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

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Abstract

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. 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, whilst collecting data for thousands of single cells. Compared to other protocols, the present protocol is relatively easy to adopt and higher-throughput. The protocol can be widely used to control and monitor single-cell signaling dynamics in other signal transduction systems in microorganisms.

Search Terms

Microfluidics; Lab on a Chip; PDMS; Lithography; Time-lapse microscopy; Fluorescence microscopy; signal transduction; signaling dynamics; transcription factor; single-cell; yeast; nucleocytoplasmic; translocation; Msn2; Gene regulation; Noise; analogue sensitive; kinase; 1-NM-PP1; PKA

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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 analogue sensitive kinases and Msn2 localization. E.K.O. supervised the projects. A.S.H., N.H. and E.K.O. wrote the manuscript.

Competing Financial Interests The authors declare that they have no competing financial interests.

Introduction

Fluorescence microscopy has become a standard laboratory tool and live-cell imaging of signaling dynamics^{1,2}, protein translocation³⁻⁷, and cell-fate choice⁸ is increasingly becoming routine⁹. This has led to a greater appreciation of the importance of dynamics in signal transduction, control of cell fate¹⁰⁻¹³, and heterogeneity¹⁴. However, traditional live-cell imaging approaches suffer from an inability to maintain constant extracellular conditions and cannot make precisely controlled perturbations.

Microfluidics largely overcomes these limitations by combining chemostatic cell culture with the ability to make perturbations with exquisite spatiotemporal control¹⁵⁻¹⁷. Additionally, since 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 behavior²² 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 expression²⁹ and how cells monitor and respond to changes in their environment³⁰. More widespread adoption of microfluidics coupled to time-lapse microscopy will help transform our understanding of signaling dynamics inside living cells.

Combining microfluidics⁷ 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 (Fig. 1)¹⁹. The device enabled us to perform 4-color quantitative time-lapse microscopy, where we followed more than 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 Msn2¹⁹.

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, easy to adopt and 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 *S. 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 kinase activity with a small molecule, 1-NM-PP1^{32,33}, without inhibiting the activity of other cellular kinases. 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 infrared³⁴ nuclear marker (NHP6a-iRFP)¹⁹. To quantify gene expression and intrinsic and extrinsic noise³⁵, we replace the ORF of Msn2 target genes with fast-maturing CFP and YFP fluorescent proteins ($\tau_{\text{maturation}} \sim 8\text{--}10$ min at 30°C) in diploid cells. A typical experiment is shown in Fig. 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 noise³⁵ 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 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 localization⁴⁴, changes in metabolite levels⁴⁵ or any other process that can be monitored with a fluorescent read-out in a range of microorganisms.

Finally, we highlight that the analogue-sensitive kinase strategy³² 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 analogue-sensitive mutations (such as Met→Gly for PKA³³) have been generated for a number of kinases including MAP kinases such as Hog1⁴⁶, kinases involved in transcription such as Cdk7/Kin28⁴⁷ and Cdk8/Srb10⁴⁷ and other important kinases such as Pho85⁴⁸, Fus3³², Cdc28³² and Snf1⁴⁹ (a partial list is given elsewhere⁵⁰). 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 cells⁶¹. In the case of 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 necessitate much more complex microfluidic devices. This highlights a trade-off between specialization on the one hand and generality, simplicity, and easy-of-use on the other hand. For example, a recent massively parallel microfluidic device¹⁸ allows for analysis of 1,152 different strains from the yeast GFP library⁴² in a single experiment. This is clearly a technical breakthrough and compared to such approaches our protocol is much lower throughput. However, such setups^{18,59} require extensive know-how and infrastructure beyond the capabilities of most labs. 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 CellAsic⁶⁶. 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 again¹⁹. More generally, we note that medium switching when using hydrophobic inhibitors such as 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 multi-layered, 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 from a company, 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 to 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 working with hydrophobic inhibitors such as 1-NM-PP1, which absorb into most other materials.

Compared to 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 less than \$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 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}. Additionally, other approaches are superior for more complicated applications such as generation of precisely defined gradients^{16,30} or sub-second medium switching⁷. Although microfluidics coupled to time-lapse microscopy is a generally powerful technique, the inclusion of microfluidics does complicate the experiment setup. However, the current protocol should be relatively simple to set up and 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 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 (Fig. 1 and 3), but can also be modified to include more or fewer channels, depending on multiplexing requirements.

Once the mask has been obtained from a company (e.g. CAD/Art Services), the silicon master mold can be fabricated in a clean room. Since 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" silicon wafer with SU-8 photoresist, exposing to UV under the transparency mask and developing in PGMEA (an organic solvent) to give 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 clean room facilities. However the fabrication of a silicon master can also be out-sourced to 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 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 also slightly higher cell loss during the experiment. At a flow rate of 1 $\mu\text{L/s/channel}$ and a height of ~100 μm , a complete change of medium occurs within ~10 s. Since the volume of a microfluidic channel (~0.6 μL per cm) is much lower than the internal volume of the solenoid valves, medium change 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 ($\sim 10\ \mu\text{m}$). Following post-exposure 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 elastomer⁶⁸. For PDMS replica molding we use Sylgard 184 (Dow Corning): PDMS is mixed with curing agent in a 1:10 (w/w) ratio, poured over the silicon master, 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. Following exposure to O₂-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). Following baking at 65°C, we then insert “adaptor-tubing” into the inlets and outlets and seal these with PDMS. This greatly eases insertion of tubing when setting up microscopy experiments and also prevents leaks from occurring during time-lapse experiments.

As a material, PDMS is optically transparent from the near-UV range into the infrared range and therefore compatible with all standard fluorescent reporters. In addition, it is gas permeable, inexpensive, hydrophobic, very easy to work with and biocompatible⁶⁸. 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 and see Supplementary Tutorial 1 and Supplementary Data 2)—We use 3-way solenoid valves and an electronic board to independently control fluid delivery to each of the five channels in which cells are grown. We connect flasks containing normal medium and perturbation medium (e.g. with 1-NM-PP1) to the valves using 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 valves⁶⁷ 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 working with hydrophobic inhibitors such as 1-NM-PP1, which absorb 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 $\sim 1\ \text{h}$ to set up and once set up it can be re-used for years. Subsequently, the valves are simply controlled in MATLAB (Box 1).

Time-lapse microscopy (Steps 28-41)—For live-cell imaging, we grow ~50 mL of cells overnight in low fluorescence medium with amino acids (LFM)^{19,71} to an OD_{600 nm} ~0.1 at 30°C. Since Msn2 activity is very sensitive to the glucose concentration²⁸, we recommend keeping cells at a low OD_{600 nm} to avoid leaky activation due to glucose deprivation, but for other purposes a higher OD_{600 nm} may be preferable. We collect cells by suction filtration instead of by centrifugation since this leads to higher cell recovery.

A microfluidic device is then washed, incubated with concanavalin A (ConA), and cells are loaded into the microfluidic channels at room temperature. Following 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 since 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). Since 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 (see Supplementary Figure 1). If there has been any carryover of treatment compound (e.g. 1-NM-PP1) due to 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 used⁷². In our hands, 1-NM-PP1 absorption into PE tubing is a major problem if PE tubing is re-used 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/channel. If new PE and ismaprene tubing is used for every experiment, any risk of carryover between experiments can be eliminated. Finally, since 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 and see Supplementary Tutorial 2 and Supplementary Data 3)

The multiplexed nature of this protocol means that each experiment can generate gigabytes of images with time-lapse information about thousands of cells. 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 this⁷³⁻⁷⁵, 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, since 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 $\mu\text{L/s/channel}$ we observe modest cell movement and cell loss due to 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 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 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-iRFP¹⁹) 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 $\times$ 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 errorbars in Fig. 2b and Msn2-mCherry intensity variability in Fig. 2a) is due to 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 total fluorescence, but have a minimal effect on mean fluorescence since 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 due to cell size variation rather than stochastic gene expression.

