# Dynamic Environmental Control in Microfluidic Single-Cell Cultivations: From Concepts to Applications

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**Microfluidic single-cell cultivation (MSCC) is an emerging field within fundamental as well as applied biology. During the last years, most MSCCs were performed at constant environmental conditions. Recently, MSCC at oscillating and dynamic environmental conditions has started to gain significant interest in the research community for the investigation of cellular behavior. Herein, an overview of this topic is given and microfluidic concepts that enable oscillating and dynamic control of environmental conditions with a focus on medium conditions are discussed, and their application in single-cell research for the cultivation of both mammalian and microbial cell systems is demonstrated. Furthermore, perspectives for performing MSCC at complex dynamic environmental profiles of single parameters and multiparameters (e.g., pH and $O_2$) in amplitude and time are discussed. The technical progress in this field provides completely new experimental approaches and lays the foundation for systematic analysis of cellular metabolism at fluctuating environments.**

## 1. Introduction

### 1.1. Microfluidic Single-Cell Cultivation (MSCC)

In recent years, novel microfluidic methods for the cultivation and investigation of cellular behavior on single-cell level have emerged (**Figure 1**A). Microfluidic systems have several advantages for example high surface to volume ratios and small cultivation volumes (nl–pl volumes), which allow fast heat and mass transfer and thus precise environmental control.[1] In microfluidic devices, cells and small cell colonies can be trapped in cultivation habitats[1] by different physical principles.[2] Besides contactless trapping such as laser trapping and negative

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dielectrophoresis (nDEP),[3] contact trapping based on hydrodynamic barrier structures in PDMS–glass devices is most widely applied.[2] Here, cells are randomly trapped in cultivation areas and cell growth is restricted by the barrier structures. The cultivation chambers on such microfluidic cultivation systems can be classified in 3D, 2D, 1D, and 0D systems (Figure 1B).[1] These microfluidic systems differ greatly in the spatial degree of freedom for cell growth and size of the growing microcolony. In 3D MSCCs, cells overlap with each other and are not in one focal plane, so that single-cell growth and physiology cannot be analyzed with full spatio-temporal resolution,[1] thus the application of these systems for dynamic single-cell analysis with full spatio-temporal resolution is not possible.[1] 2D MSCCs allow to cultivate small microcolonies with up to 1000 cells[4,5] and cell growth is restricted to a monolayer. These MSCCs are easy to handle and allow to monitor cellular behavior such as growth, cell division and morphology. Typical dimensions for 2D chambers for microbial cells are 60 µm × 50 µm,[6] whereas for cell cultures typical chamber dimensions are 250 µm × 500 µm.[7] 1D and 0D systems typically hold up to ten cells per trap[8,9] or even completely isolated single cells.[10,11] Here, cells are growing in one line that allows a reliable long-time cultivation of cells. In comparison to 2D designs, the cell loading process is difficult. Typical dimension of 1D chambers are length of 10–20 µm and width between 0.6 and 1.0 µm for microbial cells.[12] 0D MSCC systems allow cellular analysis of complete isolated single cells, but fabrication, cell tapping and long-term cultivation is still difficult and thus these systems are not yet regularly applied for microbial cells.[10] Continuous supply of culture medium and removal of products and by-products provided by perfusion allow for constant environmental conditions over long cultivation times (Figure 1C). The combination of microfluidic systems with live-cell imaging enables dynamic cell analysis in high spatio-temporal resolution (Figure 1D). Currently, such systems are frequently applied for investigating cell-to-cell heterogeneity,[13] aging and death,[8,13–15] growth,[8,16–18] cell cycle,[19] gene expression,[20–23] and various other metabolic processes.[24,25]

This review addresses MSCC under dynamically controlled environments (DCE). First, we provide a short overview regarding DCE and their nomenclature. We discuss different microfluidic concepts for the cultivation of both mammalian and microbial cell and how the choice of the cultivation chamber affects the ability to perform MSCC under DCE. Further, we

show first applications in applied biology. This review focuses on chemical environmental factors (e.g., nutrient conditions) in DCE. DCE within physical and biophysical environments (e.g., shear stress, pressure, temperature, etc.) is not addressed in detail. Finally, we present a perspective in technical and biological challenges on the implementation of complex and fluctuating environmental profiles.

### 1.2. Mimicking Environmental Conditions: Overview

Environmental conditions in natural habitats of bacteria are generally not constant but vary in concentration over the time.[26,27] In their natural habitat (e.g., soil and ocean) and artificial habitats (e.g., bioreactors) microbial cells are exposed to changing environmental stimuli. These can be classified in physical, chemical and biological stimuli (**Figure 2**A). Changes in a broad range of time scales influence the cellular physiology. Physical parameters can include changes in temperature and pressure.[28] Chemical and biological stimuli are governed through the availability of different nutrients, detrimental substances or molecules for cellular communication.[29] Based on the environmental input, cells regulate their gene networks dynamically and adjust parameters such as growth, metabolism, aging and death, evolution, cell cycle, etc. (Figure 2B).[30–33] Historically, cells are isolated from natural habitats and grown in lab-scale devices to characterize their physiology.[34] Typically, cells are cultivated in shake flasks and bioreactors, to keep environmental conditions as constant as possible.[35] However, even in these devices, consumption of nutrients and gradients through mixing[26] and the cell metabolism itself[36] lead to inhomogeneous environmental conditions and thus the analysis of cellular response to defined environmental conditions is difficult. Especially for the investigation of reason and origin of cellular heterogeneity, traditional methods have their limitations, since most of the assays rely on averaging cellular output measurements[37] and the inability for cultivations at defined environmental conditions.

The perfusion of media during conventional MSCC leads to continuous medium supply and the removal of products and by-products and thus constant environment conditions over the cultivation time (Figure 2C). In their natural habitat cells are exposed to randomly changing environmental conditions with changes of frequency and amplitude of environmental parameters (Figure 2E).[38] This includes gradients in nutrients,[39] temperature,[40] pressure,[41] pH value,[42] and oxygen level.[43] Even in simulated natural habitats such as laboratory bioreactors, cells are exposed to short-term environmental fluctuations.[26,44] Here, the fluctuations can be smaller in amplitude but can be faster in frequency.[45] To analyze the influence of environmental fluctuation in laboratory scale as well as natural habitats novel microfluidic systems must be developed to mimic fluctuating environmental conditions (Figure 2D).[46]

In literature, the term fluctuating environmental conditions is currently not well defined. Alternatively, dynamically controllable conditions,[47] dynamically changing environment,[48] and environmental fluctuations[49] are synonymously used. The corresponding systems have in common that they allow to apply specific environmental/stimulant profiles to cells that are difficult to perform with conventional MSCC devices. For better

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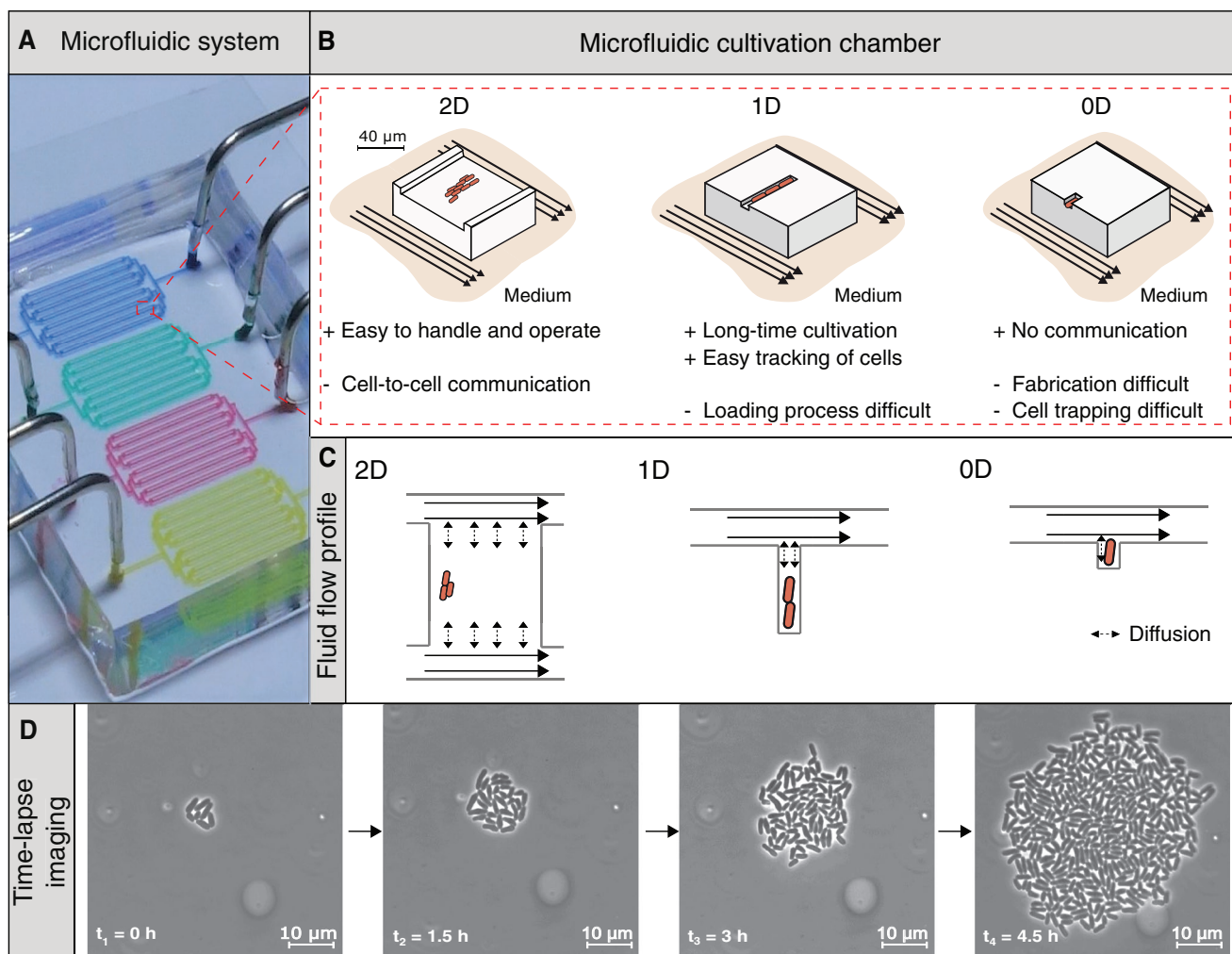
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readability, the term dynamically controlled environments (DCE) is used within this review.

