

**MICROMECHANICAL IMAGING ANALYSIS OF BULK VS. LOCAL  
PROPERTIES CONCERNING MESENCHYMAL STEM CELL  
HETEROGENEITY**

NSF Summer Undergraduate Fellowship in Sensor Technologies  
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**ABSTRACT**

Mesenchymal stem cells (MSCs) harvested from bone marrow tri-differentiation potential into osteoblasts, adipocytes, and chondrocytes along with portraying a variety of phenotypes. MSCs are a promising cell source for cartilage tissue engineering, but MSC seeded constructs have yet to match the mechanical properties of chondrocyte seeded constructs. The basis of this study is to use imaging techniques on MSCs that have already been developed to analyze chondrocytes and native articular cartilage to provide information on matrix production, cell response to load, and cell mechanical properties. This paper displays two studies. The first study used micromechanical analysis with florescent microscopy on MSCs and cartilage to study the local and bulk mechanical properties and development of the cells' extra cellular matrix (ECM). The hypothesis was that comparing the local mechanical properties to the bulk properties of MSCs and chondrocytes will explain why the MSC constructs are weaker than the chondrocyte constructs. The second part of the study used confocal microscopy to analyze cell deformation as a function of matrix production over time. The cell deformations were analyzed via a customized Matlab (Mathworks) program to act as a standardized analysis method. Analysis when complete of cell, local, and bulk tissue mechanics is expected to provide insight into the subpar bulk mechanics found in MSC constructs and determine the heterogeneity of local matrix properties.

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## **1. INTRODUCTION**

Mesenchymal Stem Cells (MSCs) harvested from bone marrow possesses the ability to differentiate into adipocytes, chondrocytes, myocytes, and osteoblasts [1 Pittenger]. This is significant because many adult stem cells have the ability to repair a variety of tissues after injury. In the area of cartilage, MSCs serve as a potential source of cells to reconstruct damaged or diseased cartilage tissue. Finding an effective method to heal cartilage has proven difficult due to the inherent nature of cartilage [2 Csak]. The slow healing rate of cartilage has contributed to the fact that its damage affects more than 20 million Americans. Thus there is a strong demand for a method to more rapidly repair cartilage. MSCs show potential to be used as a cell source for tissue engineering cartilage due to their ability to differentiate into cartilage; however, there is a problem with MSCs due to their weaker mechanical properties and heterogeneity, meaning the cells display different phenotypes and have varying likelihoods of cell differentiation. In an effort to study these unexplained problems this paper focuses on studying the micromechanical properties and bulk properties of MSCs and chondrocytes. Analysis, when complete is expected to prove that the varying likelihoods of MSC differentiation can be analyzed via the micromechanical properties of the cells and give insight into the deformation chondrocytes and MSCs experience with mechanical stimulation on a microscopic and macroscopic scale.

## **2. BACKGROUND**

### **2.1 NATURAL CARTILAGE HEALING**

Articular cartilage, the cartilage that coats the bones at joints, can handle extreme compressive, cyclic, and static loads due to its structure. However, its structure also causes it to heal slowly once damaged. The first reason for its slow repair rate is that articular cartilage is only 1% tissue by volume, while the other 99% is an extracellular matrix [3 Shulz]. The second reason that cartilage grows slowly is that it lacks both capillary and nerve connections throughout the tissue which slows down the distribution of nutrients [2 Csak]. When the cartilage matrix is damaged and the damage does not reach the bone, the adjacent chondrocytes multiply and repair the extracellular matrix [3 Shulz]. After a long period of repair, the cartilage appears the same as the rest of the tissue. When cartilage has trauma that reaches the bone, MSCs flow from the bone to the damaged site. The MSCs replace the articular cartilage with Type I and Type II cartilage. After a few weeks the MSCs differentiate into cartilage tissue that resembles the original cartilage, but has weaker mechanical properties. When the bone is ruptured from the trauma the injury receives the help of the nutrients brought by blood in addition to the MSCs to increase the rate of healing. After a few weeks the new cartilage resembles the previous tissue; however, again the tissue has weaker properties because of the MSCs that were used to make the new cartilage. Cartilage normally has the ability to withstand and repair itself under extreme dynamic loads; however, under certain circumstances cartilage loses the ability to fix itself.

### **2.2 OSTEOARTHRITIS**

Osteoarthritis is the inflammation of a joint caused by the breakdown of cartilage. Osteoarthritis is technically a repair process because as the articular cartilage is damaged new bone is formed to fill in for the lost tissue. A paper published by the British College of Physicians describes osteoarthritis as a ‘slow but efficient repair process that often compensates for the initial trauma resulting in a structurally altered, but symptom free joint’ [4 Osteoarthritis]. Osteoarthritis becomes a debilitating disease when the cartilage cannot ever make a complete recovery of its damage. Osteoarthritis is caused by obesity, age, and repetitive trauma. Primarily the disease is highly correlated to age because as we grow older the water percentage of cartilage increases while the protein percentage decreases [5 Shiel]. This trend weakens cartilage and allows it to flake or crack.

