Chapter 6

Measuring In Vivo Signaling Kinetics in a Mitogen-Activated Kinase Pathway Using Dynamic Input Stimulation

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Abstract

Determining the in vivo kinetics of a signaling pathway is a challenging task. We can measure a property we termed pathway bandwidth to put *in vivo* bounds on the kinetics of the mitogen-activated protein kinase (MAPk) signaling cascade in *Saccharomyces cerevisiae* that responds to hyperosmotic stress [the High Osmolarity Glycerol (HOG) pathway]. Our method requires stimulating cells with square waves of oscillatory hyperosmotic input (1 M sorbitol) over a range of frequencies and measuring the activity of the HOG pathway in response to this oscillatory input. The input frequency at which the pathway's steady-state activity drops precipitously because the stimulus is changing too rapidly for the pathway to respond faithfully is defined as the pathway bandwidth. In this chapter, we provide details of the techniques required to measure pathway bandwidth in the HOG pathway. These methods are generally useful and can be applied to signaling pathways in *S. cerevisiae* and other organisms whenever a rapid reporter of pathway activity is available.

Key words: MAP kinase, Microfluidics, HOG pathway, Bandwidth, Frequency-response, Kinetics

1. Introduction

MAP kinase cascades are ubiquitous highly conserved phosphorylation cascades found in signaling pathways throughout the eukaryotic kingdom (1–3). The MAP kinases regulate diverse cellular processes, including differentiation, apoptosis, and proliferation (2). These cascades generally consist of three highly conserved kinases: a MAP kinase kinase kinase (MAPKKK), a MAP kinase kinase (MAPKK), and a MAP kinase (MAPK). When a cell is exposed to an external stimuli components of the appropriate MAP kinase pathway, including upstream kinases and the MAPK cascade, are sequentially activated by phosphorylation. The phosphorylated and activated MAPK triggers appropriate

transcriptional and regulatory responses within the cell that lead to altered gene expression and protein activity (4, 5).

In *Saccharomyces cerevisiae*, the MAP kinase cascade that responds to increased external osmolarity is called the High Osmolarity Glycerol, or HOG pathway. The HOG pathway has two branches through which it receives input. One branch works through the Sho1 membrane protein and the MAPKKK Ste11. The other branch utilizes a phosphorelay system (involving the proteins Sln1, Ypd1, and Ssk1) and two semiredundant MAPKKKs (Ssk2 and Ssk22). When the HOG pathway is stimulated by increased osmolarity, the MAP kinase of the pathway, Hog1, is phosphorylated and localizes to the nucleus where it interacts with various transcription factors and begins the cell's transcriptional response to osmotic stress. The localization of Hog1 tagged with GFP (Hog1-GFP) can therefore be used as a reporter of the activity of the HOG pathway.

The HOG pathway is a well-studied system and much effort has been placed into measuring its kinetics and modeling the pathway's dynamics (6, 7). However, much of this work has been done in vitro and in silico. Here, we report a method for measuring the kinetics of all reactions in the pathway in vivo by measuring a property called pathway bandwidth (8). Pathway bandwidth puts a lower bound on the in vivo reaction rates in a cellular signaling pathway; no reaction can be slower than the pathway bandwidth.

For a signaling pathway responding to oscillatory input, the pathway bandwidth is defined as a critical frequency of input f_c above which the pathway can no longer respond faithfully to the input signal but either averages over the incoming signal or barely responds. We developed a theory and experimental technique for measuring the bandwidth of the HOG pathway, the pathway in *S. cerevisiae* which responds to hyperosmotic stress (8). To measure the bandwidth of the HOG pathway, we built a novel microfluidic device which allowed us to expose yeast cells to oscillating osmotic conditions (between 0 and 1 M sorbitol) while confined to a growth chamber. We then followed the activity of the pathway (by monitoring Hog1-GFP localization) real-time using a fluorescence microscope and measured the amplitude of this localization as the response of the pathway. The critical frequency of input f_c above which the Hog1-GFP localization was significantly reduced at steady state was taken to be the pathway bandwidth and found to be ~$4.6 \times 10^{-3}\,\text{s}^{-1}$. Furthermore, we were able to differentiate between the two input branches to the pathway and found that the Sho1 input branch is slower than the Ssk1 input branch with a bandwidth of ~$2.6 \times 10^{-3}\,\text{s}^{-1}$.

In this chapter, we describe the methods used to measure signaling pathway bandwidth. These methods can be adapted for use with a variety of signaling pathways in yeast and other organisms, and are therefore generally applicable to the study of a wide range of signaling questions.

2. Materials

2.1. Yeast Strains and Culture

1. Yeast strain ySR255 (S288C Mat a leu2Δ0 lys2Δ0 Hog1-GFP::HIS3 HTB2-mCHERRY-URA3). Htb2 was tagged with mCherry in the Hog1-GFP strain from the yeast GFP collection (9) using primers oSR421 (tactagggct-gttaccaaatactcctcctctactcaagccGGTGACGGTGCTGGTTTA) and oSR422 (aaaagaaaacatgactaaatcacaatacctagtgagtgac-TCGATGAATTCGAGCTCG) and plasmid pSR101 (mCherry-pTEF-caURA3-AMP in the pRS406 backbone (10)). Standard molecular biology and yeast transformation techniques were employed (11).

