

## Technical note

## An inverted method for culturing dissociated mouse hippocampal neurons

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## ABSTRACT

Dissociated hippocampal neuron culture has long been the model system of choice for many neuroscientists. The ability to culture dissociated hippocampal neurons from genetically modified mice provides an invaluable tool for studying many neuronal processes. In this study, we established a novel method to culture dissociated hippocampal neurons from embryonic and neonatal mice. Dissociated neurons were cultured in a microchamber between the glass coverslip and the plastic cell container without the use of glial feeder cells. Our method significantly simplifies the preparation while produces healthy and long-lived neuronal cultures that are difficult to achieve without the use of feeder cells.

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## 1. Introduction

Dissociated hippocampal neurons have always been a popular research tool for studying the central nervous system. The main reason for this high popularity is their high homogeneity. While hippocampal culture prepared from postnatal tissue contains a significant percentage of GABAergic neurons (Mynlieff, 1997, 1999), embryonic hippocampal culture consists mainly of pyramidal neurons (Banker and Goslin, 1998). Second, hippocampal neurons have well-defined shape with a single axon and numerous dendrites covered with dendritic spines (Papa et al., 1995) and they form functional synaptic connections with each other in culture (Basarsky et al., 1994). Third, the development of pyramidal neurons in culture follows a highly consistent five stage program (Dotti et al., 1988). As a result, the ability to culture dissociated hippocampal neurons is essential for many neuroscience studies.

However, preparing and maintaining dissociated hippocampal culture is far from trivial. Historically, dissociated hippocampal cultures are prepared from rat tissue (Banker and Cowan, 1977; Rothman and Cowan, 1981; Segal, 1983); one problem most often encountered is that hippocampal neurons survive for only a few days in culture at low density (Banker and Cowan, 1977). Various protocols have been developed to overcome this problem. These methods include the famous “Banker” coculture of hip-

pocampal neurons with astroglial cells (Kaech and Banker, 2006) and “ring” coculture of hippocampal neurons with cortical neurons (Fath et al., 2009); both protocols grow hippocampal neurons and supporting cells in a spatially separated format. Despite offering a stable low density culture suitable for microscope imaging, these methods are rather complicated and require the dissection of multiple brain areas. Alternatively, hippocampal neurons can be cultured directly on top of a monolayer of feeder glial cells (Forsythe and Westbrook, 1988; Yamada et al., 1989). Albeit providing a convenient alternative, this method is not applicable for immunocytochemistry or live cell imaging because neurons and supporting glial cells are intermixed. Recent advances in producing genetically modified mice have made mouse hippocampal culture a more favorable model system. However, hippocampal neurons isolated from mice are generally harder to culture than those from rats; they require a higher cell density to survive than rat cultures (Peacock et al., 1979). While a convenient protocol has been developed to maintain rat hippocampal neurons in dissociated culture (Mynlieff, 1997), it has not been established for mouse neurons.

Here we describe an easy yet effective protocol to culture embryonic or neonatal hippocampal neurons isolated from mice. No glial feeder cells are required in our protocol. The same protocol can be applied to rat neurons. Our method significantly simplifies the preparation procedure while produces neuronal culture which shows robust neuritogenesis, minimal soma aggregation, and minimal neurite fasciculation. We also demonstrated several advantages of this culturing method that make it suitable for various neuroscience studies.

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## 2. Materials and methods

### 2.1. Chemicals and reagents

Alexa Fluor 488-labeled phalloidin, Alexa Fluor 568-labeled goat-anti-rabbit secondary antibody, B27 supplement, bovine serum albumin, DAPI, Hank's balanced salt solution (HBSS), HEPES, Lipofectamine 2000, minimum essential medium (MEM), neurobasal medium, trypsin-EDTA, penicillin-streptomycin, ViraPower lentiviral expression system were purchased from Invitrogen. DNase I, D-glucose, Fluoromount, L-glutamic acid, L-glutamine, poly-L-lysine (molecular weight of 30,000–70,000), sodium tetraborate, and trypan blue were purchased from Sigma-Aldrich. Boric acid and triton X-100 were purchased from J.T. Baker. Fetal bovine serum was purchased from Biological Industries. Mouse monoclonal antibody against neuron-specific beta-III-tubulin (Tuj1; MMS-435P) was purchased from Covance. Mouse monoclonal antibody against tau (MAB3420) and rabbit polyclonal antibody against MAP2 (AB5622) were purchased from Millipore. Rabbit polyclonal antibody against GFAP (NB300-141) was purchased from Novus. DyLight 488-labeled goat-anti-mouse secondary antibody was purchased from Jackson ImmunoResearch.

