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Neuro-tracing approach to study kidney innervation: a technical note

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Neuro-tracing approach is a great option to study innervation of the visceral organs including the kidneys. Important factors contributing to the success of this technique include the choice of a neuro-tracer, and delivery methods to result in successful labeling of peripheral sensory and motor ganglia. The neuro-tracer is usually applied directly to the kidney accessed via a surgical opening of the abdominal wall under deep anesthesia. A series of local microinjections of the dye are performed followed by a wound closure, and recovery period from the surgery. An extra care should be taken to prevent neuro-tracer spillage and accidental labeling of the surrounding organs during injections of the dye. Retrograde neuro-tracers like Fast Blue do not cross synapses, therefore, only neuronal bodies located within dorsal root ganglion neurons and major peripheral ganglia will be labeled by this approach. Retrogradely labeled peripheral neurons could be freshly isolated and dissociated for electrophysiological recordings and biochemical analyses (gene and protein expression), whereas the whole fixed ganglia could be sectioned to undergo immunohisto- and immunocytochemical targeted staining.

Keywords: Fast Blue, Kidney innervation, Neuro-tracing, Retrograde labeling

Introduction

Renal innervation plays a major role in the natriuretic function via the renal tubules and regulation of salt/water excretion from the kidneys [1]. Kidney-innervating sensory neurons receive information about renal status during injury, inflammation and other pathological conditions associated with renal disease, and transfer the signaling to the central nervous system (CNS) followed by systemic sympathetic activity [2]. Sympathetic input may cause renal fluid retention and negatively affect cardiovascular function [3]. Recent studies revealed suppression of nerve-derived signaling or renal denervation to be a therapeutic approach to prevent renal fibrogenesis [4,5]. Therefore, studies focused on kidney innervation and functional significance of renal innervation are fundamental to understand the mechanisms of renal dysfunction.

Neuro-tracing approach is a great option to visualize kidney-innervating neurons, particularly those located within dorsal root ganglion (DRG) neurons and the inferior ganglion of the vagus nerve. The application of neuro-tracer to the renal cortex was first introduced by Donovan et al [6] in 1983 to study renal neurons. Subsequent studies used Fluorogold and Fast Blue (FB) to identify renal DRG neurons followed by immunohistochemical evaluation of their properties [7]. Additional studies using a neuro-tracing approach identified four different subtypes of renal DRG neurons [8] and renal efferent sympathetic neurons [9] based on their functional properties.

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The conventional neuro-tracing approach reviewed in this article is often used as the first step to identify specific populations of neurons followed by a number of functional assays. For instance, labeled DRG neurons could be further characterized by immunohistochemistry and immunocytochemistry using antibodies for neuro-peptides and other molecules [7,8,10,11]. Labeled DRG neurons could be freshly isolated and cultured for evaluation of neuronal excitability by electrophysiological recordings [8–11]. Furthermore, laser-capture microdissection of single DRG neurons could be performed to select labeled cells and evaluate gene expression using reverse transcription polymerase chain reaction [11].

Overall, this review provides an overview of the conventional neuro-tracing technique and a detailed protocol for retrograde labeling of DRG neurons innervating the kidney, as an example for practical application.

Experimental design for a neuro-tracing approach

Choice of a neuro-tracer and application method

The choice of a non-viral neuro-tracer depends on several factors including its labeling efficiency and uptake mechanisms [12,13]. Several molecules such as carbocyanine dyes, beads, dextrans, lectins, trophins, amino acides, inorganic fluorescent molecules and bacterial toxins have been previously used as non-viral neuro-tracers. Among these several options, inorganic fluorescent molecules have key features of an ideal neuro-tracer, which include its selectivity, efficiency and availability for multi-color labeling [14]. Important factors to be considered when choosing a neuro-tracer are: a) duration of time to label the target after dye application; b) duration of fluorescent labeling after uptake (how long the fluorescent labeling lasts); and c) labeling efficiency of the candidate neuro-tracer which is determined by number of labeled cells and intensity of labeling. The application methods for neuro-tracers include there main methods of dye delivery: pressure injection, iontophoretic injection, and crystal powder application [13]. The current paper describes a detailed protocol for application of FB by pressure injection method using a 27 to 32 gauge metal needle inserted into a 25 μL Hamilton syringe or crystal powder application method.

