Culture patch method for primary hippocampal neuron culture

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Abstract

In vitro culture of primary neurons, especially hippocampal neurons, is important for understanding cellular mechanisms in neurobiology. Actually, this is achieved by using culture dish or glass slide with surface coated proteins. Here, we proposed a patch method to culture primary neurons on a monolayer of gelatin nanofibers, electrospun and crosslinked on a microfabricated honeycomb frame of poly (ethylene glycol) diacrylate (PEGDA). By using such a patch method, neural networks could be formed with a minimal cell-exogenous materials contact and a maximal exposure of the cells to the medium. Interestingly, hippocampal cells, especially astrocytes, showed *in-vivo* like morphology and most of neurons were found in the porous areas inside the honeycomb compartments although the nanofibers were deposited everywhere of the frame. Finally, calcium imaging showed that primary neurons have a higher degree of neural activity on the patch than on glass.

Keywords: Nanofiber, Patch, Hippocampal neuron, Calcium imaging

1. Introduction

Primary neurons, especially the hippocampal neurons, are widely used for *in vitro* studies due to the relative simple nerve cell population and the expression of key neural phenotypic features as well as the involvement of the hippocampus in learning and memory [1-4]. In most of these studies, the primary neurons were cultured in culture dish or on glass slides with or without astrocytes [5]. It has been shown, however, that the neural growth and its functional performance are critically dependent on the culture conditions, especially the components and mechanical properties of the substrate [6, 7], while the culture dish method is not sufficiently flexible to achieve the optimal conditions. In principle, mimicking the *in vivo* cell microenvironment can lead to significant improvement of the culture conditions [8, 9].

Electrospun nanofibers were *in vivo* extracellular matrix (ECM) like which are promising for further applications in tissue engineering and regenerative medicine [10, 11]. Gertz et al showed that electrospun submicron fibers can significantly enhance neuritogenesis, maturation and the polarity formation of neurons compared to the two dimensional substrates, such as glass [12]. On the other hand, natural bio-polymers such as gelatin, which is produced by hydrolyzing collagen, should be more relevant than synthetic polymers in terms of bio-compatibility [13]. Previously, gelatin nanofibers could be used for long term expansion of human induced pluripotent stem cells (hiPSCs) [14]. Most recently, we proposed a culture patch method by using monolayer gelatin nanofibers for hiPSCs differentiation towards mature motor neurons and cardiomyocytes [15, 16]. In particular, our results showed that the culture patch method is advantageous over the conventional culture dish methods, including much up-regulated expression of neural specific genes, accelerated neuron maturation as well as easy manipulation such as plug-and-play monitoring of neuron spikes by extracellular potential recording.

In this work, we extend the culture patch method for primary hippocampal neuron culture. Monolayer of gelatin nanofibers was electrospun and crosslinked on a microfabricated honeycomb frame of poly (ethylene glycol) diacrylate (PEGDA). We show that hippocampal cells, especially astrocytes, have *in vivo* like morphology on the culture patch. Although the nanofibers were deposited everywhere of the frame, most of neurons were found in the porous areas inside the honeycomb compartments. We also show that primary neurons have a higher degree of neural activity on the patch than on glass.

2. Experimental

2.1 Fabrication of culture patch

The culture patch was fabricated by our previously reported method [15, 16]. As shown in **Fig.1**, a chromium mask with a pattern of honeycomb frame of 500 μm pitch size and 50 μm band width was produced with a micro pattern generator (μPG 101, Heidelberg Instruments). Then the mask was spin-coated with a 50 μm thick photoresist layer (AZ 40XT, MicroChem) and backside exposed with UV light. After development, the mask with resist pattern was treated in trimethylchlorosilane (TMCS) vapor for surface anti-sticking. Polydimethylsiloxane (PDMS) pre-polymer and cross-linker (GE RTV 615) at weight ratio of 10:1 was mixed completely and then poured to cover the resist layer. After curing at 80°C for 2 h, the PDMS layer was peeled off and placed on a glass slide. Then the PDMS-glass assembly was degassed for 10 min in a desiccator. A PEGDA (average Mn=250, Sigma) solution containing 1 v/v% Irgacure 2959 as photo-initiator was dropped on the edge of PDMS mould to fill the cavity between PDMS and glass by a degassing based micro-aspiration. Then PEGDA honeycomb frame was formed by curing with 30 s UV exposure (9.1 mW/cm^2). For easier handling, a 100 μm thick PEGDA ring with outer and inner diameter of 13 mm and 9 mm respectively was prepared with the similar method and mounted on the honeycomb frame with PEGDA solution as binder by UV curing.

