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Image-Based, Label-Free Methods to Detect and Quantify General and Directed T Cell Activation

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Abstract

T cell activation is an essential initial step of a targeted immune response *in vivo*. Activation of T cells *in vitro* causes increased cell proliferation and formation of cell aggregates, which were tracked using label-free imaging and analysis. T cell general activation rates in the presence of antibodies and an engineered cytokine IL-2 were compared to directed activation where T cells were also exposed to target cell spheroids.

Introduction

T cells are critical to the adaptive immune system as they recognize and destroy pathogenic cells while leaving healthy cells unharmed. Memory capabilities allow T cells to mount an effective and targeted immune response for prolonged periods of time. As such, they are a promising focus for novel cancer therapies such as antitumor monoclonal antibodies, cancer vaccines, adoptive T cell transfers and checkpoint blockades.[1] Key processes in these T cell immunotherapies are the directed activation and expansion of the T cells. Naïve T cells are activated when they encounter MHC-peptide complexes on the surface of a cell for which their T cell receptors (TCR) have high affinity. In addition to the signaling cascade created, a necessary costimulatory signal is provided by protein receptors such as CD28, which interact with the antigen presenting cell. This stimulates expression of cytokines, including IL-2, which is a critical growth factor for expansion of the T cells. As the T cells are activated, they form homotypic aggregates within the well, and proliferation rates increase.[2]

When cultured *in vitro*, T cells are activated through the addition of antigen(s), and often, IL-2. However, to strengthen the response by directing the activation, and better mimicking *in vivo* behaviors, the T cells may be cultured in the presence of target cells, which secrete several soluble factors, including the aforementioned cytokines, to increase T cell proliferation[3] and sensitize the T cells to enhance their ability to seek out target cancer cells when used in cell mediated cytotoxicity applications. Target cells cultured in two-dimensional (2D) monolayers, however, lack the cell:cell and cell:matrix communication, metabolic gradients, and polarity demonstrated *in vivo*.[4] Three-dimensional (3D) cell culture methods create environments and communication networks similar to those seen in the body which can properly stimulate the T cells.

This application note demonstrates the ability to track and quantify general and directed T cell activation using label-free imaging methods and cellular analysis. In the general method, T cells are activated using anti-CD3 and anti-CD28 antibodies which mimics stimulation by antigen presenting cells and varying concentrations of an engineered IL-2 cytokine. Directed T cell activation uses the T cells, antibodies and engineered IL-2 along with 3D bioprinted target spheroids. In this method, target cells are magnetized with a biocompatible nanoparticle assembly consisting of gold, iron oxide, and poly-L-lysine that electrostatically and nonspecifically attaches to cell membranes. The magnetized cells are then directed using mild magnetic forces to form aggregates where cells interact and build larger 3D environments with extracellular matrix (ECM) that represent native tissues. The spheroids then interact spontaneously with T cells and other components in the well. In both methods, cellular imaging and analysis were performed using a novel cell imaging multimode reader. The cells were maintained in a humidified 37 °C/5% CO_2 environment using an automated incubator and transferred to the imager at regular intervals. Proliferation rates and cellular aggregation were monitored over a six-day period as indications of T cell activation.

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 Thermo Fisher 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). CELLSTAR clear cell culture 24-well cell-repellent microplates (GBO part number 662970) 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).

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 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 Agilent BioTek Gen5 microplate reader and imager software, which also automates image capture, analysis and processing. The instrument was used to kinetically monitor T cell activity over seven days using the brightfield channel.

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 to monitor T cell activation.

Methods

General T cell activation

CD3+ T cells were thawed and prepared according to the manufacturer's recommendation. The cells were then diluted in complete RPMI 1640 medium to concentrations of either 100,000 cells/mL or 500,000 cells/mL in media containing 250 ng/mL each of anti-CD3 and anti-CD28 antibodies, in addition to IL-2 Superkine concentrations ranging from 100 to 0 ng/mL. A volume of 1 mL of the different test conditions was added to the wells of a 24-well cell repellent microplate. The plate was then placed into the BioSpa to incubate for six days at 37 ºC/5% CO_2. The BioSpa was preprogrammed such that every six hours, the plate was automatically delivered to the Cytation 5 where 4x brightfield images were captured using a 12 row by 10 column montage. Following 72 hours of incubation, the BioSpa was paused, the plates manually removed, and transferred to a sterile tissue culture hood. Spent media was slowly removed using manual aspiration and replaced with fresh media containing the same concentrations of IL-2 Superkine and antibodies as originally added. The plates were then transferred back to the BioSpa and the run continued for an additional 72 hours.

