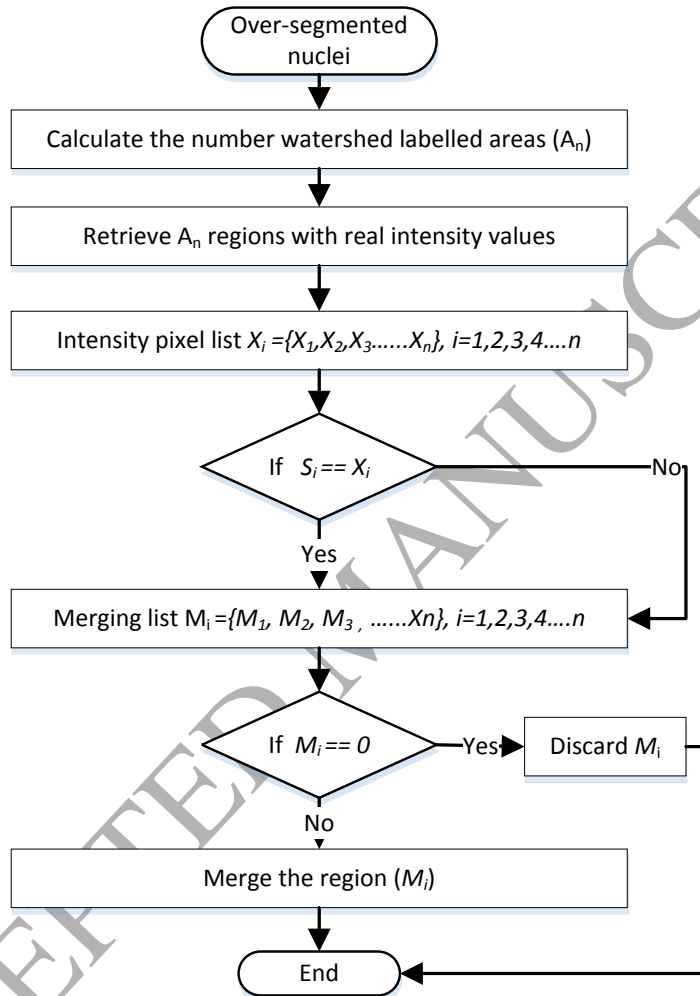


Figure 5. The flows chat of post-processing that used during the marker control watershed segmentation process.

2.9. Gray-level histograms (GLHs)

The gray-scale histogram of an image presents the compilation of grayscale value of each pixel in the image. It is presented as if each pixel is placed in a bin corresponding to the color intensity of that pixel. Then all the bins are visualized as a histogram where frequencies of different intensity levels can be seen and different quantitative measurements can be obtained based on this. The grayscale value of the pixel is within the range of 0-255; the minimum 0 states the pixel is black and 255

indicates the pixel as pure white. Let, I is a gray-scale given image and the intensity histogram is defined by a probability density function $p(k)$ as follows

$$p(k) = \frac{h(k)}{M}, \quad 0 \leq k \leq n - 1 \quad (1)$$

Where $h(k)$ is the number of pixels in the image or region of interest and k is the number of grayscale layer and M is the total number pixels in the image (image height \times image width)

The histogram bins can provide the following statistical features

Mean: the mean grayscale value of the converted grayscale image

$$Mean(\mu) = \sum_{k=0}^{n-1} kp(k) \quad (2)$$

Variance: variability of discrete measurement of the grayscale value while compared with the mean

$$Variance = \sigma^2 = \sum_{k=0}^{n-1} (k - \mu)^2 p(k) \quad (3)$$

Skewness: describes the degree of asymmetry in grayscale values in the image when compared with the mean distribution. Skewness can be positive and negative which reflect right and left asymmetry respectively:

$$Skewness = \frac{1}{\sigma^3} \sum_{k=0}^{n-1} (k - \mu)^3 p(k) \quad (4)$$

Kurtosis: reflects whether the image's grayscale values are heavy-tailed or light-tailed relative to the mean value or not.

