Cartilage Lubrication and Joint Protection by the Glycoprotein PRG4 Studied on the Microscale

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Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Mechanical Engineering and Materials Science in the Graduate School of Duke University

2010
ABSTRACT

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Abstract

Human joints are able to withstand millions of loading cycles with loads regularly more than 3 times an individual’s body weight in large part due to the unique bearing properties of articular cartilage, a strong, slippery tissue that covers the ends of long bones. PRG4 is a boundary lubricating glycoprotein present on the cartilage surface and in the synovial fluid surrounding it. While evidence that PRG4 lubricates and preserves normal joint function is strong, little is known of its effect on cartilage surface properties, the mechanism by which it lubricates, or its postulated role of preventing wear on joints. The effect of PRG4 on cartilage friction, wear, structure, morphology, and the mechanisms by which it mediates these factors are studied here. Methods to study these parameters at the microscale using atomic force microscopy are also developed.

Cartilage of mice with the Prg4 gene (which expresses PRG4) deleted is shown to be different in a number of ways from wild type cartilage. The uppermost layer is thicker and less uniform and the surface is rougher and softer. There is also a loss of proteoglycans, structural components of cartilage, from the underlying superficial tissue, and apparent tissue damage in some cases. Wear in the presence of PRG4 in shown to be significantly lower than in its absence, a finding which may have direct implications for prevention and treatment of osteoarthritis. It appears that PRG4 needs to be present in solution, not merely on the cartilage surface to have this effect, indicating that adsorption properties are important for wear prevention.
To my parents, Dave and Jean Coles,
who taught me to love to learn
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Acknowledgements

I am indebted to a number of people who both made this work possible and helped me become who I am, personally and professionally. My advisor, Stefan Zauscher, is a wonderful example to me of what a scientist should be, combining a deep insight into physical phenomena and technology with a contagious curiosity about nature. I also appreciate his care for his family and his concern for other people and am blessed to have a model who does excellent science as part of a full life. Through his influence, his lab is a positive, upbeat place to do research.

My research has been part of an interdisciplinary collaboration led by Professors Stefan Zauscher, Farshid Guilak, and Gregory Jay. Each provides unique and valuable expertise and I am grateful for the influence each of them has had on me as well as on the project. Professors Piotr Marszalek and Tod Laursen have also made themselves available on several occasions to provide input on this project and to talk about broader issues of career and life as a scientist. My research is better because of each of them.

My labmates, particularly Woo Kyung Lee, Alexei Valiaev, Marian Kaholek, and Nehal Abu-Lail helped me learn details of surface science techniques. Conversations with Yee Lam, and Robert Ducker were particularly influential in helping me think about my project as a scientist. They also helped keep me grounded and view my work from a broad perspective. Tao Chen was an attentive friend and mentor throughout his time at Duke. I have relied on Debby Chang and Eric Zhang throughout graduate school and am thankful for their camaraderie. Debby has collaborated on much of this work and has had
thousands of pieces of input ranging from the idea for wear detection with the AFM to the smallest details of presenting data. Rob Ferris and Greg Hardy have also provided thoughtful input into this project. I had the opportunity to mentor three intelligent and creative undergraduate students during the course of this work, Jason Blum, Alex Cheng, and Nissar Ahmed, and greatly appreciate their contribution.

I highly value the other friends who made graduate school a richer, more meaningful time in my life. Nathan Jenness, Andy Simnick, Soji Sájuyigbé, Jessie Rossol-Allison, Vadim Polikov, Srinath Rangarajan, Stephen Odaibo, and Michael McDougle come immediately to mind. Finally, I’m grateful to my fiancée Libby Magee, my parents, and my siblings for their love and constant support.
Chapter 1. Overview and Motivation

1.1 Introduction

Articular cartilage covers the end of bones in moveable joints, allowing them to support loads well in excess of body weight with minimal friction and wear [1]. Though cartilage is typically preserved over several decades, cartilage breakdown in osteoarthritis, due in part to mechanical wear [2,3] is a significant human health problem, affecting over 40 million people in the United States alone [4]. Coefficients of friction on hydrated cartilage tend to be in the range of 0.005-0.04 [5-9], values which are phenomenally low for the range of loads and sliding speeds that occur. The low friction on cartilage is due in large part to load sharing by interstitial fluid pressure [7]. Boundary lubrication also contributes to the low coefficients of friction on cartilage and the glycoprotein PRG4, which is present both on the cartilage surface and in synovial fluid, appears to be the most effective boundary lubricant in synovial fluid [10]. Its absence has been linked to alterations in cartilage morphology [11] and structural rearrangement possibly due to mechanical wear [12]. These studies show the importance PRG4 to joint function. However, there is a lack of evidence for the details of boundary lubrication by PRG4, its effect on surface properties, and its postulated role of preventing wear on joints.
1.2 Objectives

A series of studies of the effect of PRG4 on cartilage friction, wear, and morphology are presented in this thesis. The work is targeted toward three major objectives.

Objective 1: Measure friction on murine cartilage by colloidal probe microscopy and assess the origins and explain the mechanisms of friction measured in the model system.

Motivation 1: This work is immediately targeted towards friction measurements on cartilage of Prg4-/− mice (genetically modified animals which do not express the protein PRG4). This strain of mice was recently created and provides a unique opportunity to study the role of PRG4 in mediating cartilage surface properties. Because we measure friction by colloidal probe microscopy, the contact area is sufficiently small relative to the speed to allow measurements of friction in the absence or near absence of interstitial fluid pressurization. This condition is ideal for a focused study of boundary lubrication. While atomic force microscope probes of various geometries and surface chemistries are used as models to measure friction on a wide variety of substrates, the contributions of specific mechanisms of friction on the measurement are usually not assessed. Importantly, these mechanisms may be unique to the atomic force microscopy (AFM) setup. The approach used here is applicable to a wide range of soft and rough surfaces. Measurements of stiffness and roughness in the same test setup facilitate analysis of friction mechanisms and allow further characterization of cartilage tissue.
Objective 2: Assess friction, stiffness, and morphology of Prg4-/- cartilage at the microscale as a function of age and relate these properties to structure and morphology at the tissue level.

Motivation 2: We want to understand the role of PRG4 in promoting and maintaining normal cartilage physiology with a broader goal of understanding mechanisms of joint disease and how they can be prevented. While Prg4-/- cartilage has been visualized by light microscopy [11] and scanning electron microscopy (SEM) [12], and friction of these joints have been assessed in an arthrotripsometer [12], friction and stiffness measurements of the tissue and staining for specific components of the cartilage matrix have not. Here we measure friction, stiffness, and morphology of these cartilage surfaces as a function of age. We thus are able to both compare properties of Prg4-/- cartilage to wild-type and to track the progression of degenerative changes. Because PRG4 is present both in synovial fluid, which surrounds cartilage, and on the surface of cartilage itself, most previous tests of the effect of PRG4 on cartilage friction have lacked a true PRG4-negative sample. Here we measured friction on Prg4 knockout mice, a truly PRG4 negative test sample, and compared friction on these samples to wild-type mouse cartilage. However, we are able to measure the lubricating effect of surface-associated PRG4 only using Prg4-/- mice.

Objective 3: Measure the effect of PRG4 on surface-level cartilage wear of normal cartilage and cartilage from which proteins have been extracted.

Motivation 3: Cartilage wear prevention is not well understood and few studies of cartilage wear have been undertaken. Although in healthy cartilage, wear rates are
typically undetectably low, cartilage wear is believed to play a role in the disease process of osteoarthritis. The possibility that an understanding of natural mechanisms of wear prevention may lead to osteoarthritis motivates our study from a pathophysiological perspective. An understanding of cartilage wear prevention may lead to new biomimetic anti-wear additives, further motivating the study from an engineering perspective. To this author’s knowledge, no studies have yet measured cartilage wear at a sub-micrometer level. Measurement of nanoscale wear is important though since molecular and macromolecular scale wear is believed to contribute to osteoarthritis [13]. The upper 50-200 nm of cartilage is also chemically and structurally distinct from the bulk tissue, meaning that wear processes at the surface are likely different from those occurring in deeper zones. The present study is also the first to directly measure the effect of PRG4 on cartilage wear. Because PRG4 is present in cartilage, cartilage from which proteins have been extracted by high salt (2 M NaCl) provides a modified test system with a PRG4 negative control.

1.3 Organization

This thesis is divided into six chapters. Chapter 2 gives relevant background information on lubrication and contact mechanics and the structure and function of cartilage and PRG4. Studies based on objectives 1-3 are presented in Chapters 3-5, respectively. A number of interesting opportunities for future study have arisen through this work and these are presented in Chapter 6 along with preliminary data and conclusions of the thesis as a whole. Some material included in Chapter 2 has been
adapted from published documents [14-17] and Chapters 3 and 4 are largely composed of work published in [14] and [17], respectively.
Chapter 2. Background

2.1 Tribology

2.1.1 History of Tribology

A large number of mechanical and physiological processes involve surfaces in contact moving relative to one another. Interaction between the surfaces comes at an energetic cost and the result is a friction force acting on each surface opposite to the direction of motion. These interactions also result in wear - damage to the surfaces in the form of loss of material and other irreversible changes. Tribology is the study of interacting surfaces in relative motion and encompasses the study of both friction and wear [18].

Leonardo Da Vinci (1452-1519) is believed to be the first to have studied friction systematically. He concluded that the friction force is proportional to the normal force and independent of the contact area, findings which were rediscovered by Guillaume Amontons, who published the following laws of friction in 1699.

1: The maximum friction force is independent of the normal force, $F_F = \mu F_N$,

2: Friction is independent of contact area,

3: Friction is independent of sliding velocity.

While these laws are recognized today as holding generally true, many exceptions exist, particularly at micro and nano scales [19]. Other important early contributions to the understanding of friction were made by the renowned scientists Leonhard Euler (1707-1783), who was the first to distinguish between static and kinetic friction, and Charles-Augustin de Coulomb (1736-1806), who further clarified Amontons’ work and
identified a time-dependence of static friction with a time constant that varied depending on the materials used [19].

Revolutionary progress in the field of tribology came more recently through the work of Frank Philip Bowden and David Tabor in the 1940s, and 1950s who developed and experimentally demonstrated a model for adhesive friction [20]. They showed that the true contact area, which they assessed based on the linear relationship between electrical conductivity and cross-sectional area, varied linearly with load. Friction also increased linearly with load and with the true area of contact, leading to the adhesive model for friction,

$$F_t = SA_t,$$  \hspace{1cm} (2.1)

where $S$ is the interfacial shear strength and $A_t$ is the true area of contact. They also noted that this mechanism of friction is distinct from energy losses due to deformation of the materials involved in sliding.

According to Hertzian contact mechanics, which had been developed some 50 years prior [21], the contact area between spherical, or spherical and flat elastic contacts should increase with a $2/3$ power of load. Thus if their contacts had been elastic, Bowden and Tabor’s results would have appeared at odds with either Hertzian contact mechanics or with Amontons’ first law. They rectified this discrepancy by arguing that deformation in the metals they were working with could be expected to be primarily plastic. The true contact area would then be equal to the load divided by the yield strength of the material. Models of elastic deformation of a fractal surface [22] and a surface with a random distribution of asperity sizes [23] later showed that deformation should be proportional to
load in the case of elastic deformation as well. Thus Amontons’ first law is generally not
due to any physical property of materials but merely a consequence of stochastic
roughness.

Adhesive friction can not be directly predicted from measurements of adhesion,
which usually involve bringing two materials into contact and measuring the force
necessary to separate them. An important difference is that sliding friction does not
involve separation of materials. Instead, a contact merely moves from one area of the
interface between the same pair of materials to another. On an atomic scale this is linked
to the energy required for atoms to move from one local energetic minimum to the next
[24]. A better predictor of adhesive friction is adhesion hysteresis, the difference
between adhesion forces on approach and on separation, as shown by Jacob
Israelachvilli’s group in 1991 [25].

2.1.2 Friction

Friction arises due to forces transmitted between surfaces. In continuum
mechanical terms, forces are transmitted as tractions, defined as

\[ t = \lim_{\Delta S \to 0} \frac{\Delta F}{\Delta S}, \]

where \( S \) is an area on a surface. Oblique tractions can be resolved into normal
and tangential tractions. The force along axis \( x \) due to a surface traction \( t \) can be
calculated as

\[ F = \int_S \tilde{t} \cdot \tilde{x} \, ds. \]
A wide range of phenomena contribute to surface tractions on bodies in contact. Adhesive forces are known to influence friction as discussed in the previous paragraphs. Viscoelastic or plastic deformation and breaking of chemical bonds also costs energy and contributes directly to friction [18]. Important surface effects include debris or wear particles (“third bodies” in tribology nomenclature) which may plow through surfaces [26] and chain entanglement between polymeric surfaces [27].

An atomic scale approach is necessary to understand origins of friction. The true area which is proportional to friction is modeled more accurately as the number of interacting atoms than as the area of asperity contact [28]. Tomlinson [29] was the first to develop a mathematical model of atomic friction, well ahead of his time in 1927. In his model, one surface was modeled as completely rigid and the apposing surface was modeled as a series of atoms on springs perpendicular to the direction of motion. A number of more similar models have been used to describe friction, most notably the Frenkel-Kontorova model [30], in which atoms were linked to one another and oscillating in the plane of the surface. These models give the fundamental theoretical basis for many more recent studies [31,32]. An interesting prediction of these models is very low friction for non-commensurate atomically flat surfaces, a phenomenon which has also been observed experimentally [33]. A limitation of these models is that they do not provide information about how energy is actually lost. Though damping coefficients of the interatomic oscillations can be included in the model, these must be informed by experiment to be meaningful. Molecular dissipation mechanisms are still not well
understood but phonons (atomic vibrations) are believed to be important, as well as electron transfer in some cases [34].

2.1.3 Wear

Wear is defined as the progressive loss of substance from the operating surface of a body occurring as a result of relative motion at the surface [35]. Several different mechanisms contribute to wear, most notably adhesive wear, surface fatigue, abrasive wear, corrosion and erosion. Adhesive wear occurs when adhesive forces on an area of superficial material are stronger than cohesive forces and material is removed from a surface. This typically occurs for small particles which may become part of the opposite surface, mix with material from both surfaces, or become wear debris. Surface fatigue occurs when cracks develop as a result of the cyclic stresses in a material and lead to either delamination (for ductile materials) or surface fracture (for brittle materials). Abrasive wear is essentially grinding and occurs when a harder surface pushes into and slides along a softer one. Erosion occurs when flowing liquid or solid particles remove material from a solid surface as in sandblasting or in a deepening canyon. Corrosive wear is material loss due to the combined action of chemical degradation and interfacial sliding. Loss of material properties or material by corrosion often leaves material more susceptible to adhesive and abrasive wear [36].

Models have been developed of rates of adhesive wear [37] and abrasive wear [38], which despite being derived for very different conditions, coincidentally take the same form, \( \frac{dV}{d\ell} = \frac{KF}{3H} \), where \( V \) is the removed volume, \( \ell \) is the sliding distance, \( F \) is the load, and \( H \) is the hardness of the material being worn (the softer of the two
surfaces). In Archard’s derivation for adhesive wear, $K$ is the probability of each contact forming a wear particle, while in Rabinowicz’s derivation for abrasive wear $K$ is the cotangent of the half angle of the indenter. Practically, the value of $K$ is typically determined by experimentation.

The relationship between friction and wear is far from straightforward. Wear requires energy and the energy that causes wear comes from friction. Thus there is a minimum theoretical friction value associated with any given wear rate [18]. Because most friction is generated by mechanisms that do not result in wear, though, this relationship is not very practical. It is reasonable to conclude that a relationship exists between adhesive friction and adhesive wear and between plowing friction (which results from a hard asperity pushing through a softer material) and abrasive wear. Surface fatigue would be expected to increase with increasing friction though not necessarily linearly. More research is needed to illuminate the details of these relationships. In practical situations low friction and high wear is sometimes optimal, as for pencil lead, and high friction and low wear is optimal in other situations, as for brake pads.

### 2.1.4 Lubrication

Lubrication is the reduction of friction and wear of sliding surfaces by modification of the interface, typically the addition of a lubricating material. The two most common strategies used for lubrication are hydrodynamic lubrication and boundary lubrication. The primary mechanism of cartilage lubrication is a mechanism unique to cartilage which in this thesis will be referred to as biphasic lubrication, discussed in detail in Section 2.4.2. Squeeze film lubrication is a phenomenon closely related to
hydrodynamic lubrication which is also relevant for cartilage and will be discussed more in the context of cartilage lubrication in Section 2.4.4.

2.1.4.1 Hydrodynamic lubrication

In hydrodynamic lubrication, surfaces are separated from one another by a wedge of entrained fluid. The lift from the fluid separates the surfaces in the same way that a wedge of fluid supports a water skier on the surface of the water. The two conditions that must be met for this to occur are 1) surfaces must be angled with respect to one another to allow a wedge to develop; and 2) the velocity must be sufficient to separate the surfaces. In the hydrodynamic lubrication regime, friction is due only to the viscosity of the fluid. The range of lubrication regimes from boundary to hydrodynamic is often visualized as a Strubeck curve (developed in part by early tribologist Richard Strubeck) as seen in Figure 1.

Fluid flow between two surfaces in relative motion is described by the Reynolds equation, derived from the Navier-Stokes momentum equation and published by Osborne Reynolds (for whom the Reynolds number, the ratio of inertial to viscous forces in a fluid, is also named) in 1886 [39]. The equation relates the distance between surfaces to the relative velocity of the surfaces and the viscosity of the lubricant. Assuming unidirectional motion of surfaces, no vertical flow, and constant viscosity, the equation becomes

\[
\frac{\partial}{\partial x} \left( h^3 \frac{\partial p}{\partial x} \right) + \frac{\partial}{\partial y} \left( h^3 \frac{\partial p}{\partial y} \right) = 6U \eta \frac{dh}{dx}, \tag{2.4}
\]
where \( h \) is the height of the fluid separating the surfaces, \( p \) is the pressure, \( U \) is the relative velocity of the surfaces, and \( \eta \) is the dynamic viscosity.

The degree of hydrodynamic lubrication is often characterized by the non-dimensional parameter

\[ \lambda = h_0 / \sqrt{\sigma_A^2 - \sigma_B^2}, \]

(2.5)

where \( h_0 \) is the minimum film thickness and \( \sigma_A \) and \( \sigma_B \) is the RMS roughness of surfaces \( A \) and \( B \). When the velocity is sufficient to only partially separate the surfaces, pressure is borne partially by solid-solid contact and partially by a wedge of fluid. Mixed mode lubrication, also called thin film lubrication, may reduce friction for lambda values as low as \( \lambda = 0.05 \) [40]. Full separation of surfaces with no wear can be expected for lambda values above \( \lambda = 4 \) [41].
2.1.4.2 Boundary Lubrication

Boundary lubrication occurs when surfaces are in full solid-solid contact and are lubricated only by surface modification. A boundary lubricating layer may be a single molecular layer on each surface or may be a few molecules thick [41]. Layers thicker than a few molecules may increase friction due to shearing and plowing within the lubricant film [42]. Many lubricant additives to oils are designed to promote boundary lubrication and are classified as friction modifiers, anti-wear additives, and extreme pressure additives [43].
A general framework for boundary lubrication analogous to that developed by Stachowiak and Batchelor [41] follows. For surfaces where plastic deformation dominates, the true contact area $A_r$ is related to the yield stress $\sigma_y$ and the load $F_N$ as

$$A_r = \frac{F_N}{3\sigma_y}$$

(2.6)

Combining Equation 2.6 with Bowden and Tabor’s equation for adhesive friction (Equation 2.1) gives

$$\mu = \frac{F_F}{F_N} \approx \frac{S}{3\sigma_y}.$$  

(2.7)

Friction will be low when the interfacial shear strength is low and the material is hard. The interfacial shear strength can be minimized in two ways:

- Low shear strength materials can be used, promoting shear within the materials,
- Interactions between the surfaces can be minimized, promoting sliding between the surfaces.

Since it is typically impractical to design with materials which are both strong in compression and weak in shear, surfaces of materials which are strong in compression can be modified with materials that are weak in shear. Thus, both low surface energy (promoting sliding between apposed films) and low shear strength are desirable properties for boundary lubricants for reduction of both friction and wear [41,44].

The first approach above includes two distinct mechanisms (as classified by Stephen Hsu) [45]: sacrificial layer and low shear interlayer (diagramed in Figure 2). In
the sacrificial layer mechanism, the boundary lubricant can be sheared easily from the surface. The ease of shear corresponds to low friction and wear of the underlying surface is reduced or prevented because wear occurs in the lubricant layer instead. To be an effective sacrificial layer, the lubricant layer must be replenished more quickly than it is worn away. Anti-wear additives and extreme pressure additives (which are mechanistically similar to one another and classified by their ability to protect surfaces under high load and their corrosiveness) generally operate by reacting with surfaces to form soft sacrificial layers of soft molecules [46]. Sacrificial layers also often form by chemical reaction with the environment. One example is lubrication of steel surfaces in air by an oxide layer [45].

Low shear interlayer lubricants differ from sacrificial layers in that they are not removed from the contact interface by sliding. The most common examples are solid lubricants, most notably molybdenum disulfide and graphite, whose layered structure is essential for their ability to lubricate. The viscous boundary lubrication mechanism recently reported for porcine gastric mucin between poly(dimethylsiloxane) surfaces [47] is similar to the low shear interlayer mechanism.

The second approach, minimizing interactions between surfaces is perhaps more intuitive and is commonly accomplished in one of 3 ways. First, the surface can be modified with an adsorbed layer of material, as by friction modifiers, molecules with a long carbon chain and at least one polar group for binding to metal surfaces [43]. Hsu calls this approach a “friction modifying layer” [45]. Second, the chemistry of the surface may be altered, as by plasma treatment of poly(dimethylsiloxane) gels [48] or
passivation of diamond-like carbon films [49]. Third, the surface can be modified with a hard, wear-resistant material such as diamond-like carbon [49] (though this is arguably more a design choice than a lubrication strategy).

Figure 2: Boundary lubrication by (a) friction modifying layer, (b) sacrificial layer, and (c) low shear interlayer mechanisms.
2.2 Contact Mechanics

The field of contact mechanics explores stresses and strains near the contact region of multiple bodies in contact as a function of their topography, material properties and loading conditions [50]. Contact mechanics is closely linked to tribology, as seen in Sections 2.1.1 and 2.1.2 and has provided much of the groundwork on which the current understanding of tribology is based. Contact mechanics problems typically require numerical or finite element solutions but equations describing a number of idealized situations have been solved. Hertz [21] was the first to find analytical solutions for stresses and strains between spheres. He modeled the contact area between the spheres as occurring in a flat plane between them and stress was assumed to occur only along the loading axis. The pressure distribution across the contact was found to be

\[ p = p_o [1 - (r/a)^2]^{1/2} \]

where \( p_o \) is the maximum pressure, \( r \) is the distance from the center of the contact, and \( a \) is the contact radius [51]. Integrating the pressure over the area, the total load is

\[ F = \frac{2}{3} p_o \pi a^2 \]  \hspace{1cm} (2.8)

which, given expressions derived for the contact radius and the approach of distant points in the solid, becomes

\[ F = \frac{4E^*R^{1/2}}{3} \delta^{3/2}, \]  \hspace{1cm} (2.9)

where \( E^* \) is the reduced plane strain modulus,
\[
\frac{1}{E^*} = \frac{1-\nu_1^2}{E_1} - \frac{1-\nu_2^2}{E_2}.
\]

(2.10)

\(E\) is the young’s modulus, \(\nu\) is Poisson’s ratio, and the subscripts 1 and 2 indicate the two contacting bodies. Hertz’s formulation requires the following assumptions.

The deformation must be small compared to the size of each sphere (spheres are modeled as elastic half spaces).

The contact must be small compared to the radii of curvature (the profile is modeled as an elliptic paraboloid and only axial stress is considered).

Spheres are linearly elastic and isotropic with strains sufficiently small to conform to infinitesimal strain theory.

Surfaces are frictionless (only normal tractions are generated).

Incorporating adhesion into Hertz’s model led to the widely used Johnson, Kendall, Roberts (JKR) [52] and Derjaguin, Muller, and Toporov (DMT) [53] models. Originally thought to be conflicting, they were shown to represent opposite ends of a spectrum from long range forces and small elastic deformations (DMT model) to short range forces and large elastic deformations (JKR model) [54]. Other noteworthy contact mechanics models include Bradley’s model [55], which considers adhesive contact between rigid spheres and the Maugis-Dugdale (M-D) model [56], which is valid for the full range of short and long range adhesive forces and requires a numerical solution [19]. The appropriate model to use in each situation depends on the relative magnitude of adhesive loading and an elasticity parameter proportional to \(\mu = Rw^2 / E^{*2}z_0^3\) (Figure 3) [54,57], where \(w\) is the work of adhesion and \(z_0\) is an effective range of surface forces.
2.3 Physiology of Diarthrodial Joints

2.3.1 Diarthrodial Joints

The bones of humans and other vertebrates are linked together in a number of ways depending on anatomical need. They may be sutured together by fibrous connective tissue (as between bones in the cranium), connected directly to one another by ligamentous tissue (as between the tibia and fibula) or cartilaginous tissue (between vertebrae), or bound by a joint capsule containing synovial fluid (as in knees, hips, shoulders, etc). The final category is the only type that allows movement beyond a few degrees of flexure and are called diarthroses or diarthrodial joints. (Joints allowing no
movement are synarthroses and joints allowing little movement are amphiarthroses). Figure 4 gives a schematic showing key features of diarthrodial joints. Depending on the specific design of the joint, varying degrees of rotation and/or sliding may be allowed.

Figure 4: Schematic of a diarthrodial joint.

The joint capsule surrounding a diarthrodial joint is composed of two distinct layers [58]. The outer layer, composed of thick, dense fibrous tissue provides additional support to the joint beyond that provided by ligaments. It is continuous with the periosteum, the layer of dense connective tissue at the surface of all bones [59]. The inner layer, called the synovial membrane, lines the interior of the joint capsule and consists of an extensive matrix of collagen fibers, proteoglycans and glycoproteins [60]. It also extends to cover most intra-articular surfaces other than cartilage including bone
not capped by cartilage, ligaments, and menisci [59]. An incomplete cell layer of synovial macrophages (type A synoviocytes) and fibroblast-like cells (type B synoviocytes) occurs at the intimal region of the synovial membrane [58]. Type A cells phagocytose waste and cell debris and are involved in immune response. Type B cells produce components of the synovial membrane, including collagen and other proteins, and components of the synovial fluid, including the molecules PRG4 and hyaluronic acid (HA) [58].

Synovial fluid is a transparent colorless liquid with a viscosity similar to that of vegetable oil or motor oil [61]. Stretched between two fingers, it forms a string connecting them, a property called *spinnbarkeit* [62]. Synovial fluid is an ultrafiltrate of blood plasma with compositions of ions and small molecules comparable to that of plasma. pH and other ion concentrations are also similar to blood plasma [63]. The total protein content is approximately 28% of that of blood plasma (≈75 mg/ml [64]), with higher relative concentrations of smaller proteins [63]. As in blood, albumin is by far the most prevalent protein. HA, a flexible, linear, negatively charged polymer composed of alternating units of glucuronic acid and N-acetylglucosamine (Figure 5), is another major constituent of the synovial fluid at a concentration of 1-4 mg/ml in healthy individuals. With a molecular weight of 7 kDa to 4 MDa [65], it is primarily responsible for the viscosity of synovial fluid [62]. PRG4, which will be discussed in detail later, is present in human synovial fluid in a concentration of about 200 µg/ml [66].
2.3.2 Cartilage Structure

Cartilage tissue covers the ends of bones in diarthrodial joints and distributes localized loads across a large area and serves as a low friction, low wear bearing surface. It does not contain nerves and joint pain is sensed by nerves in the synovium, bone, or other surrounding tissues. Cartilage also does not contain blood vessels either, and nutrients are transported to cartilage by diffusion and bulk flow from the joint space [67]. Under normal conditions, cartilage is composed of 65-85% water with dissolved ions and other small solutes. The solid phase is made up primarily of collagen and proteoglycans, with collagen comprising at least 67% of the tissue’s dry weight [68]. Fixed negative charges in sulfate and carboxyl groups of the proteoglycans attract counterions, primarily Na$^+$ and Ca$^{2+}$, causing the tissue to swell through osmotic pressure. Lipids, phospholipids and other proteins and proteoglycans are also present in small amounts [69]. Chondrocytes synthesize, assemble, and maintain these extracellular matrix materials, but make up only a small fraction of the tissue (1-10%) [70].

Figure 5: Hyaluronic acid, an alternating block co-polymer of D-glucuronic acid and D-N-acetylglucosamine.
Collagen is composed of α-chains approximately 1000 residues long. The repeating sequence Gly-X-Y, where Y is often proline or hydroxyproline, allows the hydrogen bonding necessary to form a left-handed helix. These molecules combine to form a right-handed triple helix stabilized by both covalent bonds and hydrogen bonds which further aggregate, again stabilized by hydrogen bonds, to form collagen fibrils of variable thickness [71,72] (Figure 6). Collagen fibrils in cartilage are primarily composed of type II collagen with type IX and type XI collagen associated with the fibrils on the exterior [71,73,74]. It is hypothesized that type IX and XI collagen limit the size of type II collagen fibrils and studies using genetically altered mice appear consistent with this [13,71,75]. Measurements of collagen fibril diameters in cartilage range between about 10-50 nm for mice and about 10-150 nm for humans [76].

Figure 6: Hierarchical architecture of collagen fibrils. Figure adapted from [77] with permission.
Aggrecan is the predominant proteoglycan in articular cartilage and forms large complexes which are bound to and interwoven with the collagen network. The linear protein core attaches non-covalently via a link protein to HA, which is present in cartilage as well as synovial fluid, and a number of other locations in the body (Figure 7). As many as 50 aggrecan monomers may be bound to each strand of HA [78]. Large branched sugar chains, each with hundreds of strands of negatively charged chondroitin sulfate and keratin sulfate (repeating units of glucuronic-N-acetyl-galactosamine and galactose-N-acetyl-glucosamine, respectively), are arranged along the protein core of each aggrecan molecule, making each aggrecan molecule nearly 90% carbohydrate by weight [79]. Individual aggrecan units vary significantly in length due to cleavage by extracellular proteases including aggrecanases and matrix metalloproteases (MMPs) [80]. The overall molecular weight of the proteoglycan aggregates, consisting of hyaluronan plus associated aggrecan, is 50-100 MDa [81]. A number of smaller proteoglycans are also present in cartilage, playing a much smaller structural role. Most notable are the class of small leucine rich repeat proteins (SLRRPs) which are present in most connective tissue and have a role in regulating metabolism. Those in cartilage include biglycan, decorin, fibromodulin, and lumican. All but biglycan form complexes with type II collagen [80].
Figure 7: Schematic showing the structure of proteoglycan aggregates within cartilage.

