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# Generation of oligodendrocytes and oligodendrocyte conditioned medium for co-culture experiments

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**TITLE:**

# Generation of Oligodendrocytes and Oligodendrocyte-Conditioned Medium for Co-Culture Experiments

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**KEYWORDS:**

Oligodendrocytes, conditioned medium, secreted factors, cell culture, neuro-glia interactions, central nervous system, rat.

**SUMMARY:**

Herein, we display an efficient method for the purification of oligodendrocytes and production of oligodendrocyte-conditioned medium that can be used for co-culture experiments.

**ABSTRACT:**

In the central nervous system, oligodendrocytes are well-known for their role in axon myelination, that accelerates the propagation of action potentials through saltatory conduction. Moreover, an increasing number of reports suggest that oligodendrocytes interact with neurons beyond myelination, notably through the secretion of soluble factors. Here, we present a detailed protocol allowing purification of oligodendroglial lineage cells from glial cell cultures also containing astrocytes and microglial cells. The method relies on overnight shaking at 37 °C, which allows selective detachment of the overlying oligodendroglial cells and microglial cells, and the elimination of microglia by differential adhesion. We then describe the culture of oligodendrocytes and production of oligodendrocyte-conditioned medium (OCM). We also provide the kinetics of OCM treatment or oligodendrocytes addition to purified hippocampal neurons in co-culture experiments, studying oligodendrocyte-neuron interactions.

## INTRODUCTION:

Oligodendrocytes (OLs) are glial cells of the central nervous system (CNS) that generate myelin wrapping around axons. OLs originate from oligodendrocyte precursor cells (OPCs) which proliferate within the ventricular zones of the embryonic CNS and then migrate and differentiate into fully mature OLs (i.e., myelin-forming cells)[1]. OPCs are abundant during early development, but also persist in the adult brain where they represent the major proliferative cell population[2]. A single OL ensheathes multiple axons in non-excitable sections (i.e., internodes), and the edge of each myelin loop attaches to the axon forming the paranodal domain which is crucial for the insulating properties of myelin[1,3]. In between the paranodes are small unmyelinated gaps called the nodes of Ranvier. These nodes are rich in voltage-gated sodium channels ($Na_V$), allowing the regeneration and rapid propagation of action potentials through saltatory conduction[4]. This tight interaction also enables axonal energy support through neuronal uptake of lactate from OLs[5,6].

The maturation of oligodendroglial lineage cells and the myelination process are tightly regulated by their interactions with neurons[7]. Indeed, OLs and OPCs, also named NG2 cells, express an array of receptors for neurotransmitters, and can receive input from excitatory and inhibitory neurons, allowing them to sense neuronal activity that can trigger their proliferation and/or differentiation into myelinating cells[2]. In turn, OPCs/OLs secrete microvesicles and proteins into the extracellular space which alone or synergistically mediate neuromodulative and neuroprotective functions[8–12]. However, the molecular mechanisms controlling the multiple modes of interactions between oligodendroglial lineage cells and neurons are yet to be fully deciphered.

Moreover, in several CNS pathological conditions, OLs are primarily affected, thus disturbing their interaction with neurons. For instance, in Multiple Sclerosis (MS), neurological dysfunction is caused by focal demyelination in the CNS, secondary to OLs loss that can lead to axonal damage and related disability accumulation. Remyelination can take place, albeit insufficiently in most cases[13]. Progress in the last decade, due to the development of immunotherapies, have reduce the relapse rate but promoting remyelination remains to date an unmet need. As such, a better understanding of OLs role, functions and influences is of particular interest to the development of new therapies for a wide spectrum of CNS conditions.

Here, we describe the methods of OLs purification and culture. This enables precise examination of intrinsic mechanisms regulating their development and biology. In addition, such highly enriched OLs cultures allow the production of oligodendrocyte-conditioned medium (OCM), which can be added to purified neuron cultures to gain insight into the impact of OLs-secreted factors on neuronal physiology and connectivity. Furthermore, we describe how to implement an in vitro co-culture system where purified oligodendrocytes and neurons are combined together, allowing to address the mechanisms regulating (re)myelination.

## PROTOCOL:

The care and use of rats in this experiment conforms to institutional policies and guidelines (UPMC, INSERM, and European Community Council Directive 86/609/EEC). The following protocol is established for a standard litter of 12 pups.

**1. Preparation of the flasks (~5 min)**

NOTE: Perform the following steps the day before dissection in a laminar flow hood under sterile conditions.

1.1. Coat the 150 $cm^2$ flasks (T150) with filter cap (1 flask for 2 pups) using 5 mL of polyethylenimine (PEI, 100 mg/L, see protocol in **Supplementary File 1**).

1.2. Store the flasks at 4 °C overnight.

1.3. Rinse coated flasks 3 times with sterile distilled water on the day of dissection.

**2. Preparation of media (~10 min)**

NOTE: Perform the steps in a laminar flow hood under sterile conditions.

2.1. Prepare 500 mL of culture medium, consisting of Dulbecco's modified Eagle medium (DMEM) supplemented with 10% of fetal calf serum (FCS) and penicillin-streptomycin (100 IU/mL).

2.2. Prepare 20.6 mL of the enzyme digestion medium in a 50 mL tube, consisting of 20 mL of DMEM, 200 µL of DNase (50 µg/mL), 200 µL of papain (30 U/mL) and 200 µL of L-cysteine (0.24 mg/mL).

2.3. Filter-sterilize the media using a 0.22 µm filter.

2.4. Keep the media in the laminar flow hood at room temperature (RT) until dissection.

**3. Preparation for dissection (~10 min)**

NOTE: Perform the steps in a laminar flow hood under sterile conditions.

