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Comparison of commonly used retrograde tracers in rat spinal motor neurons

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Abstract

The purpose of this study was to investigate the effect of four fluorescent dyes, True Blue (TB), Fluoro-Gold (FG), Fluoro-Ruby (FR), and 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI), in retrograde tracing of rat spinal motor neurons. We transected the muscle branch of the rat femoral nerve and applied each tracer to the proximal stump in single labeling experiments, or combinations of tracers (FG-DiI and TB-DiI) in double labeling experiments. In the single labeling experiments, significantly fewer labeled motor neurons were observed after FR labeling than after TB, FG, or DiI, 3 days after tracer application. By 1 week, there were no significant differences in the number of labeled neurons between the four groups. In the double-labeling experiment, the number of double-labeled neurons in the FG-DiI group was not significantly different from that in the TB-DiI group 1 week after tracer application. Our findings indicate that TB, FG, and DiI have similar labeling efficacies in the retrograde labeling of spinal motor neurons in the rat femoral nerve when used alone. Furthermore, combinations of DiI and TB or FG are similarly effective. Therefore, of the dyes studied, TB, FG and DiI, and combinations of DiI with TB or FG, are the most suitable for retrograde labeling studies of motor neurons in the rat femoral nerve.

Key Words: *nerve regeneration; tracing efficacy; fluorescent tracers; retrograde tracing; femoral nerve; motor neurons*

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Introduction

Misdirection of regenerating axons prevents functional recovery after nerve injury. Incorrect projections to end organs fail to establish functional contacts, and occupy the pathways of appropriate axons (Brushart, 1988). A better understanding of the final and correct pathways of regenerating axons is important for the advancement of basic and clinical neuroscience.

Retrograde tracers are often used to evaluate the accuracy of reinnervation in the rat femoral nerve injury model (Brushart and Seiler, 1987; Brushart, 1990, 1993; Madison et al., 1996, 1999; Al-Majed et al., 2000; Robinson and Madison, 2003). The femoral nerve in the adult rat includes purely cutaneous sensory branches, which grow into the skin through the saphenous nerve, and mixed branches, which innervate the musculus quadriceps femoris. In the rat femoral nerve model, motor nerve fibers only exist in the muscle branches. The presence of any motor nerve fibers in the cutaneous branch after injury is considered to be incorrect reinnervation, and can be identified by retrograde tracing (Robinson and Madison, 2004). However, there is little information about the comparative efficacies of different tracers in the retrograde labeling of rat femoral nerve motor neurons (Harsh et al., 1991; Robinson and Madison, 2003, 2004).

When used in combination, it is important that the chosen retrograde tracers have similar labeling efficacies and do not interfere with each other's labeling abilities. The labeling efficacy of different retrograde nerve tracers is affected by a number of factors, including the method of application, the time required for tracing, the simultaneous use of additional tracers, and the intrinsic properties of each tracer (Richmond et al., 1994; Novikova et al., 1997; Byers et al., 2002; Puigdellívol-Sánchez et al., 2002; Hayashi et al., 2007). The same fluorescent tracer can also act differently depending on the neural pathways or types of neurons in which it is used (Maslany et al., 1992; Güntürkün et al., 1993; Deng and Rogers, 1999). Despite the abundance of commercially available fluorescent retrograde neuronal tracers, the choice of appropriate dye is often difficult (Richmond et al., 1994; Novikova et al., 1997; Vercelli et al., 2000).

The purpose of this study was to provide information on the efficacies of different fluorescent tracers, to aid in quanti-

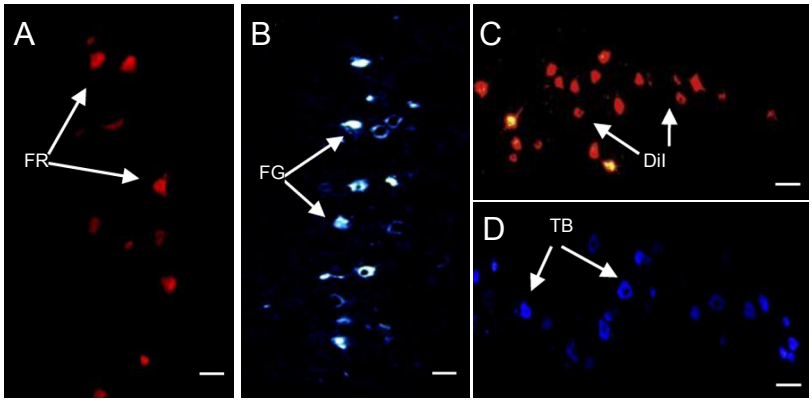

Figure 1 Photomicrographs showing retrogradely-labeled motor neurons in longitudinal sections of the rat femoral nerve; single staining 3 days after dye application.
(A) FR, red; (B) FG, white-blue; (C) DiI, red; (D) TB, deep-blue. Scale bars: 50 μm. DiI: 1,1′-Dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; FG: Fluoro-Gold; FR: Fluoro-Ruby; TB: True Blue.

