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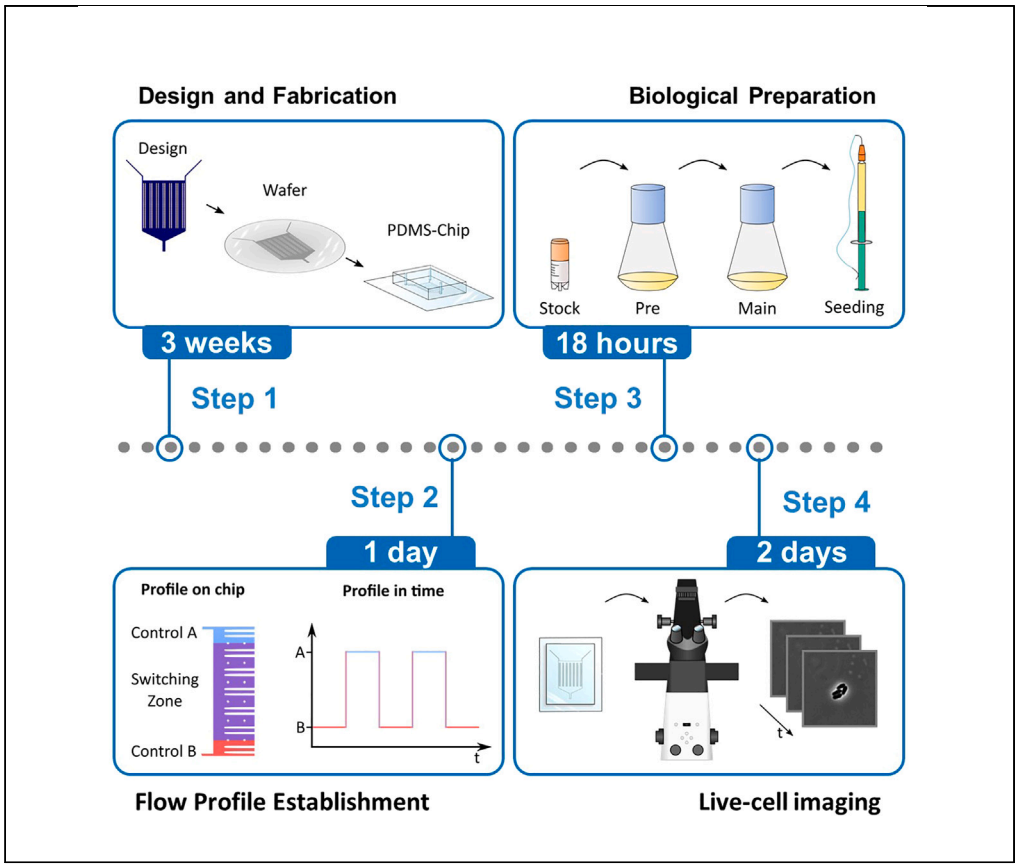
Protocol to perform dynamic microfluidic single-cell cultivation of *C. glutamicum*



Here, we present a protocol for the design, fabrication, and usage of a PDMS-based chip for dynamic microfluidic single-cell cultivation of *Corynebacterium glutamicum*. We describe steps for flow profile establishment and biological preparation. We then detail time-lapse imaging to observe reactions of *C. glutamicum* to repeated environmental changes in the range of seconds. This system can be adapted to other organisms with a cell wall and soluble non-gaseous environmental factors like nutrients.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Time-lapse live-cell imaging of microbes in rapidly changing environments

Design and fabrication of dynamic microfluidic single-cell cultivation chips

Application of fast and flexible environmental changes like pulses and oscillations

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Protocol to perform dynamic microfluidic single-cell cultivation of *C. glutamicum*

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SUMMARY

Here, we present a protocol for the design, fabrication, and usage of a polydimethylsiloxane (PDMS)-based chip for dynamic microfluidic single-cell cultivation of *Corynebacterium glutamicum*. We describe steps for flow profile establishment and biological preparation. We then detail time-lapse imaging to observe reactions of *C. glutamicum* to repeated environmental changes in the range of seconds. This system can be adapted to other organisms with a cell wall and soluble non-gaseous environmental factors like nutrients.

For complete details on the use and execution of this protocol, please refer to Täuber et al..[1]

BEFORE YOU BEGIN

Microfluidic single-cell cultivation is a technique to analyze single cells in highly controlled environments, enabling the observation of population heterogeneity and single cell reactions to stressors.[2] The protocol below describes the wet-lab procedure to design, fabricate and apply the dynamic microfluidic single-cell cultivation (dMSCC) system for *Corynebacterium glutamicum* in rapidly changing environments. The bacteria grow in monolayer growth chambers (MGCs), which are connected to supply channels. The microfluidic chip is constantly perfused with medium, delivering nutrients and removing products, which keeps the concentration of medium components constant throughout the experiment. In the presented system we are able to change the medium condition within seconds, allowing us to mimic environments with high dynamics and observe immediate reactions of cells to environmental changes. The dynamic medium exchange is mediated via pressure changes on the two medium reservoirs that are connected to the two inlets of our system. This causes the laminar boundary layer between the two media to shift across the chip, submersing the chambers in a different medium. We also used this easily adaptable protocol to cultivate other organisms, like *Saccharomyces cerevisiae*, *Escherichia coli* and *Bacillus subtilis*, increase the parallelization of experiments as well as the number of environmental conditions[3] that can be applied in our system. Furthermore, the cultivation region can be changed from MGCs to other architectures like mother machines.

Mask design

Timing: 1–3 days hands-on-time, 2 weeks in advance to include ordering time

This step will highlight the most important aspects of mask design for dMSCC using a layout editor like Clewin 4.0.

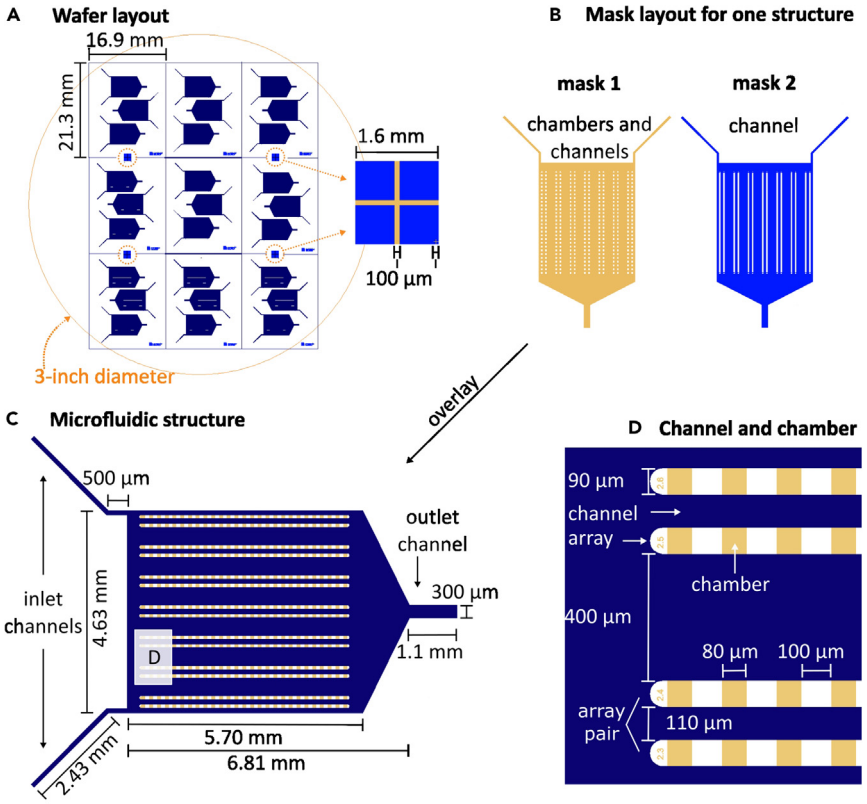

Figure 1. Mask design for dMSCC

(A): On a 3-inch-diameter illumination region, 9 separate chips can be placed. Each chip can support 3 microfluidic cultivation structures. Alignment markers (highlighted by yellow circles) are placed as far apart as possible on the wafer. The markers have a large (1.6 mm) and a small (100 µm) cross in the mask 1 (yellow) and the according negative in mask 2 (blue squares) for alignment.

(B and C) (B): Two masks need to be designed for the two-layer photolithography: mask 1 (yellow) contains the structures for the monolayer growth chambers (MGCs) as well as the channels. In mask 2 (bright blue), only the main channel structure is present. During wafer fabrication (step 3 and 5), the yellow layer is fabricated with a height of 700 nm and the blue with a height of 10 µm. The overlay of mask 1 and 2 is shown in (C). Dark blue elements are present in both masks. When placing multiple structures on one chip, a distance of approx. 2 mm between inlets and outlets should be kept.

(D) The MGCs (yellow rectangles, 80 × 90 µm) are aligned on arrays, which are divided by 110 µm wide channels. Two arrays form an array pair. Between the array pairs, the channel is 400 µm in width to compensate for flow profile inaccuracies.

Note: We order the mask with own designs from a company. As their fabrication and delivery require one to one and a half weeks until arrival, we recommend to start designing two weeks in advance of step 2.

1. Design the general mask layout.
   a. Mark the space in which the designs can be placed. It is equal to the area that can be exposed to light in the mask aligner (Figure 1A).
   b. Set four alignment markers (Figure 1A). They should be as far apart as possible to ensure precise and accurate alignment during the photolithographic fabrication and minimize the effect of local height differences during fabrication (step 5.a). Create two mask layers (Figure 1B). One layer contains a cross and the other the negative for alignment.
   c. Estimate the maximal size of a microfluidic chip. The size is limited by the size of the glass substrate used for bonding. Furthermore, space to the edges should be kept to leave space for fixing the final chip on the microscope's holder. We use a 21.3 × 16.9 mm chip on a 39.5 × 34.5 mm glass substrate.

2. Design the dynamic microfluidic structures.
   a. Create cultivation regions arranged on arrays (Figures 1C and 1D). We use monolayer growth chambers (MGCs) with a planar dimension of 80 × 90 μm that are open towards the channel.
      i. Create a square as core of the structure (Figure 1C) in mask 1 (yellow layer).
      ii. Cut out the region, where the cultivation arrays will be located (Figure 1D).

   Note: The width of the arrays (90 μm) determines the size of the cultivation chamber. The space between the cultivation arrays are the channels. We use channels with a width of 110 μm for arrays in the same cultivation environment and 400 μm channels between different cultivation environments to accommodate the laminar boundary layer between two media and its inaccuracies. For very wide channels, we use a pillar structure as shown in Figure 4 to support the PDMS ceiling.

      iii. Add rectangles with a size of 80 × 90 μm on the arrays where the cultivation chambers are supposed to be. We leave 100 μm space in between the chambers.

   Optional: Other cultivation structures like mother machines can also be applied instead of MGCs.

   b. For dynamic switching between two environmental conditions, create two inlet channels.
      i. The inlets need to be punched later on by hand with a biopsy puncher with a diameter of 0.75 mm. Therefore, the inlets need to be 2 mm apart to accommodate possible inaccuracies during punching (step 8.a).
      ii. The inlets should optimally have a distance of 2 mm to the cultivation regions, as the flow periphery like needles and tubing might otherwise cast shadows on the region of interest during live-cell imaging (step 33). Also, inserting the inlet needle applies pressure on the surrounding material, which can collapse chambers, if they are too close.