### **2.3 CURRENT PRACTICES**

Currently options for cartilage problems include arthroscopic surgery, joint replacements, and tissue transplantation. There are 1,500,000 arthroscopic operations annually to repair torn tissue such as tendons, menisci, or cartilage [3 Shulz]. However, these surgeries were deemed to be ineffective for osteoarthritis in a 2002 study at a Houston Veterans Affairs Medical Center [6 Moseley]. Joint replacements are performed to replace arthritic, damaged, or cancerous points of the joint. A total of \$15 billion worth of complete joint replacement surgeries are performed annually [3 Shulz]. Both the large costs involved in cartilage repair and demands for cartilage disease solutions are the reasons people are studying tissue engineering.

### **2.4 TISSUE ENGINEERING USING MSCs**

Many scientists in the field of tissue engineering hold high hopes for MSCs as a method to repair cartilage tissue in the human body. MSCs have the potential to differentiate into adipocytes, osteocytes, and chondrocytes. [7 Mareddy] MSCs can be isolated from many sources in the body such as liposuction fat and bone marrow. [8 Huang] [9 Guilak] This means that cartilage tissue from the body will not need to be sacrificed for cells to repair the damaged cartilage. MSCs can be relatively easily cultured and expanded while keeping their many phenotypes. The identification of a suitable cell source such as MSCs is a major step in the advancement of tissue engineering.

### **2.5 PROBLEMS WITH MSCs**

Before MSCs can be utilized for cartilage repair many steps must be taken to improve the cells’ deficiencies. First of all the standard practice for harvesting and collecting MSCs involves adhering MSCs to plastic which most other cells do not adhere. [10 Vogel] [8 Huang] This method of collecting MSCs provides a mixture of MSC phenotypes and other cell types, which creates a large hurdle when attempting to grow a homogenous population of cells. Second, MSCs lack unique cell surface markers that can be utilized to recognize the varying phenotypes of MSCs. Third, MSC constructs

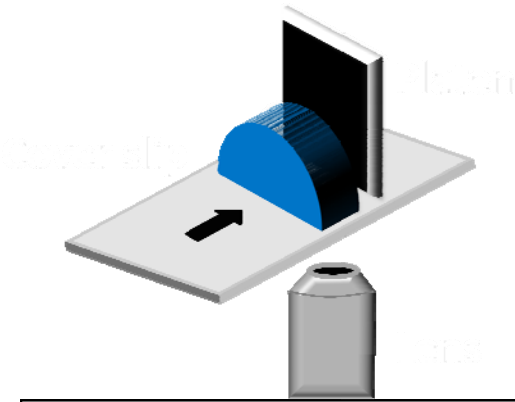
have yet to be produced that match the mechanical properties of cartilage based constructs and ultimately native cartilage.

### **3. METHODS**

Bovine MSCs and chondrocytes were harvested from three donors' femurs and tibias. The cells were then encapsulated in 2% agarose constructs 4mm in diameter and 2.25mm in thickness with density of 20million cells per mL. The cells were then cultured in a chemically defined media with or without TGF- $\beta$ 3 (CM+ or CM-). At time points of weeks 0, 1, 3, 6, and 9 the constructs were tested for biochemistry mechanical properties, and with histology. Constructs at each time point were tested for biochemistry after mechanical testing. The sGAG amount was tested with dimethylmethylene blue dye binding. The collagen amount was tested with hydroxyproline assay. The DNA was tested using PicoGreen. Three constructs were tested with an unconfined compression device from our lab at each time point to find the modulus of elasticity for constructs. One construct was fixed to 4% paraformaldehyde inside of paraffin wax used for histology at each time point. Three constructs were tested with the microscope straining device seen in Figure 1. The device has a micrometer for precise adjustments to the linear stage, load cell that can measure between  $\pm 250$ gF with precision of 0.1gF, cover slip to lay the construct, water tight PBS bath, and two platens to strain the construct. The construct was cut in half and laid on its cut portion so that the cells could be imaged by the microscope. The layout of the construct can be seen in Figure 2. The constructs were imaged at strains from 0% to 20% by increments of 4%. This means that the constructs were strained five times with 80 seconds of straining and 500 seconds of stress relation before the load reading was taken. Images were taken using a Nikon florescent microscope of the Hoechst die stain constructs while operating the microscope straining device. For more details on the microscope straining device protocol see Appendix A. The images were then stitched together using a program called Autostitch. The protocol for Autostitch can be seen in Appendix B. The stitched images were then analyzed using Vic-2D which texture-maps and compares a reference image for each sample to a strained image. This analysis technique allowed for the displacements and strains of the stress to be compared between the time points and between Chondrocytes and MSCs. In other words this method allowed for the study of the ECM by viewing the cells' strain.



