Evaluating the Structural and Functional Consequences of Traumatic Joint Injury and their Relation to NF-κB in a Non-Invasive Model of Post-traumatic Osteoarthritis

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Evaluating the Structural and Functional Consequences of Traumatic Joint Injury and their Relation to NF-κB in a Non-Invasive Model of Post-traumatic Osteoarthritis

by

Ian Matthew Berke

A dissertation presented to The Graduate School of Washington University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

January 2021
St. Louis, Missouri
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ABSTRACT OF THE DISSERTATION
Evaluating the Structural and Functional Consequences of Traumatic Joint Injury and their Relation to NF-κB in a Non-Invasive Model of Post-traumatic Osteoarthritis

by

Ian Matthew Berke

Doctor of Philosophy in Biomedical Engineering

Washington University in St. Louis, 2021

Professor Lori A. Setton, Chair

Post-traumatic osteoarthritis (PTOA) is a painful and debilitating disease of the synovial joint, characterized by degenerative changes to various joint tissues following traumatic joint injury. While several risk factors have been identified in the symptomatic progression of PTOA following injury, inflammation and NF-κB mediated changes are believed to significantly contribute to symptomatic joint dysfunction and pain. However, the temporal presentation of these pro-inflammatory signals following clinically relevant injury and their relationship to the development of symptomatic disease have not been thoroughly investigated. Therefore, there exists a critical need to better understand how these early inflammatory events following injury may contribute to the symptomatic progression of PTOA.

In the first aim of this dissertation, the temporal relationship between NF-κB activation and the development of pain-sensitivity related behaviors following joint injury was evaluated in a non-invasive murine model of PTOA. Early activation of NF-κB following traumatic knee joint injury was associated with several clinically relevant changes in animal behavior including the acute presentation of allodynia, hyperalgesia,
and transient alterations to weight bearing and ambulation/gait. Persistent unilateral allodynia was observed up to 4 months following injury. Furthermore, early NF-κB activation following injury was correlated with the severity of chronic allodynia and erosive changes to articular cartilage. Data from this aim was used to define an early therapeutic treatment window to test the efficacy of a locally delivered NF-κB inhibitor, PHA-408, following joint injury.

Given the involvement of the NF-κB pathway in inflammation and in the development of symptomatic PTOA, it was hypothesized that acute local pharmacologic NF-κB inhibition might slow the progression of symptomatic PTOA following joint injury. Findings in this aim highlighted the early chondroprotective effects of local PHA-408 following mechanical joint injury. Modest improvements in pain-sensitivity related outcomes with repeated drug treatment were also observed. Nevertheless, local delivery of PHA-408 failed to modify long-term degenerative changes to articular cartilage. Engineered drug depots were also developed for the sustained release of PHA-408. While these microparticle based depots showed the potential for sustained drug release and inhibition of NF-κB in vitro, their use did not improve measured outcomes following joint injury.

While the painful symptoms and debilitating impact of chronic conditions, such as PTOA, may be apparent to the patients who suffer from the disease, many aspects of sensory excitability which may contribute to pain, particularly in intact tissue, remain unclear. Work in the final aim of this dissertation described the development of an ex vivo imaging approach to functionally evaluate sensory neuron excitability within intact dorsal root ganglion tissues. This method was capable of simultaneously assessing functional
signaling dynamics in hundreds of neurons with single-cell resolution and was further able to evaluate alterations to neural signaling following pharmacologic modulation and injury.

Overall, this dissertation serves to improve our understanding of the symptomatic progression of PTOA following traumatic joint injury and highlights many of the challenges of intra-articular drug delivery in the disease-modifying treatment of PTOA. Furthermore, the novel imaging methods developed within this dissertation may help to elucidate the structural and functional changes to musculoskeletal and peripheral nervous system tissues following injury, respectively.
Chapter 1

Introduction

1.1 Osteoarthritis, a whole-joint disease

Osteoarthritis (OA) is one of the top three leading causes of disability in the United States where it is estimated to affect over 30 million adults\textsuperscript{1–3}. Globally more than 10\% of adults over the age of 60 suffer from symptomatic OA\textsuperscript{2,4}. In these individuals severe mobility limitations negatively impact quality of life and concurrently increase the risk for other chronic conditions such as obesity and cardiovascular disease\textsuperscript{5–8}. In addition to the direct impacts on quality of life, OA imparts a substantial economic burden on society. Within the United States, arthritis-attributed medical expenditures and earnings losses are estimated at over $300 billion annually\textsuperscript{9}. These costs account for nearly 3\% of the United States gross domestic product\textsuperscript{9–11}. Similar disease related economic impacts have been reported globally\textsuperscript{12}. While decades of research have identified several risk factors associated with an increased incidence of OA including age, gender, activity level, prior injury, obesity, and genetics, there remain no effective disease-modifying OA treatments that halt or delay OA progression in a meaningful way\textsuperscript{6,13,14}. Thus, there is great need for further scientific inquiry that aims to better understand the temporal progression of disease and develop efficacious disease-modifying treatment strategies for OA.
OA has historically been characterized by the degeneration of articular cartilage. In healthy joints, the highly organized and multi-phasic structure of articular cartilage enables its unique functional properties (Fig. 1.1). Articular cartilage is nearly 80% water by weight, the remainder of the tissue mainly consists of collagens and proteoglycans which make up the extracellular matrix (ECM), and chondrocytes which sparsely populate the ECM. Collagen is the most abundant structural macromolecule in articular cartilage and accounts for nearly 60% of tissue dry weight. Type II collagen represents roughly 90% of the collagenous ECM while minor constituents such as collagen types IX and XI further contribute to ECM stability through collagen crosslinking. The highly organized zonal microstructure of the collagenous ECM contributes to the anisotropic tensile properties of articular cartilage. The second most abundant class of macromolecule in articular cartilage are proteoglycans, these consist of negatively charged glycosaminoglycan (GAG) chains covalently bound to a protein core. The negative fixed charged density of proteoglycans results in a swelling pressure which contributes to the compressive properties of articular cartilage. Chondrocytes represent roughly 2% of

Figure 1.1: Zonal cellular and microstructural composition of healthy articular cartilage. Figure adapted from Buckwalter, Mow, and Ratcliffe. J Am Acad Orthop Surg. 1994.
total cartilage volume but are thought to be the primary mediators in cartilage homeostasis\textsuperscript{17}. Chondrocytes are largely responsible for the continued production of ECM components such as collagens and proteoglycans which are required for healthy cartilage structure and function\textsuperscript{18}. Taken together, these highly organized constituents of articular cartilage give rise to the tissue's unique functional properties which allow for decades of near-frictionless joint function.

Although OA has historically been characterized as a degenerative disease of articular cartilage, it is now well established that various joint tissues are altered in OA. For this reason, idiopathic OA is more accurately described as a whole-joint disease (Fig. 1.2)\textsuperscript{19}. For example, changes including synovial thickening and inflammation, subchondral bone remodeling and osteophyte formation, and neo-vascular innervation of joint tissues, may be observed in addition to articular cartilage degeneration\textsuperscript{19–22}. To this date, the fundamental mechanisms that drive these changes in idiopathic OA remain largely unknown.

\textbf{Figure 1.2: Inflammation contributes to OA disease progression.} Alterations to various joint tissues, and an increase in pro-inflammatory signaling contribute to the development of OA. Figure adapted from Kapoor\textsuperscript{+}. Nat Rev Rheum. 2011.
1.2 Post-traumatic Osteoarthritis

In post-traumatic osteoarthritis (PTOA) joint trauma (e.g. ligamentous rupture, meniscal tear, hyper-physiologic loading) precipitates the accelerated degeneration of joint tissues. Following traumatic joint injury, affected individuals have a nearly 10-fold increased risk of developing OA-like joint changes within 10 to 20 years of injury when compared to their uninjured age-matched counterparts\textsuperscript{23–28}. While with idiopathic OA the precise mechanical and/or biological changes that initiate disease are unknown, in PTOA the triggering events that drive pathology are known injuries. As a result, there exists a unique, early window of opportunity to study and treat acute changes that may ultimately contribute to joint degeneration, pain, and loss of function post-injury.

Following traumatic joint injury, various disease-associated changes contribute to the pathogenesis of PTOA (Fig. 1.3)\textsuperscript{28}. Principally, hyper-physiologic loading has been shown to alter chondrocyte viability, cell metabolism, and cartilage morphology within hours of loading induced injury\textsuperscript{29–37}. Additionally, shortly following injury increased levels of pro-inflammatory cytokines, such as interleukin-1 beta (IL-1\textbeta), IL-6, IL-8, and tumor necrosis factor-alpha (TNF-\textalpha), have been reported in synovial fluid and may remain elevated for several months following joint trauma\textsuperscript{38–41}. These pro-inflammatory mediators contribute to a catabolic shift in the resident cells of the joint which may have not been directly impacted by the initial injury\textsuperscript{28}. Activation of catabolic gene sets within these cells leads to increased levels of proteolytic and matrix degrading enzymes such as matrix metalloproteinases (MMPs) and aggrecanases\textsuperscript{42,43}. These enzymes directly act to
degrade the cartilage and subchondral bone ECM which, in turn, perpetuates the progression of PTOA\textsuperscript{43–49}. Knock-out studies have further highlighted these pro-inflammatory and catabolic mediators as significant contributors to disease progression in PTOA\textsuperscript{50}. Together these data suggest that manipulation of the joint microenvironment following traumatic injury may impart protective effects on articular cartilage and other joint tissues to slow the development of PTOA.

1.3 NF-κB activation in PTOA

The nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway is a rapidly inducible inflammatory signaling pathway that has been identified as a major player in the pathophysiology of several diseases\textsuperscript{51}. Dysregulation of NF-κB is known to drive chronic inflammation in conditions such as irritable bowel syndrome, chronic inflammatory airway disease, various cancer types, and rheumatoid arthritis\textsuperscript{52–55}.

Constitutive activation of NF-κB has long been implicated in the development and maintenance of rheumatoid arthritis. Histologic studies of human rheumatic synovium
have shown chronically increased expression of phosphorylated p65 (P-p65) in synovial tissues when compared to non-arthritic joints\textsuperscript{56,57}. Animal models of inflammatory arthritis have replicated these alterations seen in human joints and exhibit chronic activation of the immune system, synovial hyperplasia, neovascularization and nerve fiber sprouting in various joint tissues, and articular cartilage degeneration\textsuperscript{58–62}. Local NF-κB activation in these models has also been correlated with the development of pain-sensitivity related behaviors\textsuperscript{63}. Pharmacologic NF-κB inhibition, however, has been shown to effectively attenuate these pain-sensitivity related behaviors in inflammatory models\textsuperscript{64–66}.

While OA has long been classified as a non-inflammatory disease, recent studies have highlighted the importance of NF-κB mediated signaling and inflammation in the development of symptomatic PTOA following mechanical joint injury\textsuperscript{67–69}. While some level of loading (e.g. mechanical, osmotic) helps to maintain cartilage homeostasis, injurious loading has been shown to rapidly induce high levels of NF-κB activation in chondrocytes\textsuperscript{70–73}. It is thought this hyper-activation following injury contributes to a persistent pro-inflammatory environment that initiates NF-κB signaling in other joint tissues such as synovium\textsuperscript{42,70,74}. Ultimately, these factors contribute to a pro-inflammatory and catabolic feedback loop that drives the progression of PTOA.

Pre-clinical studies have shown NF-κB inhibition can serve to attenuate this positive feedback loop and protect against joint destruction\textsuperscript{75}. The successful use of selective IκB kinase 2 (IKK-2) inhibitors, such as 8-(5-Chloro-2-(4-methylpiperazin-1-yl)isonicotinamido)-1-(4-fluorophenyl)-4,5-dihydro-1H-benzo[g]indazole-3-carboxamide (PHA-408), highlight the utility of NF-κB inhibition as a disease-modifying treatment approach\textsuperscript{76,77}. Specifically, PHA-408, a low molecular weight (560 Da), highly selective
inhibitor of IKK-2, has been shown to reduce the severity of inflammatory arthritis in multiple preclinical animal models. However, due to the ubiquitous nature of the NF-κB pathway and associated off-target effects of systemic NF-κB inhibition, few drug candidates have been successfully implemented in the clinic. Indeed, in some treatment applications, pathway inhibition has been found to cause adverse effects such as neutrophilia, fever, and increased IL-1 release. Nevertheless, local NF-κB inhibition in PTOA remains a promising therapeutic approach due to the focal nature of the disease. For example, a phase 1 clinical trial of the IKK inhibitor, SAR113945, improved pain scores following intra-articular injection in patients with OA. Unfortunately, a phase 2a trial failed to show efficacy. In spite of this result, there is considerable evidence supporting the use of NF-κB pathway inhibitors in the treatment of OA.

1.4 Assessing the symptomatic progression of OA

Although symptomatic OA imparts a substantive social and financial burden on society, limited research has focused on the mechanisms contributing to pain in the disease state. Clinically, efforts have largely aimed to understand structural joint changes using radiographic based assessments. While these methods are valuable for disease staging, by themselves they provide no insight into pain or joint function.

Standardized functional and symptomatic self-reported scoring systems (e.g. Western Ontario and McMaster Universities Arthritis Index (WOMAC) and Knee injury and Osteoarthritis Outcome Score (KOOS)) have helped to better quantify the symptomatic effects of OA. Studies that have used these scoring systems paired with clinical imaging measures have provided valuable insight into the relationships between
joint structure changes, such as synovitis and the formation of bone marrow lesions, and symptomatic OA\textsuperscript{91,92}. However, these methods still fail to directly assess the molecular and cellular changes contributing to symptomatic OA.

The use of OA animal models for the study of symptomatic OA serves to address some limitations of clinical studies. Principally, animal models allow for the coincident and well controlled interrogation of molecular, cellular, structural, and functional sensitivity changes within the same subject\textsuperscript{93}. This approach, which may be infeasible in a clinical setting, allows for a more comprehensive understanding of the factors influencing symptomatic disease progression. Indeed, OA animal models have replicated many of the structural changes seen in symptomatic OA (e.g. synovitis, joint effusion, bone marrow lesions, sensory nerve fiber innervation)\textsuperscript{58,94–100}. Furthermore, these structural changes observed in OA models have been associated with the development of clinically relevant symptoms such as mechanical allodynia and functional limitations to ambulation\textsuperscript{101–103}. Animal models have also helped uncover molecular and functional signaling changes in the peripheral nervous system which are thought to contribute to the painful symptoms of OA\textsuperscript{104,105}. Nevertheless, further research into the temporal progression of pain-sensitivity related behaviors and functional disability in OA animal models may provide an improved basis for the treatment of symptomatic disease.

Most widely utilized small animal models for the study of OA employ surgical means to induce joint destabilization, thus they are more accurately categorized as PTOA models\textsuperscript{106}. Surgically induced injuries, such as those used by the destabilization of the medial meniscus (DMM) or anterior cruciate ligament transection (ACLT) models, ultimately lead to reproducible and progressive degeneration of joint tissues over time\textsuperscript{106–}
However, the temporal development of symptomatic disease, with implications for altered pain-sensitivity related behavior, remains unclear in these models. While standardized surgical procedures are described in the literature, surgically induced injuries can be technically challenging and rely on surgical expertise for consistent outcomes. This may, in part, explain the variability in the onset and severity of pain-sensitivity related behavioral alterations observed in surgical models. Furthermore, surgical incision related factors cannot be ruled out as contributors to alterations in pain-sensitivity related behaviors, particularly at acute timepoints. Consequently, surgical PTOA models may be limited in their ability to study pain-sensitivity related behavioral alterations shortly following injury.

Non-invasive models of PTOA allow for the study of joint injury triggered alteration to pain-sensitivity behaviors without the confounding nature of a surgical incision. These models also enable fine-tune control of injury severity depending on loading configuration, duration, and magnitude. To date, few studies have focused on the characterization of symptomatic disease progression following these non-invasive injuries, and those that have, utilize differing metrics of pain-sensitivity and behavior. For example, Anderson and colleagues have shown transient gait alterations following a non-invasive knee joint injury, however, no further changes in animal behavior were observed. Others have demonstrated unilateral changes to allodynia, hyperalgesia, and static weight bearing shortly following non-invasive injury, but the temporal progression of these changes remains unclear and largely unstudied. For these reasons, further research to interrogate the temporal presentation of pain-sensitivity...
related behaviors following non-invasive joint injury would add value to our current understanding of the symptomatic progression of PTOA.

1.5 Current treatment strategies for OA

There are currently no clinically available therapies that safely treat both the symptomatic and structural aspects of OA\textsuperscript{127,128}. Palliative treatments, such as non-steroidal anti-inflammatory drugs (NSAIDs), are the most widely utilized front line therapies to treat the symptoms of OA\textsuperscript{129}. Although NSAIDs may elicit short-term improvements in pain and function, their chronic usage can lead to cardiovascular and gastrointestinal complications\textsuperscript{130,131}.

When NSAIDs provide no palliative benefit, intra-articular corticosteroid administration is a common next step in treatment\textsuperscript{132}. While this approach has shown some efficacy in reducing acute pain and inflammation, long-term outcomes are not improved by intra-articular corticosteroid delivery\textsuperscript{133}. This may be due to the rapid clearance of low molecular weight solutes from the joint, intra-articular half-lives of corticosteroids are rarely found to exceed 12 h\textsuperscript{134}. Some data also suggest high corticosteroid concentrations may negatively impact cartilage homeostasis by reducing chondrocyte ECM biosynthesis\textsuperscript{135}.

Viscosupplements, such as hyaluronic acid derivatives, have a long history of clinical use to treat symptomatic OA, and several formulations (e.g. Gel-One, Euflexxa, Orthovisc) have been granted FDA approval for intra-articular delivery\textsuperscript{136,137}. These viscosupplements are thought to improve synovial fluid quality and reduce frictional coefficients in the joint\textsuperscript{138}. High molecular weight hyaluronan has also been shown to display anti-inflammatory and immunosuppressive properties\textsuperscript{139,140}. This is in contrast...
with the inflammatory and immune-activating properties of low molecular weight hyaluronic acid fragments. Despite the short-term palliative effects of these treatments, they still fail to substantively modify the long-term structural and symptomatic progression of OA. Given the lack of success for these current OA treatment strategies, there is an unmet need for the further development of disease-modifying OA drugs.

1.6 Intra-articular drug delivery and clearance

While systemic delivery of disease-modifying OA drugs has shown efficacy in many pre-clinical studies, systemic drug administration does not ensure ideal drug bioavailability in the affected joint. Molecular entry into the joint space is size-dependent. Smaller molecules (<10kDa) may diffuse through synovial capillaries and synovial intima and equilibrate within the synovial fluid. Larger molecules undergo size-dependent sieving and have reduced access to the joint space. Locally delivered treatment, via intra-articular injection, serves to maximize drug bioavailability within the diseased joint. The increased bioavailability of locally
administered drugs enables an overall reduction in drug dose which, in turn, reduces systemic exposure and the risk for off-target effects\textsuperscript{146}.

