

Video Article

Continuous High-resolution Microscopic Observation of Replicative Aging in Budding Yeast

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Abstract

We demonstrate the use of a simple microfluidic setup, in which single budding yeast cells can be tracked throughout their entire lifespan. The microfluidic chip exploits the size difference between mother and daughter cells using an array of micropads. Upon loading, cells are trapped underneath these micropads, because the distance between the micropad and cover glass is similar to the diameter of a yeast cell (3–4 μm). After the loading procedure, culture medium is continuously flushed through the chip, which not only creates a constant and defined environment throughout the entire experiment, but also flushes out the emerging daughter cells, which are not retained underneath the pads due to their smaller size. The setup retains mother cells so efficiently that in a single experiment up to 50 individual cells can be monitored in a fully automated manner for 5 days or, if necessary, longer. In addition, the excellent optical properties of the chip allow high-resolution imaging of cells during the entire aging process.

Video Link

The video component of this article can be found at <https://www.jove.com/video/50143/>

Introduction

Budding yeast is an important model organism for aging research¹. Until recently studying replicative aging in yeast cells was a laborious process requiring a dissection method, in which each bud was manually removed from the mother cell^{2,3}. To solve this problem, we recently presented a novel microfluidic setup able to track individual mother cells throughout their entire lifespan⁴.

In our microfluidic chip, yeast cells are trapped under soft elastomer-based micropads (see **Figure 1**). A continuous flow of medium washes away newly formed daughter cells and provides the cells with fresh nutrients. In a single experiment, up to 50 mother cells can be monitored in a fully automated manner throughout their entire replicative lifespan. Due to the excellent optical properties of the microfluidic chip, it is possible to simultaneously monitor different aspects of yeast cell biology (e.g. by using fluorescent proteins).

Compared to the classical dissection method, the microfluidic setup provides substantial advantages. It ensures a defined and constant environment during the whole aging experiment. It requires no expensive specialized equipment and can be run on any microscope equipped with automated focus and time-lapse abilities as well as temperature-control for cell cultivation. The production and operation of the microfluidic chips can be learned within a few days. In addition, cells can be directly loaded from an exponentially growing culture, an advantage over another recently published microfluidic method⁵, which requires biotinylation of mother cells.^a Combined with high-resolution imaging, the here described method can be used to measure gradual changes in cellular morphology, protein expression and localization during yeast aging in an unprecedented manner. The capability for long-term monitoring of single cells also provides unique possibilities for yeast cell cycle studies.

^aThis method has recently been optimized to remove the biotinylation from the protocol¹⁶, which was published while this manuscript was in review.

Protocol

1. Production and Preparation of a Silicon Wafer Mold

Microfluidic chips are created from a silicon wafer mold produced by soft lithography. These wafers can be reused many times to produce microfluidic chips. It is advisable that production of a respective wafer is performed by a group specialized in microfluidics⁶.

The wafer is made in a two-step photolithography process using two different layers of negative photoresist, SU-8⁷. The bottom layer is used to generate the cell trapping area (SU-8 2002; height 3–4 μm), whereas the channels are made with the top layer (SU-8 2010; height 10 μm). An impression of the wafer production process can be found in Huang *et al.*⁸. The drawings of the microfluidic chips can be obtained from the authors as well as advice on how to obtain a respective wafer.

1. Once the wafer is obtained, cut one piece of Tough-Tag into thin strips of about 0.5 mm by 3 mm with a scalpel and place them carefully on the wafer slightly on top of the channel structure between the inlet channels and the micropad array (**Figure 2A**). This will result in the formation of a large additional channel in each chip, which will not only be used during cell loading, but is also important for the stable operation of the microfluidic device.
2. Cover the glass Petri dish with a double layer of aluminum foil and put the wafer in the Petri dish. The aluminum foil will prevent PDMS from running in between the wafer and the Petri dish. If PDMS does creep in between the wafer and the glass Petri dish, the wafer will become permanently fixed in the Petri dish. Although the wafer is still useable, it will not be possible to replace the glass Petri dish if it breaks and the PDMS will need to be cut out afterwards.

