Development of a miniaturized bioreactor for neural culture and axon stretch growth

Qi Xu, Fang Chen, Yuanyuan Wang, Xiao Li and Jiping He, *Senior Member, IEEE*

***Abstract* — In this paper, a miniaturized bioreactor for accelerating nerve growth is developed to investigate potentially bi-directional peripheral neural interface based on tissue engineering. The bioreactor consisting of two parallel chambers that are extended to conduct controlled trials for optimized neuronal culture. The chamber is used to improve the micro-environment of in vitro nerve regeneration in a CO_2 incubator, whereas the process of axon stretch growth is performed by a computer controlled micro-motion system comprised of a linear motion table and a micro-stepper motor. This apparatus produces axon stretch growth by taking 1μm step every 1 minute over 1000 iterations for 1mm elongation growth per day. Results show that axons from neonatal DRG explants are grown with unidirectional polarity with a robust regeneration over 5 days in the culture chamber, whereas micro-displacement measurement indicates a satisfactory accuracy and repeatability for the implementation of computer-controlled ASG.**

Keywords—*Axon Growth Stretch; Bioreactor; Dorsal Root Ganglia Explant; Micro-motion System; Neural Culture*

I. Introduction

Researchers have been seeking methods to repair motor function for people who suffer from paralysis caused by spinal cord injury or who lost their limbs due to amputation. Particularly, major emphasis has focused on designing structures of artificial limbs with regenerated nerves and electrical wires so that these prostheses can be integrated with human body as if they were natural limbs. Accordingly, a common ideal for nervous system repair is to rebuild new neural pathways to restore the motion function based on tissue engineering.

Although targeted muscle reinnervation (TMR) provided an insight into the mechanism of neural plasticity and peripheral regeneration in humans, there are no efficient interventions for the recovery of valuable sensorimotor functions in severely paralyzed humans [1]. Recently, peripheral nerve interface (PNI) allowing the brain to control limb's movements and feel its presence has been studied [2]. Notwithstanding neurons from dorsal root ganglion (DRG) were dissociated from both embryonic and adult animals and survived well in culture medium and followed transplantation, a large number of DRGs appeared to present very irregular axon growth [3]. For this reason, some 3-D culture technologies including tailored porosity and nutrient gradient have been developed to guide neural growth direction depending on the characteristics of fetal nerve growth cone [4-5].

In order to establish PNI-based bi-directional communication between the nervous system and external devices, a challenge is particularly acute that the axons would have to grow an enormous length to create a bridge in an environment that is normally non-permissive for axon growth. In recent years, there is evidence suggesting that cell growth is induced by mechanical force. The remarkable ability of integrated central nervous system (CNS) axons had been demonstrated to grow longer distances in response to continuous mechanical tension [6]. Indeed, it has been suggested that synapsed CNS axons are able to exhibit sustained "stretch-induced growth" representing an important growth mechanism, which is in accordance with the results from other groups [7-8]. However, understanding the mechanisms of axon guidance and nerve regeneration has been one of the main priorities in the field of neuroscience. Different from growth cone extension, axon stretch growth (ASG) has been explored previously as a tissue engineering method to enable sustained growth rates of 1cm per day [9]. A standard mechanical cell system driven by an air compressor, has been commercialized to enable cell culturing under the mechanical stress. In disposable stretch chambers made with silicone, the cultured cells were subjected to slow stretching and rapid relaxation repeatedly [10].

In order to explore the application of ASG to product large living nerve conducts for PNI, an engineered bioreactor must accommodate continual elongation of the axon to adapt the increasing separation between the target and the soma [11]. In this paper, we sought to develop a miniaturized bioreactor with multiple chambers to enable neural culture and ASG allowing quantification examinations of the process by real time imaging. The ASG bioreactor consists of compatible bioreactor chambers with lanes (elongated wells), where neurons are cultured and stretched for several days, a linear motion table to apply tensile forces and a step motor drive controller with software to control microdisplacements [8].

II. Materials and Methods

A. Conceptual Framework of Tissue Engineering-Based Peripheral Neural Interface

As shown in Figure.1, the investigation of tissue engineering-based PNI includes neural culture enhancing

Research was supported by National Natural Science Foundation of China 61233015 and in part by the Fundamental Research Funds for the Central Universities (HUST: 2013YGYL004)

Qi Xu, Fang Chen, Yuanyuan Wang and Xiao Li are all with the Neural Interface & Rehabilitation Technology Research Center, Huazhong University of Science and Technology, Wuhan, P.R. China.(phone: +86-27-87557284; fax: +86-27-87543130; e-mail: xuqi@mail.hust.edu.cn)

Jiping He is with the Center for Neural Interface Design, Harrington Department of Bioengineering, Arizona State University, Tempe, AZ 85287, USA. (e-mail: jiping.he@asu.edu).

