Whitepaper

High Throughput Analysis of Spheroid Killing via the Velocyt® Flow Cytometer

Matthew Saunders, Diane Yost, Valeria Sanchez & John O'Rourke
BennuBio Inc
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Introduction

The clinical failure rates in the pharmaceutical industry are approximately 90% and this rate has remained steady since 2010[1]. While there are several reasons for the low success rates of biologics and small molecule drugs, lack of efficacy for the particular indication has recently emerged as the major reason for drug development failure[2]. To help overcome these challenges, there is a concerted effort to develop and optimize relevant advanced cell models that are more predictive of *in vivo* efficacy and to apply those models earlier in the drug discovery workflow[3]. Multicellular three-dimensional (3D) tumor spheroids are advanced models which more faithfully mimic the physiological milieu than traditional monolayer cultures and bridges *in vitro* and *in vivo* studies. While 3D cultures are a promising tool for screening and efficacy evaluation, one of the major challenges is the inability to generate and analyze large number of spheroids using a simplified workflow[4].

Spheroid assays are often measured using imaging systems, but the low number of spheroids typically analyzed may lack the statistical power needed for data driven decisions or require large number of plates, dramatically slowing the workflow. Flow cytometry is outstanding for assessing single cells, but traditional cytometers are unable to analyze particles greater than 40 microns. Therefore, spheroids require a labor-intensive dissociation protocol that often includes multiple filtration steps prior to cell staining to help prevent clogging of the cytometer. Spheroid dissociation also eliminates size-based analysis.

BennuBio's revolutionary Velocyt® instrument overcomes these limitations. The flow cell design of the Velocyt allows for particle sizes up to 500 microns to be analyzed while simultaneously resolving single cells in the same sample. This unique design uses parallel streams and acoustic standing waves to focus particles of various sizes which vastly increases the sampling rate compared to traditional, single stream flow instruments allowing for faster analysis and opening applications such as rare cell analysis. To facilitate the simultaneous analysis of multiple flow streams, the Velocyt forgoes traditional photomultiplier tube detection hardware for a simplified array-based detection technology. The integrated software converts the camara-based pixel data into established flow cytometry data files for analysis via standard FCS analysis software.

In this whitepaper, two case studies were performed to exemplify the ability of the Velocyt platform to analyze spheroid viability. The first used the well characterized apoptotic compound staurosporine to induce cell death.

The second study was an immune cell killing assay where spheroids were co-cultured with activated peripheral blood mononuclear cells (PBMCs) and spheroid viability was assessed on the Velocyt.

CASE STUDY 1: STAUROSPORINE INDUCED APOPTOSIS

METHODS

Spheroid initiation, purification, and analysis

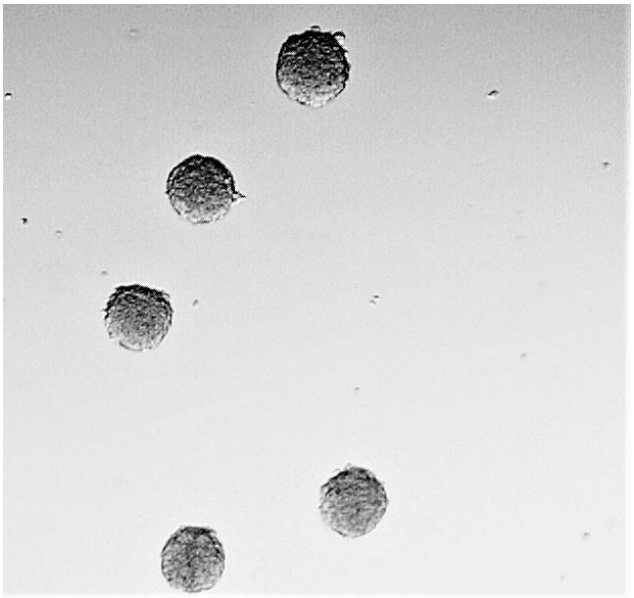
Multicellular spheroids were generated by seeding ~1 x 10^6 HCT116 cells (human colorectal carcinoma, ATCC CCL-247) in each well of a microwell 6 well dish (Stem Cell Technologies). The spheroids were cultured for 2 days in DMEM media (Gibco) supplemented with 10% calf serum (HyClone), 1X glutamax and antibiotic/antimycotic solution (Gibco). On day 3, staurosporine was added to the microwell plates at concentrations from 0.008 µM to 1 µM and untreated spheroids were used as a control. Approximately 22 hours post treatment, spheroids were harvested from the dish and were collected and purified using a 50 micron mesh filter (PluriSelect) to remove single cells. Spheroids were removed from the filter using 3 rinses with phosphate buffered saline (PBS), counted, and sized using microscopy. Approximately 3,000 intact spheroids were generated from a single 6 well dish and ranged from 80-120 microns. Spheroids were stained with Sytox Green viability stain (Thermo-Fischer) for 20 minutes. Spheroids were then sampled on the Velocyt and data was analyzed using FCS Express 7.

