*Cyto*CHECK SPAchip™ pH *single*-Detection Kit

All technical literature is available on the Internet at www.a4cell.com
Please visit the web site to verify that you are using the most current version of this Technical Bulletin. Please contact A4Cell Technical Services if you have questions on use of this system. E-mail info@a4cell.com.

Product Reference: CPS-001PC and CPS-002PD
For use with: Cell imaging platforms (confocal microscope, HCS/HCA readers are recommended) and flow cytometers.

1. General Introduction

CytoCHECK SPAchip™ assay kits are novel fluorescence assays developed by A4Cell that brings together the fields of nanotechnology and cell biology. CytoCHECK SPAchip™ are composed of fluorescently labeled silicon microparticles -SPAchips®- that can be internalized in the cytosol of cultured cells and monitor changes for long periods of time.

CytoCHECK SPAchip™ pH Single-Detection Kit allows measurement of intracellular pH levels by changes in fluorescence intensity, which allows a more comprehensive study of the living single-cell physiology and maximizes the performance of most of imaging analyzers.

CytoCHECK SPAchip™ Single-Detection kits are optimized for its use with microscopes and HCS/HCA analyzers with confocal capability and 20X or over magnification objectives, yet epi-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®.

SPAchips® are used 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; or simply add 1 µl per well to the cells in a standard 96-well plate and incubate overnight to allow SPAchip® to be incorporated in the cytosol . SPAchip® will remain in the cytosol for longer than one month to monitor the evolution of the culture.

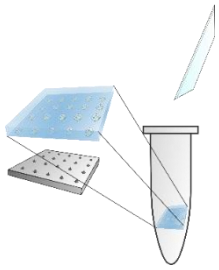
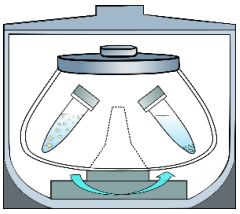
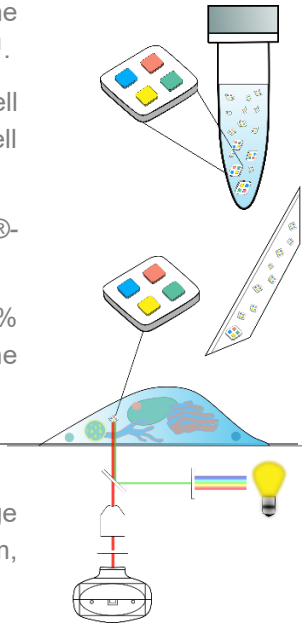
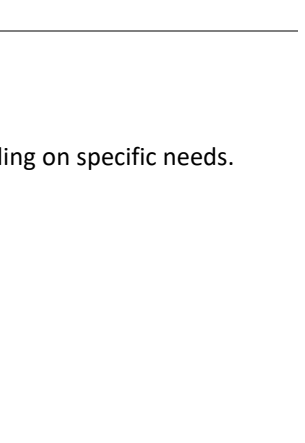
! *Storage Information*

Upon receipt, each kit can be stored protected from light at 2-6ºC. If preferred, for saving room in the fridge or cold-room, components can be stored separately. In this case, the multi-well plate can be stored at room temperature and reagent tubes (ASSAY buffer and SPAchips®) at 2-6ºC protected from light.

[1] For using CytoCHECK SPAchip™ kits in non-confocal imaging systems,
[1] CytoCHECK SPAchip™ kits are optimized for its use with adherent cells. Some cells may need a lipofection step to maximize SPAchip internalization. Please contact with A4Cell for further information or questions about your cell model.

CAIT. Campus Montegancedo, s/n, 28223, Pozuelo de Alarcón, Madrid (Spain) info@a4cell.com