3.1. Prepare 100 mL of phosphate-buffered saline without calcium and magnesium (PBS; 1x). Filter-sterilize using a 0.22 µm filter.

3.2. Prepare 50 mL of ice-cold 1x PBS solution supplemented with 750 µL of 45% glucose. Filter-sterilize using a 0.22 µm filter.

3.3. Fill a 100 mm Petri dish with 1x PBS for cleaning instruments and three 60 mm Petri dishes with ice-cold PBS-glucose for tissue harvesting. Put the petri dishes on ice until dissection.

**4. Dissection**

NOTE: Dissection is performed from male and female Wistar rat pups at postnatal day (P) 2.

4.1. In order to provide a sterile environment, make sure to clean the bench with 100% ethanol. Sterilize all surgical tools with 100% ethanol.

4.2. Gently spray the neck of the pup with 70% ethanol.

4.3. Use large surgical scissors to decapitate the animal and place the head in a 100 mm Petri dish containing ice-cold PBS-glucose.

4.4. Use curved forceps to maintain the head of the animal at eye level. Use small surgical scissors to make a small incision at the base of the skull and cut the skull following the brain midline.

4.5. Use forceps to gently peel off the two parts of the skull from the midline.

4.6. Use a small surgical spoon to remove the brain from the head cavity. Put the brain in a 60 mm Petri dish containing ice-cold PBS-glucose on ice.

4.7. Viewing under a steromicroscope, use fine forceps to remove the cerebellum, the brainstem and olfactory bulbs from cerebral hemispheres.

4.8. Use fine forceps to separate the two cerebral hemispheres. Use fine forceps to peel off the meninges. Put the cerebral cortices in a 60 mm-petri dish on ice.

NOTE: Ice-cold PBS is critical for correct meninges removal.

**5. Tissue dissociation**

NOTE: Perform the steps in a laminar flow hood under sterile conditions.

5.1. Use a sharp scalpel to finely chop the cerebral cortices. Transfer the minced tissue into a 50 mL tube containing enzyme digestion medium.

5.2. Incubate for 30 min in a humidified incubator at 37 °C under 5% $CO_2$.

5.3. Use a p1000 micropipette to gently remove the enzyme digestion medium while making sure that the cortical tissue remains at the bottom of the 50 mL tube.

5.4. Use a p1000 micropipette to add 1 mL of DMEM-10% FCS and gently triturate the tissue.

5.5. Use a 70 µm filter and a piston of a 1 mL syringe to filter the cortical tissue into a 15 mL tube.

NOTE: One can rinse residual tissue on the inner tube wall several times with DMEM-10% FCS.

5.6. Fill the 15 mL tube with DMEM-10%FCS. Centrifuge at 423 x *g* for 5 min at RT. Carefully remove supernatant and resuspend cell pellet with 2 mL of DMEM-10%FCS.

5.7. Gently triturate cell pellet with a p1000 micropipette and then with a p200 micropipette. Dilute the cell suspension with the appropriate volume of DMEM-10%FCS.

NOTE: Two brains = one T150 = 5 mL of DMEM-10%FCS.

5.8. Plate 5 mL of the cell suspension on a T150 at a density of 1 x $10^5$ cells/$cm^2$. Add 20 mL of warm DMEM-10%FCS to each T150. Incubate in a humidified incubator at 37 °C under 5% $CO_2$.

5.9. Renew half of the culture medium after 6 days in vitro (DIV) with warm DMEM-10%FCS.

**6. Shaking preparation**

6.1. Perform shaking preparation on the day before shaking in a laminar flow hood under sterile conditions.

6.2. Renew half of culture medium by adding fresh warm culture medium into the flask and incubate at 37 °C under 5% $CO_2$.

**7. Shaking**

7.1. Coat three 100 mm Petri dishes with PEI. Store them at 4 °C overnight.

7.2. Cover the flasks cap with paraffin film and put the flasks into a plastic bag. Shake flasks containing glial cells overnight at 250 rpm at 37 °C.

NOTE: First shaking is performed at 8 DIV and one can perform up to three different shakings (see **Figure 1** for timing).

**8. OL lineage cells harvesting and culture**

NOTE: These steps should be performed in a laminar flow hood under sterile conditions.

8.1. On the day after shaking, prepare Bottenstein-Sato (BS) medium according to **Table 1**.

8.2. Rinse coated Petri dishes 3 times with sterile distilled water.

8.3. Harvest flasks' supernatant containing mainly OL lineage cells but also some microglial cells and plate it on non-coated 100 mm Petri dishes.

NOTE: This step allows removal of microglial cells through differential fast adhesion on the dish surface.

8.4. Incubate the Petri dishes for 15 min in a humidified incubator at 37 °C under 5% $CO_2$.

8.5. Fill each T150 flask with 25 mL of warm freshly prepared culture medium and incubate in a humidified incubator at 37 °C under 5% $CO_2$ until the second shaking.

8.6. Transfer the supernatant from the Petri dishes into new non-coated 100 mm Petri dishes to allow adhesion of residual microglial cells.

8.7. Incubate the Petri dishes for 15 min in a humidified incubator at 37 °C under 5% $CO_2$.

8.8. Remove the supernatant, which contains non-adherent OL lineage cells, and transfer it into 50 mL tubes (supernatant from 2 Petri dishes for a 50 mL tube). Discard Petri dishes plated with microglia.

8.9. Centrifuge the supernatant for 5 min at 423 x *g*. Carefully remove supernatant and resuspend cell pellet with 1 mL of BS medium. Pool all pellets in a common 50 mL tube and adjust volume to 10 mL with BS medium.