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over

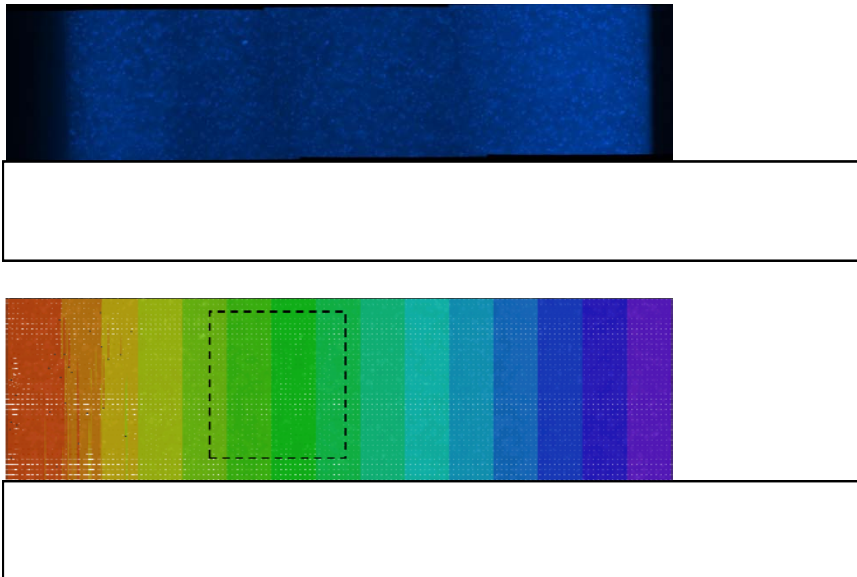
In the second part of the study, similar constructs were analyzed using confocal microscopy. Strained and unstrained constructs were imaged with a 60x objective. The images were then analyzed with a customized Matlab program seen in Appendix C. The program threshed the image via the light intensity, area size, and eccentricity. The light intensity was threshed using the Otsu method which makes an image binary by assigning each grayscale pixel a 1 or 0 in a way that minimizes the variance of each class. The area was threshed via a minimum threshold and by an upper and lower standard deviation. The eccentricity is the distance between the foci divided by the length, which was useful in filtering out the scale bar in images and non cells that were essentially lines. These methods eliminate non-cells, cells that were touching, and cells on the edge of the image. The program then outputted each of the region's orientation, major axis (length), minor axis (width), eccentricity, and aspect ratio. It is important to clarify that the major axis is

the longest axis of the ellipse, the minor axis is the width of the ellipse perpendicular to the major axis, and the orientation is the angle in degrees between the positive x-axis to the major axis. It is also important to define eccentricity as the distance between the foci of the area regions divided by the length and aspect ratio is length divided by width. This analysis method allowed for the cell deformations to be analyzed at different time points as the ECM developed.

#### 4. RESULTS

NOTE: All the results are not present because the experiments have not been completed.

The results will include a comparison between bulk mechanical properties to local mechanical properties of chondrocytes and MSCs at the time points of 0, 1, 3, 6, and 9 weeks. An example of an Autostitched hoescht stained image at 10x can be seen in Figure 3. Images like this exist for each of the strain increments for each fluorescent microscope imaged construct. The strained images were then tested with respect to an unstrained image from the construct. An example of an analysis of the strain of a construct can be seen in Figure 4. Images like this will allow the bulk properties of chondrocyte and MSC constructs to be analyzed. Figure 5 then shows that the displacement across the construct in Figure 4 is uniform across the construct. Figure 6 shows that the local displacements in the construct are not uniform from right to left even though Figure 5 showed a uniform displacement. Comparing these two types of images for chondrocytes and MSCs at various time points will allow us to compare local versus bulk properties.



