High-content screening differentiation and maturation analysis of fetal and adult neural stem cell-derived oligodendrocyte precursor cell cultures

TITLE:
High-content screening differentiation and maturation analysis of fetal and adult neural stem cell-derived oligodendrocyte precursor cell cultures

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KEYWORDS (min 6 – max 12):
oligodendrocyte precursor cells, neural stem cells, oxygen-glucose deprivation, inflammation, high-content screening, drug screening

SUMMARY (min 10- max 50 words):
We describe the production of mixed cultures of astrocytes and oligodendrocyte precursor cells derived from fetal or adult neural stem cells differentiating into mature oligodendrocytes, and *in vitro* modeling of noxious stimuli. The coupling with a cell-based high-content screening technique builds a reliable and robust drug screening system.

ABSTRACT (min 150 – max 300 words):
The main hurdle in developing drug screening techniques for assessing the efficacy of therapeutic strategies in complex diseases is striking a balance between *in vitro* simplification and recreating the complex *in vivo* environment, along with the main aim, shared by all screening strategies, of obtaining robust and reliable data, highly predictive for *in vivo* translation.
In the field of demyelinating diseases, the majority of drug screening strategies are based on immortalized cell lines or pure cultures of isolated primary oligodendrocyte precursor cells (OPCs) from newborn animals, leading to strong biases due to the lack of age-related differences and of any real pathological condition or complexity.
Here we show the setup of an *in vitro* system aimed at modeling the physiological differentiation/maturation of neural stem cell (NSC)-derived OPCs, easily manipulated to mimic pathological conditions typical of demyelinating diseases. Moreover, the method includes isolation from fetal and adult brains, giving a system which dynamically differentiates from OPCs to mature oligodendrocytes (OLs) in a spontaneous co-culture which also includes astrocytes. This model physiologically resembles the thyroid hormone-mediated myelination and myelin repair process, allowing the addition of pathological interferents which model disease mechanisms. We show how to mimic the two main components of demyelinating diseases, i.e. hypoxia/ischemia and inflammation, recreating their effect on developmental myelination and adult myelin repair and taking all the cell components of the system into account throughout, while focusing on differentiating OPCs.

This spontaneous mixed model, coupled with cell-based high-content screening technologies, allows the development of a robust and reliable drug screening system for therapeutic strategies aimed at combating the pathological processes involved in demyelination and at inducing remyelination.

INTRODUCTION (min 150 – max 1500 words; min 2 paragraphs)

In the central nervous system (CNS), myelin forming cells (oligodendrocytes, OLs) and their precursors (oligodendrocyte precursor cells, OPCs) are responsible for developmental myelination, a process which occurs during the peri- and post-natal periods, and for myelin turnover and repair (remyelination) in adulthood[1]. These cells are highly specialized, interacting anatomically and functionally with all the other glial and neuronal components, making them a fundamental part of CNS structure and function.

Demyelinating events are involved in different CNS injuries and diseases[2], and mainly act on OPCs and OLs by way of multifactorial mechanisms, both during development and adulthood. The undifferentiated precursors are driven by differentiating factors, mainly thyroid hormone (TH), in a synchronized process[3] which leads the OPC to recognize and respond to specific stimuli which induce proliferation, migration to the non-myelinated axon, and differentiation into mature OLs which in turn develop the myelin sheath[4]. All these processes are finely controlled and occur in a complex environment.

Due to the complex nature of myelination, remyelination and demyelination events, there is a great need for a simplified and reliable *in vitro* method to study the underlying mechanisms and to develop new therapeutic strategies, focusing on the main cellular player: the OPC[5].

For an *in vitro* system to be reliable, a number of factors need to be taken into account: the complexity of the cellular environment, age-related cell-intrinsic differences, physiological TH-mediated differentiation, pathological mechanisms, and the robustness of the data[6]. Indeed, the unmet need in the field is a model which mimics the complexity of the *in vivo* condition, not successfully achieved through the use of isolated pure OPC cultures. In addition, the two main components of demyelinating events, inflammation and hypoxia/ischemia (HI), directly involve other cell components that may indirectly affect the physiological differentiation and maturation of OPCs, an aspect which cannot be studied in over-simplified *in vitro* models.

