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DROPLET MICROFLUIDICS FOR BIOPROCESS ENGINEERING

KARTIK ARUN TOTLANI

DROPLET MICROFLUIDICS FOR BIOPROCESS ENGINEERING

KARTIK ARUN TOTLANI

INVITATION

To the public defence of my doctoral thesis:

DROPLET MICROFLUIDICS FOR BIOPROCESS ENGINEERING

The defence ceremony is to be held on Thursday the 21st of October 2021 at 12.30 hours in the Senaatszaal of the Aula, Delft University of Technology, Mekelweg 5, Delft.

Prior to the defence, at 12.00 hours, I will give a brief introduction to my Ph.D. research.

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Propositions

accompanying the dissertation

DROPLET MICROFLUIDICS FOR BIOPROCESS ENGINEERING

by

Kartik Arun TOTLANI

1. Microfluidics promises *Lab-on-a-Chip* yet delivers *Chip-in-a-Lab*.
2. Validation through organs-on-chips should be commonplace in pre-clinical studies.
3. Geometry-mediated strategies make droplet-based microfluidics for semi-continuous cell-cultures accessible to non-experts. (Chapter-3, this thesis).
4. Simplicity and brevity in scientific writing has been sold out to pre-empting criticism during peer review.
5. TU Delft's applied sciences' grading scheme is biased in favour of theoretical projects.
6. Instant gratification in the world of social media leads to a generation lacking scientific rigour.
7. Development of technology for rapid diagnosis of diseases deserves more media attention than self-driven cars.
8. Importance of lazy geniuses is underrated.
9. Principal Investigators should spend more of their time in the lab.
10. Owing to the funding inequity, global university rankings can't be taken seriously.

These propositions are regarded as opposable and defendable, and have been approved as such by the promotor prof. dr. ir. M. T. Kreutzer and co-promoters dr. W. M. van Gulik and dr. V. van Steijn

DROPLET MICROFLUIDICS FOR BIOPROCESS ENGINEERING

DROPLET MICROFLUIDICS FOR BIOPROCESS ENGINEERING

Dissertation

for the purpose of obtaining the degree of doctor
at Delft University of Technology,
by the authority of the Rector Magnificus prof. dr. ir. T. H. J. J. van der Hagen,
chair of the board of Doctorates,
to be defended publicly on Thursday 21 October 2021 at 12.30 hours

by

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Master of Science in Chemical Engineering,
Delft University of Technology, Delft, the Netherlands,
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Front & Back: Beautiful cover art by Khushal Kapoor and Gaurav Dhanda that captures the coalescence of nutrient droplet and cell containing droplet to establish fed-batch process on a chip.

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for my family

SUMMARY

A crucial challenge during the initial stages of bioprocess development is that tools used to screen microorganisms and optimize cultivation conditions do not represent the environment imposed at industrial scale. Inside an industrial-scale bioreactor, microorganisms are often cultivated under fed-batch conditions, where nutrients are supplied during the culture. Additionally, microorganisms continuously keep crossing zones with low and high concentrations of substrate and dissolved oxygen. However, during initial bioprocess development, growth and productivity of microorganisms are evaluated under batch conditions due to the difficulty of dynamically controlling nutrient and dissolved oxygen concentrations in screening equipment such as micotiter plates. This inconsistency in cultivation conditions often leads to selection of strains that fail to perform at industrial scale. The difficulty in continuously supplying minute amounts of nutrients to microorganisms in microtiter plates and imposing dynamic dissolved oxygen levels throughout the cultivation experiment necessitates an alternative approach. Microfluidic technology holds the potential to address this inconsistency with fidelity by offering high-throughput experimentation and excellent control over the culture microenvironment. The central theme of this Ph.D. project is the design and development of droplet-based microfluidic technology, that enable studying microorganisms under such dynamically controlled cultivation conditions. As such, the outcomes from this Ph.D. project form a foundation step towards narrowing the gap between screening and industrial-scale use, with an eye to keeping the technology sufficiently simple to be adopted by the biotechnology and bioengineering community.

We start by reviewing different types of scalable micro-bioreactor platforms that could be utilized for carrying out dynamic fed-batch and continuous cultures (chapter 2). We categorize the platforms into three types: (i) microtiter plate-based, (ii) microfluidic chamber-based, and (iii) microfluidic droplet-based. This review highlights the potential of droplet-based micro-bioreactors and motivates the development of the droplet-based systems presented in this thesis.

The first type of tool we developed is a droplet-based nanobioreactor that facilitates nutrient-controlled cultivation in fed-batch mode. Since the operation of such a device needs a reliable method for supplying nutrient droplets to cell-containing droplets, we first designed a strategy for feeding nutrient droplets. As a first goal, we developed a scalable microfluidic droplet on-demand (DoD) generator for producing monodisperse droplets, where the droplet volume is primarily dictated by the generator geometry and is independent of operating conditions (chapter 3). The DoD generator was thoroughly characterized for a range of operating conditions and flow parameters while generating droplets with a high monodispersity. By decoupling droplet formation from its transport, a reliable scale-out was achieved for the sequential generation of droplets on-demand at multiple DoD junctions in the chip. This DoD technology was used to design and develop a microfluidic tool that enables studying microorganisms under

nutrient-controlled fed-batch conditions (chapter 4). We hereby use the yeast *Cyberlindnera (Pichia) jadinii* as a model organism. The droplet-based fed-batch nanobioreactor comprises two separate DoD generators, where the first one is used for creating droplets encapsulated with microorganisms and the second one for making nutrient droplets at the desired frequency throughout the cultivation. The nutrient droplets were chemically coalesced to the cell-containing droplet, immobilized inside a trap, by temporarily de-stabilizing the droplet-droplet interface through the flow of a poor solvent around it, thereby establishing a fed-batch process. We performed nutrient-controlled cell growth experiments by varying the glucose concentration inside the nutrient droplets and by varying the frequency of droplet generation. Nutrient controlled growth inside the droplets was established by demonstrating different cell growth rates with different glucose concentrations inside nutrient droplets. The growth behaviour of the microorganisms for a different set of glucose concentrations agreed well with a simple kinetic growth model. Diverting away from continuous droplet microfluidic platforms which require sophisticated workflows and integration of multiple devices, we developed a strategy that facilitates simple operation and fabrication of devices from standard procedures. The results from this work form a proof-of-concept of long-term and nutrient-controlled growth of microorganisms inside microdroplets through a controlled supply of nutrient droplets.

The second type of microfluidic tool that we developed enables the cultivation of yeast inside microdroplets with the main supply of oxygen to the cells coming from the oil flowing around the droplets (chapter 5). We used fluorinated oil as it has a high oxygen solubility. Batch growth of *Cyberlindnera (Pichia) jadinii* was performed inside droplets under two limiting cases. For the first case, oil saturated with oxygen was flown around the droplets containing microorganisms, which showed exponential cell growth. In contrast, negligible growth was observed in the second case where the oil was saturated with nitrogen and flown around the droplets. Even though we present preliminary results on the cultivation of microorganisms under two static oxygen levels, this methodology could be further developed to impose dynamic changes in dissolved oxygen concentration as experienced by microorganisms inside an industrial-scale reactor in order to take along the sensitivity of microorganisms towards such changes during the initial stages of bioprocess development.

SAMENVATTING

Een belangrijke uitdaging tijdens de eerste fasen van de ontwikkeling van bioprocessen is dat de methoden die worden gebruikt voor het selecteren van micro-organismen en het optimaliseren van de kweekomstandigheden niet representatief zijn voor de omgeving van microorganismen op industriële schaal. Enerzijds worden in een reactor op industriële schaal micro-organismen in veel gevallen gekweekt volgens de fed-batch methode, waarbij nutriënten worden toegevoegd tijdens de kweek. Bovendien doorkruisen de micro-organismen voortdurend zones met lage en hoge substraat concentraties en opgeloste zuurstofconcentraties. Anderzijds worden tijdens screening en initiële bioprocesontwikkeling de groei en productiviteit van micro-organismen geëvalueerd in batchcultuur in microtiterplaten, zonder toevoeging van nutriënten of dynamisch wisselende niveaus van substraat en opgeloste zuurstof. Deze inconsistentie in kweekomstandigheden leidt vaak tot de selectie van stammen die niet goed presteren op industriële schaal. De moeilijkheid om voortdurend minuscule hoeveelheden voedingsstoffen toe te dienen aan micro-organismen in microtiterplaten en dynamische opgeloste zuurstofniveaus op te leggen gedurende het kweekexperiment maakt een alternatief noodzakelijk. Microfluïdische technologie biedt deze mogelijkheden en is tevens geschikt voor high-throughput experimentatie met behoud van uitstekende controle over de kweekomgeving op microschaal. Het centrale thema van dit promotieproject is het ontwerp en de ontwikkeling van druppel gebaseerde microfluïdische technologie, die het mogelijk maakt om micro-organismen te bestuderen onder dergelijke dynamisch gecontroleerde kweekomstandigheden. Daarmee zijn de resultaten van dit project een eerste stap in de richting van het verkleinen van de kloof tussen de screeningfase en het gebruik van micro-organismen op industriële schaal, terwijl er bij de ontwikkeling aandacht is geschonken aan het voldoende eenvoudig maken van de technieken om zo te kunnen worden toegepast door de biotechnologische en bio-engineeringgemeenschap.

We beginnen met een overzicht van de verschillende types schaalbare microbioreactorplatformen die kunnen worden gebruikt voor het uitvoeren van dynamische fed-batch en continue culturen (hoofdstuk 2). We delen deze platforms in drie types in: (i) microtiterplaat-gebaseerd, (ii) microfluïdisch vat gebaseerd, en (iii) microfluïdische druppel gebaseerd. Dit review illustreert de potentie van microfluïdische druppel gebaseerd systemen en motiveert de ontwikkeling ervan zoals gepresenteerd in deze thesis.

Het eerste type microfluïdisch systeem dat we ontwikkeld hebben is een nanobioreactor op basis van druppels die nutriënt gecontroleerde kweek in fed-batch modus mogelijk maakt. Aangezien de werking van een dergelijk druppel gebaseerde reactor een betrouwbare methode vereist voor de toevoer van nutriëntdruppels naar de celbevattende druppel, ontwierpen we eerst een strategie voor het genereren van dergelijke nutriëntdruppels. Hiertoe werd een schaalbare microfluïdische druppel op aanvraag (*Droplet-on-demand*, DoD) generator voor het produceren van monodisperse druppels geconstrueerd, waar het druppelvolume voornamelijk wordt bepaald door de geometrie

van de generator en onafhankelijk is van de condities (hoofdstuk 3). De DoD generator werd grondig gekarakteriseerd voor een reeks van procescondities, terwijl druppels met een hoge monodispersiteit werden gegenereerd. Door druppelvorming los te koppelen van transport werd een betrouwbare schaalbare methode bereikt voor het herhaaldelijk genereren van druppels op aanvraag op meerdere DoD generatoren op de microfluïdische chip. De DoD technologie is vervolgend gebruikt om een chip te ontwerpen en ontwikkelen die het mogelijk maakt om micro-organismen te bestuderen onder nutriënt gecontroleerde kweek in fed-batch modus (hoofdstuk 4). We hebben hierbij *Cyberlindnera (Pichia) jadinii* gebruikt als model micro-organisme. De druppel gebaseerde fed-batch nanobioreactor bestond uit twee afzonderlijke DoD generatoren waarbij de eerste werd gebruikt voor het maken van druppels met daarin de micro-organismen en de tweede voor het genereren van de voedingsdruppels met de gewenste frequentie gedurende de cultivatie. Coalescentie van de voedingsdruppels met de geïmmobiliseerde druppel met cellen werd bereikt door tijdelijk destabiliseren van het druppel-druppel interface met behulp van een oplosmiddel. Op deze manier kon een fed-batch cultivatie uitgevoerd worden. We hebben gecontroleerde celgroei experimenten uitgevoerd door de glucoseconcentratie binnen de voedingsdruppels te variëren en door de frequentie van de druppelgeneratie te wijzigen. Nutriënt gecontroleerde groei binnenin de druppels werd bereikt door aan te tonen dat de cellen verschillende groeisnelheden bij gebruik van verschillende glucoseconcentraties in de voedingsdruppels. Het groeigedrag van de micro-organismen voor verschillende glucoseconcentraties kwam goed overeen met berekeningen aan de hand een eenvoudig kinetisch groeimodel. Wij zijn er hierbij in geslaagd om een strategie voor fed-batch kweek op chip te ontwikkelen met behulp van een relatief eenvoudige opstelling bestaande uit een in de handel verkrijgbare drukpomp en microfluïdische chips die volgens standaardprocedures worden vervaardigd. De resultaten van dit werk vormen een proof-of-concept van substraat gelimiteerde cultivatie van micro-organismen binnen microdruppels door een gecontroleerde toevoer van voedingsdruppels.

