APPLICATION OF DIGITAL VOLUME CORRELATION ALGORITHM TO CELL MECHANICS

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ABSTRACT

This study developed a digital volume correlation (DVC) algorithm based on fast normalized cross-correlation to measure the 3-dimensional deformation of soft gels, which was further utilized as a force sensor for cell mechanics studies. The developed algorithm was applied to the 3-D volume images of a gel acquired by confocal microscope to measure the deformation of the gel. The gel contained uniformly-dispersed florescence-labeled microbeads so as to generate a necessary speckle pattern for cross-correlation. The developed algorithm has been validated both analytically and experimentally, and applied to investigate cell mechanics by measuring the displacement field induced by the cell motion. Then, surface traction force generated by cells can be quantified through the conventional linear elasticity theory without any further assumption.

INTRODUCTION

Cell Mechanics

Cells in a body often move from place to place to complete their functions, which is called cell migration. For cell migration, cells must apply forces to the outside tissue or extracellular matrix (ECM). Failures in generating and regulating accurate cell traction force (CTF) result in life threatening consequences [1]. CTF also contributes to tumor formation and metastasis. Therefore, a complete knowledge of CTF and the ability to measure CTF are critical.

There have been a number of approaches to measure the CTF. One of them is to mix the cells with collagen gel to make a gel disc [2,3]. Embedded cells generate CTF to causes gel contraction. CTF is evaluated by measuring the change of the gel disc diameter. However, this approach cannot measure the traction force of individual cells [4]. Harris et al. [5] have employed a thin silicone membrane to measure the CTFs generated by individual fibroblasts. Cells generate wrinkles on the membrane, and traction forces generated by individual cells are determined from wrinkles using a calibration technique [6]. However, wrinkling presents an inverse non-linear problem which doesn’t have suitable computational solution. Other researchers have used a micro-machined device consisting of an array of cantilever beams [7-9]. When CTF is applied, it bends a cantilever beam. From the extent of bending recorded, the traction force is determined based on the beam deflection-force relationship obtained through calibration.

The most popular approaches have used an elastic thin gel substrate mixed with fluorescent microbeads [10-12]. A living cell is put on the surface of the substrate and the in-plane surface deformation generated by the cell is determined from fluorescence intensity changes of the substrate. Finally, CTF is evaluated from the 2-D surface deformation of the gel by using analytical solutions. However, this is an ill-posed inverse problem which is extremely hard to solve. Most researchers have used the Boussinesq analytical solution [11,13] to convert the surface displacement field to CTFs. The Boussinesq solution gives the surface displacement of an infinite half-space due to a point surface load. Since the substrate is of finite thickness, the Boussinesq solution based on such assumption is bound to introduce errors. Yang et al. [12] evaluated the errors of the 3-D FEM analysis showing that the error increases substantially as the substrate becomes thinner than 1 mm. This indicated that it might be improper to adopt this method directly for the estimation of CTFs. Moreover, the CTF in the normal direction has been neglected in the previous methods, which turned out to be significant in this study.

Digital Image Correlation

Digital Image Correlation (DIC) is a technique to measure the deformation of materials based on the comparison of two series of images of the same sample acquired at two different stages: one series before deformation and the other after. For
this, a speckle pattern is generated on the surface of a sample. While the sample is deformed, series of images are recorded by CCD camera. The digital images of the sample contain the intensity measurements at each pixel location on the CCD array of the sample’s surface. When DIC algorithm is activated, the sub-image (also called a mask or template) is chosen from the undeformed (base) image by the user or the program. Then the DIC algorithm will find the location of the sub-image in the deformed image by comparing the intensity value of each pixel using various image processing methods such as cross-correlation (CC) [14], normalized cross-correlation (NCC) [15], gradient descent search (GDS) [16] and active contour matching (“Snakes”) [17]. By tracking the location of multiple sub-images in deformed images, the displacement fields in the deformed sample can be estimated [18]. Then, strain fields can be evaluated from the displacement fields using small or large deformation theories. Combining the load data obtained from the load-measuring device (e.g. load cell) with the strain data in consecutive images under incrementally increasing load, one is able to determine the real stress-strain relationship of the sample material which is often quite different from the nominal or engineering stress-strain relationship [19].

Among the various image processing techniques, fast normalized cross-correlation (FNCC) proposed by Lewis [20] is known to be very efficient. In FNCC, the computation for normalized cross-correlation is divided into two parts, the one with Fourier transform pair and the other without. Then, the former is calculated in frequency domain and the latter in space domain. For the computation in space domain, Lewis proposed sum tables that can significantly reduce the computation time. Sum tables are pre-computed data structures that act as lookup tables. The number of multiplications or additions required for the calculation in space domain is dramatically reduced by using sum tables. The computation time for FNCC is only about 10% of that for NCC [20]. FNCC has been successfully applied to the image processing of 2-D images.

