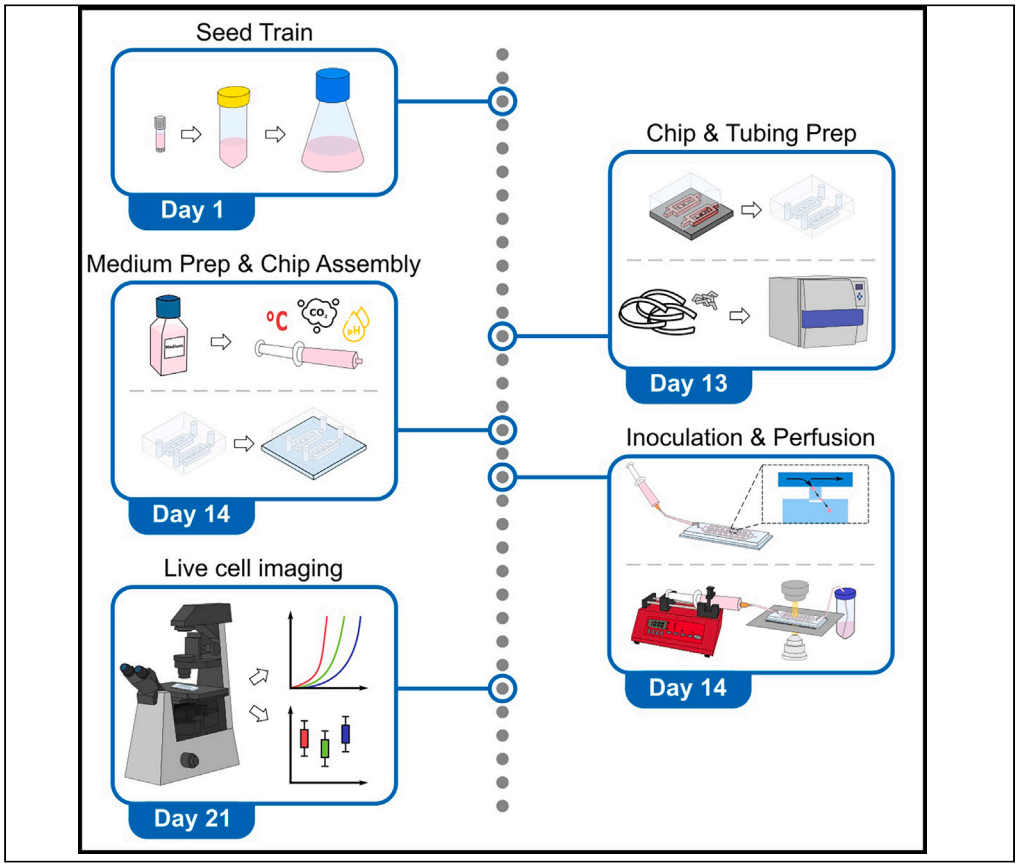
STAR Protocols

CellPress

Protocol

Protocol for microfluidic single-cell cultivation and live-cell imaging of Chinese hamster ovary suspension cell lines



Microfluidic single-cell cultivation (MSCC) is a powerful tool for investigating the cellular behavior of various cell types at the single-cell level. Here, we present a protocol specifically developed for the reliable and reproducible MSCC of industrially relevant Chinese hamster ovary (CHO) suspension cell lines. We summarize critical experimental steps from the initial seed train until the final MSCC experiment, with a special focus on pre-culture management and medium preparation, device inoculation, and the establishment of a constant medium perfusion.

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

Julian Schmitz, Boris Yermakov, Alexander Grünberger

j.schmitz@uni-bielefeld.de (J.S.)
alexander.gruenberger@kit.edu (A.G.)

Highlights

Microfluidic single-cell cultivation of mammalian suspension cell lines

Investigation of cell-to-cell heterogeneity on the single-cell level

Guidelines for the preparation of medium for mammalian single-cell cultivation

Schmitz et al., STAR Protocols 5, 103106
June 21, 2024
https://doi.org/10.1016/j.xpro.2024.103106

STAR Protocols

CellPress

Protocol

Protocol for microfluidic single-cell cultivation and live-cell imaging of Chinese hamster ovary suspension cell lines

Julian Schmitz,[1,3,4,5,*] Boris Yermakov,[2,3] and Alexander Grünberger[2,*]

[1]Multiscale Bioengineering, Faculty of Technology, Bielefeld University, Universitätsstraße 25, 33615 Bielefeld, Germany

[2]Institute of Process Engineering in Life Sciences, Microsystems in Bioprocess Engineering, Karlsruhe Institute of Technology (KIT), Fritz-Haber-Weg 2, 76131 Karlsruhe, Germany

[3]These authors contributed equally

[4]Technical contact

[5]Lead contact

*Correspondence: j.schmitz@uni-bielefeld.de (J.S.), alexander.gruenberger@kit.edu (A.G.)
https://doi.org/10.1016/j.xpro.2024.103106

SUMMARY

Microfluidic single-cell cultivation (MSCC) is a powerful tool for investigating the cellular behavior of various cell types at the single-cell level. Here, we present a protocol specifically developed for the reliable and reproducible MSCC of industrially relevant Chinese hamster ovary (CHO) suspension cell lines. We summarize critical experimental steps from the initial seed train up to the final MSCC experiment, with a special focus on pre-culture management and medium preparation, device inoculation, and the establishment of a constant medium perfusion.

BEFORE YOU BEGIN

Over the last years we developed a MSCC device that facilitates the analysis of single CHO suspension cells. While implementing live-cell imaging enables the investigation of cellular behavior with a high spatiotemporal resolution on the single-cell level, the application of microfluidics allows the precise control of the cultivation conditions.

The experimental steps outlined below will enable you to successfully perform such a MSCC experiment inside a polydimethylsiloxane (PDMS)-glass based microfluidic cultivation device particularly developed for the cultivation of CHO suspension cell lines under constant medium perfusion (Figure 1A).[1,2] Each cultivation device consists of a glass substrate and a PDMS chip containing four distinct cultivation arrays with 60 monolayer growth chambers (MGCs) respectively (Figure 1B). Metal needles and PTFE/Tygon tubing connected to the inlets and outlets are utilized to establish a constant medium perfusion through each array (Figure 1A). The overall design, geometry, and dimensions of the cultivation device with a chamber height of 8 μm and cultivation area of 200 × 200 μm^2 are optimized to ensure optimal cell trapping and retention for CHO suspension cells. Yet, by adjusting those parameters other cell types can be cultivated and investigated inside this device as well. However, the following protocol focusses on CHO cells adapted for growth in suspension only.

Contrary to the popular believe that the application of PDMS compromises ideal growth conditions for a broad variety of unicellular organisms in microfluidic cultivation devices due to e.g., adsorption of small molecules,[3] our own experiences show: It is not the chip material – it is the environment. Therefore, many parameters besides the ones afore mentioned e.g., cultivation conditions, medium

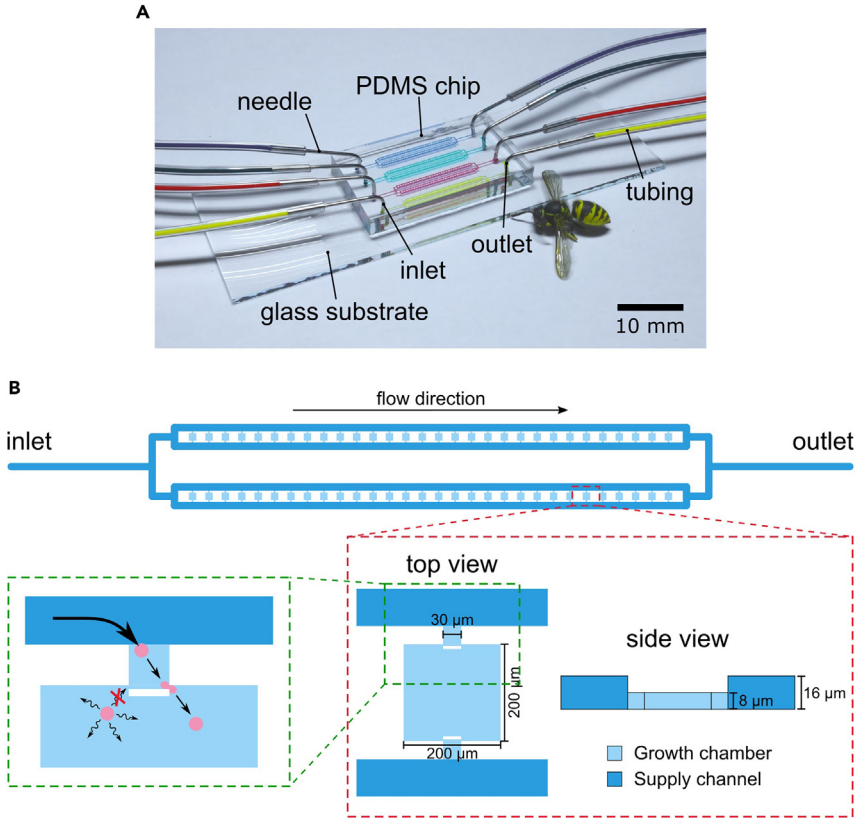


Figure 1. Microfluidic cultivation device

(A) The cultivation device consists of a PDMS chip and a glass substrate. Inlets and outlets make the microfluidic cultivation environment accessible from the outside. Using metal needles, tubing can be connected to the device to constantly perfuse it.

