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A linear programming approach to reconstructing subcellular structures from confocal images for automated generation of representative 3D cellular models

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Abstract

This paper presents a novel computer vision algorithm to analyze 3D stacks of confocal images of fluorescently stained single cells. The goal of the algorithm is to create representative *in silico* model structures that can be imported into finite element analysis software for mechanical characterization. Segmentation of cell and nucleus boundaries is accomplished via standard thresholding methods. Using novel linear programming methods, a representative actin stress fiber network is generated by computing a linear superposition of fibers having minimum discrepancy compared with an experimental 3D confocal image. Qualitative validation is performed through analysis of seven 3D confocal image stacks of adherent vascular smooth muscle cells (VSMCs) grown in 2D culture. The presented method is able to automatically generate 3D geometries of the cell's boundary, nucleus, and representative F-actin network based on standard cell microscopy data. These geometries can be used for direct importation and implementation in structural finite element models for analysis of the mechanics of a single cell to potentially speed discoveries in the fields of regenerative medicine, mechanobiology, and drug discovery.

Keywords

Automatic cell segmentation; Representative F-actin network; Confocal microscopy; Finite element model geometry

1. Introduction

While cell mechanics has been recognized as an important area of study, current computational models to interpret experimental results tend to ignore individual cellular geometries. In particular, 3D computational models could help to improve the design of experiments to characterize cell mechanical properties and interactions. This could lead to reduced times for discovery of mechano-biology principles and to faster translation of those principles from benchtop to bedside in clinically relevant devices and medications. The goal of this study is to create a fully automated algorithm capable of reconstructing the geometries of the cell membrane, nucleus, and actin stress fiber network of single cells in

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.media.2012.12.002>.

3D. We seek to accomplish this by processing fluorescent confocal microscopy images of each of those cellular components in such a way that the resulting geometries are optimized for structural analysis using finite element methods. If generated, such geometries could be utilized in various types of multiscale models to bridge the gap between the nano- and macro-scale models currently in use.

The traditional primary focus of modern medical research is the investigation of molecular biology and genetic factors in disease, which sometimes leads to a tendency to ignore changes in tissue structure and mechanics that can also lead to pain and morbidity (Ingber, 2003a). However, that lack of focus on the physical basis of disease has been changing in recent years with the growing emphasis on evidence-based medicine in US hospitals (Fielding et al., 2011; Kaufman, 2010) together with the substantial growth and maturation of the field of mechanobiology over the past decade (Butler and Wang, 2011). Indeed, there has been a great deal of effort to develop geometrically accurate 3D structural models at both the tissue and molecular levels (Biswas et al., 2009; Wu et al., 2010). However, there has been much less effort focused at the single-cell level and therefore comparatively little progress has been made toward generation of equally accurate 3D representations of the structural components of single cells.

The ability to predict the behavior of cells from their sub-micron and nanoscale structures could elucidate the mechanisms behind many tissue mechanical properties (Ingber, 2003b). For as long as there have been observations of the mechanical properties of cells, there have been models put forth to attempt to describe those observations. At the most basic level, there are two categories of these models: continuum and structure-based. Continuum models, which lack internal structure, were the first type of model utilized to describe the mechanical behavior of cells and generally consider the cell to be equivalent to a simple “balloon full of molasses” (Ingber, 2003b; Li et al., 2007). These types of models therefore make predictions with minimal use of geometric variables (Cao and Chandra, 2010; Unnikrishnan et al., 2007). Despite the growing amount of evidence in support of the importance of structural elements within cells that has been published throughout the past several decades (Bathe et al., 2008; Bursac et al., 2005; Chaudhuri et al., 2007; Deng et al., 2006; Deshpande et al., 2008; Hardin and Walston, 2004; Hawkins et al., xxxx; Hemmer et al., 2009; Ingber, 2003a,b,c; Kasza et al., 2007; Li, 2008; Mizuno et al., 2007; Pollard, 2003; Pullarkat et al., 2007; Stamenovi , 2005, 2008; Stamenovi et al., 2009; Suresh, 2007; Tseng et al., 2005), these types of models remained popular with bioengineers due to their relative simplicity and ease of implementation.

