Effects of Cross-link and Myosin Motor Concentrations on Active Muscle Gel Contraction Time and Extent

by

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Submitted to the Department of Mechanical Engineering in Partial Fulfillment of the Requirements for the Degree of Bachelor of Science in Mechanical Engineering at the Massachusetts Institute of Technology

June 2017

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ABSTRACT

The cytoskeleton is a crucial network of actin filaments that gives the cell its shape, assists in organelle organization, and allows for cell movement. Active muscle gels are a class of materials that that mimic the functionality of the cytoskeleton. Utilizing myosin II motor proteins to initiate contraction events in actin networks, active muscle gels have the unique potential of acting as microscopic actuators. Two challenges currently faced by active muscle gels are their slow contraction time and weak contraction forces. This thesis seeks to achieve contraction events in a lab setting and observe how contraction speed and extent varies with the concentration of myosin motors and alpha-actinin crosslinks.

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Acknowledgments

Firstly, I would like to thank my advisor Dr. José Alvarado for his wisdom and guidance throughout my thesis. Without his insight and willingness to answer my abundance of questions, these experiments would not be possible.

I would also like to thank Prof. Anette Hosoi for agreeing to be my thesis supervisor and helping me throughout this process. She has created an excellent, collaborative lab environment that stimulated and motivated me throughout my time there.

Lastly, I must underscore the impact my parents had on this thesis. I thank them for their continual support throughout my undergraduate studies, without which I would not have been able to even make it to this stage of my education. I truly appreciate all they have done for me throughout my education.
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1. Introduction

1.1 Motivation

Microscopic actuators have been long sought after for the purpose of performing tasks on a scale much smaller than a DC motor. Currently, most microscopic actuators are MEMS, microelectromechanical systems, which combine microscopic mechanical systems with electronics. The single-crystal silicon array actuator (Bohringer et al., 1994) and the graphene/G-0 papers actuator (Bi et al., 2013) are examples of such systems. However, these systems are often expensive, difficult to manufacture, and require an electric power source.

Meanwhile, nature provides an alternative class of microscopic actuators based on the biological proteins actin and myosin. These proteins, along with other filaments and microtubules, make up the organelle known as the cytoskeleton. This network lines the plasma membrane, allowing the cell to change shape, helping it move, and assisting in cell division. These tasks are achieved through myosin II motor proteins, which produce forces on the actin network through a process detailed below. These two proteins, actin and myosin, are thought to be the most crucial proteins to the cytoskeleton's functionality (Rayment et al., 1993).

In native muscle tissue, actin and myosin contract quite strongly, with forces up to 10 μN for a single muscle cell (Yin et al., 2005). In this tissue the proteins are highly organized into complex units called sarcomeres. This structure allows for strong and fast contractions on a macroscopic scale.

While muscle tissue's level of complexity and organization is currently not possible to replicate at the microscopic level in vitro, contractions can still be achieved in a laboratory setting. In a completely disorganized state, it may be expected that the push and pull forces of myosin motors would balance due to symmetry, resulting in no contraction. However, actin filaments strongly resist tensile forces while buckling under compressive forces. Thus, the result of this system is a net contraction (Lenz et al., 2012). In vitro, these contractions can be observed and their components isolated to determine which are necessary for contraction to occur. These in vitro systems are called active muscle gels.

1.2 Goals of These Experiments

The experiments presented here attempt to improve the functionality of active muscle gels by varying the concentrations of critical contraction components. The components explored by this study are myosin motors and alpha-actinin, a cross-link bundling protein essential to contraction. This study also attempted to streamline a procedure for producing active muscle gels, detailed below.
2. Background

2.1 Actin – Myosin Contractions

The cytoskeleton is a complex network that involves three main components: actin filaments, microtubules, and intermediate filaments. However, only actin filaments, the ones responsible for contraction, are studied in these experiments. These actin filaments are pulled together by myosin motor proteins. For contraction to occur, myosin motors must be in the presence of Adenosine triphosphate (ATP), the energy of the interaction. The process begins when ATP dissociates myosin from actin, binding to myosin’s ATPase active site. Myosin then hydrolyzes the ATP and rejoins with actin, producing a force. Actin must also be exposed to calcium ions to reveal the binding site for myosin motors (Rayment et al., 1993).

