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A simple dynamic strategy to deliver stem cells to decellularized nerve allografts

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Abstract

Background—The addition of adipose-derived Mesenchymal Stromal Cells (MSCs) to decellularized nerve allografts may improve outcomes of nerve reconstruction. Prior techniques used for cell seeding are traumatic to both the MSCs and nerve graft. An adequate, reliable and validated cell seeding technique is an essential step for evaluating the translational utility of MSC-enhanced decellularized nerve grafts. The purpose of this study was to develop a simple seeding strategy with an optimal seeding duration.

Methods—A dynamic bioreactor was used to seed rat and human MSCs separately onto rat and human decellularized nerve allografts. Cell viability was evaluated by MTS assays and cellular topology after seeding was determined by SEM microscopy. Cell density and distribution were determined by LIVE/DEAD assays and Hoechst staining at 4 different time points (6, 12, 24 and 72 hours). The validity and reliability of the seeding method were calculated.

Results—Cells remained viable at all time points, and MSCs exhibited exponential growth in the first 12 hours of seeding. Seeding efficiency increased significantly from 79.5% at 6 hours to

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Presentations

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Institution approval

This study was approved by the IACUC institutional review committee and our Institutional Review Board.

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Author Contributions:

Nadia Rbia: contributed to the conception, analysis, interpretation of the data, as well as drafting of the manuscript for submission.
Liselotte F. Bulstra: contributed to the conception, analysis, interpretation of the data, as well as the drafting of the manuscript for submission.

Allen T. Bishop: contributed to the conception and interpretation of data, as well as edited the manuscript.

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89.2% after 12 hours of seeding ($p = 0.004$). Both intra-rater ($r = 0.97$) and inter-rater reliability ($r = 0.92$) of the technique were high.

Conclusions—This study describes and validates a new method to effectively seed decellularized nerve allografts with MSCs. This method is reproducible, distributes cells homogeneously over the graft and does not traumatize the intra-neural architecture of the allograft. Utilization of this validated seeding technique will permit critical comparison of graft outcomes.

Keywords

Processed allograft; adipose derived mesenchymal stem cell; seeding technique; nerve

INTRODUCTION

Segmental injury to peripheral nerves occurs in approximately 5% of all extremity traumas.¹ However, a nerve graft substitute that is biologically equal to autologous nerve tissue has yet to be developed.² One of the most promising commercially available alternatives is the decellularized nerve allograft.³ The presence of an internal structure and extracellular matrix components (ECM) in processed allografts are critical for guiding cell migration and nerve fiber elongation.⁴ However, outcomes to date suggest that allografts are not equivalent to autografts, especially for the reconstruction of long gaps or motor nerves.^{5–7} Because Schwann cells and axons are eliminated after decellularization, the addition of supporting cells has been proposed to improve allografts. Perivascular MSCs that are derived from the stromal vascular fraction of adipose tissue have many clinical advantages, including easy accessibility, rapid proliferation and immunomodulatory effects.⁸ Multiple studies have shown that MSCs have a beneficial effect on nerve regeneration and that MSCs can potentially provide the necessary support for nerve regeneration through local production of essential growth factors.^{9,10,11}

Despite progress in our understanding of the beneficial roles of MSCs in peripheral nerve repair, differences in seeding strategies have resulted in varying outcomes and these differences have precluded comparisons of results between studies. Ikebe and colleagues demonstrated that protocols in clinical trials on MSCs varied widely and highlighted the importance of developing optimized and standardized methods.¹² An adequate cell seeding technique is essential for evaluating the potential of stem cells on nerve regeneration. Ideally, the optimal seeding technique should distribute cells homogeneously, have a high cell seeding efficiency and minimize cell/graft injury. In addition, such seeding techniques should be reproducible, easy to use and validated. For clinical use, the technique should also be rapid and user independent.¹³

Cell seeding on a solid support proceeds generally by either static or dynamic seeding.^{14,15,16} In previous studies, dynamic cell seeding resulted in more efficient and homogeneous cell seeding compared to static seeding.¹⁷

The purpose of this study was to develop a simple seeding method to efficiently seed a decellularized nerve allograft with MSCs, which would be optimally suited for clinical

strategies applications for nerve repair. We also investigated the optimal seeding duration and validated the consistency of our approach.

