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Review Article

Calcium imaging approaches in investigation of pain mechanism in the spinal cord

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ABSTRACT

The continuous advancement of microscopic imaging techniques combined with the discovery and use of more powerful calcium indicators has made calcium imaging technology much more effective and has increased its use in the study of pain circuitry. Using calcium imaging to study spinal pain mechanisms causes less damage to animals compared to electrophysiological techniques and is also able to observe the firing pattern of spinal neurons and the connections between them on a large scale. These advantages allow any changes in spinal cord circuits caused by pain transmission to be observed more effectively. This review will discuss the development of calcium indicators over the past decades as well as the various applications of calcium imaging, from *in vitro* to *in vivo* spinal cord experiments, in the study of pain circuits. We will also discuss possible directions for the study of spinal pain circuits in the future.

1. Introduction

The International Association for the Study of Pain (IASP) defines pain as an unpleasant sensory and emotional experience associated with actual or potential tissue damage, which is often described in terms of such damage. Throughout the history of pain research, many have put forth theories to delineate non-pain from pain modalities. The specificity theory states each modality (touch and pain) is encoded in separate pathways while the intensity theory states that low levels of activity encode innocuous stimuli while higher levels of activity encode noxious stimuli. The most rigorously studied theory, the gate control theory, was proposed by Ronald Melzack and Patrick Wall in 1965 and has been verified/supported in numerous studies since its inception. The gate control theory proposes innocuous stimuli activate inhibitory interneurons in the spinal cord which closes the “gates” to painful input, preventing pain sensation from traveling to the brain (Moayedi and Davis, 2013). This theory opened the door to discovering the complexity of pain circuits within the dorsal horn of the spinal cord. Development of single cell electrophysiological recording led to the characterization of many spinal cord neurons, based on their physiological and morphological properties (Light and Willcockson, 1999; Grudt and Perl, 2002). However how these neurons function in pain transmission remains unclear. The recent development of molecular genetic techniques has identified numerous molecularly-defined interneurons within the pain circuits in the spine (Abraira et al., 2017; Koch et al., 2018). Combining these molecular genetic techniques with cutting-edge calcium imaging approaches allows for observation of complex spinal circuits in real time, helping to clarify their role in pain transmission. These methods have advantages over electrophysiological methods in that calcium imaging strategies can be less invasive, in that they do not rupture or pierce the cell membrane as electrodes do. Calcium imaging can also observe large areas of neurons firing simultaneously, making it possible to study the connection between different neurons in the pain pathway. Here, we review how calcium imaging approaches have been applied to the studies of pain mechanisms in the spinal cord.

2. Calcium indicators

Both synthetic calcium dyes and genetically encoded calcium sensitive proteins(Schultz et al., 2017) have been used to study pain mechanisms in the spinal cord, with each type having its own advantages and disadvantages. In 1962, Osamu Shimomura discovered and purified a bioluminescent calcium-binding photoprotein called aequorin, which was used as the first detector of dynamic calcium changes (Shimomura et al., 1962). Subsequently, new calcium dyes, including Fura-2 and

Abbreviations: IASP, International association for the study of pain; GECIs, Genetically encoded calcium indictors; GFP, Green fluorescent protein.

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Oregon Green 488 BAPTA, have been developed that can be simultaneously loaded into many cells and tissues and used to measure calcium transients. Fura-2 is one of the most successful and popular Ca2+ indicators and is widely considered to be the standard for quantitative intracellular calcium measurement. Another popular calcium dye, Oregon Green 488 BAPTA, has more two-photon excitation absorbance efficiency compared to other fluorescein-like calcium indicators, which is advantageous in some preparations (Paredes et al., 2008). However, dye injection of these synthetic calcium dyes can damage the tissue and long-term observation can be difficult due to the dyes' inactivity over time. The use of genetically encoded calcium indictors (GECIs) can help avoid these problems.

