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4   **Dynamic environmental control in microfluidic single-cell cultivations: From concepts**

5   **to applications**

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19 **Abstract**

20 Microfluidic single-cell cultivation (MSCC) is an emerging field within fundamental as well

21 as applied biology. During the last years most MSCC were performed at constant

22 environmental conditions. Recently, MSCC at oscillating and dynamic environmental

23 conditions start to gain significant interest in the research community for the investigation of

24 cellular behavior. In this review, we will give an overview of this topic and discuss

25 microfluidic concepts that enable oscillating and dynamic control of environmental

26 conditions, with a focus on medium conditions, and demonstrate their application in single-

27 cell research for the cultivation of both mammalian and microbial cell systems. We discuss

28 further perspectives for performing MSCC at complex dynamic environmental profiles of

29 single-parameters and multiparameters (e.g. pH and O_2) in amplitude and time. The technical

30 progress in this field provides completely new experimental approaches and lays the

31 foundation for systematic analysis of cellular metabolism at fluctuating environments.

32   **1. Introduction**

33   **1.1 Microfluidic single-cell cultivation (MSCC)**

34   In recent years novel microfluidic methods for the cultivation and investigation of cellular

35   behavior on single-cell level emerged **(Figure 1A)**. Microfluidic systems have several

36   advantages for example high surface to volume ratios and small cultivation volumes (nl-pl

37   volumes), which allows fast heat and mass transfer and thus precise environmental control.[1]

38   In microfluidic devices, cells and small cell colonies can be trapped in cultivation habitats[1]

39   by different physical principles[2]: Besides contactless trapping such as laser trapping and

40   nDEP[3], contact trapping based on hydrodynamic barrier structures in PDMS-glass devices

41   are most widely applied.[2] Here, cells are randomly trapped in cultivation areas and cell

42   growth is restricted by the barrier structures. The cultivation chambers on such microfluidic

43   cultivation systems can be classified in 3D, 2D, 1D and 0D systems **(Figure 1B)**.[1] These

44   microfluidic systems differ greatly in the spatial degree of freedom for cell growth and size of

45   the growing microcolony. In 3D MSCCs, cells overlap with each other and are not in one

46   focal plane, so that single-cell growth an physiology can not be analyzed with full spatio-

47   temporal resolution[1], thus the application of these systems for dynamic single-cell analysis

48   with full spatio-temporal resolution is not possible.[1] 2D MSCCs allow to cultivate small

49   microcolonies with up to 1000 cells[4; 5] and cell growth is restricted to a monolayer. These

50   MSCCs are easy to handle and allow to monitor cellular behavior such as growth, division

51   and morphology. Typical dimensions for 2D chambers for microbial cells are 60 µm x

52   50 µm[6], whereas for cell cultures typical length is 250 µm and width of around 500 µm.[7] In

53   1D and 0D systems typically hold up to 10 cells per trap[8; 9] or even completely isolated

54   single cells[10; 11] Here, cells are growing in one line that allows a reliable long time cultivation

55   of cells. In comparison to 2D designs, the cell loading process is difficult. Typical dimension

56   of 1D chambers are length of 10 to 20 µm and width between 0.6 and 1.0 µm for microbial

57 cells.[12] 0D MSCCs system allow cellular analysis of complete isolated single cells, but

58 fabrication, cell tapping and long-term cultivation is still difficult and thus these systems are

59 not yet regularly applied for microbial cells.[10] Continuous supply of culture medium and

60 removal of products and by-products provided by perfusion allow for constant environmental

61 conditions over long cultivation times **(Figure 1C)**. The combination of microfluidic systems

62 with live-cell imaging enables dynamic cell analysis in high-spatio-temporal resolution

63 **(Figure 1D)**. Currently, such systems are frequently applied for investigating cell-to-cell

64 heterogeneity[13], aging and death[8; 13–15], growth[8; 16–18], cell cycles[19], gene expression[20–23],

65 and various other metabolic processes[24; 25].

66

67 **FIGURE 1**

68

69 This review addresses MSCC under dynamically controlled environments (DCE). First, we

70 will give a short overview regarding DCE and their nomenclature. We will discuss different

71 microfluidic concepts for the cultivation of both mammalian and microbial cell and how the

72 choice of the cultivation chamber affects the ability to perform MSCC under DCE. Further,

73 we show first applications in applied biology. This review will focus on chemical

74 environmental factors (e.g. nutrient conditions) in DCE. DCE within physical and biophysical

75 environments (e.g. shear stress, pressure, temperature etc.) will not be addressed in detail.

76 Finally, we will give a perspective in technical and biological challenges on the

77 implementation of complex and fluctuating environmental profiles.

78 **1.2 Mimicking environmental conditions – Overview**

79 Environmental conditions in natural habitats of bacteria are generally not constant but vary in

80 concentration over the time.[26; 27] In their natural habitat (e.g. soil, ocean) and artificial

81   habitats (e.g. bioreactors) microbial cells are exposed to changing environmental stimuli.

82   These can be classified in physical, chemical and biological stimuli **(Figure 2A).** Changes in

83   a broad range of time scales influence the cellular physiology. Physical parameters can

84   include changes in temperature and pressure.[28] Chemical and biological stimuli are governed

85   through the availability of different nutrients, detrimental substances or molecules for cellular

86   communication.[29] Based on the environmental input, cells regulate their gene networks

87   dynamically and adjust parameters such as growth, metabolism, aging and death, evolution,

88   cell cycle etc. **(Figure 2B)**.[30–33] Historically, cells are isolated from natural habitats and grow

89   in lab-scale devices to characterize their physiology.[34] Typically, cells are cultivated in shake

90   flasks and bioreactors, to keep environmental conditions as constant as possible.[35] However,

91   even in these devices, consumption of nutrients and gradients through mixing[26] and the cell

92   metabolism itself[36] lead to inhomogeneous environmental conditions and thus the analysis of

93   cellular response to defined environmental conditions is difficult. Especially for the

94   investigation of reason and origin of cellular heterogeneity, traditional methods have their

95   limitations, since most of the assays rely on averaging cellular output measurements[37] and

96   the inability for cultivations at defined environmental conditions.

97   The perfusion of media during conventional MSCC leads to continuous medium supply and

98   the removal of products and by-products and thus constant environment conditions over the

99   cultivation time **(Figure 2C)**. In their natural habitat cells are exposed to randomly changing

100   environmental conditions with changes of frequency and amplitude of environmental

101   parameters **(Figure 2E)**.[38] This includes gradients in nutrients[39], temperature[40],

102   pressure[41], pH value[42] and oxygen level[43]. Even in simulated natural habitats such as

103   laboratory bioreactors, cells are exposed to short-term environmental fluctuations.[26; 44] Here,

104   the fluctuations can be smaller in amplitude but can be faster in frequency.[45] To analyze the

105   influence of environmental fluctuation in laboratory scale as well as natural habitats novel

106   microfluidics systems must be developed to mimic fluctuating environmental conditions

107   **(Figure 2D)**.[46]

108   In literature, the term fluctuating environmental conditions is currently not well defined.

109   Alternatively, dynamically controllable conditions[47], dynamically changing environment[48]

110   and environmental fluctuations[49] are synonymously used. The corresponding systems have in

111   common that they allow to apply specific environmental/ stimulant profiles to cells that are

112   difficult to perform with conventional MSCC devices. For better readability, the term

113   dynamically controlled environments (DCE) is used within this review.

