Tumor spheroids -- Analysis, sorting and imaging of cell aggregates from various cell types for HTS purposes using COPAS VISION 500

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Objective

Large particle flow cytometers from Union Biometrica provide automation for the analysis and dispensing of cell clusters. Cells growing in clusters communicate with each other and behave differently than cells grown as monolayers or in suspension. Research using stem cell clusters, organoids, tumor spheres and other types of 3D cultures are important biological systems for the discovery of signals responsible for normal development as well as the abnormal disease state of solid tumors. There is enormous interest in studying how cells grow, divide and differentiate in a more natural context provided by these 3D cell culture models. Many cell types will naturally form cell clusters when given the opportunity. Using this research approach allows for cell-cell interactions to occur and provides biological insights otherwise missed when studying flat sheets of cells growing on plastic surfaces or as cells grow in isolation. Here, we demonstrate the analysis, selection and sorting of spheroids using a large object imaging flow cytometer, the COPAS VISION™. As models, we used 3D CoSeedis™ aggregates from various cell lines provided by abc Biopply. We were able to accurately dispense single, intact spheroids into 384 well plates quickly and efficiently. In contrast to traditional methods, where spheroids are sorted out by hand under a microscope, our approach offers a high-throughput manner to assess quality and sort uniform organoids/spheroids to be used for high throughput experiments.

Methods

High-throughput analysis and sorting of large and/or fragile objects in biology present a challenge for traditional flow cytometry instruments. Union Biometrica developed the BioSorter® and COPAS™ platforms to automate the analysis, sorting and dispensing of cells, seeds, beads, particles and small model organisms. It provides flexibility with regards to object size and measures several parameters: size (TOF, Time of Flight), optical density (EXT, Extinction) and the intensity of fluorescent markers. Once analyzed, objects are sorted according to user selectable criteria and they may be dispensed into stationary bulk receptacles or multi-well plates for high-throughput screening, toxicology, or sequencing. The COPAS VISION, which was used in this experiment, provides a new level of capabilities for laboratories working with large particle samples because images are collected of the objects which then can be used as surveys of individuals in a sample (population monitoring) or verification of the identity of sorted events (in multiwell plate assays). The COPAS VISION is able to analyze particles with diameters of 10-750 µm depending on the size of the flow cell in place. The flow cell diameters available are 250 micron, 500 micron, or 1000 micron. The sample is gently carried in a continuously flowing stream at a rate up to 100 objects/second.

Key features of the COPAS VISION:

- Real-time brightfield imaging
- Up to 4 excitation lasers, 4 or 8 fluorescence detectors
- Profiler™ graphically displays parameters along the axis of each particle
- Sorting by size, optical density, scatter, fluorescence and Profiler measurements
- Sorting principle by gentle air diverting, maintains sample integrity
- Collection in multiwell plates, tubes, and various receptacles

To avoid damaging or altering fragile samples, a gentle pneumatic device located after the flow cell is used for sorting and makes the instrument suitable for handling live biological materials (Fig. 1). The fluid pressurization of the instrument (up to 5 psi) is also significantly lower than those of traditional flow cytometers reducing any potential damage from shearing forces.
In this report, we describe tests with the COPAS VISION 500 for the analysis and dispensing of spheroids on the basis of size and optical density using a 488 nm excitation laser. The flow cell in this configuration has a 500 micron cross section.