GECIs can be expressed in a specific type of neuron or glial cell, facilitating a more targeted approach. One type of GECI is GCaMP, which is composed of a green fluorescent protein (GFP), calmodulin (CaM), and M13 domains, a peptide sequence from myosin light chain kinase. The GCaMP6 series of GECIs are now used in most preparations. GCaMP6m, GCaMP6s, and GCaMP6f are all GECIs for cytoplasmic free calcium in neurons and can reflect a single action potential firing. While GCaMP6m and GCaMP6s have a greater response to a single action potential, GCaMP6f is the fastest with a 142 ms half-decay time (Chen et al., 2013; Lin and Schnitzer, 2016). Another type is Red GECI named RCaMP, which replaces the green fluorescent protein to red fluorescent protein which has red-shifted excitation and emission spectra (Dana et al., 2016; Molina et al., 2018). Due to the longer wavelength for fluorescence excitation and less absorption in tissue, the Red GECI could reduce phototoxicity and enhance maximal imaging depth. In addition, the Red GECI makes it possible to combine optogenetic approach during imaging (Akerboom et al., 2013; Forli et al., 2018).

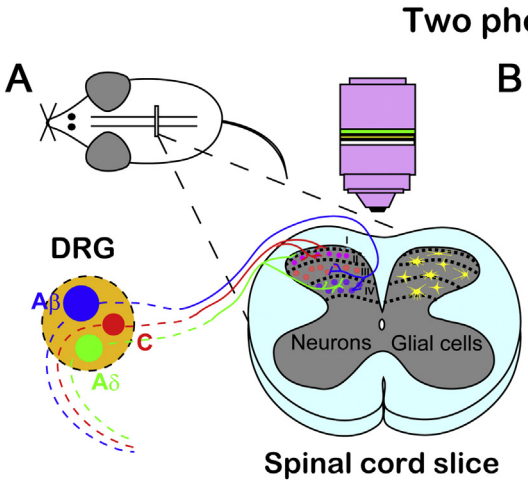
3. *In vitro* calcium imaging of the spinal cord slice preparation

Originally used for patch clamp recording studies, the spinal cord slice preparation is now commonly used for *in vitro* calcium imaging (Fig. 1). This technique allows one to study changes within the spinal cord's dorsal horn physiology, as well as any activity changes in deep spinal cord neuronal networks and glial cell interactions. *In vitro* calcium imaging of spinal cord slice preparations do not have strict experimental requirements and can use easy-loading calcium dyes. Therefore, this technique is widely used in various studies.

The spinal cord slice preparation can be easily loaded with calcium dyes. One study bulk-loaded spinal cord slices with Fura-2 dye to examine the calcium activity of dorsal spinal neurons. This technique revealed that peripheral nerve injury increased glutamate-evoked calcium mobilization within dorsal spinal neurons (Doolen et al., 2012). If one wants to examine the calcium activity of a specific type of cell, the spinal cord slice preparation can be used with GECIs (Kim et al., 2014). Crossing Advillin-Cre mice with GCaMP3 mice resulted in mice whose peripheral terminal inputs expressed Advillin-Cre/GCamP3. Using two-photon imaging to study the calcium transients of these peripheral terminals within spinal cord slices revealed peripheral nerve injury results in a loss of GABA-induced presynaptic calcium inhibition (Chen et al., 2014). In order to study neuronal networks within the spinal cord, our lab crossed Enkephalin-Cre mice with GCaMP6 mice so Enkephalin-positive inhibitory spinal cord interneurons expressed the GECI GCaMP6. Using this method, we successfully demonstrated that current injection can activate excitatory gastrin releasing peptide (GRP) neurons which, in turn, can activate Enkephalin-positive neurons. This was the first spinal calcium imaging study to demonstrate the gating function Enkephalin-positive neurons play in GRP-mediated pain signaling (Sun et al., 2017).