(B) Schematic overview over the devices channel structure with an exemplary zoom-in into a cultivation chamber with its adjacent supply channels in top and side-view.

composition, and preparation, as well as state of the inoculation culture must be adapted meticulously. For complete details on the use and execution of this protocol, please refer to Schmitz et al., 2021.[1]

Cell culture maintenance and seed train management

Timing: Start your seed train 2 weeks prior to the actual MSCC experiment

Note: This protocol has been formulated as part of our ongoing research efforts, focusing on the development, optimization, and application of MSCC systems, specifically tailored for CHO suspension cell lines, within the realm of biopharmaceutical bioprocess research and development. To ensure a high degree of comparability with industrial bioprocess standards, we employ a commercially available, chemically defined cell culture medium (TCX6D, Sartorius AG). Consequently, the precise composition of the medium remains undisclosed. While alternative media formulations (with known compositions) may be successfully utilized, it is worth noting that this could necessitate modifications to certain steps, particularly in the preparation of single-cell cultivation medium (step-by-step method details: Preparation of single-cell cultivation medium), as this protocol was specifically developed for the utilization of TCX6D medium.

⚠ CRITICAL: As the cultivation conditions and seed train management are of utmost important for the MSCC experiment, the following preparation steps must be carried out with great care to ensure an optimal experimental progression as well as a high level of reproducibility and comparability between consecutive MSCC experiments.

1. Thaw your cryopreserved cells from a uniform working cell bank.
   a. Pipette 10 mL phosphate-buffered saline into a 50 mL cultivation tube.

   Note: When pipetting liquids use sterile glass pipets with cotton wool inserted in the upper end as well as filter tips for smaller volume pipetting to minimize the risk of contamination.

   b. Remove a vial from your uniform working cell bank and gently thaw it in the palm of your hand until the ice is melted entirely.
   c. Transfer the thawed cell suspension into the previously prepared cultivation tube and resuspend gently by tilting the tube.
   d. Centrifuge the cultivation tube for 5 min at 115 × *g*.
   e. Carefully discard the cell free supernatant.

   ⚠ CRITICAL: After centrifugation, the cell pellet is not firm. Hence, discard the supernatant with care to avoid cell loss.

2. Start your initial passage.
   a. Supplement 15 mL cell culture medium with 6 mM L-Glutamine.

   Note: This step is best carried out during the centrifugation (step 1.d) to reduce the timeframe wherein the cells are not sufficiently supplied with nutrients.

   b. Carefully resuspend the cell pellet in 15 mL fully supplemented fresh cell culture medium.
   c. Draw a sample and determine the initial viable cell density and viability with an automated cell counter.
   d. Position the cultivation tube in an incubator that is set at a temperature of 37°C, provides a 5% CO_2-atmosphere as well as a relative humidity of 80%. To ensure sufficient mixing of the cell culture set the shaker to 185 rpm with a deflection of 50 mm.
   e. To not miss the exponential growth phase, determine the viable cell density periodically.
   f. Cultivate until the middle of the exponential growth phase is reached, which for our case occurs at cell densities ranging between 3.0 and 5.0 × 10^6 cells/mL.
3. Expand your cells into the second passage.
   a. Draw a sample and measure the viable cell density and viability.
   b. Calculate the needed volume of cell suspension to inoculate a working volume of 60 mL to a viable cell density of 1.5–3.0 × 10^5 cells/mL.

   Note: A working volume of 60 mL is needed for the preparation of single-cell cultivation medium (step-by-step method details: Preparation of single-cell cultivation medium) to operate all four arrays of the cultivation device for approx. a week depending on the applied flow rate during MSCC (step 15.f). You can adjust this accordingly to your application.

   c. Prepare a sterile 125 mL shake flask with fully supplemented medium.
   d. Inoculate the shake flask.
   e. Cultivate until the middle of the exponential growth phase is reached again.
4. Start your third (final) passage.
   a. Draw a sample and measure the viable cell density and viability.
   b. Calculate the needed volume of cell suspension to inoculate a working volume of 60 mL to the same viable cell density as the second passage (step 3.b).

Note: To keep each passage comparable aim for the same initial viable cell density as in the first passage (step 2.c). Try to keep the viable cell density in the same range between different seed trains to ensure reproducibility and comparability between them.

c. Draw the calculated volume into a suitable glass pipette and discard the remaining suspension.
d. Reintroduce the drawn suspension into the same shake flask and fill it with fully supplemented medium so that a working volume of 60 mL is reached.
e. Again, cultivate until the middle of the exponential growth phase is reached.

Pause point: When the third (final) passage is midst exponential phase it can be used for medium and inoculum preparation for the actual MSCC experiment. Consequently, your seed train progression determines the date of your cultivation experiment.

PDMS chip fabrication

Timing: 3 h

⚠ CRITICAL: To fabricate a PDMS chip, the following steps must be conducted under a horizontal laminar flow bench to guarantee a particle-free environment. Else the functionality of the microfluidic cultivation device will be compromised. In this step, a silicon wafer that was fabricated prior to this protocol is applied to generate a PDMS chip with the desired microfluidic structures. For detailed description of the wafer fabrication process see Blöbaum et al., 2023.[4]

5. Prepare PDMS for soft lithography of the pre-designed and already fabricated silicon wafer.

Note: In this step, we utilize generic, single-use plastic cups. However, any easily cleanable, single-use container with adequate volume can be employed here.

a. Clean a plastic cup with compressed air and place it on a scale.
b. Weigh in PDMS base and curing agent in a ratio of 10:1 according to the volume of the target chip structure.
c. Mix PDMS base and curing agent thoroughly with a clean spatula.

Note: The mixing is sufficient when the polymer is completely interspersed with air bubbles.

6. Clean the pre-designed and already fabricated silicon wafer.
   a. Use compressed air to blow away remaining particles.

Note: The silicon wafer needs to be clean and without any residue from previous chip fabrication steps to ensure the functionality of the resulting microfluidic device. Otherwise, channel structures might be blocked or chambers not sufficiently molded.

7. Pour the thoroughly mixed PDMS onto the silicon wafer.

⚠ CRITICAL: Do not exceed a thickness of 0.5–0.8 cm when pouring the PDMS onto your wafer. Otherwise introducing inlets and outlets by biopsy puncher (step 12) becomes difficult.

8. Pre-heat the oven to 80°C for the later following baking step (step 10).
9. Degas the PDMS/wafer using a desiccator.
   a. Place the laminated wafer into a desiccator and apply a vacuum.

b. Wait until all remaining air from the mixing step (step 5.c) got evacuated. Depending on your structure this step may take up to 60 min.

⚠ CRITICAL: Small air bubbles tend to get caught in the fine structures of the silicon wafer. By ventilating and re-evacuating the desiccator periodically these bubbles can be eliminated.

10. Place the coated wafer into the oven and bake it at 80°C for 2 h.

Note: We utilize this specific temperature-time combination to ensure thorough polymerization of the material as well as a standardized and reproducible fabrication process. Depending on the volume of PDMS utilized and the thickness of the cast layer (step 7 and the following CRITICAL note), other temperature-time combinations may be viable.

11. Remove the freshly baked PDMS chip from the silicon wafer using a sharp scalpel.
    a. Wait until the wafer has cooled down.
    b. Carefully cut along the edges of your chip structure and level the chip out of the remaining PDMS cast.

⚠ CRITICAL: Do not run over any structures on the silicon wafer with the scalpel or they will be irreparably damaged.

12. Introduce inlets and outlets to the PDMS chip by means of a biopsy puncher.
    a. Place the PDMS chip onto a soft underlay with the structures facing upwards.

Note: You can use PDMS as an underlay, if available black PDMS. This way your puncher is protected and identifying the target structures for punching becomes easier.

    b. Position the puncher directly above the inlets and outlet structures on your PDMS chip and punch a hole through the PDMS chip at each respective position.

⚠ CRITICAL: Fabricating the inlets and outlets to sufficient quality is paramount. This entails ensuring they are as orthogonal as possible in relation to the array, avoiding dead ends, and thoroughly removing any PDMS scraps. Subpar quality in fabrication may result in difficulties establishing a constant perfusion, an elevated risk of leakage during the MSCC experiment, thereby increasing the risk of contamination, and compromising system functionality. For further details regarding the quality standards of inlets and outlets, please refer to Täuber et al., 2021.[5]

13. Verify whether the punched inlets and outlets are of sufficient quality. Ensure that structures are not missed, and the holes are not ripped.

Cleaning the PDMS chip and glass substrate

Timing: 30 min

⚠ CRITICAL: By cleaning the previously molded PDMS chip as well as the glass substrate, a clean microfluidic chip will be at hand and potentially disturbing particles will be washed away. If cleaning is not performed thoroughly, remaining dust, hair, etc. might block on-chip structures like supply channels, making the resulting PDMS-glass chip unfeasible for application. Additionally, particles might also block the light path during microscopy. Again, the following steps must be conducted under a laminar flow bench.

14. Gather the needed materials under the laminar flow bench for the next steps.
    a. Take one petri dish, two glass substrates, the prepared PDMS chip, adhesive tape, and a wash bottle with analytical-grade 2-propanol under the laminar flow bench.
    b. Use compressed air to blow potential particles out of the petri dish.
    c. Place one of the glass substrates into the petri dish as bonding underlay for the PDMS chip.
15. Clean the PDMS chip.
    a. Use adhesive tape to remove bigger dirt particles from the PDMS chip.
    b. Take the chip into one hand and rinse the chip's surface with 2-propanol. The structures should face upwards in this step.
    c. Blow away the remaining 2-propanol with compressed air.
    d. Repeat step 15.b and 15.c until no particles or impurities can be identified anymore.

⚠ CRITICAL: Be aware of 2-propanol remaining in the chip's inlet and outlet holes. Get rid of these residues as well using compressed air.

    e. Place the cleaned PDMS chip onto the bonding underlay into the petri dish with the structures facing upwards.
16. Clean the second glass substrate with 2-propanol.
    a. Take the glass substrate into one hand and rinse the surface with 2-propanol.
    b. Blow away the remaining 2-propanol with compressed air.
    c. Repeat step 16.a and 16.b until no particles or impurities can be identified anymore.

Note: Sometimes smears remain on the glass substrate during the cleaning steps (steps 15 and 16). If this is the case, use a 2-propanol-soaked non-fuzzing wipe for cleaning.

    d. Place the cleaned glass substrate into the petri dish.

