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ORIGINAL ARTICLE

High Throughput Fluorescent Screening of Membrane Potential and Intracellular Calcium Concentration Under Variable Gravity Conditions

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Abstract In addition to the presence of specific gravity receptors in living organisms, biological membranes were found to directly respond to gravity changes. Among others, changes in membrane permeability and as a consequence in membrane potential and intracellular ion concentrations have been demonstrated mainly by using electrophysiological techniques. However, the acquired amount of data up to now is low due to technical limitations of electrophysiology in microgravity platforms. Optical techniques will be able to deliver much higher amounts of data here, especially in case high throughput techniques based on 96 well plate (or higher numbers of wells) readers can be used. In this manuscript we present a new set-up for parabolic flight campaigns based on a multi-purpose plate reader for photometric, luminescent and fluorometric measurements. In a first series of experiments during a parabolic flight campaign the system was verified for membrane potential and intracellular calcium concentration measurements of neuronal cells using fluorescent dyes.

Keywords Microgravity · Fluorescence · Membrane potential · Intracellular calcium · 96 well plate reader

Introduction

Independent on the dogma that nature has developed specified gravity sensing organs in living organisms, it has been shown that even single cells are able to respond to gravity. In some cases this has been shown to be due to specialized structures (i.e. Häder et al. 2005). In other cases, for example in neuronal cells, membrane processes are obviously directly affected by gravity (Wiedemann et al. 2011). In previous studies it has been demonstrated that the properties of action potentials depend on gravity (Meissner and Hanke 2005), as well, as the gating of ion channel can be directly changed under variable gravity conditions (Goldermann and Hanke 2001). As a consequence, membrane potential and intracellular ion concentrations should be gravity dependent what has been verified in some previous experiments (Meissner et al. 2004).

The methodological approach for these findings mainly was the use of electrophysiological techniques (Wiedemann et al. 2011), for example patch clamp, and planar lipid bilayer experiments (Klinke et al. 2000). Although these experiments delivered a number of interesting results, the total amount of data acquired in electrophysiological studies usually is small. This statement is even more correct under conditions as given in parabolic flight missions (Wiedemann et al. 2011) and other microgravity platforms. Fully automated electrophysiological set-ups up to now have only rarely been used without giving spectacular results or big amounts of data (Klinke et al. 1998; Meissner and Hanke 2002; Wiedemann et al. 2003).

Meanwhile optical techniques for the measurement of biological cell-membrane parameters have undergone a fascinating evolution. Specific fluorescent dyes have been developed to investigate, among others, membrane potential and intracellular ion-concentrations (Haugland 2005). To measure intracellular free calcium concentration is possible in high

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detail with specific dyes since years (Haugland 2005). More recently also dyes have been created to measure other intracellular ion-concentrations, i.e. sodium and potassium, too. Some first experiments have been published using singular measurements with fluorescent dyes (Meissner et al. 2004). Also, a variety of dyes have been used in microscopic experiments (Häder et al. 2004; Richter et al. 2006). Both approaches, however, again suffer from the problem of limited data point acquisition.

Mainly for pharmacological studies, systems based on optical recordings, have been developed to enable high-throughput screening of drug action. Usually these systems are based on well plates from 96 to 1,024 wells. Such plate readers can be used with photometric, luminescent or fluorometric dyes and allow the acquisition of high numbers of data-points in short time utilizing a variety of recording approaches, as there are among others photomultipliers, photosensitive diodes, or intensified cameras.

It would be tempting to use such systems in microgravity platforms to enhance the collection of data under the given complicated conditions. A first set of experiments in this direction recently has been done (Hampp, R., University of Tübingen, Germany, personal communication). We have consequently developed a set-up based on a multipurpose microwell plate reader (fluorescence, luminescence, photometric) to measure membrane potential, intracellular ion-concentrations and other membrane parameters during parabolic flights. In a first mission we have verified the system measuring membrane potential and intracellular calcium concentration in neuronal cells under conditions of changing gravity.

