CHAPTER 8

# Controlling dispersion of axonal regeneration using a multichannel collagen nerve conduit

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*Adapted from publication in Biomaterials 31 (2010): 5789-5797*

## ABSTRACT

**Background** Single channel conduits are used clinically in nerve repair as an alternative to the autologous nerve graft. Axons regenerating across single channel tubes, however, may disperse resulting in inappropriate target reinnervation. This dispersion may be limited by multichannel nerve conduits as they resemble the structure of nerve multiple basal lamina tubes. In this study, we investigated the influence of channel number on axonal regeneration using a series of 1-, 2-, 4-, and 7-channel collagen conduits and commercial (Neuragen ®) single channel conduits.
**Methods** Nerve conduits were implanted in rats with a 1cm gap of sciatic nerve. After four months, quantitative results of regeneration were evaluated with nerve morphometry and the accuracy of regeneration was assessed using retrograde tracing: two tracers being applied simultaneously to the tibial and peroneal nerves to determine the percentage of motoneurons with double projections. Recovery of function was investigated with compound muscle action potential recordings and ankle motion analysis.
**Results** Simultaneous tracing showed a significantly lower percentage of motor neurons with double projections after 2- (2.7%) and 4-channel (2.4%) conduit repair compared with single channel (7.1%) conduit repair (both $P<0.05$). The number of myelinated fibers and motoneurons were not significantly different for all types of conduit repair. Overall however, quantitative results of regeneration were superior after autograft repair.
**Conclusion** This study shows the potential influence of multichannel guidance on limiting dispersion without decreasing quantitative results of regeneration.

## INTRODUCTION

The first single lumen nerve tubes made from various synthetic and natural materials are already available for clinical use (**Chapter 5**). However, as we found in **Chapter 7**, axons regenerating across single lumen nerve tubes might disperse, resulting in inappropriate target reinnervation. Multichannel nerve tubes might limit this dispersion. In the previous Chapter we compared regeneration across single lumen and multichannel conduits made of 75:25 poly(lactic co-glycolic acid) (PLGA). Although in that study a trend towards reducing axonal dispersion after multichannel compared with single lumen PLGA nerve tube repair was found, quantitative results of regeneration were limited by extensive swelling of the conduits, possibly due to the accumulation of small degradation products that increased the osmotic value inside the conduit structure (**Chapter 6**).
We therefore developed a novel series of conduits with improved physical properties to again investigate the influence of multichannel nerve tube structure on axonal regeneration, consisting of 1-, 2-, 4-, and 7-channel conduits made from collagen using an innovative multistep molding technique (Figure 1). The fabrication

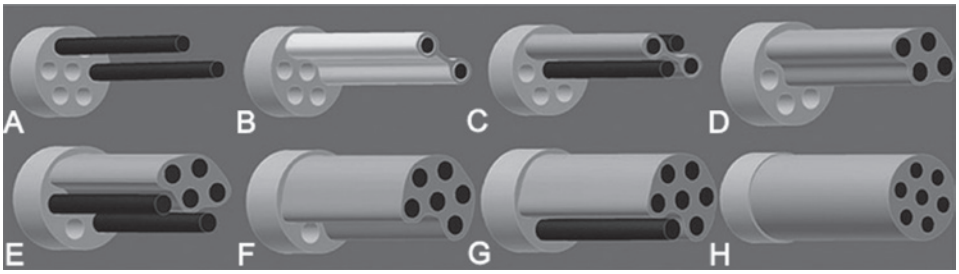



**Figure 1**

Multistep molding technique to create multichannel nerve tubes. (A) two stainless steel wires (black) were inserted through end caps (as described for the injection-molding technique, **Figure 1 Chapter 6**), (B) a collagen solution was allowed to self-assemble evenly on the wires and air-dried, (C) two additional wires were inserted into the two adjacent channels, (D) again, a collagen solution was allowed to self-assemble around the wires and air-dried, and so on for channel 5 - 7 (E-H). Obtained with permission from article Yao et al. (1).

technique and *in vitro* characterization of this series of collagen tubes have been reported separately [1].