Timeline for labeled tissue collection

The time required for the dye to label nerves and neurons innervating a certain tissue/organ varies significantly and depends on the type of the chosen neuro-tracer and structure of the targeted organ undergoing dye injections. In addition, a number of other factors should be considered. Animal species and a length of neuronal projection from the target organ (e.g., kidney) to neuro-tracer's final destination (e.g., DRG neurons) are additional critical factors influencing the time of the labeling period. The time period sufficient for a neuro-tracer to travel along the nerves and provide consistent labeling outcomes has been previously investigated and discussed [15]. For example, FB was reported to produce some labeling as early as 2 days after injections with

Table 1. Duration of labeling period upon Fast Blue application in different targets

Species of animal model	Labeling period	Application site	Labeling destination	Reference
Mouse	At least 2 days	Airway	Nodose ganglia	Kaelbere et al, *J Vis Exp* (2016) [16]
Mouse	6 days	Bladder, Prostate	DRG neurons (T13–S1)	Lee et al, *Prostate* (2016) [17]
Mouse	1 week	Nasal Mucosa	Trigeminal ganglion neurons	Mingomataj et al, *Clin Exp Allergy* (2008) [18]
Rat	10 days	Colon	DRG neurons	Yu et al, *Exp Neurol* (2012) [19]
Rat	1 week	Kidney	RESN, SCG	Vari et al, *Am J Physiol* (1999) [9]
Rat	1–4 days	Kidney	DRG neurons (T6–L2), inferior ganglia of the vagus, brainstem	Donovan et al, *Brain Res* (1983) [6]
Pig	1 week	Pyloric sphincter muscle	Gastric neurons	Zalecki, *PLoS One* (2015) [20]
Rabbit	5 weeks	Hip joint capsule	DRG neurons (L–S)	Dudek et al, *Anat Histol Embryol* (2013) [21]

Ca, caudal; DRG, dorsal root ganglion; L, lumbar; RESN, renal efferent sympathetic neurons; S, sacral; SCG, superior cervical ganglion; T, thoracic.

maximal labeling usually seen at 1–2 weeks. Table 1 summarizes available reports showing the choice of species and length of neuronal projection on the labeling periods of FB in each study [16–21]. Once the window of the labeling period is determined, the end point for tissue collection can be chosen based on the nature of the experiments planned to be done with the labeled target, as well as subsequent experimental interventions. For instance, Wang et al [8] studied functional interactions between P2Y receptors and transient receptor potential vanilloid type 1 (TRPV1) in kidney projecting sensory neurons. The group used a rat model, and applied the fluorescent tracer to the renal artery to label sensory neurons located within DRG neurons. Since they planned to proceed with immunohistochemial studies and electrophysiological recordings, 6 days of labeling period was sufficient to label sensory neurons in their study. A study by Malykhina et al [10] performed a dual-labeling procedure using 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyaine perchlorate (DiI) injected in the colon and FB in the urinary bladder in adult rats 3 to 5 days in advance before inducing trinitrobenzene sulfonic acid (TNBS) colitis in their animal model. This approach allowed sufficient time for retrograde labeling to occur, and animal model to develop experimental colitis at the same time.

Labeling procedures

Materials and reagents

Materials and reagents required for each step are listed below in alphabetical order. Alternatives can be considered for each user's convenience.