Ideally, MSCC devices should provide the possibility to create DCE with different profiles. We propose to differentiate between five different categories on how DCE can be created in microfluidic devices (Figure 2F). In the first category, during the microfluidic experiments, medium can be switched once in a step function or a pulse function and no systematic modulation is performed (Figure 2F1). Moreover, an environmental profile can be established with the same amplitude and width, only the frequency between the pulses ($f_1$ and $f_2$) is varied (pulse frequency

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**Figure 1.** Overview of a microfluidic single-cell cultivation (MSCC) chip system. A) MSCC chip, containing several cultivation arrays with monolayer cultivation chambers. B) Different cultivation chamber geometries (2D to 0D) with their advantages and disadvantages and C) fluid flow schematic of these chambers. D) Live-cell imaging of typical microbial cell colony cultivated at constant environmental conditions.

modulation (PFM)) (Figure 2F2). In the third category, the environment can be switched in a different temporal manner ($w_1$ and $w_2$) so that the pulses have different temporal resolutions (here the frequency and amplitude are constant) (pulse width modulation (PWM)) (Figure 2F3). Furthermore, the environmental profile can be modulated with different amplitudes, whereas the frequency and width of the pulses are kept constant ($a_1$ and $a_2$) (pulse amplitude modulation (PAM)) (Figure 2F4). Finally, cells can be exposed to different environments in a varying temporal manner so that the amplitude, frequency and width of the concentration varies over time in a different manner. Here, a combination of PWM, PFM, and PAM is used (Figure 2F5).

## 2. Microfluidic Setups for Dynamically Controlled Environments (DCE)

The realization of DCE in a microfluidic device can be divided into different types of technical peripheries (**Figure 3**) and the cultivation chamber (see Section 2.2). The technical periphery can be implemented either by hydrostatic pressure[50] (Figure 3A), pressure pumps[51] (Figure 3B), pneumatic micro-valves on a chip[52] (Figure 3C) or an external electric valve[53] (Figure 3D). Here, we shortly compare different concepts for the implementation of the mechanism's periphery based on the physical principle and their setup. For static differences within environments created by microfluidic gradient generators the reader is referred to recent reviews summarizing the field of microfluidic gradient generators.[54,55] Afterward, the cultivation chamber is introduced in context of a rapid DCE and an overview of the different combination of technical periphery and cultivation chambers is shortly discussed.

### 2.1. Technical Periphery

#### 2.1.1. Hydrostatic Pressure: External

The simplest realization of DCE can be done by hydrostatic pressure (Figure 3A, Setup).[50,56] For the experimental
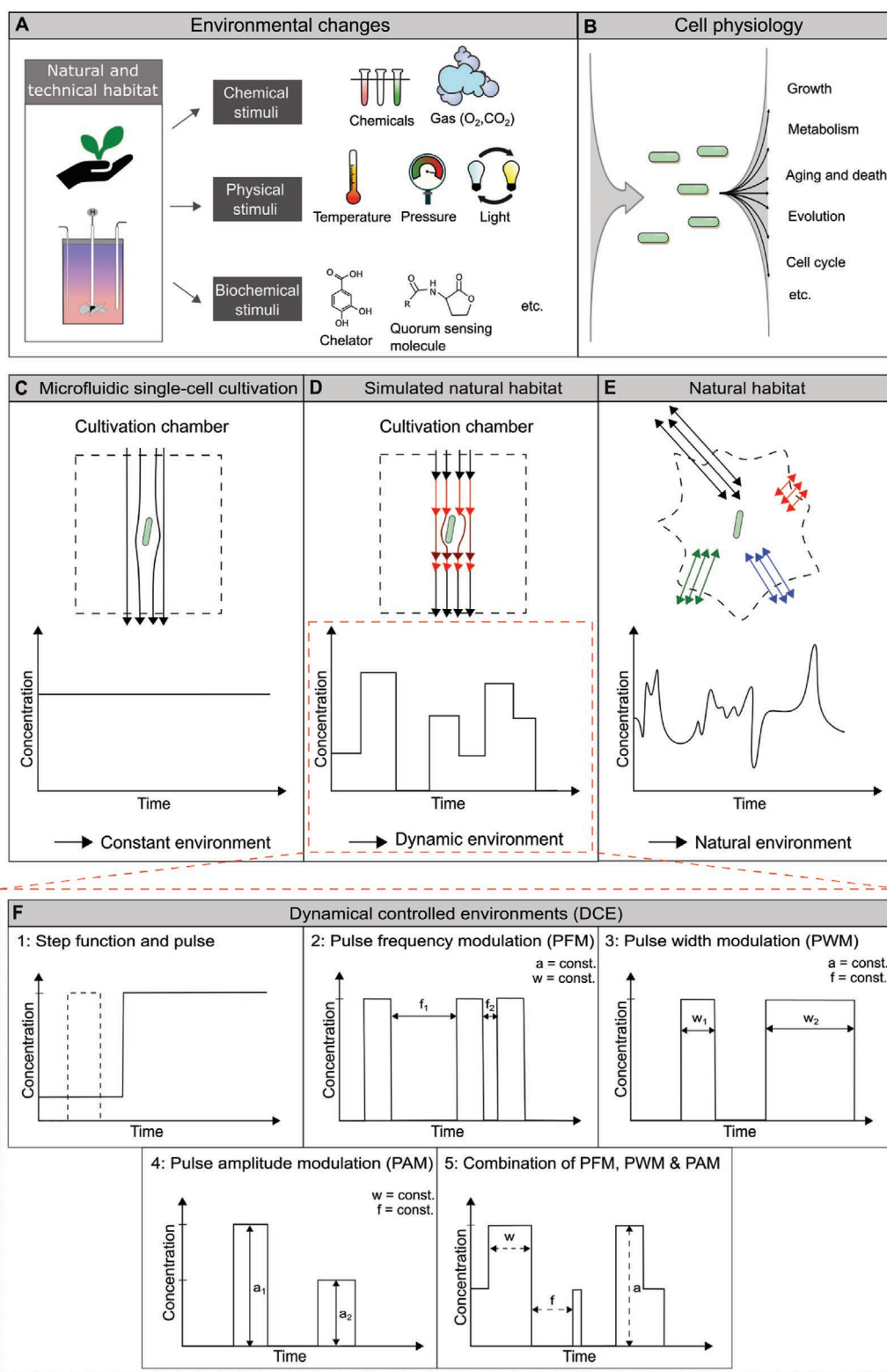
**Figure 2.** Overview of environmental factors and DCE mechanisms at single-cell level. A) The natural and technical habitat influence the environment change in physical components such as pressure, chemical stimuli like gas, and biochemical stimuli that B) influence cellular physiology for example growth, metabolism, aging, death, etc. C) In MSCC, cell cultivations can be performed at constant concentration so that the cells can grow under optimum conditions. D) In the simulated habitat the concentration in the environment varies in a fixed (defined) time frame over the cultivation time (dynamic environment). E) In natural habitats, environmental concentrations can typically change randomly over the time. F) Different DCEs: 1) step and pulse functions, 2) pulse frequency modulation (PFM), 3) pulse width modulation (PWM), 4) pulse amplitude modulation (PAM), and 5) combination of previous modulations.

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**Figure 3.** Methods for the generation of DCE at single-cell level in microfluidic chips. DCE modulation conducted with A) hydrostatic pressure method using syringe driver with motorized stage for DCE in higher minute up to hour change, B) external pressure pump in front of the microfluidic chip for rapid change between environmental conditions, C) pneumatic valves on chip used for rapid changes of environmental conditions, and D) external electric valve in front of microfluidic chip for a fast DCE change.

implementation two syringe drivers are used with different media or different concentrations of the same medium. A computer-controlled stepper motor can control the input transfers of different environments by varying the height between the two syringes or the syringes are controlled electrically (Figure 3A, Principle). Hydrostatic pressure differences are generated by the relative difference between the syringes and the surface of the fluids. Therefore, this system offers an on/off regulation for the control of the inflow in the microfluidic chip.[56] This setup is simple and easy to handle (Figure 3A, Specifications). The limitation of the setup is that only low-frequency modulations in the range of minutes can be created. This restricts the application to step functions and PAM and PWM with low resolution.

### 2.1.2. Pressure Pump: External

A similar method for the DCE offer syringe driver pumps and pressure pumps to modulate environmental conditions (Figure 3B, Setup). Here, a microfluidic chip system with several inlets is necessary so that the different concentrations are mixed on chip or a device where the multiple environment

channels lead into one inlet over a T-fitting (Figure 3B, Principle).[57] This method requires advanced periphery in form of a precise pressure driven pumping system. Nevertheless, fabrication, handling, and implementation are still quite simple and comparable to the hydrostatic pressure method (Figure 3B, Specifications).[58] In comparison to the hydrostatic pressure method, achievable frequency of the environmental modulation in a lower minute up to second range is possible.