The structure and properties of articular cartilage are depth dependent and cartilage is commonly divided into 3 zones; the superficial zone, the middle zone, and the deep zone, making up approximately 10%, 40-60%, and 30% of the tissue, respectively (Figure 8) [82]. Collagen fibrils are thinnest in the superficial zone, and typically oriented in the direction of shear. Superficial zone chondrocytes are also flattened. Fibrils are thicker in the middle zone and not consistently aligned, while cells are round. In the deep zone, cells are arranged in columns along thick collagen fibers that extend down into the calcified region [68]. Cartilage stiffness increases with depth; one study found stiffness ranging from 0.079 to 2.1 MPa from the surface to the base of the tissue [83]. Calcified cartilage, with apatite crystals randomly orientated with respect to
collagen fibers, is a transitional material between cartilage and bone [84]. With a stiffness an order of magnitude less than that of subchondral bone, it is thought to function as a buffer of intermediate stiffness between cartilage and bone, reducing stress concentrations under load [85].

Figure 8: Schematic showing collagen fibrillar orientation in superficial, middle and deep zones of cartilage and associated proteoglycans.

2.3.3 Cartilage Surface Properties

The surface of cartilage is chemically and morphologically distinct from the bulk tissue. However, the chemical identity and material properties of the upper nanometers to microns of cartilage are poorly understood. Many aspects have not been studied and a lack of consensus remains on many aspects that have.

A surface layer on articular cartilage was first observed by McConail as a bright line on the surface of cartilage by phase contrast microscopy [86]. He termed the layer the “lamina splendens.” It was widely thought to have merely been an artifact of the imaging technique [87] although a distinct surface layer was also seen on cartilage under
transmission electron microscopy (TEM) [88]. Indeed, although the lamina splendens has been further visualized by TEM [89,90], scanning electron microscopy (SEM) [91], polarized light microscopy [92] and other methods, the existence of a the lamina splendens remains controversial to this day [81].

The collagen fibrillar network within the upper few microns of cartilage is distinct from that of the rest of the superficial zone, as seen in SEM [91,93] and TEM [89] images. The term lamina splendens has been used to refer to this fibrous layer [88,93], depicted in the TEM image below (Figure 9). Because of the range of conflicting reports in the literature regarding the lamina splendens, the term “surface fibrous layer” will be used here to refer to this layer. The surface fibrous layer, 4-8 µm thick, can be separated from the underlying cartilage of human femoral heads by peeling in a longitudinal direction [91], indicating that it was only loosely connected to the underlying collagen matrix. SEM analysis did not reveal broken collagen fibrils in the underlying cartilage. Collagen fibers in the superficial zone are continuous with those in the periosteum [93] and those in the surface layer are continuous with the synovial lining [91]. Collagen fibrils in the surface fibrous layer are smaller in diameter than those in the superficial zone [91,93] and immunohistochemical analysis has indicated that the surface fibrous layer contains high concentrations of collagen I and III and a relatively low concentration of collagen II [94]. This is similar to the collagen distribution in synovial tissue [94] but in stark contrast to the composition of bulk cartilage.
A distinct non-fibrillar, acellular superficial surface layer is present superior to the fibrous surface layer [89,95-99]. This layer is resistant to hyaluronidase [99-101] and has been reported to be resistant to chondroitinase ABC [100,101], though chondroitinase ABC does at least partially expose collagen fibrils, enabling AFM imaging [89,98]. It is digested by trypsin and chymotrypsin, indicating that protein is a significant component in the layer [101]. Unlike lipids, the layer is also resistant to preparation steps for electron microscopy [102]. While this layer is also often referred to as the lamina splendens, we refer to it instead as the “surface amorphous layer” (SAL), a term which has also been used by other researchers [103-105]. It is typically measured as several tens of nanometers to a few microns thick [81,89,97,102], although a superficial
amorphous layer in excess of 100 µm, imaged on porcine knees by cryo-SEM, has been reported [105].

Until recently, the composition of the SAL was known almost exclusively based on the effect of digestion by various elements on its visual appearance in an electron microscope. Now it has also been assessed by immunolabeling and by performing assays on removed elements of the SAL. HA and fibronectin have both been detected at or near the cartilage surface by immunolabeling [90]. PRG4 has also been immunolocalized as being preferentially at or very near to the cartilage surface (Figure 10) [106]. The total amount of PRG4 in superficial zone cartilage was measured in one study as 0.5 µg/cm² [107], a coverage sufficient to form a 150 nm thick layer on a flat surface at atmospheric pressure [108].

Figure 10: PRG4 localized in by immunofluorescence in cartilage of a bovine mandibular chondyle. Note the preferential location at or near the cartilage surface. Scale bar = 100 µm. Reprinted from [106] with permission from Elsevier.
Methods to remove material from the SAL were devised only recently. In one approach, the cartilage surface was rubbed with a wet cotton plug. The plug was then rinsed in a controlled way, and the rinsing medium was then analyzed for protein, lipid, hydroxyproline, and sulphated glycosaminoglycan [103]. The removed fraction was found to contain approximately 54.5 µg/cm² lipid, 68.1 µg/cm² protein, 61.4 µg/cm² GAG; 30%, 37%, and 33% by weight respectively, assuming no additional types of material were present. Hydroxyproline was not found, indicating an absence of collagen. Since a total of 184 µg/cm² of material with density on the order of 1g/cm³ was detected, the average material removed by this procedure can be estimated as slightly less than 2 µm. This is consistent with the average SAL thickness of 1-2 µm measured by environmental SEM in the same study. Pressing a clean silicon wafer onto a cartilage surface yielded a surface whose ATR-IR spectrum was similar to that of dipalmitoyl phosphatidylcholine (DPPC), a lipid present in cartilage, suggesting that DPPC is present at the surface and is easily removed [109]. Freezing a sheet of ice onto the cartilage surface and lifting it off the surface after it froze removed more material from the surface, including HA and proteins. In this case, albumin was found to be the most prevalent protein [109].

2.3.4 Cartilage Mechanics

The properties of cartilage as a bearing tissue are unsurpassed. Human joints are able to withstand millions of loading cycles with loads regularly 3 times the individual’s body weight, sometimes 8-10 times higher [1]. Peak pressures across human joints during normal activities range between 5-20 MPa [110,111], and strains can reach 40%
Coefficients of friction are typically in the range of 0.005 to 0.04 [5-9] and coefficients of friction as low as 0.002 have been reported [113]. Although cartilage is relatively thin at, for example, 2.5 mm or less in a healthy human knee [114], cartilage effectively distributes loads across a large area on a bone by deforming to allow contact over a large area, approximately 11 cm² in human knees [115].

The stress-strain relationship for cartilage is non-linear and is poroelastic [116], rather than viscoelastic, with non-linearities coming in large part from the interaction between solid and fluid components of the tissue. Cartilage mechanics is generally described by the biphasic model [117] or the poroelastic model [116]. The poroelastic model, originally developed to study soil mechanics, considers a porous, fluid saturated, elastic medium in which fluid is allowed to flow from areas of higher pore pressure to lower pore pressure. The development of mixture theory [118] led to the formulation of the biphasic model for articular cartilage [117]. In this model, cartilage is considered to be the combination of a solid and fluid phase which are independent but interact with one another. This biphasic model describes cartilage mechanics as driven by three major internal forces: stress due to deformation of the solid matrix, fluid pressure, and frictional drag between solid and fluid phases. In the simplest formulation of the model, the solid matrix is assumed to be linearly elastic and isotropic with only 3 material coefficients needed to describe the tissue: the aggregate modulus, the Poisson’s ratio, and the hydraulic permeability. These values can all be obtained from the confined compression response of the tissue in creep or stress relaxation [119]. A number of additions have been made to biphasic and poroelastic model approaches, most notably the incorporation
of tension-compression nonlinearity of the solid matrix into the biphasic model [120], or similarly the incorporation of fiber reinforcement into the poroelastic model [121], and the treatment of ions as an additional phase [122,123].

Thus, compressive loads on cartilage are borne in most cases primarily by pressurized liquid, due to the unique material properties of cartilage [124]. The biphasic nature of cartilage, its tension compression nonlinearity, and its low hydraulic permeability, are all necessary for significant load bearing by interstitial fluid. Cartilage is much stronger in tension than in compression. (Tensile strength arises due to the high tensile stiffness of collagen fibers while compressive strength is due to swollen proteoglycans enmeshed in the collagen network [69,70,125]. Under a compressive load, fluid flows out of the contact region towards unloaded areas of the tissue. Drag between solid and fluid components results in radial stress in the solid matrix. Because cartilage is relatively strong in tension, it deforms only slightly as fluid is forced through it. Since the solid phase is relatively weak in compression, fluid supports more of the compressive stress as long as it remains pressurized. Because the hydraulic permeability of cartilage is low, this time constant is typically on the order of several minutes [124].

The depth-dependent variation of cartilage properties further accentuates load sharing by the fluid phase. The surface zone, specifically the low hydraulic permeability of the surface zone, maintains fluid pressure and decreases solid pressure by promotong drag between fluid and solid phases [126]. The hydraulic permeability of the superficial zone is larger than that of the deep zone, $3-6 \times 10^{-13}$ cm$^3$·s/g compared to $1.5-3 \times 10^{-13}$ cm$^3$·s/g [127]. Because the surface zone is softer in compression than middle or deep
zones, the ratio of tensile to compressive stiffness is relatively large. This promotes load transfer to the fluid phase while the higher compressive stiffness of lower zones prevents transfer of concentrated stress to bone even under prolonged loading. One finite element study has also found that the presence a thin soft biphasic layer above cartilage, possibly analogous to the lamina splendens, resulted in the increased load transfer to the fluid phase and more efficient distribution of loads across the cartilage surface [104].

2.4 Cartilage Tribology

2.4.1 Overview

A number of lubrication mechanisms have been shown to contribute to cartilage lubrication, which attests to the versatility of cartilage and synovial fluid in minimizing friction and wear. While disagreement remains regarding the presence and effectiveness of various lubrication mechanisms and the extent to which they occur physiologically, a picture emerges in which cartilage friction and wear is mediated by a number of cooperative mechanisms, some common in the natural world, some unique to cartilage.

2.4.2 Biphasic Lubrication

The majority of the stress between cartilage surfaces is at least temporarily borne by the incompressible fluid phase of the tissue as discussed in the previous section. Frictional stress between solid tissue components is thus dramatically reduced as long as fluid is present as a significant component of the tissue and is prevented from flowing out of the contact area. Friction increases as the fluid fraction of the tissue decreases under prolonged loading, as illustrated in Figure 11 [128]. Load support by the pressurized
fluid dissipates slowly in the healthy joint, typically on the order of minutes to tens of minutes [124,129]. High joint congruency and low hydraulic permeability, both of which may be compromised by disease, help to maintain interstitial fluid pressure [130]. A strong inverse correlation ($r^2 = 0.96$) has been established between interstitial fluid pressurization and coefficient of friction (Krishnan, 2004), using a confined compression test setup and a porous indenter. Experimental results were described well by the biphasic model. Translation of the contact area, as occurs in joint motion, allows the fluid content of the tissue to remain high provided that the rate of translation is sufficiently high relative to the diffusive velocity of fluid flow within the tissue [131], which is true for loads and speeds typically seen by cartilage [128]. Though only indirectly discussed in the last reference, it is clearly also important that the translation distance of an oscillatory load is sufficiently large relative to contact area to allow the fluid phase to be replenished.

![Figure 11](image-url)

**Figure 11:** Experimental data from [128] showing the time-dependent decrease of fluid load support ($W^f/W$) and corresponding increase of friction ($\mu_{eff}$). Reprinted with permission from Elsevier.
Charles McCutchen showed in the late 1950s that the coefficient of friction on cartilage significantly increases after prolonged loading, even if fluid remains present in the test system, and was the first to recognize the large role interstitial fluid pressure plays in load support and lubrication of joints [8,132]. He theorized that upon asperity contact, hydrostatic pressure in the joint expelled a lubricating fluid film that prevented cartilage contact in adjacent areas [133]. This process was called weeping lubrication. Later researchers, most notably Gerard Ateshian, have largely concluded that hydrostatic pressure within the cartilage reduces friction between joints by reducing the normal load on the solid matrix, effectively reducing the number of points of true solid-solid contact rather than by expelling liquid into the contact region [128]. Disagreement between Ateshian and McCutchen on this point persists to today [128,134-137]. Regardless, the coefficient of friction can be predicted as

\[
\mu_{\text{eff}} = \mu_{\text{eq}} \left[ 1 - (1 - \phi) \frac{W^p}{W} \right],
\]

(2.11)

where \( \mu_{\text{eff}} \) is the effective coefficient of friction, \( \mu_{\text{eq}} \) is the equilibrium coefficient of friction in complete absence of fluid pressure, \( \phi \) is the area fraction of solid contact, \( W^p \) is the load supported by fluid pressure, and \( W \) is the total load [128,138].

### 2.4.3 Hydrodynamic Lubrication

Many aspects of the cartilage-synovial fluid system initially appeared consistent with hydrodynamic lubrication, including the shape of the joints, the viscosity of the synovial fluid, and the low coefficient of friction measured [139,140]. Theoretical
analyses indicated that fluid film thickness that could be developed during cartilage sliding were less than the roughness of the tissue if the cartilage were assumed to be rigid, but greater than asperity heights if their deformation was accounted for (elastohydrodynamic lubrication) [141,142]. However, experimental evidence has generally been in favor of boundary or mixed lubrication regimes rather than full hydrodynamic lubrication [143]. Friction in joints has been typically measured as either independent of velocity [6] (indicative of boundary lubrication) or decreasing with increasing velocity [144] (indicative of mixed lubrication).

A systematic analysis performed recently measuring cartilage friction as a function of a range of sliding speeds and loads [145] and provides strong evidence for the occurrence of mixed lubrication in joints. Strains ranged from 5%-50%, a physiologically relevant range for load bearing joints [112] and the sliding speed was 0.1-50 mm/s. (Cartilage sliding speed in the hip during normal walking is on the order of 50 mm/s and speeds 10 times faster are seen in the glenohumeral joints of baseball pitchers [146].) A combination of boundary and mixed lubrication was observed and the onset of mixed lubrication occurred beyond a velocity of 0.1 mm/s at 15% strain and beyond a velocity of 1.0 mm/s at 50% strain in PBS. In synovial fluid, mixed lubrication began beyond 0.1 mm/s at 30% strain and beyond 0.75 mm/s at 50% strain. To the extent to which fluid film lubrication occurs in joints, it is believed to be mixed rather than full hydrodynamic lubrication.
2.4.4 Squeeze Film and Boosted Lubrication

The nature of contact between cartilage surfaces is dependent to a large extent on the path through which they are loaded. As surfaces are brought together in the presence of a fluid, contact is delayed as fluid is squeezed out from between them, a form of hydrodynamic lubrication referred to as squeeze film lubrication [41]. Because cartilage is porous, the system becomes much more complicated. Large molecules in synovial fluid, including HA and PRG4, are filtered out as synovial fluid is forced into the cartilage. This makes the synovial fluid more viscous within the contact, prolonging squeeze film times. It also leads to the build-up of solid synovial fluid components near the joint surface, which has been proposed to have a lubricating effect and has been called boosted lubrication [147].

An important and largely unanswered question in the topic of squeeze film lubrication in articular joints is how far, if at all, cartilage surfaces may become separated from one another. The thickness of a hydrodynamic film between cartilage surfaces is a good place to start [9] but since only mixed rather than full hydrodynamic lubrication typically occurs in joints, the question is often one of depletion of a trapped pool of liquid [136] rather than one of squeeze film lubrication. Ligaments and muscles provide compressive forces even across unloaded joints, holding the cartilage surfaces together under normal situations. It has been proposed that the inertia of a lower extremity during the swing-out phase of gait may be sufficient to separate joints [5], but no focused study has been performed to assess this. A mathematical model of squeeze film lubrication predicts separation in the range of hundreds of milliseconds for sufficiently large initial
heights [148]. Though a delayed increase in friction upon loading, consistent with squeeze film lubrication, has been observed experimentally, sliding has always been observed to be accompanied by a level of friction typical for initial stages of cartilage contact [9,149]. This indicates that if the cartilage surfaces ever were fully separated the squeeze film was not sufficient to keep the surfaces fully separated long enough to measure friction.

The formation of a synovial gel has been predicted by a theoretical analysis of squeeze film lubrication in a human ankle joint during walking [150], and has been experimentally reproduced by filtering synovial fluid through cartilage [151]. To this author’s knowledge, this layer has not been identified after normal loading during joint motion. It would, however, be expected to be present in nearly all experimentally analyzed joints, depending on the dissociation rate of the layer, the joint’s loading history, and the extraction procedure. A film formed by combining HA and phospholipid with properties has been found to have properties similar to those of the surface amorphous layer [109]. It may be that synovial fluid filtration by cartilage, classically called boosted lubrication, forms to a large extent the surface amorphous layer on cartilage. To this author’s knowledge, direct experimental evidence of boosted lubrication has not yet been obtained (see Section 6.5).
2.4.5 Boundary Lubrication

2.4.5.1 Overview

As experiments by a number of researchers have demonstrated, synovial fluid provides boundary lubrication which further reduces friction between hydrostatically lubricated cartilage surfaces [6,62,152,153]. The magnitude of the reduction in the coefficient of friction is typically measured as on the order of 1.5-3x [145,154-157] but has not been seen under all test conditions [6]. Since nearly all friction and wear on joints occurs in the boundary lubrication regime, successful boundary lubrication is considered important to joint health [66,158]. Similarly, boundary lubrication is the regime that controls the life of many mechanical components [159].

John Charnley was the first to propose boundary lubrication in joints [6]. He measured coefficients of friction of 0.01-0.02 on cartilage and showed that these did not arise from hydrodynamic lubrication as friction was velocity-independent. Because the biphasic mechanism of lubrication had not been determined, he proposed that nature may have discovered a system which is very slippery under boundary lubricating conditions. Later research has shown that boundary lubrication acts in combination with biphasic lubrication, though some variation in the relationship has been reported. Some experimental data shows friction reduced by a similar ratio regardless of the presence of interstitial fluid pressurization [154] as predicted in equation 2.11 in Section 2.4.2. Other studies indicate that synovial fluid has little to no effect on $\mu_{eq}$, the coefficient of friction in absence of interstitial fluid pressure [145,160]. In the latter cases, boundary lubrication apparently becomes operative as soon as fluid pressure begins to dissipate.
2.4.5.2 Lubricants

The extent to which distinct molecular constituents contribute to boundary lubrication by synovial fluid has been debated and PRG4, HA, and phospholipids have all been implicated as contributing to boundary lubrication [10]. Digesting synovial fluid with trypsin was seen to eliminate its lubricating effect in the boundary regime [152,161]. This eventually led to the isolation of lubricin, a heavily glycosylated 225 kDa glycoprotein, as the boundary lubricating constituent of synovial fluid [162-164]. Lubricin was later shown to be homologous to surface zone protein (SZP) [165,166], and these proteins will be referred to collectively as PRG4 in this document, recognizing that variations exist in mRNA splicing, post-translational modification and cellular origins [167]. More background information on the molecule PRG4 is given in Section 2.5.

Phospholipids, more traditionally thought of as boundary lubricants, were also isolated from synovial fluid and shown to lubricate in vitro [168]. In one study, phospholipids isolated from a lubricin solution were found to greatly reduce friction between two quartz plates, while other fractions of the original lubricin solution did not lubricate. It was suggested that lubricin may serve as a carrier molecule, depositing the lubricating phospholipid on articular surfaces [169]. An enzymatic study by the same group in which synovial fluid samples were incubated with enzymes to destroy HA, phospholipid, and protein agreed with this finding [170]. This enzymatic study was repeated the next year by a different group who obtained opposite results, finding that phospholipase digestion did not effect the lubricating ability of the fluid but that protein
digestion increased friction [171]. A recent study has also found that a number of phospholipid formulations lubricate cartilage more effectively than synovial fluid [172].

HA, a high molecular weight (Mw ~7 kDa to 4 MDa) [173], linear, negatively charged biopolymer, is composed of alternating units of glucuronic acid and N-acetylglucosamine (see Figure 5 ) [174]. It is a major constituent of the synovial fluid at a concentration of 1-4 mg/ml in healthy individuals, [10,175] and is primarily responsible for the viscosity of synovial fluid [72]. HA was a part of early joint lubrication models [176] and administration of exogenous HA is still used clinically to treat osteoarthritis. Some experiments have found HA to lubricate [10], while others have shown no effect [15,152]. There is also conflicting evidence as to a possible synergistic lubricating effect between PRG4 and HA [10,108,177,178], with one study finding that fragments of HA were necessary for lubrication by synovial fluid protein but that partial digestion of HA did not affect lubrication [161]. Tests on cartilage have generally shown PRG4 to be the most effective boundary lubricant, with smaller, or perhaps negligible contributions from HA and phospholipids [10,152,179].

2.4.6 Cartilage Wear

Very few studies of cartilage wear have been undertaken, which is surprising given the direct relevance of cartilage wear to joint disease. Cartilage wear lines, wear tracks that run parallel to the direction of joint motion, can be visualized on cartilage of aging joints and are believed to progress to form superficial fibrillation seen in early osteoarthritis [180,181]. While biomechanical analysis of wear lines has been informative [180] the prolonged timescale over which wear occurs in vivo limits the
studies that can be performed. Thus, most studies of cartilage wear have been performed in vitro under conditions designed to accelerate wear.

Cartilage wear was first measured by William Simon on a machine designed and built by Charles McCutchen [182]. A steel rotary file was run in reverse at a speed of 120 revolutions per minute against cartilage of human patellae and canine femoral heads. Wear was quantified both by measuring the depth into which the file penetrated the cartilage by weighing filtered wear debris. The effect of a number of cartilage sample processing steps and types of lubricant solutions were tested. However, statistically insufficient sample sizes were used to assess the role of lubricants. Synovial fluid decreased wear approximately 2x relative to normal saline. Digestion of a “synovial mucin” solution, consisting of concentrated synovial fluid constituents, with trypsin led to increased wear compared with results from treatment with undigested synovial mucin. Digestion of either synovial fluid or synovial mucin with hyaluronidase led to increased wear relative to undigested solutions. Digesting cartilage in collagenase, fixing it in formalin, and extracting proteoglycans with lanthanum chloride resulted in decreased wear while treatment with ferric chloride, which increased the cartilage stiffness, resulted in increased wear. Fibrillated cartilage also wore much faster than non-fibrillated cartilage.

A few other studies have also investigated the role of lubricants in mediating cartilage wear. Multiple studies have reported that phosphatidylcholine reduces cartilage wear [183,184] and phosphatidyl choline species with a phase transition temperature near 37°C are most effective. A cartilage-cartilage system was used in those experiments and
wear was detected by measuring GAG concentration in the bathing solution [184] or by analyzing the shape of wear particles [183]. In one study, the effect of HA on wear was also measured and yielded slightly reduced wear values relative to normal saline [184]. Radin et al. [185] measured cartilage wear under a combination of oscillatory and impact loading. While they did not see an effect of synovial fluid in reducing wear, a loss of surface integrity was correlated with a slight increase in wear, and stiffening the subchondral bone increased wear significantly. In these experiments, it appears likely that the observed wear was primarily due to impact loading, which was applied at strain rates of 8000-10000% per second, orders of magnitude higher than those occurring in natural cartilage deformation. Impact loading had been previously shown by two of the same authors to have a dramatic and detrimental effect on cartilage integrity [186]. Michael Furey has reported measurements of cartilage wear in the presence of synovial fluid, lubricin, and HA, finding that synovial fluid was effective in reducing cartilage wear but PRG4 was not particularly so [187]. HA was reported as having a variable effect. However, details of these studies have yet to be reported in peer-reviewed journals.

Lipshitz, Etheredge and Glimcher contributed significantly to the understanding of cartilage wear with a series of systematic studies [188-191]. A machine was built to wear cartilage plugs (specifically osteochondral plugs from bovine femoral chondyles) against a metal plate. They examined the effect of the roughness profile of the counterface on wear characteristics, concluding that wear was largely abrasive against rougher surfaces (smoothed with only medium grade emory cloth) but adhesive against
well polished steel. Cleaning cartilage residue off one of the smoother plates mid-
experiment did not affect the wear rate, consistent with what would be expected for
adhesive wear [191]. Wear was assessed by hydroxyproline content of removed material,
which they showed to be nearly constant through the depth of the tissue [189]. Digesting
cartilage with trypsin increased wear while incubation in CaCl₂, MgCl₂ or EDTA reduced
it [189].

More recently, John Fisher’s group has performed wear tests including
measurements of cartilage wear in a (cartilage) pin on (stainless steel) disk setup [192-
194] and the results have been characterized by biochemical analysis [192], profilometry
[193,194], and micro-MRI [193]. One particularly noteworthy finding from these studies
has been the importance of conformity between surfaces in preventing wear, a finding
with potential importance for understanding injury-induced OA, as well as
hemiarthroplasty design [193]. Finally, cartilage wear has also been detected by SEM
analysis of cartilage surfaces [155,195] and India ink staining followed by light
microscopy [180].

Biphasic lubrication appears to have a limited effect, if any, on reducing cartilage
wear. Removing the load from cartilage during a wear experiment and allowing it to
rehydrate was found in one study to have no effect on the wear rate [191]. Furthermore,
while cartilage friction becomes significantly higher during prolonged loading (see
Figure 11 on page 35), wear rates after prolonged loading have been reported as slightly
higher [182], slightly lower [191], or moderately lower [184] than the wear rates upon
initial loading. In the first two cases the contact location was static on the cartilage while
in the third case reciprocating sliding was performed with an amplitude 4x smaller than the contact diameter. These surprising findings underscore the importance of investigating friction and wear individually.

Quantitative measurements of cartilage wear have typically involved large worn volumes. The most sensitive surface level measurements to date have been performed by confocal microscopy, with a resolution limit of hundreds of nanometers [196]. However, molecular and supramolecular scale is believed to contribute to osteoarthritis [13]. Thus, there is a need for direct surface level measurements of cartilage wear, as we have developed and present here in Chapter 5.

2.5 The Molecule of Interest: PRG4

2.5.1 PRG4 Structure

PRG4 is an alternately spliced 1404 amino acid protein with 12 coding exons as schematically shown in Figure 12. It is expressed by the gene Prg4 in a number of tissues throughout the body, including superficial zone cartilage [165], synovial membrane [197], meniscus [198], tendon [167,199,200], ligament, heart, lung, and liver [167,201]. PRG4 from synovial fluid has been named lubricin [163] and in cartilage it was first called superficial zone protein [202]. Reports of the molecular weight of lubricin range between 130-280 kDa [163,164,203] while SZP has been reported as 345 kDa [202]. We note, however, that lubricin as first isolated contained an average of only 800 amino acids [203] while SZP contained 1311-1404 (splice variants had exons 4 and 5 removed) [165]. Also, an unidentified synovial fluid protein ran parallel to the 345 kDa SZP on a gel
Both lubricin and surface zone protein were first isolated from bovine joints [164,202].

Figure 12: Schematic of a PRG4 molecule highlighting important structural features. Reprinted from [165] with permission from Elsevier.

The N- and C-terminal regions of PRG4 are globular and are separated by a long, heavily glycosylated mucin-like domain encoded by exon 6 [204]. β(1-3)Gal-GalNAc oligosaccharides are O-linked to a tyrosine residues of a degenerate repeating KEPAPTT sequence, giving PRG4 a structure resembling that of a mucin [205]. The C-terminus contains a domain similar to hemopexin [165], which could potentially mediate interaction with HA [206]. It also has potential heparin and chondroitin sulfate binding sites [165]. Imaging of lubricin by SEM [203] and AFM [207] has shown it to be semi-flexible, having an extended conformation with a contour length of approximately 220 nm and some propensity for kinks in the central region. Lubricin is approximately 40% amino acid and 50% carbohydrate by weight [163]. The isoelectric point of lubricin is in the range from 7.8-8.1 [208]; the large number of negative charges associated with
carbohydrate groups is balanced by positive amino acids (primarily lysine) in the protein chain.

PRG4 forms dimers via disulfide bonding between N-terminal regions [209,210]. A networked PRG4 aggregate has also been imaged, on a graphite surface [211]. AFM imaging of PRG4 molecules on a positively charged functionalized mica surface, however, showed no aggregation beyond dimers [207] and further oligomerization beyond dimers has not been seen in solution.

2.5.2 PRG4 Function

2.5.2.1 PRG4 Behavior on Surfaces

PRG4 binds readily to a wide range of surfaces, including hydrophilic, hydrophobic, and positively and negatively charged surfaces [108,212]. Values for surface coverage have been obtained by surface plasmon resonance as 0.23 µg/cm² and 0.33 µg/cm² for –OH and –CH₃ functionalized surfaces, respectively [108], values typical for adsorbed mucin layers [213-215]. These values are only slightly lower than the 0.5 µg/cm² measured by ELISA for PRG4 binding to bovine cartilage plugs [107], meaning that cartilage does not bind PRG4 much more efficiently than uniform –OH or –CH₃ functionalized surfaces. Complicating this picture however, are findings that reduction followed by alkylation removes PRG4 from cartilage [107] but has little effect on PRG4 binding behavior on model surfaces [207].

PRG4 molecules adopt an extended, brush-like conformation on surfaces even at low solution concentrations (~100 µg/ml). This is supported by long-range steric
repulsion forces measured with AFM and SFA, and the fact that the force interactions between PRG4 coated surfaces can be described well by the Alexander de-Gennes model for polymer brushes [108,212], even at sub-monolayer coverage. Substrate surface chemistry and surface charge affect the details of PRG4 surface conformation. PRG4 adopts a loop-like conformation on uncharged hydrophobic surfaces prepared using alkane thiols and an extended, tail-like conformation on similarly prepared uncharged hydrophilic surfaces [108]. A mixture of loops and tails occurs on negatively charged mica surfaces [212] for non-reduced PRG4 and a predominantly loop-like conformation occurs for reduced PRG4 [207].

### 2.5.2.2 PRG4-Cartilage Interaction

The details of the interaction between PRG4 and cartilage are not well understood, in large part because the surface of cartilage is not well characterized. Specifically, it is not known whether PRG4 binding to cartilage is specific or non-specific or with what molecular components of the cartilage surface it interacts. However, a study using partial recombinant forms of PRG4 has shown that the C-terminal region binds to the cartilage surface while the N-terminus does not [209] (Figure 13).

