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Microfluidics-Assisted Cell Screening (MACS): an automated platform for single-cell microscopy of suspension cultures

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Abstract

Studies that rely on fluorescent imaging of non-adherent cells that are cultured in suspension, such as *Escherichia coli*, are often hampered by trade-offs that have to be made between data-throughput and imaging resolution. We developed a platform for microfluidics-assisted cell screening ('MACS') that overcomes this trade-off by enabling high-throughput and automated single-cell microscopy for a wide range of types and sizes of cells. Since cells can be rapidly sampled directly from a suspension culture, MACS bypasses the need for sample preparation and chemical fixation and therefore allows measurements without perturbing the native cell physiology. The setup can also be integrated with complex growth chambers, and can be used to enrich or sort the imaged cells. Furthermore, MACS facilitates visualization of individual cytoplasmic proteins in *Escherichia coli*, allowing low-abundance proteins to be counted using standard total internal reflection fluorescence (TIRF) microscopy. Finally, MACS can be used to impart mechanical pressure for assessing structural integrity of individual cells and their response to mechanical perturbations, or to make cells take up chemicals that otherwise would not pass through the membrane. This protocol describes the assembly of electronic control circuitry, the construction of liquid handling components, and the creation of silicon masters used to cast the microfluidics chip (4–7 d). We describe in detail how to properly cast, cure and bond the two layers of the MACS microfluidics chip (1–4 d). The operation of MACS is described and automation software is provided to integrate MACS control with image acquisition. Finally, we provide instructions for extending MACS using an external growth chamber (1 d) and how to sort rare cells of interest.

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*These authors contributed equally

TWEET A microfluidics platform for single-cell microscopy of suspension cultures

COVER TEASER Microfluidics platform to image suspension cells

AUTHOR CONTRIBUTIONS

B. O. and C. J. B., J. C. A. C, G. C. L., S. L. and D. L. performed the experiments described in this protocol, C. J. B. provided the control and analysis codes and built the system, S. B. wrote the spot detection algorithm, D. L. wrote the segmentation algorithm, E. L. designed the growth chamber and wrote the optical density measurement code, B. O., C. J. B., J. C. A. C, E. L. and J. P. wrote the paper.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details are available in the online version of the paper.

Keywords

microfluidics; single-molecule; single-cell; non-adherent cells; automated microscopy; cytometry; intracellular delivery; mechanical cell compression

INTRODUCTION

Flow cytometry has been very powerful in cell biology, allowing researchers to conveniently analyze millions of individual cells growing in standard batch culture. However, the throughput comes at the cost of such low sensitivity that many low-abundance fluorescent proteins (FPs) cannot be reliably detected. Flow cytometry also provides only limited spatial information^{1,2} both about protein localization inside cells and the sizes and shapes of the cells themselves. This is especially problematic for microbes where light scattering poorly correlates with size³, making it difficult to interpret data even for high-abundance proteins.

By contrast, microscopy is capable of detecting cellular components across a wider range of abundances while at the same time providing accurate estimates of cell size, morphology, and spatial patterns⁴. However, throughput is often much lower, making it difficult to accurately determine statistical properties, to detect rare cell states, or to condition the properties of interest to other measured properties (e.g. the distribution of protein numbers *given* a certain cell size). The amount of data required for conditional analyses can, in fact, increase exponentially with the number of properties measured. For example, breaking down data into four properties and five classes creates $5^4 = 625$ separate data sets. Some commercial imaging flow cytometers attempt to bridge this technological gap, but they are nowhere close to the image quality and multiplexing capabilities attainable by microscopy. This is partly due to the brief residence times of cells within the imaging field of view of the charged couple device (CCD) and utilization of long working distance air objectives with low magnification and numerical aperture. Just like flow cytometers, imaging flow cytometers are expensive and have high maintenance costs, and are therefore typically only available in staffed facilities. By contrast, microscopes are more generally available within labs and provide superior data quality, but typical sample sizes are smaller by many orders of magnitude.

Microscopy also has other limitations. Cells often remain under the microscope for such long time periods – especially when gathering statistics – that their properties can change, making it difficult to compare the results to batch culture. This problem has been partly addressed by using microfluidic growth chambers⁵ which have been tremendously successful in the last few years, increasing throughput substantially and sometimes achieving a uniformity of growth conditions that is comparable to batch culture⁶. However, most existing devices are very sensitive to the exact dimensions of the cells and can easily induce stress response or aging. Even when they work perfectly, the results can be hard to validate without an orthogonal method to measure the same single-cell properties (e.g. to make sure that rare events are not increased in frequency because of the growth chamber). As opposed to flow cytometry, it is difficult to isolate individual cells post-imaging, requiring highly specialized setups such as laser-capture microdissection. Finally, though

microscopy is generally more sensitive than flow cytometry for detecting low-abundance proteins, the fluorescence signal from many FP-fusions is still far below the cellular auto-fluorescence⁷. For instance, in bacteria, a large fraction of the proteome is present in just a handful copies per cell⁸. These proteins have been very difficult to analyze⁹, and include many key regulatory proteins that contribute greatly to the phenotypic heterogeneity of cells¹⁰. We recently developed a microfluidic platform, termed MACS for Microfluidics-Assisted Cell Screening, to address the limitations of flow cytometry and traditional microscopy¹¹. MACS works by injecting cells through a flow channel and gently compressing the ceiling of the channel. The advantage of this system is that it allows non-adherent cells to be immobilized for high-resolution imaging, while being able to control optimal culturing conditions for longer time periods.

MACS capitalizes on the polydimethylsiloxane (PDMS)-based, microfluidic, on-chip valve developed by Quake and coworkers¹² and consists of two layers: cells are injected through a flow layer, while a control layer that runs above – perpendicular to the flow layer – can be pressurized to collapse the flow channel ceiling on cells. Here we describe a protocol for fabricating and assembling the flow and control layers of the PDMS MACS chips using soft-lithography. In brief, we use UV light (Fig. 1a) and patterned transparency masks (Fig. 1b) to etch desired features into a layer of photo resist deposited onto clean silicon wafers to create negative master molds for each layer. We then cast the actual PDMS on each master and align them. To inject cells or collapse the control layer, we use liquids pushed out from airtight pressure tubes (PT). Introducing pressurized air into the pressure tube drives out liquid, either into the flow layer or to pressurize the control layer to push the valve downwards. The pressure supplied is regulated by two independent digital pressure regulators (P_{flow} and P_{control}), which serve as input to air solenoid valves (AV_{flow} and AV_{control}) that can switch the air flow on or off (Fig 2a). MACS is thus based on imaging cells that pass through flow channels while regulating air flow and pressure to partially or completely collapse the flow channel ceiling. Driving flow with air pressure rather than syringe pumps allows for easy streamlining and fast response times. It is possible to incorporate a growth chamber upstream of MACS, allowing for on demand microscopy coupled to real time monitoring of population density, e.g. using batch cultures or turbidostats. This is particularly convenient when studying population dynamics of multiple cell types that change in density over time.

Advantages and Limitations of MACS

The MACS setup improves the statistical power and automation capabilities of microscopy while retaining or even increasing the sensitivity of imaging of non-adherent cells. It works for a wide range of cell sizes and shapes without modifying the device dimensions, while keeping cells in the chosen growth medium with minimal perturbations until the moment of imaging. MACS is compatible with the 96-well plate format and makes it possible to capture exceedingly rare cells in a population. For *E. coli*, we have further shown that MACS allows us to exert controlled pressure to slightly flatten and widen cells, presumably by expelling some water. The diffusion of cytoplasmic proteins then slows down by orders of magnitude – enough to ensure that FPs do not move away from a diffraction-limited spot during typical exposure times in fluorescence imaging^{7,11}. The FPs thus effectively behave as if they were

temporarily fixed. As opposed to chemical fixation, this procedure does not quench the fluorescence signal, and makes it possible to accurately identify single FPs in living cells. We previously quantified this effect by applying our method to single FPs as well as FP-FP fusions, by counting photobleaching steps¹¹, and by comparing the results to other methods¹⁰, showing that the approach makes it possible to accurately count the number of fluorescent proteins per cell even when cells contain just one or two protein copies¹⁰. The flattening of cells in the z-dimension and widening in the x-y plane also helps with counting in three ways: (1) by reducing the projected autofluorescence per area unit and thereby increasing the signal-to-noise ratio for FP detection, (2) by further separating the proteins from each other to reduce the risk of spatial overlap, and (3) by making it possible to keep the whole cell in focus. Increasing the pressure further compresses cells enough to make them take up compounds that otherwise could not cross the membrane, or to evaluate cell membrane or cell wall mutants.

The simple design of the MACS chips, their straightforward operating principle, and the utilization of well-characterized valves render MACS robust and reproducible. The valves themselves can be actuated millions of times without signs of fatigue¹², and the major limitation for long-term stability of MACS is instead the eventual accumulation of debris at the intersection of the control channel and the flow channel. We therefore carried out optimizations for cell loading into the chip, surface passivation, and media preparation to minimize debris, designed cleaning protocols, and fabricated chips with an array of individual control/flow channel intersections to be able to switch to new intersections after debris builds up.

Perhaps the greatest limitation of MACS is that unlike other microfluidic platforms, such as the mother-machine⁶, MACS cannot track lineages of cells as they grow and divide. However, this comes with the advantage that the dimensions do not need to be optimized for cell retention and growth, and that the device therefore does not need to be specifically tailored to a given cell type. MACS also requires cells to be non-adherent, though problems with sticking to glass could potentially be solved by further treating the surface or sample. MACS could also cause problems with cells that are extremely sensitive to mechanical perturbations although with the samples we have tested this has not been an issue.

The MACS platform is cheap to build and compatible with a wide range of cell types and microscopes. Other bacterial strains such as *Bacillus subtilis* and *Mycobacterium smegmatis*, different yeast species such as *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, as well as red blood cells in whole blood samples can in principle also be imaged on MACS without any modifications to the device. However, the original setup was involved and required extensive tailoring. Here we present a more streamlined and standardized platform, including detailed steps for building and using the setup as well as the code for data acquisition and analysis. Although the experiments described here are carried out on *E. coli* cells, the setup works for a wide range of non-adherent cell types¹¹.

Applications

High-throughput imaging—As a proof-of-concept for the stability and throughput capabilities of MACS, we acquired unattended snapshots of approximately 10^6 *E. coli* cells in a 4-hour time window at a single valve intersection. Conventional automated xy-stages can achieve a comparable performance in terms of sampling speed, but cells are then kept on the surface for long time periods before imaging, which can change their properties. MACS, on the other hand, maintains cells in the desired medium and brings in a fresh sample every time.

