

An Image-Based Method to Detect and Quantify T Cell Mediated Cytotoxicity of 2D and 3D Target Cell Models

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Abstract

T cell mediated cytotoxicity plays an important role in a suite of new methods being developed with the goal of boosting a patient's immune system to combat cancer. In order to evaluate and optimize adoptive T cell immunotherapies, sensitive *in vitro* methods must be included in the testing process. In the procedure described here, phenotypic and quantitative assessments of 2D and 3D target cell necrotic induction were made using automated live cell imaging. It was found that direct activation of T cells produced a significantly greater cytotoxic effect than general activation suggesting that T cells can be "taught" to target and destroy specific target cells.

Introduction

CD3+CD8+ cytotoxic T lymphocytes (CTL) are the effector cells responsible for T cell mediated cytotoxicity that can act by cell-to-cell contact either by releasing granzymes and perforin or through Fas ligand mediated toxicity.¹ As part of the adaptive immune system, these cells mount targeted attacks to rid the body of a variety of compromised cells, such as cancer cells, without harming healthy cells. Counteracting this natural defense is the widely known fact that tumors develop multiple methods to avoid immune detection and create a level of tolerance against the immune cells designed to seek out and destroy cells containing foreign antigens.² For many years, the development of treatments avoided use of a patient's immune system to kill cancer cells, as immunotherapy-based treatments met with multiple clinical failures. Developing methods offer renewed hope for cancer patients. Adoptive immunotherapy techniques activates a patient's T cells *ex vivo* against tumor antigens before infusing the activated T cells back into the patient to target and destroy tumor cells selectively.³

The most popular *in vitro* method to monitor CTL effect on target cells is the cell mediated cytotoxicity (CMC) assay where T cells and target cells are added to a microplate well as a coculture. Traditionally toxicity was measured using chromium (⁵¹Cr) release from preloaded target cells. Due to problems with radioactivity disposal, and low sensitivity due to spontaneous release of the isotope from target cells⁴, newer methods were developed using microplate-based optical methods generating luminescence or fluorescence. These techniques were optimized to detect the signal from target cells plated in a uniform two-dimensional (2D) monolayer in microplate wells. With increasing adaptation of cells aggregated into a three-dimensional (3D) configuration to create a more *in vivo*-like model, cells are no longer evenly spread throughout the bottom of a well. Through the incorporation of microscopic imaging and cellular analysis, sensitive detection of induced cytotoxicity from 2D and 3D plated target cells, as well as visualization of the interplay between CTL and target cells, can be achieved.

Here, we demonstrate an automated research method to monitor and measure CTL cell mediated cytotoxicity kinetically using digital widefield microscopy. Cocultured target MDA-MB-231 breast cancer and fibroblast cells were plated in 2D and 3D format and dosed with a live cell apoptosis/necrosis reagent. T cells, activated using general or directed methods and stained with a far red tracking dye, were then added in ratios of 20, 10, 5, or 0:1 to the target cells.

The plates were then added to an automated incubator and shuttled to the digital widefield microscope, using a robotic arm, every four hours where brightfield and fluorescent images were captured for a total of seven days. Visual observation of the kinetic images enabled monitoring of CTL:target cell interactions for 2D and 3D cultured cells, while cellular image analysis allowed for calculation of CTL induced cytotoxicity during the entire incubation period.

Materials and methods

Materials

Cells and media

MDA-MB-231 epithelial breast adenocarcinoma cells (part number HTB-26) were obtained from ATCC (Manassas, VA). Human Neonatal Dermal Fibroblast cells stably expressing RFP (part number cAP-0008RFP) were purchased from Angio-Proteomie (Boston, MA). Human purified CD3+ T cells, isolated via negative selection from peripheral blood mononuclear cells (part number HM-PBMC-TCELLCD3-M) were donated by BioreclamationIVT (Westbury, NY). Advanced DMEM (part number 12491-015), RPMI 1640 medium (part number 11875-093), Fetal bovine serum, (part number 10437-036), and penicillin-streptomycin-glutamine (100X) (part number 10378-016) were purchased from ThermoFisher Scientific (Waltham, MA).

Assay and experimental components

IL-2 Superkine (Fc) (part number AG-40B-0111-C010), anti-CD3 (human), mAb (UCHT1) (part number ANC-144-020) and anti-CD28 (human), mAb (ANC28.1/5D10) (part number ANC-177-020) were donated by AdipoGen Life Sciences (San Diego, CA). SCREENSTAR 190 μ m cycloolefin filmbottom 384-well microplates (GBO part number 789836), CELLSTAR μ Clear 384-well cell-repellent surface microplates (GBO part number 781976) and the 384-Well BiO Assay Kit (GBO part number 781846, consisting of 2 vials NanoShuttle-PL, 6-Well Levitating Magnet Drive, 384-Well Spheroid and Holding Magnet Drives (2), 96-Well Deep Well Mixing Plate, 6-Well and 384-Well Clear Cell Repellent Surface Microplates), prototype 384-Well Ring Drive, and additional Cell Repellent Surface 6-Well (GBO part number 657860) were donated by Nano3D Biosciences, Inc., and Greiner Bio-One, Inc., (Monroe, NC). The Kinetic Apoptosis Kit (Microscopy) (part number ab129817) was donated by Abcam (Cambridge, MA). CellTracker Deep Red Dye (part number C34565) was purchased from ThermoFisher Scientific (Waltham, MA).

Agilent BioTek Cytation 5 cell imaging multimode reader

Cytation 5 is a modular multimode microplate reader combined with an automated digital microscope. Filter- and monochromator-based microplate reading are available, and the microscopy module provides up to 60x magnification in fluorescence, brightfield, color brightfield and phase contrast. The instrument can perform fluorescence imaging in up to four channels in a single step. With special emphasis on live cell assays, Cytation 5 features shaking, temperature control to 65 °C, CO₂/O₂ gas control and dual injectors for kinetic assays, and is controlled by integrated Agilent BioTek Gen5 microplate reader and imager software, which also automates image capture, processing and analysis. The instrument was used to kinetically monitor CTL:target cell interactions as well as cytotoxicity induction within the 2D and 3D plated target cells.

Agilent BioTek BioSpa 8 automated incubator

The BioSpa 8 automated incubator links Agilent BioTek readers or imagers together with Agilent BioTek washers and dispensers for full workflow automation of up to eight microplates. Temperature, CO₂/O₂ and humidity levels are controlled and monitored through the Agilent BioTek BioSpa software to maintain an ideal environment for cell cultures during all experimental stages. Test plates were incubated in the BioSpa to maintain proper atmospheric conditions for a period of seven days and automatically transferred to the Cytation 5 every four hours for brightfield and fluorescent imaging.

