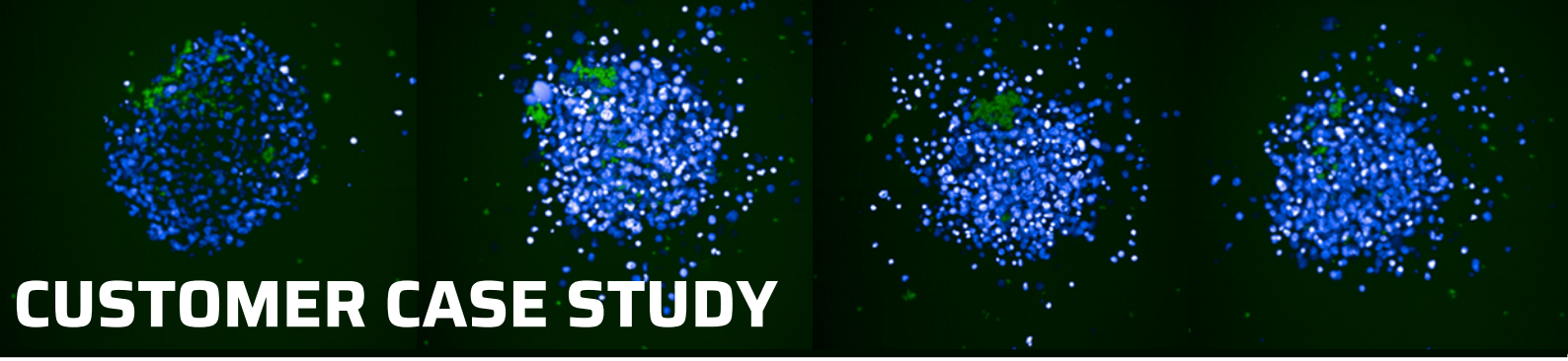

CUSTOMER CASE STUDY

Keep an eye on your 3D model cells

SpheroCHECK SPAchip® pH Green Single-Detection Kit Evaluation & Test in Human Brain Spheroids

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"The SPAchips technology provides a remarkable way to monitor cell status in real time, allowing direct visualization through microscopy. This enables researchers to extract valuable insights from both entire cell populations and individual cells. SPAchips are an excellent tool in the early drug discovery pipeline, permitting, for example, the study of a drug's effects over time without the need to perform multiple replicates to analyze multiple time points." (André Maia, i3S, Porto)

Background:

Three-dimensional (3D) cell culture models, such as spheroids and patient-derived organoids, provide a more physiologically relevant systems for studying tumor biology and drug screening. In particular, this experiment focuses on optimizing a drug screening platform for patient-derived brain tumor organoids.

Monitoring intracellular and extracellular pH is crucial for assessing **cell viability, metabolism, and response to different treatment conditions.** The **SpheroCHECK SPAchip® pH Green Single-Detection Kit** enables continuous and simultaneous pH monitoring in living 3D models, offering valuable insights into tumor microenvironment dynamics.

This study aimed to evaluate the feasibility of using **SPAchip® technology** to track **pH variations** in normal human astrocytes (NHA) spheroids, cultured in GrowDex®-T hydrogel and under free-floating conditions.

Key Features of SpheroCHECK SPAchip® pH Green Single-Detection Kit:

- Allows real-time, long-term monitoring of intracellular and extracellular pH in 3D cultures.
- Compatible with confocal microscopy, HCS/HCA analyzers, and flow cytometry.
- SPAchip® probes remain in the cytosol for over a month, reluctant exocytosis during spheroid manipulation, enabling prolonged culture assessment.
- SPAchip® can be internalized by cells prior to, along or after formation of spheroids.
- Facilitates high-throughput screening for drug discovery applications.

Methods - Experimental Setup:

Cell lines

Immortalized normal human astrocytes (NHA) (hTERT E6/E7 immortalized).

Culture Conditions

Serum-free medium supplemented with FGF, EGF, PDGF, and B27.
Non-adherent conditions to promote spheroid formation.
Spheroids embedded in GrowDex®-T hydrogel, an animal-free, nanofibrillar cellulose and water that functions as a scaffold for 3D cultures.

