Application Note

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Validation of an Image-Based 3D Natural Killer Cell Mediated Cytotoxicity Assay

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Abstract

An *in vitro* model for Natural Killer (NK) cell-mediated cytotoxicity was developed using a collagen-based scaffold 3D cell culture technology. This technology induced HCT116 cells to aggregate into tumoroids over time, which became the target cells during the cytotoxicity assay. Cytotoxicity was assessed by both phosphatidyl serine exposure (apoptosis) and plasma membrane rupture (necrosis) using fully automated workflows. Cytotoxicity was quantified using NK cells alone and with IL-2 stimulation, where a significant increase of cytotoxicity was evident. Cytotoxicity with this model was compared to HCT116 cells adhered to microplates in a conventional 2D format. The 3D model was far superior in both maintaining cell health over time and accurately depicting cytotoxic events.

Introduction

NK cells are cytotoxic lymphocytes found in peripheral blood that play a role in host defense and immune regulation. NK cells are particularly interesting in immunotherapy due to their potential to target and destroy specific cancer cells, while leaving nontarget healthy cells intact. The anticancer activity of NK cells is shown to be associated with an improved prognosis in several cancers such as colorectal cancer[1], nonsmall cell lung cancer[2], and clear cell renal cell carcinoma.[3]

To properly study the interaction between NK cells and target tumor cells, an appropriate *in vitro* model system must be established. However, much of the data published to date used cancer cells plated as a two-dimensional (2D) monolayer on the bottom of microplate wells. A growing amount of data has shown that cells cultured in this manner lack the cell:cell and cell:matrix communication, metabolic gradients, and polarity demonstrated *in vivo*.[4] The ability to perform matrix infiltration studies is also eliminated with the use of 2D cell culture. By embedding cancer cells into a three-dimensional (3D) matrix and allowing the formation of tumoroids, the shortcomings of using 2D cultured cells can be overcome as communication networks and cellular gradients observed within *in vivo* tumors are reestablished.

With the incorporation of 3D cultured cells, however, traditional methods to monitor target and NK cell interactions, and subsequent target cell killing can become problematic. Microplate reader assays designed to detect signal from cell monolayers lack the sensitivity to quantify signal from tumoroids surrounded by noncell containing areas in the well with no signal generation. By incorporating microscopy-based detection and cellular analysis, signal emanating solely from tumoroids is quantified, providing a highly robust method to detect induced toxicity within target cancer cells.

This application note describes a novel 3D NK cell mediated cytotoxicity (CMC) assay. Using the RAFT 3D cell culture system (Figure 1), HCT116 colorectal cancer cells were embedded within a collagen hydrogel of defined concentration and thickness, mimicking *in vivo* extracellular matrix (ECM). Following cell propagation to create tumoroids within the matrix, NK cells were labeled with a cell tracking dye and added to appropriate wells. Fluorescent apoptosis and necrosis probes were also added to track cytotoxic events within the tumoroids. Cellular imaging and analysis were performed at regular intervals using an automated incubator over a seven-day period to monitor NK cell induced apoptosis and necrosis of the HCT116 cells making up each tumoroid. The effect of IL-2 stimulation on these cytotoxic events was performed as well as a comparison to the same experiments performed on a monolayer of adhered HCT116 cells.

Materials and methods

Materials

Cells: HCT116 epithelial colorectal carcinoma cells (part number CCL-247) were obtained from ATCC (Manassas, VA). Negatively selected peripheral blood CD56+ CD16+ natural killer (NK) cells (part number 2W-501) were donated by Lonza (Walkersville, MD).

Assay and experimental components: The RAFT 96-well Small Kit, including reagents and absorbers, (part number 016-1R17) and LGM-3 (Lymphocyte Growth Medium-3) (part number CC-3211) were generously donated by Lonza. 96-well TC treated microplates (part number 655090) were donated by Greiner Bio-One, Inc., (Monroe, NC). CellTracker Deep Red dye (part number C34565) was procured from Thermo Fisher Scientific (Waltham, MA). Kinetic Apoptosis Kit, containing pSIVA-IANBD, a green fluorescent probe that detects phosphatidylserine exposure, and propidium iodide, a plasma cell membrane-impermeant probe (part number ab129817) was donated by abcam (Cambridge, MA). IL-2 IS (part number 130-097-744) was donated by Miltenyi Biotec (San Diego, CA).

