Technical report

Utilizing Hidex Sense for mitochondrial and cytosolic calcium measurements using the luminescent calcium reporter protein *aequorin* and the fluorescent calcium indicator Fura-2 AM

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SUMMARY

Background The calcium ion (Ca^{2+}) is a universal second messenger that regulates multiple important cell physiological functions such as cell proliferation and migration, energy metabolism, and cell death. Thus, many techniques for studying the cell biological importance of Ca^{2+} signaling have been developed. The fluorescent Ca^{2+}-binding molecule Fura-2 is a classical and a widely used Ca^{2+} reporter. Aequorin is a Ca^{2+}-sensitive luminescent protein originally derived from the jellyfish *Aequorea victoria.* When expressed in mammalian cells, wild-type aequorin localizes to the cytoplasm and can be employed for cytoplasmic Ca^{2+} measurements. Recombinant aequorin fusion proteins with specific targeting peptide sequences have been produced in order to direct the reporter protein to a desired organelle in the cell. These organelles include the nucleus, the Golgi apparatus, the endoplasmic reticulum and the mitochondria (1). The use of targeted aequorin fusion proteins has greatly influenced Ca^{2+} signaling research. Also, a fusion protein consisting of aequorin and green fluorescent protein (GFP), where aequorin excites GFP upon binding of Ca^{2+} to aequorin, has been developed to increase the light production capacity of this Ca^{2+} reporter (2). ***Aim*** In the present study, we wanted to transfer our current aequorin-based mitochondrial calcium measurement protocol from our low-throughput instrumentation to the Hidex Sense kinetic luminescence assay. We also wanted to test whether the CHO/G5A cells that stably express the wild-type aequorin and GFP in a complex and, thus, report Ca^{2+} fluxes from the cytoplasmic compartment (2) are suitable for experiments with Hidex Sense. In addition, we aimed to use the cell permeant Fura-2 AM for cytosolic Ca^{2+} measurements using the kinetic fluorescence assay of Hidex Sense. ***Conclusion*** We report here the successful employment of the Hidex Sense plate reader system for mitochondrial and cytosolic Ca^{2+} experiments using mitochondrially targeted aequorin for mitochondrial Ca^{2+} measurements and CHO/G5A cells for cytoplasmic Ca^{2+} measurements, respectively. Also, the fluorescence assay of the Hidex Sense plate reader was well suited for measurements of cytosolic Ca^{2+} using Fura-2 AM. Hidex Sense reproduced

our previous data very reliably and produced data of excellent quality in a user-friendly manner.

MATERIALS AND METHODS

Cell culture HeLa cells and the Chinese hamster ovarian (CHO/G5A) cells were cultured in DMEM medium with 1% penicillin/streptomycin and 1% L-glutamine at 37°C and 5% CO_2. ***Transfections*** TurboFect transfection reagent was used for all transfections. One day prior to transfection, 10 000 HeLa cells per well were seeded in a 96-well plate. The transfection mixture (per well) contained 10 µL OptiMEM, 0.1 µg of mitochondially targeted aequorin plasmid (mtAEQ) and 0.25 µL TurboFect. The transfection mixture was incubated as described in the manufacturer's protocol and then pipetted carefully on to the cells. ***Mitochondrial calcium measurents using recombinant aequorin*** One day after transfecting the cells with mtAEQ, the cells were washed 2-3 times in HBSS at 37°C. Then, the cells were incubated with 5 µM wild-type coelenterazine for 1 hour in the Hidex Sense machine at 37°C in the dark. Then the cells were stimulated with histamine (100 µM final concentration) at 10 seconds from the start of the measurement. Then, for inducing the maximal light production of the sample and calibrating the results, the cells were permeabilized with digitonin (100 µM final concentration) in the presence of 10 mM $CaCl_2$. For further details on experimental procedures, see reference (1). ***Cytosolic calcium measurements using CHO/G5A cells*** For cytosolic calcium measurements, we employed the CHO/G5A cell line stably expressing the GFP-aequorin chimera (G5A) which reports calcium fluxes from the cytoplasmic compartment as described in (2). For these experiments, 20 000 -30 000 cells were seeded on 96-well plates and the experiments were conducted after 24 hours. The wild-type coelenterazine reconstitution was conducted as described for mitochondrial Ca^{2+} measurements. The cells were stimulated with 50 µM ATP to induce a rise in cytosolic Ca^{2+}. ***Cytosolic calcium measurements using Fura-2 AM*** Twenty-four hours prior to the experiment, 30 000 HeLa cells per well were seeded to white 96-well plates. At the day of experiment, the cells were washed three times in HBSS buffer (37°C) and incubated for 30 minutes with 2 µM Fura-2 AM at room temperature (RT) in the dark. Then, the cells were washed twice in HBSS (RT) and incubated for 15 min in HBSS at RT, whereafter the plate was transferred to the Hidex Sense plate reader and the fluorescence assay was run. The settings for the Fura-2 fluorescence assay were "super" for the lamp power and "50" for the amount of excitation flashes. The cells were stimulated at tenth kinetic cycle with 100 µM histamine.

