Real-time kinetic viability and apoptosis detection and screening of 3D multicellular tumor spheroids using the Image Cytometer

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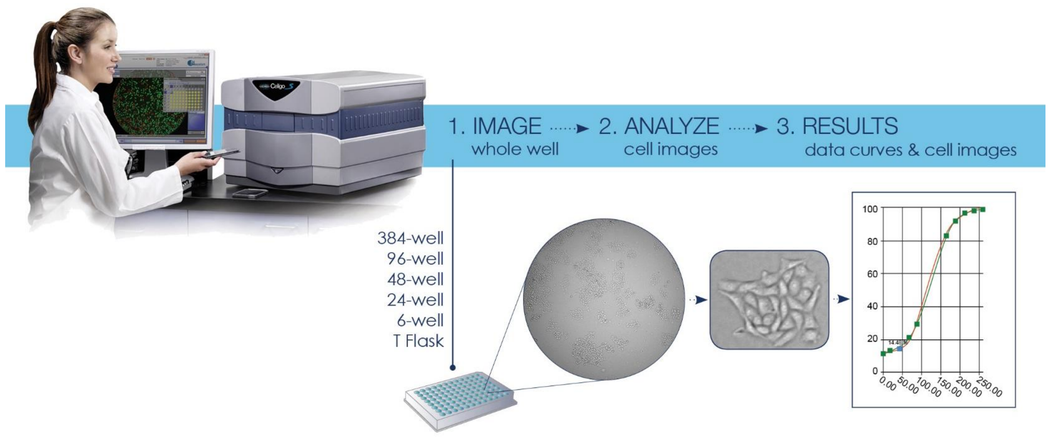
1. ABSTRACT

In the recent years, three-dimensional (3D) tumor spheroid models have been increasingly used for small molecule or antibody-based cancer therapy research. The use of 3D multicellular tumor spheroid (MCTS) models may have better representation of the complex in vivo tumor microenvironments for cancer research. Previously, we have published a novel MCTS screening method using the Celigo Image Cytometer, where we described a method for measuring of end point viability and apoptosis of MCTS in 384-well ultra-low attachment (ULA) U-bottom microplates. The spheroid size was measured kinetically over a 9-day time frame using bright field imaging, showing different growth inhibition patterns for drug-treated MCTS. It would be important to also obtain real-time viability and apoptosis MCTS results, however, there are no established methods. Herein we demonstrate the use of PI and caspase 3/7 as a method to detect viability and apoptosis in 3D MCTS. The method was initially validated by comparing the bright field kinetic growth rates of MCTS in the presence or absence of PI and caspase 3/7 stains for 16 and 21 days. Furthermore, the potential toxicity of PI was analyzed by digesting the tumor spheroids that were exposed to the reagent into single cell suspension and comparing their viabilities to untreated MCTS. By monitoring PI and caspase 3/7 fluorescent intensities in combination with spheroid size over time, the growth inhibition, viability, and apoptosis can be used to characterize MCTS in real-time. Furthermore, PI and caspase 3/7 fluorescent intensities can be correlated to the formation of a necrotic core and this can be combined with spheroid size to determine an optimal time frame for spheroid formation and cancer drug treatment. Finally, we performed a high-throughput 3D MCTS screening experiment to screen the real-time kinetic viability and apoptosis effects of 14 drug compounds (NIH/NCAT) on U87MG spheroids at different concentrations. Real-time kinetic viability and apoptosis assays are highly important for developing proper 3D cancer models, which can allow researchers to determine time-dependent drug effects that usually are not captured by end point assays. This would allow the improvement MCTS analysis method to better identify more qualified drug candidates for cancer drug discovery research.

