High-throughput microfluidics to control and measure signaling dynamics in single yeast cells

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Microfluidics coupled to quantitative time-lapse fluorescence microscopy is transforming our ability to control, measure and understand signaling dynamics in single living cells. Here we describe a pipeline that incorporates multiplexed microfluidic cell culture, automated programmable fluid handling for cell perturbation, quantitative time-lapse microscopy and computational analysis of time-lapse movies. We illustrate how this setup can be used to control the nuclear localization of the budding yeast transcription factor Msn2. By using this protocol, we generate oscillations of Msn2 localization and measure the dynamic gene expression response of individual genes in single cells. The protocol allows a single researcher to perform up to 20 different experiments in a single day, while collecting data for thousands of single cells. Compared with other protocols, the present protocol is relatively easy to adopt and of higher throughput. The protocol can be widely used to control and monitor single-cell signaling dynamics in other signal transduction systems in microorganisms.

INTRODUCTION

Fluorescence microscopy has become a standard laboratory tool, and live-cell imaging of signaling dynamics[1,2], protein translocation[3–7] and cell-fate choice[8] is increasingly becoming routine[9]. This has led to a greater appreciation of the importance of dynamics in signal transduction, control of cell fate[10–13] and heterogeneity[14]. However, traditional live-cell imaging approaches suffer from an inability to maintain constant extracellular conditions, and they cannot make precisely controlled perturbations.

Microfluidics largely overcomes these limitations by combining chemostatic cell culture with the ability to make perturbations with exquisite spatiotemporal control[15–17]. In addition, as microfluidics is highly amenable to automation and only requires small volumes of culture medium, it is ideally suited for multiplexing, which vastly improves reliability and throughput[18,19]. Combined with automated image analysis, it is therefore possible to obtain time-lapse data sets of cell signaling at a large scale[1,18–21]. From a synthetic biology point-of-view, microfluidics provides control of cellular behavior[22] including long-term control of gene expression in single cells[23,24]. Applications of microfluidics coupled to time-lapse microscopy have yielded insights into signaling dynamics[7,25–28], spatial control of gene expression[29] and how cells monitor and respond to changes in their environment[30]. More widespread adoption of microfluidics coupled to time-lapse microscopy will help transform our understanding of signaling dynamics inside living cells.

Combining microfluidics[7] with time-lapse microscopy, we previously discovered that the budding yeast general stress response transcription factor Msn2 encodes information about external stresses in its translocation dynamics[4,28]. To systematically investigate how downstream genes respond to different Msn2 dynamics, we developed an automated high-throughput microfluidic device, which we describe in this protocol[19] (**Fig. 1**). The device enabled us to perform four-color quantitative time-lapse microscopy, in which we followed >100,000 single cells over time and controlled and quantified Msn2-mCherry translocation dynamics while measuring gene expression dynamics using fluorescent reporters (CFP and YFP) with high time resolution[19,21]. From these studies, we found that it is possible to induce multiple distinct gene expression programs simply by regulating the dynamics of Msn2 (ref. 19).

Here we provide our protocol. We describe how to design and fabricate the microfluidic device, how to set up control valves for automated fluid handling and provide software to interface valves with MATLAB. We describe how to set up automated time-lapse microscopy experiments and analyze the resulting time-lapse movies. Compared with related approaches, this protocol is simple and easy to adopt, and it yields a higher throughput. With this protocol, it is possible to conduct 20 different time-lapse experiments in a single day, generating data for thousands of single cells.

Applications of the method

In this protocol, we describe how to use a multiplexed microfluidic device to investigate signaling dynamics in *Saccharomyces cerevisiae* (**Fig. 1**). This protocol will be useful for any short- to medium-term (≤6 h) perturbation experiment (involving, e.g., a change in medium such as osmotic shock or exposure to a drug) with a fluorescent readout (e.g., a change in gene expression or subcellular localization). Cells are grown in five microfluidic channels (**Fig. 1**) that can be independently controlled: thus, perturbation and control experiments can be performed side by side, or multiple conditions for the same or different strains can be investigated in a single experiment.

As an example application, we describe here a typical experiment. Msn2 nuclear localization is regulated by phosphorylation by protein kinase A (PKA)[4,31]. By expanding the ATP-binding pocket of PKA, we can selectively inhibit PKA activity with a small molecule, 1-NM-PP1 (refs. 32,33), without inhibiting the activity of other cellular kinases. The addition of 1-NM-PP1 leads to rapid nuclear localization of Msn2—so by controlling 1-NM-PP1 exposure, we can control Msn2 localization[4,19,28]. We quantify Msn2 localization using an Msn2-mCherry fusion protein and segment the nucleus using an infrared (IR)[34] nuclear marker (NHP6a-iRFP)[19]. To quantify gene expression and intrinsic and extrinsic noise[35],

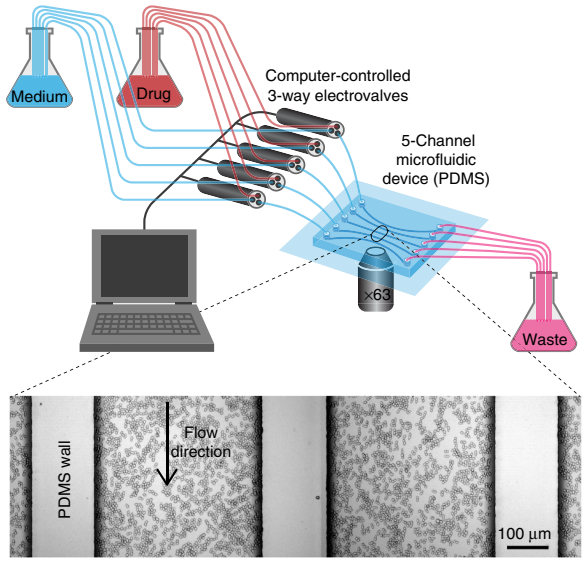

Figure 1 | Overview of the microfluidic setup. Top, microfluidic device. Five computer-controlled solenoid valves control whether normal medium or stress medium (e.g., with drug) is delivered to yeast cells grown in five microfluidic channels. Simultaneously, protein translocation dynamics and/or gene expression responses are recorded from all five channels using time-lapse microscopy. Thus, five separate perturbations can be multiplexed in a single experiment. Bottom, a cropped bright-field image of budding yeast cells growing in microfluidic channels (two images stitched horizontally together) is shown. Two full and two partial microfluidic channels (400 µm wide) are shown with a high density of cells growing in them. Each microfluidic channel is separated by PDMS walls (150 µm wide) and the direction of medium flow is from top to bottom. The top sketch is adapted from Hansen & O'Shea[19] with permission from EMBO.

we replace the open reading frame (ORF) of Msn2 target genes with fast-maturing CFP and YFP fluorescent proteins ($\tau_{maturation}$~8–10 min at 30 °C) in diploid cells. A typical experiment is shown in **Figure 2**. Cells were exposed to six 5-min pulses of 690 nM 1-NM-PP1 spaced by 10-min intervals (**Fig. 2a**,**b**), and gene expression was measured in each of ~100 single cells over time using YFP (**Fig. 2c**) and CFP (**Fig. 2d**) reporters with minimal measurement noise. In addition to studying the average level of gene expression, from these measurements we can also quantify intrinsic and extrinsic noise[35] in gene expression (**Fig. 2e**).

To keep cells immobile in the microfluidic device during time-lapse experiments, we coat the cover glass with concanavalin A (ConA)—a lectin that binds cell surface saccharides[36–38]. This works well for yeast, but it is also suitable for other microorganisms, including cyanobacteria. With the ever-increasing number of fluorescence-based sensors and reporters[39–42], our method is therefore applicable to studies involving changes in gene expression[19,28], changes in protein localization[28,43], changes in RNA localization[44], changes in metabolite levels[45] or any other process that can be monitored with a fluorescent readout in a range of microorganisms.

Finally, we highlight that the analog-sensitive kinase strategy[32] in combination with microfluidics and microscopy can be generally applied to control the activity of almost any kinase of interest with exquisite spatiotemporal resolution. The analog-sensitive mutations (such as Met→Gly for PKA[33]) have been generated for a number of kinases, including MAP kinases such as Hog1 (ref. 46); kinases involved in transcription such as Cdk7/Kin28 (ref. 47) and Cdk8/Srb10 (ref. 47); and other important kinases such as Pho85 (ref. 48), Fus3 (ref. 32), Cdc28 (ref. 32) and Snf1 (ref. 49; a partial list is given elsewhere[50]). Thus, this is a powerful method for investigating the dynamic regulation of any kinase-driven signaling pathway. Furthermore, our microfluidic method can be combined with other developments in pharmacological control of protein activity and localization[51,52] such as the 'anchor-away' method[53–55] to control and monitor the dynamics of kinase-independent signaling pathways.

Comparison with other methods

Several microfluidic methods have been described for yeast[7,23,27,30,56–60] and mammalian cells[61]. In yeast, most other methods address very specific questions[16,18,23,30]. For example, a number of recent methods elegantly enable very long-term imaging of yeast and measurement of replicative life span[56,57,62–65]. Other protocols can generate sinusoidal waves instead of step-function medium switching[16,30]. However, achieving such capabilities necessitates much more complex microfluidic devices. This highlights a trade-off between specialization on one hand and generality, simplicity and ease of use on the other hand. For example, a recent massively parallel microfluidic device[18] allows for the analysis of 1,152 different strains from the yeast GFP library[42] in a single experiment. This is clearly a technical breakthrough, and compared with such approaches our protocol is of much lower throughput. However, such setups[18,59] require extensive know-how and infrastructure beyond the capabilities of most laboratories. Furthermore, these setups may not be appropriate for very sensitive pathways such as the Msn2 pathway. There are also expensive commercial systems available, such as the ONIX system from CellAsic[66]. However, in this device, cells are retained through 'squeezing,' which induces a stress response, and hydrophobic inhibitors such as 1-NM-PP1 absorb into the material and therefore cannot be washed out again[19]. More generally, we note that medium switching when using hydrophobic inhibitors such 1-NM-PP1 can be problematic with microfluidic devices with low internal volumes (such as the ONIX system, but also other devices that retain cells through a low ceiling) and/or with a low flow rate, since 1-NM-PP1 may absorb into the device material. However, with microfluidic devices that combine a larger internal volume (100 µm ceiling) with a high flow rate such as our setup or others[7,43] this is not a problem.

Most other reported microfluidic devices are multilayered, which lengthens the photolithography process by requiring multiple spin-coating steps and accurate alignment. Our device is simply a single layer, and once the silicon master is fabricated or obtained, no specialized equipment is required for the production of microfluidic chips. The development of our device is inspired by a previously reported single Y-channel-shaped device that enables switching between two types of medium[7,38,67], which has been used to study Hog1 dynamics[7,60]. By adding five channels, our adaptation allows for more high-throughput measurements of multiple strains or conditions in a single experiment, and by automating fluid handling using solenoid valves controlled through MATLAB our setup will run in the absence of manual intervention. Thus, compared with simple Y-channel-shaped devices, our multiplexed and automated device greatly improves the experimental throughput. Our fluid handling system is made of chemically inert perfluoroelastomer—this is essential when you are working with hydrophobic inhibitors such as 1-NM-PP1, which absorb into most other materials.