Het tweede type microfluïdisch systeem dat wij ontwikkelden maakt het mogelijk gist te kweken in microdruppels terwijl de belangrijkste toevoer van zuurstof naar de cellen afkomstig is van de olie die rond de druppels stroomt (hoofdstuk 5). We gebruiken gefluoreerde olie, omdat deze een hoge oplosbaarheid van zuurstof heeft. Batchgroei van *Cyberlindnera (Pichia) jadinii* werd uitgevoerd onder twee limietgevallen. In het eerste geval werd met zuurstof verzadigde olie rond de druppels met micro-organismen geleid, met exponentiële celgroei als gevolg. de cel gedurende een langere periode in alle druppels een aanzienlijke groei vertoonden. Daarentegen werd verwaarloosbare groei waargenomen in het geval waarin olie verzadigd met stikstof rond de druppels werd geleid. Hoewel we voorlopige resultaten presenteren over de kweek van micro-organismen onder verschillende statische zuurstofniveaus, voorzien we dat deze methodologie verder kan worden ontwikkeld om fluctuaties in de opgeloste zuurstofconcentratie op te leggen zoals die worden ervaren door micro-organismen in een reactor op industriële schaal. Op deze manier kan de gevoeligheid van micro-organismen voor fluctuaties in opgelost zuurstof worden meegenomen tijdens de eerste fasen van de ontwikkeling van bioprocessen.

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1

INTRODUCTION

This doctoral dissertation is on the development of a microfluidic platform for the growth and study of microorganisms such as yeast inside microdroplets under dynamically controlled cultivation conditions. This chapter starts with a brief motivation of this development in the broader context of bioprocess development. After briefly introducing the droplet-based approach taken in this work (a detailed review is provided in Chapter 2), we introduce the research goals. We conclude by describing the outline of the dissertation.

Part of this chapter would be submitted as K. Totlani, R. J. van Tatenhoeven-Pel, M. T. Kreutzer, W. M. van Gulik, V. van Steijn. Microbioreactors for nutrient-controlled microbial cultures: Bridging the gap between early bioprocess development and industrial scale use.

1

1.1. CHALLENGES IN BIOPROCESS DEVELOPMENT

Industrial biotechnology uses microorganisms such as bacteria and yeast to transform renewable resources like agricultural feedstock into useful products, in a more sustainable manner than conventional production from fossil-based feedstocks. Microorganisms naturally synthesize antibiotics, vitamins, proteins and other valuable products but in amounts insignificant for industrial scale production. Therefore, economic feasibility of these bio-processes is typically obstructed by the low productivity of microorganisms found in nature. In order to render these processes commercially viable, microorganism's product yield can be enhanced through metabolic engineering, which necessitates a thorough understanding of organism's metabolic pathway and its regulation. Alternatively, microorganisms can also be improved by introducing random mutations in the genome, for example by exposing them to UV radiation thereby creating lots of different mutant clones. A general challenge in the development of bio-processes is the identification of the optimum combination of modified microorganisms and cultivation conditions. This requires studying the performance of a large number of mutants under dynamically controlled cultivation conditions[1]. The common bioprocess development

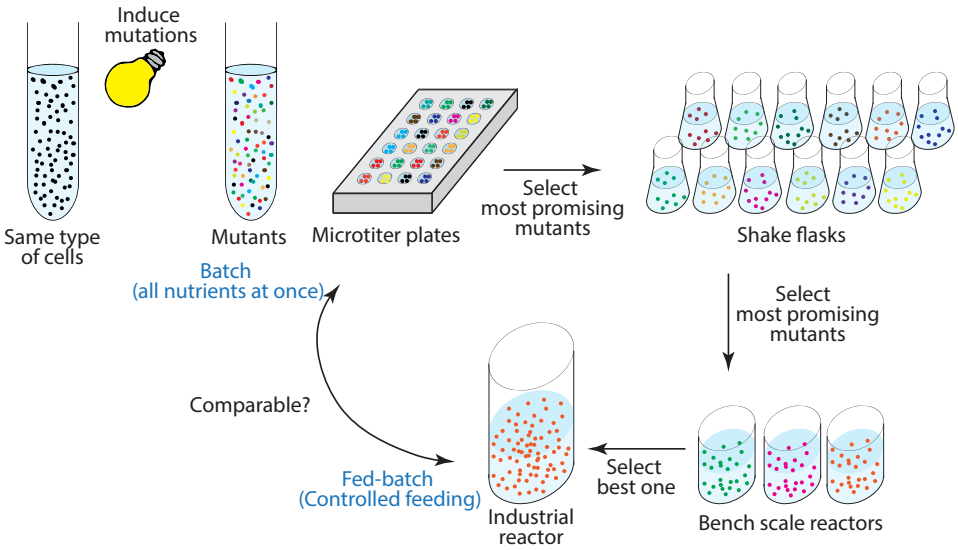

Figure 1.1.1: Schematic work flow of conventional microbial strain screening and improvement applied in bioprocess industries. The bioprocess development strategy typically begins with creation of a large number of mutants by directed or random modification. The mutants are screened for productivity in wells of microtiter plates with all nutrients present at the start and without active control over cultivation conditions. The most promising mutants found under these batch conditions are studied in shake flasks with more control over process conditions such as pH. The most promising mutants are subsequently studied in bench scale reactors with active control over nutrient supply (fed-batch) and cultivation conditions. The best performing mutants may eventually be used for industrial operation. The effectivity of the bioprocess development strategy benefits from the ability to perform screening and selection under fed-batch conditions in all steps. Microbioreactors have the potential to bridge the gap between screening and selection under batch conditions and industrial use under fed-batch conditions, by allowing nutrient-controlled fed-batch cultivation at high throughput.

strategy starts with studying a large number of modified microorganisms in the wells of microtiter plates, with a small fraction of best performers progressing to the next phase. Further selection is sequentially performed with tools that afford more control over cultivation conditions, but have a lower throughput, first with shake flasks and eventually with fully-equipped lab scale reactors, as illustrated in Figure.1.1.1. The best performer is then selected for industrial scale operation[2, 3]. Even though microtiter plates and shake flasks facilitate high throughput experimentation, they do not afford dynamic control over the cultivation conditions. Initial screening and selection is hence typically performed in batch mode where all the nutrients are present from the start of cultivation, and parameters like pH and dissolved oxygen are not actively controlled[4].

In contrast to the high-throughput experimentation tools used during initial bioprocess development and screening, fed-batch is the preferred mode of operation for majority of the industrial scale bioprocesses[7, 8]. Here, the bioreactor is continuously fed with one or more nutrients resulting in growth-limiting conditions, with nutrient feed-rate directly influencing the metabolic activity and growth rate of the microorganisms[9, 10]. Operation under fed-batch conditions is preferred over operation under batch conditions in cases of substrate inhibition[11] and production of undesired products due to excessive substrate presence (Crabtree effect)[12]. As the conversion rate of nutrients into products is a function of the environment of the microorganisms, high nutrient or product concentrations can inhibit microbial growth and limit the production of the desired product. Therefore, it is expected that microorganisms would behave differently under batch as compared to the fed-batch conditions and consequently the best performing microorganism under batch environment is almost never the best performing one in fed-batch environment. This has been demonstrated by Scheidle and co-workers wherein 224 different clones of *Hansenula polymorpha* were screened for production of green fluorescence protein (GFP) under batch as well as fed-batch conditions[5] Clearly,

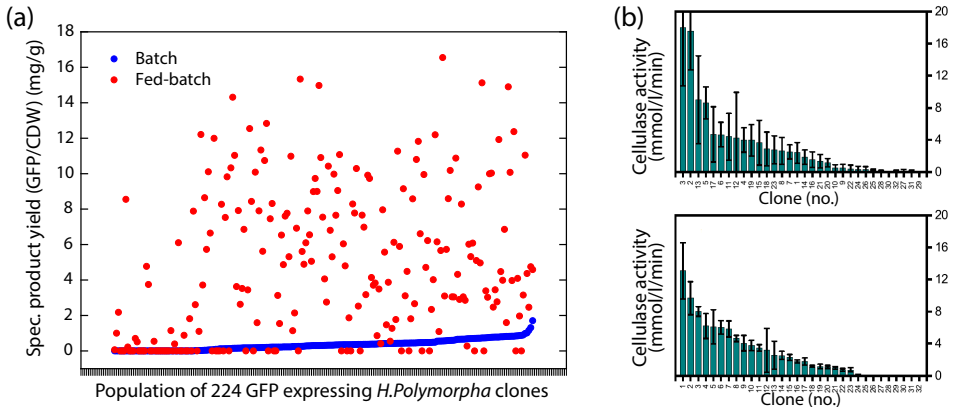

Figure 1.1.2: Comparing the performance of microorganisms under batch and fed-batch conditions, illustrating that clones that produce most under batch conditions are not the same as those that produce most under fed-batch conditions. (a) Specific yields(mg/g) of Green fluorescent protein (GFP) for 224 *Hansenula polymorpha* clones under batch (blue circles) and fed-batch (red circles) conditions. Adapted from [5]. (b) Cellulase activity from 32 *E.coli* clones cultured under batch (top) and fed-batch conditions (bottom). Adapted from [6].

there is no correlation between best-producing clones under batch and fed-batch conditions, see Figure.1.1.2(a)). Additionally, the average yield achieved under fed-batch conditions is about 14-fold higher than that achieved under batch conditions. Similar observations, although less strikingly, were recently reported by Keil and co-workers[6], who compared the performance of 32 clones of cellulase producing *E.coli* under batch and fed-batch conditions, see Figure.1.1.2(b). Both these studies clearly illustrate that identifying best performers under batch conditions with the purpose to use them under fed-batch conditions at industrial scale leads to ineffectiveness in bio-process development.

Besides nutrient levels, oxygen levels present a second inconsistency in conditions during screening and industrial scale operation. In industrial scale bioreactors, oxygen levels vary from bottom to top, since oxygen is fed from the bottom[13, 14]. During cultivation, microorganisms experience dynamically changing oxygen levels when traveling through regions of high and low concentrations of dissolved oxygen. It would therefore be beneficial to perform screening under dynamically varying oxygen levels in order to evaluate the sensitivity of different mutants towards dissolved oxygen levels. However, mimicking such a dynamic environment of dissolved oxygen is not yet possible with state-of-the-art screening tools. Microreactors, known for their unsurpassed control over fluids on the one hand and their the scalability on the other hand, offer the potential to address the inconsistency between screening and industrial use of microorganisms.

1.2. DROPLET MICROREACTORS FOR BIOPROCESS ENGINEERING

Droplet-based microfluidics is an emerging technology for numerous applications in microbiology and biotechnology[15–18] where microorganisms are encapsulated inside discrete aqueous phase microdroplets, that are formed and transported within an immiscible organic phase. Microdroplets offer compartmentalization, single-cell resolution and the possibility to add reagents for bio-chemical assays. The possibility to perform (1) a large number of experiments in parallel, (2) complex multistep and multiplexed operations on miniscule droplet microreactors, and (3) manipulation of small volumes with extensive spatio-temporal control makes droplet-based microfluidics a suitable platform for non-steady assays. Microdroplets have been used for carrying out high throughput chemical and biological experimentation where droplet operations like formation, reagent addition, on/off-chip incubation, splitting and sorting are implemented based on the requirements of the process workflow. For high throughput screening applications, millions of microdroplets encapsulated with microorganisms are typically generated, incubated, and based on the product of interest secreted in each droplet, sorting is performed for selecting best performing microbes[19]. A plethora of studies have been performed for screening of microorganism using droplet-based microfluidics. A recent review by Payne and co-workers[20] highlights the key challenges associated with the development of such droplet-based screening routines and offers perspective towards addressing them.

While most of droplet screening platforms involve addition of nutrients only in the be-

ginning of the routine (batch format), a more industrially-relevant method of screening would be to feed nutrients during the incubation in order to mimic a fed-batch process. Development of a droplet-based microfluidic platform capable of cultivating and possibly screening microorganisms in nutrient controlled fed-batch mode would require implementation of following set of operations:

1. Generation of cell-containing droplets on-demand.
2. Generation of nutrient-containing droplets on-demand.
3. Coalescence of nutrient-containing droplets with cell-containing droplets to establish nutrient feeding.
4. Long term growth(and real time monitoring) of microorganism inside droplets incubating on a microfluidic device.
5. Retrieval of the best performing microorganisms.

Studying microorganisms under dynamically controlled dissolved oxygen levels additionally requires dynamically controlling those levels throughout the incubation period. Based on the aforementioned set of conditions, research goals have been formulated in the next section.

