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Corresponding Author:	Vito Antonio Baldassarro, Ph.D. ITALY
Corresponding Author's Institution:	
Corresponding Author E-Mail:	vito.baldassarro2@unibo.it
Order of Authors:	Vito Antonio Baldassarro, Ph.D.
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TITLE:

High-Content Screening Differentiation and Maturation Analysis of Fetal and Adult Neural Stem Cell-Derived Oligodendrocyte Precursor Cell Cultures

AUTHORS AND AFFILIATIONS:

Vito Antonio Baldassarro[1]

[1]Health Science and Technologies Interdepartmental Center for Industrial Research (HST-ICIR), University of Bologna, Italy

Corresponding author:

Vito Antonio Baldassarro (vito.baldassarro2@unibo.it)

KEYWORDS:

oligodendrocyte precursor cells, neural stem cells, oxygen-glucose deprivation, inflammation, high-content screening, drug screening

SUMMARY:

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:

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:

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:

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*.

1. Solutions and reagents

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

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

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

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

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

1.6. Prepare sucrose solution: HBSS, 0.3 g/mL sucrose.

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

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

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

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

2. Dissection and NSC isolation

NOTE: 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

NOTE: 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 a 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 min 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 for 2 min at RT until it deposits at 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 T-25 or T-45 flask 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, use a T-25 flask filled with water and placed at -20 °C overnight. 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 x *g*.

2.2.12. Resuspend the pellet in the sucrose solution and centrifuge for 10 min at 500 x *g*.

2.2.13. Resuspend the pellet in BSA washing solution and centrifuge for 7 min at 400 x *g*.

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

3. Primary neurospheres

3.1. Add the growth factors (bFGF/EGF) every 2 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 x *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

NOTE: 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 x *g*.

4.1.1. Rapidly evaluate the diameter by taking pictures of the spheres using an inverted transmitted light microscope and opening them by ImageJ software.

4.1.2. Click on the **Analyze** menu and from the **Tools** window, select **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 2 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 x *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 2 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 h 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 x *g* (as indicated in step 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 3,000 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 2 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 whole spheres formation step.

NOTE: 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 min at 25 l/min before closing the chamber pipes.

9.4. Incubate the hypoxic chamber in the incubator for 3 h. 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 min 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 min 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 for 30 min at 37 °C.

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

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

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

11.1. Select the **Compartmental Analysis** algorithm from the main menu of the software (HCS Studio v 6.6.0) and select **Scan** from the main menu **Develop Assay/Scan Plate**.

11.2. In the **iDev** window, select **New** and then 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: (a) number of channels: the first one for the Hoechst nuclear staining (BGRFR_386) and one for each lineage-specific marker used in the reaction (b) select software focus on channel 1 and autofocus interval as 1 (c) select the 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 manually select the exposure time by 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 select ten fields per well in two wells per experimental condition. 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, click on the **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. Follow the workflow on the left side of the window step by step to develop the whole algorithm. First select **Process Image** for each channel and click on **Background Removal** and on the desired level.

11.10. First identify and select the nuclei by 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 whether the nuclei are well surrounded by the perimeter built by the software. It is possible to change the thresholding value and to apply segmentation algorithms to better identify single nuclei.

11.11. Once the nuclei are defined correctly, click on the following step: **Validate Primary Object**. Select **Object.BorderObject.Ch1** to avoid the analysis of nuclei at the border of each field image. Select **Object.Area.Ch1** and, by 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: Width = 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.

11.15. First click on **Object.Area.Ch1**. In the 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.

11.16. Click on **Object.AvgIntensity.Ch1**. In the 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.

11.17. Click on **Object.RingAvgIntensity** for each channel 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.

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

11.19. On the top menu, select **Population Characterization** and select **Event Subpopulation**.

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

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

11.22. Click on **Select Features to Store** in the top menu, to choose the parameters to keep in the analysis.

11.23. Select **Well Features** and move from the left list to the right only the desired parameters: (a) SelectedObjectCountPErValidField (b) %EventType1ObjectCount (c) %High_RingAvgIntensity (For each channel of the specific lineage markers).

NOTE: 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 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.

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

11.24. 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.

11.25. Write the name of the experiment and the description and once all the settings are completed, press 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 3–6 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 not be visible due to the limited space (**Figure 3A,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 10x 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 be 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 10x 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).

Table 1: List of primary and secondary antibodies

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 the 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. Since we have 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 from 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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DISCLOSURES:

The authors have nothing to disclose.

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Figure1 Click here to access/download;Figure;Figure01-Differentiation.jpg