$$Kurtosis = \frac{1}{\sigma^4} \sum_{k=0}^{n-1} (k - \mu)^4 p(k) \quad (5)$$

2.10. Grey-level co-occurrence matrix (GLCM)

The GLCM algorithm was first introduced by Haralick et al., (1973) and it was built on the conditions of probability density function of two pixels in a certain direction and distance. Basically, it is statistical approach of texture analysis which estimates the second-order joint probability $P_{\delta}(i, j)$ of the intensity values of two pixels (i and j) where δ is a distance apart along a given direction θ . In this paper, extracted ruffling image regions were 8-bit which gave 256 rows \times 256 columns GLCM dimensions for a given displacement vector. We calculated GLCM features for each ruffling image sample for all directional angles (0,45,90 and 135°). We set $\delta=4$ and then calculated five texture

descriptors. Let $P_\delta(i, j)$ be the (i, j) th entry in a normalized GLCM. The mean and standard deviations for the rows and columns of the matrix are

$$\mu_x = \sum_{i=1}^n i \sum_{j=1}^n P_\delta(i, j), \quad (6)$$

$$\mu_y = \sum_{j=1}^n j \sum_{i=1}^n P_\delta(i, j) \quad (7)$$

$$\sigma_x = \sum_{i=1}^n (i - \mu_x)^2 \sum_{j=1}^n P_\delta(i, j), \quad (8)$$

$$\sigma_y = \sum_{j=1}^n (j - \mu_y)^2 \sum_{i=1}^n P_\delta(i, j) \quad (9)$$

The descriptors are as follows

(1) Angular second moment (ASM)

$$ASM = \sum_{i=1}^n \sum_{j=1}^n \{P_\delta(i, j)\}^2 \quad (10)$$

(2) Entropy (ENT)

$$ENT = \sum_{i=1}^n \sum_{j=1}^n P_\delta(i, j) \times \log(P_\delta(i, j)) \quad (11)$$

(3) Inverse different moment (IDM)

$$IDM = \sum_{i=1}^n \sum_{j=1}^n \frac{1}{1 + (i - j)^2} P_\delta(i, j) \quad (12)$$

(4) Correlation (COR)

$$COR = \left\{ \sum_{i=1}^n \sum_{j=1}^n P_\delta(i, j) - \mu_x \mu_y \right\} / \sigma_x \sigma_y \quad (13)$$

(5) Contrast (CON)

$$Constrast = \sum_{l=0}^{n-1} l^2 \left\{ \sum_{i=1}^n \sum_{j=1}^n P_\delta(i, j) \right\}, \quad |i - k| = l \quad (14)$$

3. Results

A total of 168 confocal image samples were supplied to the proposed algorithm where 496 cells were manually annotated. The number of cells was grown on different ECM substrate were plastic (107), fibronectin (121), laminin (123) and collagen I (145). The methods were coded in MATLAB (R2014a) in a custom way. ImageJ LOCI toolbox was used to subsample the raw images by a factor 2 to speed up the processing before supplying to the MATLAB custom code (Schneider et al., 2012). The experiments were conducted using a machine with an Intel(R) Xeon(R) CPU E5-2650 v3@2.3GHz, 20 Cores Server, (25 MB Smart Cache) and 64 GB of RAM. Statistical analysis was carried out with Minitab and Microsoft Excel. Data are presented as mean \pm standard error of the mean. Significance was judged using Student's t-test and defined $p < 0.05$.

3.1. Parameter setting and segmentation results

The proposed model has two segmentation procedures and the performance of cytoplasm segmentation was depended on the success of nuclei segmentation, as nuclei were used as seeds for cytoplasm segmentation. Throughout the segmentation process, some parameters were tuned to produce good segmentation performance. Parameters used in two segmentation procedures are summarized in Tables 1.

Table 1. Parameters values used in nuclei and cytoplasm binarization steps.

Used parameters	Values
Nuclei segmentation	
MATLAB 2-D filter	Sobel
Removed object size during morphological opening	50 pixels
Remove particle size after morphological operation	400 pixels
<i>h</i> -minima level	2
Cytoplasm segmentation	
MATLAB 2-D filter	Sobel
Morphological opening	Line shaped, 2 pixels
Removed object size during morphological opening	100 pixels
<i>h</i> -minima level	5
GWDT chamfer weights method	Quasi-Euclidian

Abbreviations: GWDT-gray weighted distance transform

The subsampled images were used in both nuclei and cytoplasm segmentation algorithms. A median filter of (3, 3) values was used onto the input images. In nuclei segmentation, a mathematical morphological operation was used and the threshold image was dilated. After that, EDT was applied and a mask was made from the output of distance transform (DT) by applying h -minima transform. Then, a good mask was created using DT map before applying the watershed algorithm. In cytoplasm segmentation, the seeded nuclei were used to find the accurate position of the cytoplasm by using extended h -minima transform. Then, the GWDT was utilized with the mask of seeded nuclei preceded by applying a median filter to the cytoplasm. After that, the GWDT map was superimposed onto the mask, so that it would reveal the watershed catchment basin lines once the watershed algorithm was applied. Finally, the boundary was drawn on the labeled separated watershed regions. In the case of under- or over-segmentation, the control was given to the post-processing algorithm. Once, we got the separate nuclei and cytoplasm, the ruffling regions were extracted from the whole image. The results of whole segmentation process are displayed in Figure 6.

