A simple and fast method to image calcium activity of neurons from intact dorsal root ganglia using fluorescent chemical Ca\(^{2+}\) indicators

Yong Chen\(^1\) and Li-Yen M Huang\(^1\)

Abstract
Chemical calcium indicators have been commonly used to monitor calcium (Ca\(^{2+}\)) activity in cell bodies, i.e., somata, of isolated dorsal root ganglion neurons. Recent studies have shown that dorsal root ganglion somata play an essential role in soma–glia interactions and actively participate in the transmission of nociceptive signals. It is therefore desirable to develop methods to study Ca\(^{2+}\) activity in neurons and glia in intact dorsal root ganglia. In our previous studies, we found that incubation of intact dorsal root ganglia with acetoxymethyl dye resulted in efficient Ca\(^{2+}\) dye loading into glial cells but limited dye loading into neurons. Here, we introduce a useful method to load Ca\(^{2+}\) dyes in intact dorsal root ganglion neurons through electroporation. We found that electroporation greatly facilitated loading of Fluo-4 acetoxymethyl, Oregon green bapta-1-488 acetoxymethyl, and Fluo-4 pentapotassium salt into dorsal root ganglion neurons. In contrast, electroporation did not further facilitate dye loading into glia. Using electroporation followed by incubation of acetoxymethyl form Ca\(^{2+}\) dye, we can load acetoxymethyl Ca\(^{2+}\) dye well in both neurons and glia. With this approach, we found that inflammation induced by complete Freund’s adjuvant significantly increased the incidence of neuron–glia interactions in dorsal root ganglia. We also confirmed the actions of capsaicin and morphine on Ca\(^{2+}\) responses in dorsal root ganglion neurons. Thus, by promoting the loading of Ca\(^{2+}\) dye in neurons and glia through electroporation and incubation, Ca\(^{2+}\) activities in neurons and neuron–glia interactions can be well studied in intact dorsal root ganglia.

Keywords
Electroporation, dorsal root ganglion, calcium, calcium dye, inflammation, pain, neuron–glia interaction

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Introduction
Isolated and round-shaped cell bodies (somata) of dorsal root ganglion (DRG) neurons have been used traditionally as a model cell preparation to study electric properties of central and peripheral sensory terminals based on the assumption that the somata and terminals have similar electrical characteristics\(^1\) and somata are not actively participating in sensory signaling.\(^2\) This view has recently been challenged.\(^3\)–\(^7\) We and others have shown that DRG somata are essential in soma–glia interactions and actively participate in the transmission of nociceptive signals.\(^5\)–\(^8\)–\(^12\)

To better understand the involvement of neuronal somata and the role of somata–glia interactions in pain signaling, some recorded the activity of DRG somata using sharp electrodes and/or patch electrodes and studied changes in Ca\(^{2+}\) signaling through the injection of a salt form of fluorescent Ca\(^{2+}\) dye into a small number of neurons in intact DRGs.\(^8\)–\(^9\)\(^,\)\(^13\)–\(^17\) We bulk loaded Fluo-4 acetoxymethyl Ca\(^{2+}\) dye in L4 or L5 ganglia with sciatic nerves attached.\(^5\)–\(^6\) The approach allowed us to examine neuron–glia interactions in DRGs under different experimental conditions. This dye loading technique resulted in

\(^1\)Department of Neuroscience, Cell Biology and Anatomy, University of Texas Medical Branch, Galveston, TX, USA

Corresponding author:
Li-Yen M Huang, Department of Neuroscience, Cell Biology and Anatomy, University of Texas Medical Branch, Galveston, TX 77555, USA.
Email: LmHuang@utmb.edu
efficient Ca$^{2+}$ dye loading into glial cells. However, only a limited number of neurons were loaded. We therefore tested various Ca$^{2+}$ dye loading techniques and found that Ca$^{2+}$ dyes could be efficiently loaded in intact DRG neurons through electroporation. Conditions to optimize the study of neuron–glia interactions were explored further.

**Materials and methods**

**Animals**

Male adult (100–250 g) Sprague Dawley rats were used in this study. All experimental procedures were approved by the Institutional Animal Care and Use Committee at the University of Texas Medical Branch and were in accordance with the guidelines of the National Institutes of Health and of the International Association for the Study of Pain.