We typically achieve a throughput of 500-5,000 cells/min, depending on parameters such as magnification, trapping area, cell density etc. We typically use 250 $\times$ magnification (100 $\times$ combined with 2.5 $\times$) for single-molecule counting, and 60-100 $\times$ for high-throughput data acquisition. For larger cells, lower magnifications can be used to retain high throughput. Rather than carrying out distinct cycles of cell trapping and imaging, MACS can also run constantly in the half-open valve state to flow a stream of cells through the field of view (Fig. 5a). This sacrifices image quality somewhat but can be used to detect rare phenotypes, for example where the readout is fluorescence levels above some threshold, or the presence of a localized signal.

The throughput of MACS is limited by the time between successive rounds of cell trapping and on a longer time scale, by the eventual accumulation of debris in the field of view. Debris accumulation is only problematic during actuation of the valve and the presence of sample flow. Debris does not get stuck permanently and is eventually washed away unless pressed against. If the intersection used does get filled with debris, one of the many unused intersections on the same chip can be used for imaging, since intersections not actively in use, do not collect debris. The time between rounds of cell trapping is typically dominated by the time it takes cells to stop moving when the channel is in the closed state (t_{closed}). The cells become nearly stationary on the glass in as little as one second but can exhibit small movements up to ~10-15 sec after the chip enters the closed state. Thus, for single-molecule counting experiments we often wait 15 sec ($t_{\text{closed}} = 15$ sec), but we only wait 1-5 sec when exact localization of single molecules is not required. The optimal value for t_{closed} may also display chip-to-chip variability and depends on factors such as cell density and cell type.

If the field of view of the camera is small compared to the total cell trapping area, a substantial speed-up can be achieved by using an automated stage and moving the FOV to cover the entire trapping area. When even greater throughput is needed, it is also possible to modify the dimensions of the chip to fabricate larger valves and thus larger cell trapping areas. In principle, it could also be possible to run and image several intersections simultaneously to increase throughput further.

For a given cell density in the medium, we can also adjust the coverage within the field of view by simply changing parameters such as P_{flow} and P_{control} as well as the duration of the half-open state. Modifying cell density is particularly important for the single-molecule counting assay, because it is hard to exert enough pressure on each individual cell to slow down diffusion in areas with very high local density of cells.

Reducing the cell density can also be important, e.g. when carefully quantifying fluorescent levels between cells, since the point-spread function of light can cause light emitted from one cell to be attributed to its neighbor cells. Cells in areas of high cell density can then artificially appear brighter. On the other extreme of low cellular density (e.g. the balanced growth in bacteria), MACS can function as a concentration device since the half-open state can function locally as a ‘sieve’ and permits gathering statistics that would not be possible via traditional sample preparations (such as agar pads), which simply spread cells on cover glass surfaces.

Retrieving cells with rare phenotypes—The setup can also be used to screen large numbers of cells, identify rare cells of interest for higher-quality imaging, and then retrieve the cells from the field of view including the cell of interest into test tubes. This process enriches the ratio of cells with a rare phenotype with respect to the population. For example, it is possible to run MACS in the highest throughput mode, where cells are constantly moving through the device and imaged in video mode as they trickle by (Fig. 5a), and use real-time image analysis to identify cells of interest. By instantaneously switching to the closed state, those cells can be trapped for high-quality imaging. To illustrate this capability, we first took a cell culture of an *E. coli* strain expressing GFP, and spiked it with a few cells from an RFP expressing strain. On-the-fly image processing allowed for detection of the cell of interest (i.e., a red cell) as it appeared within the field of view (Fig. 5b). Although the detection rates depend on multiple parameters such as flow rate, cell density, and the closing properties of the valve, in our trial runs, cells diluted 1:10,000 and 1:100,000 were detected within, on average, ~3 seconds and ~30 seconds, respectively. This allows us to estimate the occurrence probability of rare phenotypes of interest, and then characterize those phenotypes with more detailed imaging.

Minor modifications to the design make it possible to retrieve the trapped cells from the device. This allows us to enrich or even directly isolate rare phenotypes (Fig. 5c). In this modified design, we control two inlets and outlets via on-chip valves (valves 1–4). During screening, the MACS device is in the half-open state and cells are imaged as they flow past the field of view to the waste (valves 3 and 4 are closed, and valves 1 and 2 are open). When a rare cell-of-interest is detected (Fig. 5b), cell flow is stopped immediately, thus switching the MACS device to the closed state. At this point, detailed images can be acquired, if needed. Next, we close all inlet and outlet valves (valves 1–4), and release the control valve (Fig. 5d). We then open valves 3 and 4 allowing oil to flow into the chip and sending the trapped volume out for collection. After growing the retrieved cells overnight and imaging them on agar pads, we observed that the RFP-expressing cells were highly enriched (Fig. 5e). Using an oil phase for cell collection provides precise control of the volume that is retrieved and serves to separate the successive collections. To facilitate the collection using the oil phase, we treated the chips with a commercial water repellent¹⁴ (Aquapel) after plasma bonding. We kept these chips at room temperature until use.

High pressure MACS for single-molecule imaging and intracellular delivery—Perhaps the most striking feature of this device is that it allows us to apply pressure to *E. coli* cells in a controlled way, such that the diffusion of cytoplasmic proteins can be slowed down

by two orders of magnitude¹¹. We believe this reflects the interesting finding that the *E. coli* cytoplasm is close to a glass transition¹⁵, where slightly compressing cells expels some of the water from the cytoplasm and appears to make the movement of cytoplasmic proteins transition from almost free diffusion to crowding.

Regardless of the underlying mechanism we have found that the slowing down of diffusion allows for visualization and even accurate counting of fluorescent proteins at exceptionally low abundances, using conventional TIRF microscopy setups^{10,11}. As opposed to chemical fixation protocols, this mechanical fixation can be achieved with no loss of fluorescence, and extensive controls with photobleaching steps and tandem dimer FPs¹¹ suggest that we do detect close to all mature fluorescent molecules.

The increased pressure also flattens cells, which improves counting in several ways. First it ensures that the autofluorescence is lower per projected area of the imaged cell, thereby reducing the background for counting. Second it can ensure that more of the cell is within the imaging focal plane. In fact, for *E. coli* growing in rich media, imaging setups typically keep most of the cell in focus, but not entirely. Mild flattening is then enough to achieve focus throughout the cytoplasm. Third, by ‘pancaking’ cells slightly the projected area is greater, which reduces the probability that individual molecules will have overlapping point-spread functions and therefore cannot be separated.

Practically, we find that the increase in effective pressure is easiest to achieve by using stiffer PDMS chips. Specifically, we recommend keeping the chips at 65 °C for three days after plasma bonding to coverslips to achieve enough stiffening to induce cytoplasmic slowing down. Unless the pressure is greatly increased, which can cause other problems, pressing the chips without such ‘aged’ chips does not cause sufficient slowing down to visualize proteins within the typical 10-100 msec integration times of the CCD camera. For single-molecule imaging it also becomes critical to properly clean the glass surfaces, or else fluorescent background spots can masquerade as actual fluorescent proteins. We recommend a cleaning protocol described by Elf *et al.* to clean glass coverslips¹⁶. Alternatively, glass coverslips can be kept under oxygen plasma for 10 min. It is also essential to wear gloves throughout since touching the involved surfaces with bare hands can cause substantial fluorescent impurities. The growth media can also introduce fluorescent particulates. For *E. coli*, we found that the standard LB broth, which is highly fluorescent, can be substituted for M9 media supplemented with 10% LB without compromising the growth rate but greatly reducing false positive counts due to fluorescent impurities. The MOPS-based rich defined medium EZRDM (Teknova) works even better for this purpose. By paying close attention to cleaning procedures at different stages, background fluorescence drops to as few as 0.3 spots/cell for a control strain that does not express any fluorescent proteins¹⁰.

Forcing the uptake of small molecules—Another feature of MACS is the observation that compressing *E. coli* cells in the closed state forces them to uptake molecules. Specifically, when we used extra stiff chips, made by plasma bonding two 10:1 PDMS layers, we could press harder on cells causing them to uptake a DNA staining dye that is normally cell impermeable (propidium iodide, Thermo Fisher) (Fig. 6 and Supplementary

Movie 1). This procedure also allowed us to distinguish wild type cells and cell wall mutants, since the latter uptake more dye at lower pressures than the wildtype.

Experimental design

In this protocol, we describe the procedures of building and using the MACS platform, focusing on the following aspects: (i) design and fabrication, (ii) operating principles, (iii) typical applications, and (iv) integration of upstream growth chambers. In the paragraphs below, we provide detailed descriptions of the different stages and we provide recommendations on how to adapt the procedures for specific experimental needs.

Microfluidic device design and fabrication—We use AutoCAD to design high-resolution transparency masks (Output city) for the pattern in the flow and control layers. We recommend maximum resolution for printing this mask, using the emulsion-side down (i.e. features are printed on the non-glossy side), to ensure minimal distances between the printed side of the mask and the PR on the wafer. A detailed, step-by-step protocol is described in the PROCEDURE section (Steps).

The silicon wafers are cleaned by sequentially squirting generous amounts of acetone, methanol and Milli-Q water (Millipore Corporation) while spinning the wafers on a spin-coater. The wafers are dried for 5 min on a 65 °C hotplate before using the spin-coater to deposit positive PR AZ10xt (AZ Electronic Materials) to a height of 10 µm on top of the wafer. The transparency mask is illuminated with UV light to etch out the desired pattern in the PR (Fig. 1a), and the wafer is subsequently heated to round off the rectangular channels into dome-shapes (aka reflow) with a final channel height of ~8.5 µm, which is essential for proper valve closure¹². We then bake the wafer on a hotplate overnight to stabilize the positive PR. We similarly build a master mold for the simpler control layer of the device, by spin-coating the negative PR SU-8 2025 (MicroChem) to a 25 µm thickness on a wafer, and UV patterning it using the transparency mask for the control channels. These steps produce the silicon masters from which the PDMS chips are molded (Fig. 1a).

PDMS is mixed from a two-part silicone elastomer kit (Sylgard 184, Dow Corning), consisting of a base (part A) and curing agent (part B) in particular weight ratios (part A:part B). To produce the MACS chips from the masters, we spin-coat 20:1 PDMS on the flow channel master at 1250 rpm for 45 sec to yield a ~65 µm thick PDMS membrane. With this thickness and PDMS ratio, the minimum pressure required to close the valve is ~5 psi. If even gentler cell handling is required, a thinner membrane can be made to allow the valve to close at lower pressures. For the control channels, we pour 5:1 PDMS onto the control layer master. After both layers are partially cured at 65 °C for 33 min, we peel the PDMS off the control layer master, punch holes through the inlets and align the control layer slabs to the flow layer. We then cure both layers for another 4 h at 65 °C to achieve thermal bonding, and cut out the now two-layer chips from the flow-layer wafer using a new razor blade. We punch holes in the two-layer chips and sonicate them in isopropanol for 30 min to remove debris (Fig. 1c) produced during hole punching. We then rinse the chips with Milli-Q water and sonicate in Milli-Q water for 30 min before leaving the chips to dry for 4 hours at 65 °C. Finally, we plasma bond the two-layer PDMS chips permanently to glass coverslips. Freshly

bonded chips are kept at room temperature for at least one day to regain the native surface properties following plasma treatment.