2. Synthetic complete yeast media (SC): 6.7 g YNB w/o AA (MP Biomedicals LLC, Pasadena, CA), 2 g CSM amino acid supplement (MP Biomedical LLC, Pasadena, CA), 20 g glucose dissolved in 1 l of water, autoclaved at 121°C at 15lb/sq of pressure.

3. 1 M Sorbitol synthetic complete media: 91.1 g of D-sorbitol is dissolved in synthetic complete yeast media, and this solution is brought up to 500 ml and sterile filtered using a 0.2-μm Supor machV membrane in the 0.75-mm filter unit from Nalgene.

4. 14 ml Polypropylene round bottom tubes.

2.2. Photolithography

1. 5,080 dpi photolithography transparency mask (Pageworks, Cambridge, MA).

2. 4″ Silicon Wafers (P(100) 0–100 Ω cm SSP 500 μm Test 100 crystal orientation, one-side polished) (University Wafers, South Boston, MA).

3. SU8 2050 (Microchem Corp., Newton, MA).

4. Propylene glycol methyl ether acetate (PGMEA) (Sigma–Aldrich, Saint Louis, MO).

5. AB-M Mask Aligner (ABM Inc., San Jose, CA).

6. Headway Spin Coater Model PWM32 (Headway Research, Garland, TX).

7. Veeco Profilometer, Model Detak 6M (Veeco Instruments, Plainview, NY).

8. 3-Aminopropyl-triethoxysilane, 99% (Sigma–Aldrich, Saint Louis, MO).

9. Hot Plate, Model EchoTherm HP30 (Torrey Pines Scientific, San Diego, CA).

2.3. Microfluidics

1. Dow Corning SYLGARD 184 Elastomer kit containing Sylgard 184 base, Sylgard 184 curing agent (polydimethylsiloxane, PDMS) (Ellsworth Adhesive Systems, Germantown, WI).

2. Cover Glass 23 × 60 mm No. 1 (VWR International Inc., Pittsburgh, PA).

3. 3M Scotch tape, Matte Finish, Magic Tape (3M, St. Paul, MN).

4. Plasma-Preen Cleaner/Etcher (Terra Universal, Fullerton, CA).

5. VWR Gravity Convection Oven Model 1300U (VWR International Inc., Pittsburgh, PA).

6. Harris Uni-Core 1.5 mm PDMS punches (Ted Pella Inc., Redding, CA).

7. 16 G 1½ and 21 G 1½ PrecisionGlide needles (Becton–Dickinson, Franklin Lakes, NJ).

2.4. Cell Loading and Adhesion

1. 1× Phosphate-buffered saline (calcium and magnesium-free, pH 7.4) (Mediatech Inc., Herndon, VA).

2. D-Sorbitol.

3. ConA loading solution: 2 mg/ml concanavalin A (conA) (supplied as a lyophilized, white powder. Essentially salt-free and carbohydrate-free, MP Biomedical LLC, Pasadena, CA), 5 mM $MnSO_4$, 5 mM $CaCl_2$, dissolved in 1× phosphate-buffered saline.

2.5. Microscopy

1. Microscope slide holder (1-mm thick aluminum, cut 1.25 × 3″ with 0.9″ × 0.9 inner square hole).

2. Lab Labeling Tape (VWR International, Pittsburgh, PA).

3. Zeiss 200M Fluorescence Microscope (Carl Zeiss MicroImaging, Inc., Thornwood, NY).

4. Orca-II-ER Camera (Hamamatsu, Bridgewater, NJ).

5. 100×/1.45 NA plan α fluor objective (Carl Zeiss MicroImaging, Inc., Thornwood, NY).

6. Metamorph (Molecular Devices, Sunnyvale, CA).

2.6. Fluid Control

1. RS-232 8-Channel 1-Amp N-Channel FET Controller Board (controlanything.com, Osceola, MO).

2. Fluidic switch, LFAA1201418H Model (The Lee Company, Westbrook, CT).

3. Intramedic tubing, Becton–Dickinson 0.86 mm inner diameter, 1.27 mm.

4. Outer diameter (Becton–Dickinson, Franklin Lakes, NJ).

5. Visual Basic software (Microsoft Visual Basic Express Edition 2008) (Microsoft, Redmond, WA).

6. Computer with available serial port.

7. DCTX-1216 12 V dc 1.2 A Wall Transformer (Allelectronics. Com, Van Nuys, CA).

8. 100 ft PVS for LIF Soft Tubing 0.042″ inner diameter (Lee Company, Westbrook, CT).

9. VWR Talon regular clamp holder (VWR International Inc., Pittsburgh, PA).

10. Screw cap tubes (15 and 50 ml Axygen Scientific, Union City, CA).

11. High vacuum grease silicone lubricant (Dow Corning, Midland, MI).

12. O-Ring stand (VWR International Inc., Pittsburgh, PA).

13. Tube clamps (VWR International Inc., Pittsburgh, PA).

14. Small binder clips for fluid control (VWR International Inc., Pittsburgh, PA).

2.7. Image and Data Analysis

1. ImageJ (http://rsbweb.nih.gov/ij/index.html).

2. Matlab (The Mathworks Inc., Natick, MA).

3. Methods

To measure signaling pathway response in vivo over different input frequencies, we use a microfluidic device that allows for rapid periodic changes in media while cells are continuously monitored under an inverted fluorescence microscope.