SPAchip® Incorporation and Analysis

SPAchip® pH Green Single-Detection Kit was incubated overnight with spheroids at a 2:1 SPAchip-to-cell ratio.

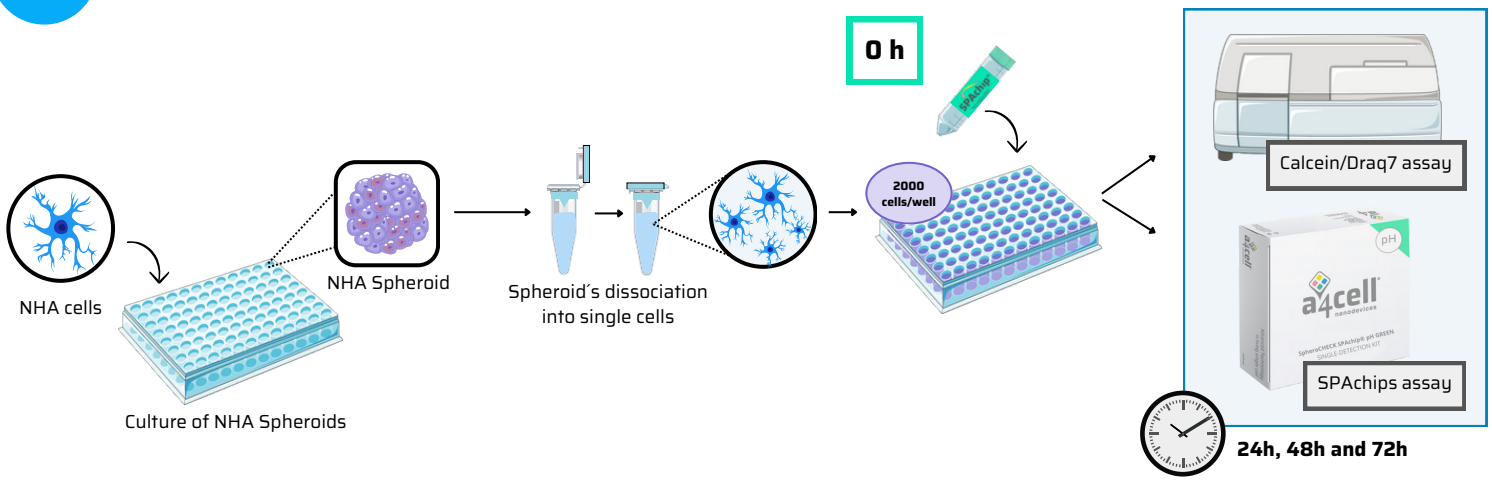
Readouts

Confocal microscopy (Opera Phenix Plus HCS System, Revvity) was used to track fluorescence intensity over time.
Image analysis was performed to quantify intracellular and extracellular pH changes.
Live/dead assays (Calcein and Draq7) were used to assess cell viability.

Experiments & Results:

1. Free Floating: testing optimal culture conditions for SPAchip® and Spheroid integration:
   a. SPAchip® In Seeding: Incorporation at the Onset of Free-Floating Spheroid Formation.
   b. SPAchip® After Seeding: Incorporation after 24 Hours of Free-Floating Spheroid Formation.
2. GrowDex®-T: SPAchip® Incorporation at 24 Hours of Spheroid embedded in GrowDex®-T Hydrogel.
3. Testing SPAchips with a positive cell death control drug (Benzethonium Chloride).

1.a Free floating: SPAchip® In Seeding



SPACHIP® INCORPORATION AT THE ONSET OF FREE-FLOATING SPHEROID FORMATION:

- **Objective:**
  - Assess the feasibility of introducing SPAchips® at 0 hours of spheroid formation in a free-floating system, ensuring uniform SPAchip® distribution as cells aggregate.
- **Experimental Approach:**
  - SPAchips® were added directly to dissociated NHA cells at the beginning of spheroid formation under non-adherent, free-floating conditions.
  - Cells aggregated into spheroids while incorporating SPAchips® within their structure.
  - Confocal microscopy was used to measure fluorescence intensity and monitor SPAchip® distribution over time.