Imaging calcium activity in spinal cord slices can also be used to study cells other than neurons, such as glia cells and astrocytes. One study loaded Fura2 calcium dye into spinal cord slices of mice where eGFP was expressed under the control of the microglia-specific protein Iba1. The majority of Iba1-eGFP labeled microglia took up the calcium dye and calcium imaging showed that the neuropeptide TLQP-21 selectively induced calcium transients in microglia cells but not in astrocytes or neurons. Previous research had shown that TLQP-21 can cause hyperalgesia and contributes to nerve injury-induced hypersensitivity in the spinal cord but the mechanism was unknown. Using calcium imaging of spinal cord slices demonstrated that TLQP-21-elicited pain hypersensitivity through a microglia mechanism (Doolen et al., 2017). In another study, Oregon Green 488 BAPTA and Fura-8 were used to dye spinal cord slices and cultured astrocytes respectively. The study found that a cannabinoid type 1 receptor (CB1-R) agonist could increase calcium transients in astrocytes of the superficial spinal dorsal horn and activation of CB1-R evoked calcium transients in cultured spinal astrocytes. These findings suggested a cannabinoid receptor-dependent mechanism for the crosstalk between astrocytes and neurons in the spinal cord (Hegyi et al., 2018).

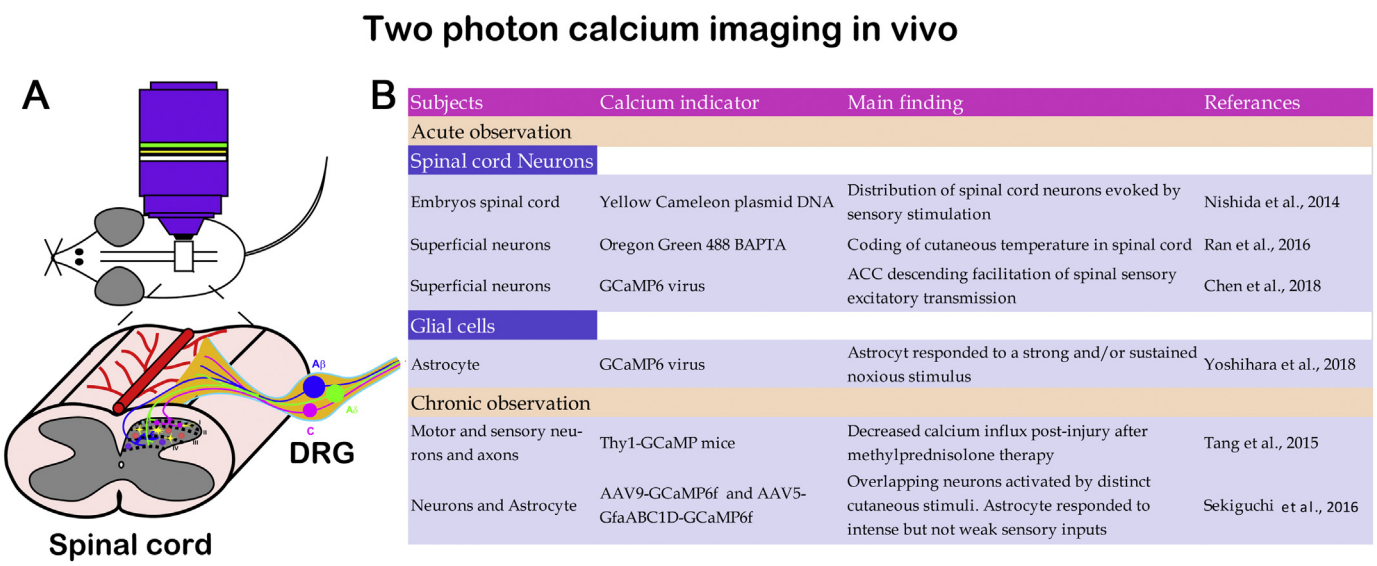
These *in vitro* studies enrich our knowledge about pain mechanisms in the spinal cord, but they have some limitations. For example, although experimental protocols focus heavily on maintaining the health of the spinal cord slice, there is no way to compare the state of a spinal cord slice with the state *in vivo*. Furthermore, with this technique the connections between the spinal cord and brain regions are interrupted, affecting the important descending inhibition or facilitation functions of certain brain regions.



