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TITLE:

Electric-Field-Induced Neural Precursor Cell Differentiation in Microfluidic Devices

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Electric fields, neural stem and progenitor cells, differentiation, polymethyl methacrylate, PMMA, microfluidic system

SUMMARY:

In this study, we present a protocol for the differentiation of neural stem and progenitor cells (NPCs) solely induced by direct current (DC) pulse stimulation in a microfluidic system.

ABSTRACT:

Physiological electric fields (EF) play vital roles in cell migration, differentiation, division, and death. This paper describes a microfluidic cell culture system that was used for a long-term cell differentiation study using microscopy. The microfluidic system consists of the following major components: an optically transparent electrotactic chip, a transparent indium–tin–oxide (ITO) heater, a culture media-filling pump, an electrical power supply, a high-frequency power amplifier, an EF multiplexer, a programmable X-Y-Z motorized stage, and an inverted phase-contrast microscope equipped with a digital camera. The microfluidic system is beneficial in simplifying the overall experimental setup and, in turn, the reagent and sample consumption. This work involves the differentiation of neural stem and progenitor cells (NPCs) induced by direct current (DC) pulse stimulation. In the stem cell maintenance medium, the mouse NPCs (mNPCs) differentiated into neurons, astrocytes, and oligodendrocytes after the DC pulse stimulation. The results suggest that simple DC pulse treatment could control the fate of mNPCs and could be

used to develop therapeutic strategies for nervous system disorders. The system can be used for cell culture in multiple channels, for long-term EF stimulation, for cell morphological observation, and for automatic time-lapse image acquisition. This microfluidic system not only shortens the required experimental time, but also increases the accuracy of control on the microenvironment.

INTRODUCTION:

Neural precursor cells (NPCs, also known as neural stem and progenitor cells) can be as a promising candidate for neurodegenerative therapeutic strategy[1]. The undifferentiated NPCs have self-renewal capacity, multi-potency, and proliferative ability[2,3]. A previous study has reported that the extracellular matrix and molecular mediators regulate differentiation of NPC. The epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) promote NPC proliferation, thus maintaining the undifferentiated state[4].

Previous studies have reported that electrical stimulation can regulate cell physiologic activities such as division[5], migration[6-8], differentiation[1,9,10], and cell death[11]. Electric fields (EFs) play vital roles in the development and regeneration of the central nervous system development[12-14]. From 2009 to 2019, this laboratory has investigated cellular responses to the application of EF in the microfluidic system[1,6-8,15-17]. A multichannel, optically transparent, electrotactic (MOE) chip was designed to be suitable for immunofluorescence staining for confocal microscopy. The chip had high optical transparency and good durability and allowed the simultaneous conduct of three independent stimulation experiments and several immunostained conditions in a single study. The microfluidic system is beneficial in simplifying the overall experimental setup and, in turn, the reagent and sample consumption. This paper describes the development of a microfluidic cell culture system that was used for a long-term cell differentiation study.

PROTOCOL:

1. Design and fabrication of the MOE chip

1.1. Draw patterns for individual polymethyl methacrylate (PMMA) layers and the double-sided tape using appropriate software (**Figure 1A, Table of Materials**). Cut both the PMMA sheets and the double-sided tape with a CO_2 laser machine scriber (**Figure 1B**).

1.1.1. Switch on the CO_2 laser scriber and connect it to a personal computer. Open the designed pattern file using the software.

1.1.2. Place the PMMA sheets (275 mm x 400 mm) or double-sided tape (210 mm x 297 mm) on the platform of the laser scriber (**Figure 2A**). Focus the laser onto the surface of the PMMA sheets or the double-sided tape using the auto-focus tool.

1.1.3. Select the laser scriber as the printer, and then “print” the pattern using the laser scriber

to start the direct ablation on the PMMA sheet or double-sided tape and obtain individual patterns on the PMMA sheet or tape (**Figure 2B**).

1.2. Remove the protective film from the PMMA sheets, and clean the surface using nitrogen gas.

NOTE: The drawing of the PMMA pattern and direct machining of the PMMA sheet were performed according to a previous report[17].

1.3. For bonding together multiple layers of PMMA sheets, stack three pieces of 1 mm PMMA sheets (Layers 1, 2, and 3), and bond them under a pressure of 5 kg/cm^2 in a thermal bonder for 30 min at 110 °C to form the flow/electrical stimulation channel assembly (**Figure 2C**).

NOTE: Different batches of commercially obtained PMMA sheet have slightly different glass transition temperature (Tg). The optimal bonding temperature needs to be tested at 5 °C increments close to the Tg.

1.4. Adhere 12 pieces of adaptors to the individual openings in Layer 1 of the MOE chip assembly with fast-acting cyanoacrylate glue.

NOTE: The adaptors are made of PMMA by injection molding. The flat surfaces at the bottom are for connecting to the MOE chip. The adaptors bearing 1/4W-28 female screw thread are for connecting white finger-tight plugs, flat bottom connectors, or Luer adaptors. Be careful when using fast-acting cyanoacrylate glue. Avoid splashing into the eyes.

1.5. Disinfect the 1 mm PMMA substrates (Layers 1–3), the double-sided tape (Layer 4), and the 3 mm optical grade PMMA (Layer 5) using ultraviolet (UV) irradiation for 30 min before assembling the chip (**Figure 1A**).

1.6. Adhere the 1 mm PMMA substrates (Layers 1–3) on the 3 mm optical grade PMMA (Layer 5) with the double-sided tape (Layer 4) to complete the PMMA assembly (Layers 1–5) (**Figure 1A**).

1.7. Prepare the clean cover glass for the assembly on the chip.

1.7.1. Fill a ten-fold dilution of the detergent in a staining jar (see the **Table of Materials**), and clean the cover glass in this detergent using an ultrasonic cleaner for 15 min.

1.7.2. Thoroughly rinse the staining jar under running tap water to remove all traces of the detergent.

1.7.3. Continue rinsing with distilled water to remove all traces of tap water, and repeat step 1.8.2 two times.

1.7.4. Dry the cleaned cover glass by blowing it with nitrogen gas.

1.8. Disinfect the PMMA assembly (Layers 1–5), the double-sided tape (Layer 6), and the cover glass (Layer 7) using UV irradiation inside a biosafety cabinet for 30 min before assembling the chip (**Figure 1A**).

1.9. Adhere the cleaned cover glass (Layer 7) to the PMMA assembly (Layers 1–5) with the double-sided tape (Layer 6) (**Figure 1A**).

1.10. Incubate the MOE chip in a vacuum chamber overnight; use the MOE chip assembly for subsequent procedures (**Figure 3**).

2. Coating poly-L-lysine (PLL) on the substrate in the cell culture regions

2.1. Prepare the polytetrafluoroethylene tube, flat-bottom connector, cone connector, cone-Luer adaptor, white finger-tight plug (also called stopper), Luer adaptor, Luer lock syringe, and black rubber bung (**Figure 4A**, **Table of Materials**). Sterilize all the above components in an autoclave at 121 °C for 30 min.

2.2. Seal the openings of the agar bridge adaptors (**Figure 1A**) with the white finger-tight plugs. Connect the flat-bottom connector to the MOE chip assembly via the medium inlet and outlet adaptors (**Figure 4B**). Connect the cone-Luer adaptor to the 3-way stopcocks.

2.3. Add 2 mL of 0.01% PLL solution using a 3 mL syringe that connects to the 3-way stopcock of the medium inlet (**Figure 4B-①**).

2.4. Connect an empty 3 mL syringe to the 3-way stopcock of the medium outlet (**Figure 4B-②**).

2.5. Fill the cell culture regions with the PLL solution. Manually pump the coating solution back and forth slowly. Close the two 3-way stopcocks to seal the solution inside the culture regions.

2.6. Incubate the MOE chip at 37 °C overnight in an incubator filled with 5% CO_2 atmosphere.

3. Preparation of the salt bridge network

3.1. Following step 2.6, open the two 3-way stopcocks and flush away the bubbles in the channels by manually pumping the coating solution back and forth in the channel using the two syringes.

3.2. Draw 3 mL of complete medium (stem cell maintenance medium consisting of Dulbecco's modified Eagle's medium/Ham's nutrient mixture F-12 (DMEM/F12), 2% B-27 supplement, 20 ng/mL EGF, and 20 ng/mL bFGF) into a 3 mL syringe that connects to the 3-way stopcock of the medium inlet (**Figure 4B-①** and **Figure 4B-③**).

3.3. Add 3 mL of complete medium to replace the coating solution in the cell culture regions. Connect an empty 5 mL syringe to the 3-way stopcock of the medium outlet (**Figure 4B-④**).

3.4. Prepare the salt bridge network (**Figure 5**).

3.4.1. Cut the black rubber bung to produce a gap, and insert the silver (Ag)/silver chloride (AgCl) electrodes through the black rubber bung and into the Luer lock syringe (**Figure 4A**).