This contraction is seen in skeletal muscle, in which actin and myosin form a complex, highly organized network of sarcomeres. Due to this organized state, the contraction is highly efficient. However, the cytoskeleton lacks such a high level of organization. Cytoskeleton contractions occur more slowly, over the course of several minutes, and more weakly, on the order of 45 nN in the case of fish keratocytes (Oliver et al., 1995). They can still perform many tasks, such as allowing cells to change shape, move, and divide. Since the complex structure of a skeletal muscle sarcomere is not currently possible to replicate in vitro, inspiration is drawn from the cytoskeleton for in vitro experimentation.

2.2 Cross – Link Proteins

One necessary accessory protein for a contractile event is the cross-link protein. The cross-link binds actin filaments to each other, creating larger actin bundles. This facilitates the pulling function of the myosin motor proteins. Many different cross-links exist, such as fascin and fimbrin, though the one used in these experiments is alpha-actinin. Alpha-actinin is a larger protein than fascin and fimbrin, allowing it to bind to actin filaments over a larger range of angles (Courson and Rock, 2010).

2.3 Active Muscle Gels

The in vitro application of this contractile system is called an active muscle gel. These gels can apply forces to pull actin filaments together into a single cluster. Should the speed and strength of the gels increase, they could be used in soft robotic applications, compliant robotic systems often inspired by living organisms.
3. Experimental Design

3.1 Methods
This study initially attempted to follow the procedure used by Alvarado and Koenderink detailed in Reconstituting Cytoskeletal Contraction Events with Biomimetic Actin-Myosin Active Gels as closely as possible. Due to lack of appropriate equipment, changes were necessary. Proteins were not purified from rabbit muscle tissue in house, but were purchased in pellet or liquid form from manufacturers and resuspended in lab. After resuspension, proteins were stored on ice at 0°C, whereas Alvarado and Koenderink snap froze proteins using liquid nitrogen and stored them at -70°C. The crosslink protein alpha actinin was substituted for fascin.

Creating an active muscle gel requires a complex procedure with many preparations. These preparations, detailed below, include resuspending proteins, creating and cleaning slides, and mixing buffer/salt solutions.

Preparing Slides
Required materials
- Microscope slide - 75x25x1 mm
- Parafilm
- Scalpel
- Tweezers
- Microscope cover slides - 22x50 mm #1
- Glass cutter
- Hot plate

To record the gel’s contraction, it is necessary to create chambers into which the gels may be inserted. These chambers are created by placing thin, cut-to-size microscope cover slides on top of microscope slides, separated by a thin layer of parafilm. The chambers are extremely thin such that the microscope can keep the entire contraction in focus. This setup creates many small (~1.0 μL) chambers per slide. These chambers are sized such that the microscope can capture activity within the entire chamber, ensuring the contraction is recorded.

Procedure
1. Cut a piece of parafilm into a 75x25 mm rectangle
2. Place this rectangle onto the microscope slide. Using the scalpel cut strips parallel to the short side of the rectangle every ~2mm. This will be the width of each of the chambers. Using tweezers, remove every other strip from the slide.
3. Using the glass cutter, cut ~2x50mm strips from the microscope cover slides.
4. Using a tissue damp with RainX, gently rub down the exposed sections of the microscope slide and the glass cover slide strips. Then wash them using a tissue damp with MilliQ water. This will clean the glass.
5. Lay one of these strips across the microscope slide, creating a glass-parafilm sandwich. 
   Tip: to ensure the strip does not shatter while cutting it from the cover slide, apply pressure on top of the strip segment that is being cut, not the larger cover slide segment, to hold the glass in place.

6. Let the chamber slide sit on a hot plate at ~100°C for approximately one minute to allow the parafilm to adhere to the glass.
   Tip: Using a small tool, such as tweezers, apply pressure on top of the cover slide where it rests over parafilm to help the adhering process. Applying pressure over areas not resting over parafilm can shatter the glass.
   Tip: Don’t leave the chamber slide on the plate too long or the parafilm will start to curl at its ends.

7. It is important to passivate the slide’s chambers to ensure that the gels do not stick to the chamber surfaces. The gels need to flow freely to obtain an optimal contraction. Fill each chamber with distilled water and dry them using compressed air.

8. Fill each chamber with 1M KOH and let the slide sit in a humid environment for 10 minutes. One such environment is a petri dish with a moist tissue. This will cause the surfaces of the chambers to become negatively charged, allowing our cleaning agent, PLL-PEG to bond more easily.

