6-2013

Studying the Material Properties of the Embryonic Chicken Heart

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Studying the Material Properties of the Embryonic Chicken Heart Using Atomic Force Microscopy

By

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Submitted in partial fulfillment of the requirements for Honors in the Department of Bioengineering

UNION COLLEGE

June, 2013

ABSTRACT

BURNETT, KYRA Studying the Material Properties of the Embryonic Chicken Heart Using Atomic Force Microscopy. Department of Bioengineering, June 2013.

ADVISOR: Professor Ramasubramanian

Congenital heart disease is the number one cause of birth defects in the United States, affecting approximately 40,000 newborns each year. Research has shown that serious structural defects of the heart can occur due to abnormal looping. Looping refers to the positional and morphological changes of the early embryo heart and is divided into different phases. Although progress has been made in identifying the genetic processes behind heart looping, the forces involved with the developmental process are still not fully understood. In this research, the chicken embryo heart was used as the experimental model and certain material properties were characterized. The Atomic Force Microscope was used to measure tissue stiffness at various locations of the heart tube. These measurements can be used to test for the presence of cytoskeletal contraction and to develop a computer model for the looping heart.

INTRODUCTION

According to the Center for Disease Control and Prevention, congenital heart disease is the number one cause of birth defects in the United States, affecting approximately 40,000 newborns each year. Today, one million adults have congenital heart defects. Research has shown that serious structural defects of the heart can occur due to abnormal looping. Although progress has been made to identify the genetic processes behind heart looping, the forces involved with the developmental process still remain poorly understood. Professor Ramasubramanian's lab does research on the forces involved with the looping process of the heart. The lab does experiments on chicken embryo hearts because they are parallel to that of human's.

Looping refers to the positional and morphological changes of the early embryo heart. This period is divided into four phases; the pre-looping phase, the dextral looping phase, the phase of transformation of a c-shaped heart loop to an s-shaped heart loop, and the phase of late positional changes of the heart. Once the embryo is incubated for 36 hours, the heart forms the shape of a straight tube, which is shown in Figure 1A . During the dextral looping phase, the ventral surface of the tube becomes the outer curvature of the c-loop, and the dorsal side of the tube becomes the inner curvature of the c-loop. This bend is shown in Figure 1B. This process occurs 36 hours to 48 hours after incubation and is referred to as c-looping. During the transformation of the c-shaped heart loop to an s-shaped heart loop, the atrium moves superior to the ventricle. This process occurs 48 to

56 hours after incubation and is referred to as s-looping, which is shown in Figure 1B though Figure 1D. Even though the forces involved with c-looping have been well researched, less is known about the forces involved with s-looping.

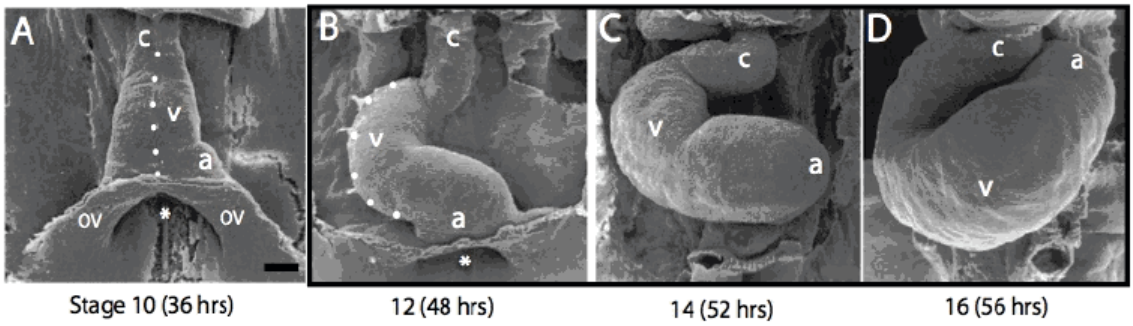

Figure 1 (A) 36 hours of incubation, which is the initiation of c-looping (B) 48 hours of incubation, which results in the final c-loop (C) 52 hours of incubation, which is midway though s-looping (D) 56 hours of incubation, which results in the final s-loop. In each stage c = conotruncus, v = ventricle, a = atrium, and ov = omphalomesenteric veins. Scale bar =100 μm. All images are from Manner (2000).