   Note: Long and thin inlet channels will lead to reduced flow at the same pressure compared to a channel with shorter and wider dimensions. However, a certain flow is needed to ensure rapid movement of the laminar boundary layer, which depends on the chosen layer heights (more details on flow profiles in step 14). We have a flow of ~ 6.4 μL/min in the presented design. With the Hagen Poiseuille equation or online tools to calculate flow in microfluidic structures, the flow through the inlet channels can be approximated during designing. Alternatively, the flow in the structure can be modelled in a CFD software to refine the design. During the first wet-lab experiments with the design (step 14), the flow can be measured using flow controllers.

   c. Create an outlet channel (Figure 1C). The outlet should be wider in dimensions than the inlets to prevent pressure build-up in front of the outlet. This can deform the PDMS and increase the chamber height. Loss of focus and decreased trapping efficiency are possible results.
   d. Duplicate the complete layout from mask 1 into mask 2. As mask 2 (Figure 1B) is supposed to show only the channel structures, delete the chamber rectangles.

Photolithographic fabrication of mold

Timing: 1–2 days

⚠ CRITICAL: For this step, use an ISO 6 clean room facility with yellow light. Particles in the master mold will change the form and local height distribution of the SU-8 negative photoresist, which can result in unusable structures. White light polymerizes the photoresist.

Note: Self-designed wafers can also be fabricated commercially.

3. Coat the silicon wafer with first layer of SU-8 (0.5), which is equal to the chamber height (700 nm) of the finished wafer.
   a. Initially, clean the silicon substrate.
      i. Prepare a bath of permonosulfuric acid by carefully mixing one part H_2O_2 (30% w/w) and two parts H_2SO_4.
      ii. Place the wafer in the acid bath for 5 min using wafer tweezers.
      iii. Take it out, rinse it thoroughly using ddH_2O and place it in a bath of ddH_2O for 5 min.
      iv. Repeat this procedure once. Dry the wafer using a wafer centrifuge (3000 rpm for 30 s) and placing it on a heating plate for 15 min at 200°C.
   b. Coat the silicon wafer with a layer of negative photoresist SU-8 (0.5).
      i. Place the wafer in a wafer centrifuge and pour the liquid photoresist in the center, so that it covers about a third of the wafer's diameter.
      ii. Centrifuge 30 s at 2500 rpm and bake the wafer afterwards for 1 min at 65°C on a heating plate.

Note: We purchased SU-8 (2) and diluted it with γ-butyrolacton to produce SU-8 (0.5).[4] SU-8 (0.5) allows to cast thinner layers that are more even than the fabrication of thinner layers by increasing the spin coating speed. You can calculate the dilution using this formula:

$$\frac{solid\ content_{SU-8\ (2)}}{solid\ content_{SU-8\ (0.5)}} \cdot Volume_{SU-8\ (2)} - Volume_{SU-8\ (2)} = Volume_{\gamma-butyrolacton}$$

For SU-8 (0.5) we use a solid content of 28.5%. We need to mix 100 mL of SU-8 (2) with 38.6 mL of γ-butyrolacton.

$$\frac{39.5\%}{28.5\%} \cdot 100\ mL - 100\ mL = 38.6\ mL$$

4. Expose coated wafer to UV-light through mask 1 (Figure 1, see step 2.a.iii).
   a. Align the coated wafer to the mask using a mask aligner. Bring the wafer in vacuum contact with the mask. Expose the wafer to near UV light (365 nm) with 58 mW cm^{-2} for 1.3 s.
   b. Bake the wafer for 5 min at 65°C and 5 min at 95°C to further crosslink the exposed SU-8 photoresist.
   c. Develop the layer for 1 min in mr-Dev 600 developer. Rinse the wafer once with acetone and once with isopropanol, by letting it flow from the wafer tweezers to the wafer edge. Isopropanol should flush away the acetone without the wafer falling dry. Finally, dry the wafer using particle-free compressed air or nitrogen. The first structures should be visible and no unlinked SU-8 residues should be left now.
5. Coat the silicon wafer with a 10-μm-layer of photoresist SU-8 (10).

⚠ CRITICAL: Tape the alignment markers on the first layer before coating. Otherwise, the position markers are not visible during alignment on the mask aligner. For the same reason, be sure to remove the tape at 65°C during the baking procedure. It is not possible to remove it after it melted.

   a. Tape each of the four alignment markers using transparent adhesive tape.
   b. Similar to step 3.b, coat the wafer using SU-8 (10) photoresist. Centrifuge for 30 s at 3000 rpm for a layer height of 10 μm. The taped regions can influence the local height distribution of the photoresist.
   c. Bake the wafer for 5 min at 65°C and 10 min at 95°C. Remove the tape as soon as the wafer is heated up to 65°C.
6. Expose coated wafer to UV-light through the mask 2 (Figure 1 – bright blue, step 2.d).
   a. Place the coated wafer and the mask 2 in the mask aligner.

b. Align the alignment markers on the wafer with their negatives on the mask. Rotate and shift the wafer accordingly, switch to the diagonal alignment marker (Figure 1A) and repeat until misalignment is decreased to a minimum. The small cross should be aligned perfectly in the end.
c. Carefully converge wafer and mask while assuring their alignment as described in step 6.a until they are in vacuum contact.
d. Expose the wafer to near UV light with 58 mW cm^{-2} for 6 s.
e. Bake the wafer for 5 min at 65°C and 10 min at 95°C to further crosslink the exposed SU-8 photoresist.
f. Develop the second layer as described in step 4.c.
g. Bake the developed wafer for 15 min at 200°C to close possible fractures in the photoresist.

Pause point: The obtained master wafer can be stored and reused indefinitely as long as the structures are not destroyed by scratches or otherwise negatively influenced.

Optional: The exact height of the chambers can be measured after wafer fabrication using a surface profiler or atomic force microscope for very low structure heights.

Note: Using the same masks, wafers with different layer heights can be fabricated, e.g., to adjust the chamber height for a different organism. Adjustments can be made by using different rotation velocities during spin coating, by diluting the photoresist with γ-butyrolacton, by repeated coating with the photoresist for very high structures, or simply by the utilization of a SU-8 with different height characteristics. Details on rotation and dilution can be found in the manufacturer's notes. When coating repeatedly, take care to bake the wafer between the coating steps.

Note: You can make a cheap and easy epoxy mold of the microfluidic structures from the molded PDMS-chips as safety-copy or for sharing structures with other research groups.

Fabrication of the microfluidic PDMS-chip

Timing: 3 h

⚠ CRITICAL: Work under a flow bench (alternatively a clean bench or clean room facility) for each step that exposes the microfluidic structures to the environment to prevent particles on the structure. Wear gloves to protect the chip from skin particles and fat.

7. Mold PDMS-structure using master wafer.
   a. Weigh 10 parts of PDMS-base and 1 part PDMS-linker in a cup and mix them thoroughly for 3 min using a plastic spatula.
   b. Clean the wafer from any particles by blowing with pressurized air. Place it in a clean petri dish.
   c. Pour the mixture onto the clean and particle free master wafer in a petri dish until it reaches a height of 3–5 mm.
   d. Remove any air bubbles from the structures by applying a low pressure environment in a desiccator for approx. 10 min. Residual air bubbles on the surface can be removed by blowing gently.
   e. Bake the air bubble-free wafer for 2 h at 80°C to polymerize the PDMS. Protect the liquid PDMS from particles.
   f. Carefully cut the PDMS on the wafer using a scalpel and peel it from the wafer using tweezers.

⚠ CRITICAL: Do not scratch the structures!

8. Prepare PDMS-chip for bonding.

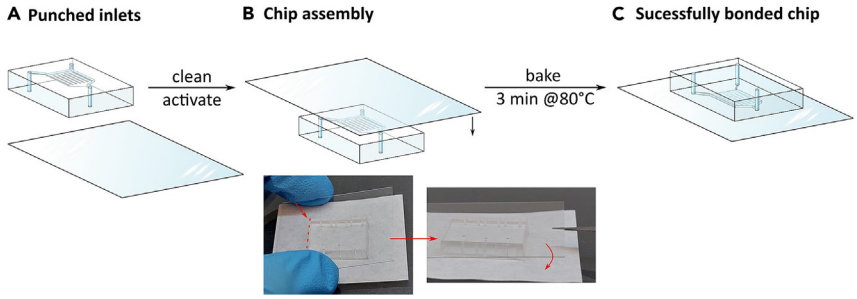

Figure 2. Chip fabrication procedure

(A) Punch in- and outlets in the PDMS-chip and clean both the chip and the glass slide before activating it.

(B) To bond the chip after activation, place the glass slide carefully on the PDMS-chip.

(C) The chip is ready to use after baking for 3 min at 80°C or letting it rest at 20°C for approximately 30 min.

a. Punch in- and outlets in the PDMS to later connect the needles (Figure 2A). Place the PDMS-chip with the structures facing upwards on another piece of PDMS, take a biopsy puncher with a diameter of 0.75 mm and punch straight in one movement from the designated inlet structure through the PDMS.

Note: The PDMS underlay will prevent ripping of the inlets. Curved inlets may lead to leakage during the experiment. Make sure that the punched PDMS remainder is not left in the inlet.

b. Clean the PDMS by washing it thrice with isopropanol and blowing it dry using compressed air. Take care to also remove any isopropanol from the punched in- and outlets.

c. Clean the glass substrate as described in 8.b. We use glass substrate with the dimensions 39.5 × 34.5 mm and a thickness of 170 µm. The dimensions can vary with the design (step 1.c), but the low thickness is important for oil immersion microscopy with the available objectives.

9. Bond PDMS structure on glass substrate.

a. Place both chip and glass substrate in a plasma generator with the future contact side facing upwards. Evacuate and apply an oxygen environment of 1 mbar. Create an oxygen plasma for 0.4 min (24 s) at 45% power (90 W).

b. Retrieve both chip and glass substrate from the plasma generator. Place the PDMS-chip with the activated side facing upwards on a particle free fabric (Figure 2B). Carefully and slowly lower the activate side of the glass substrate on the PDMS structures.

c. Observe as the glass bonds to the PDMS, which looks like adhesion between the two objects. If the bonding comes to a hold before the whole chip is bonded, use the scalpel or tweezer tip to very gently tap a non-bonded edge to solve this issue.

d. Bake the microfluidic chip for 3 min at 80°C (Figure 2C) to accelerate the bonding reaction. Alternatively, let the chip rest at 20°C for 30 min before use.

Pause point: The PDMS structure can be stored indefinitely. Bonded chips can also be stored, but they will regain their hydrophobic surface 15 min to 1 h after bonding,[5] leading to more challenging loading procedures (step 29). We recommend bonding the chip immediately before the experiment.

Note: If you want to prepare the PDMS-mixture in advance or mold multiple times a day, you can leave the mixture for approx. 6 h at 20°C or for 24 h at 4°C. As the polymerization reaction is active at 20°C, the PDMS-mixture will become increasingly viscous with time, but it is still usable for molds as long as you can properly pour it. By storing it at 4°C, you slow down the reaction, keeping the mixture fluid for a longer time.