Animal survival surgery, tissue harvest and tissue preparation

Analgesic (i.e., Buprenorphine, 0.05–0.1 mg/kg; Reckitt Benckiser Healthcare Ltd., Hull, England)
Anesthetic set up for rodents (i.e., Isoflurane vaporizer; Midmark, Kettering, OH, USA)
Aseptic gloves (Dynarex, Orangeburg, NY, USA)
Blue pads (Dynarex)
Cotton tip applicators (Puritan, Cuilford, ME, USA)
Disposable drape (Medline, Mundelein, IL, USA)
Dissecting scissors (i.e., Curved Iris Scissors)*
Forceps (one pair)*
Gauze (individually packed, sterile condition)
Hamilton syringe (25 μL volume; Hamilton, Reno, NV, USA)
Isoflurane (Baxter, Deerfield, IL, USA)
Needle holder*
A Neuro-tracer (i.e., FB; Polysciences, Inc., Warrington, PA, USA)
Phosphate-buffered saline (PBS; Life Technologies, Grand Island, NY, USA)
Surgical scissors*
Scrub (i.e., povidone-iodine scrub Betadine solution; Medline)
Skin staples (Teleflex Medical, Research Triangle Park, NC, USA)
Tissue-Teck O.C.T Compound (Sakura, Tokyo, Japan)
Tweezers (at least two pairs, preferentially 4–1/2 in, fine, curved)*
20% sucrose (Sigma-Aldrich Co. LLC., St. Louis, MO, USA)
4-0 polydioxanone (PDS) Sutures (Ethicon, Somerville, NJ, USA)
4% paraformaldehyde (PFA; Sigma-Aldrich Co. LLC)
70% EtOH in the spray bottle

Intracardial perfusion

Blue pads (Dynarex)
Dissecting scissors*
Forceps*
Isoflurane (Baxter)

Slide preparation

Cover glass (Thermo Fisher Scientific, Waltham, MA, USA)
Fluoro-gel (Electron Microscopy Sciences, Hatfield, PA, USA)
Microscope slides (SuperfrostTM Plus; Thermo Fisher Scientific)
Phosphate buffer (0.1M, pH 7.4)

Imaging

Microscope
NIS Elements imaging software (Nikon, Tokyo, Japan) or any other alternatives

Items marked with asterisks are from World Precision Instruments, Sarasota, FL, USA.

Survival surgery for neuro-tracer application

Surgical procedure to inject a neuro-tracer should be

performed in a sterile setting. The method and duration of anesthesia, as well as postoperative care should be discussed with veterinarian and approved by the Institutional Animal Care and Use Committee (IACUC) at an affiliated institution in the United States, or by other professional affiliates in the countries outside of the United States. During the surgical procedure, depth of anesthesia, animal homeostasis and recovery from anesthesia should be closely monitored. Postoperative care should include management of post-operative pain and all other potential post-surgical complications, administration of analgesics/antibiotics, and maintaining the records of post-operative care.

Here we outline the details of the surgical procedure to inject the retrograde neuro-tracer FB in the kidney. The mouse is anesthetized according to the approved animal protocol, and the depth of anesthesia should be confirmed by hind paw pinch to check plantar reflexes. Buprenorphine is administered by subcutaneous injection at a dose of 0.05–0.1 mg/kg prior to surgery for analgesia. The mouse is placed on its right lateral side, the fur is shaved, and the surgical area is prepped with providone-iodine. The kidney is exposed through a skin incision in the left flank. The neuro-tracer FB is applied by one of two methods. For the direct injection to the kidney, a total volume of up to 40 μL of 4% (weight/volume) of FB in 0.9% saline is divided into six to eight injections using a 27–32 gauge metal needle inserted into a 25 μL Hamilton syringe. For crystal powder application method, powdered FB crystals are placed on the surface of the kidney. To prevent neuro-tracer spillage, the application site is swabbed with dry cotton tip applicators after the procedure, followed by cleaning the application site and adjacent organs with PBS-soaked gauze squares. The abdominal wall, muscles and skin are readjusted and closed with 4–0 PDS suture and skin staples. Buprenorphine at a dosage of 0.05–0.1 mg/kg should be administered subcutaneously every 6 to 8 hours for 24 hours total to relieve post-operative pain.

Prevention of neuro-tracer spillage

When a neuro-tracer is injected at the application site, the leakage of neuro-tracers onto adjacent organs may occur resulting in undesired labeling [17]. Therefore, it is crucial to prevent cross-labeling of tissues during the application process, as well as to exclude any off target labeled tissues from further analyses. Precautions necessary to avoid neuro-tracer spillage have been described in the previous literature [7,10,22,23], and should be taken during the surgical procedure, tissue harvest and sectioning of the target tissues.