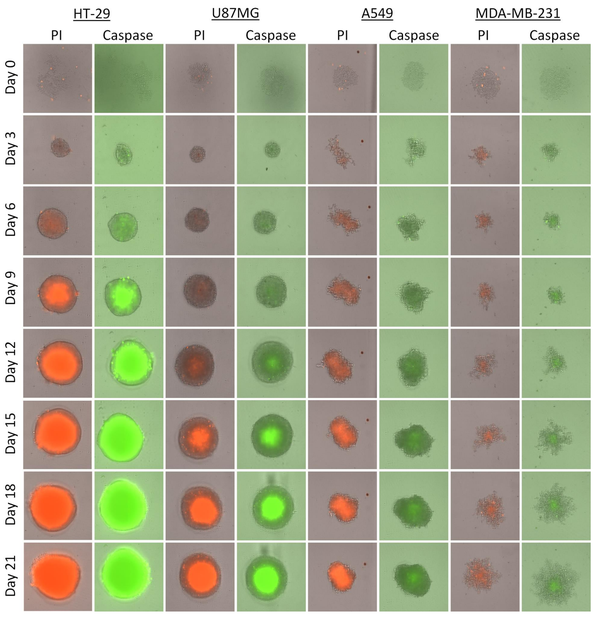
2. CELIGO IMAGING CYTOMETRY FOR 3D MCTS APOPTOSIS AND VIABILITY ANALYSIS

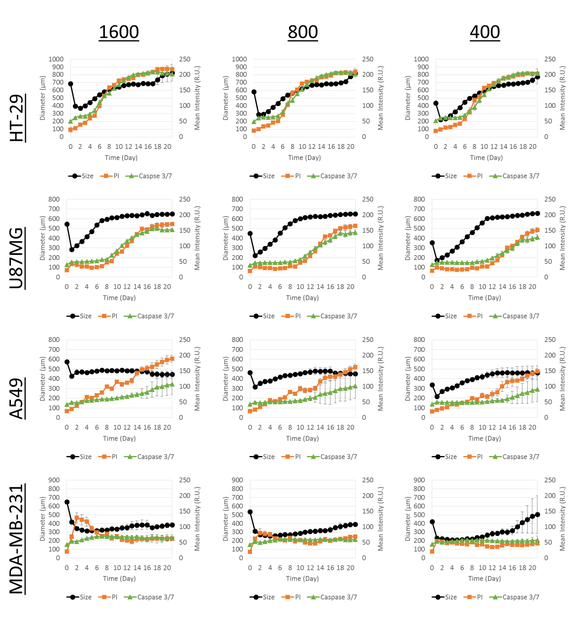


1. Celigo Imaging Cytometer is a plate-based cytometer that can scan the entire well of standard microplates and captures bright-field and fluorescent images
2. The captured images are analyzed with the Celigo software to measure size, morphology, cell count, confluence, and fluorescent intensity
3.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

3. REAL-TIME CASPASE AND PI FLUORESCENCE MONITORING FOR 21 DAYS

Stain cells with caspase 3/7 and PI separately and seed cells in 384-well ULA plates → Centrifuge the plate and allow spheroid to form over 16 days → Image and analyze spheroid size, caspase, and PI fluorescence over time

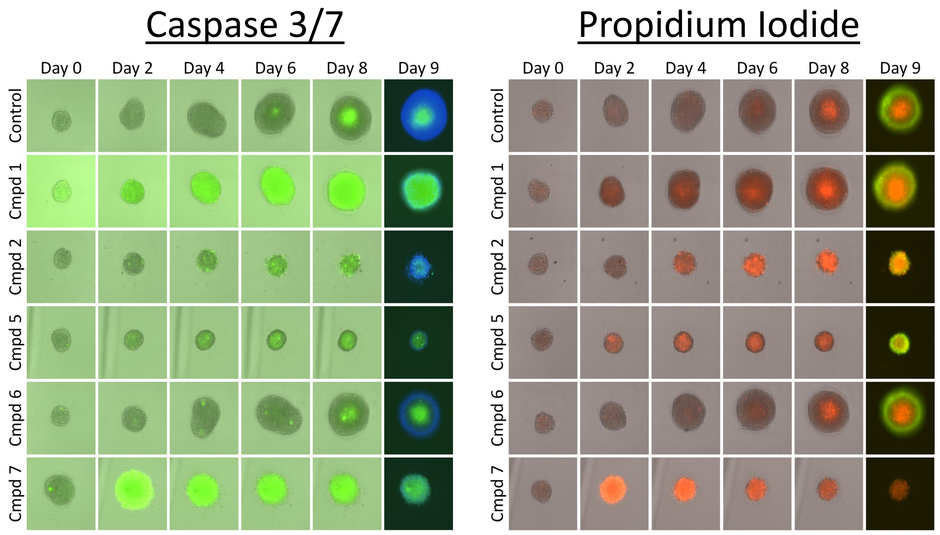




- The ability to form spheroid can dependent heavily on the cell types
- Both HT-29 and U87MG can form a tight and round spheroid at different seeding, where over 21 days, the caspase and PI fluorescence significantly increase due to necrotic core formation
- A549 formed a small spheroid but only showed increase in PI signal not caspase
- MDA-MB-231 did not form a spheroid, but lose cluster, where the cells were not healthy showing increase in PI signal in the beginning, indicating early cell death

