Development of a three-dimensional cell culture system for the enhancement of nerve axonal extension by cyclic stretch stimulation

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Abstract

Significant progress has recently been made in the development of extracellular stimulation technology for the enhancement of nerve axonal extension and network generation and regeneration in three-dimensional (3D) bioreactors for neural tissue engineering. In this study, a 3D cell culture cell culture system was developed to accelerate the regeneration of axons using cyclic stretch stimulation. A modified collagen gel was used as a scaffold to mimic the extracellular matrices of the central nervous system in the human brain. First, a cyclic stretch stimulation cell culture system was designed and fabricated in order to load uniform strain onto a 3D culture. Pheochromocytoma (PC12) cells were then mixed with the collagen gel and poured into the stretch chamber of the cell culture system. The stretch stimulation cell culture system was then used to load the cyclic tensile strain against the PC12 cells embedded in the collagen gel, where an in-situ microscopic observation was performed. Second, cyclic stretch stimulations of the PC12 cells were performed, and the 3D morphologies of the cell bodies, neurites, and axons within the PC12 cells were observed using a multi photon microscope (MPM) system. We evaluated the effectiveness of the cyclic stretch stimulation on the axonal extension of nerves in a 3D cell culture system. Finally, we confirmed the enhancement of the axonal extension and determined the optimum tensile strain and the number of cyclic stimulations required to achieve the maximum axonal extension of the PC12 cells. Using these optimum conditions—2.3% strain, 1 Hz cycles, and $1.7 \times 10^5$ times—the cyclic stretch stimulation was performed on rat cerebral cortex cells, and the effectiveness of the enhancement was also confirmed in these cells.

Keywords: Neuron, Stretch stimulation, Strain, Three dimensional cell culture system, Axonal extension, Enhancement

1. Introduction

In this study, a three-dimensional (3D) cell culture system for nerve tissue regeneration is developed, in which cyclic stretch stimulation is used in an attempt to increase the axonal extension of a nerve. The central nervous system (CNS), which consists of the brain and the spinal cord and has a lower capacity for regeneration than the peripheral nervous system (PNS). Effective treatments for damage to the CNS have not yet been established. The synergic effects of extracellular inhibition from glial scarring and intracellular inhibition leads to a decrease in the ability of axons to grow during the regeneration of nerves. Recently, biochemical researchers have established the fundamental mechanism by which regeneration is inhibited because of glial scarring and discovered medicines that can help to prevent this (Li et al., 2012; Rodriguez et al., 2014; Hara et al., 2017). However, the intracellular mechanisms which lead to a decrease in the capacity for axons to re-grow has not yet been elucidated. It has been reported that both mechanical and electromagnetic stimulation can have a positive effect on axonal extension during nerve regeneration in cell cultures. In this study, we therefore focus on developing a new stimulation method by which the mediation of intracellular inhibition can be promoted in the artificial scaffold to enhance the axonal extension of nerve cells.
Many investigations have been carried out concerned with improving the axonal extension of nerves for CNS regeneration, including mechanical (Chang et al., 2010; Franze et al., 2009; Georges et al., 2006; Haq et al., 2006; Higgins et al., 2013; Nakamachi et al., 2018; Pfister et al., 2003; Rana et al., 2010; Shibasaki et al., 2010, 2017), electromagnetic (Blackman et al., 1996; Fan et al., 2004; Komazaki and Takanu, 2007; Jung et al., 2014; Nakamachi et al., 2019, 2020), and chemical (Fan et al., 2017; Wang et al., 2017; Ren et al., 2004; MacLennan et al., 1997; Ming, et al., 1997) stimulation. Mechanical stimulation was selected for this study, as it is possible that this type of stimulation could occur via motion caused by gravity, blood pressure, or the traction force of a growth cone. Cells can convert various mechanical stimulations into biochemical signals in vivo, which are then transmitted to the cells, promoting cell activity. It has been reported in previous studies that mechanical tension can induce axonal growth and that axonal tension is tightly regulated (Ahmad et al., 2015). Shibasaki et al. reported that the growth of neurites was enhanced via the activation of the TRPV2 channel, which senses physical stimulation on the surface of cell membranes. Stretch stimulation can also be initiated by the exercise therapy that is performed as treatment after nerve injury, and axonal extension could therefore occur as a result of exercise (Meeteren et al., 1997). In this study, we focused on the use of cyclic stretch stimulation in order to increase axonal extension. Haq et al. reported that the application of stretch stimulation (4% strain at 1 Hz) to PC12 cells increased the axonal extension of these cells (Haq et al., 2006). However, these studies were carried out using two-dimensional (2D) cultures such as sheets of cells. It is therefore necessary to construct a 3D cell culture system in order to verify the effectiveness of stretch stimulation for the enhancement of axonal extension under conditions that mimic the human nervous system. This study will contribute to promote an efficient regeneration of the damaged nerve network system and the transplanted nerve cells. Collagen gel was adopted as the scaffold for use in the cell culture system, into which PC12 cells were embedded for generation of the neural network. The collagen gel plays an important role in cellular generation, differentiation, and morphogenesis as it mimics the extra-cellular matrix (Baldwin et al., 1996). Collagen gel was therefore selected to construct the framework into which the PC12 cells were embedded in this study.

