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Construction and use of a microfluidic dissection platform for long-term imaging of cellular processes in budding yeast

Daphne H E W Huberts[1,6], Sung Sik Lee[2,6], Javier Gonzáles[3], Georges E Janssens[4], Ima Avalos Vizcarra[5] & Matthias Heinemann[1]

[1]Molecular Systems Biology, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Groningen, The Netherlands. [2]Institute of Biochemistry, Zurich, Switzerland. [3]Probability and Statistics Research Group, Johann Bernoulli Institute of Mathematics and Computer Science, University of Groningen, Groningen, The Netherlands. [4]Department for the Biology of Ageing, European Research Institute for the Biology of Ageing, University Medical Centre Groningen, University of Groningen, Groningen, The Netherlands. [5]Department of Health Sciences and Technology, Zurich, Switzerland. [6]These authors contributed equally to this work. Correspondence should be addressed to M.H. (m.heinemann@rug.nl).

This protocol describes the production and operation of a microfluidic dissection platform for long-term, high-resolution imaging of budding yeast cells. At the core of this platform is an array of micropads that trap yeast cells in a single focal plane. Newly formed daughter cells are subsequently washed away by a continuous flow of fresh culture medium. In a typical experiment, 50–100 cells can be tracked during their entire replicative lifespan. Apart from aging-related research, the microfluidic platform can also be a valuable tool for other studies requiring the monitoring of single cells over time. Here we provide step-by-step instructions on how to fabricate the silicon wafer mold, how to produce and operate the microfluidic device and how to analyze the obtained data. Production of the microfluidic dissection platform and setting up an aging experiment takes ~7 h.

INTRODUCTION

Long-term microscopic imaging of single yeast cells over multiple generations is essential for unraveling the dynamics and mechanisms of important cellular processes, such as cell division and aging. However, it poses a major technical challenge because the original mother cell is rapidly outnumbered by its progeny (**Fig. 1**). We overcome this problem with a polydimethylsiloxane (PDMS)-based microfluidic device, in which yeast cells are trapped underneath micropads[1]. A continuous flow of culture medium through the channel, in which the micropads are located, ensures a constant growth environment and washes away the newly produced daughter cells, which are not retained because of their smaller size (**Fig. 1**). The alignment of the cells in a single focal plane enables high-resolution imaging of cell morphology and fluorescent labels over several days.

Applications of the method

The microfluidic platform is especially valuable for aging-related research, e.g., to determine replicative lifespan or to monitor the phenotypes of aging cells[1–3]. However, it is also well suited for other studies that require long-term tracking of cells, e.g., cell cycle studies or investigations of organelle inheritance and protein trafficking. The ability to rapidly exchange culture medium inside the platform, e.g., with external valves[4,5], can be an asset when studying cellular responses to environmental changes.

Comparison with other methods

Although there are many alternative methods for studying growing yeast cells with time-lapse microscopy[6–10], these setups are typically limited to the observation of about seven or eight generations because daughter cells are not adequately removed.

The microfluidic dissection platform is mainly used to measure the replicative lifespan of yeast cells. Until now, these types of measurements have predominantly been performed using a micromanipulation dissection method developed 50 years ago[11,12]. A major bottleneck in this classical method is that each bud needs to be manually removed, thereby making the measurement of replicative lifespan extremely labor intensive and time consuming. The researcher needs to be considerably skilled to obtain reproducible lifespan data and to avoid inducing stress in the cells[13,14]. To create periods in which the researcher can rest, cells are repeatedly stored in the refrigerator to slow down their growth rate, which may have profound effects on cell physiology, e.g., growth phase and respiration[15]. Further, as the cells need to be grown on a thick opaque agar pad, it is almost impossible to image them with high-resolution microscopy as they age.

In contrast, the microfluidic dissection platform is fully compatible with high-resolution (fluorescence) imaging. As daughter cells are automatically removed, measurement of replicative lifespan only requires about 5 d, the amount of time that is necessary for all of the initially loaded cells to complete their lifespan. The method also allows cells to be maintained under constant environmental conditions throughout the experiment. Although the cells are retained under the micropads for a prolonged period of time, the physical forces exerted on the cells by the micropads seem small and there is no induction of stress responses[1]. Indeed, similar lifespans for wild-type cells, as well as several mutants, e.g., *fob1Δ* and *sir2Δ*, were measured with the microfluidic dissection platform and the classical method[1].