Starting from a highly predictive culture system, the subsequent and more general challenge is the production of robust and reliable data. In our context, cell-based high-content screening (HCS) is the most suitable technique[7], since our aim is firstly to analyze the entire culture in an automatic workflow, avoiding the bias of choosing representative fields, and secondly to obtain the automatic and simultaneous generation of imaging-based high-content data[8].

Given that the main need is to achieve the best balance between *in vitro* simplification and *in vivo*-mimicking complexity, here we present a highly reproducible method for obtaining OPCs derived from neural stem cells (NSCs) isolated from the fetal forebrain and the adult sub-ventricular zone (SVZ). This *in vitro* model encompasses the entire OPC differentiation process, from multipotent NSC to mature/myelinating OL, in a physiological TH-dependent manner. The resulting culture is a dynamically differentiating/maturating system which results in a spontaneous co-culture consisting mainly of differentiating OPCs and astrocytes, with a low

percentage of neurons. This primary culture better mimics the complex *in vivo* environment, while its stem cell derivation allows simple manipulations to be performed to obtain the cell lineage enrichment desired.

On the contrary to other drug screening strategies using cell lines or pure cultures of primary OPCs, the method described here allows the study of the effect of pathological interferents or therapeutic molecules in a complex environment, without losing the focus on the desired cell type. The HCS workflow described permits an analysis of cell viability and lineage specification, as well as lineage-specific cell death and morphological parameters.

PROTOCOL (min 1 pag – max 10 pag):

1. Solutions and reagents

1.1 Standard medium: DMEM/F12 GlutaMAX 1 x; 8 mmol/L HEPES; 100 U/100 µg Penicillin/Streptomycin (1% P/S); 1 x B27; 1 x N-2.

1.2 Neurosphere medium: add 10 ng/mL bFGF; 10 ng/mL EGF to standard medium.

1.3 Oligosphere/OPC medium: add 10 ng/mL bFGF; 10 ng/mL PDGF-AA to standard medium.

1.4 Oligodendroctye differentiation medium: add 50 nM T3; 10 ng/ml CNTF; 1x *N*-acetyl-L-cysteine (NAC) to standard medium.

1.5 Non-enzymatic dissociation buffer: add 1% P/S to non-enzymatic dissociation buffer and keep ice cold.

1.6 Sucrose solution: HBSS, 0.3 g/ml sucrose.

1.7 BSA washing solution: EBSS, 40 mg/ml BSA, 0.02 ml/l HEPES.

1.8 Enzymatic dissociation buffer: HBSS, 5.4 mg/ml D-glucose , 15 mmol/L HEPES , 1.33 mg/ml Trypsin, 0.7 mg/ml Hyaluronidase, 80 U/ml DNase.

1.9 Cytokine mix: TGF-β1, TNF-α, IL-1β, IL-6, IL-17 and IFN-γ (20 ng/mL each).

1.10 Cytokine mix vehicle: 0.04% of the stock (10% glycerol/100 nM glycine/25 nM Tris, pH 7.3).

1.11 Oxygen-glucose deprivation medium: standard medium using DMEM w/o glucose.

2. Dissection and NSC isolation

All animal protocols described herein were carried out according to European Community Council Directives (86/609/EEC) and comply with the guidelines published in the *NIH Guide for the Care and Use of Laboratory Animals*.

Fetal and adult NSCs were isolated from E13.5 fetal forebrain or 2.5-month-old adult sub-ventricular zone (SVZ), following the Ahlenius and Kokaia protocol[9] with modifications.

2.1 Fetal NSC cultures

Before starting the dissections, prepare 1.5 ml tubes containing 150 µl of non-enzymatic dissociation buffer each, clean Petri dishes and add ice cold HBSS.

2.1.1 Collect the embryos at E13.5 - 14.5 from timed pregnant mice and place in a Petri dish containing cold HBSS.

2.1.2 Decapitate the embryos using forceps.

2.1.3 Place the heads of the embryos in clean Petri dish containing ice cold PBS and remove the skin from the skull with forceps, using magnifying glasses or a stereoscope.

