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A sequential double labeling technique for studying changes in motoneuronal projections to muscle following nerve injury and reinnervation.

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Title:

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Abstract:

The purpose of this study was to develop an anatomical technique that could directly demonstrate the motoneuron projections to the muscle both before injury and again following reinnervation. Investigation focused on the identification of a long-term retrograde fluorescent tracer that would label original motoneurons and persist long enough for reinnervating motoneurons to become labeled by a second fluorescent tracer. True Blue (TB) was evaluated as a potential long-term tracer, Fluoro-ruby (FR) and Fluoro-emerald (FE) were tested as potential short-term tracers in 45 adult Sprague-Dawley rats. In the initial phase of the study, TB was injected into the tibialis anterior (TA) muscle in 16 rats and sacrificed 1 week to 6 months later, to study its persistence. During the second stage, a short-term tracer was injected into the TA muscles bilaterally in 15 rats with survival time ranging from 4-28 days. Sequential double labeling was subsequently performed using the combination of TB and FR in 14 rats. The number and brightness of TB cells did not change over 6 months time, a period sufficient for complete reinnervation. FR and FE showed maximum labeling of motoneurons at 1 week after tracer application. In the double labeling study, we could easily distinguish double-labeled cells from those labeled only by TB or FR. These results suggest that sequential double labeling of TB and FR is a valuable method for long-term muscle reinnervation studies.

Key Words: fluorescent tracer, motoneuron, double labeling, reinnervation, True Blue, Fluoro-ruby, Fluoro-emerald

1. Introduction:

A previous study demonstrated that electrical stimulation of a denervated muscle promoted selective reinnervation by original versus foreign motoneurons (Zealear et al., 2002). These findings were based upon physiological differences between the original and reinnervating motor units. The purpose of the present study was to develop an anatomical technique which could directly demonstrate the motoneuron projections to a muscle both before injury and again following reinnervation. In order to address this aim, we considered the sequential application of two fluorescent tracers, which has been previously described (Rende et al., 1991; Bodine-Fowler et al., 1997; Popratiloff et al., 2001). The sequential application of double retrograde labeling should in theory allow an optimal evaluation of pre- and postoperative distribution of motoneurons in the same animal, avoiding counting errors due to inter-individual variability. The first tracer would be injected into the target muscle before nerve injury to label original motoneurons, and a second tracer injected after regeneration to label reinnervating motoneurons. Double-labeled cells would then identify native neurons that had reestablished connections with their original muscle.

Fluorescent retrograde tracers have been used to identify the motoneurons innervating muscles. Despite the abundance of commercially available fluorescent retrograde neuronal tracers, the choice of appropriate tracers for this application is frequently difficult (Richmond et al., 1994; Novikova et al., 1997; Vercelli et al., 2000). In order to choose a tracer which is effective for labeling motoneurons, either before or after reinnervation, an understanding of the persistency of the tracer and number of labeled cells is required. We chose to compare True Blue (TB), Fluoro-emerald (FE) and Fluoro-ruby (FR), which have been used successfully in many animal models (Haase and Payne, 1990; Fritzsch and Sonntag, 1991; Choi et al., 2002). TB was evaluated as the first long-term tracer. It is reported that the long-lasting retention of TB in labeled cell bodies provides a novel approach for experimental studies of muscle reinnervation (Skagerberg et al., 1985; Garrett et al., 1991). For double labeling with fluorescent tracers, it can be quite problematic if one tracer is visible with the same filter used for the other tracer, a phenomenon called

"bleed through" (Zhang and McClellan, 1998). In particular, if the tracers are both fluoresced through the same filter, bleed through will decrease the accuracy of counting single versus double-labeled neurons. Therefore, FR and FE were tested as potential second tracers because of their characteristic excitation and emission spectra (Table 1).

A primary concern of this investigation was whether a long-term retrograde fluorescent tracer would persist long enough in motoneurons to allow double labeling by a second fluorescent tracer injected later. In a second series of experiments, we injected a second tracer to double label neurons in order to evaluate the accuracy of reinnervation of tibialis anterior muscle after transection and repair of the sciatic nerve.

2. Materials and Methods:

2.1. Animal Care

This study was approved by the Vanderbilt Institutional Animal Care and Use Committee, and was conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals.

2.2. Labeling Procedures

2.2.1. Persistence of fluorescent tracers in labeled cell bodies

Thirty-one adult male Sprague-Dawley rats weighing 250-300 g were used in this protocol. A surgical level of anesthesia was induced by isofluorane and oxygen mixture. All surgical procedures were performed under aseptic conditions. The optimum concentration and amount of tracers used in this study were determined in preliminary experiments. Tibialis anterior (TA) muscles were exposed and 45 μl of tracer solution was distributed by injection over 3 regions within each muscle, using a Hamilton microsyringe and a 25-gauge needle. The injection needle was coupled to the microsyringe by a polyethylene tube. Prior to utilizing the needle for injection, the whole system was filled with mineral oil in order to minimize injection error arising from air compression. The tracers were delivered slowly by means of a mechanical microdrive and the needle left in place for at least 3 minutes after injection; in this way, leakage of dye from the muscle belly was avoided. A solution of 1% TB in distilled water was injected into the TA muscle in 16 rats and sacrificed at varying times 1 week to 6 months later to study its persistence. Solutions of 4% FR or 4% FE were injected into the TA muscles in 15 rats (FR: right side, FE: left side) with survival times ranging from 4-28 days.

2.2.2. Accuracy of double labeling

Fourteen rats were used in the double labeling portion of the study. The animals

were divided in three groups (Table 2).

Group 1: Five rats received the first tracer (1% TB) into the right TA muscle. These rats served as controls with sciatic nerves intact over the course of the experiment. Fourteen weeks following injection of the first tracer, the second tracer (4% FR) was injected into the same TA muscle for double labeling of the original TA motoneurons.

Group 2: Five rats received the first tracer (1% TB) into the TA muscle in order to label the original motoneurons. Two weeks following injection of the first tracer, the right sciatic nerve was isolated in the midthigh and a complete transection of the nerve was made 10 mm proximal to the trifurcation. The two nerve ends were aligned and repaired using 9-0 nylon microsutures (Ethicon^{TM}) through the epineurium. Twelve weeks following nerve repair, the second tracer (4% FR) was injected into the same TA muscle in order to label those motoneurons that had reinnervated the muscle.