2. Production of Microfluidic Chips

1. Place an empty plastic cup on the balance and tare the balance. Pour 40 g PDMS base into the plastic cup (for a 12 cm diameter glass Petri dish). Add PDMS curing agent with a disposable pipette in a w/w ratio of 1:10, *i.e.* about 4 ml.
2. Mix the PDMS and curing agent thoroughly for several minutes. This can be done with the disposable plastic pipette that was used to pipette the curing agent. It is important that the mixture is well mixed prior to pouring it on top of the wafer. Poor mixing will cause part of the PDMS not to polymerize.
3. Pour the PDMS on top of the wafer in the glass Petri dish.
4. Mixing of PDMS with the curing agent will lead to bubble formation in the PDMS mixture. These bubbles need to be removed before the PDMS polymerizes. The PDMS can be degassed after pouring it on top of the wafer by placing the Petri dish in a desiccator connected to a vacuum pump. With this setup, it takes about 30 min to remove all bubbles from the mixture.
5. Polymerize the PDMS by placing the Petri dish with the wafer on top of a hot plate at 120 °C for 1 hr and then at 65 °C for another hour.
6. Remove the aluminum foil together with the wafer and polymerized PDMS from the glass Petri dish. Peel off the aluminum foil and the thin residual layer of polymerized PDMS from the back of the wafer. The layer of PDMS on top of the wafer can then be separated from the wafer by lifting it up carefully.
7. Place the PDMS layer upside down (with the channels facing up) on the bench and cut out the single chip designs imprinted on the PDMS carefully with a scalpel. Try to retain about 3 mm of PDMS around the edges of the channels to improve attachment of the chip to the cover glass later on.
8. Punch holes in the PDMS at the ends of the inlet, outlet and side channel (**Figure 2A**) by pushing a 20 Gauge Luer stub all the way through the PDMS in a straight manner.

Make sure the PDMS chip is still lying upside down on the bench while punching the holes. This prevents PDMS from sticking to the inside of the channel, which could cause blockage of the channel.

9. After punching each hole, remove the column of PDMS in the Luer stub with tweezers before pulling the stub out again. The PDMS column could otherwise stay behind and block the just punched hole.
10. Clean the surfaces of the chip from dust particles and residual PDMS by placing scotch tape on it and immediately removing the tape again. Similarly clean the cover glass to which the chip will be attached.
11. Place the cleaned chip and cover glass below the UV lamp of the Benchtop UV-Ozone Cleaner. The sides that need to be bonded together must face the lamp.
12. Expose the PDMS and cover glass to the UV light for 6–8 min and directly afterwards put the chip on the cover glass by placing the exposed surfaces on top of each other.
13. Gently tap around the sides of the chip to promote attachment of the PDMS to the cover glass and to remove any air bubbles. Do not tap on top of the channel structure as this may cause the micropads to get attached to the cover glass as well.
14. Place the newly made microfluidic setup on a hot plate at 100 °C for 60 min. Test the strength of the bonding between the cover glass and PDMS chip by trying to lift up the edges of the PDMS chip slightly. When the PDMS block cannot be lifted from the glass surface, the bonding is successful and the chip is ready for use.

3. Preparing the Chip for Cell Loading

Failure of a microfluidic device can potentially cause leakage of medium into the microscope. To avoid this, it is advisory to use a metal holder and/or seal the sides of the chip where the PDMS meets the glass with nail polish or epoxy glue. This prevents leakage of medium in case the PDMS chip is not bonded well enough to the cover glass. Also the tubing connections can be secured around the insertion points in a similar manner to avoid leakage of medium. A home-made water sensor that switches off the syringe pump in case of leakage can be used as an extra safety precaution. Drawings of the metal holder especially designed for the microfluidic chip can be obtained from the authors.

