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APPLICATION PAPER

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 the targeted immune response in which they play. Activation of T cells causes increased cell proliferation and formation of cell aggregates, which can be tracked using label-free imaging and analysis. T cell activation rates in the presence of antibodies and an engineered cytokine IL-2 (general activation), in addition to with and without the presence of target cell spheroids (directed activation), was quantified over time.

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 components 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, in order 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 and sensitize the T cells to enhance their ability to seek out target cancer cells when used in cell mediated cytotoxicity applications[3]. 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.

Here we demonstrate 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 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 non-specifically 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 spontaneoustly with T cells and other

components in the well. In both methods, cellular imaging and analysis were performed at regular intervals using an automated incubator over a six-day period to monitor proliferation rates and cellular aggregation as indications of T cell activation.

MATERIALS AND METHODS

Materials

Cells and Media

MDA-MB-231 epithelial breast adenocarcinoma cells (Catalog No. HTB-26) were obtained from ATCC (Manassas, VA). Human Neonatal Dermal Fibroblast cells stably expressing RFP (Catalog No. cAP-0008RFP) were purchased from Angio-Proteomie (Boston, MA). Human peripheral blood mononuclear cells, purified CD3+ T cells (Catalog No. HM-PBMC-TCELLCD3-M) were donated by BioIVT (Westbury, NY). Advanced DMEM (Catalog No. 12491-015), RPMI 1640 medium (Catalog No. 11875-093), Fetal bovine serum, (Catalog No. 10437-036), and penicillin-streptomycin-glutamine (100X) (Catalog No. 10378-016) were purchased from ThermoFisher Scientific (Waltham, MA).

Assay and Experimental Components

IL-2 Superkine (Fc) (Catalog No. AG-40B-0111-C010), anti-CD3 (human), mAb (UCHT1) (Catalog No. ANC-144-020) and anti-CD28 (human), mAb (ANC28.1/5D10) (Catalog No. ANC-177-020) were donated by AdipoGen Life Sciences (San Diego, CA). CELLSTAR® clear cell culture 24-well cell-repellent microplates (GBO Catalog No. 662970) and the 384-Well BiO Assay Kit (GBO Catalog No. 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 Catalog No. 657860) were donated by Nano3D Biosciences, Inc., and Greiner Bio-One, Inc., (Monroe, NC).

Cytation™ 5 Cell Imaging Multi-Mode Reader

Cytation 5 is a modular multi-mode 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 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.

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 8 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 and automatically transferred to the Cytation 5 at designated time points to monitor T-cell activation.