Group 3: In four rats, injection of the first tracer was delayed until just after the right sciatic nerve section and repair were performed. Three days following the nerve repair, 1% TB was injected into the right TA muscle. Twelve weeks following nerve repair, the second tracer (4% FR) was injected into the same TA muscle.

2.3. Fixation, Sectioning, Microscopic Examination

After a varying length of time, each rat was perfused, under deep anesthesia, with intracardiac injection of 100 ml of 0.1 M phosphate buffered saline followed by 500 ml of 4% paraformaldehyde fixative solution (pH 7.4). The lumbar segments of the spinal cord were identified by a laminectomy from T11 to S1 vertebrae in order to trace the dorsal roots to their respective ganglia supplying the sciatic nerve. Segments L3 through L6 were removed, fixed for 24 hours in the same fixative solution, then cryoprotected in 15% sucrose in phosphate buffer overnight (both 4 ˚C). The spinal cord was frozen in isopentane cooled with liquid nitrogen. Sections of the spinal cord were cut at 30 μm in the horizontal plane using a cryostat, and mounted serially on slides. Sections were coverslipped using an antifading medium (Gel/Mount, Biomeda). Fluorescent-labeled neurons in the spinal cord were identified under a fluorescein microscope (Orthoplan 2,

Leitz) which employed a rapid light wavelength-switching system (Lambda DG-4, Sutter) and UV Enhanced Triple Band filter (82500 Chroma). In each spinal cord section, the number of cell bodies containing tracer were counted. The sum across all sections thus provided an index of the total number of labeled motoneurons projecting to the TA muscle. All labeled cells in a 30 μm section were identified and counted visually while varying the focus through the whole section. Only cells having sufficient tracer uptake and contrast to differentiate the nucleus from the surrounding cytoplasm were counted. This approach also minimized the number of cells that would be counted twice on adjacent sections.

2.4. Statistical Analysis

For comparing the number of labeled cells between groups, a one-way analysis of variance (ANOVA) using Bonferroni's post hoc correction for multiple paired comparisons was used. All statistical analyses were performed using StatView 5.0 software (SAS Institute, Inc.). Statistical significance was set at the $p < .05$ level. All data are reported as means ± standard error.

3. Results:

3.1. True Blue (TB)

Tibialis anterior muscle (TA) motoneurons labeled with TB were characterized by their blue fluorescence. They were localized in the ventral horn of the lumbar spinal cord in the region corresponding to L3 and L4. The distribution of labeled cells was found to be similar to that previously reported (Nicolopoulos-Stournaras and Iles, 1983; Swett et al., 1986). Fig. 1 shows the number of labeled cells graphed as a function of survival time. The number of labeled cells in each animal ranged from 93-146, and the mean value of labeled cells per animal across all timepoints was 117 ± 4. For survival times ranging from 1 week to 6 months, there was no significant difference found in the numbers of labeled cells. The intensity of labeling was also sufficient to identify positively labeled cells for as long as 6 months (Fig. 2). Fig. 2 shows a photograph illustrating the relative intensity of TB labeling of cells at 2 weeks versus 6 months. These results indicated that TB was a suitable long-term tracer.

3.2. Fluoro-ruby (FR)

TA motoneurons stained with FR contained fine red granular material, which filled the soma and extended along the dendrites. Fig. 3 shows the number of positively labeled FR cells observed as a function of time. FR produced reasonable staining as early as 4 days after injection and maximum labeling at 1 week. The average number of FR labeled cells at 1 week was 139 ± 3; this value was significantly larger than that observed for other survival times. When the labeling of TB was compared with FR, there was no significant difference in the number of labeled cells between FR at 1 week and TB at any timepoint. Furthermore, the location of FR cells was very similar to that of TB cells. The number of the FR-stained cells gradually decreased with fading fluorescence for times greater than one week of the four week experiment. Thus, FR staining at 1week after injection was found

to be optimum for counter-labeling of TB cells.

3.3. Fluoro-emerald (FE)

Motoneurons labeled by FE displayed green fluorescent granules which delineated the cell body and proximal dendrites. The numbers of FE cells are represented across time in Fig. 4. FE produced reasonable staining as early as 4 days after injection, with maximal labeling at 1 week. The average number of FE cells at 1 week was 107 ± 12; this value was significantly larger than for other survival times after injection. FE labeled cells at a spinal cord location similar to that observed for TB or FR labeled motoneurons. FE labeling of cells also showed the same trend as FR, peaking at 1 week and then gradually decreasing with fading fluorescence over the 4 weeks of the experiment. Thus, FE staining at 1week after injection was found to be optimum for counter labeling of TB cells. However, the overall intensity of FE staining was not as strong or consistent as TB and FR.

3.4. Double Labeling

As described above, TB was an ideal long-term tracer and FR was suitable for counter labeling of TB cells. To evaluate the accuracy of reinnervation, TB and FR were injected into the same TA muscle before and after sciatic nerve injury (Table 2). Doubly labeled cells were easily distinguished from those labeled by TB or FR alone (Fig. 5). The accuracy of reinnervation could be indexed by the number of original motoneurons labeled with TB that reconnected to the muscle and obtained the second label, FR. Thus the ratio of double-labeled to FR labeled cells was used to provide this measure.

In the control group (Group 1, Table 3), the number of TB labeled cells was 105 ± 15, which was not significantly different from that obtained in the previous experiment with a single injection of TB (117 ± 4). In contrast, the number of FR cells in the control group (80 ± 19) was significantly less than that found with a single tracer injection (139 ± 3), even though this group had an intact sciatic nerve. Also of note, the percentage of FR labeled

cells that were double-labeled was 68 ± 6%, a value smaller than expected if the entire muscle had been labeled by both tracers.

In Group 2, TB was injected prior to nerve section and reanastomosis. FR was injected later, after a waiting period for reinnervation. The number of TB labeled cells was 121 ± 10, which was not significantly different from that obtained from Group 1 or the single tracer injection. As expected, there was a decrease in the number of FR labeled cells following neurorrhaphy (65 ±10) from that of the control group (80 ± 19), although not at a significant level (p=0.424). The percentage of double labeled cells (22 ± 2%) was significantly smaller than that of the control group (Table 3), signifying a reduction in reinnervation accuracy following nerve section and repair.