There are three main objectives in this study: (1) The design and fabrication of a cyclic stretch stimulation cell culture system for the 3D culture of nerve cells using collagen gel as a scaffold (2) Evaluation of the effectiveness of cyclic stretch stimulation on the axonal extension of PC12 cells, and the identification of optimum conditions under which axonal extension is increased. (3) Performing cyclic stretch stimulation using rat cerebral cortex cells and investigating the effectiveness of this method for enhancing the axonal extension in these cells.

2. Materials and Methods
2.1 Development of the 3D cell culture system for cyclic stretch stimulation

In previous studies, PC12 cells were seeded onto a silicone sheet and loaded with cyclic tensile strain (Haq et al., 2006; Nakamachi et al., 2018). However, in this study, we aim to reproduce an environment that more closely resembles the living human brain; therefore, a 3D culture was constructed using collagen gel as a scaffold. Previous studies have reported using collagen gel to load tensile strain onto embedded cells in a 3D cell culture system (Kinoshita et al., 2008). We adopted the same stimulation strategy by applying cyclic tensile strain onto PC12 cells embedded into collagen gel in the 3D cell culture system.

The cell culture system that was designed and fabricated for this study and the photo of fabricated system are shown in Fig. 1(a) and (b). Four stretch chambers were employed in this 3D cell culture system. The stretch chamber, which consists of the stainless steel pipes, PDMS frames (stretch chamber) and the collagen gel scaffolds as shown in Fig. 1, is immersed in the medium and covered with an acrylic box to prevent the contamination. The culture area maintains a sterile environment during the experimental period. The collagen gel with the embedded cells was injected into the stretch chambers, which were hydrophilized using oxygen plasma. Polydimethylsiloxane (PDMS: SILPOT 184W/C, DuPont Toray Specialty Materials K.K.) was used to construct the stretch chamber via molding with an acrylic die, as it is highly biocompatible and extendable. The edges of the collagen gel cubes were adhered to the walls of the PDMS stretch chambers. Both ends of the PDMS stretch chamber were fixed to the transfer jig, which converts the horizontal reciprocal motion in X1 direction into vertical reciprocal motion in X2 direction using a tapered slider, stainless steel frames and a stepping motor. The reciprocal vertical motion is applied to both of the fixed ends of the PDMS stretch chamber in order to generate the cyclic tensile strain. This enables observation of the fixed points at the central area of the collagen gel. It is possible to apply an arbitrary bilateral stretch stimulation by controlling the driving distance of the stepping motor using a PC controller. The stopper prevents the compression of the collagen gel in the X1 direction, as seen in Fig. 1.
The collagen gel, together with the embedded PC12 cells, was injected into the culture section of the PDMS stretch chamber, which was then immersed in culture solution. SUS316 was used to construct the transfer jig as it has high resistance to corrosion from the culture solution. In order to simplify the attachment and detachment of the PDMS stretch chamber, thin stainless-steel pipes with outer and inner diameters of 3.5 and 3.1 mm, respectively, were inserted into holes in the stretch chamber. Those pipes were adjusted with the struts of the motion frame as shown in Fig. 1(a).