Gelatin nanofibers were prepared by electrospinning using the same protocol as previously described [8]. For much even collection of nanofibers, 10 nm thick Au layer was sputtered on the PEGDA frame. Then this PEGDA frame was fixed on a Silicon wafer as collector. 10 wt% gelatin was dissolved completely in a solvent mixture containing distilled water, ethylacetate acid and acetic acid at a volume ratio of 10:14:21. Then this gelatin solution was ejected from a syringe to the collector at a distance of 10 cm and a pumping speed of 0.2 ml/h through a stainless steel needle (23-gauge) under a bias voltage of 11 KV. After electrospinning for 15 min, the samples were removed into a desiccator overnight to remove the residual solvent. Then an ethanol solution containing 0.2 M 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and 0.2 M N-hydroxysuccinimide (NHS) was used for crosslinking the gelatin nanofibers during at least 4 h. Finally, the samples was rinsed with ethanol for three times and dried completely in a desiccator overnight to eliminate the remaining solvent.

2.2 Cell culture

Human glioblastoma cell line U-87 was cultured in Dulbecco's Modified Eagle Medium (DMEM) completed with 10% FBS and 1% penicillin/streptomycin at 37°C with 5% CO_2 supplementation for 3~4 days to reach 80% confluence. Then cells were dissociated by Trypsin at 37 °C for 3 min. Then without any additional coating, 10^5 cells in 50 µl medium were seeded on the patch in the center of the ring. After incubation at 37℃ for 1 h for cell attachment, more medium was added gently around the patch, which was then transferred back to the incubator for continued culture.

Hippocampal neurons from Wistar rats (P2-P3) were prepared in accordance with the guidelines of the Italian Animal Welfare Act, and their use was approved by the Local Veterinary Service, the SISSA Ethics Committee board and the National Ministry of Health (Permit Number: 630-III/14) in accordance with the European Union guidelines for animal care (d.1.116/92; 86/609/C.E.). The animals were anaesthetized with CO_2 and sacrificed by decapitation, and all efforts were made to minimize suffering. All substrates (Glass and patch nanofibers) were sterilized under UV light (15 min each side), soaked for 2 days in PBS and other 2 days in 100 units/ml Penicillin and 100 µg/ml Streptomycine. Finally, they were coated with 20 µg/ml Laminin (Sigma-Aldrich, St. Louis, MO, USA) overnight and washed with water before Matrigel coating (1:50 diluted in the Culture Medium) (Corning, Tewksbury MA, USA) and cell seeding. Dissociated cells were resuspended in Culture Medium: minimum essential medium (MEM) with GlutaMAX™ supplemented with 10% dialyzed fetal bovine serum (FBS, all from Thermo Fisher Scientific, Waltham, MA, USA), 0.6% D-glucose, 15 mM Hepes, 0.1 mg/ml apo-transferrin, 30 µg/ml insulin, 0.1 µg/ml D-biotin, 1 µM vitamin B12 and 2.5 µg/ml gentamycin (all from Sigma-Aldrich). A drop containing 200.000 cells was deposited per each substrate and incubated for 30 min at 37°C. The cells were then resuspended in Astrocyte Conditioned Medium (ACM) in 1:1 ratio with Neurobasal/B27 medium. After 48 hours, 2 µM cytosine-β-D-arabinofuranoside (Ara-C; Sigma-Aldrich) was added to the culture medium to block glial cell proliferation. Half of the medium was changed every 2-3 days. The neuronal cultures were maintained in an incubator at 37°C, 5% CO_2 and 95% relative humidity.

