Live Imaging of Anti-Cancer Therapy Effect in VITVO®

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Introduction

Cell-based therapies offer a relevant clinical option for the treatment of cancers which cannot be fought by conventional drugs [1]. Pre-clinical evaluation of cell-based drug approaches requires the development of biological relevant models able to mimic the clinical setting in which cell growth can influence molecular biomarkers and antigenic profiles. Three dimensional (3D) platforms have the potential to provide more physiologic environments for cell culture in drug discovery and toxicology than classical 2D culture systems and to mimic an in vivo-like context enabling testing in a human avatar environment.

VITVO® is an in vitro innovative pre-clinical testing tool, which is presented under the form of a ready-to-use device. This device is composed by a fiber-based matrix for 3D cell colonization, to mimic tissue complexity [2]. VITVO can simultaneously host target and effector cells, allowing both to evaluate the antitumor action and to identify the effective dose. In this study gene-modified adipose derived mesenchymal stromal/stem cells expressing the anticancer molecule TRAIL (AD-MSC TRAIL) [3] have been challenged for their capacity to induce apoptosis in Ewing's sarcoma (A673 tumor cell line).

In order to directly monitor the effect of AD-MSCs TRAIL on A673 Ewing's sarcoma cells at single cell level, we performed time-lapse experiment with CrestOptics X-Light V3 confocal spinning disk, starting from 24 hours (h) post-treatment and monitoring till 96 h post-treatment, following 3 different fields inside the same VITVO environment. Moreover, end-point imaging was performed on fixed samples by using CrestOptics X-Light V3 confocal spinning disk as confirmation.

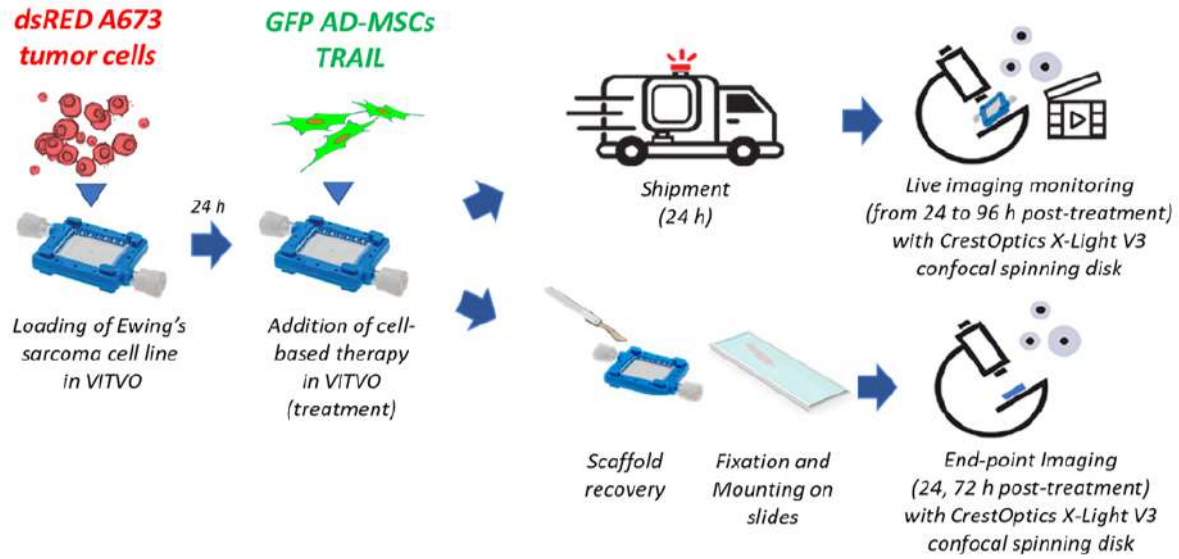


Figure 1. Experimental workflow.

Materials and methods

Cell culture: Ewing sarcoma A673 tumor cell line was purchased from ATCC (LGC Standards srl, Milan, Italy) and genetically modified by viral infection to express dsRED fluorescent protein: Retroviral vector MSCV-dsRED were transiently transfected into 293T cells together with a mixture of helper plasmid according to the jetPEI protocol (Cell Signaling Technology Inc., Danvers, MA, USA). After 48 h, conditioned media containing retroviral particles were collected to stably transduce FLYRD18 packaging cell line for the continuous production of viral particles. FLYRD18-conditioned media were used to perform 3 hits of infection on tumor cell lines (6 h of incubation/day).

