Chapter 20

Replicative Life Span Analysis in Budding Yeast

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Abstract

Identifying and characterizing the factors that modulate longevity is central to understanding the basic mechanisms of aging. Among model organisms used for research related to aging, the budding yeast has proven to be an important system for defining pathways that influence life span. Replicative life span is defined by the number of daughter cells a mother cell can produce before senescing. Over the past 10 years, we have performed replicative life span analysis on several thousand yeast strains, identifying several hundred genes that influence replicative longevity. In this chapter we describe our method for determining replicative life span. Individual cells are grown on solid media and monitored from their initial undivided state until they undergo senescence. Daughter cells are manually removed using a fiber optic needle and quantified to determine the total number of times each mother cell divides.

Key words Yeast, Aging, Replicative life span, Micromanipulation, Dissection, Mother cell, Daughter cell

1 Introduction

Aging in the budding yeast *Saccharomyces cerevisiae* was described more than 50 years ago with the observation that mother cells produce a finite number of daughter cells and then cease replication [1, 2]. This finite mitotic capacity is referred to as replicative life span [3]. A second model of aging has also been described in yeast, referred to as chronological aging. Chronological aging is a post-mitotic paradigm where yeast cells are maintained in a non-dividing, nutrient-limited state. Chronological life span is defined by the length of time that a cell retains the ability to reenter the cell cycle upon return to growth-promoting conditions [4, 5].

Life span is a key phenotype of interest in aging-related studies of yeast and other organisms. In general, genetic or environmental interventions that extend life span are considered to be more relevant than those that reduce life span, because decreased longevity can result from a variety of nonspecific factors while increased longevity requires that the intervention ameliorate the processes limiting life span in the wild type organism. Life span shortening

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interventions can be of interest in cases where a strong argument can be made that the reduction results from accelerating the normal aging process. The ability to measure life span in a reproducible and robust manner on a relatively short time-scale is one of the features that make budding yeast a particularly powerful model organism for aging-related studies. Studies from many different labs have contributed to a growing list of genes and interventions that modulate longevity, some of which play a conserved role in modulating the life span of multicellular eukaryotes.

Replicative life span is typically measured by the time and labor consuming micro dissection of daughter cells away from a dividing mother cell [6]. Methods have been developed for enriching populations of yeast for cells in their mid to late replicative life span [7–10]. One method, termed the Mother Enrichment Program, or MEP, has been modified to allow high-throughput measurement of replicative life span using genetic switches that are lethal to new daughter cells [10]. While this method holds promise, it has yet to become widely used or validated and requires several genetic modifications to the background being tested, making it less than ideal for genetic studies. Manual micromanipulation of daughter cells away from dividing mother remains the most robust and commonly used method for measuring replicative life span. To offset the labor-intensive nature inherent to this approach to measuring replicative life span, we previously developed an iterative strategy that allows moderate-throughput screening of a large numbers of interventions starting with only a small number of cells for each intervention [11], and have since demonstrated the robustness of this approach by identifying more than 50 replicatively long-lived single gene deletion mutants [12–17]. In this chapter we describe the basic underlying protocol for measuring replicative life span by manual micromanipulation of daughter cells away from dividing mothers, including our system for managing numerous such experiments simultaneously.

2 Materials

1. YPD plates: (1 % yeast extract, 2 % Bacto-Peptone, 2 % glucose, 2 % agar).
2. Synthetic Complete (SC) plates: (2 % glucose, 0.17 % yeast nitrogen base, 0.5 % ammonium sulfate, 2 % agar, amino acids). *See* Table 1 for complete list of components, including individual amino acids. Exclude appropriate amino acid for auxotrophic marker selection plates.
3. AxioScop.A1 HAL 50 or equivalent microscope configured with the following features designed for yeast dissection.

Table 1
Synthetic complete (SC) media composition

Component	Concentration (g/L)
D-glucose	20
Yeast nitrogen base (w/o ammonium sulfate or amino acids)	1.7
Ammonium sulfate, $(NH_4)_2SO_4$	5
Agar	20
Adenine	0.04
L-arginine	0.02
L-aspartic acid	0.1
L-glutamic acid	0.1
L-histidine	0.1
L-leucine	0.3
L-lysine	0.03
L-methionine	0.02
L-phenylalanine	0.05
L-serine	0.375
L-threonine	0.2
L-tryptophan	0.04
L-tyrosine	0.03
L-valine	0.15
Uracil	0.1

This table provides the mass of each component of SC solid media per unit liter

(a) Stage capable of two-dimensional (x and y) translation with horizontal and vertical demarcations every 1 mm and click stops every 5 mm.