Basic variations to the protocol can be made depending on the requirements of a given application. For instance, for specific applications, such as maximizing throughput, it may be necessary to alter the device dimensions, in which case, other parameters (such as pressure or PDMS membrane thickness or stiffness) also need to change to maintain optimal performance¹³. For the stiffer MACS chips used for mechanical slowing down of diffusion of cytoplasmic molecules, we leave the chips at 65 °C for 3 days after plasma bonding to cover glass. Alternatively, the stiffness of the PDMS membrane can be increased by using an A:B ratio greater than 20:1. In some applications, e.g. when assessing mechanical integrity of cell wall mutants or squeezing cells to force them to uptake material, even higher pressure is required. For such devices, we produce both the flow and the control channels with 10:1 or even 5:1 PDMS, and bond them together using oxygen plasma, resulting in an even stiffer chip.

Operating principle of MACS and automation—When operating MACS, the simplest scheme of flowing cells past the field of view (open state) then simply stopping the flow and collapsing the PDMS valve (closed state) yields extremely poor trapping efficiency due to the rapid displacement of liquid. Therefore, we introduce a third state that we term half-open, in which we set $(AV_{\text{flow}}, AV_{\text{control}}) = (\text{on}, \text{on})$ and then tune $(P_{\text{flow}}, P_{\text{control}})$ such that cells trickle between the cover glass and PDMS valve as a monolayer, accumulating in the field of view. After tuning the magnitude of $(P_{\text{flow}}, P_{\text{control}})$ to achieve the half-open state, MACS introduces new cells in the open state by setting $(AV_{\text{flow}}, AV_{\text{control}}) = (\text{on}, \text{off})$, accumulating cells in the field of view during the half-open state by setting $(AV_{\text{flow}}, AV_{\text{control}}) = (\text{on}, \text{on})$ and trapping cells in the FOV in the closed state by setting $(AV_{\text{flow}}, AV_{\text{control}}) = (\text{off}, \text{on})$ at which point the cells are imaged (Fig 2b). While MACS is in the closed state, the value of P_{control} can be temporarily re-adjusted prior to imaging if needed for the experiment, for example in single molecule imaging P_{control} is often increased (Fig 2c). This process of serially visiting the open, half-open and closed state then imaging, is repeated until the required number of images have been obtained. Each cycle typically takes ~2–15 s corresponding to ~240–1800 frames per hour.

Compressing cells in this controlled manner can slow down the diffusion of cytoplasmic fluorescent proteins (FPs) almost 100-fold in *E. coli* cells, allowing us to detect them on a standard total internal reflection fluorescence (TIRF) microscopy setup (see ANTICIPATED RESULTS) with minimal perturbations until the moment of imaging, as we extensively characterized elsewhere¹¹. Although this can in principle be achieved by simply using higher pressure (as in Fig. 2c), we achieved better performance by using stiffer PDMS chips rather than higher values of P_{control} , since using higher pressure increases the risk of delaminating the microfluidic chip.

Debris accumulation can dramatically limit the throughput of MACS. To minimize debris, we fabricated in-line filters on the chips, and filtered all buffers and media using 0.22 μm pore size filters (Corning). For experiments that do not require the integration of growth chambers (see separate section below), we grow cells in plastic tubes (BD Falcon, round-

bottom) instead of glass vials to prevent contaminating the sample with small glass particles, and sonicate the PDMS chips in isopropanol as described above. In addition, after setting up all connections and just prior to using the chips, we run a cleaning routine where all flow interfaces are extensively rinsed to wash away any PDMS debris stuck on the walls of the chip, as well as particulates that may form when making connections between plastic tubing and blunt end needles (Box 1). These procedures allow us to keep the same chip on the microscope and use it for multiple days.

Having a cleaning routine is essential, not only to remove debris but also to prevent carryover between successive samples. Thus the full MACS setup is a modification of the simple design in Fig. 2a, that allows for automated rinsing of all the flow paths using 10% bleach, 10% ethanol and buffer (or Milli-Q water) (Fig. 3). This full MACS setup can be broken down into three modules: the Electronic Control Base, the Cleaning Reservoir Stand, and the Pinch Valve Base. We provide designs to laser-cut acrylic bases to layout the necessary components for each module. The electronic control base contains all the electronic control circuitry as well as the digital regulators and air solenoid valves. The air solenoid valves are mounted on manifolds that share a common pressure inlet but can be independently operated to direct the air flow to a specific air-tight container. In comparison to the simple scheme from Fig. 2a, air solenoid valves have been added to direct air flow to the bottles holding the cleaning fluids on the Cleaning Reservoir Stand, as well as a valve that is used for the optional integrated growth chamber. The Pinch Valve base, which normally sits on the microscope stage, contains the two pressure tubes used to run the MACS device (Fig. 2a) and various pinch valves that direct the flow of liquids. The only modification needed to run the standard MACS protocol depicted in Fig. 2a, is to close all the pinch valves except $PV_{to\ chip}$ (Fig. 4). Furthermore, when the experiment is finished the entire sample can be disposed of by opening AV_{flow} and $PV_{to\ waste}$. At which point the whole system can be cleaned by opening $PV_{to\ PT}$, PV_{bleach} and AV_{bleach} to flow bleach, or similarly ethanol or water. Note that pinch valves provide a distinct advantage over other valve designs in that all the liquids, including the cultures, only ever contact tubing which can be easily replaced if needed.

The cleaning protocol consists of sequentially rinsing the system with 10% bleach, 10% ethanol, and buffer (or Milli-Q water) in two stages (Box 1). In the first stage of the cleaning, we fill the pressure tube with 10% bleach (open PV_{bleach} , $PV_{to\ PT}$ and AV_{bleach}) (Fig. 3), wait for 5 sec, empty the pressure tube and repeat. This gets rid of any sample that may be stuck on the walls of the pressure tube. In the second stage of cleaning, we again fill the pressure tube with 10% bleach, send a small amount of the 10% bleach to waste (open $PV_{to\ waste}$ and AV_{flow}), then flow bleach through the MACS chip for ~1.5 mins (open $PV_{to\ chip}$). We then repeat the same two-stage cleaning protocol using 10% ethanol followed by Milli-Q water or buffer. As a demonstration that this cleaning routine is effective, we used MACS to image *E. coli* expressing CFP, ran the cleaning protocol and then imaged *E. coli* expressing RFP. Not a single CFP-expressing cell was detected among ~10,000 RFP-expressing cells imaged, suggesting that the cleaning routine effectively prevents carryover between successive runs of MACS.

Integration of growth chambers—A schematic of a growth chamber incorporated upstream of MACS is shown in Fig. 7. We suggest paying attention to the aeration needs of the growing culture. This configuration does not necessarily allow for optimal aeration without other modifications, since the rigorous stirring or shaking required for optimal aeration can interfere with OD₆₀₀ measurements. For experiments that require active aeration or shaking, the OD₆₀₀ measurements can be separated from the growth flask. A detailed description of this setup is in preparation. With further modifications, it is possible to implement treatment of the samples with drugs or inducers without affecting the rest of the growing cell culture. The growth chamber can be modified to run in specialized modes, such as turbidostat or chemostat, by using peristaltic or solenoid pumps.

We believe this single, simple device serves many purposes – increasing throughput compared to conventional microscopy, ensuring that cells can be grown in the desired liquid culture until moments before imaging in 96 well plates, if desired, retrieving rare cells of interest, pressing on cells to achieve accurate mechanical fixation to count individual molecules, and squeezing cells to force them to uptake chemicals that otherwise could not pass through the membrane. Though MACS does not have the advantage of tracking cell lineages that some other microfluidic devices provide, it also does not need to be precisely tailored to the exact cell sizes and shapes of a given sample, as is required to use those devices. In fact, the same device can simultaneously be used for cells that vary >100-fold in volume. It can also serve as an orthogonal test that growth chamber methods truly reflect cell growth in suspension cultures.

MATERIALS

REAGENTS

- M9 minimal salts, 5× (Sigma-Aldrich, cat. no. M6030)
- LB broth (Sigma-Aldrich, cat. no. L3022)
- Cells of interest. We used genetically modified *E. coli* strains (MG1655 and MC4100 wild-type laboratory strain backgrounds) BO37 (MG1655 glmS::PRNAI-mCherry1-11-mKate-T1 terminator-FRT Kan FRT::pstS), BO41 (MG1655 glmS::PRNAI-GFPmut2-T1 terminatorFRT Kan FRT::pstS), and BO56 (MC4100 galK::Plac-CFP-Amp sprE-2× mNeonGreen-AP tag-FRT Kan FRT). Bacterial strains are kept at –80C as glycerol stocks. For recommendations on using other cells of interest, see Introduction. **! CAUTION** All cells should be handled by strictly following the biosafety rules. Special permissions may be required when experimenting with pathogenic bacteria.
- Bleach
- Ethanol

Making masters

- Silicon wafer (University Wafer, cat. no. ID695)
- Acetone

- Methanol
- Isopropyl alcohol
- MCC primer 80/20 (MicroChem)
- AZ 10XT, positive photoresist (AZ Electronic Materials)
- AZ 400K developer (MicroChemicals)
- SU-8 2025, negative photoresist (MicroChem)
- SU-8 2025 developer (MicroChem, cat. no. Y020100)

Making chips

- Sylgard 184 silicone elastomer kit, two-part (PDMS and curing agent) (Dow Corning)
- 1M KOH
- Sterile water

EQUIPMENT

Making the masters

- AutoCAD files (Supplementary Data file 1)
- Mask (CAD/Art Services, Inc; www.outputcity.com)
- Spin coater (Headway Research, Inc, cat. no. pwm32)
- Mask aligner (Karl Suss, cat. no. MJB3)
- Hot plate

Making the chips

- Plasma cleaner (Technics 500-II)
- Drying oven
- Dessicator
- High-Density polyethylene staining jar (Electron Microscopy Sciences, cat. no. 70319)
- Sonicator
- Fisherbrand cover glasses, 0.16 - 0.19 mm (Fisher Scientific, cat. no. 12-544D)
- Single edge razor blade box (Pack of 100) (American Safety Razor, cat. no. 66-0089)
- Aluminum foil
- Cotton swabs
- Reusable biopsy punch 0.75mm diameter (Produstrial, cat. no. 135841)

- Scotch magic tape (Scotch, cat. no. 3105)

