User Protocol

SpheroCHECK SPAchip®
pH GREEN SINGLE-DETECTION KIT

Product Reference: SS-001-PHG

For use with flow cytometers and cell imaging platforms. For research use only.

Arrays for Cell Nanodevices S.L.
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For use with fluorescence microcopy, cell imaging platforms, and flow cytometers

1. General Introduction

Spheroids are simple 3D clusters of single or multi cell types. Three-dimensional cell aggregates provide a more accurate representation of *in vivo* conditions than traditional 2D cell cultures. SpheroCHECK SPAchip® pH Green Single-Detection Kit can also be used on spheroids and 3D-biology to measure intracellular pH levels by changes in fluorescence intensity, which allows a more comprehensive study of spheroid's establishment, metabolism, and viability.

SpheroCHECK SPAchip® pH Green Single-Detection Kit enables continuous, simultaneous, and accurate monitoring of intracellular and extracellular pH in living 3D models such as spheroids. Evaluation of pH levels is key for effective quality control of living cells to warrant the standardization and reproducibility of your cell experimental methodologies and enable a more comprehensive study of the spheroid formation and its physiology.

SpheroCHECK SPAchip® pH Green Single-Detection assay kits are novel cell-based assays for living single-cell developed by A4cell that bring together the fields of nanotechnology and cell biology. SpheroCHECK SPAchip® kits are composed of fluorescently labeled silicon microparticles - SPAchip®- that can be internalized in cultured spheroids to monitor changes in specific intracellular analyte concentrations for long periods of time. Fluorescence intensity is detected from individual SPAchips harboring a pH-sensitive chemical probe covalently attached to their surface.

SpheroCHECK SPAchip® pH Green Single-Detection Kits are optimized for its use with confocal microscopes and HCS/HCA analyzers with 20X or over magnification objectives, yet widefield fluorescence microscopes and imaging system with fixed wavelength filters can be also used. Flow cytometers have additionally been validated to analyze intra and extracellular SPAchips.

SPAchip® kits can be handled as common fluorophores and chemical probes for cell biology. After resuspending them in the assay buffer (a SPAchip®-to-cell ratio of 2:1 is recommended), an overnight incubation is required to allow chips to be incorporated in the cytosol. SPAchip® will remain in the cytosol for longer than one month to monitor the evolution of the culture.

2.Contents and Storage

Each SpheroCHECK SPAchip® pH Green Single-Detection Kit contains:

Materials		Units
ASSAY SPAchip® tube (embedded in a solid fluorescence-protective soluble film)	~5x10^6 ASSAY SPAchips	1
ASSAY buffer tube (sterile, cell culture suitable)	10 mL	1
CONTROL SPAchip® tube (non-fluorescent, ready-to-use)	~5x10^5 CONTROL SPAchips/100 µL	1

Storage Information

Upon receipt, each kit should be stored protected from light at 2-8^{0}C.

SpheroCHECK SPAchip® pH Green Single-Detection Kit Workflow

	SPAchip® Assay Workflow	*Notes*
ASSAY SPAchip® Preparation	***Dissolve ASSAY SPAchip® tube film***	Thoroughly **dissolve** ASSAY SPAchip® tube solid film in assay buffer (vortexing might be required)
	↓	**Centrifugate** tubes at. 4,300 xg for 10 min. Discard supernatant and keep SPAchip® containing pellet.
	Wash with assay buffer } ***x2***	**Resuspend** the SPAchips in 1mL ASSAY Buffer. **Repeat x2**.
	↓ ***Resuspend***	**Resuspend** the ASSAY SPAchips in 100 µL ASSAY Buffer to obtain a stock solution of $2.5x10^4$ SPAchips/µL.
	↓ ***Store***	***ASSAY SPAchip® stock solution*** can be **stored** protected from light at 4^0C for up to 6 months.
Spheroid Assay	***Cell Suspension***	Seed cells (optimization will be required depending on the cell type and assay conditions) in a multi-well plate to allow slow and uniform spheroid formation[1].
	↓ ***SPAchip dilution***	**Dilute** *CONTROL* and *ASSAY SPAchip* stock solution in cell culture medium to obtain a final SPAchip® to cell ratio of 2:1.
	↓ ***Addition to cell Suspension for spheroid formation***	**Aspire** the cell medium and add SPAchip®-loaded fresh culture medium.
	↓ ***Incubation*** ↓	**Incubate** overnight in a cell incubator (37^{0}C, 5% CO_2) to allow SPAchip® internalization during spheroid aggregation
Fluorescence Quantification	***Data Acquisition***	Use green fluorescence channel to image SPAchip® detection (excitation 488 nm, emission 520 nm).
	↓ ***Data Analysis***	Analyze fluorescence intensity units and normalize the data to control conditions.