978-1-4244-7929-0/14/$26.00 ©2014 IEEE

regenerated axons from DRG, ASG enhancing axon regeneration at a rate far exceeding that of growth cone extension, and neural recording and stimulation based array electrode to provide bidirectional communication between the nervous system and the external devices. Firstly, neural culture was performed on DRG explants in compatible chambers simultaneously. Once regenerated axons bridges the gap of two populations of DRG explants located separately in the towing and stationary substrates, stretch is initiated by a linear micro-motion system for several days. In order to determine the nerve conduction across axon tract, array electrode is used to stimulate the tract and then record evoked potentials from the regenerated nerve. On the other hand, array electrode could record motor signals from the nerve to command the external devices and exert electrical stimulation on sensory axons to provide sensory feedback from artificial sensors for constructing bidirectional PNI between the subject and the external devices.

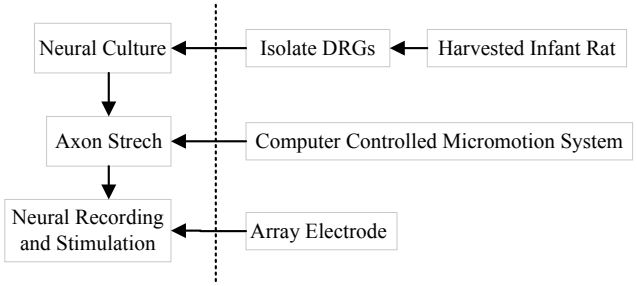


Figure 1. Concept of tissue engineering-based PNI

B. Principles of ASG operation

The ASG bioreactor was engineered to gradually apply tension to axon bundles spanning two separate substrates, i.e. towing and stationary substrates. A flexible strip of Aclar film was positioned at the bottom of the culture chamber and served as the stationary substrate, whereas the towing substrate laid underneath the moving population of cells. Generally, plated neurons extended axons from the towing substrate onto the stationary substrate via growth cone extension for several days. Then the towing substrate was pulled using a micromotion system so that the spanning axons were subjected to a gradual stretch. From a short distance between the soma and growth cones, the axons were stretched and grown into fasciculate unidirectional axon tracts with several centimeters in length.

C. Fabrication of Culture Chamber

An individual culture chamber was comprised of a stretching frame that forms a lane; an adjustable towing block that manipulates cells across the lane; a projected towing rod for external manipulation (Figure 2). A disposable stationary substrate was affixed to the bottom of the stretching frame and spanned the lane. Neural cultures were plated onto disposable towing substrates held rigidly by the towing block. Both the stationary and towing substrates were made from fluoropolymer films with moisture barrier (Aclar® 33C, Electron Microscopy Sciences Inc., Hatfield, PA, USA).

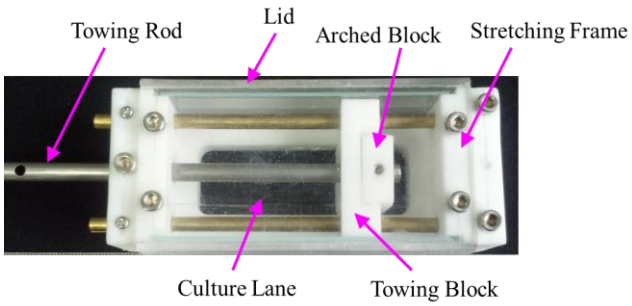


Figure 2. Prototype of the bioreactor culture chamber with stretch grown axons within the culture lanes observed by inverted microscope.

Briefly, fabrication of culture chamber prototype was performed in a machine factory. The manufactured culture chamber was 10cm in length, 4cm in width, and 3cm in height. The internal components, such as the stretching frame and towing block, were all machined from 3/8" polytetrafluoroethylene (PTFE) allowing for biocompatibility, sterilization and durability. The transparent plexiglass lid is used for light microscopy and viewing of cultures. Especially, at least 1-2mm gaps existed between the lid and two glass slides separately located on either side of the chamber. Fasteners and hardware (towing rod, guide rods, and screws) were purchased and fabricated from 304 stainless steel.

The arched block held rigidly the towing substrate and enabled stretch manipulation to achieve the desired axon elongation. Adjustment screws allowed the arched block to be independently lowered to even contact the stationary substrate. Indeed, the culture chamber was put in a CO_2 incubator (MCO-15AC, Sanyo Electric Co., Tokyo, Japan) at 37°C with 5% CO_2, whereas the gas vents were machined on each side of the chamber to provide required environment similar to petri dish for neural culture and ASG experiments.

