Chapter 3

Application of LSCM and DVC to Migrating Fibroblasts

This chapter describes the experimental setup of the substrate-cell system that will be studied in Chapter 4. In particular, the experimental procedure to produce polyacrylamide gel samples, cell culturing, mechanical testing of the substrates as well as the LSCM live cell imaging setup are described in detail below.

3.1 Experimental Procedure

Polyacrylamide gels are one of the most commonly used polymer-based substrate materials in studying cell force responses due to their mechanical tunability, optical translucency, and elastic material behavior [35]. By controlling the incorporation percentage or volume fraction of added crosslinker N, N-methylene-bis-acrylamide (BIS), the Young's modulus of each particular polyacrylamide gel can be modified with a typical modulus ranging from around 1 - 20 kPa [14, 41, 49]. Polyacrylamide substrates were prepared for all of the results shown in the subsequent chapters.

In order to control the thickness of each polyacrylamide substrate, a polyacrylamide solution of known volume was pipetted onto a clean coverslip. A chemically-activated coverslip was then laid on top of the solution causing the gel mixture to spread uniformly in-between both coverslips. After the polymerization reaction of the polyacrylamide gel was completed, the bottom coverslip was removed, and the polyacrylamide gel was left adherent to the activated bottom coverslip. The following

protocols describe how to chemically activate the coverslips followed by the preparations of the polyacrylamide films, including biological functionalization of the polyacrylamide substrates through fibronectin. Concluding this section is a description of the characterization of the fibronectin-modified polyacrylamide substrates and the employed cell culture.

3.1.1 Preparation of Activated Coverslips

Glass coverslips (Gold-Seal coverslip No. 0, Electron Microscopy Sciences) were chemically modified to allow for covalent attachment of polyacrylamide sheets using a previously established protocol [40, 35]. Briefly, coverslips were rinsed with ethanol and then placed in a sample dish containing a solution of 0.5% (v/v) 3-aminopropyltrimethoxysilane (Gelest) in ethanol for 5 minutes. The coverslips were removed from the dish and rinsed thoroughly with ethanol before being immediately submersed with the treated side facing upwards in a solution of 0.5% glutaraldehyde (Polysciences, Inc.) and water for 30 minutes. Activated coverslips were rinsed thoroughly with deionized water and left to dry for several hours at 60^o C. Treated coverslips were then covered and stored at room temperature for up to one week after the preparation.

3.1.2 Preparation of Polyacrylamide Films

Micron-sized polyacrylamide films were generated and fused to functionalized coverslips using a previously adapted protocol [14, 40]. Two different solutions of polyacrylamide (Bio-Rad, 40% w/v) and N, N-methylene-bis-acrylamide (BIS, Bio-Rad, 2.5% w/v) were mixed with distilled water to obtain substrates with different mechanical properties. The first solution contains a total volume fraction of 10% acrylamide and 0.015% BIS, whereas the second solutions consists of 10% acrylamide and 0.0075% BIS total volume fraction. By adjusting the concentration of BIS in the formulation mechanical properties of the polyacrylamide substrate was modified in a controlled manner. Next, fluorescent micro-particles (0.5 μm in diameter, carboxylate-modified, Molecular Probes) in a 2% (w/v) suspension were vortexed for 10-15 seconds and subsequently added to either polyacrylamide solution in a volume ratio of 9:100. Crosslinking was initiated through the addition of ammonium

persulfate (Sigma) and TEMED (Invitrogen). The samples were vortexed for 10 seconds, and 5-7 microliters of the acrylamide solution was pipetted on the surface of a precleaned microscope slide (No. 1, 22 mm x 50 mm, VWR). To generate thicker films, 20-40 microliters of the solution were used. 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. The entire assembly was set out to allow complete polymerization for 5 minutes, and then placed in a 60 mm Petri dish (VWR) containing distilled water for 10-30 minutes. The bottom coverslip was then peeled off using a pair of tweezers, leaving the polyacrylamide gel bonded to the activated coverslip. The polyacrylamide gel was then thoroughly rinsed with water and hydrated in a 60 mm Petri dish.

