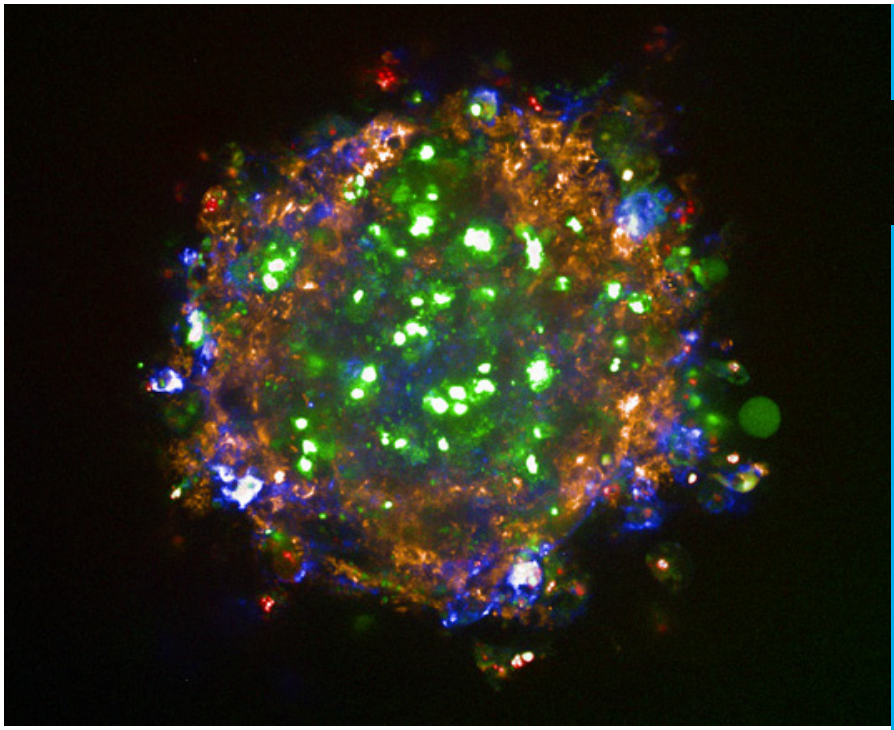


APPLICATION NOTE

Automated Liquid Handling

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Automation of 3D Spheroid Production, Cell Culture and Analysis

Introduction

Advancements in cell culture techniques to more precisely mimic in vivo systems has led to greater interest and utility of three dimensional (3D) cell culture systems, as they represent an improved model for cell migration, differentiation, survival, and growth. 3D cell culture, such as 3D microtissues, bridge the gap between traditional monolayer cultures and whole-animal systems, where traditional monolayer and monotypic (2D) cell culture and screening assays do not effectively recapitulate the more complex signaling and cellular interactions found in vivo.

Reproducible cell seeding and reliable formation of similar-sized 3D microtissues is essential to enable collection of robust data when adapting more biologically relevant, complex 3D models to high-throughput workflows. Although seeding of cells, transfer of microtissues, and medium exchange can be performed reliably using automated hand-held pipetting devices, this is not practical when production of large numbers of spheroids is needed for high-throughput studies, necessitating the adaptation of automated liquid handling workflows. Here we demonstrate a standardized, cost effective and automated means of producing and characterizing 3D spheroid microtissues using the InSphero GravityPLUS™ Hanging Drop System and the Zephyr® G3 automated workstation to automate the liquid handling steps involved in microtissue seeding and transfer to the GravityTRAP™ Plate for long-term culture and imaging. Microtissue growth was assessed with the EnSight™ multimode plate reader with well imaging directly in the GravityTRAP™ Plate. A custom-developed analysis method automatically generated brightfield images of each well to identify and measure spheroid area. This allowed for rapid monitoring of microtissue growth without necessitating the addition of a fluorescent label.

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Materials and Methods

InSphero GravityPLUS™ Hanging Drop System

The GravityPLUS™ Hanging Drop System from InSphero is a two-plate system composed of the GravityPLUS™ Plate for microtissue production, formation, and maturation in hanging drops; the second plate (GravityTRAP™ Plate) is utilized post-spheroid formation to facilitate long-term culture, compound dosing, imaging and assays (Figure 1).