D. Computer Controlled Micro-motion System

As shown in Figure.3, two compatible culture chambers for simultaneous experiments were docked to a computer controlled micromotion system consisting of a micro-stepper motor (HT23-397, Applied Motion Products Inc., Watsonville, CA, USA) controlled by a programmable motor indexer (Si2035, Applied Motion Products Inc., Watsonville, CA, USA) and a linear motion table (MLPS_3-10, Servo Systems Co., Montville, NJ, USA). An acrylonitrile-butadiene-styrene (ABS) polymer chassis was fabricated to hold the culture chambers and linear motion table. Two culture chambers were seated within the chassis underlying the linear motion table, whereas the towing rods extending from the culture chambers were fastened to the table using an ABS adaptor. Aside from the motor indexer, the bioreactor was located in a CO_2 incubator during neural culture and ASG experiments.

Axon stretch was carried out in a stepwise fashion by taking a number of displacement steps spaced by dwell times. The programmable pulse trains were output from the motor indexer to micro-stepper motor according to the preset displacement of towing substrate.

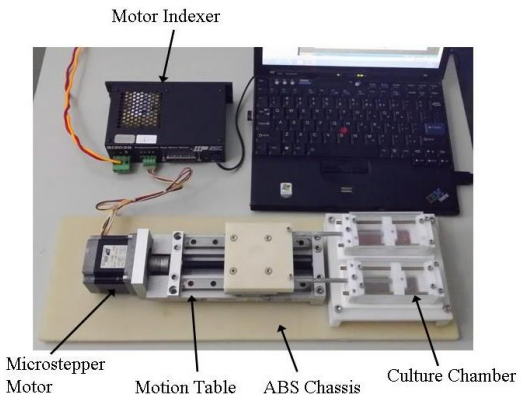


Figure 3. Axon stretch growth bioreactor system. Two compatible culture chambers were located in ABS chassis underneath the linear motion table.

The programmed parameters of the micro-stepper motor included the number of motor steps corresponding to displacement step, the number of stretching iterations, and the dwell time between two stretching iterations. Experiments were initiated to take 1μm step every 1 minute over 1000 iterations, resulting in more than 1mm stretch per day. This computer-controlled micro-motion system provided the ability to program customized profiles for ASG, and adapted the stretch rate to the axon elongation by programming displacement step or dwell time for continuous experimentation over several days or weeks.

E. Micro-displacement Measurement

Prior to axon stretching, the computer-controlled micro-motion system should be calibrated for accuracy and repeatability. A dual-frequency laser interferometer RenishawXL-80 (Canadian Measurement-Metrology Inc., Ontario, CA) with the resolution up to one nanometer was used to calibrate the accuracy of linear motion table. As shown in Figure.4, a test platform was established to determine the motion deviation of the towing block in the bioreactor from the preset displacement.

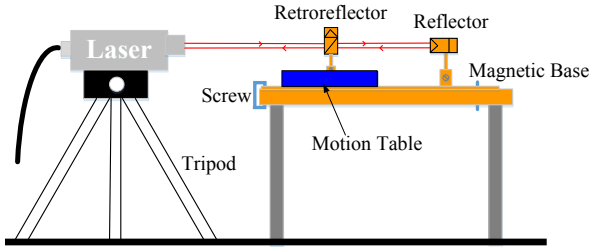


Figure 4. Schematic diagram of test platform for calibrating the micromotion system based on RenishawXL-80 laser interferometer.

The light beam from laser was divided into two beams by a splitter in the retroreflector. Then one divided beam reflected by the retroreflector tracking the towing block as the signal beam, whereas the other one traveled towards the reflector and then reflected back to the splitter as a reference beam. Based on the interference of these two beams, the micro-displacements of motion table were measured by counting the phase difference between the reference and signal beams [12]. In order to calibrate the micromotion table, the computer-controlled system was programmed to take 1μm step every 6 seconds over 500 iterations for ten cycles. Then ten groups of measured displacements were analyzed by MATLAB software (MathWorks Inc., Natick, MA, USA).

