Accurate determination and application of local strain for studying tissues with gradients in mechanical properties

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Accurate Determination and Application of Local Strain for Studying Tissues with Gradients in Mechanical Properties

by

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1.1 Examples of techniques used for the measurement of strain. (A) Overall length change following deformation for an entire sample, as determined using an extensometer, is the most simplistic strain measurement and does not reveal local deformation(s). (B) Adding markers, such as lines, to a deforming sample, then tracking the length change between the markers can reveal local strains. However, this is limited to one dimension and the overall number of localized strain measurements that can be computed is limited to the number of lines. (C) Instead of lines, squares or boxes can be added to the surface of a sample to measure two dimensional strain based on the movement of the corners or edges of the boxes.

1.2 Summary of the algorithm for computing local strain from normalized cross correlation. (A) A reference frame (left) is initially divided into several regions (black boxes). The locations of these regions is unknown in the deformed frame (right). (B) For each region (in this example, a region containing an eye), a correlation coefficient is computed for every possible position of the region template (middle) in the deformed image, resulting in a correlation matrix (right image). The maximum of the correlation matrix is the location of the region template in the deformed image (black arrow, red dot in correlation matrix image). (C) This is repeated for every region in the template image (black boxes with green center) to locate their corresponding location in the deformed image (black boxes blue centers). (D) After finding the locations of each region, several nearby regions are combined and the deformation tensor is computed by comparing the displacements of the regions before and after deformation.

1.3 The healthy adult tendon-to-bone attachment is classically divided into four distinct zones: tendon, unmineralized fibrocartilage, mineralized fibrocartilage, and bone. A toluidine blue–stained section from an adult rat supraspinatus tendon-to-bone attachment is shown on the left.
2.1 Schematic representation of how 2D-XCOR, 2D-LSF, and 2D-DDE calculate 2D deformation gradient tensors. (A) Original test image (left) and warped image (right). (B) Rigid registration yields errors due to a parallelogram fit of the midpoints of a minimum of four regions (red boxes). (C) The 2D-LSF method improves on this, but errors and reduced precision remain due to the parallelogram fit of the midpoints to calculate $F$ (blue boxes). (D) 2D-DDE accurately calculates the deformation of all four regions independently (green boxes). Black borders: matched regions; dotted borders: calculated deformations for 2D-XCOR (red), 2D-LSF (blue) and 2D-DDE (green). Definitions: $F$, deformation gradient tensor; $\gamma$, shear strain; $\lambda$, stretch ratio in $y$ direction; $\epsilon_{\alpha,x}$, error in $\alpha$ strain.

2.2 The 2D-SIMPLE Method uses the difference between the 2D deformations calculated by 2D-DDE (green boxes) and the 2D-LSF method (blue boxes) to determine an outcome we refer to as the Strain Concentration Detector (orange boxes). (A) When deformations are continuous and have no local discontinuities, the 2D-DDE and 2D-LSF methods agree and their difference is exactly 0. (B) If there is a local discontinuity in the data, the 2D-DDE and 2D-LSF methods disagree and their difference is non-zero. Definitions: $\gamma$, shear strain; $\lambda$, stretch ratio in $y$ direction; $\delta$ difference in calculation between methods.

2.3 The 2D-DDE method is often implemented on a regular grid within a strained body.
2.4 The 2D-DDE method was superior to all previously utilized methods for calculating strains in a deforming sample. In simulations where strains and rotations were known *a priori*, the 2D-DDE algorithm (green lines) calculated strains with higher precision and accuracy than 2D-XCOR (red lines) and 2D-LSF (blue lines) algorithms. For all cases, at strains less than 0.01, 2D-LSF introduces marginally less error than 2D-DDE. However 2D-DDE remain consistently more accurate and more precise through large strains while 2D-LSF introduces progressively more error. (A, B, C) The first simulation consisted of an increasing tensile incompressible deformation to a final strain of $E_{11} = 0.01$. 2D-XCOR produced errors on the order of 0.03 strain for a strain level of 0.1. In contrast, the 2D-LSF and 2D-DDE strain calculations introduced errors on the order of 0.0005 strain. (D, E, F) The second simulation consisted of a pure rotation to $\theta = 15$ in the absence of strain. 2D-XCOR was unable to accurately track displacements in this simulation, leading to large errors in the strain calculations. For this simulation, errors in strain calculation for the 2D-LSF method were on the order of 0.002 strain, while errors for the 2D-DDE method were on the order of 0.0001 strain. (G, H, I) The third simulation consisted of incompressible deformation ($E_{11} = 0.1$) combined with rotation $\theta = 15$. 2D-XCOR once again failed to track deformation, leading to large errors in strain calculations. The errors associated with the 2D-LSF and 2D-DDE algorithms were similar to those for pure rotation, with the 2D-DDE algorithm about an order of magnitude better than the 2D-LSF algorithm. Definitions: $E_{11}$, strain in 11 direction; $\theta$, rotation.

2.5 The advantages of the increased precision and accuracy of 2D-DDE over 2D-XCOR were demonstrated by cyclically stretching a PDMS sheet with a spatial gradient in stiffness. (A-D) At a low grip-to-grip strain of 0.003, 2D-DDE was able to detect a gradient in stiffness, as evidenced by gradients in the first and second principal strains, while 2D-XCOR failed to detect gradients in strain above noise. (E-H) At a grip-to-grip strain of 0.03, 2D-DDE revealed a smooth gradient in first and second principle strains. 2D-XCOR also detected the spatial gradients in strain, however the detected strains were irregular and noisy. (I-L) At a large grip-to-grip strain of 0.1, 2D-DDE detected a smooth strain gradient, with local strains greater than 0.2. In contrast, 2D-XCOR failed to detect a smooth strain gradient, demonstrating its limitations at high strains. Scale bar = 2 mm. Definitions: $E_{xx}$, strain in $xx$ direction.
2.6 The 2D-SIMPLE method accurately detected strain concentrations predictive of crack initiation formation and was able to track crack propagation. All strains are given relative to an initial grip-to-grip strain of \( E_{xx} = 1.2 \), at which the optical analysis was started. (A, B) The 2D-SIMPLE algorithm detected two developing strain concentrations (white and yellow arrows) at a low grip-to-grip strain \( E_{xx} = 0.26 \). In contrast, noise in the 2D-XCOR calculation resulted in significant uncertainty for determining the location of the strain concentrations. (C, D) At higher levels of grip-to-grip strain \( E_{xx} = 1.16 \), both algorithms were able to detect the developing crack, however, the strain concentration remained partially obscured by noise for the 2D-XCOR method. (E) The strain concentration predicted by the 2D-SIMPLE algorithm can be visualized as a crack in the material (white arrows). (E-H) As the crack forms and propagates \( E_{xx} = 1.56 \), the 2D-XCOR algorithm fails whereas the 2D-SIMPLE algorithm continues to track the crack in the material (white arrows in G). Furthermore, the second strain concentration (yellow arrow) stops developing, suggesting that the material failure at the crack (white arrows) resulted in unloading of the second concentration. Scale bar = 1 mm. Definitions: \( E_{xx} \), strain in 11 direction; \( \Delta \), 2D-SIMPLE difference.

3.1 3D-XCOR was orders of magnitude inferior at calculating strain when compared to either 3D-LSF or 3D-DDE. Although 3D-LSF had a slight advantage over 3D-DDE when strain fields were uniform, 3D-DDE was an order of magnitude superior when strain fields were complex. (A) RMS noise versus angle of rotation for a 3D body undergoing 3D rotation in one plane for 3D-XCOR, 3D-LSF, and 3D-DDE. (A, inset) Region of (A) zoomed in where 3D-XCOR maintained a reasonable calculation of strain. (C) RMS noise versus applied strain in the 11 direction for a 3D body undergoing uniform stretch in one plane for 3D-XCOR, 3D-LSF, and 3D-DDE. (E) RMS noise versus maximum stretch ratio in the 11 direction for a 3D body undergoing non-linear stretch given by equation (1) for 3D-XCOR, 3D-LSF, and 3D-DDE. (B,D,F) Regions of (A),(C),and (E), respectively, zoomed in to focus on results for only 3D-LSF and 3D-XCOR.
3.2 RMS strain error from solution versus input stretch ratio for kernel sizes and kernel spacing ranging from 11x11x11 to 45x45x45 voxels for a material stretched with a uniform strain \textit{in silico}. As kernel size increased, both 3D-LSF (red) and 3D-DDE (blue) improve their accuracy and precision. Additionally, as kernel spacing increased, the accuracy and precision of 3D-LSF increased rapidly, while 3D-DDE only improved marginally. In all cases, 3D-LSF remained slightly more accurate and more precise than 3D-DDE, with both approaching an accuracy and precision of $10^{-5}$ at a kernel size and spacing of 45x45x45. The marginal advantage of 3D-LSF at high spacing can be attributed to increased numerical precision as regions are further and further apart from one and other in a uniform strain field.

3.3 RMS strain error from solution versus input stretch ratio for kernel sizes and kernel spacing ranging from 11x11x11 to 45x45x45 voxels for a material stretched non-linearly \textit{in silico}. As kernel size increased, both 3D-LSF (red) and 3D-DDE (blue) improved their accuracy and precision marginally. However, as kernel spacing increased, the accuracy and precision of 3D-LSF decreased sharply, while 3D-DDE remained consistent. In all cases, 3D-DDE remained more accurate and more precise than 3D-LSF.

3.4 RMS error versus stretch ratio in the 11 direction for (A) noise free, (B) Poisson noise, (C,D) low and high levels of Gaussian noise, (E,F) low and high levels of salt and pepper noise, and (G,H) low and high levels of speckle noise. 3D-DDE was superior to 3D-XCOR in all cases and superior to 3D-LSF in all cases except Poisson noise and high levels of speckle or salt and pepper noise.

3.5 Stretch ratio results for an Eshelby inclusion generated \textit{in silico}. (A) Schematic of an Eshelby Inclusion, (B) 3D-SIMPLE detected a large strain concentration surrounding the inclusion, (C) True values of the stretch ratio in the Z direction closely matched the (D) 3D-DDE estimated values, while (E) 3D-LSF-estimated and (F) 3D-XCOR-estimated stretch ratios were successively worse.

3.6 Stretch ratio results for a penny shaped crack \textit{in silico}. (A) Schematic of an Eshelby inclusion. (B) 3D-SIMPLE detected and highlighted the developing crack, (C) True values of the first principal stretch ratio closely match the 3D-DDE estimated values (D), while 3D-LSF-estimated (E), and (F) 3D-XCOR-estimated stretch ratios were successively worse.

4.1 Schematic for Forward 2.5D DDE. Coordinates $x_\pi$ are allowed to rotate, translate, and deform in a three dimensional warp before projecting into two cameras with projection functions $P_1$ and $P_2$. Note that this schematic shows only two cameras, but the algorithm is not constrained to only two cameras.
4.2 Schematic for warping functions $W_A$ and $W_B$. (A) $W_A$ works in 3 steps: (1) projecting the image coordinates out to the world coordinate system, (2) allowing the world coordinate system to transform, and (3) re-projecting back into coordinates of camera $A$. (B) The warping function $W_B$ has 4 steps: (1) projecting from $B$ out to the world coordinate system, (2) allowing the world coordinate system to transform, (3) projecting back to $B$, and (4) projecting coordinates of $B$ into $A$.

5.1 Murine tendon-to-bone attachment sites were cut and milled into beams measuring approximately 60 $\mu$m long and 4.5 by 4.5 $\mu$m in diameter. (A) Dissected supraspinatus-to-humeral head complexes were fresh frozen and sectioned into 20–30 $\mu$m thick slices. (B) LCM was used to cut large beams, 250 $\mu$m by 50 $\mu$m by 20–30 $\mu$m, in the fibrocartilaginous region of the attachment where there is a gradient in mineralization. (C–E) The LCM cut beams were further milled down to the final small beams via cryo-FIB. Figure prepared by Dr. Alix Deymier and reproduced with permission.

5.2 Plots of local strain vs. position at multiple stresses (legend) for all of the samples for which local strains were measured. Dotted lines represent the calcium content as a function of position for each sample. The highest strain levels were not localized to the region closest to the tendon but instead within the beam near the gradient region. This indicates the presence of a region of high deformation within the enthesis. Strain data analyzed by John Boyle, calcium data analyzed by Dr. Alix Deymier, figure created by Dr. Alix Deymier.

5.3 Small and large grip-to-grip strains of collagen scaffolds with spatial gradients in mineral content, tested in tension. (A, B, E, F) 2D-DDE revealed a gradient in material strain for low and high grip-to-grip strains. (C, D) At low grip-to-grip strains, 2D-XCOR revealed similar trends to 2D-DDE. However, the values of strains measured were unrealistically high and are likely due to noise. (G, H) At high grip-to-grip strains, 2D-XCOR reported strains over 2. This was clearly erroneous based on visual inspection of the specimen, demonstrating the limitations of the 2D-XCOR technique for large strains in inhomogeneous samples.

5.4 Mechanical properties of collagen matrices. Toughness, modulus and strength were significantly higher in the SBF+Fet group compared with the SBF and Unmin groups. The modulus of the unmineralized group was also significantly higher than that of the SBF group. Lines above bars indicate $p < 0.05$. 

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5.5 Local mechanical properties of mineralized matrices. (a–e) Representative strain maps of (a) an unmineralized collagen matrix, (b) an ungraded SBF + Fetuin-mineralized collagen matrix, (c) an ungraded SBF mineralized collagen matrix, (d) a graded SBF+Fetuin mineralized collagen matrix and (e) a graded SBF mineralized collagen matrix. The frames shown for each sample were chosen such that the average strain in the frame was constant. The local strain analysis indicated that ungraded matrices expressed no strain field gradients. Strain was relatively constant along the lengths of unmineralized and ungraded samples. Strain decreased with increasing mineral content in the SBF+Fetuin group. Strain increased with increasing mineral content in the SBF group. (f) The average secant modulus as a function of position is shown for graded SBF and SBF+Fetuin scaffolds. Modulus increased with increasing mineral in the SBF+Fetuin group. Modulus decreased with increasing mineral in the SBF group.

5.6 (A) Plate-like mineral morphology was observed in the 10SBF group. (B) A dense coat of small mineral crystals was observed in the m10SBF group. (Outer scale bar = 10 μm, inset scale bar = 1 μm) Figure prepared by Dr. Justin Lipner and reproduced with permission.

5.7 Plots of modulus relative to mineral content demonstrate the stiffening effects of 10SBF and m10SBF (error bars represent standard deviation). The stiffening effect of m10SBF was significantly greater than 10SBF, as evidenced by a higher slope (analysis of covariance; p=0.05). Figure prepared by Dr. Justin Lipner and reproduced with permission.

5.8 Peak principal strain fields estimated from high frequency ultrasound imaging of a beating mouse heart. Four-chamber (left column: A,E,I,M,Q), long axis (middle column: B,F,J,N,R), and short axis (right column: C,G,K,O,S) views and a segmented 3D papillary muscle (PM) and chordae tendineae (CT) (D,H,L,P,T) of the heart were acquired throughout the cardiac cycle. Green-Lagrange strains were estimated using images acquired during isovolumetric contraction (A,B,C,D) as the reference (strain-free) configuration. Strain developed in the left ventricle as it contracted and blood was ejected from the heart, while the papillary muscles remained unstretched (E,F,G,H). As the heart cycle entered isovolumetric relaxation, strains in the heart wall reached peak levels on the order of 0.5 (I, J, K, L). As the heart relaxed during early ventricular filling, strain levels reduced (M,N,O,P), approaching baseline levels after late ventricular filling (Q,R,S,T). Throughout the cardiac cycle, strains in the papillary muscles (yellow arrows) were lower than those in the surrounding myocardium in the apex and base (white arrows). LV: left ventricle, RV: right ventricle, S: skin. Scale bar: 3 mm.
5.9  (A,B) Magnetic resonance images of mouse hearts showing the anatomical planes studied using 3D-DDE of ultrasound imaging volumes. (C) A schematic of the heart demonstrating the orientation of the short and long axis as well as the location of the infarction. (D,E) Peak principal strain at a specific time-point in control hearts. (F,G) Peak principal strain at this same timepoint in hearts following myocardial infarction, showing distinctly different strain patterns in both the long and short axis views. (H) Strain as a function of position along the midline of the long-axis view of the heart, showing strain attenuation in the infarcted tissue. Line corresponds to different times; position is measured from the base of the arrow in panel F. (I) Strain as a function of position along the midline of the short-axis view of the heart, showing strain attenuation in the infarcted tissue, and elevated strain in the tissue surrounding the infarct region. Lines again correspond to different times; position is measured from the base of the arrow in panel G. Scalebars: 1 mm.

5.10 The 2D-DDE and 2D-SIMPLE algorithms described the spatial and temporal patterns of embryonic wound closure, while the 2D-XCOR algorithm revealed only noise. (A, B, C) First principle strains in the radial direction away from the wound center for 90 bins around the wound (inset) with wound border marked by a circle. (A, D, G) For the circular punched wound, the first principal strain determined by 2D-DDE demonstrated an isotropic contractile ring around the wound border. A strain concentration was identified around the wound by the 2D-SIMPLE algorithm, consistent the presence on a localized isotropic contraction. (B, E, H) For the elliptical ablated wound, 2D-DDE demonstrated a localized ring of isotropic contraction and tension distal to the wound. A strain concentration was identified around the wound by the 2D-SIMPLE algorithm. (C, F, I) For the elliptical incision wound, 2D-DDE identified high tensile strain was at the leading edge of the incision and low strain in the wake of the incision. 2D-SIMPLE detected strain concentrations along the flanks of the wound. (J, K, L) 2D-XCOR failed to identify any patterns of strain at or near the wound sites. Scale bars = 200µm. Definitions: $E_{xx}$, strain in 11 direction; $\Delta$, 2D-SIMPLE difference.
5.11 Embryonic wounds were created using three methods: circular wounds created with a punch (top row), elliptical wounds created by ablation (middle row), and elliptical wounds created by incision with a micro-scalpel (bottom row). Strains were analyzed to delineate how three mechanisms combine to change the wound: (D,E,F) localized isotropic contraction around the wound, (G,H,I) passive elastic recovery of tissue distal to the wound, and (J,K,L) stretching introduced during wound creation. (A, B, C) Injuries induced by circular punching and elliptical ablation do not introduce additional deformations into the wound healing system. However, the elliptical incision method adds tension in the wake of the blade and compression ahead of the blade. (A, B, C) Localized isotropic contraction of wounds is expected at the border of the wound for all wound scenarios. (E, E, F) In response to localized isotropic contraction near the wound, regions distal to the wound are expected to be in tension, as cells near the wound pull inward to close the injury. (J, L, K) Since no additional deformations were introduced during wounding for circular punched and elliptical ablated injuries, no response to the wounding is expected in these cases. For elliptical incision injuries, however, the tissue is expected to respond to the incision deformations (C) by returning to its original state. (M, N, O) Strain concentrations are expected to arise at the wound border due to the localized isotropic contraction in all wound scenarios. For elliptical incision injuries, however, strains introduced during wounding combined with the localized isotropic contraction should result in strain concentrations primarily along the flanks of the elliptical wound.

6.1 Theoretical stresses and strains on scaffolds combining shape and stiffness gradients. Four scaffold groups were generated consisting of combinations of gradations in cross sectional area and gradations in shape (A, D, G, J). The following results for each of the four combinations was theorized: a scaffold with a uniform cross sectional area and a uniform strain would have a uniform stress (B) and uniform strain along its length (C); a scaffold with a gradient in cross sectional area but a uniform stiffness would have a gradient in stress (E) and a gradient in strain (F); a scaffold with a uniform cross sectional area but a gradient in stiffness would have a uniform stress (H) but a gradient in strain (I); and a scaffold with a gradient in cross sectional area and an inverse gradient of stiffness would have a gradient in stress (K) a uniform strain (L). The theorized stresses are based off of Equation 6.1.
6.2 Schematic procedure for the fabrication of PDMS stiffness gradients (g-PDMS). (A) Glass slides were coated with a silanizing reagent to make their surfaces hydrophobic (Sigmacote). (B) Molds were formed by clamping a Teflon insert between two silanized glass slides. (C) PDMS mixture was poured slowly into the mold. (D) The PDMS mixture was then either placed into an oven at 60°C for one hour (left) or set perpendicular on a heater ($T_{\text{surface}} = 190°C$) and crosslinked upon exposure to a temperature gradient for 1.5 h (right). (E) Excess crosslinker, oligomer and monomer were removed by rinsing in copious amounts of hexane, which swells the scaffolds and allows non-crosslinked reagents to escape. (F) Schematic of resulting uniform (left) or stiffness gradient scaffolds (right). Adapted from [1].

6.3 Results from the cell proliferation and cell density parameter study. Results demonstrated that, after 9 days in culture, too much FBS or too high initial seeding density resulted in cell confluence or even cell delamination. From these results, the optimal seeding density and FBS throughout the experiment was determined to be 2000 cells/cm$^2$ and 1% FBS, respectively.

6.4 Human mesenchymal stem cells cultured for 5 days on varying amounts of fibronectin-coated PDMS. Cells on fibronectin-coated scaffolds appeared similar in morphology to tissue culture plastic-grown cells, while those on PDMS coated scaffolds appeared rounded and did not adhere well to the surfaces.

6.5 A bioreactor capable of applying simple tensile strains to several scaffolds at once was designed in Solidworks (A) and constructed (B,C). A side view shows the overall design of the bioreactor (B) and a top down view with loaded scaffolds shows how scaffolds are configured in the bioreactor (C).

6.6 Average tensile (blue lines) and transverse (red lines) strains for uniform PDMS scaffolds stretched with an input sine wave to maximum 10% strain (A) and stretched with input sine wave to maximum 20% strain (B) on a custom designed bioreactor. Tensile strains confirm the bioreactor behaves as expected and optically estimated strains match input values.

6.7 The complete timeline of the experiment was 9 days of cell culture. Cells were first seeded onto PDMS scaffolds and allowed to attach for one day. The following day, unloaded control PDMS scaffolds were left adhered to their dishes and loaded scaffolds were transported to the bioreactor. After one additional day of static culture the scaffolds were loaded for 7 days. Specifically, scaffolds were loaded twice a day, for one hour a time, to 5% grip-to-grip strain at a rate of 0.5Hz, with an hour rest between the two loading bouts. On the seventh and final day of loading, scaffolds were fixed one hour after the second loading bout.
6.8 Validation of theoretical strains on scaffolds combining shape and stiffness gradients. Using 2D-DDE (Chapter 2), it was confirmed that measured strains were consistent with theoretical strains (Figure 6.1C,F,I,L) for all groups (A,B,C,D).

6.9 Three examples of possible outcomes for scaffolds. (A) Cells imaged along the length of a scaffold have nearly uniform expression of a gene. (B) Cells imaged along the length of a scaffold display a gradient in expression of a particular gene, from low expression on one end to high expression on the other. (C) Cells imaged along the length of a scaffold have a bimodal expression of a particular gene, with an optimal strain level at which expression is highest.

6.10 Example results for scaffolds loaded in the bioreactor showing a gradient in Runx2 expression. Images are plotted in the “jet” colormap to accentuate differences in expression.

6.11 Hypotheses for expression patterns following loading of the four groups in this study: (A) Scleraxis and Runx2 expression will be uniform in the uniform rectangle scaffolds, (B) Scleraxis and Runx2 expression will both increase with increasing stress and strain in the uniform trapezoid scaffolds, (C) Scleraxis expression will increase along the strain gradient while Runx2 expression will remain uniform in the graded rectangle scaffolds, and (D) Runx2 expression will increase along the stress gradient while Scleraxis expression will remain uniform in the graded trapezoid scaffolds. Theorized stress and measured strain for each of the four groups is shown in Figure 6.1 and Figure 6.8, respectively.
# List of Abbreviations

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<tr>
<td>2D-DDE</td>
<td>two dimensional direct deformation estimation</td>
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<td>2D-LSF</td>
<td>two dimensional least squared fit</td>
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<tr>
<td>2D-XCOR</td>
<td>two dimensional normalized cross correlation</td>
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<tr>
<td>2D-SIMPLE</td>
<td>two dimensional strain inference with measures of probable local elevation</td>
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<td>3D-DDE</td>
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<tr>
<td>LK</td>
<td>Lucas Kanade</td>
</tr>
<tr>
<td>BMP</td>
<td>bone morphogenic protein</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>EDX</td>
<td>energy-dispersive X-ray spectroscopy</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular regulated kinase</td>
</tr>
<tr>
<td>FAK</td>
<td>focal adhesion kinase</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>hMSC</td>
<td>human mesenchymal stem cell</td>
</tr>
<tr>
<td>IGF</td>
<td>insulin like growth factor</td>
</tr>
<tr>
<td>Ihh</td>
<td>indian hedge hog</td>
</tr>
<tr>
<td>MSC</td>
<td>mesenchymal stem cell</td>
</tr>
<tr>
<td>PAm</td>
<td>polyacrylamide</td>
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<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
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<td>PDMS</td>
<td>polydimethylsiloxane</td>
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<tr>
<td>Ptch</td>
<td>patched</td>
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<tr>
<td>PTHrP</td>
<td>parathyroid hormone-related protein</td>
</tr>
<tr>
<td>Runx2/Cbfa1</td>
<td>Runt-related transcription factor 2 / core-binding factor subunit alpha-1</td>
</tr>
<tr>
<td>SBF</td>
<td>simulated body fluid</td>
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<tr>
<td>Scx</td>
<td>scleraxis</td>
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<tr>
<td>Smo</td>
<td>smoothed</td>
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<tr>
<td>TAZ</td>
<td>tafazzin</td>
</tr>
<tr>
<td>YAP</td>
<td>yes associated protein</td>
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John Boyle

Washington University in Saint Louis
December 2017
Dedicated to my parents.
ABSTRACT OF THE DISSERTATION

Accurate Determination and Application of Local Strain for Studying Tissues with Gradients in Mechanical Properties

by

John Boyle

of Doctor of Philosophy in Biomedical Engineering

Washington University in St. Louis, December 2017

Research Advisor: Stavros Thomopoulos

Determination of the mechanical behavior of materials requires an understanding of deformation during loading. While this is traditionally accomplished in engineering by examining a force displacement curve for a whole sample, these techniques implicitly ignore local geometric complexities and local material inhomogeneities commonly found in biologic tissues. Techniques such as normalized cross correlation have been classically applied to address this issue and resolve deformation at the local level; however, these techniques have proven unreliable when deformations become large, if the sample undergoes a rotation, and/or if strain fields become incompatible (e.g. at or near failure).

Presented here is a toolbox of techniques that addresses the limitations of the prior state-of-the-art for localized strain estimation. The first algorithm, termed 2D direct deformation estimation (2D-DDE), directly incorporates concepts from mechanics into non-rigid registration algorithms from computer vision, eliminating the need to consider displacement fields, as
required for all of the prior state-of-the-art techniques. This results in not only an improvement in accuracy and precision of deformation estimation, but also relaxes compatibility of the deformation fields. A second algorithm, 2D Strain Inference with Measures of Probable Local Elevation (2D-SIMPLE), incorporates the results of 2D-DDE with results from algorithms that enforce strain compatibility to develop a robust detector of strain concentrations. While tracking local strain in a vinylidene chloride sheet in tension, 2D-SIMPLE detected strain concentrations which predicted the initiation of a crack in the material and the progression of the crack tip. The third and fourth algorithms generalize the two dimensional algorithms to analyze three dimensional deformations in volumetric images (3D-DDE and 3D-SIMPLE, respectively). Lastly, the 2D-DDE algorithm is modified to estimate two dimensional surface deformation from multi-view imaging systems.

The robustness and adaptability of these techniques was then validated and demonstrated on a wide variety of biomedical applications. Using 2D-DDE, a microscale compliant region was discovered at the tendon-to-bone attachment, local heterogeneity of partially mineralized scaffolds was revealed, and gradients in stiffness of partially mineralized nano-fiber scaffolds were demonstrated. Using 2D-SIMPLE, mechanisms of embryonic wound healing and associated strain localizations were elucidated. 3D-DDE confirmed the existence of strain gradients across chordae tendineae in beating murine hearts as well as demonstrated dramatic localized changes in wall deformation before and after myocardial infarction in murine hearts.

2D-DDE was also used to develop a model system to study the effects of applied stress versus the effects of applied strain on cells. The model system was first theorized by considering a system in which gradients of cross sectional area or scaffold shape were composed with
gradients in material stiffness. By combining these gradients in novel ways, it was theoretically determined that stress and strain could be locally isolated. A tensile bioreactor was constructed, techniques for fabricating scaffolds with gradients in stiffness and gradients in cross sectional area were developed, and theoretical strain gradients were confirmed experimentally using 2D-DDE. The model system was then validated for in vitro cell studies. Cell adhesion, proliferation, and viability following a seven day loading protocol were explored. Methods for determining single cell responses, which could be correlated back to a specific stress or strain states, were developed using immunocytochemistry and 2D-DDE approaches. Future studies will apply this model system to determine precise mechanotransduction responses of cells. These studies are critical to optimize stem cell tissue engineering strategies as well inform cell mechanobiology mechanisms.
Chapter 1

Introduction

The mechanical behavior and physical properties of materials are most commonly determined by deforming the material and analyzing the resulting stress-strain curve. Accurate determination of stress and strain are therefore necessary for predicting material responses to physical forces. These analyses can inform how and by how much a material will deform when subject to an applied force, if/when it will deform permanently, and when it will fracture. Stress is commonly calculated by simply dividing the measured force by the material cross-sectional area. Measurement of strain, on the other hand, is typically more difficult, particularly for heterogeneous and structurally complex materials. All measurement of strain involves comparing an initial reference configuration, or undeformed state, to a deformed state. The first device used to measure strain was the extension meter, later termed "extensometer", invented by Charles Huston in 1879. The original extensometer measured a change in the initial length of a sample by displacement of a pin on a calibrated scale [2]. Extensometers are still widely used today, however they measure deflection in more sophisticated ways, such as change of electrical resistance in a metal after stretch or deflection of a laser. While extensometers excel at determining global material properties of a sample, they fail to discern local differences of strain within a sample (Figure 1.1A).
Strain gauges, which are much smaller than extensometers and are placed directly on the deforming material, were the first devices to measure local strains. They work very similarly to extensometers in that they measure length changes, however their size and placement provide local strain measurements of the region on which they are attached. While these devices are useful for local strain measurement, each measurement requires an individual strain gauge, making them impractical for determining local strains of a large surface.

To address the limitations of extensometers and strain gages, optical or non-contact strain measurement techniques have been developed. These measurements are achieved by examining images before and after deformation, captured with a camera or similar imaging system. By considering displacements of visible features, which may be natural or artificially added to the sample, these techniques can determine local and global strains. The simplest optical strain measurement techniques involve measuring displacements of lines that are added to the sample, e.g., with dye or physical markers (Figure 1.1B,C). This technique can be expanded to measure strain in multiple dimensions by adding more sophisticated markings, such as squares. As the squares deform their corners can be used to compute a deformation tensor, yielding local two dimensional strain (Figure 1.1C).

The precise measurement of localized strain enables the study of complex non-uniform materials and informs how geometrically complex materials behave under applied loads. This is particularly useful for studying biologic tissues, which typically have heterogeneous material properties and complex structures. Examples of tissues in the body with locally varying mechanical properties include interfaces between mineralized and unmineralized tissues (e.g., the tendon to-bone-attachment) and regions of pathologic or injured tissues (e.g., infarcted myocardium). In many cases of engineering replacements for load-bearing tissues, locally
Figure 1.1: Examples of techniques used for the measurement of strain. (A) Overall length change following deformation for an entire sample, as determined using an extensometer, is the most simplistic strain measurement and does not reveal local deformation(s). (B) Adding markers, such as lines, to a deforming sample, then tracking the length change between the markers can reveal local strains. However, this is limited to one dimension and the overall number of localized strain measurements that can be computed is limited to the number of lines. (C) Instead of lines, squares or boxes can be added to the surface of a sample to measure two dimensional strain based on the movement of the corners or edges of the boxes.
varying mechanical properties are a design requirement for function. For example, the attachment of tendon to bone occurs across a functionally graded transitional tissue that dissipates stresses and allows for effective load transfer [3, 4, 5]. Evaluating if tissue engineered constructs behave mechanically similar to native tissues is necessary for tissue engineering, and accurate local strain measurement is necessary for this assessment.

1.1 Two dimensional strain estimation

1.1.1 Motivation

Analysis of images to detect and quantify spatial variations in deformation is critical for understanding morphogenesis [6], wound healing [7], tissue mechanics [8, 9, 10], and structural mechanics [11]. A standard approach for such analysis involves estimating strain fields inferred by comparing images of the same system taken at different times or under different conditions [12, 13]. At the core of this approach is an estimation and differentiation of displacement fields, often using algorithms to optimize the match between a region of a deformed image and a corresponding region of an undeformed image [13]. The mapping can be improved dramatically for large deformations through the Lucas-Kanade algorithm that applies and optimizes a “warping function” for the undeformed image (also known as the Newton-Raphson method) [14, 15, 16, 17]. These approaches have proven effective in studies of cell mechanics, where the image of a deformable medium contracted by cells is compared to images of the same medium after the cells have been deactivated or removed [18, 19, 20]. Similar approaches have been used to study collective cell motion [21], tissue morphogenesis.
[6], and tissue mechanics [8, 9, 10]. Lucas-Kanade approaches in particular have been used to improve displacement field estimation in a broad range of large strain applications [9, 22].