Note: For cleaning multiple PDMS chips, you can also place them sequentially in an isopropanol bath and a ddH_2O bath and sonicating them for 1–2 min. Do not leave PDMS in isopropanol for long, as it will swell.

Preparation of the microfluidic flow periphery

Timing: 30 min

10. Prepare the tubing.
    a. Cut one tube for each in- and outlet.

Note: The length of the inlet tubing should be sufficient to reach from the bottom of the pressurized medium reservoir to the chip without any tension in the tubing (80 cm). The outlet tubing needs a sufficient length (20 cm) to reach the waste collection without tension when placed under the microscope (step 29.e).

    b. Each tube connection to the chip is equipped with a hollow needle. The inlet tubing needs to be prepared for the attachment to the pressurized reservoirs (see Figure 3A). The final pump and tubing set-up can be seen in Figure 7.
11. Prepare the medium.
    a. Prepare the medium for growth experiments (here CGXII for *C. glutamicum*) as described in the materials and equipment section. For flow profile establishment, use diluted ink (10% v/v in water) of different colors.
    b. Filtrate the medium with syringe tip filters with a pore size of 0.2 μm to remove any particles that can alter the flow in the chip by blocking channels. You can filter the medium directly into the pressurized reservoirs.
12. Assemble tubing and medium reservoirs. Screw the pressure cap onto the medium reservoir. The tubing should reach as deep as 0.5 cm above the bottom of the reservoir.

⚠ CRITICAL: Use the same length of tubing for all experiments that use the same flow profile. Varying the tubing length will also vary the backpressure, possibly shifting the flow profile.

Note: The loading tubing can later on be used as outlet tubing to save resources.

Flow profile establishment

Timing: 1 h

13. Set-up the microfluidic experiment.
    a. Place the microfluidic chip on an inverted microscope.
    b. Connect the pressure driven pumps to the red and blue ink reservoirs. Set a low pressure (15–20 mbar) until the tubing is filled and the liquid forms a drop at the needle tip.
    c. Insert the needle into the chip's inlets. The needle should be inserted to fill 75% of the inlet, but not touching the glass bottom.
    d. Increase the pressure on both inlets briefly to 150 mbar to flood the chip with liquid and remove trapped air bubbles from the inlets and the chip.
    e. Connect the outlet tubing as soon as a drop forms on the outlet.
    f. Let the ink flow through the microfluidic structure until no air bubbles are left in the channels.
14. Establish the flow profile.
    a. Change the pressure of the two pumps, so that you can replicate the profile shown in Figure 4A. You can start with the given pressure pairs for flow profile P1 and P2. The laminar boundary layer between the red and blue liquid should be in the middle of the channel dividing the array pairs.

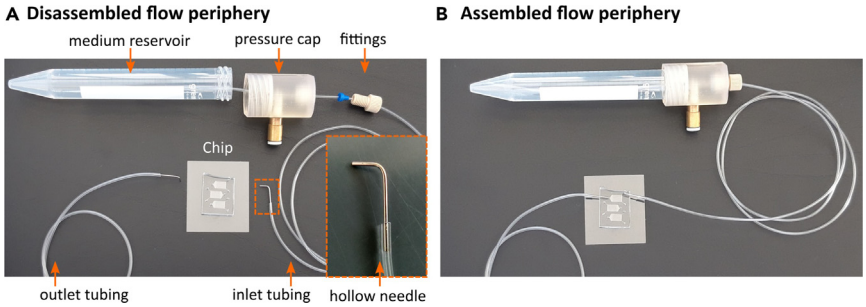

Figure 3. Flow periphery assembly

(A) The inlet tubing is threaded through the fitting and the pressure cap. A hollow needle is attached to the end, which will be connected to the microfluidic chip. The outlet is equipped with a hollow needle.

(B) When assembled, the inlet tubing reaches into the cone of the medium reservoir, not touching the bottom.

b. Test the shift between P1 and P2. For precise switches, the laminar boundary layer should shift in less than 3 s (see Methods video S1). If it takes longer, increase the inlet pressure and repeat step 14.a.

15. Program the pumps in A-i-O software according to your temporal flow profile (Figure 4B).

Pause point: The spatial flow profile has to be established only once for the given structure in the fabricated channel and chamber height with a set tubing length (80 cm) and can be applied for all future experiments.

Note: Test your established spatial flow profile on three chips with the same microfluidic structure to estimate potential shifts in the profile.

Note: The design of the microfluidic structure is designed to fit several possible operation techniques. The laminar boundary layer can be shifted between each array pair (Figure 5A). This allows to adjust, for example, the size of the control zones. Instead of oscillating between P1 and P6, an oscillation between P2 and P5 can be applied, increasing the size of the control region to four arrays on each side. Alternative operations techniques can also include the sequential profile change (Figure 5B, Methods video S2). Here, the array pairs are immersed step by step in the other liquid. The sequential profile change enables the simultaneous screening of five pulse durations in just one microfluidic structure, thereby drastically increasing experimental parallelization. Furthermore, the pump program can easily be adapted to cultivation experiments with other temporal flow profiles (Figure 5C). Next to the symmetric oscillation profile introduced in Figure 4, switches between media conditions, pulses of a medium or asymmetric oscillations are possible.

Measurement of chamber medium exchange

Timing: 1 h

Note: This step is only necessary when your experiments include very fast oscillations (<10 s) between media conditions or if you test new chamber heights. With this procedure, you can validate, that the medium inside the chamber is exchanged to 95% even during oscillation.[1]

16. Set-up the microfluidic experiment according to step 13 with the following modifications:
    a. Instead of blue and red diluted ink, use 70% (v/v) ethanol and 0.3 g/L fluorescent yellow dye in 70% (v/v) ethanol.
    b. Mount the chip on the fluorescence microscope (see step 27 and 28 for details.)

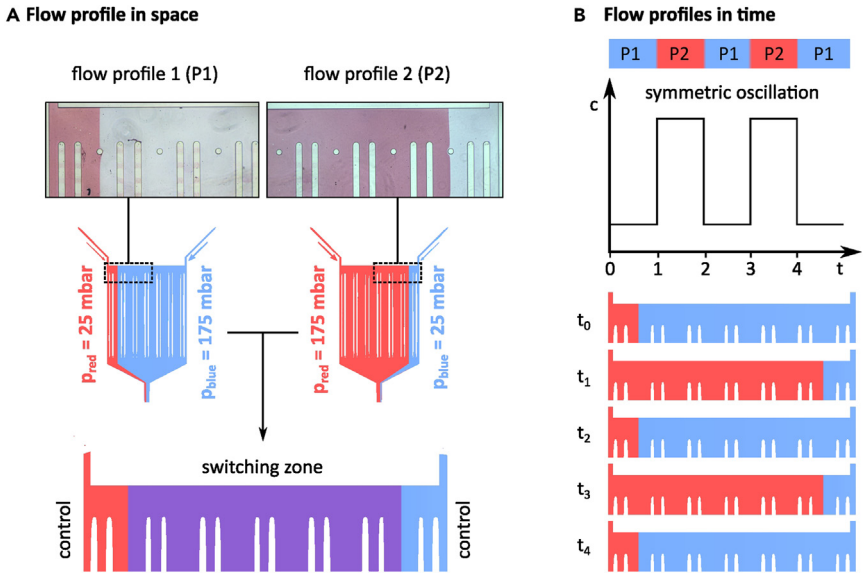

Figure 4. Flow profile establishment in space and time

(A) Two different liquids, here red and blue, are attached to the inlets. When the depicted pressure is applied to the liquid reservoirs, the liquids flow through the chip and form a boundary layer where they meet. We call the resulting spatial liquid distribution "flow profile", which can be seen in the photos. The pillars in the photos are an optional design addition, that were introduced to further support the PDMS and prevent chamber collapsing. The flow profile can be changed by changing the pressure applied on the liquids, and therefore the flow, which shifts the laminar boundary layer across the chip. With flow profile P1, only the left array pair is submersed in red liquid, while switching to P2 leaves six array pairs in red liquid. The chip can therefore be divided into control zones, which are always exposed to the same liquid and a switching zone, across which the laminar boundary layer moves when changing the flow profile.

(B) Alternating between P1 and P2 creates an oscillation of liquids for chambers in the switching zone.

17. Adjust the microscope settings.
    a. Use the YFP-channel with an intensity of 15% and an acquisition duration of 100 ms.
    b. When you do a 5-s-oscillation, the chambers will experience 5 s of fluorescent-dye-medium and 5 s of ethanol. In each medium condition, 5–10 pictures should be taken to capture the dynamics. Therefore, set the picture acquisition frequency to 0.5 s
    c. Capture at least 10 complete oscillation intervals. In this specific case, capture images for 100 s.
    d. Choose one chamber and capture the position.
18. Start the microscopic acquisition and the prepared pump program (see step 15) at the same time. There will be a short (1–3 s) delay between the pump and the acquisition due to manual starting of both programs.
19. After this experiment, measure the mean gray value of the chamber of the YFP channel in each frame to quantify the medium exchange time (Figure 6). Normalize the data to the minimum-maximum-range. This facilitates the comparison between different chamber heights or chamber designs.

Note: While we use an intensity of 15% to detect the fluorescent dye, other intensities and other dyes can be applied to measure the medium exchange in monolayer growth chambers. To compare results between different intensities and dyes, we recommend comparing the relative gray values, which are normalized to the minimum-maximum-range. If you use other intensities, take care to not over-expose the images, as information will otherwise be lost. Furthermore, the dye should not excessively stain PDMS, as this shifts the minimum and the maximum intensities.