### 2.2. Coating of coverslips

Poly-L-lysine (molecular weight of 30,000–70,000) was dissolved in the boric acid buffer (1.24 g boric acid and 1.9 g sodium tetraborate in 400 mL H<sub>2</sub>O, pH 8.5) to a final concentration of 0.1 mg/mL. Filter-sterilized (0.2 µm) poly-L-lysine solution was added onto 12 mm glass coverslips placed in a non-treated polystyrene 24-well plate. The coverslips were incubated overnight at room temperature and washed twice with sterilized H<sub>2</sub>O the following day. Washed coverslips were air dried for at least 4 h prior to use. It is important that poly-lysine coated coverslips be prepared under sterile conditions. Dry poly-L-lysine-coated coverslips can be stored at 4 °C for up to 2 weeks before use.

### 2.3. Hippocampus dissection and enzyme digestion

Pregnant dams (C57BL/6) at gestational day 18 (E18) or neonatal mice (P0) were euthanized by CO<sub>2</sub>. Embryos were collected in ice cold dissection buffer (HBSS supplemented with 10 mM HEPES and penicillin-streptomycin). Mouse brains were carefully removed from the skull according to a published protocol (Fath et al., 2009) and placed into a sterile Petri dish containing cold dissection buffer. Hippocampi were dissected from both cerebral hemispheres and meninges were thoroughly removed. Twenty isolated hippocampi were incubated at 37 °C for 30 min with 5 mL of trypsin digestion buffer (10 mM HEPES buffered 0.25% trypsin-EDTA). Fifteen minutes after starting the digestion, the tube containing hippocampi was mixed once by inversion. At the end of digestion, after hippocampal samples had sedimented, the supernatant was carefully removed. Digested hippocampi were washed three times with 5 mL dissection buffer to dilute the trypsin-EDTA. 50 µg/mL of DNase I was included in the last wash to break down DNA during the trituration step.

We certify that all animal experiments were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23), and that formal approval to conduct the experiments described has been obtained from the animal subjects review board of our institution.

### 2.4. Hippocampus trituration and centrifugation

Hippocampal tissues were gently triturated ten times with a 10 mL serological pipette to dissociate larger aggregates. Dis-

sociated hippocampi were triturated another ten times with a 10 mL serological pipette tipped with a 200 µL tip to dissociate finer aggregates. Thereafter, cells were centrifuged at 80 × g for 5 min at room temperature. After the centrifugation, the supernatant was discarded and the cell pellet was dissociated by flicking the tube and resuspended in neuronal plating medium (minimum essential medium supplemented with 5% fetal bovine serum, 0.6% D-glucose, and 2 mM L-glutamine). Viable cells were determined using a hemocytometer in the presence of 0.2% trypan blue.

### 2.5. Seeding and culturing of hippocampal neurons

The optimal density of  $3 \times 10^4$  neurons per cm<sup>2</sup> was seeded onto each 12 mm poly-L-lysine-coated coverslip in 0.5 mL prewarmed neuronal plating medium. The plating medium was replaced with neuronal maintenance medium (neurobasal medium with B27 supplement and 0.5 mM L-glutamine) 4 h after seeding. In addition, coverslips were immediately inverted following the medium replacement. Half of the neuronal maintenance medium was replaced by fresh medium every week.

