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Scientific Report:

Freezing 3D Cell Cultures In VITVO

VITVO 3D Bioreactor

Freezing 3D Cell Cultures In VITVO®

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INTRODUCTION

VITVO® is a small 3D bioreactor composed by an inner matrix 400 µm thick which can host several types of cell cultures aiming to recreate *in vitro* an *in vivo* - like microenvironment. This tool can be useful for drug testing, cell-based therapy, immuno-oncology, co-cultures studies, personalized medicine approaches and many others [1]. Previous studies have demonstrated that the cryopreservation of 3D construct differs from the cryopreservation of a single-cell suspension in terms of cells' response and viability. Notably Kang et al., analyzing the different responses to low temperatures of fibroblasts between the monolayer culture and the 3D culture, observed that the stress proteins expression in the 3D structure was more intense than those in single-cell suspension [2]. The scaffold biomaterials around the cells influence the cryopreservation effects, the close contact of cells with the surrounding materials is a determinant factor affecting the physical and chemical properties of cryopreservation [3]. These results suggest that the cryopreservation of cells in 3D structures could have advantages respect to the classical single-cell suspension method.

Here the cryopreservation of different types of 3D cell cultures was performed to validate the approach directly in the VITVO bioreactor (Figure 1). Adipose Tissue derived Mesenchymal Stromal Cells stably expressing Green Fluorescent Protein (AD-MSC GFP), pancreatic cancer cell line BxPC3 expressing GFP and Ewing's sarcoma cell line A673 expressing dsRED fluorescent protein, were cultured in VITVO for 3 days. Cell viability was first evaluated using bioluminescence assay then 10% DMSO freezing medium was added directly inside the devices before freezing them at -80°C and then in liquid nitrogen. After 6 days the devices were thawed at +37°C inside the water bath and fresh culture medium was injected to replace the freezing medium. Cell viability was monitored and compared to those obtained before the cryopreservation.

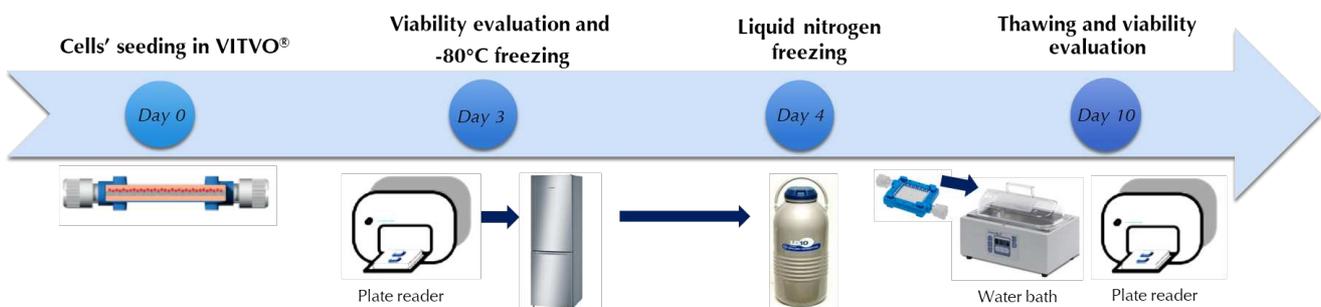


Figure 1. The approach. Experimental workflow for freezing 3D cell cultures in VITVO.

MATERIALS

- VITVO® bioreactor (Rigenerand srl)
- 2,5 mL Syringe (Becton Dickinson and Co)
- Human Adipose Tissue derived Mesenchymal Stromal Cells stably expressing Green Fluorescent Protein (AD-MSC GFP)
- BxPC3 GFP cell line
- A673 dsRED cell line
- α -MEM culture medium (Gibco) supplemented with 1% glutamine (200 mM), 0,5% Ciprofloxacin (2 mg/mL), 0,2% heparin (25.000 UL/5mL), 2,4% human platelet lysate
- IMDM culture medium (Gibco) supplemented with 10% Fetal Bovine Serum (Gibco), 1% Glutamine, 1% Penicillin-Streptomycine
- RPMI culture medium (Gibco) supplemented with 10% Fetal Bovine Serum (Gibco), 1% Glutamine, 1% Penicillin-Streptomycine
- ReadyProbes™ Cell Viability Imaging kit, Blue/Green (Invitrogen)
- Real-Time Glo™ MT Cell Viability Assay (Promega)
- CRYO.ON (DMSO) (Alchimia srl)

METHODS

3D cell culture establishment: VITVO bioreactor was first primed with culture medium alone to ensure the complete wetting of the 3D matrix, then 200.000 AD-MSC and 560.000 BXPC3 GFP and A673 dsRED cells were resuspended in 1,4 ml of α -MEM, RPMI and IMDM culture medium respectively and seeded in VITVO. For each type of cells 2 bioreactors were loaded. Cells cultures were maintained inside the incubator at +37°C and 5%CO₂ and culture medium was changed every 24 hours. At day 3 cell viability was monitored using the Real-Time Glo MT Cell Viability Assay and measuring the relative light units (RLU) using the luminometer (Glomax). Then pictures were taken with EVOS FL auto microscope with Olympus UPlanSApo 4X 0.16 objective.