Materials

Reagents

Photolithography

- SU-8 photoresist (SU-8 2100, MicroChem)
CAUTION: SU-8 photoresist is toxic and flammable. Wear appropriate PPE, handle in fume hood and avoid contact.
- PGMEA (Propylene Glycol Methyl Ether Acetate) (Sigma-Aldrich, item 484431) or SU-8 developer (Product Y020100, MicroChem).
- Isopropanol (e.g. item W292907 from Sigma-Aldrich)
- Acetone (e.g. item 439126 from Sigma-Aldrich)
- Methanol (e.g. item 32213 from Sigma-Aldrich)
CAUTION: organic solvents (PGMEA, methanol etc.) are toxic and flammable. Handle in fume hood and wear appropriate PPE.
- Silanization agent (1,1,2,2-tetrahydro(perfluorooctyl) trichlorosilane item 448931, Sigma-Aldrich). CAUTION. The silanization agent is toxic, 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 GmbH & Co.KG). CRITICAL: Can be purchased through Azer Scientific in the US. Most high NA oil immersion objectives are optimized for cover slips of the No. 1.5H thickness (170 μm).
- Razor blades (e.g. item from D109727 VWR)
- Adapter tubing (polyethylene, 1.57OD×1.14ID PE-160/10 item 64-0755 from Warner Instruments).

Low fluorescence medium (LFM)—The following ingredients are required for making LFM⁷¹. Please see “Reagents Setup” below for a recipe describing how to make and store LFM.

- (D)-Glucose (e.g. item C8270 from Sigma-Aldrich)
- × salt stock solution:
 - Ammonium sulfate ((NH₄)₂SO₄; e.g. item A4418 from Sigma-Aldrich)
 - Potassium phosphate monobasic (KH₂PO₄; e.g. item P5655 from Sigma-Aldrich)
 - Magnesium sulfate heptahydrate (MgSO₄·7H₂O; e.g. item 230391 from Sigma-Aldrich)

- Sodium chloride (NaCl; e.g. item 793566 from Sigma-Aldrich)
- Calcium chloride dihydrate (CaCl₂·2H₂O; e.g. item C5080 from Sigma-Aldrich)
- 1000× trace elements stock solution:
 - Boric acid (H₃BO₃; e.g. item B6768 from Sigma-Aldrich)
 - Copper(II) sulfate (CuSO₄; e.g. item C1297 from Sigma-Aldrich)
 - Potassium iodide (KI; e.g. item 793582 from Sigma-Aldrich)
 - Iron(III) chloride (FeCl₃; e.g. item 157740 from Sigma-Aldrich)
 - Manganese(II) sulfate (MnSO₄; e.g. item M7634 from Sigma-Aldrich)
 - Sodium molybdate dihydrate (Na₂MoO₄·2H₂O; e.g. item M1003 from Sigma-Aldrich)
 - Zinc sulfate (ZnSO₄; e.g. item Z0251 from Sigma-Aldrich)
- 1000× vitamin stock solution:
 - Biotin (e.g. item B4501 from Sigma-Aldrich)
 - Calcium pantothenate (D-pantothenic acid hemicalcium salt; e.g. item C8731 from Sigma-Aldrich)
 - *myo*-inositol (e.g. item I5125 from Sigma-Aldrich)
 - Niacin/nicotinic acid (e.g. item N0761 from Sigma-Aldrich)
 - *p*-Aminobenzoic acid (4-aminobenzoic acid; e.g. item 100536 from Sigma-Aldrich)
 - Pyridoxine hydrochloride (e.g. item P9755 from Sigma-Aldrich)
 - Thiamine hydrochloride (e.g. item T4625 from Sigma-Aldrich)
- 10× amino acid stock solution:
 - Adenine (e.g. item A8626 from Sigma-Aldrich)
 - L-Arginine (e.g. item A5006 from Sigma-Aldrich)
 - L-Histidine (e.g. item H8000 from Sigma-Aldrich)
 - L-Isoleucine (e.g. item I2752 from Sigma-Aldrich)
 - L-Leucine (e.g. item L8000 from Sigma-Aldrich)
 - L-Lysine (e.g. item L5501 from Sigma-Aldrich)
 - L-Methionine (e.g. item M9625 from Sigma-Aldrich)
 - L-Phenylalanine (e.g. item P2126 from Sigma-Aldrich)
 - L-Threonine (e.g. item T8625 from Sigma-Aldrich)
 - L-Tryptophan (e.g. item T0254 from Sigma-Aldrich)

- L-Tyrosine (e.g. item T3754 from Sigma-Aldrich)
- Uracil (e.g. item U0750 from Sigma-Aldrich)
- L-Valine (e.g. item V0500 from Sigma-Aldrich)
- Nalgene Rapid-Flow sterile filter storage bottles (item 455-1000 from Thermo Scientific)

Time-lapse microscopy

- 1-NM-PP1 (Commercially available from Cayman Chemical as item 13330. We synthesize it from 1-naphteleneacetic acid in large quantities as previously described¹⁹).
- Dimethyl sulfoxide (DMSO; e.g. item D8418 from Sigma-Aldrich)
- Concanavalin A (1 g, Type IV from *Canavalia ensiformis* as a lyophilized powder, item C2010-1g from Sigma-Aldrich)

CRITICAL: ConA from different sources can vary in activity. Type IV from Sigma-Aldrich works well in our hands.

- PBS solution (Dulbecco's phosphate buffered saline, item D8537 from Sigma-Aldrich)
- Calcium chloride (e.g. item C5080 from Sigma-Aldrich)
- Manganese(II) chloride (e.g. item M3634 from Sigma-Aldrich)
- Ethanol or reagent alcohol (e.g. item 362808 from Sigma-Aldrich)
- Yeast strain with suitable fluorescent reporters. In this example, we make use of our previously reported diploid strain¹⁹ in the W303 (*trp1 leu2 ura3 his3 can1 GAL⁺ psi⁺*) background (EY2813/ASH94): *TPK1*^{M164G} *TPK2*^{M147G} *TPK3*^{M165G} *msn4* :*TRP1/LEU2 MSN2*-mCherry *NHP6a*-iRFP: :kanMX *sip18*: :mCitrineV163A/SCFP3A-spHIS5. All previously reported yeast strains^{4,19,28} are available upon request.
- Plasmids encoding fluorescent proteins are available from AddGene:
 - Fast-folding codon-optimized⁷⁶ YFP mCitrineV163A in a pKT vector⁷¹: pKT-mCitrineV163A-HIS available from AddGene with ID 64685 at: www.addgene.org/64685/
 - Fast-folding codon-optimized⁷⁶ CFP SCFP3A⁷⁷ in a pKT vector⁷¹: pKT-SCFP3A-HIS available from AddGene with ID 64686 at: www.addgene.org/64686/
 - Codon-optimized⁷⁶ infrared iRFP³⁴ in a pKT vector⁷¹: pKT-iRFP-KAN available from AddGene with ID 64687 at: www.addgene.org/64687/

Equipment

Photolithography—CRITICAL: Most clean rooms will have all necessary equipment.