MATERIALS AND METHODS

General design

To determine if cell-seeding duration has an effect on cell attachment and seeding efficiency, cell viability, density and migration into the graft were evaluated at 4 different time points of incubation ($t= 6, 12, 24$ and 72 hours). These time points were based on literature and potential human clinical translation (preoperative planning); the critical factor was to determine the shortest and most effective seeding time possible.¹⁸ The seeding method, cell viability and effect of seeding duration were first evaluated on rat MSCs seeded onto rat nerve allografts. When the rat process was successful, the seeding method and cell viability were tested on human MSCs seeded onto human nerve allografts.

Allograft preparation

This study was approved by the IACUC institutional review committee and our Institutional Review Board. Previous work by our laboratory on decellularization techniques resulted in reduced immunogenicity, diminished cellular debris and better maintenance of the ultrastructure when compared to the commercially available processed nerve allograft.¹⁹ Sciatic nerve segments of 2 cm were therefore harvested from a total of 36 donor male Sprague-Dawley rats weighing 250–350 grams (corresponding to 59–70 days old) (Harlan, Indianapolis, IN). After Isoflurane induction, rats were euthanized with an overdose of sodium pentobarbital. Sciatic nerves were aseptically excised, cleared of peripheral fat and connective tissue and decellularized.

The human motor nerves (thoracodorsal and long thoracic nerves) were obtained from one 61-year old fresh male human cadaver. Subjects with a history of a condition that affects the peripheral nervous system were excluded. All segments were decellularized using the protocol described by Hundepool et al.¹⁹ Briefly, the samples were treated with different detergents including Triton X- 200, sulfobetaine-16, and sulfobetaine-10 and enzymatic solutions. Finally, the nerves were sterilized using γ -radiation and stored at 4°C for a maximum of 14 days before seeding. All chemicals were purchased from Sigma (St. Louis, MO, USA) and all solutions were autoclaved or filter-sterilized before use.

Mesenchymal stem cells

Rat MSCs were obtained from isogenic male Lewis rats weighing 250–350 grams (corresponding to 59–70 days old), as previously described by Kingham et al.²⁰ Sprague-Dawley donor nerves were seeded with Lewis rat MSCs to obtain a major histocompatibility complex mismatch, similar to the human allografts.²¹ After euthanasia, the inguinal fat pad was carefully dissected and minced using a sterile razor blade. The tissue was enzymatically digested for 2h at 37°C using 0.15% collagenase type 1 (Worthington, Biochemicals, Lakewood, NJ). The solution was passed through a 70- μm filter to remove undissociated tissue, neutralized by adding modified Eagle's medium (α -MEM; Invitrogen, UK) containing 10% fetal bovine serum and centrifuged at $800 \times g$ for 5 min. The stromal cell

pellet was re-suspended in advanced Minimum Essential Medium (MEM) (Life Technologies, Grand Island, NY) containing 5% Platelet Lysate (PLTMax; Mill-CreekLifeSciences, Rochester, MN), 2 mmol/L Glutamax (Life Technologies, Grand Island, NY), 2 U/ml heparin and 1% penicillin/streptomycin solution (Cellgro, Coming, NY). The cultures were maintained at subconfluent levels in a 37 °C incubator with 5% CO₂ and passaged with TrypLE (Invitrogen, UK). The multi-potency of the rat MSCs was demonstrated by differentiation into osteogenic and adipogenic lineages.

Human MSCs were isolated from abdominal lipo-aspirates from a representative 41-year-old male donor with written individual consent and institutional approval. Cell isolation and culture conditions have previously been described.^{22–24} These cells have been routinely used at our institute for clinical trial applications and have been extensively tested for cell surface markers, RNA-seq transcriptome profiles, and multi-lineage potential.^{8,22,25} All MSCs used in this study were of passage 5.

Seeding technique

The Lewis rat MSCs were then seeded onto the Sprague Dawley decellularized rat allografts at different durations of incubation (t=6, 12, 24 and 72 hours). The human MSCs were seeded onto the decellularized human allografts at one timepoint (t=12 hours). This technique involves a 9-step process as depicted in Figure 1 and described in detail in the appendix, Supplemental Digital Content 1, [INSERT HYPER LINK](#).

