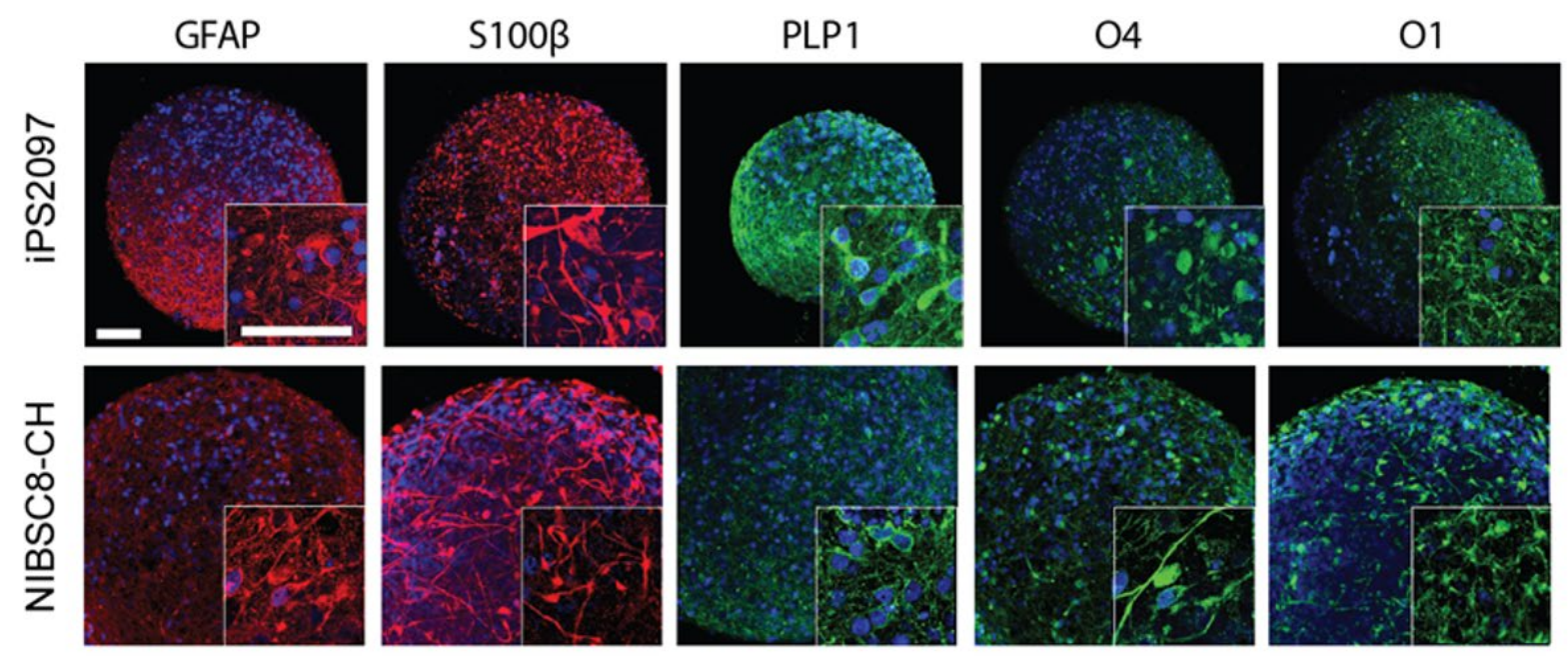
Comparison of myelin formation in two 3D mixed neuronal/glial cultures (brain spheres) derived from human induced pluripotent stem cells

Pamies, D., Wang, S.

2025



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Abstract

In the field of developmental neurotoxicity and endocrine disruption, there is a need for *in vitro* methods that are suitable for regulatory use and can measure specific changes in the human brain. In the search for a suitable, commercially available cell model, EURL ECVAM requested a contractor to evaluate the quantity and reproducibility of myelin formation in 3D mixed (neuronal/astrocyte and oligodendrocyte) cultures (brain spheres) derived from human induced pluripotent stem cells (hiPSCs). The results from neurospheres derived from the commercially available cell line were compared with results from an in-house model that was already established in the contractor's laboratory. The study shows that the investigated cell model produces myelin that can be reliably measured, but challenges remain for the reproducible measurement of increased or decreased myelination in the brainspheres upon chemical exposure.

This study contributes to the validation of suitable *in vitro* models that, when accepted for regulatory use, can reduce the amount of animals used for scientific purposes according to Directive 2010/63/EU.

1 Introduction

The alarming increase in prevalence of neurodevelopmental disorders exceeding 15% worldwide (Romero, 2021), has become a major public health concern. There is growing evidence that environmental toxicants contribute to the development of these disorders. However, the significance of exposure to xenobiotics during developmental stages is not fully understood. The Organisation for Economic Co-operation and Development (OECD), defines toxicological test guidelines to evaluate chemicals and assure human health. However developmental neurotoxicity (DNT) is not systematically studied due to the high costs ($1.4 million per substance), high number of animals (1,000 rat pups per chemical) and time-consuming experiments (between 3 – 24 months) required in the current OECD test guidelines (OECD TG 426 and US EPA 712-C-98-239). On the other hand, there are rising concerns regarding the physiological relevance of extrapolating results from animal studies to humans (Hartung and Leist 2008, Hartung 2008, Smirnova 2014), indicating that animal studies are suboptimal for many toxicological assessments. The OECD and European Food Safety Authority (EFSA) are currently developing a Guidance document (Sachana 2021 and Hernandez-Jerez 2021) to evaluate DNT effects based on an in vitro testing battery of assays8 to improve and speed up current testing towards replacing in vivo studies in the future. In addition, the EU Reference Laboratory for alternatives to animal testing (EURL ECVAM is carrying out a similar approach for a specific type of DNT compounds able to produce thyroid disruption. Myelin formation has been considered as one of the most important key events during brain development and a sensible endpoint by both DNT OECD and EURL ECVAM Thyroid experts. However, a myelin assay has not been incorporated yet in the testing batteries due to the difficulty to obtain myelin in vitro. We have combined the latest advances in new cell cultures and induced pluripotent stem cells (iPSC) to develop a 3D organotypic model for the brain (also called BrainSpheres) (Pamies 2017, Hogberg 2013). This model presents compact myelin wrapped around axons, making it an ideal tool for myelin studies. Furthermore, it allows us to generate a reliable high amount of viable BrainSpheres, which are not only homogeneous in size and shape but also present reproducible percentages of the diverse cell types. As an endpoint, after one week of chemical treatment from 7-week-old BrainSpheres to 8-week-old BrainSpheres, axon and myelin-associated protein were detected by immunocytochemistry of Neurofilament Protein NF200 and proteolipid protein 1 (PLP1) respectively (Chesnut 2021).

This study is requested by EURL ECVAM to evaluate the reproducibility of quantitative evaluation of myelin formation using 3D mixed (neuronal/astrocyte and oligodendrocyte) cultures (brain spheres) derived from human induced pluripotent stem cells (hiPSCs). This line is commercially available (UK Stem Cells Bank, NIBSC) in comparison to in-house models that are already established in the host lab.

The experiments started in January 2023, and were completed at the beginning of 2024.

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2 Materials and Methods

2.1 Cell lines used

Two human induced pluripotent stem cells (iPSC) lines were used in this study. iPS2097 line was generated at the Johns Hopkins School of Medicine at Hongyong Song laboratory. NIBSC8 were derived from the fibroblast of the lung from a female foetal donor, and purchased from the National Institute for Biological Standards and Control (NIBSC) – UK Stem Cell Bank, detailed information can be found: https://hpscreg.eu/cell-line/USCBi001-A.

2.2 Cultivation of iPSC

Induced pluripotent stem cells (iPSCs) were cultured on a 6-well plate pre-coated with 1:100 (diluted in DMEM/F12 medium) Matrigel (Corning, #356237) in mTesR plus medium (STEMCELL Tech: #100-0276) at 37°C/5% CO2.

2.3 Generation of Neural Progenitor Cells and 3D Brain Model (BrainSpheres)

Neural progenitor cells (NPCs) were generated via the formation of neural rosettes as previously described (Wen 2014), and cultured in Gibco™ PSC Neural Expansion Medium (#A1647801). At 100% confluence, NPCs were detached by scraping and counted. 2x106 cells per well were plated in 2 ml medium in non-treated 6 well-plates. Cells were grown in NPC medium for two days under constant gyratory shaking (88 rpm), allowing aggregation by using a MaxQ™ 2000 CO2 (ThermoFisher Scientific) plate shaker. Subsequently, the medium was changed to differentiation medium (Neurobasal® Electro Medium (Gibco) supplemented with 5% B-27® Electrophysiology (Gibco), 1% glutamax (Gibco), 0.02 µg/ml human recombinant GDNF (Gemini), 0.02 µg/ml human recombinant BDNF (Gemini)). Cultures were maintained at 37°C, 5% CO2 under constant gyratory shaking (88 rpm) for up to eight weeks. The differentiation medium was routinely changed every two days. Mycoplasma contaminations were checked regularly by sending the cells to Eurofins (company), see supplementary material.

