A High-Throughput Mouse Platform for Nerve Conduit Assessment

Suresh Mohan, MD[1]; Wenjin Wang MD, PhD[1]; Ivan Coto Hernández, PhD[1]; Kaiyang Yin, MS[2]; Tessa Hadlock, MD[1]; Cathryn Sundback, ScD[3]; Ulrike G.K. Wegst, PhD[2]; Nate Jowett, MD[1]

[1]Surgical Photonics & Engineering Laboratory, Department of Otolaryngology, Massachusetts Eye and Ear Infirmary, Harvard Medical School, Boston, MA, USA.

[2]Thayer School of Engineering, Dartmouth College, Hanover, NH, USA.

[3]Center for Regenerative Medicine, Department of Surgery, Massachusetts General Hospital, Harvard Medical School, Boston, MA, USA.

ABSTRACT

INTRODUCTION

Conventional histologic assessment techniques of neural regeneration such as toluidine blue staining or immunohistochemistry for neural markers are expensive, laborious, time-intensive, and can involve hazardous chemicals. Additionally, subjectivity is a concern as distinguishing nerve from surrounding tissue can be problematic in the distal segment of regenerating nerve. Transgenic murine models exist that enable stain-free axon counts, and these reporter models can be combined with the use of modern microscopy techniques to accelerate and streamline the testing of peripheral nerve conduits.

METHODS

A rapid, non-toxic, and stain-free frozen section protocol suitable for assessment of neural regeneration by brightfield and confocal laser scanning fluorescent microscopy was developed. Interposition repair of a sciatic nerve defect in Thy1.2 YFP-16 mice was performed using various types of bioengineered neural conduits, and regeneration assessed at six weeks.

RESULTS

Processing time for axon counting was shortened from two weeks to three days, and stain costs eliminated. Confocal fluorescent microscopy images revealed excellent morphology of regenerating axons, with clear elucidation of permissive vs. repulsive conduit environments.

CONCLUSIONS

A rapid and cost-efficient platform for assessment of neural regeneration suitable for testing of novel neural conduit designs has been described.

CONTACT

Suresh Mohan, MD
Massachusetts Eye and Ear Infirmary
Harvard Medical School, Boston, MA, USA
Email: suresh_mohan@meei.harvard.edu

INTRODUCTION

- Histologic assessment is fundamental to the study of nerve regeneration.
- Hematoxylin and eosin (H&E) and Masson's trichrome stains do not adequately label myelin and demonstrate nonspecific extracellular matrix staining.[1,2]
- Conventional techniques for nerve histomorphometry include osmium tetroxide (OsO_4) post-fixation and toluidine blue staining (TB) of resin-embedded sections, or immunohistochemistry (IHC)/ immunofluorescence (IF) of paraffin or frozen sections.[1-3]
- Conventional methods are labor-intensive, expensive, involve hazardous agents, and provide images that may be difficult to interpret and quantify.
- Transgenic fluorescent reporter models exist to enable stain-free imaging of axons (e.g. Thy1-YFP mouse, Thy1-GFP rat[4])
- Frozen section techniques may be used for rapid processing of transgenic nerve, with visualization using wide-field or scanning fluorescent microscopy.

METHODS

Subjects and Surgery

- Five adult female B6.Cg-Tg(Thy1-YFP)16Jrs/J mice used
- Proprietary conduits designed with a chitosan-based shell and collagen core obtained from the Dartmouth School of Engineering. [5]
- Animals underwent 15 minute surgery under isoflurane with appropriate peri-operative analgesics.
- Left hindleg shaved, incised, and dissected to find the sciatic nerve **(Fig. 1A-B)**.
- Sciatic n. transected, and 7mm conduit placed **(Fig. 1C)**.
- Single suture repair performed at both ends with 10'0 nylon.
- Conduit-nerve complexes harvested at 6 weeks after perfusion.

Histology

- Post-fixation in 2% PFA, 30% Sucrose dehydration
- Snap frozen in OCT with liquid nitrogen
- Samples transected in half lengthwise, followed by longitudinal cryosectioning of the proximal end and axial sectioning of the distal end into 20 µm sections.
- Fluoromyelin staining (1:300) per protocol.

Imaging

- Leica SP8 Confocal Microscopy at 40x magnification
- Z-stacks displayed as maximum projections
- Contrast enhancements in Fiji ImageJ software.[6]

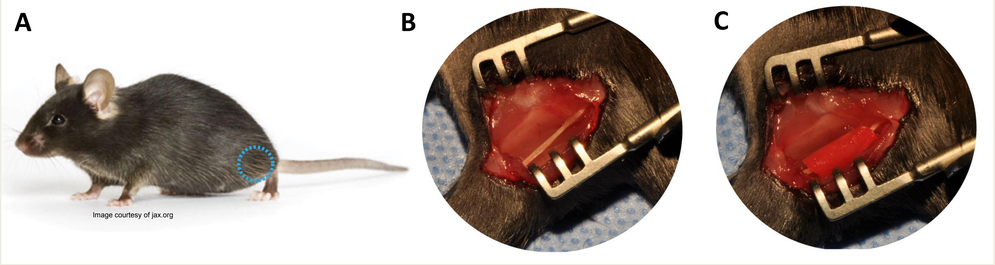


Figure 1. (A) Schematic of Thy1-YFP mice used for sciatic nerve transection. Intraoperative photos of (B) Left sciatic nerve exposed, and (C) Post-transection and conduit placement.