2.3 Scanning electron microscopy (SEM) observation

For the samples with cells, they were firstly fixed with 4% formaldehyde for 30 minutes, and rinsed three times with PBS. Then the samples were immersed in 30% ethanol solution (in distilled water) for 30 minutes. Afterward, the samples were dehydrated using graded

ethanol solutions with concentrations of 50%, 70%, 80%, 90%, 95%, and 100%, respectively, each for 10 min and dried by nitrogen gas flow. Before observation, both dehydrated cell samples and samples without cells were deposited with a 2nm thick Au layer by sputtering. Finally the samples was observed with a scanning electron microscope (Hitachi S-800) operated at 10 kV.

2.4 Calcium Imaging

The cells were loaded with 4 μM of a cell-permeable calcium dye Fluo4-AM (Life Technologies) dissolved in anhydrous DMSO (Sigma-Aldrich), stock solution 4 mM, and Pluronic F-127 20% solution in DMSO (Life Technologies) at a ratio of 1:1 in Ringer's solution (145 mM NaCl, 3 mM KCl, 1.5 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose and 10 mM Hepes, pH 7.4) at 37°C for 1 hour. After incubation, the cultures were washed and then transferred to the stage of a Nikon Eclipse Ti-U inverted microscope equipped with a piezoelectric table (Nano-ZI Series 500 μm range, Mad City Labs), an HBO 103 W/2 mercury short arc lamp (Osram, Munich, Germany), a mirror unit (exciter filter BP 465-495 nm, dichroic 505 nm, emission filter BP 515-555) and an Electron Multiplier CCD Camera C9100-13 (Hamamatsu Photonics, Japan). The experiments were performed at RT, and images were acquired using the NIS Element software (Nikon, Japan) with an S-Fluor 20x/0.75 NA objective at a sampling rate of 3-10 Hz with a spatial resolution of 256 × 256 pixels for 10 min. To avoid saturation of the signals, excitation light intensity was attenuated by ND4 and ND8 neutral density filters (Nikon).

Ca^{2+} imaging processing and analysis: The initial video was processed with the ImageJ (U. S. National Institutes of Health, Bethesda, MA) software. The image sequences were then analysed as described previously [17]. Appropriate ROIs around the cells bodies were then selected. The time course of the fluorescence intensity, $I_f(t)$, in this ROI was displayed, and any decay, which is a consequence of dye bleaching, was evaluated. The Ca^{2+} transients of each cell signal were extracted in a semi-automatic manner by selecting a threshold for the smallest detectable peak that was equal to three times the standard deviation of the baseline. $I_f(t)$ was then fitted to the original optical signal to compensate for dye bleaching, and the fractional optical signal was calculated as follows: $DF/F = (Y(t)+I_f(t))/ I_f(0)$, where $I_f(0)$ is the fluorescence intensity at the beginning of the recording.

Computation of the correlation coefficient of Ca^{2+} transient occurrence: The times, t_i, at which transient peaks occurred were used to calculate the rate of activity. The correlation coefficient of the calcium transients for neuron i and neuron j (σ_{CTij}) was computed as follows: The total recording time, T_{tot}, was divided into N intervals (1,..,n,…,N) of a duration Δt. Thus, if f_{in} and f_{jn} are the number of calcium transients of neuron i and neuron j in the time interval Δt_n, then

$$\sigma_{CT_{ij}} = \frac{\sum_n f_{in} f_{jn}}{\sqrt{\left(\sum_n f_{in}^2\right)\left(\sum_n f_{jn}^2\right)}} \quad (1)$$

such that σ_{CTij} depends on Δt and varies between 0 and 1. The range of explored values of Δt was 10 s.