**Complete Freund’s adjuvant induced inflammation**

To induce inflammation, animals were anesthetized with isoflurane (5%), and complete Freund’s adjuvant (CFA; 50 μl) was injected into the plantar surface of the left hindpaw. Solution of CFA was prepared by mixing *Mycobacterium butyricum* (10 mg/ml; Difco, Detroit, MI) in a peanut oil–saline (1:1) emulsion. Signs of localized inflammation in left hindpaw, such as redness and swelling, were seen several hours later. Ca$^{2+}$ imaging experiments were performed three to nine days after the CFA injection.

**Retrograde loading of dextran form of Ca$^{2+}$ dye**

In some rats, under isoflurane anesthesia (initially at 5% and maintained at 2%), the left sciatic nerve was transected in midthigh, and the distal cut end of the nerve was tightly ligated with 5-0 silk. The proximal cut end was soaked in 10 μl, 1% calcium green-1 dextran 3000 (Ca green dextran) solution for 30 min at room temperature. The extracellular solution contained (in mM) 115 NaCl, 5.6 KCl, 1 NaH$_2$PO$_4$, 2.0 CaCl$_2$, 1 MgCl$_2$, 11 glucose, and 25 NaHCO$_3$ and was bubbled with O$_2$.

**Chemical agents**

All Ca$^{2+}$ dyes were purchased from Thermo Fisher Scientific (Waltham, MA). Morphine was obtained from Hospira (Lake Forest, IL) and capsaicin was from Sigma (St. Louis, MO).

**Calcium imaging**

Calcium imaging studies were conducted on intact DRG preparations at room temperature. Ganglia were electroporated with the Amaxa Neucleofector II. Briefly, ganglia with or without attached sciatic nerves were put into a cuvette which contained 100 μl of extracellular solution with Ca$^{2+}$ dye for electroporation. The protocols used in the study were A003, C002, or C003. Since these three electroporation protocols did not yield significant differences in Ca$^{2+}$ dye loadings, results were combined in our data analyses. For acetoxymethyl (AM)-form Ca$^{2+}$ dyes, ganglia were incubated with the same Ca$^{2+}$ dye solution for 1 h immediately after electroporation unless stated otherwise.

The concentrations of Ca$^{2+}$ dyes used were as follows: Fluo-4 AM (0.92 mM) and Oregon Green™ 488 BAPTA-1 AM (OGB 488, 0.79 mM). To improve membrane perforation of the dyes, a final concentration of 0.4% of Pluronic F127 (dissolved in 20% in DMSO) was added into AM dye solutions. In addition, Fluo-4 pentapotassium salt (1.08 mM) and Ca green dextran (1%) were also tested.

After dye loading, a DRG was moved to a custom-built recording chamber or a MatTek culture dish, held down with a nylon mesh, and superfused with the oxygenated extracellular solution. Ca$^{2+}$ imaging experiments were performed under a Nikon confocal microscope with a 20× objective or a custom-built two-photon laser-scanning microscope based on a Coherent Laser System with a water immersion 60× objective. For the confocal microscope, the excitation wavelength was 488 nm. For the two-photon microscope, the laser system was operated at 800 nm.

The Ca$^{2+}$ images were recorded before, during, and after an electrical sciatic nerve stimulation or a bath application of chemicals, e.g., high KCl (80 mM or 55 mM) or capsaicin (10 μM). For electrical stimulation, the cut end of the sciatic nerve was inserted into a suction electrode and connected to a Master-8 stimulator through an isolator. The stimuli were 1 ms square pulses, ≤7 mA, delivered at 20 Hz. Images were analyzed off-line with a NIH ImageJ software or Nikon NIS-Elements.
Elements software. Data are presented as the relative change in fluorescence ($\Delta F/F_0$), where $F_0$ is basal fluorescence and $\Delta F = F - F_0$.

**Data analyses**

Data were expressed as means ± SE or as percentages. Student’s t and chi square tests were used to access the significance of changes. The Mann–Whitney Rank Sum Test was used when the normality test failed for the Student’s t test. Comparisons between multiple means were done with one-way analysis of variance followed by the Holm-Sidak post hoc test. A value of P < 0.05 was considered significant.

