EFFICACY OF THE FLUORESCENT DYES FAST BLUE, FLUORO-GOLD, AND DIAMIDINO YELLOW FOR RETROGRADE TRACING TO DORSAL ROOT GANGLIA AFTER SUBCUTANEOUS INJECTION

Anna Puigdellívol-Sánchez[1], Alberto Prats-Galino[1], Domingo Ruano-Gil[1], Carl Molander[2, 3]

[1] Department of Morphological Sciences. Faculty of Medicine. University of Barcelona, Spain.

[2] Department of Neuroscience, Division for Neuroanatomy and Neuronal Plasticity, Karolinska Institutet, Stockholm, Sweden

[3] Department of Surgical Sciences, Section of Rehabilitation Medicine, Karolinska Institutet, Stockholm, Sweden.

Author for correspondence: Alberto Prats Galino. Dept. of Morphological Sciences. Faculty of Medicine. University of Barcelona, c/ Casanovas nº 143, 08036 Barcelona. Tel No: 34-93-4021905, Fax No:34-93-4035260, aprats@medicina.ub.es

Number of text pages (including title page and abstract page): 17

Number of tables: 5

Number of figure pages: 1

Running headline: Skin injection of fluorescent tracers

Key words: afferent pathways, axonal transport, spinal ganglia, skin, adult rat

ABSTRACT

The present study was designed to investigate the efficacy of the fluorescent dyes Fast Blue (FB), Fluoro-Gold (FG), and Diamidino Yellow (DY) for retrograde tracing of lumbar dorsal root ganglia after their subcutaneous injection into different hindlimb digits. Injections of equal volumes (0.5µl) of 5% FB or 2% FG resulted in similar mean numbers of sensory neurones labelled by each tracer. Injection of equal volumes (0.5µl) of FB or FG in a single digit followed 10 days later by a second injection of the same volume of 5% DY into the same digit resulted in similar mean numbers of labelled sensory neurones for each of the three tracers. Furthermore, on average, 75% of all the FB-labelled cells and 74% of all FG-labelled cells contained also DY. Repeating the same experiment with an increased volume of DY (1.5µl) resulted in an increase in the mean number of double labelled profiles to 82% and 84% for FB and FG, respectively. The results show that FB, FG and DY label similar numbers of cutaneous afferents and that a high level of double labelling may be obtained after sequential injections in digits. These properties make them suitable candidates in investigations where a combination of tracers with similar labelling efficacies is needed**.**

INTRODUCTION

The method of demonstrating the axonal projections of single neurones to more than one target by retrograde tracing with fluorescent dyes that fluoresce with different colours was originally demonstrated by Van der Kooy et al. (1978) and has thereafter been used in many investigations (e.g. Kuypers et al., 1980; Catsman-Berrevoets et al., 1980; Minciacchi et al., 1985; Fritzsch and Sonntag, 1991; Harsh et al., 1991; Molander and Aldskogius, 1992). The efficacy of different individual fluorescent tracers has been tested by combined administration of more than one tracer to a nerve or to the same target region. Some of these studies have demonstrated a high degree of collocation of dyes in the cell bodies after retrograde transport, indicating similar labelling efficiency and labelling of the same population of neurones. For example, more than 95% of the trochlear nerve motoneurones in *Xenopus* tadpoles were found to be double labelled after retrograde tracing with two fluorescent dyes coupled to dextran amines (Fritzsch and Sonntag, 1991). Similar results were obtained for saphenous nerve afferents in the rat after combined application of Fluoro-Gold and DiI (Harsh et al., 1991). Different labelling efficiencies were found in other studies, however. Such differences were found for Fast Blue, propidium iodide, DAPI, bisbenzimide and Nuclear Yellow after application to the cut end of forelimb nerves in the cat (Illert et al. 1982), and for True Blue and Diamidino Yellow when tested by different ways of tracer administration to sciatic nerve motoneuron axons in the rat (Haase and Payne, 1990). Fluorescent dyes have also been used to retrogradely label dorsal root ganglia (DRG) cell bodies after injection directly in peripheral tissues such as muscle (Illert et al, 1982; O'Brien, 1989; Buisseret-Delmas, 1990; Richmond et al, 1994), or skin (O'Brien, 1989), but there is little information regarding the relative efficiency of different fluorescent retrograde tracers after cutaneous injection. Such information would be useful for quantitative studies of peripheral skin innervation, e.g. for comparing innervation pattern of different skin areas, or in quantitative studies examining reinnervation of the same skin area after nerve injury by comparing skin injections before and after the nerve injury with two tracers that can be viewed separately in sections of DRG.

The aims of this study have been to examine (i) if subcutaneous injections of different fluorescent tracers in digits can be performed in a reproducible way such that similar numbers of DRG neurones are labelled, (ii) if injections of these tracers label the

same DRG neuronal populations by describing the proportion of double labelled neurones after sequential injection of two different tracers in the same skin area. For future use in studies of reinnervation of a particular skin area after nerve injury, and with the aim to be able to label as many as possible of such regenerating sensory axons we also investigated the effect of increasing the dose of the second tracer, here in normal animals, on the proportion of double labelled neurones. The examination of the tissue is facilitated by the use of tracers that stain different parts of the neurone and fluoresce with different colours at the same excitation wavelength. For this reason we choose to examine the combination of Diamidino Yellow (DY) with either Fast Blue (FB) or Fluoro-Gold (FG).

