Application Note

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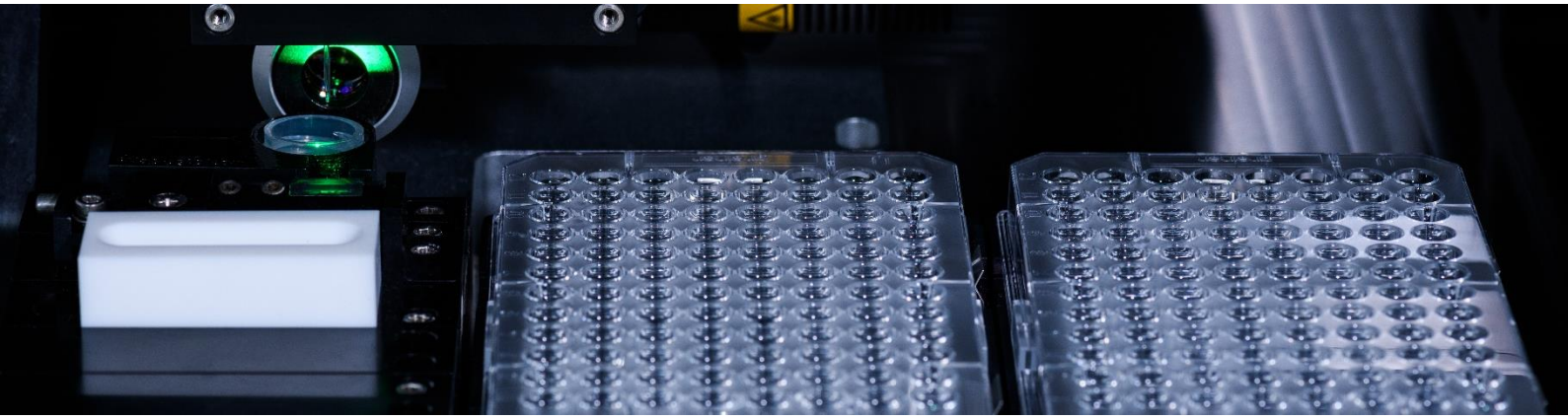

Automated 3D spheroids sorting, isolation and dispense

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Abstract

The spheroONE® is a unique large-particle isolation, sorting and dispensing instrument. The combination of precision low-volume dispensing, image-based tracking of particles within the glass dispensing capillary and automation, enables efficient isolation and sorting of cellular aggregates such as spheroids, organoids and tumoroids. The isolation process typically yields more than 92% of single spheroid dispense in each individual well of a target 96 well plate within minutes. In addition, the user can readily define isolation parameters (*i.e.* diameter and elongation of particles) enabling sorting of distinct sub-populations of cellular aggregates based on their morphology. The spheroONE® provides unprecedented means toward 3D cellular model standardization and automated handling.

Introduction

3D spheroids have gained significant interest in high throughput screening due to their inherent clinical relevance (Nunes, 2018). This is especially relevant in modelling solid tumors, as 3D spheroids present features such as spatial architecture, diffusion barrier, differential gene expression and drug resistance, rendering them the model of choice in anti-tumor drug screenings (Costa, 2016). To yield reproducible assays, pre-selection of spheroids based on their size, morphology and internal organization is critical. There is to date no tractable means to isolate and manipulate cellular aggregates (Singhera, 2017). By merging key technologies, ie. precision nanoliter-volume drop-on-demand dispensing, robotics, imaging and software automation, Cellenion has developed spheroONE®, an innovative platform to sort, isolate and dispense large-particles such as 3D spheroids, organoids and tumoroids. Here, we highlight the capabilities of the platform by demonstrating our ability to isolate spheroids displaying user-defined dimensions and morphological characteristics from a bulk heterogeneous population.