Group 3 was included to examine whether residual TB tracer persisted at the application site and was taken up by regenerating motoneurons. This was accomplished by injecting TB shortly after nerve section but before any reinnervation had occurred, thus ensuring that only reinnervating motoneurons could be labeled. FR was injected later, after a period of time to allow reinnervation, like Group 2. FR cells were identified in this group (41 ± 11), suggesting that reinnervation had occurred. This value was smaller than those of Groups 1 and 2, although not at a significant level (p=0.0657; p=0.2396). No TB labeled cells was found in this group. Apparently, TB did not persist long enough at the original application site to double label regenerating motoneurons in this animal model.

As illustrated in Fig. 6, all labeled motoneurons in Group 1were distributed consistently between spinal cord levels L3 and L4, comparable to that found following single injection of tracers. Likewise, the TB cells labeled prior to neurorrhaphy in Group 2 showed the same pattern of distribution. In contrast, FR cells labeled after neurorrhaphy were distributed more widely from L3 to L6, corresponding to the whole sciatic nerve motoneuron pool. A similar change in the pattern of distribution was found along the mediolateral axis after nerve section and repair. Specifically, TB cells of Groups 1 and 2, and FR cells of the control group were typically located laterally in the ventral horn, whereas FR cells in Group 2 were observed both medially and laterally in the ventral horn. This pattern suggested that both tibial and peroneal motoneurons had randomly

reinnervated the TA muscle after nerve anastomosis.

4. Discussion:

4.1. Muscle Injection

Retrograde labeling of motoneurons can be achieved by tracer application to the cut end of a motor nerve or by tracer injection directly into an intact muscle or nerve. We chose the muscle injection method because the purpose of this study was to develop an anatomical technique which could directly demonstrate the motoneuron projections to a muscle both before injury and again following reinnervation. The muscle injection of tracer is also indicated for a long-term reinnervation study, because this method prevents neuronal death induced by peripheral axon damage that can occur with direct nerve injection. On the other hand, it has been suggested that direct tracer injection into muscles or nerves results in less effective neuronal labeling and large variation in the number of retrogradely labeled neurons (Richmond et al., 1994). In order to label the maximum number of motoneurons and reduce variability, the injection technique requires that the tracer be exposed to as many motor endplates as possible. Thus, we injected each tracer into three different areas within each TA muscle to more evenly fill the muscle. The number of labeled cells by each tracer ranged from 107 ± 12 to 139 ± 3 at each optimum labeling time. These findings are comparable to those using horseradish peroxidase (HRP) applied to the cut end of the nerve innervating the TA muscle. This approach is viewed as the “gold standard method” for retrograde motoneuron labeling (Nicolopoulos-Stournaras and Iles, 1983; Peyronnard et al., 1986). Our results indicate that muscle injection of TB, FR or FE represents a reasonable method for labeling of TA motoneurons.

4.2. Persistence of True Blue

In the ideal sequential double labeling model to study reinnervation, one of the prerequisites would be that labeling by the first tracer, applied before nerve injury, should

identify the entire original motoneuron population of the muscle at the end of the follow-up period. Garrett et al. (1991) reported that long-lasting retention of cellular TB had no effect on the survival of labeled motoneurons. Our present study also demonstrated that the number of labeled TB cells is constant over 6 months survival time and that TB can be reliably used as a long-term tracer for spinal motoneuron labeling.

In theory, another confounding factor in a sequential double labeling model is re-uptake of the first tracer (Puigdellivol-Sanchez et al., 2003). If the first tracer remains available at the injection site for later re-uptake, regenerating axons from foreign motoneurons could be double labeled by both the first and second tracer. Puigdellivol-Sanchez et al. proposed that experimental designs utilizing fluorescent tracers to study reinnervation after nerve injury should include tests to check whether re-uptake for each tracer is significant. To examine this possibility, we injected TB into the TA muscle 3 days after nerve section and repair and looked for any TB labeled cells after reinnervation was complete. No TB cell was found in any animal of this protocol; therefore we conclude that a re-uptake phenomenon is not a concern.

4.3. Combination of TB and FR

It has been reported that dextran conjugates such as FR and FE are very effective for short-term retrograde labeling of motoneurons (Novikova et al., 1997). We tested FR and FE as tracers for counter-labeling of TB cells in this study. FR and FE exhibited maximum labeling at 1 week and significant reduction in number of labeled cells 2 to 4 weeks after injection. This decrease of labeled neurons has been previously attributed to leakage or degradation of the dyes (Novikova et al., 1997; Choi et al., 2002). Results of this study indicated that the optimum time for labeling of FE or FR was one week after injection. More importantly, the finding that the number of FR and FE cells at 1 week was similar to the number of TB-labeled cells also supports their usefulness in this sequential double labeling study.

In this study, we did not test Fluoro-gold (FG), which is well known as a retrograde

tracer. FG has been reported for double labeling of motoneurons in animal models (Novikova et al., 1997; Popratiloff et al., 2001). However, using TB and FG together would present practical difficulties in identifying double-labeled cells. Both tracers label the cytoplasm and their distinct fluorescent appearance occurs under the same ultraviolet wavelength, making them difficult to distinguish. FR and FE were selected as the second tracer because they have different fluorescent characteristics from that of TB. For double labeling with fluorescent tracers, it can frequently be problematic if one tracer is visible with the filter for the other tracer. In order to prevent bleed through, the two tracers chosen for double labeling should have widely separated spectra. As such, FR is preferable to FE, because the excitation and emission spectra of FR are more distinct from that of TB (Table 1). We conclude that TB and FR are the best combination for sequential double labeling to study muscle reinnervation of rat hindlimb muscles.

4.4. Potential Problems of this Technique

It is reported that Fast Blue, which is a related substance of TB, is not acceptable as a pre-surgery long-term tracer because motoneurons were labeled in unexpected locations of the facial nucleus following facial nerve transection and repair (Popratiloff et al., 2001). This investigation may have implications regarding possible spurious labeling of TB in our reinnervation experiment. However, we were not able to detect any change in the distribution of TB cells in the reinnervating groups when compared to the control group. Although we cannot fully rule out the possibility of false labeling by TB, this tracer may be distinguished from Fast Blue in this regard.

When using sequential application of fluorescent tracers to evaluate muscle reinnervation after nerve injury, the second tracer should account for the reinnervating population. Ideally, labeling by the first tracer should not interfere with the labeling ability of the second tracer (Puigdellivol-Sanchez et al., 2003). However, in our experience, labeling by FR was significantly reduced when injected subsequent to a TB injection in the innervated animal model (139 cells versus 80). Although we are unable to

definitively conclude that TB had a selective toxic effect on FR uptake, this result suggested that the previous application of TB affected the subsequent labeling with FR. Examination of muscle specimens at TB injection sites was consistent with the idea that there was some muscle damage and encapsulation that occurred with the first injection of TB.