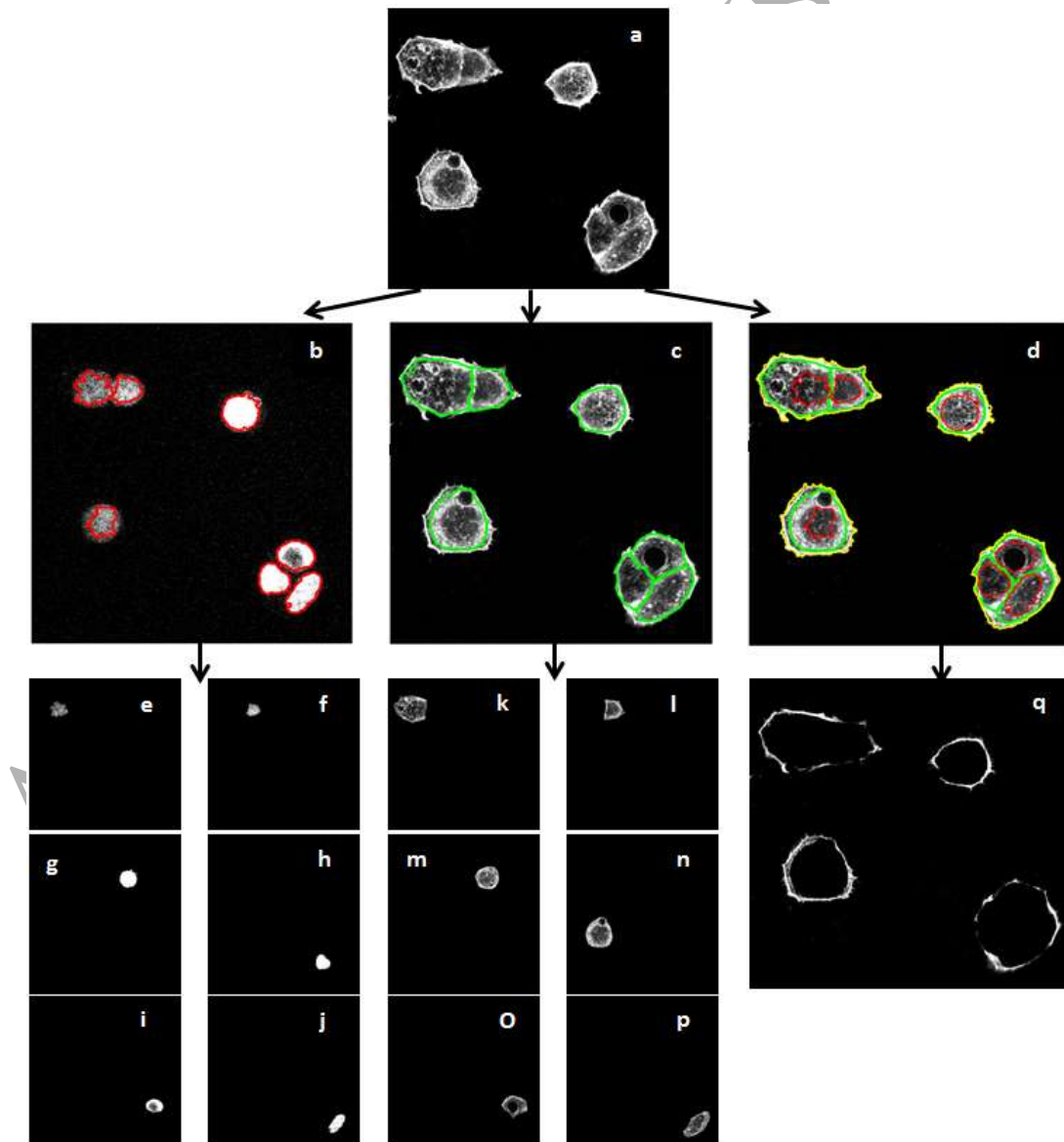


Figure 6. The summary of segmentation results, (a) combined image sample of two channels, (b) the segmented nuclei after nuclei segmentation step, the nuclei are outlined with red color line, (c) the segmented cytoplasm after cytoplasm segmentation step, the cytoplasmic areas are outlined by green color line, (d) ruffling regions are highlighted using yellow line along with nuclei and cytoplasm, (e-f) separated nuclei as single sample, (k-p) separated cytoplasmic areas as single sample, and (q) the identified ruffling regions separated from whole cell image.