For reference, the equipment we use is given below:

- Spin coater (e.g. Headway Spin Coater model PWM32).
- Silicon wafers (4-inch diameter, 0-100 Ω -cm, 500 μ m thickness, Test grade, item 452 at University Wafers, South Boston, MA).
- 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 GmbH or item 82050-596 from VWR).
- Appropriate design software (e.g. Auto Cad, Adobe Illustrator etc. 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 (The Mathworks).
- Serial-to-USB converter (Tripp-lite USA-19HS - Keyspan High-Speed USB to Serial Adapter. 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 www.controlanything.com).
- 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 www.controlanything.com).
- Solenoid valves (3-way 12 volts LFYA1228032H Y-valve in perfluoroelastomer, the Lee Company). CRITICAL: While other valves are available, LFYA1228032H valves have a low internal volume (22 μ L) and are inert to hydrophobic inhibitors like 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 Inc.).

- 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 Inc).
- Tweezers (e.g. 5-SA tweezers, S95307 Aven Tools 18062ER from Fisher Scientific).
- Dissection scissors (e.g. item 25874-105 from VWR).
- Vacuum desiccator attached to vacuum pump (e.g. item 250-028 from Jencons but any will work).

Time-lapse microscopy

- Ismaprene tubing (Ismatec PharMed BPT (ID 0.51 mm, wall 0.85 mm), item SC0305 from IDEX Corporation).
- PE tubing (Polyethylene tubing, 0.050" OD/0.034" ID, item BPE-T90 from Instech Solomon).
- Valve adaptor tubing (Polyethylene tubing (1.57OD × 1.14ID mm), PE-160/10. Cat 64-0755 from Warner Instruments).
- 20G needle (PrecisionGlide™ needle 20G (0.9 mm × 25 mm) REF 305175 from BD).
- Immersion Oil (use immersion oil appropriate for microscope and objective. We use Carl Zeiss Immersol immersion oil 518F, item 12-624-66A from Fisher Scientific).
- 1 mL Luer-Lok™ disposable syringes (item 309628 from BD Biosciences).
- Suitable inverted fluorescence microscope with environmental incubation chamber, automated stage, and auto-focus 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 EM-CCD camera (Evolve 512, Photometrics) and an oil-immersion objective (63×, NA 1.4, oil Ph3, Plan-Apochromat).

Reagent Setup

Time-lapse microscopy

- ConA solution. Prepare and filter-sterilize a 1 M CaCl₂ solution in water and a 1 M MnCl₂ solution in water. Obtain 1 g of ConA^{36,37} (type IV from Sigma-Aldrich C2010). Adjust PBS solution pH to 6.5. On ice, gently dissolve ConA powder in 5 mL PBS at pH 6.5, 40 mL H₂O, 2.5 mL 1 M CaCl₂ solution and 2.5 mL 1 M MnCl₂ solution. Once fully dissolved, aliquot the 20 mg/mL ConA solution into

200 μ L aliquots and store these at -20°C 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 activity is too high or low, adjust the concentration accordingly. Handle powder gently on ice and carefully adjust the pH (a pH between 6 and 7 is important for high ConA activity³⁶⁻³⁸). Freeze thawing or long-term storage at 4°C leads to loss of activity.

- Low fluorescence medium (LFM) with amino acids. We recommend LFM for microscopy since its autofluorescence is within 10% of water⁷¹. Note that LFM lacks riboflavin and folic acid. However, for less sensitive application standard synthetic complete (SC) yeast medium is also acceptable. Per liter, LFM medium contains: 20 g glucose (2% glucose), 5 g $(\text{NH}_4)_2\text{SO}_4$, 1 g KH_2PO_4 , 500 mg $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 100 mg NaCl, 100 mg $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 500 μg H_3BO_3 , 40 μg CuSO_4 , 100 μg KI, 200 μg FeCl_3 , 400 μg MnSO_4 , 200 μg $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 400 μg ZnSO_4 , 2 μg biotin, 400 μg calcium pantothenate, 2 mg myo-inositol, 400 μg niacin/nicotinic acid, 200 μg p-aminobenzoic acid, 400 μg pyridoxine-HCl, 400 μg thiamine-HCl, 30 mg L-isoleucine, 150 mg L-valine, 40 mg adenine, 20 mg L-arginine, 20 mg L-histidine, 100 mg L-leucine, 30 mg L-lysine, 20 mg L-methionine, 50 mg L-phenylalanine, 200 mg L-threonine, 100 mg L-tryptophan, 30 mg L-tyrosine and 20 mg uracil.

To make 1 L of LFM medium, mix 100 mL 10 $\times$ custom YNB stock, 100 mL 10 $\times$ amino acid stock, 40 mL 50% glucose stock and 760 mL H_2O and then sterilize by filtration through Nalgene filter. Once filter sterilized – the same Nalgene filter can be re-used if properly handled with sterile techniques – we store LFM at 4°C , where it is stable for more than six months.

To make 1 L of 10 $\times$ amino acid stock, mix amino acid and nucleobase powders in a autoclave-safe glass bottle: 300 mg L-isoleucine, 1500 mg L-valine, 400 mg adenine, 200 mg L-arginine, 200 mg L-histidine, 1000 mg L-leucine, 300 mg L-lysine, 200 mg L-methionine, 500 mg L-phenylalanine, 2000 mg L-threonine, 1000 mg L-tryptophan, 300 mg L-tyrosine and 200 mg uracil. Add ~ 600 mL H_2O , microwave and swirl until fully dissolved and then fill up to 1 L. Sterilize by sterile-filtration or by autoclaving. Store at 4°C .

To make 100 mL 1000 $\times$ trace elements stock, mix element powders in autoclave-safe glass bottle: 50 mg H_3BO_3 , 4 mg CuSO_4 , 10 mg KI, 20 mg FeCl_3 , 40 mg MnSO_4 , 20 mg $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 40 mg ZnSO_4 . Fill to 100 mL and sterilize by autoclaving – solution may be cloudy so do not filter-sterilize. Store at room temperature indefinitely.

To make 100 mL 1000 $\times$ vitamin stock, mix powders in beaker: 200 μg biotin, 40 mg calcium pantothenate, 200 mg myo-inositol, 40 mg niacin/nicotinic acid, 20 mg p-aminobenzoic acid, 40 mg pyridoxine-HCl, 40 mg thiamine-HCl. Dissolve and fill to 100 mL. Sterilize by filtration into Nalgene sterile filter storage bottle. Store bottle at 4°C .

To make 1 L of 20× salt stock, mix powders in autoclave-safe glass bottle: 100 g $(\text{NH}_4)_2\text{SO}_4$, 20 g KH_2PO_4 , 10 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 2 g NaCl, 2 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$. Fill to 1 L and microwave and stir if necessary to fully dissolve. Sterilize by autoclaving. Store at room temperature indefinitely.

To make 500 mL 50% (w/v) glucose solution, add 250 mL H_2O to beaker and a stirrer bar. Heat and stir solution. Add 250 g glucose and dissolve. Fill to 500 mL and sterilize by autoclaving. Store at room temperature.

To make 1 L 10× custom YNB stock, mix 500 mL 20× salt stock, 10 mL 1000× trace elements stock, 10 mL 1000× vitamin stock and 480 mL H_2O . Sterilize by filtration into Nalgene sterile filter storage bottle. Store bottle at 4°C.