Structure-based models, on the other hand, are comprised of one or more networks of discrete structural elements that work in harmony to determine the mechanical responses of cells. These models tend to utilize Finite Element Analysis (FEA) to allow for analysis of complicated cellular and subcellular geometries. Many single-cell Finite Element Models (FEMs) rely on idealized geometries (Karcher et al., 2003; Peeters et al., 2005; Unnikrishnan et al., 2007), however recent efforts have incorporated geometries obtained from image segmentation. The first efforts to generate accurate 3D representations of subcellular structural components using image segmentation techniques focused primarily on nuclei (Funnell and Maysinger, 2006; Gladilin et al., 2008), and the most advanced structure-based cellular mechanics models to date utilize stacks of confocal photomicrographs of a cell to generate 3D model structures. There have been a small number of these types of models proposed in the last several years (Dailey et al., 2009; Slomka and Gefen, 2010), each of which have been important advances towards the development of a fully representative 3D model of single cell mechanics. However, none of those models has been constructed with entirely non-idealized geometries for all mechanically relevant components of a cell.

Few 3D single cell models have included any form of cytoskeletal elements inside the cells (Slomka and Gefen, 2010); yet even though these models represent a significant step towards reality, they still rely on the manual addition of a limited number of cytoskeletal components. There has not yet been a system put forth in the literature that is either fully automated or capable of reconstructing any elements of the cytoskeletal networks of cells in a representative manner. The goal of this study is to present such a fully automated cellular geometric reconstruction system based on 3D confocal microscopy images of single subconfluent cells.

2. Methods

2.1. Data acquisition: Cell culture, staining, and imaging

Primary rat aortic vascular smooth muscle cells (VSMCs) obtained from female Sprague Dawley rats are used in this study. The cells are cultured in high glucose Dulbecco's Modified Eagle's Medium (DMEM) (HyClone Laboratories, Logan, UT, USA) with an antibiotic solution of penicillin and streptomycin (HyClone Laboratories) added to a concentration 0.5%, and an antimycotic solution of amphotericin B (HyClone Laboratories) added to a concentration 0.5%. Cells are cultured in T75 cell culture polystyrene flasks and maintained in an incubator at 37 °C and 5% CO₂ with fresh media being exchanged every other day. VSMCs are utilized between passages five and eight. Once the cells reach about 90% confluency, they are trypsinized with a solution of 0.25% trypsin and 0.02% ethyldiaminetetraacetic acid (EDTA) in 1X HBSS without sodium bicarbonate, calcium, or magnesium (Mediatech, Manassas, VA, USA) and seeded at 7000 cells/cm² on 25 mm diameter glass coverslips (VWR, Radnor, PA, USA) coated with 50 µg/mL type I rat tail collagen (BD Biosciences, Bedford, MA, USA) 24 h prior to seeding. The cells are then cultured for 3–5 days to reach about 25% confluency.

Upon reaching 25% confluency, cells are fixed with 4% paraformaldehyde (Sigma–Aldrich, St. Louis, MO, USA) at 37 °C for 10 min. After fixation, cells are treated with 130 nM AlexaFluor 488 phalloidin (Invitrogen, Eugene, OR, USA) at room temperature for 15 min to visualize filamentous actin (F-actin), rinsed three times with phosphate buffered saline (PBS) (MP Biomedicals, Solon, OH, USA), and then mounted onto glass slides using SlowFade® Gold antifade reagent with DAPI (Invitrogen) to visualize the nucleus. The cells are then imaged using an Olympus PLAPON60XO 60× oil objective (NA = 1.42) on an Olympus IX81 inverted microscope equipped with a DSU spinning disc confocal unit and a Hamamatsu Imagem CCD camera (Hamamatsu Photonics K.K., Hamamatsu City, Japan). Image stacks are taken using a Nyquist step size of 200 nm between image planes for maximum resolution in the z-direction as calculated by the microscope controller software (MetaMorph® for Olympus Basic, Version 7.7.1.0, Molecular Devices, Sunnyvale, CA, USA).

It should be noted that several types of microscopy were originally considered for this study. Atomic Force Microscopy (AFM) is capable of atomic-level resolution, but was eliminated from consideration due to its topographical nature and therefore inherent inability to image intracellular structures more than a few nanometers below the apical surface of a cell. Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM) also provide more-than-sufficient levels of resolution for imaging subcellular structures; however, each was ultimately eliminated from consideration due to limitations of the imaging environment and sample preparation. Electron microscopy usually requires samples to be imaged in an arid (i.e. non-aqueous) vacuum chamber and bombarded by an incident electron beam. Because electrons must pass through the specimen, TEM requires a very thin (40–90 nm thick) section which is difficult to accomplish with biological materials using traditional ultramicrotomy methods of sample preparation. In order to process the samples to

