MINIREVIEW

Approaches to study yeast cell aging and death

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Abstract

For millennia, yeast has been exploited to obtain fermentation products, such as foods and beverages. For *c.* 50 years, yeast has been an established model organism for basic and applied research, and more specifically, for *c.* 15 years, this unicellular organism has been applied to dissect molecular mechanisms of cell aging and programmed cell death. In this review, we present an overview of approaches to study cell aging and death in yeast, including lifespan assessments, calorie restriction, cell viability, survival, and death markers.

Introduction

The yeast *Saccharomyces cerevisiae* is a common eukaryal model organism in the fields of genetics, biochemistry, and molecular and cell biology; in fact, more than 50 000 scientific articles have been published so far using yeast as model system. Yeast has played a pivotal role in the understanding of basic cellular processes such as cell cycle regulation (Hartwell, 2002), intracellular trafficking (Nakano, 2004; Bowers & Stevens, 2005), protein folding regulation (Lindquist, 2002; Coughlan & Brodsky, 2005), and many others. Most benefits of using yeast are based on its short generation time, convenient and cheap experimental setups, straightforward genetic approaches, and, more recently, high-throughput methodologies (Petranovic & Nielsen, 2008). Additionally, yeast has some surprising similarities (homologues and orthologues) with mammalian (including human) cells, which makes it effective to model human diseases (Karathia *et al.*, 2011).

Two different paradigms of aging have been developed using yeast: replicative life span (RLS) and chronological life span (CLS) (Longo *et al.*, 2012). The former is based on the replicative potential of individual cells (obtained by counting the number of buds a single mother cell can bear), while the latter measures the survival of a cellular population in the postmitotic, nondividing phase. The yeast RLS thus resembles the RLS observed in mammalian cells, such as fibroblasts and lymphocytes, which undergo a fixed number of population doublings when maintained in culture. On the other hand, the CLS is more suited to model aging of postmitotic tissues, and of mammalian cells that do not divide, such as neurons, or have long nonmitotic resting phases.

It is interesting to note that in spite of the obvious differences underpinning the two lifespan paradigms, most mutations that increase RLS, such as those affecting the Ras-PKA or Tor-Sch9 pathways, do the same for CLS and vice versa (Longo *et al.*, 2012), while others have been reported to have opposite roles [e.g. Sir2, (Fabrizio *et al.*, 2005)]. More recently, a method to monitor the growth and aging of a yeast colony (clonal life span) based on isolation of single cells by micromanipulation and individual seeding on separate plates has been proposed (Mazzoni *et al.*, 2012).

Yeast RLS

Saccharomyces cerevisiae is a budding yeast that has the peculiarity to undergo an asymmetric cell division giving rise to an easily recognizable bigger mother cells and a smaller emerging daughter cell (bud). Robert K. Mortimer used this characteristic almost 50 years ago (Mortimer & Johnston, 1959) to set up a protocol to count the number of cell divisions a single cell can successfully accomplish (Fig. 1). In this procedure, the emerging buds are carefully removed as they appear by micromanipulation until the time, surprisingly, that the mother cell stops dividing. The total number of cell divisions a single cell can successfully complete is then scored. To statistically validate the result, the entire process must be repeated many times (normally 40) to determine the average RLS of a certain genotype and/or condition. Fluctuations are observed within isogenic cells and may reflect random differences between cells and/or damage resulting from the micromanipulation procedure. In addition, as the average RLS is *c.* 20 divisions for wild-type DBY746 strain and yeast doubles every 2 h, the experiments are usually interrupted by storage, thus adding the possibility that cold sensitivity may differentially affect specific genotypes. Using this method, significant discoveries have been made; such as the finding that Sir2 impairment is sufficient to decrease the RLS (Lin *et al.*, 2000). For more details on the RLS methodology and its successes in the search of the aging pathways please refer to Bishop & Guarente (2007) and Longo *et al.* (2012).