2. CytoCHECK SPAchip™ pH Single Detection Kit Workflow:

	SPAchip® Assay Workflow	Notes	
ASSAY SPAchip® Preparation	***Dissolve ASSAY SPAchip® tube***	Thoroughly **dissolve** ASSAY *SPAchip*® tube solid film in assay buffer (vortexing might be required)	
	Wash with Assay Buffer ***x2***	**Centrifugate** tubes at. 4300xg for 10 min. Discard supernatant and keep *SPAchip*®-containing pellet.	
	Resuspend	**Resuspend** the *SPAchips*® in 1mL ASSAY Buffer. **Repeat x2**.	
	Store	**Resuspend** the ASSAY *SPAchips*® in 100 µL ASSAY Buffer to obtain a stock solution of $2.5x10^4$ *SPAchips*®/µL. *ASSAY SPAchips*® stock solution can be **stored** protected from light at 4ºC for at least 6 months.	
Cell Assay	***Cell Culture***	Seed cells in the multi-well plate provided with the kit. Incubate the cells up to 70-80% confluence[1].	
	SPAchip Dilution	**Dilute** *CONTROL* and *ASSAY SPAchips*® in cell culture medium to obtain a final SPAchip to cell ratio of 2:1.	
	Addition to Cell Culture	**Aspire** the cell medium and add *SPAchip*®-loaded fresh culture medium.	
	Incubation	**Incubate** overnight in a cell incubator (37ºC, 5% CO_2) to allow *SPAchip*® internalization by the cells.	
Fluorescence Quantification	***Data Acquisition***	Use green fluorescence channel to image SPAchip® detection (Excitation 488 nm, Emission 520 nm)	
	Data Analysis		

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

3. Contents and Storage (CPS-001PC and CPS-002PD)

Each CytoCHECK SPAchip™ pH *single*-Detection Kit contains:

Materials			Units
Sterile multi-well plate suitable for cell culture (24-, 96- or 384-well optical bottom plate, depending on the item)	96-well	384-well	1
Sterile ASSAY buffer tube	3 mL		1
CONTROL SPAchips® (non-fluorescent, ready to use)	~10^4 *CONTROL SPAchips*®/4 uL		1
ASSAY SPAchips® tube (embedded in a fluorescence-protective soluble film)	~2.5x10^6 *ASSAY SPAchips*®		1

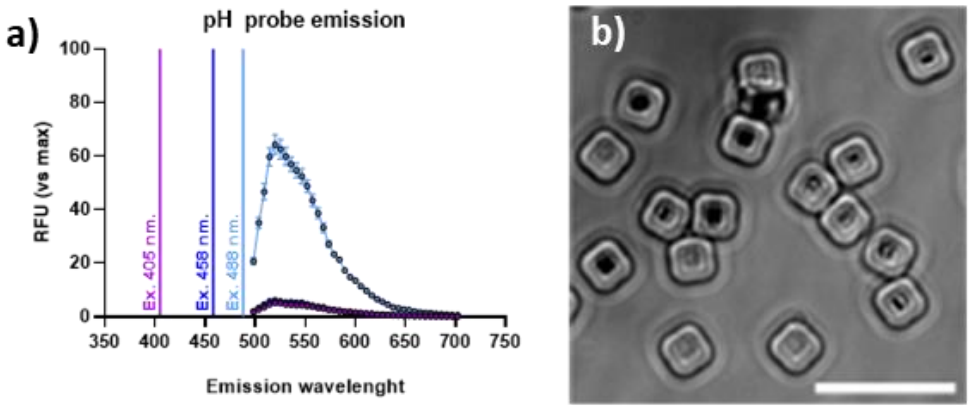


***Figure 1**: **a)** Emission spectra of pH--sensing probes when excited at the indicated wavelength. **b)** Brightfield image of SPAchips® once in solution (Image from Torras et al. 2015 DOI: 10.1002/adma.201504164) Scale bar = 10 µm.*

4. 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 facilities
- Cell culture media (phenol red free is recommended)
- Cell imaging system (HCS/HCS, Confocal microscope, imaging plate reader, etc…). At least 20x magnification objectives are required for quantitative analysis.
- Image analysis software (Contact A4Cell Staff for support)

If quantitative analysis is required, it will be necessary:

- Intracellular pH calibrators

5. Assay procedure

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

A. *ASSAY SPAchips*® preparation

1- Add 1 mL of assay buffer to *ASSAY SPAchips*® tubes and mix until complete solubilization of the membrane (vortexing is desirable).

2- Centrifugate the tubes at approx. 4300 xg (8000 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. Repeat steps 1 and 2.

4- Carefully, aspire and discard supernatant (**Look out not to aspire the pellet!!!**). 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 SPAchips*® can be stored at 2-6ºC protected from light for up to 6 months.

B. Assay protocol (standard protocol for 96-well plate)[2]:

NOTE: CONTROL and ASSAY SPAchip® dilutions suggested in this section are suggested for 96-well plates. Optimization might be required for different cell types or multiwell plates.

1- Seed cells of interest in the multi-well plate provided with the kit following standard protocols. (See an example of plate template in Figure 2)

2- Incubate the cells until 70-80% confluence is reached, approximately 10^4 cells/well (times may vary depending on the cell type).

3- Prepare a 1:100 dilution of the non-fluorescent *CONTROL SPAchips*® in cell culture medium. Mix thoroughly (vortex). Do not spin the tubes.