1. Place the microfluidic chip in the metal holder, with silicone gel between the glass of the chip and the metal parts of the holder to create a waterproof seal. Screw the nuts gently, not to break the glass.
2. Connect thin tubes (ca. 5-15 cm long) to the side channel and the outlet channel of the chip. The use of tweezers makes it easier to insert the tubing in the punched holes.
3. Fill a 50 ml Luer-Lok syringe with culture medium, remove the air from the syringe and connect it sequentially to a syringe filter, a 20 Gauge Luer stub, a short thick tube (ca. 2 cm long) and a thin tube. Make sure that the thin tube is long enough to span the distance between the syringe pump and the microscope stage.
4. Place the syringe in the syringe pump, fast forward the pump until the thin tube is completely filled with medium.
5. Push the thin tube connected to the syringe into the inlet channel of the chip and let the medium flow through the chip at 10 $\mu\text{l}/\text{min}$ (**Figure 2B**). Collect the medium leaving the chip (e.g. in a Petri dish). The medium will run out via the side channel because of the resistance difference between the Tough-Tag made large side channel and the outlet channel.
6. Place the chip in the microscope stage and set the focus of the microscope on the pads.

The use of oil immersion objectives is preferred over that of water immersion objectives because oil will not dry out over the time-course of the experiment.

Depending on the microscope there might be issues with retaining the focus during the initial hours of the experiment. It is therefore advisable to leave the chip for one to two hours in the microscope stage so it can settle down before loading cells and starting the experiment.

4. Loading of Yeast Cells into the Microfluidic Chip

1. Connect a 5 ml Luer tip syringe to a 20 Gauge Luer stub and a thick tube (ca. 3 cm).
2. Take a sample of the cell culture to be loaded into the chip.

The preferred cell count for loading is between $1-5 \times 10^6$ cells per ml. Cultures with higher cell counts need to be diluted before loading. When culturing cells on YPD, cell loading efficiency can be improved many-fold if cells are first washed with and resuspended in minimal medium containing a similar glucose percentage before loading. During operation of the chip, YPD medium can again be safely used.

3. Load approximately 1 ml of the cell suspension into the syringe and remove all the air from the syringe.
4. Decrease the flow rate of the syringe pump to 0.5 $\mu\text{l}/\text{min}$. The low and continued flow enforced by the syringe pump during loading (in step 4.5) will push non-attached cells out via the side channel (**Figure 2C**).
5. Connect the syringe containing the cell suspension to the tube of the outlet channel. Load the cells by pressing on the plunger of the syringe gently. Observe the cells coming in and settling underneath the micropads via the ocular or via the computer screen.
6. Maintain pressure on the plunger until sufficient cells settle underneath the pads. The optimum load is 1-3 cells per pad. As cells settle underneath the pads randomly, it is quite common that a few pads do not contain any cells and other pads are completely filled with cells.
7. Disconnect the syringe used for cell loading and the thick tube connected to it from the thin tubing of the outlet channel, flush the side channel for a couple of minutes at higher flow rates (10 $\mu\text{l}/\text{min}$) to remove air bubbles and/or cells that did not yet exit the chip (**Figure 2B**). If cells are not removed from the side channel, they can start to grow in the side channel and interfere with the experiment later on.
8. Put the flow of the syringe pump back down to 0.5 $\mu\text{l}/\text{min}$ and close the side channel off by connecting it to a thick tube (ca. 5 cm long) containing a catheter plug at its end (**Figure 2D**).
9. Increase the flow to a final flow rate of 1-5 $\mu\text{l}/\text{min}$. Flow rates may vary depending on medium and yeast strain.
10. Start a movie with the microscope and camera settings suitable for the goal of the experiment. Make sure that the speed of the microscope stage is set to such a low speed that the oil can follow. For determination of replicative lifespan, image cells every 10 min. To protect cells from the minute amounts of UV light emitted by the halogen lamp, which is used during DIC acquisition, it is advisable to place an additional UV filter in the light path. When using fluorescence microscopy, it is preferred to image the cells less frequently (e.g. every 20 min) to avoid phototoxic effects. The experiment is completed when all cells loaded at the start of the experiment are dead, which under normal conditions takes up to 5 days. It is recommended to regularly check the focus of the images during the experiment and if necessary adjust it.

Representative Results

In this protocol, cells are loaded into the microfluidic chip directly from mid-exponential culture. To ascertain whether the age distribution of cells trapped in the microfluidic chip is similar to that of the culture prior to loading, cells were stained with wheat agglutinin conjugated to FITC (WGA-FITC) to visualize bud scars. As can be seen in **Figure 3**, the entrapment of cells under the micropads of the microfluidic chip is not biased to cells of a certain age.