Some tips to assess the RLS

The yeast strains of interest are streaked from frozen stocks onto a rich YPD plate to obtain at least two colonies for each yeast strain that will be further analyzed. A sample of each colony to be analyzed is used to create a small patch of cells on a fresh YPD plate. After growth, using a micromanipulator suitable for yeast dissection (Singer semi-automated micromanipulation microscope or Zeiss Axioscope), about 40–50 cells from each patch are transferred to different coordinates on the same YPD plate. Cells are separated by micromanipulation, and after about 2 h of incubation, at least 20 virgin daughter cells (per strain) are analyzed. Plate drying must be avoided at this time (by Parafilm sealing of the plates or storing plates in a humidified chamber). Daughter cells are then removed by micromanipulation every 2–3 h, and data are recorded for statistical analysis. Alternatively, a microfluidics method that allows trapping of the mother cells and removal of the daughter cells provides automated determination of RLS and uses microscopy imaging (including fluorescence) to study the whole aging process (Lee *et al.*, 2012). An additional benefit of this method is that the environment can be kept constant during the experiment.

Yeast CLS

Yeast cultures grown on liquid media will divide reaching a certain cell density and then will enter the postdiauxic phase. Cells maintained in this condition will survive for a variable time ranging from a few days to several weeks. Factors affecting the survival time include incubation temperature, nutrient availability, pH, acetic acid concentration (Werner-Washburne *et al.*, 1996; Burtner *et al.*, 2009; Mirisola & Longo, 2012), and genotype (Fig. 1). It is commonly believed that CLS mimics the hypometabolic starvation condition of higher eukaryotes (Fabrizio *et al.*, 2005); however, this is incorrect as cells grown on synthetic dextrose media reach their maximum cell density within 48 h but remain in a high metabolic state (Gray *et al.*, 2004) for the subsequent three to five days and only then begin to die (50% survival is normally reached after 6–7 days by the widely used DBY746 yeast strain; Fabrizio *et al.*, 2001). It is important to note that wild-type cells do not die because they starve; in fact, both the intracellular glycogen and the extracellular ethanol (and other nutrients) remain high for the entire survival period (Fabrizio *et al.*, 2005). It has instead been demonstrated that chronologically aging yeast cells die exhibiting apoptotic markers (Herker *et al.*, 2004). Yeast survival in the wild may have taken advantage of the ability of yeast to cope with huge changes in nutrient availability, including fluctuations of nitrogen and carbon sources. In unfavorable natural conditions, apoptosis may have been an important adaptive mechanism that was selected to ensure the survival of some members of the clonal population. The substances released by the dying cells enhance the survival of the clones in the population by providing nutrients (Herker *et al.*, 2004), thus these findings are consistent with the existence of an altruistic aging program (Fabrizio *et al.*, 2004).

Many genotypic variations have been associated with chronological extension of life span. Although some of them can be considered specific for yeasts or fungi, some have also been found in higher eukaryotes, including mammals. Briefly, there are two major prochronological aging pathways discovered in yeast that have been confirmed in mammals. One is the Tor/S6K pathway (Fabrizio *et al.*, 2001), which responds to amino acid and glucose availability, the second one is the Ras/adenylate cyclase (AC)/PKA pathway (Longo *et al.*, 1999; Fabrizio & Longo, 2003), which senses glucose but is also influenced by other nutrient availabilities (Fontana *et al.*, 2010). These two pathways have been demonstrated to converge on the Rim15 kinase and on the transcription

factors Msn2/4 and Gis1 (Reinders *et al.*, 1998; Pedruzzi *et al.*, 2000, 2003; Cameroni *et al.*, 2004; Wei *et al.*, 2008, 2009). Interestingly, in addition to stress responsive genes, these transcription factors control both intracellular and extracellular carbon source balances (Bonawitz *et al.*, 2007; Wei *et al.*, 2008). It must be noted that the histone deacetylase Sir2, originally discovered as a modulator of RLS, also converges on Msn2/4 activity regulation (Fabrizio *et al.*, 2005; Medvedik *et al.*, 2007; Smith *et al.*, 2007). These findings suggest that metabolic regulation by Msn2/4 plays a pivotal role longevity regulation.