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 1, treated with 600 µL NanoShuttle-PL overnight at 37 °C/5% CO_2. After incubation, cells were trypsinized, washed, and incubated for 3 to 5 minutes at 37 °C/5% CO_2. 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 ECM formation took place during an eight-hour incubation at 37 °C/5% CO_2. After incubation, the cells and ECM were broken up and resuspended in complete advanced DMEM medium, in preparation for bioprinting spheroids in a 24-well plate (Figure 1).
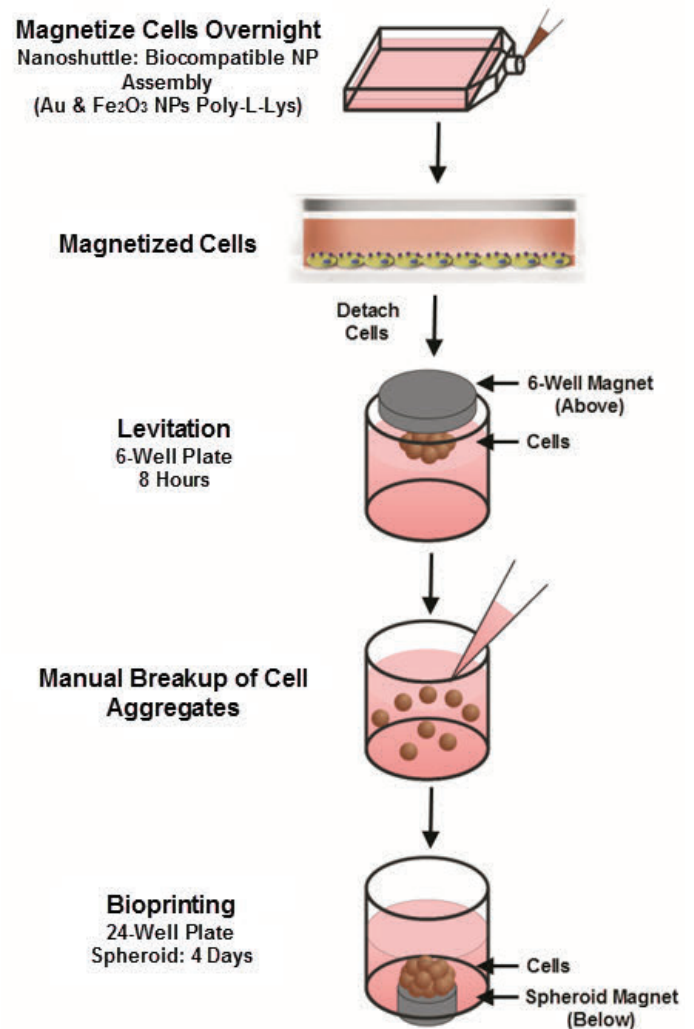

Figure 1. Preparation of target cells for directed T cell activation.

Directed 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: (A) 100% MDA-MB-231; (B) 75% MDA-MB-231 and 25% fibroblasts; (C) 50% MDA-MB-231 and 50% fibroblasts; (D) no cells. A final test condition included wells with (E) media only. 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% CO_2 for four days where the cells aggregated into multiple 3D spheroids within each well (Figure 2).
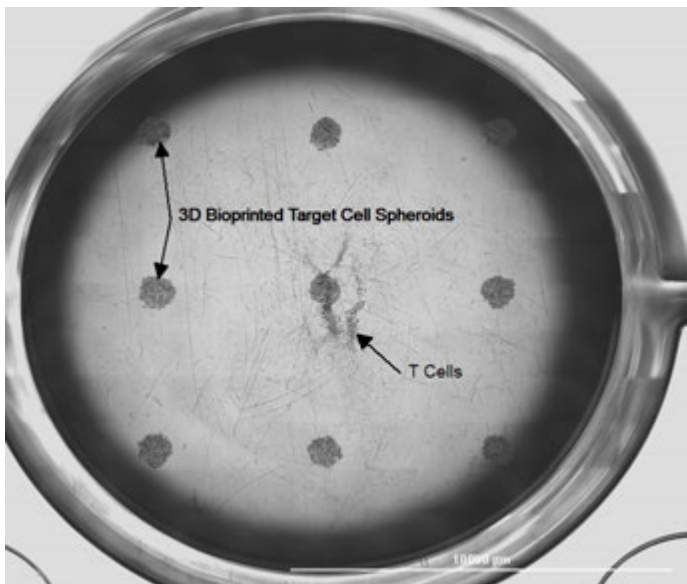

