APPLICATION NOTE

Quality Control of Organoid and Tumoroid 3D Cell Clusters with FlowCam

FlowCam® CORNING

INTRODUCTION

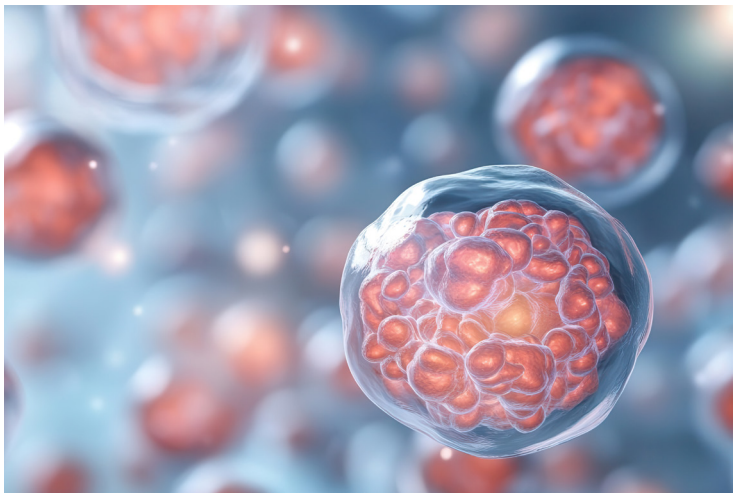
How organoids are generated from inducible pluripotent stem cells (iPSCs) is a delicate and carefully guided process. To direct differentiation toward specific cell types of the target tissue, various growth factors, signaling molecules, and cell nutrients must be combined in the culture media[1]. Further, the physical 3D environment in which cells are grown is crucial because it directly influences cellular functions, including growth, differentiation, migration, and survival. Some 3D cell clusters require a scaffolding matrix, like Corning® Matrigel® matrix or synthetic hydrogels, to support cell migration, cell-cell interactions, and self-organization[2]. In contrast, other types of 3D cell clusters proliferate more efficiently without extracellular matrices[3]. Some rely on stable, spatially confined environments and thrive in static culture conditions. Others thrive in dynamic culture conditions and require exposure to more physiologically relevant stimuli, like fluid flow, shear stress, and oxygen gradients[4]. Regardless of the culture process, effective monitoring of 3D cell cluster size, shape, and morphology metrics is needed to ensure conditions are appropriately tuned and the desired developmental cell cluster fate is achieved.

Traditional manual microscopic analysis of 3D cell clusters is time-consuming and limited

Developing optimized 3D cell cluster culture methods requires regular observation under a microscope to assess size, shape, and cellular arrangement. Traditional microscopic analysis of cultures relies on qualitative visual inspection followed by digital analysis of only a handful of individual cell clusters. Characterization of 3D cell clusters using manual microscopy requires specialized expertise, is time-consuming, and because of the limited sample size, fails to capture the full diversity of 3D aggregate morphological attributes. The ability to monitor 3D cell cluster cultures temporally, non-invasively, and with automation throughout the culturing process, therefore, becomes paramount for optimizing small-scale 3D cell cluster culture methods and for developing the large-scale organoid production methods required for translation to pre-clinical and clinical applications.

Automated flow imaging microscopy (FIM) of 3D cell clusters is fast and reliable

Utilizing advanced imaging technologies to monitor 3D cell cluster morphology and cellular behavior in real-time can provide valuable



insights into optimization and quality control. Flow Imaging Microscopy (FIM) with FlowCam offers a high-throughput method for assessing organoid morphology, analyzing cellular heterogeneity, and informing overall system health. As at-line culture samples flow through the FlowCam microfluidic imaging system, high-resolution digital images of individual 3D cell clusters are captured, and precise particle property metrics are calculated. The biological quality of organoids can be immediately determined, and efficient cell culture process strategy decisions can be made (**Figure 1**).