⚠ CRITICAL: Do not touch the surface of your cleaned PDMS chip or glass substrate. If it happens accidentally, start again with step 15.

Pause point: As long as PDMS chip and glass substrate are placed in a particle free environment e.g., a closed petri dish, both can be stored for multiple weeks. Therefore, preparing more than one chip as a backup in case problems occur during the following procedures is advised.

Tubing and needle preparation

Timing: 2 h

⚠ CRITICAL: The actual MSCC experiment is performed in a non-sterile environment within a regular laboratory room. Because CHO cell culture is especially prone to contaminations when no special precautions are taken, every material that comes in direct contact with the cells or the cultivation medium must be sterilized to minimize the risk of contaminations.

17. Cut the tubing for your experiment to an appropriate length depending on your experimental setup and the distance between your pumps and the microfluidic device when it is positioned on the microscope stage (Figure 2).
    a. Prepare one PTFE tube to establish perfusion for every inoculated cultivation array, one PTFE tube for cell loading, and one PTFE tube for subsequent rinsing.

Note: As PTFE tubes are rigid, add approx. 25% to the required length of your tubing for better handling.

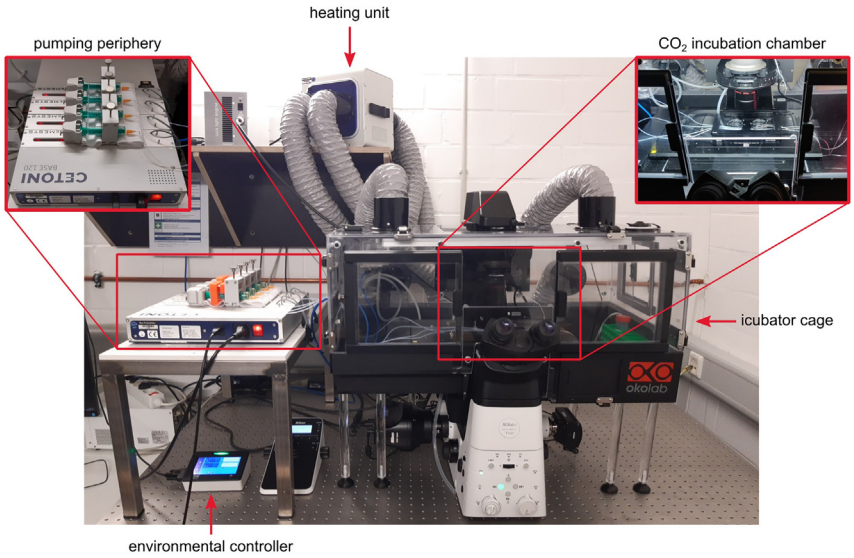


Figure 2. Experimental setup for a MSCC consisting of an automated live-cell imaging microscope as well as the respective pumping periphery and environmental control

b. Prepare one Tygon tube to lead the flow-through into waste containers for every inoculated cultivation array.

18. Gather two needles for every inoculated cultivation array, one needle for cell loading, and one needle for purging.
19. Autoclave PTFE tubing and needles.
    a. Place the PTFE tubing and needles into separate glass petri dishes.
    b. Autoclave the glass petri dishes for 20 min at 121°C at 1 bar overpressure.

Note: As the Tygon tubing is only meant to connect the microfluidic cultivation device with the waste containers, these tubes do not have to be sterilized and can just be stored without any precautions.

Pause point: Autoclaved needles and PTFE tubing can be stored for multiple weeks for later use as long as they stay sterile. To ease the preparation prior to an experiment, having sterile needles and tubing in stock is advised.

Setting up the microscope

Timing: 30 min

⚠ CRITICAL: By adjusting the microscope and its periphery before the microfluidic cultivation device is installed, the temporal delay between inoculation of the device and starting the live-cell imaging should be minimized so that no aspect of cellular behavior on-chip is missed out. Here, a special focus lies on fine-tuning the light path and adjusting the environmental conditions.

20. Adjust environmental conditions to your needs.
    a. Pre-heat the microscope's incubator cage to the need cultivation temperature. In case of CHO cells, adjust it to 37°C.

Note: Heating up the whole microscope setup takes some time. Therefore, start at least 30 min prior to the microfluidic single-cell cultivation with this step.

⚠ CRITICAL: If there is a temperature sensor inside your microscope cage incubator, place it close to the microfluidic cultivation device so that it monitors the temperature at the most relevant position of your cage incubator.

b. Regulate your atmosphere to a certain CO_2 concentration. Using TCX6D medium, 5% CO_2 concentration is needed for a proper pH value.

21. Configure your microscope.
    a. Turn on your live-cell imaging microscope and the computer periphery.
    b. Adjust the correct focus level.
    c. Select the 40× objective for your experiment and center the optical path by Köhler illumination.

Note: As you need a microfluidic cultivation device for this step, you can use an old device from a previous experiment.

d. If needed, also center the phase ring for optimal phase contrast microscopy.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
2-Propanol (Analytical)	VWR International GmbH	Cat#: 20842.312 (Europe)
KH_2PO_4	Carl Roth GmbH + Co. KG	Cat#: 3904.1 (Europe)
L-glutamine	Carl Roth GmbH + Co. KG	Cat#: 3772.3 (Europe)
Na_2HPO_4	Th. Geyer GmbH & Co. KG	Cat#: 007056-1KG (Europe)
NaCl	VWR International GmbH	Cat#: X190-1KG (Europe)
PDMS (Sylgard 184 Silicone Elastomer Kit)	Dow Corning, Inc.	Cat#: 1024001 (Global)
Cell culture medium (TCX6D)	Sartorius AG	Cat#: 1070-0001 (Global)
Experimental models: Cell lines		
CHO-K1	ATCC	CCL-61, adapted to growth in suspension
Software and algorithms		
CETONI Elements	CETONI GmbH	v20231205
NIS-Elements Advanced Research	Nikon Europe B.V.	5.21.02
Other		
Adhesive tape	Tesa SE	Cat#: TE-001594-ST (Europe)
Automated cell counter	Innovatis AG	Cedex AS20
Cage-type incubator for microscope	Okolab S.R.L.	OKO-H201
Camera for microscope (FX CMOS - Nikon DS-Qi2)	Nikon Europe B.V.	Cat#: MQA17599 (Europe)
Cannula (0.80 × 120 mm)	B. Braun SE	Cat#: 4665643 (Germany)
Cannula (0.90 × 50 mm)	B. Braun SE	Cat#: 4667093 (Germany)
Centrifuge	Heraeus GmbH	Megafuge 1.0
Clean room wipe (non-fuzzing, Spec-Wipe® 7)	VWR International GmbH	Cat#: 115-0043 (Europe)
CO_2 incubation chamber for microscope	Okolab S.R.L	H201-K-Frame GS26-M
Desiccator	Th. Geyer GmbH & Co. KG	Cat#: 9042143 (Europe)
Glass substrate (76 × 26 × 1 mm microscope slides)	VWR International GmbH	Cat#: 631–1552 (Europe)
Horizontal laminar flow cabinet (AEOLUS H)	Azbil Telstar, S.L.U.	Cat#: 135–2611 (Global)
Incubator	Mytron GmbH	-
Lab-Shaker	Kühner AG	ES-X
Light source (diascopic phase contrast)	Nikon Europe B.V.	Cat#: MEE59920 (Europe)
Microscope (Nikon Eclipse Ti2-E, inverted)	Nikon Europe B.V.	Cat#: MEA54000 (Europe)
Objective (CFI P-Apo DM 40× Lambda Ph2/ 0.95)	Nikon Europe B.V.	Cat#: MRD30405 (Europe)
Oven	Memmert GmbH + Co. KG	UN30 Single Display
Perfect Focus System (TI2-N-ND-P)	Nikon Europe B.V.	Cat#: MEP59394 (Europe)

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Petri dish (150 × 20 mm)	Sarstedt AG & Co. KG	Cat#: 82.1184.500 (Europe)
Petri dish (92 × 16 mm)	Sarstedt AG & Co. KG	Cat#: 82.1472 (Europe)
Pipette tips (Filter tip, 1,250 µL, transparent, Biosphere)	Sarstedt AG & Co. KG	Cat#: 70.1186.210 (Europe)
Plasma cleaner (40 kHz, 200 W)	Diener electronic GmbH & Co. KG	Femto
Plug connections (90° curvature; ID: 0.34 mm; OD: 0.65 mm)	Gonano Dosiertechnik GmbH	Cat#: GGA7R23-90 (Germany)
Precision tips (90° Curvature; ID: 0.33 mm; OD: 0.65 mm)	Nordson Corporation	Cat#: 7018316 (Global)
Puncher (0.75 mm)	Welltec	Cat#: 504529 (Global)
Scalpel	C. Bruno Bayha GmbH	Cat#: 233-5202 (Germany)
Scalpel blade	C. Bruno Bayha GmbH	Cat#: 233-5544 (Germany)
Shakeflask (Flat-base, 125 mL)	TriForest Enterprises, Inc.	Cat#: FPC0125S (Global)
Spatula (Disposable stirring spatula)	BRAND GmbH & Co. KG	Cat#: 759800 (Europe)
Sterile filter (0,22 µm)	Sarstedt AG & Co. KG	Cat#: 83.1826.001 (Europe)
Syringe (Henke-Ject 1 mL)	Henke Sass Wolf GmbH	Cat#: 4010-200V0 (Germany)
Syringe (Norm-Ject 20 mL)	B. Braun Melsungen AG	Cat#: NJ-4606205 (Germany)
Syringe (Norm-Ject 50 mL)	B. Braun Melsungen AG	Cat#: 4616502F (Germany)
Syringe pump (neMESYS)	CETONI GmbH	Cat#: A3921000123 (Germany)
Tube (50 mL)	Sarstedt AG & Co. KG	Cat#: 62.547.254 (Europe)
TubeSpin Bioreactor 50	TPP Techno Plastic Products AG	Cat#: 87050 (Europe)
Tubing (PTFE; ID: 0.5 mm; OD: 1.6 mm)	Gebr. Rettberg GmbH	Cat#: 101761009 (Europe)
Tubing (Tygon; ID: 0.51 mm; OD: 1.52 mm)	OMNILAB-LABORZENTRUM GmbH & Co. KG	Cat#: 1210111 (Global)
Vacuum pump	LABOPORT, KNF	-

MATERIALS AND EQUIPMENT

Phosphate-buffered saline

Reagent	Final concentration / mM	Amount
NaCl	155,17	8 g
KH_2PO_4	1,54	2,9 g
Na_2HPO_4	5,11	0,24 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: Adjust pH to 7.2 and autoclave for sterilization.