Two research groups have performed binding assays for PRG4 on cartilage. A radiolabeled binding assay showed the surface concentration of PRG4 increasing linearly up to a point of saturation [162]. Because only PRG4 in solution was labeled, this result could potentially mean either an increase in the total PRG4 at the cartilage surface or exchange between PRG4 in solution and that on the surface. A more recent study in
which binding was quantitatively assessed by ELISA showed that soaking explants from healthy cartilage in PRG4 solutions did not lead to additional PRG4 binding [107].

PRG4 binds preferentially to the cartilage surface and binds poorly to the cut sides of the tissue [107,209], consistent with the theory that the articular cartilage surface contains a finite number of binding sites for PRG4. After removal of PRG4 (by high salt, SDS, reduction and alkylation, or HAase) the original layer (0.5 µg/cm²) can be restored by soaking in a solution containing PRG4 [107]. After digestion with HAase, even more PRG4 could be adsorbed than had been present initially. This is possibly because more binding sites were opened up by HAase, a highly abrasive chemical that digested nearly all of the proteoglycan content in the 0.6 mm cartilage discs used.

Figure 13: Schematic of PRG4 adsorption on cartilage highlighting: cartilage binding by the C-terminus [209], dimerization of the N-terminus [209], and extensive negative charges associated with glycosylation in the protein’s central mucinous domain [204].

Hydrophobic interactions, often a factor in protein adsorption are also expected to be a factor in PRG4-cartilage interaction. Contact angles of the normal articular surface can approach 100 degrees [216] and the surface is partially composed of lipid [109].
Circumstantial evidence favoring the possibility of PRG4-cartilage interaction via hydrophobic interactions is seen in the similarity of concentration-dependent lubrication by PRG4 on cartilage and methyl functionalized model surfaces. On cartilage, lubrication by PRG4 becomes effective at a concentration above 10-20 µg/ml, with the lowest coefficient of friction seen at 50 µg/ml (friction at higher concentrations was not dramatically larger) [160], nearly identical to the concentration dependence seen on the hydrophobic model surfaces [15]. However, PRG4 can be removed from cartilage by high salt concentrations, which strengthen hydrophobic interactions [209].

2.5.2.3 Lubrication by PRG4

Relatively little is known about the mechanism by which PRG4 lubricates and conflicting evidence exists regarding conditions under which it may be expected to lubricate. It is unknown to what extent the presence of PRG4 modifies surface properties by becoming an integral part of the cartilage surface, which motivates our study of surface properties of \textit{Prg4/-} cartilage. One study has reported the formation of a lubricating film on cartilage that originated from synovial fluid and continued to lubricate even after the synovial fluid was exchanged with buffer [62]. In another study, though, synovial fluid needed to be present in solution to lubricate effectively [217]. To explain this apparent discrepancy, it is informative to consider the differences in the test setups between those two studies. In the first case, friction was measured on excised dog ankles as they were rotated through an arc of 36° under a load of 20-80 lbs [62,218]. In the second case 3 mm diameter chondral plugs from the femoral patellar groove of calves
were linearly oscillated against a glass counterface with a constant strain of 30% [217]. Squeeze film loading conditions occur in the presence of synovial fluid in the first case but not in the second, providing a possible explanation for the difference in the observations. Boundary lubrication by synovial fluid is apparently more effective (reducing friction by 2-3x) in cases where articulating surfaces are statically loaded, then slid [157,217], and less effective (reducing friction by 0.15-0.5x) in cases where surfaces are slid into contact [131,155]. Clearly, a study directly exploring the effect of loading path on boundary lubrication would be a valuable addition to this literature.

PRG4 lubricates a range of articulating surfaces under boundary lubrication conditions, including cartilage-cartilage [10] cartilage-glass [162,217] latex-glass [171,219] and mica-mica [212]. It has not been seen to lubricate surfaces functionalized with poly-lysine or monolayers of amino or hydroxyl terminated thiols [108,212]. In addition to reducing friction, PRG4 has been found to inhibit synovial cell overgrowth, reduce adhesion of synoviocytes to tissue culture polystyrene [11], and prevent cartilage-cartilage integration [220].

The effect of PRG4 on the frictional properties of the cartilage surface itself, has not been shown consistently in previous studies. Removing the surface layer by rubbing the cartilage surface with a cotton plug soaked in SDS [103], rubbing the surface layer away by prolonged sliding [221], and cutting off the upper surface of cartilage [222] all did not result in a detectible increase in the coefficient of friction under boundary lubricating conditions. Removal of PRG4 from the cartilage surface by NaCl extraction increased the frictional properties by only about 20% in a system where whole synovial
fluid reduced friction by a factor of 3 relative to PBS [223]. A decrease in friction did correlate with SZP expression in one study though [224]. A possible explanation for this apparent discrepancy may be a transition from a friction modifying layer regime to a sacrificial layer regime (see Figure 2 on page 17). Pressures applied in the latter study were relatively low at 0.1 MPa, possibly low enough to preserve a lubricin layer that would otherwise have been sheared away.

Some insight into PRG4 function has been gained by measuring lubrication following chemical modifications on PRG4. Lubrication is lost following enzymatic removal of the glycosylations of the mucin-like domain [204]. This observation is in line with the notion that hydration, associated with carbohydrate groups, contributes to lubrication [204,225] as has been seen in polymeric systems [226-228]. Cleavage of the protein backbone by chymotrypsin digestion causes a significant loss of lubrication accompanied by a loss of steric repulsion between PRG4 layers [207]. Chemical reduction followed by alkylation to remove disulfide bonds also results in a loss of lubrication with no apparent difference in the extent of PRG4 adsorption or steric repulsion [207].

Wear protection by PRG4 has not been shown directly on cartilage prior to the work presented in this thesis (4.3), though wear measurements by William Simon [182] provide indication of wear prevention by PRG4, as discussed in Section 2.4.6. PRG4 can also prevent wear between mica surfaces [212]. Wear protection on mica was also obtained with reduced and alkylated PRG4 (which could no longer form disulfide bonds) even though friction forces measured between layers of reduced and alkylated PRG4
were much higher [212]. Digestion of adsorbed layers by the protease chymotrypsin, however, dramatically increased both friction and wear and resulted in a significant decrease in the thickness of the adsorbed layer [207].

### 2.5.2.4 Chondroprotection by PRG4

Evidence for chondroprotection by PRG4 has come from three major sources: studies of *Prg4* knockout mice, studies of CACP patients, and a single study of intra-articular injection of PRG4 in a rat model for OA [229].

Camptodactyly-arthropathy-coxa vara-pericarditis syndrome (CACP), a rare autosomal recessive disorder has been linked to truncating mutations of PRG4 [230]. The disease phenotype and study of synovial fluid from CACP patients has given some clues towards the role of PRG4 *in vivo*. It is characterized by synovial hyperplasia (overgrowth) and non-inflammatory cartilage degradation and joint failure. Patients with this disorder have flexion contractures of phalangeal joints at an early age and develop a non-inflammatory arthropathy which leads to precocious joint failure [231]. Synovial fluid from patients with CACP does not have the ability to lubricate in the boundary mode; rather it lubricates similarly to normal saline [211]. Interestingly, one disease causing mutation on PRG4 involves the loss of just 8 amino acids, including one cysteine. This may indicate that intra or inter-molecular bonding is necessary for PRG4 to function properly [232].

Mice lacking *Prg4* exhibit changes similar to those of CACP patients, including an abnormal cartilage surface, synovial hyperplasia, and eventual joint failure [11]. Joint
shape and structure appear normal at birth but evidence of abnormal protein deposition along the articular cartilage surface appears within the first week. Synovial hyperplasia occurs by two weeks and progresses with age. By four months, mice exhibit joint swelling and gait abnormalities. By 12 months, joint shape is highly abnormal with destruction of articular cartilage, bony overgrowth (osteophytes), and subchondral cyst formation. Whole joints of these mice show reduced friction in a pendulum friction tester and the superficial collagen network is disrupted [211]. Further characterization of the knockout mouse strain with an emphasis on cartilage surface properties is presented in Chapter 4.

2.6 Osteoarthritis

2.6.1 Summary of the Disease

Osteoarthritis (OA) is a degenerative joint disease affecting over 40 million people in the United States alone [4]. A range of degenerative phenotypes are classified as OA, and the American College of Rheumatology has defined it as a “heterogeneous group of conditions” rather than a single condition [233]. The disease progression involves a vicious cycle of abnormal mechanical stresses, and biochemical imbalances and inflammation that lead to a loss of proteoglycans, disruption of the collagen network, and alterations in other joint tissues such as the synovium and subchondral bone [2,234]. Matrix degeneration generally begins in superficial cartilage and progresses to deeper regions of the tissue [235] and early degenerative changes include softening and fibrillation of superficial tissue [236]. Fibrillation progresses into fissures that extend
into the middle zone and cartilage erosion follows. Cartilage is eventually eroded down to the bone, resulting in joint deformity, osteophytes (bone spurs), and severe joint pain.

Varied and often interrelated factors contribute to the onset and progression of the disease, including hereditary, developmental, metabolic, and mechanical factors [237]. Factors can be broadly classified as (a) drivers of abnormal stresses on the joint and (b) drivers of aberrant cartilage physiology [234]. Healthy cartilage exists in a balance between synthesis and degradation but degradation becomes imbalanced and uncontrolled in OA. Cartilage breakdown occurs primarily via two families of enzymes abbreviated MMP (matrix metalloproteinases which break down collagen and other proteins) and ADAMTS (a disintegrin and metalloproteinase with thrombospondin motif, a class of aggrecanases). These are upregulated by pro-inflammatory cytokines, most notably IL-1 and TNF-α, which, along with their receptors, are upregulated in OA [234]. These cytokines also promote cartilage breakdown by downregulating inhibitors of degradative enzymes and stimulating the release of nitric oxide, which promotes chondrocyte apoptosis [234], downregulation of anabolic factors, and upregulation of catabolic factors [238]. Increased catabolic activity in early OA is accompanied by increased anabolic activity but catabolic factors eventually dominate [239]. Age-related factors contribute to the inability of anabolic pathways to check and compensate for cartilage deterioration in early OA. The sensitivity of chondrocytes to growth factors decreases with age and the quality of matrix material synthesized also declines [240]. For example, GAG molecules tend to be smaller and link proteins tend to be less functional.
Chondrocyte gene expression varies in response to mechanical loading and loading is necessary for normal cartilage function. For example, fixing canine knee joints to prevent movement has been shown to result in a significant loss of proteoglycan content. However, abnormally large mechanical stimuli administered to chondrocytes stimulate inflammation, leading to cartilage breakdown [241]. At least partially for this reason, muscle weakness, ligament stiffness and, more generally, joint instability are significant risk factors for OA [242]. Tears in menisci and ligaments often lead to OA [241] and ligament transection and meniscal resection are commonly used to induce arthritis in animal models [243]. It has been was shown that following ACL transection (a common animal model for OA), immediately immobilizing the joint prevents development of OA [244], a point which underscores the importance of mechanical stimuli in OA.
Figure 14: Degenerative changes occurring in osteoarthritis result in reinforcing feedback loops. Other feedback loops operative in osteoarthritis but not depicted here include inflammation triggered by products of cartilage breakdown and bone hardening as part of the disease process which results in abnormal cartilage loading.

Much regarding the inter-related degenerative processes of OA remains controversial. For more detail, the reader is referred to a number of excellent reviews on the topic [234,245-249]. However, a simplified schematic of the current understanding of the osteoarthritis disease process is presented here (Figure 14). Cartilage degeneration
in OA results in a vicious cycle that ultimately increases cartilage degeneration. Other
vicious cycles are also at play in osteoarthritis beyond those depicted. Products of
cartilage breakdown are thought to irritate synovial membrane tissue, promoting
inflammation, feeding back into the positive feedback loop depicted above [250].
Hardening of subchondral bone and formation of new bone also occur [251], apparently
in response to degenerative changes to cartilage [252], and in turn increase stresses on
chondrocytes (abnormal loading), feeding back into the degenerative cycle.

2.6.2 Relationship Between PRG4 and Osteoarthritis

Multiple studies have related PRG4 concentration to various markers of arthritis.
In one study, low PRG4 concentrations were measured in human joints after ACL injury,
a risk factor for OA. PRG4 levels rebounded over the course of a 1 year. Pro-
inflammatory cytokines were highly expressed after injury and decreased to normal
levels after 100-200 days [158]. In another study, ACL transection (a standard model for
OA) led to cartilage degradation, a 73% decrease in PRG4 concentration, and increased
friction measured in a pendulum friction tester [253]. Boundary lubrication by synovial
fluid from rabbits after ACL/PCL transection has been measured as less effective than
that from healthy joints. PRG4 concentration was seen to decrease progressively during
the first 3 weeks after which it became too low to detect by the Western blot. Also,
various markers of cartilage degradation – CII peptide, 5-D-4, 9A4, and elastase – were
found in the synovial fluid of effected joints [66]. In an antigen (Freud’s adjuvant and
BSA) -induced arthritis model, whole joint lubrication and PRG4 concentration were
seen to decrease sharply corresponding with an initial increase of pro-inflammatory
cytokines. Localizing PRG4 by immunohistochemistry showed decreased expression of PRG4 by chondrocytes in diseased tissue but apparently increased staining in the cartilage surface layer [254]. Direct intraarticular injections of recombinant PRG4 were shown to slow cartilage degeneration in a rat model of OA, suggesting that PRG4 can play a chondroprotective role in the joint [229]. A leading theory to at least partially explain these changes is that reduction of friction by PRG4 is keeps stresses on the solid matrix within healthy levels, preventing wear [12]. In relation to Figure 14, PRG4 is hypothesized to act against the cycle of osteoarthritis by reducing abnormal loading.
Chapter 3.  Measuring Friction, Stiffness, and Roughness on Mouse Cartilage by Atomic Force Microscopy

[Work presented in this Chapter was a collaborative effort between Jason Blum, Dr. Eric Darling, Dr. Farshid Guilak and Dr. Gregory Jay, Dr. Stefan Zauscher, and myself [14]. All parties contributed to discussions of the experimental approach and interpretation. Jason Blum performed the analysis of internal friction.]

3.1 Background and Motivation

Our first major study focused on the development and characterization of a method for *in situ* friction measurement on murine cartilage using colloidal probe microscopy. Although colloidal probe microscopy had been used previously to measure friction on a wide variety of surfaces [255,256], including bovine cartilage [257], ours are the first friction measurements on murine cartilage. We also present limited analysis of the mechanisms by which friction was generated in these systems.

In this study we measured sliding friction between a chemically functionalized microsphere and the cartilage of the murine femoral head, with normal loads ranging incrementally from 20 nN to 100 nN, a sliding speed of 40 µm/s, and a scan size of 64 µm. Under these test conditions, hydrostatic pressurization and biphasic load support in the cartilage are minimized (Section 2.4.2) and provide frictional measurements that predominantly reflect boundary lubrication properties. Friction coefficients measured on murine tissue (0.25±0.11) were similar to those measured on porcine tissue (0.23±0.09) and were in general agreement with measurements of boundary friction on cartilage by other researchers. Using the colloidal probe as an indenter, the elastic mechanical
properties and surface roughness were measured in the same configuration. Friction was characterized according to relevant mechanisms of friction generation found in the literature. We found that interfacial shear provides the principal mechanism of friction generation (energy dissipation), with little to no friction resulting from plowing forces, collision forces, or energy losses due to normal deformation. Our measurement approach can be applied to future studies of cartilage friction and mechanical properties on genetically altered mice or other small animals.

In the genetic age, there is great potential for focused studies of cartilage lubrication on tissues from genetically altered animals. However, the size of these cartilage specimens precludes mounting in a standard friction tester. Colloidal probe microscopy [258], a modified atomic force microscopy [259] (AFM) technique, facilitates the measurements of such small samples, eliminating this limitation [257]. In the present study, this technique, which predominantly measures boundary lubrication properties, was used to measure friction in situ on the cartilage of the murine femoral head. We demonstrate the feasibility of these measurements and validate the approach by comparing these results to AFM measurements on porcine cartilage. Roughness and indentation properties were also measured on each cartilage surface, providing further knowledge of the factors influencing cartilage friction in the boundary regime. The methods developed here were later applied to study cartilage of Prg4−/− mice (Chapter 4).
3.2 Materials and Methods

3.2.1 Cartilage Samples

C57BL/6J mice (N=9, 20 weeks old) were sacrificed and frozen whole at -80º C until thawed for measurements. Both male and female mice were used. One femur was extracted from each mouse by carefully cutting away the surrounding tissue. The femur was then affixed to a polystyrene Petri dish by embedding its distal end in hot melt glue (Arrow Fastener Co., Saddle Brook, NJ). The orientation of the femoral head was kept constant by placing both epicondyles of the knee flat on the base of the dish. Friction was measured on the anterior portion of the femoral head (Figure 15).

Porcine cartilage used for this study was taken from the lateral section of the patella midway between superior and inferior regions. Fresh knees of female pigs (N=5, 2-3 years old) were obtained from a local slaughterhouse. Visually normal chondral samples approximately 1 cm in diameter were cut away, wrapped in gauze soaked with phosphate-buffered saline (PBS, Gibco, Carlsbad, CA), and frozen at -80ºC until thawed for measurements. Sections were mounted to Petri dishes with hot melt glue (Arrow Fastener Co., Saddle Brook, NJ). All samples were kept in PBS at room temperature throughout testing.
3.2.2 Atomic Force Microscopy

AFM is a versatile tool used for high resolution imaging on a molecular scale [259] as well as detection of nanoscale normal [260] and lateral [261,262] forces between a probe and a surface. Forces are typically detected by reflectance of a laser into a quadrant photodiode [262]. Modifying standard AFM cantilevers with a spherical particle [256] provides a contact more suitable for measuring interfacial interactions on soft surfaces. An MFP-3D AFM (Asylum Research, Santa Barbara, CA) was used for all measurements.

Quantitative friction measurement by atomic force microscopy (AFM) requires both normal and lateral calibration constants [263,264]. Normal force spring constants were determined from the power spectral density of thermal noise fluctuations in air [265,266] by the MFP-3D software provided by Asylum Research. Normal photodiode sensitivity was measured as the slope of the constant compliance regime of the normal force curve on a glass slide [260]. Lateral spring constants were calculated analytically.
from normal spring constants [267] using cantilever dimensions obtained by optical and scanning electron microscopy. Lateral photodiode sensitivity was calculated by measuring the slope of the “stick-slip” region of a small friction loop (50-200 nm) on glass [268]. Since the stick-slip effect was not seen in fluid, the lateral sensitivity of the lever was first measured in air, then offset to account for differences in laser path length and spatial density of reflected signal between fluid and non-fluid environments according to the ratio of normal sensitivities measured in air. This offset factor was 67±5%.

Friction was measured by tracking the lateral deflection signal as the probe scanned over the surface with a scan angle of 90° [262], with trace and retrace signals averaged to eliminate any crosstalk between normal and lateral deflection. The probe was raster scanned across a 64 by 64 µm area of articular cartilage at 40 µm/s with 16 scan lines and 512 points per scan line captured. Each area was scanned 5 times at applied normal loads increasing incrementally from 20 nN to 100 nN. Preliminary studies showed that repeated scanning under these conditions at applied normal loads up to 250 nN did not detectably influence measured friction. Three scan areas were measured per joint. The effects of scan rate and scan size on friction were also assessed. These tests were done in previously untested areas at an applied normal load of 100 nN.

3.2.3 Colloidal Probe Preparation

Friction and indentation measurements were performed by colloidal probe microscopy using triangular silicon nitride AFM cantilevers (spring constant ≈ 0.58 N/m, Veeco, Santa Barbara, CA) (Figure 16). Borosilicate microspheres (10±1 µm diameter,
Duke Scientific Corporation, Palo Alto, CA) were attached near the free end of the AFM cantilever with Norland optical adhesive #81 (Norland Products inc., Cranbury, NJ). The spherical probe serves as a model of a single asperity and the contact area during sliding (estimated as 1-3 μm$^2$ by Hertz contact analysis) is well within the range of asperity sizes seen in topographical images (see Figure 23 on page 77). Probes were functionalized with tri-ethylene glycol to provide uniform surface chemistry and minimize biofouling. To this end, they were coated with 45 nm of gold over a 5 nm adhesion layer of chromium in a thermal evaporator and soaked overnight in a 1 mM SH-(CH$_2$)$_{11}$-EG$_3$ (ProChemia, Poland) in ethanol to allow formation of tri-ethylene glycol terminated monolayers [269].

Figure 16: Scanning electron microscopy image of an AFM cantilever with a 10 μm glass sphere attached.
3.2.4 Roughness

Topography of the cartilage surface was measured concurrently with friction and a second-order flattening procedure was applied to eliminate topography variation from the curvature of the femoral head and other orientation effects; furthermore, the root mean squared (RMS) roughness was recorded. As surface roughness measurements may depend on resolution, sample rate, sample size, and deformation [270], we individually assessed the influence of each of these factors. Colloidal probe measurements were compared to pyramidal tip measurements (with significantly smaller expected contact areas), sample rates and sample sizes were varied, and topography scans were compared over the full range of normal loads used for friction measurements.

3.2.5 Indentation

The elastic modulus of the superficial zone was determined using AFM indentation [271,272]. Within each scan location, a 4x4 array of force-indentation curves was captured. A force trigger of 100 nN and an indentation rate of 1 µm/s was used for all indentations. Approach portions of force curves were analyzed by the Harding Sneddon model [273], a Hertzian contact equation modified to model indentation of a rigid sphere into a semi-infinite elastic solid (Equation 3.1),

\[
F = \frac{4ER^{1/2}}{3(1-\nu^2)}\delta^{3/2},
\]  

(3.1)

where \( F \) is the applied force, \( E \) is the compression modulus, \( R \) is the radius of the indenter, \( \delta \) is the indentation, and \( \nu \) is Poisson’s ratio.
Minimal adhesion was seen in the approach portion of the force curves (Figure 17), supporting the use of a Hertzian analysis (see Figure 3 on page 20). Deformations are larger than those assumed in the formulation of the Hertz model, leading to some errors in measurement. The roughness (on the order of 100 nm) and the anisotropy of the layer lead to further unquantified errors. Because cartilage at this scale is an anisotropic thin layer of unknown composition and unknown thickness overlaid on a biphasic material, precise measurement of material properties is difficult. However, the modulus calculated by the Hertz model gives a characteristic material property useful for comparison between samples. Poisson’s ratios of 0.2 and 0.03, determined for murine cartilage [274] and the superficial zone of porcine cartilage [275], were used for the fit.
Figure 17: (a) Sample indentation data. (b) Young’s modulus calculated from a linear fit to indentation data raised to the $2/3$ power. The contact point is taken as the point at which this linear fit crosses the x-axis.
Data was fit to a linearized version of equation 3.1 derived by raising both sides of the equation to the 2/3 power and substituting $\delta$ with an estimated indentation $\Delta$ that differs from the absolute indentation by a constant. This gives Equation 3.2, which can be fit without knowledge of the constant since $E$ is the only unknown term in the slope 

$$F^{2/3} = C^* - \left[ \frac{4ER^{1/2}}{3(1-v^2)} \right]^{2/3} \Delta. \quad (3.2)$$

The absolute indentation is obtained from Equation 3.1 once the modulus has been found [277]. Thus, we have a consistent means of deriving the contact point based on information from the bulk sample rather than from the contact itself. Adhesion energy was calculated as the total area of the retract portion of each force curve below the zero force line (Figure 18).
Figure 18: Adhesion energy is calculated as the area of the retract portion of each force curve below zero force.

Custom Matlab code (Appendix A) was written to convert raw data output from the Asylum AFM to force v. separation data, set the force in the region of the force curve far from the surface to zero, and calculate the area of retract curve below zero (adhesion energy) by trapezoidal integration.

3.2.6 Imaging

Samples were imaged in contact mode using pyramidal AFM tips (spring constant \( \approx 0.12 \) N/m, Veeco, Santa Barbara, CA) functionalized with tri-ethylene glycol (see section 2.3). The applied load was limited to 5 nN. Other imaging parameters were varied to maximize image quality.
3.2.7 **Statistical Analysis**

Comparisons between the two data sets (murine vs. porcine) were made using a 2-tailed t-Test. Regression analyses were performed using StatView version 5.0.1. All results are reported as mean ± standard deviation.

3.3 **Results**

Friction force was nearly independent of scan rate from 10 to 100 µm/s (Figure 19a) with a trend toward higher friction at lower rates. Friction depended weakly on sliding distance for distances above 5 µm (Figure 19b). Deviations from this trend at small sliding distances may have arisen because mechanical and chemical properties of the surface on those scales are not representative of the whole tissue. Regression analyses of data from scans with applied loads of 100 nN showed a statistically significant correlation with adhesion energy (Figure 20, p = 0.003, r² = 0.3) but not with roughness (p = 0.99) or indentation depth (p = 0.32).
Figure 19: (a) Friction force measured on three different joints at various scan rates (applied load = 100 nN, scan size = 64 µm). (b) Friction force measured on three different joints at various scan sizes (normal load = 100 nN, scan rate = 40 µm/s). Only single error bars are shown, representing typical standard deviations of the 16 scan lines averaged for each data point.
Figure 20: Friction force on murine cartilage plotted as a function of mean adhesion energy for each scan location. The applied normal load is 100 nN.

The measured roughness of mouse cartilage was $201\pm76$ nm (scan size: 64 µm, applied load: 20 nN) and was load dependent. Increasing the load to 100 nN decreased measured roughness by about 13%. Roughness measured on porcine cartilage (scan size: 64 µm, applied load: 20 nN) was $325\pm114$ nm, about 50% higher than on murine cartilage. Although the resolution and sample rate used here were sufficient to describe the system, a consistent increase in measured roughness as a function of scan size was observed. Approach portions of indentation curves were fitted well by the Hertz model, with correlation coefficients typically above 0.99. Compressive moduli were $354\pm158$ kPa and $44\pm42$ kPa on murine and porcine tissue, respectively.
Figure 21: Representative graph showing friction forces plotted as a function of load on one sample. Different symbols represent different scan locations. Error bars represent standard deviations of the 16 scan lines averaged for each data point.

The dependence of friction on applied load was linear within the scatter in all cases. Typical results are shown in Figure 21. This system appears to be well described by Derjaguin’s modification of Amontons’ law [279], giving the load on the system as a sum of the external load and the “internal” adhesive load,

$$F = \mu(L_0 + L) = F_0 + \mu L,$$

(3.3)

where $F$ is the friction force, $L$ is the normal load, and $\mu$ is the coefficient of friction. The subscript “0” indicates forces present at zero applied load. Coefficients of friction were thus interpreted as the slope of friction force plotted versus normal force and were $0.25 \pm 0.11$ for murine samples and $0.23 \pm 0.09$ for porcine samples (Figure 22).
$F_0$ values were 3.0±3.5 nN on murine specimens and 2.8±3.5 nN on porcine specimens. Coefficients of friction did not differ significantly ($p = 0.68$) between species.

![Bar graph showing coefficients of friction for murine and porcine cartilage.](image)

Figure 22: Coefficients of friction measured on murine cartilage are comparable to those measured on porcine cartilage ($p = 0.68$).

The underlying fibrillar structure of the tissue was evident in AFM imaging (Figure 23). It was not visible uniformly though, consistent with characterization of the cartilage surface as either “amorphous” or “fibrillar” by earlier researchers [280].
Figure 23: A representative AFM image taken with an EG3-functionalized pyramidal probe shows the topography of the cartilage surface. (Scan rate = 70 µm/s, applied normal load ≈ 5 nN).

3.4 Discussion

In this study, lubrication mechanisms other than boundary lubrication were minimized, allowing focused study of this mechanism. The time constant for interstitial fluid pressurization is given by

$$\tau = \frac{a^2}{H_a k}$$

(3.4)

where $a$ is a characteristic length scale for fluid flow, $H_a$ is the aggregate modulus, given by $H_a = \lambda + 2\mu$ in terms of Lamé constants or $H_a = E(1-\nu)/(1+\nu)(1-2\nu)$ in terms of Young’s modulus and Poisson’s ratio, and $k$ is the hydraulic permeability [119]. Using a typical hydraulic permeability for the superficial zone of $4.5 \times 10^{-13} \text{ cm}^3 \cdot \text{s/g}$ [127], an
average Young’s modulus found here by indentation of 350 kPa, and a typical contact radius of 1 µm predicted by the Hertz model gives an estimated time constant of 6 ms.

and thus scales directly with the contact area, as has been verified experimentally [281]. A characteristic contact time is given by the contact radius divided by the speed of 40 µm/s as 25 ms, several times larger than the estimated time constant. Furthermore, the measured frictional properties were generally independent of scan rate over a large range (Figure 19a), which would not be expected if friction were measured in the presence of interstitial fluid pressurization [124].

Figure 24: Schematic illustrating the four possible mechanisms of friction considered in this work: (a) Interfacial friction, (b) internal friction, (c) plowing friction, and (d) friction due to collisions with asperities.

In the near absence of biphasic lubrication under microscale contacts, boundary lubrication is expected to be dominant. Additional lubrication mechanisms potentially
contributing to friction reduction in vivo are also expected to be minimized in this configuration. Specifically, hydrodynamic lubrication by PBS on cartilage is only expected at much higher speeds than those used here [145] and the prolonged sliding of the probe is not amenable to squeeze film lubrication. Four possible contributions to the friction (all in the boundary regime) were measured were identified and analyzed in depth (Figure 24). A general model for boundary friction attributes friction to interfacial effects, arising from molecular interactions between surface components as they move over each other, and internal effects, due to inelastic deformation and recovery of the material [26]. Additionally, plowing friction can result when a hard asperity sliding through a softer surface pushes the softer material forward, resulting in an asymmetric pressure distribution at the contact interface (due to either plastic or viscoelastic effects) [282]. Finally, collisions between an AFM probe and the asperities of a rough surface can lead to an apparent dependence of friction on roughness that is not present at the macroscale [283].

Neither plowing nor collision forces were seen to contribute significantly to friction measured in our system. Plowing friction, resulting from a rigid sphere sliding through a viscoelastic material, has been modeled and is expected to scale linearly with sliding velocity [284]. We observed little or even negative dependence on scan rate (Figure 19a), which suggests that plowing friction likely contributes little to the overall friction. This result is consistent with the fact that the angle of attack of the spherical probe is relatively small. A plowing effect due to plasticity is improbable also since articular cartilage regularly withstands pressures much larger than those applied here.
(predicted by Hertz analysis to be tens of kPa) without damage [111]. Furthermore, there was no correlation between the frictional properties and cartilage roughness (p=0.99), suggesting that asperity collisions did not dominate friction.

