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ORIGINAL ARTICLE

PMT (Photomultiplier) Clinostat

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Abstract In order to enable kinetic online measurements in cell cultures during exposure to altered gravitational stimulation a new device has been constructed. The analysis principle is based on photon counting with a photomultiplier tube (PMT). The system can work in a rotating mode (clinostat principle) as well as in 1 g conditions. Biological verification was successfully performed with a Luminol-based luminescence measurement of the immune reaction of mammal macrophages. An identical methodical approach is foreseen for an ISS experiment (TRIPLE LUX). Alteration of the rotation speed of the PMT clinostat (60 and 2 rotations per minute—rpm) resulted in a speed-dependent decrease of the luminescent signal, contributing to the current discussion whether and how fast rotation of a sample around one axis perpendicular to the direction of the g-vector provides the condition of functional weightlessness or omnilateral mechano-(gravi-) stimulation.

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Abbreviations

rpm	Rotations per minute
DLR	German Aerospace Center
PBS	Phosphate Buffered Saline
PMT	Photomultiplier Tube

Introduction

Gravitational biologists investigate the effects of altered gravity on a biological system. In order to simulate the condition of "weightlessness" a commonly used method is rotating a horizontally positioned sample around an axis perpendicular to the direction of the *g*-vector (clinostat principle: Briegleb 1988, 1992).

Luminescence measurement is a widely applied method in biology for detection and quantification of biomolecules in certain assay systems for example ELISA and Western blots. In a common plate reader online measurements of the desired luminescence are possible. However, such kind of reader does not allow a kinetic measurement of a system in a different environment: in the past, a system was exposed, the reaction had to be stopped and one endpoint measurement was done inside the reader.

In order to enable online kinetic measurements of luminescent signals of biological systems in "functional weightlessness" we combined the photomultiplier and the clinostat techniques. The result is a portable "photomultiplier clinostat" (PMT clinostat). The measurement method is identical to the one which will be used

in an upcoming space experiment on ISS (TRIPLE LUX) dealing with the cellular immune response. Consequently, biological verification of our PMT clinostat device was performed by the chemiluminescence of the Luminol–H_2O_2–horseradish peroxidase system, measuring the oxidative burst reaction of mammal macrophages of the cell line NR8383.

Material and Methods

Designed was a system for online luminescence measurements. The system is able to house cells in media and provide clinorotation of varying speed. A general black box model of the system can be seen in Fig. 1 and the corresponding technical details and specifications in Table 1.

Photomultiplier Tube (PMT) and Clinostat

The luminescence was measured by a PMT (Hamamatsu, Hamamatsu City, Japan), which can operate with the luminescence of all wavelengths between 300 and 650 nm. The PMT is read out with a frequency counter (Fig. 2), which can be connected to a laptop. With the software COUNTER the resulting data are directly read out. A reading of the actual luminescence can be done every second.

The PMT is combined with a clinostat (see Fig. 2). The clinostat consists of a motor (Faulhaber, Stuttgart, Germany) with an integrated tachometer, which can be run with varied speed, depending on the input voltage: 7.9 V corresponds to 60 rpm, 0.8 V to 2 rpm. The variation of the speed is verified by measuring the frequency at a tachometer. The motor is connected to a VLP-1303 PRO Voltcraft power supply (Hirschau, Germany) (see Fig. 2). A drill chuck in which all kinds of cuvettes with diameters between 5 mm and 20 mm can be fixed and centred in the middle of the rotational axis is attached to the motor (see Fig. 3). Currently, the machine runs with a cylindrical 5 cm long glass cuvette with 2 mm radius. Apart from the measuring window, the cuvette is coloured white inside, for better refraction, and black at the outside in order to reduce stray light inside the machine. For the same purpose, the clinostat is assembled in a black anodized aluminium box. The walls and the lid of the box are constructed as overlapping segments which again reduces stray light. The cuvette and the PMT are centred in the middle of the rotation axis. The distance between cuvette and measuring window can be varied by moving the whole motor with attached cuvette back and forth with respect to the motor block (Fig. 3). Additionally, the position of the PMT can be changed via a sledge on which the PMT is mounted.