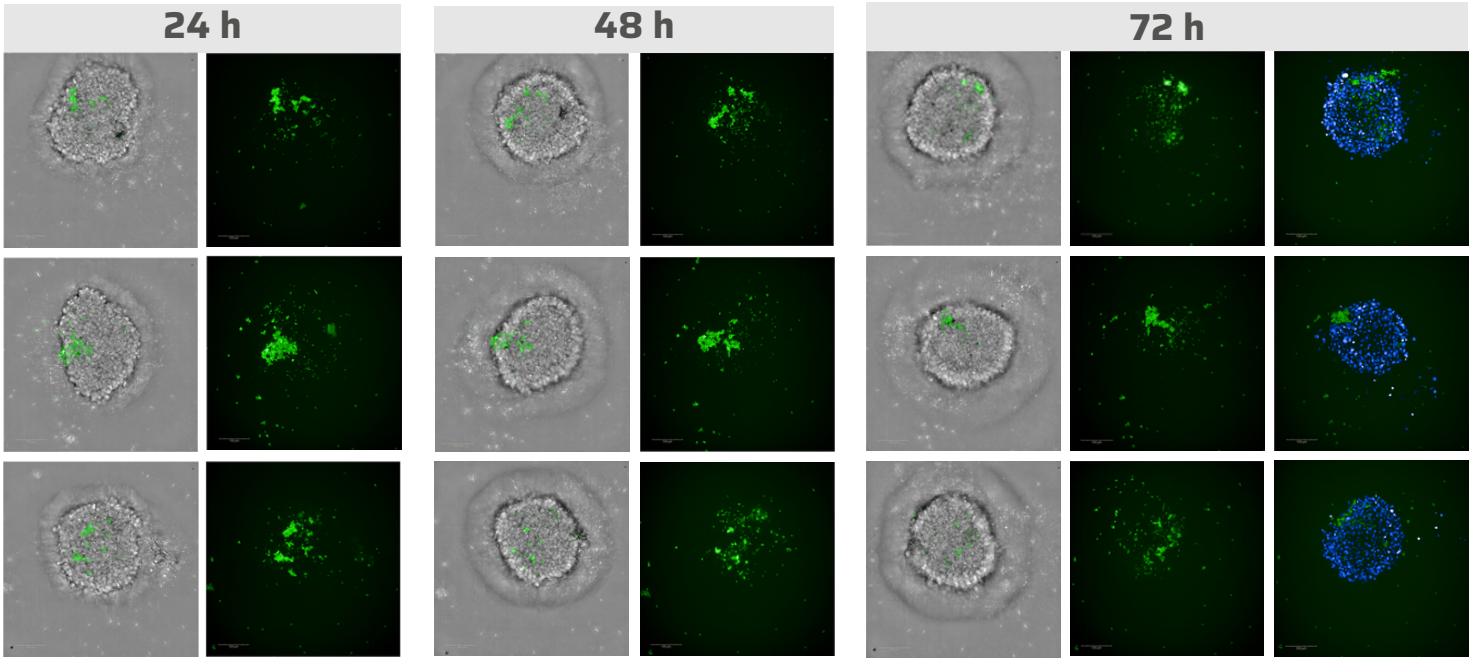
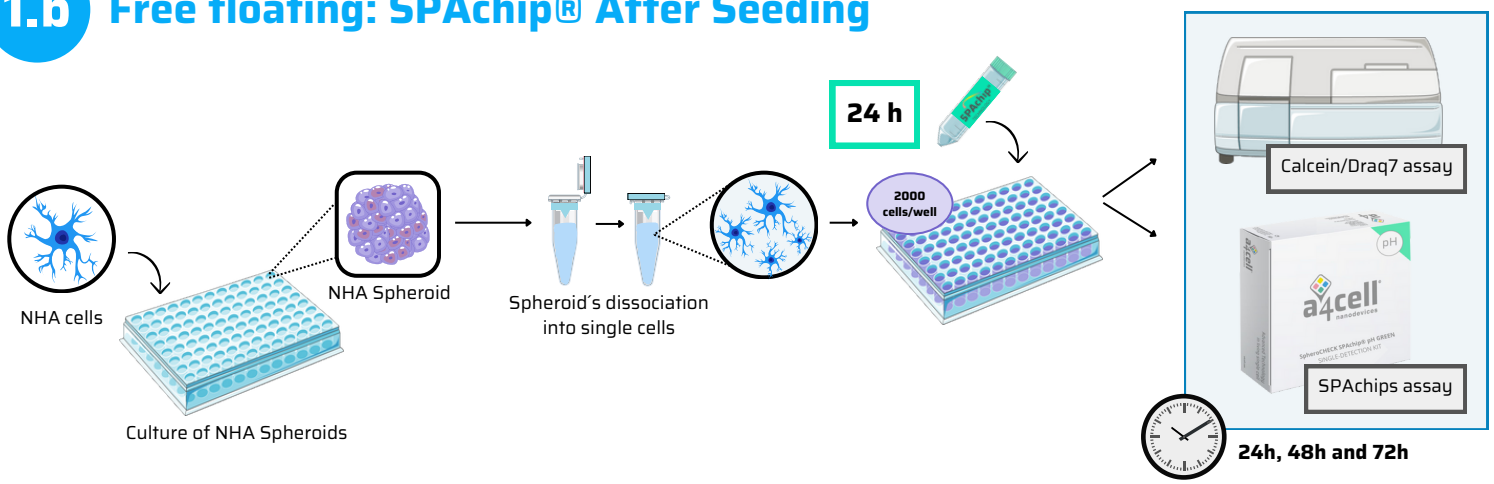