### 2.6. Hippocampal neuron transfection and transduction

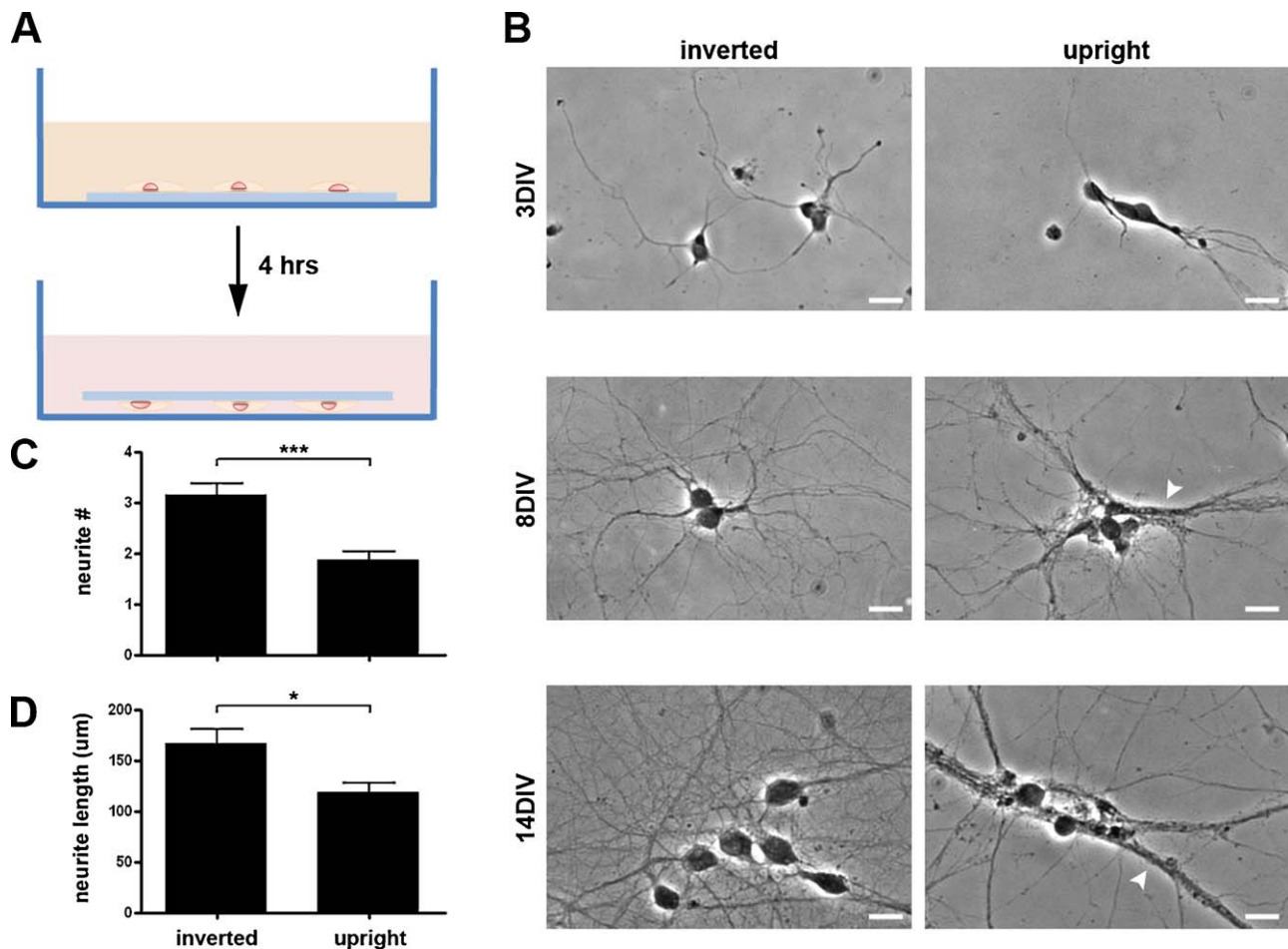
The mβ6p-YFP plasmid (YFP driven by the beta-III-tubulin promoter) was introduced into hippocampal neurons using Lonza nucleofector (Lonza, Switzerland) immediately after dissociation. Transfected neurons were seeded and cultured as described above. Alternatively, dissociated hippocampal neurons on the inverted coverslip were flipped upside down and transfected with pEGFP-N2 at 2 or 4 days *in vitro* (2DIV or 4DIV) using Lipofectamin 2000 or transduced with ViraPower lentivirus expressing mCherry at 5DIV according to the manufacturer's direction. Lipofectamine-containing medium was replaced with fresh neuronal maintenance medium and coverslips with attached neurons were re-inverted 6 h post-transfection.