Table 1 Technical data of the PMT clinostat

Technical data	
Working temperature	4–70°
Relative humidity	20–95%
Sensitivity (PMT)	350–600 nm
Rotation speed	0–100 rpm
Flexible and stepless change of rotation speed	
Sample volume	Cuvettes of 1 ml volume
Size	370 × 100 × 100 mm (with lid)
Input voltage	0–15 V
Input frequency	47–63 Hz

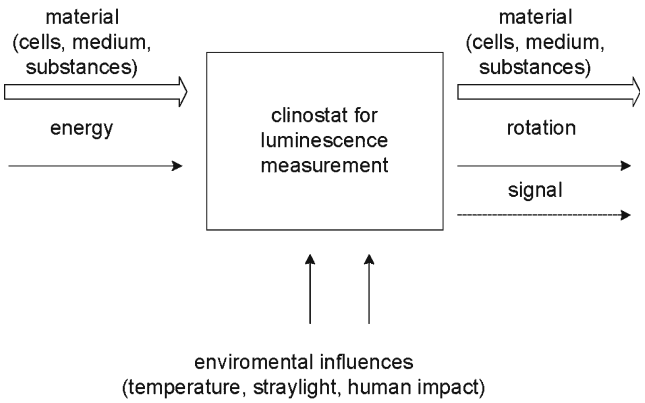

Fig. 1 Black-box model of the described machine: cells, media and activating substances will be put in, and if desired, rotated. The output luminescence signal will be measured. Environmental influences have to be hold as small as possible

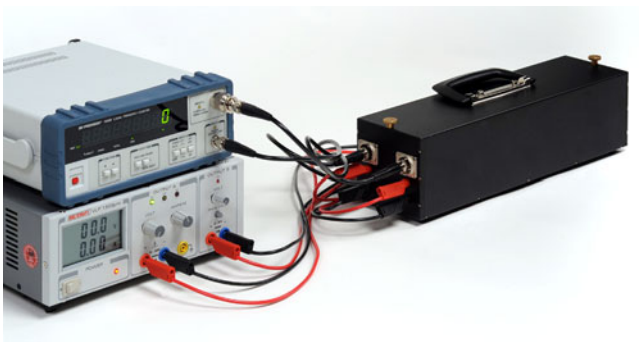

Fig. 2 Configuration of clinostat (*right*) power supply (*bottom left*) and frequency counter (*top left*)

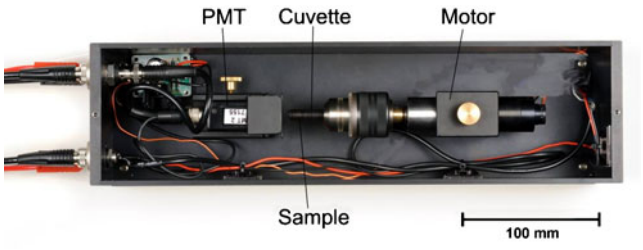

Fig. 3 Clinostat with a PMT, containing the photomultiplier tube, cuvette to house the samples, drill chuck to fix the samples and a motor for rotation. The motor can be moved to vary the distance of the sample cuvette and the sensor

Rotation always generates a centrifugal force (Fig. 4) which leads to residual acceleration inside the cuvette. This acceleration reaches its maximum at the largest radius at the border of the cuvette (Klaus et al. 1998). The acceleration can be calculated, depending on the rotational speed, with the help of Formula 1.

$$a = \varpi^2 \cdot r = (2\pi f)^2 \cdot r = \left(\frac{2\pi}{T}\right)^2 \cdot r$$
$$g = 9.81 \cdot a \qquad (1)$$

with a = acceleration [m/s^2], f = frequency of rotation [Hz], T = period [s], r = radius [m], ω = rotational speed [rpm]