Methods

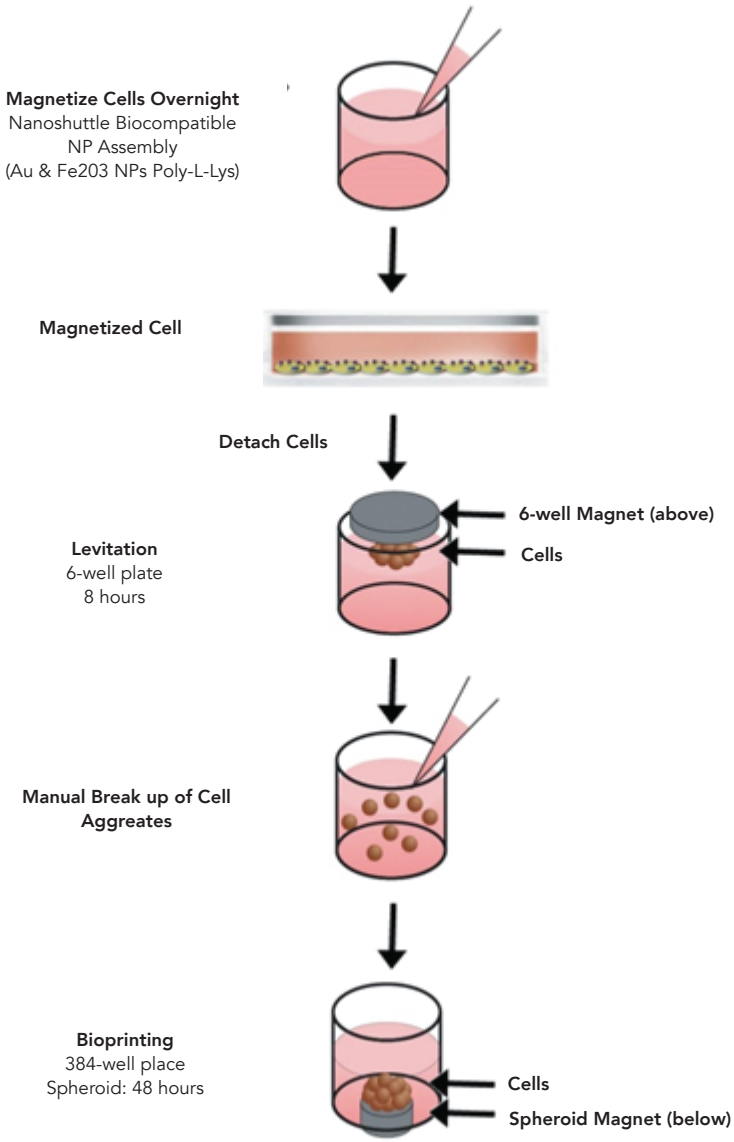
General T Cell Activation

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 100-0 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 °C/5% CO_2. The BioSpa was pre-programmed 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.

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 3-5 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 $1.2x10^6$ 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.

Figure 1. BIO assay kit protocol



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 5).

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 5). The plate was then placed back into the BioSpa to incubate for six days. The BioSpa was again pre-programmed to capture a 12 x 10 image montage from each test well every six hours.

Results and Discucssion

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.