Materials and methods

Automated large-particle isolation and dispensing
A spheroONE® instrument equipped with precision nanoliter-volume drop-on-demand dispensing system, Nano Dispense Capillaries (NDC, *ca.* Ø = 400 µm), an optical system (*incl.* high resolution camera, dark field illumination module), an x-y-z axis system and a dedicated software, was used throughout the following experiments.

Cell culture
HEK cells were cultured in DMEM supplemented with 10% FBS and antibiotics (Penicillin/streptavidin). HEK spheroids were prepared by liquid overlay cell culture in ultra-low attachment (ULA) flasks (Nunclon sphera, Thermo Fisher) under constant agitation (100-150 rpm) for 3-7 days. Culture media was exchanged every second day.

Sample preparation
Samples (3mL) containing spheroid suspension (500-1500 spheroids/mL) in PBS solution were strained (Uberstrain, 70µm) prior to uptake to remove small cellular debris. The strained samples were loaded under sterile conditions into dedicated pressurized sample reservoirs, then transferred into the spheroONE® for processing.

Live/dead assay
4 hours post-isolation and dispense, cell viability was assessed by fluorescent imaging and analysis of spheroids stained using Hoechst 33342 and propidium iodine (PI).

Results and discussion

Single spheroid isolation

A schematic representation of the isolation process can be seen in **Figure 1**. Prior to isolation, to ensure accurate isolation, the NDC is placed in front of the instrument's camera and an automatic mapping procedure is undertaken. The mapping procedure (*ca.* tracking of up to 100 objects inside the NDC while continuously dispensing drops) allows precise determination of the ejection zone (**Fig. 1a**), corresponding to the area filled by the volume of the next drop to be dispensed. To consider the possible sedimentation of particles inside the dispensing capillary during axis movement, the software implements a sedimentation zone (**Fig. 1a**). Once the mapping is done, the isolation process performed by the spheroONE® goes as follows (**Fig. 1b**): (i) the capillary is placed in front of the camera, (ii) before making the next drop, an image is acquired, (iii) the image is processed and analyzed, and (iv) the software automatically determines if the next drop contains a single spheroid matching user-defined isolation parameters (in which case the axis system moves the NDC on top of the next target well to dispense the drop containing the spheroid then comes back in front of the camera) or not (in which case the drop is dispensed directly into a recovery tube).