make them electrically conductive for SEM, it is often necessary to coat them in harsh chemicals such as heavy metal salts and silver or osmium. While it is possible to image biological samples using electron microscopy techniques, the sample preparation and imaging environment (in particular the non-aqueous nature) are capable of producing artifacts (i.e. altering their structure) (Echlin, 2009) that could cause 3D reconstruction of those images to be inaccurate using the image processing techniques utilized in this study. Confocal microscopy does not provide the same level of resolution as any of the aforementioned techniques; however, with a maximum lateral resolution of approximately 180 nm and maximum axial resolution of roughly 500 nm (Spring et al., 2004; David, 2007) it is still sufficiently capable of imaging the structurally relevant subcellular components at the whole-cell level. Ultimately, confocal microscopy was chosen for this study due to its ability to image cells in their native aqueous environment, its non-destructive nature, its relative low-cost compared to electron microscopy, and the fact that it is generally considered to be the standard modality for cytoskeletal imaging. An additional benefit of this imaging technique is that it may be utilized to image live cells. This allows for imaging a cell for which mechanical characterization is also obtained, thus enabling direct validation of eventual models.

2.2. Image pre-processing

All images are saved and analyzed as 8-bit grayscale images in TIF format, at a size of 256×256 pixels. For each image, the F-actin data and the data for the nucleus of the cell are stored in separate image stacks. Each image stack is then deconvolved using MetaMorph® for Olympus Premier Offline (Version 7.7.0.0) using a 3D deconvolution algorithm based on measured point spread functions using a single iteration. For all further image processing, pixel intensities for each image stack are loaded into MATLAB (Release 2010b, MathWorks®, Natick, MA, USA) as a 3D matrix, creating a voxel map of each image channel. The matrix is then scaled in each dimension to match the dimensions of the sampled volume in cubic micrometers, so that each voxel is $1 \mu\text{m} \times 1 \mu\text{m} \times 0.2 \mu\text{m}$. Our image to model conversion process and algorithm is detailed below; a flow chart summarizing the entire process is provided in Fig. 11.

2.3. Construction of nucleus and cell boundary meshes

Segmentation of a single cell nucleus and cell body on a black background are relatively straightforward problems from a computer vision perspective, and there are many approaches one can conceivably plug in at this stage of our pipeline to build satisfactory boundary meshes for the nucleus and cell body (Lin et al., 2003, 2005; Russell et al., 2009). For simplicity, we employed a straightforward thresholding approach, since we found that this delivered amply sufficient results for the segmentation of the images used in this study, and since the generation of representative actin fibers later in the pipeline is our primary focus. Of course, more sophisticated techniques from the literature could be used to make the segmentation more robust (e.g. to handle multiple cells per image) if necessary.

For segmenting the nucleus, we normalize the nucleus voxel map so voxel intensities belong to the range $[0,1]$, and we select all voxels having intensity at least 0.25 (empirically chosen, Fig. 1b). The resulting 3D binary matrix is then dilated (Fig. 1c) to remove boundary gaps, and interior gaps in the matrix are filled (Fig. 1d). All objects lying along the xy border of the voxel map are then removed and the matrix is smoothed with a diamond shaped erosion element (Fig. 1e). Finally, we identify all connected components and retain the one containing the most voxels (Fig. 1f).

Segmentation of the cell boundary from the data is conducted in almost exactly the same manner, except we begin with a voxel map that is the actin voxel map smoothed with a 3D

Gaussian filter, giving a voxel map that is relatively intense across the entire cell. After completing the steps above to produce a 3D binary matrix representing the cell body (using a segmentation threshold of 0.05, empirically chosen), we turn on any additional voxels that are selected in the 3D nucleus matrix above, in order to ensure that the entire volume of the nucleus lies within the volume of the cell body. Finally, due to the nature of the 2D cell culture techniques employed in this study, it is assumed that there is no empty space underneath any part of the cell that is not part of the cell. Therefore, we fill the image matrix downward by turning on any voxel that has a voxel turned on above it (in the +z direction).