CRITICAL. We strongly recommend storing LFM at 4°C, where it is stable for more than six 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 1000× vitamin stock should also be stored at 4°C.

- 70% ethanol. Mix ethanol or reagent alcohol with water in 70:30 (v/v) ratio and store at room temperature.
- 1-NM-PP1 stock. We prepare 1-NM-PP1 stocks in DMSO at 1000× concentration (e.g. a 3 mM stock for experiments where a 3 μM concentration is needed) and store these as aliquots at -20°C. 1-NM-PP1 is extremely stable and 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 5080 dpi. CAD/Art Services provides masks at 20,000 dpi.

Device holder for microscopy—We use a metal holder to hold microfluidic device. Any machine shop should be able to prepare this. The dimensions for our holder are given online (Supplementary data 4).

Suction filtration setup—We use a Millipore system (Millipore kit item XX1002530 contains everything needed) with nitrocellulose filters (mixed cellulose esters, 0.8 μm , 25 mm, item 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 out-sourced to a company and once a master mold has been obtained it can be re-used indefinitely. SIMTech offers these services (<http://www.simtech.a-star.edu.sg/smf/>), as do a number of other companies (see also <http://stanford.edu/group/foundry/Services.html>). Since 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) from company (we use CAD/Art Services). Steps 2-14 for fabrication of silicon wafers can also be outsourced to companies (see above).
2. Choose an SU-8 photoresist appropriate for the desired height. We recommend SU-8 2050 for ~ 50 μm height and SU-8 2100 for ~ 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 ~ 100 μm height using SU-8 2100.

CRITICAL STEP. Steps 3-14 need to be performed in a clean room to prevent contamination by dust etc. Avoid introducing air bubbles when 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 wafer by incubation in an acetone bath ideally with sonication for 5 min (Fig. 5a). Wash wafer with methanol and then isopropanol. Dry wafer with nitrogen gun and then bake at 200°C for 5 min to remove any leftover solvent.
4. Prepare spin coater. Line the bowl of the spin coater with aluminium foil to facilitate cleaning (Fig. 5b) and place 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 ca. 4-5 mL SU-8 2100 to the centre of the wafer (Fig. 5c). Use the following spin program: Ramp up to 500 rpm at 100 rpm/s acceleration. Hold for 5 s. Ramp up to 3000 rpm at 300 rpm/s acceleration. Hold for 45 s. Then ramp down.
6. Soft bake. Pre-bake the wafer on level 65°C hot plate for 5 min, then soft-bake at 95°C for 20 min and cool down to 65°C before proceeding (Fig. 5d).
CRITICAL STEP: any air bubbles should be removed during the soft-bake by tapping with needle tip or tip of tweezers.
7. (Recommended) Edge bead removal. Place the coated wafer back on the chuck of the spin coater and spin at 400 rpm for 60 s. As the wafer is spinning remove edge by placing a cotton swab tip soaked in PGMEA or SU-8 developer on the edge (Fig. 5e). Re-soak the cotton swab tip as necessary. Bake wafer on 65°C hot plate for 2 min to remove any residual PGMEA.
8. Exposure. While the exact conditions will depend on instrument and 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/cm² (a lower exposure energy should be used if a long-pass filter is not used). On the mask aligner we use, this works out to an exposure time of ~21 s. Place transparency mask on top of wafer substrate, add 360 nm long-pass filter and expose.

9. Post-exposure bake. Place the wafer on a level 65°C hot plate. Ramp hot plate up to 95°C and hold for 11 min. Latent visible image should appear during post-exposure bake (Fig. 5f).

CRITICAL STEP: cool down wafer slowly for example by switching off the hot place – rapid cooling can cause cracks in the SU-8 film.

?TROUBLESHOOTING.

10. Development. Prepare PGMEA bath for development and place on shaker. Submerge wafer with the SU-8 coated side facing upwards. Gently rock the PGMEA bath with wafer at 90 rpm for 10 min (Fig. 5g). To check whether development is complete remove wafer and use spray bottle to spray isopropanol on wafer. If a white film is observed, this indicates underdevelopment. In that case, put the wafer back into the PGMEA bath and develop for longer.
11. Once development is complete, rinse with isopropanol and dry with nitrogen gun.
12. (Recommended) Hard bake. Place wafer on level hot plate and heat to 150°C for 20 min and then cool down slowly (Fig. 5h). CRITICAL STEP: Performing hard bake will increase durability and can heal any surface cracks.
13. (Recommended) Characterization. To ensure that mold pattern is without defect, the wafer mold should be inspected using a simple transmitted light microscope and a profilometer to characterize height etc.
14. (Recommended) Silanization. Place wafer in petri dish without lid inside a desiccator together with a small vial containing ~500 µL silanization agent 1,1,2,2-tetrahydro(perfluorooctyl)trichlorosilane. Incubate in dessicator attached to a vacuum pump under reduced pressure overnight.

CAUTION. Silanization agent is toxic and corrosive. Wear PPE and work in fume hood.

PAUSE POINT. After these steps, the wafer mold is stable and 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 cover glass.

15. Place wafer in petri dish with mold pattern facing up.

16. Mix PDMS and curing agent in a 10:1 ratio (w/w) in 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 disposable plastic spoon and degas in vacuum desiccator.

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

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

19. Once PDMS degassing is complete (when foaming/bubbling ceases), place lid over petri dish containing wafer and uncured PDMS and place petri dish in an oven at 65°C. Make sure the surface is level. Cure in 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 a couple of minutes for it to cool down. With a razor blade, cut through PDMS against wafer and circle the entire SU-8 pattern (Fig. 6b). Be careful to keep ~1 cm from cutting area to SU-8 pattern since accidentally cutting SU-8 pattern with 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. Then carefully peel off 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 re-used for years.

?TROUBLESHOOTING.

21. After PDMS peeling, place PDMS on cutting mat with 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 (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 the punched out PDMS is fully removed.

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

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 cleaned PDMS devices and cover glasses upright with the side to be bonded exposed inside plasma chamber. Close plasma chamber and turn on vacuum. Switch gas

to pure oxygen. Wait until a pressure of 300 Torr (400 mbar) is reached. Turn on oxygen plasma and tune instrument. Expose for 12 s at ~60 W (Fig. 6d). Turn off vacuum and wait for instrument to reach standard pressure. As soon as possible, take out plasma chamber and carefully place PDMS chips on cover glasses (Fig. 6e). The channel side should face cover glass. Place on soft material (e.g. layers of paper tissues) and gently press PDMS device against cover glass until fully bound. The PDMS chip is fully bonded to the cover glass when all air bubbles between glass and PDMS have disappeared.

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

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

25. Cut adapter tubing (PE-160/10 (1.57 mm outer diameter; 1.14 mm inner diameter, Warner Instruments) into pieces of ~8-10 mm in length. Take out microfluidic device and place 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 vs. 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 small amount of mixed PDMS (from Step 16) around each inlet and outlet to seal tubing (Fig. 6g).

26. Place each microfluidic device with sealed inlet and outlet tubing in 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) and they can be stored for months. It is more efficient to make a large number of devices in a single setting. Store 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 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 low fluorescence medium (LFM) and grow for >8 h at 30°C. At night, calculate how many OD_{600 nm} units of cells to add (assuming a doubling time of 90 min) to a 250 mL conical flask containing 50 mL LFM so that the culture reaches an OD_{600 nm} of 0.1 at the desired time the following day assuming a doubling time of 90 min. Grow this culture overnight at 30°C with shaking at 180 rpm.

