Development of a Computational Pipeline and Associated Quantitative Assessment of Superficial Dorsal Horn Activity Across Pain States

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Abstract

The superficial dorsal horn (SDH) is a critical site for pain processing and regulation. A large proportion of synaptic activity within the SDH is produced by subnetworks of local interneurons, which are responsible for the integration of sensory information received from peripheral afferents. Investigation of these subnetworks in rodent models is aimed to further elucidate the SDH’s role across models of chronic pain at the subnetwork level. Through epifluorescent microscopy on acute SDH slices, intracellular calcium increases were recorded as a marker for action potential firing. Functional neural populations within the SDH were quantified using a series of glutamate challenges on each slice, to identify all active neurons and respective evoked response magnitudes. Responses within sham control animals were compared to an anterior cruciate ligament transection model of osteoarthritic pain, as well as to a spared nerve injury model of neuropathic pain. A developed custom image processing pipeline combined MATLAB and ImageJ-Fiji Macro Language scripts with peer-reviewed, open-source toolboxes to quantify changes in SDH subnetwork excitability across pain models. The developed pipeline quantitatively assessed image reconstruction quality following parallel application of popular image denoising techniques. Non-local means denoising was observed to improve image quality significantly more than the other methods, and was prescribed to the full dataset. The presented harmonized pipeline serves as a novel assay for specific and multidimensional evaluation of SDH circuitry. No significant differences were observed in evoked SDH network response following two levels of glutamate challenges across experimental groups. Future experiments will use these pipeline approaches to investigate whether there are differences in spontaneous SDH network activity between the experimental groups, which may underlie differential pain sensitivities in these chronic pain conditions.
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This thesis is dedicated to Dorothy and Keith, my loving grandparents.
Table of Contents

Abstract .................................................................................................................................................. 2
Acknowledgements ................................................................................................................................. 3
Table of Contents .................................................................................................................................. 4
List of Abbreviations ............................................................................................................................. 5
Introduction .............................................................................................................................................. 6
  1.1 Investigation of Diverse Mechanisms of Pathological Pain ......................................................... 6
  1.2 - Somatosensory Morphology and Experimental Areas of Interest ............................................. 7
  1.3 Nociceptive Neuronal Networks in the Spinal Dorsal Horn ......................................................... 9
  1.4 Mechanisms of Central Hypersensitization and Spontaneous Pain ........................................... 13
  1.5 Inflammatory Pain ......................................................................................................................... 14
  1.6 Neuropathic Pain ......................................................................................................................... 15
  1.7 Subnetwork Analysis Across Models of Chronic Pain ................................................................. 16
  1.8 Computational Approaches to Calcium Imaging Data Analysis in the Spinal Cord ................. 17
Purpose .................................................................................................................................................... 22
  2.1 Objective 1: Construction of Harmonized Image Preprocessing Pipeline ............................... 22
  2.2: Objective 2: Quantitative Assessment of Evoked Potentials Across Chronic Pain Models ....... 23
Methods .................................................................................................................................................... 25
  3.1 Animals and Tissue Preparation .................................................................................................... 25
  3.2 Pain Model Experimental Design .................................................................................................. 26
  3.3 Calcium Imaging Recording ........................................................................................................ 27
  3.4 Image Processing and Analysis .................................................................................................... 28
  3.5 Statistical Analysis ....................................................................................................................... 31
Results ..................................................................................................................................................... 32
  4.1a Developing a Harmonized Image Processing Pipeline ............................................................... 32
  4.1b Image Denoising Method Comparison ....................................................................................... 40
  4.2 Quantifying Evoked Subnetwork Activity in the Superficial Dorsal Horn ............................... 44
Discussion ............................................................................................................................................... 51
  5.1 Development of a Harmonized Image Processing Pipeline ....................................................... 51
  5.2 Image Quality Assessment ............................................................................................................ 53
  5.3 Studying Image Analysis Techniques & Future Directions in Tool Development ................... 54
  5.4 Quantifying Evoked Potentials of SDH Neural Subnetworks .................................................... 55
  5.5 Limitations and Future Directions of Subnetwork Characterization ........................................ 56
Conclusions .............................................................................................................................................. 60
References ................................................................................................................................................ 61
Appendix A: Supplementary Table 1. Statistics Summary Table ....................................................... 67
Appendix B: Objective 1 MATLAB and ImageJ/FIJI Scripts ............................................................... 67
### List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>ACLT</td>
<td>anterior cruciate ligament transection</td>
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<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
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<tr>
<td>AuC</td>
<td>area under the curve</td>
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<tr>
<td>CCD</td>
<td>charge-coupled device</td>
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<tr>
<td>CICR</td>
<td>calcium-induced calcium release</td>
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<td>DRG</td>
<td>dorsal root ganglia</td>
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<tr>
<td>GABA</td>
<td>gamma-aminobutyric acid</td>
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<tr>
<td>IJM</td>
<td>ImageJ macro language</td>
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<tr>
<td>JVM</td>
<td>Java memory space</td>
</tr>
<tr>
<td>KCC2</td>
<td>chloride potassium symporter 2</td>
</tr>
<tr>
<td>NIQE</td>
<td>naturalness image-quality evaluator</td>
</tr>
<tr>
<td>MIN1PIPE</td>
<td>miniscope 1-photon imaging pipeline</td>
</tr>
<tr>
<td>OA</td>
<td>osteoarthritis</td>
</tr>
<tr>
<td>PSNR</td>
<td>peak signal-to-noise ratio</td>
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<tr>
<td>ROI</td>
<td>region of interest</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
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<tr>
<td>SDH</td>
<td>superficial dorsal horn</td>
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<tr>
<td>SNR</td>
<td>signal-to-noise ratio</td>
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<td>TIFF</td>
<td>tag image file format</td>
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Introduction

1.1 Investigation of Diverse Mechanisms of Pathological Pain

The lack of effective and sustainable management strategies for chronic pain is an ongoing concern which spans many professional disciplines and industries. This pervasive issue is partially due to the lack of a precise understanding of the many mechanisms which underpin chronic pain states, and how they deviate from the adaptive phenomenon of acute pain. Whereas acute pain serves as a warning system for impending damage from present noxious environmental stimuli, chronic pain typically either lacks this prerequisite exposure or persists following cessation of stimuli or initial trigger injury (Kuner & Flor, 2016). As such, chronic pain may be considered a pathological response as opposed to ‘physiological’. The etiology of these chronic pain states include hallmark dynamic shifts in the somatosensory system’s network dynamics and corresponding cellular and molecular mechanisms. These dynamics and mechanisms are notably diverse when considering all the conditions which fall under a standard chronic pain diagnosis; factors such as past injury, autoimmune disorders, opioid use, and comorbid diagnoses such as diabetes may all contribute to the development of certain chronic pain conditions over others. As one may suspect, the effective treatment of diverse chronic pain conditions requires correspondingly diverse treatment targets, which is a common goal across research, clinical, and community environments that has yet to be fully achieved.

The heterogeneous nature of chronic pain further extends to the demographics of those who suffer from it; women, veterans, and Indigenous Peoples are examples of clinical groups that are disproportionately affected by chronic pain conditions in Canada, amongst many others who are at greater risk of developing a chronic pain condition due to systemic inequity.
and marginalization (Health Canada, 2020). Indigenous Peoples in Canada suffer from the highest prevalence of chronic pain under the age of 65 (Meana, Cho, & Desmeules, 2004), and suffer from osteoarthritis (OA) at twice the rate of non-Indigenous Canadians (Barnabe et al., 2015). The host of factors contributing to this example of disproportionate burden of disease remain far from completely understood; as mentioned, the effective diagnosis and management of chronic pain conditions requires diverse biological, psychological, and social analyses. Primary research of the biological foundations of chronic pain conditions may provide a foundation of technical knowledge for complementary research to contextualize and ultimately translate these findings to highly relevant developments in clinical care and corresponding policy. Comparing and contrasting under-explored chronic pain neurobiological features such as network dynamics may propel further characterization and distinction of common chronic pain differential diagnoses, as well as further clarify the discrete separation between the adaptive and disruptive conditions of acute and chronic pain, respectively. Conducting research at the subnetwork level may effectively profile heterogeneous function of local neural circuits across pain models. As such, this approach may further elucidate the roles of implicated neural aggregates and developing novel treatment strategies.

1.2 - Somatosensory Morphology and Experimental Areas of Interest

When discussing the biological mechanisms of chronic pain disorders, it is important to properly distinguish terms of study such as pain and nociception. While pain as a technical term refers to the experience, “associated with, or resembling that associated with, actual or potential tissue damage” (Raja, 2020), nociception is dedicated to the biological cascade of events in reaction to sensory neurons being exposed to noxious stimuli. The study of
nociception is set within the broader research area of general somatosensation, of which it has been defined as an ‘aversive subtype’ (Loutit, Vickery, & Potas, 2020; Ross, 2011). In healthy mammalian nervous systems, sensory neurons in the periphery detect and transduce the required noxious stimuli into a neuronal response, and propagate the resultant action potential signals towards the central nervous system for higher level integration and stimuli response. Specifically, sensory afferents project to the dorsal horn (Todd, 2010), which is a primary somatosensory integrative region of the spinal cord. Where the grey matter of the spinal cord is organized into ten discrete laminae (Rexed, 1952), the first six form the dorsal horn. This categorization is appropriate on both structural and functional levels, where each lamina is responsible for the reception and integration of different types of somatosensory information. Superficial laminae, which includes lamina I and II, are primarily responsible for nociceptive signaling (Peirs & Seal, 2016), and as such are specific, yet popular sites of intervention for the development of novel analgesics. Ascending nociceptive information received by the dorsal horn is initiated by the responses of specialized primary nociceptors, which are nerve cell endings that are responsible for signal transduction of noxious stimuli to trigger the afferent nociceptive pathway (Purves, 2001). These peripheral nociceptors are categorized according to their axons, where their myelination and diameter impact their respective signal transmission qualities. Of the somatosensory axon subtypes, Aδ myelinated axons and C fiber unmyelinated axons have notable roles in nociceptive signal transmission. Due to their myelination, Aδ fiber axons have relatively higher conduction velocity and are thus much faster-acting than C fiber axons. While Aδ axons conveys both nociceptive and general somatosensory signal with emphasis on intense mechanical stimuli, slow and thin C fibers play important roles in
polymodal nociception and low-threshold sensations of light touch (Bourinet et al., 2014; Purves, 2001). The organized manner of signals entering the dorsal horn through the dorsal root ganglia (DRG) is a reminder of the sheer diversity of information gathered by nociceptors, inviting consideration to the afferent nociceptive pathway’s integrative capabilities as well as its transmissive ones.

