

**Prophylactic Dosing of Myofascial Release in a Human
Fibroblast Model of Wound Closure**

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Dedication

I dedicate this work to my family. It is their love and support that led me through my medical education and will keep me strong beyond these years.

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Abstract

Myofascial release (MFR) allows clinicians to directly stretch and palpate soft tissue restrictions, improving tissue elasticity, and maximizing range of motion. Research has focused on MFR following repetitive motion strain (RMS), however there is no known application of prophylactic MFR. Utilizing in vitro strain models we will investigate the role of prophylactic MFR in regulating fibroblast wound healing. **We hypothesize that MFR treatments will have greater efficacy when used prior to the repetitive motion strain, increasing the rate of wound healing.**

Human fibroblasts were seeded onto 6-well collagen-I bioflex plates, strained with the Flexcell vacuum compression system. Sub-confluent cell constructs were wounded using sterile 1ml pipette tips to create an area devoid of cells. Spatial wound edge changes were monitored to determine closure rate at 0, 12, 24, 36 and 48 hours. Pooled data for 36 hours demonstrated that RMS closed 32% faster than the combined RMS+MFR and 30.5% faster than the non-strain control, $p < 0.05$.

This meant the data did not support the hypothesis, but prophylactic stretching has been shown to prevent and reduce injury in

other models. Prophylactic MFR requires additional studies to expand our model to include multiple dosed treatments with a stronger emphasis on prevention vs. healing.

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Introduction

The field of sports medicine may include a variety of disciplines, but it is muscle injuries, which account for the majority of activity related injuries among sports players, physical laborers and the military. The benefits of prophylactic stretch intervention and its effect on injury reduction have been well documented. Studies have demonstrated how stretching before strenuous exercise can reduce both muscle strain and injury. Research has shown that there are great benefits to scheduled fitness, strength training, stretching and proper warm-up, and that each of these is critical to the prevention of musculotendinous injuries. The rationale follows that stretching and training increase the muscle elasticity, a necessary part of proper training and strengthening [1, 2, 3, 4]. Modern clinicians including osteopathic physicians, chiropractors, physical therapist and rolfers utilize manual manipulative techniques to relieve and treat musculoskeletal pain, somatic dysfunction, physical trauma and disability [5]. One of the most commonly utilized techniques among osteopathic physicians is that of myofascial release (MFR) and its researched applications have been widespread. During these treatment cycles the physician applies direct pressure to the area of

interest loading the tissue with a constant force and thereby causing the restriction to give way. Benefits have been demonstrated with numerous ailments from fibromyalgia, chronic back pain and sprains [6, 7, 8], to fields such as Cardiology, Emergency Medicine and Oncology [5]. In each situation the unique application of MFR has demonstrated positive and measurable benefits to patient outcomes.

Myofascial release focuses on the fascia, a continuous connective tissue that unites the entire body [5, 9]. Principally this fascia is composed of fibroblasts cells, which serve as both biomechanical receptors and producers of the extracellular matrix proteins [9, 11]. Through their dual role as both mechanosensor cells and cytokine mediators, fibroblasts become the perfect target for soft tissue wound healing. The principles of MFR are based on the understanding that injury to the fascial layer creates fibrosis in the tissue and the application of MFR to these tissues allows the fibrotic restrictions to release [5]. Depending on the technique and practitioner, treatments tend to last from 90 to 120 seconds with patients often experiencing immediate and long lasting pain relief [9].

The implication of such techniques to the general physician is evident as repetitive motion related soft tissue injuries have become

the most common traumatic injuries encountered in the primary care setting [10]. It should be no surprise as repetitive motion injuries originate from any number of commonplace activities such as swimming, running, martial arts, and construction work. These activities bring patients into the physician's office with carpal tunnel syndrome, lower back pain, tension neck syndrome, rotator cuff syndrome, sciatica, epicondylitis, tendinitis, and carpet layer's knee. And the repetitive motion strain affects the patient at the cellular level causing microtears that lead to further inflammation and somatic dysfunction. Our previous in vitro fibroblast modeling has demonstrated MFR down regulates inflammatory cytokine secretions, decreases apoptosis induction, attenuates the damage induced from repetitive motion injury [12] and was shown to have a significant ($p < 0.05$) positive impact on the rate of wound closure compared to a RMS only treated groups [13].