It is questionable whether the amount of reinnervation after nerve injury is smaller than that of original innervation (Bodine-Fowler et al., 1997; Gramsbergen et al., 2000; Puigdellivol-Sanchez et al., 2002). Our result showed that the number of FR cells in reinnervation Groups 2 and 3 tended to be lower than that in the innervated control Group 1 (65 and 41cells versus 80). In comparing FR labeling in the two neurorrhaphy groups, it was interesting to note that there was a reduction in the number of labeled cells if the TB injection was made after denervation (Group 3) rather than before (Group 2). We do not have an explanation for this decrease in FR labeled cells. It might be speculated that the damage occurring with TB injection may be more profound in the inactive, denervated muscle than the active , innervated muscle due to differences in clearance of the agent. Further studies should be required to assess the possibility that TB interferes with the ability to label reinnervating motoneurons.

4.5. Efficacy of This Technique for Reinnervation Study

Previous studies (Rende et al., 1991; Bodine-Fowler et al., 1997) evaluated the accuracy of reinnervation of rat hindlimb muscles after transection and repair of the sciatic nerve using a sequential double labeling technique. About 20-30% of the original motoneurons were found to have successfully reinnervated the target muscle following transection and repair of sciatic nerve in the adult rat. In our study, 22% of the original TA motoneurons reinnervated the muscle after transection injury and repair, consistent with these prior reports.

It should also be noted that the control group exhibited a failure to double-label all motoneurons, even though the sciatic nerve was intact. The percentage of double-labeled

cells in the normal control group was 68 ± 6 %, which was lower than expected. However, because this value was similar to that reported by other authors (77 ± 6%) (Bodine-Fowler et al., 1997), it may reflect an inherent limitation of the muscle injection technique. A modification of the technique might further improve this result. It may be possible to obtain more complete labeling of the entire muscle and greater overlap in the distributions of the two tracers by injecting continuously while withdrawing the needle, rather than bolus tracer injections as performed in this study and others.

5. Conclusions:

The intramuscular injection of retrograde fluorescent tracers TB, FR and FE were effective in labeling the TA muscle motoneurons in rats. These tracers have differing optimum times for labeling. TB was an excellent long-term tracer with sufficient persistence. FR and FE showed maximum labeling of motoneurons at 1 week after tracer application. The combined use of TB and FR to label cells before and after injury to the sciatic nerve gave the greatest labeling of original and reinnervating motoneurons from the TA muscle. We propose that the combination of TB and FR might be useful for sequential double labeling experiments in the reinnervation study of rat hindlimb muscles, and should be considered for use in other reinnervation research.

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References

Bodine-Fowler SC, Meyer RS, Moskovitz A, Abrams R, Botte MJ. Inaccurate projection of rat soleus motoneurons: a comparison of nerve repair techniques. Muscle Nerve 1997;20:29-37.

Choi D, Li D, Raisman G. Fluorescent retrograde neuronal tracers that label the rat facial nucleus: a comparison of Fast Blue, Fluoro-ruby, Fluoro-emerald, Fluoro-Gold and DiI. J Neurosci Methods 2002;117:167-172.

Fritzsch B, Sonntag R. Sequential double labelling with different fluorescent dyes coupled to dextran amines as a tool to estimate the accuracy of tracer application and of regeneration. J Neurosci Methods 1991;39:9-17.

Garrett WT, McBride RL, Williams JK, Jr., Feringa ER. Fluoro-Gold's toxicity makes it inferior to True Blue for long-term studies of dorsal root ganglion neurons and motoneurons. Neurosci Lett 1991;128:137-139.

Gramsbergen A, Ijkema-Paassen J, Meek MF. Sciatic nerve transection in the adult rat: abnormal EMG patterns during locomotion by aberrant innervation of hindleg muscles. Exp Neurol 2000;161:183-193.

Haase P, Payne JN. Comparison of the efficiencies of true blue and diamidino yellow as retrograde tracers in the peripheral motor system. J Neurosci Methods 1990;35:175-183.

Nicolopoulos-Stournaras S, Iles JF. Motor neuron columns in the lumbar spinal cord of the rat. J Comp Neurol 1983;217:75-85.

Novikova L, Novikov L, Kellerth JO. Persistent neuronal labeling by retrograde fluorescent tracers: a comparison between Fast Blue, Fluoro-Gold and various dextran conjugates. J Neurosci Methods 1997;74:9-15.

Peyronnard JM, Charron LF, Lavoie J, Messier JP. Motor, sympathetic and sensory innervation of rat skeletal muscles. Brain Res 1986;373:288-302.

Popratiloff AS, Neiss WF, Skouras E, Streppel M, Guntinas-Lichius O, Angelov DN. Evaluation of muscle re-innervation employing pre- and post-axotomy injections of fluorescent retrograde tracers. Brain Res Bull 2001;54:115-123.

Puigdellivol-Sanchez A, Cabre-Valero A, Prats-Galino A, Navarro X, Molander C. On the use of fast blue, fluoro-gold and diamidino yellow for retrograde tracing after peripheral nerve injury: uptake, fading, dye interactions, and toxicity. J Neurosci Methods 2002;115:115-127.

Puigdellivol-Sanchez A, Prats-Galino A, Ruano-Gil D, Molander C. Persistence of tracer in the application site--a potential confounding factor in nerve regeneration studies. J Neurosci Methods 2003;127:105-110.

Rende M, Granato A, Lo Monaco M, Zelano G, Toesca A. Accuracy of reinnervation by peripheral nerve axons regenerating across a 10-mm gap within an impermeable chamber. Exp Neurol 1991;111:332-339.

Richmond FJ, Gladdy R, Creasy JL, Kitamura S, Smits E, Thomson DB. Efficacy of seven retrograde tracers, compared in multiple-labelling studies of feline motoneurones. J Neurosci Methods 1994;53:35-46.

Skagerberg G, Bjorklund A, Lindvall O. Further studies on the use of the fluorescent retrograde tracer True Blue in combination with monoamine histochemistry. J Neurosci Methods 1985;14:25-40.

Swett JE, Wikholm RP, Blanks RH, Swett AL, Conley LC. Motoneurons of the rat sciatic

nerve. Exp Neurol 1986;93:227-252.