MATERIALS AND METHODS

Twenty-six adult female Sprague Dawley rats, weighing 200-350g were used. All the animals have been obtained from Harlan Interfauna Iberica S.A., maintained in the Animal Care Service, Faculty of Medicine, University of Barcelona, and treated in compliance with the ethical guidelines of this Center. Anaesthesia was initiated with ether and then continued with chloral hydrate (300mg/kg.) during surgical procedures and perfusion.

Unilateral injections showed that no contralateral DRG labelling of the injected tracer occurred. This enabled the use of bilateral experiments, thereby reducing the number of animals used. Different digits were used in the different experimental situations, although not at the same time in the same paw.

Injection of tracers

All injections were performed with Hamilton syringes with 25 gauge needles. An operating microscope was used for optimal control of the needle introduction and to ensure tracer injection in the central plantar part of the distal phalanx of the digit. In a few cases there was a slight leakage from the injection opening. In those cases, the leaked tracer was aspirated with the syringe and reinjected with the aim to obtain equal injections between animals. In experiments where the injection in a digit was repeated 10 days later, the second injection was made in the same place and with the aim to cover an area identical to that covered by the first injection.

Rats used to compare the DRG labelling after subcutaneous injection of FB and FG were injected with either 0.5µl 2% FG (Fluorochrome Inc.; n=6), or 0.5µl 5% FB (Sigma; n=6) in the distal phalanx of the second digit.

Rats used to evaluate double labelling of FB and DY were first injected with 0.5µl 5% FB in the fourth or fifth digit. Ten days later, the previously injected digit was re-injected with either 0.5µl (fourth digit, n=7) or 1.5µl (fifth digit, n=5) 5% DY (EMS, Polyloy). The second injection was made in the same place as the first injection.

Rats used to evaluate double labelling after subcutaneous injection of FG and DY were first injected with 0.5µl 2% FG in the fourth or third digit. Ten days later, the previously injected digit was re-injected with either 0.5µl (fourth digit, n=6) or 1.5µl 5% DY (third digit, n=6).

Fixation, sectioning, microscopic examination, and analysis

Five days after the final tracer injection the rats were re-anaesthetised as above and a thoracotomy was performed. After an intracardiacal injection of 1000 IU of heparin/kg b.w. the rats were perfused through the ascending aorta with 100 ml of warm 0.9% saline followed by 1000 ml cold 4% paraformaldehyde and 10% sucrose in 0.1M phosphate buffer (PB) at pH 7.40 for twenty minutes. The DRGs L3-5 were removed. The L2 and L6 ganglia were not removed, as previous unpublished observations showed that less than five labelled cells per rat would be expected to be found in these ganglia after injections in digits 2-5 when examining every fifth section. For the same reason, the DRG L3 was excluded of counting after injection in digits 3,4 or 5. The DRGs were postfixed for three hours in the same fixative and then kept in 15% sucrose in PB at 4ºC overnight, cut on a cryostat at 16 µm thick horizontal sections that were all thaw-mounted on chromalum-gelatin-coated (5%) slides, and finally coverslipped using an antifading solution containing 1% para-phenylenediamine and 10% PBS in glycerol.

The skin of the injected paws was peeled off and directly examined under epifluorescent illumination, to determine the distribution of tracer near the injection site. DRG sections were examined in a Olympus Vanox fluorescent microscope, using appropriate filter combinations (ultraviolet light filters: DM 400 dichroic mirror and UG1 excitation filter, which gives 365nm excitation and 420nm emission wave lengths;

and violet light filters: DM 455 dichroic mirror and BP 405 excitation filter, which gives 405nm excitation and 455nm emission wave lengths).

All neuronal profiles with an identifiable nucleus were counted in every fifth serial section. Counting was performed within two hours after tissue sectioning to avoid possible bias from spread of label to surrounding cells in the ganglion (see Results).

Neurones identified as FB-labelled showed an intense blue fluorescence in the cytoplasm and usually also in the nucleus, whereas neurones identified as DY-labelled showed an intense yellow fluorescence in the nucleus, and less staining in the cytoplasm**.** Double-labelled FB/DY neurones were best seen with the violet light filters, as ultraviolet light filters tended to visualise DY less well. As FB-labelled neurones also show some nuclear labelling, it was sometimes difficult to identify FB/DY double-labelled neurones. In those cases, identification was facilitated by exposing the sections with violet light filters for up to half a minute, until the nuclear FB had faded and the DY could be observed more clearly. The DY nuclear labelling was found to be less intense but more resistant to fading than FB. The cytoplasmic FB labelling was still clearly visible after this procedure. Neurones identified as FG-labelled showed reddish labelling restricted to the cytoplasm with ultraviolet light filters. Although FG did not label the nucleus, the cytoplasmic labelling was often so intense that it tended to obstruct identification of DY nuclear labelling. In such cases, the presence of DY could be confirmed using violet light filters that excite DY, but FG only to a very small extent.

Vascular spread of the tracer from the injection site was suspected in cases where all DRG cells showed a very faint FB or FG labelling. This was also visible in ganglia without retrograde labelling. The difference in labelling intensity between these cells and those labelled from axonal transport was very striking, and was not considered to cause any problem regarding identification. Such vascular spread was not observed for DY in any case.

