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The chronological life span of *Saccharomyces cerevisiae* to study mitochondrial dysfunction and disease

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ABSTRACT

Saccharomyces cerevisiae has played an important role as a model system to understand the biochemistry and molecular biology of mammalian cells. The genetic tools available and the short life span have also made *S. cerevisiae* a powerful system to study aging. The yeast chronological life span (CLS) is a measure of the survival of a non-dividing population of cells, and thus can model aging of mammalian non-dividing cells but also of higher eukaryotic organisms. The parallel description of the pro-aging role of homologs of Akt, S6 kinase, adenylate cyclase, and Tor in yeast and in higher eukaryotes, suggests that findings in the *S. cerevisiae* will be valuable to understand human aging and diseases. Moreover, the similarities between mitochondria and age-dependent mitochondrial damage in yeast and mammalian cells indicate that *S. cerevisiae* is a valuable model to study mitochondrial dysfunction and diseases that involve this organelle. Here, we describe the use of *S. cerevisiae* CLS in combination with three methods to quantify age-dependent mitochondrial damage and the accumulation of mitochondrial DNA mutations.

1. Introduction

1.1. Saccharomyces cerevisiae as a model organism to study aging

As a unicellular eukaryote, *S. cerevisiae* has the advantage of being the simplest and shortest-lived organism among the major aging model systems (chronological mean life span is 6–15 days depending on the genetic background used; see section below). Because of the extensive genetic and molecular biology techniques available and wide characterization and often-conserved roles of its genes, baker's yeast is an ideal organism in which to model aging and diseases [1].

Increasing evidence points to similarities in age-dependent phenotypes including mitochondrial damage in yeast and mammalian cells. When limited to a non-fermentable carbon source, yeast is forced to obtain energy exclusively from mitochondrial respiration. The time yeast take to form a visible colony on a non-fermentable carbon source plate increases with age. In addition, the percentage of cells that can use non-fermentable carbon sources and therefore respiration for growth decreases with age [2]. Also, inactivation of the mitochondrial aconitase by superoxide increases with age in yeast [3,4] and in higher organisms [5]. Furthermore, both nuclear and mitochondrial DNA (mtDNA) mutations increase with aging in mammalian dividing cells [6] and post-mitotic cells [7] and preliminary evidences suggest that mtDNA mutations also increase in aging yeast (Longo et al., unpublished results).

Several methods are available to identify and quantify specific nuclear and mitochondrial DNA mutations on a variety of experimental settings in higher organisms [8–11]. Although the mouse is a very valuable organism to model diseases and study the effect of mutations on cancer and aging, its life spans is long, and the procedures to measure DNA mutations are complex. Therefore, yeast represents a powerful and simple system to complement more sophisticated models in the study of the role of DNA mutations in aging and diseases.

1.2. Chronological life span

Our laboratory introduced a method to measure life span in yeast based on the survival of populations of non-dividing yeast cells in a mostly high-metabolism non-dividing state, i.e., the chronological life span (CLS) [3,12,13]. This method differs from the replicative life span, which measures the reproductive potential of individual yeast mother cells [14], and better resembles the methods used to measure life span in higher eukaryotes. Experimentally, the organisms are grown in synthetic complete glucose medium (SDC), and kept in the same culture until at least 90% of the population has died. During the growth phase (approximately 10 h), energy is produced mainly by the fermentative catabolism of glucose. When most of the glucose is exhausted, the population switches to a prevalently respiratory metabolism which utilizes in part the ethanol produced during the growth phase. Yeast cells stop dividing after 24–48 h and survive for 5–7 days while

