A novel image cytometric analysis method for T cell-mediated cytotoxicity of 3D tumor spheroids

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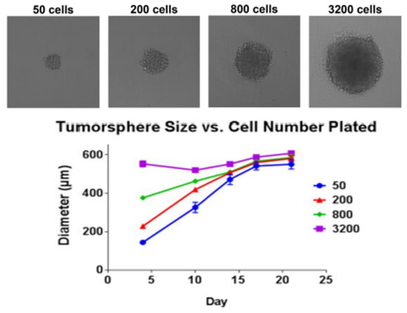
1. ABSTRACT

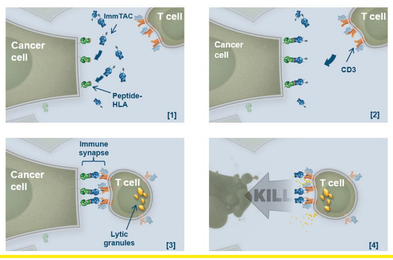
Cell-mediated cytotoxicity assays have been frequently performed to characterize cancer cytotoxic potential of immune cells, antibodies, and drug compounds. Traditionally, these assays are performed using release assays such as Cr^{51} (radioactivity), Calcein (fluorescence), or LDH (enzymatic). However, release assays have limitations such as the handling of hazardous material, the indirect measurement of cell death leading to an under estimation of cytotoxicity, the requirement for a large volume of cell sample, and the inability to visually confirm, image and track the assay kinetically. In the recent years, Celigo image cytometry has been used to perform high-throughput cell-mediated cytotoxicity assays using a direct cell counting method where cancer cells (Target) are stained with Calcein AM. Upon the addition of effector immune cells with the fluorescently stained Target cells in the presence or absence of antibody or drug compounds, the Celigo image cytometer is used to capture bright-field and fluorescent images and analyze the change in Target cell count over time to determine the cytotoxicity percentage. In general, these assays are performed in a 2D cultures. The 2D assays for cancer drug discovery have had some difficulty in identifying more qualified drug candidates for clinical testing, thus there has been an increase in interest of performing cytotoxicity assays in 3D tumor models. Traditional 3D spheroid analysis methods require the use of standard microscopy, which is time-consuming and subjective. Celigo imaging cytometer has also been used to rapidly analyze drug effects on 3D tumor spheroids. In this work, we demonstrate a novel method of analyzing T cell-mediated cytotoxicity on 3D tumor spheroids in the presence of absence of ImmTAC molecules, which redirect higher T cell killing. In this experiment, MDA-MB-453 GFP expressing breast cancer cells are used to form tumor spheroids in an ultra-low attachment plate in 2.5% matrigel. The spheroids are then treated with primary T cells at 1:10 and 1:50 E:T ratios, as well as 10, 1, 0.1, 0.01, and 0.001 nM ImmTAC. The results showed a dose response effect of T cell killing with the addition of ImmTAC molecules by measuring spheroid size in GFP fluorescence and viability using Propidium Iodide (PI). The image cytometry method was able to monitor changes in spheroid size and fluorescence over time and rapidly quantify T cell killing, which was as high as 75%. The captured bright-field and fluorescent images also clearly showed the cytotoxicity effect from the combination of T cells and ImmTAC. The ability to screen cytotoxic effects of immune cells, antibody, and drug compounds on 3D tumor spheroids can provide an alternative tumor model for identifying more qualified cancer drug candidates for drug discovery campaigns.

2. CELIGO IMAGING CYTOMETRY FOR SPHEROID T-CELL MEDIATED CYTOTOXICITY



1. Celigo Imaging Cytometer is a plate-based cytometer that can scan the entire well of standard microplates and captures bright-field and fluorescent images
2. The captured images are analyzed with the Celigo software to measure size, morphology, cell count, confluence, and fluorescent intensity
3. The measured parameters are used to generate cell proliferation kinetic data, GFP/RFP expression, tumor spheroid size change, DNA cell cycle analysis, apoptosis, and ADCC cytotoxicity results

3. WHAT IS THE IMMTAC MOLECULE?



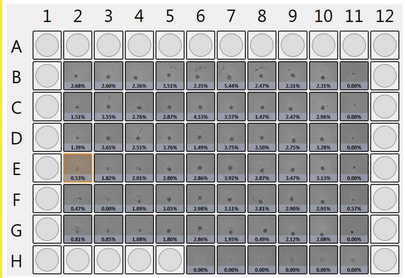
1. ImmTACs recognize and strongly bind to cancer cells expressing a peptide-HLA target
2. Circulating T cells are recruited to the tumor site by interacting with the anti-CD3 fragment free end of the ImmTAC molecule
3. The ImmTAC is a bridging molecule between the cancer cells and T cells, forming an optimized immune synapse
4. The T cells are then activated to release the cancer cell killing lytic granules, inducing cancer cytotoxicity