Table 1. Materials and reagents used for cell culturing

Materials and reagents	**Source**
Neurobasal® Electro Medium	Gibco #A1413701
B-27® Electrophysiology	Gibco #A1413701
Glutamax	Gibco #35050038
Human recombinant GDNF	PeproTech #450-10-100ug
Human recombinant BDNF	PeproTech #AF-450-02

Source: David Pamies

2.4 Chemical exposure

The BrainSpheres (were exposed to Cuprizone (Sigma Aldrich, #14690), Bisphenol A (BPA; Sigma Aldrich, #239658), and Acetominophen (Sigma Aldrich, ST. Louis, MO, USA, #I4883) at concentrations ranging from 0.1 µM to 100 µM. Stock solutions of the chemicals were prepared in DMSO. BrainSpheres were exposed for seven days at seven week of differentiation. Medium containing fresh chemicals or vehicles (either in DMSO or in 50% ethanol in culturing medium) was changed every 48 h. DMSO final concentration in medium was maintained at 0.1% [32]. Experiments were performed in two independent batches. BrainSpheres were maintained until they reached 8 weeks of age, with the treatment initiated at 7 weeks and continued until they were 8 weeks old.

- Cuprizone (Positive)
- Bisphenol A (Positive)
- Acetaminophen (Negative)

Table 2. Information on the chemicals tested

Chemicals	**CAS-numbers**	**Lot-batch numbers**	**Purity**	**Control**
Cuprizone	370-81-0	BCCJ1026	≥95% (TLC)	Positive
Bisphenol A	80-05-7	Le073192507	≥99.0%	Positive
Acetaminophen	103-90-2	SLCB2770	≥99.0%	Negative

Source: David Pamies

Table 3. Information on the chemicals tested

Chemicals	**Concentration stock solution**	**Concentration work solutions**
Cuprizone	10 mM	0.1 µM, 1 µM, 10µM, 50 µM, 100 µM
Bisphenol A	200 mM	0.1 µM, 1 µM, 10 µM, 100 µM
Acetaminophen	100 mM	0.1 µM, 1 µM, 10µM, 50 µM, 100 µM

Source: David Pamies

Bisphenol A and Acetaminophen were dissolved in DMSO.

Cuprizone was dissolved in the culture medium or 50% ethanol in medium.

Table 4. Information on the solvents used

Chemical	CAS-numbers	Lot-batch numbers	Purity
DMSO	370-81-0	BCCJ1026	≥95% (TLC)
Ethanol	64-17-5	No annotated.	≥95% (TLC)

Source: David Pamies

The vehicle controls were either with DMSO or culture medium or 50% Ethanol in medium.

The concentration of DMSO was 0.1 %.

2.5 Cell viability assessment

Cell viability was assessed using the resazurin assay to select sub-cytotoxic concentrations with potential to disrupt myelination in BrainSpheres without inducing general cytotoxicity. At the end of the exposure (8 weeks), BrainSpheres were transferred to 24-well plates in 500 µL of differentiation medium. Then, 5 µL of a 1 mg/mL resazurin sodium salt (Sigma-Aldrich) stock solution in 1× PBS was added to each well, and plates were kept on a gyratory shaker at 88 rpm in a humidified incubator at 37 °C with 5% CO2. After incubation for three hours, 100 µL aliquots of sample medium were transferred to a 96-well plate, and the fluorescence of the resazurin metabolite (resorufin) was measured in a multi-well fluorometric plate reader (Cytofluor Multi-well Plate Reader Series 4000, Perspective Biosystem, Framingham, MA, USA) at 530 nm/590 nm (excitation/emission). Cell viability was determined for three replicates by normalisation of absorbance measurements from the test samples to the vehicle (DMSO) control samples. Blank subtraction was performed with normal media without cells.

Two independent experiments with 3 technical replicates were performed. Replicates are defined as independent wells in a 6-well plate.

2.6 Immunocytochemical and confocal imaging

BrainSpheres were fixed for 30 min with 4% paraformaldehyde, washed three times with 1× PBS for 10 min each, then incubated in blocking solution (5% normal goat serum (NGS) and 4% 1× Triton in 1× PBS) for two hours on a shaker at room temperature. BrainSpheres were then incubated with primary antibodies diluted in PBS containing 5% NGS and 1% 1× Triton at 4 °C for 24 h.

BrainSpheres were washed with PBS five times for 30 min each and further incubated for one hour with secondary antibodies (goat anti-mouse Alexa Fluor 488 IgG (Invitrogen) or goat anti-rabbit Alexa fluor 568 IgG (Invitrogen, Waltham, MA, USA), 1:200 in PBS containing 5% NGS) on a shaker at room temperature. BrainSpheres were then washed with PBS five times for 30 min each, and nuclei were stained with Hoechst 33342 Trihydrochloride Trihydrate (Invitrogen, 1:10,000 in PBS) for 30 min on a shaker. BrainSpheres were then mounted on glass slides using a mounting medium (Immu-Mount, Thermo Scientific, Waltham, MA, USA). Images were taken using a confocal microscope (Zeiss LSM 700 Confocal III and Zeiss LSM 780 GaAsP) and visualised in ZEN Imaging software (Zeiss, Jena, Germany).

We used primary antibodies PLP1 (Bio-Rad MCA839G) and Neurofilament 200 (Sigma-Aldrich N4142-2ML), as well as secondary antibodies Alexa Donkey anti-mouse 488 (ThermoFisher Scientific A21202) and Alexa Goat anti-rabbit 594 (ThermoFisher Scientific A11037). Antibody in flow cytometry experiment: Anti-MBP-FITC (Miltenyibiotec; 130-120-341), Anti-PLP-APC (Miltenyibiotec; 130-120-275).

2.7 Myelin quantification

The myelin marker proteolipid protein 1 (PLP1) was evaluated by measuring total fluorescence in ImageJ after immunocytochemistry. Confocal images were taken at 63x for at least 10 Brianspheres per condition. To calculate the corrected total cell fluorescence (CTCF) the following formula was used: CTCF = integrated density-area of selected cell X Mean fluorescence of background readings). Data is expressed as percent of CTCF to the vehicle (DMSO) control.

2.8 Brainspheres dissociation and flow cytometry

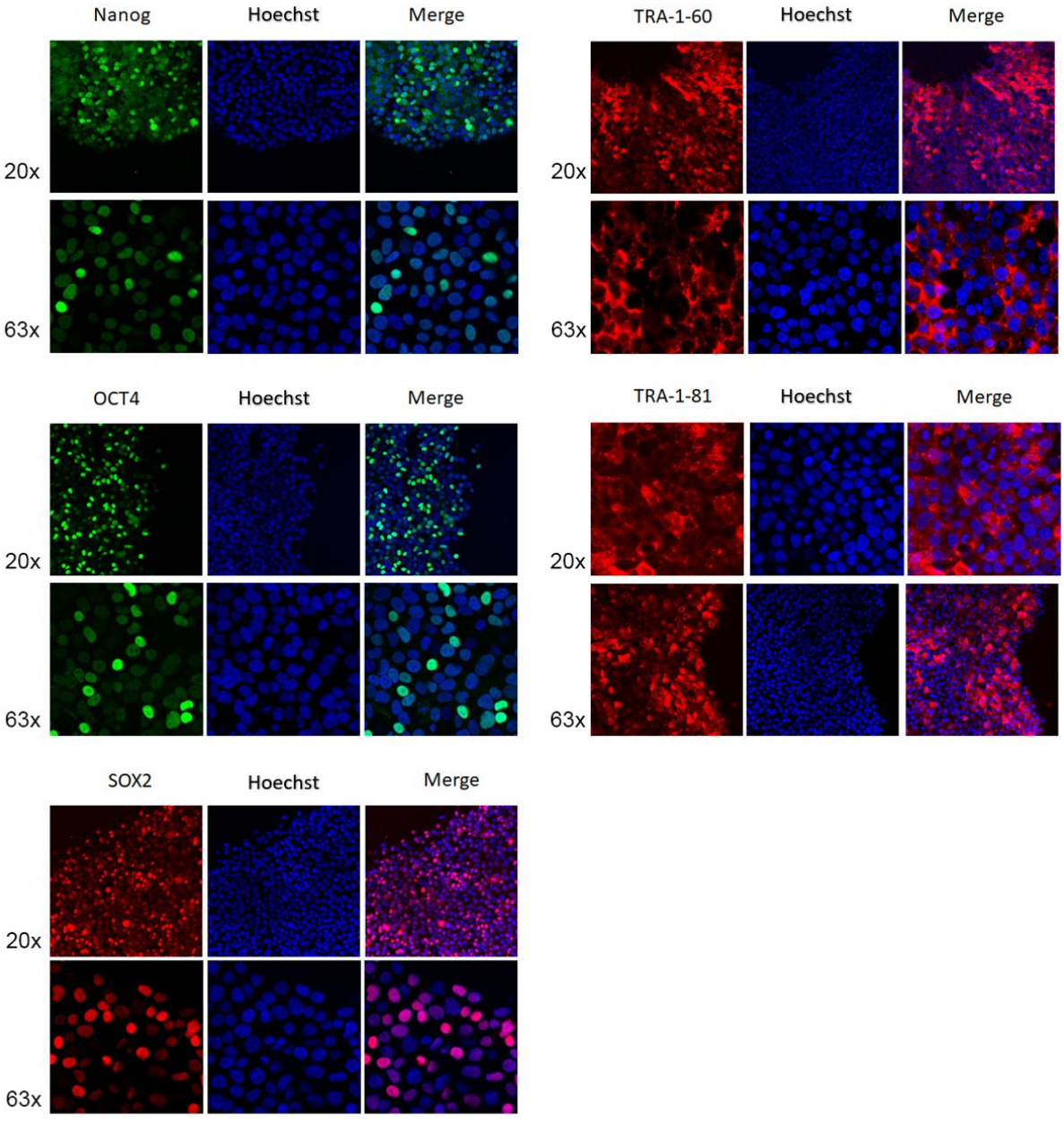
BrainSpheres were dissociated using the Papain Dissociation buffer (StemCell Tech: #07465). Briefly, BrainSpheres were washed twice with DPBS (Gibco), and cultures were resuspended in papain/DNAse solution containing 10 U of papain. BrainSpheres were incubated for up to 3 h at 37°C on a shaker. After full dissociation, the cell suspension was spun down at 300 × g for 5 min at RT. Then, the pellet was washed with DPBS once, and then fixed and permeabilised in the fixation and permeabilization buffer. Mix well and incubate for 30 minutes in the dark in the refrigerator (2–8 °C). Centrifuge cell suspension at 300×g for 5 minutes and aspirate supernatant completely. Wash with 1 ml PEB and centrifugate 300×g for 5 min and aspirate the supernatant. Resuspend up to 1.000.000 nucleated cells per 98 µL of buffer (PEB) and add 2 µL of the antibody. Mix well and incubate for 10 minutes in the dark in the refrigerator (2–8 °C). Wash cells by adding 1–2 mL of PBS and centrifuge at 300×g for 5 minutes. Aspirate supernatant completely. Add 300 uL PBS and analyse with flow cytometry.