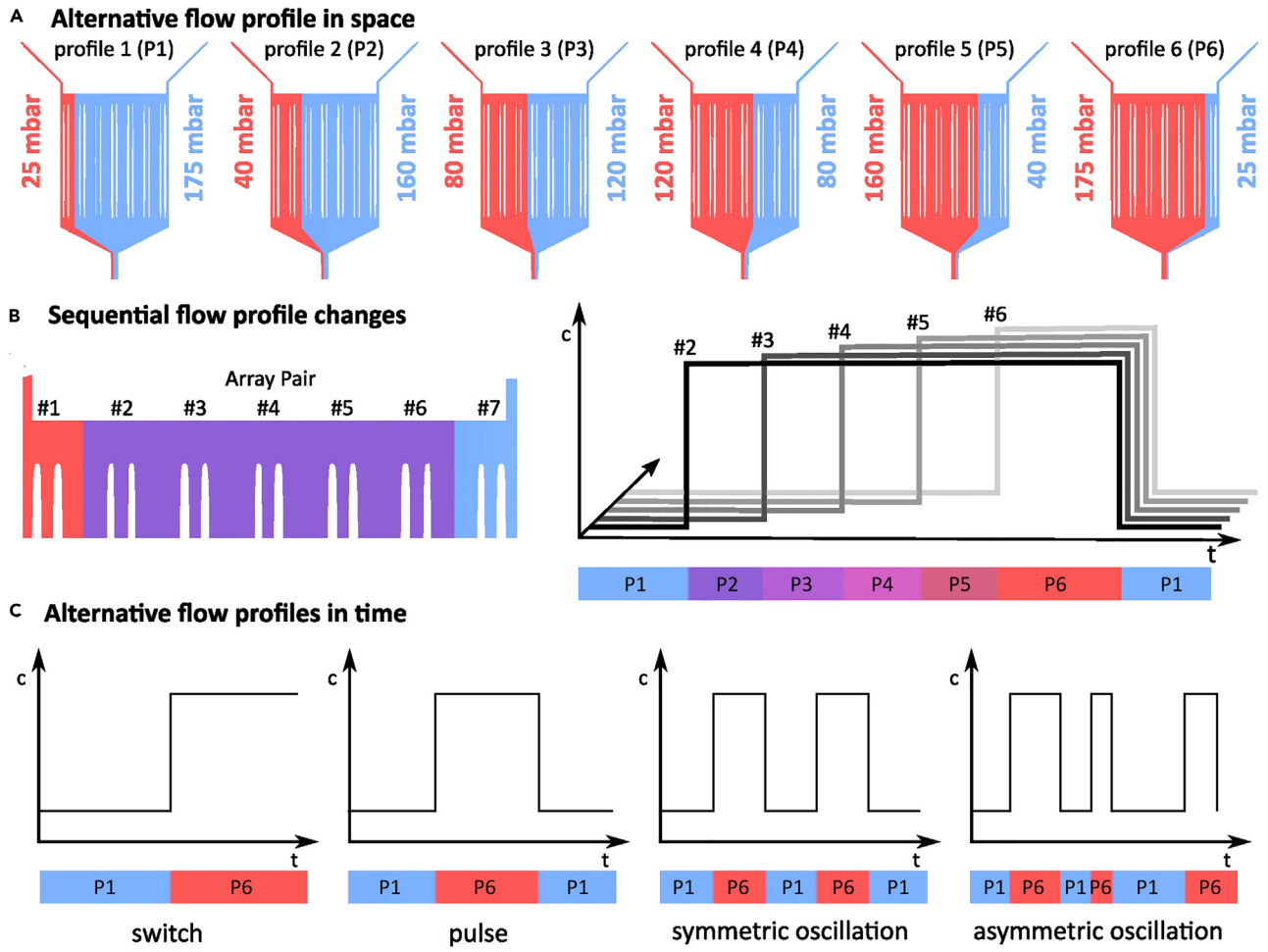

Figure 5. Flow profile variations

(A) Different pressure pairs on the medium reservoirs lead to different spatial flow profiles (P1-P6), allowing the adaption of the medium exchange to different experimental needs.

(B) For example, a sequential change in flow profiles can be applied to test 5 different pulse durations in one experiment, as the array pairs are sequentially immersed in the red liquid.

(C) Profiles can also be changed in time to resemble switches, pulses, symmetric or asymmetric oscillations for chambers in the switching zone.

Working cell bank of *C. glutamicum*

Timing: 1 day

Note: To reduce biological differences between experiments, we use glycerol stocks as a working cell bank that was prepared from the same main culture.

20. Prepare CGXII-medium for 10 mL preculture in a 100 mL shaking flask and for 30 mL main culture in a 300 mL shaking flask (see Materials and equipment for CGXII).

Note: We prepare the medium maximally 24 h before use. Until then, we store it at 4°C.

21. Inoculate the preculture from your *C. glutamicum* master cell bank and cultivate it for 16 h at 30°C and 120 rpm on a shaker with a shaking throw of 25 mm.
22. Inoculate your main culture from the preculture with $OD_{600} \approx 0.5$. Cultivate the main culture at 30°C until it reaches the early exponential phase ($OD_{600} \approx 2–3$).

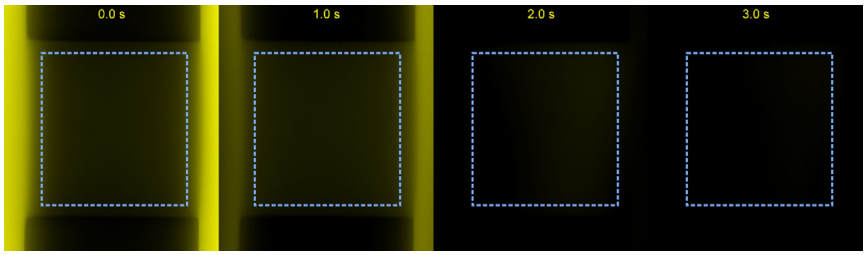

Figure 6. Measurement of chamber medium exchange

After switching from fluorescent dye to ethanol, the fluorescence intensity decreases from the left panel to the right within seconds

The mean gray value within the blue rectangle is measured to quantify the medium exchange.

23. Prepare the cryostocks.
    a. Cool the main culture on ice. Mix the main culture with sterile and cold (4°C) glycerol in a 50 mL reaction tube. The glycerol content should be 25% (v/v) in the final mixture.
    b. Aliquot the mixture to micro-reaction tubes and immediately freeze them in liquid nitrogen.
    c. Store the working cell bank at −80°C.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
γ-Butyrolactone	Carl Roth	Cat#: HN98.3
Mr-dev 600 negative photoresist developer	Micro resist technology	mr-Dev 600
PDMS	Dow Corning	Sylgard 184 Silicone
SU-8 (2) negative photoresist	Kayaku (formerly MicroChem)	SU-8 (2)
SU-8 (10) negative photoresist	Kayaku (formerly MicroChem)	SU-8 (10)
$(NH_4)_2SO_4$	Carl Roth	Cat#: 3746.1
Urea	Carl Roth	Cat#: 3941.1
KH_2PO_4	Chemsolute	Cat#: 1648.1000
K_2HPO_4	Carl Roth	Cat#: P749.1
$MgSO_4 \times 7\ H_2O$	Carl Roth	Cat#: 8283.2
MOPS	Carl Roth	Cat#: 6979.3
D-Glucose monohydrate	Carl Roth	Cat#: 6780.1
Citric acid	Carl Roth	Cat#: 5110.3
$CaCl_2 \times 2\ H_2O$	Carl Roth	Cat#: 5239.2
D-Biotin	Carl Roth	Cat#: 3822.1
$FeSO_4 \times 7\ H_2O$	Carl Roth	Cat#: P015.1
$MnSO_4 \times H_2O$	Carl Roth	Cat#: 4487.1
$ZnSO_4 \times 7\ H_2O$	Carl Roth	Cat#: K301.1
$CuSO_4 \times 5\ H_2O$	Carl Roth	Cat#: 8175.1
$NiCl_2 \times 6\ H_2O$	Carl Roth	Cat#: 4489.1
KOH	Carl Roth	Cat#: 6751.1
Yellow fluorescent dye	Lanxess	Macrolex® Fluorescent Yellow 10GN
Deposited data		
Microfluidic time-lapse images	This work	Figshare: https://doi.org/10.6084/m9.figshare.22140272
Mask Design for dMSCC (CelWin file)	This work	Figshare: https://doi.org/10.6084/m9.figshare.22140365

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Fiji Macro for pre-processing	This work	Figshare: https://doi.org/10.6084/m9.figshare.22140341
Fiji Macro for cell counting	This work	Figshare: https://doi.org/10.6084/m9.figshare.22724981
Experimental models: Organisms/strains		
Corynebacterium glutamicum	DSMZ	ATCC 13032
Software and algorithms		
CleWin Layout Editor	MESA+ Institute	Version 4.0.1
NIS-Elements Advanced Research	Nikon	5.21.02
Fiji (is just ImageJ)	Schindelin et al.[6]	1.53t
Fluigent All-in-One 2019	Fluigent	19.0.0.2
Microfluidic Automation Tool 2019	Fluigent	19.0.0.1
Other		
Camera	Nikon	FX CMOS - Nikon DS-Qi2 (Cat#: MQA17599)
Cage-type incubator for microscope	OKO Lab	OKO-H201
Cuvettes, PS semi-micro single use	Brand	Cat#: 759015
Filter Cube YFP (Ex: 500/24 nm; DM: 520 nm; Em: 542/27 nm)	Nikon	YFP-2427B (Cat#: MXR00707)
Glass substrate	Schott	D 263 R T eco (39.5 × 34.5 × 0.175 mm)
Immersion oil	Nikon	Type N for Microscopy 8cc
Light source (Diascopic phase contrast)	Nikon	Cat#: MEE59920
Light source (Episcopic fluorescence)	Lumencor	Sola SE II Set
Mask	Deltamask	4-inch lithography mask
Mask aligner	Süss MicroTec	MJB3
Microscope, inverted	Nikon	Nikon Eclipse Ti2-E (Cat#: MEA54000)
Objective, CFI P-Apo DM λ 100× Oil, DM PH3	Nikon	Cat#: MRD30205
Perfect Focus System TI2-N-ND-P	Nikon	Cat#: MEP59394
Photometer OD600 DiluPhotometer	Implen	Cat# OD600-1020
Plasma cleaner	Diener electronics	Femto
Plug connections, 90° curved, 0.33 mm inner diameter	Nordson EFD	Cat#: 7018316
Pressure cap	Fluigent	Cat#: P-CAP15-LP
Pumps, pressure driven from Line-up EZ series	Fluigent	Cat#: EX-01000001 MFCS-EX
Puncher (0.75 mm)	WellTech	Cat#: 504529
Silicon wafer, 4-inch	Microchemicals	Cat#: WSA40525155B1314SNN2
Tubing, 0.51 × 0.51 mm, Tygon®	Omnilab	Cat#: 5205508

MATERIALS AND EQUIPMENT

We use the minimal medium CGXII[7] without the MOPS buffer in microfluidic experiments, as it is chemically defined and can easily be adapted to the research question. When oscillating the carbon source availability, oscillate between the medium described below and the same medium without glucose. We changed the iron chelator from protocatechuic acid to citric acid, because protocatechuic acid was found to function as carbon source in microfluidic experiments.[8]

CGXII medium

Mix the CGXII medium from the stock solutions below in the given concentration in a sterile environment.

Reagent	Final concentration	Amount
CGXII base (1×)	1×	46 mL
D-Glucose (500 g/L)	40 g/L	4 mL

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Continued		
Reagent	Final concentration	Amount
$CaCl_2$ (1000×)	0.09 mmol/L	50 μL
Biotin(1000×)	1 μmol/L	50 μL
Citric acid (1000×)	1 μmol/L	50 μL
Trace element solution (1000×)	1×	50 μL
Total	**N/A**	**50,2 mL**

Store at 4°C for a maximum of 24 h.

CGXII base		
Reagent	Final concentration / mmol/L	Amount
$(NH_4)_2SO_4$	151	20 g
Urea	83	5 g
KH_2PO_4	7	1 g
K_2HPO_4	6	1 g
$MgSO_4 \times 7\ H_2O$	1	0.25 g
MOPS (only for shaking flask)	200	42 g
ddH_2O	N/A	918 mL
Total	**N/A**	**918 mL**

Autoclave for 20 min at 121°C and 1 bar overpressure for sterilization. Store at 20°C for maximally one year or until precipitate forms.

Note: Adjust pH to 7 using KOH and autoclave for sterilization. The base will yield 1 L of medium after adding every supplement. For microfluidic experiments, no MOPS-buffer is needed as the pH is not altered by cell metabolism.