Hysteresis between approach and retract portions of force curves indicated that tissue recovery after deformation was not completely elastic (Figure 17a). The frictional component arising from inelastic deformation of the tissue was estimated from this hysteresis [285] and was found to be only a small fraction of the total friction (see section 3.5.1). Therefore we conclude that interfacial shear is the major component of the frictional forces measured here.

Adhesion energy can affect interfacial friction [286], although the relationship is highly material dependent. Here, friction depended slightly on adhesion energy (Figure 18). For many materials, adhesion hysteresis, the energy lost in the formation and breaking of adhesive bonds between surfaces, has been shown to affect friction more strongly than adhesion force or adhesion energy [287]. Adhesion hysteresis may be velocity dependent since molecules at the contact interface are able to achieve more intimate and stronger interactions when they have more time to interact with each other. The high friction forces at very low sliding speeds (Figure 19a) may be due to the formation of stronger interactions between the sliding surfaces on those time scales.

The coefficients of friction measured on murine cartilage ($\mu=0.23$) are in good general agreement with microscale measurements of porcine cartilage ($\mu=0.25$, Figure 22) or previous measurements on bovine cartilage ($\mu=0.15$) [257]. They also agree well with macroscale measurements of cartilage friction after significant time ($\mu = 0.14-0.57$).
These findings suggest that cartilage frictional properties may not vary significantly among species, despite large changes in “scale”. This is consistent with other mechanical properties of cartilage, such as compressive and tensile moduli, which are relatively consistent among mammals ranging in size from mice to cows [274,289,290]. The ability to perform direct, in situ measurement of frictional and elastic mechanical properties, as shown here, upon genetically modified mice, will likely provide new detailed insight into the mechanisms that control cartilage lubrication in health and disease.

3.5 Supplemental Information

3.5.1 Estimation of Internal Friction

As surfaces slide past each other under load, they are alternately compressed and allowed to recover, a process that involves energy loss (hysteresis) for all real materials. The resulting friction force has been derived for elastic materials [285] and is given by

\[ F = \beta \left( \frac{9}{128R} \right)^{2/3} \left( \frac{1-\nu^2}{E} \right)^{1/3} W^{4/3}, \]

where \( \beta \) is the elastic loss fraction, \( R \) is the radius of the probe, \( \nu \) is Poisson’s ratio, \( E \) is the elastic modulus, and \( W \) is the load. In our case, all variables are known except the elastic loss fraction, which can be estimated from force curve hysteresis.

Hysteresis was seen between approach and retract portions of force curves in this study and is expected to be due to a combination of adhesion and elastic hysteresis. While hysteresis occurring as the tip pulls away from the surface is due only to adhesion,
hysteresis in the indentation region is due to both effects in an unknown proportion. It is known that elastic hysteresis will be greater in regions of deeper indentation; i.e. each unit of normal displacement deforms a larger area of the tissue. Adhesive effects, however, will be smaller; i.e. each unit of normal displacement changes the contact area by a smaller amount. For consistency, we estimated the elastic loss fraction here as the hysteresis in the upper 75% of each force curve divided by the total area under the approach curve (Figure 25). We noted that this estimated loss fraction increased with increasing indentation rate, which would be expected of an effect due to elastic hysteresis but is the opposite of what would be expected for a trend due to adhesive effects.

![Figure 25: Typical force curve highlighting the region used for estimation of the elastic loss fraction.](image)

Using typical values from this experiment and a normal load of 100 nN, the friction forces predicted by Equation 3.5 ranged from 0.3 to 1.2 nN, and thus account for
approximately 2-3 % of the total friction we measured; however, multiple factors complicate this analysis. First a static analysis of the loss fraction underestimates the true loss fraction by 2-3 times [291]. Second, while indentation curves had a constant rate of 1 µm/s, normal deformation of a point on the tissue during lateral sliding occurred at a variable rate dependent on both the depth of the probe and the exact part of the sphere in contact with that point. For a particle directly in line with the apex of the probe, the maximum normal indentation rate may have been as high as 10 µm/s (from Hertz contact analysis) if the tissue were relatively soft and the maximum load of 100 nN were applied. We have seen that increasing the indentation rate to 10 µm/s increases the loss fraction by about 2 times and note that most normal indentation will take place at a rate much closer to 1 µm/s. While this analysis is far from exact, we are confident that internal friction is not responsible for much more than 10% of the total friction we have measured.
3.5.2 The Effect of the Indentation Rate on Bulk Hysteresis and Adhesion Hysteresis

![Graph showing the effect of indentation rate on bulk hysteresis and adhesion hysteresis.](image)

Figure 26: Bulk hysteresis and adhesion hysteresis measured as a function of indentation rate.

The effect of indentation rate on bulk hysteresis and adhesion hysteresis was tested on cartilage of the femoral heads of mice sacrificed at 20 weeks of age (Figure 26). Bulk hysteresis is expected to be small at low indentation rates since below a certain strain rate the tissue can be considered elastic – non-linearities in the stress-strain relationship become negligible for very low indentation rates. Measurements at very low indentation rates were more susceptible to variability, as from particulate matter interfering with the laser signal in the AFM, and this may be the cause for the variation seen in bulk hysteresis at low indentation rates.
Adhesion in this system is likely due to molecular entanglement. At very high indentation rates, there is limited time for entanglements to form, meaning low adhesion. At very low indentation rates, there is time for molecular entanglements to gently unwind, requiring less energy than quickly pulling them apart. Thus, we see that adhesion energy increases with indentation rate, then decreases.

### 3.5.3 Contamination from Mounting Medium

Cyanacrylate glues are often used for fixing biological samples for mechanical testing [292,293]. A UV-visible spectrophotometer was used to detect particulate matter leached from cyanacrylate glue (Krazy Glue, Inc., New York, NY) and hot melt glue (Arrow Fastener Co., Saddle Brook, NJ) when they were placed in liquid. A drop of each glue was placed in a Petri dish and allowed to harden. PBS was then added to the dish and left for 5 hours (not an unusual length of time for a set of AFM measurements on a limb). A Petri dish containing only PBS was used as a control. It was observed that upon addition of PBS the cyanacrylate glue layer turned from clear to white. After 1 hour, a film could be seen at the top of the fluid above the cyanacrylate sample. The film was white and flaky and some places and clear in others. By hour 5, the film could no longer be seen. No visible changes occurred in the fluid kept with the hot melt glue. No absorbance was detected in the visible range. Absorbance was detected in both test samples in the UV range with much greater absorbance by the sample soaked with cyanacrylate glue (Figure 27).
Figure 27: Absorbance of liquids soaked in the presence of hot melt and cyanoacrylate glues.

3.5.4 Effect of scan size, sample rate, and normal force on roughness measurements.

Scan size, sample rate, and normal force may all influence roughness measurements. Effects of scan size and scan rate were analyzed by dividing images into equal sections and analyzing the roughness of individual sections. To assess the effect of scan size, each image was divided into quarters, then each smaller image was divided into quarters, etc. Thus, a 64 µm image yielded 4 32x32 µm images, then 16 16x16 µm images etc. The RMS roughness of each division was calculated and averaged to remove effects due to variations in location. The general trend seen was towards higher roughness calculated from larger images, as expected. However, in some cases, this trend leveled off in the range of 50 µm (Figure 28).
A similar process was used to assess the effect of sample rate on roughness. Each full image was again divided into quarters but in this case 256x256 point images were obtained from 512x512 point images by taking every other point along each axis. Again, 4 new images were obtained from each larger image in each division and RMS roughness values were averaged together to eliminate random variations. The sample rate used for these images appeared easily sufficient to characterize surfaces (Figure 29).
Figure 29: Measured RMS roughness as a function of the pixel resolution of the image.

The effect of normal force on roughness measurement was assessed using height data corresponding to friction measurements. These data were captured with a colloidal probe and normal forces ranging from 20nN – 13nN. 27 total images were analyzed, 3 for each joint, and measured roughness decreased an average of 13% over that force range. Sample results are plotted in Figure 30.
3.5.5 The effect of surface roughness on measured friction

Although no effect of roughness on friction was seen by regression analysis, closer analysis of individual scan lines indicates that topography does in fact contribute somewhat to measured friction. This is illustrated in the sample friction trace in Figure 31. In that case, the probe appears to get “stuck” during the trace at a distance of about 35 μm. This is likely due to collision with the asperity seen in the corresponding height image. Though this effect was observed in a few cases, both a visual assessment of scan lines and the regression analysis of friction as a function of roughness indicate that this is not a significant factor in the total friction measurement.
Figure 31: (a) Friction trace and retrace and (b) corresponding height trace from a scan line showing the influence of topography on friction. The arrow highlights the locally high friction measurement.
Chapter 4. Properties of Prg4 Knockout Joints as a Function of Age

[Work presented in this Chapter was a collaborative effort between Jason Blum, Dr. Ling Zhang, Dr. Matthew Warman, Dr. Gregory Jay, Dr. Farshid Guilak, Dr. Stefan Zauscher, and myself [17]. Jason Blum wrote the Matlab code used for lateral calibration of AFM cantilevers and performed lateral calibration of several cantilevers. Matthew Warman created the strain of mice used Ling Zhang maintained it in Gregory Jay’s lab. Farshid Guilak, Gregory Jay, Stefan Zauscher, and I planned experiments and interpreted data.]

4.1 Background and Motivation

In this study, we assessed the role of PRG4 in joint lubrication and chondroprotection by measuring friction, stiffness, surface topography, and subsurface histology of the hip joints of Prg4-/- and wild-type mice. Initial studies have shown that mice lacking Prg4 develop joint problems as they age, including an abnormal cartilage surface, synovial hyperplasia, and eventual joint failure [11]. Joint shape and structure appear normal at birth but evidence of abnormal protein deposition along the articular cartilage surface appears within the first week. Synovial hyperplasia occurs by two weeks and progresses with age. By four months, mice exhibit joint swelling and gait abnormalities. By 12 months, joint shape is highly abnormal with destruction of articular cartilage, bony overgrowth (osteophytes), and subchondral cyst formation. Whole joints of these mice show reduced friction in a pendulum friction tester [211].

We used AFM to measure the frictional properties, stiffness, and topography of cartilage of Prg4-/- and wild-type mice aged 2-16 weeks and relate these measurements to histological appearance. The low coefficients of friction which have often been measured on cartilage are due in large part to load sharing by pressurization of the
interstitial fluid, which makes up the majority of the tissue [128]. Boundary lubrication can further reduce friction, and possibly wear, in regions of joint contact, acting independently of interstitial fluid pressure [131]. Lubrication by interstitial fluid pressurization is dependent on the biphasic time constant of the tissue [294], which becomes very small for small contact areas (on the order of milliseconds or smaller for microscale contacts). Thus, AFM allows measurement of cartilage friction in near or complete absence of interstitial fluid pressurization [14,257], making it an excellent tool for the focused study of boundary lubrication properties.

Truncating mutations in \textit{Prg4} are responsible for the rare, autosomal recessive camptodactyly-arthropathy-coxa vara-pericarditis syndrome (CACP) [230]. Patients with this disorder have flexion contractures of phalangeal joints at an early age and develop a non-inflammatory arthropathy which leads to precocious joint failure [231]. In addition, synovial fluid from patients with CACP shows no ability to lower friction in a latex-glass bearing which can be effectively lubricated by normal synovial fluid [211]. Since cartilage from CACP patients has not been available for study, cartilage from \textit{Prg4}-/- mice provides a novel experimental model for PRG4 absence in humans.

There evidence that PRG4 slows the progression of osteoarthritis (OA), as discussed in more detail in Section 2.6.2. PRG4 downregulation has been seen in animal models of OA [253,295], a low PRG4 concentration has been observed in human patients following anterior cruciate ligament injury, a significant risk factor for OA [158], and intraarticular injections of recombinant PRG4 have been shown to slow cartilage degeneration in a rat model of OA
OA is a multifactorial disease with complex etiologies involving hereditary, developmental, metabolic, mechanical, and other factors. The disease progression, discussed in more detail in Section 2.6.1, involves a combination of abnormal mechanical stresses and biochemical imbalances that lead to a loss of proteoglycans, disruption of the collagen network, and alterations in other joint tissues such as the synovium and subchondral bone [2,234]. Matrix degeneration generally begins in superficial cartilage and progresses to deeper regions of the tissue [235]. A better understanding of the role of PRG4 in preventing cartilage damage and degeneration may lead to improved treatment of joint diseases.

4.2 Materials and Methods

4.2.1 PRG4 Null Mouse Model

C57/Bl6J Prg4-/- mice and wild-type controls were sacrificed by carbon dioxide asphyxiation followed by cervical dislocation at 1 of 4 ages, 2, 4, 10, or 16 weeks, in accordance with protocols approved by the Institutional Animal Care and Use Committee. The strain of Prg4 knockout mice used in this research was created at Case Western Reserve [11] and the phenotype was explored in two earlier research papers [11,296].

Limbs were then stored at -80º C until analysis. Seven Prg4-/- and 7 wild-type of each age were used. Femurs were extracted with the aid of a stereomicroscope and rinsed with phosphate buffered saline (PBS) before measurements were performed.
4.2.2 Histologic Analysis and Scoring

After AFM measurements, femurs were fixed in 10% formalin, decalcified in Cal-ex (Fisher Scientific, Fair Lawn, NJ), dehydrated in graded solutions of ethanol in water, infiltrated with xylenes, and finally infiltrated with Paraplast embedding medium according to a standard protocol. Sections 6 µm thick were cut with a microtome and placed on glass microscope slides. The tissue was sliced distal to proximal, parallel to the axis of the femoral neck and the femoral neck was used as a reference point to gauge the depth of the slices. Slices 50-70% through the femoral neck were chosen for analysis as they included the approximate region measured in AFM. Sections were stained with Harris hematoxylin, fast green FCF, and Safranin-O. Quantitative measurements of images were performed using ImageJ (National Institutes of Health, Bethesda, MD). If adequate sections were not obtained from the femur measured in the AFM, the opposite limb (eight data points) or, if necessary, a limb from a new mouse (one data point) was used. Detailed methods of histology techniques used are given in Appendix C.

The surface of the joint typically stained for fast green FCF only and the surface layer thickness was measured from the point where Safranin-O staining gave way to only fast green staining to the joint surface. Uncalcified cartilage thickness was measured from the tidemark to the base of the surface layer. Both surface layer thickness and uncalcified cartilage thickness were measured at three points across an image of the anterior load-bearing region of the joint. Full cartilage thickness was measured along the axis of the femoral neck. Semi-quantitative scores for degenerative changes were assigned based on images of 3 areas of each joint (anterior, medial, and posterior regions.
of load bearing cartilage) by graders, who were blinded to age and the *Prg4* genotype. A modification of a previously reported [297] scoring system for mouse cartilage, including additional changes such as pericellular loss of proteoglycans and surface layer morphology, was used. Articular cartilage structure was scored from 0-4, surface layer morphology was scored from 0-3, and pericellular loss of Safranin-O staining was scored from 0-4. (Table 1). These scores were combined for a total score of 0-11.
Table 1: Scoring system for qualitative analysis of histologically stained micrographs.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Score</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Articular surface degeneration</td>
<td>0</td>
<td>Normal</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>Undulating articular surface with no fibrillation</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Mild fibrillation or severely roughened underlying surface involving &lt; half of articular surface</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Mild fibrillation or severely roughened underlying surface involving &gt; half of articular surface</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>Clefts, cartilage loss, or fibrillation through the superficial zone</td>
</tr>
<tr>
<td>Surface proteinaceous layer fibrillation and overgrowth</td>
<td>0</td>
<td>Smooth</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>Mild fibrillation of surface layer</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Severe fibrillation of surface layer, mild overgrowth or cellular infiltrate</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Severe overgrowth or cellular infiltrate</td>
</tr>
<tr>
<td>Pericellular loss of Safranin-O staining</td>
<td>0</td>
<td>No pericellular loss of staining</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>Mild loss of staining around &lt; 50% of cells</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Mild loss of stain around ≥ 50% of cells or moderate loss of staining around &lt; 50% of cells</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Moderate loss of staining around ≥ 50% of cells or severe loss of staining around &lt; 50% of cells</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>Severe loss of staining around ≥ 50% of cells</td>
</tr>
</tbody>
</table>

4.2.3 Atomic Force Microscopy

AFM measurements were performed with an MFP-3D AFM (Asylum Research, Santa Barbara, CA) on the anterior region of the femoral head as we have reported previously [14]. All measurements were done in PBS at room temperature. Frictional properties and compressive moduli were measured using custom AFM cantilevers with
10 μm spherical tips prepared by gluing borosilicate microspheres (Duke Scientific Corporation, Palo Alto, CA) to the end of triangular silicon nitride cantilevers (spring constant ≈ 0.58 N/m, Veeco, Santa Barbara, CA). These were functionalized with octadecane thiol (SH-(CH2)_{17}-CH3, Sigma Aldrich, St. Louis, MO), chosen for its methyl functionality as a first order approximation of natural cartilage surface chemistry [216]. Normal force spring constants were found using the MFP-3D software provided by Asylum Research [265,266] and lateral calibration constants were calculated by the wedge method [264], using a 30° silicon wedge [298] (Advanced Scanning Probe Solutions, Enschede, Netherlands) and equations modified for spherical probes [255]. A fresh probe was used for each cartilage sample.

The effective elastic modulus, an aggregate measure of stiffness at the surface of the tissue, was determined by indenting cartilage specimens to a maximum force of 100 nN with a constant velocity of 1 μm/s and fitting the approach to a Hertzian contact model for a hard sphere against an infinite plane [273], a method that has been used previously to measure stiffness of articular cartilage [97,257]. Indentation depths ranged from an average of 340 nm on 10 week old wild-type mice to an average of 980 nm on 10 week old Prg4-/− mice. Sixteen measurements were taken across each of three 50 x 50 μm areas on the joint and averaged. Friction measurements were performed subsequent to all stiffness measurements at the same three locations on each joint. Friction was measured at 16 scan lines across each 50 x 50 μm area and repeated with normal loads ranging incrementally from 20 to 100 nN (20, 40, 60, 80, and 100 nN), alternating between increasing and decreasing load. The coefficient of friction (COF) at each
location was defined as the slope of friction force vs. normal force, and the mean value was recorded as the COF for the joint.

Subsequent to stiffness and friction testing, surfaces were imaged using softer AFM cantilevers with sharp tips (spring constant $\approx 0.12$ N/m, nominal tip radius 20 nm, Veeco). These cantilevers were gold coated and functionalized with tri-ethylene glycol, (SH-(CH$_2$)$_{11}$-(OCH$_2$CH$_2$)$_3$OH, Sigma Aldrich), to minimize adhesion of cartilage material to the probe. Images were taken in contact mode and contact forces were kept low (approximately $\leq 2$ nN) to minimize distortion of the tissue due to the contact force. Root-mean-square roughness was calculated from these images after a second order flattening procedure to remove effects of the curvature of the femoral head.

**Statistics.** AFM data and semi-quantitative measurements of histological images were analyzed by Student’s t-test and histological scores were analyzed by Mann-Whitney U to compare between genotypes at each age. Trends of histological scores with respect to age were tested by Spearman’s rank correlation. The proportion of joints exhibiting distinct abnormal features were compared by Fisher’s exact test with Bonferroni correction where applicable. Statistical significance was reported at the 95% confidence level ($\alpha=0.05$).
Figure 32: Sample micrographs of histologically stained cross-sections of cartilage from the femoral heads of wild-type and Prg4-/- mice ages 2, 4, 10, and 16 weeks. Arrowheads indicate delamination of the surface layer (i), a Safranin O–positive feature in the surface layer (ii), a cell in the surface layer (iii), pericellular loss of Safranin O staining (iv), and absence of the surface layer, leaving the underlying cartilage exposed to damage (v). Bars = 50 µm.
Table 2: Summary of qualitative grading of histologically stained micrographs (mean ± standard deviation). Asterisks indicate statistical significance (p < 0.05) between genotypes.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Age</th>
<th>Wild-type</th>
<th>Prg4-/-</th>
</tr>
</thead>
<tbody>
<tr>
<td>Articular surface degeneration</td>
<td>2 weeks</td>
<td>0.64 ± 0.63</td>
<td>1.75 ± 1.27*</td>
</tr>
<tr>
<td>score</td>
<td>4 weeks</td>
<td>0.46 ± 0.34</td>
<td>1.75 ± 0.87*</td>
</tr>
<tr>
<td></td>
<td>10 weeks</td>
<td>0.46 ± 0.27</td>
<td>1.71 ± 0.34*</td>
</tr>
<tr>
<td></td>
<td>16 weeks</td>
<td>0.36 ± 0.32</td>
<td>1.75 ± 0.74*</td>
</tr>
<tr>
<td>Surface layer</td>
<td>2 weeks</td>
<td>0.07 ± 0.12</td>
<td>1.71 ± 0.62*</td>
</tr>
<tr>
<td>fibrillation and overgrowth score</td>
<td>4 weeks</td>
<td>0.39 ± 0.32</td>
<td>2.04 ± 0.53*</td>
</tr>
<tr>
<td></td>
<td>10 weeks</td>
<td>0.14 ± 0.28</td>
<td>1.93 ± 0.55*</td>
</tr>
<tr>
<td></td>
<td>16 weeks</td>
<td>0.18 ± 0.19</td>
<td>1.68 ± 0.43*</td>
</tr>
<tr>
<td>Pericellular loss of Safranin-O</td>
<td>2 weeks</td>
<td>0.29 ± 0.62</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>staining score</td>
<td>4 weeks</td>
<td>0.62 ± 0.59</td>
<td>1.52 ± 1.82</td>
</tr>
<tr>
<td></td>
<td>10 weeks</td>
<td>2 ± 0.69</td>
<td>2.62 ± 1.27</td>
</tr>
<tr>
<td></td>
<td>16 weeks</td>
<td>1.1 ± 1.44</td>
<td>3.05 ± 0.68*</td>
</tr>
</tbody>
</table>

4.3 Results

4.3.1 Histologic Analysis and Scoring

Histology of Prg4-/- cartilage revealed both surface and subsurface abnormalities (Figure 32). The surface layer and underlying cartilage surface of Prg4-/- cartilage were highly irregular relative to wild-type for all ages measured, with no detectable effect of age (Table 2). Pericellular proteoglycan loss increased significantly as a function of age and was significantly higher in Prg4-/- joints at 16 weeks. Overall degeneration scores were significantly higher for Prg4-/- joints than wild-type for all animals older than two weeks (Figure 33). These trends did not appear to vary significantly as a function of the location on the joint.
Figure 33: Total degeneration score, an aggregate score of articular cartilage structure, surface layer morphology, and loss of Safranin O staining, at each age tested for wild-type and Prg4-/- mice. Seven mice of each genotype were studied per age group. Values are the mean ± SD. * = P < 0.05.

Figure 34: Thickness of uncalcified cartilage at ages 10 and 16 weeks for wild-type and Prg4-/- mice. Seven mice of each genotype were studied per age group. Values are the mean ± SD. * = P < 0.05.
Figure 35: Percentages of joints of Prg4-/- mice (joints of 7 mice at each age; only 1 joint was tested per mouse) exhibiting distinct characteristics. None of these features were observed in joints of WT mice. † = P < 0.05.

The thickness of the surface layer averaged 4.2 ± 3.5 μm in Prg4-/- and 2.4 ± 0.8 μm in wild-types and did not vary as a function of age. Measurements of uncalcified cartilage depth in 10 and 16 week old wild-type and Prg4-/- joints showed that the natural process of cartilage mineralization was slowed [299] (Figure 34). Younger joints were not included in this analysis as most did not have visible tidemarks. Full cartilage thickness was also measured from the micrographs, but no thickness differences between wild-type and Prg4-/- joints were resolved. The enlarged surface layer of Prg4-/- cartilage was seen to delaminate in some places and was completely absent in other areas, giving way to an underlying cartilage surface that appeared abnormally rough (Figure 32). Cells were seen within the surface layer in four cases and Safranin-O positive features were also observed on Prg4-/- cartilage in nine cases (Figure 35), suggesting the
presence of glycosaminoglycans (GAGs). Safranin-O positive features were typically present over 5% or less of the joint surface but in 1 case were present over approximately 20% of the linear surface analyzed. None of these features were observed on wild-type joints. Fisher’s exact test with a Bonferroni correction was used to compare the incidence of each of these features with respect to genotype and to compare rates of occurrence on younger (2-4 weeks) and older (10-16 weeks) Prg4-/- mice. Surface layer delamination and Safranin-O positive surface features were shown to occur at a rate significantly higher than on wild-type joints and Safranin-O positive surface features were found to be significantly more likely in older mice.

Figure 36: Representative atomic force microscopy images of cartilage from the anterior femoral head of wild-type and Prg4-/- mice ages 2, 4, 10, and 16 weeks. Bars = 10 µm.
4.3.2 Atomic Force Microscopy

AFM imaging allowed surfaces to be visualized at higher resolution (Figure 36). *Prg4-/-* joint surfaces showed increased surface roughness as compared to wild-type surfaces (Figure 37) and generally were characterized by large numbers of rounded features on the scale of approximately 1 μm. Wild-type surfaces, meanwhile, had more linear surface features. Large collagen bundles were visible in 4 of 7 AFM images of 2-week-old and in 2 of 7 images of 4-week-old *Prg4-/-* joints. A structure similar to these thick fibrillar bundles was only observed a single 16-week-old wild-type surface. The appearance of such structures predominantly on surfaces of young *Prg4-/-* mice may relate to the reordering of the collagen fibrillar structure of *Prg4-/-* mice aged 0-2 weeks previously observed by transmission electron microscopy [211].

The COF was slightly higher on *Prg4-/-* joints than on wild-type joints at 16 weeks and was similar at younger ages (Figure 38). The stiffness of wild-type surfaces increased significantly with age, a trend that was not observed on *Prg4-/-* surfaces (Figure 39).
Figure 37: RMS roughness of cartilage surfaces determined by atomic force microscopy. Seven mice of each genotype were studied per age group. Values are the mean ± SD. * = P < 0.05.
Figure 38: Coefficient of friction on surfaces of wild-type and Prg4−/− cartilage surfaces. Seven mice of each genotype were studied per age group. Values are the mean ± SD. * = P < 0.05.

Figure 39: Stiffness of wild-type and Prg4−/− cartilage surfaces measured by atomic force microscopy. Seven mice of each genotype were studied per age group. Values are the mean ± SD. * = P < 0.05.
4.4 Discussion

The findings of this study indicate that the presence of PRG4 plays an important role in preserving natural joint structure and functional properties. *Prg4/-* mice exhibited significant changes in articular cartilage properties, including enlargement and roughening of the surface layer, irregularities in cartilage structure, and age-related changes in the compressive modulus. Cartilage of *Prg4/-* mice was also characterized by a pericellular loss of proteoglycans and delayed tidemark progression. This degenerative phenotype caused by the lack of *Prg4* is similar to that observed clinically with CACP and many of these changes are also similar to osteoarthritic degeneration.

The measurements performed here show a limited effect of PRG4 on frictional properties and suggest that the PRG4 present on the surface of wild-type cartilage does not provide significant boundary lubrication. PRG4 is present in both synovial fluid and superficial cartilage, and PRG4 in solution has been shown to effectively decrease the COF of both cartilage and non-biological bearings in previous studies [163,219,300]. The effect of PRG4 on the frictional properties of the cartilage surface itself, however, has not been shown consistently in previous studies. For example, removing the surface layer by rubbing the cartilage surface with a cotton plug soaked in SDS [103], rubbing the surface layer away by prolonged sliding [221], and cutting off the upper surface of cartilage [222] all did not result in a detectible increase in the coefficient of friction under boundary lubricating conditions. There is, however evidence that the frictional properties of the cartilage surface are influenced by levels of *Prg4* expression [224,301]. Removal
of PRG4 from the cartilage surface by NaCl extraction increased the frictional properties by only about 20% in a system where whole synovial fluid reduced friction by a factor of 3 relative to PBS [223]. It should be noted that the measurements performed in the present study were of the effect of a chronic rather than sudden absence of PRG4. Friction on these joint surfaces was affected both by the presence or absence of PRG4 and by degenerative and/or compensatory changes that had occurred in the joint, as seen by AFM and histology. The structural and morphological changes that occurred in Prg4-/− joints may have had the general effect of increasing friction, resulting in a significant difference in friction only at 16 weeks when the most damage had occurred to the joints.

Whole joint friction measurements on Prg4-/− joints have also been performed, finding moderately higher friction in Prg4-/− joints than heterozygous joints [211]. Friction coefficients measured on the cartilage surface in our study are more than two orders of magnitude higher than shown in whole joint tests [211], a difference we primarily attribute to the presence of interstitial fluid pressurization in the joints. Although interstitial fluid pressurization provides a significantly greater influence on the frictional properties of the joint than boundary lubrication by PRG4, it is clear that the absence of PRG4 results in significant joint abnormalities and degenerative changes. These findings suggest that relatively small changes in boundary friction may have significant effects on cartilage physiology over the long term. While increased shear loading of cartilage promotes expression of various cartilage constituents, including
collagen, aggrecan, and PRG4 [302,303], it can also have deleterious effects, such as an increase in the production of oxidants which contribute to cartilage degeneration [304].

Alternatively, the influence of PRG4 on joint health may be through mechanisms that do not involve friction directly. There is evidence that PRG4 dissipates strain energy within synovial fluid in addition to lubricating joint surfaces [296]. It further functions as an anti-adhesive, preventing cartilage integrative repair in vitro [220]. PRG4’s ability to form a surface covering, as it does on surfaces with a wide range of chemical functionalities [108,212], may provide an important property for normal joint function by conferring wear resistance in addition to boundary lubrication. We observed that the surface layer was smooth and continuous on wild-type cartilage but was highly variable, sometimes delaminated, and occasionally absent on Prg4-/ cartilage. With a superficial surface coating, high forces occurring at a single point on the surface will be dissipated, reducing the pressure exerted at any single point on the underlying surface. The formation of PRG4 dimers [209,210] and possibly further networked structures [211] likely contributes to this dissipative mechanism. The occurrence of microstructural damage in the PRG4 layer rather than in the collagen proteoglycan matrix would be beneficial for two reasons. First, PRG4 can be readily replenished but cartilage does not repair quickly, if at all [305]. Second, damage to the cartilage matrix may trigger chondrocytes to produce catabolic cytokines and matrix-degrading proteases as in early stage OA [306].