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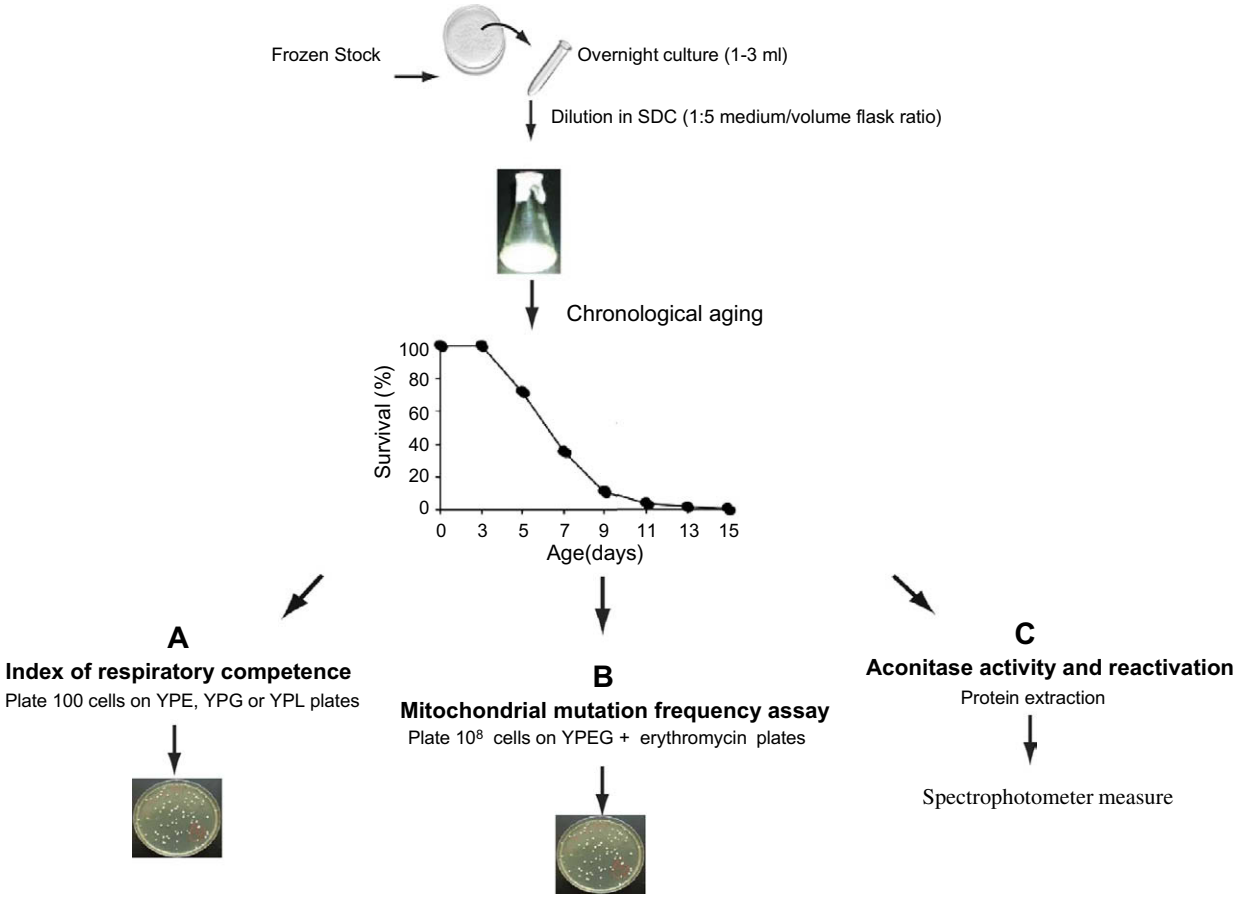

Fig. 1. Scheme representing the use of chronological life span to monitor mitochondrial damage. Few colonies are inoculated from a fresh plate in 1–3 ml of SDC. The overnight culture is diluted in 10–50 ml of SDC. Cell survival is determined every 2 days by diluting the yeast cultures and plating them onto rich-medium (YPD) plates to measure the colony forming units (CFUs). CFUs are counted after 2–3 days. Alternately the cells are switched to water and the cultures are washed every 2 days. (A) IRC. The same number of cells is harvested from the yeast culture, diluted and plated onto YPE, YPG or YPL. CFUs are counted after 2–3 days. The IRC is calculated based on the number of viable cells as measured by the CFUs assay. (B) Detection of mtDNA point mutations. yeast cells (10^8) are harvested from the SDC solution, washed and plated onto YPEG + erythromycin plates. The erythromycin resistant CFUs are scored after 10 days. Mutation frequency is calculated as the number of erythromycin resistant colonies/total number of colonies scored on YPD plates. (C) Aconitase activity. Aliquots of cells are harvested from the SDC cell culture and subjected to cellular lysis. The whole protein extract is used to measure spectrophotometrically the enzyme activity.

maintaining high metabolic rates for the first 3–4 days [15]. The aging organisms are maintained at 30 °C with shaking, and their viability is monitored by harvesting aliquots of cells at regular times and plating them onto rich-medium plates. The viability is scored by counting the number of cells able to form colonies but can also be measured by live/dead staining (FUN-1) or by measuring the proteins released by cells into the medium [15]. An additional advantage of the CLS, is that it mimics environmental conditions encountered by microorganisms in their natural environment, where they survive as non-dividing populations in the presence of limited external nutrients [16].