With professional athletes and many physical activities the patient is well aware of the repetitive motion injury they are about to endure from exercise. This puts them in the unique position to take steps to reduce and prevent injury before it happens by seeking out prophylactic treatment. After a literature search we were unable to

find any studies that had experimented with the use of prophylactic MFR as a method to reduce injury and decrease recovery time. Such an approach if found successful, would have significant implications in athletic medicine and the reduction of injuries. The goal of this study was to develop and evaluate the use of prophylactically dosed MFR to our existing RMS model and evaluate its effect of wound closure rate. **We hypothesize that such MFR treatments will have a greater efficacy when used prior to the injury and repetitive motion strain, increasing the rate of wound healing.**

Research Materials and Methods

Cell Culture

Normal human dermal fibroblasts (NHDF) were obtained from Cambrex Laboratories (East Rutherford, New Jersey) and cultured in Dulbecco's modified Eagles Medium (DMEM) supplemented with 2% fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37°C at 5% CO₂ and relative 100% humidity. Every other day the medium was replaced with fresh pre-warmed growth medium. Subconfluent cultures (typically acquired in 7 to 10 days) were passed at a ratio of 1:3 until the necessary number of cells was achieved; all experiments utilized cell passages between 4 and 12.

In Vitro Strain Apparatus

All strain profiles were conducted utilizing the Flexercell FX-4000 Tension Plus System developed by Flexcell International Corporation (Hillsborough, NC). This apparatus encompasses a computer controlled vacuum system, which directed controlled vacuum pressure to a 4-plate baseplate. The individual plates were sterile plates, each with six flexible collagen I-coated elastomeric-bottomed

wells. 25 mm BioFlex® loading station platens were placed beneath the flexible membrane to allow an equibiaxial strain across the membrane. Programming the strain magnitude, duration, direction and frequency of the negative pressure induced by the system created the desired strain profile. The deformation of the collagen induced by the vacuum causes the attached fibroblasts to be similarly strained (Fig. 1).

Figure 1

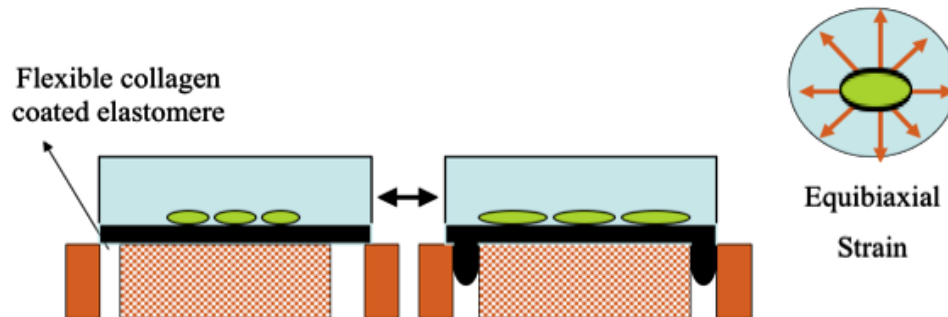


Diagram of Bioflex plate and strain apparatus. Green ovals represent fibroblasts attached to collagen wells. Under each well is placed a 25mm platen. When the vacuum load is applied the membrane is pulled down and over the platen in the direction of the black arrows as shown on the right diagram.

Strain Profiles and Scratch Wound Assay

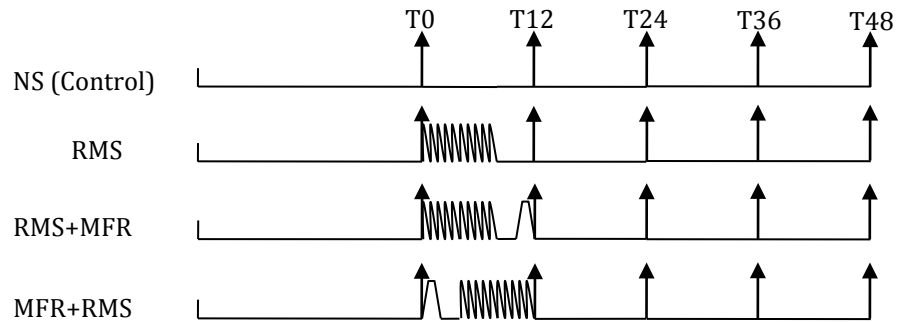
NHDF cells were seeded at a concentration of 1×10^5 cells per well onto the collagen I-coated Bioflex plates. Previous experiments have shown that fibroblasts underwent growth arrest once full confluence was reached on this substrate. As a result, once the cultures had reached 70-80% confluence at roughly 24 hours, the 2% FBS medium was replaced with a reduced 0.2% FBS medium to induce quiescence.