B

Subjects	Calcium indicator	Main finding	Referances
DRG			
Central terminal	Pirt-GCaMP3 mice	RVM descending 5-HT mechanism of central terminal sensitization underlying chronic pain	Kim et al., 2014
Central terminal	GCaMP3 mice	Loss of GABA induced presynaptic calcium inhibition after peripheral nerve injury	Chen et al., 2014
Spinal cord Neurons			
Enkephalin+ neurons	GCaMP6 mice	Gating function of Enkephalin+ neurons in GRP-mediated pain signaling transition	Sun et al., 2017
Superficial neurons	Fura-2	Enhancement of Glutamate evoked calcium transition after peripheral nerve injury	Doolen et al., 2012
Glial cells			
Microglia	Fura-2	Microglia mechanism of pain hypersensitivity induced by TLQP-21-elicited nerve injury	Doolen et al., 2017
Astrocyte	Oregon Green 488 BAPTA	Cannabinoid receptor-dependent mechanism between astrocyte and neurons	Hegyi et al., 2018

Fig. 1. *In vitro* calcium imaging on spinal cord slice. (A).Schematic illustrating the spinal cord slice preparation for calcium imaging. The colored dot lines show the removed peripheral DRG. (B) Selected pain mechanism studies by using spinal cord slice calcium imaging *in vitro*.



Subjects	Calcium indicator	Main finding	Referances
Acute observation			
Spinal cord Neurons			
Embryos spinal cord	Yellow Cameleon plasmid DNA	Distribution of spinal cord neurons evoked by sensory stimulation	Nishida et al., 2014
Superficial neurons	Oregon Green 488 BAPTA	Coding of cutaneous temperature in spinal cord	Ran et al., 2016
Superficial neurons	GCaMP6 virus	ACC descending facilitation of spinal sensory excitatory transmission	Chen et al., 2018
Glial cells			
Astrocyte	GCaMP6 virus	Astrocyt responded to a strong and/or sustained noxious stimulus	Yoshihara et al., 2018
Chronic observation			
Motor and sensory neurons and axons	Thy1-GCaMP mice	Decreased calcium influx post-injury after methylprednisolone therapy	Tang et al., 2015
Neurons and Astrocyte	AAV9-GCaMP6f and AAV5-GfaABC1D-GCaMP6f	Overlapping neurons activated by distinct cutaneous stimuli. Astrocyte responded to intense but not weak sensory inputs	Sekiguchi et al., 2016

Fig. 2. *In vivo* calcium imaging on spinal cord. (A).Schematic illustrating the spinal cord of live animal for calcium imaging. (B) Selected pain mechanism studies by using spinal cord calcium imaging *in vivo*.

4. Calcium imaging *in vivo* in mice

Besides using imaging approaches on *in vitro* spinal cord tissues, numerous studies also use live animals as a model to study the spinal function in pain transmission. Currently, there are two main ways to conduct calcium imaging *in vivo*. One is acute observation, where the animal is under anesthesia and the spinal cord is directly exposed for 2-photon imaging (Fig. 2). One study bulk-loaded neurons in the superficial spinal cord with Oregon Green 488 BAPTA to investigate the coding of cutaneous temperature in the spinal cord by 2 photon calcium imaging (Ran et al., 2016). In another study, Oregon Green 488 BAPTA was utilized to observe evoked responses to mechanical stimulation of the paw and spontaneous calcium transients in superficial dorsal horn neurons (Johannssen and Helmchen, 2010). Another group used Oregon Green 488 BAPTA and astrocyte-specific indicator SR101 to investigate the function of glia. It was shown that electrical hind paw stimulation induced calcium transients in double labeled astrocytes within the superficial dorsal horn (Cirillo et al., 2012).

Besides calcium dye, calcium responses can be imaged in mice who, as an embryo, had *in utero* electroporation introduce Yellow Cameleon plasmid DNA into their spinal cord. This method has been used to study the calcium response to pinch, brush and heat stimulation in the mouse spinal dorsal horn (Nishida et al., 2014). But a major disadvantage of *in utero* electroporation is that it causes negative developmental effects. To overcome this disadvantage, a GCaMP6 virus was used to label the superficial dorsal horn neurons or glial cells in adult mice. Calcium activity was visualized by 2 photon confocal microscopy (Chen et al., 2018; Yoshihara et al., 2018).