Following size-dependent entry into the joint, molecular efflux shows a similar size dependence. Small molecules, like acridine orange (370 Da), have short intra-articular half-lives ($t_{1/2} \sim 30$ min), whereas larger macromolecules such as hyaluronic acid ($3.0 \times 10^6$ Da) are cleared more slowly ($t_{1/2} \sim 24$ h)\textsuperscript{147-151}. Intra-articular half-lives for NSAIDs and soluble steroids cluster around 1 to 4 h. These values illustrate the challenges facing intra-articular therapy, especially for chronic or progressive conditions where sustained drug concentrations are required for therapeutic efficacy\textsuperscript{152}.

There are however various biophysical phenomena that can be exploited to overcome some of the hurdles of intra-articular drug delivery\textsuperscript{153}. For example, recent research has revealed the importance of charge-based interactions between locally delivered molecules and negatively charged cartilage ECM. Bajpayee and Grodzinsky have shown positively charged macromolecules exhibit enhanced molecular diffusivity and residence times within healthy cartilage ECM\textsuperscript{154}. This effect of cationic potential in cartilage targeting is not without its limitations\textsuperscript{155}. Specifically, supercharged-GFP variants, modified to have increased cationic electrostatic potential, had improved cartilage permeability at lower, but non-zero, net charges. Interestingly, chondrocyte uptake was enhanced in variants with increased cationic charge\textsuperscript{156}. These results suggest drug biophysical properties should be carefully considered when assessing the joint tissue and resident cell targeting potential of intra-articular therapeutic approaches\textsuperscript{157}.

Disease related changes to other joint structures also play a role in the clearance of locally delivered molecules. Most notably, synovial thickening is thought to reduce
molecular efflux from the intra-articular space. Despite the presence of increased vascularization in arthritic joints, literature consistently suggests functionally impaired clearance of multiple solutes. Radiolabeling studies were the first to highlight these alterations with disease and revealed significantly reduced intra-articular clearance rates of $^{123}$I and $^{131}$I-labeled human serum albumin (66.5 kDa) in patients with OA. Similar disease associated changes to intra-articular clearance have been observed in PTOA animal models. Altered lymphatic structure and function have also been implicated in modulating intra-articular clearance in diseased joints. Surprisingly, functional deficits in lymphatic mediated clearance are despite increased lymphatic vessel formation reported in arthritic joints. Research by Doan and colleagues has also demonstrated the clearance modulating role of knee joint innervating venous and lymphatic vascular structures in the absence of disease.

Taken together, these findings reveal the benefit of intra-articular drug delivery for the treatment of OA and further emphasize the wide variety of factors that influence molecular transport from arthritic joints. When assessing and/or modulating molecular efflux from the joint, special care must be taken in considering the full array of parameters that are known to impact intra-articular transport phenomena.

### 1.7 Engineered drug delivery strategies for OA

Engineered drug delivery strategies primarily seek to overcome the clearance based limitations of small molecule and protein delivery to the joint. Historically, hydrogel and particle based drug depots have been the most widely utilized carriers for intra-articular therapies. These approaches heavily rely on the fundamental molecular weight based clearance properties that largely govern molecular efflux from the joint.
Despite the seeming simplicity of this phenomenon, its exploitation has resulted in increased intra-articular residence times for locally delivered drugs through the use of particle and hydrogel based sustained release drug depots\textsuperscript{138,169,170}. Clinical application of these depots (e.g. Lipotalon, Zilretta/FX-006) have prolonged the palliative effects of locally delivered drugs\textsuperscript{138,171–175}.

While a wide variety of drug delivery depots have been shown to improve intra-articular residence times and the subsequent therapeutic efficacy of locally administered drugs, polymeric microparticles are generally thought to be better suited for intra-articular delivery due to their high degree of tunability\textsuperscript{176}. Specifically, polymeric particles can be synthesized with larger diameters, when compared to lipid or dendrimer based particles, which help to increase their passive size-based retention within the joint\textsuperscript{170,177}. Furthermore, synthetic and natural polymeric depots can deliver a diverse set of cargo (e.g. siRNA, virus, protein, imaging agents, small molecules) with a high degree of control over drug release and particle degradation kinetics\textsuperscript{178}.

The research in this dissertation employs both synthetic and natural polymeric microparticles synthesized with amphiphilic PEG-block-PLGA copolymer and silk fibroin (SF), respectively. Prior work has shown PEG-PLGA drug depots can efficiently encapsulate small hydrophobic drugs and persist in the joint for up to 4 weeks following intra-articular injection\textsuperscript{179,180}. SF, derived from the \textit{Bombyx mori} silkworm, has high biocompatibility, non-immunogenic degradation products, and is FDA approved in some biomedical applications\textsuperscript{181}. Depending on processing conditions, SF biomaterial properties (e.g. pore size, stiffness, beta-sheet content, degradation kinetics) can be fine-tuned for use in different applications and have shown the ability to entrap and release
compounds of various physiochemical properties. Specifically, low molecular weight hydrophobic compounds, such as Rhodamine B, have been previously incorporated into SF microparticles with high loading efficiencies and favorable extended release profiles. Furthermore, our group has shown SF microparticles serve to increase intra-articular dwell times of low molecular weight cargo. Given these characteristics of PEG-PLGA and SF microparticles, and their successful use in sustained release drug depot applications, they were selected as favorable polymeric microparticle candidates for the sustained release of the hydrophobic small molecule IKK-2 inhibitor, PHA-408.

1.8 Sensory neuron signaling and imaging

While functional assessments of peripheral sensitivity (e.g. von Frey and Hargreaves tests) are valuable in assessing pain-sensitivity changes resulting from disease or injury, these methods alone do not provide direct insight into functional signaling alterations at the cellular level. Sensory neurons, which consist of a cell body/soma, axon, and peripheral axon terminals/receptors, innervate peripheral tissues and are responsible for receiving and transmitting sensory input. These afferent fibers relay unipolar sensory signals in response to thermal, chemical, pressure, and noxious stimuli through spinal nerves to the dorsal root ganglion (DRG). DRGs contain the cell bodies of sensory neurons which further transmit sensory stimuli to the dorsal horn of the spinal cord. Although the basic structure and function of DRG neurons have are well characterized, the mechanisms by which sensory stimuli are encoded and transmitted by these cells remain unclear. Therefore, a better understanding of signal transduction within DRG neurons is required.
Typical *in vitro* methods for the functional assessment of DRG neuron excitability, such as whole cell patch clamping, require surgical removal of the DRG via axotomy followed by subsequent enzymatic and mechanical disruption\textsuperscript{192,193}. While this is useful for preparing DRG neurons for monolayer culture, such techniques substantially modify the native cellular microenvironment by reducing physiologically relevant cell-cell and cell-matrix interactions that modulate cellular excitability *in vivo*\textsuperscript{194,195}. Furthermore, patch clamping and similar current or voltage querying methods are limited to measuring changes in individual cells at any one time\textsuperscript{191,196}. While similar approaches, such as extracellular single unit recordings, may assess cellular *in situ* electrophysiology with minimal tissue disruption, these techniques still have limited throughput which may impact their ability to accurately represent heterogeneous cell populations\textsuperscript{189}. Nevertheless, these tissue preparations and electrophysiological methods remain the gold standard for understanding ion channel dynamics\textsuperscript{191}.

Recent advancements in imaging have allowed for the simultaneous capture and analysis of ion and ion channel dynamics in diverse cell populations\textsuperscript{197}. Fura-2 has been the most widely utilized fluorescent dye for this purpose\textsuperscript{198}. However, Fura-2 and similar ultra-violet excited dyes are often not suitable for long-term or repeat analysis of ion dynamics due to ultra-violet induced phototoxicity\textsuperscript{197,198}. Additionally, the use of exogenous fluorescent dyes are somewhat restricted in intact tissues due to dye loading and diffusion based limitations\textsuperscript{197}. The development of genetically encoded calcium indicators (GECIs), such as those from the GCaMP family, enable spatiotemporal control of cell-type specific indicator expression\textsuperscript{199}. These genetically encoded indicators of neuronal activity when paired with high speed imaging allow for the study of cell signaling
dynamics with minimal chemical or mechanical disturbance to the native cellular environment. The research in this dissertation uses these recent advances in imaging and genetic techniques to develop a novel imaging modality to assess the excitability of sensory neurons within acutely isolated intact DRG tissues.

1.9 Dissertation structure and aims

1.9.1 Aim 1: NF-κB-mediated effects on behavior and cartilage pathology in a non-invasive loading model of post-traumatic osteoarthritis (Chapter 2)

To better understand the temporal activation of NF-κB and its relation to the development of symptomatic PTOA following knee joint injury, a murine non-invasive tibial loading model of PTOA was used. Emphasis was placed on outcome measures that track longitudinal changes to pain-sensitivity related behavior as well as concurrent NF-κB activation via in vivo bioluminescent imaging. Findings in Chapter 2 highlighted the temporal relationship between joint injury, NF-κB activation, and the development of pain-sensitivity related behaviors. In addition, Chapter 2 results were used to define a therapeutic treatment window, which in turn was used to test the therapeutic efficacy of locally delivered PHA-408 following joint injury in Aim 2.

1.9.2 Aim 2: Intra-articular delivery of an NF-κB inhibitor in a non-invasive loading model of post-traumatic osteoarthritis (Chapter 3)

Given the involvement of the NF-κB pathway in the development of PTOA, it was hypothesized that acute local pharmacologic NF-κB inhibition would slow the progression of PTOA following joint injury. In Chapter 3 the same longitudinal outcome measures of pain-sensitivity and NF-κB activation, described in Chapter 2, were used to evaluate the efficacy of locally delivered PHA-408 following joint injury. Work also characterized and
explored the effectiveness of two drug depots for the sustained release of PHA-408. Findings in Chapter 3 highlighted the early chondroprotective effects of local PHA-408 administration following mechanical joint injury. Modest improvements in pain-sensitivity related outcomes with repeated drug treatment were also observed. Nevertheless, local delivery of PHA-408 failed to prevent the long-term degeneration of articular cartilage. While engineered drug depots showed the potential for sustained drug release, their use did not improve measured outcomes following joint injury.

1.9.3 Aim 3: Electric field stimulation for the rapid functional assessment of alterations to intact dorsal root ganglion neuron excitability (Chapter 4)

Sensory neuron signaling is required for the sensation of physical stimuli. Yet, many aspects of sensory excitability, particularly in intact tissue, remain unclear. The primary goal of this aim was to describe and validate the development of an ex vivo imaging approach to functionally assess sensory neuron excitability within intact dorsal root ganglion tissues. Results showed the described novel imaging method was reproducible and that cells responded to applied electric field amplitudes in a voltage dependent manner. Electric field stimulated responses were also shown to be, in part, driven by tetrodotoxin-sensitive voltage-gated sodium channels. Finally, this novel method was used in a proof of concept study to assess excitability of sensory neurons following peripheral nerve injury. With injury, neurons had reduced sensitivity to electric field mediated stimuli, furthermore, ex vivo observations were correlated with in vivo assessments of peripheral sensitivity. Ultimately, these methods may help to further interrogate the neurophysiologic basis of sensory neuron signaling and guide the development of improved therapeutic approaches in ion channel mediated diseases.
Chapter 2

NF-κB-mediated effects on behavior and cartilage pathology in a non-invasive loading model of post-traumatic osteoarthritis

2.1 Introduction

As discussed in Chapter 1, OA is one of the leading causes of disability in the United States. Approximately 12% of all patients seeking treatment for symptomatic OA have had prior joint trauma\(^1\). Following traumatic joint injury, roughly half of affected individuals will develop post-traumatic osteoarthritis (PTOA) within 10-20 years\(^{24,26}\). Pathological changes following traumatic joint injury are varied but may include alterations to joint mechanics, inflammation, cell death, and altered extracellular matrix properties\(^{28}\). Many of these changes, which occur shortly following injury, are thought to play a pivotal role in initiating the long-term development of symptomatic PTOA\(^{30,39–41}\). A better understanding of these acute post-injury changes, and their relationship to the development of symptomatic disease, may help to define optimal strategies for therapeutic intervention in PTOA.

Pre-clinical animal models of arthritis serve as a useful tool for understanding the pathophysiology of disease\(^{108,201,210,202–209}\). Generally, these models may be classified by their means of initiation which may include chemically, surgically, non-invasively/overuse, and genetically induced arthritis\(^{30,106}\). Within each of these categories, there are an assortment of models that produce arthritis at varying severities and over differing timescales. In this work, we have chosen to use a non-invasive loading model of PTOA.
to study behavioral changes that occur following an acute traumatic joint injury. This model was selected for its consistent and reproducible injury, disease-severity tunability (dependent on loading parameters), and the non-invasive nature of the procedure. Furthermore, the mechanism of injury initiation, overloading induced joint instability, is relevant to a large number of clinical PTOA cases.

Loading models accurately capture many of the features of traumatic joint injury which may lead to PTOA in humans\textsuperscript{30}. Following injurious tibial compression, knee joints exhibit rapid alterations to joint biomechanics, synovium, cartilage, and bone\textsuperscript{29,30,42,73,207,208,211,212}. Knee joint loading has been shown to trigger robust NF-κB activation in cartilage and synovium within hours of injury\textsuperscript{68}. Inflammatory signaling mediated via NF-κB is thought to play a crucial role in the development of PTOA\textsuperscript{51,67,75}. NF-κB activation following injury has also been directly implicated in the development of chronic pain-related sensitivity\textsuperscript{63,66,213–215}. Thus, further work to define the role of NF-κB signaling in the symptomatic progression of PTOA is warranted.

In this chapter, our goal was to longitudinally examine the relationships between the development of pain-related behavioral changes and NF-κB activation \textit{in vivo} in a non-invasive murine tibial compression model of PTOA.
2.2 Methods

2.2.1 Animal and injury model

All procedures were performed with approval of the Washington University Institutional Animal Care and Use Committee. Transgenic FVB.Cg-Tg(HIV-EGFP,luc)8Tsb/J mice (Fig. 2.1A) (The Jackson Laboratory, Bar Harbor, ME) were acquired (male, 9-12 weeks of age) and acclimated to testing equipment for 1 week. Mice in cohort 1 were randomly assigned to sham control (CTRL, n=5) or joint injury (INJ, n=6). An additional group of mice (male, 9-12 weeks of age, n=5/group) were used to assess how knee joint injury influenced gait (section 2.2.8). To induce joint injury, mice were anesthetized under isoflurane (1-3% inhalation, 2 L/min oxygen) and right knees were loaded as previously described by Rai and colleagues on a materials testing system (Instron ElectroPulse E1000, Norwood, MA). Briefly, tibiae were positioned vertically with the knee upwards in deep flexion between custom-made fixtures and were then subjected to 60 cycles of 12 N axial compressive load (54 N/sec with 10 sec hold at 0.5 N between cycles). This protocol reproducibly induced a sudden drop in the axial force recorded on the time-force curve and increased anterior-posterior joint laxity (Fig. 2.1B,C) via a joint laxity test in a separate cadaveric test cohort (Appendix A, Supplementary Methods). CTRL animals were placed within the test frame and their knees were subjected to 10 min of static axial compressive load at 0.5 N. Animals were returned to their home cage and allowed to freely ambulate upon recovery.
2.2.2 *In vivo* luminescence imaging of NF-κB activity

To assess local NF-κB activation following joint injury, *in vivo* bioluminescent imaging was performed. At each imaging timepoint (Fig. 2.1D) mice received an injection of D-luciferin (150 mg/kg i.p., Sigma-Aldrich, St Louis, MO). Mice were anesthetized 10 minutes following D-luciferin injection and whole-body luminescent images were acquired with an *in vivo* imaging system (10 sec exposure, IVIS 50, PerkinElmer, Waltham, MA). NF-κB-driven luminescence was quantified in both ipsilateral and contralateral knees within an 8x10 mm elliptical region of interest (ROI) that was placed around the knee joint.

![Diagram](image)

**Figure 2.1: Study design.** (A) Representative NF-κB response element of FVB.Cg-Tg(HIV-EGFP, luc)8Tsb/J mice. (B) Custom-made mount for *in vivo* loading to induce non-invasive joint injury and corresponding time versus force and displacement curves showing one cycle of compressive loading with characteristic one-time mid-cycle drop in the time versus force curve coinciding with ligament injury. (C) Injured joints have increased anterior-posterior joint laxity as measured by a joint laxity score. (D) Study timeline showing repeated imaging and behavioral assessments. Mean±SD (n=10/group), Mann Whitney test; ***=p<0.001.
2.2.3 Pain-related hindpaw sensitivity

Mechanical allodynia was evaluated in both the ipsilateral and contralateral hindpaws using von Frey filaments. Filaments ranging from 0.02-2.0 g were applied to the plantar region of a stationary, non-rearing, animal using the Chaplan “up-down” method\textsuperscript{186,217}. Briefly, the starting filament (0.4g) was applied for 3 sec or until the animal had a positive paw-withdrawal response (guarding, paw flick, lick, and/or jump seen in Fig. 2.2A). Based on the prior response either the next weaker (positive response) or stronger (negative response) filament was then applied. Following the first change in response four additional filament applications were applied (using the up-down method). Provided no change in response occurred, filaments were applied until the weakest or strongest filament produced either a positive or negative response, respectively.

Figure 2.2: Graphical overview of select behavioral assessments. Representative still images of positive withdrawal responses (arrowpoint) for von Frey (A) and peripheral thermal sensitivity (B) assessments. (C) Schematic of murine gait arena, enables simultaneous recording of sagittal and ventral views.
2.2.4 Pain-related hyperalgesia

The threshold for contact hyperalgesia was tested at the knee joint using a small animal algometer (SMALGO, Bioseb, Vitrolles, FRA). Mice were scruffed with one hand and the hindpaw was held with the knee at a ~30° flexion angle. The tip of the SMALGO force transducer was applied to the lateral aspect of the knee at ~30 g/sec until the animal showed an escape response (flinch, head jerk, and/or squeak, to a maximum of 400 g)\textsuperscript{218}. Three independent measurements were obtained and averaged for each hindlimb on each mouse on all behavioral testing days. One animal within the INJ group showed extreme hypersensitivity/discomfort to light knee palpation on days 2 and 8 following loading and thus was excluded from hyperalgesia testing on those days.

2.2.5 Hindpaw thermal sensitivity

Mice were gently scruffed and the plantar side of the tested paw was placed on a hot/cold plate (50/0°C, BIO-CHP, Bioseb) while the other paw was placed on a room temperature plexiglass surface at the same height. Mice were held stationary until a clear paw withdrawal occurred or a 20 or 30 sec threshold was reached on the hot and cold plate, respectively (Fig. 2.2B)\textsuperscript{219}. Latency to withdrawal was recorded and three independent measures were collected for each hindpaw at each temperature. Animals were allowed a one minute recovery period between consecutive hindpaw measurements.

2.2.6 Static weight bearing

Mouse hindlimb static weight bearing was measured using an incapacitance meter (BIO-SWB-TOUCH-M, Bioseb). Mice were placed in a small transparent enclosure with their hindpaws resting on two independent force plates and their forepaws resting on an
angled wall of the enclosure. Independent force measurements from both hindpaws were collected simultaneously over a 3 sec interval. Three independent measurements were obtained and averaged for each mouse on all behavioral testing days.