Automated Seeding and Spheroid Formation Workflow

On day zero, the GravityPLUS™ Hanging Drop plates are seeded by the Zephyr G3 Automated Workstation with 40 µL of cell suspension (e.g., HepG2 cells) through the SureDrop™ Inlet (supplementary video). Newly seeded cultures are transferred manually to the incubator. Within 2-3 days, a single microtissue "spheroid" matures in each drop. Note that the time for spheroid formation depends on cell type and should be monitored daily by microscopic evaluation (Figure 2).

Post-spheroid formation, microtissues are harvested and transferred into the companion GravityTRAP™ Culture and Assay plate. This process has been automated on the Zephyr G3 automated workstation (Figure 3). The GravityTRAP™ plates are pre-wetted (supplementary video) with cell culture media; then the spheroids are transferred to the plate. All automated pipetting was accomplished within a sterile environment, as the Zephyr G3 instrument fits easily within a standard tissue culture hood. (An optional centrifugation step was performed to improve transfer of the spheroid tissue to the small central well of the GravityTRAP™ Plate).

Subsequent cell culture and assay experimentation steps can be automated on the Zephyr G3 platform, including media changes, assay compound dosing and supernatant sampling or addition of buffers for tissue lysis allowing for analyses of cellular markers. The proprietary non-adhesive coating of the GravityTRAP™ Plate allows for culturing over weeks without attachment and decomposition of the microtissues. Cultures can be treated with compounds and imaged directly in the GravityTRAP™ Plate.

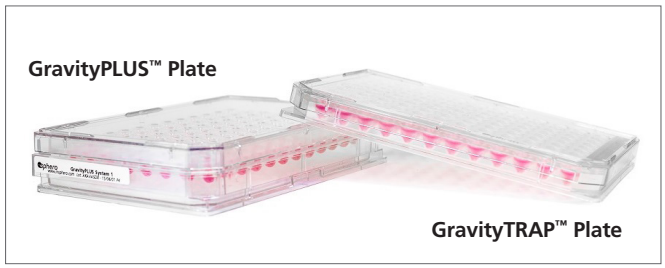


Figure 1. InSphero Hanging Drop System consisting of the GravityPLUS™ Plate and the GravityTRAP™ Plate.

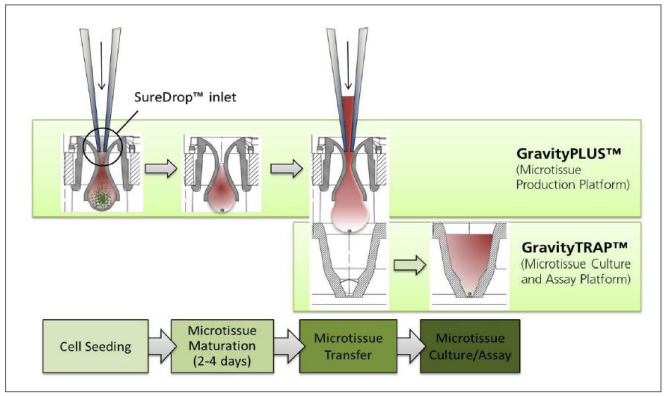


Figure 2. 3D microtissue formation using the InSphero GravityPLUS™ Hanging Drop System. All major steps of the workflow were automated using the Zephyr G3 automated workflow to demonstrate reliable, reproducible high-throughput production and maintenance of 3D tumor spheroids.

