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Scientific Report:

Anti-Mitochondria Immunohistochemistry On ParaffinEmbedded Human Glioblastoma Neurospheres Cultured In VITVO

VITVO 3D Bioreactor

2019

Anti-Mitochondria Immunohistochemistry On Paraffin-Embedded Human Glioblastoma Neurospheres Cultured In VITVO®

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INTRODUCTION

Glioblastoma is the most frequent and aggressive primary malignant brain tumor in adults. Despite the increasing knowledge about this tumor, to date no targeted therapy has been formally validated as effective in clinical trials [1].

VITVO® is a small and portable bioreactor developed to recreate a 3D tissue-like structure in a closed system easy to use and suitable for several applications [2]. To test an immunohistochemistry approach primary glioblastoma cells (GBM) were loaded in VITVO and cultured for one month in serum-free medium generating neurospheres. After 30 days 3D matrix with neurospheres was removed from the device, formalin-fixed and paraffin embedded for immunohistochemistry (IHC) staining using anti-human mitochondria antibody (Figure 1).

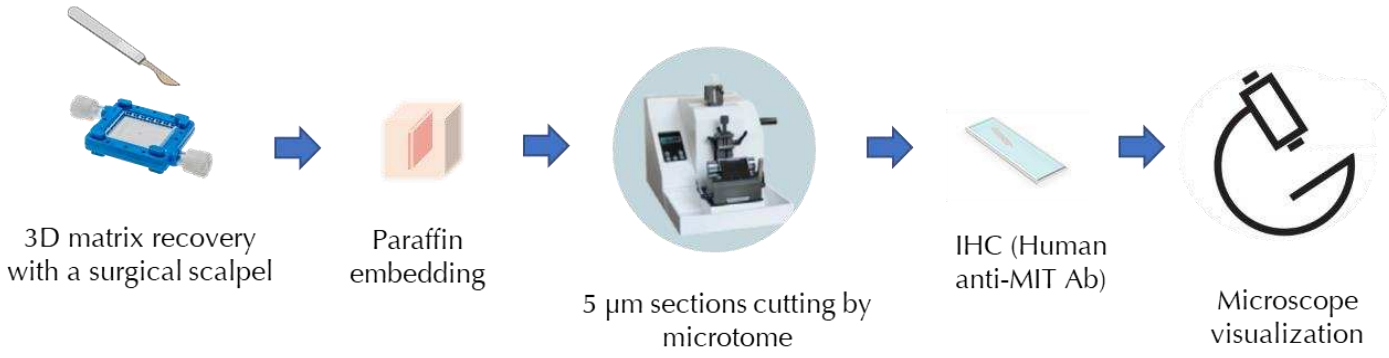

Figure 1. The approach. *Workflow of VITVO processing for immunohistochemistry readout.*

MATERIALS

- VITVO® bioreactor (Rigenerand srl)
- Human primary glioblastoma cells (GBM)
- 2,5 mL Syringe (Becton Dickinson and Co)
- Formalin solution, neutral buffered 10% (Sigma-Aldrich)
- Ethanol 99% (Sigma-Aldrich)
- Dulbecco's Phosphate Buffer Saline w/o Calcium w/o Magnesium (PBS) 10X (EuroClone)
- Histoclear (Sigma-Aldrich)
- Paraffin
- Superfrost Plus™ Adhesion Microscope Slides (Thermo Fisher Scientific)
- "Humidity chamber"
- PAP Pen (Vector Laboratories)
- Proteinase K kit (Vector Laboratories)
- Blocking solution: 60% Maleic acid (Sigma-Aldrich), 20% Blocking Buffer (Roche) 10%, 20% New Born Calf Serum (EuroClone)
- Antibody dilution buffer: 90% PBS 1X + 0,1% BSA (Sigma-Aldrich), 10% Normal Goat Serum (Vector Laboratories)
- Anti- Human Mitochondria Antibody MAB 1273 (Merck)
- Anti-mouse biotinylated secondary antibody (Vector Laboratories)
- Quenching solution: 250 mL PBS 1X, 50 mg Azide Sodium (Merck), 825 µL Hydrogen Peroxide (Sigma-Aldrich)
- RTU vectastain Avidin-Biotin Complex kit (Vector Laboratories)
- DAB substrate kit (Vector Laboratories)
- Mayer's Hematoxylin solution
- Bluing solution: 3.85 g Lithium Carbonate (Sigma-Aldrich) in 250 mL distilled water
- 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 $5{,}6*10^5$ GBM 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, was changed every 48 hours, then every 24 hours. The 3D cell culture was real time monitored using EVOS FL auto microscope with Olympus UPlanSApo 4X 0.16 objective. After 30 days of culture neurospheres were fixed for immunohistochemistry staining.

3D matrix fixation and embedding: Neutral buffered 10% formalin solution was directly injected inside VITVO using a syringe and incubated for 20 minutes at room temperature (RT). Then, 3D matrix was recovered cutting the oxygenation membrane with a surgical scalpel and placed in a falcon tube for a washing with PBS. Over night incubation in 70% ethanol solution was performed. 3D matrix was dehydrated incubating in ethanol solutions from 80% to 100%, 15 minutes each solution, then in HistoClear and in liquid paraffin for 1 hour at +70°C. Then the matrix was placed vertically to the mould and liquid paraffin was added. Sample was RT dried before freezing at -20°C. 5 µm thick sections were cut using microtome, collected on Superfrost Plu Adhesion Microscope Slides and incubated overnight at +37°C.