The total number of labelled neurones obtained from every fifth section of DRG L3-5 is presented in the tables. No corrections were made for the possibility of counting split cells twice in different sections. The number of labelled neurones found after injections of different single tracers in different animals were compared by Student's t-test after checking that the counts showed a normal distribution with the Kolmogoroff-Smirnoff test. The numbers of labelled neurones found after sequential injections of two

tracers in the same animal were analysed by means of paired t-test. Percentages were compared between groups using the Mann-Whitney U test.

RESULTS

Specificity of injection site

Examination of the skin and subcutaneous parts of the paws showed that the distribution of the injected tracers was similar between cases and always restricted to the injected digits, with the main distribution in their most distal parts. In experiments where two tracers had been injected in the same digit, a diffuse spot containing both tracers was observed.

Spread of label in sections

Retrogradely labelled cells were found to be sparsely distributed within the ganglionic tissue. Limited spread of FB and DY was seen to surrounding glia (earliest after 3 hours; Fig 1A) and to the nuclei of surrounding neurones (earliest after 24 hours) after sectioning, resulting in clusters of labelled glia and neurones. No spread of FG was observed to surrounding glia or neuronal profiles within the first days after sectioning.

Comparison of the number of labelled neurones after injection of FB and FG

Variable numbers of labelled DRG neurones were found in the L3-5 DRGs after injections of either 0.5µl FB (Fig. 1A) or 0.5µl FG (Fig. 1B-D) in the tip of the second digit (Table I). When comparing results from several cases, no significant difference between FB and FG could be demonstrated ($p=0.58$).

Double labelling after sequential injections of FB and DY

Injection of 0.5µl FB in digit 4 followed ten days later by a second injection of 0.5µl DY in the same digit resulted in variable numbers of labelled neurones in the L4-5 DRGs (table II, Fig. 1A). No significant difference was found when the total numbers of cells labelled by FB were compared with those labelled by DY ($p=0.64$).

After injection of 0.5µl FB followed by 0.5µl DY, an average of 59% of all labelled neurones (FB, DY and FB/DY) were double labelled, 22% contained only FB, and 19% contained only DY (table II, Fig. 1A). From these data it follows that approximately 75% of all neurones labelled with FB by the initial injection became labelled also with DY after the second injection. When the amount of injected DY was increased to 1.5µl, the percentage of all FB-labelled neurones that became labelled also with DY increased to about 82% (table III). There was no statistical difference between the percentage of FB/DY double labelled cells after injection of 0.5 µl DY (74.9%) and 1.5 µl DY (81.8%), respectively (p=0.68).

Double labelling after sequential injections of FG and DY

Injection of 0.5µl FG in digit 4 followed ten days later by a second injection of 0.5µl DY in the same digit resulted in variable numbers of labelled neurones in the L4-L5 DRGs (table IV). No significant difference was found (p=0.66) when the numbers of cells labelled by FG (mean =96.3) were compared with the numbers labelled by DY (mean =91.8; table IV).

After injection of 0.5µl FG followed by 0.5µl DY, an average of 61% of all labelled neurones (FG, DY and FG/DY) were double labelled, 22% contained only FG, and 18% contained only DY (table IV, Fig. 1B-D). From these data it follows that about 74% of all neurones labelled with FG by the initial injection became labelled also with DY after the second injection. When the amount of injected DY was increased to 1.5µl, the percentage of all FG-labelled neurones that became labelled also with DY increased to 84% (table V). There was no statistical difference between the percentage of FG/DY double labelled cells after injection of 0.5 µl DY (74.5%) and 1.5 µl DY (84.3%), respectively (p=0.26).

DISCUSSION

This study has examined some problems and consequences associated with the use of subcutaneous injections of fluorescent dyes to trace sensory afferents to the DRG. It appears to be the first systematic comparison of different fluorescent tracers for this purpose. Fluorescent tracers have the main advantage of simplicity in that they may be observed directly on the mounted sections, without further histological processing. They

may be used as an alternative to horseradish peroxidase conjugated to wheatgerm agglutinin, which has previously been used to trace primary afferents to DRG after subcutaneous injection to the hindlimb digits (Molander and Grant, 1985), but requires either enzyme-histochemical or immune-histochemical processing. Furthermore, fluorescent tracers are relatively easy to combine with immunocytological techniques if further characterisation of the labelled cells is desired (cf. Skirboll et al., 1984).

Injection site

Examination of the injection sites using the fluorescent microscope showed remaining dye in an area very close to where the injection had been performed. This area was similar in extent for the three tracers studied. Even though this does not rule out that some of the dye may have reached axons further away, it is an indication that it is possible to obtain axonal uptake and retrograde transport from a restricted cutaneous region and that spread of dye is a minor problem. Furthermore, the use of the digits offers an area with natural boundaries that make them particularly suitable when several injections need to be performed in the same defined cutaneous region and spread of tracers to adjacent territories must be avoided.

Concentration and amount of tracer injected

We used a concentration of 5% FB since we found it efficient in a previous study (Molander and Aldskogius, 1992). For FG, concentrations ranging from 2 to 5% have been used (Schmued and Fallon, 1986; Harsh et al., 1991; Novikova et al., 1997; Madison et al., 1996). We used a concentration of 2% FG in the double labelling experiments, as preliminary experiments showed that the use of this concentration in combination with 5% FB yielded similar mean numbers of labelled neurones. For DY, we decided to use 5% rather than 2% which is the concentration used by many other investigators (Keizer et al, 1983; Innocenti et al, 1986, Craig et al, 1989) because 2% DY has been reported to have a lower tracer efficiency than 3% FB (Horikawa et al., 1986) or 2% True Blue (Haase and Payne, 1990).