Despite these advantages, the microfluidic dissection platform has certain limitations. It is difficult to track lineages of cells because daughter cells are only occasionally retained. For these types of experiments, other methods are recommended (see e.g., refs. 6,9–11). In addition, it is not possible to select for virgin daughter cells before starting the aging experiment. Although this may seem to be a crucial disadvantage, it has only a minor effect on the experimental outcome, as the majority of cells in an exponentially growing
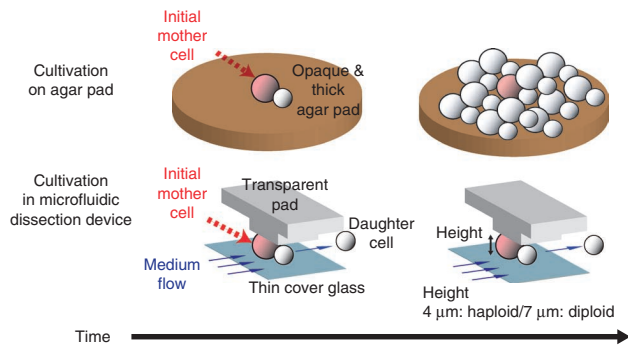
Figure 1 | Removal of daughter cells is essential for lifelong tracking of aging yeast cells. When grown on an agar pad, an aging cell (in red) becomes quickly overgrown by its progeny. In the microfluidic dissection platform, daughter cells are removed by a continuous flow of the culture medium, making it possible to track an aging cell during its entire lifespan. The height underneath the micropads can be adapted to trap cells with different cell sizes, e.g., use 4 μm for haploid cells and 7 μm for diploid cells.

liquid culture are newly born or relatively young (i.e., 54–59% of the cells never budded before and 27% budded once[16]).

Recently, two methods were published with capabilities similar to the setup presented here[2,17]. Although the method described by Xie *et al.*[17] suffers from the disadvantage that the cells need to be biotinylated before the experiment, the microfluidic device published by Zhang *et al.*[2] closely resembles our experimental setup.

Experimental design

Design of the microfluidic device. The microfluidic dissection platform consists of a Y-shaped microchannel with an array of micropads (**Fig. 2**). Inside the array, the micropads are positioned in a Riga pattern to optimize loading of the cells (**Fig. 2a**). The dimensions of each micropad are 30 μm × 15 μm. The side channel positioned upstream of the micropad array (**Fig. 2b**) is relatively much wider and higher compared with the medium channel and is primarily used during cell loading to remove excess medium. To increase the measurement throughput of the microfluidic dissection method, it is possible to design a silicon wafer mold with a set of identical microfluidic channels arranged in a parallel fashion (**Fig. 3**).

Fabrication of the silicon wafer mold. The microstructures of the microfluidic dissection platform are produced by casting PDMS against a silicon wafer mold[18]. This mold is produced using UV photolithography. In this process, layers of light-sensitive material (SU-8 photoresist) are spin coated on a silicon wafer. Masks are then used to expose specific parts of the wafer to UV and create microstructures by light-induced cross-linking. The height of the structures produced is determined by the height of the applied SU-8 layer.

Three SU-8 layers and photomasks (**Fig. 2a**) are needed to fabricate the different structures of the microfluidic dissection platform on top of a silicon wafer. The thickness of the first SU-8 layer determines the height underneath the micropads. For haploid cells, the bottom layer of the photoresist should be around 4 μm high, which is similar to the diameter of these cells. It is possible to adapt the thickness of this layer to accommodate cells of different diameters, e.g., using a 7-μm-thick layer for diploid cells. The main channel

with micropads is formed using a 10-μm-high second layer of SU-8. The final third layer of photoresist is relatively high (100 μm) to produce the side channel. Silicon wafer molds can be used many times over and only a single wafer is needed to produce up to 18 individual platforms simultaneously.

Setting up an aging experiment. Although the microfluidic dissection platform has been used for yeast from various strain backgrounds, e.g., CEN.PK, YSBN6, W303 and BY4741, it may be necessary to optimize the experimental settings, e.g., flow rate and cell load, for different experimental conditions. For example, for YSBN6, we generally use a flow rate of 3 μl min⁻¹, whereas for CEN.PK, we recommend a flow rate of 5 μl min⁻¹. This variation in flow rate may be due to differences in the average cell size between the different strains, mutants and growth conditions[19,20]. In addition, certain yeast cells, e.g., W303 or particular mutants, have a tendency to form clumps that interfere with cell loading. If these clumps are not resolved by vortexing, one can opt to use sonication before cell loading[1].

The use of synthetic media is strongly recommended because cell retention is greatly reduced in the presence of rich media, such as yeast peptone dextrose (YPD). Because cell loading is also greatly reduced when the platform is preflushed with 1% (wt/vol) BSA solution before cell loading, we attribute the reduced cell retention with YPD to the presence of proteins/peptides in this type of medium. However, it is possible to perform experiments with rich medium. In this case, an additional step of washing and temporarily resuspending cells in synthetic medium is required before loading the cells into the microfluidic setup. A continuous flow of rich medium can then be used during the experiment.

