An Image-Based Method to Detect and Quantify T Cell Mediated Cytotoxicity of 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 either in a cell-to-cell contact or through cytokine production. As part of the adaptive immune system, these cells mount targeted attacks to rid the body of a variety of threats 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. 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.

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 release from pre-labeled target cells. Due to problems with radioactivity disposal, and low sensitivity due to spontaneous release of the isotope from target cells, 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, sensitivity 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 measure CTL cell mediated cytotoxicity in vitro 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 either 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 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 incubation period. Prior to image analysis, all images were automatically preprocessed to remove background signal. 3D image processing also included creating a 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 microscope reader combined with an automated digital microscope. Filter- and monochromator-based microscope readings are available, and the microscope module provides up to 44 filters for 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 assay, Cytation 5 features shaking, temperature control at 5°C, CO2/CO2, 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 system 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 BioSpa readers or imagers together with washers and dispensers for full workflow automation of up to eight microplates. Temperature, CO2/CO2 and humidity are 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 (Figure 3A-B). This T cell aggregation was in marked contrast to the 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 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 define 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. Cytoskeleton Imaging of 2D cytotoxic target cell induction. 4x overlaid CY5 and PI images. Red CY5 channel showing stained E-cells and yellow propidium iodide channel showing signal from necrotic cell probe following (A) 0, (B) 4, (C) 9, and (D) 164 hour co-culture incubation periods.

Figure 5. Brightfield/CYT5/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) 4, (C) 9, and (D) 164 hour co-culture incubation periods.

Quantification of Target Cell Cytotoxicity

Following image capture, T cell induced target cell cytotoxicity levels were quantified. Object mode analysis of differential signal patterns from the PI probe. Object size cutoff values were set to exclude single necrotic T cells, and include small cell aggregates observed at the 2D tumoroid edge(apart from increasing cytotoxicity. From the analysis, the number of necrotic 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 x-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 and superkine with cytotoxicity induction of T cells activated with 100% MDA-MB-231 cells, complemented with superkine. The PI necrotic cell probe was used to detect and quantify target cell death in the presence of T cell binding.

Figure 7. Effect of T cell concentration. Comparison of cytotoxic target cell induction by T cells added to wells at a constant cell density of 30,000 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) 20, (B) 10, and (C) 5:1 ratio cell to target cell ratios.

Figure 8. Cell confluence determination using image analysis. A. Images showing monolayer confluence and percent confluence determination. Pixels not included in confluent 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) 135 hour incubation periods. B. Plot of kinetic brightfield image percent confluence due to 3D tumoroid disintegration.

In this setup, T cell induced cytotoxicity can also be measured from brightfield images when incorporating a 3D tumoroid model. Tumoroids break apart over time in response to increasing T cell effect, releasing aggregates created as the 3D tumoroid disintegrated in response to increasing cytotoxicity.

Figure 9. Comparison of cytotoxic target cell induction between test conditions.

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 cultured with fibroblasts in the activation process.
2. The 3D tumoroid 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.