### 2.1.3. Pneumatic Valve on a Chip

Pneumatic microvalves, also called "Quake valves" were developed by Stephen Quake in 2000.[59] They use the effect of the deformation of polydimethylsiloxane (PDMS) to control the flow so that a change can develop actively and passively.[59,60] Microfluidic valves on a chip can be opened and closed through the variation of the flow rate of liquid or air (Figure 3C, Setup). The continuous regulation of the valve can be controlled with the manipulation of airflow, here pressure on chip, so that the external pressure is constant.[61]

Pneumatic microvalves are fabricated using multilayer soft lithography in a microfabrication step with a soft polymer, at

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least two polymer layers are required for the microvalve. One layer contains the microfluidic channel for the liquid flow. The other one is the control layer which deflects this part into the microfluidic channel and stops the flow (Figure 3C, Principle).[62] In comparison to the hydrostatic pressure approach, high frequency oscillations of the environment can be obtained (Figure 3C, Specifications). The switching between different environmental conditions takes only a few milliseconds so that oscillations can be created in a range of milliseconds.[63] The fabrication of the microfluidic chip and implementation as well as the handling is very time consuming. A few research groups utilize syringe drivers for the manipulation of airflow of the valves. As a result, pressure fluctuations in flow occurs and the regulation of the syringe pumps is slower in comparison to pressure pumps.[64] All in all, pneumatic valves allow a high resolution in frequency of DCE and amplitude of DCE in a range of seconds and milliseconds.[65]

### 2.1.4. External Electric Valve

An alternative method for the implementation of DCE offer external electric valves (Figure 3D, Setup). These valves are computer-controlled and are installed in front of the inlets of the microfluidic channel so that a T-type setup is used to combine the different environment reservoirs and the channel inlet (Figure 3D, Principle). The switching time of the external electric valve between two different states takes only a few milliseconds.[66,67] The change of the environment inside the channel is not instantaneously with switching of the valve because of the dead volume of the connecting tube between the valve and the microfluidic device.[53,68] It is important to choose a tube with a low cross-section area and a short length of a few centimeter so that the dead volume in the tube can be changed fast. Depending on the flow rate for example 1 µL s$^{-1}$, a temporal resolution of around 10 s can be experimentally realized with a microfluidic channel with a height of ≈100 µm and a volume of ≈0.6 µL cm$^{-1}$.[67,68] The DCE can oscillate in a range of seconds so that different feed streams can be feed in short time-scales. The handling of this method is also simple and easier to implement than pneumatic valves (Figure 3D, Specifications). In summary, this method is simple to handle and a high resolution in frequency and amplitude of DCE in a range of seconds is possible.[68]

Additionally, studies are reported, where DCE, mainly step profiles, were performed manually.[24,69,70] Here, the operator/experimenter replaces the inlet channel or tubing by an alternative inlet filled with different medium connected to syringe pump systems. This method is often used when single medium changes are performed. Applications range from changes in carbon sources[71,72] to studies with potential effectors such as antibiotics.[72–74] Although this method is quite simple in handling, the implementation of oscillating conditions and defined pulses is hardly possible. Furthermore, the manual interruption of live-cell experiments bears the risk to disturb experimental conditions for example through air, contaminations, etc. Therefore, this method is not further discussed within this review.
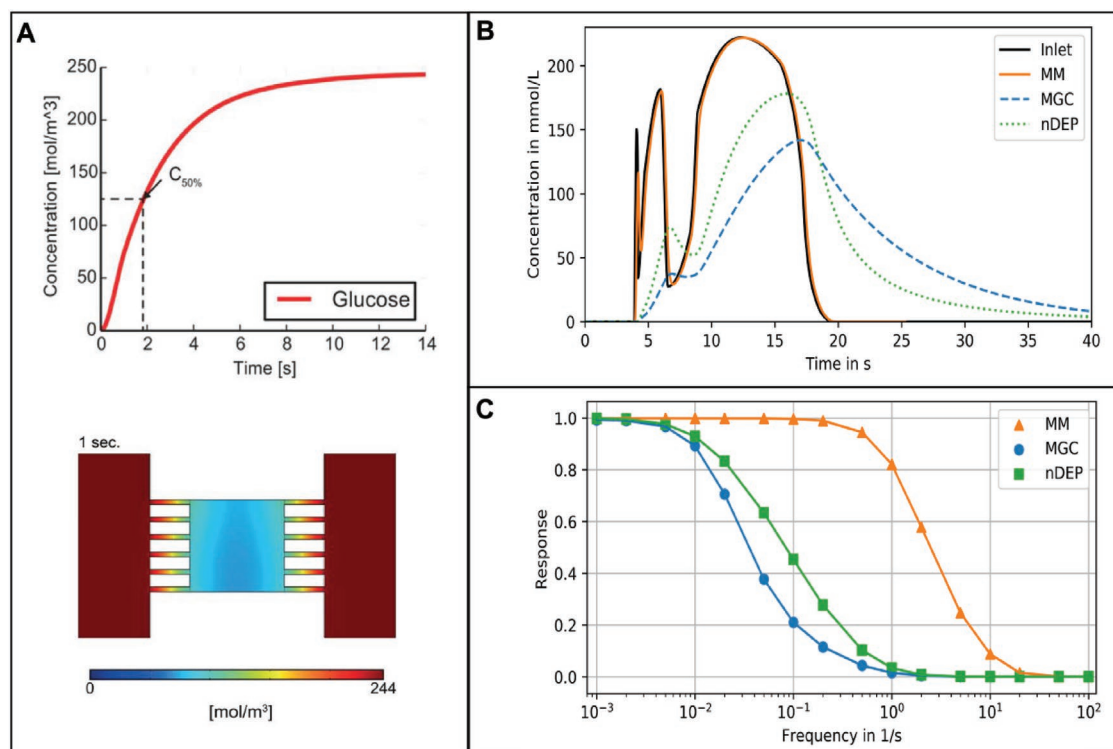
## 2.2. Cultivation Chamber

Whereas the technical periphery (see the last section) plays an important role in the realization of DCE within MSCC, the cultivation chamber is the most important factor when cells shall be cultivated under rapidly changing DCE.

For 2D chambers (Figure 1B) nutrient availability can be deteriorated, and exchange of the complete chamber volume ranges between 10 s and minutes (**Figure 4**A). This depends on chamber geometry and size, type of supply (convection and diffusion), and the number of nutrient inlets[4,75,76] because the nutrient exchange in the cultivation chamber occurs only via diffusion. The exchange of nutrients within the chamber is the limiting factor for high-frequency DCE applications. In contrast, Wang et al. have demonstrated that the complete volume change in a 1D structure per diffusion is in the lower seconds range (≈1 s).[8] 0D structures have the fastest volume exchange because medium exchange occurs mainly through convection. The complete volume exchange requires about 100 ms depending on the geometry of the trap and flow rate of the surrounding media flow.[62]

The "quality" of reproduction of a defined environmental profile generally depends on the cultivation chamber design which is important for a high precision of the DCE. Ho et al.[76] have investigated this in detail by advanced computational fluid dynamics (CFD) modeling studies. In a first step, they investigated which cultivation chamber design can be used to create DCE at second and subsecond resolution. They analyze if typical lifelines of cell metabolism observed in large-scale bioreactors can be analyze on single-cell level.[44] As shown in Figure 4B, a typical concentration profile features three concentration peaks with increasing width and amplitude. Ho et al. found that 1D systems are able to accurately reproduced this entire signal. Both nDEP and 2D systems completely miss the first peak and can by far not reproduce the amplitude of the second peak (44% for nDEP and 17% for 2D). In a next step Ho et al.[76] systematically investigate features of which frequency in DCE profiles can be produced at the cell surface on the microfluidic chip and cultivation chamber designs. Therefore, they use sinus signals with different frequencies. The resulting bode plot (Figure 4C) reveals frequency ranges for which environmental changes can be precisely reproduced so that for a high reproduction of lifelines an amplitude signal loss of maximum 5% is requisite. In 2D cultivation chambers (here chambers have one nutrient supply channel) the amplitude signal with a period of 200 s is damped less by 5%. In comparison, DCE in 1D cultivation systems such as the mother machine[8] can reproduce an amplitude signal with a period of 5 s with an amplitude loss of less than 5%. Despite the significant advantage in mass transfer, 1D cultivation systems have to be chosen carefully. Recently, it was shown by Yang et al.[12] that design and geometry of the cultivation channel can impact growth of microbial cells significantly.

The choice of the technical periphery and cultivation chamber conform of the depending requirements. It is important that the change of the environmental conditions is significantly lower than biological relevant timescales. For DCE with frequencies in lower minutes and second ranges the choice of the cultivation chamber is important, since medium change from main and nutrient channels to cultivation chambers are

**Figure 4.** DCE in microfluidic cultivation chambers. A) Simulation of glucose change in 2D cultivation chamber. After 6 s glucose concentration equilibrium is reproduced in chamber. Reproduced with permission.[4] Copyright 2019, Wiley-VCH. B) Reproduction of environmental profile with different cultivation chamber designs.[76] C) Bode diagram compares responses for three chamber designs.[76]

often restricted by diffusion.[75] If a step function is of interest all combinations of chamber designs and technical periphery can be used. Similarly, if DCE with times in hours is the aim all combinations of technical periphery and cultivation chamber designs are possible. In these cases, the diffusion driven mass transport within the cultivation chambers has a temporal delay over a few seconds but can be neglected. For DCE with changes in minutes up to seconds the choice of the technical periphery and cultivation chamber is crucial. Here, microfluidic cultivation chambers with short volume exchange times must be chosen such as 1D systems like the mother machines and 0D like traps. 2D cultivation chambers as well as 1D chambers can be chosen if an oscillation in a minute range is the aim. For the technical periphery, pressure pumps can be used for rapid DCE because of the fast switching times (≈milliseconds) between different concentrations of the pumps in combination with 1D and 0D cultivation chambers. Pneumatic valves on a chip and external electric valves have in combination with 1D or 0D chambers the advantage that an environmental change in a range of seconds is possible.