1

1.3. RESEARCH GOALS

The main goal of this Ph.D. thesis is development of droplet-based microfluidic tools that enable studying cells under dynamically controlled cultivation conditions. The first type of tool we engineered is a fed-batch droplet microreactor that enables nutrient-controlled fed-batch cultivation. Its development critically hinges on the development of a method that enables a robust on-demand supply of nutrient droplets to the cell-containing droplet. Once the nutrient feeding strategy was developed in the first research goal, the next step was the application of the designed droplet on-demand generator for carrying out nutrient limited fed-batch cultivation of microorganisms such as yeast. The second type of tool we engineered enables studying cells under different oxygen tensions. Taken together, the three goals addressed in this thesis are:

- **Droplet on-demand generator**

  Generating multiple monodisperse droplets on-demand at different locations on a microfluidic chip at the same instance for non-steady droplet based assays.

  This research goal has been addressed in Chapter 3 of this dissertation.

- **Fed-batch droplet nanobioreactor**

  Development of a droplet-based microfluidic platform for cultivation of microorganisms where their growth rate can be controlled by nutrient feeding.

  This research goal has been addressed in Chapter 4 of this dissertation.

- **Microfluidic tool for dissolved oxygen**

  Design and development of a microfluidic static droplet array where growth of microorganisms can be studied under different concentrations of dissolved oxygen.

  This research goal has been addressed in Chapter 5 of this dissertation.

1.4. DISSERTATION OUTLINE

This doctoral dissertation comprises of 6 chapters including this introduction chapter. The outline of the dissertation is as follows:

- Chapter 2 describes an overview of different types of microbioreactor technologies that can be used for more dynamic continuous and fed-batch cultivation experiments. Three different types of microbioreactor platforms have been reviewed in this chapter: (i) microtiter plate-based platforms, (ii) microfluidic chamber-based platforms, and (iii) microfluidic droplet-based platforms. The chapter concludes with limitations for each of these types.

- Chapter 3 presents the design and development of a robust and scalable microfluidic droplet on-demand (DoD) generator for the production of monodisperse water-in-oil droplets. The core idea is that volume of generated droplets is primarily governed by the geometry of the DoD generator and independent of flow conditions. By decoupling the *formation* and the *transport* of the droplets, efficient scale-out is possible for producing multiple droplets on-demand that could be further used as reagent droplets for non-steady droplet-based bio-chemical assays on a chip.

- Chapter 4 is on the development of a droplet-based fed-batch nanobioreactor that enabled studying and growth of *Cyberlindnera jadinii* under nutrient-limited fed-batch conditions. Microorganisms are encapsulated inside the droplets and repeatedly fed with nutrient-containing droplets to establish a fed-batch process. An innovative aspect of this platform is the integration of droplet on-demand generators described in Chapter 3 with on-demand coalescence achieved by temporary injection of a poor solvent.

- Chapter 5 is a short chapter on the development of a microfluidic platform where yeast cells are cultivated inside microdroplets while, the continuously flowing oil phase is used as a source and sink for dissolved oxygen (DO). The goal was to perform batch growth of *Cyberlindnera jadinii* under two different concentrations of dissolved oxygen by leveraging the high oxygen-carrying capacity of fluorinated oil. This chapter presents preliminary results on the development of a microfluidic chip that could be used to impose dynamic dissolved oxygen levels such as those experienced by microorganisms inside an industrial-scale reactor.

- Chapter 6 summarizes the main findings and conclusions of the work carried out in this doctoral dissertation as well as provides outlook and recommendations for future research that could be carried out.

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2

MICROBIOREACTORS FOR NUTRIENT-CONTROLLED CULTURES

Microbioreactors for nutrient-controlled microbial cultures: Bridging the gap between early bioprocess development and industrial scale use

In industrial strain improvement programs large numbers of mutants are generated through random mutagenesis or automated strain construction, followed by high-throughput screening to select the ones that perform best for use at industrial scale. At industrial scale, strict nutrient-controlled fed-batch conditions are imposed as a means to control the metabolic activity and growth rate of the microorganism, thereby enhancing the expression of the product of interest. Although, it is known that microorganisms that perform best under these strictly controlled fed-batch conditions are not the same as the ones that perform best under uncontrolled batch conditions, screening and selection is predominantly performed under batch conditions. Tools that afford high-throughput on the one hand and dynamic control over cultivation conditions on the other hand are not yet available. Microbioreactors offer the potential to address this problem, resolving the gap between the bioprocess development and industrial application. In this chapter, we highlight the current state-of-the-art of microbioreactors that offer the potential for high-throughput screening of microorganisms under controlled conditions. We classify them into: (i) microtiter plates based platforms, (ii) microfluidic chamber based platforms, and (iii) microfluidic droplet based platforms. We also highlight some challenges that each of these platforms face.

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2.1. INTRODUCTION

In the experiments performed with state-of-the-art tools in early stage bioprocess development and mutant screening routines, the process control over the experiment is inversely proportional to the achieved throughput[1]. Over the years, several technologies have been developed to address this shortcoming and transform the conventional bioprocess development strategy. The control over fluids in microfluidic channels offers the potential to overcome the trade-off between throughput and level of control through the development of microbioreactors that enable studying a large number of modified microorganisms in parallel under dynamically controlled fed-batch conditions. An ideal microbioreactor should: (1) enable dynamic supply of nutrients and control over pH and dissolved oxygen, (2) enable integration with analytics to quantify biomass and products of interest and allow for selection and retrieval of best performers (3) be scalable thereby enabling high throughput experimentation, and (4) be robust and simple for easy adoption by the industry.

In this chapter, we present an overview of the emerging microbioreactor technologies, with the focus on nutrient feeding strategies. For more general reviews on microbioreactors or larger miniaturized bioreactors, we refer to Hemmerich et. al.[1], Hegab et. al.[2] and Schäpper et.al.[3]. We start by presenting different types of modified microtiter plate based platforms, which are commonly used for mutant screening in industrial biotechnology. Next, we review microfluidic chamber based platforms where microorganisms are immobilized in geometrical constrictions and grown in a dynamic and nutrient-controlled manner. Finally, droplet-based microfluidics has been discussed as a potential technology for performing (semi) continuous cultivation of microorganisms. We believe that these miniaturized fermentation platforms can play a drastic role in accelerating the bioprocess development by reducing gap between conditions imposed during screening phase and that inside the industrial scale bioreactor.

2.2. MICROBIOREACTORS FOR NUTRIENT-CONTROLLED CULTIVATION OF MICROORGANISMS

2.2.1. MICROTITER PLATE-BASED PLATFORMS

In the recent years, significant effort has been made to modify microtiter plates to enable cultivation of microorganisms in fed-batch mode with passive and active control over nutrients. One strategy to continuously supply glucose to cells in the wells is by producing glucose inside the wells through enzymatic conversion of starch by glucoamylase, both added to the culture medium at the start of cultivation[4, 5]. Different supply rates of glucose can be achieved by using different concentrations of the enzyme. A different strategy is to connect pairs of wells by a microchannel, with one well acting as a reservoir for nutrients, and the other as a cultivation chamber, see Figure.2.2.1(a). The use of a polyacrylamide gel inside the connecting microchannel enables the slow and steady supply of nutrients to the culture well, with the feed rate depending on the diffusional properties of the gel [6]. Another strategy for nutrient release into the culture chamber is by loading wells with a silicon elastomer that contains crystals of glucose, see Figure.2.2.1(b). In these fed-batch microtiter plates (FeedPlate), release of glucose from the elastomer into the culture well is driven by a difference in osmotic pressure (steps 1, 2,

and 3)[7, 10, 11]. Since the silicon elastomer at the bottom of the wells hinders optical monitoring during cultivation, Habicher and co-workers[8] designed glucose-releasing rings in order to make the wells accessible for online monitoring, see Figure.2.2.1(c). Apart from passive diffusion based release strategies, active supply strategies have been developed such as the microfluidic BioLector system by m2p-labs[9, 12, 13]. Figure.2.2.1(d) illustrates two wells of a microtiter plate that are connected through a microchannel, with the flow from the nutrient well to the culture well actively controlled through pneumatic actuation. Controlled dosage is achieved by first filling the pump chamber with nutrient solution through opening and closing parts of the microchannel with an inte-

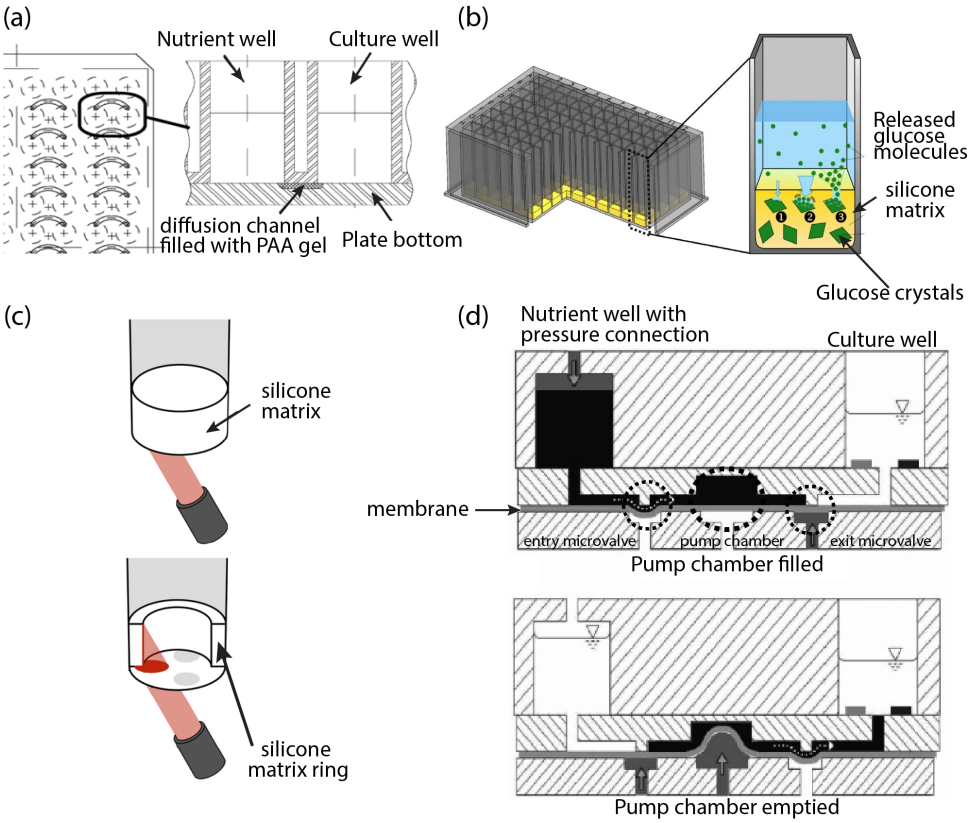

Figure 2.2.1: Different types of modifications made to conventional microtiter plates to enable fed-batch cultivation. (a) Fed-batch microtiter plate showing a pair of wells connected by a polyacrylamide gel filled microchannel. One well acts as a cultivation chamber and the other as a reservoir for nutrients, with the nutrient supply controlled by the diffusional properties of the gel. Adapted from [6]. (b) Fed-batch microtiter plate with a glucose containing silicone matrix at bottom of each well. Adapted from [7]. (c) Adaptation of the concept in (b) from a disc to a glucose releasing ring, enabling optical accessibility during cultivation. Adapted from [8]. (d) Fed-batch microtiter plate with the bottom replaced by a PDMS microfluidic chip that connects pairs of wells (microfluidic Biolector). Precise delivery of nutrients from the nutrient well to the culture well is achieved through pneumatic activation of membrane-based valves, which allows the pump chamber to be filled first, before the nutrient solution is pumped into the culture well. Adapted from [9].

grated micropump that inflates/deflates an elastic membrane, and subsequently emptying the pump chamber into the culture well. This technology allows fed-batch fermentations with a predetermined feeding profile[9]. Besides, it enables actively pH-controlled cultivations. A commercially available modified microtiter plate system (Micro-matrix) developed by Applikon Biotechnology (http://www.applikon-bio.com, Delft, the Netherlands) offers 24 parallel fed-batch fermentations with the possibility to feed nutrients whilst controlling parameters such pH, temperature and dissolved oxygen.

While different modifications to existing microtiter plate-based platforms have enabled potential options to carry out nutrient-controlled fed-batch cultivation experiments at microscale, there are several challenges that each of these platforms face before they are ready for adoption in biotechnological screening routines. Firstly, enzymatic glucose release based systems are strongly influenced by the operation parameters such as pH and temperature as the activity of the enzyme depends on them. Additionally, enzymatic release feeding can only be used for glucose controlled fed-batch cultivations. Diffusion based feeding mechanisms also depend on environmental factors such as media, pH, temperature and geometrical factors as in the case of PAA filled microchannel[6]. Establishing nutrient feeding profiles could be difficult to implement in diffusion-based release strategies as little control can be achieved in the release rate of glucose through the silicone matrices on the bottom of the plate or through the disks floating inside the microwells. Additionally, multiple glucose crystals can be released at once due to the osmotic pressure difference, leading to random dynamics of the glucose concentration. Finally, embedding microfluidic channels at the bottom of the standard microtiter plate as in the case of Microfluidic BioLector[9], could pose difficulties in fabrication. The combination of Microtiter plates and microfluidic channels, thereby replacing the base of the plate with embedded micro-pumps and valves, increases the complexity of the system. Additionally, systems like the Microfluidic Biolector offer a maximum of 44 to 48 fed-batch experiments per plate, which could be treated as relatively low-throughput in screening routines.While some of the modified microtiter plates discussed above integrate microfluidic channels, microbioreactors constructed as complete microfluidic devices present an interesting alternative and are discussed next.