3.4.2. Replace the white fingertight plug with the Luer adaptor, and inject 3% hot agarose to fill the Luer adaptor.

NOTE: For the preparation of the hot agarose, dissolve 3 g of agarose powder in 100 mL of phosphate-buffered saline (PBS) and sterilize in an autoclave at 121 °C for 30 min.

3.4.3. Connect the Luer lock syringe to the Luer adaptor. Inject 3% hot agarose through the black rubber bung to fill the Luer lock syringe using the syringe with needle. Allow 10 to 20 mins for the agarose to cool down and solidify.

NOTE: In order to increase the volume capacity of the agarose, the Luer lock syringe is mounted on the Luer adaptor (**Figure 4** and **Figure 5**). Then, the large electrodes are inserted into the Luer lock syringe. The electrode is capable of providing a stable electrical stimulation for the long-term experiment.

4. Preparation of mNPCs

4.1. Culture the mNPCs[1] in the complete medium in a 25T cell culture flask at 37 °C in an incubator filled with 5% CO_2 atmosphere. Subculture the cells every 3–4 days, and perform all experiments with cells that have undergone 3–8 passages from the original source.

4.2. Transfer the cell suspension to a 15 mL conical tube, and spin-down the neurospheres at 100 × *g* for 5 min. Aspirate the supernatant, and wash the neurospheres with 1x Dulbecco's PBS (DPBS). Spin-down the neurospheres at 100 × *g* for 5 min.

4.3. Aspirate the 1x DPBS and then resuspend the neurospheres in the complete medium. Mix thoroughly and gently.

4.4. Add 1 mL of the neurosphere suspension using a 1 mL syringe that connects to the 3-way stopcock of the outlet (**Figure 4B-②**).

5. Setup of the microfluidic system for DC pulse stimulation (Figure 6)

5.1. Install the cell-seeded MOE chip onto the transparent ITO heater that is fastened on a programmable X-Y-Z motorized stage.

NOTE: The ITO surface temperature is controlled by a proportional–integral–derivative controller and maintained at 37 °C. A K-type thermocouple is clamped between the chip and the ITO heater to monitor the temperature of the cell culture regions within the chip. The MOE chip is installed on a programmable X-Y-Z motorized stage and is suitable for automatic time-lapse image

acquisition at individual channel sections. The fabrication of the ITO heater and the setup of the cell culture heating system have been described previously[18,19].

5.2. Infuse the mNPCs by manual pumping into the MOE chip via the medium outlet. Incubate the cell-seeded MOE chip on the 37 °C ITO heater for 4 h.

5.3. After 4 h, pump the complete medium through the MOE chip via the medium inlet at a flow rate of 20 µL/h using a syringe pump.

NOTE: The mNPCs are grown and maintained in the chip for an additional 24 h before EF stimulation to allow cell attachment and growth. The waste liquid is collected in an empty 5 mL syringe connected to the 3-way stopcock of the outlet, shown as "waste" in **Figure 6A**. The MOE microfluidic system configuration is shown in **Figure 6**. This microfluidic system provides a continuous supply of nutrition to the cells. The complete fresh medium is continuously pumped into the MOE chip to maintain a constant pH value. Therefore, the cells can be cultured outside a CO_2 incubator.

5.4. Use electrical wires to connect an EF multiplexer to the MOE chip via the Ag/AgCl electrodes on the chip. Connect an EF multiplexer and a function generator to an amplifier to output square-wave DC pulses with a magnitude of 300 mV/mm at a frequency of 100 Hz at 50% duty cycles (50% time-on and 50% time-off) (**Figure 6B**).

5.4.1. Connect the electrical wires to the EF multiplexer. Connect the electrical wires to the MOE chip via the Ag/AgCl electrodes.

5.4.2. Connect the EF multiplexer to the amplifier using electrical wires. Connect the function generator to the amplifier and the digital oscilloscope.

NOTE: The EF multiplexer is a circuit that includes the impedance of the culture chamber in the circuit and connects all individual chambers in a parallel electronic network. Each of the three culture chambers is electrically connected in serial to a variable resistor (Vr) and an ammeter (shown as µA in **Figure 6A**) in the multiplexer. The electric current through each culture chamber is varied by controlling the Vr, and the current is shown on the corresponding ammeter. The electric field strength in each cell culture region was calculated by Ohm's Law, $I = \sigma EA$, where I is the electric current, σ (set as 1.38 $S \cdot m^{-1}$ for DMEM/F12[20]) is the electrical conductivity of the culture medium, E is the electric field, and A is the cross-sectional area of the electrotactic chamber. For the cell culture region dimension shown in **Figure 1**, the electric current is ~87 mA and ~44 mA for DC and DC pulse at 50% duty cycle, respectively.

5.5. Subject the mNPCs to square DC pulses with a magnitude of 300 mV/mm at the frequency of 100 Hz for 48h. Continuously pump the complete medium at a rate of 10 µL/h to supply adequate nutrition to the cells and to maintain a constant pH value in the medium.

6. Immunofluorescence assays of mNPCs after pulsed DC stimulation

NOTE: In this step, all reagent is pumped via the medium inlet using a syringe pump.

6.1. After 3, 7, or 14 days in vitro (DIV) culturing after seeding[1], wash the cells with 1x PBS at a flow rate of 25 µL/min for 20 min.

6.2. Fix the cells with 4% paraformaldehyde (PFA). Pump 4% PFA into the chip at a flow rate of 25 µL/min for 20 min to replace the 1x PBS. To replace the 4% PFA, wash the cells with 1x PBS at a flow rate of 25 µL/min for 20 min.

6.3. Pump 0.1% Triton X-100 into the chip at a flow rate of 50 µL/min for 6 min to permeabilize the cells. Reduce the flow rate to 50 µL/h for an additional 30 min to react with the cells. To replace the 0.1% Triton X-100, wash the cells with 1x PBS at a flow rate of 50 µL/min for 6 min.

6.4. Block the cells with PBS containing 1% bovine serum albumin (BSA) to reduce nonspecific antibody binding. Pump 1% BSA into the chip at a flow rate of 50 µL/min for 6 min. Reduce the flow rate to 100 µL/h and pump for 1 h.

6.5. Pump the antibodies for double immunostaining into the chip at a flow rate of 50 µL/min for 6 min, and incubate the chip for 18 h at 4 °C. Wash the cells with 1x PBS at a flow rate of 50 µL/min for 15 min.

6.6. Pump the Alexa Fluor-conjugated secondary antibodies into the chip at a flow rate of 50 µL/min for 6 min. Reduce the flow rate to 50 µL/h, and pump the antibodies for 1 h at room temperature in the dark. Wash the cells with 1x PBS at a flow rate of 50 µL/min for 15 min.

6.7. For nuclear staining, pump Hoechst 33342 into the chip at a flow rate of 20 µL/min for 10 min at room temperature in the dark. Wash the cells with 1x PBS at a flow rate of 50 µL/min for 15 min.

6.8. After immunostaining, observe the cells using a confocal fluorescence microscope.

7. Image analysis and data processing

7.1. Analyze the fluorescent images using software with built-in measurement tools (see the **Table of Materials**).

7.2. Compare the Hoechst-counterstained nuclei (total number of cells) in the control and treatment groups, and calculate the percentage of cells expressing each phenotypic marker.

REPRESENTATIVE RESULTS:

The detailed configuration of the MOE chip is shown in **Figure 1**. The microfluidic chip provides a beneficial approach for reducing the experimental setup size, sample volume, and reagent volume. The MOE chip was designed to perform three independent EF stimulation experiments and several immunostaining conditions simultaneously in a single study (**Figure 3**). In addition, the MOE chip, which has a high optical transparency is suitable for confocal microscopy examinations. The MOE chip is also designed to investigate the effects of different cell culture conditions (e.g., multiple EF stimulation, several drugs, different coating substrate, multiple series of cells) simultaneously in a single experiment.

The mNPCs were exposed to square-wave DC pulses (magnitude 300 mV/mm at a frequency of 100 Hz). The DC pulse stimulation was conducted for 48 h. The differentiated cells were immunostained with Tuj1 (neuron-specific class III β-tubulin), glial fibrillary acidic protein (GFAP to identify astrocytes), and oligodendrocyte marker O4. After the DC pulse treatment, the mNPCs expressed significantly high numbers of neurons (Tuj1+ cells) at DIV 7. At DIV 3, astrocytes (GFAP+ cells) were present at relatively higher levels in the stimulation groups than in the control (CTL) group. Compared with the CTL group, oligodendrocytes (O4+ cells) were significantly higher in the stimulation group at DIV 7 and DIV 14 (**Figure 7**). These results show that the DC pulse stimulation resulted in mNPCs differentiating into neurons, astrocytes, and oligodendrocytes simultaneously in stem cell maintenance medium. These results suggest that the MOE microfluidic system is suitable for a long-term cell differentiation study by microscopy.