1.1.2 Previous state of the art

Cross correlation

Two dimensional cross correlation (2D-XCOR) is the most widely used strain estimation algorithm in biomedical applications. Cross correlation is performed by diving a template image into several regions and finding a best match region in a deformed image. Specifically, cross correlation finds these best match regions by computing a correlation coefficient for each pixel in the corresponding search region and then finding for the maximum value in the resulting correlation matrix. The correlation coefficient is computed using equation 1.1, where $T$ is a reference image and $I$ is a deformed image. The maximum value of the correlation matrix corresponds to the best match of the template region and can be used to compute a displacement between the two images [23].

$$r = \frac{\sum_i \sum_j (T_{i,j} - \bar{T}) (I_{i,j} - \bar{I})}{\sqrt{\left(\sum_i \sum_j (T_{i,j} - \bar{T})^2\right) \left(\sum_i \sum_j (I_{i,j} - \bar{I})^2\right)}}$$  \hspace{1cm} (1.1)

Following image registration using normalized cross correlation, strain fields can be estimated from the displacements of the midpoints of each region, often through calculation of a deformation gradient tensor that can be used to relate the spatial gradient of displacement fields to the strain fields:
The technique for computing local strain with cross correlation is summarized in Figure 1.1.2.

An important shortcoming of cross correlation is that it is a rigid image registration technique. As discussed in more detail in Chapter 2, the algorithm assumes that the texture in the search region is identical to the texture in the template image and that it has not changed shape, which is an obvious shortcoming when attempting to estimate deformation. However, cross correlation is relatively robust to small shape changes and therefore performs reasonably well under small strain conditions. Biologic tissues, however, often undergo large strains which render rigid techniques, including cross correlation, unsuitable for reliably estimating strain for these cases.

Cross correlation also struggles to properly find search regions when samples have rotated. Because the standard cross correlation algorithm does not account for rotation, only translation, it will fail to properly match pixel intensities after a region has rotated. Expansions of the basic cross correlation algorithm can account for this by first computing the displacement of a region and then, assuming the rotation is small, refine the estimate by computing a cross correlation matrix for a third parameter, $\theta$, the rotation about the centroid of the search region. This technique, however, still relies on the texture being similar enough before rotation that cross correlation can reliably find the correct region in the deformed and rotated image. It also increases the computational cost because additional correlations need to be computed following the standard cross correlation algorithm [24].
Figure 1.2: Summary of the algorithm for computing local strain from normalized cross correlation. (A) A reference frame (left) is initially divided into several regions (black boxes). The locations of these regions is unknown in the deformed frame (right). (B) For each region (in this example, a region containing an eye), a correlation coefficient is computed for every possible position of the region template (middle) in the deformed image, resulting in a correlation matrix (right image). The maximum of the correlation matrix is the location of the region template in the deformed image (black arrow, red dot in correlation matrix image). (C) This is repeated for every region in the template image (black boxes with green center) to locate their corresponding location in the deformed image (black boxes blue centers). (D) After finding the locations of each region, several nearby regions are combined and the deformation tensor is computed by comparing the displacements of the regions before and after deformation.
Many techniques exist to refine cross correlation to sub-pixel displacements. The most widely used techniques involve fitting functions around the maximum value of the correlation matrix, then finding the maximum of the fit function to a defined numerical precision [25]. Many function types can be used, but the most common are quadratic. Alternative techniques exist which implement a Newtonian optimization following cross correlation. These methods work by employing a displacement based warping function, described in section 1.1.2, following cross correlation for the purpose of obtaining sub-pixel refinements [26].

**Lucas Kanade algorithm from computer vision**

A commonly used technique for image correlation in the field of computer vision is the Lucas-Kanade (LK) method for optical flow estimation [15]. Rather than doing a comprehensive search for a template within a search region in a corresponding image, the Lucas-Kanade algorithm seeks to iteratively minimize an energy equation by modulating parameters of a warping function that relate the template to the corresponding image. Specifically, the algorithm seeks to minimize the following equation:

\[
\sum [I(W(x; p)) - T(x)]^2
\]

where \(T\) is a template image, \(I\) is an input image, and \(W(x; p)\) is a warping function with parameters \(p\) that can be modulated. By taking a Taylor expansion of this energy equation around \(p\), an expression for iterative parameter updates \(\Delta p\) can be found:

\[
\Delta p = H^{-1}\sum_x \left[ \nabla I \frac{\partial W}{\partial p} \right]^T [T(x) - I(W(x; p))]
\]
where $H$ is a second order approximation to the Hessian matrix:

$$H = \left[ \nabla I \frac{\partial W}{\partial p} \right]^T \left[ \nabla I \frac{\partial W}{\partial p} \right]$$

Using this equation iterative parameter updates of $p$ can be computed until the norm $\|\Delta p\|$ drops below a defined threshold. For a modified and computationally efficient inverse compositional version of this algorithm, each successive parameter update of $p$ is given by the composition of $W(x; p)$ and $W(x; \Delta p)$:

$$W(x; p) \leftarrow W(x; p) \circ W(x; \Delta p)^{-1}$$

Importantly, the requirements of the warp, $W(x; p)$ are (1) that it is differentiable with respect to $p$ and (2) that it can be inverted and then composed with the current estimate or in other words the set of warps must form a group [15].

**Estimation of deformation with least squares fit Lucas-Kanade**

Two common warping functions that are used with the Lucas-Kanade algorithm to estimate deformation are a rigid translation and an affine transformation given by the following equations, respectively:

$$W(x; p) = \begin{bmatrix} 1 & 0 & p_1 \\ 0 & 1 & p_2 \end{bmatrix} \begin{bmatrix} x \\ y \end{bmatrix}$$

$$W(x; p) = \begin{bmatrix} 1 + p_1 & p_3 & p_5 \\ p_2 & 1 + p_4 & p_6 \end{bmatrix} \begin{bmatrix} x \\ y \end{bmatrix}$$
Solving for the parameters $p$ of either of these warping functions results in displacement estimations ($p_1$ and $p_2$ in equation 1.7 and $p_5$ and $p_6$ in equation 1.8) which can be used to compute deformation gradient tensors given equation 1.2. Since these techniques still rely on a least squared fit of the displacement gradient, we term this group of techniques the Least Squared Fit Lucas Kanade methods (2D-LSF-LK, or 2D-LSF).

Similarly to cross correlation, these techniques rely on displacement estimation to determine deformation gradient tensors. For the rigid body translation warping function (Equation 1.7), while their implementations differ, the result is nearly identical to cross correlation: regions are rigidly matched and displacement information is obtained. An important distinction, however, for the affine transformation (Equation 1.8) is that the additional parameters allow for non-rigid registration between the template and deformed image. Specifically, in the affine transformation, the first four parameters of the warp ($p_1$, $p_2$, $p_3$, and $p_4$) permit pixels within a region to move relative to one and other or for the entire region to change shape. Allowing shape change of a region during registration increases registration precision and therefore displacement estimation relative to rigid registration techniques. Since the prior state of the art relies on displacement estimation for deformation estimation, this improvement in image registration improves strain estimation accuracy and precision [13].

Improvement of these techniques will be greatly beneficial to the biomedical field, where deformations are often large, inhomogeneous, and may contain concentrated areas of deformation. This will lead to improvements in identifying and charactering tissues with variations in material properties, like the tendon-to-bone attachment. Aside from characterizing native tissue, these techniques could be applied to tissue engineered scaffolds. For many tissue engineered scaffolds, it is critical to match the mechanical properties of the scaffolds
to those of the native tissue; verification of this requires highly precise and accurate deformation estimation techniques. Lastly, concentrations of deformation are challenging to analyze with current techniques, yet they are critical to analyze because they lead to cracks and subsequent material failure.

1.1.3 Current approach

Although these aforementioned techniques have enabled progress in numerous research areas, they are subject to large errors when deformation is high or localized. Specifically, any inaccuracies from the displacement estimates are amplified by the numerical derivatives needed to estimate strain fields [12, 13]. Furthermore, minor mis-tracking of displacements can lead to an artifact that is typically indistinguishable from a region in which strain is high. Many techniques exist for incorporating the physics of the specific class of phenomena being imaged in an effort to improve strain calculations [27]. The focus of this thesis, however, was to develop a simple and general algorithm, without making assumptions about the mechanics of the material, for improving the accuracy of strain estimates. As described in Chapters 2 and 3, we achieved this by developing the “Direct Deformation Estimation” algorithms (2D-DDE and 3D-DDE), and associated algorithms for distinguishing regions of elevated strain from displacement mapping artifacts, the “Strain Inference with Measures of Probable Local Elevation” (2D-SIMPLE and 3D-SIMPLE) algorithms.
1.2 Three dimensional (volumetric) strain estimation

1.2.1 Motivation

Characterizing the mechanical behavior of biologic tissues requires the precise and accurate determination of strain fields. While two dimensional methods are ubiquitously used in this characterization, they inherently assume that the deformation on the surface correlates with the behavior of the bulk of the material. While two dimensional strain estimation techniques can be combined with imaging modalities that penetrate into the bulk of a material to analyze the interior structure, the results from applying these two dimensional techniques to three dimensional structures are difficult to interpret for several reasons. First, the two dimensional imaging plane discards most of the information associated with a three dimensional structure. Second, out of plane motion often results in an inability to track structures as they move in an out of the imaging plane, resulting in failure of image registration algorithms to find an appropriate solution. Third, if structures remain in the imaging plane their out of plane motion may be interpreted as deformation rather than motion, resulting in erroneous deformation estimation. To address these issues, full volumetric datasets should be combined with three dimensional deformation analysis techniques to determine the mechanical behavior of three dimensional structures.
1.2.2 Prior state of the art

Digital volume correlation

Deformation or strain estimation of volumetric data sets is typically accomplished using digital volume correlation [28, 29, 30, 31, 32, 33, 34]. These algorithms, similarly to their two dimensional counterparts, divide a reference image into several smaller volumes and search for where these volumes have displaced to a corresponding deformed image. Following registration of the volumes, coordinate systems are differentiated to determine deformation fields or strain fields. The most widely implemented class of these algorithms is three dimensional cross correlation (3D-XCOR). 3D-XCOR registers images by computing a similarity measure for all nearby pixels in a corresponding image, then considers the maximum of the correlation matrix as the location of the corresponding image. The similarity measure, termed the correlation coefficient, is computed with the following equation:

\[
r = \frac{\sum_i \sum_j \sum_k (T_{i,j,k} - \overline{T}) (I_{i,j,k} - \overline{I})}{\sqrt{\left(\sum_i \sum_j \sum_k (T_{i,j,k} - \overline{T})^2\right) \left(\sum_i \sum_j \sum_k (I_{i,j,k} - \overline{I})^2\right)}}
\]  

(1.9)

Techniques for sub-pixel estimation of displacements following three dimensional cross correlation are similar to those of two dimensional cross correlation. These techniques involve creating interpolation functions around the local maxima of the correlation matrix and determining the maximum on the refined grid. Interpolation functions are often taken to be quadratic. Alternative techniques exist that employ a Newtonian optimization following cross correlation to determine sub-pixel displacements [35, 34]. Three dimensional cross correlation, like its two dimensional analogue, is a rigid registration technique and subject
to issues similar to its two dimensional analogue. More specifically, it is unable to register image volumes after large deformations or rotations. This renders it not suitable for large deformations or volumes undergoing rotation.

**Three dimensional (volumetric) Lucas Kanade**

The three dimensional version of the Lucas Kanade algorithm is identical to its two dimensional analogue (Section 1.1.2) with the exceptions that the warped and template images are volumetric images rather than two dimensional images and the warping functions are updated to be three dimensional rather than two dimensional. Two three dimensional warping functions that are used with the volumetric Lucas-Kanade algorithm to estimate deformation are a rigid translation and an affine transformation given by the following equations, respectively:

\[
W(x; p) = \begin{bmatrix}
1 & 0 & 0 & p_1 \\
0 & 1 & 0 & p_2 \\
0 & 0 & 1 & p_3
\end{bmatrix}
\begin{bmatrix}
x \\
y \\
z
\end{bmatrix}
\] (1.10)

\[
W(x; p) = \begin{bmatrix}
1 + p_1 & p_4 & p_7 & p_{10} \\
p_2 & 1 + p_5 & p_8 & p_{11} \\
p_3 & p_6 & 1 + p_9 & p_{12}
\end{bmatrix}
\begin{bmatrix}
x \\
y \\
z
\end{bmatrix}
\] (1.11)

Solving for the parameters \( p \) of either of these warping functions results in three dimensional displacement estimations (\( p_1, p_2, \) and \( p_3 \) in equation 1.10 and \( p_{10}, p_{11}, \) and \( p_{12} \) in equation 1.11) which can be used to compute deformation gradient tensors given equation 1.2. Since these techniques still rely on a least squared fit of the displacement, we term this group of techniques the Least Squared Fit Lucas Kanade methods (3D LSF-LK, or 3D-LSF).
techniques have been used to refine sub-pixel displacements following 3D-XCOR [35, 34] or own their own to estimate displacements [36].

Similarly to 3D-XCOR, these techniques rely on displacement estimation to determine deformation gradient tensors. The rigid body translation warping function (Equation 1.10) still suffers from the same downfalls of cross correlation, despite its different implementation. An important distinction, however, for the affine transformation (Equation 1.11) is that the additional parameters allow for non-rigid registration between the template and deformed image. Specifically, in the affine transformation, the first nine parameters of the warp \( p_1, p_2, p_3, p_4, p_5, p_6, p_7, p_8, \) and \( p_9 \) permit voxels within a region to move relative to one and other or for the entire region to change shape or to rotate. Allowing shape change during registration improves registration accuracy which in turn improves displacement estimation. Naturally, since deformation estimation in the prior state of the art relies on displacement estimation this also results in an increase in accuracy and precision of deformation estimation. Improvement of these techniques has the potential to improve several areas of biomedical research. One area is cardiac research. Current volumetric deformation estimation techniques do not have enough resolution to resolve and study small structures in the heart, like the chordae tendineae. Improved three dimensional deformation estimation could also lead to new techniques to compare between healthy and diseased state hearts.

1.2.3 Current approach

The current state of the art techniques are accurate and efficient for certain applications but are limited in general applicability because they use rigid registration [36, 29], require
inherent assumptions about a material or the strain fields, [28, 30], or require strain compatibility [31]. Although methods exist for refining three-dimensional strain fields, these rely heavily upon post hoc regularization that tends to mask strain concentrations and efforts to overcome this limitation are a topic of intense focus [30, 37, 38]. By building on our work estimating 2D strains using warped digital image correlation (Chapter 2), we develop a technique which estimates deformation in volumetric images without consideration of displacement fields (Chapter 3). The result is the first computationally efficient and unconstrained 3D strain estimation algorithm for full volumetric data sets, which reliably determines strains within tissue volumes without material assumptions or the imposition of strain compatibility.

1.3 Use of spatial gradients in stress and strain for engineering complex tissue interfaces

Load-bearing tissues with spatial gradients in mechanical properties cannot be characterized or synthesized without accurate determination of local strain. For example, the healthy tendon-to-bone attachment is a transitional tissue that facilitates the transfer of load from compliant tendon to stiff bone. This tissue is able to accomplish this transfer of load across a two order of magnitude stiffness difference through gradients in structure, composition, and mechanical properties [39, 5, 40, 41]. Following injury and repair with traditional surgical techniques, this natural transitional tissue is not reformed and is instead replaced by an abrupt interface between tendon and bone. The failure to recapitulate the graded nature of the natural tissue leads to high repair site failure rates [42]. Therefore, tissue engineering
strategies have attempted to create functionally graded materials for enhancing tendon-to-bone repair, and characterization of such scaffolds requires accurate assessment of the local mechanical properties.

1.3.1 The healthy tendon-to-bone attachment

The adult tendon-to-bone attachment was classically divided into four distinct regions: tendon, unmineralized fibrocartilage, mineralized fibrocartilage, and bone (Figure 1.3). However, more recent evidence has demonstrated that the tendon-to-bone attachment contains gradients in material and mechanical properties rather than distinct zones. Tendon consists of highly aligned collagen fibers. As they insert into the bony interface, these fibers become less aligned creating a gradient in the fiber alignment distribution from tendon to bone [5]. Bone is highly mineralized while tendon is unmineralized; across the attachment, there is a gradual reduction of mineral content as it transitions from bone, to mineralized fibrocartilage, to fibrocartilage, and finally to tendon proper [39]. Bone and tendon are rich in type I collagen, while the fibrocartilaginous region also includes type II collagen [40]. Collectively, these gradients in structure and composition contribute to the tissue’s ability to carry load across a two order of magnitude difference of modulus without the emergence of stress concentrations [5, 39, 40].

1.3.2 Tissue engineering the tendon-to-bone attachment

Efforts to engineer the tendon-to-bone attachment have focused on generating functionally graded tissues through cell-guided formation and/or through direct scaffold fabrication. Studying the development of the tendon-to-bone attachment has been instructive for how
Figure 1.3: The healthy adult tendon-to-bone attachment is classically divided into four distinct zones: tendon, unmineralized fibrocartilage, mineralized fibrocartilage, and bone. A toluidine blue–stained section from an adult rat supraspinatus tendon-to-bone attachment is shown on the left.

cells may form such a complex tissue. During fetal and postnatal development of the tendon-to-bone attachment, gradients in cell signaling factors, cell phenotypes, and material properties define the blueprint for a functional adult tendon-to-bone attachment [43, 44, 45, 46, 47]. Local mechanical cues are instrumental in driving the formation of this tissue. Removal of force across the tendon-to-bone attachment, e.g., by spaceflight or muscle paralysis, results in a disorganized tissue with poor mechanical properties [48]. It is therefore unsurprising that many of the biochemical signals involved in tendon-to-bone attachment development have been found downstream of mechanical stimuli.

There are a number of factors associated with tendon, tendon-to-bone attachment, and bone formation. Scleraxis (Scx) is a tendon-specific transcription factor associated with developing and mature tendons and is often used as a marker of tenogenesis [49, 50, 51, 52]. Runx2 is a transcription factor that is considered a central regulator of bone development and
differentiation [53] and is often used as a marker of osteogenesis. At the interface between tendon and bone, expression of a number of factors have been reported, including Scx, Runx2, and chondrogenic factors such as Sox9 and Gli1. *In vivo* and *in vitro* studies have associated expression of these factors with mechanical loading and a gradient in loading may drive a gradient in cell differentiation [40]. These gradients and tissue formation markers may be useful for developing *in vitro* analogues of the tendon-to-bone attachment for use in basic and translational studies of tendon-to-bone repair.

Traditionally, fabrication of tissue engineered scaffolds has been approached in a uniform manner. Researchers typically attempt to engineer a uniform tissue without consideration of local heterogeneity. However, local heterogeneity is critical for engineering tissue interfaces like the tendon-to-bone attachment, which have gradients in cell phenotypes and mechanical behavior. There have been numerous attempts to recapitulate the complex arrangement of the natural tendon tendon-to-bone attachment using biochemical [54] or material gradients [132, 56, 57, 58]. Alternatively, there have been studies creating multiphasic scaffolds, with each individual region tuned for a corresponding zone in the tendon to bone attachment [59, 60, 61]. Rather than approaching the problem from a materials point of view, other groups have investigated if chemical gradients of biologic factors are sufficient to pattern cells into the corresponding cell types of the tendon-to-bone attachment [62, 54].

While somewhat successful in recapitulating various structural aspects of the native tissue, few prior attempts to engineer the tendon-to-bone attachment have investigated incorporating mechanical stimuli into the design. Among these studies, Thomopoulos *et al.* used collagen scaffolds in either tension or tension and compression while Morita *et al.* studied gradients in applied strain, but only investigated tenogenesis [63, 64]. Since mechanical
signaling has been shown to be critical for proper development of the tendon-to-bone attachment [48], in Chapter 6 we propose an approach which relies on mechanical gradients in stress or strain in the absence of chemical or material signals to engineer a tendon-to-bone attachment. When employed, this technique will reveal how mechanical stimuli can control cellular differentiation in a graded fashion and also lay the groundwork for future studies which incorporate graded mechanical stimuli along with material and chemical stimuli.

1.4 Scope and procedure of the dissertation

This thesis describes the development of highly precise and accurate 2D and 3D strain estimation methods that do not rely on strain compatibility, previous knowledge of the material’s properties, or assumptions about the deformation. In Chapter 2 these techniques are introduced in two dimensions and in Chapter 3 they are expanded to three dimensional volumes. In Chapters 2 and 3, these techniques are used as a foundation for a new technique capable of detecting local strain concentrations. In Chapter 4, a theoretical framework for applying these techniques to multi-view imaging modalities is explored. The efficacy of these techniques is tested in a wide variety of biomedical applications in Chapter 5, including mechanical characterization of the tendon-to-bone attachment, tissue engineered tendon-to-bone attachments, wound healing, and myocardial infarction. Lastly, in Chapter 6, these techniques are used to develop a novel in vitro system to drive mesenchymal stem cells differentiation via independently controlled gradients of stress and strain.
1.5 Specific aims

Aim 1

Develop two dimensional techniques that integrate mechanics theory with cutting edge computer vision algorithms for the direct estimation of strain from a sequence of images.

Hypothesis

Strain estimation techniques that directly incorporate mechanics concepts with computer vision techniques will allow for direct estimation of strain and result in more accurate and precise estimation when compared to techniques which rely on displacement estimation.

Rationale

The prior state of the art techniques in optical strain estimation rely on taking derivatives of displacement fields computed from digital image registration. Any noise introduced in the system by minor mis-tracking of a region is amplified when derivatives of the displacement fields taken to compute deformation. Displacement-based algorithms also pose additional constraints on the computation, namely strain compatibility. While enforcement of compatibility may smooth out noise, it also has the effect of smoothing out strain fields which are non compatible, such as those near cracks or highly concentrated strain. After careful consideration of how the digital image registration is computed, it is clear that mechanics can be directly incorporated into the image registration algorithm for direct estimation of deformation without consideration of displacement. Incorporation of mechanics concepts into the computer vision algorithms and image registration step will provide higher resolution strain estimation, yield more accurate strain estimation, and allow for relaxation of strain compatibility.
Study Design

Algorithms which directly incorporate deformation concepts into image registration algorithms will be developed and implemented in the MATLAB programming language. Following implementation, the algorithm will be used to determine deformations on images deformed \textit{in silico} so that true deformation fields are known. Average root mean square (RMS) error from true values of deformation will be computed for each estimated strain and results will be compared to the prior state of the art. Following validation, the algorithm will be tested on a real data set consisting of a PDMS sheet with a stiffness gradient and results will be compared to the prior state of the art.

Anticipated Results

Algorithms directly incorporating mechanics into computer vision techniques will be more accurate and precise than the prior state of the art.

Broader Impact

Local strain measurement is critical to understanding the mechanics of complex, non-uniform materials and are not limited to biologic applications. For example, glacial rifts and fronts of earthquakes begin as strain localizations before failure and estimates of strain at these fronts could be critical for understanding glacial mechanics. Furthermore, the method would be useful in monitoring of civil structures. We are hopeful that the increased accuracy and precision of these techniques will enable easy strain estimation and strain concentration detection across many fields.

Aim 2
Expand two dimensional techniques that directly integrate mechanics into cutting edge computer vision algorithms to three dimensions to directly estimate strain from volumetric image sequences.

Hypothesis

Expansion of two-dimensional strain techniques to fully volumetric image sequences will allow for more accurate precise and more precise accurate determination of full three dimensional strain tensors compared to techniques which rely on displacement estimations.

Rationale

Volumetric strain estimation is critical to understanding the internal mechanical behavior of materials. Many biologic materials are non-heterogeneous; therefore, the mechanical behavior of their surfaces differs from the mechanical behavior of their interior. While two dimensional techniques such as ultrasound are capable of viewing internal structures of materials, they only reveal a two dimensional slice of that internal structure and are susceptible to out of plane motion. Therefore, algorithms which provide the most accurate, precise, and regularization free measurement of full field volumetric strains are crucial for understanding the mechanical behavior of complex non-heterogeneous three dimensional structures.

Study Design

Three dimensional algorithms which directly incorporate deformation concepts into image volume registration algorithms will be developed and implemented in the MATLAB programming language. Following implementation, the algorithm will be used to determine deformations on image volumes deformed in silico so that true deformation fields are known.
Average root mean square (RMS) error from true values of deformation will be computed for each estimation and compared to the prior state of the art for incompressible deformation, pure rotation, and combined rotation and deformation. The algorithms will then be compared on image volumes with artificially added noise since many volumetric imaging techniques are inherently noisy.

**Anticipated Results**

Three dimensional algorithms directly incorporating mechanics into computer vision techniques will be more accurate and precise than the prior state of the art. This trend will also apply to noisy image volumes.

**Broader Impact**

Volumetric strain estimation is not limited to biologic applications. Mechanical engineers working with complex non-heterogeneous structures may also be interested in internal strains or strain concentrations of their composite materials. Similarly, geologists studying plate tectonics are interested in how geologic materials are moving and deforming in three dimensions within the earth’s crust and would also benefit from advanced volumetric strain analysis techniques.

**Aim 3**

Develop a bioreactor system to evaluate how mesenchymal stem cells respond to gradients in stress and/or strain.

**Hypothesis**
The mechanical effects of stress and strain can be independently applied to cells in a graded fashion by carefully controlling the material properties and the shape of scaffolds in a tensile bioreactor system.

Rationale

While many previous studies have demonstrated mesenchymal stem cell differentiation in response to applied mechanical loads, the methods of applied load, surface presentation, amount of applied load, and differentiation results have all varied widely. This study seeks to develop a system which simplifies these results by controlling, in a graded fashion, for a continuous spectrum of independently controlled stress or strain. Such a system will not only be capable of revealing if cells respond to stress or strain but may also reveal precise bands of stress or strain that are optimal for passively applied mechanical signals for mechanotransduction-aided differentiation. This system will also control for surface presentation so that cells are responding differentially to the applied mechanical stimulation rather than their local cell adhesion environment.

Study Design

A model system capable of independently applying stress or strain to cells will be developed and validated. Validation will include preliminary experiments demonstrating cell viability and the potential to control differentiation based on mechanical signals alone.

Anticipated Results

The model system will demonstrate the ability to passively isolate mechanical stress or strain in a tensile bioreactor while maintaining cell viability.

Broader Impact
While it is well understood that mesenchymal stem cells respond to forces in their environment, it is not known how they respond to stress versus strain. Determining the differential response of cells to these independent stimuli will not only demonstrate the efficacy of complex tissue engineering using mechanics alone, but will also reveal precisely what aspect of the mechanical stimuli cells respond to. Dysregulation of mechanical signaling is also implicated in many disease states, including cancer. The development of this model system will allow for future studies that may reveal if stress and/or strain environment is implicated in these disease states. This information will be critical for understanding mechanotransduction signaling pathways which will guide future mechanotransduction-based tissue engineering strategies.
Chapter 2

Accurate and precise methods for estimating strain in two dimensions

Portions of this chapter were adapted from:

2.1 Abstract

When mechanical factors underlie growth, development, disease, or healing, they often function through local regions of tissue where deformation is highly concentrated. Current optical techniques to estimate deformation can lack precision and accuracy in such regions due to challenges in distinguishing a region of concentrated deformation from an error in displacement tracking. Here, we present a simple and general technique for improving the accuracy
and precision of strain estimation and an associated technique for distinguishing a concentrated deformation from a tracking error. The strain estimation technique improves accuracy relative to other state-of-the-art algorithms by directly estimating strain fields without first estimating displacements, resulting in a very simple method and low computational cost. The technique for identifying local elevation of strain enables for the first time the successful identification of the onset and consequences of local strain concentrating features such as cracks and tears in a highly strained tissue. More generally, the analytical methods we have developed provide a simple tool for quantifying the appearance and magnitude of localized deformation from a series of digital images across a broad range of disciplines.

2.2 Introduction

Mechanical characterization of inhomogeneous and/or geometrically complex biological tissues requires precise and accurate determination of strain fields. A standard approach for such analysis, termed optical strain estimation, involves estimating strain fields inferred by comparing images before and after a deformation [12, 13]. Optical strain estimation techniques work by examining before and after images, captured with a camera or similar imaging system, of a deforming sample and comparing the length change of the sample to determine strain. By considering displacements of visible features, which may be natural or artificially added to the sample, these sets of techniques can determine local or global strains. The simplest optical strain measurement techniques involves measuring displacements of lines artificially added to the sample. More sophisticated techniques often utilize digital image correlation or image registration and can measure strain in multiple dimensions. Applications of optical strain estimation include understanding morphogenesis [6], wound healing
(Section 5.7 and [7]), tissue mechanics (Section 5.2 and [8, 9, 10, 91]), tissue engineering (Section 5.3, Section 5.4 and Chapter 6), and structural mechanics [11].

In the most simple one-dimensional case, engineering strain is defined as the change in length of a sample, \( \Delta L \), divided by the original length, \( L \):

\[
\epsilon = \frac{\Delta L}{L}
\]  

(2.1)

In finite strain or large strain theory, an alternate formulation of strain is more commonly used, the stretch ratio, \( \lambda \), which is the deformed length, \( l \) divided by the original length, \( L \):

\[
\lambda = \frac{l}{L}
\]  

(2.2)

The stretch ratio, \( \lambda \), is related to the engineering strain by the following equation:

\[
\epsilon = \lambda - 1
\]  

(2.3)

Multidimensional strain estimation most commonly relies on first estimating the deformation gradient tensor, \( \mathbf{F} \). The deformation gradient tensor is an infinitesimal measure that describes the shape change of an infinitesimal region of a deforming body. It is analogous to the one dimensional stretch ratio, \( \lambda \), and related to the deformed and undeformed coordinate systems by the following equation:

\[
d\mathbf{x} = \mathbf{F}d\mathbf{X}
\]  

(2.4)
The Green-Lagrange strain tensor, \( \mathbf{E} \), which is analogous to engineering strain in one dimension, \( \epsilon \), can then be calculated from \( \mathbf{F} \) using the following equation:

\[
\mathbf{E} = 0.5(\mathbf{F}^T \mathbf{F} - \mathbf{I})
\]  

(2.5)

where \( \mathbf{I} \) is the second order identity tensor.

### 2.2.1 Prior state of the art

Digital image correlation is a well-established technique for determining strain fields on the surfaces of deforming materials [13]. The technique involves matching patterns between pairs of images to estimate the displacement of certain regions or features on a sample [13, 17].

The correlation is computed using equation 1.1, where \( \mathbf{T} \) is a reference image and \( \mathbf{I} \) is a deformed image. The peak of the correlation matrix corresponds to where the template region best matches the pixel intensities in the search region, yielding a new position for where the search region in the template image displaced to [23]. Both spatial domain and frequency domain approaches for cross correlation have been studied, with the latter being more computationally efficient and generally preferred [23].

Traditionally, strain calculations are performed after digital image correlation by binding the midpoints of matched regions to form quadrilateral elements. The initial and displaced positions of the points are used to estimate the deformation gradient tensor \( \mathbf{F} \), by taking derivatives of the undeformed \( \mathbf{X} \) and deformed \( \mathbf{x} \) positions, yielding \( d\mathbf{X} \) and \( dx \) respectively for each set of bound midpoints. Deformation is then computed using a least squares fit (LSF) of equation 2.4 [13]. Strain is then estimated from \( \mathbf{F} \) using equation 2.5. We term this technique of estimating strain normalized cross correlation (2D-XCOR).
A central limitation of 2D-XCOR is that it based on a rigid registration technique. 2D-XCOR searches for exactly matched regions between successive frames and assumes they only displace over time and do not deform or change orientation. Because of this, it is not suitable for tracking large deformations or samples undergoing orientation changes such as rotations.

