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Generalised Image Processing Method for Quantitative Analysis of Nucleus, Cell and Focal Adhesion Clusters

Abstract: Focal adhesion clusters (FAC) are dynamic and complex structures that help cells to sense physicochemical properties of their environment. Research in biomaterials, cell adhesion or cell migration often involves the visualization of FAC by fluorescence staining and microscopy, which necessitates quantitative analysis of FAC and other cell features in microscopy images using image processing. Fluorescence microscopy images of human umbilical vein endothelial cells (HUVEC) obtained at 63x magnification were quantitatively analysed using ImageJ software. A generalised algorithm for selective segmentation and morphological analysis of FAC, nucleus and cell morphology is implemented. Further, a method for discrimination of FAC near the nucleus and around the periphery is implemented using masks. Our algorithm is able to effectively quantify different morphological characteristics of cell components and shows a high sensitivity and specificity while providing a modular software implementation.

Keywords: Focal adhesion clusters, algorithm, fluorescence, quantitative analysis, ImageJ, sensitivity, specificity, location selective segmentation, masks.

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1 Introduction

With the advent of digitalization of microscopy images, image processing has become a key tool for the analysis of the same. In the last decade, huge strides have been made in the acquisition and processing of camera images for computer vision and perception. While the field of biomedical image processing has seen the application of many of these techniques, most of the analysis is by visual inspection focusing on qualitative aspects.

Recently, progress has been made in automated detection and characterisation of cellular components in microscopy images using image processing frameworks like ImageJ, Matlab, among others. Horzum et al., proposed a step by step method for FAC detection and analysis using ImageJ [1]. We replicated the method and found that while it performed quite well for red, green or blue colours, it was less sensitive to other colours as it splits the images into RGB constituents as the first step. Buskermolen et al. performed a similar automatic detection of FAC, nucleus and cell using marker-controlled segmentation, where their focus was on single cell characteristics [2]. Carpenter et al. developed a complete framework for batch processing for automated detection of cellular components [3], whereas Berginski et al. focuses on the spatiotemporal dynamics of FAC in living cells [4].

While the above methods taken together have led to marked improvements in the quantification and characterisation of cellular components, none of them individually had all the methods required to fulfil our goals of high sensitivity, modular software implementation, and spatial selective segmentation.

In a model study using specific Polyelectrolyte multilayer (PEM) coatings to control endothelial cell adhesion and growth on implant surfaces we evaluate an integrated tool for cell analysis.

2 Methodology

PEM coatings for cell culture experiments were prepared and physico-chemical characterization studies were carried out [5]. In our work, we used the coatings to study the adhesion and proliferation of HUVEC.

35,000 HUVEC (Promocell) were seeded per well of 3.5cm^2 area on non-coated microtiter plates as negative controls (NC), tissue culture treated (TCT) microtiter plates as

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positive controls (PC) and PEM1 (SP0), PEM2 (SN0), PEM3 (SP10) and PEM4 (SN10) coated microtiter plates. Immunofluorescent staining was performed to detect cell (actin/phalloidin), nucleus (DAPI) and focal adhesion (paxillin) morphologies.

8-bit fluoroscent images of control and PEM coatings were captured using Zeiss Axio Observer 63x oil immersion objective with a numerical aperture of 1.4 oil DIC M27. The images have a resolution of 2752 x 2208 pixels, covering a corresponding area of 198.32 µm x 159.12 µm. They were then analysed using ImageJ framework.

2.1 Detection and Analysis of Focal Adhesion Clusters

The primary goal of the work was the detection and quantitative analysis of Focal Adhesion Clusters (FAC) and extraction of the morphological features. We developed a new algorithm for Hue Saturation Value (HSV) based detection for the same as follows (Figure 1):

1. Subtract Background: A rolling ball background subtraction of radius 50 pixels is applied to the color image to compensate for unevenness in the background illumination [6].
2. Color Thresholding Segmentation: Color thresholding using the HSB stack is executed on the image. Auto select or manually select the range of colors for detection of FAC on train images. This range is then kept constant for all the other images.
3. The result of the color thresholding is then converted into a mask and thus distinct segments are formed.
4. The resulting segments are then converted into a binary image.
5. Morphological operation is performed using erode, dilate and close operators.
6. Analyse Particles with size thresholding of 10 pixels is executed.