Twenty-four hours after the low-serum medium was added, a modeled scratch wound was induced via a sterile 1000 μ l pipet tip that removed the fibroblast cells from the membrane within its strike width. The result was a “wound” strike of approximately 2 mm by 25 mm or a 50 mm² area completely devoid of cells. The remaining debris and medium were removed with sterile phosphate buffer saline (Sigma Aldrich St. Louis, MO) washed in triplicate. Once the washes were completed, fresh 2% FBS serum-medium was reintroduced to each well and the plates were imaged as a time point of 0 hours and then subjected to one of the four strain paradigms (Fig 2, 3A, 3B). The groups were then reimaged at 24, 36, and 48 hours.

1. *Control (No-strain)*: cells that did not receive any strain during the duration of the experiment Represented immobilized tissue after injury.
2. *RMS (Repetitive Motion Strain)*: cells subjected to RMS strain profile, which consisted of a cyclic strain at 0.625 hertz (cycles/second) through a 10% strain. The strain's duration lasted 8 hours. Represented those who ignored treatment after injury and completed an RMS activity.
3. *RMS+MFR (RMS plus Myofascial Release)*: cells subjected to the 8 hour RMS strain profile followed three hours later by MFR stain profile for 60 seconds and sampled at 12, 24, 36 and 48 hours post injury. The MFR strain consisted of a slow loading strain increased at a 3% a second for two seconds, held constant at roughly 6.6% strain for 54 seconds and then decreased by -1.5% per second for 4 seconds. Represented those who ignored treatment after injury and completed an RMS activity, but did seek treatment following injury in the form of MFR therapy.
4. *MFR+RMS*: cells subjected to the 60 second MFR profile followed three hours later by RMS profile. Represented those

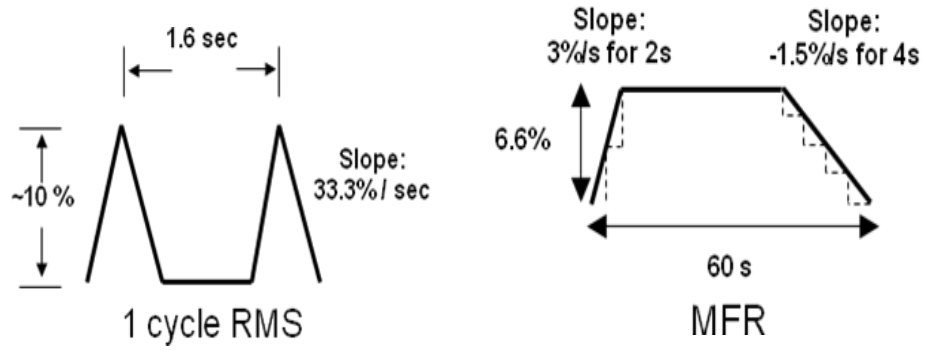
who seek treatment after a wound but before a known RMS activity.

Figure 2



Strain time-line for the four treatment groups: non-strain (NS) control, repetitive motion strain (RMS), Repetitive motion strain followed by myofascial release (RMS+MFR) and myofascial release followed by repetitive motion strain (MFR+RMS). The cells were wounded and imaged immediately and that point acted as time zero. Time points 12, 24, 36 and 48 hours were imaged based on time zero.

Figure 3



(A) Specifics of strain profile repetitive motion strain (RMS) and for (B) Myofascial Release (MFR).

Each strain paradigm was tested on a separate Bioflex plate with six wells. The entire experiment included two replicate runs on separate days, where for each run, one full set of 6 wells was tested for each paradigm. This set of experiments did not include a MFR only control. This choice was made because during prior published experiments with a similar model it was found that there was consistently no significant difference between the MFR control and other experimental groups negating the include it in further.