3 Results

3.1 Characterisation of iPSCs from NIBSC8 cell line

To ensure the cells were performing adequately, several quality controls were conducted. On one hand, pluripotent markers were examined to confirm the pluripotent capabilities of the cells **(Figure 1)**. Immunohistochemistry for the markers studied on the iPSC NIBSC8 line showed positive results as desired. The cells were positive for Nanog, OCT4, SOX2, TRA-1-60, and TRA-1-81, which are commonly used pluripotent markers. This indicates that the quality of the iPSCs and their capability to differentiate into various cell types should be satisfactory.

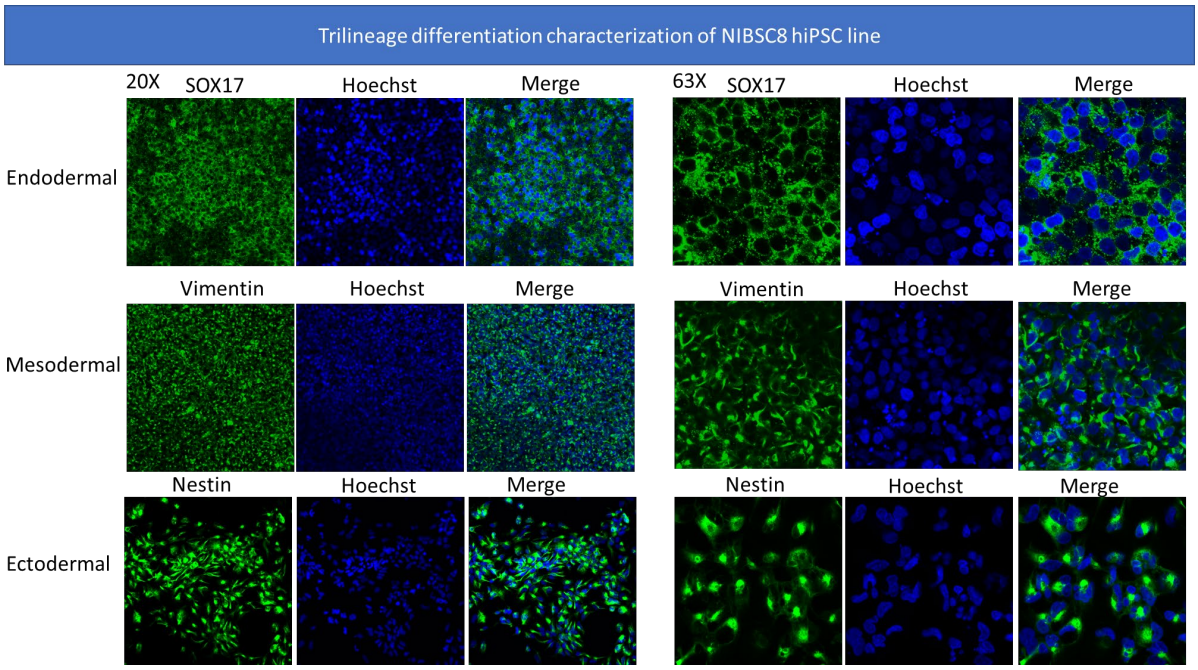
Figure 1. Representative **images of NIBSC8 hiPSCs stained for pluripotent markers**: Nanog; OCT4; SOX2; TRA-1-60; TRA-1-81.



Source: Shan Wang.

To ensure that the cells perform well under differentiation conditions, we also conducted a trilineage differentiation characterisation. In this experiment, we used different media to differentiate the cells into endodermal, mesodermal, and ectodermal cell layers. Immunohistochemistry for endoderm (SOX17), mesoderm (Vimentin), and ectoderm (Nestin) showed positive results for the specific lineage markers **(Figure 2)**. The results demonstrated that this iPSC line is capable of differentiating into all three lineages as expected.

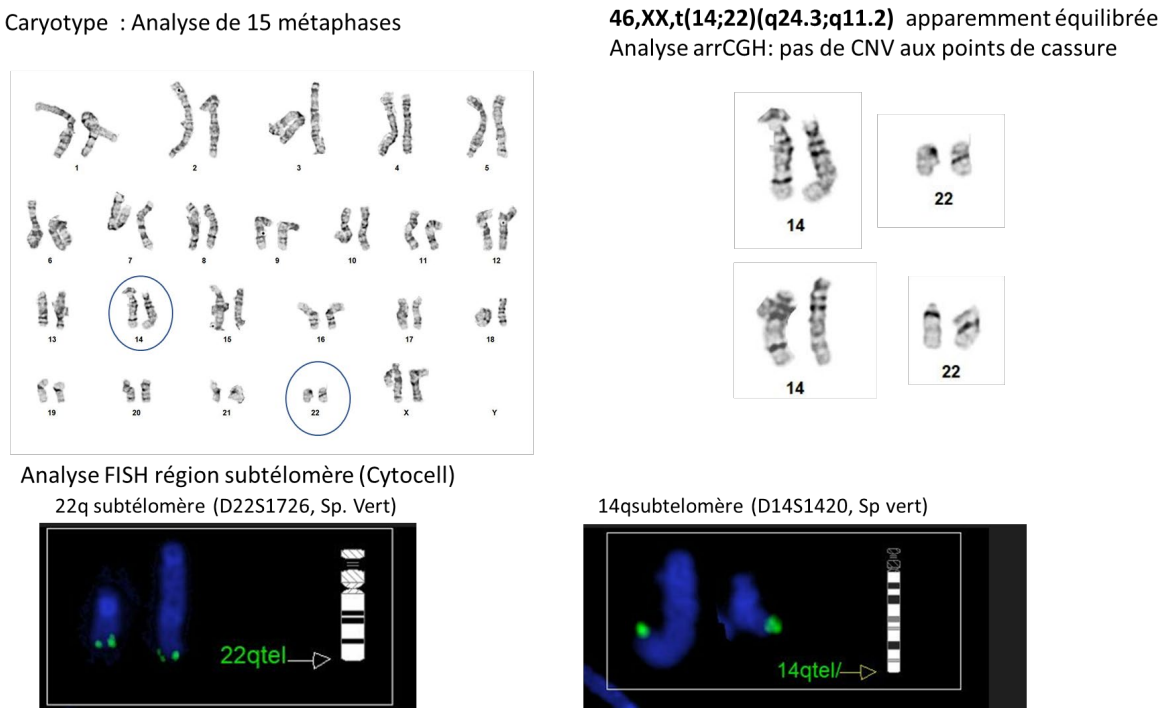
Figure 2. Representative **images of NIBSC8 hiPSCs stained for trilineage marker**: Endoderm (SOX17), Mesoderm (Vimentin) and Ectoderm (Nestin).



Source: Shan Wang.

NIBSC8 was meant to be a control line, extracted from a healthy donor, but sometimes during reprogramming genetic alteration may occur. In order to determine the genome stability of the cells we did a Karyotyping check **(Figure 3)**. Karyotyping results showed a translocation 46,XX,t(14;22)(q24.3;q11.2). This abnormality was not detected in previous tests as it does not involve any gene loss, but rather a translocation between chromosomes. This translocation has been observed in some reprogram lines, but has not shown significant problems in the differentiation or performance of the cells.