Agilent BioTek Cytation 5 cell imaging multimode reader: Cytation 5 is a modular multimode microplate reader combined with automated digital microscopy. Filter- and monochromator-based microplate reading are available, and the microscopy module provides a wide variety of magnification in fluorescence, brightfield, color brightfield, and phase contrast using objectives ranging from 1.25x to 60x. 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 temperature control to 65 °C, CO_2/O_2 gas control, and dual injectors for kinetic assays, and is controlled by integrated Agilent BioTek Gen5 microplate reader and imager software. The imager and software were used to capture brightfield and fluorescent images for 2D and 3D CMC assays and quantify the level of NK-induced cytotoxicity.

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 8 microplates. Temperature, CO_2/O_2, 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 and automatically transferred to the Cytation 5 at designated time points, as noted in a previous application note, to monitor cytotoxicity in 2D- and 3D-cultured cells.

Methods

2D- and 3D-target, and NK-effector cell preparation: On Day 0, HCT116 target cells were mixed with the prepared RAFT Collagen I suspension and dispensed to 96-well TC-treated microplates in a volume of 240 µL to yield 1,200 cells/well. The cell plate was incubated at 37 °C/5% CO_2 for 15 minutes, followed by addition of the absorbers in the RAFT plate, and a second 15-minute incubation at 37 °C/5% CO_2 during which the RAFT process concentrated the collagen density to a physiologically relevant strength. The absorbers were then removed and 100 µL of new medium was added to the concentrated cell/collagen hydrogel (Figure 1). Cells were propagated at 37 °C/5% CO_2 in the plates for seven days to allow tumoroid creation through cell doubling, with media exchanges every two days. On Day 6, a total of 3,000 cells/well of HCT116 cells intended for 2D culture were added to a separate 96-well TC-treated microplate and allowed to attach overnight.

On Day 7, negatively selected peripheral blood CD56+ CD16+ NK cells were prepared. Cryopreserved NK cells were thawed and diluted in LGM-3 (Lymphocyte Growth Medium-3) per the manufacturer's protocol, then stained with the fluorescent CellTracker Deep Red dye. The NK cells were diluted to a concentration that equaled a 20:1 or 10:1 ratio of final HCT116 populations per well in media containing either abcam Kinetic Apoptosis Kit pSIVA-IANBD and propidium iodide reagents and 500 U/mL human IL-2 IS, or apoptosis kit reagents alone, and added to the 3D tumoroids and the 2D-cultured cells.

Automated cell mediated cytotoxicity process: After the diluted NK cell mixtures were added to the 96-well 3D and 2D test plates, the plates were placed into the BioSpa 8 at 37 °C/5% CO_2 for an incubation period of 120 hours. The BioSpa method was programmed such that plates were automatically moved to the Cytation 5 every two hours, where fluorescent imaging was carried out to monitor NK effector cell induced cytotoxicity (Figure 2). The Cytation imaging chamber was also maintained at 37 °C/5% CO_2 to ensure consistent environmental cell conditions. Imaging channels included in the experimental procedure were as follows:

- **GFP:** pSIVA-IANBD fluorescent probe binding to externally exposed phosphatidyl serine (PS) on apoptotic cells
- **Propidium iodide (PI):** PI intercalating dye bound to necrotic cell DNA
- **CY5:** CellTracker Deep Red stained NK cells.

3D CMC images were captured using a 4x objective and an 11-slice z-stack with 10 µm steps, while 2D CMC images were captured using a 10x objective and a 2 × 2 image montage.