2.2.2. MICROFLUIDIC CHAMBER-BASED PLATFORMS

One of the first microfluidic bioreactors for carrying out nutrient-controlled cultivation was developed by Balagaddé and co-workers[14]. The design of one of the six microfluidic circuits integrated on a single chip is shown in Figure.2.2.2(a). Cells are continuously circulated in the growth chamber (loop) through the use of an integrated peristaltic pump constructed from pneumatically actuated membrane valves, also known as Quake valves. A solution of nutrients is periodically pumped into the growth chamber by opening and closing parts of the circuit using such valves, while effluent is removed from the growth chamber. This allows continuous long-term cultivation of cells under nutrient-controlled conditions, with the cell growth rate directly controlled through the dilution rate, i.e. the rate at which the chamber volume is refreshed by the nutrient solution. An alternative strategy that does not require the integration of peristaltic pumps to supply nutrients and induce mixing was presented by Jensen[15]. They developed a microbioreactor with an external syringe pump for nutrient supply, while mixing in the

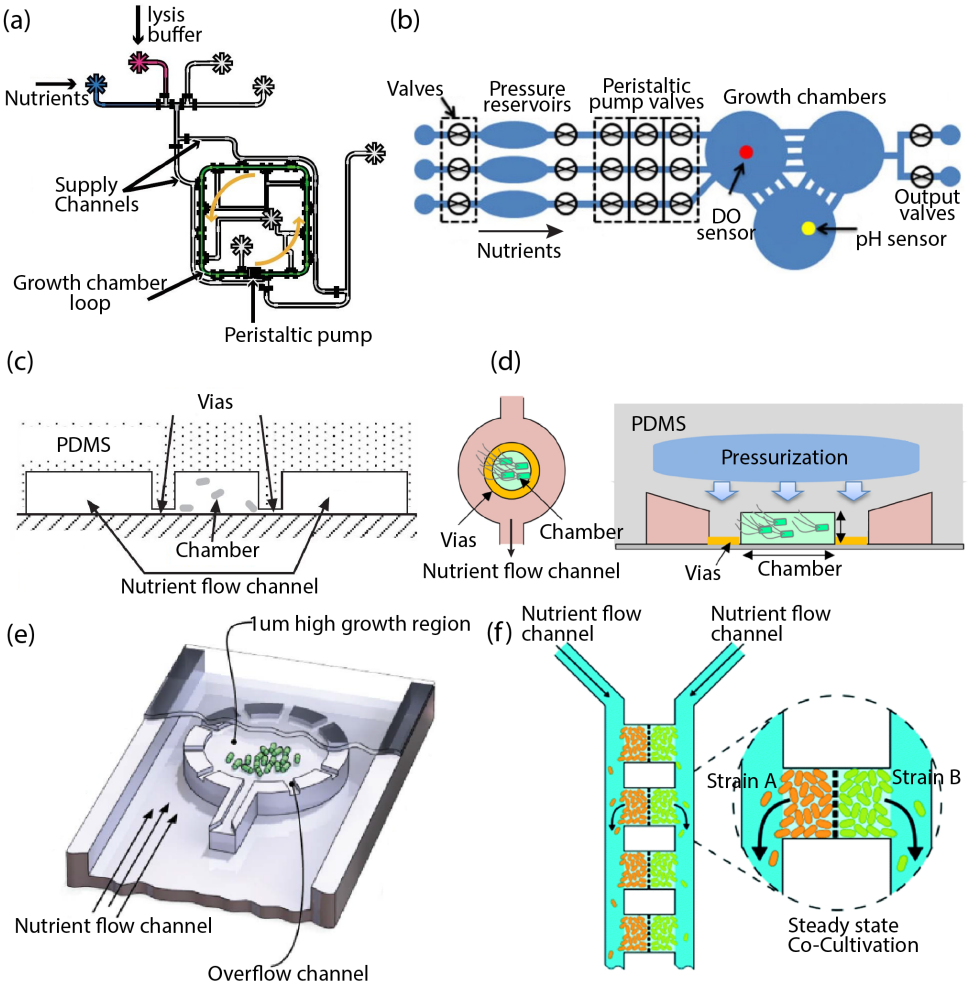

Figure 2.2.2: Microfluidic chamber-based strategies for nutrient-controlled microbial cultures. (a) Microfluidic chemostat in which cells are cultured in a growth chamber loop, with the cell suspension circulating using an integrated peristaltic pump. Nutrient solution is periodically supplied to growth chamber loop, while effluent is removed, through opening and closing of on-chip Quake valves (black lines). Occasionally, lysis buffer is pumped into segments of the growth chamber to remove cells that adhere to the walls. Adapted from [14]. (b) Fed-batch microbioreactor with cells cultured in growth chambers and nutrients regularly supplied by pumping nutrient solution to the growth chambers through pneumatic actuation of the valves of the integrated peristaltic pumps. Dissolved oxygen and pH were measured online by the installed sensors. Adapted from [16] (c) Microfluidic chemostat in which cells are cultured in chambers, with nutrients supplied from the surrounding nutrient flow channels by diffusion through the shallow vias. The cells can be trapped in and released from the chambers by inflating the channels in the elastomeric device (PDMS) through an increase in pressure. Adapted from [17] (d) Microfluidic chemostat with a similar working principle as in (c), but with the number of cells trapped inside the chambers automatically diluted by in/deflating the vias through de/repressurizing the channel above the growth chambers. Adapted from [18]. (e) Microfluidic chemostat in which cells are cultured in a flow-through chamber, with the nutrient-supply being controlled through the nutrient concentration. Once the chamber is populated, cells exit through the overflow channel, allowing non-restricted continuous growth. Adapted from [19]. (f) Microfluidic chemostat based a on similar principle as in (e), allowing studies on co-cultures. Adapted from [20].

culture chamber was achieved by a ringed magnetic stir needle. We note that the obtained mode of operation in the above two examples, with cells being studied under so-called chemostatic conditions, is commonly used in biotechnology to characterize cell physiology and study product formation under constant conditions and at different imposed growth rates. This mode of operation differs from the earlier discussed mode that allows cells to be studied under fed-batch conditions, with nutrient solution added but no effluent removed, leading to an increase in cultivation volume until the cultivation chamber is full and thus a finite cultivation time. An example of a microfluidic fed-batch bioreactor was presented by Bower and co-workers[16]. The device comprises of three independent input channels which are connected to growth chambers via pressurized fluid reservoirs, see Figure.2.2.2(b). The fed-batch process is achieved by partially filling up the chambers with cell solution, followed by the periodic supply of nutrients to the cells in the chambers through the actuation of the on-chip valves, until the maximum working volume is occupied and the fed-batch process is complete.

A different strategy that requires less advanced integrated micro-pumps and valves is to culture cells inside perfusable chambers. An example of such a device, developed by Groisman and co-workers[17], is shown in Figure.2.2.2(c). The chambers in which the cells are trapped are perfused by two surrounding nutrient supply channels. The shallow vias that connect the chambers to the supply channels ensure fluid to be exchanged, while cells remain trapped. The rate of diffusion of nutrients through these vias is much faster than the rate of nutrient consumption by the cells, such that the nutrient concentration in the chamber equals that in the supply channels, allowing direct control over nutrient-limited growth conditions. The use of an elastomeric material such as PDMS provides the means to load cells into the chambers, by injecting a cell solution into the device and subsequently pressurizing the device allowing cells to enter the chambers through the inflated vias. As cells remain trapped inside the chambers and do leave as effluent, this type of perfusable device can be seen as a retentostat, a special type of chemostat in which the cells are retained. A method that does allow continuous regulation of the number of cells inside the chambers during cultivation is one that enables controlled inflation of the vias. This can be achieved by depressurizing a separate channel above the chambers, see Figure.2.2.2(d). Automatic dilution of cells is then achieved using a feedback loop, with the pneumatic actuation controlled based on online measurements of the number of cells in the chambers [18, 21]. While the primary feed strategy in the above two examples is based on diffusion, flow-through chambers have also been developed. Cells are trapped inside shallow chambers located in a main channel, while nutrient solution is flown around and through the chamber, as illustrated in Figure.2.2.2(e). Nutrient-limited growth conditions are primarily controlled through the concentration of nutrient solution, supplied using an external pump. The shallow nature of the chambers facilitates cells to remain trapped and to grow in a two-dimensional fashion, enabling accurate monitoring at single cell resolution. Besides in- and outflow, the perforations in the chambers also allow cells to leave the chambers once they are populated. One of the first types of devices developed by Grunberger and co-workers enabled monitoring the growth of *E.coli* and *C. glutamicum* under dynamically controlled conditions[19]. Similar type of strategies even enable studies on co-cultures of cells, see Figure.2.2.2(f)[20]. For extensive reviews on applying chamber-based mi-

crofluidic devices for studying morphology, heterogeneity, growth and communication of microorganisms in a high-throughput manner and at single-cell resolution, we refer to [22–24].

Microfluidic chamber based devices offer a great platform for performing nutrient-controlled cultivation of microorganisms. However, there are number of limitations these systems face. Due to the presence of a solid wall, the chambers are susceptible to formation of biofilm. Secondly, since the chamber based micro-bioreactors operate in single phase and every chamber is not isolated from each other, cross-contamination of nutrients and microorganisms could occur across different chambers which could hamper the experiment. Due to lack of complete compartmentalization and cross-talk between cultivation chambers, quantification of secreted product during screening routines also becomes difficult. A challenge while operating the microfluidic device is that, large number of inlet ports are typically required as compared to number of experiments that could be performed on that device. This could lead to increased complexity of the device on increasing the number of experiments per chip. Since, microfluidic chamber based platforms contain solid walls to separate every cultivation experiment, it is difficult to accomodate increasing reactor volumes as in the case of semi-continuous cultivations. Increasing the microreactor volume becomes much easier with droplets as compared to solid chambers. Droplet based microfluidics can facilitate nutrient-controlled growth of microorganisms where the nutrient feeding strategy could be coalescing smaller nutrient droplets to the cell-containing droplets during cultivation. This platform is discussed in the next section.

2.2.3. MICROFLUIDIC DROPLET-BASED PLATFORMS

The potential to use droplets as cultivation environments has been outlined decades ago[34]. The precise generation and control of droplets in microfluidic devices led to the development of droplet-based microbioreactors. Most efforts so far focus on batch processes, with all components encapsulated at the start. The typical workflow then comprises the generation of millions of droplets with cells and nutrients encapsulated, incubation of the droplets off-chip, and reinjection in a separate chip to analyse and sort the droplets [35–37]. While providing a high throughput, this type of workflow with off-chip incubation makes it cumbersome to periodically supply nutrients to all individual droplets[38]. An alternative workflow that may facilitate periodic supply of nutrients is based on on-chip (or in-tube) incubation. While operations on droplets can be precisely performed when operating microfluidic devices under steady state conditions, the inherent non-steady nature of such microbioreactors with different droplet operations to enable regular nutrient supply nevertheless makes their development an outstanding challenge.