(A) Structural design of the stretch chamber using elastic FE analysis

It is possible that the bottom of the collagen gel could become detached from the stretch chamber during the stretching process by deflection in the $X_3$ direction. Therefore, the deflection of the bottom part of the PDMS stretch chamber was analyzed in order to minimize any deflection occurring in the $X_3$ direction. Elastic finite element (FE) analysis was carried out to predict the deformation, strain, and stress occurring in the stretch chamber. In order to generate a uniform tensile strain, distortion of the cubic culture region was minimized by reducing the deflection taking place at the bottom surface of the stretch chamber, which also prevents the possible detachment. Through several FE calculations by adopting the cut-off sizes from the bottom of PDMS stretch chamber, we found the adequate condition to prevent the distortion as shown in in Fig. 2(a). An area of 10.0×10.0×1.0 mm was used to contain the collagen gel scaffold for culture in the PDMS stretch chamber, with the entire PDMS chamber covering 31.5×12.0×4.0 mm. The thickness of the wall of chamber was set at 1 mm to avoid the PDMS tearing and the collagen gel leaking. In order to load the stretching, four...
thin stainless-steel pipes were set onto the struts of the moving platform, and four holes were made in the PDMS stretch chamber. The four pipes were then inserted into the holes of the PDMS stretch chamber, in which the PC12 cells and the collagen gel scaffold were fixed as shown in Fig. 1(a) and 2(a). The elastic FE analysis was carried out using the commercial FE analysis code, ANSYS, Cybernet System Inc., to find the optimum conditions under which the distortion and deflection of the PDMS stretch chamber could be minimized. The bilateral stretch stimulation induced by the reciprocal motion of the struts was applied in the PDMS stretch chamber for the FE analysis, by loading the displacement onto the holes on the opposite outer surface. As demonstrated in Fig. 1(a) and 2(a), any positive displacements in the $X_1$ direction were forced onto the right-hand cylindrical surfaces at the two holes, and the negative displacements occurred on the two left-hand cylindrical surfaces. The collagen gel was connected to the bottom and the inner walls of the PDMS stretch chamber. Both end struts were then connected to the thin stainless-steel pipes and the outer half of each of the four thin stainless-steel pipes was connected to the PDMS stretch chamber. We assumed that the collagen gel was sufficiently adhered to the bottom and inner walls of the PDMS stretch chamber. Figure 2(b) shows the results of the analysis when a bilateral stretch displacement of 0.592 mm is applied to the surface boundaries of the four struts in the $X_1$ direction, from which it was confirmed that deflection of the bottom surface in the $X_3$ direction was prevented.

In order to confirm the complete adhesion of the collagen gel to the walls of the PDMS stretch chamber under stretch stimulation, preliminary experiments were carried out with 4% strain at 1 Hz. After 24 hours of loading, it was confirmed that the collagen gel was completely adhered to all of the four PDMS stretch chambers, as shown in Fig. 2(b). The shape of the PDMS stretch chamber could thus be determined, as shown in Fig. 2(a).

Table 1  Material properties of 3D cell culture system used in ANSYS.

| Material       | PDMS | SUS316 | SUS04 | Collagen gel |
|----------------|------|--------|-------|--------------|
| Young’s modulus (Pa) | $6.0 \times 10^6$ | $19.3 \times 10^{10}$ | $19.3 \times 10^{10}$ | $9.3 \times 10^{10}$ |
| Poisson’s ratio | 0.49 | 0.28   | 0.29  | 0.49         |

Fig. 2  Stretch chamber; (a) Schematic drawing (Unit: mm), (b) Strain $E_{11}$ (Displacement: 0.592 mm).

(B) Determination of the region under observation using hyperelastic FE analyses and validation

The strain that was generated in the collagen gel scaffold in the PDMS stretch chamber was not uniform. However, there should be a uniform strain in the selected observation area of the collagen gel culture region as shown Figures 2(a) and (b). We therefore needed to identify an area in which Lagrange tensile strains $E_{11}$ were uniform, such as $2.0 \pm 0.1\%$, $4.0 \pm 0.1\%$, and $6.0 \pm 0.1\%$, which were determined via FE analysis using PAM-CRASH, ESI Co., Ltd. (Nakamachi et al., 2017). Figure 3(b) shows the FE meshes of the stretch chamber, the collagen gel scaffold, and the stainless steel pipes with struts. For the collagen gel structure, we used fine FE mesh, the 0.2 mm cube eight-node solid element, which is enough to confirm the uniformity of collagen gel scaffold. Considering the symmetry of the deformations on the $X_2$-$X_3$ and $X_1$-$X_3$ planes, a quarter of the structure was selected for the analysis, as shown in Fig. 2(a) and Fig. 3(b). The collagen gel shared connecting nodes with the stretch chamber at the interface. The struts of the cell culture system and the stainless-steel pipes in the stretch chamber have a higher elastic modulus than ones of PDMS and PC12 cells. Therefore, the struts of the cell culture system and the stainless-steel pipe of the stretch chamber were combined and represented as...
one rigid pole for the analysis. We used the hyperelastic constitutive law, which shows a highly nonlinear stress-strain relationship, for the collagen gel, and the elastic constitutive law for PDMS chamber. The material properties of the collagen gel was newly determined using the stress-strain relationship in the range of strain between 0.0 and 0.10 as shown in Fig. 3(a) in the previous study by Takeda et al. (Takeda et al., 2018). We obtained the initial tangent modulus μ as 1.97 kPa and the nonlinear coefficient of exponential function A as 29. The forced displacement in the X₁ direction against the pole generates the objective strain in the collagen gel. The results for the 2.0, 4.0, and 6.0% stretches based on FE analysis are shown in Fig. 3(c) – 3(e), from which a 3D area of 3.6×2.0×0.5 mm was determined the observation area, as shown in Fig. 3(b). The multiphoton excitation fluorescence microscope (MPM, TCS SP8 Multi-Photon, Leica Geosystems) was used to observe morphologies and distributions of PC12 cells in this selected observation area, in which MPM system can observe several times with the 443×443×500 μm visualization domain. The characteristics of MPM visualization will be shown in section 2.3.