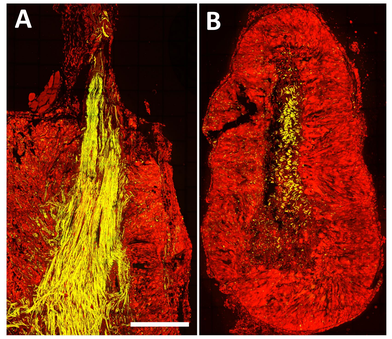
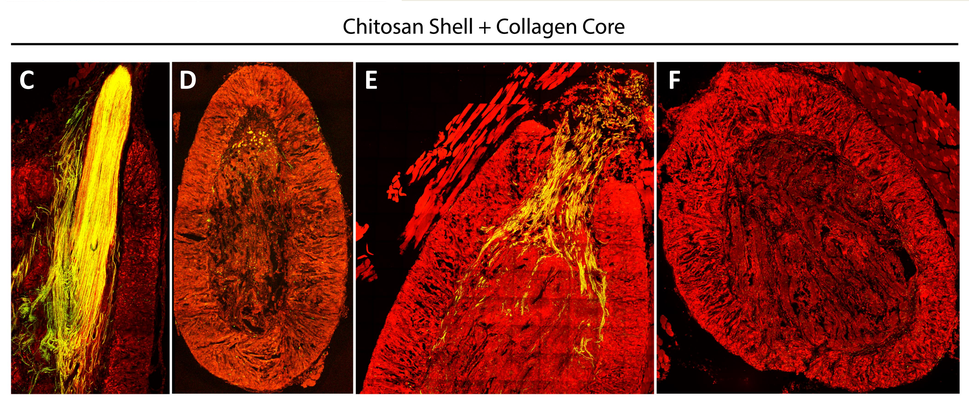


Figure 2. (A), (C), (E) Proximal longitudinal sections of conduits. (B), (D), (F) Distal axial sections. Permissive nature of chitosan shell-only conduit seen as axons traverse through conduit in longitudinal section (2A), with evidence of axonal penetration through to distal segment (2B). Conversely, a growth-restrictive environment is seen in shell + core conduits (2C-F). One model of growth restriction shows a 180 degree reversal of growth out of the conduit (2C), reflected by sparse axonal penetration into the distal conduit (2D). An alternate resistive pattern shows evasion of the core with splaying of axons around the periphery (2E), corroborated by nearly no axons seen at the distal conduit (2F). Fluoromyelin Red was seen to not only stain myelin (not shown), but act as an effective counterstain for labelling of the conduit shell and core. YFP (Axons) – yellow, Fluoromyelin Red (Background) – Red. Scale bar, 500 µm.



RESULTS AND DISCUSSION

- Processing time for axonal assessment was shortened from 2 weeks to three days, and stain costs eliminated.
- Excellent qualitative morphological detail of axonal regeneration patterns obtained (**Fig. 2**).
- Due to their tendency towards robust neural regeneration, use of rodents to study nerve conduit performance has been called into question.[7]
- Herein we demonstrate that discrimination between permissive and repulsive conduit environments may be clearly visualized in a transgenic mouse model
- Histological assessment is a critical adjunct to functional assessment of conduit performance in rodent models as axons may grow around a conduit to re-innervate an end organ
- The present model is more efficient than conventional techniques using rats in that:
  - Multiple conduit designs can be feasibly assessed in a short time frame and with limited resources
  - Conduit performance can be assessed as early as 3-4 weeks after implant
  - Smaller footprint (5 mice per cage)
  - Less labor-intensive tissue processing
  - Leg autotomy rate in mice is low, minimizing subject loss
  - Quantitative analysis of axon regeneration is facilitated by label expression only within viable axons

CONCLUSIONS

A rapid and cost-efficient platform for assessment of neural regeneration suitable for testing of novel neural conduit designs has been described.

REFERENCES

1. Carriel V, Garzon I, Alaminos M, et al. Evaluation of myelin sheath and collagen reorganization pattern in a model of peripheral nerve regeneration using an integrated histochemical approach. Histochem Cell Biol 2011;136:709-17.
2. Raimondo S, Fornaro M, Di Scipio F, et al. Chapter 5: Methods and protocols in peripheral nerve regeneration experimental research: part II-morphological techniques. Int Rev Neurobiol 2009;87:81-103.
3. Hunter DA, Moradzadeh A, Whitlock EL, et al. Binary imaging analysis for comprehensive quantitative histomorphometry of peripheral nerve. J Neurosci Methods 2007;166:116-24.
4. Magill CK, Moore AM, Borschel GH, et al. A new model for facial nerve research: the novel transgenic Thy1-GFP rat. Arch Facial Plast Surg 2010;12:315-20.
5. Yin K, Divakar P, Hong J, et al. Freeze-cast Porous Chitosan Conduit for Peripheral Nerve Repair. MRS Advances 2018. (Accepted)
6. Schindelin J, Arganda-Carreras I, Frise E, et al. Fiji: an open-source platform for biological-image analysis. Nat Methods 2012;9:676-82.
7. Kaplan HM, Mishra P, Kohn J. The overwhelming use of rat models in nerve regeneration research may compromise designs of nerve guidance conduits for humans. J Mater Sci Mater Med 2015;26:226.