Figure 2. 24-well plate well showing co-culture of T cells and bio-printed magnetized 3D target spheroids prior to commencement of directed activation. T cells added in a 10:1 ratio to target cells previously aggregated into 3D spheroids.

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 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 for holding the spheroids, and replaced with fresh media containing the T cells, antibodies, and Superkine as previously described (Figure 2). The plate was then placed back into the Agilent BioTek BioSpa to incubate for six days. The BioSpa was again preprogrammed to capture a 12 × 10 image montage from each test well every six hours. Manual exchange of media, superkine, and antibodies was again performed after 72 hours as explained for general T cell activation.

Image processing and analysis

Following capture of individual image tiles, an Image Stitching step using the criteria in Table 1 was applied in Gen5 to create final images encompassing the entire well.

Table 1. Brightfield image stitching parameters.

Image Stitching Parameters	
Registration Channel	Brightfield
Fusion Method	Linear blend
Crop Stitched Image	Checked
Downsize Final Image	27.03%

Quantitative analysis to determine the extent of proliferation and cell aggregation was then performed using the parameters identified in Table 2.

Table 2. Confluence measurement parameters.

Confluence Analysis Parameters	
Data In	Stitched [Brightfield]
Lower Value	Unchecked
Upper Value	Checked (7500)
Metric of Interest	Confluence

Results and discussion

General T cell activation imaging

Kinetic brightfield imaging was carried out using the previously described settings to demonstrate the ability to track and quantify T cell activation.

When visually comparing the images captured of T cell activation, higher levels of cell proliferation, indicated by an increase in darker, highly confluent portions of the image, and aggregation, indicated by dense circular areas of concentrated cells, were observed in wells with T cells cultured with antibodies and IL-2 Superkine (Figures 3C and 4C) compared to those cultured with antibodies alone (Figures 3A and 4A). Video of T cell activation over time is available at http://bit.ly/t-cell. This phenomenon confirms the high levels of T cell activation that can be attained when using the IL-2 Superkine, previously reported in literature.[5] The results also confirm the ability of the Cytation 5 to monitor T cell activation in a label-free manner.
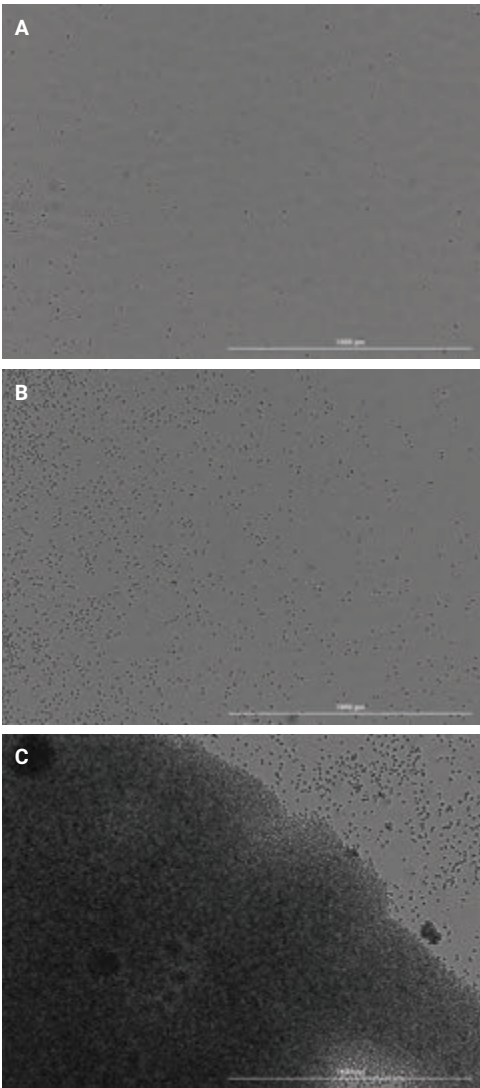
Figure 3. Brightfield imaging of general T cell activation proliferation and aggregation, 100,000 cells/well. 4x images of (A) 0 ng/mL IL-2 Superkine, 144 hours; (B) 100 ng/mL IL-2 Superkine, 0 hours; (C) 100 ng/mL IL-2 Superkine, 144 hours incubation.