9. Rinse the KOH out of the chambers and dry with compressed air. Fill each chamber with PLL-PEG and allow the slide to sit in a humid environment for 45 minutes.

10. Rinse the PLL-PEG out of the chambers and dry with compressed air.

**Figure 1:** Active muscle gel microscope contraction slide. The thin glass cover strip rests perpendicular to the parafilm strips. The chambers can be filled from either of the two exposed sized and sealed with silicone grease to prevent fluid evaporation.
Preparing Troubleshooting Slides

Required materials
- Microscope slide - 75x25x1 mm
- Parafilm
- Scalpel
- Tweezers
- Hot plate

When these experiments did not achieve the expected results, troubleshooting was required. It was theorized that one of the substances in the gel was causing the actin proteins to misfold and create aggregates, unable to contract in the presence of myosin II motors. Creating new active muscle gels to test for every variable became tedious and a wasteful. To alleviate the repetitiveness of mixing gels and mitigate the cost of proteins used for troubleshooting, a new slide technique was developed. These slides consisted of several square parafilm sections ~1cm x 1cm. These slides resembled gel slides, though the squares were scaled up and no glass top layer was used. The gels were then mixed directly under the microscope, such that it could be observed whether the actin proteins misfolded as a direct result of adding a given substance.

Procedure
1. Cut a piece of parafilm into a 75x25 mm rectangle
2. Place this rectangle onto the microscope slide. Using the scalpel cut the parafilm into three equally spaced strips parallel to the long side.
3. Using tweezers, remove the middle strip from the slide.
4. On a separate surface, cut strips of parafilm ~20x2mm. Place these strips parallel to the short side of the microscope slide every ~8mm, creating 8x8 mm square patterns.
5. Let the chamber slide sit on a hot plate at ~100ºC for approximately one minute to allow the parafilm to adhere to the glass
   Tip: Using a small tool, such as tweezers, apply pressure on top of the cover slide where it rests over parafilm to help the adhering process. Applying pressure over areas not resting over parafilm can shatter the glass.
   Tip: Don’t leave the chamber slide on the plate too long or the parafilm will start to curl at its ends.
Figure 2: Active muscle gel microscope troubleshooting slide. The chambers in this slide and surrounded on all sides by parafilm. Since there is no glass cover slip and fluid will evaporate, these slides cannot be used for experiments with large length-scales.

Preparing Proteins

Required tools
- 0.5 – 10 μL pipette
- 2 – 20 μL pipette
- 20 – 200 μL pipette
- 100 – 1000 μL pipette
- Pipette tips
- Silicone grease
- 600 μL test tubes
- Painters tape

Required materials
- Alpha-Actinin Cytoskeleton Inc.
- Alexa Actin 568 Thermo Fisher
- Skeletal Muscle Actin Thermo Fisher
- Myosin II Motor Protein Sigma Aldrich

All proteins used were extracted from rabbit muscle. For storage purposes, these proteins have been centrifuged into condensed pellets, which need to be mixed with fluid to be usable. At this state, these proteins should be stored in a <10% humidity environment at 4°C. Once resuspended, they should be kept on ice at 0°C where they will be stable for a few weeks, or snap frozen in liquid nitrogen and stored at -70°C, where they will be stable for one year.

Procedure
1. To resuspend alpha-actinin, mix it with distilled water to dilute it to 1mg/ml. For these experiments, 50 μg of protein were mixed with 50 μL of distilled water.
2. The myosin motor protein is shipped in suspended form at 10mg/mL. Distribute the proteins into 50 μL aliquots and store at -20°C, where they are stable for one year.
3. To resuspend muscle actin, mix it with 2mM Tris pH 8.0 to a concentration of 2mg/mL. Distribute the proteins into 100 μL aliquots and store at -70°C, where they are stable for one year. In lyophilized form, store the proteins at -20°C.

4. The Alexa actin is shipped in suspended form at 10mg/mL. Distribute the proteins into 2 μL aliquots and store at -70°C, where they are stable for one year.