3.2. Analysis of GLH features

The means and standard deviations of gray-level histogram parameter values were calculated for separated ruffling regions to analyze the complexity of the grayscale distribution. The results of GLH parameters are presented in Table 2. Mean and variances of control and maspin do not carry much information, but skewness and kurtosis values indicate that plastic cells with maspin has the highest skewness and kurtosis value while it is also showing high energy and low entropy value (data not shown). It clearly shows that while maspin was present in the cells, the grayscale parameters are uniformly distributed. But for plastic, the values of skewness and kurtosis are highest in compared with others. The lower value of kurtosis for control images show that GLH distributions were dominated by extreme values that maspin images.

Table 2. Characteristics of gray-level histogram parameter values of ruffling regions for four different extracellular matrix (ECM) substrates (plastic, collagen I, laminin, and fibronectin) on control (no maspin) and maspin (with maspin) experimental images^a

GLH	Plastic (n = 43)		Collagen I (n = 30)		Laminin (n = 46)		Fibronectin (n = 49)	
	control	maspin	control	maspin	control	maspin	control	maspin
Mean	1.24 (0.09)	1.23 (0.09)	1.20 (0.07)	1.23 (0.1)	1.48 (0.27)	1.39 (0.19)	1.37 (0.13)	1.26 (0.09)
Variance	0.70 (0.49)	1.04 (0.42)	0.77 (0.42)	0.72 (0.55)	2.29 (1.42)	1.82 (0.95)	1.67 (0.75)	1.12 (0.47)
Skewness	4.39 (1.04)	5.34 (1.25)	5.09 (0.79)	4.23 (0.89)	3.72 (1.30)	3.99 (1.09)	3.96 (0.89)	4.63 (0.79)
Kurtosis	26.98 (13.63)	33.73 (14.05)	31.58 (9.29)	23.70 (9.54)	18.38 (13.28)	20.36 (12.89)	19.37 (8.47)	25.73 (9.18)

^aAll values are presented as mean±SD from number of image samples (n)

3.3. Analysis of GLCM features

Figure 7 presents the GLCM parameters comparison between control and maspin using student t-test. It is plausible to mention that control cells are showing more polarized characteristics as they have high ASM, CON, COR and IDM values and low ENT values. But on the other hand, maspin cells

show more regular and non-polarized characteristics. It is evident from the correlation values that maspin cells are more compact as they have lower COR values. From visual inspection, we also confirmed that there was dense meshwork of cellular actin at the periphery. Similar types of measurements have been found in Lan et al., (2015) where microtubules patterns were quantified using GLCM parameters.