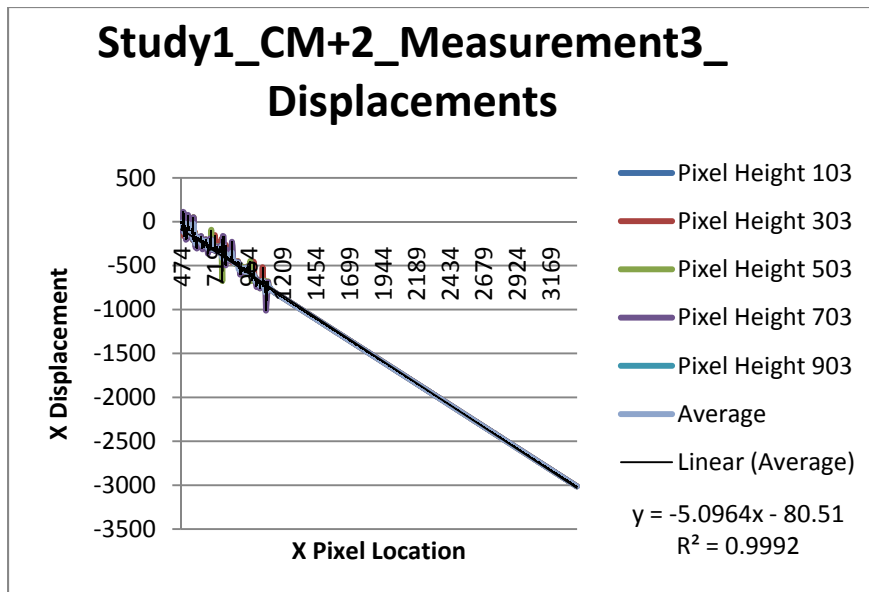


Figure 5: Analysis of Figure 4 at different y-pixel heights showing that the x-displacement across the construct is linear.

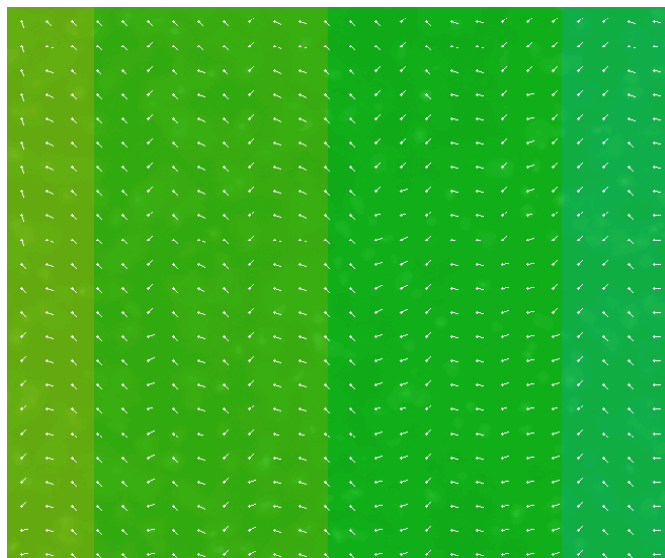
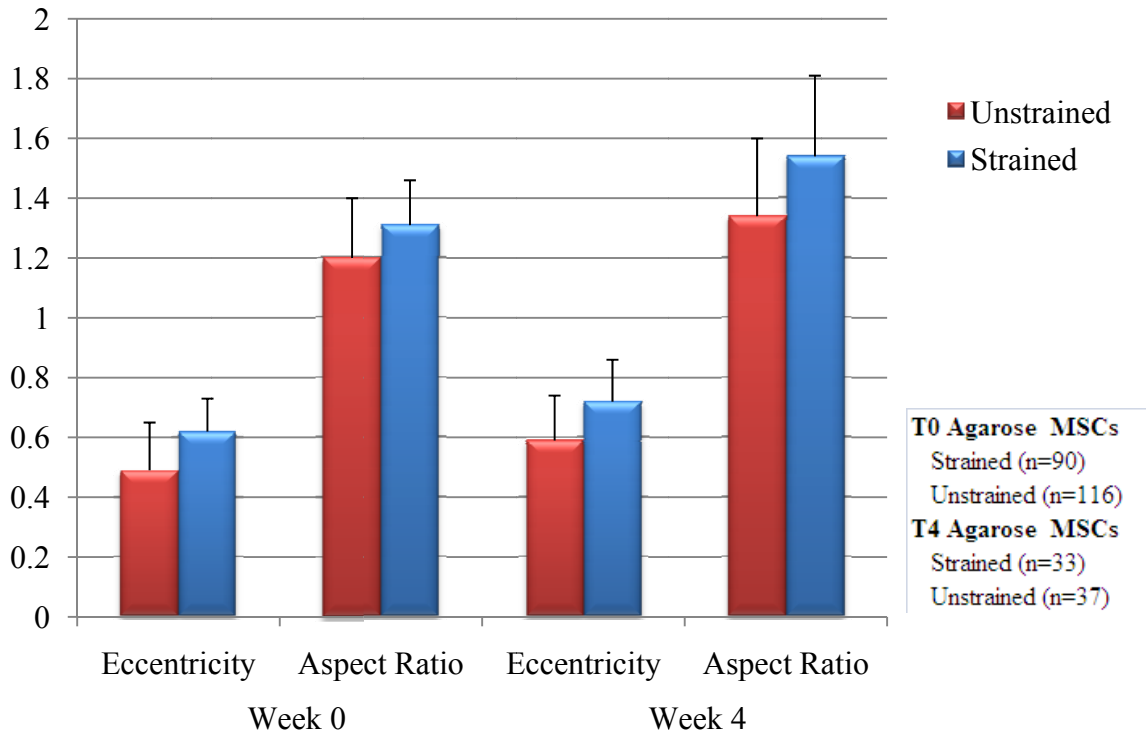


Figure 6: A zoomed in image of Figure 4 with the arrows showing the direction of maximum displacement.