The strain magnitude, frequency and duration in our strain paradigms were modeled based on a roofer repeatedly hammering nails. The frequency was based on his hammer swings with the duration being an 8 hour workday. The RMS injury paradigm was modeled to reflect scenarios that working class laborers may encounter in daily activity.

The specific parameters of MFR modeling were empirically determined through videomorphometric analyses of clinically applied manual medicine treatments that were applied directly after an injury was sustained [13]. When combined the strain groups they represented a patient seeking treatment from injury and the continued overuse of the affected area. It is recognized that there were various

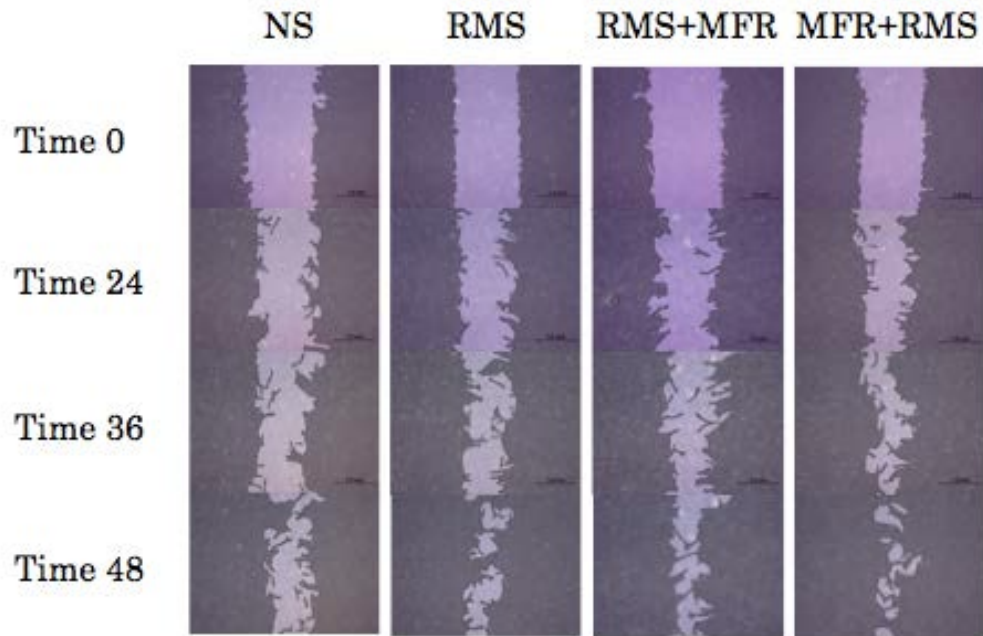
limitations in using such in vitro models when correlated to in vivo wound healing and biomechanics. One such limitation was how our model only included a single cell type known to contribute to healing. In a true in vivo setting various cell types would be involved with such an injury and aid in the rate of wound repair. However, such effects are beyond the scope of this project and we instead focused on providing reproducible and controllable methods to investigate fibroblast wound healing in response to our biomechanical strain profiles.

Photomicrography and Wound Healing Rate Quantification

Digital images were captured for each wound area using 40x magnification, which produced a 7 mm² viewable area of the wound (Fig 4). At 0, 12, 24, 36 and 48 hours images were taken at a specific location using grids placed on the underside of the Bioflex wells. Phase contrast images were captured using an IX71 Olympus inverted microscope and a DP71 camera (Olympus America Inc, Melville, NY). The wells were coded upon initial imaging to blind the observer from the experimental conditions. Wound margins were determined and outlined by a single observer using Adobe Photoshop CS3 version 10.0

(Adobe Systems Inc, USA). Digital images were then analyzed using Image J 1.40g (National Institute of Health, USA; <http://rsb.info.nih.gov/ij>) and Cell Profiler (Broad Institute, USA) to measure the area of the wound at that stage of healing (in square mm). Fibroblast wound closure rates were determined as a rate of change in area over time for each well. Wound closure rates were then normalized to the averaged non-strain closure rate prior to statistical analysis.

Figure 4



Representative photomicrographs of NS, RMS, RMS+MFR and MFR+RMS human fibroblasts immediately after wounding (time 0 hours), 24 hours, 36 hours and 48 hours. All images were captured at 40x magnification and the scale bar shown indicates 1.0mm.