Before placing the microfluidic device in the microscope stage, it is advisable to always check the bonding between the PDMS and the coverslip. Detachment of the PDMS from the coverslip can lead to spillage of culture medium and cause serious damage to the microscope, especially when the microfluidic platform runs unsupervised for prolonged time periods. A good precautionary measure is sealing the edges where the PDMS and the coverslip meet with epoxy glue. Other connections, e.g., that between the polytetrafluoroethylene (PTFE; e.g., Teflon) and the microbore
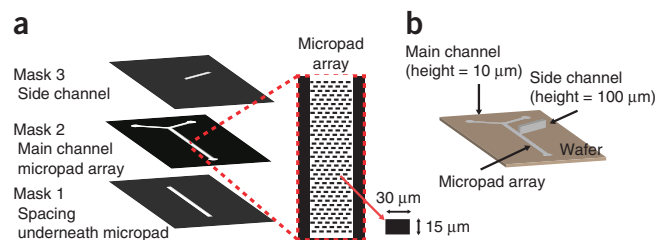

Figure 2 | Design of the silicon wafer mold. (**a**) The silicon wafer mold is made by photolithography using three different layers of SU-8 photoresist and masks. The first mask is required to produce the spacing between the micropads and coverslip. The second mask is used to create the Y-shaped medium channel and the array of micropads. The micropads (see inset) are positioned in a Riga pattern to optimize cell loading. The side channel is created with a third mask containing a simple straight line. The thickness of each SU-8 layer determines the height of each structure. (**b**) Overview of the channel structure in the microfluidic dissection platform. It contains a Y-shaped medium channel (height = 10 μm), an array of micropads (height = 4 μm) for trapping yeast cells and a large side channel (height = 100 μm) to remove excess medium during loading of the cells.

Figure 3 | Illustration of the most important steps in the protocol. (**a**) An example of a wafer carrying different channel designs, such as single Y-shaped channel designs (bottom), multiplexed Y-shaped channel design (top) and an alternative design in which the side channel is omitted (center), which makes it possible to add even more channels in a single microfluidic dissection platform. (**b**) Holes are pierced at the ends of the outlet, inlet and side channel with a Luer stub. (**c**) The column of PDMS is removed from the Luer stub before pulling it back out again. (**d**) After exposure to UV light in the ozone bench cleaner, attachment of the PDMS chip to the coverslip is promoted by gently tapping on the sides of the chip. (**e**) PTFE microbore tubing is inserted in the microfluidic device with tweezers. (**f**) A 50-ml syringe is prepared for an experiment by filling it with the medium and sequentially connecting a syringe filter, 20-gauge Luer stub, short piece of Tygon tubing and PTFE tubing to it.
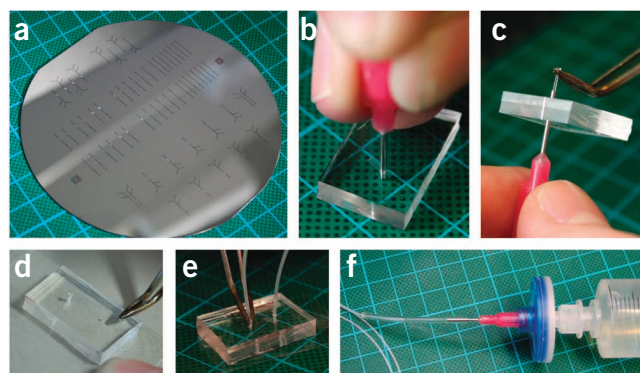
tubing (Steps 36 and 42), can be secured with epoxy glue or nail polish. In addition, it is advisable to use a holder for the coverslip, in which spilled medium can be collected.

An overview of the procedure. Below, we describe the fabrication of the silicon wafer mold, the fabrication of a microfluidic chip and its use. Once the silicon wafer mold is produced, which is a task that can also be fulfilled by a specialized laboratory or a commercial company, many microfluidic chips can be produced from the same mold. Thus, the first 16 steps of the below-described protocol do not need to be performed every time an actual long-term imaging experiment is done.