In addition, CLS studies can be performed in water by switching the cells from SDC medium to water after 72 h [13]. These experimental conditions induce a low metabolism stationary phase and represent a form of severe caloric restriction (CR) that allows the yeast to survive much longer than when maintained in SDC. In fact, the mean life of wild-type strain DBY746 in water is approximately 2–3 times longer than in SDC (15–20 days). These conditions also mimic extreme starvation situations encountered by yeast in nature. Both the paradigms (SDC or water) can be used for mitochondrial damage analysis. However, incubation in water is particularly useful for studies of mitochondrial function during aging, since it extends the monitoring of the index of respiratory competence (IRC, described in the following paragraphs) for several weeks and allows a better correlation between IRC and cell death [2].

1.3. CLS to model human mitochondrial diseases

Chronological life span has been used to study mitochondria-related diseases such as Friedreich's ataxia, an hereditary neurodegenerative disease associated with cardiomyopathy and diabetes. Deficiency of frataxin, a mitochondrial protein involved in the assembly of the iron–sulfur clusters and cellular anti-oxidant protection, seems to underlie this disease [17]. Isaya and co-workers employed a method similar to the one described in this manuscript to study the activity of the yeast frataxin [18]. By studying mutations that specifically impair ferroxidation of yeast frataxin and reduce chronological life span, they described a primary role for frataxin in iron detoxification, a crucial function to take in consideration while designing therapeutic strategies to treat Friederich's ataxia.

Our laboratory combined the chronological life span with several techniques to quantify the age-dependent mitochondrial damage and the accumulation of mtDNA mutations. In the following paragraphs, we present and discuss these methods: index of respiratory competence, erythromycin resistance assay, aconitase activity assay. A broad view of the steps currently in use in our group to perform these assays can be observed in Fig. 1.

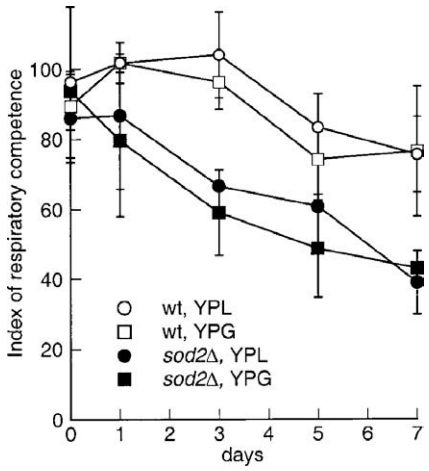

Fig. 2. Index of respiratory competence (IRC) in wild-type and mutants lacking mitochondrial superoxide dismutase (sod2Δ). Wild-type and sod2Δ mutants were grown in complete minimal glucose medium (SDC) and switched to water on day 3. At the indicated times an aliquot of cells was removed from each flask, serially diluted, and plated onto YPD, YPG and YPL plates. The samples on day "0" were taken when the cells were actively growing. The IRC was calculated as (number of colonies formed on YPG or YPL)/(number of colonies formed on YPD). The IRC for *sod2Δ* mutants decreased steadily with time. This result indicates that the mitochondria in *sod2Δ* were undergoing time-dependent changes that eventually prevented their proper function [2].

1.4. Index of respiratory competence (IRC)

The ability of yeast to grow by either respiration or fermentation offers a particularly important feature for the study of mitochondrial function. In fact, *S. cerevisiae* can survive for several days by fermenting glucose even in the absence of mitochondrial respiration. As long as glycolysis can occur, respiration incompetent strains (petite mutants) can remain viable. When mitochondrial respiration is intact, yeast can also grow by respiration using non-fermentable carbon sources such as glycerol, lactate or ethanol. This ability is a remarkably useful feature used to determine whether mitochondria are extensively damaged at a point when the cell is still viable. We have defined the percentage of cells able to grow in both fermentable (glucose) and non-fermentable (ethanol, glycerol or lactate) carbon sources as the "index of respiratory competence" (IRC) [2]. For example, if all the cells that grow using glucose as a carbon source also grow utilizing lactate, it indicates that all the mitochondria are functional and the IRC is 100%. IRC has been used to characterize yeast lacking mitochondrial superoxide dismutase [2] (Fig 2). By day 7 of the life span, whereas close to 80% of wild-type cells maintained the ability to grow by utilizing the non-fermentable carbon sources lactate and glycerol, the IRC was only 40% for mutants lacking the mitochondrial superoxide dismutase *SOD2* (Fig 2), suggesting that IRC can reflect the respiration status of mitochondria.