Freezing VITVO: Freezing medium was prepared with the appropriate culture medium for each cell type: 50% Fetal Bovine Serum, 40% culture medium, 10% CRYO.ON (DMSO). Using a syringe 1,4 mL of freezing medium was injected inside the devices and they were rapidly frozen at -80°C for 24 hours. Then VITVO devices were positioned inside a cryobox and frozen in a liquid nitrogen container for 6 days. Also longer period (up to 2 months) in liquid nitrogen were tested successfully (not shown).

Thawing VITVO: devices were incubated in the water bath at +37°C for some minutes to thaw the liquid inside, then 2 mL of culture medium was injected for the total replacement of the freezing medium. VITVO devices were put into the incubator for 4-5 hours to let cells to recover.

Cell viability and morphology evaluation: Real-Time Glo MT Cell Viability Assay reagent was added to check viability. Cell cultures morphology was monitored using EVOS Fl auto microscope. Moreover, 1 drop/mL of ReadyProbes Cell Viability Imaging kit (Blue/Green) was added to A673 dsRED cultures: NucBlue live reagent stains nuclei of all cells and was detected with DAPI filter, NucGreen Dead reagent stains only the nuclei of dead cells and is detected with GFP filter.

RESULTS AND DISCUSSION

Three different cell cultures have been established in VITVO 3D bioreactor. Cell viability was evaluated using a bioluminescence assay (Real Time Glo) before and after cryopreservation. The Relative Light Units (RLU) values indicate that the cryopreservation did not affect cell viability: the values obtained after thawing are comparable or even higher than those obtained before freezing (Figure 2). Thanks to VITVO's peculiarities it is possible to directly inject inside the device the freezing medium in order to cryopreserve the 3D cultures in liquid nitrogen without any damage neither to the device structure nor to the cell cultures.

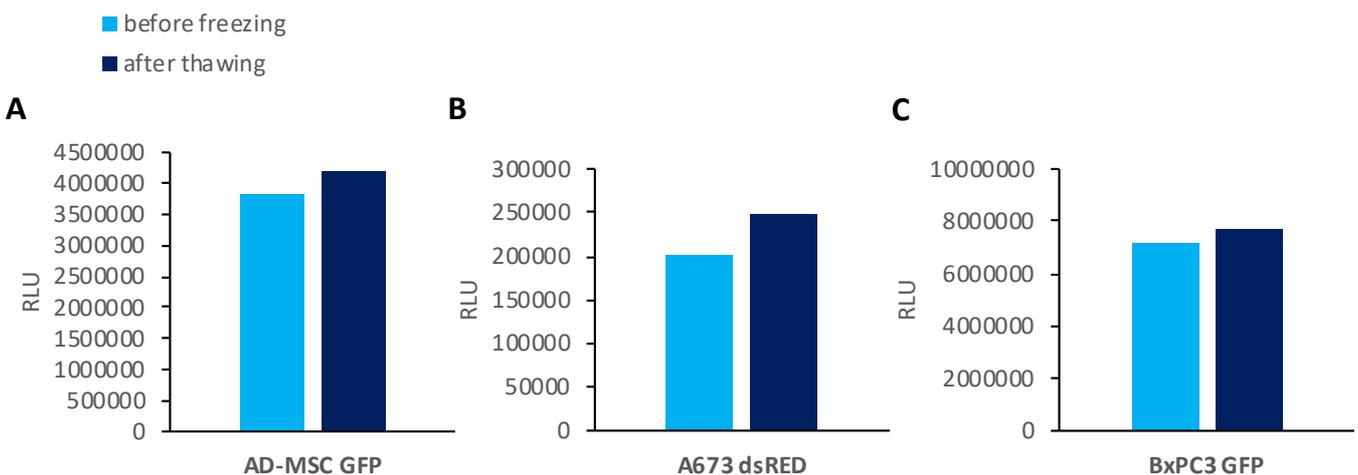


Figure 2. Relative Light Unit measurements. RLU values before freezing (light blue) and after thawing (dark blue) measured for AD-MSC GFP (A), A673 dsRED (B) and BxPC3 GFP (C).

Pictures were taken at different time points, before and after the cryopreservation, with EVOS FL auto microscope to monitor cells morphology and growth (Figure 3). ReadyProbes Cell Viability Imaging kit, Blue/Green was used only for A673 dsRED since the AD-MSC and the BxPC3 cell line were GFP positive. This supravital staining allows a real time monitoring of cell viability: the nuclei of all cells were stained in blue and the nuclei of dead cells were stained in green.