Our automated segmentation technique was compared to manual segmentation by 10 individuals (Fig. 2). For the manual segmentation, each individual was asked to trace the cell and nuclear boundary on a single 2D image plane using a table monitor. The superimposed results of these manual segmentations matched the 2D cross-section of our automated 3D segmentation well (Fig. 2). In addition to comparison with manual segmentation, we used a cell outliner plugin (<http://rsbweb.nih.gov/ij/plugins/cell-outliner.html>) for the popular ImageJ image analysis package. The automated tools in this plugin were unable to consistently segment the cell into a single connected region. The plugin's semiautomated tools with significant manual input were necessary to generate segmentation of reasonable quality. For the cells shown in Fig. 2, the semi-automated tools produced outlines that were $1.0 \pm 0.3 \mu\text{m}$ from the average manual segmentation result. Our automated approach resulted in outlines that were $2.5 \pm 0.5 \mu\text{m}$ from the average manual segmentation result. The VSMCs used in these experiments are 50–80 μm long and 20–30 μm wide and their nuclei have a diameter of about 15 μm . The results for each individual cell and nucleus in Fig. 2 are summarized in Table 1. It should be noted that the MATLAB Image Processing Toolbox Cell Segmentation Demo provides automated cell segmentation outlines in 2D. Our approach is essentially a 3D generalization of this approach. Therefore, cell segmentation outlines obtained by our method will match these exactly.

In order to create isosurfaces of the cell boundary and nucleus, each image matrix is down-sampled using an empirically chosen percentage of matrix size and then smoothed using a 3D Gaussian filter. Down-sampling is necessary at this stage as an additional method of smoothing in order to reduce the size of elements in the mesh to a range that allows for accurate geometric representation of the data without unnecessarily increasing computational intensity or generating elements of poor quality to make the mesh fit unnecessarily minute geometric details. Three-dimensional isosurfaces composed of three-node triangular faces are then generated for the cell boundary and nucleus. On average, the time required for generation of the cell boundary and nucleus isosurfaces is approximately 1 min on an Intel® Xeon® 5160 dual core CPU at 3.00 GHz with 4.00 GB RAM.

2.4. Generation of representative actin fibers: a linear programming approach

In this section, we describe our novel algorithmic framework for generating a representative network of actin fibers. The strategy presented is not to reconstruct the exact fibers in the original image, but rather to generate a statistically *representative* reconstruction of entire fiber network. This is due to the limited resolution of fluorescence confocal microscopy that prevents high resolution visualization of individual F-actin fibers together with the inherent complexity of the actin stress fiber network. Most previous work focuses on *exact* reconstruction; however a representative reconstruction is appropriate here primarily for two reasons. First is the need to limit computational complexity when using the resulting geometries in finite element software. Second is that a representative geometry is likely sufficient to produce an accurate mechanical finite element model. Moreover, the generation of an exact reconstruction of the entire actin network based on any imaging modality is much less computationally tractable due to the inherent complexity of the network itself.

We begin by generating a large set of candidate fibers, from which we will then select a smaller subset as our representative network. A fiber belongs to our large candidate set if (1) it is a straight line segment, (2) it lies entirely within the cytosolic space of the cell, which we define as inside the cell boundary mesh but outside the nucleus boundary mesh, and (3), if its endpoints are nodes in the cell boundary mesh. Since the number of fibers satisfying these conditions can still easily number in the millions, we randomly sample at most 10,000 such fibers to form our candidate set. We denote these fibers $f_1, \dots, f_n$. If the nucleus and cell boundary meshes are stored in appropriate spatial data structures (e.g. binary space partition trees), then these n candidate fibers can be generated quite efficiently.

Let us now think of forming a *synthetic* 3D image by taking a linear superposition $x_1 f_1 + \dots + x_n f_n$ of our candidate fibers. At a high level, our algorithm can be considered a type of regression method that finds a set of coefficients $x_1, \dots, x_n$ (each a real number in the range 0–1) resulting in a synthetic image that most closely matches the actual *measured* 3D confocal actin image. The coefficient x_i for each fiber f_i represents the extent to which fiber f_i is present in the solution. This can be regarded as a “fuzzy” measurement of fiber presence or equivalently as the probability of f_i being present. In some sense, we would like to find the best projection of our measured confocal image into the basis described by our n candidate fibers.

We compute $x_1, \dots, x_n$ by solving a large linear program as described here. After solving for $x_1, \dots, x_n$ we must then decide which fibers to actually include in our actin network. The ideal method for this task is to flip a biased coin for each fiber f_i including f_i in the final network with probability x_i . This approach gives a synthetic image that matches, in expectation, the distribution output by the linear program. In several applications, however, we may wish to limit the number of fibers present in the final network; for example, if the network is to be used in a finite element model, then it may prove computationally intractable to include too many fibers. In this case, two different approaches can be used: we can either select all fibers f_i for which x_i is at least some specified threshold T , or we can regard $x_1, \dots, x_n$ as a probability distribution and randomly sample some specified number K of fibers, where fiber f_i is sampled with probability $x_i / \sum x_i$. The difficulty with the former approach is picking an appropriate threshold T , and with the latter approach the difficulty lies in choosing an appropriate value for K . We set the threshold in order to limit the generated actin network to only the most representative fibers and limit the computational complexity of the finite element models ultimately generated from our geometry. The threshold value we used typically resulted in the acceptance of approximately 135–450 fibers per cell.