1.5. Mitochondrial mutation frequency assay

Erythromycin is a macrolide antibiotic that specifically inhibits its mitochondrial translation [19,20]. Specific point mutations in the *rib2* and *rib3* loci, the mitochondrial genes for the large ribosomal RNA (21rRNA), can confer resistance to the drug [21–23]. For this reason, acquisition of erythromycin resistance is a convenient, direct measurement of mtDNA point mutations.

In order to study age-dependent mitochondrial DNA damage, the erythromycin resistance assay can be coupled to the chronological life span method. The conditions used in the erythromycin resistance assay can be adapted from the protocol of Chi and Kolodner [24] modified by Shadel and co-workers [25].

1.6. Aconitase activity and reactivation

Aconitase, a mitochondrial enzyme sensitive to superoxide, isomerizes citrate to isocitrate, a key intermediate of the citric acid cycle. Loss of aconitase activity is an intracellular indicator of oxidative damage in a variety of degenerative diseases and aging [5,26]. Aconitase can be reversibly inactivated through the oxidation of its 4Fe–4S cluster. Superoxide can cause the reversible release of one iron atom from the 4Fe–4S cluster but the enzyme can then be reactivated through iron–sulfur cluster reduction in the presence of sulfur and iron [27]. The portion of the aconitase activity that can be reactivated by iron and sulfur, thereby, serves as an indirect but sensitive method to measure mitochondrial superoxide levels in both bacteria [28] and mammalian cells [29]. By measuring aconitase activity during various stages of the chronological lifespan, we estimated the level of superoxide in yeast aging. The reversibly inactivated portion of aconitase activity increased progressively with aging in wild-type cells, indicating that mitochondrial superoxide level increases with chronological age (Fig. 3)[4,15]. Another study revealed the importance of mitochondrial superoxide dismutase SOD2 during aging [2]. The dramatic age-dependent decrease of aconitase activity on cells lacking SOD2 indicates that superoxide produced by mitochondria inactivates aconitase if not removed by enzymatic activity of the mitochondrial superoxide dismutase. In addition, we identified other proteins that impact aconitase activity: mutations in *SCH9* (a pro-aging serine–threonine kinase) and *CYR1* (adenylate cyclase) delay the reversible inactivation of aconitase [4,15]. Fig. 3 shows the application of the aconitase assay with chronological life span

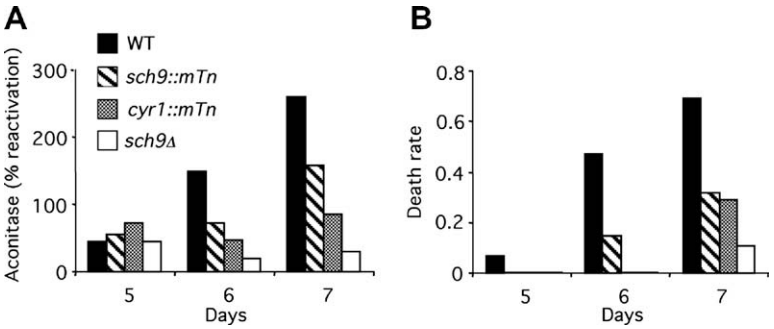

Fig. 3. The aconitase assay and the chronological life span have been exploited to study the effect of mutations that reduce activities of *cyr1* and *sch9*.(A) Mitochondrial aconitase percent reactivation after treatment of whole-cell extracts of yeast removed from cultures at day 5 through 7 with agents (iron and Na_2S) able to reactivate superoxide inactivated [4Fe–4S] clusters. (B) Death rate reported as the fraction of cells that lose viability in the 24-h period following the indicated day. The percent reactivation of aconitase was lowest in long-lived mutants and highest in wild-type cells and correlated with death rates [4].

to characterize the role of life span extension mutations in *CYR1* and *SCH9* in age-dependent oxidative stress. Aconitase activity was measured spectrophotometrically, as described by Racker [30] and Gardner et al. [29].

1.7. Conclusion

The versatility of the yeast model, the simplicity of the methodology, the effectiveness of combining the CLS assay with one or more of the tools described above, and cost-effective of the techniques employed make the chronological life span a valuable method to study the fundamentals of mitochondrial dysfunction.