An alternative to rigid registration techniques is non-rigid (i.e., deformable image) registration, which allows an image to deform and change orientation during image registration. Non-rigid approaches involve optimization to minimize an energy function iteratively:

\[
\sum [I(W(X; p)) - T(X)]^2
\]

where \(T(X)\) is a template image, and \(I(W(X; p))\) is an image, \(I\), warped by a defined warping function \(W(X; p)\) whose warping parameter \(p\) can be modulated. By taking a Taylor expansion of the energy equation and solving for an incremental update, the Lucas Kanade (LK) algorithm iterates using the following increments for \(p\):

\[
\Delta p = H^{-1} \sum_x \left[ \nabla I \frac{\partial W}{\partial p} \right]^T [T(X) - I(W(X; p))]
\]

until the norm \(\|\Delta p\|\) drops below a defined threshold \([15]\). In this equation, \(H\) is the Gauss-Newton approximation to the Hessian matrix:

\[
H = \left[ \nabla I \frac{\partial W}{\partial p} \right]^T \left[ \nabla I \frac{\partial W}{\partial p} \right]
\]

and successive updates of \(p\) are given by:

\[
p \leftarrow p + \Delta p
\]
The Lucas Kanade algorithm, however, is computationally expensive, due to the requirement that the gradient of the input image $\nabla I$, the Jacobian $\partial W/\partial p$ of the warp, and the Hessian matrix $H$ must be recomputed every iteration. The Lucas Kanade inverse compositional algorithm improves upon this by inverting the roles of the template and input images so that the these quantities can be precomputed on the template image and at an initial warp of $W(X; 0)$ \[15\]. With this new approach we have the following energy equation:

$$\sum [T(W(X; \Delta p)) - I(W(X; p))]^2$$ \hspace{1cm} (2.10)

By first assuming that a warp $W(X; 0)$ is the identity warp, then taking the Taylor expansion of Equation 2.10, and lastly solving for $\Delta p$, an expression for an incremental update to $p$ is obtained:

$$\Delta p = H^{-1} \sum_x \left[ \nabla^T \frac{\partial W}{\partial p} \right] T \left[ I(W(X; p) - T(X)] \right. \hspace{1cm} (2.11)$$

where $H$ is the Hessian matrix:

$$H = \left[ \nabla^T \frac{\partial W}{\partial p} \right] T \left[ \nabla^T \frac{\partial W}{\partial p} \right] \hspace{1cm} (2.12)$$

and successive updates are computed by:

$$W(X; p) \leftarrow W(X; p) \circ W(X; \Delta p)^{-1}$$ \hspace{1cm} (2.13)

This approach allows pre-computation on the gradients on the template image, as well as computation of the Jacobians of the warp $W$ at $(X; 0)$. As shown by Baker and Matthews and by Hager and Belhumeur, the inverse compositional algorithm is equivalent to the original Lucas Kanade algorithm since the initial estimate of the parameters is approximately correct [15, 65].
Following optimization, displacement parameters from $\mathbf{p}$ are used to estimate strains from a least squared fit of equation (2.4). We refer to this method as the 2D Least Squared Fit (2D-LSF) method [14, 15, 16, 17]. These approaches have proven effective in studies of cell mechanics [18, 19, 20], collective cell motion [21], tissue morphogenesis [6], tissue mechanics [8, 9, 10], and large strain applications [9, 22].

A central limitation of estimating strain fields using existing digital image correlation methods, like 2D-LSF and 2D-XCOR, is the need to take numerical derivatives on the undeformed and undeformed coordinate systems or the displacement fields. Small errors in mis-tracking are amplified when derivatives are taken of these fields. Additionally, errors may arise from sample rotation, image noise, local strain discontinuities, and large deformation.

2.3 Methods

2.3.1 Derivation of 2D-DDE algorithm

We present here a novel technique to circumvent the LSF deformation gradient tensor calculation based on the midpoints in Eq. (2.4). The new method allows the intrinsic calculation of $\mathbf{F}$ during digital image registration by careful consideration of the warp parameters during the Lucas Kanade registration. By removing the calculation in Eq. (2.4), this new method is more precise, less susceptible to noise, and more computationally efficient (Figure 2.1).

Considering each region $(i)$ with initial undeformed coordinates $\mathbf{X}^{(i)}$ and parameter vector $\mathbf{p}^{(i)}$ solved for using equations 2.10 to 2.13, a linear form for the warping function is chosen.
$W^{(i)} (X^{(i)}; p^{(i)})$:

$$W^{(i)} (X^{(i)}; p^{(i)}) = A^{(i)} (p^{(i)}) [X^{(i)}1]^T$$

(2.14)

where $A^{(i)} (p^{(i)})$ is an affine transformation with parameters $p^{(i)}$:

$$A^{(i)} (p^{(i)}) = \begin{bmatrix} 1 + p_1^{(i)} & p_3^{(i)} & p_5^{(i)} \\ p_2^{(i)} & 1 + p_4^{(i)} & p_6^{(i)} \\ 0 & 0 & 1 \end{bmatrix}$$

(2.15)

The warping function in 2.14 computes deformed image coordinates $x^{(i)}$:

$$[x^{(i)}1]^T = A^{(i)} (p^{(i)}) [X^{(i)}1]^T$$

(2.16)

Since the deformation gradient tensor $F$ is an affine transformation that relates the infinitesimal vector $dX$ in a reference configuration to a corresponding infinitesimal vector $dx$ in a deformed configuration, equation 2.16 is analogous to equation 2.4. Since they are analogous, $F$ can be directly extracted from $A^{(i)} (p^{(i)})$ by ignoring the displacement parameters in equation 2.15, $(p_5^{(i)}$ and $p_6^{(i)})$ and removing the final row:

$$F^{(i)} = \begin{bmatrix} 1 + p_1^{(i)} & p_3^{(i)} \\ p_2^{(i)} & 1 + p_4^{(i)} \end{bmatrix}$$

(2.17)

Considering multiple search regions across the reference image, each with a centroid $Y^{(i)}$ in the coordinate system of the reference image and each at acquired at a time $t_j$ we obtain an
expression for the full deformation field over space and time:

\[
F^{(i,j)}(Y^{(i)}, t_j) = \begin{bmatrix}
1 + p_1^{(i,j)} & p_3^{(i,j)} \\
p_2^{(i,j)} & 1 + p_4^{(i,j)}
\end{bmatrix}
\] (2.18)

The deformation field is then known by \( F^{(i,j)} \) without regularization, least squared estimation of the displacement field, or numerical derivatives of displacement estimates.

In order to quickly compute region coordinates \( X^{(i)} \) from global image coordinates, \( Y^{(i)} \), normalization transformation matrices \( N^{(i)} \) were computed for each region \( (i) \) such that:

\[
Y^{(i)} = N^{(i)}X^{(i)}
\] (2.19)

Each \( N^{(i)} \) was created using the following equation:

\[
N^{(i)} = \begin{bmatrix}
k_1^{(i)} & 0 & Y_1^{(i)} \\
0 & k_2^{(i)} & Y_2^{(i)} \\
0 & 0 & 1
\end{bmatrix}
\] (2.20)

where \( k_1^{(i)} \) and \( k_2^{(i)} \) are the kernel sizes of region \( (i) \) and \( Y_1^{(i)} \) and \( Y_2^{(i)} \) are the centroids of the region in the image coordinates for the 1 and 2 dimensions. Combining equations 2.16, 2.20, and 2.19 yields an equation relating the undeformed volumetric image coordinates \( Y^{(i)} \) to the deformed volumetric image coordinates \( y^{(i)} \):

\[
[y^{(i)}]^{T} = N^{(i)}A^{(i)}(p^{(i)})N^{-1}(i)[Y^{(i)}]^{T}
\] (2.21)

circumventing the need to keep track of each region’s coordinate system.
Figure 2.1: Schematic representation of how 2D-XCOR, 2D-LSF, and 2D-DDE calculate 2D deformation gradient tensors. (A) Original test image (left) and warped image (right). (B) Rigid registration yields errors due to a parallelogram fit of the midpoints of a minimum of four regions (red boxes). (C) The 2D-LSF method improves on this, but errors and reduced precision remain due to the parallelogram fit of the midpoints to calculate $F$ (blue boxes). (D) 2D-DDE accurately calculates the deformation of all four regions independently (green boxes). Black borders: matched regions; dotted borders: calculated deformations for 2D-XCOR (red), 2D-LSF (blue) and 2D-DDE (green). Definitions: $F$, deformation gradient tensor; $\gamma$, shear strain; $\lambda$, stretch ratio in $y$ direction; $\epsilon_{\alpha,x}$, error in $\alpha$ strain.

### 2.3.2 Derivation of the 2D-SIMPLE algorithm

The Strain Inference with Measures of Probable Local Elevation (2D-SIMPLE) method for determining strain concentrations was then developed by considering the difference between the 2D-DDE and 2D-LSF solutions. Convergence on a common solution in $\|\Delta p\|$ involves
translation and deformation – all components of $\Delta \mathbf{p}$ must converge on a local minimum for the solution to be accepted. Therefore, the 2D-LSF method can be independently coupled with 2D-DDE to provide robust criteria for smoothness and continuity. Conversely, disagreement of the solutions suggests emergence of a strain concentration. To detect these concentrations, a simple difference approach was employed (Fig 2.2. A, B):

$$\Delta = E_{DDE} - E_{LK}$$ (2.22)

This method is analogous to a spatial high pass filter of the strain field. To construct the high pass filter, consider subtracting the calculated strain for a particular correlated element from the average strain calculated over some small region $\Omega$:

$$\frac{1}{\Omega} \int_{\Omega} \epsilon_{xx} d\Omega - \epsilon_{xx} = \delta_{xx}$$ (2.23)

where $\delta_{xx}$ is the strain concentration in the $xx$ direction and $\epsilon_{xx}$ is the strain in the $xx$ direction. We can then define the average strain over the region $\Omega$ as $\epsilon^*_{xx}$:

$$\frac{1}{\Omega} \int_{\Omega} \epsilon_{xx} d\Omega = \epsilon^*_{xx}$$ (2.24)

Then by assuming small strain:

$$\lambda_{xx} = \epsilon_{xx} + 1$$ (2.25)

$$\lambda^*_{xx} = \epsilon^*_{xx} + 1$$ (2.26)

Combining Eq. 9-12:

$$\lambda^*_{xx} - \lambda_{xx} = \delta_{xx}$$ (2.27)
Which is analogous to the tensor equation:

\[ \mathbf{F}^* - \mathbf{F} = \Delta \]  

(2.28)

Where \( \mathbf{F}^* \) is \( \mathbf{F}_{DDE} \) and \( \mathbf{F} \) is \( \mathbf{F}_{LK} \) and \( \Delta \) is a strain concentration matrix.

\[ \begin{bmatrix} 1 & \gamma \\ 0 & \lambda \end{bmatrix} \begin{bmatrix} 1 & \gamma \\ 0 & \lambda \end{bmatrix} = \begin{bmatrix} 1 & \gamma \\ 0 & \lambda \end{bmatrix} \]

\[ = \begin{bmatrix} 0 & 0 \\ 0 & 0 \end{bmatrix} \]

\[ \begin{bmatrix} 1 & \gamma \\ 0 & \lambda \end{bmatrix} \begin{bmatrix} 1 & \gamma \\ 0 & \lambda \end{bmatrix} = \begin{bmatrix} 1 + \delta & \gamma \\ 0 & \lambda \end{bmatrix} \]

\[ = \begin{bmatrix} \delta & 0 \\ 0 & 0 \end{bmatrix} \]

Figure 2.2: The 2D-SIMPLE Method uses the difference between the 2D deformations calculated by 2D-DDE (green boxes) and the 2D-LSF method (blue boxes) to determine an outcome we refer to as the Strain Concentration Detector (orange boxes). (A) When deformations are continuous and have no local discontinuities, the 2D-DDE and 2D-LSF methods agree and their difference is exactly 0. (B) If there is a local discontinuity in the data, the 2D-DDE and 2D-LSF methods disagree and their difference is non-zero. Definitions: \( \gamma \), shear strain; \( \lambda \), stretch ratio in \( y \) direction; \( \delta \) difference in calculation between methods.

2.3.3 A note on the compatibility of strain fields averaged over finite regions

The 2D-DDE method is more accurate than the Lucas Kanade displacement-based or standard cross-correlation methods for estimating average strains over discrete regions of finite size. This accuracy is attained by estimating deformation gradient tensors without consideration of displacement fields. We emphasize here that displacement fields can be calculated
uniquely from 2D-DDE estimates of strain fields only in special cases, and that if a displacement field is required the Lucas Kanade displacement-based approach is a better choice.

The reason for this relates to the problem of “strain compatibility” that is well known in mechanics: unique components of a displacement field can be determined from the more numerous components of a strain field only if the spatial variations of these strain fields satisfy certain conditions. For example, for linearized strains in two dimensions, the components of the strain tensor $\epsilon(x, y)$ in a Cartesian $(x, y)$ coordinate frame must satisfy (e.g., [66]):

$$\frac{\partial^2 \epsilon_{xx}(x, y)}{\partial y^2} - 2\frac{\partial^2 \epsilon_{xy}(x, y)}{\partial x \partial y} + \frac{\partial^2 \epsilon_{yy}(x, y)}{\partial x^2} = 0. \quad (2.29)$$

However, rather than reporting a continuous strain field $\epsilon_{\alpha\beta}(x, y)$ that must satisfy the compatibility relations, the 2D-DDE method reports components of strain $\bar{\epsilon}_{\alpha\beta}^{(i)(j)}$ averaged over a region of dimensions $L \times L$ at each position $\{i, j\}$, usually on a regular grid (e.g., Figure 2.3):

$$\bar{\epsilon}_{\alpha\beta}^{(i)(j)} = \frac{1}{L^2} \int_{(i-1)L}^{iL} \int_{(j-1)L}^{jL} \epsilon_{\alpha\beta}(x, y) dx dy \quad (2.30)$$

As shown below, a compatible strain field averaged over an array of finite, discrete regions, does not in general satisfy the compatibility relations. On the one hand, this means that finding a unique displacement field that satisfies a 2D-DDE-estimated strain field is usually not possible. On the other, this means that 2D-DDE is never constrained by specific models or interpolations of strain fields.

The reason for this relates to computation of the second derivatives in Equation 2.29: the finite difference approximation to $\frac{\partial^2 \bar{\epsilon}_{\alpha\beta}^{(i)(j)}}{\partial x^2}$ equals the continuous value of $\frac{\partial^2 \epsilon_{\alpha\beta}}{\partial x^2}(x_0, y_0)$ at the...
center \((x_0, y_0)\) of region \((i, j)\) only under special conditions. The finite difference approximation of this term for the 2D-DDE-estimated average strain fields is:

\[
\frac{\partial^2 \bar{\epsilon}_{yy}^{(i)}(j)}{\partial x^2} = \frac{\bar{\epsilon}_{yy}^{(i)}(j+1) - 2\bar{\epsilon}_{yy}^{(i)}(j) + \bar{\epsilon}_{yy}^{(i)}(j-1)}{L^2}
\]  
(2.31)

and, for \(x_0 = L(j + \frac{1}{2})\) and \(y_0 = L(i + \frac{1}{2})\), and \(L\) sufficiently small, the finite difference approximation is:

\[
\frac{\partial^2 \epsilon_{yy}(x_0, y_0)}{\partial x^2} \approx \frac{\epsilon_{yy}(x_0 + L, y_0) - 2\epsilon_{yy}(x_0, y_0) + \epsilon_{yy}(x_0 - L, y_0)}{L^2}
\]  
(2.32)

The approximation in Equation 2.32 will, for a sufficiently smooth function, equal the exact second derivative in the limit of \(L\) approaching zero. The approximation in Equation 2.31 to approach this same value for all choices of \(x_0\) and \(y_0\) \((i\ \text{and}\ j)\) if the average value of \(\epsilon_{yy}(x, y)\) in a region happens to equal the value at the center of the region (that is, \(\bar{\epsilon}_{yy}^{(i)} = \epsilon_{yy}(x_0, y_0)\)),

Figure 2.3: The 2D-DDE method is often implemented on a regular grid within a strained body.
or if the difference between the two varies in specific ways, such as:

\[
\epsilon_{yy}^{(i)(j)} = \epsilon_{yy}(x_0, y_0) + f_1(y_0) + x_0f_2(y_0) + C_1
\]  

(2.33)

where \(C_1\) is an arbitrary constant and \(f_1\) and \(f_2\) are functions of \(y_0\) only. Similar relations can be derived for the other terms in Equation 2.29. The consequence is that, no matter how fine the discretization, discrete derivatives of 2D-DDE-estimated strain fields should never be forced to meet the compatibility equations. As a simple example, consider the following strain field that satisfies Equation 2.29:

\[
\epsilon_{\alpha\beta} = \begin{bmatrix}
0 & 2Ax^3y \\
2Ax^3y & Ax^4
\end{bmatrix}
\]  

(2.34)

In the absence of any experimental or measurement error, the averaged strains that the 2D-DDE algorithm should report are the following:

\[
\begin{align*}
\bar{\epsilon}_{xy}^{(i)(j)} &= \frac{1}{L^2} \int_{(i-1)L}^{iL} \int_{(j-1)L}^{jL} 2Ax^3y \, dx \, dy \\
&= 2AL^4 \left( (j - \frac{1}{2})^3 + \frac{1}{4}(j - \frac{1}{2}) \right) (i - \frac{1}{2}) \\
\bar{\epsilon}_{xx}^{(i)(j)} &= 0 \\
\bar{\epsilon}_{yy}^{(i)(j)} &= \frac{1}{L^2} \int_{(i-1)L}^{iL} \int_{(j-1)L}^{jL} Ax^4 \, dx \, dy \\
&= AL^4 \left( (j - \frac{1}{2})^4 + \frac{1}{2}(j - \frac{1}{2})^2 + \frac{1}{80} \right)
\end{align*}
\]  

(2.35)
Note that these can be written in terms of $x_0$ and $y_0$ as:

$$\bar{\epsilon}^{(i)(j)}_{xy} = 2A \left( x_0^3 + \frac{x_0 L^2}{4} \right) y_0 = \epsilon_{xy}(x_0, y_0) + 2Ax_0y_0 \left( \frac{L}{2} \right)^2$$

$$\bar{\epsilon}^{(i)(j)}_{yy} = Ax_0^4 + AL^2 \left( \frac{x_0^2}{2} + \frac{L^2}{80} \right) = \epsilon_{yy}(x_0, y_0) + AL^2 \left( \frac{x_0^2}{2} + \frac{L^2}{80} \right) \quad (2.37)$$

Finite difference approximations to the derivatives in the compatibility equation are:

$$\frac{\partial^2 \bar{\epsilon}^{(i)(j)}_{xy}}{\partial x \partial y} \approx \frac{1}{4L^2} \left( \bar{\epsilon}^{(i+1)(j+1)}_{xy} - \bar{\epsilon}^{(i-1)(j-1)}_{xy} + \bar{\epsilon}^{(i-1)(j+1)}_{xy} + \bar{\epsilon}^{(i+1)(j-1)}_{xy} \right)$$

$$= 6AL^2(j^2 - j + \frac{2}{3}) = 6Ax_0^2 + \frac{5}{2}AL^2 \quad (2.38)$$

$$\frac{\partial^2 \bar{\epsilon}^{(i)(j)}_{yy}}{\partial x^2} = \frac{\bar{\epsilon}^{(i)(j+1)}_{yy} - 2\bar{\epsilon}^{(i)(j)}_{yy} + \bar{\epsilon}^{(i)(j-1)}_{yy}}{L^2}$$

$$= 12AL^2(j^2 - j + \frac{1}{2}) = 12Ax_0^2 + 3AL^2 \quad (2.39)$$

Substituting into the compatibility relation Equation 2.29 yields:

$$\frac{\partial^2 \epsilon_{xx}(x,y)}{\partial y^2} - 2 \frac{\partial^2 \epsilon_{xy}(x,y)}{\partial x \partial y} + \frac{\partial^2 \epsilon_{yy}(x,y)}{\partial x^2} = -2 \left( 6Ax_0^2 + \frac{5}{2}AL^2 \right) + 12Ax_0^2 + 3AL^2$$

$$= -2AL^2$$

$$\neq 0 \quad (2.40)$$

In this example, and in general, forcing strain compatibility into the direct estimation of strain fields would be incorrect.

From the above example it is clear that forcing strain compatibility is not always appropriate and may result in the incorrect calculations of strains. Strain compatibility was originally introduced into digital image correlation to ensure that strain fields were continuous and that minor mis-tracking would not introduce errors into the strain field [66]. With the improved
accuracy and precision of 2D-DDE coupled with the direct calculation of strain by neglecting displacements, strain compatibility can be relaxed, allowing incompatible, non-continuous, strain fields. Detecting when strain fields become incompatible is the motivation for the derivation of the 2D-SIMPLE method. The 2D-SIMPLE method, in essence, looks at the difference between strain fields in which compatibility is not enforced (2D-DDE) and strain fields in which compatibility is enforced (2D-LSF). By comparing the two, the 2D-SIMPLE method is able to detect precisely when and where strain fields become incompatible and non-smooth. In the example considered above, 2D-SIMPLE would calculate the non-zero difference between the two results (Eq. 2.40).

\subsection*{2.3.4 In silico validation}

To compare 2D-DDE to the prior state of the art, an \textit{in silico} validation was performed on idealized data. Briefly, a marbled texture was warped by computing displacement fields from defined deformation fields for three cases: an incompressible deformation, a pure rotation, and combined rotation and deformation. Following displacement field computation, new coordinates for each pixel were computed. This results in new, non-rectangular, unevenly spaced coordinate system. To create a successive image, interpolation must be performed on the new, non-gridded image using the grid of the original image. This is analogous to a camera, which has stationary pixels and light from a deforming sample projected onto the camera sensor on the same grid, regardless of how the sample is deforming. Interpolation on the non-gridded coordinate system of the deformed image was performed using the scattered interpolant function in Matlab. This function first computes a Delaunay triangulation of the deformed coordinate system, then performs linear interpolation for each requested point by computing a weighted average of the closest deformed pixel intensities. Finally, this was
repeated for 100 frames to create a final image sequence of a gradual deformation over time to final values of $E_{11} = 0.1$ for deforming images and $\theta = 15^\circ$ for rotating images.

Mean absolute error plots were computed comparing 2D-XCOR, 2D-LSF, and 2D-DDE to true values of strain known from the initial deformation fields using the following equation:

$$RMSE_{\text{Error}} = \sqrt{\frac{\sum_{i=1}^{n}(\hat{y}_i - y_i)^2}{n}}$$  \hspace{1cm} (2.41)

where $\hat{y}_i$ is the known true value for each strain estimate, $y_i$ is the estimated value for each technique, and $n$ is the number of estimates. Error bars are computed as one standard deviation of the error values. Lastly, a mean and a standard deviation are plotted for each value of increasing strain for each estimate of strain.

### 2.3.5 Experimental validation

**Fabrication and testing of PDMS scaffolds with gradients in stiffness**

In order to validate the 2D-DDE algorithm, PDMS sheets (N=3) with gradients in stiffness were fabricated according to published methods [1]. Briefly, Sylgard 184 PDMS was mixed at two base:curing agent ratios, 10:1 and 20:1. Silanized glass slides and a Teflon spacer were used to create a mold. The two PDMS mixtures were then poured into the mold such that the 10:1 mixture was on the bottom and the 20:1 mixture was on the top. Filled molds were placed on top of a hot plate at 120C for 90 minutes so that a temperature gradient developed along the mold, creating a gradient in cross linker activation and a subsequent gradient in stiffness. Polymerized PDMS scaffolds were rinsed in hexane to swell the scaffold and remove residual crosslinkers, preventing further polymerization. Scaffolds were then sprayed lightly
with black latex spray paint to produce a random surface speckle texture, and placed in a custom designed cyclic tensile machine. Scaffolds were pulled in tension to 10% grip-to-grip strain at a rate of 0.1 Hz. Videos of the test were captured using an Illunis VMV-8M camera for subsequent strain analysis.

Testing of vinylidene chloride sheets

In order to validate the 2D-SIMPLE algorithm, commercially available vinylidene chloride sheets (Saran Premium Wrap, SC Johnson) were coated in white latex paint and allowed to dry overnight (N=2). After drying, the sheets were cut into 20 x 5 mm$^2$ sheets and sprayed lightly with black latex spray paint to produce a random surface speckle texture. Sheets were griped using spring clamps and loaded in tension at a strain rate of 0.1 %/s using a materials testing frame (Instron Electropuls E1000). Videos of the test were captured using an Illunis VMV-8M camera for analysis.

2.4 Results

2.4.1 2D-DDE is simpler, more precise, and more accurate than existing algorithms

Optical strain measurements utilize texture matching to estimate deformation. The basic texture-matching algorithm divides an initial reference image into several regions and finds the best-matching region in a deformed image. The most-widely-implemented class of algorithms, 2D-XCOR, search for matching regions without considering how the shape of the
individual undeformed texture regions change (Figure 2.1A,B) [67]. Strain fields can be estimated from the displacements of the midpoints of each region, often through calculation of a deformation gradient tensor that can be used to relate the spatial gradient of displacement fields to the strain fields (Figure 2.1B).

Although not widely used in the biomedical and engineering communities, the Lucas Kanade (LK) method is broadly applied in computer vision to improve region-matching (Figure 2.1A,C) [14, 13, 15, 17]. Rather than matching regions of identical size and shape, the LK method optimizes a warping function for each region to improve the matching. Displacements of the midpoints of each region are then used to estimate the deformation gradient tensor and strain fields to arrive at the 2D-LSF method [13]. This can reduce errors in strain estimation in cases of large deformations by two orders of magnitude (Figure 2.4B,E,H). Alternate deformable image registration techniques have been developed and utilized in biomedical applications which improve upon LK displacement estimation, however all of these alternative techniques require a LSF of the displacement field [68, 69].

We found that errors could be reduced another order of magnitude by incorporating continuum mechanics directly into the LK algorithm. During the Newtonian optimization performed to solve for the LK method, a warping function must be defined to describe the change from an undeformed to a deformed image, and is usually chosen for efficiency and accuracy in estimation of displacement fields [13]. We defined a warping function that could be directly related to the deformation gradient tensor (see Section 2.3.1 for details). Using this approach, referred to as 2D-DDE, the deformation gradient tensor is intrinsically calculated as part of the region-matching algorithm, without the need to take numerical derivatives (Figure 2.1A,D)
When tested against idealized images with deformation fields known \textit{a priori}, the 2D-DDE approach out-performed 2D-LSF and 2D-XCOR approaches substantially in pure incompressible deformation (Figure 2.4A), pure rotation with no deformation (Figure 2.4D), and incompressible deformation with rotation (Figure 2.4G). Three advantages of the 2D-DDE approach are: (i) improved accuracy due to a unique deformation gradient tensor associated with each region (Figures 2.1 and 2.4) (ii) increased precision due to circumvention of a least squares estimation of the deformation gradient tensor (Figures 2.1 and 2.4), and (iii) improved simplicity because least squares estimation based on image motion is eliminated.

To demonstrate the utility of the increased accuracy and precision, we analyzed deformation of PDMS samples fabricated with gradients in crosslinking and hence gradients of material stiffness, and coated with a speckle pattern to track deformations. Using videos taken as specimens were cyclically strained between 0 and 10\% grip-to-grip strain, the 2D-DDE algorithm detected smooth spatial gradients of both axial strain and lateral contraction along the sample, corresponding to the expected stiffness gradient at a grip-to-grip strain of only 0.003 (Figure 2.5A and C). These patterns were evident long before detection using 2D-XCOR (Figure 2.5B, D). Both methods detected strain gradients at a grip-to-grip strain of 0.03, but the strain fields predicted by 2D-XCOR were irregular, with regions of high strain abutting regions of low strain (Figure 2.5F). At higher strain levels, the 2D-XCOR methods failed to capture a meaningful strain field, while the 2D-DDE algorithm continued to identify the smooth gradient, even with peak strains over 0.2 (Figure 2.5I-L).

Many algorithms exist for improving the smoothing and improving the accuracy of displacement fields [70]. As these were not applied in this example, the example does not represent
the limitations of 2D-XCOR. However, it does represent the strength and simplicity of 2D-DDE strain tracking which is inherently sensitive, does not require smoothing/averaging, has higher resolution, and tracks to much higher strain levels.

2.4.2 2D-SIMPLE identifies strain localizations and predicts crack formation

The irregular strain pattern in Figures 2.5F and 2.5H represents a well-known challenge with 2D-XCOR methods. Slight mistracking of a texture region leads to zones of over-estimated strain abutting zones of underestimated strain. If strain fields are known to be smooth, this can be fixed by simply averaging over several regions. However, in the absence of such information, a strain field such as that in Figures 2.5F and 2.5H might lead to the erroneous conclusion that strain concentrations existed in these regions and that the material did not have a smooth stiffness gradient. Although the 2D-DDE method is able to reveal the smooth material gradient, detection of strain concentrations remains difficult because strain concentration detection methods must rely on post processing filtering techniques to detect local features.

The 2D-SIMPLE algorithm achieved robust prediction of strain localization and crack formation through a metric based upon differences between predictions of the 2D-DDE and 2D-LSF methods (Figure 2.2A,B; details in Section 2.3.2). The output of this algorithm is a tensor whose principal components reveal strain concentrations. As a demonstration, a vinylidene chloride sheet with a speckle pattern on its surface was pulled to failure. The peak principal value of $\Delta$, termed the strain concentration detector, $\Delta_f$, identified strain localizations leading to cracks earlier and with more certainty than 2D-XCOR (Figure 2.6).
A,B, white arrow). As the primary region of strain localization inferred using $\Delta I$ cracked, $\Delta I$ continued to increase over the uncracked ligaments (Figure 2.6C). Further, as the crack developed, neighboring strain concentrations halted their development and unloaded, demonstrating transfer of stress to the propagating crack (yellow arrows in Figure 2.6). In contrast, the strain concentration was difficult to identify above noise using 2D-XCOR methods (Figure 2.6D, white arrow), 2D-XCOR returned unreasonable strains of 200% while cracks were propagating (Figure 2.6F), and the 2D-XCOR method failed to track deformations after the crack had formed (Figure 2.6F,H).

2D-SIMPLE identifies when the strain field arising from the best estimates for displacements differ significantly from the piece-wise constant 2D-DDE strain field that best represents how a defined portion of an image has deformed. Estimating displacements based upon the 2D-DDE strain fields, as is required for the former, has the advantage of providing a compatible strain field, meaning that it corresponds to a unique displacement field [66]. However, imposing compatibility is not in general appropriate for piece-wise constant averaged strain fields (Section 2.3.3).

### 2.5 Discussion

The 2D-DDE and 2D-SIMPLE methods can identify strain concentrations that are very difficult to detect with any previously published method and are appealing due to the simplicity of their implementation. The 2D-SIMPLE method detected strain concentrations on the order of 0.005, long before they were evident using 2D-XCOR (Figure 2.6A). Strain localizations were predictive of crack initiation, and are therefore useful for applications ranging
across biomaterial design and structural engineering. We are unaware of any other techniques to detect strain concentrations as robustly or predict the onset of fracture with this precision and accuracy.

The 2D-DDE method showed improvement in accuracy, precision, and resolution over previously employed techniques and maintained this through high strains. This renders the method particularly suitable for biologic systems, which often endure large and inhomogeneous strains [6, 7, 8, 9, 10]. 2D-DDE also demonstrated sensitivity sufficient to differences in strain as small as 0.001 (Figure 2.5). The method is insensitive to movements and rotations of a specimen and is relatively robust against image noise. The method is therefore suitable for analysis of low resolution/noisy images (e.g., from magnetic resonance imaging). Other methods for deformable image registration employ more flexibility than the simple affine transformation used here [15]; however, the 2D-DDE method is the first to take into account the formulations of mechanics directly into the image correlation algorithm, and thus delivers improved accuracy. Importantly, as with other texture based methods including 2D-XCOR and 2D-LSF, 2D-DDE is limited by image resolution and the size of the smallest image feature: the region size cannot be known a priori and must be adjusted for each sample based on the image resolution and the size of the smallest texture feature.

The general applicability of 2D-DDE and 2D-SIMPLE are demonstrated in Chapters 5 and 6. In Chapter 5, a modified version of 2D-DDE, adapted to analyze one dimensional manifolds, reveals elevated local strain between tendon and bone at the micrometer scale. 2D-DDE also aided tissue engineering studies by estimating large deformations to determine local material properties of scaffolds made of synthetic or natural materials. In a separate study, 2D-SIMPLE aids the investigation of how embryonic wound healing provides a robust healing response. Lastly, in Chapter 6, a model system to investigate the differentiation potential of
passively applied stress and strain is developed. During validation of this system, 2D-XCOR fails to reliably estimate strains while 2D-DDE reliably estimates strain, effectively enabling the study to proceed.