Setting up the chips

- 2-mL Screw-Cap Microcentrifuge Tubes, Nonsterile (VWR, cat. no. 89004-300)
- Universal fit screwcaps with O-rings, Assorted, Nonsterile (VWR, cat. no. 16466-084)
- 21-gauge blunt end needles, 1" length (McMaster-Carr, cat. no. 75165A681)
- 0.75-mm diamond drill bit - 10 Pack (Lasco, Amazon)
- Dremel 483 1/32" chuck collet (Dremel, Amazon)
- 25-mL general purpose 5-min epoxy (ITW Devcon, cat. no. 14250)
- Micro soft-wire cutter (Hakko, cat. no. CHP-170)
- VWR lab tape (VWR, cat. no. 89097-990)
- Pliers (Amazon)
- Tygon microbore medical tubing (Tygon, cat. no. AAD04103)

DAC board and Electronic Control

- Arduino Uno rev. 3 (Adafruit, cat. no. 50)
- Digital to analog converter, i2c (Adafruit, cat. no. 935)

MOSFET board

- Custom fabricated printed circuit board (Seeed studio) (Supplementary Data file xx)
- DC barrel jack 2 × 5.5 mm (Digikey, cat. no. CP-002AH-ND)
- Screw terminal block 12 pos, 3.5 mm (Digikey, cat. no. 277-1729-ND)
- Axial resistor, 1 kΩ 1/4W (Digikey, cat. no. 1.0KQBK-ND)
- Screw terminal block 3 pos, 5.0 mm (Digikey, cat. no. 277-1578-ND)
- General purpose diode, 1 kV 1A (Digikey, cat. no. 1N4007FSCT-ND)
- 5 mm red LEDs (Digikey, cat. no. 754-1274-ND)
- N-channel MOSFET, TO-251AA package (Digikey, cat. no. RFD3055LE-ND)

Power and connectors

- 24-volt power supply (McMaster-Carr, cat. no. 70235K99)
- Engineer Inc PA-20 precise universal wire terminal crimping tool (Amazon)
- DC barrel switch (Adafruit, cat. no. 1125)
- 22 AWG stranded wire - black (McMaster-Carr, cat. no., 8054T13)

- 22 AWG stranded wire - red (McMaster-Carr, cat. no. 8054T13)
- 20-24 AWG female pin crimp (Digikey, cat. no. A32523-nd)
- 20-24 AWG male pin crimp (Digikey, cat. no. A33280-nd)
- Female 2-position TE Amp Micro MATE-N-LOK receptacle (Digikey, cat. no. A30269-ND)
- Male 2-position TE Amp Micro MATE-N-LOK receptacle (Digikey, cat. no. A30293-ND)

Air pressure fittings and control

- Digital pressure regulators (SMC Corporation, cat. no. ITV0030-2UBL)
- Air solenoid valves, normally closed 24 volts (SMC Corporation, cat. no. s070b-5dc)
- Solenoid blanking plate (SMC Corporation, cat. no. SS070B-10A)
- 4-port solenoid base (SMC Corporation, cat. no. SS073B01-04C)
- Male Luer lock to 3/32" barb adapter (Nordson Medical cat. no. MTLL007-9)
- T junction push-to-connect fitting, for 5/32" OD tubing (SMC Corporation, cat. no. KQ2T03-00)
- 4 mm polyurethane tubing (SMC Corporation, cat. no. TU0425B-33)
- M3 to 4 mm barb adapter (SMC Corporation, cat. no. M-3AU-4)
- 1/4-28 UNF to 5/32 push-to-connect adapter (Hydradyne, cat. no. 68PLP-5/32-4LT)
- 4 mm OD, 2.5 mm id polyurethane tubing (SMC Corporation, cat. no. TU0425B)
- M5 to 4 mm push-to-connect adapter (SMC Corporation, cat. no. KQ2H04-M5)

Liquid Handling

- GL45 bottle cap with two 1/4-28 threaded holes (VICI-Jour, cat. no. Jr-s-11001)
- Normally closed pinch valve (Clippard, cat. no. NPV1-1C-01-24)
- Normally open pinch valve (Clippard, cat. no. NPV1-1O-01-24)
- Y junction barbed fitting, for 1/16" ID tubing (Nordson Medical, cat. no. Y210-1)
- Pyrex 100 ml media bottles with GL45 cap (VWR, cat. no. 16157-103)
- Pyrex 250 ml media bottle with GL45 cap (VWR, cat. no. 16157-136)
- 1/32" ID Silicone tubing (McMaster-Carr, cat. no. 9628T54)

Fasteners

- Steel screw #2-56 $\times$ 3/8" (McMaster-Carr, cat. no. 90272A079)
- Steel screw #4-40 $\times$ 3/8" (McMaster-Carr, cat. no. 90272A108)
- Steel screw #4-40 $\times$ 1/2" (McMaster-Carr, cat. no. 90272A110)
- Steel screw #4-40 $\times$ 5/8" (McMaster-Carr, cat. no. 90272A112)
- Steel narrow hex nut #4-40 (McMaster-Carr, cat. no. 90760A005)
- Steel female threaded standoff #4-40 $\times$ 1" L $\times$ 1/4" OD (McMaster-Carr, cat. no. 91125A439)
- Steel female threaded standoff #4-40 $\times$ 1-1/2" L $\times$ 1/4" OD (McMaster-Carr, cat. no. 91125A471)
- Nylon female threaded standoff #4-40 $\times$ 3/8" L $\times$ 1/4" OD (McMaster-Carr, cat. no. 96110A008)
- Nylon screw #4-40 $\times$ 3/8" (McMaster-Carr, cat. no. 93135A275)
- Nylon screw # 4-40 $\times$ 3/16" (McMaster-Carr, cat. no. 93135A271)

Setting up the Growth Chamber

- Flat-bottom glass vial, 40 ml (Chemglass, cat. no. CG-4902-08)
- Cap, White, solid top, 24-400, with 0.125" PP/PTFE/Silicone SURE-LINK™ Septa (Chemglass, cat. no. CG-4911-21)
- PEEK tubing (Thermo Scientific, cat. no. 03-050-412)
- Custom laser-cut acrylic vial holder
- LED holders (<http://www.tme.eu/>, cat. no. RTM-5020-B)
- Steel screw #4-40 $\times$ 1-1/2" L (McMaster-Carr, cat. no. 92196A032)
- Steel screw #4-40 $\times$ 1-1/4" L (McMaster-Carr, cat. no. 92196A032)
- Acrylic solvent cement (Scigrip, cat. no. 10308)
- Nylon washer 0.112" ID $\times$ 0.206" OD (McMaster-Carr, cat. no. 90295A340)
- Nylon unthreaded spacers 1-3/16" L $\times$ 3/16" OD (McMaster-Carr, cat. no. 94639A733)
- Nylon unthreaded spacers 1/8" L $\times$ 3/16" OD (McMaster-Carr, cat. no. 94639A702)
- Nylon unthreaded spacers 3/16" L $\times$ 3/16" OD (McMaster-Carr, cat. no. 94639A704)
- Nylon unthreaded spacers 1/4" L $\times$ 3/16" OD (McMaster-Carr, cat. no. 94639A706)
- Magnetic disc (McMaster-Carr, cat. no. 57005K84)

- 7 mm × 2mm Magnetic PTFE-coated stir bars (Big Science Inc, cat. no. SBM-0702-CMB)
- 40 mm 12-volt computer fan (Scythe, cat. no. SY124020L)
- 1-1/2" wide double sided poster tape (3M brand, cat. no. 4032)
- Photodiode 900 nm 3 mm w/filter (Digikey, cat. no. 475-1079-ND)
- Emitter IR 860 nm 100 mA radial (Digikey, cat. no. 475-1200-ND)
- 47 ohm 1/4W Resistor (Digikey, cat. no. S47CACT-ND)
- 1.5 K ohm 1/4W Resistor (Digikey, cat. no. 1.50KXBK-ND)
- Adafruit Perma-Proto quarter-sized breadboard (Adafruit, cat. no. 589)
- Colored 22-gauge stranded wire ([Amazon.com](https://www.amazon.com))
- Soldering tools
- Electrical tape and/or heat shrink insulation
- Spectrophotometer and cuvettes
- TIRF microscope

Software

- Matlab (...)
- AutoCAD(...)
- Arduino IDE (...), see Equipment Setup
- Plugins
- Scripts, see Supplementary Data files
- ...

REAGENT SETUP

- Growth media:
- 10% (vol/vol) Bleach
- 10 % (vol/vol) Ethanol

EQUIPMENT SETUP

Installing the software

- Download the Arduino IDE from arduino.cc and install.
- Download the 12-bit I2C DAC Arduino repository and save in your Arduino libraries folder. https://github.com/adafruit/Adafruit_MCP4725
- Download the modified Micro-Manager Arduino firmware, MacsController.ino, open with the Arduino IDE and upload the sketch to your Arduino.

- Download the MacsGUI.jar Micro-Manager plugin and move it to the mmplugins directory in your Micro-Manager install directory.
- Open Micro-Manager and follow the directions on the Micro-Manager wiki to install the Arduino using the Hardware configuration wizard but ignore the steps involving installation of the firmware. Be sure to install all peripheral devices, i.e. Arduino-Switch, Arduino-Shutter, Arduino-Input and Arduino-Dac{1,2}. Save the configuration.
- Start the MACS Controller software by clicking MACS Controller in the Micro-Manager Plugins menu.

▲ **CRITICAL** When an operation initiated with the MACS Controller software is finished it will display the status: Finished/Ready. If another button is pressed before that message is displayed, the previous command will be cancelled and the new command will start.

▲ **CRITICAL** At any time, all the pressure regulators can be set to zero by clicking Pressure Off and operations initiated with the MACS Controller software can be stopped by pressing Stop.

Notes for Advanced Users:

- The MACS setup state and pressures can be manually set using the Direct Control tab.
- Different default settings and hidden parameters can be defined by creating a file called macs_config.properties and pasting it in the mmplugins directory. All possible parameters with their default values are defined in the all_macs_config.properties file. Simply paste the parameters you wish to modify into the macs_config.properties file and update their values in that file.
- For users with programming experience, we provide the source code which can be modified to define custom procedures and hardware interfaces (Supplementary Data file xx).

PROCEDURES

Ordering the masks ● TIMING ~1-3 d

- 1| Download the provided AutoCAD files (Supplementary Data file 1) for mask designs and submit them to a printing service to obtain transparency masks for the control and the flow layers. We order our masks at but suppliers like ... and ... are good alternatives. Typically the commercial vendor will take several days to send the transparency masks.

Cleaning the silicon wafers ● TIMING ~30 min

- 2| Setup the spin coater to run two consecutive stages. Configure stage one for spreading with angular frequency: 500 rpm, ramp: 100 rpm/s, time: 15 s. Configure stage two for spin-coating with angular frequency: 3000 rpm, ramp: 500 rpm/s, time: 45 s. These are the default settings unless stated otherwise.

