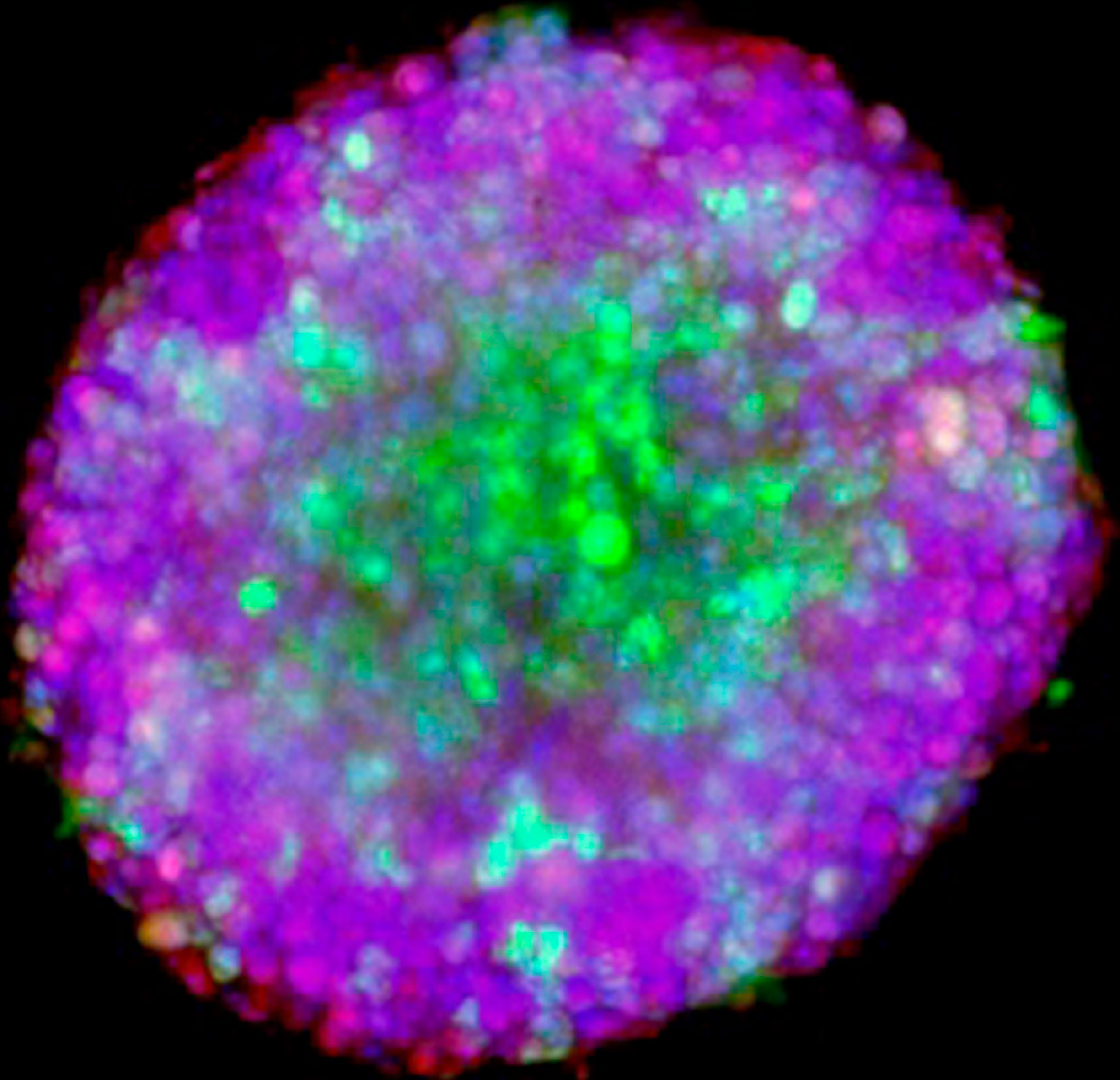
TECHNICAL NOTE

AUTOMATION-ASSISTED WASHING OF MULTICELLULAR 3D SPHEROIDS.

Automated supernatant aspiration and dispensing in cell culture microplates with the HydroSpeed™ plate washer.



TECAN.

INTRODUCTION.

Staining and fixation are basic cell biology techniques that allow the detailed examination and analysis of e.g. cultured cells. Fixation helps to preserve cellular morphology, while specific cellular structures can be visualized by different staining procedures.

