Utilizing multiphoton imaging and integrative clearing to reveal sex differences in neuroimmune interactions after nerve injury

Zachary W. Castillo, Michael D. Burton*

With the constant development of multiphoton microscopy, our ability to observe complex and dynamic biological processes deeper within living tissue, is steadily improving. Researchers use multiphoton microscopy, because experiments can be conducted with little to no invasiveness or tissue damage over a long period of time with no photodamage (Mancuso et al., 2009). This allows for the introduction of tissue into the context of a three-dimensional (3D) environment in which visualization of cellular activation and interaction is viable. By circumventing a distorted reconstruction with limited z-stacks, multiphoton imaging provides enhanced spatiotemporal resolution.

Peripheral injury, immune cell activation, and the development of pain: Peripheral nerve injury is the result of trauma that disrupts the peripheral nervous system which can result in neuropathic pain. Circulating monocytes and tissue macrophages play a key role in facilitating maladaptive pain processing following nerve injury (Zhuo et al., 2011; Peng et al., 2016; Szabo-Pardi et al., 2021a). Recent studies have shown that macrophages demonstrate direct molecular crosstalk with sensory neurons to facilitate neuropathic pain (Yu et al., 2020) and use of proximity, morphology, can be used to determine interaction and activation states (Szabo-Pardi et al., 2021b).

This communication is an integral process of both the induction of pain as a tool of self-preservation and is also important to its transition into maladaptive chronic pain in certain instances (Grace et al., 2021). Previous studies have found sex differences in the prevalence and perception of pain in males versus females and have implicated neuroimmune crosstalk (Mogil, 2012). For this reason, discovering the sex-dependent role that macrophages have in tissue injury is critical as the incidence of macrophage-dependent chronic pain in females is lower than in males (Agalave et al., 2021; Rudjito et al., 2021). For these reasons, identifying sex differences in macrophage biology will serve as a foundation for future studies which seek to use the immunological component of pain regulation as a tool for a tailored approach to therapeutics. Recently our group has utilized intravital imaging techniques with transgenic reporter mice and multiphoton microscopy (Szabo-Pardi et al., 2019). To conceptualize the immune response to injury we adapted these methods to be able to study tissues pertinent to sensitization (Szabo-Pardi et al., 2021b). Through optical clearing (ScaleS1), we were able to visualize tdTomato-tagged macrophages via multiphoton microscopy and advanced 3D image analysis to provide a deeper understanding of interactions in peripheral nervous tissue after injury.

Multiphoton imaging: intravital and cleared tissue: In a previous study, we developed a novel in vivo technique using multiphoton microscopy to assess the ability of the endotoxin, lipopolysaccharide (LPS) FITC-conjugated (LPS-FITC) to bind to its receptor, toll-like receptor-4 (TLR4), expressed on peripheral fibroblast. We used a genetic reporter mouse line, Fibroblast specific protein 1(FSP1): cre crossed with Rosa26CreERT2 (FSP1CreERT2). For proof-of-concept experiments, we showed that after injection of LPS-FITC, FSP1CreERT2 fibroblasts interact and uptake LPS-FITC. In contrast, whole-body TLR4 knockouts did not interact or uptake LPS-FITC after injection. This unique approach enables us to create detailed, time-lapse videos and/or images of proteins interacting with live cells that allowed for improved comprehension of how proteins can alter cellular behavior and kinetics of those interactions. Studying protein-receptor interactions in vivo provides advantages by allowing the clear capture of how cells respond to a stimulus in real time, and in their native microenvironment without the unpredictable influence of post-mortem tissue extraction.