Detection of labelled neurones in the DRG

In most cases, the identification of FB, FG, and DY-labelled neurones was easy

with the filter combinations and criteria used. Several potential complications need to be considered, however. Injected tracers may diffuse to the blood stream and result in hematogeneous labelling of DRG neurones. This phenomenon has previously been noted after application of fluorescent dyes to hindlimb nerves as diffuse labelling in DRG neurones in remote territories such as the trigeminal ganglia (Molander and Aldskogius, 1992). In this study, however, the presence in some cases of a very faint FB or FG staining in most DRG cells of the examined lumbar DRGs was considered to indicate vascular spread and uptake. Furthermore, the presence of this kind of labelling in the L3 DRG after injections in digits 3,4 and 5, which do not give rise to typical retrograde labelling in this ganglion, supports the hypothesis of a systemic diffusion rather than local diffusion from retrogradely labelled neurons. It was possible to use these cases as there was a clear difference in intensity from the retrogradely labelled cells.

A second potential problem is diffusion of dye from retrogradely labelled neurones to surrounding glia and neurones, occurring later than three hours after sectioning. The problem of the spread of the dye has previously been described to be associated to the use of aqueous mounting media (Shmid et al, 1983) which would explain why it is considered to be a problem that occurs after sectioning rather that before. In our material, neuronal clusters were never seen before one day after sectioning. We were able to avoid this problem by performing counts within two hours after sectioning. Furthermore, the sparse distribution of neurones also strongly speaks against early diffusion of labelling from retrogradely labelled neurones.

A third problem was identifying double-labelled FB/DY cells when the FB nuclear labelling was very intense. This was solved by allowing some fading to occur by prolonging the illumination time. As the sections were constantly observed during the fading process and since the number of labelled cells was usually less than ten in each section and therefore easy to control, we do not think that any neurones identified as FB-labelled were lost in the counts. Such exposure to light while observing the slide to allow a restricted fading of FB has been reported previously (Innocenti et al., 1986). Furthermore, it was evident that FG is easier than FB to separate from DY as alternating filter combinations could be used for FG/DY in cases where identification was difficult.

FG is better visualised with ultraviolet filters than with violet filters, whereas vice versa is true for DY.

Finally, a potential technical problem is degradation of dye in the DRG. Less labelling was reported in rat motoneurones retrogradely labelled with FG at four weeks compared to one week after tracer application to a peripheral nerve, whereas the number of FB neurones appeared to remain unchanged up to 24 weeks (Novikova et al, 1997). Whether degradation occurs at similar rates in sensory neurones appears to be unknown. In the axolotl (Wigston and Kennedy, 1987), the number of motoneurones retrogradely labelled with DY and HRP was found to be similar after 7-11 days. After 2-3 months, however, the number of DY-labelled cells exceeded the number of HRP labelled cells, suggesting local spread of DY to adjacent motoneurones. Assuming that the rat and the axolotl are similar in this regard, this indicates that degradation of DY does not need to be considered at the survival times used in this study. It remains to be demonstrated, however, whether DY becomes degraded in sensory neurones after long survival times.

Double labelling

Sequential injections of two tracers into the same digit separated by 10 days resulted in double-labelled neurones and neurones labelled with either tracer alone. The majority of labelled neurones were identified as double labelled, indicating that the same afferents had transported both injected tracers. Mean percentages of double-labelled cells were found to be similar when comparing the ability of the DY to label the cells previously labelled with FB (74.9%) or with FG (74.3%).

Single labelled neurones indicate those that were either exposed to only one of the tracers, or transported DY less efficiently as a result of a toxic effect from the first injection of FB or FG. Even though a toxic effect can not be ruled out, variable uptake due to differences in injected area seems to be the most likely explanation, considering that the majority of labelled neurones were double labelled. Garrett et al. (1991) suggested a toxic effect of FG resulting in cell death at long survival times. The finding by Novikova et al. (1997), however, that the number of FB-labelled motoneurones stays unchanged for long periods of time after combined FB and FG administration argues against toxicity as a general problem.

It may be argued that the failure to get a 100% double labelling with FB/DY or with FG/DY in experiments where the same amounts of the two tracers had been injected, is attributed to selective labelling of different populations of sensory neurones, or to different tracer efficiencies. However, no differences were found when comparing the mean total number of neurones labelled with each tracer, indicating that the dyes have similar labelling efficiencies. Furthermore, the finding that injection of a greater amount of DY tended to result in an increase in the percentage of double labelled neurones suggests that non-overlapping injection territories rather than differences in selectivity caused the less than total overlap. It can not be excluded, however, from the findings in the present study that certain functional subpopulations of afferents are left unlabelled by all three tracers.

Cell counts

Results from experiments where the same volumes of the three tracers were injected in the same digit showed that even if variation was considerable (maximal S.D. up to 38.4) the mean absolute number of labelled cells was similar for the three tracers (range 91.8 to 100.4), using 6-7 cases per group. This indicates that reproducible means may be obtained in experiments where the investigated tracers are combined using a reasonable number of animals.