Our methods seek to minimize the discrepancy between the synthetic 3D image obtained by the linear combination $x_1 f_1 + \dots + x_n f_n$ of candidate fibers, and the actual measured 3D confocal actin image. To give a mathematical characterization of this discrepancy, we discretize the interior of the cell into a 3D volumetric grid of regularly-spaced voxels (currently separated by five pixels from their neighbors). We will compute the local discrepancy between the synthetic and measured images at each of these voxels and sum the results to obtain our global objective. Since we want to match not only the pixel intensity at each voxel of the measured image but also the “directionality” of the fiber texture at this voxel, we further subdivide each voxel into 8 *directional voxels* (dvoxels), each representing a “bow tie” shaped angular range of 45° of directionality in the xy plane of the voxel (Fig. 3). Although in principal we could subdivide each voxel into dvoxels that each represent 3D angular regions, we believe this is probably not worth the substantial extra computational overhead it would create, since very little information about texture in the z direction can be

computed due to the limited z resolution of the confocal microscopy relative to the thickness of the cells utilized in this study. We denote the set of all dvoxels $d_1, \dots, d_m$.

For each dvoxel d_j , let a_j denote its intensity in the measured confocal image; that is, a_j reflects the amount of 2D textural directionality in the angular range of the xy plane and 3D location corresponding to the dvoxel d_j . We compute a_j as follows: we first isolate the z image plane of the dvoxel and apply a 2D Gaussian filter of size 33×33 centered at its (x, y) location, thereby extracting a 33×33 image of the local 2D neighborhood surrounding d_j (Fig. 7a and b). We then perform a 2D FFT on this image (Fig. 7c), and sum the magnitudes of all the points in the FFT image corresponding to d_j 's angular range (Fig. 7d). Note that the bright points in a 2D FFT image indicate angles that are perpendicular to texture directionality, so each dvoxel represents textures internally at a 90° rotation to their original orientation. Since the middle point of the 2D FFT image is shared between all 8 angular ranges, it contributes $1/8$ to each of them. Fig. 7e–m shows the angular contribution of all dvoxels co-located at a single voxel in the measured actin image.

If desired, Gabor filters could also be used instead of a 2D FFT to measure texture directionality at each relevant angle within a voxel. However, although Gabor filters are more efficient from a computational standpoint, they can only measure texture in a specific direction at a specific frequency, and in our application, although we know the direction of interest, we do not know which frequency to look for a priori. The 2D FFT, on the other hand, measures texture content in multiple directions and frequencies simultaneously, so it is our tool of choice. Note that the dvoxels are regularly spaced in 3D although each one only reflects directionality in a local 2D horizontal plane around itself. Ideally, a 3D FFT would be used to fully characterize the 3D texture for each voxel. However, due to the limited z -resolution of confocal microscopy and the flat character of adherent cells, there was not sufficient textural information in the z -direction to warrant the extra computational cost of the 3D FFT.

We have now computed the intensity a_j of our 3D measured actin image at every dvoxel d_j . Next, we compute the intensity s_j in our synthetic image at this dvoxel. To do this, let A_{ij} denote the intensity of just candidate fiber f_i at dvoxel d_j . That is, A_{ij} is small if fiber f_i either lies far from d_j or does not run in a direction compatible to the angular range represented by d_j . We compute A_{ij} just as we computed a_j above, only starting with a 3D image consisting of a black background on which only fiber f_i is drawn, modeled as a cylinder of diameter 1 pixel, with the intensity of each voxel along its path set to the volume of the cylinder passing through the cubical volume represented by the voxel. Since we measure A_{ij} and a_j the same way, these two values are directly comparable. Fig. 8 shows an example of the computation of A_{ij} for a specific fiber f_i and at all the dvoxels d_j corresponding to a common voxel. For a synthetic image formed from a linear combination $x_1 f_1 + \dots + x_n f_n$ of fibers, the total

contribution at dvoxel d_j across all fibers is given by $s_j = \sum_i A_{ij} x_i$. While each dvoxel only accounts for directionality in local 2D horizontal plane, fibers are 3D objects that can cross through multiple dvoxels at different z -heights. Therefore, 3D fibers that match the local 2D texture along their 3D extent will be favored for inclusion.