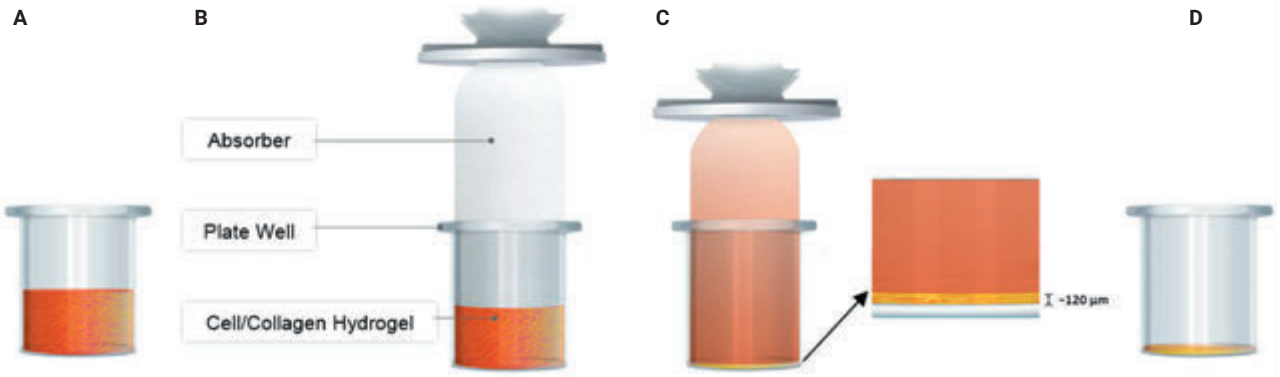

Figure 1. Creation of 3D cell/collagen hydrogel using RAFT System. (A) Cell/collagen mix dispensed to wells of tissue culture (TC) treated plate. (B) Absorber insertion into plate well. (C) Absorption of medium, concentrating collagen and cells to *in vivo* strength, creating an ~120 µm thick hydrogel. (D) Removal of absorber prior to dispense of fresh cell medium.

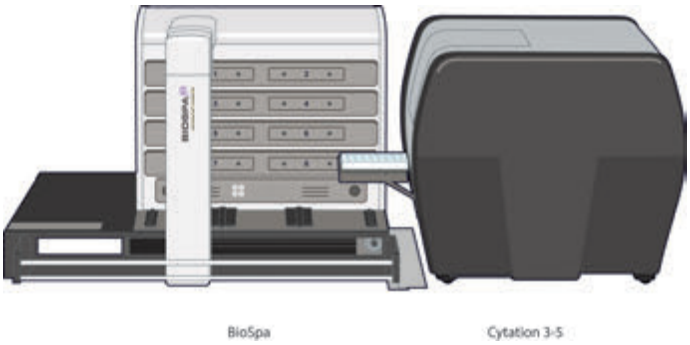

Figure 2. Agilent BioTek BioSpa live cell analysis system consisting of Agilent BioTek BioSpa 8 automated incubator (left) and Agilent BioTek Cytation 5 cell imaging multimode reader (right) used to automate the cell-mediated cytotoxicity assays.

Results and discussion

Tumoroid formation conformational imaging

Brightfield imaging was performed to confirm that 3D tumoroids formed over the 7-day incubation period via HCT116 propagation.

As shown in Figure 3, HCT116 cells initially suspended in the RAFT hydrogel matrix continuously divided over the 7-day incubation period to create multicellular structures. 3D growth and tumoroid assembly was also confirmed by images captured at separate z-heights in Figures 3A to 3C. Here, cells within the different tumoroids captured in the image were seen to be in focus at varying z-planes, confirming that the formed tumoroids existed in a 3D configuration and not a 2D layer of cells within the hydrogel. A final in focus image was then able to be created using the z-projection capabilities in the Agilent BioTek Gen5 software to improve downstream cellular analysis (Figure 3D).

3D NK cell-mediated cytotoxicity imaging

Following NK cell addition to the 3D plates, z-stacked images were automatically captured of HCT116 tumoroids every 2 hours over the entire 120-hour incubation period from 20:1 and 10:1 NK treated positive control wells, as well as negative control wells containing no NK cells (Figure 4).

By using overlaid final projections of the z-stacked images, NK cell interactions with HCT116 tumoroids, in addition to apoptotic and necrotic cell induction, were tracked in a kinetic fashion for each test condition.