We believe the Safranin-O poor surface layer observed in histology images of wild-type joints, as also seen by earlier researchers [191] to be the lamina splendens,
described in more detail in Section 0. Because the protein PRG4 is concentrated at the cartilage surface, it is believed by many to be a major component of the lamina splendens, though direct chemical assessment of the lamina splendens has not yet been possible. \textit{Prg4/-} mice showed significant accumulation of surface material beyond that seen on wild-type surfaces, likely composed of adsorbed proteins from synovial fluid [11]. The surface layer on \textit{Prg4/-} mice sometimes contained cells and localized Safranin-O positive areas, features which were not seen on wild-type mice. The source of the Safranin-O positive formations seen within the surface layer is unclear but may have been metabolized proteoglycans from within the cartilage matrix [307].

The cartilage stiffness measured at the microscale represents an aggregate stiffness of the surface layer and superficial zone cartilage. Using average measurements of surface layer thicknesses from our histology and assuming that the surface layer is softer than the underlying cartilage, we conclude from the analysis of Perriot and Barthel [308] that stiffness values are typically dominated by the surface layer and somewhat influenced by the underlying tissue stiffness on both wild type and \textit{Prg4/-} joints. The effect of the underlying surface on stiffness values is smaller on \textit{Prg4/-} joints because the surface layer is thicker. The proteoglycan loss in \textit{Prg4/-} mice was predominantly localized in the pericellular region, suggesting that it was mediated primarily by the chondrocytes. The mechanism of proteoglycan loss remains to be determined but most likely involves the upregulation of matrix metalloproteinases and aggrecanases in response to both mechanical and biochemical factors [309-311]. Our observations are
intriguing as the loss of cartilage stiffness in the superficial zone is one of the earliest signs of cartilage damage in early stage OA [235,312,313].

PRG4 appears to have a limited role in reducing the COF on the cartilage surface of mammalian joints when only present on the articular surface. Despite this, it is apparently necessary for preservation of surface integrity, superficial stiffness, and glycosaminoglycan content. Further exploration of the mechanisms by which PRG4 protects joints may lead to improved treatments for OA.
Chapter 5. Wear Measurements on Cartilage

[Work presented in this Chapter is an ongoing collaborative effort between Dr. Debby Chang, Dr. Ling Zhang, Dr. Farshid Guilak, Dr. Gregory Jay, Dr. Stefan Zauscher, and myself [14]. Debby Chang first developed the wear measurement technique on collagen surfaces and took the SPR data used in the data analysis, Gregory Jay and Ling Zhang have provided cartilage samples and lubricin, and Farshid Guilak, Gregory Jay, and Stefan Zauscher have been involved in planning the study.]

5.1 Background and Motivation

Articular cartilage, which lines the ends of bones in moveable joints, distributes loads across joints and allows them to support large loads without damage over several decades. However, cartilage breakdown in osteoarthritis (OA) eventually leads to damage to bone and surrounding tissues and painful movement. While the etiology of OA is unknown in most cases, a number of interrelated factors are at play in the disease progression and contribute to the likelihood of disease onset (see also Section 2.6). Mechanical factors play a significant role in osteoarthritis progression and increased intra-articular stress (which may arise from obesity, joint instability, joint incongruity, or other factors), is a significant risk factor for OA [246]. Cartilage wear is widely theorized to contribute to the initiation and progression of osteoarthritis [12,196,314] but the process remains poorly understood. Other mechanical factors contributing to osteoarthritis include altered cell expression of a number of signaling molecules and cell apoptosis, the effects of which have been better characterized to date [315].

Most studies of cartilage wear have been performed in vitro under conditions designed to accelerate wear. Sliding has typically been either cartilage on cartilage
or cartilage on steel [182,188,189,191-194], which was shown to give adhesive rather than abrasive wear for sufficiently smooth surfaces [191]. Wear depth has been detected either by a measurement of the surface topography or a biochemical assay. Measured wear has been on the order of several microns or more. However, the wear that is theorized to contribute to early stage osteoarthritis occurs on a much smaller scale [13] and the necessary surface level measurements of cartilage wear have not yet been made. Here, we induce surface level wear of articular cartilage surfaces and are able to assess the wear depth with a resolution of about 10 nm.

Measurement of cartilage wear at nano- to micro-scales is particularly important because the cartilage surface is structurally and chemically distinct from the bulk. The collagen fibrillar network within the upper few microns of cartilage is distinct from that of the rest of the superficial zone, as seen in SEM [91,93] and TEM [89] images. Collagen fibrils in the surface fibrous layer are smaller in diameter than those in the superficial zone, [91,93] and immunohistochemical analysis has indicated that the surface fibrous layer contains high concentrations of collagen I and III and a relatively low concentration of collagen II [94]. This is similar to the collagen distribution in synovial tissue [94] but dissimilar to the composition of bulk cartilage. A distinct non-fibrillar, acellular superficial surface layer is present superior to the fibrous surface layer at the cartilage surface [89,95-99] and has been called the surface amorphous layer (SAL). This layer is several tens of nanometers to a few microns thick [81,89,97,102] and is digested by trypsin and chymotrypsin, indicating that protein is a significant component in the layer [101]. In one study, the layer was removed by rubbing a cotton swab soaked
in detergent along the surface [103]. The removed fraction contained approximately 54.5 µg/cm² lipid, 68.1 µg/cm² protein, 61.4 µg/cm² GAG; 30%, 37%, and 33% by weight respectively, assuming no additional types of material were present. Other studies report the presence of HA [90,109], fibronectin [90], phospholipids [109], albumin [109], and PRG4 [106].

PRG4 is a glycoprotein synthesized by superficial zone chondrocytes and synovial fibroblasts present in synovial fluid in a concentration of approximately 200 µg/ml [66]. It has been shown to lubricate a variety of articulating surfaces under boundary lubrication conditions, including cartilage-cartilage [10] cartilage-glass [162,217] and latex-glass [171,219]. Conflicting evidence exists on the role of PRG4 in mediating friction when not present in solution, i.e. when only PRG4 bound to a cartilage surface is present. Removing the surface layer by rubbing the cartilage surface with a cotton plug soaked in SDS [103], rubbing the surface layer away by prolonged sliding [221], and cutting off the upper surface of cartilage [222] did not result in a detectible increase in the coefficient of friction under boundary lubricating conditions. There is, however, evidence that the frictional properties of the cartilage surface are influenced by the level of Prg4 expression, [224,301] and digestion of cartilage with trypsin has been shown to increase friction [179]. As discussed in Section 2.5.2.3, this may indicate that PRG4 functions as a friction modifying layer under some conditions and as a sacrificial layer in others.

Wear protection by PRG4 has not been measured directly. However, SEM analysis of cartilage from a genetic knockout not expressing PRG4 showed superficial
reorientation of collagen fibrils, believed to result from wear [12]. Wear in the presence of “synovial mucin”, filtered solid components of synovial fluid was higher than that in the presence of synovial fluid alone and was increased by approximately 2 times when the synovial mucin was digested by trypsin [182], indicating that protein present in synovial fluid reduced wear.

As discussed in Section 2.4.2, cartilage friction is mediated to a large extent by biphasic lubrication where compressive loads on cartilage are borne by pressurized interstitial fluid [124]. As fluid pressure dissipates, load is transferred increasingly to solid components, with a corresponding increase in friction [7]. Surprisingly, biphasic lubrication appears to have a limited effect, if any, in reducing cartilage wear. In one study, removing the load from cartilage during a wear experiment and allowing it to rehydrate did not affect the wear rate [191]. Although cartilage friction becomes significantly higher during prolonged loading (see Figure 11 on page 35), a significantly higher wear rate due to the depletion of interstitial fluid pressurization has not been seen. Instead, wear rates after prolonged loading have been reported as slightly higher [182], slightly lower [191], or moderately lower [184] than the wear rates upon initial loading. In the first two cases the contact location was static on the cartilage, while in the third case reciprocating sliding was performed with an amplitude 4 times smaller than the contact diameter, all conditions under which fluid pressure would be expected to deplete. These findings underscore the importance of measuring wear directly as there is generally no consistent correlation between friction and wear [18].
5.2 Methods

5.2.1 Cartilage

Femurs of six C57BL/6J wild type mice (one femur per mouse) that had been sacrificed at age 16 weeks were used. Mice were sacrificed and frozen whole at -80°C until thawed for measurements. Femurs were extracted with the aid of a stereomicroscope and PBS was cartilage was kept hydrated in PBS following separation of the hip. The femur was cut just above the knee joint and the distal extremity was refrozen. Femurs were then mounted in a lid of a 60 diameter tissue culture polystyrene Petri Dish (Falcon) by gluing the distal end of the femur to the Petri dish, as described previously. A silicone O-ring, inner diameter 0.25 in. (Pressure Seals, Inc., South Windsor, CT) was glued around the femoral head using hot melt glue. This allowed specimens to be tested in approximately 125 µl of fluid, a relatively small volume.

5.2.2 Test Conditions

Testing was performed in either PBS (pH 7.4, without CaCl₂ or MgCl₂, Invitrogen, Grand Island, New York) or in 200 µg/ml purified human lubricin. Protease inhibitors, 1mM PMSF (Sigma-Aldrich), 2mM EDTA (Sigma-Aldrich), 5mM benzamidine HCl (Sigma-Aldrich), were added to PBS to prevent cartilage breakdown as experiments lasted several hours. Measurements were performed first in PBS, in the presence of residual PRG4 on the cartilage surface, then in the PRG4 solution, then again in PBS. Cartilage was then incubated in 2M NaCl in PBS for 20 min on a rotating table to extract the PRG4 from the surface [209]. Test conditions along with abbreviations are
summarized below in Table 3. The AFM cantilever was also incubated in 2M salt for 20 minutes. Measurements in PBS and PRG4 were then repeated.

Table 3: Summary of the conditions measured in this study.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Solution</th>
<th>Processed in NaCl?</th>
<th>Cartilage Surface</th>
<th>Order Measured</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>PBS</td>
<td>No</td>
<td>Normal cartilage surface layer</td>
<td>1\textsuperscript{st}</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Yes</td>
<td>Modified cartilage with reduced PRG4 content</td>
<td>4\textsuperscript{th}</td>
</tr>
<tr>
<td>LUB</td>
<td>200 µg/ml lubricin in PBS</td>
<td>No</td>
<td>Normal cartilage surface layer + loosely associated PRG4</td>
<td>2\textsuperscript{nd}</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Yes</td>
<td>Modified cartilage with both surface bound and loosely associated PRG4</td>
<td>5\textsuperscript{th}</td>
</tr>
<tr>
<td>PBS_LUB</td>
<td>PBS</td>
<td>No</td>
<td>Normal cartilage surface layer</td>
<td>3\textsuperscript{rd}</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Yes</td>
<td>Modified cartilage with surface bound PRG4 only</td>
<td>6\textsuperscript{th}</td>
</tr>
</tbody>
</table>

5.2.3 Atomic Force Microscopy

Borosilicate beads (10 µm diameter, Duke Scientific, Palo Alto, CA) were glued to tipless silicon atomic force microscopy cantilevers (AppNano, Santa Clara, CA) and functionalized with octadecane thiol as we have described previously [17]. Cantilever spring constants were nominally 20-95 N/m and were typically measured as 50-60 N/m. SEM images were taken of all cantilevers prior to thiol functionalization to ensure that the surfaces were smooth and free of adhesive residue. A typical SEM image is shown in Figure 40.
Cartilage was worn by imaging an 8x8 µm area 30 consecutive times under a 50 µN load with a sliding speed of 100 µm/s at a scan angle of 90°. As is typical in AFM imaging mode, the cantilever overscans the area of interest slightly along the fast scan axis for a total area of 9.27 µm x 8.00 µm or 9.27 µm x 8.25 µm for piezo travel. Each image thus consists of 128 forward and reverse scanlines with a total sliding distance of approximately 7.1 cm. A 22 µm area in which wear images were centered was imaged before and after the wear experiment using the same AFM cantilever. Scan parameters for those images were: sliding speed: 75 µm/s; normal load: 1 µN, resolution: 256 points x 256 lines, scan angle: 0°. We found that post-wear images were most consistent and gave the best topographical detail when they were performed at a 90° angle relative to wear images. Thus, pre-wear and post-wear images were performed with a 0° scan angle. The trace and retrace of height images were averaged to correct for the shift due to lateral deformation of samples during imaging. The pre-wear image was subtracted from the post-wear image and the difference was planefit based on the outer 2 µm of the image.
sufficiently far from the center of the image where wear had occurred. There was typically very little wear debris present in this outer area. Planefitting was done using a custom Matlab code, included in Appendix 1.

5.2.4 Details of the Measurement Technique

The AFM cantilever likely is fouled by material from the cartilage surface in the process of scanning, and fouling likely affects the wear properties. To assess this effect, wear was measured by repeated scanning on three cartilage samples. A new cantilever was used for each sample. We found that measured wear was relatively constant beyond the first location imaged. Furthermore, wear after several hours of experiments was comparable to that induced in the second location tested. Thus, all further measurements were recorded after a minimum of one run-in scan.
Figure 41: Worn depth measured in consecutive scans on cartilage from hips of 16 week old C57BL/6J mice. Wear was induced on sample 3 after 7 hr and 9 hr of scanning and worn depth was found to be 42 nm and 38 nm, respectively (not shown).

We performed control measurements to assess the extent to which measured differences between pre-wear and post-wear images are due to plastic deformation or stress-relaxation. Control measurements in which the AFM tip was held in contact at a normal force of 50 µN without lateral movement for 14 minutes, the time required for a full set of 30 scratches, were performed on three samples. Measured wear depth was within the error of the measurement, 1.4 ± 8.4 nm.
5.2.5 Statistics

Differences between conditions were assessed by a one-way repeated measures ANOVA followed by Fisher’s PLSD. Correlations between measured parameters were assessed by regression analysis. Statistical analyses were preformed in Statview version 5.0.1.

5.3 Results

Our experiments were designed to test both wear protection by PRG4 in solution and wear protection by surface-bound PRG4. The effect of PRG4 in solution on wear is seen by comparing PBS to LUB (undetectable effect) or to PBS_LUB on unmodified cartilage or comparing PBS_LUB to LUB on salt-extracted cartilage (Figure 42). Differences were not resolved on unmodified cartilage but a significant wear reduction due to PRG4 in solution was seen on salt-extracted cartilage. The wear-reducing effect of surface-bound PRG4 is seen by comparing PBS_LUB to PBS on salt extracted cartilage. Similar wear occurs for each condition, which means we are not able to detect a measurable effect of surface associated PRG4 on wear under these conditions. Salt-extraction had the trend of increasing both friction and wear regardless of solution. The difference in wear between unmodified and salt-extracted cartilage is statistically significant for the PBS data point and the difference in friction is statistically significant for both PBS and PBS_LUB. No statistically significant differences in stiffness were detected. Friction and stiffness under each condition are given in Figure 43 and Figure 44, respectively.
Figure 42: Wear depth under all conditions measured. * indicates a statistically significant difference. $\alpha = 0.05$.

Figure 43: Friction under all conditions measured. * indicates a statistically significant difference. $\alpha = 0.05$. 
Figure 44: Stiffness under all conditions measured. No statistical significance was seen between data points. \( \alpha = 0.05 \).

Regression analyses between all combinations of friction, wear, and stiffness were performed. Only one statistically significant correlation was found, a slight positive correlation between friction and wear (\( p = 0.02, r = 0.28 \)). The averaged data tells only part of the story though. Performing regression analyses on each limb individually shows a strong positive correlation on 3 of 6 samples (\( p < 0.001, r > 0.80 \), data not shown) while no correlation was seen between friction and wear for the other samples (\( p > 0.05, r < 0.3 \)).

5.4 Discussion

Boundary lubrication, including both friction and wear prevention, may occur by a number of mechanisms, as discussed in section 2.1.4.2. The cartilage wear protection
by PRG4 in solution shown here, combined with the lack of wear protection by surface-bound PRG4 is consistent with a sacrificial layer mechanism of boundary lubrication. In a sacrificial layer lubrication mechanism, shear occurs within a lubricating layer which can be removed easily from the surface. Wear occurs primarily in the lubricant layer, protecting underlying surfaces.

The following conditions need to be met for sustained wear prevention by the sacrificial layer mechanism: (1) the surface layer needs to be replenished more quickly than it is worn away, (2) stresses transmitted to the underlying surface need to be sufficiently distributed to prevent wear. The adsorption of PRG4 to model functionalized model surfaces measured by surface plasmon resonance confirms that PRG4 adsorbs quickly, (approximately half of the layer thickness forms in the first 2 seconds) at the concentration (200 µg/ml) used in this study (Figure 45). This is consistent with the first requirement above. The second requirement above is consistent with a broader view of cartilage architecture, where the surface zone is designed to distribute stresses seen by deeper zones, and cartilage as a whole is designed to effectively distribute stresses onto bones (see Section 2.3.4). PRG4 may also lubricate by other mechanisms under other loading conditions. Specifically, the friction modifying layer mechanism may be operative under pressures not sufficient to shear the PRG4 layer away [225]. The pressures under which each mechanism is operative and the effectiveness under a range of loading conditions should thus be studied further.
Cartilage wear prevention by a sacrificial layer mechanism may not be unique to PRG4. All known constituents of the SAL – PRG4, albumin, phospholipids, fibronectin, HA, and loose proteoglycans - can be readily replenished from synovial fluid. While this layer is not particularly effective at reducing friction [221], it could nonetheless theoretically reduce wear by a sacrificial layer mechanism. This possibility needs to be tested further (see Section 6.4) and has potential significance for the design of disease modifying agents targeted towards surface level mechanical forces. The critical design criterion for this sacrificial layer would be reduction of both shear and normal forces transmitted to the underlying cartilage surface, not necessarily reduction of friction.
The processing step in 2 M salt was performed to yield a PRG4 negative control. High salt concentrations, which shield electrostatic forces and alter protein folding by favoring hydrophobic interactions, have been reported to completely remove PRG4 from cartilage surfaces [209] (though another study has found it to be less successful in PRG4 removal [107]). The extent to which PRG4 was removed here has not yet been directly assessed. Folding, and potentially also binding, of other proteins is expected to be affected and high salt is known to remove some proteoglycans from cartilage, though the amount of proteoglycan removed is relatively low compared to other methods of protein extraction [209].

The usefulness of PRG4 as a disease modifying agent for OA will depend on the concentration dependence of wear prevention. It could be used to both prevent and treat the disease. Inflammation, as occurring in cartilage injury, results in a decrease in PRG4 expression and/or a abundance of secreted PRG4 [316]. For example, PRG4 levels were reduced in humans by about 4 times (from about 200 µg/ml to about 50 µg/ml) following ACL injury, a significant risk factor for OA [158]. If PRG4 prevents wear more effectively at a solution concentration of 200 µg/ml than at 50 µg/ml, it could potentially be added to inflamed joints to prevent later development of OA. If PRG4 also prevents wear more effectively at concentrations higher than 200 µg/ml than at 200 µg/ml, it could also potentially be added to joints of OA patients to inhibit the vicious cycle of mechanical and chemical alterations that make up the disease process (see Figure 14 on page 58). Instead of using PRG4 directly to treat or prevent OA, a molecule could be
designed that is turned over more slowly than PRG4 but lubricates by a similar mechanism. Initial follow-up studies are discussed in Section 6.4.

In conclusion, this study shows that PRG4 reduces mechanical wear on cartilage. Friction tended to correlate with wear. This study contributes to growing evidence that PRG4 prevents degenerative changes in cartilage [12,17,229]. The presence of PRG4 in solution reduced wear much more than did its presence on the surface alone, consistent with a sacrificial layer mechanism of boundary lubrication and indicating that absorption properties are an important factor in reducing wear in this system.
Chapter 6. Conclusions and Future Work

6.1 Conclusions

Approaches to measure friction and wear on murine cartilage at the microscale have been developed in this thesis, including analysis of factors contributing to friction measured. These approaches have been used to study the effect of PRG4 on the boundary lubricating properties of articular cartilage and the physiological effects of a prolonged absence of PRG4 on joint friction, stiffness, and morphology. Finally, we tested the ability of PRG4 to reduce mechanical wear induced by a functionalized AFM probe and correlated these measurements to stiffness and friction.

In the first study (Chapter 3), we found that friction measured on murine cartilage by our colloidal probe microscopy technique was similar to friction on porcine cartilage and comparable to cartilage friction measurements in the absence of interstitial fluid pressurization performed by other researchers. We also showed that internal friction, plowing, and asperity collision had minimal effects on the friction measured in our system. Instead, the friction measured in this system is due primarily to interfacial shear. Plowing and asperity collision in particular can arise due to the AFM probe instead of the properties of the surface. The finding that these effects are minimal eliminates one possible source of error from future experiments using the same technique.

In the second study (Chapter 4), we found that deletion of the Prg4 gene results in significant structural and biomechanical changes in the articular cartilage with age, indicating that the PRG4 glycoprotein plays a significant role in preserving normal joint
structure and function. Histologic analysis of $Prg4^{-/-}$ joints showed an enlarged, fragmented surface layer of variable thickness with Safranin-O positive formations sometimes present, a roughened underlying articular cartilage surface, and a progressive loss of pericellular proteoglycans. Friction was significantly higher on Prg4-/- cartilage at 16 weeks but statistically significant differences in friction were not detected at younger ages. The elastic modulus of the cartilage was similar between $Prg4^{-/-}$ and wild-type cartilage surfaces at young ages but wild-type cartilage showed increasing stiffness with age with significantly higher moduli than $Prg4^{-/-}$ cartilage at later ages.

In the third study (Chapter 5), we found that wear in the presence of PRG4 was significantly lower than in its absence under the conditions tested, a finding which may have direct implications for prevention and treatment of osteoarthritis. It appears that PRG4 needs to be present in solution, not merely on the cartilage surface, to have this effect, pointing to a sacrificial layer mechanism of lubrication and the importance of adsorption properties for effective PRG4 function.

6.2 Proposed topic 1: Self-healing properties of PRG4 layers

**Motivation 1:** Our work shows that PRG4 is effective in reducing wear on NaCl-extracted cartilage surfaces only when present in solution, consistent with a sacrificial layer mechanism of boundary lubrication. We propose to build on this work by testing the properties of these sacrificial layers on model surfaces.

**Hypothesis 1.1:** Wear of PRG4 layers is reduced when PRG4 is present in solution.
**Approach 1.1:** PRG4 readily forms layers on a wide range of model surfaces. We will measure the effect of PRG4 in solution on wear of PRG4 layers on methyl functionalized model surfaces, which we have previously used to study PRG4 adsorption, and lubrication. Monolayers will be prepared as described in Section 4.2.3. Surfaces will be incubated for 5 minutes in 200 µg/ml purified human PRG4 in PBS, washed exhaustively in PBS, then scratched using a protocol similar to that described in Section 5.2 in PBS and in the PRG4 solution. AFM imaging parameters will be adjusted to cause a significant amount of wear on the surface. Wear will be assessed by subtracting the average height of the worn area from that of the surrounding unscratched area.

**Hypothesis 1.2:** PRG4 on model surfaces and on cartilage is replaced by PRG4 from solution when the layer is worn.

**Approach 1.2:** If the experiments proposed in specific aim 1.1 show a reduction in wear in the presence of PRG4, we will design experiments to verify that wear is reduced in the layer because PRG4 adsorbed from solution to replace that removed by wear. For experiments on model surfaces, surface preparation and approach will be identical to that described in approach 1.1 with the exception that fluorescently tagged PRG4 will be used in place of non-fluorescent PRG4. The samples will then be imaged by fluorescent microscopy. (The size of the worn area will also be increased as needed to allow clear detection by fluorescence microscopy.) AFM analysis of wear will be used to confirm that the fluorescently tagged molecules behave similarly to normal PRG4 under these conditions. If difference in adsorption of fluorescent PRG4 can be clearly resolved on model surfaces, the experiment will be repeated on cartilage.
6.3 Proposed topic 2: Morphology and stiffness of superficial cartilage in health and disease

**Motivation 2:** The properties of the superficial layers on cartilage, particularly the uppermost SAL (see Section 0) have not been well characterized. This is a significant impediment to the understanding of cartilage friction and wear. Assessment of this layer in the early stages of osteoarthritis could also lead to techniques for early disease prevention. Specifically, any morphological changes will likely be linked to chemical changes, potentially leading to a minimally invasive biopsy for osteoarthritis.

**Hypothesis 2.1:** The SAL is softer than the underlying tissue and does not significantly affect cartilage friction.

**Approach 2.1:** Stiffness, friction, and topography will be measured on 2 types of modified surfaces. (1) Cartilage will be cut at an angle approximately 20° from parallel to the articular surface (measurements will be performed along the cut to assess properties down to approximately 10 µm below the surface). (2) The SAL will be removed by rubbing with cotton soaked in 10% SDS [103]. This procedure will be performed multiple times on the same cartilage samples to assess the extent to which the process alters properties of underlying cartilage (by removing proteoglycans, for example). Measurements on these samples will be compared to those on unmodified cartilage. If the change in stiffness due to the SAL removal method is small, we will fit indentation data from unmodified cartilage to a thin-layer model, using the stiffness on cartilage from which the SAL has been removed as the stiffness of the underlying material.
Hypothesis 2.2: The content of PRG4, albumin, and HA in the superficial layers of articular cartilage varies as a function of depth.

Approach 2.2: We will attempt to peel the surface fibrous layer and surface amorphous layer (see Section 0) from the surface of articular cartilage by repeating the method of Teshima et al. [91]. If successful, we will use fluorescent tagged secondary antibodies to detect PRG4, albumin, and HA on en bloc sections by confocal microscopy on cartilage from 3 test groups (1) unprocessed, (2) surface amorphous layer removed by rubbing with a cotton swab, (3) surface fibrous layer peeled away. Cartilage from large animals, such as cows or pigs will be used.

Hypothesis 2.3: Molecular scale superficial changes in cartilage morphology occur in osteoarthritis (OA) and can be detected from the earliest stages of OA.

Approach 2.3: We will capture AFM images of visually normal cartilage and visually osteoarthritic cartilage. Features of AFM images will be quantitatively characterized by RMS roughness and skewness and the number of occurrences of distinct features, such as visible collagen fibrils and round globules. If differences between AFM images can be quantitatively distinguished, cartilage at a range of stages of OA progression will be measured. After AFM imaging, joints will be histologically processed and stained and a Mankin score will be assigned to each surface. Care will be taken to assess a location near that imaged by AFM for histological scoring. Scores from AFM images and histology will then be correlated.

AFM images will be captured using an Asylum MFP3D. SNL cantilevers (Veeco Metrology, Santa Barbara, CA) with nominal spring constant 0.12 nN/nm will be coated
in a 15 nm layer of gold and functionalized with a self-assembled monolayer of tri-ethylene glycol terminated thiol as described previously. The 15 nm thickness of the gold coating is chosen to ensure full coverage of the tip without significantly increasing the tip’s radius of curvature. Square images with edge length 5 µm (sufficiently small that resolution will be limited primarily by the tip radius and by cartilage deformation) and 50 µm (to give a larger view of the surface) will be captured.

6.4 Proposed topic 3: Details of cartilage wear protection

Motivation 3: Addition of PRG4 to joints has been proposed as a palliative treatment for OA [158,229]. It is not known under what conditions this is indicated or what volume of PRG4 should be optimally added.

Hypothesis 3.1: PRG4 reduces cartilage wear in a concentration dependent manner.

Approach 3.1: We will measure wear in the presence of recombinant human PRG4 at 0, 50, 200, and 500 µg/ml. 50 µg/ml is chosen as a minimum concentration needed to form monolayers on both hydrophobic and hydrophilic model surfaces [108]. Location-to-location variation of area is a significant source of error in this measurement technique. While each data point was an average of only 2 measurements in Chapter 5, here, we will reduce the effect of location-to-location error by measuring wear on 5 locations in each solution. Osteochondral plugs from the femoral grooves of pigs will be used as there is little variation in the gross topography of the surface at the microscale, allowing imaging and wear in several locations to be automated and allowing AFM analysis over a large range of the surface without remounting. Although wear on murine
cartilage in our experimental setup remains fairly constant over time (see Figure 41), the order will be alternated between increasing and decreasing PRG4 concentrations.

**Hypothesis 3.2:** Wear protection by PRG4 is linked to its structure as a mucinous glycoprotein.

**Approach 3.2:** We will test the role of synovial mucin (a mucin not present in joints which lubricates similarly in a model system [317]), albumin, HA, and DPPC (a protein, long chain sugar molecule, and a phospholipid present in joints), in mediating joint wear to assess the extent to which wear protection by PRG4 is due to its mucinous structure. The latter two molecules have been previously shown to reduce wear in a cartilage-on-cartilage system [184]. If wear protection by PRG4 is relatively effective compared to the other molecules tested, the molecular details of the wear prevention mechanism will be tested using chemically altered PRG4 molecules or genetically controlled variants of recombinant PRG4.

### 6.5 Proposed Topic 4: Revisiting the role of boosted lubrication in cartilage lubrication

**Motivation 4:** Several studies confirm that PRG4 acts as a boundary lubricant on cartilage (see section 2.5.2.3). However, surface associated PRG4 alone has a limited affect on friction (see Figure 38 on page 106). An analysis of the literature gives a possible explanation. Lubrication by synovial fluid appears to be more effective in cases where surfaces are loaded under squeeze film conditions in the presence of lubricants, then oscillated [157,217] than when surfaces are brought into contact while oscillating [131,155]. Lubrication by a filtrate of synovial fluid following squeeze film loading, a
mechanism of cartilage lubrication called boosted lubrication [147], which has been theoretically analyzed [150] but never directly tested, may provide the explanation.

**Hypothesis 4.1:** Cartilage lubrication by synovial fluid in the boundary regime is dependent on the path through which the surfaces are brought into contact.

**Approach 4.1:** Friction will be measured between osteochondral plugs using an ELF [10]. Cartilage samples will be loaded in one of two ways. 1: Samples will be loaded uniaxially with no lateral motion. 2: Samples will be oscillated with respect to one another as they are loaded. In both cases, the creep deformation of the samples will be monitored and friction testing will not begin until the displacement has reached a steady state.

**Hypothesis 4.2:** Components of the thin layer on the surface of cartilage observed by a number of researchers [89,95-99] readily exchange with components of synovial fluid under a combination of cyclic loading and sliding.