To confirm the uniformity of the tensile strain in the cell culture region, the distribution of the tensile strain in the stretch chamber was measured. Particles with an average size of 100 μm were spread onto the surface of the collagen gel, and displacements of 0.316 mm, 0.644 mm, and 0.984 mm were applied to generate tensile strains of 2.0, 4.0 and 6.0% in the region under observation. The particles were photographed before and after stretching was carried out, using a micro lens camera (AF-S VR Micro-Nikkor 105 mm f/2.8G IF-ED, Nikon Co.). The resolution of image was less than one μm. Using the image processing software, the displacements of particles were calculated using the position of particle centers, and finally the Lagrange strain \( E_{11} \) was evaluated using the displacement field. It is confirmed that average strains of 2.05%, 4.08%, and 6.06% were occurred in the 3.6×2.0 mm area on the collagen gel scaffold surface. By comparison between measured strain and FE analyses result, we concluded that the guaranteeing the uniformity and validation of system was achieved with enough accuracy.

We did not analyze to predict the stress and strain occurred in the cell at the micro-scale, because of enormous time consumption. It required the multi-scale FE analyses such as our work (Nakamachi et al., 2017).

Fig. 3  Results of FE analyses; (a) Stress-strain relationship of collagen gel, (b) FE model of stretch chamber, (c) strain \( E_{11} \) (Displacement: 0.316 mm), (d) strain \( E_{11} \) (Displacement: 0.644 mm), (e) strain \( E_{11} \) (Displacement: 0.984 mm).

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2.2 Evaluation of the PC12 axonal extension loaded using stretch stimulation

2.2.1 Conditions used in the stretch stimulation

The PC12 cells (RBRC-RCB0009, Lot No. 053) were provided by the RIKEN Cell Bank. Dulbecco’s modified Eagle’s medium (DMEM, D6046, Sigma-Aldrich Japan Co. LLC., Japan), and supplemented with 10 % horse serum (16050-122, Gibco®, Life Technologies NZ Ltd., Japan), 5% fetal bovine serum (SH30396.03, HyClone Laboratories Inc., U.S.A.), and an antibiotic (Antibiotic Antimycotic Solution (100x), Stabilized, A5955, Sigma-Aldrich Japan Co. LLC., Japan) was used as the culture medium.

In this study, the collagen gel Cellmatrix® Type I-A (631-00653, Nitta Gelatin Inc., Japan) was used for the 3D culture of the PC12 cells. The stretch chamber, which was constructed using PDMS, has a low affinity for collagen gel because the surface of the chamber is hydrophobic. The inner wall of the PDMS chamber was therefore hydrophilized using oxygen plasma in order to prevent detachment. Conditions used in the hydrophilization treatment include an oxygen flow rate of 50 sccm, an atmospheric pressure of 20 Pa, an RF output of 75 W, and an irradiation time of 3 s, as referred to in the report by Okada (Okada et al., 2011).