114   Ideally, MSCC devices should provide the possibility to create DCE with different profiles.

115   We propose to differentiate between five different categories on how DCE can be created in

116   microfluidic devices **(Figure 2F)**. In the first category, during the microfluidic experiments,

117   medium can be switched once in a step function or a pulse function and no systematic

118   modulation is performed **(Figure 2F – 1)**. Moreover, an environmental profile can be

119   established with the same amplitude and width, only the frequency between the pulses (f_1 and

120   f_2) is varied (pulse frequency modulation (PFM)) **(Figure 2F – 2)**. In the third category, the

121   environment can be switched in a different temporal manner (w_1 and w_2) so that the pulses

122   have different temporal resolutions (here the frequency and amplitude are constant) (pulse

123   width modulation (PWM)) **(Figure 2F – 3)**. Furthermore, the environmental profile can be

124   modulated with different amplitudes, whereas the frequency and width of the pulses are kept

125   constant (a_1 and a_2) (pulse amplitude modulation (PAM)) **(Figure 2F – 4)**. Finally, cells can

126   be exposed to different environments in a varying temporal manner so that the amplitude,

127   frequency and width of the concentration varies over time in a different manner. Here, a

128   combination of PWM, PFM and PAM is used **(Figure 2F – 5)**.

129

130   **FIGURE 2**

131

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 cultivation chamber). The technical periphery can be implemented either by hydrostatic pressure[50] **(Figure 3A)**, pressure pumps[51] **(Figure 3B)**, pneumatic microvalves on a chip[52] **(Figure 3C)** or an external electric valve[53] **(Figure 3D)**. Here, we will 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 create by microfluidic gradient generators the reader is referred to recent reviews summarizing the field of microfluidic gradient generators.[54; 55] Afterwards, the cultivation chamber will be introduced in context of a rapid DCE and overview of the different combination of technical periphery and cultivation chambers will be 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 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 electric **(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

155 range of minutes can be created. This restricts the application to step functions and PAM and

156 PWM with low resolution.

2.1.2 Pressure pump - external

158 A similar method for the DCE offer syringe driver pumps and pressure pumps to modulate

159 environmental conditions **(Figure 3B – Setup)**. Here, a microfluidic chip system with several

160 inlets is necessary so that the different concentration are mixed on chip or a device where the

161 multiple environment channels lead into one inlet over a T-fitting **(Figure 3B – Principle)**.[57]

162 This method requires advanced periphery in form of a precise pressure driven pumping

163 system. Nevertheless, fabrication, handling and implementation are still quite simple and

164 comparable to the hydrostatic pressure method **(Figure 3B – Specifications)**.[58] In

165 comparison to the hydrostatic pressure method, achievable frequency of the environmental

166 modulation in a lower minute up to second range is possible.

2.1.3 Pneumatic valve on a chip

168 Pneumatic microvalves, also called "Quake valves" were developed by Stephen Quake in

169 2000.[59] They use the effect of the deformation of polydimethylsiloxane (PDMS) to control

170 the flow so that a change can develop actively and passively.[59; 60] Microfluidic valves on a

171 chip can be opened and closed through the variation of the flow rate of liquid or air **(Figure**

172 **3C – Setup)**. The continuous regulation of the valve can be controlled with the manipulation

173 of airflow, here pressure on chip, so that the external pressure is constant.[61]

174 Pneumatic microvalves are fabricated using multilayer soft lithography in a microfabrication

175 step with a soft polymer, at least two polymer layers are required for the microvalve. One

176 layer contains the microfluidic channel for the liquid flow. The other one is the control layer

177 which deflects this part into the microfluidic channel and stops the flow **(Figure 3C –**

178 **Principle)**.[62] In comparison to the hydrostatic pressure approach, high frequency oscillations

179   of the environment can be obtained **(Figure 3C – Specifications)**. The switching between

180   different environmental conditions takes only a few milliseconds so that oscillations can be

181   created in a range of milliseconds.[63] The fabrication of the microfluidic chip and

182   implementation as well as the handling is very time consuming. A few research groups utilize

183   syringe drivers for the manipulation of airflow of the valves. As a result, pressure fluctuations

184   in flow occurs and the regulation of the syringe pumps is slower in comparison to pressure

185   pumps.[64] All in all, pneumatic valves allow a high resolution in frequency of DCE and

186   amplitude of DCE in a range of seconds and milliseconds.[65]

187   *2.1.4 External electric valve*

188   An alternative method for the implementation of DCE offer external electric valves **(Figure

189   3D – Setup)**. These valves are computer-controlled and are installed in front of the inlets of

190   the microfluidic channel so that a T-type setup is used to combine the different environment

191   reservoirs and the channel inlet **(Figure 3D – Principle)**. The switching time of the external

192   electric valve between two different states takes only a few milliseconds.[66; 67] The change of

193   the environment inside the channel is not instantaneously with switching of the valve because

194   of the dead volume of the connecting tube between the valve and the microfluidic device.[53;

195   68] It is important to choose a tube with a low cross-section area and a short length of a few

196   centimeter so that the dead volume in the tube can be changed fast. Depending on the flow

197   rate for example $1 \, \mu l \, s^{-1}$, a temporal resolution of around 10 seconds can be experimentally

198   realized with a microfluidic channel with a height of ~ 100 µm and a volume of ~ 0,6 µl cm⁻

199   ¹.[67; 68] The DCE can oscillate in a range of seconds so that different feed streams can be feed

200   in short time-scales. The handling of this method is also simple and easier to implement than

201   pneumatic valves **(Figure 3D – Specifications)**. In summary, this method is simple to handle

202   and a high resolution in frequency and amplitude of DCE in a range of seconds is possible.[68]

203

204   **FIGURE 3**

205

206   Additionally, studies are reported, where DCE, mainly step profiles, were performed

207   manually.[24; 69; 70] Here, the operator/ experimenter replaces the inlet channel or tubing by an

208   alternative inlet filled with different medium connected to syringe pump systems. This

209   method is often used when single medium changes are performed. Applications range from

210   changes in carbon sources[71; 72] to studies with potential effectors such as antibiotics[72–74].

211   Although this method is quite simple in handling, the implementation of oscillating conditions

212   and defined pulses is hardly possible. Furthermore, the manual interruption of live-cell

213   experiments bears the risk to disturb experimental conditions for example through air,

214   contaminations, etc. Therefore, this method will not further be discussed within this review.