MATERIALS

REAGENTS
- Computer-aided design (CAD) software (e.g., AutoCAD)
- Photoresist SU-8 2005 (MicroChem) **! CAUTION** Photoresists are highly flammable. Wear safety goggles, gloves resistant to organic acids and protective clothing while handling photoresists. Avoid contact with eyes, skin or clothing.
- Photoresist SU-8 2010 (MicroChem) **! CAUTION** Photoresists are highly flammable. Wear safety goggles, gloves resistant to organic acids and protective clothing while handling photoresists. Avoid contact with eyes, skin or clothing.
- Photoresist SU-8 100 (MicroChem) **! CAUTION** Photoresists are highly flammable. Wear safety goggles, gloves resistant to organic acids and protective clothing while handling photoresists. Avoid contact with eyes, skin or clothing.
- SU-8 developer (MicroChem) **! CAUTION** 1-Methoxy-2-propyl acetate is toxic. Wear protective gear while handling it.
- Polished silicon wafer (4 inch, Prologsemico)
- Transparency masks for photolithography ▲ **CRITICAL** Printing resolution of the masks should be above 65,024 d.p.i.
- DC Sylgard 184 elastomer (PDMS, Mavom BV, cat. no. 1060040) ▲ **CRITICAL** DC Sylgard elastomer contains PDMS elastomer base and a curing agent. PDMS base is very viscous and sticky. It is therefore advisable to wear gloves when handling PDMS base. Minor amounts of spilled PDMS base can be cleaned up using paper towels. Curing agent and PDMS need to be thoroughly mixed to ensure homogenous polymerization.
- Silicone grease (GE Bayer silicones; Sigma-Aldrich, cat. no. 85403-1EA)
- PTFE microbore tubing (0.012 inch inner diameter (i.d.) × 0.030 inch outer diameter (o.d.); Cole Parmer, cat. no. EW-06417-11)
- Tygon microbore tubing (0.030 inch i.d. × 0.090 inch o.d.; Cole Parmer, cat. no. EW-06418-03)
- *Saccharomyces cerevisiae* strain in liquid culture (e.g., BY4741; Euroscarf, cat. no. Y00000) in yeast nitrogen base (YNB; Formedium, cat. no. CYN0201) with glucose (D(+)-glucose monohydrate, 2% (wt/vol); Merck, cat. no. 1.08342.1000), if necessary supplemented with complete supplement mixture

EQUIPMENT
- Clean room facility (only for production of silicon wafer mold)
- Spin coater (Karl Suss, cat. no. CT 60; only for production of silicon wafer mold)
- Mask aligner (Karl Suss, cat. no. MA6; only for production of silicon wafer mold)
- Hot plate (Prazitherm, cat. no. PZ60)
- Balance (Sartorius corporation, cat. no. ED4202S)
- Vacuum pump (KNF Neuberger, cat. no. N 022 AN.18)
- Vacuum chamber (VWR International, cat. no. 467-2115)
- Cutting mat (A4; Qconnect, cat. no. KF01135)
- Benchtop UV-ozone cleaner (NOVA Scan, cat. no. PSD-UVT)
- Syringe pump (Harvard Pump 11 Elite; Harvard Apparatus, cat. no. 70-4505)
- Microscope (fluorescence) with autofocus, time-lapse abilities and preferably an automated *xy* control stage (e.g., Nikon Eclipse Ti-E)
- Microscope temperature control system (e.g., Life Imaging Services, Cube & Box)
- Metal coverslip holder (self-made)
- Statistical analysis program with Kaplan-Meier analysis (e.g., R software, free download online)
- Glass Petri dishes (120/20 mm, VWR International, cat. no. 391-2850)
- Coverslips (22 × 40 mm, CBN Labsuppliers, cat. no. 190002240)
- Aluminum foil
- Weighing boats
- Serological pipette, 5 ml (VWR International, cat. no. 612-1245)
- Scotch tape (VWR International, cat. no. 819-1460)
- Scalpel (VWR International, cat. no. 233-5334)
- Luer-lock syringes, 50 ml (sterile; BD, cat. no. 300137)
- Syringes, 5 ml (Sterile, Luer tip; VWR International, cat. no. 613-1599)
- Tweezers (VWR International, cat. no. 232-2132)
- Luer stubs (gauge size 20; Instech Solomon, cat. no. LS20)
- Syringe filters (pore size 0.20 μm; Sigma-Aldrich, cat. no. 16534K)
- Stainless steel catheter plug, 20 gauge × 12 mm (Instech Solomon, cat. no. SP20/12)
- Plastic Petri dishes (VWR International, cat. no. 391-0892)
- Nail polish (transparent)
- Epoxy glue (Bizon, hardware store)

PROTOCOL

PROCEDURE

Design of the silicon wafer mold ● TIMING 4–8 h

1| Use CAD software to generate designs for the dark-field photolithography masks. A single mask needs to be generated for each layer. Our design contains three layers (**Fig. 2a**). Different patterns can be made on a single wafer mold (e.g., 18 chip designs on a 4-inch wafer).