29. Prepare all media. For a ~3 h, five-channel experiment, ~100-150 mL of LFM is needed depending on 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 overnight at 30°C.

CRITICAL STEP. Incubating LFM overnight at 30°C is essential to avoid air bubbles forming 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 freezer and gently thaw on ice 1 h before experiment. Once fully thawed, add 200 µL PBS (pH 6.5) and 600 µL H₂O to the 200 µL ConA solution and mix to obtain 1 mL final solution of 4 mg/mL ConA.

CRITICAL STEP. It is important to gently and slowly thaw ConA on ice to prevent a 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 microscope set-up at least 1 h before experiment. Turn on microscope and heat up incubation chamber to 30°C. Connect valve control system to computer, open MATLAB and establish serial connection to valves. Cut PE tubing for inlets and outlets (Fig. 7a). 5 pieces are needed for each inlet and outlet, so 15 total. These should be around 50-100 cm long depending on microscope setup. We keep flasks with inlet LFM and outlet waste outside of microscope incubation chamber, so tubing must be long enough to reach these. Cut 5 pieces of ismaprene tubing (~12 cm) and attach to valve outlets (Fig. 7b). Cut small pieces of PE tubing and attach to other end of ismaprene tubing with 20G needle (Fig. 7c).

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

33. Wash microfluidic device. Wash each microfluidic channel with ~200 µL 70% ethanol by inserting syringe through PE tubing and gently pushing liquid through channels (Fig. 7d). Then wash each channel with ~200 µL H₂O. Dry off excess liquid with Kim wipes. Finally load each channel with ~200 µL 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 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 syringe to flush channel until the air bubble has been removed again. During wash steps, apply gentle pressure from syringe – too high pressure can break the PDMS walls between the channels.

?TROUBLESHOOTING.

34. Prepare 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 1000× stock in DMSO at -20°C and add it just before use). Connect PE tubing with flasks containing medium. Tape tubing to flasks (Fig. 7f). To start flow, insert 1 mL syringe with 20G needle into PE tubing and draw out liquid (Fig. 7g). Then attach PE tubing to valve inlets and outlets. Flow rate is controlled by gravity. Switch between inlets a few times using 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 medium. It is important to tape PE tubing to medium flasks (Fig. 7f). If tubing ends move above medium, air will be sucked in and when these air bubbles reach microfluidic channels, cells will be lost.

35. Load cells into microfluidic device. Collect cells when they have reached the right OD_{600 nm} (we use 50 mL at OD_{600 nm} 0.1 so 5 OD units of cells). We prefer collecting cells by suction filtration rather than centrifugation. Attach the suction filtration system (Millipore XX1002530) to house vacuum and pour 50 mL cell culture over a nitrocellulose filter at room temperature. As soon as the cell culture has been filtered, remove nitrocellulose filter with cells and quickly re-suspend cells in 500 µL 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). Then load cell suspension into each microfluidic channel using syringe (Fig. 7d) (~100 µL per channel). Incubate 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 microscope with flow of LFM to keep cells in a chemostatic environment and to avoid stress.

36. Load microfluidic device on microscope. Carefully, place microfluidic device on holder inside microscope incubation chamber on paper towels to absorb any spillage. Carefully, move board and valves into 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 the medium is constantly flowing from ismaprene tubing to avoid introducing air bubbles.

37. Attach outlets. Attach PE tubing to outlet waste flask and tape it. Move flask above microscope chamber and start flow by drawing liquid from PE tubing with syringe (Fig. 7g). Carefully attach outlet PE tubing to microfluidic device outlets – make sure PE tubing is stably inserted to avoid leakage. Move outlet flask to below microscope chamber (Fig. 7i). The flow rate is controlled by gravity – the height difference between

the inlet and outlet flasks governs this. A height difference of ~ 30 cm yields a flow rate of $\sim 1 \mu\text{L/s/channel}$ in our hands.

38. Begin microscopy. Add immersion oil to objective. Move objective to the straight part of the microfluidic channels and focus to find cells (should look as shown in brightfield image in Fig. 1). Tape tubing and valves to 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 stage positions in each microfluidic channel and the auto-focus 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 homogenous exposure, since 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 2.5 min time resolution (10 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 $\pm 1.75 \mu\text{m}$), 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 images and remove and dispose of the microfluidic device as glass disposal (although the microfluidic device can be re-used, washing is cumbersome and cell retention is significantly reduced³⁸). Clean objective with lens cleaner. We recommend disposing of tubing that has been in contact with 1-NM-PP1, but all other tubing can be re-used. Wash valves and ismaprene tubing with 70% 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 Fig. 2a. A number of software packages are available for this⁷³⁻⁷⁵. 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.

7TROUBLESHOOTING

Troubleshooting

Troubleshooting advice can be found in Table 1.

Anticipated Results

The anticipated results will depend on the experiment in question. Using the analogue sensitive PKA^{as}-system to control Msn2 dynamics and 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 whilst collecting two fields-of-view per channel and ~200 cells per field-of-view, thus generating data for ~2000 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 whilst 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 account²¹. 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 transduction²¹. 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}.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank M. McClean and S. Ramanathan for help with setting up the original Y-channel microfluidic device. We thank D. MacLaurin and E. Zwiebach-Cohen for discussions. We thank the O'Shea lab for discussions and comments on the manuscript. This work was performed in part at the Center for Nanoscale Systems at Harvard University, a member of the National Nanotechnology Infrastructure Network (NNIN), which is supported by the National Science Foundation under NSF award no. ECS- 0335765. The Howard Hughes Medical Institute supported this work.

References

1. Selimkhanov J, et al. Accurate information transmission through dynamic biochemical signaling networks. *Science*. 2014; 346:1370–1373. [PubMed: 25504722]
2. Batchelor E, Loewer A, Mock C, Lahav G. Stimulus-dependent dynamics of p53 in single cells. *Molecular systems biology*. 2011; 7
3. Cai L, Dalal CK, Elowitz MB. Frequency-modulated nuclear localization bursts coordinate gene regulation. *Nature*. 2008; 455:485–90. [PubMed: 18818649]
4. Hao N, Budnik BA, Gunawardena J, O'Shea EK. Tunable Signal Processing Through Modular Control of Transcription Factor Translocation. *Science*. 2013; 339:460–464. [PubMed: 23349292]