1.3 Nociceptive Neuronal Networks in the Spinal Dorsal Horn

As nociceptive signals travel along the afferent pathway to reach the dorsal horn, their modulation and integration within spinal neuronal networks can shape nociceptive outputs to the brain. When considering spinal mechanisms of nociception, understanding how the dorsal horn encodes modality-specific information remains largely unknown (Gatto et al., 2021). It is understood that the integration of these nociceptive inputs is performed by intricate subnetworks of largely excitatory interneurons within the dorsal horn (Todd, 2010), which composes the first site of synaptic relay as it exits the periphery. These glutamatergic interneuron subnetworks communicate internally across the superficial region of the dorsal horn closest to the DRG entrance, an area specific for nociceptive transmission and integration, as well as connect to deeper parts of the dorsal horn through ventral dendritic connections (Kosugi et al., 2013). Excitatory interneuron subnetworks charged with receiving incoming signal from the DRG are accompanied by smaller, yet still diverse populations of inhibitory gamma-Aminobutyric acid- (GABA-)ergic and glycinergic interneurons. This deeper dorsal horn region, largely populated by inhibitory glycinergic interneurons, contributes to a delicate balance between excitatory and inhibitory signaling which serves as a primary regulator of input received by projection neurons which pass integrated nociceptive signal towards the
brain (Lee, Ratté, & Prescott, 2019). Nociceptive signaling from afferents onto excitatory neural circuits is modulated within the dorsal horn primarily by inhibitory interneurons, where they determine a threshold for nociceptive information that may be transmitted to dorsal horn projection neurons via polysynaptic signaling (Lee, Ratté, & Prescott, 2019; Peirs & Seal, 2016). Modulation of signal has been observed to operate through local circuits with high degrees of intraregional connectivity, with key connections between specialized interneuron populations (Kato et al., 2013; Stachowski & Dougherty, 2021) and laminar location (Abraira et al., 2017; Kato et al., 2013). These circuits become maladaptive in chronic pain states to drive symptoms such as allodynia, where pain is triggered by normally nonpainful mechanical stimuli.

The dorsal horn’s catalogue of specialized cell populations and their respective assignments within a network architecture of such intricate detail compels rigorous investigation. As discussed, the dorsal horn’s structure and function alludes to the breadth of somatosensory signal modalities which it effectively encodes for ascending projection to the brain. While these two aspects of the SDH’s characterization, structure and function, each provide crucial information with regards to the region’s principal mechanisms across the spectrum of health to disease, it is argued that they cannot stand alone to achieve complete understanding. Understanding these aspects may most directly begin with using cell morphology and electrical output in the form of spiking patterns as classifiers to predict continued function and trends in plasticity over time (Prescott & De Koninck, 2002). Where neurochemical indicators have also been commonly used as classifiers for specialized cell populations, observed differences in spiking patterns collected by electrophysiological techniques are accompanied by a rich history of experiments using immunohistochemistry
(Todd, 2010). This latter method is used to visualize distributions of biomarkers across laminae to further elucidate laminar and specialized cell population specific roles in nociceptive signal integration and transmission at cellular and subcellular levels. As an example, primary evidence demonstrating lamina I and lamina II being significantly implicated in nociception includes staining for calcitonin gene-related peptide, a neuropeptide released by peptidergic nociceptive fibres terminating within the SDH (Lorenzo et al., 2008). More specifically, classification of excitatory and inhibitory interneuron populations within the SDH has been studied through staining for their principal neurotransmitters (Todd, 2017), which include glutamate as well as GABA and glycine, respectively. These interneuron groups have also been extensively studied using cell-type markers such as transcription factors (Gutierrez-Mecinas et al., 2019; Todd, 2017). The tandem use of standard immunohistochemistry and genetic interventions to investigate neurochemical classes of SDH subpopulations has generated a precise understanding of the differential roles of SDH cell populations in nociceptive processing through analysis of validated gene expression (Chamessian et al., 2018). Identified excitatory and inhibitory cell populations are further subdivided into an immense array of specialized subtypes which are transcriptionally and subsequently neurochemically different, each with unique contributions to information processing and transmission within and beyond the SDH. However, the above genetic and neuroanatomical methods come with the limitation of the collection of static data types, as well as discrepancies in matching transcriptional and neurochemical classes to functional classes. Where these identified groups of interneuron populations must work in concert over time to fulfil their respective processing roles within the
SDH, reliable classifiers and combinations thereof should respect their dynamism across conventional and pathological nociceptive systems.

As mentioned, while it is generally agreed upon that the SDH is primarily composed of excitatory interneurons (Todd, 2010; West et al., 2015, Dickie et al., 2019), consistent methods of predicting their wide range of activity have yet to be achieved. A seminal study performed in 2002 by Prescott and De Koninck observed correlation between morphology and firing pattern of locally arborized cells found in lamina I with novel quantification of four different firing patterns in this region (tonic, phasic, single spike, and delayed onset). As spiking patterns are argued to reflect efficiency of functional connectivity and individual cellular contribution to larger circuits (Santos et al., 2007), it was determined that the SDH is diverse in specialized cell populations and their corresponding functions. It must be considered, however, that there is similar diversity in the proposed alternative methods to predict these observed firing patterns. The use of functional class as sorting criteria to predict cellular response has been supported by experiments investigating interneurons within the SDH (Harding, Boivin, & Salter, 2020), but also dismissed in favour of afferent input as a more robust predictor (Dougherty & Chen, 2016), as well as previously addressed neuropeptide expression (Dickie et al., 2019). Investigation of local circuit function within the SDH has thus produced understanding of the region’s morphological and functional diversity, but has yet to reach sufficient understanding to disentangle and fully define the connections between subnetwork structure and function, presenting considerable difficulty in expanding the understanding of action potential firing patterns and coding beyond the subcellular and cellular levels. While interventions at said levels provide a cogent response to questions posed at that same scale, they insufficiently
describe dynamics which occur at the population level (Grienberger et al., 2012). Similarly, where population-level trends may be observed using genetic and neurochemical classes of cell population subtypes, their use alone provides detailed, but static representations of dynamic nociceptive mechanisms. This leaves a problematic knowledge gap when considering high-level informational processing in functional somatosensory circuits, which in turn drives behaviour. As such, there is considerable opportunity to further investigate the dynamics of the SDH’s local circuits to further refine its functional profile as the spinal cord’s primary integrative hub.

1.4 Mechanisms of Central Hypersensitization and Spontaneous Pain

As neural subnetwork dynamics are incompletely understood in healthy somatosensory systems, they are even less understood across chronic pain pathologies. As mentioned, when discussing the conventional function of nociceptive systems, the balance between excitatory and inhibitory signaling conducted by SDH interneurons is the product of their high degree of plasticity. Made possible by the system’s nature as a diverse ensemble of microcircuits, this adaptivity has the dual capability of serving as a similarly multipartite substrate for the induction and maintenance of chronic pain states. Historical literature thoroughly summarizes common molecular cascades which induce chronic pain states, where increased nociceptive activity is produced by functional shifts within the SDH (Kuner & Flor, 2016). More specifically, chronic pain states have been widely observed to be accompanied by reduced synaptic inhibition (Coull et al., 2003; Lee, Ratté, & Prescott, 2019). Where this compromised inhibition’s role was established as a key modulator contributing to network imbalance, excitatory interneurons within the spinal dorsal horn are exposed to higher levels of signal output. This increased output is propagated to projection neurons within the superficial laminae. Lack of
inhibitory modulation in this regard has been argued to disproportionately affect excitatory interneuron subtypes (Lee, Ratté, & Prescott, 2019). This maladaptive propagation of low-threshold signal has been widely attributed to behavioural manifestations of chronic pain states such as hypersensitivity and spontaneous pain (Dedek et al., 2019; Keller et al., 2007). The co-opting of novel propagated signal within the context of an induced chronic pain state may summarize the central sensitization that is characteristic of several chronic pain manifestations, with particular emphasis on mechanical hypersensitivity (Latremoliere & Woolf, 2009). While recalling that the induction and maintenance of these states have molecular bases, it is worth recognizing them as key determinants of greater network disruption. Specifically, it has been observed that downregulation of KCl transporter chloride potassium symporter 2 (KCC2) following neuropathic injury impacts local transmembrane anion gradients, driving anionic shifts which drive net excitability across lamina I (Coull et al., 2003), providing ideal conditions to introduce additional propagation of low-threshold signal. This mechanism is also relevant in inflammatory pain, where KCC2 is similarly downregulated following peripheral nerve injury (Lalisse et al., 2018); downregulation across both syndromes have been observed to be driven by brain-derived neurotrophic factor. The resulting increase of intracellular chloride inhibits glycineric and GABAergic signaling, further driving local hyperexcitation (Chen & Marvizon, 2020). Additional molecular cascades are responsible for the perpetuation of hyperexcitability, which becomes increasingly pertinent to discuss when examining specific chronic pain syndromes and their lasting hyperexcitable state.
1.5 Inflammatory Pain

Inflammatory pain is a common subtype of pathological pain, and can be driven by the sensitization of primary sensory neurons following injury to axons of DRG cells resting in the periphery (Basbaum et al., 2009; West et al., 2015). This peripheral sensitization occurs even after inflammation from acute injury subsides (Burma, 2017). It was also observed through these experiments that excitatory cholecystokinin neurons play a vital role in the transmission of heat hypersensitivity following inflammatory injury. While inflammatory pain model circuitry within the dorsal horn recruited a more diverse set of excitatory interneurons when a mechanical allodynic state was induced, investigations of spontaneous activity of subnetwork neurons in concert and focus on broader circuit dysfunction has yet to be conducted.

