Flow Cytometry Technologies

Utilizing a 200 µm nozzle tip to sort HEK293 spheroids and human colorectal cancer tumoroids for downstream characterization

Mitch Klomp, Thermo Fisher Scientific, 145 Mountain Ave, Fort Collins, CO, 80524

Abstract

Cell sorters utilize different sized nozzle tips to manage sort pressure and sort speed which impact the size of the particles that can be sorted. On the Invitrogen™ Bigfoot™ Spectral Cell Sorter, the 200 µm nozzle tip is used to sort highly complex and large particles for downstream applications. Spheroid and tumoroid culture models are used in regenerative medicine, cancer biology, and stem cell research to imitate *in vivo* models more closely than traditional 2D cell cultures. Here, we show how HEK293 spheroids were grown in suspension culture to a size exceeding 80 µm in diameter as confirmed by the Invitrogen™ Attune™ Cytpix™ Flow Cytometer. HEK293 spheres were harvested and sorted into well plates that contained PMA-activated U937 macrophages. Similarly, mixed cultures of GFP$^+$ and GFP$^-$ HuCo1044 tumoroids were grown using the Gibco™ OncoPro™ Tumoroid Culture Medium Kit until clusters were at least 50 µm in diameter and purified GFP$^+$ tumoroids were sorted into well-plates for downstream characterization. The Bigfoot Spectral Cell Sorter is currently the only cell sorter on the market with the flexibility to sort using a 70 µm in the morning while sorting large particles with the 200 µm nozzle tip in the afternoon. Here, we describe unique applications for sorting large particles safely and conveniently using the 200 µm nozzle tip.

Materials and methods

Cell culture preparation

HEK293-RFP$^+$ cells were grown in Gibco™ MEM™ supplemented with 10% fetal bovine serum (FBS) and 1X penicillin/streptomycin (P/S). Cells were grown to confluency as adherent cells for a minimum of three passages before being harvested and 50,000 cells were added to each well of a non-adherent 12 well plate to generate spheroids. After growing for 72 hours, spheroids were visually inspected via an Invitrogen™ Evos™ M7000 Imaging System for size and spheroid density. Spheres were harvested and concentrated via centrifugation at 400 x g for 5 minutes. Spheres were resuspended in 10 mL of cold DPBS. Human colorectal 1044 tumoroids (HuCO1044) were a kind gift from Colin Paul (Thermo Fisher Scientific). The OncoPro™ Tumoroid Culture Medium Kit was supplemented following the manufacturer's recommendation in addition to adding 1X P/S. Further cell culture aliquots were supplemented with 10 mM Y-27632. Tumoroids were seeded at a cell density of 1.0x10^6 / mL in OncoPro™ Tumoroid Cell Culture Medium with 2% v/v cold, Gibco™ GelTrex™ Matrix. Tumoroids were fed every 2-3 days by collecting the cells in a conical tube and washing the flask with unsupplemented DMEM/F12 and added to the collected cells. Cells were concentrated via centrifugation at 400 x g and resuspended in an appropriate volume of OncoPro medium plus 10 mM T-27632. tumoroids were counted via a Countess 3 and re-seeded at an appropriate volume in a non-adherent cell culture flask. Ice cold 1% v/v Gibco™ GelTrex™ Matrix was added to the flask. After several feeds, and after confirming the size of the tumoroids, the cells were harvested, washed several times with cold DPBS (-/-), and resuspended in Gibco™ StemPro™ Accutase™ containing 10 mM Y-27632. The tumoroids suspension was triturated 10 times before incubating in a 37° C hot water bath for 10 minutes. Post-incubation, cells were triturated at least 20 times before concentrating via centrifugation. Tumoroids were counted prior to sorting. U937 cells were cultured in RPMI supplemented with 10% FBS and 1X P/S. 24 hours prior to sorting spheroids or tumoroids for cell killing assays, U937 had been grown to confluency and harvested. 50,000 U937 cells were seeded into six wells of a 12 well plate. Invitrogen™ eBioscience™ Cell Stimulation Cocktail PMA/Ionomycin were diluted to 1X in three of the wells containing U937 cells.