▲ **CRITICAL STEP** To link PDMS channel imprints to specific structures on the silicon wafer mold for later use, we recommend adding a label near each pattern in the drawing.

2| Create transparency masks using a high-resolution film printing. Alternatively, order chrome photomasks from a commercial supplier (e.g., Finelineimaging).

! CAUTION High-resolution printing (at least 65,024 d.p.i.) is required for the fabrication of the micropads.

Fabrication of the silicon wafer mold ● TIMING 4–5 h

3| Dehydrate the silicon wafer by baking it at 150 °C for 20 min on a hot plate. This enhances adhesion of the SU-8 photoresist.

▲ **CRITICAL STEP** Steps 3–16 need to be performed in a clean room facility to prevent contamination of the silicon wafer mold with dust particles.

4| Spin-coat SU-8 2005 on the wafer at 4,000 r.p.m. for 30 s to fabricate the first layer (**Fig. 4**). This layer needs to be 4 μm high for haploid yeast cells.

! CAUTION Photoresists are highly flammable. Wear appropriate protective gear when handling them. Avoid contact with eyes, skin or clothing.

5| Soft-bake the wafer at 95 °C for 2 min.

▲ **CRITICAL STEP** Make sure that the hot plate is standing level during all soft-baking steps to ensure uniform baking.

6| Place the first mask and the SU-8–coated wafer in the mask aligner. Expose to UV (350–400 nm) radiation with a dose of 105 mJ per cm^2.

7| Postbake the UV-exposed wafer at 95 °C for 3 min.

8| Develop the wafer by immersion in SU-8 developer for 1 min. Clean and dry the wafer with filtered air and hard-bake it at 150 °C for 20 min.

**! CAUTION** SU-8 developer is toxic. Wear appropriate protective gear.

▲ **CRITICAL STEP** We recommend checking the SU-8 microstructures under a microscope directly after fabrication of each layer.

? TROUBLESHOOTING

9| Spin-coat SU-8 2010 at 3,500 r.p.m. for 30 s on top of the wafer (**Fig. 4b**). This second SU-8 layer needs to be 10 μm high and will create the micropads.

10| Soft-bake the wafer at 95 °C for 3 min before UV exposure.

Figure 4 | Fabrication of the silicon wafer mold and microfluidic dissection platform. (**a**–**e**) Work scheme showing production of the silicon wafer mold and the microfluidic dissection platform. (**a**–**c**) SU-8 photoresist layers are spin coated on top of a silicon wafer mold, exposed to UV and developed to create the Y-shaped medium channel, micropads and side channel. (**d**) PDMS is mixed in a 1:10 weight to weight ratio with a curing agent and poured on top of the silicon wafer mold, followed by baking for 1 h at 65 °C and 1 h at 120 °C to promote polymerization of PDMS. (**e**) After polymerization, the single chip designs are cut out using a scalpel. Holes are pierced at the end of the side channel, at the outlet and at one of the inlets of the medium channel. The PDMS is then bonded with a glass coverslip by exposure to UV light for 8 min. (**f**) A microfluidic dissection platform is ready for use.
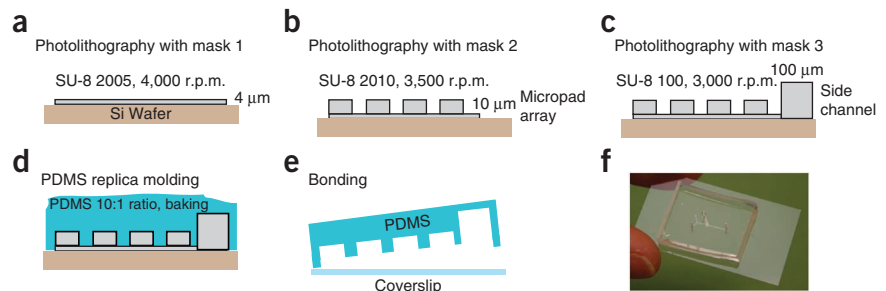
11| Align the wafer and the mask for the second layer using the mask aligner. Expose it to UV radiation with a dose of 120 mJ per cm^2.

12| Postbake the wafer at 95 °C for 4 min and develop the wafer for 2–3 min by immersing it in the SU-8 developer. Clean and dry the wafer with filtered air. Hard-bake the wafer at 150 °C for 20 min.

13| Spin-coat SU-8 100 on top of the wafer at 3,000 r.p.m. for 30 s. (**Fig. 4c**) This third layer will be 100 μm and is necessary to create the side channel.

14| Prebake the wafer at 65 °C for 10 min, followed by soft-baking at 95 °C for 30 min.

15| Align the wafer and the third mask using the mask aligner. Expose the wafer to UV radiation with a dosage of 320 mJ per cm^2.