RESULTS AND DISCUSSION

Drug-Induced Loss of Spheroid Viability can be Measured by the Velocyt

Staurosporine is a well characterized cell permeable drug that induces apoptosis by inhibiting protein kinase C. To see the effect of staurosporine treatment on spheroids, we incubated HCT116 derived spheroids with various staurosporine concentrations. Untreated purified HCT116 spheroids formed tight, uniformed spheroids with very few single cells (Figure 1A). In contrast, the 1 µM staurosporine treated spheroids were darker and the spheroid edges were more ruffled which are morphological characteristics of reduced spheroid viability (Figure 1B). The increase in single cells may be due to apoptotic cells shedding from the spheroid. To quantitate spheroid viability, spheroids were stained with Sytox Green dye which enters cells that have compromised membranes and binds to DNA. Spheroids were sampled on the Velocyt and ~700 to 2,000 spheroids were analyzed in each sample.

1A.



1B.

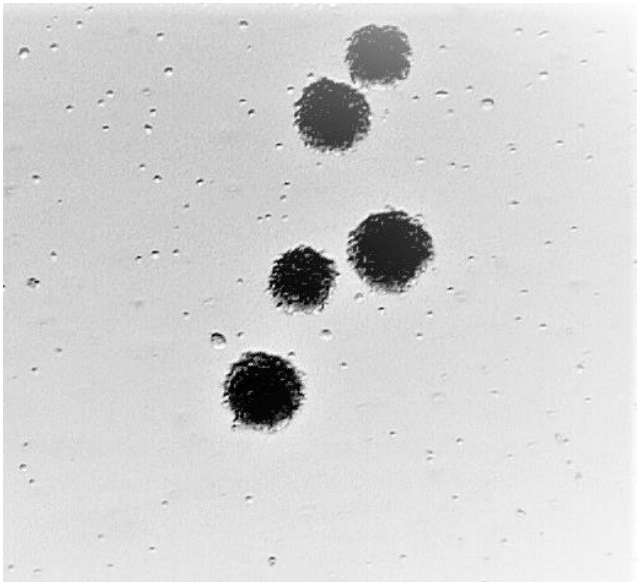
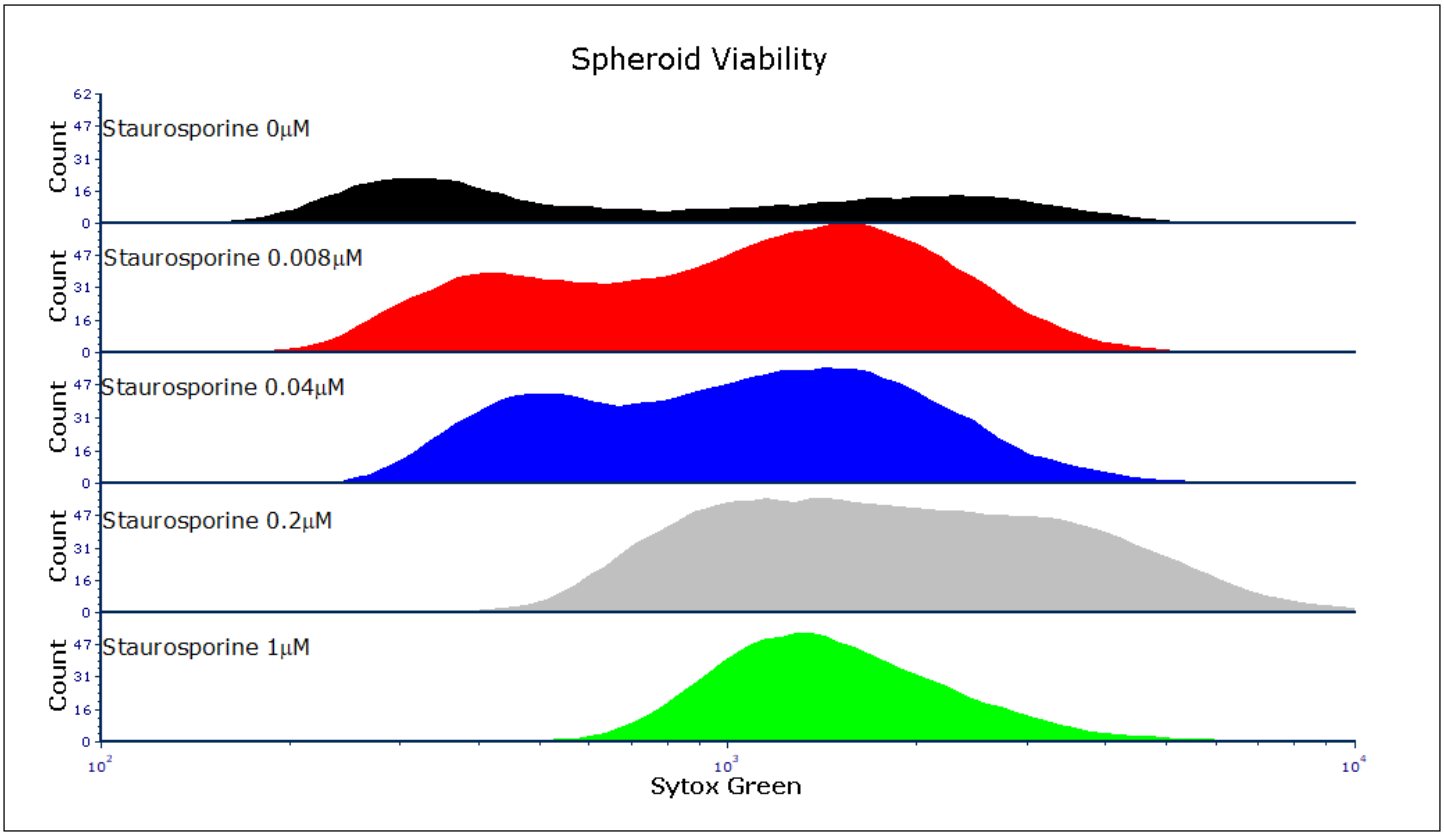