FIGURE LEGENDS:

Figure 1: The detailed configuration of the multichannel optically transparent electrotactic chip. (**A**) Exploded view of the MOE chip assembly. The MOE chip consists of PMMA sheets (50 mm x 25 mm x 1 mm), double-sided tape (50 mm x 25 mm x 0.07 mm), adaptors (10 mm x 10 mm x 6 mm), optical grade PMMA sheet (50 mm x 75 mm x 3 mm), double-sided tape (24 mm x 60 mm x 0.07 mm), and a cover glass (24 mm × 60 mm). There are three cell culture chambers in the MOE chip. The MOE chip has connecting holes for the medium inlet/outlet and the agar salt bridges. Cells were cultured in the cell culture region (width 3 mm x length 42 mm x height 0.07 mm). **Figure 1A** has been modified from Chang et al.[6]. (**B**) Photograph of the MOE chip comprising adaptors, PMMA sheets, double-sided tape, and cover glass. Abbreviations: MOE= multichannel optically transparent electrotactic; PMMA = polymethyl methacrylate.

Figure 2: The fabrication and assembling processes of the MOE chip. (**A**) The designed patterns of the PMMA sheets or double-sided tape were fabricated using laser micromachining. (**B**) The individual PMMA sheets were cut by a CO_2 laser scriber. (**C**) The multiple layers of the cleaned PMMA sheets were bonded together by a thermal bonder. Abbreviations: MOE= multichannel optically transparent electrotactic; PMMA = polymethyl methacrylate; CO_2 = carbon dioxide.

Figure 3: A photograph of the MOE chip. This figure has been modified from Chang et al.[6]. Abbreviation: MOE= multichannel optically transparent electrotactic.

Figure 4: Medium and electrical connection to the MOE chip. (**A**) Photograph of the components for the medium flow network and the EF network in the MOE microfluidic system, including the PTFE tube, flat-bottom connector, cone connector, cone-Luer adaptor, white finger-tight plug, Luer adaptor, Luer lock syringe, black rubber bung, and the Ag/AgCl electrodes. (**B**) Photograph of the configuration for the medium flow network. Abbreviations: MOE= multichannel optically transparent electrotactic; EF = electric field; PTFE = polytetrafluoroethylene; Ag = silver; AgCl = silver chloride.

Figure 5: A photograph showing the MOE chip on a microscope. Abbreviations: MOE= multichannel optically transparent electrotactic; Ag = silver; AgCl = silver chloride; ITO = indium–tin–oxide.

Figure 6: The configuration and the system used for the DC pulse stimulation. (**A**) The configuration of the entire system for the DC pulse stimulation. The syringes connected to the MOE chip were used for medium infusion and waste efflux. The DC pulse in the chip was provided by a power supply conducted through the Ag/AgCl electrodes. The device setup was installed on the X-Y-Z motorized stage of an inverted phase-contrast microscope equipped with a digital camera. (**B**) A photograph showing the setup on a laboratory bench. Abbreviations: MOE= multichannel optically transparent electrotactic; Ag = silver; AgCl = silver chloride; ITO = indium–tin–oxide; EF = electric field.

Figure 7: Differentiation of the mNPC cells in the control group (CTL) and in the DC pulse stimulation group at DIV 3, 7, and 14. The percentage of neuron (Tuj1+ cells), astrocytes (GFAP+ cells), and oligodendrocytes (O4+ cells) in (**A–C**) the CTL group and (**D–F**) in the stimulation (DC pulses) group. This figure has been published by Chang et al.[1]. Abbreviations: CTL: control; DC = direct current; Tuj1 = neuron-specific class III β-tubulin; GFAP = glial fibrillary acidic protein; O4 = oligodendrocyte marker O4.

DISCUSSION:

During the fabrication of the MOE chip, the adaptors are attached to the Layer 1 of the MOE chip with fast-acting cyanoacrylate glue. The glue is applied to 4 corners of the adaptors, and then pressure is applied evenly over the adaptors. Excess amount of glue must be avoided to ensure complete polymerization of the glue. Moreover, the completed MOE chip assembly is incubated in a vacuum chamber. This step helps to remove the bubbles between the PMMA layer, the double-sided tape, and the cover glass.

The choice of the electrode material is based on the fact that chloride ions, which are abundantly present in the medium, are the electrolytic products flowing through the cell culture region. During the EF stimulation experiment, the pH around the electrodes remained constant. A simpler configuration using platinum (Pt) as the electrode material electrolyzes water and generates hydrogen ions (H^+) and hydroxide ions (OH^-) at the positive electrode and the negative electrode, respectively, inducing pH changes in the culture region. Avoiding the use of Pt

electrodes circumvents the problem of pH changes during the EF stimulation experiment.

The hot agarose and bubble-free agarose are essential during the preparation of the salt bridge network. The hot agarose has high fluidity and can be easily injected into the salt bridge network. Connect the Luer lock syringe to the Luer adaptor after injecting the 3% hot agarose into the Luer adaptor. During this step, the agarose will be pushed up into the Luer lock syringe so that a bubble-free firm connection of the salt bridge network can be achieved. Bubbles in the salt bridges increase the electrical resistance and hence, the anticipated electric current cannot be reached. After the agarose injection, it is important to wait for the agarose to cool down and solidify at room temperature for 10–20 min to prevent the formation of solidified agarose debris in the cell culture region.

The MOE chip is placed onto an ITO heater that is locked on a programmable X-Y-Z motorized stage. The entire system is built onto an inverted phase-contrast microscope equipped with a digital camera to monitor cell differentiation within the cell culture regions in the chip. It is convenient to observe the cell morphology and acquisition of the automatic time-lapse images in the MOE microfluidic system outside an incubator. This microfluidic system not only shortens the required experimental time, but also increases the accuracy of control on the microenvironment.

The mNPC cells grow as a suspension in culture media. However, mNPCs adhering to the PLL-coated plate in the MOE chip are critical for differentiation. Neurospheres formed by 30–40 cells are preferred for initiating mNPC differentiation. Overgrowth of mNPCs will impair cell survival during the differentiation process. Furthermore, after the pulsed DC stimulation, the immunofluorescence staining experimental can be affected by the flow rate. Hence, use several flow rates for different steps to avoid detaching cells during the wash.

In this study, a limitation of this technique is that the MOE chip cannot be reused because of the difficulty in thorough cleaning of the chip. However, the MOE chip can be placed under a phase-contrast microscope or a scanning confocal microscope directly. The water-tight design of the reported microfluidic system ensures that buffer/medium evaporation does not occur, maintaining the accurate concentration of the buffer/medium and the corresponding electrical properties. By reducing reagent volumes and the corresponding operation time, the MOE microfluidic system provides an efficient approach for studying cell differentiation.

A previous study has shown that EGF and bFGF promote NPC survival, expansion, and maintenance in the undifferentiated state[4]. In this study, the DC pulses induced the differentiation of the mNPCs in the stem cell maintenance medium that contained EGF and bFGF. Previous studies have reported that EF promotes differentiation of NPCs into neurons and/or astrocytes in differentiation medium without EGF and bFGF[14,21,22]. These results show that the mNPCs differentiated into neurons, astrocytes, and oligodendrocytes after the DC pulse stimulation. They also suggest that simple DC pulse treatment could control the fate of NPCs. With further optimization on the stimulation time, EF strength, or duty cycle, DC pulses may be applied to manipulate NPC differentiation and may be used for the development of therapeutic

strategies that employ NPCs to treat nervous system disorders.

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DISCLOSURES:

The authors have nothing to disclose.

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21 Chang, K. A. et al. Biphasic electrical currents stimulation promotes both proliferation and differentiation of fetal neural stem cells. *PLoS One.* **6** (4), e18738 (2011).
22 Lim, J. H., McCullen, S. D., Piedrahita, J. A., Loboa, E. G., Olby, N. J. Alternating current electric fields of varying frequencies: effects on proliferation and differentiation of porcine neural progenitor cells. *Cell Reprogram.* **15** (5), 405–412 (2013).