Figure 3. Karyotyping of the NIBSC8 line



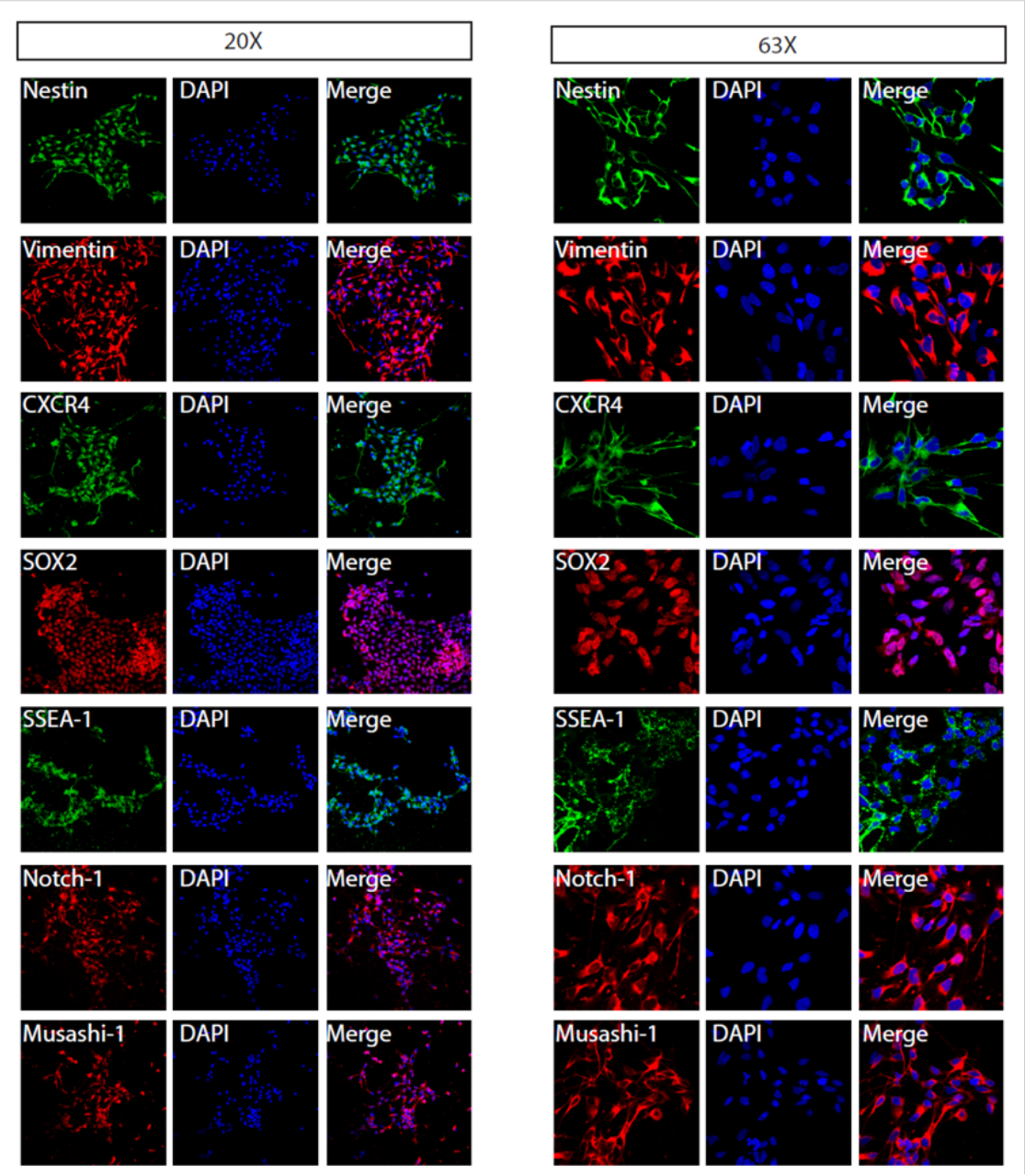
Source: Shan Wang.

3.2 Characterisation of NPCS from NIBSC8 cell line

To evaluate whether the production of NPCs was adequate, two quality control methods were established: one based on immunohistochemistry and the other on flow cytometry.

Firstly, immunohistochemistry was performed to study common NPC markers using a commercial kit (R&D # SC025). NPCs were fixed with 4% paraformaldehyde as described in the methods section. The immunostaining process was then conducted following the specified protocols. The stained samples were imaged using a Leica LSM780 confocal microscope. The differentiated NPCs tested positive for all the studied NPC markers **(Figure 4)**. The presence of these markers indicates that the NPCs are of adequate quality.

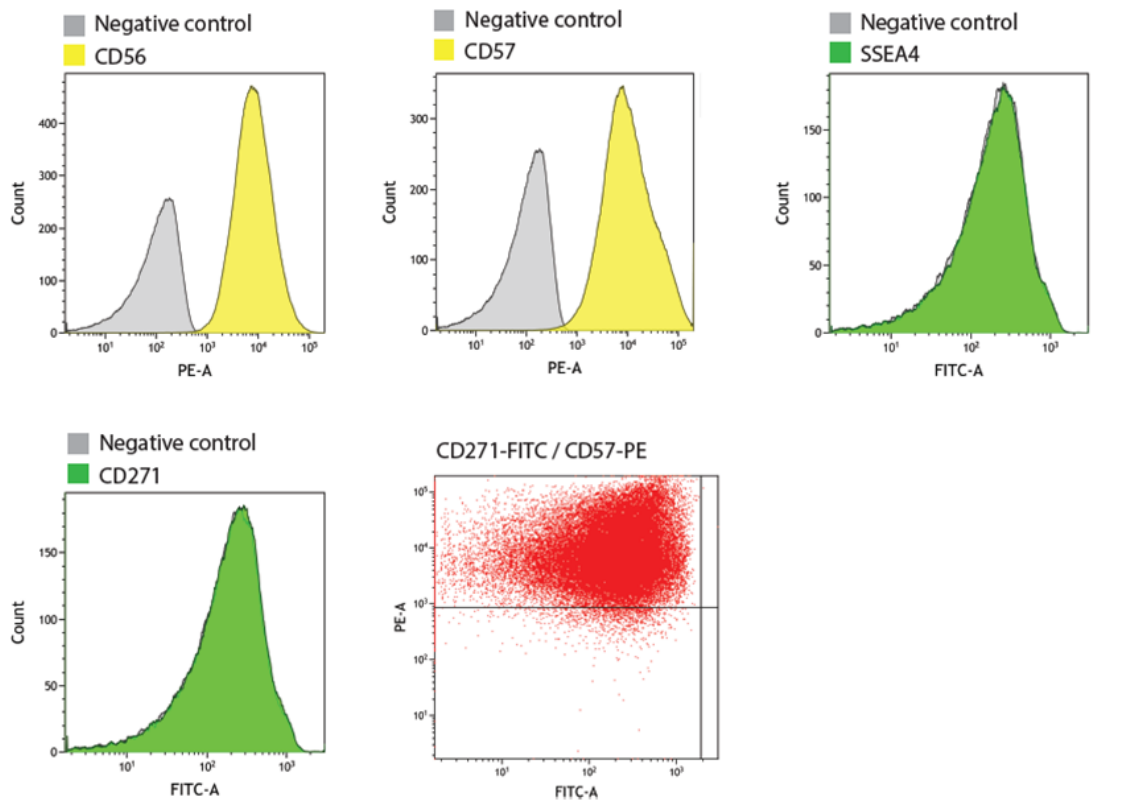
Figure 4. Representative **images of NPCs differentiated from NIBSC8 hiPSCs stained for NPC markers**: Nestin; Vimentin; CXCR4; SOX2; SSEA-1; Notch-1; Musashi-1.



Source: Shan Wang.

Secondly, flow cytometry analysis was conducted to further confirm the quality and presence of NPC markers. This assessment distinguished between neuroepithelial cells (CD57 positive), neural crest cells (CD271 positive), and undifferentiated cells (SSEA4 positive). It also identified the absence of non-neuronal lineage cells (CD56 positive). The results showed that the majority of the cells (>99%) were from the neuronal lineage, with all being neuroepithelial cells. The number of neural crest cells was less than 0.1% **(Figure 5)**. These results indicate that all the cells generated using the protocol were NPCs. The combined results of both methods demonstrated that the NPCs produced were of sufficient quality, meeting the established standards.

Figure 5. Flow cytometry data showing that NPCs showed expression of neural lineage markers (CD56; CD57), whereas no expression of neural crest markers (CD271) or pluripotent markers (SSEA4).



Marker	Cell type	Percentage
CD56	Neural linegae	99 %
CD57	Neural epithelial cells	99 %
CD271	Neural crest cells	0 %
SSEA4	Pluripotent cells	0 %
CD271/CD57	-	0 %

Source: Shan Wang.

3.3 Characterisation of Brainspheres generated from NIBSC8 cell line

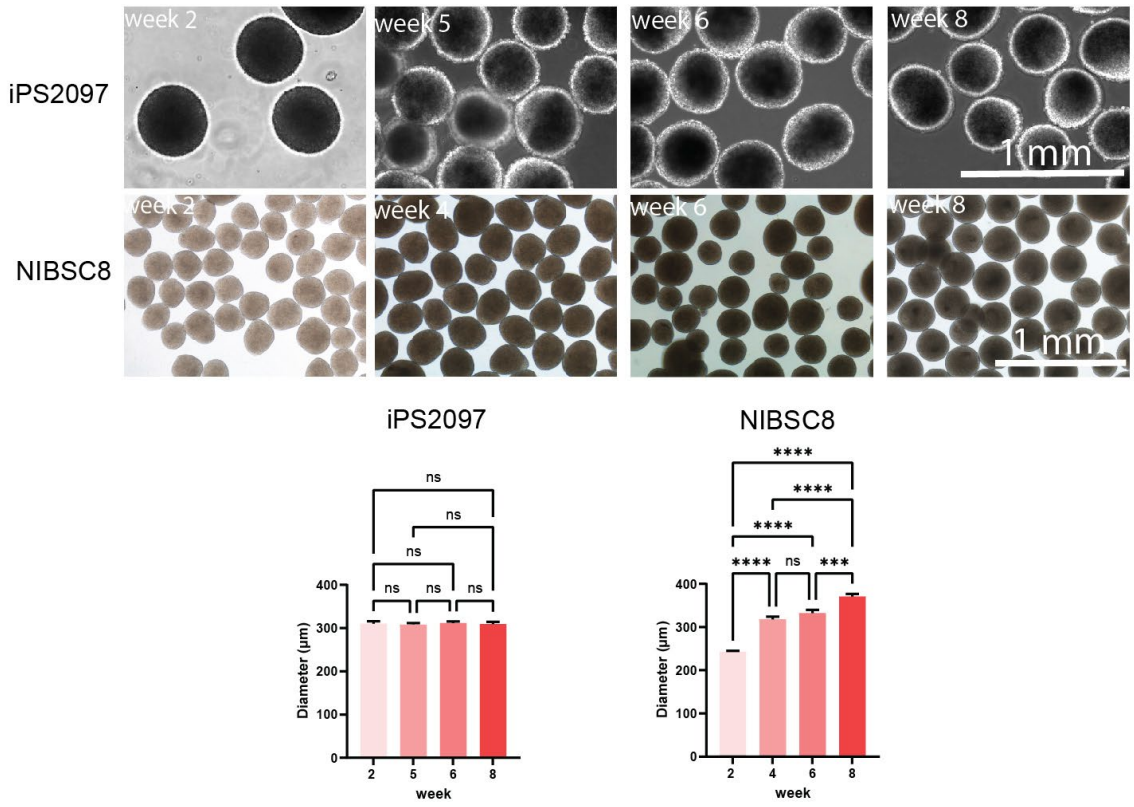
To understand the differences in forming brain spheres with both lines, various parameters were studied, including size, gene expression, and cell composition, to evaluate the effectiveness of the differentiation method in the NIBSC8 line.