Osteoarthritis (OA), a common example of inflammatory pain, is a degenerative form of arthritis which involves maladaptive plasticity in the central and peripheral nervous system over time (Brederson et al., 2018). Where it is common for long term OA risk to increase in a joint with past trauma (Tawonsawatruk et al., 2018), surgically induced OA via joint injury is a popular research method in animal models to study inflammatory pain mechanisms. OA may remain a latent, asymptomatic condition for extended periods of time before onset of aberrant nociceptive activity is detectable in clinical settings (e.g., mechanical allodynia) (He et al., 2017). As such, surgically induced OA regimes accommodating long-term symptomatic disease development may be considered optimal as animal research methods approach applicability to human systems (Tawonsawatruk et al., 2018). Specifically, anterior cruciate ligament transection (ACLT) has become a popular method to induce inflammatory trauma to the joint,
and introduces distinctive changes to gait post-surgery, consistently exacerbating joint
degradation over time (Teeple et al., 2013).

1.6 Neuropathic Pain

An interesting comparison to inflammatory pain is neuropathic pain, which is a
distinctive chronic pain state defined by direct damage to somatosensory nervous system tissue
(Wang & Wang, 2003). Central sensitization induced by peripheral nerve injury can produce
allodynia, hyperalgesia, and spontaneous pain (Polgar & Todd, 2008). Resulting changes in local
circuitry dynamics occur over time upon induction of these chronic pain states, where initial
waves of activity which occur immediately post-injury dampen to retrograde signaling
complexes, perpetuating symptoms over the long term (West et al., 2015). The functional
profiles of neuropathic pain states feature spontaneous spike bursts in lamina I, observed to be
distinct from healthy controls where this dynamic was not observed (Keller et al., 2007).

Peirs and colleagues (2021) similarly addressed neuropathic pain through two spared
nerve injury (SNI) models, which involve transecting two of three terminal branches of the
sciatic nerve. This induces a robust neuropathic model of pain which displays early and includes
long-term changes in behaviour (Decosterd & Woolf, 2000), and is argued to not result in loss of
neurons when implicating laminae I-III of the dorsal horn (Polgar & Todd, 2008). Peirs and
colleagues (2021) observed a complex recruitment regime within the SDH during induction of a
neuropathic state via SNI; protein kinase C neurons play a key role in the recruitment of
transient neurons within the subnetwork to indirectly propagate signal to projection neurons,
ultimately producing neuropathic mechanical allodynia in their mouse model. These findings,
along with previously mentioned interactions between diverse cell populations in the SDH,
point towards a complex integration of signal that likely extends beyond understanding of mechanical allodynia. Use of spatiotemporal data may further characterize the dynamics of this indirect system as an active neural circuit.

### 1.7 Subnetwork Analysis Across Models of Chronic Pain

Quantitative assessment of active neural subnetworks requires consideration of techniques which parse structural and functional activity as collections of cells. Peirs and colleagues’ series of experiments thoroughly examined relationships between pain pathology and cell morphology, animal behaviour, and single cell function to produce a foundational structural profile of the dorsal horn, with connections to functional roles (2021) that complement previous literature on the region’s population diversity. As discussed above, further refining the SDH’s functional signatures across chronic pain models requires additional investigation using spatiotemporal data across the entire subnetwork. It is proposed that this may be done through calcium imaging recordings of acute spinal tissue slices, where it is possible to observe spontaneous activity and evoked potentials of local subnetworks across laminae I and II.

Calcium imaging is a technique which produce traces of subnetwork activity specific enough to form the readout of the functional responses of individual cells (Greinberger et al., 2012). Where free calcium ions compose signals which are responsible for a wide range of cellular messaging in mammalian nervous systems (Greinberger & Konnerth, 2012), cytosolic calcium concentration may serve as an indicator for action potential firing. Increases in calcium concentration within the somatic cytosol following postsynaptic potentials and action potentials are dependent on the function of voltage-gated calcium channels, which is
supplemented by released free calcium from ryanodine-sensitive stores within the cell (Harding, Boivin, & Salter, 2020). In calcium imaging, these changes in intracellular calcium concentration are observed through changes in fluorescence intensity produced by pretreating the recorded tissue with calcium-binding fluorophores (Harding, Fung, & Bonin, 2020). This method reports calcium changes in the entirety of the cell cytosol, features high spatial resolution, and enables monitoring of neural subnetwork activity across space and time.

Use of calcium indicators and other pharmacological interventions highlight the roles that certain receptors play in cellular activity, where molecular interventions act as ligands to elicit cell responses. Distribution of these receptors across specialized cell populations may also indicate their degrees of contribution to larger system events (Doolen et al., 2012; Harding, Fung, & Bonin, 2021). It has been observed in previous literature that calcium dynamics in the brain follow complex spatiotemporal dynamics (Lee et al., 2016), where plastic networks exhibit shifts in calcium-induced calcium release (CICR) activity to modulate development. This observation was followed by the hypothesis that similar maladaptive shifts drive developmental and psychiatric disorders; recent investigation of CICR within the spinal cord illustrates that this mechanism may influence circuit activity within lamina I and II (Harding, Boivin, & Salter, 2020).

With the use of calcium dynamics as a proxy for observing overall synaptic responses and action potential firing within neural subnetworks, there is the opportunity to monitor evoked responses over time to gain understanding of cellular functional profiles. Stimulation of excitatory receptors may maximize cell output, where the maximum active and viable cell population may be identified and studied. For example, glutamate, an excitatory neurotransmitter expressed by the superficial dorsal horn’s excitatory interneurons and
nociceptive afferents (Todd, 2010), can be administered to activate glutamate receptors (Hartwick, Hamilton, & Baldridge, 2008). Glutamate receptors are permeable to free calcium, and this relationship is the primary driver of glutamate-induced calcium influx. This influx occurs where glutamate drives synaptic firing, where resultant synaptic depolarization activates voltage-gated calcium channels during action potential firing to drive greater increases in cytosolic calcium.

Whereas structure and function of local subnetworks within the SDH differ upon induction of different chronic pain states (Kuner & Flor, 2016; Peirs et al., 2021), it is proposed that the analysis of spatiotemporal dynamics of superficial laminae neural circuits may further elucidate pathological functional profiles. Using calcium imaging and in vitro application of increasing concentrations of glutamate, the functional activity of these neural populations may be visualized, and their dynamic shifts quantitatively compared between naïve and pathological pain states for further divergent qualities.

1.8 Computational Approaches to Calcium Imaging Data Analysis in the Spinal Cord

The development of a specialized image preprocessing pipeline increases capacity for the low-cost and standardized processing of large volumes of image-based neural data. A review of open-source tools in image processing to develop an accessible and precise analysis regime was conducted to identify accessible options for calcium imaging data analysis, as well as the experimental need for new or updated tools or processing regimes in this regard. The following needs were identified:
i) The regime must have the capacity to be fully automated, with minimal required supervision when processing multiple selected recording videos.

Spinal calcium imaging data analysis often involves manual, user-driven image contrast adjustment and cell ROI tracing to provide exact image segmentation across the entire recording length. While these methods are accessible to those without coding experience, they are labour intensive and prone to bias and other forms of human error when treating large volumes of data. The development of an image processing pipeline with the use of custom scripting to achieve automation of these processes mitigates these issues; large datasets may be processed and analyzed in detail in a standardized manner.

ii) The regime must additionally accommodate large volumes of image-based neural data (>2 gigabytes per image recording, with an unspecified number of sequentially processed recordings).

An additional need for this dataset includes considerable capacity for the processing of large image-based files, where image recordings that often surpass 2 gigabytes exceed many programs’ set Java Heap Memory (JVM) limits (which may be considered as the working memory of a given program). This limit often interferes with image recording processing and storage, especially when computationally expensive strategies are used to process and analyze large datasets. Calls for simple, interpretable regimes which may be easily integrated into new and existing experimental protocols (Stringer & Pachitariu, 2019) include this desired outcome, where computational tools directly address and mitigate system capacity limits while effectively processing and analyzing image-based data.
iii) The regime must respect the dynamic nature of biological neural networks, with emphasis on the quantification of time-dependent trends.

Unlike other neural imaging processes such as the common use of functional magnetic resonance imaging (fMRI) on the brain or brain structures, calcium imaging at cellular and cell subnetwork levels do not usually have a priori ROIs. Rather, image frames contain diverse spreads of cells and puncta between individual recordings; the treatment of this neural imaging data must fall within a narrow window of precision to allow this variability while maintaining accurate ROI identification.

In addition, the regime must be capable of detailed time series analysis of image-based neural data. Characterization of neural ensembles are arguably incomplete when only collecting and comparing static measures of their function. As an example, sparse-firing neurons may greatly contribute to local and global network dynamics, however, are often excluded from manual cell tracing as well as by automatic thresholding functions due to their overall low relative fluorescence across the entire recording (Stringer & Pachitariu, 2019). The inclusion of time as a key quantitative variable will allow for more biologically realistic analysis of neural network function.

iv) The regime must be capable of specific quantitative reporting of incoming and outgoing image quality metrics.