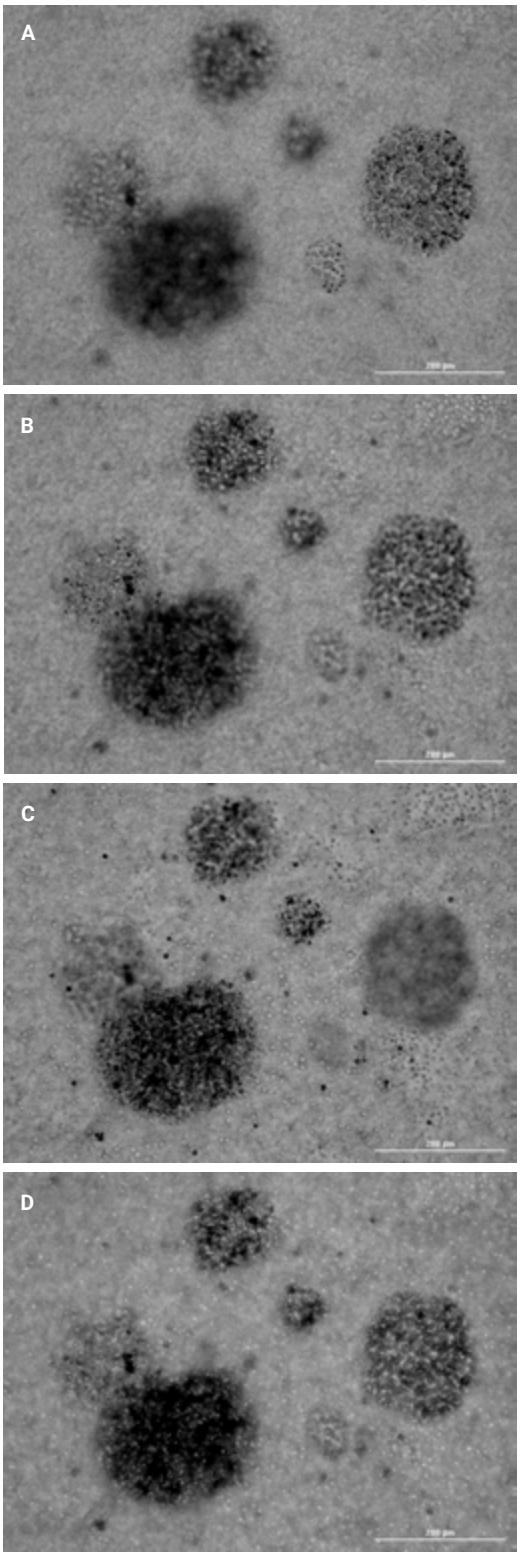

Figure 3. 3D Tumoroid formation conformational imaging. (A,B,C) Brightfield image captured following a 7-day incubation in plate wells using 10x objective at three separate z-heights within the RAFT hydrogel. (D) Final z-projected image of tumoroids.