215

216   **2.2 Cultivation chamber**

217   Whereas the technical periphery (see last chapter) plays an important role in the realization of

218   DCE within MSCC, the cultivation chamber is the most important factor when cells shall be

219   cultivated under rapidly changing DCE.

220   For 2D chambers (**Figure 1B**) nutrient availability can be deteriorated, and exchange of the

221   complete chamber volume ranges between 10 seconds and minutes **(Figure 4A)**. This

222   depends on chamber geometry and size, type of supply (convection, diffusion) and number of

223   nutrient inlets[4; 75; 76] because the nutrient exchange in the cultivation chamber occurs only via

224   diffusion. The exchange of nutrients within the chamber is the limiting factor for high-

225   frequency DCE applications. In contrast, Wang *et al.* have demonstrated that the complete

226   volume change in a 1D structure per diffusion is in the lower seconds range (~ 1 second).[8]

227   0D structures have the fastest volume exchange because medium exchange occurs mainly

228   through convection. The complete volume exchange requires about 100 milliseconds

229   depending on the geometry of the trap and flow rate of the surrounding media flow.[62]

230   The "quality" of reproduction of a defined environmental profile generally depends on the

231   cultivation chamber design which is important for a high precision of the DCE. Ho *et al.*[76]

232   have investigated this in detail by advanced CFD modelling studies. In a first step, they

233   investigated which cultivation chamber design can be used to create DCE at second and sub-

234   second resolution. They analyze if typical life-lines of cell metabolism observed in large-scale

235   bioreactors can be analyze on single-cell level.[44] As shown in **Figure 4B**, a typical

236   concentration profile features three concentration peaks with increasing width and amplitude.

237   Ho *et al.* found that 1D systems are able to accurately reproduced this entire signal. Both

238   nDEP and 2D systems completely miss the first peak and can by far not reproduce the

239   amplitude of the second peak (44% for nDEP and 17% for 2D). In a next step Ho *et al.*

240   systematically investigate features of which frequency in DCE profiles can be produced at the

241   cell surface on the microfluidic chip and cultivation chamber designs. Therefore, they use

242   sinus signals with different frequencies. The resulting bode plot **(Figure 4C)** reveals

243   frequency ranges for which environmental changes can be precisely reproduced so that for a

244   high reproduction of life-lines an amplitude signal loss of maximum 5% is requisite. In 2D

245   cultivation chambers (here chambers have one nutrient supply channel) the amplitude signal

246   with a period of 200 seconds is damped less by 5%. In comparison, DCE in 1D cultivation

247   systems such as the mother machine[8] can be reproduced an amplitude signal with a period of

248   5 seconds with an amplitude loss of less than 5%. Despite the significant advantage in mass

249   transfer, 1D cultivation systems have to be chosen carefully. Recently it was shown by Yang

250   *et al.*[12] that design and geometry of the cultivation channel can impact growth of microbial

251   cells significantly.

252

253   **FIGURE 4**

254

255   The choice of the technical periphery and cultivation chamber conform of the depending

256   requirements. It is important that the change of the environmental conditions is significantly

257   lower than biological relevant timescales. For DCE with frequencies in lower minutes and

258   second ranges the choice of the cultivation chamber is important, since medium change from

259   main and nutrient channels to cultivation chambers are often restricted by diffusion.[75] If a

260   step function is of interest all combinations of chamber designs and technical periphery can be

261   used. Similarly, if DCE with times in hours is the aim all combinations of technical periphery

262   and cultivation chamber designs are possible. In these cases, the diffusion driven mass

263   transport within the cultivation chambers has a temporal delay over a few seconds but can be

264   neglected. For DCE within changes in minutes up to seconds the choice of the technical

265   periphery and cultivation chamber is crucial. Here, microfluidic cultivation chambers with

266   short volume exchange times must be chosen such as 1D systems like the mother machines

267   and 0D like traps. 2D cultivation chambers as well as 1D chambers can be chose if an

268   oscillation in a minute range is the aim. For the technical periphery, pressure pump can be

269   used for rapidly DCE because of the fast switching times (~ milliseconds) between different

270   concentrations of the pumps in combination with 1D and 0D cultivation chambers. Pneumatic

271   valves on a chip and external electric valves have in combination with 1D or 0D chambers the

272   advantage that an environmental change in a range of seconds is possible.

273

274   **3. Applications**

275   Several dynamic single-cell cultivation proof-of-concept studies were performed in the last

276   years. They differ in the cultivation chamber, technical periphery, modulation methods and

277 applied environmental profile and the application. Examples are chosen to demonstrate both,

278 technical feasibility as well as novelty in the application to perform cellular experiments that

279 are not possible or difficult to perform with conventional technologies such as shaking flasks

280 or lab-scale bioreactors. In shaking flasks and bioreactors only average measurements are

281 possible to determine, not single cell values. **Figure 5** and **6** gives an overview of these

282 studies, which will be discussed in the next section in more detail.

283

284 **FIGURE 5**

285

286 **FIGURE 6**

287

288 **3.1 Hydrostatic pressure - external**

289 Fracassi *et al.* used a microfluidic device with a motorized syringe driver which had an

290 automatic control in real time for analyses of gene expression of tetracycline inducible

291 promotor in CHO tetO7-YFP cells **(Figure 7A – Setup)**. The DCE between tetracycline-rich

292 and standard growth medium were generated by PWM/ PFM in 15 to 1000 minutes intervals

293 **(Figure 7A – Environmental profile)**. The microfluidic device consists of several 2D

294 cultivation chambers that contain small CHO colonies in a monolayer. They showed that the

295 cells expressed an average value of 50% of d2EYFP after 3500 minutes **(Figure 7A –**

296 **Representative results)**.[50]

297 The same research group modified the setup so that the motorized stage of the syringe could

298 regulate desired concentration 0%, 50% and 100% in a pulse range from 15 to 100 minutes.

299 This way, they also investigated the gene expression of tetracycline inducible promotor of

300 CHO and could demonstrated a set-point regulation of gene expression in a proof of concept
301 study.[56; 77]

302 Fiore *et al.* used hydrodynamic pressure for the control of the GAL1 promoter in *S.*
303 *cerevisiae*. The regulation of DCE between galactose and glucose adjusted over a negative
304 feedback loop with different controllers so that pulses were generated by PWM/ PFM in 15 to
305 500 minutes intervals.[78] In the same research group, Menolascina *et al.*, used a similar setup
306 with an on/ off actuation for the induction of GAL1 promotor in *S. cerevisiae*. The DCE
307 between galactose and glucose were generated by PWM/ PFM in 8 to 350 minutes intervals.
308 They revealed a control mechanism of the expression of a protein in a yeast cell
309 population.[79]

310 A similar concept used Luke *et al.* for the fluid flow with a Dial a Wave system (DAW)
311 which allows a temporal control of medium and generated a combination of the two inputs
312 defined by the user. They analyzed the effect of limiting nitrogen concentrations onto cell
313 proliferation and chlorophyll autofluorescence of the microalgae *C. sorokiniana*. DCE with
314 and without nitrogen were generated by PFM/ PWM in 50 minutes to 5 hours interval.[80]