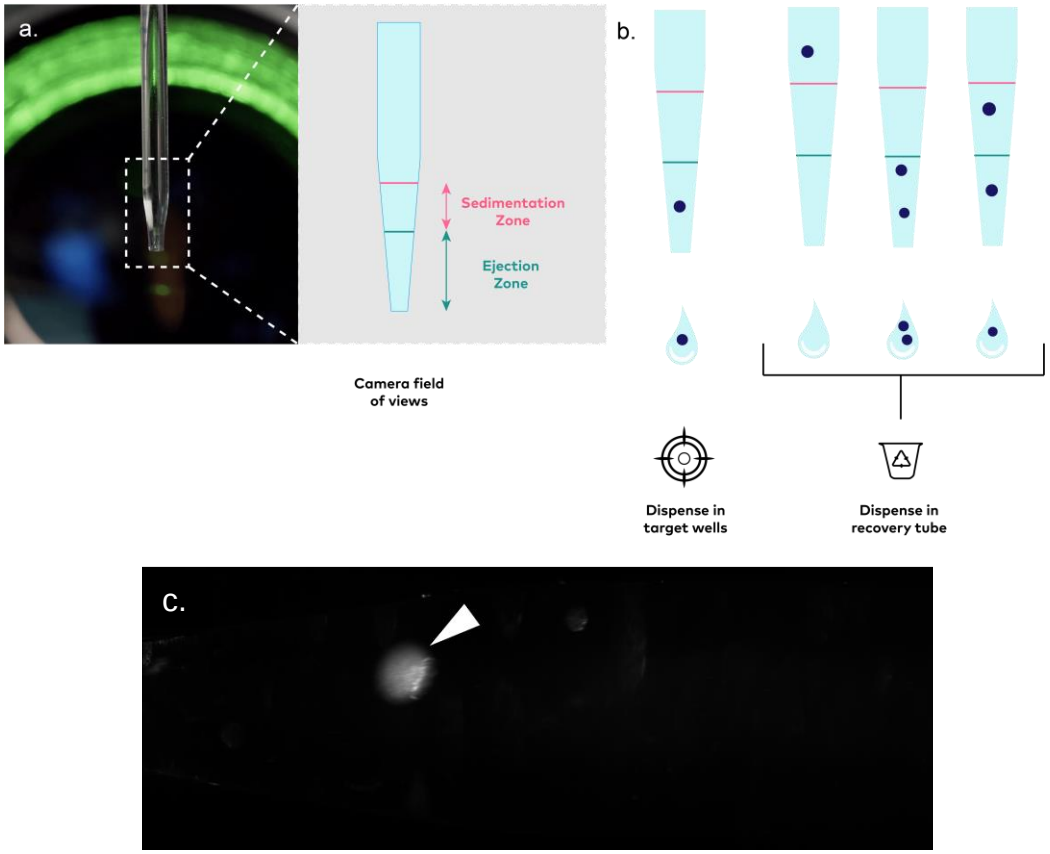

Figure 1: *Isolation process.* ***a****. Nano dispense capillary image with schematic representation of the ejection and sedimentation zones.* ***b****. Schematic representation of isolation process. Isolation case: single particle detected in the ejection zone, no particle in the sedimentation zone. Discard case: no particles detected in the ejection zone (left) or multiple particles detected in the ejection zone (middle) and particle detected in the sedimentation zone (right).* ***c****. spheroONE® image of a cellular aggregate (white arrow) in the NDC under dark field illumination.*

To illustrate the isolation process, a suspension of HEK spheroids (500-1500 spheroids per mL in PBS) was loaded in the spheroONE® (**Fig. 2a**). The sample reservoir was put under pressure (about 200 mBar), and the system was primed using a dedicated task to ensure the tubing were filled with solution and free of any air bubbles. Detection and isolation parameters were set, and a mapping performed to determine the ejection zone. A 96 U-bottom well plate, pre-filled with 50 µL of media, was placed on the target plate holder. An isolation run was started, leading to the isolation and dispense of single spheroids in each individual well of the target well plate (**Fig. 2b**). Sorting of a sub-population was enabled by setting defined size and elongation parameters (**Fig. 2c**).