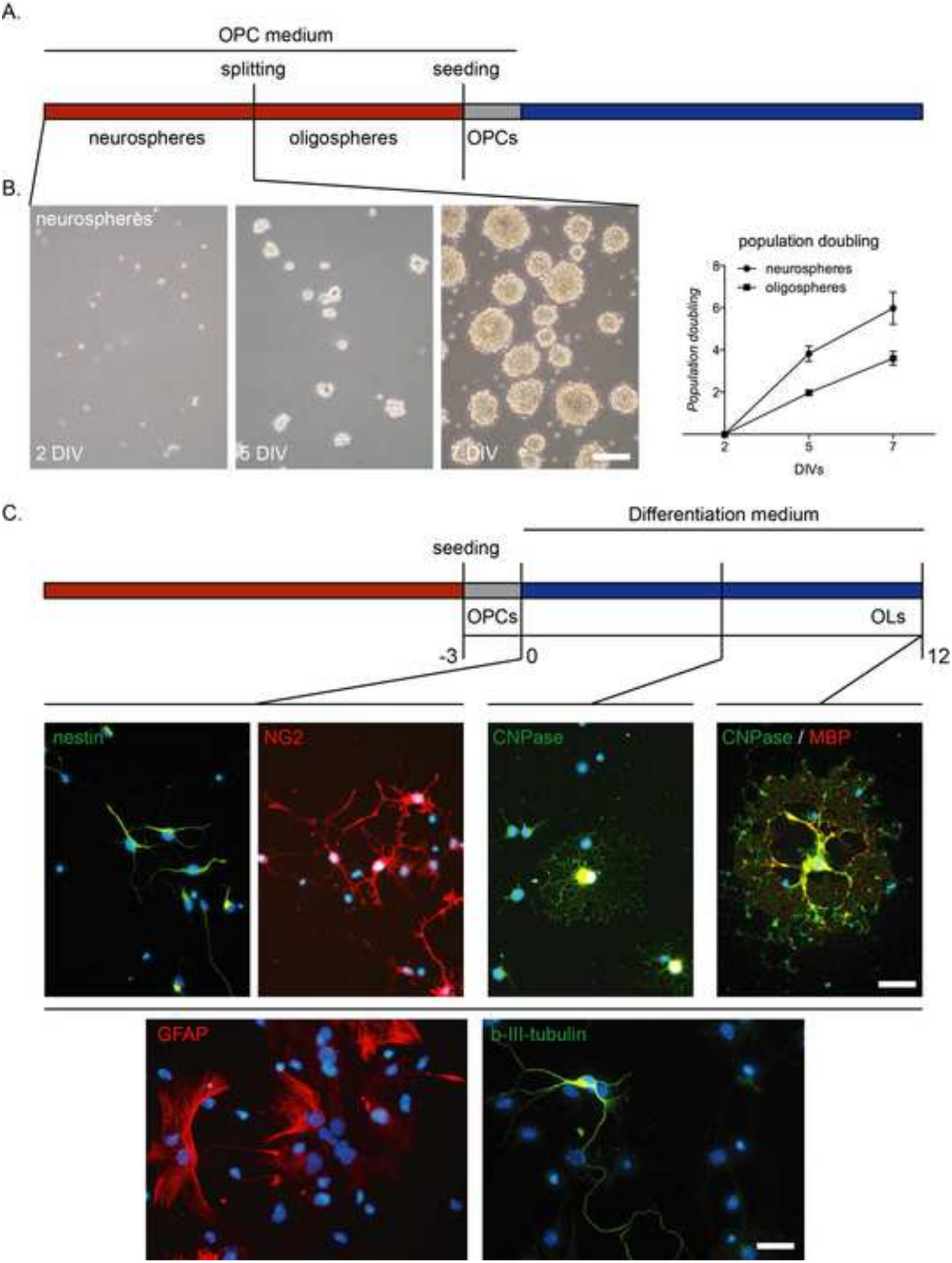
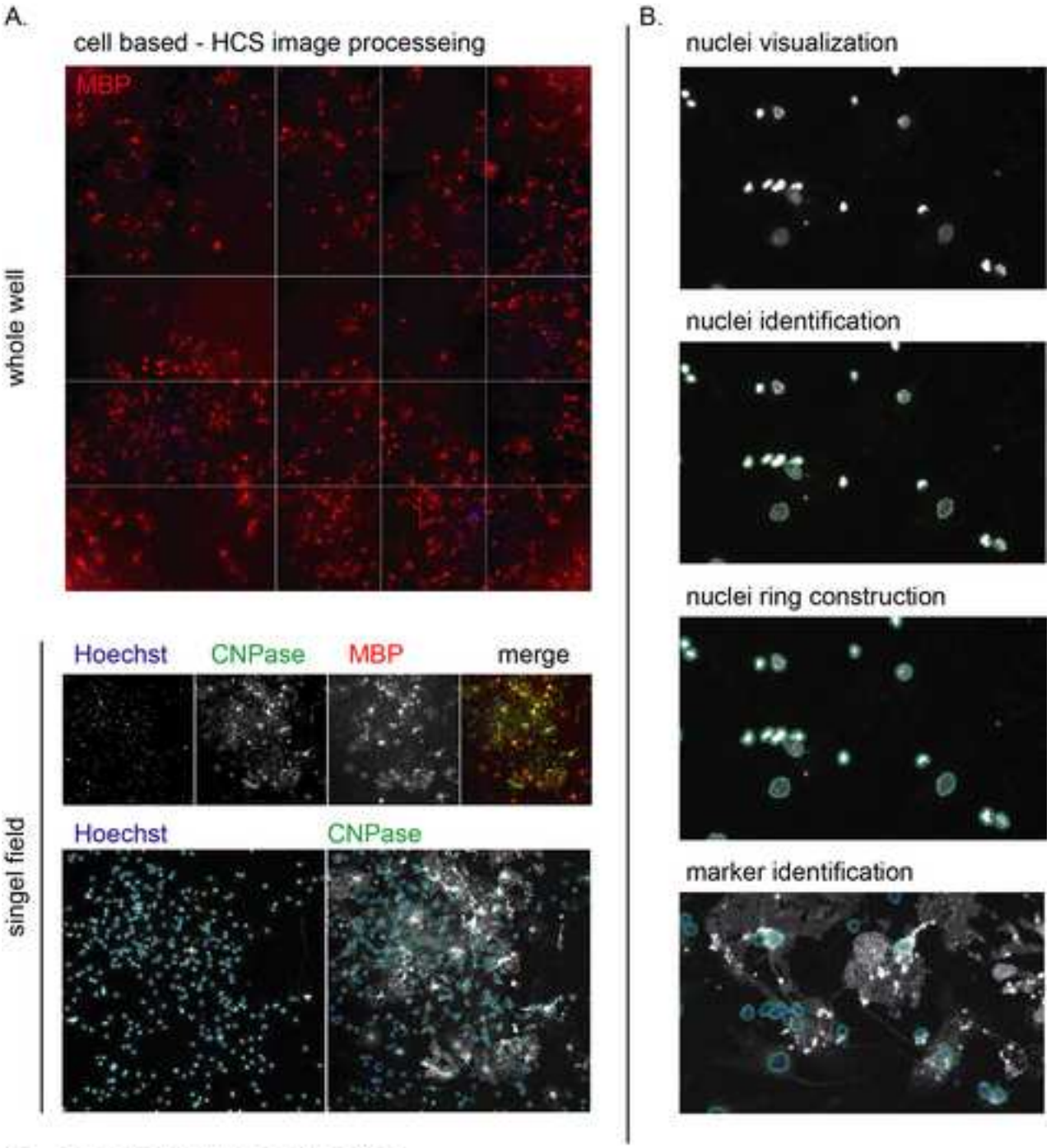