8.9. Determine cell density counting cells under a microscope.

NOTE: A cell density between 3 x $10^5$/mL and 5 x $10^5$/mL should be obtained.

8.10. Add 20 mL of BS if cell density is higher than or equal to 4 x $10^5$/mL to obtain a final volume of 30 mL, or add only 10 mL of BS if cell density is less than 4 x $10^5$/mL to obtain a final volume of 20 mL.

8.10. Plate two or three pre-coated 100 mm Petri dishes with 10 mL of cell suspension. Incubate in a humidified incubator at 37 °C under 5% $CO_2$.

8.11. Clear the debris from the Petri dishes by refreshing all of the BS medium 2 h later.

NOTE: Examine the culture under the microscope before and after clearing to verify cell density and efficiency of debris removal.

8.12. Incubate for 2 days in BS medium in a humidified incubator at 37 °C under 5% $CO_2$.

NOTE: Examine the culture under the microscope. The confluence should be 70% to 80%.

**9. OCM production**

NOTE: Perform these steps in a laminar flow hood under sterile conditions.

9.1. Prepare NB-B27low medium according to **Table 2**.

9.2. Renew culture medium with 10 mL of warm NB-B27low medium. Incubate for 2 days in a

humidified incubator at 37 °C under 5% $CO_2$.

9.3. Harvest the OCM, i.e., supernatant containing OL secreted factors. Filter-sterilize OCM using a 0.22 µm filter.

NOTE: Store OCM at 4 °C for a maximum of 2 months.

**10. OCM addition**

NOTE: Steps should be performed in a laminar flow hood under sterile conditions. OCM can be added to purified hippocampal neuron cultures prepared according to the following protocol[14], and obtained by adding, 24 h after isolation, the anti-mitotic agents uridine and 5-fluorodeoxyuridine (5 µM) for 36 h.

10.1. At 3 DIV, remove all neuron culture medium containing anti-mitotic agents and add 500 µL of fresh warm OCM.

10.2. Renew half of medium every 3 days with freshly made warm NB-B27.

NOTE: Such cultures can be maintained up to 21 DIV.

**11. Addition of OL to purified hippocampal neuron culture**

NOTE: Perform the following steps in a laminar flow hood under sterile conditions. OLs can be added to purified hippocampal cultures obtained the same way as described above.

11.1. Prepare co-culture medium according to **Table 3**.

11.2. Retrieve the OL culture at 70% to 80% confluence. Rinse with 2 mL of warm 1x PBS.

11.3. To detach the cells, add 2 mL of 0.25% trypsin to a 100 mm Petri dish.

11.4. Incubate for 5 min in a humidified incubator at 37 °C under 5% $CO_2$.

11.5. Add 2 mL of DMEM-10% FCS to block the enzymatic reaction. Harvest the supernatant containing OL lineage cells.

11.6. Centrifuge at 423 x g for 5 min at RT. Carefully remove supernatant and resuspend the cell pellet in warm co-culture medium to obtain a concentration of 1.25 x $10^5$ cells/mL.

11.7. Add OL to purified hippocampal neurons culture by removing 200 µL of neuron culture medium and adding 200 µL of cell suspension per well (2.5 x $10^4$ cells/well) of a 24-well plate.

11.8. Refresh half of co-culture medium every 2–3 days.

NOTE: Co-cultures can be maintained up to 24 DIV.

**REPRESENTATIVE RESULTS:**

In this protocol, OL lineage cells are purified from glial cultures by shaking off astrocytes and microglia. Purity and phenotypic examination of OL cultures can be assessed by immunostaining with glial markers[15]. Analysis of the expression of different markers indicated that OL cultures were mostly pre-OLs with 90% ± 4% of $O4^+$ cells, 85% ± 7% $NG2^+$ cells, and 4.7% ± 2.1% of $PLP^+$ cells, while 7.2% ± 2.5% of cells were $GFAP^+$ astrocytes (mean ± S.D., n = 3; **Figure 2**). In addition, 4.6% ± 0.7% of cells were $CD11b^+$ microglial cells (mean ± S.D., not shown).

OCM produced from such cultures can be added at 3 DIV to purified hippocampal neuron cultures. This treatment promotes the clustering of nodal proteins, consisting of $Na_v$ channels associated with Neurofascin 186 and Ankyrin G along the axon of hippocampal GABAergic neurons before myelination, at 17 DIV (**Figure 3A**,**B**). Of note, electrophysiological recordings revealed that these clusters are associated with an increased conduction of action potentials[14]. In addition, expression of phosphorylated intermediate filament protein H stained by Smi31 is increased in OCM-treated hippocampal neurons (**Figure 3A**). Oligodendroglial secreted factors are therefore implicated in neuronal maturation and physiology.

Myelination of hippocampal neurons can be studied through addition of OL at 14 DIV. From 20 DIV to 24 DIV, immunostaining of myelin markers, such as proteolipid protein (PLP) allows visualization of myelin segments (**Figure 4**).

**FIGURE LEGENDS:**

**Figure 1: Protocol timeline of OL lineage cells isolation and OCM production.** After dissecting out cerebral cortices from P2 Wistar rats (step 4), perform tissue dissociation to culture glial cells (step 5). At 8, 12 and 15 DIV (i.e., days before shaking), renew half medium with warm DMEM-10% FCS (step 6). The next day, shake glial cultures overnight at 250 rpm at 37 °C (step 7.2). Harvest supernatant containing OL lineage cells and few microglia cells and plate it for 15 min in a humidified incubator at 37 °C under 5% $CO_2$ (steps 8.3 to 8.7). Centrifuge the supernatant for 5 min at 423 x *g*, resuspend cell pellet with BS and incubate for 2 days in a humidified incubator at 37 °C under 5% $CO_2$ (steps 8.8 to 8.12). To produce OCM, incubate for 2 days in NB-B27low (step 9). To isolate OLs for co-culture experiments, detach cell using trypsin (step 11.3).

