B232 Monitoring of T cell invasions assay using a 3D spheroid model

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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 workflow for the generation of 3D tumor spheroids, co-cultured with T-cells as a proof-of-concept model for CAR T assays. Activated peripheral blood mononuclear cells (PBMCs) were added to spheroids and their activity monitored over time using high-content imaging. To optimize the workflow, we developed an image analysis approach that uses deep learning to accurately segment biological objects of interest and machine learning to quantify the T-cell induced phenotypic changes in the spheroids using only brightfield images.

Our results show the feasibility of using AI-based analysis workflows to predict the efficacy of T-cells. A robust deep-learning model was first trained to recognize spheroids and generate masks of the whole spheroids and their edges. Extracted measurements from the masks were then used to classify the spheroids using machine-learning approaches, where distinct phenotypic changes of the spheroids were observed compared to controls, allowing for analysis of the T-cell efficacy. To further elucidate which features dominate the classification, we quantified and compared features such as area, form factor, total intensity, and grey level non-uniformity between the different treatment groups.

Methods

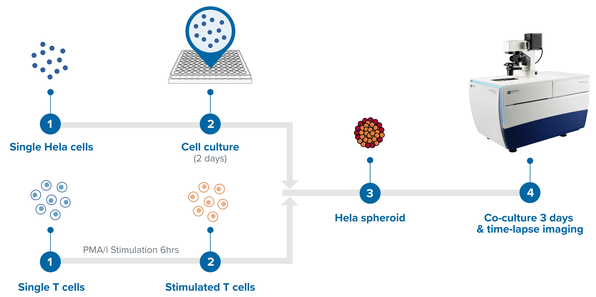


Figure 1. ImageXpress Micro Confocal High-Content Imaging System and Workflow.

Methods

- 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

Deep-learning based image segmentation using 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).

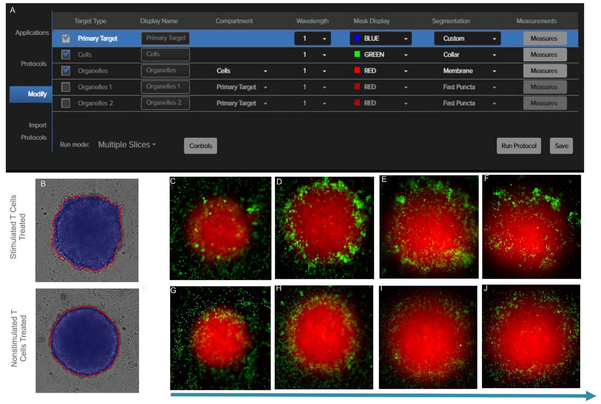


Figure 2. (A) Primary Target, Cells and Organelles models used in IN Carta to generate the masks for spheroids and the edge of the spheroids; (B) The mask of the edge (red) and the spheroid (blue) treated with stimulated T cells and nonstimulated T cells at late stage; The fluorescent image blending 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 cell (G) at 0hr; (H) at 18hr; (I) at 48hr; (J) at 68hr.

Analysis

Machine-learning based image classification using phenoglyph

Measurements extracted from the resulting masks were then used in Phenoglyphs (Figure 3A) to generate a model for spheroid classification. In Phenoglyphs, one 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).

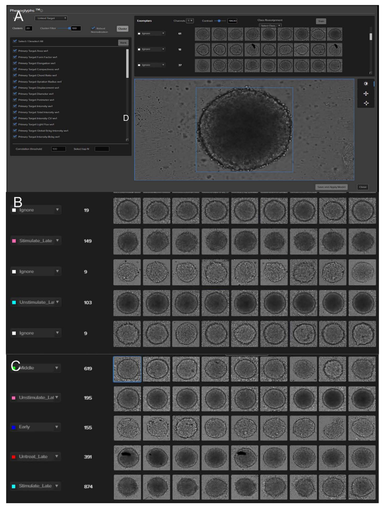


Figure 3. (A) Screenshot of IN Carta Phenoglyphs classifier; (B) The clustering generated from intensity related features with 50 clusters (only a subset shown); (C) The final classification generated from the trained phenoglyph model.

Step 1: 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.

Step 3: Examine the classified images.

Results

Cell induced phenotypic changes of Hela spheroid

Some interesting observations are worthy of discussion before we jump into our analysis results. The edges of the simulated T cells treated spheroids, forming bulge structures around the edges, are bumpier than unstimulated T cells treated spheroids (Figure 2B), which is aligned with the presence of more stimulated T-cells within the spheroids compared to unstimulated T-cells case (Figure 2C–2J).

We used our trained model to predict all the spheroids at the final time point. The model accurately predicted 63 out of 65 wells (Figure 4E), with prediction accuracy of about 97% (Figure 4). Thus, artificial intelligence (AI)-based label-free prediction of spheroid evolution stemmed from T-cell infiltration is possible with high accuracy.

Results

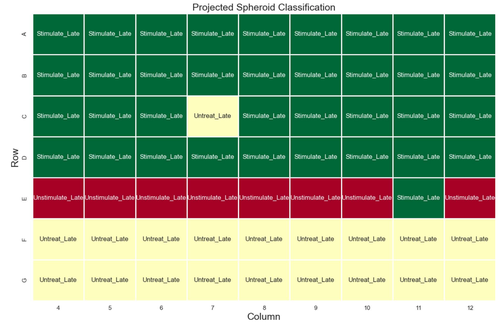
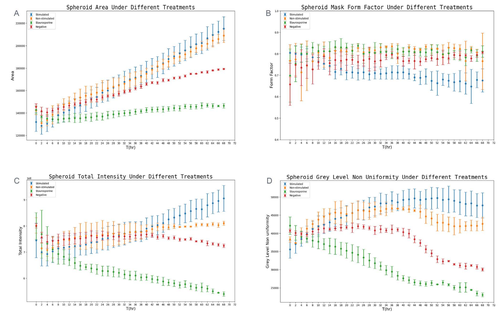


Figure 4. The projected spheroid classification from Phenoglyphs model.

Measurements of phenotype related features

To further investigate how the treatments impact the phenotypes of the spheroids over time, we plotted 4 of the key features from the top 20 features. Here we focus on the divergence of the stimulated curve (blue) from the rest of the curves. Overall, stimulated T-cell curves began to diverge as early as 22 hours for the form factor feature, at approximately 50 hours for total intensity, and at 38 hours for grey level non-uniformity features. The area feature was the least affected between stimulated and non-stimulated groups. The holistic trends align with the penetration of T-cells which started at around 18 hours.



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 classify the spheroids into 5 classes.
- Four key features are outlined over time suggesting useful information for future similar experiments.