Jakiela and co-workers[25] presented the first example of a droplet-based chemostat by addressing this challenge through the development of a device that comprises different circuits that can be isolated from each other with the use of off-chip solenoid valves. Cells were encapsulated inside droplets, which were moved back and forth in the main channel of the device. Each droplet regularly entered a circuit in which effluent was removed from the droplets through controlled break-up, as illustrated in the top of Figure.2.2.3(a). The resulting droplets were supplied with nutrients in another circuit in

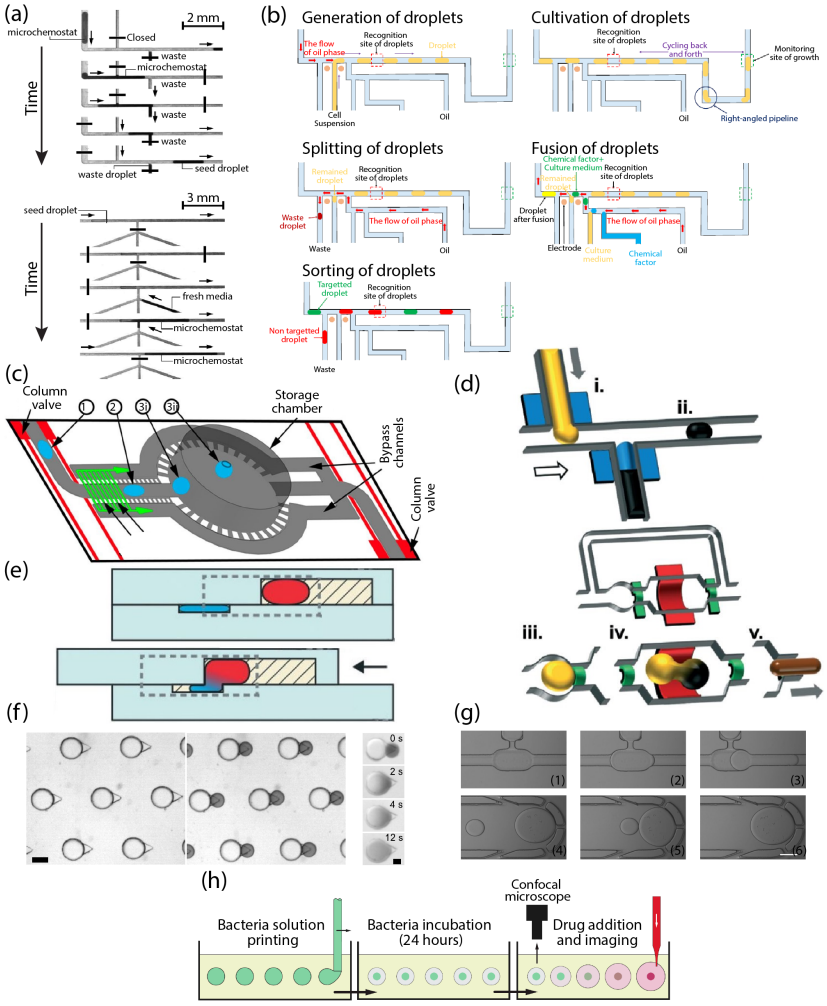

Figure 2.2.3: Droplet-based microfluidic strategies that could be used for nutrient-controlled microbial cultures. (a) Oscillating trains of microdroplet chemostats with *E.coli* colonies growing inside them. After every incubation cycle, each microchemostat droplet is broken up into a seed and a waste droplet, after which a fresh nutrient droplet is coalesced with the seed droplet, mimicking a traditional chemostat. Adapted from [25]. (b) Similar type of approach to study cells under chemostatic conditions, with an addition sorting step. Adapted from [26]. (c) Schematic of one of the chambers in a multiplex device, in which a cell-containing droplet is immobilized and supplied with nutrient-containing droplets to enable cell studies under fed-batch conditions. Adapted from [27] (d) Formation of cell-containing and nutrient-containing droplets through the use of T-junctions controlled through integrated pneumatic valves (top). Chamber for the immobilization of droplets (middle), opened and closed through actuation of integrated pneumatic valves (middle). Strategy to immobilize a cell-containing droplet in a chamber and merge it with a nutrient-containing droplet (bottom). Adapted from [28] (e) Microfluidic SlipChip device illustrating loading of the two droplets in the wells (top). On moving the upper plate with respect to the lower plate the droplets are merged (bottom). Adapted from [29] (f) Passive droplet trapping and coalescence within surface energy wells grooved on the floor of the microfluidic channel. Adapted from [30] (g) Fed-batch droplet-based nanobioreactor comprising of droplet-on-demand junctions (top) for the controlled supply of nutrient droplets to a cell-containing droplet immobilized in a trap (bottom). Adapted from [31, 32]. (h) Channel-free approach in which cell-containing droplets are bio-printed in a yield stress fluids. This approach has the potential subsequently supply reagents (e.g. drugs/nutrients) to the cell-containing droplets and study cells under controlled conditions. Adapted from [33].

which they were coalesced with nutrient droplets that were generated on demand, as illustrated in the bottom of Figure.2.2.3(a). More recently, Jian and co-workers[26] developed a similar automated droplet-based chemostat, including a sorting step based on monitoring biomass growth via OD measurements (see Figure.2.2.3(b)). An alternative to guiding cell-containing droplets to a nutrient-delivery location is to spatially immobilize the cell-containing droplets and supply them with nutrients. One of the first examples of such a strategy was by presented by Leung and co-workers[27]. The device comprises 95 chambers, which can all be individually addressed through the pneumatic actuation of integrated Quake valves. After loading a cell-containing droplet inside each chamber of this multiplex device, nutrient droplets can be generated on demand and guided to the chambers as illustrated in Figure.2.2.3(c), enabling studies under fed-batch conditions. A similar strategy based on immobilization of droplets in chambers and controlled supply of reagents through pneumatic actuation of integrated valves has used by the group of Chang-Soo Lee[28, 39, 40] for the cultivation of cells and the development of bio-chemical assays, see Figure.2.2.3(d)).

The above examples show a trend towards enhanced control over cultivation environment by developing sophisticated devices with integrated/external values, operated through multi-step actuation schemes. There is also another, almost opposite, trend visible in literature, in which control is achieved through the use (passive) geometrical features in order to keep device architecture and operation as simple as possible. An elegant example was demonstrated by Ismagilov and co-workers[29, 41], who compartmentalized droplets inside chambers, which can be joined by manually sliding the top and bottom half of these so-called *SlipChips* towards each other, as illustrated in Figure.2.2.3(e). Baroud and co-workers[30] explored the use of cavities in the floor/ceiling of a microchannel in which droplets squeezed between the floor and ceiling can relax their shape, and thereby remain trapped. These cavities can be designed such that each allows immobilization of a cell-containing droplet, while leaving room for another droplet to be loaded for controlled supply, as illustrated in the left panel of Figure.2.2.3(f). After loading those droplets (middle panel), supply is achieved by imposing coalescence (right panel). This is done by flowing a solvent through the channel in which the surfactant used for stabilization of the interfaces is less soluble. This strategy so far has been used for drug toxicity studies of cells with a single delivery (of drugs)[30]. Whether it can be used for repeated supply of nutrients to enable nutrient-controlled growth experiments under fed-batch conditions is yet to be explored. A recent example of a droplet-based microbioreactor does allow the repeated and controlled supply of nutrients to a cell-containing droplet immobilized in a chamber was demonstrated in the lab of the authors[31]. The design of the geometry in which the nutrient droplets are produced allows a robust periodic on-demand supply of droplets in a device free of valves, just through the use of a commercially available pressure pump[32], see Figure.2.2.3(g). The authors demonstrated the cultivation of cells under fed-batch conditions, with the growth rate of the cells inside the immobilized cell-containing droplet controlled by the concentration of the nutrient-containing droplets. Besides the droplet-based approaches in microfluidic channels, we conclude by highlighting a channel-free approach in which cell-containing droplets are bio-printed in a yield-stress fluid. Nelson and co-workers[33] developed a bioprinting method to study the response to drugs

injected into the droplets after 24h of incubation, see Figure.2.2.3(h). Since the printhead enables injection of nutrients or extraction of effluent, this relative unexplored strategy is also potentially interesting for high-throughput studies of microorganisms under nutrient-controlled growth conditions.

Even though droplet microfluidics offer an interesting platform for carrying out nutrient-controlled cultivation with feeding of nutrients, there are certain open challenges before which this can be readily adopted. One of the first challenges with the analytics and process control in microdroplet format. On-line monitoring and control of process parameters such as pH, dissolved oxygen and nutrient concentration can be difficult during the cultivation inside droplets. Quantifying concentrations of secreted metabolites of interest, which forms the heart of any screening routine, can be difficult to achieve in a non-invasive way. One way to achieve this is by using a fluorescent-based readout at the end of fed-batch cultivation. Further on, implementing control over dissolved oxygen and assuring that the cultivation does not run under oxygen limitations can be challenging in droplet based cultivation experiments. Fluorinated oils which often serve as the continuous phase can be used as oxygen source during the fed-batch cultivation[42]. A second challenge is to establish a simple and robust feeding strategy such that the technology can be easily adapted by non-experts[43]. As aforementioned, microfluidic droplet arrays are useful in carrying out non-steady state assays, in which nutrients can be added semi-continuously. However, these droplet arrays use multiple membrane based pneumatic valves which make the devices less robust, difficult to fabricate and possibly inhibit its easy adaptation by the biotechnology and bioprocess engineering community. The barrier of adoption of droplet microfluidic methods by non-experts can be reduced by embedding the complicated chip operation workflow and associated experimental paraphernalia in “chip-in a box” type of systems[44]. A third challenge in implementation of droplet-based microfluidic formats for long-term nutrient-limited fermentation is the possible leakage of molecules through the oil-water interface of the droplet[45, 46]. Leakage of nutrients or secreted metabolites could not just lead to uneven growth rates but also selection of false positives during fed-batch strain selection. A number of studies have been performed in order to investigate mass transport through the oil-water interphase where droplets are incubated inside PDMS devices. The nature of the molecules inside the droplets and the surfactants at the oil-water interphase are hypothesized as the most important parameters in understanding and controlling the leakage of molecules through microdroplets.

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3

DROPLET ON DEMAND GENERATOR

Scalable microfluidic droplet on-demand generator for non-steady operation of droplet-based assays

We developed a microfluidic droplet on-demand (DoD) generator that enables the production of droplets with a volume solely governed by the geometry of the generator for a range of operating conditions. The prime reason to develop this novel type of DoD generator is that its robustness in operation enables scale out and operation under non-steady conditions, which are both essential features for the further advancement of droplet-based assays. We first detail the working principle of the DoD generator and study the sensitivity of the volume of the generated droplets with respect to used fluids and control parameters. We next compare the performance of our DoD generator when scaled out to 8 parallel generators to the performance of a conventional DoD generator in which droplet volume is not geometry-controlled, showing its superior performance. Further scale out to 64 parallel DoD generator shows that all generators produce droplets with a volume between 91% and 105% of the predesigned volume. We conclude the chapter by presenting a simple droplet-based assay in which the DoD generator enables sequential supply of reagent droplets to a droplet stored in the device, illustrating its potential to be used in droplet-based assays for biochemical studies under non-steady operation.

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3.1. INTRODUCTION

Microscale droplets are being used as miniaturized reaction vessels for a variety of applications in the fields of biotechnology, chemical engineering, and material science. Some of these applications include high throughput screening of microorganisms[1–5], drugs[6], antibiotic toxicity[7] and combinatorial bio-chemical spaces[8, 9], while other applications use microdroplets to construct artificial cells[10, 11] or other advanced materials[12–15]. The development of microdroplet-based assays as an alternative to traditional assays is driven by the ongoing desire for massively parallel experimentation[16, 17], with an unprecedented throughput and a small sample volume as key enablers[2]. Over the past two decades, methods have been developed to produce[18–20], navigate[21], infuse[22], dilute[23], combine[24], split[25], store[26, 27], incubate[28] and sort[2] microdroplets. Notably, these operations have almost exclusively been developed for steady flow conditions. However, assays are inherently non-steady, as they typically require sequential execution of multiple of these operations. A common strategy to utilize these steady operations in a non-steady assay is to decouple them and execute them separately in a sequence, typically in separate dedicated devices, each one operated under steady flow conditions[29]. Although this decoupling strategy has proven to be powerful for large screens[3, 30], the number of operations possible during the screen is limited, often resorting to studying systems under batch conditions with all reagents present from the beginning, with end point measurements only. The application window of such droplet-based assays would hence significantly widen when developing an alternative strategy in which different operations can be integrated in the same device, operated in a non-continuous fashion. For example, a microdroplet-based assay that would be very powerful is one that enables sequential addition of reagents to droplets containing cells and studying their behaviour over time under (semi) continuous conditions. However, the required intermittent addition of reagents to droplets stored in the device presents an important hurdle. In fact, integrating long-term culturing in a controlled environment with technology accessible to non-experts is seen as one of the great outstanding challenges in the field[31]. While a few successful examples have been demonstrated that overcome the integration challenge using sophisticated devices with valves (integrated in the device or in the external connections to it)[8, 9, 32–35], the complexity of these devices and their use prevents their widespread adoption. In this paper, we address this hurdle by presenting a scalable droplet on-demand approach that enables the sequential delivery of reagent droplets of predefined volume to many locations in the assaying device at the same time using commercially available equipment.

Droplet on-demand (DoD) techniques in unbounded systems are well-established and find their widespread use, for example in the printing industry. One of the first DoD implementations in a microfluidic chip was provided by Attinger and co-workers[36]. While most developments so far focused on single on-demand generators[37–40], several scaled-out DoD approaches have been proposed[41, 42]. Guzowski and co-workers[41], for example, developed a scaled-out device, generating droplets on demand in 16 parallel channels, using just one continuous and one dispersed phase inlet, controlled by two external valves, with the volume of the droplets controlled by the duration the valves were opened. A foreseen challenge in adopting this approach in a more complicated device is that small difference in the hydrodynamic resistance across the device may lead

to differences in volume and timing of the generated droplets: a well-known problem in the scale out of multiphase microfluidic devices[43]. Similar to making continuous droplet generation methods more robust in operation through geometric design[44–49], we present an innovative design that enables the generation of droplets with a volume solely governed by the geometry for a range of operating conditions. This insensitivity to local conditions enables robust scale out and the generation of droplets of the same volume at the same time at different locations in the device using an on-demand change in pressures in the fluid reservoirs from which the device is fed. In order to facilitate adoption of the here presented DoD approach within the community, we used commercially available equipment and microfluidic devices fabricated using routine protocols.