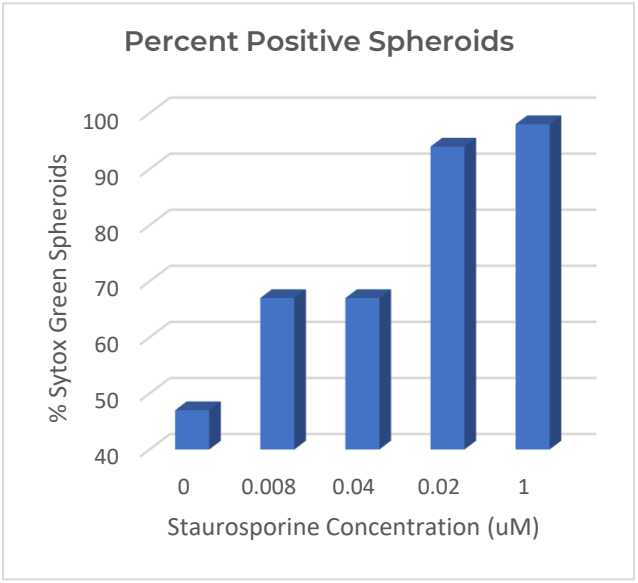
Figure 1 – Generation of HCT116 Spheroids. The human colorectal carcinoma cell line HCT116 were cultured in a 6 well microwell dish at a concentration of 100-200 cells per microwell for three days. Staurosporine a various concentrations were added to the cells and cells were analyzed ~22 hours after addition of drug. A) Control spheroids. B) Spheroids treated with 1 mM of staurosporine.