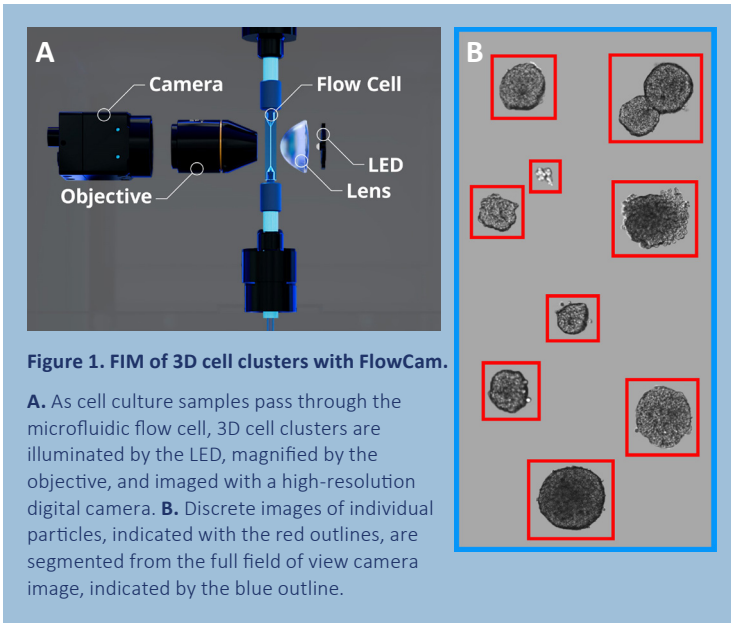


Figure 1. FIM of 3D cell clusters with FlowCam.

A. As cell culture samples pass through the microfluidic flow cell, 3D cell clusters are illuminated by the LED, magnified by the objective, and imaged with a high-resolution digital camera. **B.** Discrete images of individual particles, indicated with the red outlines, are segmented from the full field of view camera image, indicated by the blue outline.

Using FlowCam to evaluate the impact of technology selection on iPSC 3D cell cluster expansion

FlowCam can be used to rapidly assess 3D iPSC culture growth with real-time morphological analysis.

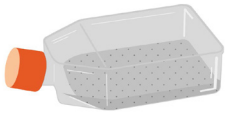
Analysis Goal and Outcome

In this study, we set out to understand the impact of technology selection on iPSC aggregate formation and expansion. Utilizing Corning® Elplasia® technology, static 3D culture in vessels with Ultra-Low Attachment (ULA) surface coating, or dynamic 3D culture in shaker flask technology, iPSC aggregate counts, morphologies, and size distributions were rapidly quantified using FIM with FlowCam. Highly efficient assessment of 3D cell cultures with FlowCam provided high-throughput data in real-time via high-resolution imaging that far surpassed the inefficiency of manual image acquisition and analysis.

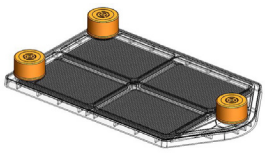
Materials and Methods

iPSC Cell Culture: Normal human iPSCs (iXCells Biotechnologies) were expanded in 2D using Corning Synthemax® II-SC Substrate (SMII) coated Corning CellBIND® surface cell culture flasks, harvested into single cells and seeded into various Corning vessels for 3D aggregate culture. mTeSR™ Plus Medium (STEMCELL Technologies) was used for all 2D and 3D cultures with 10 µM Dihydrochloride (Y-27632, Sigma) added during seeding and removed after 24h.

- *Corning Elplasia® 3D culture:* Corning Elplasia 12K flask (Elplasia 12K) or Corning Elplasia 48K vessel (Elplasia 48K) seeded at 500 cells per microcavity (6 x 10^6 and 24 x 10^6 iPSCs, respectively.)



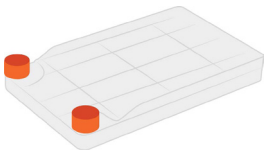
Corning® Elplasia® 12K Flask



Corning® Elplasia® 48K Vessel

- *Static ULA 3D culture:* Corning 75 cm^2 U-shaped flask with ULA surface coating seeded at 6 x 10^6 iPSCs. Corning CellSTACK® 1-chamber with ULA surface seeded at 24 x 10^6 cells.

Corning® 75 cm2 U-Flask with Ultra-Low Attachment Surface

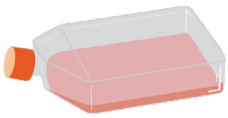


Corning® CellSTACK® 1-Chamber with Ultra-Low Attachment Surface

- *Dynamic 3D culture:* Corning 125 mL Erlenmeyer flask seeded at 3 x 10^6 cells in 20 mL total volume and placed on an orbital shaker (1.9 cm orbital diameter) set to 70 rpm.

Corning® 125 mL Erlenmeyer Flask

- *2D control:* Corning Synthemax coated CellBIND surface 175 cm^2 flask seeded at 10 x 10^3 cells per cm^2.