First, a minimal but sufficient amount of a neuro-tracer should be applied to the intended application site. It should be noted that a greater amount of the neuro-tracer may increase the chances of leakage and unwanted labeling of surrounding tissues. On the other side, a lesser amount of the neuro-tracer may result in deficient labeling of neuronal targets. Therefore, optimization of the tracer amount is essential for successful labeling of kidney innervation. In case of dual or multi-organ labeling, each neuro-tracer should be applied using a separate syringe (for the injection method) or forceps (for the crystal powder method) [22]. For the injection method done by using either a pressure-injection system or a manual plunger, the injection should be made as slowly as possible, and the needle should be left inserted for a minute in the tissue to prevent the leakage of the dye [10,24]. An absorbing material like gauze is recommended to be used to isolate the surgical site during dye injections to prevent the spillage [8,10]. After injection, the application site should be swabbed by dry cotton tip applicators to soak up residual neuro-tracers [11,17]. Then both the application site and adjacent organs are swabbed with PBS-soaked cotton tip applicators. Separate cotton tip applicators should be used for the application site and adjacent organs to prevent cross-labeling of the tissues. Given that neuro-tracer uptake by target organs can stain the site of neuro-tracer uptake [10], errant labeling in adjacent organs can be revealed by using a dissecting microscope during the process of tissue harvest. Lastly, errant labeling should be identified by examining sectioned slides of both target and adjacent organs under a fluorescent microscope. Tissue slides with errant labeling should be excluded from further analysis [7,17] (Fig. 1).

Post-labeling procedures

Harvest and preparation of labeled tissues for analysis

On the day when the labeled ganglia/neurons are collected, it is strongly recommended that the target organ