Vercelli A, Repici M, Garbossa D, Grimaldi A. Recent techniques for tracing pathways in the central nervous system of developing and adult mammals. Brain Res Bull 2000;51:11-28.

Zealear DL, Rodriguez RJ, Kenny T, Billante MJ, Cho Y, Billante CR, Garren KC. Electrical stimulation of a denervated muscle promotes selective reinnervation by native over foreign motoneurons. J Neurophysiol 2002;87:2195-2199.

Zhang L, McClellan AD. Fluorescent tracers as potential candidates for double labeling of descending brain neurons in larval lamprey. J Neurosci Methods 1998;85:51-62.

Figure Legends

Fig. 1. Average number of motoneurons labeled by True Blue (TB) at each time point following tracer injection.

Fig 2. Photograph illustrating the relative intensity of TB labeling of cells at 2 weeks versus 6 months. Scale bar indicates 50 μm.

Fig. 3. Average number of motoneurons labeled by Fluoro-ruby (FR) at each time point following tracer injection.

Fig. 4. Average number of motoneurons labeled by Fluoro-emerald (FE) at each time point following tracer injection.

Fig. 5. Section demonstrating labeled cells with excitation of TB in (A) and FR (B). 1: single-labeled TB neuron in A. 2: single-labeled FR neuron in B. 3: double-labeled neuron by TB and FR. Scale bar indicates 50 μm.

Fig. 6. Rostro-caucal distribution of labeled cells in control group (Group 1: left column) and reinnervation group (Group 2: right column).

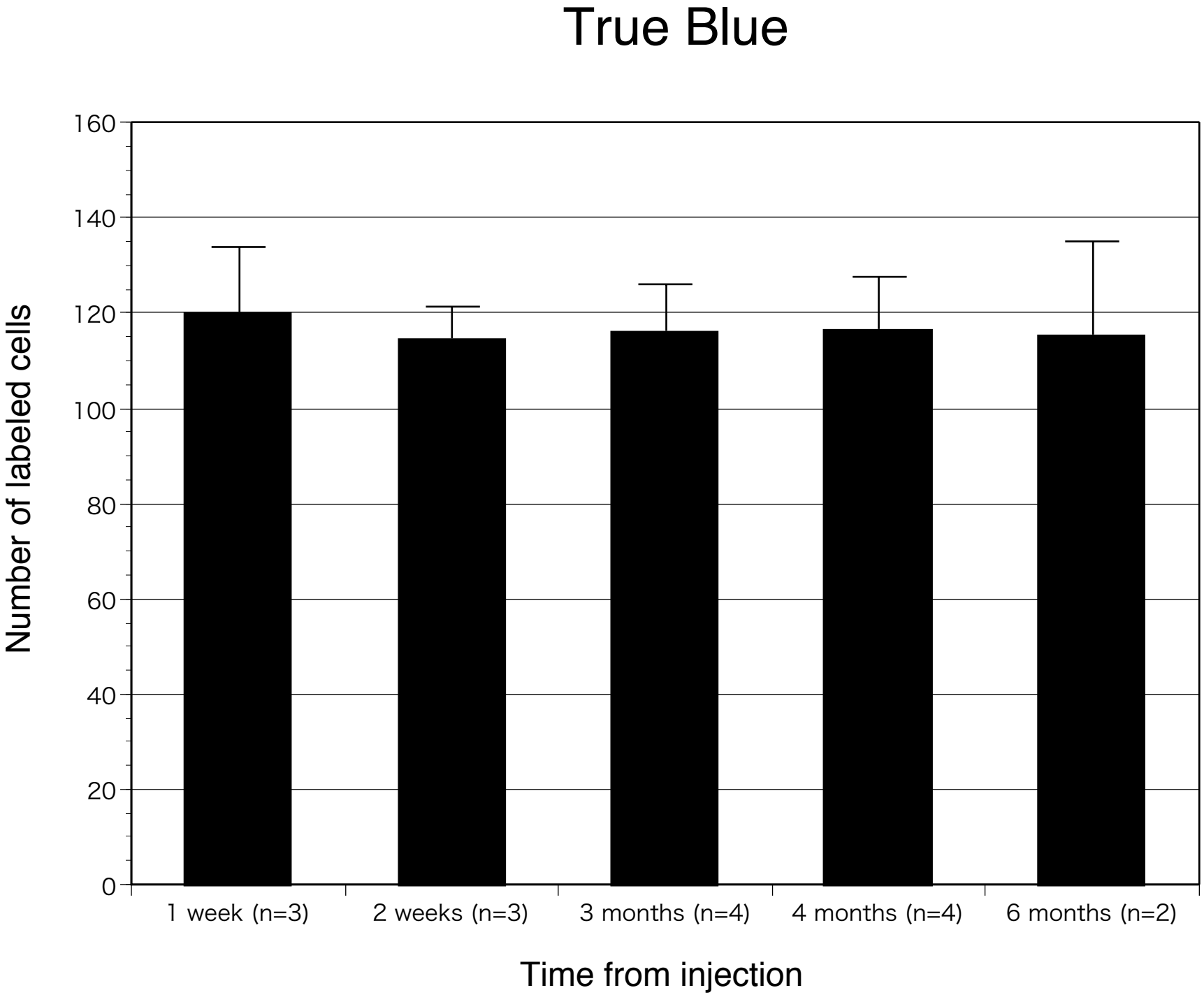
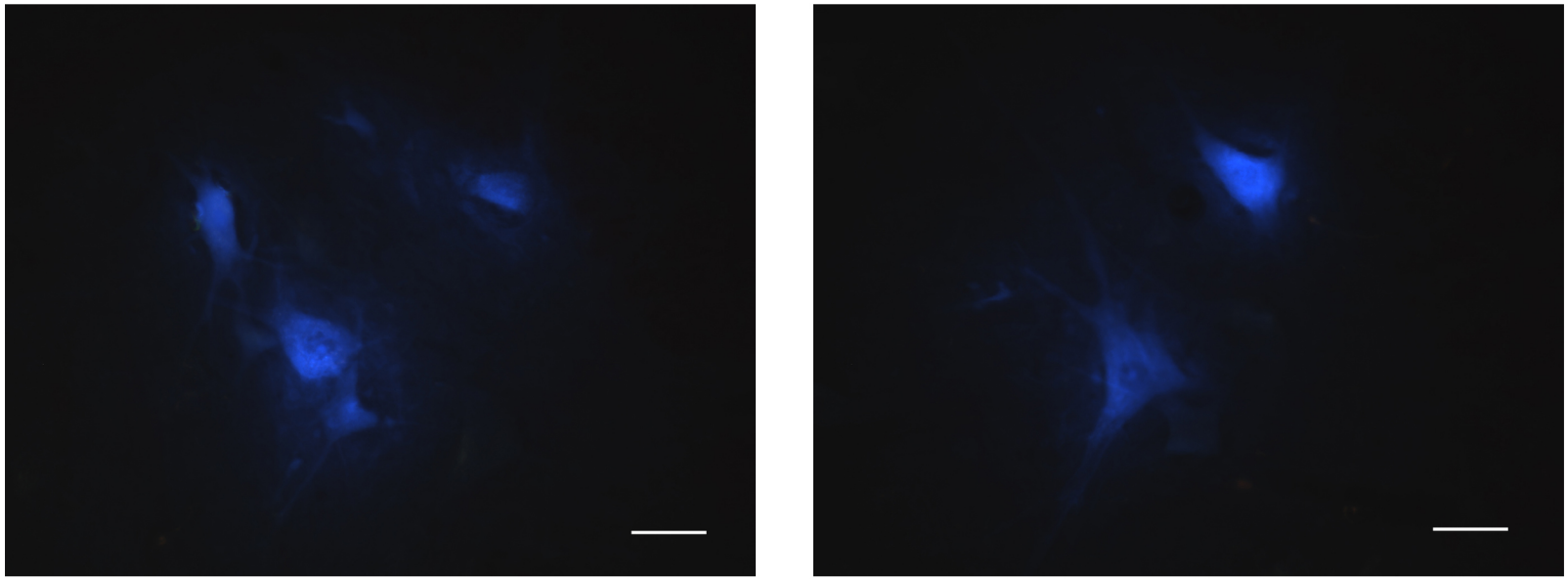