**Figure 2: OL lineage cells phenotype in cultures.** Images were acquired using a confocal microscope. Maximum intensity projections are presented. (**A**) OL cultures contain mostly pre-OLs (i.e., expressing only NG2 (red), or both O4 (green) and NG2 (red); cells expressing both markers are indicated with yellow stars), but also some immature OLs (i.e., only expressing O4 and not NG2; white stars). (**B**) Few mature OLs (i.e., $PLP^+$; green) and few astrocytes ($GFAP^+$ cells; red) are found in OL lineage cell cultures. Scale bars = 25 µm.

**Figure 3: Representative applications.** (**A**, **B**) Hippocampal neurons treated with OCM at 3 DIV and fixed at 17 DIV express phosphorylated intermediate filament protein H (Smi31; green; panel

A). GABAergic neurons, identified by glutamate decarboxylase isoform of 67 kDa (GAD67) expression (white), display accumulation of Ankyrin G and $Na_v$ sodium channels (red; panels A and B, respectively) at the axon initial segment and form Ankyrin G and $Na_v$ clusters along their axon (panels A and B, respectively). Scale bars = 25 µm.

**Figure 4: Representative applications.** OL lineage cells added to hippocampal neuron culture at 14 DIV myelinate some hippocampal axons, here fixed at 23 DIV (PLP as a myelin marker; green). Nodes of Ranvier ($Na_v$; red) are observed in between myelin segments. Scale bar = 25 µm.

**Table 1: Preparation of Bottenstein-Sato (BS) media.**

**Table 2: Preparation of NB-B27low and NB-B27 media.**

**Table 3: Preparation of co-culture media.**

**DISCUSSION:**

Here, we provide a detailed protocol to obtain highly enriched oligodendroglial lineage cell cultures from mixed glial cultures, adapted from a previously published method[16], and the subsequent production of OL-conditioned medium. This shaking technique is not expensive, can be repeated three times and is optimal to obtain high quantity of purified OLs, as cells cultured in Bottenstein-Sato (BS) medium containing PDGFα proliferate. Glial cells are prepared using cerebral cortices of Wistar rats at P2, a time point at which a vast majority of the oligodendroglial lineage cells are pre-oligodendrocytes expressing NG2 and O4[15]. Of note, OL lineage maturation is similar at P2 in mouse and rat, and this protocol can also be used to isolate mouse pre-oligodendrocytes[17].

After shaking the mixed glial cell cultures, detached cells consist mainly of oligodendroglial lineage cells, but also some microglial cells and few astrocytes. Microglial cells are removed through differential adhesion on uncoated Petri dishes. Of note, removal efficiency can be improved by performing an additional adhesion step. However, about 5% of microglial cells are still found in enriched oligodendroglial cell cultures, as well as 5% to 9% of astrocytes. It is possible to decrease contamination from astrocytes to less than 5% by performing an additional immuno-panning step using O4 antibody-coated Petri dishes; for a detailed protocol see supplemental information in Freeman et al.[14]. The removal of debris 2 h after plating oligodendroglial cells is a critical step, which relies on the strength of the flow applied with the pipet. At this step, it is important to examine the culture under the microscope before and after clearing to verify the efficiency as the presence of too much debris may impair cell viability and growth. Of note, it is also important to use freshly made BS medium, otherwise it could alter oligodendrocytes survival. In addition, purified cells survive only up to 6 days after plating. Indeed, it is known that other glial cells and neurons promote OPC survival and proliferation or differentiation through secreted factors or direct contacts[2,18].

Other methods allow OLs isolation immediately after brain dissociation, using immunolabelling with O4 antibody followed by fluorescent-activated cell sorting by flow cytometry (FACS) or

magnetic-activated cell sorting (MACS). In addition, GFP-positive OPCs or GFP-positive oligodendrocytes can be purified by fluorescent-activated cell sorting from *PDGFαR:GFP* or *PLP:GFP* mice, respectively[19,20]. These sorting methods are more relevant for studying physiological state of oligodendrocytes compared to cultures treated with growth factors which could alter their phenotype. Notably, fluorescent-activated cell sorting has been used for gene-profiling approaches in the normal physiological state and demyelinating conditions[21]. As cell survival could be altered by cell sorting, it is better to perform functional assays immediately after sorting.

We have shown that OL cultures can be detached and added to purified hippocampal neuron cultures at 14 DIV. Such OL-neuron co-culture allows the study of early steps of myelination which starts during the first week of co-culture (Dubessy, unpublished results). Other models of OL-hippocampal neuron myelinating co-culture have been achieved by adding oligodendrocytes immediately after sorting[22,23]. Furthermore, we produced OCM to further dissect OL–neuron interactions and address the role of OL-secreted factors on neuron cultures. By using this technique, we demonstrated that hippocampal GABAergic neuron subtypes (i.e., parvalbumin$^+$ and/or somatostatin$^+$) can form clusters of nodal proteins along their axon which are induced by OCM prior to myelination[14,24]. Mass spectrometry analysis of OCM has unraveled several secreted proteins and led to identify oligodendroglial Contactin-1 that in synergy with extracellular matrix proteins mediates early steps of nodal clustering[24]. Primary cultures are useful models that allow the assessment of oligodendroglial lineage cell differentiation and interactions with neurons. However, other approaches have also been developed to evaluate OL functions and myelination, demyelination and remyelination from ex vivo cerebellar organotypic slice cultures[25,26], and in vivo studies, notably with zebrafish and tadpole models[27] which are needed in final steps of pre-clinical studies.