**Approach 4.2:** Radiolabeled PRG4 and HA will be obtained. Cartilage will be incubated in the presence of these molecules (1) in absence of load (2) under oscillatory uniaxial loading and unloading and (3) under loading and unloading accompanied by sliding. The extent to which radiolabeled molecules become associated with the cartilage surface in each case will be compared. If these experiments show promise, lubrication of joints in rolling motion could also be tested.
Appendix A. Matlab Codes Used to Process Friction, Indentation and Wear Data

AFM data for all studies were saved as .ibw files by Asylum’s MFP3D software. These were exported as text and processed using MATLAB. The function asylumload.m is a simple script that separates this text file into channels saved by the Asylum software. Friction was processed using the file TMRfromtext.m or similar code. In many cases, scan lines need to be removed for analysis (e.g. where the tip was clearly not tracking well with the cartilage surface. The files TMRfromtext.m, TMRfromtextautokeepall.m, TMRfromtextauto.m, and TMRfromtextlinebylineselect provide varying levels of filters to determine whether scan lines are automatically included in the analysis or plotted for the user to evaluate. TMRfromtextloop.m was written to batch process friction data and save the results. Force curves were fit to the Hertzian indentation model using the code Boris2.m which calls the function rsq.m to calculate the goodness of fit. Adhesion_Energy.m and hysteresis_energy_friction.m were used to calculate adhesion hysteresis and bulk hysteresis, respectively.

Analysis of wear for Chapter 5 was performed using the script file wear22um.m, which calls functions asylumload.m and planefitwoselected.m. Height and friction of wear images were analysed using the script heightandlat.m, which includes all scan lines in the image.

TMRfromtextloop.m
% Written by Jeff Coles, 6-2007
% Instructions to extract TMR values from sets of asylum images.
% % 1) Save all images as text. This program will extract data from sets of % files that have the same filename (different extensions).
% 2) Ensure that the following options are set correctly
% a) for loop (line 22) includes the full range of extensions you
% want to use.
% b) outputrow calls the right program (lines 32-35). The difference between the
% programs is that one automatically uses all points in a file while
% the other asks the user for input.
% c) in the program that is called TMRfromtextnosave, TMRfromtext, or
% TMRfromtextlinebyline, make sure scanpoints, friction, and
% friction2 are defined correctly for the number of total layers saved
% in each text file.
% 3) Enter "TMRfromtextloop('filestem')", where the filestem is the filename
% without numerical or filetype extensions, in the command window and press
% enter. For the file R482loc10003.txt, for example, you would enter
% "TMRfromtextloop('R482loc1')"

function output = TMRfromtextloop(filestem)
for f = 0:4 %change depending on the total number of files you want to analyze
    if f < 10
        filename = [filestem '000' num2str(f) '.txt'];
    elseif f < 100
        filename = [filestem '00' num2str(f) '.txt'];
    else
        filename = [filestem '0' num2str(f) '.txt'];
    end

    % ensure that filepath is correct
    isitthere = exist(filename,'file');
    if isitthere == 0
        disp(['!!Could not find file ', filename '!!']);
        continue
    end

    % outputrow = TMRfromtextautokeepall(filename); % does not ask user to select points to use and keeps
    % all friction curves
    % outputrow = TMRfromtextauto(filename); % does not ask user to select points to use but asks about
    % questionable friction curves
    % outputrow = TMRfromtext(filename); % asks user to select points to use on image
    % outputrow = TMRfromtextlinebyline(filename); % asks user to select points to use on each line

    % not sure what this did
    if outputrow(1) ~= 0
        output(f+1,1:length(outputrow)) = outputrow;
    end

    % reorder output appropriately if scans alternated between ascending and
    % descending set point values (checks by comparing friction value in first
    % and third rows and won't work if there are fewer than three rows
    if exist('output') == 1
        if output(3,1) < output(1,1)
            output = flipud(output);
            disp('OUTPUT HAS BEEN REORDERED!!');
        end
    end
end
%saves file if analysis was successful
if exist('output') == 1
    savename = [filestem 'sum'];
    save(savename,'output');

    %checks to insure file saved
    isitthere = exist(savename,'file');
    if isitthere == 0
        disp('file saved');
    end
end

TMRfromtextautokeepall.m
%Written for quick analysis of a large number of files in conjunction with
code TMRfromtextloop

%Keeps all lines and does not ask user about questionable friction curves
%Cuts data a specified number of points from beginning and end
%Does not display TMR for each image
%Does not ask user to wait while calculating

function TMRmatrix = TMRfromtextauto(filename)
close all;

 cutpoints = 11; % # of points to cut from each end of each scan line

 TMR = 0; % resets TMR
 scanlinesomited = 0; %keeps track of lines not included

 %ensure that filepath is correct
 isitthere = exist(filename,'file');
 if isitthere == 0
     dispstr = ['!!ERROR Could not find file ', filename '!!'];
     disp(dispstr);
 else

     %load file and define height and friction data
     a = load(filename);
     dimensions = size(a);
     scanpoints = dimensions(1)/6;
     scanlines = dimensions(2);
     height = rot90(a(1:scanpoints,:));

     %make sure the following section reads friction trace and retrace.
     %Also, make sure the number defining 'scanpoints' is appropriate for
     %the number of channels saved
     %friction = rot90(a(2*scanpoints+1:3*scanpoints,:));
     %friction2 = rot90(a(3*scanpoints+1:4*scanpoints,:));
     %height2 = rot90(a(scanpoints+1:2*scanpoints,:));
     friction = rot90(a(4*scanpoints+1:5*scanpoints,:));
friction2 = rot90(a(5*scanpoints+1:6*scanpoints,:));

% option to display each image to user to aid user decision on what data to use
% imagesc(height);
% fprintf('Showing height image, click to accept\n');
% waitforbuttonpress;
% imagesc(friction);
% fprintf('Showing friction trace image, click to accept\n');
% waitforbuttonpress;
% imagesc(friction2);
% fprintf('Showing friction retrace image\n');

% option to select data points to use
% select = input('hit enter to include all points, \n  "m" to select points with the mouse, or \n  "k" to select points with the keyboard:  ','s');
% points chosen become top left and bottom right corner of rectangular
% scan area. Actual points will be inside clicked points.
% if select == 'm'
%     disp('select top left, bottom right corner of rectangle to define area to include');
%     [xpoints,ypoints] = ginput(2)
%     firstline = ceil(ypoints(1));
%     lastline = floor(ypoints(2));
%     firstpoint = ceil(xpoints(1));
%     lastpoint = floor(xpoints(2));
% elseif select == 'k'
%     fprintf('
Of scan lines 1-%1.0f, ',scanlines);
%     firstline = input('
enter the first line you would like to include:  ');
%     lastline = input('
Enter the last scan line you would like to include:  ');
%     fprintf('
There are %1.0f points per scan line.  ',scanpoints);
%     firstpoint = input('
Enter the first point you would like to include:  ');
%     lastpoint = input('
Enter the last point you would like to include:  ');
% else
%     firstline = 1; lastline = scanlines; firstpoint = cutpoints+1; lastpoint = scanpoints-cutpoints;
% end

% x currently just 1 per data point but could be adjusted according to
% length scale for true recreation of trace/retrace plot
x = firstpoint:lastpoint;

% cycles through each scan line
for i = firstline:lastline
    trace = friction(i,firstpoint:lastpoint);
    retrace = friction2(i,firstpoint:lastpoint);

    % the offset is the value by which the midpoint of the graph deviates from y = 0
    offset = (mean(trace)+mean(retrace))/2;
% fits for trace and retrace
% slope and intercept 1 and 2 are of trace and retrace, respectively
p = polyfit(x,trace,1);
slope(1) = p(1);
intercept(1) = p(2);
Ltrace = slope(1)*x + intercept(1);

p = polyfit(x,retrace,1);
slope(2) = p(1);
intercept(2) = p(2);
Lretrace = slope(2)*x + intercept(2);

% adjusts trace and retrace to compensate for offset, tilt
for j = 1:length(trace)
    tracenorm(j) = trace(j) - offset + (mean(x) - x(j))*slope(1);
    retracenorm(j) = retrace(j) - offset + (mean(x) - x(j))*slope(2);
end

% automatically accepts curves if and only if min of trace is not lower
% than max of retrace (lines do not cross) and curves never deviate from their mean value by
% more than a certain factor. All other curves must be individually
% accepted or rejected by the user
TMR(i) = (mean(tracenorm) - mean(retracenorm))/2; % adds all curves to TMR matrix
end
end
TMRmatrix = [mean(TMR),std(TMR)];
% fprintf('%n%ncalculation is complete!!')
% fprintf('%n%ntmr = %g', TMRmatrix(1));
% fprintf('%n%ntmr standard deviation = %g', TMRmatrix(2));
% fprintf('%n%noffset = %g', TMRmatrix(4));
% end

TMRfromtextauto.m
% TMR matrix % much code in common with frictionranda. Written only for files with image
% 1 height, image 2 trace, image 3 retrace. Also gives offset value of
% graphs.

function TMRmatrix = TMRfromtextauto(filename)
close all;
TMR = 0; % resets TMR
scanlinesomited = 0; % keeps track of lines not included

% ensure that filepath is correct
isitthere = exist(filename,'file');
if isitthere == 0
    dispstr = ['!!ERROR Could not find file ', filename '!!'];
    disp(dispstr);
else

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% load file and define height and friction data
a = load(filename);
dimensions = size(a);
scanpoints = dimensions(1)/6;
scanlines = dimensions(2);
height = rot90(a(1:scanpoints,:));

% make sure the following section reads friction trace and retrace.
% Also, make sure the number defining 'scanpoints' is appropriate for
% the number of channels saved
% friction = rot90(a(2*scanpoints+1:3*scanpoints,:));
% friction2 = rot90(a(3*scanpoints+1:4*scanpoints,:));
% height2 = rot90(a(scanpoints+1:2*scanpoints,:));
friction = rot90(a(4*scanpoints+1:5*scanpoints,:));
friction2 = rot90(a(5*scanpoints+1:6*scanpoints,:));

% option to display each image to user to aid user decision on what data to use
imagesc(height);
fprintf('Showing height image, click to accept\n')
waitforbuttonpress;
imagesc(friction);
fprintf('Showing friction trace image, click to accept\n')
waitforbuttonpress;
imagesc(friction2);
fprintf('Showing friction retrace image\n')

% option to select data points to use
% select = input('hit enter to include all points, \n "m" to select points with the mouse, or \n "k" to select points with the keyboard: ','s');
% points chosen become top left and bottom right corner of rectangular
% scan area. Actual points will be inside clicked points.
% if select == 'm'
% disp('select top left, bottom right corner of rectangle to define area to include');
% [xpoints,ypoints] = ginput(2)
% firstline = ceil(ypoints(1));
% lastline = floor(ypoints(2));
% firstpoint = ceil(xpoints(1));
% lastpoint = floor(xpoints(2));
% else if select == 'k'
% fprintf('nOf scan lines 1-%1.0f, ','scanlines);
% firstline = input('nenter the first line you would like to include: ');
% lastline = input('nEnter the last scan line you would like to include: ');
% fprintf('nThere are %1.0f points per scan line. ','scanpoints);
% firstpoint = input('nEnter the first point you would like to include: ');
% lastpoint = input('nEnter the last point you would like to include: ');
% else
  firstline = 1; lastline = scanlines; firstpoint = 1; lastpoint = scanpoints;
% end

%x currently just 1 per data point but could be adjusted according to
%length scale for true recreation of trace/retrace plot
x = firstpoint:lastpoint;
k = 0; %initializes loop for TMR vector

cycles through each scan line
disp('Calculating, please wait')
for i = firstline:lastline
  trace = friction(i,firstpoint:lastpoint);
  retrace = friction2(i,firstpoint:lastpoint);
%
% the offset is the value by which the midpoint of the graph deviates from y = 0
offset = (mean(trace)+mean(retrace))/2;
%
% fits for trace and retrace
%slope and intercept 1 and 2 are of trace and retrace, respectively
p = polyfit(x,trace,1);
slope(1) = p(1);
intercept(1) = p(2);
Ltrace = slope(1)*x + intercept(1);

p = polyfit(x,retrace,1);
slope(2) = p(1);
intercept(2) = p(2);
Lretrace = slope(2)*x + intercept(2);
%
% adjusts trace and retrace to compensate for offset, tilt
for j = 1:length(trace)
  tracenorm(j) = trace(j) - offset + (mean(x) - x(j))*slope(1);
  retracenorm(j) = retrace(j) - offset + (mean(x) - x(j))*slope(2);
end
%
% automatically accepts curves if and only if min of trace is not lower
% than max of retrace (lines do not cross) and curves never deviate from their mean value by
% more than a certain factor. All other curves must be individually
% accepted or rejected by the user
if min(tracenorm) > mean(retracenorm) && mean(tracenorm) > max(retracenorm) &&
max(tracenorm) <5*mean(retracenorm) && min(retracenorm) > 5*mean(retracenorm) &&
(abs(mean(TMR)/mean(tracenorm)-1)<0.3 )|| i==firstline
  k=k+1;
  TMR(k) = (mean(tracenorm) - mean(retracenorm))/2; %includes curve in evaluation iff it meets
  conditions
  %tracenormcounter(k,:,1) = tracenorm;
  %tracenormcounter(k,:,2) = retracenorm;
else
% plots adjusted trace and retrace
hold off;
plot(tracenorm);
hold on;
plot(retracenorm,'r');
title(num2str(i))
xlabel('points')
ylabel('Lateral signal (V)')

fprintf('
should line %1.0f, i)
keep = input('nbe included in the calculation, y or n? ','s');
if keep == 'y'
k = k+1;
TMR(k) = (mean(tracenorm) - mean(retracenorm))/2;
%tracenormcounter(k,:,1) = tracenorm;
%tracenormcounter(k,:,2) = retracenorm;
else
    scanlinesomited(i) = i;
end

if keep == 'x'
    break;
end
end
end
TMRmatrix = [mean(TMR),std(TMR),scanlinesomited];

% fprintf('

Calculation is complete!!')
fprintf('

TMR = %g

TMR standard deviation = %g', TMRmatrix(1));
%f fprintf('
offset = %g', TMRmatrix(4));
% end

TMRfromtext.m
% TMR matrix % much code in common with frictionranda. Written only for files with image
% 1 height, image 2 trace, image 3 retrace. Also gives offset value of
% graphs.

% Calls function select area

function TMRmatrix = TMRfromtext(filename)

close all;

TMR = 0; % resets TMR
scanlinesomited = 0; % keeps track of lines not included

% ensure that filepath is correct
isitthere = exist(filename,'file');
if isitthere == 0
disp('!!ERROR Could not find file ', filename '!!');
    return
end
% load file and define height and friction data
a = load(filename);
dimensions = size(a);
scanpoints = dimensions(1)/6;
scanlines = dimensions(2);
height = rot90(a(1:scanpoints,:));

% when height retrace is not saved, also need to change scanpoints to
% dimensions(1)/3
friction1 = rot90(a(4*scanpoints+1:5*scanpoints,:));
friction2 = rot90(a(5*scanpoints+1:6*scanpoints,:));

% user selects area to include
[firstline, lastline, firstpoint, lastpoint] = selectarea(height, friction1, friction2, scanlines, scanpoints)

% x currently just 1 per data point but could be adjusted according to
% length scale for true recreation of trace/retrace plot
%x = firstpoint:lastpoint;
k = 0; % initializes loop for TMR vector

cycles through each scan line
disp('Calculating, please wait')
for i = firstline:lastline
    trace = friction1(i,firstpoint:lastpoint);
    retrace = friction2(i,firstpoint:lastpoint);
    [tracenorm, retracenorm] = normalizefriction(trace, retrace);
%
% the offset is the value by which the midpoint of the graph deviates from y = 0
% offset = (mean(trace)+mean(retrace))/2;
%
% fits for trace and retrace
% % slope and intercept 1 and 2 are of trace and retrace, respectively
% p = polyfit(x,trace,1);
% slope(1) = p(1);
% intercept(1) = p(2);
% Ltrace = slope(1)*x + intercept(1);
%
% p = polyfit(x,retrace,1);
% slope(2) = p(1);
% intercept(2) = p(2);
% Lretrace = slope(2)*x + intercept(2);
%
% adjusts trace and retrace to compensate for offset, tilt
% for j = 1:length(trace)
%     tracenorm(j) = trace(j) - offset + (mean(x) - x(j))*slope(1);
%     retracenorm(j) = retrace(j) - offset + (mean(x) - x(j))*slope(2);
% end
%
% automatically accepts curves if and only if min of trace is not lower
% than max of retrace (lines do not cross) and curves never deviate from their mean value by
% more than a certain factor. All other curves must be individually
% accepted or rejected by the user

if min(tracenorm) > mean(retracenorm) && mean(tracenorm) > max(retracenorm) && max(tracenorm) < 5*mean(tracenorm) && min(retracenorm) > 5*mean(retracenorm) && (abs(mean(TMR)/mean(tracenorm)-1)<0.3 | i==firstline)
    k=k+1;
    TMR(k) = (mean(tracenorm) - mean(retracenorm))/2; %includes curve in evaluation iff it meets conditions
    %tracenormcounter(k,:,1) = tracenorm;
    %tracenormcounter(k,:,2) = retracenorm;
else
    %plots adjusted trace and retrace
    hold off;
    plot(tracenorm);
    hold on;
    plot(retracenorm,'r');
    fprintf('Should line %1.0f be included in the calculation, y or n? ','s');
    if keep == 'y'
        k = k+1;
        TMR(k) = (mean(tracenorm) - mean(retracenorm))/2;
        %tracenormcounter(k,:,1) = tracenorm;
        %tracenormcounter(k,:,2) = retracenorm;
    else
        scanlinesomited(i) = i;
    end
end

%saving files
TMRmatrix = [mean(TMR),std(TMR),scanlinesomited];
%marker = findstr('!', filename);
%savename = [filename(1:marker-1) 'sum'];
%save(savename,'TMRmatrix');

% fprintf('
Calculation is complete!!
')
fprintf('
TMR = %g
', TMRmatrix(1));
% fprintf('
TMR standard deviation = %g', TMRmatrix(2));
% %end

TMRfromtextlinebylineselect.m

function TMRmatrix = TMRfromtextlinebylineselect(filename)
close all;

TMR = 0; % resets TMR
scanlinesomited = 0; % keeps track of lines not included

% ensure that filepath is correct
isitthere = exist(filename,'file');
if isitthere == 0
    disp(['!!ERROR Could not find file ', filename '!!']);
    return
end

% load file and define height and friction data
a = load(filename);
dimensions = size(a);
scanpoints = dimensions(1)/6;
scanlines = dimensions(2);
height = rot90(a(1:scanpoints,:));
friction1 = rot90(a(4*scanpoints+1:5*scanpoints,:));
friction2 = rot90(a(5*scanpoints+1:6*scanpoints,:));

% when height retrace is not saved, also need to change scanpoints to
% dimensions(1)/3
friction1 = rot90(a(4*scanpoints+1:5*scanpoints,:));
friction2 = rot90(a(5*scanpoints+1:6*scanpoints,:));

% user selects area to include
[firstline, lastline, firstpoint, lastpoint] = selectarea(height, friction1, friction2, scanlines, scanpoints);

% x currently just 1 per data point but could be adjusted according to
% length scale for true recreation of trace/retrace plot
x = firstpoint:lastpoint;
k = 0; % initializes loop for TMR vector

% cycles through each scan line
disp('Calculating, please wait')
for i = firstline:lastline
    trace = friction1(i,firstpoint:lastpoint);
    retrace = friction2(i,firstpoint:lastpoint);
    [tracenorm, retracenorm] = normalizefriction(trace, retrace);
    % automatically plots trace and retrace in all cases
    hold off;
    plot(tracenorm);
    hold on;
    plot(retracenorm,'r');
    fprintf('
how many sections on line %1.0f',i);
    keep = input('would you like to include (0, 1, or 2, type 8 to include full line, 9 to break)?    ');
    if keep == 0
        scanlinesomited(i) = i;
    end
    continue;
end
if keep == 1
  k = k+1;
  disp('select start, then end of section to keep')
  [keypts, values] = ginput(2); keypts = round(keypts);  %need integer values for assignments
  tracenorm = tracenorm(keypts(1):keypts(2));
  retracenorm = retracenorm(keypts(1):keypts(2));
  TMR(k) = (mean(tracenorm) - mean(retracenorm))/2;
elseif keep == 2
  k = k+1;
  disp('select start, then end of first section to keep, then start, then end of second section')
  [keypts, values] = ginput(4); keypts = round(keypts);
  tracenorm = [tracenorm(keypts(1):keypts(2)),tracenorm(keypts(3):keypts(4))];
  retracenorm = [retracenorm(keypts(1):keypts(2)), retracenorm(keypts(3):keypts(4))];
  TMR(k) = (mean(tracenorm) - mean(retracenorm))/2;
end
if keep == 8
  k = k+1;
  TMR(k) = (mean(tracenorm) - mean(retracenorm))/2;
end
if keep == 9
  break;
end
end

%saving files
TMRmatrix = [mean(TMR),std(TMR),scanlinesomited];
%marker = findstr('.', filename);
%savename = [filename(1:marker-1) 'sum'];
%save(savename,'TMRmatrix');

% fprintf('
\n\n\nCalculation is complete!!')
fprintf('
\n\n\nTMR = %g

', TMRmatrix(1));
% fprintf('
TMR standard deviation = %g', TMRmatrix(2));
% fprintf('
offset = %g', TMRmatrix(4));
% %end

Boris2.m
% The function used to process indentation curves for first two papers,
% created from script borisfixed

% calls function rsq

% Inputs:
% File name and path eg folder_name\filename calls files
% 'filename0000.dat'...'filename0015.dat' in folder 'folder_name'

%Enter kz in nN/nm
%function Output = borisfixed2(path, kz)
%function [Iatforce Output] = borisfixed2(path, kz) %second output added to show indentation at specific forces

close all;
% Invols adjustment
% If the correct defl invols value was not initially entered, enter both values here
trueinvols = 100;   %(nm/V)
falseinvols = 100;  %(nm/V)

% Enter additional parameters
kz = .58;     %nN/nm
v = 0.2;     %Poisson's ratio
r = 0.000005;     %Indenter radius, m

path = 'KOVAGE\16wk++\R681\';     %include last front slash
root = 'R681loc1E';
firstnum = 14;
lastnum = 14;

for d = firstnum:lastnum

%finds filename
if d < 10
    ext = ['000' num2str(d)];
else
    if d < 100
        ext = ['00' num2str(d)];
    else
        ext = ['' num2str(d)];
    end
end
filename = [path root ext '.dat'];

%gives error message if file doesn't exist
isitthere = exist(filename,'file');
if isitthere == 0
    dispstr = ['!!ERROR Could not find file ', filename];
    disp(dispstr);
else

%Loads data if possible
a = load(filename);
defl = a(:,1)/falseinvols*trueinvols;
raw = a(:,2);

%Adjust for Constant Compliance, kz
separation = - raw + defl; %flips sign of piezo movement so it will truly be "separation
force=kz*defl;

%Zero Separation
separation = separation - min(separation);
%Adjust for Piezo Drift, Zero Force
[maxforce, cutoff] = max(force);
sepslope = separation(1:floor(cutoff/2)); %fits data to first half of approach curve (presumably flat)
forceslope = force(1:floor(cutoff/2));
fit = polyfit(sepslope, forceslope, 1);
force = force - fit(2) - fit(1)*separation;

%Separate Approach and Retract
force1 = force(1:cutoff); %force1 is approach
force2 = force(cutoff:length(force)); %force2 is retract
separation1 = separation(1:cutoff);
separation2 = separation(cutoff:length(raw));

%cuts forces above any specific force value if needed
forcecutoff = max(find(force1<1.5e-7));
force1 = force1(1:forcecutoff);
separation1 = separation1(1:forcecutoff);

% %sloppy way to cut off forces above any specific force value if needed
% for i = 1:length(force1)
% if force1<2e-7
% force2(i) = force1(i);
% separation2(i) = separation1(i);
% end
% end

% Plot Force v. Separation
figure;
hold off;
plot(separation1*1e6, force1*1e9);
hold on
plot(separation2*1e6, force2*1e9, 'r');
title(['Separation v. Force for curve number ' num2str(ext)]);
xlabel('Separation(um)');
ylabel('Force (nN)');
legend('approach', 'retract');

force23 = (force1.^2).^(1/3); %fits upper 75% of the curve
fitrange = find(force1 > 0.25*max(force1));
contact = min(fitrange); %the smallest index that meets the criterion is the initial contact point
yfit = force23(contact:length(force23));
xfit = separation1(contact:length(separation1));
p = polyfit(xfit, yfit, 1);
fit23 = p(1)*xfit+p(2);

E = 0.75*(1-v^2)*r^(-0.5)*(-p(1))^(3/2);

% %plots fit portion of force*(2/3)
% figure
%      plot(xfit, fit23, 'o')
%      hold on
%      plot(xfit, yfit)

% calculates contact point
C=p(2)/((4/3)*(r^.5)*E/(1-v^2))^(2/3);
indentation1 = separation1 - C;  % indentation 2 (retract) is not calculated)

% extracts positive indentation portion of curve
fitrange = find(indentation1<0);
contact = min(fitrange);
indshow = indentation1(contact:length(indentation1));  % measured indentation from contact point to limit
forceshow = force1(contact:length(force1));  % measured force from contact point to limit

% variation: finds indentation seen at specific force
% indicator(d+1) = length(find(forceshow<2e-8))
% latforce(d+1) = mean(indshow(indicator(d+1):indicator(d+1)+1));

% plots experimental data with fit
figure;
plot(indshow, forceshow, 'r.')
hold on
fit = fit23.^((3/2));  % calculates actual fit from linear fit
xlabel('indentation')
ylabel('force')

if length(indshow)>length(fit)
    plot(indshow(length(indshow)+1 - length(fit):length(indshow)), fit)
    % calculates r squared value for forceshow v. fit
    rsquared = rsq(forceshow(length(forceshow)+1 - length(fit):length(forceshow)),fit);
else
    plot(indshow.fit(length(fit)+1 - length(indshow):length(fit)))
    rsquared = rsq(forceshow.fit(length(fit)+1 - length(forceshow):length(fit)));
end

% option to check whether each curve is acceptable
% question = ['Does curve ' ext ' look acceptable (n for no, x to exit)??'];
% check = input(question,'s');

% if check == 'x'
%    break
% end
% if check == 'n'
%    continue
% end
Ematrix1(d+1) = E;
R21(d+1) = rsquared;
I1(d+1) = -min(indentation1);
end
end
if exist('Ematrix1') == 1

    %removes points that deviate by 2.5 standard deviations or more
    k=1;
    for i = 1:length(Ematrix1)
        %     if Ematrix1(i) > mean(Ematrix1)+2.5*std(Ematrix1)|Ematrix1(i) < mean(Ematrix1)-
        2.5*std(Ematrix1)
            %         disp(['force curve from file number 00' num2str(i-1) ' removed ']);
            %         fprintf('because E calculated to be');
            %         disp(num2str(Ematrix1(i)));
            %         continue
        end
        Ematrix(k) = Ematrix1(i);
        R2(k) = R21(i);
        I(k) = I1(i);
        k = k+1;
    end
    %output is a 3x(data points) matrix with E as the first row, R^2 as the second row
    Output(1,:) = Ematrix;
    Output(2,:) = R2;
    Output(3,:) = I;
end

rsq.m
%calculates r squared value according to function in microsoft excel
function rsquared = rsq(x,y)

    % x and y must be the same lengths
    if length(x) ~= length(y)
        disp('!!Error in R squared calculation: vectors must be the same lengths!!')
        return
    end

    %calculation of numerator
    numerator = 0;
    denom1 = 0;
    denom2 = 0;
    for i = 1:length(x)
        numerator = numerator + (x(i) - mean(x))*(y(i) - mean(y));
        denom1 = denom1 + (x(i) - mean(x))^2;
        denom2 = denom2 + (y(i) - mean(y))^2;
    end

    r = numerator/(sqrt(denom1*denom2));
    rsquared = r^2;

Adhesion_Energy.m
% This function outputs the maximum adhesion and adhesion energy for a series of force
% curves (must all be saved as with the same base filename).
% The first and last file number to be called need to set by the user in
% the beginning lines of this function. Inputs are text files with the
% deflection in nm in the first column and Z- piezo movement in the second
% column. Force curves are zeroed and corrected for tilt based on the part
% of the curve far from contact and plotted as force v. nominal (contact
% point is not determined) separation. The region in which adhesion energy
% is calculated is marked with an 'x' on either end.
%
% Outputs are max adhesion force and adhesion energy for each force curve
% analyzed.
%
% Example: adhesion_energy('Indentation\KOVAGE\2wk++\L722\L722loc1E', .4)
% calculates adhesion force and adhesion energy from the force curves saved
% in the file L722loc1E0000.txt, L722loc1E0001.txt, L722loc1E0002.txt, etc.
%
% The code currently assumes that the area where adhesion occurs will be
% more than 100 points long, a safe assumption for the data I was working
% with but not necessarily true for other data.
%
% Written by Jeff Coles, modified from the file hysteresis_energy_friction.m
% written by Jason Blum

function output = adhesion_energy(filestem, kz)

close all

% User inputs - ensure these are correct
firstnum = 0000;
lastnum = 0020;
maxnoise = 0.1; %max distance from zero line counted as 0 for purposes of defining
% intercept for adhesion hysteresis calculation (in NANOMETERS). 0.1 (100
% pN) used for J. Biomech. study.
maxadlength = 6; % This is the maximum length over which an adhesion energy will
% be calculated (in MICRONS). 6 um was used for the J. Biomech. study.
% For curves with adhesion below the zero line over a longer distance, only
% the first maxadlength microns

% End user inputs

for d = firstnum:lastnum

% Assign file extension to each file
if d < 10
    ext = ['000' num2str(d)];
else
    if d < 100
        ext = ['00' num2str(d)];
    else
        ext = ['' num2str(d)];
    end
end

filename = [filestem ext '.dat'];
%gives error message if file doesn't exist
isitthere = exist(filename,'file');
if isitthere == 0
    dispstr = ['!!ERROR Could not find file ', [filestem ext '.dat'], ' in folder ', path '!!'];
    disp(dispstr);
else

    a = load(filename);
    % 1st column of data is deflection
    % 2nd column of data is Zpiezo

    defl = a(:,1);
    Z = a(:,2);
    clear a % must clear variables as you go or it takes up too much memory

    force = kz*defl;
    s = - (Z - defl); %flips sign of Zpiezo data, subtract out deflection to find separation
    clear Z defl

    %Zero Separation
    s = s - min(s); % sets minimum separation equal to zero

    %Adjust for Piezo Drift, Zero Force
    [maxforce, cutoff] = max(force); %cutoff is the index between trace and retrace, where deflection is maximum
    sepslope = s(1:floor(cutoff/4)); %fits data to first quarter of approach curve (presumably flat)
    forceslope = force(1:floor(cutoff/4)); %cuts force data to same length
    fit = polyfit(sepslope,forceslope,1);
    force = force - fit(2) - fit(1)*s; %rotates force data according to fit to first quarter of approach curve

    %Separate Approach and Retract
    f1 = force(1:cutoff); %force1 is approach
    f2 = force(cutoff:length(force)); %force2 is retract
    s1 = s(1:cutoff); %s1 is separation approach
    s2 = s(cutoff:length(force)); %s2 is separation retract