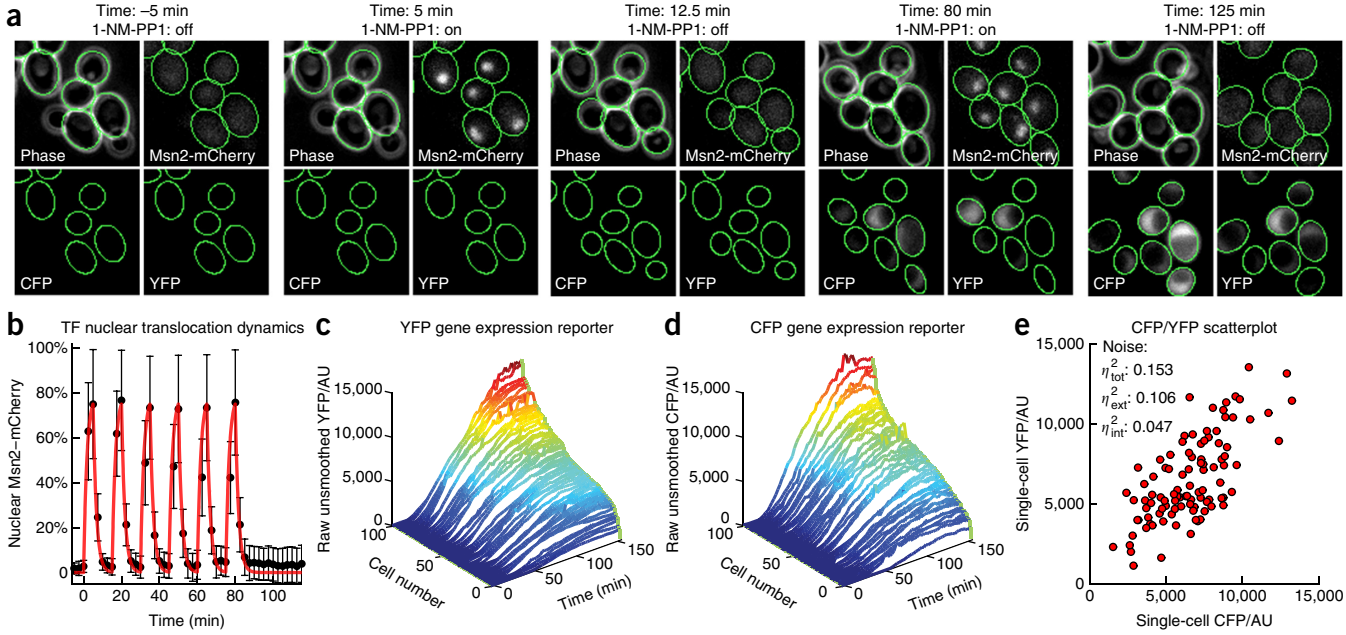

Figure 2 | A typical experiment. (**a**) Raw phase-contrast (top left), Msn2-mCherry (top right), CFP expression (bottom left) and YFP expression (bottom right) images are shown for five time points. Small (99 × 99 pixels) sections of full images are shown. The cell segmentation is overlaid in green. (**a**–**e**) The raw data in **a** and the quantified data in **b**–**e** are from the same time-lapse experiment. Images have been contrast adjusted, but not adjusted for photobleaching, which is why later Msn2-mCherry and YFP images appear less bright. CFP shows much lower photobleaching than YFP, and therefore it appears brighter in later frames. Msn2-mCherry nuclear localization is visible as nuclear foci in frames 2 and 4. Cell growth and movement is visible between frames. The cell segmentation algorithm (**Supplementary Tutorial 2**) fits the yeast cell phase ring to an ellipse. Frames with slight segmentation errors are deliberately shown to illustrate the limits of our segmentation algorithm. As the phase ring is outside the cell, for quantification of CFP and YFP expression ellipses with 3 pixels smaller *a* and *b* values are used (*a* and *b* define the size and shape of an ellipse). This is why the ellipses in the bottom images are smaller than those in the top images. Cell-to-cell variability in CFP and YFP expression is detectable both in the raw data and in **c**,**d**. (**b**) Protein translocation dynamics. This plot shows Msn2-mCherry translocation dynamics in response to six 5-min pulses of 690 nM 1-NM-PP1 spaced by 10-min intervals, which leads to ~75% of maximal Msn2 nuclear localization. Raw data (black dots) and error bars (s.d.) are from 101 single cells, and the red line shows a fit to the data. Much of the observed Msn2-mCherry variability is due to the nucleus moving in and out of focus. (**c**,**d**) Single-cell time traces of the YFP (**c**) and CFP (**d**) gene expression reporters. Raw, unsmoothed data in arbitrary units (AU) are shown. As can be seen, accurate time-trace quantification of gene expression is possible even without smoothing the single-cell traces. Further, the dynamics of promoter activity may be inferred from the single-cell time traces[78]. (**e**) By following both CFP and YFP gene expression dynamics in the same single cell, their covariance can be computed. Thus, we can calculate both intrinsic and extrinsic noise[35]. Each dot in the scatterplot is the maximum CFP and YFP from one single cell. The above experiment was performed with strain EY2967/ASH189 (ref. 21). **Supplementary Figure 1** shows a control experiment, in which the same plots are shown without 1-NM-PP1 treatment. In this case, we observe no gene activation, indicating that gene activation is Msn2- and 1-NM-PP1–specific.

Compared with more complex devices, our simpler setup requires less time to set up each experiment—for short- to medium-term experiments (≤5–6 h), this is crucial. We routinely perform four ~2.5-h time-lapse experiments per day, thereby sampling 20 conditions or strains in a day. Furthermore, for flow-rate control many other approaches (summarized in Crane *et al.*[57]) use air pressure, syringe pumps or expensive commercial equipment. Our setup simply uses gravity—by adjusting the relative height difference between the input medium flask and waste flask, the flow rate can be accurately and reproducibly controlled. Therefore, by eliminating the demands for expensive and complicated equipment, our method is easy to adopt and very inexpensive—the total cost per experiment is <$5.

Limitations

The main limitation of this protocol is cell retention for long-term imaging (>6 h). ConA-mediated cell retention slowly begins to fail after ~4 h of flow depending on the flow rate, and gradual loss of individual cells is observed. Furthermore, even a single cell eventually generates a very large colony. Therefore, for long-term imaging (>6 h), alternative approaches that selectively retain only mother cells are recommended[56,57,62–64]. In addition, other approaches are superior for more-complicated applications such as the generation of precisely defined gradients[16,30] or sub-second medium switching[7]. Although microfluidics coupled to time-lapse microscopy is a generally powerful technique, the inclusion of microfluidics does complicate the experimental setup. However, the current protocol should be relatively simple to set up, and it does not require expensive or complex equipment. We have verified this method for budding yeast and cyanobacteria and the method may work for other microorganisms, but we have not tested it.

Experimental design

Design and fabrication of silicon master mold (Steps 1–14). A silicon wafer master mold is used for making polydimethylsiloxane (PDMS) chips. To fabricate the silicon master, a mask is required (**Fig. 3**). Our negative transparency mask is available online (**Supplementary Data 1**), and several companies print masks at a resolution sufficient for photolithography. Our design consists of five 400-µm-wide channels separated by 150-µm-wide walls (**Figs. 1** and **3**), but it can also be modified to include more or fewer channels, depending on multiplexing requirements.

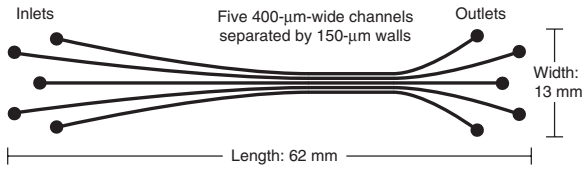

Figure 3 | Device design. The transparency mask with annotation is shown (**Supplementary Data 1**). The device consists of five 400-µm-wide channels, in which cells are grown, separated by 150-µm walls. It is a negative mask, so areas in black are exposed to UV during photolithography.

Once the mask has been obtained from a company (e.g., CAD/Art Services), the silicon master mold can be fabricated in a cleanroom. As our device contains only a single layer, no mask alignment is necessary during photolithography and the silicon master is therefore simply produced by spin-coating a 4-inch silicon wafer with SU-8 photoresist, exposing it to UV light under the transparency mask and developing it in PGMEA (propylene glycol methyl ether acetate; an organic solvent) to yield the final silicon master mold (**Fig. 4**). SU-8 photoresist cross-links under UV exposure, such that any unexposed SU-8 will be washed away during development, leaving only the desired pattern. Most universities have cleanroom facilities. However, the fabrication of a silicon master can also be outsourced to various companies (e.g., SIMTech). We use SU-8 2100 to generate a pattern with a height of ~100 µm, but a different height can simply be obtained by using another photoresist and by changing the spin-coating program accordingly (e.g., SU-8 2050 for a height of ~50 µm). In general, smaller heights lead to lower flow velocities and better cell retention. Higher flow velocities enable more rapid change of medium, but they also result in slightly higher cell loss during the experiment. At a flow rate of 1 µl per second per channel and a height of ~100 µm, a complete change of medium occurs within ~10 s. As the volume of a microfluidic channel (~0.6 µl/cm) is much lower than the internal volume of the solenoid valves, medium changing inside the microfluidic channel is almost instantaneous. At ~100-µm height, we have not observed clogging during an experiment—something that can be a common issue with very low heights (≤10 µm). After postexposure baking, if the recommended hard bake (Step 12) and silanization (Step 14) steps are included, the resulting silicon master mold is indefinitely stable and can be used for PDMS replica molding for years.

Soft lithography: fabrication of microfluidic chips (Steps 15–26). Once the silicon master mold has been obtained, the microfluidic device is fabricated through replica molding of PDMS elastomer[68]. For PDMS replica molding, we use Sylgard 184 (Dow Corning): PDMS is mixed with curing agent in a 1:10 (wt/wt) ratio, poured over the silicon master, and then degassed and cured at 65 °C (**Fig. 4**). Once cured, PDMS adopts a permanent and stable solid structure. Cured PDMS is then carefully peeled off the silicon master, and holes are punched for inlets and outlets. Next, the PDMS chip is bonded to the cover glass. After exposure to O_2 plasma, both the PDMS chip and cover glass will undergo surface activation[69,70], and they can subsequently be permanently bonded to form the desired microfluidic device (**Fig. 4**). After baking at 65 °C, we then insert 'adapter tubing' into the inlets and outlets and seal these with PDMS. This greatly eases the insertion of tubing when setting up microscopy experiments, and it also prevents leaks from occurring during time-lapse experiments.

As a material, PDMS is optically transparent from the near-UV range into the IR range, and it is therefore compatible with all standard fluorescent reporters. In addition, it is gas-permeable, inexpensive, hydrophobic, very easy to work with and biocompatible[68]. PDMS is therefore well suited for both cell culture and fluorescence microscopy.

Once a microfluidic device has been made, it can be stored for months without an observable loss in ConA-mediated cell retention (in a Petri dish to avoid dust accumulation). We find it more efficient to make a large number of devices at once and to store them rather than making a new device for each experiment.