D-Glucose 500 g/L stock solution		
Reagent	Final concentration	Amount
D-Glucose monohydrate	550 g/L	550 g
ddH_2O	N/A	To 1 L
Total	**N/A**	**1 L**

Autoclave for 20 min at 121°C and 1 bar overpressure for sterilization. Store at 20°C for max. one year.

Note: As we use the monohydrate of glucose for the stock solution, 550 g are needed for a stock solution of 500 g/L of glucose.

Note: Solve the glucose completely before fine adjusting the volume. Please note, that heat sterilization causes degradation of glucose.[9]

Citric acid 1000× stock solution		
Reagent	Final concentration	Amount
Citric acid	1 mmol/L	0.019 g
ddH_2O	N/A	To 100 mL
Total	**N/A**	**100 mL**

Autoclave for 20 min at 121°C and 1 bar overpressure for sterilization. Store at 4°C in 1 mL aliquots for max. one year.

$CaCl_2$ 1000× stock solution		
Reagent	Final concentration	Amount
$CaCl_2 \times 2\ H_2O$	0.09 mol/L	1.32 g
ddH_2O	N/A	To 100 mL
Total	**N/A**	**100 mL**

Autoclave for 20 min at 121°C and 1 bar overpressure for sterilization. Store at 4°C in 1 mL aliquots for max. one year.

Biotin 1000× stock solution		
Reagent	Final concentration	Amount
D-Biotin	1 mmol/L	0.02 g
ddH_2O	N/A	To 100 mL
Total	**N/A**	**100 mL**

Sterilize by filtration through syringe tip filter with a 0.2 μm pore size. Store at −20°C in 1 mL aliquots for max. one year.

Trace elements 1000× stock solution		
Reagent	Final concentration / mmol/L	Amount
$FeSO_4 \times 7\ H_2O$	36.0	1.0 g
$MnSO_4 \times H_2O$	59.2	1.0 g
$ZnSO_4 \times 7\ H_2O$	3.5	0.1 g
$CuSO_4 \times 5\ H_2O$	1.9	0.031 g
$NiCl_2 \times 6\ H_2O$	0.1	0.002 g
ddH_2O	N/A	To 100 mL
Total	**N/A**	**100 mL**

Sterilize by filtration through syringe tip filter with a 0.2 μm pore size. Store at 4°C in 1 mL aliquots for max. one year.

⚠ CRITICAL: $NiCl_2$ is toxic and a health hazard. According to H and P phrases, avoid skin contact by wearing protective gloves. Rinse thoroughly with water if you had contact.

STEP-BY-STEP METHOD DETAILS

This section describes how to set up the actual dynamic microfluidic single-cell experiment after preparations are completed. The preparations in "before you begin" can be applied to multiple dMSCC experiments, as long as the chip design does not change.

Seed train

Timing: 20 h

This step describes the preculture procedure for each microfluidic cultivation experiment. A reproducible seed train from the working cell bank to the chip seeding ensures reproducible starting conditions for microfluidic cultivations.

⚠ CRITICAL: Do not refreeze a glycerol stock once thawed. It is possible to prevent thawing by keeping the glycerol culture on dry ice.

1. For the preculture, inoculate 10 mL CGXII-medium in a 100-mL-baffled shaking flask from a glycerol stock aliquot and incubate over night at 30°C on a shaker at 120 rpm.
2. From the preculture, inoculate the main culture at an OD_{600} of 0.05–0.1. Incubate the main culture on a shaker at 30°C for 3–4 h, until the OD_{600} increased by at least a 1.5-fold.

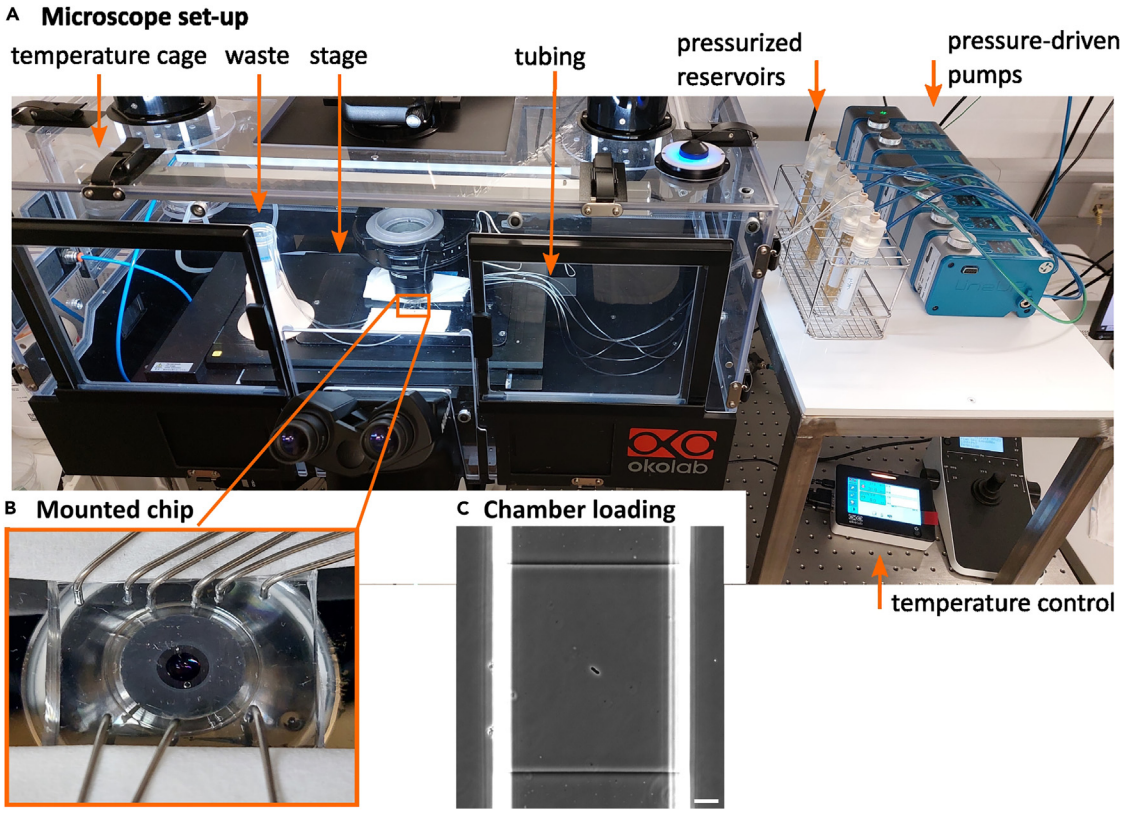

Figure 7. Experimental set-up for dynamic microfluidic single-cell cultivation

(A) This photo shows the complete experimental set-up at the microscope. Note, that the tubing needs to be sufficiently long (approx. 80 cm), that it will not put any tension on the chip during stage movement (step 10.a).

(B) The chip is connected via hollow needles to the pressurized reservoirs and the waste. Place a tissue beneath the needles to soak up any potential leakage. Take care, that the needles and tubing do not obscure the imaging region.

(C) Optimally, one single bacterial cell is caught in the center of the chamber after the loading is finished. The scale bar in C is 10 μm.

Note: Inoculate the main culture before preparing the microfluidic chip and the media for the optimal temporal workflow.

Live-cell imaging

Timing: 1 day, 3 h hands-on-time

The aim of this step is to start the dynamic microfluidic cultivation and the time-lapse live-cell imaging.

3. Turn on the temperature control of the microscope cage (Figure 7A). Let the objects inside the cage warm up, so that no temperature-based material expansion takes place during measurement.
4. Prepare the flow periphery (step 10), the medium (step 11) and the microfluidic chip (step 8 and 9).
5. Connect the periphery and place the mounted chip as shown in Figure 7A.
6. Give a drop of immersion oil on the 100× oil objective (Figure 7B).
7. Place the chip mounted on a holder on the microscope's stage and fixate the holder.

⚠ CRITICAL: If the holder is not properly fixed, this will result in a shifting region of interest during live-cell imaging.

Note: We use a self-fabricated holder that has a cut-out in the size of our glass substrate. We tape our chip to the holder from below (see Figure 8). If you use a similar system, take care to tape the chip to the holder on its entire perimeter. This prevents possible leakage to flow into the microscope. Furthermore, the tape should be on the very edge of the chip (Figure 8A). Otherwise, the immersion oil touching the edges will move along the tape on touch. This thins the oil film, leading to focus problems during live-cell imaging.

⚠ CRITICAL: Not all immersion oils are suitable for longer (> 4 h) imaging, increasing in viscosity with time and thus leading to focus problems. If you use a different oil than proposed in this protocol, try its performance during a longer imaging session first.

8. Load the cells into the microfluidic chambers.
   a. Fill a 1-mL-syringe with the bacteria suspension from the main culture.
      i. If the OD_{600} exceeds 0.3–0.4, dilute it accordingly using medium.
      ii. Connect the syringe to the loading tubing and fill the tube with suspension.
   b. Insert the needle connected to the syringe with the suspension into the outlet (see Figure 1C) of the microfluidic structure.
   c. Flush the structure very gently with suspension and remove emerging droplets from the inlets with a tissue.
   d. Check at least 10 chambers across the control and switching zones to see, if enough chambers contain an adequate number (1–4) of cells (Figure 7C).
   e. After loading, do not remove the drops formed on the inlets.

   Note: If the number of chambers with loaded cells is too low, flush a little more forcefully in a pulsating manner, so that the flexible PDMS is raised slightly. If the loading is still insufficient, insert an air bubble into the channels and move them back and forth along the chamber arrays. An air bubble can be inserted via the outlet by reversing the flow or via the inlet by re-inserting the needle without a drop on the needle's tip.

   Optional: Remove any cells present in the channels and inlets by flushing the chip with a syringe of medium. Insert the medium-needle into the outlet. This procedure decreases the risk of inlets overgrowing with bacteria during the cultivation.

   f. Start the pumps with a pressure of 15–20 mbar.
   g. When the first drop of medium forms at the needle tip, insert the needles into the inlets.

   Note: Merging the drops on the needle tip and the inlet will assure an air bubble-free connection. Air bubbles in this stage of loading will most likely lead to overcrowding of the chambers (Figure 14A).

   h. Increase the pressure of the pumps to the level that has been chosen as pressure pair of flow profile P1 in step 14.
   i. Attach the outlet tubing when the first drop forms on the outlet.
   j. Fixate the tube's end to the waste container using tape. Make sure, that all tubing is not under tension.
9. Center and focus the light path using Köhler illumination and adjust the phase ring according to the manufacturer's manual.
10. Set the phase contrast channel with a light intensity of 10% and the exposure time of 100 ms for the acquisition.
11. Adjust the frequency of acquisition to 10 min.
12. Search for chambers with one to four cells in both control regions and the switching zone and save the positions for time-lapse imaging.