Materials and Methods

Materials

The membrane potential dye Di-4-ANNEPS and the calcium dye Fura-2 AM were obtained from Invitrogen. Purified water was from a laboratory distillery. DMEM-Ham's F12 medium, fetal bovine serum (FBS), trypsin, penicillin and streptomycin were obtained from Biochrom AG (Berlin, Germany). All other substances used were from Sigma-Aldrich (Steinheim, Germany) at least of pa purity. The mechanical components were purchased from Bosch Rexroth AG (Lohr am Main, Germany). The black 96 well plates (or microplate) with clear bottom were purchased from Packard Instrument Company (Meriden, CT, United States).

Cell Culture

For this experiment, the human neuroblastoma cell line SH-SY5Y was used. It was first reported in 1978 (Biedler et al. 1978). The cells can have two different phenotypes: the neuroblastoma cells with small, spherical cell bodies and epithelia-like cells with larger, flat bodies (Ross et al. 1983). By treatment with retinoic acid the cells can be differentiated to neuron-like cells (Abemayor 1992; Sidell et al. 1998) which can be identified by developed neurites (Påhlman et al. 1984). The SH-SY5Y cell line is a robust cell line and it is frequently used in parabolic flights by several teams (Wiedemann et al. 2011; Rösner et al. 2006). The adherent SH-SY5Y cells were cultivated in DMEM-Ham's F12 liquid medium with stable glutamine, 10 % heat inactivated FBS and 100 U penicillin and 100 µg streptomycin per ml at 37 °C with 6.5 % CO_2.

Twenty four hours before each flight day, the cells were transferred from the T25 culture flasks to 96-well plates with clear bottom. For an optimal fluorescence yield it was aimed at confluent monolayers in each of the 96 wells. An 80 % confluent T25 culture flask with SH-SY5Y was sufficient to prepare a single 96 well plate.

Assay Preparation for DI-4-ANEPPS and Fura-2 AM

Dye Preparation

For each flight day only one dye was used. The dye was dissolved with dimethyl sulfoxide (DMSO) in a ratio of 1 µg:1 µl to obtain a stock solution (2 mM for Di-4-Anepps; 1 mM for Fura-2 AM). This stock solution was frozen in 2 µl aliquots. For loading of the cell, this stock solution was mixed with the Ca^{2+}-free wash buffer (Tables 1 and 2) to obtain an end concentration of approx. 4 µM (Di-4-Anepps), respectively 2 µM (Fura-2 AM). For a complete 96 well plate 10 µl stock solution was mixed with 4990 µl wash buffer.

Table 1 Imaging buffer with Ca^{2+}

Substance	Concentration [mM]
NaCl	140
KCl	2
$MgCl_2$	2.5
$CaCl_2$	1
HEPES	1
Glucose	10
Sucrose	10
pH 7.3 (with NaOH)	

Table 2 Wash buffer with Ca^{2+}

Substance	Concentration [mM]
NaCl	140
KCl	2
$MgCl_2$	2.5
HEPES	1
Glucose	10
Sucrose	10
pH 7.3 (with NaOH)	

Dye Protocol

- Remove cell culture medium from wells, replace with 200 µl wash buffer
- Wash twice with 200 µl wash buffer
- 50 µl of the fluorescence-dye-solution per well. Incubate in darkness for 30 min at room temperature
- Wash twice with 200 µl wash buffer, avoid strong light
- Fill the wells completely with imaging buffer (Table 1), storage in darkness

Sealing

After the dyeing protocol was finished, the plates were tightly closed with adhesive optical sealing tape (Adhesive PCR Film, Thermo Fisher Scientific, USA) for 96 well plates (first level of containment). The wells were completely filled with imaging buffer and the sealing tape was carefully attached to minimize air bubbles. A second level of liquid containment was requested for safety reasons, for that, clear adhesive tape (Scotch® Cystal Tape, 3M Corporation, USA) was used to seal the clear lid of the plate to the microwell body (see Fig. 1).