There are many approaches to studying the development of the heart. In this study, the heart is treated as a material, therefore it is important to understand its key mechanical properties. A mechanical property that our lab is interested in is the strength of a material. By definition, the strength of a material is the ability of the material to withstand forces without breaking or failing. There are a variety of different types of forces that can be applied to materials, however, each of them cause different stresses or strains on the material. The stress of a material is defined by the amount of force applied to the material divided by the cross sectional area of the material, while strain is the change in shape and size of the material over the original material's shape and size. When a force is applied to

a material, the material will develop an internal resistance to that applied force. The magnitude of this internal resistance is dependent upon the stiffness of the material, which is an important value that is studied.

Stress versus strain curves are used commonly to find the stiffness of a material by determining their slopes. Different types of materials will produce different types of curves. For example, the stress-strain curve for a material like copper is shown in Figure 2. This curve can be divided into two sections, separated by the yield point; the linear curve is the elastic region and the downwards shaped curve is the plastic region. In the elastic region, the material will deform during loading and return to its original shape when the load is removed. In the plastic region, the material will deform during loading, and when the load is removed the material does not return to its original shape. A plastic deformation results in permanent change of the material because the material's molecular bonds are fractured. For a material like the heart, the stress-strain curve that is represented is a positive exponential curve and does not exhibit properties that are in the plastic region. However, the curve indicates that these materials are initially compliant and become progressively stiffer as the load is increased. This behavior can be compared to stretching a rubber band; initially it is very easy to deform, but it becomes much stiffer as elongation increases.

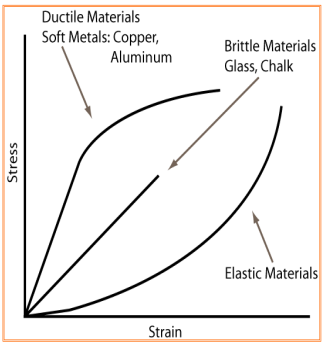

Figure 2. The stress-strain curves for different categories of materials. The chicken embryo heart can be categorized in the elastic materials.

Not only is the heart categorized as an elastic material when a force is being applied to it, but it also falls into the category of being a viscoelastic material. By definition, a material that returns to the original shape yet takes a longer time to restore to its original position. Unlike an elastic material that has the same slope when the material loads and unloads, a viscoelastic material will load and unload in different ways when energy is released from the material. Residual stresses, stresses that remain in the material after an applied force has been removed, are common for viscoelastic materials. Viscoelastic materials also have more stiff properties when an applied force is loaded fast and less stiff properties when the applied force is loaded slowly. Understanding the viscoelastic factors of the heart is important when analyzing the stiffness of the heart.

To find the stiffness of the chicken embryo heart, an Atomic Force Microscope (AFM) was used. There are three main components to this instrument: the microscope stage, the control electronics and the computer. The microscope stage consists of the piezoelectric scanner, the sample holder and the force sensor. The piezoelectric scanner on the AFM moves the sample holder over the sample surface, while the force sensor

senses the force between the sample holder and the sample surface. On the sample holder is a cantilever that has a nanometer sized tip used for scanning images at the atomic level. The control electronics generate signals to drive the scanner and digitalized signals coming back from the AFM. Lastly, the computer is used to display images and set specific parameters on the AFM software. Not only can the AFM scan topographical images, it can also produce force-displacement curves by indenting the heart with the tip of the AFM probe. From this method, slopes are obtained from the force-displacement curves that equal the stiffness of the material being studied.

By determining the stiffness value of the heart, the value will ultimately help improve the computer model shown in Figure 3 that replicates the s-looping phase of cardiac looping. The finite element model has certain values that are controlled, and obtaining a more accurate stiffness value to the heart will make our model more precise. Recent studies have also shown that c-looping hearts have areas that undergo cytoskeletal contractions that are stiffer than non-contracting areas. Therefore, we can improve the model further by determining the different stiffnesses in various areas of the heart and applying those numbers to our initial parameters. Finding multiple stiffness values, instead of setting the model to only a uniform stiffness, will make our model heart more accurate.