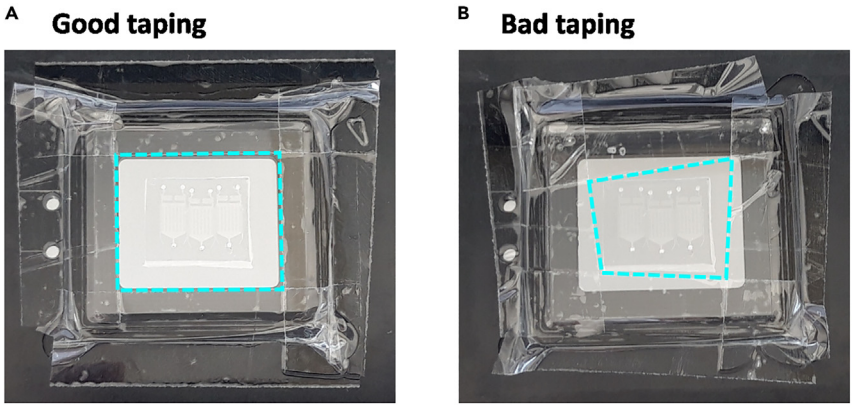

Figure 8. The microfluidic chip is attached to the bottom side of the holder using adhesive tape

(A and B) The tape should stay clear of the cut-out window (A). The chip should not be taped as depicted in (B), as this will lead to contact of the adhesive tape with the immersion oil film, resulting in focus problems.

Note: Depending on the number of positions, the experiment's duration, the acquisition frequency and the number of fluorescent channels, a lot of hard drive storage space will be needed for the data. Depending on the field of view one image has a size of approx. 12 MB. For an experiment observing one channel for 20 h with images taken every 10 min for 80 positions, 115 GB will be necessary. The size can optionally be significantly decreased by acquiring only the region of interest (the chamber with an additional radius of 10–15 μm) and not the whole field of view.

Note: The image frequency depends strongly on the research question. As we analyze the growth of *C. glutamicum*, which has a medium-dependent growth rate of 0.46–0.61 1/h,[8] we acquire an image every 10 min to have approx. 10 images per division.

13. Start the acquisition and the pump program (step 15) at the same time.

⚠ CRITICAL: Check the running experiment for focus loss after 1 h. Readjust the focus if necessary.

14. End the acquisition after the planned experiment duration and tidy up.
    a. Stop the image acquisition and the pump program.

Optional: After finishing the experiment, you can check the spatial flow profile to see, if the experiment was subjected to technical flaws. Turn off the pumps, exchange one medium reservoir with an ink reservoir. Turn the pumps on and set the pressures according to flow profile P1 and P2. Observe the critical regions for the correct spatial flow profile (Figure 4A). This works best with dark ink.

    b. Discard the chip and the tubing. Both will be inactivated by autoclaving.
    c. Detach the needles from the tubing and collect them in isopropanol.
        i. Once enough needles are collected, place them in ultrasonic bath for 90 min.
        ii. Discard the isopropanol and rinse the needles with ddH_2O water.
        iii. Remove any loose syringe adapters, as the isopropanol dissolves the glue.
        iv. Place the needles in water in an ultrasonic bath for 90 min.
        v. Discard the water and repeat step 14.c.iv.
        vi. Autoclave the needles in a glass petri dish for sterilization.
    d. Remove the oil film from the objective's lens with a soft tissue. Wipe the objective with an isopropanol-soaked tissue afterwards.

e. Clean the pressure caps and the tubing adaptors by washing them with dH_2O.

Image pre-processing

Timing: 3 h, 30 min hands-on-time

The aim of this step is to extract the usable data and prepare the images for further systematic image and data analysis. As a lot of digital storage space is required for time-lapse live-cell imaging, it is sensible to discard data that is not entirely usable.

15. Discard data not fit or necessary for the analysis during the cropping process in the microscope software.

Note: Some cells might get washed out of the chamber during the experiment. The data for this position cannot be used for cell number-based analysis or cell tracking. In this case, it can be discarded.

Note: If you chose to record the whole field of view, you can crop the edges of the acquired picture, so that only the chamber but not the channel is visible. This can decrease the image size by 50%.

16. Save the image sequences in a usable file format for data analysis.

Note: We use OME-TIFF stacks or Nikon-specific nd2 stacks, which are saved by using the command "split multipoint". Each stack contains the image data of one chamber.

17. We have the experience that the automated cell detection works best if no refraction from the chamber structures is visible in the region of analysis (Figure 9). Therefore, we pre-process the images using Fiji and a self-tailored macro (you can find the macro in the key resources table). The macro's steps can also be applied manually, as described in the following steps.
    a. Stabilize the movement of the images in a stack. We have good experience with using the plugin "Image Stabilizer" with a reference area on the PDMS structures.
    b. Rotate the chamber so that is straight.
    c. Crop the image stack so that no refraction from the chamber is visible anymore.
    d. Save the stack as TIFF file.
18. Discard data not fit or necessary for the analysis during the cropping process in the microscope software.

Image analysis

Timing: 3 h, 30 min hands-on-time

Note: In this section, we showcase one possible way to detect and count microbial cells from live-cell imaging pictures using a FIJI macro, which can be found in the key resources table. It applies k-means clustering and watershed algorithms prior to detection with "Analyze particles". This macro is specialized in detecting bacterial cells that are evenly dark in a phase contrast picture and it works best if cells have a bright halo in the picture to be clearly distinguishable from each other. It is simple to install and to use.

For some bacteria, it can be sensible to use other available tools for cell detection, like Omnipose[10] that can be integrated into FIJI using the "PTBIOP" plugin. For tracking cells in time, you can use TrackMate in FIJI or a python environment[11] which offers a wide band of detection options and tracking algorithms. An alternative tool for segmentation and tracking of bacteria in a python

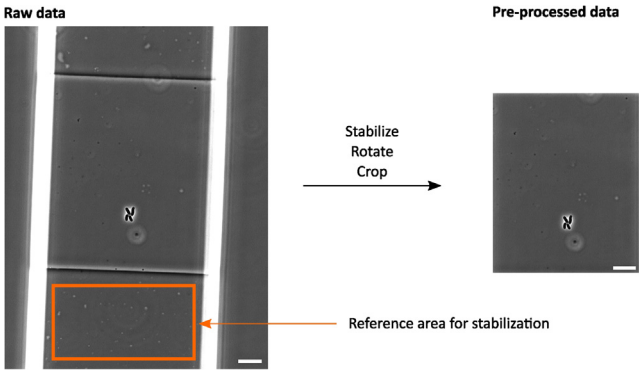

Figure 9. Data pre-processing
The raw data can be stabilized, rotated and cropped for further processing and analysis. Choose a region on the PDMS as reference for stabilization. The scale bar is 10 μm.

environment is DeLTA.[12] For tracking single cells in time, we recommend higher image frequencies (e.g., every 2 min) for better tracking results.

19. Working in FIJI, load the image stack from step 37.
20. Open the macro "colony segmentation" (see key resources table) by dragging the file into FIJI.
21. Set the initial image analysis parameters (Figure 10A).
    a. Draw a region of interest around the colony. You can also choose to analyze the whole chamber but note that this will take longer to analyze.
    b. Press "Run" in the macro editor.
    c. Set the boundaries for circularity and area for cell detection. Tick "Average values per frame" and "Single-cell values". If you experience difficulties in detection of cells, increase to number of clusters. Press "ok" to continue.
    d. In "Set Measurements", select the values that you want to have measured. Mean gray values are especially important for the analysis of fluorescence data. Press "ok" to continue.
22. Create a binary mask for cell detection (Figure 10B).
    a. After clustering, the macro will request you to set a threshold. The threshold result should look like Figure 10B. Press "ok" to continue. If the macro segments the background and not the cells, check if the box "black background" is ticked under Process > Binary > Options.
    b. The macro will prompt you to set the adjustable watershed for splitting cells that were wrongly connected during clustering (see red box in Figure 10B). Low values will split more cells, higher values less. You can use the preview to find the right value before continuing.
23. Detect the cells with "Analyze Particles" (Figure 10C). Some cells might not be counted (see red box in Figure 10C). You can either count them by hand with the multipoint tool or re-run the macro with different settings in clusters and detection boundaries (step 41).
24. Save the results table for further analysis.

EXPECTED OUTCOMES

Microbes in both their natural environment and in bioprocesses are faced with rapidly changing environments and nutrient availability. Previously, we were able to observe that a decrease in growth rate of *C. glutamicum* during nutrient oscillation is also dependent on the frequency of the applied starvation and not only on the starvation duration.[1] In the example presented in this protocol, we grew *C. glutamicum* in CGXII medium with 4% (w/v) glucose as carbon source. The control chamber experienced a stable environment, while we switched between a glucose containing and a glucose free medium every hour for the chamber in the switching region. For both the control and the switching region, we expected the colony to grow until it reached the chambers edge (Figure 11, control at

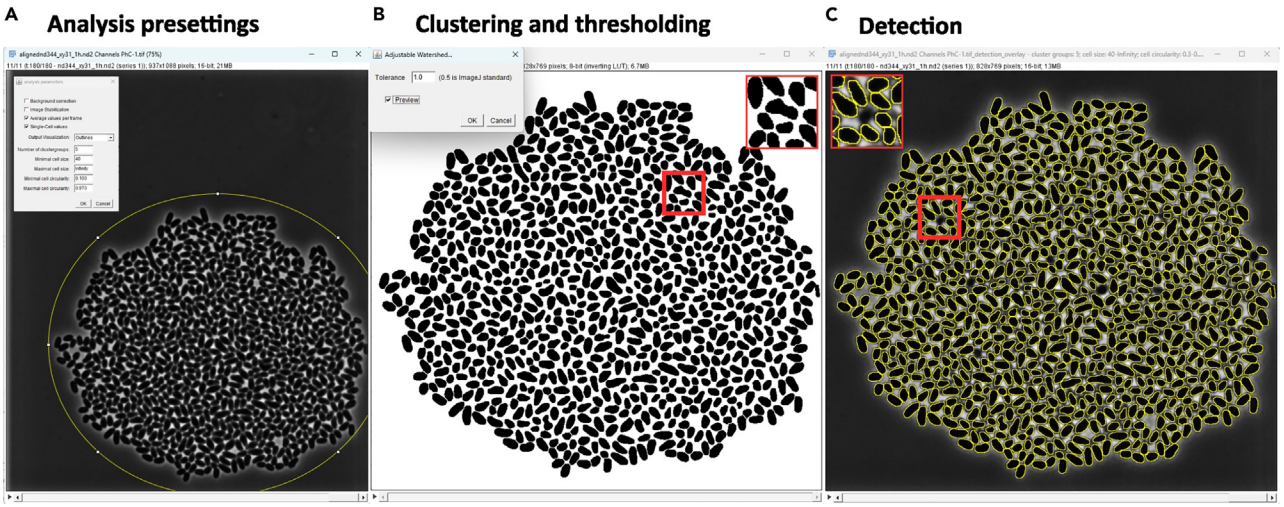

Figure 10. Image analysis using a colony segmentation macro in FIJI

(A) The analysis parameters like cell size, cell circularity and the region to analyze can be set in the beginning of the analysis.

(B) The colony segmentation macro binarizes the phase contrast picture by applying k-means clustering, thresholding and an adjustable watershed algorithm. The latter should be set to split cells that are still counted as one, as can be seen in the red box.

(C) The binary mask is then analyzed with "Analyze particles" to detect the cells. Cell detection sometimes misses a cell (red box). For this example, 849 cells were detected by the macro, failing to detect 7 and under-segmenting 4 cell pairs.