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

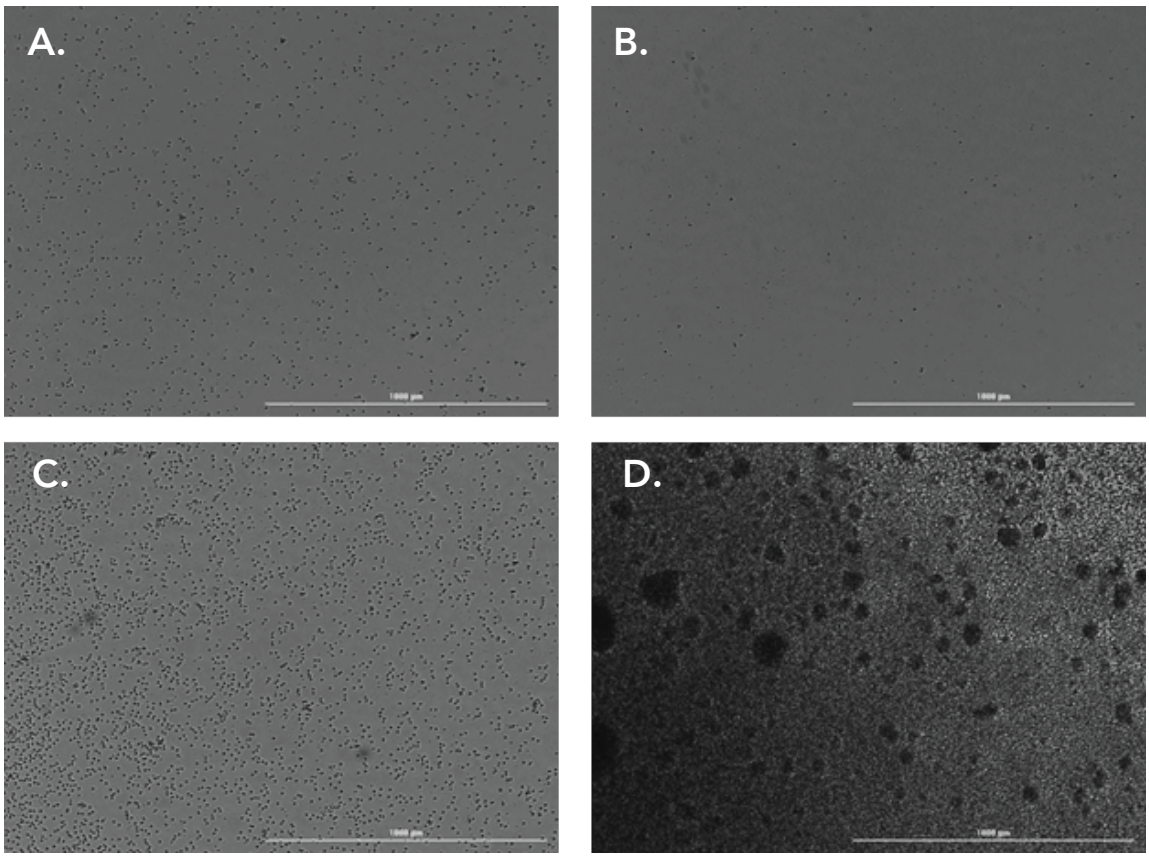
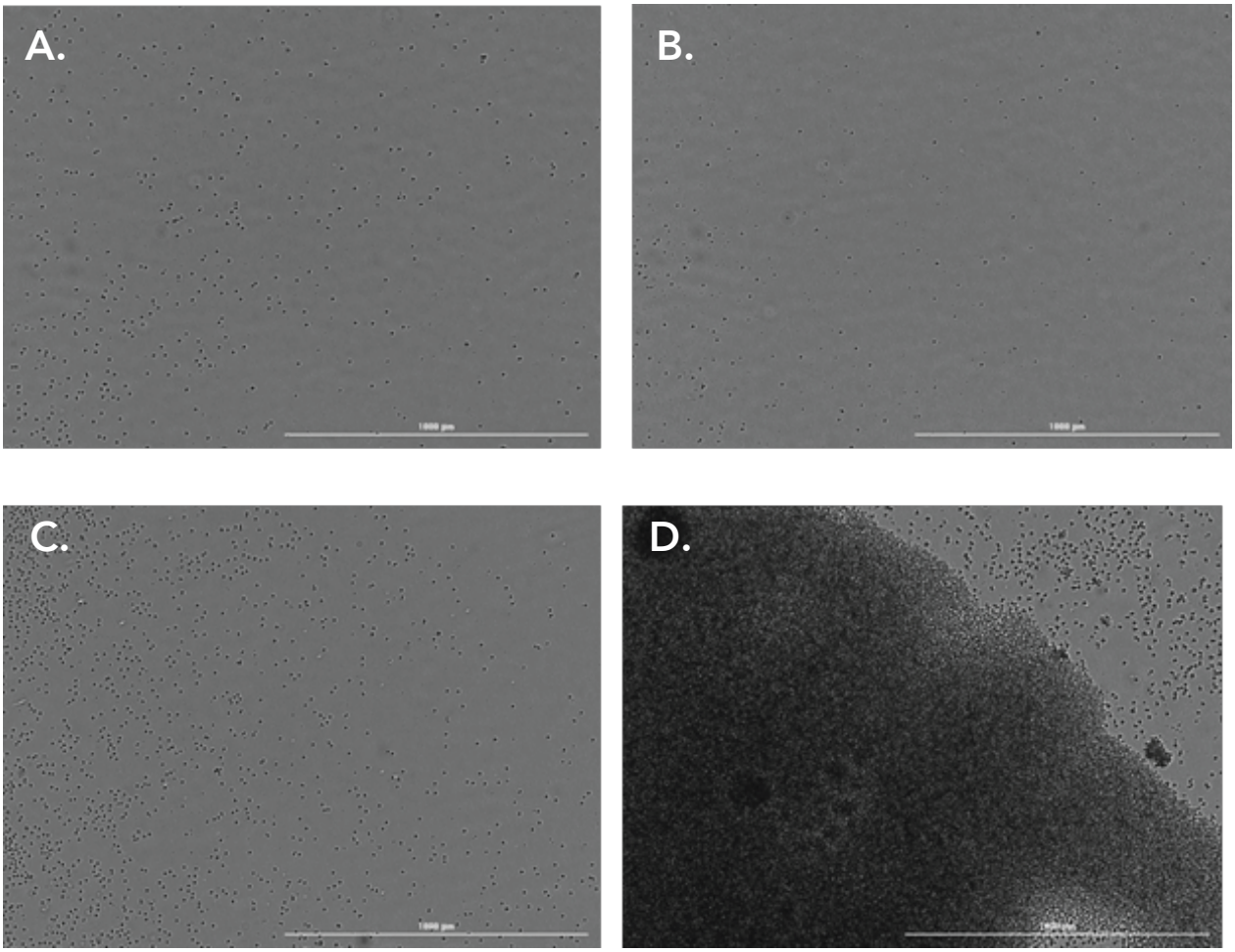