Figure2

Click here to access/download;Figure;Figure02-Differentiation_HCS.jpg



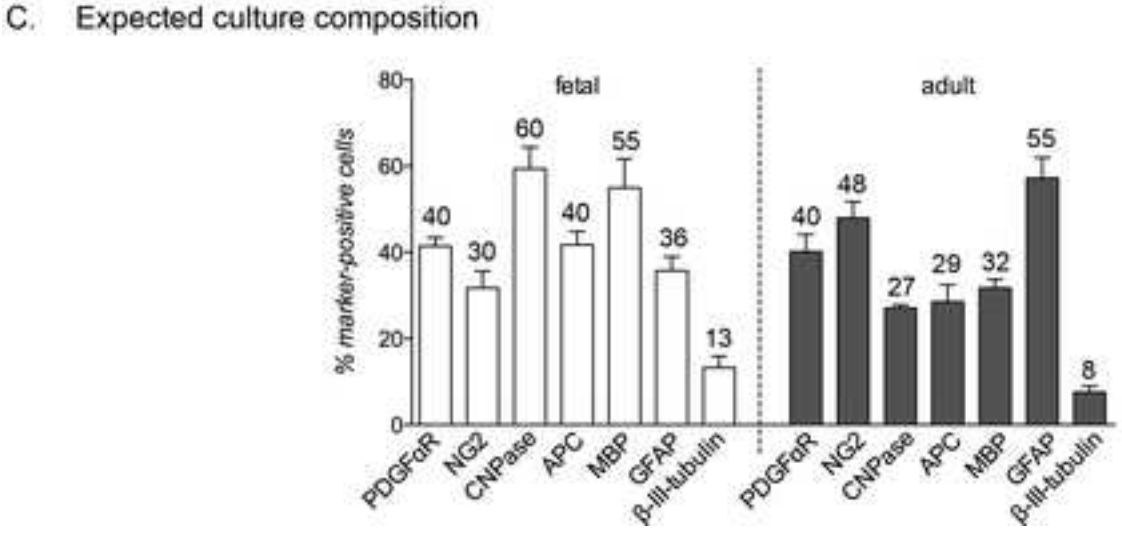


