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Retrograde labeling of regenerating motor and sensory neurons using silicone caps

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HIGHLIGHTS

- Description of two detailed approaches to the retrograde labeling of peripheral nerves.
- The novel approach is validated using both neuron counts and labeling intensity.
- The novel approach to retrograde labeling significantly reduces the time required for the procedure.

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ABSTRACT

Background: Retrograde labeling permits the investigation of the number, distribution and axonal projections of neurons in the peripheral nervous system. The well technique for labeling peripheral nerves consists of incubating the exposed peripheral nerve in a well for one hour, a time intensive technique. However, other techniques that inject tracers directly into the nerve or muscle may result in variable labeling depending on nerve preparation and location of injection.
New method: We describe a method of retrograde labeling peripheral nerves that increases tracer uptake and improves labeling efficiency. This technique utilizes a silicone cap over the nerve that is kept in place with fibrin glue, permitting closure of the incision with the cap in place, mitigating the need to wait one hour for back-labeling as with the standard well technique.
Results: In the rat common peroneal nerve, the new silicone cap technique, compared to the standard well technique, labeled 405 ± 11 (SEM) vs. 378 ± 21 motoneurons and 953 ± 40 vs. 948 ± 57 sensory neurons. These counts were not statistically different. Labeling intensity was greater in DRG neurons with the silicone cap technique, but this difference was not evident in motoneurons.
Comparison with existing method: Retrograde-labeling with silicone caps labels an equal number of motor and sensory neurons in comparison with the standard well technique and labels sensory neurons with greater intensity.
Conclusions: Retrograde-labeling with silicone caps reliably labels neurons and significantly decreases the time required for labeling, reducing anesthetic exposure and improving the efficiency of the technique.

1. Introduction

Retrograde-labeling techniques are valuable in neuroscience and have been used in many studies to investigate the size and distribution of neuronal pools and the architecture of axonal projections in the central (Bentivoglio et al., 1979, 1980; Shipley, 1985) and peripheral nervous system (Mesulam and Brushart, 1979; Mesulam et al., 1980; Richmond et al., 1994; Novikova et al., 1997; Choi et al., 2002). The retrograde labeling technique has emerged as an important method of investigating peripheral nerve regeneration because retrograde labeled neurons that have regenerated

Abbreviations: CP, common peroneal; FG, fluorogold; RG, retrograde; DRG, dorsal root ganglia.

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their axons can be counted to localize and quantify the regeneration of peripheral nerve axons following injury (Brushart, 1993; Sulaiman and Gordon, 2000; Guntinas-Lichius et al., 2007; Hayashi et al., 2007a).

Early studies used agents such as horseradish peroxidase (HRP) for retrograde labeling, which required histochemical or immunocytochemical processing after labeling (Kristensson and Olsson, 1971; De Groat et al., 1978; Mesulam and Brushart, 1979; Mesulam et al., 1980; Janjua and Leong, 1981; Brushart and Seiler, 1987). More recently, HRP has been largely replaced by cheaper fluorescent tracers which are easier to use as they require no post-labeling processing (Brushart, 1990; Al-Majed et al., 2000; Brushart et al., 2005; Huang et al., 2013). Fluorescent tracers have been used to label both motor and sensory neurons in several different models of peripheral nerve injury including nerves of the lower limb (Brushart, 1990; Wood et al., 2012; Placheta et al., 2014; Kemp et al., 2015b), upper limb (Aszmann et al., 2002, 2004), cranial nerves (LaVail et al., 1993; Choi et al., 2002; Guntinas-Lichius et al., 2007; Placheta et al., 2015) and others (Marfurt et al., 1989; Gordon and Richmond, 1990; Hayashi et al., 2007a). Several techniques have been used to retrograde label neurons including injection of dye directly into the muscle or nerve (Sagot et al., 1998; Haenggeli and Kato, 2002; Hayashi et al., 2007a). These techniques are highly dependent on the location of injection and risk dye leakage through the site of injection, potentially increasing labeling variability and decreasing specificity (Janjua and Leong, 1981; Prodanov et al., 2005; Katada et al., 2006; Hayashi et al., 2007b; Tosolini et al., 2013).

The standard well technique, which involves a one-hour incubation of a transected peripheral nerve in a petroleum jelly well containing either fluorescent dye (Richmond et al., 1994; Hayashi et al., 2007a) or fluorescent crystals (Al-Majed et al., 2000; Sulaiman and Gordon, 2000; Boyd and Gordon, 2001), overcomes many of these limitations. However, while this technique results in robust labeling, an incubation period is required prior to wound closure to avoid exposure of the crystals to other tissues, making the technique time-intensive and limiting the number of animals that can be labeled daily. A less time-intensive retrograde labeling technique is required that continues to ensure labeling specificity while avoiding the lengthy incubation period.