Replicative lifespan can simply be determined by counting the number of buds that are produced by a single mother cell. This data is transformed into a lifespan curve by plotting the percentage of viable cells against the number of buds produced. Statistical tests, such as those performed by Kaerberlein, *et al.*⁹, can be used to compare lifespan data from different experiments. **Figure 4** shows an example of lifespan curves obtained for a WT BY4741 yeast strain and two mutants (*sir2 Δ* , *fob1 Δ*).

Movie 1 Time-lapse movie of a single WT BY4741 yeast cell growing on YPD medium. Images were taken every 10 min. The cell produces a total of 30 buds before it dies. Scale bar, 5 μm . [Click here to view movie.](#)

An important advantage of the microfluidic method is the ability to use continuous high-resolution imaging. To monitor changes in mitochondrial morphology as cells age, an aging experiment was performed with BY4742 yeast cells expressing ILV3-GFP, which is targeted to the mitochondria. **Figure 4** gives an overview of mitochondrial morphology for the same set of cells at different replicative ages (0 buds, 10 buds and prior to death). The morphology changes seen in mid-aged cells resemble those reported in literature before¹⁰. Although in this particular representative experiment, the flow rate of the medium was not optimal and daughter cells were retained with a relatively high frequency in

contrast to **Movie 1**, the quality of the experiment was still sufficient to analyze mitochondrial morphology in 49 individual cells over their entire lifespan.

Movie 2 Time-lapse movie of a single BY4742 yeast cell expressing ILV3-GFP, which is used to visualize the mitochondria. Images were taken every 30 min. Both the brightfield as well as the GFP images were background corrected before merging the two channels into a single image using ImageJ. Scale bar, 5 μm . [Click here to view movie.](#)

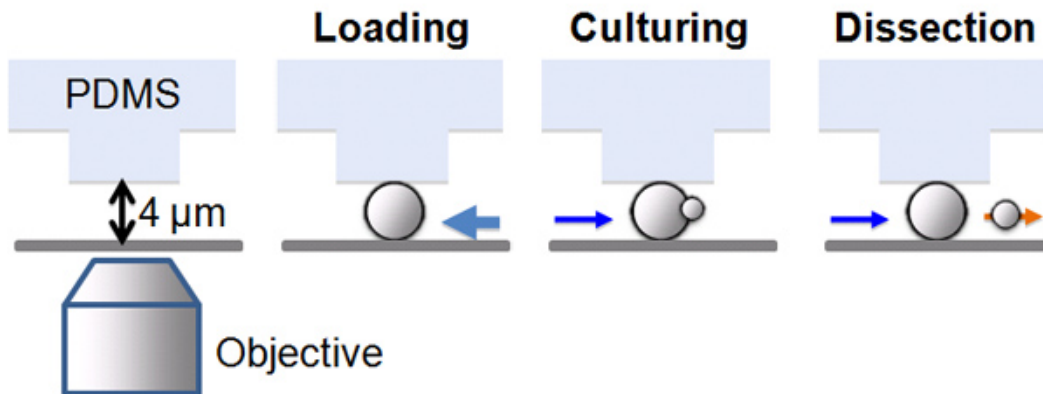


Figure 1. Schematic overview of the microfluidic setup. The height difference between the PDMS micropad and cover glass is similar to the diameter of a yeast cell (about 3–4 μm). **Loading:** Yeast cells are loaded under the PDMS micropads. **Culturing:** Fresh medium flows continuously over the trapped cells (blue arrow). **Dissection:** Emerging daughter cells are flushed away by the flow of medium (orange arrow).