Corning® Synthemax® II-SC Substrate coated Corning CellBIND® 175 cm2 Flask

iPSCs were cultured for 72 hrs. in a 37°C, 5% CO_2 humidified incubator.

3D cell cluster analysis using traditional manual microscopy:
Images of aggregates in-culture were obtained with an inverted microscope at 24h and 72h time points using 4X objective in brightfield mode. Representative images at 2X magnification were analyzed manually for aggregate size and shape using the ImageJ measuring program.

Automated 3D cell cluster analysis using FIM with FlowCam:
iPSC aggregates were collected at 72h and processed for FIM analysis:

- Real-time image and data analysis with FlowCam: 1 mL iPSC aggregate samples in Corning Phosphate Buffered Saline were run in triplicate using FlowCam 8100 (Yokogawa Fluid Imaging Technologies) configured with 4X magnification objective, FOV 600 flow cell, a sample flow rate of 1 mL/min and ~90 sec. run time.

Results and Discussion

Representative images of 72h iPSC aggregates in Corning 3D cell culture technologies, or 2D culture control flask, were obtained with a manual microscope using a 4X objective in brightfield mode **(Figure 2**). Image acquisition and analysis time required several hours of dedicated expert operator attention. Rounded iPSC spheroid morphology was observed in all 3D culture conditions, with Corning Elplasia technology generating uniform sized and shaped cultures throughout, while other 3D culture technologies showed the formation of various aggregate sizes.

While in-culture image analysis of iPSC aggregates was informative about the health and status of the cultures across technologies, the rapid image capture of iPSCs with FlowCam showed high-resolution particle morphologies and provided cumulative size distribution profiles of the overall in-process culture sample in real-time (**Figure 3**).

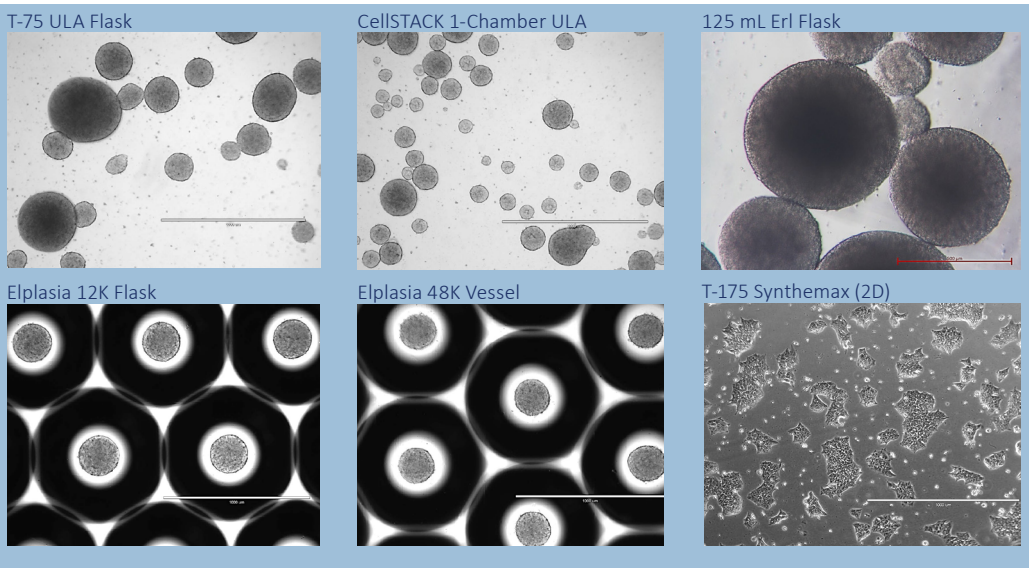


Figure 2. Traditional manual microscopy reveals that spherical iPSC aggregates form and expand in 3D culture.

Representative images of 3D aggregates across technologies obtained with an inverted microscope at 72h time points using 4X magnification in brightfield mode (scale bar = 1000 µm). Note: T-175 Synthemax culture is 2D. The process of manual image acquisition and analysis took several hours.

Cumulative distribution curves showed differences in uniformity across technologies, with the highest degree of uniformity observed in the Corning Elplasia technologies. Static ULA culture in the Corning 75 cm^2 U-shaped flask and the CellSTACK 1-chamber with ULA surface, and dynamic culture in the 125 mL Erlenmeyer flask show greater variability and higher maximum aggregate diameter than Elplasia 12K and Elplasia 48K technologies.