2.1.4 Once the brain is visible and cleared of skin, squeeze it out by applying pressure at the sides with forceps.

2.1.5 Remove the cerebellum, keep only the forebrain and remove the meninges with forceps.

2.1.6 Place the isolated tissue in the non-enzymatic dissociation buffer and repeat the dissection steps with the other embryos. Insert the tissue from 2 – 3 animals into each tube containing the buffer.

2.1.7 Incubate at 37°C for 15 minutes under continuous shaking.

2.1.8 After incubation, add 850 µl of standard medium and mix by pipetting until the suspension is free of clumps.

2.1.9 If non-dissociated tissue is still visible, wait 2 min at RT until it deposits on the bottom of the tube.

2.1.10 When dissociation is complete, count the cells and plate them in suspension at a density of 10 – 50 cells/µl in a t25 or t45 flask (Nunc) containing 10 – 30 ml of neurosphere medium, kept in a vertical position to avoid cell adhesion. The vertical position will allow the cell suspension cultures to avoid cell attachment.

2.2 Adult NSC cultures

2.2.1 Sacrifice animals by cervical dislocation.

2.2.2 Collect brains from 4 – 5 mice in a 50 ml tube containing ice cold HBSS.

2.2.3 Place the brain on a cold sterile surface. For this purpose, a t25 flask filled with water and placed at -20°C overnight can be used. At the time of the experiment, cover the flask with sterile aluminum foil.

2.2.4 Place the brain ventral side downwards, in rostro-caudal direction, and remove the olfactory bulbs using a razor blade.

2.2.5 Using a razor blade, cut 2 – 3 coronal slices of 1 mm thickness, from the cortex to the optical chiasma.

2.2.6 Place the slices on the cold surface in a ventro-dorsal position and identify the corpus callosum and the two lateral ventricles.

2.2.7 Using magnifying glasses or a stereoscope, isolate the walls of the lateral ventricles, taking care not to carry pieces of the corpus callosum.

2.2.8 Put the isolated tissue in the enzymatic dissociation buffer (5 – 10 ml) and incubate at 37°C for 15 min.

2.2.9 Mix the solution, pipetting several times (at least 50), and incubate again at 37°C for 10 min.

2.2.10 Neutralize the trypsin by adding 5 ml of standard culture medium and filter the solution using a 70 µm filter.

2.2.11 Centrifuge the filtered solution for 5 min at 400 × g.

2.2.12 Resuspend the pellet in the sucrose solution and centrifuge for 10 minutes at 500 × g.

2.2.13 Resuspend the pellet in BSA washing solution and centrifuge for 7 minutes at 400 × g.

2.2.14 Resuspend the pellet in the standard culture medium, count the cells, and perform plating as described above (2.1.10).

3. Primary neurospheres

3.1 Add the growth factors (bFGF/EGF) every two days.

3.2 Every 4 – 6 days (depending on cell density), change half of the medium as follows:

3.2.1 Transfer the entire cell suspension to a 15 or 50 ml tube.

3.2.2 Centrifuge for 5 min at 400 × g.

3.2.3 Remove half of the volume.

3.2.4 Add the same amount of fresh medium, gently mix by pipetting, and add growth factors.

4. Oligospheres

Oligodendrocyte differentiation is performed following the Chen protocol[10] with modifications.

4.1 When the neurospheres reach a diameter of 100 – 150 µm, they are ready to be passed. To do so, transfer the entire cell suspension to a 15 or 50 ml tube, and centrifuge for 5 min at 400 × g.

> 4.1.1 The diameter can be rapidly evaluated by taking pictures of the spheres using an inverted transmitted light microscope and opening them by ImageJ software.
>
> 4.1.2 Click on “Analyze” menu and from the “Tools” window choose “Scale bar”
>
> 4.1.3 Set 150 µm as “Width in microns” and compare the scale bar with the spheres.

4.2 Remove the entire volume by inversion and resuspend the pellet in 180 µl of fresh standard culture medium. Pipette 50 times to allow disaggregation of the spheres.