Development of Digital Volume Correlation

Recently, the microscopes having optical sectioning capability such as confocal, multi-photon, or image deconvolution microscopes were frequently adopted for materials studies, especially for biological materials. These microscopes can create 3-D volume images that provide much better insight into the 3-D structures of biological tissues or cells than conventional microscopes. Since the 3-D images contain all intensity information of the pixels within the volume, it might be possible to evaluate the deformation inside the volume using similar algorithm to DIC. In order to analyze the 3-D volume images, the DIC algorithm need to be extended to 3-D, herein, called Digital Volume Correlation (DVC).

In this study, a DVC algorithm was developed adopting FNCC as a measure of the similarity of two volume images. All calculation schemes, including sum tables, were extended to 3-D. The developed DVC algorithm has the capability to track the movement of multiple sub-volumes to determine the 3-D displacement fields in a series of images of deformed gel sample. Strain fields are determined by using finite strain tensors. And, using the elastic constants determined by uniaxial compression test, all 3-D stress components within the volume domain can be evaluated from strain tensors. This algorithm was successfully validated by evaluating the 3-D strain fields of the gel sample using the 3-D volume images acquired by a confocal microscope.

The developed DVC algorithm was applied to cell mechanics to measure the surface traction force induced by the cell. During cell locomotion on a soft substrate containing fluorescent microbeads, the deformed 3-D volume images of the substrate are obtained by confocal microscope, and 3-D displacement fields are estimated by the DVC algorithm. From the 3-D displacement fields, the 3-D stress fields, the 3-D stress distribution, and the surface traction forces, generated by the cell, can be accurately determined. Since it adopts tensorial elastic constitutive equations, stresses and traction forces can be calculated explicitly without any assumptions. Also, the accuracy of the solution is independent of the substrate thickness. As well, the computation time is much less than the Boussinesq solution. Direct measurement of the deformation in z-direction made it possible to achieve better resolution in the range of 0.5 to 1 μm compared to 1-10 μm of previous studies [10,21,22].

EXPERIMENTAL VALIDATION

In order to verify the capability of the developed DVC algorithm in evaluating the strain field of 3-D volume images, the testing protocol in ref. [23] was adopted. Briefly, the agarose gel containing microbeads was compressed while the volumes images were recorded by a confocal microscope. The recorded images were analyzed using developed DVC algorithm and the results are compared with the experimental data.

Sample Preparation

Agarose sols (agarose in the melting state) were firstly prepared by dispersing agarose powders (J. T. Baker) in a standard 0.5 TBE buffer (Tris/Borate/EDTA, PH 8.0), to form 1% (w/v) solution of agarose. The agarose solution was then heated to 95 °C for 1 hr and mixed with red fluorescence-labled polystyrene beads (Invitrogen) of 1 μm in diameter. The nominal volume fraction of the fluorescent microbeads in the mixture was 0.5% (v/v). The mixture was poured into a Teflon mold mounted on a glass plate. Samples were left at room temperature for 20 min for gelation. The formed agrose gel specimens have a cylindrical shape with 8 mm in diameter and 4 mm in height. Preliminary tests showed the added fluorescent microbeads had a negligible effect of on the mechanical properties of agrose gel. The gels were immersed in the buffer to prevent swelling or shrinking during the test.

Uniaxial Compression Test

A custom-built micro loading frame was integrated to the inverted microscope as shown in Fig. 1. Samples were
compressed uniaxially with nominal strain increments of 1-2 % using a micrometer head with a resolution of 1 μm, while the 3-D volume images are recorded simultaneously by a CCD camera equipped on the confocal microscope. The maximum nominal strain applied was 10%. A series of volume images were analyzed using the DVC algorithm with a sub-volume size of 64×64×64 pixels (or 29 x 29 x 29 μm³; 1 pixel is equivalent to 0.45 μm) to evaluate the displacement fields (u₁, u₂ and u₃) at the center of each sub-volume. Fig. 2 shows the image of a sub-volume obtained by the confocal microscope. The emitted fluorescent light from the microbeads caused the light intensity variations within the gel, which played the role of patterns necessary for cross-correlation. By tracking the sub-volumes within a volume image, displacement field in the deformed image was determined.

Fig. 1 Schematic of micro loading frame installed on inverted microscope for uniaxial compression test.

Fig. 2 Confocal image of sub-volume in the agarose gel.

Fig. 3 Vertical displacement field in a deformed volume image evaluated by DVC algorithm.

