

Application of quantitative morphological cytometry for evaluation of shear stress: potential for HCS systems.

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ABSTRACT

Shear stress is known to have a significant effect on the state of cellular differentiation. It also induces morphologic responses including changes to cytoskeletal organization subsequently leading to changes in cell shape. In fact, fluid shear stress caused by blood flow is a major determinant of vascular remodeling and can lead to development of atherosclerosis. The morphological changes are usually evaluated using boundary-based shape descriptors or binary geometrical moments on manually segmented cells. Although any one of the many automated segmentation methods could be employed, these techniques are known to be complex and time consuming, and often require user input to operate properly, which is especially problematic for HCS systems. Therefore, development of robust, quantitative morphological measurements that are not dependent on precision and reproducibility of segmentation is extremely important for a substantial improvement of shear-stress analysis. The goals of this study were to find simple morphological descriptors that could be applied to cells isolated by tessellation in order to enable a high-throughput screening of morphological shear-stress response, and to determine the amount of fluid shear stress to which endothelial cells were exposed on the basis of changes in their morphology. The proposed technique is based on the monitoring of changes in cytoskeleton organization using texture descriptors, rather than on quantifying cell-boundary modifications. We showed that objects identified by Voronoi tessellation carried enough information about cytoskeleton texture of individual cells to create a robust classifier. Our approach provided higher discriminant and predictive powers, and better classification capability, than traditional boundary-based methods. The robustness of classification in the presence of segmentation difficulties makes the proposed approach particularly suitable for automated HCS systems.

Keywords: image cytometry, microscopy, high-content screening, image analysis, automated classification.

1. INTRODUCTION

The change of shape by endothelial cells coinciding with differentiation is a well-known response to shear stress and has been described in many reports¹⁻⁵ (Figure 1), but the provided quantitative evaluation has been incomplete and difficult to reproduce. The described changes in morphology include polarization of the cells and cell elongation in the direction of the shear vector. The quantitative analysis of morphological changes triggered by shear stress has been mostly limited to calculation of a form factor (defined as $4\pi \cdot \text{area} / \text{perimeter}^2$). Although the form factor, which was introduced to shear stress studies by Nerem and his colleagues in '80s⁵⁻⁸, is intuitively understandable and seemingly easy to calculate, in practice it is hard to determine and ambiguous owing to its boundary dependence. Therefore, development of a robust yet simple method for fast evaluation of morphological changes in cells exposed to shear stress, in a way that is not dependent on precisely segmented boundaries can be an important contribution to automated shear-stress analysis.

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2. MATERIALS AND METHODS

2.1. Cell cultures

Bovine aortic endothelial cells (BAECs) (Vec Technologies, Rensselaer, NY) were grown in commercially available flasks (Falcon, Becton Dickinson) until they reached ~80% confluence. Subsequently the cells were transferred to Y-shaped flow chambers (μ -Slide, Integrated BioDiagnostics GmbH, München, Germany) which were pretreated with media 24 hours before seeding the cells. BAECs were then seeded by adding 200 μ l of suspended cells at a concentration of 5×10^5 cells/ml. The cells were kept in the flow chamber 24-48 hours prior to the experiments. Subsequently, cells were subjected to different levels of shear stress using a peristaltic pump connected to the chamber slides:

- Group 1 was subjected to laminar shear stress of 2 dynes/cm²
- Group 2 was subjected to laminar shear stress of 15 dynes/cm²
- Group 3 was subjected to laminar shear stress of 30 dynes/cm²

After exposure to the fluid shear stress for 24 hours the cells were stained with Alexa-488-phalloidin and 4.5 μ l Hoechst 33342 (Molecular Probes – Invitrogen Corp., Eugene, OR).

2.2. Automated imaging

Automated imaging was performed using an IC100 imaging cytometer (Beckman Coulter, Miami, FL). The nuclear staining was imaged using a 350/50-nm dichroic band-pass excitation filter, and a 457/17-nm emission filter. Alexa 488 was imaged using 484/15-nm excitation, and a 517/30-nm emission filter. Within each group, ca. 20000 cells were imaged.