4.3 Add 810 µl of fresh standard culture medium, count the cells, and re-plate them as described for the neurospheres.

4.4 Add bFGF/PDGF-AA 10 ng/ml every two days.

4.5 Every 4 – 6 days (depending on cell density), change half of the medium as follows:

4.6 Transfer the entire cell suspension to a 15 or 50 ml tube.

4.7 Centrifuge for 5 min at 400 × g.

4.8 Remove half of the volume.

4.9 Add the same amount of fresh medium, gently mix by pipetting, and add growth factors.

5. Plate coating

5.1 Poly-D,L-ornithine / laminin coating: at least two days before plating the OPCs, add 50 µg/ml poly-D,L-ornithine solution, diluted in PBS, to each well (40 µl/well for 96-well plates) and incubate at RT overnight.

5.2 The following day, remove the liquid and wash three times with distilled sterile water.

5.3 Let the plates dry at RT overnight. The following day, add a laminin solution diluted in PBS (5 µg/ml; 40 µl/well for 96-well plates) and incubate for 2 hours at 37°C.

6. Cell seeding

6.1 When the oligospheres reach a diameter of 100 – 150 µm, they are ready to be dissociated and seeded on the poly-D,L-ornithine / laminine coated plates. To do so, transfer the entire cell suspension to a 15 or 50 ml tube, and centrifuge for 5 min at 400 × g (as indicated at 4.1)

6.2 Remove the entire volume by inversion and resuspend the pellet in 180 µl of fresh standard culture medium. Pipette 50 times to allow disaggregation of the spheres.

6.3 Add 810 µl of fresh standard culture medium and count the cells.

6.4 Remove the laminin solution from the wells and plate the cells at 3000 cell/cm^2 density (100 µl/well for 96-well plates).

7. OPC differentiation induction

7.1 After 3 days, remove the entire medium and add the same volume of oligodendrocyte differentiation medium.

7.2 Change half of the medium every 4 days and add fresh differentiation mix (T3/CNTF/NAC) every two days.

8. Induction of inflammation-mediated differentiation block

8.1 After neurosphere dissociation and oligosphere production (section 4), add the cytokine mix to the culture medium and keep oligospheres exposed to cytokines for the wole spheres formation step.
Note that the volume depends on the number of cells, since for the spheres forming cells are seeded at 10 – 50 cells/µl.

8.2 If the medium needs to be changed, change the entire volume and add the cytokine mix once more.

9. Induction of oxygen-glucose deprivation cell death

9.1 At -1 DIV (2 days after cell seeding in multiwell plates), remove the medium and conserve it in a new multiwell plate.

9.2 Add half the volume (50 µl for 96-well plates) of OGD-medium (OGD group) or fresh medium (control group). The half amount of volume is used to reduce the exchange of oxygen between the liquid and the air.

9.3 Place the OGD group cultures in an airtight hypoxia chamber saturated with 95% N_2 and 5% CO_2. To achieve saturation of the chamber, let the gas mixture flow for 6 minutes at 25 l/min before closing the chamber pipes.

9.4 Incubate the hypoxic chamber in the incubator for 3 hours. The control group and plates containing the medium removed and conserved at step 9.1 should also be left in the incubator.

9.5 Remove the glucose-free (OGD group) or the new medium (control group) and add the medium removed and conserved at step 9.1.

10. Immunocytochemistry

10.1 At the desired time point, fix the cells with cold 4% paraformaldehyde for 20 min at RT.

10.2 Wash twice with PBS (10 minutes of incubation for each wash at RT).

10.3 Incubate with primary antibody mix (Table 1), diluted in PBS triton 0.3%, overnight at 4°C.

10.4 Wash twice with PBS (10 minutes of incubation for each wash at RT).

10.5 Incubate with secondary antibody (Table 1) solution diluted in PBS triton 0.3% adding Hoechst 33258 (Thermo Fisher Scientific) for 30 minutes at 37°C.

10.6 Wash twice with PBS (10 minutes of incubation for each wash at RT).

11. HCS analysis of cell viability, lineage composition and lineage-specific cell death

The HCS representative images and workflow are shown in **Figure 2A,B**.