Methods

Overview

This work uses three workflows which are shown in Figure 1.

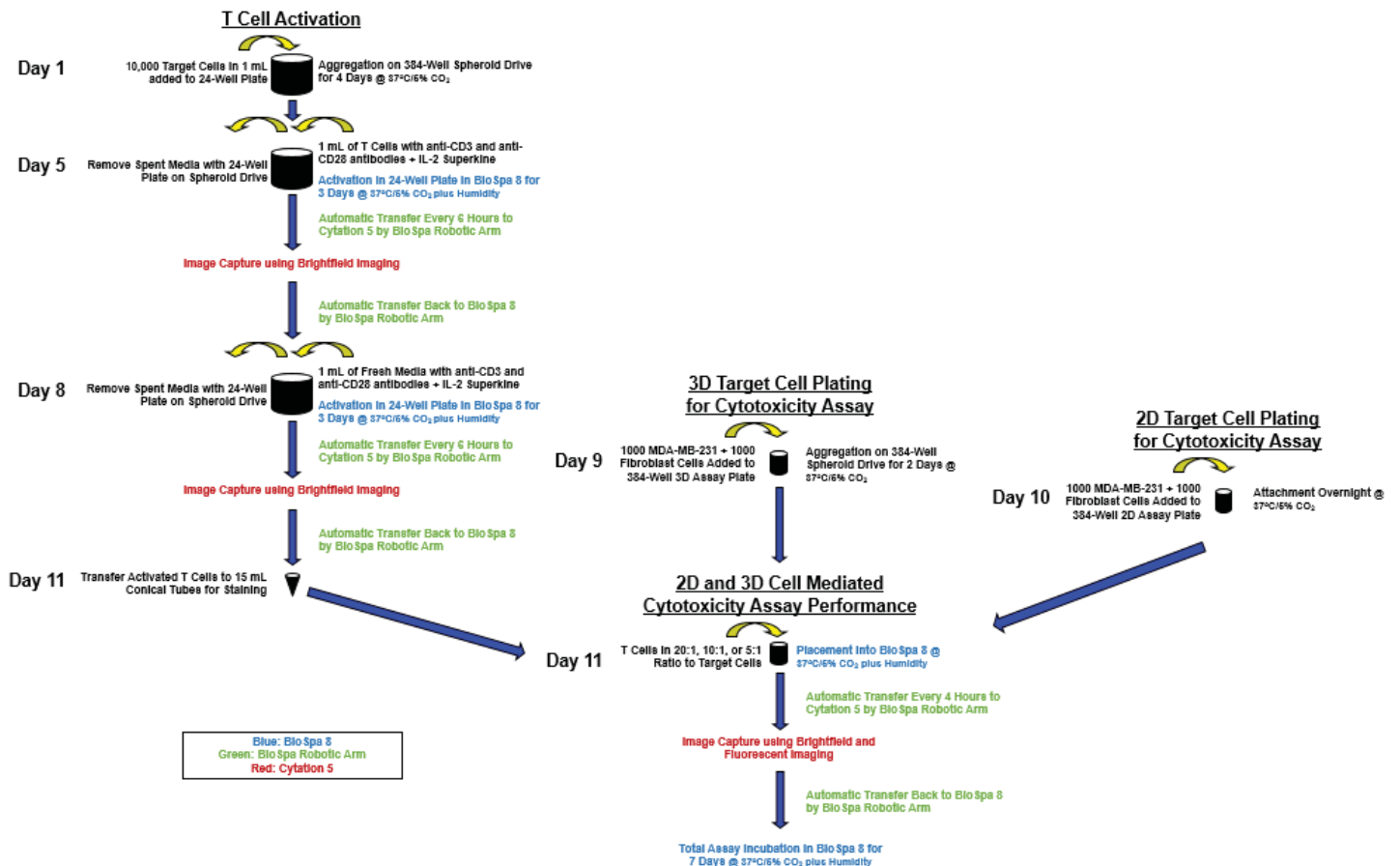


Figure 1. T cell activation and cell mediated cytotoxicity assay workflow.

The first workflow on the left side of Figure 1 involves T cell activation where CD3+ T cells are exposed to the MDA-MB-231 target cells which have been bioprinted into spheroids using magnetic fields (see "3D target cell preparation" for further detail.) The activated T cells are then stained with CellTracker Deep Red dye and used in either a bioprinted 3D spheroid-based cytotoxicity assay or another using plated cells. The CellTracker dye allows visualization of the T cells attacking the target cells, while propidium iodide dye allows for quantification of target cell death associated with plasma membrane rupture.

3D target cell preparation

T-75 flasks of MDA-MB-231 or fibroblast cell cultures were cultured to 80% confluence, then as illustrated in Figure 2, treated with 600 μ L NanoShuttle-PL overnight at 37 °C/5% CO₂. After incubation, cells were trypsinized for 3 to 5 minutes at 37 °C/5% CO₂.

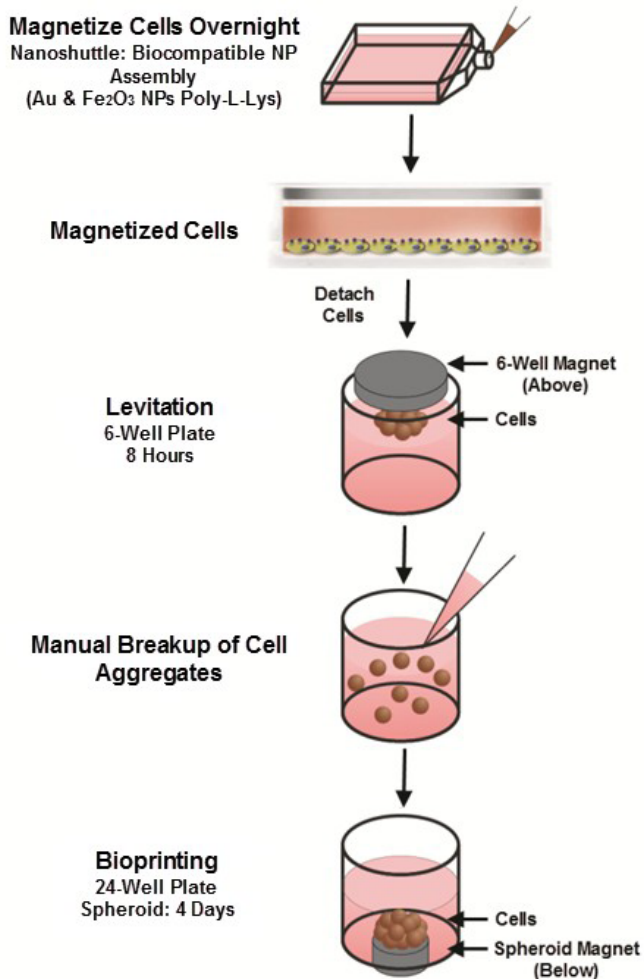


Figure 2. Bioprinting procedure used to create 3D spheroids for T cell activation.