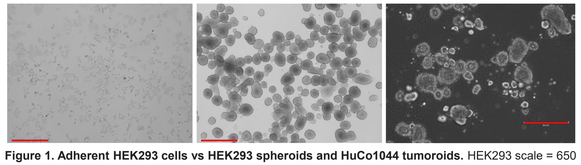

Figure 1. Adherent HEK293 cells vs HEK293 spheroids and HuCo1044 tumoroids. HEK293 scale = 650 um and HuCO1044 scale = 300 um. Images show the difference in size and cell morphology between adherent and 3D culture

Test method(s)

A four laser Attune CytPix Flow Cytometer was used to assess cellular morphology, diameter (µm), circularity, eccentricity, and fluorescence intensity of RFP for HE293 spheroids or GFP for HuCo1044 tumoroids. Scatter voltages were optimized on the Attune CytPix Flow Cytometer using image analysis and cellular characterization to help minimize sample loss when setting up the large particle sort. A 9 laser Bigfoot Spectral Cell sorter was used to sort 100 spheroids or tumoroids utilizing the 200 µm nozzle tip at 6 psi. The optimal drop delay value was confirmed using an aliquot of either HEK293 spheres of HuCo1044 tumoroids. The EVOS M7000 Imaging System was used to assess cell death of the spheroids and tumoroids in the cell killing assay. The magnifications utilized for the assay ranged from 10X (scale bar = 275 µm) to 20X (scale bar = 150 µm). FCS Express was used to analyze data from the Bigfoot Spectral Cell Sorter.

Results

Figure 2. Characterization of HEK293 spheroids for size and morphology using image-enhanced flow cytometry

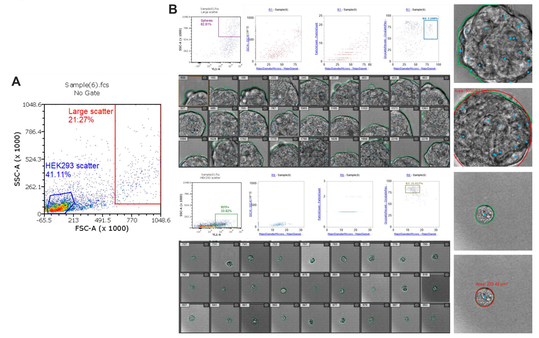

Figure 2. HEK293-RFP$^+$ cells and spheroids were analyzed on the Attune CytPix Flow Cytometer. This characterization of adherent cells vs spheroids allowed for the correct setting of scatter voltages for large particles over 80 µm in diameter. The Attune CytPix Flow Cytometer also provided deeper insights into the morphology and physical characteristics of the spheroids which is important for understanding tumor microenvironments for downstream analyses.

Figure 3. Sorting HEK293-RFP$^+$ spheroids for co-culture with macrophage-like U937 cells to demonstrate a functional killing assay

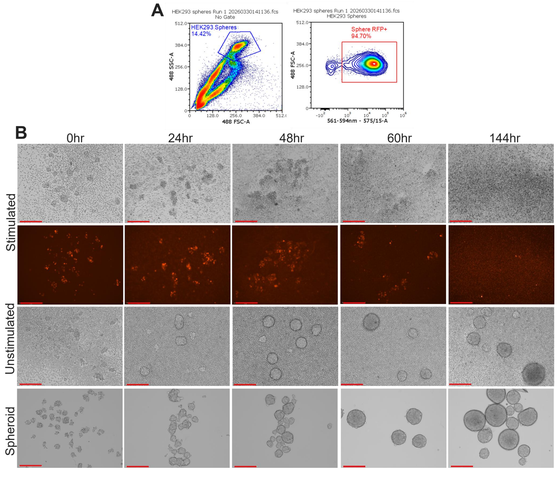

Figure 3. A. 100 HEK293-RFP$^+$ spheroids were sorted using the 200 µm nozzle tip on the Bigfoot Spectral Cell Sorter. Spheroids were sorted into 12-well plates seeded with activated or naïve U937 cells. As a control, spheroids were sorted into media alone. B. The co-cultured cells were imaged daily to assess damage to the spheroids from the macrophage-like U937 cells relative to unstimulated controls. Spheroid damage and cell death began to occur as early as 24 hours and was complete by 60 hours while there was no noticeable phagocytosis occurring to the spheroids co-cultured with unstimulated U937 cells. Images taken at 10X magnification, scale bars = 275 µm. Images representative of two, independent experiments. Images representative of 10 images for each time point across three wells.

Figure 4. Characterization of human colorectal cancer 1044 (HuCo1044) tumoroids for size and morphology using image-enhanced flow cytometry

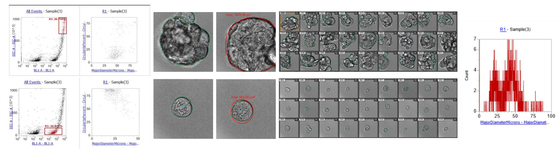

Figure 4. Human Colorectal Cancer 1044 cells (HuCo1044) expressing GFP were grown in OncoPro Tumoroid cell culture medium before being harvested and characterized on the Attune CytPix Flow Cytometer. The size, morphology, proliferation state, GFP fluorescence, and distribution of tumoroids are important factors when assessing downstream experiments for anti-tumor drugs or cancer assays..

