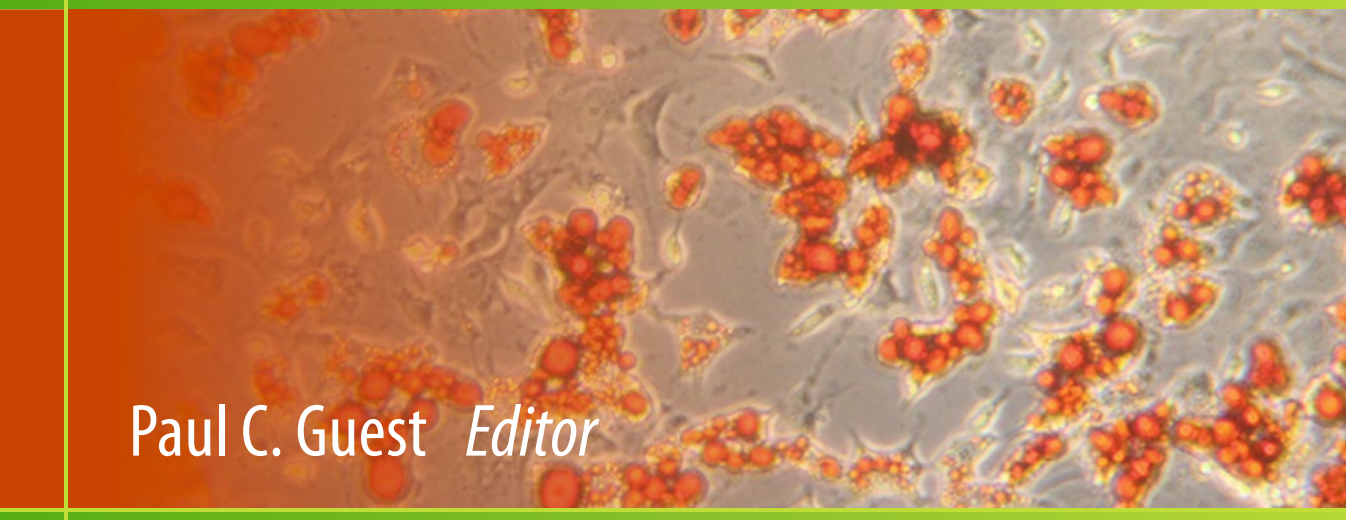
Methods in Molecular Biology 2138

Springer Protocols



Paul C. Guest *Editor*

Clinical and Preclinical Models for Maximizing Healthspan

Methods and Protocols

Humana Press

Chapter 16

Assays for Monitoring the Effects of Nicotinamide Supplementation on Mitochondrial Activity in *Saccharomyces cerevisiae*

Ivan Orlandi and Marina Vai

Abstract

The single-celled yeast *Saccharomyces cerevisiae* is one of the most valuable laboratory models that has been used successfully to identify factors and pathways involved in several cellular processes, the counterparts of which are evolutionarily conserved. Furthermore, it is also a powerful tool for analyzing the effects of molecules of nutraceutical interest with the view of leading to human health benefits and improving the quality of aging. In this context, we present some of the methods that have allowed us to assess the beneficial influence of a form of vitamin B3, namely nicotinamide, on mitochondrial functionality during yeast chronological aging. Mitochondrial dysfunctions are considered to be hallmarks of aging, and of several metabolic and neurodegenerative diseases. More specifically, these methods concern the determination of the respiratory parameters in intact cells in order to evaluate the efficiency of mitochondrial respiration in concert with the risk of superoxide anion (O_2^-) production, which results from inefficient respiration. In addition, we describe fluorescent staining specific for O_2^- detection and mitochondrial membrane potential, as well as a simple clonogenic assay based on the ability of cells to grow on a carbon source that requires a functional mitochondrial metabolism.