Compared to Horzum et al.'s method, we do not split the image into RGB channels for image analysis. This ensures that non-primary i.e. non RGB images, for example from cameras used in histology, can be similarly detected using our algorithm on the complete colour spectrum.

FAC are key elements to understand the adhesion of cells onto surfaces. Depending on size and localisation they may be distinguished in different classes. Hence, we are interested in detecting FAC on the cell periphery and nucleus region, necessitating a method to discriminate between them. Using the same image, the afore-mentioned algorithm was executed to detect the nucleus and its surrounding areas. Two sets of masks, one each for the nucleus and adjoining areas and one for the periphery were created. The binary images for FAC were then ANDed with the corresponding masks to obtain location-selective segmentation of FAC. The flowchart for the location selective segmentation is shown in Figure 2.

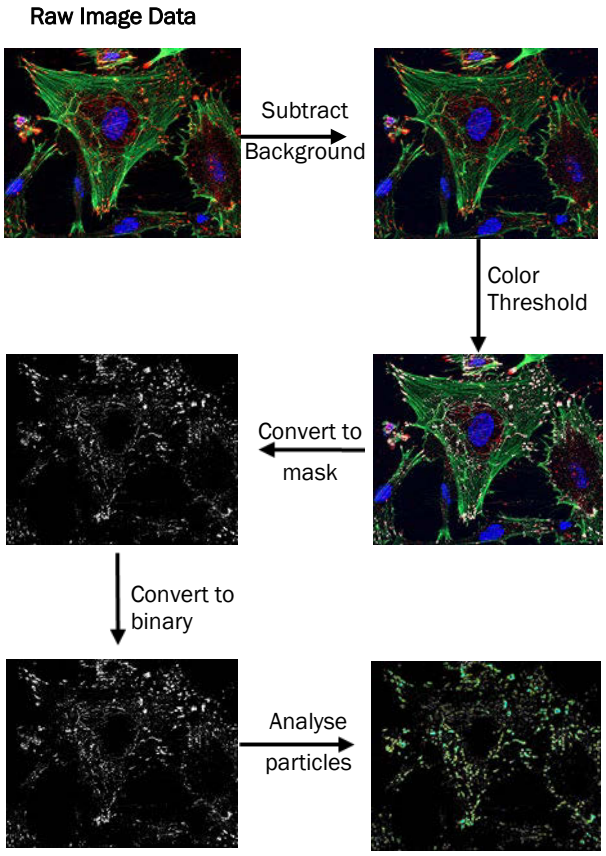

Figure 1: Flow chart of HSV method

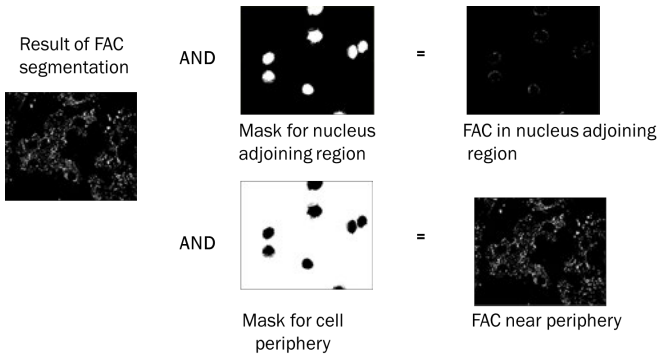

Figure 2: Location Selective Segmentation

2.2 Detection and Analysis of Nucleus and Cell Cytoskeleton

The algorithm explained in 2.1, with the exception of Step 1, was also used to detect the nucleus. Background subtraction is skipped as it was observed that the nucleus in many cases was part of the background rather than foreground. Instead of tuning the colour band to the orange-red stain of FAC, we tuned the colour threshold to the purple band to detect the nucleus. A minimum size threshold of 3000 pixels corresponding to 16 μm^2 was used to classify the masks as nucleus. Similar approach with a higher size threshold of 10000 pixels was used to detect the cytoskeleton. Data analysis and statistical tests were performed with Microcal Origin.