- 3| Place a silicon wafer on the spin coater with the reflective side up. To clean the wafer, run the spin coater and during the second step generously squirt acetone onto the wafer. Repeat this process with methanol and then isopropyl alcohol.

! CAUTION Methanol is highly toxic and can cause blindness or death. Wear appropriate laboratory clothing when handling it and use it in a fume hood.

- 4| Place the clean silicon wafer with the shiny side up on a hotplate at 95 °C for 5 minutes.

Master for the control layer ● TIMING ~1 h

- 5| Place a cleaned silicon wafer on the spin coater.
- 6| Pour enough SU-8 2025 over the silicon wafer to cover half the surface.

! CAUTION Photoresists are highly toxic. Wear appropriate laboratory clothing when handling it and use it in a fume hood.

- 7| Run the spin coater according to the configuration in Step 2.
- 8| Pre-exposure bake: place the SU-8 coated silicon wafer with the SU-8 side up on a hotplate at 65 °C for 1 minute, then transfer the wafer to another hotplate at 95 °C for 5 minutes.
- 9| Cool the wafer to room temperature (xx-yy ° C).
- 10| Align the control layer mask to the coated silicon wafer using the mask aligner.
- 11| Expose the wafer on the mask aligner to a total dose of 150-160 mJ/cm² xx nm UV light (For example: expose the wafer for 6 seconds using a 25 mW/cm² mask aligner power density).
- 12| Post-exposure bake: Place the wafer with the feature-side up on a hotplate at 65 °C for 1 minute, then transfer the wafer to another hotplate at 95 °C for 5 minutes.
- 13| Cool the wafer to room temperature.
- 14| Develop the wafer using SU-8 developer until the features appear (~5 min).

? TROUBLESHOOTING

! CAUTION Photoresist developers are highly toxic and reactive. Wear appropriate laboratory clothing when handling it and use it in a fume hood.

- 15| Rinse the wafer with isopropyl alcohol until the channel design is clearly visible. The control layer master is now formed.
- 16| Hard bake: place the master, feature side up, on a hotplate at 150 °C for 5 minutes.

PAUSE POINT—Hard-baked masters can be stored at room temperature in a dust-free environment for up to xx months/years.

Master for the flow layer ● TIMING ~1 h plus 8 h hard baking (overnight)

- 17| Place a clean silicon wafer on the spin coater.
- 18| Pour enough MCC primer 80/20 onto the silicon wafer to cover half the surface and run the spin coater according to the configuration in Step 2.

! CAUTION This reagent is highly toxic and reactive. Wear appropriate laboratory clothing when handling it and use it in a fume hood to prevent inhalation.

- 19| Pour enough AZ 10XT onto the silicon wafer to cover half the surface and run the spin coater according to the configuration in Step 2.

! CAUTION Photoresists are highly toxic. Wear appropriate laboratory clothing when handling it and use it in a fume hood.

- 20| Pre-exposure bake: place the silicon wafer with the coated side up, on a hotplate at 110 °C for 3 minutes.
- 21| Cool the wafer to room temperature.
- 22| Align the flow layer mask to the coated silicon wafer using the mask aligner.
- 23| Expose the wafer on the mask aligner to a total dose of 1425 mJ/cm² xx nm UV light (for example: expose the wafer for 57 seconds using a 25 mW/cm² mask aligner power density)
- 24| Develop the master for 5 min in a 4:1 solution of water to AZ 400K developer (v/v).

? TROUBLESHOOTING

! CAUTION Photoresist developers are highly toxic and reactive. Wear appropriate laboratory clothing when handling it and use it in a fume hood.

- 25| Rinse the wafer with water until the channel designs are clearly visible. The master is now formed.
- 26| Reflow: place the master with feature side up on a hotplate at 150 °C for 5 minutes.
- 27| Hard bake the master overnight by placing it feature side up on a hotplate at 260 °C.

■ PAUSE POINT Hard-baked masters can be stored at room temperature in a dust-free environment for up to xx months/years.

Making the PDMS control layer ● TIMING ~1 h

- 28| Make a circular aluminum foil cup for the control master using the base of a 10-cm diameter petri dish as a mold. To support the fragile base of the aluminum foil cup, place it in the lid of the petri dish.
- 29| Place the control layer master in the aluminum foil cup.

- 30| Mix 42.5 g PDMS monomer with 8.5 g curing agent (5:1 PDMS to curing agent (w/w)), mix well using a
- 31| Pour 35 g of the 5:1 PDMS mixture over the master. At the same time, use a cotton swab to gently press the master against the bottom of the cup to prevent PDMS from leaking underneath the master. This makes ~5 mm thick chips.
- 32| Place the PDMS mixture in a desiccator chamber until all the bubbles have been removed (~ 30 minutes).

? TROUBLESHOOTING

Making the PDMS flow layer ● TIMING ~30 min

- 33| Place the flow layer master on a spin coater. Change the angular frequency in the centrifugation setup of the second stage in Step 2 to 1250 rpm.
- 34| Make a 20:1 PDMS to curing agent mixture and pour enough of the mixture onto the center of the master to cover half its surface. Run the spin coater. This makes ~65 µm thick PDMS coating.
- 35| Place the master in a plastic petri dish with the PDMS coated side facing up.

Thermal bonding of the control and the flow layers ● TIMING ~5-14 h

- 36| Place both masters in a drying oven at 65 °C for 33 minutes then move them to room temperature.

▲ **CRITICAL STEP** This step is time-sensitive. If left in the oven for longer than 33 minutes, the PDMS will cure completely and the layers will not bond.

- 37| Carefully peel the PDMS off the control layer master.
- 38| Cut out the chips using a single edge razor blade.

▲ **CRITICAL STEP** Use a new razor blade since a dull blade can tear the thin spin-coated PDMS layer.

- 39| Using a 0.75 mm biopsy punch, make holes through the valve inlets on the control layer.
- 40| Carefully adhere a strip of scotch magic tape to the cast-side of the PDMS chips and then remove the tape in order to remove any fibers that might have fallen on this face of the chip.
- 41| Place the PDMS control layer slabs on top of the PDMS covered flow layer master, being careful to align the features on both layers. Namely, the channels of the control layer must be perpendicular to the channels of the flow layer.

▲ CRITICAL STEP

- 42| Place the petri dish holding the flow layer master in a drying oven at 65 °C for 4 hours.

- 43| Using a new razor, cut out the bonded, two-layer PDMS chips and carefully peel the chips off the flow layer master.

▲ **CRITICAL STEP** Use a new razor blade since a dull blade can tear the thin spin-coated PDMS layer.

- 44| Using a 0.75 mm biopsy punch, make holes through the inlets and outlets of the flow channels.

▲ **CRITICAL STEP** Do not punch through the holes made in step 39, as they are only meant to go through the control layer.

- 45| Submerge the PDMS chips in a beaker of isopropyl alcohol and sonicate for 45 minutes at room temperature.
- 46| Remove the PDMS chips from the isopropyl alcohol, rinse with Milli-Q water, then submerge in a beaker of Milli-Q water and sonicate for 45 minutes.
- 47| Use pressurized air to blow any excess liquid from the PDMS chips.
- 48| Place the PDMS chips in a covered petri dish and dry in the oven at 65 °C for 4 hours.

■ **PAUSE POINT** Chips can be stored in the petri dish at room temperature for several days.

Preparing the cover glass for plasma bonding ● TIMING ~2 h

- 49| Place several cover glasses in a beaker of 1M KOH and sonicate for 30 minutes.

! **CAUTION** Necessary precautions should be taken when handling and disposing of highly basic substances.

- 50| Remove the cover glasses from the KOH and rinse with Milli-Q water. Then submerge them in a beaker of Milli-Q water and sonicate for 30 minutes.
- 51| Remove the cover glasses from the water and blow with clean pressurized air to remove excess water.
- 52| Place the cover glasses in a drying oven at 65 °C for 15 minutes.

■ **PAUSE POINT** Cleaned cover glasses can be stored at room temperature in a dust-free environment for up to xx weeks/months/years.

Plasma Bonding ● TIMING ~2 h

- 53| Configure the plasma cleaner by doing a mock plasma treatment with an empty plasma chamber. Turn on the vacuum, flow oxygen into the chamber, wait for ~ 2 min to allow the pressure to stabilize then adjust the chamber pressure to 200 mTorr. Set the plasma power to 100 W, wait 11 seconds then turn off the plasma power.
- 54| Place several clean cover glasses and PDMS chips with the feature side up, in the plasma cleaner and run with the above settings.

- 55| Immediately after removing from the plasma cleaner, carefully place one of the cover glasses, plasma treated side down, on top of the feature side of one of the PDMS chips. Tap gently on the cover glass if needed. Do not apply pressure since this can cause the collapse of flow channels hence their irreversible closure. Repeat until all PDMS chips have been bonded.

▲ **CRITICAL STEP** Binding between the cover glass and PDMS happens very quickly and is practically irreversible. So, make sure to carefully center the cover glass over the PDMS chip since the cover glass cannot be re-positioned after contact with the PDMS.

- 56| Place the chips, PDMS side up, on a hotplate at 150 °C for 5 minutes.
- 57| Put the bonded MACS chips in a covered petri dish and place in a drying oven at 65 °C. Unless you are planning to use the chips for single molecule imaging, remove the chips from the oven after one hour and leave them at room temperature for 24 hours before using. For single molecule imaging, leave the chips in the oven for 3 days to allow the PDMS to stiffen.

■ **PAUSE POINT** The bonded MACS chips can be stored in the petri dish at room temperature many weeks. The PDMS does however slowly cure over time, so chips stored for extended periods tend to be stiffer.

Ordering custom components ● TIMING ~2-5 d

- 58| Download the Gerber files, contained in the “Mosfet_board” folder (Supplementary Data file xx), for the MOSFET circuit board and submit to a Printed Circuit Board (PCB) manufacturer for construction. It is important to note that PCB manufacturing can take several days and shipping may take even longer depending on the location of the PCB manufacturer.
- 59| Download the laser cutter design file “combined_macs_parts.ai” (Supplementary Data file xx), and have the design cut using 1/4” clear acrylic. Laser cutting services can take several days to cut the design.
- 60| (optional) If planning to use the growth chamber, download the laser cutter design file for the vial holder “acrylic vial holder.ai” (Supplementary Data file xx), and have the design cut using 0.118” black acrylic.

Assembly of the MOSFET Board ● TIMING ~2-5 h

- 61| Solder the LEDs to the circuit board and clip off the leads. Next, solder the resistors on the bottom side of the board, being careful not to touch the LEDs with the soldering iron. Solder the MOSFETs and diodes in places. Finally, solder the screw terminal blocks and DC barrel jacks to the PCB (Supplementary Fig. 1).