Collagen is a naturally occurring protein that is ubiquitous among mammalian species. It is an important component of the nerve tissue matrix (epineurial, perineurial and endoneurial sheaths/ basal lamina tubes) and plays an important role in the regeneration process [2]. Collagen has been used before in the fabrication of nerve tubes [3], and there are also commercially available single channel collagen conduits (Neuragen®, Integra, NeuroMatrix® and Neuroflex®, both Stryker).

To investigate the influence of channel number on axonal regeneration we implanted the 1-, 2-, 4-, and 7-channel conduits in a 1cm gap of the rat sciatic nerve model. After 16 weeks of implantation, quantitative results of regeneration were analyzed with compound muscle action potential (CMAP) recordings and quantitative nerve morphometry. In a different experimental group qualitative restuls of regeneration were analyzed with simultaneous retrograde axonal tracing and ankle motion analysis. Finally, results were compared to repair with an autograft and repair with a commercially available conduit (Neuragen® from Integra).



## MATERIALS AND METHODS

### Fabrication of multichannel nerve conduits

1-, 2-, 4- and 7-channel collagen conduits were fabricated using a multistep molding technique (Figure 1). Similar mold assemblies to the ones used for the fabrication of multichannel PLGA nerve tubes (**Chapter 6**) were used (with wires inserted through endcaps), except that for 2- and 4-channel conduits wires of 530μm in diameter were used and for 7-channel conduits wires of 410μm in diameter. The distance between the 2 end caps was 1cm and the end-caps were layered to cre-

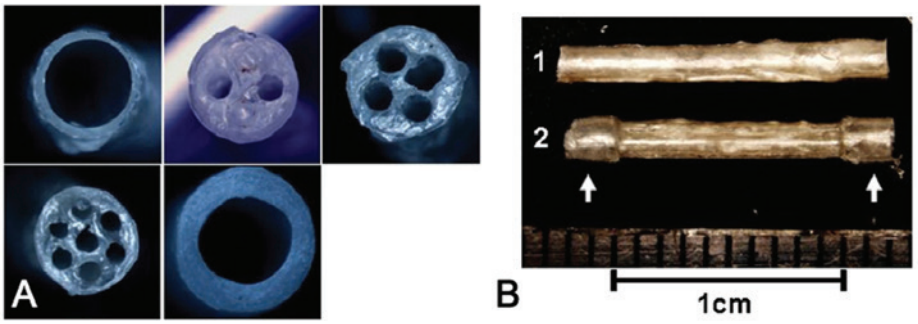



**Figure 2**
(A) transverse images of 1-, 2-, 4- and 7-channel collagen conduits and the NeuraGen ® (Integra) conduit, (B) longitudinal images of single channel and multichannel conduit with the sleeves at the end for nerve stump insertion.