## 3. Applications

Several dynamic single-cell cultivation proof-of-concept studies were performed in the last years. They differ in the cultivation chamber, technical periphery, modulation methods, applied environmental profile and the application. Examples are chosen to demonstrate both, technical feasibility as well as novelty in

the application to perform cellular experiments that are not possible or difficult to perform with conventional technologies such as shaking flasks or lab-scale bioreactors. In shaking flasks and bioreactors only average measurements are possible to determine, not single-cell values. **Figures 5** and **6** present an overview of these studies, which is discussed in the next section in more detail.

### 3.1. Hydrostatic Pressure: External

Fracassi et al.[50] used a microfluidic device with a motorized syringe driver which had an automatic control in real time for analyses of gene expression of tetracycline inducible promotor in CHO tetO7-YFP cells (**Figure 7**A, Setup). The DCE between tetracycline-rich and standard growth medium were generated by PWM/PFM in 15–1000 min intervals (Figure 7A, Environmental profile). The microfluidic device consists of several 2D cultivation chambers that contain small CHO colonies in a monolayer. They showed that the cells expressed an average value of 50% of d2EYFP after 3500 min (Figure 7A, Representative results).

The same research group modified the setup so that the motorized stage of the syringe could regulate the desired concentration, 0%, 50%, and 100%, in a pulse range from 15 to 100 min. This way, they also investigated the gene expression of tetracycline inducible promotor of CHO and could demonstrated a set-point regulation of gene expression in a proof of concept study.[56,77]

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| Method | Chamber | | | Tech. periphery | DCE | | | Specification | Application | Ref. |
|---|---|---|---|---|---|---|---|---|---|---|
| | 2D | 1D | 0D | | PWM | PFM | PAM | | | |
| Hydrostatic pressure - external | x | - | - | On-off actuation | x | x | - | **Interval:** 8 - 350 min **Environments:** galactose and glucose | Induction of GAL1 promotor in *S.cerevisiae* | [79] |
| | x | - | - | PI, MPC and ZAD controller | x | x | - | **Interval:** 15 - 500 min **Environments:** galactose and glucose | Control of GAL1 promotor in *S.cerevisiae* | [78] |
| | x | - | - | On-off actuation | x | x | - | **Interval:** 15 - 1000 min **Environments:** tetracycline rich and standard medium | Gene expression of tetracycline inducible promotor in CHO tetO7-YFP cells | [50] |
| | x | - | - | Motorized syringe for desired concentration | x | x | - | **Interval:** 15 - 100 min **Environments:** standard medium and standard medium with tetracycline | Gene expression of tetracycline inducible promotor in CHO AA8 tet-off cells | [56; 77] |
| | x | - | - | Dial a Wave (DAW) | x | x | - | **Interval:** 50 min - 5 h **Environments:** medium with and without nitrogen | Analysis of the cell pro-liferation and chlorophyll autofluorescence in *C. sorokiniana* | [80] |
| | - | x | - | DAW | - | x | - | **Interval:** 4 h **Environments:** glucose and lactose | Induction of lac operon in *E.coli* | [47] |
| | - | - | x | Programmable syringe pumps | x | x | - | **Interval:** 6h/ 2h **Environments:** 2% and 0.1% glucose | Gene regulation of glucose in *S. cerevisiae* | [81] |
| Pressure based principles - external | x | - | - | Onix system (CellASIC) | - | x | - | **Interval:** 90 sec **Environments:** LB medium and LB medium with sorbitol | Induction of hyper-osmotic shock in *E.coli* | [58] |
| | x | - | - | Onix system (CellASIC) | - | x | x | **Interval:** 4 min/ 10 min **Environments:** 2, 5, 10, 100 mM glucose | Analysis of cytosolic free glucose in *S. cerevisiae* | [82] |
| | x | - | - | Fluidic wave generator | x | - | - | **Interval:** 0.75 - 4.5 h **Environments:** glucose and galactose | Gene expression of GAL1-yECFP fusion gene in *S. cerevisiae* | [48] |
| | - | x | - | Syringe pumps | - | x | - | **Interval:** 1 h **Environments:** SMM medium and SMM medium with IPTG | Control of sigma factor activity for RNA polymerase in *B. subtilis* | [57] |
| | x | - | - | Multilayer device, pressure driven pump | x | x | - | **Interval:** 3 - 12 h **Environments:** cycloheximide and cell media (concentration gradient) | Analyse of cycloheximide (CHX) gradients of Vero cells | [51] |

**Figure 5.** DCE studies with hydrostatic pressure and pressure pumps.

Fiore et al.[78] used hydrodynamic pressure for the control of the GAL1 promoter in *Saccharomyces cerevisiae*. The regulation of DCE between galactose and glucose was adjusted over a negative feedback loop with different controllers so that pulses were generated by PWM/PFM in 15–500 min intervals. In the same research group, Menolascina et al.,[79] used a similar setup with an on/off actuation for the induction of GAL1 promotor in *S. cerevisiae*. The DCE between galactose and glucose were

| Method | Chamber | | | Tech. periphery | DCE | | | Specification | Application | Ref. |
|---|---|---|---|---|---|---|---|---|---|---|
| | 2D | 1D | 0D | | PWM | PFM | PAM | | | |
| Pneumatic valve on a chip | - | - | x | Multilayer chip with integrated pumps and valves | x | - | - | **Interval:** 40 sec **Environments:** HBS medium with Ionomycin and HBS medium | Analysis of $Ca^{2+}$ dynamics in Jurkat T-cells | [62] |
| | x | - | - | Three-layer microfluidic chip | - | x | - | **Interval:** 30 - 150 sec **Environments:** carbachol and normal cell medium | Analysis of $Ca^{2+}$ dynamics in HEK-293 cells | [63] |
| | x | - | - | Additional external electric valves | x | - | - | **Interval:** 15 - 160 sec **Environments:** FBS concentration profile | Induction of FBS in the viability and migrational response in MDA-MB-231 cells | [65] |
| | x | - | - | Additional external electric valves | - | x | - | **Interval:** 20 - 200 sec **Environments:** 100 µM $H_2O_2$ + RPMI medium and RPMI medium | Induction of calcium signalling in Jurkat T cells | [83] |
| | x | - | - | Constant inflow over a syringe pump, three-layer chip | x | x | - | **Interval:** 57 - 360 sec **Environments:** 1 µM fluorescence dye in TE buffer and DMEM medium | Induction of periodic cell staining in C2C12 cells | [52] |
| | x | - | - | Multilayer microfluidic chip with integrated pumps | x | x | - | **Interval:** 9 - 14 min **Environments:** fluorescein and medium | Analysis of pheromone induced mitrogen-activated protein kinase in *S. cerevsiae* | [84] |
| | x | - | - | Multilayer microfluidc chip | - | x | - | **Interval:** 100 min (sinus wave) **Environments:** TNF with a maximum concentration of 3 ng/ml | Analysis of transcription factor NFκB in mouse 3T3 fibroblasts | [85] |
| | x | - | - | Two-layer microfluidic chip | x | x | - | **Interval:** 2 - 8 h **Environments:** no methionine and methionine-containing medium | Induction of repressible pMET17 promoter for YFP expression in *S. cerevisiae* | [86] |
| External electric valve | x | - | - | 3-way solenoid valves | x | x | - | **Interval:** 10 sec - 5 min **Environments:** medium with and without 1-NM-PP1 | Control of Msn2 dynamics in *S. cerevisiae* | [67; 88] |
| | x | - | - | Computer controlled three-way valves | x | x | - | **Interval:** 5 - 8 min/ 20 min **Environments:** normal and sorbitol-enriched medium | Induction of HOG1 promotor for the osmotic stress in *S. cerevisiae* | [53; 87] |
| | x | - | - | Separation of flow and growing cells through a diffusive cellulose membrane | x | x | - | **Interval:** 10 - 15 min **Environments:** SCD + 10x Met and SCD + 0x Met | Induction of GAL1 and MET3 promotor in *S. cerevisiae* | [68] |