Figure3

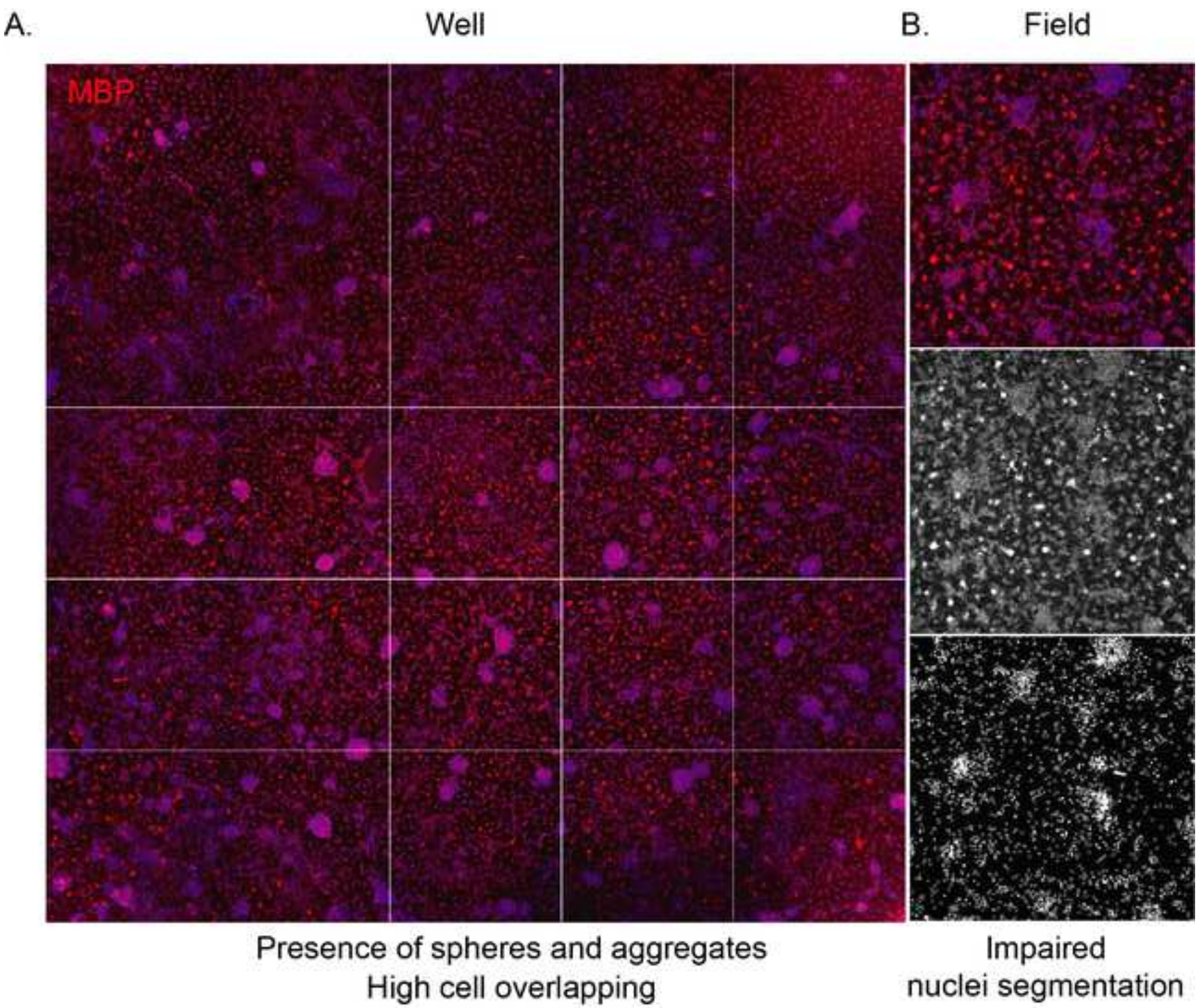
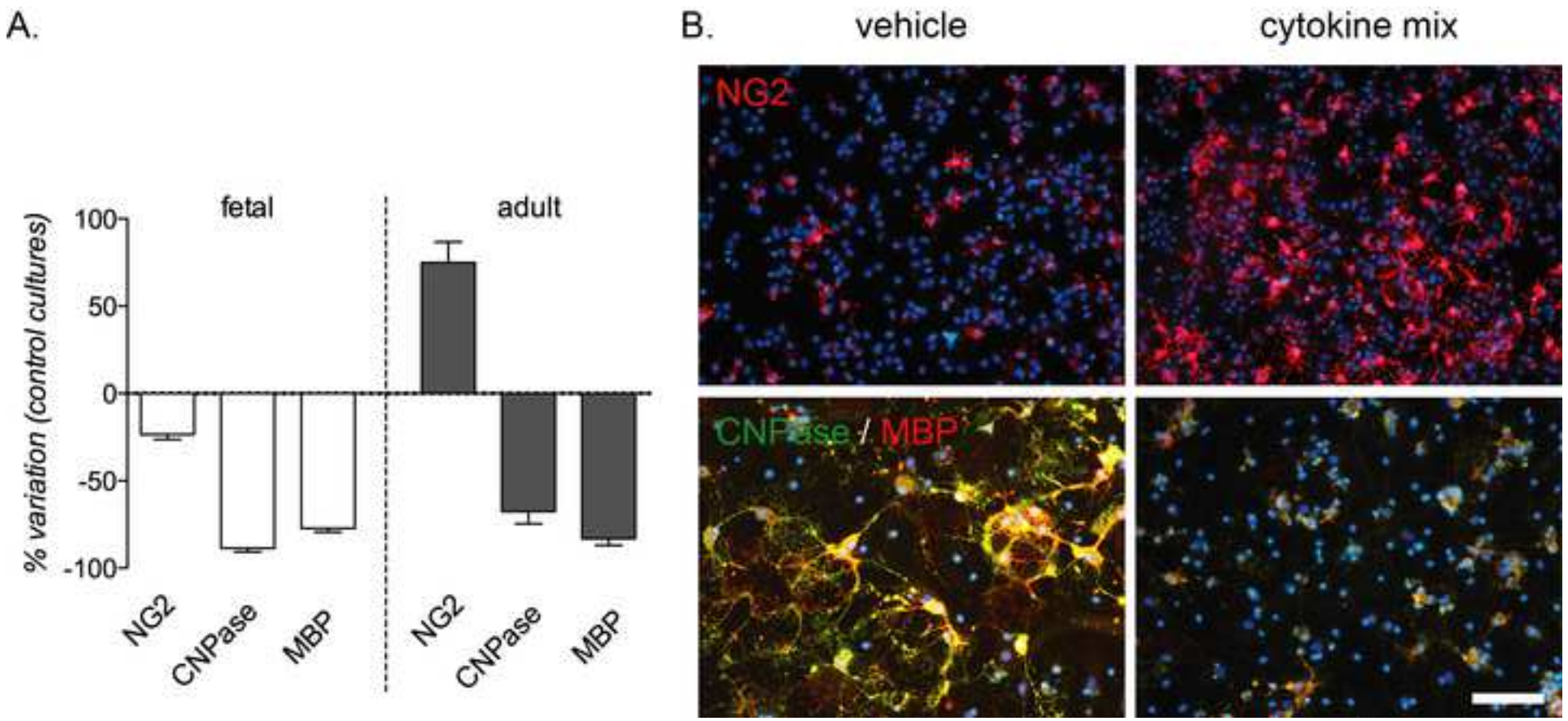
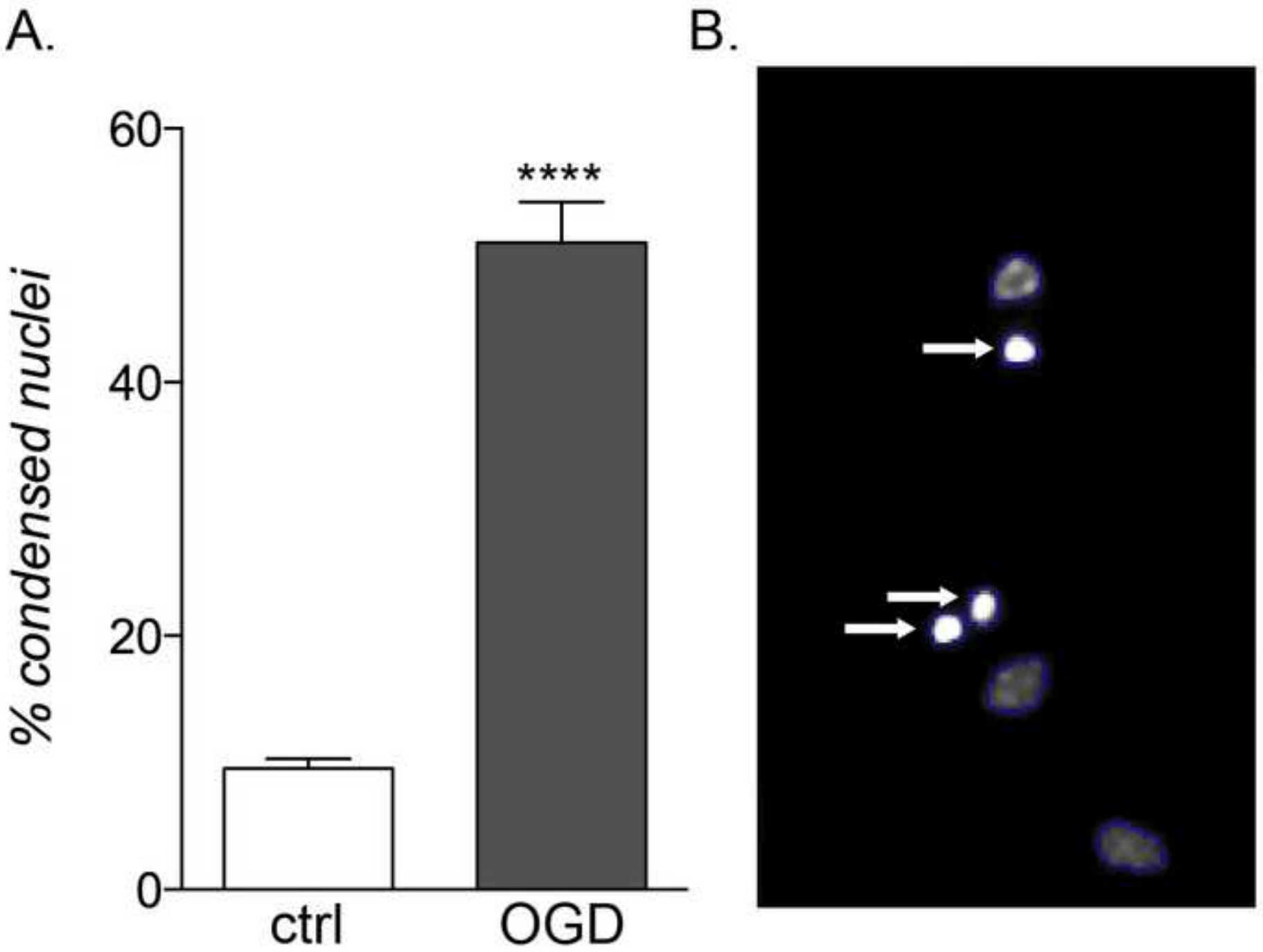