Key words *Saccharomyces cerevisiae*, Nicotinamide, Chronological aging, Mitochondrial respiration, O_2^-, Fluorescence microscopy

1 Introduction

Nicotinamide (NAM), the water-soluble amide form of vitamin B3, is a key component of the metabolic pathways required for the production of nicotinamide adenine dinucleotide (NAD^+) [1]. NAD^+ is a coenzyme involved in all essential anabolic, catabolic, and bioenergetics pathways as well as an obligate co-substrate for sirtuins. The sirtuins are a family of evolutionarily conserved NAD^+-dependent deacetylases that consume NAD^+ and generate NAM in carrying out their enzymatic activity [2]. Sirtuin functions influence metabolic health and longevity both in humans and in

Paul C. Guest (ed.), *Clinical and Preclinical Models for Maximizing Healthspan: Methods and Protocols*, Methods in Molecular Biology, vol. 2138, https://doi.org/10.1007/978-1-0716-0471-7_16,

yeast [3]. Consequently, the possibility of modulating NAD^+ bioavailability through vitamin B3 supplementation has become of great interest in order to produce beneficial outcomes for human health and aging. We investigated the effects of NAM supplementation in the context of yeast chronological aging [4], the established model for simulating aging of post-mitotic quiescent mammalian cells [5]. We found that NAM enables cells to acquire metabolic features that favor a better long-term survival by preserving functional mitochondria [4]. It is well known that maintenance of the quality and functionality of mitochondria ensures an enhanced longevity and prevents the appearance and development of several diseases. Thus, methods that accurately estimate the functional state of mitochondria are in the center of procedures required to assess the effectiveness of treatments with lifespan or healthspan-extending effects.

Here, we describe some protocols for analyzing mitochondrial functionality in yeast at different levels. These protocols include methods that allow measurement of respiratory parameters (basal respiration, non-phosphorylating respiration, maximal respiratory capacity and respiratory efficiency) in intact cells and to determine the respiratory competence index [6] and to evaluate in vivo the mitochondrial membrane potential and the presence of O_2^-, by fluorescent staining.

2 Materials

2.1 Yeast Strains and Growth Conditions

1. Experiments can be performed using the wild type yeast strain W303-1A (*MATa ade2-1 his3-11,15 leu2-3112 trp1-1 ura3-1 can1-100*) (*see* **Note 1**).
2. Minimal medium: Yeast nitrogen base without amino acids, containing 2% w/v glucose (*see* **Note 2**).
3. Rich medium agar plates: 2% w/v bacto peptone, 1% w/v yeast extract and 2% w/v agar, containing either 2% w/v glucose (YEPD) or 3% w/v glycerol (YEPG) as the carbon source.

2.2 Reagents

1. ISOTON® II diluent (Beckman Coulter).
2. Phosphate-buffered saline (PBS) (pH 7).
3. 500 mM nicotinamide (NAM) stock solution in sterile water.
4. Triethyltin bromide (TET).
5. Carbonyl cyanide 3-chlorophenylhydrazone (CCCP).
6. Antimycin A.
7. 17.5 mM 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6$) stock solution in ethanol (Molecular Probes, Invitrogen).
8. 5 mg/mL dihydroethidium (DHE) stock solution.

9. Acid-washed 0.45 μm glass beads (Sigma-Aldrich).
10. Complete™, EDTA-free Protease Inhibitor Cocktail (Roche).
11. Phenylmethanesulfonyl fluoride (PMSF).
12. Cell resuspension buffer: 10 mM HEPES (pH 7.4) containing 5% glucose.

2.3 Assays

1. K-HKGLU Glucose Assay Kit (Megazyme, Southern Cross Rd, Irishtown, Bray, Co. Wicklow, A98 YV29, Ireland).
2. Pierce™ BCA Protein Assay Kit (ThermoFisher Scientific Frankfurter Strasse, 64293 Darmstadt, Germany).

2.4 Equipment

1. Shaking water bath.
2. Sonicator.
3. Z2 Coulter Counter-Particle Count and Size Analyzer.
4. UV-visible spectrophotometer.
5. "Clark-type" oxygen electrode in a thermostatically controlled chamber (Oxygraph System).
6. Oxygraph Plus software.
7. Eclipse E600 fluorescence microscope equipped with a Digital Sight DS Qi1 camera (Nikon).