(b) 10 cm petri plate mount on the stage.

(c) 50 μm fiber optic needle (from Cora Styles) mounted with two-dimensional (y and z) controls for micromanipulation of cells on the plate surface.

3 Methods

For the methods detailed in this chapter, we refer to yeast cultured at 30 °C on YPD medium. In principle, these methods can be used to measure replicative life span for yeast on virtually any desired

solid medium and temperature. For dietary restriction, medium containing a reduced concentration of glucose (typically 0.5 % or 0.05 % instead of the 2 % found in YPD) or an alternative carbon source (e.g., 3 % glycerol instead of 2 % glucose) can be used. For pharmacological experiments, compounds of interest can be added to the YPD medium at the desired concentration. In these cases, the experiments are performed as described, except cells should be patched onto the alternative medium beginning the night prior to positioning cells on life span plates (Subheading 3.3).

3.1 Design Replicative Life Span Experiment

This section outlines considerations to take when designing a replicative life span experiment, including the number of cells to include, the number of plates needed for a given number of cells, and how to divide cells among life span plates.

1. Determine the strains and number of mother cells to be assayed. A typical replicative life span experiment in the Kaeberlein Lab contains 30 strains with 20 mother cell life spans determined for each strain, for a total of 600 mother cell life spans per experiment. Multiple biological replicates are often assayed for the same strain in order to obtain 40, 60, 80, or 100 mother cell life spans in the same experiment.
2. Code the strain so that the dissectors are blind to the identity of each strain. We use a simple numerical code (strain 1, strain 2, … strain 30) for each experiment.
3. Determine the number of life span plates needed for the experiment, and how strains will be divided among the plates. The following points should be taken into consideration:
   (a) Each life span plate will typically contain 60 cells encompassing three strains with 20 cells each. However, a plate can hold upwards of 100 vertically oriented cells and smaller or larger cell sets per strain if needed for a particular experiment design.
   (b) If one experiment contains haploid strains of both mating types, make sure that each plate contains only *MATa* or *MATα* cells. Secreted mating factor from cells of one mating type can impact cell cycle control in cells of the opposite mating type and alter replicative life span.
   (c) Each experiment should be designed such that only one media condition is tested per plate.

3.2 Pour Plates, Prepare Strains, and Perform Initial Quality Control

Prior to beginning the quantification of replicative life span, prepare all media needed for the experiment, streak all strains from frozen stocks, and perform initial quality control to ensure that the strains used for analysis have the expected genotypes. Strains are always freshly streaked from frozen stocks prior to each experiment to ensure consistency between strains and between experiments,

and to minimize the accumulation of spontaneous mutations that can result from maintaining live strains.

1. Prepare initial YPD plates, quality control plates, pre-patch plates, and life span plates (*see* Subheading 2 for base YPD and SC plate recipes).
   (a) Initial YPD plates: Prepare enough YPD plates to streak all strains from the frozen stock. Up to 8 strains can be streaked onto each YPD plate.
   (b) Quality control plates: Prepare enough YPD plates to patch all strains for quality control. Up to 10 strains can be patched onto each plate. For each YPD plate, prepare one selection plate corresponding to each genetic marker used in the experiment. Common markers include *KanMX*, which is selected using YPD + G418, and auxotrophic markers (*HIS3*, *LEU2*, *URA3*, etc.), which are selected using SC media lacking the appropriate amino acid. Plates containing 3 % glycerol in place of glucose can also be included to determine whether a strain is capable of undergoing respiration. To test mating type, patched cells are replica plated onto lawns of a specific "mating test" strain which contains an auxotrophy complementary to those in the strain being tested (e.g., a strain auxotrophic for arginine is used for the BY background, which is auxotrophic for histidine, uracil, leucine, methionine, and lysine). The mating test strain should be of the opposite mating type as that expected for the strain to be tested. After strains are allowed to mate for 12–24 h at 30 °C, they are then replica plated onto basic synthetic media (SC media with no amino acids added). Only cells which successfully mate will be capable of growing on basic media.
   (c) Pre-patch plates: For each media type used in the experiment, prepare enough additional plates so that all strains can be pre-grown on the appropriate media prior to being transferred to the plates used in the experiment. This allows strains to acclimate to the media for several cell cycles prior to starting life span measurement (*see* **Note 1**).
   (d) Life span plates: Pour life span plates a minimum of 72 h prior to selection of virgin cells (Subheading 3.4) to allow sufficient drying time (*see* **Note 2**).
2. Streak all strains for single colonies onto initial YPD plates from frozen stocks 5 days prior to selection of virgin cells (Subheading 3.4). Strains with severe growth defects can be streaked from frozen stocks on preceding days as needed to ensure that colonies from all strains are ready on the same day. From this point forward, strain designators assigned in Subheading 3.1, **step 2** (strain 1, strain 2, etc.) should be used to keep the life span blinded.