Staining and fixing cells or cellular aggregates for endpoint analysis following a successful experiment is a time-consuming and laborious task, typically performed manually using standard workflow protocols. These protocols involve multiple cycles of incubation with different reagents and subsequent washing steps. Fixation aims to preserve cellular architecture by stopping enzyme activity and stabilizing proteins and cellular structures. Commonly used fixatives include paraformaldehyde (PFA), glutaraldehyde and methanol, each one having specific attributes tailored to different experimental needs. Staining is employed to enhance contrast in microscopic images, making specific cellular compartments or structures more visible and distinguishable. Staining protocols must be optimized for 3D cellular aggregates to ensure even distribution of reagents and consistent results across wells. This involves careful control of reagent volumes, incubation times and washing steps to minimize variability. Washing the specimen is a critical step. It helps to remove excess reagents to ensure clear and accurate visualization of cellular structures while maintaining cell integrity to achieve reproducible results. Errors can often occur during this process, potentially affecting the quality and reliability of the experimental results. These errors often revolve around procedural steps such as manual pipetting of liquids and equipment handling. One way to overcome these issues is to use automated washing systems, such as Tecan's HydroFlex™ and HydroSpeed plate washers. These systems not only help to increase throughput for drug discovery and screening applications, but also help to standardize washing procedures, enhancing consistency and reproducibility, and minimizing well-to-well variability across a microplate. Controlled aspiration and dispensing reduce the risk of cell loss and have a significant effect on the generation of scientifically relevant, comparable data. This technical note describes the integration of the HydroSpeed plate washer into the staining and fixation processes for 3D multicellular spheroid cultures, due to its aspiration and dispensing properties which can be performed in various microplate formats (Figure 1).

MATERIALS AND METHODS.

Handling.

Image acquisition and monitoring of the spheroids, and quality control of the processing steps were performed using a Spark® Cyto multimode reader and SparkControl™ software. A HydroSpeed plate washer was used for all aspiration steps in the workflow, as well as for dispensing phosphate-buffered saline (PBS) during the washing process. Downstream analysis was performed with the proprietary Image Analyzer™ software.

Workflow.

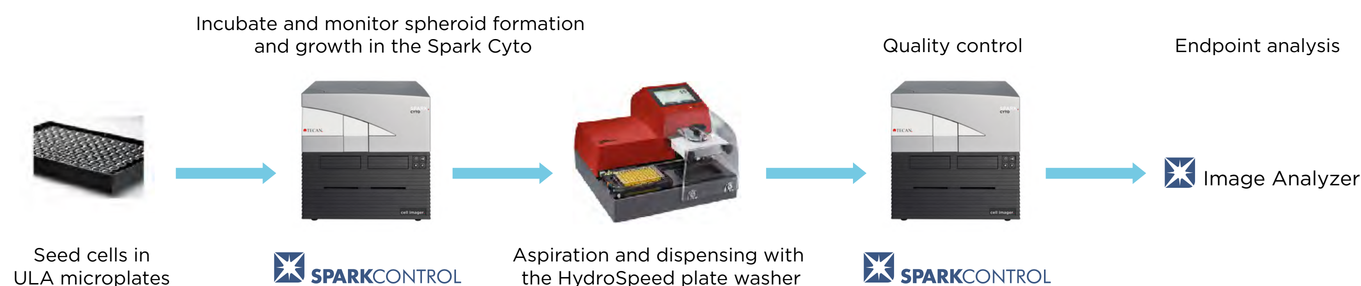


Figure 1: Visualization of the workflow.

Cell culture.

Passage 8 (p8) HeLa-GFP cells (GenTarget/Gentaur; #SC034-Puro) were expanded in an F75 flask in Gibco™ RPMI 1640 medium (Thermo Fisher Scientific, #21875034) supplemented with 1 mM Gibco sodium pyruvate (100 mM; Thermo Fisher Scientific, #11360070), 2 mM Gibco L-glutamine (200 mM; Thermo Fisher Scientific #25030024), 10 mM Gibco HEPES (1 M; Thermo Fisher Scientific, #15630080), 1X Gibco antibiotic-antimycotic (100X; Thermo Fisher Scientific, #15240062) and 10 % Gibco fetal bovine serum (FBS; Thermo Fisher Scientific #10500064). Cells were maintained in a humidified environment at 37 °C and 5 % CO_2.