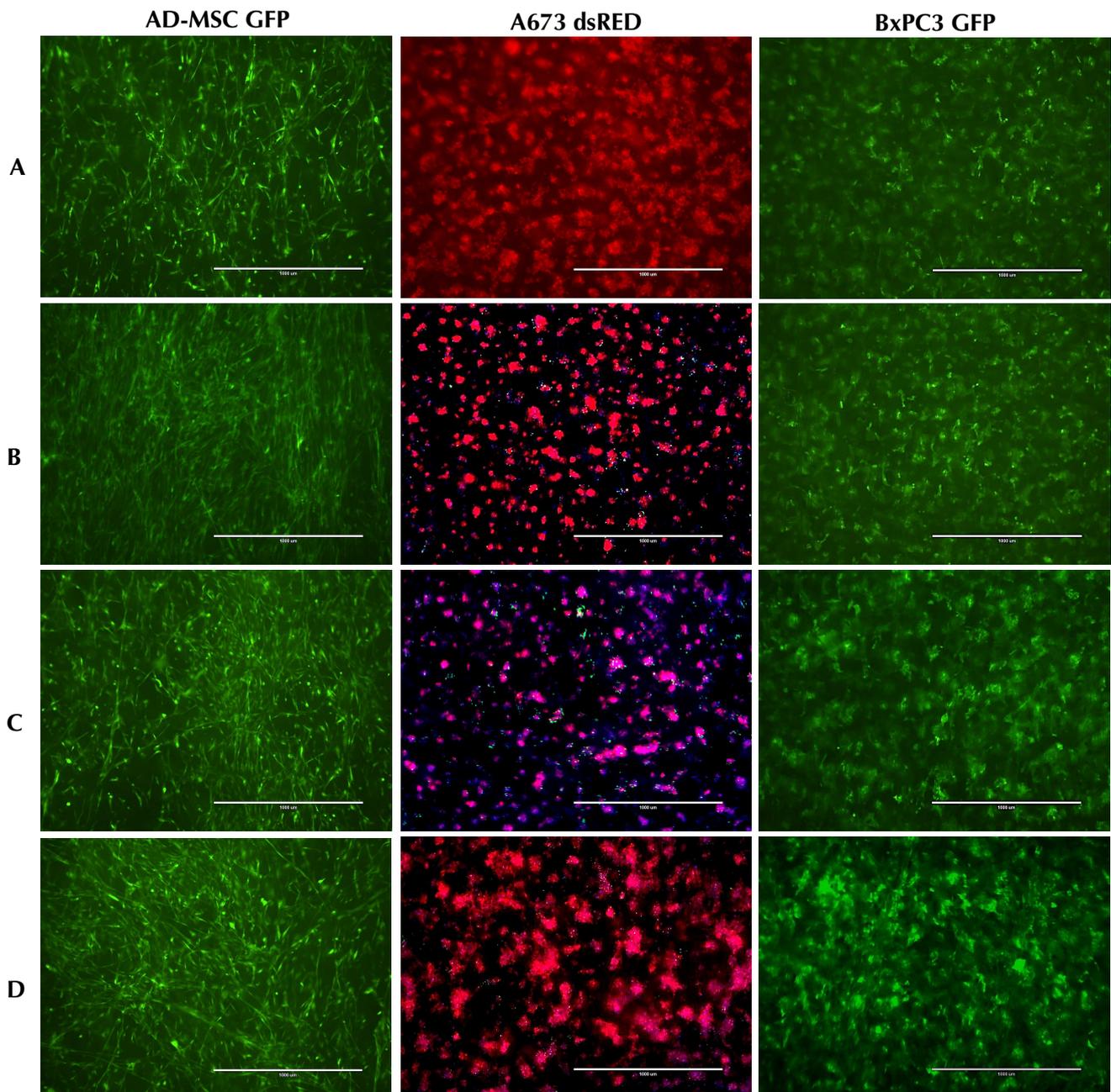


Figure 3. Microscope monitoring before and after cryopreservation. (A)

AD-MSC, A673, BxPC3 VITVO 3D matrix colonization before freezing. (B) Cells morphology after 4-5 hours from thawing. In A673 dsRED stained with ReadyProbes Cell Viability Imaging kit, Blue/Green. (C) Cells morphology after 24 hours from thawing. (D) Cells morphology after 48 hours from thawing.

Scale bar = 1 mm, 4X magnification

Before cryopreservation cells well colonized the VITVO 3D matrix (Figure 2A). After thawing all cell types showed their typical morphology (Figure 2B); in A673 dsRED culture, the ReadyProbes Cell Viability Imaging kit, allowed to confirm a good cell viability after thawing as only few green dead cells were detected (Figure 2B, middle column). At 24 and 48 hours from thawing cells were viable, retaining their morphology and showing a growth recovery. These data suggest the feasibility of the cryopreservation approach of in VITVO 3D cultures. This approach could be useful to better preserve 3D cultures in terms of their structure and functionalities. The presence of the scaffold keeping cells in place, together with the possibility to avoid trypsinization and centrifugation needed for classical single cells cryopreservation, may considerably reduce cellular stress. In VITVO cryopreservation of well-established 3D cell culture may be exploited for biobanking, inter-laboratories shipping needs or other research purposes.

CONCLUSIONS

VITVO 3D bioreactor allows an easy cryopreservation of the 3D cell culture with a good recovery after thawing. The device can support the freezing procedure without any structural damage even for longer time offering a useful tool for biobanking, sample shipment and other research purposes.

REFERENCES

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