▲ **CRITICAL STEP** It is important to solder in this order to avoid difficulty accessing components with the soldering iron.

! **CAUTION**

- 62| Insert the DC barrel switch into the DC barrel jack, flip the switch to the off position and connect the switch to a 24-volt power supply.

Assembly of the Digital Analog Converter (DAC) circuit ● TIMING ~1-3 h

- 63| Solder the 6-pin male header to the DAC board.
- 64| Connect the two DAC boards to the half-sized breadboard (Fig. 8)
- 65| Using jumper wires, connect the SDA/SCL (i2c) pins of both DAC boards to one of the rails on the periphery of the breadboard and connect the VDD and GND of the DAC boards to the rails on the other side of the breadboard. Then wire the A0 pin of one of the DAC boards to VDD. Next attach screw terminals, for connecting the pressure regulator, and wire the VOUT pins of the DAC board to the screw terminals (Fig. 8)

Assembly of the Electronic Control Base ● TIMING ~3-6 h

- 66| Attach four air solenoid valves to a 4-valve manifold. Next, attach one (or two if planning to use the growth curve) to the inlet side of a second 4-valve manifold and fill the remaining slots with solenoid valve blanks.
- 67| Screw an M3 to 4 mm barb adapter to the outlet ports of the air solenoid valves, but not the blanks.
- 68| Screw an M5 to 4 mm OD push-to-connect adapter to one of the manifold supply ports, and screw the plug included with the manifold to the other supply port.
- 69| Connect the air valve manifolds, the MOSFET board, the Arduino Uno and the two pressure regulators to the Electronic Control Base (Supplementary Fig. 2).
- 70| Use short jumper wires to connect the pins of the Arduino Uno to the corresponding spots on the MOSFET board and the DAC circuit (Fig. 8).
- 71| Crimp and connect female MATE-N-LOK receptacles to the leads of the five solenoid valves.
- 72| Cut ten ~6" lengths of stranded 22 AWG wire for connecting the solenoid valves to the MOSFET board (Fig. 8). Crimp and connect male MATE-N-LOK receptacles to each of the five pairs of wires. Connect these wires to their corresponding solenoid valves (Fig. 8).
- 73| Cut two ~10" lengths of 4 mm polyurethane tubing and use this tubing to connect the supply ports of the pressure regulators together with a T-junction. Connect the remaining branch of the T-junction to the filtered, pressurized air.
- 74| Use two ~3" lengths of 4 mm polyurethane tubing to connect the outlet ports of the pressure regulators to the supply ports of the corresponding solenoid valve manifolds.

Assembly of the Pinch Valve Base ● TIMING ~3-6 h

- 75| Connect the three acrylic components of the Pinch Valve Base using #4-40 × 3/8" L screws and #4-40 × 1" L standoffs then attach the pinch valves with #2-56 × 3/8" L screws (Supplementary Fig. 3).
- 76| Crimp and connect female MATE-N-LOK receptacles to the leads of the six pinch valves.
- 77| Measure the distance (L) from the microscope stage, where the Pinch-Valve Base will sit, to the MOSFET board, being sure to leave enough slack to account for stage movements. Cut 12 pieces of 22 AWG stranded wire of length L. Crimp and connect male MATE-N-LOK receptacles to one end of each of the six pairs of wires. Use these wires to connect the pinch valves to the MOSFET board (Fig. 8).

Assembly of the Cleaning Reservoir Stand ● TIMING ~30 min

- 78| Assemble the Cleaning Reservoir Stand using four #4-40 × 1.5" L standoffs (Supplementary Fig. 4).
- 79| Cut a short piece (~6") of green PEEK tubing and attach to the smaller diameter inlet on the GL45 VICI-jour cap using the connector provided with the cap. Connect the 1/4-28 UNF to 5/32" push to connect fitting to the other inlet. Screw the cap onto a 100 mL GL45 threaded media bottle.
- 80| Use 4 mm (~5/32") OD polyurethane tubing to connect the outlet port of the bleach solenoid valve to the push-to-connect fitting on the bleach reservoir in the Cleaning Reservoir Stand.
- 81| Repeat the two preceding steps for the ethanol and water solenoid valves.
- 82| Cut a ~6" piece of green PEEK tubing and attach it to the smaller diameter inlet on the GL45 VICI-jour cap using the connector provided with the cap. Screw the cap onto a 250 mL GL45 threaded media bottle. This will serve as the main waste bottle.

Assembly of the growth chamber upstream of MACS (optional) ● TIMING ~4-6 h

- 83| To assemble the top of the acrylic vial holder, mount the LED Holders to the LED and TRANSMISSION sockets noting that the washer and bolt should be attached on the side with the engraved text. Next, slot the acrylic pieces together, being sure to include the shelf in the center. Use tape to hold the acrylic pieces tightly together. Finally, use a syringe to dispense acrylic glue into the seams between components and allow the glue to set as per the manufacturer's instructions (Supplementary Fig. 7).
- 84| To assemble the base of the vial holder, tap #4-40 holes in the acrylic bottom panel. Next, attach a 40-mm computer fan to the base using #4-40 × 1-1/4" L screws, using 1/8" spacers for the top and 3/16" spacers for the bottom. Finally,

attach a magnetic disk to the top of the fan using double-sided poster tape (Supplementary Fig. 7).

- 85| Connect the top of the vial holder to the base using 1-3/16" spacers and #4-40 × 1-1/2" screws.
- 86| Slide the leads of the detector (photodiode) and emitter (LED) into the plastic LED holder inserts. Use 5-minute epoxy to glue them in place and let dry for at least 15 minutes. Then slide them into their corresponding LED holder on the side of the vial holder (Supplementary Fig. 7).
- 87| Connect the stirrer (fan) to the 3.3V power pin on an Arduino Uno. Connect the emitter (LED) and a 47 Ω resistor in series across the 5V power pin of the Arduino. Connect the detector (photodiode) and 1.5 kΩ resistor in series across the 5V power pin of the Arduino. Measure the voltage drop across the 1.5 kΩ resistor using analog pin A4 on the Arduino (Supplementary Fig. 7).
- 88| Drill a 3/64" hole in the periphery of the cap of a 40-mL glass vial. Cut a piece of green PEEK tubing ~4.5" long and push it through the hole until it is nearly touching the bottom of the vial.
- 89| Following the same procedure described for the pressure tube, insert two 21-gauge blunt end needles into the cap, then make a cup around the cap with tape. Fill the cup with epoxy to hold the PEEK tubing and the blunt end needles in place. Let the epoxy fully harden.
- 90| Attach a normally closed pinch valve to the external fill slot on the Pinch Valve Base with two #2-56 × 3/8". Insert a y-junction at the inlet to the master pinch valve and connect to the pinch valve tubing. Insert a ~1" piece of green PEEK tubing into the free end of the pinch valve tubing.
- 91| Use 1/32" ID silicone tubing to connect the PEEK tubing on the growth chamber cap to the external fill pinch valve.
- 92| Connect the tubing from a normally open pinch valve to one of the needle inlets of the growth chamber, this will allow passive aeration when the culture is not actively being pumped out of the growth chamber.
- 93| Cut a ~3" piece of 4 mm polyurethane tubing, connect a Male Luer to 3/32" barb fitting to one end and attach the other end to the outlet of the growth chamber solenoid valve. Connect a 21-gauge blunt needle to the Luer fitting. Use Tygon tubing to connect this needle to the final inlet to the growth chamber.
- 94| The two pinch valves and the corresponding air solenoid valve are controlled by a single Arduino pin via the MOSFET board. So, connect the leads of the pinch valve and solenoid valve together, then wire them to the remaining screw terminal slots on the MOSFET board (Fig. 7).

Making the pressure tubes ● TIMING ~3h

- 95| Using a 0.75 mm diamond drill bit, drill a hole in the cap of a VWR 2mL screw cap microcentrifuge tube.

- 96| Using wire cutters, carefully cut the Luer lock off a 21-gauge blunt end needle, strip all the plastic off the needle and insert it through the hole in the microcentrifuge cap.
- 97| Wrap VWR lab tape around the outside of the cap, as shown in Supplementary Fig. 5, being sure to adjust the blunt end needle so that at least ¼" is exposed above the lip formed by the tape. Put this to the side for now.
- 98| Using a 9/64" drill bit, drill a hole through the bottom of the 2-mL VWR microcentrifuge tube (Supplementary Fig. 5).
- 99| Wrap VWR lab tape around the base of the drilled pressure tube, forming a cup. Place the Luer lock side of a 21-gauge blunt end needle into the cup. Put this to the side for now.
- 100| Mix 5-minute epoxy in a weigh boat.
- 101| Attach a 14-gauge needle to a 10-mL syringe and remove the plunger from the syringe.
- 102| Squeeze the epoxy into the top of the syringe and replace the barrel carefully.
- 103| With the needle pointing up, slowly depress the syringe until the epoxy starts to come out of the needle.
- 104| Use this syringe to dispense epoxy into the tape cups on the cap and base of the VWR centrifuge tubes (Supplementary Fig. 5). It may be necessary to hold the Luer lock needle against the base of the centrifuge tube while the epoxy sets.

▲ **CRITICAL STEP** Make sure that the epoxy is fully hardened before using the pressure tubes. This can take several hours even for the 5-minute epoxy.

■ **PAUSE POINT** The pressure tubes can be left essentially indefinitely at room temperature.

Connecting the tubing on the Pinch Valve Base ● TIMING ~30-45 min

- 105| Snap the two pressure tubes into the pressure tube holder on the Pinch Valve Base (Fig. 9).
- 106| Connect silicone pinch valve tubing using barbed Y-junctions, attach green PEEK tubing at the outlets of the pinch valves and connect to the outlet of the flow layer pressure tube (Supplementary Fig. 6). Connecting the pinch valve tubing to the Y-junctions can be challenging. Slowly twisting the tubing side to side can help push it over the barb.

▲ **CRITICAL STEP** It is important to push the tubing fully over the barb to avoid leaks.

- 107| Measure the distance (L) from the Cleaning Reservoir Stand to the Pinch Valve Base and cut four pieces of 1/32" ID silicone tubing of length L. Use this tubing to connect the washing fluid reservoirs and waste bottle to their corresponding pinch valves. Use the short pieces of green PEEK tubing as adapters between the 1/32" ID silicone tubing and the pinch valve tubing.