L-Glutamine stock solution

Reagent	Final concentration / mM	Amount
L-Glutamine	200	5,846 g
ddH_2O	N/A	To 200 mL
Total	**200**	**200 mL**

Sterilize by filtration through syringe tip filter with a 0.2 µm pore size. Store at −20°C in 50 mL aliquots for max. one year. Alternatively store at −4°C for max. one month.

STEP-BY-STEP METHOD DETAILS

The following steps for starting a MSCC experiment with CHO suspension cells are listed and described in chronological order. However, some of them include incubation times so that all steps interlock.

Preparation of single-cell cultivation medium

Timing: 3–4 h

In this section, we outline the steps for preparing single-cell cultivation medium. It starts with obtaining conditioned medium and supplementing fresh medium, followed by incubation and the process of combining the medium portions. Finally, we describe how to fill syringes and connect them to tubing, facilitating the use of the single-cell medium for MSCC.

Note: Since MSCC is a relatively new field of research there are currently no commercial, chemically defined cell culture media available that provide optimal growth conditions on a single-cell level. Therefore, a mixture consisting of 50% (v/v) fresh, fully supplemented medium and 50% (v/v) conditioned medium is utilized to perform MSCC experiments. The conditioned medium originates from the exponential growth phase of the third (final) passage of the seed train. To guarantee comparable quality of conditioned medium, reproducible seed train management is of utmost importance. We developed the following medium preparation protocol to ensure a suitable growth environment and guarantee the reproducibility of consecutive cultivations (see before you begin: cell culture maintenance and seed train management).

⚠ CRITICAL: To minimize the risk of contaminations, work under a clean bench.

1. Obtain conditioned medium from your seed train.
   a. Aliquot 50 mL cell suspension from the final passage of the seed train equally into two 50 mL centrifugation tubes.

   Note: Put the shake flask back into the incubator, as the remaining 10 mL cell suspension will be needed later to draw inoculum for the cultivation.

   b. Centrifuge the two tubes containing the suspension at 200 × g for 5 min.
   c. Remove the syringe stamps of two 20 mL disposable syringes and mount 0.22 μm syringe filters in front of each syringe.
   d. Position one of the syringes on top of a fresh cultivation tube, pour 25 mL of cell-free supernatant into it and reinstall the syringe stamp (Figure 3A).

   ⚠ CRITICAL: Make sure to keep the syringe on top of the cultivation tube during the stamp installation, as the applied pressure will push liquid through the filter.

   e. Sterile filtrate the entire volume.
   f. Repeat the process for the second syringe.
   g. Close both cultivation tubes containing the conditioned medium.

   Note: Depending on your cell density, you will not be able to gain 50 mL cell-free conditioned medium from 50 mL cell suspension. In that case, increase the volume of cell suspension for centrifugation and filtration.

2. Prepare fresh medium with glutamate supplements.
   a. Prepare 50 mL fresh cell culture medium supplemented with 6 mM L-glutamine (Figure 3B).
   b. Aliquot 50 mL supplemented fresh medium equally into two 50 mL cultivation tubes.
3. Incubate the four cultivation tubes (Figure 3C) for 2 h at 37°C, 5% CO_2, and 80% relative humidity on a shaker at 185 rpm with a deflection of 50 mm.

   Note: Incubation in a 5% CO_2 atmosphere ensures that the pH value of each respective medium aliquot is equilibrated at 7.2.

   ⏸ **Pause point:** You should have four 50 mL cultivation tubes in total: two containing 25 mL conditioned medium and two containing 25 mL fully supplemented fresh medium (Figure 3C).

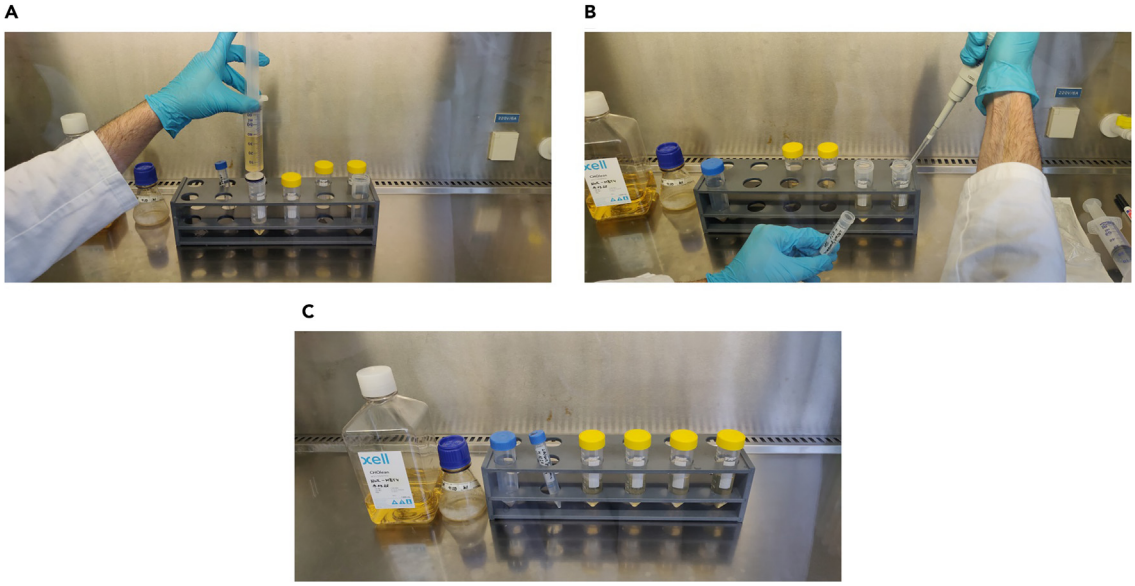


Figure 3. Preparation of conditioned medium and fresh medium

(A) Sterile filtration of one aliquot of conditioned medium drawn from the exponential growth phase of the third passage.

(B) Supplementation of the fresh medium with 6 mM L-Glutamine.

(C) Overview of the expected result after you reach the pause point: Two aliquots of conditioned medium and two portions of fresh medium in cultivation tubes.

4. After the incubation period, combine the conditioned medium with the fresh medium.

⚠ CRITICAL: Introducing air bubbles into the liquid as well as room air into the headspace of the cultivation tubes will potentially result in altering the pH value of the prepared medium and thus renders the prior incubation step obsolete. Therefore, each aliquot is handled in closed containers and accessed via cannulas (Figure 4A). Make sure to keep the cannula steady and inside the liquid the entire time to avoid turbulences and to minimize the amount of air bubbles introduced into the syringe.

a. Puncture the membranes of each cultivation tube located at the lid with a 120 mm long cannula.
b. Draw the first conditioned medium portion carefully into a 25 mL syringe through the cannula (Figure 4B).
c. Remove the cannula from the filled syringe and mount the syringe onto the cannula of the first cultivation tube containing the fresh medium (Figure 4C).
d. Unite the conditioned medium with the fresh medium by slowly moving the syringe stamp down (Figure 4C).
e. Repeat this procedure for the two remaining cultivation tubes.

Pause point: Now you should have two cultivation tubes each containing 50 mL of a mixture consisting of 50% (v/v) conditioned medium and 50% (v/v) fresh medium. This mixture will further be referred to as single-cell cultivation medium (Figure 4D).

5. Prepare four syringes with single-cell cultivation medium for MSCC.
   a. Insert a sterile needle and syringe adapter into your sterile perfusion PTFE tube.

Note: As the outer diameter of your needle is bigger than the inner diameter of your PTFE tube, inserting the needle is tricky. If the tip of your tube gets worn out by trying to insert

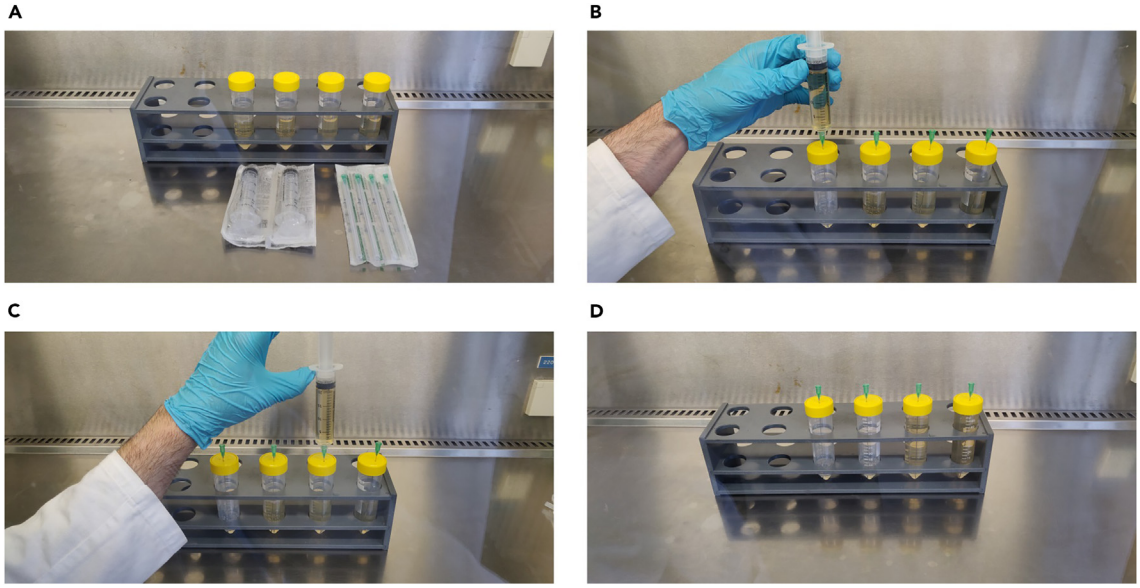


Figure 4. Preparation of single-cell cultivation medium

(A) Overview of the needed material for the preparation of single-cell cultivation medium: two-times 25 mL conditioned and sterile-filtrated medium, two-times 25 mL supplemented fresh medium, two 20 mL disposable syringes, four 120 mm long cannulas.