2.3. Calculation of traditional shape descriptors

128 cells in each group were segmented manually from the microphotographs. The binarized images were subsequently analyzed using Image-Pro Plus, Version 5.1 (Media Cybernetics, Silver Spring, MD, USA). The following parameters were computed for every cell⁹:

- form factor = $4\pi \cdot \text{area} / \text{perimeter}^2$
- roundness = $4 \cdot \text{area} / (\pi \cdot \text{maximal diameter}^2)$
- aspect ratio = $(\text{maximal diameter}) / (\text{minimal diameter})$
- convexity = $(\text{convex perimeter}) / \text{perimeter}$
- solidity = $\text{area} / (\text{area polygon})$
- compactness = $\frac{1}{\text{maximal diameter}} \sqrt{\frac{4}{\pi} \cdot \text{area}}$
- extent = $(\text{net area}) / (\text{bounding rectangle})$

2.4. Dirichlet Tessellation (Voronoi Diagram)

A Voronoi diagram is the partitioning of a plane with n points into convex polygons such that each polygon contains exactly one generating point (seed) and every point in a given polygon is closer to its own generating point than to any other¹⁰. For our analysis, the centers of nuclei are chosen as the seed (center) points. Cytoskeleton images were tessellated using Voronoi diagrams with irregular tessellation using these seeds. An example Voronoi diagram is shown in Figure 2.

2.5. Texture analysis

A primary and common tool for texture analysis was introduced by Haralick¹¹, who used gray-level co-occurrence matrices to quantify the number of occurrences at various distances and angles of pixel intensity values with respect to each other. The so-called Haralick texture features are then used to extract 14 low- and high-frequency texture properties. In our analysis, 12 of these features were used to extract texture information from images. 12 Haralick features

multiplied by 3 different distances, represented as value and range of each feature gave 72 scalars which were used for the subsequent analysis. The tessellated cytoskeleton images were placed on a uniform, black background of 384-by-384 pixels, so background pixels did not contribute to feature values, which would otherwise make them significantly biased.

3. RESULTS AND DISCUSSION

Overall changes in cellular morphology and organization of cells in the population as a result of shear-stress exposure were readily visible by eye, as shown in representative microscopy images in Figure 1. Linear discriminant analysis (LDA) was employed to find a linear classifier which would allow identification of the shear-stress level on the basis of cell-boundary deformation quantified by the classical descriptors. However, the LDA was unable to distinguish between cells sampled from 2 and 15 dynes/cm² populations (Figure 3A). Test performed by the 0.632-bootstrap algorithm using 25 replications showed an average classification error on the order of 45%.

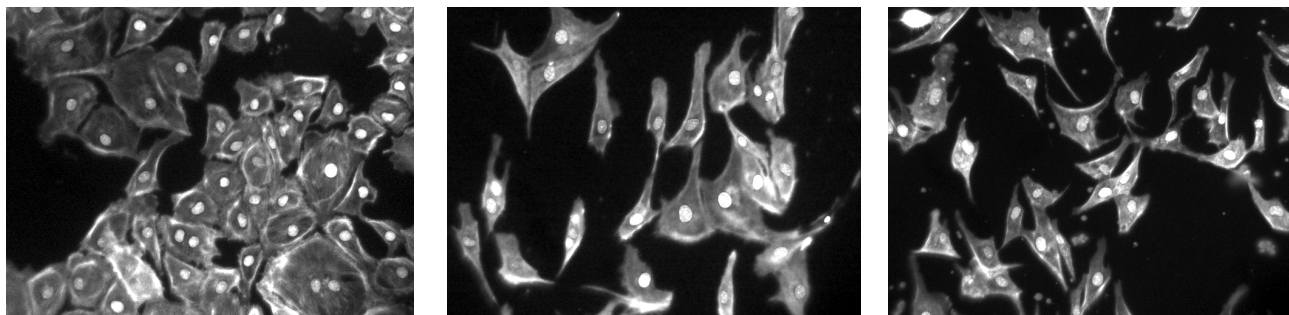


Figure 1. Representative images of bovine endothelial cells exposed to 2, 15, and 30 dynes/cm² of liquid shear stress.