More recently, we explored the potential of this technique by adapting it ex vivo, by isolated tissues extracted from the peripheral nervous system to assess the neuroimmune response to injury with animals that have tdTomato in LysM positive macrophages, LysMcre × Rosa26CreERT2LSL (LysMCreERT2LSL). Using the spared nerve injury model of neuropathic pain, an injury model where two branches of the sciatic (the peroneal and tibial nerve) were transected, leaving the sural nerve left intact (Decosterd and Woolf, 2000), this model leads to robust pain states and is immune cell-dependent. Macrophages in the dorsal root ganglia (DRG) and sciatic nerve (ScN) both play key roles in the initiation and maintenance of neuropathic pain. Hence, by clearing whole DRGs and ScNs within 5 days post-surgery, we were able to image intact tissue collected from reporter animals using multiphoton microscopy. Cleared, agorase embedded DRGs and ScNs were imaged using an Olympus MPE-RS TWIN multiphoton microscope with dual excitation lasers in combination with a 25× MPE water-immersion objective. Proper multiphoton visualization of neuroimmune interactions is made possible through reconstruction into a 3D model which allows us to determine the spatial resolution of macrophage tissue infiltration/proliferation, as well as the spatial relationship between neuronal and non-neuronal immune cells. In this study, we found that males have higher LysMCreERT2 macrophage counts in the lumbar DRGs, expanding on current literature which suggests females have alternative neuroimmune mechanisms which contribute to pain states. Additionally, we found dynamic changes in the morphology of these macrophages, with spared nerve injury-inducing a pro-inflammatory, or M1 polarized, phenotype as measured by cell shape. These findings are important to improve our understanding of the sex-dependent distribution and the role of other immune cells in the development of pain. Much of our current understanding in macrophage functionality is based on soluble factors, such as cytokine production and cell surface protein expression. While useful in providing a framework to isolate the molecular component of macrophage activation in response to injury, bridging the gap between functionality and physical characteristics will elucidate the full scope of their involvement in tissue injury.

Literature has shown that polarized macrophages express distinct morphological changes differentiating into M1 and M2 phenotypes, pro-inflammatory or anti-inflammatory respectively. These macrophages are associated with an elongated morphology while M1 morphology is associated with a flattened morphology (Bertani et al., 2017). These can be measured in the third dimension as both are ellipsoids which form a spectrum with a perfect sphere linking the two. For our experiment, we used Imaris software (Oxford instruments, version 9.0.1) as our 3D image analysis software to understand the dynamics of macrophage recruitment, activation, and morphology after injury (Figure 1). Pixels are converted into voxels from which a computer-generated representation of the original 3D object is created for the channel of interest and from which volumetric and morphological data is derived. The drawbacks of prototypical immunohistochemistry such as occurrences of non-specific antibody binding or fluctuation in fluorescence intensity across trials are circumvented with genetically tagged reporter animals. This consistency in fluorescent labeling can be carried over to the analysis. Traditional imaging acquisition and analysis hold limitations when analyzing this marker of phenotype for several reasons. Tissue depth and resolution of the acquired images are critical to obtaining the granularity which optimizes the use of 3D image analysis. Proper depth of the image stacks are critical for optimal visualization and localization of cells in the context of their natural environment. Further, it can improve the accuracy of automated analysis. By fully capturing the tissue you avoid processes and fragments of cells in the edge of the frame which can either require manual intervention to correct or a filter which may result in the unintended exclusion of captured cells. When recreating the object of interest in a 3D space, the
resolution of the image affects the detail in which morphology is represented in the analysis. With 3D image analysis, this enhanced surface detail can yield insight into activation state associated morphological alterations. These techniques and analysis can also be used to provide a more comprehensive data set than that obtained from 2D image analysis across different research interests. With spatiotemporal analysis, tracking algorithms can be utilized which allow the tracking of objects of interest within a time-resolved data set in 3D space. This allows you to generate quantitative data on the movement and trajectory of moving objects individually and in relation to each other. Like the surfaces created for measuring morphology, a similar technique can be used to trace and detect dendrites and synapses in neurons as well as the processes of other structures such as endothelial cells, astrocytes, and microglia. The dynamic changes in cell shape which can occur in response to stimuli can also affect the morphology of these processes which speaks to the need for the full utilization of the technique. 2D image reconstruction of finite z-stacks can provide skewed information due to the variation in section recovery and cellular density throughout selected regions of interest. Optical clearing techniques circumvent this issue, facilitating the imaging of deep tissues by minimizing light refraction. Many clearing methods exist and for our purposes, we chose Scale S1 as it has been shown to avoid tissue expansion, preserve lipids and provide a safe immersion medium for objectives (Hama et al., 2015).