Spherical Inclusion Test

Another verification test was performed by measuring the deformation fields around hard spherical particle under uniaxial compressive loading. Spherical polymethylmethacrylate (PMMA) beads (Sigma–Aldrich, MO) of 100 μm diameter were added to the mixture before casting. Confocal images around a PMMA bead were recorded during incremental compressive loading. The nominal strain increment was 2 %. The size of scanning volume was 512×512×512 pixels, and the pixel size was 0.45 μm in all three directions. The confocal images were analyzed by using the proposed DVC algorithm with a sub-volume size of 64×64×64 pixels. The contour map of in Fig. 4(a) presents the vertical (u₃) displacement field on the meridian plane. The experimentally measured displacement field in Fig. 4 was compared to the analytical solution of the equivalent linear-elasticity problem. Adopting the solution of the sliding inclusion problem under uniaxial loading [24], the vertical displacement around the spherical particle was plotted in Fig. 4(b) which shows a good agreement with DVC result.

The results of both verification tests (uniaxial compression and spherical inclusion tests) indicated that the proposed DVC algorithm is well suited for the measurements of uniform and non-uniform 3-D deformation fields of the gel.
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Using the DVC algorithm, cell-induced deformation field, displacement field, and strain field in the gel can be estimated successively. Then the 3-D stress field can be calculated using the linear elastic theory (Hooke's law) without any assumption. Finally the CTF on the surface can be accurately determined.

Sample Preparation

Polyacrylamide substrate attached to glass coverslip was prepared by adopting previously established protocols [10,25] with slight modification. First, glass coverslips were chemically modified to allow for covalent attachment of polyacrylamide substrate. Then, polyacrylamide substrates were generated in two different cross-linking density. Briefly, solutions of polyacrylamide (40% w/v, Bio-Rad) and N, N-methylene-bis-acylamide (BIS, 2.5% w/v, Bio-Rad) were mixed with distilled water to obtain 10% acrylamide and 0.015% BIS, and 10% acrylamide and 0.0075% BIS. By adjusting the concentration of BIS in the formulation, substrates with varying mechanical properties can be created. To these solutions, red fluorescent microbeads (0.5 μm, Molecular Probes) were added in a volume ratio of 9:100. The acrylamide solution was pipetted on the surface of a precleaned microscope slide. The activated surface of the coverslip was then placed on top of the acrylamide droplet, causing the solution to flatten under the weight of the coverslip. In order to promote cell attachment to polyacrylamide films, a saturating density of fibronectin was conjugated to the gel surface using the heterobifunctional crosslinker, sulfo-SANPAH (Pierce Chemicals).

Sample Characterization

The mechanical properties of the substrates were determined by performing compression tests on cylindrical polyacrylamide substrates. The same test scheme as the uniaxial compression test was used for the characterization of polyacrylamide substrates. For each volume fraction of BIS crosslinker used, 6-8 samples of each were tested. The specimens were compressed with a nominal strain increment of 1 %. Force variation was monitored for 5 minutes at each increment in order to detect any time-dependent behavior of the material. The total applied nominal compressive strain was 15 %. After complete loading, the specimen was unloaded using the same strain increments to record the entire loading-unloading cycle. Figure 5(a) presents force-time curves from incremental loading tests. All curves demonstrate negligible time-dependent behavior. Figure 5(b) shows the typical nominal stress-nominal strain curve from loading and unloading cycles of the uniaxial compression test. It also suggests that there is very little hysteresis in loading and unloading. Elastic modulus was calculated from stress-strain curve. Table 2 summarizes the test results for two different crosslinker volume fractions.

Cell culture

3T3 fibroblasts transfected with a GFP-actin vector were cultured in Dulbecco’s Modified Eagle Medium (DMEM)
supplemented with fetal bovine serum, streptomycin, and penicillin. For all experiments, cells were first treated with Mitotracker Deep Red. Mitotracker dyes provide a second method for tracking the location of cells on the material as well as cell viability. Cells were incubated on the substrate for 8-12 hours before imaging.

**Confocal microscopy and time-lapse imaging**

Three-dimensional image stacks were acquired using a Nikon C-1 confocal system mounted on an inverted optical microscope. Confocal stacks were acquired at every 30 minutes for several hours with a resolution of 512 x 512 x H (X x Y x Z) pixels, where H ranges from 120 – 250 pixels. Typical imaging areas were between 150-200 μm²; images with a larger field of view were captured before and after experiments to ensure that measured displacements were not the result of contributions from neighboring cells.