    % Find max adhesion
    [maxadhesion minimumindex] = min(f2);
    maxadhesionlist(d+1) = maxadhesion*-1e9; %writes each max adhesion value to an array

    % Define first and last indices for adhesion hysteresis calculation
    % (avoids inflation of adhesion hysteresis by thousands of points
    % just barely below the zero line due to noise
    [ignore maxindex] = max(s2);
    intercepts = find(abs(f2*1e9)<maxnoise); % indices for retract that hit zero (actually retract that hits 100 pN or less)
    adstart = intercepts(1); % first point adhesion calculated from is the first within 100 pN of 0 force
    for i = 2:length(intercepts)
        if intercepts(i) - intercepts(i-1) > 100 %identifies gap of 100 points or more between indices with values close to zero

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adend = intercepts(i);  %last point adhesion calculated from is the first within 100 pN of 0 after a break of 10 or more points
   disp(['file # ' num2str(d) ': assigned adhesion points based on proximity to zero line'])
   break
end
end

%case where an end adhesion point isn't found because
%retract curve doesn't come back to zero
if i == length(intercepts)
   adend = max(find(abs(s2*1e6 - maxadlength - s2(adstart)*1e6)<.01));  %if second adhesion point not found, finds point a user-defined distance past first adhesion point.  6 um was used for j. biomech. study.
   disp(['file # ' num2str(d) ': assigned adhesion end point as 6 um past adhesion start point'])
end

%case where end adhesion point isn't found and the retract portion
%of the curve is less than 6 um long.
if isscalar(adend) == 0
   adend = length(s2);  %final point counted for adhesion is simply last point in retract curve
   disp(['file # ' num2str(d) ': assigned adhesion end point as as end of data!!'])
end

figure
plot(s1,f1, 'r')
hold on;
plot(s2,f2)
plot(sepslope, forceslope)
plot(s2(adstart), f2(adstart),'x')   %puts x at start point of adhesion caculation
plot(s2(adend), f2(adend),'x')       %puts x at end point of adhesion caculation
%area(s2(adstart:adend),f2(adstart:adend))       %fills in adhesion area (doesn't always work)
adhesionenergy(d+1) = -trapz(s2(adstart:adend),f2(adstart:adend))*1e15; %femtoJoules
   title(['file # ' num2str(d) ', Max ad = ' num2str(maxadhesionlist(d+1)) ' nN, ' 'AE = ' num2str(adhesionenergy(d+1)) ' fJ'])
end
end

%Writes everything to output variable
output(1,:) =  maxadhesionlist;
output(2,:) =  adhesionenergy;

%saves output
savename = [filestem 'ad'];
save(savename,'output');

%checks to ensure file saved
isitthere = exist([savename '.mat'],'file');
if isitthere == 0
   disp('file not saved');

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else
    disp(['output saved as ' savename])
end

hysteresis_energy_friction.m
(Written by Jason Blum, Pratt Fellow 2007-2007)

%Code skeletons, especially load file loops, are credited to Jeff Coles.
%
%MATLAB file that quantifies hysteresis in force curves and calculates friction due to inelastic
%deformation.
%
%Input is .dat force curve file, output is 7x(number of files) matrix of Elastic Modulus, coefficient of
%determination for the Elastic Modulus, maximum indentation, adhesion hysteresis energy, bulk hysteresis
%energy, hysteretic loss fraction, and COF due to inelastic deformation.
%--------------------------------------------------------------------------------------------------

clc
clear all
close all

path = 'm9\';   %include last front slash
root = 'M9RJ2loc3E';
firstnum = 0000;
lastnum = 0000;

for d = firstnum:lastnum
    if d < 10
        ext = ['000' num2str(d)];
    else
        if d < 100
            ext = ['00' num2str(d)];
        else
            ext = ['' num2str(d)];
        end
    end
    filename = [path root ext '.dat'];
    %gives error message if file doesn't exist
    isitthere = exist(filename,'file');
    if isitthere == 0
        dispstr = ['!!ERROR Could not find file ', [root ext '.dat'], ' in folder ', path '!'];
        disp(dispstr);
    else
        a = load(filename);
        % 1st column of data is deflection
        % 2nd column of data is Zpiezo
        defl = a(:,1);
        Z = a(:,2);
clear a % must clear variables as you go or it takes up too much memory

```matlab
kz = .4;   %nN/nm
v = 0.2;   %Poisson's ratio
r = 5e-6;  %Indenter radius, m
force = kz*defl;
separation = -(Z - defl); %flips sign of Zpiezo data, subtract out deflection to find separation
clear Z defl
%Zero Separation
separation = separation - min(separation); % sets minimum separation equal to zero
s = separation;
clear separation
%Adjust for Piezo Drift, Zero Force
[maxforce, cutoff] = max(force);      % cutoff is the index between trace and retrace, where deflection is maximum
f1 = force(1:cutoff);                   %force1 is approach
f2 = force(cutoff:length(force));       %force2 is retract
s1 = s(1:cutoff);
s2 = s(cutoff:length(force));

%%% Use this section to fit with the retract data
check = 1;
flatstart = max(s)/3*1e6;
% flatstart = 1.2;
while check == 1
% separation in micrometers above which the data is a straight line
% this number may need to be changed frequently because it seems to switch between 2, 3, and 4 randomly
q = find(s*1e6>flatstart); % indexes of values beyond flatstart that are used in the fit
sfit = s(q(find(q>cutoff)));      % fits data to the retract curve, making the data flat and at zero force
forcefit = force(q(find(q>cutoff)));
if length(sfit)==0 length(forcefit)==0
    file_with_error = ['root' '000' num2str(d)];
    disp([file_with_error ' --- Fit error. Automatically decreased the variable flatstart to ' num2str(max(s)*1e6-.1)])
    flatstart = flatstart-.1;
    continue
end
driftfit = polyfit(sfit,forcefit,1);
force = force - polyval(driftfit,s);
forcefit = polyfit(sfit,force(q(find(q>cutoff))),1); % fit to the retract
if abs(forcefit(1)) > 2e-20
    file_with_error = ['root' '000' num2str(d)];
    disp([file_with_error ' --- Fit error. Automatically increased the variable flatstart to ' num2str(flatstart+.1)])
    flatstart = flatstart+.1;
    continue
end
% sfit = 0; % uncomment this to force use of the approach in the fit

if (max(sfit) - min(sfit)) < max(s2)/4 % if points used in the fit span less than 1/4 of the maximum separation, use the approach
    check2 = 1;
end
```

file_with_error = [root '000' num2str(d)];
disp([file_with_error ' --- Using approach for fit'])
flatstart = max(s)/3*1e6;

while check2 == 1
    file_with_error = [root '000' num2str(d)];
    [maxforce, cutoff] = max(force);
    w = find(s*1e6>flatstart);
    sepslope = s(find(w<cutoff));
    for seslope = force(find(w<cutoff));
        forcefit = polyfit(sepslope,forceslope,1);
        force = force - polyval(forcefit,s);
    end
end

check = 0;
end
end
end
end
end
end
end
end

file_with_error = [root '000' num2str(d)];
disp([file_with_error ' flatstart = ' num2str(flatstart)])
clear sepslope forceslope driftfit q
%%%%%%%%%%%%%%%%%%%

% Separate Approach and Retract
f1 = force(1:cutoff); % force1 is approach
f2 = force(cutoff:length(force)); % force2 is retract
s1 = s(1:cutoff);

% % Trim the huge unused portion of data
% trimmer1 = min(find(s1*1e6<4)); % comes before the max force cutoff
% trimmer2 = min(find(s2*1e6>4)); % comes after the max force cutoff
% f1 = f1(trimmer1:length(f1));
% s1 = s1(trimmer1:length(s1));
% f2 = f2(1:trimmer2);
% s2 = s2(1:trimmer2);
% cuts forces above any specific force value if needed
forcecutoff = max(find(f1<1.5e-7)); % finds the max index with force below 150 nN
f1 = f1(1:forcecutoff); % cuts off the indexes beyond the forcecutoff index
s1 = s1(1:forcecutoff);
forcecutoff2 = max(find(f2<1.5e-7));
f2 = f2(1:forcecutoff2);

s2 = s2(1:forcecutoff2);
\text{s1atmax} = \text{s1}(\text{forcecutoff}); \\
\text{s2indexatmax} = \text{round}(\text{mean}(\text{find}(\text{abs}(\text{s2} - \text{s1atmax})*1e6<.001))); \\
\text{s2atmax} = \text{s2}(\text{s2indexatmax});

%% Plot Force v. Separation 
figure 
subplot(2,3,1) 
plot(s1*1e6,f1*1e9, s2*1e6, f2*1e9, 'r'); 
% title(['Separation v. Force for curve number ' num2str(\text{ext})]) 
% xlabel('Separation(\text{mum})'); 
% ylabel('Force (\text{nN})'); 
% legend('approach', 'retract'); 
% axis([0 3 -\text{inf} \text{inf}]) 
force23 = f1.^(2/3);  % This code utilizes the Hertz indentation equation set to the 2/3 power 
% fits upper 75\% of the curve 
fitrange = find(f1 > 0.25*\text{max}(f1));  % only uses the linear part of the force curve 
forcestart = \text{min}(\text{fitrange});  % the smallest index that meets the criterion 
sepcon1 = s1(\text{forcestart});  % the separation value at smallest index that meets the criterion 
forcestart2 = \text{round}(\text{mean}(\text{find}(\text{abs}(\text{s2} - \text{sepcon1})*1e6<.01)));  % the index of retract where \text{s1} = \text{s2} 
sepcon2 = s2(\text{forcestart2}); 
\text{y} = \text{force23}(\text{forcestart:length(\text{force23})}); 
\text{x} = \text{s1}(\text{forcestart:length(s1)}); 
\text{p} = \text{polyfit}(\text{x}, \text{y}, 1); 
\text{fit23} = \text{polyval}(\text{p},\text{x});  % \text{fit23} is the fit raised to the 2/3 power (linear fit to the line) 
\text{E} = 0.75*(1-v^2)*r^(-1/2)*(-p(1))^{(3/2)};  % Young's Modulus of the indented surface, calculated with the slope of the line 
fitresiduals = \text{y} - \text{fit23};  % Used to judge when adhesion hysteresis is taking over instead of bulk hysteresis

%% plots fit portion of force^(2/3) with fit 
subplot(2,3,2) 
plot(\text{x}*1e6, \text{fit23}, 'r', \text{x}*1e6, \text{y}) 
% legend('linear fit','approach data') 
% title('Separation vs. Force'\text{^}(^2/^3^)) 
% xlabel('Separation (\text{mum})') 
% ylabel('Force (\text{nN})') 
% subplot(2,3,3) 
% plot(\text{x}*1e6,\text{fitresiduals},'kx') 
% title('Residuals for Force'\text{^}(^2/^3^) \text{ fit}') 
% xlabel('Separation (\text{mum})') 
% ylabel('Force (\text{nN})') 

% calculates contact point 
\text{C} = \text{p(2)}/((4/3)^*(r^*.5)*E*(1-v^2))^{(2/3)}; 
\text{indentation1} = \text{s1} - \text{C};  % indentation 2 (retract) is not calculated 
\text{i1} = \text{indentation1}*1e6; 

% extracts positive indentation portion of curve 
ifitrange = find(\text{i1}<0); 
\text{contact} = \text{min}(\text{ifitrange}); 
\text{indshow} = \text{i1}(\text{contact:length(i1)});  % measured indentation from contact point to limit 
\text{forceshow} = \text{f1}(\text{contact:length(f1)})*1e9;  % measured force from contact point to limit
% plots experimental data with fit
forcefit = fit23.*(3/2)*1e9; % calculates actual fit from linear fit
if length(indshow)>length(forcefit)
    subplot(2,3,4)
    plot(indshow, forshow, 'g.', indshow(length(indshow)+1:length(forcefit)), forcefit)
    title('Force vs. Indentation')
    xlabel('Indentation (\mum)')
    ylabel('Force (nN)')
else
    subplot(2,3,4)
    plot(indshow, forshow, 'g.', indshow(length(forcefit)+1:length(indshow)), forcefit)
    rsquared = rsq(forshow(length(forcefit)+1:length(indshow)), forcefit)
end
%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
%% Adhesion hysteresis energy
[maxadhesion minimumindex] = min(f2);
[ignore maxindex] = max(s2);
intercepts = find(abs(f2*1e9)<.1); % indexes for retract that hit zero
if sum(intercepts) == intercepts(1) % if there is only one intercept, use 3 micrometers as the other intercept
    intercepts(2) = max(find(abs(s2*1e6 - 3)<.01));
elseif sum(intercepts) == intercepts(1) % if there is only one intercept and there are no data points beyond 3 micrometers, use the farthest separation
    intercepts(2) = maxindex;
elseif intercepts(1) > minimumindex % if the find command was too strict and didn't find an intercept to the left of min(f2), widen the range
    intercepts(1)= min(find(abs(f2*1e9)<.1));
    intercepts = [min(intercepts) min(intercepts(find(intercepts>minimumindex)))];
elseif intercepts < minimumindex & max(s2) > 3e-6
    intercepts = [min(intercepts) max(find(abs(s2*1e6 - 3)<.01))];
elseif intercepts < minimumindex & max(s2) < 3e-6
    intercepts = [min(intercepts) maxindex];
elseif s2(min(intercepts(find(intercepts>minimumindex)))) < 3e-6
    intercepts = [min(intercepts) min(intercepts(find(intercepts>minimumindex)))] ;
else
    intercepts = [min(intercepts) max(find(abs(s2*1e6 - 3)<.01))];
end
% figure
% plot(s2(intercepts),f2(intercepts),'kx',s2,f2,'r',s2,0,'g',s1,f1,'b')
lengthadhesion = intercepts(2) - intercepts(1); % N*m = J
adhesion_hysteresis = intercepts(2) - intercepts(1);
trapz(s2(intercepts(1):round(lengthadhesion/1000):intercepts(2)),f2(intercepts(1):round(lengthadhesion/1000):intercepts(2))); % N*m = J
%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
%% Bulk hysteresis energy
lengthbulk1 = fitrange(end) - fitrange(1);
lengthbulk2 = -(s2indexatmax - forcestart2);
% this final subplot is supposed to show the filled bulk area, but is extremely hard to get to work, so this is incomplete right now
% subplot(2,3,6)
% for i = 1:(length(1:bottomf2index)/length(fitrange)):bottomf2index
% BaseValue = f2(round(i));
% area(s1(flipud(fitrange))*1e6,f1(flipud(fitrange)), BaseValue)
% hold on
% end
% plot(s1*1e6,f1*1e9,s2*1e6, f2*1e9, 'r');
% title('Bulk Hysteresis')
% xlabel('Separation (m)')
% ylabel('Force (N)')
% axis([0 .25 -15 max(f1)*1e9])

end
k=1;
for i = 1:length(Ematrix1)
    if Ematrix1(i) > mean(Ematrix1)+2.5*std(Ematrix1) | Ematrix1(i) < mean(Ematrix1)-2.5*std(Ematrix1)
        disp([file ext. num2str(i) ' removed'])
        continue
    end
    Ematrix(k) = Ematrix1(i);
    R2(k) = R21(i);
    I(k) = I1(i);
    AdhEn(k) = AdhEn1(i);
    BulEn(k) = BulEn1(i);
    BETA_fc(k) = BETA_fc1(i);
    Mu_Elastic(k) = Mu_Elastic1(i);
    MaxAdhesion(k) = MaxAdhesion1(i);
    k = k+1;
end
Output(1,:) = Ematrix;
Output(2,:) = R2;
Output(3,:) = I;
Output(4,:) = AdhEn;
Output(5,:) = BulEn;
Output(6,:) = BETA_fc;
Output(7,:) = Mu_Elastic;
format short e
Output;
MaxAdhesion = flipud(rot90(MaxAdhesion))*-1e9
close all

wear22um.m
%Performs standard image analysis for wear as of 7-20-2010. Subtracts before
%image from after image based on planefit of outer 4 um section of 22 um square
%image. Analyzes the change in volume within the central 4 um, 6 um, and 8 um of the
%image.

%Calls functions:
%asylumload, planefitwoselected

clear all;
close all;

%Filename - include path from working directory with slash
path = '7-10\L467\';
filestem = 'LUBloc3';

prefilename = [path filestem 'before0000.txt'];
postfilename = [path filestem 'after0000.txt'];

%How many total images in the pre-scratch file (height trace, height retrace, deflection trace, etc.)?
%Assumes that height trace is first image and height retrace is second
imgtotpre = 4;
% How many total images in the pre-scratch file (height trace, height retrace, deflection trace, etc.)?
% Assumes that height trace is first image and height retrace is second
imgtotpost = 4;
% Length of one side of image in microns (assumes square image, also assumes pre, post images are the
% same size)
edgelength = 22;
% Note = ";

% End user

%%%%%End user inputs%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
% Load pre-scratch image
pretrace = asylumload(prefilename, 3, imgtotpre, 'u');
preretrace = asylumload(prefilename, 4, imgtotpre, 'u');
preimgdata = (pretrace+preretrace)/2;
preimgdatasize = size(preimgdata);
% Plot Pre-scratch Image
figure;
axisum = (0:edgelength/(length(preimgdata)-1):edgelength);
imagesc(axisum, axisum, preimgdata); xlabel('distance (um)'); ylabel('distance (um)'), title('Pre-scratch Image');
colormap(gray(256)); hold on; axis xy;

% Load Post-Scratch Image
posttrace = asylumload(postfilename, 3, imgtotpost, 'u');
postretrace = asylumload(postfilename, 4, imgtotpost, 'u');
postimgdata = (posttrace+postretrace)/2;
postimgdatasize = size(postimgdata);
% Plot Post-Scratch Image
figure
axisum = (0:edgelength/(length(postimgdata)-1):edgelength); %define axes in um
imagesc(axisum, axisum, postimgdata); xlabel('distance (um)'); ylabel('distance (um)'); title('Post-scratch image');
colormap(gray(256)); hold on; axis xy;

difference = postimgdata-preimgdata;
differenceplanefit = planefitwoselected(difference, 2, 127, 2, 127);
differenceplanefit = planefitwoselected(difference, round(preimgdatasize(1)*1/11),round(preimgdatasize(1)*10/11),round(preimgdatasize(2)*1/11),round(preimgdatasize(2)*10/11)); % points around an 18x18 um square at the center of the 22x22 um square are selected.
figure; imagesc(differenceplanefit*1e9); colorbar;
% Show 4x4 um area at center
xsquare = [preimgdatasize(2)*9/22, preimgdatasize(2)*13/22, preimgdatasize(2)*13/22, preimgdatasize(2)*9/22];
ysquare = [preimgdatasize(1)*9/22, preimgdatasize(1)*9/22, preimgdatasize(1)*13/22, preimgdatasize(1)*9/22];
hold on; plot(xsquare,ysquare, 'r'); title([filestem 'difference image, data in nm']); xlabel('xdata, pixels (size is 22 um square');

% savefigure
if exist([path 'processing'], 'dir') == 0
    mkdir([path 'processing'])
end
saveas(gcf, [path 'processing' filestem 'diffimg.jpg'])

% show 2x8 um areas at ends of scratches
xrect1 = [preimgdatasize(2)*7/22, preimgdatasize(2)*15/22, preimgdatasize(2)*7/22, preimgdatasize(2)*15/22];
yrect1 = [preimgdatasize(1)*3/22, preimgdatasize(1)*3/22, preimgdatasize(1)*6/22, preimgdatasize(1)*6/22];
hold on; plot(xrect1,yrect1, 'b');
xrect2 = [preimgdatasize(2)*7/22, preimgdatasize(2)*15/22, preimgdatasize(2)*7/22, preimgdatasize(2)*15/22];
yrect2 = [preimgdatasize(1)*16/22, preimgdatasize(1)*16/22, preimgdatasize(1)*19/22, preimgdatasize(1)*19/22];
hold on; plot(xrect2,yrect2, 'b');

% calculate depth
centerdifference4um = differenceplanefit(round(preimgdatasize(1)*9/22):round(preimgdatasize(1)*13/22),round(preimgdatasize(2)*9/22):round(preimgdatasize(2)*13/22));
avdepth4um = -mean(mean(centerdifference4um))
centerdifference6um = differenceplanefit(round(preimgdatasize(1)*8/22):round(preimgdatasize(1)*14/22),round(preimgdatasize(2)*8/22):round(preimgdatasize(2)*14/22));
avdepth6um = -mean(mean(centerdifference6um))
centerdifference8um = differenceplanefit(round(preimgdatasize(1)*7/22):round(preimgdatasize(1)*15/22),round(preimgdatasize(2)*7/22):round(preimgdatasize(2)*15/22));
avdepth8um = -mean(mean(centerdifference8um))
summary = [avdepth4um avdepth6um avdepth8um];
save([path 'processing' filestem '.txt'], 'summary', '-ascii')

Asylumload.m
%% Inputs
% * Name of text file exported from Asylum MFP3D,
% * Number of image
% * Total number of images (eg 3rd image out of a total of 7 images)
% * Frame direction, should be either 'u' or 'd' for "frame up" or "frame down"

%% Outputs
% Data from a single image that, when plotted, is oriented as it appears
% when the IBW file is opened in Igor.

% IMPORTANT NOTE: Data saved using frame down is not always saved in the
% same orientation. Data captured using frame up will always be read

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function data = asylumload(filename, imgnumber, imgtot, frame)
    alldata = load(filename);
    sizes = size(alldata);
    points = sizes(1)/imgtot; % define points and lines separately for non-square images
    lines = sizes(2);

    % Checks that sizes above is divided by the right number for a square image
    if points ~= sizes(2)
        disp('Warning: extracted image is not square!! Check to ensure the correct value was entered for image
tot when calling function asylumload')
        fprintf('The file %s appears to contain %6.0f ra ther than %6.0f images', filename, sizes(1)/sizes(2), imgtot)
    end

    % checks that imgnumber is less than total # of images
    if imgnumber>imgtot
        disp('Error: The image number must be less than or equal to the total number of images')
    end

    % gives output for frame up or frame down
    data = flipud(rot90(alldata(1+points*(imgnumber-1):points*imgnumber,:)));
    if frame == 'd'
        data = rot90(rot90(data));
    elseif frame ~= 'u'
        disp('Error: the 4th input to the function Asylum load must be either u or d for frame up or frame down')
    end

 Planefitwoselected.m

% Corrects a 2-D height image based on a planefit to the image with a
% user-determined square area of the image not included. Called by
% function wear22um and used to process data for Chapter 5 of thesis.
function planefitdata = planefitwoselected(data,icutmin,icutmax,jcutmin,jcutmax)

        [ilimit, jlimit] = size(data);
        a11 = 0; a12 = 0; a13 = 0; a21 = 0; a22 = 0; a23 = 0; a31 = 0; a32 = 0; a33 = 0; b11 = 0; b21 = 0; b31 = 0;
        for i = 1:ilimit
            for j = 1:jlimit
                if (i < icutmin | i > icutmax | j < jcutmin | j > jcutmax) % if data point is outside the square
                    a11 = a11 + i^2;
                    a12 = a12 + i*j;
                    a13 = a13 + i;
                    a21 = a21 + i*j;
                    a31 = a31 + i;
                    a32 = a32 + i*j;
                end
            end
        end

        a11 = a11 / ilimit;
        a12 = a12 / ilimit;
        a13 = a13 / ilimit;
        a21 = a21 / ilimit;
        a22 = a22 / ilimit;
        a23 = a23 / ilimit;
        a31 = a31 / ilimit;
        a32 = a32 / ilimit;
        a33 = a33 / ilimit;
        b11 = b11 / jlimit;
        b21 = b21 / jlimit;
        b31 = b31 / jlimit;

        % planefits data using equations from
        % initializes variables for summations
        % planefits data using equations from
        [ilimit, jlimit] = size(data);
        % initializes variables for summations
        a11 = 0; a12 = 0; a13 = 0; a21 = 0; a22 = 0; a23 = 0; a31 = 0; a32 = 0; a33 = 0; b11 = 0; b21 = 0; b31 = 0;
        for i = 1:ilimit
            for j = 1:jlimit
                if (i < icutmin | i > icutmax | j < jcutmin | j > jcutmax) % if data point is outside the square
                    a11 = a11 + i^2;
                    a12 = a12 + i*j;
                    a13 = a13 + i;
                    a21 = a21 + i*j;
                    a31 = a31 + i;
                    a32 = a32 + i*j;
                end
            end
        end

        a11 = a11 / ilimit;
        a12 = a12 / ilimit;
        a13 = a13 / ilimit;
        a21 = a21 / ilimit;
        a22 = a22 / ilimit;
        a23 = a23 / ilimit;
        a31 = a31 / ilimit;
        a32 = a32 / ilimit;
        a33 = a33 / ilimit;
        b11 = b11 / jlimit;
        b21 = b21 / jlimit;
        b31 = b31 / jlimit;

        % planefits data using equations from
        % initializes variables for summations
        % planefits data using equations from
        [ilimit, jlimit] = size(data);
        % initializes variables for summations
        a11 = 0; a12 = 0; a13 = 0; a21 = 0; a22 = 0; a23 = 0; a31 = 0; a32 = 0; a33 = 0; b11 = 0; b21 = 0; b31 = 0;
\[ a_{22} = a_{22} + j^2; \]
\[ a_{23} = a_{23} + j; \]
\[ a_{31} = a_{31} + i; \]
\[ a_{32} = a_{32} + j; \]
\[ a_{33} = a_{33} + 1; \]
\[ b_{11} = b_{11} + i \cdot \text{data}(i,j); \]
\[ b_{21} = b_{21} + j \cdot \text{data}(i,j); \]
\[ b_{31} = b_{31} + \text{data}(i,j); \]

\[
\text{end}
\]
\[
\text{end}
\]
\[
\text{end}
\]

\[
A = \begin{bmatrix} a_{11}, a_{12}, a_{13}; a_{21}, a_{22}, a_{23}; a_{31}, a_{32}, a_{33}\end{bmatrix};
\]
\[
B = \begin{bmatrix} b_{11}; b_{21}; b_{31}\end{bmatrix};
\]
\[
X = \text{inv}(A) \cdot B;
\]

%Planefit applied to matrix "data"
\[
[\text{ilength} \ j\text{length}] = \text{size(data)};
\]
\[
\text{for } i = 1:\text{ilength}
\]
\[
\text{for } j = 1:\text{jlength}
\]
\[
\text{correction}(i,j) = X(1) \cdot i + X(2) \cdot j + X(3);
\]
\[
\text{end}
\]
\[
\text{end}
\]

\[
\text{planefitdata} = \text{data} - \text{correction};
\]

\text{heightandlat.m}
%This function retrieves the height of a scan (averaging both trace and retrace)
%and the average trace minus retrace value (raw friction signal). It is currently
%assumed that height trace and retrace are saved as the first and second images of
%four and the lateral signal is saved as the third and fourth. The
%code outputs both average values for the full area of the image and the
%average value for the central area of the image with height and width each
%equal to 1/2 of that of the original image.

% Note: Changed to only output TMR of whole image

%function output = heightandlat(filestem)

%clear
close all
clear summary

%User Inputs
directory = '10-10\R1768\friction scans\lub'; %separating the filename this way makes it easy to save
images and variables in the same folder the images are in
filestem = 'lubbloc1'; %included in case user wants to run code
%as a script instead of a function
firstext = 1;
lastext = 30;

%Create directory to save output
if exist([directory '\heightandlat'], 'dir') < 1
mkdir([directory 'heightandlat'])
end

for f = firstext:lastext
  % Assemble filenames based on user inputs
  if f < 10
    filein = [directory '/' filestem '000' num2str(f) '.txt'];
  else
    if f < 100
      filein = [directory '/' filestem '00' num2str(f) '.txt'];
    else
      filein = [directory '/' filestem '0' num2str(f) '.txt'];
    end
  end
  e = exist(filein,'file');
  if e == 0
    dispstr = ['Could not find file ' filein];
    disp(dispstr);
    if f == lastext
      return
    else
      continue
    end
  end
  % Load file
  a = load(filein);
  dimensions = size(a);
  scanpoints = dimensions(1)/6;
  scanlines = dimensions(2);
  % Load file and define height and friction data
  htt = rot90(a(1:scanpoints,:))*1e6;  % Height information converted to microns
  middlehtt = htt(scanlines/4+1:3*scanlines/4,scanpoints/4+1:3*scanpoints/4);  % Selects areas in the center of matrices with height and width half of the full matrices
  htr = rot90(a(scanpoints+1:2*scanpoints,:))*1e6;
  middlehtr = htr(scanlines/4+1:3*scanlines/4,scanpoints/4+1:3*scanpoints/4);  % Selects areas in the center of matrices with height and width half of the full matrices
  latt = rot90(a(4*scanpoints+1:5*scanpoints,:))*1e3;  % Lateral information converted to mV
  middlelatt = latt(scanlines/4+1:3*scanlines/4,scanpoints/4+1:3*scanpoints/4);  % Selects areas in the center of matrices with height and width half of the full matrices
  latr = rot90(a(5*scanpoints+1:6*scanpoints,:))*1e3;
  middlcelatr = latr(scanlines/4+1:3*scanlines/4,scanpoints/4+1:3*scanpoints/4);  % Selects areas in the center of matrices with height and width half of the full matrices
  avht(f+1) = (mean(mean(htt))+mean(mean(htr)))/2;
  middleavht(f+1) = (mean(mean(middlehtt))+mean(mean(middlehtr)))/2;
  TMR(f+1) = (mean(mean(latt))-mean(mean(latr)))/2;
  middlcelTMR(f+1) = (mean(mean(middlelatt))-mean(mean(middlcelatr)))/2;
end
% Output = avht';
% Output = TMR';
output = [avht;middleavht;TMR;middlcelTMR]';  % Transposed to save in columns instead of rows
%save([directory 'heightandlat' filestem 'fullht,middleht,fullTMR,middleTMR.txt'], 'output', 'ascii', 'tabs');
%disp('data saved')
Appendix B. Lateral Calibration

The wedge method for lateral calibration [264] was used for calibrated friction measurements reported in Chapter 4 and Chapter 5 using equations modified for spherical probes [255]. In this method, the cantilever is scanned along a flat surface and a sloped surface and a calibration factor is calculated from a force balance on the lever and the lateral signal detected during scanning. Friction loops 30° silicon wedge fabricated by focused ion beam milling (Advanced Scanning Probe Solutions, Enschede, Netherlands). Fabrication of the wedge and its use for calibration of various probes has been published [298]. A schematic of the wedge with accompanying sample lateral data is shown below in Figure 46. The wedge was cleaned before each use. To this end it was sonicated at power 2 for 10 minutes in 0.5% (w/v) SDS in deionized water. It was then scanned along the edge of the wedge as shown in figure at 10 µm/s with the slow scan axis disabled. The scan up button was used to start the scan and the set point was incremented down from the maximum force that would be used in the experiment to zero using the user function RampSetPoint.ipf.
The following Matlab programs were written by Jason Blum (Pratt Fellow, 2007-2008) and used to calculate lateral calibration factors used in Chapter 4 and Chapter 5. Lateral calibration reported in Chapter 3 was accomplished by finding the lateral spring constant and lateral sensitivity sensitivity separately. A small (~ 200 nm) friction loop was captured on a glass surface in air and the following code was used to find the lateral sensitivity from the data. An average of 4 or more values was used for each calibration.