3 Results and Observations

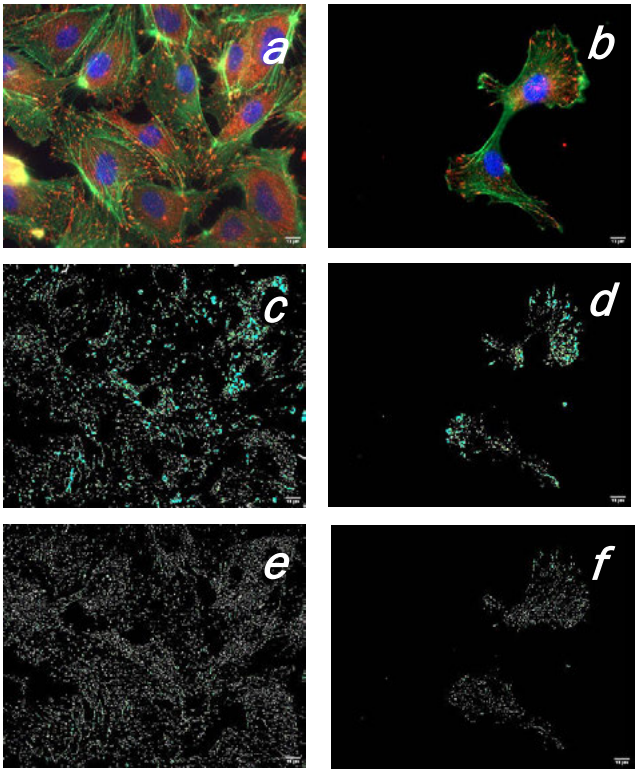

Figure 3: a, b) Original Images c, d) Overlay of detected FAC using HSV method e, f) Overlay of detected FAC using RGB method. Scale bar corresponds to 10μm

Figure 3 compares the results of the detection of FAC using [1] and our method. Fig 3.a shows smaller FAC particles in larger numbers, due to over-segmentation by the various sharpening operators. Our method is less prone to over-segmentation and is more sensitive to the reddish-orange staining instead of only the red channel.

Figure 4 shows the location-selective segmentation achieved using a combination of methods described in 2.1 and 2.2 to yield a separation of FAC into those near the nucleus and those in the periphery. Figure 5 shows the average number and average size of FAC per cell, detected around the nucleus and the cell periphery for different coatings. The results obtained from location selective segmentation show that cells on SP10 coating have higher FAC count per cell. That result corresponds with the results for cell activity normalised to cell count obtained by Rudt et al. [5]. One way ANOVA tests were carried out for data in Fig. 5. It showed that population means for cells on SP10 and SP0 are significantly different to NC (and partly to PC) for both parameters, area and count, for both regions, with $p = 0.05$. Population means of SN10 differs from NC in FAC count in the nucleus region whereas SN0 differs from NC for FAC area in both the regions, at $p = 0.05$. However, there was no statistically significant difference among the sample coatings themselves except for the count between SN10 and SP0 around the nucleus region.

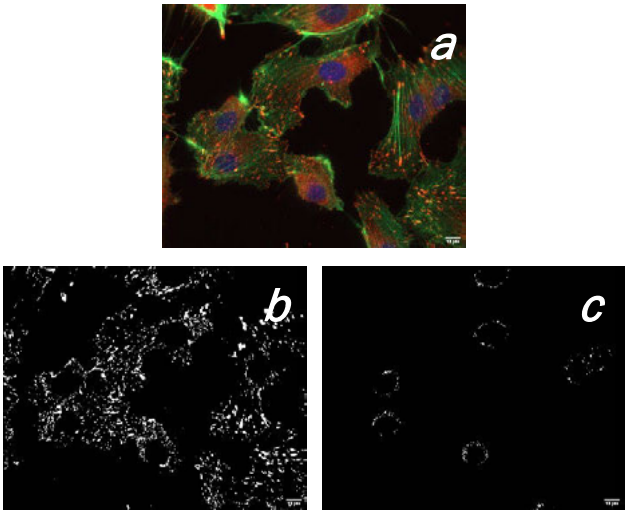

Figure 4: a) Original Image b) FAC detected near the periphery c) FAC detected near the nucleus. Scale bar corresponds to 10 μm

As can been seen from Figure 6, our method is also able to detect nucleus with a high degree of accuracy and also cell structures to a limited extent. The above-mentioned results were consistent across different types of coatings and differences in contrast and brightness of the images.

