HIGH PURITY
NEW ENGLAND

Scientific Report:

Human Primary Glioblastoma Neurospheres Cultured In VITVO 3D Bioreactor

VITVO 3D Bioreactor

2019

RIGENERAND
Steps Ahead In Cell Technology

Application Note

VITVO®

Human Primary Glioblastoma Neurospheres Cultured In VITVO® 3D Bioreactor

Stella Frabetti[1], Olivia Candini[1], Elisa Gregianin[1], in collaboration with Giacomo Pavesi[2], Alberto Feletti[2], Malvina Prapa[3], Chiara Chiavelli[3], Francesco Cavani[4] and Carla Palumbo[4]

[1]3D Cell Culture Technologies Department, Rigenerand srl, Medolla (MO), Italy

[2]Neurosurgery Civil Hospital of Baggiovara, Modena, Italy

[3]Cellular Therapies Laboratory, Department of Medical and Surgical Sciences for Children & Adults, University Hospital of Modena and Reggio Emilia, Italy

[4]Department of Biomedical, Metabolic and Neural Sciences, University of Modena and Reggio Emilia, Italy

INTRODUCTION

Glioblastoma is the most common primary malignant brain tumor due to its invasive nature and failure of standard radio and chemotherapy protocols [1]. In the last years growing efforts have been made to find targeted therapy based on tumors molecular features but, to date, no one of these approaches have been validated as effective in clinical trial [2]. In this scenario, VITVO® 3D bioreactor may offer a useful tool able to recreate a tissue-like structure *in vitro*, providing more physiologically relevant information on cell responses to a variety of stimuli and contributing to bridge the gap between traditional 2D monolayer cell culture and clinical trial [3].

Thanks to a collaboration with Division of Oncology, Department of Medical and Surgical Sciences for Children & Adults, University-Hospital of Modena and Reggio Emilia, human primary glioblastoma cells (GBM) from surgical specimen were collected and maintained in culture with glioblastoma stem cell selective media. In the appropriate media these cells naturally organize themselves in neurospheres. In VITVO bioreactor it has been possible to culture GBM neurospheres for one month and to follow cells' growing both in real time by fluorescence microscope and in end point by histology.

MATERIALS

- Human primary glioblastoma cells (GBM)
- GSC selective culture media (DMEM-F12 1:1, 1% penicillin-streptomycine, 1% L-Glutamine, supplemented with B27 (1X) w/o VitA, hFGF (20 ng/mL), hEGF (20 ng/mL))
- VITVO® bioreactor (Rigenerand srl)
- 2,5 mL Syringe (Becton Dickinson and Co)
- ReadyProbes™ Cell Viability Imaging kit, Blue/Green (Invitrogen)

- Formalin solution, neutral buffered 10% (Sigma-Aldrich)
- PBS Phosphate Buffered Saline solution (Gibco)
- Ethanol 99% (Sigma-Aldrich)
- HistoClear (Sigma-Aldrich)
- Paraffin
- Methyl Methacrylate (Sigma-Aldrich)
- Methylacetate (Sigma-Aldrich)
- Mayer's Hematoxylin solution
- Eosin solution
- DPX Mountant for histology (Sigma-Aldrich)

METHODS

3D cell culture model: VITVO bioreactor was first primed with empty culture medium to ensure the complete wetting of the 3D matrix, then 560.000 human primary glioblastoma cells were resuspended in 1,4 ml of culture medium and injected in VITVO.

To visualize cells by fluorescence microscope to follow cellular growth 1 drop/mL of ReadyProbes Cell Viability Imaging kit (Blue/Green) was added to culture medium. For the first week of culture, medium was changed every 48 hours, then every 24 hours.

Matrix fixation and embedding: After one month 3D matrix with cells was fixed injecting formalin solution neutral buffered 10% directly inside VITVO and incubating for 20 minutes at room temperature. Then 3D matrix was recovered using a surgical scalpel, washed once with PBS in falcon tube and incubated over night in 70% ethanol solution. VITVO matrix was cut by scissors in order to process one half for paraffin embedding and the other half for methyl methacrylate embedding to compare the two different techniques (Figure 1).