3. Incubate freshly streaked plates at 30 °C for 2 days to allow cells to form colonies.
4. For each strain, pick a single colony from the initial YPD plate and patch onto the quality control YPD plate. Take care to select colonies of the appropriate size based on the phenotype of the strain. For example, select small colonies for slow-growing strains that have a tendency to acquire mutations that suppress the slow growth phenotype. Select large colonies for strains that tend to lose their mitochondrial DNA, resulting in the formation of petite colonies.
5. Incubate patched quality control plate at 30 °C overnight to allow patched cells to grow.
6. Replica-plate each quality control plate to each type of selection plate.
7. Incubate selection plates at 30 °C overnight.
8. Assess growth on selection plates to verify that each strain contains expected markers. Remove strains that do not display expected markers and redesign or restart experiment if needed.
9. Two days prior to selection of virgin cells (Subheading 3.4), pick a single colony from the initial YPD plate and patch onto the appropriate pre-patch plate.
10. Incubate pre-patch plates at 30 °C overnight to allow patches to grow.
11. The evening before selection of virgin cells, pick a small quantity of cells from the edge of each patch on the pre-patch plate and patch to one side of the appropriate life span plate as shown in Fig. 1a. Be sure to patch lightly so that cells do not become overgrown prior to initiating the life span experiment. Up to six patches may be included on a single life span plate.
12. Incubate life span plate at 30 °C overnight to allow patches to grow.

3.3 Position Cells on Life Span Plates

In this section individual cells will be arrayed in a single vertical line for rapid manipulation and quantification of number of divisions. To mark which patch each set of cells came from, the fiber optic needle is used to physically punch holes in the agar of the plate, with a single hole marking cells from the first patch, two holes marking cells from the second patch, and so on. To making counting easier, each set of 20 cells is divided into 2 sets of 10 cells separated by a horizontal line of needle holes. Figure 1 provides an illustration of the life span plate setup for one 20-cell set.

1. Place life span plate on the microscope plate mount with the patches to the left.
2. Mark the edge of the plate relative to the microscope stage so that the plate can be identically positioned in future steps.

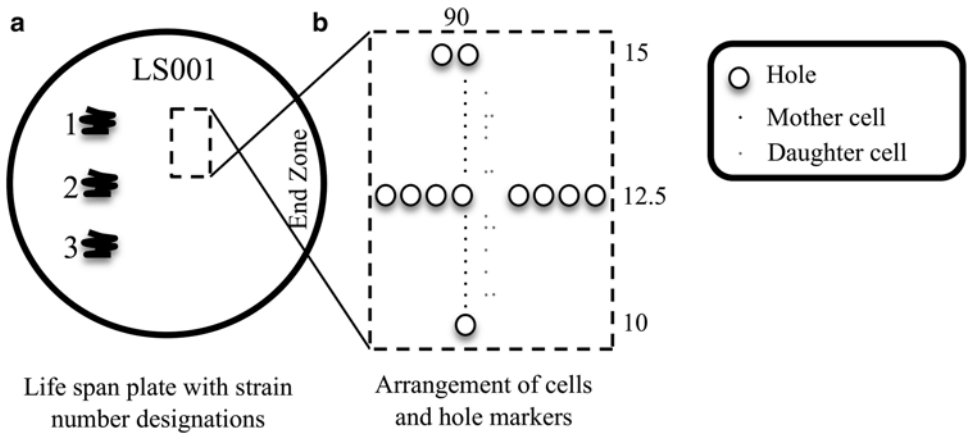

Fig. 1 Life span plate layout. (**a**) Each life span plate has three main areas: (1) patched cells for growth prior to analysis, (2) the life span analysis area, which contains cells arrayed for division quantification and marker holes, and (3) the "End Zone," where excess cells are discarded well away from the analysis area to prevent colonies from obscuring cells included in experiment. (**b**) Expanded diagram of cell analysis area