Size was measured in both the original line iPSC2097 and the NIBSC8 line over time. Bright field images were taken at different differentiation stages, and the diameters of the spheres were measured. GE01 showed a very stable size after 2 weeks, with a diameter of approximately 300 µm **(Figure 6)**. In contrast, the NIBSC8 line displayed a gradual increase in size, starting with a diameter

of 250 µm at 2 weeks and slowly reaching around 350 µm at 8 weeks **(Figure 6)**. Overall, despite these small differences, the aggregates were quite similar at 7 weeks, where the assay began.

Figure 6. Brainspheres generated from NIBSC8 showed increased **diameter over development** (week 2; week4; week6; week8).

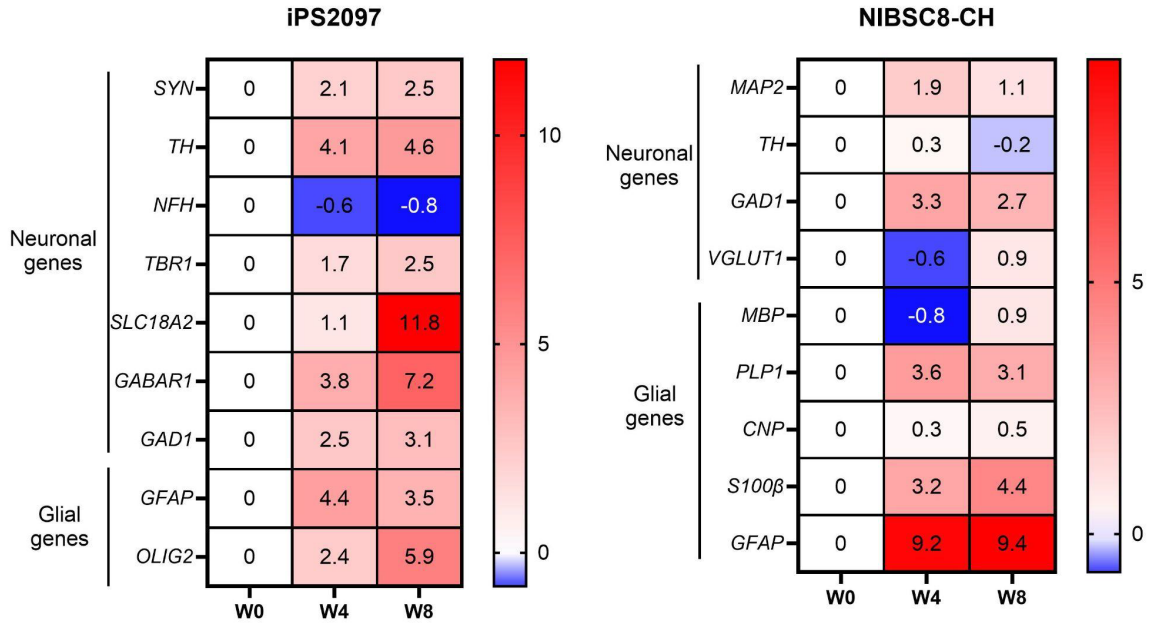
The size of 8-week-old Brainspheres from NIBSC8 showed comparable size compared to iPS2097.



Source: Shan Wang.

Gene expression was also studied for several general markers of both neurons and glia, and the results were compared with previous data. Some discrepancies in gene selection were noted due to the iPS2097 data being obtained from Johns Hopkins laboratory. Overall, most of the genes studied showed an increase in expression of neuronal and glial genes over the differentiation process, as expected **(Figure 7)**. However, some differences were found between the lines. While iPS2097 only showed a decrease in NFH expression, NIBSC8 exhibited a decrease in the expression of MBP and VGLUT at week 4 (W4), followed by a small increase at week 8 (W8) compared to the baseline (W0) cells **(Figure 7)**. The most striking result was the very low expression of the dopaminergic marker TH in NIBSC8-derived BrainSpheres **(Figure 7)**. We are not sure how these results may influence the test method.

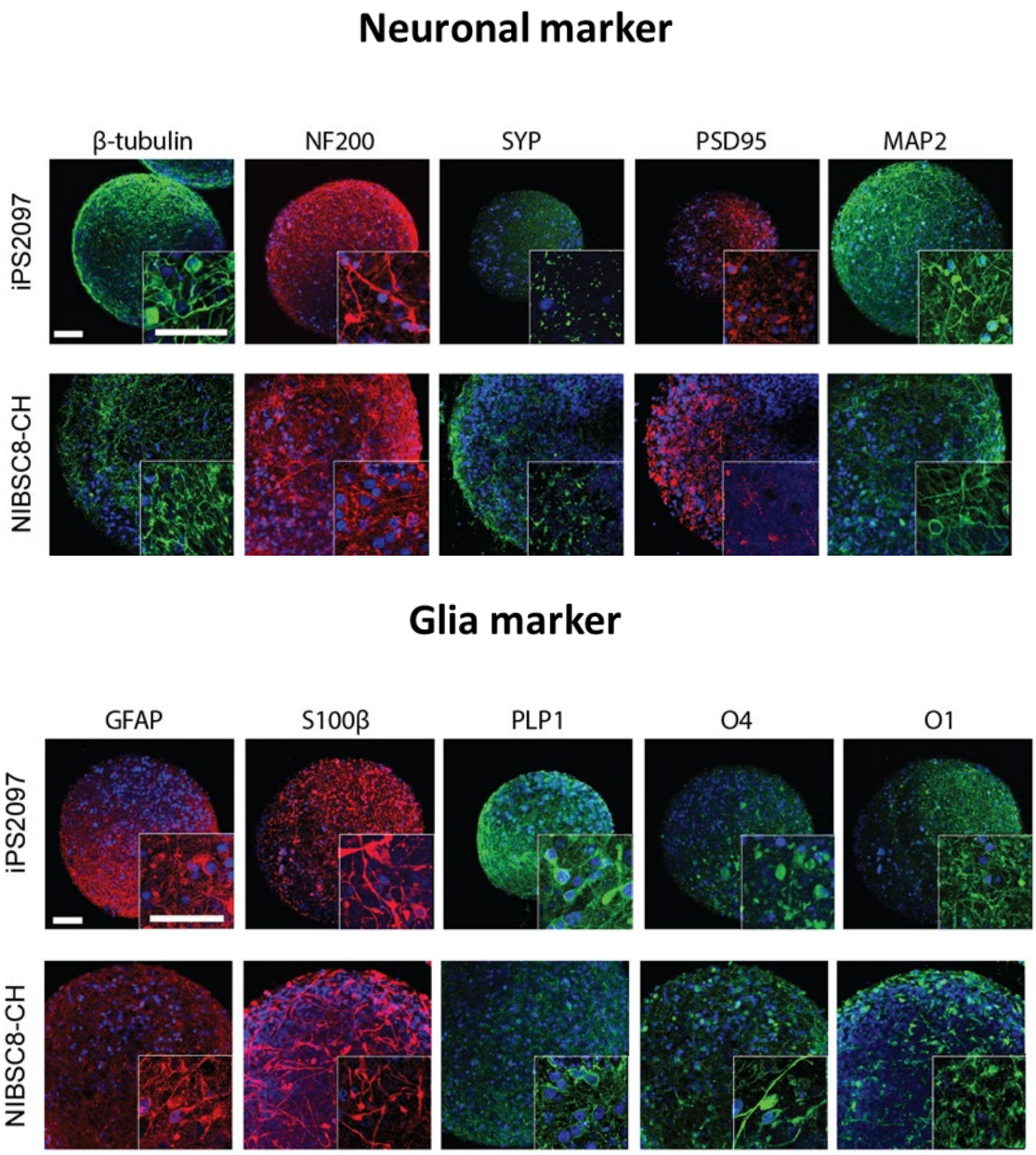
Figure 7. Gene (neuronal and glial) **expression pattern of iPS2097 and NIBSC8 BrainSpheres** during development (week 4, week 8). Heatmap was made based on the qPCR data. The colour represents log2 (fold change).



Source: Shan Wang.

Neuronal and glial markers were also studied using immunohistochemistry followed by confocal microscope image acquisition. These experiments showed that both lines showed positive signals for the neuronal markers (β-Tubulin, NF200, SYP, PSD95, and MAP1) (Figure 8). In terms of glial markers, GFAP, S100b, PLP1, O4, and O1, both lines showed positive signals (Figure 8).

Figure 8. Similar to iPS2097 BrainSpheres, N8 **BrainSpheres showed positive signals for neuronal markers** b-tubulin; NF200; Synaptophysin, PSD95 and MAP3, as well as glial markers GFAP; S100b; PLP1; O4; O1



Source: Shan Wang.