### 2.7. Indirect immunofluorescence staining and image acquisition

Cells on coverslips were fixed with 3.7% formaldehyde in PBS (prewarmed to 37 °C) for 15 min at 37 °C after 7–14 days in culture. After fixation, cells were washed three times with PBS at room temperature and permeabilized with 0.25% triton X-100 for 5 min at room temperature. After being washed three times with PBS, cells were blocked for 1 h at room temperature with 10% BSA. Coverslips with cells were incubated for 1 h at 37 °C with antibodies against GFAP (1:2000), MAP2 (1:4000), Tau-1 (1:200), or Tuj1 (1:2000) diluted in 2% BSA. After being washed three times with PBS, cells were incubated with DyLight 488- or Alexa Fluor 568-labeled secondary antibodies (1:1000), Alexa Fluor 488-labeled phalloidin (1:40), and DNA-binding dye DAPI (5 µg/mL) for 1 h at 37 °C in the dark. Coverslips with cells were washed three times with PBS and mounted with Fluoromount onto glass slides. Fluorescence images were acquired with an Olympus IX-71 inverted microscope equipped with a CoolLED fluorescent light source and a Hamamatsu ORCA-R2 camera. A 20 × 0.75 N.A. and a 60 × 1.35 N.A. plan apochromat objective lenses were used to collect fluorescent images. A 20 × 0.4 N.A. phase contrast objective lens was used to collect phase contrast images.

### 2.8. Statistical analysis

All statistical analyses were performed using GraphPad Prism 4. Values are expressed as means ± standard error of the mean. Significant differences between the means were calculated with the



**Fig. 1.** The inverted culture shows improved neuronal culture condition. (A) The schematics of the inverted culture preparation. (B) Phase contrast images of E18 mouse hippocampal neurons grown in the inverted (left column) and typical upright (right column) conditions. 3 days *in vitro* (3DIV, top row), 8DIV (middle row), and 14DIV (bottom row) hippocampal neuron cultures are shown. Excessive neurite fasciculation can be observed in upright culture (arrowheads). All scale bars represent 20  $\mu\text{m}$ . (C) Quantification of the number of neurites per neuron in 3DIV inverted and upright cultures. (D) Quantification of the total neurite length per neuron in 3DIV inverted and upright cultures. \*\*\* $p < 0.001$ , two-tailed Student's *t*-test; \* $p < 0.05$ , two-tailed Student's *t*-test. More than 50 neurons were analyzed per condition.

two-tailed Student's *t*-test or one-way ANOVA followed by Tukey's multiple comparison tests.