(B) 25 mL of conditioned medium are drawn into a disposable syringe.

(C) The 25 mL of conditioned medium are injected into one of the cultivation tubes holding 25 mL fresh medium.

(D) After repeating this step, two-times 50 mL of single-cell cultivation medium should be at hand.

the needle, cut off the tip of the tube with a scalpel and try it again, otherwise the connection between tube and needle might become leaky during the MSCC experiment.

b. Mount a fresh 20 mL syringe on top of the cannula inside the cultivation tube containing the single-cell cultivation medium (Figure 5A).
c. Carefully draw 20 mL of medium into the syringe. Again, try to avoid turbulences and the introduction of air bubbles.
d. Dismount the filled syringe from the cannula, invert the syringe and carefully remove trapped air bubbles by slowly moving the syringe stamp upwards with a rotating motion (Figure 5B).

⚠ CRITICAL: The removal of entrapped air bubble is crucial as those could potentially get stuck inside the supply channels of the microfluidic cultivation device and compromise medium flow and thus cultivation conditions on-chip.

e. Fill the syringe adapter with single-cell cultivation medium until a droplet arises at its top (Figure 5C).
f. Produce a small droplet of medium at the tip of the syringe and connect it to the syringe adapter without introducing air into the system (Figure 5C).
g. Repeat steps 5.a – 5.f until four syringes are filled with single-cell cultivation medium.

6. Prepare a 1 mL single-cell cultivation medium syringe for rinsing the supply channels of the microfluidic cultivation device.
   a. Repeat the aforementioned steps 5.a – 5.f with a 1 mL syringe.

⏸ Pause point: At this point you should have four syringes with single-cell cultivation medium prepared for MSCC and one syringe with single-cell medium for rinsing the cultivation device after inoculation.

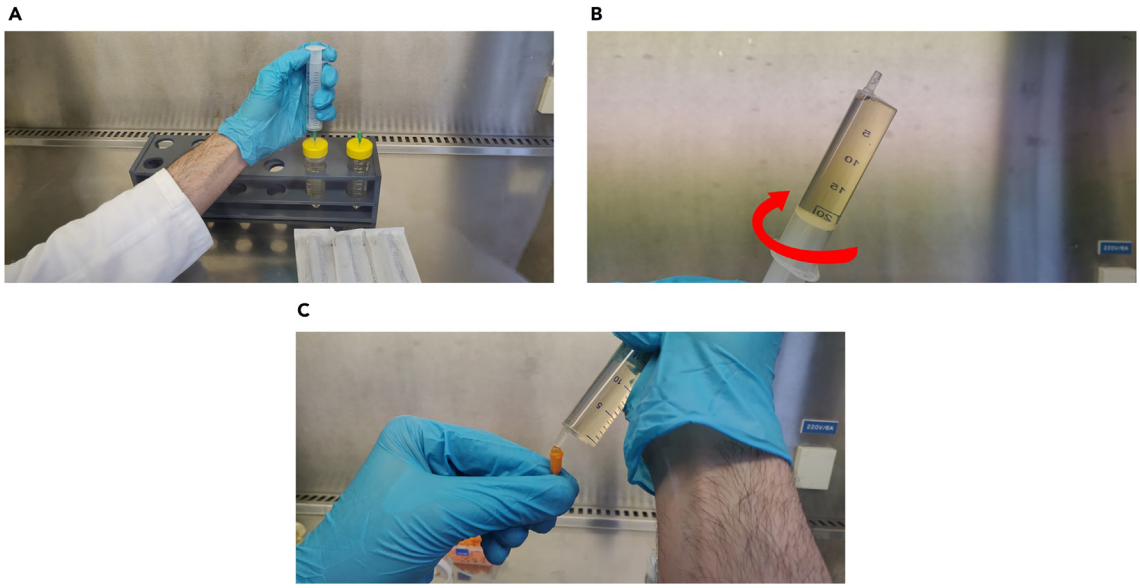


Figure 5. Preparation of single-cell cultivation medium containing syringes

(A) 20 mL of single-cell cultivation medium are slowly drawn into a single-use syringe to avoid the introduction of air bubbles to the medium.

(B) To get rid of potentially arising air bubbles, the medium containing syringe is inverted, and the air bubbles are removed by slowly moving and rotating the stamp.

(C) The syringe adapter is filled with medium until a droplet forms at its top. Afterwards the wetted syringe is connected to the adapter without introducing any air.

Bonding of the PDMS-glass device

Timing: 30 min

In this section, we outline the concluding steps of fabricating a microfluidic cultivation device. This includes activating the surfaces of the PDMS chip and glass substrate using oxygen plasma technology, followed by the assembly of the device. We emphasize essential and time-critical steps to ensure the creation of a clean, accurately aligned, and functional PDMS-glass based device.

Note: The subsequent assembly of the chip and glass substrate relies on oxygen plasma technology, requiring the use of an oxygen plasma generator (plasma cleaner). Our selected model delivers a maximum power output of 200 W at a frequency of 40 kHz. In our case, pure oxygen is used as the source to generate the oxygen plasma. Adjustments to parameters (step 7.d) may be necessary depending on the technical specifications of the device and the gas utilized. Furthermore, pre-heating the device may be necessary depending on its configuration; if required, ensure this step is completed before proceeding further.

7. Activate the surfaces of the PDMS chip and the glass substrate for subsequent assembly of the final microfluidic cultivation device.
   a. Place the clean PDMS chip and the glass substrate into the plasma generator.

   ⚠ CRITICAL: Ensure that the surfaces to be activated are uncovered, do not overlap, and that the microfluidic structures are facing upwards.

   b. Apply a vacuum to degas the activation chamber of the plasma generator by means of a vacuum pump.

c. Once the remaining pressure reaches 0.2 mbar, introduce oxygen into the activation chamber at a pressure of 1 mbar and a flow rate of 15 sccm.

⚠ CRITICAL: Wait until vacuum pump and oxygen inflow reach an equilibrium state. In our case the equilibrium pressure is 1.0 mbar.

d. Activate the surfaces of the PDMS chip and glass substrate for 24 s with 45% of the maximal power of the plasma generator (90 W).

Note: The utilization of oxygen plasma renders the surfaces of the materials hydrophilic, thereby also serving as a biological sterilization measure.

e. Switch of the oxygen inflow, stop the vacuum pump, and re-ventilate the activation chamber.

⚠ CRITICAL: If ventilation happens abruptly, the chip and glass substrate might move erratically or, in the worst-case scenario, flip over, necessitating the repetition of the fabrication, cleaning and activation step. If your plasma generator does not possess a ventilation valve, add a simple needle valve with an upstream particle filter to the ventilation port of your generator so that you can regulate the re-ventilation manually.

f. Remove the activated PDMS chip and glass substrate from the activation chamber and place them back into the particle-free petri dish.

⚠ CRITICAL: Do not touch the activated surfaces and prohibit potential particle contamination of the PDMS chip and glass substrate by limiting the time both parts are exposed to particle-containing room air.

8. Assemble the activated PDMS chip and the glass substrate.
   a. Place the petri dish under the laminar flow bench for a particle-free environment.
   b. Take the PDMS chip and place it with the activated side facing downwards onto the glass substrate.

Note: Try to align PDMS chip and glass substrate rectangular to each other so that cultivation chambers are not tilted during live-cell imaging. This makes image analysis easier.

   c. If bonding of PDMS chip and glass substrate is not sufficient, apply gentle pressure to the edges of the PDMS chip.

⚠ CRITICAL: Never apply pressure directly to the microfluidic structures of your chip. Otherwise, channels and chambers might collapse.

   d. Place the freshly assembled microfluidic cultivation device into the oven at 80°C for 2 min to strengthen the bonding of PDMS chip and glass substrate.
9. Place the microfluidic cultivation device on your automated live-cell imaging microscope.
10. Check the microfluidic cultivation device for any residue particles or dirt that might affect the microfluidic single-cell cultivation.

⚠ CRITICAL: After bonding the chip, the following steps must be executed promptly. Activating the surfaces of the PDMS chip and glass substrate leaves them hydrophilic which massively facilitates inoculation of the fabricated microfluidic cultivation device and replacement of any air inside the supply channels and cultivation chambers. After 15 min the surfaces start to turn hydrophobic again[6] which results in more difficult inoculation and entrapment of air on-chip.

Preparing the inoculation culture

Timing: 10 min

In this section, we elucidate the steps required to obtain and prepare the inoculation culture, ensuring its suitability for inoculating the microfluidic cultivation device.

⚠ CRITICAL: At this point of experimental progression, you should have everything prepared except the inoculation culture. Since CHO suspension cells are susceptible regarding the exposure to suboptimal environmental conditions, the preparation of the inoculation culture is carried out last.

11. Prepare a cell suspension containing syringe for inoculation of the microfluidic cultivation device.
    a. Take your shake flask with the remaining 10 mL cell suspension out of the incubator and under your clean bench.
    b. Insert a sterile needle and syringe adapter into your sterile cell loading PTFE tube.
    c. Mount a 50 mm long cannula onto the syringe and carefully draw cell suspension into the syringe until it is filled entirely.
    d. Invert the syringe and gently remove entrapped air bubbles by rotating the syringe stamp gently (step 5.d)
    e. Remove the cannula and mount the prepared tubing via a connector (step 5.f).