Immunohistochemistry protocol:

- Histoclear solution three incubation of 10 minutes
- 100% ethanol twice for 5 minutes
- 95% ethanol twice for 5 minutes
- 70% ethanol 3 minutes
- 50% ethanol 1 minute
- three washes with PBS 1X
- proteinase K solution 1X 5 minutes at RT
- two washes with PBS 1X 5 minutes each
- mark slides perimeter using PAP pen
- incubation with blocking solution (100 µL/slide) inside the “humidity chamber”, 45 minutes at RT
- two washes with PBS 1X 5 minutes each
- anti human-mitochondria primary antibody (1:200) 100 µL/slide inside the “humidity chamber” at +4°C overnight
- three washes with PBS 1X 5 minutes each
- anti-mouse biotinylated secondary antibody (1:200) 100 µL/slide inside the “humidity chamber” for one hour at RT

- three washes with PBS 1X 5 minutes each
- quenching solution for 30 minutes at RT
- three washes with PBS 1X 5 minutes each
- incubation with 1 drop of ABC substrate for 1 hour at RT
- three washes with PBS 1X 5 minutes each
- DAB substrate solution 100 µL/slide 2-3 minutes)
- distilled water for 5 minutes
- Mayer's Hematoxylin solution for 3 minutes
- two washes with distilled water
- incubation in the bluing solution 20 seconds
- one wash with distilled water 30 seconds
- dehydration incubating in ethanol solutions from 50% to 100%, one minute/solution
- Histoclear solution for one minute
- DPX mountant for histology and coverslides

RESULTS AND DISCUSSION

Glioblastoma cells have grown inside VITVO forming neurospheres, well engrafted in the 3D matrix. 30 days after seeding cells were formalin-fixed and paraffin-embedded, immunohistochemistry staining was performed using an anti-human mitochondria antibody. Negative control sample (isotype) was stained with anti-mouse secondary antibody only (Figure 2B).

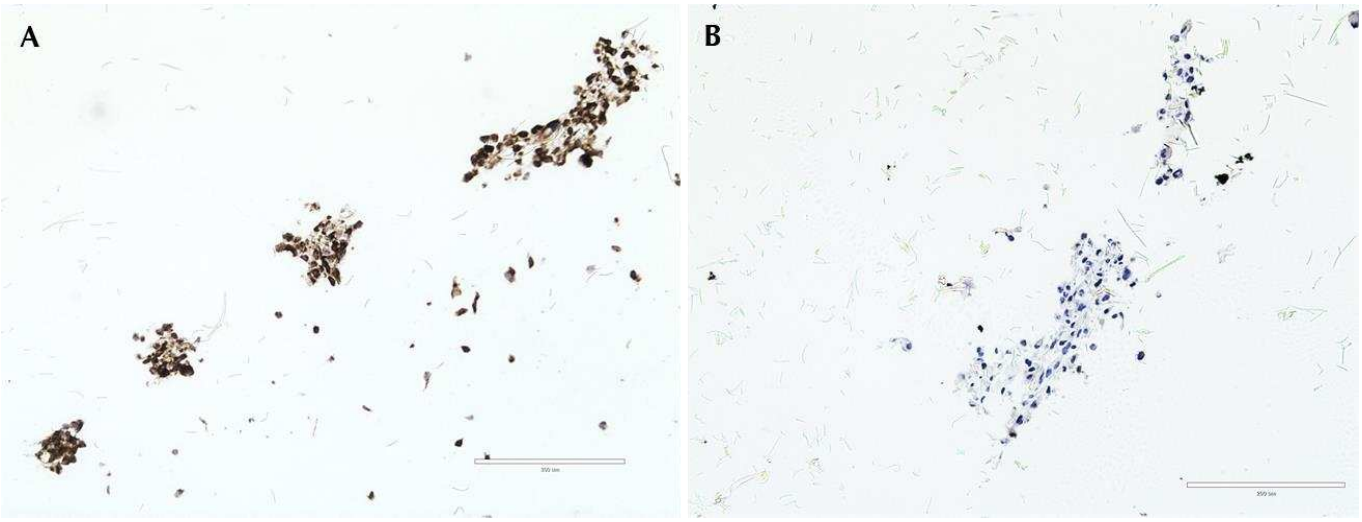

Figure 2. Anti-MIT IHC staining. *Paraffin sections of GBM neurospheres in VITVO matrix showing brown signal in cells' cytoplasm (A) and negative control (B). 20X magnification, scale bar = 200 µm.*

IHC staining was successfully performed: the presence of brown staining in the cells' cytoplasm (Figure 2A), absent in the negative control (Figure 2B), indicates that the signal detected is specific for mitochondrial epitope so the antibody was neither impeded nor linked by the matrix fibers.

CONCLUSIONS

These data show that VITVO bioreactor is suitable for immunohistochemistry downstream application. Here spheroidal structures grown in VITVO for one month were formalin fixed directly inside the device, then the matrix was recovered, paraffin-embedded and processed to demonstrate the feasibility of IHC approach. Features of VITVO allow to easily and safely perform the first steps of the IHC procedure. VITVO matrix can be processed like a tissue sample following the usual protocol without any risk of degradation.

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. 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

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