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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 vivo*, image informatics based point-of-care expert system and quantification of various complex architectures in organisms.

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, Hessler, Bhandary, & Martin, 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 (Matuszewski Jean, van Delft, van der Kammen, & Collard, 1999). Novel imaging techniques (*e.g.* fluorescent microscopy, con-

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focal 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, Anisowicz, Hendrix, Thor, & Neveu, 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, Contreras, Latha, Rosen, & Zhang, 2006), and more recently protein interactions of maspin (Endsley et al., 2011). Many studies suggest that maspin is present at the cell surface (Bass, Wagstaff, Ravenhill, & Ellis, 2009; Cella et al., 2006; Endsley et al., 2011; Khalkhali-Ellis & Hendrix, 2007; Law et al., 2005; Ngamkitidechakul, Burke, O' Brien, & Twining, 2001; 2003; Pemberton et al., 1997; Ravenhill, Wagstaff, Edwards, Ellis, & Bass, 2010; Seftor et al., 1998; Sheng et al., 1996). But, exogenous activities of maspin are still debatable (Teoh, Whisstock, & Bird, 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, Lan, Zhang, Li, & Huang, 2015), combined method of gray-level-histogram (GLHs), a gray-level cooccurrence matrix (GLCM) (Lu, Huang, Wang, & Shang, 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, Sintorn, Erlandsson, Borgefors, & Bengtsson, 2004), watershed (Mukherjee, Ray, & Acton, 2004), mean shift method (Debeir, Van Ham, Kiss, & Decaestecker, 2005), level set algorithms (Nath, Palaniappan, & Bunyak, 2006), active contour models (Kayser et al., 2009), sliding band filter (Quelhas, Marcuzzo, Mendonça, & Campilho, 2010), and iterative thresholding (Cai, Yang, Cao, Xia, & Xu, 2014). To overcome the limitation of over-segmentation of the watershed method, marker-controlled watershed techniques have been developed to improve the performance (Arco, Górriz, Ramírez, Álvarez, & Puntonet, 2015; Fenistein, Lenseigne, Christophe, Brodin, & Genovesio, 2008; Plissiti, Nikou, & Charchanti, 2011; Koyuncu, Arslan, Durmaz, Cetin-Atalay, & Gunduz-Demir, 2012; Yang, Li, & Zhou, 2006). Before applying a segmentation method, morphological analysis is helpful to extract useful information from the cellular images (Amini, Veilleux, & Villemure, 2010; Plissiti et al., 2011). A two-step binarization method is proposed to split the clumped nuclei (LaTorre, Alonso-Nanclares, Muelas, Peña, & DeFelipe, 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, Wählby, Bengtsson, & Zaltsman, 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.