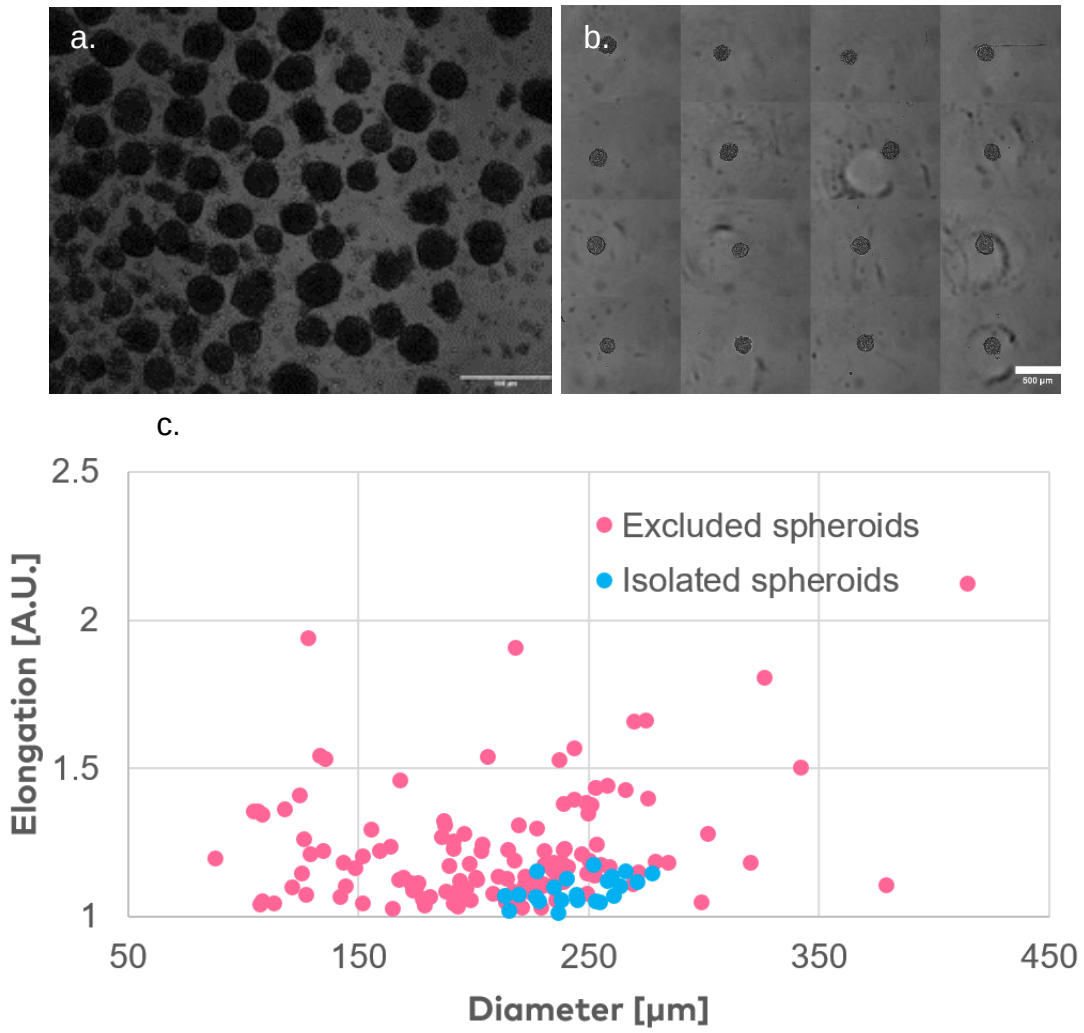

Figure 2: *Spheroid isolation.* ***a****. Microscopy image of the spheroid suspension before sorting and isolation.* ***b****. Stitched images of spheroid isolated into individual wells.* ***c****. Graphical representation of the size (diameter) and shape (elongation) of isolated spheroids (Pink dots) and excluded spheroids (Blue dots). (scale bar = 500µm).*

The isolation process was highly reproducible as illustrated by the accuracy of isolation achieved in four independent isolation runs (**Fig. 3**). On average, 91% (CV < 2%) single spheroids accuracy was achieved.