Figure4





Antibody	Species	Dilution
anti-β-III-tubulin (R&D system)	mouse	2.125
anti-GFAP (Dako)	rabbit	0.7361111
anti-NG2 (Millipore)	rabbit	0.2847222
anti-PDGFαR (Santa Cruz Biotechnology)	rabbit	0.25
anti-CNPase (Millipore)	mouse	0.3888889
IgG2b anti-APC, clone CC1 (Calbiochem)	mouse	0.1111111
Anti-MBP (Dako)	rabbit	0.2152778
Anti-nestin (Millipore)	mouse	0.3888889
Alexa Fluor 488-conjugated anti mouse (ThermoFisher Scientific)	donkey	0.3888889
Alexa Fluor 647-conjugated anti- mouse IgG2b (ThermoFisher Scientific)	goat	0.3888889
Alexa 568-conjugated anti-rabbit (ThermoFisher Scientific)	donkey	0.3888889

Name of Material/Equipment	Company	Catalog Number	Comments/Description
96-well plates - untreated	NUNC	267313	
B27 supplement (100x)	GIBCO	17504-044	
basic Fibroblast Growth Factor (bFGF)	GIBCO	PHG0024	
BSA	Sigma-Aldrich	A2153	
Ciliary Neurotropic Factor (CNTF)	GIBCO	PHC7015	
DMEM w/o glucose	GIBCO	A14430-01	
DMEM/F12 GlutaMAX	GIBCO	31331-028	
DNase	Sigma-Aldrich	D5025-150KU	
EBSS	GIBCO	14155-048	
Epidermal Growth Factor (EGF)	GIBCO	PHG6045	
HBSS	GIBCO	14170-088	
HEPES	GIBCO	15630-056	
Hyaluronidase	Sigma-Aldrich	H3884	
IFN-γ	Origene	TP721239	
IL-17A	Origene	TP723199	
IL-1β	Origene	TP723210	
IL-6	Origene	TP723240	
laminin	GIBCO	23017-051	
N-acetyl-L-cysteine	Sigma-Aldrich	A9165	
N2 supplement (50x)	GIBCO	17502-048	
Non-enzymatic dissociation buffer	GIBCO	13150-016	
PBS	GIBCO	70011-036	
Penicillin / Streptomycin	Sigma-Aldrich	P4333	
Platelet Derived Growth Factor (PDGF-AA)	GIBCO	PHG0035	
poly-D,L-ornitine	Sigma-Aldrich	P4957	
TGF-β1	Origene	TP720760	
TNF-α	Origene	TP723451	
Triiodothyronine	Sigma-Aldrich	T2752-1G	
Trypsin	Sigma-Aldrich	T1426	

Editorial comments:

Changes to be made by the Author(s):

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

I followed the editor suggestion and fixed the few spelling errors. However, I want to notice that the manuscript was checked by a professional English Language proofreader and, if needed, I can provide a certificate.

2. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: N-2 (Thermo Fisher Scientific, Waltham, MA, USA); flask (Nunc); Billiups-Rothenberg, Inc., Del Mar, CA;

I apologize for the mistake; I removed all the commercial language throughout the text.

3. 2.2.1: Please do not highlight any steps describing euthanasia.

I removed the highlighting from the text describing euthanasia.

4. For centrifugation conditions, use format: 400 × g or 1,000 × g.

I changed the format as indicated.

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

As indicated, I add more details about the protocol throughout the text and specifically for the software section.

6. 6.1: How do you determine that the oligospheres have reached a diameter of 100-150 µm?

I added a description of a rapid evaluation of the diameter at 4.1, replicated for 6.1.

7. 8.1: What is the final volume of the well contents (as you have stated the concentration of each cytokine in the mix is 20 ng/mL)?

The volume depends on the cell number, since cells are seeded as 10 - 50 cells/µl for spheres formation as explained during neurospheres and oligospheres protocol. I added a note at 8.1.

8. 11.1: Which software is this? If readers/viewers have some other software, what operations should they perform with their software?

The software is specific for the HCS and directly linked at the hardware. I added the name of the software.

9. Please convert 11.11 into a note if there are no actions to be performed.

I updated the software section, better describing also this point.

10. Please highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

I have tried to reduce as much as possible to stay in the 3 pages limit.

11. As we are a methods journal, please add to the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:

a) Critical steps within the protocol
b) Any limitations of the technique
c) How physiologically relevant the mixture of fetal and adult brain OPCs would be for drug screening and other applications.

As indicated by the Editor and the Reviewers, I updated the Discussion section.
One of the main limitations was already described (less uniformity of the mixed cultures; last paragraph before conclusions) and I clearly added now the fundamental limitation suggested also by the Reviewer 1 (lacking axon myelination). This is now followed by the physiological relevance of the fetal/adult cultures in a translational view.

12. In the reference list, please do not abbreviate any journal names. Do capitalize the first letters of all the words in the journal title.

I updated the bibliography according to the Editor's instructions

13. Where is Table 1 (list of primary and secondary antibodies)?

I apologize for the missing table, I updated it during the first submission but apparently it did not worked.

Reviewers' comments:

Reviewer #1:
Manuscript Summary:
The author describes a protocol for differentiation of oligodendrocytes from adult or fetal neural progenitor cells. The differentiation protocol is described in an easy to follow way. It is reproducible as presented. The author couples this differentiation protocol with a high content screening design in which the quantification in automatic. There are a few issues with the description of the workflow.

Major Concerns:
1- Though the author discussed the complex cellular environment better mimicking the in vivo, he fails to discuss the culture does not produce myelin and lacks the interaction between neurons and oligodendrocytes. This, of course, does not diminish the importance of the work however an important limitation to be discussed.

I agree with the Reviewer's suggestion, and I implemented the discussion of the method limitations.

2- Although the author mentions approximately 40% astrocytes in line 388, for adult NSCs it looks more like more than half of the cells are astrocytes. In addition, beta-III-tubulin-positive cells looks like above 10% for the fetal cultures. It may be helpful to give the numbers as a table or to print on the graph.

I agree with the Reviewer's comment and I add the percentage for each cell type in fetal and adult-derived cultures in the figure.
I also commented this aspect in the text (Results section) and figure legend.

3- The automated quantification is not described in enough detail. First, the software used was not stated. Therefore, all the descriptions are vague as we cannot replicate them.

I apologize for di missing details and I updated the software section as suggested by the Reviewer.

4- Are the images pre-processed? For example, was histogram stretching applied?

All the HCS software-based image processing is now described in the procedure.

5- How are the false positive nuclear rings identified? In figure 2, there are several nuclei that overlap with the marker (looks like MBP but not stated but clearly are not positive for the marker i.e they maybe the nuclei of underlying astrocytes. What are the criteria for calling a ring positive? Is it possible to distinguish between a bright spot that falls into the ring vs a general low but diffuse signal? They both may give about the same average intensity.

I agree with the Reviewer that this is a critical point and must be better clarified.
The positive ring is chosen by the "average intensity" of the lineage-specific marker. To do so, it is important to go through the sample images, during the mini scan section, select the rings of true-positive cells and nuclei randomly overlapping the staining and define the threshold based on this observation. This is now explained in the updated software procedure.
Note that the HCS is a balance between rapid and robust results avoiding the operator bias and a percentage of error occurring by automated analysis. This error is reduced at the minimum by the good cell seeding and good staining.

Minor Concerns:
1- It will be easier to interpret figures if the colors of the markers were stated. For example, MBP (red).

Labeling markers are always stated, I understand that the confusion may derive by the double staining and I modified the pictures giving the specific color for the specific marker.

Reviewer #2:
Manuscript Summary:
Dr. Baldassarro proposes a protocol able to obtain OPCs differentiated from NSCs isolated from SVZ at embryonic and adult stages. The author claim that this protocol should be very useful because of the equilibrium between the content of OPCs (very useful for the study of one of the contingents acting in demyelination) and the presence of a significant amount of astrocytes (40%) which is intended to add a physiological surplus to this experimental paradigm.