Cells were removed from the flasks and added to the 6-well cell repellent plate at a concentration of 1.2×10^6 cells/well. A 6-well magnet drive was placed atop the well plate to levitate the cells, where aggregation and extracellular matrix (ECM) formation took place during an eight-hour incubation at 37 °C/5% CO₂. After incubation, the cells and ECM were broken up, resuspended, and combined together at equal concentrations in complete advanced DMEM medium.

For T cell activation, 3D spheroids were bioprinted in a 24-well cell repellent microplate using a 384-well spheroid magnet drive (see "Directed and general T cell activation").

A modified procedure associated with Figure 2 was used to prepare 3D spheroids for the cytotoxicity assay. The procedure was the same until the spheroid bioprinting was conducted. Instead of bioprinting in a 24-well plate as for T cell activation, the assay used 384-well plates such that a single spheroid was bioprinted in each well. To each well of the 384-well cell repellent microplate, a total of 2,000 cells (1,000 MDA-MB-231 and 1,000 fibroblasts) were added. The microplate was incubated at 37 °C/5% CO₂ for 48 hours to allow the cells to aggregate into cocultured tumoroids within each well.

2D target cell preparation

T-75 flasks of MDA-MB-231 or fibroblast cell cultures were cultured to 80% confluence. Cells were then trypsinized for 3 to 5 minutes at 37 °C/5% CO₂ and removed from the flasks. Following centrifugation, the cells were resuspended and combined together at equal concentrations in complete advanced DMEM medium. A total of 2,000 cells (1,000 MDA-MB-231 and 1,000 fibroblasts) were added to wells of a 384-well TC treated microplate intended for 2D cell culture (Figure 2). The microplate was incubated at 37 °C/5% CO₂ overnight to allow the cells to attach to the wells.

Directed and general T cell activation

A total of 10,000 target cells and media were added to 24-well cell repellent plate wells for each experimental condition as follows (Figure 2). Directed activation: (A) 100% MDA-MB-231; (B) 75% MDA-MB-231 and 25% fibroblasts; (C) 50% MDA-MB-231 and 50% fibroblasts; general activation: (D) no cells. Total volume was 1 mL for wells in each test condition. The 24-well plate was then placed atop a 384-well spheroid magnet drive and incubated at 37 °C/5% CO₂ for four days where the cells aggregated into multiple 3D spheroids within each well (Figure 3). Note that the magnet drive is designed for 384-well densities, such that the expanded size of a 24-well plate well provides nine (9) separate spheroids/well.

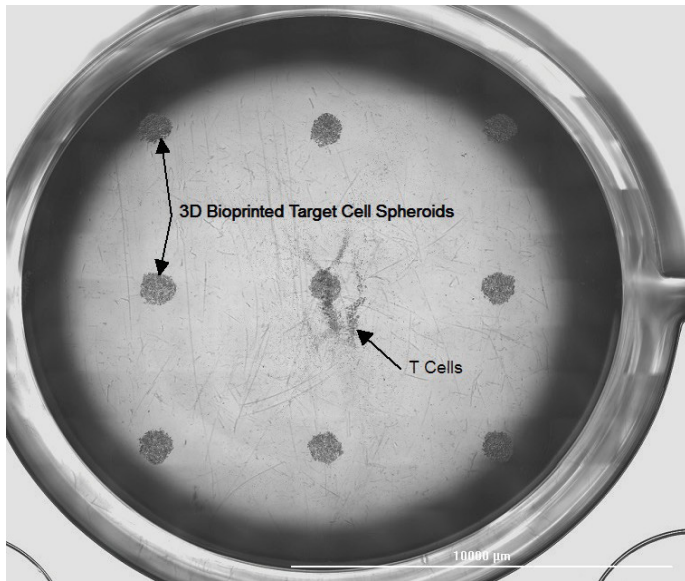


Figure 3. 24-well plate well showing coculture of T cells and bioprinted magnetized 3D target spheroids prior to commencement of directed activation. T cells added in a 10:1 ratio to target cells previously aggregated into 3D spheroids (~1,000 μm ID).

Following spheroid aggregation, T cells were prepared at a concentration of 100,000 cells/mL in RPMI medium containing 100 ng/mL IL-2 Superkine (Fc) (superkine) along with 250 ng/mL each of anti-CD3 and anti-CD28 antibodies. Spent media was then aspirated while the plate remained on the magnet drive to secure the spheroids, and replaced with fresh media containing the T cells, antibodies, and superkine as previously described. The plate was then placed back into the BioSpa to incubate for six days. The BioSpa was preprogrammed to capture a 12 \times 10 image montage

from each test well every six hours. Manual exchange of media, IL-2 Superkine, and antibodies was performed after 72 hours. The directed activation procedure over the six days serves to not only activate the T cells, but also teaches them to recognize target cell antigens allowing for targeted cytotoxicity. General activation follows the same procedure, but uses no target cells, thus there should be no targeted cytotoxicity.⁵

T cell staining and addition

Upon completion of the activation process, the 24-well plate containing the T cells and magnetized target cells was placed back on the 384-well magnet drive. The T cells were then removed from each well and transferred to a separate 15 mL conical tube for staining with the CellTracker Deep Red dye allowing for differentiation from the target cells during the cytotoxicity experiment. Dye, at a concentration of 1 μM , was added to the tubes and incubated at 37 $^{\circ}\text{C}$ /5% CO_2 for 45 minutes. The tubes were then centrifuged for 15 minutes at 200 RCF. Media containing the excess dye was then removed and replaced with fresh RPMI medium. Stained T cells from each activation condition were then diluted in RPMI medium containing 10 $\mu\text{L}/\text{mL}$ of the propidium iodide necrosis probe from the Kinetic Apoptosis kit. The cells were then added to the 384-well 2D or 3D cell culture plates, already containing a total of 2,000 target cells, in concentrations equaling 40,000 cells/well, 20,000 cells/well, or 10,000 cells/well (Figure 1). These concentrations created ratios of 20:1, 10:1, or 5:1 T cells to target cells in each well. Untreated negative control wells were also included to examine basal target cell cytotoxicity levels over time. Table 1 illustrates the final plate layout.