5. Yissachar N, et al. Dynamic Response Diversity of NFAT Isoforms in Individual Living Cells. *Molecular Cell*. 2013; 49:322–330. [PubMed: 23219532]
6. Cai HQ, et al. Nucleocytoplasmic Shuttling of a GATA Transcription Factor Functions as a Development Timer. *Science*. 2014; 343:1329–+.
7. Hersen P, McClean MN, Mahadevan L, Ramanathan S. Signal processing by the HOG MAP kinase pathway. *Proceedings of the National Academy of Sciences of the United States of America*. 2008; 105:7165–7170. [PubMed: 18480263]
8. Imayoshi I, et al. Oscillatory Control of Factors Determining Multipotency and Fate in Mouse Neural Progenitors. *Science*. 2013; 342:1203–1208. [PubMed: 24179156]
9. Spiller DG, Wood CD, Rand DA, White MRH. Measurement of single-cell dynamics. *Nature*. 2010; 465:736–745. [PubMed: 20535203]
10. Behar M, Hoffmann A. Understanding the temporal codes of intra-cellular signals. *Current opinion in genetics & development*. 2010; 20:684–693. [PubMed: 20956081]
11. Levine JH, Lin YH, Elowitz MB. Functional Roles of Pulsing in Genetic Circuits. *Science*. 2013; 342:1193–1200. [PubMed: 24311681]
12. Purvis JE, Lahav G. Encoding and Decoding Cellular Information through Signaling Dynamics. *Cell*. 2013; 152:945–56. [PubMed: 23452846]
13. Castillo-Hair SM, Igoshin OA, Tabor JJ. How to train your microbe: methods for dynamically characterizing gene networks. *Current Opinion in Microbiology*. 2015; 24C:113–123. [PubMed: 25677419]
14. Sanchez A, Golding I. Genetic determinants and cellular constraints in noisy gene expression. *Science*. 2013; 342:1188–93. [PubMed: 24311680]
15. Bennett MR, Hasty J. Microfluidic devices for measuring gene network dynamics in single cells. *Nature Reviews Genetics*. 2009; 10:628–638.
16. Ferry MS, Razinkov IA, Hasty J. Microfluidics for Synthetic Biology: From Design to Execution. *Methods in Enzymology*, Vol 497: *Synthetic Biology, Methods for Part/Device Characterization and Chassis Engineering*, Pt A. 2011; 497:295–372.
17. Sackmann EK, Fulton AL, Beebe DJ. The present and future role of microfluidics in biomedical research. *Nature*. 2014; 507:181–189. [PubMed: 24622198]
18. Denervaud N, et al. A chemostat array enables the spatio-temporal analysis of the yeast proteome. *Proceedings of the National Academy of Sciences of the United States of America*. 2013; 110:15842–7. [PubMed: 24019481]
19. Hansen AS, O'Shea EK. Promoter decoding of transcription factor dynamics involves a trade-off between noise and control of gene expression. *Molecular systems biology*. 2013; 9
20. Tseng P, Weaver WM, Masaeli M, Owsley K, Di Carlo D. Research highlights: microfluidics meets big data. *Lab on a chip*. 2014; 14:828–32. [PubMed: 24473594]
21. Hansen AS, O'Shea EK. Limits on Information Transduction through Amplitude and Frequency Regulation of Transcription Factor Activity. *Elife*. 2015 in press.
22. Prindle A, et al. Rapid and tunable post-translational coupling of genetic circuits. *Nature*. 2014; 508:387–391. [PubMed: 24717442]
23. Uhlenhof J, et al. Long-term model predictive control of gene expression at the population and single-cell levels. *Proceedings of the National Academy of Sciences of the United States of America*. 2012; 109:14271–14276. [PubMed: 22893687]
24. Menolascina F, et al. In-vivo real-time control of protein expression from endogenous and synthetic gene networks. *PLoS computational biology*. 2014; 10:e1003625. [PubMed: 24831205]
25. Sorre B, Warmflash A, Brivanlou AH, Siggia ED. Encoding of Temporal Signals by the TGF-beta Pathway and Implications for Embryonic Patterning. *Developmental Cell*. 2014; 30:334–342. [PubMed: 25065773]
26. Hao N, et al. Regulation of cell signaling dynamics by the protein kinase-scaffold Ste5. *Molecular Cell*. 2008; 30:649–56. [PubMed: 18538663]
27. Mettetal JT, Muzzey D, Gomez-Urbe C, van Oudenaarden A. The frequency dependence of osmo-adaptation in *Saccharomyces cerevisiae*. *Science*. 2008; 319:482–484. [PubMed: 18218902]

28. Hao N, O'Shea EK. Signal-dependent dynamics of transcription factor translocation controls gene expression. *Nature Structural & Molecular Biology*. 2012; 19:31–U47.
29. Cohen MS, Ghosh AK, Kim HJ, Jeon NL, Jaffrey SR. Chemical Genetic-Mediated Spatial Regulation of Protein Expression in Neurons Reveals an Axonal Function for Wld(S). *Chemistry & Biology*. 2012; 19:179–187. [PubMed: 22365601]
30. Bennett MR, et al. Metabolic gene regulation in a dynamically changing environment. *Nature*. 2008; 454:1119–1122. [PubMed: 18668041]
31. Gorner W, et al. Nuclear localization of the C2H2 zinc finger protein Msn2p is regulated by stress and protein kinase A activity. *Genes & Development*. 1998; 12:586–97. [PubMed: 9472026]
32. Bishop AC, et al. A chemical switch for inhibitor-sensitive alleles of any protein kinase. *Nature*. 2000; 407:395–401. [PubMed: 11014197]
33. Zaman S, Lippman SI, Schnepfer L, Slonim N, Broach JR. Glucose regulates transcription in yeast through a network of signaling pathways. *Molecular systems biology*. 2009; 5
34. Filonov GS, et al. Bright and stable near-infrared fluorescent protein for in vivo imaging. *Nature biotechnology*. 2011; 29:757–U133.
35. Elowitz MB, Levine AJ, Siggia ED, Swain PS. Stochastic gene expression in a single cell. *Science*. 2002; 297:1183–1186. [PubMed: 12183631]
36. Agrawal BBL, Goldstein IJ. Protein-Carbohydrate Interaction .7. Physical and Chemical Studies on Concanavalin a Hemagglutinin of Jack Bean. *Archives of biochemistry and biophysics*. 1968; 124:218. [PubMed: 5661602]
37. Senear DF, Teller DC. Thermodynamics of Concanavalin-a Dimer-Tetramer Self-Association - Sedimentation Equilibrium Studies. *Biochemistry*. 1981; 20:3076–3083. [PubMed: 7248268]
38. Bisaria A, Hersen P, McClean MN. Microfluidic Platforms for Generating Dynamic Environmental Perturbations to Study the Responses of Single Yeast Cells. *Yeast Genetics: Methods and Protocols*. 2014; 1205:111–129.
39. Strack RL, Song WJ, Jaffrey SR. Using Spinach-based sensors for fluorescence imaging of intracellular metabolites and proteins in living bacteria. *Nature protocols*. 2014; 9:146–155. [PubMed: 24356773]
40. Bermejo C, Haerizadeh F, Takanaga H, Chermak D, Frommer WB. Optical sensors for measuring dynamic changes of cytosolic metabolite levels in yeast. *Nature protocols*. 2011; 6:1806–1817. [PubMed: 22036883]
41. Dean KM, Palmer AE. Advances in fluorescence labeling strategies for dynamic cellular imaging. *Nature Chemical Biology*. 2014; 10:512–523. [PubMed: 24937069]
42. Huh WK, et al. Global analysis of protein localization in budding yeast. *Nature*. 2003; 425:686–691. [PubMed: 14562095]
43. Petrenko N, Chereji RV, McClean MN, Morozov AV, Broach JR. Noise and interlocking signaling pathways promote distinct transcription factor dynamics in response to different stresses. *Molecular Biology of the Cell*. 2013; 24:2045–57. [PubMed: 23615444]
44. Zid BM, O'Shea EK. Promoter sequences direct cytoplasmic localization and translation of mRNAs during starvation in yeast. *Nature*. 2014; 514:117–121. [PubMed: 25119046]
45. Paige JS, Nguyen-Duc T, Song WJ, Jaffrey SR. Fluorescence Imaging of Cellular Metabolites with RNA. *Science*. 2012; 335:1194–1194. [PubMed: 22403384]
46. Westfall PJ, Thorner J. Analysis of mitogen-activated protein kinase signaling specificity in response to hyperosmotic stress: use of an analog-sensitive HOG1 allele. *Eukaryotic cell*. 2006; 5:1215–28. [PubMed: 16896207]
47. Liu Y, et al. Two cyclin-dependent kinases promote RNA polymerase II transcription and formation of the scaffold complex. *Molecular and Cellular Biology*. 2004; 24:1721–35. [PubMed: 14749387]
48. Carroll AS, Bishop AC, DeRisi JL, Shokat KM, O'Shea EK. Chemical inhibition of the Pho85 cyclin-dependent kinase reveals a role in the environmental stress response. *Proceedings of the National Academy of Sciences of the United States of America*. 2001; 98:12578–83. [PubMed: 11675494]
49. Shirra MK, et al. A chemical genomics study identifies Snf1 as a repressor of GCN4 translation. *The Journal of biological chemistry*. 2008; 283:35889–98. [PubMed: 18955495]