). An overview of the 9 steps is as follows:

1. Nerve allograft harvest
2. Nerve decellularization¹⁹
3. Cell culture and proliferation according to cell type.
4. Removal of harmful remnants of decellularization process by placing nerve grafts in cell culture medium and soak for 2 hours.
5. Combine nerves with the cells in a 15 mL TubeSpin® Bioreactor tube containing 10 ml cell culture medium and 1 million cells/nerve, with a maximum of 4 nerve segments/tube.
6. Place tubes horizontally in a bioreactor rotator system (Revolver™ Labnet, Edison, USA) and secure the tubes. Balance the tubes on the rotator (maximum of 6 tubes per side) with a rotation axis of 30°.
7. Turn rotator on at fixed speed 18 rpm to remove the gravitational component and place the rotator in an incubator at 37 degree Celsius (98.6 Fahrenheit) and 5% CO₂.
8. Incubate for 12 hours.
9. Place seeded nerves in a culture dish after 12 hours of bioreactor seeding.

Cell viability

MTS-assay—To determine the influence of the graft and possible remnants of the decellularization process and seeding strategy on the viability of the MSCs, MTS assays (Aqueous One Cell Proliferation Assay, Promega, Madison, USA) were performed on MSC seeded nerves according to the manufacturer's instructions. The wells with the nerve samples were pHEMA (Poly(2-hydroxyethyl methacrylate)) coated to prevent migration of cells to the well. Wells filled with medium and MTS reagent only served as negative control and for subtraction of the background signal. Wells incubated with MSCs served as positive control. MTS assays were performed in triplicate for each group at multiple time points after incubation (t= 1, 2, 3 and 7 days). Results were analyzed using a microplate reader (SpectraMax Plus 384, Molecular Devices, Sunnyvale, USA) at an absorbance wavelength of 490 nm.

Seeding efficiency, cell density and distribution

Scanning Electron Microscopy (SEM)—Seeded samples (n=1 per time point) were fixed in 2% Trump's fixative solution (37% formaldehyde and 25% glutaraldehyde) and processed for SEM. Samples were rinsed, fixed and desiccated with (i) PBS (2×), (ii) water (2×), (iii) 10% ethanol, (iv) 30% ethanol, (v) 50% ethanol, (vi) 70% ethanol, (vii) 90% ethanol, (viii) 95% ethanol, and (ix) 100% ethanol (2×). Samples were then placed in a critical point dryer while still in 100% ethanol and subsequently attached to an aluminum stub by double-sided conductive tape. The final preparation included sputter-coating for 90 seconds with gold-palladium. Images of the surface of the graft were obtained at 3 different magnifications (150x, 500x and 1800x)(Hitachi S-4700 field emission SEM). Seeding efficiency, determined as the percentage of graft coverage, and cell morphology were observed by one investigator. To obtain inter-rater and intra-rater reliability of the seeding efficiency, results were compared with pilot data from two independent investigators at two different points in time.

Live/Dead stain—After incubation at 37°C for 6, 12, 24 and 72 hours, seeded samples (n=3 per time point) were stained using a standard LIVE/DEAD® Cell Viability Assay (Invitrogen, Life Technologies, Grand Island, USA) following manufacturer's instructions. Live cells were identified by the incorporation of the membrane permeable Calcein AM stain within a cell, whereas dead cells were identified by the binding of Ethidium homodimer-1 to the nucleic acids of cells with damaged plasma membranes. The cells were visualized using confocal microscopy (Zeiss LSM 780; Zeiss).

Hoechst stain—Following the Live/Dead stain, seeded samples (n=2 per time point) were Hoechst 33342 stained (Invitrogen, Life Technologies, Grand Island, USA). The seeded grafts were longitudinal visualized using confocal microscopy (Zeiss LSM 780; Zeiss). Subsequently, to evaluate migration into the graft, samples (n=1 per time point) were suspended in OCT (Tissue-Tek, Sakura, Torrance, USA) and snap frozen. 15-µm cross sections of different levels of the nerve were evaluated for the presence of cell nuclei (Hoechst 33342 stained) to determine cell penetration into the graft.

Image analysis

All images were analyzed using Image J (NIH, Bethesda, USA). For SEM images, to determine the seeding efficiency, images were converted to 8-bit and the “MaxEntropy” automatic thresholding method was applied. “MaxEntropy” used the mean of grey levels as the threshold.²⁶ The fraction below the threshold (the part of the graft not covered by cells) was then calculated using the “Measure Area Fraction” function and was calculated by subtracting this fraction 100%. In the Live/Dead stained samples, the number of live and dead cells were determined per microscopic field. The number of positive Hoechst stained cells (nuclei) were manually counted. All Hoechst stained samples were also post-fixed with 10% Neutral Buffered Formalin (Thermo Fisher Scientific, Massachusetts), wrapped in foil and stored at 4°. After the final time-point all nerves were transferred to a 48 well plate and fluorescence was quantified using a microplate reader (SpectraMax Plus 384, Molecular Devices, Sunnyvale, USA) at an absorbance wavelength of 340/460 nm.