The collagen gel, which is highly transparent and therefore ensures easy microscopic observation, was prepared using the following method. A collagen solution was prepared by mixing an acid extract of collagen derived from porcine tendons at pH 3.0 and a concentration of 3.0 mg/ml with a concentrated culture solution and a buffer solution for reconstitution and cell suspension. The PC12 cells were then added to the culture medium, and 50 ng/ml Nerve Growth Factor (NGF) (NGF 2.5S, 13257-019, Thermo Fisher Scientific K.K., Japan) was also added in order to prepare a cell suspension. The resulting cell suspension had a density of 2.5×10⁶ cells/ml. The collagen solution was prepared by pouring 100 μl of the solution into the four chambers and incubating in a 5% CO₂ incubator at 37 °C for 30 min.

2.2.2 Evaluation of the effect of the stretch stimulation on the axonal extension of the PC12 cells

The stretch stimulation (4% strain, 1 Hz) was found to increase the axonal extension of the PC12 cells when 2D cell cultures were used, as previously reported (Haq et al., 2006). We aimed to find the optimum conditions for cyclic stretch stimulation in order to enhance the axonal extension of the nerve cells in 3D cell cultures by using a newly designed and fabricated cell culture system.

The effects of different tensile strains $E_{11}$ and the number of stretch cycles carried out on axonal extension were evaluated under selected conditions of cyclic stretch stimulation. Three tensile strains were adopted; 2.0, 4.0 and 6.0%, and three cyclic repetitions of stretch stimulation; 1.0×10⁵, 2.0×10⁵, and 3.0×10⁵ at 1 Hz. The PC12 cells were cultured for a total of 96 hours, including the stimulation period. From the beginning of culture, the continuous cyclic stretch stimulation with 1 Hz interval was loaded. It means that the total cyclic number corresponds to the period of stimulation time with the unit second. The number 1.0×10⁵ means 1.0×10⁵ seconds, 27.78 hours, 1.16 day. After completing the required number of cyclic stretch stimulations, the stretch chambers with the stimulated PC12 cells were settled in the culture medium chamber of 3D cell culture system under “stress free condition” until the total culture time 96 hours. We carried out four tests of cyclic numbers, 0, 1.0×10⁵, 2.0×10⁵, and 3.0×10⁵ in each stretch stimulation condition simultaneously using our system as shown in Fig.1(a). The control was set at zero stimulation cycles, meaning that no stimulation was conducted. Three times were spent for each tensile strain $E_{11}$ condition, such as 2.0, 4.0 and 6.0% strain. Statistical analytical results of 12 conditions using total 36 collagen gel scaffold samples are shown in Fig. 6(a) - (c), in which MPM observations with 443×443×500 μm visualization domain were carried out three times in different areas of each sample.

2.3 Measurement methodology of nerve axonal extension

The effectiveness of using stretch stimulation for PC12 cells was assessed quantitatively. 3D images of the PC12 cells in the collagen gel were taken with MPM system. MPM system performs fluorescence observation by exciting a specific molecule with a number of photons and can take a 3D image by exciting photons that are only in the focal plane using infrared light. Our MPM system provides two kinds of images, MPM image by using two photons whose wave length is between 700 nm and 1000 nm, and the confocal image by using single photon, 405 nm (UV blue) or 488 nm.
(Visible green). PC12 cells were stained using the Milli-Mark™ FluoroPan neuronal marker (MAB2300X, Merck KGaA, Germany) and DAPI solution (340-07971, Dojindo Laboratories, Japan). MAB2300X stains were used to identify the axons, dendrites, spines, nuclei, and the body of the PC12 cells. DAPI stains were used to investigate the nuclei of the PC12 cells. The PC12 cells were observed in all three selected areas of the collagen gel sample, covering a size of 443×443×500 μm. The resolution of MPM observation is 0.43 μm in both X₁ and X₂ directions of tomography plane, and 0.85 μm in the vertical direction X₃. The lens is HCX IRAPO L 25x/0.95 WATER and the magnification is 250. We adopted the confocal image because of clear photo using the wave length 405 nm (UV blue) for DAPI and 488 nm (Visible green) for MAB2300X. For the stretch chamber was set with the MPM system so that the stretching occurred in the direction of the X₁-axis. The effectiveness of the stretch stimulation in increasing axonal outgrowth was then evaluated from the confocal images.

The axonal length and the orientation angle are defined in Fig. 4. The image analysis software packages LAS X (Leica Application Suite X, Leica Microsystems, Japan) and Image J (Schneider et al., 2012) were used to measure the extent of the axonal extension and the orientation of the PC12 cells. The length by which the axons were extended was measured based on the criteria and definitions given below:

1. The cells should not be in contact with any other cells.
2. Measurements were performed on only one axon for each cell.
3. Only the longest axon was selected if several neurites extended from a cell.
4. The axonal length was defined as the distance in a straight line from the tip of the axon to the root of the cell body surface, as shown in Fig. 4.
5. Neurites with a length of 30 μm or more were defined as axons.
6. If the axons branched into several directions, only the longest branch was selected.