50. Elphick LM, Lee SE, Gouverneur V, Mann DJ. Using chemical genetics and ATP analogues to dissect protein kinase function. *ACS chemical biology*. 2007; 2:299–314. [PubMed: 17518431]
51. Rakhit R, Navarro R, Wandless TJ. Chemical Biology Strategies for Posttranslational Control of Protein Function. *Chemistry & Biology*. 2014; 21:1238–1252. [PubMed: 25237866]
52. McIsaac RS, et al. Fast-acting and nearly gratuitous induction of gene expression and protein depletion in *Saccharomyces cerevisiae*. *Molecular Biology of the Cell*. 2011; 22:4447–4459. [PubMed: 21965290]
53. Haruki H, Nishikawa J, Laemmli UK. The anchor-away technique: Rapid, conditional establishment of yeast mutant phenotypes. *Molecular Cell*. 2008; 31:925–932. [PubMed: 18922474]
54. Belshaw PJ, Ho SN, Crabtree GR, Schreiber SL. Controlling protein association and subcellular localization with a synthetic ligand that induces heterodimerization of proteins. *Proceedings of the National Academy of Sciences of the United States of America*. 1996; 93:4604–4607. [PubMed: 8643450]
55. Geda P, et al. A small molecule-directed approach to control protein localization and function. *Yeast*. 2008; 25:577–594. [PubMed: 18668531]
56. Huberts DHEW, et al. Construction and use of a microfluidic dissection platform for long-term imaging of cellular processes in budding yeast. *Nature protocols*. 2013; 8:1019–1027. [PubMed: 23640166]
57. Crane MM, Clark IB, Bakker E, Smith S, Swain PS. A microfluidic system for studying ageing and dynamic single-cell responses in budding yeast. *Plos One*. 2014; 9:e100042. [PubMed: 24950344]
58. Rowat AC, Bird JC, Agresti JJ, Rando OJ, Weitz DA. Tracking lineages of single cells in lines using a microfluidic device. *Proceedings of the National Academy of Sciences of the United States of America*. 2009; 106:18149–18154. [PubMed: 19826080]
59. Taylor RJ, et al. Dynamic analysis of MAPK signaling using a high-throughput microfluidic single-cell imaging platform. *Proceedings of the National Academy of Sciences of the United States of America*. 2009; 106:3758–3763. [PubMed: 19223588]
60. Sott K, Eriksson E, Goksor M. Acquisition of single cell data in an optical microscope. *Lab on a Chip Technology: Biomolecular Separation and Analysis*. 2009:151–166.
61. Kellogg RA, Gomez-Sjoberg R, Leyrat AA, Tay S. High-throughput microfluidic single-cell analysis pipeline for studies of signaling dynamics. *Nature protocols*. 2014; 9:1713–1726. [PubMed: 24967621]
62. Zhang Y, et al. Single cell analysis of yeast replicative aging using a new generation of microfluidic device. *Plos One*. 2012; 7:e48275. [PubMed: 23144860]
63. Lee SS, Avalos Vizcarra I, Huberts DH, Lee LP, Heinemann M. Whole lifespan microscopic observation of budding yeast aging through a microfluidic dissection platform. *Proceedings of the National Academy of Sciences of the United States of America*. 2012; 109:4916–20. [PubMed: 22421136]
64. Xie Z, et al. Molecular phenotyping of aging in single yeast cells using a novel microfluidic device. *Aging Cell*. 2012; 11:599–606. [PubMed: 22498653]
65. Ryley J, Pereira-Smith OM. Microfluidics device for single cell gene expression analysis in *Saccharomyces cerevisiae*. *Yeast*. 2006; 23:1065–1073. [PubMed: 17083143]
66. Lee PJ, Helman NC, Lim WA, Hung PJ. A microfluidic system for dynamic yeast cell imaging. *Biotechniques*. 2008; 44:91–95. [PubMed: 18254385]
67. McClean MN, Hersen P, Ramanathan S. Measuring In Vivo Signaling Kinetics in a Mitogen-Activated Kinase Pathway Using Dynamic Input Stimulation. *Yeast Genetic Networks: Methods and Protocols*. 2011; 734:101–119.
68. Qin D, Xia Y, Whitesides GM. Soft lithography for micro- and nanoscale patterning. *Nature protocols*. 2010; 5:491–502. [PubMed: 20203666]
69. Hillborg H, et al. Crosslinked polydimethylsiloxane exposed to oxygen plasma studied by neutron reflectometry and other surface specific techniques. *Polymer*. 2000; 41:6851–6863.
70. McDonald JC, et al. Fabrication of microfluidic systems in poly(dimethylsiloxane). *Electrophoresis*. 2000; 21:27–40. [PubMed: 10634468]

71. Sheff MA, Thorn KS. Optimized cassettes for fluorescent protein tagging in *Saccharomyces cerevisiae*. *Yeast*. 2004; 21:661–670. [PubMed: 15197731]
72. Wang JD, Douville NJ, Takayama S, ElSayed M. Quantitative Analysis of Molecular Absorption into PDMS Microfluidic Channels. *Annals of Biomedical Engineering*. 2012; 40:1862–1873. [PubMed: 22484830]
73. Gordon A, et al. Single-cell quantification of molecules and rates using open-source microscope-based cytometry. *Nature Methods*. 2007; 4:175–181. [PubMed: 17237792]
74. Lamprecht MR, Sabatini DM, Carpenter AE. CellProfiler(TM): free, versatile software for automated biological image analysis. *Biotechniques*. 2007; 42:71–75. [PubMed: 17269487]
75. Doncic A, Eser U, Atay O, Skotheim JM. An Algorithm to Automate Yeast Segmentation and Tracking. *Plos One*. 2013; 8
76. Grote A, et al. JCat: a novel tool to adapt codon usage of a target gene to its potential expression host. *Nucleic Acids Research*. 2005; 33:W526–W531. [PubMed: 15980527]
77. Kremers GJ, Goedhart J, van Munster EB, Gadella TWJ. Cyan and yellow super fluorescent proteins with improved brightness, protein folding, and FRET Forster radius. *Biochemistry*. 2006; 45:6570–6580. [PubMed: 16716067]
78. Zechner C, Unger M, Pelet S, Peter M, Koepl H. Scalable inference of heterogeneous reaction kinetics from pooled single-cell recordings. *Nature Methods*. 2014; 11:197–202. [PubMed: 24412977]

Box 1**Controlling valves in MATLAB**

To use MATLAB to control electrovalves, make sure the board is plugged in and the USB connection inserted into the computer (see also Supplementary Tutorial 1 for more detailed guidelines). The first step is establishing a serial connection. Run script “Windows_open_valves.m” if on a Windows-based computer and run 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 again 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:

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

Or to switch OFF all valves use:

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

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

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

Or to turn valve 4 OFF use:

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

Combining the “toggleValves.m” function with the “pause” function in MATLAB, fluid delivery programs are easily made. E.g. the simple program

```
for i=1:6

    toggleValves([0,0,1],s1); %all ON

    pause(300);

    toggleValves([0,0,0],s1); %all OFF

    pause(600);

end
```

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 Fig. 2.