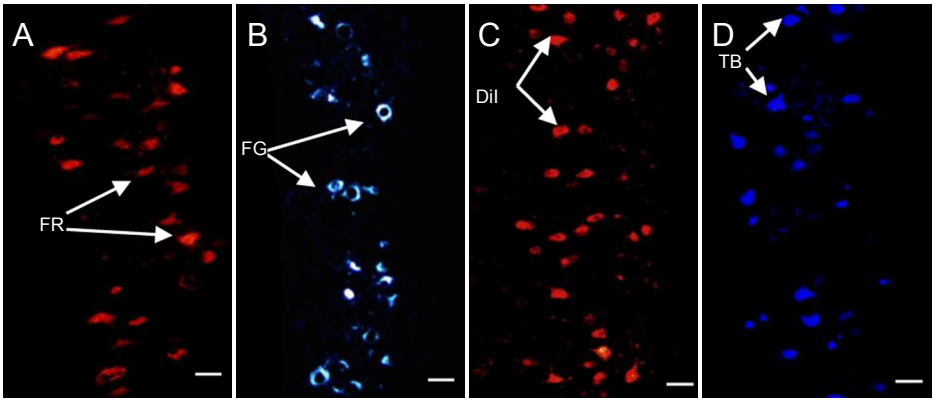

Figure 2 Photomicrographs showing retrogradely-labeled motor neurons in longitudinal sections of the rat femoral nerve; single staining 7 days after dye application.
(A) FR, red; (B) FG, white-blue; (C) DiI, red; (D) TB, deep-blue. Scale bars: 50 μm. DiI: 1,1′-Dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; FG: Fluoro-Gold; FR: Fluoro-Ruby; TB: True Blue.

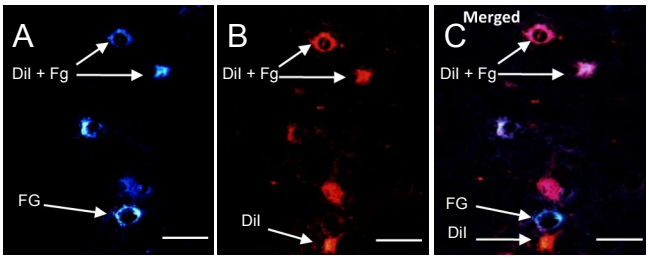

Figure 3 Photomicrographs showing retrogradely-labeled motor neurons in longitudinal sections of the rat femoral nerve; double staining with FG-DiI 7 days after dye application.
(A) FG, white-blue; (B) DiI, red; (C) merge, FG-DiI double-labeled cells appeared pink. Scale bars: 50 μm. DiI: 1,1′-Dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; FG: Fluoro-Gold; FR: Fluoro-Ruby; TB: True Blue.

tative investigations of motor axon regeneration specificity. To this end, we compared the labeling efficacy of four commonly used fluorescent dyes, True Blue (TB), Fluoro-Gold (FG), Fluoro-Ruby (FR), and 1,1′-Dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI), alone and in combination, using retrograde tracing of motor neurons in the rat femoral nerve.

Materials and Methods

Animal preparation

Thirty young adult female Sprague-Dawley rats (specific pathogen free, aged 8–10 weeks, weighing 200–240 g), were obtained from the Animal Experimental Center of Peking University People's Hospital (Beijing, China; license No. SYXK 2011-0010). For all surgical procedures, the animals were deeply anesthetized with sodium pentobarbital (30 mg/kg) via intraperitoneal injection. The study was performed in strict accordance with Chinese guidelines for the care and use of experimental animals, and approved by the Research Ethics Committee at Peking University People's Hospital. Unilateral application of the retrograde nerve tracers to the muscle branch of the rat femoral nerve showed no contralateral spinal motor neuron labeling, so we were able to perform bilateral experiments and reduce the number of animals used.

