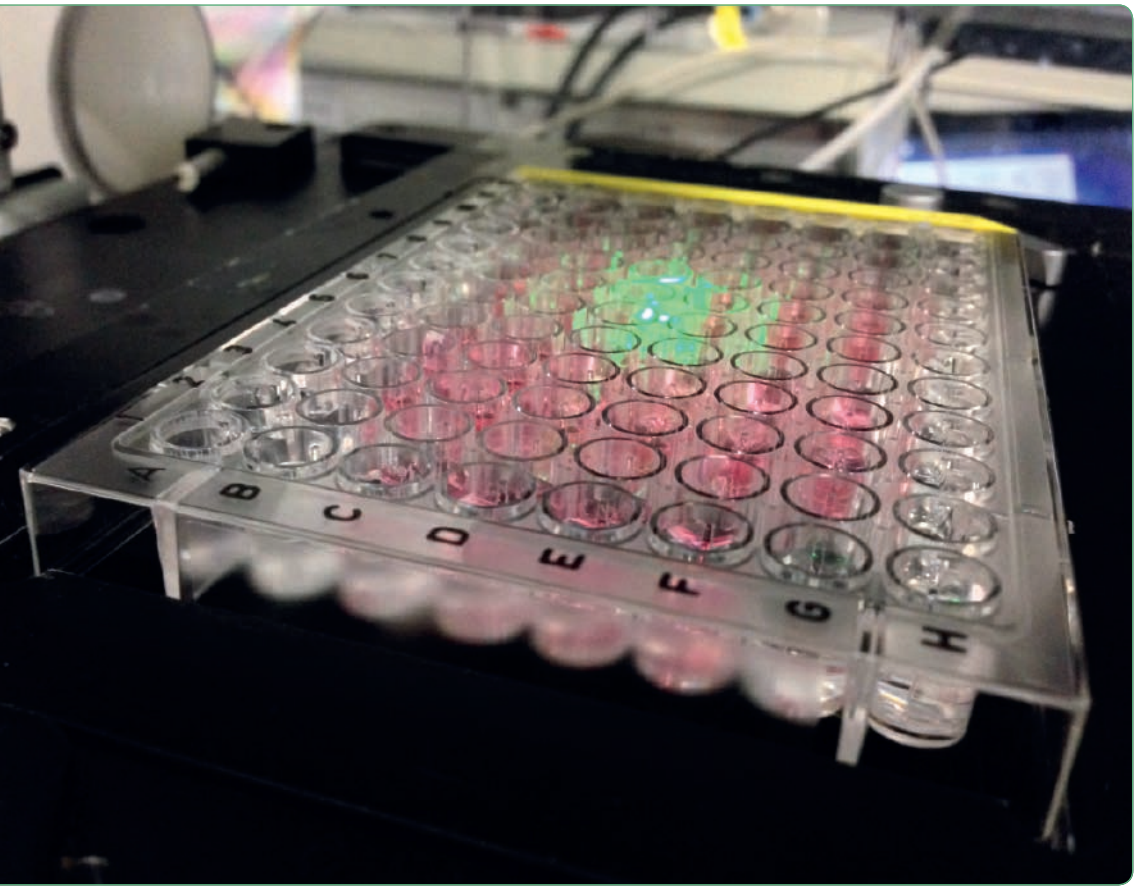
Intracellular O_2 Measurements

Fluorescence Microscopy with Nanosensors

The combination of a soluble cell-penetrating oxygen sensor and an optoelectronic accessory unit for standard fluorescence microscopes allows for a simple and robust determination of the intracellular oxygen content in cell samples. An advantage of the system is its ability to overcome interfering background signals. The system evaluates the phosphorescence decay time even from weak and interfered signals and relates them to the oxygen concentration. For demonstration, cells were treated with carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP) and antimycin A and the corresponding changes in the intracellular oxygen content were recorded.