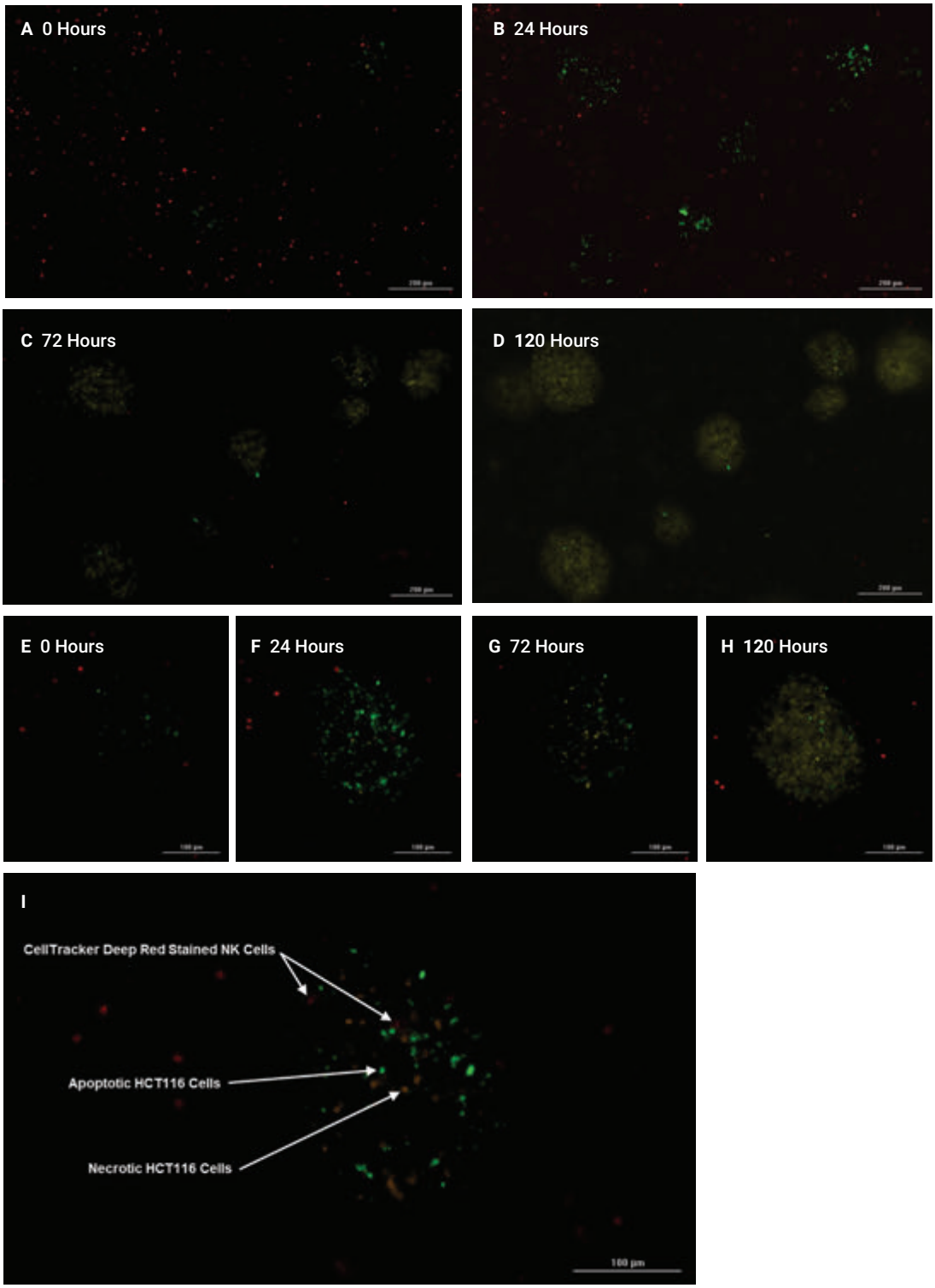

Figure 4. Imaging of NK cell-induced apoptosis and necrosis within HCT116 tumoroids. (A to D) Fluorescent overlaid final projected multiple tumoroid images captured using a 4x objective, and (E to H) zoomed single tumoroid images following treatment with a 20:1 ratio of IL-2 stimulated NK cells and 0, 24, 72, or 120 hour incubations, respectively. (I) Projected, zoomed image demonstrating green pSIVA-IANDB stained apoptotic cells, yellow propidium iodide stained necrotic cells, and red CellTracker Deep Red labeled NK cells.

3D NK cell apoptosis and necrosis induction analysis

Using the z-stacked images, Gen5 software automatically preprocessed the samples to remove excess background signal and prepare the image for quantitative analysis. Two separate cellular analysis steps were conducted to place object masks around areas within the image meeting primary analysis criteria in either the GFP or PI imaging channel. Minimum and maximum object sizes and threshold fluorescence values were set such that only apoptotic or necrotic areas within target tumoroids were identified (Figures 5A and 5B).

The graphs in Figures 5C and 5D demonstrate how the cell area within tumoroids treated with NK cells, identified with the green fluorescent pSIVA-IANBD probe, increases rapidly over the first 24 hours of exposure, then decreases back to basal levels. As external phosphatidyl serine (PS) exposure is a hallmark of early apoptotic activity, and the reagent binds to externally exposed PS, the observed phenomenon is consistent with expected results as apoptosis is initially induced within treated tumoroid cells. As NK cells continue to interact with tumoroids, cells become increasingly necrotic and PS once again internalizes within the lipid bilayer. This is confirmed by the increase in signal from the fluorescent cell impermeable PI probe (Figures 5E and 5F). Cellular necrosis leads to loss of membrane integrity, allowing PI to bind to the nucleus.