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None of the authors have competing interests or conflicting interests.

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Figure 1



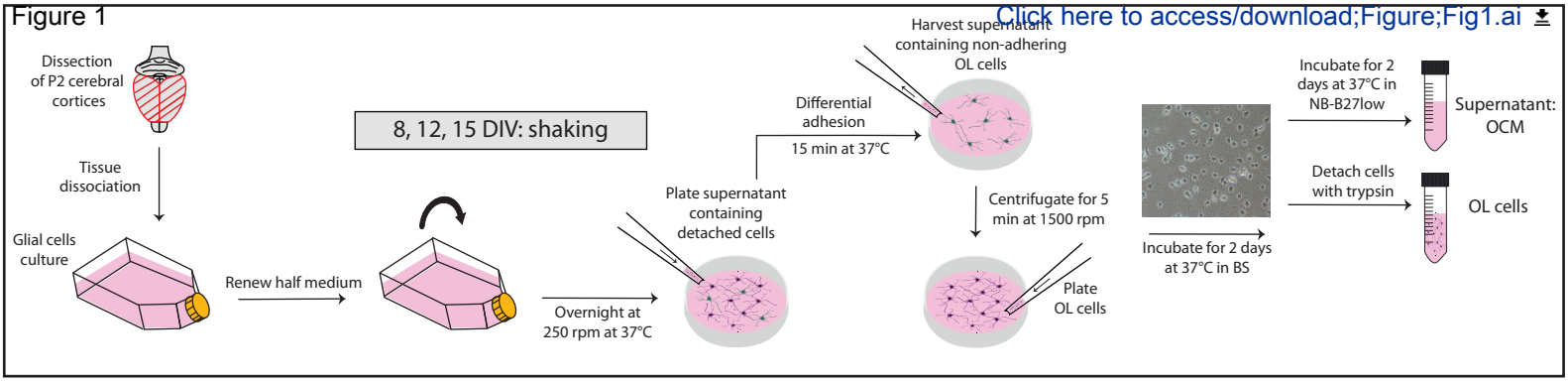


Figure 2

Click here to access/download;Figure;Fig2.psd

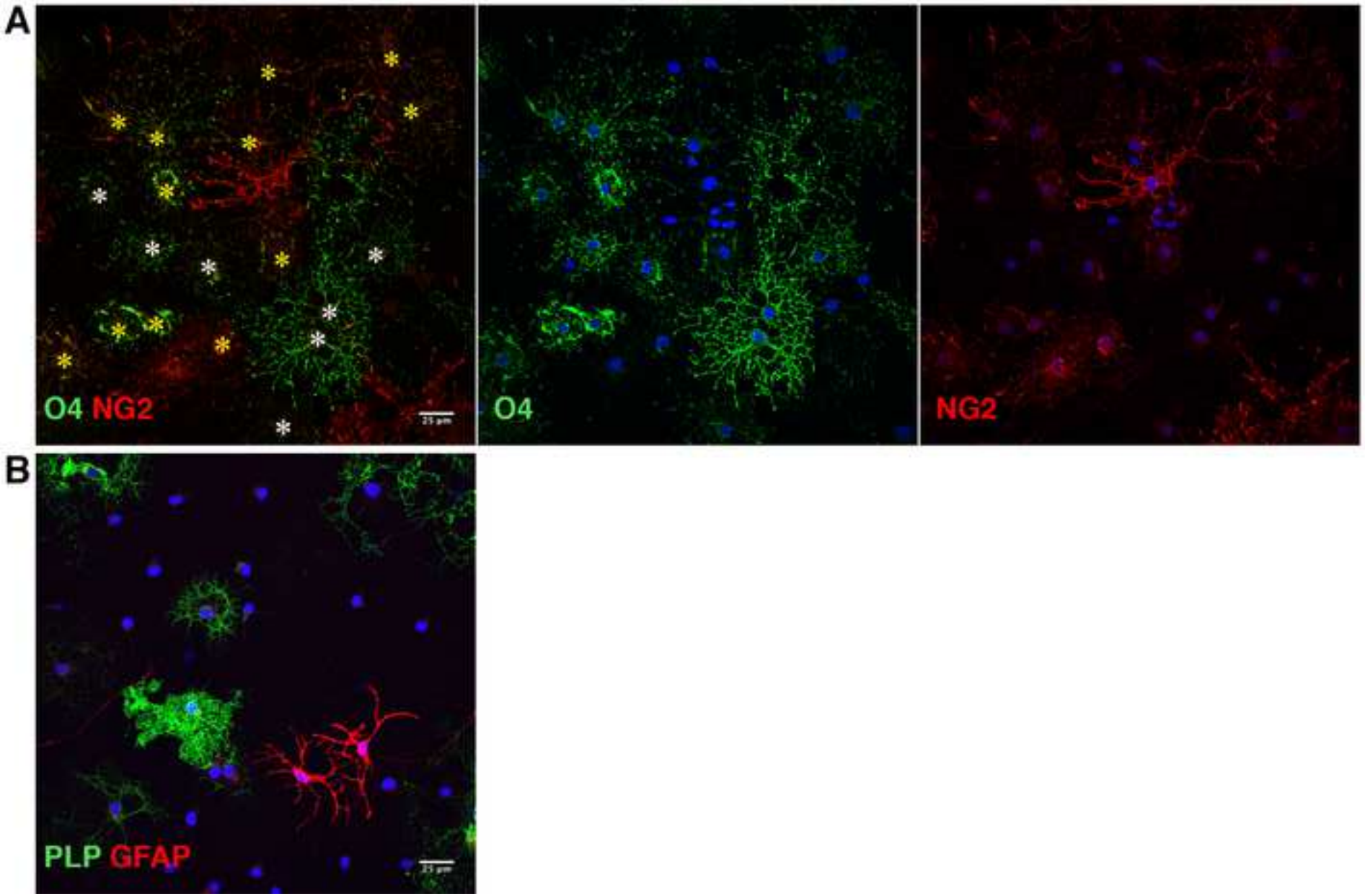


Figure 3

Click here to access/download;Figure;Fig3.psd

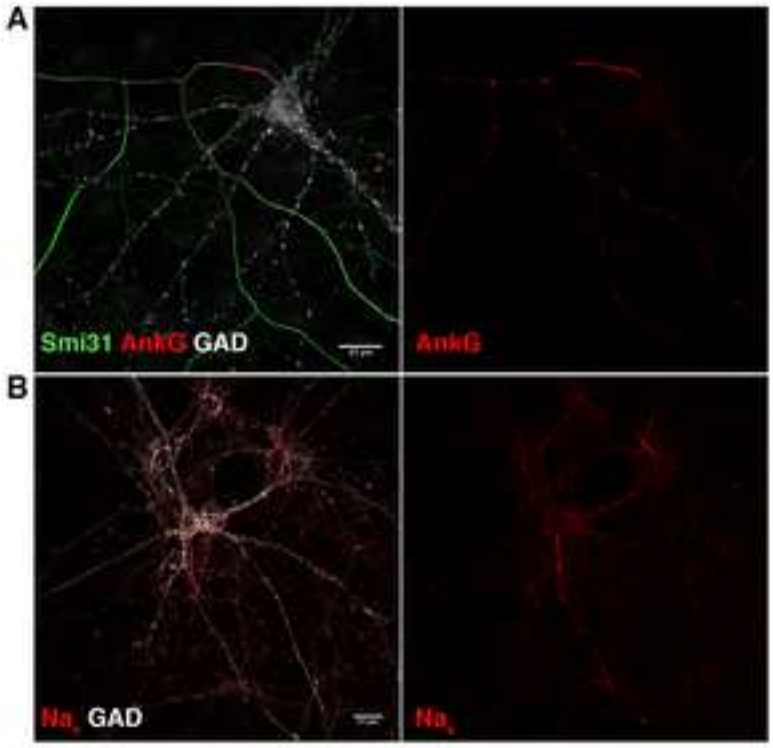
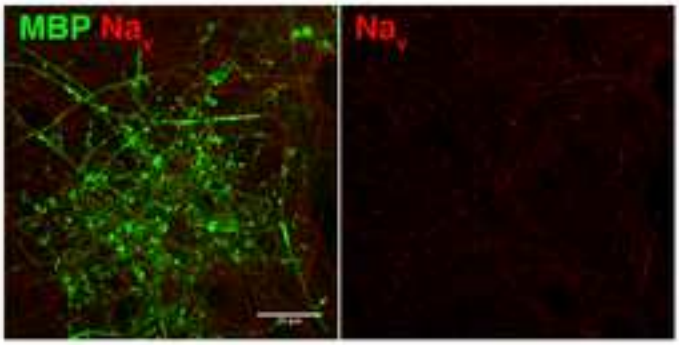


Figure 4

Click here to access/download;Figure;Fig4.psd



| **Bottenstein-Sato (BS) media** | **Final concentration** |
|---|---|
| | |
| Dulbecco's Modified Eagle Medium | |
| Penicillin-Streptomycin | 100 IU/mL |
| apo-Transferrin human | 100 µg/mL |
| BSA (Bovine Serum Albumin) | 100 µg/mL |
| Insulin | 5 µg/mL |
| PDGF | 10 ng/mL |
| Progesterone | 62 ng/mL |
| Putrescine dihydrochloride | 16 µg/mL |
| Sodium selenite | 40 ng/mL |
| T3 (3,3′,5-Triiodo-L-thyronine sodium salt) | 30 ng/mL |
