Simplified and high throughput analysis of intact 3D models using large particle imaging cytometry

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Introduction

Three-dimensional (3D) models, such as organoids and spheroids, are indispensable in translational and clinical research, providing a physiologically relevant platform to study complex biological processes and advance therapeutic development. Despite their potential, the high-throughput analysis of these models remains a significant challenge. Traditional methods often restrict organoid and spheroid cultures to multiwell plates, where each well houses a single particle. This limitation drastically reduces the number of 3D structures that can be analyzed per treatment, potentially leading to underrepresentation of biological variability, compromised data reliability, and a lack of statistical power. Such constraints hinder the ability to detect subtle differences or trends, which are critical for robust scientific conclusions.

The advantages of growing organoids and induced pluripotent stem cell (iPSC) aggregates in suspension cultures are numerous. These include scalability, adaptability to bioreactors, and applicability across various research and therapeutic contexts. However, analyzing these 3D suspension cultures presents its own set of challenges. Current workflows are labor-intensive and inefficient, often requiring the transfer of single particles to multiwell plates for imaging, paraffin embedding for histological analysis, or dissociation into single cells for flow cytometry. These steps not only introduce variability but also increase the risk of losing valuable information.

To address these challenges, we employed the Velocyt cytometer by Bennubio, which is uniquely designed to analyze intact 3D models directly from suspension and static cultures. The Velocyt leverages parallel stream acoustic flow technology combined with camera-based optics, effectively merging the imaging capabilities of microscopy with the speed and convenience of flow cytometry.

In this study, we present two case studies that highlight the versatility and efficiency of the Velocyt. The first involves a longitudinal assessment of size distribution and viability of 3D cultures grown in stirred bioreactors under different media formulations. The second utilizes microwell technology to perform immune cell killing assays, measuring both immune cell penetration and 3D culture viability. These examples underscore the Velocyt's ability to simplify workflows and its value in diverse 3D applications, offering a robust solution for the challenges inherent in high-throughput 3D model analysis.

Methods

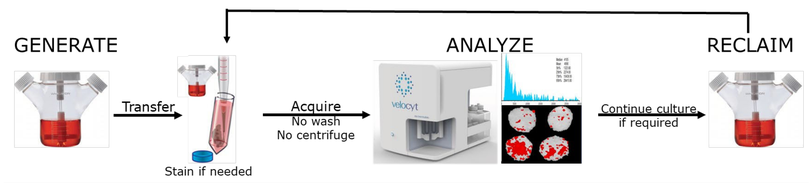
Bioreactor media optimization study - HCT116 colon cancer cells were seeded into spinner flasks at 1e5 cells per mL into 30mL of media containing either 0% serum, 2% serum, or 10% serum. Following 1 day of incubation, 5 mL aliquots were removed from each flask, stained with ethidium homodimer III and caspase substrate and analyzed on the Velocyt. This process was repeated over 2 additional days.

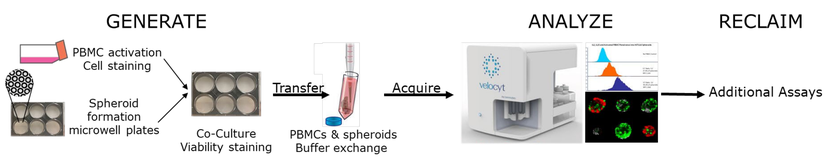
Co-culture assay – HCT116 spheroids of ~200 microns in width were generated using microwell plates. PBMCs were cultured for 3-days in media containing different combinations of IL-2, IL-15 and activation reagents (ImmunoCult, Stem Cell Technologies). Prior to co-culture, PBMCs were labeled with CytoTell UltraGreen (AAT Bioquest). Labeled PBMCs were co-cultured overnight with various E:T ratios. Co-cultures were stained with ethidium homodimer I and spheroids were purified from PBMC using filtration. Additional washes were performed to remove loosely bound PBMCs

Data analysis – Samples were acquired on the Velocyt using Kytos™ software. Initial analysis including gating of spheroids, size and shape analysis, image thresholding, image generation and image based fluorescent parameters were performed using VeloView™ software (Bennubio). Additional analysis were done using FCS Express software (Dotmatics) using FCS 3.0 files auto-generated during data acquisition on the Velocyt.