Figure 1

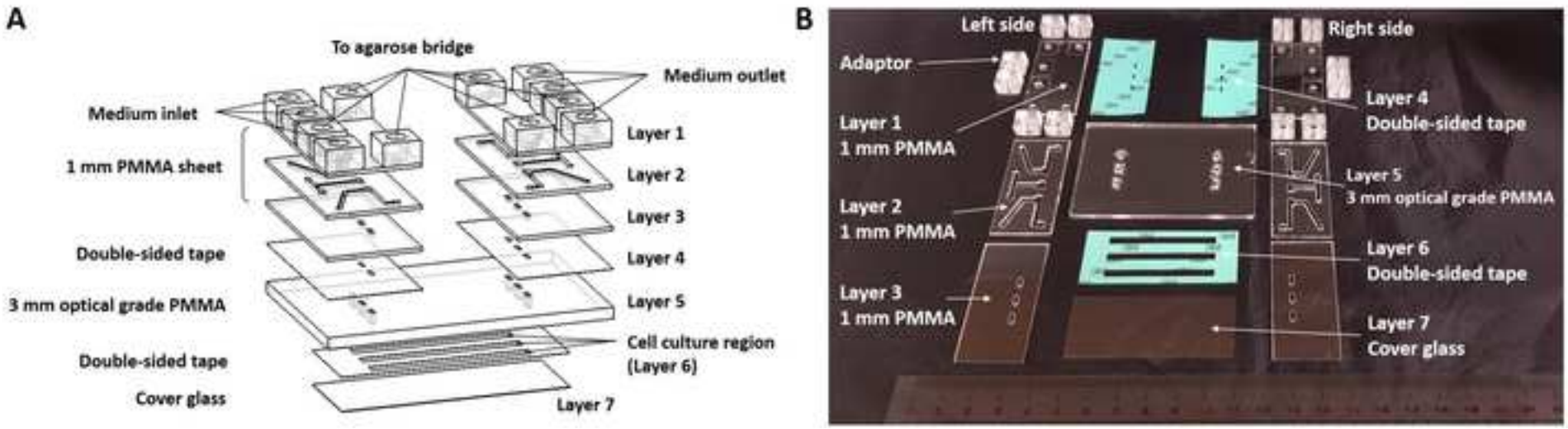


Figure 2

Click here to access/download;Figure;Fig_2.jpg

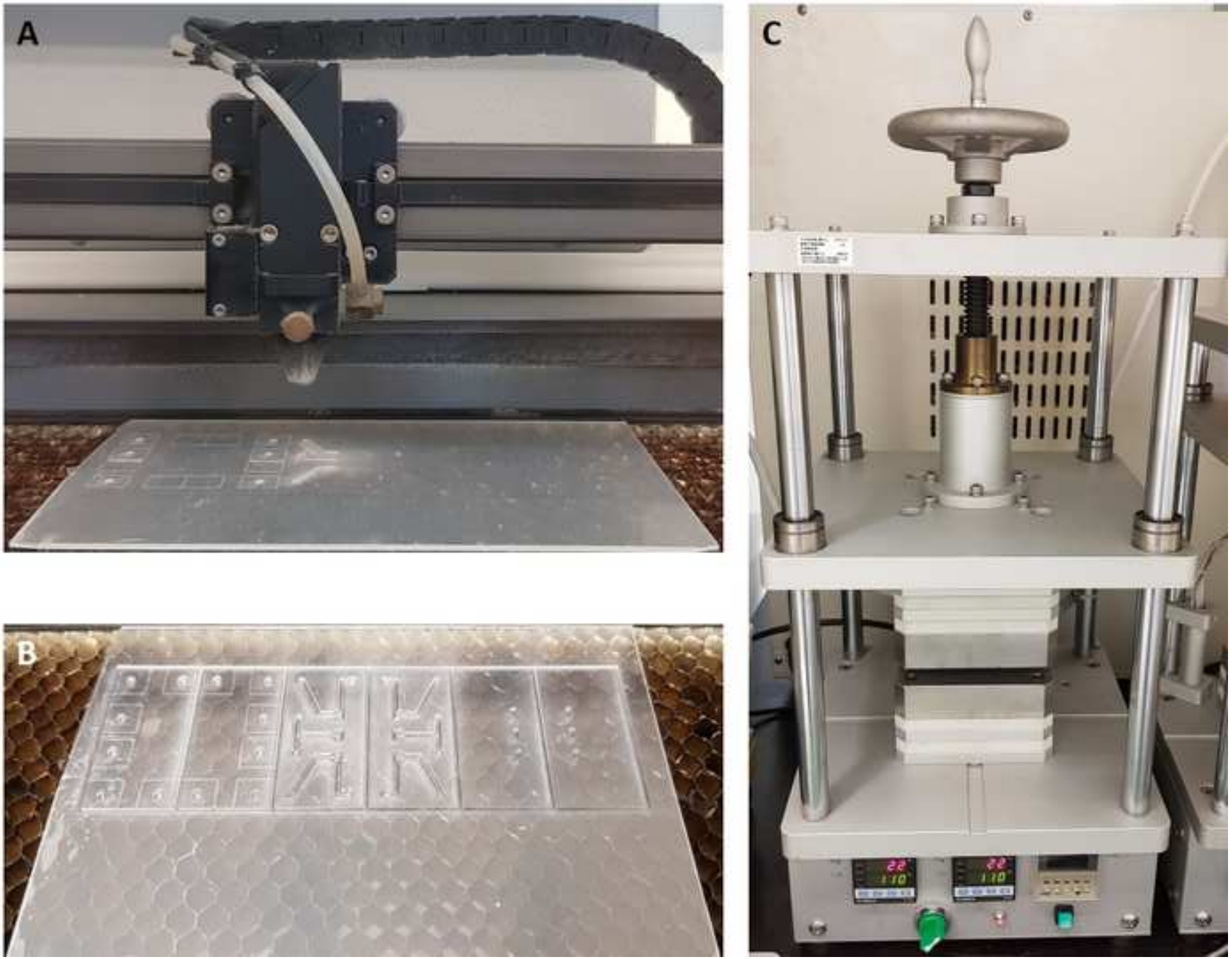


Figure 3

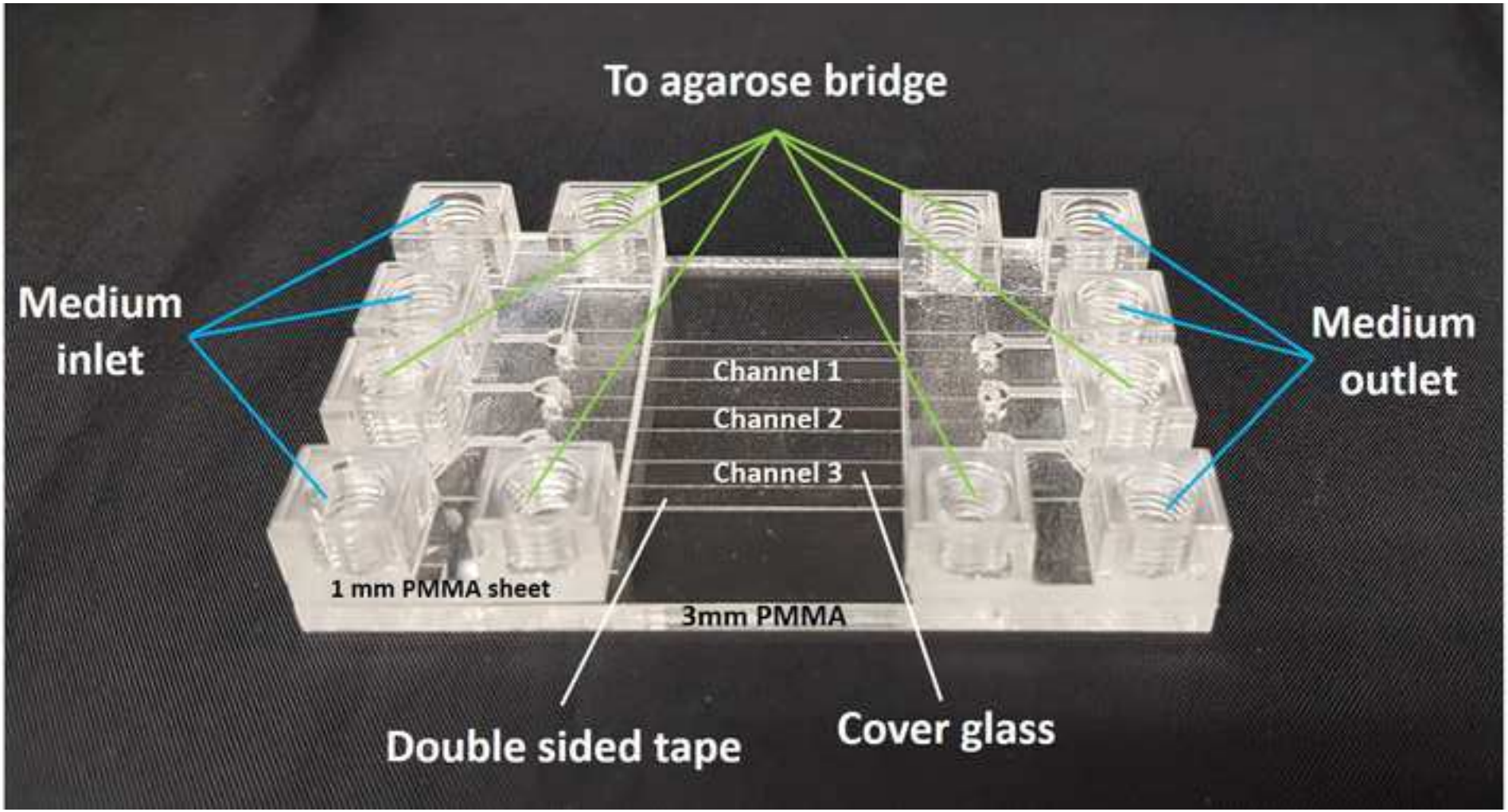
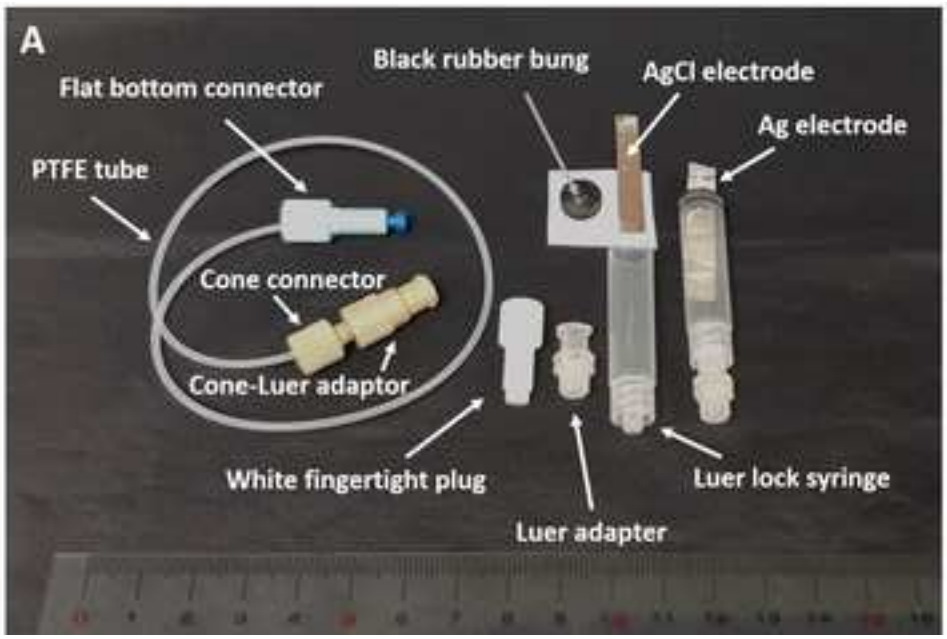