Setting up solenoid valves for fluid control (Step 27). We use three-way solenoid valves and an electronic board to independently control fluid delivery to each of the five channels in which cells are grown (**Supplementary Tutorial 1** and **Supplementary Data 2**). We connect flasks containing normal medium and perturbation medium (e.g., with 1-NM-PP1) to the valves using polyethylene (PE) tubing (**Fig. 1**). The electronic board then controls which of the two inlets supplies the outlet, which is connected to the microfluidic device through ismaprene tubing. A number of electro-fluidic solenoid valves[67] are available; we use LFYA1228032H from The Lee Company. These valves benefit from a minimal internal volume (22 µl) and excellent chemical resistance (made from FFKM perfluoroelastomer). This is essential when you are working with hydrophobic inhibitors such as 1-NM-PP1, which absorbs into most hydrophobic materials (e.g., PE tubing) and subsequently slowly releases, thereby preventing true medium switching. We provide a simple step-by-step tutorial (**Supplementary Tutorial 1**), aimed at researchers without previous electronics experience, on how to set up the valve control system and interface it with MATLAB, and we also provide the necessary MATLAB code (**Supplementary Data 2**). In total, it should take ~1 h to set up, and once it is set up it can be reused for years. Subsequently, the valves are simply controlled in MATLAB (**Box 1**).

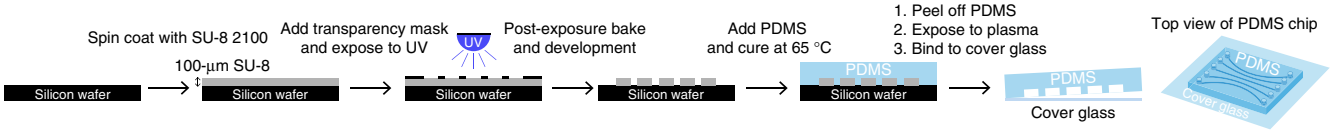

Figure 4 | Photolithography and soft lithography overview. Overview of protocol Steps 2–26. During the photolithography steps, a 4-inch silicon wafer is spin coated with SU-8 photoresist to a height of ~100 µm. Subsequently, it is exposed to UV light under a transparency mask. The photoresist pattern that is exposed to UV cross-links, whereas the unexposed photoresist is washed away during development to give the desired channel pattern. During the soft lithography steps, PDMS is poured over the silicon master mold, cured and then peeled off; holes are then punched for inlets and outlets. After plasma exposure, PDMS is bonded to a cover glass to yield the final microfluidic device (far right).

Box 1 | Controlling valves in MATLAB

To use MATLAB to control electrovalves, make sure that the board is plugged in and that the USB connection is inserted into the computer (see also **Supplementary Tutorial 1** for more detailed guidelines). The first step is establishing a serial connection. Run the script `'Windows_open_valves.m'` if on a Windows-based computer and run the script `'Mac_open_valves.m'` if on a Mac (scripts are provided in **Supplementary Data 2**). Once this is done, a serial connection (`s1`) is established and the valves can be controlled through MATLAB. To close the serial connection, use `'fclose(s1)'`.

The valves are programmed by sending ASCII characters. First ASCII character 254 is sent to enter command mode and then an ASCII character of 0–15 is sent to turn ON/OFF the desired valve. The function `'toggleValves.m'` controls whether each valve is turned ON or OFF. For example, to switch ON all valves use the following command:

```
>> toggleValves([0,0,1],s1)
```

Or to switch OFF all valves use the following command:

```
>> toggleValves([0,0,0],s1)
```

To switch ON/OFF a single valve use the following command (e.g., turn valve 3 ON):

```
>> toggleValves([3,1],s1)
```

Or to turn valve 4 OFF use the following command:

```
>> toggleValves([4,0],s1)
```

Combining the '`toggleValves.m`' function with the '`pause`' function in MATLAB, fluid delivery programs are easily made. For example, use the simple program as follows:

```
for i=1:6
    toggleValves([0,0,1],s1); %all ON
    pause(300);
    toggleValves([0,0,0],s1); %all OFF
    pause(600);
end
```

This delivers six 5-min pulses (5 min = 300 s) of treatment followed by 10-min intervals. This is the 1-NM-PP1 delivery program used to generate **Figure 2**.

Time-lapse microscopy (Steps 28–41). For live-cell imaging, we grow ~50 ml of cells overnight in low-fluorescence medium (LFM) with amino acids[19,71] to an optical density at 600 nm (OD_{600}) of ~0.1 at 30 °C. As Msn2 activity is very sensitive to the glucose concentration[28], we recommend keeping cells at a low OD_{600} value to avoid leaky activation of Msn1 and Msn2 target genes because of glucose deprivation; however, for other purposes a higher OD_{600} value may be preferable. We collect cells by suction filtration instead of by centrifugation, as this leads to higher cell recovery.

A microfluidic device is then washed, incubated with ConA and cells are loaded into the microfluidic channels at room temperature (~22 °C). After a brief incubation, the microfluidic device is mounted on the microscope stage maintained at 30 °C by the microscope incubation chamber and the flow is started. It is important to be quick and careful here, as the Msn2 pathway is very sensitive and seemingly small perturbations can cause Msn2 nuclear localization. For example, mechanical perturbations such as bumping of the microfluidic device or tubing can cause Msn2 nuclear localization. However, if care is taken, Msn2 will be entirely cytoplasmic at the beginning of an experiment. Even if a pathway unrelated to Msn2 is being studied, unnecessary cell stress should always be avoided. Furthermore, care must be taken when inserting tubing into the microfluidic inlets and outlets. It is important to keep a constant medium flow to avoid introducing air bubbles and to securely insert the tubing to avoid leakage during an experiment.

During time-lapse experiments, there is a trade-off between time resolution and the number of stage positions that can be recorded at. On our microscope (Zeiss AxioObserver Z1), it takes ~13.5 s for the microscope stage to move to a position, focus and collect phase contrast images and images in four fluorescent channels (including a *z*-stack of Msn2-mCherry). As we collect two stage positions per microfluidic channel, this limits our time resolution to ~2.5 min during time-lapse acquisition. This is sufficient for quantifying gene expression dynamics, but for other purposes, such as natural transcription factor dynamics during stress exposure, a finer time resolution may be necessary[3,4,28,43]. Furthermore, it is important to incorporate this delay into the pre-programmed valve control script in MATLAB. In our case, we use a 27-s delay between 1-NM-PP1 treatment in one microfluidic channel and the next to account for the time required to image two stage positions in each channel.

In general, it can be useful to include one microfluidic channel as a no-treatment control (**Supplementary Fig. 1**). If there has been any carryover of treatment compound (e.g., 1-NM-PP1) because of absorption into tubing, this can then be detected through the no-treatment control experiment. It is especially important to control for absorption of small molecules into tubing or PDMS if a very hydrophobic treatment compound is used[72]. In our hands, 1-NM-PP1 absorption into PE tubing is a major problem if PE tubing is reused between experiments,

but 1-NM-PP1 absorption into PDMS is not and 1-NM-PP1 washout is rapid at a flow rate of 1 µl/s per channel. If new PE and ismaprene tubing is used for every experiment, any risk of carryover between experiments can be eliminated. Finally, as the flow is driven by gravity (determined by the height difference between the inlet and outlet medium flasks), it is important to optimize which flow rate yields the optimal balance between rapid medium switching and minimal flow-driven cell loss by repeating the experiment with multiple different flow rates.

Analysis of time-lapse movies (Step 42). The multiplexed nature of this protocol means that each experiment can generate gigabytes of images with time-lapse information about thousands of cells (**Supplementary Tutorial 2** and **Supplementary Data 3**). Thus, it is desirable to automate the image analysis process, which includes segmentation of cells, tracking between frames and quantification of fluorescence. A number of open-source programs are available for this[73–75], although in many cases it is worth investing the time to develop code optimized for a more specialized purpose. We use our own custom-written code in MATLAB (**Supplementary Data 3**). Yeast cells are relatively simple to segment from phase-contrast images, as their contours show up as a bright, white phase ring. We segment by fitting ellipses to this phase ring. Although computationally expensive, this is very robust (**Fig. 2a**). The optimal tracking algorithm will depend on how much movement is observed during the movie. For 4 h or shorter experiments at a flow rate of 1 µl per second per channel, we observe modest cell movement and cell loss because flow is generally limited to ≤10% of cells. Therefore, it is essential to allow for some level of cell loss in the tracking algorithm. We simply match the closest cells between frames, unless there is no close cell in the subsequent frame, in which case our tracking algorithm aborts tracking for the cell in question.

For tracking and segmentation purposes, another consideration is which cells to count. In the case of *S. cerevisiae*, cells divide asymmetrically by budding. Budding daughter cells are frequently outside the focal plane (**Fig. 2a**), and we therefore only analyze mother cells that are already present during the beginning of the time-lapse experiment. Although most cells will be in the same focal plane, it may be necessary to manually exclude out-of-focus cells from the analysis.

For quantification of nuclear localization, one approach is to segment the nucleus using a nuclear marker (e.g., we use Nhp6a-iRFP[19]) and then quantify the mean transcription factor intensity (e.g., Msn2-mCherry) inside the nucleus. This approach is sensitive to slight errors in nuclear segmentation. Instead, we find the approach[3,28] of quantifying nuclear localization using the 10 (or 15 for diploids) brightest pixels in the cell much more robust (for a 63× objective—if a different magnification is used, the number of pixels used should be adjusted accordingly). Much of the variability observed in nuclear localization in diploid cells (e.g., the error bars in **Fig. 2b** and Msn2-mCherry intensity variability in **Fig. 2a**) is because of the nucleus moving in and out of focus during image acquisition, and this can partially be overcome by recording a *z*-stack series and using a maximum *z*-projection image for quantification of nuclear localization. To quantify gene expression, one approach is to quantify total fluorescence per cell. We quantify gene expression as the mean pixel intensity across the entire cell for several reasons. First, what matters to the cell is generally the protein concentration, not the absolute number. Second, any slight segmentation error will strongly affect the total fluorescence, but it will have a minimal effect on mean fluorescence as the fluorescent reporter is evenly distributed throughout the cytoplasm (**Fig. 2a**). Third, when considering cell-to-cell variability, if total fluorescence is used, a lot of the observed variability is attributable to cell size variation rather than stochastic gene expression.

MATERIALS

REAGENTS

Photolithography

- SU-8 photoresist (MicroChem, SU-8 2100) ! **CAUTION** SU-8 photoresist is toxic and flammable. Wear appropriate personal protective equipment (PPE), handle it in a fume hood and avoid contact.
- PGMEA (propylene glycol methyl ether acetate; Sigma-Aldrich, item no. 484431) or SU-8 developer (MicroChem, product no. Y020100)
- Isopropanol (e.g., Sigma-Aldrich, item no. W292907)
- Acetone (e.g., Sigma-Aldrich, item no. 439126)
- Methanol (e.g., Sigma-Aldrich, item no. 32213) ! **CAUTION** Organic solvents (PGMEA, methanol and so on) are toxic and flammable. Handle them in a fume hood and wear appropriate PPE.
- Silanization agent (1,1,2,2-tetrahydro(perfluorooctyl) trichlorosilane; Sigma-Aldrich, item no. 448931) ! **CAUTION** The silanization agent is toxic and corrosive and can cause serious eye damage. Ensure that you wear PPE and work in a fume hood.