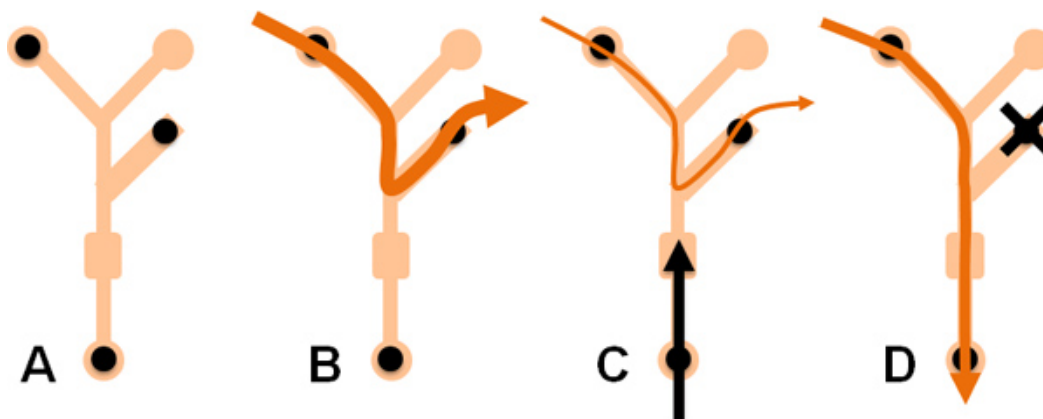


Figure 2. Schematic illustration of the channel design of the microfluidic chip. **A.** Black circles indicate the location of the punched holes at the end of the inlet, outlet and side channel. **B.** Medium flows through the inlet channel (10 $\mu\text{l/min}$) and exits via the larger side channel (orange arrow). There is no flow through the outlet channel, because of the resistance difference between the side and outlet channel. **C.** Cells are loaded into the chip (black arrow) via the outlet channel. The flow through the side channel is reduced to 0.5 $\mu\text{l/min}$. **D:** After flushing the side channel, the side channel is blocked and medium starts to flow over the cells trapped underneath the micropads (1–5 $\mu\text{l/min}$).

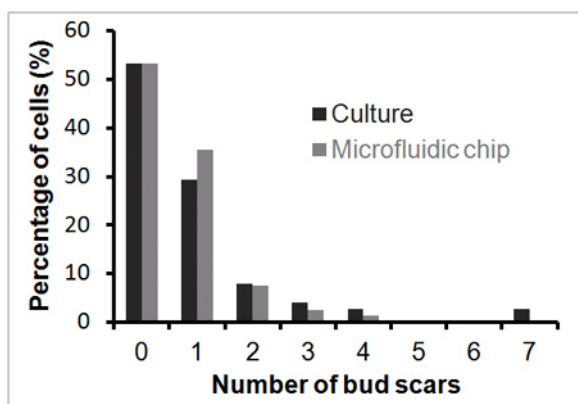


Figure 3. Cell loading onto the chip is not biased towards a certain cell age. A mid-exponential YSBN6 WT culture (10^7 cells/ml) was labeled with WGA-FITC (2 μ l of 5 mg/ml stock solution per 10^6 cells) for 1.5 hr. Cells were then put on a glass slide or loaded into the microfluidic chip. Z stacks were made (15 z-slices at 0.8 μ m distance) at an excitation wavelength of 470 nm using a 63X magnification water immersion objective. The number of bud scars on each cell was counted manually. Bud scars were determined from 75 cells in the culture and 79 cells in the microfluidic chip.

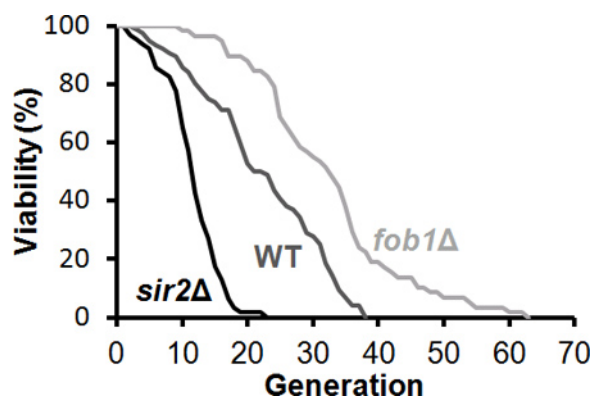


Figure 4. Examples of life span curves obtained for BY4741 WT (n=76), *sir2Δ* (n=63) and *fob1Δ* (n=58) using the here described microfluidic device. Both deletions have a strong, yet opposite, effect on the measured median replicative lifespan of the yeast cells. For each experiment, the strains were grown overnight in YPD medium to stationary phase and then allowed to resume exponential growth by dilution into fresh YPD medium and incubation at 30 °C for 3 hr before starting the experiment. Data was obtained from Lee *et al.*⁴.