3 Methods

3.1 NAM Supplementation to S. cerevisiae Cells at the Diauxic Shift

1. Grow yeast cells in 200–250 mL minimal medium at 30 °C in the shaking water bath.
2. Monitor cell growth over time by collecting aliquots of the culture and by counting cell number using the Coulter counter (*see* **Note 3**).
3. Plot cell number against time on a semilog scale to define the growth profile (Fig. 1).
4. In parallel, measure the extracellular glucose levels by collecting 1 mL aliquots of the culture.
5. Centrifuge at 12,000 × *g* for 5 min at room temperature (RT) to obtain cell-free medium samples (*see* **Note 4**).
6. To define the time of extracellular glucose exhaustion when the diauxic shift occurs (the time-point of NAM supplementation), measure the concentration of glucose in the cell-free medium according to the steps below.
7. Add 100 μL of cell-free medium into a cuvette containing 2 mL deionized water and 100 μL of solution 1 (provided in the K-HKGLU Assay Kit).

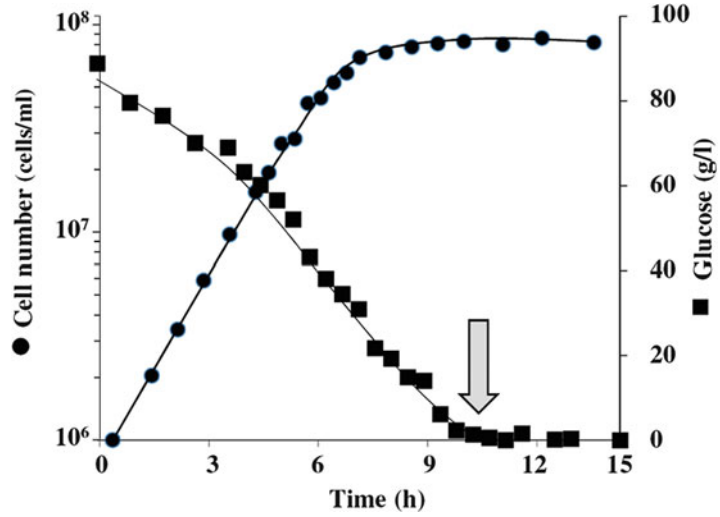


Fig. 1 Growth curve of W303-1A cells on minimal medium/2% glucose determined by counting cell number over time. In parallel, extracellular concentration of glucose measured in medium cell-free samples collected at different time-points is shown. One representative experiment is shown. The arrow indicates the time-point of NAM supplementation

8. In parallel prepare a blank sample with 2.1 mL deionized water and 100 μL solution 1.
9. Add 100 μL solution 2 containing $NADP^+$/ATP (provided in the kit).
10. Mix and, after 5 min, read the absorbance (A1) in the spectrophotometer at 340 nm.
11. Add 20 μL suspension 3 containing hexokinase plus glucose-6-phosphate dehydrogenase (provided in the kit) to start the reactions.
12. Mix and read the absorbance of the samples at 340 nm after 5 min, corresponding to the end of the reaction (A2).
13. Determine A2 − A1 and subtract the absorbance difference of the blank from that of the sample, obtaining ΔAD-glucose.
14. The value of glucose concentration will be 0.6634 × ΔAD-glucose (g/L).
15. Plot the glucose concentration against time (Fig. 1).
16. When glucose is no longer detectable, split the culture and add NAM at a final concentration of 5 mM from the stock solution, maintaining sterile conditions.

3.2 Determination of Respiratory Parameters of Intact Cells

1. Starting from the time-point of NAM addition (diauxic shift), collect culture samples every 2–3 days from NAM-treated and untreated cultures, and prepare 2 mL cell suspension at a concentration of 5×10^6/mL (*see* **Note 5**).
2. Transfer the cell suspension into the Oxygraph chamber set at 30 °C.
3. Measure the oxygen consumption (routine respiration; J_R) by recording data at sampling intervals of 1 s using the Oxygraph Plus software and acquire data for 10 min.
4. After J_R determination, assess the non-phosphorylating respiration (J_{TET}) due to proton leakage by stopping the measurement and adding the FoF1-ATPase inhibitor (TET) at 37.5 mM final concentration into the Oxygraph chamber and restart measurements.
5. Acquire oxygen values for 10 min (*see* **Note 6**).
6. To determine the maximal/uncoupled respiratory capacity (J_{MAX}), stop the measurement and add the uncoupler CCCP at 10 μM final concentration in 100 mM ethanol to provide a non-limiting substrate for respiration.
7. Use the values of J_R, J_{TET} and J_{MAX} to determine other respiratory parameters (*see* **Note 7**).
8. Finally, estimate the non-mitochondrial oxygen consumption by adding 2 M antimycin A.
9. Determine respiratory rates from the slope of a plot of O_2 concentration against time, divided by the cellular concentration or the parameters can be expressed per mg of cellular protein.
10. If respiratory rates are expressed relative to protein concentration, recover the cells from the Oxygraph chamber and collect by centrifugation for 5 min at 12,000 × *g* at RT.
11. Discard the supernatant and resuspend the pellet in 100 μL PBS, containing 1 mM PMSF and complete EDTA-free protease inhibitors.
12. Add 100 μL glass beads and break the cells by shaking on a vortex mixer for 5 cycles of 1 min interspersed with cooling for 1 min on ice.
13. Centrifuge 5 min at 12,000 × *g* at RT and transfer the supernatant to a new tube.
14. Determine the protein concentration with the BCA Protein Assay Kit, or a similar kit, according to the manufacturer's instructions.