2.6 Immunofluorescence

Cells were fixed in 4% paraformaldehyde containing 0.15% picric acid in phosphate-buffered saline (PBS), saturated with 0.1 M glycine, permeabilized with 0.1% Triton X-100, saturated with 0.5% BSA (all from Sigma-Aldrich) in PBS and then incubated for 1h with primary antibodies: mouse monoclonal glial fibrillary acidic protein (GFAP)(Sigma-Aldrich) and anti-β-tubulin III (TUJ1) mouse monoclonal antibody (Covance, Berkeley, CA). The secondary antibodies were goat anti-mouse immunoglobulin (Ig) G1 Alexa Fluor® 488, goat anti-mouse IgG2a Alexa Fluor® 594, (all from Life Technologies) and the incubation time was 30 min. Nuclei were stained with 2 µg/ml in PBS Hoechst 33342 (Sigma-Aldrich) for 5 min. All the incubations were performed at room temperature (20-22°C). The cells were examined using a Leica DM6000 fluorescent microscope equipped with DIC and fluorescence optics, CCD camera and Volocity 5.4 3D imaging software (PerkinElmer, Coventry, UK). The fluorescence images were collected with a 20x magnification and 0.5 NA objective. When necessary z-stack images were acquired with slice spacing of 0.5µm. Image J by W. Rasband (developed at the U.S. National Institutes of Health and available at http://rsbweb.nih.gov/ij/) was used for image processing.

The theoretical number of expected cells for a homogeneously distributed culture was calculated as follow:

$$\mathrm{ExpCell} = (A_{in}/A_{tot}) \times \mathrm{n}Cell_{tot} \quad (2)$$

where A_{in} is the area inside the frame, A_{tot} is the total area of the honeycomb and $nCell_{tot}$ is the total number of cells observed in the entire honeycomb.

Then the variation percentage of the real average cell numbers in the centre of honeycomb compartment from the theoretical value could be calculated as:

$$\%\Delta n_{cell} = \frac{nCell_{in} - ExpCell}{ExpCell} \quad (3)$$

where $nCell_{in}$ is the observed number of cells inside the hexagon. The positive or negative values indicate the increase or decrease of cell numbers comparing to the theoretical number of expected cells.

2.7 Statistical analysis

Data are shown as the mean ± s.e.m from at least three neuronal cultures. For the morphological analysis of immunofluorescence images, n refers to the number of images analysed, and the number in brackets refers to total number of cells analysed. All the cells were counted not in the entire field of view, but considering the hexagon of the patch as a unit. The quantified activity (frequency and Cross-correlation) and morphological data were analysed with Student's-t test or Mann-Whitney test using the software SygmaPlot 10.0. The number of replicates and statistical tests used for each experiment are mentioned in the respective figure legends or in the Results. Significance was set to *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

3. Results and discussion

The culture patch can be easily handled with a tweezer (Fig.1E), facilitating a large number of biological analyses. After crosslinking, the electrospun gelatin nanofibers exhibited a net structure (Fig.2) with an average nanofiber diameter of about 500 nm and pore sizes of less than 8 μm [15, 16]. These pore sizes are smaller than the size of a single cell forsupporting cell growth, but large enough to minimize the cell-exogenous materials contact and to maximize the exposure area of cells.

We firstly cultured brain-original glioblastoma U87-MG cells on the culture patch. Due to the comparable material density to the culture medium, the culture patch could be immerged in the medium but has no tight contact with the surface of the medium container, suggesting an off-ground culture condition. Figure 3 shows SEM images of U87-MG cells after 2 days' culture on a patch. Unlike in conventional culture, the most of cells on the patch were entirely exposed to medium. We found, however, that the nuclei were mostly on the topside of the nanofibers, due probably to the fact that the pore sizes of the patch are smaller than that of nuclei so that no cell could migrate to the bottom side of nanofibers. Interestingly,

the spreading of cells was largely conditioned by the morphology of nanofibers, and the nanofibers could be wrapped due to cell-fiber interaction. This would suggest an outstanding cell-compatibility of our culture patch.

