Automated, Image-Based T Cell Mediated Cytotoxicity Assessments using 2D and 3D Target Cell Models

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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 activate 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 co-culture. Traditionally, toxicity was measured using chromium (^{51}Cr) release from preloaded target cells. Due to problems with radioactivity disposal, and low sensitivity due to spontaneous release of the isotope from target cells[4], 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 method to monitor and measure CTL cell mediated cytotoxicity kinetically using digital widefield microscopy. Co-cultured target MDA-MB-231 breast cancer and fibroblast cells were plated in 2D format and 3D bioprinted spheroids, 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. Prior to image analysis, all images were automatically pre-processed to removing background signal. 3D image processing also included creating a z-projected final image containing only the most in-focus information prior to removing background signal.

BioTek Instrumentation

Figure 1. BioSpa 8 and Cytation 5 integrated as the BioSpa Live Cell Imaging System.

Cytation™ 5 Cell Imaging Multi-Mode Reader: Cytation 5 is a modular multi-mode 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, CO_2/O_2 gas control and dual injectors for kinetic assays, and is controlled by integrated Gen5™ Microplate Reader and Imager Software, which also automates image capture, analysis and processing. The instrument was used to kinetically monitor CTL-target cell interactions as well as cytotoxicity induction within the 2D and 3D plated target cells.

BioSpa™ 8 Automated Incubator: The BioSpa 8 Automated Incubator links BioTek readers or imagers together with washers and dispensers for full workflow automation of up to eight microplates. Temperature, CO_2/O_2 and humidity levels are controlled and monitored through the 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.

Assay Procedure

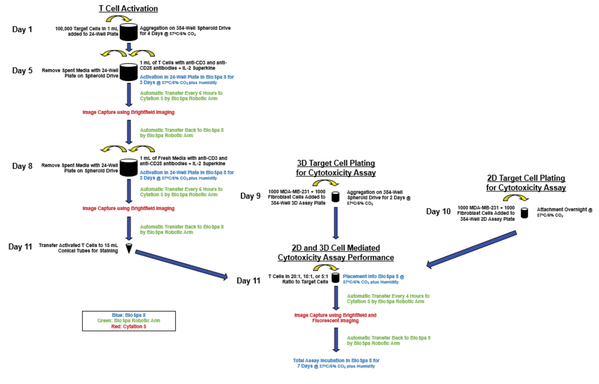

Figure 2. T cell activation and cell mediated cytotoxicity procedure.

Image-Based Detection of Co-Cultured Cell Interaction

Over the seven day assay incubation period, activated T cells (red fluorescence) sought out and clustered around the antigen presenting target cells through antigen-receptor binding in 2D and 3D formats (Figures 3A-B). This T cell aggregation was in marked contrast to the more even distribution at time 0. When images from the propidium iodide (PI) channel were overlaid with those from the brightfield channel, one can observe that yellow fluorescent signal from the PI necrotic cell probe originates from the same target cells with bound T cells (Figure 3C-D), confirming the downstream cytotoxic effect of T cell:target cell binding.

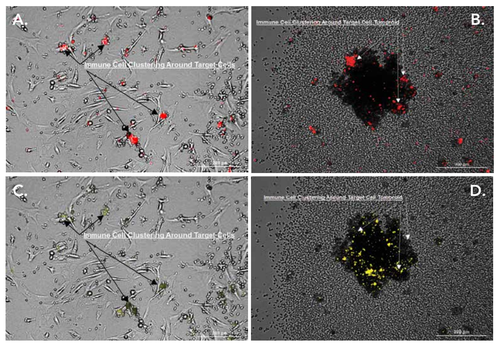

Figure 3. Cellular activity over 24 hours. Brightfield/Cy5 4x images showing T cell clustering and binding to **(A)** 2D or; **(B)** 3D target cells. Brightfield/PI 4x images showing necrotic **(C)** 2D or; **(D)** 3D target cells in response to T cell binding.

Kinetic Imaging of T Cell Mediated Target Cell Cytotoxicity Induction

To determine the kinetics of T cell induced cytotoxicity within the target cells, imaging was performed at regular intervals throughout the incubation period. As extended incubations were required to determine the full cytotoxic effect of T cell addition, brightfield and fluorescent images were captured every four hours for a total of seven days. Figures 4 and 5 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.