315 Kaiser *et al.* utilized the hydrostatic pressure with the DAW system for a dual-input mother
316 machine chip for the induction of lac operon in *E. coli* cells. The DCE between glucose and
317 lactose containing media were generated every 4 hours by PFM. They could show that cells
318 expressed lacZ-GFP from the lac promotor by switching the carbon source from glucose to
319 lactose.[47] Crane and co-workers[81] used programmable syringe pumps for the gene
320 regulation in *S. cerevisiae*, upon DCE between different glucose concentrations. Here, a
321 microfluidic system called ALCATRAS (a long-term culturing and trapping system) was used
322 with 6-hour pulses of high glucose (2 % glucose) and 2-hour pulses of low glucose
323 concentration (0.1 % glucose) medium by PWM/ PFM. They could show that yeast upon
324 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 hours by PFM/ PWM. The shortest pulses of CHX were 3 hours. 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 shown that CHX had a toxicity 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.* 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 minutes and 10 minutes wash process with a MES buffer by PFM/ PWM. The increasing concentrations were 2 mM, 5 mM, 10 mM and 100 mM glucose. They could shown the reversible changes of cytosolic free glucose in individual cells.[82]

Rojas *et al.* 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 seconds intervals.[58]

In an alternative approach Bennett and co-workers 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 to 4.5 hours intervals.[48]

350   Park *et al.* used syringe pumps for the regulation of time pulses of 1 hour to analyze sigma

351   factor activity for RNA polymerase of *B. subtilis* in mother machines (1D chambers). Using a

352   switch from SMM medium to SMM medium with IPTG, they could shown that alternative

353   sigma factors activate the core RNA polymerase dynamically. [57]

354

355   **FIGURE 7**

356

357   **3.3 Pneumatic valves on a chip**

358   Kim *et al.*[52] used pneumatic valves on the chip with a resolution of the oscillation periods

359   between 57 and 360 seconds **(Figure 8A – Setup)**. Therefore, the device was generated in

360   three layers. The fluid layer and also the microfluidic channel were composed of the top and

361   bottom layer. The layer between, the middle layer, is a membrane layer with a thickness of

362   20 µm. In this setup two valves were used, one with the fluorescence dye and the other one

363   with a clear solution. As a model system C2C12 cells was utilized in the channel for cell

364   staining and were stained with a fluorescence dye which was cell permeable through the

365   membrane with a high affinity to nucleic acids so that the fluorescence dye bonded there, and

366   the fluorescence intensity increased. By opening the first valve the fluorescence intensity in

367   the cell nucleus increased to a constant value over a time period of 25 seconds. After this the

368   second valve was opened and valve one was closed. Upon medium change, the fluorescence

369   intensity decreased in this phase because of photobleaching or dye dissociation. In

370   consequence of this "open and close process" a periodically feeding of the fluorescence dye

371   was possible with a total oscillation period of 57 seconds for both states with a flow rate of

372   4 µL min^{-1} **(Figure 8A – Representative results)**. Kim *et al.* could shown the induction of

373   periodic cell staining in C2C12 cells with a DCE between 1 µM fluorescence dye in TE buffer

374   and DMEM medium by PFM/ PWM.[52]

375   Educator and coworkers[62] also used multilayer microfluidic chips with integrated pumps and

376   valves for the analysis of Ca^{2+} dynamics in Jurkat T-cells. The medium exchange in the 0D

377   structures takes only 100 milliseconds. The DCE between HBS with ionomycin and HBS

378   were generated by PWM in 40 seconds intervals. They demonstrated the viability of cells and

379   the ionophore mediated intracellular Ca^{2+} flow analysis.

380   A three layer microfluidic chip with integrated valves for the analysis of Ca^{2+} dynamics in

381   HEK-293 cells was used by Gonzalez *et al.* The DCE between cell medium and medium with

382   carbachol were generated by PFM 30 to 150 seconds intervals.[63]

383   Park *et al.*[65] used a similar approach compared to Wheeler *et al.* to an investigate FBS

384   concentration of MDA-MB-231 cells. Therefore, they additionally implemented external

385   electric valves for DCE ranges between 15 and 160 seconds by PWM. The chambers (2D)

386   have a large width of 1000 µm so that the mediums exchange in the chamber only occurs by

387   diffusion. They could shown that FBS concentration profile induced migrational response.[65]

388   He *et al.*[83] utilized also additional external electric valves for the induction of calcium

389   signaling in Jurkat T cells. The DCE between RPMI medium and RPMI medium with

390   100 µM H_2O_2 were generated by PFM in 20 to 200 second intervals.[83]

391   The investigation of pheromone induced mitogen-activated protein kinase in *S. cerevisiae* was

392   performed by Falconnet *et al.*.[84] They used a two-layer microfluidic chip with integrated

393   vales and integrated peristaltic pumps for a DCE between fluorescein and medium. The DCE

394   were generated by PFM/ PWM in 9 to 14 minutes intervals.[84]

395   Piehler *et al.* used multilayer microfluidic chips for the analysis of transcription factor NFκB

396   in mouse 3T3 fibroblasts. The DCE of TNF with a maximum concentration of 3 ng ml^{-1} were

397   generated by PFM in 100 minutes periods of a sinus wave.[85]

398   In an alternative approach Kusen *et al.* used also a two-layer microfluidic chip with integrated

399   valves for the induction repressible pMET17 promoter for YFP expression in *S. cerevisiae.*

400   The DCE between methionine containing medium and no methionine medium were generated

401   by PFM/ PWM in 2 to 8 hours intervals.[86]

3.4 External electric valve

403   Uhlendorf *et al.* analyzed the high osmolarity glycerol signaling cascade in *S. cerevisiae* with

404   an external electric valve at a microfluidic device **(Figure 8B – Setup)**. Here, the native

405   promoter pSTL1 was chosen for the expression of a fluorescence reporter. The promotor of

406   the high osmolarity glycerol cascade was activated due to the osmotic stress. The microfluidic

407   device consisted of open box chambers with a height of 3.1 μm with two inlets and outlets.