Figure 1: Free floating system with SPAchips® added at 0 hours of spheroid formation. Images were taken using confocal microscopy (Opera Phenix Plus HCS System, Revvity) with 20X objective at 24, 48, and 72 hours after SPAchip® addition. SpheroCHECK SPAchip® pH Green Single-Detection Kit (2:1) in green channel and Hoescht (1:1000) in blue channel.

1.b Free floating: SPAchip® After Seeding



SPACHIP® INCORPORATION AFTER 24 HOURS OF FREE-FLOATING SPHEROID FORMATION:

- **Objective:**
  - Assess the feasibility of introducing SPAchips® 24 hours after spheroid formation begins in a free-floating system, ensuring effective chip uptake in pre-formed structures.
- **Experimental Approach:**
  - NHA cells were allowed to aggregate into spheroids for 24 hours under non-adherent, free-floating conditions.
  - After 24 hours, SPAchips® were added to assess their penetration and retention in formed spheroids.
  - Confocal microscopy was used to monitor fluorescence intensity and chip distribution over time.

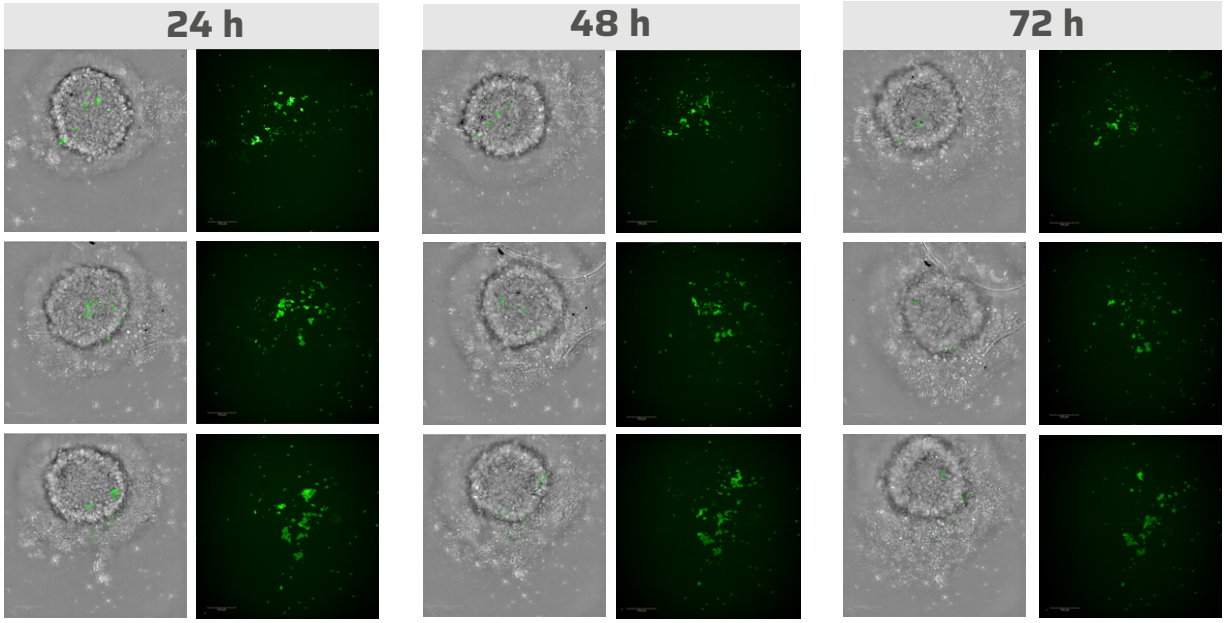


Figure 2: Free floating system with SPAchips® added after 24 hours of spheroid formation. Images were taken using confocal microscopy (Opera Phenix Plus HCS System, Revvity) with 20X objective at 24, 48, and 72 hours after SPAchip® addition. SpheroCHECK SPAchip® pH Green Single-Detection Kit (2:1) in green channel.

SPHEROID LIVE/DEAD ASSAY IN FREE-FLOATING CONDITIONS:

- **Objective:** Evaluate spheroid viability in free-floating conditions.
- **Live/Dead Assay:** Spheroids were analyzed using Calcein (viable cells cytosol staining) and Draq7 (dead nuclei staining) to assess viability.

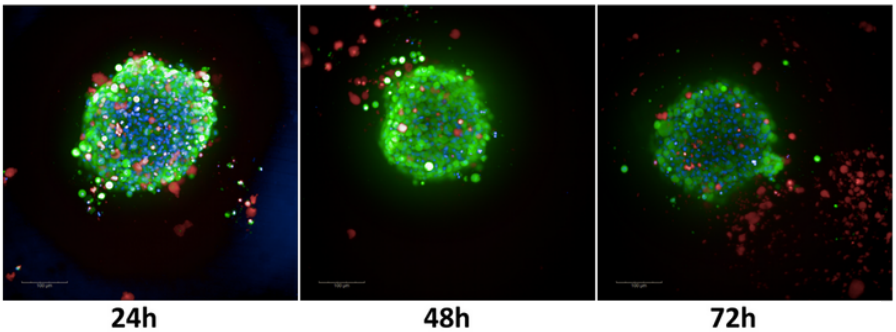
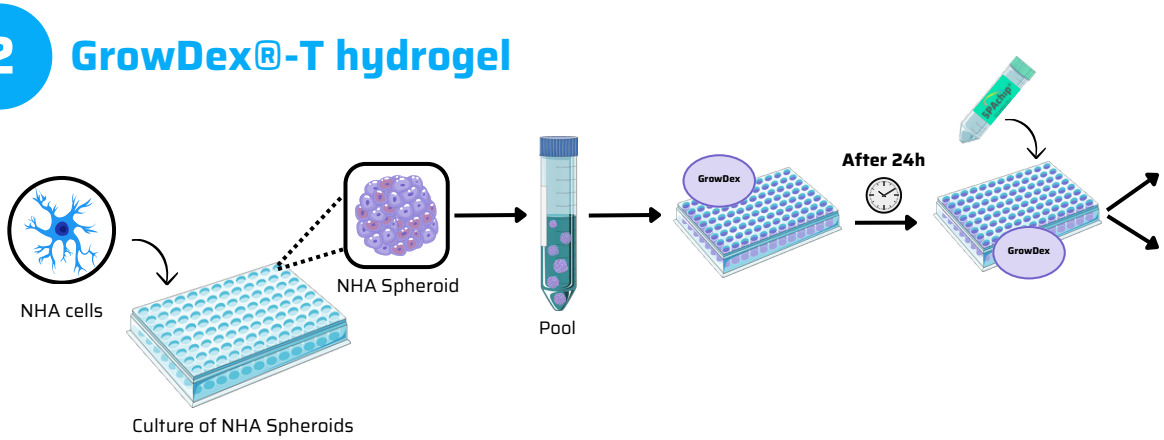