Some tips to assess the CLS

The population mean and maximal survival are usually determined by measuring the clonogenic survival of yeast cultures upon chronological aging. Here, the ability of yeast cells to form new colonies on rich media can be quantified as colony-forming units (CFU). Survival is then reported as the percentage of CFU measured at day 3 (conventionally referred to as the 100% survival point). Complementary approaches to assess yeast cell survival, such as the FUN-1 assay measuring the metabolic activity of yeast cells (see below), are used to confirm the age-dependent decline in clonogenic survival (Fabrizio & Longo, 2003).

CLS measures clonogenic survival in the high metabolic postdiauxic phase. It is well known that both the mean and the maximum survival time are affected by the medium used and the strain tested. For instance, rich media normally ensure a much longer viability than synthetic media [SDC, synthetic dextrose complete, (Werner-Washburne *et al.*, 1996)]. This is probably due to the prolonged slow growth phase after the YPD-based cultures have reached the high-density status. In addition, YPD cultures tend to be more permissive to the 'adaptive regrowth' phenomenon, that is, the restart of growth of a few surviving cells in a chronologically old culture (Fabrizio *et al.*, 2004), and are therefore more prone to artifacts. For these reasons, SDC medium is the preferred choice. The experiments start by streaking the strain of interest from a frozen stock onto YPD-rich medium. After 2 days of growth, some cells are transferred onto YPGly medium to be sure that the strain still has functional mitochondria. Petite strains, which have lost functional mitochondria, are not capable of growing on nonfermentable carbon sources. This is an important step to perform as the lack of functional mitochondria profoundly affects CLS. Cells from the YPGly plate are then inoculated into 1 mL of SDC medium for an overnight incubation. This preculture is then diluted to an initial density of $1–2 \times 10^6$ cells mL^{-1} (corresponding to an OD$_{600}$ of 0.1–0.2) in 10–50 mL of synthetic complete medium containing 2% glucose (SDC). Yeast cultures are incubated (30 °C, volume/medium ratio of 5 : 1, shaking at 220 r.p.m), and after *c.* 10 h of growth, the glucose concentration in the medium drops and yeast switches to a respiration-based metabolism. After this 'diauxic shift', yeast starts using the ethanol produced during the fermentative phase through mitochondrial oxidative phosphorylation. In the postdiauxic phase, metabolic rates remain high until day 5–6. The highest optical density, which is obtained around day 3, varies from strain to strain from 7 to 15 OD$_{600}$. Different yeast strains may have a different mean as well as maximum life span. For example, mean survival of wild-type strains ranges from 6–7 days (DBY746/SP1) to 15–20 days (S288C/BY4700) in SDC medium (Fabrizio *et al.*, 2005).

As already mentioned, yeast cell cultures, as well as bacterial cultures, may start dividing again when the number of survivors drops to 1% (of day 3, 100% value), giving rise to phenomena called adaptive regrowth and gasping, respectively (Zambrano & Kolter, 1996; Fabrizio *et al.*, 2004). As this may lead to misinterpretation of the results, especially for the calculation of the maximum life span, two alternative protocols have been developed. The first one is water incubation. Briefly, after the cultures have reached their maximal density by standard protocol, the cells are harvested by centrifugation, and after a washing step with water, they are incubated in water. This procedure is repeated every 2 days. In this way, the metabolites released by the dead cells are washed away and cannot be used by the survivors. This strategy effectively eliminates the regrowth; however, as it corresponds to an extreme calorie restriction (CR), it is useless when the effects of nutrients or of certain metabolic pathways are the focus. Alternatively, *in situ* viability may be used (Hu *et al.*, 2013). Briefly, aliquots of 2-day-old liquid culture of, *for example,* a trp$^-$ strain are plated on many SDC plates lacking tryptophan. The plates are incubated at 30 °C but, as the essential amino acid tryptophan is missing, no growth is observed. In a timely manner (e.g. every other day), two of these plates are removed from the incubator, supplemented with tryptophan and put back in the incubator. As all the auxotrophies are now complemented, cells can start dividing. CFUs are scored after 2 days of additional incubation. The survival curve is calculated as the percentage of CFU with respect to CFU at day 2–3, the latter as usual, referred to as 100% survival. Alternatives include amino acids for other auxotrophies (typically leucine, histidine), or plating on agar plates lacking carbon and nitrogen sources (thus mimicking the extreme starvation condition resulting from water incubation) and then adding YPD or SDC instead of just one amino acid every other day. As amino acid availability may affect the survival, the comparison of yeast strains