A673 were cultivated in Iscove (Euroclone, Padmington, UK), supplemented with 10% fetal bovine serum (FBS, PAA, Pasching, Austria), 1% glutamine (200 mM), and 1% penicillin-streptomycin (104 UI/mL and 10 mg/mL; both from Euroclone).
AD-MSC TRAIL were obtained as previously described [3].
For the co-culture setting, Iscove supplemented with 10% FBS, 1% glutamine and 1% penicillin-streptomycin was used.

VITVO loading: VITVO bioreactors (Rigenerand srl, Medolla, MO, Italy) were first primed with 1.4 ml of media alone by using a 2.5 ml syringe (Becton Dickinson and Co, Franklin Lakes, NJ, USA) to ensure a complete wetting of 3D matrix. Next, the A673 target cells (500.000/VITVO) were resuspended in 1 ml of culture medium and seeded in VITVO, and after 24 h the effector AD-MSC TRAIL (50.000/VITVO) was added in VITVO following the loading procedure already used for A673 cell line.
Co-culture of effector : target (1:10) was maintained in culture till 96 h.

Sample fixation: Co-coltures were stopped at 24 h and 72 h post-treatment by injection in VITVO of cold PFA 4% fixing solution (Merck, KGaA Darmstadt, Germany) and incubated for 15 minutes (min) at room temperature (RT). After a PBS washing, 3D matrix was collected by cutting the edges of the transparent membrane and the 3D matrix with a surgical scalpel. The matrices were mounted on slides (seeding side upward) with a drop of mounting solution and a coverslip.

Microscope monitoring and 3D imaging: Time lapse acquisitions have been performed with a Nikon Ti2 Eclipsed equipped with CrestOptics X-Light V3 confocal spinning disk, Okolab incubator at 37°C (H301-K-FRAME, kindly provided by Okolab), Celesta laser source (lambda used 470 and 545; Lumencor) and Prime 95B camera (11 um pixel size, 1608x1608 pixels, Photometrics). For 72 h long-term live imaging, Nikon 4x Plan Apo objective (air, 0.2 NA) has been used with a Z step of 10 um for a total Z volume of 500 um. Three different areas in the VITVO device have been monitored with acquisition rate of 30 min for the first 6 h, then every hour for the rest of the video. Imaging of living cells at the end of the video has been performed with a 4x Plan Apo (air, 0.2 NA) with a Z step of 10 um for a total Z volume of 450 um.

Acquisitions of fixed samples, collected at specific time points (24 h and 72 h post treatment), have been performed with CrestOptics X-Light V3 confocal spinning disk by using a 10x Plan Apo (air, 0.45 NA) objective with a Z step of 2 um for a total Z volume of 100-150 um.

Results and discussion

In order to real-time monitor the capability of AD-MSCs TRAIL to induce the apoptotic effect on Ewing's sarcoma A673 cells over time, the co-culture was maintained into the Okolab incubator and continuously imaged till 96 h post-treatment.

In the Figure 2, the full field of view (FOV) in XYZ dimensions collected 24 h post-treatment is shown. Thanks to the 25 mm diagonal FOV of the camera and to the ability to penetrate inside the sample without losing the spinning disk confocality, it was possible to visualize and record cells that homogeneously colonize the entire thickness of the 3D matrix.