3. Position the stage so that the fiber optic needle is near the edge of the plate away from cells (see the "End Zone" in Fig. 1a). Punch several deep holes through the surface of the plate to ensure that the needle is clean and free of cells.
4. Using the fiber optic needle, pick up ~40 cells from the first patch and drop near a predetermined position in the top center of the life span plate. For our microscope, all life spans are set up with the first set of cells beginning at the 90 mm horizontal and 10 mm vertical position (Fig. 1b).
5. Ensure that the needle is clear of cells by touching the tip to the surface several times.
6. Punch a single hole to mark the starting position for cells from the first patch on this plate (Fig. 1b). Be careful not to allow any cells to fall into the hole. If they do, they will form a colony that will overtake and obscure the cells being examined for life span.
7. Pick 10 individual cells and position them in a vertical line toward the center of the plate, leaving ~100 μm (2–3 needle widths) between cells (Fig. 1b). Cell clusters can be separated by lightly touching the needle to the plate surface over the cells and gently tapping the side of the microscope to cause the needle to vibrate.
8. Ensure that the needle is clear of cells by touching the tip to the surface several times.
9. Punch a horizontal line of eight holes several needle widths beyond the line of 10 cells. These holes are used to indicate

the halfway point for the first strain. Leave a gap between the center-most holes (Fig. 1b) to allow a continuous needle sweep while cleaning up excess cells at the end of each replicative life span score point (Subheading 3.4, **step 6**).

10. Pick 10 additional individual cells and position them in a vertical line toward the center of the plate (Fig. 1b).
11. Separate 5–10 additional cells and leave them off to the side. These cells will be used to replace cells that fail to divide during the first round of division. More cells can be added for strains with defects in cell division.
12. Ensure that the needle is clear of cells by touching the tip to the surface several times.
13. Punch two horizontal holes several needle widths beyond the second line of 10 cells. These holes will mark the starting position for cells from the second patch on this plate (Fig. 1b).
14. Discard excess cells in the End Zone.
15. Repeat the Subheading 3.3, **steps 7** through **14** for all remaining strains in the experiment.
16. Once all cells have been positioned, wrap life span plates in Parafilm and incubate at 30 °C for 90–120 min.

3.4 Select Virgin Cells for Life Span Analysis

The cells arrayed in Subheading 3.3 were picked directly from a growing patch. As such, there is no way to know how many times each cell divided prior to selection. Following the 90–120 min incubation in Subheading 3.3, **step 16**, each arrayed cell will have divided once on average. To obtain a population with a defined replicative life span, newly budded (virgin) daughter cells are picked for analysis, and the original cells arrayed in Subheading 3.3 discarded.

1. Prepare replicative life span score sheets. Score sheets typically consist of a grid with each cell represented by a single row, and each time point represented by a single column. In our current practice, scores are tracked using a spreadsheet program on a netbook equipped with a number pad. Each plate is assigned a separate file. This allows scores to be immediately digitized and uploaded into a replicative life span database for storage and analysis. In the past, graph paper was used to record replicative life span data, with one plate represented per sheet, and all data manually entered at the end of the experiment. Figure 2 provides a representative score sheet for one strain.
2. Place the first plate in the life span analysis on the microscope stage and locate the first strain (e.g., the single hole punched at location 90, 10 in Subheading 3.3, **step 6**).