| T4 (L-Thyroxine) | 40 ng/mL |

Table 2



| NB-B27low media | Final concentration |
|---|---|
| | |
| Neurobasal | |
| B27 supplement | 0.5x |
| L-glutamine | 0.5 mM |
| Penicillin-Streptomycin | 100 IU/mL |

| NB-B27 media | Final concentration |
|---|---|
| | |
| Neurobasal | |
| B27 supplement | 1x |
| L-glutamine | 0.5 mM |
| Penicillin-Streptomycin | 100 IU/mL |

| Co-culture media | Final concentration |
|---|---|
| | |
| Dulbecco's Modified Eagle Medium | 1 vol |
| Neurobasal | 1 vol |
| B27 supplement | 1x |
| Penicillin-Streptomycin | 100 IU/mL |
| apo-Transferrin human | 50 µg/mL |
| Biotin | 10 ng/mL |
| BSA (Bovine Serum Albumin) | 50 µg/mL |
| Ceruloplasmin | 100 ng/mL |
| Hydrocortisone | 0.05 µM |
| Insulin | 5 µg/mL |
| N-Acetyl-L-cysteine | 5 µg/mL |
| Progesterone | 6.2 ng/mL |
| Putrescin | 16 µg/mL |
| Recombinant Human CNTF | 0.1 ng/mL |
| Sodium selenite | 5 ng/mL |
| T3 (3,3′,5-Triiodo-L-thyronine sodium salt) | 40 ng/mL |
| Vitamin B12 | 27.2 ng/mL |

