Growth And Imaging Of Neural Stem Cells In VITVO®

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INTRODUCTION

So far current pharmaceutical treatments poorly tackle neurological disorders and the development of new therapeutic approaches is relevant to broaden our capability to cure them. For this reason, *in vitro* models and advances in neural stem cells (NSCs) research play a crucial role to shed light on the diseases' pathophysiology and further characterize cellular and molecular mechanisms underlying the growth and differentiation of neural tissues. The neurosphere assay is an accepted model for the study of NSC self-renewal and their differentiation into neural cell phenotypes (neurons, astrocytes and oligodendrocytes), and of neurite outgrowth [1].

Moreover, switching to 3D *in vitro* platforms offers the possibility to better mimic the *in vivo* physiological behavior of cells, and to recapitulate a higher level of complexity with highly-predictive models. VITVO® is a ready-to-use device consisting of a close bioreactor with a fiber-based 3D matrix, which offers a mechanical support for a free 3D cell colonization able to recreate real tissue architectures.

In this study, neural stem cells from mouse's fetal brain (E13.5) were cultivated in VITVO and investigated in their morphology and differentiation respect to 2D culture (Figure 1). VITVO showed the potential to strongly impact and improve the value of collected data, also in the perspective of a drug development process for neurological disorders.

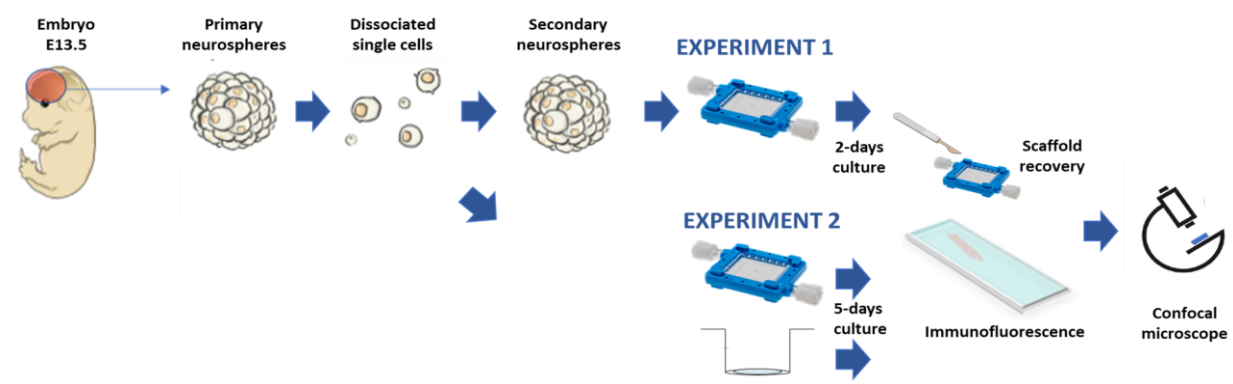


Figure 1. Experimental workflow. *Neurospheres and dissociated NSCs were seeded in VITVO and in 2D wells. Neurosphere structures, cell morphologies and differentiation markers were imaged after immunofluorescence, using confocal microscope.*

MATERIALS AND METHODS

Tissue culture: Neural stem cells (NSCs) were isolated from the fetal brain tissue of C57BL6/J mice, then cultivated in plate as primary neurospheres and dissociated into single cells as previously reported [2]. Neurospheres were maintained as cultures in suspensions in flasks in NSCs culture medium (DMEM F12 Glutamax, 1x B27, 1 x N2, 8 mmol/L HEPES, 100 U/100 µg Penicillin/Streptomycin, 20 ng/ml bFGF, 20 ng/ml EGF). Primary neurospheres were dissociated and seeded back in suspension to generate secondary neurospheres, which were loaded in the VITVO devices. In another set of experiments primary neurospheres were dissociated and single cells were plated on coverslips or seeded in VITVO.

2D cell culture establishment: Single cells were seeded at 3000 cells/cm^2 in a glass coverslip in 24 well plate and coated with poli-D,L-ornitin (50 µg/ml) and laminin (5 µg/ml) (Sigma-Aldrich). 5-days culture was maintained by changing half of the medium every 2 days.

3D cell culture establishment: VITVO® (Rigenerand srl, Medolla (MO), Italy) devices were primed with culture medium to wet the 3D matrix, and then secondary neurospheres (almost 20 spheres) (Experiment 1) or dissociated NSCs (50000 cells) (Experiment 2) were resuspended in 1.4 ml of culture medium and injected into the device with a 2.5 ml syringe (Becton Dickinson). Two (neurospheres) or five (single cells) days cultures were maintained by changing half of the medium every 2 days.