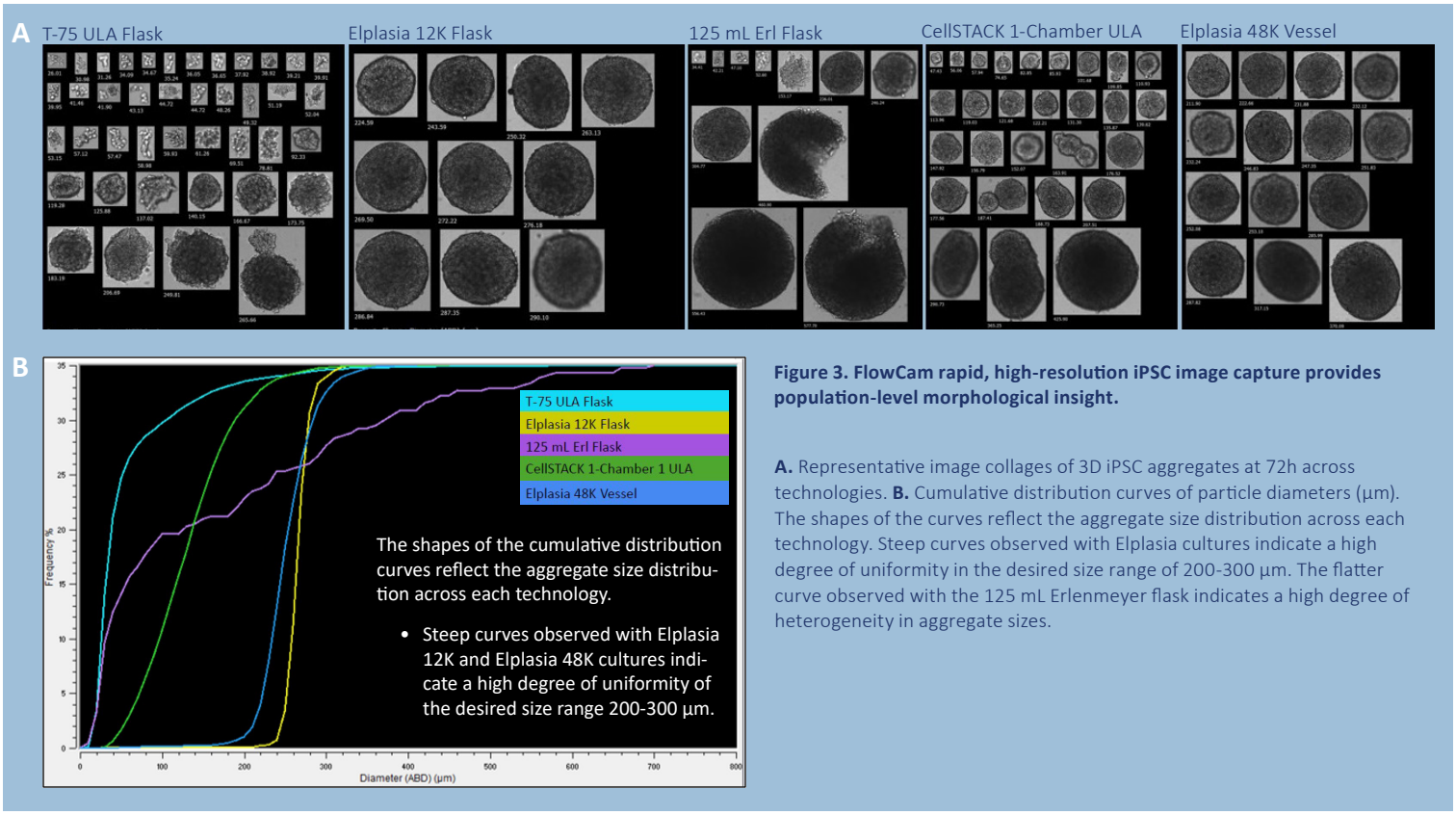


Figure 3. FlowCam rapid, high-resolution iPSC image capture provides population-level morphological insight.

A. Representative image collages of 3D iPSC aggregates at 72h across technologies. **B.** Cumulative distribution curves of particle diameters (µm). The shapes of the curves reflect the aggregate size distribution across each technology. Steep curves observed with Elplasia cultures indicate a high degree of uniformity in the desired size range of 200-300 µm. The flatter curve observed with the 125 mL Erlenmeyer flask indicates a high degree of heterogeneity in aggregate sizes.

FIM with FlowCam eliminates the aggregate size measurement bias inadvertently applied with manual microscopy.