The data in this section will allow us to assess cell deformation as a function of matrix production over time. There will be the average eccentricities, aspect ratios, and orientation of cells at various time points using the confocal microscope and the customized Matlab program in Appendix C. An example of a completely analyzed image sample can be seen in Appendix D. Figure 7 then shows the cumulated data for many analyzed MSC agarose constructs from weeks 0 and 4.





## 5. DISCUSSION AND CONCLUSIONS

The results from the first part of the study with the microscope straining device will allow for comparing the local and bulk micromechanical properties of MSCs to chondrocytes. The local properties will be observed with the help of Vic-2D and the bulk properties will be observed with the help of an unconfined-compression device. Comparing the local properties to the bulk properties will hopefully explain why the MSCs have a lower module of elasticity than the chondrocytes.

The results from the second part of the study will allow for assessing the cell deformation as a function of matrix production over time. This portion of the study will also show the effects of stress shielding. Stress shielding normally occurs within weeks 0 and 8 weeks when the ECM protects the cells from deformations. The ECM prevents the cells from deforming because the ECM is stiffer than the agarose in which the cells reside. This is of interest because stress shielding prevents the cells from receiving some of the loading signals during mechanical stimulation in bioreactors. Loading signals are extremely important with cartilage and MSCs because they tell the cells to produce

different proteins. This portion of the study will also give insight into the experience of the deforming cells during mechanical stimulation.

## **6. RECOMMENDATIONS**

I recommend that in the future the florescent microscope straining device be redesigned. The platens should be flush to the bottom of the PBS bath so that soft constructs do not seep between the platens and cover slip. The micrometer should be motorized so that there is no human error with adjusting the strain on the constructs. Adjustments to the PBS bath should be made so that it is easier to place the constructs in between the platens. The load cell on the microscope straining device should be attached to a DAQ so that the readings are accurately recorded. This redesigned device will be more precise and allow for more representative comparisons between time trials and samples.

I recommend that in the future a confocal microscope straining device be created. Currently the strain is placed on the construct by placing the construct in between two slides and squeezing the construct with a rubber band. A device similar to the florescent microscope straining device should be made for the confocal microscope so that the strain can be adjusted during imaging. This device would allow for tracking cells while they are strained and during stress relaxation.

## **7. ACKNOWLEDGMENTS**

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  - a. [10 Vogel]

## APPENDIX A: MICROSCOPE IMAGING PROTOCOL

This protocol is utilized to study how MSCs and cartilage cells grown in constructs deform under strain at various time points. The time points will be weeks 0, 1, 3, 6, and 9. At each time trial there will be three CM- and three CM+ constructs tested.

### Step A: Hoescht Stain

1. Measure the diameter and thickness of the construct.
2. Cut the construct in half across the diameter.
3. Measure the radius of each of the halves.
4. Place the two halves of the construct in separate capsules
5. Add 1mL of PBS to each capsule.
6. Add 2 $\mu$ L of Hoescht stain to the capsules.
7. Wait 30 minutes for stain to set. Wrap the capsules in aluminum foil.
8. Remove the stain from the capsules via pipette.
9. Add 1mL of PBS to the capsules. Wrap the capsule in aluminum foil.