with different auxotrophies must be carefully evaluated (Gomes *et al.*, 2007). One practical possibility to avoid possible artifacts is to equalize the auxotrophies between the strains under analysis transforming yeast cells with an empty plasmid carrying the missing markers, thus balancing any difference in auxotrophies (Hu *et al.*, 2013).

CR, the only known intervention capable of positively affecting the life span in a wide range of species, can be effectively mimicked in these experiments by reducing the glucose concentration from 2% to 0.5–0.1%, or even to 0% (Fabrizio & Longo, 2007). In these conditions of caloric restriction from mild to extreme, yeasts will live longer (extended CLS), entering a state of low metabolic activity. Incubation in water has the additional advantage of reducing the possibility of the adaptive regrowth, as discussed above.

Mutation rate detection during CLS

As with RLS, CLS is found to be associated with various mutations, and this work has led to the discovery of the roles of the oncogene homologue Sch9 and superoxide in age-dependent mutations (Madia *et al.*, 2008, 2009). One method used to assess appearance of mutations as a function of CLS is based on the selection of spontaneous inactivation of the permease *CAN1* gene (Chen *et al.*, 1998). This permease is responsible for the uptake of extracellular arginine and its toxic analogues, such as canavanine. Incubation of yeast cells in the presence of canavanine (60 mgL^{-1}) will not allow survival unless a mutation impairing that permease occurs (Guthrie & Fink, 1991). As canavanine and arginine compete for the same transport system, the selective medium must lack arginine, which is a nonessential amino acid. For this reason, arginine auxotrophs cannot be used in this test. From a practical point of view, the experiment is performed at the same time as a standard CLS measurement. Whenever a small amount of the culture is serially diluted to measure the CFU (normally with a bi-daily frequency), a larger aliquot containing *c.* 2×10^7 cells is collected, washed with sterile water, and plated on media lacking arginine, but containing 60 mgL^{-1} of canavanine. The mutation frequency is then calculated as the number of Can^r colonies with respect to the total number of viable cells scored each plating day in the absence of canavanine (YPD plates). When Chen and coworkers sequenced the mutated *CAN1* locus obtained in this way, they found point mutations (65%), frameshifts (25%), and other more complex events (10 %) (Chen *et al.*, 1998).

When point mutations are the main focus of the research, it is possible to measure the *reversion rate of stop codons*. One of the most commonly used methods is based on the ability to acquire tryptophan prototrophy.

DBY746, a widely used aging research yeast strain, has a C-T mutation in codon 403 of the *TRP1* gene, leading to a stop amber codon that results in tryptophan auxotrophy (Capizzi & Jameson, 1973). The procedure is quite similar to the inactivation of Can1 method, but in this case, only the few point mutations that revert the amber stop codon to a coding one will be detected, and for this reason, a much higher number of cells must be plated (10^8) onto SDC-Trp plates.

For detecting frameshifts mutations, lysine prototrophy of an engineered Lys$^-$ yeast strain is used. The *lys2ΔBgl*II allele, obtained by Klenow treatment at the *Bgl*II restriction site, is inserted at the *lys*2 locus resulting in a lysine auxotrophy that is only reverted by mutations restoring the original reading frame (Chen *et al.*, 1998).