Figure 3. Brightfield imaging of general T cell activation proliferation and aggregation, 100,000 cells/well. 4x images of (A) 0 ng/mL IL-2 Superkine, 0 hours; (B) 0 ng/mL IL-2 Superkine, 144 hours; (C) 100 ng/mL IL-2 Superkine, 0 hours; (D) 100 ng/mL IL-2 Superkine, 144 hours incubation.



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 2D and 3D) compared to those cultured with antibodies alone (Figures 2B and 3B). Video of T cell activation over time is available at www.biotek.com. This phenomenon confirms the high levels of T cell activation which 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.

Quantification of activation was also performed. Individual image tiles were first stitched together using the following criteria (Table 1).

Table 1. Brightfield image stitching parameters

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

Analysis of the complete images was then carried out. Proliferation levels were determined via confluence measurements using changes in brightfield signal (Table 2). Pixels within the image containing cells will have lower numbers in the brightfield channel compared to non-cell containing pixels. The percentage of identified pixels compared to the total equaled the image confluence

Table 2. Confluence measurement parameters

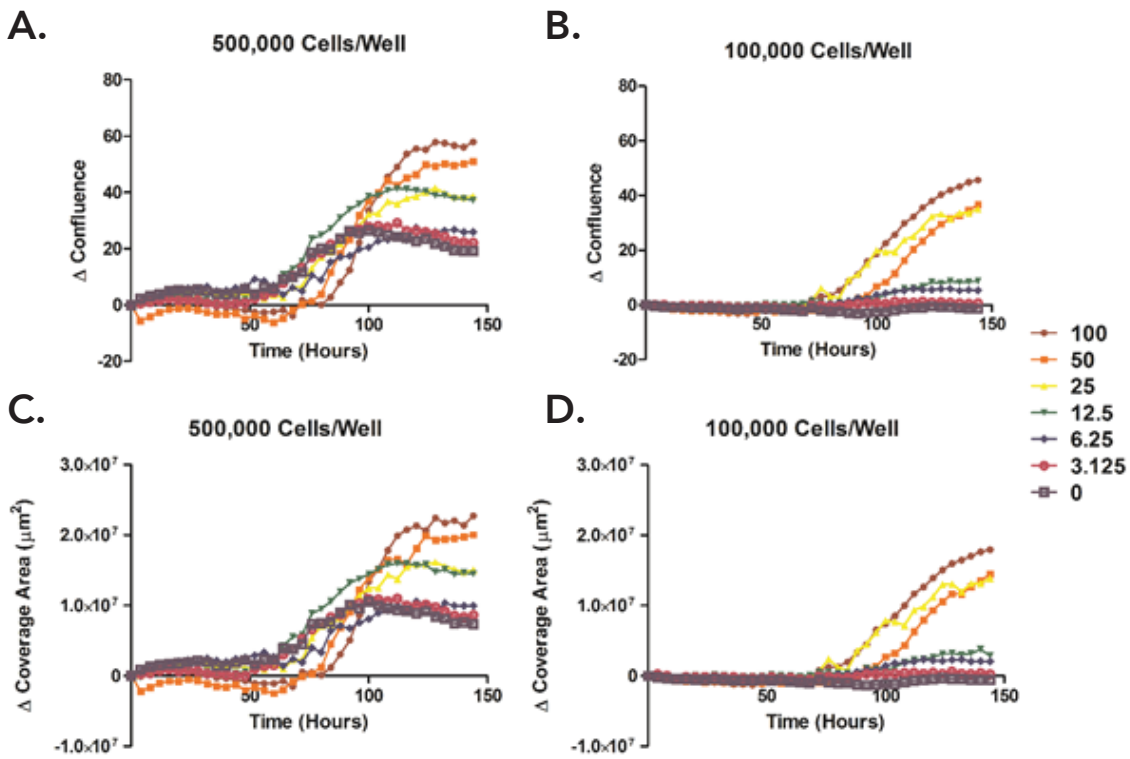
Confluence Analysis Parameters	
Data In	Brightfield
Lower Value	Unchecked
Upper Value	Checked (15000)
Metric of Interest	Confluence

Cellular analysis of cell aggregation was also completed. Using changes in brightfield signal as with confluence measurements, in addition to the fact that aggregated T cells have a larger size in microns than non-aggregated cells, object masks were placed around any cell clumps regardless of size using the parameters in Table 3. The total area covered by the combined aggregates was then reported.

Table 3. Clumped cell measurement parameters

Primary Cellular Analysis Parameters	
Channel	Brightfield
Threshold	1500
Background	Light
Split Touching Objects	Unchecked
Fill Holes in Masks	Checked
Min. Object Size	50 µm
Max. Object Size	1500 µm
Include Primary Edge Objects	Checked
Analyze the Entire Image	Checked
Advanced Detection Options	
Background Flattening Size	10000 µm (Rolling Ball diameter)
Image Smoothing Strength	0 Cycles of 3x3 average filter
Evaluate Background On	85 % of Lowest Pixels
Primary Mask	Use Threshold Mask
Metric of Interest	
Object Sum Area	

Figure 4. Quantification of general T Cell activation. Change in percent confluence for (A) 500,000 cells/well and (B) 100,000 cells/well; and change in initial cell coverage area for (C) 500,000 cells/well and (D) 100,000 cells/well T cell concentrations when treated with 250 ng/mL anti-CD3 and anti-CD28 antibodies in addition to 100-0 ng/mL IL-2 Superkine concentrations.



The kinetic curves seen in Figure 4A and B confirm the visual results previously explained in Figures 2 and 3. T cell proliferation, demonstrated by changes from initial confluence measurements, can be tracked and quantified using label-free imaging and Gen5 image analysis. Addition of the IL-2 Superkine also enhances T cell proliferative rates in a dose dependent manner. Final confluence values show a 3-fold increase when using 500,000 cells/well and a 50-fold increase when using 100,000 cells/well. These findings further validate the ability of the IL-2 Superkine to activate T cells not only when using high cell concentrations, but if lower cell numbers are desired for use in downstream applications.