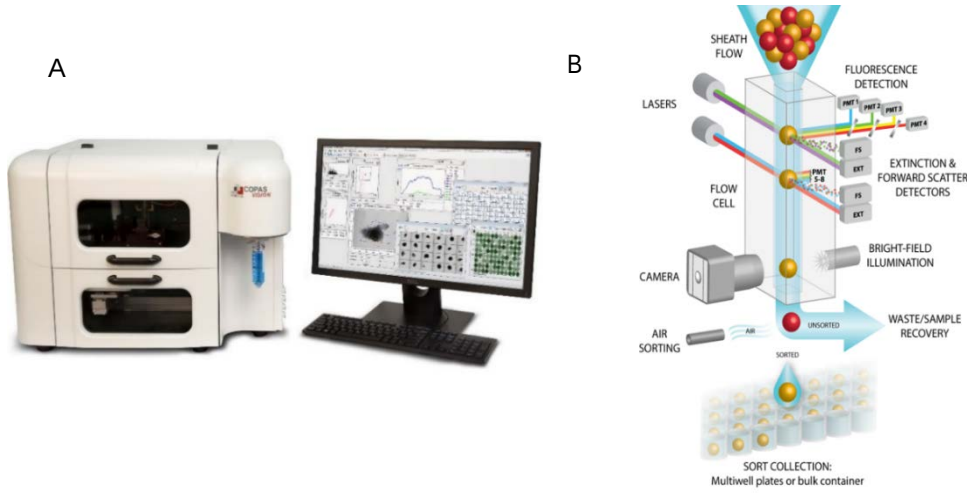


Figure 1. Analysis and sorting of objects inside the flow cell.
A) COPAS VISION instrument and FlowPilot software interface. Contamination is managed by the enclosed sorting area, built-in UV light and HEPA filter. The small footprint of the instrument also allows easy installation into many conventional biosafety cabinets. B) Objects are carried through the flow cell by a liquid stream while their physical properties are being measured. Convergence of the sheath and sample fluid allows "hydrodynamic focusing" of the objects, causing them to go through the center of the flow cell along their longitudinal axis. Inside the flow cell objects are illuminated by a 488nm and/or other solid-state lasers to measure the object's optical properties of: Size (TOF), Optical Density (EXT), Scatter (FSC and SSC) and multiple fluorescence (FLU) parameters. Those objects meeting sort criteria determined by the operator are permitted to drop into the collection device of choice, while those that do not are diverted to waste/recovery using a pneumatic sorting device.

COPAS VISION analysis, sorting and imaging

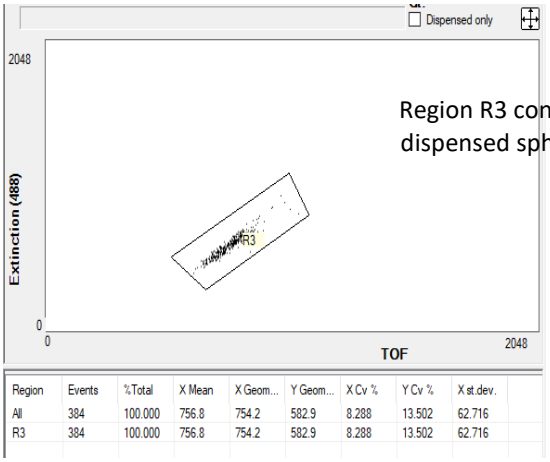
To demonstrate the use of COPAS VISION for high-throughput assay setup with spheroids, tests were performed with cell aggregates from the following cell lines:

#	Cell type	Tissue	Aggregation type
1	WiDr	colon	spheroidal
2	LNCaP	prostate	spheroidal
3	A-549	lung	spheroidal
4	BT-474	breast	spheroidal
5	HT-29	colon	spheroidal

Table 1. Spheroid cell lines tested on COPAS VISION 500. Cell aggregates were generated using 3D CoSeedis™ technology from abc Biopply

HT-29 colon spheroids will be used as a representative case study in this technical note and will be discussed in more detail. After harvesting the spheroids from the 3D CoSeedis™ agarose disks in a single short centrifugation step, the spheroids were pooled together in PBS buffer into a 50 ml conical sample cup and attached to the COPAS VISION 500. As optical measurements of the spheroids were collected with the 488 nm laser, a dual parameter dot plot of TOF (~size) vs EXT (optical density) was generated where every spheroid was represented as a dot in the plot. A region was then drawn around the population of HT-29 spheroids to be sorted (trapezoid region R3 shown in Figure 2).

Figure 2. FlowPilot dotplot display showing gate region selected for sorting HT-29 spheroids



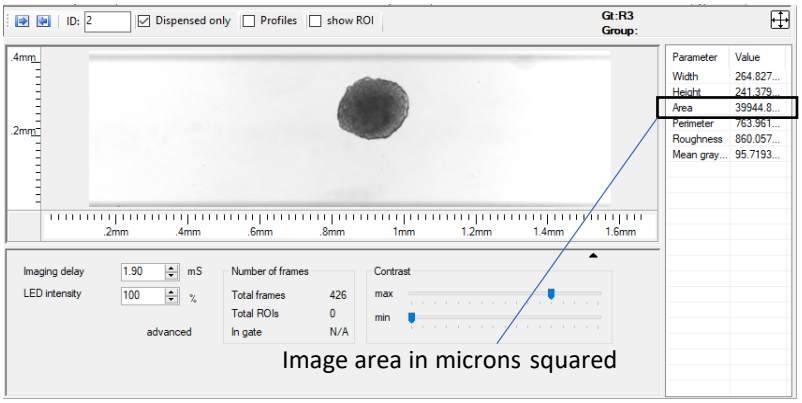


Figure 3. FlowPilot image window showing processed/analyzed single HT-29 spheroid.