Figure 4



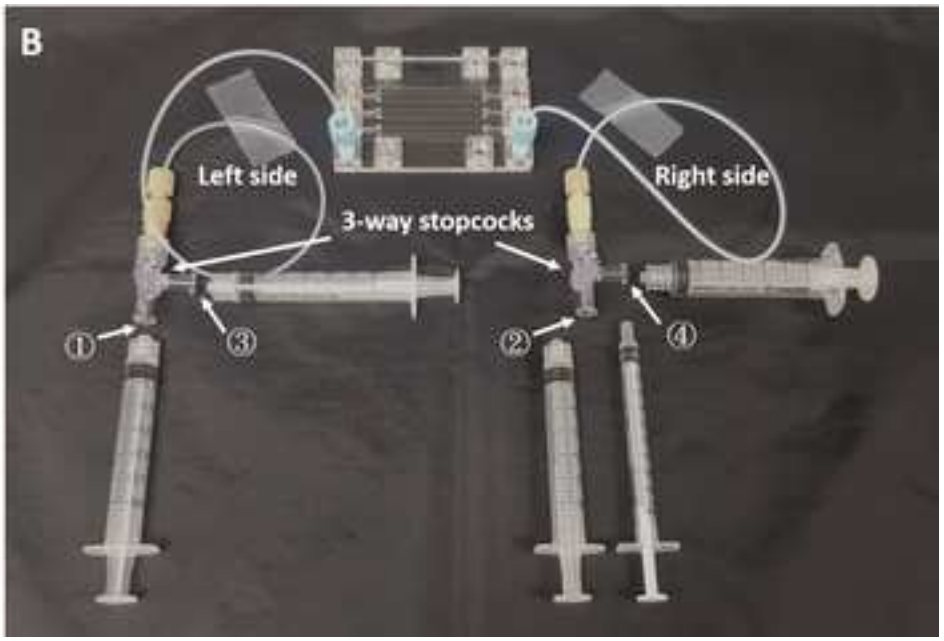


Figure 5

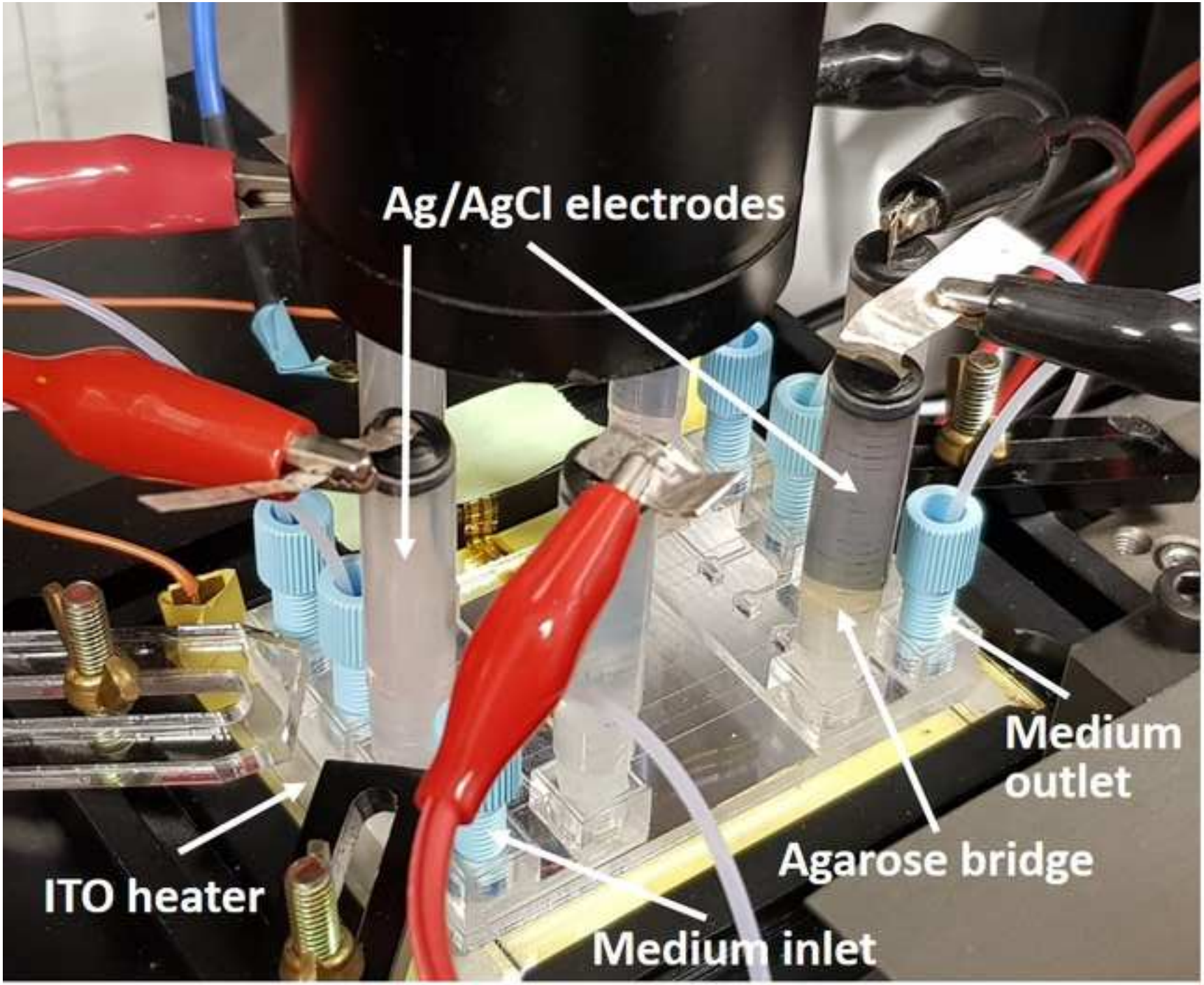
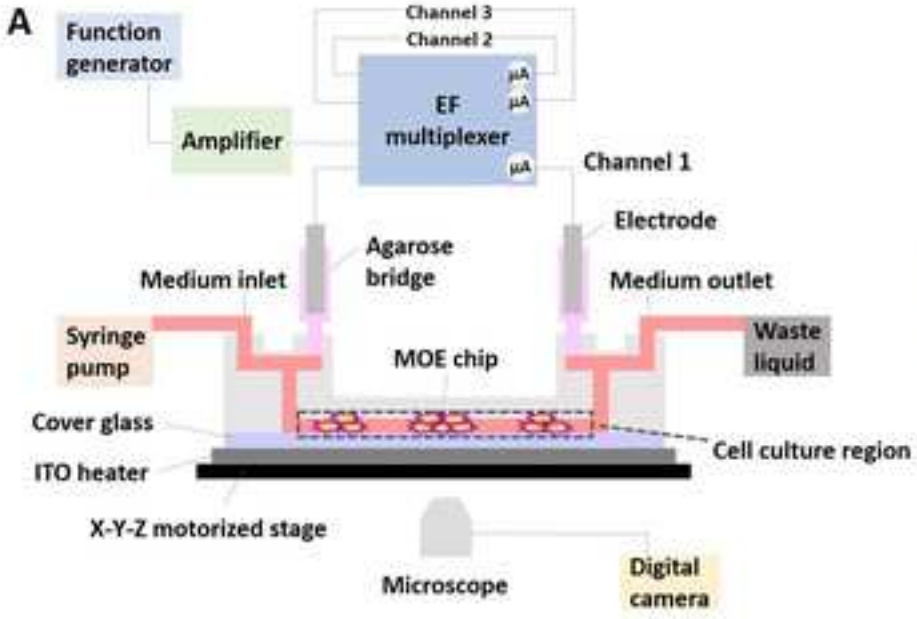