All the aforementioned studies are based on the calcium imaging method of acute observation. The advantages of acute observation are that the operations are simple and suitable for one-time observation. However, direct exposure of the spinal cord by surgery often causes unintended damage and irritations to the spinal cord and surrounding tissues, which renders the animal unsuitable for repetitive testing. In order to solve this problem, small imaging chambers have been implanted at the L4-L5 spinal vertebra level, corresponding to sacral spinal cord for chronic observation. The spinal windows allow the spinal activity to be imaged immediately as well as at later imaging sessions (Farrar and Schaffer, 2014; Tang et al., 2015). Using this method, sequential spinal cord images have been taken in a range of minutes to days after spinal cord injury (Farrar et al., 2012). Another significant advantage of this method is the capability of conducting imaging experiments in awake animals. By using the spinal window to image the calcium responses of neurons and glial cells in the spinal cord, it was found that anesthesia suppressed calcium signal responses that were induced by the different sensory stimuli (Sekiguchi et al., 2016).

5. Trouble shooting

Although anesthesia has some negative effects on the animal's condition or the excitatory of neurons and glial cells, imaging under anesthesia appears to be most widely used. The commonly used anesthetic in spinal cord calcium imaging are isoflurane (Johannssen and Helmchen, 2010; Farrar et al., 2012; Farrar and Schaffer, 2014; Sekiguchi et al., 2016; Chen et al., 2018), urethane (Johannssen and Helmchen, 2010; Nishida et al., 2014; Ran et al., 2016), and katamine +xylazine (Tang et al., 2015; Yoshihara et al., 2018). However, the best way to imaging calcium signal response is also using the awake animals. Recent developments of molecular genetic techniques have revealed numerous specific and molecularly defined inhibitory interneurons (*e.g.* B5–I, DYN, Galanin, nNOs, NPY, PV, Ret, TRPV1) and excitatory interneurons (*e.g.* CCK, Calb2, GRP, GRPR, NPRA, PKC γ, RORα, SOM, vGluT3) in the spinal circuits for pain, touch and itch (Koch et al., 2018). These known molecular markers can be used in conjunction with calcium-sensitive genetically encoded proteins to selectively label different types of neurons. This specific labeling allows one to study what influence these defined neurons have on the activity of spinal cord pain circuits. In our previous study of spinal pain circuits, we used GCaMP6 expression in enkephalin-positive neurons to examine their role in gating spinal circuits for touch and itch (Sun et al., 2017). However, due to the presence of myelinated fiber bundles in the superficial spinal cord and the maximum depth of microscopy observation, we cannot clearly observe GCaMP6-expressing neurons within the deep lamina of the spinal cord. To maximize imaging depth, the animal can be slightly rotated to expose more lateral portions of the spinal cord and help avoid the obstructive myelinated fibers which decrease the resolution and contrast of the imaging (Johannssen and Helmchen, 2010). Three-photon imaging can also be used to obtain much higher resolution and depth than 2-photon imaging (Ouzounov et al., 2017). To achieve stable imaging of spinal neurons, one must compensate for any movement from the animal's heart beat and breathing. For acute observation, movement can be compensated by fixing the vertebral column with a firm metal clamp, slightly elevating the animal from the

heating pad to achieve sufficient stability. Additionally, a layer of agarose and a coverslip have been shown to dampen tissue pulsations (Johannssen and Helmchen, 2013). If a spinal chamber implant is used, positioning the chamber as close to the exposed spinal cord as possible can reduce unintended instabilities (Sekiguchi et al., 2016). Another possible complication is that the excitation light from the microscopy laser may be phototoxic to cells and tissues. In general, using longer wavelengths for fluorescence excitation is better for sample tissue health; blue light is more harmful compared to red or infrared light. Not surprisingly, photobleaching rates in 2-photon excitation, which usually uses high peak excitation intensities, are higher than single photon excitation in biological imaging (Patterson and Piston, 2000). So it is of great importance to minimize the excitation intensities, which may significantly reduce signal-to-noise ratio during imaging (Icha et al., 2017).

6. Future perspectives

Calcium imaging approaches have been used to successfully investigate pain signaling in the spinal cord. With the recent progress in genetic, optogenetic, chemogenetic and other related fields, we are no longer limited to the use of spinal cord slices and can now study pain at the whole neuronal circuit level.