### 2.2.7 Spontaneous activity

Freely selected activity was assessed by tracking mouse movement within a custom arena (40 cm x 40 cm x 40 cm, matte black P95 acrylic, TAP Plastics, Stockton, CA) for 30 min during the dark portion of a 12 h light-dark cycle; all tracking was performed without the presence of investigators or other personnel and with illumination by red light\(^{220,221}\). Movement was recorded (Sony Handycam HDR-CX405, Sony Corp, New York, NY) and each 30 min video was analyzed (EthoVision, Noldus, Wageningen, NLD) to determine the difference in distance traveled, average speed, and percent time stationary from baseline for each animal.

### 2.2.8 Spatiotemporal gait testing

A separate cohort of mice underwent longitudinal spatiotemporal gait testing to determine how non-invasive knee injury alters joint function during free, unprompted, ambulation. A modified custom-built gait arena (Fig. 2.2C; 76 cm long x 7 cm wide x 13 cm tall, extruded acrylic, TAP Plastics) was used to acquire spatiotemporal gait data as described previously\(^{222-224}\). During gait collection, animals voluntarily explored (i.e. not forced/prompted) the arena at self-selected velocities and recordings were collected with a high-speed video camera (FASTCAM Mini UX50, Photron, San Diego, CA) at 1000 frames per sec. Trials with consistent walking speed and at least three complete gait cycles were analyzed. Longitudinal gait data were collected 2, 7, 14, and 36 days following joint injury. Spatiotemporal gait parameters including duty factor, duty factor
imbalance, and spatial symmetry were calculated using Automated Gait Analysis Through Hues and Areas (AGATHA) open source software\textsuperscript{223–226}.

### 2.2.9 Histology

Mice were euthanized at the terminal timepoint (113 days) and ipsilateral knees collected for histology. All knees were fixed in 4\% paraformaldehyde for 48 h, decalcified for 72 h using Immunocal\textsuperscript{TM} (Fisher Scientific, Waltham, MA), immersed in 30\% w/v sucrose for 24 h, and cryoembedded in Tissue-Tek\textsuperscript{®} O.C.T. (Sakura Finetek, Torrance, CA). Serial coronal sections (8 \textmu m) of ipsilateral knees from all groups were acquired, and one section representing the most severe evidence of arthritic changes was chosen for staining with H&E, Safranin O, and fast green. Stained sections were graded to consensus by two blinded graders using the Osteoarthritis Histopathology Assessment System (OARSI) within the medial and lateral femoral condyle (MFC/LFC) as well as the medial and lateral tibial plateau (MTP/LTP)\textsuperscript{227}.

### 2.2.10 Statistical analysis

Statistical analyses were performed using GraphPad Prism 8.0 (GraphPad Software, La Jolla, CA). A repeated measures two-way ANOVA with Bonferroni’s post-hoc was used to test for differences between CTRL and INJ at each timepoint for all longitudinal measures. \textit{In vivo} imaging and 50\% withdrawal threshold data were log-transformed (base 10) to better meet ANOVA assumptions. A repeated measures two-way ANOVA with Bonferroni’s post-hoc was used to test for differences in cartilage OARSI scores at each cartilage site between CTRL and INJ groups. The relationship between NF-κB luminescence and allodynia was tested via Pearson correlation on log-transformed (base 10) data (Appendix A, Supplementary Methods). For velocity
dependent gait parameters a linear regression was fit to CTRL animals and residual values were calculated\textsuperscript{225,226,228,229}. Following, a two-way ANOVA with Bonferroni’s post-hoc was used to test for differences in gait outcomes at each timepoint between CTRL and INJ groups. Statistical significance was set at p < 0.05.
2.3 Results

2.3.1 Joint injury leads to increased NF-kB-driven luminescence

A transient increase in NF-kB-driven luminescence was observed in INJ ipsilateral limbs when compared to ipsilateral limbs from CTRL mice on days 1 and 3 following joint injury (Fig. 2.3A,B). Within injured ipsilateral limbs, luminescence intensity peaked within the knee ROI on day 3 and decreased thereafter. There was no evidence of differences in NF-kB-driven luminescence in the contralateral joints between CTRL and INJ animals.

2.3.2 Joint injury alters sensitivity to evoked mechanical but not thermal stimuli

Non-invasive joint injury resulted in several changes to pain-related sensitivity and behavior in mice. Increased hindpaw sensitivity to mechanical stimuli (allodynia) was observed following joint injury as evidenced by a reduced 50% hindpaw withdrawal threshold at days 2, 8, and 15 following joint injury (Fig. 2.3C). Following day 15, the 50% withdrawal threshold in the INJ group returned to levels that were consistent with those in the CTRL group, although a reduced 50% hindpaw withdrawal reemerged in the INJ group on day 113. There were no differences between 50% withdrawal thresholds of CTRL and INJ groups within contralateral hindpaws. Within a 4 week period following loading, several animals in INJ group exhibited promoted guarding in their ipsilateral hindpaw. In some animals, this behavior persisted for the remainder of the study. Furthermore, increased NF-kB signaling was associated with reduced withdrawal thresholds in the first week of the study (Fig. 2.3D).
Joint injury also led to transient hyperalgesia in the ipsilateral limbs of animals in the INJ group; reduced hyperalgesia thresholds were observed on days 2 and 8 following joint injury compared to animals in the CTRL group (Fig. 2.3E). There were no observed differences in hyperalgesia thresholds for contralateral limbs between mice in the CTRL and INJ groups.
Withdrawal latency from a hot (Fig. 2.4A) or cold (Fig. 2.4B) thermal stimuli at the hindpaw were not found to differ between CTRL and INJ groups. Within groups, response latencies were similar in ipsilateral and contralateral limbs (Appendix A, Fig. A.1), generally, latencies were also found to decrease over the course of the study.

Figure 2.4: Thermal sensitivity at the hindpaw. Non-invasive knee joint injury did not alter sensitivity to a hot (A, 50°C) or cold (B, 0°C) stimulus at the hindpaw. C=contralateral, repeated measures two-way ANOVA.
2.3.3 Joint injury alters non-evoked behaviors

Mice in the INJ group exhibited reduced weight bearing on their ipsilateral hindlimb on days 2 and 8 following joint injury (Fig. 2.5). For the remainder of the study weight bearing between CTRL and INJ groups were equivalent.

Animals in the INJ group showed a trend of reduced freely selected activity on day 2 following joint injury, corresponding trends in average speed and percent time stationary were also observed (Fig. 2.6A-D). Ambulatory levels within individual mice, particularly within the CTRL group, were quite variable throughout the study, additionally, ambulation decreased with time within 30 minute open field trials (Appendix A, Fig. A.2).
Several gait parameters were transiently altered with joint injury. Duty factor (Fig. 2.7A), stride length, and stance width were linear related to animal velocity, therefore, residuals were used for the analysis of these data. Due to inconsistent gait patterns (e.g. hopping and non-weight bearing) 2 days following joint injury, animals of the INJ group could not undergo digitization using AGATHA software. One week following joint injury animals in the INJ group had a reduced ipsilateral hindpaw duty factor residual and a corresponding increased duty factor residual for their contralateral hindpaw (Fig. 2.7B,C). This imbalance between hindpaws was sustained until day 14 post-injury (Fig. 2.7D). Forepaw duty factors were not influenced by hindpaw injury (Fig. 2.7E,F). There were also no observed differences in animal walking speed between groups following joint injury (Fig. 2.7G). Spatial symmetry, which highlights unilateral imbalances in the step:stride length ratio, was altered one week following injury in hindpaws only (Fig. 2.7H,I). No differences were observed in fore or hindlimb measures of stance width or bilateral measures of step length (data not shown).

Figure 2.6: Non-invasive joint injury results in a modest reduction to spontaneous activity. (A) Representative open field centroid traces of CTRL and INJ mice. (B-D) Following INJ mice showed a transient trend of decreased distance traveled and corresponding alterations to average speed and percent time stationary in each 30 min recording. Mean±SEM, repeated measures two-way ANOVA.
Figure 2.7: Non-invasive joint injury alters spatiotemporal gait parameters. (A) Duty factor is dependent on velocity, concentric squares highlight reduced ipsilateral hindlimb duty factor 7 days post INJ. (B and C) Individual hindlimb duty factor residuals (C=contralateral) and hindlimb duty factor imbalance (D) were transiently altered following injury. (E and F) Forelimb duty factors and animal velocity (G) were not altered by joint injury. Hindlimb gait became spatially asymmetric (H), no spatial alterations to gait were observed in the forelimbs (I). Repeated measures two-way ANOVA w/ Bonferroni’s post-hoc; *=p<0.05, **=p<0.01, ****=p<0.0001.
2.3.4 Joint injury leads to histological development of PTOA

Grading of articular cartilage revealed significant damage to select joint regions following joint injury. Knee joints from animals of the INJ group showed evidence of severe articular degeneration in the medial compartment of the joint compared to knee joints from the CTRL group (Fig. 2.8A,B); median OARSI scores in MFC were 0.5 and 6 in CTRL and INJ joints respectively (p<0.001), median scores within the MTP were 1.75 in CTRL and 6 in INJ joints (p<0.01). In several cases, complete loss of cartilage on the MFC and MTP was observed at the terminal timepoint. The LFC and LTP of animals in the INJ group had median OARSI grades of 3 and 1.5, respectively, compared to median scores of 0.5 and 1 in CTRL joints. Qualitatively, knee joints of the INJ group showed signs of inflammation, fibrosis, synovial hyperplasia, and osteophyte formation when compared to joints from the CTRL group.

![Figure 2.8](image)

**Figure 2.8. Long-term degenerative changes to articular cartilage following injury.** (A) Representative histology shows full thickness articular defects in injured knee joints 113 days following injury (B) INJ knees had elevated OARSI scores in the medial femoral condyle (MFC) and medial tibial plateau (MTP), scale bar = 500 µm. LFC=lateral femoral condyle, LTP=lateral tibial plateau. Repeated measures two-way ANOVA w/ Bonferroni’s post-hoc; **=p<0.01, ***=p<0.001.
2.4 Discussion

In this work, we showed non-invasive knee joint injury in mice led to both transient and long-term pain-related sensitivity and behavior changes consistent with those seen in humans following acute traumatic joint injury\textsuperscript{28}. Specifically, injury induced NF-κB activation within the knee joint was associated with the onset of pain-related sensitivity, gait alterations, weight bearing imbalances, and cartilage degeneration. Together these data provide novel insight into the temporal progression of symptomatic disease within this non-invasive murine model of PTOA.

We and others have previously shown injection models of inflammatory arthritis result in transient changes to peripheral sensitivity following joint injury\textsuperscript{63,93,220}. Similarly, surgical destabilization models of PTOA consistently result in hindpaw allodynia when compared to sham operated animals; however, the onset, presentation, and duration of this mechanical allodynia is varied and different from that observed in this non-surgical PTOA model\textsuperscript{103,109,113,230}. For example, Temp and colleagues showed mice experience a biphasic pain-sensitivity phenotype following medial meniscus transection\textsuperscript{113}. Specifically, acute allodynia primarily attributed to surgery resolved within one week and a second onset of allodynia was observed four weeks after surgery. Withdrawal thresholds within this second pain-sensitivity phase were of similar magnitude to those observed within the one week post-surgery window. These results are in contrast to those reported within this study; following non-invasive injury we observed sustained allodynia for two weeks. At later timepoints withdrawal thresholds remained slightly lower in INJ ipsilateral hindpaws, however, these thresholds were never as low as those observed in the two week post-injury period. Recent work by Heegde and colleagues corroborates the findings of the
current study and shows that mechanically-induced joint injury is associated with a transient and early onset of hindpaw allodynia\textsuperscript{124}.

In addition to the development of chronic allodynia, we report a transient decrease in the mechanical threshold to noxious stimuli at the injured knee joint. This acute unilateral alteration is contrary to the chronic mechanical hyperalgesia observed following surgically induced destabilization of the medial meniscus\textsuperscript{218}. The mechanisms that contribute to these pain-related sensitivity alterations in differing models of PTOA remain unknown. Further scientific inquiry should aim to shed light on the phenotypic changes to sensory neurons that innervate structures within the injured knee joint; these studies may better delineate the mechanistic differences in pain-sensitivity between PTOA models\textsuperscript{105,231}. In this study no bilateral sensitivity alterations were observed following injury. This may suggest a lack of bilateral changes to the peripheral and central nervous system following mechanically induced joint injury. This is in contrast to observations made in injection models of inflammatory arthritis which have shown increased bilateral expression of neuropeptides such as substance P and calcitonin gene related peptide in the dorsal root ganglia and dorsal horn of the spinal cord shortly following injury\textsuperscript{232,233}. These changes have been reported to be influenced by commissural spinal interneuron crosstalk and increased systemic levels of pro-inflammatory cytokines\textsuperscript{234}. To date, there have been no reports of increased systemic pro-inflammatory cytokines within this non-invasive model of PTOA.

A novel finding of this study highlighted the early changes to non-evoked behaviors of activity in gait/ambulation and weight bearing imbalance in stance. Prior studies have not characterized the temporal progression of such behavioral changes following non-
invasive joint injury\textsuperscript{109,110,126}. Anderson and colleagues showed slight alterations (6% reduction in duty factor for ipsilateral hindpaws and corresponding, but non-significant, 7.3% increase in duty factor for the contralateral hindpaw) to gait parameters 1 day following non-invasive joint injury\textsuperscript{126}. In this study, we were unable to characterize changes shortly following joint injury due to severe gait abnormalities including limb disuse. Despite this, gait alterations one week post-injury appeared to be larger, with a 14.7% reduction in ipsilateral hindpaw and 9.9% increase in contralateral hindpaw duty factor, respectively. Furthermore, alterations were sustained for two weeks (-3.9% and +3.5% for ipsilateral and contralateral hindpaws, respectively) following joint injury. This may suggest that the temporal progression of factors influencing behavioral alterations are only just beginning 1 day following injury and more severe behavioral phenotypes/abnormalities are present at slightly later timepoints within one week of knee joint injury. Additionally, this study utilized a freely selected gait assessment as opposed to the forced treadmill running assessment used by Anderson and colleagues which may further explain these observed differences. Others have shown similar transient alterations to gait following chemically, genetically, and surgically induced arthritis\textsuperscript{109,110,230,235,236}.

By 3 weeks following joint injury CTRL and INJ groups appear to be similar in most behavioral measures assessed in this study. This is despite persistent alterations to joint morphology and mechanics (e.g. development of osteophytes, cartilage lesions, synovial hyperplasia, reduced range of motion)\textsuperscript{29–31,68,211}. With that said, these findings point to consistent changes in behavior under conditions that minimize animal fatigue and external stresses. Mice are widely believed to temporarily mask pain-related behaviors

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during periods of prompted stimulation or stress such that activity changes associated with freely selected behaviors may be of particular value to understanding symptomatic joint pathology in murine models\textsuperscript{122}. Future studies might benefit from assessments that allow for quantification of freely selected behaviors including animal rearing, running wheel use, and place preference\textsuperscript{122,237}. With this in mind, a careful understanding and pairing of complementary behavioral tests that assess different aspects of pain-related sensitivity in disease are important for accurate and consistent characterization of symptomatic profiles in animal models of PTOA.

\textit{In vivo} bioluminescent imaging enabled longitudinal quantification of bulk NF-κB pathway activation following knee joint injury. Data suggested that early NF-κB signaling was correlated (Appendix A, Fig A.3) with the acute and chronic presentation of hindpaw allodynia. Early increases in NF-κB-driven luminescence were also associated with more severe cartilage erosion at the terminal timepoint (Appendix A, Fig. A.4). These data suggest acute NF-κB signaling following injury may be predictive of the symptomatic and structural progression of PTOA. The week long elevation in NF-κB-driven luminescence observed in this study is in contrast to changes observed following surgically induced knee joint injury. In studies using surgically induced PTOA models, joint injury has been shown to elicit a prolonged elevation in NF-κB-driven luminescence for up to 6 weeks\textsuperscript{118,238}. This suggests the systemic effects of surgery, with consequences for the prolonged elevation of inflammatory mediators, may elicit sustained alterations in joint inflammation and associated pain-related sensitivity in these widely utilized surgical models of PTOA. It is therefore imperative to consider these factors when comparing outcomes across differing pre-clinical animal models of PTOA.
In conclusion, the non-invasive model of PTOA described in this study may be valuable for studying the early pathological changes following acute traumatic joint injury and how they relate to pain-related sensitivity and behavioral changes without the confounding nature of a surgical incision. Nevertheless, further work to better dissect the temporal development and presentation of pain and sensitivity alterations in PTOA should be performed to help direct future treatment strategies that may prevent both the histological and painful progression of disease.

2.5 Acknowledgements

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Chapter 3

Intra-articular delivery of an NF-κB inhibitor in a non-invasive loading model of post-traumatic osteoarthritis

3.1 Introduction

In Chapter 2 we showed that acute NF-κB activation within the knee joint was coincident with increased mechanical sensitivity at the hindpaw. Additionally, we showed that transient activation of NF-κB was associated with the development of several other evoked and non-evoked behavioral changes that were not observed following sham loading within the control (CTRL) group. Longitudinal in vivo imaging of NF-κB activation, as described in Chapter 2, allowed us to determine an appropriate window for therapeutic intervention following joint injury. In this chapter, we sought to inhibit NF-κB signaling in the knee joint following injury via various local delivery strategies.

Increased NF-κB activity has been implicated in the development of pain following peripheral injury and recent data suggest NF-κB may be directly responsible for sensitization in some pain-related ion channels\textsuperscript{213–215}. Furthermore, human studies have shown that patients with worsening symptomatic OA have increased mRNA and protein level expression of p-65, MMPs, and IL-1β within the knee joint\textsuperscript{239}. NF-κB activation via inflammatory mediators has also been shown to play a causal role in the increased production of neurotrophic factors such as nerve growth factor (NGF)\textsuperscript{240}. Indeed, these neurotrophic factors are known to induce further context dependent NF-κB signaling and play a pivotal role in injury induced sensitization\textsuperscript{241,242}. Additionally, these factors have
been observed at high concentrations in synovium and cartilage following joint injury\textsuperscript{58,243–251}. While aberrant NF-κB signaling is thought to drive the excessive production of pro-algesic factors in symptomatic OA, many studies have sought to elucidate the role of these pro-algesic molecules independently of NF-κB\textsuperscript{252–254}. While these approaches have shown analgesic benefits in arthritic patients adaptation of therapies, such as tanuzamib, beyond clinical trials has been limited due to long-term deleterious effects on cartilage and bone\textsuperscript{252,255,256}. Therefore, strategies that target pain and inflammatory signaling pathways via alternative mechanisms are of interest in the disease-modifying treatment of symptomatic OA.