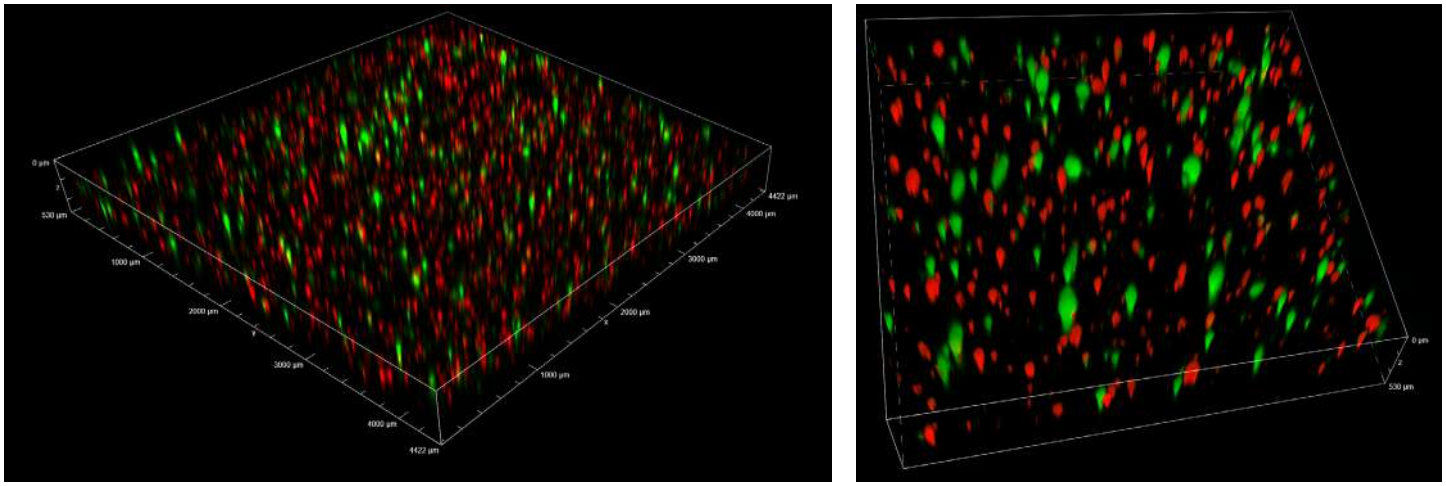


Figure 2. 3D view of the entire FOV acquired with spinning disk at 24 h post-treatment. FOV with XY=4422x4422 µm, Z=530 µm. 3D matrix thickness was entirely acquired. Tumor target A673 cells are shown in red and AD-MSCs TRAIL in green.

Three VITVO areas were monitored along the Z axis and over time in order to record movies. A representative entire movie of one of three areas enables to appreciate the intense activity of AD-MSCs TRAIL (green) moving towards A673 tumour cells (red), which showed a dramatic change in their cell morphology (Figure 3).

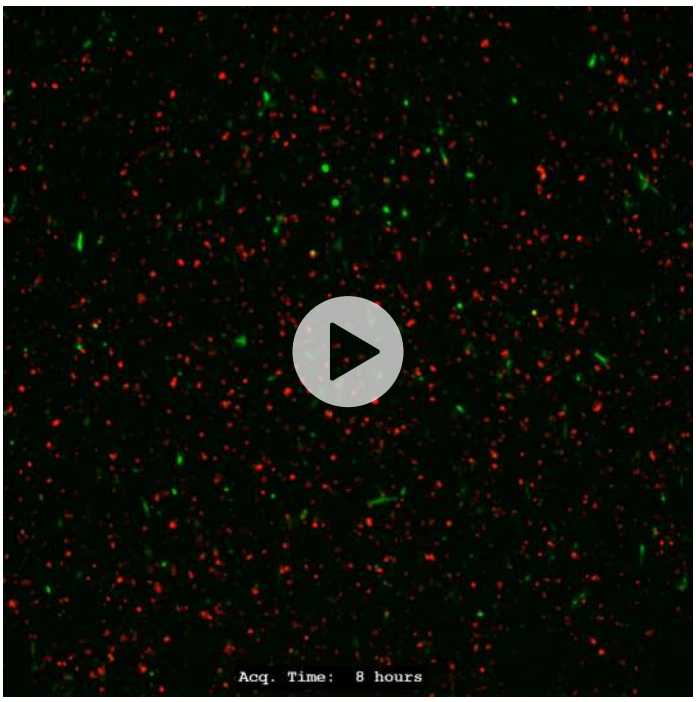
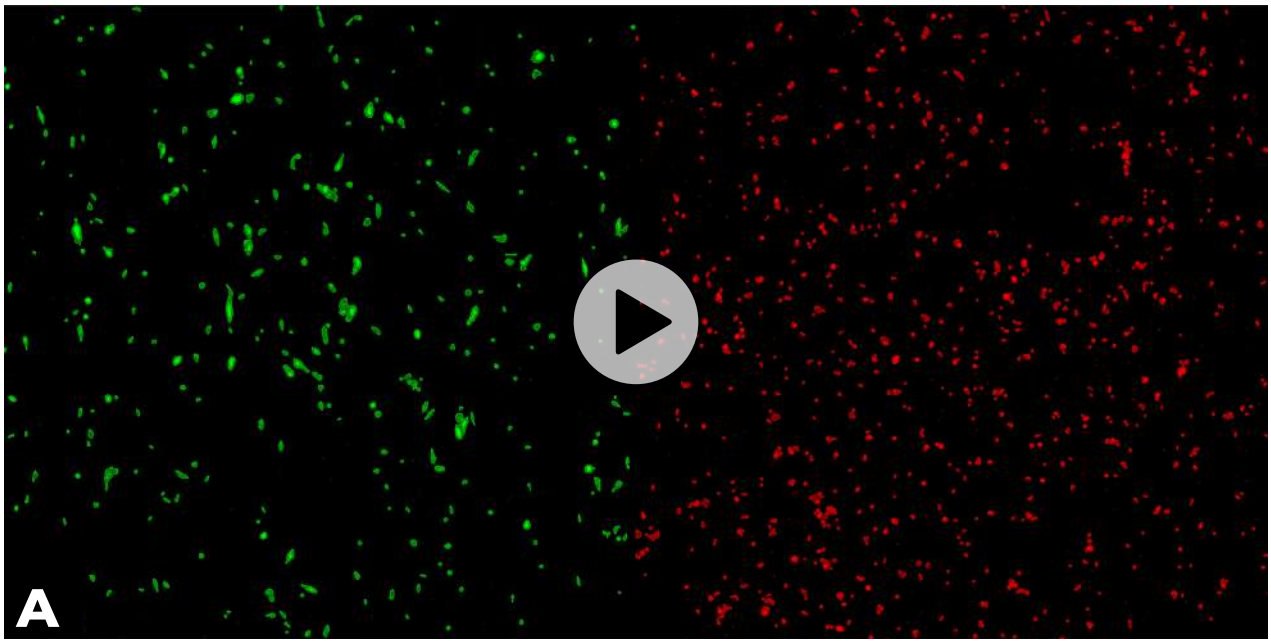