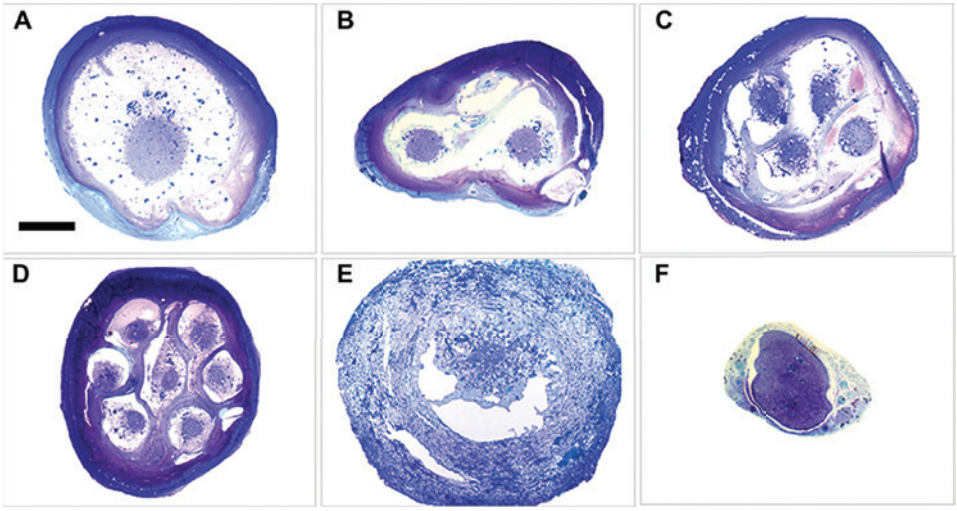




ate a 1mm sleeve at the ends for insertion of the proximal and distal nerve ends. A solution of type I collagen (12mg/ml, in 10mM HCL, derived from bovine Achilles tendon by pepsin and acid extraction; purity 90%) was allowed to self-assemble around the wires in multiple steps (Figure 1) and was air dried. The collagen was then treated with a cross-linking solution of EDC (30mM) and NHS (10mM) in 2-morpholinoethanesulfonic acid solution (50mM; pH 5.5) overnight. After washing with $NaH_2PO_4$ (0.1M) and distilled water, the collagen was freeze-dried on the wires. Molds and wires were removed from the collagen conduits after freeze-drying. The same procedure was performed to fabricate 1-channel conduits, only a single stainless steel wire of 1.5mm in diameter was used. Samples of the different conduits are presented in Figure 2 A and B.

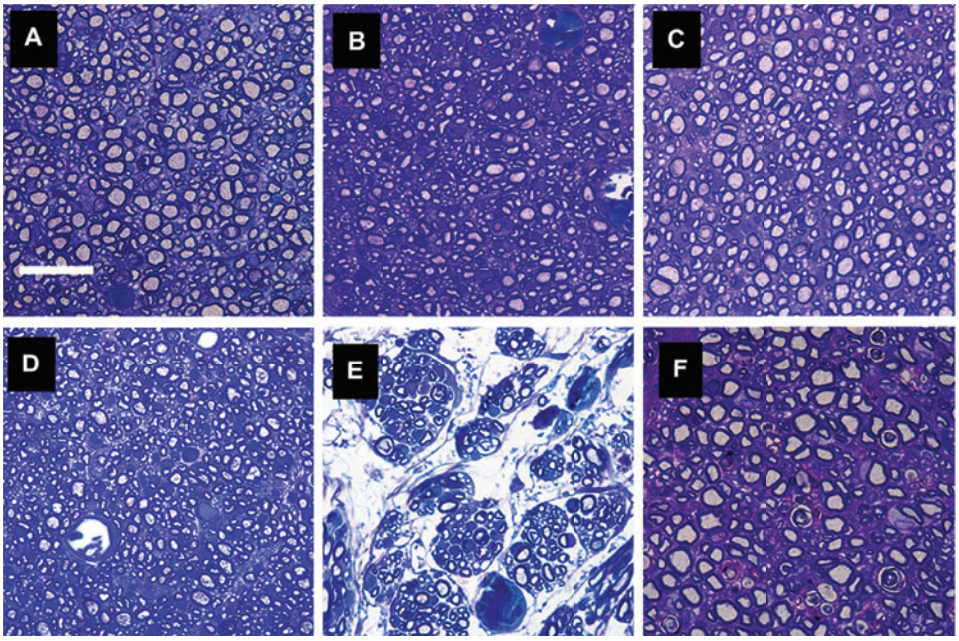
## Animal procedures and experimental groups