Given the role of NF-κB activation in the development of OA, it has been identified as a promising druggable target for the treatment of disease. Preclinical studies have shown protective effects of selective IκB kinase 2 (IKK-2) inhibitors, such as 8-(5-Chloro-2-(4-methylpiperazin-1-yl)isonicotinamido)-1-(4-fluorophenyl)-4,5-dihydro-1H-benzo[g]indazole-3-carboxamide (PHA-408) (Fig. 3.1), highlighting their potential as disease-modifying OA drugs\textsuperscript{76,77}. Specifically, PHA-408, a low molecular weight (560 Da), highly selective inhibitor of IKK-2, has been shown to prevent the development of inflammatory arthritis in streptococcal cell wall and lipopolysaccharide preclinical animal models\textsuperscript{76}. Furthermore, direct inhibition of the NF-κB signaling pathway has been shown to have analgesic benefits following inflammatory injuries\textsuperscript{66,257–259}. However, off-target effects upon systemic delivery and rapid clearance of small molecules from the joint following intra-articular delivery have limited their therapeutic effect\textsuperscript{51,260}. The proven efficacy of IKK-2 inhibitors when administered systemically motivates the development of sustained release intra-articular delivery strategies for the treatment of OA. These
strategies may help to reduce the deleterious effects of repeated systemic administration and prolong drug residence times within the joint.

Sustained release drug depots have shown promise in clinical translation for the treatment of OA\(^{175,261}\). Poly(lactic-co-glycolic) (PLGA), is FDA approved, and has long been utilized as a polymeric drug carrier for its tunable sustained drug release characteristics; however, acidic breakdown products from the polymer are known to lead to inflammation. Amphiphilic block polymers combining polyethylene glycol (PEG) and PLGA can efficiently encapsulate small hydrophobic drug molecules for sustained release. These block polymers also have reduced inflammatory effects due to the incorporation of PEG which serves to mask the inner PLGA core\(^{180}\). Prior work has shown that PEG-PLGA microparticles can remain entrapped in the synovial cavity for up to 4 weeks following intra-articular injection\(^{179}\). Similarly, silk fibroin (SF) microparticles have been shown to improve joint residence times of locally injected drugs by nearly 4-fold.
when compared to non-particle formulations\textsuperscript{184}. SF also exhibits limited immunogenicity, tunable temporal bioresorbable characteristics, and the potential for sustained release of low molecular weight hydrophobic drugs\textsuperscript{183,262}.

In Chapter 3, work aimed to investigate the disease-modifying potential and biodistribution of locally delivered PHA-408 following non-invasive mechanical joint injury. Additionally, we formulated and explored the efficacy of PEG-PLGA and SF microparticles for the sustained release of PHA-408 to determine if a single intra-articular dose of a sustained release preparation may influence the development of symptomatic PTOA.

3.2 Methods

3.2.1 Joint injury and drug delivery

All procedures were performed with approval of the Washington University Institutional Animal Care and Use Committee. Transgenic FVB.Cg-Tg(HIV-EGFP, luc)8Tsb/J mice (male, 9-12 weeks of age) were used in the four animal cohorts described in this chapter.

A preliminary study was used to determine intra-articular PHA-408 dosing. In this work, mice received a single intra-articular injection of mono-iodoacetate (0.1 mg/5 µL, MIA) and two subsequent intra-articular injections of PHA-408 (0.1 µg/ 5 µL, n=4) or vehicle (saline+0.1% DMSO, n=3) at 0 and 48 h following MIA injection. Drug treatment had minimal impact on joint localized NF-κB signaling (Fig. 3.2A). However, early delivery of PHA-408 reduced hindpaw allodynia (Appendix B, Fig. B.1) and appeared to have modest protective effects on articular cartilage one month following MIA injection (Fig.
Based on these data, PHA-408 dosing was increased in hopes of reducing localized NF-κB-driven luminescence. To first determine the efficacy of local NF-κB inhibition following mechanical joint injury, mice were randomly assigned to naive (CTRL, n=4), joint injury (INJ, n=6), or joint injury plus treatment (INJ/Tx, n=5) groups. Following joint injury, as described in Chapter 2, mice received a single intra-articular injection of PHA-408 (3 µg/5 µL, sterile saline + 0.1% DMSO, Alchem Pharmtech Inc, Monmouth Junction, NJ) or vehicle (sterile saline + 0.1% DMSO) and were sacrificed 48 h post-injury (Fig. 3.3) to study the immediate cellular effects of joint injury and drug treatment on articular cartilage (described in Section 3.2.6).

A separate cohort of mice was used to test for drug related effects using longitudinal measures described in Chapter 2. Mice from cohort 1 (Chapter 2) were randomly assigned to joint injury plus treatment (INJ/Tx, n=5). Animals in the INJ/Tx group received two intra-articular injections of PHA-408 (3 µg/5 µL), one immediately following injury and another 48 h post-injury.
To test the effect of a sustained release drug formulation, mice in cohort 3 received a single intra-articular injection of PHA-408 loaded PEG-PLGA microparticles (INJ/TxMP, 3 µg/5 µL; synthesis described in Section 3.2.3), PHA-408 (1 µg/5 µL), or vehicle (in vivo imaging n=2-5 group/timepoint; hindpaw sensitivity n=2 group/timepoints) and were tracked for 1 week to assess changes in NF-κB-driven luminescence and hindpaw sensitivity. Finally, to test the biodistribution and clearance behaviors PHA-408 (described in Section 3.2.7), mice in cohort 4 received two intra-articular injections of PHA-408 (3 µg/5 uL) or vehicle (0 and 48 h) following knee joint injury (n=2-5 per group/timepoint).

3.2.2 Gene expression

Gene expression was assayed using qPCR on an Applied Biosystems™ StepOnePlus™ Real-Time PCR System (Software v2.3, Foster City, CA). Briefly, immortalized human synovial sarcoma cells (SW982, ATCC, Manassas, VA) were seeded (300,000 cells/well) on a 6-well plate and allowed to grow until 90% confluency (37°C, 5% CO2). Cells were then co-treated with recombinant human interleukin-1 beta (1 ng/mL; IL-1β, R&D Biosystems, Minneapolis, MN) and PHA-408 (0.1 to 10 µM). Following a 6 h incubation period, cells were lysed using RLT buffer (Qiagen, Hilden, CA).
DEU) + 1% mercaptoethanol. RNA isolation was performed using the QIAGEN™ Mini kit (Qiagen). Briefly, samples were homogenized using a QIAshredder™ column and RNA was then bound to an RNeasy spin column. The sample was then washed, and DNA was digested using DNase I. RNA was eluted with RNAse-free water and RNA concentration and purity were determined using the 260/280 ratio in a NanoDrop™ system (ThermoFisher, Waltham, MA). RT-PCR was performed using the iScript cDNA Synthesis Kit (BioRad, Hercules, CA). qPCR was used to detect amplification of tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6), and interleukin-8 (IL-8) (Applied Biosystems, Foster City, CA) using ∆∆Ct; GAPDH and ACTB were used as housekeeping genes and data were normalized to untreated controls.

### 3.2.3 PEG-PLGA microparticle formulation

PEG-PLGA microparticles were fabricated using a coaxial microfluidic phase separation method by Dr. Era Jain. A 2% w/v PEG-b-PLGA (PEG 2kDa, PLGA 11.5kDa, Sigma-Aldrich, St. Louis, MO) solution prepared in dichloromethane (DCM) was flowed through an inner needle (31 G) at 0.2 mL/h and a 5% w/v polyvinylalcohol (PVA) solution was passed through an outer concentric needle (16 G). PEG-PLGA droplets were collected in a Teflon™ bath in 5% w/v PVA under constant stirring. Following washing and sieving (70 μm nylon filter) microparticles were vacuum dried and stored. Drug loaded microparticles were prepared by adding PHA-408 to the DCM/PEG-PLGA (1:10 drug:polymer ratio) phase prior to microparticle preparation. Microparticles were imaged and their average diameter was calculated by measuring ≥100 microparticles per experimental run using ImageJ™ (National Institutes of Health, Bethesda, MD).
3.2.4 Silk fibroin microparticle formulation

Silk fibroin (SF) solution was prepared as previously described\textsuperscript{181,264}. Briefly, whole silk cocoons were cut into dime sized pieces and boiled in 0.025M sodium bicarbonate for 30, 60, or 120 min for sericin residue removal. Silk was then washed (20 min, 3X, diH\textsubscript{2}O) and allowed to dry overnight. Dried silk was solubilized in 9.3M lithium bromide (4h, 60°C) at a 20\% w/v ratio. The solution was transferred into a dialysis cassette (MWCO 3500, Thermo Scientific) and dialyzed against 5 L of diH\textsubscript{2}O for 48 h with constant stirring, water was exchanged every 12 h. Dialysate was then removed and centrifuged (Sorvall Evolution RC, Thermo Fisher) at 12,700 RCF (20 min, 4°C) to remove aggregates and debris. The solution was diluted to a final 6\% (w/v) solution with diH\textsubscript{2}O and stored at 4°C.

SF microparticles were prepared by mixing 3 mL extracted SF (30, 60 or 120 min) with 12 mL of 5\% PVA (1:4 silk:PVA ratio) and casting the mixture in a polystyrene petri dish (100 mm) to form a film. The film was re-suspended in diH\textsubscript{2}O and microparticles were washed, centrifuged, and lyophilized. PHA-408 loading was performed post microparticle synthesis by mixing PHA-408 in ethanol (EtOH, 2 mg/mL) with microparticles (1:10 drug:microparticle ratio) for 3 days under constant stirring. EtOH was then allowed to evaporate, microparticles were subsequently washed with EtOH, lyophilized, and stored at 4°C. Microparticle synthesis was performed by Dr. Burcin Yavuz at Tufts University.

3.2.5 In vitro release and drug bioactivity

For quantification of drug release via high performance liquid chromatography (HPLC) 10 mg of PHA-408 loaded microparticles were incubated with 1 mL of phosphate
buffered saline (PBS), pH 7.4 at 37°C. At regular time intervals PBS was collected and an equivalent volume of fresh PBS was added. Samples were stored at -80°C for later analysis. Collected aliquots were analyzed via HPLC (Agilent 1200 system, Agilent Technologies, Santa Clara, CA) to measure released PHA-408. HPLC analyses were performed by Dr. Burcin Yavuz at Tufts University. Following extraction in dimethyl sulfoxide (DMSO) samples were run through an Agilent Poroshell 120 EC-C18 column (2.7 μm, 4.6mm×50mm, Agilent Technologies) at 25°C. A mobile phase of methanol:water with 0.1% trifluoroacetic acid (60:40) was used at a flow rate of 0.75 mL/min and UV absorption was measured at 260 nm. Drug concentration in aliquots was determined by comparison to a standard curve.

To determine drug bioactivity following release from microparticles, PHA-408 loaded microparticles (10 mg/mL) were suspended in Dulbecco’s Modified Eagle Medium (DMEM), supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin, and placed in a Transwell™ insert at 37°C. At regular time intervals, aliquots of media incubated with PHA-408 loaded microparticles were collected and an equivalent volume of fresh media was added to each Transwell™. NF-κB inhibition from released PHA-408 in microparticle incubated media was measured using a SW982 reporter cell line transduced with lentivirus carrying an NF-κB luciferase response element construct (pHAGE NFκB-TA-LUC-UBC-GFP-W, Addgene, Watertown, MA). Following transduction (1 μL, 4.3 multiplicity of infection), cells were plated and allowed to attach. Media was then supplemented with 1 ng/mL of recombinant human interleukin-1 beta (IL-1β, R&D Biosystems, Minneapolis, MN) to induce an inflammatory response as measured by increased luciferase expression in the transduced cells. Some wells received dosing
with an aliquot of media incubated with PHA-408 loaded microparticles as described above, and the luminescence signal was measured following a 24 h incubation period (Enspire, PerkinElmer) using Bright-Glo™ (Promega, Madison, WI). Bioluminescence values were normalized to IL-1β only treated controls.

### 3.2.6 In situ live-dead cellular imaging

Intact femurs from animals in cohort 2 were harvested 48 h following joint injury for in situ live-dead imaging (Fig. 3.4A-C). Knees were grossly dissected from mice by cutting through the mid-femoral diaphysis. Muscle and connective tissue were then removed from the around the knee joint, patellar tendon and medial/lateral collateral ligaments were transected, and the knee joint was disarticulated via tensile overload. Care was taken to avoid any contact with articular cartilage and samples were continuously irrigated with hypertonic (400 mOsm) media during dissection. Jeweler forceps were used to remove medial and lateral menisci as well as posterior/anterior cruciate ligament.

![Figure 3.4: In situ live-dead imaging setup.](image)

Following staining, intact femurs where placed atop a #1.5 coverslip and fixed in custom holder (A) such that the femoral shaft was positioned at a ~30° angle with respect to the x-axis and both condyles made complete contact with the coverslip as shown from the sagittal plane. (B) Representative coronal histology section and (C) corresponding max intensity projections (100µm z-stack) of fluorescence images show cell nuclei in the articular cartilage of both the lateral (left) and medial (right) femoral condyles (LFC/MFC) in the transverse imaging plane, scale bar = 200 µm.
remnants. Cleaned femurs were stained with Hoechst 33342 (10 µM, ThermoFisher), Calcein-AM (5 µM, Invitrogen, Carlsbad, CA), and Propidium Iodide (PI, 5 µM, ThermoFisher) for 1 h at 37°C. Following staining, intact femurs were imaged (FV1200, Olympus, Tokyo, JPN). Z-stacks (100 µm thickness) were acquired of each femoral condyle. Hoechst+ and PI+ cell number per condyle was determined via an automated thresholding-based ImageJ script.

3.2.7 Biodistribution of PHA-408 following intra-articular injection

Mice (n=2-5 per group/timepoint) in cohort 4 underwent compressive joint injury followed by intra-articular injections of either PHA-408 (3 µg/5 µL) or vehicle (5 µL) immediately following and 48 h post-injury. At terminal timepoints (30 min, 2 h, 3 d, 7 d) 1 mL of blood was collected by cardiac puncture and serum was obtained following centrifugation. Lymph nodes (inguinal and lumbar), knees, and urine were collected at the time of sacrifice. Lymph nodes were suspended in 500 µL acetonitrile (ACN), and homogenized (Mini-BeadBeater™, BioSpec, Bartlesville, OK). Knees were flash frozen in liquid nitrogen, homogenized (BioPulverizer™, BioSpec), and suspended in 2 mL of ACN. Samples were then vortexed for 2 h and centrifuged to collect clarified ACN. Drug concentrations in serum, urine, lymph nodes, and knee tissue were estimated via LC-MS upon an Agilent 1200 series HPLC instrument and an Agilent 6410 triple-quadruple mass spectrometer operated in positive electrospray ionization mode (collision gas flow rate: 11 L/min, source block temperature: 300°C). Samples (20 µL) were injected into a C18 column (4.6×100 mm, 3.5 µm, Agilent) at 37°C using a gradient elution method (Water and ACN mix) at a flow rate of 0.65 mL/min. PHA-408 was quantified by multiple reaction monitoring (Q1/Q3, 560.246/514.113) with a 51V collision energy. LC-MS
analyses on ACN extracted tissues were performed by Dr. Burcin Yavuz at Tufts University. High levels of background signal were detected in lymph nodes of vehicle injected mice, thus a noise floor was defined for lymph node tissues (mean+2*SD, vehicle injected mice).

3.2.8 *In vivo* luminescence imaging of NF-κB activity

To assess local NF-κB activation following joint injury, *in vivo* bioluminescent imaging was performed as described in Chapter 2, Section 2.2.2.

3.2.9 Pain-related hindpaw sensitivity

The effects of local NF-κB inhibition on the development of mechanical allodynia following joint injury were using von Frey filaments as described in Chapter 2, Section 2.2.3.

3.2.10 Pain-related hyperalgesia

The threshold for contact hyperalgesia was tested at the knee joint using a small animal algometer as described in Chapter 2, Section 2.2.4.

3.2.11 Hindpaw thermal sensitivity

Hindpaw thermal sensitivity to hot and cold stimuli were tested using methods previously described in Chapter 2, Section 2.2.5.

3.2.12 Static weight bearing

Alterations to static weight bearing were assessed using methods described in Chapter 2, Section 2.2.6.
3.2.13 Spontaneous activity

Freely selected activity during the animal dark cycle was tracked as previously described in Chapter 2, Section 2.2.7.

3.2.14 Histology

Following sacrifice (113 days post-injury), ipsilateral knees were collected for histology and processed as described in Chapter 2, Section 2.2.9. Stained sections were graded to consensus by two blinded graders using OARSI scoring\textsuperscript{227}.

3.2.15 Statistical analysis

Statistical analyses were performed using GraphPad Prism 8.0 (GraphPad Software, La Jolla, CA). A one-way ANOVA with Dunnett’s post-hoc was performed to test for differences in $\Delta\Delta C_i$ values in SW982 cells, all comparisons were made to IL-1$\beta$ only treated controls. A repeated measures two-way ANOVA with Bonferroni’s post-hoc was used to test for differences in Hoechst$^+$ and PI$^+$ cell counts between CTRL, INJ, and INJ/Tx femoral condyles. A repeated measures two-way ANOVA with Bonferroni’s post-hoc was used to test for differences between INJ and INJ/Tx groups at each timepoint for all longitudinal measures. \textit{In vivo} imaging and 50% withdrawal threshold data were log-transformed (base 10) to better meet ANOVA assumptions. A repeated measures two-way ANOVA with Bonferroni’s post-hoc was used to test for differences in cartilage OARSI scores at each cartilage site between INJ and INJ/Tx groups. Statistical significance was set at $p < 0.05$. 
3.3 Results

3.3.1 PHA-408 effectively reduced IL-1β induced inflammatory signaling in vitro

The small molecule inhibitor of IKK-2, PHA-408, reduced inflammatory signaling following co-stimulation with IL-1β in vitro. Shortly after stimulation with the inflammatory mediator IL-1β, PHA-408 treated cells showed a reduction in transcriptional levels of the inflammatory and immunomodulatory genes TNF-α, IL-6, and IL-8 (Fig. 3.5A-C).

3.3.2 Characterization of an NF-κB reporter cell line

Transduced SW982 cells expressed GFP in a viral titer dependent manner (Fig. 3.6A). Furthermore, transduction was stable following multiple passages (Fig. 3.6B) and IL-1β stimulated NF-κB-driven luminescence showed a dose (Fig. 3.6C) and time (Appendix B, Fig. B.2) dependent decrease with PHA-408 mediated NF-κB inhibition.
3.3.3 Local delivery of PHA-408 exhibits chondroprotective effects shortly following joint injury

Shortly following joint injury an increase in PI staining was observed in fluorescent images of the MFC (Fig. 3.7A). Cell numbers in the mid-contact region of femoral condyle articular cartilage, as assessed by Hoechst+ staining, were not different between groups (Fig. 3.7B). Injured knee joints had an increased number of PI+ cells within the MFC when compared to CTRL; however, PI+ cells were reduced in INJ/Tx MFCs when compared to untreated INJ MFCs (Fig. 3.7C).