10 h). After that, cells would still divide, but the cell count in the chambers will stay the same. We chose to illustrate our dynamic microfluidic system with an 1 h glucose oscillation to highlight that the growth of the cells in the oscillation chamber is abruptly impaired during perfusion with glucose free medium (Figure 11, blue growth curve, Methods video S3). Next to the cell count, the colony area can also be plotted against time, showing even more clearly the impaired growth during starvation (see supplement Figure S1). Faster oscillations, as fast as 5 s, are also possible.[1,3]

LIMITATIONS

The dynamic microfluidic single-cell cultivation presented in this study is meant to observe the growth of microbes in switching media conditions. It is a simple and easy-to-use design compared to other microfluidic set-ups that use pneumatic valves and multi-layer chip fabrication processes (for example[13]). However, dMSCC is restrained by limitations inherent to microfluidic cultivations in general as well as technical limitation in the dynamic environments.

First of all, dMSCC is limited to organisms that can be trapped in the chamber. Cells can change their shape during the experiment, so that they eventually become smaller and get washed out. Organisms without a rigid cell wall will not be retained. Very small microbes can also not be retained. Fabricating flatter chambers (lower than 500 nm) increases the chance for chamber collapse, thereby decreasing the number of functioning MGCs substantially. It is possible to decrease the chamber size, so that the PDMS chamber ceiling is better supported by the walls. However, this also decreases the size for colony observation.

When choosing the MGC as cultivation region, the observation time of the microcolony is limited to the time point, when it reaches the chamber edges.[3] After this, the colony cannot be analyzed via cell count, but has to be analyzed via single-cell tracking.[12]

Similar to other microfluidic live-cell imaging experiments with constant medium perfusion, cultivation conditions in the chip are different to those in shaking flasks. The differences lie in cultivation mode, aeration principle, material and mixing.[14] For example, in the perfusion cultivation mode,

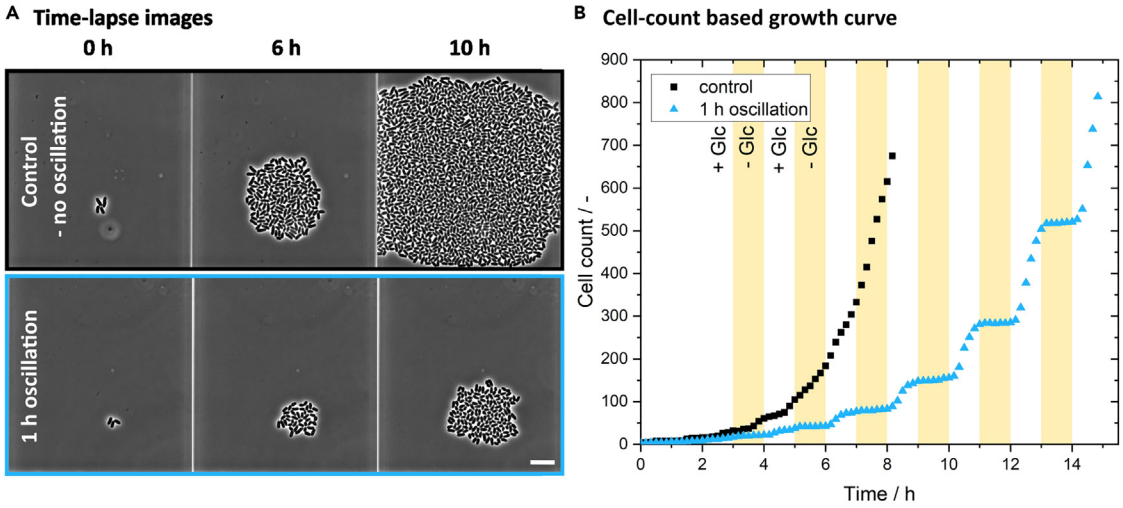

Figure 11. Expected Results of dMSCC in life-cell imaging for oscillating glucose availability

(A) Single cells grow into a microcolony. Depending on the oscillation applied by dMSCC, growth is impaired during the glucose-free conditions (blue box).

(B) Growth inhibition can also be observed in the cell-count based growth curves of the two chambers, where the yellow stripes indicate glucose-free growth time for the blue curve.

the medium is exchanged every few seconds. While this allows us to perfectly control the medium in its composition throughout the experiment, beneficial secreted factors and signal molecules are washed out. This can alter or even compromise the growth of this microbe in perfused microfluidic cultivation systems.

The medium reservoir size limits the experiment time. Medium reservoirs can be refilled during the experiment, but this bears the danger of introducing air bubbles and contaminations in the medium, as well as shifting the flow profile for a half a minute. The alternative would be to use larger reservoirs. Sensitive medium components will, however, start degrading, changing the medium conditions for the organism. Very long experiments with high imaging frequency will run into a limitation of digital storage space.

Furthermore, the single-cell analysis in dMSCC is currently limited to the optical readout of the microscopes. Analysis of substrate consumption, production or application of Omics-technology is not possible with this specific microfluidic set-up. In other systems, optical traps are used for single-cell extraction from the microfluidic chip for further analysis,[15] while other microfluidic techniques like droplets can be analyzed with mass spectrometry.[16]

Specifically for dMSCC, the environmental oscillation is limited to non-gaseous, soluble factors like C-source, N-source, pH and inducer molecules. Oscillations in solved oxygen or carbon dioxide are not possible due to the gas permeability of PDMS. Furthermore, the combination of possible oscillation environments is limited to two media conditions with this design. There is also no possibility to mix the media to achieve gradual transition from one medium to the other.

When conducting very fast oscillations (5s), it is not possible to capture the fast dynamics in multiple chambers because of image acquisition time. Therefore, a balance between a high temporal resolution with very few chambers or a low temporal resolution with many chambers has to be found, which is dependent on the research question. It has to be noted that very frequent image intervals can have an influence on the microbial growth behavior, especially when using fluorescent proteins.[17,18]

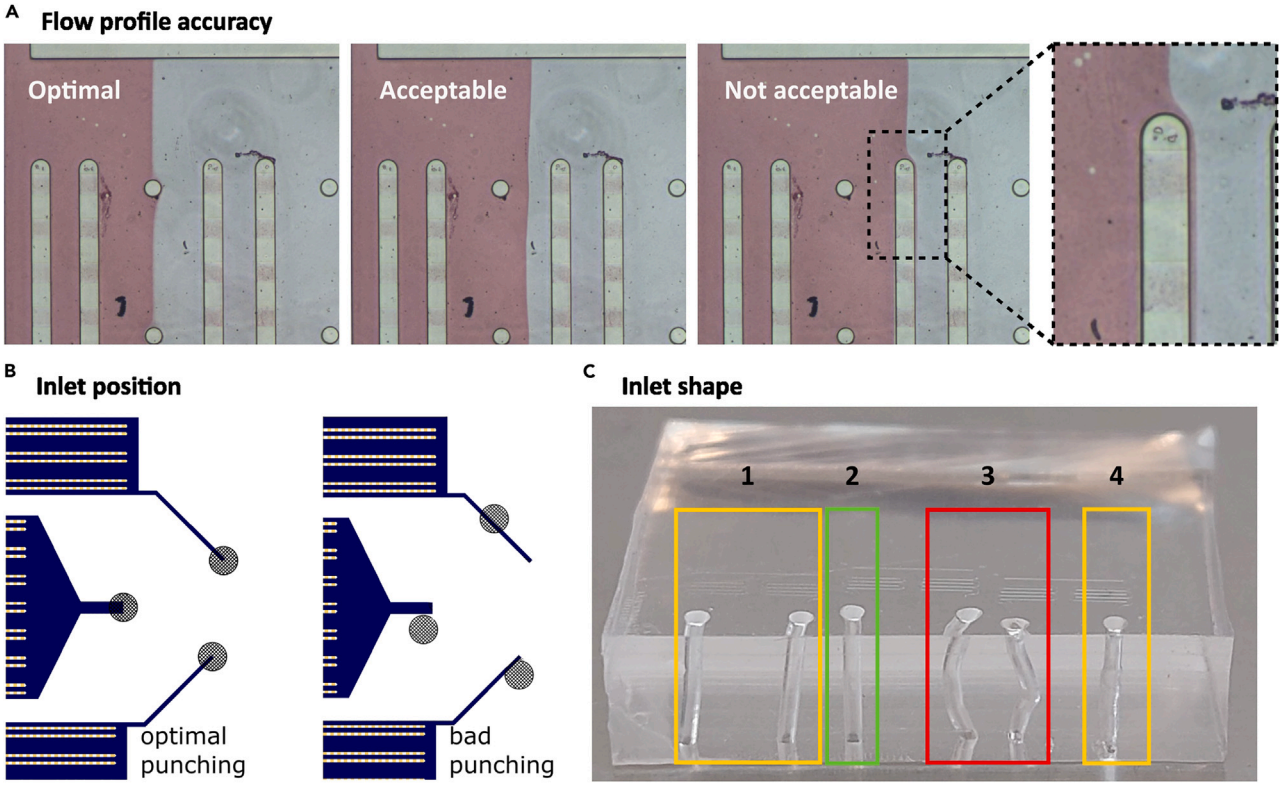

Figure 12. Possible causes for unreliable or inaccurate spatial flow profiles

(A) The laminar boundary layer can be shifted.

(B) Inaccurate inlet punching can lead to a profile shift.

(C) The inlet shape influences possible leakage. Inlet 2 is a perpendicular, straight and not ripped. Inlets in 1 are slightly tilted, but leakage is unlikely. Inlet 4 is ripped at the bottom, which might lead to leakage. The inlets in 3 are curved, which will lead to leakage.

TROUBLESHOOTING

In the following section, we describe the five challenges that we frequently experience in our daily lab work with dMSCC and how we tackle them. More inspirations on how to perform and troubleshoot microfluidic cultivation in general can be found in Täuber et al. 2021.[19]

Problem 1

The spatial flow profile is inaccurate or unreliable (see Figure 12A). This is most frequently observed during flow profile establishment or after the dynamic microfluidic cultivation. There, it can be identified by unexpected growth behavior of microbes or by the post-experimental flow profile check (see step 34).

Potential solution

The flow profile is inaccurate or unreliable if the flow is altered compared to the initial establishment of the spatial flow profile. Potential solutions are:

- Punch the inlets accurately and orthogonally (see Figure 12B). This is facilitated if the in- and outlets are far enough apart, which can be influenced during chip design (step 2.b.i).
- Curved inlets lead to leakage at the needle (Figure 12C). This also alters the flow. Take care to punch straight inlets and that the inlet needles are not askew (step 8.a).
- Particles or cell aggregates can block channels and thereby alter the flow profile. This risk can be decreased by filtrating the medium before use (step 11.b). It is also possible, that crystals form at the laminar boundary layer, because the two media cause precipitation where they meet on the chip.