408   The osmotic stress was activated by DCE between normal and sorbitol-enriched media by

409   PFM/ PWM. Pulses between 5 and 8 minutes were generated for the visualization of the

410   osmotic shock with a 20-minute relaxation period afterwards **(Figure 8B – Environmental**

411   **profile)**. The control loop for the input media was repeated every 6 minutes. This method is a

412   real-time control of the single-cell level over 15 hours to analyze the gene expression. The

413   single-cell control was more difficult than the population control because the fluorescence

414   value in single cells had more fluctuation as the population, so that defined cells varied over a

415   long time period from the population. The single-cell control could reduce the fluctuations of

416   the population so that the noise at the single-cell level decreased.[53; 87]

417   Hansen *et al.* used also external electric valves in combination with hydrodynamic pressure

418   for the control of Msn2 dynamics in *S cerevisiae*. The DCE between medium with and

419   without 1-NM-PP1 were generated by PFM/ PWM in 10 seconds up to 5 minutes intervals.[67;

420   88]

421   In an alternative approach Charvin *et al.* used a microfluidic device to separate the flow and

422   the growth channel trough a diffusive cellulose membrane. Upstream the microfluidic device

423   an external electric valve was used for the DCE between SCD medium + 10x Met and SCD

424   medium + 0x Met by PFM/ PWM. They could shown that GAL 1 and MET3 promotors turn

425   on and off very quickly in *S. cerevisiae* with pulses of 10 to 15 minutes intervals.[68]

426

427   **FIGRUE 8**

428

429   In the demonstrated examples, mainly PFM and PWM were used.[47; 48] Many studies combine

430   both modulations so that they can control the width of the pulses or created different

431   frequencies. Only a few studies varied the amplitude (PAM) as well as the frequency and

432   width of the environmental conditions.[82] PAM is important to reproduce the nutrient

433   fluctuations as in a bioreactor. The emulation of complex environments such as natural

434   habitats, requires the change of several environmental factors at the same time, but was not

435   demonstrate for application yet.

436   ## 4. Technical and biological perspective

437   The shown examples demonstrate how microfluidic experiments with DCE can be

438   implemented and performed. Technological progress will allow the implementation of more

439   complex environmental profiles for example the continuously variation of the amplitude or

440   DCE between more than two solutions.

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

452 Another interesting aspect is the environmental control of chemical stimuli such as gas, for
453 example oxygen, concentrations during microfluidic single-cell cultivation.[91] A first concept
454 was established by Mauleon *et al.*[92] They developed a microfluidic gas channel that can
455 generated an oxygen switch. The oxygen profile can be switched in a two-step function from
456 95% oxygen concentration to 0% oxygen for 4 respectively 10 minutes.[91; 92] Another
457 research group created a microfluidic device based on a multilayer chip with integrated
458 differential oxygenator to control the dissolved oxygen over the cultivation of aerobic and
459 anaerobic cultures as well as cell culture.[93]

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

465 Furthermore, automated programmable microfluidic platforms that combine image-based

466 single-cell analysis and DCE with feedback control are emerging.[50; 56; 78–80] Here, cellular

467 behavior is controlled by real time image analysis of cells, coupled to dynamic environmental

468 control.[96; 97] As soon as cellular threshold values are reached defined medium pulses are

469 triggered (referred as negative feedback control).[44; 45; 66; 67] Fracassi *et al.*[50] implemented a

470 negative feedback control to control tetracycline of CHO cells. This was realized with a relay

471 and PI controller so that an oscillation of the set point was generated. In the experiments half

472 of the fluorescence intensity was determined for the set point with the analysis of 180 minutes

473 without tetracycline. The regulation with the relay and PI controller shows an oscillation of

474 the fluorescence intensity around the set point value. The relay controller has an undamped

475 oscillation so that the actual set point value could not be achieved. In contrast with a PI

476 controller the oscillation decreased over the time in this case and in the end the set point was

477 achieved.[50] A review, discussing progress and challenges within feedback controlled

478 microfluidic experiment was recently published by Lugagne *et al.*.[98]

479 Technical progress will not only allow to investigate cellular behavior at various

480 environmental conditions but enable the cultivation and analysis of microbes at natural habitat

481 like environmental conditions. Finally, this will increase the portfolio of cultivation protocols

482 and workflows to completely new fields of applications. Currently only 1% of the microbes

483 grow under lab conditions because of the constant and incongruous environmental conditions.

484 MSCC in combination with DEC might enable to cultivate and analyze currently

485 uncultivatable microbes, often referred to microbial dark matter.[99; 100]

486

5. Conclusion and outlook

488 MSCCs with DCE have a high potential for the analyzing and understanding of cellular

489 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 concepts 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 can not 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 to millisecond. 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, gas exchange) is necessary to be able to emulate more complex profiles. This will lay the foundation for the

514   investigation of cellular behavior at complex environmental changes prevailing in natural and

515   artificial cultivation habitats.

516

Conflict of Interest

518   The authors declare that they have no financial/ commercial conflict of interest.

522

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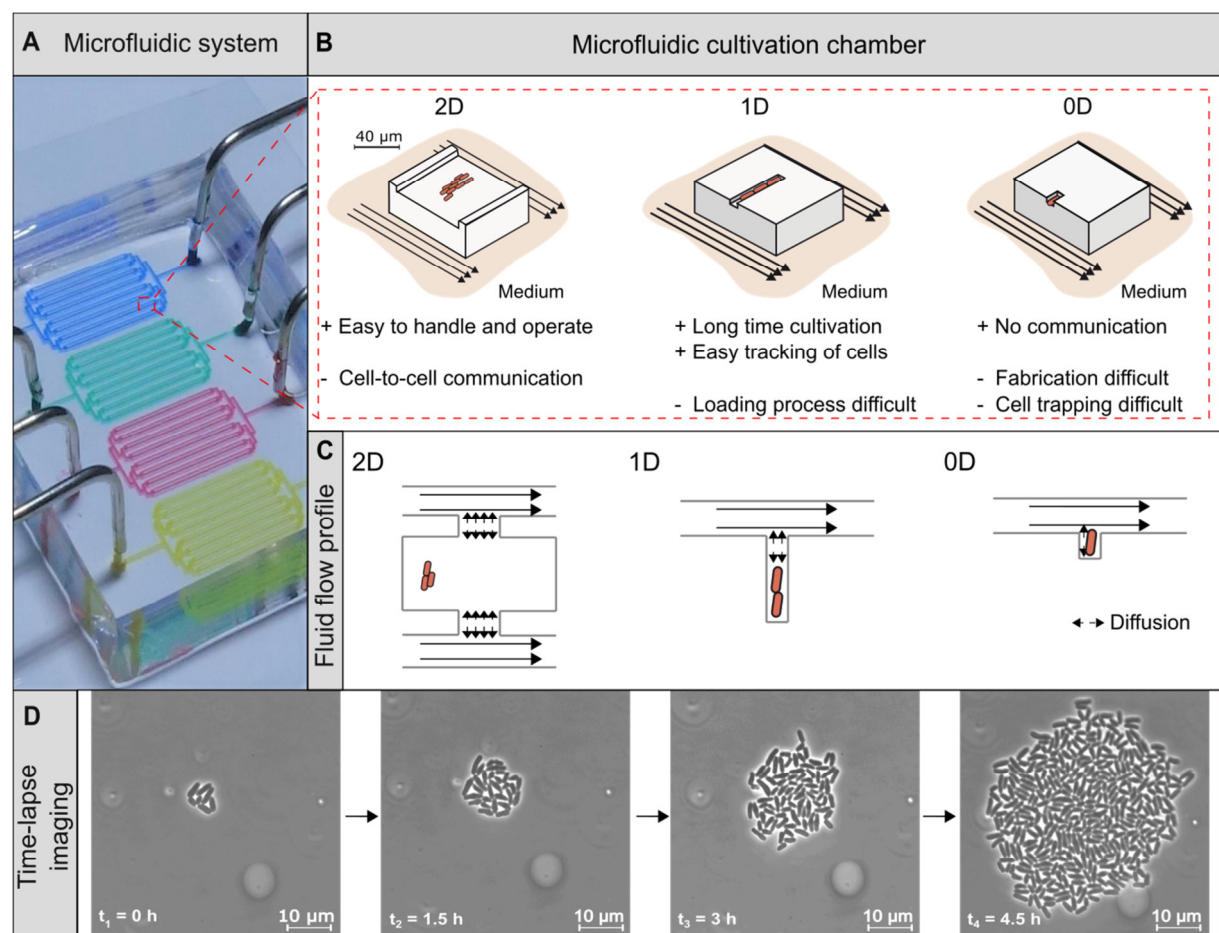
Figure 1: Overview of a microfluidic single-cell cultivation (MSCC) chip system. A) MSCC chip, containing several cultivation arrays with monolayer cultivation chambers. B)