The axonal length was then calculated using Eqs. 1 and 2:

\[
\| \mathbf{L} \| = \sqrt{L_{1}^2 + L_{2}^2 + L_{3}^2} \tag{1}
\]

\[
\mathbf{L} = L_{1}\mathbf{e}_1 + L_{2}\mathbf{e}_2 + L_{3}\mathbf{e}_3 \tag{2}
\]

As shown in Fig. 8, the starting point was defined using the coordinates (0, 0, 0), and the tip of the axon as the coordinate \((L_{1}, L_{2}, L_{3})\). The direction of axonal extension is defined as the angle of orientation \(\theta_1\) against the direction of the stretch \(\mathbf{e}_1\), which is expressed using Eq. 3:

\[
\theta_1 = \cos^{-1} \frac{L_{1}}{\| \mathbf{L} \|} \tag{3}
\]
2.4 Rat cerebral cortex cell culture

In Section 2.2, we used imitation PC12 nerve cells to find the optimum conditions for cyclic stretch stimulation in order to increase axonal extension. Many features of PC12 cells are advantageous for use in cell cultures, such as the high rate of proliferation and the ease of use. However, to verify the applicability of this method to “real” nerve cells, cells from the cerebral cortex of a rat, which can achieve quick nerve network generation and regeneration, were used as primary CNS cells in order to confirm the increase caused by the cyclic stretch stimulation.

Frozen rat cerebral cortex cells from embryonic day 17 (033-24871, Lot No. PTP4143) were provided by FUJIFILM Wako Pure Chemical Co. The cells were allowed to thaw for 30 minutes and were recovered using a neuron dissociation solution (291-78001, FUJIFILM Wako Pure Chemical Co., Japan). A cell suspension of 4.0×10^6 cells/ml was prepared using neuron culture medium (148-09671, FUJIFILM Wako Pure Chemical Co., Japan), which was then mixed with a collagen solution to produce a collagen gel in the stretch chamber of the cell culture system. The primary cells were cultured for 96 hours in the neuron culture medium with NGF under the optimum conditions for cyclic stretch stimulation that were obtained from the previous experimentation with PC12 cells. The neuron culture medium was exchanged with NGF at 48 hours. The primary cells were observed using MPM system in the same manner as for PC12 cells, except the observation domain 1110×1110×500 μm and the magnification 100. The resolutions of image processing in X1, X2 and X3 direction were 1.08, 1.08 and 0.85 μm.

3. Results and Discussion
3.1 Evaluation of the axonal extension of PC12 cells under cyclic stretch stimulation

Figure 5 is a top view of the multi-focus images of the PC12 cells obtained by the MPM system in all conditions. The multi-focus images mean the z-stack image of “Reica MPM system,” in which z corresponds to X1 coordinate. The increase in axonal extension was measured based on the morphological images of the PC12 cells. Figure 6(a) - (c) shows the relationship between the axonal extension and the number of cyclic stimulations under strains of 2.0, 4.0 and 6.0%, respectively. The process was repeated with 1.0×10^3, 2.0×10^3, and 3.0×10^3 cycles of stimulation performed at 1 Hz. The letter “N” in the captions for Fig. 6(a) - (c) describes the number of cells forming the axon, and the “0” stimulation time describes the control.