True Blue
160
140
120
100
80
60
40
20
0
Number of labeled cells
1 week (n=3)
2 weeks (n=3)
3 months (n=4)
4 months (n=4)
6 months (n=2)
Time from injection

Fig. 1



2 weeks

6 months

Fig. 2

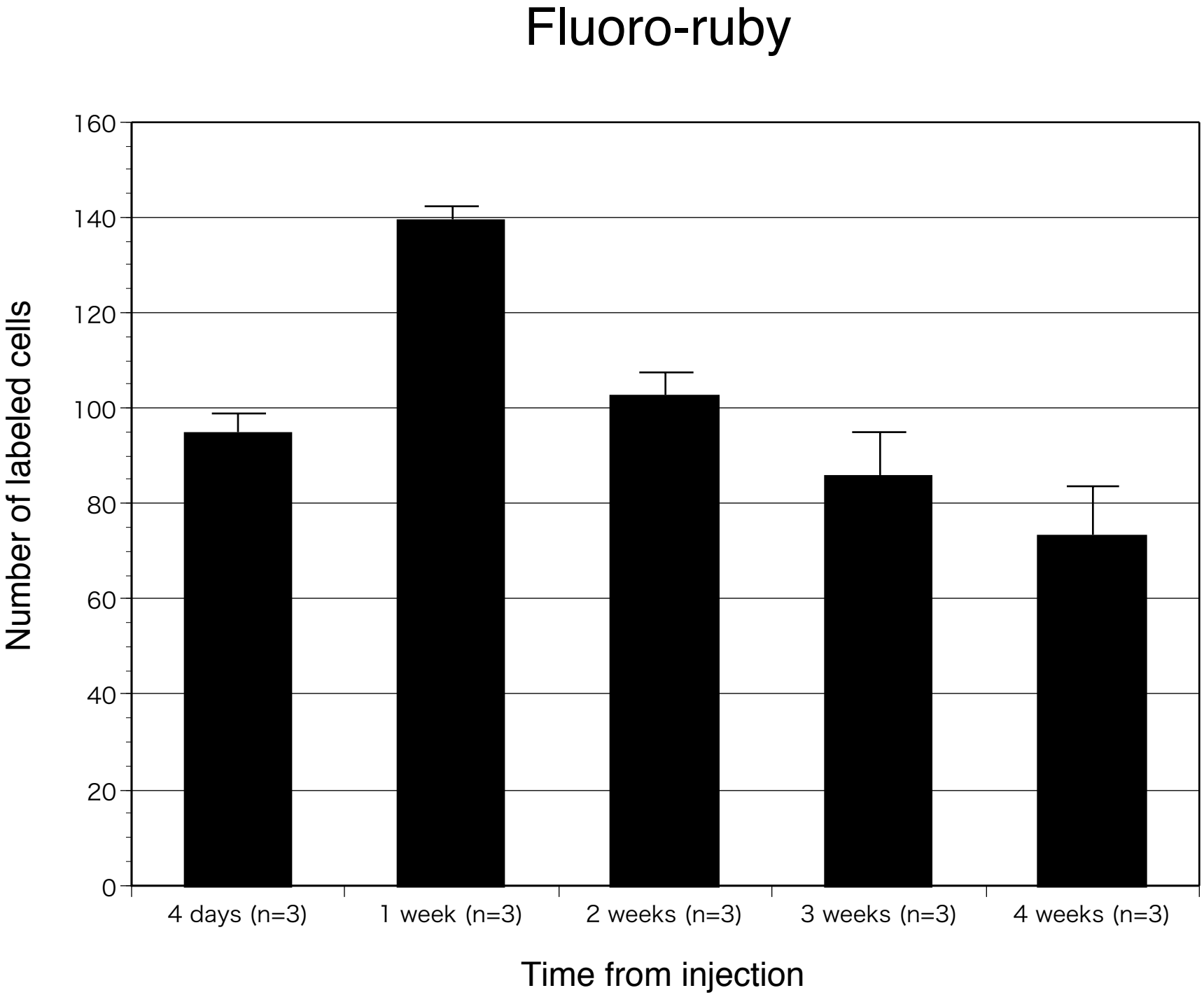

Fluoro-ruby
160
140
120
100
80
60
40
20
0
Number of labeled cells
4 days (n=3)
1 week (n=3)
2 weeks (n=3)
3 weeks (n=3)
4 weeks (n=3)
Time from injection