11.1 Choose the “Compartmental Analysis” algorithm from the main menu of the software (HCS Studio v 6.6.0) and chose “Scan” from the main menu “Develop Assay/Scan Plate”.

11.2 In the “iDev” window chose “New” and select “General intensity Measurement Tool” from the “Develop Assay” template.

11.3 Click on “Create” on the right side of the menu, selecting the 10x objective.

11.4 This will open the “Configure acquisition” menu. In this window select the following parameters:
- number of channels: the first one for the Hoechst nuclear staining (BGRFR_386) and one for each lineage specific marker used in the reaction
- select software focus on channel 1 and autofocus interval as 1
- select your plate model from the list

11.5 From the acquisition menu, look at the quality of the staining in different wells and different fields and select manually the exposure time, selecting “fixed exposure time” in the menu.

11.6 Once the acquisitions parameters are set, select “Mini Scan” on the top of the menu and chose ten fields per well in two wells per experimental conditions. This will allow the set-up of all the analysis parameter in a subset of fields for the entire plate.

11.7 When the mini scan is finished, clik on “Configure Assay Parameter” to configure the algorithm of the analysis.

11.8 Click on “Configure groups” in the right side of the window and drag-and-drop the wells of the miniscan. Click on the “Add” button in the “Groups” sub-section to configure the different groups.

11.9 The left side of the window is the workflow that should be follow step by step to develop the whole algorithm. First Select “Process Image” for each channel, clicking on “Background removal” and on the desired level.

11.10 First identify and select the nuclei by the nuclear staining. Click on “Identify Primary Object – Channel 1” to select the real nuclei and avoid analyzing artifact and debris. For this purpose, zoom in a representative picture of nuclear staining and check if nuclei are well surrounded by the perimeter build by the software.
It is possible to change the thresholding value and to apply segmentation algorithms to better identify single nuclei.

11.11 Once nuclei are defined correctly, click on the following step: “Validate Primary Object”.
Select “Object.BorderObject.Ch1” to avoi the analysis of nuclei at the border of each field image.
Select “Object.Area.Ch1” and, moving the “low” and “high” bars on the histograms, remove all the identified debris or big-objects corresponding to aggregates or artifacts.

11.12 Check all the mini scan representative images of all the experimental conditions to be sure that the selected parameters fit with all of them.

11.13 Click on “Identify spots” for each channel corresponding to the specific lineage markers, and select the Ring values: Widht = 3 and Distance = 0. This will allow the identification of the cytoplasmatic fluorescence.

According to the cell density these values can be adapted. The software will automatically avoid the overlapping between adjacent rings.

11.14 Select “Reference Levels” in the workflow to build the analysis. The setting of the reference levels will allow the automatic counting of condensed nuclei, based on the nuclear size and nuclear staining intensity, and of specific marker-positive cells, based on the cytoplasmatic fluorescence identified by the Ring.
First click on “Object.Area.Ch1”. In your mini scan images select a condensed nucleus and move the “LOW” bar on the histograms in order to select as “condensed” all the nuclei under this size.

11.15 Click on “Object.AvgIntensity.Ch1”. In your mini scan images select a condensed nucleus and move the “HIGH” bar on the histograms in order to select as “condensed” all the nuclei above this fluorescence intensity.

11.16 Click on “Object.RingAvgIntensity” for each channels of lineage specific markers. Select in your mini scan images a positive cell and move the “HIGH” bar on the histograms in order to select as “positive” all the cells above this fluorescence intensity.

11.17 Check all the mini scan representative images of all the experimental conditions to be sure that the selected parameters fit with all of them.

11.18 On the top menu, select “Population Characterization” and select “Event Subpopulation”.

11.19 As “Type 1” Event, select “ObjectAreaCh1” on the left list, than click on “AND >” button and finally select “ObjectAvgIntensityCh1”. This will allow the identification of condensed nuclei, as a combination of low area and high intensity.