Table 1. 2D and 3D cell-mediated cytotoxicity assay plate layout.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
A	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a
B	b	b	b	b	b	b	b	b	b	b	b	b	b	b	b
C	c	c	c	c	c	c	c	c	c	c	c	c	c	c	c
D	d	d	d	d	d	d	d	d	d	d	d	d	d	d	d

■	100% Directed Activation
■	75% Directed Activation
■	50% Directed Activation
■	General Activation
■	Inactivated

a	20:1 T Cell:Target Cell Ratio
b	10:1 T Cell:Target Cell Ratio
c	5:1 T Cell:Target Cell Ratio
d	0:1 T Cell:Target Cell Ratio

Cell-mediated cytotoxicity assay automated procedure



Figure 4. Agilent BioTek BioSpa live cell imaging system, including Agilent BioTek BioSpa 8 and Agilent BioTek Cytation 5.

2D and 3D assay plates, containing T cells and target cells, were added to the BioSpa 8, as part of the BioSpa live cell imaging system (Figure 4), with atmospheric conditions previously set to 37 °C/5% CO₂. Water was also added to the pan to create a humidified environment, which was monitored. The Agilent BioTek BioSpa software was set such that the plates were automatically transferred to the Cytation 5 for brightfield and fluorescent imaging of the test wells every four hours for a total of seven days. Table 2 explains the imaging carried out with each channel. For 2D plated cells, a single 4x magnification image was taken with each channel to capture a representative population of cells per well. Laser autofocus was incorporated to ensure proper focusing on the target cell layer as well as the most efficient focusing procedure. For 3D plated cells, since the cells within the 3D target cell spheroids existed on multiple z-planes, a z-stack consisting of five slices was captured with each channel. Laser autofocus was again incorporated. Two images each were taken below and above the decided upon focal plane.

Table 2. Cell imaged per imaging channel.

Imaging Channel Target	
Brightfield	All cells
PI	Necrotic cells
CY5	T cells

2D and 3D image processing

Following capture, 2D and 3D images were processed prior to analysis. 2D images underwent preprocessing to remove background signal from each channel using the settings in Table 3.

Table 3. 2D image preprocessing parameters.

2D Image Preprocessing Parameters			
Channel	Apply Image Preprocessing	Background	Rolling Ball Diameter
Brightfield	No		
PI	Yes	Dark	Auto
CY5	Yes	Dark	Auto

For 3D images, first a z-projection of the images captured in the z-stack was carried out to create a final image containing only the most in-focus information (Table 4).

Table 4. 3D Z-projection criteria.

3D Image Stching Parameters	
Method	Focus shaking
Size of Max. Filter	11 pixels
Top Slice	0 µm from focal plane
Bottom Slice	-53.8 µm from focal plane

Preprocessing of the projected image was then performed to again remove background signal from each channel (Table 5).

Table 5. 3D image preprocessing parameters.

3D Image Preprocessing Parameters			
Channel	Apply Image Preprocessing	Background	Rolling Ball Diameter
Brightfield	No		
PI	Yes	Dark	Auto
CY5	Yes	Dark	Auto

Cellular analysis of 2D and 3D processed images

Cellular analysis was carried out on the processed images to determine the total signal emanating from necrotic target cells using the criteria in Table 6.

Table 6. Necrotic cell identification criteria.

Necrotic Cell Identification Criteria		
	2D Analysis	3D Analysis
Channel	Tsf[Propidium Iodide]	Tsf(ZProj[Propidium Iodide])
Threshold	Auto (-6)	5,000
Background	Dark	Dark
Split Touching Objects	Checked	Checked
Fill Holes in Masks	Checked	Checked
Min. Object Size	10 μm	10 μm
Max. Object Size	4,000 μm	100 μm
Include Primary Edge Objects	Unchecked	Unchecked
Analyze Entire Image	Checked	Checked
Advanced Detection Options		
Rolling Ball Diameter	1,000 μm	75 μm
Image Smoothing Strength	20	0
Evaluate Background On	5% of lowest pixels	5% of lowest pixels
Analysis Metric		
Metric of Interest	Cell count	Object Sum Int[Tsf[ZProj [Propidium Iodide]]]

An additional image analysis step was performed on the 3D images to determine the extent to which target cell spheroids disintegrated following T cell treatment (Table 7).

Table 7. Spheroid disintegration criteria.

3D Image Analysis Parameters	
Data In	Tsf(ZProj[Brightfield])
Threshold (Lower Value)	Unchecked
Threshold (Upper Value)	Checked (2,500)
Analysis Metric	
Metric of Interest	Confluence

Results and discussion

Image-based detection of cocultured cell interaction

T cells, activated using direct and general activation procedures, were added to the target cells in concentrations equaling 20:1, 10:1, 5:1 and 0:1 to start the cell-mediated cytotoxicity assay. To monitor the interaction of the cocultured cells, plates were imaged immediately following T cell addition and every four hours subsequent throughout the entire seven-day incubation period.

As assay incubation times increase, it is apparent that activated T cells (red fluorescence) seek out and cluster around the antigen presenting target cells through antigen-receptor binding in both 2D and 3D formats (Figure 5A). This T cell aggregation is in marked contrast to the more even distribution of red fluorescence at time 0.