In this study a total of 72 adult female Lewis rats, weighing between 190–220gr, were used. All experimental procedures were conducted according to animal care guidelines of the Mayo Foundation Institutional Animal Care and Use Committee. In the first experimental group, for the study of nerve morphometry and CMAP recording, 48 rats were randomly assigned to 6 subgroups: autograft, 1-, 2-, 4- and 7-channel collagen conduits, and commercial single channel conduit (Neuragen®, Integra Life Sciences Corporation, USA). In the second experimental group on simultaneous retrograde tracing and ankle motion analysis, 24 rats were randomly assigned to four subgroups: autograft, 1-, 2- and 4-channel conduits. In this latter group, treadmill training (running speed 15m/min, duration 10 min) was performed, one week post operation, and then 4 times per week for 8 weeks in total to prevent formation of contractures.




## Figure 3

Microscopic images (5x magnification) of sections stained with toluidine blue taken through the middle of an (A) 1-channel, (B) 2-channel, (C) 4-channel, and (D) 7-channel collagen conduit, (E) a Neuragen® single channel conduit and (F) autograft. Scale bar, 500μm.




## Figure 4

Microscopic images (40x magnification) of sections stained with toluidine blue taken through the middle of an (A) 1-channel, (B) 2-channel, (C) 4-channel, and (D) 7-channel collagen conduit, (E) a Neuragen® single channel conduit, and (F) autograft. Scale bar, 30μm.

## Surgical procedure

Rats were anesthetized using 80mg/kg of ketamine and 5mg/kg of xylazine that were injected intraperitoneally. Dissection was performed with the aid of a Zeiss operating microscope (Carl Zeiss, Inc., Oberkochen, Germany). The left sciatic nerve was exposed and isolated at the midthigh level using a dorsal-lateral approach. A 5mm segment of the sciatic nerve was resected before the bifurcation of the nerve into the tibial and peroneal nerve branches. The proximal and distal nerve ends were inserted 1mm into the 12mm long tubes with 10-0 monofilament nylon sutures (Ethilon; Ethicon, Inc., Piscataway, NJ). The wound was subsequently closed in layers. The same procedure was performed for autologous nerve graft repair, except that a 1cm segment of sciatic nerve was transected and microsurgically repaired with 10-0 monofilament nylon sutures.

## Nerve morphometry

After 16 weeks of implantation, in all animals of the first experimental group, the graft was re-exposed and fixed *in situ* with a Trump solution (4% formaldehyde and 1% glutaraldehyde in phosphate buffered solution) for 30 minutes [4]. The graft was resected and placed in the same fixative overnight. Specimens (2mm) at the midpoint of the graft were collected and embedded in spur resin. Sections (1μm) were cut with a glass knife on an ultramicrotome (Leica EMUC6 ultracut, Wetzlar, Germany). The sections of each specimen were stained with toluidine blue or 1% phenylenediamine for nerve morphometry. Nerve morphometry was performed on an image analysis system (see **Chapter 4** and **7** for more detailed description of the method). Briefly, the inner and outer borders of myelinated fibers was manually drawn for at least 500 myelinated fibers at 63x magnification in randomly selected areas in the slide to determine the number of myelinated fibers, the density of myelinated fibers, the mean diameter of myelinated fibers and the mean myelin thickness. SS