The techniques described are useful outside of biology as well. Glacial rifts and fronts of earthquakes begin as strain localizations before failure [71, 72], and estimates of strain concentrations at these fronts might prove useful. Further, the method would be useful in monitoring of civil structures. We are hopeful that the simplicity of 2D-DDE and 2D-SIMPLE will enable easy strain estimation and strain concentration detection across many fields.
Figure 2.4: The 2D-DDE method was superior to all previously utilized methods for calculating strains in a deforming sample. In simulations where strains and rotations were known a priori, the 2D-DDE algorithm (green lines) calculated strains with higher precision and accuracy than 2D-XCOR (red lines) and 2D-LSF (blue lines) algorithms. For all cases, at strains less than 0.01, 2D-LSF introduces marginally less error than 2D-DDE. However 2D-DDE remain consistently more accurate and more precise through large strains while 2D-LSF introduces progressively more error. (A, B, C) The first simulation consisted of an increasing tensile incompressible deformation to a final strain of $E_{11} = 0.01$. 2D-XCOR produced errors on the order of 0.03 strain for a strain level of 0.1. In contrast, the 2D-LSF and 2D-DDE strain calculations introduced errors on the order of 0.0005 strain. (D, E, F) The second simulation consisted of a pure rotation to $\theta = 15$ in the absence of strain. 2D-XCOR was unable to accurately track displacements in this simulation, leading to large errors in the strain calculations. For this simulation, errors in strain calculation for the 2D-LSF method were on the order of 0.002 strain, while errors for the 2D-DDE method were on the order of 0.0001 strain. (G, H, I) The third simulation consisted of incompressible deformation ($E_{11} = 0.1$) combined with rotation $\theta = 15$. 2D-XCOR once again failed to track deformation, leading to large errors in strain calculations. The errors associated with the 2D-LSF and 2D-DDE algorithms were similar to those for pure rotation, with the 2D-DDE algorithm about an order of magnitude better than the 2D-LSF algorithm. Definitions: $E_{11}$, strain in 11 direction; $\theta$, rotation.
Figure 2.5: The advantages of the increased precision and accuracy of 2D-DDE over 2D-XCOR were demonstrated by cyclically stretching a PDMS sheet with a spatial gradient in stiffness. (A-D) At a low grip-to-grip strain of 0.003, 2D-DDE was able to detect a gradient in stiffness, as evidenced by gradients in the first and second principal strains, while 2D-XCOR failed to detect gradients in strain above noise. (E-H) At a grip-to-grip strain of 0.03, 2D-DDE revealed a smooth gradient in first and second principle strains. 2D-XCOR also detected the spatial gradients in strain, however the detected strains were irregular and noisy. (I-L) At a large grip-to-grip strain of 0.1, 2D-DDE detected a smooth strain gradient, with local strains greater than 0.2. In contrast, 2D-XCOR failed to detect a smooth strain gradient, demonstrating its limitations at high strains. Scale bar = 2 mm. Definitions: $E_{xx}$, strain in $xx$ direction.
Figure 2.6: The 2D-SIMPLE method accurately detected strain concentrations predictive of crack initiation formation and was able to track crack propagation. All strains are given relative to an initial grip-to-grip strain of ($E_{xx} = 1.2$), at which the optical analysis was started. (A, B) The 2D-SIMPLE algorithm detected two developing strain concentrations (white and yellow arrows) at a low grip-to-grip strain ($E_{xx} = 0.26$). In contrast, noise in the 2D-XCOR calculation resulted in significant uncertainty for determining the location of the strain concentrations. (C, D) At higher levels of grip-to-grip strain ($E_{xx} = 1.16$), both algorithms were able to detect the developing crack, however, the strain concentration remained partially obscured by noise for the 2D-XCOR method. (E) The strain concentration predicted by the 2D-SIMPLE algorithm can be visualized as a crack in the material (white arrows). (E-H) As the crack forms and propagates ($E_{xx} = 1.56$), the 2D-XCOR algorithm fails whereas the 2D-SIMPLE algorithm continues to track the crack in the material (white arrows in G). Furthermore, the second strain concentration (yellow arrow) stops developing, suggesting that the material failure at the crack (white arrows) resulted in unloading of the second concentration. Scale bar = 1 mm. Definitions: $E_{xx}$, strain in 11 direction; $\Delta$, 2D-SIMPLE difference.
Chapter 3

Accurate and precise methods for estimating strain in three dimensions

Portions of this chapter were adapted from: John J. Boyle Arvin Soepriatna, Frederick Damen, Roger A. Rowe, Robert B. Pless, Attila Kovacs, Craig J. Goergen, Stavros Thomopoulos, Guy M. Genin (2017). Accurate and noise insensitive strain mapping enables ultrasound analysis of cardiac function in three dimensions. Under Review

3.1 Abstract

Tracking deformation of organs, tissues, and cells from time-resolved volumetric medical imaging and microscopy stacks is a pressing need for the next generation of diagnostic and mechanobiological tools. A critical barrier is that, because these volumetric images are inherently noisy, current strategies require either regularization and smoothing schemes that
sacrifice spatial resolution or assumptions about material properties that are difficult to validate. Here, we present and validate the first three-dimensional (3D) method for estimating mechanical strain directly from raw 3D image stacks with no regularization or assumed material model. We demonstrate increased accuracy and precision of these techniques over the prior state of the art on two *in silico* models: a penny shaped crack and an Eshelby inclusion. The method shows promise for broad application to dynamic medical imaging modalities, including high frequency ultrasound, tagged magnetic resonance imaging, and confocal fluorescence microscopy.

### 3.2 Introduction

Mechanical characterization of inhomogeneous and/or geometrically complex three-dimensional (3D) biological tissues requires precise and accurate determination of strain fields. The problem of determining strain fields on the interior of a deforming structure can be addressed using various well established techniques based on digital image correlation (DIC). The technique involves matching patterns between pairs of images to estimate the displacement of certain regions or features on a sample [36]. The most advanced DIC techniques, although limited to two-dimensional (2D) manifolds, have proven effective for applications such as tracking deformations in dynamic magnetic resonance (MR) images of the heart and brain (Section 5.5, Section 5.6, and [28]). However, use of these 2D techniques on images acquired in samples that undergo 3D deformation can lead to data that are difficult to interpret for several reasons. First, analysis limited to 2D manifolds is insufficient to understand the complex mechanics of a 3D structure because it discards most of the information. Second, out-of-plane motion introduces error into strain calculations as features move in and out of
the imaging plane. Finally, small errors in displacement estimation can be amplified when numerical gradients are taken, leading to uncertainty in the interpretation of deformation fields that contain substantial strain gradients relative to the pixel size or that contain strain concentrations. Fully volumetric strain estimation on three dimensional volumetric image sequences overcomes all of these limitations and enables determination of the mechanics of the bulk of a material rather than its surface.

Three dimensional strain estimation most commonly relies on first estimating the deformation gradient tensor, $F_{3D}$. Since the formulation of the deformation gradient tensor does not rely on the number of dimensions, the three dimensional deformation gradient tensor is directly related to the two dimensional deformation gradient tensor from equation 2.4:

$$dx = F_{3D}dX$$ (3.1)

The Green-Lagrange strain tensor, $E_{3D}$, which is analogous to engineering strain in two dimensions, $E$, can then be then calculated from $F_{3D}$ using the following equation:

$$E_{3D} = 0.5(F_{3D}^T F_{3D} - I_{3D})$$ (3.2)

where $I_{3D}$ is the second order identity tensor.

### 3.2.1 Prior state of the art

In the prior state of the art strain estimation on volumetric images typically utilized digital volume correlation. Several approaches exist that use a digital volume correlation technique to estimate full field 3D strains [28, 29, 30, 31, 32, 33, 34]. These algorithms divide an
initial reference volume into several regions and then search for the best-matching region in a deformed volume. The most widely implemented class of these algorithms is a volumetric normalized cross correlation which searches for a matching region by computing a similarity measure for all nearby pixels and then finding the maximum. The similarity measure, termed the correlation coefficient, is found using equation 1.9.

Traditionally, estimation of strain from three dimensional stacks of images is performed after digital volume correlation by analyzing deformation of a mesh constructed by binding the midpoints of matched regions into elements [36, 28, 29, 30, 31]. The initial and displaced positions of the midpoints are used to estimate the deformation gradient tensor, $F_{3D}$, in each element. $F_{3D}$ relates a material vector $dX$ in the undeformed reference configuration to the corresponding spatial vector $dx$ in the deformed configuration $F_{3D}$ is typically estimated using a least squares fit (LSF) of equation 3.1. We term this technique three dimensional normalized cross correlation (3D-XCOR). As with 2D-XCOR, a central limitation of 3D-XCOR is that it is a rigid registration technique. 3D-XCOR searches for exactly matched regions between successive frames and assumes they only displace over time and do not deform or change orientation. Because of this, it is not suitable for tracking large deformations or samples undergoing orientation changes, like a rotation.

Alternative to rigid registration techniques, non-rigid or deformable image registration techniques allow an image to deform and change orientation during image registration. Non-rigid volumetric image registration approaches involve expanding on their two dimensional analogues (Section 2.2.1). We expand the two dimensional energy equation to three dimensions by considering volumetric images and a three dimensional warping function:

$$
\sum [T_{3D}(W_{3D}(X; p)) - I_{3D}(W(X; p))]^2
$$

(3.3)
where $T_{3D}(W_{3D}(X; p))$ is a volumetric template image, warped by an identity warp $W_{3D}(X; 0)$ and $I_{3D}(W_{3D}(X; p))$ is a volumetric input image, $I_{3D}$, warped by a defined three dimensional warping function $W_{3D}(X; p)$ whose warping parameter $p$ can be modulated. Similarly to the Lucas-Kanade (LK) inverse compositional algorithm [15], it is iterated upon using the following increments for $p$:

\[
\Delta p = H^{-1}_{3D} \sum_x \left[ \nabla T_{3D} \frac{\partial W_{3D}}{\partial p} \right]^T [I_{3D}(W_{3D}(X; p)) - T_{3D}(X)]
\]  

(3.4)

until the norm $\|\Delta p\|$ drops below a defined threshold. In this implementation $H_{3D}$ is the Gauss-Newton approximation to the three dimensional Hessian matrix:

\[
H_{3D} = \left[ \nabla T_{3D} \frac{\partial W_{3D}}{\partial p} \right]^T \left[ \nabla T_{3D} \frac{\partial W_{3D}}{\partial p} \right]
\]  

(3.5)

and successive updates of $\Delta p$ are given by:

\[
W_{3D}(X; p) \leftarrow W_{3D}(X; p) \circ W_{3D}(X; \Delta p)^{-1}
\]  

(3.6)

Following optimization, displacement parameters from $p$ are used to estimate strains from a least squared fit of equation (3.1). This technique improves strain estimation compared to 3D-XCOR by allowing shape change during registration thereby improving registration and displacement estimates. Registration is also improved by allowing the region to rotate, which also contributes to improved registration accuracy and therefore improves displacement and strain estimation. We refer to this method as the 3D Least Squared Fit Lucas Kanade (3D-LSF).

A central limitation of estimating strain fields using existing digital image correlation methods is the need to take numerical derivatives after estimating displacements. Additionally,
errors arise from sample rotation, image noise, local strain discontinuities, and large deformation.

3.3 Methods

3.3.1 Derivation of the 3D-DDE algorithm

We present here a novel technique to circumvent the LSF deformation gradient tensor calculation based on the midpoints in Eq. (3.1). The new method allows the intrinsic calculation of $F_{3D}$ during digital image registration by careful consideration of the warp parameters during the LK registration.

Considering each region $(i)$ with initial undeformed coordinates $X_{(i)}$ and parameter vector $p_{(i)}$ solved for using equations 3.3 to 3.6, a linear form for the warping function $W_{3D}^{(i)}(X_{(i)}; p_{(i)})$ is chosen as:

$$W_{3D}^{(i)}(X_{(i)}; p_{(i)}) = A_{3D}^{(i)}(p_{(i)}) [X_{(i)}1]^T$$

where $A_{(i)}(p_{(i)})$ is an affine transformation with parameters $p_{(i)}$:

$$A_{3D}^{(i)}(p_{(i)}) = \begin{bmatrix}
1 + p_1^{(i)} & p_4^{(i)} & p_7^{(i)} & p_{10}^{(i)} \\
p_2^{(i)} & 1 + p_5^{(i)} & p_8^{(i)} & p_{11}^{(i)} \\
p_3^{(i)} & p_6^{(i)} & 1 + p_9^{(i)} & p_{12}^{(i)} \\
0 & 0 & 0 & 1
\end{bmatrix}$$

(3.8)
The warping function in 3.7 then computes deformed image coordinates $x^{(i)}$:

$$[x^{(i)}]^T = A_{3D}^{(i)}(p^{(i)})[X^{(i)}1]^T$$  \hspace{1cm} (3.9)

Since the deformation gradient tensor $F_{3D}$ is an affine transformation that relates the infinitesimal vector $dX$ in a reference configuration to a corresponding infinitesimal vector $dx$ in a deformed configuration, equation 3.9 is analogous to equation 3.1. Since they are analogous, $F_{3D}$ can be directly extracted from $A_{3D}^{(i)}(p^{(i)})$ by ignoring the displacement parameters in equation 3.8, $(p_{10}^{(i)}, p_{11}^{(i)}, p_{12}^{(i)})$ and removing the final row:

$$F_{3D}^{(i)} = \begin{bmatrix}
1 + p_{1}^{(i)} & p_{4}^{(i)} & p_{7}^{(i)} \\
 p_{2}^{(i)} & 1 + p_{5}^{(i)} & p_{8}^{(i)} \\
p_{3}^{(i)} & p_{6}^{(i)} & 1 + p_{9}^{(i)}
\end{bmatrix}$$  \hspace{1cm} (3.10)

Considering multiple search regions across the reference image, each with a centroid $Y^{(i)}$ in the coordinate system of the reference volumetric image and each at acquired at a time $t_j$ we obtain an expression for the full deformation field over space and time:

$$F_{3D}^{(i,j)}(Y^{(i)}, t_j) = \begin{bmatrix}
1 + p_{1}^{(i,j)} & p_{4}^{(i,j)} & p_{7}^{(i,j)} \\
p_{2}^{(i,j)} & 1 + p_{5}^{(i,j)} & p_{8}^{(i,j)} \\
p_{3}^{(i,j)} & p_{6}^{(i,j)} & 1 + p_{9}^{(i,j)}
\end{bmatrix}$$  \hspace{1cm} (3.11)

The deformation field is then known by $F_{3D}^{(i,j)}$ without regularization, least squared estimation of the displacement field, or numerical derivatives of the displacement fields.
In order to quickly compute region coordinates $X^{(i)}$ from global image coordinates, $Y^{(i)}$, normalization transformation matrices $N_{3D}^{(i)}$ were computed for each region $(i)$ such that:

$$Y^{(i)} = N_{3D}^{(i)}X^{(i)}$$ (3.12)

Each $N_{3D}^{(i)}$ was created using the following equation:

$$N_{3D}^{(i)} = \begin{bmatrix} k_1^{(i)} & 0 & 0 & Y_1^{(i)} \\ 0 & k_2^{(i)} & 0 & Y_2^{(i)} \\ 0 & 0 & k_3^{(i)} & Y_3^{(i)} \\ 0 & 0 & 0 & 1 \end{bmatrix}$$ (3.13)

where $k_1^{(i)}$, $k_2^{(i)}$, and $k_3^{(i)}$ are the kernel sizes of region $(i)$ and $Y_1^{(i)}$, $Y_2^{(i)}$, and $Y_3^{(i)}$ are the centroids of the region in the image coordinates for the 1, 2, and 3 dimensions. Combining equations 3.9, 3.13, and 3.12 yields an equation relating the undeformed volumetric image coordinates $Y^{(i)}$ to the deformed volumetric image coordinates $y^{(i)}$:

$$[y^{(i)}]^{T} = N_{3D}^{(i)}A_{3D}^{(i)}(p^{(i)})N_{3D}^{-1}^{(i)}[Y^{(i)}]^{T}$$ (3.14)

circumventing the need to keep track of each region’s coordinate system.

### 3.3.2 Derivation of the 3D-SIMPLE algorithm

A key difference between the 3D-DDE and 3D-LSF fits is that that latter imposes compatibility upon the strain field by forcing the strain field to reduce to a unique set of displacements. As described in Chapter 2, this is an inappropriate constraint in cases when a strong strain
gradient exists because the average displacement of a volume of an image, estimated using cross-correlation, is not in general equal to the displacement of the mid-point of that volume. The difference between strain estimates arising from 3D-DDE and 3D-LSF is therefore a sensitive measure of the existence of a strain gradient. We term this difference the 3D-SIMPLE method for determining strain concentrations.

3D-SIMPLE was developed by considering the difference between the 3D-DDE and 3D-LSF strain estimates. Convergence on a common solution in $\|\Delta p\|$ involves translation and deformation – all components of $\Delta p$ must converge on a local minimum for the solution to be accepted. Therefore, the 3D-LSF method can be independently coupled with 3D-DDE to provide robust criteria for smoothness and continuity. Conversely, disagreement of the solutions suggests emergence of a strain concentration. To detect these concentrations, a simple difference approach is employed:

$$\Delta_{3D} = E_{3D\cdot DDE} - E_{3D\cdot LSF} \quad (3.15)$$

This method is analogous to a spatial high pass filter of the strain field. To construct the high pass filter, consider subtracting the calculated strain for a particular correlated element from the average strain calculated over some small region $\Omega$:

$$\frac{1}{\Omega} \int_{\Omega} \epsilon_{xx} d\Omega - \epsilon_{xx} = \delta_{xx} \quad (3.16)$$

where $\delta_{xx}$ is the strain concentration in the $x$-direction and $\epsilon_{xx}$ is the strain in that direction. We can then define the average strain over the region $\Omega$ as $\epsilon^*_{xx}$:

$$\frac{1}{\Omega} \int_{\Omega} \epsilon_{xx} d\Omega = \epsilon^*_{xx} \quad (3.17)$$
Then by assuming small strain:

\[ \lambda_x = \epsilon_{xx} + 1 \]  

(3.18)

\[ \lambda^*_x = \epsilon^*_{xx} + 1 \]  

(3.19)

Combining Equations (3.16)-(3.19):

\[ \lambda^*_x - \lambda_x = \delta_{xx} \]  

(3.20)

Which is analogous to the tensor equation:

\[ F^*_3D - F_{3D} = \Delta_{3D} \]  

(3.21)

Where \( F^*_3D \) is \( F_{3D-DDE} \) and \( F_{3D} \) is \( F_{3D-LSF} \) and \( \Delta_{3D} \) is a three dimensional strain concentration matrix.

### 3.3.3 Efficient warping of three dimensional volumes from spatially varying deformation tensors and determination of Lagrangian Strains

Novel computational methods were developed to efficiently warp volumetric images while precisely determining the true strain at every spatial location. In two dimensions, this was simple: an initial image was deformed using displacements calculated from deformation or displacement equations to find a new coordinate system. Using that new, non-rectangular coordinate system, a Delaunay triangulation was computed to determine nearest neighbors
for linear interpolation (Section 2.3.4). However, the three dimensional Delaunay triangulation is very computational expensive and in fact was computationally prohibitive. To circumvent this, new methods were devised for coordinate system inversion. This was done so that interpolation will be performed on a rectangular grid, circumventing the need for a Delaunay triangulation at each step. Corresponding methods for determining true strain at every location despite coordinate system inversion were also developed.

We present here a computationally efficient way to take an input image and apply an arbitrary deformation to it over time. The challenge arises from the fact that traditional formulations for deformation involve a set of initial predefined coordinates $X$ that change position over time to new coordinates $x$. To translate this to warping an image, we assigned pixel intensities to the original coordinates to create a static image, then used the deformation tensor to displace these pixel intensities to new coordinates. To create a warped image from this data, the deformed image needs to be interpolated on to determine what would look like in the original, or camera, coordinate system. This is analogous to how a real sample would appear as it deforms to a camera: the material coordinate system, (the sample) deforms and displaces over time while the camera continues to capture images in the camera coordinate system. The basic algorithm to simulate this \textit{in silico} is described in Chapter 2. In the current chapter, a significantly less computationally expensive version, which inverts the roles of deforming the material coordinate system and the observational coordinate system allowing the camera coordinate system to deform and the material coordinate system to remain the same, is described. This efficiency, while not meaningful when considering 2D images, is extremely important for 3D implementation where calculations may take more than an order of magnitude longer than their 2D counterparts.
Deforming the material over time

To begin, consider Equation 3.1 where $F$ is a 3D deformation gradient tensor, then consider that each new coordinate in $x$ is only a function of its initial position and current time:

$$F = f(X, t) \tag{3.22}$$

then integrate to find the position at any time:

$$\int dx = \int f(X, t) dX \tag{3.23}$$

to arrive an an equation for the new coordinates $X$ for any $x$ and $t$. As an example, arbitrarily choose the first component of $F$ to be the the following non-linear function of position and time:

$$F = f(X, t) = \begin{bmatrix} AtX^2 + 1 & 0 & 0 \\ 0 & 1 & 0 \\ 0 & 0 & 1 \end{bmatrix} \tag{3.24}$$

Then considering the only component that is a function of $X$ and $t$ is $F_{11}$, and integrating it to find the position at any time:

$$\int dx = \int (AtX^2 + 1) dX \tag{3.25}$$

This then yields the following equation for $x$

$$x = \frac{AtX^3}{3} + X + C(t) \tag{3.26}$$
Knowing that at \( t = 0 \) there is no deformation \( x(t = 0) = X \) yields that \( C(t) = 0 \). Simplifying Equation 3.26 results in the following for the current position of any coordinate \( X \) at time \( t \):

\[
x = \frac{AtX^3}{3} + X
\]

(3.27)

To create a sequence of images which displace pixels according to the above equations it is obvious to use interpolation functions which interpolate between pixel values to artificially warp an initial image. In the case of a normal camera system, the sample has an initial coordinate system \( \mathbf{X} \) that deforms to a new coordinate system \( \mathbf{x} \), while the camera and it’s coordinate system, \( \mathbf{X}_C \), remain stationary. For each time \( t \), we create an interpolation function based on the current pixel coordinates given by equation 3.23 or 3.26: \( I_x \). We then interpolate on that function using the stationary camera coordinates \( \mathbf{X}_C \) to get a new image \( I_t \):

\[
I_t = I_x(\mathbf{X}_C)
\]

(3.28)

Using equations 3.23 and 3.28, one can generate a series of images warped by any arbitrary deformation, such as the non-linear deformation given by equation 3.26. Assuming that displacement estimation is always perfect, these images can be then used to evaluate the accuracy and precision of any strain estimation algorithm using equation 3.22 knowing the initial position of the tracked region and the current time \( t \). Of course, however, the assumption of perfect displacement tracking is not appropriate since strain estimation implicitly involves displacement tracking as well. It is therefore important to also consider if the strain estimation algorithm is correctly estimating strain at an instantaneous time and location \( (F(x, t)) \), regardless of the original coordinates of the region element used in strain estimation. Since in the above solution the deformation is a function of the original coordinates and time (equation 3.22), we must find an initial coordinate \( \mathbf{X} \) for every instantaneous
coordinate $x$. This can be accomplished for any arbitrary function by iteratively minimizing the following energy equation given any value of $x$:

$$E = (f(X,t) - x)^2 \quad (3.29)$$

In the case of the arbitrary equation given in 3.24 we have:

$$E = \left( \frac{AtX^3}{3} + X - x \right)^2 \quad (3.30)$$

**Inverting the roles of the material and observational coordinate systems**

A more computationally efficient version of this problem can be accomplished by inverting the roles of the initial coordinates $X$ and the instantaneous coordinates $x$. Inversion of the problem removes the Delaunay Triangulation during image interpolation and allows direct calculation of the initial coordinates given the starting coordinates without minimizing an arbitrary function. This can be achieved by working backwards from above and inverting the roles of the coordinates during the interpolation. To start, assume that the original coordinate system of the sample $X$ is a proportionally related to the camera coordinates $X_C$:

$$X \sim X_C \quad (3.31)$$

or for simplicity that they are equivalent

$$X = X_C \quad (3.32)$$
then consider the inversion the interpolation step given by equation 3.28:

\[ I_t = I_{X_C}(x) = I_X(x) \]  \hspace{1cm} (3.33)

This results in an interpolation that is now based on a coordinate system that the user can define: \( X_C \). By defining this coordinate system to be rectangular, the interpolations can be performed using a simple lookup method rather than performing a Delaunay triangulation at each time step. Equations 3.23 and 3.33 can then be used to warp the images for each time \( t \). As a consequence, however, the known deformation at each location must be reconsidered since the roles of \( X \) and \( x \) have been inverted only for interpolation, while keeping the roles consistent for finding positions. The result of this will be that the output images will have inverse deformation fields and inverted positions. To start swap \( X \) and \( x \) in equation 3.23:

\[
\int dX = \int f(x, t)dx
\]  \hspace{1cm} (3.34)

to obtain an equation for the original position \( X \) for every \( x \). To obtain the deformation field, next divide each side by \( dx \) and consider equation 3.1:

\[
\frac{dX}{dx} = f(x, t) = F^{-1}
\]  \hspace{1cm} (3.35)

Equation 3.35 demonstrates that the deformation field will be given by the inverse of the input deformation field. Repeating this for the example arbitrary function given in 3.24 yields that the original position of each region \( X \) is a function of the current position \( x \):

\[
X = \frac{Ax^3}{3} + x
\]  \hspace{1cm} (3.36)
and that the Eulerian deformation $F_{11}$ is

$$F_{11} = f(X, t) = \frac{1}{AtX^2 + 1} = \frac{1}{At \left( \frac{Atx^3}{3} + x \right)^2 + 1}$$

(3.37)

Lastly, if interested in determining the Lagrangian strain accuracy and precision, it is imperative to determine where each region has displaced to, and then calculate the strain at that location. This, however, is implicitly known because the original location of all regions $X$ is chosen before strain estimation begins. Therefore, it can be directly calculated using 3.37.

One should be aware that, when inverting the roles of the coordinate systems in the interpolation, the desired deformations are also inverted. For example, in the forward computation with the given example deformation field in 3.24, assuming $t > 0$, when $A > 0$ the material will stretch and when $A < 0$ the material will compress. For the inverted computation, when $A < 0$ the material will stretch and when $A > 0$ the material will compress.

Inverting the roles of the coordinate systems in this case improves computational efficiency in three areas:

1. Delaunay Triangulation of the deforming coordinate system is avoided and all interpolations can be performed on a rectangular grid. (Removal of $O(n \log(n))$ per image calculation where $n = \text{number of pixels}$)

2. Calculation of a priori Eulerian strains does not rely in minimizing an arbitrary function to find the original location of all pixels.

3. Calculation of Lagrangian strains is simplistic because original locations of all regions are implicitly known.
3.3.4 Efficient warping of three dimensional volumes from spatially varying displacement fields and determination of Lagrangian Strains

The above methods were extended to invert the roles of the Lagrangian and Eulerian coordinate systems for a given displacement function rather than a given deformation tensor. To achieve this for any arbitrary function, first consider an arbitrary displacement function:

\[ \mathbf{x} = \mathbf{X} + \mathbf{u}(\mathbf{X}, t) \quad (3.38) \]

As described above, inverting the roles of \( \mathbf{X} \) and \( \mathbf{x} \) results in a system where the interpolated coordinates lie on a rectangular grid, circumventing the need for a Delaunay Triangulation prior to interpolation. The resulting deformation function then becomes:

\[ \mathbf{X} = \mathbf{x} + \mathbf{u}(\mathbf{x}, t) \quad (3.39) \]

Then, taking the partial derivative with respect to \( \partial \mathbf{x} \):

\[ \frac{\partial \mathbf{X}}{\partial \mathbf{x}} = \mathbf{I} + \frac{\partial (\mathbf{u}(\mathbf{x}, t))}{\partial \mathbf{x}} \quad (3.40) \]

Next, inverting and simplifying Equation 3.40 results in an expression for the deformation at every coordinate as a function of the current position:

\[ \mathbf{F} = \left( \mathbf{I} + \frac{\partial (\mathbf{u}(\mathbf{x}, t))}{\partial \mathbf{x}} \right)^{-1} \quad (3.41) \]
The resulting expression demonstrates that the true deformation at an instantaneous location is a function of the Jacobian of the displacement function. In practice, Jacobians were too complicated to calculate by hand, so the MATLAB symbolic toolkit was employed to calculate the Jacobians of the displacement functions.

### 3.3.5 Displacement models for in silico simulations

#### Penny shaped crack simulation

Strain fields of a penny shaped crack were generated using the methods outlined in Section 3.3.4 where \( u(X, t) \) is given by [73]:

\[
\begin{align*}
    u(X, t) &= t u(X) \quad (3.42) \\
    u_r &= -\frac{v \sigma r}{E} + \frac{(1 + v) \sigma r}{\pi E} \left\{ (1 - 2v) \left( \frac{\alpha p_2^2 - a^2}{p_2^2} - \sin^{-1} \frac{a}{p_2} \right) + \frac{2a^2|z|\sqrt{a^2 - p_1^2}}{p_2^2 (p_2^2 - p_1^2)} \right\} \quad (3.43) \\
    u_z &= \frac{\sigma z}{E} + \frac{2(1 + v)}{\pi E} \left\{ 2(1 - v) \left( \frac{z}{|z|} \sqrt{a^2 - p_1^2} - z \sin^{-1} \frac{a}{p_2} \right) + z \left( \sin^{-1} \frac{a}{p_2} - \frac{\sqrt{p_2^2 - a^2}}{p_2^2 - p_1^2} \right) \right\} \quad (3.44) \\
    p_1 &= \frac{1}{2} \left( \sqrt{(a + r)^2 + z^2} - \sqrt{(a - r)^2 + z^2} \right) \quad (3.45) \\
    p_2 &= \frac{1}{2} \left( \sqrt{(a + r)^2 + z^2} + \sqrt{(a - r)^2 + z^2} \right) \quad (3.46)
\end{align*}
\]
Eshelby inclusion simulation

Strain fields of an Eshelby inclusion were generated using the methods outlined in Section 3.3.4 where \( u(X, t) \) is given by [73, 74]:

\[
\begin{align*}
\mathbf{u}(X, t) &= t\mathbf{u}(X) \\
u_i &= \frac{(1 + \nu) a^3}{2(1 - \nu)E} \left\{ \frac{2p^T_{ik}x_k + p^T_{kk}x_i}{15R^5} \left( 3a^2 - 5R^2 \right) + \frac{p^T_{jk}x_jx_kx_i}{R^7} \left( R^2 - a^2 \right) + \frac{4(1 - \nu)p^T_{ik}x_k}{3R^3} \right\} \\
R &= \sqrt{x_kx_k} \\
p^T_{ij} &= \frac{E}{(1 + \nu)} \left\{ \varepsilon^T_{ij} + \frac{\nu\varepsilon^T_{kk}\delta_{ij}}{(1 - 2\nu)} \right\}
\end{align*}
\]

### 3.3.6 Validation methods

Mean absolute error plots were computed comparing 3D-XCOR, 3D-LSF, and 3D-DDE to the true values of strain known from the initial deformation fields using the following equation:

\[
RMSE_{\text{error}} = \sqrt{\frac{\sum_{i=1}^{n}(\hat{y}_i - y_i)^2}{n}}
\]

where \( \hat{y}_i \) is the known true value for each strain estimate, \( y_i \) is the estimated value for each technique, and \( n \) is the number of estimates. Error bars are computed by one standard deviation of the error values. Lastly, a mean and a standard deviation are plotted for each value of increasing strain for each estimate of strain.
RMS Error plots were also computed for varying amounts of artificially added noise. Varying amounts of Gaussian, Poisson, salt and pepper, or speckle noise were added to each volumetric image using built-in Matlab functions for adding image noise.

3.4 Results

3.4.1 3D-DDE was more accurate and precise than displacement-based methods

We first tested the ability of 3D-DDE on results of in silico experiments to establish its ability to resolve non-uniform strain fields arising from a spatially- and temporally-varying deformation gradient tensor, $F$. As an example, we studied Equation (3.24) where $A$ was a parameter controlling the maximum deformation, $t$ was time ranging from 0 to 1, and $x$ was a Cartesian coordinate in the undeformed reference image. This spatially quadratic field was chosen as a best-case scenario for competing 3D-XCOR methods because the strain fields estimated using displacements of the midpoints of tracked regions converge to the strain fields at the center of such regions (Chapter 2). Note that computationally efficient generation of successive volumetric images of these strain fields was non-trivial (see Section 3.3.3). Using this deformation field for all cases, regardless of size of spacing of the search regions used to track deformation, 3D-DDE was more accurate and more precise than both standard 3D-XCOR and displacement-based tracking (3D-LSF) (Figure 3.1). Although displacement-based approaches were just as accurate as 3D-DDE for uniform strain fields (Section 3.4.2), direct strain estimation was superior for all cases involving strain gradients.
Figure 3.1: 3D-XCOR was orders of magnitude inferior at calculating strain when compared to either 3D-LSF or 3D-DDE. Although 3D-LSF had a slight advantage over 3D-DDE when strain fields were uniform, 3D-DDE was an order of magnitude superior when strain fields were complex. (A) RMS noise versus angle of rotation for a 3D body undergoing 3D rotation in one plane for 3D-XCOR, 3D-LSF, and 3D-DDE. (A, inset) Region of (A) zoomed in where 3D-XCOR maintained a reasonable calculation of strain. (C) RMS noise versus applied strain in the 11 direction for a 3D body undergoing uniform stretch in one plane for 3D-XCOR, 3D-LSF, and 3D-DDE. (E) RMS noise versus maximum stretch ratio in the 11 direction for a 3D body undergoing non-linear stretch given by equation (1) for 3D-XCOR, 3D-LSF, and 3D-DDE. (B,D,F) Regions of (A),(C),and (E), respectively, zoomed in to focus on results for only 3D-LSF and 3D-XCOR.
3.4.2 Direct Estimation of strains is in general more accurate and precise than displacement based methods except when considering uniform strain fields

During the expansion of the strain estimation algorithm from two dimensions to three dimensions, great care was taken to reduce computational complexity so that the digital volume correlation algorithm was highly efficient and computations could be executed in a practical amount of time. Improvements to the code were several fold: 1) the coordinate system of each tracked region were normalized by centering about 0 with a range of 1 in each dimension, 2) parameter updates during image registration were adjusted to all be of similar order, and 3) all interpolations were inverted to always be performed on the original image to circumvent scattered data interpolation and the need for Delaunay triangulations.

