T Cell Invasions Assay Using a 3D Spheroid Model

Zhisong Tong[1], Angeline Lim[1], Misha Bashkurov and Oksana Sirenko[1]
Molecular Devices[1]

Introduction

High-content, imaging-based T-Cell invasions assay

T-cell therapies are designed to help our immune system eliminate cancer cells. Those include CAR T-cells (Chimeric Antigen Receptor engineered T-cells), tumor infiltrating lymphocytes (TIL), and other genetically modified T-cells. In recent years, the field of cell therapy has started to expand, including the launch of the first CAR T-cell therapies to treat blood cancer in 2017, which was a critical milestone in this field. Despite its boom, the discovery of novel immunotherapies that specifically enhance T-cell response against cancer cells remains a challenging task limited by the absence of robust in vitro models to evaluate these immunotherapies throughout their development.

In the past, these models have been limited to the use of suspension cells and 2D cell monolayers. The use of CAR T-cells on solid tumors has been lagging due to challenges that include tumor heterogeneity, immunosuppressive microenvironments, and the lack of unique tumor antigens that can be recognized by the CAR-T cells. As such, the ability to screen for CAR T-cells (e.g. with CRISPR) that effectively target and kill tumors is an area of active research.

Here we describe a method for assessment of T-cell effect and penetration into the multi-cellular 3D tumor spheroids as a proof-of-concept model for in vitro CAR T assays. Spheroids were formed from Hela cell lines in 96-well round bottom plates. We activated the human peripheral blood mononuclear (PBMC) cells with PMA/ionomycin for 6 hours, then treated the spheroids with stimulated PBMC for 72 hours. Samples were imaged with a confocal imager every 2 hours for a 72 hour period. High-content imaging and analysis allowed us to observe and measure phenotypic changes in cancer spheroids and the process of T-cell penetration over a period of 72 hours. To optimize the workflow, we developed an image analysis approach that uses a deep learning-based segmentation model and a machine learning-based classification model to quantify the T-cell induced phenotypic changes in the spheroids using brightfield images. Moreover, the information associated with the penetration of T-cell into the 3D spheroids was explored by calculating the coordinates of the penetrated T-cells and T-cell movements from the nearest edge of the spheroids. Overall, our results show that the 3D spheroid models and the high-content analysis workflow may potentially be used as a metric to evaluate the efficacy of cell therapy in vitro.

Methods

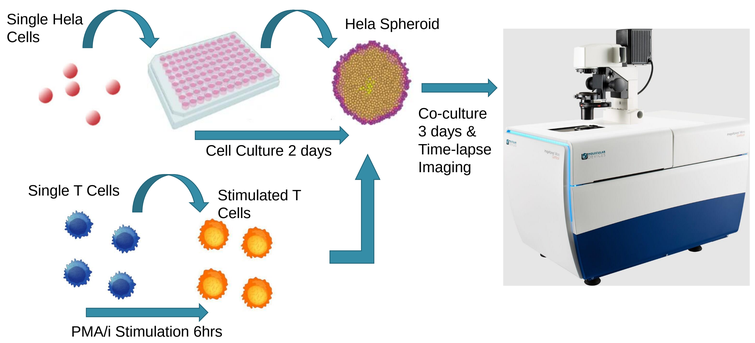


Figure 1 ImageXpress Micro Confocal High-Content Imaging System and Workflow.

- Hela cells were stained with MitoTracker Red before seeding in 96-well round-bottom plate to form spheroids for 2 day. After 2 days, the thawed PBMC/T cells were stimulated in PMA/i for 6 hours and stained with CellTracker Green before adding to the spheroids for co-culture. We also added unstimulated T cells, staurosporine and no treatment groups as controls. The time-lapse live imaging were then performed on spheroids and T cells every two hours.
- We used the ImageXpress Micro Confocal High-Content Imaging System equipped with spinning disk confocal and sCMOS camera to capture the 3D structures of the whole spheroids.

Analysis

Image segmentation analysis using DL-based SINAP

To improve the workflow for the T-cell based assay, we developed a custom analysis pipeline to assess phenotypic changes in spheroids using only images acquired in brightfield using SINAP (Figure 2A). The 2D projection images in the TL channel (Best Focus Plane) were used to train and generate a SINAP model to mask the spheroid region. We created separate segmentation masks for spheroids and the edges because we observed that untreated spheroids form smoother boundaries compared to the treated spheroids (Figure 2B).