Where entire datasets may be heterogenous in image quality even within experimental groups, a processing and analysis regime must be capable of identifying image-based data that is of objectively low quality. Identifying imaging recordings which have outlying image quality
metric values (e.g., signal-to-noise ratio and derivative measures) may provide more accurate groupwise comparisons of large sets of image-based data. In addition, assessment of image quality across stages of image processing may validate the given experimental protocol. As image processing aims to effectively reconstruct an image’s signal by mitigating noise introduced by several sources (biological, electronic, optical) (Aaron & Chew, 2021), integrating quantitative image quality reporting capability into an image processing and analysis regime gives rise to a final protocol that is well-fitted to a given dataset. This results in higher confidence that trends observed during groupwise comparison are minimally biased by a given image processing and analysis protocol, which can be of drastic influence if not properly implemented and interpreted (Aaron & Chew, 2021; Stringer & Pachitariu, 2019).

Purpose

This study seeks to identify and study the functional profiles of local subnetworks within the SDH across neuropathic and inflammatory models of chronic pain (compared to an inflammatory sham group) through the development of computational tools to analyze epifluorescent calcium imaging data. This will be done through the (1) construction of a harmonized image preprocessing pipeline which combines peer-reviewed image assessment and processing toolboxes with custom MATLAB and ImageJ Macro Language (IJM) functions, and quantitative assessment of spatiotemporal calcium trace data reflecting (2) evoked activity across chronic pain models.

2.1 Objective 1: Construction of Harmonized Image Preprocessing Pipeline

Previously used methods of calcium imaging analysis including manual tracing and analysis of trial recording regions of interest are subjective, time-consuming and provide data of limited dimensionality. Otherwise stated, these methods are only able to label manually
traced regions of interest as a binary readout of either “active” or “inactive” for the length of a recording. Development of an automated preprocessing pipeline which conserves and integrates temporal parameters of identified active cell regions-of-interest (ROIs) may more accurately present relative subnetwork activity across experimental levels (e.g. chronic pain model, level of glutamate challenge). Automation of preprocessing measures provides standardized, low-cost analysis of large volumes of imaging data, which will increase analysis capacity and reproducibility. The harmonized image preprocessing pipeline will be easily adjustable and appropriate for the analysis of any calcium imaging dataset.

2.2: Objective 2: Quantitative Assessment of Evoked Potentials Across Chronic Pain Models

To determine if there are quantitative differences in dorsal horn calcium responses across neuropathic, inflammatory, and acute control models of pain, recording trials with 10µM and 25µM glutamate challenges will be used to characterize respective subnetwork evoked activity. Quantitative assessment of evoked activity across models will provide a functional baseline for future study of spontaneous activity across chronic pain models. Calcium imaging recordings will be analyzed to identify and average calcium response curves and the magnitude of their response relative to experimental glutamate challenges and experimental models through calculation of area under the curve (AuC). Maximized output of subnetwork activity may elucidate finer functional profiles of analyzed subnetworks through elevated number of active cell ROIs per tissue slice.
Figure 1. Project workflow, with described study objectives: (1) construction of harmonized image preprocessing pipeline, (2) quantitative assessment of evoked activity across models of chronic pain.
Methods

Tissue preparation and calcium imaging recordings were performed by Dr. Erika Harding and Samuel Fung at the University of Toronto. Calcium imaging recording data was shared with Stephanie Norlock at Carleton University to be processed and analyzed. Data analysis using computational methods provided multidimensional, time-dependent findings aimed to characterize activity signatures of identified neural subnetworks.

3.1 Animals and Tissue Preparation

Calcium recording experiments were performed using male adult C57BI/6 mice (24-30 weeks). Anterior cruciate ligament transection (ACLT), ACLT sham, and SNI surgeries were performed at the University of Calgary prior to delivery to University of Toronto for terminal experiments. Terminal experiments were approved by the University of Toronto Animal Care Committee and performed in accordance with animal care regulation and policies of the Canadian Council on Animal Care. Mice were kept on a 12:12 hour light:dark cycle in groups of one to four mice per cage, with food and water provided ad libitum.

Mice were anaesthetized with 2mg/kg chloral hydrate (Sigma Aldrich, USA), and cardiac perfusion of ice-cold protective sucrose solution was performed. This dissection solution contained (mM): 50 sucrose, 92 NaCl, 15 D-Glucose, 26NaHCO3, 2.5 KCl, 1.25 NaH2PO4, 0.5 CaCl2, 7 MgSO4, 1 kynurenic acid, bubbled with 5% CO2/95% O2 (pH 7.3, 310 mOsm) (Hildebrand et al., 2014; Harding et al., 2020). Dissection of the mouse vertebral column procured a section of spinal cord from the lumbar region, which was placed in ice old, oxygenated dissection solution. L4-L6 was isolated from the rest of the lumbar sample following trimming of dorsal roots and dura, and the ventral side of the L3-L6 section was glued to an
agar block (4% agarose in distilled water) to be sliced in ice-cold sucrose solution with a VT 1200S vibratome (Leica Microsystems). Tissue was sliced at parasagittal orientation and at 300µm thickness. Slices were incubated in a modified dissection solution replacing kynurenic acid with cell-permeable calcium indicator Oregon Green Bapta-1 (50µg in 5mL dissection solution, ThermoFisher, USA) at 34°C for 40 minutes before being cooled passively to room temperature for 30 minutes. After the 70-minute incubation period, the incubation solution was replaced with room temperature dissection solution without kynurenic acid.

3.2 Pain Model Experimental Design

Comparison of neural subnetwork dynamics across chronic pain conditions were performed using an inflammatory osteoarthritis model induced by ACLT (n=8), as well as a neuropathic SNI model (n=5). Experimental ACLT group was accompanied by a sham surgery group (n=5).

An osteoarthritic condition of inflammatory pain was induced in mice by ACLT to induce an inflammatory chronic pain state over the course of 12 and 13 weeks (Mousseau et al., 2018). Neuropathic SNI model rats underwent surgery to induce a neuropathic pain state, where sural and tibial branches of the sciatic nerve were ligated and transected (Zhang et al., 2021). Mice were housed individually for two hours to allow for sensitization to develop prior to euthanasia and spinal cord dissection.

3.3 Calcium Imaging Recording

Oregon Green Bapta-1 AM (OGB-1 AM) (Thermofisher USA) was prepared using a modified protocol from Thermofisher. AM conjugation allowed this fluorophore to be membrane-permeable, where cells were bulk-loaded with indicator across treated tissue. 50 ug
of OGB-1 AM was dissolved to 5 mM in 100% DMSO (8 µL) and 4 µL of 10% w/v Pluronic F-127 was added to increase solubility. This solution was then added to 5 mL of dissection solution lacking kynurenic acid. After incubation, slices were removed from solution and allowed to de-esterify for 40 minutes at room temperature in dissection solution lacking kynurenic acid. Slices were then placed under an upright Olympus BX51WI microscope with a 40x water immersion objective and white light arc lamp. Blue light was separated using a bandpass filter and sent up to a charge-coupled device (CCD) camera (Teledyne QImaging, USA) via a dichroic mirror, and filtered using a bandpass filter (Chroma, USA). The CCD camera was connected to a computer, and controlled by Ocular (Teledyne QImaging, USA). Video was acquired at a rate of 1.67 frames per second. The following experimental procedure was followed: a harp was placed over a spinal cord slice, and the slice was then allowed to equilibrate for 15 minutes to reduce drift. A recording area was then determined by locating the myelin tracts, and aligning to a recording area of laminae I-II. Baseline recording of spontaneous activity was performed for 6 minutes. A second 6-minute recording was performed, where 10µM glutamate is added to the perfusion solution 2 minutes into the trial. Following completion of the second recording, the glutamate was washed out of the bath following a 20-minute rest period prior to the third recording. The third 6-minute recording similarly includes application of 25µM glutamate 2 minutes into the recording. For each experimental condition except for naïve mice, ipsilateral and contralateral recordings were performed.

3.4 Image Processing and Analysis

Construction of an automated harmonized preprocessing pipeline prepared calcium imaging recordings for individual and groupwise analysis through use of peer-reviewed analysis
tools and custom scripting in MATLAB (Mathworks Inc.) and ImageJ Macro Language (IJM) via FIJI/ImageJ’s plugin, ImageJ-MATLAB (Schindelin, Arganda-Carreras, & Frise, 2012). Specifically, this custom script was developed to be accompanied by MIN1PIPE (Lu et al., 2018), an open-source calcium signal processing toolbox selected for its additional motion control capabilities, seamless data import into MATLAB workspace, and automatic cell ROI selection. Automation of the harmonized pipeline was achieved through nested loops within custom MATLAB script, housing MIN1PIPE, ImageJ-MATLAB recruiters, and other custom MATLAB code, with capability to select one or multiple recording files within a file directory folder. Recording files had 16-bit greyscale tag image file format (TIFF) stack formatting, with frame dimensions of 4.00 inches in width and 2.24 inches in height. In their 16-bit resolution, recording files were imported with respective file sizes ranging from 1.8GB to 2.2GB.