Velocyt's Simplified Spheroid/Organoid Workflow

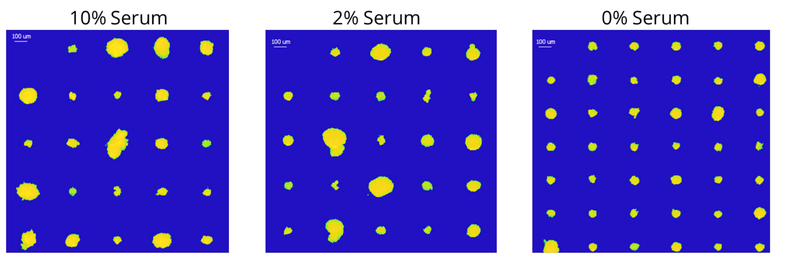
Below are the workflows for the two case studies illustrating the simplicity, flexibility and versatility of the Velocyt. Additional assays using scaffold embedded samples and low attachment plates have been validated on the Velocyt. Hundreds to thousands of 3D particles are imaged in less than 5 minutes and samples are recovered intact and viable for longitudinal studies or to perform additional analysis.

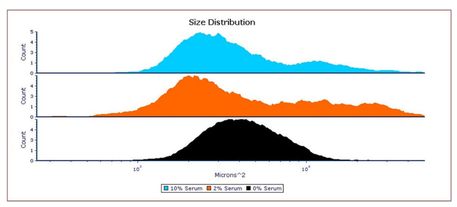


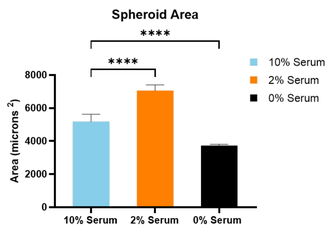


Bioreactor Media Optimization Study

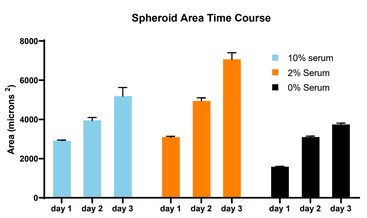
Spheroid area 3 days after cell seeding







Spheroid area time course



2% Serum

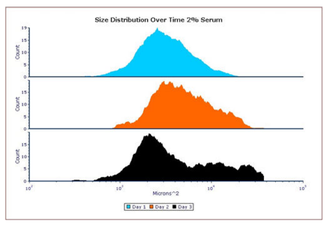
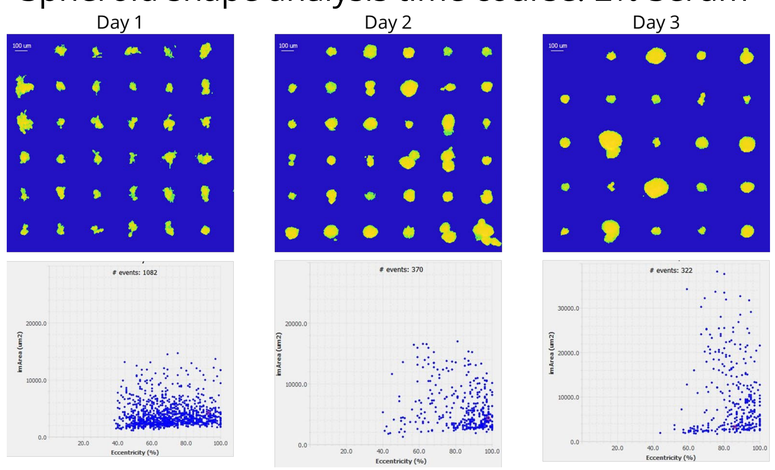
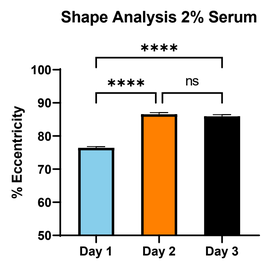


Figure 1 – Size Analysis. Cells were seeded into spinner flasks and aliquots were removed at days 1, 2 & 3. Data were acquired on the Velocyt and events over 50 microns in width were further analyzed. The scatter image galleries were generated with our VeloView software and show a small portion of the total events. Scale bar is 100 microns. One way ANOVA was performed to determine significance and error bars are SEM.