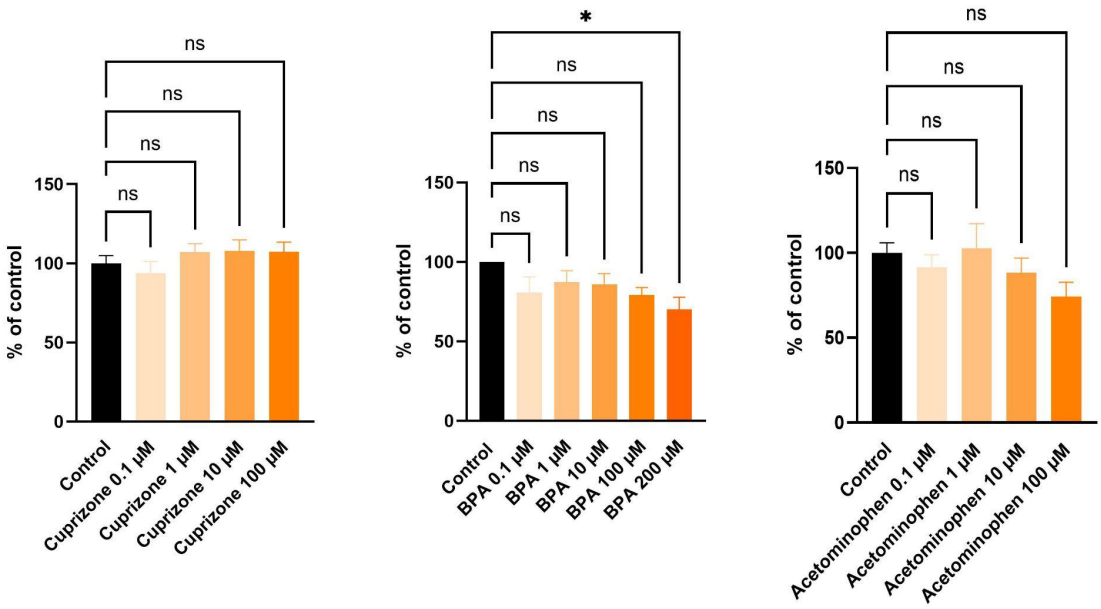
3.4 Results from cytotoxicity test or other type of test performed to confirm absence of interference

To determine the appropriate dose range for the myelin assay quantification, cytotoxicity was assessed using the Resazurin assay. BrainSpheres were differentiated for 7 weeks in differentiation media before being exposed to varying concentrations of Cuprizone, Acetaminophen, and BPA. The concentration range tested was 0.1 µM to 100 µM for Cuprizone and Acetaminophen, and up to 200 µM for BPA **(Figure 9)**. The exposure period lasted one week, consistent with the exposure time used in the test method.

According to the results of the Resazurin assay, only the 200 µM concentration of BPA resulted in significant cytotoxicity **(Figure 9)**. In contrast, all other tested concentrations of BPA, as well as all tested concentrations of Cuprizone and Acetaminophen, did not exhibit significant cytotoxicity. These findings suggest that while high levels of BPA can induce cytotoxic effects, Cuprizone and Acetaminophen, within the tested concentration range, are not cytotoxic under the same conditions.

Figure 9. Resazurin assay for cell viability.

Data are represented as mean ± standard deviation (SD). Data were collected from three independent batches. Statistical analysis: One-way ANOVA with P values *P < 0.05, **P < 0.01, and ***P < 0.001. Data sets are compared to control. Cuprizone was dissolved in 50% ethanol in culture medium.



Source: Shan Wang.

3.5 PLP1 quantification after test compounds

The total fluorescence of the myelin marker PLP1 was measured after a 1-week exposure of BrainSpheres to cuprizone, BPA, and acetaminophen. The measurements were conducted as previously described. Three independent experiments were performed for each compound.

Plate layout of the 6-well plate is shown below:

Vehicle Control (0.1% DMSO)	Acetaminophen 0.1 µM	Acetaminophen 1 µM
Acetaminophen 10 µM	Acetaminophen 50 µM	Acetaminophen 100 µM

Vehicle Control (0.1% DMSO)	Bisphenol A 0.1 µM	Bisphenol A 1 µM
Bisphenol A 10 µM	Bisphenol A 100 µM	X

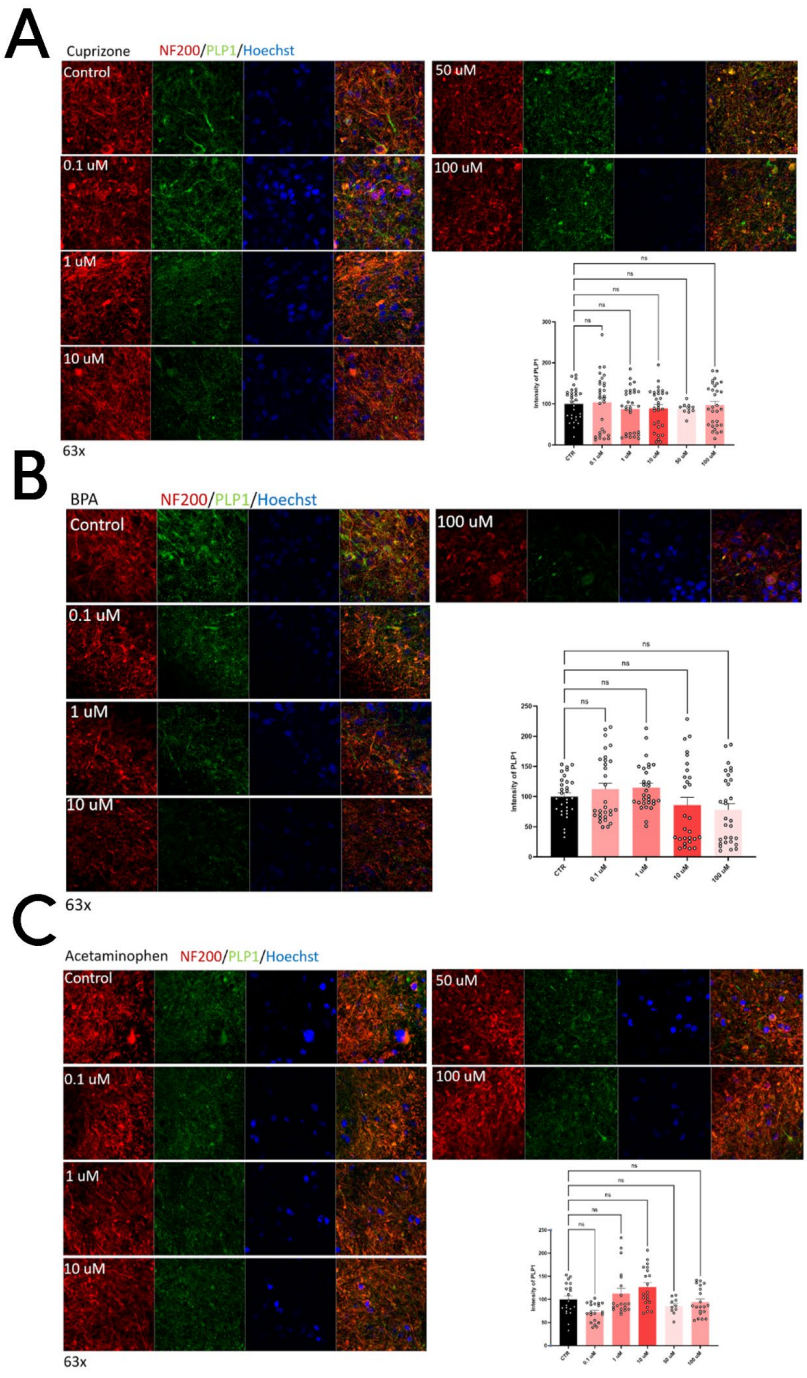
Vehicle Control (Culture medium or with 50% ethanol in medium)	Cuprizone 0.1 µM	Cuprizone 1 µM
Cuprizone 10 µM	Cuprizone 50 µM	Cuprizone 100 µM

The results, represented by the combination of the three experiments, indicate that no significant changes were found after the exposure to any of these compounds **(Figure 10)**. However, a closer examination revealed high variability between experiments **(Figure 11)**. This variability could be due to several factors: it could be that the cell line does not behave the same way as previously used lines, it could be due to operator differences since the technique to acquire the images requires

specific practice, or it could be that new compounds stocks present some differences with the previously assessed.

Figure 10. PLP1 levels of BrainSpheres treated with Cuprzione (A), Acetaminophen (B) and BPA (C) were measured by immunocytochemistry.

Intensity of PLP1 was normalised to control. Data were collected from three independent batches for BPA and Cuprizone, and two independent batches for Acetaminophen. Data are represented as mean ± standard deviation (SD). Statistical analysis: One-way ANOVA with P values *P < 0.05, **P < 0.01, and ***P < 0.001. Cuprizone was dissolved in culture medium (1 batch), and 50% ethanol in culturing medium (2 batches).