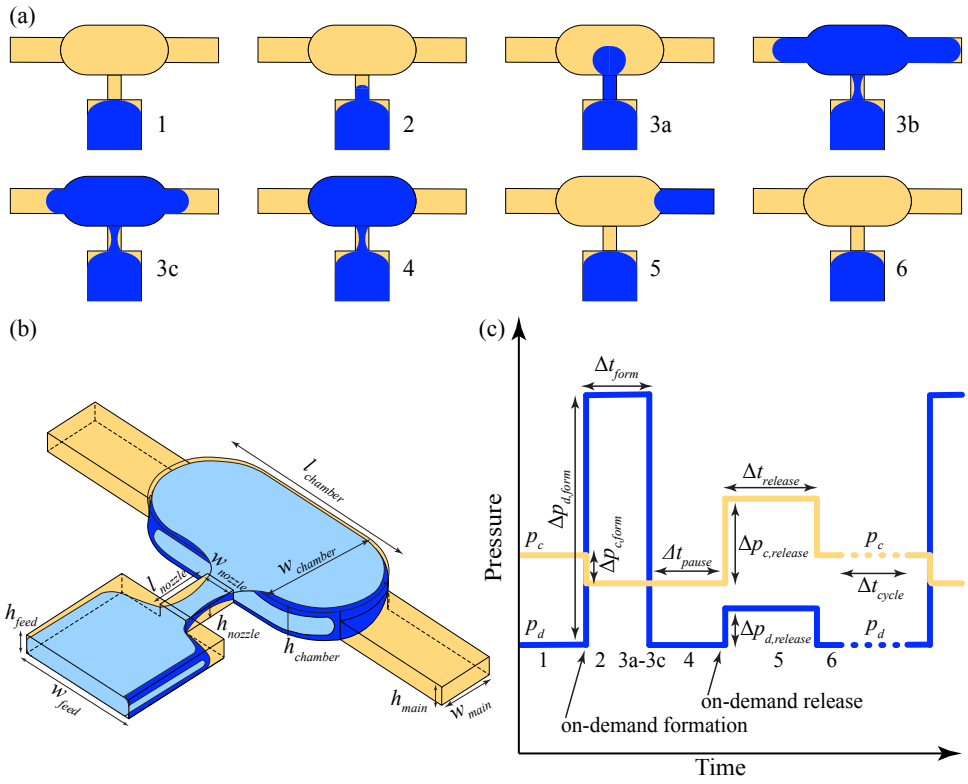

Figure 3.1.1: Droplet on-demand generator working principle and design. (a) Illustration of a DoD cycle, showing the dispersed phase steadily pressed against the nozzle (1), the dispersed phase filling the chamber (2-3c), the interfaces of the dispersed phase steadily pressed against the entrance of the main channel (4), the release of a droplet with a volume similar to the volume of the chamber into the main channel (5), after which the new interface is steadily pressed against the nozzle (6) ready for a new DoD cycle. (b) Design of the DoD generator, illustrating the constrictions in width and height, between the feed channel of the dispersed phase and the chamber, and between the chamber and the main chamber. These two Laplace pressure barriers enable decoupling the formation of a droplet from its release, such that the generated droplet volume solely depends on chamber volume for a range of operating conditions. (c) Pressure profiles used during a DoD cycle, showing that formation and release of a droplet are initiated through two separate pressure pulses.

The working principle of our droplet on-demand generator is illustrated in Figure.3.1.1(a), showing that the interface is steadily pressed against a nozzle (1) until, at will, the pressure in the fluid reservoir of the dispersed phase is temporarily elevated. This on-demand *formation pulse* pushes the interface through the nozzle (2) such that the dispersed phase starts filling the chamber (3a). From here two modes of operation are possible, depending on the used control parameters: either the dispersed phase steadily fills the chamber until full or it initially spills into the main channel (3b) and subsequently retracts back into the chamber (3c). Importantly, both these modes result in a filled chamber, with the interfaces steadily pressed against the entrance of the main channel (4). By elevating the pressure in the fluid reservoir of the continuous phase, the dispersed phase is pushed from the chamber into the main channel. This on-demand *release pulse* leads to pinch-off and the release of a droplet (5) with a volume similar to the volume of the chamber. After release, the pressures are adjusted to their initial values, the new interface being steadily pressed against the nozzle (6), ready for the next DoD cycle. Two innovative aspects of the here introduced two-step *formation and release* DoD approach are (1) the constrained growth of the droplet to a geometry-controlled (chamber) volume and (2) the decoupling between formation and release. While the use of chambers[46, 50] and the decoupling of formation and release based on flow modulation[41, 51–53] have been demonstrated before, it is the here introduced combination that makes the on-demand generation insensitive to the pressure up and downstream of the generator, enabling the robust scale out.

We start this chapter by presenting a proof-of-principle for single droplet on-demand generation, showing the robustness of the approach, with regard to operating conditions and used fluids. We next demonstrate the scalability of this approach by implementing the DoD design in 8 parallel channels, illustrating the importance of the chamber by comparing parallel DoD devices with and without chambers. Next, we demonstrate further scale out to 64 parallel channels, fed from just two fluid reservoirs, one for the continuous and one for the dispersed phase, through the use of a single (commercially available) pressure pump. We conclude this chapter with an outlook on the use of the DoD generator in droplet-based assays, by demonstrating sequential feeding of reagent droplets to a droplet stored on a chip.

3.2. MATERIALS AND METHODS

3.2.1. DEVICE DESIGN

The three-dimensional design of the microfluidic DoD generator is shown in Figure.3.1.1(b), with two key features being the nozzle and the chamber. Firstly, the constriction between the feed and the chamber (i.e. nozzle) enables one to (1) steadily press the interface against the nozzle and initiate *formation* when raising the pressure above the Laplace pressure associated with the difference in height and width between feed and nozzle. Secondly, the constriction between chamber and main channel (2) facilitates confinement of the growth of the dispersed phase to the chamber and its subsequent *release* from the chamber into the main channel, again by changing the pressure in accordance with the Laplace pressure. Since these two Laplace pressure barriers act as valves[54], allowing the interface to only pass when the driving pressure (temporarily)

exceeds the (static) Laplace pressure associated to the barriers, we hypothesize that the volume of the dispersed phase in the chamber after the *formation pulse* does not depend on the actual values of the strength and duration of the pulse. Even when the dispersed phase initially spills into the main channel and halts within a few channel widths from the chamber, it is expected to retract back into the chamber. This is again understood by considering the static Laplace pressure: the curvature of the interface in the main channel is higher than that in the chamber, such that the disperse phase flows back. Similarly, we do not expect the volume of the droplet after the *release pulse* to depend on the exact settings of the pulse. In order to prevent unwanted formation or release of droplets due to pressure fluctuations present in the system, we constricted the channels not just in width, but also in height. The chamber, nozzle and main channel therefore have different heights, as shown in Figure.3.1.1(b).

All DoD junctions presented in this paper have the same dimensions. The main channel is 50 μm wide (w_{main}) and 35 μm high (h_{main}), the nozzle is 25 μm wide (w_{nozzle}), 25 μm high (h_{nozzle}) and 50 μm long (l_{nozzle}), and the feed of the dispersed phase is 100 μm wide (w_{feed}) and 35 μm high (h_{feed}). Of key importance are the dimensions of the chamber, because they impose the volume of the generated droplets. The chamber is 100 μm wide ($w_{chamber}$), 40 μm high ($h_{chamber}$), and 200 μm long ($l_{chamber}$) such that its volume is about V_{ch} = 0.71 nL. The chosen dimensions were based on initial tests performed in devices with different nozzle and chamber dimensions, with the ones with highly constricted nozzles and large chambers leading to premature droplet pinch-off, i.e. before the chamber was completely filled. The full design of the here used DoD generator is made available as an AutoCAD file in Section.3.A.

3.2.2. DEVICE OPERATION

A pressure flow controller (MFCS-4C 1000 mbar, Fluigent) was used to control the injection of the working fluids into the chips. The ports on this controller were connected to reservoirs containing the fluids using silicone tubing. PTFE tubing (0.5 mm ID, 1/16 inch OD, 60 cm in length, Kinesis) was used to connect the reservoirs to the tubing glued to the inlets of the chips with the use of PEEK connectors. Care was taken to use the same height difference between the outlet of the chips and the liquid levels in the reservoirs in order to have comparable contributions of the hydrostatic pressure between different sets of experiments. Relatively large reservoirs (15 ml Eppendorf tubes) were used to ensure a negligible change in liquid level over the course of an experiment.

The pressure pump was controlled using a custom made MATLAB script, which is provided in Section.3.B. In each set of experiments, we ran the script to carry out 50 DoD cycles with predefined pressure profile in an automated fashion. This profile is characterised by p_d, p_c, $\Delta p_{d,form}$, $\Delta p_{c,form}$, Δt_{form}, Δt_{pause}, $\Delta p_{d,release}$, $\Delta p_{c,release}$, $\Delta t_{release}$, and Δt_{cycle}, as defined in Figure.3.1.1(c). While the main working principle has been explained before, with an increase in the dispersed phase pressure ($\Delta p_{d,form}$) initiating formation and an increase in continuous phase pressure ($\Delta p_{c,release}$) initiating release, we did not yet explain the rationale behind the base line pressures p_d and p_c and the jumps $\Delta p_{c,form}$ and $\Delta p_{d,release}$. The base line pressure p_c is chosen in order to obtain a desired (background) flow rate of the continuous phase through the main channel when the device is running steadily between droplet generation events. As the chosen

value of p_c determines the local pressure in the chamber, the baseline pressure p_d is adjusted in accordance. In experiments, p_d was tuned manually in such a way that it resulted in the interface being steadily pressed against the nozzle. While the choice of p_c is not stringent, p_d should be chosen such that the interface neither gets pushed back in to the feed channel nor through the nozzle. In the experiments performed, this was relatively straightforward. To ensure that the continuous phase does not prematurely break the neck of the dispersed phase by pushing it forward into the chamber after the formation pulse, we reduced the background flow of the continuous phase using $\Delta p_{c,form}$ when initiating formation. When initiating release, an extra pressure of $\Delta p_{d,release}$ is added to the dispersed phase in order to avoid the dispersed phase being pushed back into the feed channel. With this in mind, we noticed in initial experiments that the window of operation is relatively large for the release stage. In the experiments, we hence focus on the sensitivity of the droplet volume with respect to the operating parameters associated with the formation stage.

3.2.3. DEVICE FABRICATION

The microfluidic devices were fabricated using standard soft lithographic methods[55]. To obtain channels with three different heights in the same device, we spun three layers of the negative photoresist SU-8 (micro resist technology GmbH) on top of each other on a 4 inch silicon wafer and exposed each layer to near UV (EVG 620, EV Group) through a separate mask. These masks were designed using AutoCAD 2015 (Autodesk) and printed on transparencies using a high resolution printer (CAD/Art (Oregon, USA)). The first mask featured all channels of the microfluidic device, while the channel connecting the feed of the dispersed phase to the chamber (i.e. the nozzle) was not included on the second mask in order to make a nozzle with a constriction in height. The third mask only contained the chamber in order to make it higher than all other channels. The designs of the three masks are made available in Section.3.A. Unless stated otherwise, microfluidic devices were constructed from a 25 μm thick first layer (SU-8 3025), a 10 μm thick second layer (SU-8 3005), and a 5 μm thick third layer (SU-8 3005). After spin-coating a layer, it was soft baked, exposed, and post baked, following the guidelines provided by the manufacturer. The resulting wafer was then developed with mr-Dev 600 (micro resist technology GmbH), washed with isopropyl alcohol, spin dried, hard baked at 150°C for 30 minutes, and slowly cooled down on a hot plate to avoid cracks in the SU-8 structures. Before using the wafer as a master for replica moulding in PDMS, its surface was silanized by exposing it to vapours of 1H,1H,2H,2H-perfluorooctyl- trichlorosilane in a depressurized desiccator.

PDMS devices were made by mixing 80 g of PDMS elastomer and 8 g of curing agent (Dow corning, Slygard 184 elastomer kit), degassing the mixture and pouring it over the master placed in a 5 inch petridish. The PDMS mixture was cured at 70°C for 3 hours in an oven, gently removed from the wafer, and cut to size. The inlets and outlets were punched with a 1.5 mm biopsy punch and the resulting PDMS moulds were washed with ethanol to remove dust and debris. PDMS moulds and PDMS spin coated glass slides (25 mm × 75 mm) were exposed to an oxygen plasma (Harrick, PDC-002) for 140 s at a pressure of 0.2-0.4 mbar and subsequently pressed together to bond them. The obtained microfluidic devices were then baked at 140°C for at least 4 hours to regain the

hydrophobicity of PDMS. Small pieces of PEEK tubing (0.02 inch ID,1/16 inch OD, 2 cm in length , IDEX Health and Science) were inserted into the inlets and glued tight.