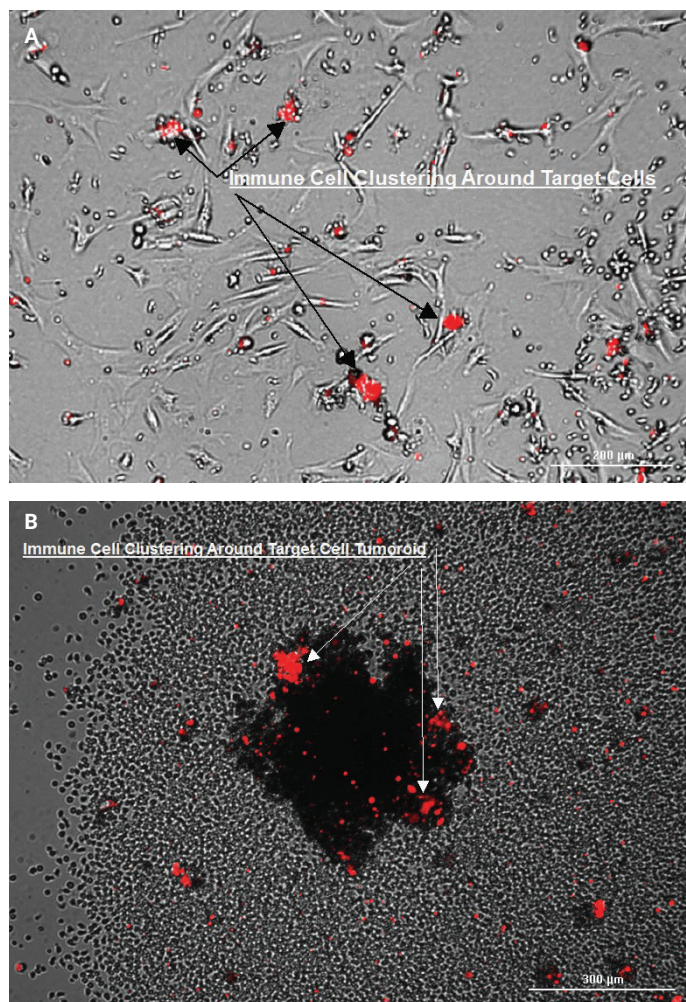


Figure 5. Brightfield/CY5 imaging of cellular interaction. 4x brightfield and CY5 images showing T cell clustering and binding to (A) 2D or; (B) 3D target cells. Time = 24 hours.

When images from the PI channel are overlaid with those from the brightfield channel one can observe that yellow fluorescent signal from the propidium iodide necrotic cell probe originates from the same target cells with bound T cells (Figure 6). This confirms the downstream cytotoxic effect of T cell binding to the target cells.

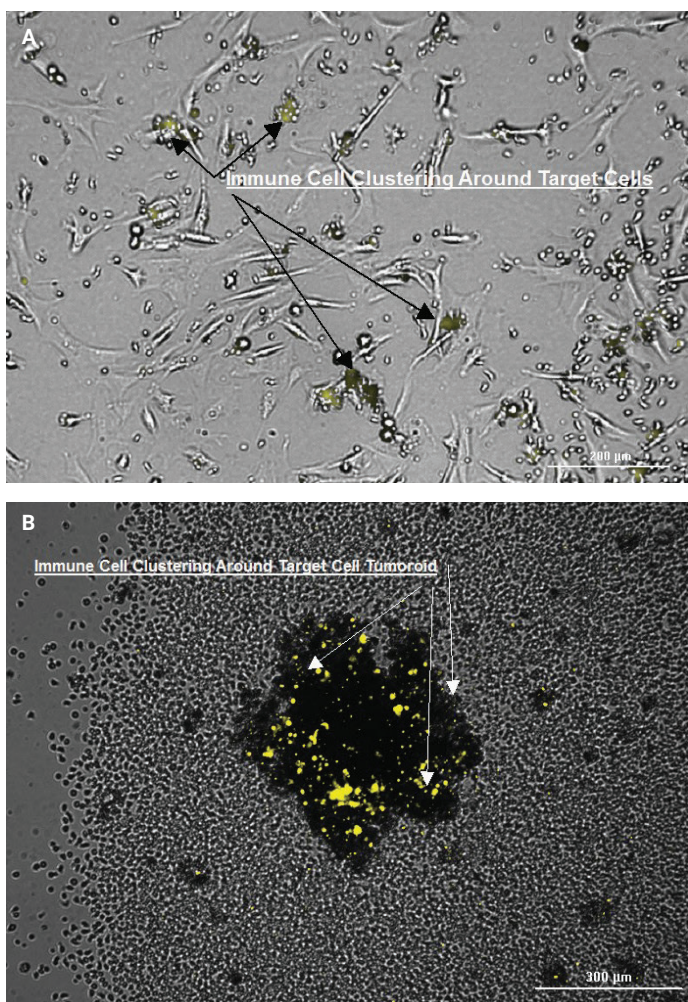


Figure 6. Brightfield/PI imaging of cellular interaction. 4x brightfield and PI images showing necrotic (A) 2D or; (B) 3D target cells in response to T cell binding. Time = 24 hours.

Kinetic imaging of T cell-mediated target cell cytotoxicity induction

In order to determine the kinetics of cytotoxicity induction within the target cells, imaging must be carried out at regular intervals throughout the entire incubation period. As the full cytotoxic effect may not be reached until days after T cell addition, it is also essential that cells be allowed to interact for multiple days. The environmental controls of the Cytation 5 and BioSpa 8, as well as automatic transfer of test plates from incubator to imager, allow kinetic analysis to be completed without compromising cell health. In the experiments performed here, brightfield and fluorescent images were captured every four hours for a total of seven days. Figures 7 and 8 demonstrate the iterative cytotoxic effect that T cells, directly activated in the presence of 100% MDA-MB-231 cells and added at a 20:1 ratio, have on 2D and 3D cultured target cells, respectively.

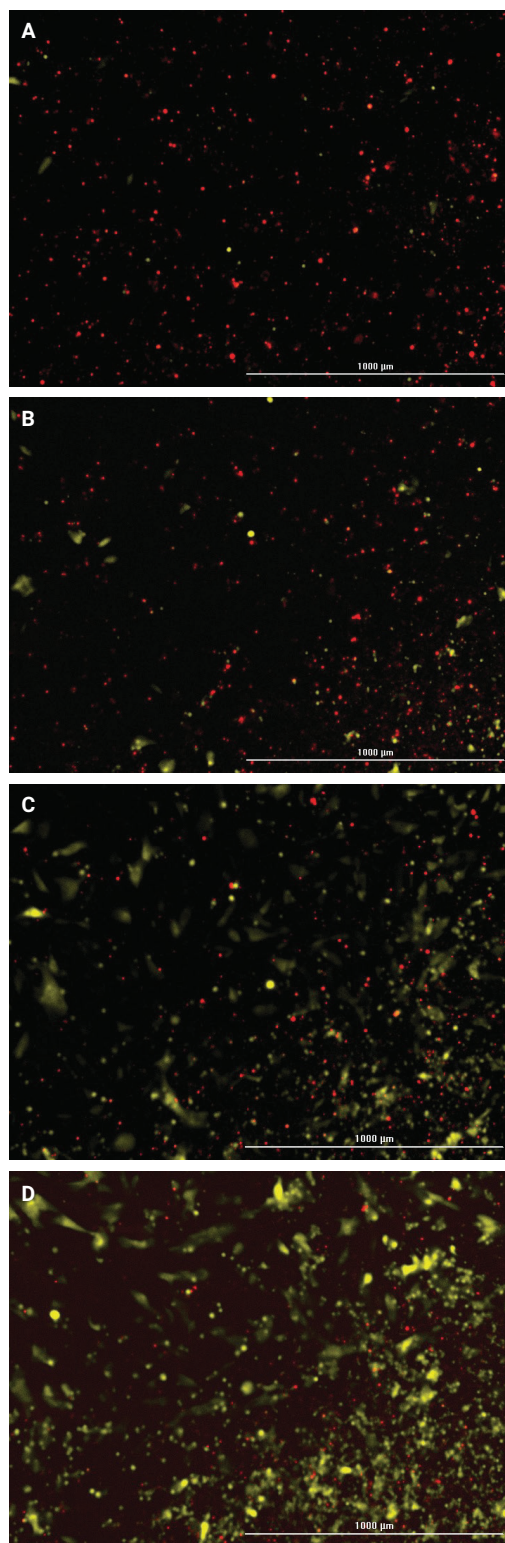


Figure 7. CY5/PI imaging of 2D cytotoxic target cell induction. 4x overlaid CY5 and PI images showing stained T cells and signal from propidium iodide necrotic cell probe following (A) 0; (B) 48; (C) 96; and (D) 168 hour coculture incubation periods.