	Mother		RLS
	1	n 0 0 0 n 0 0 1 1 1 0 0 0 2 1 2 1 2 2 2 2 1 2 1 2 1 2 1 0 0 0 0 0 0 0 0 0 0 x U	27
	2	n 0 0 0 2 1 0 1 0 1 0 1 2 1 1 2 1 2 1 2 1 1 0 2 2 1 2 1 2 2 1 1 2 2 1 0 0 0 x S	39
	3	n 0 0 0 1 1 0 1 1 1 1 1 2 1 2 1 1 2 1 0 1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 x U	18
	4	n 0 0 0 1 1 0 2 0 1 1 1 0 2 0 1 1 2 1 2 2 1 0 2 2 2 2 2 2 2 1 1 2 2 1 0 0 0 x U	40
	5	n 0 0 0 1 1 1 1 1 1 0 2 1 2 0 2 1 2 1 2 2 1 1 2 2 0 1 0 0 0 0 0 0 0 0 0 0 0 0 x U	28
	6	n 0 0 0 1 2 0 1 1 1 1 1 1 2 1 1 2 1 1 1 1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 x U	19
	7	n 0 0 0 1 1 1 1 0 0 1 1 2 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 x L	8
	8	n 0 0 0 2 1 0 1 0 0 1 0 1 2 1 1 1 2 1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 x U	14
	9	n 0 1 0 2 1 1 1 2 1 0 2 1 2 1 2 1 2 1 2 1 1 1 1 1 2 1 1 1 1 1 1 1 0 0 0 0 0 x U	37
Strain	10	n 0 0 0 1 1 0 1 1 1 1 1 1 2 1 1 1 2 1 2 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 x U	18
1	11	n 0 0 0 2 1 0 2 0 0 1 0 1 2 1 1 0 2 1 2 2 1 1 1 2 1 0 2 1 0 0 0 0 0 0 0 0 0 x U	27
	12	n 0 0 0 1 0 0 2 0 1 1 2 1 2 1 2 1 2 2 2 2 1 1 1 2 2 2 2 1 2 2 1 0 0 0 0 0 0 x C	39
	13	A n 0 0 0 1 1 1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 x U	3
	14	A n 0 0 0 1 0 1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 x U	2
	15	A n 0 0 0 n 0 1 0 0 0 1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 x U	2
	16	A n 0 0 1 1 1 0 1 0 0 2 1 2 1 2 1 2 1 2 1 1 0 1 0 1 1 0 0 0 0 0 0 0 0 0 0 0 x U	23
	17	A n 0 0 1 1 1 0 1 0 0 2 1 2 0 2 0 2 1 2 1 1 1 1 2 1 2 2 1 0 0 0 0 0 0 0 0 0 x U	28
	18	A n 0 0 0 n 0 0 0 2 1 2 2 2 1 0 1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 x U	11
	19	A n 0 0 0 n 0 2 1 L O S T	
	20	A n 0 0 2 1 0 1 2 0 0 2 2 2 0 2 1 1 2 2 1 1 1 0 1 0 0 0 0 0 0 0 0 0 0 0 0 0 x U	24

Fig. 2 Representative score sheet. The replicative life span score sheet consists of rows representing individual cells within the experiment and columns representing time points. Each point in the grid represents the number of divisions that were recorded for the corresponding cell on the corresponding round of quantification

3. Examine each cell individually and perform the following actions:
   (a) If the cell has divided, separate the newly formed daughter cell from the original cell using the needle. Leave the daughter cell and move the original cell a few needle widths to the right to be discarded. On the score sheet, record that a virgin daughter cell was selected by entering "n" in the box for the first time point of the appropriate cell.
   (b) If the original cell has not yet divided, or if the new daughter cannot be removed, first examine the extra set of cells that were set off to one side in Subheading 3.3, **step 11**. If a virgin daughter can be separated from one of these cells, use it to replace the undivided original cell. Move the undivided original to the right to be discarded and record that a virgin daughter cell was selected by entering "n" on the score sheet.

(c) If a virgin daughter cell cannot be obtained from either the original cell or one of the extra cells, move the original cell a few needle widths to the left and record that a virgin cell was not selected by entering "A" on the score sheet. The cells for a given strain are often rearranged so that all "A" cells are grouped consecutively in the array (e.g., positions 8–10). This ensures that all "A" cells are dealt with appropriately during the next round of scoring, and not treated as newly dividing virgin cells.

4. Separate any remaining extra cells for the current strain. Leave five to ten individual cells and move the rest to the side to be discarded. These extra cells will be used later to replace selected cells that fail to divide.
5. Continue until all cells on the current plate have been examined.
6. Clean up excess cells by sweeping the needle across the surface of the plate. Collect and discard all cells except selected virgin daughter cells, undivided original cells, and the few remaining extra cells. All discarded cells on a plate can be simultaneously collected in this fashion and moved to the End Zone (*see* **Note 3**).
7. Repeat the Subheading 3.4, **steps 2** and **6** for all remaining plates in the experiment.