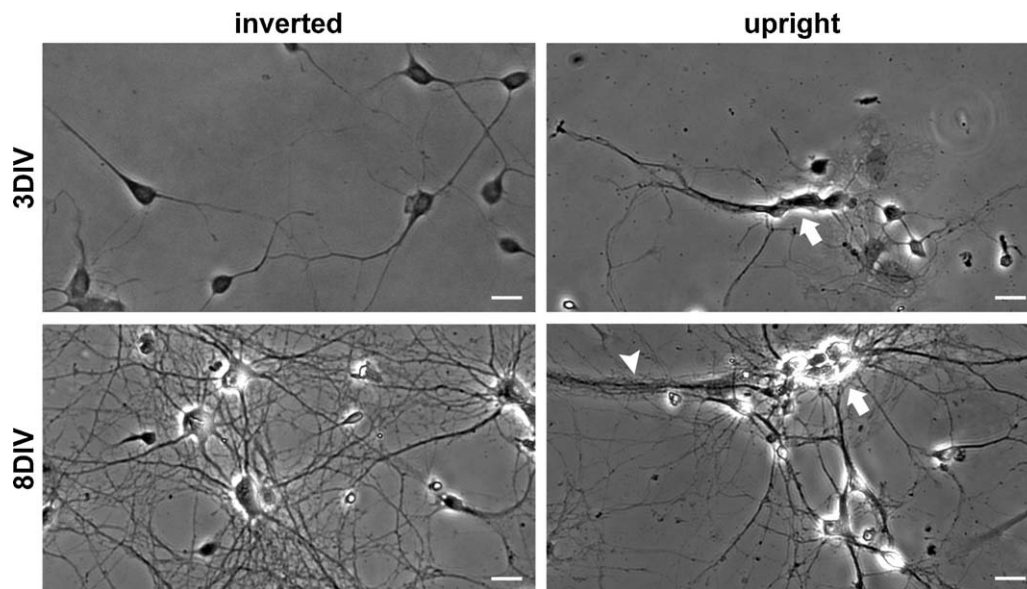
### 3. Results and discussion

Despite its high popularity, primary hippocampal culture has been known for its difficulty in preparation and maintenance. To simplify the preparation procedure, we developed this protocol which maintains hippocampal neurons underneath a glass coverslip (Fig. 1A). Dissociated hippocampal neurons were initially seeded onto poly-L-lysine-coated glass coverslips in serum-containing medium (neuronal plating medium). The use of poly-L-lysine as coating material instead of collagen or laminin significantly reduces the preparation cost. Neuronal plating medium was exchanged to a serum-free neurobasal medium plus B27 supplement (Brewer et al., 1993) (neuronal maintenance medium) 2–8 h post-seeding. The timing of medium exchange is crucial as shorter incubation in neuronal plating medium impedes cell attachment and prolonged incubation impedes both cell attachment and neuriteogenesis (Supplemental Fig. 1). Note that the antibiotic mixture of penicillin and streptomycin was removed from neuronal plating medium and neuronal maintenance medium. This is because penicillin is a known antagonist against GABA<sub>A</sub> receptor (Oliver et al., 1977; Pickles and Simmonds, 1980) and this may lead to a less physiological state of the culture. The coverslip with attached neurons was inverted immediately

after medium exchange. There is no need to place spacers (e.g. paraffin dots) between the coverslip and the cell container as in Banker coculture (Kaeche and Banker, 2006), this further simplifies the culture preparation. We observed far less neurite fasciculation in our “inverted” culturing condition compare to the typical “upright” culturing condition (Fig. 1B). In addition, neurons in the inverted culture extend longer and more numerous neurites (Fig. 1C and D); this indicates the inverted culture supports a more robust neuriteogenesis process. The benefit of our inverted culture is also evident in more matured neurons. Dissociated hippocampal neurons in the typical upright culture showed excessive soma aggregation after one week of growth. However, neurons in the inverted culture did not show such problem (Fig. 1B). Furthermore, neurons were unable to survive for more than 2 weeks in the typical upright culture. Neurons in our inverted culture, on the other hand, showed robust dendritic spine formation (judging from phalloidin staining), minimal neurite fragmentation (judging from beta-III-tubulin staining), and continue to develop after 3 weeks (Fig. 3C). The exact protocol can be used to culture neurons isolated from neonatal mice and the benefit of our inverted culture is still evident (Fig. 2).

We tested the seeding density in our inverted culture, and the optimal seeding density is around  $3 \times 10^4$  neurons per  $\text{cm}^2$ . Cell density ranging from  $1 \times 10^4$  to  $5 \times 10^4$  neurons per  $\text{cm}^2$  is practicable. We recommend the use of non-treated plastic cell container instead of the tissue culture-treated cell con-