F. Preparation of Bioreactor Chamber

The towing substrate was cut from 8.5" x 11" sheet of Aclar film (33C 50μm, Electron Microscopy Sciences Inc., Hatfield, PA, USA). A sharp blade was used to cut the substrates to slightly shorter than the lane width of 20mm to allow 1-2mm clearance on both sides. The towing substrate was lightly sanded on either side using 1000-grit waterproof sandpaper (MATADOR, Germany) to facilitate axon growth from the towing substrate onto the stationary substrate. A 70mm×30mm strip was cut from Aclar film (33C 198μm, Electron Microscopy Sciences Inc., Hatfield, PA, USA) and served as the stationary substrate. The culture chamber and substrates were cleaned with laboratory detergent, rinsed thoroughly with purified dH_2O, and sterilized by 75% ethanol for 30 minutes under UV light on a super clean bench. The culture chamber and substrates were allowed to air dry within a sterile tissue culture hood.

Prior to each culture experiment, the miniaturized chamber was assembled from all substrates within the sterile hood. First, with the towing and arched blocks in their full upright positions, two sides of two blocks next to each other were coated with silicon (Windshield & glass sealer #81730, PERMATEX Inc., USA) using sterile cotton-tipped swabs, and then the non-sanded portion of towing substrate was inserted into the interspace between the arched and towing blocks so that the towing substrate was glued on the towing block. Next, the stationary substrate was attached to the bottom of the stretching frame in the bioreactor chamber, whereas excess silicon and air pockets were removed by slightly depressing a dry swab against the glued substrate. The stationary substrate was used for the attachment of growth cones and served as a window of viewing cultures. About an hour afterwards two glass slides were glued to both sides of the culture chamber. Since silicon leaches acetic acid possibly toxic to neurons, the culture chamber was left to dry under UV light within the sterile hood for at least 36 hours before conducting neural cultures..

G. Neural Culture

Experiments were performed using DRG neurons isolated from Sprague-Dawley infant rats. All animal protocols were approved by the Ethics Committee for animal research, Huazhong University of Science and Technology, China. At least 10-20 DRG explants were pipetted from the petri dish and then plated directly onto the edge of the sanded towing culture substrate by using a stereomicroscope. The culture lane was filled with DMEM medium (HyClone, Logan, UT) supplemented with 10% FBS (HyClone, Logan, UT), 50 μg/mL 2.5S nerve growth factor (R&G), 1% penicillin/streptomycin, and the mitotic inhibitors constituted of 10mM uridine (Sigma) and 10mM 5-fluoro-2-deoxyuridine (FdU). The cultures in the bioreactor chamber were viewed in real time by using the inverted microscope (Olympus CKX41, Olympus Inc.), and then recorded by a Nikon TE2000-S inverted microscope.

III. Results

A. Displacement Measurement

The computer-controlled micromotion system was programmed to take 1μm step every 6 seconds over 500 iterations for ten cycles. As shown in Table I, the mean of 1μm step measured by a laser interferometer was between 0.982μm and 1.005μm with maximal mean error of 0.017μm. The maximal standard deviation (STD) for ten cycles was 0.0269μm. The probability histogram of 5,000 data for measuring 1μm step was given in Figure.5, where the fitted curve of probability density was well agreement with normal distribution with the mean and STD of 0.992μm and 0.0028μm, respectively.

TABLE I. MEAN AND STANDARD DEVIATION OF REPEATED MEASURES ON ONE MICROMRTER STEP WITHIN TRAVEL RANGE OF 500 MICROMETERS

Cycle	1	2	3	4	5	6	7	8	9	10
Mean (μm)	0.982	0.986	0.977	0.994	0.991	0.988	0.999	1.005	0.999	0.994
STD (μm)	0.1838	0.0193	0.0223	0.0245	0.0217	0.0269	0.0174	0.0234	0.0168	0.0153

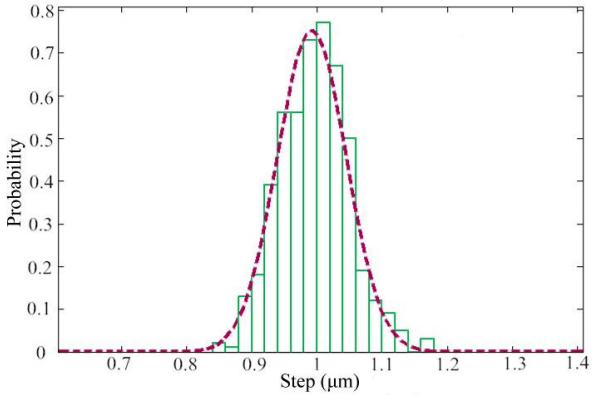
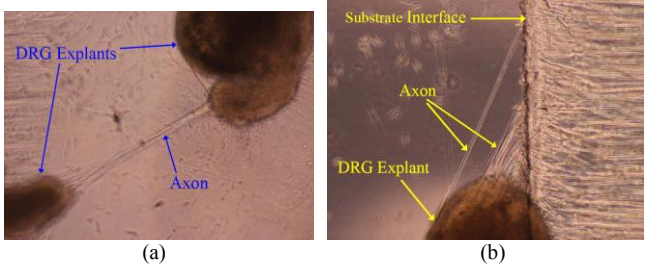


Figure 5. Probability histogram of step measures by taking 1μm step over 500 iterations for ten cycles.