Finally, gross chromosomal rearrangements are monitored by replacing the *HXT13* gene (a member of hexose transporter family) with the *URA3* gene, in an otherwise Ura$^-$ strain (Chen & Kolodner, 1999). The inserted *URA3* gene is therefore positioned on the same chromosome as *CAN1*, but 7.5 kb more telomeric. 10^8 cells are harvested and plated on synthetic plates lacking arginine in the presence of both canavanine (60 mgL^{-1}) and 5-fluoro-orotic acid (5-FOA, 1 mgL^{-1}). The only cells capable of surviving in this condition are those that have lost the entire region containing both the *CAN1* and the *HXT13* loci (Chen & Kolodner, 1999).

Yeast cell viability and cell death

Determining the viability of growing and aging yeast cultures has given important insights into cellular mechanisms of yeast cell death and aging (Carmona-Gutierrez *et al.*, 2010; Hu *et al.*, 2013). Various methods have been used to determine cell viability, cytotoxicity, and cell death, including assays based on the analysis of growth, clonogenicity, oxidative stress, morphological markers of cell death, and metabolic activities (Braun *et al.*, 2010; Wloch-Salamon & Bem, 2013). This section aims at giving an overview of major complementary approaches and their useful combination. For a more comprehensive list of methods assaying yeast cell viability and cell death, the reader can refer to a very recent review on this subject (Wloch-Salamon & Bem, 2013).

Assessment of growth

Yeast cell viability can be determined by measuring the ability of yeast cultures to grow on culture media. This approach is based on the rationale that decreased growth rates or decreased culture densities during stationary phase are consequences of decreased cell viability in a given yeast culture, as compared with a control culture.

For instance, expression of cytotoxic proteins (e.g. human neurotoxic proteins) or treatment with cytotoxic chemicals (e.g. hydrogen peroxide) may result in delayed cell cycle progression and increased incidence of cell death (Perrone *et al.*, 2008; Braun *et al.*, 2010). Both may affect growth rates and culture densities during stationary phases. Growth analyses can be performed both on solid agar plates and in liquid cultures. Measurement of growth on agar plates can be taken as spot dilution assays performed manually or as automated spotting assays using robotics (Gitler, 2008; Braun *et al.*, 2010). In a spot dilution assay, cultures are spotted in serial dilutions on agar plates. Growth is compared among the different spotted cultures by qualitatively determining growth at the various dilution steps. In an automated spotting assay, cultures were spotted on agar plates in 384-well format based on a single dilution, and spots were analyzed for growth or nongrowth, and growth can be further quantified by software that is measuring colony sizes and densities (Costanzo & Boone, 2009). Applying these automated spotting assays, large-scale growth analyses can be performed using either the yeast knock-out or the yeast ORF collections. For instance, human neurotoxic proteins, which are also cytotoxic for yeast (e.g. TDP-43), were systematically expressed in the yeast knockout collection, and the growth abilities in the knockout strains were consequently determined by spotting assays (Armakola *et al.*, 2011). Alternatively, in high-throughput yeast plasmid overexpression screens, yeast strains expressing neurotoxic proteins were transformed with yeast ORF collections, and growth abilities upon overexpression of these ORFs were also determined by spotting assays (Fleming & Gitler, 2011). In some yeast knockout and overexpression strains, the cytotoxicity of the human neurotoxic proteins was increased or decreased. Using both approaches, yeast genes with human homologues were identified, which are involved in the execution and relief of neurotoxicity (Willingham *et al.*, 2003; Giorgini *et al.*, 2005; Sun *et al.*, 2011; Treusch *et al.*, 2011; Armakola *et al.*, 2012).