### Implications and conclusion

The methods described here provide benefit over existing methods to visualize the activation and spatiotemporal interactions between cells. Performing *in vivo* allows for real-time visualization of cells binding and interacting with other cells and molecules which is indicative of activation with specificity to an insult. In addition, using multiphoton microscopy in this experimental setting reduces the rate at which photobleaching occurs, allowing for continuous and longer-lasting sessions which hold significant value when being applied in studies investigating the rate of metabolism or long-term activation. When studying cell populations not localized near the skin, the clearing of whole tissues in situ can reveal neuroimmune interactions that have been overlooked in the past. In addition to measuring morphology-dependent phenotype changes, 3D analysis packages such as Imaris provide an array of multifaceted tools to quantify different aspects of cells in a 3D space. The combined use of our genetic reporter animals, tissue clearing, and multiphoton microscopy serve as a powerful tool for investigating cell activation and spatiotemporal relationships and provides the framework for users to modify to suit their research interests.

This work was supported by NIH grant K22NS096030 (to MDB), American Pain Society Future Leaders Grant (MDB), Rita Allen Foundation Award in Pain (to MDB), and The University of Texas System STARS program research support grant (to MDB).

### References

Agalave NM, Rudjito R, Farinotti AB, Khonsari PE, Sandor K, Nomura Y, Szabo-Pardi TA, Urbina CM, Polada V, Price TJ, Erlendsson Harris H, Burton MD, Kultima K, Svensson CI (2021) Sex-dependent role of microglia in disulfitide high mobility group box 1 protein-mediated mechanical hypersensitivity. Pain 162:446-458.  
Bertani FR, Mozetic P, Foramonti M, Iuliani M, Ribelli G, Fantino F, Santini O, Torini G, Trombeta M, Businaro L, Selci S, Rainer A (2017) Classification of M1/M2 polarized human macrophages by label-free hyperspectral reflectance confocal microscopy and multivariate analysis. Sci Rep 7:8985.  
Decosterd I, Woolf CJ (2000) Spared nerve injury: an animal model of persistent peripheral neuropathic pain. Pain 87:149-158.  
Grace PM, Tawfic VL, Svensson CI, Burton MD, Loggia ML, Hutchinson MR (2021) The neuroimmunology of chronic pain: From rodents to humans. J Neurosci 41:855-865.  
Hama H, Hicki H, Namiki K, Hoshida T, Kurokawa H, Ishidate F, Kaneko T, Akagi T, Saito T, Saito T, Miyawaki A (2015) ScaleS: an optical clearing palette for biological imaging. Nat Neurosci 18:1518-1529.  
Mancuso JJ, Larson AM, Wensel TG, Saggau P (2009) Multiphoton adaptation of a commercial low-cost confocal microscope for live tissue imaging. J Biomed Opt 14:034048.  
Mogil JS (2012) Sex differences in pain and pain inhibition: multiple explanations of a controversial phenomenon. Nat Rev Neurosci 13:859-866.  
Peng J, Gu N, Zhou L, B Eyo U, Murugan M, Gan WB, Wu Li (2016) Microglia and monocytes synergistically promote the transition from acute to chronic pain after nerve injury. Nat Commun 7:12029.  
Rudjito R, Agalave NM, Farinotti AB, Lundsback P, Szabo-Pardi TA, Price TJ, Harris HE, Burton MD, Svensson CI (2021) Sex- and cell-dependent contribution of peripheral high mobility group box 1 and TLRA in arthritis-induced pain. Pain 162:459-470.  
Szabo-Pardi TA, Agalave NM, Andrew AT, Burton MD (2019) In vivo two-color 2-photon imaging of genetically-tagged reporter cells in the skin. J Vis Exp doi: 10.3791/59647.  
Szabo-Pardi TA, Agalave NM, Burton MD (2021a) The role of microglia versus peripheral macrophages in maladaptive plasticity after nerve injury. Neural Regen Res 16:1202-1203.  
Szabo-Pardi TA, Syed UM, Castillo ZW, Burton MD (2021b) Use of integrated optical clearing and 2-photonimaging to investigate sex differences in neuroimmune interactions after peripheral nerve injury. Front Cell Dev Biol 9:624011.  
Yu X, Liu H, Hamel KA, Morvan MG, Yu S, Leff J, Guan Z, Braz JM, Basbaum AI (2020) Dorsal root ganglion macrophages contribute to both the initiation and persistence of neuropathic pain. Nat Commun 11:264.  
Zhuo M, Wu G, Wu Li (2011) Neuronal and microglial mechanisms of neuropathic pain. Mol Brain 4:85.