Image preprocessing steps implemented prior to data import into MIN1PIPE were designed to maximize image signal-to-noise ratio, conserving significant components of object intensity over time while minimizing recording artifacts and random noise. While the constructed harmonized pipeline prioritized portability between data and processing systems, preprocessing steps were to be determined with comparison of quantitative scores derived from blind and reference dependent image quality indicators. To prescribe effective preprocessing steps for given recording datasets, an image quality assessment function written in MATLAB compared image quality outcomes of the respective application of common image filtering techniques employed by FIJI/ImageJ. Image filtering techniques to reduce noise included gaussian blur filtering, median filtering, mean filtering, and difference of gaussian filtering of varying magnitude. Following initial code execution via MATLAB, ImageJ-MATLAB
(Schindelin, Arganda-Carreras, & Frise, 2012) opened the given recording in FIJI/ImageJ and automatically applied a specified FIJI/ImageJ macro which included respective filtering techniques, data resolution standardization, and rigid body image stack registration. Rigid body image stack registration was implemented using StackReg (Thévanaz, Ruttimann, & Unser, 1998), where each frame in the recording was aligned to minimize drift. There is opportunity within the primary MATLAB script to select which FIJI/ImageJ macro should be recruited at this timepoint; several simple macros were written in IJM to reflect basic image preprocessing steps, and were recruited using this method to create iterations of differentially preprocessed recordings for direct comparison in MATLAB. IJM macros were written manually, however, they are capable of being generated using FIJI/ImageJ’s record function. As such, the described protocol is designed to be accessible to those who may not be familiar with coding in IJM or MATLAB languages. Following execution of the selected macro, the preprocessed recording was saved to the specified system directory as a multipage TIFF stack, and FIJI/ImageJ was ordered to end its active session. The image quality assessment function continued to automatically import this new preprocessed recording version into the MATLAB workspace, and began reference-independent and reference-dependent image quality assessments. Naturalness Image Quality Evaluator (NIQE), a no-reference image quality score, was respectively calculated for iterations of preprocessed recordings. NIQE is a peer-reviewed blind image quality assessment algorithm which does not require prior knowledge of typical image distortions or image scoring data (Mittal et al., 2013). NIQE was used in this context as a quality assessment of the raw calcium image recording, assessing local image distortions in reference to a global natural scene statistic model. Reference-dependent metrics such as signal-to-noise ratio (SNR),
the Rose Criterion, and peak signal-to-noise ratio were calculated using the preprocessed recording in reference to an imported, raw version of the same recording dataset. Peak signal-to-noise ratio served as a summative comparison of error between two images and was used in this context to assess reconstruction quality following preprocessing measures. The Rose Criterion described the quality of contrast-to-noise ratio, referring specifically to the detection limit of an imaging system (Bao & Chatziioannou, 2010). Should a given SNR value be less than 3 to 7, there is insignificant confidence in detection ability given the implicated image-based dataset (Bushberg et al., 2002). Assessment results were saved as components of a collective image quality assessment data structure, which was automatically saved to the system directory by the MATLAB function for comparison across preprocessing interventions.

Following adoption of preferred preprocessing techniques as described, a preprocessed version of the recording was saved as a TIFF stack within the system directory. The primary MATLAB harmonized pipeline code retrieved this preprocessed recording to be imported into the MATLAB workspace by MIN1PIPE toolbox. At this stage, input variables were set to spatially downsample by a degree of four, resulting in two-dimensional TIFF stack slices being reduced from 1749 pixels in width and 978 pixels in height with 16-bit resolution to approximately 437 pixels in width and 244 pixels in height. This downsampling was set to the general capacity of the system processing the recordings, in addition to the flexible JVM space allocated to MATLAB and ImageJ/FIJI which may be set manually ahead of time. While input variables were capable of temporally downsampling the recording, this was not performed. MIN1PIPE produced a two-dimensional matrix of identified cell ROI activity over the length of the recording, with dimensions $n_{cells} \times m_{stack\ frames}$. Custom scripting was applied to these matrices to
ensure candidate ROIs identified by MIN1PIPE reflected the activity of viable cells (i.e., minimal noise). This matrix was saved within the file directory as a solitary MATLAB data file to be recalled during groupwise analysis after being indexed based upon experimental group identity (e.g., glutamate challenge level, pain model).

3.5 Statistical Analysis

Mean PSNR and NIQE values of sample recordings were qualitatively compared across denoising methods. Residuals of mean PSNR values were plotted to qualitatively assess for normality. A one-way ANOVA with Bonferroni adjustment was performed to quantitatively compare mean PSNR values across denoising methods.

Groupwise assessment of individual recording calcium trace matrices was performed using custom MATLAB scripts, where individual and groups of recordings were recalled to compare within-slice recordings across glutamate challenges, as well as groupwise comparison of activity across chronic pain models and inflammatory sham group recordings. Average evoked response curves were produced for each group, and an average area under the curve (AuC) was calculated for each animal. Residuals of mean AuC values were plotted to qualitatively assess for normality. Groupwise comparison of mean AuC was performed using a two-way ANOVA, testing for main effects of glutamate level and experimental group in addition to a test for interaction between main effects.
Results

4.1a Developing a Harmonized Image Processing Pipeline

The developed image processing pipeline design included a targeted literature review and subsequent beta testing of peer-reviewed, open-source computational tools which addressed the identified regime needs. Beta testing used individual recordings from the experimental dataset, and was conducted in MATLAB and Fiji/ImageJ, noting processing time, manual parameter tuning requirements, and data format. Using a modified image processing framework from Stringer & Pachitariu (2019) and Robbins et al. (2021) (Figure 2), as well as identified dataset needs, the tools that were assessed primarily focused around image registration and ROI identification steps of analysis.

Image registration, or the process of aligning frames in a multidimensional

Figure 2. Calcium image preprocessing to optimize cell region-of-interest (ROI) identification. (A) Recording image stack is registered using rigid body registration to reduce image drift, and standardize image resolution (B) Recording image stack is denoised to improve image contrast and eventual image segmentation to distinguish cell ROI candidates from image background (C) Recording image stack is imported into the MATLAB workspace, and greyscale images are converted to respective matrices representing pixelwise relative fluorescence values (where dark blue represents low fluorescence, and yellow and red represent increasing fluorescence).
series of image recordings, is an important step in image processing which allows for the accurate identification of cell ROIs. The process corrects for dimensional drift caused by the microscope or biological factors, minimizing the possibility of signal contamination between cell ROIs or between cell ROIs and the neuropil (Figure 2A). A popular method of image registration for imaging at the subnetwork level is rigid body registration, where a given registration program rotates and translates images in a stack without rescaling or otherwise changing the imaging data itself (Thévenaz, 2011). There are several options for image registration available as MATLAB functions and Fiji/ImageJ plugins which have been peer-reviewed and are publicly available for experimental use. Despite the wide initial selection, many published tools were initially developed for use on a specific project, leaving little flexibility for other applications (e.g., tools only allowing analysis of human clinical imaging data despite tool operations being suitable for data at a cellular scale).

Elastix (Klein et al., 2010) is a Fiji/ImageJ plugin which was initially considered due to its selection of registration options available, as well as a robust graphics user interface and detailed system directory navigation features. Unfortunately, Elastix was observed to have limited efficacy when handling rigid body image registration techniques for 2D images. In addition, it was determined that the automated use of Elastix as a Fiji/ImageJ plugin through ImageJ-MATLAB was too computationally expensive for large volumes of data. The operating system quickly ran out of JVM, or ‘working memory’, when sequentially handling large-scale image recordings. An additional option that was considered included MATLAB’s `imregister` command, which is not designed specifically for medical or scientific imaging but provides highly detailed 2D and 3D registration of greyscale images (Mathworks, 2012). When tested on
a random sample recording from the experimental dataset with the use of an average intensity projection image as a static reference, this command’s performance was observed to be computationally expensive and time intensive with over two hours of processing time per recording.

Finally, TurboReg/StackReg (Thévenaz, 2011) are ‘sister’ ImageJ/Fiji plugins that are commonly used for the registration of microscopy image stacks or videos. While both TurboReg and StackReg are capable of rigid body registration (where StackReg’s function is dependent on TurboReg code in the system directory), TurboReg provides more options for data format and reference-based image registration. StackReg is specifically designed for image stacks and may be considered a lower capacity ‘front-end’ of TurboReg. While using ImageJ-MATLAB to interface ImageJ/Fiji and the MATLAB workspace, it was a significant challenge using and manipulating tools like TurboReg and StackReg to respect operating system JVM limits, as well as MATLAB’s internal allotted JVM. These tools were designed to primarily run with an open graphics user interface (GUI), which consumes a significant amount of a program or system’s JVM; custom scripting to automate ‘headless’ function of these tools (e.g., running the programs without launching the GUIs) to preserve system capacity was not accessible when using ImageJ-MATLAB interfacing. Both plugins are functional for single recording processing through ImageJ-MATLAB, however, StackReg was observed to provide effective registration of multiple image recordings at low computational cost. Due to its more robust features, TurboReg was observed to lag and crash when processing large volumes of image-based data, interrupting the automated image processing operation. As such, StackReg was included in the final harmonized pipeline due to its lightweight and effective function.
Identifying cell ROIs when processing calcium imaging recordings is a task which demands most of the discussed needs for calcium imaging data processing. High-capacity, biologically realistic, and accurate cell ROI identification is prerequisite to the effective characterization of recorded neural ensembles. There are several open-access and peer-reviewed calcium imaging data analysis packages which often address several stages of image processing and analysis, offering ‘end-to-end’ services which provide isolated calcium transients following the input of raw image recording files. Due to their high processing capacity and capability for automation, Suite2p (Pachitariu et al., 2016), EZCalcium (Cantu et al., 2020), and MIN1PIPE (Lu et al., 2018) all presented as attractive options. EZCalcium, while computationally robust, is a GUI-based tool that presented similar issues to registration tools such as TurboReg while batch processing, limiting the capacity for sequentially processing large volumes of data even while using automated functions. Suite2p was a more lightweight option than EZCalcium due to its modular script design and less dependency on user GUIs, however, its setup was not as straightforward as desired for inclusion in an accessible harmonized pipeline. MIN1PIPE was selected for incorporation into the harmonized processing and analysis pipeline as it best met the described dataset needs through its design and resulting function. During beta testing, it was observed that the tool was extremely simple to install with limited dependencies on additional external or internal MATLAB toolboxes, could run in a completely headless manner, and had a concise and easily manipulable set of tuning parameters to suit individual user data. While MIN1PIPE does offer ‘end-to-end’ service to isolate calcium transients of identified cell ROIs like EZCalcium, the open-access code is also designed in a modular fashion allowing for accessible omission of certain processing and analysis steps such as spike deconvolution. As
such, MIN1PIPE is an attractive option for a harmonized pipeline, where there is little risk of image processing measures being duplicated or the given images being otherwise overprocessed. It was also observed that MIN1PIPE addressed heterogeneity of image frame quality within recordings, pooling stable and unstable frames when assessing cell ROI locations over the entire recording time course. The algorithm output produced by MIN1PIPE includes low-dimensional ROI calcium transient matrices which may be easily used with custom MATLAB scripting and is immediately savable to the system directory.