These figures also demonstrate that untreated HCT116 tumoroids maintain high cell viability and negligible observable apoptotic and necrotic increases over time, with or without IL-2 stimulation. This further validates that exposure to NK cells causes initial HCT116 cellular apoptosis followed by secondary necrosis, and also agrees with previously published literature results.[5]

From the data, it is apparent that the higher NK cell ratio does not affect the kinetics of either PS inversion or plasma membrane rupture, but induces a proportional increase in both phenotypes according to the relative amount of NK cells. Finally, stimulation by IL-2 was also shown to significantly increase NK-induced cytotoxicity compared to unactivated NK cells at both ratios, again agreeing with previous literature findings.[6]

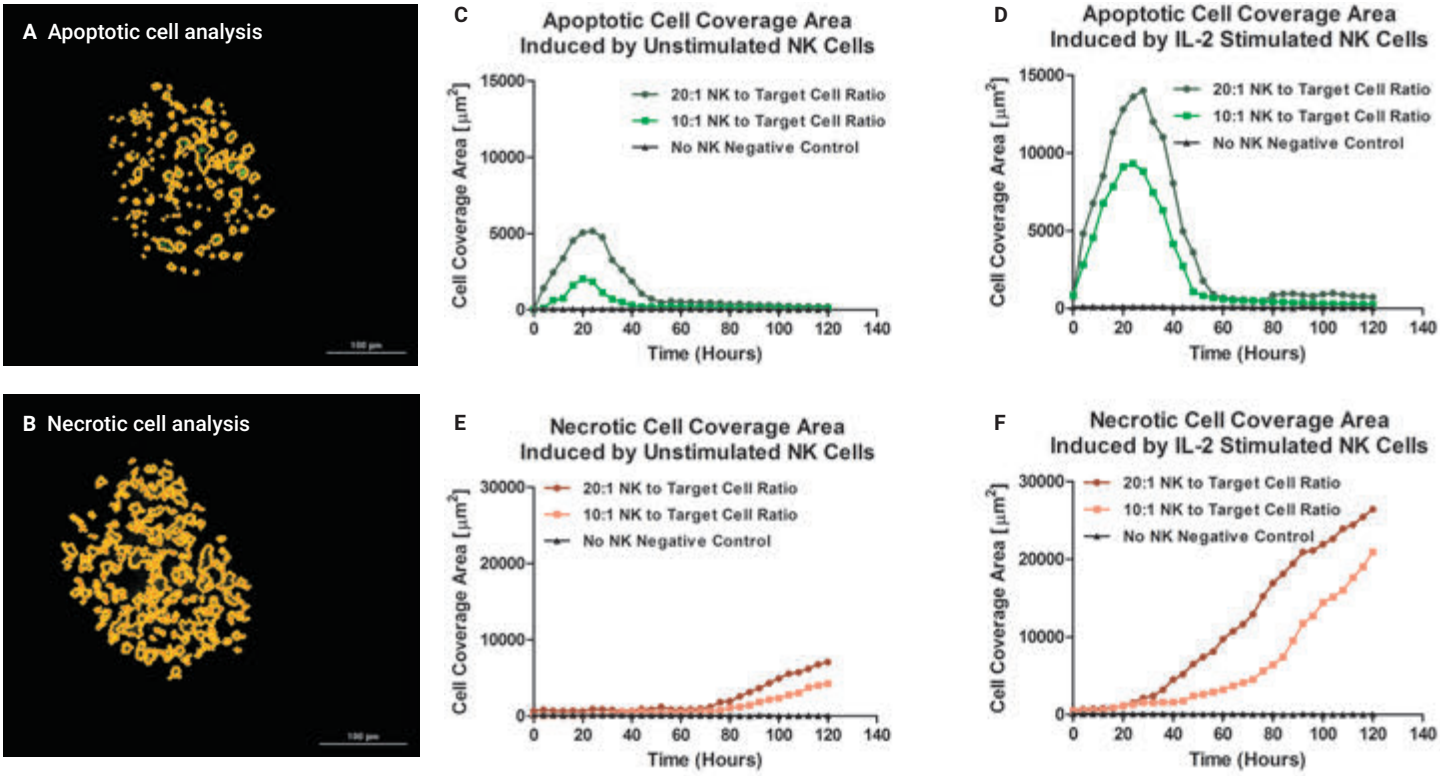

Figure 5. 3D NK CMC analysis. Agilent BioTek Gen5 software placed object masks around (A) apoptotic or (B) necrotic cells during individual analysis of zoomed images of a single tumoroid. Total cell coverage area calculated from object masks placed around all apoptotic cells within all tumoroids in the field of view following interaction with (C) unstimulated or (D) IL-2 stimulated NK cells, and necrotic cell coverage area following interaction with (E) unstimulated or (F) IL-2 stimulated NK cells at each timepoint.

2D NK cell-mediated cytotoxicity imaging