**Figure 6.** DCE studies with pneumatic valves on the chip and external electric valves.
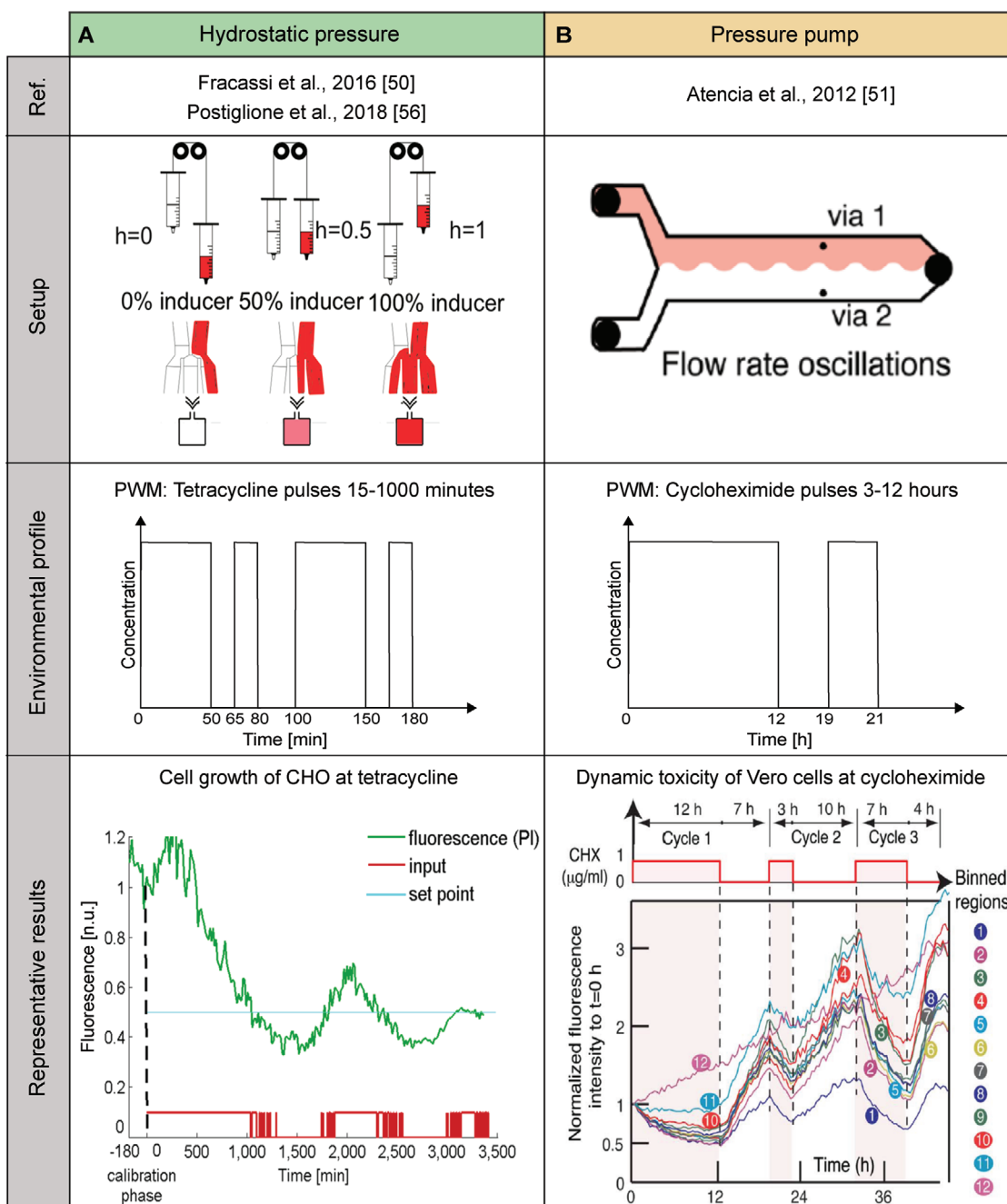
**Figure 7.** Applications for hydrostatic pressure and pressure pumps for the modulation of DCE at single-cell level. A) Application of Fracassi et al.[50] illustrates practical implementation of syringe driver with motorized stage. Environmental profile shows DCE in 15–1000 min time range of tetracycline pulses. The representative result show DCE of tetracycline impact on cell growth of CHO. Reproduced with permission.[50,56] Copyright 2019, American Chemical Society. B) Application from Atencia et al.[51] illustrates microfluidic chip design with two inlets and pressure pump for DCE between 3 and 12 h. The representative result illustrates dynamic toxicity of Vero cells by cycloheximide pulses. Reproduced with permission.[51] Copyright 2019, Royal Society of Chemistry.

generated by PWM/PFM in 8–350 min intervals. They revealed a control mechanism of the expression of a protein in a yeast cell population.

A similar concept used Luke et al.[80] for the fluid flow with a dial a wave system (DAW) which allows a temporal control of medium and generated a combination of the two inputs defined by the user. They analyzed the effect of limiting

nitrogen concentrations onto cell proliferation and chlorophyll autofluorescence of the microalgae *Chlorella sorokiniana*. DCE with and without nitrogen were generated by PFM/PWM in 50 min to 5 h interval.

Kaiser et al.[47] utilized the hydrostatic pressure with the DAW system for a dual-input mother machine chip for the induction of lac operon in *Escherichia coli* cells. The DCE between

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glucose and lactose containing media were generated every 4 h by PFM. They could show that cells expressed lacZ-GFP from the lac promotor by switching the carbon source from glucose to lactose. Crane et al.[81] used programmable syringe pumps for the gene regulation in *S. cerevisiae*, upon DCE between different glucose concentrations. Here, a microfluidic system called ALCATRAS (a long-term culturing and trapping system) was used with 6 h pulses of high glucose (2% glucose) and 2 h pulses of low glucose concentration (0.1% glucose) medium by PWM/PFM. They could show that yeast upon glucose limitation shows a reduction of nuclear Msn2p-GFP.[81]

### 3.2. Pressure Pumps: External

Atencia et al.[51] used a multilayer gradient generator with pressure pumps for the analysis of cycloheximide (CHX) gradients in Vero cells (Figure 7B). They created three pulsing cycles of a total time scale of 43 h by PFM/PWM. The shortest pulses of CHX were 3 h. The microfluidic device had two inlets, one for the cell media and one for CHX. The concentration of CHX was varied within the cultivation area by a change of the inlet pressure (Figure 7B, Setup). They could show that CHX had a toxic effect on Vero cells. CHX in different concentration were exposed to the cells during cultivation (Figure 7B, Representative result).

The CellASIC system (Merck KGaA, Darmstadt, Germany) is a commercially available pressure-driven single-cell cultivation systems that allows to switch between five different input conditions with different inlets for each input. Bermejo et al.[82] applied the CellASIC systems for the investigation of *S. cerevisiae* with a reversible change of cytosolic free glucose. They applied glucose pulses with increasing concentration with a regular interval of 4 and 10 min wash process with an MES buffer by PFM/PWM. The increasing concentrations were 2, 5, 10, and $100 \times 10^{-3}$ M glucose. They could show the reversible changes of cytosolic free glucose in individual cells.

Rojas et al.[58] also used the CellASIC system for the analysis of hyperosmotic shock in *E. coli* with a DCE of LB medium and LB medium with sorbitol by PFM with 90 s intervals.

In an alternative approach Bennett et al.[48] used a fluidic waveform generator for the gene expression of GAL1-yECFP fusion gene in *S. cerevisiae* which are cultivated in a monolayer (2D chambers). The fluidic waveform generator can switch and mix the medium inputs, similar to a pressure driven pump. The DCE between glucose and galactose were generated by PWM in 0.75–4.5 h intervals.

Park et al.[57] used syringe pumps for the regulation of time pulses of 1 hour to analyze sigma factor activity for RNA polymerase of *Bacillus subtilis* in mother machines (1D chambers). Using a switch from SMM medium to SMM medium with IPTG, they could show that alternative sigma factors activate the core RNA polymerase dynamically.

### 3.3. Pneumatic Valves on a Chip

Kim et al.[52] used pneumatic valves on the chip with a resolution of the oscillation periods between 57 and 360 s (**Figure 8**A,

Setup). Therefore, the device was generated in three layers. The fluid layer and also the microfluidic channel were composed of the top and bottom layer. The layer between, the middle layer, is a membrane layer with a thickness of 20 μm. In this setup two valves were used, one with the fluorescence dye and the other one with a clear solution. As a model system C2C12 cells were utilized in the channel for cell staining and were stained with a fluorescence dye which was cell permeable through the membrane with a high affinity to nucleic acids so that the fluorescence dye bonded there, and the fluorescence intensity increased. By opening the first valve the fluorescence intensity in the cell nucleus increased to a constant value over a time period of 25 s. After this the second valve was opened and valve one was closed. Upon medium change, the fluorescence intensity decreased in this phase because of photobleaching or dye dissociation. In consequence of this "open and close process" a periodically feeding of the fluorescence dye was possible with a total oscillation period of 57 s for both states with a flow rate of 4 μL min$^{-1}$ (Figure 8A, Representative results). Kim et al. could show the induction of periodic cell staining in C2C12 cells with a DCE between $1 \times 10^{-3}$ M fluorescence dye in TE buffer and DMEM medium by PFM/PWM.[52]

Wheeler and co-workers[62] also used multilayer microfluidic chips with integrated pumps and valves for the analysis of Ca$^{2+}$ dynamics in Jurkat T-cells. The medium exchange in the 0D structures takes only 100 ms. The DCE between HBS with ionomycin and HBS were generated by PWM in 40 s intervals. They demonstrated the viability of cells and the ionophore mediated intracellular Ca$^{2+}$ flow analysis.

A three-layer microfluidic chip with integrated valves for the analysis of Ca$^{2+}$ dynamics in HEK-293 cells was used by Gonzalez et al.[63] The DCE between cell medium and medium with carbachol were generated by PFM in 30–150 s intervals.

Park et al.[65] used a similar approach compared to Wheeler et al. to investigate FBS concentration of MDA-MB-231 cells. Therefore, they additionally implemented external electric valves for DCE ranges between 15 and 160 s by PWM. The chambers (2D) have a large width of 1000 μm so that the medium exchange in the chamber only occurs by diffusion. They could show that FBS concentration profile induced migrational response. He et al.[83] also utilized additional external electric valves for the induction of calcium signaling in Jurkat T-cells. The DCE between RPMI medium and RPMI medium with $100 \times 10^{-3}$ M H$_2$O$_2$ were generated by PFM in 20–200 s intervals.

The investigation of pheromone induced mitogen-activated protein kinase in *S. cerevisiae* was performed by Falconnet et al.[84] They used a two-layer microfluidic chip with integrated vales and integrated peristaltic pumps for a DCE between fluorescein and medium. The DCE were generated by PFM/PWM in 9–14 min intervals.

Piehler et al.[85] used multilayer microfluidic chips for the analysis of transcription factor NFκB in mouse 3T3 fibroblasts. The DCE of TNF with a maximum concentration of 3 ng mL$^{-1}$ were generated by PFM in 100 min periods of a sinus wave.