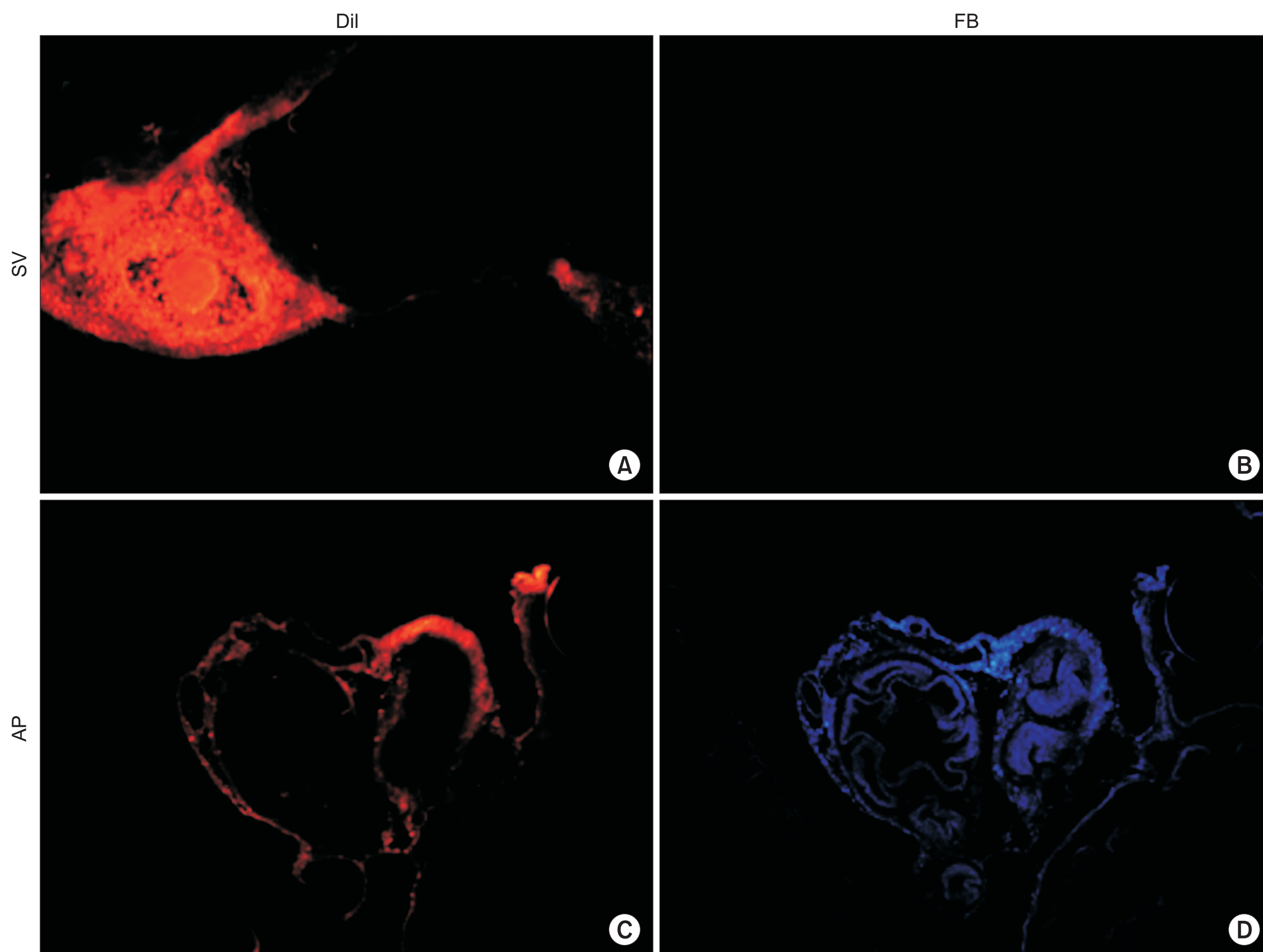

Figure 1. Fluorescent microscopic findings of neuro-tracer spillage. Microscopic observation of SV (A, B) and AP (C, D) after an application of DiI to the bladder and FB to the prostate. AP, anterior prostate; DiI, 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyaine perchlorate; FB, Fast Blue; SV, seminal vesicle.

injected with dyes and adjacent organs are collected for potential spillage of the dyes *in vivo* after the surgery. For example, the kidney, spleen, pancreas and stomach can be considered for collection. If there is any other tissue that may have been affected by neuro-tracer spillage, that tissue should be collected as well for further analysis. Please note that by keeping the orientation of the organs for dissection as close to their original way as possible, the location of neuro-tracer spillage may be more easily identified. After the dissection, all tissues are washed with PBS and fixed in 4% PFA in phosphate buffer (0.1 M, pH 7.4) overnight at 4°C. They are then transferred to 20% sucrose in phosphate buffer for at least 48 hours at 4°C for cryoprotection. For cryosectioning at –20°C, tissues are immersed in Tissue-Teck O.C.T Compound and frozen at –20°C. The organ blocks are cut into 10 μm serial sections using a cryostat. At least 5 sections per each tissue should be collected for analysis. The slides could be imaged under the fluorescent microscope to select the tissues with and without labeling.

Isolation and sectioning of DRG neurons

DRG neurons from an adult mouse can be dissected either by spinal column isolation [20] or open access from the dorsal side of the spine. The former procedure is described in depth by Sleigh et al [25]. Briefly, the pelt is pulled up and fully removed to expose the musculature followed by spinal column isolation. Each spinal segment can be identified by using two methods. First, as Sleigh

et al [25] suggested, the most caudal ribs can be labeled with a fine marker pen; thoracic segment (T) 13 is found just caudal to the ribs. Second, the spinal column can be harvested with ribs intact, which allows the spinal segments to be counted. Extraneous muscle, fat, nerves and other soft tissues are removed. Then DRG neurons can be harvested with or without transverse excision of the spinal column by pulling out dorsal root nerves with caution.

The other way to dissect DRG neurons is to gain open access to the spinal cord from the dorsal side of the spine. The euthanized mouse is usually decapitated to drain the blood. The dorsal pelt is removed to expose the musculature, and the access to the spine is gained via the cervical segments followed by the opening of the dorsal spine all way down to the lumbar segments. Spinal nerves along with the spinal column are pulled aside making DRG neurons visible for isolation. DRG neurons are processed the same way as the other tissues for subsequent analyses, as described above. However, the blocks of DRG neurons should be cut into 10 μm serial sections with all sections or every other section to be collected (preferable) due to small diameter of the ganglia.