RESULTS

Mitochondrial calcium measurements Linear downscaling of most of the steps in our previous experimental protocol (using 12-well plates) was sufficient for successful mitochondrial calcium measurements with the Hidex Sense plate reader (using 96-well plates). Only the protocol for transfecting the cells with the mitochondrially targeted aequorin plasmids (mtAEQ) was slightly adjusted for optimal transfection efficiency. The sensitivity of Hidex Sense luminometer was excellent, which provided for a good signal-to-noise ratio and optimal counts per second (CPS) readings (Fig. 1). Also, we found that white 96-well plates

were more suitable for this application than the clear plates. The white plates had slightly higher basal CPS readings but the increase in CPS during cell stimulation was greatly amplified, which led to improved signal-to-noise ratio as compared to the clear plates. The raw data was analyzed as described in (1) and the results were well in line with our previous findings, with very little variation (Fig. 2). For mitochondrial Ca^{2+} measurements, Hidex Sense provided for an approximately 10-fold increase in the throughput and an improved quality of the data. ***Cytosolic calcium measurements*** We successfully used the Hidex Sense luminescence assay for monitoring Ca^{2+} signals in the CHO/G5A cells, and the fluorescence assay for monitoring Ca^{2+} signals in Fura-2 loaded HeLa cells. The CHO/G5A cell line is a convenient tool for measuring robust changes in cytosolic Ca^{2+} signals but in contrast to the mitochondrial Ca^{2+} measurements, the raw data cannot be converted to absolute Ca^{2+} concentrations. This shortcoming is due to the lack of calibration values for the G5A GFP-aequorin coplex [see reference (1) for more information on aequorin measurements and calibration, and reference (2) for information on CHO/G5A cells]. However, the lack of possibility for calibration of the results when using CHO/G5A cells causes limitations only when the cells are subjected to treatments that would alter the cell count as shown in Fig. 2, left panel. In experiments where the cytosolic Ca^{2+} signals are modulated by treatments that do not alter the cell count (Fig 2, right panel) the CHO/G5A cell assay was very user-friendly and produced reliable data with good consistency.

The measurements using Fura-2 loaded HeLa cells were simple to carry out with the Hidex Sense kinetic fluorescence assay by following a standard Fura-2 protocol, and the obtained data was of good quality with low variation.

CONCLUSIONS AND REMARKS

Hidex Sense is well suited for mitochondrial calcium measurements using the recombinant aequorin approach when conducting an experiment where only a single stimulation is needed. The number of stimulations with different agonists during one run is limited because there are only two dispensers, which is a limiting factor for more complex cell physiological experiments (the second dispenser is reserved for the calibration buffer). Previously, both dispensers could not be used during one kinetic measurement which compromised the throughput as the crucial calibration step of the experiment had to be conducted in a separate kinetic run. This caveat was later fixed by a software update, which then allowed for all of the steps that are needed for an experiment to be done during one run. Without this update, the experimental procedures would have been more time-consuming and less user-friendly. The amount of dispensers could be increased to allow more than two different stimulations during an experiment. This would allow more complexity in the experimental design, which often is needed in more advanced cell physiological experiments. Nevertheless, the current kinetic luminescence setup of the Hidex Sense plate reader can be very effectively employed for aequorin-based mitochondrial calcium measurements using single-agonist stimulations, and the data is of excellent technical quality (Fig. 2).

In addition, the tested cytosolic Ca^{2+} measurement applications are well suited for the Hidex Sense plate reader. With both the luminescence assay and the fluorescence assay, we were able to generate high-quality data with great reproducibility (Fig. 3, Fig. 4).

REFERENCES

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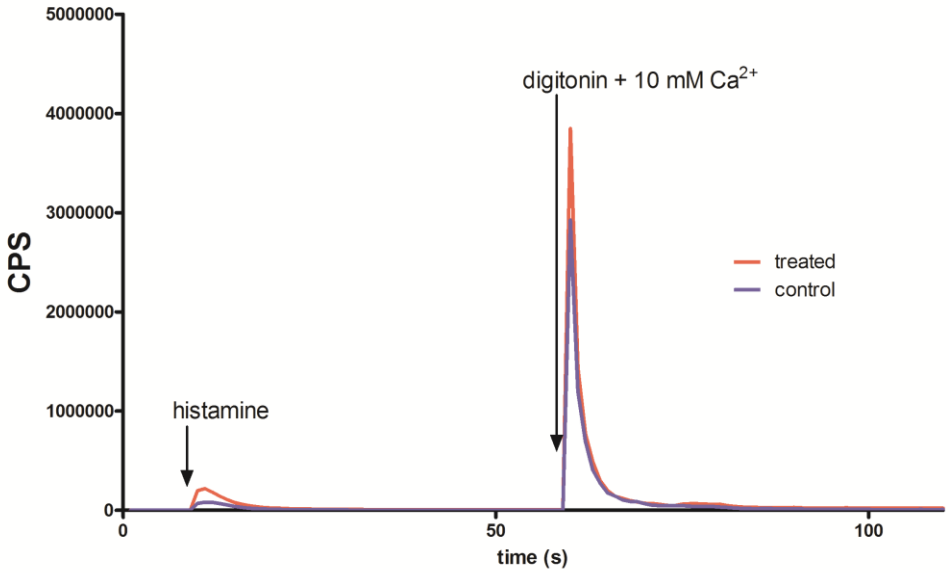

Figure 1. Representative traces showing the raw values during the whole time course of a mitochondrial calcium measurement in HeLa cells. CPS = counts per second.