Figure 6



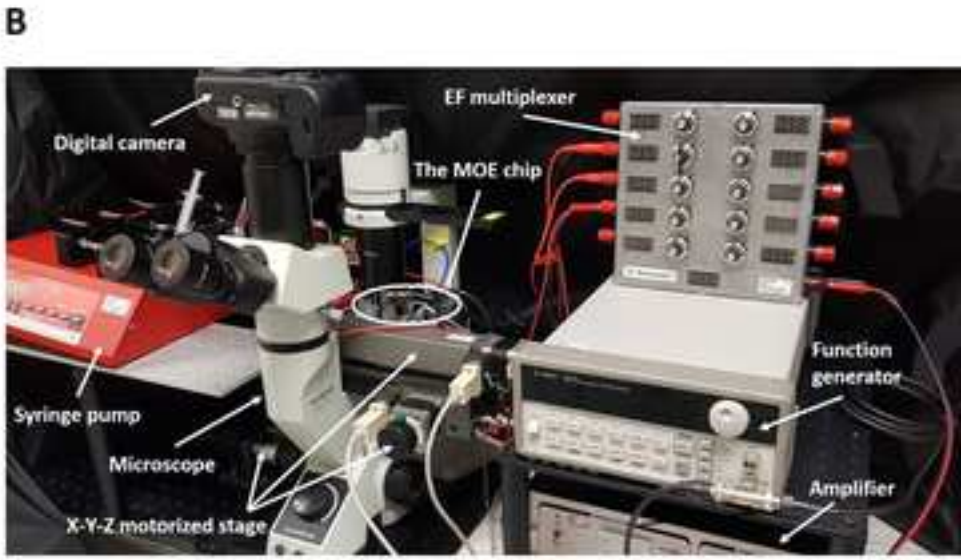
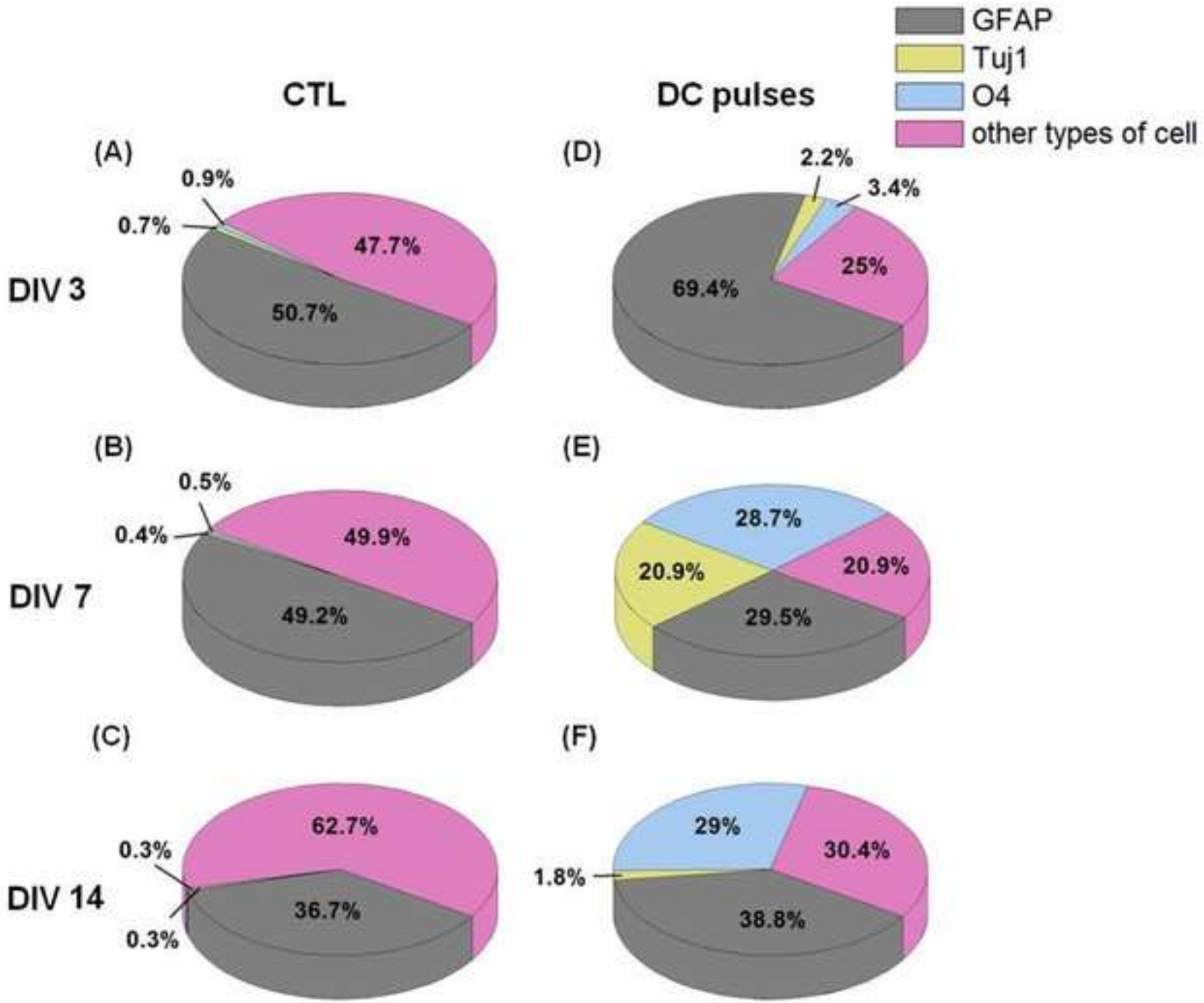


Figure 7



Name of Material/ Equipment	Company	Catalog Number
1 mm PMMA substrates (Layers 1-3)	BHT	K2R20
15 mL plastic tube	Protech Technology Enterprise Co., Ltd	CT-15-PL-TW
3 mL syringe	TERUMO	DVR-3413
3 mm optical grade PMMA (Layer 5)	CHI MEI Corporation	ACRYPOLY PMMA Sheet
3-way stopcock	NIPRO	NCN-3L
5 mL syringe	TERUMO	DVR-3410
Adaptor	Dong Zhong Co., Ltd.	Customized
Agarose	Sigma-Aldrich	A9414
Amplifier	A.A. Lab Systems Ltd	A-304
AutoCAD software	Autodesk	Educational Version
B-27 supplement	Gibco	12587-010
Basic fibroblast growth factor (bFGF)	Peprotech	AF-100-18B
Black rubber bung	TERUMO	DVR-3413
Bovine serum albumin (BSA)	Sigma-Aldrich	B4287
Centrifuge	HSIANGTAI	CV2060
CO_2 laser scriber	Laser Tools and Technics Corp.	ILS-II
Cone connector	IDEX Health & Science	F-120X
Cone-Luer adaptor	IDEX Health & Science	P-659
Confocal fluorescence microscope	Leica Microsystems	TCS SP5
Digital camera	OLYMPUS	E-330