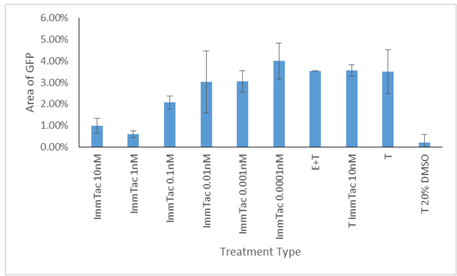
4. T-CELL MEDIATED CYTOTOXICITY ON 3D TUMOR SPHEROIDS PROTOCOL

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C	1:10	10nM ImmTac	1nM ImmTac	0.1nM ImmTac	0.01nM ImmTac	0.001nM ImmTac	0.0001nM ImmTac	ImmTac E+T	10nm ImmTac + T	T alone	20% DMSO	
D		10nM ImmTac	1nM ImmTac	0.1nM ImmTac	0.01nM ImmTac	0.001nM ImmTac	0.0001nM ImmTac	ImmTac E+T	10nm ImmTac + T	T alone	20% DMSO	
E		10nM ImmTac	1nM ImmTac	0.1nM ImmTac	0.01nM ImmTac	0.001nM ImmTac	0.0001nM ImmTac	ImmTac E+T	10nm ImmTac + T	T alone	20% DMSO	
F	1:50	10nM ImmTac	1nM ImmTac	0.1nM ImmTac	0.01nM ImmTac	0.001nM ImmTac	0.0001nM ImmTac	ImmTac E+T	10nm ImmTac + T	T alone	20% DMSO	
G		10nM ImmTac	1nM ImmTac	0.1nM ImmTac	0.01nM ImmTac	0.001nM ImmTac	0.0001nM ImmTac	ImmTac E+T	10nm ImmTac + T	T alone	20% DMSO	
H						T cells only	T cells only	T cells only	T cells only	T cells only	T cells only	

- MDA-MB-453 GFP cells are used to form 3D tumor spheroids in ultra-low attachment 96-well plates
- When the spheroids are fully formed after 4 days, T cells are added into the wells at 10:1 and 50:1 E:T ratios
- Different concentrations of ImmTAC are also added to the wells at triplicate
  - 10, 1, 0.1, 0.01, 0.001, 0.0001 nM of ImmTAC
  - Control wells
    - Tumor spheroid and T cells only, no ImmTAC
    - Tumor spheroid with only ImmTAC 10 nM, no T cells
    - T cell only
    - Tumor spheroid with 20% DMSO

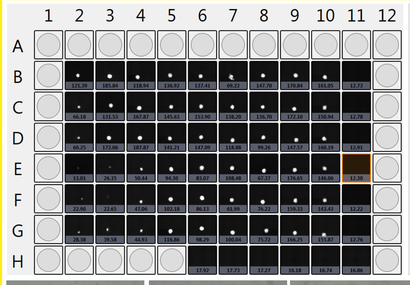
5. TUMOR SPHEROID GFP AREA-DEPENDENT CYTOTOXICITY MEASUREMENT

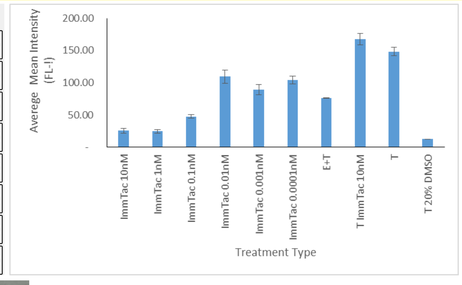


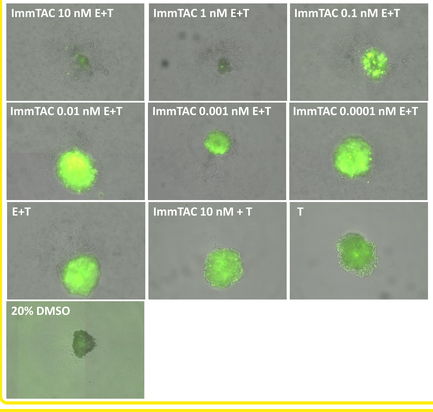


- Using the tumor spheroid fluorescence analysis, the GFP positive area was measured, which showed that as the concentration of ImmTAC increased, the area of the tumor spheroid decreased, indicating T cell killing
- In addition, the control wells showed correlated results as expected
  - Tumor spheroid and T cells only, no ImmTAC: **Showed no cytotoxicity**
  - Tumor spheroid with only ImmTAC 10 nM, no T cells: **Showed no cytotoxicity**
  - T cell only: **Showed no cytotoxicity**
  - Tumor spheroid with 20% DMSO: **Showed high cytotoxicity**