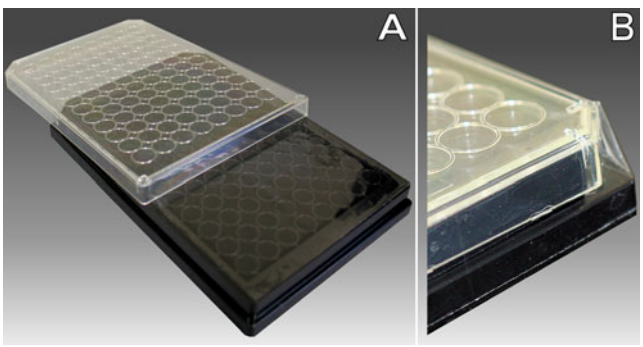

Fig. 1 The completely sealed 96 well plate (microplate) by means of adhesive optical film (plate) and adhesive tape (lid)

Set-Up

To build the setup, a FLEXstation I from Molecular Devices (Sunnyvale, CA, United States), controlled by the software SOFTmax® PRO, was used. The FLEXstation™ is a benchtop fluorescence, spectroscopic and luminescence reader which can handle plates up to 384 wells. In addition, a liquid handling system is integrated for kinetic measurements.

The optical system (see Fig. 2) consists of a xenon flash lamp, two holographic diffraction grating monochromators to optimize excitation and emission wavelength, additional excitation bandpass and emission cut-off filters to reduce the amount of stray light, and a PMT (photomultiplier) detector. The components are connected by optical fibers. The FLEXstation has two read heads, bottom and top, for cell based assays and solution-phase measurements. The temperature of the microplate reading chamber can be controlled up to 45 °C above ambient (with operation temperature between 15–35 °C).

To be usable in the European parabolic flight program, the FLEXstation had to comply with mandatory safety regulations. Therefore a setup was buildt according to the *RG-Rules and Guidelines* 2009–2 issued by Novespace.

The FLEXstation was integrated in a rack which was made from 45 × 45 mm t-groove aluminum profiles, 45 × 45 mm and 45 × 90 mm brackets and 45 × 45 mm cubic connectors (see Figs. 3 and 4). The rack was attached to a solid 10 mm aluminum base plate. As a safety margin, the rack had to withstand a calculated load of 9 g, therefore the complete setup weighed 88 kg, whereas the FLEXstation only contributed 29.5 kg.

The outer walls of the rack were made from aluminum and polycarbonate sheets (sides: 2 mm; top: 5 mm). Between the aluminum profiles and the outer walls, two 20 mm wide separate strips of sticky silicone gasket were used to completely seal the setup, in addition all boreholes with screws and cables were sealed with silicone. The sealing was needed for two reasons. First, safety of the plane, as the risk of accidentally releasing liquid in the aircraft must be minimized, and second, for a future use of genetically modified organisms (GMO) in the aircraft, as a total containment for GMO is a mandatory regulation by the local authorities.

The plate reader can be accessed by a lockable, sealed door. For the case of cabin depressurization a burst disc had to be integrated into the access door for quick pressure compensation.