Figure 3.6: Characterization of NF-κB luciferase reporter cell line. SW982 cells were transduced with an NF-κB responsive lentivirus at varying viral concentrations. (A) Increasing viral concentration led to increased levels of GFP+ cells when assessed via fluorescence microscopy (top) and flow cytometry (bottom). (B) Cells remained stably transduced with passaging (1 µL virus, green box represents mean±SD at P0). (C) NF-κB-driven luminescent shows a dose dependent response with increasing PHA-408 concentration when co-stimulated with IL-1β (1 µL virus, 4.3 MOI).
Figure 3.7: Short-term chondroprotective effect of PHA-408 following loading. (A) Representative maximum intensity projected images of the medial femoral condyle (MFC) from each group. (B) No differences in lateral femoral condyle (LFC) or MFC cell number were observed 48 h post joint injury. (C) INJ led to an increase in PI⁺ cells in the MFC, however, INJ/Tx MFC PI⁺ cell count was reduced when compared to INJ only. Scale bar = 100 µm, compass rose: L=lateral, A=anterior, M=medial, P=posterior. Repeated measures two-way ANOVA w/ Bonferroni's post-hoc; **=p<0.01, ****=p<0.0001 vs CTRL; #=p<0.05 INJ vs INJ/Tx.
3.3.4 Characterization of PEG-PLGA and SF microparticles and in vitro drug release

PEG-PLGA microparticles had an average diameter of 11.9±7.6 µm (Fig. 3.8A,C) and a 37% drug loading efficiency (final drug loading ratio 1:27, drug:microparticle). PEG-PLGA microparticles exhibited sustained release characteristics with limited burst release in vitro, their estimated drug release half-life was 19.7±3.2 days (Fig. 3.8D). Furthermore, 100% of loaded drug was eventually released from PEG-PLGA microparticles into PBS release buffer as particles degraded.

SF microparticles had an average diameter of 13.4±7.1 µm (Fig. 3.8B,C) and drug loading efficiencies of greater than 99% across all formulations (final drug loading ratio 1:10, drug:microparticle). SF microparticles showed the potential for sustained release for over two months in vitro; however, only ~10% of loaded PHA-408 was released from SF microparticles into PBS release buffer in all formulations (Fig. 3.8D). The average SF microparticle drug release half-life was 16.2±2.4 days.
PHA-408 released from both PEG-PLGA and SF microparticles remained bioactive and effectively inhibited IL-1β induced NF-κB signaling in a reporter cell line (Fig. 3.8E). NF-κB inhibition from microparticle conditioned media was similar to inhibitory levels observed with 10 µM concentration of free drug.

Figure 3.8: Characterization of PEG-PLGA and SF microparticles. Representative bright field (A) and SEM (B) images of PEG-PLGA and SF microparticles, respectively. (C) Size distribution of PEG-PLGA and SF drug loaded microparticles. All particle formulations showed continuous release of PHA-408 for 80 days (D) and PHA-408 released from microparticles showed sustained inhibition of IL-1β induced NF-κB activation in SW982 reporter cells (E), inhibition was near levels seen with 10 µM of free drug. Scale bar = 100 µm.
3.3.5 Local delivery of PHA-408 following joint injury has modest effects on NF-κB activity and animal behavior

Locally delivery of PHA-408 following joint injury did not significantly alter the transient rise of NF-κB-driven luminescence within injured knees; however, NF-κB activity was elevated in INJ/Tx joints 14 days following knee joint injury when compared to INJ untreated joints (Fig. 3.9A,B). The development of allodynia shortly following injury was similar in INJ and INJ/Tx groups (Fig. 3.9C). Withdrawal thresholds in the INJ/Tx group

![Figure 3.9: Intra-articular injection of PHA-408 follow joint injury has modest effects on NF-κB activity and animal behavior following knee joint injury. (A and B) NF-κB activity in drug treated joints was elevated two weeks following joint injury. INJ and INJ/Tx animals showed similar 50% withdrawal (C) and escape (D) thresholds. (E) INJ/Tx mice had a more rapid recovery towards uniform static weight bearing. Repeated measures two-way ANOVA w/ Bonferroni’s post-hoc; ***=p<0.001, ****=p<0.0001.](image-url)
did show a trend towards recovery at later timepoints, however, these were not significantly different from INJ 50% withdrawal thresholds (99/113 d, p=0.09, INJ v INJ/Tx). INJ and INJ/Tx groups had similar thresholds to noxious mechanical stimuli at the injured knee joint (Fig. 3.9D). Drug treatment improved static weight bearing in the INJ/Tx group 8 days following injury when compared to the INJ, untreated, group (Fig. 3.9E).

Alterations to free ambulation following joint injury were similar in INJ and INJ/Tx groups (Fig. 3.10A-C). On average, distance traveled in both groups remained below baseline for 8 weeks following knee joint injury.

![Figure 3.10: Treatment does not modify freely selected ambulation.](image)

Figure 3.10: Treatment does not modify freely selected ambulation. (A) Animal matched centroid traces at baseline and 2 days following knee joint injury. INJ/Tx animals had similar alterations to their change in distance traveled (B) and percent time stationary (C) when compared to the INJ group. Mean±SEM, repeated measures two-way ANOVA w/ Bonferroni’s post-hoc.

No alterations to thermal sensitivity at the ipsilateral hindpaw were observed with the presentation of a hot stimulus between INJ and INJ/Tx groups (Fig. 3.11A). Interestingly, animals in the INJ group had reduced latencies to withdrawal from a cold thermal stimulus, however, this was prior to knee joint injury and subsequent drug treatment (Fig. 3.11B).

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3.3.6 Drug treatment does not improve long-term articular cartilage outcomes

Drug treatment was not effective in modifying the long-term degeneration of articular surfaces in the joint (Fig. 3.12A,B). Similar OARSI scores were observed in INJ and INJ/Tx joints 16 weeks following knee joint injury (Fig. 3.12C). A trend of increased degeneration in the medial joint compartment was consistent between groups.

Figure 3.12: Drug treatment does not influence long-term degenerative changes to articular cartilage following injury. Representative histology of INJ (A) and INJ/Tx (B) knees. (C) OARSI scores showed similar levels of degeneration to articular surfaces of INJ and INJ/Tx knee joints in both the lateral and medial compartments of the joint 16 weeks following joint injury. Repeated measures two-way ANOVA w/ Bonferroni’s post-hoc.

3.3.7 PHA-408 is rapidly cleared from the knee joint following intra-articular administration

Due to the limited efficacy of locally delivered free PHA-408 following joint injury, we hypothesized short joint residence times of the low molecular weight drug might be
contributing to the observed outcomes. Following intra-articular injection, low molecular weight drugs are rapidly cleared from the joint via the circulatory and lymphatic systems. Despite suspension based PHA-408 injections (Appendix B, Fig. B.3), drug was rapidly cleared from the joint space to draining lymph nodes (Fig. 3.13A). PHA-408 was detected within injected knee joints for 72 h following the first intra-articular injection (Fig. 3.13B). The estimated intra-articular half-life for PHA-408 was 41.2 min. Drug was not detected in knee joints one-week post-injection. Drug levels above the lower detection limit (Appendix B, Fig. B.4) persisted in contralateral, uninjected, limbs for 2 h following

Figure 3.13: Drug biodistribution and clearance. (A) Schematic highlighting tissues removed for biodistribution and clearance assessments following intra-articular injection with PHA-408 (t=0 and 48 h). Drug was rapidly cleared from (B) knee tissue, as well as (C) inguinal and (D) lumbar lymph nodes, (E) serum, and (F) urine following repeated intra-articular injections at t=0 h and t=48 h. Solid line=lower limit of detection, dashed line=noise floor.
injection. Average drug concentrations within injected limbs were 17.8 µM and 4.0 µM at 30 min and 2 h timepoints, respectively. Drug levels in major draining lymph nodes, serum, and urine all exhibited a rapid reduction in drug concentration with time following intra-articular injection (Fig. 3.13C-F).

3.3.8 Sustained release of PHA-408 does not overtly improve measured outcomes over freely administered drug

Given the rapid clearance of suspension based PHA-408, we hypothesized local delivery of sustained release PHA-408 loaded PEG-PLGA microparticles may elicit protective effects against joint injury-induced NF-κB signaling and pain-related behavioral changes shortly following injury. A trend of increased NF-κB-driven luminescence was observed in knees of INJ/Veh, INJ/Tx, and INJ/TxMP treated groups as compared to baseline (Fig. 3.14A). Trends in 50% withdrawal thresholds were similar in INJ/Veh, INJ/Tx, and INJ/TxMP groups (Fig. 3.14B). Thus, treatment with free drug or a sustained release preparation did not overtly resolve pain-related sensitivity or inhibit an increase in NF-κB activity shortly following joint injury. Furthermore, a sustained release SF microparticle formulation did not prolong drug residence time within the knee joint following intra-articular administration (Appendix B, Fig. B.5)

Figure 3.14: PEG-PLGA microparticles do not overtly improve early PTOA related outcomes. One-time drug treatment in soluble or MP formulation did not alter the onset of NF-κB signaling in the knee joint (A), or the development of allodynia (B) following knee joint injury.
3.4 Discussion

Work in this chapter described the effects of locally delivered PHA-408 following knee joint injury. Data suggested treatment with PHA-408 acutely protected chondrocytes from injury initiated cell death. Despite these observed early effects on articular cartilage, PHA-408 only modestly influenced the development of pain-sensitivity related behaviors following knee joint injury. Furthermore, PHA-408 did not prevent the long-term histological development of PTOA. Finally, we showed PHA-408 loaded PEG-PLGA and SF microparticles provided sustained release of bioactive drug; however, local delivery of drug-loaded PEG-PLGA and SF microparticles, respectively, did not alter imaging/sensitivity outcomes or improve drug residence times within the joint following injury. These results highlight the challenges of translating promising small molecule drug candidates from in vitro development to animal models of disease.

Shortly following knee joint injury we observed increased chondrocyte membrane permeability within the MFC of loaded knee joints. In comparison, changes within the LFC were minimal. These early differences in medial/lateral chondrocyte health aligned with histologic articular cartilage outcomes assessed 16 weeks following injury which showed increased cartilage degeneration within the medial joint compartment. We hypothesize acute delivery of PHA-408 following injurious loading may have reduced chondrocyte necroapoptosis mediated cell death\textsuperscript{72,271–273}. Indeed, others have shown similar chondroprotective effects of NF-κB inhibition following joint injury\textsuperscript{67,68,77,164,274}. Nevertheless, these acute protective effects were inconsequential to long term joint health. This may be in part due to the severity and chronic destabilizing nature of this injury model\textsuperscript{35,43,70,275,276}.
Data described in Chapter 2, and prior work by Yan and colleagues, informed the PHA-408 dosing strategy used in this chapter. Yan and colleagues showed cyclical loading of the murine knee joint, albeit at a lower force amplitude of 6 N, led to robust phosphorylation of p65 in chondrocytes within 12 h of loading, with additional P-p65 observed in synovium 48 h following knee joint injury. In Chapter 2 we showed longitudinal NF-κB-driven luminescent signal rapidly increased in a similar timeframe following injurious loading. Signal peaked 3 days following knee joint injury and quickly returned towards baseline by day 7, where it stayed for the remainder of the study. Given these pathway kinetics, it seemed drug treatment immediately following injury may elicit favorable disease-modifying outcomes. Indeed, Yan and colleagues further showed two time (0 & 48 h post-injury) intra-articular administration of NF-κB inhibiting siRNA nanoparticles following knee joint injury modestly reduced chondrocyte expression of P-p65, chondrocyte apoptosis, and cartilage lesion size; locally delivered siRNA also entirely prevented the development of knee joint hyperalgesia. As eluded to previously, the contrasting results in observed outcomes between these studies may be explained by the severity of loading induced knee joint injury. While 12 N cyclic loading of the knee joint consistently leads to ligamentous rupture and subsequent joint destabilization, 6 N loading has not been reported to induce such changes. These significant and long-lasting changes to joint biomechanics following 12 N injury may warrant an increase in drug dosing frequency and duration. To date, the long-term efficacy of drug treatment following the 12 N injury model used in this study has remained elusive. However, preliminary dosing studies we performed in the MIA model of arthritis suggested acute delivery of PHA-408 may protect articular cartilage from MIA
induced changes. Together these data highlight the potential utility of infrequent/acute NF-κB inhibition in the absence of a persistent destabilizing injury and suggest alternative/additional dosing timepoints may be required following traumatic joint injuries that chronically alter joint biomechanics\textsuperscript{77,164,278}.

Given the chronic destabilizing nature of the injury model used in this study and the observed clearance kinetics of free PHA-408 from the knee joint, we hypothesized a sustained release drug depot might help to prolong intra-articular drug residence times and improve measured outcomes described in this chapter. Large polymeric micron-sized drug carriers have long been shown to have increased residence times when compared to freely administered solutes\textsuperscript{168,172,173,184}. Additionally, both PEG-PLGA and SF microparticles have been used for the sustained release of various low molecular weight hydrophobic drugs making them ideal carriers for the sustained release of PHA-408\textsuperscript{179,183,279,280}. Indeed, \textit{in vitro} studies showed both microparticle formulations facilitated the sustained release of PHA-408 and released drug showed effective inhibition of inflammatory mediator induced NF-κB signaling. However, preliminary results following the local delivery of drug loaded PEG-PLGA microparticles did not show an overt impact on measured outcomes shortly following knee joint injury. Furthermore, drug levels within the joint were below the lower detectable limit (1.99 nM) of our assay following local delivery of drug loaded SF microparticles. This was despite a more than 15-fold increase in total delivered/encapsulated drug. Given these factors, the long-term effects of PHA-408 laden sustained release depots were not studied.

Acute treatment with PHA-408 led to increased NF-κB-driven luminescence in INJ/Tx mice; although this result was somewhat surprising, similar observations have
been previously reported following the administration of small molecule NF-κB inhibitors, such as bortezomib and BAY 11-7082, in inflammatory disease states\textsuperscript{82}. Given this, the acute effect of PHA-408 on NF-κB-driven luminescence was further explored in the absence of knee joint injury. Results showed intra-articular delivery of PHA-408 or vehicle control both led to slightly increased NF-κB-driven luminescence within injected knee joints when compared to contralateral knees (Appendix B, Fig. B.6), however, these levels were still far below those observed in INJ and INJ/Tx knees. The influence of high local drug concentrations may elicit cytotoxic effects which is a further concern following intra-articular injection (Appendix B, Fig. B.7). Nevertheless, these results highlight an inability to inhibit bulk knee joint NF-κB-driven luminescence following local administration of PHA-408. Based on this, and the rapid rise and subsequent fall of NF-κB-driven luminescence following injury, it seems possible NF-κB-driven luminescence at the whole joint level in this reporter mouse line is not entirely representative of signaling kinetics within relevant tissues such as articular cartilage and synovium. To this point Kobayashi and colleagues have reported increased NF-κB activation in articular chondrocytes 8 weeks following a knee joint destabilizing injury, this far exceeds the week long rise in signaling we observed in this study\textsuperscript{87}. Thus, NF-κB-driven luminescence in this mouse line may have limited sensitivity in measuring injury and drug treatment induced changes to knee joint tissues and is a limitation of this longitudinal study. Future cross-sectional studies, that rely on histological outcomes rather than longitudinal \textit{in vivo} imaging, may help to better dissect the spatiotemporal activation of NF-κB in specific joint tissues post-injury\textsuperscript{238}. Indeed, in a simplified \textit{ex vivo} organ culture model, we observed effective inhibition of NF-κB signaling with application of PHA-408 (Appendix B, Fig. B.8). In spite of the results reported in this
dissertation, positive structural disease-modifying effects observed in less severe PTOA models, such as the destabilization of the medial meniscus model, support the use of local NF-κB inhibition for the treatment of PTOA.

In this study intra-articular administration of PHA-408 led to modest alterations in pain-sensitivity related behaviors following knee joint injury. Interestingly, INJ/Tx mice showed a trend towards increased 50% withdrawal thresholds in their ipsilateral hindpaw at late timepoints within this study. This suggests acute treatment with PHA-408 following knee joint injury may have had some long-term effect on pain-sensitivity even following drug clearance from the joint. Others have shown similar peripheral sensitivity modulating effects of anti-inflammatory drugs following traumatic joint injury; however, little work has focused on the specific role of NF-κB inhibition in mediating pain-sensitivity related behaviors in PTOA animal models. Early delivery of NF-κB inhibitors has been shown to impart similar long-lasting effects in inflammatory and neuropathic pain models by modulating the local inflammatory environment at the site of injury and by influencing the expression of nociception related proteins, such as the voltage gated sodium channels NaV1.7 and NaV1.8, in tissue innervating sensory neurons. Recent work has also highlighted the putative role P-p65 plays in the direct gating of NaV1.7 activity in sensory neurons. These data suggest there is a role for careful modulation of NF-κB in the treatment of painful inflammatory conditions such as PTOA.

In general, the analgesic effects of disease-modifying OA drugs are poorly understood. While many pre-clinical studies seek to characterize the structural influences of drug treatment, they often overlook drug related impacts on pain-related behaviors.
In some instances, however, the opposite has been true. For example, great promise had been placed on the clinical development of tanezumab, a humanized monoclonal NGF antibody, for the analgesic treatment of knee OA, however, its widespread adaptation has been limited due to long-term deleterious effects on articular cartilage and bone quality\textsuperscript{252,255,256}. Indeed, subsequently conducted animal studies have shown tanezumab may accelerate the onset of articular defects and osteophyte formation following joint injury. Presumably, degenerative changes are accelerated due to increased joint loading on the injured limb in the presence of the analgesic effects of tanezumab\textsuperscript{296,297}. With this in mind, future work should simultaneously aim to better understand and treat both the structural and symptomatic progression of OA.

In conclusion, the work described in this chapter showed the potential for PEG-PLGA and SF microparticles to act as sustained release drug depots for the small molecule NF-κB pathway inhibitor, PHA-408. Results also highlighted the short-term chondroprotective effects of local PHA-408 delivery and the modest behavioral modifying effects of these treatments.

![Figure 3.15: Traumatic joint injury triggers a pro-inflammatory/algesic cascade which contributes to the symptomatic progression of PTOA.](image-url) Following traumatic joint injury, local NF-κB activation contributes to the increased production and release of pro-inflammatory/algesic/catabolic molecules by the resident cells of the joint which contribute to erosive changes to joint structures. Increased intra-articular levels of these factors may induce nerve fiber sprouting and sensitization, in part, through NF-κB mediated signaling.
impact repeated drug treatment had on longitudinal pain-sensitivity related behavioral outcomes following joint injury. Drug treatment, however, did not influence the long-term structural development of PTOA in injured knee joints. While this study ultimately failed to show substantial long-term structural disease modification via local PHA-408 delivery, the results still provide valuable insight into the temporal development of symptomatic PTOA following knee joint injury. This work also highlights many of the hurdles which must be overcome for successful small-molecule intra-articular drug delivery. Functional assessments of pain-sensitivity related behaviors, such as those employed in this study, should continue to be utilized to better understand what factors contribute to the symptomatic progression of conditions such as OA. Hopefully, these approaches will help to inform the future development of efficacious treatment strategies that slow or halt the structural and symptomatic progression of musculoskeletal pathologies.