In an alternative approach Kusen et al.[86] used also a two-layer microfluidic chip with integrated valves for the induction repressible pMET17 promoter for YFP expression in *S. cerevisiae*. The DCE between methionine containing medium
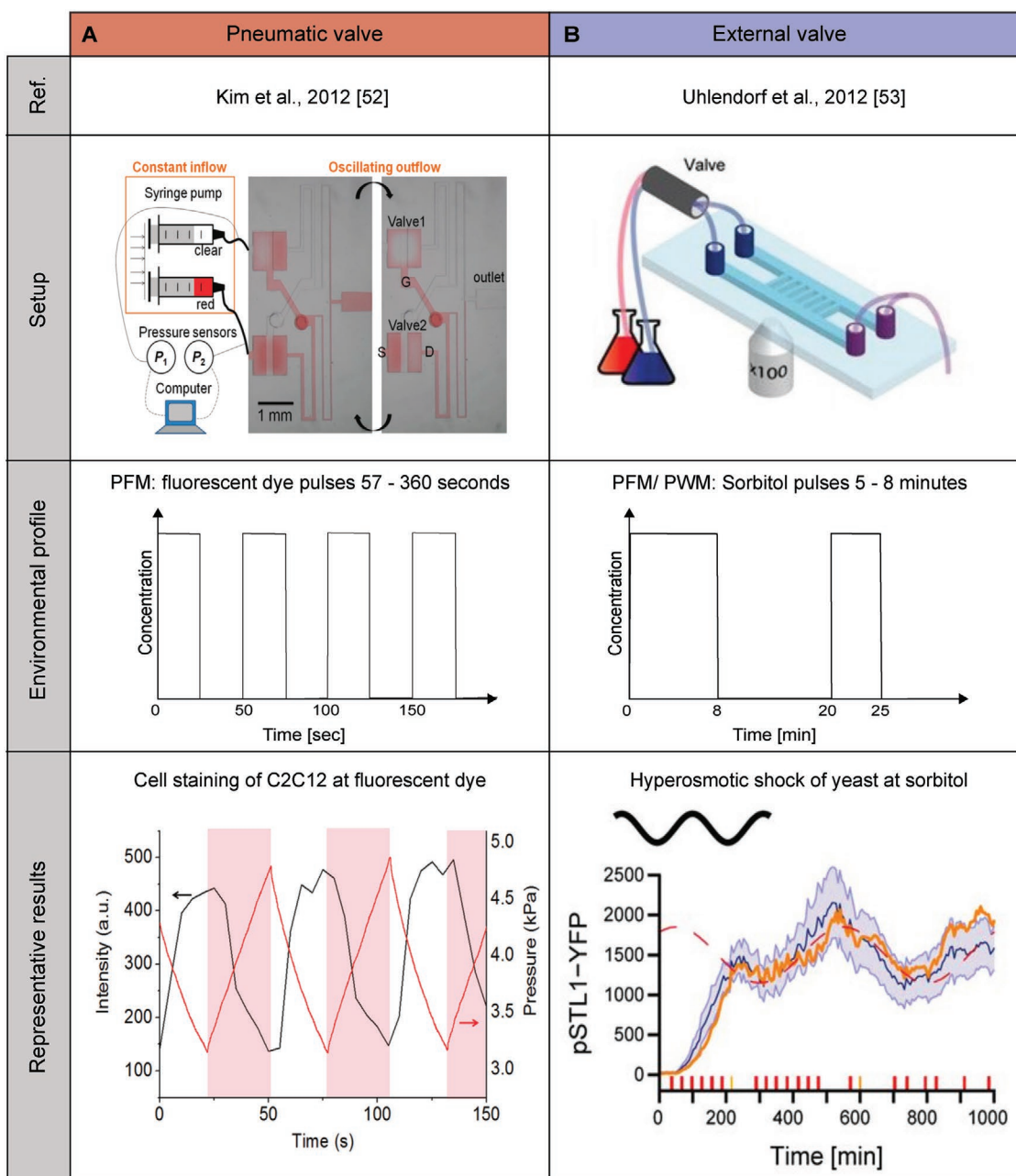
**Figure 8.** Applications for pneumatic valves and external electric valves for the modulation of DCE at a single-cell level. A) Application from Kim et al.[52] illustrates the setup of the pneumatic valves on the microfluidic chip. The white solution is deionized water and the red solution is deionized water with red food dye. The environmental profile shows the DCE in a 57–360 s range. The representative result shows the oscillation of the fluorescence dye for the cell staining of C2C12 cells. Reproduced with permission.[52] Copyright 2019, American Chemical Society. B) Application from Uhlendorf et al.[53] shows the setup of the electric valve in a microfluidic device. The environmental profile shows the DCE with 5–8 min pulses of sorbitol. The representative result shows the hyperosmotic shock of yeast. Reproduced under the terms of the CC BY 4.0 International License.[53] Copyright 2012, The Authors, Published by National Academy of Sciences of the United States of America.

and no methionine medium were generated by PFM/PWM in 2–8 h intervals.

### 3.4. External Electric Valve

Uhlendorf et al.[53,87] analyzed the high osmolarity glycerol signaling cascade in *S. cerevisiae* with an external electric valve at a microfluidic device (Figure 8B, Setup). Here, the native promoter pSTL1 was chosen for the expression of a fluorescence reporter. The promotor of the high osmolarity glycerol cascade was activated due to the osmotic stress. The microfluidic device consisted of open box chambers with a height of 3.1 µm with two inlets and outlets. The osmotic stress was activated by DCE between normal and sorbitol-enriched media by PFM/PWM. Pulses between 5 and 8 min were generated for

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the visualization of the osmotic shock with a 20 min relaxation period afterwards (Figure 8B, Environmental profile). The control loop for the input media was repeated every 6 min. This method is a real-time control of the single-cell level over 15 h to analyze the gene expression. The single-cell control was more difficult than the population control because the fluorescence value in single cells had more fluctuation as the population, so that defined cells varied over a long time period from the population. The single-cell control could reduce the fluctuations of the population so that the noise at the single-cell level decreased.

Hansen et al.[67,88] used also external electric valves in combination with hydrodynamic pressure for the control of Msn2 dynamics in *S. cerevisiae*. The DCE between medium with and without 1-NM-PP1 were generated by PFM/PWM in 10 s up to 5 min intervals.

In an alternative approach Charvin et al.[68] used a microfluidic device to separate the flow and the growth channel trough a diffusive cellulose membrane. Upstream the microfluidic device an external electric valve was used for the DCE between SCD medium + 10x Met and SCD medium + 0x Met by PFM/PWM. They could show that GAL 1 and MET3 promotors turn on and off very quickly in *S. cerevisiae* with pulses of 10–15 min intervals.

In the demonstrated examples, mainly PFM and PWM were used.[47,48] Many studies combine both modulations so that they can control the width of the pulses or created different frequencies. Only a few studies varied the amplitude (PAM) as well as the frequency and width of the environmental conditions.[82] PAM is important to reproduce the nutrient fluctuations as in a bioreactor. The emulation of complex environments such as natural habitats, requires the change of several environmental factors at the same time, but was not demonstrate for application yet.

## 4. Technical and Biological Perspective

The shown examples demonstrate how microfluidic experiments with DCE can be implemented and performed. Technological progress will allow the implementation of more complex environmental profiles for example the continuous variation of the amplitude or DCE between more than two solutions.

Woodruff et al.[66] generated a microfluidic device where oscillating conditions with different period and amplitude are possible. They implemented a microfluidic setup for the creation of short pulses of 1 s up to 1 h in PWM/PAM mode. Eight different mass flows can be mixed over mixing channels of six inlet solutions.[66] Li et al.[89] developed an automatic microfluidic device where with square pulses of five stock solutions. The functionality was demonstrated with fluorescence dye in five different concentrations. All square pulses had a temporal resolution of 30 s. Zhang et al.[90] created a multilayer microfluidic chip with peristaltic pumps and valves for a high throughput live cell analysis under dynamical ligand conditions for example Jagged1, DLL1, EGF, PACAP, CXCL, and PDGF. They cultivated mouse fibroblast cells and HSCs under daily changing ligand combinations over 6 days and found synergistic and antagonistic signal interactions.

Another interesting aspect is the environmental control of chemical stimuli such as gas concentrations during microfluidic single-cell cultivation, for example oxygen.[91] A first concept was established by Mauleon et al.[92] They developed a microfluidic gas channel that can generate an oxygen switch. The oxygen profile can be switched in a two-step function from 95% oxygen concentration to 0% oxygen for four respectively 10 min.[91,92] Another research group created a microfluidic device based on a multilayer chip with integrated differential oxygenator to control the dissolved oxygen over the cultivation of aerobic and anaerobic cultures as well as cell culture.[93]

First microfluidic devices that allow the dynamic change of physical stimuli have also been reported. Peng et al.[94] established a cooling and heating microfluidic device where temperature changes within 10 s between 2 and 37 °C can be performed.[94] A general overview regarding temperature control in microfluidic systems is reviewed by Miralles et al.[95]

Furthermore, automated programmable microfluidic platforms that combine image-based single-cell analysis and DCE with feedback control are emerging.[50,56,78–80] Here, cellular behavior is controlled by real time image analysis of cells, coupled to dynamic environmental control.[96,97] As soon as cellular threshold values are reached defined medium pulses are triggered (referred as negative feedback control).[44,45,66,67] Fracassi et al.[50] implemented a negative feedback control to control tetracycline of CHO cells. This was realized with a relay and PI controller so that an oscillation of the set point was generated. In the experiments half of the fluorescence intensity was determined for the set point with the analysis of 180 min without tetracycline. The regulation with the relay and PI controller shows an oscillation of the fluorescence intensity around the set point value. The relay controller has an undamped oscillation so that the actual set point value could not be achieved. In contrast with a PI controller the oscillation decreased over the time in this case and in the end the set point was achieved.[50] A review discussing progress and challenges within feedback controlled microfluidic experiment was recently published by Lugagne et al.[98]