798   Different cultivation chamber geometries (2D to 0D) with their advantages and disadvantages

799   and C) fluid flow schematic of these chambers. D) Live-cell imaging of typical microbial cell

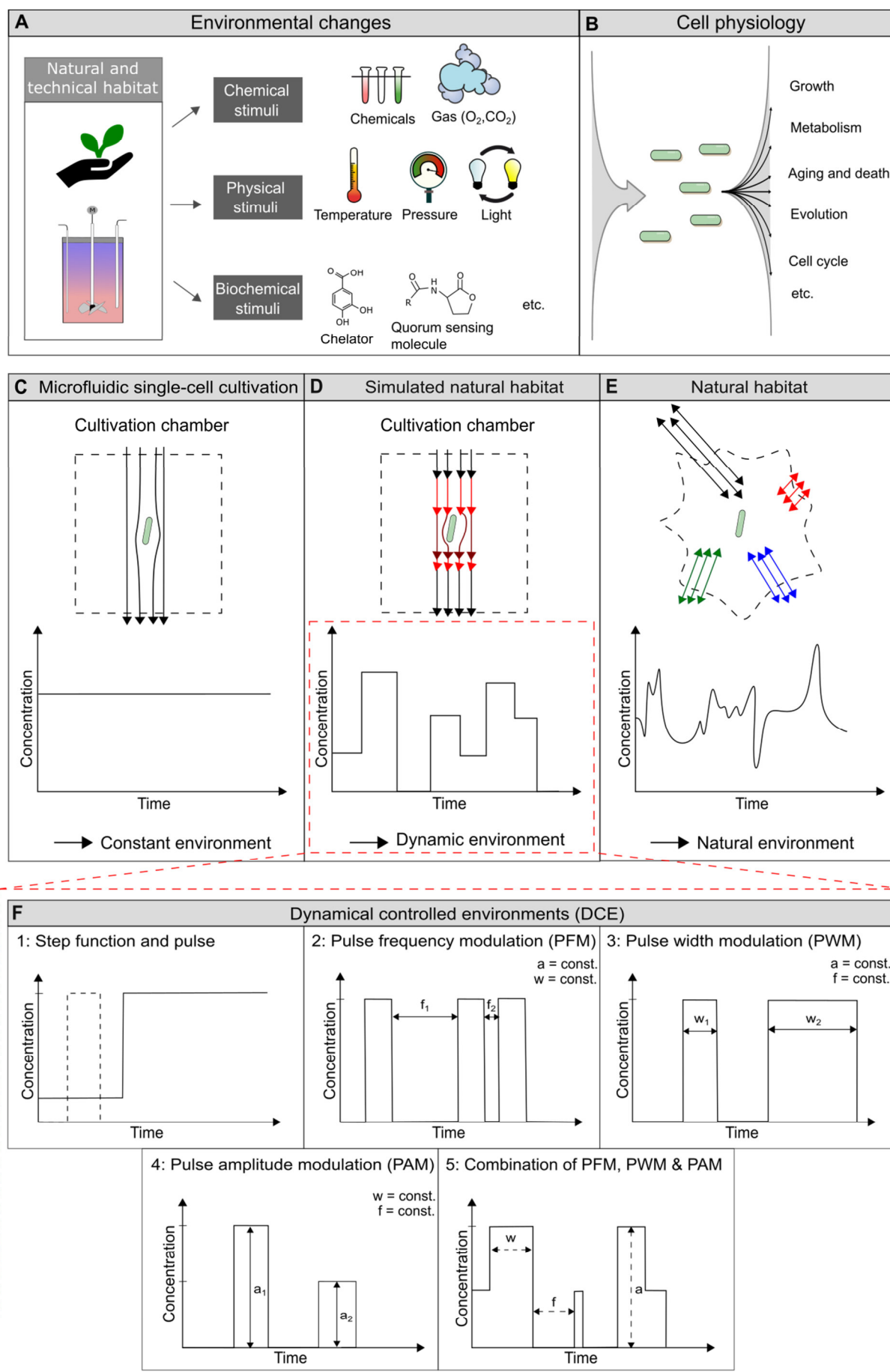800   colony cultivated at constant environmental conditions.

801

802   **FIGURE 2: Overview of environmental factors and DCE mechanisms at single-cell level.**

803   A) The natural and technical habitat influence the environment change in physical

804   components like pressure, chemical stimuli like gas and biochemical stimuli, that B) influence

805   cellular physiology for example growth, metabolism, aging and death etc. C) In MSCC, cell

806   cultivations can be performed at constant concentration so that the cells can grow under

807   optimum conditions. D) In the simulated habitat the concentration in the environment varies

808   in a fixed (defined) time frame over the cultivation time (dynamic environment). E) In natural

809   habitats, environmental concentrations can typically change randomly over the time. F)

810   Different DCEs: (1) step and pulse functions, (2) pulse frequency modulation (PFM), (3)

811   pulse width modulation (PWM), (4) pulse amplitude modulation (PAM) and (5) combination

812   previous modulations.