Statistical analysis

Cell counts were performed at least three times and results were expressed as the mean $\pm$ SD. The data was analyzed using the one-way analysis of variance (ANOVA) with Bonferoni's *post hoc* correction for multiple comparisons. For non-normally distributed data the Kruskal-Wallis test was used. To assess the validity and reliability of these measurements, the inter-rater and intra-rater reliability of this method was calculated using the intraclass correlation coefficient (ICC). A value of $p < 0.05$ was considered statistically significant.

RESULTS

Cell viability

Results from MTS assays demonstrate that all cells have robust mitochondrial metabolic activity at all time points as is expected from actively proliferating cells. MTS absorbance increases between days 1 (average 0.65 ± 0.33) and 3 (1.27 ± 0.06), while decreasing between days 3 and 7 (0.82 ± 0.13), consistent with a more active proliferative phase during the first few days when MSCs settle on the decellularized construct.

Cell density, distribution and seeding efficiency

Scanning Electron Microscopy—All following results are based on rat-tissue. To evaluate cell morphology and coverage of cells seeded on the graft, SEM images were made. Manual quantification of the cell distribution was not possible, as the cells formed aggregates on the graft. MSCs attaching to the decellularized nerve allograft formed flat (layered) sheets. Figure 2 depicts the seeded nerve allografts at the four time points and at 3 different magnifications (300, 100 and 30 μ m). The cells appeared evenly distributed throughout the nerve grafts and the number of cells/percentage of coverage gradually increased. Cell morphology was consistent throughout the time course of the experiment. Objective fraction analysis of the grey levels (seeding efficiency) indicates that there is a significant ($p = 0.004$) mean increase of 79.5% at $t=6h$ to, the longer seeding durations, 89.2% at $t=12h$, 88.1% at $t=24h$ and 89.7% at $t=72h$ (figure 3).

Live/Dead stain—Cells remained viable on the nerve allograft at all time points. Figure 4 shows Live/Dead staining of MSCs on nerve allografts at the 4 different time points. From 6 to 12 hours in culture, the live cell count showed a 2-fold increase in cell attachment from 200 to 395 cells per microscopic field, while from 12 to 24 hours cell counts only marginally increased to 453 cells per microscopic field. Time point 72h showed a decreased cell count of 225 cells per field. However, these differences in cell number were not significant ($p=0.620$ and $p=0.826$) (Figure 5). At 12h, the nerve allograft was completely covered indicating proliferation of cells on the graft, while only a few dead cells were seen.

Hoechst stain—Figure 6 depicts nuclei of cells stained with Hoechst dye and figure 7 presents the corresponding manual cell count. An increase in cell numbers was seen between time points 6 and 12 hours. A 12-hour seeding duration exhibited greater cell attachment and proliferation when compared to 6 hours. No striking differences were found between 12 and 24 hours of seeding and a decrease was noted between 24 and 72 hours. None of the differences were significant ($p=0.198$).

To control for manual counting errors, fluorescence intensity was also analyzed using a microplate reader after fixation of the cells (Figure 8). The fluorescence data show trends in cell proliferation during the first 24h that are similar to those observed for manual cell counts, but differences in cell counts over time were not significant ($p=0.392$).

MSCs did not migrate on a large scale but some positive staining was seen in the center of the graft, indicating that MSCs are dynamically seeded with a bioreactor can penetrate into the graft (figure 9).

Technique reliability

Analysis of seeding efficiency of 3 different SEM images that were seeded for 12 hours, resulted in 90.2%, 88.4% and 87.6% and demonstrated a high intra-rater reliability ($r = 0.97$). Inter-rater reliability between two investigators was also high ($r = 0.92$).

DISCUSSION

Prior techniques used for stem cell seeding in peripheral nerve research have primarily used static-seeding methods, such as the injection method (table 1).^{4,27–34} Aside from the known disadvantages of static seeding, the process of (micro) injection can be traumatic to both stem cells and delicate intra-neural architecture and may lead to abnormal cell distribution. Additionally, possible aggregation of cells due to needle passage can result in clusters of cells in the vicinity of the injection point. Moreover, injecting cells into small acellular nerves can be difficult.^{35,36} An alternative to the static injection technique is desirable, in particular for seeding of decellularized nerve allografts as they are delicate and abundant in extracellular matrix proteins. In this study, we describe a novel and technically straightforward seeding method that effectively and consistently seeds acellular nerve allografts in a dynamic bioreactor.