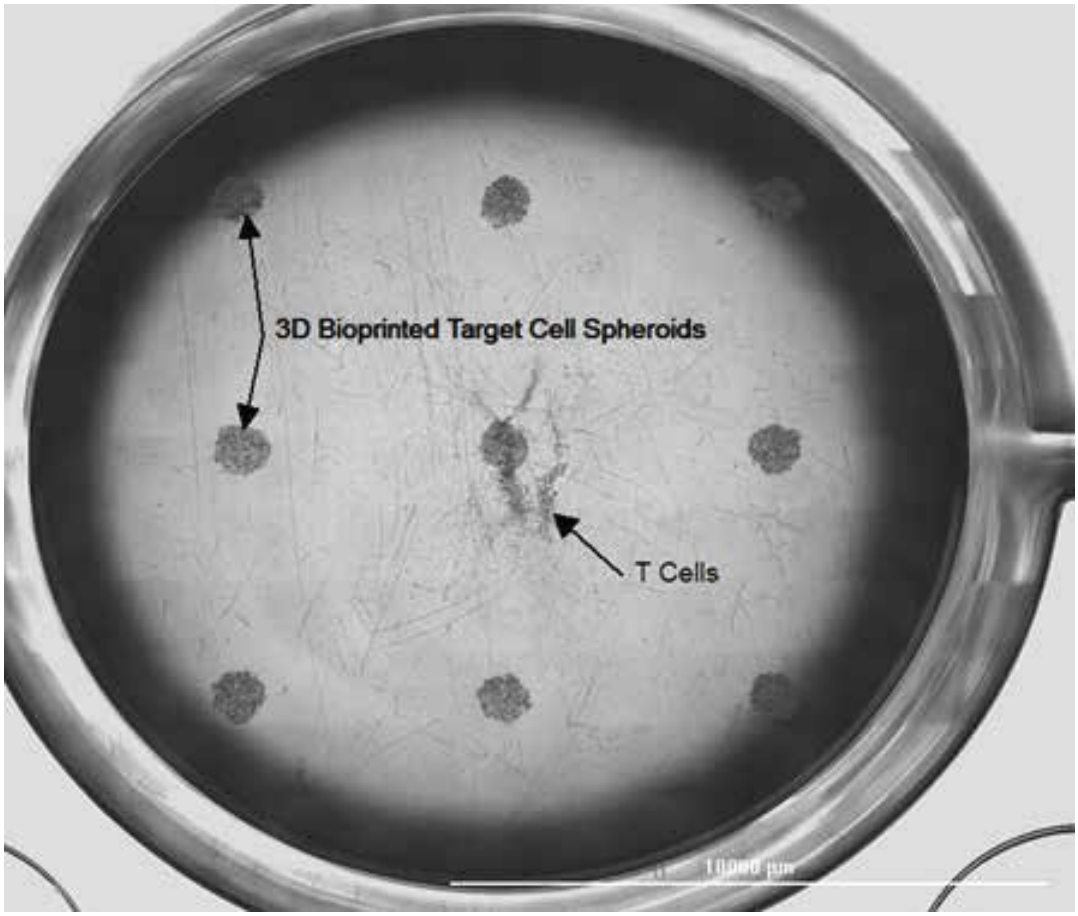
Similar results were seen when performing cellular analysis of T cell aggregates. The total area covered by clumped cells also increases over time in a dose dependent manner according to the concentration of IL-2 Superkine used for activation. This validates the findings of Sabatos, et al., 2008, and the ability to incorporate the combined imaging and analysis method when using T cell aggregation to track T cell activation.

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 non-specific 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.