Next, hippocampal neurons were isolated from postnatal wistar rats (P1-P3) and seeded on both laminin coated culture patch and glass slide for comparison. In order to examine the relative abundance of neurons and glial cells, cultures were stained with antibodies for neurons with TUJ1 and for glial cells with glial fibrillary acidic protein (GFAP) after 8~10 days *in vitro* (DIV). Fig.4 A and B showed the GFAP staining images of cells under normal FBS culture medium, and an obvious morphology difference of astrocytes between cells on culture patch and on glass could be observed. As we know that the *in vivo* morphology of astrocytes is stellate-shaped with many processes arising from the soma [18]. However, most astrocytes on glass presented flattened morphologies with very few branches (91.4±1.9% flattened and 8.6±1.9% *in vivo* like astrocytes), while on culture patch, GFAP positive astrocytes presented more *in vivo* like morphology (39.1±4.1% flattened and 60.9±4.1% *in vivo* like astrocytes), as shown in Fig.4C. Then to ensure good neural viability, we used the Astrocyte Conditioned Medium (ACM) for cell culture. As shown in Fig.5 A and B, glial cells adhered to the culture patch as well as to glass and formed a supporting layer, above which neurons formed a network. Besides, astrocytes on both culture patch and glass showed obviously increased *in vivo* like morphology and much decreased flattened morphology, which could be related to the effect of astrocyte conditioned medium. But there were still flattened astrocytes observed on glass even under the effect of astrocyte conditioned medium. Both of the results suggested that compared to the conventional 2D glass culture, the culture patch with monolayer porous gelatin nanofibers could provide not only a much lower stiffness, but also an *in vivo* neural ECM like environment with three-dimensional permeability for the hippocampal neurons, thus facilitate the cells to keep the *in vivo* state and function.

Puschmann et al [19] found that contaminating astrocytes on nanofiber substrate were less reactive than on 2D substrate, leading to a less fraction of proliferating astrocytes on nanofibers. Nevertheless, our statistic results (Fig.5C) showed that the portion of neurons on patch was smaller than that on glass, although the percentage of astrocytes on patch and on glass was similar, which could also be explained by the use of ACM. With culture patch, the effect of ACM should be largely improved due to the enhanced exchange between cells and medium on monolayer nanofibers, resulted into the relative lower percentage of neurons on culture patch. During experiment, we also found that cells were more inclined to stay on the

porous area in the center of hexagon structure, but not on PEGDA frame. Statistic results (Fig.5D) showed that there was a variation of 12.4±3.9% more cells in the center of hexagon structure for the patch, while 2.5±1.4% less for the glass, from the theoretical value (in the case of assumed homogeneous cell distribution), which further proved the advantage of this monolayer porous nanofiber net.

The spontaneous electrical activity of hippocampal neurons was recorded by Calcium imaging. The fluorescence images of the Fluo-4-loaded neuronal cultures on culture patch and on glass were shown in Fig.6 A and B respectively. Neural activity was evaluated by recording their calcium transients (DF/F), obtained by acquiring fluorescence images at 3~10 Hz for 10~20 min (Fig.6C and D). The neural activity on culture patch was ~20% more frequent than that on glass as shown in Fig.6E, even though the amount of neurons on patch was fewer (Fig.5C). The synchronization of neural signals could be represented by the mean correlation coefficient of the calcium transients. However, the synchrony of neural activity did not show obvious difference on patch and on glass (Fig.6F).

4. Conclusion

We have demonstrated a culture patch method for primary hippocampal neurons culture. This method is advantageous over the conventional culture dish methods since it allows not only minimizing the cell-exogenous materials contact but also maximizing the cell-medium exchange under off-ground culture condition. We found that astrocytes on the patch exhibited much more *in vivo* like morphologies comparing to that on a glass slide. We also found that neurons were more active on the patch than on glass. Finally, we believe that our culture patch method is reliable for more systematic studies of primary hippocampal neurons due to its flexibility and versatility.

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Figure captions

Figure 1. Culture-patch fabrication. (A) Mould fabrication by backside UV lithography and PDMS casting; (B) Fabrication of PEGDA honeycomb microframe by aspiration-assisted molding. (C) Binding of a PEGDA ring and backside Au deposition. (D) Electrospinning of gelatin nanofiber. (E) Photograph of a culture patch handled with a tweezer.

Figure 2. SEM images of the culture patch. After electrospinning and crosslinking, the nanofibers on PEGDA microframe formed a monolayer net with high porosity and pore size smaller than 8 μm.

Figure 3. SEM images of U87 cells on culture patch after 48 h culture from top-view and bottom-view, respectively.

Figure 4. (A, B) Immunofluorescence images of hippocampal neurons after 8~10 DIV in FBS culture medium on culture patch (A) and on glass (B) respectively, stained with glial fibrillary acidic protein (GFAP, green). (C) Percentage of astrocytes with flattened and *in-vivo* like morphology on culture patch and on glass respectively.