Major Concerns:
1-the quality of the images is very low and difficult to be properly evaluated;

As the Reviewer may note, all the images produced by a standard epifluorescence microscope are of a standard good quality (e.g. Figure 1). However, HCS machine takes pictures at 10x objective (for the described protocol) and produces images of the reported quality.

2-description of procedure is sometimes very superficial (ex.: composition of dissociation buffer is just described at the Table without any reference in the text, etc.). It is not conceivable the use of sentences like this one: "The entire protocol takes around 30 days, depending on the age of the animals and the experimental goals" (lines 488-489).

I apologize if some passages sound superficial to the Reviewer. I hope that Reviewer understands that, as he stated, it is a complex procedure with different variables that should be taken in consideration. The example of the protocol timing is complex, since if the user decides to work only on fetal cells or only on adult cells the timing is highly variable, according also to the seeding density. The age of the adult animal, also, may make a variation in time of more than one week with old animals. It is not possible to describe the exact time for each variables combination. However, I added more details about the different variables.

I do not understand the dissociation buffer concern. Non-enzymatic dissociation buffer is a commercial product (please, see the table of the products), while enzymatic dissociation buffer composition is described in detail in section 1 (solution and reagents). Both of them are used in the protocol, the “non-enzymatic” for the fetal tissue (see 2.1, firs sentence; 2.1.6) dissociation and the “enzymatic” for the adult (2.2.8). I do not understand where the Reviewer noted the superficiality of the description.

3-the coexistence of nesting and NG2 co-stainings is suggested by the author (lines 378-382; experiment illustrated in Figure 2) but not at all clearly demonstrated. Many of the problems derived of the lack of co-stainings for the evaluation of GFAP vs nestin/NG2 positive cells, for example. Additional staining (PDGFRalpha) would be very useful to clarify this point. Is the author sure about the lack of predicts in this cultures?

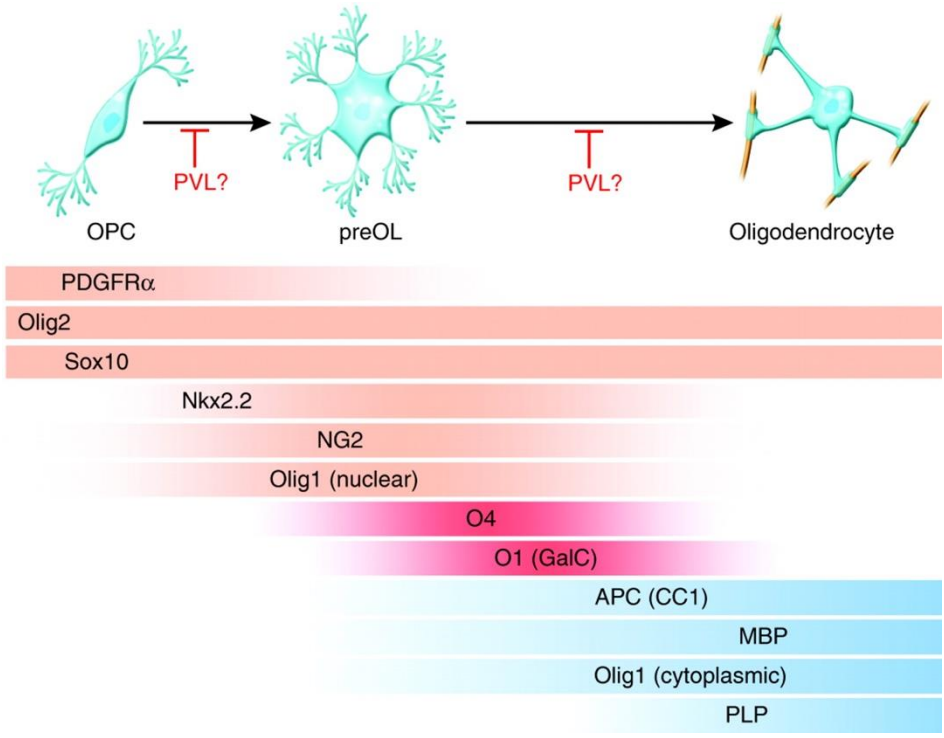
Please, note that throughout the whole comment text, maybe the Reviewer missed the figure order. The figure with the culture characterization with different stainings is figure 1.
Since when plated (-3 DIVs) most of the cells are positive for nestin and for NG2 we suggested this. I agree that, since double staining was not performed, I cannot state the co-existence of the two markers. I now added a clear statement of the goal of the protocol, to describe only the final cultures composition.

As the Reviewer perfectly knows, the differentiating OPCs cultures are NOT defined as black-or-white system (see figure 2 in Silbereis et al., 2010), but markers progressively disappear and appear and, in some stages, they can co-exists (e.g. all the MBP-positive cells are also CNPase-positive) or they can be in the transitions sate (e.g. between NG2- and CNPase-positive cells).

The objective of this protocol is not the study of the shades of these markers and, of course, each user can adopt the protocol to their main differentiation study goal. Moreover, the system was already well characterized in previous studies (Baldassarro et al., Stem Cell Research, 2019) and here I showed also the percentage of PDGFaR-positive cells (Figure 2) that, in cells differentiating from the multipotent NSCs population, identify OPCs.

Here the markers are used to quantify the percentage of cells in a specific population at the end of the differentiation phase.

Ref:



Silbereis JC, Huang EJ, Back SA and Rowitch DH. Towards improved animal models of neonatal white matter injury associated with cerebral palsy. Dis Model Mech, 2010. 3:678-88.