An identical analysis has been performed for the data containing Haralick descriptors calculated with manually segmented cells. An application of LDA produced three distinct clusters representing three groups of cells (Figure 3B). Verification of this classifier by 0.632-bootstrap algorithms demonstrated much higher predictive power than the one found for boundary-based descriptors: the average error rate was estimated to be on the order of 17%. The texture-based classifier was also able to distinguish between cells cultured in low- (2 dynes/cm²), and medium-level (15 dynes/cm²) fluid-stress environments.

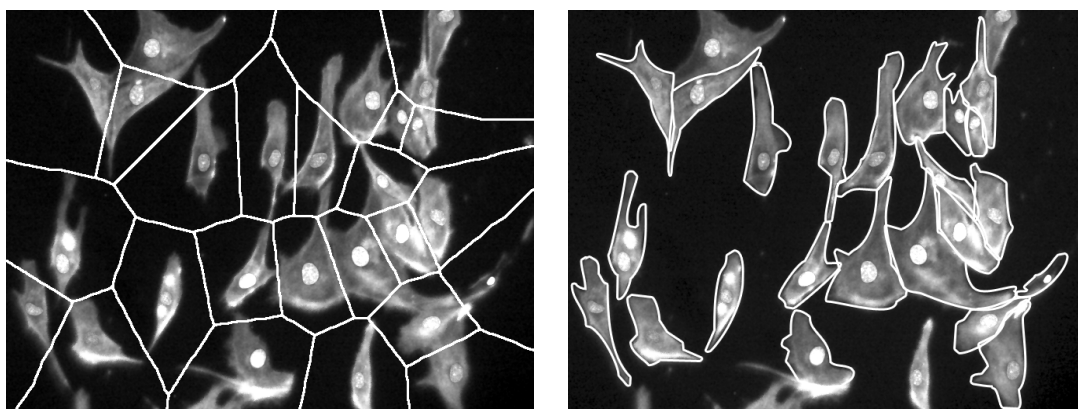
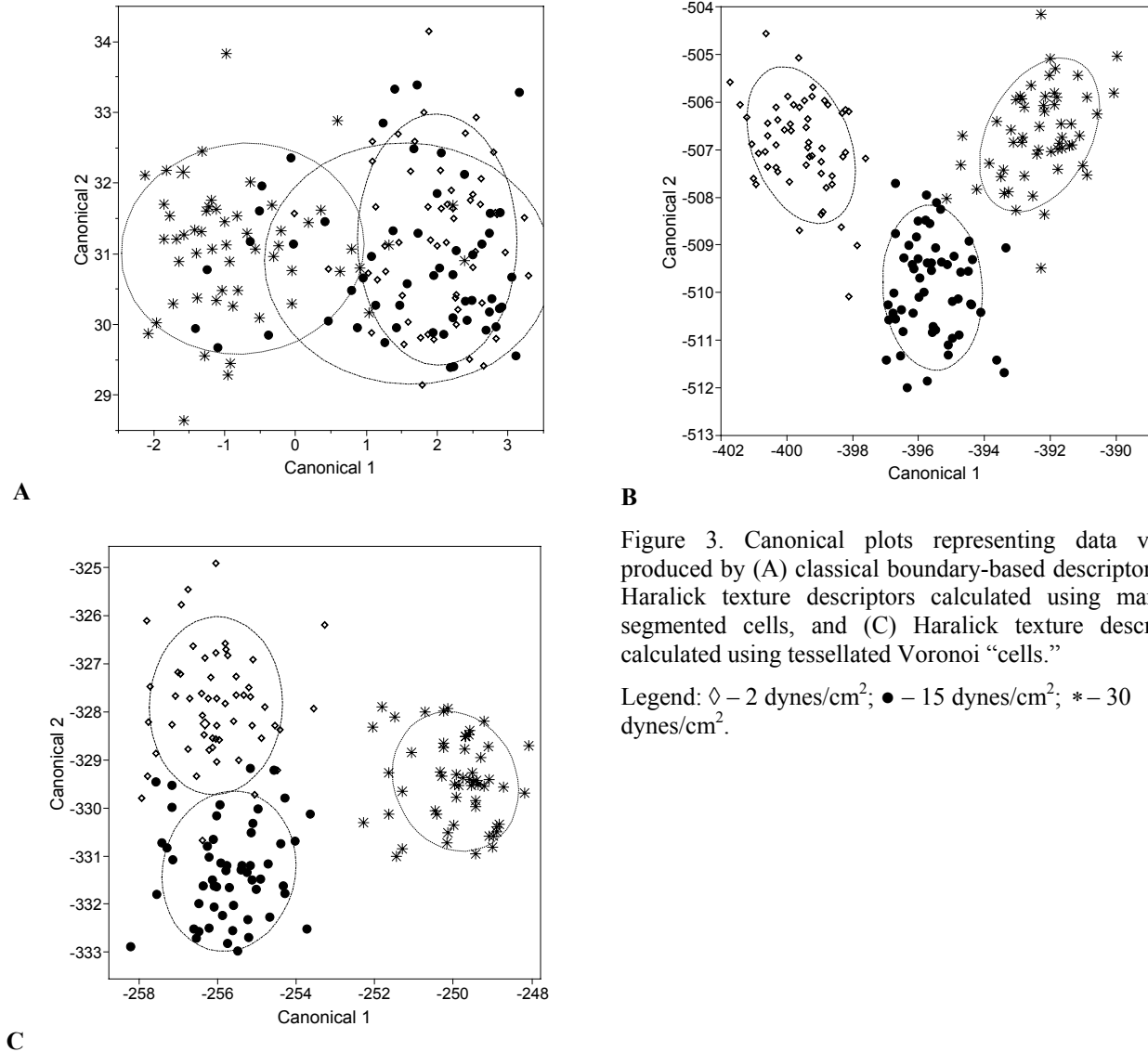


