CHAPTER

Microfabricated Chambers as Force Sensors for Probing Forces of Fungal Growth

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CHAPTER OUTLINE

Abstract

The mechanical properties of fungal cells influence their growth, division, morphogenesis, and invasiveness. These cells are characterized by high internal turgor pressure contained by a stiff but elastic cell wall. In here, we describe simple and versatile methods to measure forces of fungal growth, turgor pressure, and elastic moduli of fungi cell wall, using microfabricated polydimethylsiloxane wells of varying stiffness as single cell force sensors. We demonstrate the strength of this method with the rod-shape fission yeast *Schizosaccaromyces pombe* and highlight how it may be implemented for studying mechanical properties of other walled cells.

INTRODUCTION

The mechanical properties of cells contribute to the regulation of numerous biological processes ranging from differentiation, cell division, and tissue morphogenesis (Engler, Sen, Sweeney, & Discher, 2006; Fink et al., 2011; Heisenberg & Bellaiche, 2013). While animal cells mechanics rely principally on membrane tension, actin cortex, and on adhesion (Salbreux, Charras, & Paluch, 2012), walled cells of bacteria, fungi, and plants depict markedly different mechanical properties. These cells possess high internal turgor pressure, which is osmotically generated, and are encased by a rigid cell wall (Gow, Brown, & Odds, 2002; Harold, 2002). In fungi, these properties have been suggested to regulate core processes such as growth, cytokinesis, size, shape, and invasion (Bastmeyer, Deising, & Bechinger, 2002; Boudaoud, 2003; Lo et al., 1997; Minc, Boudaoud, & Chang, 2009; Proctor, Minc, Boudaoud, & Chang, 2012), yet our knowledge of how these mechanical aspects are regulated and how they contribute to these processes remain understudied.

Historically, the mechanics of walled cells have been assessed by different methods, such as assaying the effect of extracellular osmolarity, impalement of pressure probes to derive turgor values, or by indenting cell wall with atomic force microscopy (AFM) tips to assess mechanical properties of the wall (Bastmeyer et al., 2002). AFM has provided numbers for cell wall elastic moduli, but discrepancies of over 2 orders of magnitude with other methods have been reported in the literature (Smith, Zhang, Thomas, Moxham, & Middelberg, 2000; Touhami, Nysten, & Dufrene, 2003). These discrepancies could arise from the models used to fit AFM force–displacement curves, which may depend extensively on parameters such as cell wall depth or indentation tip size (Vella, Ajdari, Vaziri, & Boudaoud, 2012a, 2012b). Other methods such as the use of strain gauges or waveguide microscopy have also been proposed to measure growth force of certain fungi (Bastmeyer et al., 2002), but these rely on complicated setups, and prevent obtaining large data sets and performing detailed live-cell imaging.

Here, we describe the use of microfabricated polydimethylsiloxane (PDMS) chambers made by simple soft lithography to assess the forces of fungal growth, turgor pressure values, and the elastic modulus of the cell wall (Minc, Boudaoud, et al., 2009). Advantages of this microchamber approach are: (1) it is possible to quickly

obtain large data sets necessary for quantitative analysis; (2) it is possible to perform time-lapse live imaging of intracellular components inside cells, while measuring forces; (3) the approach is simple and inexpensive; (4) this method should be adaptable for many different contexts and walled-cell types.

In our initial study (Minc, Boudaoud, et al., 2009), we used the rod-shape fission yeast *Schizosaccaromyces pombe*, because of its quantitative growth habits and excellent genetics (Chang & Martin, 2009). These cells elongate along a polarized growth axis from 7 to 14 μm after which they divide. Their cell wall is composed in part by alpha- and beta-glucan sugar chains, which are being synthesized and remodeled by specific enzymes at cell tips for tip growth, or in the cell middle during cytokinesis and septation (Cortes et al., 2005; Ishiguro, 1998). This wall has a homogenous thickness of roughly 200 nm and is stressed and deformed by internal osmotic pressure which is regulated in part by the glycerol synthesis machinery (Aiba, Yamada, Ohmiya, & Mizuno, 1995). We pioneered two assays to derive (i) the elastic modulus of the cell wall and (ii) the force–velocity of single fission yeast cells that we used to derive turgor pressure values. These numbers were highly instructive in testing quantitative models for cell growth and cytokinesis in fission yeast (Minc, Boudaoud, et al., 2009; Proctor et al., 2012).

