Flow Cytometry pH Measurement with CytoCHECK SPAchip® pH Single-Detection kits

Intracellular pH Analysis in Living Cells by new Lab-in-a-Cell Devices based on Spachip® Technology.

BACKGROUND

Many cell processes, as those regulating metabolism or proliferation, are highly sensitive to pH changes. For instance, enzymatic activities are generally optimal over a narrow pH margin and heavily decrease above and below it [1]. In this regard, net charge and structure of macromolecules also depend on proton concentration and maintaining intracellular proton fluxes is crucial for energetic metabolism [2][3]. On the other hand, alterations in cell metabolism also induce changes in cytosolic and extracellular pH. Thus, Warburg effect in cancer cells leads to increased glucose uptake and lactic acid production, which in turn alters extracellular and intracellular pH. Therefore, developing new tools for measuring proton fluxes in cells is appealing for a better understanding of cell physiology.

CytoCHECK SPAchip® assay kits are novel fluorescence assays developed by A4Cell that bring together the fields of nanotechnology and cell biology. CytoCHECK SPAchip® kits are composed of silicon microparticles -SPAchip® [4]- that can be internalized in the cytosol of cultured cells and monitor changes for long periods of time. CytoCHECK SPAchip® kits are optimized for its use with flow cytometers and fluorescence microscopes.

Here we feature the ***CytoCHECK SPAchip® pH single-detection kit*** for simultaneous measurement of cytosolic and extracellular pH in **fluorescence microscopy** and **flow cytometry (FC)**. SPAchip® are small (3x3x1 µm) silicon oxide chips functionalized with a fluorescent pH-sensing probe.

Cellomics reagents can be used in flow cytometry experiments to analyze intracellular pH levels. Relevant information on the role cell energetic state can help to elucidate the metabolic effect of different compounds in cell signaling pathways. CytoCHECK SPAchip® pH Single-Detection kit allows to perform real-time dynamic cell-based assays by flow cytometry measurements, which allows a more comprehensive study of the cell physiology processes where proton fluxes are involved and maximizes the performance of flow cytometers.

GOAL

To demonstrate intracellular pH monitorization in living single cells under different treatment conditions (dose-response curves) affecting oxidative phosporilation by using SPAchip® nanodevices in flow cytometry.

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MATERIALS

- CytoCHECK SPAchip® pH Single-Detection Kit
- Tissue-culture treated 96-well plates (Falcon)
- 293T cells (CRL-, ATCC)
- DMEM cell culture medium(Gibco) supplemented with FBS, gentamycin, L-Glutamine.
- TrypLE (Gibco)
- Intracellular pH Calibration Buffer Kit (Invitrogen)
- Antimycin A
- CytoFlex (Beckman Coulter)

METHODS

1. **CytoCHECK SPAchip® Calcium-Detection Kit preparation:** Assay SPAchips were dissolved in Assay Buffer contained in the kit, centrifuged, pelleted and resuspended again in Assay Buffer following A4cell's procedure.
2. **Cell Culture:** 293T cells were seeded independently in triplicates in a 96-well plate by adding 70 000 cells in 100 µl medium and allowed to adhere for 24h.
3. **SPAchip® addition:** a SPAchip®-to-cell ratio of 2:1 was added to each indicated well by diluting 140000 SPAchips in 100 µl of fresh medium. Additional control conditions with no SPAchips were supplemented with 100 µl fresh medium. Cells were incubated overnight to allow them to uptake the SPAchips.
4. **Cell Harvest:** media was removed and 100µl TrypLE were used to harvest cells from plate. Cells were collected in flow cytometry tubes and 100 µl fresh media were used to block TrypLE action.
5. **Treatments:** Cells in suspension were treated with Nigericin/Valinomycin (10µM) prepared in the appropriate Intracellular pH Calibration buffer for the control conditions as depicted in Fig. 1. Antimycin A (10 µM) diluted in DMEM was incubated in indicated samples for 15 min prior flow cytometry analysis.
6. **Flow Cytometry:** Samples were analyzed in an CytoFlex (Beckman Coulter) flow cytometer. SPAchip® fluorescence was analyzed by using the standard FITC detection (Ex: 488 nm, Em: 520 nm) in the flow cytometers. FSC and SSC were used to identify cells and extracellular SPAchips.