MIXING SUBSTANCES

Long term storage substances – can stay at room temperature indefinitely

Create the following stock substances:
- KCl 4.6M
- HEPES pH 7.4 1M
- Tris HCl pH 8 1M
- CaCl₂ 1M
- MgCl₂ 1M
- KOH 1M

Mix the following:
- HK₅₀ ~25mL
  - 0.5 mL HEPES pH 7.4 1M
  - 6.8 mL KCl 4.6M
  - 17.7 mL H₂O

- HK₁₂₅₀ ~25mL
  - 0.5 mL HEPES pH 7.4 1M
  - 0.5 mL KCl 4.6M
  - 17.7 mL H₂O

Long term storage substances – can stay at -20°C for ~1 year

Resuspend the following from their containers:
- Dithiothreitol (DTT) ~1mL
- MgATP 0.1M ~1mL
- PLL-PEG at 50x concentration ~1mL
- Creatine Phosphate (CP) ~ 1mL
Short term storage substances – can stay at 2°C for <1 week

Mix the following:

- Alexa Actin / Actin (AA*)
  - 50.0 μL Actin 2 μg/μL
  - 1.0 μL Alexa Actin 568 10 μg/μL

- Myosin with HK (M*)
  - 246.7 μL HK1250
  - 20.0 μL Myosin 10 μg/μL

Resuspend the following from their containers:
Creatine Phosphokinase (CK) at 50x concentration ∼ 200 μL
  - Using a balance, weigh out ∼1mg of CK
  - Mix with HK50 to a concentration of 5 μg/μL

Day-of Substances

Mix the following:
5xBuffer (5xB)
  - 20.0 μL HEPES pH 7.4 1M
  - 2.0 μL MgCl₂ 1M
  - 2.0 μL CaCl₂ 1M
  - 1.0 μL Dithiothreitol (DTT)
  - 1.0 μL MgATP 0.1M
  - 10.0 μL Creatine Phosphate (CP) 1M
  - 164.0 μL H₂O (MilliQ)

Mixing Active Gels

1. Mix 100 μL active gel
  - 42.0 μL H₂O
  - 20.0 μL 5xB
  - 2.0 μL CK 50x
  - 12.0 Alpha Actinin
  - 20.0 AA*
  - *At this point the mixture is stable. The contraction won’t begin until the next step. Once myosin is added, time is of the essence*
  - 4.0 μL M*

2. Pipette ∼2 μL into microscope slide chamber.

3. Using mid-viscosity silicone grease, hastily seal the two exposed sides of the chamber. This will prevent the gel from evaporating.

4. Record video on microscope (description below) using green fluorescent light to observe the fluorescently marked rhodamine actin.
Tip: To save time in transferring the protein from test tube to chamber to microscope, set the slide on the microscope before adding myosin. Focus in on the desired contraction chamber and place a tape outline on the microscope stage. The microscope can easily slide into place, constrained by this tape outline. The outline will facilitate focusing in on the correct contraction chamber once the gel begins contracting.

3.2 Apparatus
For these experiments, a Nikon Eclipse TE2000-U microscope with a Nikon Plan Fluor 4x objective was used to view contraction events. This microscope utilized a QImaging wLS LED Illumination Unit giving it the ability to view objects with green fluorescent light. To record the microscope data, a Photometrics Prime sCMOS Camera was used. Micromanager, an ImageJ software package, was utilized to analyze the camera’s data.

The controller on the illumination unit was set to 20% green light and 0% blue and red light. The binning time in Micromanager was set to 2000ms. This was determined to be sufficient exposure to observe the proteins. Using the Multi-D Acq. command, the camera recorded a new image every 30 seconds for 90 minutes. The fluorescent imaging attachment was paired with the camera using the trigger cable and a BNC cable (via “expose out”) to only allow the fluorescent light to shine when the camera recorded an image. This reduced overall light exposure on the Alexa Actin. Since fluorescent light can damage the fluorescent marker in an effect called photobleaching, this reduced possible negative effects of using fluorescent light.
4. Results

The experiments presented in this thesis encountered many difficulties shedding light on several useful phenomena. Initially, proteins from Cytoskeleton, Inc. were used to mix muscle gels. Proteins procured from this manufacturer included actin, alpha-actinin and myosin II. However, contractions with these proteins were difficult to achieve. The best contractions were weak and hardly noticeable. We suspect that the actin proteins were misfolding prior to contraction. This misfolding caused hydrophobic portions of the filaments to be exposed to water, forcing them to attach to the hydrophobic portions of other actin filaments. This would cause the actin to form misfolded clumps prior to the introduction of myosin, preventing the possibility of contraction. It then became the goal of these experiments to determine which chemical or chemicals was causing the actin to misfold.