Rapid changes in media are difficult to achieve in conventional microfluidic devices. Our device has stimulating (1 M sorbitol) and nonstimulating media entering through the two inlets of a Y-shaped flow cell, as shown in Fig. 1. The flow of the media to the flow cell is gravity-driven and the flow velocity within the flow cell is proportional to the pressure drop ΔP between the inlet and outlet (12). The dimensions of the flow cell are shown in Fig. 4, which is a diagram of the mask used to make the flow cell. At these length scales and with an average flow rate of 7,500 $\mu m^3/s$ the Reynolds number Re of the fluids in the flow chamber stays $Re < 2,300$ and therefore flow in the flow cell is laminar (12, 13). The only mixing occurs by diffusion which scales as $\sqrt{(Dx/u)}$ with D representing the diffusion constant of the media, u the speed of the laminar flow, and x the distance from the point of union of the two fluids, measured along the direction of the flow. Near the point where the two fluids meet, mixing is minimal. The pressure difference between the two fluids is changed by using a computer-controlled
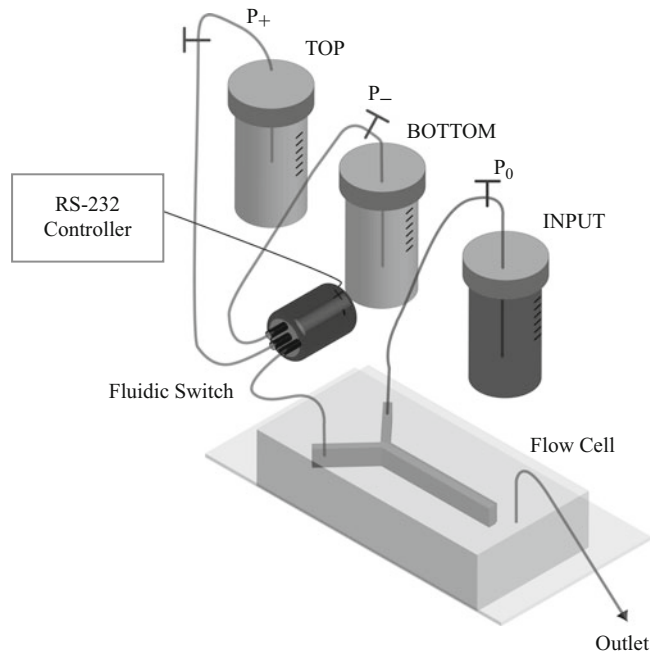
Fig. 1. One of the input arms of the Y-shaped flow cell is fed by a stimulating media (1 M sorbitol synthetic complete yeast media, *dark gray*) contained in the reservoir INPUT at a hydrostatic pressure head P_0. The other arm is fed by nonstimulating media (synthetic complete yeast media, *light gray*) from one of the two reservoirs, TOP at a hydrostatic pressure head P_+ or BOTTOM at P_-. The choice between the two reservoirs, TOP or BOTTOM, is made by a fluidic switch which is controlled using the RS-232 controller. When reservoir BOTTOM is chosen, the fluid from reservoir INPUT fills most of the chamber, while when TOP is chosen, the fluid from TOP fills the chamber. Periodic changes in which reservoir (TOP or BOTTOM) feeds nonstimulating media to the flow cell allow a change in the environment of the cells at a tunable period T.

fluidic switch to change the reservoir being used. By changing the relative pressure between the stimulating and nonstimulating media we can sweep the separation line across the width of the flow cell. This allows us to rapidly switch the conditions to which the cells in the flow cell are exposed. The media can be changed as frequently as twice per second without perturbing cell adhesion. Cell adhesion is achieved by functionalizing the glass coverslip with conA as described below. conA is a lectin which binds specifically to mannosyl and glucosyl residues in the yeast cell wall (14). Appropriate alignment is achieved by observing the interface between the stimulating and nonstimulating media in real time by using phase contrast microscopy as detailed below. Due to the difference in refractive index between the two fluids the interface is clearly visible (Fig. 2).

During the course of the experiment cells are stimulated with programmed input waves. The switching is controlled by a RS-232 relay controller which controls power to the fluidic switch.
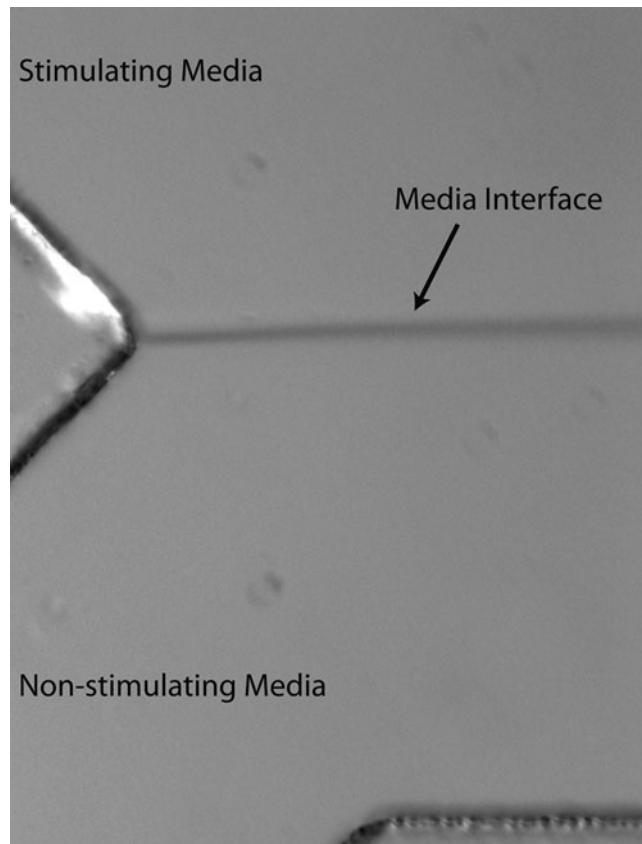
Fig. 2. Picture of the interface between stimulating (1 M sorbitol) and nonstimulating media in the flow cell under phase microscopy at ×40 magnification. The inlets where media enters the flow cell are on the *left* of the picture.