For every spheroid that passes through the flow cell, a real-time brightfield image was captured with the on-board CMOS camera. After data collection the images were processed and

information about the surface area, perimeter, roughness and mean grey scale was determined. Below (Figure 3) is the image plot showing one spheroid from the HT-29 colon spheroid sample. On the right-hand side of the plot is a listing of the processed image data for this particular event.

In this example, the HT-29 spheroids were sorted into 384-well plates. After sorting, the sorted spheroids were displayed in a plate view representation for inspection, as shown in Figure 4. The average time to fill a 384 well plate with a single spheroid per well was about 5 minutes, or a little better than 1 per second.

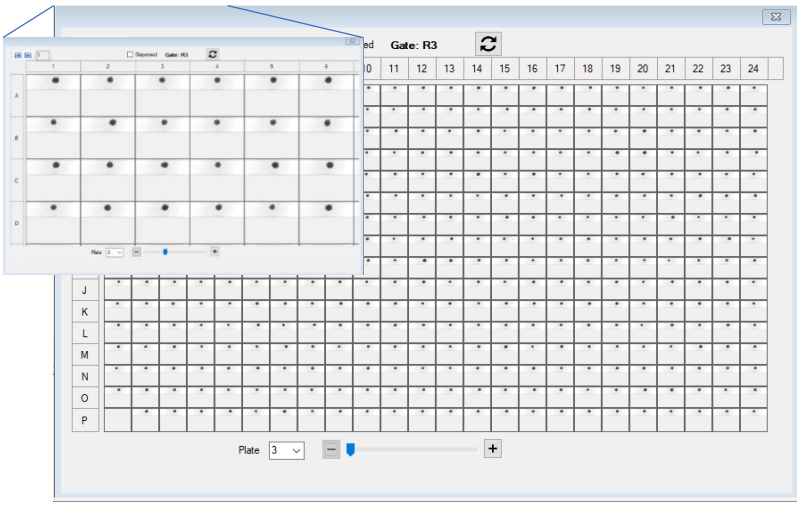


Figure 4. FlowPilot plateview representation. HT-29 spheroids dispensed one per well to 384-well plate.

The sorted HT-29 cell line spheroids can also be visualised as ROI images (regions of interest) as shown in Figure 5. The software removes the background and retains that part of the image that was used for image processing. This allows for a quick means of verifying whether the image processing parameters were correctly set, to verify whether the gating strategy is satisfactory, and to inspect morphology differences in the sample. Because every spheroid has a unique ID number, every event can be traced back to where its location is in the dot plot, in the plate view, and well location coordinates, allowing for easy adjustment of the sort parameters.

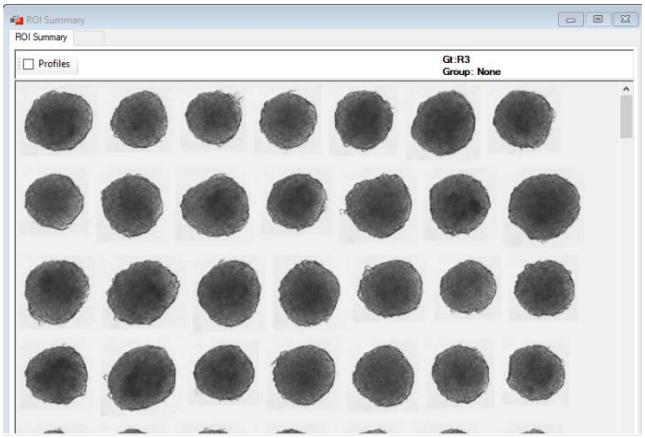


Figure 5. ROI (regions of interest) representation. The processed images are collected for easy visualization.

This experiment was repeated in a similar way for the other cell lines. In total thirty-five 384-well plates were filled with one spheroid per well for the various cell lines. For every cell line, when possible, 4x 384 plates were filled. The spheroids were generated using two different matrix formats of the 3D CoSeedis™ platform (0.5x0.5 and 1x1, abc Biopply).