⚠ CRITICAL: From this point onward, you should proceed as quick as possible because of the susceptibility of CHO cells to suboptimal environmental conditions.

Inoculation of the cultivation device and establishment of constant medium perfusion

Timing: 15–30 min

In this section, we outline the essential steps for the proper commissioning of the microfluidic cultivation device. We commence with the inoculation procedure, followed by rinsing of the cultivation array, and culminate with the establishment of a constant medium perfusion to facilitate long-term MSCC under constant and controlled environmental conditions.

Note: Compared to traditional cultivation systems the inoculation process of microfluidic cultivation devices is more intricate and plays a more important role regarding the quality of the data as well as ease of its evaluation and analysis after the experiment. Ideally every microfluidic growth chamber should be seeded with one single cell so that an isogenic population can proliferate over the course of the cultivation from one common mother cell.

12. Mount the microfluidic cultivation device on the automated stage of your microscope.
13. Start with the inoculation of the first array of the microfluidic cultivation device.
    a. Take the 1 mL syringe containing the inoculation culture and move the syringe stamp downwards until a droplet of cell suspension builds up at the needle.
    b. Position the needle in the outlet of the array you want to inoculate.

⚠ CRITICAL: It is paramount to handle both the aforementioned and subsequent needle insertion and removal steps with great care. Applying excessive force could potentially tear the material, leading to leakage, thereby compromising system functionality, and increasing the risk of contamination.

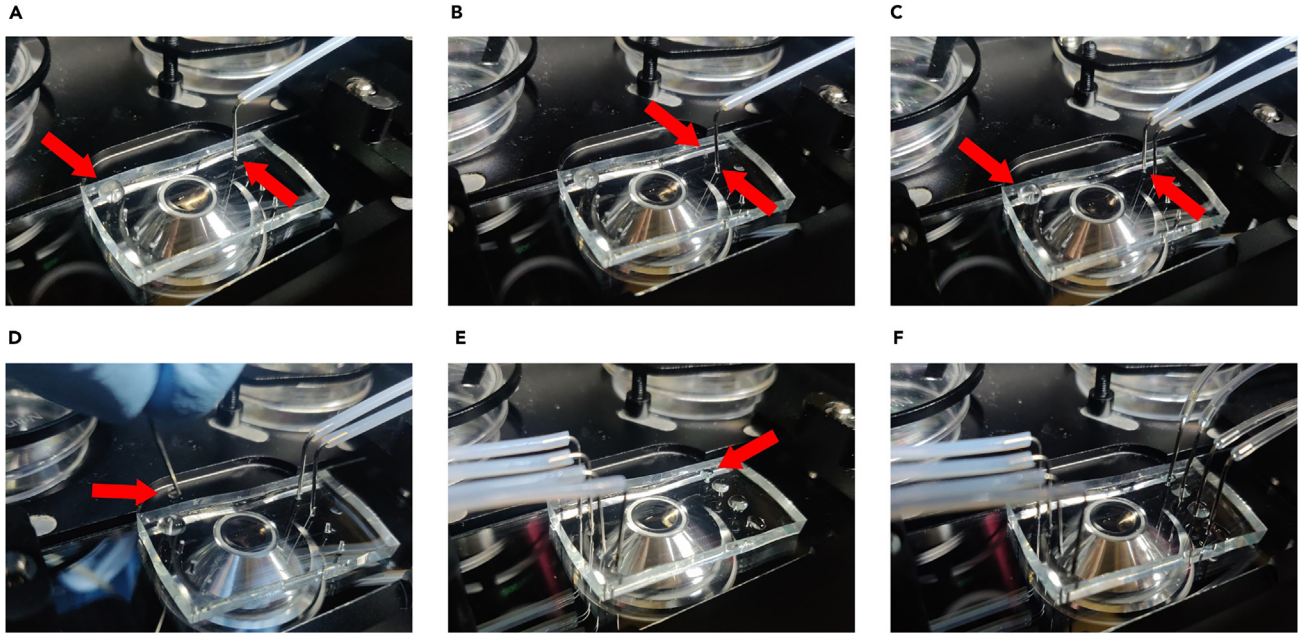


Figure 6. Inoculation of the microfluidic cultivation device and subsequent establishment of constant perfusion

(A) A cultivation array is flushed with cell suspension from the outlet until a droplet is formed at the belonging inlet.

(B) After sufficient cell loading, the needle of the inoculation syringe is place into the next array's outlet.

(C) By means of a medium containing syringe, the previously inoculated array is rinsed to get rid of remaining cells and air bubbles until a clear droplet forms at the inlet.

(D) A wetted needle is connected to the inoculated array by the exclusion of air.

(E) After establishing constant perfusion of the inoculated arrays, a clear droplet should form at the arrays' outlets.

(F) Waste-tubing is connected to the outlet to collect the flow-through.

c. Carefully fill the array with cell suspension until a droplet forms at the respective inlet (Figure 6A).

⚠ CRITICAL: Because mammalian cell lines sediment relatively fast it is advised to periodically invert the inoculation syringe to avoid overloading the array.

d. Move the cell suspension back and forth through the supply channel to push cells into the cultivation chambers.

Note: If you have difficulty achieving a satisfactory loading you can utilize entrapped air as a barrier which will guide cells into the respective MGC. For this remove the droplet on the inlet with a microscope tissue and suck air into the supply channel.[2]

e. When the array is sufficiently inoculated connect the inoculation syringe to the next outlet (Figure 6B).

Note: During the entire process described above asses the progression of cell inoculation on the microscope's monitor.

14. Rinse the inoculated array to get rid of remaining cells and air bubbles from the supply channels.
    a. Take the 1 mL syringe containing single-cell cultivation medium and move the syringe stamp downward until a droplet forms at the needle.
    b. Position the needle inside the outlet of the array to be rinsed.

c. Flush the array until a clear droplet of liquid forms at the respective inlet (Figure 6C).

⚠ CRITICAL: If the droplet is cloudy there are still air bubbles trapped inside the supply channel and therefore rinsing was not sufficient. Insert a sterile metal needle into the inlet and move it up and down to loosen air bubbles stuck on the inlet's walls.

d. Leave the clear droplet on top of the inlet.

15. Establish a constant perfusion of the inoculated cultivation array.
    a. Take one medium containing syringe and move the syringe stamp downwards until a droplet forms at the needle.
    b. Position the wetted needle so that its droplet unifies with the droplet remaining on the inlet (Figure 6D).

⚠ CRITICAL: This step is critical to avoid the reintroduction of air bubbles into the rinsed arrays.

    c. Connect the needle with the device's inlet.
    d. Manually flush the array with medium until a clear droplet forms on top of the respective outlet (Figure 6E).
    e. Use the microscope to check if there is air remaining in the supply channel. If so, continue flushing the array manually.
    f. Mount the medium containing syringe into your pump setup and establish the perfusion with your desired flow rate, in our case 2 µL/min are sufficient.
    g. Repeat steps 15.a – 15.f with the remaining arrays.

Note: Keep an eye on the outlet of the already perfused array during loading of the remaining arrays. The lack of a droplet after a while indicates problems with your perfusion.

16. Connect the waste-tubing to the inoculated cultivation arrays.
    a. Insert a sterile needle into your waste-tubing.
    b. Wait until a clear droplet forms at the outlet due to constant perfusion.
    c. Connect the prepared waste-tubing to the outlet (Figure 6F).
    d. Place the waste-tubing into a waste container.

Note: Use an individual waste container for each array so that you can relate any potentially occurring problems e.g., concerning the perfusion to the affected array.

Live-cell imaging

Timing: 45 min

In the following section, we delineate the steps to configure the live-cell imaging to achieve optimal image and, consequently, data quality. We elaborate on the acquisition settings and frequency, as well as the process of selecting suitable regions of interest.

Note: After successful inoculation of the microfluidic cultivation device and starting constant medium perfusion, live-cell imaging settings must be adjusted, and regions of interest on-chip must be identified for subsequent monitoring with the automated microscope.

17. Adjust the exposure time and light intensity for phase contrast microscopy to 100 ms and 15%.
18. Select regions of interest for subsequent live-cell imaging.

a. Identify interesting cultivation chambers and center them in the display window for best focus stability.

Note: For single-cell analysis, cultivation chambers with only one or few initial starting cells are most interesting. Cells should look vital, show a round morphology and do not feature any unnatural morphologies.

b. Add interesting positions to your acquisition list.

⚠ CRITICAL: When saving positions for acquisition, include the x-, y-, and z-value of the respective position into the list for best focus results over long-term cultivations.

Optional: To check whether air is introduced to your microfluidic cultivation device during the experiment, also select the inlet of each inoculated cultivation array for constant live-cell imaging.

19. Set live-cell imaging frequency.
    a. Decide on an interval for microscopic imaging. For CHO cells 20 min is sufficient.

⚠ CRITICAL: Keep in mind that acquiring microscopic images takes time and the more positions you want to monitor the longer one imaging interval takes. Therefore, the duration of one imaging interval must go with your imaging frequency.

    b. When the experimental duration is known, set a temporal limit to the image acquisition to prevent unnecessary image data.

⚠ CRITICAL: Check if the data storage capacity of your computer periphery is sufficient for the planed live-cell imaging experiment. If not, delete old data to circumvent sudden software failure.

Closing steps

Timing: 10 min

In this section, we outline precautionary measures and final steps to mitigate experimental failure and ensure smooth experimental progression.

Note: Before finally starting the live-cell imaging you should check certain potential sources of error respectively take precautions to prohibit common problems.

20. Check tubing for potential sources of error.
    a. Fix the inlet tubing to the automated microscope stage using adhesive tape to prevent leakage at the device's inlets because of tension stress (Figure 7A).
    b. Check if the outlet tubing leads into a waste container to prevent flooding of your microscope (Figure 7B).
21. Start your live-cell imaging experiment!
22. Check after one or two imaging loops (alternatively after 1 h) if your experiment is running as planned.
    a. Check the focus of the already taken images.
    b. Check your pump periphery and the outlet tubing/waste containers for the expected flow through.
    c. Check the chip's inlets and outlets for leakages.
    d. Check the temperature and atmosphere of your microscope incubator.