Results and Discussion

Automation Maintains Spheroid Reproducibility and Increases Process Efficiency

Compared to manual seeding and handling, automating 3D spheroid formation, continuous culture, and imaging processes results in comparable cell seeding accuracy, microtissue formation efficiency, and size uniformity while minimizing hands-on time and reducing contamination risk for high-throughput spheroid production runs. The transfer support frame (Figure 3, Product # CS-10-001, InSphero AG) allows the Zephyr G3 instrument to precisely align the GravityPLUS™ Plate over the GravityTRAP™ Plate for optimal transfer of the spheroids. Additional benefits of using automation (not shown) include higher-throughput

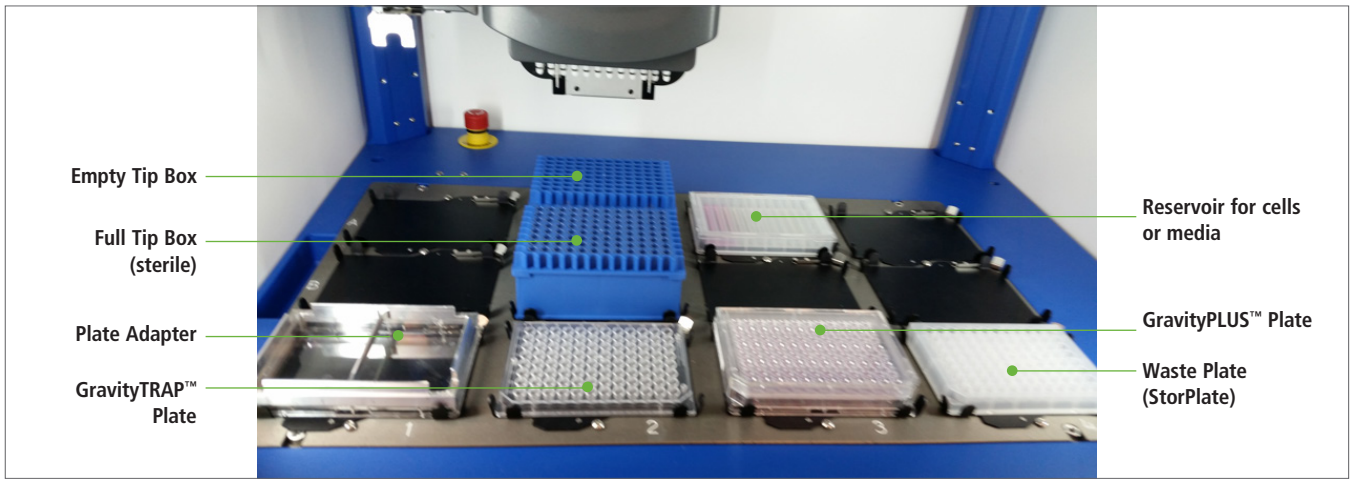


Figure 3. Zephyr G3 deck setup for all automated pipetting steps, initial cell seeding of the GravityPLUS™ Hanging Drop plate, GravityTRAP™ culture plate preparation (wash) and subsequent harvesting of microtissues from the GravityPLUS™ Hanging Drop plate and transfer into the companion GravityTRAP™ Culture and Assay plate. A sterile environment was preserved, as the instrument fitted within a standard tissue culture hood.

workflows, integrated spheroid growth detection and assay development workflows.

Automatic Spheroid Detection and Size Measurement with the EnSight Multimode Plate Reader

Utilizing the EnSight's user-friendly, yet powerful, Kaleido™ data acquisition and analysis software (Figure 4 and 5), a custom spheroid measurement analysis method was written to:

- Detect the presence of a spheroid within a well
- Automatically autofocus and image cultures directly in the GravityTRAP™ plates (brightfield, single-plane)
- Detect the edges of the well and spheroid
- Measure area, shape and contrast of the detected spheroid
- Export the data in the preferred format

Automated 2D imaging and size analysis was performed using the EnSight multimode plate reader in order to characterize 3D tissue size and growth directly in the GravityTRAP™ Plate using a custom-developed measurement protocol. Kaleido software uses pre-determined focus settings to generate brightfield images of the tissue cultures, and automatically analyzes the images by first detecting the edges of the well and the spheroid tissue (Figure 5). Spheroid number and area data can be exported automatically to the analysis program of choice. A comparative study of manual and automated plating on the Zephyr G3 platform was done to assess spheroid seeding variability for each plating method. 3D HepG2 spheroid cultures were imaged and measured on day four after being transferred to GravityTRAP™ Plate 3 days post seeding. The cultures that were seeded and transferred using the Zephyr G3 instrument had regularly shaped, consistent spheres. We demonstrate similar seeding consistency (3000, 6000 and 9000 cell densities) between manual and automated methods (Figures 6 and 7) leads to similar uniform growth. This was determined by raw areas (square microns) exported from the spheroid measurement analysis method measured on the EnSight plate reader.