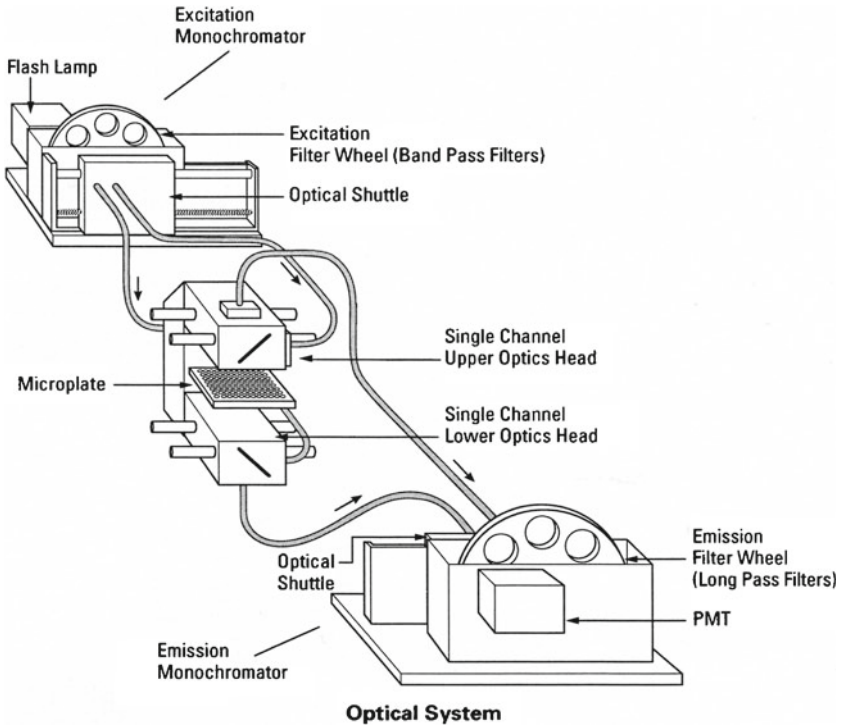

Fig. 2 Schematic of the optical system of the FLEXstation. Picture from FLEXstation user manual PN 0112–007-Rev. C (Molecular Devices)

The laptop was attached on the top aluminum plate by 20 × 20 mm t-groove aluminum profiles and brackets. The data cable and the power cord are the only connection to the inside of the rack. The holes were also sealed with silicone. To minimize the needed sockets in the aircraft, a multi-plug was directly attached to the top plate. All equipment was connected to this multi-plug and via a mandatory 30 mA ground fault interrupter (GFI) with emergency-stop button it was connected to the aircraft power supply.

To use as much as possible of the parabolic flight maneuver, the needed software operations were semi-

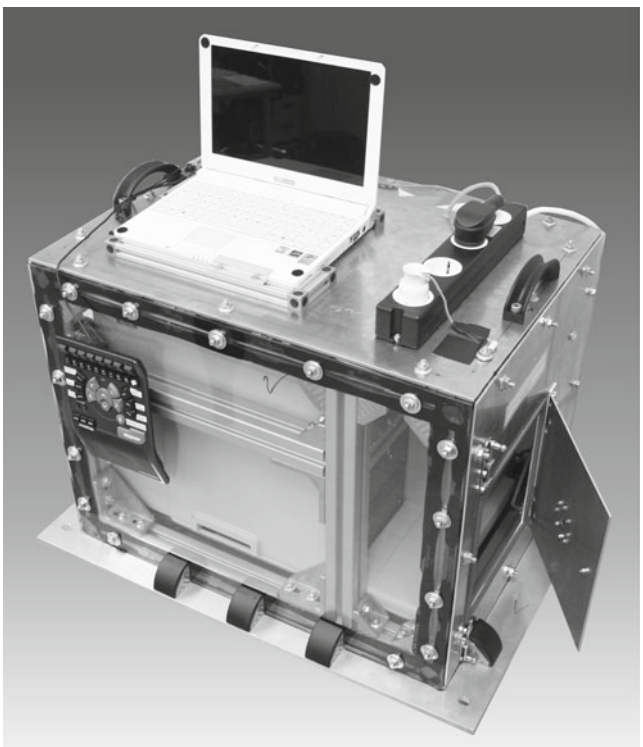

Fig. 3 The main rack. To comply with the mandatory safety regulations (liquid handling and use of GMO), the FLEXstation was integrated in a completely sealed rack. It can be accessed by a lockable, sealed door