**Results and Discussions**

Cell induced 3-D displacement fields are determined from confocal volume stacks using DVC algorithm at each time increment. Instantaneous z-directional displacement contour under the cell is depicted in Fig 6(a), presenting the significant variation of displacement field depending on the location. A sectioned contour image along the major axis of the cell shown in Fig. 6(b) illustrates that displacement is highly concentrated at focal adhesions (F.A. in the figure). As the color contours and color bar indicate, the displacement resolution that could be detected by the proposed method is less than 0.5 μm, which is much better than most of the previous studies [10,21,22,26,27]. The variation of the u, v, and w (x-, y- and z-directional displacements) as well as the total displacement (the magnitude of displacement vector) under F.A. in the thickness direction is presented in Fig. 6(c) which shows that the displacement decayed very rapidly as the distance from the top surface increases. At the depth of 10 μm, all directional displacements are less than 15% than those at the top surface. Therefore, previous studies that determine the CTF by measuring the in-plane displacement of the bottom surface of substrate employing inverted microscope [28,29] might not be able to yield accurate result as the substrate thicknesses were larger than 10 μm.

Once the entire displacement field is determined the strain tensor of the material substrate is computed using a displacement-gradient technique. The stress tensor $\sigma_{ij}$ is then determined through the materials constitutive relations based on the material properties determined from the above characterization tests. Hence, stresses can be calculated using linear elasticity as:

$$\sigma_{ij} = 2\mu \varepsilon_{ij}$$  \hspace{1cm} (1)

where $\mu$ is shear modulus. $\varepsilon_{ij}$ is Lagrangian strain tensor as:

$$\varepsilon_{ij} = \frac{1}{2} \left[ \frac{\partial u_i}{\partial x_j} + \frac{\partial u_j}{\partial x_i} + \sum_{k=1}^{3} \frac{\partial u_k}{\partial x_k} \frac{\partial X_k}{\partial X_i} \right]$$  \hspace{1cm} (2)

Finally, traction forces are calculated along the top surface plane, directly beneath the cell using the known Cauchy relationship:

![Fig. 6](image-url)
\[ T_i = \sigma_j n_j \]  

where \( T_i \) are the 3-D surface traction vectors, and \( n_j \) the surface normal vectors. Fig. 7 presents contour plot of instantaneous Mises stress induced by the cell in the sectioned substrate. The stress just below the focal adhesion point is the highest at around 180 Pa, which decreases rapidly as the distance from that point increases.

![Fig. 7. The contour plot of instantaneous Mises stress induced by the cell on soft substrates (E = 0.82 kPa).](image)

Table 1  Mean and standard deviation values for measured strain fields under uniaxial compression for 3 different strains.

<table>
<thead>
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<th>Strain</th>
<th>Measured Strain</th>
<th>Mean</th>
<th>Std. Dev.</th>
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<tbody>
<tr>
<td>( \varepsilon_{11} ) (%)</td>
<td>-1.5 x 10^{-2}</td>
<td>2.8 x 10^{-2}</td>
<td></td>
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<tr>
<td>( \varepsilon_{22} ) (%)</td>
<td>2.5 x 10^{-2}</td>
<td>3.3 x 10^{-2}</td>
<td></td>
</tr>
<tr>
<td>( \varepsilon_{33} ) (%)</td>
<td>-3.0</td>
<td>-3.05</td>
<td>0.15</td>
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<td>( \varepsilon_{11} ) (%)</td>
<td>-3.4 x 10^{-2}</td>
<td>3.4 x 10^{-2}</td>
<td></td>
</tr>
<tr>
<td>( \varepsilon_{22} ) (%)</td>
<td>4.2 x 10^{-2}</td>
<td>4.5 x 10^{-2}</td>
<td></td>
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<tr>
<td>( \varepsilon_{33} ) (%)</td>
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<table>
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<td>( \varepsilon_{11} ) (%)</td>
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<td>5.8 x 10^{-2}</td>
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<td>( \varepsilon_{22} ) (%)</td>
<td>8.5 x 10^{-2}</td>
<td>7.3 x 10^{-2}</td>
<td></td>
</tr>
<tr>
<td>( \varepsilon_{33} ) (%)</td>
<td>-10.0</td>
<td>-9.86</td>
<td>0.35</td>
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Table 2  Elastic modulus of polyacrylamide substrate for different crosslinker volume fractions from uniaxial compression tests.

<table>
<thead>
<tr>
<th>Crosslinker Volume Fraction</th>
<th>Young's Modulus (kPa)</th>
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<tr>
<td>0.015% BIS</td>
<td>9.64 ± 1.12</td>
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<tr>
<td>0.0075% BIS</td>
<td>0.82 ± 0.23</td>
</tr>
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CONCLUSIONS

Digital volume correlation (DVC) algorithm is applied to cell mechanics to measure the cell-induced displacement in the soft gel. The measured displacement can be converted to the CTF using linear elasticity theory without any assumption.

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