As a consequence of these optimizations the accuracy and precision of the algorithm, especially with respect to the displacement estimation, was improved. In the process, certain conditions arose in which displacement based strain estimation was superior to direct strain estimation, specifically in the case of uniform deformation fields.

While both direct strain estimation techniques (3D-DDE) and non-rigid displacement based techniques (3D-LSF) remained far superior to normalized cross correlation (3D-XCOR) (Figure 3.1) the combined effect of the computational improvements to the code also had an unexpected side effect: they improved displacement tracking and displacement based strain estimation. Further examination revealed certain cases, such as linear deformation or rigid body motion, where non-rigid displacement based strain estimation (3D-LSF) became more accurate and precise than direct methods (3D-DDE) (Figure 3.1B,D). To test the hypothesis
that this may be a result of increased numerical precision arising from improved displacement tracking, the size and spacing of each kernel region was systematically varied. For image sequences with constant deformation, when individual regions were spaced further and further apart, the numerical error from taking a derivative of the displacement field decays exponentially and displacement based tracking improves exponentially (Figure 3.2). Further, as the regions are made larger and larger, both displacement based tracking and direct estimation improved, likely due to more information available to the algorithm for precise determination of both region displacement and deformation. Interestingly, in the case of linear deformation fields or rigid body rotation, direct estimation was still inferior to displacement based tracking even with very closely spaced regions (Figure 3.2 and Figure 3.1).

We note that these special conditions exist under which displacement-based techniques can outperform deformation-based techniques, but that in these cases all methods provide relatively accurate strain estimates. It is somewhat obvious that when the deformation gradient is uniform, by varying the region kernel size and the distance between each region tracked, numerical precision can be optimized for displacement-based strain estimation. However, for increasingly nonuniform fields, the spatially varying terms of Equation 3.1 deviate further from the solution so that direct estimation, which does not rely upon numerical gradient estimation, is consistently more precise and accurate.

3.4.3 3D-DDE was relatively insensitive to noise

Because noise is inherent to volumetric imaging modalities such as ultrasound, magnetic resonance, and confocal fluorescence imaging, techniques for strain estimation from 3D image
Figure 3.2: RMS strain error from solution versus input stretch ratio for kernel sizes and kernel spacing ranging from 11x11x11 to 45x45x45 voxels for a material stretched with a uniform strain \textit{in silico}. As kernel size increased, both 3D-LSF (red) and 3D-DDE (blue) improve their accuracy and precision. Additionally, as kernel spacing increased, the accuracy and precision of 3D-LSF increased rapidly, while 3D-DDE only improved marginally. In all cases, 3D-LSF remained slightly more accurate and more precise than 3D-DDE, with both approaching an accuracy and precision of $10^{-5}$ at a kernel size and spacing of 45x45x45. The marginal advantage of 3D-LSF at high spacing can be attributed to increased numerical precision as regions are further and further apart from one and other in a uniform strain field.
Figure 3.3: RMS strain error from solution versus input stretch ratio for kernel sizes and kernel spacing ranging from 11x11x11 to 45x45x45 voxels for a material stretched non-linearly in silico. As kernel size increased, both 3D-LSF (red) and 3D-DDE (blue) improved their accuracy and precision marginally. However, as kernel spacing increased, the accuracy and precision of 3D-LSF decreased sharply, while 3D-DDE remained consistent. In all cases, 3D-DDE remained more accurate and more precise than 3D-LSF.
stacks must be robust to noise. To evaluate the relative robustness of 3D-DDE, the non-linear warping function of Equation (3.24) was applied to artificially generated volumetric images, and varying amounts of Gaussian, Poisson, salt and pepper, or speckle noise were added to each volumetric image in the sequence. 3D-DDE was relatively insensitive to small amounts of Gaussian noise and was superior to both 3D-LSF and 3D-XCOR calculations (Figure 3.4A,C). When applied to image sequences with larger amounts of Gaussian noise, 3D-DDE provided the most consistently accurate strain estimation, but its advantages over 3D-LSF or 3D-XCOR were less pronounced (Figure 3.4D). For moderate salt-and-pepper and speckle noise, 3D-DDE remained accurate and precise across all strain values while 3D-LSF lost accuracy and precision with increasing strain and 3D-XCOR performed poorly at all strain levels (Figure 3.4E,G). Sufficiently high levels of added salt-and-pepper or speckle noise caused all three methods to fail. For added Poisson noise, 3D-LSF was more accurate and more precise than 3D-DDE, but nevertheless provided only a crude approximation of strain, while 3D-XCOR failed to estimate a reasonable strain value (Figure 3.4B).

3.4.4 3D-DDE accurately estimates strains in representative 3D strain fields

To benchmark 3D-DDE against strain fields representative of an isolated contractile cell, displacement fields of an Eshelby inclusion were used to nonlinearly warp a volumetric volume in silico (Figure 3.5) [74]. Note that, as above, warping and determining the true a priori deformation fields in a computationally efficient manner was non-trivial (Section 3.3.5). 3D-DDE replicated the actual strains arising from the Eshelby solution accurately, with 3D-LSF and 3D-XCOR each performing successively worse (Figure 3.5). The central challenge of distinguishing tracking errors from true regions of elevated strain was overcome through
Figure 3.4: RMS error versus stretch ratio in the 11 direction for (A) noise free, (B) Poisson noise, (C,D) low and high levels of Gaussian noise, (E,F) low and high levels of salt and pepper noise, and (G,H) low and high levels of speckle noise. 3D-DDE was superior to 3D-XCOR in all cases and superior to 3D-LSF in all cases except Poisson noise and high levels of speckle or salt and pepper noise.
3.4.5 3D-DDE and 3D-SIMPLE could identify and characterize singular strain fields

Displacement fields surrounding a penny shaped crack were used to nonlinearly warp a volumetric image in silico following the procedures used for the Eshelby solution (Figure 3.6, Section 3.3.5) [75]. During loading, the crack, initially ellipsoidal due to a pre-load, extended...
Figure 3.6: Stretch ratio results for a penny shaped crack *in silico*. (A) Schematic of an Eshelby inclusion. (B) 3D-SIMPLE detected and highlighted the developing crack, (C) True values of the first principal stretch ratio closely match the 3D-DDE estimated values (D), while 3D-LSF-estimated (E), and (F) 3D-XCOR-estimated stretch ratios were successively worse.

into a more spherical ellipsoid. Again, 3D-DDE identified the input strain field faithfully, with 3D-LSF and 3D-XCOR performed successively worse (Figure 3.6). 3D-SIMPLE identified regions of elevated strain and high strain gradient, including both the singular crack tips and the displacing fracture surfaces (Figure 3.6).

3D-DDE provided estimates of spatially-varying 3D strain fields with accuracy and precision exceeding those of all other existing methods of which we are aware. These improvements were especially pronounced for the inherently noisy image volumes that arise from medical imaging modalities such as ultrasound and enabled 3D strain analysis of a beating mouse heart (Section 5.5). 3D-DDE estimated these strain fields without the ad hoc constraints and
post hoc regularization that other methods require, such as rigid registration [36, 29] specific
material constitutive laws [28, 30], and strain compatability of averaged displacement fields
[31]. We note that the requirement that strains estimated over a grid be compatible is
inappropriate when strong strain gradients exist [76]. Although other technologies could
be tailored to be at least as accurate as 3D-DDE for uniform strain fields, 3D-DDE out-
performed state-of-the-art techniques when strain concentrations and gradients existed.

3.5 Discussion

The key differences between 3D-DDE and standard cross-correlation techniques are: 1) op-
timal 3D warping of undeformed images prior to cross-correlation and 2) direct calculation
of the deformation gradient. The latter factor enabled a robust identification of strain con-
centrations and strain gradients. The 3D-SIMPLE metric, which measured the difference
between 3D-DDE estimates and strains calculated using an advanced, warped extension of
standard cross-correlation, enabled the first fully automated detection of strain concentra-
tions in 3D volumetric images, both at the poles of a contracting ellipsoidal inclusion and in
the vicinity of a stressed penny-shaped crack.

Although results showed how advanced strain mapping techniques can now enable dynamic
and regularization-free 3D strain analysis of tissue structures in vivo, the method has several
shortcomings that bear mention. Acquisition of volumetric images remains challenging. We
found a kernel size of 15x15x15 voxels to be the minimum for reliable strain estimation, and
the sampling rate of current commercial 3D ultrasound probes are not adequate for identify-
ing subtle features within a strain field. In one real world example, described in Section 5.5,
this limitation was overcome by taking advantage of the periodicity and reproducibility of a
heart beat to construct 3D volumes from 2D slices. This yielded a high resolution volumetric time series of composite heartbeats (Section 5.5). Our experiments required specialized apparatus not typically available in a clinical ultrasound suite, although others have overcome this limitation by focusing on nearly quasi-static images (e.g., [77]).

3D-DDE shows promise for estimation of full 3D strain tensors in both biomedical and engineering applications. The potential to identify cracks and strain concentrations is of potential value in applications such as earthquake fault analysis with ground-penetrating radar (e.g., [78]) and inspection of aerospace composites [79, 80, 81]. The method is also of potential value to our efforts to extend current 2D strain analysis of brain motion to 3D, where a major unmet challenge is to identify the origins of strain concentrations near attachment points (e.g., [82, 83, 84]). In all such applications, we believe that the accuracy and precision of 3D-DDE and the reliability check afforded by 3D-SIMPLE will improve our ability to interpret the distribution of strains on the interior of biological and engineered structures.
Chapter 4

Methods for estimating two dimensional surface strain from a stereo vision system

4.1 Abstract

The tracking of surface deformation of geometrically complex three dimensional structures is critical for the next generation mechanical understanding of complex biologic structures and biologic materials. Single view techniques, such as 2D-DDE, excel at estimating deformation on a surface parallel to the imaging plane. However, motion or deformation out of this plane is incorrectly estimated by 2D-DDE due to lack of three dimensional information. To alleviate this, stereo or multi-view systems are required to resolve three dimensional positioning information. The prior state of the art techniques in multi-view deformation estimation relies on a distinct three step process for deformation estimation: 1) independent registration, 2) triangulation, and 3) deformation estimation. Inspired by our previous work, presented in Chapters 2 and 3 which directly incorporated registration and deformation information into
deformation estimation, we theorize here two techniques for simultaneous image registration, three dimensional triangulation, and deformation estimation in a multi-view system. The strengths and weaknesses of the two theorized implementations are compared with specific regard to continuum mechanics assumptions and deformation estimation.

4.2 Introduction

While the two dimensional strain estimation techniques described in Chapter 2 have a broad range of applications, they are limited to strain estimation that occurs in a plane parallel to a camera or imaging sensor. Surface deformations occurring outside of this plane are viewed by the single camera as an affine or projective transformation that is misconstrued by the two dimensional algorithms as a deformation, when in reality it may be a three dimensional translation or three dimensional rotation of the surface. To address this issue, stereo vision systems, which consider three dimensional motion of the plane, must be used along with methods for estimating the two dimensional deformation of the sample surface.

Traditional techniques for estimating two dimensional strain from three dimensional surfaces rely on triangulating a three dimensional position of a surface through a series of multi-view images. Displacements of several tracked regions are combined following registration and triangulation to form a three dimensional displacement field. Similarly to how two dimensional algorithms work, this three dimensional displacement field is then differentiated to arrive at a final deformation [13]. While there are a wide variety of techniques to accomplish the image registration as well as the triangulation of the registered points, they are all inherently flawed in that they must accept image registration noise as well as three dimensional triangulation noise, both of which are exacerbated when derivatives of the displacements are taken. Much
of the research in this field has been aimed at reducing these sources of noise rather than developing a new framework for determining deformations from stereo vision systems [13].

Following our earlier work, which directly incorporated mechanics concepts into the image registration algorithms, we develop here a framework for two dimensional surface strain estimation from a three dimensional surface. This framework uses a stereo vision system that directly incorporates mechanics into the image registration algorithms without consideration of displacements. Due to the inherent complexity in incorporating mechanics into these approaches, two algorithms were considered: a computationally expensive version which does not compromise in terms of mechanics assumptions or accuracy and a computationally efficient version which makes some compromises with respect to mechanics assumptions to achieve efficient calculation.

### 4.3 A theoretical framework for direct strain estimation from a stereo vision system

Both algorithms begin by considering a calibrated camera system. The calibrated cameras view a two dimensional surface in a three dimensional space from multiple angles. Each camera views a projection of the surface $\pi$. The coordinates of the surface, $x_\pi$, are projected into each camera, $i$, with projection functions, $P_i$. These projection matrices are calculated from the camera calibration, and the structure of the plane is calculated using published techniques to create structure from multi-view systems [85]. The goal of the algorithm is to minimize a function where coordinates from $x_\pi$ are projected into each camera before
and after a three dimensional warping function parameterized with three dimensional rotation, three dimensional translation, and two dimensional surface stretch (Figure 4.1). More specifically, the goal is to minimize the following energy equation:

$$E = \sum_{x_{ni}} \sum_{i} [I_i(P_i(W^{3D}(x_{ni}; p))) - T_i(P_i(x_{ni}))]^2$$  \hspace{1cm} (4.1)$$

with respect to warping parameters $p$, where $P_i$ is a 3D to 2D projection function for camera $i$, $W^{3D}$ is a three dimensional warping function, $I_i$ is an input image from camera $i$, and $T_i$ is a template image from camera $i$. We define the projection functions $P_i$ as:

$$P_i = P_i(x_{ni}; K_i, R_i, t_i) = K_i \begin{bmatrix} \cdots \cdots \cdot R_i & -R_i t_A \cdots \cdots \cdot 0 0 0 1 \end{bmatrix} \begin{bmatrix} x_{ni} \\ y_{ni} \\ z_{ni} \\ 1 \end{bmatrix}$$  \hspace{1cm} (4.2)$$

where $R_i$ is a three dimensional rotation matrix from the plane $\pi$ to camera $i$ that can be parametrized with any three dimensional rotation formulation, (e.g. quaternion, Rodrigues, or Eulerian parameterizations), and $t_i$ is a three dimensional translation vector from the plane $\pi$ to the camera center in world units. The three dimensional warping function is defined to similarly have a three dimensional rotation, $\delta R$, and three dimensional translation, $\delta t$, but also include a two dimensional surface stretch $\delta \lambda$:

$$W^{3D} = f(x_{ni}; \delta \lambda, \delta R, \delta t) = \delta \lambda \begin{bmatrix} \cdots \cdots \cdot \delta R & -\delta R \delta t \cdots \cdots \cdot 0 0 0 1 \end{bmatrix} \begin{bmatrix} x \\ y \\ z \\ 1 \end{bmatrix}$$  \hspace{1cm} (4.3)$$
where $\delta \lambda$ is a surface deformation matrix with pure shear parameterized by $\lambda_1$, $\lambda_2$, and $\gamma$:

$$
\delta \lambda = \begin{bmatrix}
1 + \lambda_1 & \gamma & 0 & 0 \\
\gamma & 1 + \lambda_2 & 0 & 0 \\
0 & 0 & 1 & 0 \\
0 & 0 & 0 & 1 \\
\end{bmatrix}
$$

(4.4)

Following the original implementation of the Lucas-Kanade algorithm [16] the current estimate of $p$ is assumed to be known and we are seeking an update to $p$ as $\Delta p$:

$$
p \leftarrow p + \Delta p
$$

(4.5)

which makes the energy equation become:

$$
E = \sum_{x_p} \sum_i \left[ I_i(P_i(W^{3D}(x_p; p + \Delta p))) - T_i(P_i(x_p)) \right]^2
$$

(4.6)

then taking a first order Taylor expansion and solving for the update $\Delta p$:

$$
\Delta p = H_{2.5D}^{-1} \sum_{x_p} \sum_i \left[ \nabla I_i \frac{\partial P_i}{\partial x_p} \frac{\partial W^{3D}}{\partial p} \right]^T \left[ T_i(P_i(x_p)) - I_i(P_i(W^{3D}(x_p; p))) \right]
$$

(4.7)

where $H_{2.5D}$ is a two and half dimensional Hessian matrix approximated by:

$$
H_{2.5D} = \sum_{x_p} \sum_i \left[ \nabla I_i \frac{\partial P_i}{\partial x_p} \frac{\partial W^{3D}}{\partial p} \right]^T \left[ \nabla I_i \frac{\partial P_i}{\partial x_p} \frac{\partial W^{3D}}{\partial p} \right]
$$

(4.8)

Following optimization, a deformation tensor, $F$ is then directly known:

$$
F_{2.5D} = \delta \lambda
$$

(4.9)
We term this algorithm Forward 2.5D DDE (F25D-DDE) since it is a forward implementation of the original Lucas Kanade algorithm expanded to work with a three dimensional motion as well as a two dimensional surface deformation estimation. A schematic for this algorithm is shown in Figure 4.1.

Figure 4.1: Schematic for Forward 2.5D DDE. Coordinates $x_\pi$ are allowed to rotate, translate, and deform in a three dimensional warp before projecting into two cameras with projection functions $P_1$ and $P_2$. Note that this schematic shows only two cameras, but the algorithm is not constrained to only two cameras.

Examining these equations and comparing them to earlier versions, note that the gradients of the images, $\nabla I_i$, which are used in both the calculation of the update and the calculation of the Hessian, are taken on the input image rather than on the template image $\nabla T$ (Equations 2.11 and 2.12 versus 4.7 and 4.8). Because of this, these gradients and the Hessian matrix must be computed at each iteration. For 2D and 3D the roles of the template and the input images could be inverted (Chapter 2, Chapter 3, and [15]) to develop a computationally efficient algorithm where the gradients depend only on the template and can therefore be precomputed. However, Baker and Matthews demonstrate that this is impossible for the 2.5D case because the gradients of the images following projections in 2.5D are non-equivalent [86].
This is apparent if the energy equation is converted to include a warp on the template: in that case, an identity warp $W^{3D}(x; 0)$ cannot be defined on the template because there is also a projection (see section 2.2.1 and reference [86]). In the next section, we explore generalizing the algorithm to invert the roles of the input image and template images for a more computationally efficient algorithm.

4.4 A theoretical framework for computationally efficient direct strain estimation from a stereo vision system

Several groups have generalized the Lucas Kanade algorithms to develop an efficient 2.5D algorithm. To achieve this, these groups converted the problem into a mapping from a 2D texture space to a 2D image [86, 87, 88]. These groups achieved this in two ways: (1) extending the set of parameters $p$ so that they include the identity warp, then enforcing priors on the results with an expanded energy equation [88] and (2) embedding the three dimensional shape of $x$ and the camera projection matrix $P$ into the warp, converting it into a 2D warp on the image coordinates $W(x; p)$, with an adjusted update function given by [87]:

$$W(x; p) \leftarrow W(x; p) \circ W(x; p^*)^{-1} \circ W(x; p^* + \Delta p)$$  (4.10)

where $p$ is all parameters and $p^*$ is the set of parameters pertaining only to the camera projections. The authors found that this generally works best when $p^* \approx p$, which is not always the case and can result in reduced precision and accuracy [87].
Here, we compare the two implementations by considering how they affect strain tracking. Using the first approach and enforcing priors (ref. [88]), assumptions must be made about the mechanics. One assumption is to enforce strain compatibility. However, as shown in Chapter 2, if the algorithm is constrained to enforce strain compatibility, errors may be introduced. Additionally, a multi-view surface estimation implementation of SIMPLE cannot be developed with strain compatibility. Alternate priors that could be used would be to assume a certain form of the deformation or motion of the planes; however, this requires making assumptions on the deformation or about the material behavior, which also constrains the algorithm. Because of this, we take the approach of Romdhani et al. to develop a computationally efficient algorithm [87].

To begin, a two camera system with cameras $A$ and $B$, both with normalized camera coordinates is considered. Since normalized camera coordinates are considered, it is reasonable to assume that the intrinsic camera matrices $K_i$ of each camera are already accounted for in all calculations [85]. An energy equation, which compares the image intensity differences in a coordinate system of a single reference camera (which is chosen arbitrarily to be camera $A$), is then minimized. The particular energy equation used here consists of the sum of the squared differences in image intensity over all pixels in camera $A$ for image intensity in camera $A$ and in camera $B$. This energy equation is introduced with identity warps on the template images so that the inverse compositional algorithm can be readily applied:

$$
E = \sum_{x_A} \left( (I_A(W_A(X_A; p)) - T_A(W_A(X_A; p^*))^2 + [I_B(W_{AB}(X_B; p)) - T_B(W_{AB}(X_B; p^*))]^2 \right)
$$

(4.11)

where $W_A(X_A; p)$ is a warping function on the coordinates of camera $A$, $X_A$, with parameters $p$, that returns coordinates in $A$ after deformation, $x_A$, $W_A(X_A; p)$ is a similar warping function but with initial parameters $p^*$, $W_{AB}(X_B; p)$ is a warping function on coordinates
that returns coordinates in camera $A$ after deformation, and $W_{AB}(X_B; p^*)$ is a similar warping function but with initial parameters $p^*$. Compared to Equation 4.6, the warping functions in this energy equation have the camera projections built in. It is important to realize the parameters of the projection matrices are now part of the parameters of the warp, $p$.

To define the warping functions, $W_A(X_A; p)$ and $W_{AB}(X_B; p)$, a previous implementation is followed [89] and begins by considering a calibrated stereo rig viewing a static plane, and that plane is said to induce a homography between the two views [85]. Considering a calibrated stereo rig with world coordinates at the center of the plane $\pi$, the projection matrices are:

\[
P_A = \begin{bmatrix} R_A & -R_A t_A \\ 0^T_3 & 1 \end{bmatrix}
\]

\[
P_B = \begin{bmatrix} R_B & -R_B t_B \\ 0^T_3 & 1 \end{bmatrix}
\]

where $R_A$ and $R_B$ are rotation matrices from $A$ or $B$ to $\pi$ parameterized by the Rodrigues rotation angles and $t_A$ and $t_B$ are three dimensional translation vectors from $A$ or $B$ to $\pi$. These projection matrices are specifically chosen so that their inverses are equal to:

\[
P_A^{-1} = \begin{bmatrix} R_A^T & t_A \\ 0^T_T & 1 \end{bmatrix}
\]

\[
P_B^{-1} = \begin{bmatrix} R_B^T & t_B \\ 0^T_T & 1 \end{bmatrix}
\]
Next consider a world plane $\pi$ with coordinates parameterized by

$$\pi = (n^T_\pi, d)$$  \hspace{1cm} (4.16)$$

such that the following equation is satisfied for all points on that plane:

$$n^T_\pi X_\pi + d = 0$$  \hspace{1cm} (4.17)$$

Every point $X_\pi$ is then sensed by each camera as:

$$X_A = P_A X_\pi = (R_A X_\pi - R_A t_A)$$  \hspace{1cm} (4.18)$$

$$X_B = P_B X_\pi = (R_B X_\pi - R_B t_B)$$  \hspace{1cm} (4.19)$$

We rewrite equation 4.18 by combining with 4.17 as:

$$X_A = P_A X_\pi = (R_A X_\pi - R_A \frac{t_A n^T_\pi}{d} X_\pi)$$  \hspace{1cm} (4.20)$$

then simplifying to obtain a relation for how the plane is sensed by camera $A$ before deformation:

$$X_A = \left( R_A - R_A \frac{t_A n^T_\pi}{d} \right) X_\pi$$  \hspace{1cm} (4.21)$$

Next consider that the plane may be in motion. Following a rigid body motion of the plane, the new plane is defined by:

$$\pi^* = (n^*_\pi, d^*)$$  \hspace{1cm} (4.22)$$
parameterized by a three dimensional rotation, $\delta R$, and a three dimensional translation $\delta t$. This transformation is defined such that:

$$x_\pi = \delta R X_\pi - \delta t$$  \hspace{1cm} (4.23)

or similarly:

$$X_\pi = \delta R^T x_\pi + \delta R^T \delta t$$  \hspace{1cm} (4.24)

This result is combined with equation 4.17, simplified, and converted to homogeneous coordinates in 4 dimensions to obtain:

$$X_\pi = \left( \delta R^T + \delta R^T \frac{\delta t n_T}{d} \right) x_\pi$$  \hspace{1cm} (4.25)

Since the projection is equivalent before and after the three dimensional warp, equation 4.21 can be modified after the motion of the plane

$$x_A = \left( R_A - R_A \frac{t_A n_T^T}{d} \right) x_\pi$$  \hspace{1cm} (4.26)

Then, by chaining together 4.21,4.25, and 4.26:

$$x_A = \left( R_A - R_A \frac{t_A n_T^T}{d} \right) \left( \delta R^T + \delta R^T \frac{\delta t n_T}{d} \right)^{-1} \left( R_A - R_A \frac{t_A n_T^T}{d} \right)^{-1} X_A$$  \hspace{1cm} (4.27)

This is a warping function for how the reference camera, camera A, views the plane before and after a three dimensional rotation and a three dimensional translation. This equation is the form

$$x_A = H X_A$$  \hspace{1cm} (4.28)

where $H$ is a homography induced by the plane from $X_A$ to $x_A$ [89, 85].
To obtain a similar warping function for $X_B$ to $x_a$, begin by combining 4.19 and 4.18:

$$X_A = R_A R_B^T X_B + R_A(t_B - t_A) \quad (4.29)$$

and similarly for after the warp:

$$x_A = R_A R_B^T x_B + R_A(t_B - t_A) \quad (4.30)$$

Next consider an equation for the projection of $x_\pi$ into camera $B$, which is similar to equation 4.26:

$$x_B = \left( R_B - R_B \frac{t_B n_\pi^T}{d} \right) x_\pi \quad (4.31)$$

and similarly for before the deformation:

$$X_B = \left( R_B - R_B \frac{t_B n_\pi^T}{d} \right) X_\pi \quad (4.32)$$

then chaining 4.32, 4.31, and 4.25 and inserting into 4.30 a final equation for the warp from $X_B$ to $x_A$ is realized as:

$$x_A = R_A R_B^T \left( R_B - R_B \frac{t_B n_\pi^T}{d} \right) \left( \delta R^T + \delta R^T \frac{\delta t n_\pi^T}{d} \right)^{-1} \left( R_B - R_B \frac{t_B n_\pi^T}{d} \right)^{-1} \left( R_B - R_B \frac{t_B n_\pi^T}{d} \right) X_B + R_A(t_B - t_A) \quad (4.33)$$

which demonstrates this plane is constrained under a plane and parallax homography of the form:

$$x_A = HX_B + \rho e_A \quad (4.34)$$

where $\rho$ is the parallax displacement and $e_A$ is the projection of the epipole under $P_B$ [89, 85].
Up until now, for simplicity, the world coordinate transformation has been strictly defined to be parametrized by a three dimensional rotation matrix $\delta R$ and a three dimensional translation $\delta t$, with no deformation. To expand upon this and arrive at our final warping functions, a new parametrization is considered that includes deformation: a two dimensional surface stretch $\delta \lambda$, a three dimensional rotation $\delta R$, and a three dimensional translation $\delta t$. Importantly, the two dimensional surface stretch has parameters for normal strain $\lambda_1$ and $\lambda_2$, and shear strain $\gamma$:

$$
\delta \lambda = \begin{bmatrix}
1 + \lambda_1 & \gamma & 0 \\
\gamma & 1 + \lambda_2 & 0 \\
0 & 0 & 1
\end{bmatrix}
$$

Next, modifying the equation that defines the motion of the plane to include deformation of the plane:

$$
x_\pi = \delta \lambda \delta R x_\pi - \delta t
$$

or similarly:

$$
X_\pi = \delta R^T \delta \lambda^{-1} x_\pi + \delta R^T \delta \lambda^{-1} \delta t
$$

Combining this with 4.17:

$$
X_\pi = \left( \delta R^T \delta \lambda^{-1} + \delta R^T \delta \lambda^{-1} \frac{\delta t n_\pi^T}{d} \right) x_\pi
$$

Lastly this new plane warp equation is used to update the warping functions, 4.27 and 4.33:

$$
x_A = \left( R_A - R_A \frac{t_A n_\pi^T}{d} \right) \left( \delta R^T \delta \lambda^{-1} + \delta R^T \delta \lambda^{-1} \frac{\delta t n_\pi^T}{d} \right)^{-1} \left( R_A - R_A \frac{t_A n_\pi^T}{d} \right)^{-1} X_A
$$
\[ x_A = R_A R_B^T \left( R_B - R_B \frac{t_B n_B^T}{d} \right) \left( \delta R^T \delta \lambda^{-1} + \delta R^T \delta \lambda^{-1} \frac{\delta t n_B^T}{d} \right)^{-1} \left( R_B - R_B \frac{t_B n_B^T}{d} \right)^{-1} X_B + R_A (t_B - t_A) \]

(4.40)

to arrive at a final warping functions for use in equation 4.11. Taking a closer look at these warping functions, it is clear that the warping function \( W_A \) works in 3 steps: (1) projecting the image coordinates out to the world coordinate system, (2) allowing the world coordinate system to transform, and (3) re-projecting back into coordinates of camera \( A \) (Figure 4.2A). Similarly, the warping function \( W_B \) has four steps: (1) it projects from \( B \) out to the world coordinate system, (2) allows the world coordinate system to transform, (3) projects it back to \( B \), and (4) projects coordinates of \( B \) into \( A \) (Figure 4.2B). Using these warping functions following optimization, we directly know the deformation of each plane with equation 4.9. This algorithm has an update rule given by 4.10 and by taking the first order Taylor expansion of 4.11 and following similar logic to section 4.3, as calculated by Romdhani, the iterative update rule and Hessian matrices can be easily computed [87]. We term this algorithm Inverse 2.5D DDE (I25D-DDE) since it is an inverse and computationally efficient version of DDE.

### 4.5 Discussion

We developed a forwards algorithm (F25D-DDE) and an inverse algorithm (I25D-DDE) for estimating three dimensional position and orientation as well as two dimensional surface deformation. F25D-DDE was based on the original Lucas-Kanade algorithm, which is computationally expensive due to the requirement that image gradients and steepest descent
images must be computed for each iteration [15]. For the single camera version of this problem, the Lucas Kanade inverse compositional algorithm inverts the roles of the template and input image so that the image gradients and steepest descent images can be computed on the template rather than on the continuously updating input image [15]. Importantly, forwards and inverse algorithms have been proven to be equivalent for this single camera case and we implement them in the two dimensional and three dimensional algorithms (2D-DDE in Chapter 2 and 3D-DDE in Chapter 3). However, as demonstrated by Baker and Matthews, the forwards and inverse algorithms are not equivalent for the multiple camera case [86]. Several groups attempted to overcome this shortcoming by expanding on the original Lucas-Kanade algorithm by converting the problem into a mapping from a 2D texture space to

Figure 4.2: Schematic for warping functions $W_A$ and $W_B$. (A) $W_A$ works in 3 steps: (1) projecting the image coordinates out to the world coordinate system, (2) allowing the world coordinate system to transform, and (3) re-projecting back into coordinates of camera A. (B) The warping function $W_B$ has 4 steps: (1) projecting from B out to the world coordinate system, (2) allowing the world coordinate system to transform, (3) projecting back to B, and (4) projecting coordinates of B into A.
a 2D image [86, 87, 88]. From a mechanics perspective, we decided that the approach of Romdhani et al. would be superior, since we would not have to enforce priors about the warp, such as strain compatibility, to develop an algorithm. The completed algorithm, I25D-DDE, uses the projection matrices of each camera as well as parameters of the plane to create a 2D-2D projective mapping for each camera, effectively solving the problem by including the parameters of projection into the warping function.

Examining the implementation of I25D-DDE more closely, several points of interest become apparent. First, the algorithm can be extended beyond two cameras if we keep one camera, (camera A in the current implementation) as a reference camera. Second, the calculated warping functions depend on the parameters of motion and deformation of the plane \((\delta R, \delta t, \delta \lambda)\) but also on the parameters of the projections and the orientation of the plane \((R_A, t_A, R_B, t_B, n_B, d)\), where \(p^*\) is the set of parameters pertaining to the camera projections and plane orientation. This has several implications on the computational complexity and resulting strain estimation. The Jacobians \(\partial W/\partial x\) will depend on the parameters of the projection and on the parameters of each plane. In practice, Romdhani found that the algorithm works best when the full set of parameters \(p\) is in the vicinity of the initialized parameters \(p^*\), or mathematically when \(p \approx p^*\) [87]. While this should hold true for each iteration during optimization, it will likely not hold true for each successive frame. As a consequence, the projection parameters \(p^*\) and the Jacobians of the warp \(\partial W/\partial x\) will need to be recomputed for each successive frame to maintain accuracy and precision. This results in a slight increase in the computational complexity. Furthermore, the identity warp for the template in camera \(B\) will return coordinates in camera \(A\), so it is not a true identity warp and will have to be interpolated. This is important to consider because the algorithm relies on the gradients of the template image and the gradients will have to be computed.
on a projection of the template and will be approximations of the true gradient, which will reduce accuracy and precision [86].