Figure 3. Representative movie recorded in one of the three VITVO areas with spinning disk. The deconvolved Z-maximum intensity projection of each time frame is shown.

On the Z-sum intensity projection images created along the movie, the area covered by both AD-MSCs TRAIL (green fluorescence) and A673 tumor cells (red fluorescence) were quantified by using binary images through the identification of regions of interest (ROIs) around fluorescent cells (NIKON NIS Elements software tool). A representative movie is shown in Figure 4A. In order to evaluate the killing effect induced by AD-MSCs TRAIL to A673 tumour cells, the total area covered by cells was measured and quantified over time (Figure 4B). As shown in the graph curve, red covered area decreases firstly to 50% after 48 h post-treatment, furtherly reducing to 8% at 72 h post-treatment and then to 2% at 96 h post-treatment. The observed trend suggests that the pro-apoptotic process was effectively activated being entirely monitored in the considered time frame. On the contrary, AD-MSCs TRAIL maintain a good area coverage over time. The slight decrease to 35% at 96 h post-treatment may be explained by the capability of cells to migrate towards still-viable tumor cells, considering also their intense activity observed in Figure 3 movie.



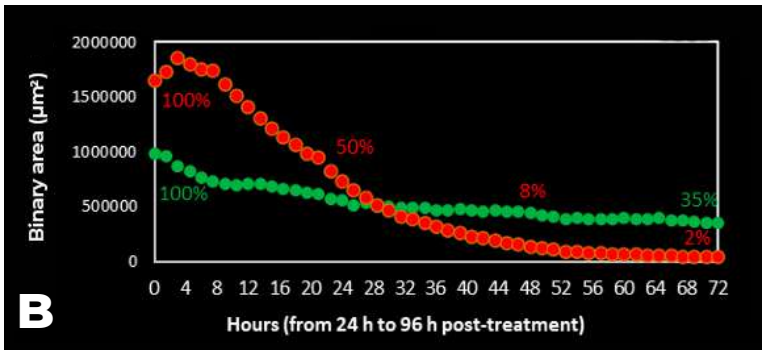


Figure 4. Montage and binary area measurements along the movie. A representative video of the two binary areas (green and red) (A). The total areas covered by cells from the first (24 h post-treatment) up to the last time frame (96 h post-treatment) were measured and quantified by using the following calculation:

(% = (binary area (μm^2)$_{each\ time\ point}$*100)/ binary area (μm^2)$_{starting\ point}$).