6. TUMOR SPHEROID GFP INTENSITY-DEPENDENT CYTOTOXICITY MEASUREMENT

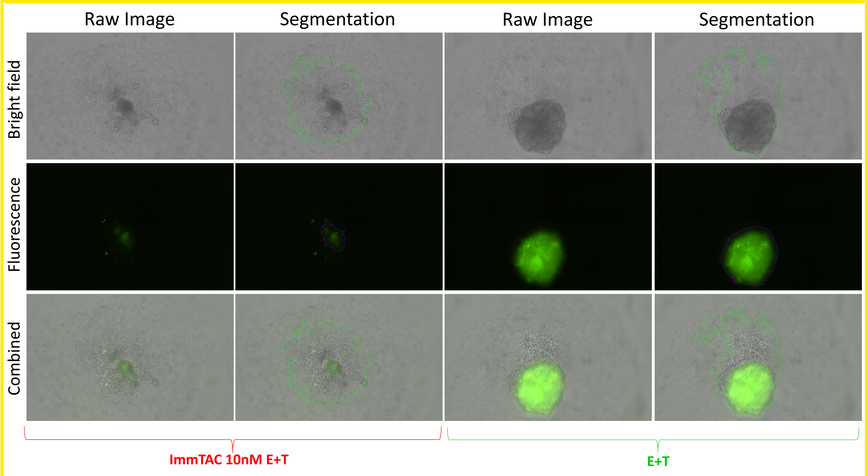




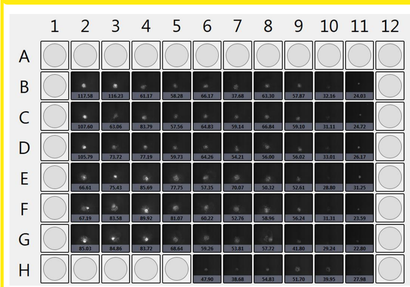


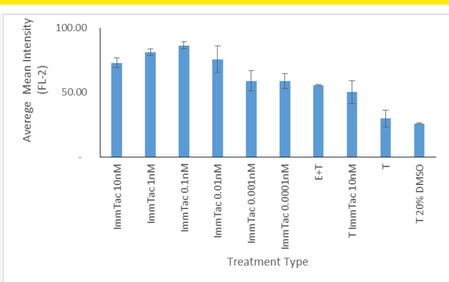
- Celigo was also used to measure the GFP fluorescent intensity within the spheroids
- The results showed that T cell mediated cytotoxicity diminished the GFP fluorescence due to cell killing
- The bright-field and fluorescent overlay images are shown on the left
- Visually, high ImmTAC concentration 10 and 1 nM showed complete disintegration of the MDA-MB -453 tumor spheroid
- As ImmTAC concentration increased, the GFP fluorescent intensity decreased
- The DMSO treatment still showed larger tumor spheroid, but the GFP fluorescence was dramatically reduced

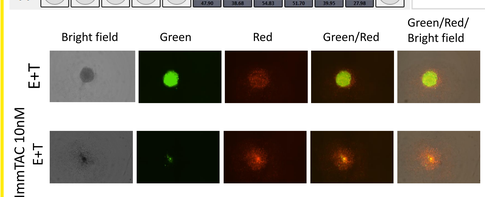
7. T-CELL MEDIATED CYTOTOXICITY ANALYSIS METHOD



8. TUMOR SPHEROID PI INTENSITY-DEPENDENT CYTOTOXICITY MEASUREMENT







- The treated tumor spheroids were also stained with propidium iodide to measure dead cells
- The control spheroid only showed a slight level PI intensity, while the treated showed high level
- The differences in PI intensity between control and treated are not very high

9. SUMMARY AND CONCLUSION

- The Celigo detected a decrease in both spheroid size and a GFP intensity and an increase in Propidium Iodide intensity upon co incubation of MDA MB 453 spheroids with ImmTAC activated T cells
- The Celigo can read a single channel spheroid assay in less that 5 minutes and a two color channel spheroid assay in less that 10 minutes
- This could allow screening of ImmTACs effects in a robust, efficient and objective manner