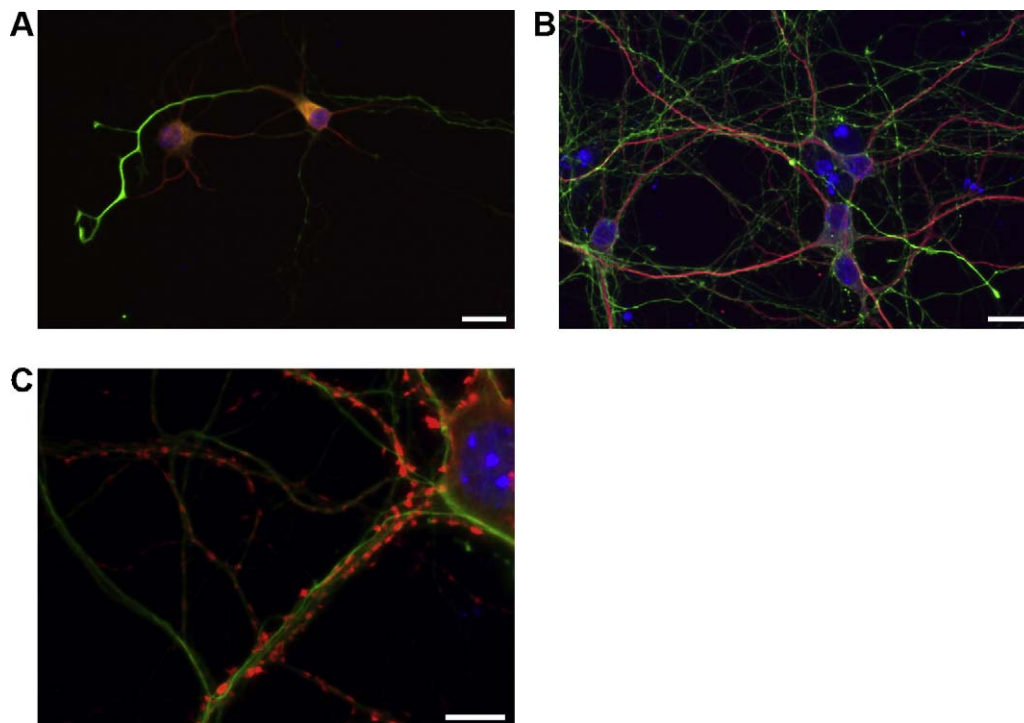




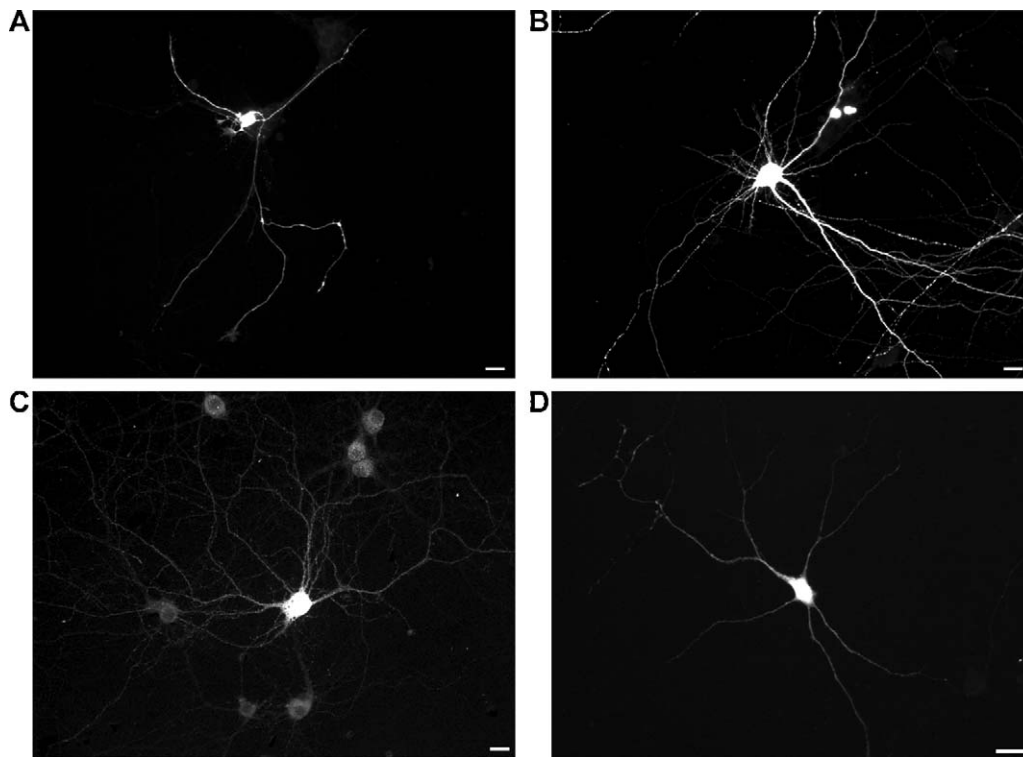
**Fig. 2.** The inverted culture can be used to culture neonatal hippocampal neurons. Phase contrast images of P0 mouse hippocampal neurons grown in the inverted (left column) and upright (right column) conditions. 3DIV (top row) and 8DIV (bottom row) neonatal hippocampal neuron cultures are shown. Excessive neurite fasciculation (arrowheads) and soma aggregation (arrows) can be observed in the upright culture. All scale bars represent 20  $\mu\text{m}$ .

tainer. This is because we observed an infrequent but unusual “squashed” morphology when tissue culture-treated cell containers were used (Supplemental Fig. 2). These squashed neurons are viable and continue to grow. However, this is not a normal neuronal morphology and most likely arose from the electrostatic interaction between the positively charged poly-L-lysine surface and the negatively charged tissue culture-treated surface.