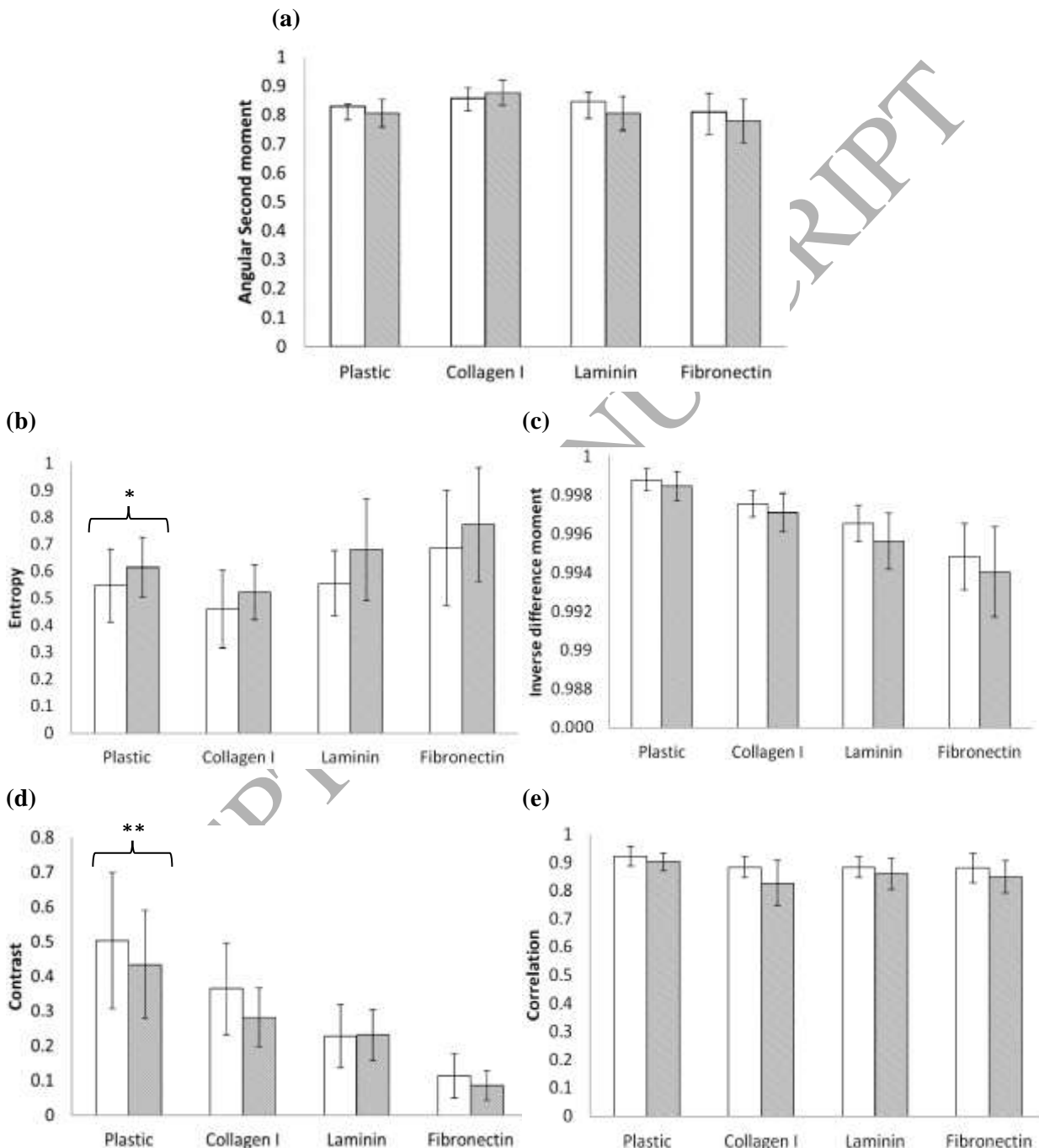


Figure 7. Texture analysis results of selected gray-level co-occurrence matrix (GLCM) parameters: (a) energy, (b) entropy, (c) inverse difference moment (IDM), and (e) contrast for maspin exposure for four different extracellular matrix (ECM) substrates (plastic, collagen I, laminin, and fibronectin).

White bars represent control experiments while colored bars present the maspin experiments. *, $p < 0.01$; **, $p < 0.005$.

3.4. Maspin effects on cellular concentrations of G and F actin

The influence of maspin on the cellular levels of globular and filamentous actin was investigated, as this can be related to cell motility. Biochemical fractionation was used to separate soluble G-actin and insoluble F-actin from MCF7 cells stably expressing pcDNA-3.2 or pcDNA3.2-Maspin, that were plated on plastic, collagen I, laminin or fibronectin (Figure 8). MCF7-pcDNA-Maspin cells were found to have increased levels of F-actin in comparison to MCF-7-pcDNA-3.2. The greatest increase (34%) was observed in cells grown on plastic or collagen I. MCF-7-pcDNA3.2-Maspin cells grown on plastic, collagen I and laminin showed an increase in G-actin content in comparison to control cells. All cells grown on fibronectin showed equal expression of G-actin. Generally, maspin caused an increase in the cellular content of both G-actin and F-actin.

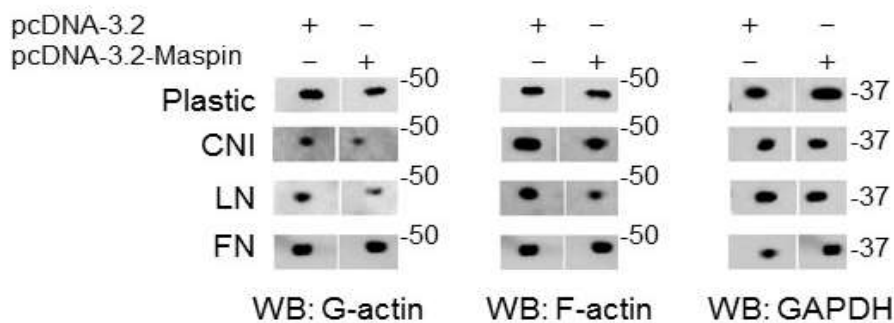


Figure 8: Biochemical fractionation of actin components. MCF-7 stably transfected with pcDNA-3.2 or pcDNA3.2-Maspin were grown on isolated ECM components: plastic, collagen I (CNI), laminin (LN), or fibronectin (FN). Cell lysates were separated into soluble (containing G-actin) and insoluble (containing F-actin) fractions. 10 μ g of the soluble fraction and 30 μ g of the insoluble fraction were separated by SDS-PAGE and detected on western blots using anti-actin (200ng/ml) or anti-GAPDH (1:5000). Staining was performed on three independent samples and only the representative images were selected.