Immunofluorescence protocol (IF): VITVO 3D matrix was collected after having cut the edges of the transparent membrane and the 3D matrix with a surgical scalpel. VITVO 3D matrices and the 2D coverslips were put in different wells of a plate and processed in parallel with the IF protocol. Fixation was performed with cold 4% PFA in PBS incubated for 15 min at room temperature (RT). Blocking solution with 1% BSA in PBS, 1% normal serum donkey was incubated for 1 h at RT. Primary antibody was diluted in 0.1% Triton X-100 in PBS (according to manufacturer recommendations) and incubated for 2 h at RT. 2 washes with PBS (10 mins each) were followed by secondary antibody incubation in 0.1% Triton X-100 and PBS (diluted accordingly to manufacturer's recommendation) for 30 mins at 37°C. The nuclei staining was obtained with Hoechst 33258 dye (1 µg/ml diluted in PBS-0.3% Triton-X 100, Life Technologies) added to secondary antibody solutions. After 2 washes with PBS (10 mins each), the matrices were mounted on slides with a drop of phenylendiamine and a coverslip.

Imaging: Images were acquired with confocal microscope Nikon Ti-E A1R, 40x objective magnification. For each z-stack, 1 µm cross-sectional images were taken in increments along the Z-axis to encompass the entire structure volume. Image processing were performed with Nis-Elements AR 3.2 software.

RESULTS AND DISCUSSION

Neurospheres growth in VITVO was inspected by analyzing their structure, cell morphology and differentiation. VITVO allows the cultivation of NSCs, seeded either with dissociated cells or neurospheres. VITVO 3D matrix is an inert and biocompatible fiber-based scaffold with a thickness of 400 µm. Cells can freely colonize both fibers and the empty volume among them which represents about 90% of the total volume. Considering that NSCs require strictly controlled culture conditions and the *in vivo* stiffness of the brain is crucial for the diffusion of nutrients/growth factors, VITVO matrix alone offers such a mechanical support to make possible the penetration of molecules and nutrients. Secondary neurospheres were seeded and hosted in VITVO matrix, by maintaining structures, cell-to-cell interactions and network, mimicking their typical features (Figure 2). After 48 h, cells maintained the undifferentiated state (green nestin-positive cells) and showed neurites outgrowth.

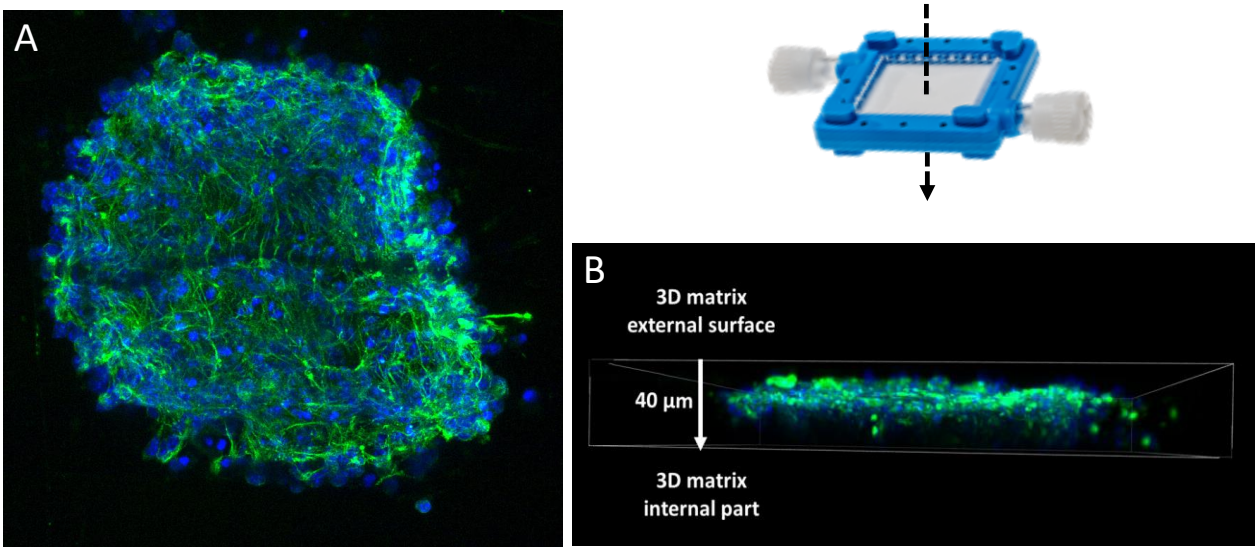


Figure 2. Immunofluorescence of neurospheres grown in VITVO. *NSCs neurospheres grown inside VITVO were probed with an anti-nestin antibody and a secondary antibody Alexa488-conjugated (green). Hoechst (blue dye) stained nuclei and revealed the distribution of the total cell population. 3D tissue-like structures were imaged using Nikon Ti-E A1R confocal microscope, with 40X objective magnification. Sequential scanning over the whole 3D structure has improved resolution capacity. (A) Maximum intensity projection. (B) 40-µm thickness in the 3D matrix.*