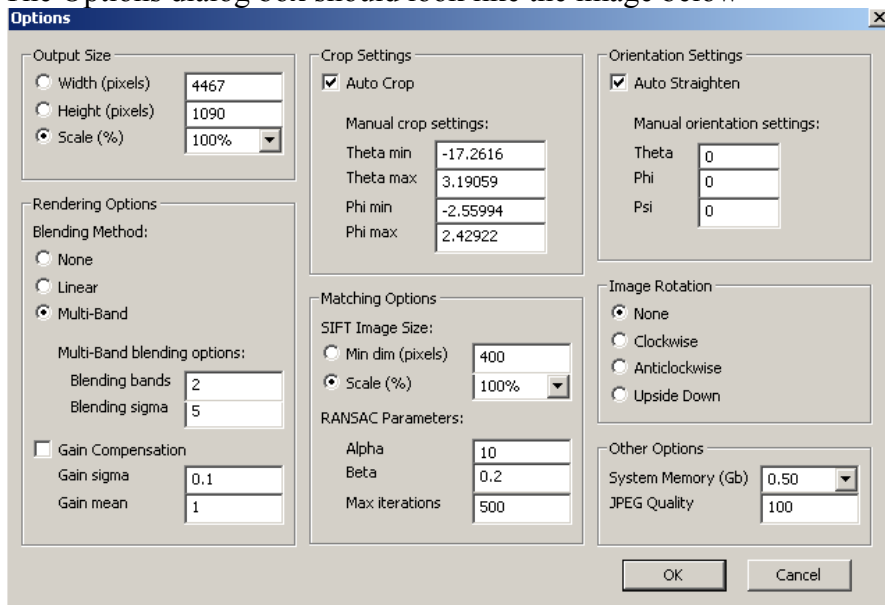
### Step B: Microscope Imaging

1. Fill the microscope straining device's (MSD's) water bath with PBS.
2. Lay the cut edge of the construct in the MSD.
3. Screw the MSD to the microscope stage.
4. Plug in the load cell display.
5. Record the load cell zero.
6. Screw the micrometer so that the plungers just touch the construct.
  - a. This is done by adjusting the micrometer until the load reading decreases below the load cell zero.
7. Take a "Series of Pictures" to get reference images
  - a. At 10x take Florescent DAPI setting images across the construct.
    - i. Adjust the exposure time of the microscope each time the magnification of the microscope or displacement of the construct changes by clicking Auto Exposure in the middle of the construct.
    - ii. Ensure that there is overlap of 1/8<sup>th</sup> of the image after each time the microscope stage is moved so that the image stitching software can operate effectively.
  - b. At 2x take a Florescent DAPI setting image of the construct.
  - c. At 2x take a Phase image of the construct.
    - i. Do not turn off the lamp until the end of the experiment.
  - d. After all the pictures, block the light source so that photo bleaching is prevented.
8. Adjust the micrometer by 0.10mm over an 80 second time period, which is approximately 4% strain.
9. After 8 minutes 20seconds from adjusting the micrometer, record the load cell reading.
10. Take another "Series of Pictures"
11. Repeat steps 8-10 five times (until 20% strain is achieved) this means that there are a total of 6 image series

## APPENDIX B: AUTOSTITCH IMAGING PROTOCOL

This protocol is used to prepare the images from the Microscope Imaging Protocol for Vic-2D analysis. The directions must be followed so that the images maintain the highest resolution possible and to allow for accurate Vic-2D analysis.

1. Download the Windows Demo Version of Autostitch from [www.cs.ubc.ca/~mbrown/autostitch/autostitch.html](http://www.cs.ubc.ca/~mbrown/autostitch/autostitch.html)
2. Close all other programs. This program takes a lot of memory when working with large image files
3. Click *Edit* and then *Options*
  - a. Under *Output Size*-select *Scale %* and change the drop down menu to **100%**
  - b. Under *Matching Options* and *SIFT Image Size* and change the drop down menu to **100%**
  - c. Under *Other Options* change *System Memory (Gb)* to **.50**
  - d. Under *Other Options* change *JPEG Quality* to **100**
  - e. The Options dialog box should look like the image below



4. Click *File* and then *Open*
  - a. Open images (.jpg) in order that you want to stitch together
5. Save the stitched image for Vic-2D analysis.
6. Trouble shooting
  - a. If the stitched image does not look correct then retry step 4

## APPENDIX C: CONFOCAL IMAGING ANALYSIS CODE

```
%-----  
%Jeffrey Perreira  
%UPENN ORL  
%Strain Analysis  
%This program takes a jpg, thresholds areas by statistics, thresholds  
%eccentricities, finds statistics on cells, saves the image file, and  
%writes statistics to a specified XLS file in a sheet named the image name  
%StrainAnalysis_07-28-09_BlobAnalysis_Methods.m  
%-----  
clc  
clear all  
close all  
%-----  
%Step 1: Modify Each Time  
  
%Specify File to Write To  
XLSfilename='t0 1% HA';  
XLSfilename=char(XLSfilename);  
  
%SPECIFY METHOD  
%Column 1: Intensity Threshold  
%Column 2: Minimum Area  
%Column 3: Lower Area Std.  
%Column 4: Higher Area Std.,  
%Column 5: Eccentricity Threshold  
%Column 6: Region definition for Area threshold  
%Column 7: ConvexArea Threshold  
  
%Method 1:  
Method(1,:)=[1.000, 50.000, 2.000, 2.000, 1.000, 8.000, 5.000];  
%Method 2:  
Method(2,:)=[1.500, 90.000, 1.000, 2.000, 1.000, 4.000, 5.000];  
%Method 3: Same as Method 1 with eccentricity threshold  
Method(3,:)=[1.000, 50.000, 2.000, 2.000, 0.925, 8.000, 5.000];  
%Method 4: Same as Method 2 with eccentricity threshold  
Method(4,:)=[1.500, 90.000, 1.000, 2.000, 0.925, 4.000, 5.000];  
%Method 5: Best blob analysis method as of 7/11/09  
Method(5,:)=[1.250, 90.000, 1.000, 3.000, 0.925, 8.000, 1.000];  
%Method 6: Created for Greg's microsphere analysis  
Method(6,:)=[1.000, 40.000, 1.000, 5.000, 0.925, 8.000, 1.000];  
  
%Select a method  
M=Method(5,:);
```

```

%-----
% STEP 2: OPEN FILE

% open file prompt
[filename, pathname, filterindex] = uigetfile('*.jpg', 'Open Image File');

% if user hits cancel, exits script
if filterindex == 0
    returnL
else
    name = strcat(pathname,filename)
end
% open the file
img = imread(name);

%Find image pixel height and width
pictureSize=size(img);
pictureW=pictureSize(2);
pictureH=pictureSize(1);

%-----
% STEP 3: EDIT IMAGE

% Create grayscale image
img = rgb2gray(img);
figure(1)
imshow(img);
title('Grayscale')

% Create black and white image
% Create a threshold level
level = M(1)*graythresh(img);
%Instances of 1 are the cell, 0 is the background
img = im2bw(img,level);
figure(2)
imshow(img)
title('Thresholded Using Otsu Method')

%Threshold image's small areas
%M(6) for either 8 or 4
img2=bwlabel(img,M(6));
Area0=regionprops(img2,'area');
indxb = find([Area0.Area] > M(2));
img3 = ismember(img2,indxb);
figure(3)
imshow(img3);

