Mitochondrial oxidant generation follows oxygen deprivation and re-oxygenation

Daniel Pastor-Flores and Tobias Dick
German Cancer Research Center (DKFZ), Division of Redoxregulation (A160)

- **Measure fluorescent yeast conlonies on agar**
- **Investigate normoxic and hypoxic conditions using the CLARIOstar® plate reader with atmospheric control unit (ACU)**
- **Measure multiple parameters employing the flexibility of the LVF-monochromator™**

Introduction

Yeast is a popular model organism because it is easy to genetically modify, it is robust to differing environments and it is a eukaryote. Yeast can be studied under aerobic conditions when plated on agar plates. The Singer Instruments ROTOR device pins colonies of yeast, fungi or bacteria onto agar plates in 96, 384 or 1536 plate format and enables to study colonies in high-throughput. Here, different yeast clones were pinned onto a plate resulting in 384 measure points to determine the organism's response to varying oxygen concentrations. The CLARIOstar microplate reader detected differences in yeast autofluorescence, citrate synthase 2 expression reported by mCherry and a redox-sensitive roGFP2-based probe.

Assay Principle

Yeast clones expressing mito-roGFP2-Tsa2ΔC$_R$, a mitochondrial redox-sensitive sensor[1], as well as non-modified yeast was pinned onto agar plates by the ROTOR (Singer Instruments) resembling the layout of a 384 well plate. The roGFP2 probe changes its excitation spectrum in response to H_2O_2: the oxidized probe is excitable at 405 nm and the reduced form at 488 nm. The ratio of oxidized and reduced signal is used to report on the oxidation state of the probe. In addition to the mitochondrial redox probe, yeast cells expressed a citrate synthase 2 (Cit2) fusion protein with mCherry. Cit2 is upregulated upon activation of the retrograde pathway, a common marker of mitochondrial dysfunction. In order to correct for the growth of yeast colonies, autofluorescence of yeast NAD(P)H was acquired.

Materials & Methods

- Yeast cells (strain BY4742) with with genomically integrated roGFP2-Tsa2ΔC$_R$ and mCherry-fussion tag in C-terminal of endogenous Cit2
- Synthetic-defined-Agar in empty plates, pinned with yeast colonies (ROTOR robot to with a 96-Pad, Singer Instruments)
- CLARIOstar microplate reader, BMG LABTECH

Experimental procedure
Yeast cells were grown at 30°C for 24 hours after pinning and then exposed to changing ambient concentrations of oxygen as displayed in Fig. 1. The experiment was performed with 24 replicates. Agar only served as blank and non-modified yeast colonies as negative control.

Instrument settings

Top read, number of flashes:	40
Focal height:	7.0
Settling time:	0.1
roGFP2 (measured with filters)	
Excitation 1 (oxidized roGFP2):	400-10
Excitation 2 (reduced roGFP2):	482-16
Dichroic:	LP504
Emission:	520-10
Gains:	691 (Ex 1); 504 (Ex 2)
NAD(P)H autofluorescence (LVF monochromator)	
Excitation:	340-15
Dichroic:	auto 393.8
Emission:	460-20
Gain:	1376
Cit2-mCherry (LVF monochromator)	
Excitation:	570-15
Dichroic auto:	593.8
Emission:	620-20
Gain:	2780

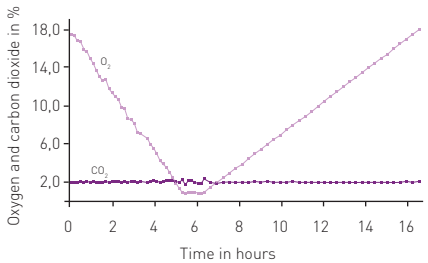

Fig. 1: Oxygen and carbon dioxide levels in the course of the experiment.