4 Summary

In this work, we first reimplemented the method described in [1]. Due to the problems of over-segmentation, we not only implemented a novel method for improving the detection of FAC, but also extended it to detect cell nucleus and cell outline. In addition, we also implemented a location selective segmentation using region growing, to distinguish between

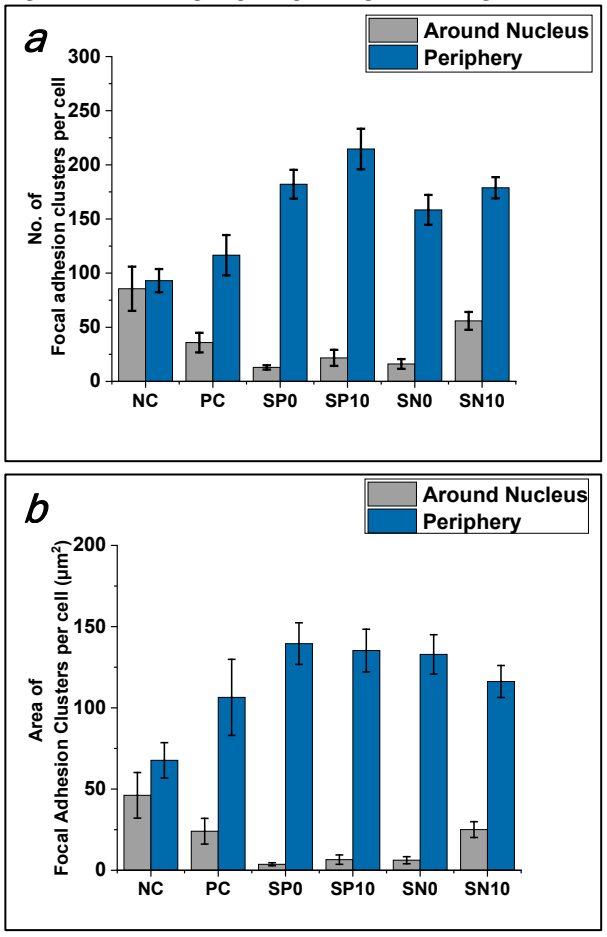

Figure 5: a) No. of FAC/cell around nucleus and on the periphery b) Area of FAC/cell (μm^2) around nucleus and on the periphery. Error indicators represent standard error of the mean of 25 samples. Numbers of cells analysed were minimum 71 to 340 per coating conditions.

FAC near the nucleus and near the periphery. The method is also robust to fluctuations in contrast and brightness. Cell nuclei which are normally part of the image background are detected with a high degree of accuracy.

As we do not have the ground truth for the acquired images, we do not have any benchmarks to compare our results with. We also intend to extend our work to detect other FAC structures of interest in other cell types.

Author Statement

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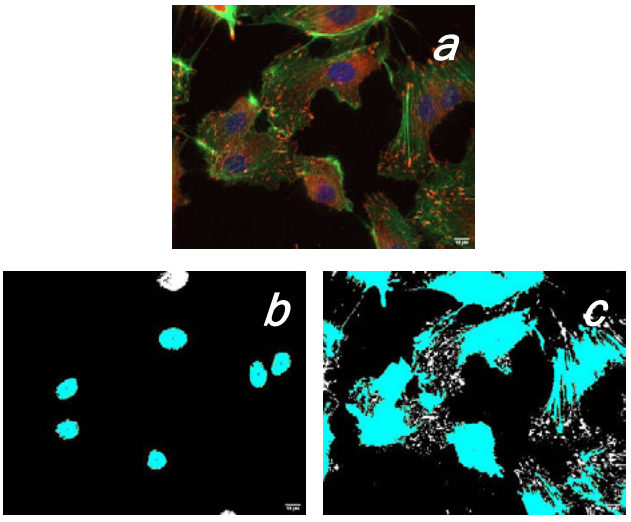

Figure 6: a) Original image b) Nucleus detection c) Cell area detection. Scale bar corresponds to 10 µm

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