With many different chemicals and proteins being mixed together to create a gel, it was difficult to isolate which ones caused the issue when only observing the gel after all the components were mixed. Thus, a new procedure was developed to identify the problem. This technique involved using the troubleshooting microscope slide procedure detailed in the methods section, first dispensing the actin onto a square in the troubleshooting slide. Each other component was added to the gel while the Prime camera was recording at a rate of 2 fps. The video was then observed to see whether the gel changed from a homogeneous mixture to a heterogeneous mixture of clumps. Through this testing, it was determined 5xB was causing this phenomenon. After breaking 5xB into its components and adding them one by one, it was determined that the buffer, HEPES pH 7.4 1M, and the salts, MgCl₂ caused these issues. (Figure 3)
Tests were performed to isolate the components which caused actin filaments to misfold. Adding HEPES to a solution of actin, rhodamine actin, alpha-actinin, and CK (a) yielded aggregates (b) that hindered myosin driven contraction. Adding MgCl$_2$ to a solution of actin, rhodamine actin, alpha-actinin, and CK (c) yielded more, smaller aggregates (d) that also hindered contraction.

Other buffers were tested to determine whether a substitute could be made. These included Tris pH 8 1M and Imidazole pH 7.6 1M. Imidazole caused slight clumping, but not nearly as severely as HEPES. Tris caused almost no clumping. (Figure 4)
Various components were added to solutions of actin, rhodamine actin, alpha-actinin, and CK to see if a substitute for HEPES could be made. (a) HEPES pH 7.4 1M generated severe aggregation. (b) Imidazole pH 7.6 1M created noticeable aggregates but not as thoroughly as HEPES. (c) Tris pH 8.0 1M resulted in mild aggregation.

Various concentrations of MgCl$_2$ were used to determine if concentration was an issue. Since Mg is a necessary component for myosin II activation, no direct substitute could be made. Though various concentrations were attempted, all caused various levels of clumping among the actin proteins.

We observed that the substances that caused this clumping were of a lower pH than other substances that did not cause clumping. To determine whether pH was a factor, the pH of these substances was brought to 8.5 through adding NaOH. These substances were then added to active gels, resulting in minimal clumping. However, the gels were still not able to contract. Since the optimal pH for contraction is considered to be ~pH 7 (Ikeuchi et al., 1992), this is consistent with the observation that the gels were not able to contract correctly.
Figure 5: To determine whether pH was hindering contraction, MgCl$_2$, a substance with pH $\sim$6, was brought to a pH of 9.0 and added to a solution of actin, rhodamine actin, alpha-actinin, and CK (left). No noticeable change occurred after the salts were added (right), though contraction still did not occur. While this pH increase may have prevented MgCl$_2$ from misfolding proteins, the increased pH appeared to prevent contraction.

An interesting behavior was observed with the Cytoskeleton Inc. proteins. Using these proteins, no contraction occurred with homogeneous starting conditions. However, these experiments did achieve strong contractions with inhomogeneous starting conditions (Figure 6). These contractions were different from those observed by Alvarado and Koenderink, contracting non-uniformly and without local contractions. Understanding how these proteins contract despite their starting conditions could provide further insight into the methods by which these gels contract.
Figure 6: Microscope images of an active muscle gel created using the procedure outline in Appendix A. The initial frame (left) had a significant lack of homogeneity but was still able to contract decisively within 150 minutes (right). This procedure is the only one that produced contractions from inhomogeneous starting conditions.

Having exhausted most options, the possibility that Cytoskeleton Inc.’s proteins were not suitable for these experiments was considered. To determine whether this was the case, proteins were replaced with those from other manufacturers. Actin was purchased from Thermo Fisher. Rhodamine actin was replaced with Thermo Fisher’s Alexa Act 568, a similar protein instead using an Alexa Fluor dye. Myosin was purchased from Sigma Aldrich. Cytoskeleton Inc.’s alpha-actinin was used, as no other manufacturer carried a suitable substitute. Whereas the Cytoskeleton Inc. proteins were shipped in a lyophilized state, the new proteins were shipped in suspended form.