Soft lithography: fabrication of microfluidic device in PDMS

- PDMS and curing agent (Sylgard 184 silicone elastomer kit from Dow Corning)
- Microscope cover glasses (60 × 85 mm, no 1.5H (170 µm ± 5 high precision) from Paul Marienfeld KG) ▲ **CRITICAL** These can be purchased through Azer Scientific in the United States. Most high-numerical aperture oil-immersion objectives are optimized for coverslips of the no. 1.5H thickness (170 µm).
- Razor blades (e.g., item from D109727 VWR)
- Adapter tubing (polyethylene, 1.57 mm outer diameter × 1.14 mm inner diameter PE-160/10 item 64-0755 from Warner Instruments)

Low-fluorescence medium (LFM)[71]

- D-Glucose (e.g., Sigma-Aldrich, item no. C8270)
- Ammonium sulfate ($(NH_4)_2SO_4$; e.g., Sigma-Aldrich, item no. A4418)
- Potassium phosphate monobasic (KH_2PO_4; e.g., Sigma-Aldrich, item no. P5655)
- Magnesium sulfate heptahydrate ($MgSO_4{\cdot}7H_2O$; e.g., Sigma-Aldrich, item no. 230391)
- Sodium chloride (NaCl; e.g., Sigma-Aldrich, item no. 793566)
- Calcium chloride dihydrate ($CaCl_2{\cdot}2H_2O$; e.g., Sigma-Aldrich, item no. C5080)
- Boric acid (H_3BO_3; e.g., Sigma-Aldrich, item no. B6768)
- Copper(II) sulfate ($CuSO_4$; e.g., Sigma-Aldrich, item no. C1297)
- Potassium iodide (KI; e.g., Sigma-Aldrich, item no. 793582)
- Iron(III) chloride ($FeCl_3$; e.g., Sigma-Aldrich, item no. 157740)
- Manganese(II) sulfate ($MnSO_4$; e.g., Sigma-Aldrich, item no. M7634)
- Sodium molybdate dihydrate ($Na_2MoO_4{\cdot}2H_2O$; e.g., Sigma-Aldrich, item no. M1003)
- Zinc sulfate ($ZnSO_4$; e.g., Sigma-Aldrich, item no. Z0251)
- Biotin (e.g., Sigma-Aldrich, item no. B4501)
- Calcium pantothenate (D-pantothenic acid hemicalcium salt; e.g., Sigma-Aldrich, item no. C8731)
- *myo*-Inositol (e.g., Sigma-Aldrich, item no. I5125)
- Niacin/nicotinic acid (e.g., Sigma-Aldrich, item no. N0761)
- *p*-Aminobenzoic acid (4-aminobenzoic acid; e.g., Sigma-Aldrich, item no. 100536)

- Pyridoxine hydrochloride (pyridoxine·HCl; e.g., Sigma-Aldrich, item no. P9755)
- Thiamine hydrochloride (thiamine·HCl; e.g., Sigma-Aldrich, item no. T4625)
- Adenine (e.g., Sigma-Aldrich, item no. A8626)
- L-Arginine (e.g., Sigma-Aldrich, item no. A5006)
- L-Histidine (e.g., Sigma-Aldrich, item no. H8000)
- L-Isoleucine (e.g., Sigma-Aldrich, item no. I2752)
- L-Leucine (e.g., Sigma-Aldrich, item no. L8000)
- L-Lysine (e.g., Sigma-Aldrich, item no. L5501)
- L-Methionine (e.g., Sigma-Aldrich, item no. M9625)
- L-Phenylalanine (e.g., Sigma-Aldrich, item no. P2126)
- L-Threonine (e.g., Sigma-Aldrich, item no. T8625)
- L-Tryptophan (e.g., Sigma-Aldrich, item no. T0254)
- L-Tyrosine (e.g., Sigma-Aldrich, item no. T3754)
- Uracil (e.g., Sigma-Aldrich, item no. U0750)
- L-Valine (e.g., Sigma-Aldrich, item no. V0500)
- Nalgene rapid-flow sterile filter storage bottles (Thermo Scientific, item no. 455-1,000)

Time-lapse microscopy

- 1-NM-PP1 (commercially available from Cayman Chemical as item no. 3330. We synthesize it from 1-naphteleneacetic acid in large quantities, as previously described[19])
- DMSO (e.g., Sigma-Aldrich, item no. D8418)
- Concanavalin A (ConA; 1 g, type IV from *Canavalia ensiformis* as a lyophilized powder; Sigma-Aldrich, item no. C2010-1g) ▲ **CRITICAL** ConA from different sources can vary in activity. Type IV from Sigma-Aldrich works well in our hands.
- PBS solution (Dulbecco's PBS; Sigma-Aldrich, item no. D8537)
- Calcium chloride ($CaCl_2$; e.g., Sigma-Aldrich; item no. C5080)
- Manganese(II) chloride (e.g., Sigma-Aldrich, item no. M3634)
- Ethanol or reagent alcohol (e.g., Sigma-Aldrich, item no. 362808)
- Yeast strain with suitable fluorescent reporters. In this example, we make use of our previously reported diploid strain[19] in the W303 (*trp1 leu2 ura3 his3 can1 GAL*$^{+}$*psi*$^{+}$) background (EY2813/ASH94): *TPK1*^{M164G} *TPK2*^{M147G} *TPK3*^{M165G} *msn4*Δ::*TRP1/LEU2 MSN2*-mCherry *NHP6a*-iRFP::kanMX *sip18*::mCitrineV163A/SCFP3A-*spHIS5*. All previously reported yeast strains[4,19,21,28] are available upon request from the authors
- Fast-folding codon-optimized[76] YFP mCitrineV163A in a pKT vector[71]: pKT-mCitrineV163A-HIS available from AddGene with ID no. 64685 at http://www.addgene.org/64685/
- Fast-folding codon-optimized[76] CFP SCFP3A (ref. 77) in a pKT vector[71]: pKT-SCFP3A-HIS available from AddGene with ID no. 64686 at http://www.addgene.org/64686/
- Codon-optimized[76] IR iRFP[34] in a pKT vector[71]: pKT-iRFP-KAN available from AddGene with ID no. 64687 at http://www.addgene.org/64687/

EQUIPMENT

Photolithography

- Spin coater (e.g., Headway spin coater, model PWM32) ▲ **CRITICAL** Most cleanrooms will have all necessary equipment. For reference, the equipment we use is given in this section.
- Silicon wafers (4-inch diameter, 0–100 Ω·cm, 500 μm thickness, test grade, item 452 at University Wafers)
- Hot plates (e.g., HP30 hot plates from Torrey Pines Scientific)
- Mask aligner (e.g., Karl Suss MJB4 from SUSS MicroTec)
- Profilometer (e.g., P-16+ Contact stylus profiler from KLA-Tencor)
- Optical microscope (e.g., Eclipse ME600L from Nikon)
- Petri dishes (e.g., 145/20 mm Petri dishes item 639102 from Greiner Bio-One or item 82050-596 from VWR)
- Appropriate design software (e.g., Auto Cad, Adobe Illustrator and so on. This is only necessary if a modified design is desired)

Solenoid valve control

- Soldering iron and solder (any will work)
- Wire cutter (any will work)
- Heat shrink tubing (recommended)
- Computer (either PC or Mac) with USB port and MATLAB software (Mathworks)
- Serial-to-USB converter (Tripp-lite USA-19HS - Keyspan High-Speed USB to Serial Adapter. It can be purchased from CDW as item 555201. See also http://www.tripplite.com/high-speed-usb-to-serial-adapter-keyspan~USA19HS/)
- Control board (F81 RS-232 8-channel 1-Amp N-channel FET controller board (item F81) from National Control Devices, LLC)
- Quick start kit with power supply, serial cable and RSIO serial interface board (QS12 +12-Volt quick start kit (item QS12) from National Control Devices, LLC)
- Solenoid valves (three-way 12 volts LFYA1228032H Y-valve in perfluoroelastomer, the Lee Company) ▲ **CRITICAL** Although other valves are available, LFYA1228032H valves have a low internal volume (22 μl) and are inert to hydrophobic inhibitors such as 1-NM-PP1, which can absorb into other materials.

Soft lithography: fabrication of microfluidic device in PDMS

- Planetary mixer and degasser with disposable cups (e.g., THINKY ARE-250 Mixer from THINKY USA)
- Oven with level surfaces (any lab-grade oven with adjustable temperature settings will work)
- Plasma exposure (we use a Plasma-Prep II plasma etcher from SPI)
- Cutting mat (any will work, but one with a grid pattern is helpful; we use an ALVIN cutting mat)
- Hole puncher (Harris Uni-Core 2.00 sold, e.g., as item 15076 from Ted Pella)
- Tweezers (e.g., 5-SA tweezers, S95307 Aven Tools 18062ER from Fisher Scientific)
- Dissection scissors (e.g., item no. 25874-105 from VWR)
- Vacuum desiccator attached to a vacuum pump (e.g., item no. 50-028, from Jencons but any will work)

Time-lapse microscopy

- Ismaprene tubing (Ismatec PharMed BPT (inner diameter 0.51 mm, wall 0.85 mm), item SC0305 from IDEX)
- PE tubing (polyethylene tubing, 0.050 inch outer diameter/0.034-inch inner diameter, item BPE-T90 from Instech Solomon)
- Valve adapter tubing (polyethylene tubing (1.57 mm outer diameter × 1.14 inner diameter), PE-160/10; Warner Instruments, cat. no. 64-0755)
- Needle, 20-G (PrecisionGlide needle 20-gauge (0.9 mm × 25 mm) REF 305175 from BD)
- Immersion oil (Use immersion oil appropriate for your microscope and objective. We use Carl Zeiss Immersol immersion oil 518F; Fisher Scientific, item no. 12-624-66A.)
- 1-ml Luer-Lok disposable syringes (BD Biosciences, item no. 309628)
- Suitable inverted fluorescence microscope with environmental incubation chamber, automated stage and autofocus function. Any such microscope should work. We use a Zeiss AxioObserver Z1 inverted microscope with both Colibri LED and DG4 Lamp excitation and Zeiss Definite Focus for focusing. Our setup uses an electron-multiplying charge-coupled device (EM-CCD) camera (Evolve 512, Photometrics) and an oil-immersion objective (63×, numerical aperture (NA) 1.4, oil Ph3, Plan-Apochromat)

REAGENT SETUP

ConA solution Prepare and filter-sterilize a 1 M $CaCl_2$ solution in water and a 1 M $MnCl_2$ solution in water. Obtain 1 g of ConA[36,37] (type IV from Sigma-Aldrich). Adjust the pH of the PBS solution to 6.5. On ice, gently dissolve ConA powder in 5 ml of PBS at pH 6.5, 40 ml of H_2O, 2.5 ml of 1 M $CaCl_2$ solution and 2.5 ml of 1 M $MnCl_2$ solution. Once it is fully dissolved, divide the 20 mg/ml ConA solution into 200-μl aliquots and store these at −20 or −80 °C. The aliquots can be stored for at least a year at −80 °C without an observable loss of efficiency. ▲ **CRITICAL** ConA activity can vary from batch to batch, and proper activity is essential to retain cells under flow during the microfluidic experiments. If the activity is too high or too low, adjust the concentration accordingly. Handle the powder gently on ice and carefully adjust the pH (a pH between 6 and 7 is important for high ConA activity[36–38]). Freeze-thawing or long-term storage at 4 °C leads to loss of activity.