## Compound muscle action potential recording

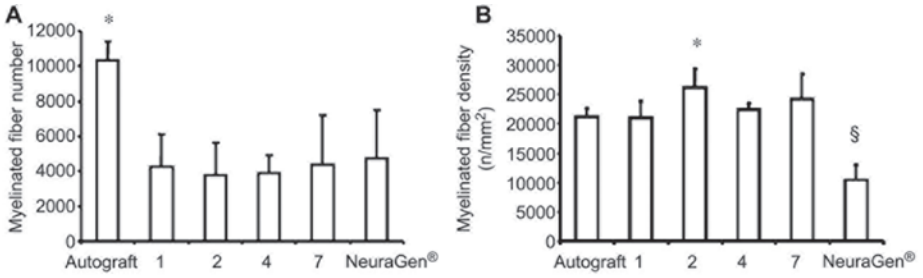
In all animals of both experimental groups, CMAPs were recorded before operation, 6, 8, 10, 12 and 16 weeks after nerve conduit implantation. Briefly, animals were first anesthetized with the  procedure described above (surgical procedure), then CMAPs were recorded with an electromyography machine (Nicolet Viking IV; Viasys Healthcare, Inc., Conshohocken, PA) in the tibial and peroneal nerve-innervated foot muscles of the left limb (see **Chapter 4** and **7**). Needle recording electrodes were placed in the plantar or dorsal foot muscles referenced to needle electrodes placed distally in the foot digits. Needle-stimulating electrodes were placed directly posterior to the tibia with approximately 5mm between the distal cathode and proximal anode. The stimulating electrodes were adjusted locally to produce the maximal CMAP amplitude. The stimulus was increased incrementally to produce a supramaximal response. CMAPs were recorded and analyzed for the amplitude of the action potential.

## Ankle motion analysis

In the second experimental group, ankle motion was analyzed before surgery, 1 week, 6 weeks, 12 weeks and 16 weeks after repair. The method as described in **Chapter 3** was applied only with the use of a different 2D ankle angle model. Three markers (instead of four) were placed on bony landmarks of the left leg: the tibia, the lateral malleolus, and the fifth metatarsal (the calcaneus as markation point was excluded in this model). Markers were tattooed to enhance the reproducibility of marker placement. Rats were filmed in a 1 meter long plexiglass runway with a black box on one end, which was alternately switched to the other end to get the rats to walk. The animal walking was filmed using a 60Hz digital camera (Dinion XF CCD Camera; Bosch Security Systems, Fairport, New York). After filming, the digital videos were processed using motion analysis software (Vicon Peak, Centennial, Colorado) that automatically tracks the markers on the leg of the rat in each frame of the video. The ankle joint motion was analyzed and the value of the ankle angle was compared at different moments during the step cycle: midstance (MSt), the moment the right foot in the air crosses the left foot in the stance (that bears the weight); terminal stance (TS), the moment the left foot comes off the runway (in normal animals, the moment of maximum plantar flexion); and midswing (MSw), the moment the left foot crosses the right foot in the stance (in normal animals, the moment of maximal dorsiflexion). Data for the ankle angles were reported in degrees of the intersection angle of the line connecting tibia and lateral malleolus and the line connecting lateral malleolus and fifth metatarsal.