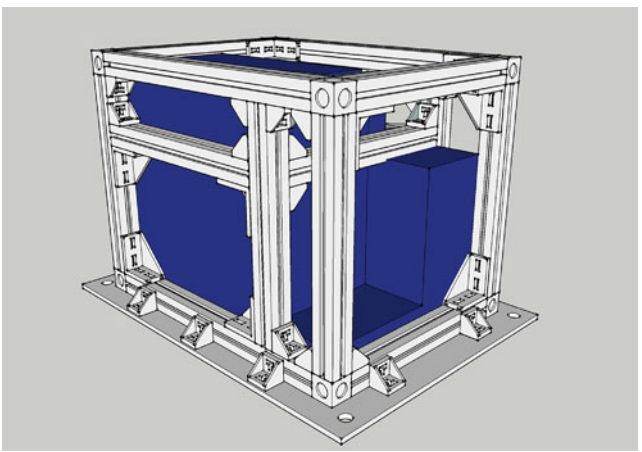

Fig. 4 Schematic of the main rack (without the outer walls). The rack structure was made from 45 × 45 mm t-slotted aluminum profiles and brackets. The FLEXstation was secured in all directions

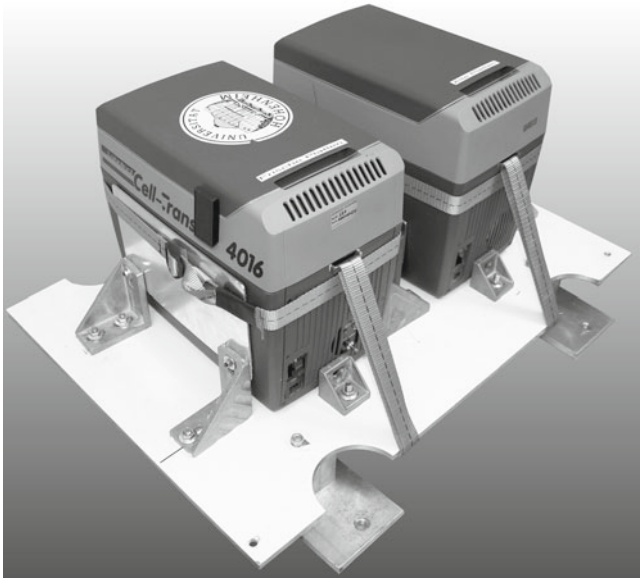

Fig. 5 The incubator rack. Until use, the 96 well plates were stored in a 37 °C incubator. A second, unpowered box was used to store the used plates. In addition to the supporting structure, the incubators were secured with lashing straps

automated by using a programmable keyboard (Fightpad from Listan GmbH & Co. KG, Glinde, Germany) which allowed complex keyboard and mouse commands to be executed with a single keystroke (e.g. labeling of the recordings, setup of a new recording, ...). The keyboard was attached to the main rack with industrial type hook and loop fastener.

A second rack was constructed to house two incubator boxes for proper storage of the 96 well plated during flight (see Figs. 5 and 6). Until use, the plates were stored in a portable 37 °C incubator (Cell-Trans 4016 from Labotect Labortechnik GmbH, Göttingen, Germany). A similar thermoelectric container was used unpowered as a waste container for the used plates (TC-07 from Waeco International GmbH, Emsdetten, Germany). The rack was made from solid 10 mm aluminum plates with legs made from solid 60 × 60 mm aluminum blocks. 45 × 90 mm aluminum brackets with 3 mm aluminum sheets and additional lashing belts were used to fix the incubator boxes to the base plate. The complete incubator rack weighed 20 kg.

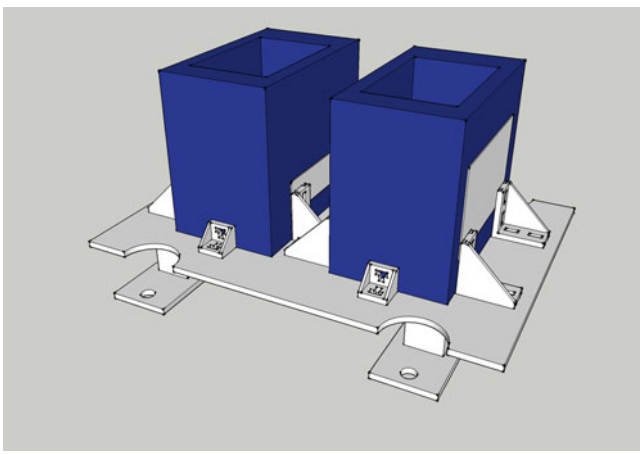

Fig. 6 Schematic of the incubator rack. The rack structure was made from aluminum sheets and 45 × 90 mm brackets