Figure 3: Spheroid live/dead assay in free-floating conditions. Images were taken using confocal microscopy (Opera Phenix Plus HCS System, Revvity) with 20X objective.

- Calcein (2.5 µM)
- Draq7 (1:1000)
- Hoescht (1:1000)

2 GrowDex®-T hydrogel



SPACHIP® INCORPORATION AT 24 HOURS OF SPHEROIDS EMBEDDED IN GOWDEX-T HYDROGEL:

- **Objective:**
  - Evaluate the incorporation and functionality of SPAchips® when added 24 hours after spheroid embedded in GrowDex®-T hydrogel, assessing their penetration and distribution within the 3D scaffold.
- **Experimental Approach:**
  - A pool of NHA spheroids were embedded in GrowDex®-T hydrogel and allowed to settle for 24 hours.
  - After 24 hours, SPAchips® were introduced to analyze their ability to penetrate the hydrogel and integrate into spheroids.
  - Confocal microscopy was used to quantify SPAchip® fluorescence intensity and investigate the internalization and localization of SPAchip® within the spheroids.

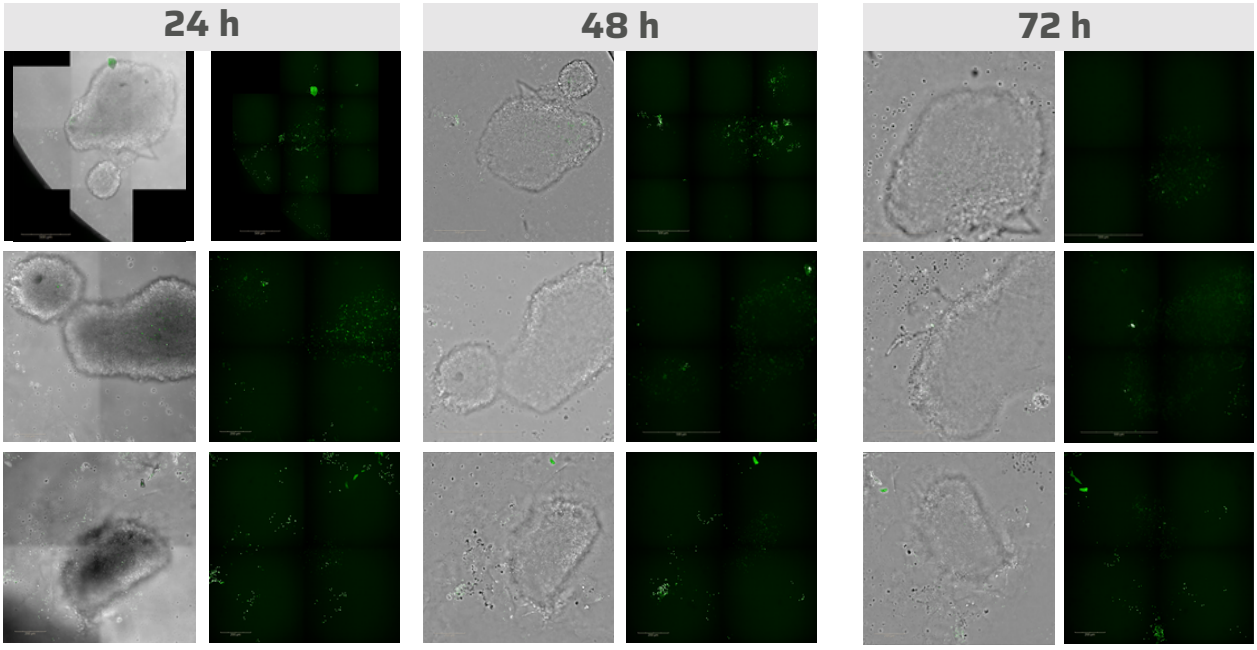


Figure 4: SPAchip® Incorporation in spheroids embedded in GrowDex®-T Hydrogel. Images were taken using confocal microscopy (Opera Phenix Plus HCS System, Revvity) with 20X objective at 24, 48, and 72 hours after SPAchip® addition. SpheroCHECK SPAchip® pH Green Single-Detection Kit (2:1) in green channel.