11.20 In the same window, deselect all the Scan Limits.

11.21 Click on “Select Features to Store” in the top menu, to choose the parameters to keep in the analysis.

11.22 Select “Well features” and move from the left list to the right only the desired parameters:
- SelectedObjectCountPErValidField
- %EventType1ObjectCount
- %High_RingAvgIntensity (For each channel of the specific lineage markers).

Note that this analysis will give as readout the total number of cells, the percentage of condensed nuclei and the percentage of lineage-specific positive cells for each analyzed marker on the total cell number.
If the the percentage of the different lineages is needed only on live cells, is it possible either to keep the value “High_RingAvgIntensity” for the channel (absolute number of positive cells) and recalculate the percentage on total cell numbers after the subtraction of the percentage of dead cells.

Alternatively it is possible to remove the dead cells from the analysis setting the same parameters used to identify condensed nuclei (11.14 – 11.15) on the nuclei validation step (11.11).

11.23 Select "Scan Plate" from the main top menu and click on the plate symbol on "Scan Setting" sub-menu on the top section to identify the well to analyze.

11.24 Write the name of your experiment and the description and once all the setting is completed, click on the "play" symbol.

REPRESENTATIVE RESULTS

The first phase of the culture may vary in duration, depending on seeding density and on whether the spheres are of fetal or adult origin. Moreover, oligospheres display a reduced population doubling compared to neurospheres (**Figure 1B**). Moreover, spheres production from adult tissue is slower and it may take 2 - 3 weeks to generate oligospheres compared to fetal that may take 1 -2 weeks, depending on the seeding density.

Once seeded, the entire differentiation phase of the cultures can be monitored using lineage-specific antibodies. Since the objective of this protocol is to study the final phase of the differentiation, the culture composition at 0 DIVs is not presented. However, during the first culture phase, cells will be still nestin-positive, representing neural precursors, and the majority of cells are also NG2-positive (OPCs)[11]. CNPase-positive cells, corresponding to the preOL stage, will be detectable three to six days after T3-mediated differentiation induction, while MBP-positive cells will appear between 6 and 12 DIVs (mature OLs; see **Figure 2C** for the cultures composition at the end of the differentiation phase).

The HCS analysis allows the detection of each single cell in the culture through the nuclear staining and the analysis of the fluorescence intensity in the remaining channels (**Figure 2A,B**).

The composition of the culture at the end of the differentiation phase (12 DIVs) differs depending on whether the cultures are of fetal or adult origin, with fetal cultures more responsive to T3-mediated differentiation and reaching a higher percentage of mature OLs[12].

Throughout the entire culture process, around 40 – 50% of the cells are astrocytes (GFAP-positive cells), while a small percentage (less than 0 - 10%) are neurons (beta-III-tubulin-positive cells; **Figure 2C**). The culture composition may vary of a 10% between different culture preparations. Adult and fetal cultures differ for the yield of mature OLs production at the end of the differentiation phase, with fetal cells showing high percentage of mature OLs, low percentage of precursors and around 30 - 40% of astrocytes. On the other hand, adult cultures present more astrocytes (around 45 - 55%) and less differentiated cells after 12 DIVs of differentiation induction.

To allow the software to recognize the cells and to provide a proper unbiased analysis of the culture composition, it is important that the seeding density is correct, avoiding overlapping between adjacent cells. When NSC-derived OPCs are seeded at high density, they tend to aggregate very fast, leading to the entire surface of the well being occupied by astrocytes after a few days. Moreover, mature OLs with their characteristic spider-net shape will be not visible due to the limited space (**Figure 3 A, B**).

The inflammation-mediated differentiation block is reproducible by this *in vitro* assay, and generates a strong decrease in preOLs and mature OLs detected by CNPase and MBP staining in both fetal and adult cultures. An increase in the number of OPCs also occurs in adult cultures (**Figure 4A,B**). The cytokine mix composition was chosen from *in vivo* experiments in a rat model of multiple sclerosis[13], and was tested as an *in vitro* model for the inflammation-mediated differentiation block occurring in this disease.

While fetal and adult OPCs show the same vulnerability to inflammatory cytokine exposure, only fetal-derived cultures are sensitive to OGD toxicity (**Figure 5A,B**), showing an increase in cell death and differentiation impairment due to their different metabolic profile[14].