The cell flask was briefly rinsed with 10 ml of PBS (Sigma-Aldrich, #L0615). Cells were incubated with PBS + trypsin-EDTA (0.5 %, Thermo Fisher Scientific, #15400054) at 37 °C and 5 % CO_2 for 5 minutes, to detach the cells and harvest them in their respective main experimental media. After centrifugation at 1,200 rpm for 3 minutes, the cell pellet was resuspended in media, and the cell suspension was counted for cell seeding.

Spheroid formation.

HeLa-GFP cells were seeded in a 96-well, round bottom, ultra-low attachment (ULA) microplate (Corning® #4520) at five different cell densities – 6,000, 3,000, 1,500, 750, and 375 cells per well in 150 µl media – Additionally, HeLa-GFP cells were seeded in a 384-well, round bottom, ULA microplate (Corning #4516) with 4,000 cells per well in 80 µl media - both plates were centrifuged at 1,200 rpm for 2 minutes. The microplates were then placed in an incubator at 37 °C and 5 % CO_2 for 48 hours.

Staining and fixation.

The post-experimental workflow was automated by integration of a HydroSpeed plate washer for all aspiration and washing steps during the spheroid staining and fixation process. Briefly, the medium was aspirated using the set-up depicted in Figure 2 to give a resulting residual volume of ~50 µl. Hoechst 33342 staining solution (Thermo Fisher Scientific, #62249) was prepared to a working concentration of 30 µg/ml. 50 µl of the working solution was manually added to each corresponding well using a multichannel pipette, resulting in a final concentration of 15 µg/ml (1:1 dilution). MitoTracker™ Red CMXRos staining solution (Thermo Fisher Scientific, #M7512) was prepared to a working solution of 400 nM. 50 µl of the working solution was added to each corresponding well in the same fashion as before to give a final concentration of 200 nM (1:1 dilution). The staining solution was aspirated to a minimum residual volume after approximately 1 h of incubation at 37 °C and 5 % CO_2 and washed once with 100 µl PBS. For fixation of the spheroids, 150 µl of 4 % paraformaldehyde (PFA) was added to each well manually, followed by incubation for approximately 1 h at room temperature. The spheroids were then washed three times with 100 µl PBS. The process was fully automated on the HydroSpeed washer, sequentially aspirating each well and then dispensing the PBS washing solution (Figure 3). The volume was altered to 150 µl PBS for the final dispensing step and subsequent storage of the plate.

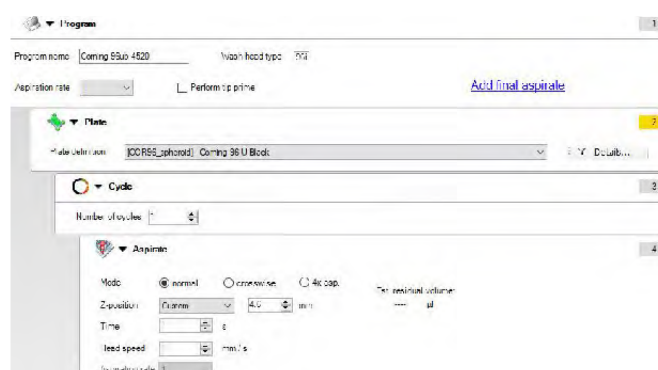


Figure 2: HydroControl settings for aspiration of supernatant from a 96-well round bottom microplate. Aspiration with this setting generates a remaining residual volume of 50 µl per well.

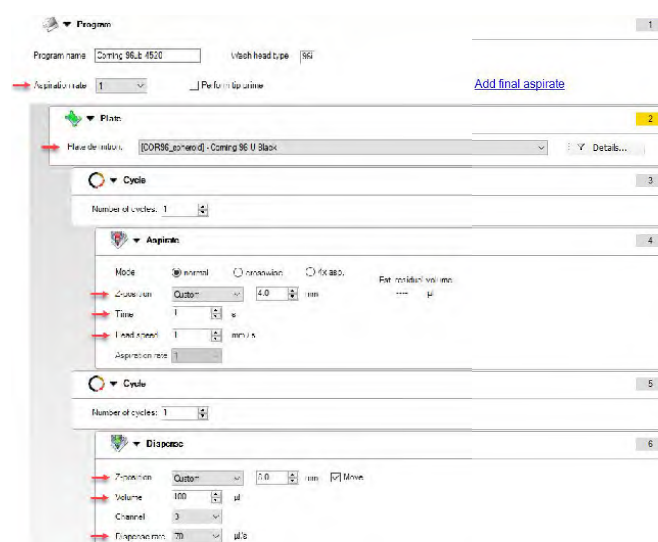


Figure 3: HydroControl settings for the washing protocol of the 96-well plate using the HydroSpeed plate washer. The most important settings are highlighted with red arrows.