```

```

title('Eliminated Bottom Areas')

%Histogram of Areas
img4=bwlabel(img3,8);
Area0=regionprops(img4,'area');
Area1=struct2cell(Area0);
Area1=cell2mat(Area1);
StandardDev=std(Area1);
Average=mean(Area1);
y=hist(Area1,20);
MIN=min(Area1);
MAX=max(Area1);
x=(MIN+(MAX-MIN)/20):(MAX-MIN)/20:MAX;
figure(4)
bar(x,y)
title('Histogram of Areas')
xlabel('Areas')
ylabel('Quantity of Occurances')

%Upper and Lower Bounds
%Lowerbound area
indxl = find([Area0.Area] > Average-M(3)*StandardDev);
img5 = ismember(img4,indxl);
figure(5)
imshow(img5)
title('Eliminated Lower Standard Deviation')
%Upperbound area
img6=bwlabel(img5,8);
Area0=regionprops(img6,'area');
indxu = find([Area0.Area] < Average+M(4)*StandardDev);
img7 = ismember(img6,indxu);
figure(6)
imshow(img7)
title('Eliminated Upper Standard Deviation')

%Eliminate cells on border of image
img8=bwlabel(img7,8);
indx=[img8(1,:),img8(pictureH,:),img8(:,1),img8(:,pictureW)];
indx=sort(indx,'ascend');
indx=unique(indx);
img9 = ~ismember(img8,indx);
figure(7)
imshow(img9)
title('Eliminated Border Areas')

%Eliminate cells with high eccentricities

```



```

img10=bwlabel(img9, 8);
Eccentricity0=regionprops(img10, 'eccentricity');
indx = find([Eccentricity0.Eccentricity] < M(5));
img11 = ismember(img10,indx);
figure(8)
imshow(img11)
title('Eliminated High Eccentricities')

%Eliminate cells with holes, abnormal outshoots, or multiple cells
img12=bwlabel(img11, 8);
ConvexArea0=regionprops(img10, 'ConvexArea');
Area0=regionprops(img10, 'Area');
indx = find([ConvexArea0.ConvexArea] < [Area0.Area]*M(7)*4/pi);
img13 = ismember(img12,indx);
figure(9)
imshow(img13)
title('Eliminated via ConvexArea')

%-----
%STEP 4: ANALYZE BLOBS

%Label each cell with a different number
img14=bwlabel(img13,8);
Area=regionprops(img14,'area');
Image=img14;
imshow(Image);

%Regionprops measures a set of properties for each labeled region
%Length in pixels of major axis
Length=regionprops(Image,'majoraxislength');
Length=[Length.MajorAxisLength];
%Length in pixels of minor axis
Width=regionprops(Image,'minoraxislength');
Width=[Width.MinorAxisLength];
%Eccentricity is the ratio of the foci and the major axis length. 0=circle
Eccentricity=regionprops(Image,'Eccentricity');
Eccentricity=[Eccentricity.Eccentricity];
%Orientation is the angle (degrees) between the x-axis and the major axis
Orientation=regionprops(Image,'Orientation');
Orientation=[Orientation.Orientation];
%Aspect Ratio is the long side divided by the short side
AspectRatio=Length./Width;%
% %-----
% %Step(5)
%
%Consolidate data