Fig. 3

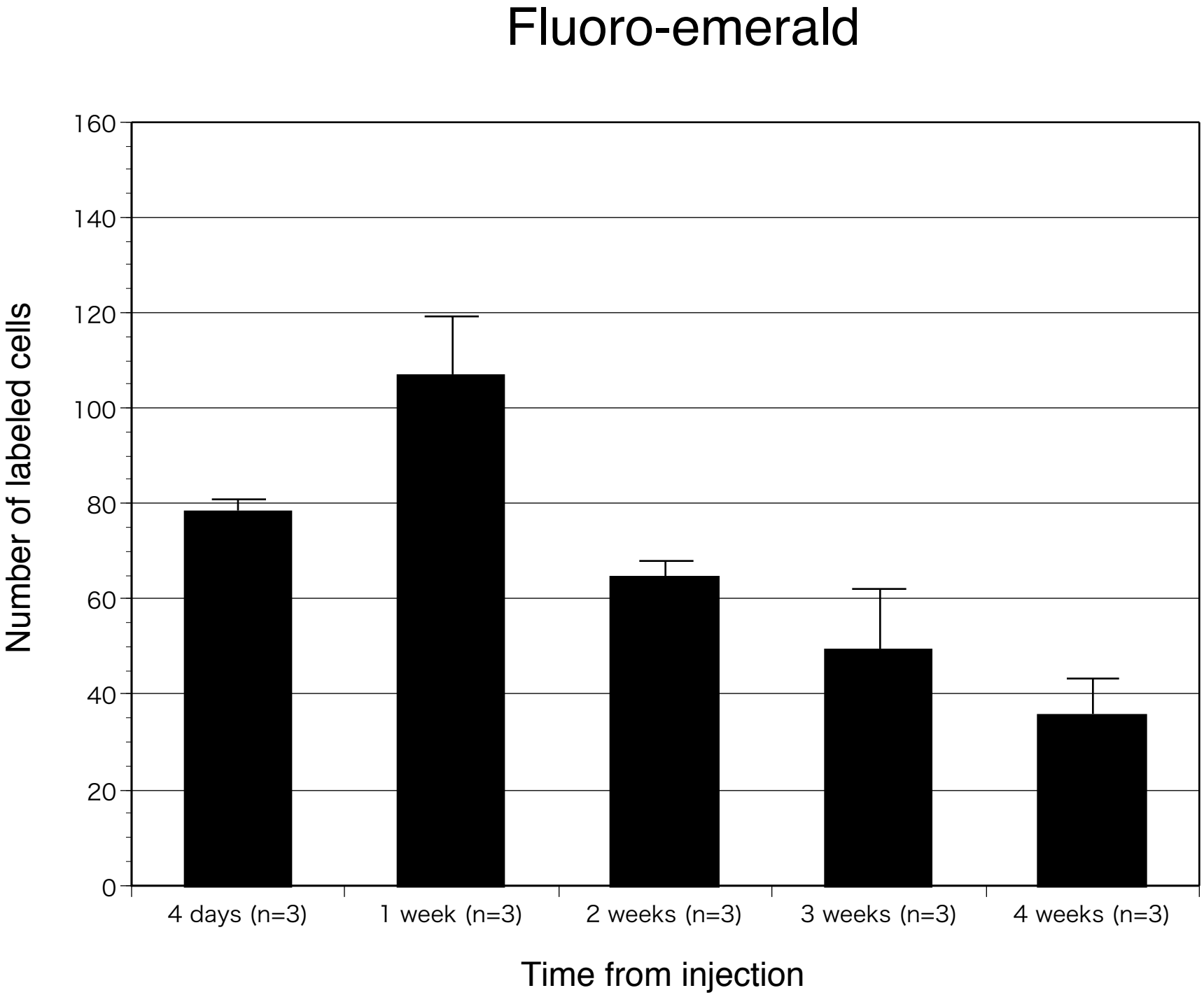

Fluoro-emerald
160
140
120
100
80
60
40
20
0
Number of labeled cells
4 days (n=3)
1 week (n=3)
2 weeks (n=3)
3 weeks (n=3)
4 weeks (n=3)
Time from injection