**Results**

**Electroporation significantly improves the loading of Fluo-4 AM Ca$^{2+}$ indicator into DRG neurons**

In our previous studies of neuron–glia interactions,\(^5,6\) we loaded Fluo-4 AM into neurons and glial cells in whole DRGs through 1 h incubation period. The method allowed us to load Fluo-4 AM into glia. In contrast, only a limited number of neurons were loaded. To improve the Ca$^{2+}$ dye loading in neurons, DRGs first underwent electroporation, followed by 1 h dye incubation. We found that electroporation greatly increased dye loading in neurons but had little effect on loading in glial cells (Figure 1). To further confirm that the electroporation-treated labeled neurons are functional, we studied the effect of electroporation on the number of labeled neurons in response to a high concentration (80 mM) of KCl treatment. KCl is known to reliably depolarize neurons causing Ca$^{2+}$ influx into functional neurons.\(^18\)

Electroporation followed by incubation was found to increase Fluo-4 AM labeled neurons responding to high KCl by 4.9 fold (Figure 1(b)). In addition, we found that Fluo-4 AM can be efficiently loaded into neurons through electroporation without postincubation (Figure 1(b)). These results suggest that electroporation significantly improves Fluo-4 AM loading into DRG neurons in intact DRGs. On the other hand, AM dye incubation can label glia well without electroporation.

The improvement of dye loading also allowed us to study the time-dependent changes in intracellular Ca$^{2+}$ in different neurons in intact DRGs. Following electroporation and incubation of Fluo-4 AM, we determined the dynamic changes in fluorescence in response to high KCl treatment (Figure 2). The fluorescence intensity peaked ~35 s after high KCl application. The average peak fluorescence Ca$^{2+}$ response ($\Delta F/F_0$) was increased by ~69% (Figure 2).

![Figure 1. Electroporation increases Fluo-4 AM loading in intact DRG neurons.](image)

Figure 1. Electroporation increases Fluo-4 AM loading in intact DRG neurons. (a) Examples of pseudocolor intracellular Ca$^{2+}$ images in cells responding to bath application of 80 mM KCl in intact DRGs with or without electroporation (EP) of Fluo-4 AM, followed by 1 h dye incubation. Arrows indicate the neurons responding to high KCl treatment. Scale bar: 50 µm. Color-coded intensity calibration bar is shown below. (b) Electroporation increased the number of DRG neurons responding to high KCl stimulation (3.90 ± 2.10%, w/o EP, n = 3 DRGs; 19.10 ± 3.86%, w/EP; n = 4; *P < 0.05). In the absence of postincubation, electroporation of Fluo-4 AM yielded a similar percentage of neurons responding to KCl (16.90 ± 0.04% EP only, n = 4 DRGs; P > 0.05 compared with EP + incubation). Note that glia were labeled well with 1 h Fluo-4 AM incubation. Electroporation of Fluo-4 AM had minimal effect on glia labeling.

**Electroporation also improves loading other AM and salt forms of Ca$^{2+}$ dye in DRG neurons**

To determine whether neuronal dye loading by electroporation is dye dependent, a number of other Ca$^{2+}$ dyes were tested. We found that electroporation of OGB-488 AM was equally efficient in producing functional dye loaded DRG neurons (Figure 3(a)). In previous studies,
a salt form Ca\(^{2+}\) indicator, e.g., Fluo-4 pentapotassium, was loaded into a small number of neurons by direct intracellular injection.\(^{19}\) About 26% of neurons responded to high KCl following electroporation (Figure 3(b)). In contrast to Ca\(^{2+}\) AM dyes, the loading of Fluo-4 salt into glia was rather limited.