Technical progress will not only allow to investigate cellular behavior at various environmental conditions but enable the cultivation and analysis of microbes at natural-habitat-like environmental conditions. Finally, this will increase the portfolio of cultivation protocols and workflows to completely new fields of applications. Currently only 1% of the microbes grow under lab conditions because of the constant and incongruous environmental conditions. MSCC in combination with DCE might enable to cultivate and analyze currently uncultivatable microbes, often referred to microbial dark matter.[99,100]

## 5. Conclusion and Outlook

MSCCs with DCE have a high potential for analyzing and understanding cellular mechanisms at defined fluctuating environments. These tools will for the first-time allow to emulate environmental conditions of natural and simulated natural habitats. We have reviewed numerous studies that established microfluidic setups for generating DCE and demonstrated their application for the analysis of cellular systems. The systems

differ in their ability of dynamic control in period of the amplitude signal. Whereas simple setups allow to change environments in an on/off manner as well as a medium change within hours, advanced setups allow to pulse medium in time frames of seconds. Most of the setups and applications shown here are proof of concept studies. Currently, except the CellASIC system, the systems are not commercially available and rely on time-consuming and expensive clean-room fabrication processes. To perform systematic cellular studies under DCE several improvements need to be done. This includes a detailed characterization of the flow regimes and nutrient exchange of cultivation chambers. In most of the cases, microfluidic systems cannot be transferred directly for the investigation of different organisms and need adjustment of cultivation chamber design. For the most shown examples, only selected cellular parameter for example fluorescence expression of single cells were analyzed. Therefore, novel image analysis workflows need to be implemented, to be able to analyze complex cellular parameters, such as growth rates, division times and heterogeneity within fluorescence coupled metabolic processes. On a long-term, these novel methods need to be integrated into biological experimental workflows.[101]

The choice of pneumatic valves on chip or pressure pumps and 1D cultivation chambers are the most promising combination for rapid and precise environmental changes in a range of seconds. Combined with emerging feedback control strategies, these setups will allow the cultivation of cells at various (nutrient) environmental profiles. In future, the development of setups for multiparameter changes (multiple nutrient changes and gas exchange) is necessary to be able to emulate more complex profiles. This will lay the foundation for the investigation of cellular behavior at complex environmental changes prevailing in natural and artificial cultivation habitats.

## Conflict of Interest

The authors declare no conflict of interest.

[1] A. Grünberger, W. Wiechert, D. Kohlheyer, *Curr. Opin. Biotechnol.* **2014**, *29*, 15.
[2] R. M. Johann, *Anal. Bioanal. Chem.* **2006**, *385*, 408.
[3] F. S. O. Fritzsch, K. Rosenthal, A. Kampert, S. Howitz, C. Dusny, L. M. Blank, A. Schmid, *Lab Chip* **2013**, *13*, 397.
[4] A. Grünberger, C. Probst, S. Helfrich, A. Nanda, B. Stute, W. Wiechert, E. von Lieres, K. Nöh, J. Frunzke, D. Kohlheyer, *Cytometry, Part A* **2015**, *87*, 1101.
[5] W. Mather, O. Mondragón-Palomino, T. Danino, J. Hasty, L. S. Tsimring, *Phys. Rev. Lett.* **2010**, *104*, 881.
[6] C. Probst, A. Grünberger, N. Braun, S. Helfrich, K. Nöh, W. Wiechert, D. Kohlheyer, *Anal. Methods* **2015**, *7*, 91.
[7] P. Shah, I. Vedarethinam, D. Kwasny, L. Andresen, M. Dimaki, S. Skov, W. E. Svendsen, *Sens. Actuators, B* **2011**, *156*, 1002.
[8] P. Wang, L. Robert, J. Pelletier, W. L. Dang, F. Taddei, A. Wright, S. Jun, *Curr. Biol.* **2010**, *20*, 1099.
[9] D. Lindemann, C. Westerwalbesloh, D. Kohlheyer, A. Grünberger, E. von Lieres, *RSC Adv.* **2019**, *9*, 14040.
[10] C. Probst, A. Grünberger, W. Wiechert, D. Kohlheyer, *Micromachines* **2013**, *4*, 357.
[11] A. Dal Co, M. Ackermann, S. van Vliet, *J. R. Soc., Interface* **2019**, *156*, 20190182.
[12] D. Yang, A. D. Jennings, E. Borrego, S. T. Retterer, J. Männik, *Front. Microbiol.* **2018**, *9*, 871.
[13] S. Uphoff, *Proc. Natl. Acad. Sci. USA* **2018**, *115*, E6516.
[14] L. Robert, J. Ollion, J. Robert, X. Song, I. Matic, M. Elez, *Science* **2018**, *359*, 1283.
[15] M. Dormeyer, S. Lentes, P. Ballin, M. Wilkens, S. Klumpp, D. Kohlheyer, L. Stannek, A. Grünberger, F. M. Commichau, *DNA Repair.* **2018**, *63*, 10.
[16] C. Dusny, A. Schmid, *Environ. Microbiol.* **2015**, *17*, 1839.
[17] S. Unthan, A. Grünberger, J. van Ooyen, J. Gätgens, J. Heinrich, N. Paczia, W. Wiechert, D. Kohlheyer, S. Noack, *Biotechnol. Bioeng.* **2014**, *111*, 359.
[18] F. Si, G. Le Treut, J. T. Sauls, S. Vadia, P. A. Levin, S. Jun, *Curr. Biol.* **2019**, *29*, 1760.e7.
[19] M. B. Elowitz, S. Leibler, *Nature* **2000**, *403*, 335.
[20] N. M. Toriello, E. S. Douglas, N. Thaitrong, S. C. Hsiao, M. B. Francis, C. R. Bertozzi, R. A. Mathies, *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 20173.
[21] J. Ryley, O. M. Pereira-Smith, *Yeast* **2006**, *23*, 1065.
[22] J. Stricker, S. Cookson, M. R. Bennett, W. H. Mather, L. S. Tsimring, J. Hasty, *Nature.* **2008**, *456*, 516.
[23] M. R. Atkinson, M. A. Savageau, J. T. Myers, A. J. Ninfa, *Cell* **2003**, *113*, 597.
[24] N. Mustafi, A. Grünberger, D. Kohlheyer, M. Bott, J. Frunzke, *Metab. Eng.* **2012**, *14*, 449.
[25] S. van Vliet, A. Dal Co, A. R. Winkler, S. Spriewald, B. Stecher, M. Ackermann, *Cell Syst.* **2018**, *6*, 496.e6.
[26] A. R. Lara, E. Galindo, O. T. Ramírez, L. A. Palomares, *Mol. Biotechnol.* **2006**, *34*, 355.
[27] P. B. Pearman, A. Guisan, O. Broennimann, C. F. Randin, *Trends. Ecol. Evol.* **2008**, *23*, 149.
[28] W. L. Nicholson, P. Fajardo-Cavazos, J. Fedenko, J. L. Ortíz-Lugo, A. Rivas-Castillo, S. M. Waters, A. C. Schuerger, *Appl. Environ. Microbiol.* **2010**, *76*, 7559.
[29] A. Melbinger, M. Vergassola, *Sci. Rep.* **2015**, *5*, 15211.
[30] B. Koskella, M. Vos, *Annu. Rev. Ecol. Evol. Syst.* **2015**, *46*, 503.
[31] A. N. Brooks, S. Turkarslan, K. D. Beer, F. Y. Lo, N. S. Baliga, *Wiley Interdiscip. Rev.: Syst. Biol. Med.* **2011**, *3*, 544.
[32] R. F. Rosenzweig, J. Adams, *BioEssays.* **1994**, *16*, 715.
[33] M. Pigliucci, *Trends Ecol. Evol.* **1996**, *11*, 168.
[34] J. Monod, *Annu. Rev. Microbiol.* **1949**, *3*, 371.
[35] J. Hemmerich, S. Noack, W. Wiechert, M. Oldiges, *Biotechnol. J.* **2018**, *13*, 1700141.
[36] N. Paczia, A. Nilgen, T. Lehmann, J. Gätgens, W. Wiechert, S. Noack, *Microb. Cell Fact.* **2012**, *11*, 122.
[37] M. E. Lidstrom, M. C. Konopka, *Nat. Chem. Biol.* **2010**, *6*, 705.
[38] C. K. Byun, K. Abi-Samra, Y.-K. Cho, S. Takayama, *Electrophoresis* **2014**, *35*, 245.

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[39] J. Liu, A. Prindle, J. Humphries, M. Gabalda-Sagarra, M. Asally, D.-Y. D. Lee, S. Ly, J. Garcia-Ojalvo, G. M. Süel, *Nature* **2015**, *523*, 550.

[40] K. Saarinen, J. Laakso, L. Lindström, T. Ketola, *Ecol. Evol.* **2018**, *8*, 2901.

[41] S. K. Walia, T. D. Chugh, *Indian J. Pathol. Bacteriol.* **1979**, *22*, 153.

[42] S. Calsamiglia, A. Ferret, M. Devant, *J. Dairy Sci.* **2002**, *85*, 574.

[43] R. L. Morris, T. M. Schmidt, *Nat. Rev. Microbiol.* **2013**, *11*, 205.

[44] C. Haringa, W. Tang, A. T. Deshmukh, J. Xia, M. Reuss, J. J. Heijnen, R. F. Mudde, H. J. Noorman, *Eng. Life Sci.* **2016**, *16*, 652.