3.5 Acknowledgements

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Chapter 4

Electric field stimulation for the rapid functional assessment of alterations to intact dorsal root ganglion neuron excitability

4.1 Introduction

A diverse set of somatosensory neurons are required for physical perception. Sensations of touch, temperature, taste, and pain are all transmitted via sensory neurons, but the mechanisms by which these sensory stimuli are encoded and transmitted remain unclear. For this reason, there is great need to further interrogate the neurophysiologic basis of sensory neuron signaling. The functional assessment of cellular neurophysiology has traditionally relied on manual patch clamp based techniques. While this approach remains the gold standard for understanding the electrophysiological basis of ion channel dynamics and action potential propagation, these methods are limiting due to their relative technical difficulty and low throughput. When paired with calcium- or voltage-sensitive fluorescent indicators, optical imaging allows for the simultaneous capture and analysis of cellular signaling in diverse cell populations. Additionally, the development of genetically encoded calcium indicators (GECIs), such as GCaMP6s, allows for the spatiotemporal control of cell-type specific calcium indicator expression. In these
imaging-based approaches, calcium flux is used as a surrogate trailing measure of action potential propagation and can be influenced by a wide variety of ion channels (Fig. 4.1). Nevertheless, GECIs have given investigators the freedom to reliably study \textit{in vivo} and \textit{in vitro} neural signaling dynamics without the need for invasive patch clamping or exogenous dye loading\textsuperscript{199}.

Following acute isolation of dorsal root ganglion (DRG) tissues by axotomy, \textit{in vitro} techniques typically require DRG dissociation by enzymatic digestion and mechanical disruption. Prior work has shown even short-term incubation of neural cells with low-concentration enzymatic agents, such as trypsin or papain, may reduce cell viability and the expression of structurally and functionally relevant proteins\textsuperscript{299}. Furthermore, this physical disruption of native tissue architecture alters the intrinsic cell-cell and cell-matrix interactions that are present \textit{in vivo}; indeed, these interactions have been shown to

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**Figure 4.1: Sensory neuron classifications and their ion channels.** Sensory neurons exit the dorsal horn of the spinal cord, through the dorsal root (DR). Cell soma reside in the dorsal root ganglion (DRG) and can be generally classified into four sub-categories by size, conduction velocity, and protein/ion channel expression. C-fibers are small diameter, peptidergic or non-peptidergic neurons which are primarily implicated in the sensing of noxious stimuli. Lightly myelinated Aδ fibers are also implicated in sensitivity to noxious thermal, mechanical, and chemical stimuli. Larger Aα and Aβ myelinated fibers are primarily implicated in proprioception and non-noxious mechanosensation. All neuron subtypes express a variety of ion channels (TRP, NaV, KV, CaV, etc.) which are essential for appropriate action potential initiation and transmission, alterations in cellular expression of these ion channels can alter functional excitability.
influence neuronal excitability as well as gene and protein expression\textsuperscript{194,300,301}. Long-term monolayer culture of DRG neurons also often requires the exogenous administration of growth factors, such as nerve growth factor and glial cell line-derived neurotrophic factor, but these have been shown to directly impact cell phenotype\textsuperscript{302–304}. Extended culture periods following isolation have also been shown to alter cell-cell interactions through satellite cell proliferation; while anti-mitotic drugs may be administered \textit{in vitro} to prevent this proliferation, their use negatively impacts sensory neuron structure and function\textsuperscript{305,306}. These factors, amongst others, may influence neural cell responses \textit{in vitro}. Hence, there is a need to develop alternative \textit{in vitro} methods for assessing sensory neuron excitability in acutely isolated DRGs.

The work in this chapter describes the development of an electric field stimulation mediated cellular imaging method for the direct interrogation of Ca\textsuperscript{2+} signaling dynamics in intact acutely isolated DRG tissues. This method allows for the rapid and simultaneous characterization of functional excitability in hundreds of sensory neurons. Using this technique, we show an ability to measure dose-dependent changes to electric field stimulated Ca\textsuperscript{2+} signaling in the presence of a voltage-gated sodium channel inhibitor and an ability to detect differences in neuronal excitability in a peripheral nerve injury model. Taken together, the described system shows potential for the study of pharmacologically and injury-induced alterations to large and heterogeneous neural populations without the need for dramatic perturbation to the DRG neurons’ native microenvironment.
4.2 Methods

4.2.1 Animal model

All procedures were performed with approval of the Washington University in St Louis Institutional Animal Care and Use Committee. Transgenic Advillin-CreERT2 (Cre/+, Stock No: 032027, The Jackson Laboratory, Bar Harbor, ME) mice were crossed with STOP-GCaMP6s (+/-, Stock No: 028866, The Jackson Laboratory) mice. Cre-positive mice received three consecutive doses of tamoxifen (i.p, daily, 75 mg/kg, Sigma-Aldrich, St Louis, MO) to induce GCaMP6s expression in Advillin expressing neurons (Fig. 4.2A,B). GCaMP6s was chosen for its high signal to noise ratio and slower temporal dynamics which enable its use in widefield, spinning-disk confocal, imaging.

Figure 4.2: Experimental design. (A) Sensory neuron specific expression of GCaMP6s was attained by crossing STOP-GCaMP6s (+/-) and Advillin-CreERT2 (Cre/-) mice. (B) Robust GCaMP expression in DRG neurons was observed 3 weeks following tamoxifen (tam, 75 mg/kg, IP, 3X) dosing. (C) Intact DRGs were placed within an EFS recording chamber beneath a nylon mesh and were continuously perfused with fresh artificial cerebrospinal fluid (aCSF, 2 mL/min). (D) Representative cellular traces of the normalized GCaMP fluorescence ($\Delta F/F$) and corresponding images (E), with increasing voltage amplitude (5-100V). The inset depicts the applied excitatory pulse train that was used at each voltage (10 Hz, 1 ms pulse width); frames corresponding to each numbered peak are shown with color-coded cellular regions of interest.
Three weeks following tamoxifen dosing mice were utilized for electric field stimulation experiments. Mice were maintained on a 12-h light-dark cycle with access to food and water ad libitum.

4.2.2 Dorsal root ganglion (DRG) isolation

Mice were deeply anesthetized under isoflurane (3% inhalation, 2 L/min oxygen, n=6, 16-20 wks). A transcardial perfusion was performed with artificial cerebrospinal fluid (aCSF, 124 mM NaCl, 5 mM KCl, 20 mM HEPES, 10 mM d-glucose, 1.3 mM MgCl$_2$, 1.5 mM CaCl$_2$, 26 mM NaHCO$_3$, 1.25 mM NaH$_2$PO$_4$) after which DRGs (T13-L5) were isolated via a dorsal approach. Following a midline dorsal incision, paraspinal musculature, connective tissue, and fat were carefully removed with micro-dissection scissors to reveal the dorsal bony elements of the spine. Micro-rongeurs were then used to perform a multi-level laminectomy to expose the spinal cord, which was gently repositioned to expose the DRGs at each spinal level. Fine forceps were used to grasp the distal nerve root and gently remove the DRG from the neuroforamen, and spring scissors were used to carefully cut the nerve roots proximal and distal to the DRG. Isolated DRGs were then incubated in aCSF and used for EFS experiments within 8 h of isolation.

4.2.3 Electric field stimulation (EFS)

Intact DRGs were placed in the center of a recording chamber (RC-RC49MFSH, Warner Instruments, Hamden, CT), secured by a nylon mesh, and perfused with aCSF (2 mL/min) (Fig. 4.2C). Two parallel, linear platinum electrodes connected to a pulse generator (Grass SD9, Natus Neuro, Warwick, RI) were inserted at opposite ends of the recording chamber and DRGs were stimulated with bipolar square pulse waves (1 ms, 10
Hz, 1 sec on/ 29 sec off) at voltage amplitudes of 5-100V (Fig. 4.2D); these EFS parameters were chosen for their ability to evoke robust evoked Ca2+ transients within intact DRGs as have been reported previously\textsuperscript{190,309–311}. The geometry of the recording chamber ensured the generation of a uniform electric field between electrodes\textsuperscript{190}. Intact DRGs were imaged (Plan Apo λ 10X, 0.45 NA) to collect GCaMP6s fluorescence (488/525 nm, ex/em, 10 Hz) upon a spinning disk confocal microscope (Ti2-CSU-X1, Nikon Instruments, JPN) (Fig. 4.2E). Time series recordings at a single imaging plane (~20 µm from tissue basal surface, 2.5 µm z-thickness) were acquired. Single recordings were completed in 6 min; DRGs were allowed to recover within the recording chamber for 5 min followed by a repeated exposure to EFS in order to evaluate the repeatability of response and to confirm viability of tested neurons.

4.2.4 Tetrodotoxin inhibition

To test the ability of the described EFS imaging technique to be used for drug screening in intact DRGs, the potent voltage-gated sodium channel (VGSC) inhibitor tetrodotoxin (TTX, Tocris, Minneapolis, MN) was used as described here\textsuperscript{312}. DRGs underwent one voltage sweep (5-100 V) as described, followed by incubation with increasing concentrations of TTX (5 min incubation, 0.01-10 µM), and a repeat of the EFS voltage sweep protocol was performed.

4.2.5 Peripheral nerve injury

Peripheral nerve injury was induced in female Advillin-GCaMP6s mice (16 wks, n=2, [Cre/+,-/], n=2 [+/-,+/+-]) as described\textsuperscript{313,314}. Mice were anesthetized under isoflurane (1-3% inhalation, 2 L/min oxygen), and the left hindlimb was prepped with sterile technique. A 1 cm skin incision parallel to the femur was made at the mid-thigh to
expose the muscle fascia and blunt dissection was used to expose the sciatic nerve below the biceps femoris (Fig. 4.3A,B). The sciatic nerve was then tightly ligated with three ligatures (6-0 chromic gut suture), with 1 mm spacing, proximal to the sciatic (Fig. 4.3C). Muscle, fascia, and subcutaneous tissues were closed with 5-0 vicryl suture and skin closed was closed with 4-0 nylon suture. Following recovery from anesthesia animals were returned to their home cage (≤5 mice/cage) and allowed to freely ambulate. Following nerve injury, mice were aged one month prior to use in EFS experiments which were performed on L3-L5 DRG.

4.2.6 Hindpaw mechanical sensitivity

Hindpaw sensitivity to light-touch was evaluated bilaterally using von Frey filaments as described in section 2.2.3.

4.2.7 Hindpaw thermal sensitivity

Hindpaw sensitivity to a hot stimulus (55°C) was tested using methods previously described in section 2.2.5.
4.2.8 Data analysis

Following stimulation of DRGs in the EFS chamber, digital images of DRG responses were compiled into high-speed video format. Within a single frame corresponding to the period preceding EFS stimulation, manual tracing of all visible cell bodies was performed in ImageJ (NIH, Bethesda, MD). Fluorescent intensity values within each cell region of interest (ROI) over time were exported and analyzed using a custom MATLAB (MathWorks, Natick, MA) script. Briefly, fluorescent signal prior to each EFS stimulation was used to calculate baseline fluorescence within each cell ROI ($F_0$). This baseline value was used to calculate $\Delta F/F_0$, where $\Delta F$ is the rise in fluorescence due to EFS at each stimulation voltage. A piecewise cubic interpolating polynomial was used to correct for drift in baseline fluorescence with time prior to each stimulus using the local minimum ($F_0$) within each ROI prior to EFS. A peak-detecting algorithm was used to detect calcium responses following each voltage stimulus, and the threshold for a positive response was set to 20% of an individual neuron’s maximum $\Delta F/F_0$ peak height. This on-off binarization of sensory neuron Ca2+ signaling, when assessed by GCaMP fluorescence, has been previously reported by Hartung and Gold. For each DRG, the percent of responding neurons at each voltage amplitude was calculated by normalizing the number of responding neurons at each amplitude by the highest number responding across all amplitudes.
4.2.9 Statistical analysis

All statistical analyses were performed in Prism 8 (GraphPad, San Diego, CA). A repeated measures two-way ANOVA with Bonferroni post-hoc was used to test for differences in percent responding cells at each voltage between trials in repeatability experiments. A constrained sigmoidal fit was performed on population response data to determine a half-maximal response voltage (EV<sub>50</sub>) and area under the curve (AUC) was calculated. A paired student t-test was used to compare EV<sub>50</sub> and AUC values between trials in reproducibility experiments. To test for an effect of TTX dose on cellular responses, a repeated measures one-way ANOVA with Dunnett’s post-hoc was used to compare EV<sub>50</sub> and AUC values at each TTX dose to the 0 µM untreated control. A repeated measures two-way ANOVA with Bonferroni post-hoc was used to test for differences in behavioral outcomes at each timepoint. To test for differences in EV<sub>50</sub> and AUC values amongst ipsilateral and contralateral DRGs following peripheral nerve injury an unpaired student t-test was performed. To investigate the influence of cell size on EFS responsiveness following TTX treatment, neurons were sorted by soma diameter into three groups (diameter < 20 µm, 20 µm < diameter < 25 µm, diameter > 25 µm) and a repeated measures two-way ANOVA was used to compare EV<sub>50</sub> values between conditions. Relationships between 50% withdrawal threshold in hindpaw sensitivity trials collected prior to sacrifice and AUC were tested via linear regression followed by an F-test. Statistical significance was set at p < 0.05.
4.3 Results

4.3.1 EFS responses are reproducible

In DRGs harvested from Advillin-GCaMP6s mice, we identified on average more than 200 individual GCaMP expressing soma within each imaging field (221±64). Following EFS, an increase in GCaMP fluorescence intensity was transiently observed (Fig. 4.4A), individual cellular responses at each voltage amplitude were well conserved between trials (Fig. 4.4B). Neurons were largely quiescent at EFS amplitudes below 20 V, with only 2.4 and 22.3% of cells responding at EFS voltage amplitudes of 5 and 10 V on average, respectively (Fig. 4.4C). Within reproducibility validation experiments, there were no detectable differences in percent responsive cells at each voltage in sequential EFS trials (Voltage p<0.0001, Trial p=0.201, n=13 DRGs with n≥113 cells per DRG). Based on observed voltage-response behaviors, data were fit with a sigmoidal function on logarithmically transformed voltage amplitudes, and subsequent voltage-response data are presented as linear-log plots. Furthermore, there were no detectable differences between EV_{50} (p=0.923) or AUC (p=0.252) values between individual trials (Fig. 4.4D,E). EV_{50} values were on average 17.2 and 17.1 V in first and second EFS trials, respectively.
Figure 4.4: EFS produces similar responses in serially stimulated intact DRG neurons.

(A) Neurons within intact DRG tissues showed increased GCaMP6s fluorescence shortly following EFS.

(B) Representative traces of an individual neuron in sequential EFS trials, all detected responses are marked by a closed or open circle in the first and second trials, respectively. The dotted line represents the peak height cutoff, which is 20% of the maximum peak height.

(C) In subsequent trials, DRG neurons responded similarly to voltage sweeps, and there were no differences in the percent of responding neurons at any voltage (Repeated measures two-way ANOVA; Voltage p<0.0001, Trial p=0.201, mean±SEM). No significant alterations were observed in EV50 (p=0.923) (D) or AUC (p=0.252) (E) values. Repeated measures two-way ANOVA w/ Bonferroni’s post-hoc to test for differences between trials at each voltage amplitude, paired student t-test to test for differences between trials in EV50 and AUC (n=13 DRG, with n ≥ 113 cells per tissue). Mean±SEM, sigmoidal fit ± 95% CI.
4.3.2 Small molecule sodium channel inhibition reduces EFS induced signaling

To test for an ability to use the described EFS imaging method as a drug screening platform, DRGs (n=5 with n≥138 cells per DRG) were treated with the voltage-gated sodium channel inhibitor, TTX. Following TTX treatment, DRG neurons showed a marked reduction in EFS evoked Ca2+ signaling (Fig. 4.5A,B). This reduced cellular responsiveness led to a rightward shift in the voltage-response curve with increasing concentrations of TTX (Fig. 4.5C). A dose-dependent increase in EV$_{50}$ was observed with increasing TTX dose (p<0.0001, Fig. 4.5D). Significantly increased EV$_{50}$ values were observed at 1 (p=0.0006) and 10 µM (p<0.0001) TTX doses compared to the untreated negative control. Prior to TTX treatment, the average EV$_{50}$ within DRGs was 13.4 V, with TTX treatment these values increased to 51.5 and 60.6 V at 1 and 10 µM TTX doses, respectively. A corresponding dose-dependent reduction in AUC values were observed with increasing TTX dose (p<0.0001); significant reductions in AUC were observed with
1 (p=0.0003) and 10 µM (p<0.0001) TTX doses when compared to the untreated negative control (Fig. 4.5E). TTX-sensitive voltage-gated ion channels are expressed robustly on soma of differing size and sensory-modality; to investigate the effect of TTX inhibition across these neural sub-populations soma were stratified by cellular diameter. Soma size (p=0.924) did not influence EV$_{50}$ values with TTX inhibition (p=0.007) (Appendix C, Fig. C.1).

Figure 4.5: Tetrodotoxin inhibits EFS mediated Ca$^{2+}$ signaling in intact DRG neurons. (A) Post EFS (100 V) GCaMP6s fluorescence dynamics were noticeably reduced with tetrodotoxin (TTX) treatment, and average $\Delta F/F$ (B) values across all cellular regions of interest (n=163 for displayed representative traces) within DRG tissues had reduced amplitude with TTX treatment. Data from one representative DRG tissue is shown. (C) TTX incubation led to a dose dependent decrease in EFS mediated cellular response (mean±SEM). (D) An increase in EV$_{50}$ was observed at 1 (p=0.0006) and 10 µM (p<0.0001) TTX doses while AUC (E) decreased at 1 (p=0.0003) and 10 µM (p<0.0001) TTX doses when compared to untreated controls. Repeated measures one-way ANOVA w/ Dunnett’s post-hoc was used to test for differences between individual TTX doses and the untreated 0 µM control; **=p<0.01, ****=p<0.0001. Dotted line represents 0 µM control, (n=5 DRG, with n ≥ 138 cells per DRG).
4.3.3 Peripheral nerve injury resulted in unilateral hypoesthesia

Surgically induced nerve injury resulted in clear alterations to animal behavior including impaired motor function upon ambulation in the injured hindlimb which began to improve 14 days following surgery. Following peripheral nerve injury, animals exhibited a decreased sensitivity to light touch in the ipsilateral hindpaw on days 7 (p<0.0001), 14 (p<0.0001), 21 (p=0.0011), and 28 (p<0.0005) when compared the contralateral hindpaw (Fig 4.6A). Furthermore, an increased latency to withdrawal from a hot thermal stimulus was observed at the ipsilateral hindpaw on days 7 (p=0.0149), 14 (p<0.0066), 21 (p<0.0001), and 28 (p=0.0022) when compared to the contralateral hindpaw (Fig 4.6B).