At first, we found the data shows no characteristics of “normal distribution,” so we employed “non-parametric study.” We adopted Steel-Dwass test as shown in Figs. 6(a) - (c). As shown in Fig. 6(a), under conditions of a 2.0% strain at 1 Hz, the median of axonal length exceeded that of the control in all conditions. A significant difference in the axonal extension was only seen in comparison with the control in the case where stimulation was carried out 2.0×10^3 times (*p<0.05, Steel-Dwass test). When 4.0% strain was used at 1 Hz, the median of the axonal length exceeded that of the control in all cases, as seen in Fig. 6(b), which was similar to the results obtained using a 2.0% strain. However, a significant difference was obtained compared with the control when the stimulation was carried out 1.0×10^3 times (*p<0.05, Steel-Dwass test). As seen in Fig. 6(c), different results were obtained at 6.0% strain for 1 Hz, showing a decrease in the median of axonal length in all the samples. No significant difference in axonal extension was confirmed in comparison with the control (p>0.05, Steel-Dwass test). Data shows a large standard deviation, however the data, which shows a significant difference, has a large number of axonal lengths between the first quartile and the median as shown in Fig. 6(a) and (b). The more the axonal length distributes between the first quartile and the median, it shows the significant difference, such as, 2.0×10^3 in Fig. 6(a) for 2.0% strain and 1.0×10^3 in Fig. 6(b) for 4.0% strain. Dotti and Nakamachi reported that the effects of cyclic stretch stimulation are mainly observed in the early stages of axonal formation (Nakamachi et al., 2018; Dotti et al., 1998). Dotti defined the early stage (from Stage 1 to Stage 3) as 1.5 day (1.29×10^6 cyclic number) in 2D culture, and Nakamachi 2 day (1.73×10^6 cyclic number) in 3D culture. In this study, the enhancement in axonal extension was confirmed at 2.0% strain and 4.0% strain, both in the early stages of axonal formation; at 2.0×10^3 times with a 2.0% strain and 1.0×10^3 times with a 4.0% strain, respectively. We concluded that stimulation carried out with 6.0% strain does not enhance axonal formation, even in the early stages. It has been suggested that there is a combination of thresholds, consisting of both the strain and the number of stretch cycle. The synergetic effect concerned with the fatigue with the strain and the cyclic number on the enhancement and the inhibition of axonal extension.

Next, we found the optimum conditions for axonal growth using response surface methodology, where the strain and
the number of cycles were adopted as variables in the experimental design and the axonal growth was the objective function. Figure 7 shows the response surface. The optimum conditions that were identified using this method were 2.3% strain ($E_{11}$) and $1.7 \times 10^5$ times. Figure 8(a) and (b) show 3D and multi-focus images of the PC12 cells in the collagen gel which were cultured for 96 hours under the optimum conditions for stretch stimulation, including the period of stimulation. Figure 9 shows the results of a statistical study of the measured axonal extension under the optimum conditions for stimulation. As shown in Fig. 9, it was confirmed that there was a significant increase in axonal extension compared to the control (**$p<0.01$, Mann-Whitney $U$ test). It shows no extreme large increase compared with results shown in Fig. 6, however the significant difference of axonal extension was confirmed.

We found the difference from the result of 2D cell culture, in which the optimum condition of cyclic stretch stimulation for the enhancement of axonal extension was 4.0% strain, 1 Hz and 96 hours culture (Haq et al., 2006; Nakamachi et al., 2018). In the 2D in vitro cell culture, PC12 cells were seeded and cultured on the silicon rubber sheet. In this study, PC12 cells were seeded in the collagen gel scaffold and the stress and strain distributions in 3D culture region are difference with the case of 2D. The stress condition in 2D cell culture is “plane stress” condition, which means that the stress in the perpendicular direction against the sheet plane is zero. Our 3D cell culture imitates more correctly the brain structure than the case of 2D cell culture. As a result, the stress in 3D culture environment affects more severely than the case of 2D and consequently the threshold value of cyclic strain was less than 2D case, such as 4% in 2D culture and 2.3% in 3D culture. This threshold value of cyclic stretch strain can be applied to the mechanical stimulation technology for the nerve network regeneration of damaged nerve cells.

Fig. 5 Multi focus images of PC12 cells (Bar: 100 μm); (a) 2.0% strain, (b) 4.0% strain, (c) 6.0% strain, (1) 0 times, (2) $1.0 \times 10^5$ times, (3) $2.0 \times 10^5$ times, (4) $3.0 \times 10^5$ times.
Fig. 6  Axonal length of PC12 cells vs. number of stretch cycles; (a) 2.0% strain (0 time: N=114, 1.0×10⁵ times: N=91, 2.0×10⁵ times: N=94, 3.0×10⁵ times: N=100), (b) 4.0% strain (0 time: N=100, 1.0×10⁵ times: N=106, 2.0×10⁵ times: N=92, 3.0×10⁵ times: N=105), (c) 6.0% strain (0 time: N=84, 1.0×10⁵ times: N=111, 2.0×10⁵ times: N=94, 3.0×10⁵ times: N=103), *p<0.05, Steel-Dwass test.