Monitoring.
The whole plate was initially imaged using the Spark Cyto with the 4x objective and a center field of view (FOV) in the brightfield (BF) and green fluorescence (FL) channels before staining. The plate was then imaged in the BF, and green, blue, and red FL channels after staining. Additional imaging was performed following the final washing steps, to document any potential spheroid loss during processing. The plate layout is shown in Figure 4; the last two columns (11 and 12) were intentionally left out because a further seeding density dilution might not result in spheroid formation.

RESULTS AND DISCUSSION.

Processing plates for better visualization of the experimental outcome can be very laborious, especially when working with a high sample load. Manual aspiration of multiple 96- or 384-well microplates and subsequent pipetting of fluorescent stains or antibody labeling can be time consuming and requires intensive effort from researchers. The same applies to washing the plates for further fixation and final storage. An automated process enables fast and precise aspiration of the supernatant, with the aspiration settings adjusted depending on the plate type and cultivation method. Monitoring of the microplate was performed with the Spark Cyto before and after critical processing steps, such as staining and washing of the spheroids.

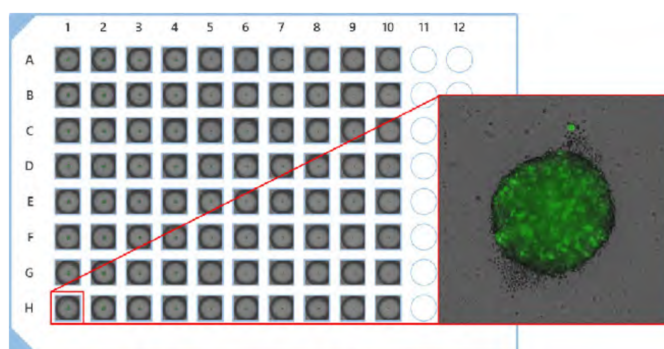


Figure 4: Matrix plate view of the microplate with HeLa-GFP spheroids with decreasing cell densities from left to right. The plate layout is depicted before post-processing (staining and fixation) was performed; the red box illustrates a zoom-in section of well H1.

The spheroids were incubated with Hoechst (rows C and D), MitoTracker Red (rows E and F) and a combination of both (rows G and H). As shown in Figure 5, the staining of the spheroids is already visible and the remaining dye in the wells can be seen, especially for the red dye (rows E-H). This is because washing of the dyes was performed after imaging.

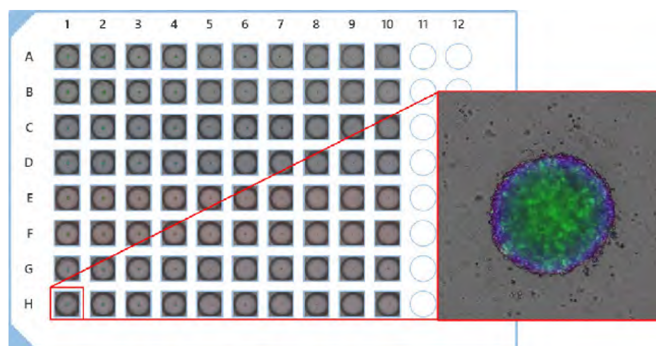


Figure 5: Matrix plate view of the microplate with HeLa-GFP spheroids. An overview of the microplate after cell staining was performed; the red box illustrates a zoom-in section of well H1.

After staining, the spheroids were fixed with 4 % PFA and subsequently washed thoroughly with PBS to ensure the remaining dye and fixative were fully removed from each well. The results can be seen in Figure 6, which depicts an overview of the microplate after complete post-processing. Only three of the spheroids were lost (golden boxes) during these final washing steps, either due to the specimen sticking to the walls of the wells or being aspirated.

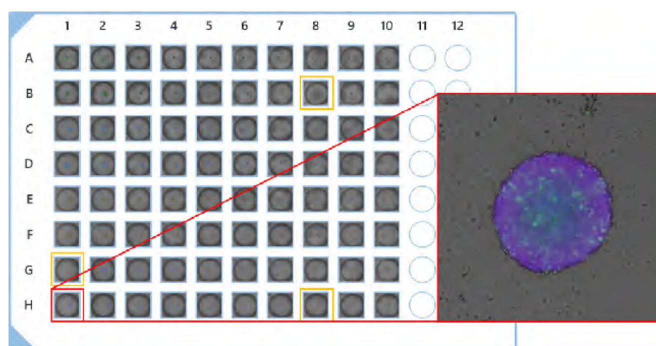


Figure 6: Matrix plate view of the microplate with HeLa-GFP spheroids. An overview of the microplate after post-processing (staining and fixation). Three spheroids were lost during the final washing step, indicated by the golden boxes; the red box illustrates a zoom-in section of well H1.