2. Materials

2.1. Chronological life span

1. SDC liquid medium (see Note 4.1.1): 0.18% yeast nitrogen base without amino acids and ammonium sulfate (Becton, Dickenson and Company), 0.5% ammonium sulfate, 0.14% NaH_2PO_4, and 0.173% complete amino acid mix (discussed later). Dissolve all components almost completely in water and adjust pH to 6.0 with NaOH. Autoclave and add dextrose to a final concentration of 2% before use (discussed later).
2. Complete amino acid mix with adenine and uracil (Sigma–Aldrich): 80 mg/L adenine, 80 mg/L uracil, 80 mg/L tryptophan, 80 mg/L histidine–HCl, 40 mg/L arginine–HCl, 80 mg/L methionine, 40 mg/L tyrosine, 1200 mg/L leucine, 60 mg/L isoleucine, 60 mg/L lysine–HCl, 60 mg/L phenylalanine, 100 mg/L glutamic acid, 100 mg/L aspartic acid, 150 mg/L valine, 200 mg/L threonine and 400 mg/L serine. This is a modification of the original recipe [1].
3. Yeast extract/peptone dextrose solid medium (YPD): 10 g/L bacto-yeast extract (Becton, Dickenson and Company), 20 g/L bacto-peptone (Becton, Dickenson and Company), 20 g/L bacto-agar (Becton, Dickenson and Company). Dissolve in water. Autoclave and add dextrose to a final concentration of 2%. Mix well and pour plates.
4. Autoclaved glucose (20% (w/v)) stock solution
5. Autoclaved 18 MΩ Milli-Q grade water is used for all media preparations and dilutions for CFUs assay.
6. 100 × 15 mm Petri dishes (VWR).

2.2. Index of respiratory competence (IRC)

The same materials described in Section 2.1 are used, except for points 3 (YPD plates) and 4 (glucose stock solution).

1. Yeast extract/peptone ethanol solid medium (YPE): ethanol is used as a carbon source instead of glucose. The same recipe as above, but ethanol is added to a final concentration of 2%.
2. Yeast extract/peptone glycerol solid medium (YPG): glycerol is used instead of glucose. Glycerol (Sigma–Aldrich) is added to the yeast extract/peptone solution while stirring, reaching a final concentration of 3%. A 30% (w/v) autoclaved glycerol stock solution is used.
3. Yeast extract/peptone lactate solid medium (YPL): the non-fermentable carbon source used is lactate. The compound is added to the final concentration of 2%. 60% (w/w) sodium DL-lactate (Sigma–Aldrich) is used.

2.3. Erythromycin resistance mitochondrial mutation frequency assay

The same materials described in Section 2.1 are used, excluding the points 3 (YPD plates) and 4 (glucose stock solution).

1. Yeast extract/peptone ethanol/glycerol solid medium (YPEG) containing erythromycin (2 mg/mL) (see Note 4.3.1). For this medium, both ethanol and glycerol are used instead of dextrose. Erythromycin is dissolved in the ethanol solution (see Note 4.3.2). The glycerol stock solution is added to the yeast extract/peptone solution while stirring, followed by the ethanol with the dissolved antibiotic. The final concentration of both glycerol and ethanol is 3%.
2. (−)-Erythromycin hydrate, 96% (Sigma–Aldrich).
3. 150 × 15 mm Petri dishes (VWR) (see Note 4.3.3).

2.4. Aconitase activity

1. Lysis buffer [50 mM Tris pH 7.2, 150 mM NaCl, 5 mM EDTA and 0.2 mM protease inhibitor phenylmethylsulfonyl fluoride (PMSF) (VWR)].
2. 0.05 mm acid-washed glass beads (VWR).
3. Reaction mixture: 50 mM Tris–HCl, pH 7.5, 5 mM sodium citrate, 0.6 mM $MnCl_2$, 0.2 mM $NADP^+$ (Sigma–Aldrich) and 2 U of $NADP^+$ isocitrate dehydrogenase (Sigma–Aldrich).
4. For 4Fe–4S cluster reactivation experiments: add to the cuvette containing all the reagents required for the aconitase assay 1 mM ferric sulfate ($FeSO_4$) and 1 mM sodium sulfide (Na_2S) (Sigma–Aldrich).