14.1 DESIGNING MICROWELLS TO BEND AND CONFINE FISSION YEAST CELLS

This section describes the methods used to fabricate PDMS microwells (Minc, Boudaoud, et al., 2009; Minc, Bratman, Basu, & Chang, 2009; Weibel, Diluzio, & Whitesides, 2007) which will serve to (i) buckle fission yeast cells to compute cell wall elastic modulus and (ii) to grow these cells in constraining chambers to derive force–velocity relationships and turgor pressure. These wells are made of PDMS of different elasticity to extend the range of force measurement. The fabrication procedure follows three steps: soft lithography, PDMS molding, and PDMS elasticity calibration. In what follows, we describe detailed material and procedure for each of these steps.

14.1.1 Soft lithography
14.1.1.1 Materials
Computer with drawing software; 2 in. diameter silicon wafer (Universitywafers, 2″ or 4″ N(111) SSP Test Grade Quality), 6 cm diameter petri dish, SU8 2005 resist and developer (MicroChem); acetone and isopropanol; access to clean room facility (with spin coater, hot plates, sonication bath, and UV lamp).

1. *Designing photo-transparency mask*. The design of patterns is done on a computer assisted drawing program (CAD). Available programs include AutoCAD (free for students) and QCAD (freeware). The design includes several arrays of 500×500 circles. Each array is made of circles of a fixed diameter (10, 12, 15, 20, 25, 30, and 50 μm) spaced apart by 5 μm. For these ranges of size,
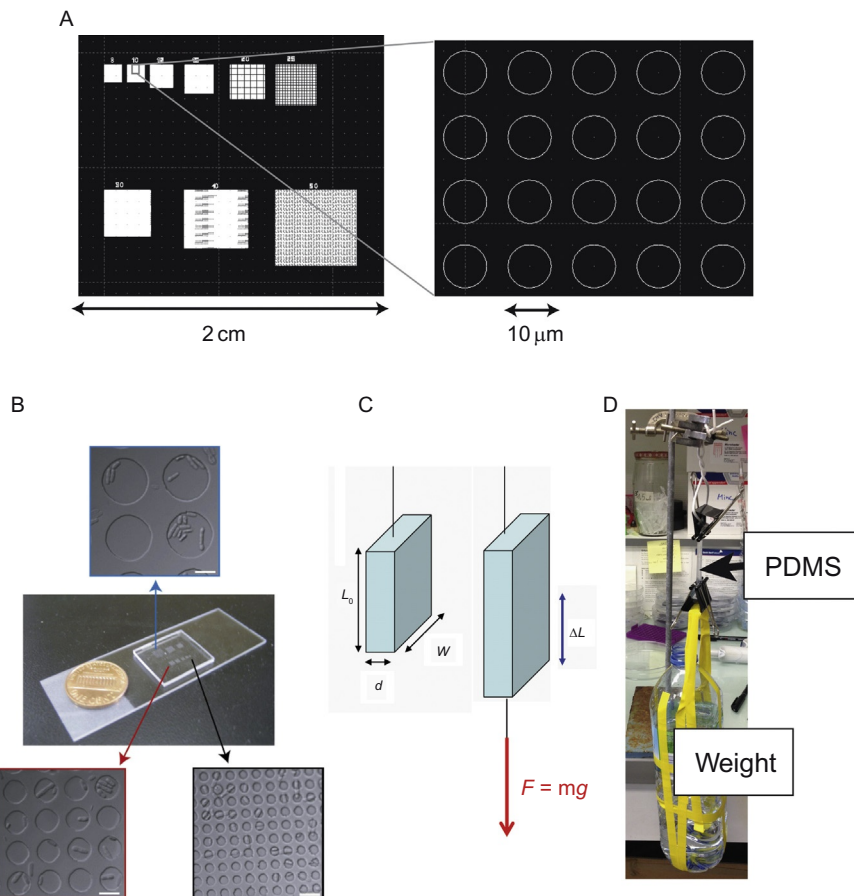
FIGURE 14.1