Imaging and analysis of labeled DRG neurons

Retrograde labeling allows to identify DRG neurons innervating the kidney. Usually, not all DRG neurons in given sections are neuro-tracer labeled. Additionally, the labeled neurons may show different levels of fluorescent intensity (Fig. 2). Characterization of DRG neurons innervating specific targets and the functional significance of that innervation have been studied with great interest [7,10]. Briefly, DRG neurons that play a major role in nociception are known to be either Aδ- or C-fiber afferents, which are generally small- or medium-sized cells. The area of labeled DRG neurons can be measured using NIS Elements Imaging software. For this approach, only the population having cell bodies with symmetric shapes and nuclei should be included in the analysis [17]. This strategy will prevent double-counting of the same DRG neurons. Note, that it was previously reported that the kidney has preferential innervation from ipsilateral DRG neurons [8]. Interestingly, other visceral organs mostly showed symmetrical innervation from both ipsilateral and contralateral DRG neurons [15,17].

Anticipated appearance of the injection site

The neuro-tracer itself is not cell type-specific, and will label a variety of cells which are physically exposed to the tracer (Fig. 3) [26]. Therefore, blood vessels, nerve fibers, neurons, and other cells can be labeled at the site of the injection. However, once the tracer is picked up by the nerve endings, it will label only neurons having axonal terminals at the site of dye injection. Peripheral nerve endings residing at the neuro-tracer application site uptake the applied neuro-tracer allowing it to travel through the nerve bundles to reach the cell bodies of peripheral ganglia.

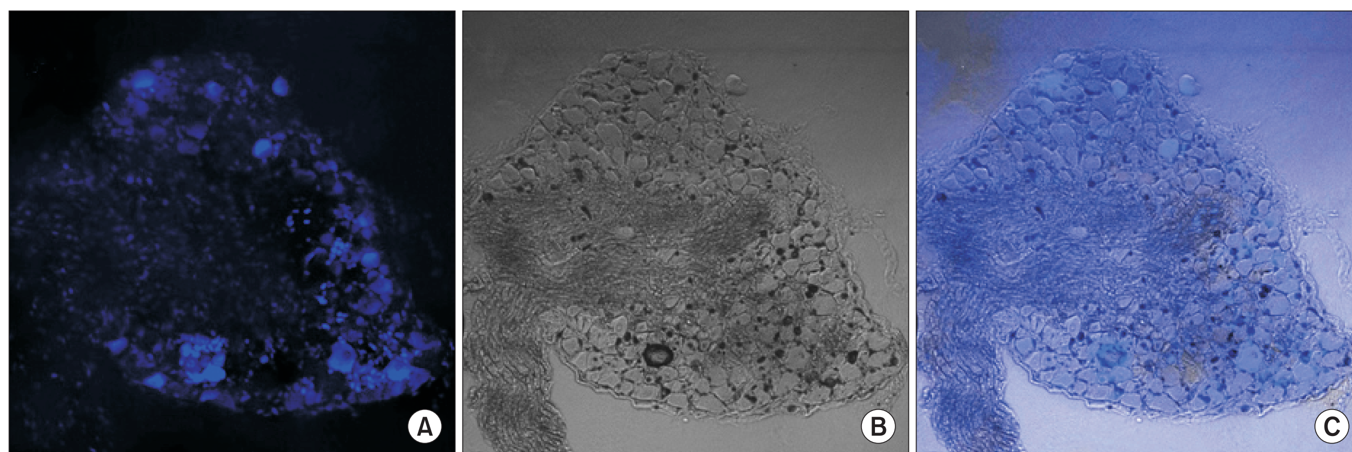

Figure 2. Fluorescent microscopic findings of labeled dorsal root ganglion (DRG) neurons innervating the kidney. Fast Blue-labeled (A), bright-field (B), and merged image (C) of DRG neurons in the spinal segment of thoracic 13.