In untreated spheroids, 40% of the spheroids stained positive for Sytox Green (Figure 2A). A high apoptotic background of untreated HCT116 spheroids was reported by Herrmann[5] and suggests that optimization of culture media, time and spheroid size is required to reduce spontaneous cell death in the HCT116 spheroids. In treated spheroids, there is a clear concentration dependent increase in the number of spheroids that were positive for Sytox Green staining with 98% of spheroids positive for Sytox Green in the 1 µM treated samples. Further analysis of the treated spheroids showed that the median fluorescence intensity (MFI) between the 0.008 µM and 0.2 µM samples increased about 2-fold (Figure 2C). Since the spheroids were nearly identical in size, this data suggests that a higher number of cells in the 0.2 µM treated spheroids were positive for Sytox Green. The 1 µM treated spheroids were more fragile during handling and had more single cells in the culture that stained positive for Sytox Green (See Figure 1). The shedding of Sytox Green positive cells from these spheroids may reflect the slight decrease in the MFI compared to the 0.2 µM samples.

2A.



2B.



2 C.

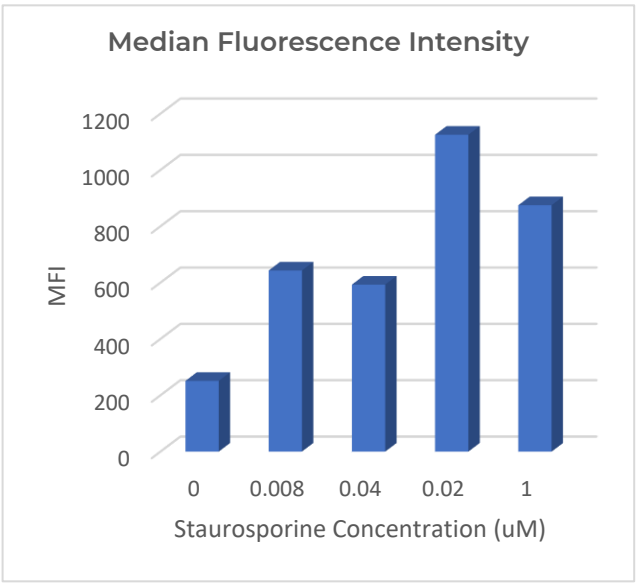


Figure 2 – Spheroid Viability after Staurosporine Treatment. Spheroids treated for ~22 hours with the indicated concentration of staurosporine were stained with the viability dye Sytox Green for 20 minutes.
Spheroids were analyzed on the Velocyt. A) The stacked histograms indicate the increased staining of spheroids with increasing concentration of staurosporine. B) The graph shows the percent positive spheroids in each treatment group. C) The graph illustrates the median fluorescence intensity from each treatment group.

CASE STUDY 2: IMMUNE CELL KILLING OF SPHEROIDS

METHODS

Spheroid initiation and purification

Multicellular spheroids were generated by seeding 200,000 H1650 cells (human lung adenocarcinoma cell line ATCC CRL-5883) in DMEM media (Gibco) supplemented with 10% calf serum (HyClone), 1X glutamax and antibiotic/antimycotic solution (Gibco) in 100 mm tissue culture dishes coated with 1.5% agarose to inhibit cell attachment. After 5 days, spheroids were collected and harvested by settling in 50 mL tubes for one hour and the enriched spheroids were combined and placed in flasks and cultured overnight. The next day, spheroids were harvested from the flasks and spheroids were collected and purified using a 50 micron mesh filter (PluriSelect) to remove single cells. Spheroids were removed from the filter using 3 rinses with PBS and counted using microscopy.

PBMC culture and activation

Cryopreserved PBMCs pooled from healthy human donors were purchased from Cellero. Cells were thawed and rested overnight in DMEM media (Gibco) supplemented with 10% fetal bovine serum (Gibco). Rested cells were plated at a concentration of 1 x 10^6 cells per mL and T-cells were activated by the addition of ImmunoCult Human CD3/CD28 solution (Stem Cell Technologies). After 3-days, cells were counted and stained with CD4, CD8 and CD25 antibodies conjugated to the fluorochromes FITC, PE and PE-Cy-5 respectively (BioLegend) and samples were run on the Velocyt to assess T-cell activation.