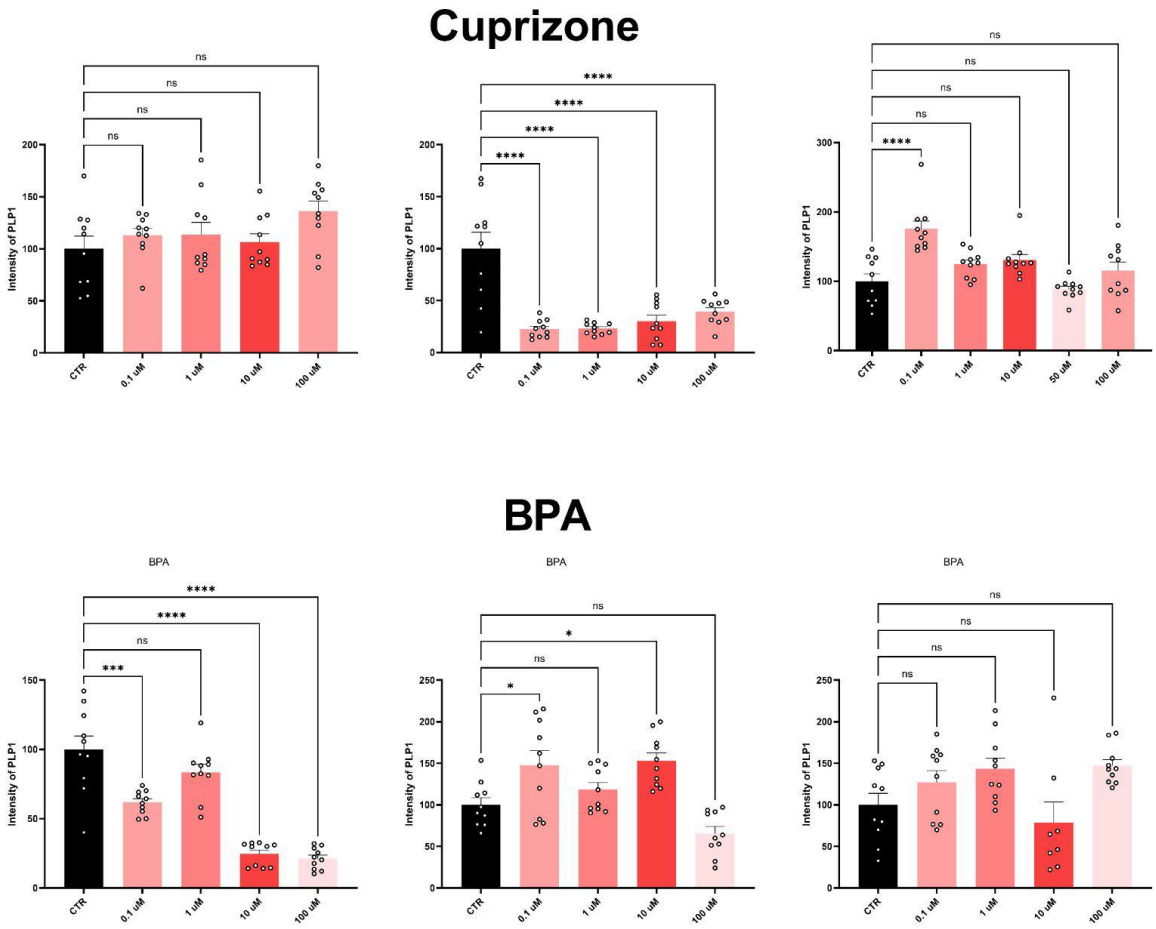


Source: Shan Wang.

If we analyse the experiments independently, we can clearly see that the variation between experiments is quite high **(Figure 11)**.

Figure 11. PLP1 levels of BrainSpheres treated with Cuprzione, BPA were measured by immunocytochemistry.

Data was plotted in separate batches. Data are represented as mean ± standard deviation (SD). Statistical analysis: One-way ANOVA with P values *P < 0.05, **P < 0.01, and ***P < 0.001.

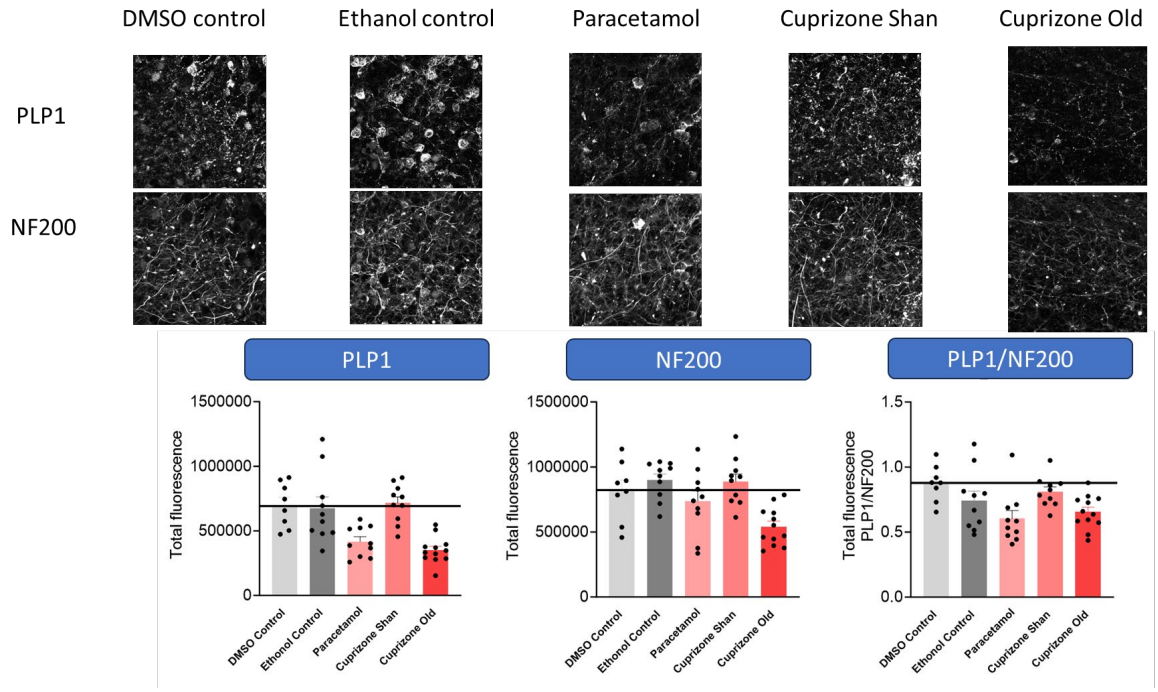


Source: Shan Wang.

These results were quite different to the ones obtained before with the iPS2097 line. For that reason, we also tried old dilution from Chesnut et al manuscript and new cuprizone.

Figure 12. PLP1 levels of BrainSpheres treated with Cuprzione stock made in the US (Cuprizone old).

Cuprizone stock made by Shan in UNiL (Cuprizone Shan), Paracetamol were measured by immunocytochemistry.



Source: Shan Wang.

In this case, we observed a decrease in iPS2097 with the previously diluted Cuprizone, but not with the new dilution of Cuprizone. However, we also found a decrease in our negative control, Paracetamol, and a slight decrease in NF200. This could indicate that this concentration was somewhat cytotoxic.

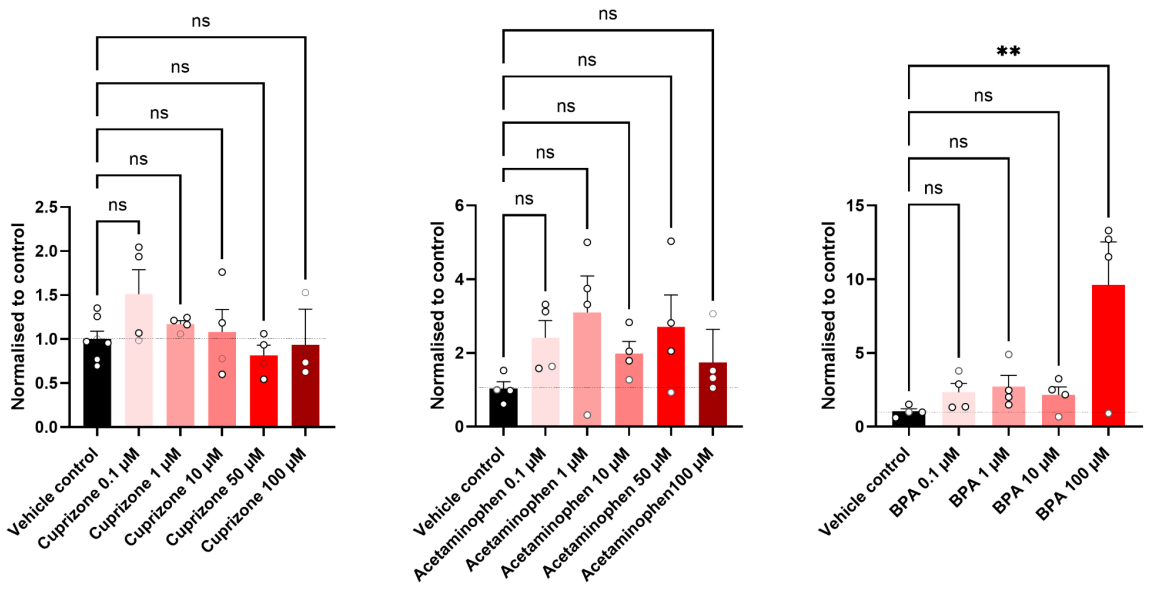
It is difficult to take conclusion, but we believe that different factors may influence in this variability between experiments (see discussion). The technique is highly operator-dependent, which introduces variability into the measurements. Finding a more robust quantification method will help improve the assay's development. We also believe that differences between cell lines can introduce additional variability in the measurements.