FIGURES AND TABLE LEGENDS:

Figure 1: Neural stem cell-derived oligodendrocyte precursor cell culture setup and differentiation protocol. (**A**) Scheme of the experimental procedure. (B) Representative images of neurospheres at 2, 5 and 7 DIVs, and graph showing the population doubling of neurospheres and oligospheres. Scale bar: 100 µm. (**C**) Representative images of seeded oligosphere-derived OPCs showing the different stages of differentiation, from nestin and NG2-positive cells at 0 DIV (neural precursor / OPCs), through CNPase-positive cells at 6 DIVs (preOLs) and CNPase/MBP double positive cells at the end of the differentiation phase (12 DIVs; mature OLs). GFAP-positive cells (astrocytes) and a small percentage of beta-III-tubulin positive cells (neurons) are present throughout the entire culture. Scale bars: 20 µm.

Figure 2: Cell-based high-content screening analysis workflows and expected differentiation readout. (**A**) Representative images of HCS acquisition of an entire well (96-well plate) and an isolated single field acquired with a 10 x objective of a 12 DIVs culture of NSC-derived OPCs. (**B**) HCS analysis workflow steps including nuclei (objects) visualization, identification, and construction of nuclei ring to identify the cytoplasmic staining and marker identification. (**C**) Graph showing the expected culture composition at the end of the differentiation phase (12 DIVs). Markers for OPCs (PDGFαR, NG2), preOLs (CNPase, APC), mature OLs (MBP), astrocytes (GFAP) and neurons (β-III-tubulin) are shown for both fetal- and adult-derived cultures. Rounded off percentages for each cell markers are included in the graph, note that this is a representative experiment and percentages may different about 5 – 10%.

Figure 3: Representative high-content screening images of a high-density culture. (**A**) Representative image of a well (96-well plate) image acquired by 10 x objective and marked for MBP expression at the end of the differentiation phase (12 DIVs). (**B**) Representative extracted field image highlighting the presence of aggregated cells and overlapping nuclei.

Figure 4: Expected effect of cytokine treatment on fetal- and adult-derived OPC cultures. (**A**) Graph showing the percentage of variation of fetal- and adult-derived OPC cultures compared to standard cultures, including the quantification of OPCs (NG2), preOLs (CNPase) and mature OLs (MBP) at the end of the differentiation phase (12 DIVs). (**B**) Representative images of adult

cultures at the end of the differentiation phase (12 DIVs) treated with vehicle or cytokine mix and marked for NG2 or CNPase / MBP. Scale bar: 20 µm.

Figure 5: Expected effect of OGD exposure on fetal-derived OPC cultures. (**A**) Graph showing the percentage of condensed nuclei quantified by cell-based HCS in control (ctrl) and OGD-exposed cultures. (**B**) Representative images of HCS-processed objects highlighting the identified condensed nuclei (white arrows).

DISCUSSION

The complex nature of myelination/remyelination processes and demyelinating events makes the development of predictive in vitro systems extremely challenging. The most widely used *in vitro* drug screening systems are mostly human cell lines or primary pure OL cultures, with increasing use of more complex co-cultures or organotypic systems[15]. Even if such systems are coupled with high content technologies, pure OL cultures remain the method of choice when developing screening platforms[16].

The spontaneous mixed culture described here represents a useful *in vitro* system which takes all main variables into account: physiological T3-mediated OPC differentiation, pathological interferents with the process, other cellular components and age-related differences. The procedure contains a number of variables deriving from the origin of the cells (age of the animal) and the spheroids formation and manipulation. In fact, a critical step is the cell density of NSCs seeding after the isolation from the tissue, since in the optimal condition a single sphere should derive from a single proliferating cell. Seen that isolated NSCs tend to aggregate and that they need their own secreted paracrine factors, seeding them in a range of 10 - 50 cells/µl, in a t25 or t75 flask, is the best compromise to avoid cell aggregation but still allowing cells to communicate by secreting factors.