Co-culture and spheroid analysis

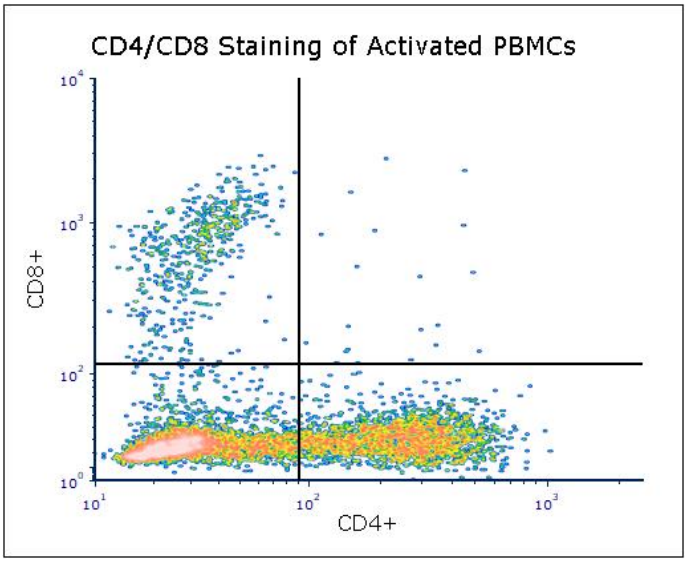
In order to calculate the effector to target cell ratio, the number of cells per spheroids were calculated by dissociating a known number of spheroids and counting cells on a Cellometer Auto 1000 (NexCelom). Co-cultures were set up with an effector to target cell ratio of 10:1. Spheroids and PBMCs cultured alone were used as controls. Co-cultures were added to flasks and incubated for 3-days before analysis. After incubation, the cultures were placed through a 300 micron filter (PluriSelect) to remove large spheroid and aggregates. Spheroids were separated from PBMCs by settling for one hour and the supernatant containing the PBMCs were centrifuged and added back to the spheroids prior to staining. Co-cultures and control samples were stained with Sytox Green viable stain (Thermo-Fischer) for 20 minutes and run on the Velocyt.

RESULTS AND DISCUSSION

Immunophenotyping Primary PBMCs using the Velocyt

The Velocyt as configured for this assay can analyze particle sizes from 3-300 microns in size. To exemplify the Veolcyt's use for immunophenotyping, which is required for our immune cell killing application, a pooled sample of PBMCs from healthy human donors were analyzed. PBMCs were activated using an anti-CD3/CD28 antibody solution and cultured for 3-days. To assess the activation status of the T-cells, an aliquot from the culture was stained using a combination of FITC labeled CD4, PE labeled CD8 and PE-Cy5 labeled CD25. As seen in Figure 3A, a typical ratio of CD4 to CD8 cells were measured (greater than 1). The CD4 and CD8 positive T-cells were then assessed for the percentage of CD25 which is a marker of T-cell activation. Nearly all the CD4 and CD8 T-cells were also positive for CD25 whereas the non-T cells (CD4-/CD8- cells) were mostly negative. These activated PMBCs were then used for the immune cell spheroid killing assay.

3A.