Connecting tubing to the pressure tubes and PDMS chip ● TIMING ~30 min

- 108|** Cut a ~3" piece of 4 mm polyurethane tubing, connect a Male Luer to 3/32" barb fitting to one end and attach the other end to the outlet of the flow layer solenoid valve. Connect a 21-gauge blunt needle to the Luer fitting.
- 109|** Measure the distance (D) from the flow layer solenoid valve to the flow layer pressure tube. Cut a piece of Tygon tubing of length D, attach one end to the blunt end needle at the outlet of the flow layer solenoid valve and attach the other to the inlet on the cap of the flow layer pressure tube (Fig. 9).
- 110|** Repeat the preceding two steps for the control layer pressure tube.
- 111|** Using wire cutters, carefully cut the Luer lock off a 21-gauge blunt end needle. Bend the needle 90°. Prepare three 90°-bent needles in total.
- 112|** Cut a piece of Tygon tubing of length ~5", insert one of the 90°-bent needles into one end of the tubing and connect the other end of the tubing to the outlet of the 'To Chip' pinch valve. Insert the bent needle into the flow layer inlet (Fig. 9).
- 113|** Cut a piece of Tygon tubing of length ~3". Insert one of the 90°-bent needles into one end of the tubing and connect the other end to the outlet of the Control Layer Pressure Tube (Fig. 9).
- 114|** Fill the control layer pressure tube with Milli-Q water. Wait for gravity to completely fill the tubing at the outlet of the control layer pressure tube, at which point the water will be dripping slowly out of the blunt end needle. Attach this blunt end needle to the control layer inlet (Fig. 9).
- 115|** Cut a ~10" long piece of Tygon tubing for the flow layer outlet. Insert a 90°-bent needle into one end of the tubing and insert the other end into the 40 mL MACS waste bottle. Insert the blunt end needle into the flow layer outlet (Fig. 9).

Running MACS ● TIMING ~2-8 h

- 116|** Place the MACS chip from Step 63 on the microscope stage. MACS can be mounted on a stage holder that accepts 75 mm × 25 mm cover slides using the adapter design provided. Download the Adobe Illustrator file (chip mounting adapter.ai, Supplementary Data file xx) and have the adapter cut with a laser cutter (Step 65).
- 117|** Open the direct control pane, set the Control Layer Pressure to 30 psi and the MACS State to closed then click apply. Wait until the control layer has been filled with water (aka dead-end filling), which may take up to 15 minutes. The filling will be evident by a change in contrast of the control channel.

▲ CRITICAL STEP Watch out for possible delamination of the chip. If the two PDMS layers are poorly bonded, this will cause all the water to leak rapidly from the edge of the chip. If this happens, the chip must be replaced.

- 118| Optional: If planning an automated acquisition, open the multi-dimensional acquisition software in Micro-Manager, select the parameters for your acquisition and save the acquisition settings in an .xml file.
- 119| Engage the Live View in Micro-Manager and focus on the position where the flow layer channel being used intersects the control layer channel being used (Fig 5d).
- 120| Select the parameters for your configuration in the Run MACS tab.
- 121| Clean the MACS system as described in Box 1
- 122| Remove the top and pipette 600 μ L of your sample into the pressure tube of a previously cleaned MACS setup.
- 123| Begin by clicking the inject sample button, which will flow the sample from the pressure tube into the MACS chip at the specified Flow Layer Pressure (... mbar) for the specified Inject Cells time (... sec).
- 124| While observing the field of view in the Live View window, click the Run MACS button.
- 125| If the parameters are correct for the given experiment, then cells will be seen flowing past the field of view while in the open state, they will accumulate in the half open state (see Figure 5a), will be trapped in the closed state, and finally will be pressed with Imaging Pressure. If cells are not properly trapped in the closed state, adjust the parameters.

? TROUBLESHOOTING

- 126| Optional: Once the cells have been loaded and the parameters have been optimized, select the acquisition file (.xml) from Step 112 by pressing Choose File under the acquisition pane. Once a file has been selected, the Run Acquisition Button will become enabled. Press Run Acquisition to begin capturing snapshots of the cells with MACS.
- 127| Clean the MACS system as described in Box 1.

Running a growth chamber experiment with MACS (optional) ● TIMING ~2-18 h

- 128| Download the folder: “MACS Growth Curve Code” (Supplementary Data file xx).
- 129| Open the Arduino IDE and upload “od_measurement.ino” to an Arduino Uno.
- 130| Open Matlab and change directories to the location of the downloaded MACS growth curve code.
- 131| Run growth_chamber_macs.m. Note that all output files from this program will be saved to an output directory created on the desktop by default.

- 132|** In the first GUI, enter the strain name and any desired notes into the corresponding fields then click next. This information will be saved to a text file in the output directory.
- 133|** In the second GUI, click the Single Measure button and wait for the Transmission value (T) and OD value (OD) to be updated (this takes about 10 seconds). Ensure that the photodetectors are responding to variations in incident light by manually blocking the opening between the IR LED and the photodetector.
- 134|** Add 35 mL of growth media and a sterile magnetic stir-bar to the sterile growth chamber vial. Make sure the stirrer is running. If not, it may be necessary to slightly push the fan to break static friction and initiate rotation.
- 135|** Provide the maximum transmission value expected during the experiment by pressing Measure to Max, this will enter the most recently measured transmission value into the box next to MaxT. This value will be used to properly scale the figure axes for the experiment. It does not affect the measurements or output.
- 136|** Enter the desired measurement interval, in seconds, into the 'Time btw measurements' panel. Measurement intervals should be longer than 2 seconds. We typically...
- 137|** Inoculate the growth media.
- 138|** To begin timed measurements, click the Start button. The first measurement will take ~10-15 seconds to appear on the OD plot. All proceeding measurements will take place after the specified measurement interval.
- 139|** Injected the culture into the MACS pressure tube by directly setting the Arduino-Switch state in micromanager to "flow culture from the growth chamber into the pressure tube". Once the pressure tube has been filled, the MACS setup can be controlled with the MACS controller plugin. This process can be repeated at set intervals along the growth curve as dictated by experimental requirements.
- 140|** Calibrate the system if a calibration has not been performed for the given combination of growth media and cells of interest, or if the growth chamber has been idle for longer than ~1 month. To calibrate the detectors, grow your cells of interest to stationary phase then measure its OD on a spectrophotometer and the transmission value obtained with the growth chamber setup. Add these numbers to an excel spreadsheet. Dilute the culture with minimal growth media and repeat the measurements. For best results, try to obtain calibration points with increments of at most $OD_{600} = 0.2$.

? TROUBLESHOOTING

Troubleshooting advice can be found in Table 1

● TIMING

Step 1, ordering the masks: ~1-3 d

Steps 2-4, cleaning the silicon wafers: ~30 min

Steps 5-16, master for the control layer: ~1 h

Steps 17-27, master for the flow layer: ~1 h plus 8 h hard baking (overnight)

Steps 28-32, making the PDMS control layer: ~1 h

Steps 33-35, making the PDMS flow layer: ~30 min

Steps 36-48, thermal bonding of the control and the flow layers: ~5-14 h

Steps 49-52, preparing the cover glass for plasma bonding: ~2 h

Steps 53-57, plasma bonding: ~2 h

Steps 58-60, ordering custom components: ~2-5 d

Steps 61-62, assembly of the MOSFET board: ~2-5 h

Steps 63-65, assembly of the digital analog converter (DAC) circuit: ~1-3 h

Steps 66-74, assembly of the electronic control base: ~3-6 h

Steps 76-77, assembly of the pinch valve base: ~3-6 h

Steps 78-82, assembly of the cleaning reservoir stand: ~30 min

Steps 127-138, assembly of the growth chamber upstream of MACS (optional): ~4-6 h

Steps 83-92, making the pressure tubes: ~3 h

Steps 93-95, connecting the tubing on the pinch valve base: ~30-45 min

Steps 96-103, connecting tubing to the pressure tubes and PDMS chip: ~30 min

Steps 110-119, running MACS: ~2-8 h

Box 1, cleaning MACS: ~10-45 min

Steps 128-140, running a growth chamber experiment with MACS (optional): ~2-18 h

ANTICIPATED RESULTS

After setting up MACS, one should be able to run automated image acquisition of non-adherent cells, which are temporarily immobilized. For a short segment of an experiment where cells were imaged at a rate of ~100,000 cells/hour, see Supplementary Movie 2. This technique can be applied to count single-molecules in bacteria, and detect, enrich as well as sort rare phenotypes, applications for which we provide examples below.

For the single-molecule counting measurement, we used an *E. coli* strain where a low-abundance protein (SprE) had been fused to GFP (mNeonGreen) at its native chromosomal locus. An exogenous copy of a second fluorescent protein (CFP) on the chromosome served

as a segmentation marker and allowed identification of cells and their boundaries (Fig. 10). For this experiment, we used chips that had been cured for 3 days at 65 °C after fabrication (Step 57) and thus the chips were stiffer. Cells were injected, accumulated in the half open state, and trapped with an imaging pressure (P_{control}) of 30 psi. Approximately 15 seconds after trapping, cells were imaged using a 50 mW 514-nm laser with HILO modality on an EMCCD camera at video rate (33 Hz, i.e. 30 msec exposure time). We then used the provided MATLAB code, *AnalyzeMacsInteractively.m*, to analyze the data, see Supplementary Manual for a detailed description of using the code. Under these growth conditions, the average number of SprE per cell was ~14 and the population-wide distribution of SprE abundance is close to Poissonian.

Running MACS in the continuous mode, we detected *E. coli* cells that are proficient in plasmid conjugation – a leading cause of horizontal gene transfer and the spread of antibiotic resistance genes. Because the expression of transfer (*tra*) genes is associated with higher metabolic burden and increased sensitivity to male-specific phages, many conjugative plasmids are naturally repressed¹⁸. For example, under laboratory conditions, it is known that plasmid R1 turns on the conjugation machinery at frequencies below 10^{-3} per cell and generation¹⁹, but even order-of magnitude estimates have proven challenging because the events are so rare. To identify these rare events we placed a *cfp* gene at the very end of the transfer operon and used MACS to identify cells expressing CFP (data not shown). Our preliminary data suggests that the *tra* operon is activated at a frequency of approximately 10^{-6} per cell and generation, under our conditions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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EDITORIAL SUMMARY

This protocol describes a microfluidics platform, termed MACS, for single-cell microscopy of suspension cultures. MACS uses controlled pressure valves to temporarily immobilise the cells, allowing for highthroughput and high-resolution imaging.

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Box 1**Cleaning MACS ● TIMING ~10-45 min**

The MACS system should be thoroughly cleaned before and after use. This is because... This box describes the general cleaning procedures.

- 1** If using a new pressure tube, remove the cap, pipette 600 µL of Milli-Q water into the tube and draw a line on the pressure tube to mark the position of the meniscus. Then empty the pressure tube.
- 2** Before running a full cleaning protocol, establish the parameters for filling the pressure tube with cleaning fluids. Begin by choosing very short active filling durations for all cleaning fluids, e.g. ~3 seconds.