Variability in total cell counts after injections in the same digit might be due to inter-animal differences in innervation density and/or to differences between areas covered by the injection. Even though the number of animal observations is small, the tendency of finding either low or high numbers of labelled cells labelled with each of the tracers in individual animals indicates that such inter-animal differences in innervation density may exist (c.f. table IV). However, two cases shown in table II (R77 l and R37 l) show opposite dominance regarding tracers, indicating that differences between injections also have to be considered. Variability is evident also when counts are expressed as percentages of double labelled cells, although the variability becomes reduced when a higher amount of DY is injected. Thus, FB/DY experiments showed 75% ±12.8, and FG/DY showed 74% ±12.0 after injection of the same amounts of tracers. However, when a greater amount of the second tracer (DY) was injected, thereby increasing the DY area overlapping with the FB or FG area in the digit,

percentages showed smaller variation (84% ±8.2 for FB/DY, and 81% ±7.5 for FG/DY). Thus, our results indicate that similar means of both absolute numbers and percentages can be obtained with a low number of rats with the different combinations of tracers investigated.

Surprisingly, the number of labelled DY neurones was found to be smaller than the number of FB cells when a higher dose of DY was injected 10 days after FB injection (tables III). We have no good explanation for this finding.

Usefulness of the technique for studies of normal innervation and of reinnervation after nerve injury

This is the first demonstration that subcutaneous injections of different fluorescent tracers can be used to show a similar labelling efficacy in primary afferent cell bodies. Previous studies have shown that FB, FB and DY may differ with regard to numbers of retrogradely labelled non-sensory neurones after injection in target tissue. Thus, comparable numbers of retrogradely labelled motoneurones were found after application of FB and FG to cut nerve ends, whereas dissimilar numbers were found following intramuscular injection (Richmond et al., 1994). Three times more facial nucleus neurones were found to be labelled with FB compared to DY after injection in the rat snout (Horikawa and Powell, 1986). In another study, in which FB and DY were injected in the anterior chamber of the eye, only 32% of all labelled cells contained DY, suggesting less labelling efficacy of DY compared to FB in this system (Hendry et al., 1986).

The finding that FB, FG, and DY label similar numbers of primary afferent neurones indicates that the three tracers can be injected simultaneously to label primary afferents in different cutaneous territories in studies where tracers with similar labelling efficacy are needed, for instance when cell quantification is required. Furthermore, provided that the neurones show similar transport capacity in the normal state and after regeneration, the tracers are potential candidates for use in combinations to study the accuracy of sensory reinnervation to the skin after injury**.** FB or FG might be used to label the original population of neurones innervating a specific cutaneous territory before nerve injury. After nerve injury and subsequent regeneration, DY injected in the

same territory could be used as a second tracer to label afferents that have regenerated. Double-labelled cells in the DRG would indicate selective reinnervation of the previously innervated territory. We show 75-93% double labelled neurones in the normal experiments where the greater amount of the second tracer was injected, indicating an acceptable rate of double labelling for investigations of changes after injury, provided that the changes are of a notable magnitude.

Further potential problems associated with the use of sequential injections of fluorescent dyes for studies of primary afferent regeneration accuracy after nerve injury are cellular degradation of the dyes at long survival times, possible toxic effects of FB, FG and DY, and the possibility that injected dyes remain available for uptake in the cutaneous injection area also when regenerating fibres reach their former targets. These problems are now under investigation.

ACKNOWLEDGEMENTS

We thank Mrs. Dolors Fuster and Nieves Cayuela for excellent technical assistance and the special collaboration of Mary Cayuela. We also want to thank Dr. Francesc Calafell for the statistical assessment and Dr Josep Mª Estañol and Professor Oriol Bachs for their collaboration in obtaining pictures. This project was supported with the grants of the Secretaría de Estado de Universidades e Investigación, Programa Sectorial de Promoción General del Conocimiento, project No PM96-0059, the Fundació Marató TV3, project No 003/97 and the Swedish Medical Research Council, project No. 11566. We are grateful to professor Gunnar Grant and doctor Peter Shortland for valuable comments.

REFERENCES

Akintude, A. and Buxton, D.F. (1992) Quadruple labelling of brain-stem neurones: a multiple retrograde fluorescent tracer study of axonal collateralization, J. Neurosci. Meth., 45: 15-22.

Buisseret-Delmas, C. and Buisseret, P. (1990) Central projections of extraocular muscle afferents in cat. Neurosci. Lett., 109: 48-53.

Catsman-Berrevoets, C.E., Lemo, R.N., Verburgh, C.A., Bentivoglio, M. and Kuypers, H.G. (1980) Absence of callosal collaterals derived from rat corticospinal

neurones. A study using fluorescent retrograde tracing and electrophysiological techniques, Exp. Brain. Res., 39 (4): 433-40.

Craig, J.R., Linington, A.J. and Kniffki, K.D. (1989) Significant differences in the retrograde labelling of spinothalamic tract cells by horseradish peroxidase and the fluorescent tracers fast blue and diamidino yellow, Exp.Brain.Res., 74: 431-436.

Fritzsch, B. and Sonntag, R. (1991) 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.Meth., 39: 9-17.

Garret, T.W., McBride, R.L., Williams, J.K. and Feringa, E.R. (1991) Fluoro-Gold's toxicity makes it inferior to True Blue for long-term studies of dorsal root ganglion neurones and motoneurons, Neurosci. Lett., 128: 137-139.