B. Neural Culture

Fig.6a showed that two DRG explants plated on the towing substrate exhibited robust axon growth in the culture chamber over two days, whereas axons was grown from DRG explant on the stationary substrate via growth cone extension onto the sanded towing substrate with unidirectional axon polarity over five days (Fig.6b). It is seen that the bioreactor chamber is capable of supporting axon growth for further ASG experiments.



(a) (b)

Figure 6. Live imaging of axon growth via growth cone extension in the culture chamber. (a) Axons were grown from two DRG explants plated on the towing substrate over two days. (b) Regenerated axons from a DRG explant plated on the stationary substrate were adhered to the sanded towing substrate.

IV. Conclusion

We developed a new systems allowing for the exploration of peripheral nerve interface based on tissue engineering method. Moreover, the simultaneous application of multiple compatible chambers has great potential in controlled trials for optimized neuronal culture. The bioreactor chamber is fabricated allowing for biocompatibility and ease of sterilization, and then located in a commercial CO_2 incubator to ensure the required environment for neural culture. It is seen that the computer-controlled micro-motion table with high accuracy and repeatability can be used to implement custom axon stretch profiles. Future works include conducting ASG experiment and exploring array electrode for nerve stimulation and recording under careful consideration of being compatible with each other.

Acknowledgment

Authors wish to express sincere thanks to Ang Ke and Dr. Jun Ma for their help in the fabrication and neural culture.

References

[1] T. A. Kuiken, G. Li, B. A. Lock, R. D. Lipschutz, L. A. Miller, K. A. Stubblefield, and K. B. Englehart, "Targeted muscle reinnervation for real-time myoelectric control of multifunction artificial arms," *Journal of the American Medical Association,* vol.301, no.6, pp.619-628, Feb. 2009.

[2] D. K. Cullen and D. H. Smith, "Bionic connections," *Scientific American,* pp. 52-57, Jan. 2013

[3] D. H. Smith, "Stretch growth of integrated axon tracts: extremes and exploitations," *Progress in Neurobiology,* vol. 89, no. 3, pp. 231-239, Nov. 2009

[4] N. Li, Q. Zhang, S. Gao, Q. Song, R. Huang, L. Wang, L. Liu, J. Dai, M. Tang and G. Cheng, "Three-dimensional graphene foam as a biocompatible and conductive scaffold for neural stem cells," *Scientific Report*, 2013, 3:1604

[5] H. R. Irons, D. K. Cullen, N. P. Shapiro, N. A. Lambert, R. H. Lee and M. C. LaPlaca, "Three-dimensional neural constructs: a novel platform for neurophysiological investigation," *Journal of Neural Engineering,* vol. 5, no. 3, pp. 333-341, Sep. 2008

[6] D. H. Smith, J. A. Wolf, and D. F. Meaney, "A new strategy to produce sustained growth of central nervous system axons: continuous mechanical tension," *Tissue Engineering,* vol.7, no. 2, pp.131-139, Jul. 2001

[7] A. G. Robling, D. B. Burr, and C. H. Turner, "Partitioning a daily mechanical stimulus into discrete loading bouts improves the osteogenic response to loading," *Journal of Bone and Mineral Research*, vol. 15, no. 8, pp. 1596-1602, Aug. 2000

[8] P. Lamoureux, S. R. Heidemann, N. R. Martzke and K. E. Miller, "Growth and elongation within and along the axon," *Developmental neurobiology*, vol.70, pp. 135-149, Oct. 2010

[9] J. R. Loverde, R. E. Tolentino, and B. J. Pfister, "Axon stretch growth: the mechanotransduction of neuronal growth," *Journal of visualized experiments*, vol. 54, e2573, Aug. 2011

[10] http://menicon-lifescience.com/english/shellpa.html

[11] J. R. Loverde, V. C. Ozoka, R. Aquino, L. Lin, and B. J. Pfister, "Live imaging of axon stretch growth in embryonic and adult neurons," *Journal of Neurotrauma*, vol.28, no. 11, pp.2389-2403, Nov. 2011

[12] C. R. Steinmetz, "Sub-micron position measurement and control on precision machine tools with laser interferometry," *Precision Engineering,* vol.12, no.1, pp.12-24, Jan. 1990