3.6 Exploring other techniques to quantify PLP1 and MBP

In order to try to find a less biased and operator independent technique, Elisa and Flow cytometry were tried. Cells were treated the same way as for immunocytochemistry assay. According to the results of PLP1 ELISA, 100 uM BPA led to significantly increased levels of PLP1, whereas all the other concentrations of BPA, Cuprzione and Acetaminophen did not show altered levels of PLP1 **(Figure 13)**. We also observed a big variability between experiments. It is possible that standardisation and optimisation of the procedure will help to obtain better results.

Figure 13. PLP1 levels of BrainSpheres treated with Cuprzione, Acetaminophen and BPA were measured by ELISA.

Data were collected from two independent batches. Data are represented as mean ± standard deviation (SD). Statistical analysis: One-way ANOVA with P values *P < 0.05, **P < 0.01, and ***P < 0.001. Cuprizone was dissolved in the culture medium.



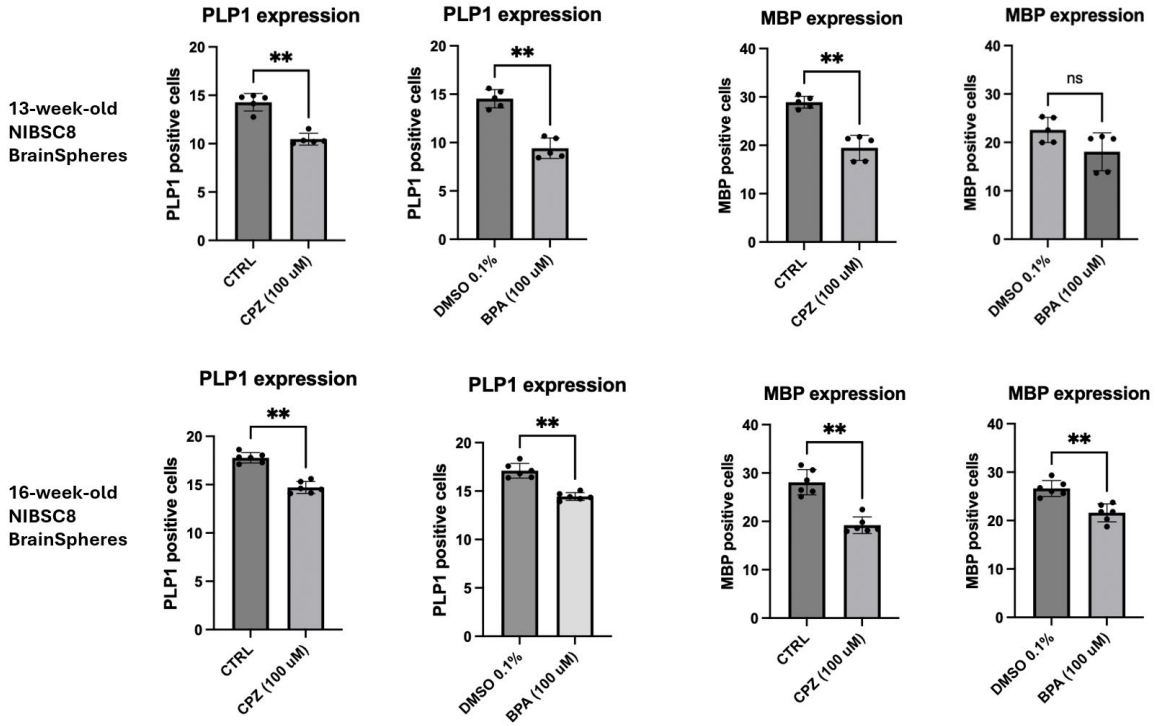
Source: Shan Wang.

Flow cytometry was also performed at different weeks of differentiation using the NIBSC8 line. The results showed a significant decrease in PLP1 and MBP levels after exposure to 100 µM Cuprizone and a decrease in PLP1 levels after exposure to 100 µM BPA at 13 weeks in BrainSpheres **(Figure 14)**. Additionally, we observed decreases in both markers following both treatments at 16 weeks of differentiation **(Figure 14)**.

These results still need to be confirmed, but they suggest that this method could potentially be adapted, as it is less biassed and provides automatic quantification. Implementing flow cytometry as a standard technique could help improve the accuracy and reliability of our measurements.

Figure 14. PLP1 levels of BrainSpheres treated with 100 uM Cuprzione and 100 uM BPA were measured by flow cytometry.

Data were collected from one batch. Data are represented as mean ± standard deviation (SD). Statistical analysis: unpaired T-test. *P < 0.05, **P < 0.01, and ***P < 0.001. Cuprizone was dissolved in the culture medium.



Source: Massimo Frangiamore

3.7 Establishment of acceptance criteria

Table 5. Proposed acceptance criteria

Experimental design steps	**Acceptance criteria**
Characterise human induced pluripotent stem cells (hiPSCs) from a commercial line	Cells need to express Stem cells markers (e.g., OCT4, Nanog, SOX2, TRA-1-60, TRA-1-81) and able to differentiate into the 3 lineages: endodermal (SOX17); mesodermal (vimentin), ectodermal (nestin).
Differentiation to Neuronal Progenitors	Cells required to express neural progenitor markers and present high percentage of neuronal lineage (e.g.; CD56, CD57. > 90%) and lower percentage of neural crest cells (e.g., CD271, SSEA4. < 5%)
Concentration selection is based on resazurin assay.	Concentration used needs to present no cytotoxic effects.
Perform immunocytochemistry, or flow cyotmetry to detect myelin-associated protein using proteolipid protein 1 (PLP1).	Not possible to establish at this point.

Source: David Pamies and Shan Wang.

4 Conclusions and discussion

The results indicated that the NIBSC8 line performs well as an iPSC line, displaying the typical characteristics of healthy iPSCs, including the presence of key pluripotency markers. These cells also demonstrate the capability to differentiate into the three germ layers: mesoderm, ectoderm, and endoderm. Differentiation into neural progenitor cells (NPCs) was optimal, generating nearly 100% NPCs that express key NPC markers such as Nestin, Vimentin, CXCR4, SOX2, SSEA-1, Notch-1, and Musashi-1. Size comparison between iPS2097 (Original cell line) and NIBSC8-CH shows very small differences. This indicates that NIBSC8 is a good starting source for generating BrainSpheres. The generation of BrainSpheres from NIBSC8 cells also appears optimal. Despite slight differences in the gene expression of some markers, the cells were able to produce aggregates of similar size with the expression of all relevant markers for the assay.

Assessment of myelin disruption based on the PLP1 total fluorescence assay has yielded contradictory results. Data from NIBSC8 line BrainSpheres did not show statistically significant results after exposure to our positive controls (Cuprizone and BPA). Additionally, there was strong variability between experiments. While sometimes we observe a decrease, sometimes there were not changes. We believe that this variation could stem not only from the cell line itself but also from issues with Cuprizone dilution. When comparing old and new dilutions on the original cell line, results were more consistent with expectations.

We also believe that the method is highly operator-dependent, which introduces bias and makes the assay less suitable for regulatory purposes. Therefore, we have explored other possibilities such as high-content automated image analysis (not shown in this report), ELISA, and flow cytometry. Of these three methods, flow cytometry has proven to be the most effective. Using flow cytometry, we observed decreases in both MBP and PLP1 levels after exposure to Cuprizone and BPA. However, more work is needed to establish appropriate acceptance criteria.

In this context, we have a year and a half remaining on a Swiss National Fund grant to develop a better method for studying myelin disruption, in which we will continue with this work. Additionally, we are now part of a funded EFSA project aimed at incorporating the assay into the developmental neurotoxicity (DNT) battery. For this project, we will use another commercial line, IRM90, and all future work will be conducted with this line. We plan to move forward with flow cytometry while continuing to optimise ELISA and high-content image analysis. Our goal is to find a robust, less operator-dependent method for studying myelin disruption in the near future.

We are preparing a manuscript on the reproducibility of our model, in which we have compared the capability of four different hiPSC lines to generate BrainSpheres across three different laboratories. We expect this manuscript to be published soon. Some of the data presented in this report will be included in the upcoming publication.

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List of abbreviations and definitions

Abbreviations	
Nanog	Homeobox protein NANOG (hNanog) is a transcriptional factor that helps embryonic stem cells (ESCs) maintain pluripotency by suppressing cell determination factors
OCT4	Octamer-binding transcription factor, a transcription factor that regulates the pluripotency of embryonic stem cells
SOX2 and SOX17	Sex determining region Y-box 2 or box 17
TRA-1-60 and TRA-1-81	Cell surface antigens expressed along with SSEA-3, SSEA-4 in human embryonic stem cells, embryonal carcinoma cells and induced pluripotent stem cells (iPS)
NIBSC	National Institute for Biological Standards and Control (NIBSC)
hiPSC	Human induced pluripotent stem cells
NPCS	Neural Progenitor Cells
CXCR4	a G-protein-coupled chemokine receptor also known as fusin or CD184
SSEA-1 and SSEA-4	Stage-specific embryonic antigen 1 and 4
Notch-1	Neurogenic locus notch homolog protein 1
Musashi-1	RNA-binding protein Musashi homolog 1
CD56, CD57 and CD271	Cell surface markers, cluster of differentiation 56, 57 and 271.
NF200	Neurofilament protein 200
Synaptophysin	An integral membrane glycoprotein of 38 kDa
PSD95	Postsynaptic density protein 95
MAP3	Microtubule-associated protein 3
GFAP	Glial Fibrillary Acidic Protein

Abbreviations

S100b	A calcium-binding protein found in astrocytes, glial cells, adipose tissue, cardiac cells, and skeletal muscles.
PLP1	Proteolipid protein 1 is a form of myelin proteolipid protein
MBP	Myelin basic protein
BPA	Bisphenol A

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