Collectively, when examining these two implementations of a stereo vision DDE algorithm, the theory demonstrates that if we want to simultaneously register surfaces using two cameras while intrinsically computing deformation, it is likely that one must compromise in accuracy and precision, enforce strain compatibility, and/or settle for an increase in computational cost. Both algorithms will be implemented in future studies and compared using computer generated data, where true strains are known, to evaluate if the reduction in accuracy and precision from the use of the more computationally efficient algorithm is warranted. Alternatively, a hybrid algorithm may be developed where we first estimate the parameters using the efficient algorithm, and then refine the parameters using the more precise but more computationally expensive algorithm.

A major advantage of the new algorithms is that, compared to other multiple view surface strain algorithms, the implementations described in this chapter do not depend on the estimation of three dimensional position by triangulation following registration [13]. As shown previously for 2D-DDE and 3D-DDE, removal of reliance on displacement estimation should increase accuracy and precision of strain estimation. In the case of multiple view surface strain estimation, this advantage will be even more apparent than 2D and 3D implementations, for several reasons. First, in other approaches, images are registered independently of one and other, introducing two independent sources of noise from two independent registrations. The algorithms presented here simultaneously register the two images using a single set of parameters, which is less susceptible to registration noise. Second, other algorithms must triangulate the two registrations to arrive at a displacement parameter for each plane, which the current algorithm does not need to do, further reducing a source of noise. Lastly,
the algorithms presented in this chapter do not require taking derivatives of displacement fields to arrive at a deformation estimation. In fact, some of this noise could be removed by introducing a third algorithm based on our described implementations that does not rely on estimation of surface deformation during registration, but instead uses a least squared fit of the displacements calculated during simultaneous registration. This approach would remove the independent image registration noise as well as the triangulation noise.
Chapter 5

Applications of Strain Estimation Algorithms

5.1 Abstract

The algorithms developed in Chapters 2 and 3 made no assumptions about the mechanical behavior of the material being analyzed. Because of this, they are suitable for determining strain in a wide variety of biomedical applications. We present here six examples using these algorithms in various settings and demonstrate their utility for providing insight into a wide range of biological questions. The examples include analysis of the micro-mechanics of the tendon-to-bone attachment, tissue engineering materials characterization, detection of local mechanical behavior in cardiac tissue, analysis of cardiac tissue following myocardial infarction, and insights into the mechanisms of embryonic wound healing.
5.2 Micro-mechanical properties of the tendon-to-bone attachment

Portions of this section were adapted from:

5.2.1 Introduction

The enthesis is a specialized tissue at the tendon-to-bone interface that connects two mechanically dissimilar materials: tendon, a compliant proteinaceous material with high toughness, and bone, a hard mineralized tissue with a significantly higher modulus. Compositional and structural features of the tendon-to-bone attachment at the micrometer scale are relatively well understood, but corresponding mechanics, especially in physiologically relevant tension loading, is lacking. Modeling efforts have suggested that the gradient structures at the tendon-to-bone attachment result in unique mechanical behaviors, such as the presence of a region of increased compliance near the interfacial region [90]. However, due to the difficulty in preparing small, mechanically testable samples of the attachment containing both mineralized and unmineralized tissue, information on the attachment mechanics at the micrometer scale during physiologically relevant loading conditions have been impossible to obtain. The goal of this study was to determine how the micrometer gradient structure at the attachment site participates in dissipation of applied forces and stress concentrations in healthy and pathological tissues.
5.2.2 Methods

Beam preparation

Murine tendon-to-bone attachment sites were progressively cut and shaped using a variety of techniques until a final beam size of approximately 60 µm long and 4.5 by 4.5 µm in diameter. Beams were within the fibrocartilaginous region of the attachment, where there is a gradient in mineral content (preparation methods are summarized in Figure 5.1 and details in the full text [91]).

AFM mechanical tensile testing

Mechanical testing of the tendon enthesis beams was conducted using atomic force microscopy coupled with scanning electron microscopy. Testing details can be found in the full text [91].

Strain analysis

Experimental determination of the deformation field within tendon-to-bone attachments is instructive in relating mechanical behavior to composition. While using full featured 2D-DDE would have been ideal for this study, the imaging constraints and nature of the sample led us to develop a one-dimensional manifold version of the strain tracking algorithm. Reasons for this were several-fold:

1. The total width of the beams was approximately 25 pixels, which is approaching the minimum required for DDE.
Figure 5.1: Murine tendon-to-bone attachment sites were cut and milled into beams measuring approximately 60 µm long and 4.5 by 4.5 µm in diameter. (A) Dissected supraspinatus-to-humeral head complexes were fresh frozen and sectioned into 20–30 µm thick slices. (B) LCM was used to cut large beams, 250 µm by 50 µm by 20–30 µm, in the fibrocartilaginous region of the attachment where there is a gradient in mineralization. (C–E) The LCM cut beams were further milled down to the final small beams via cryo-FIB. Figure prepared by Dr. Alix Deymier and reproduced with permission.
2. The beams were relatively featureless, which made tracking deformations at arbitrary locations on the sample difficult.

3. The few features which could be readily tracked on the beam did not deform by an appreciable amount.

Due to the difficult nature of using 2D-DDE on these beams, a tangential algorithm using the 2D-DDE displacement tracking was created. Briefly, regions with defining features, which would be readily trackable, were manually selected by a user, starting from one end of the beam and progressing to the other. On each beam, about 5-6 regions were selected for tracking. Using the initial locations of these selected points, a one dimensional manifold was created from the two dimensional image space, starting from the first user selected point, the cumulative sum of the Euclidean distances between the centroids neighboring regions was used to create the manifold. Due to the relatively large distance between these tracked regions, this one dimensional strain approach was precise and accurate, similar to the kernel size versus box spacing study completed for 3D-LSF (Figure 3.2). While this approach severely limited the resolution of the strain field result, it enabled the measurement of strain in these samples, which would have been impossible using conventional methods.

Using this technique, approximately five locations along the length of each specimen were chosen based on identification of distinct features that were tracked using an image registration algorithm. For each successive frame, the regions were tracked using a Newton-Raphson-like method that computes pixel displacements (Chapter 2). The manifold was broken into individual segments bounded by a tracked region on either side for 1-dimensional strain calculations. Segment lengths were calculated by the Euclidean distance formula using pixel displacements of the tracked regions. Lagrangian strains were determined by comparing the lengths of the segments at the start of the test to the length of the segment at each successive
frame, resulting in a single measure of Lagrangian strain for each segment at each frame. Aberrations and distortions caused by the SEM were determined to minimally affect the image at the energy, magnification, and raster rate used here and were therefore ignored.

5.2.3 Results

Local strains were measured for 9 out of the 11 beams that were imaged in situ with the SEM during loading. Of these 9 samples, 4 beams (3 WT and 1 KO) exhibited measurable local strains above 7%. Notably, the position of maximum strain was not located in the unmineralized region, but rather consistently appeared in between the mineralized and unmineralized regions in the area associated with the mineral gradient (Figure 5.2). Monitoring the exact determination of high compliance locations along the tendon-to-bone gradient was limited by the relatively low spatial resolution of the local strain analysis. However, all samples clearly displayed a high-strain region associated with the transition from mineralized to unmineralized (Figure 5.2).

5.2.4 Discussion

The tendon enthesis is a complex hierarchical tissue. Although the attachment site exhibits unique compositional and structural features at the micrometer scale, mechanical tests have never been performed at this scale due to difficulty in preparing and testing small samples. Micrometer-scale beams of the tendon enthesis were prepared and tested under uniaxial tension to failure using a suite of micro- and nano-tools. The samples exhibited elastic moduli and strengths that were dramatically higher than those measured in testing performed on the entire humerus-supraspinatus complex. These mechanical properties are expected to
Figure 5.2: Plots of local strain vs. position at multiple stresses (legend) for all of the samples for which local strains were measured. Dotted lines represent the calcium content as a function of position for each sample. The highest strain levels were not localized to the region closest to the tendon but instead within the beam near the gradient region. This indicates the presence of a region of high deformation within the enthesis. Strain data analyzed by John Boyle, calcium data analyzed by Dr. Alix Deymier, figure created by Dr. Alix Deymier.
be a result of scaling effects that eliminate certain deformation modes. Beam moduli were found to be dependent on local composition using a beam bending model that incorporated spatial variations in beam composition. Image correlation analysis of local strains indicated the presence of a compliant region near the mineral gradient. This region, whose existence had been previously proposed [92, 93], must be caused by micrometer-scale structures such as changes in collagen orientation rather than mineral content or macroscale features. Importantly, by increasing local deformation at the interface between the dissimilar materials, the tissue was able to absorb greater amounts of energy without failing, thus maintaining its integrity.

5.2.5 Acknowledgments

Dr. Alix Deymier performed the experiments for this study and drafted the manuscript.

5.3 Tunability of collagen scaffold mechanics by multiple modes of mineral localization

Portions of this section were adapted from:

5.3.1 Introduction

Biologic tissues, such as bones and teeth and their interfaces with tendons and ligaments, achieve high strength and stiffness through a highly mineralized hierarchical composite structure. Although significant variations exist in the micrometer-scale mineral volume fractions and the millimetre-scale shape of the tissues, the basic building block of these tissues is the same: nanometre-scale mineralized collagen fibrils [94]. Collagen molecules (300 nm in length and 1.5 nm in diameter) have triple helical structures that self-assemble into well-organized fibrils. These fibrils contain repeating regions of densely packed collagen (i.e. overlap zones) and regions of loosely packed collagen (i.e. gap zones). During the mineralization process, mineral can deposit either within the gap zones of the fibrils, on the surface of the fibrils or on the surface of the tissue [95, 96, 97, 98, 22]. Importantly, the particular location of the mineral relative to the collagen will have significant effects on the mechanics of the collagen fibril; although mineralization occurs at the nanometer scale, it affects the mechanical behavior at the tissue level [99]. Therefore, proper design and synthesis of mineralized collagen scaffolds with appropriate mechanical properties must finely control the deposition of mineral within the collagen nano and microstructure.

Developing a collagen–mineral composite with mechanical gradients similar to those seen at the tendon-to-bone attachment is important for improving repairs [100, 101]. Recently,
techniques have been developed to make functionally graded materials for replacement of interfacial tissues however many of these techniques focus on modifying properties of polymer-based scaffolds [102, 103]. Some work has been done examining collagen scaffolds, which utilize native proteins rather than artificial polymers, with gradients in mineralization chemistry, but not specifically mineral content [104], whereas others have directly created mineral gradients on tendon tissue [57]. These results demonstrate that, although there is a clinical need for controlled mineralization for engineered tissues with functional gradients, there is little research being devoted to this topic.

Spatially tuning the mineral content and mechanical properties of a hydroxyapatite–collagen scaffold would allow for the creation of graded scaffolds for repair of the tendon-to-bone attachment. Two mineralization methodologies were used: immersion in simulated body fluid (SBF) and immersion in SBF with size-excluding fetuin. The addition of fetuin mineralization was hypothesized to lead to more mineral deposition within (rather than on) the scaffold leading to superior bonding between mineral and collagen. This was expected to result in a greater increase in stiffness and toughness compared with collagen mineralized without fetuin.

### 5.3.2 Methods

**Fabrication and testing of collagen scaffolds with gradients in stiffness**

Collagen scaffolds with gradients in stiffness were created using reconstituted collagen and simulated body fluid-induced mineralization according to a published procedures (N=4)[100]. Briefly, lyophilized collagen (Elastin Products Company, product no. C857) was dissolved in a dilute solution of hydrochloric acid, homogenized, degassed, and pumped into cylindrical
casts (4 mm). Collagen casts were polymerized in TES buffer (135 mM N-tris(hydroxymethyl)-methyl-2-aminoethane sulfonic acid, 30 mM NaCl, and 30 mM Na2PO4 in distilled water; pH 7.5) at 37°C for 1 hr and then allowed to soak at room temperature overnight in de-ionized water [100]. Following soaking, collagen scaffolds were dehydrated in 95% ethanol and then allowed to air dry overnight. Scaffolds were placed in 10X simulated body fluid solution with or without 5 mg/ml fetuin at a pH of 7.4 for mineralization[100]. Uniform scaffolds were left in solution for 1 hour or 24 hours for solutions containing no fetuin or for solutions containing fetuin, respectively. For generation of graded scaffolds, scaffolds were slowly drawn out of their respective solution to create a gradient in mineralization[105]. Following mineralization, scaffolds were dehydrated a second time in 95% ethanol and allowed to air dry overnight. Scaffolds were then sprayed lightly with Verhoff’s stain to produce a random surface speckle texture. For mechanical testing, scaffolds were loaded in tension in a PBS bath (37°C) at a strain rate of 0.1 %/s using a materials testing frame (Instron Electropuls E1000). Videos of the test were captured using an Illunis VMV-8M camera for subsequent strain analysis in Matlab.

**Computational methods**

Strains were analyzed using the 2D-DDE and the 2D-SIMPLE algorithms. Detailed methods and theory for these methods are described in Chapter 2.
5.3.3 Results

2D-XCOR versus 2D-DDE for collagen scaffolds

While the fabricated collagen scaffolds were relatively tactile and could be easily handled, they did strain to beyond physiological levels: scaffolds would easily strain beyond 100%. Because of this, the estimation of surface strain on the scaffolds with older cross correlation or displacement based methods was not as effective as 2D-DDE. 2D-DDE accurately detected a gradient in strain between the top and the bottom of the scaffold, demonstrating a mineral-induced gradient in stiffness along the length of the scaffold (Figure 5.3A,B). In contrast, 2D-XCOR demonstrated unrealistically high strains, likely due to errors resulting from slight rotation of the sample during testing (Figure 5.3C,D). These errors were exacerbated at high grip-to-grip strains with the 2D-XCOR technique (Figure 5.3G,H), whereas 2D-DDE tracked a local strains as high as 0.18 (Figure 5.3E,F).

Mechanical properties of surface mineralization versus intra-fibrillar mineralization

Following demonstration that mineral gradients could be achieved and characterized using 2D-DDE, we sought to determine the effect of the protein fetuin on collagen mineralization and mechanics. Fetuin is a protein known to inhibit crystal formation, however due to it’s relatively large size it is excluded from the collagen intra-fibrillar space. Therefore, by size exclusion, fetuin can only inhibit surface mineralization and not intra-fibrillar mineralization [106]. Prior modeling studies have suggested that intra-fibrillar mineralization results in a stiffer and tougher collagen matrix compared to surface mineralization. SBF+Fetuin
Figure 5.3: Small and large grip-to-grip strains of collagen scaffolds with spatial gradients in mineral content, tested in tension. (A, B, E, F) 2D-DDE revealed a gradient in material strain for low and high grip-to-grip strains. (C, D) At low grip-to-grip strains, 2D-XCOR revealed similar trends to 2D-DDE. However, the values of strains measured were unrealistically high and are likely due to noise. (G, H) At high grip-to-grip strains, 2D-XCOR reported strains over 2. This was clearly erroneous based on visual inspection of the specimen, demonstrating the limitations of the 2D-XCOR technique for large strains in inhomogeneous samples.
Figure 5.4: Mechanical properties of collagen matrices. Toughness, modulus and strength were significantly higher in the SBF+Fet group compared with the SBF and Unmin groups. The modulus of the unmineralized group was also significantly higher than that of the SBF group. Lines above bars indicate p < 0.05.

mineralization enhanced the mechanical properties of the collagen scaffolds compared to unmineralized scaffolds. Surprisingly, SBF alone resulted in diminished material properties (Figure 5.4).

2D-DDE strain analysis of collagen matrices with gradients in mineral content

Local strain analysis using 2D-DDE demonstrated no gradient in strain for any of the uniformly mineralized samples (Figure 5.5A,B,C). However, a gradient in strain was seen in
Figure 5.5: Local mechanical properties of mineralized matrices. (a–e) Representative strain maps of (a) an unmineralized collagen matrix, (b) an ungraded SBF + Fetuin-mineralized collagen matrix, (c) an ungraded SBF mineralized collagen matrix, (d) a graded SBF+Fetuin mineralized collagen matrix and (e) a graded SBF mineralized collagen matrix. The frames shown for each sample were chosen such that the average strain in the frame was constant. The local strain analysis indicated that ungraded matrices expressed no strain field gradients. Strain was relatively constant along the lengths of unmineralized and ungraded samples. Strain decreased with increasing mineral content in the SBF+Fetuin group. Strain increased with increasing mineral content in the SBF group. (f) The average secant modulus as a function of position is shown for graded SBF and SBF+Fetuin scaffolds. Modulus increased with increasing mineral in the SBF+Fetuin group. Modulus decreased with increasing mineral in the SBF group.

matrices with gradients in mineral: SBF+fetuin mineralization had lower strains in the mineralized regions compared to the unmineralized regions whereas SBF mineralization showed the opposite trend (Figure 5.5D,E). Combining the strain data with stress calculations from the mechanical test revealed similar trends for the local tensile modulus for the graded samples. Mineralization led to an increase in the modulus of SBF+Fetuin samples whereas mineralization led to a decrease in the modulus of SBF alone samples (Figure 5.5F).
5.3.4 Discussion

The past two decades have produced major advances in the development of engineered structural biological materials to replace damaged tissues. Research in mineralized tissue engineering has led to successes that are now reaching the clinical sector [107, 108]. Many of these bone replacement materials focused on recreating the mineralized collagen structure and were therefore composites of collagen and hydroxyapatite mineral [96, 109, 110, 111]. These composites can be assembled in a variety of ways, but the two most common were (i) immersion of collagen scaffolds in a calcium- and phosphate-containing solution [109, 112, 113, 114, 115, 116] and (ii) mixing of hydroxyapatite nanoparticles with collagen before polymerization [113, 117, 118, 119]. In the case of the former methodology, a number of mineralization techniques have been employed. These included simply immersing collagen scaffolds in solutions such as SBF or adding proteins, peptides or other chemicals to help control the mineralization process [97, 57, 106, 120, 121, 122, 123, 124, 125]. Simple immersion of collagen leads to surface deposition of mineral either on the surface of fibrils, creating extrafibrillar mineral, or on the surface of dense collagen scaffolds. The addition of components such as fetuin inhibits surface mineralization, leading to mineral deposition either within fibrils, i.e. intrafibrillar mineralization, or within dense collagen scaffolds. This, in turn, had significant effects on the mechanical properties of the scaffolds [99]. The goal of this study was to create collagen matrices with gradients in mechanical properties for the repair of tendon-to-bone injuries. The mechanical properties were controlled by controlling the location and quantity of mineral deposited on the surfaces of and within collagen matrices. Tuning the mechanics of these matrices via mineralization may allow for tailored mechanical and cellular responses at interfacial repair sites.
When mechanically tested in uniaxial tension, the SBF Fet-mineralized matrices exhibited an increased modulus compared with the unmineralized matrices, as expected for a reinforced composite (Figure 5.4). However, the SBF-mineralized matrices showed a decrease in modulus compared with both the unmineralized and SBF Fet-mineralized scaffolds. In the presence of a mineral coating, a few micrometers thick on the collagen matrix surface, one would expect the modulus of the composite to increase according to the Voigt model (i.e. a parallel arrangement of compliant collagen matrix with a stiff mineral layer). Assuming a mineral volume fraction of approximately 25%, estimated from bright-field images, an increase in modulus of over three orders of magnitude would be expected for the matrices of this study. However, the clear decrease in modulus with SBF mineralization indicates that other factors dominated the mechanical response of these scaffolds.

The results contained here demonstrate the necessity of accurately calculating relatively high level strains for determining the mechanical properties of partially mineralized collagen scaffolds. 2D-DDE enabled the precise characterization of local strain in collagen matrices and revealed a number of unexpected material behaviors, revealing the utility of 2D-DDE in characterizing tissue engineered samples.

5.3.5 Acknowledgments

Dr. Lester Smith and Dr. Alix Deymier performed the bulk of the experimental work for this project, including synthesis of collagen matrices with gradients in mineral, material characterization, and the data analysis. Dr. Stephen Linderman assisted with experiments. Dr. Justin Lipner helped develop methods to determine the secant modulus.
5.4 The mechanics of PLGA nanofiber scaffolds with biomimetic gradients in mineral for tendon-to-bone repair

This section was adapted from:

5.4.1 Introduction

At the interface between compliant tendon and stiff bone, a gradient in mechanical properties serves to mitigate stress concentrations [126, 4]. The two main components of this transitional tissue are nanofibers of collagen and nanometer-scale plates of a stiff, carbonated, hydroxylapatite (“mineral”). Stiffening of the collagen occurs via a monotonic rise in mineral content [90, 127]. The increasing mineral content results in a stiffness increase from $\sim 400$ MPa to $\sim 20$ GPa, a difference of almost two orders of magnitude [128, 129]. Using the healthy tendon-to-bone attachment system as a guide, we created a polymer-hydroxylapatite nanofiber-based material with gradients in mineral content, and investigated the mechanical effects of these gradients.

In this study, we used electrospinning to create scaffolds of aligned nanofibers, and deposited mineral in a graded fashion on the fibers using modifications of a biomimetic ion solution known as “simulated body fluid” (SBF). We synthesized mineral gradients over the
fibers by submerging the scaffolds into SBF. Two different mineralization formulations were used to create mineral coats that were compositionally similar but morphologically different [130, 131]. The first formulation resulted in plate-like mineral morphology and has been used previously to generate nanofiber scaffolds with gradients in mineral [105]. The second formulation resulted in a dense mineral morphology and was described in a study investigating the effects of homogeneous (i.e., non-graded) mineralization of nanofiber scaffolds [130]. We asked whether either of the mineralization methods we developed holds potential to achieve adequate stiffening of the scaffold.

We hypothesized that mineral of both types would stiffen the polymer networks, manifesting itself as lower strains in the mineralized regions and larger calculated moduli. Furthermore, we hypothesized that the stiffening effect would depend on the morphology of the mineral, with denser coatings leading to a more potent stiffening effect. Testing of this hypothesis required development of the homogenization bounds and of a technique for estimating spatial gradations in elastic modulus in a graded scaffold. Understanding the stiffening mechanisms of mineral on nanofiber polymer scaffolds is critical for the development of mechanically competent scaffolds for tendon-to-bone tissue engineering. We adapted a newly developed digital image correlation algorithm to measure local strain patterns and analyzed these strain fields to estimate the relationship between mineral volume fraction and mechanical properties.

### 5.4.2 Methods

**Scaffold synthesis and characterization**

Fibrous scaffolds with gradients in mineral content were generated using electrospinning and two different simulated body fluid (SBF) solutions (10 times SBF (10SBF) and modified
10SBF (m10SBF)) [130, 131]. Scaffold mineral content was determined using EDX and details of scaffold characterization can be found in [132].

**Scaffold mechanical testing and analysis**

Uniaxial tensile tests were performed using an Instron Electropuls E1000 (Norwood, MA) with custom grips, and analyzed in MATLAB (MathWorks, Natick, MA). Samples were tested under uniaxial tension at quasi-static conditions, with a constant strain rate of ~0.4% per second. Video was captured concurrently at 3 frames per second with a resolution of 1360 × 1024 pixels (Olympus DP70).

Video was analyzed using the 2D-DDE method (Chapter 2). The local moduli were determined from linear regressions of the engineering stress and regional strain. EDX data of Calcium (Ca) and Carbon (C) were used to calculate Ca/(Ca+C) as a proxy for relative mineral content. A small number of samples had local defects due to fabrication issues or mishandling. These defects were readily apparent during testing through the appearance of local strain concentrations. Any samples that displayed this behavior were excluded. The mineral volume was then plotted against the average modulus for each transverse slice, and if the modulus measurements did not indicate zones of failure (negative moduli) the data were included in a larger dataset for each mineralization method (m10SBF 4/5 included, 10SBF 7/7). This larger dataset was analyzed for the overall trends, and a linear fit was found to estimate the relationship between modulus and mineral.
5.4.3 Results

Mineral deposited using 10SBF was distinctly plate-like and diffuse, arranged in florets over fibers. In contrast, mineral deposited using m10SBF was dense and largely conformal to the fibers, except for some bunched, bead-like accumulations similar to those seen on fracture surfaces in natural bone [133] (Figure 5.6). Mineral morphology strongly affected the mechanical properties of the scaffolds. When the graded scaffolds were pulled in tension, strain was higher on the low mineral content ends than the high mineral content ends. The elastic modulus of scaffolds generated using both coating methods increased with increasing mineral content. This relationship was statistically significant based on linear regression analysis (Figure 5.7). A high correlation coefficient was found for the m10SBF group, indicating that the variation in modulus can largely be explained by changes in mineral content for this group. In contrast, a relatively low correlation coefficient was found for the 10SBF group, indicating that the variation in modulus can only partially be explained by changes in mineral content for that group. In other words, the denser mineral coat produced by m10SBF led to more rapid stiffening compared to the plate-like 10SBF coat (Figure 5.7).

5.4.4 Discussion

In this work, we estimated the stiffening effects of two different mineralization methods on nanofiber polymer scaffolds and compared these to mechanical models to measure how efficient the stiffening was. This is the first study to rigorously examine the mechanics of nanofiber PLGA scaffolds with gradients in mineral. New methods were developed to determine the local mechanical properties and the mineral volume fractions. We found that both mineralization methods stiffened the scaffolds, but that their magnitudes varied
Figure 5.6: (A) Plate-like mineral morphology was observed in the 10SBF group. (B) A dense coat of small mineral crystals was observed in the m10SBF group. (Outer scale bar = 10 µm, inset scale bar = 1 µm) Figure prepared by Dr. Justin Lipner and reproduced with permission.

Figure 5.7: Plots of modulus relative to mineral content demonstrate the stiffening effects of 10SBF and m10SBF (error bars represent standard deviation). The stiffening effect of m10SBF was significantly greater than 10SBF, as evidenced by a higher slope (analysis of covariance; p=0.05). Figure prepared by Dr. Justin Lipner and reproduced with permission.
substantially, with modified simulated body fluid (m10SBF) giving a more potent effect than simulated body fluid (10SBF). When compared to the composite bounds we developed, the stiffening by mineralization achieved using 10SBF proved to be weaker than the lowest possible stiffening predicted by homogenization theory, indicating that mineral was not well connected to the scaffold. In contrast, mineralization using m10SBF achieved stiffening that was nearly an order of magnitude greater than 10SBF, supporting our initial hypotheses.

5.4.5 Acknowledgments

Dr. Justin Lipner carried out the experiments for this project and drafted the manuscript.

5.5 3D-DDE reveals the mechanics of the deforming myotendinous junction of the chordae tendineae

Portions of this section were adapted from:

John J. Boyle, Arvin Soepriatna, Frederick Damen, Roger A. Rowe, Robert B. Pless, Attila Kovacs, Craig J. Goergen, Stavros Thomopoulos, Guy M. Genin (2017). Accurate and noise insensitive strain mapping enables ultrasound analysis of cardiac function in three dimensions. Under Review
5.5.1 Introduction

Many pathologies in load-bearing and load-producing biologic tissues lead to abnormal mechanical behavior and poor tissue function. For example, myocardial infarction leads to local changes in myocardial tissue stiffness and loss of heart function. However, quantifying these abnormalities for medical diagnosis is often impossible because of the limited precision and accuracy of existing tools for estimating strain fields from medical imaging scans. State of the art digital image correlation (DIC) techniques, which match patterned features between pairs of images to estimate displacement fields over time, are most accurate when displacement fields are two-dimensional (2D), with all displacement occurring in the plane of the original image [134, 135, 36, 28, 29, 30, 31, 37]. Accuracy suffers when three-dimensional (3D) displacements cause image features to move into or out of the imaging plane, as occurs commonly when using static medical imaging equipment to track motion of tissues within the body [76, 82]. As a consequence, 2D methods applied to 3D medical image stacks have not yet succeeded in robustly identifying the altered strain fields that underlie many pathologies.

Digital volume correlation (DVC) techniques can overcome this challenge by tracking displacement in 3D, but these, too, suffer from limitations on accuracy and precision. DVC techniques typically estimate displacement fields over time via a volumetric cross correlation (3D-XCOR) approach that maximizes the similarity between groups of voxels in initial “reference” imaging volumes and subsequent “deformed” imaging volumes. Strain fields can be estimated from the gradient of these estimated displacement fields, but these estimates suffer from the well-known challenge of taking numerical gradients of noisy data: numerical differentiation magnifies small errors in displacement tracking. This problem is exacerbated for DVC relative to 2D DIC because of limited resolution in the z-direction: modern imaging stacks typically have higher resolution within each imaging plane or “slice” than between
slices. Existing techniques therefore have to impose regularization, either by smoothing or by making guesses about the mechanical properties of the tissue being imaged [28, 30]. Furthermore, existing techniques do not take advantage of modern tools of computer vision, do not warp reference volumes when searching for their counterparts in deformed imaged volumes [36, 29] and require imposition of strain compatibility upon averaged fields [31]. Overcoming all of these limitations currently requires post hoc regularization that tends to mask strain concentrations [30, 37, 38].

We therefore used an unconstrained 3D strain estimation algorithm for full volumetric data sets which reliably determines strains within tissue volumes without material assumptions or regularization. The method, 3D-DDE, estimates deformation gradient fields directly from a new warping function that maps targeted regions in the reference image volumes to their counterparts in deformed image volumes (Chapter 3). 3D-DDE yielded superior accuracy, noise-insensitivity, and precision compared to existing displacement-based methods, and furthermore identified regions of tissue with high strain gradients.

5.5.2 Methods

Experimental methods for capturing volumetric time series of a cardiac and respiratory-gated volumetric murine ultrasound

Ultrasound images of a beating heart were acquired in vivo from a healthy adult male C57BL/6 wild-type mouse using a high-frequency small animal ultrasound system (Vevo2100, FUJIFILM VisualSonics Inc.). Prior to ultrasound imaging, the mouse was anesthetized with 3.0% isoflurane and room air at 1.5 L/min. After removing hair from the left ventral thorax using a depilatory cream, the mouse was positioned supine on a heat-modulated imaging
stage (FUJIFILM VisualSonics Inc.). The animal’s paws were secured to gold-plated stage electrodes to monitor ECG and respiration signals. Ophthalmic ointment was applied to the eyes to prevent drying of the corneas. Throughout imaging, the mouse’s body temperature was monitored closely using a rectal temperature probe and maintained between 34-37C. Isoflurane levels were adjusted around 2.0% to keep respiration rates above 50 breaths per minute and maintain a stable heart rate. The Purdue Animal Care and Use Committee approved all studies.

To obtain a series of spatially dependent 2D cine loops of the heart throughout the cardiac cycle, a 40 MHz linear array ultrasound probe (MS550D) was attached to a linearly translating step motor (3D Acquisition Motor). The transducer was positioned perpendicular to the base-apex axis of the heart to acquire cross sectional views of the left ventricle. An in-house MATLAB script was then used to translate the transducer with a 180 µm step size from the apex to the base of the heart, acquiring cine loops at each sequential location. Both respiratory and cardiac gating was used to minimize breathing artifacts and to acquire 2D cine loops of the heart with a high temporal resolution of approximately 1 ms. Spatially adjacent cine loops were imported into MATLAB and temporally matched to digitally reconstruct a volumetric mouse heart dataset with temporally-synced information. Linear interpolation was used to resample the ultrasound data to isotropic voxels of 60µm resolution. The preprocessed volumetric data was then used for the strain calculation methods.

**Computational methods**

Strains were analyzed using the 3D-DDE and the 3D-SIMPLE algorithms. Methods and theory of these methods are described in Chapter 3.
5.5.3 Results

In vivo ultrasound of a beating murine heart reveals the mechanics of the deforming myotendinous junction of the chordae tendineae

3D-DDE identified strain fields from noisy in vivo volumetric imaging data were acquired by high frequency ultrasound imaging of a murine heart. Volumes were assembled from 2D ultrasound slices and were synchronized to ECG signal using post processing techniques, allowing for the estimation of full 3D strain fields (Figure 5.8). Taking the isovolumetric contractile state as a reference, large strains evolved over the cardiac cycle, gradually relaxing to the reference state during diastolic filling (Figure 5.8 A-O).

The papillary muscles, which assist in the opening and closing of the atroventricular valves, underwent high strains. However, the chordae tendineae, which connect the papillary muscle to the atroventricular valves, showed little straining over the course of a cardiac cycle. This was expected due to the high stiffness mismatch of the chordae compared to the softer myocardium. In the vicinity of the chordae tendineae to papillary muscle insertion site, a substantial change in strain was noted, with a transition from the highest to lowest tensile principal strains evident (Figure 5.8 H,L,P).