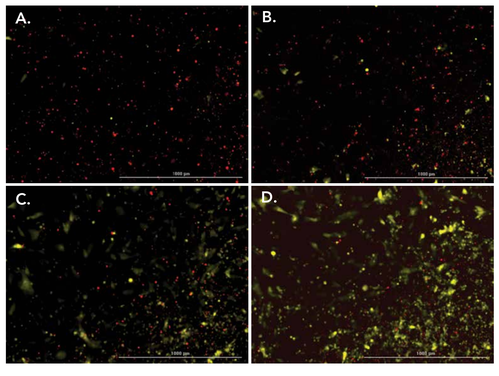

Figure 4. CY5/PI imaging of 2D cytotoxic target cell induction. 4x overlaid CY5 and PI images. Red CY5 channel showing stained T cells and yellow propidium iodide channel showing signal from necrotic cell probe following **(A)** 0; **(B)** 48; **(C)** 96; and **(D)** 168 hour co-culture incubation periods.

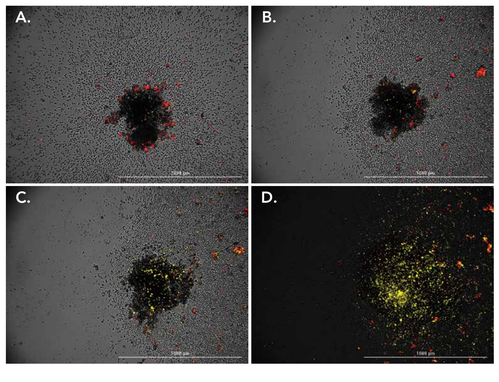

Figure 5. Brightfield/CY5/PI imaging of 3D cytotoxic target cell induction. 4x overlaid brightfield, CY5 and PI images. Brightfield channel showing aggregated co-cultured target cell tumor spheroids, red CY5 channel showing stained T cells and yellow propidium iodide channel showing signal from necrotic cell probe following **(A)** 0; **(B)** 48; **(C)** 96; and **(D)** 168 hour co-culture incubation periods.

Quantification of Target Cell Cytotoxicity

Following image capture, T cell induced target cell cytotoxicity levels were quantified. Object masks were placed around cells meeting minimum threshold signal criteria from the PI probe. Object size cutoff values were set to exclude single necrotic T cells, and include small cell aggregates created as the 3D tumoroid disintegrated in response to increasing cytotoxicity. From the analysis, the number of necrotic cells per image was calculated for 2D cultured target cells. Per 3D cells, the total PI signal within all object masks per image was quantified as the cells exist on multiple z-planes. The values (cell count or total PI signal) calculated at each timepoint were then automatically normalized and plotted to evaluate potential differences in induced target cell cytotoxicity between test conditions.

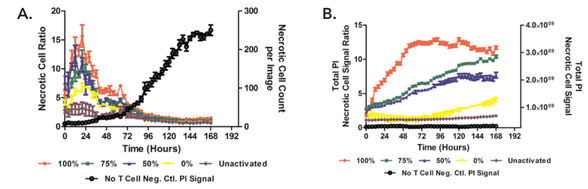

Figure 6. Activation protocol cytotoxicity induction analysis. Comparison of cytotoxic target cell induction by T cells activated in the presence of antibodies, IL superkine, and varying cell type ratios including unactivated T cells. Necrotic cell count or total PI signal over time from untreated negative control target cells plotted on right y-axis. Results shown for T cells incubated with **(A)** 2D; or **(B)** 3D cultured target cells.

Figure 6 shows 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. 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. In the 2D model (Figure 6A), 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 three 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 6B), necrotic ratios of total signal from the PI probe continue to increase or plateau over the course of the kinetic run as cell health is better maintained in the untreated 3D cell model.