Figure 2. Images of tessellated microphotographs (left) and manually segmented endothelial cells (right). The center of nuclei were chosen as the seed points. This was made possible owing to different staining of nuclei. Cytoskeleton images (in a separate color channel) were tessellated by Voronoi diagrams using these seeds.

In the third step, a linear classifier was built on the basis of the texture measurements obtained from the Voronoi “cells” (polygons) produced by the automated tessellation process. The error of 26% was similar to that observed during the classification of the data vectors originating from the manually segmented cells. The classification system, unlike the boundary-based method, has still been able to identify the difference between low- and medium- stress environments (Figure 3C).

Application of LDA showed that both the boundary-based shape descriptors and the texture descriptors were capable of identifying cells exposed to the highest shear-stress level (30 dynes/cm²). However, quantification based on cell contours failed to identify differences between the moderate shear-stress group and the low-stress group. In contrast, quantification based on the cytoskeletal texture correctly identifies the majority of cells belonging to all three groups. The classifiers were obviously unable to successfully recognize the fluid-stress environment of every cell. This was expected since information about the specific microenvironments that every cell experienced was not accessible. Therefore bulk pressure values have been used for training and validation of the classifiers.



B

Figure 3. Canonical plots representing data vectors produced by (A) classical boundary-based descriptors, (B) Haralick texture descriptors calculated using manually segmented cells, and (C) Haralick texture descriptors calculated using tessellated Voronoi "cells."

Legend: \diamond – 2 dynes/cm²; \bullet – 15 dynes/cm²; $*$ – 30 dynes/cm².

C

The texture-based classification has been demonstrated to have two important advantages over analysis of cell contours for quantification of shear stress: texture descriptors are more sensitive to the lower amount of shear, and they are not dependent on correct identification of the cell boundaries.

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