In this study, results from MTS assays indicate that the seeding does not affect MSC metabolic activity. SEM images showed that the rotating motion of the bioreactor enabled

cells to distribute homogeneously over the graft and yielded a maximum seeding efficiency of 89.7%. This method also minimizes cell injury, as demonstrated by the Live/Dead stain. Furthermore, bioreactor based cell seeding is reproducible and not traumatic to intra-neural architecture.

A secondary goal of this study was to determine the optimal seeding duration. After 12 hours the surfaces of the allografts were entirely covered with cells, and a significantly greater cell attachment and proliferation (seeding efficiency) was exhibited compared to shorter seeding durations. No statistical differences were found between 12, 24 and 72 hours of seeding, which is consistent within all outcome measurements as well as previous observations showing that MSCs exhibit exponential growth in the first days of seeding.²⁵ Although not statistically significant, live cell counts showed a decrease at 72 hours of incubation. This is secondary to the death of cells that do not attach to the nerve graft; although the exact effect is unknown, these dead cells could potentially secrete harmful signals. Also, longer incubation times could theoretically increase cell damage from the bioreactors rotational forces. We therefore hypothesize no further decrease in cell counts after 72 hours of seeding when nerves are placed in a culture dish in the incubator after 12 hours of seeding in the bioreactor and cell media is changed after 72–96 hours.

Infiltration was studied by Hoechst stains of cross-sections and MSCs did not migrate on a large scale. The presence of flat sheets of cells occluding the superficial pores may have limited the ability of MSCs to penetrate the inner aspects of the grafts. In contrast, it has been hypothesized that longer culture periods (weeks) may allow cells to proliferate and migrate toward the center of the graft.³⁷ While mechanisms underlying the neurotrophic potential of MSCs remain largely unknown, it is postulated that the local production of growth factors promote neuronal survival, and help guide axons during regeneration.^{9,10} Although the transition from MSC to Schwann cell has been reported previously, other investigators have reported regeneration with few remaining MSCs and in the absence of Schwann cell-like differentiation, suggesting alternative mechanisms of support, such as enhancing host repair mechanisms.^{11,38,39} Growth factors could penetrate the graft by diffusion and therefore the role of MSC migrating into the nerve allograft may not be an important variable. Zhao et al. compared the effect of MSCs injected inside or outside an decellularized nerve graft and concluded that supplementing MSCs around nerve grafts was effective and did not destroy the graft while having the same effect on nerve regeneration as injecting MSCs inside the grafts.³¹ Another concern with numerous cells in the lumen of the graft is that the cells would block the nerve growth cone during regeneration.

One limitation of the study is the lack of direct comparison to other seeding methods. Static seeding techniques yield efficiencies of approximately 10–25% while dynamic techniques result in efficiencies, ranging from 60% to 90%.⁴⁰ Drawbacks of dynamic seeding strategies in general include prolonged seeding time in low-speed rotational systems and potential cell damage in high-speed rotational systems.¹⁷ Our proposed bioreactor strategy does take longer than the static injection method, but decreases the potential risk of damage to both the cells and the nerve graft while improving seeding efficiency. Furthermore, dynamic seeding protocols allow the clinician to evaluate the seeded graft prior to transplantation, and when used for research purposes, the cells can be labeled and tracked. Other limitations include

our small sample size and manual cell counts. The 3D structure of the nerves impedes automated cell counts and evaluation of infiltration of the cells was complicated by freeze and sectioning artifacts of the samples. These limitations notwithstanding, all outcome measurements were consistent and reproducible.

Leferink et al. and Tan et al. demonstrated the importance of seeding parameters.^{41,42} There is extensive literature, which have repeatedly found dynamic techniques to be superior to static techniques in terms of cell seeding homogeneity and cell density.^{43,44} Most of the older studies seeded synthetic conduits that do not have an inner matrix structure. The nerve allograft, however, is more vulnerable and may be more difficult to seed adequately. Jesuraj et al. demonstrated this in their study, the insertion of a 24-gauge needle in cold-preserved acellular nerve grafts resulted in tearing of the epineurium and this would allow the cells to escape the nerve graft.²⁹ With the proposed dynamic bioreactor seeding method, there is no risk of damaging of the cells or the graft, which makes it a more reliable method.