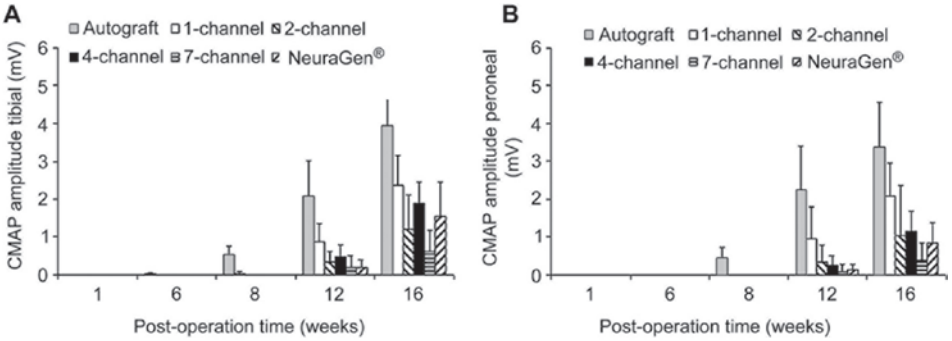
## Simultaneous retrograde tracing

In the second experimental group, after 16 weeks, all animals were anesthetized for simultaneous retrograde tracing (same method as described in **Chapter 7**). The nerve graft and distal tibial and peroneal nerve branches were exposed. First the peroneal nerve was transected and the proximal end was placed in a cup with 5% diamidino yellow (DY) (EMS-Chemie, Mannedorf, Switzerland) solution for 20 minutes. After that, the nerve end was cleaned, then sutured into and covered by surrounding fat tissue to prevent tracer leakage and cross-contamination. Then the tibial nerve was transected and the proximal nerve end was placed in a cup with 5% fast blue (FB) (EMS-Chemie, Mannedorf, Switzerland) solution for 20 minutes. Again, the nerve end was cleaned and then sutured into and covered by surrounding fat tissue. Six days after tracer application, the animals were transcardially perfused with 4% paraphomaldehyde and 10% sucrose in phosphate buffered solution (PBS). Spinal cord segments L1 to L6 were removed and post-fixed overnight. Sagittal longitudinal 30μm-thick sections were cut on a cryostat (LEICA Cryostat, CM3050S, Nussloch, Germany) at -20$^0$C. Slides were immediately evaluated under a fluorescent microscope (Axioplan 2; Carl Zeiss, Inc.). Neuronal profiles with blue cytoplasm and a dark nucleus were counted as FB-labeled, profiles with a yellow nucleus and dark cytoplasm as DY-labeled, and profiles with a yellow nucleus and blue cytoplasm as FB-DY-double-labeled profiles. All profiles in all sections




## Figure 5

Results of nerve morphometry after autograft, 1-, 2-, 4-, 7-channel collagen conduit and Neuragen® single conduit repair for (A) the mean number of myelinated fibers (*, P<0.01, vs all the conduit groups) and (B) the mean myelinated fiber density (*, P< 0.05, vs the autograft, single channel and 4-channel tube graft groups; §, P<0.01, vs all the other groups).




## Figure 6

Results of the mean amplitude for compound muscle action potentials recorded in plantar and dorsal foot muscle respectively (A and B).

were counted. The percentage of double projections to both the tibial and peroneal nerve branch was calculated (as in **Chapter 7**) by dividing the total number of double-labeled neurons by the total number of neurons (single-labeled (DY and FB) and double-labeled neurons).

### Statistics

The data were expressed as means ± SD and analyzed by using oneway ANOVA (post-hoc Bonferroni) with SPSS version 17.0 software package (SPSS Inc., Chicago, IL, USA). P values less than 0.05 were considered statistically significant.

## RESULTS

### Nerve morphometry

Successful regeneration (defined for the presence of myelinated axons at the mid of the conduit) was observed in 39 out of 40 cases of conduit repair (in one 2-channel conduit graft only fibrous tissue was present). Figures 3 and 4 show microscopic images (respectively taken at 5x and 40x magnification) of sections taken through the mid of all 6 types of grafts.

The mean number of fascicles (channels filled with myelinated axons) was one for the single channel conduits, 1.6 ± 0.8 for the 2-channel conduits, 3.75 ± 0.4 for the 4-channel conduits, and 6 ± 1 for the 7-channel conduits. The mean number of myelinated fibers was not significantly different between conduit groups (Figure 5A), but was significantly higher after autograft repair (10348 ± 1038, vs all conduit groups, $P<0.01$). The mean density of myelinated fibers (Figure 5B) was significantly higher for the 2-channel conduit group (vs autograft, 1-channel conduit, commercial conduit, $P<0.05$), and significantly lower for the commercial conduit group (vs all the other groups, $P<0.01$).