The controller interfaces with a custom-written Visual Basic program. The frequency and duty cycle of the input wave are adjustable. Image acquisition is controlled by using a multidimensional acquisition program. In our particular experiments, we used MetaMorph's multidimensional acquisition feature to acquire differential interference contrast (DIC), mCherry, and GFP images at fixed intervals. Autofocusing is achieved using Metamorph's built-in autofocusing software in the mCherry channel. Emission from GFP is visualized at 528 nm (38 nm bandwidth) upon excitation at 490 nm (20 nm bandwidth) and emission of mCherry is visualized at 617 nm (73 nm bandwidth) upon excitation at 555 nm (28 nm bandwidth). Images are processed using a custom-written ImageJ program to identify the nucleus of each cell using thresholding in the mCherry channel and then measuring the Hog1-GFP signal that is colocalized in that region. The ImageJ macro returns intensity and location data for each feature analyzed. A custom-written Matlab program is then used to analyze the ImageJ data, identify cells throughout the time course, and measure Hog1-GFP nuclear

localization for each cell. The response of the pathway is then computed as the average of the amplitudes across the time course. Finally, the bandwidth of the pathway is computed using Matlab's curve fitting utility to fit the amplitude data across different input frequencies f_i to the equation:

$$X = \sqrt{\frac{G}{1 + (f_i \tau_c)^2}} + \delta, \tag{1}$$

where $f_c = 1/\tau_c$ is taken as the critical input frequency, or pathway bandwidth. G represents the gain of the system. The term δ takes into account a constant offset that is the result of changes in autofluorescence due to changes in cell size as water enters and leaves the cell.

It is also consistent to fit across input periods T to the equation:

$$X = A \frac{\left(1 - e^{-k_{on} T/2}\right)\left(1 - e^{-k_{off} T/2}\right)}{\left(1 + e^{-\left(k_{on} + k_{off}\right) T/2}\right)} + \delta, \tag{2}$$

where the larger of k_{on} or k_{off} is taken as the pathway bandwidth f_c. Here, A represents the gain of the system and δ is again a constant offset due to cell size change.

3.1. Timeline

The timeline for running a flow cell experiment is given here. Several of the steps need to be performed days and hours before the microscopy experiment is started:

Prior to day 1: Prepare the microfluidic mask. This mask can be reused many times to make multiple flow cells.

Day 1: Pour uncured PDMS into flow cell molds. Cure in convection oven overnight.

Day 2: Cut out the plasma bond PDMS flow cell. Cure overnight. Inoculate yeast into 4 ml of synthetic complete media to grow to saturation overnight.

Day 3: Run the flow cell experiment.

Five hours prior: Reinoculate yeast from the saturated culture approximately 5 h before you would like to begin microscopy.

One hour prior: Prepare the setup shown in Fig. 1 approximately 1 h before you would like to begin microscopy.

Immediately before: Check the line, load cells into the flow cell.

During: Set up a time course acquisition using the appropriate multidimensional acquisition software.

After: Thoroughly clean the setup.

Following days: Image processing and data analysis

3.2. Fabrication of the Microfluidic Mask (see Note 1)

1. Sonicate silicon wafers for 5–10 min in an acetone bath. Rinse with isopropyl alcohol. Dry wafers with a nitrogen gun. Bake wafer at 200°C for at least 10 min to remove moisture. Cool wafer with nitrogen gun until it is cool to the touch.

2. Spin coat the wafer with desired thickness of SU8 2050. For our masks we coated the wafers to approximate thickness of 100 μm using a Headway Spin Coater Model PWM32. The spin program is as follows:

   Step 1: Speed 500 rpm/s, Ramp 100 rpm/s, 10 s

   Step 2: Speed 1,000 rpm/s, Ramp 300 rpm/s, 30 s

   Step 3: Speed 0 rpm/s, ramp 500 rpm/s, 0 s

3. Prebake coated wafer at 65°C for 10 min.

4. Using a 28-gauge needle and syringes filled with PGMEA, carefully remove the SU8 edge on the wafer while the wafer is spinning at 1,000 rpm on the Headway Spin Coater.

5. Place the wafer at 95°C for 50 min, allow wafer to cool to 65°C by changing hot plate temperature to 65°C and waiting for temperature to adjust (approximately 15 min on Torrey Pines HP30 hot plates).

6. Cover the wafer with a 360-nm long-pass filter and the transparency mask and expose for 1 min at 25 mW power on the AB-M Mask Aligner (see Note 2).

7. Put wafers at 65°C (1 min), allow hot plate temperature to ramp to 95°C (approximately 7 min), keep wafers at 95°C for 10 min. Allow temperature to ramp back down to 45°C (approximately 20 min). Move wafer to the bench and allow cooling at room temperature for 5–10 min.