16| Postbake the wafer at 95 °C for 4 min and develop the wafer by immersing it in the SU-8 developer for 10 min. Clean and dry the wafer with filtered air. Hard-bake the wafer at 150 °C for 20 min.
▲ **CRITICAL STEP** The silicon wafer mold (**Fig. 3a**) is then ready to be used as a master for numerous cycles of PDMS replica molding. The production of the silicon wafer mold can also be outsourced.

Replica molding of PDMS ● TIMING 3 h
17| Wrap the inside of the bottom part of a glass Petri dish in a double layer of aluminum foil. Make sure that the layer of the aluminum foil is straight before putting the wafer mold on top of it.

18| Weigh 40 *g* of PDMS in a weighing boat. Add curing agent to the PDMS in a 1:10 weight-to-weight ratio (**Fig. 4d**). Mix them vigorously to ensure that the PDMS will polymerize uniformly.

19| Gently pour the PDMS on top of the mold. The layer of PDMS on top of the mold should be ~3 mm thick.

20| Place the PDMS in a vacuum chamber to remove all air bubbles. This takes about 30 min.

21| After degassing, place the PDMS on top of a hot plate for 1 h at 65 °C, followed by 1 h at 120 °C.

22| Take the glass Petri dish with cured PDMS from the hot plate and let it cool for a few minutes. Lift the aluminum foil from the Petri dish.

23| Carefully peel off the aluminum foil and the thin layer of PDMS on the back of the mold. The PDMS layer with the channel imprints on top of the wafer can then gently be lifted from the wafer mold. This completes PDMS replica molding. Additional replicas can be made by repeating Steps 17–23.
■ **PAUSE POINT** Cured PDMS slabs can be stored for months. They need to be kept clean and dust free (e.g., by storage in a glass Petri dish).

Fabrication of the microfluidic device ● TIMING 2 h
24| Place the PDMS slab on a cutting mat with the channel imprints facing upward.

25| Cut out a channel imprint with a scalpel.
▲ **CRITICAL STEP** Leave about 3 mm of PDMS on all sides of the channel for optimal attachment to the coverslip.

26| At the end of the side channel, push a 20-gauge Luer stub down in a right angle through the PDMS (**Fig. 3b**).
▲ **CRITICAL STEP** If the channel imprint is facing upward, it will be easier to punch the hole precisely at the end of the channel. Shining bright light on the PDMS, e.g., with a flashlight, as well as using a dark cutting mat below the PDMS, makes it easier to see the ends of the channels.

27| Remove the residual PDMS from the Luer stub and pull the stub back out (**Fig. 3c**).
▲ **CRITICAL STEP** Removal of the punched out PDMS is essential to avoid blockage of the channel.

28| Repeat Steps 26 and 27 for the outlet and for one of the inlets of the microfluidic channel.

29| Apply Scotch tape on top of the channel imprint and lift it off again to remove dust particles. Further, clean a coverslip by adding and removing a piece of Scotch tape.
▲ **CRITICAL STEP** Removal of dust and other dirt is important for proper attachment of the PDMS to the coverslip.

30| Put the cleaned PDMS and coverslip in the benchtop UV-ozone cleaner. Set the temperature at 40 °C and expose the cleaned PDMS and coverslip for 8 min to UV light.
▲ **CRITICAL STEP** Make sure that the side with the channel imprint is directly exposed to the UV light, so that it can bond with the coverslip.

31| After UV exposure, immediately bring the exposed surfaces together (**Fig. 4e**) and gently tap on the edges of the PDMS to promote bonding (**Fig. 3d**).
! CAUTION Small amounts of ozone can be released upon opening the benchtop UV-ozone cleaner. When inhaled, ozone is harmful.

32| Move the device to a hot plate at 100 °C for 1 h (**Fig. 4f**).

33| Gently try to lift up the sides of the PDMS from the glass coverslip. If this is not possible, then the bonding is sufficient and the microfluidic device is ready.
■ **PAUSE POINT** Microfluidic devices can be stored for several months at room temperature (20–25 °C) without any special precautions. The coverslips are fragile; they should preferably be stored in a box with paper tissues to protect them from breaking.

Setting up an aging experiment ● **TIMING** 3 h
34| Insert 10-cm long pieces of 0.012-inch (i.d.) PTFE tubing at the end of the side channel and outlet of the microfluidic device (**Fig. 3e**).
▲ **CRITICAL STEP** Use tweezers if necessary.

35| Fill a 50-ml Luer-lock syringe with the desired culture medium.