Harsh, C., Archibald, S.J. and Madison, R.D. (1991) Double-labelling of saphenous nerve neurone pools: a model for determining the accuracy of axon regeneration at the single neuron level, J.Neurosci.Meth., 39: 123-129.

Haase, P. and Payne, J.N. (1990) Comparison of the efficiencies of the true blue and Diamidino yellow as retrograde tracers in the peripheral motor system, J.Neurosci.Meth., 35: 175-183.

Hendry, L.A., Hill, C.E. and Watters, D.J. (1986) Long-term retention of Fast Blue in Sympathetic neurones after axotomy and regeneration-demonstration of incorrect reconnections, Brain Res., 376: 292-298.

Horikawa, K. and Powell, E.W. (1986) Comparison of techniques for retrograde labelling using the rat's facial nucleus, J.Neurosci.Meth., 17: 287-296.

Illert, M., Fritz, N., Aschoff, A. and Hollander, H. (1982) Fluorescent compounds as retrograde tracers compared with horseradish peroxidase (HRP). II. A parametric study in the peripheral motor system of the cat, J. Neurosci. Meth., 6 (3): 199-218.

Innocenti, G.M., Clarke, S. and Kraftsik, R. (1986) Interchange of callosal and association projections in the developing visual cortex, J.Neurosci., 6 (5): 1384-1409.

Keizer, K., Kuypers, H.G., Huisman, A.M. and Dann, O. (1983) Diamidino Yellow Dihydrocholoride (DY:2HCl), a new fluorescent retrograde neuronal tracer, which migrates only very slowly our of the cell, Exp.Brain.Res., 51:179-191.

Kuypers, H.G., Bentivoglio, M., Castsman-Berrevoets, C.E. and Bharos, A.T. (1980) Double retrograde neuronal labelling through divergent axon collaterals, using two fluorescent tracers with the same excitation wavelength which label different features of the cell, Exp Brain Res., 40 (4): 383-92.

Minciacchi, D., Molinari, M., Bentivoglio, M. and Macchi, G. (1985) The organisation of the ipsi- and contralateral claustrocortical system in rat with note on the bilateral claustrocortical projections in cat, Neuroscience., 16 (3): 557-76.

Molander, C. and Grant, G. (1985) Cutaneous projections from the rat hindlimb foot to the substantia gelatinosa studied with transganglionic transport of WGA-HRP, J. Comp. Neurol., 237:476-484.

Molander, C. and Aldskogius H. (1992) H.: Directional specificity of regenerating neurons after peripheral nerve crush or transection and epineurial suture. A sequential double-labelling study in the rat, J. Restor. Neurol. and Neurosci., (4): 339-344.

Novikova, L., Novikov, L. and Kellerth, O. (1997) Persistent neuronal labelling by retrograde fluorescent tracers: a comparison between Fast Blue, Fluoro-Gold and various dextran amines, J.Neurosci.Meth., 74: 9-15.

O'Brian, C., Woolf, C., Fitzgerald, M., Lindsay, R. and Molander, C. (1989) Differences in the chemical expression of rat primary afferent neurons which innervate skin, muscle and joint, Neuroscience, 32 (2): 493-502.

Skirboll, L., Hökfelt, T., Norell, G., Phillipson, O., Kuypers, H.G. and Bentivoglio, M. (1984) A method for specific transmitter identification of retrogradely labelled neurons: immunofluorescence combined with fluorescence tracing, Brain Res., 320 (3-2): 99-127.

Schmid, H., Taylor, D.C.M. and Pierau, F.K., (1983) Tracing of sensory neurones and spinal motoneurones of the pigeon by injection of fluorescent dyes into peripheral nerves, Cell Tissue Res., 232: 9-19.

Richmond, F.J.R, Gladdy, R., Creasy, J.L., Kitamura, S., Smits, E. and Thomson, D.B. (1994) Efficacy of seven retrograde tracers, compared in multiple-labelling studies of feline motoneurons, J.Neurosci.Meth., 53: 35-46.

Schumued, L.C. and Fallon, J.H. (1986) Fluoro-Gold: a new fluorescent retrograde axonal tracer with numerous unique properties, Brain Res., 377: 147-154.

Van der Kooy, D., Kuypers, H.G. and Catsman-Berrevoets, C.E. (1978) Single mammillary body cells with divergent axon collaterals. Demonstration by a simple, fluorescent retrorade double labelling technique in the rat, Brain Res., 158:189-196.

Wigston, J. and Kennedy, P.R. (1987) Selective reinnervation of transplanted muscles by their original motoneurons in the axolotl, J. Neurosci., 7 (6): 1857-1865.

Table I. Total numbers of labelled neuronal profiles counted in DRGs L3-5 after injections of FB or FG in digit 2.

Rat	FB total	Rat	FG total
19	43	26	90
23	56	29	71
25	47	56	59
28	102	57	81
37	91	58	66
45	86	59	98
Mean	70.8		77.5
S.D.±	25.2		14.9

Table II. Numbers and percentages of neuronal profiles counted in DRG:s L4-5 containing either FB, DY, or both FB and DY (FBDY) following sequential injections of 0.5µl FB and 0.5µl DY in digit 4. Total numbers of FB labelled cells (FBDY+FB) and DY labelled cells (FBDY+DY) have been calculated. The percentage of FB cells that became also labelled with DY, [FBDY/(FBDY+FB)], is also presented in the last column of the table.