Quality control of calcium imaging processing and analysis mitigates contamination of isolated calcium signal from identified cell ROIs, where image segmentation and ROI identification steps in image processing may be influenced by background noise or neuropil fluorescence. This ‘mistracing’ combines signal from multiple structures, which may consequently contaminate desired somatic signal or result in false positive cell ROIs. Where poor reported image quality may serve as an objective indicator for the integrity of identified cell ROI populations (i.e., confidence in low contamination, false ROI identification), a mechanism to report image quality was needed. The MIN1PIPE package also enabled motion correction scoring, where the algorithm assesses relative improvement of three-dimensional image drift before and after image processing. This scoring is conducted across the entire recording in a framewise manner and is useful to qualitatively monitor improvements in custom preprocessing measures implemented before data input into MIN1PIPE (e.g., comparison of image registration methods). Export of cell ROI calcium transients into a three-dimensional matrix (cell ROI x recording frame x relative fluorescence value) allows for accessible quality control with custom scripting following implementation of the MIN1PIPE algorithm, as well as
convenient data storage and indexing for the sequential analysis of large, discrete pieces of data.

Despite the efficacy of ImageJ-MATLAB, StackReg, and MIN1PIPE, there are aspects of image processing and analysis that were still unaddressed when solely using these programs. To meet dataset needs, custom scripting in MATLAB and ImageJ/FIJI was required. These scripts served several purposes, including logistical improvements such as enhanced pipeline automation and data management, as well as quantitative assessment of image recordings and extracted calcium signal. An important example includes custom scripting to determine if an incoming image recording is in greyscale format, and if not, to convert the image type to greyscale from RGB (colour) format (Figure 2C). Greyscale format is required for several types of processing and analysis measures for intensity images, and is not the same as an image simply being in black and white; this is a common mistake made during visual inspection of imaging data which has been mitigated by custom script.

In addition, a robust MATLAB function was developed to meet the need for improved quality control through automated quantitative assessment of incoming large-scale, image-based fluorescence data. The function, initially written to apply additional denoising measures to incoming calcium imaging recordings prior to MIN1PIPE, uses quantitative image quality indicators to compare and prescribe optimal denoising methods to a given recording (Figure 2B). Where previous literature has argued that the efficacy of popular image denoising methods may vary depending on factors such as initial signal-to-noise ratio (Robbins et al., 2021), comparison of image reconstruction quality validates image processing and analysis protocols which may vary widely between experiments and other discussed factors (i.e.,
biological, optical, and electronic sources of error). Assessment of global image quality before and after differential application of denoising procedures is conducted using estimated signal-to-noise ratio and application of the Rose criterion, as well as PSNR. The use of NIQE to determine optimal applied intensity of gaussian, median, and non-local means filtering provides an additional indicator to optimize image denoising and meets the need for pipeline automation through its ability to discern image quality without an opinion-based scoring database (Mittel et al., 2013).

Where the third identified dataset need discussed above involved capacity to address biologically realistic and time-dependent networks, an intermediate measure was required between image preprocessing and analysis of extracted signal. This was implemented using custom MATLAB script. The script assessed candidate ROIs identified by MIN1PIPE to exclude candidates whose mean relative fluorescence decreased over the course of the recording. Candidate ROIs whose mean relative fluorescence was net positive of any magnitude were retained.

The resulting harmonized pipeline consists of four interdependent scripts across MATLAB and Fiji/ImageJ platforms, and recruits three peer-reviewed, open-source tools (StackReg, ImageJ-MATLAB, and MIN1PIPE) (Figure 3) (Appendix B). Unless the user wishes to modify set parameters within the script to suit their own project, the only required input to the pipeline is their greyscale image-based data in TIFF or video (.avi) format.
Figure 3. Calcium image processing workflow, as part of a harmonized image processing pipeline. Using custom MATLAB script (1), raw calcium image recordings in the form of multipage TIFF stacks are analyzed within the system directory for basic file parameters (e.g. frame size, frame quantity, and file location). This script recruits ImageJ/FIJU using ImageJ-MATLAB (Schindelin, Arganda-Carreras, & Frise, 2012) to apply image preprocessing measures using a custom ImageJ/FIJU macro (2). The macro’s outputs, which include the preprocessed image recording and an average intensity projection image, are then saved to the system directory. These outputs are recalled by the general image processing MATLAB script (1) and imported directly to the MATLAB workspace as image objects. A custom image quality assessment MATLAB function (3) is recruited by the general image processing script. The function uses the average intensity projection image to generate and save quantitative metric outputs to the system directory and the MATLAB workspace for the general script’s use. The general script applies the denoising method recommended by the function to the calcium image recording, and it is then passed to MIN1PIPE calcium image processing toolbox (Lu et al., 2018) (4) for further processing and region of interest (ROI) identification. MIN1PIPE’s output includes MATLAB matrices containing calcium traces of identified candidate cell ROIs over time. MIN1PIPE output is returned to the general image processing function to assess candidate cell ROIs for false positive results.
4.1b Image Denoising Method Comparison

Custom MATLAB script allowed for the automated denoising of calcium image recordings and also collected quantitative image quality metrics to report the efficacy of image reconstruction. To determine which popular method of image denoising would be most effective for this dataset, methods were respectively applied to 24 calcium image recordings across all experimental groups. These calcium image recordings were from three animals of experimental groups using datasets that included both glutamate challenge levels. Upon initial visual inspection of the average intensity projections of denoised recordings, striking differences in denoising outcomes were observed (Figure 4A). Gaussian filtering, a local denoising filter, resulted in a very ‘smoothed’ average intensity projection, eliminating almost all low-intensity fluctuations across the entire recording. Median filtering, another local denoising filter, exhibited similar results, however application of this method resulted in substantial local image distortions. Non-local means filtering did not exhibit significant image distortion and preserved low-intensity contributions to the global fluorescence topology of the average intensity projection. As such, non-local means filtering initially appeared to be minimally disruptive to the image’s signal relative to other denoising methods.

This initial visual inspection provided context for subsequent quantitative indicators of effective image denoising. While non-local means filtering preserved image signal with minimal disruption, it was also found to improve image quality significantly better than the local denoising options (Figure 4B). A repeated measures one-way ANOVA was performed using the mean PSNR values of sampled denoised recordings using each described method. It was observed that mean PSNR differed in a statistically significant manner between denoising
methods\(F(2,46) = 48, p<.001\). Post hoc analysis with Bonferroni adjustment presented mean PSNR values were statistically different across all three groups, with non-local means filtering having a significantly higher mean PSNR than both gaussian blur (3.6 (99% CI, 3.3 to 3.9), \(p<.001\)) and median filtering (5.1 (99% CI, 4.7 to 5.4), \(p<.001\)) values. Additionally, the mean PSNR value of the gaussian blur results was significantly higher than that of the median filtering results (1.47 (99% CI, 1.2 to 1.7), \(p<.001\)). All 24 sample image recordings passed the Rose Criterion. As such, the image recordings met minimum SNR requirements for further image preprocessing and processing measures.

Interestingly, when denoised images were assessed independently using NIQE, the efficacy of the local denoising methods differed from that found using PSNR comparisons. Median filtering produced a lower mean NIQE score, more similar to that of non-local means filtering, whereas gaussian filtering produced a higher, suboptimal mean NIQE score (Figure 4C). This difference may be explained by the type of error that respective denoising methods mitigate, where improved PSNR may indicate reduction in noise or error due to lossy compression for a given image, and improved NIQE may indicate mitigation of several types of possible image distortion (Mittal et al., 2013). Given that non-local means filtering produced significantly higher mean PSNR and low overall mean NIQE (Figure 4C) compared to the other two methods, the image processing pipeline used this denoising method for all calcium imaging recording data.

Following selection of non-local means filtering as the denoising method of choice, NIQE was also used as a quantitative indicator to assess image quality across varied degrees of non-local means filtering (Figure 4D). The automated MATLAB image quality function used NIQE to
apply non-local means filtering to sampled calcium imaging recording average intensity projections using a gradient of degrees of smoothing. This gradient was developed using the estimated standard deviation of noise of a given recording as a central reference point. From this analysis, the pipeline’s optimized denoising procedure included application of non-local means filtering with 0.0013 degrees of smoothing for all calcium imaging recordings in the dataset.
Figure 4. Quantitative assessment of image denoising methods, as applied to rodent spinal dorsal horn calcium imaging data. A) Topographical plots of an average intensity projection of a spinal dorsal horn calcium imaging recording across image denoising methods. Representative calcium imaging recording is included in the SNI experimental group animal before glutamate challenge. B) Comparison of mean peak signal-to-noise (PSNR) ratios of image reconstructions following denoising of 24 calcium imaging recordings across experimental groups following one-way ANOVA. Recordings were from three randomly selected animals across all groups (9 SNI, 9 OA sham, 6 OA). Non-local means filtering was observed to produce processed images with significantly higher PSNR values compared to gaussian blur and median filtering techniques. There was no significant difference in PSNR values between gaussian blur and median filtering image outcomes. C) Naturalness image quality evaluator (NIQE) scores of denoised calcium imaging recordings plotted against respective PSNR scores across image denoising methods (n = 24 calcium image recordings). D) NIQE values across non-local means filtering intensity gradient, applied to spinal dorsal horn calcium imaging average intensity projection.