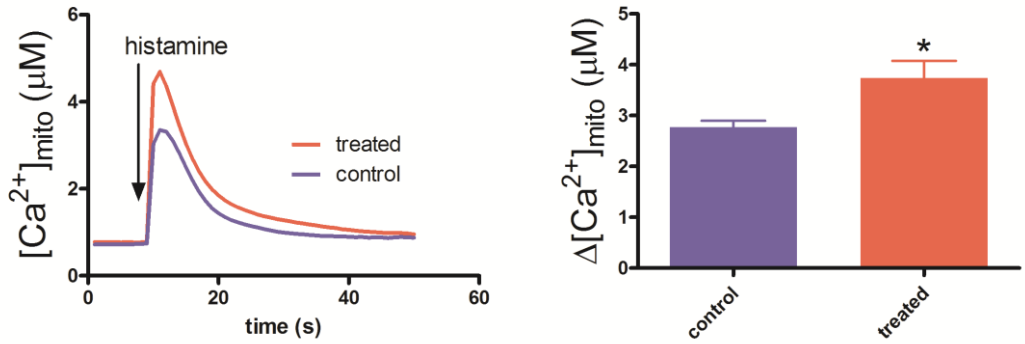

Figure 2. *Left panel*: Representative traces showing calibrated mitochondrial calcium concentrations during the first 50 seconds of the same experiment as in Figure 1. *Right panel*: Combined and quantified results showing the change (basal subtracted from the peak) in mitochondrial calcium during histamine (100 µM final concentration) stimulation. Data is expressed as mean with S.E.M, n=7, *P < 0.05.

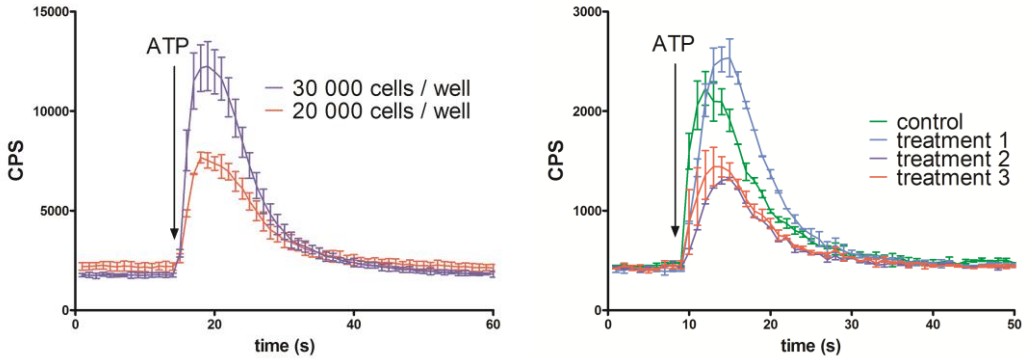

Figure 3. Cytosolic calcium measurements using CHO/G5A cells. *Left panel:* Thirty thousand versus 20 000 CHO/G5A cells per well were plated 24 hours prior to the experiment and stimulated with 50 µM adenosine triphosphate (ATP). The cell count greatly affects the light production of the sample. *Right panel:* Representative experiments showing consistency in how different treatments affect Ca^{2+} signaling in CHO/G5A cells. The traces are averages of 2-3 experiments with +/- S.E.M.

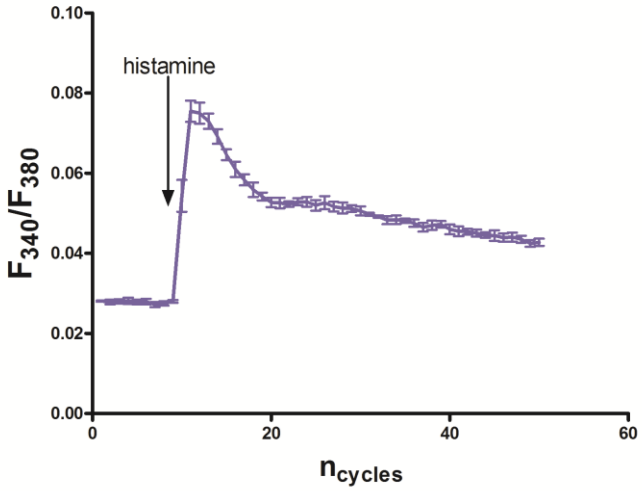

Figure 4. An averaged trace from Fura-2 loaded HeLa cells showing the effect of histamine-stimulation (100 µM). The error bars show +/- S.E.M from 4 individual measurements.