| Product |
|---|
| 5-fluorodeoxyuridine |
| B27 supplement |
| D-(+)-Glucose solution |
| DNase (Deoxyribonuclease I) |
| Dulbecco's Modified Eagle Medium |
| Ethanol 100% |
| Ethanol 70% |
| Fetal Calf Serum |
| L-cysteine |
| Neurobasal |
| Papain |
| Penicillin-Streptomycin |
| Phosphate Buffered Saline without calcium and magnesium |
| Polyethylenimine (PEI) |
| Tetraborate decahydrate |
| Trypsin |
| Uridine |

| **Bottenstein-Sato (BS) media** |
|---|
| apo-Transferrin human |
| BSA (Bovine Serum Albumin) |
| Dulbecco's Modified Eagle Medium |
| Insulin |
| PDGF |
| Penicillin-Streptomycin |
| Progesterone |
| Putrescine dihydrochloride |
| Sodium selenite |
| T3 (3,3′,5-Triiodo-L-thyronine sodium salt) |
| T4 (L-Thyroxine) |

| **Co-culture media** |
|---|
| apo-Transferrin human |
| B27 supplement |
| Biotin |
| BSA (Bovine Serum Albumin) |
| Ceruloplasmin |
| Dulbecco's Modified Eagle Medium |
| Hydrocortisone |
| Insulin |

| |
|---|
| N-Acetyl-L-cysteine |
| Neurobasal |
| Penicillin-Streptomycin |
| Progesterone |
| Putrescin |
| Recombinant Human CNTF |
| Sodium selenite |
| T3 (3,3′,5-Triiodo-L-thyronine sodium salt) |
| Vitamin B12 |

| **Tools** |
|---|
| 0.22 µm filter |
| 1 mL syringe |
| 100 mm Petri dish |
| 15 mL tube |
| 50 mL tube |
| 60 mm Petri dish |
| 70 µm filter |
| Binocular microscope |
| Curved forceps |
| Fine forceps |
| Large surgical scissors |
| Scalpel |
| Shaker |
| Small surgical scissors |
| Small surgical spoon |
| T150 $cm^2$ flask with filter cap |

| **Animal** |
|---|
| P2 Wistar rat |

| Company |
| --- |
| Sigma |
| ThermoFisher |
| Sigma |
| Worthington |
| ThermoFisher |
| Sigma |
| VWR Chemicals |
| ThermoFisher |
| Sigma |
| ThermoFisher |
| Worthington |
| ThermoFisher |
| ThermoFisher |
| Sigma |
| Sigma |
| Sigma |
| Sigma |

| Company |
| --- |
| Sigma |
| Sigma |
| ThermoFisher |
| Sigma |
| Peprotech |
| ThermoFisher |
| Sigma |
| Sigma |
| Sigma |
| Sigma |
| Sigma |

| Company |
| --- |
| Sigma |
| ThermoFisher |
| Sigma |
| Sigma |
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| ThermoFisher |
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| Sigma |
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| Sigma |

| **Company** |
|---|
| Sartorius |
| Terumo |
| Dutscher |
| Corning Life Science |
| Corning Life Science |
| Dutscher |
| Miltenyi Biotec |
| Olympus |
| Fine Science Tools |
| Fine Science Tools |
| Fine Science Tools |
| Swann-morton |
| Infors HT |
| Fine Science Tools |
| Bar Naor Ltd |
| Dutscher |

| **Company** |
|---|
| Janvier |

| Catalog number |
| --- |
| F0503 |
| 17504044 |
| G8769 |
| LS002139 |
| 31966021 |
| 32221-M |
| 83801.360 |
| 10082147 |
| C7352 |
| 21103049 |
| LS003126 |
| 15140122 |
| A1285601 |
| P3143 |
| B9876 |
| Sigma |
| U3750 |

| Catalog number |
| --- |
| T1147 |
| A4161 |
| 31966021 |
| I5500 |
| AF-100-13A |
| 15140122 |
| P8783 |
| P5780 |
| S5261 |
| T6397 |
| T1775 |

| Catalog number |
| --- |
| T1147 |
| 17504044 |
| B4639 |
| A4161 |
| 239799 |
| 31966021 |
| H4001 |
| I5500 |

| |
|---|
| A8199 |
| 21103049 |
| 15140122 |
| P8783 |
| P5780 |
| 450-13 |
| S5261 |
| T6397 |
| V6629 |

| Catalog number |
|---|
| 514-7010 |
| 1611127 |
| 193100 |
| 734-1867 |
| 734-1869 |
| 067003 |
| 130-095-823 |
| SZX7 |
| 11152-10 |
| 91150-20 |
| 14008-14 |
| 233-5528 |
| |
| 91460-11 |
| BN2706 |
| 190151 |

| Catalog number |
|---|
| RjHAn:WI |

November 20th, 2019

Dear Editor,

We would like to thank you for considering a revised version of our manuscript entitled "Generation of oligodendrocytes and oligodendrocyte conditioned medium for co-culture experiments" JoVE60912.

We would like to thank the reviewers, whose comments and suggestions allowed us to clarify and improve our manuscript.

We provide below a line-by-line response to each of the editorial and reviewer comments.

We hope that the revised version of our manuscript answers in a satisfactory manner the points raised by the reviewers and the editor, and that it is now suitable for the next step for publication in JoVE.

Best regards,

Nathalie Sol-Foulon

## Editorial Comments:

• Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.

Please track the changes within the manuscript to identify all of the edits: *we put in orange the modified text in the manuscript.*

• **Protocol Detail:**
1) 5.8, 8.7, 11.8: Convert centrifuge speed to g.
*We indicated g values in 5.8, 8.7 which is now 8.9 and 11.8.*

• **Protocol Numbering:** All steps should be lined up at the left margin with no indentations. Please add a one-line space after each protocol step.
*We modified the layout as asked*

• **Protocol Highlight:** Please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps.