4. REAL-TIME IMAGES OF CASPASE AND PI OF DRUG TREATED 3D TUMOR SPHEROIDS

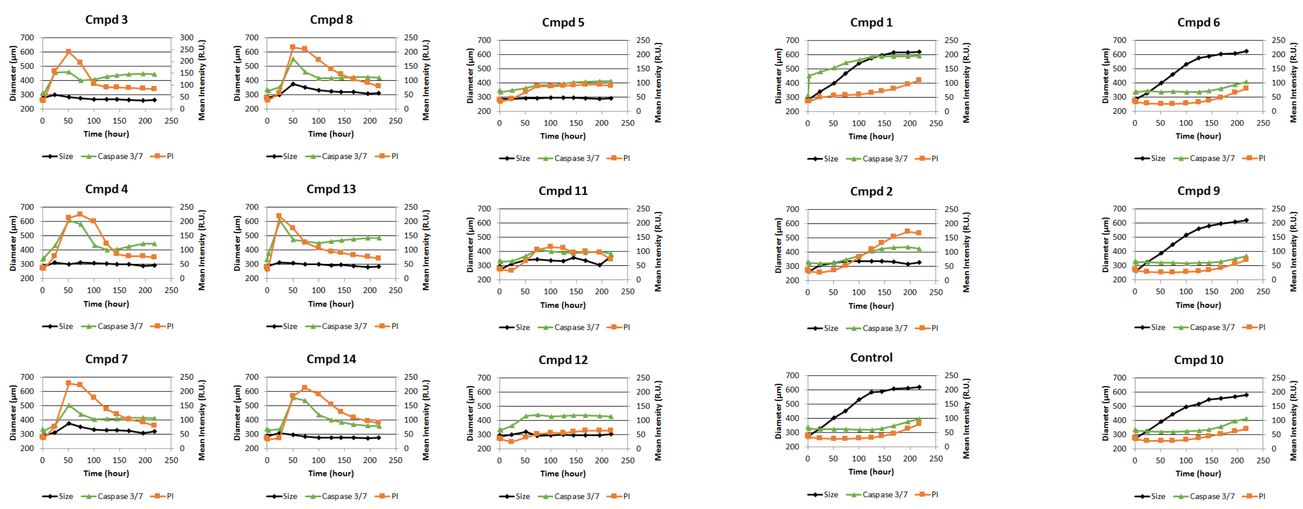
Stain the cells with PI and caspase 3/7 separately → Seed 500 cells/well in 4 ULA plates → From spheroid in 4 days then treat with 14 different cmpds → Image and analyze spheroid size, caspase, and PI FL daily for 9 days



ID	Compound Name	Primary MOA
Cmpd 1	Sunitinib malate	VEGFR-1/2/3 Inhibitor
Cmpd 2	Paclitaxel	Tubulin depolymerization inhibitor
Cmpd 3	Romidepsin	Histone Deacetylase (HDAC) Inhibitor
Cmpd 4	Panobinostat	Histone Deacetylase (HDAC) Inhibitor
Cmpd 5	GSK-2126458	PI3Kalpha/beta/delta/gamma Inhibitor
Cmpd 6	Cisplatin (A)	DNA Alkylating Agent
Cmpd 7	Bortezomib	Proteasome Inhibitor
Cmpd 8	Carfilzomib	Proteasome Inhibitor
Cmpd 9	Cisplatin (B)	DNA Alkylating Agent
Cmpd 10	Sorafenib	VEGFR-1/2/3 Inhibitor
Cmpd 11	Trametinib	Mek 1/2 inhibitor
Cmpd 12	BEZ-235	mTOR inhibitor
Cmpd 13	Staurosporin	Protein Kinase C inhibitor
Cmpd 14	17-AAG	Hsp90 inhibitors

- The tested drug compounds were group into 5 categories
  - Highly cytotoxic, cytostatic, apoptotic, necrotic and no effect
  - Cmpd 1 is apoptotic
  - Cmpd 2 is necrotic
  - Cmpd 5 is cytostatic
  - Cmpd 6 is no effect
  - Cmpd 7 is highly cytotoxic
- Each compound showed changed in caspase and PI fluorescence over time

5. REAL-TIME APOPTOSIS AND VIABILITY EFFECT OF DRUGS ON TUMOR SPHEROIDS



- Highly cytotoxic drugs (cmpd 3, 4, 7, 8, 13, and 14) showed early increase in caspase and PI fluorescence
- Cytostatic drugs (cmpd 5, 11, and 12) showed growth inhibition, but both caspase and PI did not increase
- Apoptotic (cmpd 1) showed increase in both size and caspase fluorescence
- Necrotic (cmpd 2) showed increase in PI fluorescence not caspase, indicating necrotic death
- Drugs (cmpd 6, 9, and 10) showed no noticeable effect