The resulting force with different rotational speeds [rpm] for the chosen cuvette is shown in Fig. 4. At 60 rpm and a radius of 2 mm there is a maximal residual force of 8×10^{-3} *g* at the border of the cuvette, while at 2 rpm the residual force is only 9×10^{-6} *g*. Figure 4 also shows the time it takes, for different rotation speeds, to achieve the averaging of the *g*-vector. If this time is longer than the reaction time of the investigated biological systems, it will be permanently stressed due to omnilateral gravi/mechano-stimulation. If the reaction time is lower than the presentation time of the *g*-stimulus, the organism can not sense the gravitational force (rotating *g*-vector) any more. At 60 rpm averaging occurs in half a second, at 2 rpm this takes 15 s, which is supposed to be significantly longer than the reaction time of the organism (less than 2 s, own parabolic flight experiments, data not published jet).

The PMT clinostat can be installed into an incubator, thus allowing experiments under controlled temperature conditions.

Handling of Biological Samples

The mammal (*Rattus norvegicus*) macrophage cell line NR8383 was chosen as biological reference system for testing the suitability of our constructed device. By activating the cells with opsonised Zymosan, macrophages respond with a typical oxidative burst reaction meaning that they produce certain species of reactive oxygen (ROS). ROS can be detected by adding Luminol and horse radish peroxidise as a catalyst (Huber 2007 modified after Allen 1986). Luminol, in the presence of ROS, undergoes a conformation change during which energy is transferred in form of blue light. Given a constant number of reacting cells, the amount of light indicates the strength of the cellular reaction to a changed experimental condition.

The cells were cultivated in culture medium (37°C, 5% CO_2) (Huber 2007). Before each test, cells were harvested and their concentration/ml was adjusted to 7×10^5/ml. 560 µl of this stock was used for each

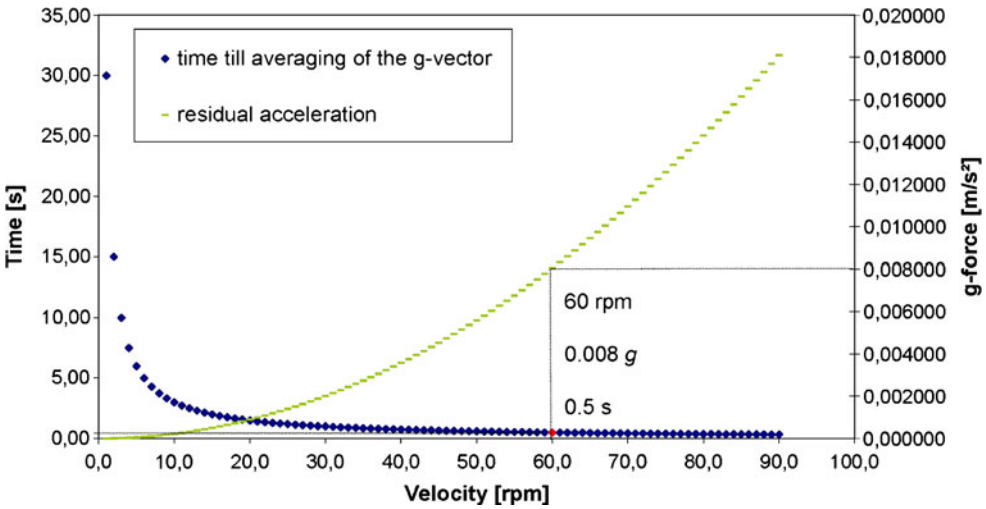

Fig. 4 Dependence between rotation speed, *g* force and the time for changing the direction of the *g*-vector in a rotating clinostat. If the reaction time of the organism is lower than the averaging time, the organism can sense the change of the direction of the *g*-vector and is permanently stressed (*green* residual acceleration; *blue* averaging time, meaning the time the organism has available to detect the change of the *g*-vector)