	Absolute numbers of counted profiles					Relative numbers of counted profiles			
Rat	FB	DY	FBDY	**FBDY +DY**	**FBDY +FB**	%FB	%DY	%FBDY	**FBDY / FBDY+FB**
R37 l	51	0	84	**84**	**135**	37.8	0.0	62.2	**62.2**
R77 r	19	17	102	**119**	**121**	13.8	12.3	73.9	**84.3**
R77 l	2	64	25	**89**	**27**	2.2	70.3	27.5	**92.6**
R78 r	29	6	84	**90**	**113**	24.4	5.0	70.6	**74.3**
R78 l	37	2	62	**64**	**99**	36.6	2.0	61.4	**62.6**
R79 r	40	29	59	**88**	**99**	31.2	22.7	46.1	**59.6**
R79 l	12	25	97	**122**	**109**	9.0	18.7	72.4	**89.0**
Mean	27.1	20.4	73.3	**93.7**	**100.4**	22.1%	18.7%	59.1%	**74.9%**
S.D.±	15.9	20.6	24.7	**18.8**	**32.2**	13.0	22.5	15.6	**12.8**

Table III. Numbers and percentages of neuronal profiles counted in DRGs L4-5 containing either FB, DY or both FB and DY (FBDY) following sequential injections of 0.5µl FB and 1.5µl DY in digit 5. Total numbers of FB labelled cells (FBDY+FB) and DY labelled cells (FBDY+DY) has been calculated. The percentage of FB cells that became also labelled with DY, [FBDY/(FBDY+FB)], is also presented in the last column of the table.

	Absolute numbers of counted profiles					Relative numbers of counted profiles			
Rat	FB	DY	FBDY	**FBDY +DY**	**FBDY +FB**	%FB	%DY	%FBDY	**$\frac{\text{FBDY}}{\text{FBDY+FB}}$**
50 r	20	1	56	**57**	**76**	26.0	1.3	72.7	**73.7**
50 l	21	5	95	**100**	**116**	17.4	4.1	78.5	**81.9**
51 r	5	15	66	**81**	**71**	5.8	17.4	76.7	**93.0**
51 l	17	9	112	**121**	**129**	12.3	6.5	81.2	**86.8**
151 l	54	35	151	**186**	**205**	22.5	14.6	62.9	**73.7**
Mean	23.4	13	96	**109**	**119.4**	16.8%	8.8%	74.4%	**81.8%**
S.D.±	16.3	11.9	34.0	**43.9**	**48.3**	7.2	6.2	6.4	**7.5**

Table IV. Numbers and percentages of neuronal profiles in DRGs L4-L5 containing either FG, DY or both FG and DY (FGDY) following sequential injections of 0.5µl FG and 0.5µl DY in digit 4. Total numbers of FG labelled cells (FGDY+FG) and DY labelled cells (FGDY+DY) has been calculated. The percentage of FG cells that became also labelled with DY, [FGDY/(FGDY+FG)], is also presented in the last column of the table.

	Absolute numbers of counted profiles					Relative numbers of counted profiles			
Rat	FG	DY	FGDY	**FGDY +DY**	**FGDY +FG**	%FG	%DY	%FGDY	**$\frac{\text{FGDY}}{\text{FGDY+FG}}$**
136 l	15	27	69	**96**	**83**	13.5	24.3	62.2	**82.1**
137 l	14	22	61	**83**	**75**	14.4	22.7	62.9	**81.3**
138 l	16	29	49	**78**	**63**	17.0	30.8	52.1	**75.4**
139 l	38	1	39	**40**	**77**	48.7	1.3	50.0	**50.6**
140 l	21	36	92	**128**	**113**	14.1	24.2	61.7	**81.4**
151 r	42	1	125	**126**	**167**	25.0	0.6	74.4	**74.8**
Mean	23.8	19.3	72.5	**91.8**	**96.3**	22.1%	17.3%	60.5%	**74.3%**
S.D.±	12.8	14.9	31.5	**33.0**	**38.4**	13.7	13.0	8.8	**12.0**

Table V. Numbers and percentages of neuronal profiles in DRGs L4-L5 containing either FG, DY or both FG and DY (FGDY) following sequential injections of 0.5μl FG and 1.5μl DY in digit 3. Total numbers of FG labelled cells (FGDY+FG) and DY labelled cells (FGDY+DY) has been calculated. The percentage of FG cells that became also labelled with DY, [FGDY/(FGDY+FG)], is also presented in the last column of the table.

	Absolute numbers of counted profiles					Relative numbers of counted profiles			
Rat	FG	DY	FGDY	**FGDY +DY**	**FGDY +FG**	%FG	%DY	%FGDY	**$\frac{\text{FGDY}}{\text{FGDY+FG}}$**
92 r	16	65	57	**122**	**73**	11.6	47.1	41.3	**78.1**
92 l	14	81	49	**130**	**63**	9.7	56.2	34.0	**77.8**
93 r	10	25	145	**170**	**155**	5.6	13.9	80.6	**93.5**
93 l	9	36	132	**168**	**141**	5.1	20.3	74.6	**93.6**
94 r	7	32	19	**51**	**26**	12.1	55.2	32.8	**73.1**
94 l	4	72	35	**107**	**39**	3.6	64.9	31.5	**89.7**
Mean	10.0	51.8	72.8	**124.7**	**82.8**	7.9%	42.9%	49.1%	**84.3%**
S.D.±	4.0	21.6	48.1	**40.2**	**48.7**	3.3	19.1	20.4	**8.3**