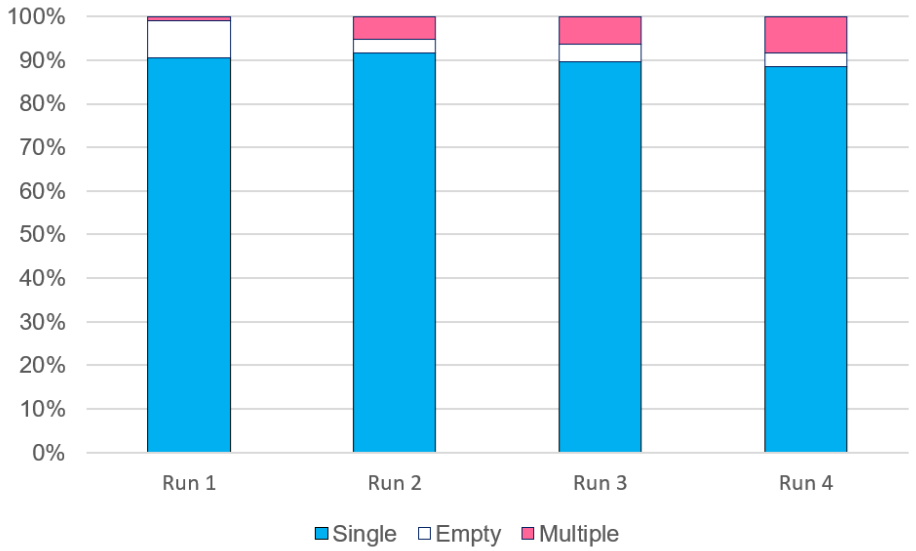

Figure 3: *Graphical representation of isolation accuracy as a percentage per run. (Blue) single spheroid per well, (White) no spheroid per well, (Pink) more than one spheroid per well.*

Spheroid sorting by size

To further illustrate the ability of the spheroONE® to sort spheroids by size, a number of additional runs using distinct sets of isolation parameters were undertaken. In the first isolation run, spheroids with diameters ranging from 100-500 µm were isolated. The second run used slightly narrower parameters and isolated the largest spheroids with diameters ranging from 350-500 µm. The third run isolated smaller spheroids with diameters ranging from 200-250 µm.

After isolation, spheroids were imaged using brightfield microscopy and their average diameter measured from these images. As expected, the first run led to highly heterogeneous single spheroids with measured diameters from 160 to 669 µm (**Fig. 4a, d**). The second run successfully isolated the largest spheroids with diameters from 277 to 550 µm (**Fig. 4b, d**). The third run led to highly homogeneous spheroids ranging from 151 to 238 µm (**Fig. 4c, d**). The discrepancies between the diameters measured using microscopy and spheroONE®'s dark field images were explained by an over estimation of the spheroid diameter using dark field illumination due to light diffusion forming a halo around the outer edge of the spheroids.