4.2 Quantifying Evoked Subnetwork Activity in the Superficial Dorsal Horn

Application of the harmonized preprocessing tool to the described experimental dataset resulted in multiple individual recordings being removed due to low image quality. This low quality manifested primarily at the stage of ROI identification through the use of MIN1PIPE, where the algorithm would not identify any or very few (i.e. less than 5) viable cell ROI candidates over the course of the recording. Isolated cell ROI candidate traces in the latter case were often not viable, where they were either quite noisy or experienced considerable rundown of signal over time. Approximately 60% of the collected image recordings were retained following image preprocessing and processing measures for groupwise analysis. Retained image recordings were evenly distributed across all animals in experimental groups, and only three animals were lost due to low image quality, with one animal missing from each group. As such, groupwise analysis proceeded with a total of 14 animals (osteoarthritis model = 6, osteoarthritis sham = 4, spared nerve injury = 4).
Assessment of candidate cell ROIs from MIN1PIPE was conducted to exclude those that could be considered as outliers or not biologically realistic (e.g., exhibiting significant ‘rundown’ of signal without any evoked response). This was done by visually inspecting the fit of averaged candidate cell ROI traces across animals when generating lines of best fit reflecting second, third, and fourth degree polynomials. It was observed that only fourth degree polynomials conserved biologically realistic glutamate response dynamics across several recordings. As such, each candidate cell ROI was fitted to a fourth degree polynomial line of best fit, and the latter’s global slope was measured. Traces whose lines of best fit were net positive were retained, and those whose lines of best fit had net negative slope values were excluded from groupwise analysis.

Identified cell ROI population volumes per tissue slice for each experimental group across all animals were observed as follows: 11.47 from osteoarthritis image recordings (10.58 cell ROIs per slice from 10µm glutamate challenge and 12.36 cell ROIs per slice from 25µm glutamate challenge), 41.41 from osteoarthritis sham image recordings (17.33 cell ROIs per slice from 10µm glutamate challenge and 65.5 cell ROIs per slice from 25µm glutamate challenge), and 12.4 from the spared nerve injury image recordings (13.17 cell ROIs per slice from 10µm glutamate challenge and 11.62 cell ROIs per slice from 25µm glutamate challenge).

Retained cell ROIs for each image recording were stored together in common matrices for groupwise analysis. Each matrix was averaged along its first dimension, the number of cell ROIs in a given image recording matrix, to produce an averaged calcium response curve for each recording file. These averaged calcium response curve vectors were similarly averaged
again to produce average responses for each animal, and then again to produce average responses, with associated standard errors, across experimental groups and glutamate level.

Upon comparison of average normalized calcium signal across experimental groups, it was observed that they all exhibited a clear response to the 25µm glutamate challenge (Figure 5). Average evoked responses were observed to increase consistently following glutamate challenge application at 150 seconds into the recording – a timepoint where glutamate had sufficiently perfused into the recording chamber and tissue slice to reach the target SDH neurons. The responses may be observed to peak between 275-325 seconds into the recording before a considerable reduction in response at the end of the evoked potential trial.
Figure 5. Average normalized fluorescent calcium signals from dorsal horn ROIs in spinal cord sections from osteoarthritis (n = 6), osteoarthritis sham (n = 4), and spared nerve injury (n = 4) experimental groups, included evoked responses following 25µM glutamate challenge, plotted with respective timewise standard error. Timepoint for glutamate challenge to perfuse onto target tissue at sufficient concentration estimated to be approximately 150 seconds into calcium imaging recording, following a washout period from previous 10µM glutamate challenge.
Figure 6. Average normalized evoked calcium response across osteoarthritis (n = 6), osteoarthritis sham (n = 4), and spared nerve injury (n = 4) experimental groups following 10µm and 25µm glutamate challenges. Glutamate challenges were applied sequentially, with 15-minute washouts between increasing glutamate challenge trials. Timepoint for glutamate challenge to perfuse onto target tissue at sufficient concentration estimated to be approximately 150 seconds into each recording.
The pattern of an elevated plateau calcium response was also observed during the initial 10 µm glutamate challenges across all groups (Figure 6). It therefore appears that identified viable SDH cells are responsive and resilient to increasing levels of glutamate challenge, where there is no perceivable decrease in response as the maximum glutamate challenge is applied following intermediate glutamate challenge and washout. It is noted that the osteoarthritis sham group does exhibit a higher degree of variability, with particular attention brought to the maximum glutamate challenge subset, compared to the osteoarthritis model and spared nerve injury model groups. The magnitude of evoked responses were quantified through calculating area under the curve (AuC) from 150 seconds onwards for the normalized average response of each experimental animal (Figure 7). When comparing mean AuC values across experimental groups and glutamate challenge level, the maximum glutamate challenge elicited a higher mean response. A two-way ANOVA was performed using the mean AuC values of averaged animal calcium traces across experimental groups and glutamate challenge. It was observed that mean AuC was not significantly different across experimental groups \((F(2,22) = 1.186, p = .324)\). There were no significant interactions present between glutamate level and experimental group \((F(2,22) = 0.298, p = .745)\), contributing to a valid main observed effect of glutamate. Across experimental groups, there was a significantly higher mean AuC in response to the 25 µm challenge compared to the 10 µm challenge \((F(1,22) = 5.210, p = .032)\) (Appendix A).
Figure 7. Mean glutamate-evoked area under the curve calculated from average normalized evoked calcium responses of superficial dorsal horn ROIs across osteoarthritis (n = 6; SEM 10 = 36.79, SEM 25 = 46.10), osteoarthritis sham (n = 4; SEM 10 = 35.12, SEM 25 = 75.72), and spared nerve injury (n = 4; SEM 10 = 24.93, SEM 25 = 25.05) experimental groups following 10µm and 25µm glutamate challenges.
Discussion

5.1 Development of a Harmonized Image Processing Pipeline

This study sought to quantify evoked subnetwork dynamics within the SDH across inflammatory and neuropathic models of chronic pain. This was implemented following the development of a custom harmonized image processing pipeline. The resulting pipeline incorporated best practices from peer-reviewed computational tools and custom scripting in MATLAB and ImageJ/Fiji Macro Language to automatically isolate and quantify large volumes of individual calcium transients for further groupwise analysis.

As mentioned, the final design of the presented harmonized image processing pipeline has struck a careful balance between accessibility and robust data processing and analyses. The selection of peer-reviewed tools in the pipeline such as ImageJ-MATLAB, StackReg, and MIN1PIPE were similarly included as programs that were effective, but lightweight with regards to computational expense (e.g., required processing capacity for a given operating system, time required to process given datasets). Their integration with custom scripting and across programming platforms presented a considerable need for the formats of their inputs and outputs to be accessible to users across most steps of image processing. As previously mentioned, MIN1PIPE does offer ‘end-to-end’ service like other similar calcium imaging toolboxes, however its modular script design and accessible output variables ultimately made it a superior option in this regard. Results are easily interpretable and accessible for use by subsequent custom scripts and other programs, and may be easily extracted at any stage of processing for user assessment. This quality of simple integration with other parts of the pipeline reduces overall computational expense as well as risk for error, where certain
transformations of image-based data for analysis or storage purposes may introduce unwanted distortions such as compression.

The final pipeline is capable of automatically producing high-dimensional, time-dependent calcium transients from large volumes of raw calcium imaging data. Where automation presents accessibility challenges to those unfamiliar with coding but in need of high-capacity image processing, the pipeline effectively replaces calcium image analysis steps such as manual tracing. This considerably reduces human error in these data analyses, where there is no opportunity for human bias or error in identifying cell ROIs or quantifying their activity. It is clear that the development of this lightweight harmonized pipeline fills a considerable gap in available analysis options for calcium imaging processing between laborious manual tracing and calculations, and ‘one-click’ calcium imaging toolboxes which risk disuse or misuse due to their inaccessibility. As such, the development of the presented tool could be considered a sustainable approach to quantifying cell subnetwork activity, where best practices in peer-reviewed tools are integrated and mobilized by additional custom scripts to be used for a variety of experimental applications.

Limitations of the present version of the pipeline includes chronic working memory issues with regards to MATLAB and StackReg, respectively. MATLAB’s ‘leaky memory’ issues, where it does not consistently clear temporary variable information, can sometimes interrupt sequential processing of large-volume data files. This has been mitigated using prompts to clear JVM and large unneeded variables upon each sequential iteration of the pipeline. This ‘leaky memory’ is a chronic but not formally recognized issue with MATLAB, and is a common topic of discussion across its userbase. StackReg’s inability to run in a ‘headless’ manner when
integrated with ImageJ-MATLAB similarly interferes with processing capacity due to its large JVM requirement. Further fine-tuning of the interfacing between MATLAB and Fiji/ImageJ in future versions of the pipeline may solve this issue, as well as the use of custom JVM clearing functions in ImageJ/Fiji. For this study’s dataset, processing capacity in this regard was not prohibitive to implementing Objective 2, however, these changes may be of interest for future use of the pipeline with even larger volumes of image-based data.

5.2 Image Quality Assessment

The development of an image quality assessment function within the image processing pipeline allowed for the prescription of a denoising method ‘of best fit’ to be used on the calcium imaging recording dataset analyzed in Objective 2. This feature is a great contributor to the aforementioned sustainability of the developed image processing pipeline, where the image quality metrics used are not specific to calcium imaging or even neural data, and may be integrated into a large number of image-based experimental workups.

Quality and quantity of denoising filters were assessed on a subset of calcium image recordings across all experimental groups and glutamate challenge levels based on both original image and image reconstruction quality. This was quantified using NIQE and PSNR, respectively. Comparison of qualitative or visual denoising filter outcomes with quantitative outcomes (i.e. NIQE and PSNR values) was striking not only in determining that the use of non-local means filtering was significantly more effective, but also how these contrasting outcomes led to that conclusion. Where visual inspection of average intensity projections in Figure 4B did indicate which denoising methods ‘over-smoothed’ or distorted data, it must be noted that without quantitative indicators, the function of effective denoising methods such as non-local means
filtering may be overlooked or misinterpreted. Minimal disruption of image signal observable through qualitative visual inspection of average intensity projection topology may have suggested lack of effect if not for the robust PSNR results indicating otherwise. This is a poignant example demonstrating the need for widespread objective image quality assessment and optimization in image-based data processing and analysis.