10× amino acid stock To make 1 liter of stock, mix the following amino acid and nucleobase powders in an autoclave-safe glass bottle: 300 mg of L-isoleucine, 1,500 mg of L-valine, 400 mg of adenine, 200 mg of L-arginine, 200 mg of L-histidine, 1,000 mg of L-leucine, 300 mg of L-lysine, 200 mg of L-methionine, 500 mg of L-phenylalanine, 2,000 mg of L-threonine, 1,000 mg of L-tryptophan, 300 mg of L-tyrosine and 200 mg of uracil. Add ~600 ml of H_2O, microwave it and swirl until it is fully dissolved, and then fill up the volume to 1 liter with water. Sterilize by sterile-filtration or by autoclaving. Store the stock at 4 °C for at least 6 months.

1,000× trace elements stock To make 100 ml of stock, mix the following element powders in an autoclave-safe glass bottle: 50 mg of H_3BO_3, 4 mg

of $CuSO_4$, 10 mg of KI, 20 mg of $FeCl_3$, 40 mg of $MnSO_4$, 20 mg of $Na_2MoO_4{\cdot}2H_2O$ and 40 mg of $ZnSO_4$. Fill it to 100 ml with water and sterilize by autoclaving; the solution may be cloudy, so do not filter-sterilize it. Store it at room temperature indefinitely.

1,000× vitamin stock To make 100 ml of stock, mix the following powders in a beaker: 200 µg of biotin, 40 mg of calcium pantothenate, 200 mg of *myo*-inositol, 40 mg of niacin/nicotinic acid, 20 mg of *p*-aminobenzoic acid, 40 mg of pyridoxine·HCl and 40 mg of thiamine·HCl. Dissolve the powders and fill the beaker to a final volume of 100 ml with water. Sterilize the solution by filtration into Nalgene sterile filter storage bottle. Store the bottle at 4 °C for at least 1 year.

20× salt stock To make 1 liter of stock, mix the following powders in an autoclave-safe glass bottle: 100 g of $(NH_4)_2SO_4$, 20 g of KH_2PO_4, 10 g of $MgSO_4{\cdot}7H_2O$, 2 g of NaCl and 2 g of $CaCl_2{\cdot}2H_2O$. Fill the solution to a final volume of 1 liter with water and microwave and stir it if necessary to fully dissolve. Sterilize by autoclaving. Store it at room temperature indefinitely.

50% (wt/vol) glucose solution To make 500 ml of solution, add 250 ml of H_2O to a beaker and add a stirrer bar. Heat and stir the solution. Add 250 g of glucose and dissolve it. Fill the solution to a final volume of 500 ml and sterilize by autoclaving. Store the solution at room temperature indefinitely.

10× Custom YNB stock To make 1 liter of stock, mix 500 ml of 20× salt stock, 10 ml of 1,000× trace elements stock, 10 ml of 1,000× vitamin stock and 480 ml of H_2O. Sterilize the stock by filtration into Nalgene sterile filter storage bottle. Store the bottle at 4 °C for at least 6 months.

LFM with amino acids Per liter, the LFM medium contains: 20 g of glucose (2% (wt/vol) glucose), 5 g of $(NH_4)_2SO_4$, 1 g of KH_2PO_4, 500 mg of $MgSO_4{\cdot}7H_2O$, 100 mg of NaCl, 100 mg of $CaCl_2{\cdot}2H_2O$, 500 µg of H_3BO_3, 40 µg of $CuSO_4$, 100 µg of KI, 200 µg of $FeCl_3$, 400 µg of $MnSO_4$, 200 µg of $Na_2MoO_4{\cdot}2H_2O$, 400 µg of $ZnSO_4$, 2 µg of biotin, 400 µg of calcium pantothenate, 2 mg of *myo*-inositol, 400 µg of niacin/nicotinic acid, 200 µg of *p*-aminobenzoic acid, 400 µg of pyridoxine·HCl, 400 µg of thiamine·HCl, 30 mg of L-isoleucine, 150 mg of L-valine, 40 mg of adenine, 20 mg of L-arginine, 20 mg of L-histidine, 100 mg of L-leucine, 30 mg of L-lysine, 20 mg of L-methionine, 50 mg of L-phenylalanine, 200 mg of L-threonine, 100 mg of L-tryptophan, 30 mg of L-tyrosine and 20 mg of uracil.

To make 1 liter of LFM medium, mix 100 ml of 10× custom YNB stock, 100 ml of 10× amino acid stock, 40 ml of 50% (wt/vol) glucose stock and 760 ml of H_2O, and then sterilize it by filtration through a Nalgene filter. Once the solution is filter-sterilized—the same Nalgene filter can be reused if it is properly handled with sterile techniques—we store LFM at 4 °C, where it is stable for >6 months. ▲ **CRITICAL** We recommend LFM for microscopy, as its autofluorescence is within 10% of that of water[71]. Note that LFM lacks riboflavin and folic acid. However, for less-sensitive applications, standard synthetic complete yeast medium is also acceptable. ▲ **CRITICAL** We strongly recommend storing LFM at 4 °C, where it is stable for >6 months. Long-term storage of LFM at room temperature causes decay (medium becomes slightly yellow). This interferes with microscopy. 10× amino acid stock, 10× custom YNB stock and 1,000× vitamin stock should also be stored at 4 °C.

70% (vol/vol) ethanol Mix ethanol or reagent alcohol with water at a 70:30 (vol/vol) ratio, and store it at room temperature indefinitely.

1-NM-PP1 stock We prepare 1-NM-PP1 stocks in DMSO at 1,000× concentration (e.g., a 3 mM stock for experiments in which a 3 µM concentration is needed), and store these as aliquots at −20 °C. 1-NM-PP1 is extremely stable and it can be freeze-thawed without degrading. 1-NM-PP1 aliquots in DMSO at –20 °C can be stored indefinitely.

EQUIPMENT SETUP

Transparency mask for photolithography Obtain negative film photolithography transparency mask from a company. Our mask file is available online (**Supplementary Data 1**), and several companies offer this service (we use CAD/Art Services, http://www.outputcity.com/). The resolution should be at least 5,080 d.p.i. CAD/Art Services provides masks at 20,000 d.p.i.

Device holder for microscopy We use a metal holder to hold the microfluidic device. Any machine shop should be able to prepare this. The dimensions for our holder are given in **Supplementary Data 4**.

Suction filtration setup We use a Millipore system (Millipore kit no. XX1002530 contains everything needed) with nitrocellulose filters (mixed cellulose esters, 0.8 µm, 25 mm, item no. AAWG02500 from Millipore).

Silicon wafer master mold Steps 2–14 describe how to fabricate the silicon wafer master mold. If desired, these steps can also be outsourced, and once a master mold has been obtained it can be reused indefinitely. SIMTech offers these services (http://www.simtech.a-star.edu.sg/smf/), as do a number of other companies (http://stanford.edu/group/foundry/Services.html). As the companies that offer this service are constantly changing, see also http://en.wikipedia.org/wiki/List_of_microfluidics_related_companies for an up-to-date list.

PROCEDURE

Photolithography: fabrication of silicon wafer master mold ● TIMING 2–4 h

1| Obtain photolithography transparency mask (**Supplementary Data 1**; we use CAD/Art Services). Steps 2–14 for fabrication of silicon wafers can also be outsourced (Experimental design).

2| Choose an SU-8 photoresist that is appropriate for the desired height. We recommend SU-8 2050 for an ~50-µm height and SU-8 2100 for an ~100-µm height. Instructions are available from the MicroChem website (http://www.microchem.com/Prod-SU82000.htm). Below, we detail the steps for obtaining microfluidic channels of an ~100-µm height using SU-8 2100. ▲ **CRITICAL STEP** Steps 3–14 need to be performed in a cleanroom to prevent contamination. Avoid introducing air bubbles when you are working with SU-8, and make sure that all surfaces (e.g., hot plates) are level.
! CAUTION SU-8 photoresists are toxic and flammable. Make sure that you wear proper PPE and work inside a fume hood.

3| (Recommended) Clean the wafer by incubation in an acetone bath, ideally with sonication, for 5 min (**Fig. 5a**). Wash the wafer with methanol and then isopropanol. Dry the wafer with a nitrogen gun and then bake it at 200 °C for 5 min to remove any leftover solvent.

4| Prepare the spin coater. Line the bowl of the spin coater with aluminum foil to facilitate cleaning (**Fig. 5b**), and place the wafer on the chuck center and make sure that it sticks by applying vacuum.

5| *Spin coating*. To obtain a thickness of ~100 µm, we use SU-8 2100. Add ~ 4–5 ml of SU-8 2100 to the center of the wafer (**Fig. 5c**). Use the following spin program: ramp up to 500 r.p.m. at 100 r.p.m./s acceleration, hold for 5 s, ramp up to 3,000 r.p.m. at 300 r.p.m./s acceleration, hold for 45 s, and then ramp down.