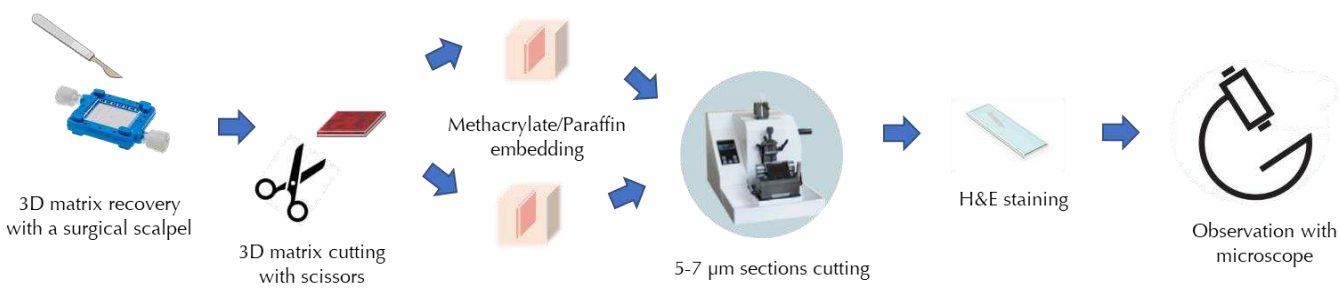

Figure 1. The approach. *Workflow of VITVO processing for histological read-out.*

For paraffin embedding dehydration was performed incubating 3D matrix in ethanol solutions from 70% to 100%, then in HistoClear and in liquid paraffin for 1 hour at 70°C.

For methyl methacrylate embedding after dehydration the following protocol has been applied:

- 1:1 solution 100% ethanol 100% methyl methacrylate for 10 minutes
- 100% methyl methacrylate twice for 10 minutes
- Polymerizing solution composed by methyl methacrylate 100 mL, Arkopal N100 15 mL, benzoyl peroxide 3 g. To start the polymerization reaction 0,2% of the following solution (diphenylalanine 1:15 polyethylene glycol 200) was added to the polymerizing solution.
- 24 hours incubation at +4°C to allow methyl methacrylate polymerization

7µm thick slices were cut by microtome both for paraffin and methyl methacrylate embedded half matrix. Methyl methacrylate sections were collected on glycerinated albumin glass slides, covered with polyethylene film and pressed overnight at 60°C.

Hematoxylin and Eosin Staining: Methyl methacrylate slides were first incubated in methylacetate twice for 5 minutes, then both methacrylate and paraffin sections were stained following the same protocol:

- HistoClear solution three times for 10 minutes
- 100% ethanol twice for 5 minutes
- 95% ethanol twice for 5 minutes
- 70% ethanol 3 minutes
- 50% ethanol 1 minute
- Wash with distilled water
- Mayer's Hematoxylin solution 3 minutes
- Three quick washes in distilled water
- 95% ethanol 1 minute
- Eosin solution 20 seconds
- 95% ethanol 20 seconds
- 100% ethanol 20 seconds
- HistoClear solution 1-5 minutes
- DPX Mountant for histology 2 drops/slide and cover with coverslide
- Overnight drying under chemical hood

RESULTS AND DISCUSSION

Cells viability inside VITVO was monitored using ReadyProbes Cell Viability Imaging kit (Blue/Green) fluorescence supravital staining. The progressive 3D matrix colonization by labeled cells was directly observed under a fluorescence microscope. Pictures were taken at different time points with EVOS FL auto microscope with Olympus UPlanSApo 4X 0.16 objective (Figure 2). Glioblastoma cells have been able to engraft and colonize VITVO 3D matrix, organizing to form neurospheres. Neurospheres have grown inside 3D matrix and aggregated each other forming bigger structures along time (Figure 2D). Cell viability was real-time evaluated during culture, showing a very low level of mortality at each time point, as demonstrated by the low number of died-green cells compared to the viable-blue cells (Figure 2 A-D).