As mentioned earlier, we regard the problem of computing a representative actin fiber network as a type of inverse problem, where we seek to find a linear superposition of

candidate fibers $\sum_i x_i f_i$ (with $0 \leq x_i \leq 1$ for each $i = 1, \dots, n$) that minimizes the global discrepancy with our measured confocal image. The local error between synthetic and

measured images at dvoxel d_j is $e_j = |a_j - s_j|$. Our goal is to minimize the total error $\sum_j e_j$, yielding the optimization problem:

$$\begin{aligned}
&\text{Minimize: } \sum_j e_j \\
&\text{Subject to: } e_j \geq a_j - s_j \quad \text{for all } j=1, \dots, m \\
&\quad e_j \geq s_j - a_j \quad \text{for all } j=1, \dots, m \\
&\quad s_j = \sum_i A_{ij} x_i \quad \text{for all } j=1, \dots, m \\
&\quad 0 \leq x_i \leq 1 \quad \text{for all } i=1, \dots, m.
\end{aligned}$$

As the objective and all constraints above are linear, this is a linear program, which can be solved relatively efficiently in practice, even for large instances. Note that the definition $e_j = |a_j - s_j|$ has been split into a pair of linear constraints – a standard trick in formulating linear programs. Another natural objective might be to minimize the quadratic error function

$\sum_j e_j^2$, but this is much more computationally prohibitive given the extremely large instances we are dealing with.

One further advantage of the linear programming framework above is that we can place length constraints on the fibers in our solution. Ideally, we would like the actin network to consist of relatively long fibers, say of average length at least some threshold L (we did not use this in our current study because the fiber lengths in our results seemed reasonable), letting L_i denote the length of fiber f_i . We can write this constraint as

$$\left(\sum_i x_i L_i\right) / \left(\sum_i x_i\right) \geq L, \text{ which can be re-written as the linear constraint } \sum_i (L_i - L) x_i \geq 0.$$

We anticipate that this general linear programming approach for decomposing a complicated object into a linear combination of basis elements of large average size may have applicability in other domains beyond the present application of actin network reconstruction; for example, it could be used in signal processing to develop an interesting type of low-pass filter that decomposes a signal into sinusoidal waves that on average have low frequency.

3. Results

3.1. Cell imaging

The deconvoluted images of the cell used in the analysis are shown in Fig. 4 with separated actin and nucleus images shown on the left in each pane, and a distance between image planes of 200 nm. In addition, further examples of the segmentation technique on other cells are provided in the Supplemental material. Note that a portion the nucleus still clearly appears in the uppermost image planes whereas the actin network, though present, is much more difficult to distinguish. This is a result of “bleed-through” in the z direction due to the intense brightness of the DAPI stain coupled with the limited axial resolution of the spinning disk confocal microscope. Fig. 5 shows a 3D reconstruction of the cell generated in MetaMorph® displaying orthogonal views of the cell with the actin network shown in green and the nucleus shown in blue for use as a comparison to the image processing results.

3.2. Nucleus and cell boundary meshes

The results of nucleus and cell boundary segmentation are shown as a z stack in Fig. 4 and as a 3D mesh in Fig. 6. Note in the front and side views, that the synthetic cell is taller in the z direction than the original data shown in Fig. 5. This phenomenon is a result of an extra dilation of the actin image data in the z direction, which is performed to ensure sufficient cytosolic space above the nucleus for elements above the nucleus to be of sufficient quality for eventual use in finite element models. On average, the time required for generation of the

cell boundary and nucleus isosurfaces is approximately 1 min on an Intel® Xeon® 5160 dual core CPU at 3.00 GHz with 4.00 GB RAM.

3.3. Generation of representative actin fibers

The results of dvoxel processing are shown in Fig. 7. Fig. 7a shows the location of the 33×33 pixel sampling unit on the original image. Fig. 7b shows the result of negating the influence of neighboring sampling units using a Gaussian filter, and Fig. 7c shows the result of the 2D FFT applied to Fig. 7b. The mask of a single dvoxel is shown in Fig. 7d and e–l show the result of the application of the mask for each dvoxel to Fig. 7c with the sum of all intensity magnitudes for each dvoxel shown in brackets. The angular contributions of all dvoxels co-located at a single voxel are shown in Fig. 7m.