To determine if the sorting process had an effect on cell survival a viability test using Promega's CellTiter-Glo® 3D Cell Viability Assay was performed. Data is shown and described in Figure 6. For every spheroid sample generated a luminescence test measuring viability was performed at day 0 (D0) and day 2 (D2) after sorting. The results indicated no significant differences resulting from the sorting process.

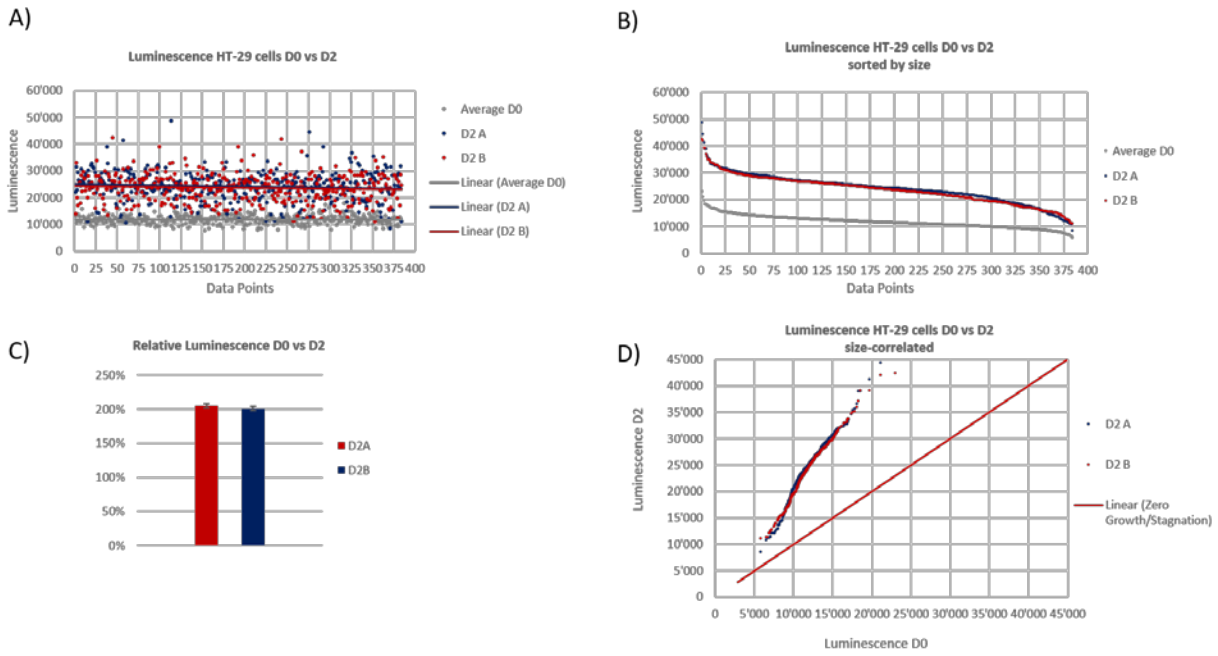


Figure 6: Viability assay on separated HT-29 (colon tumor) spheroids.

Figure 6 shows that the total luminescence is greater for the day 2 spheroids when compared with the day 0 spheroids indicating that they remained viable and increased their metabolic ATP level outputs as expected for growing/dividing cells. The data represents analysis for two 384-well plates of HT-29 colon tumor spheroids and shows that sorting did not have a negative effect on the general metabolism of the HT-29 cell.

Table 2 (below) shows a representative summary of the data collected for the five tumor cell lines tested. These include size, area, "roughness" and time to fill a plate. Other features could also be extracted from the data for analysis, characterization and reporting.