### Compound muscle action potential recording

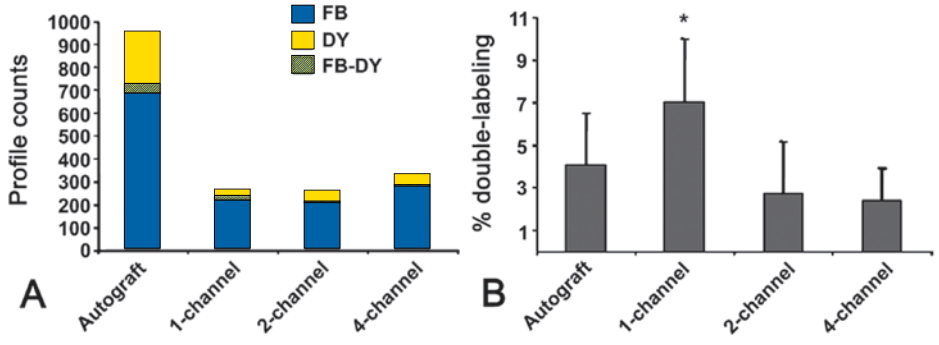
The first CMAPs were detected earlier after autograft repair compared with conduit repair (Figure 6) and the amplitudes at 12 weeks were significantly higher than for all other groups ($P<0.01$). At 16 weeks, the CMAP amplitudes of the 1- and 4-channel conduit groups were slightly larger than for 2- and 7-channel conduit groups, although not significantly different.

### Simultaneous retrograde tracing

After autograft, 1-, 2- and 4-channel conduit repair, FB-, DY-, and FB-DY-labeled profiles in the anterior horn in the spinal cord were found intermingled, as reported in **Chapter 7**. The total number of labeled profiles was not significantly different after 1-, 2- and 4-channel conduit repair, but was significantly higher after autograft repair ($P<0.01$). Interestingly, the percentage of double-labeled neurons was significantly smaller after 2-channel (2.7% ± 2.9%) and 4-channel conduit (2.4% ± 1.5%) repair, compared with single channel conduit repair (7.1% ± 2.7%) (both $P<0.05$), indicating less axonal dispersion after multichannel nerve tube repair.

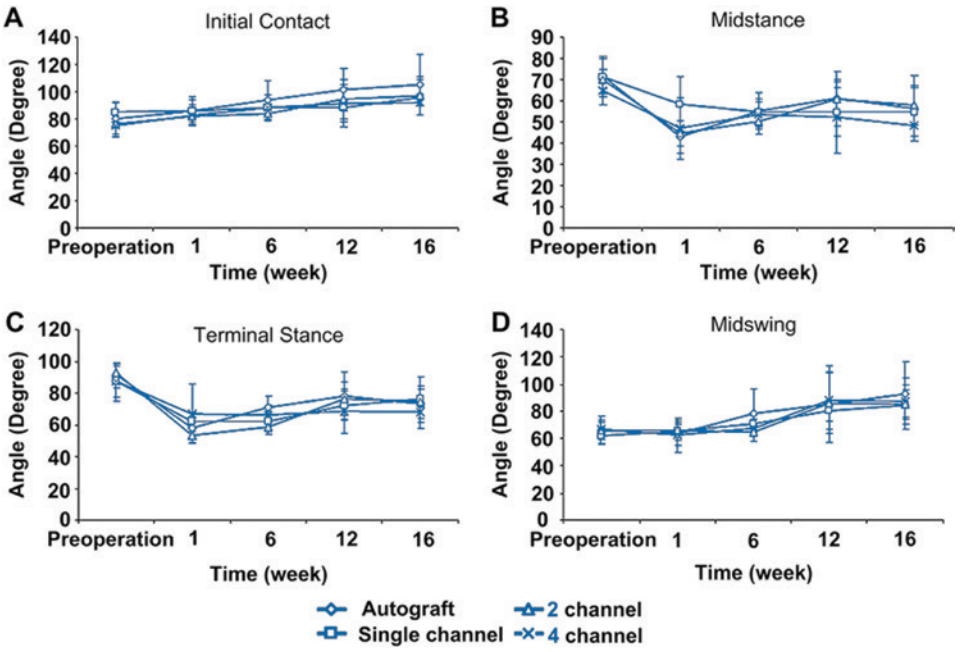
### Ankle motion analysis

The ankle angle at terminal stance and midstance was significantly decreased one week after the surgery in all experimental groups (Figures 8B and 8C), and slowly recovered in time, but did not recover fully. The angle at midswing did not significantly change after surgery, but significantly increased in time. At 16 weeks there were no significant differences between all experimental groups for the different angles.