Automated washing in a 384-well Black/Clear Round Bottom Ultra-Low Attachment microplate with single spheroids.
This section illustrates the customized compatibility of the HydroSpeed for post-processing procedures. A 384-well microplate was selected and evaluated for the washing process to highlight the ease of use of the HydroSpeed in a high throughput setting. An essential step for the successful implementation of automated aspiration is establishing the optimal settings for each plate type. This includes finding a z-position that is high enough to leave the sample undisturbed while simultaneously reducing the residual volume per well to a minimum.

The HydroControl settings are displayed in Figure 7. All aspiration and dispensing steps were performed successfully with the HydroSpeed. The overall washing process was quick and easy, and all spheroids remained in place (Figure 8).

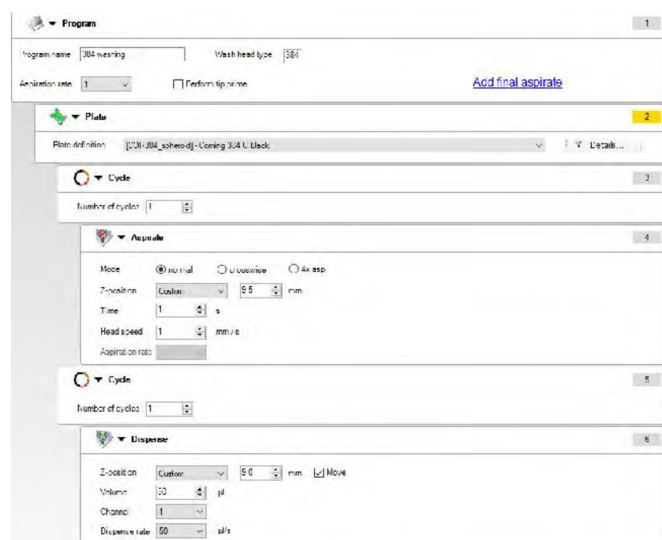


Figure 7: HydroControl settings for the washing protocol of a Corning 384-well microplate using the HydroSpeed plate washer.

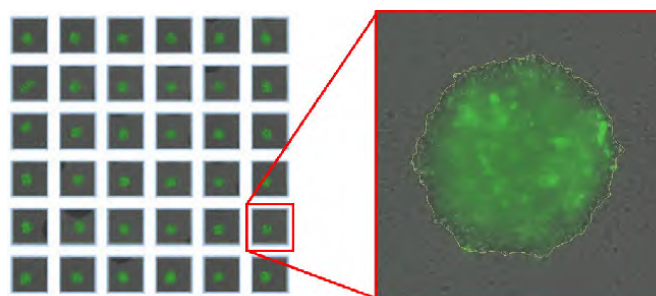


Figure 8: Matrix view of a section of the 384-well microplate with HeLa-GFP spheroids created with the SparkControl software during imaging. The images shown were taken after fixation with 4 % PFA. The image in the red box was acquired with the Spark Cyto using the 10x objective and a center FOV; included is the BF segmentation mask (yellow line).

CONCLUSIONS.

The HydroSpeed has been successfully used for post-experimental processing of spheroids, including washing steps. This technical note demonstrates that it can be used for both 96-well and 384-well round bottom microplates with single multicellular 3D spheroids. Integrating the HydroSpeed into the staining and fixation workflow offers the following benefits:

1. Liquid and equipment handling errors are minimized.
2. Residual volumes are identical in each well.
3. Consistency and reproducibility are increased.
4. Minimal well-to-well variability improves standardization, saving time and reducing the number of consumables required.

ABOUT THE AUTHOR.

Eric Lutsch, MSc. *is an Application Scientist at Tecan Austria. He studied medical biology at the University of Salzburg to achieve his master's degree, focusing on regenerative biology and aging, conducting stem cell research, and establishing workflows for 3D cell culture, such as spheroids. Eric has experience in 3D cell culture, tissue engineering, microfluidics, and low-cost device design and production. He joined the sales and marketing team at Tecan in 2023, with an emphasis on the Spark Cyto multimode reader and its imaging and detection applications.*

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