5.3 Studying Image Analysis Techniques & Future Directions in Tool Development

Formal study of computational tools and analysis techniques regarding image-based data processing in the life sciences is an emerging topic of interest (Aaron & Chew, 2021; Stringer & Pachitariu, 2019). Most development in this area is driven by individual need for computational or analytical tools to be used for a given dataset or project, with many presently available open-source tools being further developed over time to lend flexibility for others’ use. The presented harmonized pipeline (Appendix B) and its modules was developed with this trend in mind with the added consideration that many individuals wishing to use it in the future may not know how to extensively code in MATLAB. As such, the existing version of the harmonized pipeline specifically includes interfacing to ImageJ/Fiji to allow for any custom macro to be included or edited to suit a user’s desired image processing regime. Where ImageJ/FIJI scripting is possible using open-source wrappers and other minor script adjustments (e.g., Javascript, Groovy, R, Python), custom macros can be developed in ImageJ/Fiji using the record function without the user coding at all. This accessibility measure serves as another sustainability feature of the presented pipeline whose individual modules or entire script may be used for future projects.
A final consideration for future directions of the developed pipeline focuses on the image quality assessment function. Where the function has been successful in rapidly assessing image reconstruction quality in an automated fashion (i.e. without the need for user supervision or intervention), there is opportunity for further development to achieve intelligent function. NIQE was selected as a holistic image quality assessment indicator primarily due to its ability to perform while blind, or without any single image reference or repository of scored images. This image quality assessment function could indeed be adapted to suit an image quality assessment algorithm that uses repositories of images with human opinion-based scores to train with. This may result in more robust assessment of image quality, and allow for the algorithm to discriminate against or even automatically correct more complex or specific sources of error for neural image data processing (e.g., learning anatomical markers in an image to correct for image orientation).

5.4 Quantifying Evoked Potentials of SDH Neural Subnetworks

Lack of significant differences observed in evoked response magnitude across experimental models of inflammatory and neuropathic pain was reflected both in comparison of average response (Figures 5 and 6) as well as direct comparison of mean AuC (Figure 7). The clear presence of increasing evoked response across experimental groups following sequential applications of glutamate challenge demonstrates presence of viable, reactive SDH subnetworks following experimental inductions of chronic pain states. While results indicate no significant difference between the osteoarthritis sham and experimental models, it could be considered that similar responses across groups may indicate a degree of global resilience following different types of substantial damage to the nervous system. While hypersensitivity
through increased average evoked response was not observed, neither was a decreased or erratic one observed.

Findings from Objective 2 may support the future use of glutamate challenges as a means of assessing subnetwork activity (Doolen et al., 2012; Harding, Fung, & Bonin, 2021), but may also prompt further investigation using other, more modest stimulation protocols. This could mitigate certain common response dynamics observed across groups such as reduction of response towards the end of recording. This dynamic suggests accumulation of intracellular calcium due to sustained synaptic activity and neuronal firing during continuous glutamate administration. Lower concentrations of glutamate, short puffs of locally-applied glutamate, or alternative methods of generating more modest action potential firing could be used. This could produce more acute calcium transients with observable event dynamics as opposed to the sustained increase in activity observed in Objective 2 results. Treatments which induce synaptic inhibition such as bicuculine or strychnine (Yaksh, 1989) or electric stimulation of the primary afferents of the dorsal root (Jeftinija, Urban, & Kojic, 1993) serve as compelling examples whose experimental use may further understanding of superficial SDH subnetwork dynamics.

5.5 Limitations and Future Directions of Subnetwork Characterization

Application of the developed harmonized image processing pipeline resulted in a considerable number of image recordings being excluded due to poor image quality. As mentioned, circumstances leading to these exclusions largely consisted of compromised image quality prohibiting tools such as MIN1PIPE from executing when prompted. In this respect, poor image quality may have additionally contributed to the non-local means filter from Objective 1
performing significantly better on the sample dataset. It must be considered that a proportion of the sample image recordings used for the image quality assessment experiment were not included in Objective 2’s groupwise analyses, however, there was equal representation from all animals used in Objective 1’s experiment.

Previous literature does note the utility of non-local denoising methods for lower resolution images (Robbins et al., 2021) compared to popular local denoising filters such as gaussian blur and median filtering. As mentioned, types of error in image-based neural data can become quite complex and the product of multiple biological and technical sources. Despite the harmonized image processing tool’s success at assessing and optimizing image quality for cell ROI identification and analysis, the largest limitation of Objective 2’s outcome remains to be its substantial amount of error. Attempts to mitigate this issue is reflected in the harmonized pipeline’s design. Reviews of fluorescence image-based data processing and analysis processes often conclude at the point of cell ROI signal extraction, whether the pipeline outputs use calcium traces or deconvoluted spike trains to study subnetwork dynamics (Aaron & Chew, 2021; Robbins et al., 2021; Stringer & Pachitariu, 2019). Experimentally relevant signal, often taking the form of cell ROI transients or spike trains, is not widely addressed at these stages. Similarly, historical use of MIN1PIPE has resulted in users applying their own significant event criteria for further analysis (Barkai et al., 2021) as well as relying on the manual ROI selection feature to accurately navigate and analyze their data (Bagramyan et al., 2021). This inspired the custom scripting to assess candidate ROIs, as described in Objective 1’s methods.

The osteoarthritis sham’s maximum glutamate challenge recordings were observed to have considerably high variance. This group was the only one without a normal distribution,
making it difficult to assess for main effects between experimental groups and glutamate challenge. It also resulted in ipsilateral and contralateral tissue slices being placed together for groupwise comparison, as separating them would have resulted in underpowered statistical analyses. It should be noted that using manual ROI selection and associated calcium signal analyses on ipsilateral slices only, some significant differences were found for glutamate-evoked calcium responses between experimental groups (Bonin lab, data not shown). Given that many changes in excitability at the level of the superficial dorsal horn are observed ipsilaterally across chronic pain models (Goff, Burkey, & Jasmin, 1997), future experiments using contralateral slices as controls only are likely to exhibit different results. Due to aforementioned quality issues, certain metrics such as total cell count or event-specific dynamics were not a point of focus due to the over- and under-selection of candidate cell ROIs by MIN1PIPE because of this discussed issue with image quality and consequences on image segmentation. While measures to assess and exclude nonviable cell ROIs were reviewed to minimize bias (i.e., inspection of excluded candidate cell ROIs), the widespread quality control issues may place bias against experimental groups with relatively higher variability or lower overall image quality as they would likely produce less viable cell ROIs. Calculating mean AUC across glutamate challenges and experimental groups provided a more holistic means of quantifying evoked response magnitude, where described bias was mitigated despite unequal variance between groups. The findings of Objective 2 provide evidence against differential magnitude of evoked SDH cellular responses across models of chronic pain, however, the above limitations should encourage further investigation in this regard.
There is considerable opportunity to continue this research given the outcomes of Objectives 1 and 2 of this study. Studying spontaneous activity over time in these subnetworks using similar methods could provide valuable information concerning functional baselines across chronic pain states, increasing understanding of their perpetuation over time.

Spontaneous activity has been observed to increase following induction of chronic pain states (e.g., peripheral nerve injury) (Keller et al., 2007), followed by the onset of shifted circuit dynamics even when the initial injury no longer produces acute symptoms. This increase in spontaneous activity may manifest through factors such as increased firing rate, synchronous firing, and reduced firing thresholds (Roza et al., 2016). Assessment of spontaneous activity provides the opportunity to investigate determinants of spontaneous pain, particularly when used as a baseline for evoked potentials within the circuit. It could be hypothesized that viable, responsive SDH neurons within chronic pain subnetworks would exhibit increased spontaneous activity compared to shams or controls.

Studying evoked potentials could be continued using this project’s methods in addition to other discussed experimental techniques such as optogenetics to begin to parse the many different cell types which respectively contribute to subnetwork dynamics. Additional experiments to explore these relationships could include RNA sequencing or immunohistochemistry to supplement understanding at the subcellular level. It may also be valuable to consider replicating experiments using electrophysiology methods at the subnetwork level such as with multielectrode arrays. Where calcium imaging has considerable spatial resolution, population-level electrophysiology may capture time-sensitive subnetwork event dynamics. A final consideration is that it may be of interest to continue investigations
using female rodent models; as sexual dimorphisms have been increasingly observed across rodent models of chronic pain (Dedek et al., 2022), sex as a biological variable should be considered in all preclinical and clinical pain studies.

**Conclusions**

The presented harmonized image processing pipeline provides a novel and accessible tool for neural and life sciences image data processing and analysis. Its development met considerable and ongoing experimental need for objective image quality assessment in life sciences research, and serves as a sustainable option which may replace and challenge historical manual analysis methods and ‘end-to-end’ imaging analysis toolboxes, respectively. There were no significant differences in evoked response magnitude in the superficial SDH across osteoarthritis model, osteoarthritis sham, and spared nerve injury experimental groups. However, all groups demonstrated clear response to increasing levels of glutamate challenge, supporting the efficacy of these challenges in activating superficial SDH functional subnetworks. Future approaches to study modestly evoked and spontaneous SDH excitability are therefore needed to identify potential subtle changes in SDH signalling across models of chronic pain.
References


Gutierrez-Mecinas M, Davis O, Polgár E, Shahzad M, Navarro-Batista K, Furuta T, Watanabe M, Hughes DI, Todd AJ. Expression of Calretinin Among Different Neurochemical Classes of Interneuron in the


### Appendix A: Supplementary Table 1. Statistics Summary Table

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<th>Test</th>
<th>P value</th>
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<td>Figure 7</td>
<td>Interaction between Experimental Group and Glutamate Challenge Level</td>
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### Appendix B: Objective 1 MATLAB and ImageJ/FIJI Scripts

Dropbox Link to View-Only Scripts: https://www.dropbox.com/home/Stephanie%20Thesis