Microfabrication and calibration of polydimethylsiloxane (PDMS) microwells for force measurement. (A) Global view of transparency mask design depicting arrays of microwells with different sizes, and close up on the local structure of the array and spacing between wells. After printing the inside of the circles are transparent. (B) Picture of a PDMS chamber on a glass coverslip and close up view of different wells with different diameters with fission yeast cells inside (bars = 20 μm). (C) Schematic explaining the strain–stress assay to derive the elastic modulus of PDMS. (D) Photograph of a simple setup to perform this experiment.

these designs are printed on plastic transparencies with resolution of 12,700 dpi, which have a black background and are transparent inside the circles (Fig. 14.1A).

2. *Photolithography*. Chambers are made from PDMS using a positive master composed of SU8. Masters were prepared using standard lithography methods for SU8 microfabrication (Weibel et al., 2007). A positive master is a hardened structure of SU8 such that the desired geometric shapes extend as posts from a silicon wafer. SU8 is an epoxy-based negative photoresist: when it is exposed to

UV light it becomes insoluble to the photoresist developer and the unexposed portion of the photoresist is dissolved by the developer.

14.1.1.2 Procedure

1. Clean the silicon wafer by rinsing with acetone in a sonication bath for 2 min, subsequently rinse with isopropanol and DI water. Dry it with air and bake at 200 °C for 5 min on hotplate.
2. Put SU8 onto the wafer by using a transfer pipette (∼1 ml for each square inch) and spin it. Rotation speeds depend on the type of SU8 used as well as desired thickness of the coating. To obtain 5 μm high features, the basic procedure involves a SU8-2005 and a first spin of 20 s at 500 rpm, with an acceleration of 500 rpm s^{-1} and a subsequent spin of 45 s at 3000 rpm with the same acceleration.
3. Bake the resist on a hot plate for about 1 min at 65 °C and 1 min at 95 °C.
4. Create patterns on the SU8 by covering the wafer with the transparency mask using a dedicated UV insulator (or mask aligner). It is important to ensure that the transparency remains well plastered onto the SU8 layer (this can be achieved by placing a heavy and clean quartz glass on top of the transparency). The optimal time of exposure will depend on wavelength and intensity of the light and has to be adapted. Low exposure will make features that do not stick well to the substrate and detach, while too much exposure will generate features with poor spatial definition.
5. The sample is then baked again for 1 min at 65 °C and 1 min at 95 °C, immersed in a glass dish containing developer for 2 min, and transferred to another glass dish containing clean developer for 30 s. The wafer is then rinsed with isopropyl alcohol and dried on the spin coater (1 min at 1000 rpm). A final bake at 150 °C for 10 min is then performed on the hot plate.
6. The master is then observed on a bright-field reflected light microscope to check the shape and size of SU8 posts.
7. An overnight exposure of the master with vapors of silane (tridecafluoro-(1,1,2,2-tetrahydrooctyl)-1-trichlorosilane, United Chemical Technologies, T2492) is then performed to prevent the PDMS to stick too much to the SU8 (see hereafter). This is done by placing the SU8 masters together with a small flask containing 200 μl of silane in a vacuum desiccator overnight.
8. Masters can be stored in small petri dish for long times.

14.1.2 **Creating PDMS chambers from master**

PDMS chambers can be replicated many times from a positive SU8 master. PDMS is highly suitable for biological applications as it is optically clear, gas permeable, and nontoxic to cells.

14.1.2.1 Materials

Sylgard 184 base and curing agent (Fisher, NC9644388), plastic cups, plastic stirring rod, timer, scale, vacuum desiccator, 65 °C oven, and a scalpel.