3.2.4. WORKING FLUIDS

Unless stated otherwise, experiments were done with demi water as the dispersed phase and HFE-7500 (Novec, 3M) as the continuous phase, with 0.1 v/v % Picosurf-1 (Sphere fluidics) dissolved in HFE-7500 to stabilise the interfaces and ensure full wetting of the walls by the continuous phase. The fluid properties for this system at room temperature are: 1 mPa.s and 1.24 mPa.s for the viscosities of the dispersed and continuous phase, respectively, and 2.4 mN/m for the interfacial tension.

3.2.5. IMAGE ACQUISITION AND ANALYSIS

For each set of experiments, 50 DoD cycles were captured using a TIS Cam (DMK 33UJ003, The Imaging Source) mounted on an internally illuminated microscope (Axiovert S100, Zeiss). Images were captured through a combination of a 5× objective and a 0.63× mount objective. Images were acquired at a resolution of 3856 px × 500 px and at a frame rate of 1 fps. To avoid unnecessary acquisition of images in experiments with long cycle times, a custom-made MATLAB script was used to only acquire images during the DoD cycles, by sending a trigger signal to the camera right before the signal to initiate the formation pulse was sent to the pressure pump. In addition, a high speed camera (phantom V9, Vision Research) was used to capture the dynamics of the DoD formation process.

Images were processed to determine the length, L, of the droplets after they were fully released into the main channel. The length was subsequently used to determine the volume using $V = [h_{main} w_{main} - (4-\pi)(2/h_{main} + 2/w_{main})^{-2}](L - w_{main}/3)$ established in earlier work[56]. For each set of 50 DoD cycles, we calculated the average and standard deviation of the volume. We used the coefficient of variation (CoV), defined as the ratio between the standard deviation and the average, as a measure for the monodispersity.

3.3. RESULTS AND DISCUSSION

3.3.1. SINGLE DROPLET ON-DEMAND: ROBUSTNESS IN OPERATION

In order to test the robustness in operation of the on-demand droplet generation, we conducted experiments in which we varied the main control parameters. We start by detailing how the strength of the pressure pulse ($\Delta p_{d,form}$) and the duration of the pressure pulse (Δt_{form}) affect the sensitivity of the volume of the generated droplets and their monodispersity.

The effect of pulse strength is first detailed for the dynamics of the DoD process, before showing its influence on resulting droplet volume. Figure.3.3.1(a) illustrates the dynamics for different pulse strengths as series of three microscopy images, one during filling, one before release from the chamber, and one after release into the main channel. For the lower pulse strengths of 15 and 20 mbar, the dispersed phase fills the chamber until it is full, after which the dispersed phase steadily resides inside the chamber, until it is released. For larger pulse strengths, we observe that the dispersed phase initially over-

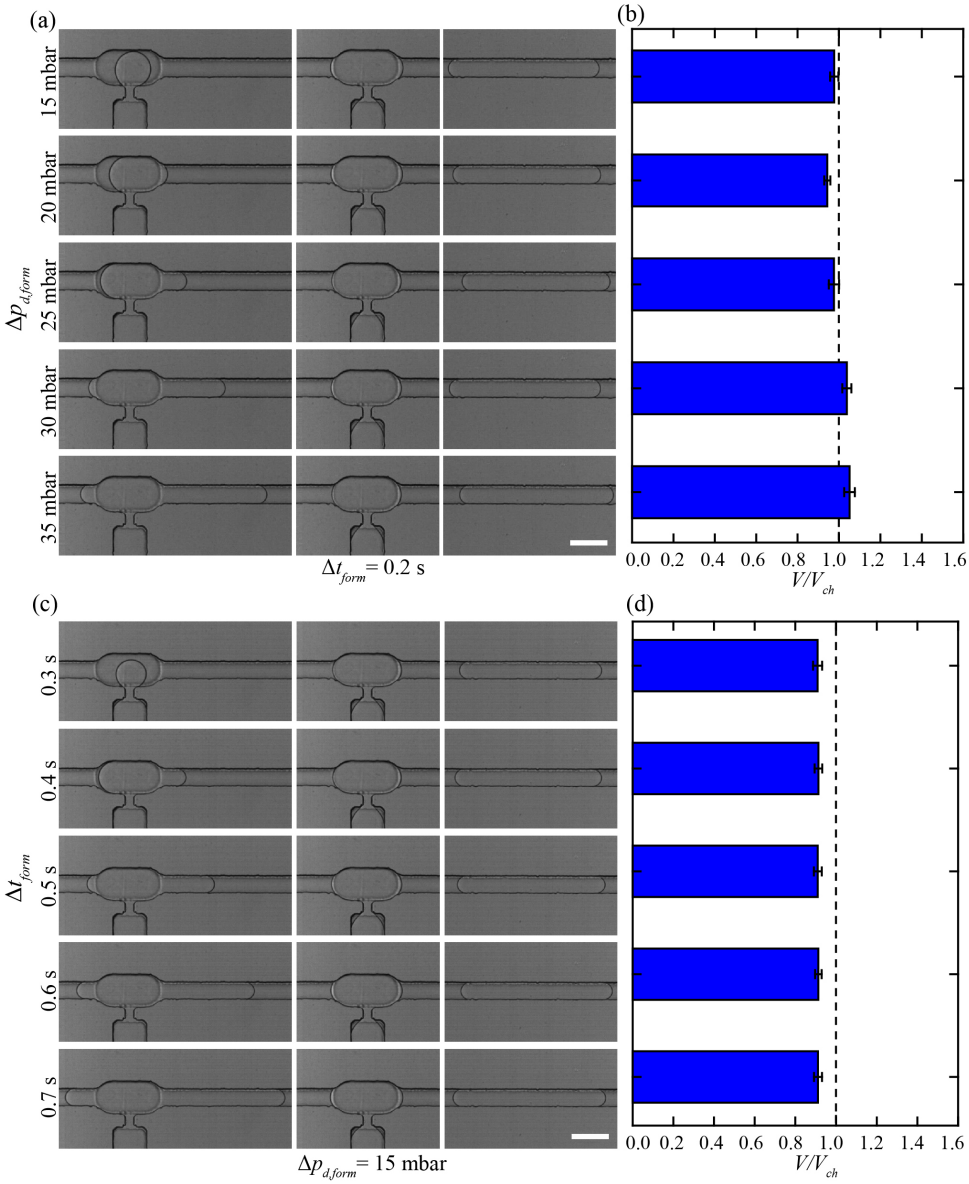

Figure 3.3.1: Characterization of the DoD generator, showing its robustness in operation with respect to two main control parameters. (a) Effect of pulse strength ($\Delta p_{d,form}$) on the dynamics of droplet generation, illustrated by micrographs taken during filling (left), before release (middle), and after release (right). Scan Figure.3.D.1 for the corresponding movie. (b) Resulting normalized droplet volume for 50 DoD cycles, with the error bars representing the standard deviation. (c) Effect of pulse duration (Δt_{form}) on the dynamics of droplet generation (scan Figure.3.D.2 for the corresponding movie) with the resulting normalized volumes in (d). Pressure profile: p_d = 22 mbar, p_c = 30 mbar, $\Delta p_{c,form}$ = 3 mbar for $\Delta p_{d,form}$ variation and p_d = 26 mbar, p_c = 36 mbar, $\Delta p_{c,form}$ = 2.8 mbar for Δt_{form} variation. The other parameters that define the pressure profile were kept constant at Δt_{pause} = 15 s, $\Delta p_{d,release}$= 5 mbar, $\Delta p_{c,release}$ = 12 mbar, $\Delta t_{release}$ = 5 s, Δt_{cycle} = 8 s. Scale bars in (a) and (c): 100 μm.

flows the chamber, spilling into the main channel, with larger pulse strengths leading to larger overshoots as evident from the left column in Figure.3.3.1(a). After this overshoot, the interfaces halt within a few channel widths from the chamber. At that instant, the dispersed phase pressure in the main channel is higher than in the chamber due to the curvature of the interfaces imposed by the main channel. As a result, the dispersed phase flows back into the chamber. Despite the overshoot for larger pulse strengths and the difference in dynamics for low and high pulse strengths, the formation pulse results in the same steady outcome: a chamber filled with the dispersed phase, from which a droplet can be released upon a second pulse.

For pulse strengths between 15 to 35 mbar, we find that the generated droplets have a volume (V) similar to the volume of the chamber (V_{ch}), as shown in Figure.3.3.1(b), with all corresponding CoVs below 0.03. Pulse strengths below 15 mbar were not sufficiently powerful to push the dispersed phase through the nozzle. This is in line with the order of magnitude of the (over) pressure required to push the interface quasi-statically through the nozzle, which is about 4 mbar as estimated from the Laplace law, $\gamma(2/w_{nozzle} + 2/h_{nozzle})$. Although not shown, the droplet formation process works in a similar fashion for pulse strengths of 40 and 45 mbar and device operation was insensitive to pulse strengths up to 45 mbar. For larger pulse strengths (at Δt_{form}= 0.2 s), we observed that the interface penetrated all the way to the exit of the downstream channel such that retraction back into the chamber did not occur.

The effect of the duration of the pulse (Δt_{form}) on the dynamics of DoD generation is illustrated in Figure.3.3.1(c). The behaviour is very similar to that observed for different pulse strengths; the chamber is gradually filled with the dispersed phase for short pulses, while an initial overshoot and subsequent retraction occurs for longer pulses. The resulting droplet volume is insensitive to the applied pulse duration for the here reported range of durations, see Figure.3.3.1(d), with corresponding CoVs below 0.03. Outside the reported range, the interface was not pushed through the nozzle for shorter pulses, while it overshot to the exit for longer pulses. We note that the droplet volumes in Figure.3.3.1(d) are systematically lower than the chamber volume. With the pre-formed droplets in the chamber similar in volume in Figure.3.3.1(a) and Figure.3.3.1(c), we expect that the lower value is caused by partial backflow of dispersed phase into the nozzle before complete pinch-off during release.

We conclude the experiments on pulse strength and pulse duration by returning to our original hypothesis that the volume of the dispersed phase in the chamber after the *formation pulse*, and the volume of the generated droplet after the *release pulse*, have a low sensitivity with respect to the actual values of the pressure pulses used, as long as the driving pressure is raised above the static Laplace pressure associated to the barriers for a sufficient amount of time. The data presented in Figure.3.3.1 confirms that (i) droplets are not generated for driving pressure below the static Laplace pressure, (ii) droplets are generated for higher pressures, with the performance of the generator, characterized in terms of droplet volume and variations therein, having a low sensitivity to the actual settings of the pressure pulse, for pulse strengths and pulse durations in the range between 15-45 mbar and 0.3-0.7 seconds. This low sensitivity enables different DoDs in a scaled-out device to produce similar sized droplets, even when the local pressures near the different DoDs are different.

To further substantiate the robustness in operation of the DoD generator, we studied the sensitivity of the volume of the generated droplets with respect to the time between two droplet formation cycles (Δt_{cycle}). The volume of the generated droplets is insensitive for the here studied range as shown in Figure.3.3.2. The CoV for Δt_{cycle} = 10 s and 60 s is 0.02, while it is 0.06 for Δt_{cycle}= 300 s. In this set, we observed slight differences in the location of the interfaces when pressed against the entrance of main channel in different DoD cycles, which may be caused by (long term) variations in the base line pressures.

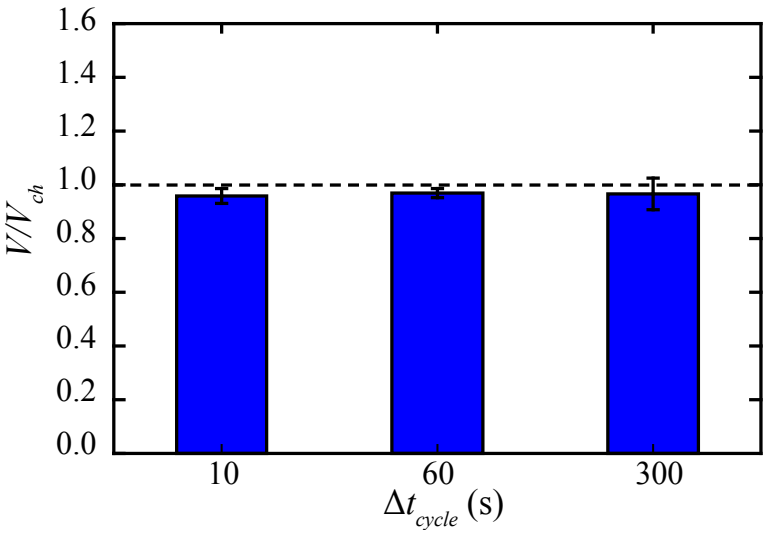

Figure 3.3.2: Effect of cycle time (Δt_{cycle}) on generated droplet volume and monodispersity. Pressure profile: p_d = 26 mbar, p_c = 52 mbar, $\Delta p_{d,form}$ = 20 mbar, $\Delta p_{c,form}$ = 3 mbar, Δt_{form} = 0.2 s, Δt_{pause} = 30 s, $\Delta p_{d,release}$= 5 mbar, $\Delta p_{c,release}$ = 12 mbar, $\Delta t_{release}$ = 5 s.