To further confirm both the migration capability of AD-MSCs TRAIL and their anti-tumor activity, we focused on a small area to create 3D movies collecting all dynamic events. AD-MSCs TRAIL actively migrated inside VITVO 3D matrix by surrounding A673 tumor cells, which dramatically changed their morphology till to form apoptotic bodies before death (Figure 5A). Cell tracks and measurements are showed in Figure 5B. The polar graph indicates that AD-MSCs TRAIL (GFP) were about three times faster than A673 tumor cells (dsRED) (speed <0.015 um/s and <0.0050 um/s, respectively).

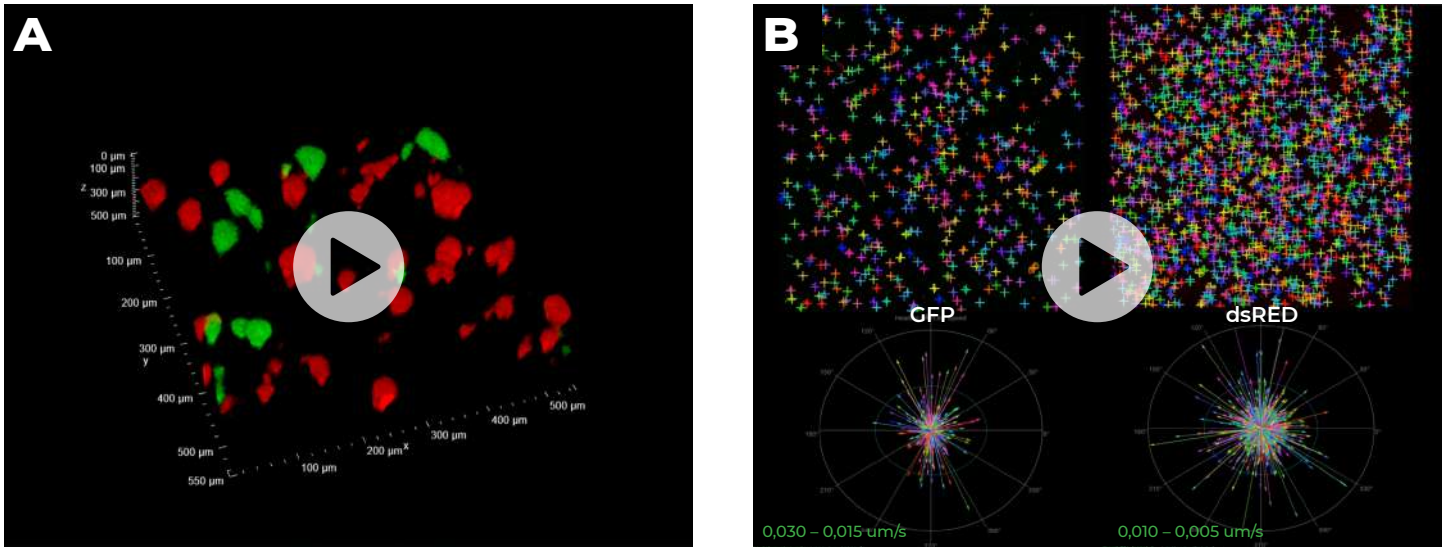


Figure 5. A) 3D movie in a zoomed area of interest acquired with spinning disk. B) Cell tracking movie and polar graph measurements.

To confirm that the apoptotic effects observed were not due to laser exposure during the video-recording, we compared the laser exposed area (FOV (i)) with a not-exposed area inside VITVO (FOV (ii)). Z-stack acquisitions performed by using the same settings used during the time lapse, allow to confirm that both area coverage and cell density were comparable between FOV (i) and (ii), as shown in the 3D render visualization (Figure 6).