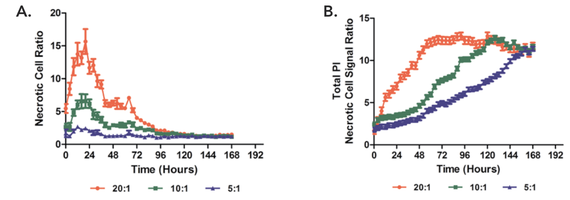

Figure 7. Effect of T cell concentration. Comparison of cytotoxic target cell induction by T cells added to wells at concentrations of 40,000 cells/well (20:1 ratio), 20,000 cells/well (10:1 ratio), 10,000 cells/well (5:1 ratio), and 0 cells/well (negative control). Results shown for T cells incubated with **(A)** 2D; or **(B)** 3D cultured target cells.

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 added to 2D and 3D plated target cells in 20:1, 10:1, and 5:1 T cell to target cell ratios. Per Figure 7, kinetic responses of T cell-mediated cytotoxicity are consistent with activation protocol comparison results for 2D and 3D models (Figure 6), as well as *in vivo* testing results[5]. The effect of T cell concentration is also confirmed, as the highest target cell cytotoxic induction is seen from wells containing a 20:1 ratio of T cells to target cells.

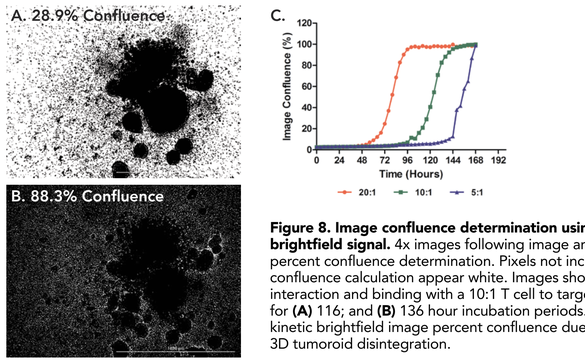

Figure 8. Image confluence determination using brightfield signal. 4x images following image analysis and percent confluence determination. Pixels not included in confluence calculation appear white. Images shown after cell interaction and binding with a 10:1 T cell to target cell ratio for **(A)** 116; and **(B)** 136 hour incubation periods. **(C)** Plot of kinetic brightfield image percent confluence due to 3D tumoroid disintegration.

T cell induced cytotoxicity can also be measured from brightfield images when incorporating a 3D target cell model. Tumoroids break apart over time in response to the cytotoxic T cell effect, releasing cells and extracellular matrix (ECM) into the well, causing images to darken in appearance. Using image analysis, pixels below upper threshold criteria are included in percent confluence calculations (Figures 8A and B). 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 8C 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.

Conclusions

1. Direct activation of T cells produced a significant increase in cytotoxicity compared to general activation using no target cells, whereas a diminishing effect was evident if the target cells were co-cultured with fibroblasts in the activation process.
2. The 3D cell model was proven to maintain superior cell health throughout long kinetic runs compared to 2D cell models.
3. Cytotoxicity may be quantified using propidium iodide or brightfield (label-free) methods.
4. BioSpa Live Cell Imaging System allows for walk-away automation of the 7-day kinetic cytotoxicity assay.

1. Janeway, C.A. Jr.; Travers, P.; Walport, M.; Shlomchik, M.J. T Cell-Mediated Immunity. *Immunobiology: The Immune System in Health and Disease*, 5th edition [Online]; Garland Science: New York, 2001; https://www.ncbi.nlm.nih.gov/books/NBK27101/ (accessed Oct 24, 2017). 2. Mahoney, K.M.; Rennert, P.D.; Freeman, G.J.; Combination cancer immunotherapy and new immunomodulatory targets. *Nat Rev Drug Discov.* **2015**, *14*, 561-584. 3. Perica, K.; Varela, J.C.; Oelke, M.; Schneck, J. Adoptive T cell immunotherapy for cancer. *Rambam Maimonides Med J.* **2015**, *6*(1), 1-9. 4. Zaritskaya, L.; Shurin, M.R.; Sayers, T.J.; Malyguine, A.M. New flow cytometric assays for monitoring cell-mediated cytotoxicity. *Expert Rev Vaccines.* **2010**, *9*(6), 601-616. 5. Zangle, T.A.; Burnes, D.; Mathis, C.; Witte, O.N.; Teitell, M.A. Quantifying biomass changes of single CD8+ cells during antigen specific cytotoxicity. *PLoS One.* [Online] **2013**, http://journals.plos.org/plosone/ article?id=10.1371/journal.pone.0068916 (accessed Oct 24, 2017).