3.5 Quantify Replicative Life Span

Once virgin cells have been selected and arrayed, life span quantification can begin. The virgin cells selected in Subheading 3.4 are the individuals that will be followed for replicative life span, and will be referred to from this point forward as the "mother cells." This process consists of repeated rounds of incubating the life span plates and quantifying the number of divisions completed by each cell during incubation. The incubation time is targeted to allow each cell to divide 1 to 2 times. As the life span progresses and cell division slows, the incubation time is increased. An incubation time of 2 h is typically used for the first ~16 divisions and then increased in 1 h increments to 4 h (*see* **Note 4**).

During these rounds, the plates are kept at 30 °C during the incubation period and at room temperature while being scored. Plates can be stored at 4 °C overnight without affecting replicative life span to prevent cell division and allow researchers to rest. Each experiment should be advanced by a minimum of one round every day, with at least three rounds of dissection per plate optimal, including on weekends. Under no circumstances should an experimental plate to be stored at 4 °C for more than one day without incubation and dissection.

1. Incubate life span plates at 30 °C for a number of hours appropriate for the current stage of the life span and rate of cell division. If this is the first round of life span quantification, incubate for 2 h.

2. Place the first plate in the life span on the microscope stage and locate the first strain (i.e., the single hole punched at location 90, 10 in Subheading 3.3, **step 6**).
3. Examine each cell individually and perform the following actions:
   (a) If this is one of the first few rounds of the life span and a virgin cell has not yet been selected for the current position (i.e., the original cell is still present set off to the left, and an "A" was recorded for the previous time point on the score sheet), perform the actions outlined in Subheading 3.4, **step 3** for this cell.
   (b) If no bud is visible for the cell, record "0" on the score sheet. If the cell has not yet divided, and this is the third "0" recorded, replace the cell with a virgin cell and record "n" on the score sheet to indicate that a new virgin cell was selected at this time point. The virgin cell can be taken either from a neighboring cell within the life span, or from one of the extra cells. Refer to cells 1, 15, 18, and 19 in Fig. 2 for examples.
   (c) Remove all daughter and granddaughter cells from the mother cell by lightly touching the needle to the cells and gently tapping the side of the microscope. The mother cell will almost always be the largest cell present.
   (d) Determine the number of divisions that the mother cell has undergone since the last time point. Daughter and granddaughter cells will begin to divide as soon as they have reached sufficient size, so the number of cells present is not equivalent to the number of divisions. The pattern of cells present can be used to determine the number of mother cell divisions (*see* Fig. 3). Record the appropriate score on the score sheet.
   (e) Occasionally, older mother cells will lose the ability to complete cytokinesis and daughter cells cannot be removed (Fig. 3b). In this case, it is difficult to determine whether a new cell was produced by the mother cell or the attached daughter cell. Unless a definitive determination can be made that the removed cell was produced by the mother cell, remove any loose cells and record "0" on the score sheet.
   (f) Move all non-mother cells off to the right to be discarded.
   (g) If no cell is present at the current position, indicate that the cell has been lost by entering "LOST" on the score sheet for the current position and highlighting the row associated with that cell. Punch a single hole in the plate to indicate that a cell has been lost in that location. This cell will be excluded from analysis (e.g., cell 19 in Fig. 2).

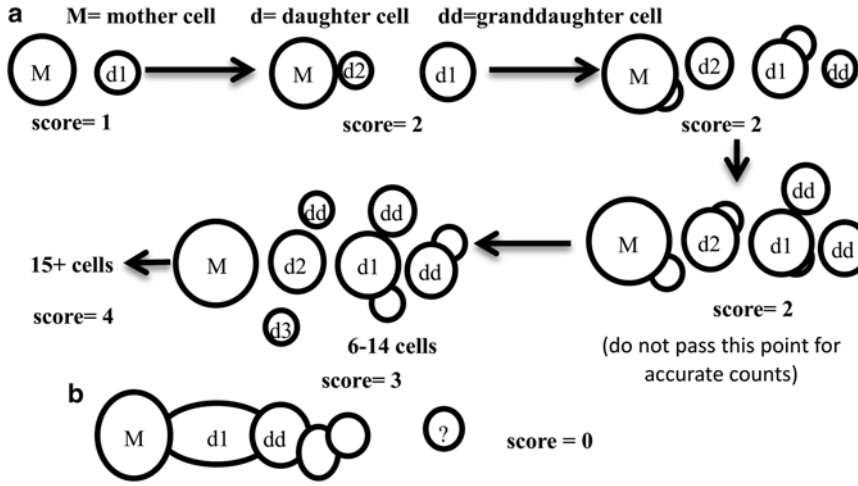

Fig. 3 Scoring number of mother cell divisions. Daughter cells produced by the mother cell will often begin dividing during the incubation time, complicating the quantification of mother cell divisions. (**a**) Reference chart for determining number of mother cell divisions based on cell pattern. (**b**) Mother cells that fail to complete cytokinesis are scored as having undergone 0 divisions for all future time points, because any cells which detach are unlikely to arise from the original mother cell