Selection of fluorescent tracers

We determined optimum tracer concentrations in preliminary experiments. In the present study, we used four retrograde fluorescent tracers: 2% TB (diaceturate salt; Sigma-Aldrich, St. Louis, MO, USA) in distilled water, 4% FG (Fluorochrome LLC, Denver, CO, USA) in cocodylic acid (pH 3), 10% FR (Fluorochrome LLC) in distilled water, and 15% DiI (Sigma-Aldrich) in 100% ethanol. For double-labeling experiments, we selected the high-efficacy tracers from the single labeling experiments: FG-DiI (4% FG and 15% DiI), and TB-DiI (2% TB and 15% DiI).

Tracer application

All animal experiments were performed in a specific pathogen-free animal laboratory using a binocular surgical microscope and standard microsurgical techniques. Rats were

anesthetized and the inguinal region was shaved. The inguinal approach was used to expose the muscle branch of the rat femoral nerve on one side, which was transected 5-mm distal to the femoral nerve bifurcation. The proximal stump of the transected muscle nerve was dipped into a very small polyethylene tube containing a retrograde tracer or tracer combination. The same procedure was then performed on the contralateral side with a different tracer or combination. Each individual tracer was applied to 12 femoral nerves, and each combination was applied to six femoral nerves. A total of 24 rats were used in the single labeling experiments: FG or DiI alone, $n = 12$ (right side, FG; left side, DiI); TB or FR alone, $n = 12$ (right side, TB; left side, FR). The remaining six rats were used in the double labeling experiments: FG-DiI and TB-DiI, $n = 6$ (right side, FG-DiI; left side, TB-DiI). The tube was sealed with a mixture of vaseline and silicone grease to prevent leakage, and the stump of the nerve was placed in the tracer for about 2 hours. The tube was then removed, and the tip of the severed branch was rinsed in saline and sealed with silicone grease. Finally, the wound was closed using 4-0 nylon sutures.

Tissue preparation

For the single-labeling experiments, 12 of the 24 rats were sacrificed 3 days after tracer application, and the remaining 12 after 1 week, to allow sufficient time for the dyes to travel back to the motor neurons. The six rats in the double-labeling experiment were sacrificed 1 week after tracer application. The animals were deeply anesthetized and perfused through the heart with 200-mL warm saline followed by 4% paraformaldehyde. The lumbar spinal cord, which contains all femoral nerve motor neurons (Brushart and Seiler, 1987), was removed, post-fixed for 6 hours in 4% paraformaldehyde, then stored in 20% sucrose in phosphate buffer. The cord was quick-frozen in dry ice, and stored at −80°C until sectioning. Serial 25-μm longitudinal sections were cut in a freezing microtome onto gelatin-coated glass slides, dried, and overlaid with coverslips using Prolong (P-7481, Molecular Probes, OR, USA) to prevent quenching of fluorescence.

Motor neuron quantification

The spinal cord sections were viewed under a fluorescence microscope (BX51TR, Olympus, Tokyo, Japan) with a camera (DP70, Olympus), by two independent observers unaware of the experimental treatment. The following Olympus mirror units were used: U-MWG2 (excitation filter 510–550 nm, dichroic beamsplitter 570 nm, barrier filter 590 nm) for DiI and FR, U-MWU2 (excitation filter 330–385 nm, dichroic beamsplitter 400 nm, barrier filter 420 nm) for TB and FG. All labeled cells per 25 μm section were counted by varying the focus throughout the section. For the double-labeling experiments, the sections were photographed using two different mirror units, the two photomicrographs were superimposed, and motor neurons were identified as either single- or double-labeled. Only motor neurons with obvious labeling, notably different from background fluorescence, were counted. Counting variation between the observers was approximately 2%. Counts were adjusted for split cells using the Abercrombie correction (Abercrombie, 1946).

Statistical analysis

Differences in the number of labeled neurons were compared between groups using one-way analysis of variance (ANOVA) followed by *post hoc* test with Bonferroni correction. Analysis was performed using SPSS 17.0 software (SPSS, Chicago, IL, USA). $P < 0.05$ was defined as statistically significant. All results are reported as the mean ± SEM.