Using a repurposed procedure, contractions were observed with the new proteins. It was observed that adding MgCl$_2$ and HEPES to these new gels resulted in no noticeable misfolding. This resulted in much better contractions, similar to those observed by other researchers, but on a smaller scale (Alvarado et al., 2013). These researchers observed that local contractions, those bringing together small groups of actin filaments, occurred first. These were followed by global contractions, bringing together all of the locally contracted clumps into one major clump. With the new proteins, these experiments were only able to observe local contractions, with slight hints of global contraction.

In an attempt to achieve global contractions, concentrations were changed for myosin II motor proteins and alpha-actinin crosslinks. It was found that increasing myosin motors generally resulted in no contraction from the gel. Changing the level
of alpha-actinin crosslinks changed the nature of the contraction, but not its strength. Adding more crosslinks dragged proteins from larger areas together, but didn’t contract them as completely. (Figure 7)

Figure 7: Once contraction was achieved with different manufacturers’ proteins, the concentrations of alpha-actinin and myosin were varied to determine their effects on contraction. Myosin concentrations chosen were 0.015 mg/mL, 0.030 mg/mL, and 0.060 mg/mL. Alpha-actinin concentrations were 0.06 mg/mL, 0.12 mg/mL, and 0.18 mg/mL. At myosin concentration of 0.06 mg/mL, contraction was tangentially noticeable. At lower alpha-actinin concentrations, the contractions occurred on a strictly local scale. At higher alpha-actinin scales, the contractions were observed on a more global scale. (a) no contraction (b) strong global (c) no contraction (d) weak global (e) strong global (f) no contraction (g) weak local (h) strong local (g) moderate local
One peculiar phenomenon observed through these experiments was that contractions appeared to occur in test tubes, even when they did not occur in chambers under the microscope. Since only \( \sim 10\% \) of the mixed gel is inserted into the contraction chamber, the other 90% remained in its test tube on a rack. After only a few hours, a visible clump of proteins could be observed. This lead to the hypothesis that the glass chambers prevented contraction. However, even using the troubleshooting microscope slides, which have no top seal, no strong contractions were observed. The protein aggregates in the test tubes were imaged. (Figure 8)

Figure 8: It was often observed that while contraction did not occur in the microscope slide chamber, a clump would appear in the original test tube, where a majority of the active gel was left. These clumps appeared to be full contractions, but this was not confirmed since the entire solution in the test tube was not imaged.
5. Conclusions and Future Work

There are several key conclusions that can be drawn from these experiments. The first is that the Cytoskeleton Inc. proteins are not suitable for the current experimental procedure. Since the buffer (HEPES) and magnesium (MgCl₂), both crucial ingredients for a contraction, cause the actin filaments to misfold, no contraction will be possible with the current procedure. Unless a substitute buffer not tested in these experiments is found to work, and another method of delivering magnesium to myosin motors is developed, Cytoskeleton Inc. actin should not be used in these experiments. Since local contractions were achieved with Cytoskeleton Inc. alpha-actinin crosslinks, these may be suitable for experimentation. However, these crosslinks may be the reason that global contractions did not occur. It is unknown whether Cytoskeleton Inc. myosin motors will achieve contraction, as they were never isolated in these experiments and cannot be directly observed, as they lack a fluorescent marker.

Through the experiments with Thermo Fisher and Sigma Aldrich proteins, it can be deduced that increasing the myosin motor protein concentration past 0.06 mg/mL will prevent contraction. No contractions, local or global, were achieved in these experiments with a myosin concentration at or beyond this level.

These experiments also showed that increasing the crosslink concentration changed the nature of the contraction from a strictly local contraction to a global/local mix. As the concentration of alpha-actinin increased, the contractions of groups occurred over larger areas (global nature) but did not seem to contract into less dense clusters (local nature).

Further experimentation could be conducted to observe whether the Cytoskeleton Inc. Alpha Actinin prevents global contraction. Substituting this protein with another manufacturer’s fascin crosslinks could shed light on this issue. Changing actin concentration could also have a great effect on the contraction. This has yet to be tested with the current experimental procedure. Varying this concentration could help to optimize the contraction. It should also be investigated why contractions appeared to occur in test tubes but not in microscope slide chambers. Lastly, understanding how Cytoskeleton Inc. proteins achieved contractile behavior despite inhomogeneous starting conditions could provide useful knowledge on this topic. Exploring any of these could result in a number of useful discoveries within the active muscle gel field.
6. Appendices

Appendix A

The following procedure outlines how to achieve noticeable contraction with Cytoskeleton Inc. proteins despite inhomogeneous starting conditions. The necessary change is to replace all instances of HEPES pH 7.4 1M with either imidazole 1M or Tris pH 8.0 1M.