Fig. 7  Response surface for the axonal extension length of PC12 cells.
3.2 Evaluation of primary cell axonal extension of loaded with stretch stimulation

Figure 10 shows the 3D images and the multi-focus images of rat cerebral cortex cells obtained by MPM system after 96 hours of culture under control conditions and optimum cyclic stretch stimulation conditions of 2.3% strain carried out $1.7 \times 10^5$ times at 1 Hz, which were obtained using PC12 cells. The axonal extension was measured based on these images in both cases. Figure 11 shows the length by which the axons were extended; “N” means the number of cells forming the axon. It was confirmed that the cyclic stretch stimulation enhanced the axonal extension of the primary cells, because a significant increase was seen in the results for axonal length compared with the control ($^*p<0.01$, Mann-Whitney U test).

Figure 12 shows the histogram for axon orientation against the stretch direction using the cosine of the $X_1$ direction ($n_1$) for the PC12 cells and the primary cells. If the direction cosine $n_1$ close to one, it means incline toward the stretch direction $X_1$. Figure 12 shows that there was no significant difference between those affected by the stretch stimulation and the control. There was no significant tendency for inclination toward the stretch direction $X_1$ in any of the cells, although there was a very slight tendency for inclination toward the stretch direction in the case of stretch simulation.

In addition, axons of longer length were often observed in the primary cells compared to the PC12 cells; therefore only axons with an extension greater than 200 $\mu$m were selected for the statistical study of the orientation tendency. The threshold value of 200 $\mu$m was identified in a previous study (Nakamachi et al., 2014), in which the influence of cell interval on axonal extension was neglected. Figure 13 shows the relationship between the number of cells with axonal lengths greater than 200 $\mu$m, and the direction cosine ($n_1$). It is apparent that the axons of primary cells have tendency to grow to coincide with the stretch direction $X_1$. The results of the numerical analyses carried out by Takeda et al. demonstrated that the stress in axons that are inclined coincides with the stretch direction tended to be higher than that of axons that are inclined perpendicular to the stretch direction (Takeda et al., 2018). In this experiment it was observed that the axons that were inclined toward the stretch direction were more stimulated and grew longer than those inclined toward the perpendicular against the stretch direction. These results suggest that the cyclic stretch stimulation of nerve cells in a 3D cell culture system affects the axonal orientation and the extension length, meaning that the more an axon is inclined towards the stretch direction $X_1$, the more the axon will extend.
Fig. 10  3D image (left) and multi-focus image (right) of cerebral cortex by using MPM system (Unit: μm); (a) Control, (b) 1.7×10^5 times of 2.3%-1 Hz stretch stimulation.

Fig. 11  Axonal Length of cerebral cortex by 1.7×10^5 times with 2.3%-1 Hz stretch stimulation (Control: N=125, Stretch: N=159, *p<0.01, Mann-Whitney U test).

Fig. 12  Histogram of n_1; (a) PC12 cells (left: Control, N=83, right: Stretch, N=76), (b) cerebral cortex cells (left: Control, N=125, right: Stretch, N=159).
4. Conclusion

In this study, we aimed to enhance the axonal extension of nerves by applying cyclic stretch stimulation to both PC12 cells and rat cerebral cortex cells in the 3D stretch chamber of a cell culture system. The following results were obtained.

(1) We designed and fabricated a cyclic stretch stimulation cell culture system, which could be used to load uniform $E_{11}$ strain onto cells that were embedded in collagen gel.

(2) Strain was applied at 2.0, 4.0, and 6.0% with $1.0 \times 10^5$, $2.0 \times 10^5$, and $3.0 \times 10^5$ times at 1 Hz for 96 hours. As a result, a significant increase was observed in axonal extension compared to the control at 2.0% strain carried out $2.0 \times 10^5$ times and 4.0% strain carried out $1.0 \times 10^5$ times. These results suggest that both the amount of strain and the number of stimulations conducted affected axonal extension. In addition, we found the optimum conditions for the stimulation of PC12 cells to be 2.3% strain and $1.7 \times 10^5$ times in order to enhance axonal extension. An experiment was carried out to verify these optimum conditions and a significant effect confirmed in terms of axonal extension as compared with the control.

(3) Using the optimum conditions identified for use with the PC12 cells, cells from the cerebral cortex of a rat were loaded using cyclic stretch stimulation. A significant axonal extension was confirmed compared to the control. It was also observed that the axons of primary cells with a length greater than 200 $\mu$m were inclined more significantly towards the direction of stretch, $X_1$, as compared to the control.

In the future, the 3D cyclic stretch stimulation cell culture system could be adopted as a new neuro-therapy device for nerve network regeneration.

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