experiment. Cell medium was mixed with Luminol (165 µl) (Sigma), peroxidase (33 µl) (Merck) and Zymosan (230 µl) (Sigma) and injected into the cuvette. Immediately, the reading of the PMT was started, while the motor of the clinostat was turned on to the desired speed. For the 1 *g*—control, the same measurement was conducted, however without starting the clinostat. Additionally the reaction of non-activated cells, where PBS (buffer solution) instead of Zymosan was used, was compared to the response of cells activated with Zymosan. Each measurement was running for 50 min to 1 h. The signal height of samples with different cell concentrations was compared. Additionally, the signal height with the same cell concentration and different rotation speeds of the clinostat was analyzed.

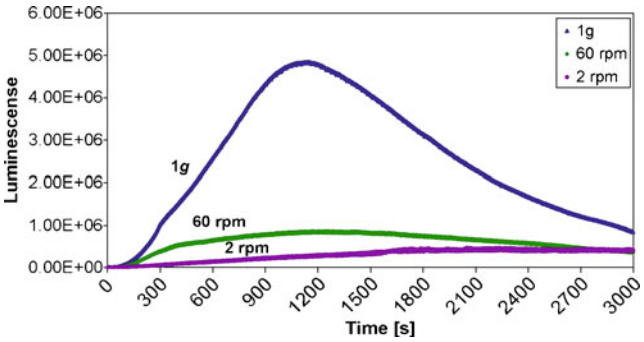

Fig. 6 Impact of different clinostat speeds (0 rpm (1 *g*), 60 rpm and 2 rpm) on the oxidative burst reaction of cell line NR8383 (cell concentration (7×10^5/ml), measured in the PMT clinostat. The cellular reaction at 60 rpm is drastically reduced compared to the reaction at 1 *g*. At 2 rpm the signal is again reduced to such an amount that only a very low reaction is recognizable

Results

1 g Experiment Parameters

The validity of our PMT clinostat device was tested by using the chemiluminescence of the Luminol–H_2O_2–horseradish peroxidase system, an established method to quantify the oxidative burst reaction of mammal macrophages. Figure 5 shows a typical oxidative burst reaction under 1 *g* conditions, in which cells were activated with Zymosan at t = 0. The luminescence signal achieves a peak after 15–20 min corresponding to the maximum activity of the cells in this experiment. Within 1 h the luminescent signal decreases back to under the noise level in the order of 100 signals/min.

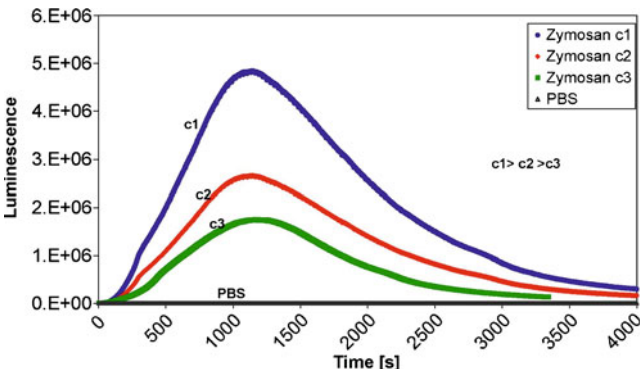

Fig. 5 Typical oxidative burst reaction of macrophage cell line NR8383 under 1 g conditions. Comparison of **a** different cell concentrations (Zymosan activated: c1 = 7×10^5/ml; c2 = 4×10^5/ml, c2 = 3×10^5/ml) and **b** activated versus non activated cells (PBS, c = 7×10^5/ml). Non activated cells show no reaction. As expected a higher cell concentration leads to a higher signal due to the fact that more cells produce a higher amount of ROS. One characteristic curve for demonstration of the effect is shown

As expected, different concentrations of activated cells lead to a different signal height as a higher number of cells produce a stronger signal. Non activated cells, to which PBS instead of Zymosan was added, show no response (see bottom line, PBS).