Additional considerations to enhance labeling outcomes

Intracardial perfusion

Unless harvested tissues are designated for cell culture and expression profile assay, cardiac perfusion with fixatives is highly recommended prior to the tissue harvest. Rapid preservation of the tissue using fixative provides rigidity for many tissues including the kidneys. Please note that cervical dislocation should not be considered as an option for euthanasia to isolate the neural tissues because the procedure may damage DRG neurons and other neural structures. A detailed protocol and video clip for intracardial perfusion are available at http://www.jove.com/video/3564/whole-animal-perfusion-fixation-for-rodents [27].

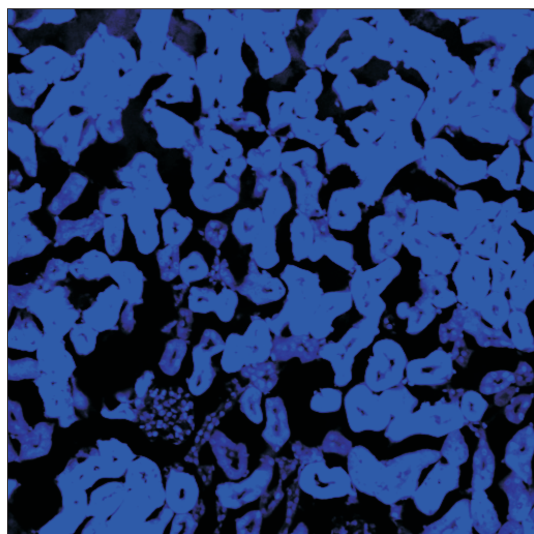

Figure 3. Image of injection site of Fast Blue (FB) in the kidney. A section of kidney after injection of FB.

Briefly, the mouse should be fully anesthetized, and the anesthetic effect should be confirmed by hind paw pinch to check plantar reflexes. The chest is then opened via a 5–6 cm lateral incision just beneath the rib cage. The diaphragm is carefully opened via a small incision using the curved, blunt scissors until the pleural cavity is exposed. The lung is displaced and the rib cage is incised up to the collarbone on both the ipsilateral and contralateral sides. The sternum is lifted, and any connective tissue surrounding the heart is removed. The posterior end of the left ventricle is located and pierced using iris scissors. A 15-gauge blunt or olive-tipped perfusion needle is inserted in the right atrium to allow blood exit the vasculature. After the blood draining, the catheter is perfused with PBS first followed by slow perfusion with 4% PFA for