4. Once every cell has divided at least once for a given strain, all extra cells can be discarded.
5. Once all cells have been dissected and scored on a plate, clean up all excess cells as described in Subheading 3.4, **step 6**.
6. Review the scores given for each strain. For a given strain, if three rounds of quantification have been completed without a single cell division (i.e., all scores are recorded as "0" for all cells for three concurrent rounds), the strain is considered dead. Proceed to Subheading 3.6.
7. Wrap the plate in Parafilm and return to 30 °C.
8. Repeat the Subheading 3.5, **steps 1**–**7** for all remaining plates in the current experiment.
9. Review the score sheet for the completed time point. If the majority of strains were given a score of "0" or "1," increase the incubation time by 1 h to a maximum of 4 h.
10. Repeat the steps outlined in Subheading 3.5 until all strains have completed 8 concurrent hours of incubation without a single cell division.

3.6 Complete Replicative Life Span Measurement

For a given cell set, each cell is continually monitored until all cells in that set have ceased dividing. Once a set of cells is judged "dead" in Subheading 3.5, **step 6**, perform the steps outlined in this section to finalize the data.

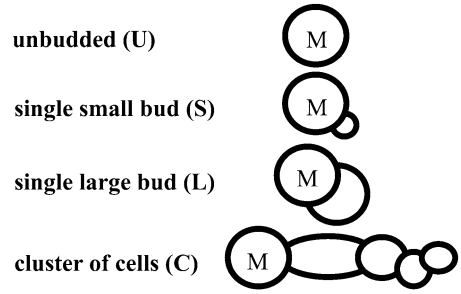

Fig. 4 Terminal cell states. Yeast cells can cease dividing at various points in the cell cycle. Provided are codes used to indicate common terminal cell states

1. Record "x" in the next available time point for all cells in the dead cell set except cells labeled as "LOST."
2. Cross off the patch number on the life span plate to indicate that quantification for that cell set has been completed.
3. Cells can cease division at various points in the cell cycle. Terminal phenotypes of mother cells can be recorded if desired. Our lab uses the following designations in the score sheets: "U" for unbudded, "S" for single small bud, "L" for single large bud, or "C" for cluster of cells (as in the case when a mother cannot perform cytokinesis and forms a string of connected cells). *See* Fig. 4 or a representation of each terminal cell state.
4. Once all cell sets in an experiment have been judged dead, the experiment is complete.

3.7 Manage Multiple Experiments Simultaneously

The previous sections describe the protocol for determining the replicative life span for cells included in one experiment. We have developed a system to simultaneously monitor replicative life span for multiple experiments. Our current setup consists of four dedicated microscopes with a 5th microscope for overflow, and a team of approximately 20 individuals, each contributing to replicative life span dissection part-time. At present, the team typically completes 1,200–1,400 mother cell life spans per week.

Analysis for a single experiment typically requires 3–4 weeks. The workload decreases over time as cell division slows and dissection is ceased on strains that undergo senescence. By staggering start dates, we are able to pair new experiments that require a large amount of dissection time with older experiments that require less time. On a typical week, two 600–800 cell experiments are started, with timing planned such that selection of virgin cells (Subheading 3.4) occurs on Monday for the first experiment and Tuesday for the second, allowing the maximum number of rounds of quantification to be completed prior to the first weekend.

Experiment	Incubation Time	Incubation Start Time	Dissection Start Time	Rounds Done Today	Note
485	4	9:00	1:00	I	
486	4	9:30	1:30	I	
487	3	10:00	1:00	I	
488	2	10:30	12:30	I	
489	2	12:00	2:00	II	
490	1.5	10:30	12:00		Pick daughters

Fig. 5 Example chart for managing multiple experiments simultaneously. When multiple experiments at different stages of analysis are progressing in parallel, a common area to record the current status of each is useful. For each experiment, we track the current incubation time each round, the start of the current incubation step, the time at which the next round of division scoring should begin, the number of rounds completed on the current day so far, and any notes relevant to the current or next scoring round. In the Kaeberlein lab, this information is recorded on a white board and updated as experiments are progressed through rounds of replicative life span quantification

The following section describes our process for managing multiple experiments at different stages of analysis simultaneously.