Clinostat Speed

In order to test whether fast or slow rotation of the clinostat has a direct influence on the result, we varied the speed of clinorotation. Figure 6 shows characteristical luminescence reactions at different rotation speeds. It is obvious that clinorotation in general dramatically reduces the amount of produced ROS in Zymosan-activated cells. The maximum of the luminal reaction at 1 *g* is more than 5 times higher than at 60 rpm. Furthermore, it is demonstrated that the speed of clinorotation has a clear impact on the signal response. Cells rotated with 2 rpm show an even lesser signal and thus a further decrease of ROS production than cells at 60 rpm. Control measurements at 60 rpm, 2 rpm and 0 rpm (1 *g*) with non activated (PBS instead of Zymosan) cells showed no reaction.

Discussion

The signal detection of the constructed PMT clinostat was very sensitive, meaning the detected signal maximum (between 1×10^5 and 5×10^6 signals/s) was far above the noise level (1×10^2 signal/s). The signal could be read out every second. This allows very fast monitoring of signal changes and therefore enables a detailed kinetic visualisation of the luminescence signal, which

is an indicator of ROS production and thus activation status of the macrophages (Huber 2007)

The biological verification also led to adequate results. By choosing different cell concentrations the detected signals differed as expected—meaning a higher signal peak was reached with a higher cell concentration at otherwise same experimental conditions. Consequently, the cell concentration has to be carefully adjusted before start of the experiment.

Changing the rotation speed of the clinostat proofed that the rotation mode has a strong impact on the response of the test system. Clinorotation resulted in a dramatic decrease of the cell signal. This could be an indication of a decrease of the immune response of the investigated macrophages, supporting previous data (Huber 2007), also see review by Ullrich et al. (2008). Similar experiments in real microgravity, e.g. during parabolic flights, are currently under investigation and will finally prove the suitability of this chosen simulation approach. Furthermore, speed of rotation is of high importance as it has an obvious effect on the signal strength and thus the radical production of the macrophages. Cells rotated with 2 rpm responded with only half of the signal strength compared to cells rotated at 60 rpm. This gives clear evidence that the clinostat parameters (diameter of the sample, speed of rotation) have to be considered and defined carefully. Figure 4 shows that at a rotation with 60 rpm every 0.5 s the direction of the *g*-vector is inverted. At 2 rpm this inversion takes 30 s. Therefore, the cells have a significantly longer time, in which they can sense the change of the *g*-vector. This might result in an omnilateral stressor if the cells are rotated too slowly, caused by the permanent change of the direction of the gravity force.

The obtained results must be critically discussed and corresponding control experiments included. What is achieved—omnilateral gravistimulation (as it was also shown in highly specialized plant statocytes on a slow rotating clinostat of 1–2 rpm (Hensel and Sievers 1980)) or functional weightlessness? In any case, simulation demands for final verification experiments in real microgravity.

Our newly constructed device allows the investigation of further questions. As the clinostat can be stopped and restarted within seconds, the organisms' reaction to abrupt (or slow) changes of gravity can be studied. Therefore, acceleration profiles, like the ones during parabolic flights can be investigated.

Evaluation of the PMT Clinostat

The new device allows online kinetic measurements with a high time resolution and very sensitive signal detection. Future developments might be parallel measurements, for example with a moving PMT, and placement within a heating box to guarantee a transportable controlled temperature environment. This approach would allow biological measurements at almost every place, for example on a centrifuge for studying the reactions to hypergravity, or within a rocket or airplane. In addition to the applied luminol assay our new PMT clinostat can be used for further kinds of luminescence analyses within the detection range of the PMT. By integrating an emission source, e.g. a fluorescent lamp, it might even be possible to perform fluorescence measurements.

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