Results

Retrogradely labeled motor neurons in the femoral nerve were observed with all four fluorescent tracers. Cytoplasm of neurons labeled with TB or FG displayed deep-blue and white-blue fluorescence, respectively, and those labeled with FR or DiI showed red fluorescence (**Figures 1**, **2**). The FG-DiI combination resulted in white-blue and red fluorescence (**Figure 3**), and TB-DiI double-labeled cells showed deep-blue and red fluorescence (**Figure 4**).

FR labeled significantly fewer neurons than the other tracers 3 days after application ($P < 0.05$). There were no significant differences in the number of motor neurons labeled by TB, FG, and DiI at 3 days ($P > 0.05$); however, at 1 week, the number of FR-labeled neurons was significantly greater than that at 3 days ($P < 0.05$) (**Figure 5**). The number of labeled neurons was also greater at 1 week than at 3 days for the remaining three dyes but this did not reach significance ($P > 0.05$). At 1 week, no significant differences were observed between the four fluorescent tracers ($P > 0.05$). At both time points, FG labeled the most neurons, and FR the fewest.

The double labeled motor neurons in the TB-DiI and FG-DiI groups represented 96.0% and 95.3% of all motor neurons, respectively (**Figure 6**). No significant difference in the number of labeled cells was observed between the two double-label groups, or between the double-label groups and FG, TB or DiI single-label groups ($P > 0.05$).

Discussion

Retrograde labeling with different dyes is an important method by which to distinguish motor from sensory neurons and to explore the relationship between the spinal cord and peripheral nerves. Radioactive tracer materials have been pivotal in understanding axoplasmic transport, and enabled direct observation of axonal transport. To obtain functional recovery after injury, injured peripheral nerve fibers must grow into the correct target organ (Hayashi et al., 2007). Using retrograde labeling and other anatomical methods it is possible to determine the accuracy of reinnervation in the rat femoral nerve injury model. However, the range of application methods and retrograde tracers available makes it difficult to select the most suitable dyes. Our goal in the present study was to test the labeling efficacy of different nerve retrograde tracers to find appropriate tracer combinations for retrograde labeling in the rat femoral nerve.

There are many ways by which retrograde labeling of motor neurons can be achieved, including immersion of the transected stump in the tracer, or injection of the tracer directly into the intact nerve, muscle or skin. In the present study, we chose the first method, since it has been shown that tracer injection into nerve or muscle leads to changes in the number of labeled motor neurons and is less effective in neuronal tracing (Richmond et al., 1994). To maximize labeling and reduce variability, we used the tracers at their most effective concentrations and dipped the stump of the transected nerve into the tracer for about 2 hours. Previous studies have used TB at concentrations of 1–5% (Deng and Rogers, 1999; Katada et al., 2006; Žele et al., 2010), FG at 2–5% (Deng and Rogers, 1999; Al-Majed et al., 2000), FR at 2–10% (Choi et al., 2002; Katada et al., 2006; Žele et al., 2010), and DiI at 2–10% (Richmond et al., 1994; Choi et al., 2002; Žele et al., 2010). In addition, we chose recovery periods of 3 days or 1 week to allow sufficient time for retrograde transport to occur but avoid neuronal death from axonal damage. Retrograde tracing time longer than 2 weeks after application of FG or FR results in fewer labeled motor or dorsal root ganglion neurons than at 1 week, presumably owing to fading fluorescence or neuronal death (Novikova et al., 1997; Choi et al., 2002; Welin et al., 2008).

In our single labeling experiments, TB, FG and DiI labeled similar numbers of motor neurons, at both 3 days and 1 week after dye application. This indicates that the three tracers have similar efficacies in motor neuron labeling in the rat femoral nerve. In comparison, FR labeled significantly fewer cells 3 days after application, and slightly fewer cells than the other tracers 1 week after application, although the difference was no longer significant. These results suggest that FR undergoes slow retrograde axonal transport. One study also reported that FR labeled less than half of the dorsal root ganglion neurons that were labeled by TB, FG, DiI or Diamidino Yellow (counted 10 days after application) (Žele et al., 2010). FR is a conjugate of the hydrophilic polysaccharide dextran. It is characterized by good water solubility, and is non-toxic and relatively inert. FR can be transported by retrograde and anterograde transport in living axons, but it is most effective as an anterograde tracer (Schmued et al., 1990). Optimal anterograde labeling is obtained 6–14 days after application, whereas effective retrograde labeling takes 10–15 days (Schmued et al., 1990; Vercelli et al., 2000; Choi et al., 2002; Katada et al., 2006). The fluorescent excitation and emission profiles of FR are distinct from those of FG and TB, making FR the most suitable tracer for multi-label studies (Zhang and McClellan, 1998; Katada et al., 2006). However, FR fades more quickly than FG, restricting its use to short-term experiments (Novikova et al., 1997; Choi et al., 2002) or as a secondary tracer in sequential labeling studies (Novikova et al., 1997; Choi et al., 2002; Katada et al., 2006). FR can be used at different concentrations, usually 5–10% or higher. It can also be applied directly as crystals (Al-Majed et al., 2000). Higher concentrations of dextrans (up to 20%) result in slightly greater labeling sensitivity (Vercelli et al., 2000).