While manual image acquisition and analysis and FlowCam characterization of aggregates captured at 72h both showed an increase in iPSC aggregate diameter over time across all 3D culture conditions, the average diameter values confirmed by the two methods differed, likely because of the differences in total aggregates analyzed by each method (**Figure 4**). For the 125 mL Erlenmeyer flask (125 mL), 5 times more aggregates were measured with FlowCam. The average aggregate diameter confirmed with FlowCam was 249 µm (n=93), ~31% less than that confirmed manually (359 µm; n=18). Similarly, for T-75 ULA aggregates collected at 72h, rapid high-resolution imaging using FlowCam analyzed 55 times more aggregates than manually and confirmed the average aggregate diameter was 115 µm (n=1,045), 44% smaller than that confirmed with manual analysis (205 µm; n=19). For the CellSTACK 1-chamber ULA surface vessel, FlowCam characterized over 250 times more aggregates than those counted manually and confirmed a mean diameter of 143 µm (n= 4,902) compared to 167 µm (n=18).

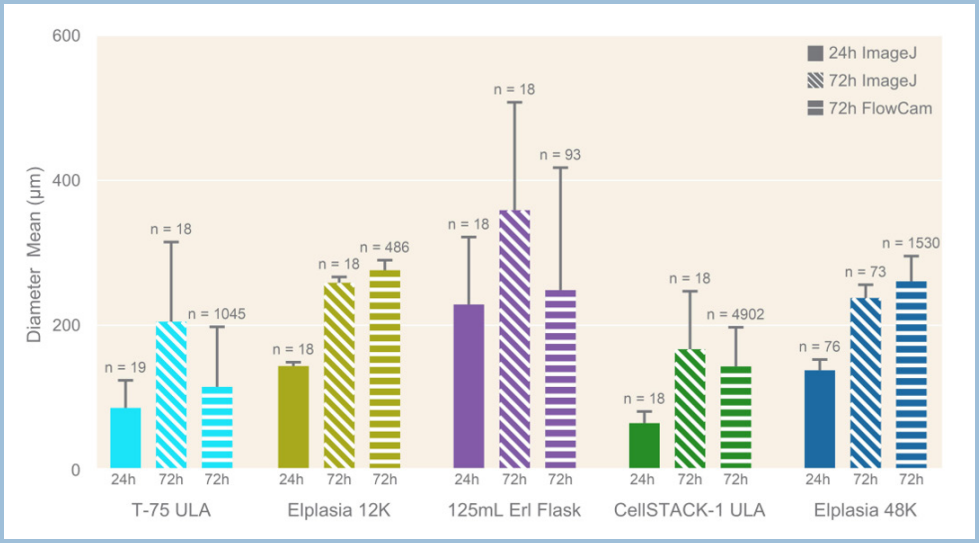


Figure 4. High-throughput FlowCam analysis of iPSC aggregate growth confirms in-culture image analysis in 90 seconds.

Average aggregate diameters of representative images (n=19 to n=76) attained through manual image acquisition and analysis (ImageJ) at 24h and 72h showed aggregate growth over time across technologies (Analysis time: Several hours). FlowCam analysis of many more particles at 72h (n=93 to n=4,902) confirmed in-culture image analysis in a fraction of the time (Analysis time: ~90 seconds). Higher maximum aggregate diameters were observed in static ULA (Corning 75cm^2 U-shaped flask and Corning CellSTACK-1 chamber) and dynamic 125mL Erlenmeyer flask culture systems compared to Elplasia 12K and Elplasia 48K culture systems.

Discrepancies in diameter mean values can be explained by the high-frequency percent of small aggregates (20 µm to 60 µm range) identified with FlowCam that are missed with manual microscopy. Insufficient data collected with manual microscopy, therefore, may result in misleading information. For example, while 3D cell cluster growth was observed with the Corning 75 cm^2 U-shaped flask with ULA surface coating and with the 125 mL Erlenmeyer flask, the extent of reported growth might be exaggerated if looking only at manual image analysis generated data.

The average diameter sizes confirmed with FlowCam and manual analysis were less disparate for the Elplasia 12K flask, Corning CellSTACK 1-chamber with ULA surface, and Elplasia 48K vessel, despite differences in overall aggregate counts, supporting the observation that these platforms yield more uniform particle size distributions compared to the Corning 75 cm^2 U-shaped flask with ULA surface coating and the 125 mL Erlenmeyer flask (**Figure 5**).