14.1.2.2 Procedure
1. Mix over 50 g of PDMS base and curing agent in a precise ratio. This ratio can range from 5:1 to 30:1 to generate PDMS with varying elasticity (5:1 will make it rigid and 30:1 will make it more deformable). Mix vigorously with a transfer pipette or a plastic rod.
2. De-gas mixture by placing into a vacuum desiccator. Apply vacuum until bubbles disappear.
3. Apply a fraction of the PDMS slowly onto the side of the wafer. Once the wafer is coated completely by a few millimeters of PDMS, let it settle onto the wafer for 10 min. Bake at 65 °C for at least 4 h. Note that baking temperature and time will influence the rigidity of the PDMS.
4. The remaining of the PDMS is poured in an empty large petri dish to generate a large piece of material which will serve for elastic modulus measurement (see here after).
5. Cut the PDMS off the masters (typically a 3×3 cm^2 piece, bigger than a 22×22 mm^2 coverslip) with a blade and carefully peel it off the master using a tweezer. Place the PDMS with the wells facing up in a petri dish. The PDMS slab can be stored for long periods of time (Fig. 14.1B).

14.1.3 Calibrating PDMS elastic properties
In our assays, PDMS deformation is used as a measure of the force generated by the cells, and thus a requirement of the method is to carefully compute the elastic modulus of the PDMS used for assembling the chambers. PDMS with elastic moduli, E_{PDMS}, ranging from 0.1 to 1.5 MPa can be generated. It is however important to note that these values will not only depend on PDMS/crosslinker ratio but also on the baking time, temperature, and possibly other factors. Thus, it is crucial to assemble the PDMS wells on the SU-8 master with the same PDMS used for the calibration. This is done using a simple strain–stress experiment on large pieces of PDMS on which we measure the deformation as a function of the external force (Fig. 14.1C and D).

14.1.3.1 Materials
High metallic rod, metal clips, calibrated weights (could be a bottle filled with different amount of water), micrometer, and ruler.

14.1.3.2 Procedure
1. Cut a large piece of PDMS of about 5 cm in length, 1 cm in width, and 5 mm in depth. The size of this piece will influence the force you have to apply to deform it, and thus for rigid PDMS it is advised to cut thinner pieces. Measure carefully the width, w and depth, d with a micrometer.
2. Clamp the PDMS with two metal clips on both sides of the piece and attach one clip to a hanger attached to the rod (see Fig. 14.1D). Measure carefully with the ruler, the distance between the metal clips, L_0.

3. Attach to the other metal clip a given weight, m, which will cause the PDMS piece to stretch. Measure the new distance between clips ($\Delta L + L_0$).
4. Repeat with increasing weights to reach deformation values of $\Delta L/L_0$ of around 30–40%.
5. The stress–strain relationship is given by:

$$mg = E_{PDMS} \frac{\Delta L}{L_0} wd \qquad (14.1)$$

with g the standard earth acceleration ($g = 9.81$ m s^{-2}). The slope of the plot of m as a function of $\Delta L/L_0$ should yield a straightforward estimation of E_{PDMS}.

14.2 BUCKLING ASSAY ON SINGLE FISSION YEAST CELLS TO COMPUTE CELL WALL ELASTIC MODULUS

The measurement of the cell wall surface modulus, σ_{cw}, relies on an assay in which single cells are buckled in deformable chambers (Fig. 14.2A). The surface modulus has units of N m^{-1} and is linked to the elastic modulus and the height of the cell wall, h, through the relationship: $\sigma_{cw} = hE_{cw}$. The force needed to buckle a rod-shape cell is given by:
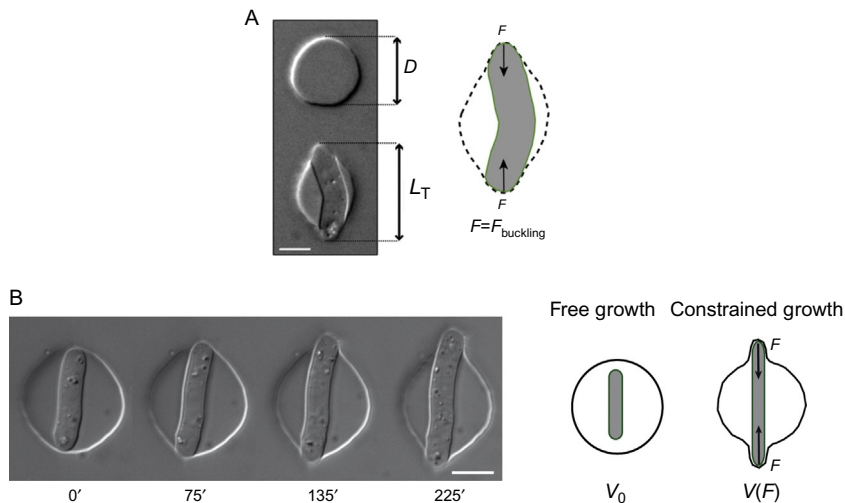