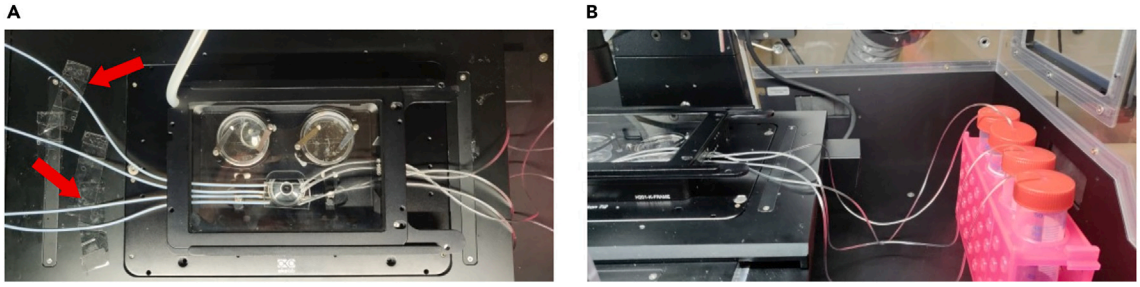


Figure 7. Closing steps to guarantee issue-free MSCC
(A) Inlet tubing is fixed to the automated microscope stage to prevent stress tension on the inlet.
(B) The positioning of outlet tubing in the waste containers is checked to prevent flooding of the microscope.

EXPECTED OUTCOMES

If executed properly, the protocol will result in a stable long-term cultivation of single CHO suspension cells inside your MGC (Figure 8A, Methods video S1). Due to growth promoting medium conditions, growth of the cells will show an exponential behavior until the dimensions of the cultivation chamber lead to spatial restrictions. If cell retention performs sufficiently, no cells will leave the cultivation chamber during the experiment.

Microcolony growth rates can be determined from the respective growth curves (Figure 8B). Depending on the initial cell number, the MGC are overgrown at different stages of the MSCC experiment. On the morphological level cellular diameter distribution can be determined throughout a microcolony (Figure 8C) and changes can be monitored over the course of cultivation or investigated under the influence of different environmental conditions like osmolality.[7]

Addressing a more specific scientific questions, the investigation of cellular heterogeneity is enabled applying the presented MSCC experiment. On single-cell level the high spatiotemporal resolution allows to investigate single-cell doubling times of the same microcolonies (Figure 8D), showing highly comparable doubling time distributions. Yet also outliers are observable that have highly prolonged single-cell doubling times. When looking at the lineage tree of an isogenic microcolony (Figure 8E), single-cell analysis via live-cell imaging allows easy identification of heterogeneous growth behavior.

LIMITATIONS

When cultivating CHO suspension cells in a microfluidic cultivation device, our protocol allows reproducible single-cell experiments. However, when the microfluidic chip is partially blocked by air or by residual particles the cultivation conditions on-chip are affected. Presumably, trapped cells are still supplied with sufficient nutrients, yet environmental control is restricted. Thus, the validity of the achieved results is questionable. Here, identification of the impaired cultivation conditions is very important so that the resulting data can be realized as biased.

To get CHO suspension cells to grow in isolation, the applied single-cell cultivation medium must be a mixture of fresh medium and already conditioned medium from the seed train. Consequently, the composition of the single-cell cultivation medium is unknown and therefore hardly reproducible. This limitation can only be solved by designing tailored media for single-cell applications.

With the current design of the cultivation chamber, it is possible to withhold randomly moving cells inside the chamber.[2] Despite that, cells still might leave the cultivation chamber occasionally, which results in distinct bends in the respective growth curves and thereby lead to artificially depressed growth rates when determined graphically by counting the cell number.

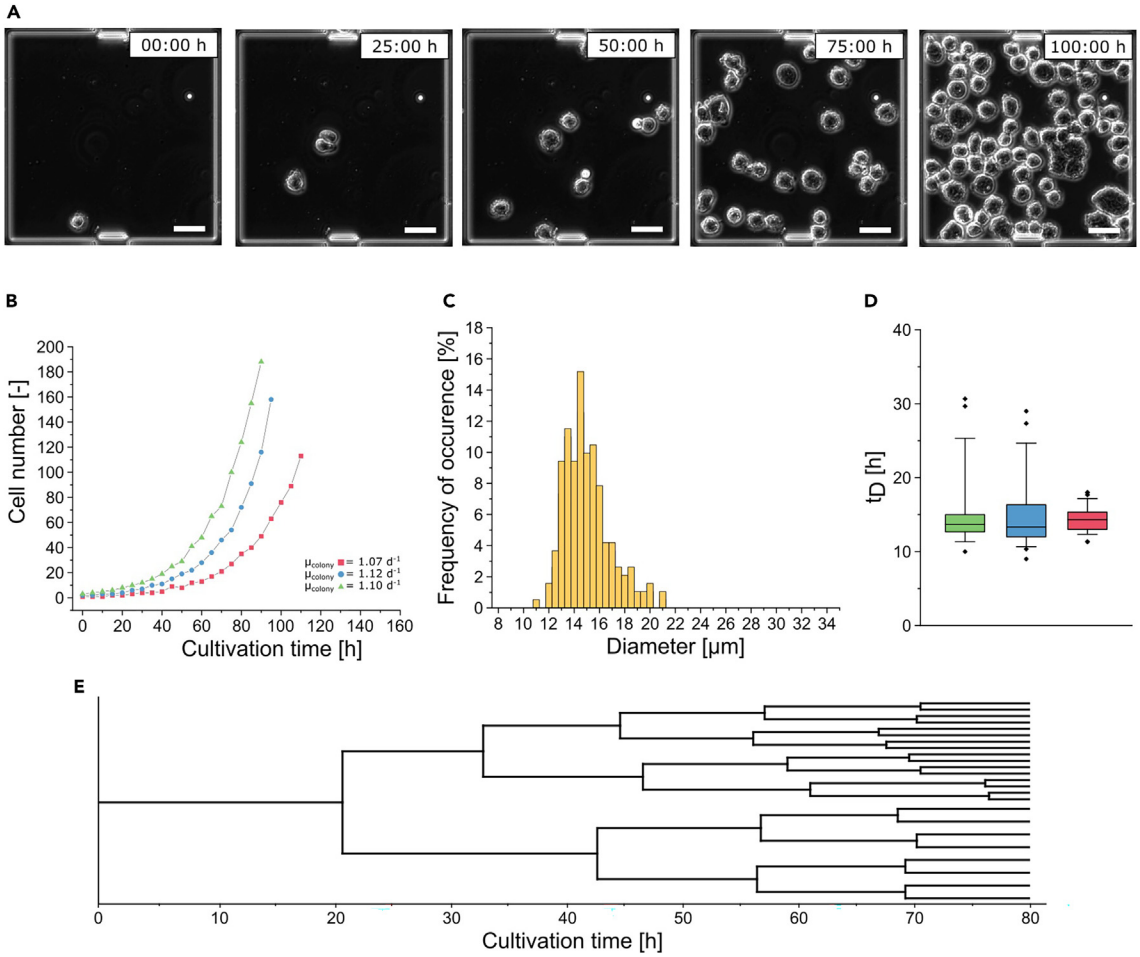


Figure 8. Single-cell data from MSCC experiment

(A) Time-lapse image sequence of one initial CHO cell to a microcolony over an experimental duration of 100 h (scale bar = 30 µm).

(B) By counting the cell number at specific cultivation times, growth curves can be recorded, and different cultivation chambers can be compared.

(C) By determining the diameter of single cells at a certain cultivation time, the cellular diameter distribution of a microcolony can be addressed.

(D) Single-cell doubling times can be determined and compared within the isogenic microcolony or with other microcolonies. Depicted as horizontal line is the median of all determined doubling times, the colored segment marks the interquartile range from 25% to 75%. The whiskers represent the 10% and 90% percentile and the tilted squares mark rare cellular events.

(E) By tracking single cells, a lineage tree can be recorded. This way differences in division behavior can be analyzed with incorporating the respective family relation.

Another limitation consists of the evaluation of the cultivation data. If analyses are not coupled to optical readouts such as cell morphology or fluorescence signals, analysis is not possible since no sampling of single cells is possible. Therefore, no genomic or transcriptomic analyses are performable and due to the high perfusion rate any secreted products are to highly diluted for proteomic or metabolomic analyses of the flow-through.

Currently, image analysis and thereby analysis of the cultivation data concerning key performance indicators like growth rate, morphology, or productivity of single cells is done manually.[8] For higher throughput, more sophisticated image analysis pipelines are needed that combine automated cell identification and prospectively cell tracking.[9]

The here presented protocol is optimized in its conditions and preparational steps for the cultivation of CHO suspension cells and their specific requirements towards the cultivation environment. In

general, the cultivation of other in suspension growing cell lines as well as adherent growing cells is possible,[10] but the cultivation conditions must be adapted to these changed requirements.

TROUBLESHOOTING

In the following the most frequently occurring problems during the cultivation of CHO suspension cells in a PDMS-glass based microfluidic cultivation device are described and approaches to solve them are given. For more general information on troubleshooting concerning microfluidic cultivation experiments, the reader is explicitly referred to Täuber et al., 2021.[5]

Problem 1

You notice a lag phase at the beginning of your MSCC experiment that extends beyond the expected time needed by the cells to adapt to the new cultivation environment.