SPHEROID LIVE/DEAD ASSAY IN GROWDEX®-T HYDROGEL:

- **Objective:** Evaluate spheroid viability in GrowDex®-T conditions.
- **Live/Dead Assay:** Spheroids were analyzed using Calcein (viable cells cytosol staining) and Draq7 (dead nuclei staining) to assess viability.

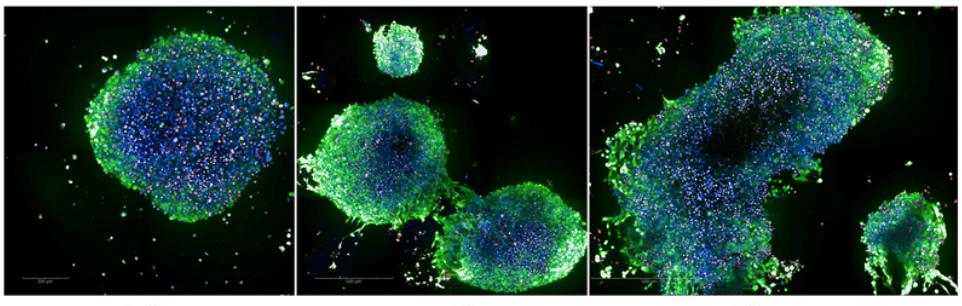


Figure 5: Spheroid live/dead assay in GrowDex®-T conditions. Images were taken using confocal microscopy (Opera Phenix Plus HCS System, Revvity) with 20X objective.

- Calcein (2.5 µM)
- Draq7 (1:1000)
- Hoescht (1:1000)

3 Testing SPAchips with a positive cell death control drug (Benzethonium Chloride)

The study evaluates the **effects of benzethonium chloride (BZT)**, a positive cell death control drug used in drug screening approaches.
To monitor intracellular physiological changes, the **SpheroCHECK SPAchip® pH Green Single-Detection Kit** was used to measure and monitor intracellular pH variations over time. This approach provides valuable insights into how the drug influences the integration and detection of SPAchips in the context of cell death.