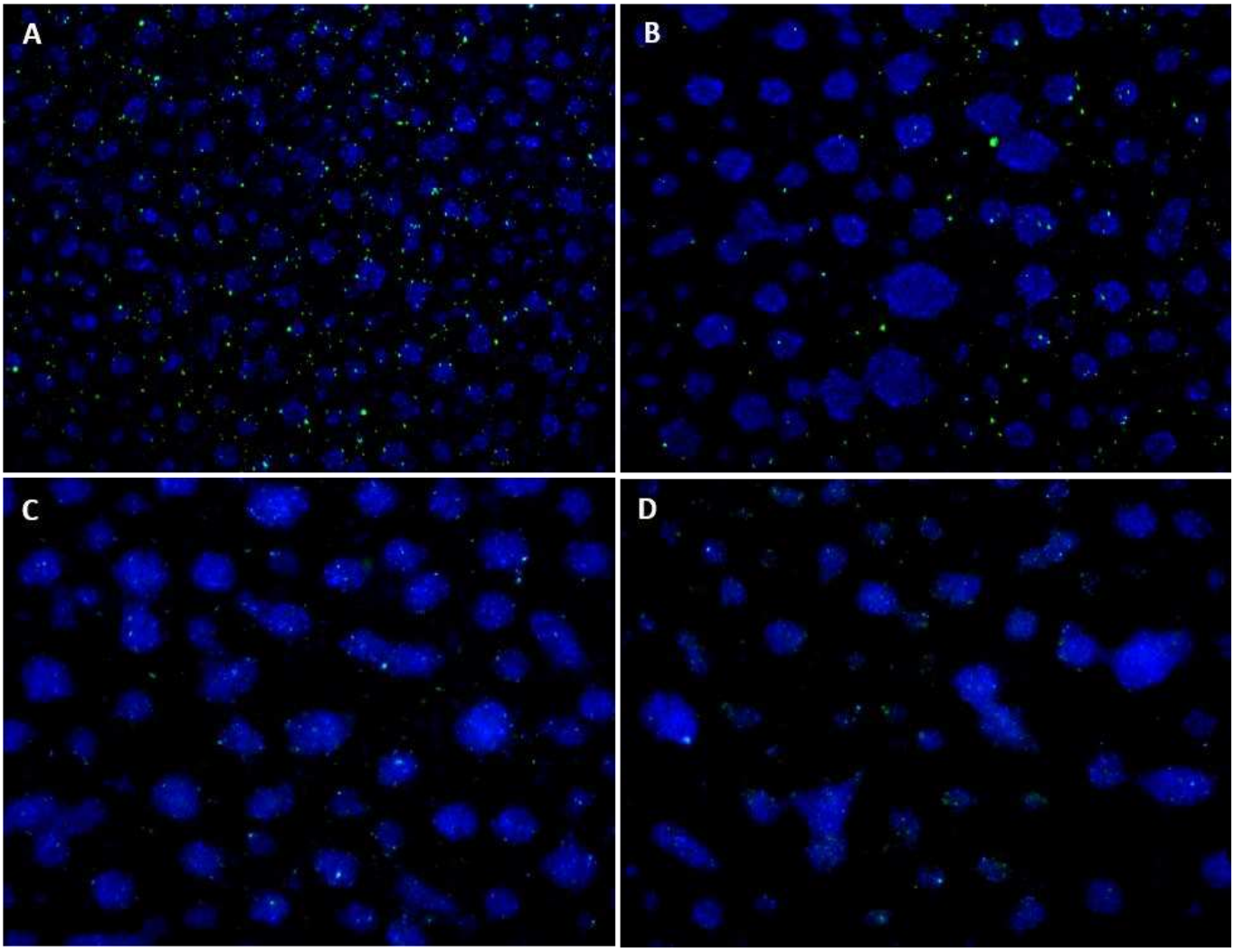

Figure 2. Cell culture monitoring by fluorescence. *Viability staining NucBlue Live reagent stains the nuclei of all the cells and NucGreen Dead stains only the nuclei of cells with compromised plasma membrane integrity. Growth and viability monitoring at 5 days (A), 13 days (B), 21 days (C) and 30 days (D) after seeding. 4X magnification.*

At day 30 3D matrix was recovered from VITVO and processed for histological evaluation using two different embedding methods. Paraffin and methyl methacrylate sections were hematoxylin and eosin stained and visualized with EVOS FL auto microscope. Different sizes of neurospheres well engrafted in 3D matrix thickness have been observed (Figure 3 A-D). The largest one showed a diameter >400 µm with a half of its structure anchored to the matrix fibers (Figure 3B).