5.5.4 Discussion

The results provided the first glimpse into the mechanical structure of key connections between stiff and compliant tissues in the heart. Although no gold standard result exists for comparison, the strain fields measured by 3D-DDE are qualitatively as expected. High strains in the ventricular wall correlated with ventricular ejection, and relatively small strains
Figure 5.8: Peak principal strain fields estimated from high frequency ultrasound imaging of a beating mouse heart. Four-chamber (left column: A,E,I,M,Q), long axis (middle column: B,F,J,N,R), and short axis (right column: C,G,K,O,S) views and a segmented 3D papillary muscle (PM) and chordae tendineae (CT) (D,H,L,P,T) of the heart were acquired throughout the cardiac cycle. Green-Lagrange strains were estimated using images acquired during isovolumetric contraction (A,B,C,D) as the reference (strain-free) configuration. Strain developed in the left ventricle as it contracted and blood was ejected from the heart, while the papillary muscles remained unstretched (E,F,G,H). As the heart cycle entered isovolumetric relaxation, strains in the heart wall reached peak levels on the order of 0.5 (I, J, K, L). As the heart relaxed during early ventricular filling, strain levels reduced (M,N,O,P), approaching baseline levels after late ventricular filling (Q,R,S,T). Throughout the cardiac cycle, strains in the papillary muscles (yellow arrows) were lower than those in the surrounding myocardium in the apex and base (white arrows). LV: left ventricle, RV: right ventricle, S: skin. Scale bar: 3 mm.
were observed in the stiff chordae tendineae. These results were expected due to the high stiffness mismatch of the chordae and the more compliant myocardium, and supported our hypothesis. More broadly, results showed that, as in other severe material mismatches in physiology and nature (e.g. [126, 90, 136]), mechanisms appear to be in place to limit local elevations of strain near the interface, such as at the insertion of chordae tendineae into the papillary muscle in the wall of the heart. The absence of local strain concentrations at the points of insertion, where gross wall strains change substantially, suggest a future target for the mechanically-based diagnosis of structural pathologies related to valve function. This exercise demonstrated that our technique could readily resolve small structural differences effectively.

5.5.5 Acknowledgments

Arvin Soepriatna and Frederick Damen carried out the experimental work for this project as well as the pre-processing of the data for analysis.

5.6 3D-DDE discerns differences in cardiac wall mechanics between healthy and post-myocardial infarction hearts

Portions of this section were adapted from:
John J. Boyle, Arvin Soepriatna, Frederick Damen, Roger A. Rowe, Robert B. Pless, Attila Kovacs, Craig J. Goergen, Stavros Thomopoulos, Guy M. Genin (2017). Accurate and
noise insensitive strain mapping enables ultrasound analysis of cardiac function in three dimensions. *Under Review*

### 5.6.1 Introduction

Building on our prior work on investigating the mechanics of a murine heart (Section 5.5), we performed the first full thickness strain mapping of a mouse heart wall and showed spatial variations of strain associated with both anatomical features and myocardial infarction. Clear changes to the strain fields over a full cardiac cycle enabled diagnosis of the extent and mechanical consequences of myocardial infarction. These features of 3D-DDE suggest promise for enabling quantitative diagnosis using dynamic and non-invasive technologies such as ultrasound.

### 5.6.2 Methods

**Experimental methods for capturing volumetric time series of a cardiac and respiratory-gated volumetric murine ultrasound**

Image volumes were captured using techniques described earlier (Section 5.5.2). In preparation for myocardial infarction induction surgery, the mice were ventilated via endotracheal intubation and connected to a small animal ventilator (SomnoSuite, Kent Scientific Inc.). The ventilator supplied air to the lungs with a target inspiratory pressure between 16-18 cm H2O and a minimum peak-end expiratory pressure between 3-5 cm H2O to prevent pneumothorax during surgery. The mice were subjected to a left mini-thoracotomy by making a small incision between the 2nd and 3rd rib. Once the incision was made, a rib cage retractor
was used to carefully expose the left ventricle without damaging the left lung. The pericardium was then dissected and excess pericardial fat removed to visualize the left anterior coronary artery. Once the coronary artery was clearly visualized, an 8-0 suture was looped around the artery, and the two ends of the suture were tightened to permanently occlude the vessel to induce ischemia. The rib cage and the skin were then sutured separately, and the mice remained connected to the ventilator until they regained their natural breathing pattern and were mobile. Buprenorphine (0.05-0.2 mg/kg) was administered subcutaneously prior to and periodically for 48 hours after surgery. The mice were then allowed to recover for two weeks before ultrasound images of the remodeled heart were acquired.

**Computational methods**

Strains were analyzed using the 3D-DDE and the 3D-SIMPLE algorithms. Methods and theory of these methods are described in Chapter 3.

**5.6.3 Results**

*In vivo ultrasound of post myocardial infarction hearts reveals dramatic differences in wall strain when compared to controls*

3D-DDE provided a robust metric to quantify differences between healthy and two-week post-myocardial infarction (PMI) murine hearts. Using the same techniques of combining 2D slices synchronized to an ECG signal, we estimated full field strain fields in healthy control and PMI murine hearts. Strain fields estimated using 3D-DDE were dramatically reduced in magnitude in the infarcted region of the heart wall in PMI hearts; these regions of
attenuated strain were not evident in control hearts (Figure 5.9 D,E,F,G). Peak 3D principal Green-Lagrange strain was reduced by approximately 90% in these regions during systole compared to control hearts (Figure 5.9 H,I). In addition to this absolute measure of stiffening from infarction, a relative measure was evident, with strains in the infarct regions disproportionately lower than those in the surrounding healthy tissue.

5.6.4 Discussion

The technique also quantified differences between healthy and infarcted myocardium. Myocardial infarctions are known to cause local remodeling of injured heart tissue [137, 138]. 3D-DDE quantified these effects and showed the scar tissue to be significantly stiffer than the surrounding healthy tissue: the stiff scar tissue resulted in strains that were substantially lower than those in either the surrounding healthy tissue or in the same region of healthy controls. Interestingly, these areas of very low strain were abutted by regions of elevated strain compared to controls, possibly indicating an adaptive response in the surrounding tissue. Future studies involving these techniques could be used to study this remodeling over time. The current study demonstrates that measurable responses exist and support the use of high frequency ultrasound as a diagnostic tool.

5.6.5 Acknowledgments

Arvin Soepriatna and Frederick Damen performed the experiments for this project and the pre-processing of the data for analysis.
Figure 5.9: (A,B) Magnetic resonance images of mouse hearts showing the anatomical planes studied using 3D-DDE of ultrasound imaging volumes. (C) A schematic of the heart demonstrating the orientation of the short and long axis as well as the location of the infarction. (D,E) Peak principal strain at a specific timepoint in control hearts. (F,G) Peak principal strain at this same timepoint in hearts following myocardial infarction, showing distinctly different strain patterns in both the long and short axis views. (H) Strain as a function of position along the midline of the long-axis view of the heart, showing strain attenuation in the infarcted tissue. Line corresponds to different times; position is measured from the base of the arrow in panel F. (I) Strain as a function of position along the midline of the short-axis view of the heart, showing strain attenuation in the infarcted tissue, and elevated strain in the tissue surrounding the infarct region. Lines again correspond to different times; position is measured from the base of the arrow in panel G. Scalebars: 1 mm.
5.7 Novel mechanisms of embryonic wound healing revealed by 2D-SIMPLE and 2D-DDE algorithms

Portions of this section were adapted from:

5.7.1 Introduction

Embryonic wounds can heal without scarring and therefore serve as model systems for regenerative healing strategies and fetal surgery [139]. Central unresolved issues surround identification of the contractile field around a wound perimeter shortly after wounding, and of how surgical technique affects this behavior [7]. The dominant model suggests that a local ring contracts isotropically [7], but the strain fields needed to support this model have never before been imaged successfully. We therefore studied differences between three wound types in early stage chick embryos: (i) circular wounds created with a punch [7], (ii) elliptical wounds created by ablation, and (iii) elliptical wounds created by incision with a micro-scalpel [7]. We sought to delineate three sources of tissue strain in vicinity of wound: (i) isotropic contraction of the wound, (ii) passive elastic recovery of tissue distal to the wound, and (iii) stretching introduced during wound creation (Figure 5.11).
5.7.2 Methods

Embryonic injury models

Videos for elliptical incision and circular punched embryonic injury models were obtained from a previously described experiment [7]. All wounds were made at early embryonic time points where cells do not reside on a substrate (Hamburger-Hamilton 5-6). Linear ablated wounds were created using the Gastromaster microsurgery device (Xenotek Engineering) with white tips, which lyses cells with no direct mechanical contact [140].

Computational methods

Strains were analyzed using the 2D-DDE and the 2D-SIMPLE algorithms. Methods and theory of these methods are described in Chapter 2.

5.7.3 Results

2D-DDE and 2D-SIMPLE algorithms reveal previously ambiguous mechanisms of embryonic wound healing

The 2D-DDE method succeeded in identifying the time course of straining, while 2D-XCOR revealed only noise (Figure 5.10). For the circular punched wound, the first principal strain showed a contractile ring around the wound border, consistent with the isotropic contraction model [7] (Figure 5.10A, D, ). The 2D-SIMPLE algorithm detected a strain concentration around the wound consistent with localized isotropic contraction [7] (Figure 5.10G). For
the elliptical ablated wound, a localized isotropic contractile ring again formed, and small amounts of tension distal to the wound were evident (Figure 5.10B,E). The 2D-SIMPLE algorithm again detected a strain concentration around the wound (Figure 5.10H). In contrast, the micro-scalpel incision showed elevated tensile strain at the leading edge of the incision, and very low strain in the wake of the incision (Figure 5.10C,F). Strain concentrations were detected along the flanks of the wound, and subsequent analysis revealed these to arise from shearing of the wound flanks (Figure 5.10I). Results suggest that the mode of incision, rather than the shape of the wound, dictated where strains were localized during closure/contracture. As discussed below, this supports predictions of the isotropic contraction model and sheds light on some basic mechanisms of fetal wound healing.

5.7.4 Discussion

2D-DDE and 2D-SIMPLE quantified features of embryonic wound healing that were previously undetectable, and in addition enabled a qualitative picture of the effect of wound type. The interplay of isotropic constriction, passive elastic recovery, and stretching introduced during wound creation became apparent, providing insight into wound healing mechanisms and fetal surgery [7, 141, 142]. The strain fields associated with circular and elliptical ablated wounds exhibited a trade-off between localized isotropic contraction and distal tissue tension, with no additional effects of the wounding process (Figure 5.10 and Figure 5.11). The scalpel-incision, however, left tension in the wake of the wound, compression ahead of the wound, and shear abutting the flanks. Cells surrounding the wound reacted to reach a homeostatic state, which combined with the effects of the localized isotropic contraction to
Figure 5.10: The 2D-DDE and 2D-SIMPLE algorithms described the spatial and temporal patterns of embryonic wound closure, while the 2D-XCOR algorithm revealed only noise. (A, B, C) First principle strains in the radial direction away from the wound center for 90 bins around the wound (inset) with wound border marked by a circle. (A, D, G) For the circular punched wound, the first principal strain determined by 2D-DDE demonstrated an isotopic contractile ring around the wound border. A strain concentration was identified around the wound by the 2D-SIMPLE algorithm, consistent the presence on a localized isotropic contraction. (B, E, H) For the elliptical ablated wound, 2D-DDE demonstrated a localized ring of isotropic contraction and tension distal to the wound. A strain concentration was identified around the wound by the 2D-SIMPLE algorithm. (C, F, I) For the elliptical incision wound, 2D-DDE identified high tensile strain was at the leading edge of the incision and low strain in the wake of the incision. 2D-SIMPLE detected strain concentrations along the flanks of the wound. (J, K, L) 2D-XCOR failed to identify any patterns of strain at or near the wound sites. Scale bars = 200µm. Definitions: $E_{xx}$, strain in 11 direction; $\Delta$, 2D-SIMPLE difference.
Figure 5.11: Embryonic wounds were created using three methods: circular wounds created with a punch (top row), elliptical wounds created by ablation (middle row), and elliptical wounds created by incision with a micro-scalpel (bottom row). Strains were analyzed to delineate how three mechanisms combine to change the wound: (D,E,F) localized isotropic contraction around the wound, (G,H,I) passive elastic recovery of tissue distal to the wound, and (J,K,L) stretching introduced during wound creation. (A, B, C) Injuries induced by circular punching and elliptical ablation do not introduce additional deformations into the wound healing system. However, the elliptical incision method adds tension in the wake of the blade and compression ahead of the blade. (A, B, C) Localized isotropic contraction of wounds is expected at the border of the wound for all wound scenarios. (E, E, F) In response to localized isotropic contraction near the wound, regions distal to the wound are expected to be in tension, as cells near the wound pull inward to close the injury. (J, L, K) Since no additional deformations were introduced during wounding for circular punched and elliptical ablated injuries, no response to the wounding is expected in these cases. For elliptical incision injuries, however, the tissue is expected to respond to the incision deformations (C) by returning to its original state. (M, N, O) Strain concentrations are expected to arise at the wound border due to the localized isotropic contraction in all wound scenarios. For elliptical incision injuries, however, strains introduced during wounding combined with the localized isotropic contraction should result in strain concentrations primarily along the flanks of the elliptical wound.
induce wound closure (Figure 5.10). Results suggest that ablating and punching are less disruptive to embryonic wounds than scalpel incisions, and show that the method of wounding has a strong effect on the initial stages of the wound healing response.

5.7.5 Acknowledgments

Dr. Matthew Wyczalkowski performed the experimental work for this study.

5.8 Discussion

These six examples from a wide variety of biomedical applications collectively demonstrate the robustness and general applicability of the newly developed algorithms over a wide variety of imaging modalities and biologic tissues. Of note, a modified one dimensional version of 2D-DDE was capable of resolving local strains despite a lack of texture to determine micro-mechanical properties of the tendon to bone insertion. 2D-DDE was capable of resolving large deformations suitable for determining mechanical properties of tissue engineered scaffolds which are capable of undergoing strains as large as 150%. The high resolution and robustness to noise allowed for 3D-DDE to detect subtle mechanical changes in noisy ultrasound images in three dimensions. Furthermore, 3D-DDE detected dramatic changes between pre- and post-myocardial infarction in noisy ultrasound image volumes. Lastly, 2D-SIMPLE was capable of detecting strain concentrations during embryonic wound healing, providing insight into the mechanisms behind driving this biologic process. Future studies will continue to explore more applications for this toolbox of techniques for measurement of local strain.
Chapter 6

Control of stress and strain gradients for mechanoactive tissue engineering of the tendon-to-bone attachment

6.1 Abstract

Spatial control of cell differentiation is critical for successful regeneration of complex hierarchical tissues such as the tendon-to-bone attachment. Among the myriad of biophysical and biochemical cues that can drive differentiation, recent work has highlighted how the local environment around a cell can control its behavior. Locally defined mechanical stimuli can therefore prove useful for driving the formation of complex, spatially varying, tissues. However, it remains unclear how to distinguish between the effects of external forces acting on cells and forces generated by the cell itself. Although it has been shown that cells actively respond to stresses or strains originating from their environment, it has not been determined whether cell deformation or external stress exerted on the cell drives their response. In the
current study, we designed and validated an *in vitro* model system that could precisely control local stress and strain to study mechanotransduction. 2D-DDE (Chapter 2) enabled the validation of this system by providing robust and accurate strain estimates that the prior state of the art could not resolve.

### 6.2 Introduction

Numerous studies have reported a multitude of differentiation effects on mesenchymal stem cells as a result of passively applied stresses and strains [143, 144, 63, 145, 146]. However, no study we are aware of has attempted a comprehensive evaluation with isolated gradients in passively applied mechanical stress or strain. To investigate the independent effects of stress and strain, we theorized and validated a model system which has independent control of stress and strain in a continuously graded fashion, thereby creating a system which is internally controlled, comprehensively investigates every stress or strain state between two fixed values, and has identical surface and chemical presentation to all of the cells. We use this model system to study the effects of pure mechanotransduction without the addition of soluble differentiation factors. To simplify and facilitate this study, we focus it on tendon-to-bone attachment tissue engineering.

#### 6.2.1 The tendon-to-bone attachment

The tendon-to-bone attachment is a musculoskeletal structural tissue that facilitates the transfer of load from the compliant tendon to the stiff bone. Importantly, the natural tissue achieves this transfer of load across a two order of magnitude stiffness difference without
potentially damaging stress or strain concentrations. This is accomplished through gradients in material properties, mechanical properties, and cell phenotype [92, 147, 39]. Following injury and repair with traditional surgical techniques, this natural transitional tissue is not reformed and instead replaced by an abrupt interface between tendon and bone. This failure to recapitulate the graded nature of the natural tissue which does not shield from stress or strain concentrations results in a nearly 94% re-tear rate [42]. Therefore, strategies that improve tendon-to-bone healing by restoring the transitional nature of the natural tissue have the potential to greatly improve surgical outcomes.

Material and mechanical gradients in the healthy adult tendon-to-bone attachment

The natural tendon-to-bone attachment consists of several gradually changing material properties. Tendon tissue consists of well aligned collagen fibers. These fibers become less aligned and more random as they transition from tendon to bone, where their orientations become fully random [5]. There is also a gradient in mineral content: while bone tissue is highly mineralized and tendon tissue is fully unmineralized, the tendon-to-bone attachment region bridges this difference by having a gradient in mineralization [39]. There are also gradients in structural proteins. While both bone and tendon are very rich in collagen I, the interfacial region is also rich in collagen II [40]. Type X collagen is localized to the developing tendon-to-bone attachment and is evident in a band of cells on the bone side of the tendon-to-bone attachment. While collagen X is typically localized to hypertrophic chondrocytes in the growth plate before mineralization, at the tendon-to-bone attachment expression persists after hypertrophic chondrocytes are gone suggesting collagen X plays a role in maintaining the mineralized interface [41].
Strain across the tendon to bone tendon-to-bone attachment is also graded. Tendon tissue, which is relatively compliant, deforms 2-5% during normal function, while bone tissue strains less than 1%. In the tendon-to-bone attachment region, there is strong evidence that strains are higher than that of either tendon or than that of bone, likely providing an energy absorption region increasing the toughness and reducing stress or strain concentrations [3]. Although not measured experimentally, modeling studies demonstrate the advantages of a gradient in strain [4].

The bulk shape of the tendon to bone tendon-to-bone attachment also affects the distribution of stress the system. The force generated by the muscle is carried by the tendon, tendon-to-bone attachment, and the bone. However, stresses carried by each tissue vary greatly. Tendon cross sectional area, tangential to transmitted force, is relatively small compared to bone. Across the tendon-to-bone attachment region, the cross sectional area becomes larger as the interfaces splays out over the bone surface where it connects to the humeral head [148]. Lastly, the bone has a larger cross sectional area than the tendon proper and the tendon-to-bone attachment. Because the transmitted force across these tissues is the same, this change in cross sectional area also results in a gradient of stress across the tendon-to-bone attachment. This idea was theoretically confirmed by modeling the tendon-to-bone attachment region [4]. These studies demonstrate that the bulk shape of the tendon-to-bone attachment is sufficient to create gradients in the mechanical environment across the tendon-to-bone attachment.
Chemical gradients during development of the tendon to bone tendon-to-bone attachment

During development of the tendon-to-bone attachment, many biologic factors are localized in a gradient fashion. Four biochemical factors that are chemically graded and precisely spatially controlled during tendon-to-bone attachment development are parathyroid hormone-related protein (PTHrP), Indian hedgehog (Ihh), Scleraxis (Scx), and type X collagen (Col X) [40]. PTHrP is localized between the tendon proper and the transitional tissue that inserts into the underlying bone. Due to its nature of regulating growth plate maturation, PTHrP is believed to be important to maintaining the mineralized interface during development by maintaining chondrocyte proliferation, blocking chondrocyte maturation, and blocking whole tendon-to-bone attachment mineralization [45, 46]. Ihh is expressed in the transitional zone of the attachment by pre-hypertrophic and hypertrophic chondrocytes [43, 44]. Via interaction with Patched (Ptch) and Smoothened (Smo), Ihh stimulates synthesis of PTHrP. PTHrP expression then blocks further expression of Ihh, creating a negative feedback loop for fine control of the developing tendon-to-bone attachment [43, 44]. Scleraxis (Scx) is a transcription factor associated with mature tendons and found in tendon progenitor cells [49, 50, 51, 52]. Blitz recently demonstrated that Scx was necessary for initiation and development of deltoid tiberosity which is the attachment site of deltoid tendon on the humerus [47].

6.2.2 Mechanotransduction

Mechanotransduction is the process by which cells convert mechanical signals to chemical activity. Many theories exist for how cells convert mechanical signals to chemical activity
including nuclear shape change and the dynamic stability of actin fibers [149]. Most, however, include the same basic cytoskeletal elements and adhesive proteins that link the cell to its surroundings. Cells experience forces that they generate internally or that are externally imposed upon them. Internal forces are generated as myosin slides along actin filaments, similarly to how a muscle contracts. Since these forces are actively generated by the cell, they will be referred to as active stresses in this text. Forces that are externally imposed upon the cell will be called passive stresses in this text, since they are passive with respect to the cell. These forces may occur locally, e.g., via a neighboring cell, or at a distance, e.g., a bone deforms as organisms walk upon the ground.

**Active mechanotransduction**

In a seminal 2006 paper, Engler *et al.* demonstrated that naïve stem cells could respond and differentiate to their physical environment in the absence of other chemical signals. More specifically, it was demonstrated that cells on compliant substrates became neurogenic, cells on stiffer substrates became myogenic, and cells on the stiffest substrates became osteogenic, all without other endogenous factors. Importantly, it was also demonstrated that this was a function of the cell force generating machinery: the actin-myosin complex [150]. Since this type of mechanotransduction relies on the cell force generating machinery, it is commonly referred to as active mechanotransduction. The transduction of these physical cues to biochemical responses is still relatively unknown, but recent studies have implicated cellular localization of the hippo-pathway effectors yes-associated protein (YAP) and tafazzin (TAZ) as central regulators of these processes [151]. Further research identified several regulators
of actin dynamics which can potently control YAP/TAZ expression, demonstrating that cytoskeletal mechanics play an important role in this mechanotransduction signaling cascade [152].

**Passive mechanotransduction**

In contrast to active mechanotransduction, passive mechanotransduction is the result of forces or deformations on a cell applied at a distance, such as from the surrounding cells or tissue. In the context of the tendon-to-bone tendon-to-bone attachment, Schwartz et al. demonstrated passive mechanotransduction was critical for proper tendon-to-bone attachment maturation: paralyzing the force generating muscle with botox during development results in formation of a functionally degraded tendon-to-bone attachment [48]. In tissue engineering paradigms, these forces or deformations can also be applied by a scaffold to the cells via bioreactors. Tissue engineering studies utilizing passive mechanotransduction to scaffolds have yielded a wide variety of cell phenotypes, depending on cells used, scaffolds used, chemical factors included, and other methodological inputs [143, 144, 63, 145, 146].

**Mechanotransduction is necessary for the proper development of the tendon-to-bone attachment**

Recent studies by Schwartz et al. have demonstrated that removal of mechanical stimuli from the tendon-to-bone attachment by paralyzing muscles with botox leads to impaired tendon-to-bone attachment development [48]. Following removal of mechanical stimulation, the resulting tissue is less organized and has highly impaired mechanical properties. While it is currently not known exactly how muscle unloading impairs tendon-to-bone attachment
development, it may be because of decreased mechanotransduction signals. In fact, the four chemical factors localized to the tendon-to-bone attachment described above (Section 6.2.1) are known to be mechanosensitive. PTHrP has been implicated in mechanotransduction pathways [153]. Ihh expression in chondrocytes culture is upregulated in response to tensile stretching and required for increased proliferation [154] independently of PTHrP. In vitro loading modulates Scx expression in tenocyte cultures [155]. Loss of loading results in decreased Scx. Mendias et al. found that treadmill loading upregulates Scx [156]. Lastly, Col X expression is increased in response to mechanical loading [157].

Mechanotransduction has also been implicated in driving bone development [158]. One factor, runt-related transcription factor 2 or core-binding factor subunit (Runx2/Cbfa), has long been established as a central regulator of bone development and differentiation [53]. It is therefore not surprising to find that Cbfa/Runx2 is downstream of mechanical signaling pathways [159]. More recent research suggests upregulation of Cbfa/Runx2 in response to mechanical stimuli is controlled by a cascade involving focal adhesion kinase (FAK) and extracellular regulated kinase 1/2 (ERK1/2) implicating focal adhesion dynamics in the Cbfa/Runx2 cascade [160, 161].

6.2.3 Prior attempts for tissue engineering of tendon-to-bone attachment

Traditionally, tissue engineering approaches have not incorporated complexity in scaffold and bioreactor designs. Specifically, researchers have attempted to engineer uniform tissues without consideration for local heterogeneities that may exist in the native tissues. This local heterogeneity is even more important to consider at tissue interfaces like the tendon-to-bone
attachment, which have local distinct tissues such as tendon and bone as well a functionally graded tissues that connect them. While there have been numerous attempts to recapitulate the complex arrangement of gradients of material properties and cell phenotypes of the natural tendon-to-bone attachment, involving biochemical and/or mechanical gradients, none have succeeded entirely.

**Material and chemical gradients for tissue engineering of the tendon-to-bone attachment**

Many studies have focused on partially mineralizing scaffolds in a graded fashion. Lipner *et al.* created polymer based electrospun scaffolds coated gradients in mineral content that mimicked the gradient in mineral content found in the natural tendon-to-bone attachment [132]. Smith *et al.* also created scaffolds with gradients in mineral content, but with natural collagen instead of synthetic polymers [56]. Of note, the two studies by Smith *et al.* and Lipner *et al.* used the strain estimation techniques described in Chapter 2 and are given as examples in Chapter 5 (Section 5.4 and Section 5.3). Qu *et al.* demonstrated that natural tendon tissue could be partially mineralized as well [57]. Chatterjee *et al.* partially mineralized PEG hydrogel scaffolds and found that they differentially induced osteoblast differentiation [58]. In general, these approaches were complex in nature because they not only required techniques to deliver localized mineralization, but also required care to properly present the chemistry of mineralization.

Similar to mineral gradients, gradients in growth factors have also been explored for tendon-to-bone attachment engineering. Wang *et al.* used opposing gradients of bone morphogenetic protein 2 (BMP-2) and insulin like growth factor 1 (IGF-1), encapsulated in microspheres and demonstrated opposing gradients of osteogenic and chondrogenic differentiation, respectively.
Similarly, Sharma et al. found that tenogenic or osteogenic differentiation could be controlled by presentation of precise surface stiffness and cell adhesion proteins [54]. However, the full complexity of cell signaling molecules present at the tendon-to-bone interface is not fully known, and must be taken into account when developing these tissue engineering solutions.

A different materials approach for tendon-to-bone attachment engineering is the development of multiphasic scaffolds with discrete regions corresponding to the different regions of the tendon-to-bone attachment. Spalazzi et al. developed a triphasic scaffold with regions corresponding to tendon, fibrocartilage, and bone. These three regions were seeded with fibroblasts, chondrocytes, and osteoblasts. Following implantation they found that mineralization was confined only to the bone layer, demonstrating local confinement of the cells and potential as a therapeutic approach for tendon-to-bone repair [59, 60, 61]. While promising, these scaffolds are partitioned into discrete regions which do not accurately reflect the graded nature of the natural tissue.

Studies using scaffold loading for gradient engineering have also been conducted. Thomopoulos et al. found that localized expression of tenogenic or chondrogenic factors varied on the same scaffold depending on if the local region was in tension or in tension combined with compression [63]. Using gradients in applied strain, Morita et al. was able to determine a precise band of strain for optimal tenogenic differentiation, but did not examine other differentiation lineages [64]. These studies are attractive for their relative simplicity but have not been widely explored.
Mechanotransduction for tendon to bone attachment tissue engineering

While mechanical signaling has been shown to be critical for proper tendon-to-bone attachment development (Section 6.2.2), we are currently unaware of any studies that attempt to use only mechanotransduction for tendon-to-bone attachment tissue engineering. However, there are several studies which focus on using mechanotransduction for engineering the various tissues present within the native tendon-to-bone attachment: tendon, fibrocartilage, and bone. Kuo et al. demonstrated upregulation of tenogenic markers by mesenchymal stem cells on cyclically loaded collagen seeded gels [143]. Huang et al. demonstrated mesenchymal stem cells underwent osteogenesis on cyclically loaded PDMS surfaces coated in fibronectin [144]. Alternatively, Baker et al. used compressed nanofibers in cyclic tension to demonstrate fibrochondrogenesis of mesenchymal stem cells [146]. Abousleiman et al. seeded human umbilical veins with mesenchymal stem cells and after cyclic tension and found upregulation of collagen type I as well as close mechanical properties within an order of magnitude of native tendon [145]. Thomopoulos et al. found that cyclic loading under tension of collagen gels resulted in MSC tenogenesis while tension and compression encouraged traits more similar to fibrochondrogenesis [63].

6.3 Overview of approach

6.3.1 Design criteria

To test the hypotheses on how cells respond to gradients in passively applied stress and strain, an experimental system was designed with the following design criteria:
1. To simplify mechanical application and interpretation, a two-dimensional culture system must be used. Although a three-dimensional system would be preferred since it is closer to how cell experience mechanical forces \textit{in vivo}, the use of a three-dimensional system would render precise mechanical application and interpretation difficult.

2. For the theories outlined in Section 6.3.2 to be tested, a material in which the scaffold modulus can be reliably modulated in a graded fashion is required.

3. The scaffold must be bio-compatible and promote cell adherence, or easily modifiable to promote cell adherence without impacting or changing the scaffold mechanics.

4. A bioreactor which is capable of applying pure cyclic tensile strain to many scaffolds simultaneously must be fabricated.

5. Cells used in the experiments should be clinically relevant, consistent, and easily obtained.

6. Assays which provide single cell, highly localized data will be critical to correlate localized scaffold mechanics to individual cellular responses.

6.3.2 Theory for isolating applied stress and strain

A theoretical framework for applying independent gradients in stress or strain to cells was developed. To start, recall from basic linear mechanics that stress is defined as

\[
\sigma = \frac{F}{A}
\]  

(6.1)
where $\sigma$ is the stress, $F$ is the force, and $A$ is the cross sectional area. Stress can also be related to the material properties of the scaffold with the constitutive equation:

$$\sigma = E\epsilon$$

(6.2)

where $E$ is the modulus of the material and $\epsilon$ is the engineering strain. Next these equations are generalized so that each variable is a function of position along the length of the material:

$$\sigma(x) = \frac{F(x)}{A(x)}$$

(6.3)

$$\sigma(x) = E(x)\epsilon(x)$$

(6.4)

In an *in vitro* system, the cross sectional area of a scaffold can be controlled by changing shape of the scaffold and the local modulus of a scaffold can be controlled by modifying fabrication methods. These approaches were combined to create a theory for independent gradients in stress or strain.

For a scaffold in uniaxial tension in a quasi-static state, in the direction of the applied load, the force $F(x)$ is constant for each individual scaffold:

$$F(x) = constant$$

(6.5)

Starting with the most simplistic case and ignoring any boundary conditions, consider a scaffold with no change in cross sectional area along its length and no change in modulus along its length (rectangular uniform, RU)):

$$A_{RU}(x) = constant$$

(6.6)
\[ E_{RU}(x) = constant \] (6.7)

It can then be deduced that:

\[ \sigma_{RU}(x) = constant \] (6.8)
\[ \epsilon_{RU}(x) = constant \] (6.9)

This case is illustrated in Figure 6.1A,B,C.

Next consider a scaffold that has a gradient in cross sectional area, which can be achieved by using a trapezoidal shape, but a uniform modulus (trapezoidal uniform, TU):

\[ A_{TU}(x) = f_A(x) \] (6.10)
\[ E_{TU}(x) = constant \] (6.11)

It can then be deduced that:

\[ \sigma_{TU}(x) = f_\sigma(x) \] (6.12)
\[ \epsilon_{TU}(x) = f_\epsilon(x) \] (6.13)

In the case of the trapezoidal shape described above, the function for cross sectional area is a simple linear model:

\[ A_{TU}(x) = f_A(x) = A_0 + ax \] (6.14)

where \( A_0 \) is the cross sectional area at the smallest end and \( a \) is an arbitrary scale factor determined by the slope of the edges of the trapezoid. Expressions for \( \sigma_{TU}(x) \) and \( \epsilon_{TU}(x) \) can then be determined from equations 6.3 and 6.4. This scaffold has a gradient in strain and a gradient in stress and is illustrated by Figure 6.1D,E,F.
Next consider a scaffold with a uniform cross sectional area, but a gradient in modulus along its length (rectangular gradient, RG):

\[ A_{RG}(x) = constant \]  
\[ E_{RG}(x) = f_E(x) \] (6.15) (6.16)

then from 6.3 we get that

\[ \sigma_{RG}(x) = \frac{A_{RG}}{F} = constant \] (6.17)

however

\[ \epsilon_{RG}(x) = E_{RG}(x)\sigma_{RG} = f_\epsilon(x) \] (6.18)

thereby creating a scaffold with uniform stress in the direction of loading but a gradient in strain. A scaffold of this type is illustrated in Figure 6.1G,H,I.

Lastly, the gradients in cross sectional area and modulus can be combined to create a hybrid scaffold (trapezoid gradient, TG):

\[ A_{TG}(x) = f_A(x) \] (6.19)
\[ E_{TG}(x) = f_E(x) \] (6.20)

then combining with Equation 6.3

\[ \sigma_{TG}(x) = \frac{A_{TG}(x)}{F} = f(x) \] (6.21)
and
\[ \varepsilon_{TG}(x) = E_{TG}(x)\sigma_{TG}(x) = f_\varepsilon(x) \quad (6.22) \]

Upon closer inspection, however, there exists particular forms of \( E_{TG}(x) \) and \( \sigma_{TG}(x) \) which will render \( \varepsilon_{TG}(x) \) equal to a constant.