4-Dilute ASSAY SPAchips® stock solution in cell culture medium to obtain a final SPAchip® to cell ratio of 2:1. Alternatively, simply calculate 1 uL of stock per assay well and dilute 1:100 in cell culture medium. Vortex the *ASSAY SPAchips*® tube right before using it. Pipette up-and-down twice to homogenize the ASSAY *SPAchips*® solution and do not spin the tube.

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

5- In the multi-well plate, aspire the cell medium and add 100 µL of *CONTROL SPAchips*® dilution to the control wells C1 and C2. 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.

6- Incubate overnight in a cell incubator to let the cells to internalize the *SPAchips*®. Internalization rate may vary depending on the cell subtype but should be over 25%.

7- If quantification is needed, use the first column of the plate for calibrating the system (controls and intracellular pH calibrators, see example of plate template below (Figure 2). In such case, follow calibrator manufacturer's instructions.

8- 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 proper conditions between each measurement and change the medium every 24-48 hours, depending on the cell subtype.

[2] For cells resistant to SPAchips internalization, lipofection may be necessary. Please, contact A4Cell for further information.

	CONTROL SPAchips 1	pH C.1	Sample 2.1								
	CONTROL SPAchips 2	pH C.2	Sample 2.2								
	pH A.1	Sample 1.1	Etc…								
	pH A.2	Sample 1.2									
	pH B.1	Sample 2.1									
	pH B.2	Sample 2.2									

Figure 2: Example of 96-well plate configuration with positive controls and calibrators

C. *SPAchip*® Data Collection

- *Image Acquisition*:

1- Use transmitted light to localize at least three fields of interest per well (if using fluorescent light sources use long-wavelength light -561nm and over- in order to avoid photobleaching of the pH-sensitive probes).

2- Use z-stacks to capture and set the best focus and the z-position of each *SPAchip*®. In confocal systems, reflected light can be used to visualize the *SPAchips*® and the cells. Brightfield images or cell masking stains are recommended for intracellular quantification.

3- Adjust the acquisition parameters of the test probes: Ex 488/Em 520 (FITC fluorescence filter or similar) to avoid pixel saturation. If using calibrators, use pH 7.5 wells to adjust this value.

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

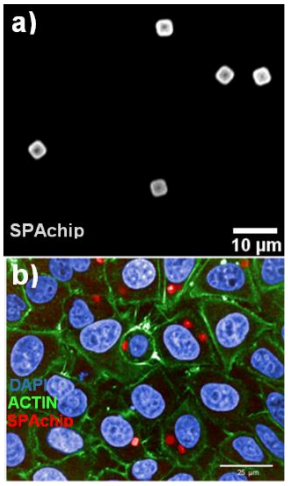


Figure 4: a) Fluorescence signal of SPAchips® in an imaging plate with cell culture medium. b) Representative image of HeLa cells with internalized SPAchips® (red) stained with DAPI (blue) and phalloidin (green).

- *Flow Cytometry Analysis*:

1- Use FCS and SSC to locate *SPAchips*® and cells independently by their size and complexity.

2- Create fluorescence histograms for green channel. Use cells treated with *CONTROL SPAchips*® to set the fluorescence thresholds in both populations (free *SPAchips*® and cells).

3- Analyze a tube of control cells treated with *ASSAY SPAchips*® to verify the fluorescence threshold set in step 2 for gate "Cells". Positive cells will appear as a separate peak in the histogram.

D. Quantitative analysis

1- **A)** 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.

B) For flow cytometry assays: Analyze each sample with the adjusted gating parameters and quantify green channel detection (mean ± SD or median ± rSD).

2-Quantify the mean fluorescence of extra and intracellular *SPAchip*® populations.

3- Export your data to a spreadsheet software.

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

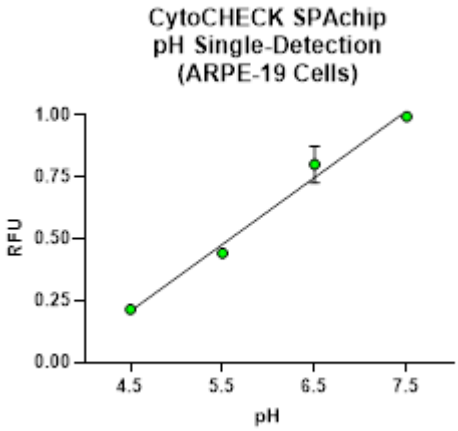


Figure 5: Graph showing normalized relative fluorescence intensity values of intracellular *CytoCHECK SPAchip*™ pH Single Detection at different pH conditions using intracellular