Resuspending Proteins

Required tools
- 0.5 – 10 µL pipette
- 2 – 20 µL pipette
- 20 – 200 µL pipette
- 100 – 1000 µL pipette
- Pipette tips
- Silicone grease
- 600 µL test tubes
- Painters tape

Required materials
- Rhodamine Muscle Actin Cytoskeleton Inc.
- Alpha-Actinin Cytoskeleton Inc.
- Myosin II Motor Protein Cytoskeleton Inc.
- Skeletal Muscle Actin Inc.

All proteins used were extracted from rabbit muscle. For storage purposes, these proteins have been centrifuged into condensed pellets, which need to be mixed with fluid to be usable. At this state, these proteins should be stored in a <10% humidity environment at 4°C. Once resuspended, they should be kept on ice at 0°C where they will be stable for a few weeks, or snap frozen in liquid nitrogen and stored at -70°C, where they will be stable for one year.

Procedure
1. To resuspend rhodamine muscle actin, mix it with distilled water to dilute it to 10 mg/ml. For these experiments, 20 µg of protein were mixed with 2 µL of distilled water.
2. To resuspend alpha-actinin, mix it with distilled water to dilute it to 1 mg/ml. For these experiments, 50 µg of protein were mixed with 50 µL of distilled water.
3. To resuspend myosin motor protein, mix it with distilled water with 1 mM DTT to dilute it to 10 mg/ml. For these experiments, 1 mg of protein was mixed with 100 µL of 1 mM DTT diluted with distilled water.
4. To resuspend muscle actin, mix it with distilled water to dilute it to 10 mg/ml. For these experiments, 1mg of protein was mixed with 100 μL of distilled water.

**MIXING SUBSTANCES**

**Long term storage substances – can stay at room temperature indefinitely**

Mix the following:
- IK$_{1250}$ ~25mL (previously HK$_{1250}$)
  - 0.5 mL Imidazole pH 7.4 1M
  - 6.8 mL KCl 4.6M
  - 17.7 mL H$_2$O
- IK$_{50}$ ~25mL (previously HK$_{50}$)
  - 0.5 mL Imidazole pH 7.4 1M
  - 0.273 mL KCl 4.6M
  - 23.250 mL H$_2$O

**Long term storage substances – can stay at -20°C for ~1 year**

Mix the following:
- Globular Buffer (GB) at 10x concentration ~2mL
  - 40 μL 1M Tris HCl pH8
  - 4 μL 1M Na2ATP
  - 4 μL 1M CaCl$_2$
  - 4 μL 1M DTT
  - 1948 μL H$_2$O

**Short term storage substances – can stay at 2°C for <1 week**

Mix the following:
- Rhodamine Actin / Actin (RA*)
  - 66.0 μL GB
  - 20.0 μL Actin 10 μg/μL
  - 2.0 μL Rhodamine Actin 10 μg/μL
- Myosin with IK (M*)
  - 246.7 μL IK$_{1250}$
  - 20.0 μL Myosin 10 μg/μL

Resuspend the following from their containers:
- Creatine Phosphokinase (CK) at 50x concentration ~ 200 μL
  - Using a balance, weigh out ~1mg of CK
  - Mix with IK$_{50}$ to a concentration of 5 μg/μL
**Day-of Substances**

Mix the following:

- 5xBuffer (5xB)
  - 20.0 μL Imidazole pH 8.0 1M
  - 2.0 μL MgCl₂ 1M
  - 2.0 μL CaCl₂ 1M
  - 1.0 μL Dithiothreitol (DTT)
  - 1.0 μL MgATP 0.1M
  - 10.0 μL Creatine Phosphate (CP) 1M
  - 164.0 μL H₂O (MilliQ)

When using Tris, replace **all** instances of imidazole in the above procedure with Tris pH 8.0 1M.

**Mixing Active Gels**

- Mix 100 μL active gel
  - 42.0 μL H₂O
  - 20.0 μL 5xB
  - 2.0 μL CK 50x
  - 12.0 μL Alpha Actinin
  - 20.0 RA*
  - *At this point the mixture is stable. The contraction won’t begin until the next step. Once myosin is added, time is of the essence*
  - 4.0 μL M*
7. References