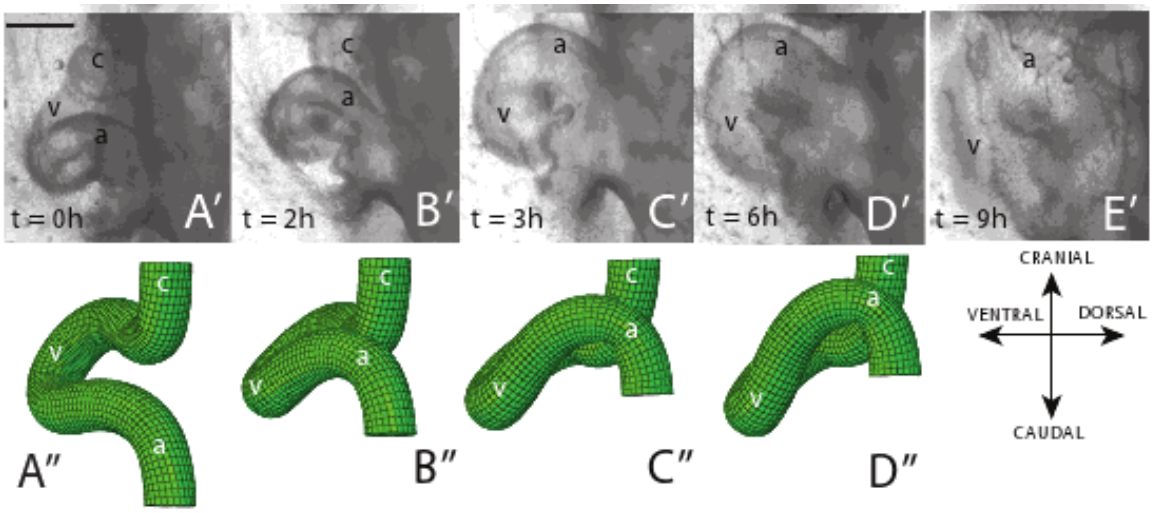

Figure 3. Morphological changes during s-looping. (A'–E') Embryo hearts at time points indicated. (A''-D'') Finite element model of embryo hearts for time points corresponding from (A'-E'). v = primitive ventricle, a = common atrium, c = conotruncus. Scale bars: 400 µm.

METHODS

Before force spectroscopy was used on the AFM, the sample was imaged using topographical modes. There were two possible ways to prepare the heart samples; the first method involved using live embryos and the second involved isolated heart culture. In the first method, the embryo sat in a plate of ager and the AFM's contact mode was used to scan images across the embryo. The advantage to this method was that the heart was in its most natural setting when being indented, while the disadvantage was getting the probe to indent the surface of the heart. Naturally, the splanchnopleure lies anterior to the heart and needed to be remove for the AFM probe to scan the actual surface of the heart. The second method involved isolated heart culture. In this method, the heart was cut away from the embryo and placed on a glass slide. The advantage of this method was

learning that the sample only contained heart tissue, while the disadvantage was the way the heart was secured onto the slide. With this method, the heart dried on the glass slide without any chemical interactions. Relying on the material-surface interaction to be strong enough to withstand the probe moving across the heart during imaging was a major disadvantage.

For this study, the method used involved the isolated heart cultures. When taking topographical images, non-contact mode was the best mode because impacted the surface of the sample in a less forceful way than contact mode. In non-contact mode, the tip of the probe oscillated a certain distance away from the surface of the sample and was not always in direct contact with the sample's surface. Instructions to scan images of samples on the AFM using non-contact mode are found in the Appendix section. Basic Force Spectroscopy was also used to find force curves that could ultimately determine the stiffness values of the heart. Instructions for cantilever based indentations are also found in the Appendix section.