8. Put wafer in PGMA with sporadic stirring for 10 min or until unexposed SU8 is removed. Rinse with isopropyl alcohol when it is clear that the unexposed SU8 has been removed. Dry wafer with the nitrogen gun (see Note 3).

9. Measure mask features using a contact profilometer.

10. Place mask in an appropriately sized petri dish.

11. Incubate the mask in a fume hood in an enclosed vacuum chamber at 6 mmHg pressure for 3 h with two to three drops of 3-aminopropyl-triethoxysilane in a separate disposable aluminum tray (see Note 4).

3.3. Flow Cell Preparation (see Note 5)

1. Flow cell masks patterned with SU8 are constructed on 4″ silicon wafers using standard photolithography techniques for microfluidics (12). Once the microfluidic mask has been made (see above) it can be reused many times to make multiple flow cells. The design of the mask is shown in Fig. 3.

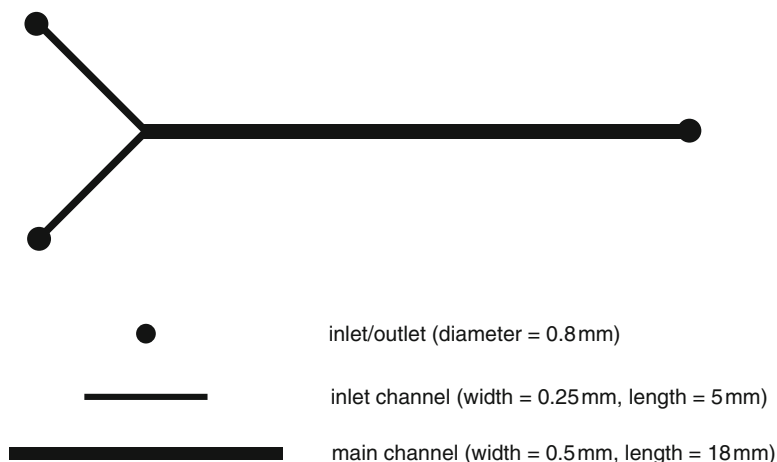2. Place the mask in a petri dish if you have not done so already.

inlet/outlet (diameter = 0.8 mm)

inlet channel (width = 0.25 mm, length = 5 mm)

main channel (width = 0.5 mm, length = 18 mm)

Fig. 3. Diagram of the mask pattern used to make the PDMS flow cell. The height of the channels is 100 μm. The height is set by how thickly the SU8 is applied as explained in Subheading 3.2.2.

3. Prepare PDMS by mixing curing agent and polymer in a 1:9 ratio by weight (see Note 6).

4. Degas the PDMS in a vacuum desiccator. The amount of pressure is not important, but will affect the amount of time required to degas the PDMS. Be cautious that your mixing container is not overfull, or the PDMS will bubble over during degassing.

5. Pour the PDMS carefully into the petri dish so that new air bubbles are not created. If bubbles form, remove them carefully with a 21 G 1/2 gauge needle without scratching the mask.

6. Cure the PDMS at 65°C overnight.

7. Using a razor blade, cut out a 15 × 40 mm rectangle of PDMS surrounding the flow cell design. Cut gently without pressure so as not to break the mask. When the PDMS separates from the underlying mask remove the rectangle of PDMS from the mold. The mold can now be reused by simply refilling the hole made in the cured PDMS (see Note 7).

8. Punch holes for the inlets and outlets using a PDMS puncher.

9. Clean the PDMS block with scotch tape. With the feature side face up on the bench, apply tape being careful not to touch the feature-side of the block with your gloves. Repeat this three times (see Note 8).

10. Plasma clean the PDMS block and a 23 × 60 mm coverslip for 30 s at 100 W plasma power at 30 mTorr base pressure and 200 mTorr process pressure.
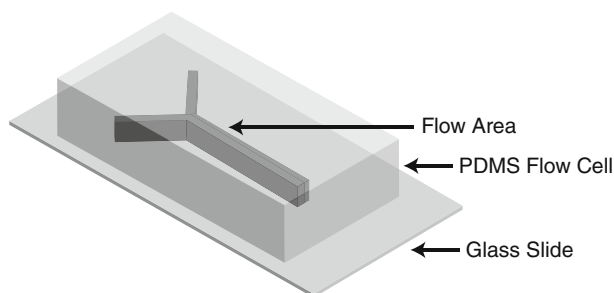
Fig. 4. The microfluidic flow cell after plasma cleaning and bonding to the cover glass.

11. Apply the PDMS to the coverslip feature-side down to create the finished microfluidic flow cell shown in Fig. 4.

12. Cure the flow cell in the convection oven at 65°C for several hours or overnight (see Note 9).

3.4. Preparation of Yeast Samples

1. Grow yeast cells to saturation overnight in standard yeast synthetic complete (SC) yeast media (11) at 30°C with shaking (OD600 ~2).

2. Reinoculate cells into fresh media several hours before the experiment is started. Reinoculate at a low density in SC media in a conical flask such that cells are in early exponential growth phase (OD600 ~0.05 for haploid yeast) just before being loaded into the flow cell (approximately 100 μl in 25 ml of SC media for our strains and media).

3.5. Preparation of the Switch and Liquid Handling

1. The experimental setup with the liquid containers for feeding the flow cell and switching media in the flow cell is shown in Fig. 1. Punch a hole in the plastic top of two 50 ml screw cap tubes and one 15 ml screw cap tube with a 16 G 1/2 gauge needle.