Previously, silicone (Alant et al., 2013) and polyethylene caps (Sagot et al., 1998; Haenggeli and Kato, 2002) have been described to prevent dye leakage following tracer application, but the technique was not described in detail for the purposes of reproducing the procedure and it has not been compared directly to the standard well technique. Here we describe a labeling technique using silicone caps, which isolates fluorescent crystals to the transected nerve while permitting wound closure, mitigating the need for a long incubation period. Once in place, the silicone cap is sealed with tissue glue, preventing leakage to adjacent tissues when the wound is closed. Silicone caps have the potential to improve the efficiency of retrograde-labeling, increase the intensity of labeling and decrease the time-associated costs of the procedure. We directly compare the silicone cap technique to the standard well technique, describe our experience with both procedures and provide technical notes to maximize the quality and specificity of labeling with both techniques.

2. Materials and methods

2.1. Experimental animal model

Six adult Sprague-Dawley rats (Charles River, Montreal, QC) weighing 220–250 g at the time of surgery were used. Each rat had both the left and right common peroneal nerve retrograde labeled in order to compare the two techniques within the same rat. Rats received both water and standard rat chow (Purina, Mississauga, ON) ad libitum, and were housed with a 12:12 h light:dark cycle. All rats were maintained in a temperature and humidity controlled environment. All surgical procedures were executed in an aseptic manner under an operating microscope and were approved by The Hospital for Sick Children Laboratory Animal Services (LAS), which adhered to the Canadian Council on Animal Care guidelines.

2.2. Experimental design

In order to evaluate and compare both techniques, we used a common peroneal (CP) nerve model that has been well established in the literature (Boyd and Gordon, 2002; Udina et al., 2010; Wood et al., 2012, 2013; Placheta et al., 2014; Elzinga et al., 2015). Rats were operated on bilaterally, with the right (CP) nerve labeled using the standard well technique with a petroleum jelly (white petroleum jelly) well. The left CP nerve was labeled using a technique with a silicone cap. In both methods, Fluorogold tracer was employed as this fluorescent dye was shown by others to provide robust labeling in a CP nerve model (Kemp et al., 2015a; Placheta et al., 2015; Richmond et al., 1994; Wood et al., 2012). All rats were allowed to recover from the surgical procedures and were then returned to their cages. Three days following retrograde labeling rats were anesthetized and transcardially perfused with fixative and the lumbar spinal cord and L4/L5 DRGs were harvested for sectioning and counting of motor and sensory neurons.

2.3. Surgical procedures for retrograde labeling using the standard well technique

The right sciatic nerve was exposed through a mid-lateral thigh incision and the CP nerve was isolated and dissected away from the surrounding nerves and connective tissue. The CP nerve was then transected 10 mm distal to the biceps femoris branch. To construct the petroleum jelly well, we first placed the petroleum jelly in a 1 mL syringe with a 25-gauge half-inch needle, which permitted accurate distribution of the petroleum jelly. The well was then constructed by placing two layers of petroleum jelly around the periphery of a small piece of plastic paraffin film (approximately $5 \times 5\ mm^2$) adjacent to the transected nerve tip while ensuring that the nerve does not come in contact with the petroleum jelly as this may decrease labeling by preventing dye uptake (Fig. 1A). The proximal nerve stump was then carefully draped over the well so that the nerve tip was centered within the well and another layer of petroleum jelly was placed over the existing well walls to ensure no leakage occurred around the nerve (Fig. 1B). It was necessary to ensure that while the petroleum jelly was being placed that it came in contact with the Parafilm and nerve stump, otherwise leakage of the well occurred. The well was then filled with 10 μL of 4% solution of FluoroGold (FG: Fluorochrome LLC, Denver, CO) dissolved in sterile saline solution (Fig. 1C) and the nerve was left within the well for one hour, after which the well was removed and staining was confirmed visually by observing yellow staining of the exposed nerve tip (Fig. 1D). The proximal stump and wound were then rinsed three times with sterile saline to remove any residual dye and prevent staining of the adjacent muscle or nerve. Incisions were closed and, following recovery, animals were returned to their cages. The time of completion for the procedure, from anesthetizing the animal to returning the animal to the cage, is approximately 90 minutes.

2.4. Silicone cap construction

Prior to retrograde labeling with the silicone cap technique, the caps were prepared. In order to permit the nerve to be placed within the silicone cap, the inner diameter of the tubing was selected to fit the nerve to be labeled (Table 1). The appropriately sized silicone