```

```
SUM(:,1)=Length;
SUM(:,2)=Width;
SUM(:,3)=Eccentricity;
SUM(:,4)=Orientation;
SUM(:,5)=AspectRatio;

%Print data to file
characters=size(filename);
characters=characters-4;
XLSsheet=filename(1:characters(2));
xlswrite(XLSfilename,SUM,XLSsheet);

%Print image to a .xsl file
s=' Matlab.jpg';
s=char(s);
AfterImageFilename=[filename(1:characters(2)),s];
imwrite(Image, AfterImageFilename, 'jpg');
```

## Appendix D: Matlab Imaging Analysis Example

Appendix D shows an example analysis of a confocal image utilizing the customized Matlab program in Appendix C. Figure 8 shows a strained MSC construct at week 0 in 2% Agarose gel. Figure 9 shows the outputted Matlab image after Figure 8 has been analyzed by Matlab. Figure 10 labels the cells seen in Figure 9. The program then outputs the length, width, eccentricity, orientation, and aspect ratio of each cell as seen in Table 1.

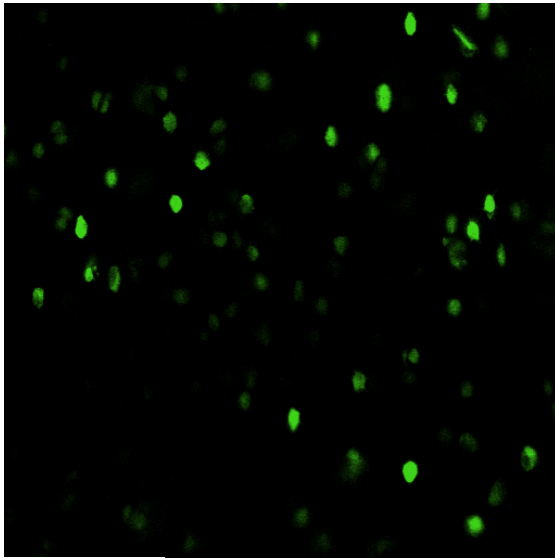


Figure 8

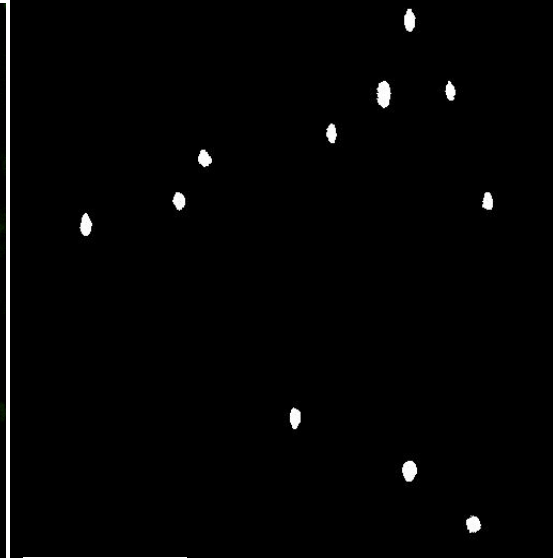


Figure 9

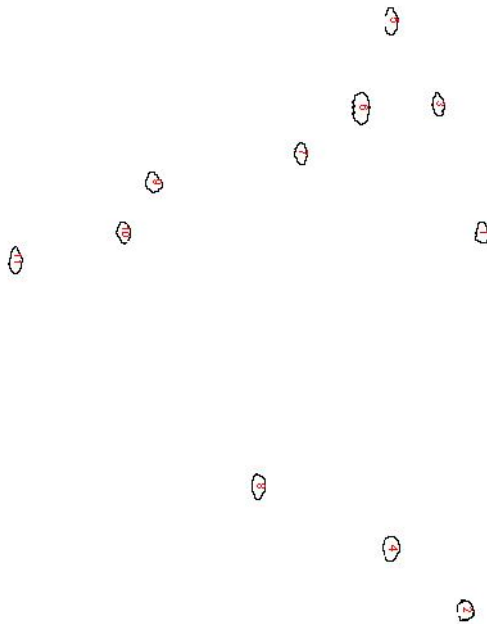


Figure 10

Table 1

Cell #	Length	Width	Eccentricity	Orientation	Aspect Ratio
1	20.67	10.57	0.86	89.86	1.96
2	16.25	11.31	0.72	82.22	1.44
3	15.61	12.00	0.64	69.42	1.3
4	19.29	10.29	0.85	89.42	1.87
5	17.95	9.29	0.86	87.39	1.93
6	25.02	12.87	0.86	89.99	1.94
7	19.2	13.67	0.7	87.25	1.4
8	20.84	10.29	0.87	89.19	2.03
9	17.58	8.93	0.86	83.25	1.97
10	15.38	13.62	0.46	77.78	1.13
11	16.8	9.89	0.81	88.34	1.7
Average	18.60	11.16	0.77	84.92	1.70
Standard Deviation	2.84	1.67	0.13	6.43	0.32