Figure 5. (A, B) Immunofluorescence images of hippocampal neurons after 8~10 DIV in astrocyte conditioned medium on culture patch (A) and on glass (B) respectively, stained with TUJ1 (red), GFAP (green) and Hoechst 33342 (blue). (C) Proportion of neurons (TUJ1-positive) and astrocytes (GFAP-positive) on culture patch and on glass respectively. TUJ1- and GFAP-negative cells are referred to as "other". (D) Variation percentage of the real average cell numbers in the center of honeycomb compartment from the theoretical value, on culture patch and on glass respectively. The theoretical value was calculated from an assumed homogeneously cell distribution (n=691 couple of neurons from 8 experiments for culture patch; n=407 couple of neurons from 7 experiments for glass; $p<0.01$ Student's-t test).

Figure 6. (A, B) Fluorescence images of neuronal cultures loaded with 4 μM Fluo-4-AM calcium indicator on culture patch (A) and on glass (B), respectively. (C, D) Calcium transients on culture patch (C) and on glass (D) respectively, for 4 selected neurons. (E) Frequency of neural spikes on culture patch and on glass, respectively (n=110 neurons from 8 experiments for culture patch; n=70 neurons from 3 experiments for glass; $p<0.01$ Mann-

Whitney test). (F) Mean correlation coefficient of the calcium transients and slow calcium signals (n=599 couple of neurons from 8 experiments for culture patch; n=782 couple of neurons from 3 experiments for glass).

Figure 1

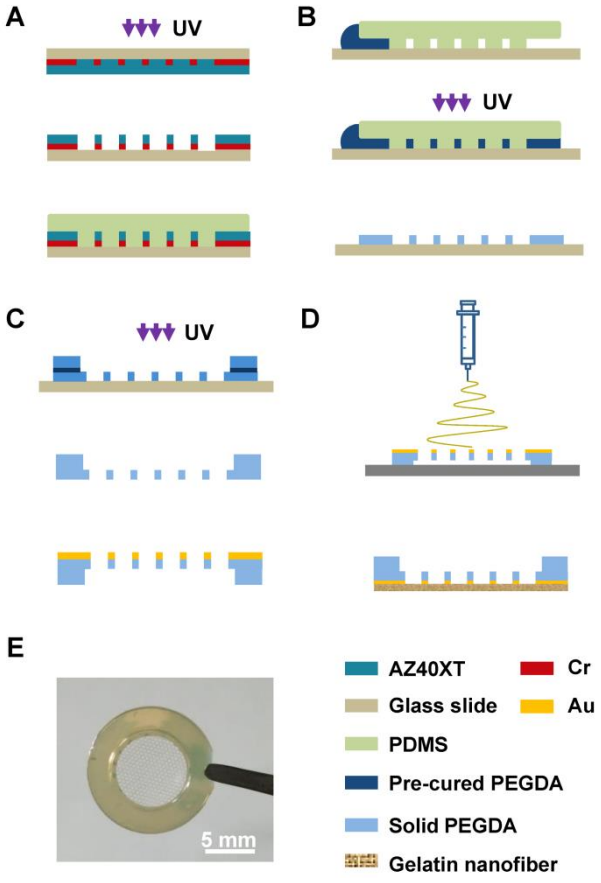
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Figure 2