Measurements of culture growth in liquid media are taken by recording growth curves and culture densities based on photometric measurement of optical densities at 600 nm (OD_{600}), nephelometric measurement of light scattering (nephelometric turbidity), or measurements of cell densities (cells mL^{-1}) by automated cell counters (e.g. CASY cell counter or Beckman Coulter Counter). Growth curves can be recorded for small- to medium-scale analyses using a photometer or cell counter. Large-scale analyses can be performed using a microplate nephelometer, such as a Bioscreen, where you can record growth curves in a 96-well format (Gey *et al.*, 2008).

Assessment of clonogenic survival and cell death

The number of surviving and dead cells of a giving culture (solid or liquid) can be determined using a slightly modified CLS method (clonogenic approach), which is also based on the (in-) ability of cells to form new colonies (Madeo *et al.*, 2002). Here, the cell densities (cells mL^{-1}) of the cultures are determined (using an automated cell counter), and a defined number of cells (e.g. 500) are plated on agar plates with ideal nutrient compositions, such as rich medium with glucose as carbon source (YPD). The number of colonies (colony-forming unit, CFU) is determined after 2 days of incubation and correlates with the survivability of the plated yeast culture (Fabrizio *et al.*, 2001; Büttner *et al.*, 2007, 2011; Eisenberg *et al.*, 2009; Ruckenstuhl *et al.*, 2009; Braun *et al.*, 2011; Hu *et al.*, 2013). The bottleneck of this technique is the fact that only a limited number of cultures can be measured simultaneously. Even when using automated cell counter and automated colony counter, these analyses are used for low- to medium-throughput analyses.

An alternative way to measure clonogenicity is based on time-lapse photomicroscopy (Palermo *et al.*, 2007, 2010). Here, cell suspensions containing a defined number of cells are poured on a thin layer of YPD agar on a microscope slide. After 24 h, viable and unviable cells are scored on the basis of their ability to form microcolonies. In contrast to the classical clonogenic approach, which is restricted to counting the colony-forming surviving cells, this approach has the advantage of measuring both the dividing (alive) and nondividing (dead) cells at the same time.

Measuring intracellular reactive oxygen species (ROS)

Cytotoxicity and cell death are often correlated with the accumulation of intracellular ROS, including the superoxide anion, hydroxyl ion, and hydrogen peroxide (Perrone *et al.*, 2008; Ayer *et al.*, in press). These detrimental species are produced, for instance, by a disturbed mitochondrial respiratory chain, by ER stress or as a result of peroxisomal dysfunction (Jungwirth *et al.*, 2008; Perrone *et al.*, 2008; Ayer *et al.*, in press). Increased levels of ROS are detected with ROS-sensitive stains, such as dihydroethidium, dihydrorhodamine 123 (DHR123), and dichlorodihydrofluorescein diacetate (H_2-DCF-DA) (Madeo *et al.*, 1999). Plasma membranes are permeable to these chemicals, which are oxidized by cellular ROS to fluorescent products, for example the red fluorescent ethidium, and the green fluorescent rhodamine 123 and dichloroflu-
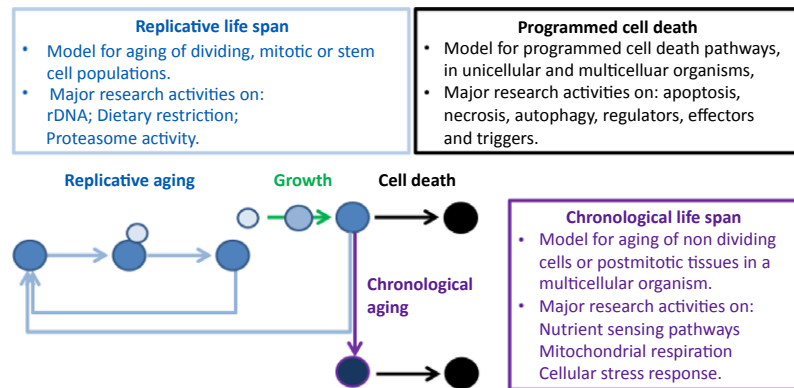
Fig. 1. Yeast as a model for replicative aging, chronological aging and cell death. Replicative life span (RLS) (blue), chronological life span (CLS) (purple) and programmed cell death (black) are parts of the yeast life cycle and differentiation, making yeast a suitable model to study these pathways.