FG, introduced in 1986, is a water soluble crystalline tracer (Schmued and Fallon, 1986). The spectral characteristics of FG vary with pH. When dissolved in phosphate buffered saline (pH 7.40), FG has a wide-band ultraviolet excitation and produces a golden-yellow fluorescence. In the present study, FG was dissolved in cocodylic acid and produced a white-blue fluorescence (Al-Majed et al., 2000), which is more appropriate for double-labeling experiments than golden-yellow, since it is more reaDiIy differentiated from the fluorescence produced by DiI. We found that FG stained the greatest number of motor neurons at both time points examined. FG is effectively transported from the stump of the transected nerve and is considered the most useful and effective marker in experiments that require fast neuronal labeling (Richmond et al., 1994; Choi et al., 2002).

TB (related chemically to Fast Blue) is less water soluble than FG and dextran conjugates. It can be transported over long distances and primarily labels the neuronal cytoplasm (Choi et al., 2002; Hayashi et al., 2007). TB produces a deep blue fluorescence, which is observed with wideband ultraviolet excitation (excitation: 365 nm; emission: 420 nm). Labeling using TB results in similar staining of the cytoplasm and dendritic processes. Katada et al. (2006) reported that the label can survive for long periods (at least 6 months) – much longer than that of FG or dextran conjugates (Novikova et al., 1997). Garrett et al. (1991) found that long-lasting retention of cellular TB had no effect on the survival of labeled motor neurons. However, TB may have a selective toxic effect on FR uptake in double-labeling experiments (Katada et al., 2006). In the present study, TB did not influence the labeling efficacy of the other tracers in the double-labeling experiments.

DiI is a lipophilic carbocyanine dye that results in a red fluorescence with a tetramethylrhodamine isothiocyanate filter set. It can label fixed cells by diffusing through phospholipid membranes but, importantly, it can also label living cells by axonal transport. DiI diffuses rapidly *in vivo* (6 mm/d) but slowly in fixed tissue at room temperature (2 mm/mon) (Holmqvist et al., 1992). It is non-toxic and can be used for both anterograde and retrograde labeling (Haugland, 1996). Some tracers, such as DiI or 4-(4-(dihexadecylamino)styryl)-N-methylpyridinium iodide) (DiA), are used at high concentrations, so that passive diffusion of the tracers into cells can occur down local concentration gradients. Others can be used as crystals, applied directly to the stump of the nerve (Hayashi et al., 2007). DiI is not water-soluble and may be used in its crystalline form, or dissolved in ethanol, dimethylsulfoxide or dimethylformamide. Dissolving the crystals in grease is reported to increase the diffusion rate by 50% (Vercelli et al., 2000). In the present study, we found that DiI (15% in 100% ethanol) has a similar efficacy to TB and FG, in agreement with other studies (Vercelli et al., 2000; Popratiloff et al., 2001; Žele et al., 2010).

For double-labeling with fluorescent dyes, it is a problem if both tracers are visible under the same filter, a phenomenon called "bleed-through" (Zhang and McClellan, 1998), which reduces the accuracy of counting single- versus double-labeled cells. To avoid this situation, the dyes chosen for