3. Methods

3.1. Chronological life span in SDC

1. Using an applicator stick, streak a small portion of the frozen stock onto a YPD plate and incubate at 30 °C for 2–3 days (see Note 4.1.2).
2. Inoculate a few colonies (4–5) into 1–2 mL of SDC medium in glass tubes and grow overnight. Dilute the overnight culture to an initial density of $1–2 \times 10^6$ cells/mL (OD_{600} of 0.1–0.2) in 10–50 mL of SDC. Incubate at 30 °C in flasks with a volume/medium ratio of 5:1, shaking at 220 rpm (see Notes 4.1.3–4).
3. After 3 days, start monitoring survival by measuring the ability of an individual yeast cell/organism to form a colony (colony forming units [CFUs]) (see Note 4.1.5). Normally, cultures are serially diluted to reach a $1:10^4$ dilution in sterile distilled water, and 10 μL of the same dilution are plated in duplicate onto two halves of a YPD plate (starting from cell culture density $OD_{600} = 10$ roughly 100 cells are plated). Particular care must be taken to avoid spreading the diluted culture over the line that marks the separation between the two halves of a plate. Two dilutions per culture are used routinely to reduce fluctuations due to manual error. The plates are incubated at 30 °C to allow the growth of the colonies. CFUs are counted after 2–3 days (see Note 4.1.6). In our classic chronological aging study, the number of CFUs at day 3 is usually considered to be the initial survival (100% survival) and is used to determine the age-dependent percent survival (see Note 4.1.7).
4. Monitor CFUs every 48 h by adjusting the dilution factor and the volume plated according to the mortality rate. Continue until survival reaches 1–10% to record a time value that approximates the maximum survival time (see Notes 4.1.8–9).

3.2. Chronological life span in water (CR)

1. Proceed as in Section 3.1, steps 1–3.
2. Harvest cells by centrifuging at 1400 RCF for 5 min at room temperature in sterile polypropylene tubes. Resuspend the pellet in a volume of sterile distilled water and centrifuge as before (see Notes 4.1.10–11).

3. Wash cells two more times and resuspend them in a volume of sterile distilled water.

4. Pour the culture into the original flask used for incubation after rinsing it with sterile water.

5. Repeat steps 2–4 every 48 h.

6. Monitor CFUs as described above every 48 h.

3.3. IRC

Both the paradigms (survival study in SDC or water) can be applied to study IRC.

1. Proceed as in Section 3.1, steps 1 and 2, or Section 3.2, steps 1–5.

2. From the same flasks that have been used for the classic chronological aging study described above, harvest a new aliquot of cells in order to monitor their ability to form colonies in presence of only non-fermentable carbon sources. Cultures are serially diluted to reach a $1{:}10^4$ dilution in sterile distilled water, and 10 μL of the same dilution are plated in duplicate onto two halves of YPE, YPG, and/or YPL plates as previously described. Two dilutions per culture are also performed, and the plates are incubated at 30 °C. CFUs are counted after 3 days (see Note 4.2.1).

3. Monitor CFUs every 48 h by adjusting the dilution factor and the volume plated according to the colonies counted scored the previous counting and the mortality rate determined thanks to CFUs scored in YPD plates.

4. IRC is calculated as colonies on non-fermentable carbon source plates (YPE, YPG, or YPL) divided by colonies on fermentable carbon source plates (YPD) times 100%.

3.4. Erythromycin resistance mitochondrial mutation frequency assay

Both paradigms (survival study in SDC or water) can be applied to determine mitochondrial mutation frequency.

1. Proceed as in Section 3.1, steps 1 and 2, or Section 3.2, steps 1–5.

2. From the same yeast culture flasks that have been used for the classic chronological aging study described in Subheading 3.1, harvest 1×10^8 cells (considering density $OD_{600} = 10$ the required volume is 1 mL) using sterile polypropylene tubes. Cells are water washed and plated onto the whole surface of the YPEG + erythromycin plates (see Note 4.3.3). Two aliquots per culture are plated routinely to reduce the fluctuations due to manual error. The plates are incubated at 30 °C. Erythromycin-resistant colonies are counted after 10 days (see Note 4.3.4).

3. Monitor CFUs every 48 h by adjusting the dilution factor and the volume plated according to the colonies counted scored the previous counting and the mortality rate determined thanks to CFUs scored in YPD plates.

4. Mutation frequency is calculated as the number of erythromycin resistant colonies/the total number of colonies scored on YPD plates (see Note 4.3.5).

3.5. Aconitase activity

Both the paradigms (survival study in SDC or water) can be applied to measure aconitase activity.