[45] C. Haringa, H. J. Noorman, R. F. Mudde, *Chem. Eng. Sci.* **2017**, *157*, 159.

[46] C. Haringa, R. F. Mudde, H. J. Noorman, *Biochem. Eng. J.* **2018**, *140*, 57.

[47] M. Kaiser, F. Jug, T. Julou, S. Deshpande, T. Pfohl, O. K. Silander, G. Myers, E. van Nimwegen, *Nat. Commun.* **2018**, *9*, 212.

[48] M. R. Bennett, W. L. Pang, N. A. Ostroff, B. L. Baumgartner, S. Nayak, L. S. Tsimring, J. Hasty, *Nature* **2008**, *454*, 1119.

[49] G. Fiore, A. Matyjaszkiewicz, F. Annunziata, C. Grierson, N. J. Savery, L. Marucci, M. Di Bernardo, *ACS Synth. Biol.* **2017**, *6*, 507.

[50] C. Fracassi, L. Postiglione, G. Fiore, D. Di Bernardo, *ACS Synth. Biol.* **2016**, *5*, 296.

[51] J. Atencia, G. A. Cooksey, L. E. Locascio, *Lab Chip* **2012**, *12*, 309.

[52] S.-J. Kim, R. Yokokawa, S. C. Lesher-Perez, S. Takayama, *Anal. Chem.* **2012**, *84*, 1152.

[53] J. Uhlendorf, A. Miermont, T. Delaveau, G. Charvin, F. Fages, S. Bottani, G. Batt, P. Hersen, *Proc. Natl. Acad. Sci. USA* **2012**, *109*, 14271.

[54] D. Irimia, D. A. Geba, M. Toner, *Anal. Chem.* **2006**, *78*, 3472.

[55] X. Wang, Z. Liu, Y. Pang, *RSC Adv.* **2017**, *7*, 29966.

[56] L. Postiglione, M. Santorelli, B. Tumaini, D. Di Bernardo, *IFAC-PapersOnLine* **2016**, *49*, 14.

[57] J. Park, M. Dies, Y. Lin, S. Hormoz, S. E. Smith-Unna, S. Quinodoz, M. J. Hernández-Jiménez, J. Garcia-Ojalvo, J. C. W. Locke, M. B. Elowitz, *Cell Syst.* **2018**, *6*, 216.e15.

[58] E. Rojas, J. A. Theriot, K. C. Huang, *Proc. Natl. Acad. Sci. USA* **2014**, *111*, 7807.

[59] A. K. Au, H. Lai, B. R. Utela, A. Folch, *Micromachines* **2011**, *2*, 179.

[60] G. M. Whitesides, *Nature* **2006**, *442*, 368.

[61] J. Shin, H. Park, B. van Dang, C.-W. Kim, S.-J. Kim, *RSC Adv.* **2015**, *5*, 23239.

[62] A. R. Wheeler, W. R. Throndset, R. J. Whelan, A. M. Leach, R. N. Zare, Y. H. Liao, K. Farrell, I. D. Manger, A. Daridon, *Anal. Chem.* **2003**, *75*, 3581.

[63] A. M. Gonzalez-Suarez, J. G. Peña-Del Castillo, A. Hernández-Cruz, J. L. Garcia-Cordero, *Anal. Chem.* **2018**, *90*, 8331.

[64] S.-J. Kim, R. Yokokawa, S. Takayama, *Lab Chip* **2013**, *13*, 1644.

[65] E. S. Park, M. A. Difeo, J. M. Rand, M. M. Crane, H. Lu, *Biomicrofluidics* **2013**, *7*, 011804.

[66] K. Woodruff, S. J. Maerkl, *Anal. Chem.* **2018**, *90*, 696.

[67] A. S. Hansen, N. Hao, E. K. O'Shea, *Nat. Protoc.* **2015**, *10*, 1181.

[68] G. Charvin, F. R. Cross, E. D. Siggia, *PLoS One* **2008**, *3*, e1468.

[69] G. Schendzielorz, M. Dippong, A. Grünberger, D. Kohlheyer, A. Yoshida, S. Binder, C. Nishiyama, M. Nishiyama, M. Bott, L. Eggeling, *ACS Synth. Biol.* **2014**, *3*, 21.

[70] R. Hornung, A. Grünberger, C. Westerwalbesloh, D. Kohlheyer, G. Gompper, J. Elgeti, *J. R. Soc., Interface* **2018**, *15*, 20170713.

[71] N. Mustafi, A. Grünberger, R. Mahr, S. Helfrich, K. Nöh, B. Blombach, D. Kohlheyer, J. Frunzke, *PLoS One* **2014**, *9*, e85731.

[72] J. Hage-Hülsmann, A. Grünberger, S. Thies, B. Santiago-Schübel, A. S. Klein, J. Pietruszka, D. Binder, F. Hilgers, A. Domröse, T. Drepper, D. Kohlheyer, K.-E. Jaeger, A. Loeschcke, *PLoS One* **2018**, *13*, e0200940.

[73] N. Q. Balaban, J. Merrin, R. Chait, L. Kowalik, S. Leibler, *Science* **2004**, *305*, 1622.

[74] C. E. M. Krämer, W. Wiechert, D. Kohlheyer, *Sci. Rep.* **2016**, *6*, 32104.

[75] C. Westerwalbesloh, A. Grünberger, B. Stute, S. Weber, W. Wiechert, D. Kohlheyer, E. von Lieres, *Lab Chip* **2015**, *15*, 4177.

[76] P. Ho, C. Westerwalbesloh, E. Kaganovitch, A. Grünberger, P. Neubauer, D. Kohlheyer, E. von Lieres, *Microorganisms* **2019**, *7*, 105.

[77] L. Postiglione, S. Napolitano, E. Pedone, D. L. Rocca, F. Aulicino, M. Santorelli, B. Tumaini, L. Marucci, D. Di Bernardo, *ACS Synth. Biol.* **2018**, *7*, 2558.

[78] G. Fiore, G. Perrino, M. Di Bernardo, D. Di Bernardo, *ACS Synth. Biol.* **2016**, *5*, 154.

[79] F. Menolascina, G. Fiore, E. Orabona, L. de Stefano, M. Ferry, J. Hasty, M. Di Bernardo, D. Di Bernardo, *PLoS Comput. Biol.* **2014**, *10*, e1003625.

[80] C. S. Luke, J. Selimkhanov, L. Baumgart, S. E. Cohen, S. S. Golden, N. A. Cookson, J. Hasty, *ACS Synth. Biol.* **2016**, *5*, 8.

[81] M. M. Crane, I. B. N. Clark, E. Bakker, S. Smith, P. S. Swain, *PLoS One* **2014**, *9*, e100042.

[82] C. Bermejo, F. Haerizadeh, H. Takanaga, D. Chermak, W. B. Frommer, *Nat. Protoc.* **2011**, *6*, 1806.

[83] L. He, A. Kniss, A. San-Miguel, T. Rouse, M. L. Kemp, H. Lu, *Lab Chip* **2015**, *15*, 1497.

[84] D. Falconnet, A. Niemistö, R. J. Taylor, M. Ricicova, T. Galitski, I. Shmulevich, C. L. Hansen, *Lab Chip* **2011**, *11*, 466.

[85] A. Piehler, N. Ghorashian, C. Zhang, S. Tay, *Lab Chip* **2017**, *17*, 2218.

[86] P. M. Kusen, G. Wandrey, C. Probst, A. Grünberger, M. Holz, S. Meyer Zu Berstenhorst, D. Kohlheyer, J. Büchs, J. Pietruszka, *ACS Chem. Biol.* **2016**, *11*, 2915.

[87] J. Uhlendorf, A. Miermont, T. Delaveau, G. Charvin, F. Fages, S. Bottani, P. Hersen, G. Batt, *Methods Mol. Biol.* **2015**, *1244*, 277.

[88] A. S. Hansen, E. K. O'Shea, *Mol. Syst. Biol.* **2013**, *9*, 704.

[89] Z. Li, S.-J. Kim, *Sci. Adv.* **2019**, *5*, eaat3080.

[90] C. Zhang, H.-L. Tu, G. Jia, T. Mukhtar, V. Taylor, A. Rzhetsky, S. Tay, *Sci. Adv.* **2019**, *5*, eaav7959.

[91] M. D. Brennan, M. L. Rexius-Hall, L. J. Elgass, D. T. Eddington, *Lab Chip* **2014**, *14*, 4305.

[92] G. Mauleon, C. P. Fall, D. T. Eddington, *PLoS One* **2012**, *7*, e43309.

[93] R. H. W. Lam, M.-C. Kim, T. Thorsen, *Anal. Chem.* **2009**, *81*, 5918.

[94] J. Peng, C. Fang, S. Ren, J. Pan, Y. Jia, Z. Shu, D. Gao, *Int. J. Heat Mass Transfer* **2019**, *130*, 660.

[95] V. Miralles, A. Huerre, F. Malloggi, M.-C. Jullien, *Diagnostics* **2013**, *3*, 33.

[96] P. Demling, C. Westerwalbesloh, S. Noack, W. Wiechert, D. Kohlheyer, *Curr. Opin. Biotechnol.* **2018**, *54*, 121.

[97] R. Chait, J. Ruess, T. Bergmiller, G. Tkačik, C. C. Guet, *Nat. Commun.* **2017**, *8*, 1535.

[98] J.-B. Lugagne, M. J. Dunlop, *Curr. Opin. Syst. Biol.* **2019**, *14*, 1.

[99] C. Lok, *Nature* **2015**, *522*, 270.

[100] H. Ledford, *Nature* **2015**, *76*, 2445.

[101] C. Dusny, A. Grünberger, *Curr. Opin. Biotechnol.* **2020**, *63*, 26.