3.3 Determination of the Index of Respiratory Competence (IRC)

1. To determine the IRC over time [6], collect 1 mL samples of treated and untreated cultures every 2–3 days starting from the time of NAM addition.
2. Determine the cell concentration using the Coulter counter.
3. Prepare diluted cellular suspensions of the cultures in sterilized deionized water at a final concentration of 10^4 cells/mL.
4. Plate identical 100 µL samples of cellular suspensions on YEPD and YEPG plates.
5. Incubate at 30 °C until complete growth of the colonies (generally 3–4 days).
6. Calculate the % IRC for each time-point as colonies on YEPG divided by colonies on YEPD multiplied by 100.
7. Plot IRC values against days.

3.4 Fluorescence Microscopy to Assess Mitochondrial Membrane Potential and Morphology

1. To analyze mitochondrial membrane potential and morphology at designed time-points [7], harvest culture samples corresponding to 10^6 cells by centrifugation for 5 min at 12,000 × *g* at RT (*see* **Note 8**).
2. Remove the medium and resuspend the cells in cell resuspension buffer.
3. Prepare a fresh 17.5 µM working solution of $DiOC_6$ in ethanol starting from the 17.5 mM stock solution.
4. Add the working solution of $DiOC_6$ to the cell at 175 nM final concentration.
5. Incubate the samples at RT 30 min.
6. Prepare the slide for the analysis with 5 µL cell suspension and observe using fluorescence microscopy with a 500–520 nm fluorescence filter (*see* **Note 9**).
7. Acquire digital images from at least 500 cells with the DS Qi1 camera and use identical exposure times to compare different samples (Fig. 2) (*see* **Note 10**).

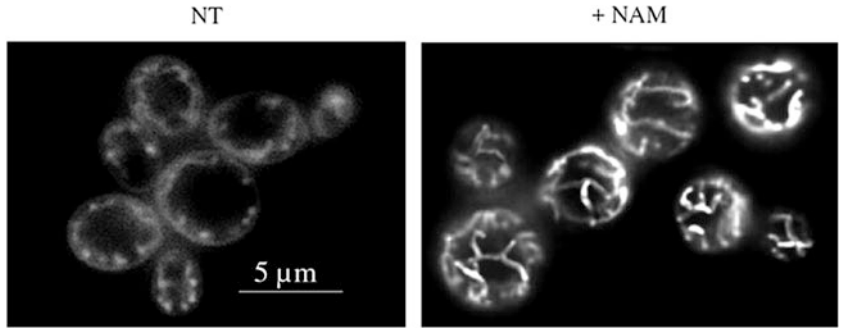


Fig. 2 Representative images of mitochondria stained with $DiOC_6$ of NAM-treated and untreated cultures (NT) after 6 days from the diauxic shift. Mitochondria of NAM-treated cells display a tubular morphology and bright fluorescence indicative of a proper functional state [4]

3.5 Fluorescence Microscopy to Analyze O_2^- Accumulation

1. To detect O_2^-accumulation [8], harvest culture samples at designed time-points.
2. Add DHE directly to the cell culture samples at a final concentration of 5 μg/mL using the 5 mg/mL aqueous stock solution.
3. Incubate the samples at RT for 10 min.
4. Prepare the slide for the analysis with 5 μL of the cell suspension and observe using fluorescence microscopy with a 565 nm fluorescence filter.
5. Acquire digital images by using the camera as described for $DiOC_6$ staining.
6. To obtain the frequency of ethidium stained cells (O_2^- accumulating cells), analyze approximately 1000 cells for each sample.