We also determined changes in intracellular Ca\(^{2+}\) fluorescence in L4 or L5 DRG neurons in response to electrical stimulation of sciatic nerves. The DRG preparation has been electroporated and then incubated with OGB488 AM. Following electric stimulation, the Ca\(^{2+}\) fluorescence in neurons robustly increased and reached to its peak within 1–2 s. The fluorescence quickly dropped when the stimulation was stopped and then gradually returned to the baseline (Figure 4). We found that the average peak increase was 35.00 ± 5.00% (\(\Delta F/F_0; n = 5\)). These results suggest that both axons and somata of DRG neurons are functional following electroporation. Using Fluro-4 AM, we previously showed that neurons and glia interact.\(^{5,6}\) To determine whether neuron–glia interactions are maintained when DRGs undergo electroporation, Ca\(^{2+}\) changes in glia were also determined. Glial Ca\(^{2+}\) was increased with a delay (Figure 4), similar to our studies without electroporation.\(^5\) Thus, neuron–glia interactions are indeed preserved.

We repeated the experiments using high KCl stimulation (Figure 5). As expected, the rates of Ca\(^{2+}\) fluorescence increase in neurons and glia following chemical, e.g., KCl, stimulation are slower than those following electrical stimulation. The delay in Ca\(^{2+}\) fluorescence increase between neurons and glia persisted. Following CFA, neuron–glia interactions were preserved, and the interactions occurred in more neuron–glia cell pairs. Six neuron–glia interactions were found in 76 KCl responsive neurons (7.9%) in normal rats, and 11 interactions were seen in 46 KCl responsive neurons (23.9%) in CFA rats.
Dextran Ca\(^{2+}\) indicators respond to high KCl stimulation in very few rat DRG neurons

One exception to the success in functional preservation using electroporation was the use of dextran form Ca\(^{2+}\) indicator, i.e., Ca green dextran. Although we were able to load the dextran dye in intact DRGs, only a very low percentage of neurons (3.72 ± 0.42%; \(n = 5\)) responded to high KCl treatment (Figure 6(a)). We also used retrograde transport instead of electroporation to load Ca green dextran in DRGs. Neurons with retrograde loaded dye did not respond to high KCl treatment (Figure 6(b)). Thus, the failed KCl experiments are not dye loading method dependent. Others have reported that in adult rats none of the DRG neurons that underwent retrograde loading of Ca green dextran responded to sciatic nerve electric stimuli or bath application of high KCl.\(^{20}\) Since peripheral or CNS neurons loaded with dextran Ca\(^{2+}\) dyes do respond to manipulations such as KCl or electrical nerve stimulation,\(^{21-23}\) the observations suggest that dextran Ca\(^{2+}\) dyes are not suitable for studying Ca\(^{2+}\) functions in DRG neurons. Reasons underlying the failure are unclear.

Electroporation facilitates studies of pain signaling processing in intact DRG neurons

To determine whether the electroporation technique facilitates the study of pain signaling processing in intact DRG neurons, we studied the effect of capsaicin in DRGs treated with electroporation of Fluo-4 salt. Capsaicin was found to increase Ca\(^{2+}\) concentrations in many small and medium DRG neurons (Figure 7(a)), consistent with the studies obtained from the distribution of capsaicin receptors in DRGs.\(^{24-27}\)
Thus, the increase in Ca$^{2+}$ dye loading with electroporation is likely to help us characterize functions of capsaicin receptor activation in intact DRG neurons.

We also studied the morphine effects on DRG neurons underwent electroporation with Fluo-4 AM (Figure 7(b)). The effects of morphine on KCl-induced Ca$^{2+}$ increases in cells treated with and without morphine were studied. Morphine was found to block the KCl-induced Ca$^{2+}$ fluorescence in DRG neurons by $\sim$80%, a result consistent with the observation that morphine blocks voltage-dependent Ca$^{2+}$ channels in DRG neurons.$^{28}$

**Discussion**

In this study, we found that electroporation of intact DRGs greatly facilitates the loading of Ca$^{2+}$ dyes simultaneously into individual neurons without affecting their physiological functions. By combining electroporation and incubation of AM Ca$^{2+}$ dye, we were able to study neuron–glia interactions efficiently in intact DRGs. The major advantages of this approach are its simplicity and high efficiency.

Bulk AM Ca$^{2+}$ dye loading of CNS neurons has been successful in in vitro and in vivo preparations.$^{29-31}$ It is unclear the reason for poor loading of AM Ca$^{2+}$ dye in intact DRG neurons in the absence of electroporation. One of the possibilities is that each soma of DRG neuron is tightly surrounded by a layer of SGCs, which form a functional unit enclosed by a connective tissue sheath.$^{32}$ This results in a physical barrier for Ca$^{2+}$ dye to gain access to DRG neurons.