36| Connect a syringe filter and a 20-gauge Luer stub, followed by ~3-cm-long piece of 0.030-inch (i.d.) microbore tubing and a piece of PTFE tubing (**Fig. 3f**).
▲ **CRITICAL STEP** Make sure that the PTFE tubing is long enough to cover the distance between the syringe pump and the microscope stage.

37| Place the syringe in the syringe pump and press 'Fast Forward' on the pump to rapidly fill the PTFE tube with the medium. Insert the end of the PTFE tube in the inlet of the microfluidic device.

38| Fix the microfluidic device on the microscope stage and flush it using a flow rate of 10 µl min^{-1} (**Fig. 5a**). Wait for the medium to exit via the side channel and collect it in a Petri dish.

39| Attach a 10-cm-long piece of Tygon microbore tubing to a 5-ml syringe using a 20-gauge Luer stub, and then load 1 ml of a yeast cell suspension into the syringe. Remove excess air.
▲ **CRITICAL STEP** Use cell densities between 1×10^6 and 5×10^6 cells per ml when loading cells into the microfluidic device to avoid clogging the array of micropads with cells.

40| Set the flow rate of the syringe pump to 0.5 µl min^{-1}. Use the 0.030-inch (i.d.) microbore tubing on the syringe with cells to connect it to the PTFE tubing at the end of the medium channel.

**41|** Gradually increase the pressure on the plunger until cells begin to settle underneath the micropads (**Fig. 5a**). When sufficient cells are collected underneath the pads, slowly release the pressure again.
▲ **CRITICAL STEP** Cell loading usually requires some practice. An average of 1–3 cells per pad is considered an optimal load for an aging experiment (**Fig. 5b**).
? TROUBLESHOOTING

42| Carefully remove the 5-ml syringe from the outlet. Set the flow rate back to 10 µl min^{-1} to flush out cells from the side channel. This takes ~10 min.

Figure 5 | Operation of the microfluidic dissection platform. (**a**) Schematic overview showing how to start up an experiment. Culture medium is flushed through the microfluidic platform (top). It exits via the side channel, which is much larger compared with the medium channel. The flow rate is set at 10 μl min^{-1}. (Middle) The flow rate is reduced to 0.5 μl min^{-1} and cells are loaded into the microfluidic dissection platform via the outlet of the medium channel. Excess medium containing nonretained cells flows out through the side channel. When sufficient cells are trapped underneath the micropads, the side channel is flushed for about 10 min at 10 μl min^{-1} to remove cells. (Bottom) The flow is reduced again to 0.5 μl min^{-1} and the side channel is blocked using a catheter plug. Culture medium will now flow through the entire medium channel and simultaneously supply the cells trapped underneath the micropads with fresh medium, while removing daughter cells. (**b**) Example of BY4741 cells expressing Ilv3-GFP loaded underneath the micropads at the start of an experiment. Most pads contain 1–3 cells. Cells were grown in YNB containing 2% (wt/vol) glucose and complete supplement mixture. Scale bar 10 μm. (**c**) Images of a single aging mother cell (YSBN6) with bud at various replicative ages in YNB containing 2% glucose. The numbers on the top of each image indicate the number of buds, including the bud in the image, the cell produced. The final image shows the cell morphology after cell death (cross symbol). Apparent is the increase in cell size and the appearance of wrinkles on the cell surface. Scale bar 4 μm.
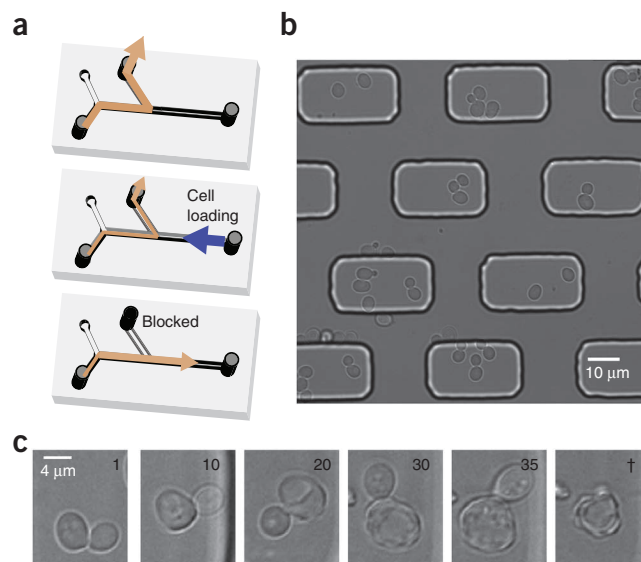
43| Reduce the flow rate again to 0.5 μl min^{-1}. Insert a catheter plug in a 5-cm-long piece of Tygon microbore tubing and connect the tubing with plug to the side channel to close it off (**Fig. 5a**).
▲ CRITICAL STEP Make sure that the flow rate is reduced upon closing off the side channel. If it is not reduced, there will be a sudden increase in pressure inside the chip that will dislodge cells from the pads.