Figure 4. Brightfield imaging of general T cell activation proliferation and aggregation, 500,000 cells/well. 4x images of (A) 0 ng/mL IL-2 Superkine, 144 hours; (B) 100 ng/mL IL-2 Superkine, 0 hours; (C) 100 ng/mL IL-2 Superkine, 144 hours incubation.

Quantification of activation was also performed. As seen in Figures 3C and 4C, highly proliferative cells that have aggregated together appear as dark areas within the image compared to bacground areas containing no cells. The difference in brightfield signal for pixels containing these two conditions is taken advantage of to perform image analysis. By setting a threshold such that only pixels containing darker proliferative, aggregated cells are included, confluence measurements can be created to track T cell activation. The generated curves are then normalized by subtracting initial confluence values at time 0 from those calculated at each subsequent timepoint.
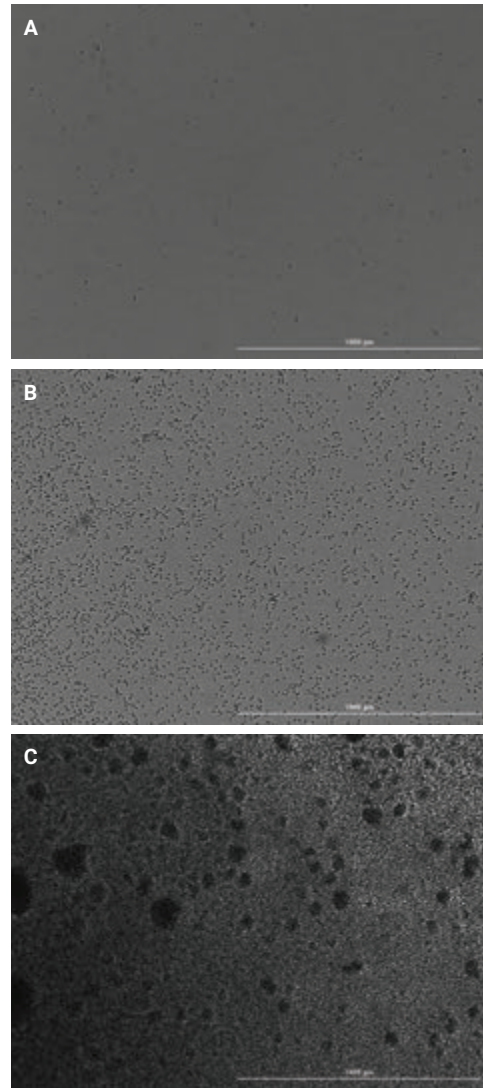
The results from Figure 5A, using 100,000 cells/well, discernible changes in confluence are seen after approximately 96 hours of incubation with the highest concentrations of IL-2 Superkine tested and continue in a consistent manner, reaching a peak after 144 hours, or six days of incubation. Lower IL-2 Superkine concentrations tested, as well as the negative control, have little to no effect on activation, as expected.
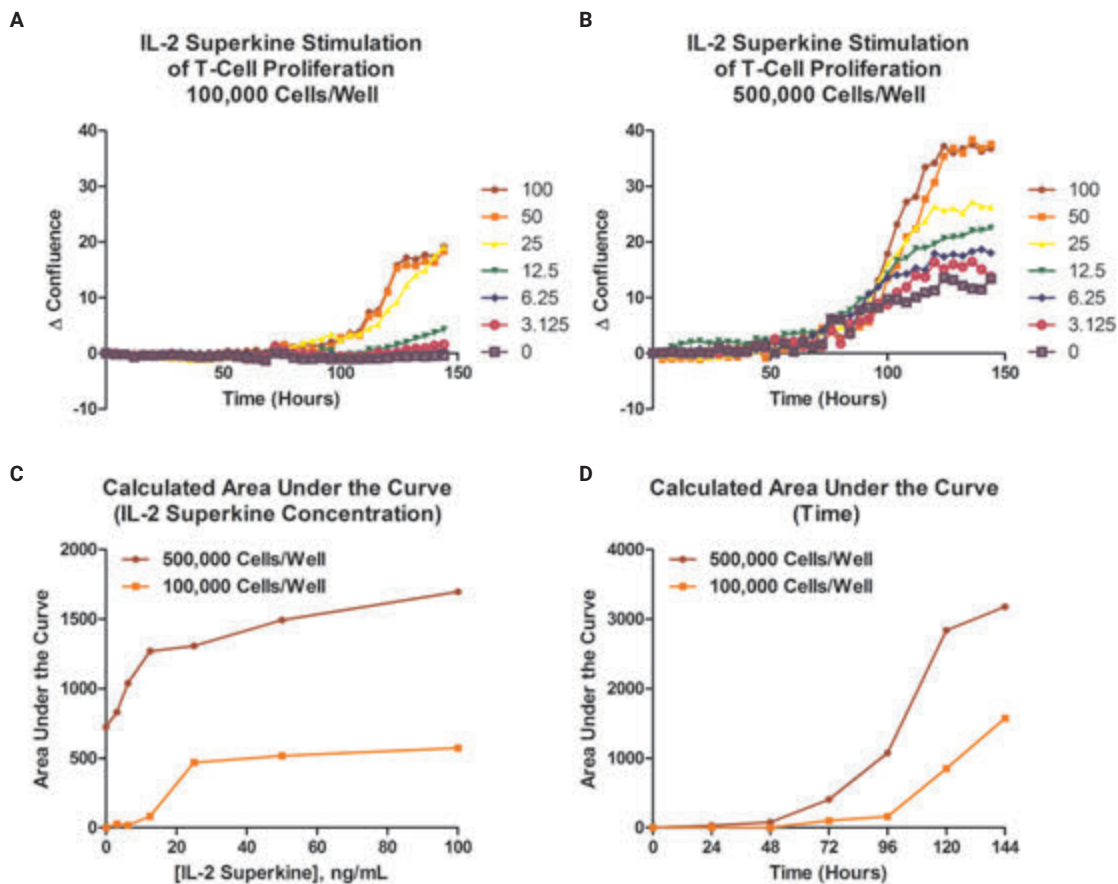
Figure 5. Quantification of general T cell activation. Change in percent confluence for (A) 100,000 cells/well and (B) 500,000 cells/well. Area under the curve plots generated from (C) IL-2 Superkine concentration specific delta confluence curves and (D) delta confluence values for variable superkine concentrations at specific incubation timepoints.