The ability to process many more particles and perform high-throughput data analysis in real-time with FlowCam provides an objective, more complete assessment of the aggregate population in a fraction of the time required by manual microscopy (**Figure 6**). Compared to the several hours required for the manual acquisition of representative images and image analysis, high-throughput FlowCam analysis of iPSC aggregate growth can be confirmed in 90 seconds.

CONCLUSIONS

FIM with FlowCam provided rapid processing of 3D iPSC aggregates across Corning culture technologies with efficient real-time analysis. Compared to manual microscopy and analysis, the overall analysis time was greatly reduced from several hours to less than 2 minutes.

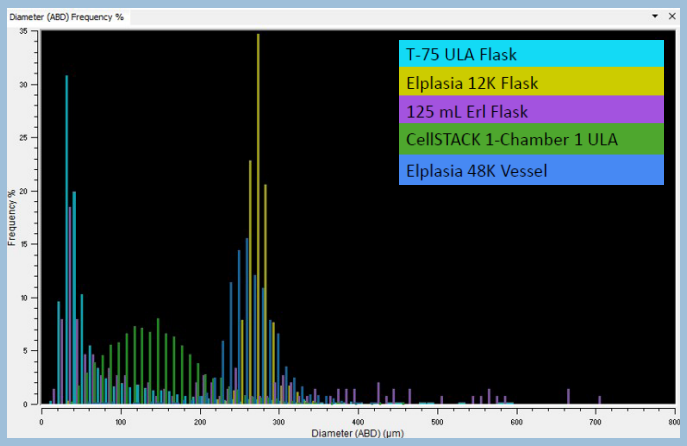


Figure 5. FlowCam particle size distribution analysis highlights the range of iPSC aggregate diameters across Corning technologies.

Elplasia 12K and Elplasia 48K cultures show a more even size distribution of aggregates within the target 200-300 µm size range. The Corning CellSTACK 1-chamber with ULA surface coating cultures are broadly, yet evenly, distributed across 20-240 µm size range. The Corning 75 cm^2 U-shaped flask with ULA surface coating and 125 mL Erlenmeyer flask cultures show right skewed distributions suggesting aggregates are highly variable in size with most aggregates <100 µm.

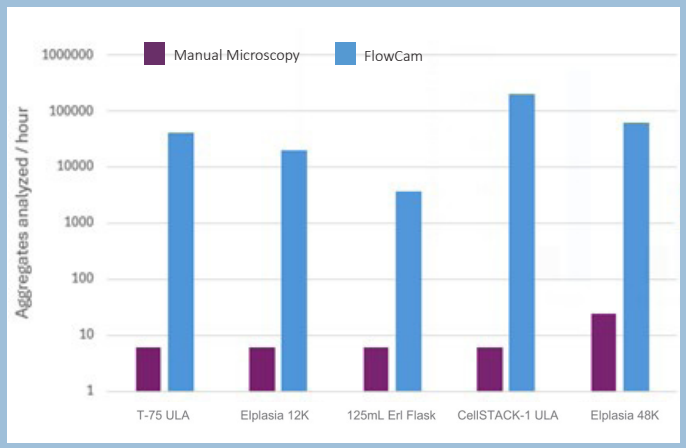


Figure 6. Time savings and statistical strength of FlowCam vs. manual microscopy.

Representation of the time required to analyze 3D cell aggregates with manual microscopy and analysis versus FlowCam. A conservative estimate of 3 hours per sample was used for manual microscopy, and the actual real-time of 90 seconds was used for FlowCam.

High-resolution FlowCam images and particle size distribution analysis showed the variability in 3D aggregate morphologies and size uniformities that can be achieved using the various Corning technologies for both static and dynamic culture conditions. While similar trends were observed with manual microscopy, between 5 and over 200 times more aggregates were measured with FlowCam, bringing greater depth and statistical confidence to the population characteristics analysis.

FlowCam provides an efficient measurement system for assessing iPSC culture optimization methods. As demonstrated in the study with Corning 3D cell culture technologies, FlowCam readily identifies differences in aggregates grown in static and dynamic conditions. Further, the fast, reliable, measurements that FlowCam offers can help ensure critical cell culture attributes are maintained while expansion processes are being tested.

The ability to rapidly process 3D cell cluster samples and gain real-time morphological analysis, compared to labor-intensive and time-consuming approaches of manual microscopy and image analysis is what makes FlowCam invaluable in establishing robust small- and large-scale organoid culture protocols.

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