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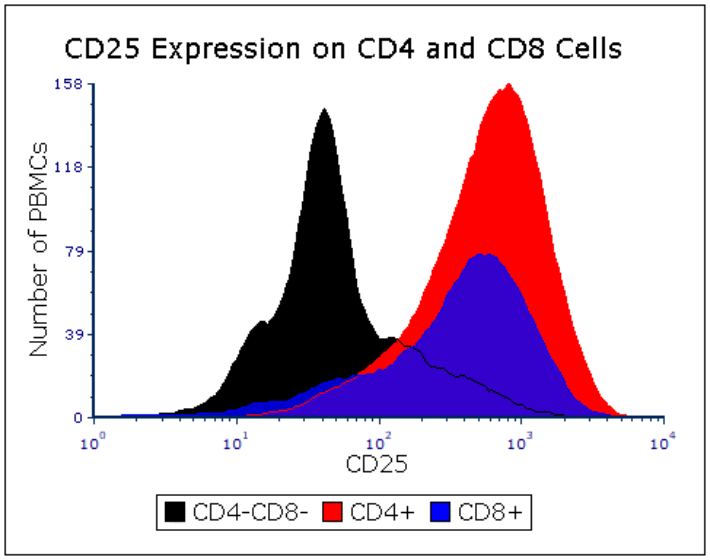
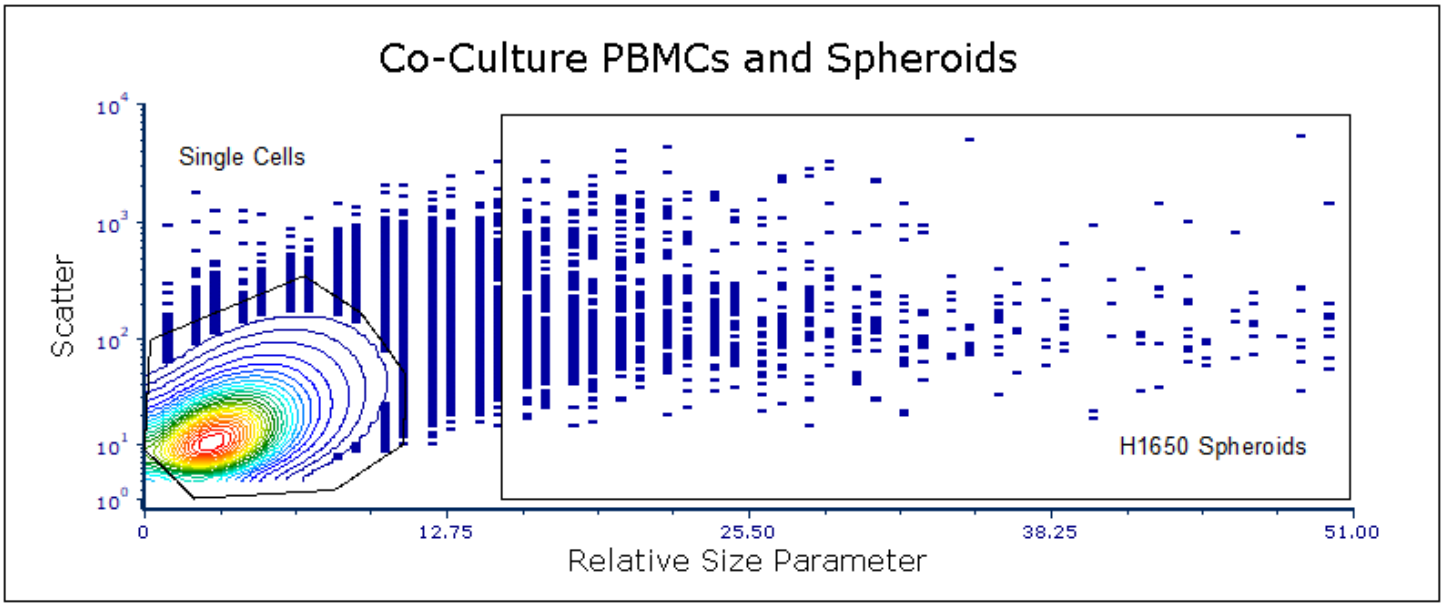


Figure 3 – Activation of PBMCs. PBMCs were plated at a concentration of 1 x 10^6 cells per ml and T-cells were activated by stimulating the CD3/CD28 signaling pathway. After 3-days, cells were counted and stained with CD4, CD8 and CD25 antibodies conjugated to the fluorochromes FITC, PE and PE-Cy-5 respectively. Samples were run on the Velocyt.

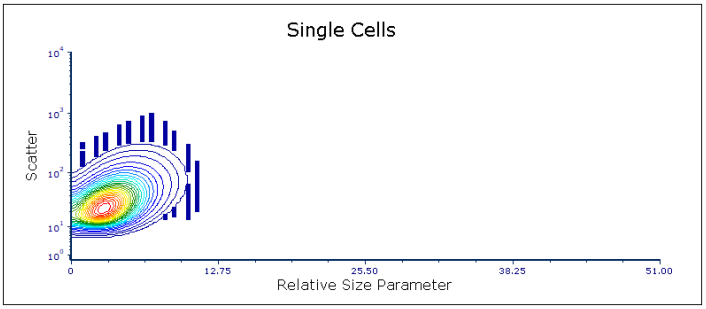
Immune-Cell Killing of Spheroids and Relative Differences Spheroid Size can be Measured on the Velocyt

To analyze co-cultures, effector cells need to be easily identified from the target cells. In conventional flow cytometry, the target cells including spheroids are often encoded with fluorescent membrane dyes to discriminate them from the effector cells. Spheroids analyzed on the Velocyt can be discriminated from immune cells using sizing and scatter parameters (Figure 4). This greatly simplifies the workflow and can provide additional spheroid sizing data that is not possible using current flow cytometers.

4A.



4B.