2. Label one 50 ml tube TOP and fill it with 45 ml of synthetic complete yeast media (SC). Label one 50 ml tube INPUT and fill it with 45 ml of 1 M sorbitol synthetic complete media. Fill the 15 ml tube with 13 ml of SC and label it BOTTOM.

3. Suspend the tubes on the ring stand using the clamp holders. The TOP tube should be the highest, followed by the INPUT, and then the BOTTOM tube.

4. Connect the RS-232 controller to your computer through the desired COM serial port. Attach a power source to the RS-232 controller but do not plug it in yet.

5. Open the Switch Control software. The interface for the software is shown in Fig. 5 (see Note 10).
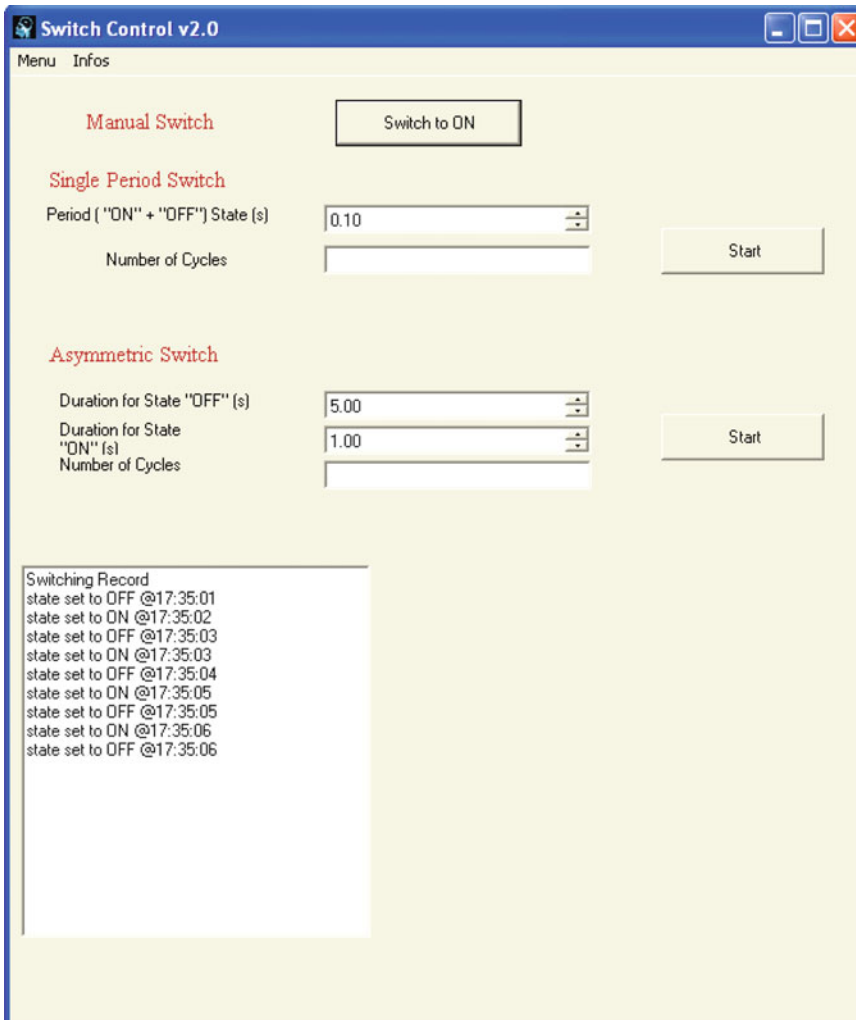
Fig. 5. The Visual Basic software user interface for controlling the RS-232 relay controller. The user is able to define both symmetric and asymmetric input waves. The switching times are recorded and can be saved from "Menu." The "OFF" state refers to when the flow cell is filled with media from the TOP reservoir and the "ON" state refers to when the flow cell is filled with media from the INPUT reservoir (1 M sorbitol).

6. Connect the switch to the RS-232 microcontroller relay R1. Connect a power source to the switch.

7. Plug in power sources for the switch. Check that the COM port is communicating with the RS-232 controller by switching the switch on and off several times using the software.

8. Attach 60 cm of soft tubing with inner diameter 0.042″ to the outlet of the Lee switch (see Note 11). With the other end of the tubing in a conical flask full of sterile water clean and remove bubbles from the switch by running MiliQ water through both outlets using 10 s "ON," 10 s "OFF" switching for about 5–10 min.

9. Insert 60 cm of the intramedic tubing into each media tube. Allow media to flow to the bottom of the tubing before clamping the tubing with a binder clip (see Note 12).

10. Attach 5 cm of the soft tubing to the inlets of the Lee switch.

11. Insert the end of the tubing from the TOP tube into the soft tubing attached to the upper inlet on the switch. Insert the end of the tubing from the BOTTOM tube into the soft tubing attached to the lower inlet on the switch. Insert 2″ of intramedic tubing into the soft-tubing attached to the outlet of the Lee switch.

12. To ensure that there are no air bubbles trapped in the switch use the software to turn the switch to the "ON" state. Unclamp the BOTTOM tube and allow media to flow until there are no bubbles. Repeat for the TOP tube with the switch in the "OFF" state (see Note 13).