FIGURE 14.2

Assays to compute cell wall surface modulus and force–velocity of single fission yeast cells. (A) Picture of a cell buckled in a deformable chamber. The force for buckling the cell balances the force deforming the chamber. (B) Cell growing and deforming a microwell, as the deformation increases the force increases. These time lapse serve to derive force–velocity relationships.

$$F_{\mathrm{B}} = \frac{\pi^2 R^3 \sigma_{\mathrm{cw}}}{L_{\mathrm{T}}^2} \qquad (14.2)$$

with L_{T} the distance between cell tips along the force axis and R, the cell's radius.

This force balances the force for deforming the PDMS well of diameter D which is given by (Minc, Boudaoud, et al., 2009):

$$F = \frac{8}{3} E_{\mathrm{PDMS}} R (L_{\mathrm{T}} - D) \qquad (14.3)$$

In what follows we describe the detailed procedure to buckle cells in chambers and to measure relevant parameters.

14.2.1 Materials
Glass slide, 22×22 mm^2 coverslip, exponentially growing fission yeast culture, microscope with $100 \times$ objective.

14.2.2 Procedure
1. Start from the PDMS slab containing the microwells and place it on a glass slide with wells facing up (Fig. 14.1B). The slab must have a surface larger than a 22×22 mm^2 cover glass.
2. Spin down 1 ml of an exponentially growing culture of fission yeast cells and concentrate to 50 μl typically (use a similar protocol as if you were imaging yeast cells between a normal glass slide and a cover glass).
3. Place a 1 μl drop of cell suspension on the PDMS slab and rapidly cover with a 22×22 mm^2 coverslip. This should cause a large number of cells to immediately bend inside the little wells. Immediately monitor the cells under a routine microscope with $40 \times$ magnification. If the cells are not bent, push gently with finger tips onto the coverslip.
This approach should be very efficient. Troubleshooting includes reducing the volume of the drop (down to 0.5 μl for instance) and adapting cell density. A too dense drop will cause many cells to enter the same well, and a low density will result in few bent cells.
4. Quickly go to a microscope with high magnification ($100 \times$ Oil objective). If the microscope is inverted flip the glass slide to have it on top of the PDMS, if the microscope is upright keep the PDMS up. Depending on cell length and on the elasticity of the PDMS you may obtain a mix of these three situations:
(i) The cell is buckled but does not deform the chamber, (ii) the cell is buckled and deforms the chamber, and (iii) the cell remains straight and deforms the chamber. The only useful case is situation (ii).
5. Take pictures of bent cells that cause chamber deformation and pictures of empty chambers in the near surrounding. The empty chambers will serve to compute the initial diameter of the chambers. Scan through the z-axis using the focus knob to

ensure that both cell tips are well inside the chamber (it may occur than one cell tip slips out the well).
6. Repeat the same experiment with different PDMS elasticity and cell length to span a large range of measurement (in our previous work we used a *cdc25-22* mutants which display longer cells).
7. Analyze images, using image J, to compute cell diameter, length along the force axis, and chamber deformation. The surface modulus for each cell can then be computed using the following formula:

$$\sigma_{\mathrm{cw}} = \frac{8}{3\pi^2} \frac{L_{\mathrm{T}}^2}{R^2} (L_{\mathrm{T}} - D) E_{PDMS} \tag{14.4}$$

14.3 SINGLE CELL FORCE–VELOCITY; DETERMINATION OF STALL FORCE AND TURGOR PRESSURE

In this assay we use the same general concept, with the major difference that the cells grow inside chambers, and that we deduce the forces associated with growth from the dynamic deformation of the chamber. The force is still obtained from Eq. (14.3) and the elongation rate of single cells is computed from the evolution of cell length as a function of time.