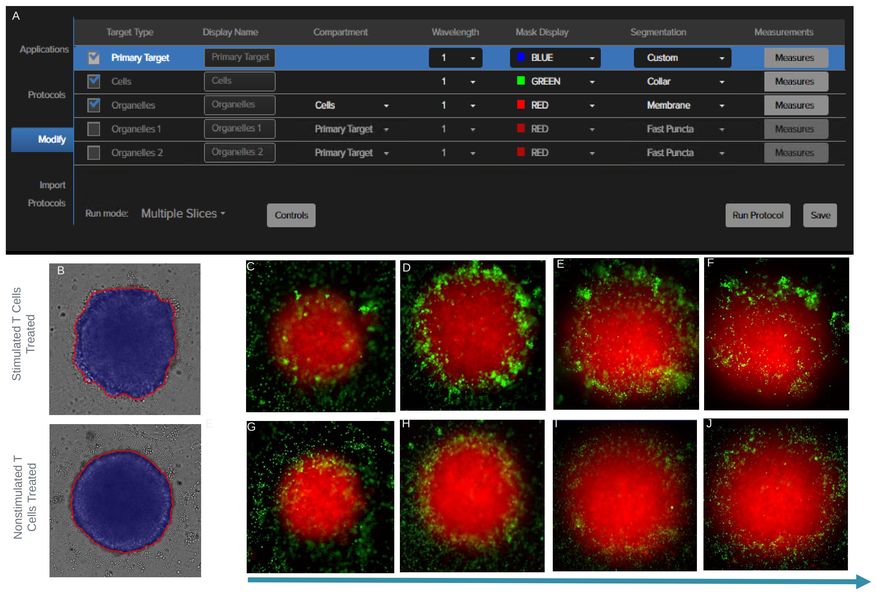
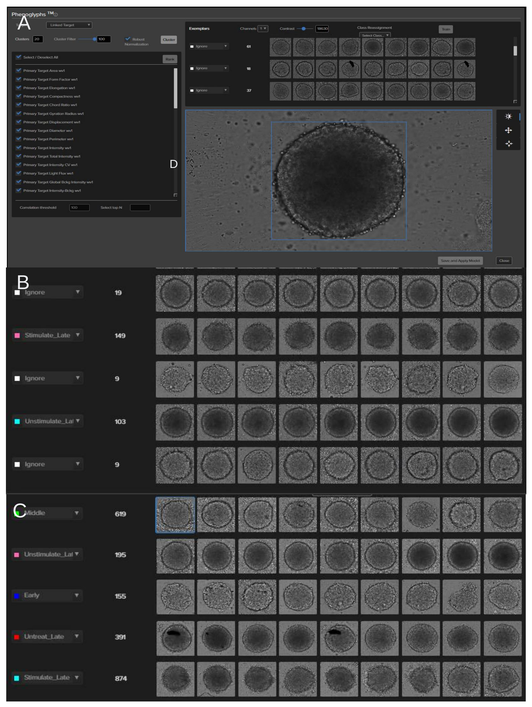


Figure 2 (A) Primary target, cells and organelles models were used in IN Carta to generate the masks for spheroids and the edge of the spheroids; (B) The mask of the edge (red) and the spheroid (blue) treated with stimulated T-cells and nonstimulated T-cells at late stage; The fluorescent image blending of mitotracker (spheroid, red) and celltracker (T-cells, green) treated with stimulated T-cell (C) at 0hr; (D) at 18hr; (E) at 48hr; (F) at 68hr; treated with unstimulated T-cells (G) at 0hr; (H) at 18hr; (I) at 48hr; (J) at 68hr.

Image classification analysis using ML-based phenoglyphs

Measurements extracted from the resulting masks were then used in IN Carta® Phenoglyphs™ Software Module (Figure 3A) to generate a model for spheroid classification. In Phenoglyphs, we started with a clustering tool to label the appropriate classes (step 1) before training a classifier model (step 2). Figure 3B shows that two sets of images after clustering were labeled with *stimulate T-cells late stage* and *unstimulate T-cells late stage*. After the labeling was done, we trained the model and examined the results (Figure 3C)



Step1: Label the measurements to generate training sets using clustering tool. We then labeled the training sets into 5 categories, including *early stage*, *middle stage*, *stimulated T-cells late stage*, *unstimulated T-cells late stage* and *no T-cells late stage* with reassignment of the images if necessary and proceeded to the next step.

Step 2: Choose top 20 measures and train the classifier model using training sets. Ranking tool sorts all measurements with significance scores after the clusters were manually labeled. We chose top 20 measurements with 95% correlation threshold to avoid overfitting of the resulting classifier model and proceeded with training of the model using a subset of measurements.