Figure 1. Overview of microfluidic setup

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. Below, a cropped brightfield image of budding yeast cells growing in microfluidic channels (two images stitched horizontally together). 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. Top sketch is adapted from Hansen & O'Shea¹⁹ with permission from EMBO.

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 timepoints. Small (99×99 pixels) sections of full images are shown. The cell segmentation is overlaid in green. 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 appears brighter in later frames. Msn2-mCherry nuclear localization is visible as nuclear foci in frame 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. Since 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. This is why the bottom panel ellipses are smaller than the upper panel ellipses. Cell-to-cell variability in CFP and YFP expression is detectable both in the raw data and in **(c-d)**.

b) Protein translocation dynamics. In this example, Msn2-mCherry translocation dynamics in response to six 5-min pulses of 690 nM 1-NM-PP1 separated by 10-min intervals which leads to $\sim 75\%$ of maximal nuclear localization is shown. Raw data (black dots) and errorbars (standard deviation) 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 is 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-traces⁷⁸.

e) By following both CFP and YFP gene expression dynamics in the same single cell, their co-variance can be computed. Thus, we can calculate both intrinsic and extrinsic noise³⁵. Each dot in the scatterplot, is the max CFP and YFP from one single cell. The above experiment was performed with strain EY2967/ASH189²¹. Supplementary Fig. 1 shows a control experiment, where 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.

Figure 3. Device design

The transparency mask with annotation is shown (see also Supplementary Data 1). The device consists of five 400 μm wide channels, where cells are grown, separated by 150 μm walls. It is a negative mask, so areas in black are exposed to UV during photolithography.

Figure 4. Photolithography and soft lithography overview

Overview of protocol Steps 2-26. During the photolithography steps a 4" Si wafer is spin-coated with SU-8 photoresist to a height of $\sim 100\ \mu\text{m}$. Subsequently, it is exposed to UV light under a transparency mask. The photoresist pattern 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 and holes punched for inlets and outlets. Following plasma exposure, PDMS is bonded to a cover glass to yield the final microfluidic device (right most).

Figure 5. Photolithography steps: fabrication of silicon wafer master mold

- a)** Step 3: clean wafer in acetone.
- b)** Step 4: Prepare spin coater. Line bowl with aluminium foil to facilitate cleaning.
- c)** Step 5: Pour ca. 4-5 mL SU-8 2100 on 4" wafer and spin.
- d)** Step 6: Soft bake. Place spin-coated wafer on 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 wafer at 1000 rpm and remove edge by pressing a cotton swab tip soaked in PGMEA against the wafer edge as illustrated.
- f)** Step 9: Post-exposure bake. Place wafer on level 65°C hotplate and then ramp up to 95°C for 11 min. Features will begin to appear.
- g)** Step 10: Development. Place wafer in PGMEA bath with shaking and incubate for 10 min or until development is complete.
- h)** Step 12: Hard bake. After development, perform hard bake. The wafer is now finished.

Figure 6. Soft lithography steps: fabrication of microfluidic device

- a)** Step 17: Pour mixed PDMS on silicon wafer master mold in petri dish.
- b)** Step 20: Once cured at 65°C, cut out PDMS chip with razor blade taking care not to cut the SU-8 pattern.
- c)** Step 21: Punch holes for inlets and outlets using 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 surfaces.
- e)** Step 23-24: After plasma treatment, place PDMS chip featured-side down on cover glass and gently press. Then incubate at 65°C for >1 h.
- f)** Step 25: Insert a small piece (~8-10 mm) of “adaptor tubing” into each inlet and outlet.
- g)** Step 25: Seal “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 at 65°C, this provides a seal and prevents leaks.
- h)** Step 26: Close-up of final device. Following incubation at 65°C for >2 h, the final microfluidic device is obtained.

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 20G needle and then insert PE tubing piece into ismaprene tubing. This is the piece that goes into the microfluidic device inlets and should be short (~ 5mm) and have a diagonal rather than blunt end.
- d)** Step 33: Wash microfluidic device using 1 mL syringe with 20G needle and PE tubing attached to needle by gently flushing each channel with 70% ethanol, 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 medium and safely tape everything down.
- g)** Step 34: To start flow from medium, insert syringe with 20G needle into PE tubing end and pull. Then insert PE tubing into the solenoid valve inlets.
- h)** Step 36: Move board, valves and microfluidic device into 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 PE tubing to waste flask the setup should look as shown.
- j)** Step 40: Illustration of setup during time-lapse acquisition.
- k)** Full view of setup. Flow rate is controlled by height difference between inlet medium and waste flask.

Table 1
Troubleshooting table

Step	Problem	Possible reason	Solution
9	Cracks in 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 SU-8 pattern	Insufficient adhesion of SU-8 to wafer	Include additional wafer cleaning step before Step 4. We sometimes perform oxygen plasma cleaning of wafer immediately prior to SU-8 coating (150 mTorr O ₂ at 60 W for 2 min with a Technics Plasma Stripper Model 220).
		SU-8 pattern detaches during PDMS peeling	Ensure that razor blade cut is complete and be gentler whilst 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 PDMS chip and cover glass.	Apply only gently pressure on 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 flow rate by reducing height difference between inlet and outlet flasks. Unless very quick medium switching is required, a much lower flow rate may be fine.
		Defective ConA	Change ConA stock. We sometimes observe dramatic batch-to-batch variation in activity.
	Sudden loss of all cells in field-of-view	Air bubble	Make sure all LFM/medium is pre-heated to 30°C before experiments.
		Air bubble	When washing 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 microfluidic device holder	Pre-warm 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 experiment	Tubing coming loose during experiment	Securely attach PE tubing to microfluidic device. Cutting PE tubing ends sharply and diagonally makes secure insertion much easier. Avoid blunt ends.
	PDMS delamination	Insufficient bonding of PDMS to cover glass	Properly clean PDMS and cover glasses prior to plasma exposure. It may be necessary to optimize oxygen plasma exposure time (both too brief and too long exposure times can cause this).
			When sealing adapter tubing in Step 25, also add mixed PDMS around PDMS chip to seal it to the cover glass.
	Msn2 is nuclear prior to 1-NM-PP1 treatment	Leftover 1-NM-PP1 from previous experiment absorbed into tubing	Do not re-use 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 ismaprene tubing.
		Clogged tubing	Occasionally, we observe nuclear Msn2 if tubing is clogged or improperly positioned. Change tubing and keep 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	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 flow rate.
42	Cell segmentation or tracking errors during image analysis	Segmentation or tracking algorithm not optimal	Optimize 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: ~3-6h (variable).
Step 27: Setting up control valves: 1 h
Steps 28-29: Preparing a time-lapse microscopy experiment (day before): variable.
Steps 30-41: Time-lapse microscopy experiment: <2 h + imaging time.
Step 42: Analysis of time-lapse movies: variable.