Results & Discussion

Yeast was exposed to varying levels of oxygen (Fig. 1) and the redox state of mitochondrial redox probe was investigated. During the initial period of oxygen decrease (0 –1.2 h) from 18 % down to 12 %, the redox state of mitochondrial H_2O_2 probe was not influenced by the decreasing oxygen[2]. This might be explained by a favored oxygen supply to mitochondria over the cytosol in case of fluctuating oxygen concentrations. At lower oxygen saturation, the amount of the reduced probe increased as reported by lower roGFP2 ratios. While the oxygen is kept at 1 % (5-6.5 h), the probe persists in its reduced form and gets oxidized only in the phase of re-oxygenation. The immediate probe oxidation as soon as more oxygen is available further points to the high priority of mitochondrial oxygen supply.

Rev. 08/2017
Keywords: **ACU, Autofluorescence, Multiplexing, Redox processes, roGFP, Yeast**

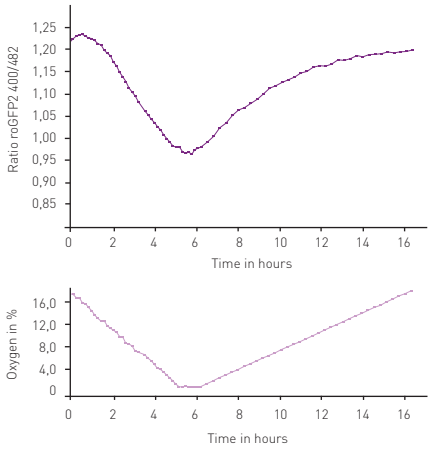

Fig. 2: The influence of oxygen availability on the redox state of a mitochondrial peroxiredoxin-based probe. Oxygen pressure modulates the redox state of a genomic-integrated Mito-roGFP2-Tsa2ΔC$_R$ probe. roGFP2 oxidation is represented by 400nm/480nm ratio and indicative of H_2O_2 generation.

A pathway that is induced upon inhibition of mitochondrial respiration is the retrograde pathway. It reports mitochondrial failure to the nucleus. One protein synthesized as a result of pathway activation is Cit2.
In the course of oxygen deprivation, Cit2 expression is slightly reduced whereas it remarkably increases during reperfusion (Fig. 3). Whether the Cit2 increase is due to reperfusion or if it is a delayed response to hypoxic conditions, remains to be elucidated.

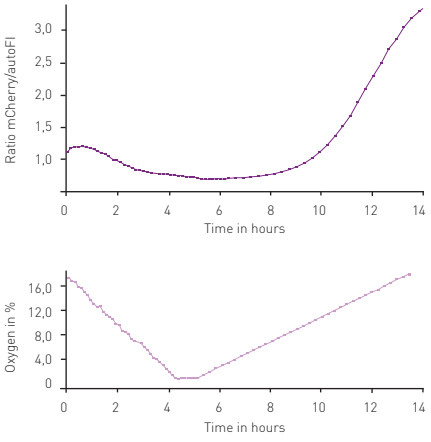

Fig. 3: Influence of oxygen availability on Cit2 expression as reported by CIT2-mCherry fusion protein and corrected for yeast growth by NAD(P)H autofluorescence.

Conclusion

The pinning of yeast or bacteria onto agar plates in a standard SBS format allows studying colonies in high throughput format. Pinned colonies can be analyzed using fluorescent markers that are read at a microplate reader such as the CLARIOstar. The device is designed to meet both needs: flexibility and sensitivity. Flexibility is achieved by the LVF monochromator allowing for the choice of any wavelength and bandwidth. The extraordinary sensitivity of the system can be enhanced by the use of filters. This way the output of the experiment can be maximized as demonstrated here: redox state, H_2O_2 levels and NAD(P)H autofluorescence.

References

1. Bruce Morgan, et al. (2016) Real-time monitoring of basal H_2O_2 levels with peroxiredoxin-based probes *Nature Chemical Biology* **12**, 437–443 (2016) doi:10.1038/nchembio.2067
2. Pastor-Flores D., Dick T. (2017) Monitoring yeast mitochondria with peroxiredoxin based redox probes – The Influence of oxygen and glucose availability. *Interface Focus*, DOI:10.1038/nchembio.2067