Digital oscilloscope	Tektronix	TDS2024
Double-sided tape	3M	PET 8018
Dulbecco's modified Eagle's medium/Ham's nutrient mixture F-12 (DMEM/F12)	Gibco	12400024
Dulbecco's phosphate-buffered saline (DPBS)	Gibco	21600010
EF multiplexer	Asiatic Sky Co., Ltd.	Customized
Epidermal growth factor (EGF)	Peprotech	AF-100-15
Fast-acting cyanoacrylate glue	3M	7004T
Flat bottom connector	IDEX Health & Science	P-206
Function generator	Agilent Technologies	33120A
Goat anti-mouse IgG H&L (Alexa Fluor 488)	Abcam	ab150117
Goat anti-rabbit IgG H&L (Alexa Fluor 555)	Abcam	ab150086
Hoechst 33342	Invitrogen	H3570
ImageJ software	National Institutes of Health	1.48v
Indium–tin–oxide (ITO) glass	Merck	300739
Inverted phase contrast microscope	OLYMPUS	CKX41
K-type thermocouple	Tecpel	TPK-02A
Luer adapter	IDEX Health & Science	P618-01
Luer lock syringe	TERUMO	DVR-3413
Mouse anti-GFAP	eBioscience	14-9892

Oligodendrocyte marker O4 antibody	R&D Systems	MAB1326
Paraformaldehyde (PFA)	Sigma-Aldrich	P6148
Phosphate buffered saline (PBS)	Basic Life	BL2651
Poly-L-Lysine (PLL)	SIGMA	P4707
Precision cover glasses thickness No. 1.5H	MARIENFELD	107242
Programmable X-Y-Z motorised stage	Tanlian Inc	Customized
Proportional–integral–derivative (PID) controller	Toho Electronics	TTM-J4-R-AB
PTFE tube	Professional Plastics Inc. Taiwan Branch	Outer diameter 1/16 Inches
Rabbit anti-Tuj1	Abcam	ab18207
Syringe pump	New Era Systems Inc	NE-1000
TFD4 detergent	FRANKLAB	TFD4
Thermal bonder	Kuan-MIN Tech Co.	Customized
Triton X-100	Sigma-Aldrich	T8787
Ultrasonic cleaner	LEO	LEO-300S
Vacuum chamber	DENG YNG INSTRUMENTS CO., Ltd.	DOV-30
White fingertight plug	IDEX Health & Science	P-316

Comments/Description
Polymethyl methacrylate (PMMA), http://www.bothharvest.com/zh-tw/product-421076/Optical-PMMA-Non-Coated-BHT-K2Rxx-xx=-thickness-choices.html
Conical bottomed tube with cap, assembled, presterilized 3 mL oral syringes, without needle
Optical grade PMMA
Sterile disposable 3-way stopcock
5 mL oral syringes, without needle
PMMA adaptor
Agarose, low gelling temperature
High voltage amplifier
Drafting
B-27 supplement (50x), minus vitamin A
Also called recombinant human FGF-basic
From 3 mL oral syringes, without needle
Blocking reagent Centrifuge Purchased from http://www.lttcorp.com/index.htm
One-piece fingertight 10-32 coned, for 1/16" OD natural
Luer Adapter 10-32 Female to Female Luer, PEEK
Leica TCS SP5 user manual, http://www3.unifr.ch/bioimage/wp-content/uploads/2013/10/User-Manual_TCS_SP5_V02_EN.pdf
Automatic time-lapse image acquisition

Measure voltage or current signals over time in an electronic circuit or component to display amplitude and frequency.
Purchased from http://en.thd.com.tw/

DMEM/F-12, powder, HEPES

DPBS, powder, no calcium, no magnesium

Monitor and control the electric current in individual channels

Also called recombinant human EGF

Strength instant adhesive (liquid)

Flangeless male nut Delrin, 1/4-28 flat-bottom, for 1/16" OD blue

High-performance 15 MHz synthesized function generator with built-in arbitrary waveform capability

Goat anti-mouse IgG H&L (Alexa Fluor 488) preadsorbed

Goat polyclonal secondary antibody to rabbit IgG - H&L (Alexa Fluor 555), preadsorbed

Nuclear staining

Analyze the fluorescent images

For ITO heater

For cell morphology observation

Temperature thermocouples

Luer adapter female Luer to 1/4-28 male polypropylene

For agar salt bridges

Astrocytes marker

Oligodendrocytes marker

Fixing agent

Washing solution

Coating solution

https://www.marienfeld-superior.com/precision-cover-glasses-thickness-no-1-5h-tol-5-m.html

Purchased from http://www.tanlian.tw/ndex.files/motort.htm

Temperature controller

White translucent PTFE tubing

Neuron marker

NE-1000 programmable single syringe pump

Cover glass cleaner

Purchased from http://kmtco.com.tw/

Permeabilized solution

Ultrasonic steri-cleaner

Vacuum drying oven

1/4-28 Flat-Bottom, https://www.idex-hs.com/store/fluidics/fluidic-connections/plug-teflonr-pfa-1-4-28-flat-bottom.html

Dear Editor and reviewers,

The authors appreciate your kind suggestions regarding our manuscript. We want to thank you for giving us the opportunity to revise our manuscript according to the valuable comments of the editor and reviewers. We are answering comments as the following:

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations at first use.

Answer:

Thanks for your friendly reminder. The revision (blue text of the revised manuscript) has been made according to the suggestions of the editors.

2. Unfortunately, there are sections of the manuscript that show overlap with previously published work. Please revise the following lines: 77-96, 120-125, 244 (The required)-248, 266-268, 299-301, 328 (After the)-332, 332 (Our)-336, 346-348, 351-354, 370-374.

Answer:

The revision (blue text of the revised manuscript) has been made according to the suggestions of the editors.

3. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

Answer:

The revision (blue text of the revised manuscript) has been made according to the suggestions of the editors.

4. 1.2: How big are the PMMA sheets?

Answer:

We added the descriptions in 1.2 of the revised manuscript, “1.2.3 Place the PMMA sheets (275 × 400 mm) or double-sided tape (210 × 297 mm) on the platform of the laser scriber (Figure 2A).”

5. 1.4: How is the temperature to be determined for thickness of the raw PMMA sheets?

Answer:

We usually bond three layers of 1 mm PMMA sheets in a thermal bonder for 30 min at 110 °C. Different batch of commercially obtained PMMA sheet has slightly different glass transition temperature (Tg). The optimal bonding temperature needs to be tested at 5 °C increments relative to the Tg.

6. Note after 1.5: what bears 1/4W-28 female screw thread? The adaptor?

Answer:

We modified the descriptions in 1.5 in the revised manuscript, “NOTE: The adaptors are made of PMMA by injection molding. The flat surfaces at the bottom are for connecting to MOE chip. The adaptors bear 1/4W-28 female screw thread are for connecting white fingertight plug, flat bottom connector or Luer adaptor.”

7. 2.1: How do you prepare these components; what are their specifications? If you have described them elsewhere, please cite a reference.

Answer:

We added the descriptions in 2.1 of the revised manuscript, “The detail of these components is shown in the material list.”

8. 4.1: Please move text about mNPCs from Prof. Tang K. Tang to the Acknowledgements section.

Answer:

The revision has been made according to the suggestions of the editors.

9. Note after 5.4: Please ensure that all steps that will help readers/viewers understand how to do the experiment are explained and filmed. For example, how to use the EF multiplexer to monitor the electric current.

Answer:

We added the descriptions in 5.4 of the revised manuscript to explain the steps to use the EF multiplexer and to monitor the electric current.

5.4.1. Connect the electrical wires to the EF multiplexer.
5.4.2. Connect the electrical wires to the MOE chip via the Ag/AgCl electrodes.
5.4.3. Connect the EF multiplexer to the amplifier using electrical wires.
5.4.4. Connect the function generator to the amplifier and the digital oscilloscope.

NOTE: The EF multiplexer is a circuit that includes the impedance of the culture chamber in the circuit and connects all individual chambers in a parallel electronic network. Each of the three culture chambers is electrically connected in serial to a variable resistor (Vr) and an ammeter (shown as mA in Figure 6A) in the multiplexer. The electric current through each culture chamber is varied by controlling the Vr, and the current is shown on the corresponding ammeter. The electric field strength in each cell culture region was calculated by Ohm's Law, $I= \sigma EA$, where I is the electric current, σ (set as 1.38 $S \cdot m^{-1}$ for DMEM/F12[1]) is the electrical conductivity of the culture medium, E is the electric field, and A is the cross-sectional area of the electrotactic chamber.

10. Representative Results: the second paragraph (lines 314-323) may be more helpful as a note in the protocol at the relevant place.

Answer:

We added the corresponding descriptions in the revised manuscript.

3.5.3, "NOTE: The Luer lock syringe is mounted on the Luer adaptor (Figure 4 and Figure 5) to increase the volume capacity of the agarose. In this way, relatively large electrodes can be inserted into the agarose to provide stable electrical stimulation to the cells for a long period of time."

5.1, "NOTE: The MOE chip is installed on a programmable X-Y-Z motorized stage and is suitable for automatic time-lapse image acquisition at individual channel sections…."