814   **FIGURE 3: Methods for the generation of DCE at a single-cell level in microfluidic**

815   **chips.** DCE modulation conducted with A) hydrostatic pressure method using syringe driver

816   with motorized stage for DCE in higher minutes up to hours change, B) external pressure

817   pump in front of the microfluidic chip for rapid change between environmental conditions, C)

818   pneumatic valves on chip used for rapid changes of environmental conditions and D) external

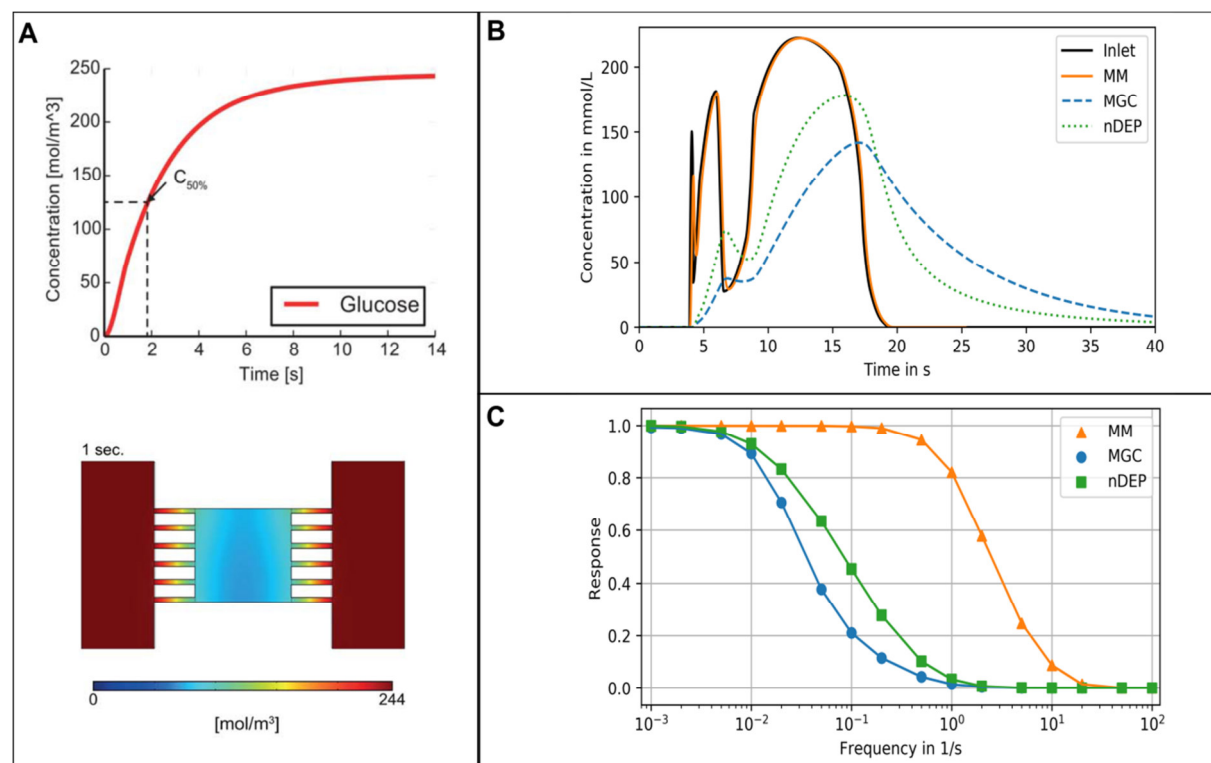819   electric valve in front of microfluidic chip for a fast DCE change.

820

821   **FIGURE 4: DCE in microfluidic cultivation chambers.** A) Simulation of glucose change

822   in 2D cultivation chamber. After 6 seconds glucose concentration equilibrium is reproduced

823   in chamber. Reproduced with permission.[4] Copyright 2019, Wiley-VCH. B) Reproduction of

824   environmental profile with different cultivation chamber designs.[76] C) Bode diagram

825   compares responses for three chamber designs.[76]

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 proliferation 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]

826

827   **FIGURE 5: DCE studies with hydrostatic pressure and pressure pumps.**

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 18 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]

828

829 **FIGURE 6: DCE studies with pneumatic valves on the chip and external electric valves.**
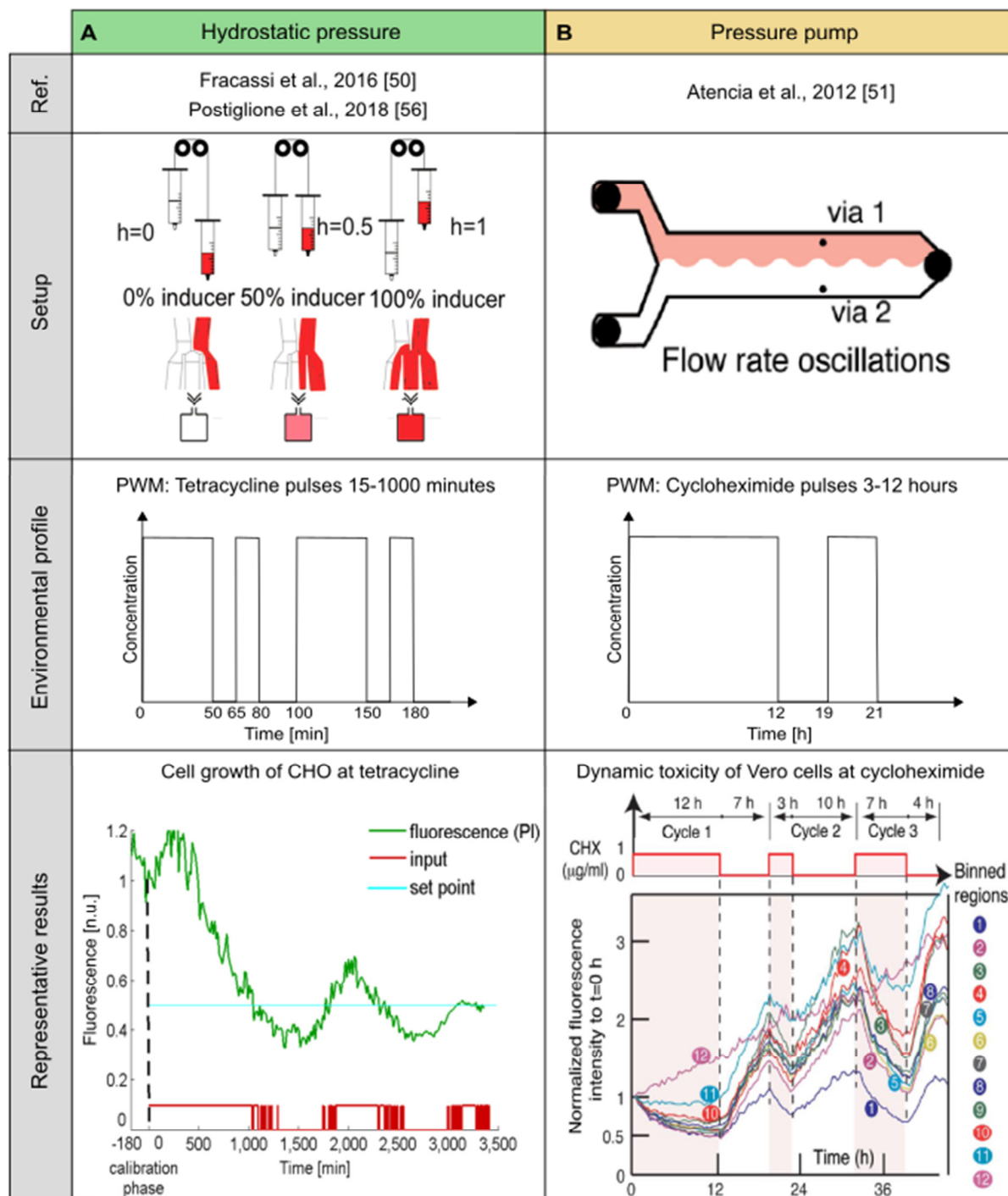
830
831 **FIGURE 7: Applications for hydrostatic pressure and pressure pumps of the**