Sep3: Examine the classified images.

Figure 3 (A) Screenshot of IN Carta Phenoglyphs software module ; (B) The clustering generated from intensity related features with 50 clusters (only a subset shown); (C) The final classification generated from the trained phenoglyphs model.

3D T-cell penetration analysis using 3D CME

To further characterize the T-cell penetration into the 3D spheroids, we used 3D custom module editor (CME) to segment and detect the T-cell's location within the spheroids. Briefly, we were able to segment each T-cell and extracted information like 3D position, area and intensity from generated objects. We then segmented the whole spheroid with the mask and the distance to the edge of the mask was transformed into a 16-bit grayscale image where the intensity value indicates the 3D distance to the nearest edge of the mask. At last, the 3D penetration distance of T-cell within the spheroid was measured by aligning the T-cell mask with the16-bit grayscale image.

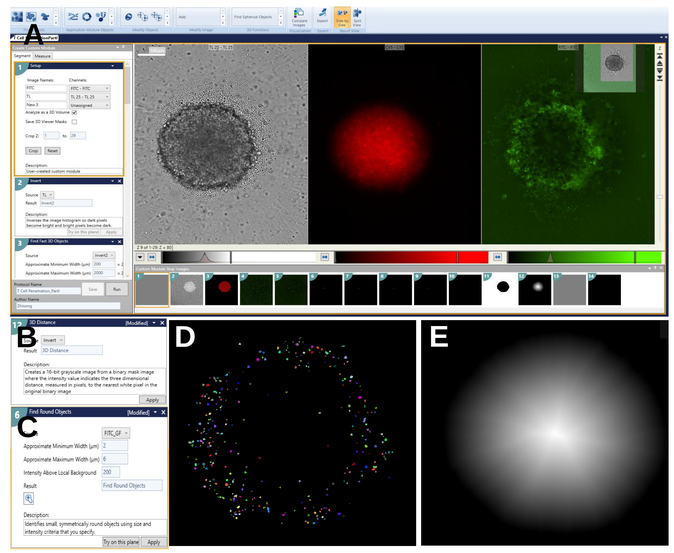


Figure 4 (A) The CME interface with transmitted light (TL) channel (left), mitotraker (middle) and T-cell**(right); (B) The *3D Distance* module; (C) *Find Round Objects* module; (D) Mask of** T-cells **generated from the image in (A); (E) 16-bit grayscale image generated from the TL image.**

The object-level and well-level (sum) T-cell z-position and penetration distance into the spheroids are outlined in Figure 5. The object-level and well-level data of activated T-cell clearly shows that the penetration distances of T-cell into the spheroids climb higher throughout the incubation time, as well as z-position, while the non-activated T-cell only penetrate into the superficial surface of spheroids.

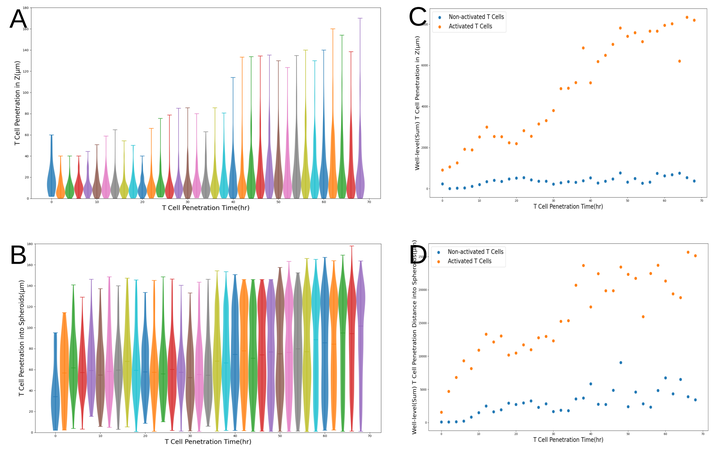


Figure 5 (A) Violin plot of object-level T-cell penetration in Z; (B) Scatter plot of well-level T-cell penetration in Z; (C) Violin plot of object-level T-cell penetration distance into spheroids; (D) Scatter plot of well-level T-cell penetration distance into spheroids.

Conclusion

- We used time-lapse, high-content imaging to monitor the growth and phenotypic changes of T-cell-treated 3D spheroids.
- We successfully generated SINAP models to apply masks to the whole spheroid and the edge of the spheroid.
- We also trained a model in Phenoglyphs to categorize the spheroids into 5 classes.
- We successfully used 3D CME modules to analyze the T-cell penetration distance into the spheroids.