Figure 4.6: Surgically induced peripheral nerve injury reduces mechanical and thermal sensitivity at the injured hindpaw. (A) Reduced mechanical sensitivity in the ipsilateral hindpaw was observed 7 (p<0.0001), 14(p<0.0001), 21(p=0.0011), and 28 (p<0.0005) days following nerve injury when compared to contralateral hindpaws. (B) Increased latency to withdrawal from a hot thermal stimulus (55°C) at the ipsilateral hindpaw was observed 7 (p=0.0149), 14(p=0.0066), 21(p<0.0001), and 28 (p=0.0022) days post-injury when compared to the contralateral hindpaw. Repeated measures two-way ANOVA w/ Bonferroni’s post-hoc; *=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001.
4.3.4 EFS reveals increased voltage response threshold of ipsilateral DRGs following peripheral nerve injury

The excitatory behaviors of ipsilateral DRG neurons were altered following surgically induced peripheral nerve injury. A rightward shift in the voltage response curve was observed in ipsilateral DRGs (Fig. 4.7A). A corresponding increase to EV$_{50}$ (p=0.0106) and decrease in AUC (p=0.0224) was apparent in ipsilateral DRGs when compared to their level matched contralateral DRGs (Fig. 4.7B,C). Furthermore, reduced sensitivity at the hindpaw corresponded with decreased AUC values (Fig. 4.7D).

Figure 4.7: EFS reveals increased voltage response threshold in ipsilateral DRGs following peripheral nerve injury. (A) One month following nerve injury acutely isolated DRGs exhibited a rightward shift in their voltage response behavior. (B) Ipsilateral DRGs had an increased EV$_{50}$ (p=0.0106) and (C) decreased AUC (p=0.0224) values when compared to contralateral DRGs. (D) Decreased sensitivity at the hindpaw corresponded with reduced AUC values upon ex vivo assessment (p=0.048, F-test). Paired student t-test, *p<0.05. Mean±SEM, sigmoidal fit±95% CI.
4.4 Discussion

Here, we describe the development of a novel ex vivo imaging technique for the simultaneous visualization and functional assessment of hundreds of sensory neurons in intact DRG tissues. By utilizing genetic techniques to drive the robust expression of GCaMP6s in Advillin-expressing neurons, we showed an ability to reproducibly capture voltage-dependent EFS mediated signaling dynamics; furthermore, we showed an ability to measure pharmacologically induced alterations to functional signaling upon voltage mediated EFS. Finally, this method was shown to detect a change in ex vivo DRG responses consistent with pain-sensitivity related behavioral changes in a surgically induced nerve injury model. These results highlight the potential utility of the described method.

While enzymatic digestion and mechanical dissociation of DRG neurons following axotomy remains the gold standard preparation for in vitro experiments of isolated sensory neurons, alterations to cellular phenotype have been documented in neurons when plated in monolayer culture. In addition to obvious artifact introduced by cellular dissociation, the spatial organization of individual soma and axons within the DRG are lost with cellular isolation. Kim and colleagues have previously shown that with nerve injury, increased calcium signaling occurs in adjacent soma mediated in part through CX43-expressing glial cells, an observation that is lost in dissociated cultures due to disruption of neuron-glial cell interaction. Furthermore, the effects of dissociation may change with time in culture: glial cells have been shown to progressively separate from sensory neuron soma over a one week culture period. Extended culture periods often require the addition of exogenous factors, such as mitotic inhibitors or growth factors, to
prevent over-proliferation of satellite cells and improve viability in sensory neurons, respectively. Mitotic inhibitors have been shown to reduce antero- and retro-grade neuronal axonal transport in vitro and animal models have shown their systemic administration may result in chronic pain\textsuperscript{305,316}. The inclusion of supplemented growth factors in vitro increases basal intracellular production and extracellular release of calcitonin gene-related peptide upon stimulation. In vivo studies have also highlighted the potential for nerve growth factor to benefit the survival of small-diameter neurons, whereas the pro-survival effects of neurotrophin-3 have been shown in larger-diameter proprioceptive neurons\textsuperscript{317–319}. These factors may further contribute to a shift in cell population with time in prolonged sensory neuron monolayer culture.

Prior work has shown EFS can be utilized for the simultaneous and unbiased activation of action potentials in large numbers of sensory neurons\textsuperscript{309,320–322}. Fouillet and colleagues utilized EFS in the development of an in vitro drug screening platform and showed that with increasing doses of TTX, dissociated monolayer cultures of DRG sensory neurons exhibited reduced responses to voltage-mediated stimuli, similar to the results presented in this study\textsuperscript{309}. Others have shown the importance of both high-voltage-gated calcium channels and VGSCs in the generation of EFS mediated Ca\textsuperscript{2+} influx\textsuperscript{323}. Similar work has utilized micro-electrode arrays for the in vitro assessment of various pharmacologic modulators of neuronal activity measuring Ca\textsuperscript{2+} mobilization in response to voltage stimuli\textsuperscript{324}. We believe, however, the method shown in the current study provides important advantages over these prior techniques for its abilities to simultaneously assess firing rates in hundreds of cells while maintaining tissue integrity and single-cell firing resolution. Furthermore, the described method does not require
exogenous dye loading and instead relies on Cre-driven expression which reduces EFS experimental prep-times and ensures fluorescence cell-type-specificity and robust genetically encoded protein expression. This is in contrast to exogenous dye loading which relies on non-specific diffusion-based cellular uptake or micropipette injection\textsuperscript{197}.

Surgically induced peripheral nerve injury resulted in desensitization to mechanical and thermal stimuli at the injured hindpaw. These behavioral alterations following tight ligation were similar to those reported following either sciatic nerve crush or transection\textsuperscript{325–327}. Following peripheral nerve injury in this model, we also observed alterations in EFS mediated DRG neuron excitability. Others have noted reduced transcriptional and protein level expression of voltage-gated sodium channels Nav1.1, Nav1.6, Nav1.7, Nav1.8, and Nav1.9 in DRGs following peripheral nerve injury which may partly explain our findings for increased EV\textsubscript{50} in ipsilateral DRGs\textsuperscript{328–330}.

While the described method has some intrinsic benefits over standard dissociation based protocols, DRG isolation still requires axotomy, and as a result alterations to neuron excitability using this method are representative of somatic changes. While alterations within the soma are linked to changes at the site of peripheral sensory innervation, this method as described does not allow for the direct interrogation of fully intact sensory units. A further concern is that the electrical impedance of the isolated DRG may result in a heterogeneous electric field across the tissue. These differences, however, are expected to have little effect given the dimensions of individual cells in proportion to the DRG tissue. Additionally, observations in this study were consistent across neurons within DRG of varying size.
In conclusion, these data suggest an ability to use EFS in intact DRGs to simultaneously study the functional signaling dynamics in hundreds of neurons with single-cell resolution. Using the genetically encoded calcium indicator GCaMP6s together with standard spinning-disk confocal microscopy, we were able to assess alterations to EFS induced population responses of DRG neurons following pharmacologic modulation and injury. Taken together, these results suggest the described imaging approach possesses the sensitivity required for the functional assessment of DRG neuron excitability ex vivo.

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Chapter 5

Conclusions and Future Directions

5.1 Conclusions

This dissertation aimed to better understand how traumatic joint injury, and subsequent NF-κB activation influences the symptomatic progression of PTOA. In Chapter 2, data highlighted the transient alterations to whole joint NF-κB signaling shortly following non-invasive knee joint injury. Work also established the temporal presentation of various evoked and non-evoked pain-sensitivity related behaviors including the development of chronic allodynia as well as short term alterations to ambulation/gait. Severe joint degeneration was also observed four months following traumatic knee joint injury.

Given the observed changes to whole joint NF-κB signaling and pain-sensitivity related behaviors following joint injury, we hypothesized early and local NF-κB inhibition would attenuate symptomatic and structural disease progression. Within Chapter 3, work described the synthesis and characterization of SF and PEG-PLGA microparticles for the sustained release of the NF-κB inhibitor, PHA-408. Both microparticle formulations effectively entrapped PHA-408 and exhibited sustained drug release for over two months \textit{in vitro}. Drug released from microparticles was further shown to be bioactive and reduced inflammatory mediated signaling in an NF-κB luciferase reporter cell line. While intra-articular delivery of free PHA-408 provided a modest acute chondroprotective benefit, its local delivery had limited influence on the development of pain-sensitivity related behaviors, and did not reduce the extent of degenerative changes to articular cartilage at
late timepoints following knee joint injury. Furthermore, intra-articular delivery of sustained release formulations did not overtly impact early imaging or behavioral outcomes. Drug clearance studies showed PHA-408 was rapidly cleared from the knee joint following local delivery, this may explain the drugs limited influence on the outcomes measured in this dissertation.

Together, these data provide valuable insight into the symptomatic progression of PTOA in a non-invasive murine model. Specifically, this work serves to provide a baseline set of pain-sensitivity related behavioral changes that future studies may further interrogate. It also establishes the utility of this model for the study of symptomatic PTOA given its clear behavioral phenotype and clinically relevant mechanism of disease initiation. Finally, this work highlights the challenges of intra-articular small molecule delivery in the disease-modifying treatment of PTOA.

Additional work in this dissertation sought to develop improved methods for the rapid and unbiased en bloc functional assessment of DRG neurons. Work in Chapter 4 established an Advillin-GCaMP6s mouse line which was used to establish ex vivo methods to assess sensory neuron excitability to electric field stimulation within intact DRG tissues. The described high-throughput technique was shown to reproducibly measure Ca2+ activity following electric field induced action potential generation. Data further showed this method could be used to detect cellular signaling alterations in response to peripheral nerve injury and pharmacologic ion channel inhibition. This technique may help researchers improve an understanding of neural signaling while retaining tissue structural organization. More generally, this approach serves as a new tool for the rapid and large-scale ex vivo recording and assessment of neural activity.
5.2 Future Directions

This dissertation provides additional understanding into the temporal progression of symptomatic disease in a non-invasive murine model of PTOA. Nevertheless, the mechanisms that contribute to both the acute and long-term development of pain-sensitivity related behaviors remain elusive. We hypothesized that chronic activation of NF-κB within the injured joint would contribute to persistent pain-sensitivity related behaviors. This, however, was not supported by the data in this dissertation. To more closely probe how longitudinal NF-κB activity within joint tissues affects the development of pain-sensitivity behaviors, cell-type specific transgenic approaches that modulate or sense NF-κB activation within particular joint tissues may be of benefit.67,331,332 Furthermore, studies targeted to investigate the symptomatic impacts of musculoskeletal injury should make use of non-evoked behavioral assessments that minimize animal handling and external stressors as these factors have been shown to influence outcomes.122,333

While local delivery of PHA-408 provided no long-term benefit to joint health within this dissertation, it remains unclear as to whether alternative dosing strategies would provide a therapeutic benefit given the rapid onset and severity of degenerative changes observed in this model.119 Additional studies are suggested to more thoroughly investigate the disease-modifying potential of PHA-408, and similar NF-κB inhibitors, in the context of PTOA. Such studies may benefit from using more slowly progressing PTOA models, or earlier endpoints in the described model, paired with more frequent drug dosing if their goal is to highlight drug efficacy.77,106,142,164,274,334
Although it is well established that NF-κB plays a prominent role in inflammation and associated pain-sensitivity, pharmacologic treatment strategies that target known upstream mediators of nociception (e.g. voltage-gated and mechanosensitive ion channels) may be of benefit in treating symptomatic PTOA\textsuperscript{14}. Indeed, the use of ion channel agonists such as capsaicin and resiniferatoxin have shown benefit in reducing OA pain\textsuperscript{335,336}. However, the pharmacologic modulation of other ion channels in symptomatic OA remains a promising and understudied avenue for the analgesic treatment of disease\textsuperscript{14,254,290,337,338}. As an extension of the work in this dissertation, DRGs were isolated from mice 4 months following knee joint injury and immunostained (Appendix B, Supplementary Methods) for the voltage-gated sodium channels NaV1.8; preliminary results suggested a marked increase in the pain-related ion channel when compared to DRGs isolated from age matched uninjured controls (Appendix B, Fig. B.9). Thus, local treatments that directly antagonize these channels may provide favorable symptomatic relief and should be further explored in the context of this PTOA model\textsuperscript{337,339}. Additional molecular and cellular profiling of the nervous system within this non-invasive model is also warranted to identify novel analgesic targets in PTOA. Future studies may utilize the novel \textit{ex vivo} functional and structural (Appendix D, Fig. D.1-D.4) imaging methods developed in this dissertation to interrogate DRG neuron sensitivity or morphometric changes to knee joint structure following injury, respectively. If paired with retrograde/antegrade labeling techniques, the described methods may also be used to assess functional sensitivity and structural organization of DRG neurons known to specifically innervate distal joint structures\textsuperscript{340,341}. Together these strategies may provide
for a more comprehensive understanding of how degenerative changes within the joint influence nociception and could lead to improved treatments for symptomatic PTOA.
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APPENDIX A

Supplementary methods and results from Chapter 2

Joint laxity testing

Murine cadavers (male, 9-12 weeks of age, n=10) underwent cyclic compressive loading (as described in Section 2.2.1) of a randomly assigned knee joint to induce ligamentous injury. Hindlimbs were then dissected and scored by three blinded investigators for anterior-posterior laxity (0=naive, 1=increased anterior-posterior laxity). Summed scores for each joint were calculated and CTRL and INJ joint scores were compared via a Mann Whitney test.

Correlation of longitudinal measures

To test for relationships between early NF-κB-driven luminescence and longitudinally measured behavioral outcomes Pearson correlation coefficients were calculated. In vivo imaging and 50% withdrawal threshold data were log-transformed (base 10). All data were then normalized by each animals' baseline values, represented as the difference from baseline, and the area under the curve was calculated in early (0 to 7/8 d) and late (15 to 113 d) study windows. Data were then z-scored and Pearson correlation coefficients were calculated. Early and late windows were chosen based on observed NF-κB luminescence temporal dynamics.

To test for relationships between OARSI scores and early NF-κB-driven luminescence as well as chronic behavioral changes, summed OARSI scores (LFC+LTP+MFC+MTP) were calculated. Area under the curve was calculated within a chronic (57 to 113 d) study window for baseline corrected behavioral measures. Data
were then z-scored and Pearson correlation coefficients were calculated. The chronic window used in this analysis was based on previous reports of cartilage degeneration within this model which have shown OARSI scores remain similar between 56 and 112 d following non-invasive mechanical injury\textsuperscript{120,277}. 
Figure A.1: Within groups thermal latencies are bilaterally similar. (A and B) Latency to withdrawal from a hot (50°C) stimulus at the hindpaw in CTRL (time p<0.0001, limb p=0.123, interaction p=0.989) and INJ (time p=0.0002, limb p=0.328, interaction p=0.228) groups. (C and D) Latency to withdrawal from a cold (0°C) stimulus at the hindpaw in CTRL (time p<0.0011, limb p=0.707, interaction p=0.889) and INJ (time p=0.0220, limb p=0.199, interaction p=0.493) groups. Mean±SD, repeated measures (both factors) two-way ANOVA.

Figure A.2: Measures of ambulation within short recording periods are highly variable. (A) Individual distance traveled traces within a 30 min recording period highlight the highly variable ambulatory behaviors within CTRL mice. Missing datapoint within INJ group due to animal death. (B) Animal distance traveled within 5 minute time bins decreased over the duration of the 30 minute open field assessment, representative data is shown from baseline assessments prior to injury, mean±SD.
Figure A.3: Relationships between early NF-κB and behavior. Early NF-κB luminescent scores were correlated with early and late alldynia withdrawal-scores (A) and late hyperalgesia withdrawal-scores (B). No significant correlations between early NF-κB luminescent-scores and weight bearing- (C), hot- (D), or cold-scores (E) were observed within early or late time windows. Pearson correlation coefficient, $r^2$, and p-values are indicated. Two datapoints in thermal correlations are missing due to instrumentation malfunction on day 0, thus baseline corrected values could not be calculated.

Figure A.4: Relationships between NF-κB, behavior, and histological outcomes. (A) Early NF-κB luminescent scores were correlated with terminal OARSI-scores. (B) OARSI-scores were correlated with chronic alldynia withdrawal-scores. (C) No significant correlations between terminal OARSI-scores and chronic hyperalgesia- (C), weight bearing- (D), hot- (E), or cold-scores (F) were observed. Pearson correlation coefficient, $r^2$, and p-values are indicated. Two datapoints in thermal correlations are missing due to instrumentation malfunction on day 0, thus baseline corrected values could not be calculated.
APPENDIX B

Supplementary methods and results from Chapter 3

Dorsal root ganglia immunostaining

At the terminal timepoint, 113 days following knee joint loading, animals were sacrificed by exsanguination and subsequently transcardially perfused with 4% PFA. Ipsilateral lumbar level 4 dorsal root ganglia (DRG) were then isolated, post-fixed (4 h, 4°C), cryoprotected in 30% sucrose (overnight, 4°C), flash-frozen in optimal cutting temperature compound, and cryosectioned (n=4 CTRL, n=3 INJ, n=3 INJ/Tx) at 7 µm. Slides with the largest number of cell bodies were selected from each DRG and underwent antigen retrieval (citrate buffer, 30 min, 90°C, pH 6.0), and permeabilization (0.2% Triton, 20 min). Samples were then blocked in 5% goat serum + 0.2% Triton (30 min) and subsequently stained in blocking buffer with primary antibody (rabbit anti-NaV1.8, 1:100, Alomone Labs, ASC-016, overnight, 4°C) or isotype (rabbit IgG) control. Samples were then washed (3X, PBS+ 0.1% Tween20), stained with secondary antibody (goat anti-rabbit Alexa 488, 1:300, Invitrogen, 1 h) in blocking buffer, washed (3X) again, and nuclei were counterstained with DRAQ5 (1:1000, 10 min). Samples were then coverslipped and imaged upon a Lecia DM6 confocal microscope at 20X (ACS APO 20x/0.60 IMM). Z-stacks (5 steps, 2.2 µm step-size) were taken of all DRG and maximum intensity projections were used for subsequent analysis. Image analysis was performed in ImageJ, all intact cell soma within each imaging field were manually traced (n=676 CTRL, n=367 INJ, n=311 INJ/Tx) and mean fluorescent intensity (MFI) and area were
calculated. MFI values were then compared between groups using a one-way ANOVA with Tukey’s post-hoc. Significance was set at p<0.05.
Figure B.1: Repeated acute intra-articular administration of PHA-408 reduces allodynia in the MIA injection model of arthritis. Following intra-articular injection of mono-iodoacetate (0.1 mg, MIA) repeated intra-articular delivery of PHA-408 (0.1 µg, 0 and 48 h, Tx) reduced ipsilateral hindpaw allodynia when compared to mice receiving a Veh injection, no sensitivity alterations were observed between groups in contralateral (C) hindpaws. Mean±SD, repeated measures two-way ANOVA w/ Bonferroni’s post-hoc; *=p<0.05, ***=p<0.001.

Figure B.2: Reporter cell responses are stimulus- and time-dependent (A) SW982 NF-κB luciferase reporter cell line was responsive to IL-1β and lipopolysaccharide (LPS), one-way ANOVA w/ Dunnett’s post-hoc. (B) NF-κB-driven bioluminescent signal is increased if PHA-408 treatment is delayed following stimulation with IL-1β (1 ng/mL). Data is normalized to IL-1β-only treated control (dotted line=mean of non-treated wells). Mean±SD, one-way ANOVA w/ Bonferroni’s post-hoc; *=p<0.05, ***=p<0.01, **=p<0.001.
Figure B.3: PHA-408 suspension size. (A) PHA-408 drug particles were readily visible via epifluorescent microscopy at the injected concentration (0.6 mg/mL). (B) Particle diameter was 2.09±0.53 µm. Scale bar = 20 µm.