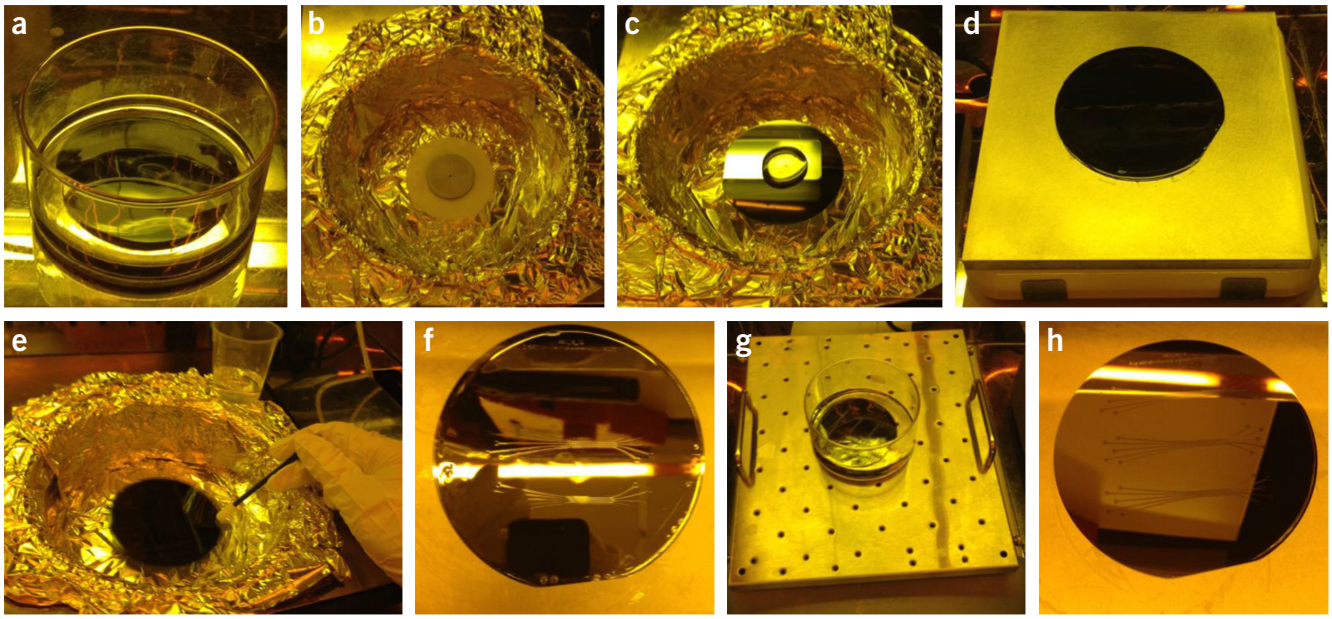

Figure 5 | Photolithography steps: fabrication of silicon wafer master mold. (**a**) Step 3: clean the wafer in acetone. (**b**) Step 4: prepare the spin coater. Line the bowl with aluminum foil to facilitate cleaning. (**c**) Step 5: pour ~4–5 ml of SU-8 2100 on the 4-inch wafer and spin it. (**d**) Step 6: soft bake. Place the spin-coated wafer on a level hot plate and bake. Be sure to remove any air bubbles by tapping with the tip of, e.g., a needle. (**e**) Step 7: edge bead removal (recommended). Spin the wafer at 1,000 r.p.m. and remove the edge by pressing a cotton swab tip soaked in PGMEA against the wafer edge, as illustrated. (**f**) Step 9: postexposure bake. Place the wafer on a level 65 °C hotplate and then ramp up to 95 °C for 11 min. Features will begin to appear. (**g**) Step 10: development. Place the wafer in a PGMEA bath with shaking, and incubate it for 10 min or until development is complete. (**h**) Step 12: hard bake. After development, perform a hard bake. The wafer is now finished.

6| *Soft bake*. Prebake the wafer on a level 65 °C hot plate for 5 min, and then soft-bake it at 95 °C for 20 min and cool it down to 65 °C before proceeding (**Fig. 5d**).
▲ **CRITICAL STEP** Any air bubbles should be removed during the soft bake by tapping with a needle tip or the tip of tweezers.

7| *(Recommended) Edge bead removal*. Place the coated wafer back on the chuck of the spin coater, and spin it at 400 r.p.m. for 60 s. As the wafer is spinning, remove the edge by placing a cotton swab tip soaked in PGMEA or SU-8 developer on the edge (**Fig. 5e**). Resoak the cotton swab tip as necessary. Bake the wafer on a 65 °C hot plate for 2 min to remove any residual PGMEA.

8| *Exposure*. Although the exact conditions will depend on the instrument and on the UV source power, this step should be performed according to the MicroChem guidelines (http://www.microchem.com/Prod-SU82000.htm). To eliminate radiation below 350 nm, we use a 360-nm long-pass filter and an exposure energy of 430 mJ/cm^2 (a lower exposure energy should be used if a long-pass filter is not used). On the mask aligner that we use, this works out to an exposure time of ~21 s. Place the transparency mask on top of the wafer substrate, add a 360-nm long-pass filter and expose.

9| *Postexposure bake*. Place the wafer on a level 65 °C hot plate. Ramp the hot plate up to 95 °C and hold it at this temperature for 11 min. Latent visible image should appear during postexposure bake (**Fig. 5f**).
▲ **CRITICAL STEP** Cool down the wafer slowly, for example, by switching off the hot plate; rapid cooling can cause cracks in the SU-8 film.
? TROUBLESHOOTING

10| *Development*. Prepare a PGMEA bath for development, and place it on a shaker. Submerge the wafer with the SU-8-coated side facing upward. Gently rock the PGMEA bath with the wafer at 90 r.p.m. for 10 min (**Fig. 5g**). To check whether development is complete, remove the wafer and use the spray bottle to spray isopropanol on the wafer. If a white film is observed, this indicates underdevelopment. In that case, put the wafer back into the PGMEA bath and develop it for a longer time.

11| Once development is complete, rinse the wafer with isopropanol and dry it with a nitrogen gun.

12| *(Recommended) Hard bake.* Place the wafer on a level hot plate and heat it to 150 °C for 20 min, and then cool it down slowly (**Fig. 5h**).
▲ **CRITICAL STEP** Performing the hard bake step will increase durability, and it can heal any surface cracks.

13| *(Recommended) Characterization.* To ensure that the mold pattern is without defects, the wafer mold should be inspected using a simple transmitted light microscope and a profilometer.

14| *(Recommended) Silanization.* Place the wafer in a Petri dish without the lid inside a desiccator together with a small vial containing ~500 µl of the silanization agent 1,1,2,2-tetrahydro(perfluorooctyl)trichlorosilane. Incubate the wafer in a desiccator attached to a vacuum pump under reduced pressure overnight.
! **CAUTION** The silanization agent is toxic and corrosive. Wear appropriate PPE and work in a fume hood.
■ **PAUSE POINT** After these steps, the wafer mold is stable, and it can be used and stored for years in a Petri dish or similar at room temperature.

Soft lithography: replica molding of PDMS and fabrication of microfluidic device ● **TIMING variable, 3–6 h**
! **CAUTION** The following steps involve organic solvents and elastomers, which are flammable, toxic and carcinogenic. Use proper PPE, work in a fume hood if possible and wear gloves. In addition to protecting the researcher, gloves furthermore prevent oils from the skin interfering with PDMS curing and bonding to the cover glass.
15| Place the wafer in a Petri dish with the mold pattern facing up.

16| Mix PDMS and curing agent in a 10:1 ratio (wt/wt) in a disposable plastic cup. If a planetary mixer is available, use this for mixing and degassing (we use a THINKY ARE-250 mixer with a 30-s mixing and 30-s degassing program, and 100-ml disposable cups). If not, manually mix extensively with a disposable plastic spoon and degas it in a vacuum desiccator.

17| Pour mixed PDMS over the wafer in a Petri dish to a height of ~5 mm (**Fig. 6a**). Pour it gently to avoid the formation of air bubbles. Degas it in a vacuum desiccator until air bubbles disappear. This often takes ~20 min.
▲ **CRITICAL STEP** More PDMS will be needed the first time, as you have to cover the entire Petri dish. Subsequently, ~30 g of PDMS is suitable for a height of ~5 mm.

18| *(Recommended) Cover glass cleaning.* Pour ~300 ml of isopropanol into a beaker (enough to fully submerge the cover glasses). Wash the cover glasses in acetone using a spray bottle, and then store the cover glasses in an isopropanol bath until needed in Step 22.
▲ **CRITICAL STEP** Cleaning the cover glasses improves plasma bonding to PDMS.

19| Once PDMS degassing is complete (when foaming and bubbling ceases), place the lid over the Petri dish containing the wafer and uncured PDMS, and place the Petri dish in an oven at 65 °C. Make sure that the surface is level. Cure it in an oven for at least 2 h.
■ **PAUSE POINT** After curing, the whole Petri dish can be stored indefinitely at room temperature.

20| After curing, take Petri dish out of oven and wait for a couple of minutes for it to cool down. With a razor blade, cut through PDMS against the wafer and circle the entire SU-8 pattern (**Fig. 6b**). Be careful to keep ~1 cm buffer area between the cutting area and SU-8 pattern, as accidentally cutting the SU-8 pattern with a razor blade can destroy it. Once you have cut a circle around the SU-8 pattern, carefully wedge the blade under the PDMS until you can grab PDMS with a finger. Next, carefully peel off the PDMS mold from the master surface.
▲ **CRITICAL STEP** It is important to be careful during PDMS peeling to leave the SU-8 pattern intact. If this part is done carefully, the same SU-8 master can be reused for years.
? TROUBLESHOOTING

21| After PDMS peeling, place the PDMS on the cutting mat with the channel features facing up. Cut each device into a PDMS rectangle, leaving ~5 mm between the PDMS edge and the edge-most channels. Punch holes for all inlets and outlets with Harris Uni-Core 2.00 (the punched hole diameter will be 2.4 mm; **Fig. 6c**). Be careful to punch straight holes at the exact end of each channel, and make sure that the punched out PDMS is fully removed.

22| Hold each hole-punched PDMS chip with tweezers, and wash it with acetone and then isopropanol from the spray bottle. Next, dry it with a nitrogen gun. For each PDMS device, also dry a cleaned cover glass from Step 18 with a nitrogen gun.

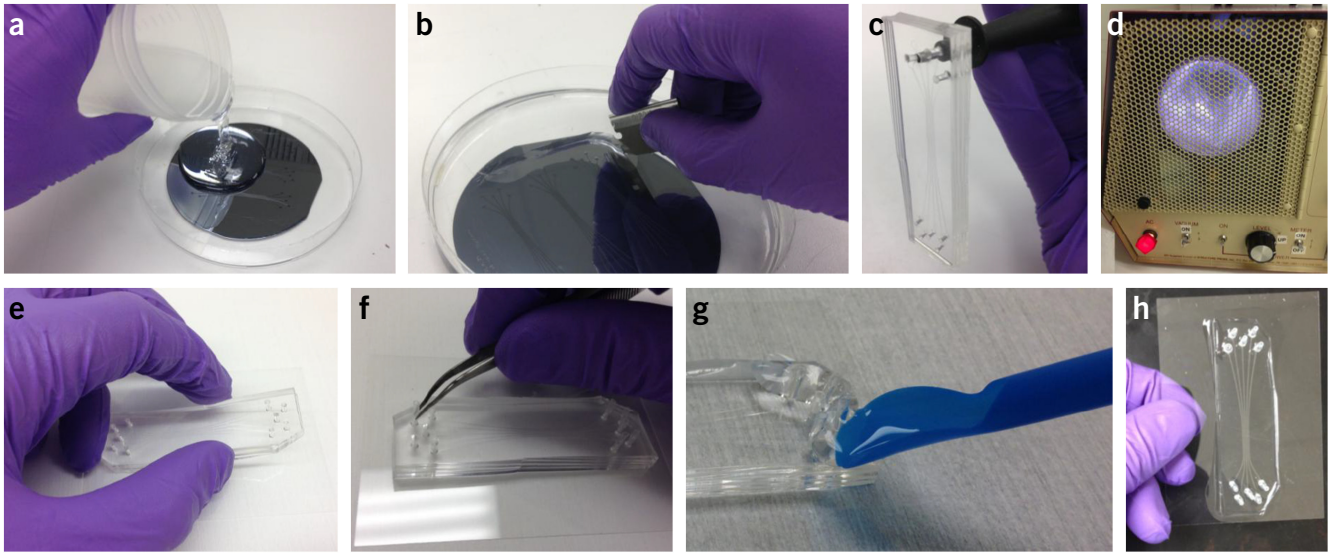

Figure 6 | Soft lithography steps: fabrication of microfluidic device. (**a**) Step 17: pour mixed PDMS on silicon wafer master mold in a Petri dish. (**b**) Step 20: once it is cured at 65 °C, cut out the PDMS chip with a razor blade, taking care not to cut the SU-8 pattern. (**c**) Step 21: punch holes for inlets and outlets using a Harris Uni-Core 2.00 to finish the PDMS chip. (**d**) Step 23: expose both PDMS chips and cover glasses to oxygen plasma to activate the surfaces. (**e**) Steps 23 and 24: after plasma treatment, place the PDMS chip featured-side down on a cover glass and gently press it. Next, incubate it at 65 °C for >1 h. (**f**) Step 25: insert a small piece (~8–10 mm) of 'adapter tubing' into each inlet and outlet. (**g**) Step 25: seal the adapter tubing with PDMS. Use a plastic spoon (shown in blue) to pour a small amount of PDMS around each inserted piece of 'adapter tubing'. After curing it at 65 °C, this provides a seal and prevents leaks. (**h**) Step 26: close-up of the final device. After incubation at 65 °C for >2 h, the final microfluidic device is obtained.