13. Clean a brand new flow cell by injecting the cell with 70% ethanol followed by sterile water by syringe injection. Make sure to fill the flow cell with water completely, so that the inlets and outlets are covered with fluid to prevent air bubbles.

14. Attach the flow cell to the metal slide holder with lab tape.

15. Tape the Lee company switch to the microscope stage.

16. With the flow cell on the microscope stage, insert the intramedic tubing from the fluidic switch outlet into the flow cell inlet closest to you (see Fig. 1).

17. Unclamp the intramedic tubing from the INPUT tube and insert it into the other flow cell inlet.

18. Allow the flow cell to fill with media.

19. Cut 8″ of intramedic tubing and insert it into the flow cell outlet. Allow this tubing to fill with media. Put the end of the tubing into the waste collection container and fill the waste container with 50 ml of sterile water. Make sure that the end of the outlet tube is completely submerged (see Note 14).

20. Unclamp all tubing if you have not done so already.

21. By changing the relative heights of the TOP, BOTTOM, and INPUT tubes set the line under the microscope using phase contrast microscopy so that it matches the diagram in Fig. 6 in the "ON" and "OFF" states. The "ON" state means that the flow cell is filled with the INPUT media and "OFF" means that the flow cell is filled with media from the TOP tube (see Note 15).

22. Once the line is set you are ready to load cells into the flow cell. Clamp all tubing to avoid leaks and remove the flow cell in its holder from the microscope.

From Fluidic Switch

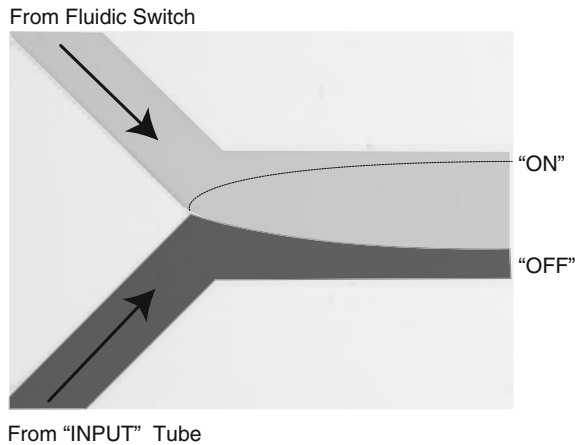"ON"

"OFF"

From "INPUT" Tube

Fig. 6. Top view of flow cell. The figure shows the appropriate orientations of the media interface for the "ON" and "OFF" states. The input is "ON" when the flow cell is filled with media from the INPUT reservoir (*dark gray*) and "OFF" when the flow cell is filled with media from the TOP reservoir (*light gray*).

3.6. Preparation of the conA Loading Solution

1. Gather the appropriate supplies: 45 ml of sterile H_2O, 2.5 ml of 1 M $CaCl_2$ in H_2O, 2.5 ml of 1 M $MnSO_4$ in H_2O, and conA (MP Biomedicals cat. no. 150710/CAS #11028710/ EC #2342582 supplied as a lyophilized white powder. Essentially salt-free and carbohydrate-free, molecular weight not available).

2. Mix the water, $CaCl_2$, and $MnSO_4$. The pH of this solution should be between 6 and 7.

3. Dissolve conA to a concentration of 20 mg/ml in this solution.

4. Dilute this conA solution in sterile water to make a 2 mg/ml solution for use in the flow cell (see Note 16).

3.7. Loading Cells into the Microfluidic Chip

1. Clean flow cell with 70% ethanol by injection. Use a syringe that has intramedic tubing slipped over a 16 G 1/2 gauge needle to syringe inject the ethanol. Then inject MiliQ water. Use 1–2 ml of ethanol and water.

2. Load the 2 mg/ml conA solution into the flow cell using a syringe. Allow conA to incubate in the flow cell at room temperature for 15 min (see Note 17).

3. While conA is incubating spin down cells (3000 g) growing in a 14-ml falcon tube (~4 ml of cells in log-phase growth.)

4. Resuspend cells in 4 ml sterile water, and spin down again. Discard the supernatant. Resuspend cells in remaining supernatant by gently shaking the tube. If needed add a few hundred microliters of water to the cells.

5. When the conA is incubated, load the cells.

6. Allow cells to sit in the flow cell for 5 min at room temperature on the bench.

7. Check that cells have stuck at 40× magnification. Stuck cells will not move when the flow cell is gently tapped.

8. Put the flow cell back on the microscope and replace the appropriate tubing. Recheck the media interface.

9. Unclamp all tubing and check for leaks. Make sure that the switch is in the "OFF" position.

3.8. Microscopy Time Course (see Note 18)

1. With the switch in the "OFF" position allow cells to equilibrate for 20 min.

2. Switch the microscope objective to 100×.

3. Set the parameters for the time course on the microscope software. Set the software so that the stage position, time, and channel of each image are recorded in a log file.

4. Set stage positions to acquire in the middle of the flow channel. This is important as the middle of the channel is not exposed to diffusion region near the media interface and therefore sees the appropriate input signal.

5. Once cells have equilibrated start the multidimensional acquisition and then start fluid switching.

6. Acquire a DIC (10 ms), mCherry (50 ms), and GFP (200 ms) exposure at each time point. Take pictures every n seconds where $n = $ (the period of the switching)/10 (see Note 19).