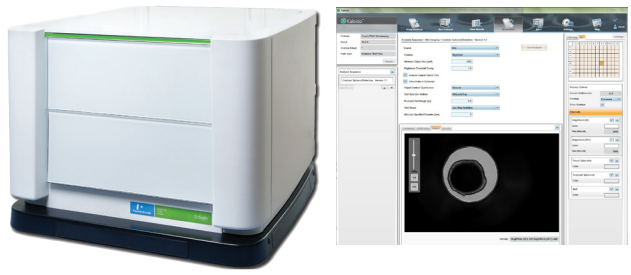


Figure 4. EnSight multimode detection plate reader and Kaleido data acquisition and analysis software. It is the first benchtop system to offer well-imaging alongside label-free and labeled detection technologies.

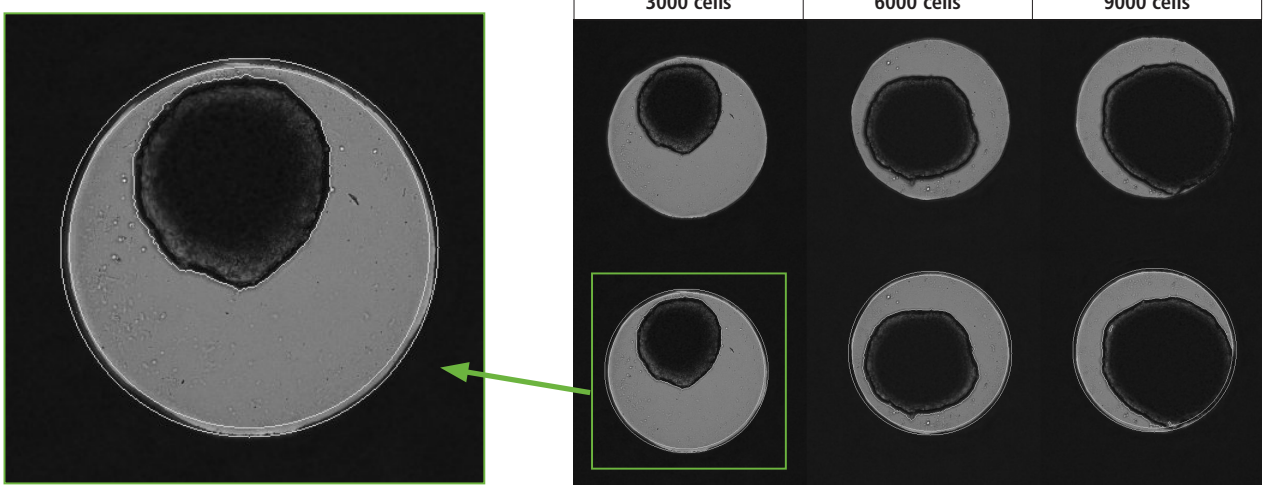


Figure 5. Representative images of 3D HepG2 (hepatocyte) spheroid cultures taken with the EnSight plate reader. The white lines drawn by the spheroid measurement analysis method illustrate the edges of the GravityTRAP™ plate wells and the edges of the spheroid. The analysis software can distinguish spheroid formation that rests against the edge of the well. The more cells initially seeded, the larger the tissue area. The diameter of the well in the GravityTRAP™ plate is 1 millimeter. These images were cropped and compiled into a montage using ImageJ Software (NIH).