3.5. Evaluation of segmentation algorithms

To avoid the over-segmentation in cytoplasm it was needed to provide perfect nuclei seed to the cytoplasm segmentation procedure. Both procedures forwarded the under- or over-segmented images to the post-processing algorithm (shown in figure 9a). Once the segmented labelled images were obtained (shown in figure 9b), it calculated the number of watershed flooded area and retrieved them as sub-images with original intensity from the original image (shown in figure 9c). The final

segmented images after post-processing routine were confirmed by human annotation and evaluation (shown in Figure 9.d).

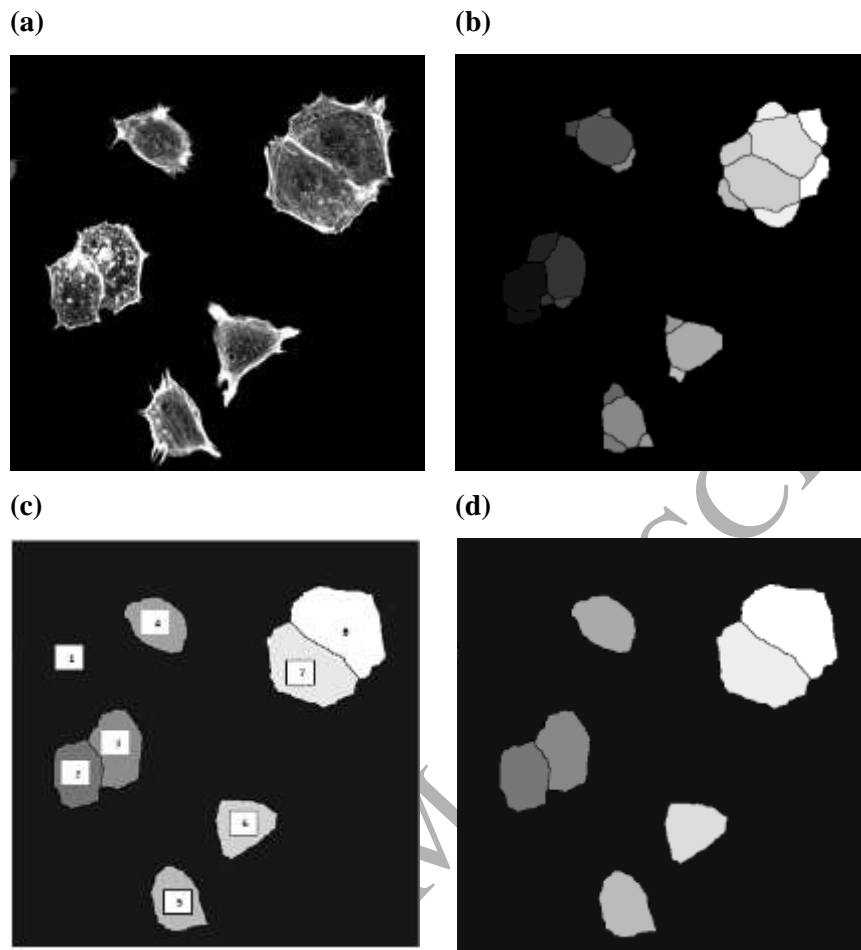


Figure 9: The post-processing method for proposed marker-controlled watershed segmentation (MCWS) method, a) the filtered grayscale image, b) the over-segmented cytoplasm, c) identified regions during the post-processing method based on which area, and d) final segmented cytoplasm after applying the post-processing algorithm.

4. Discussion

This paper presents a quantitative image segmentation method that includes marker control watershed segmentation and two texture analysis method to analyze the cellular cytoskeleton of the cells. We developed several *in vitro* experiments of tumor cell line MCF-7 with different maspin exposure on different ECM substrates (plastic, collagen I, laminin and fibronectin) to support our computational evidence. Moreover, this paper provides a quantitative image analysis method that can segment nuclei, cytoplasm and ruffling regions of single and as well as clumped cells, while the segmentation

results were validated using domain expert's knowledge. The method integrated two texture analysis methods: GLH and GLCM to find some important information about the shape and behavior of the ruffling region.