1. To keep track of the current status of each experiment, prepare a chart or table with columns for experiment name, current length of incubation time between rounds, incubation start time, targeted incubation end time, rounds of quantification completed on the current day, and current notes (*see* Fig. 5). We use a whiteboard in the microscope room, so that all team members can quickly refer to the current work status and so that the current status can be quickly updated as needed.
2. Designate three sections of the 30 °C incubator as "In progress," "Done," and "Incubating."
3. At the beginning of a day of replicative life span analysis, move life span plates for all experiments to the "Incubating" section of the 30 °C incubator. On setup days (Monday and Tuesday) some experiments may be left at 4 °C until setup is done, as time and personnel permit.
4. Update the status board as follows:
   (a) Change the incubation start time to the current time.
   (b) Change the target time to begin the next round of quantification based on both the current length of incubation required for each experiment, and the amount of incubation time that each experiment received at the end of the previous day.

(c) Reset the number of rounds completed today to 0.

(d) Add any experiments that are starting today to the board.

5. When the dissection start time for the first experiment arrives, move all plates for that experiment to the "In progress" section of the 30 °C incubator.
6. Take the first plate in the experiment back to the microscope and quantify the number of daughter cells produced by each mother cell on that plate (Subheading 3.5). We use a spreadsheet program in a shared network folder and a netbook computer at each microscope so that all team members have access to all score sheets at all times. For paper score sheets, some other method of sharing will have to be employed.
7. Return completed plate to the "Done" section of the 30 °C incubator. If this was the first plate in the experiment, change the incubation start time to reflect the current time, and the target dissection time to reflect the beginning of the next dissection time point. Add a tally mark to the number of rounds completed today.
8. Repeat Subheading 3.7, **steps 5**–7 for all remaining plates in the current experiment.
9. Once scoring is completed for an experiment, move all life span plates for that experiment to the "Incubating" section of the 30 °C incubator.
10. Continue scoring experiments and updating the board throughout the day.
11. At the end of the dissection time for the day, perform the following steps:

    (a) Move life span plates for all experiments to 4 °C.

    (b) Update the "Dissection Start Time" section of the status board to reflect the time that the plates were moved to 4 °C. This information will be used the following day to determine how much remaining incubation time is needed for each experiment.

    (c) Fill in any notes that will be relevant to the person starting the experiments in the morning.

3.8 Analyze Data

This section describes using data collected in the replicative life span assay described above to generate basic life span statistics and survival curves for a given cell set.

1. Sum the divisions across each row of the replicative life span score sheet to obtain the total number of divisions undergone by each individual cell in the experiment (Fig. 2). For each cell set, this will provide a list of replicative ages at death (i.e., senescence) for each individual in that set.

2. The list of individual replicative life spans can be used directly to generate basic life span statistics, such as mean, median, or standard deviation. The Wilcoxon Rank Sum test can be used with the lists of individual replicative life spans from two cell sets to calculate the probability that the two cell sets came from the same population. The mean replicative life span for wild type yeast in the BY4742 background is about 26 divisions.
3. To generate survival curves, complete the following steps:
   (a) Tally the number of individuals in each set that lived to each replicative age.
   (b) Invert the tally to determine the proportion of individuals within the population alive at each progressively increasing number of divisions.
   (c) Plot the proportion of individuals alive at each replicative age (*y*-axis) against replicative age (*x*-axis).

4 Notes

1. Plan to patch MAT*a* and MATα strains on different plates to avoid exposure to of strains to opposite mating factors. Up to 10 can be patched to a single plate.
2. Plates should be left unwrapped, face up on the bench with lids on. Cells are difficult to manipulate with the fiber optic needle if the plates are too wet. This drying period may include the time during which cells are patched and grown on the life span plate prior to life span analysis. If the plates will not be used immediately following the 72 h drying period, wrap in Parafilm and store inverted to minimize desiccation.
3. When moving cells on the life span plates, be sure not do drag them long distances, as this will often result in a cell falling off the needle and creating a colony, which can be problematic later in the life span. Lift the cells fully off the plate when moving long distances.
4. Note that the incubation time is approximate, and variations of 30–60 min from the desired incubation time are often acceptable, as long as most mother cells have not produced more than two daughter cells since the previous time point.

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