In particular, we can trace projection neurons from the spinal cord to express GECIs in the brain and study the communication between spinal cord and brain. Combined with optogenetic or chemogenetic peripheral stimulation, we may further discover how pain or touch signals reach the brain and how they are controlled. Therefore, calcium imaging strategies have become a powerful tool to explore pain circuitry and other neuronal circuits.

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References

Abraira, V.E., Kuehn, E.D., Chirila, A.M., Springel, M.W., Toliver, A.A., Zimmerman, A.L., Orefice, L.L., Boyle, K.A., Bai, L., Song, B.J., Bashista, K.A., O'Neill, T.G., Zhuo, J., Tsan, C., Hoynoski, J., Rutlin, M., Kus, L., Niederkofler, V., Watanabe, M., Dymecki, S.M., Nelson, S.B., Heintz, N., Hughes, D.I., Ginty, D.D., 2017. The cellular and synaptic architecture of the mechanosensory dorsal horn. Cell 168 (1–2), 295–310 e219. https://doi.org/10.1016/j.cell.2016.12.010.

Akerboom, J., Carreras Calderon, N., Tian, L., Wabnig, S., Prigge, M., Tolo, J., Gordus, A., Orger, M.B., Severi, K.E., Macklin, J.J., Patel, R., Pulver, S.R., Wardill, T.J., Fischer, E., Schuler, C., Chen, T.W., Sarkisyan, K.S., Marvin, J.S., Bargmann, C.I., Kim, D.S., Kugler, S., Lagnado, L., Hegemann, P., Gottschalk, A., Schreiter, E.R., Looger, L.L., 2013. Genetically encoded calcium indicators for multi-color neural activity imaging and combination with optogenetics. Front. Mol. Neurosci. 6, 2. https://doi.org/10.3389/fnmol.2013.00002.

Chen, T.W., Wardill, T.J., Sun, Y., Pulver, S.R., Renninger, S.L., Baohan, A., Schreiter, E.R., Kerr, R.A., Orger, M.B., Jayaraman, V., Looger, L.L., Svoboda, K., Kim, D.S., 2013. Ultrasensitive fluorescent proteins for imaging neuronal activity. Nature 499 (7458), 295–300. https://doi.org/10.1038/nature12354.

Chen, J.T., Guo, D., Campanelli, D., Frattini, F., Mayer, F., Zhou, L., Kuner, R., Heppenstall, P.A., Knipper, M., Hu, J., 2014. Presynaptic GABAergic inhibition regulated by BDNF contributes to neuropathic pain induction. Nat. Commun. 5, 5331. https://doi.org/10.1038/ncomms6331.

Chen, T., Taniguchi, W., Chen, Q.Y., Tozaki-Saitoh, H., Song, Q., Liu, R.H., Koga, K., Matsuda, T., Kaito-Sugimura, Y., Wang, J., Li, Z.H., Lu, Y.C., Inoue, K., Tsuda, M., Li, Y.Q., Nakatsuka, T., Zhuo, M., 2018. Top-down descending facilitation of spinal sensory excitatory transmission from the anterior cingulate cortex. Nat. Commun. 9 (1), 1886. https://doi.org/10.1038/s41467-018-04309-2.

Cirillo, G., De Luca, D., Papa, M., 2012. Calcium imaging of living astrocytes in the mouse spinal cord following sensory stimulation. Neural Plast 2012, 425818. https://doi.org/10.1155/2012/425818.

Dana, H., Mohar, B., Sun, Y., Narayan, S., Gordus, A., Hasseman, J.P., Tsegaye, G., Holt, G.T., Hu, A., Walpita, D., Patel, R., Macklin, J.J., Bargmann, C.I., Ahrens, M.B., Schreiter, E.R., Jayaraman, V., Looger, L.L., Svoboda, K., Kim, D.S., 2016. Sensitive red protein calcium indicators for imaging neural activity. Elife 5. https://doi.org/10.7554/eLife.12727.