	Summary of Analysis	Values accross plates				
	plate ID	TOF	um2	Circumverance	Img. Roughness/roundness	Time to fill 384 wells (s)
A549	A549 D0A 0.5	639.6	20,662	597	695	289
	A549 D0B 0.5	648.4	24,069	682	648	337
	A549 D22A 0.5	647.4	24,090	687	645	384
	A549 D23B 0.5	647.2	23,353	675	635	485
	Average	**645.6**	**23043.4**	**660.2**	**655.7**	**373.7**
	STD	**3.5**	**1406.4**	**36.9**	**23.2**	**72.3**
	SEM	**0.54%**	**6.10%**	**5.59%**	**3.54%**	**19.33%**
BT474	BT474 0.5	512.0	18,812	525	848	287
	BT474 D0B 0.5	508.7	18,340	514	847	337
	Average	**510.3**	**18576.1**	**519.4**	**847.5**	**311.9**
	STD	**1.6**	**236.2**	**5.1**	**0.0**	**24.9**
	SEM	**0.32%**	**1.27%**	**0.98%**	**0.00%**	**8.00%**
	BT474 D22B 0.5	507.8	18,251	516	844	327
	BT474 day2 0.5	512.6	18,694	520	850	288
	Average	**510.2**	**18472.5**	**518.3**	**846.8**	**307.9**
	STD	**2.4**	**221.2**	**2.1**	**3.1**	**19.6**
	SEM	**0.47%**	**1.20%**	**0.40%**	**0.36%**	**6.35%**
HT29	HT29 day 0 0.5 (1)	754.9	26,895	585	610	290
	HT29 day 0 0.5 (2)	748.4	25,945	557	587	349
	Average	**751.6**	**26420.2**	**571.0**	**598.2**	**319.3**
	STD	**3.2**	**474.9**	**14.0**	**11.5**	**29.3**
	SEM	**0.43%**	**1.80%**	**2.45%**	**1.92%**	**9.17%**
	HT29 day 0 0.5 (3)	756.8	35,542	720	853	353
	HT29 day 0 0.5 (4)	761.9	34,910	742	800	241
	Average	**759.3**	**35226.1**	**731.4**	**826.5**	**296.7**
	STD	**2.6**	**315.6**	**11.0**	**26.5**	**55.8**
	SEM	**0.34%**	**0.90%**	**1.50%**	**3.20%**	**18.79%**
LNCaP	LNCaP D0A 0.5	644.1	26,946	648	800	233
	LNCaP D0B 0.5	623.3	26,995	645	808	261
	Average	**633.7**	**26970.6**	**646.4**	**803.8**	**247.0**
	STD	**10.4**	**24.4**	**1.7**	**4.2**	**13.9**
	SEM	**1.65%**	**0.09%**	**0.26%**	**0.53%**	**5.63%**
	LNCaP D22A 0.5	636.1	26,790	642	809	262
	LNCaP D22B 0.5	656.0	27,461	651	805	242
	Average	**646.0**	**27125.5**	**646.6**	**806.9**	**252.1**
	STD	**10.0**	**335.5**	**4.8**	**2.2**	**9.9**
	SEM	**1.54%**	**1.24%**	**0.74%**	**0.27%**	**3.92%**
WiDr	WiDr tumor colon 0.5 (1)	706.4	25,772	654	772	273
	WiDr tumor colon 0.5 (2)	708.8	19,170	521	572	865
	WiDr tumor colon 0.5 (3)	695.7	23,606	636	742	778
	WiDr tumor colon0.5 (4)	695.3	23,580	650	714	848
	WiDr tumor colon 0.5 (5)	700.7	23,544	624	766	812
	WiDr tumor colon 0.5 (6)	698.6	23,863	628	725	1,970
	Average	**700.9**	**23255.8**	**618.9**	**715.2**	**924.3**
	STD	**5.1**	**1987.7**	**45.1**	**67.4**	**510.1**
	SEM	**0.73%**	**8.55%**	**7.28%**	**9.43%**	**55.19%**

Table 2. Representative data set for cell lines tested on COPAS VISION 500.

Conclusions

These experiments demonstrate that the COPAS VISION is very suitable for the handling of spheroidal cell aggregates generated from several different tumor cell lines using the 3D CoSeedis™ platform. We demonstrated that sorting spheroids is fast, reproducible and does not harm the integrity of the spheroids. The automated analysis and sorting process is gentle and does not influence morphology or viability. A luminescence tests at D0 and D2 demonstrated that the cells are viable and grow.

The COPAS VISION provides a level of automation to the process of handling cell aggregates allowing for increased throughput and eliminates any biases that might be introduced by the researcher. The integrated image processing software allows for accurate determination of surface area, perimeter, and other parameters of every dispensed spheroid for easy data interpretation in high throughput screening assays. The data is collected and analyzed electronically and made available for easy incorporation into presentations and reports.

The COPAS VISION instrument brings the method of flow cytometry to the analysis and sorting of spheroids which are otherwise too large and fragile for analysis on conventional single-cell flow cytometers. This instrument brings the advantages of flow cytometry – statistically meaningful data, large unbiased data sets, multiparametric analysis and imaging– to experiments using 3D-biology.