Parabolic Flight Protocol

A 96 well plate was used for a set of 5 parabolas as the exchange of the plate was only allowed in the 4 to 8 min breaks between these sets (ESA 2005). Strips of clear adhesive tape were used to attach the plate to the drawer of the reading chamber to secure the microplate during microgravity. Therefore column 1 and 12 were not used for the measurement as these columns were covered by the adhesive tape. Before the first parabola of each set, three 1 g recordings were performed to calibrate the optical system of the FLEXstation to the used microplate. As the FLEXstation has to perform several mechanical operations which could not be shortened (e.g. the operation of the filter wheels with approx. 3 s), the measurements protocols for the fluorescence dyes had to be adapted accordingly to fit into the 22 s stages of micro- and hypergravity during a parabola. For this first experiment, we tried to find a balance between the obtained amount of data points (number of wells) and the quality of the optical measurements (number of repeated measurements for averaging during a single recording) to fit in 20 s. Depending on the used dye the number of repeats per well had to be adjusted to get a good fluorescence signal. As repeats cost time it was tried to find a good balance between the quality of the fluorescence signal and the number of repeats.

General settings of the FLEXstation Temperature 37 °C; Endpoint; Fluorescence; Bottom read; Plate: Black/ clearbottom

Fura-2 AM Excitation wavelengths 340 and 380 nm; emission wavelength 510 nm; Sensitivity 4; wells to read: rows 5–9

Di-4-ANEPPS Excitation wavelength 475 nm; emission wavelength 617 nm; Sensitivity 6; Wells to read: rows 5–11

As the drawer of the FLEXstation moves out after each measurement, which cannot be switched off by the user, only every second gravity phase during a single parabola could be used in endpoint measurements,

therefore 1 g and 0 g or 2 g-in and 2 g-out were recorded during a single parabola.

Results

As the plates could only be exchanged before the first parabola and during the five long lasting (4–8 min) 1 g-phases, 7 plates were prepared for each flight day with the seventh as reserve. Each plate was used for a set of 5 parabolas. Due to the timing limitations of the FlexStation described above, alternate recording were made in 1 g and 0 g (recording 1) and 2 g-in and 2 g-out (recording 2). For each set of 5 parabolas the mean fluorescence values of the different gravity phases were compared with the corresponding mean values of the 1 g in-flight measurements. At the end the normalized values of all parabolas (with 1 g set to 100 %) were statistically analyzed.

Di-4-Anepps (See Fig. 7)

The fluorescence was significantly increased by 1.0 % during the first 2 g-phase (2 g-in). For Di-4-ANEPPS, an increase of 1 % is equitable to a depolarization of approx. 10 mV (Haugland 2005). A non-significant decrease in fluorescence to 99.73 % compared to the in-flight 1g measurements was observed during the microgravity phase (0 g). This corresponds to a hyperpolarization of approx. 3 mV. The non-significant decrease to 99.34 % during the second 2 g-phase (2 g-out) corresponds to a hyperpolarization of approx. 6 mV. In addition the fluorescence of the first 2 g phase is significantly increased compared to the 0 g- and the 2 g-out-phase.

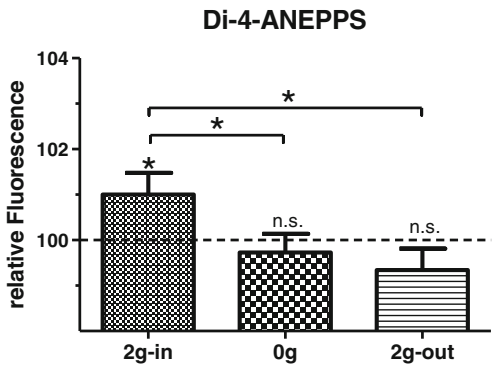

Fig. 7 Changes in fluorescence of di-4-ANEPPS-stained SY-SY5Y cells. Mean ± SD. *p(2g-in) = 0.035, *p(2g-in to 0g) = 0.043, *p(2g-in to 2g-out) = 0.013, paired t-test (n = 31, 6720 data points). The different gravity phases are normalized against the 1g in-flight measurements (*dotted line*)