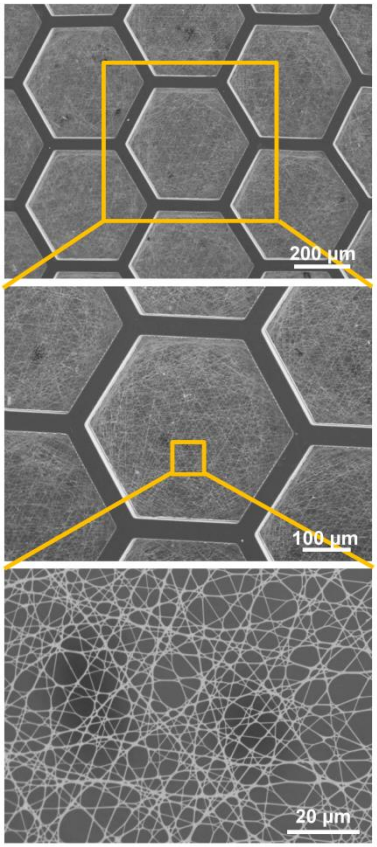


Figure 3

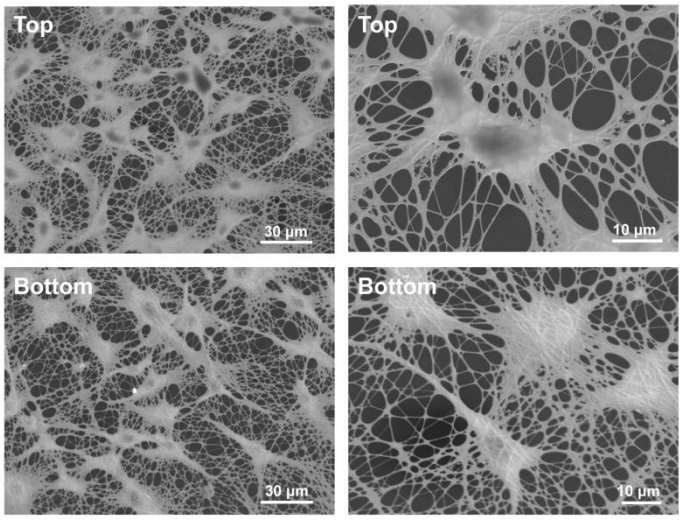
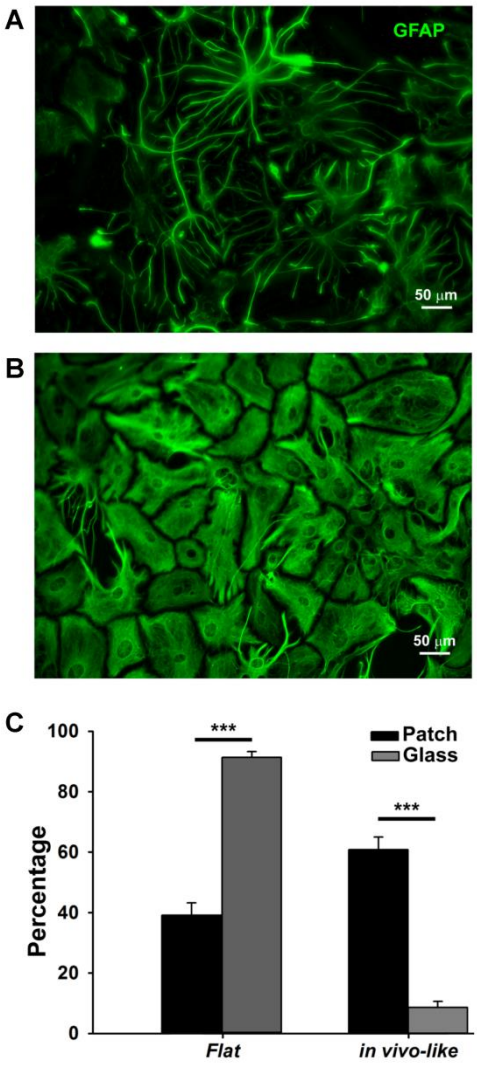


Figure 4


A
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Patch
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in vivo-like