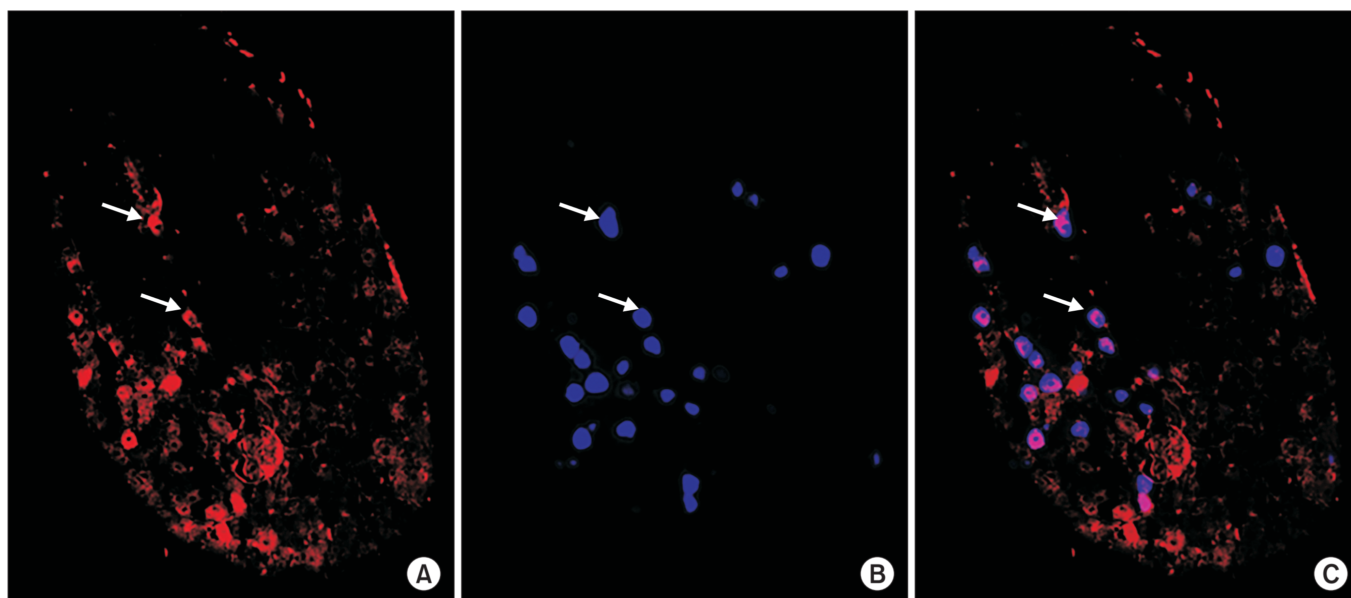

Figure 4. Images of labeled convergent dorsal root ganglion (DRG) neurons. DiI-labeled DRG neurons innervating the colon (A), Fast Blue (FB)-labeled DRG neurons innervating the urinary bladder (B) and dual labeling of DRG neurons innervating both the colon and the urinary bladder (C) in the spinal segment of lumbar 6. DiI was injected in the distal colon and FB was injected in the urinary bladder. DiI, 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyaine perchlorate. Arrows indicate convergent DRG neurons innervating the urinary bladder and colon.

in vivo fixation of the tissues.

Integrity of labeling

In general, FB provides reproducible and robust labeling outcomes [12,16]. The best efficiency of FB uptake is achieved by following the manufacturer's direction for storage conditions and dissolving method. Even if the uptake of neuro-tracers occurs properly, labeled cells can fade out after the sections are placed on slides. This can be prevented by using anti-fade mounting media fluorogel. In addition, minimal duration of light exposure during imaging may help with the loss of fluorescent signal. It was previously discussed that combination or sequential application of different neuro-tracers may result in deficient labeling [12]. However, both FB and DiI are known to provide a long-lasting efficient labeling, and are considered to be the efficient dyes (Fig. 4).

Conventional neuro-tracers (fluorescent dyes) vs. retrograde trans-synaptic viral tracers

Neurotropic viruses delivering genetic material with fluorescent protein have been recently highlighted as a relatively new technique of neuro-tracing [14,28]. Understanding pros and cons of conventional neuro-tracers vs. retrograde trans-synaptic viral tracers is fundamental for research design. Major difference between the two techniques is that only viral tracers can cross the synapses, therefore, result in labeling of both post- and presynaptic neurons of interest [29]. Cross-synaptic features of viral tracers enable visualization of organ-specific neuronal population in the higher centers of the nervous system like the spinal cord and brain [28,30]. Using the virus-based tracers require to consider a packaging size of the viral vectors and trans-synaptic efficiency, as well as a burden to handle the virus. More than three different non-viral neuro-tracers can be applied to each target organ, and then labeled neurons can be visualized by confocal microscope.

Summary and future directions

This technical note intended to outline a detailed practical protocol for application of a neuro-tracing technique. Conventional neuro-tracing approach can be a useful tool to study kidney innervation, and also to evaluate a potential neural cross-talk between the kidneys and other organs. Visualization of renal neurons also provides an opportunity to study functional properties of these cells and their role in kidney function. Furthermore, a neuro-tracing technique is a great tool to determine disease-associated changes in renal innervation in variable animal models of acute kidney injury (AKI), chronic kidney disease (CKD), diabetic mellitus (DM), and other diseases. Therefore, analysis of neuro-tracer labelled renal neurons followed by additional analyses may uncover physiological significance of renal neurons in the progression/resolution of renal diseases, and provide a better understanding of the mechanisms underlying renal pathologies.

Conflicts of interest

All authors have no conflicts of interest to declare.

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