Doolen, S., Blake, C.B., Smith, B.N., Taylor, B.K., 2012. Peripheral nerve injury increases glutamate-evoked calcium mobilization in adult spinal cord neurons. Mol. Pain 8, 56. https://doi.org/10.1186/1744-8069-8-56.

Doolen, S., Cook, J., Riedl, M., Kitto, K., Kohsaka, S., Honda, C.N., Fairbanks, C.A., Taylor, B.K., Vulchanova, L., 2017. Complement 3a receptor in dorsal horn microglia mediates pronociceptive neuropeptide signaling. Glia 65 (12), 1976–1989. https://doi.org/10.1002/glia.23208.

Farrar, M.J., Schaffer, C.B., 2014. A procedure for implanting a spinal chamber for longitudinal in vivo imaging of the mouse spinal cord. J. Vis. Exp.(94). https://doi.org/10.3791/52196.

Farrar, M.J., Bernstein, I.M., Schlafer, D.H., Cleland, T.A., Fetcho, J.R., Schaffer, C.B., 2012. Chronic in vivo imaging in the mouse spinal cord using an implanted chamber. Nat. Methods 9 (3), 297–302. https://doi.org/10.1038/nmeth.1856.

Forli, A., Vecchia, D., Binini, N., Succol, F., Bovetti, S., Moretti, C., Nespoli, F., Mahn, M., Baker, C.A., Bolton, M.M., Yizhar, O., Fellin, T., 2018. Two-photon bidirectional control and imaging of neuronal excitability with high spatial resolution in vivo. Cell Rep. 22 (11), 3087–3098. https://doi.org/10.1016/j.celrep.2018.02.063.

Grudt, T.J., Perl, E.R., 2002. Correlations between neuronal morphology and electrophysiological features in the rodent superficial dorsal horn. J. Physiol. 540 (1), 189–207. https://doi.org/10.1113/jphysiol.2001.012890.

Hegyi, Z., Olah, T., Koszeghy, A., Pisticelli, F., Hollo, K., Pal, B., Csernoch, L., Di Marzo, V., Antal, M., 2018. CB1 receptor activation induces intracellular Ca(2+) mobilization and 2-arachidonoylglycerol release in rodent spinal cord astrocytes. Sci. Rep. 8 (1), 10562. https://doi.org/10.1038/s41598-018-28763-6.

Icha, J., Weber, M., Waters, J.C., Norden, C., 2017. Phototoxicity in live fluorescence microscopy, and how to avoid it. Bio. Essays 39 (8). https://doi.org/10.1002/bies.201700003.

Johannssen, H.C., Helmchen, F., 2010. In vivo Ca2+ imaging of dorsal horn neuronal populations in mouse spinal cord. J. Physiol. 588, 3397–3402. https://doi.org/10.1113/jphysiol.2010.191833. Pt 18.

Johannssen, H.C., Helmchen, F., 2013. Two-photon imaging of spinal cord cellular networks. Exp. Neurol. 242, 18–26. https://doi.org/10.1016/j.expneurol.2012.07.014.

Kim, Y.S., Chu, Y., Han, L., Li, M., Li, Z., LaVinka, P.C., Sun, S., Tang, Z., Park, K., Caterina, M.J., Ren, K., Dubner, R., Wei, F., Dong, X., 2014. Central terminal sensitization of TRPV1 by descending serotonergic facilitation modulates chronic pain. Neuron 81 (4), 873–887. https://doi.org/10.1016/j.neuron.2013.12.011.

Koch, S.C., Acton, D., Goulding, M., 2018. Spinal circuits for touch, pain, and itch. Annu. Rev. Physiol. 80, 189–217. https://doi.org/10.1146/annurev-physiol-022516-034303.

Light, A.R., Willcockson, H.H., 1999. Spinal laminae I-II neurons in rat recorded in vivo in whole cell, tight seal configuration: properties and opioid responses. J. Neurophysiol. 82 (6), 3316–3326. https://doi.org/10.1152/jn.1999.82.6.3316.

Lin, M.Z., Schnitzer, M.J., 2016. Genetically encoded indicators of neuronal activity. Nat. Neurosci. 19 (9), 1142–1153. https://doi.org/10.1038/nn.4359.