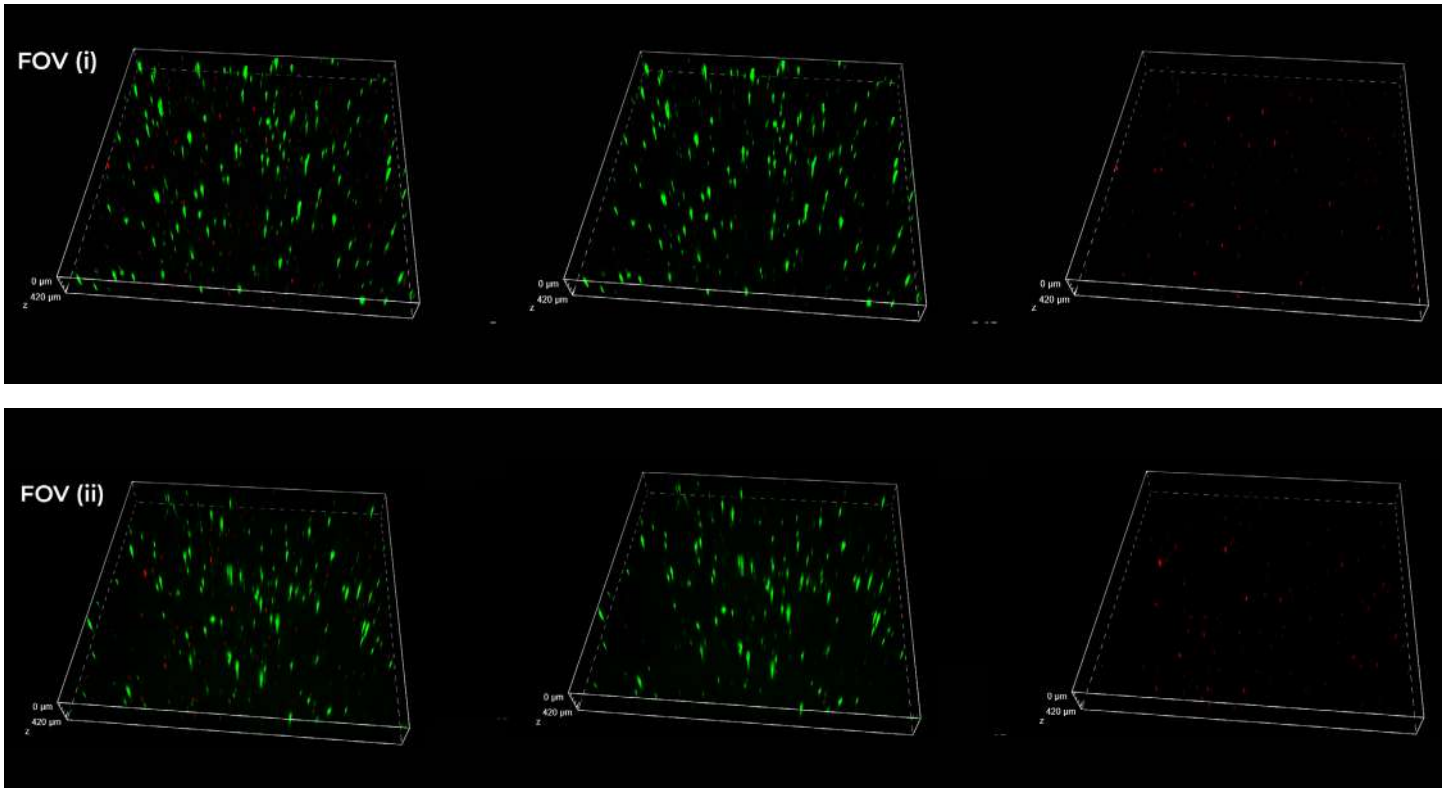


Figure 6. 3D views collected in VITVO at 96 h post-treatment by spinning disk acquisitions. FOV (i) of a VITVO exposed area during time lapse movie and FOV (ii) of a not-exposed area (4x objective magnification).

Moreover, to exclude bleaching side effect, the signal intensity of red fluorescent cells was quantified. Specifically, 3D objects detected by fluorescence intensity thresholding showed a comparable distribution between the two different FOVs, confirming that the bleaching did not affect the intensity levels (Figure 7).