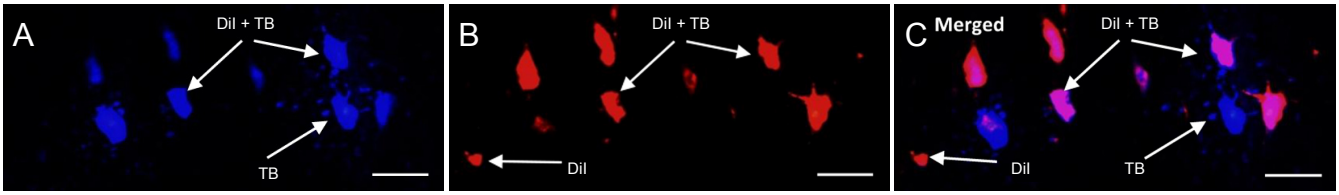

Figure 4 Photomicrographs showing retrogradely-labeled motor neurons in longitudinal sections of the rat femoral nerve; double-staining with TB-DiI 7 days after dye application.
(A) TB, deep blue; (B) DiI, red; (C) merge, TB-DiI double-labeled cells appeared pink. Scale bars: 50 μm. DiI: 1,1′-Dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; FG: Fluoro-Gold; FR: Fluoro-Ruby; TB: True Blue.

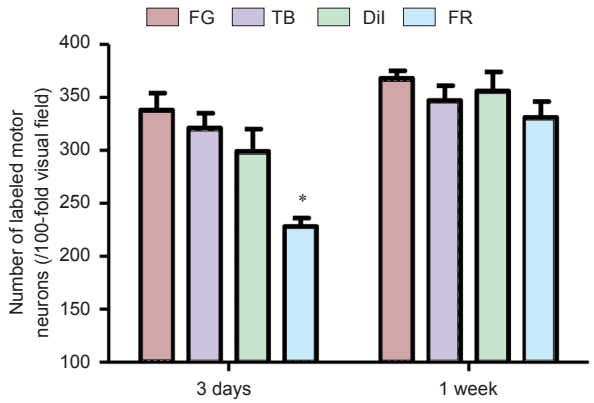

Figure 5 Number of motor neurons in rat femoral nerve following single labeling using FG, TB, DiI and FR, 3 days and 1 week after tracer application.
The results are presented as the mean ± SEM (n = 12 femoral nerves per group). *P < 0.05, *vs.* other groups at 3 days (one-way analysis of variance and *post-hoc* test with Bonferroni correction). DiI: 1,1′-Dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; FG: Fluoro-Gold; FR: Fluoro-Ruby; TB: True Blue.

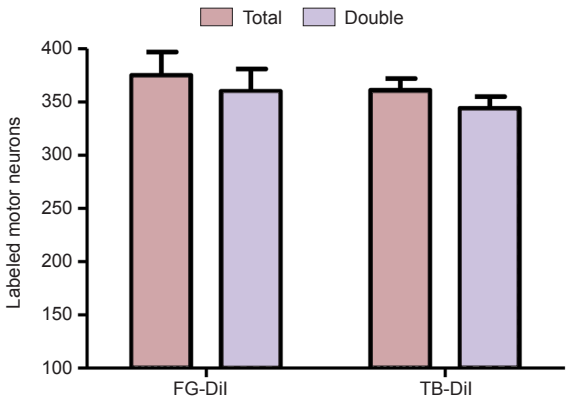

Figure 6 Number of motor neurons double-labeled using FG-DiI and TB-DiI, 1 week after tracer application in the rat femoral nerve.
Data are expressed as the mean ± SEM (n = 6 femoral nerves per group). One-way analysis of variance and *post-hoc* test with Bonferroni correction were used to compare differences in number of labeled neurons between groups. DiI: 1,1′-Dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; FG: Fluoro-Gold; FR: Fluoro-Ruby; TB: True Blue.

double-labeling experiments should have widely separated spectra. Therefore, in our double-labeling experiments, we did not test the combination FG-TB, which would present difficulties in identifying double-labeled neurons, as both tracers label the cytoplasm, and fluoresce under the same ultraviolet wavelength.

We found that the simultaneous application of DiI and FG or TB to the cut muscle nerve resulted in 95% or more double-labeled neurons, not different from the numbers of neurons labeled when each tracer was applied separately. Therefore, the dyes TB, FG and DiI label similar numbers of rat femoral nerve motor neurons, and their individual labeling efficacy is not reduced when applied in combination.

In conclusion, we have shown here that the tracers TB, FG and DiI label a virtually identical population of motor neurons at 3 days and at 1 week after their application to the transected end of the muscle branch of the rat femoral nerve. In double-labeling experiments, the efficacies of TB-DiI and FG-DiI were no lower than when used separately, and the tracer pairs labeled an almost-overlapping neuronal population. This makes the combinations of TB and DiI, and FG and DiI, suitable for double retrograde labeling of femoral nerve motor neurons in the rat.

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Conflicts of interest: *None declared.*

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