44| Set the flow rate between 1 and 5 μl min^{-1}. Start image acquisition.
▲ CRITICAL STEP The optimal flow rate needs to be determined by experience and vary according to yeast strains or culture media.
? TROUBLESHOOTING

? TROUBLESHOOTING
Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Step	Problem	Possible reason	Solution
8	Poor pattern definition of microstructures	Improper UV exposure of the wafer	Check the mode of contact between photomask and wafer in the mask aligner. The hard contact mode is recommended
			Check the photomask for dust, which could have created a gap between the mask and SU-8 layer
	Detachment of SU-8 microstructures	Poor adhesion between the SU-8 layer and wafer	Increase the dehydration time in Step 3
41	Cells do not load underneath the pads	Too much pressure on the micropads	Carefully reduce the pressure on the syringe and/or reduce the flow rate of the syringe pump
		PDMS pads are glued to the coverslip	When placing the PDMS on top of the coverslip (Step 31), take care not to push down on the pads
		Cells are suspended in rich culture medium, e.g., YPD	Spin down cells (5 min, 3,000*g* at room temperature), wash and resuspend in minimal medium. After loading the cells, the experiment can be run using YPD

(continued)

TABLE 1 | Troubleshooting table (continued).

Step	Problem	Possible reason	Solution
44	All cells are lost after running the platform for *x* hours	The flow rate of the medium is too high	Reduce the flow rate of the medium. The optimal flow rate may differ between experimental conditions, e.g., different medium or yeast strain
	Cells appear in different focal planes underneath the pads	The PDMS is (partially) detached from the coverslip	The microfluidic dissection platform is no longer functional. Terminate the experiment to avoid spillage of medium into the microscope
	Appearance of large cell clumps in the pad region	After loading the cells, the medium in the side channel was not sufficiently flushed	After loading the cells, make sure all the medium in the side channel is refreshed before closing it off (Step 41). Alternatively remove the tube of the side channel and insert a catheter plug in its place
	The focus is lost during the movie, despite using autofocus	The platform is still settling in the microscope stage at the start of the experiment	Place the microfluidic device in the microscope stage and prewarm the microscope incubator 1 h before loading the cells
		When using a water-immersion objective, the water evaporated during the experiment	Preferably use immersion fluids, such as oil or glycerin, as these cannot dry out over the time course of the experiment
	Poor removal of daughter cells	The flow rate of the medium is too low	Increase the flow rate
			Check if the connections between the tubing are watertight. If not, use nail polish and/or epoxy glue to seal them
			Make sure that the syringe pump is active and that the side channel is closed off
	Premature death of cells during the experiment	Phototoxicity	Halogen lamps can emit small amounts of UV light. Insert a UV filter in front of the light path
			Reduce the intensity or frequency of fluorescence exposure

● **TIMING**
Steps 1 and 2, design of the silicon wafer mold: 4–8 h
Steps 3–16, fabrication of the silicon wafer mold: 4–5 h
Steps 17–23, replica molding of PDMS: 3 h
Steps 24–33, fabrication of the microfluidic device: 2 h
Steps 34–44, setting up an aging experiment: 3 h

ANTICIPATED RESULTS
Cell imaging
Changes in morphology and fluorescent markers during aging can be analyzed using the standard image analysis software and techniques[1,3]. For example, using the microfluidic dissection platform, we imaged the morphology of single cells at various replicative ages (**Fig. 5c**) and showed that there is substantial heterogeneity in the morphology of aging yeast cells[1].

Measurements of replicative lifespan
Lifespan data are collected by counting the total number of buds produced by a single cell during its life. Alternatively, one can note the amount of time that a cell was viable. The lifespan curve can then be generated by plotting the fraction of viable cells versus the number of buds produced or time[21]. For the mutants and strains tested, the replicative lifespans measured with the microfluidic platform are similar to those reported with the classical microdissection method[1,22,23].

    If desired, the data from mother cells that are accidently washed out during the experiment can be incorporated in the lifespan curve using Kaplan-Meier analysis[24]. Even though it is not possible to record the time of death for these cells, they carry information about cell viability up to the point at which they were washed out. Integration of such information into

the lifespan curve improves the quality of the data by increasing the number of observations and counteracts effects that washing out of cells may have on the measured replicative lifespan[25]. Moreover, the use of survival analysis techniques, such as Kaplan-Meier analysis, may be essential to faithfully compare the outcome of experiments with different cell retention. Lifespan curves generated with Kaplan-Meier analysis can be compared using the log rank test[26].

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