orescein. The higher the levels of ROS within a culture, the stronger the fluorescence measured within the treated culture. Fluorescence intensities can be recorded by fluorescence microcopy and can be quantified by a fluorescence plate reader or flow cytometer (Madeo *et al.*, 1999; Büttner *et al.*, 2007; Eisenberg *et al.*, 2010). The big advantages of these methods are their simplicity and their usefulness for medium- to large-throughput analyses. For instance, oxidative stress during chronological aging of 96-well batch cultures can be easily measured using a fluorescence plate reader or an automated flow cytometer. Upon application of these ROS measurements, one should consider the different chemical reactivities of the ROS-sensitive stains. Whereas dihydroethidium is somewhat specific for superoxides, DHR123, and H_2-DCF-DA react poorly with superoxides but with a broader range of other ROS, and DHR123 staining further depends on the mitochondrial membrane potential (Halliwell & Whiteman, 2004). These may lead to different results. For instance, replicatively aged wild-type cells demonstrated increased levels of ROS detected by dihydroethidium staining, whereas when using H_2-DCF-DA, the ROS levels remained unchanged (Lam *et al.*, 2011).

Morphological markers of cell death

Cell death is accompanied by morphological alterations. These can be used as markers for the determination and characterization of cell death and for the discrimination between different cell death subroutines, such as apoptosis and necrosis (Carmona-Gutierrez *et al.*, 2010). Apoptotic yeast cells can be identified with 'terminal deoxynucleotidyl transferase dUTP nick end-labeling' (TUNEL) that visualizes DNA fragmentation, a hallmark of apoptotic cell death (Madeo *et al.*, 1997). Here, yeast cells are fixed, the cell wall is digested by treatment with one of the commercially available enzymes (lyticase, zymolyase, glusulase or cytohelicase extracted from the bacterium *Arthrobacter luteus* or from the snail *Helix pomatia*), and the plasma membrane needs to be permeabilized to enable access of the enzyme terminal deoxynucleotidyl transferase and the fluorescence-labeled dUTP to the fragmented DNA. DNA fragments are then enzymatically labeled with dUTP. Labeled nuclei are counted by fluorescence microscopy or flow cytometry. Alternatively, apoptotic yeast cells can be visualized by Annexin V staining which detects the externalization of phosphatidylserine to the outer leaflet of the plasma membrane, an early hallmark of apoptosis (Madeo *et al.*, 1997). In contrast to the TUNEL assay, the cells remain unfixed, but in order to enable accessibility of the labeled Annexin V to the plasma membrane, the yeast cell walls have to be digested enzymatically. Due to the high costs of the necessary chemicals, TUNEL and Annexin V assays are more suitable for confirmative experiments of data obtained by complementary methods.

Necrotic yeast cells are characterized by disintegrated membranes (Eisenberg *et al.*, 2010). The chemical propidium iodide (PI) is a 'vital dye' that enters dead yeast cells, binds to nucleic acids, and produces red fluorescence that can be determined using fluorescence microcopy, a fluorescence plate reader, or flow cytometry (Büttner *et al.*, 2007). PI and Annexin V staining can be combined, enabling the discrimination between apoptosis and necrosis (Büttner *et al.*, 2007). Yeast cells that only stain for Annexin V are apoptotic, cells that only stain for PI are necrotic, and Annexin V/PI double-positive cells can be discriminated as secondary necrotic or late apoptotic, in which apoptosis was initiated but ultimately succumbed to necrotic subroutines.