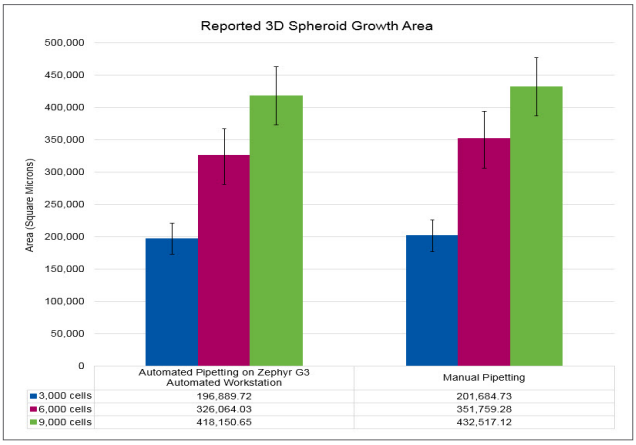


Figure 6. Uniformity of HepG2 spheroid growth of various seeding densities from automated and manual pipetting platforms. Manual plating was performed with a multi-channel pipette (one column/8 wells at once). 3D spheroid growth was assessed on day four. The raw area data (square microns) shown is exported from the spheroid measurement analysis method measured on the EnSight plate reader.

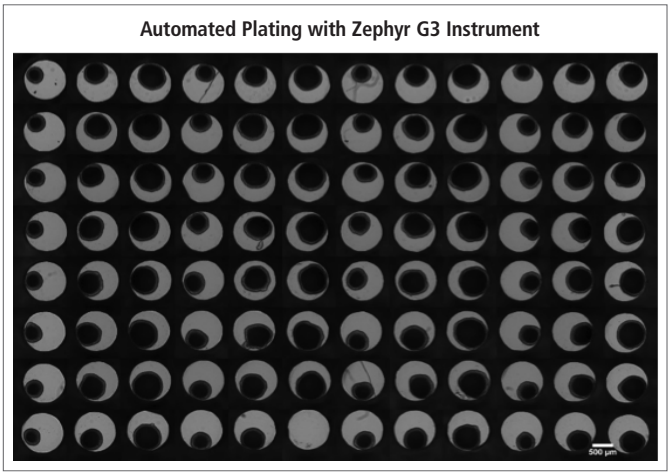


Figure 7. Reproducible automated HepG2 spheroid production using the Zephyr G3 Instrument. Image results are 4 days post seeding; transferred to GravityTRAP™ Plate on day 3. All wells were plated in groups of three columns at once - from left-to-right in the order of 3000, 6000, and 9000 cells per well. Individual images were generated by the EnSight plate reader, cropped and compiled to 96-well microplate orientation using a macro and ImageJ Software (NIH).

Conclusion

Automation allows for highly reproducible cell seeding, yielding equivalent-sized microtissues, demonstrating the compatibility of the GravityPLUS™ Hanging Drop System with high-throughput liquid handling systems. The combination of compact hardware size and thoughtful automation programming enable the system to maintain sterility throughout all automated pipetting steps. The Zephyr G3 Automated Workstation's compact size permits its use within a standard tissue culture hood. Further reducing potential contamination, plates and reservoir lids are removed by the gripper arm and remain elevated during relevant pipetting, ensuring that the lid and plate/reservoir remain sterile with minimal possible cross-contamination.

Imaging 3D microtissues in the GravityTRAP™ Plate with the EnSight multimode plate reader allows for rapid identification of the presence or absence of spheroids within each well and provides relative size metrics and analysis. Additionally, cells can be maintained and grown in GravityTRAP™ plates for further downstream assays and analyzed on the EnSight for efficient high-throughput assay development. While we used the Zephyr G3 automated workstation, the InSphero GravityPLUS™ Hanging Drop System could also be automated on a JANUS® G3 automated workstation with an 8 tip Varispan arm.

This automated workflow solution from PerkinElmer and InSphero provides an accurate, high-throughput cell culture platform enabling further understanding of drug interaction and efficacy in compound screening campaigns.

	Part Number
GravityPLUS™ Hanging Drop System 96–well, pack of 10	ISP-06-001
GravityPLUS™ Hanging Drop System 96-well, pack of 3	ISP-06-010
GravityPLUS™ Plate 96-well, pack of 10	ISP-03-001
GravityPLUS™ Hanging Drop System Adapter	CS-10-001

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