The final results of our image processing are shown in Fig. 9, showing the cell periphery (gray), the nucleus (blue), and a representative actin stress fiber network (green). The intensity of the color of each fiber correlates to the decision variable x_p , with brighter fibers having higher scores. Results of our image processing algorithm are shown for additional cells in Fig. 10. The generation of actin fibers takes on average 11.75 ± 7.5 h to complete on an Intel® Core® i7 CPU 860 at 2.80 GHz with 8 cores and 16 GB RAM. The maximum time required for generation of actin fibers in this study was 15.0 h.

4. Discussion

4.1. Potential applications

As mentioned above, the synthetic actin stress fiber networks generated in this study do not exactly match those of the original images they were generated from. This is intentional due to the fact that the geometries generated in this study are produced with the primary goal of utilization in finite element models for structural analysis. Therefore, it is imperative that the geometries be both an accurate representation of the original geometry yet sufficiently simple that the models can be solved in a reasonable time-frame on the average high-end consumer PC workstation. It is with this criterion in mind that we choose to generate a *representative* reconstruction of the actin stress fiber network rather than an exact replica. This strategy also lends itself toward the potential for the generation of “average” synthetic cells that may be able to represent an entire phenotype in structural finite element models.

The principles used in the fiber generation algorithm may also be applicable for the generation of representative tissue-level structures as well, especially in non-invasive imaging techniques. One such example is the potential reconstruction of representative muscle fibers from ultrasound images, where resolution limitations can make exact reconstructions difficult. Such reconstructions could be incorporated into structural or dynamic finite element analysis, where exact reconstructions are not a necessity.

4.2. Limitations

We assume all fibers are straight line segments, although our methods can in principle all be generalized to handle more complicated geometric fiber shapes at the expense of additional computational cost. We also assume that any node on the cell boundary mesh is a potential point of attachment for an actin fiber, which is somewhat artificial. Ideally, one would identify through immunocytochemical staining and further image analysis a more specific set of “integrin” sites at which fibers are likely to attach.

The linear programming method for actin fiber generation used in this study is currently somewhat slow. The computation time is dependent on the size of the cell being solved. Therefore, smaller cells will tend to take less time than larger ones to process. Increasing the

level of downsampling will speed up the computation. In addition, this algorithm could be made faster through the utilization of more efficient spatial data structures.

The final, and most significant limitation identified by the authors lies in the imaging modality. Because the resolution of confocal microscopy is approximately 500 nm in the *z*-direction (Spring et al., 2004), it is much easier to distinguish the directionality of subcellular components laterally than axially. For instance, if an actin stress fiber were oriented in exactly the *z*-direction, it would appear in the data only as a series of disjointed dots rather than a solid line and would be ignored by the 2D FFT that sets the basis for directionality within the present algorithm. Therefore, this technique is best suited for analysis and representation of adherent cells in 2D culture which tend to be much wider than they are tall. In order to modify the present algorithm to best analyze and reconstruct the components of cells in 3D culture, a 3D FFT would likely need to be utilized, which would be both more computationally expensive and require greater axial resolution in the original image data set than the current 2D FFT approach.

5. Summary and conclusions

This paper presents an automated method for generation of structural components of single cells based on 3D stacks of confocal microscope images for use in structural finite element analysis (Fig. 11). The major contribution of this study is the novel technique presented for generation of a representative actin stress fiber network.

Cell and nucleus boundaries are segmented using simple thresholding techniques. Generation of a representative actin stress fiber network is achieved by analyzing a random distribution of all geometrically feasible fibers within the segmented geometries and using a linear optimization problem to select appropriate fibers based on the directionality of the image stack at each point as measured by a 2D FFT. For qualitative validation, analysis of seven 3D confocal image stacks of adherent vascular smooth muscle cells grown in 2D culture is performed.

Recent models have been proposed that allow for near-realistic representation of single cell geometries for finite element analysis. However, the method presented here is the first fully automated technique that can both segment 3D geometries of the cell boundary and nucleus and generate a representative F-actin network. These cell geometries can then be directly imported for use in finite element analysis of single cell mechanics. Models of this type are currently uncommon in biomedical research due to several factors, but could potentially be used to speed discoveries in the fields of regenerative medicine, mechanobiology, and drug discovery. This method promises to lower a substantial hurdle toward the use of such models by providing reconstruction of cytoskeletal networks in an automated and representative manner.