Metabolic activity as a measure of cell survival

Yeast cell viability can further be determined by measuring metabolic activity, based on the rationale that metabolically active cells are alive and metabolically inactive cells are considered to be dead. For instance, the two-color fluorescence probe FUN-1 can cross-intact and disintegrated plasma membranes, resulting in a diffuse cytoplasmic green fluorescence stain (Fannjiang *et al.*, 2004; Teng & Hardwick, 2009). In contrast to metabolically inactive cells, FUN-1 is converted into metabolically active cells by an unknown enzymatic reaction, and actively transported into the vacuole, where it forms a compact red fluorescent bar. Counting the number of green and red fluorescent cells by fluorescence microscopy or flow cytometry allows for the determination of dead and living cells within a tested culture, whereas measuring green and red mean fluorescence by a fluorescence plate reader determines the proportion of dead and living cells.

Critical comparison of complementary methods and their useful combinations

Measuring yeast growth, clonogenicity, oxidative stress, morphological markers of cell death, and metabolic activity are complementary methods with specific advantages and disadvantages. For instance, decreased growth rate could result for different reasons, including increased incidence of cell death, cell cycle deterioration, or decreased metabolic activity due to altered abilities to use different carbon sources. Clonogenic measurements of cell viability enable the quantification of cells within a culture that are definitely alive (because they form new colonies); however, the rationale that cells, which are unable to form new colonies, are dead is untrue for some situations. It is also possible that (some) of these cells are still alive but that colony formation is somehow blocked. Whereas clonogenic survival measurements potentially underestimate the number of living cells, using morphological markers of cell death could result in a marked underestimation of dead cells in a given culture, because not all dead cells demonstrate these morphological hallmarks (Wloch-Salamon & Bem, 2013). Measurement of cell viabilities by metabolic assays potentially overestimates living cells, because dead cells may be able to maintain some metabolic activities unless the cellular membranes disintegrate; and oxidative stress measurement cannot discriminate between living and dead cells because conversion of the ROS-sensitive dyes occurs in both living and dead cells.

Due to the different properties of the described methods, combinations of different approaches are very advisable. Growth analyses are unbeatable for genome-wide analyses and screens. Based on their results, clonogenic approaches could be combined with measurements of oxidative stress or metabolic viability measurements (e.g. Fabrizio & Longo, 2003; Fannjiang *et al.*, 2004; Herker *et al.*, 2004; Büttner *et al.*, 2008). The measurement of morphological cell death markers could be used as a supplement to discriminate between different subroutines of cell death (e.g. Büttner *et al.*, 2007; Eisenberg *et al.*, 2009; Braun *et al.*, 2011). Such useful combinations enable very deep insights into cellular mechanisms of cell death and aging (Fig. 1).

Outlook

Yeast has been a model organism for many years and for many purposes, including basic and biomedical research, and biotechnological applications (Petranovic & Nielsen, 2008). The ever expanding yeast toolbox now contains efficient and easily applicable tools and methods for molecular, cellular, biochemical, and genetic manipulations. To this, recent advances in omics technologies, bioinformatics and mathematical modeling have added valuable insights, datasets, and models that in the future will be used to study yeast cell aging and death (Munoz *et al.*, 2012). To create possibilities for more comparative studies, and to exploit systems biology approaches (that integrate molecular biology results and large datasets with bioinformatics and modeling), we recommend a community effort for standardization of experimental protocols and methods, including comparison of standardly used yeast strains, stress/death inducers, and cultivation conditions. These comparisons should be used to generate large experimental datasets, for example, transcriptome, proteome, and metabolome, in conditions that are specifically of interest for the yeast cell aging and death communities. The obtained data, as well as standardized protocols should be deposited and stored in a dedicated user-friendly database, and this platform should be used to collect, organize, and retrieve relevant information, as well as a resource for bioinformatics and modeling. We believe that this approach will allow for a more complete view of the pathways governing yeast cell aging and death, and their regulation.

Acknowledgement

Authors' contribution

M.G.M., R.J.B. and D.P. contributed equally to this work.

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