The thickness of each sample was measured by vertical slicing of the acquired volumetric confocal images, and calculating the distance from the top layer of fluorescent particles to the bottom layer of fluorescent beads. Sample thicknesses were controlled by adjusting the total volume of acrylamide mixture used.

3.1.3 Functionalization of Polyacrylamide Substrates with Fibronectin (FN)

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). Adopting a previously outlined procedure [14], polyacrylamide gel samples were briefly dried in air to remove any excess water before 200 µl of sulfo-SANPAH (1.0 mg/ml) were deposited on the surface of the film. The sample was then exposed to unfiltered UV light from a high-pressure mercury lamp (Oriel Q 100W at 5 A, $>$ 10 min warm up time) at a distance of 10 inches away from the sample for 7.5 minutes. The darkened sulfo-SANPAH solution was subsequently removed from the surface of the gel and replaced with another 200 µl aliquot solution of sulfo-SANPAH and irradiated for another 7.5 minutes for a total of 15 minutes of UV exposure. The samples were then rinsed vigorously with water for 5 minutes, and adhered to the bottom of 60 mm Petri dishes (Becton Dickinson) by applying a thin layer of vacuum grease (Dow Corning) around the perimeter

of the unmodified side of the coverslip. The samples were rinsed twice with phosphate buffered saline (pH 7.4), and covered with a solution of fibronectin (FN, 0.2 mg/ml, Millipore) and left undisturbed overnight at 4^o C. Following overnight incubation, the substrates were rinsed three times with a phosphate buffered saline (PBS) solution and sterilized by rinsing with ethanol before use.

3.1.4 Characterization of Fibronectin-Modified Films

A comparison of the relative concentration of covalently attached fibronectin on samples made with varying percentages of crosslinker was conducted using a bicinchoninic acid (BCA) assay (Sigma). In this assay, the relative protein concentration is exhibited as a color change in the sample solution upon the addition of the copper/BCA reagents. The degree of color change can be quantified by measuring the absorbance at 542 nm using a plate reader. A set of fibronectin-modified samples made with varying crosslinker (BIS) percentages (3-4 total samples for each percentage) were prepared as described above, and placed in a 6-well plate. However, fluorescent particles were not added to the polyacrylamide samples in the BCA assays in order to avoid unintentional complications, since the microparticles partially fluoresce at the absorbance wavelength of the plate reader. Negative controls consisting of unmodified samples made with each crosslinker percentage were also prepared. All samples were then treated with the copper/BCA reagent for 1 hour at 60^o C, followed by an absorbance reading. All tests were repeated twice. The absorbance reading for both sets of samples showed similar absorbance values, and lie within the standard deviation calculated from the tested samples. These results confirm that cells are responding to the difference in mechanical properties and not differences in the chemical composition of the surfaces.

3.1.5 Cell Culture

Prior to depositing cells, fibronectin-modified gel samples were equilibrated in a growth medium at 37^o C for 15 minutes. Swiss 3T3 fibroblasts transfected with a green-fluorescent-protein-actin (GFP-actin) vector (from Scott Frasier's laboratory) were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum, 50 μg/ml streptomycin, and 50 μg/ml

penicillin. For all experiments, cells were first treated with Mitotracker Deep Red (Invitrogen) for 45 minutes before passaging with trypsin. Mitotracker dyes accumulate in actively respiring mitochondria providing a second method in addition to the GFP-actin vector for tracking the location of cells on the material as well as showing cell viability. Cells were plated at a concentration of $\sim$ 40,000 cells/coverslip, and were incubated on samples for 8-12 hours before imaging.