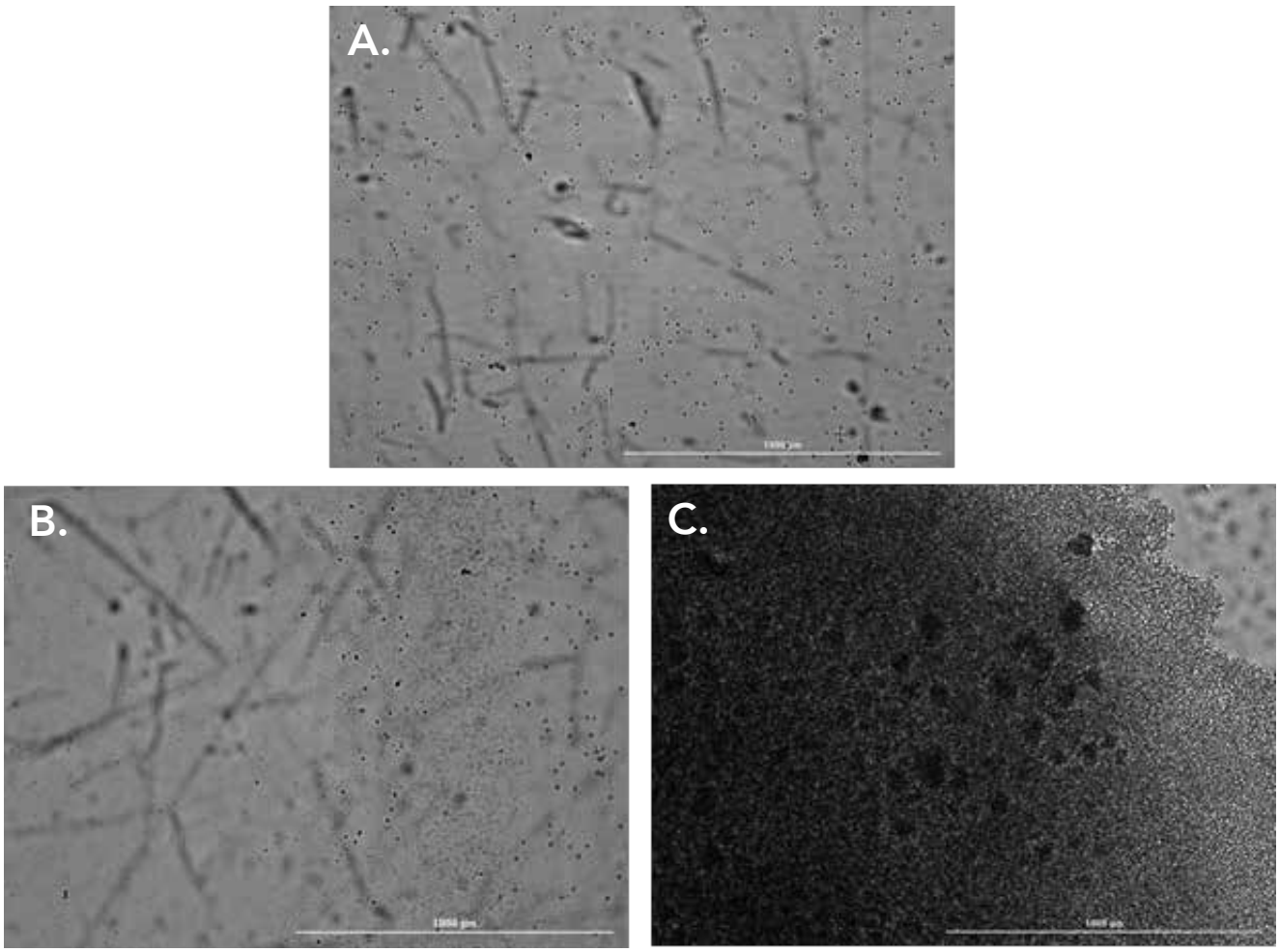
Following addition of the T cells to the wells of the 24-well plate containing the bioprinted target cell spheroids, kinetic label-free imaging was performed. Figure 5 illustrates placement of the target cell spheroids and T cells in each of the test wells for directed activation at time 0 of the incubation period.

Figure 5. 24-well plate well showing co-culture 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.