## Figure 7

Results of simultaneous tracing for the mean number of FB-labeled, DY-lableed, and FB-DY double-labeled profiles (A) and percentages of double-labeled motoneurons (B) after autograft, 1-, 2-, and 4-channel conduit repair.




## Figure 8

Recovery of ankle motion for the angles at (A) initial contact, (B) midstance, (C) terminal stance, and (D) mid-swing after autograft, 1-, 2-, 4- and 7-channel and Neuragen® conduit repair.

## DISCUSSION

This study shows the influence of multichannel structure on limiting axonal dispersion without decreasing the quantitative results of regeneration. The numbers of regenerated myelinated axons and retrogradely labeled profiles were not significantly different for the different types of conduits despite the reduction in the total cross-sectional area for axons to grow into for multichannel nerve tubes (with ratios 1-channel versus 2-, 4- and 7-channel conduits of 4.1, 2.1 and 1.9, respectively). Also, almost all channels contained fascicles with myelinated fibers, contrary to our previous study (**Chapter 7**) in which only 3 out of 7 channels were filled with myelinated axons.

As for the limiting influence of multichannel structure on axonal dispersion; although the reduction might be small (with a 3x smaller percentage double projecting motoneurons after 4-channel conduit repair compared with single channel collagen conduit repair (2.4% vs 7.1%), it is important to realize that this percentage only indicates part of the axonal dispersion that occurs during regeneration across the conduit. In addition, there may be motoneurons with single projections that have dispersed and regenerated to the wrong target organ (See Figure 1, **Chapter 10**). This can be investigated for example with sequential retrograde tracing (**Chapter 7**). Also, the percentages double projections may have decreased in time due to pruning of misdirected collaterals in favor of correctly directed ones. The mechanism of pruning has been investigated in detail for motor versus sensory regeneration [5, 6], but may also affect the reinnervation of plantar and dorsiflexion muscles by tibial and peroneal motoneurons respectively. In our previous study (**Chapter 7**), in which the observation period was 8 weeks instead of 16 weeks, we also found much higher percentages of double projections (16.9% after multichannel nerve tube repair and 21.4% after single lumen nerve tube) compared with the percentages found in the present study. Pruning might thus be a mechanism to later correct for misdirection. Ideally however, initial targeting should be optimal. More channels will probably not completely solve the problem of misdirection, for as axons are also misdirected after autograft repair (**Chapter 4**), probably due to the dispersion of regenerating axons at the two coaptation sites [7], but modifying the multichannel conduit by for example adding supportive cells, growth factors in microspheres [8], and/or viral vectors expressing growth factors to attract axons into the distal pathways may overcome this problem of dispersion at the nerve-channel and channel-nerve junctions (for overview of different modifications, see **Chapter 5**). At the same time, these modifications can also increase the number of axons that regenerate into the conduit. An additional advantage of the multichannel conduit thereby is that it provides more internal lining for cell attachment and/or the controlled release of growth factors compared with single lumen conduits.

Finally, it should be noted that the quantitative results of regeneration in our study were still superior after autograft repair. Although this did not result in a better functional outcome, it is important to realize that an ideal alternative for the auto-

graft should perform better than the autograft. Only in selective cases of nerve graft repair, for example in the repair of small nerve defects (<3cm) in small nerves (for example digital nerves), the advantages of a conduit that is right-off-the-shelf available can outweigh the advantages of an autograft that consists of longitudinally orientated collagen tubes that contain Schwann cells.

## CONCLUSION

This study clearly demonstrates the influence of multichannel guidance on limiting axonal dispersion without decreasing quantitative results of regeneration.





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