There are several characteristics of our inverted culture system that can be beneficial to neuroscience applications. First, hippocampal neurons rapidly underwent axon-dendrite polarization in the inverted culture (Fig. 3A and B). Second, actin-rich dendritic spines can be readily detected in the inverted culture after 3 weeks *in vitro* (Fig. 3C). Furthermore, neuron-containing coverslips can be flipped over (i.e. neurons facing up instead of being sandwiched) without damaging the attached neurons. We showed that flipped over



**Fig. 3.** Hippocampal neurons in the inverted culture undergo axon-dendrite polarization and dendritic spine formation. 3DIV (A) and 8DIV (B) mouse hippocampal neurons immunofluorescence stained with antibodies against dendrite-specific protein MAP2 (red) and axon-specific protein tau (green). DAPI-stained nuclei (blue) are shown for soma visualization. (C) The proximal dendrite of a 21DIV mouse hippocampal neuron is shown stained with phalloidin (red), antibody against neuron-specific beta-III-tubulin (green), and DAPI (blue). Scale bars represent 20  $\mu\text{m}$  in A, B and 10  $\mu\text{m}$  in C.



**Fig. 4.** Hippocampal neurons in the inverted culture can be readily transfected or transduced. (A) E18 mouse hippocampal neurons transfected with the pEGFP-N2 plasmid using Lipofectamine 2000 at 2DIV and fixed at 5DIV or (B) transfected at 4DIV and fixed at 14DIV. (C) E18 mouse hippocampal neurons transfected with the mβ6p-YFP plasmid using Lonza Nucleofector immediately after dissociation and fixed at 14DIV. (D) An E18 mouse hippocampal neuron transduced at 5DIV with lentivirus carrying the red fluorescent protein mCherry and fixed at 13DIV is shown. All scale bars represent 20 μm.

neurons can be transfected using liposome-based method (Fig. 4A and B), transfected using electroporation (Fig. 4C), or transduced using virus-based method (Fig. 4D). Coverslips with attached neurons can be re-inverted after the required treatment has completed. The ability of our inverted culture to proceed normally through axon-dendrite polarization and dendritic spine formation, as well as tolerate physical manipulation makes it suitable for various neuroscience studies.

There are two possible reasons for the improved neuron survival and neuritogenesis using our inverted culture. First, the microenvironment created by the glass coverslip and the plastic cell container can reduce the oxygen level in the culture. Lowering oxygen level in the neuronal culture has been documented to promote neuronal survival and growth (Brewer and Cotman, 1989). Second, the confined microenvironment increases the concentration of trophic factors secreted by neurons or glial cells. We have confirmed the presence of astrocytes in the inverted culture using antibody against glial fibrillary acidic protein (GFAP) (Supplemental Fig. 3A). In our hands, the ratios of neurons and astrocytes in the inverted culture are statistically indistinguishable from those of the upright culture (Supplemental Fig. 3B).

The major advantage of our inverted culture is that no feeder cells are needed. This significantly reduces the workload and eliminates the need to precisely time feeder glia and neuron cultures. In addition, by culturing the neurons directly on the glass coverslips, our inverted culture system can be applied to high resolution imaging techniques such as differential interference contrast or confocal microscopy. Furthermore, primary neurons on the glass coverslips can tolerate moderate amount of handling thus making experimental manipulation such as transfection or virus transduction possible. However, it is important to note that our culturing method is by no mean a replacement for the low-density coculture methods (Fath et al., 2009; Kaech and Banker, 2006). Our culture is intended for

researches that aim for a median-density culture ( $1 \times 10^4$  to  $5 \times 10^4$  neurons per  $\text{cm}^2$ ). While neurons in the inverted culture developed numerous dendritic spines after 3 weeks *in vitro* (Fig. 3C), the applicability of the inverted culture on electrophysiology studies is currently not known. We plan on investigating this possibility in the near future.

In conclusion, we presented a simplified protocol for the preparation of mouse primary hippocampal neurons. Our method significantly simplifies the preparation procedure, reduces the reagent cost, but at the same time produces healthy neuronal cultures which show robust neuritogenesis, minimal soma aggregation, and minimal neurite fasciculation. Neurons in the inverted culture exhibit normal axon-dendrite polarization, dendritic spine formation, and can be readily transfected or transduced. These results demonstrate the inverted culture as a valid culturing method for various neuroscience studies.

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#### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.neures.2011.01.002](https://doi.org/10.1016/j.neures.2011.01.002).

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