The same analysis parameters were also tested with wells containing 500,000 T cells/well. Activating higher concentrations of T cells in the same well is preferable for certain downstream applications. Therefore, it is essential that correct confluence measurements can also be performed under these conditions. Consistent increases in confluence are also seen for 500,000 cells/well (Figure 5B) across the entire incubation period. Yet increases in confluence begin earlier, around 72 hours, compared to 96 hours using 100,000 cells/well. In addition, negative control wells exhibit distinguishable changes in proliferation and cell aggregation not seen when using lower cell concentrations. These two phenomena can be attributed to the higher T cell concentration such that cells in closer proximity to each other will create a higher basal level of activation. Then, when in the presence of the IL-2 Superkine, induced activation will also begin following a shorter incubation period.

The effect of [IL-2 Superkine] and time can be further elicited from the data by performing area under the curve calculations from kinetic IL-2 Superkine concentration curves, as well as variable superkine concentrations at specific timepoints. It is apparent from Figure 5C that there is a significant T cell activation using 500,000 T cells/well with or without IL-2 Superkine. The relative responses for the two conditions appear similar in their response to IL-2 Superkine dize, but the higher T cell/well condition demonstrates approximately a 3-fold increase in activation at higher [IL-2 Superkine]. Finally, the curves in Figure 5D, for both 500,000 and 100,000 cells/well, illustrate the cumulative effect that time has on T cell proliferation and aggregation, and the importance of activating T cells for the appropriate incubation period to attain the best possible results in downstream applications.