1) The highlighting must include all relevant details that are required to perform the step. For example, if step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be included in the highlighting.
2) The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next.

3) Please highlight complete sentences (not parts of sentences). Include sub-headings and spaces when calculating the final highlighted length.
4) Notes cannot be filmed and should be excluded from highlighting.

• **Discussion:** JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.

*We slightly modified the discussion in accordance with this recommendation.*

• **Tables:** Table 1 should be made in the table of materials, Table 2 should be Table 1 etc.

• **Commercial Language:** JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. Examples of commercial sounding language in your manuscript are ThermoFisher, Sigma, Peprotech, Neurobasal, PEI (Sigma, P3143) etc ?
1) The above names are listed in Table 2, 3, 4; List all products names in the table of materials instead.
2) Please also check supplementary files.

• **Table of Materials:** Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials/software in separate columns in an xls/xlsx file. Please include items such as animal strain, microscopes, surgical tools, items from the supplementary file etc.

• If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."

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## Comments from Peer-Reviewers:

**Reviewer #1:**
Manuscript Summary:
This is a detailed and useful protocol from experts in the field of myelination and cultures of oligodendrocyte lineage cells.

Major Concerns:

no

Minor Concerns:
4.4: Should be ice cold PBS instead of PBS-glucose?

*Ice-cold PBS-glucose has been added in the text in 4.4 and 3.3.*

5.1 Cerebral cortices and not cerebellar.

*Correction has been done: "Use a sharp scalpel to finely chop the cerebral cortices. "*

5.6 Reference of the 70 µm filter
*Reference is now indicated in the Table of Materials.*

7. Shaking:
The reference of the flasks should be indicated, without aerated cap?

*Culture flasks have a filter cap, reference is indicated in the Table of Materials*

The reference of the rotary shaker should be indicated.

*The rotary shaker is from Infors HT company as indicated in the Table of Materials, but our shaker is old and we do not have a more precise reference.*

I guess that cells from 6 X flasks of primary culture will be plated in 3 X 100 mm-Petri dishes.

*Yes, this is the cells from 6 X flasks that are plated in two or three dishes.*

8.10 What is the cell density for the plating of OL lineage cells in the coated 100 mm-Petri dishes?

*The cell density for plating is better explain now in 8.11 to 8.14.*

How long does it take to get 70-80 % confluence?

*A 70-80% confluence is reached after 2 days in culture in BS medium. This is now indicated in 8.17 Note.*

P9, lane 9 To be precisely indicated: This treatment promotes the clustering of nodal proteins…before myelination, at DIV17?

*We added at 17 DIV in the text.*

Legend Figure 2: Images are not "Orthogonal projections of Z-series" but Maximum intensity projections.

*Correction has been done, see P10*: "Maximum intensity projections are represented"

In (A) indicate with arrows cells expressing both O4 and NG2 and immature cells only expressing O4.

*Cells expressing both markers are indicated with yellow stars, and cells only expressing O4 with white stars.*

Figure 3: (A) indicate that the OCM medium was added at DIV3 and hippocampal neurons fixed at DIV17.

*We added this in the figure legend as asked*: "Hippocampal neurons treated with OCM at 3 DIV and fixed at 17 DIV"

(B) indicate that OL lineage cells were added at DIV14 and the hippocampal culture fixed at DIV 17-24?

*We now put this result in figure 4 and add the fixation time i.e. 23 DIV in the figure legend.*

(B) I only observed a single node of Ranvier with Nav channels clustered between two myelin segments. As a matter of comparison between the two types of protocol, the same nodal marker should be used (AnkyrinG or Nav).

*We replace this image by another one in figure 4 with better nodes of Ranvier staining (see Nav staining). We also add another image showing Nav channel clustering induced by OCM in figure 3B.*

**Reviewer #2:**
Manuscript Summary:
The protocol is well written and in sufficient detail. Basically, it represents a change from the usual protocol since 3 shakings instead of 1 are included. I would recommend to the authors to check the % of microglia in their protocol. Also I would recommend (I consider this as essential) that they report on the efficiency of their method

Major Concerns:
See above

*We checked the % of microglia by using CD11b staining, see P9*: "In addition, 4.6 ± 0.7 % of cells were $CD11b^+$ microglial cells (mean±sd, not shown)."

*We now better explain the efficiency of the method see 8.12, 8.13 and 8.17 steps*

Minor Concerns:
Several mistakes (ie 7D??? pg 9 steps 11 not 10 for the trypsin step) and adding the reference for rat and mouse staging =Janvier

*We corrected the mistakes and the reference for rat is in the table of materials.*

# Polyethylenimine (PEI) Solution Protocol

1. **Prepare Borax buffer:**
   1.1. Dissolve 14.3 g of sodium tetraborate decahydrate in 950 ml of sterile water.
   1.2. Maintain the solution under constant agitation for 30 min.
   1.3. Adjust pH to 8.3.
   1.4. Add 50 ml of sterile water to the solution for a final solution of 1l.
   1.5. Filter-sterilize the solution using a 0.22 µm filter.

2. **Prepare PEI solution (20X):**
   2.1. Dissolve 2g of PEI in 200 ml of borax buffer.
   2.2. Maintain the solution under constant agitation for 30 min.
   2.3. Add 800 ml of borax buffer to the solution for a final solution of 1l at 2g/l.
   2.4. Filter-sterilize the solution using a 0.22 µm filter.
   2.5. Store at 4°C.

NOTE: Dilute 20X stock PEI solution in sterile water to obtain 1X working PEI solution. Coating can be performed for 30 min at 37°C or overnight at 4°C. Due to PEI toxicity, rinse coating three times with sterile water before cell plating.