832 **modulation of DCE at single-cell level.** A) Application of Fracassi *et al.*, 2016 illustrates

833 practical implementation of syringe driver with a motorized stage. Environmental profile

834 shows DCE in 15 to 1000 minutes time range of tetracycline pulses. These representative

835 result shows DCE of tetracycline impact on cell growth of CHO. Reproduced with

836 permission.[50; 56] Copyright 2019, American Chemical Society. B) Application from Atencia

837   *et al.*, 2012 illustrates microfluidic chip design with two inlets and pressure pump for DCE

838   between 3 and 12 hours. The representative result illustrates dynamic toxicity of Vero cells by

839   cycloheximide pulses. Reproduced with permission.[51] Copyright 2019, Royal Society of
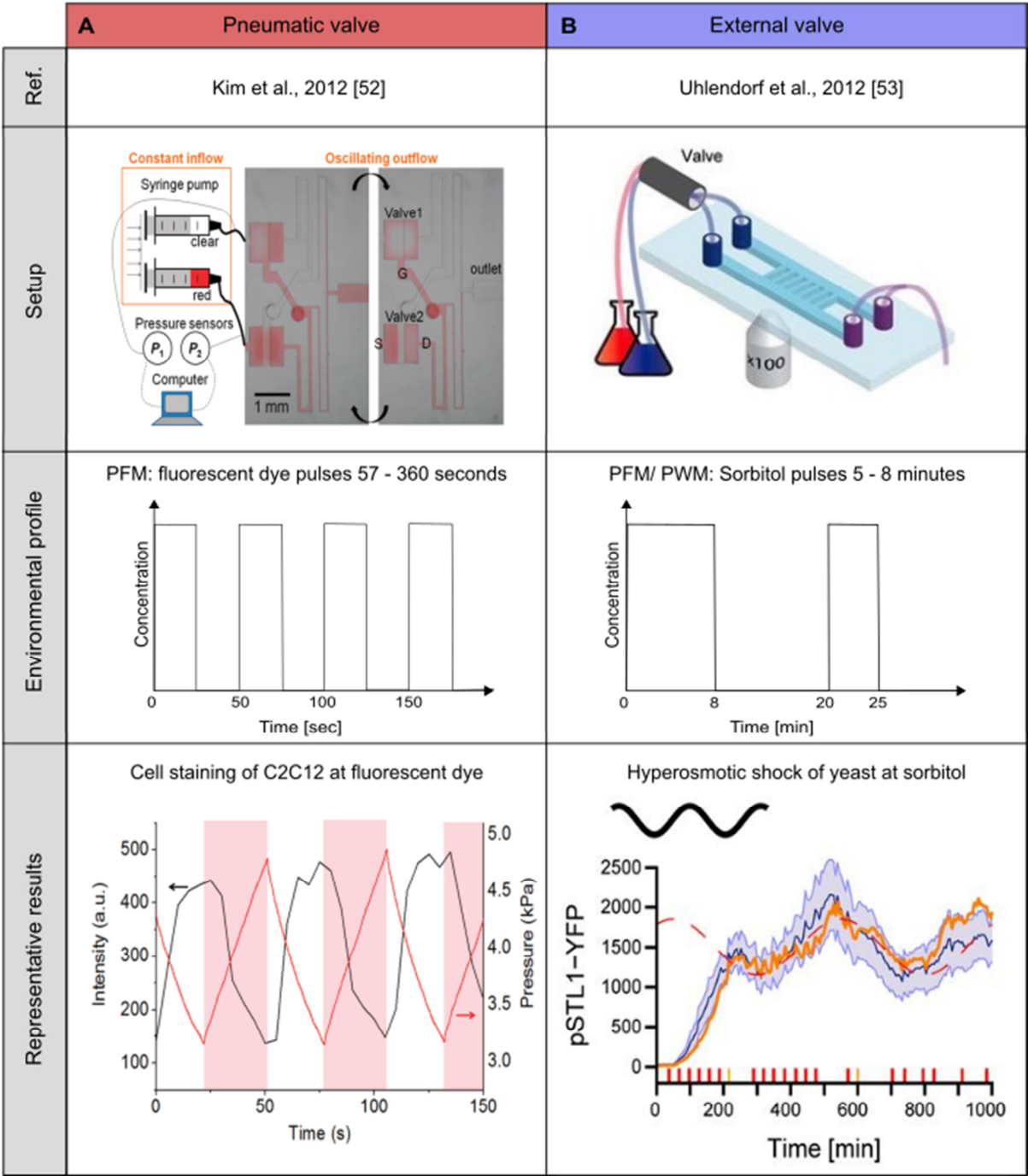
840   Chemistry.

841

842   **FIGURE 8: Applications for pneumatic valves and external electric valves of the**

843   **modulation of DCE at a single-cell level.** A) Application from Kim *et al.*, 2012 illustrate the

844   setup of the pneumatic valves on the microfluidic chip. The white solution is deionized water

845   and the red solution was deionized water with red food dye. The environmental profile shows

846   the DCE in a 57 to 360 seconds range. The representative result shows the oscillation of the

847   fluorescence dye for the cell staining of C2C12 cells. Reproduced with permission.[52]

848   Copyright 2019, American Chemical Society. B) Application from Uhlendorf *et al.*, 2012

849   shows the illustration of the setup of the electric valve in a microfluidic device. The

850   environmental profile shows the DCE with 5 to 8 minutes pulses of sorbitol. The

851   representative result shows the hyperosmotic shock of yeast. Reproduced with permission.[53]

852

853   **Author biographies and photographs**

854

855   Sarah Täuber is a Ph.D. student at the University of Bielefeld. She received her Master's

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860

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870

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876 **Table of contents**

877 Microfluidic single-cell cultivation (MSCC) at oscillating and dynamic environmental

878 conditions is of note in the research community for the investigation of biologically relevant

879 issues. In this review, we give an overview of this topic, discuss the MSCC setups for the

880 realization and their application in single-cell research. Concluding, we discuss further

881 perspective of performing MSCC under complex dynamic environmental conditions.

882 **Keyword** Dynamic control environment

883 S. Täuber, E. von Lieres and A. Grünberger[*]

884 **Dynamic environmental control in microfluidic single-cell cultivations: From concepts**

885 **to applications**

886 ToC figure

887