1. Proceed as in Section 3.1, steps 1 and 2, or Section 3.2, steps 1–5.

2. Harvest 5 mL cells from the same flasks have been used for chronological life span study.

3. The protein extracts are obtained by glass bead lysis under argon. The cells are lysed in a mixture consisting of one volume of lysis buffer and an equal volume of 0.5 mm acid-washed glass beads (see Section 2.4). Resuspend the cell pellet in 0.25 mL of lysis buffer and 0.3 g glass beads. Argon is bubbled through the samples before vortexing (see Note 4.4.1). Vortex for six cycles of 30 s followed by 2 min of cooling.

4. Microcentrifuge the extracts at 4 °C for 15 min.

5. After centrifugation, aliquot the supernatants, freeze them quickly and store at −70 °C (see Note 4.4.1).

5. Aconitase activity is measured spectrophotometrically [2]. Add approximately 50 μg of protein extract to the reaction mixture (Section 2.4). The enzyme activity is followed as absorbance change at 340 nm at 25 °C due to NADPH production by isocitrate dehydrogenase, using isocitrate produced by the aconitase reaction (see Note 4.4.2). The slop is measured during a 5-min linear portion of assay.

6. For 4Fe–4S cluster reactivation experiments, add 1 mM ferric sulfate ($FeSO_4$) and 1 mM sodium sulfide (Na_2S) to the cuvette containing all the reagents required for the aconitase assay.

4. Notes

4.1. Chronological life span

1. In our experiments, we use SDC medium [13] instead of the rich-medium YPD commonly used for stationary phase characterization studies [31,32]. Our choice is mainly justified by the observation that the YPD medium induces continued slow growth after day 2 and fluctuations in cell viability, probably due to a re-entry of part of the yeast population to the cell cycle. This problem is minimized in the SDC medium.

2. Ideally, viability experiments should be started using yeast that have been recovered from frozen stocks and grown on YPD plates for 2–3 days only. Incubation of the master plates at 4 °C, especially for an extended period of time (>1 week) and for particular sensitive mutants, might affect survival significantly.

3. The standard procedure in our laboratory is to start experiments by inoculating a few colonies instead of a single one to reduce the effect of stochastic components on life span. The selection of the size of the flasks used for aging studies performed in SDC depends on the life expectancy of the strains used. Bigger flasks (250 mL) are recommended for long-term experiments (>3 weeks), to avoid medium evaporation. In case of oxygen-sensitive mutants, we recommend using more SDC volume in order to limit the contact of the cells with the environment oxygen inside the flask.

4. Experiments are normally performed at 30 °C. Incubation at higher temperatures (37 °C) causes cellular stress and could reduce yeast survival.

5. We have confirmed that the progressive decline in CFUs over time reflects a loss in cell viability, observing an increase in the proteins released in the medium by dead and damaged cells and also using a live-dead assay (FUN-1) [15].

6. Older cells tend to re-enter the cell cycle more slowly than young ones. At the more advanced stages of a survival experiment, incubate the YPD plates for an additional day at 30 °C to avoid the underestimation of viability.

7. Usually, the number of CFUs at day 3 is selected as initial survival (100%), since in our wild-type strains DBY746 and SP1, the population density does not normally increase after day 3, indicating that the great majority of the cells have stopped dividing. When working with strains or mutants that are particularly short-lived, it is recommended to monitor survival daily starting at day 1. This is effective if the chronological life span study is coupled to a mitochondrial damage analysis. In fact, some mutants exhibit a particular sensitivity to mitochondria damage, showing a decrease in the index of respiratory competence already at day 3.

8. In our studies, we have noticed that after more than 99% of wild-type DBY746 and SP1 strains grown in SDC die, in about 50% of the cases, a subpopulation is able to utilize the nutrients

realised by dead cells and grow back [33]. Under these conditions, the survival can increase as much as 100-fold, allowing an easy detection of the re-growth. However, the possibility that a small fraction of cells in the culture starts dividing again before survival reaches 1% remains.

9. In some genetic backgrounds (i.e., BY4741), survival rate usually levels out after reaching approx 5–10%. It is not clear whether this is due to the selection of extremely long-lived subpopulations or, more probably, to the re-growth of a fraction of the population able to utilize the nutrients released by dead cells.

10. For chronological aging studies performed in water, we recommend to start the experiment with day 3 SDC cultures that have reached a proper concentration. The use of cells that had not grown optimally can invalidate the survival in water.

11. With water culture, the risk of contamination is higher compared to SDC experiments. We recommend particular care in manipulating yeast cultured in water.