CONCLUSION

This study describes and validates a convenient and technically feasible method for effective seeding of cells onto decellularized nerve allografts using a dynamic bioreactor. This method can also be applied to seed other grafts or tubular structures. Cells are homogeneously distributed over the graft, this method also minimizes cell injury, is reproducible, is operator independent and is not traumatic to the graft or the stem cells. Twelve hours of seeding appears to be the optimal time to obtain a homogenous seeding. Utilization of a validated, reproducible and effective seeding technique permits consideration of rigorously testing of cell-enhanced allografts for a range of biological parameters during optimization for possible use of these hybrid grafts in clinical applications for nerve repair.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Seeding technique

This technique involves a 9-step process as depicted above and described in detail in the appendix, Supplemental Digital Content 1, [INSERT HYPER LINK](#). A) Nerve graft harvest, B) Nerve decellularization, C) Cell culture and isolation, D) Bioreactor seeding, E) Static storage before grafting. The seeding method, cell viability and effect of seeding duration were first evaluated on rat MSCs seeded onto rat nerve allografts. Secondly, the seeding method and cell viability were tested on human MSCs seeded onto human nerve allografts. Used with permission of Mayo Foundation for Medical Education and Research. All rights reserved. MAYO© 2016.

Figure 2. Scanning electron microscopy images of MSC seeded nerve graft

Scale bars = 300μm (150x), 100μm (500x) and 30μm (1800x). The MSC seeded nerve graft is depicted here in 3 different magnifications. Illustrating the cell morphology and coverage of the unseeded decellularized graft (t=0) and after 6, 12, 24 and 72 hours of seeding. The unseeded nerve at time-point 0 shows the longitudinal structure of the epineurium of the decellularized nerve. The later time-points show the attached cells as flat (layered) sheets covering (and possibly penetrating) the epineurium. Cell morphology was consistent throughout the time course of the experiment; cells appeared evenly distributed throughout the nerve grafts and the number of cells gradually increased.

Figure 3. Seeding efficiency

Seeding efficiency was determined as the percentage of graft coverage imaged with Scanning electron microscopy. Seeding efficiency increased significantly from 79.5% at 6 hours to 89.2% after 12 hours of seeding ($p = 0.004$). No significant differences were found between time points 12, 24 and 72 hours.

Figure 4. Live/Dead staining of MSC seeded allografts

Cellular viability and density is demonstrated after seeding the nerve allografts for A)6 hours, B)12 hours, C)24hours and D)72 hours. The cells were visualized using a confocal scanning microscopy at 40x magnification. Live cells were stained green and dead cells red.

Figure 5. Live/Dead cell count

Average manual live and dead cell count per microscopic field per seeding duration. Error bars represent the standard error of the mean.

Figure 6. Hoechst longitudinal images

Cell nuclei were Hoechst stained after seeding the nerve allografts for A)6 hours, B)12 hours, C)24hours and D)72 hours. The cells were visualized using a confocal scanning microscopy at 20x magnification.

Figure 7. Hoechst cell count

Shows the average manual Hoechst cell count per microscopic field per seeding duration. Error bars represent the standard deviation or standard error of the mean as indicated.

Figure 8. Hoechst fluorescence intensity
Demonstrates the differences in fluorescence intensity between different seeding durations as measured using a microplate reader.

Figure 9. Hoechst cross-sectional images

A frozen cross-section is depicted after 12 hours of seeding and imaged at 5x magnification (inset = 10x). Samples of later time-points contained freeze and sectioning artifacts and are therefore not depicted.

Table 1

Stem cell delivery methods in peripheral nerve literature.

Author (year)	Seeding method	Application	Cell source	Concentration (cells/ml)
di Summa (2010)	Injected into	Fibrin nerve conduits	Schwann cells	2×10^6
Zhang (2010)	Micro-injection	Xenogeneic acellular nerve matrix	AMSCs	5×10^6
Lui (2011)	Micro-Injection	Acellular nerve allografts	AMSCs	2×10^6
Jesuraj (2011)	Injection	Acellular nerve graft	Schwann cells	1×10^6
Nijhuis (2013)	Injection	Vein conduit	BMSC	3×10^6
Zhao (2014)	In fibrin glue (injected around nerve)	Acellular nerve	BMSC	5×10^5
Ozan (2016)	Injection	Autograft	AMSCs	1×10^6
Klein (2016)	Static onto	Type 1 collagen conduit	AMSCs	2×10^6
Sowa (2016)	Infused into lumen	Gelatin hydrogel	AMSCs	1×10^4