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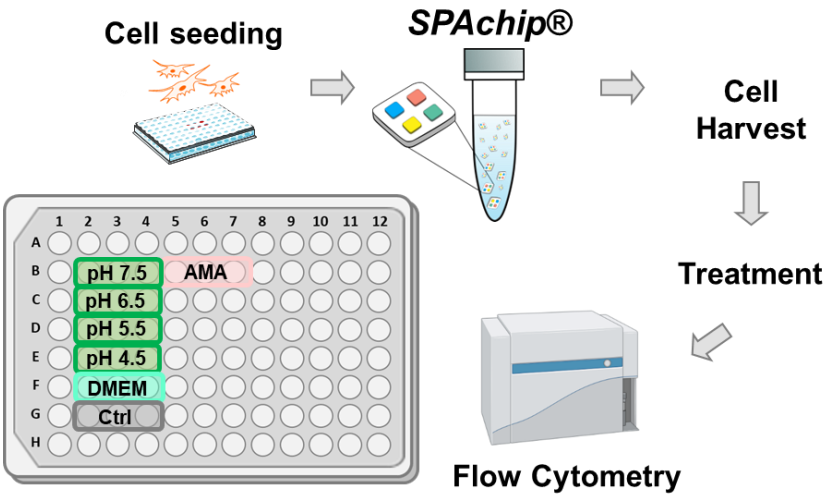

Fig.1. CytoCHECK SPAchip® pH Single-Detection use in Flow Cytometry.
Diagram depicting flow cytometry experimental design. A 96-well plate was used to seed 70000 293T cells in each well. After cell adhesion (24h), SPAchip® were added to indicated wells at a 2:1 SPAchip®-to-cell ratio. Samples included intracellular pH Calibration controls (pH 4.5 to 7.5), cells in DMEM medium, cells treated with Antimycin A (AMA) and control conditions with no SPAchips. Cells were harvested with TrypLE, treated when necessary and analyzed in the flow cytometer.

RESULTS

Extracellular SPAchip® populations can be easily identified by flow cytometry in the cell culture suspension by size and complexity (FSC vs SSC) (**Fig.2**). SPAchip are characterized for having a very defined distribution in terms of size due to the tight fabrication control. Control cells can be used to gate cell populations (**Fig. 3A**). Once extracellular SPAchip® and cell populations have been gated accordingly, it is possible to discriminate fluorescence intensity ranges of extracellular and cytosolic SPAchip® to interpolate pH values in the FITC channel.

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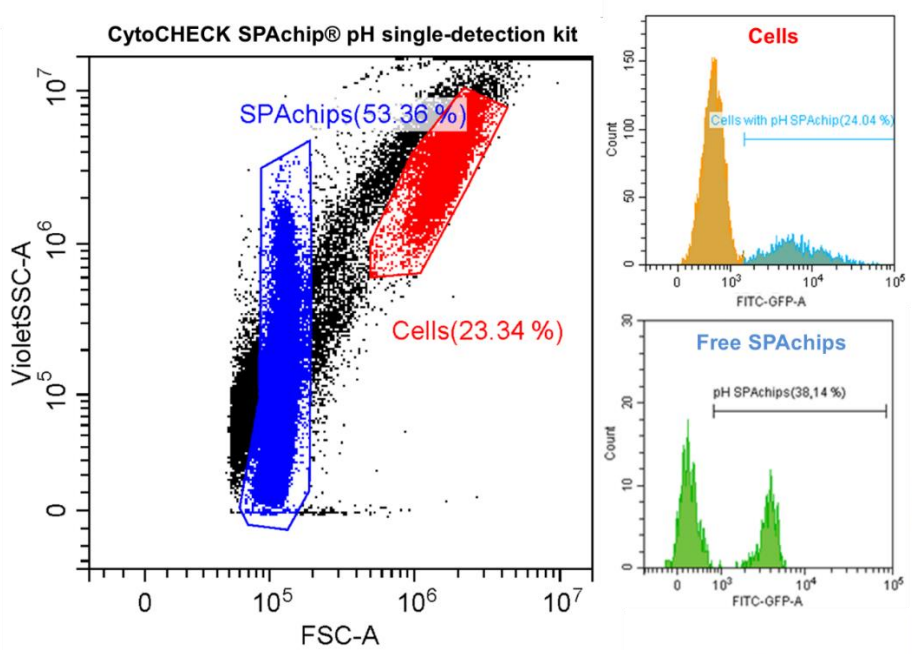