The results shown in Table 2, Figure 7 and 8 have biological significances in case of maspin dynamics in cellular and subcellular level. It has been reported previously that maspin triggers several cellular processes which get reflected on cell behavior and cytoskeletal architecture (Odero-Marah et al., 2003; Qin & Zhang, 2010; Lara et al., 2012; Al-Mamun et al., 2016a). In a recent attempt, Al-Mamun et al., (2016b) showed that an image processing tool can be used to quantify three cellular parts: nuclei, cytoplasm and ruffling regions to observe the changes in shape and behavior. The study considers descriptors including area, perimeter, integrated optical density, fractal dimension and circularity to quantify each cellular part. In this paper, we focused on the cellular periphery machinery and built a quantitative method that combines the image segmentation and two texture analysis methods to quantify the ruffling area textures. GLH and GLCM texture parameters were calculated and analyzed in Figure 7. A biochemical fractionation experiment was used to separate soluble G-actin and insoluble F-actin on four different ECM substrates for stably expressing pcDNA-3.2, pcDNA3.2-Maspin. The results confirm the computational results that the thick rim around the tumor cells are found due to the presence of maspin. This finding aligns with the proposed second pathway by Endsley et al., (2011), that maspin remains exogenously with uPAR and $\beta 1$ integrin complex and facilitates enhanced migration capabilities. Then, the maspin-uPA complex binds to (or associates with) the uPAR- $\beta 1$ integrin complex, thereby inducing a conformational or lateral mobility change in uPAR, which causes an altered physical association (and/or activation state) with $\beta 1$ integrin, eventually resulting in decreased cell migration and/or increased cell adhesion. However, the first pathway remains to be tested and verified that maspin binds to uPA and localizes on the cell surface by maspin-uPA binding to uPAR. In this case, uPAR and $\beta 1$ integrin do not reside in a complex. Then, the maspin -uPA-uPAR complex associates with and inactivates $\beta 1$ integrin (via the G-helix of maspin binding to $\beta 1$ integrin), thus reducing cell migration potentially by increasing cell adhesion.

By looking at the texture parameters value we found a common ground to show that cells without having maspin are non-polarized. This phenomenon is also confirmed by biochemical fractionation where we saw that maspin cells grown on plastic, collagen I and laminin showed an increase in G-actin content in comparison to the other cell types. Also, cells transfected with wild type maspin showed a slight increase in the content of both G-actin and F-actin which corresponds with its phenotype of a thick actin periphery and large flattened phenotype. It supports the finding that MCF7 cells stably expressing wild type maspin significantly increased cell adhesion by $113 \pm 5\%$ on a laminin matrix and by 45 to 76% on either collagen I or fibronectin matrices, in comparison to cells expressing vector only (Al-Mamun et al., 2016a). Also, GLCM values from our current analysis, we

saw that ruffling regions of control cells (without maspin) are polarized and elongated as their entropy value is lower.

To evaluate the segmentation method, we compared our segmentation results with the level set method. The level set method is a popular and established numeric method for segmenting shapes and contours (Osher & Fedkiw, 2003, Li, et al., 2010). For keeping the experiments simple, we replaced the binarization by the level set method proposed by Li et al., (2010). Specifically, the method adopted Distance Regularized Level Set Method (DRLSE) algorithm where several parameter ($\lambda, \mu, \Delta t, \alpha$) values were used. We ran the algorithms with four parameters and fixed the values by an iterative process. $\lambda = 5, \mu = 0.02, \Delta t = 5.0$ and $\alpha = 1.5$. It has been discussed in LaTore et al., (2013) that large values of α may lead to boundary leakage, in our experiments we choose low alpha values in a range (0.5 – 2.5). Moreover, we used DT map for nuclei segmentation while we used DT and GWDT both for cytoplasm segmentation for validation purpose. Table 3 summarizes the average segmentation accuracy for three cell parts using without GWDT, with GWDT and level set when compared with manually detected cells. When there is an error or over-segmentation occurred, it means the error occurred in at least one part of segmentation. To presents the accuracy we divided it into different error. Type-I error refers to cells had faced over-segmentation (they have been divided into multiple parts when they should have not). Type-II error occurs when the algorithm detected background noise as a cell in the binarization process. Finally, Type-III error represents the image samples which were rejected because of intensity problem.

Table 3. Validation of segmentation accuracy and comparison of three methods used during binarization process: a model without gray-weighted distance transforms (GWDT), a model with GWDT and level set method.

	Model without GWDT	Model with GWDT	Level set
Number of manually detected cells	496	496	496
Correctly Segmented (%)	81.05	92.54	91.54
Type-I error: Over-segmentation (%)	11.50	2.22	3.13
Type-II: Noise detected as cells (%)	2.62	0.8	0.8
Type-III error: intensity problem (%)	4.83	4.43	4.43

Table 3 indicates that model without using GWDT in cytoplasm segmentation gives the lowest accuracy, over-segmentation could not be avoided, but a model with GWDT performs slightly better than level set method. But when we tuned the parameters in the level set method, it was seen that the method was really sensitive to the image sample that had intensity problems and that results in a slightly higher percentage of over-segmentation.