The supply of molecular oxygen (O_2) is crucial for cell growth and differentiation [1]. Abnormal O_2 content induces and indicates dysfunctions. Energy-consuming biochemical processes can be monitored by measuring changes of the O_2 content [2].

Therefore, there is substantial interest in simple probes and methods to measure intracellular O_2. Optical oxygen measurements using cell-penetrating probes, the phosphorescence of which is quenched, have been described previously [3]. With increasing O_2 content in the probe microenvironment, the phosphorescence decay time and the intensity of the signal decrease. The corresponding O_2 concentration is evaluated using a calibration curve. For practical purposes, it is favorable to use the decay time as a measurement value. In contrast to the intensity, the decay time does not depend on the probe concentration and the excitation intensity. Furthermore, it is far less interfered by sample absorption, scattering, background fluorescence, and by photo bleaching of the probe.



A major challenge was the introduction of such O_2 probes into cells without causing too much harm or stress. This problem has been overcome by the recently developed self-loading probes.

These nanoparticle sensors are taken up by cells by means of phagocytosis. A detailed characterization is given in [4]. These nanoparticles are commercially available (MitoImage Probe $NanO_2$, Luxcel Biosciences, Ireland). They were primarily intended for fluorescence lifetime imaging (FLIM) or in combination with time-resolved fluorescence plate readers. However, microsecond FLIM microscopes are expensive and rare, while plate readers only give average information for the entire sample. These facts have impeded a widespread application of such nanosensors until now. A more reasonable setup for the use of the $NanO_2$ sensor is a standard fluorescence microscope in combination with an optoelectronic accessory unit to measure phosphorescence decay times. The used system was originally developed for spherical O_2 microprobes doped with a phosphorescent dye. It applies a dedicated two-frequency phase modulation technique for the separation of specific phosphorescence signals from interfering background fluorescence arising from the sample [2]. Especially for nanoprobes with naturally low signal amplitudes this feature is crucial. To prove that this setup is suitable for the use with the $NanO_2$ sensor, the respiratory response of the cells to uncoupling and inhibiting stimuli were examined and compared with published plate reader data [4].

Material and Methods

An Olympus IX81 inverted fluorescence microscope with a climate control chamber (ACU02, Evotec,

Germany) was equipped with a 531/40, 555, 635 nm filter set. Green light is preferable for excitation, since violet or blue light induces photochemical side reactions in culture media, likely induced by riboflavin [5].

The oxygen measurement system ("OPAL", Colibri Photonics, Germany) consists of a detector, a green LED as excitation source and a control electronic module linked to a computer. The detector unit comprises a photomultiplier and a confocal tube to mask signals that arise out of the focal plane. The detector was mounted on the microscope's camera port and the LED source was connected with the light guide of the microscope fluorescence lamp.

Murine myeloma cells (P3 x 63Ag8.653, ATCC CRL-1580) were cultured in RPMI 1640 medium without phenol red (Biochrome, Berlin, Germany) supplemented with 10% FCS, 2 mM glutamine, 50 µM mercaptoethanol in a standard flat bottom 96 well plate [6]. For O_2 measurement, cells were loaded by adding the $NanO_2$ sensor to the medium (5 µg/ml) and incubating for 24 h in a CO_2 incubator (37 °C, 5% v/v CO2, 95% relative humidity). Prior to the measurement cells were washed once with fresh medium.

Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP) and antimycin A were prepared as 10 mM stock solutions in dimethyl sulfoxide (DMSO) and then diluted with PBS (phosphate buffered saline) 1:999 and 1:99, respectively. The addition of 20 µl of these solutions to 200 µl culture medium resulted in concentrations of approximately 1 µM FCCP and 10 µM antimycin A in a well.