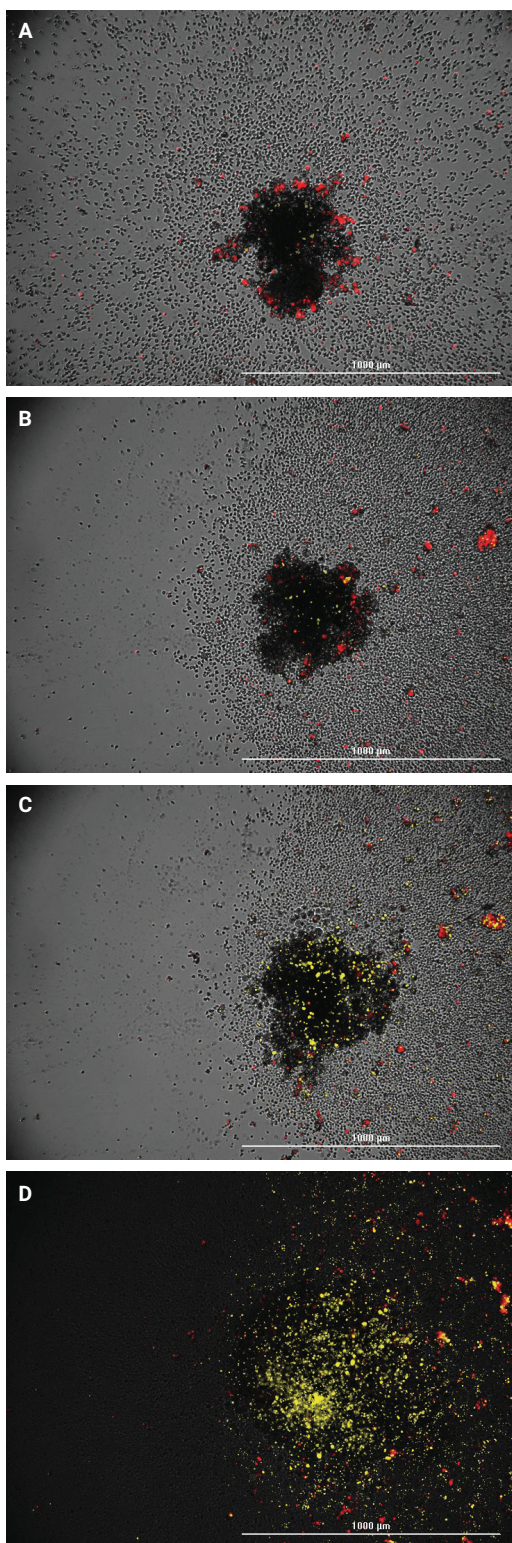


Figure 8. Brightfield/CY5/PI imaging of 3D cytotoxic target cell induction. 4x overlaid brightfield, CY5, and PI images showing stained T cells and signal from propidium iodide necrotic cell probe following (A) 0; (B) 48; (C) 96; and (D) 168 hour coculture incubation periods.

Quantification of target cell cytotoxicity

Following image capture, the level of T cell-induced target cell cytotoxicity was then quantified.

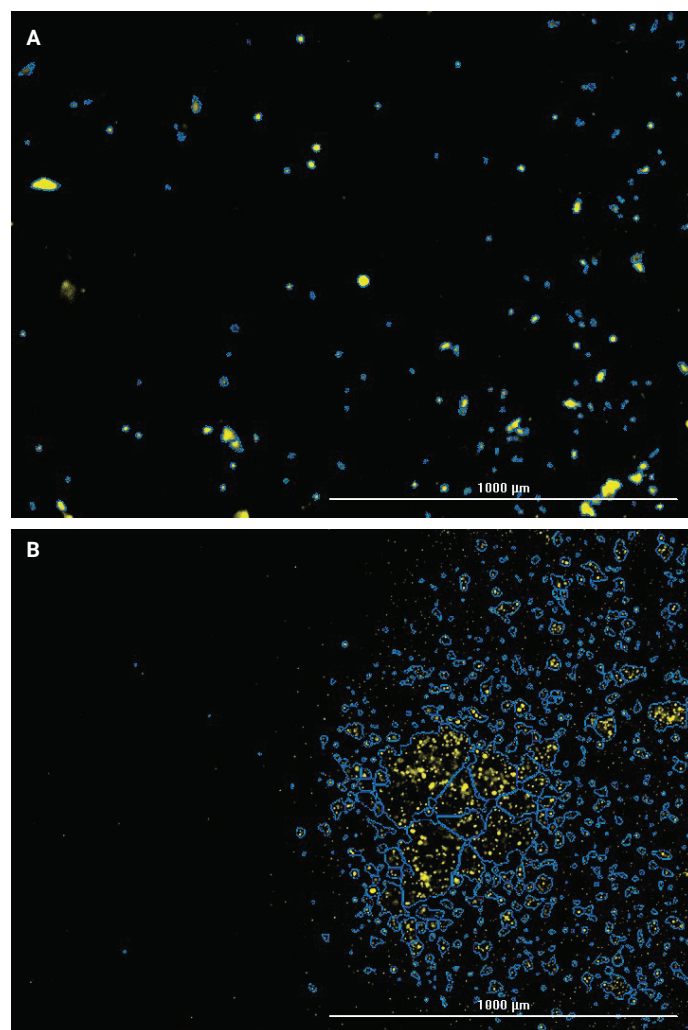


Figure 9. Cellular analysis of target cell cytotoxicity. 4x images showing fluorescence from propidium iodide necrotic cell probe following 96-hour incubation. Object masks (in blue) placed around (A) 2D and; (B) 3D cultured target cells meeting cellular analysis criteria.