Directed T cell activation imaging

By adding specific antibodies and stimulatory cytokines, T cells attain an activated state, and are prepared to seek out antigen expressing invading cells. However, without being primed to recognize specific antigens, the immune response will be nonspecific and therefore diminished in potency. With the incorporation of specific target cells during the activation process, T cells not only become activated, but are tuned to recognize antigens expressed by target cancer cells. The ability to perform, monitor, and quantify directed T cell activation, therefore, is highly desirable.

Following addition of the T cells to the wells of the 24-well plate containing the bioprinted target cell spheroids, kinetic label-free imaging was performed. Figure 2 illustrates placement of the target cell spheroids and T cells in each of the test wells for directed activation at time 0 of the incubation period.

Images captured from wells containing T cells activated with 250 ng/mL anti-CD3 and anti-CD28 antibodies and 100 ng/mL IL-2 Superkine, in addition to MDA-MB-231 cancer cell spheroids, when compared to negative control wells containing nonactivated T cells, once again demonstrate the ability to monitor activation through label-free imaging, as well as the capacity of the IL-2 Superkine to activate T cells even in the presence of co-cultured target cells.

Plots of changes in confluence values and area coverage (data not shown) also confirm that proliferation and cell aggregation can be quantified when using a co-cultured cell model. Furthermore, the results illustrate that equivalent levels of T cell activation are achieved from directed and general T cell activation.
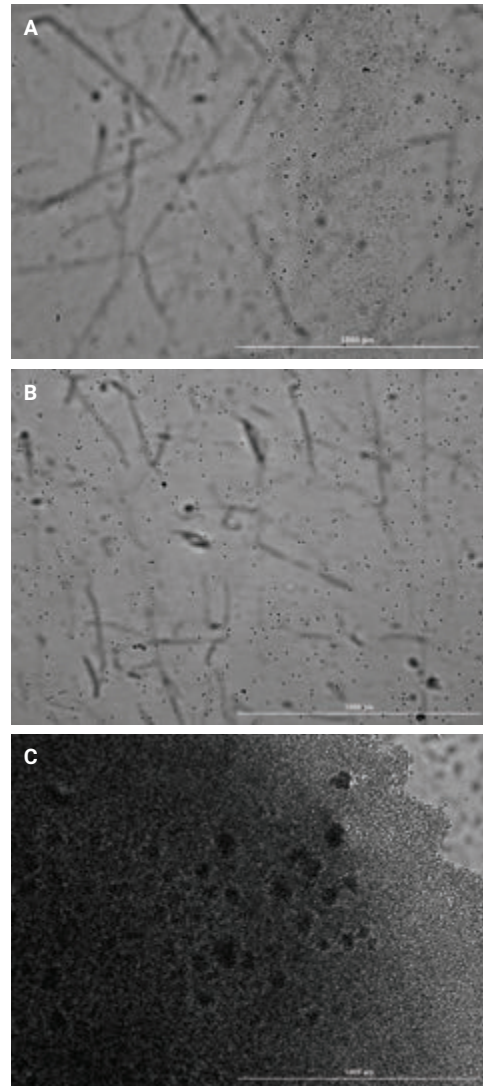

Figure 6. Brightfield imaging of directed T cell activation proliferation and aggregation, 100,000 cells/well. 4x images of (A) 100 ng/mL IL-2 Superkine plus target cells, 0 hours; (B) 0 ng/mL IL-2 Superkine, 144 hours; (C) 100 ng/mL IL-2 Superkine plus target cells, 144 hours incubation.

Conclusion

Through the incorporation of the Agilent BioTek BioSpa 8 automated incubator and Agilent BioTek Cytation 5 cell imaging multimode reader, kinetic imaging can be used to monitor cell proliferation and aggregation as phenotypic metrics of T cell activation. The two phenotypes are then quantified using image and cellular analysis tools in Agilent BioTek Gen5 microplate reader and imager software. Directed activation can also be easily performed by adding magnetized target cells prior to activation. The combination of appropriate target and therapeutic cell models, a potent activation cocktail, and walk-away, label-free imaging creates an ideal method to monitor this critical first step in the immunotherapy treatment process.

References

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