Spheroid shape analysis time course: 2% Serum





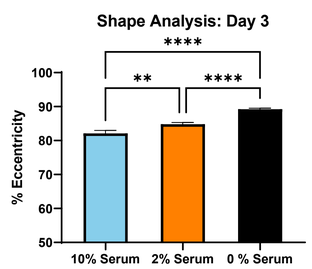
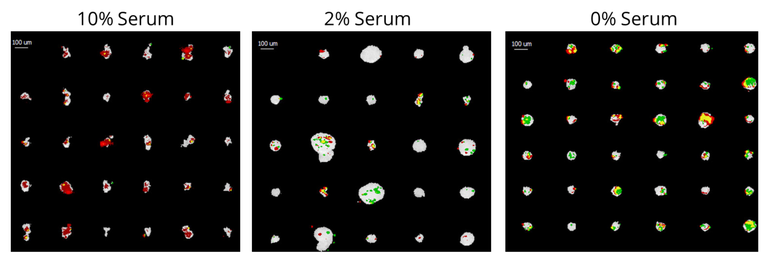


Figure 2 – Shape Analysis. Cells were seeded into spinner flasks and aliquots were removed at days 1, 2 & 3. Data were acquired on the Velocyt and events over 50 microns in width were further analyzed. The scatter image galleries were generated with our VeloView software and show a small portion of the total events. The eccentricity vs area dot plots were generated with VeloView. One way ANOVA was performed to determine significance and error bars are SEM.

Spheroid health 3 days after cell seeding



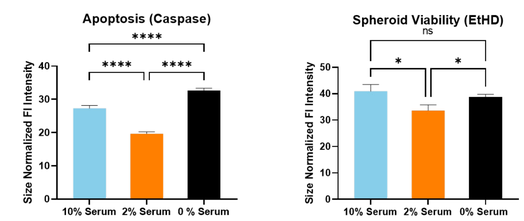
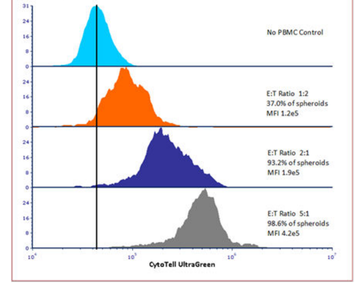
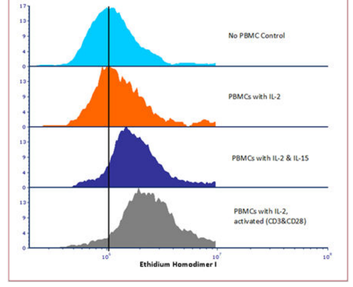


Figure 3 – Spheroid Health. Cells were seeded into spinner flasks and aliquots were removed at day 1,2 & 3. Spheroids were stained with a caspase activation dye (green) and ethidium homodimer (red). Co-localized signal is yellow. The image galleries were generated with our VeloView software and show a small portion of the total events, and the graphs represent the integrated fluorescent values normalized by area. One way ANOVA was performed to determine significance and error bars are SEM.

3D immune cell killing assay





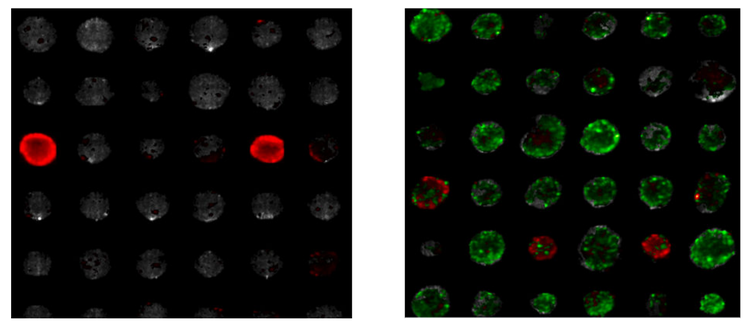


Figure 4 Immune cell killing. Left histograms shows increasing PBMC penetrance into spheroids with increasing E:T ratios. Right histogram shows the impact of PBMC culture conditions on their ability to kill 3D particles. Partial image gallery of spheroid immune cell killing without PBMC co-culture or with an E:T ratio of 5 to 1. PBMCs are green and non-viable cells in spheroids are red.

Conclusions

Bioreactor studies:

1. The Velocyt analyzed size, shape and viability of samples drawn from flasks with no sample prep other than staining.
2. Spheroids generated in media containing 2% serum were larger, more circular and had higher viability than other media formulations.
3. Spheroids generated without serum had a higher percentage of caspase + particles consistent with serum and growth factors withdrawal initiating the apoptotic pathway.
4. Additional optimization including seeding density, agitation rate and monitoring growth for longer periods of time are ongoing.

Immune cell killing assays:

1. The Velocyt easily analyzes both immune cell penetrance and spheroid death using a simple and rapid workflow.
2. Activation through stimulation of the CD3/CD28 pathways were required to unleash maximum 3D killing potential although there was little difference in PBMC penetrance based on culture conditions.
3. Immune cell penetrance was determined by effector to target ratio.