Fig. 4

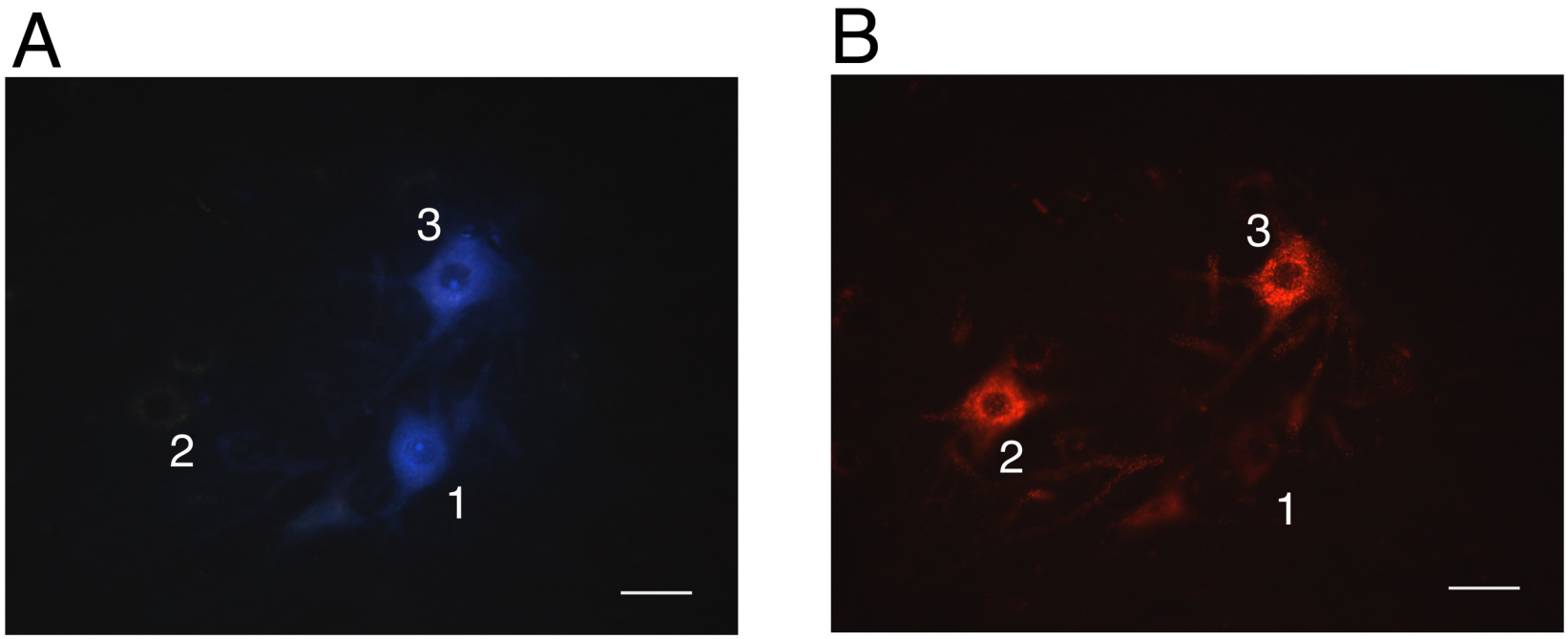


Fig. 5

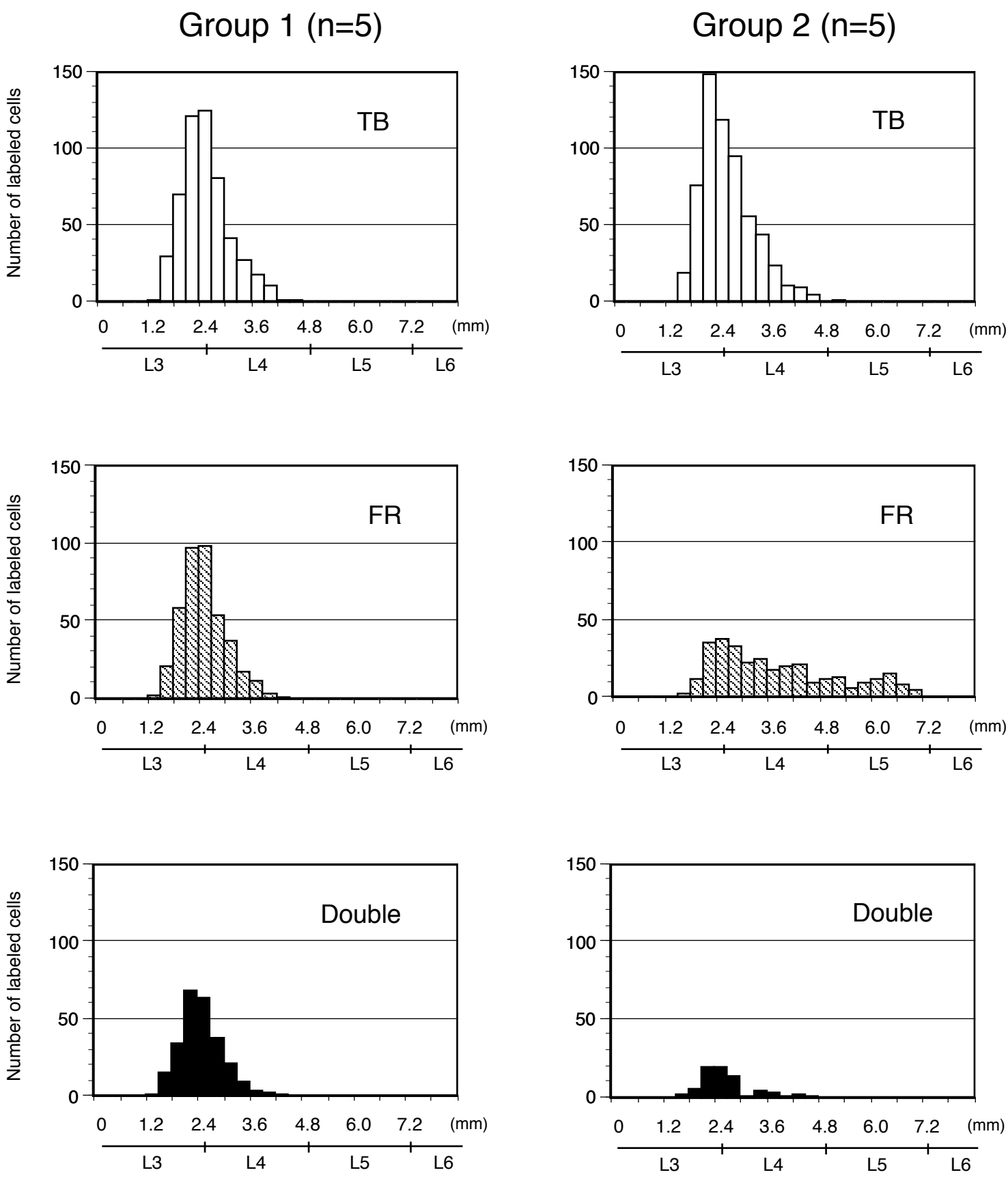

Group 1 (n=5)
Group 2 (n=5)
Number of labeled cells
150
100
50
0
TB
FR
Double
0
1.2
2.4
3.6
4.8
6.0
7.2
(mm)
L3
L4
L5
L6

Fig. 6

Table 1

Characteristics of fluorescent tracers

Tracer	Synonyms	Catalogue #	Excitation (nm)	Emission (nm)
True Blue (TB)	1,2-Bis(5-amidino-2-benzofuranyl) ethylene diaceturate salt	Sigma, T5891	386	420
Fluoro-emerald (FE)	dextran, fluorescein, 10,000 MW, anionic, lysine fixable	Molecular Probes, D1817	492	520
Fluoro-ruby (FR)	dextran, tetramethylrhodamine, 10,000 MW, lysine fixable	Molecular Probes, D1820	552	590

Table 2

Summary of the double labeling procedures

Group	0 weeks	2 weeks	14 weeks	15 weeks
Group 1 (n=5)	Injection (TB)	/	Injection (FR)	Perfusion
Group 2 (n=5)	Injection (TB)	Cut & Repair Nerve	Injection (FR)	Perfusion
Group 3 (n=4)	/	Cut & Repair Nerve Injection (TB)*	Injection (FR)	Perfusion

*TB injection was performed 3 days after nerve repair.

Table 3

Results of doble labeling

Group	Total TB cells	Total FR cells	Double labeled cells	% of double labeled cells*
Group 1 (n=5)	105 ± 15	80 ± 19	52 ± 9	68 ± 6
Group 2 (n=5)	121 ± 10	65 ± 10	14 ± 2	22 ± 2
Group 3 (n=4)	0 ± 0	41 ± 11	0 ± 0	0 ± 0

*(Double labeled cells/ Total FR cells) X 100.