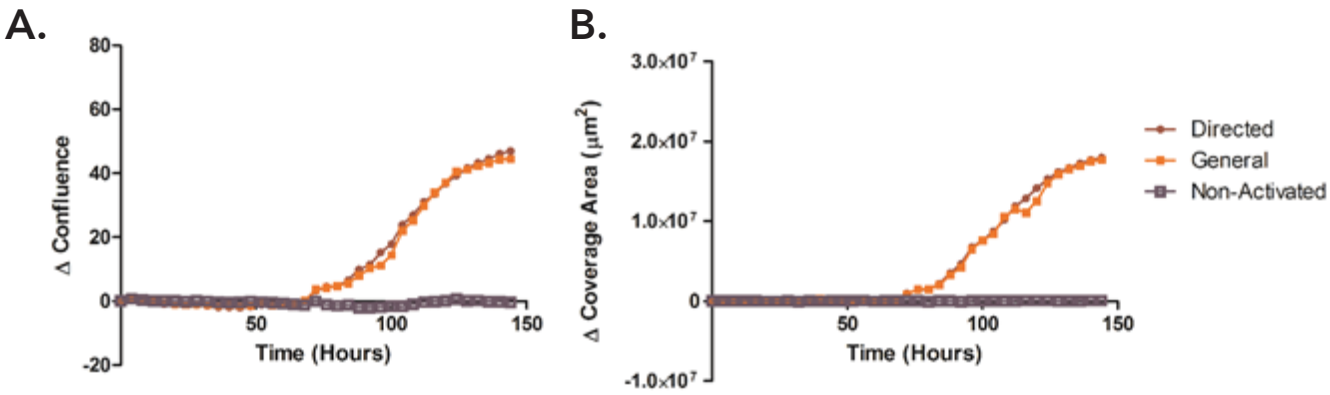
Following addition of the T cells to the wells of the 24-well plate containing the bioprinted target cell spheroids, kinetic label-free imaging was performed. Figure 5 illustrates placement of the target cell spheroids and T cells in each of the test wells for directed activation at time 0 of the incubation period.

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.



Plots of changes in confluence values and area coverage (Figure 7) 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 7. Quantification of general and directed T cell activation. (A) Change in percent confluence and (B) change in initial cell coverage area for 100,000 cells/well T cell concentrations when activated with 250 ng/mL anti-CD3 and anti-CD28 antibodies in addition to 100 ng/mL IL-2 Superkine with (Directed) or without (General) target MDA-MB-231 cells, or non-activated.



Conclusions

Through the incorporation of the BioSpa 8 and Cytation 5, 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 Gen5 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

1.Topalian, S.L.; Weiner, G.J.; Pardoll, D. M. Cancer Immunotherapy Comes of Age. J Clin Oncol. 2011, 29(36), 4828-4836.

2. Sabatos, C.A.; Doh, J.; Chakravarti, S.; Friedman, R.S.; Pandurangi, P.G.; Tooley, A.J.; Krummel, M.F. A Synaptic Basis for Paracrine Interleukin-2 Signaling during homotypic T cell interaction. Immunity, 2008, 29(2), 238-248.

3. Ge, Q.; Palliser, D.; Eisen, H.N.; Chen, J. Homeostatic T cell proliferation in a T cell-dendritic cell coculture system. Proc Natl Acad Sci USA, 2002, 99(5), 2983-2988.

4. Hirschhaeuser, F.; Menne, H.; Dittfeld, C.; West, J.; Mueller-Klieser, W.; Kunz-Schughart, L.A. Multicellular tumor tumoroids: an underestimated tool is catching up again. J Biotechnol. 2010, 148(1), 3-15.

5. Levin, A.M.; Bates, D.L.; Ring, A.M.; Krieg, C.; Lin, J.T.; Su, L.; Moraga, I.L.; Raeber, M.E.; Bowman, G.R.; Novick, P.; Pande, V.S.; Fathman, C.G.; Boyman, O.; Garcia, K.C. Exploiting a Natural Conformational Switch to Engineer an Interleukin-2 Superkine. Nature, 2012, 484(7395), 529-533.

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