▲ CRITICAL STEP This should be done regularly and must be done every time a cleaning fluid reservoir is changed to avoid overfilling the pressure tube.

? TROUBLESHOOTING

- 3** Under fill panel, click Bleach and observe the height of the fill. It should be slightly above the mark on the side of the pressure tube. If the system has been sitting for more than a few hours, it may be necessary to fill the tube multiple times, emptying between each fill, to remove air from the tubing and get a stable reading of the fill volume.
- 4** If the volume dispensed into the pressure tube is too low, increase the fill duration, if it is too high decrease the duration.
- 5** Repeat the previous two steps with ethanol and Milli-Q water, with a slightly large fill volume for each subsequent cleaning fluid to ensure that no bleach or ethanol is left on the walls of the pressure tube after cleaning.
- 6** Click the Full button under Run Cleaning Protocol to run a full wash with bleach, ethanol and water respectively. During this procedure, the pressure tube will first fill and empty multiple times for each liquid. Next it will inject the cleaning fluid through the MACS chip before finally emptying the remaining fluid to waste. Note that at any time, the pressure tube can be washed with a single cleaning fluid by clicking Bleach, Ethanol or Water in the Run Cleaning Protocol panel.

Figure 1. MACS fabrication

(a) Schematics summarizing photolithography, device making and bonding. After the control layer is prepared (left, shown are steps 23, 24, 26 and 34 in the procedures section), it is aligned and bonded onto the flow layer (middle, shown are steps 11, 14, and 31 in the procedures section). After the two layers adhere, the two-layer PDMS chip is bonded to a cover glass (right, shown are steps 41 and 55 in the procedures section). (b) Transparency masks for flow (left) and control (right) layers. Scale bar is 2 mm. The inset shows the in-line filters in more detail. (c) Cut through at the inlets after hole punching. **Top.** Without isopropanol treatment, there are PDMS crumbs stuck to the channel (marked with a red arrow). **Bottom.** After isopropanol treatment, the debris is removed from the inlet holes. Scale bars are 0.75 mm.

Figure 2. Operating principle of MACS

(a) The basic MACS setup consists of two digital pressure regulators, P_{flow} and P_{control} , two air solenoid valves, AV_{control} and AV_{flow} , and two pressure tubes. P_{flow} (P_{control}) supplies air to the inlet of AV_{flow} (AV_{control}) which is actuated to start/stop the flow of air into a pressure tube that is connected to the flow (control) channel of the PDMS chip. (b) MACS works by serially visiting three states of the PDMS valve: open, half-open and closed. Once P_{control} and P_{flow} are tuned so that cells flow in a monolayer past the FOV when $(AV_{\text{control}}, AV_{\text{flow}}) = (\text{on}, \text{on})$, the three PDMS valve states can be achieved simply by actuating AV_{control} and AV_{flow} . (c) By using higher pressure or stiffer chips, MACS can be used for mechanical slowing down of diffusion within cells (orange ovals), allowing for the visualization of single cytoplasmic molecules as small as individual proteins (stars).

Figure 3. Overview of full MACS setup

Schematic representation of the three modules comprising the standard MACS setup, including the required liquid and air connections. Electronic Control Base: Pressurized air is connected to the inlet of the pressure splitter and fed into two independent digital pressure regulators, P_{flow} and P_{control} . These pressure regulators supply pressurized air to air solenoid valves – mounted on 4-valve manifolds– which are electronically actuated to direct the flow of air to air-tight pressure tubes or cleaning-liquid reservoirs. Note: the Electronic Control Base also contains an Arduino Uno, MOSFET board and electronics breadboard (not

pictured). Pinch Valve Base: Fluids from the cleaning reservoirs and the pressure tube are directed through tubing via pinch valves. For instance, by actuating $PV_{to\ chip}$ and $PV_{to\ waste}$ and applying pressure to the pressure tube, fluid from the pressure tube can either be dumped directly to waste or sent to the MACS microfluidic chip. In between samples, the MACS setup is cleaned with cleaning fluids from the Cleaning Reservoir Stand by opening both $PV_{to\ PT}$ and the appropriate cleaning fluid pinch valve, i.e. PV_{bleach} , then applying pressure to the reservoir, i.e. $P_{flow} > 0$ and AV_{bleach} on. Note that for applications such as integrating upstream growth cultures, an optional Pinch Valve ($PV_{external\ fill}$) and air solenoid valve (AV_{growth}) are used.

Figure 4. Flow chart for operation of MACS

The protocol begins by running a full cleaning procedure with 10% bleach, 10% ethanol and water to clean the PDMS chip and flow path. Then, using a pipette, 600 μ L of a cultured sample is loaded into the flow pressure tube. This sample is injected into the PDMS chip by flowing first to waste for a very brief interval and then flowing into the chip. Next all the valves are closed before setting the pressures to 20 psi. The $AV_{control}$ valve is opened to flow cells past the FOV. This is the open state. Next $AV_{control}$ is opened, this puts pressure on the PDMS valve. If the pressures were chosen correctly for the sample, then cells will flow past the field of view as a monolayer and accumulate under the PDMS valve. If this does not happen, the pressures need to be re-adjusted until a monolayer of cells is seen. At this point, the chip is in the half-open state. Finally, after accumulating cells in the half-open state,

AV_{flow} is closed and the cells are trapped in the FOV. This is the closed state. If the experiment requires single molecule imaging, the value of P_{control} can be temporarily increased, applying additional pressure to the trapped cells. Next, cells are imaged under the scope, and if P_{control} was adjusted for imaging, it should be returned to its previous value. This cycle of open, half-open and closed followed by imaging can then be repeated for as many cycles as needed, without tuning pressures. Once imaging of the sample has been completed, the culture is expelled to waste and the setup is cleaned. At which point new samples can be loaded and the procedure repeated.

Figure 5. Capturing rare phenotypes and their retrieval using MACS

(a) Cells continuously flowing through the field of view in the half-open state. Scale bar (white), 25 μm . **(b)** The cell of interest – an RFP-expressing cell, which we diluted by a factor of 100,000 using GFP-expressing cells – is captured in the closed state and is detected (circled). Scale bar (white), 10 μm . **(c)** Minor modification of MACS enables cell retrieval.

Left. Masks for flow (top) and control (bottom) layers. Scale bars are 1 mm. The flow-focusing feature is shown within the semi-transparent gray box. This feature is optional, and allows for the cells to trickle through the central region of the flow channel and prevent cell accumulation at the channel sides. It also allows for adjusting the cell density on the FOV: when the side streams (of media) are stronger, the middle stream gets thinner, thereby diluting the cells at the FOV. **Right.** Schematics of cell retrieval. The volume that is trapped when valves 1–4 are closed (with the control valve open) is shown by the red dashed line.

(d) Circled is the captured cell of interest in Fig. 5b within the trapped volume, which is outlined by the red dashed line. Shown is bright field and RFP fluorescence images overlaid. Scale bar (white) is 40 μm . **(e)** When we collected the trapped volume using the oil phase, grew cells overnight and imaged them on the agar pad; the RFP-expressing cells were enriched. This scheme allows for the immediate retrieval of cells at low cell densities. At high densities, a second round is necessary to achieve 100% purity. Scale bar (white), 2 μm . Figure is modified from figure 4 of reference ¹¹.

Figure 6. Dye uptake upon pressing

The *E. coli* cells are first mixed with propidium iodide and then squeezed by the control layer valve of the MACS device. Top and bottom panels show two different cells (a) brightfield channel (b) propidium iodide fluorescence channel right after pressing (c) propidium iodide fluorescence channel 9 min after pressing. Scale bar is 1 μm .

Figure 7. Integration of growth chambers with MACS

Schematic showing sampling from a growth chamber where we can monitor the optical density of a growing culture in real time. Passive aeration of the culture is achieved through the normally open (NO) pinch valve ($PV_{\text{growth vent}}$), which is connected to open air by default. To flow from the growth chamber to the PT, we open AV_{growth} and $PV_{\text{external fill}}$ (see Fig. 3) and close $PV_{\text{growth vent}}$. The detector – placed at 180° with respect to the emitter – measures the transmission of light through the sample, which serves as a metric for optical density (OD_{600}) after calibration¹⁷.

Figure 8. Electrical connections for the MACS setup

Commands are sent from a computer to an Arduino Uno microcontroller which translates those commands into electrical signals. These electrical signals actuate MOSFETS on a MOSFET board (green PCB shown above) to control the pinch valves and air solenoid valves. The pressure of P_{flow} and P_{control} is set with an analog voltage level between 0-5V. The voltage corresponding to the desired pressure is sent from the Arduino to external digital to analog converter (DAC) over an i2c bus. These DACs should be connected as shown in the breadboard above. Finally, we note that for the optional growth chamber, AV_{growth} , $PV_{\text{growth vent}}$ (normally open) and $PV_{\text{external fill}}$ are actuated by the same MOSFET, so should be electrically tied together as shown. Figure made with Fritzing.

Figure 9. Fully assembled MACS setup

(a) Overview of the entire assembled MACS setup with the Cleaning Reservoir Holder in the top left, the Pinch Valve Base directly below that, the stage holder on the front left with the PDMS chip attached and the Electronics Control Base on the top right. (b) A zoomed in view of the PDMS chip and the Pinch Valve Base. The fluid in the control tube has been dyed red and the fluid from the flow tube has been dyed green. The inlets of the PDMS chip are connected to Tygon tubing using bent 21 gauge blunt-end needles.

Figure 10. Single-molecule counting in *E. coli*

A. The analysis code outlines the cell boundary and displays the spots detected from single molecules of SprE-mNeonGreen within individual cells. **B.** Population-wide distribution of SprE abundance. The experimental data (blue bars) is shown overlaid with a theoretical Poisson distribution (red dots) of the same average.

TABLE 1

Troubleshooting table

Step	Problem	Possible reason	Solution
14, 24	The design does not appear	Master is not fully developed	Gently shake the master while in the developer
32	Numerous bubbles remain in PDMS	Insufficient vacuum pressure for the desiccator	Obtain a stronger vacuum pump
	A few bubbles remain in the PDMS	The last few bubbles may get trapped in the PDMS	Use filtered pressurized air to gently blow on the bubble, allowing the bubble to be released
125, Fig. 4	Cells are not flowing past as a monolayer	The cells did not reach to the intersection	Increase the duration of cell injection to the MACS chip from the pressure tube
		The PDMS valve is completely closed preventing any flow in the flow layer	Decrease the control pressure (P_{control}) in 0.1 psi steps, until monolayer of cells is observed
		The PDMS valve is not closed enough allowing cells to flow freely past	Increase the control pressure (P_{control}) in 0.1 psi steps, until monolayer of cells is observed
Box 1 step 2	Pressure tube will not fill with cleaning liquids	The tubing is clogged	Replace the tubing