4 Notes

1. This strain was chosen for its high respiratory capacity that makes it an excellent model for studying the dynamics of mitochondrial respiration during chronological aging [9].
2. Prepare media according to the published guidebook [10]. In order to avoid auxotrophy starvation, add supplements in excess to a final concentration of 200 mg/L, except for leucine at 500 mg/L [5]. Fill cultures to no more than 20% of the flask volume to ensure adequate aeration.
3. Collect 1 mL samples and sonicate 2 or 3 cycles of 10 s. Dilute a known volume of the sample in 10 mL Isoton II. If a Coulter counter is not available, determine growth by monitoring the increase in the absorbance over time at 450 nm with a spectrophotometer.
4. Cell-free medium samples can be stored at −80 °C until used.
5. Before NAM supplementation, collect culture samples as controls. If the oxygen consumption is low, the cellular concentration can be increased to 10^7 cells/mL.
6. To ensure that the concentration of TET is not limiting for complete inhibition, it is possible to assess the optimal level of TET by performing preliminary determinations using up to 225 mM TET.
7. It is possible to calculate the net respiration (netR), which is an estimate of the coupled respiration, by subtracting J_{TET} from J_R. Then, netR can be used to calculate the net routine control ratio (netR/J_{MAX}), which expresses the fraction of the electron transfer system utilized to drive ATP synthesis [11]. In

addition, the respiratory reserve capacity, which is the ratio of uncoupled respiration to basal respiration (J_{MAX}/J_R), can be determined.

8. Avoid sonication of the samples to be stained.
9. If the background fluorescence is high, carry out 2–3 washes with 100 μL 10 mM HEPES, pH 7.4, containing 5% glucose.
10. It is possible to estimate $DiOC_6$ signal, which is proportional to the membrane potential, by using an image analysis software under condition of acquiring unsaturated images.

References

1. Bogan KL, Brenner C (2008) Nicotinic acid, nicotinamide, and nicotinamide riboside: a molecular evaluation of NAD^+ precursor vitamins in human nutrition. Annu Rev Nutr 28:115–130
2. Houtkooper RH, Pirinen E, Auwerx J (2012) Sirtuins as regulators of metabolism and healthspan. Nat Rev Mol Cell Biol 13 (4):225–238
3. Imai S, Guarente L (2014) NAD^+ and sirtuins in aging and disease. Trends Cell Biol 24 (8):464–471
4. Orlandi I, Pellegrino Coppola D, Strippoli M, Ronzulli R, Vai M (2017) Nicotinamide supplementation phenocopies *SIR2* inactivation by modulating carbon metabolism and respiration during yeast chronological aging. Mech Ageing Dev 161(Pt B):277–287
5. Longo VD, Shadel GS, Kaeberlein M, Kennedy B (2012) Replicative and chronological aging in *Saccharomyces cerevisiae*. Cell Metab 16 (1):18–31
6. Parrella E, Longo VD (2008) The chronological life span of *Saccharomyces cerevisiae* to study mitochondrial dysfunction and disease. Methods 46(4):256–262
7. Koning AJ, Lum PY, Williams JM, Wright R (1993) DiOC6 staining reveals organelle structure and dynamics in living yeast cells. Cell Motil Cytoskeleton 25(2):111–128
8. Madeo F, Fröhlich E, Ligr M, Grey M, Sigrist SJ, Wolf DH et al (1999) Oxygen stress: a regulator of apoptosis in yeast. J Cell Biol 145 (4):757–767
9. Ocampo A, Liu J, Schroeder EA, Shadel GS, Barrientos A (2012) Mitochondrial respiratory thresholds regulate yeast chronological life span and its extension by caloric restriction. Cell Metab 16(1):55–67
10. Amberg DC, Burke D, Strathern J (2005) Methods in yeast genetics: a Cold Spring Harbor Laboratory course manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. ISBN-10: 0879697288
11. Gnaiger E (2012) Mitochondrial pathways and respiratory control. An introduction to OXPHOS analysis, 4th edn. OROBOROS MiPNet Publications, Innsbruck. 2014: 4000 prints. ISBN-13: 978-3-9502399-8-0. https://pdfs.semanticscholar.org/1d1c/e797c78fcdab9de8641ca311d457b03c3a87.pdf