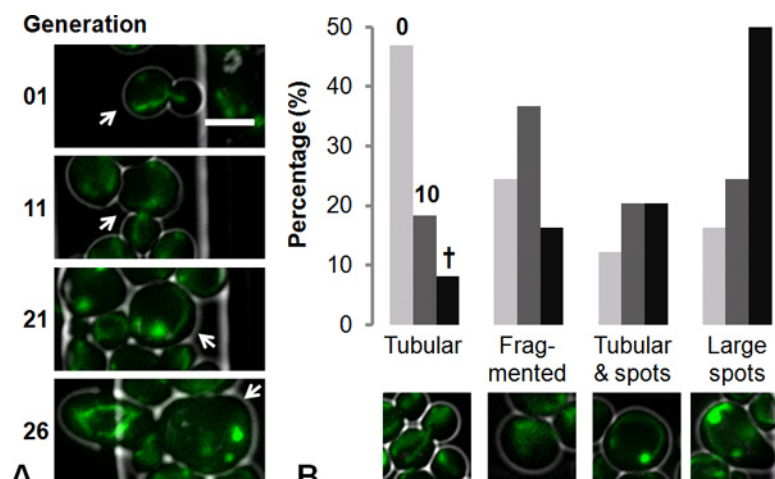


Figure 5. Mitochondrial morphology as a function of age. **A:** A representative example of age-associated changes in mitochondrial morphology in a single cell (white arrow). All images are scaled identically. Scale bar, 5 μ m. **B:** Mitochondrial morphologies observed in a set of 49 cells before producing their first bud (0), after 10 buds (10) and prior to death ($\dagger$). An example image of each morphology class is included. The presented data was obtained during a single experiment and the same set of cells was used for scoring morphology at each time point. Mitochondria were visualized in a BY4742 strain expressing a GFP-tagged version of the mitochondrial protein ILV3 (obtained from the GFP collection database¹⁵). Both the brightfield as well as the GFP images were background corrected before merging the two channels into a single image using ImageJ.

Discussion

The microfluidic method described here is an important novel tool in aging research as it enables simple and automated generation of yeast replicative lifespan data in combination with continuous high resolution imaging. These attributes are major improvements over the experimental possibilities of the classical dissection method, yet there are a few limitations of the method that need to be taken into account.

Note that the determined replicative lifespan can be affected by the efficiency of the retention of mother cells: Every cell kept under a micropad has a certain probability to be washed out from the chip (e.g. the budding cell of a neighboring cell could push it away from the micropad). The integrated probability of a cell to be washed out increases with increasing lifespan. Thus, it is more likely that short-lived cells completed their life cycle before being washed away than long-lived cells. The fact that similar lifespan data was generated with the microfluidic setup⁴ as with the classical microdissection method¹¹⁻¹³ indicates that this potential issue is only minor, if not irrelevant.

It is important to note that with the microfluidic setup it is not possible to select virgin daughter cells at the start of the experiment, in contrast to the classical dissection method. Nevertheless when loading cells from an exponentially growing liquid culture into the chip, the majority of the loaded cells are newly born or relatively young (i.e. about 54% of the cells never budded before and around 27% once)¹⁴. This means that lifespans are underestimated by 1 to 2 generations at most. In the exceptional case that the age structure of the cell population is significantly altered, it is possible to enrich the cell population for virgin daughters prior to loading, e.g. by gradient centrifugation or elutriation.

The microfluidic chip can in principle be used to study haploid yeast strains in different culture media. However, different yeast strains and/or media may require some optimization of flow rate to allow efficient retention of mother cells. As cells are retained based on their size, it is not possible to load yeast strains that are significantly larger or smaller than 3-4 μm in diameter, such as diploid yeast cells. However by producing a wafer with different photoresists, microfluidic chips could be generated with a different spacing between the cover glass and micropads to allow loading of these differently sized cells.

Although the protocol presented here describes how to make a chip with a single channel, it is possible to add multiple channels in a single chip and run parallel experiments simultaneously. Depending on the number of desired channels in a single chip, it may be necessary to create an adapted wafer in which the channels are spaced more closely together.

Disclosures

The authors declare that they have no competing financial interests.

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