4-there are very general assumptions with no detailed descriptions (ex.: the percentage of astrocytes present in the culture, if there are differences between the cultures derived from embryonic NSCs and from adult ones, the timing differences between both types of cultures). It is not possible that cellular composition and timing for such a complex differentiation process do not vary from cultures derived from embryonic NSCs to the adult ones (in purified OPC primary cultures, the difference would be as much as three times more...!). There are no quantifications (see lines 388-390), not descriptive data (average numbers with SDs/SEMs, statistical analysis): this is intended to be a protocol and data should be very orientating for future users (as examples, see: Duncan et al., 2012; Medina-Rodrigues et al., 2013).

I agree with the Reviewer: that fetal and adult cultures are different. In fact, this is one of the main points of the protocol, to compare two different cultures in the most standardized way.
The first sentence of the Result section is "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" and now I added comment about the longer duration of the adult one.
However, after the cell seeding, the protocol follows the same timing.

The difference in doubling time between neurospheres and oligospheres (two different phases of the culture) is also stated in the text with a dedicated graph (Figure 1B).

In Figure 2 the culture composition of both fetal and adult cultures at the end of the differentiation phase is clearly stated with a dedicated graph (Figure 2C). Now I added also comment about this in the text.

5-It is not very clear why if OPCs are seeded in high density, the culture derive in astrocytic massive production (lines 394-396; experiments illustrated in Figure 3).

Please note that this is always a mixed culture, with astrocytes as the main proliferative cell type, since OPCs are induced to differentiate. In fact, while the OPCs are differentiating and the few neurons are post-mitotic, astrocytes keep replicating and, when in high number, rapidly generate a continuum layer.
I added this as an example of bad situation that can be produced by seeding an high number of cells and I think it can be useful for researcher approaching this in vitro model for the first time.

6-Inflammatory conditions are not described in detail.

I do not understand this point. In the text it is present the cytokine mix composition (1.9 cytokine mix preapration) and the treatment (8. Induction of inflammation-mediated differentiation block).
The choice of those cytokines and the rationale behind their effect it is also justified in the results section (penultimate paragraph).

7-the surprising data of similar survival to inflammatory conditions (experiments illustrated in Figure 5) are not sufficiently discussed (line 406).

Please, note that figure 5 does not refer to inflammatory conditions, but to OGD experiment conducted on fetal OPCs. In these cultures, the survival in strongly challenged by OGD exposure.

8-Introduction and Discussion seem very biased for this reviewer: while in the Introduction the option of organotypic slice cultures is systematically forbidden (and in the Discussion it is just cited -lines 454-456-), almost the entire Discussion just contemplates previous works of the author´s group.

As Reviewer stated, for the author was worth to mention the organotypic cultures. However, they were not "systematically forbidden" but "systematically avoided" in the introduction, since this model do not represent a cellular model comparable to cell lines, primary OPCs and NSCs-derived OPCs. Moreover, for the limited space for this section in a method manuscript it is not possible to discern the whole portrait of non-in vivo models.
Since the journal instructions state to focus the discussion on the protocol (critical steps, limitation, troubleshooting, applications, significance respect alternative methods) and to avoid the replication of existing experiments, and since the representative results are originally presented in the figures, I added all the model characterization references, coming from previous studies, in the discussion. It is useful to understand the potential of the system and, for new users, if it fits the experimental questions.
The discussion has been modified and, as editor requested, shortened.

Minor Concerns:
-Introduction should be completed with relevant bibliography (Dincman et al., 2012; Medina-Rodrigues et al., 2013; others). It is quite surprising that the work of maybe the two more active groups working with adult OPCs (Antel, deCastro) are missed in the manuscript, as well as extremely important data recently obtained by the groups of Williams and Castello-Branco: all these would significantly increase the quality of the manuscript and put proposed protocol in the real perspective to be balanced.

I apologize for the missing updated bibliography. I would only remind that this is not a review, and even the editor requested to shorten the discussion focusing more about the method itself.
I updated the text (in the discussion where contextualization according to other methods is requested) according to the Reviewer's comment and suggested references, within the limited space requested by the journal.

-It is quite naïve to still refer myelination and remyelination as identical events (lines 463-464): Franklin et al. have clearly discarded this in later work (Franklin & french-Constant, 2017) than the cited...!

I agree with the Reviewer's comment. In fact, it is already underlined that it was described that myelination and remyelination are different events.
However, it is useful to cite the recapitulation hypothesis to clarify the context. This is, in fact, cited in different recent reviews of other groups describing myelination and remyelination and still a debated and discussed point.

See, for example:

Guo et al., 2020. Medical Hypotheses. Doi: 10.1016/j.mehy.2019.109522
Voskuhl et al., 2020. PNAS. Doi: 10.1073/pnas.1821306116
Laitman et al., 2018. Mult Scler J Exp Transl Clin. doi: 10.1177/2055217318806527
Bove and Green, 2017. Neurotherapeutics. Doi: 10.1007/s13311-017-0577-0

Moreover, the recapitulation hypothesis is still at the base of a big in vitro bias: using fetal/neonatal-derived primary OPCs to test molecules aimed to stimulate remyelination in adulthood. This is a key point at the base of the presented method.

Following the Reviewer's comment, I better clarified that point in the introduction, and I added the reference.

-bFGF is an old-fashion form to refer to FGF2 (along the entire paper, Table included).

I do not agree with the Reviewe's comment, since bFGF is still widely used and alternatively to FGF2.
I choose this form since it is also used by the company selling the product.

Please see recent papers on relevant journal using the "bFGF" nomenclature:

Shakya et al., 2020. Scientific Reports. Doi: 10.1038/s41598-020-65572-2
Oyane et al., 2020. Royal Society of Chemistry. Doi: 10.1039/C9RA06906B

-the mixture of ornithine/laminin used is poorly described.

I do not understand this point. The full protocol on how to prepare the coating ornithine/laminin is fully described in section 5 as follow:

"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."

I do not understand which detail is missing.