The main limitations of the technique is the lacking of a functional axonal myelination and a direct interaction with neurons, since the method takes into account only the OPC differentiation until the stage of mature OLs: CNPase/MBP-double positive cells with a spider net morphology. For this purpose, primary OPCs cultured on isolated dorsal root ganglia is still the main methodology[17]. However, the possibility to differentiate these cells from animals at any age is a fundamental point in the translational process, since it allows the test of compounds and noxious stimuli on cells isolated from the age of interest. As described here, in fact, NSCs can be isolated from both the fetal and the adult brain. Since developmental myelination and remyelination in adulthood share the same objective, i.e. to reach the nude axon and create the myelin sheath, it was originally hypothesized that the two processes were identical in every aspect, generating the so called recapitulation hypothesis[18]. However, it is now clear that the two processes cannot be considered equal and that cell-intrinsic age-related differences are present and should be taken into account when choosing the most suitable in vitro model for the experimental question[19]. Adult NSCs-derived OPCs, in fact, show strong differences in physiological TH-driven differentiation and vulnerability to noxious stimuli[14,20] as well as primary OPCs[21,22]. There is also heterogeneity of OPCs and OLs population in adult tissues, of particular relevance for

pathological conditions[23]. Protocols for primary OPCs isolation from adult tissues are available[24] and should be considered when the experimental question is addressed to molecules acting on remyelination in adulthood.

The differentiation of OPCs from NSCs permits the in vitro representation of the entire differentiation process, from undifferentiated precursor to mature OL. This process resembles the *in vivo* condition, where TH is the main driver of the process, acting through specific nuclear receptors, and it permits experimental interference with this mechanism to mimic pathological conditions in a translational view[13].

The final fundamental characteristic of the model is the constant presence of astrocytes throughout the entire culture. While this makes the culture more difficult to analyze, its complex cell composition constitutes a distinct advantage. The manner in which astrocytes contribute to the response to noxious events in mixed neuronal cultures[25] is widely known, and the absence of this main component of the CNS makes the in vitro system poorly predictable and translatable. On the other hand, for this characteristic, NSC-derived cultures have the disadvantage of being less uniform than single-cell type systems, and this may lead to a biased analysis. However, the cell-based HCS technique allows an analysis of the entire culture and of all the cell populations, removing also the randomization of representative fields for analysis. Assuming that the cell culture used for the experiment is of a reliable seeding quality, the HCS will give a full picture of the experimental conditions, generating statistically robust data and a number of automatic fluorescence-based analyses.

In conclusion, the current protocol describes the procedure for the isolation and differentiation of NSC-derived OPCs from fetal and adult brain. The entire protocol takes around 30 days, depending on the age of the animals and the experimental goals. In particular, spheres formation form adult origin may take double time compared to fetal ones, at the same seeding density. The time of 15 days (from -3 to 12 DIVs) after the seeding on 2D surface for the differentiation induction is, however, a fixed time in all the conditions. The full protocol allows the study of the entire TH-mediated differentiation process in a complex cellular environment, interference through specific pathological mechanisms (i.e. inflammatory cytokines and HI) and the consequent testing of new strategies aimed at overcoming these issues. The coupling of the culture model with the HCS technique generates a robust and translatable screening platform.

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The authors have nothing to disclose.

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Antibody	Species	Dilution
anti-β-III-tubulin (R&D system)	mouse	1:3000
anti-GFAP (Dako)	rabbit	1:1000
anti-NG2 (Millipore)	rabbit	1:350
anti-PDGFαR (Santa Cruz Biotechnology)	rabbit	1:300
anti-CNPase (Millipore)	mouse	1:500
IgG2b anti-APC, clone CC1 (Calbiochem)	mouse	1:100
Anti-MBP (Dako)	rabbit	1:250
Anti-nestin (Millipore)	mouse	1:500
Alexa Fluor 488-conjugated anti mouse (ThermoFisher Scientific)	donkey	1: 500
Alexa Fluor 647-conjugated anti- mouse IgG2b (ThermoFisher Scientific)	goat	1:500
Alexa 568-conjugated anti-rabbit (ThermoFisher Scientific)	donkey	1:500

Table 1: List of primary and secondary antibodies