The techniques used to first harvest and then isolate the hearts from the chicken embryos were similar to the methods described by Manning and McLachlan. The chicken eggs were incubated for two days at 38 degrees Celsius. The eggs were then harvested so that they could be prepared for isolated heart culture. The eggs were first cracked in a glass petri dish under a sterile hood so that the heart was not exposed to anything in the environment that might affect its survival. Once the egg was cracked, the albumen was removed. Filter papers with holes were then put on top of the yolk where the embryo was. The filter paper was then removed and cleaned off with Phosphate Buffer Saline (PBS).

The filter paper and embryo then went into a smaller petri dish with PBS. A metal ring was placed over the filter paper and embryo to hold them down. In the experiments, the hearts were removed from the embryo and were put on a glass slide. The removal of the heart occurred in several steps. All hearts first had the splanchnopleure removed with small forceps, and the dorsal mesocardium severed with a glass micropipette. The heart was cut on the omphalomesenteric veins (located at the caudal end) and on the outflow tract (located at the cranial end) using mircoscissors. This process was done when the heart was in a C-shape, which was 48 hours after incubation. Because the isolated heart needed the rest of the embryo to keep it functioning, the experiments were done within 25 minutes after the heart was removed from the embryo.

When the heart dried on the glass slide, the surface interaction between the heart and the glass was extremely strong and the heart stayed in place while the tip of the cantilever was moving over the sample. If this technique moves the sample in the future, agar will be put on the slides to prevent the sample from sliding. The ager can be helpful to use because it supplies the heart with nutrients and provides mechanical support for the heart.

RESULTS

As of now, only the Asylum Research Calibration Sample has been imaged using the AFM, which is shown in Figure 4. For future work, the isolated Stage 12 hearts placed on glass sides will be scanned. Once the biotech equipment is added to the AFM, scanning liquid samples will be possible, which means the heart attached to the embryo can be

scanned.

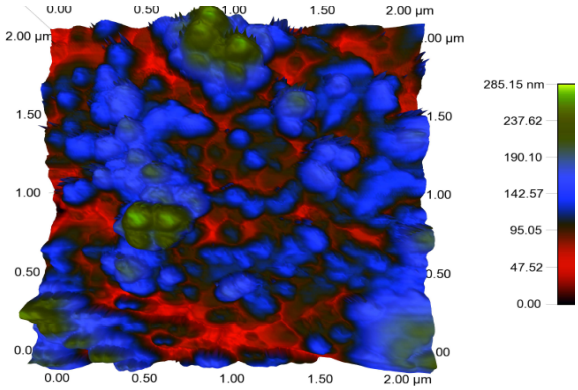

Figure 4. The AFM scan of a the Asylum Research Calibration Sample using non-contact mode. Coloring is by height, low to high is black/red to green.

DISCUSSION

Knowing the stiffness of the heart was important because the material stiffness of the cantilever probe had to be equal to the stiffness of the heart for the AFM scanning to properly work. In a case where the tip of the probe on the cantilever was too stiff, compared to the heart, the heart would have indented a large amount. This would have caused no deflection in the cantilever tip. In a case where the probe tip was too soft, the heart would not be indented, which would have caused too much deflection in the probe tip. Because the chicken embryo heart is estimated to have a stiffness of 0.06 N/m, the probe that will be used for scanning and indenting the heart is the TR400PSA, which has

a spring constant range of 0.02 N/m – 0.08 N/m. Because these probes are very flexible, they have to be used in liquid samples to be stabilized. However because the isolated heart samples attached to the glass slide were dry samples, a probe with the spring constant of 40 N/m was used so that an interaction between the tip of the probe and the heart could be made. In future studies, indentations of the heart should be done with the TR400PSA probe in PBS and the sample should be set up using the whole embryo method described previously in the Method section.

During the experiments, the probes used had pyramidal shaped tips and issues occurred due to their shape. The problems that arose were that the sharp tips easily damaged the surfaces of the sensitive cells and they did not evenly distributed forces when they were applied to the cells of the heart. To prevent damage to the cell, experiments can be done on probes that have bulbs attached to them that are 20 um. Because the heart is 400 um in length, the bulbs attached to the tips will evenly distributed enough force onto an area of the heart so that the heart tissue will not get damaged.