Moayedi, M., Davis, K.D., 2013. Theories of pain: from specificity to gate control. J. Neurophysiol. 109 (1), 5–12. https://doi.org/10.1152/jn.00457.2012.

Molina, R.S., Qian, Y., Wu, J., Shen, Y., Campbell, R.E., Hughes, T.E., Drobizhev, M., 2018. Understanding the Ca 2+ -dependent fluorescence change in red genetically encoded Ca 2+ indicators. bioRxiv 435891. https://doi.org/10.1101/435891.

Nishida, K., Matsumura, S., Taniguchi, W., Uta, D., Furue, H., Ito, S., 2014. Three-dimensional distribution of sensory stimulation-evoked neuronal activity of spinal dorsal horn neurons analyzed by in vivo calcium imaging. PLoS ONE 9 (8), e103321. https://doi.org/10.1371/journal.pone.0103321.

Ouzounov, D.G., Wang, T., Wang, M., Feng, D.D., Horton, N.G., Cruz-Hernandez, J.C., Cheng, Y.T., Reimer, J., Tolias, A.S., Nishimura, N., Xu, C., 2017. In vivo three-photon imaging of activity of GCaMP6-labeled neurons deep in intact mouse brain. Nat. Methods 14 (4), 388–390. https://doi.org/10.1038/nmeth.4183.

Paredes, R.M., Etzler, J.C., Watts, L.T., Zheng, W., Lechleiter, J.D., 2008. Chemical calcium indicators. Methods 46 (3), 143–151. https://doi.org/10.1016/j.ymeth.2008.09.025.

Patterson, G.H., Piston, D.W., 2000. Photobleaching in two-photon excitation microscopy. Biophys. J. 78 (4), 2159–2162. https://doi.org/10.1016/S0006-3495(00)76762-2.

Ran, C., Hoon, M.A., Chen, X., 2016. The coding of cutaneous temperature in the spinal cord. Nat. Neurosci. 19 (9), 1201–1209. https://doi.org/10.1038/nn.4350.

Schultz, S.R., Copeland, C.S., Foust, A.J., Quicke, P., Schuck, R., 2017. Advances in two photon scanning and scanless microscopy technologies for functional neural circuit imaging. Proc. IEEE Inst. Electr. Electron. Eng. 105 (1), 139–157. https://doi.org/10.1109/JPROC.2016.2577380.

Sekiguchi, K.J., Shekhtmeyster, P., Merten, K., Arena, A., Cook, D., Hoffman, E., Ngo, A., Nimmerjahn, A., 2016. Imaging large-scale cellular activity in spinal cord of freely behaving mice. Nat. Commun. 7, 11450. https://doi.org/10.1038/ncomms11450.

Shimomura, O., Johnson, F.H., Saiga, Y., 1962. Extraction, purification and properties of Aequorin, a bioluminescent protein from the luminous hydromedusan, Aequorea. J. Cell. Comp. Physiol. 59 (3), 223–239. https://doi.org/10.1002/jcp.1030590302.

Sun, S., Xu, Q., Guo, C., Guan, Y., Liu, Q., Dong, X., 2017. Leaky gate model: intensity-dependent coding of pain and itch in the spinal cord. Neuron 93 (4), 840–853. e845. https://doi.org/10.1016/j.neuron.2017.01.012.

Tang, P., Zhang, Y., Chen, C., Ji, X., Ju, F., Liu, X., Gan, W.B., He, Z., Zhang, S., Li, W., Zhang, L., 2015. In vivo two-photon imaging of axonal dieback, blood flow, and calcium influx with methylprednisolone therapy after spinal cord injury. Sci. Rep. 5, 9691. https://doi.org/10.1038/srep09691.

Yoshihara, K., Matsuda, T., Kohro, Y., Tozaki-Saitoh, H., Inoue, K., Tsuda, M., 2018. Astrocytic Ca(2+) responses in the spinal dorsal horn by noxious stimuli to the skin. J. Pharmacol. Sci. 137 (1), 101–104. https://doi.org/10.1016/j.jphs.2018.04.007.