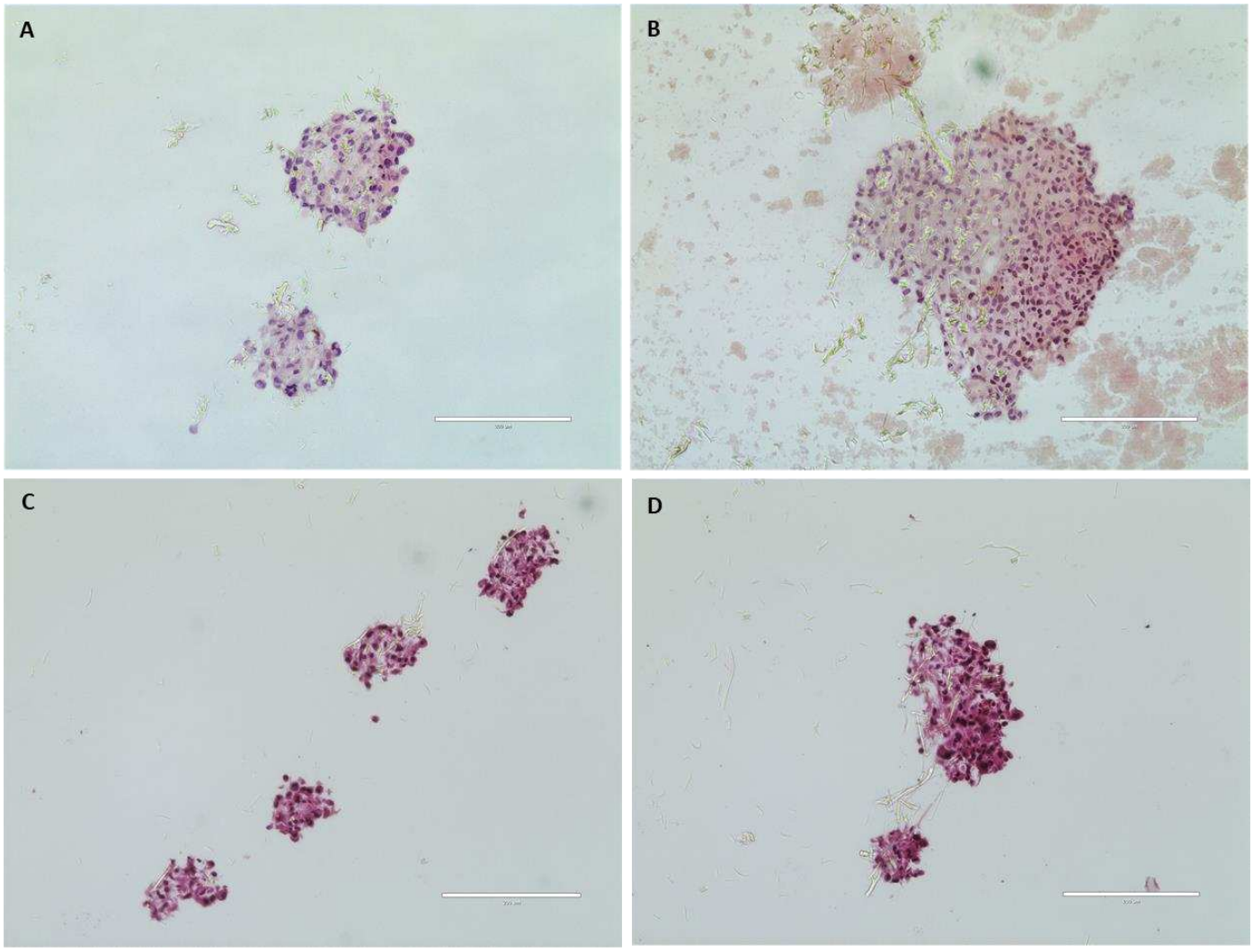

Figure 3. Neurospheres' Hematoxylin and Eosin staining. *Methyl methacrylate (A-B) and paraffin (C-D) sections, 20X magnification, scale bar 200 µm.*

CONCLUSIONS

These results suggest that VITVO matrix offers a reliable support for long term culture also for large spheroidal structures; in fact, as shown by hematoxylin and eosin staining, neurospheres have a good morphology and did not show necrotic core (Figure 3B).

In conclusion the shape of the VITVO inner core may allow an improved and sustained cells aggregation with the further advantage to keep in place cell aggregates for their further histological characterization.

REFERENCES

1. Touat M, Idbaih A, Sanson M, Ligon KL. Glioblastoma targeted therapy: updated approaches from recent biological insights. Ann Oncol. 2017;28(7):1457–1472. doi:10.1093/annonc/mdx106
2. Golinelli, G., Grisendi, G., Prapa, M. et al. Targeting GD2-positive glioblastoma by chimeric antigen receptor empowered mesenchymal progenitors. Cancer Gene Ther (2018) doi:10.1038/s41417-018-0062-x
3. Candini O, Grisendi G, Foppiani EM, et al. A Novel 3D In Vitro Platform for Pre-Clinical Investigations in Drug Testing, Gene Therapy, and Immuno-oncology. Sci Rep. 2019;9(1):7154. Published 2019 May 9. doi:10.1038/s41598-019-43613-9

RIGENERAND Srl
Via Maestri del Lavoro, 4
41036 Medolla (MO) ITALY
Tel. +39 0535 187.61.72
www.rigenerand.it

For asking a quotation or finding the distributors of our products, visit our website at: www.rigenerand.it/vitvo