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APPENDIX

AFM AC AIR INSTRUCTIONS

1) Turn the Controller Box on

2) Start up Asylum Research software

3) Choose Mode Master-Simple-Topography Air

4) Put the tip on the cantilever

    a) Loosen Middle Screw

    b) Slide probe in using tweezers

    c) Don’t push probe all the way in

    d) Tighten screw (1/4 more after resistance)

5) Put cantilever holder into head

6) Put the head onto the stage

    a) Move legs all the way up before putting head on

    b) Check stage to see if it is aligned properly

7) Head security check

8) Turn on fiber optic light

9) Turn on camera (icon)- Choose input-composite “upper one”- the camera will now be in view

10) Laser Alignment

a) Maximize Sum- to approximately 7.0 units

b) Deflection = 0

11) Auto Tune (resonant frequency of cantilever)

a) Tune tab: Low-50k, High- 400k

b) Target Amplitude- 1.00 V, Target Percent- -5.0%

12) Soft Engage (Main Tab)

a) Set Point = 950 mV; Gain = 10, Scan Size = 2.0 um

b) Click engage (Z voltage should shoot up) – move Z voltage down until ding sound (0.95)- Keep tuning until Z-voltage is at 30 V (left)

c) If wavering z voltage- increase drive amp by +1 V.

13) Close lid

14) Set Point

a) Lower the set point using the hamster wheel

b) The phase should “jump” and be at least 700mV

15) Do Scan!

16) To fix all scales, right click before image comes into view

17) Once load and unloading lines are produced below the graph, lower set point between values of 500-700V

18) “Slow Scan disabled”

a) Make lines closer to each other by changing highlighted portions in the Main Tab

b) My recommendations- lower set point (make sure it is between 500-700V), lower gain, raise driver amplitude

AFM CANTILEVER BASED INDENTATIONS

1) Turn the Controller Box on

2) Start up Asylum Research software

3) Choose Mode Master-Simple-Topography Contact Mode

4) Put the tip on the cantilever

a) Loosen Middle Screw

b) Slide probe in using tweezers

c) Don't push probe all the way in

d) Tighten screw (1/4 more after resistance)

5) Put cantilever holder into head

6) Put the head onto the stage

a) Move legs all the way up before putting head on

b) Check stage to see if it is aligned properly

7) Head security check

8) Turn on fiber optic light

9) Turn on camera (icon)- Choose input-s video- the camera will now be in view

10) Laser Alignment

a) Maximize Sum- to approximately 7.0

b) Deflection = -0.2

11) Soft Engage (Main Tab)

a) Set Point = -0.19 V; Gain = 10, Scan Size = 2.0 um

b) Click engage (Z voltage should shoot up) – move Z voltage down until ding sound - Keep tuning until Z-voltage is at 70 V

c) If wavering z voltage- increase drive amp by +1 V.

12) Close lid

13) Force Tab

a) Move force bar up (bar located on the left of the tab)

b) Velocity = 1.0 um/s ; Trigger Point = 0.5 nN; Force Distance = 1um

c) Trigger Options are set to Positive and Relative

d) Trigger set to 'Deflvolts'

e) Single Force

14) Set Sensitivity- Virtual Def

a) Obtain markers by pressing “Ctrl and I” and placing them on the straight red line

b) Right click- Choose Virtual Def line- Fit Stays- Remove Fit

c) Record Virtual Def #

15) Set Sensitivity- InVols

a) Single Force

b)Trigger is set to ‘InVols (z sensor)’

c) Obtain markers by pressing ‘Ctrl and I’ and placing them on the red slope

d) Record Defl Invols #

16) Withdraw & back away from the surface (approximately 4 spins)

17) Deflection = 0

18) Thermal Scan

a) Obtain markers by pressing “Ctrl and I” and placing them on the peak

b) Set Frequency value to Zoom Center value

c) Check off Show Fit box

d) Initialize Fit (repeat step c and step d twice)

e) Fit Thermal Data and Record Spring Constant #