Statistical Analysis

Wound healing data were collected and assessed for each strain paradigm in two experiments (N=6 per group/per experiment). Data are shown as mean +/- standard error of the mean and the four treatment groups were compared by unpaired, two-tailed T-tests using Prism 4.03 (GraphPad Software, Inc., San Diego, California). Group means with $p < 0.05$ were considered to be significantly different. Different lowercase letters above bars in Fig indicated significant differences among respective groups.

Results

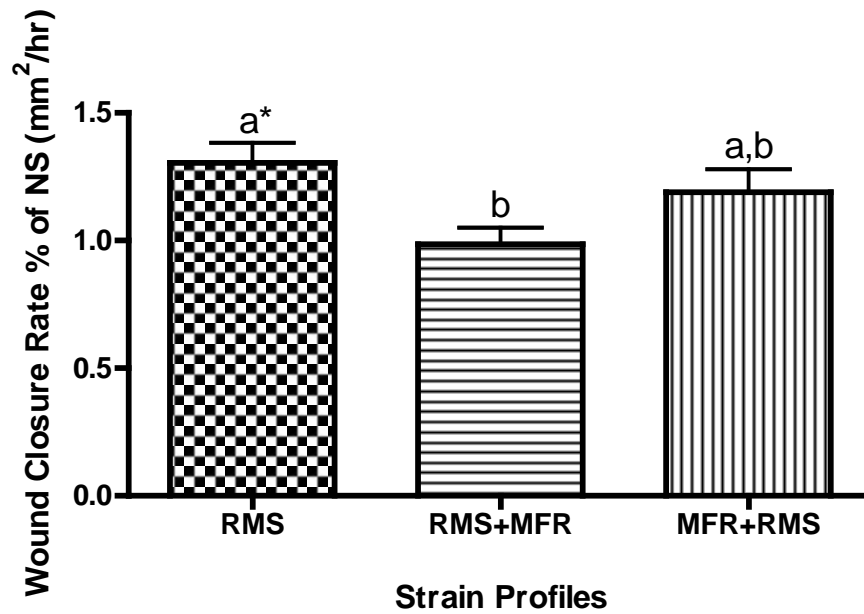
Combined Meta-Analysis

A meta-analysis was completed for the 36-hour time point. The experimental data was normalized as a percent of non-strain. This was accomplished through calculating the non-strain average for each experimental trial and then using the value to normalize the strain group data associated with that individual trial (Fig. 5). Thirty six-hours was chosen because the wound edges had begun to significantly approximate and close at the 48 hour time point making the measured values of closure rate unreliable. Each strain paradigm was represented by 12 wells (n=12).

The combined data showed that RMS groups closed 32% faster than combined RMS+MFR treated group when both sets of data were normalized. This was denoted in Figure 5 with the letters "a" and "b". RMS was also found to close at a rate 30.5% faster than non-strain control (n=12; p<005), denoted by the "*" above the bar. There was no significant difference in closure rate when RMS was compared to combined MFR+RMS or when combined RMS+MFR was compared to combined MFR+RMS. This was denoted by "a,b" above the MFR+RMS bar (Fig. 5). No significance was found

when both combined RMS+MFR and combined MFR+RMS were compared to the non-strain control.

Figure 5



Meta-analysis of wound closure rate (n=12) as a percent of non-strain control for both experiment 1 and 2 at 36 hours. Different letters (a, b) denote significant difference among groups (p<0.05) * Indicates a significant difference of strain profile compared to control (p<0.05).

Discussion

In this study we investigated the effect of prophylactic myofascial release in fibroblast wound healing through an in vitro injury strain model. To the best of our knowledge this was the first study to experiment with a model of pre-injury myofascial release application. We hypothesized that MFR treatments will have a greater efficacy when used prior to injury and repetitive motion strain resulting in increased rate of wound healing.