3.2 Mechanical Testing of the Substrate Material

The mechanical properties of the substrates were determined by performing both unconfined and confined compression testing on cylindrical polyacrylamide specimens using a custom-built compression setup [17]. The typical sample dimensions were 8 mm in diameter and 4 mm in height. The displacements during each compression increment were controlled using a digital micrometer with a resolution of 1 µm. The resulting nominal force was measured using a 10 g load cell (A.L. Design, NY). For each volume fraction of polyacrylamide crosslinker 6-8 samples were tested in both confined and unconfined uniaxial compression.

For unconfined tests, gel samples were cast in a circular washer secured to the bottom of a 60 mm diameter plastic Petri dish. Following polymerization ($\sim$ 2-5 minutes), the washer was removed from the dish and the sample was hydrated and left covered at room temperature overnight to ensure hydrostatic (swelling) equilibrium. Prior to compression, the alignment of the setup with the sample was inspected to ensure pure compression along the nominal loading axis. The samples were compressed between the top platen of the compression setup and the bottom of the Petri dish with a nominal strain increment of 1-2%. Force values were obtained continuously during each 5-minute increment in order to detect any time-dependent relaxation of the material during the compression. The typical total applied compressive strain was $\sim$ 13-15%. After complete loading, the sample was successively unloaded using the same strain increments to record the entire loading-unloading cycle. Figure 3.1 shows the raw data for an incremental loading cycle highlighting negligible time-dependent material behavior, whereas Figure 3.2 shows the loading-unloading stress strain curve for a typical sample.

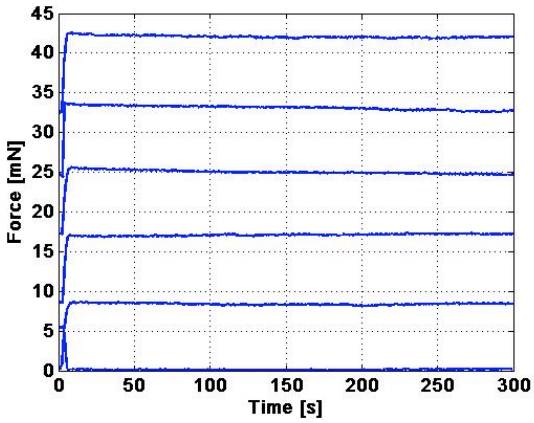

Figure 3.1: Representative force history plot during uniaxial compression experiments on a polyacrylamide gel showing negligible time-dependent material behavior.

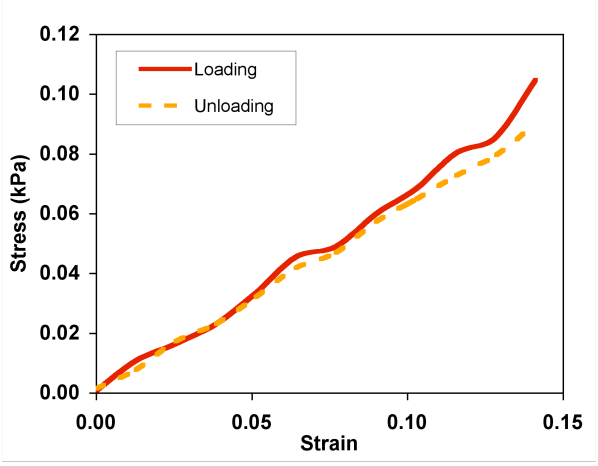

Figure 3.2: Representative loading and unloading stress-strain plot of a polyacrylamide gel, highlighting the linear elastic material response with negligible hysteresis.

The Young's modulus for the polyacrylamide samples was calculated from each stress-strain curve as $E = \sigma_{33}/\epsilon_{33}$ ($\sigma_{11} = \sigma_{22} = 0$), where σ and ϵ denote the uniaxial engineering stress and engineering strain. Table 3.1 summarizes the unconfined compression test results for two different crosslinker volume fractions.

Crosslinker Volume Fraction	Young's Modulus (kPa)
0.015% BIS	9.64 ± 1.12
0.0075% BIS	0.82 ± 0.23

Table 3.1: Young's modulus values for polyacrylamide substrates with different crosslinker volume fraction.