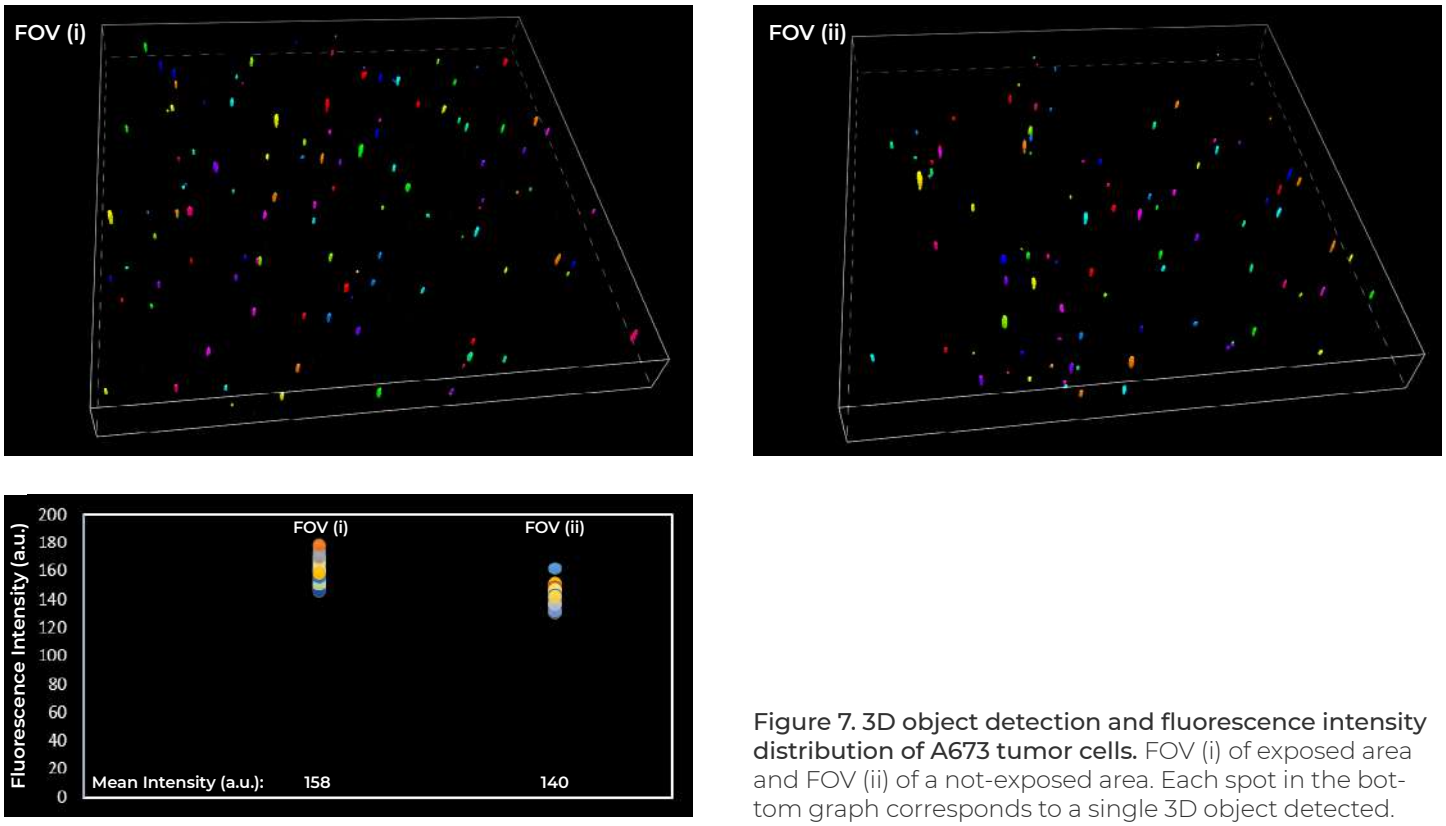


Figure 7. 3D object detection and fluorescence intensity distribution of A673 tumor cells. FOV (i) of exposed area and FOV (ii) of a not-exposed area. Each spot in the bottom graph corresponds to a single 3D object detected.

Data obtained from live monitoring of co-cultures in VITVO were compared with end-point readouts from fixed samples at specific time points post-treatment (24 and 72 h, Figure 8). 3D comparison among three different FOVs confirmed a homogeneous distribution of cells without any significant inter-field difference. These data point out that AD-MSCs TRAIL were persisted over time, instead of A673 tumor cells which massively decreased in their signal and cell density (Figure 8).