Potential solution

- Seed train management: The most likely explanation for a prolonged lag phase comes from your seed train management (before you begin, step 1–4). As cells are transferred to a completely new cultivation environment one could expect them to need some adaption time. However, to counteract this it is of utmost importance that your cells are in the exponential growth phase when you prepare the inoculum (step 11). In case of your inoculum cells that are in exponential growth phase can faster adapt to the new cultivation environment so that transferring them into your microfluidic cultivation device should not influence their growth behavior. Looking at your single-cell cultivation medium it is important to obtain the conditioned part of the mixture from a culture being in exponential phase as well (step 1). Only this way you can guarantee the presence of growth promoting signaling molecules that enables single cells to grow despite their isolation. Using conditioned medium from stationary phase might even have growth prohibiting effects on your MSCC experiment.
- Technical Setup: Ensure that the technical peripherals are correctly adjusted and functioning properly. Specifically, check if the temperature remains consistent at the desired setpoint, confirm the gas atmosphere maintains the desired CO_2 concentration, and ensure the perfusion is sufficient (before you begin, step 20). Small oversights, such as leaving the doors of the microscope incubator open, can lead to instabilities in cultivation parameters.

Problem 2

During the time-course of a MSCC experiment, you observe air bubbles arising in your microfluidic cultivation device that travel through the supply channels and disturb your intended medium flow.

Most of the times, disturbing air bubbles occur in the inlet of your chip. This can have multiple reasons, from remaining air after device inoculation to degassing of CO_2 from the pre-saturated medium. Once there are small air bubbles remaining for the process of inoculation or the connecting of your pumping periphery to your microfluidic cultivation device, they can serve as initial starting point for more air to arise just like an initial nucleus in crystallization.

Potential solution

- Prevention: Ensure meticulous execution of the manual rinsing procedure (step 14) and the establishment of constant perfusion (step 15). Before initiating live-cell imaging, verify the absence of any air within your microfluidic device using the microscope.
- Flow rate increase: Increasing the applied perfusion flow rate temporarily can dislodge trapped air bubbles from the microfluidic cultivation device. However, it is essential to proceed cautiously as this method raises internal pressure, thereby increasing the risk of leaks and cell loss, and alters the cultivation conditions. Alternatively, a slight permanent increase in the flow rate can be implemented to help mitigate the degassing of CO_2 from the saturated single-cell cultivation medium (step 15.f).

- Atmosphere regulation: As CO_2 buffered medium has an increased CO_2 saturation in comparison to the outer atmosphere it can also be the case that the solved CO_2 degasses from your single-cell cultivation medium and forms gas bubbles. In this case you should regulate the outer atmosphere of your microscope periphery accordingly (before you begin, step 20.b).

Problem 3

When checking for your cell density before starting the seeding procedure of MSCC experiment, your main culture has the wrong cell density and is either in early exponential phase or late exponential/stationary phase.

Potential solution

- Waiting: When your seed train is in early exponential phase, waiting until it reaches midst exponential phase is the best option (step 11). MSCC might also work with cells and conditioned medium from early exponential phase, but results might not be comparable with prior performed MSCC experiments. You should always use seed trains that are in a comparable growth phase, as it not only influences the state of your inoculum but also the quality of your conditioned medium.
- Restart: When your seed train is in late exponential or stationary phase it will have negative effects on the state of your cells and the quality of your conditioned medium. Therefore, using such a seed train for MSCC experiments makes no sense and starting a new seed train is the only option.

Problem 4

During inoculation and/or establishment of a constant perfusion, debris was introduced into the MSCC device, thereby blocking supply channels either by sedimenting inside or attaching to the walls of the channels.

Potential solution

- Prevention: If debris is introduced during inoculation, it is likely that the viability of the inoculation culture is not sufficiently high, resulting in the presence of cell fragments and other contaminants. To address this issue, it is crucial to regularly assess the viability of the culture during seed train management and particularly at the time of inoculation culture preparation. Ideally, the viability should consistently exceed 95%, with a minimum threshold of 90% deemed acceptable (before you begin, step 1–4).
- Diluting the inoculum: If the viability falls below the afore mentioned thresholds the inoculation culture can be diluted with fresh medium to reduce the overall amount of debris, although this may complicate the inoculation procedure. It is important to note that this solution should only be utilized as a last resort, as it can exert additional stress on the inoculation culture, alter its state by delaying the start of the experiment, and reduce overall comparability with previous experiments.
- Centrifugation and filtration: In case of debris being introduced through the single-cell cultivation medium, the medium can be centrifuged at 200 × g for 5 min and then sterile filtered with a 0.22 μm filter once again (step 1.b-e). However, this process necessitates an additional incubation period of 2 h at 37°C, 5% CO_2, and 80% relative humidity on a shaker at 185 rpm to achieve the desired pH value. This solution should also be considered a last resort, as it is subject to the same afore mentioned drawbacks.

Problem 5

The Focus is lost during the MSCC thereby impeding the data and image quality.

Potential solution

- Prevention: The optimal approach to addressing this issue is to prevent it from occurring altogether. Therefore, it is advisable to remain by the microscope after the start of the experiment to assess the quality of the experimental setup. Specifically, this involves monitoring the stability of perfusion and ensuring that no external forces are exerted on the microfluidic device by the connected periphery during the movement of the automated microscope stage. Additionally, with some manufacturers perfect focus systems (PFS) can be applied to guarantee stable focus during long-term experiments. However, it is crucial to verify that the PFS was configured properly.
- Readjustment: Image acquisition can be temporarily halted, allowing for manual readjustment of the focus plane for each position saved in the acquisition list. However, the effectiveness of this solution is contingent upon the duration of focus loss. When it occurs right after starting the experiment, readjusting the focus is still reasonable. If loss of focus is noticed after a few hours the quality of the data is already too compromised so that readjusting will not help improving the subsequent data analysis. Any modification during a running experiment leads to a change in image acquisition frequency which might further compromises the quality of the data, thereby complicating subsequent analyses, even more. Hence, the reasonability of this solution must be evaluated on a case-by-case basis.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Julian Schmitz (j.schmitz@uni-bielefeld.de).

Technical contact

Further information and requests for technical issues should be directed to and will be fulfilled by the technical contact, Julian Schmitz (j.schmitz@uni-bielefeld.de).

Materials availability

This study did not generate new unique reagents.

Data and code availability

The datasets supporting the current study have not been deposited in a public repository because of the immense size of the respective image data (multiple gigabytes) but are available from the corresponding author on request.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2024.103106.

ACKNOWLEDGMENTS

We acknowledge support for publication costs by the Open Access Publication Fund of Bielefeld University and the Deutsche Forschungsgemeinschaft (DFG).

AUTHOR CONTRIBUTIONS

Conceptualization, J.S.; investigation, J.S. and B.Y.; formal analysis and validation, J.S. and B.Y.; visualization, J.S. and B.Y.; resources, A.G.; supervision, J.S. and A.G.; writing – original draft, J.S. and B.Y.; writing – review and editing, J.S., B.Y., and A.G.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

1. Schmitz, J., Täuber, S., Westerwalbesloh, C., von Lieres, E., Noll, T., and Grünberger, A. (2021). Development and application of a cultivation platform for mammalian suspension cell lines with single-cell resolution. Biotechnol. Bioeng. *118*, 992–1005. https://doi.org/10.1002/bit.27627.

2. Schmitz, J., Stute, B., Täuber, S., Kohlheyer, D., von Lieres, E., and Grünberger, A. (2023). Reliable cell retention of mammalian suspension cells in microfluidic cultivation chambers. Sci. Rep. *13*, 3857. https://doi.org/10.1038/s41598-023-30297-5.

3. Halldorsson, S., Lucumi, E., Gómez-Sjöberg, R., and Fleming, R.M.T. (2015). Advantages and challenges of microfluidic cell culture in polydimethylsiloxane devices. Biosens. Bioelectron. *63*, 218–231. https://doi.org/10.1016/j.bios.2014.07.029.

4. Blöbaum, L., Täuber, S., and Grünberger, A. (2023). Protocol to perform dynamic microfluidic single-cell cultivation of C. glutamicum. STAR Protoc. *4*, 102436. https://doi.org/10.1016/j.xpro.2023.102436.

5. Täuber, S., Schmitz, J., Blöbaum, L., Fante, N., Steinhoff, H., and Grünberger, A. (2021). How to Perform a Microfluidic Cultivation Experiment-A Guideline to Success. Biosensors *11*, 485. https://doi.org/10.3390/bios11120485.

6. Eddington, D.T., Puccinelli, J.P., and Beebe, D.J. (2006). Thermal aging and reduced hydrophobic recovery of polydimethylsiloxane. Sensor. Actuator. B Chem. *114*, 170–172. https://doi.org/10.1016/j.snb.2005.04.037.

7. Romanova, N., Schmitz, J., Strakeljahn, M., Grünberger, A., Bahnemann, J., and Noll, T. (2022). Single-Cell Analysis of CHO Cells Reveals Clonal Heterogeneity in Hyperosmolality-Induced Stress Response. Cells *11*, 1763. https://doi.org/10.3390/cells11111763.

8. Schmitz, J., Hertel, O., Yermakov, B., Noll, T., and Grünberger, A. (2021). Growth and eGFP Production of CHO-K1 Suspension Cells Cultivated From Single Cell to Laboratory Scale. Front. Bioeng. Biotechnol. *9*, 716343. https://doi.org/10.3389/fbioe.2021.716343.

9. Scherr, T., Seiffarth, J., Wollenhaupt, B., Neumann, O., Schilling, M.P., Kohlheyer, D., Scharr, H., Nöh, K., and Mikut, R. (2022). microbeSEG: A deep learning software tool with OMERO data management for efficient and accurate cell segmentation. PLoS One *17*, e0277601. https://doi.org/10.1371/journal.pone.0277601.

10. Höving, A.L., Schmitz, J., Schmidt, K.E., Greiner, J.F.W., Knabbe, C., Kaltschmidt, B., Grünberger, A., and Kaltschmidt, C. (2021). Human Blood Serum Induces p38-MAPK- and Hsp27-Dependent Migration Dynamics of Adult Human Cardiac Stem Cells: Single-Cell Analysis via a Microfluidic-Based Cultivation Platform. Biology *10*, 708. https://doi.org/10.3390/biology10080708.