Kinetic montage images were captured from test plates containing 2D cultured HCT116 and NK cells or HCT116 cells alone using the aforementioned automated monitoring procedure (Figure 6).

Following image preprocessing and stitching, individual primary analyses were once again carried out using either the GFP or PI channel on the final complete images. Gen5 placed object masks around individual apoptotic or necrotic cells per image (Figures 7A and 7B).

It is evident in Figure 7 that the peak of maximum PS exposure is delayed in the 2D format to approximately 40 hours, which is approximately 2x longer relative to 3D. Yet, the onset of plasma membrane rupture occurs much more rapidly in 2D relative to 3D, and by the time of peak PS exposure there is already significant plasma membrane rupture. Conversely, the HCT116 cells in the tumoroids appear to first undergo PS exposure, then plasma membrane rupture consistent with the progression of apoptotic programmed cell death induced by NK cell activity. In addition, after 60 hours, the number of apoptotic and necrotic cells begins

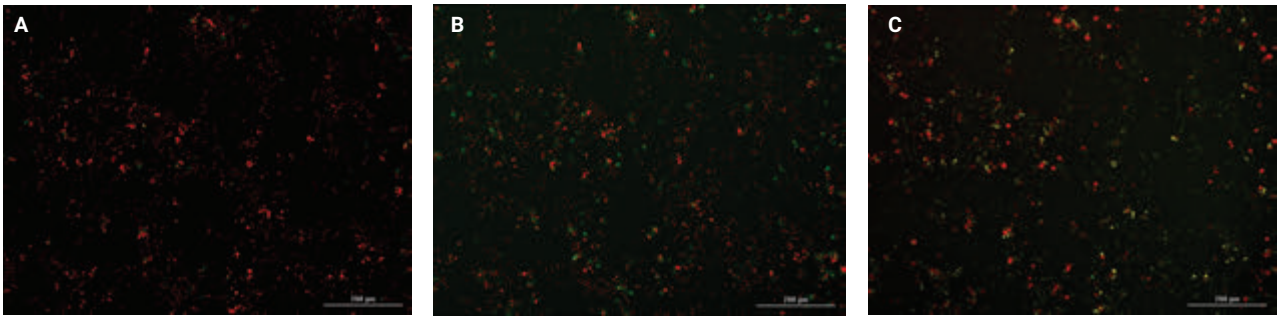

Figure 6. NK cell induced apoptosis and necrosis within 2D HCT116 cells. Final stitched images following treatment with a 20:1 ratio of IL-2 stimulated NK to 2D cultured HCT116 cells following (A) 0, (B) 24, or (C) 120-hour incubations. Green: pSIVA-IANBD stained apoptotic cells; yellow: propidium iodide stained necrotic cells; red: CellTracker Deep Red labeled NK cells.