FIGURE LEGENDS

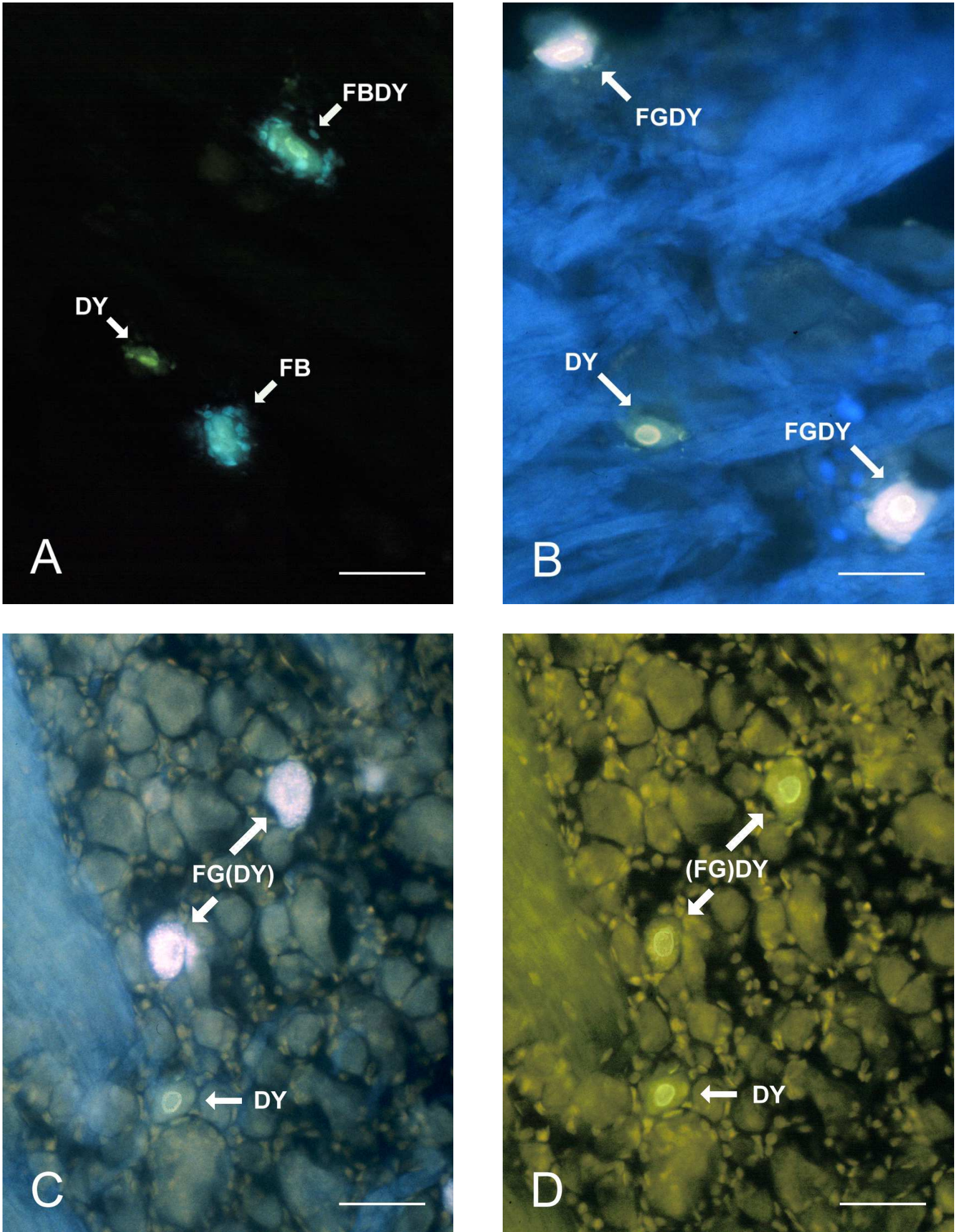
Fig 1. Photomicrographs of longitudinal sections from dorsal root ganglia showing retrogradely labelled neuronal profiles after subcutaneous injections of fluorescent tracers. Scale bar=50 μm in all sections.

A. Fast blue (FB) single labelled cell profile showing nuclear and cytoplasmic labelling. Diamidino Yellow (DY) single labelled profile showing nuclear labelling surrounded by a perinuclear halo. Double labelled cell profile showing DY labelling in the nucleus and FB in the cytoplasm (FBDY). Labelling has diffused to small nuclei of satellite cells surrounding neuronal cell bodies. Violet light filters.

B. Double labelled cell profiles with FG and DY (FGDY). Note FG cytoplasmic labelling through which a DY-labelled nuclear contour may be observed. Note also the single labelled DY cell profile. Ultraviolet light filters.

C. FG labelling visualised by the ultraviolet light filters, in this case hiding a DY-labelled nuclear profile, FG(DY). Note also single DY-labelled profile.

D. Same section as in C using the violet light filters. Note decrease of FG labelling intensity and the appearance of DY labelling in double labelled profiles, (FG)DY, compare with panel C. Note also single DY-labelled cell profile.


FBDY
DY
FB
A
FGDY
DY
FGDY
B
FG(DY)
DY
C
(FG)DY
DY
D