This contribution of the proposed work in terms of maspin biology and experts and intelligent systems are five folds. First, the quantification results obtained from this study supports that maspin resides exogenously in cellular environment to show its potential engagement in reducing cell migration and enhancing cell adhesion. Second, the current version of the method is compared with the level set method over Al-Mamun et al., (2016b). Third, it included an explicit texture analysis method (both GLH and GLCM) that confirmed the specific changes of ruffling regions due to maspin. Fourth, it presented an *in vitro* experimental data support to our computational hypothesis that maspin acts as cellular cytoskeleton regulator during tumor growth. Fifth, this method offers comprehensive quantification of the segmented objects to make the proof based decision support system for subcellular image cytometry domain. Nevertheless, this method has some solid strength both computationally and biologically. As an intelligent system, this method is capable of taking detailed subcellular-level confocal microscopic images as input and providing quantification about their textures. The existing quantitative image tools for subcellular images are more subjects specific, where our method offers a flexible framework. Another strength of our method is scalability, our model can take temporal image as input and can provide the texture quantification of a series of images. However, the presented method has some limitations. Firstly, our current model did not consider the precise measurements of filopodia, lamellipodia, stress fiber, and focal adhesions. Secondly, it could not provide cell-ECM adhesion measurement due to lack of data collection. Thirdly, the presented MCWS method presented here can segment the subcellular images from our experiments, but care should be taken to use this method to the histopathological nuclei images. Fourthly, this paper does not offer a classification technique due to the lack of insufficient reference dataset.

5. Conclusion

In conclusion, it is seen in the current practices that manual assessment and quantification of histopathological, cellular and subcellular microscopic images are labor intensive and prone to interobserver variations. It is plausible from the great deal of interest in current scientific groups that computational methods can significantly improve the objectivity and reproducibility of the both quantitative and qualitative evaluation of such complex image samples. In align with the interest; the present work has combined image segmentation and texture analysis techniques to quantify the cellular cytoskeletal changes in terms of shape and behavior. It is demonstrated that reduction of cell migration effects of maspin can be facilitated by cellular cytoskeletal changes due to maspin presence. Researchers have been trying to investigate subcellular cell images to extract both qualitative and descriptive information from nuclei, cytoplasm, and other parts (LaTorre et al., 2013). But clumped nuclei and cytoplasm, uneven illumination of images and subject specific limitations of image tools challenge the quantitative image analysis tool (Xing and Yang 2016). Moreover, methods like marker

controlled watershed can be easily implemented, but sometimes it is not easy to obtain correct marker detection while separating the clumped cells. On the other hand deformable models, clustering, and graph-based methods might be considerably better than the conventional segmentation methods, but users need to pay high computational costs when applying to whole images (Xing and Yang 2016).

Overall, the proposed method can be used by biologist, pathologist, cancer specialist and computer scientists to perform similar types of quantification studies on subcellular complex images. In this work, we develop an algorithm for segmenting nuclei of both isolated and confluent cell taken from confocal microscope which are used for explaining complex mechanisms in tumor growth. But, this kind of quantitative image tool can also be applied in conventional wide field fluorescent microscopes that available from various laboratories. Moreover, this method can be used in histopathology diagnostic laboratories where they are routinely used for morphological analysis of cells (Krishnan et al., 2012). Furthermore, this method can serve as a key method for tracking active cells in live animals in vivo, real-time view of complex biological processes like oncogenic signaling, tumor metabolism, and the complex architecture of a developing organism (Damiano et al., 2014; Han et al., 2015; Shah et al., 2015; Gerhold et al., 2015).

In future, the additional research features of this method can be five folds from both biological and intelligent system point of view: 1) this method can be extended by including the temporal images of different maspin exposures to validate the in vitro hypothesis that maspin decreases tumor cell migration by reducing EMT process, 2) the segmentation method can include the prior biological knowledge as input of the systems, so that the biologist can use the quantification for decision making, 3) the method can add a classification method, but prior that we will create a benchmark dataset especially for subcellular cytoskeleton images, 4) this method will be improved with using parallel or distributed computing techniques to improve the running time cost, and 5) a graphical user interface (GUI) will be made for the ease of biologist to upload the subcellular images to our database. The GUI will increase the usability for the users especially, who are not a subject matter experts of cellular and subcellular images.

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