After a 5-days culture of dissociated NSCs, lineage markers analysis (βIII-tubulin for neurons, GFAP for astrocytes, CNPase for mature oligodendrocytes) highlighted a different distribution of neural derivatives and the capability of differentiation compared to 2D culture (Figures 3-4). In VITVO cells did not require the coating with poli-D,L-ornitin and laminin of 2D surface, and cells were more homogeneously distributed. NSC-derived neurons and astrocytes displayed poorly-elongated morphologies, different from cells laying on a 2D surface. Moreover, in VITVO cells were organized in little clusters and their interactions were established in a 3D volume.

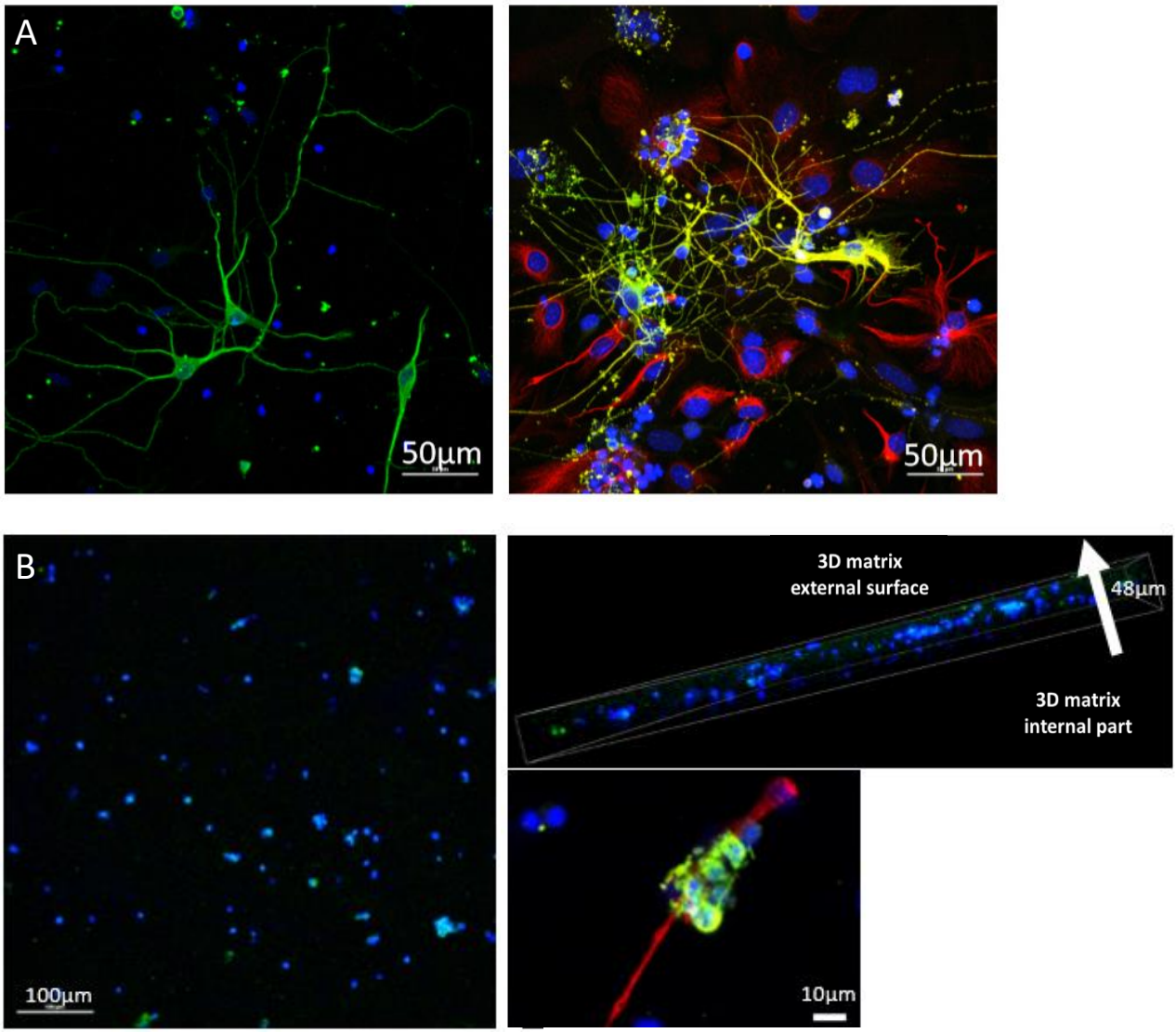
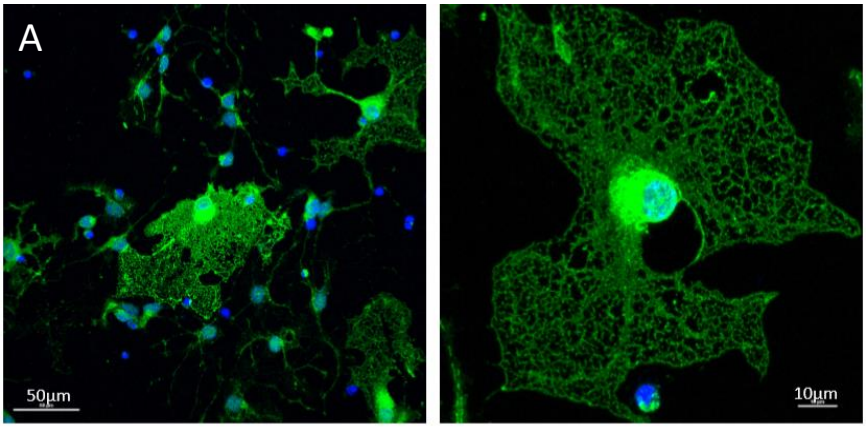