7. Periodically check the experiment for leaks.

8. Periodically check that the "ON" and "OFF" states of the line are correct by pausing the multidimensional acquisition and monitoring switching by eye using phase microscopy. This will require movement of the stage to monitor different regions of the flow cell, thus the stage position of interest must be marked in the microscopy software.

3.9. Cleaning the Setup

1. Once the experiment is over, clamp all tubing. Remove all tubing and clean with 70% ethanol by injection from a syringe with a 16 G 1/2 gauge needle.

2. Repeat cleaning of the switch using sterile water as described previously.

3.10. Image Processing (see Note 20)

1. We used a custom-written ImageJ macro to threshold on the Htb2-mCherry images and then measure the colocalization of Hog1-GFP with these nuclear regions. However, any program which allows you to collect intensity data for Hog-GFP in the cell's nucleus will work.

2. Run the ImageJ macro to extract values for Hog1-GFP colocalization with the Htb2-mCherry tagged nucleus.

3. Allow the ImageJ macro to run (see Note 21).

4. This macro returns an excel file which contains the intensity data for both the GFP and mCherry channels.

5. The Matlab program takes the intensity data, a background file (found by measuring the average background in each image slide using ImageJ), and the log file recorded by the multidimensional acquisition routine.

6. Use the Matlab program to reconstruct time traces for the nuclear intensity for each cell in the experiment.

7. Calculate the amplitude of the cellular response to the input by finding the mean amplitude of each cell (over the first few periods in the experiment so that photobleaching does not have a significant effect) then computing the mean and standard deviation of these means.

3.11. Bandwidth Measurement

1. Amplitude distributions are collected for Hog1 localization over a range of input frequencies.

2. The bandwidth is found using Matlab's Curve Fitting Toolbox and fitting the amplitude data to Eq. 1 or 2. The variable f_c is taken to be the pathway bandwidth.

4. Notes

1. All fabrication steps for creation of the microfluidic mask were performed in a nanofabrication facility with an appropriate clean room. Similar facilities are available at many universities and research institutes.

2. Failure to filter wavelengths below 350 nm will result in overexposure of the top portion of the SU8 resist film leaving negative sidewall profiles or T-topping. If you see these features, then check whether you have used the correct bandpass filter. Alternatively, reduce exposure time.

3. Problems in the fabrication process often become apparent during development. If the developed mask pattern does not remain in contact with the wafer or there is excessive cracking this indicates an under cross-linking condition and can be corrected by increasing the exposure time or increasing the postexposure bake.

4. Silanization of the mask is an optional step. However, it allows for easy removal of the polymerized PDMS used for creation

of the flow cell and is highly recommended to increase the useful lifetime of the mask.

5. Always wear nitrile gloves when handling PDMS. Oil from your skin can compromise the polymerization of the PDMS.

6. This is most easily done in a disposable plastic container (such as a drinking cup) because leftover PDMS will eventually ruin most containers. Aim for about 10 ml of PDMS the first time you use the mask. During subsequent flow cell construction you will need less PDMS to fill the mold because not all cured PDMS is removed after each round of construction. Mix the PDMS *very* thoroughly with a plastic disposable fork.

7. Cracking masks is inevitable. It is recommended to have two to three identical masks on hand so that breaking a mask does not cause experimental delays.

8. If desired, the flow cell can be stored in this state (with tape covering the attachment side) for several days before plasma cleaning and bonding to the coverslip.

9. This curing step can help remove small bubbles than might have formed between the PDMS and the coverslip. Do not cure for longer than overnight, as PDMS will shrink with excessive drying affecting the channels and seal with the coverslip.

10. Visual Basic code for controlling the switch is available upon request.

11. Vacuum grease can be used to ease insertion of the switch outlet into the tubing. We have found that we do not need additional connectors for connecting tubing to the switch or to the microfluidic chamber. Vacuum grease can also be used to seal minor leaks.

12. This may require using a syringe with a 21 G 1/2 needle to start flow. Be cautious when clamping the tubing with the binder clip. Care must be taken to prevent leaks as they are messy and expensive around microscopy equipment.

13. This step is crucial. Even small bubbles will aggregate in the switch becoming very large over the course of the experiment until they eventually release and perturb fluid flow or cell adhesion upon reaching the interior of the flow cell.

14. If the outlet tube is not submerged in an waste container containing a large amount of liquid the pressure in the flow cell with change drastically over the course of the experiment altering the alignment of the interface between the stimulus and nonstimulus.

15. It is often easier to set the line using a lower magnification than used to observe cells over the course of the experiment. Try 40× magnification and phase illumination for setting the line.

16. This conA solution may be stored at $-20°C$ indefinitely and thawed immediately before use. It can be refrozen several times before its efficacy is reduced. Storage in aliquots of about 500 µl is recommended.

17. When loading flow cells always make sure that the flow cell outlets and inlets are covered with large droplets of MiliQ water to prevent drying and air bubble formation.

18. We used Metamorph imaging software to control the microscopy time course. We used the built-in multidimensional acquisition utility to acquire images at programmed stage positions, time points, and wavelengths. However, the instructions are easily modified to work with any microscope control software.

19. Pictures were never acquired more rapidly than once every 10 s. To maintain focus throughout the experiment we used Metamorph's autofocus routine to autofocus on the mCherry labeled nucleus once every few timepoints.

20. Matlab and ImageJ codes are available upon request.

21. The thresholding values for the mCherry channel might need to be manually adjusted so that they find the cell nucleus correctly. The values depend on the background of your camera.

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