Using the optimized image analysis criteria described in Table 6, object masks were placed around cells meeting minimum threshold signal criteria from the PI necrotic cell probe (Figure 9). As T cells have a smaller size compared to the target cells in either 2D or 3D format, the minimum object size cutoff value was set such that single necrotic T cells were not included in the analysis. This can be seen in Figures 9A and 9B.

A phenomenon also observed in the kinetic images of the 3D CMC assay is that the tumoroid began to disintegrate in response to increasing cytotoxicity, releasing groups of cells into the surrounding media. While smaller than the intact tumoroid body, these aggregates remain larger than individual T cells and also emit signal from the PI necrotic cell probe, therefore are included in the final analysis (Figure 9B).

From the analysis performed, the number of necrotic cells per image was calculated for 2D cultured target cells. When cultured in 3D, cells within the tumoroid and smaller aggregates exist on multiple z-planes. Therefore, to quantify induced cytotoxicity with the greatest level of accuracy, the total PI signal within all object masks per image was quantified. The values (cell count or total PI signal) calculated at each timepoint were then automatically divided by the value calculated at time 0 in Gen5 software. In this way small variances between replicates were normalized. Following analysis, the results were plotted to evaluate whether differences were seen in induced target cell cytotoxicity between test conditions. The graphs in Figure 10 show the calculated data for T cells added to test wells in a 20:1 ratio, activated in the presence of 100%, 75%, 50% or 0% MDA-MB-231 cells, compared to unactivated T cells.

From Figure 10 it is evident that T cell-induced cytotoxicity increases in terms of the degree of directed cell activation in both 2D and 3D cell models. T cells activated in the presence of 100% MDA-MB-231 cells elicit the highest level of cytotoxicity, while those activated only in the presence of antibodies and superkine elicit the lowest increase in necrotic cell numbers per image over basal necrotic cell numbers. The models differ in their kinetic responses, however. In the 2D model (Figure 10A), T cell-mediated cytotoxicity peaks at about 24 hours after addition of the activated T cells, as witnessed by the ratio of necrotic cells from wells containing T cells to necrotic cell numbers from negative control wells. Any further necrosis beyond about 3 days is due to the limitations of the 2D model as noted by the increased necrosis over time evident in the negative control. Conversely, in the 3D model (Figure 10B), necrotic ratios of total signal from the PI probe continue to increase or plateau over the course of the kinetic run due to the fact that cell health is much better retained in the untreated 3D cell model.

Analysis of necrotic cell induction was then performed on wells containing T cells directly activated in the presence of 100% MDA-MB-231 cells and then added to 2D and 3D plated target cells for the CMC assay in ratios of 20:1, 10:1, 5:1. A negative control was also included where target cells were untreated.

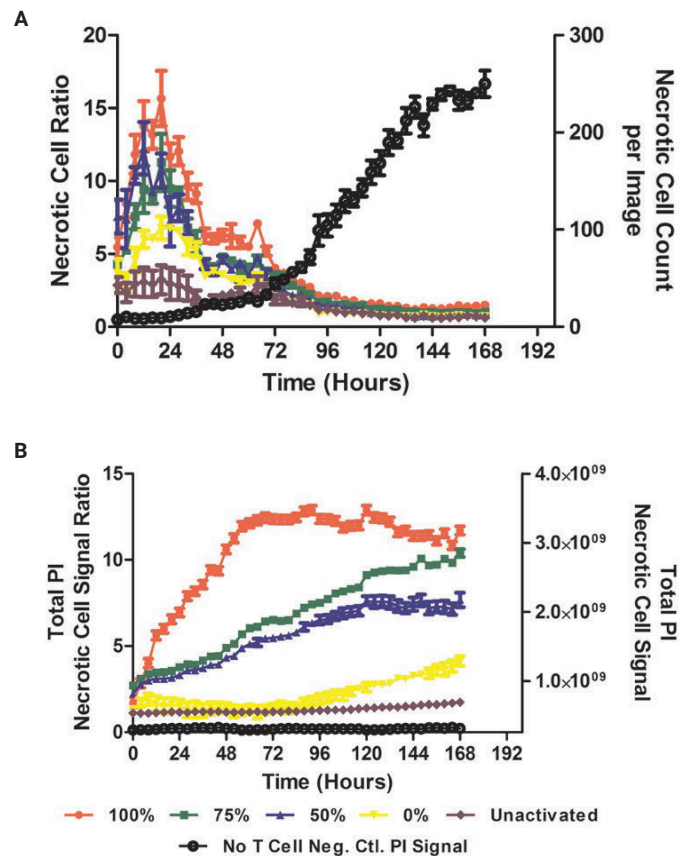


Figure 10. Activation protocol cytotoxicity induction analysis. Comparison of cytotoxic target cell induction by T cells activated in the presence of anti-CD3 and anti-CD28 antibodies, superkine, and 100% MDA-MB-231 cells, 75% MDA-MB-231/25% fibroblast cells, 50% MDA-MB-231/50% fibroblast cells, or no cells. Data for unactivated T cells also included and plotted using left y-axis. Necrotic cell count or total PI signal over time from untreated negative control target cells plotted on right y-axis. Results shown for T cells incubated with (A) 2D cultured target cells; or (B) 3D cultured target cells for seven days.

It is evident from Figure 11 that kinetic responses of T cell-mediated cytotoxicity for different ratios of T cell to target cell for both 2D and 3D models are obtained over time. These findings are consistent with the previous results from the activation protocol comparison (Figure 10), as well as results reported with *in vivo* testing.