Figure B.4: LC-MS PHA-408 tissue standard curves. Standard curves are shown for each tissue type, the inset shows one representative lymph node sample with a ~4.5 min elution time.
Figure B.6: NF-κB activation due to intra-articular injection of PHA-408 is minimal, but not reduced when compared to vehicle control. Naive mice received one intra-articular injection (t=0) of either PHA-408 (3ug/5uL, n=5) or vehicle control (saline+0.1% DMSO, n=4). Intra-articular injection of PHA-408 or vehicle led to an increase in joint localized NF-κB-driven luminescence in injected knees when compared to contralateral (C) knees, however, local delivery of PHA-408 did not lead to a reduction in NF-κB-driven luminescence when compared to vehicle control. Mean±SD, repeated measures two-way ANOVA w/ Bonferroni’s post-hoc to test for differences between all treatment groups; *p<0.05. Dotted and solid lines represent average ROI radiance values for INJ and INJ/Tx ipsilateral knee joints 3 days post-injury, respectively.

Fig. B.5: A sustained release microparticle did not improve PHA-408 concentrations within the joint. Following one-time local delivery of PHA-408 loaded SF microparticles (50 µg PHA-408,120 MB formulation, t=0 h), drug was not detected within (A) injected knee joint tissues 24, 48, 72, or 96 h post-injection. Peak concentration in (B) serum and (C) urine were detected 48 h post microparticle injection (n=2-3 per timepoint). Concentrations from suspension based PHA-408 (3 µg, t=0 and 48 h) intra-articular injections are plotted for reference. Mean±SD.
Figure B.7: Small molecule NF-κB inhibition influences cell viability in a dose dependent manner. (A) SW982 cells had reduced viability after 24 hr treatment with either PHA-408 or SC-514, a similar effect was observed with increasing concentrations of (B) drug loaded SF microparticles. Mean±SD.

Figure B.8: Ex vivo PHA-408 treatment reduces NF-κB signaling, nitric oxide release, and sGAG loss following an inflammatory stimulus in musculoskeletal organ cultures. (A) Experimental timeline. (B) NF-κB luminescence over time and (C) area under curve (AUC) from murine functional spinal unit organ cultures. (D) Nitric oxide (NO) and (E) sGAG released into media. (F) Tissue associated sGAG. Mean±SD, one- or two-way ANOVA w/ Dunnett’s post-hoc, *p<0.05 vs vehicle control.
Figure B.9: Alterations to NaV1.8 protein expression in L4 ipsilateral dorsal root ganglia neurons with injury. At the terminal timepoint, ipsilateral dorsal root ganglia (DRG) were isolated from the L4 spinal level and stained for the voltage gated sodium channel NaV1.8. (A) Representative DRG from CTRL, INJ, and INJ/Tx groups show increased NaV1.8 staining intensity (B) in INJ and INJ/Tx DRG, however, INJ/Tx DRG had reduced staining intensity when compared to their untreated INJ counterparts. (C) NaV1.8 expression was highest in smaller DRG neurons. One-way ANOVA w/ Tukey’s post-hoc to test for differences between all groups; *p<0.05.
Figure B.10: Ipsilateral to contralateral comparisons. Given the study design, ipsilateral to contralateral=C comparisons were made within each timepoint. (A) NF-κB-driven luminescence, (B) 50% withdrawal threshold, (C) escape threshold, and latency to a (D) hot or (E) cold stimulus. Repeated measures two-way ANOVA w/ Bonferroni's post-hoc; *=p<0.05, **=p<0.01, ****=p<0.0001.
APPENDIX C

Supplementary results from Chapter 4

**Figure C.1:** Soma size does not influence neural responsiveness to EFS with TTX inhibition. (A) Responses in neurons of varying soma size were similar across TTX concentrations. Percent response values in each size group represent a portion of all responding neurons (mean±SEM). (B) No size-based differences were observed in EV$_{50}$ at any individual TTX concentration (TTX p=0.007, Size p=0.924). Repeated measures two-way ANOVA; n=5 DRG, with n ≥ 138 cells per DRG.
APPENDIX D

Optically clearing musculoskeletal tissues: methods
development and application

Introduction

The gold standard approach for assessing degeneration in musculoskeletal tissues has long been histology\textsuperscript{227}. Following a study endpoint, tissues designated for histological evaluation must be processed following a protocol that coincides with their ultimate embedding medium. This usually requires fixation, followed by a multiweek decalcification process and subsequent dehydration before embedding\textsuperscript{342}. Then samples must undergo multiple resource intensive tasks including: sectioning, staining, imaging, and scoring\textsuperscript{343}. This method has been invaluable in understanding how anatomy changes with disease; however, biology does not exist within the confines of a several micron thick section. As such, recent approaches have aimed to improve our three-dimensional understanding of biology by offering new tissue processing methods that support optical light transmission through thick tissues ("clearing")\textsuperscript{344}. By pairing old and new tissue clearing techniques with multi-photon or light-sheet microscopy many groups have been able to image intact samples and uncover intricacies of three-dimensional spatial biology that were overlooked or unresolvable in micron thick sections\textsuperscript{344–348}. The majority of clearing techniques, however, have not been developed to address the unique challenges of musculoskeletal tissues\textsuperscript{349–353}. Joints have a variety of tissue types with diverse physical properties in close proximity which pose as hurdles to clearing and imaging intact specimens\textsuperscript{354}. However, by using a combination of clearing techniques paired with
fluorescent small molecules it may be possible to uncover morphometric changes to the joint following injury with an improved three-dimensional context. The work described in this appendix establishes clearing methods that have been optimized for imaging through musculoskeletal tissues. These methods are applied in a non-invasive murine model of PTOA to illustrate the early changes to joint morphology following joint injury.

Methods

Optical clearing method screening

Several aqueous and non-aqueous tissue clearing methods were first screened in porcine cartilage plugs (Fig. D.1). Plugs (4 mm diameter) were biopsied from load-bearing

Figure D.1: Clearing method development. (A) Graphical schematic of tissue clearing pipelines. (B) Representative images of tissues in respective clearing solutions (grid = 1 mm). (C and D) Representative SHG image stacks and MFI of signal throughout collected cartilage plug z-stacks. Mean±SEM, n=3 plugs/group.
regions of the tibial plateau. Transverse sections of the patellar tendon were also collected. Following isolation, tissues were fixed in 4% PFA (4 h, 4°C) and washed in PBS before clearing. All following steps were performed in solution volumes of roughly ten-times that of the sample of interest, 15 or 50 mL polypropylene screw-top Falcon™ tubes are typically preferred. **Aqueous.** TDE: Samples cleared in 2,2’-thiodiethanol (TDE, Sigma-Aldrich, St. Louis, MO) underwent sequential immersion in increasing concentrations of TDE in PBS (10%, 25%, 50%, 70%, 80%, and 100% vol/vol). The final concentration of TDE can be varied to tune its refractive index (RI) for imaging depending on sample RI\(^{355}\). Similar RI tunability can be attained with SeeDB, an aqueous fructose-based clearing protocol\(^{356}\). **Non-aqueous.** Following dehydration in graded tetrahydrofuran (THF, pH 9.0, 4°C, Sigma-Aldrich) and dichloromethane (DCM, 25°C, Sigma-Aldrich), samples were immersed in their respective clearing solution. **MS:** Samples were immersed in methyl-salicylate (MS, Sigma-Aldrich) until clear, and then mounted in MS\(^{354}\). **BABB:** Samples were placed in benzyl alcohol-benzyl benzoate (BABB; 1:1 vol/vol, Sigma-Aldrich) until the samples were clear to the naked eye, followed by mounting in fresh BABB, the refractive index of BABB can be tuned by varying the BA:BB ratio\(^{357–360}\). **DBE:** Following dehydration samples were immersed in dibenzyl ether (DBE, Sigma-Aldrich) until clear and were then mounted in fresh DBE\(^{361}\). Special care should be taken when using these non-aqueous agents; polystyrene products should be avoided.

Non-aqueous clearing methods appeared to more consistently reduce tissue opacity in cartilage and tendon; thus, they were used in further optimization for the *en bloc* imaging of murine knee joints. It has been previously reported that decalcification
and dehemification of musculoskeletal tissues may serve to reduce RI mismatch induced photon scattering and absorption, respectively\textsuperscript{351,362}. Therefore, we investigated how these tissue constituents impacted an ability to resolve propidium iodide stained chondrocytes deep within intact knee joints. Mice (12-20 weeks of age, FVB.Cg-Tg(HIV-EGFP,luc)8Tsb/J, Jackson Laboratory, Bar Harbor, ME) were euthanized via exsanguination and transcardially perfused with 4\% PFA. Intact knee joints were harvested and post-fixed overnight (4\% PFA, 4°C). Tissue surrounding the joint capsule was carefully trimmed and samples were then processed as outlined in Figure D.2A. A subset of knee joints were decalcified with 10\% ethylenediaminetetraacetic acid (EDTA, pH 8.0, 37°C, 2 days, Sigma-Aldrich). Cell nuclei were stained with propidium iodide (PI, 10 \(\mu\)g/mL, 25°C, 1 day, Sigma-Aldrich)\textsuperscript{352,363}. An additional group of knee joints were treated to remove heme (“dehemed”) in 25\% N,N,N’,N’-Tetrakis(2-Hydroxypropyl)ethylenediamine (Quadrol + 1\% Triton X-100, 37°C, 1 d, Sigma-Aldrich). Samples were then dehydrated in graded THFs and immersed in DBE as previously described.

To illustrate the utility of this imaging technique, a non-invasive model of PTOA was used. Right knees of mice (12-16 weeks of age, FVB.Cg-Tg(HIV-EGFP,luc)8Tsb/J) underwent 60 cycles of either 6 or 12 N compressive loading\textsuperscript{29}. Mice were then sacrificed either 7 or 14 days following injury. A subset of mice undergoing 12 N loading were sacrificed 28 days following injury. Following exsanguination (under isoflurane anesthesia, IACUC# 19-1032) mice received a transcardial perfusion of Lectin-DyLight 649 (0.1 mg/ 100 \(\mu\)L, 1230/655-710 nm ex/em, Vector Labs, Burlingame, CA) to label vascular surfaces at the time of sacrifice\textsuperscript{364–366}.

[143]
Visible light stereomicroscopy

Following clearing, tissues were immersed in their final clearing agents within a glass petri dish and imaged via a visible light stereomicroscope (MZ125, Leica Microsystems, Buffalo Grove, IL) to qualitatively assess clearing efficacy (grid = 1.0 mm).

Multiphoton microscopy

Cleared cartilage plugs were secured in a custom-made imaging chamber with two-part epoxy and immersed in their respective clearing solution. Full-thickness second harmonic generation (SHG, 880/420-460nm ex/em) images were acquired from the superficial articular surface upon an upright multi-photon microscope (SP8 DIVE, Leica Microsystems). All imaging parameters were kept constant between groups.

Cleared murine joints were imaged within the sagittal plane, intact joints were mounted with the medial side of the joint facing upwards towards the imaging objective. Serial planar images were collected from the medial collateral ligament (MCL) to a subsequent depth of 1 mm. All imaging parameters were kept constant between groups for clearing optimization and fluorescent PI (1045/630-660 nm, ex/em) and SHG (1045/510-530 nm, ex/em) signals were simultaneously collected. Scanning parameters in other images were chosen to balance image quality, scanning time, and file size. Multiphoton imaging was performed using a long working distance multi-immersion objective (FLUOTAR L 16x IMM CORR VISIR, NA=0.6, WD = 2.5 mm, Leica Microsystems).

Image processing and analysis

To aid in clearing protocol screening, quantitative analyses were performed on acquired images. The mean fluorescent intensity (MFI) at each imaging plane was
calculated within a region of interest (ROI). The selected ROI was defined to include articular surfaces of the femur and tibia within PI image stacks and the entire imaging field within SHG image stacks. Degenerative changes to synovium and articular cartilage were assessed to illustrate the potential utility of the described imaging techniques. Within planar images of the medial anterior synovium, a synovial ROI was defined (200 x 50 µm)\textsuperscript{119}. Cell nuclei within the ROI were manually counted to determine a nuclear number per unit area and thickness of the intimal synovial layer was manually measured at five locations within each ROI using ImageJ. Fluorescent images were also graded using a standard synovitis scoring system by two blinded investigators\textsuperscript{119,367}.

To assess focal alteration to cartilage, chondrocyte nuclei within a manually defined volume of interest (VOI) were segmented using an automated ImageJ code. VOIs were defined to include all tissues within 30 µm of the medial femoral condyle superficial surface. Following VOI segmentation images were cropped to reduce computational time. A three-dimensional Gaussian kernel was applied (2 x 2 x 4 voxels) followed by a rolling ball filter (20 voxel diameter) to minimize the impact of spatially varying background signal on binarization. Images were then binarized and a three-dimensional watershed and morphological opening (1 voxel structuring element) were used to help reduce overconnected binarized nuclei. Centroid positions for all nuclear objects could then be identified. A custom MATLAB script was used to calculate Euclidian centroid-centroid distances between all nuclear objects and these data were used to determine the three nearest neighbors of all nuclei. From these data, and observations of planar images, it was apparent 12 N injury led to a well-conserved focal lesion within the anterior region of
the medial femoral condyle which was more closely integrated within a smaller VOI (300 x 30 x 100 µm). Nuclei per unit volume were calculated within this VOI.

Results and Discussion

Optical clearing increased qualitative sample transparency (Fig. D.1B), less opaque samples had increased depth-resolved SHG signal (Fig. D.1C,D). Decalcification of intact murine joints improved sample transparency, particularly in calcified joint structures, while heme removal had a minimal qualitative impact on sample clarity (Fig. D.2C). Decalcified samples had a trend of increased PI (Fig. D.2E) and SHG (Fig. D.2F)

Figure D.2: Clearing optimization within murine joints. (A) Graphical schematic of generalized knee clearing pipeline. (B) Steps and time to completion for each protocol following tissue fixation. (C) Representative brightfield images of murine joints cleared using each protocol (grid = 1 mm). (D) Field of view (FOV) used in subsequent sagittal plane image stacks. (E and F) Representative depth resolved images and (G and H) quantification of propidium iodide (PI) and second harmonic generation (SHG) images, respectively. Signal intensity within the defined region of interest (ROI) are plotted as well as a qualitative z-depth limits of cellular and collagen morphology resolution. Mean, n=2-3 knees/group. PM=posterior meniscus, AM=anterior meniscus, MFC=medial femoral condyle, MTP=medial tibial plateau, MCL=medial collateral ligament, ACL=anterior cruciate ligament, PCL=posterior cruciate ligament.
signal at deeper imaging depths within the imaging ROI. The qualitative limit of cellular resolution within non-decalcified samples was roughly 500 µm from the MCL, in decalcified samples individual PI stained nuclei could be seen at imaging depths of nearly 800 µm (Fig D.2G), similarly, fibrillar collagen structures could be seen at depths of over 1000 µm from the medial collateral ligament in decalcified samples (Fig D.2H). Furthermore, homogeneity of PI and SHG signal intensity across the imaging field was improved in decalcified samples.
Following knee joint injury (Fig. D.3A), joints tended to have increased synovial thickness and planar cell density within the intimal layer of the anterior synovium (Fig. D.3B,C). Synovitis scores were similar to those previously reported within this non-invasive loading model at early timepoints\textsuperscript{119}. A modest focal reduction in three-dimensional chondrocyte density was observed within the anterior region of the MFC (300 x 30 x 100 μm VOI) of 12 N loaded knee joints (Fig. D.3D-F).

**Figure D.3:** *En bloc* imaging enables structural interrogation of joint tissues through optical sectioning. (A) Right knees underwent 6 or 12 N cyclical loading and mice were sacrificed within 14 days of knee joint injury and were subsequently cleared using a decalcification-based protocol. (B and C) Anterior synovium of injured knee joints trended towards having increased thickness, cell nuclei per unit area, and higher histopathological scores. (D) Representative nuclear centroid maps of the medial femoral condyle display position and average distance to each nuclear objects’ three nearest neighbors, the outlined region in the 12 N image highlights a focal reduction in cellularity. (E and F) Nuclear object detection within a 30x300x100 μm volume of interest in the anterior region of the medial femoral condyle revealed a trend of reduced cell number per unit volume in 12 N loaded joints. Mean±SD, n=3 knees/group.
Morphological alterations to synovial vascular structures were observed 4 weeks following knee joint injury (Fig. D.4A,B). Additional early changes to cellularity were observed in other joint structures (e.g. posterior medial meniscus) following 12 N injury (Fig. D.4C). The described methods were also compatible with other tools (Fig. D.4D,E) frequently used within musculoskeletal research (e.g. fluorescent reporters, dynamic bone labeling).

These data suggest optical clearing may serve as a first-pass method to assess changes in musculoskeletal tissue structures in novel animal models where the spatial position of a phenotype may be unknown. This technique is of particular value in focal diseases such as PTOA where different sectioning planes may show vastly different degrees of degeneration\textsuperscript{35,36}. Others have emphasized this limitation of gold standard...
histopathological assessments in the context of PTOA. For example, Glasson and colleagues highlight the high degree of degenerative changes following ACL transection in the posterior aspect of the tibial plateau. These erosive changes are outside of the typical mid-contact region which is typically assessed histologically within the coronal plane. Thus, sections scored from the mid-contact region within ACL transection knees might not accurately represent the full extent of joint degeneration. Non-destructive imaging techniques, such as those described here, may help to overcome these limitations. Following identification of an ideal sectioning plane or region of interest, standard histological techniques can still be used to assess morphometric, phenotypic, and cellular changes via more well-established measures.

Despite the described benefits of clearing and en bloc imaging, the technique is not without limitations. Clearing methods have mostly been developed to improve en bloc imaging in soft tissues with relatively homogenous tissue constituents. The heterogeneity of tissues in the joint presents several hurdles for which the developed tissue clearing methods are unable to fully overcome. For example, the RI mismatch between bone and DBE was apparent in non-decalcified samples where bone appeared to be more opaque than surrounding tissues. Decalcification helps to reduce the RI of bone thereby homogenizing the RIs within the joint space. Ultimately this reduces the number of scattering events for incident and emitted photons and improves axial resolution. However, there remains structural and RI inhomogeneities throughout these cleared tissues which result in uneven illumination due to differing light-path interactions. This makes robust quantification of acquired images difficult. For this reason, intensity-based quantification, particularly over large fields-of-view, has limited accuracy.
Feedforward intelligent imaging controls may help to improve signal-to-noise by actively modulating laser intensity and detector gain depending on previously collected imaging data. Recent advances in deep-learning neural network based segmentation have also sought to address these image analysis based shortcomings and have led to more accurate quantification of such volumetric imaging datasets\textsuperscript{369}. Nevertheless, *en bloc* imaging serves as a valuable companion method to investigators within the musculoskeletal field for assessing the three-dimensional microscale changes to intact tissues and organs without the immediate need for laborious histological techniques.

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