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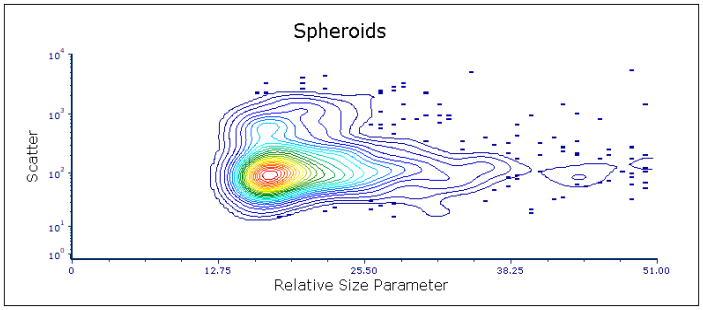

Figure 4 – Resolution of PBMCs and Spheroids in Co-Culture Assays. Co-culture assays were run on the Velocyt. Scatter and sizing parameters were used to resolve the single cells and spheroids. A) Contour plot showing all events. Gates were drawn around the single cell and spheroid populations. Contour plots using the single cell gate (B) and the spheroid gate (C) shows the separation of the two populations.

Spheroids were analyzed for cells containing compromised membranes using Sytox Green viability dye. Figure 5 shows that 42% of the spheroids co-cultured with activated PBMCs (green histogram) stained positive for Sytox Green. In contrast, only 5% of the control spheroids (red histogram) contained Sytox Green positive cells. The greater number of spheroids containing Sytox Green positive cells is also shown by a 3-fold increase in the median fluorescent intensity of spheroids co-cultured with activated PMBCs compared to control spheroids.

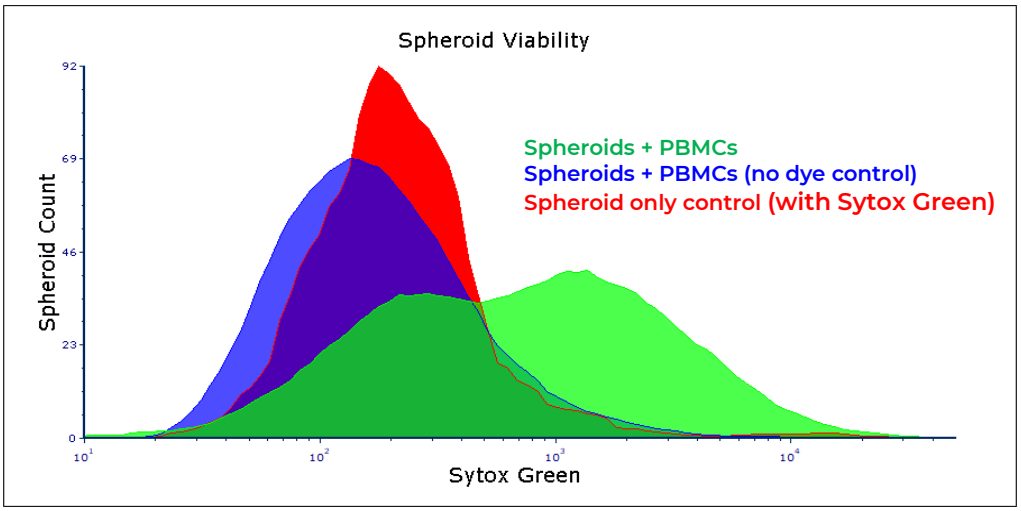

Figure 5 – Spheroid Viability after Co-Culture with Activated PBMCs. Spheroids were co-cultured with activated PBMCs for three days. Spheroids and cells were harvested and stained with the viability dye Sytox Green to assess compromised cellular membranes in the spheroids. The co-cultured spheroids (green) show an increased number of Sytox Green positive spheroids compared to spheroid culture in the absence of PBMCs (red) and an unstained control (blue).

Microscopic evaluation showed that the co-cultured spheroids were smaller in size than the control spheroids. To show that the Velocyt can assess relative size differences, we visualized spheroids using a contour plot (Figure 6). The x-axis is a size parameter, and the y axis is the Sytox Green viability parameter. The black contour lines represent the spheroids co-cultured with the PBMCs and the red lines represents the control spheroids.

As observed in Figure 5, a larger percentage of co-cultured spheroids were positive for Sytox Green compared to controls spheroids as can be seen by the number of contour lines above the horizontal gate (Figure 6). Of more interest, the control sample contains many larger spheroids compared to the co-cultured spheroids as represented by the number of contour lines to the right of the vertical gate. Median fluorescent intensity analysis shows an approximate 2-fold reduction is spheroid size in the co-cultured spheroids consistent with our microscopic analysis.