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**Figure 4.** Neuron–satellite glial cell interactions triggered by electrical nerve stimulation. Sciatic nerve stimulation at 20 Hz increased intracellular Ca$^{2+}$ in DRG neurons and glial cells. The DRG with attached sciatic nerves was electroporated and incubated with OGB 488-AM. (a) Pseudocolor images of fluorescence changes in a neuron (N) and a glial cell (G). The number at the top or bottom in each frame indicates the time at which the image was taken. Scale bar: 20 μm. (b) Time courses of relative fluorescence changes, i.e., ΔF/F$_0$ in the neuron and glial cell. F$_0$ is the basal fluorescence in either the neuron or the glial cell before nerve stimulation. The horizontal line indicates the period of nerve stimulation. Nerve stimulation evoked intracellular Ca$^{2+}$ increase in the neuron first and then in the glial cell with a delay of $\sim$2.0 s.

**Figure 5.** Inflammation increases the incidence of neuron–glia interactions. Inflammation was induced by injecting CFA into the left hindpaw of a rat. DRGs from control and CFA rats were taken out and electroporated and incubated with Fluo-4 AM. (a) Example pseudocolor images of intracellular Ca$^{2+}$ changes in DRG neurons from a control (upper) and a CFA (lower) rat in response to bath application of 80 mM KCl. High KCl increased the intracellular Ca$^{2+}$ first in the neuron (N) and then in glial cells (G1 and G2) with a delay. The number at the top or bottom in each frame indicates the time the image was taken. Time courses of relative fluorescence changes, i.e., ΔF/F$_0$, in the neuron and in the glial cells are shown on the right. The horizontal lines indicate the period of high KCl application. For the images of the CFA rat, high KCl induced similar responses with those in the control rat both in the neuron and in the glial cell. Scale bars: 30 μm. Time courses of relative fluorescence changes are also shown on the right. (b) Neuron–glia interactions were significantly increased in DRGs of CFA rats. In response to high KCl stimulation, 7.9% KCl responsive neurons interacted with glia in control and 23.9% in CFA rats interacted.
The number of neuron–glia interacting pairs induced by high KCl is increased in DRGs in CFA-induced inflamed rats (Figure 5). We have previously shown that CFA-induced inflammation enhances the expression of P2X7 receptors in rats, and the neuron–glia interactions depend on the P2X7 receptors in SGCs in DRGs.\textsuperscript{5,6} The roles of P2X7R in neuron–glia interactions in CFA rats can now be further determined with the improved Ca\textsuperscript{2+} dye loading in intact DRGs.

The results that capsaicin causes an increase in Ca\textsuperscript{2+} signaling in small and medium cells and the \textmu-opioid receptor agonist, morphine, significantly inhibits high KCl-induced Ca\textsuperscript{2+} increase in DRG neurons are consistent with published findings.\textsuperscript{33–36} These observations suggest that the electroporation Ca\textsuperscript{2+} dye loading method should be useful for studying drug effects in intact DRGs in pain research.

Recently, genetic Ca\textsuperscript{2+} sensors have become available for studying Ca\textsuperscript{2+} changes in virus-transfected neurons and/or in mutant mice.\textsuperscript{37–39} Using these sensors, it is possible to probe the Ca\textsuperscript{2+} activities in specific subpopulations of sensory neurons.\textsuperscript{40} However, these approaches usually take weeks or months to produce results. Compared with genetic approaches, Ca\textsuperscript{2+} dye loading through electroporation and incubation cannot target specific populations of neurons. On the other hand, in combination with specific pharmacological tools, electroporation and incubation of Ca\textsuperscript{2+} dyes can efficiently provide general ideas about Ca\textsuperscript{2+} signaling in subpopulations of DRG neurons. We can then use the observations as a prelude to understanding Ca\textsuperscript{2+} signaling in a specific subpopulation of DRG neurons using genetic Ca\textsuperscript{2+} sensors.

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Author Contributions
YC and LMH designed research. YC performed experiments. YC and LMH wrote the paper.

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