12. In our laboratory the common method for transferring yeast cells to solid media is spreading the suspension of cells on the plate thanks a spreader. A spreader can be easily obtained holding a glass rod over a bunsen burner. When the glass is pliable it can be bended to form a triangle. Prior to use, the spreader is sterilized by dipping the triangle end in 70% ethanol and igniting it in a flame. Before applying to a cell suspension, the spreader must be cooled by touching the surface of the agar plate. The use of an inoculating turntable, consisting of a solid base and a freely rotating platform, facilitates the plating [34].

4.2. I.R.C.

1. Some yeast mutants could show a slower growth in the presence of non-fermentable carbon sources compared to those in YPD plates, although their mitochondrial respiration is still efficient. We recommend incubating plates for additional days to allow proper growth.

4.3. Erythromycin resistance mitochondrial mutation frequency assay

1. The proper solubilization of the erythromycin is essential. The erythromycin solubility is poor in water, higher in ethanol. We recommend dissolving the drug in the ethanol solution. Be sure that the antibiotic is completely dissolved, then pour the alcoholic solution into the plate mixture while stirring.

2. We have noticed that some yeast mutants display resistance to erythromycin. The effect of this antibiotic on the yeast survival should be tested before the experiment, in case the drug concentration needs to be increased. In our experiments, we did not use antibiotic concentration higher than 4 mg/ml due to a solubility problem (see Note 4.3.1).

3. Especially when the mortality rate is increasing, a very high number of cells and therefore yeast culture volumes are required to obtain a significant number of erythromycin resistant colonies. For this reason, we recommend plating the yeast cells on the whole surface of the dish. In addition, we suggest using larger Petri dishes in the last stages of the chronological aging study.

4. We have observed that some yeast mutants, particularly long-lived mutants, need more than 10 days to form visible colonies in the presence of erythromycin. We suggest counting CFUs on different days in order to identify the best day for the colonies counting. Because of the length of the experiment, the risk of contaminations and dehydration of the YPEG erythromycin plates is high. To get around this issue, we recommend particular care during the manipulation of erythromycin plates and advise the use of more YPEG solution when pouring the Petri dishes, in order to obtain plates that are less thin. In our experiences, the plates became too dry to support further growth after 3 weeks.

5. To calculate the mutation frequency, we divide the number of colonies scored on YPEG + erythromycin plates by the colonies scored on YPD plates. *S. cerevisiae* can survive for several days by fermenting glucose whether or not the mitochondria are functional. Thus, considering the colonies grown on YPD plates, in our assay we determine the mitochondria mutations occurring in the whole population, as well as in cells that could be deficient in mitochondrial respiration. In order to asses the mutation frequency of a cell population whose mitochondrial respiration is still efficient, the cells harvested from the SDC culture can be also plated onto YPEG without erythromycin, and the mutation frequency calculated as follows: number colonies counted on YPEG + erythromycin/colonies counted on YPEG. Alternately, colonies can be directly replicated from YPD plates to fresh YPEG plates.

4.4. Aconitase activity and reactivation

1. Because of the instability of 4 iron–4 sulfur (4Fe–4S) clusters in air, the extraction procedures must be performed as rapidly as possible minimizing the contact with environment oxygen. Furthermore, we recommend thawing the aliquots kept at −70 °C only once and immediately before the assay.

2. Aconitase assay kits are also commercially available. Similarly to our experimental conditions, the Aconitase-340 Assay System (Oxis Research) utilizes the coupled reaction of citrate to isocitrate and isocitrate to α-ketoglutarate to quantify enzyme activity. Under these assay conditions, the rate of NADH production is a measure of aconitase activity.

5. Equipment and supply

5.1. Chronological life span

Equipment:

- Media preparation: autoclave, pH-meter, magnetic stir bar and stir plate.
- Culturing yeast: bunsen burner, 30 °C incubator shaker (New Brunswick Scientific), 30 °C incubator, spectrophotometer (λ = 600 nm), microcentrifuge, vortex (Fisher Scientific).

Supplies: 100 × 15 mm Petri dishes (VWR), glass tubes, glass flasks, polypropylene tubes, wood applicator sticks (VWR), spreader, inoculating turntable (Fisher Scientific) (see Note 4.1.12).

5.2. IRC

Equipment: see Section 5.1.
Supply: see Section 5.1

5.3. Erythromycin resistance mitochondrial mutation frequency assay

Equipment: see Section 5.1.
Supply: see Section 5.1, besides 150 × 15 mm Petri dishes (VWR).

5.4. Aconitase activity and reactivation

Equipment: spectrophotometer (λ = 320 nm), refrigerated microcentrifuge, argon cylinder.
Supply: polypropylene tubes.

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