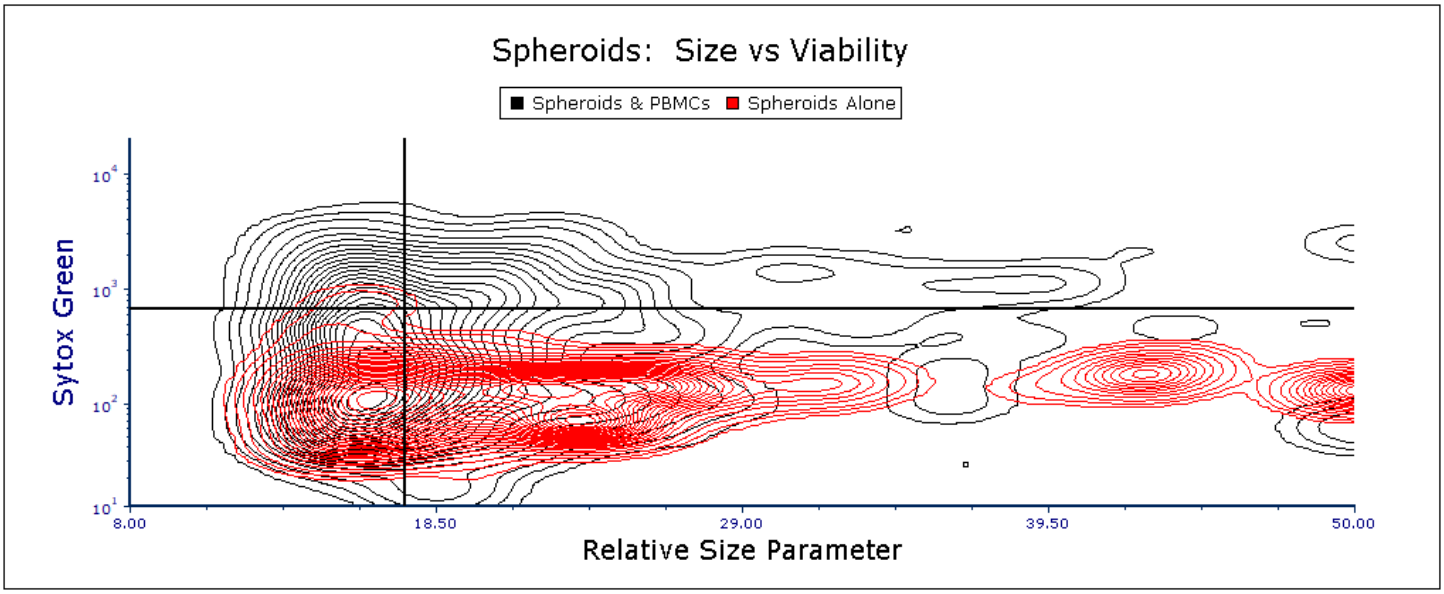

Figure 6 - Spheroids Co-Cultured with PBMCs are Smaller than Controls. Spheroids cultured with PBMCs are shown in the black contour plot while control spheroids are in the red. The y-axis shows viability as measured by Sytox Green while the x-axis shows relative size. The horizontal gate shows the Sytox Green positive spheroids while the vertical gate represents larger spheroids.

Conclusion

Pharmaceutical researchers require improved tools for assessing the *in-vitro* effectiveness and behavior of possible therapies. The Velocyt instrument combines the power of 3D multi-cellular models with the speed, throughput, and multiplexing capability of flow cytometry to provide accurate, statistically relevant insights into drug behavior and effectiveness. This paper demonstrates one such application, a spheroid killing assay. Using two different spheroid forming cell types (colorectal carcinoma and adenocarcinoma) and two different apoptotic agents (staurosporine and PBMCs), these simple assays show that it is possible to rapidly characterize apoptosis activity in live tumor spheroids grown in bulk. The Velocyt is the only instrument that can provide these powerful analytical insights and is therefore poised to become an essential part of the pharmaceutical researcher's toolkit.

References

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BennuBio Inc
6610 Gulton Ct NE
Albuquerque, NM 87109
USA

www.bennubio.com

info@bennubio.com