23| *Plasma treatment*. The exact protocol will depend on the plasma instrument available. We use a Plasma-Prep II (from SPI) and the following protocol: Carefully place the cleaned PDMS devices and cover glasses upright with the side to be bonded exposed inside plasma chamber. Close the plasma chamber and turn on vacuum. Switch the gas to pure oxygen. Wait until a pressure of 300 Torr (400 mbar) is reached. Turn on the oxygen plasma and tune the instrument. Expose for 12 s at ~60 W (**Fig. 6d**). Turn off vacuum and wait for the instrument to reach standard pressure. As soon as possible, take out the plasma chamber and carefully place the PDMS chips on cover glasses (**Fig. 6e**). The channel side should face the cover glass. Place it on soft material (e.g., layers of paper tissues), and gently press the PDMS device against the cover glass until it is fully bound. The PDMS chip is fully bonded to the cover glass when all air bubbles between the glass and PDMS have disappeared.

! CAUTION Cover glasses are very thin, and they can break when the PDMS device is pressed against them, so take great care.

24| Place each plasma-bonded microfluidic device in an oven at 65 °C for at least 1 h.

25| Cut the adapter tubing (PE-160/10; 1.57 mm outer diameter and 1.14 mm inner diameter, Warner Instruments) into pieces of ~8–10 mm in length. Take out the microfluidic device and place it on soft material. Gently press one piece of tubing into each inlet and each outlet (**Fig. 6f**; despite the apparent outer diameter mismatch (1.57 versus 2.4 mm), the adapter tubing should fit tightly into inlets and outlets). Then, with a plastic spoon or equivalent (blue spoon in **Fig. 6g**), pour a small amount of mixed PDMS (from Step 16) around each inlet and outlet to seal the tubing (**Fig. 6g**).

26| Place each microfluidic device with a sealed inlet and outlet tubing in an oven at 65 °C for at least 2 h, but preferably overnight. Steps 15–26 should be repeated whenever more microfluidic devices are needed.

■ PAUSE POINT Microfluidic device fabrication is now complete (**Fig. 6h**). Devices can be stored for months. It is more efficient to make a large number of devices in a single setting. Store the devices in Petri dishes to avoid dust accumulation.

Setting up control valves ● TIMING 1 h

27| Setting up the valve control system requires a small amount of soldering. We provide a detailed step-by-step tutorial aimed at someone without previous soldering and electronics experience (**Supplementary Tutorial 1**). Setting up the control valves should take ~1 h, and it only needs to be done once. An example of how to interface valves with MATLAB is shown in **Box 1**. We also provide the MATLAB source code used to control the valves (**Supplementary Data 2**).

Preparing a time-lapse microscopy experiment ● TIMING variable

▲ **CRITICAL** To prepare a time-lapse experiment, Steps 28 and 29 should be performed the day before.

28| In the morning, inoculate the relevant yeast strain in LFM and grow it for >8 h at 30 °C. At night, calculate how many OD_{600} units of cells to add (assuming a doubling time of 90 min) to a 250-ml conical flask containing 50 ml of LFM so that the culture reaches an OD_{600} of 0.1 at the desired time the following day. Grow this culture overnight at 30 °C with shaking at 180 r.p.m.

29| Prepare all media. For an ~3-h, five-channel experiment, ~100–150 ml of LFM is needed, depending on the flow rate. Prepare a flask with LFM without treatment and flasks for each treatment (e.g., 1-NM-PP1). Also prepare a flask with LFM for outlet waste. Incubate these flasks overnight at 30 °C.

▲ **CRITICAL STEP** Incubating LFM overnight at 30 °C is essential to avoid the formation of air bubbles during the experiment.

Setting up a time-lapse microscopy experiment ● TIMING <2 h to set up + imaging time

30| Take out a tube containing 20 mg/ml ConA solution from the freezer and gently thaw it on ice 1 h before the experiment. Once it is fully thawed, add 200 µl of PBS (pH 6.5) and 600 µl of H_2O to the 200-µl ConA solution and mix to obtain 1 ml of final solution of 4 mg/ml ConA.

▲ **CRITICAL STEP** It is important to gently and slowly thaw ConA on ice to prevent the loss of activity. If ConA is stored at 4 °C, the solution should be relatively fresh (<1 month old) to ensure optimal immobilizing activity.

31| Prepare the microscope setup at least 1 h before experiment. Turn on the microscope and heat up the incubation chamber to 30 °C. Connect the valve control system to the computer, open MATLAB and establish serial connection to the valves. Cut the PE tubing for inlets and outlets (**Fig. 7a**). Five pieces are needed for each inlet and outlet, for a total of 15. These should be ~50–100 cm long depending on the microscope setup. We keep flasks with inlet LFM and outlet waste outside of the microscope incubation chamber, and thus tubing must be long enough to reach these. Cut five pieces of ismaprene tubing (~12 cm) and attach them to valve outlets (**Fig. 7b**). Cut small pieces of PE tubing and attach them to the other end of the ismaprene tubing with a 20-gauge needle (**Fig. 7c**).

32| Prepare the microfluidic device. Tape the microfluidic device to a microscope holder with Scotch tape. Cut off excess adapter tubing so that it is level with PDMS. Cut a piece of PE tubing (~10–15 cm), attach it to a 20-gauge needle and attach the needle to a 1-ml syringe. Cut the tubing in such a way that the end is diagonal rather than blunt—this greatly eases insertion.

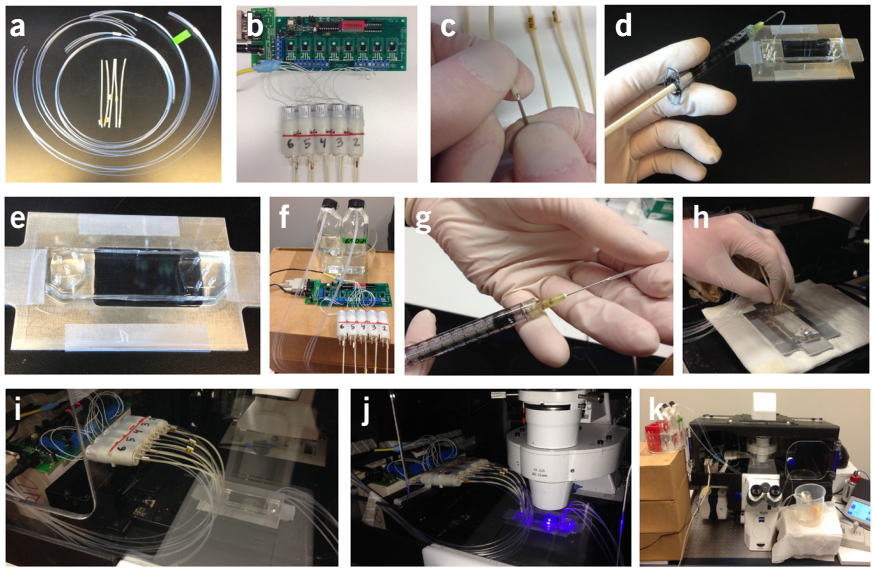

Figure 7 | Time-lapse microscopy experiments. (**a**) Step 31: prepare PE tubing for inlets and outlets and ismaprene tubing for valves. (**b**) Step 31: attach ismaprene tubing to valve outlets. (**c**) Step 31: insert a small piece of PE tubing on a 20-gauge needle and then insert the PE tubing piece into the ismaprene tubing. This is the piece that goes into the microfluidic device inlets, and it should be short (~5 mm) and have a diagonal rather than a blunt end. (**d**) Step 33: wash the microfluidic device using a 1-ml syringe with a 20-gauge needle and PE tubing attached to the needle by gently flushing each channel with 70% (vol/vol) ethanol and water, and then loading ConA solution. (**e**) Step 33: ideally always keep inlets and outlets covered with liquid to avoid introducing air bubbles. (**f**) Step 34: insert PE tubing into the medium and safely tape everything down. (**g**) Step 34: to start flow from the medium, insert the syringe with the 20-gauge needle into PE tubing end and pull it. Then insert the PE tubing into the solenoid valve inlets. (**h**) Step 36: move the board, valves and microfluidic device into the microscope chamber and insert ismaprene tubing with PE ends into the microfluidic device inlets taking care to avoid air bubbles. (**i**) Step 37: once the outlets have been connected via the PE tubing to the waste flask the setup should appear as shown. (**j**) Step 40: illustration of the setup during time-lapse acquisition. (**k**) Full view of the setup. Flow rate is controlled by height difference between the inlet medium and the waste flask.

33| Wash the microfluidic device. Wash each microfluidic channel with ~200 µl of 70% (vol/vol) ethanol by inserting the syringe through the PE tubing and gently pushing the liquid through the channels (**Fig. 7d**). Next, wash each channel with ~200 µl of H_2O. Dry off excess liquid with Kimwipes. Finally, load each channel with ~200 µl of 4 mg/ml ConA solution. Ensure that all inlets and outlets are fully covered by ConA solution (**Fig. 7e**), and incubate at room temperature for at least 5 min and up to 2 h before loading the cells. We do not observe a strong relationship between ConA incubation time and cell retention.
▲ **CRITICAL STEP** Avoid introducing air bubbles—as an air bubble travels through the channel, it will take out all adhered cells with it. If an air bubble is introduced before cell loading, use the syringe to flush the channel until the air bubble has been removed again. During wash steps, apply gentle pressure from the syringe—too high a pressure can break the PDMS walls between the channels.
? TROUBLESHOOTING

34| Prepare the tubing and LFM/medium for inlets and outlets. Add 1-NM-PP1 or equivalent to stress medium at the desired concentration (we store 1-NM-PP1 as a 1,000× stock in DMSO at –20 °C, and add it just before use). Connect PE tubing with flasks containing the medium. Tape the tubing to the flasks (**Fig. 7f**). To start the flow, insert a 1-ml syringe with a 20-gauge needle into PE tubing and draw out the liquid (**Fig. 7g**). Then attach PE tubing to valve inlets and outlets. The flow rate is controlled by gravity. Switch between inlets a few times using the 'togglevalves.m' script in MATLAB to remove any air bubbles. Ensure that valves are off before proceeding.
▲ **CRITICAL STEP** Avoid reusing PE tubing for 1-NM-PP1 treatments; old PE tubing will cause the release of residual 1-NM-PP1 into the medium. It is important to tape the PE tubing to medium flasks (**Fig. 7f**). If tubing ends move above the medium, air will be sucked in and when these air bubbles reach microfluidic channels, cells will be lost.