Figure 5

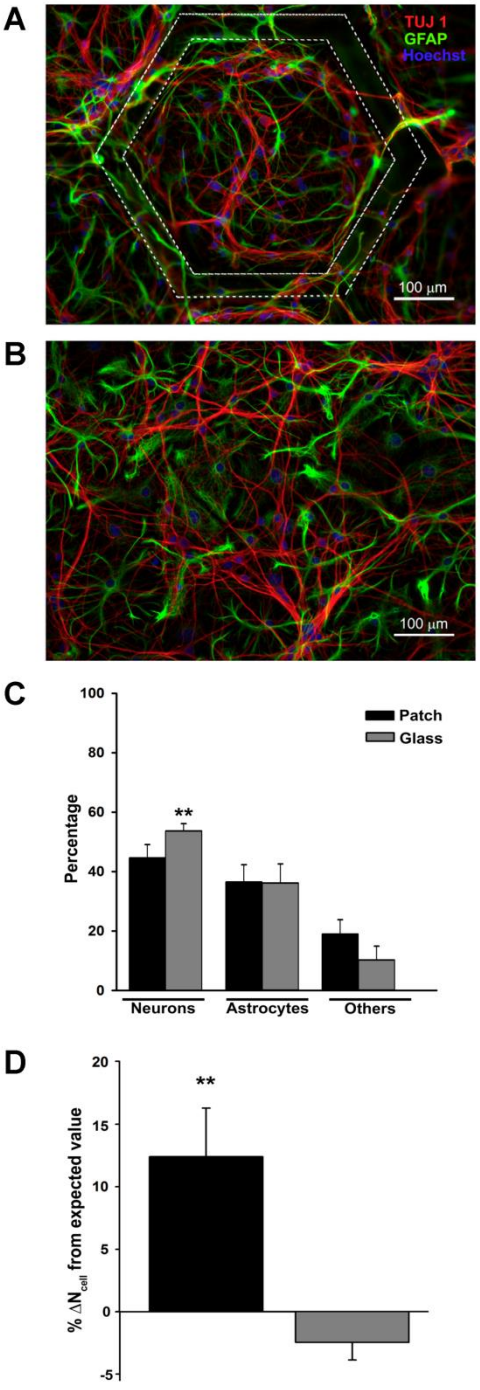
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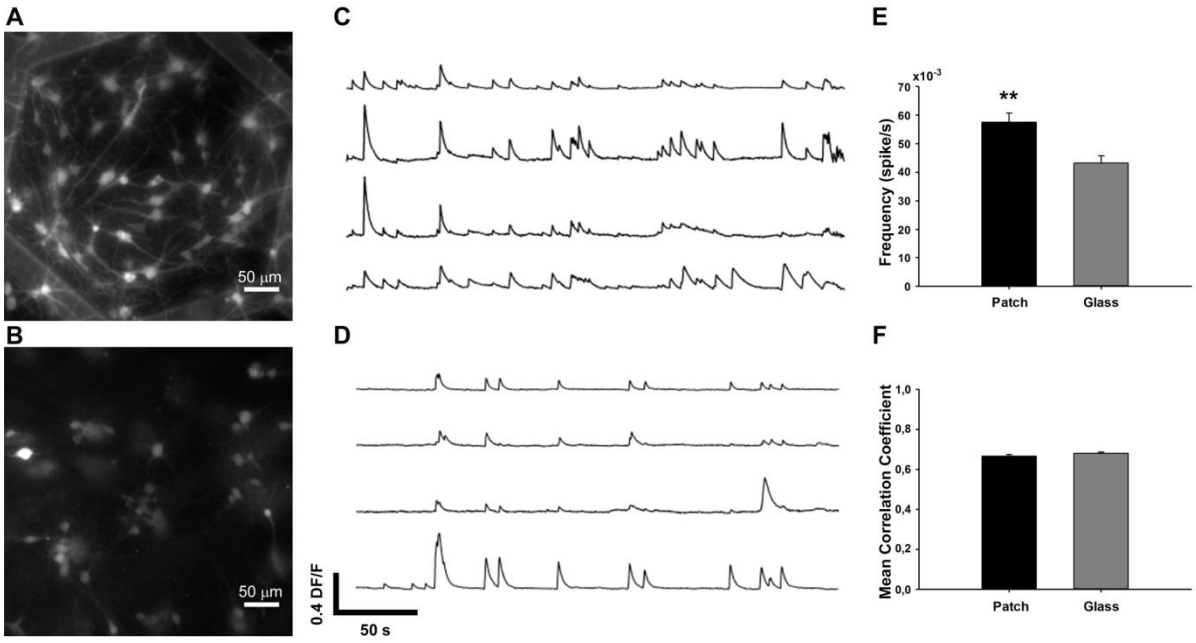
Figure 6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0,2
0,0
Mean Correlation Coefficient
Patch
Glass