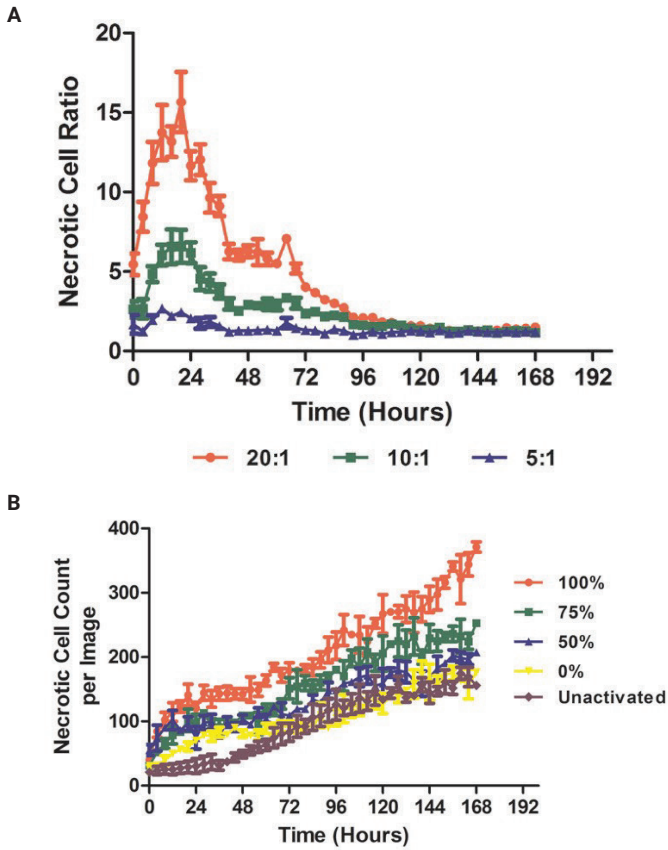


Figure 11. Effect of T cell concentration. Comparison of cytotoxic target cell induction by T cells added to wells at concentrations of 40,000 cells/well (20:1 ratio), 20,000 cells/well (10:1 ratio), 10,000 cells/well (5:1 ratio), and 0 cells/well (negative control). Results shown for T cells incubated with (A) 2D; or (B) 3D cultured target cells for seven days.

Finally, the effects of directed activation can also be measured using the brightfield channel when target-cells are cultured in 3D. This is due to the fact that in response to the cytotoxic T cell effect, tumoroids break apart over time, or explode, releasing cells and ECM within the well. Using the confluence measurement capabilities of Gen5 and the optimized metrics in Table 7, the extent of tumoroid disintegration can then be quantified. Only pixels within each image with a brightfield signal below the upper threshold criteria are included in the percent confluence calculation. When viewed in Gen5, outlier pixels are seen as white (Figure 12).

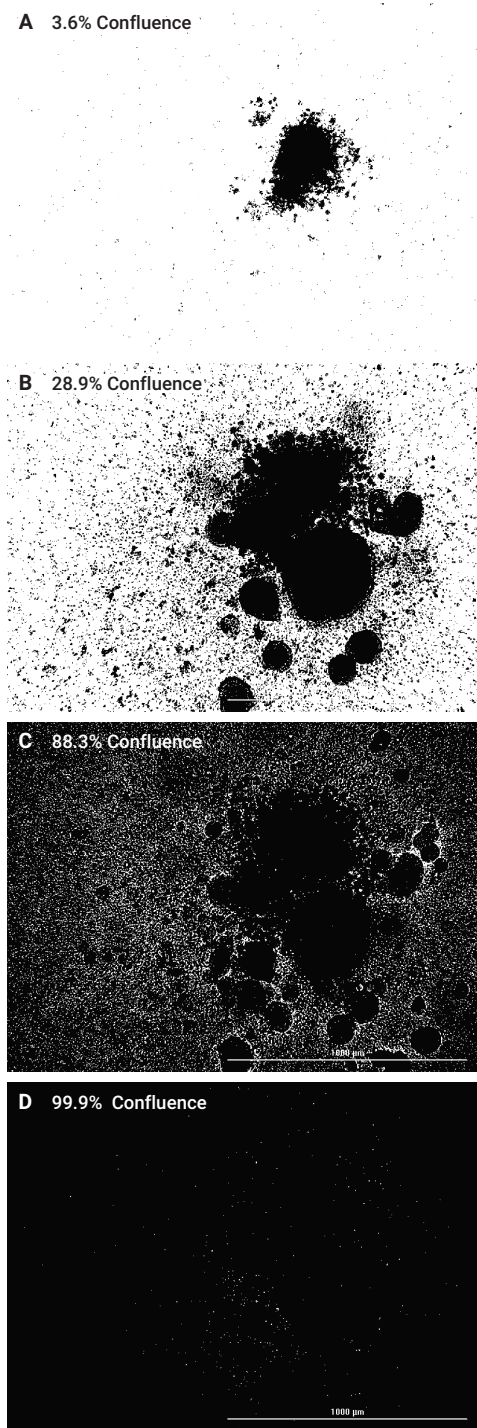


Figure 12. Image confluence determination using brightfield signal. 4x brightfield images following image analysis and % confluence determination. Pixels not included in confluence calculation appear white. Images shown after cell interaction and binding with a 10:1 T cell to target cell ratio for (A) 72; (B) 116; (C) 136; and (D) 168 hour incubation periods.

Percent confluence values can then be plotted over time to visualize the kinetics of tumoroid disintegration in response to increasing T cell to target cell ratios.

The curves in Figure 13 illustrate how plotting confluence over time explains the kinetics of the final effect. As would be expected, higher concentrations of activated T cells destroy the tumoroid faster than lower concentrations. Untreated tumoroids also show little change in confluence due to the fact that little to no cellular toxicity is seen (Figure 10B) allowing the tumoroid to remain intact during the seven day incubation period.

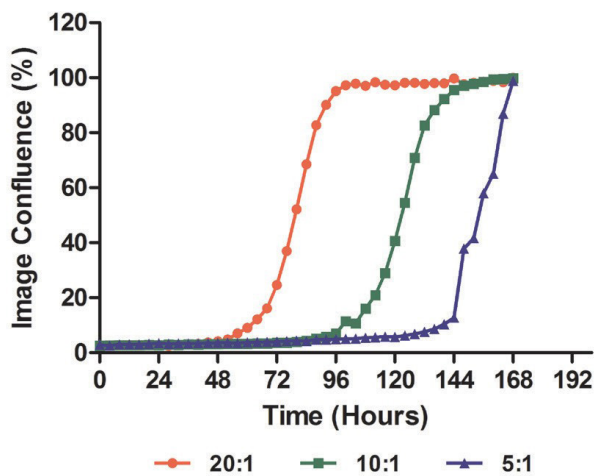


Figure 13. Kinetic percent image confluence quantification. Plot of kinetic brightfield image percent confluence due to 3D tumoroid disintegration.

Conclusion

It was found that direct activation of T cells, where they were exposed to target cells over extended periods *in vitro*, produced a significant increase in cytotoxicity compared to general activation using no target cells. Furthermore, a diminishing effect was evident if the target cells were cocultured with fibroblasts in the activation process: the greater the ratio of fibroblasts, the less cytotoxicity evident. This suggests that T cells can be instructed in the activation process to seek out and destroy target cells.

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The 3D cell model was far superior to the 2D cell model as cell health was maintained throughout the long kinetic runs. Cytotoxicity could be quantified using propidium iodide that measure plasma membrane rupture or with brightfield (label-free) that measured confluence increase by spheroid disaggregation.

The Agilent BioTek BioSpa system, comprised of an automated CO₂ incubator shuffling microplates to the Agilent BioTek cell imaging reader, allows for walk-away automation of the 7-day kinetic cytotoxicity assay.

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