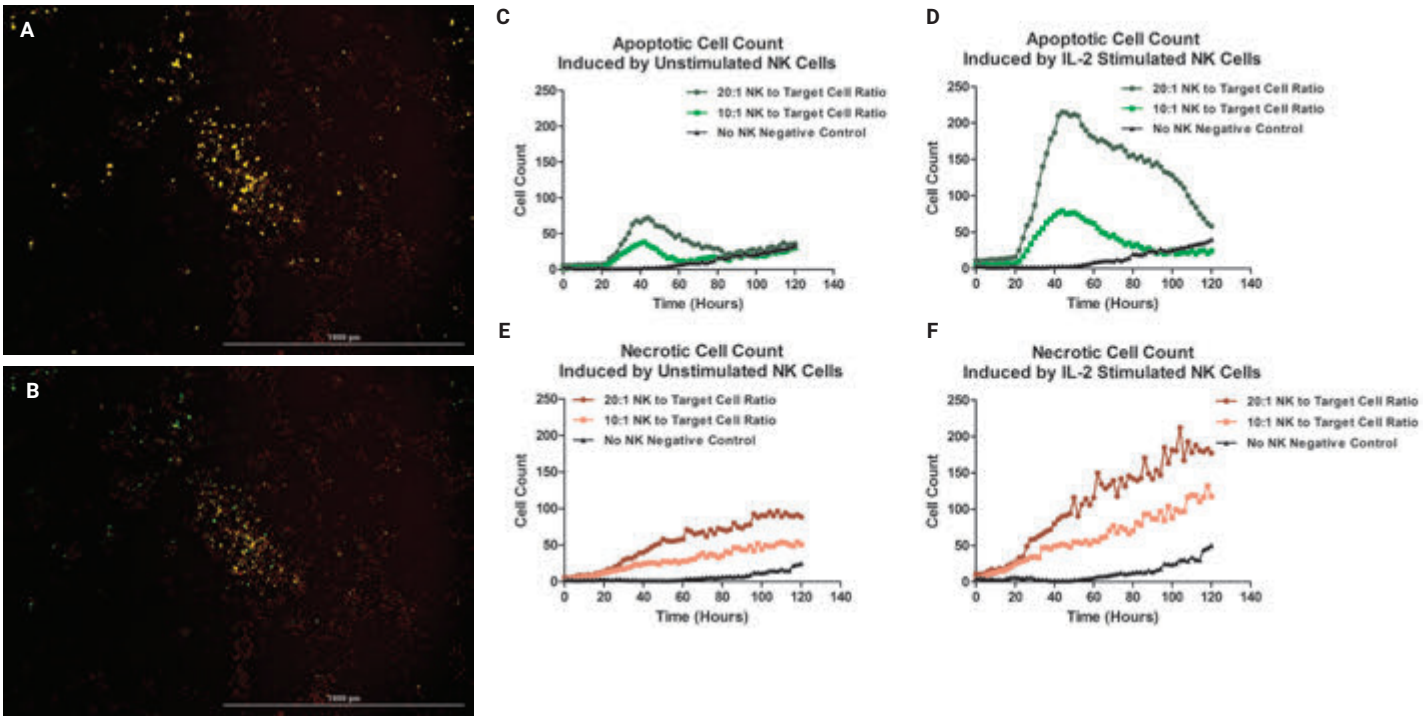

Figure 7. 2D NK CMC analysis. Agilent BioTek Gen5 software placed object masks around (A) apoptotic or (B) necrotic cells during individual analysis of images captured from 3D test plates. Total cell numbers calculated from object masks placed around all apoptotic cells per image following interaction with (C) unstimulated or (D) IL-2 stimulated NK cells; and necrotic cells following interaction with (E) unstimulated or (F) IL-2 stimulated NK cells at each timepoint.

to increase in the NK cell negative control. At 120 hours, there are approximately the same number of apoptotic cells in all conditions. At the same timepoint, there are significant levels of necrotic cells in the negative control: approximately 50% of the number demonstrated in the 10:1 ratio; and approximately 25% of the 20:1 ratio.

Conclusion

The RAFT 3D Cell Culture System provides a useful model for NK cell-mediated cytotoxicity assays. The assay workflow is enabled through automation using the Agilent BioTek BioSpa live cell analysis system providing walk-away kinetic imaging and quantification of both apoptosis (PS exposure) and necrosis (plasma membrane rupture). It was found that the 3D cell model incorporating aggregated HCT116 cells into tumoroids embedded in a collagen matrix was far superior to HCT116 cells adhered in a monolayer (2D) to microplates. The cells remained completely viable over a period of 120 hours in the negative control and NK cell-mediated cytotoxicity progressed kinetically in a manner consistent with programmed cell death. Conversely, in adhered cells, both plasma membrane rupture and PS exposure occurred in the same time frame and the negative control contained large proportions of cytotoxic cells during the latter half of the kinetic experiment.

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