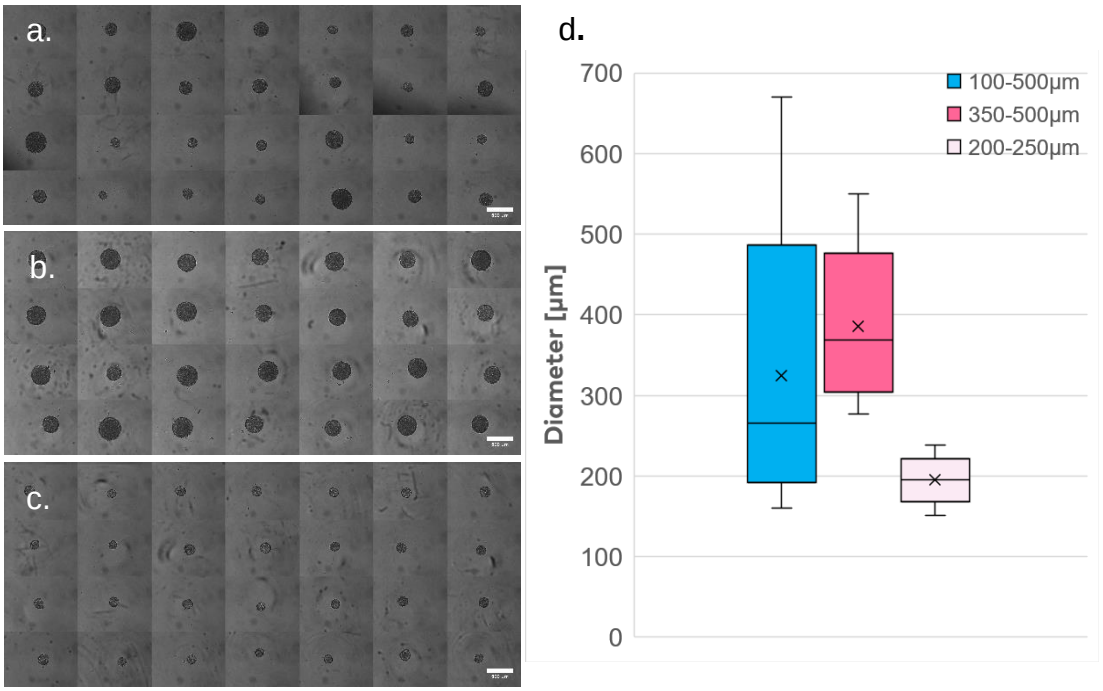

Figure 4: *Spheroid sorting by size.* ***a****. Microscopy image of sorted spheroids ranging from 100-500 µm in diameter.* ***b****. Microscopy image of sorted spheroids ranging from 350-500 µm in diameter* ***c****. Microscopy image of sorted spheroids ranging from 200-250 µm in diameter.* ***d****. Graphical representation of the size distribution of sorted spheroids. Measures were made from microscopy images using default segmentation tools. (scale bar = 500 µm).*

Viability post isolation

To evaluate the viability of spheroids after spheroONE® isolation, a live/dead staining was also performed 4 hours post-isolation (**Fig. 5**). Isolation and dispensing did not compromise the integrity of the cellular aggregates nor their viability (**Fig. 5**). Overall, viability was measured to be above 97% (**Fig. 5b**).

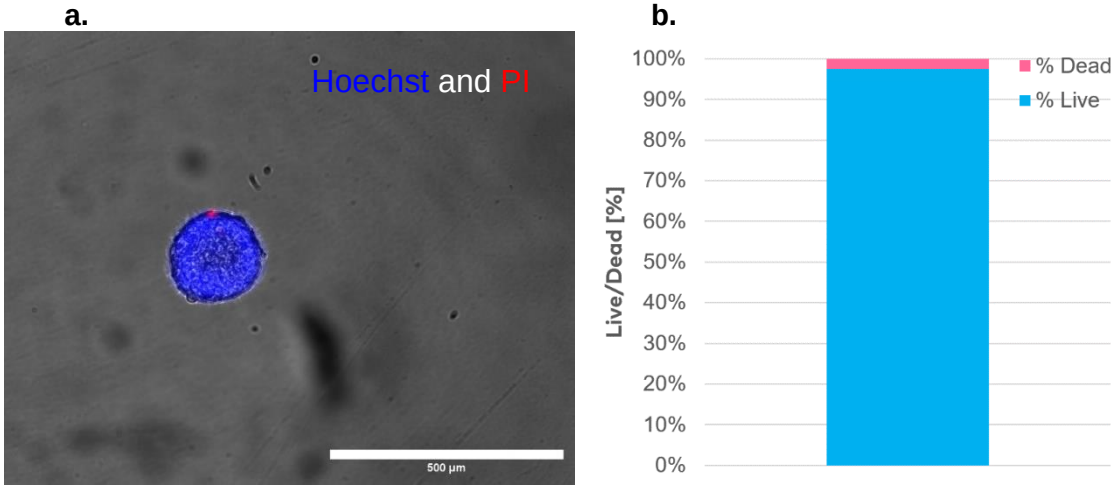

Figure 5: *Spheroid viability post-dispense.* ***a****. Microscopy image of isolated spheroid, stained with Hoechst and PI.* ***b****. Graphical representation of isolated spheroid viability 4 hours post-dispense. Analysis showed > 98% viability after isolation. (scale bar = 500 µm).*

Conclusion and future direction

The results highlight the capabilities of the spheroONE® platform by demonstrating its ability to reproducibly isolate single spheroids with high accuracy (up to 92% single spheroid per well) and sort them according to user-defined characteristics (size and shape). Finally, the integrity of spheroids post isolation was verified using a viability assay which demonstrated that spheroONE® technology did not damage those cellular aggregates or the cells within those during their sorting and isolation. Those capabilities show the potential of this instrument to become an enabling technology toward the establishment of standardized complex *in vitro* 3D models that will drastically accelerate drug discovery and eventually reduce/replace some animal experimentations.

References

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