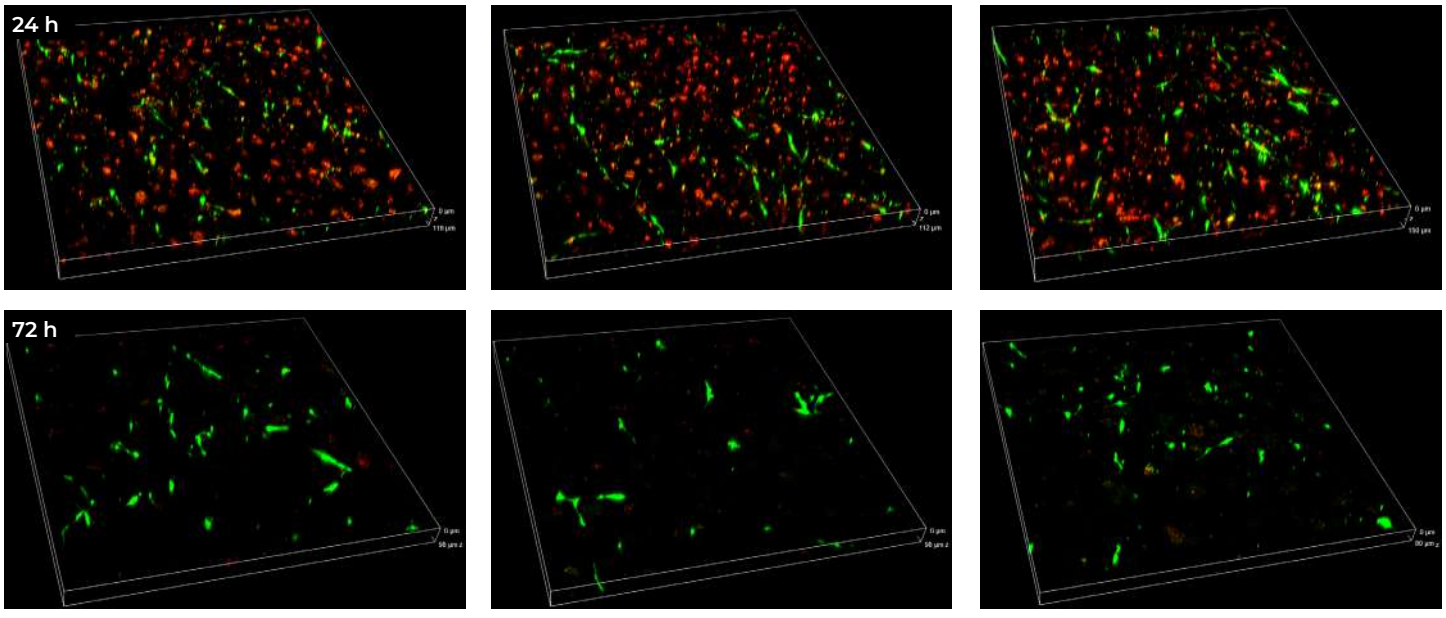


Figure 8. Z-stacks on VITVO FOVs collected and fixed at different time points with spinning disk. 3 different FOVs corresponding to 24 h and 72 h post-treatment fixed sample, respectively (10x objective magnification).

Conclusions

This study shows VITVO like a suitable tool for easily, directly and continuously monitoring a co-culture over days, in order to deeply inspect the effect of a cell-based therapy (AD-MSCs TRAIL) on tumor target (A673 Ewing's sarcoma cells). Thanks to CrestOptics's spinning disk imaging technology, a great deal of data was collected both at full FOVs and at single cell level over time at high resolution. Culture changes in a 3D environment, such as apoptotic effect and cell migration capability, can be easily measured and quantified.

The possibility to monitor living cell culture over time offers the advantage of modifying and adjusting conditions during the entire duration of the experiment, also to select appropriate time-points for endpoint studies. Moreover, multiple data collection from the same VITVO eliminates both the need to use one device for each end-point readout and the variability among replicates.

By using this approach of live imaging spinning disk, drug responses, interactions among cells and tissues can be monitored and analyzed to provide a more robust and complete picture of dynamic biological processes through greater spatial and temporal understanding, compared to fixed endpoint studies. In VITVO imaging of antitumor actions can offer novel intriguing opportunities in drug discovery and development by improving predictivity and accelerating pre-clinical process timelines.

References

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