35| Load cells into the microfluidic device. Collect the cells when they have reached the right OD_{600} value (we use 50 ml at an OD_{600} of 0.1, so 5 OD units of cells). We prefer collecting cells by suction filtration rather than by centrifugation. Attach the suction filtration system (Millipore XX1002530) to house vacuum, and pour 50 ml of cell culture over a nitrocellulose filter at room temperature. As soon as the cell culture has been filtered, remove the nitrocellulose filter with cells and quickly resuspend the cells in 500 µl of LFM in a microcentrifuge tube by pipetting up and down. Wash away all ConA by flushing each microfluidic channel with LFM using syringe (~200 µl per channel). Next, load the cell suspension into each microfluidic channel using the syringe (**Fig. 7d**; ~100 µl per channel). Incubate the cells in the microfluidic device for 5 min at room temperature. After 5 min, wash each microfluidic channel with LFM. The syringe pressure with which cells are washed with LFM determines subsequent cell density in each channel—pressing the syringe too tightly can lead to low cell density, but pressing it too gently can lead to overcrowding of the microfluidic channel.
▲ **CRITICAL STEP** After this step, the microfluidic device should immediately be loaded onto the microscope with a flow of LFM to keep the cells in a chemostatic environment and to avoid stress.

36| Load the microfluidic device on the microscope. Carefully place the microfluidic device on the holder inside the microscope incubation chamber on paper towels to absorb any spillage. Carefully move the board and valves into the incubation chamber maintained at 30 °C—take care not to detach any tubing. Attach each ismaprene tubing outlet to each microfluidic device inlet under constant medium flow (**Fig. 7h**). Dry off any spillage.
▲ **CRITICAL STEP** When inserting ismaprene tubing into device inlets, make sure that the medium is constantly flowing from the ismaprene tubing to avoid introducing air bubbles.

37| Attach the outlets. Attach the PE tubing to the outlet waste flask and tape it. Move the flask above the microscope chamber and start the flow by drawing the liquid from the PE tubing with a syringe (**Fig. 7g**). Carefully attach the outlet PE tubing to the microfluidic device outlets; make sure that the PE tubing is stably inserted to avoid leakage. Move the outlet flask to below the microscope chamber (**Fig. 7i**). The flow rate is controlled by gravity; the height difference between the inlet and outlet flasks governs this. In our hands, a height difference of ~30 cm yields a flow rate of ~1 µl per second per channel.

38| *Begin microscopy*. Add immersion oil to the objective. Move the objective to the straight part of the microfluidic channels and focus to find cells (should look as shown in the bright-field image in **Fig. 1**). Tape the tubing and the valves to the chamber so that nothing moves during image acquisition.
▲ **CRITICAL STEP** Cell handling and loading will inevitably introduce some stress. Therefore, before starting the microscope acquisition, allow cells to stay in the device with constant LFM flow for at least 20–30 min to recover from stress and adapt to the microfluidic culturing condition.

39| *Pick stage positions*. Use microscope software (we use Zeiss AxioVision 4.8) to pick the stage positions in each microfluidic channel and the autofocus function to maintain focus (we use Zeiss Definite Focus). Pick positions as close as possible to each other to avoid excessive stage movement during time-lapse acquisition. We use ~5 fields-of-view as separation between positions. We avoid positions immediately adjacent to the PDMS wall of the microfluidic channel (**Fig. 1**) to ensure homogeneous exposure, as the flow velocity immediately adjacent to the PDMS wall is slower.

40| *Time-lapse movie acquisition* (**Fig. 7j**,**k**). This will depend on the microscope, software and experiment in question. For reference, in a typical experiment, we acquire two field-of-view positions per microfluidic channel at a 2.5-min time resolution (ten positions total) using the following exposure settings: phase-contrast (10 ms), YFP (50 ms, Zeiss Colibri LED at 505-nm excitation using Zeiss filter cube HE 46 (EX BP 500/20, BS FT 515, EM BP 535/30), intensity: 100%), CFP (100 ms, Zeiss Colibri LED at 445 nm using Zeiss filter cube HE 47 (EX BP 436/25, BS FT 455, EM BP 480/40), intensity: 100%), iRFP (400 ms, Sutter DG-4 lamp using filter cube 32 Alexa Fluor 680/Cy5.5 (EX BP 665/45, BS FT 695, EM BP 725/50)), RFP (3 × 400 ms z-stack series (focal plane ± 1.75 μm), Zeiss Colibri LED at 590 nm using Zeiss filter cube HE 64 (EX BP 587/25, BS FT 605, EM BP 647/70), intensity: 100%).
? TROUBLESHOOTING

41| *Clean up*. Save the images and remove and dispose of the microfluidic device as glass disposal (although the microfluidic device can be reused, washing is cumbersome and cell retention is markedly reduced[38]). Clean the objective with lens cleaner. We recommend disposing of tubing that has been in contact with 1-NM-PP1, but all other tubing can be reused. Wash valves and ismaprene tubing with 70% (vol/vol) ethanol in between experiments.

Analyzing time-lapse movies ● TIMING variable

42| Analyzing movies involves segmentation of cells in each frame and tracking of cells between frames, followed by quantification of fluorescence in each single cell over time, as illustrated in **Figure 2a**. A number of software packages are available for this[73–75]. We have found it easier to write our own software. We illustrate the main steps involved in segmenting and tracking single cells in **Supplementary Tutorial 2** and provide MATLAB code (**Supplementary Data 3**) for these steps. **Supplementary Tutorial 2** is aimed at a researcher without previous image analysis experience.
? TROUBLESHOOTING

? TROUBLESHOOTING

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

TABLE 1 | Troubleshooting table.

Step	Problem	Possible reason	Solution
9	Cracks in the SU-8 pattern	Rapid cooling can cause thermal stress	In all steps involving heating on hot plates, slowly ramp the temperature up or down to avoid thermal stress
20	Detachment of the SU-8 pattern	Insufficient adhesion of SU-8 to the wafer	Include an additional wafer cleaning step before Step 4. We sometimes perform oxygen plasma cleaning of wafer immediately before SU-8 coating (150 mTorr O_2 at 60 W for 2 min with a Technics Plasma Stripper Model 220)
	PDMS is still attached to the SU-8 pattern and/or wafer		Ensure that the razor blade cut is complete, and be more gentle while peeling off PDMS. Perform recommended hard bake (Step 12) and silanization (Step 14)
33	Walls between microfluidic channels break down during washing steps	Too much pressure or too weak bonding between the PDMS chip and the cover glass	Apply only gentle pressure on the syringe during wash steps. Perform all recommended cleaning steps during soft lithography (Step 18) and optimize oxygen plasma exposure time (Step 23)
40	Excessive cell loss or movement during time-lapse experiment	High flow rate	Lower the flow rate by reducing the height difference between the inlet and outlet flasks. Unless very quick medium switching is required, a much lower flow rate may be fine

(continued)

TABLE 1 | Troubleshooting table (continued).

Step	Problem	Possible reason	Solution
		Defective ConA	Change the ConA stock. We sometimes observe dramatic batch-to-batch variation in activity
	Sudden loss of all cells in the field of view	Air bubble	Make sure that all LFM and medium is preheated to 30 °C before experiments
		Air bubble	When washing the microfluidic device and loading cells, take care not to introduce air bubbles. These can sit around and only move through microfluidic channels during time-lapse experiments
	Focus is lost during time-lapse acquisition	Thermal expansion of the microfluidic device holder	Prewarm the microfluidic device holder to 30 °C. Wait for 20–30 min before starting time-lapse acquisition to allow everything to heat up
		Unstable setup during stage movement	If the stage positions are too far apart or if device holder or tubing is not stable during acquisition, this can happen. Tape everything down
	Leakage during the experiment	Tubing comes loose during the experiment	Securely attach the PE tubing to the microfluidic device. Cutting the PE tubing ends sharply and diagonally makes secure insertion much easier. Avoid blunt ends
	PDMS delamination	Insufficient bonding of the PDMS to the cover glass	Properly clean the PDMS and cover glasses before plasma exposure. It may be necessary to optimize the oxygen plasma exposure time (both too-brief and too-long exposure times can cause this)
			When sealing the adapter tubing in Step 25, also add mixed PDMS around the PDMS chip to seal it to the cover glass
	Msn2 is nuclear before 1-NM-PP1 treatment	Leftover 1-NM-PP1 from previous experiment absorbed into the tubing	Do not reuse PE tubing that has been exposed to 1-NM-PP1. If an extremely high concentration of 1-NM-PP1 has been used (e.g., >10 μM), it may be necessary to also replace the ismaprene tubing
		Clogged tubing	Occasionally, we observe nuclear Msn2 if the tubing is clogged or improperly positioned. Change the tubing and keep the tubing path straight
	Too few (or too many) cells in microfluidic channels	Improper cell loading	Load more (or fewer cells) during Step 35. Apply less (or more) pressure when washing with fresh LFM during Step 35
	Washout of 1-NM-PP1 is too slow	The flow rate is too low	Washout of 1-NM-PP1, as measured by Msn2 localization, should not take more than 2–3 min. If it takes longer, increase the flow rate
42	Cell segmentation or tracking errors during image analysis	Segmentation or tracking algorithm not optimal	Optimize the scoring parameters for cell segmentation and cell tracking. Also, remove cells that are out-of-focus from the analysis. See also **Supplementary Tutorial 2**

● TIMING

Steps 1–14, fabrication of silicon wafer master mold: 2–4 h
Steps 15–26: replica molding of PDMS to make microfluidic devices: ~3–6 h (variable)
Step 27, setting up control valves: 1 h
Steps 28 and 29, preparing a time-lapse microscopy experiment (day before): variable
Steps 30–41, setting up a time-lapse microscopy experiment: <2 h + imaging time
Step 42, analysis of time-lapse movies: variable

ANTICIPATED RESULTS

The anticipated results will depend on the experiment in question. By using the analog-sensitive PKA^{as}-system to control Msn2 dynamics and to measure gene expression (**Fig. 2**) as an example, this protocol should allow a single researcher to expose cells to, e.g., five different Msn2 pulse frequencies (**Fig. 2a**,**b**) in a single experiment while collecting two fields of view per channel and ~200 cells per field of view, thus generating data for ~2,000 cells in a single experiment. With a good image acquisition system, reporter gene expression can be quantified with minimal measurement noise (**Fig. 2c**,**d**). If experiments are well planned, a single researcher readily can set up four experiments in a day, sampling 20 different experimental conditions or strains while obtaining time-lapse data for up to 10,000 single cells. With this amount of data, it becomes possible to not just study the average behavior of a cell population but to take the entire probability distribution into account[21]. For further single-cell example applications of this protocol, we refer the reader to previous work on protein translocation dynamics[4,28], transcription factor–induced gene expression[19,28] and information transduction[21]. Time-lapse data for transcription factor activation and gene expression (**Fig. 2**) can further enable the development of mathematical models to understand gene expression dynamics[19,28].

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS A.S.H. developed the multiplexed microfluidic device, the automated fluid control system, developed MATLAB code and wrote the protocol. N.H. developed the original method of using microfluidics to control analog-sensitive kinases and Msn2 localization. E.K.O'S. supervised the projects. A.S.H., N.H. and E.K.O'S. wrote the manuscript.

COMPETING FINANCIAL INTERESTS The authors declare no competing financial interests.

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