The climate chamber was adjusted to 37 °C, 5% v/v CO_2 and saturated humidity. The solutions of FCCP and antimycin A were also preheated to 37 °C. To perform an O_2 measurement experiment, light was focused on the cell layer within a well of the microtiter plate using a 20x objective and dimmed transmitted light. Then, the transmitted light was switched off and the emission light path was switched to the camera port with the detector module. Switching on the LED source started the measurement. For calibration, two wells were used: One in which 0% oxygen content was maintained by the addition of a small amount of sodium sulfite which consumes all dissolved O_2. A decay time value of 49.2 µs was measured in this well. The second well was used to determine the lifetime at 100% of air saturation (21% v/v O_2 calibration point). At 37 °C and 1013 mbar air pressure, 100% of air saturation corresponds to 210 µM O_2 [7]. From this well culture medium was gently removed, and the well with cells attached to the bottom was dried for several minutes resulting in complete cell death. Then, 100 µl of preheated air-saturated water were added to the well and the resulting decay time of 23.0 µs was taken as the value for 100% air saturation. For the actual measurement, a further well with cells was used. To measure the initial (basal) oxygen concentration, the LED excitation source was switched on for approximately 7 s, the decay time recorded (at a frequency of 1 Hz), and the mean value calculated as one data point. After that FCCP was added and measurement points were recorded at 4 - 5 min intervals. After 10 min antimycin was added. The curve resulting from this protocol is shown in Figure 1.

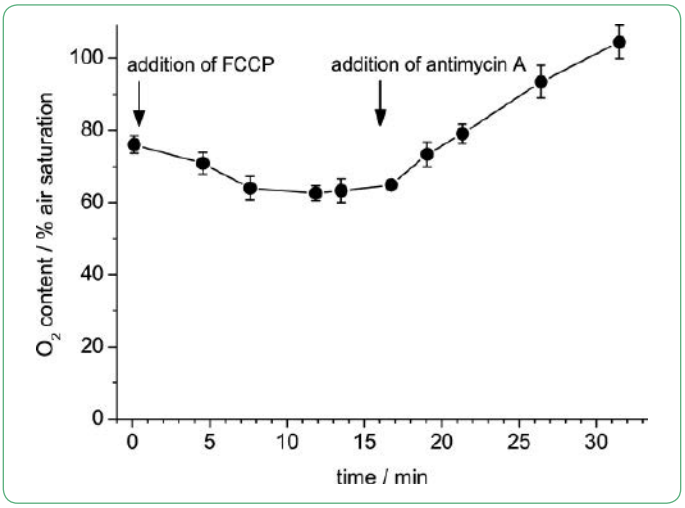


Fig. 1 Changes in intracellular oxygen content upon addition of FCCP (1 µM) and antimycin A (10 µM). 100% air saturation corresponds to 210 µM O_2 at 37 °C. The error bars correspond to approximately seven single measurements (1 s each) per data point

Results and Discussion

Four minutes after the addition of FCCP to the resting cells, cellular O_2 levels dropped from 76 to 71% of air saturation. After 12 min from the start a minimal O_2 concentration of 63% was reached. This change reflects FCCP uncoupling of the respiratory chain leading to increased oxygen consumption and cell deoxygenation compared to the basal state (resting cells). 3 min after the addition of antimycin A, the O_2 concentration increased to 73% and 12 min. later, it reached 100% of air saturation. This is because antimycin A effectively blocked the respiration and selfdeoxygenation of the cells. The results match those obtained with $NanO_2$ probes on a fluorescence reader. This clearly shows that the used setup is very capable of detecting signals from cells stained with sensors and converting them into O_2 concentration. To prevent photodamage of the cells it was important to switch off the excitation between the measurements. Otherwise, when monitored under continuous illumination the cells loaded with the $NanO_2$ sensor suffer significant stress, seen as pronounced swelling, lack of self-deoxygenation and characteristic responses to metabolic stimuli such as FCCP (data not shown).

Summary

For the measurement of the intracellular O_2 content cells were incubated with self-loading phosphorescent probes. Using a fluorescence microscope with an accessory unit for the determination of phosphorescence decay times, O_2 content in live respiring cells and dynamic changes induced by stimulation was quantified.

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