In order to determine the Poisson's ratio for each polyacrylamide gel, cylindrical specimens were cast and polymerized in a confined Teflon sleeve 15 mm in diameter and about 8 mm in height. Samples were hydrated following the same protocol as described above. The samples were compressed following the same loading-unloading protocol used for the unconfined test. Using the determined Young's modulus value of the unconfined test case and observing that further compression beyond an initial compression strain of $\sim 0.25\%$ was not possible (due to the Poisson effect), Poisson's ratio was determined to be $\sim$ 0.48 - 0.5 according to the following equation:

$$\overline{E} = \frac{\sigma_{33}}{\epsilon_{33}} = \frac{(1-\nu)}{(1+\nu)(1-2\nu)}E, \tag{3.1}$$

where $\overline{E}$ denotes the measured confined compression modulus, ν is the Poisson's ratio, and E is the Young's modulus as determined from unconfined compression test. From this set of experiments, Poisson's ratio was taken to be 0.5, and the material behavior is described as a linearly elastic, isotropic, incompressible for all traction calculations.

3.3 Live Cell Imaging

Three-dimensional image stacks were acquired using a Nikon C-1 confocal system mounted on a TE-2000-U inverted optical microscope. A 40x CFI planar fluor air objective with a numerical aperture of 0.6 was used in all experiments. Three laser lines were used to image the cells and the fluorescent microparticles: an argon (488 nm) laser for the GFP-actin, a green helium neon (543 nm) for the microparticles inside the polyacrylamide gels, and a red helium neon (633 nm) illuminating Mitotracker Deep Red for mitochondrial labeling. Confocal stacks were acquired every 35 minutes for several hours at a resolution of 512 x 512 x Z voxels (x_1 x x_2 x x_3), where Z ranges from 120 250 pixels (voxels). Typical imaging areas were between 150-200 μm^2 in-plane (x_1, x_2) with imaged volume depth of $\sim$ 15 - 20 μm. 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. Physiological conditions were maintained during all times by housing the entire confocal microscope inside a custom-built temperature-controlled chamber. The temperature was controlled using a feedback controlled heater, Air-Therm ATX Air Heater Controller (World Precision Instruments), and cell media PH-levels were maintained by the addition of arterial gas (5% CO_2, 20% O_2, 75% N_2) into the chamber.

3.4 Establishing LSCM and DVC Resolution for Polyacrylamide Gels

Chapter 2 already presented the resolution of the LSCM-DVC technique, however, all measurements in the development of this technique were performed at room temperature. Here, several zero-load (baseline) measurements were performed on fibronectin-modified polyacrylamide gels for both crosslinker volume fraction levels at 37^o C to establish the maximum resolution of the technique for softer materials at elevated temperatures. Table 3.2 shows representative results of the fibronectin-modified polyacrylamide gels side by side with the earlier presented no-load DVC results from an agarose gel. As can been seen in Table 3.2, the standard deviation of the experimental uncertainties at room temperature and 37^o C are similar in magnitude, highlighting subpixel or submicron resolution.

	25^o C	37^o C
u_1 [voxel]	0.0605	0.0289
u_2 [voxel]	0.0541	0.0282
u_3 [voxel]	0.2106	0.187
ϵ_{11} (%)	6.39 x 10^{-3}	6.59 x 10^{-2}
ϵ_{22} (%)	9.80 x 10^{-3}	6.04 x 10^{-2}
ϵ_{33} (%)	0.260	0.526

Table 3.2: Standard deviation values for measured displacement fields in the undeformed condition for agarose gels at 25^o C and polyacrylamide gels at 37^o C

This chapter describes the experimental setup to perform live cell imaging experiments using laser scanning confocal microscopy including the fabrication of fibronectin-modified polyacrylamide gel substrates with embedded submicron fluorescent particles. The next chapter presents the quantitative findings of cell induced material deformations during cell movement as imaged by laser scanning confocal microscopy and analyzed by digital volume correlation.