Calortiz.m: File is used after wedge calibration data acquisition to select usable data and correct for absolute deflection drift. References outliertrimmer.m (A3.1) and getalphaoortiz colloidal.m (A3.2).

Input is contact mode scan data with 10 channels captures: height trace and retrace, deflection trace and retrace, z scanner trace and retrace, deflection set point + deflection trace and retrace, and lateral trace and retrace. Outputs are the COF of the flat region, COF of sloped region, calibration constant, and the standard deviations associated with each.

```
clear
clc

close all;

% user input
k = .54;
```
deflINVOLS = 32;
VLOslope = 0;
R = 5; % tip radius in micrometers
t = 0.6; % tip thickness in micrometers
filein = 'M9WedgeCal0001.txt'; % file name, including directory tree

% finds number of points and lines (ensure number of images correct)
a = load(filein);
sizes = size(a);
points = sizes(1)/10;
lines = sizes(2);

% defines ranges for images
% columns are scan lines
height1 = rot90(a(1:points,:));
height2 = rot90(a(1+points: 2*points,:));
defl1 = rot90(a(1+2*points: 3*points,:));
defl2 = rot90(a(1+3*points: 4*points,:));
zscan1 = rot90(a(1+4*points: 5*points,:));
zscan2 = rot90(a(1+5*points: 6*points,:));
user1 = rot90(a(1+6*points: 7*points,:));
user2 = rot90(a(1+7*points: 8*points,:));
fric1 = rot90(a(1+8*points: 9*points,:));
fric2 = rot90(a(1+9*points: 10*points,:));

% creates a loads vector by loading user input
% the load is the same for entire trace and retrace
% the load starts at maximum and decreases incrementally
deflection = user1(1, :);
figure
plot(1:lines, deflection)
title('absolute deflection')
xlabel('line #')
ylabel('deflection')

% After pulloff, there should be evidence of drift. This shows up as a nonzero deflection.
% Code trims the abs. deflection right at pulloff. The deflection right after pulloff is the maximum time drift.
% The drift is assumed to be linear and a fit is made. This drift fit is then subtracted from the abs. deflection.
% The deflection is then multiplied by spring constant and deflection sensitivity to obtain a loads vector.
check = 1;
while check == 1
  for i = 1:lines-1
    if (user1(1,i+1) - user1(1,i)) > 0
      finalindex = i;
      check = 0;
      break
    end
  end
maxdrift = user1(finalindex+1);
deflection = deflection(1, finalindex);
dummyx = [1 finalindex];
dummyy = [0 maxdrift];
drift = polyfit(dummyx, dummyy, 1);
driftline = polyval(drift, 1:finalindex);
deflection = deflection - driftline;
loads = k*deflINVOLS*deflection;

% plots first and last deflection lines
tdeflectionline1 = defl1(1,:);
tdeflectionlinelast = defl1(lines,:);
rdeflectionline1 = defl2(1,:);
rdeflectionlinelast = defl2(lines,:);
figure
hold on
plot(tdeflectionline1); plot(tdeflectionlinelast, 'r');
plot(rdeflectionline1); plot(rdeflectionlinelast, 'r');
title(['First (blue) and last (red) deflection curves, scan #' num2str(f)])

% user selects sloped and flat regions of first scan
disp('select start and end points of flat region')
[xflat, yflat] = ginput(2);
xflat(1) = floor(xflat(1));
xflat(2) = ceil(xflat(2));
disp('select start and end points of sloped region')
[xsloped, ysloped] = ginput(2);
xsloped(1) = floor(xsloped(1));
xsloped(2) = ceil(xsloped(2));

plot(xflat, yflat, 's', 'MarkerEdgeColor', 'k', 'MarkerFaceColor', 'g', 'MarkerSize', 12)
plot(xsloped, ysloped, 's', 'MarkerEdgeColor', 'k', 'MarkerFaceColor', 'g', 'MarkerSize', 12)

% finds and plots friction signal averages for sloped and flat sections
for i = 1:xflat(2)-xflat(1)+1
    fric1flat(i) = mean(fric1(:,i+xflat(1)-1));
    fric2flat(i) = mean(fric2(:,i+xflat(1)-1));
end

for i = 1:xsloped(2)-xsloped(1)+1
    fric1sloped(i) = mean(fric1(:,i+xsloped(1)-1));
    fric2sloped(i) = mean(fric2(:,i+xsloped(1)-1));
end

figure
plot(fric1flat)
hold on
plot(fric2flat, 'r')
title('Flat friction region')

figure
plot(fric1sloped)
hold on
plot(fric2sloped, 'r')
title('Sloped friction region')
plot(fric2sloped,'r')
title('sloped friction region')

Wf(f-firstext+1) = (mean(fric1flat) - mean(fric2flat))/2;
Ws(f-firstext+1) = (mean(fric1sloped) - mean(fric2sloped))/2;
deltaf(f-firstext+1) = (mean(fric1flat) + mean(fric2flat))/2;
deltas(f-firstext+1) = (mean(fric1sloped) + mean(fric2sloped))/2;

% fits slopes of curves v. applied normal load
% finds statistics of data

% flat region, halfwidth
x = loads; xL = 'load (nN)'; % xL is the label for the x axis
y = Wf; yL = 'Wf (V/nN)'; % yL is the label for the y axis
Ti = 'Wf'; % Ti is used in the title of plots
n = length(loads); % # of points in original data set before trimming
outliertrimmer(x, y, n, xL, yL, Ti) % statistical function

dWf = fit(1);  % slope
Adf = fit(2)/fit(1);  % calculates adhesion as the x-offset
sdWf = stddev_slope; % standard deviation of dWf

% sloped region, halfwidth
x = loads; xL = 'load (nN)';
y = Ws; yL = 'Ws (V/nN)';
Ti = 'Ws';
n = length(loads);
outliertrimmer(x, y, n, xL, yL, Ti)
dWs = fit(1);
Ads = fit(2)/fit(1);
sdWs = stddev_slope;

% delta difference
deltadiff = deltas - deltaf;
x = loads; xL = 'load (nN)';
y = deltadiff; yL = '/delta_s - /delta_f (V/nN)';
Ti = '/delta_s - /delta_f (V/nN)';
n = length(loads);
outliertrimmer(x, y, n, xL, yL, Ti)
ddeltadiff = fit(1);
sddeltadiff = stddev_slope;

tiptype = input('If the tip was sharp, type s. If the tip was colloidal, type c. ');  

if tiptype == s
alpha = getalphaortizsharp(dWs, ddeltadiff, theta, dWf, sdWf, sdWs, sddeltadiff)
else
alpha = getalphaortizcolloidal(dWs, dWf, ddeltadiff, theta, R, t, sdWs, sdWf, sddeltadiff)
end
end
Outlinetrimmer.m
Statistical analysis of data selected by calortiz.m. Creates linear fits and 95% confidence bands. Trims values outside of confidence bands, refits (this happens only once, i.e. only one trim).

Input is the halfwidth and offset data for the given region and plot axis labels and title. Outputs fit data and standard deviation. Feeds getalphaortizcolloidal.m.

function [fit stddev_slope E] = outliertrimmer(x, y, n, xL, yL, Ti)
    % x - x axis data
    % y - y axis data
    % n - number of data points
    % xL - x axis label
    % yL - y axis label
    % Ti - graph title
    df = n-2;
    fit = polyfit(x, y, 1);
    fitline = polyval(fit, x);
    residuals = y - fitline;
    null(1:n) = mean(y);
    ssreg = sum((y-fitline).^2);
    ssnull = sum((y-null).^2);
    rsquared = 1 - ssreg/ssnull;
    syx = (sum((y-fitline).^2)/(n-2)).^(1/2);
    slope = fit(1);
    stddev_slope = syx/sum((x-mean(x)).^2);
    intercept = fit(2);
    stddev_intercept = syx*(sum(x.^2)/(n*sum((x-mean(x)).^2)))^(1/2);
    ssy = sum(y.^2) - (sum(y)).^2/n;
    ssxy = sum(x.*y) - (sum(x)*sum(y)/n);
    ssx = sum(x.^2) - (sum(x)).^2/n;
    b = ssxy/ssx;
    se = sqrt((ssy - b*ssxy)/(n-2));
    t = tvalues(df);
    E = t*se*sqrt(1+1/n+(x-mean(x)).^2/ssx);

    figure
    plot(x,y,'x',x,fitline,'r.-',x,fitline+E,'--k',x,fitline-E,'--k')
    legend('data','Fit', '95% confidence bands')
    title([Ti ', r^2 = ' num2str(rsquared)])
    xlabel([xL])
    ylabel([yL])

    check = 1;
    toomany = n;
    while check == 1
        maxindexy = length(y);
        counter2 = maxindexy;
        for i = 1:maxindexy;
            if counter2 <= maxindexy
                break
            end
        end
    end
if (y(i) > fitline(i) + E(i)) | (y(i) < fitline(i) - E(i))
    counter2 = 0;
    toomany = toomany - 1;
    if toomany <= n - (n/5)
        disp('Too many data points trimmed') % won't let you trim more than 20% of the data points
        return
    end
    y(i) = [];
    x(i) = [];
    null(i) = [];
end
end

if (y(end) < fitline(i) + E(i)) & (y(i) > fitline(i) - E(i))
    check = 0;
end
end

% redo fit and confidence bands
% creates a linear fit
if toomany ~= n
    n = length(y);
    df = n-2;
    fit = polyfit(x, y, 1);
    fitline = polyval(fit, x);
    residuals = y - fitline;
    null(1:n) = mean(y);
    ssreg = sum((y-fitline).^2);
    ssnull = sum((y-null).^2);
    rsquared = 1 - ssreg/ssnull;
    syx = (sum((y-fitline).^2)/(n-2)).^(1/2);
    slope = fit(1);
    stddev_slope = syx/sum((x-mean(x)).^2);
    intercept = fit(2);
    stddev_intercept = syx*sum((x-mean(x)).^2);
    ssy = sum(y.^2) - (sum(y)).^2/n;
    ssxy = sum(x.*y) - (sum(x)*sum(y)/n);
    ssx = sum(x.^2) - (sum(x)).^2/n;
    b = ssxy/ssx;
    se = sqrt((ssy - b*ssxy)/(n-2));
    t = tvalues(df);
    E = t*se*sqrt(1+1/n+(x-mean(x)).^2/ssx);
figure
plot(x,y,'x',x,fitline,'r.-',x,fitline+E,'--k',x,fitline-E, '--k')
legend('data','Fit', '95% confidence bands')
title(['Ti ' w/ outliers removed, r^2 = ' num2str(rsquared)])
xlabel(['xL'])
ylabel(['yL'])
end
tvalues.m
This function is used in outliertrimmer.m to create 95% confidence bands using the student’s t distribution. Input is the degrees of freedom associated with the data being currently used in outliertrimmer.m. Outputs the t value needed to create 95% confidence bands.

function t = tvalues(df,x,y)
% t values for P = .025 up to n = 42, aka df = 40. this, initially, is a 40 element vector
tvalues = [12.706 4.303 3.182 2.776 2.571 2.447 2.365 2.306 2.262 2.201 2.179 2.160 2.145 2.131
2.120 2.110 2.101 2.093 2.086 2.080 2.074 2.069 2.064 2.060 2.056 2.052 2.048 2.045 2.042 2.037
2.035 2.032 2.030 2.028 2.026 2.024 2.023 2.021];

% use linear fits of t table data for n > 42
for i = 41:60
    tvalues(i) = (2 - 2.021)/(60-40)*(i-40) + tvalues(40);
end

% t up to df = 80
for i = 61:80
    tvalues(i) = (1.990 - 2)/(80-60)*(i-60) + tvalues(60);
end

% t up to df = 100
for i = 81:100
    tvalues(i) = (1.984 - 1.990)/(100-80)*(i-80) + tvalues(80);
end

% t up to df = 120
for i = 101:120
    tvalues(i) = (1.980 - 1.984)/(120-100)*(i-100) + tvalues(100);
end

% t up to df = 140
for i = 121:140
    tvalues(i) = (1.977-1.980)/(140-120)*(i-120) + tvalues(120);
end

% t up to df = 160
for i = 141:160
    tvalues(i) = (1.975-1.977)/(160-140)*(i-140) + tvalues(140);
end

% t up to df = 180
for i = 161:180
    tvalues(i) = (1.973-1.975)/(180-160)*(i-160) + tvalues(160);
end

% t up to df = 200
for i = 181:200
    tvalues(i) = (1.972-1.973)/(200-180)*(i-180) + tvalues(180);
end

% t up to df = 250
for i = 201:250
tvalues(i) = (1.969-1.972)/(250-200)*(i-200) + tvalues(200);
end

% note that the next given value for t is at df = inf.
% df = 10,000 will be sit in as infinity here to create a linear fit

for i = 251:10000
    tvalues(i) = (1.960-1.969)/(10000-250)*(i-250) + tvalues(250);
end
t = tvalues(df);

getalphaortizcolloidal.m
Takes fit data and standard deviation from outliertrimmer.m and uses the wedge method equations to find the lateral calibration constant. Also finds coefficients of friction on the sloped and flat regions of wedge and propagates error using the partial derivative technique.

---------------------------------------------
function [alpha1, sa1, alpha2, sa2, uslope1, su1, uslope2, su2, uflat1, suflat1, uflat2, suflat2] = getalphaortizcolloidal(dWs, dWf, ddeltadiff, theta, R, t, sdWs, sdWf, sddeltadiff)
theta = theta*pi/180;
a = sin(theta)*cos(theta);
b = -ddeltadiff/dWs;
c = sin(theta)*cos(theta) - R*sin(theta)/(R*(1+cos(theta)))+t/2);
uslope1 = (-b + sqrt(b^2-4*a*c))/(2*a);
uslope2 = (-b - sqrt(b^2-4*a*c))/(2*a);
duddiff1 = 1/(2*a)*(1/dWs + ((ddeltadiff/dWs)^2 - 4*a*c)\n(-1/2)*1/dWs^2*2*ddeltadiff);
duddiff2 = 1/(2*a)*(1/dWs - ((ddeltadiff/dWs)^2 - 4*a*c)\n(-1/2)*1/dWs^2*2*ddeltadiff);
dudWs1 = 1/(2*a)*(-ddeltadiff/dWs^2 + ((ddeltadiff/dWs)^2 - 4*a*c)\n(-1/2)*ddeltadiff^2/dWs^3);   
dudWs2 = 1/(2*a)*(-ddeltadiff/dWs^2 - ((ddeltadiff/dWs)^2 - 4*a*c)\n(-1/2)*ddeltadiff^2/dWs^3);
 su1 = ((duddiff1*sdddeltadiff)^2 + (dudWs1*sdWs)^2)^(1/2); % std. dev. of sloped friction coefficient 1
 su2 = ((duddiff2*sdddeltadiff)^2 + (dudWs2*sdWs)^2)^(1/2); % std. dev. of sloped friction coefficient 2
 dadWs1 = -uslope1*(cos(theta)^2-uslope1^2*sin(theta)^2)^(-1)*(dWs)^(-2);
dadu1 = 1/dWs*((cos(theta)^2-uslope1^2*sin(theta)^2)\n(-1)*((uslope1*scos(theta))^2-\nuslope1^2*2*sin(theta)^2)*(-2))^2*uslope1*scos(theta)*\nsin(theta)^2))^(-1) + uslope1*((cos(theta)^2-\nuslope1^2*2*sin(theta)^2)*(-2))^2*uslope1*scos(theta)*\nsin(theta)^2))^(-1) + uslope2*((cos(theta)^2-\nuslope2^2*2*sin(theta)^2)*(-2))^2*uslope2*scos(theta)*\nsin(theta)^2))^(-1) + uslope2*((cos(theta)^2-\nuslope2^2*2*sin(theta)^2)*(-2))^2*uslope2*scos(theta)*\nsin(theta)^2))^(-1)

alpha1 = (1/dWs)*scos(theta);  alpha2 = (1/dWs)*(scos(theta))^2;  sa1 = ((dadWs1*sdWs)^2 + (dadu1*scos(theta))^2))/(1/2); % std. dev. of alpha 1
sa2 = ((dadWs2*sdWs)^2 + (dadu2*scos(theta))^2))/(1/2); % std. dev. of alpha 2

uflat1 = alpha1*dWf;
uflat2 = alpha2*dWf;
suflat1 = ((dWf*sa1)^2 + (alpha1*sdWf)^2)\n^ (1/2); % std. dev. of flat friction coefficient 1
suflat2 = ((dWf*sa2)^2 + (alpha2*sdWf)^2)\n^ (1/2); % std. dev. of flat friction coefficient 2
end
Appendix C. Detailed Histology Methods

Paraffin-embedded histology was performed according to the following protocol, compiled by Tim Griffin and Inchan Youn.

Day 1: Dissection and Fixation
  Decalcification (time depends on
  Days 2-5: sample size)
  Dehydration, Infiltration, and
Day 5: Embedding*
Day 6: Sectioning*, Staining, and Mounting*

*potential stopping points

1. Dissection
   a. Spray mouse legs with 70% Ethanol.
   b. Cut the skin around knee with scissors and move cut distally.
   c. Cut the muscle along the medial and lateral tibial shaft, and cut the muscles that attach near the knee joint.
   d. Expose the thigh and cut muscle along the anterior and posterior femoral shaft.
   e. If exposing knee joint surface, use PBS (Hank’s Balanced Salt Solution) to keep tissue hydrated. First cut patellar tendon, LCL, and MCL. Then distend the joint and insert the scalpel (#11 blade) anteriorly between the femoral condyles with the blade faced toward the femur. Rotate blade left and right to cut the ACL and PCL.
   g. Cut the tibia and femur mid-shaft.

2. Fixation
   a. Fill small vial with formaldehyde buffered solution (Ricca Chem. Corp. cat. # 3190-1).
   b. Place dissected specimen in vial and store at 4°C for 12-24 hrs. Do not store >48 hrs.

3. Decalcification
   a. Pour formaldehyde into labeled waste container.
   b. Pour decalcifying solution (Cal-EX®, #CS510-1D, Fisher Scientific) back into vial.
   Store at 4°C for 24-48 hrs (small samples, such as epiphysis of tibia or femur) or 48-72 hrs (intact knee joint with muscles removed).
4. Dehydration
   a. Turn on heat on for wax pot now. Double-check that embedding dry oven is turned on and that the temperature is 56 – 60°C.
   b. Trim sample, if needed (check decalcifying process by trimming bone shaft).
   c. Dump decalcifying solution down drain with water running. Dehydrations can be prepared with tap water and Ethyl Alcohol USP (200 proof).

Note: dehydration times can be increased up to 1 hr for samples bigger than 5 mm x 5 mm.

<table>
<thead>
<tr>
<th>Solution</th>
<th>Time</th>
<th>Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>30% Ethanol</td>
<td>20 min.</td>
<td>Room temp.</td>
</tr>
<tr>
<td>50% Ethanol</td>
<td>20 min.</td>
<td>Room temp.</td>
</tr>
<tr>
<td>75% Ethanol</td>
<td>20 min.</td>
<td>Room temp.</td>
</tr>
<tr>
<td>90% Ethanol</td>
<td>20 min.</td>
<td>Room temp.</td>
</tr>
<tr>
<td>100% Ethanol</td>
<td>20 min.</td>
<td>Room temp.</td>
</tr>
<tr>
<td>100% Ethanol</td>
<td>30 min.</td>
<td>Room temp.</td>
</tr>
</tbody>
</table>

   d. If tissue samples contain air bubbles, place vials with loosened lids in a vacuum chamber and turn on vacuum line for a couple minutes. Re-pressurize chamber by gently pulling the front red nozzle forward. (Caution: this process can distort the tissue)

5. Infiltration
   a. If samples have been stored at 4°C, bring it up to room temperature and then place in fresh 100% Ethanol for 15 minutes.
   b. If continuing from directly from dehydration, pour 1/2 of the Ethanol solution out of the vial and fill the remaining half with Xylenes (#VW8500-3, VWR International).

Xylenes must be used in the fume hood and discarded in a labeled waste container located in the fume hood.

Note: Infiltration time also depends on the size of the sample. Each step can be increased up to 30 minutes.

<table>
<thead>
<tr>
<th>Solution</th>
<th>Time</th>
<th>Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xylenes and 100% Ethanol (1:1)</td>
<td>15 min.</td>
<td>Room temp.</td>
</tr>
<tr>
<td>Xylenes</td>
<td>30 min.</td>
<td>Room temp.</td>
</tr>
<tr>
<td>Xylenes</td>
<td>30 min.</td>
<td>Room temp.</td>
</tr>
</tbody>
</table>

6. Embedding
   a. Dump 1/2 of the xylenes out of the vial (into waste container!) and fill the remaining half of the vial with melted paraffin wax.
### Solution

<table>
<thead>
<tr>
<th>Solution</th>
<th>Time</th>
<th>Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xylenes and Paraffin (1:1</td>
<td>45 min.</td>
<td>56 – 60°C in dry oven</td>
</tr>
<tr>
<td>*dispose of in xylenes waste jug</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Paraffin</td>
<td>45 min.</td>
<td>56 – 60°C in dry oven</td>
</tr>
<tr>
<td>*dispose of in jug for wax waste</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Paraffin</td>
<td>60 min.</td>
<td>56 – 60°C in dry oven</td>
</tr>
<tr>
<td>*dispose of in jug for wax waste</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Paraffin</td>
<td>90 min. or up to 18 hrs overnight</td>
<td>56 – 60°C in dry oven</td>
</tr>
<tr>
<td>*dispose of in jug for wax waste</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note: times are somewhat flexible

b. Place tweezers in oven with vials. Fill metal embedding tray with liquid paraffin (pea-size amount) and use tweezers to place tissue sample in wax. Gently warm the tray and tweezers with the heat gun (low setting).
c. Position sample (medial side down for sagittal sections and posterior side down for frontal sections). Move tray to cool spot on counter and let wax on bottom of tray begin to harden. Immediately press green screen onto sample tray, and add more wax to fill green screen. Check for bubbles—use new set of warm tweezers to remove bubbles if necessary. Move sample to a cool spot. Clean wax off countertop with razor blade.
d. Let sample cool at least 3-24 hrs. To remove metal tray from sample, place tray on metal table in cold room for 5 min and loosen. *stopping point

### 7. Sectioning and Flattening

a. **1 hour prior to sectioning**: empty water from tissue float bath, wipe clean, and fill with filtered water. Turn bath on to level “5” (45-50°C) Two factors are important when preparing the water bath: temperature and cleanliness. Any residue of soap or oil will decrease the surface tension of the water and dramatically reduce its stretching ability and may cause the section to sink.
b. Turn on dry oven in Tissue Culture Room at least 30 min. prior to placing the first sample in the oven.
c. Block off edges of embedded wax sample with razor blade as follows:

d. Create sections using a microtome (Reichert-Jung, model 2030) and disposable blades. To maximize blade use, begin using far left side of blade. Mark blade area used after each sectioning session and adjust blade position to next unused area of blade for each new session. Section depth should be set to ~5μm. Once the section block is locked into place, begin sectioning until cartilage and/or
bone is reached. Sections (i.e. ribbons) are collected from the blade edge using two small, flat paint brushes. Ribbons are removed from the cutting area and placed in the water bath. Ribbons can be placed on a glass slide and viewed under a light microscope to determine the anatomical position of the tissue sample. If viewed quickly, sections can be re-floated. Slides hold ~ 4-5 ribbon sections. Once the desired ribbon sections are reached, ribbons should be floated onto a specially coated glass slide (Superfrost/Plus microscope slides, #12-550-15, Fisher Scientific). Immediately place slide into the slider warmer for approximately 6 hrs at 37°C. Label the slide using pencil. Keep track of the number of ribbons cut throughout the sectioning process to facilitate determining the anatomical position of the blade. This information is also important for determining the anatomical distribution of ribbon sections being analyzed.

e. When finished sectioning, clean ribbon plate with xylenes to remove wax residue.

8. Staining: Hematoxylin, Safranine-O & Fast Green
   a. Deparaffinize slide sections as follows:
      Note: protocol for 5μm paraffin sections on glass slides. Use grey slide rack and green solution chamber.

<table>
<thead>
<tr>
<th>Solution</th>
<th>Time</th>
<th>Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>*Xylenes (can be old)</td>
<td>5 min.</td>
<td>Room temp.</td>
</tr>
<tr>
<td>dry sample in hood after treatment (3-5’)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Xylenes (can be old)</td>
<td>5 min.</td>
<td>Room temp.</td>
</tr>
<tr>
<td>dry sample in hood after treatment (3-5’)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Xylenes (new!!)</td>
<td>5 min.</td>
<td>Room temp.</td>
</tr>
<tr>
<td>dry sample in hood after treatment (3-5’)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* During first xylenes treatment, make Weigert’s Iron Hematoxylin solution by mixing equal parts of Part A and Part B of Weigert’s Iron Hematoxylin Set (Ht 10-79, Sigma-Aldrich). 0.5-1.0 ml per slide. Use disposable pipette to dispense solution into orange capped plastic tube—be sure to use a fresh pipette for each solution! Confirm that stain is active by placing a couple drops of mixed Weigert’s solution into water. Solution should become purple tinted, not brown.
b. Hydrate slides as follows. For small numbers of slides (<5), place drops on slides. For larger numbers, use rack.

<table>
<thead>
<tr>
<th>Solution</th>
<th>Time</th>
<th>Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>100% Ethanol</td>
<td>2 min.</td>
<td>Room temp.</td>
</tr>
<tr>
<td>50% Ethanol</td>
<td>2 min.</td>
<td>Room temp.</td>
</tr>
<tr>
<td>Tap water</td>
<td>2 min.</td>
<td>Room temp.</td>
</tr>
</tbody>
</table>

*Remove extra water from slide. If any samples need to be removed, do so now.

c. Hematoxylin staining:

<table>
<thead>
<tr>
<th>Solution</th>
<th>Time</th>
<th>Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weigert’s Iron Hematoxylin (fresh, pre-mixed batch)</td>
<td>3 min.</td>
<td>Room temp.</td>
</tr>
<tr>
<td>Pour off and rinse in tap water (in rack)</td>
<td>2 min.</td>
<td>Room temp.</td>
</tr>
<tr>
<td>Differentiate in Acid Alcohol</td>
<td>15 sec!!</td>
<td>Room temp.</td>
</tr>
<tr>
<td>Pour off and rinse in fresh batch of tap water (in rack)</td>
<td>3 min.</td>
<td>Room temp.</td>
</tr>
</tbody>
</table>

*Remove extra water from slide.

d. Fast Green staining:

<table>
<thead>
<tr>
<th>Solution</th>
<th>Time</th>
<th>Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.02% aqueous fast green</td>
<td>3 min.</td>
<td>Room temp.</td>
</tr>
<tr>
<td>Pour off fast green and pour 1% Acetic Acid over slide at angle</td>
<td>~5 sec (brief!)</td>
<td>Room temp.</td>
</tr>
<tr>
<td>Rinse with tap water</td>
<td>Brief</td>
<td>Room temp.</td>
</tr>
</tbody>
</table>

*Remove extra water from slide.

e. Safranine-O staining:

<table>
<thead>
<tr>
<th>Solution</th>
<th>Time</th>
<th>Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1% aqueous safranine-O</td>
<td>3 min. (max)</td>
<td>Room temp.</td>
</tr>
<tr>
<td>Pour off &amp; rinse with 100% Ethanol (hold slide at angle-rinse front and back of slide, dab vertically)</td>
<td>Until extra red stain is removed from slide</td>
<td>Room temp.</td>
</tr>
</tbody>
</table>

*Remove extra water from slide and let dry at steep angle 10-20 min (shield from dust).

Stain legend:

- Nuclei: black or dark blue
- Bone & muscle (collagen): green
- Cartilage and mast cell granules (GAGs): orange or red
9. Mounting coverslips
   a. Once slide is dry, place it in xylenes (batch in green solution chamber from
deparaffinizing step ok to use). Position slide vertically with a slight angle. Insert
2 slides
      initially.
   b. Open Permount (Fisher Scientific, SP15-100) and insert (squeezed) disposable
pipette.
   c. Remove one slide (with tweezers). Hold slide in left hand—do not place the
bottom of the slide on any surface (want to keep xylenes on the bottom of the
slide in case permount overflows to the bottom).
   d. Quickly dispense one drop of permount per sample. Gently place a coverslip
over permount. Lower coverslide to that it makes contact at a slight angle.
   e. Rest slide edge on paper towel and use tweezers to push bubbles to the edges of
the slide. Begin by gently pushing in the center of the slide—bubbles will move
after a couple seconds. Don’t push too hard or permount could leak out of the top
edge. Make sure the coverslip is distributed evenly over all the samples. Do not
spend more than about 1 min removing bubbles (permount begins to set).
   f. Dip kimwipe into xylenes and wipe the bottom of the slide. Edges may be
wiped carefully to either remove some permount or to slightly re-align coverslip
with slide. If permount gets on kimwipe, throw away and use new kimwipe. Wipe
bottom with clean, dry kimwipe. Lay slide on clean, flat surface. Place a new slide
in the xylenes and repeat steps c-f for the next slide that had been sitting in the
xylenes.
   g. Let slides set.
References


Biography

Jeff is originally from Potsdam, New York and lived in Bandung, Indonesia from age 11-17. He was homeschooled from kindergarten through high school. He then attended the University at Buffalo, where he majored in mechanical engineering and minored in biology. There he developed an interest in biomaterial interfaces while working Hui Meng’s fluid dynamics lab and Robert Baier’s biomaterials lab. Through discussions with professors Kemper Lewis and Robert Baier he decided to pursue graduate research in the topic. In the application process, he met Stefan Zauscher, who would become his mentor at Duke, and identified a strong overlap in research interests. A Pratt-Gardner fellowship supported Jeff’s first year of study at Duke. There, Jeff benefited from intelligent and creative mentors and coworkers in a highly collaborative research environment. He authored or co-authored the following papers while in graduate school at Duke.