Fig. 2. CytoCHECK SPAchip® pH Single-Detection data from Flow Cytometry. Population gating based on FSC and SSC channels to define extracellular SPAchips and cells. Fluorescence intensity distribution in FITC channels for cells and extracellular "free" SPAchips.

CytoCHECK SPAchip® pH-single detection kit detected a significant decrease in cytosolic pH in cells treated 15 minutes with Antimycin A, with minor changes in cell medium, likely because of glycolytic boost and pyruvate production after electron transport inhibition (**Fig. 3B**). Bars represent the mean ± SD of two experiments in triplicate. Statistical comparison vs Control ***p<0.001 (Sidak test). This novel techniques allows to compare intracellular pH values to the cell culture conditions to study the proton fluxes in living single cells as never before.

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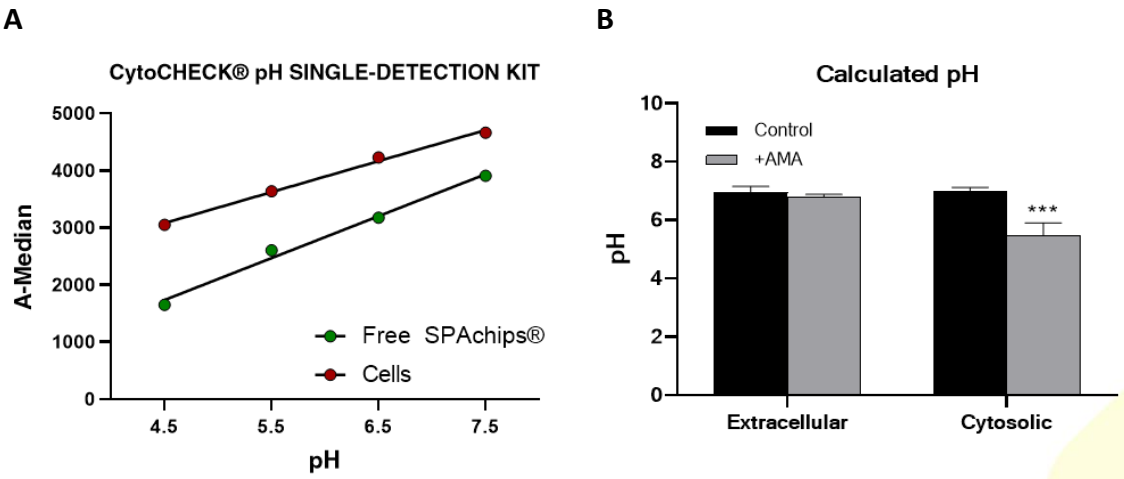

Fig. 3. CytoCHECK SPAchip® pH Single-Detection results by Flow Cytometry.
A) Interpolation curve obtained from intracellular pH calibration control conditions adapted to extracellular SPAchips and cytosolic signals. **B)** Calculated pH of extracellular SPAchips showing a value of 7 with no significant difference between cells in DMEM and Antimycin A-treated. SPAchip® allowed to show how intracellular pH significantly dropped below a value of 6 when treated with Antimycin A. ***p<0.001

CONCLUSIONS

CytoCHECK SPAchip® pH-single detection technology is a new reliable and accurate tool for performing cell analysis in flow cytometry. Its capability to detect pH changes in the cytosol and cell environment simultaneously makes our technology a valuable tool for:

- Basic cell biology and cell physiology studies.
- Quality Control in bio-industrial processes based in cell cultures, where controlling extracellular pH affects product yield, as recombinant antibody or AAV production.

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REFERENCES

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SPAchip® technology offers new insights into intracellular proton concentration analysis by flow cytometry. SPAchip® kits are the unique tool to characterize differential pH levels between the cytosol and the extracellular medium to study proton fluxes

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