[1]This cell culture protocol only provides a guideline, and it should be modified depending on specific needs. Please, read whole protocol before starting.

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3. Materials to be Supplied by the User

- Pipettes and pipette tips (1-10 µL, 2-20 µL, 20-200 µL, 200-1000 µL).
- Multi-channel pipettes and pipette tips (50-300 µL) (not mandatory but desirable).
- Vortex mixer.
- Mini-centrifuge.
- Reagent reservoirs.
- Cell culture conditions:
  - Cell culture facilities.
  - Cell culture plate (multi-well): Akura™ 384 ImagePro well plate (InSphero) or other type of ULA (Ultra-Low Attachment surface) microplate to be able to work with spheroids.
  - Cell culture media (phenol red free is recommended) according to specific cell line.
- Cell imaging system (HCS/HCS, Confocal microscope, imaging plate reader, etc.) with the appropriate fluorescence excitation and emission wavelength filters. At least 20x magnification objective is required for quantitative analysis.
- Image analysis software (Contact A4cell staff for support).

<u>If quantitative analysis is required, it will be necessary:</u>

- Intracellular pH calibrators.

4.Assay Procedure

NOTE: Work under sterile conditions. Protect the tubes from light, specially from UV light.

a. ASSAY SPAchip® Preparation:

1. Add 1 mL of assay buffer to ASSAY SPAchip® tubes and mix until complete solubilization of the membrane (vigorous **vortexing** required).

2. Centrifuge the tubes at approx. 4,300 xg (8,000 RPM in a 6 cm minispin rotor) for 10 minutes.

3. Carefully, aspire and discard supernatant and wash pellets with 1 mL of solubilization medium (**Look out not to aspire the pellet!**). Repeat steps 1 and 2.

4. Carefully, aspire and discard supernatant. Resuspend the pellet in **100 µL of assay buffer** to obtain a SPAchip® stock solution. This results in approximately **$2.5x10^4$ SPAchips/µl**. Once prepared, ASSAY SPAchip® tube can be stored at 2-8^{0}C protected from light for up to 6 months.

It is essential to properly dissolve the SPAchip® solid film for the success of the assay.

b. SPHEROID ASSAY PROTOCOL (standard protocol for 384-well plate to work in 3D models: Spheroids):

NOTE: ASSAY and CONTROL SPAchip® dilutions suggested in this section are suggested for InSphero Akura™ 384 ImagePro plates. Optimization might be required for different cell types or multiwell plates. If using a different plate type, steps 1-5 may be skipped.

Goal: To demonstrate intracellular pH monitorization in 3D spheroid models under different treatment conditions for studying cancer and metabolic diseases by using SPAchip® nanodevices with fluorescence microscopy techniques.

1. **Pre-wetting t**he wells of the InSphero Akura™ 384 ImagePro multi-well plate by add 50 µL PBS 1x per well.

2. **Centrifuge** InSphero Akura™ 384 ImagePro multi-well plate at 1,200 rpm for 2 mins.

3. **Incubate** 24 hours at 37^{0}C, 5% CO2 and humidity.

4. After 24 hours, **centrifuge** InSphero Akura™ 384 ImagePro multi-well plate at 1,200 rpm for 2 mins.

5. Carefully **discard** PBS1x, along the top of the plate (left side).

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6. **Add** 50 µL of the cellular suspension, adjust your cell number according to expected cell growth:

- 300-500 cells/well are generally used for a slow spheroid formation (e.g. using Hek293 cells, see **Table 1**).
- Note 1: to form spheroids with uniform size, gently pipette up and down the cell suspension before adding the 50 µL to the well.
- Note 2: Place the tip of the pipette on the edge, sliding the volume along the wall slowly into the well. To perform this, tilt the multi-well plate slightly towards its top-most.
- Note 3: Proper aspiration for multiple wells is only possible in rows, not columns.

7. **Add** SPAchips® following ASSAY SPAchip® preparation and ASSAY protocol:

- Prepare a 1:100 dilution of the non-fluorescent CONTROL SPAchips in cell culture medium. Mix thoroughly (vortex). Do not spin the tubes.
- Dilute ASSAY SPAchip® stock solution in cell culture medium to obtain a final SPAchip® to cell ratio of 2:1 (spheroid cell number example for HEK293 cells on **Table 1**). Vortex the ASSAY SPAchip® tube right before using it. Pipette up-and-down twice to homogenize the ASSAY SPAchip® solution and do not spin the tube.