Future directions of research include investigation of the use of a random sampling approach using the *Metropolis* algorithm to sample fibers using a random walk and the use of a mixture modeling approach for fiber generation based on the *Expectation Maximization* algorithm. Either of these methods, as well as the presented method, could also potentially be used for generation of representative networks of more geometrically complex cytoskeletal components such as microtubules or intermediate filaments.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AFM	Atomic Force Microscopy
DMEM	Dulbecco's Modified Eagle's Medium
F-actin	filamentous actin
FEA	Finite Element Analysis
FEM	Finite Element Model
FFT	fast Fourier transform
NA	numerical aperture
SEM	Scanning Electron Microscopy
TEM	Transmission Electron Microscopy
VSMC	vascular smooth muscle cell

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Fig. 1.

Segmentation sequence shown for segmentation of one plane of the cell boundary from actin image data: (a) original image, (b) after thresholding, (c) after dilation, (d) after filling, (e) after smoothing, (f) after retaining only the largest component.

Fig. 2.

Comparison of automated segmentation results (red line) against superposition of the results of 10 independent manual segmentations (greyscale shaded region) performed using a tablet monitor on the same image planes by tracing the outline of the cell (a–c) and corresponding nucleus (d–f). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 3. Illustration of a dvoxel. Note that the directions associated with a dvoxel form a symmetric pair of sectors each representing 22.5° of the circle.

Fig. 4.

Image stack of deconvoluted confocal images alongside the results of our segmentation algorithm. The actin image (top) and nucleus image (bottom) are shown on left in each pane, and the outline of the segmented volumes of these objects are shown alongside to the right. The distance between image planes is 200 nm. Scale bar = 20 μm . Additional examples using other cells can be found in the online Supplemental material.

Fig. 5.
Orthogonal views of 3D reconstruction of the actin stress fiber network (green) and nucleus (blue) of the cell generated in MetaMorph®.

Fig. 6. Results of image analysis showing cell periphery and nucleus (shown in blue). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 7. Dvoxel processing. (a) Original image with 33×33 pixel sampling unit shown. (b) Gaussian filter applied in sampling unit. (c) 2D FFT of Gaussian filter. (d) Mask applied at one dvoxel. (e–l) Results of 2D FFT multiplied by the mask of each dvoxel with the sum of all intensity magnitudes shown in brackets. (m) Angular contribution of all dvoxels co-located at a single voxel; note that the dvoxel represents textures internally at a 90° rotation to their original orientation, due to the way the 2D FFT produces its output.

Fig. 8.

Computation of A_{ij} for a specific fiber f_i and at all dvoxels, d_j , corresponding to a common voxel. (a) 2D cross-section of 3D cell image containing only fiber f_i and showing a box around the voxel of interest. Note that the end-points of the fiber do not extend to the edges of the cell in this image plane as the fiber is tilted in 3D and not fully contained in this particular image plane. (b) Contents of the box in (a) zoomed in showing the local 2D neighborhood of the voxel of interest containing fiber f_i . (c) 2D FFT of (b). (d) Measurement of aggregate intensity in (c) in each of the eight directions corresponding to dvoxels at our voxel of interest. Observe that the highest intensity is perpendicular to the direction of the fiber in (b).

Fig. 9. Results of image analysis showing cell periphery, nucleus (shown in blue), and representative actin stress fiber network (shown in green). Intensity of fiber color correlates to decision variable x_i , with brighter fibers having higher scores. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 10. Results of our algorithm for various other VSMCs. Original confocal images of each are shown on the left of each pair with the results of image analysis on the right; all scale bars are 20 μm .

Fig. 11.

Flow chart summarizing conversion process from a confocal image of a single cell to a representative model structure.

Table 1

Average and standard deviation (in microns) of the difference between manual segmentation and semi-automated and automated segmentations for the cells and nuclei shown in Fig. 2. The manual segmentation results are the average obtained from manual tracing by 10 individuals.

	Semi-automated ImageJ plugin	Automated segmentation
Cell A	0.7 (± 0.3)	2.5 (± 0.5)
Cell B	1.0 (± 0.3)	2.9 (± 0.7)
Cell C	1.2 (± 0.8)	1.9 (± 0.4)
Nucleus D	0.2 (± 0.03)	1.1 (± 0.1)
Nucleus E	0.3 (± 0.1)	1.2 (± 0.1)
Nucleus F	0.2 (± 0.1)	1.1 (± 0.1)