Figure 5. Sorting HuCo1044 tumoroids for co-culture with macrophage-like U937 cells to assess tumor killing potential

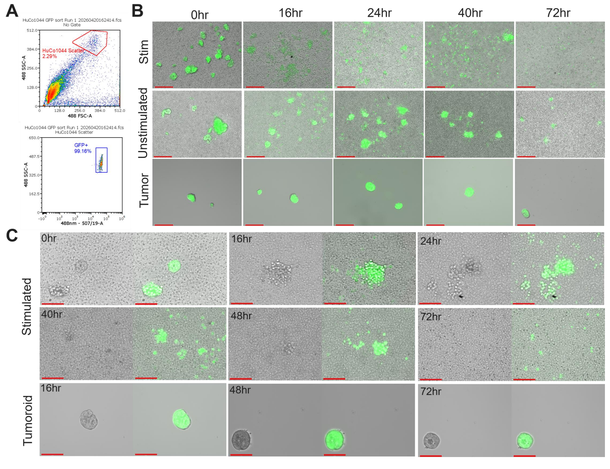

Figure 5. A. 100 HuCo1044-GFP$^+$ tumoroids were sorted into 12-well plates containing stimulated or unstimulated U937 cells using the 200 µm nozzle tip on the Bigfoot Cell Sorter. Tumors were also sorted into media without U937 cells as a control. B. Phagocytosis of tumors or tumor growth was assessed using an EVOS M7000 Imaging System over the course of 72 hours. Phagocytosis of the HuCo1044 cells was noticeable within 24 hours and clearance of the tumoroids was completed within 72 hours. HuCo1044 tumoroids co-cultured with unstimulated U937 cells did not undergo phagocytosis or cell killing while tumoroids cultured without U937 cells continued to proliferate. Images taken at 10X magnification, scale bars = 275 µm. C. For closer analysis, each well containing HuCo1044 tumoroids co-cultured with stimulated U937 cells was imaged at 20X magnification. Phagocytosis of tumoroids began around 16 hours post-sort with tumor clusters falling apart. Macrophages began to fluoresce green due to phagocytizing GFP$^+$ tumoroids which began to diffuse across the field of view for each well. Non-co-cultured HuCo1044 tumoroid were imaged as controls to detail the morphology and lack of cell death. Scale bars at 20X = 150 µm. Images representative of two, independent experiments. Images representative of 10 images for each time point across three wells.

Figure 6. OncoPro Tumoroid Cell Culture Medium was utilized to grow primary human tumoroids (available through Thermo Fisher Scientific). The 200 µm nozzle tip on the Bigfoot Spectral Cell Sorter operates at 6 psi for optimal performance when sorting large or delicate cells for downstream applications.

Conclusions

- Reagents and instrumentation needs to keep pace with the growing demand for 3D cell culture models and applications. 3D cell culture models, cell culture reagents, and tumoroid guides are commercially available through Thermo Fisher Scientific
- The Attune CytPix Flow Cytometer is an invaluable tool in characterizing large particles for size, morphological features, and size distribution prior to further downstream applications. The use of image-enhanced flow cytometry provides deeper insights into the characteristic of every particle. A customizable and adjustable camera ensures accurate and fast images of every cell of interest.
- Sorting individual spheroids or tumoroids is possible using the 200 µm nozzle tip for the Bigfoot Spectral Cell Sorter. Drop delay confirmations using cells of interest enable confidence in large particle sorting. The low pressure of the 200 µm nozzle tip ensures large particles remain viable during and after the sort. Efficient sorting into well plates is made faster when using InfiniSort to continuously sort into well plates by keeping the sample in the sample line and avoiding sample loss between sorts.
- Large particles sorted using the Bigfoot Spectral Cell Sorter remain intact during and after the sort. They retain their tumoroid characteristics such that downstream anti-tumor assays can be performed. Large particles that are not co-cultured with activated cells not only remain intact during the sort but remain viable and continue to proliferate after the sort.

References

1. Paul, C.D., Yankaskas, C., Shahi Thakuri, P. *et al.* Long-term maintenance of patient-specific characteristics in tumoroids from six cancer indications. *Sci Rep* **15**, 3933 (2025). https://doi.org/10.1038/s41598-025-86979-9

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