***Optimization may be required depending on the cell type and assay conditions.**

Hours	Spheroid Diameter (µm)	Number of cells / well	SPAchip® Number / well (2:1 ratio)
0	N/A	400	-
24	~200	800	1,600
48	~500	1,600	3,200

Table 1: HEK293 spheroid formation. Table show the estimated number of cells per well and estimated spheroid diameter (µm) over time.

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- In the multi-well plate, aspire the cell medium and add 100 µL of CONTROL SPAchip® dilution to the control wells (see **Figure 1**). Vortex the tube right before use. Add 100 µL ASSAY SPAchip®-containing fresh culture medium per well. Homogenize the solution by pipetting up and down often.

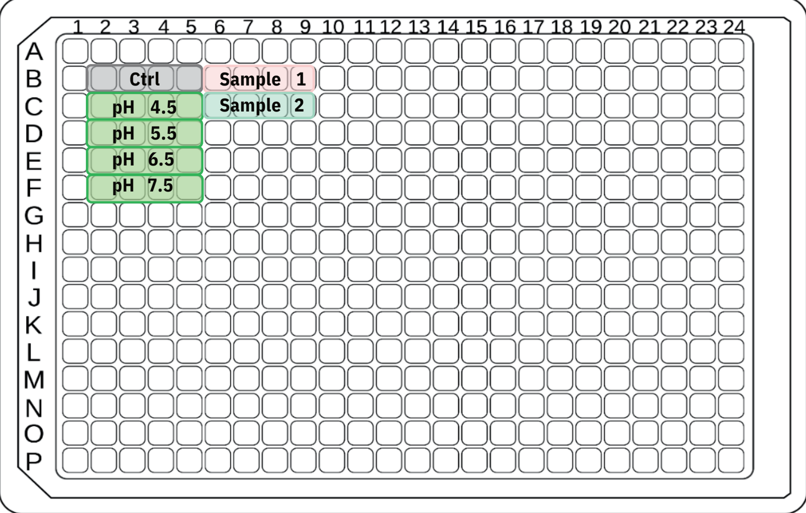


Figure 1: Example of 384-well plate configuration with positive controls and calibrators.

8. **Centrifuge** InSphero Akura™ 384 ImagePro multi-well plate at 1,200 rpm for 2 mins.

9. **Incubate** the multi-well plate with the cells and SPAchip® overnight at 37^{0}C, 5% CO_2 and humidity to let the spheroid form and the cells to internalize the SPAchip®. Keep the plate slightly tilted on some kind of holder (or another plate) about 30^{0} degree angle (from the top of the plate) to improve the spheroid maturation process. SPAchip® internalization rate may vary depending on the cell subtype.

10. Every 2-3 days, carefully **change** the medium by aspirating and gently pipetting up and down. If desired, additional SPAchip® can be added to the spheroid.

- Note 1: to obtain a final SPAchip® to cell ratio of 2:1, calculations need to be done considering the division rate of the cell line**.**

11. When spheroids reach the desired and appropriate size, proceed to the desired experiment setup and image acquisition.

12. If quantification is needed, use some wells of the plate for calibrating the system (controls and intracellular pH calibrators depicted in rows C, D, E, F in **Figure 2**). In such case, follow calibrator manufacturer's instructions.

- See an example of plate template in **Figure 1** where *sample 1* and *2* are cells of interest; *ctrl* wells are non-fluorescent SPAchips; wells with different pH in rows *C, D, E* and *F* are intracellular calibration wells where measurements should be interpolated.

13. Perform the experiment with your read-out platform. For long-time multiple-measurements assay (for example, monitorization during a week or month), keep the plate in appropriate condition between each measurement and change the medium every 2-3 days, depending on the cell subtype.

c. SPAchip® Data Collection

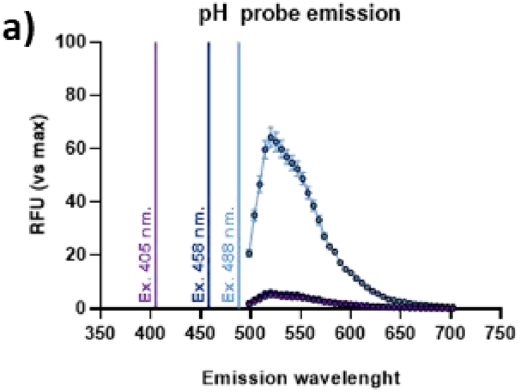
Image Acquisition

1. Use transmitted light to localize the spheroids of interest (if using fluorescent light sources use long-wavelength light -561 nm and over- in order to avoid photobleaching of the pH- sensitive probe).