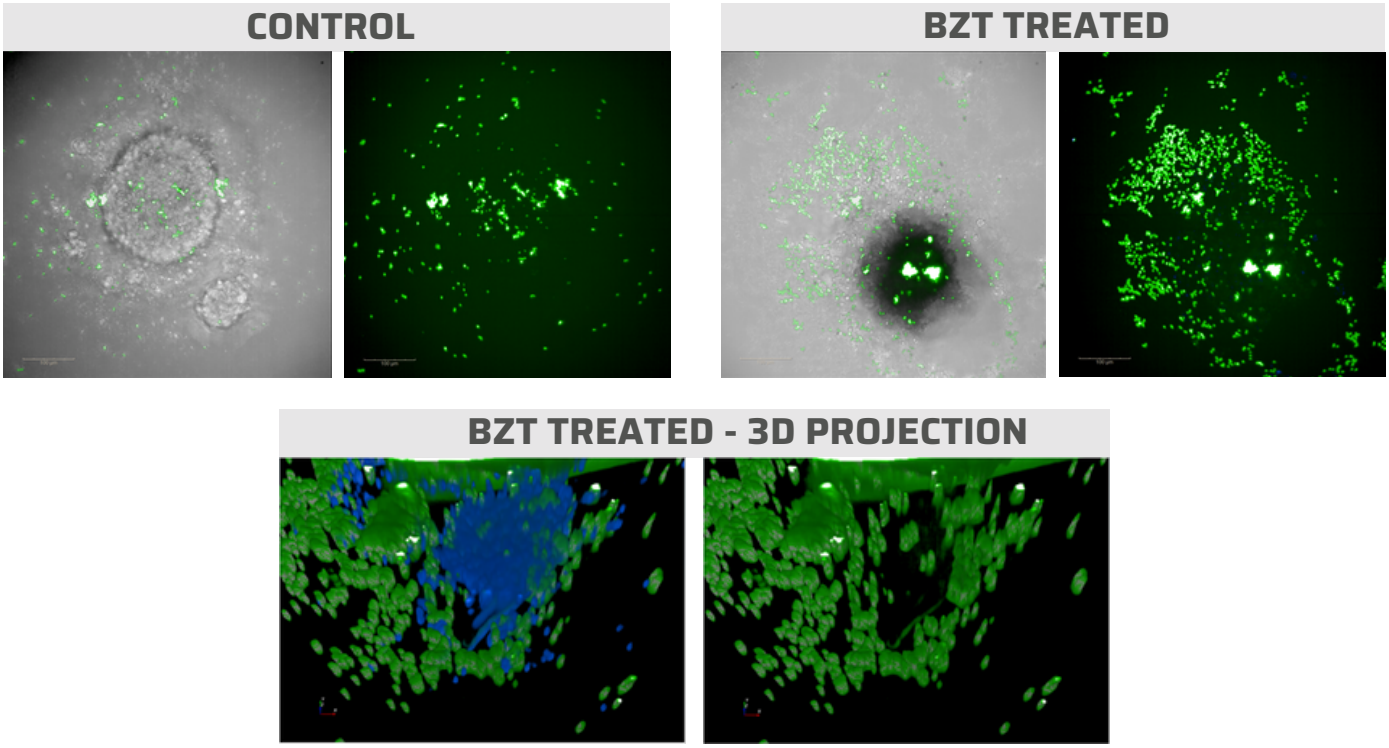


Figure 6: Testing SPAchips with a positive cell death control drug (Benzethonium Chloride). Images were taken using confocal microscopy (Opera Phenix Plus HCS System, Revvity) with 20X objective. SpheroCHECK SPAchip® pH Green Single-Detection Kit (2:1) in green channel and Hoescht (1:1000) in blue channel.

Key Observations and Conclusions:

✓ **Effective organoid immobilization** in GrowDex®-T at 0.2%, ensuring stability for long-term studies.
✓ **Easy and efficient media exchange over time**, supporting sustained culture conditions.
✓ **SPAchips® successfully penetrate GrowDex®-T**, reaching cells within the spheroid for accurate intracellular pH monitoring.
✓ **Fluorescence intensity of SPAchips® can be tracked over time** using confocal microscopy and image analysis, enabling precise pH measurement.

A4CELL ADDITIONAL STUDIES: EXPLORING FURTHER POSSIBILITIES

At A4cell, we are dedicated to unlocking the full potential of SPAchip® technology, continuously pushing its limits — **SpheroCHECK SPAchip® pH Green Single-Detection Kit** offers remarkable capabilities within 3D models. Based on this study, we suggest the following recommendations:

- **Data quantification and analysis:** While this experiment has confirmed the successful internalization and functionality of the SPAchip® in spheroids, the most valuable insights come from **quantitative data analysis**. The SPAchip® technology enables **precise measurement of intracellular pH fluctuations across the different layers of the spheroid**, providing a comprehensive view of cellular responses within the 3D structure. Proper data quantification will be crucial for extracting meaningful conclusions and optimizing the drug screening process.
- **Effective dissolution of the SPAchip® solid film**: Ensuring the complete and uniform dissolution of the SPAchip® solid film is **essential** for the success of the assay. Proper preparation guarantees even distribution of the SPAchips within the culture, allowing **accurate and reproducible pH measurements**.