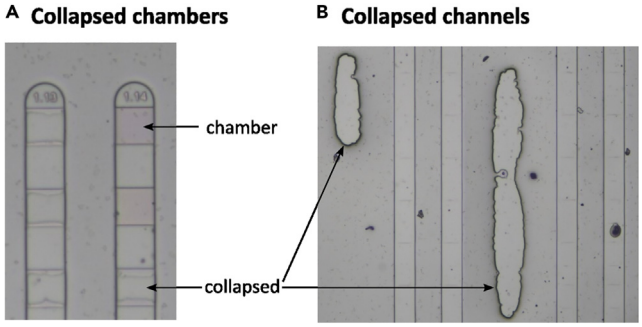

Figure 13. Collapsed chambers (A) and microfluidic channels (B)

- Take care to establish an accurate flow profile in step 14.a. Test this profile in three separate microfluidic structures to determine its reliability. If you still observe large shifts (Figure 12A), increase the pressure for higher flow. This can stabilize the profile.
- Repeated usage of the tubing fittings will wear them out. We found that older tubing fittings often decrease in the size of their inner diameter. This decreases the diameter of the tubing as well, leading to a different flow at the same pressure settings. Changing the tubing fittings when a decrease in diameter is noticed, is therefore beneficial for the reliability of the flow profile.

Problem 2

The MGC on the microfluidic chip collapsed (Figure 13). Under the microscope, no chamber can be observed and no colored light refraction from the chambers can be seen with the eye. This issue arises frequently directly after chip bonding (step 9) or during the loading process (step 29). Chambers tend to collapse when the PDMS is too close to the activated glass substrate (step 9.a). The problem increases with decreasing chamber height. The collapse can be caused by pressure being applied onto the chip during chip assembly (step 9.c) or during tubing connection (step 13.c and 29.b).

Potential solution

The risk of chambers collapsing can be decreased with these potential solutions:

- During chip assembly (step 9.b), lay the glass substrate onto the PDMS-chip and slowly converge them.
- If you need to touch the chip because the bonding stopped before it was completed (step 9.c), do not touch directly onto the chamber region.
- If it is possible for your organism, use a higher chamber height (step 3). However, an increase in chamber height can decrease the trapping efficacy during loading (step 29) and during cultivation (step 33).
- Let the chip rest for 10 min after activation (step 9.a) in a particle free environment e.g., flow bench, clean bench or petri dish. This will decrease the hydrophilicity of the activated PDMS, decreasing the attractive forces between chip and glass. However, the bonding can be weakened, possibly leading to chip de-attachment.
- Avoid large temperature fluctuations between bonding (step 9.b) and loading (step 29).

Problem 3

The trapping efficacy is not optimal for the experiment. Either too many or too few cells were trapped in the chamber during loading (see Figure 14A and step 29) or cells cannot be retained in the chambers during the experiment (Figure 14B). Too many cells in one chamber are not optimal if only one single cell and its descendants are the focus of the experiment. Furthermore, the time until the microcolony outgrows the MGC is decreased and consequently the time frames for cell

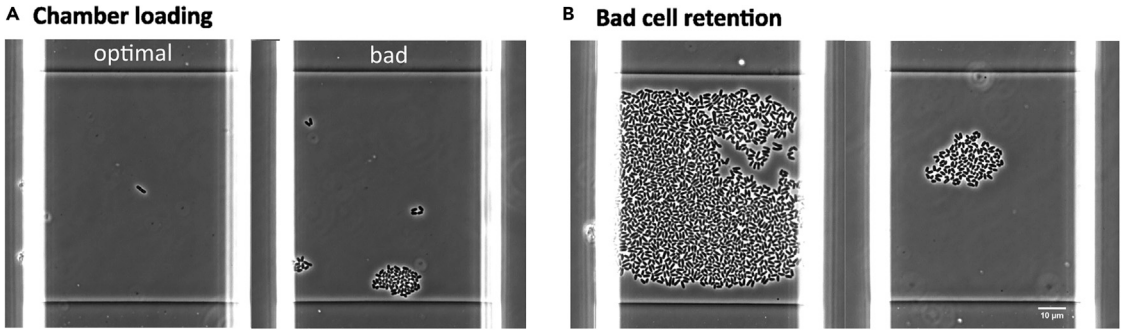

Figure 14. Trapping efficacy of *C. glutamicum* in MGCs

(A) During loading, optimally one cell is trapped in the middle of the MGC. The chamber is overloaded if multiple cells are trapped. Trapping of cells close to the edge is also not optimal, as the colonies will outgrow the chambers very early in the course of cultivation.

(B) Bad cell retention during the cultivation can be identified by holes in the microcolony (left) or by colonies, which have an asymmetric shape (right). Here, flow through the chamber overcame the friction between cell and chamber, dragging cells along. The scale bar is 10 µm.

count-based analysis of the growth behavior. With too few cells being caught in the chambers across the whole microfluidic structure, comparability and statistic relevance decrease. It can occur that no cells are observable for the control measurements. If cells are washed out during the experiment, difficulties during data analysis arise because the cell count is influenced and tracks of single microbes are cut short, as they are washed out too early.

Potential solution

If you have too many cells caught in one chip, fabricate a new one and take care to follow these tips.

- Dilute the seeding culture to OD_{600} of 0.3. Vortex the dilute to split up cell clusters.
- Avoid air bubbles during the loading and connection procedure (step 29).

If you did not catch enough cells during loading, try any of these tips:

- Load your cells with a little more pressure during step 29.
- Try inserting an air bubble. This re-directs the flow through the microfluidic chip, and sometimes also through the chambers.
- Let your main culture grow to a higher OD_{600} or concentrate it by centrifugation.

If you lose cells during the experiment, you can do the following:

- When searching for positions to capture during the experiment (step 12), check if any of the cells are moving in the chambers. If they do, do not select the chamber for time-lapse imaging, because the cells are very likely to be flushed out of the chamber.
- Place your alignment markers on the mask design closer to the edge of the wafer 1.b). During wafer fabrication, local height differences cannot be avoided around the (alignment marker 5.a). Alternatively, leave more space between alignment marker and cultivation regions.
- Fabricate a wafer with a lower chamber height for better cell retention (step 3).
- Check, that the chamber walls are properly bonded to the glass substrate (step 9.c). Otherwise, the PDMS can be lifted by the pressure inside the chip, allowing cells to escape.
- If the backpressure in the chip is too high, the PDMS chamber ceiling can be lifted. Therefore, decrease the back pressure by designing wider outlet channels (step 2.c) or by decreasing the applied pressure used for flow profile establishment (step 14).

Problem 4

The image quality is an important factor for successful data analysis. Problems can arise from shadows on the imaging region, which can be cast by needles and tubing being too close to the

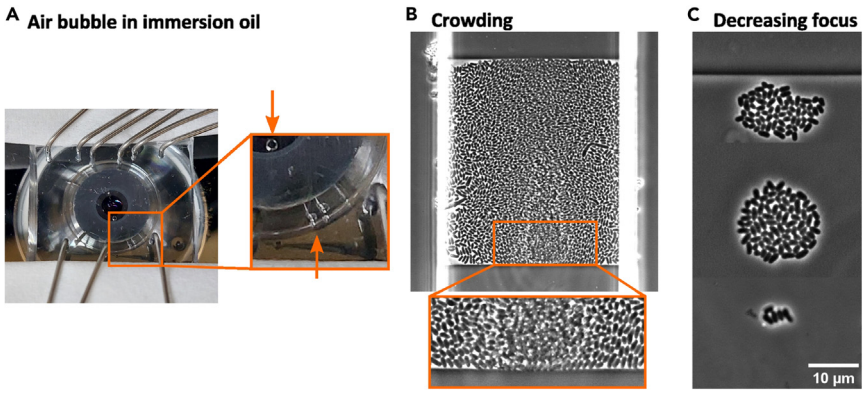

Figure 15. Experimental factors that influence the raw data quality

(A) Air bubbles in the immersion oil film obscure the light path and can shift the focus.

(B) In crowded chambers, microbes can disrupt the monolayer. In this region, cell segmentation is not possible.

(C) For cell detection, images with defined features need to be captured. Keeping the focus during the experiment is therefore very important.

region of interest (step 2.a.ii) or by leakage flowing over the region of interest (step 8.a). Another common problem is a spatial shift of the microscopic picture during the experiment, which results from a non-fixed holder (step 28). Furthermore, air bubbles in the immersion oil can move into the light path, obscuring the view on the region of interest and shifting the focus (see Figure 15A and step 28). The focus can additionally be affected the experimental set up or cells leaving the focal plane.

Potential solution

In case of obstacles in the light path (shadows and leakage), or shifting images, the following countermeasures can be applied for the running or for the next experiment.

- Design the inlet and outlet channels in a way that leaves enough space to the regions of interest. This prevents shadows on the imaging region.
- Punch straight and vertical inlets (Figure 12C). This will prevent leakage and minimize shadows.
- Check your puncher if you have problems with leakage. If you notice traces of use, it can help to replace the puncher.
- Punch the inlets on a soft underlay, preferably PDMS, to prevent ripping and therefore leakage.
- Double-check that the microscope's holder is properly fixed with screws.

If the cells are not in the focal plane (Figure 15B and 15C), three countermeasures can be taken.

- Decrease the inlet pressure (step 14). Slower flow will result in decreased backpressure; therefore, it decreases the PDMS deformation of the MGC and the cell might grow in a monolayer again.
- Increase the width of your outlet channel (step 2.c) to decrease backpressure build-up.
- Sometimes, cells in very crowded chambers also leave the focal plane (Figure 15B). This happens, if it takes less force to deform the PDMS than to push other bacteria away for growth. In this case, a slightly higher chamber height (step 3) can resolve the issue, as cell movement becomes easier again.

If a focus gradient, drift or sudden loss of focus is observed during the experiment, try the following:

- Make sure that you have enough oil on your lens. If you can observe that the oil drop between glass slide and lens is partially sheared away during stage movement, apply more oil. Take care that the oil is not lost at e.g., the slide's edge. Another reason for this phenomenon can be that the oil changed its viscosity during the experiment and needs to be exchanged for another type of oil.

- Before starting the time-lapse imaging, let the equipment heat up to the cultivation temperature of your microbes (step 26). Thermal expansion of material can shift the focus.
- Make sure that the microfluidic chip is fixed horizontally to the microscope's holder and that the holder is placed perfectly horizontally in the microscope. Small height deviations on one side will lead to a focus gradient across the region of interest.
- After loading the chip (step 29), look for air bubbles in the immersion oil between the lens and the glass substrate. Remove existing air bubbles by moving the stage until the air bubble is on the outer-edge of the objective.

When using an auto focusing system, like Nikon's PFS, we take care to:

- Keep the PFS-setting constant for all positions.
- Keep the captured chamber in the center of the acquisition frame. We noticed the PFS shifting the focus when bonded PDMS was in the center.
- Avoid channel clogging by cells. This happens especially in the late stages of the experiment. Similar to the bonded PDMS, the PFS shifts the focus.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Alexander Grünberger (alexander.gruenberger@kit.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

The dataset, code and microfluidic design generated during this study are available at the repositories stated in the key resources table. The published article includes all datasets and code generated or analyzed during this study.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2023.102436.

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AUTHOR CONTRIBUTIONS

Conceptualization, investigation, software, writing – original draft, visualization, L.B.; methodology, validation, writing- review & editing, S.T.; conceptualization, writing – review & editing, supervision, A.G.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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