The prophylactic application of MFR did not improve wound closure rates once normalized to the non-strain control and supported the null-hypothesis. The combined data demonstrated significance between the RMS group and combined RMS+MFR as well as RMS to that of the non-strain control. We did not see the increased wound closure rate that we have observed in prior models when MFR followed repetitive motion strain. While we had been unable to find existing studies of prophylactic MFR use, multiple studies had discussed the use of stretching in wound reduction. Many had showed improvement through warm-up stretching [1, 2, 3, 4], however there was literature that found a similar lack of statistically attributable improvement [14,

15, 16]. The stretching and massage to which these athletes were exposed was similar in nature to the MFR technique we utilized. It was unusual however that we did not observe improved wound closure in the RMS followed by MFR model. Our previous research had shown such a result using a similar experimental modeling [13].

The discrepancy between our modeling in this experiment and previous experiments may lay in the complex nature of wound healing itself. The experimental model itself may not have tested components important to the stretch response mechanism such as extracellular matrix, different cell types, transport vessels and other components, which might contribute to wound closure. Experimental error potentially played a role as well. Visual inspection of the individual wells found no significant difference in cell density among groups but the cells were only sampled at a small-centralized location of the wound edge. When the full length of the wound was visualized we observed complete wound closure at certain regions of the wound edge. This premature closure likely contributed to the data inconsistency and may have demonstrated that the cells settled in different densities within the regions of the well. We were concerned that this data did not agree with our prior findings, however the findings were consistent

even among initial testing experiments not included in the final analysis. This led us to believe that there must be some unknown difference between this modeling and previous models. Our previous studies were also analyzed at 48 hours, which may have had a significant impact on the results. As discussed previously, we were unable to do this based on premature wound closure and were unsure if this may have existed in prior experiments as well. In future experiments it would be useful to image the entire wound edge to better reduce the sampling error that may be caused by focusing on such a specific location of the wound edge as well as consider expanding the wound width to avoid premature closure.

Despite these limitations, we believed this data suggested that MFR applied pre- and post RMS does not affect wound closure. Research has shown wound healing to involve a mix of mechanisms including apoptosis, inflammation, secretory response, migration and replication [10, 12, 17, 18]. Although we did not observe enhanced wound closure with our modeled strains, it does not preclude the possibility that MFR may potentially reduce the risk of injury in differently modeled situations. Our model only applied a single 60-second MFR treatment where many of the other pre-stretch therapies

utilized multiple segmented and repeated treatments [1, 2, 3, 4]. We believe that to fully appreciate the true effect of prophylactic MFR application additional strain studies are necessary to expand on our model to include multiple dosed MFR treatment, varied MFR strain patterns or the use of animal models focusing the emphasis on prevention of injury instead of wound healing.

Our experimental in-vitro model did not support our hypothesis and prophylactic myofascial release was not able to increase the rate of wound closure. We think that it would be valuable to run additional experiments in the future to determine which specific mechanisms of wound closure may have played a role in our observations and to deduce the difference seen with this wound closure model compared to our previous models.

Future Direction

During the course of this project there were two significant areas for future expansion on this experimental model: the inclusion of a replication and migration inhibitor, and varying the MFR strain profile.

The inclusion of a replication and migration inhibitor would involve including two groups, one with an integrin inhibitor to control for migration and second group with a proliferation inhibitor to control for replication. These groups would help determine the relationship between wound closure and cellular proliferation verses that of cellular migration. Such research would direct future treatment strategies through the inclusion or modification of different biomechanical strains or chemical therapies to aid wound closure.

The second alternative would involve inducing various strain paradigms in place of the existing modeled MFR treatment. Such a change would determine whether alternative biomechanical strains including a difference in duration, frequency or magnitude could result affect wound healing. The current model is based on specific observed treatments and changing this variable may help glean the implication

and importance of the actual strain in cellular remodeling, replication and migration, and more accurately reflect the differences found among providers

Conclusions

Although this experiment supported the null hypothesis it did provide a useful example of an idea, which logical in theory, did not however translate well to the in vitro model. The unexpected outcome seen in this experiment may have multiple layers of complexity. The fibroblasts may have been cultured in slightly different densities, contributing to different closure rates. The cells may have been exposed to unintended strain as they were loaded and unloaded off of the strain apparatus. Or the blinding process could have been faulty with the observer not truly unbiased. There are many plausible arguments for what has happened within this experiment. We did our best to control for variation and insure consistency between the two final experiments and our laboratory will continue to actively research both myofascial release and repetitive motion injuries into the future. The data and findings from this project will help to expound upon our knowledge base and provide insight into future experiment ideas and models.

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