Examining the case for the linear cross sectional area, as in the case of an isosceles trapezoid (Equation 6.14) and combining an equation for the modulus of the material that will be required for a constant strain can be obtained:

\[ E_{TG}(x) = \frac{C}{(A_0 + ax)} \quad (6.23) \]

This demonstrates that a linear gradient of modulus inversely proportional to the cross sectional area of the scaffold will result in a scaffold with a uniform strain. This scaffold is illustrated by Figure 6.1J,K,L.

### 6.4 Methods

#### 6.4.1 Biomaterial design and fabrication

**Choice of biomaterial**

Two initial candidate materials and one hybrid material were considered for this study: Polydimethylsiloxane (PDMS), polyacrylamide (PAm), and a Polydimethylsiloxane/ polyacrylamide hybrid (PDMS/PAm). Polyacrylamide (Pam) is a widely studied bio-compatible polymer for mechanotransduction experiments. Most studies using polyacrylamide have
Figure 6.1: Theoretical stresses and strains on scaffolds combining shape and stiffness gradients. Four scaffold groups were generated consisting of combinations of gradations in cross sectional area and gradations in shape (A, D, G, J). The following results for each of the four combinations was theorized: a scaffold with a uniform cross sectional area and a uniform strain would have a uniform stress (B) and uniform strain along its length (C); a scaffold with a gradient in cross sectional area but a uniform stiffness would have a gradient in stress (E) and a gradient in strain (F); a scaffold with a uniform cross sectional area but a gradient in stiffness would have a uniform stress (H) but a gradient in strain (I); and a scaffold with a gradient in cross sectional area and an inverse gradient of stiffness would have a gradient in stress (K) a uniform strain (L). The theorized stresses are based off of Equation 6.1.
investigated the role of actively generated cell stresses on cell behavior and how cells respond to the local substrate stiffness (typically in the absence of chemical signals) [150, 162, 163, 151, 152]. Polyacrylamide surfaces are easily modified to promote cell adhesion using Sulfo-SANPAH (sulfosuccinimidyl 6-(4-azido-2-nitrophenylamino)hexanoate) as a crosslinking agent [150, 162, 163, 151, 152]. Simple experimental methods exist for creating gradients in cross linking activity of PAm resulting in gradients in substrate stiffness. A typical approach to achieve this includes spatially controlling the amount of UV light the scaffold receives during crosslinking [164]. However, PAm scaffolds themselves are not physically tractable and are very difficult to handle due to their highly compliant hydrogel structure. Cell active mechanotransduction on PAm scaffolds has been very extensively studied and much is known about how stiffnesses controls stem cell differentiation in the absence of strain [150, 162, 163, 151, 152].

Polydimethylsiloxane (PDMS) is another common bio-compatible polymer often used in cell mechanics studies [162, 165, 163, 166, 167, 64]. PDMS biofouls very readily, so surface modification can be carried out simply by immersing the material in a solution that includes cell adhesion proteins [1, 64]. The stiffness of PDMS is also easily modified by either controlling the ratio of crosslinker to monomer or by controlling temperature-dependent activation of the crosslinker. By combining these two stiffness-modulating paradigms, it is possible to create scaffolds with precisely controlled stiffness gradients [1]. The role of PDMS in cell-active mechanotransduction, however, is of current debate in the field [162, 163]. Prior reports have presented conflicting views on how the stiffness of PDMS may influence cell differentiation. However, these studies are hard to interpret because PDMS directly influences cell differentiation. The lack of a consensus on how PDMS may mechanically regulate stem cell differentiation and fate is therefore a limitation of any study using PDMS as a scaffold material.
Due to the inherent limitations of both PDMS and PAm, a hybrid material consisting of a PAm surface covalently bonded to a PDMS bulk scaffold was considered. This hybrid material would have high tractability due to the bulk of the scaffold being PDMS as well as a high degree of control of the local cell environment, as the cells interact only with the PAm layer. One group presented a study in which polyacrylamide was covalently bound to a PDMS base [168]. However, when attempting to implement this protocol, it was discovered that PAm would only bind to stiff PDMS substrates and that the binding was inconsistent, rendering it unsuitable for the proposed studies (data not shown).

Due to its non-tractability, PAm could not be used for this study. While a PDMS/PAm hybrid material was considered and would have been ideal, the inconsistency of chemical binding introduced too many issues in practice to be viable for these studies. Therefore, pure PDMS was chosen as the scaffold material for subsequent experiments.

**PDMS scaffold fabrication**

PDMS scaffolds in uniform or graded fashion were fabricated according to published techniques [1]. Briefly, PDMS (Sylgard 184, Dow Corning) was thoroughly mixed at various base to curing ratios and placed under vacuum to degas the resulting solution. Glass slides were coated with a chlorinated organopolysiloxane in heptane to render their surfaces hydrophobic (Sigmacote, Sigma-Aldrich). Degassed PDMS solutions were carefully poured between a mold consisting of two silanized glass slides and a 2 mm thick Teflon spacer, and assembled together with four metal clamps (Figure 6.2). Two different permutations of PDMS base:curing premix were used with two different permutations of crosslinking methodologies. In the first group, the entire mold was filled with 1:10 base:crosslinking PDMS premixture and placed in an oven at 60°C for 90 minutes. In the second group, the mold was first
Figure 6.2: Schematic procedure for the fabrication of PDMS stiffness gradients (g-PDMS). (A) Glass slides were coated with a silanizing reagent to make their surfaces hydrophobic (Sigmacote). (B) Molds were formed by clamping a Teflon insert between two silanized glass slides. (C) PDMS mixture was poured slowly into the mold. (D) The PDMS mixture was then either placed into an oven at 60°C for one hour (left) or set perpendicular on a heater ($T_{surface} = 190°C$) and crosslinked upon exposure to a temperature gradient for 1.5 h (right). (E) Excess crosslinker, oligomer and monomer were removed by rinsing in copious amounts of hexane, which swells the scaffolds and allows non-crosslinked reagents to escape. (F) Schematic of resulting uniform (left) or stiffness gradient scaffolds (right). Adapted from [1].

Filled halfway with 1:10 base:cure PDMS premix, then the remaining half was filled with 1:20 base:cure PDMS premix. The resulting mold was placed in an upright position on a hot plate heated to 190°C. This configuration results in a vertically increasing temperature gradient and subsequent gradient in crosslinking density.

Following crosslinking, scaffolds were rinsed twice in copious hexane. At room temperature, PDMS will cross-link given enough time. Hexane swells PDMS scaffolds and allows
excess crosslinking reagents to escape, preventing further polymerization of the scaffolds and thereby ensuring the cross-linker density gradients and stiffnesses from initial heating were permanent. Scaffolds were next placed in a desiccator overnight to remove residual hexane from swelling. Following desiccation, the scaffolds were trimmed to remove any excess material at the edges, then cut into their final shapes. Uniform scaffolds were cut into either rectangles or into isosceles trapezoids. Graded scaffolds were cut into the same shapes but trapezoids were always cut such that the more compliant region correlated with the larger area of the trapezoid.

Scaffolds were next placed into the bottom of six or twelve well plates and allowed to adhere to the surface of the tissue culture plastic overnight. PDMS scaffolds are smooth and readily adhere to the bottom of the tissue culture plastic if both are sufficiently dry. We found that leaving the scaffolds to adhere to the plastic overnight resulted in a strong enough bond that could survive rinses over several weeks. Importantly, the adhesion was reversible, so scaffolds could be easily detached with a small amount of force for handling and loading into the bioreactor. Before surface modification, scaffolds were sterilized by immersion in 70% ethanol for 45 minutes, followed by three PBS rinses to remove residual ethanol.

**PDMS surface modification**

PDMS surfaces easily biofoul and readily absorb proteins in solution. This property lends PDMS to easy surface modification by simply biofouling the surface of the PDMS with cell adhesion molecules. To determine how this influences cell adhesion, PDMS was immersed in various concentrations of fibronectin in PBS, from 0 $\mu$g/mL to 12.5 $\mu$g/mL. Cells were imaged over time to determine how well they adhered to the surfaces of the scaffolds. There was little to no difference in cell adhesion for solutions containing 1 $\mu$g/mL of fibronectin.
and higher. Cells adhered to the surfaces and spread in a fashion similar to tissue culture plastic. While cells did adhere to PDMS surfaces without fibronectin, their morphology was distinctly different from the fibronectin-treated PDMS adhered cells, resembling a more rounded morphology with less focal adhesions (Figure 6.4.2). In many cases, however, cells on fibronectin-coated scaffolds formed clusters and delaminated from the scaffold surfaces. Therefore, an additional assay was conducted to address this problem, as detailed in Section 6.4.2.

For subsequent experiments, surface modification was achieved with the following protocol: First, PDMS was incubated with 2.5 mg/mL human fibronectin (R&D Systems, Minneapolis MN, 1918-FN) in PBS at 4°C overnight. Next, on the following day scaffolds were warmed to room temperature for 30 minutes and then rinsed three times with PBS for 5 minutes each time. PDMS surface modification was apparent following incubation with fibronectin: scaffolds were significantly more hydrophilic following biofouling with fibronectin. After three additional PBS rinses to remove any unattached fibronectin from solution, scaffolds were immediately used for cell culture.

### 6.4.2 Mesenchymal stem cell seeding and viability

Off the shelf cryopreserved Poietics normal human bone marrow derived human mesenchymal stem cells (hMSC) were chosen as the primary stem cell source for all experiments (Lonza Group, Basel Switzerland). hMSCs were chosen for their well documented multipotency, direct applicability and translatability to humans, and overall consistency.
Cell proliferation and cell density parameter choice

A pilot study was performed to determine the optimal cell density and fetal bovine serum (FBS) concentration to inhibit cell proliferation, which could result in undesirably high cell densities. High cell densities were associated with the undesirable delamination of cells from the PDMS surface and the creation of cell bundles. To assay proliferation and initial cell seeding density, cells were serum starved to 0% FBS then cultured on fibronectin modified PDMS with either 5%, 2% or 1% FBS and at initial concentrations of 10k cells/cm$^2$, 5k cells/cm$^2$, or 1k cells/cm$^2$. Cells were cultured for a total of 9 days on the scaffolds and imaged at days 1, 4, 7, and 9. For simplicity, results from only days 1 and 9 are shown. Results confirmed that at very high cell densities and high FBS concentrations, cells delaminated and formed clusters. However, at lower cell densities and lower FBS concentrations, cells remain adhered to the scaffold. Results also indicated that, after 9 days, with an initial density of 1k cells/cm$^2$, cell distributions remained separate and sparse. At an initial cell density of 5k cells/cm$^2$, with an FBS concentration of 1%, the cells formed confluent layers, which was sub-optimal for local cellular analysis. Based on these pilot studies, a concentration of 2k cells/cm$^2$ was defined as an optimal initial cell density, yielding enough cells for analysis without reaching confluence after 9 days in culture (Figure 6.3).

Culture conditions

hMSCs were expanded in culture prior to all experiments. Cells were allowed to expand in culture using human Mesenchymal Stem Cell Growth BulletKit Medium (Lonza Group, Basel Switzerland) for up to 7 days or 3 passages, whichever came first. After initial expansion, vials of 500k cells were cryopreserved using 20% FBS, DMEM, and 5% DMSO for
Figure 6.3: Results from the cell proliferation and cell density parameter study. Results demonstrated that, after 9 days in culture, too much FBS or too high initial seeding density resulted in cell confluence or even cell delamination. From these results, the optimal seeding density and FBS throughout the experiment was determined to be 2000 cells/cm$^2$ and 1% FBS, respectively.

Figure 6.4: Human mesenchymal stem cells cultured for 5 days on varying amounts of fibronectin-coated PDMS. Cells on fibronectin-coated scaffolds appeared similar in morphology to tissue culture plastic-grown cells, while those on PDMS coated scaffolds appeared rounded and did not adhere well to the surfaces.
individual experiments. After the initial expansions and cryopreservation, cells were recon-
stituted in Dulbecco’s Modified Eagle Medium containing 1.5g/L glucose, 10% FBS, 0.2%
penicillin/streptomycin, and 0.2% Amphotericin-B and allowed to expand for up to three
more days in culture before a four day serum starvation to 0% FBS. Following serum star-
vation, cells were seeded onto the scaffolds in Dulbecco’s Modified Eagle Medium containing
1.5g/L glucose, 1% FBS, 0.2% penicillin/streptomycin, and 0.2% Amphotericin-B 1% at
2,000 cells/cm$^2$. From our initial studies on cell proliferation and cell density (Section 6.4.2),
it was determined that 1% FBS and 2,000 cells/cm$^2$ was optimal for restricting proliferation,
having an initial cell density that yields enough cells for statistical certainty, and does not
have a confluent layer of cells (which would make individual cell analysis difficult).

6.4.3 Bioreactor design and validation

A tensile bioreactor was designed, tested, and validated for use in this project (Figure 6.4.3).
To verify control of strain, the bioreactor was programmed to impose 10% and 20% strains
to scaffolds at 0.1Hz. Using 2D-DDE (Chapter 2), the average strain on the scaffold was
calculated and confirmed to match the input (Figure 6.4.3).

In order to validate theoretical strain patterns (Section 6.3.2) were achieved in our *in vitro*
bioreactor optical strain estimation techniques were utilized. Briefly, scaffolds were prepared
according to methods described in Section 6.4.1. Scaffolds were then sprayed with an aerosol
paint to apply a random pattern to the surface for strain tracking. Scaffolds were loaded
into the bioreactor (Section 6.4.3) and tensile strains varying from 5% to 15% were applied
to the scaffolds at 0.5 Hz. During straining, videos were captured of the test using a high
resolution camera at 8 frames per second (Model RMV-8050, Illunis Camera, Minnetonka,
Figure 6.5: A bioreactor capable of applying simple tensile strains to several scaffolds at once was designed in Solidworks (A) and constructed (B,C). A side view shows the overall design of the bioreactor (B) and a top down view with loaded scaffolds shows how scaffolds are configured in the bioreactor (C).
Figure 6.6: Average tensile (blue lines) and transverse (red lines) strains for uniform PDMS scaffolds stretched with an input sine wave to maximum 10% strain (A) and stretched with input sine wave to maximum 20% strain (B) on a custom designed bioreactor. Tensile strains confirm the bioreactor behaves as expected and optically estimated strains match input values.

MN). 2D-DDE methods from Chapter 2 were used to estimate applied strains. Importantly, 2D-DDE enabled the measurement and confirmation of the theorized strain patterns: the prior state of the art (2D-XCOR) estimated erroneous and unrealistic strains (Figure 2.5).

6.4.4 Experimental design and outcome measures

Loading protocol and timeline

Cells were seeded on scaffolds and cultured for 9 days: 2 days under static load and 7 days of 5% cyclic loading. Seven days was chosen as the time frame for loading because robust expression of tenogenic and osteogenic transcription factors is expected after 7 days [169, 170, 171]. 5% grip-to-grip strain was chosen for loading to accommodate the range of local strains expected on gradient scaffolds. Specifically, we found that 5% grip-to-grip strain
Figure 6.7: The complete timeline of the experiment was 9 days of cell culture. Cells were first seeded onto PDMS scaffolds and allowed to attach for one day. The following day, unloaded control PDMS scaffolds were left adhered to their dishes and loaded scaffolds were transported to the bioreactor. After one additional day of static culture the scaffolds were loaded for 7 days. Specifically, scaffolds were loaded twice a day, for one hour a time, to 5% grip-to-grip strain at a rate of 0.5 Hz, with an hour rest between the two loading bouts. On the seventh and final day of loading, scaffolds were fixed one hour after the second loading bout.

resulted in a range of local strains from 12% to 0% on gradient scaffolds; this is expected to capture the ideal strain band for tenogenesis which is near 7% strain [64]. Cells were seeded onto the PDMS scaffolds and left to adhere for one day. The following day, half of the scaffolds were left adhered the dishes and served as unloaded controls while the other half were carefully placed into the bioreactor. Because the procedure for transporting the scaffolds to the bioreactor may affect cells behavior, an additional day of static culture was allowed (in the bioreactor) before beginning the loading protocol (Figure 6.4.4). Scaffolds were subjected to two daily bouts of loading to 5% grip-to-grip strain at a rate of 0.5 Hz. Between each loading bout, scaffolds were ”rested” for one hour. On the final day of loading, scaffolds were fixed one hour after the second bout of loading.

**Immunocytochemistry and image analysis**

Following completion of the loading protocol, scaffolds were processed for immunocytochemistry using standard techniques. Scaffolds were rinsed in PBS and fixed with 4%
paraformaldehyde for 5 minutes. Following fixation, scaffolds were rinsed three times with PBS and then incubated with a blocking and cell permeabilization solution containing 2% donkey serum and 0.1% Triton X100. Scaffolds were then incubated with a primary antibody solution containing 1/500 goat polyclonal IgG anti-Scleraxis (Santa-Cruz Biotechnology, sc-87425), 1/500 rabbit polyclonal IgG anti-RUNX2 (Santa-Cruz Biotechnology, sc-10758), 2% donkey serum, and 0.1% Triton X100 overnight at 4°C. The following day, samples were allowed to warm to room temperature for 30 minutes, after which they were incubated in a secondary antibody solution containing 1/500 Alexa Fluor 546 conjugated Donkey anti-rabbit IgG (ThermoFisher Scientific A10040), 1/500 Alexa Fluor 488 conjugated Donkey anti-goat IgG (ThermoFisher Scientific a11055), 2% donkey serum, and 0.1% Triton X100 for 1 hour at room temperature. Following incubation with the secondary antibody solution, samples were rinsed 3x with PBS, inverted onto glass coverslips, and mounted with Vectashield mounting medium with DAPI. Scaffolds were then imaged using a Nikon Ti-E Eclipse inverted spinning disc confocal microscope.

Two sets of images were captured for each sample. First, a whole scaffold image was captured by scanning across the entire length of the scaffold in both the x and y directions to visualize every cell in the scaffold, with an average of about 200 individual images for each scaffold. Due to small differences in the focus plane across the length of the relatively large scaffold, comparison of expression levels of assayed proteins was inconsistent with this imaging approach. Therefore, each large composite image was supplemented with individually focused images along the length of each scaffold, to ensure optimal focus. These two imaging approaches revealed broad cell density and general expression patterns (from composite images) as well as localized expression patterns (from individual images).
6.5 Results and discussion

6.5.1 Validation of local control of stress and strain

Due to the relatively high amounts of applied tensile strain, traditional optical strain estimation methods such as normal cross correlation (2D-XCOR) revealed unrealistic strain patterns on the stiffness-graded rectangular PDMS scaffolds (Figure 2.5A,C,E,G). Because of this, 2D-DDE, developed in Chapter 2, was required to accurately reveal the true local strain fields (Figure 2.5B,D,F,H).

2D-DDE confirmed that, for each group, the strain patterns inferred from the combination of the shape and stiffness gradients were correct (Figure 6.1 and Figure 6.8). In uniform rectangular scaffolds, there was no gradient in strain (Figure 6.1A,B,C and Figure 6.8A). In uniform trapezoidal scaffolds, there was a gradient in strain associated with the change in cross sectional area of the scaffold, and therefore the relative stress (Figure 6.1D,E,F and Figure 6.8B). In gradient rectangular scaffolds, there was a gradient in strain associated with the prescribed gradient in stiffness (Figure 6.1G,H,I and Figure 6.8C). Lastly, for the scaffolds with inversely imposed gradients in cross sectional area and stiffness, no gradient in strain was observed, as expected based on theoretical considerations presented in section 6.3.2 (Figure 6.1J,K,L and Figure 6.8D). Overall, theoretical strains matched strains measured experimentally for all scaffold groups.
Figure 6.8: Validation of theoretical strains on scaffolds combining shape and stiffness gradients. Using 2D-DDE (Chapter 2), it was confirmed that measured strains were consistent with theoretical strains (Figure 6.1C,F,I,L) for all groups (A,B,C,D).
6.5.2 Expected outcomes for cellular mechanotransduction responses

In order to determine local stem cell differentiation responses to stress and strain, local expression patterns Runx2 and Scleraxis will be examined along the length of each sample to determine osteogenesis and tenogenesis, respectively. Samples with a uniform distribution of stress or strain are expected to show uniform expression patterns while those that have a non-uniform stress or strain are expected to have unique localized expression patterns. Three possible outcomes are shown schematically in Figure 6.9. Example images from cells loaded in the bioreactor are shown in Figure 6.10. Expression patterns will then be associated with strain profiles for each scaffold group scenario to determine the precise levels of strains that elicit particular responses.

We hypothesize that Runx2 expression will increase with increasing stress but not strain and that Scleraxis expression will increase with increasing strain but not stress. The basis for these hypotheses is the stress and strain states present within the body: in the bone, high stress caused by micro damage is believed initiate a bone repair cascade ending with osteogenesis to repair the bone surface [172]. In this case there is high stress, however strain is relatively low (compared to tendon) since bone is relatively stiff. Likewise, strain is more likely to be a driver of tenogenesis due to the high levels of strain in healthy tendons (when compared to bone). In fact, Morita et al. determined a precise band of strain for optimal tenogenic differentiation at around 7% although in their model system it is difficult to resolve the ambiguity of stress versus strain [64].

To confirm this, for each of the four groups, we expect the following results: (1) Scleraxis and Runx2 expression will be uniform in the uniform rectangle (Figure 6.11A), (2) Scleraxis and Runx2 expression will both increase with increasing stress and strain in the uniform
Figure 6.9: Three examples of possible outcomes for scaffolds. (A) Cells imaged along the length of a scaffold have nearly uniform expression of a gene. (B) Cells imaged along the length of a scaffold display a gradient in expression of a particular gene, from low expression on one end to high expression on the other. (C) Cells imaged along the length of a scaffold have a bimodal expression of a particular gene, with an optimal strain level at which expression is highest.
Figure 6.10: Example results for scaffolds loaded in the bioreactor showing a gradient in Runx2 expression. Images are plotted in the "jet" colormap to accentuate differences in expression.

trapezoid (Figure 6.11B), (3) Scleraxis expression will increase along the strain gradient while Runx2 expression will remain uniform in the graded rectangle (Figure 6.11C), and (4) Runx2 expression will increase along the stress gradient while Scleraxis expression will remain uniform in the graded trapezoid (Figure 6.11D).

Preliminary outcomes (N = 1-2 per group) were obtained to test these hypotheses. Qualitative results suggest that cells differentially express Runx2 along stress gradients, but not strain gradients. More specifically, Runx2 expression increased with increased applied stress, but applied strain had no effect on Runx2 expression. Due to ongoing issues with visualizing Scleraxis expression, no results for Scleraxis expression were obtained. Confirmation of these preliminary results will require repetition of the experiment as well as quantification of the relative expression levels.
Figure 6.11: Hypotheses for expression patterns following loading of the four groups in this study: (A) Scleraxis and Runx2 expression will be uniform in the uniform rectangle scaffolds, (B) Scleraxis and Runx2 expression will both increase with increasing stress and strain in the uniform trapezoid scaffolds, (C) Scleraxis expression will increase along the strain gradient while Runx2 expression will remain uniform in the graded rectangle scaffolds, and (D) Runx2 expression will increase along the stress gradient while Scleraxis expression will remain uniform in the graded trapezoid scaffolds. Theorized stress and measured strain for each of the four groups is shown in Figure 6.1 and Figure 6.8, respectively.
6.5.3 Conclusions

The results demonstrate validation of a new in vitro model system which can be used to independently modulate stress and strain. The use of immunocytochemistry for individual cell analysis in conjunction with 2D-DDE strain estimates provides extremely precise control and determination of cell response to mechanical stimuli. This control will allow for mechanistic study of how cells respond to local mechanical inputs. Importantly, stress or strain can be presented to cells in a graded fashion on each scaffold, allowing for development of functionally graded tissue such as the tendon-to-bone attachment. In contrast to currently published tissue engineering techniques, this approach can be modified for more complex patterning simply by further changing the geometry of the scaffold or the stiffness configuration. Following validation of the hypotheses illustrated in Figure 6.11, these ideas will be further explored and confirmed with supplementary techniques. More specifically, the scaffold will be broken into discrete pieces instead of left whole, and qPCR will be performed on the individual pieces. While this will not provide the high resolution that the optical techniques described within this chapter, it will provide robust and quantitative data to test the hypotheses. Future studies will also probe mechanosensitive factors, such as actin, myosin, and focal adhesions, to investigate the cytoskeletal mechanisms involved in these responses.

The newly developed methods will allow for both mechanistic mechanobiology studies and applied tissue engineering studies. These studies can lead to rapid and high throughput study of cellular responses to a wide variety of mechanical stimuli at precisely defined local stress or strain levels, with particular relevance to mesenchymal stem cell differentiation. These studies may also lead to an increase in the basic understanding of cellular response to stress and strain due to the model’s ability to effectively isolate them.
Chapter 7

Conclusions and future directions

7.1 Summary of the dissertation

The previous chapters described an effort to develop, implement, and evaluate the efficacy of techniques that estimate strain directly, by incorporating mechanics into non-rigid image registration algorithms. Two versions of this approach were developed and validated: a two dimensional surface version (2D-DDE) and a three dimensional fully volumetric version (3D-DDE). Both versions were based on established algorithms developed for solving the optical flow problem in computer vision [15]. Estimates of strain with either 2D-DDE or 3D-DDE on datasets with known strain fields revealed dramatically improved accuracy and precision compared to the prior state of the art. Both 2D-DDE and 3D-DDE were constructed such that they do not require enforcement of priors for robust strain estimation. Furthermore, relaxation of these constraints on strain estimation allowed for the development of complementary algorithms capable of robustly detecting highly localized elevated strains: 2D-SIMPLE and 3D-SIMPLE. Using real-world laboratory data, 2D-SIMPLE demonstrated the ability to precisely predict crack initiation and propagation on vinylidene chloride sheets in tension. In silico 3D-SIMPLE demonstrated the ability to detect strain concentrations
forming around a penny shaped crack and around a developing Eshelby inclusion. Lastly, a theoretical framework was developed for implementing a DDE-like algorithm using images from stereo or multi-view vision systems. Following implementation and validation in future studies, this algorithm will be capable of directly estimating surface strains of three dimensional objects without consideration of the three dimensional position or pose of the object.

To demonstrate robustness and general applicability of the algorithms, they were applied to interpret data from a wide range of biomedical experiments:

1. A microscale compliant region was discovered at the tendon-to-bone interface by using a modified version of 2D-DDE.
2. Local heterogeneity of partially mineralized collagen scaffolds were revealed by 2D-DDE.
3. Gradients in stiffness of partially mineralized nano-fiber scaffolds were revealed by 2D-DDE.
4. 3D-DDE confirmed the existence of strain gradients across chordae tendineae in beating murine hearts.
5. Dramatic localized changes in heart wall deformation were revealed due to myocardial infarction in murine hearts using 3D-DDE.
6. Mechanisms of embryonic wound healing and associated strain localizations were demonstrated using 2D-SIMPLE.
7. A model system isolating passively applied stress and strain for studying cell mechanotransduction was developed and validated using 2D-DDE.
Collectively, these eight examples demonstrate the utility and adaptability of 2D-DDE, 3D-DDE, and SIMPLE for a wide variety of biomedical applications.

In the last example application listed above, a model system for studying passively applied stress or strain on cells was theorized, developed, and validated. The model system allows for local control of stress and strain on cell-friendly scaffolds. Cells are seeded onto scaffolds and cyclically loaded in tension. Cells can selectively be subjected to no gradient in stress or strain, a gradient in only stress, a gradient in only strain, or a combined stress and strain gradient. Control of local stress and strain was achieved by controlling scaffold stiffness and geometry and validated using 2D-DDE. Furthermore, scaffold and protocol parameters were optimized for cell attachment, cell density, and cell viability. Preliminary results using the model system motivate future tendon-to-bone enthesis tissue engineering experiments using local mechanical stimuli to promote spatially graded stem cell differentiation.

7.2 Limitations and future directions

Chapters 2 and 3 develop the 2D-DDE and 3D-DDE algorithms. 2D-DDE and 3D-DDE extend techniques first established in the computer vision field [15, 16] to biomechanics. As such, they are subject to the same inherent limitations of the initial approaches. The first limitation is that the algorithms are based on a Newtonian optimization which assumes that, when beginning optimization, the initial guess is approximately equal to the global minimum. In practice, this translates to an inability to reliably determine large increments of deformation and an inability to determine deformation following a large increment of translation. In the future, the algorithm will be expanded to cope with this limitation in two manners. First, an extension of the original Lucas-Kanade algorithm, the Lucas-Kanade pyramidal
approach, will be implemented [173]. This algorithm expands the optimization such that it is performed initially on downsampled images until convergence, then optimization is refined on the original high quality images until convergence is again reached. This will not only result in an ability to handle large increments of translation, but may also allow for even more precise refinement of local strain. Second, a global preregistration step will be added that allows for arbitrarily large rigid body motions between successive frames. This will alleviate the requirement that images are continuously captured, which in many cases (e.g., microscopy) can be experimentally difficult to achieve.

A second limitation is that 2D-DDE and 3D-DDE are based on matching image intensities and achieve the registration by examining the structure of the gradients of the intensities. As a result, they are limited to analyses where the geometric shape change of the image intensity between the template and input image is directly correlated to the deformation. This becomes problematic when image intensities, and therefore textures, are varying on a scale outside of the deformation of interest. One example of this is the determination of deformation of a sheet of cells. In this case, the measurement of deformation of a collection of cells in the sheet by DDE requires that every cell deforms identically and there is no local variation. However, since cells are mechanically inhomogeneous, each cell deformation will be unique and their collective deformation will be unique and based on the local deformations. In such a case, DDE will not be able to reliably estimate the strain for a collection of cells at this scale. A similar case can be found when trying to determine the deformation of sparsely stained collagen networks at the fiber level: the deformation of the collagen network does not necessarily correlate to the local movement of the collagen fibers. Due to the initial formulation of DDE, these limitations cannot be overcome and alternative techniques must be used.
Chapter 4 presented two theoretical frameworks for implementing a two dimensional / three dimensional hybrid version of DDE using multiple views. However, the theoretically valid techniques have not yet been implemented and therefore could not yet be compared to the prior state of the art. As described in 4, both techniques make trade-offs: either the computation required to estimate the strain is expensive or the accuracy of strain measurement is impaired. In the future, these algorithms will be implemented in MATLAB and compared to the current state of the art using synthetic data, where true deformations are known, and real-world experimental data, as we did for 2D-DDE and 3D-DDE. In the current state of the art proves to be superior to the new methods, an alternate approach will be explored. A recent paper by Benhimane and Malis modified the original minimization approach of the Lucas Kanade technique by using a second order minimization instead of a first order minimization [174]. As a result of the second order minimization, the necessity of computing the Hessian matrix was removed without loss of generality and the algorithm was more computationally efficient. To implement this algorithm, however, Benhimane and Malis had to carefully choose the parameterizations of their warping functions or the computation of the Jacobians would have been computationally expensive. Specifically, they choose their parametrization to be a set of linearly independent matrices in the Special Linear group, which consists of only matrices that have a determinant equal to 1. While they demonstrated that their technique was able to track positions, it remained to be determined if this parameterization would be suitable for expansion to include deformation, so that deformation can be intrinsically determined during image registration.

Chapter 6 detailed the development of a model system for studying mechanotransduction, with precise control of passively applied stress or strain. While the model system was fully developed and validated, it has not yet been used to test a specific mechanotransduction
hypothesis. Future studies will explore mechanically-induced mesenchymal stem cell differentiation and spatially graded differentiation towards developing tendon-to-bone attachment tissue engineering. Despite the fine control of local stress and strain that this model system affords, there are a number of inherent limitations. First, the system is limited to two dimensional cell culture and loading. This design choice was made to simplify the relationship between the mechanics and the material: for a three dimensional application, the true cross sectional area in tension must also consider the local volume fraction of cells and the local porosity of the substrate; extending these techniques to the third dimension requires precise control of these factors. A second limitation is the relatively simple patterns of stress and strain that were implemented; future future work should include more complex scenarios. For example, the effect of stress/strain concentrations could be studied by creating a hole in the center of the scaffold.

7.3 Conclusions

Overall, this work details the development, implementation, and usage of new algorithms for the study of deformation in biology. Techniques in this new toolbox gain their advantages over the prior state of the art from the incorporation of mechanics directly into cutting edge computer vision algorithms. As a result, these algorithms are not only more accurate, more precise, and higher resolution, but they are also less constrained. Removal of these constraints, such as the enforcement of strain compatibility, resulted in the development of a new class of algorithms (2D-SIMPLE and 3D-SIMPLE) which are capable of detecting strain localizations and predicting failure. The development of a multiview analog of these techniques demonstrates the importance of considering mechanics at every step of the strain
estimation pipeline and how computational efficiency may impair the estimation of strain. The six examples of strain estimation described provide a small overview of the myriad of biological processes that can be explored with these techniques as well as their general applicability. Lastly, the development and validation of a model system for studying the effects of stress and strain on cellular differentiation was enabled by these techniques. Additionally, the model system has the potential to answer a myriad of mechanobiology questions including if cells respond differently to stress or strain. Collectively, this work introduces a new toolbox of techniques for studying the relationship of deformation to biology.
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