2. Use Z-stacks to capture and set the best focus and the Z position of each SPAchip®. Brightfield images or cell masking stains are recommended for intracellular quantification.

3. Adjust the acquisition parameters of the test probes: Ex 488 nm /Em 520 nm (FITC fluorescence filter or similar) to avoid pixel saturation. If using calibrators, use pH 7.5 wells to adjust this value. See fluorescence spectrum for SPAchips in **Figure 2**.

4. Launch the experiment and save the images. Do not change settings parameters while acquisition.



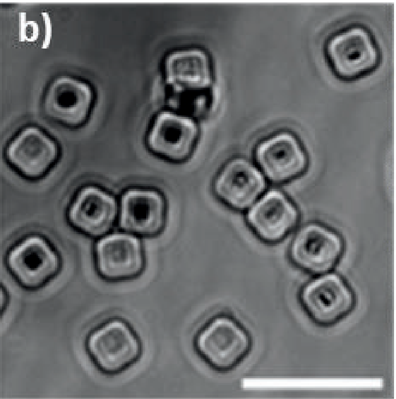


Figure 2: **a)** Emission spectra of SpheroCHECK SPAchip® pH Green Single-Detection kit probe when excited at the indicated wavelength. **b)** Brightfield image of SPAchips once in solution (Image from Torras et al. 2015 DOI: 10.1002/adma.201504164). Scale bar=10 µm.

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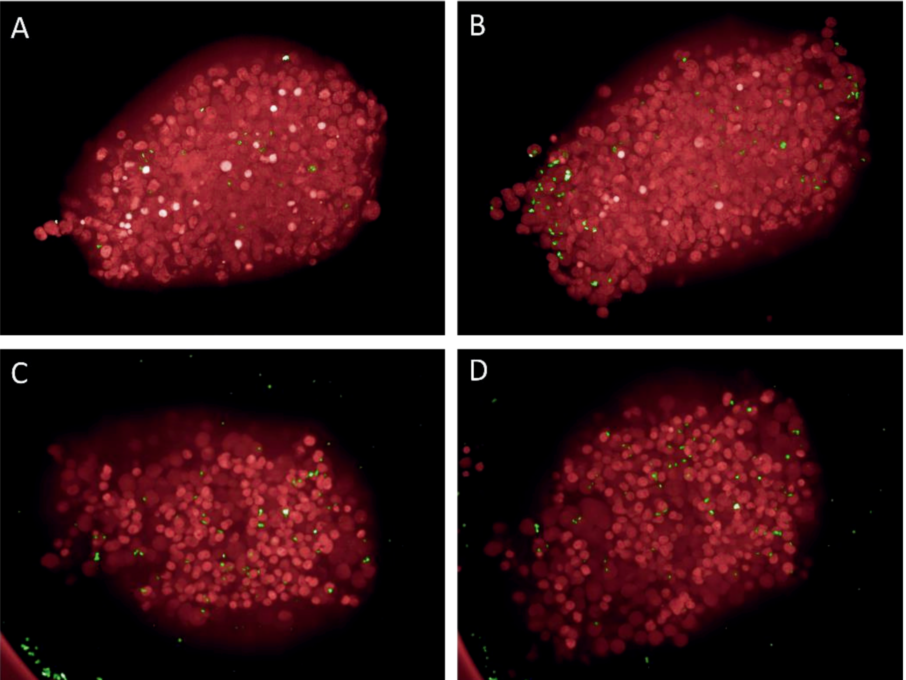

Figure 3: HEK293 spheroid formation. Timelapse of HEK293 cell line (from Human Embryonic Kidney) spheroid formation with internalization of SpheroCHECK SPAchip® pH Green Single-Detection Kit. DRAQ5™ in red stained live nuclei. SPAchip® technology allows pH measurement at the different time points of spheroid formation. HEK293 cells were incubated with SpheroCHECK SPAchip® pH Green Single-Detection Kit to allow spheroid formation at 24 **A)**, 28 **B)**, 48 **C)**, and 52 hours **D)**.

d. Quantitative Analysis

1. **Image Analysis:** select SPAchip® fluorescent channel signal and measure the intensity of segmented Regions of Interest (ROIs) in every SPAchip® with the image analysis software. Quantify the mean fluorescence of extra and intracellular SPAchip® populations.

1. **Export** your data to a spreadsheet software.

2. To quantify intracellular pH, use the fluorescence of the calibration wells to plot fluorescence units *vs* calibration pH. Interpolate the values of the sample wells to obtain the intracellular pH value of the sample wells.

** Contact A4cell staff for assistance with imaging setup and data analysis. Ask for our **cell biology image analysis guide** at **labservices@a4cell.com** .*