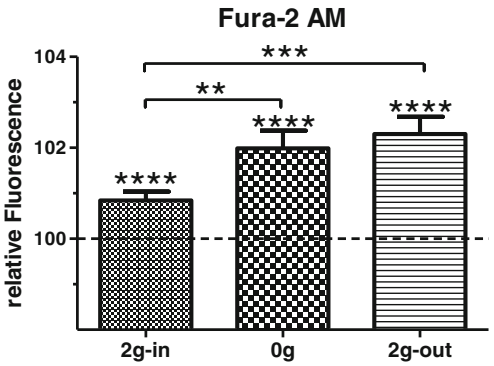

Fig. 8 Changes in fluorescence of Fura-2 AM-stained SY-SY5Y cells. Mean ± SD. ****p < 0.0001, **p = 0.0088, ***P = 0.0008, paired t-test. (n = 31, 3200 data points)

Fura-2 AM (See Fig. 8)

To monitor the change of free intracellular Ca^{2+}, the ratio of the excitation wavelengths (340 and 380 nm) was used (Haugland 2005). The fluorescence of all three non-1 g-phases, 2 g-in, 0 g and 2 g-out are significantly increased by 0.8 %, 2.0 % and 2.3 % compared to the 1g in-flight measurements.

Discussion and Outlook

It is obvious from the presented results, that high throughput optical measurements can be performed under parabolic flight conditions. Even after four hours, the signal strength of the used dyes was stable, no difference could be observed between the plate used at the beginning of the flight day and the last used plate.

Using the fluorometric dye di-4-ANNEPS and a 96-well multi-purpose reader it could be shown that the membrane potential of neuronal cells depolarizes at higher gravity and show a tendency to hyperpolarization under microgravity. Additionally it is shown that the intracellular calcium concentration rises as well under hyper- as under microgravity. This is surprising, however, might be a memory or hysteresis effect of calcium influx stimulation by the 1.8 g phase at the entrance of each parabola. According to that, it has to be taken into account, that in parabolic flights in principle oscillating gravity is given (Klink et al. 2011),

dependent on the time scale of the experiment. Only for short time processes (seconds or shorter), the different periods of gravity can be looked at separately. This can be also seen in comparing the presented results to former drop-tower experiments (Meissner et al. 2004), were no hypergravity phase is given before the microgravity period. There, the membrane potential also hyperpolarized under microgravity, however, intracellular calcium concentration decreased. According to the fact that different platforms were used, and due to the problem of a strictly limited number of data points from the drop tower (see introduction), it is difficult to compare the results, and additional experiments will be necessary.

The cells used in the presented experiments are human neuroblastoma cells which can be re-differentiated to neuron like cells (Abemayor 1992; Sidell et al. 1998). It would be useful to perform additional experiments with these cells to find out, whether the change in ion channel repertoire during re-differentiation (Tosetti et al. 1996, 1998) effects the cellular response to gravity changes. Also, the use of other cells will be necessary for comparison, as well as additional experiments using a platform which delivers plain microgravity or plain hypergravity. For hypergravity in a centrifuge this is in principle no problem, however, for microgravity experiments either the short period (4 s) drop-tower, future balloon experiments (1 min) or orbital systems must be used. In any case the need of new set-ups for these platforms is obvious.

As has been demonstrated at least in parabolic flights the use of high throughput technology is possible. According to recent developments, a possible new set-up should be designed for use by multiple users. Additionally, such a system could be based on an intensified camera as is used by some new readers (i.e. Hamamatsu μCell 2011), instead of a scanned photomultiplier, which further more would increase the number of data-point which can be measured.

Nevertheless, even with the presented hardware a variety of additional questions can be examined with such a system including pharmacological and binding studies, as well as kinetic measurements.

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