14.3.1 Materials

Plasma cleaner, glass slide, 22×22 mm^2 coverslip, exponentially growing fission yeast culture, poly-lysine solution (Sigma, P7280, 1 mg ml^{-1} in water), lectin (Sigma, L-2380, 100 µg ml^{-1} in 0.1M Phosphate Buffer Saline), parafilm. Microscope with $100 \times$ objective and automated X–Y stage.

14.3.2 Procedure

1. Start from the PDMS slab containing the microwells and place it on a glass slide with wells facing up.
2. Just before use, activate the PDMS with a plasma cleaner (Harrick Plasma). Insert the PDMS into the chamber of the plasma cleaner for 45–60 s under vacuum. This critical step makes the wells hydrophilic and negatively charged which improves subsequent coating.
3. Place a large drop (1 ml) of poly-lysine on a piece of parafilm, and place the PDMS, wells facing down onto the drop. Let coating occur for 20–30 min and subsequently dry the PDMS surface with clean air.
4. Repeat the same procedure with the lectin solution for 10 min, dry, and repeat again with media for 5 min and dry again.
5. Spin down 1 ml of a culture of fission yeast and concentrate to 50 µl typically.
6. Place a drop of 2–3 µl of yeast suspension, and gently cover with a 22×22 mm^2.

7. Let the cells sediment 5–10 min into the bottom of chambers and go to the microscope. Follow the same procedure as in Section 14.2 to adjust your observation setup to the type of microscope (upright or inverted). Note that a motorized stage is required for collecting large data sets.

8. Observe and select positions where cells are well attached at the bottom of a microwells but in which cell length has not yet reached the diameter of the chamber.

9. Start filming cells growing with a frequency of 10 min typically. It is important to track for each cell, growth rates without external force from the chamber, v_0, and subsequent growth rate, $v(F)$, when the cell is deforming the chambers and growing under force. This method should allow keeping cells happy and growing for 3–4 h, without major activation of stress pathways (Minc, Boudaoud, et al., 2009).

10. Analyze movies by tracking cell length as a function of time. For fission yeast, it is important to select for cells that are already growing from both ends (using a polarity fluorescent marker or from tracking fiducial marks on the cell surface like birth scars) and to monitor the onset of mitosis at which cells cease growth (using a strain with fluorescently labeled tubulin, for instance).

11. Repeat experiments in different PDMS elasticity and compile at the population level $v(F)/v_0$ as a function of F, which stands for the force–velocity behavior. The simplest model for turgor driven growth predicts a linear behavior:

$$\frac{v(F)}{v_0} = \left(1 - \frac{F}{\pi R^2 P}\right) \qquad (14.5)$$

where P is turgor pressure, which can thus be computed from linearly fitting the force–velocity curve. The stall force F_s, at which growth should cease then corresponds to $v(F_s) = 0$ (Minc, Boudaoud, et al., 2009).

CONCLUSIONS

The method described here allows assaying cell wall mechanical properties and forces generated by tip growth in yeast. The resolution of the method as described here should be on the order of 1 N m^{-1} for the wall surface modulus and should be valid up to 50 N m^{-1}. The external forces that can typically be resolved are within $0.5 \ \mu N$ and may be measured at values reaching $50–70 \ \mu N$. We thus emphasize that this method should be readily transposable to study mechanics in walled cells which grow by tip extension (rod-shaped bacteria, fungal hyphae, or pollen tubes, for instance). Changes in chamber size and geometries should further allow transferring this approach to cells with other growth modes and shapes. More quantitative understanding of walled cell mechanics will bring important new insights into the basics of cell growth, morphogenesis, and mechanisms of host invasion.

Acknowledgments

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