5.3, "NOTE: The MOE microfluidic system configuration is illustrated in Figure 6. This microfluidic system provides a continuous supply of nutrition to the cells. The complete fresh medium is continuously pumped into the MOE chip to maintain a constant pH value. Therefore, the cells can be cultured outside a CO_2 incubator."

5.4, "NOTE: The EF multiplexer is a circuit that includes the impedance of the culture chamber in the circuit and connects all individual chambers in a parallel electronic network.…."

11. As we are a methods journal, please revise the Discussion (3-6 paragraphs) to add the following with citations:
a) Any limitations of the technique
b) The significance with respect to existing methods

Answer:

The revision has been made according to the suggestions of the editors. We added the descriptions in the discussion of the revised manuscript, "In this study, the limitations of the technique are that the MOE chip cannot be reused because of the difficulty in thorough cleaning of the chip. However, the MOE chip can be placed under a phase-contrast microscope or a scanning confocal microscope directly. The water tight design of the reported microfluidic system ensures that buffer/medium evaporation does not occur, maintaining the accurate concentration of the buffer/medium and the corresponding electrical properties. By reducing reagent volumes and the corresponding operation time, the MOE microfluidic system provides an efficient approach for studying cell differentiation. A previous study has shown the EGF and bFGF promote NPC survival, expansion, and maintenance at the undifferentiated state[2]. In our study, the DC pulse induced the differentiation of the mNPCs in the stem cell maintenance medium that contained EGF and bFGF. Previous studies have reported that EF promotes differentiation of NPCs into neuron and/or astrocytes in differentiation medium that excludes EGF and bFGF[3-5]. Our results showed that the mNPCs differentiated into neurons, astrocytes, and oligodendrocytes after the DC pulse stimulation. These results suggest that simple DC pulse treatment could control the fate of NPCs. With further optimization on the stimulation time, electric-field strength, or duty cycle, DC pulses may be applied to manipulate NPC differentiation and may be used for the development of therapeutic strategies that employ NPCs to treat nervous system disorders."

12. Please do not abbreviate journal titles in the reference list.

Answer:

The revision has been made according to the suggestions of the editors.

Reviewers' comments:

Reviewer #1:

Minor Concerns:

1. In line 409 and 410, the authors claim that their microfluidic system

brings down experimental time and increases experimental accuracy. It would be nice if the authors could back this statement by some relevant data, comparing this method with other technologies by defining appropriate performance metrics. Moreover, the term 'experimental accuracy' needs to be clearly defined.

Answer:

The water tight design of the reported microfluidic system ensures that buffer/medium evaporation does not occur, maintaining the accurate concentration of the buffer/medium and the corresponding electrical properties.

The multichannel design of the system allows multiple conditions (multiple EFs and/or types of medium) to be tested in a single experiment. We modified the descriptions in the abstract and the discussion of the revised manuscript, **"**This microfluidic system not only shortens the required experimental time but also increases the accuracy of control on the microenvironment."

2. In line 418 and 419, the authors state that their system provides an efficient approach for studying cell differentiation. A concise comparison with multiple other existing methods would help to substantiate the claims.

Answer:

The revision has been made according to the suggestions of the reviewer. We added the descriptions in the discussion of the revised manuscript, "A previous study has shown the EGF and bFGF promote NPC survival, expansion, and maintenance at the undifferentiated state[2]. In our study, the DC pulse induced the differentiation of the mNPCs in the stem cell maintenance medium that contained EGF and bFGF. Previous studies have reported that EF promotes differentiation of NPCs into neuron and/or astrocytes in differentiation medium, which excludes EGF and bFGF[3-5]. Our results showed that the mNPCs differentiated into neurons, astrocytes, and oligodendrocytes after the DC pulse stimulation."

3. Figure 2D is not very clear, a better high-resolution image is needed. Some of the text within the image is not legible.

Answer:

Thanks for your friendly reminder. The revision has been made according to the suggestions of the reviewer. Figure 2D is modified as Figure 3 of the revised manuscript.

4. A clearer image for figure 3C would be helpful.

Answer:

The revision has been made according to the suggestions of the reviewer. Figure 3C is modified as Figure 5 of the revised manuscript.

Reviewer #3:

Major Concerns:

1. There are lots of articles about the electric field stimulation on the cells for neuron differentiation using different pulse width, frequency, magnitude, or waveform.

Why was the stimulation pulse with a frequency of 100 Hz and magnitude of 300 mV/mm selected?

Answer:

We thank the editors for the comment. A previous study had reported that the PC 12 are subjected to rectangular pulse with amplitudes of 200 mV and 400 mV at a frequency of 100 Hz. The strength of the applied DC EF has been known to be significant for the differentiation of PC12 cells[6]. Therefore, in our study, the mNPCs are subjected to square DC pulses with a magnitude of 300 mV/mm at a frequency of 100 Hz.

2. The current equation of Ohms's law was employed without considering the continuous flow of media which may affect the potential distribution in the medium.

Therefore, it needs to explain the reason of selecting the flow velocity of medium 10 ul/h.

Answer:

The medium that was supplied when applying the electric current is identical to that before the current is applied. Therefore there is no composition change, and hence the Ohm's law should apply.

Please note that the unit of uL/h is for flow rate but not for flow velocity. The flow rate is chosen because of the following considerations:

The flow rate of 10 μL/h corresponds to the flow velocity of 0.01 mm/sec in the cell chamber (cross-section area is 0.07 mm x 3 mm = 0.21 mm^2). With such a slow velocity, the shear that may be incurred by the liquid flow is expected to be extremely low.

A previous study has shown the EGF and bFGF help maintain the undifferentiated state of NPC[2]. In our study, the replenishing of EGF and

bFGF at a flow velocity of 0.01 mm/sec ensures the cells are continuously in contact with the maintenance medium.

3. Fig. 5 showed the final result of the mNPC differentiation by showing only percentage.
To show the reliability of the suggested system, why doesn't authors provide the statistical results with averages and standard deviations.

Answer:

For each experiment, we randomly picked 10 areas in the cell culture region and counted the cells. In each region, we observed 3-5 spheres and took the high-resolution images. Each image has 40-100 cells, and the image (field-of-view) has an area of 390 μm×390 μm. All images were taken at the same magnification (×40), and the experiment was performed for at least three times. In order to compare each phenotypic marker between the control and treatment groups, the data are expressed as the percentage of positive cells for each marker.

Minor Concerns:

1. It needs to provide the difference of the MOE chip detail from the established chip in their literature [17].

Answer:

We thank the reviewer for the comment. In Ref. 17, the microfluidic network produces dual EFs regions in multiple single channels. In each channel, two regions, one with EF and one without EF, are provided. The single channels are connected in series electrically, and only one EFs is provided in a single experiment.

In the reported MOE chip, the multiple channel are connected in parallel so that different EFs can be provided in individual channels. The difference between the multichannel optically-transparent electrotactic (MOE) chip and the multichannel dual-electric-field (MDF) chip, as shown below:

	MOE chip	MDF chip
Microscope type	Suitable for confocal microscope and inverted phase-contrast microscope examinations	Suitable for inverted phase-contrast microscope examinations
EF stimulation	Suitable for multiple EF stimulation simultaneously	Suitable for single EFs stimulation

	in a single experiment	
Cell culture medium	Three types of culture medium in individual channel	Single type of culture medium

2. How much concentration of PLL solution?

Answer:

We added the descriptions in 2.5 of the revised manuscript, "Add 2 mL of 0.01% PLL solution using a 3 ml syringe that connects to the 3-way stopcock of the medium inlet (Figure 4B-①)."

REFERENCES:

1 Fuhr, G. & Shirley, S. G. Cell handling and characterization using micron and submicron electrode arrays: state of the art and perspectives of semiconductor microtools. *Journal of Micromechanics and Microengineering.* **5** (2), 77-85, (1995).

2 Kim, Y. H. *et al.* Differential regulation of proliferation and differentiation in neural precursor cells by the Jak pathway. *Stem Cells.* **28** (10), 1816-1828, (2010).

3 Ariza, C. A. *et al.* The influence of electric fields on hippocampal neural progenitor cells. *Stem cell reviews and reports.* **6** (4), 585-600, (2010).

4 Chang, K. A. *et al.* Biphasic electrical currents stimulation promotes both proliferation and differentiation of fetal neural stem cells. *PLoS One.* **6** (4), e18738, (2011).

5 Lim, J. H., McCullen, S. D., Piedrahita, J. A., Loboa, E. G. & Olby, N. J. Alternating current electric fields of varying frequencies: effects on proliferation and differentiation of porcine neural progenitor cells. *Cell Reprogram.* **15** (5), 405-412, (2013).

6 Kimura, K., Yanagida, Y., Haruyama, T., Kobatake, E. & Aizawa, M. Electrically induced neurite outgrowth of PC12 cells on the electrode surface. *Medical & biological engineering & computing.* **36** (4), 493-498, (1998).