Figure 3. Neurons (green) and astrocytes (red) in 2D and VITVO show different distribution and cell morphology. *NSCs grown on 2D glass surface (A) and in VITVO 3D matrix (B) were probed with anti-βIII-tubulin (green) and anti-GFAP (red) antibodies. Hoechst (blue) dye stained nuclei. Structures were imaged using Nikon Ti-E A1R confocal microscope, with 40X objective magnification.*



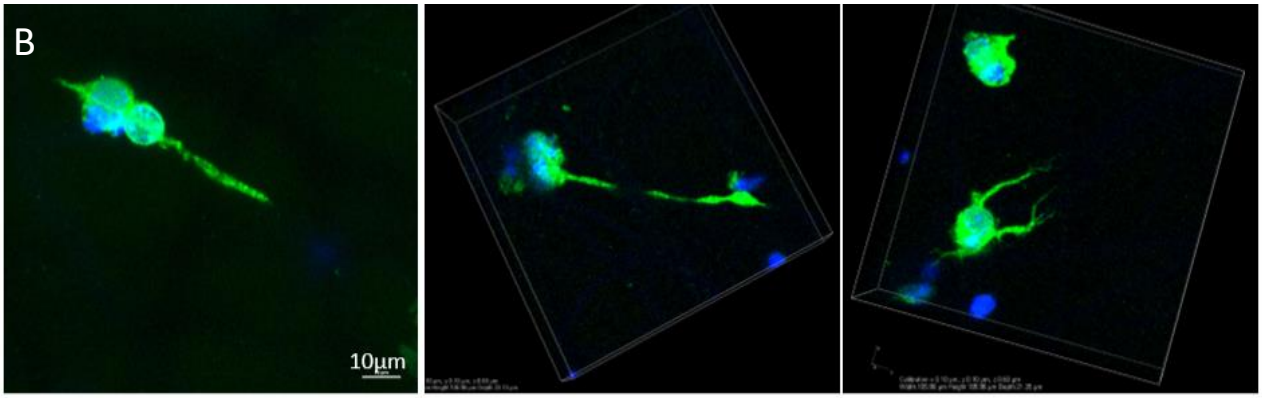


Figure 4. Oligodendrocytes in 2D and VITVO reveal a different cell morphology. *NSCs grown on 2D glass surface (A) and 3D VITVO matrix (B) were probed with mature marker CNPase (green). Hoechst (blue) dye stained nuclei. Structures were imaged using Nikon Ti-E A1R confocal microscopy, 40X objective magnification.*

CONCLUSIONS

These data demonstrate that VITVO is a suitable system for growing 3D neurospheres and obtaining complex 3D structures without adding growth factors, considering the necessity of strictly-controlled culture conditions for neural stem cells.

Therefore, VITVO offers the opportunity to study the 3D spatial connections among different neuronal populations and how they communicate each other, representing a more physiological and reliable model compared to 2D culture.

REFERENCES

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