Before further characterizing the performance of the DoD generator, we comment on the feeding frequencies (or throughput) that can be achieved in the context of its intended use. One of the foreseen areas of its application is in bioprocess engineering, where it can be used for long term cultivation of cells under sequential-batch/fed-batch/semi-continuous conditions, with control over the produced metabolites through the controlled supply of nutrients. The required feeding frequency then depends on the desired growth rate of the cells. The simplest estimate of the order of magnitude of the required feeding frequency is obtained by considering a chemostat, for which the required volumetric flow rate of nutrients (F) solely depends on the volume in which the cells are cultured (V_r) and the desired cell growth rate (μ_{cell}) as $F = V_r\ \mu_{cell}$. Feeding nutrient droplets of volume V at a frequency f, the required frequency simply equals $f = (V_r/V)\mu_{cell}$. For typical nutrient-controlled growth rates of the order of 0.1 h^{-1} and nutrient volumes of the order of 10 - 100 times the cell culture volume, the expected feeding frequency is of the order of 1 - 10 droplets per hour. This is well in the range of feeding frequencies possible with the DoD generator, with its maximum generation frequency primarily depending on the time it takes for the interface to retract back from the main channel into the chamber after the formation pulse. This typically occurs within 5 - 10

seconds, such that we used 15 - 30 seconds of pause time (Δt_{pause}) in the experiments reported in Figure.3.3.1 and Figure.3.3.2 to ensure that the dispersed phase is steadily pressed against the entrance of the main channel before releasing it. Considering also the other durations in a DoD cycle ($\Delta t_{form} < 1$ s, $\Delta t_{release} \sim 5$ s, $\Delta t_{cycle} \sim 10$ - 300 s), the maximum generation frequency is about one droplet per minute.

3.3.2. SINGLE DROPLET ON-DEMAND: ROBUSTNESS IN USED FLUIDS

To test the versatility of the DoD generator in dispensing different types of aqueous fluids, we performed experiments in which we systematically changed the viscosity of the dispersed phase from 1 mPa.s to 220 mPa.s by adding glycerol (Sigma Aldrich) to demi water in different weight percentages (0-90%). While we kept all control parameters the same except for the one varied in the measurement series in Figure.3.3.1, we found that the strength ($\Delta p_{d,form}$) and duration (Δt_{form}) of the formation pulse, as well as the equilibrium pressures (p_c, p_d), needed adjustment for each viscosity. More specifically, a higher viscosity required a higher strength and/or duration of the pulse for it to be of sufficient power to push the interface through the nozzle. Additionally, higher viscosities did not show the overshoot, as observed for the experiment with demi-water, increasing the window of operation. Although different settings were needed for different viscosities (see Table.3.C.1), it is important to stress that, as before, a range of operating conditions could easily be identified in which droplet volume was insensitive to the operating conditions. Thus, the generated droplets have a volume closely resembling the volume of the chamber, with CoVs below 0.02, regardless of the dispersed phase viscosity, as shown in Figure.3.3.3(a).

A key aspect in the design of droplet-based microfluidic devices is ensuring that droplets, during and after their formation, are not in direct contact with the microchannel walls, as the resulting contact lines and hysteresis in their motion generally makes operation of the device amendable for control. To ensure full wetting of the microchannel walls by the continuous phase in the so far reported experiments, we used 0.1 v/v % Picosurf-1 as a surfactant. To test device operation under partially wetting conditions, we also performed one experiment without surfactant. The static contact angle as measured by dispensing a droplet of pure HFE-7500 on an untreated PDMS surface submerged in demi-water is 74°, while the interfacial tension between the fluids is about 48 mN/m. Although droplets were reasonably monodisperse (CoV below 0.06), the volume of the droplets was significantly smaller than the volume of the chamber, see Figure.3.3.3(b). Figure.3.3.3(c) illustrates the dynamics of the droplet release process under fully wetting and partially wetting conditions. Before the release pulse, the chamber is completely filled with the dispersed phase in both cases. As soon as the release pulse is applied we observe contact line pinning under partially wetting conditions, whereas the interface moves fluently under fully wetting conditions. This hysteric behaviour is in line with the higher required release pulse strength ($\Delta p_{c,release}$) = 25 mbar versus 10 mbar. Due to interface pinning near the exit of the chamber, the dispersed phase is partially pushed back into the nozzle before pinch-off occurs, resulting in droplets smaller than the chamber volume, see also the corresponding movie (scan Figure.3.D.3). This data set illustrates the importance to work under fully wetting conditions for an intended operation of the DoD generator. In case it is not possible to choose the combination of

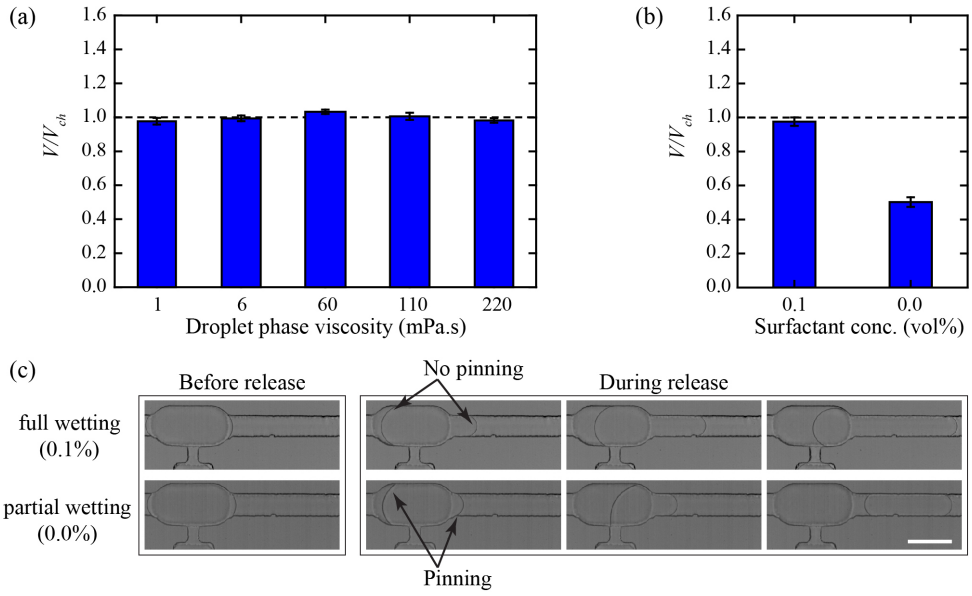

Figure 3.3.3: Characterization of the DoD generator, showing its robustness in operation with respect to use of different fluids. (a) Droplet volumes (average and standard deviation) for different viscosities of the dispersed phase. (b) Droplet volumes (pure demi-water) generated with oil with and without surfactant, showing the importance to ensure full wetting conditions for the DoD generator to operate as intended. (c) Corresponding time series, showing the dispersed phase is fluently pushed out the chamber for the fully wetting case, while contact line pinning causes the dispersed phase to be partially pushed back into the nozzle, resulting in droplets that are smaller than the chamber volume. Scan Figure.3.D.3 for the corresponding movie. Used pressure profile for full wetting case: $\Delta p_{d,form}$ = 40 mbar, Δt_{form} = 0.2 s. For rest of the settings see caption for Figure.3.3.1. Used pressure profile for partially wetting case: p_d = 54 mbar, p_c = 35 mbar, $\Delta p_{d,form}$ = 40 mbar, $\Delta p_{c,form}$ = 2 mbar, Δt_{form} = 0.2 s, Δt_{pause} = 15 s, $\Delta p_{d,release}$= 5 mbar, $\Delta p_{c,release}$ = 30 mbar, $\Delta t_{release}$ = 5 s, Δt_{cycle} = 8 s. Scale bar: 100 μm.

working fluids such that the continuous phase fully wets the walls, one may be able to modify the roughness and chemical nature of the microchannel walls to achieve this.

3.3.3. SCALING OUT DROPLET ON-DEMAND: IMPORTANCE OF DECOUPLING FORMATION AND RELEASE

After a thorough characterization of a single DoD generator, we demonstrate that its robustness in operation enables the scale out by presenting a device in which droplets are generated at 8 parallel generators, all fed from one fluid reservoir for the continuous phase and one for the dispersed phase through the use of a single pressure pump. To illustrate the importance of the here introduced decoupling strategy through inclusion of the chamber in the design, we also fabricated a device without chambers, see Figure.3.3.4(a). This device was fabricated using the same fabrication protocol, with omission of the third layer. The obtained data for 50 DoD cycles shows that the volume of the droplets closely resembles the volume of the chamber for the 8 DoD generators with chamber, with values of the monodispersity within 0.03. By contrast, large variations in

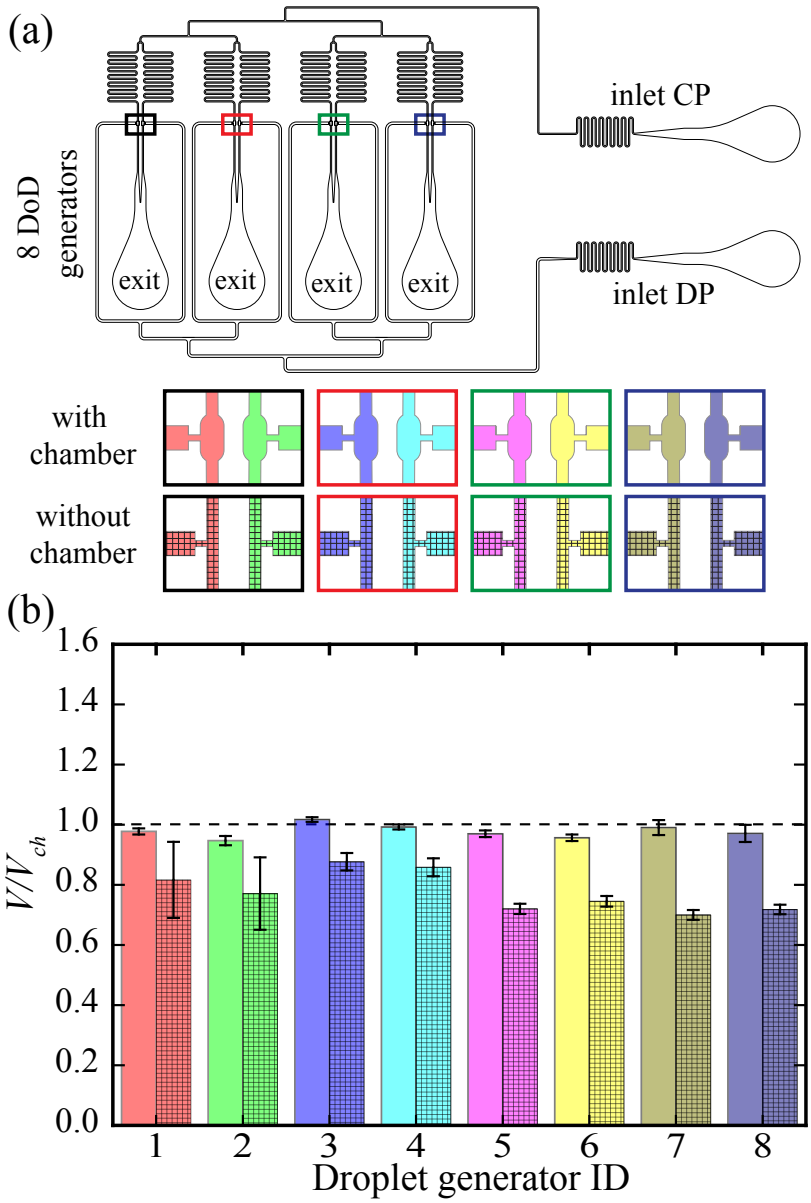

Figure 3.3.4: Scale out of the DoD generator. (a) Design of the device with 8 parallel DoD generators, all fed from one fluid reservoir for the continuous phase and one for the dispersed phase through the use of a single pressure pump. (b) Volume of 50 DoD cycles and their monodispersity (error bars), for a device with chambers (unhatched) and without chambers (hatched), demonstrating the importance of the chamber in the design. Used pressure profile for device with chambers: p_d = 28 mbar, p_c = 38 mbar, $\Delta p_{d,form}$ = 45 mbar, $\Delta p_{c,form}$ = 3.7 mbar, Δt_{form} = 0.4 s, Δt_{pause} = 30 s, $\Delta p_{d,release}$= 0 mbar, $\Delta p_{c,release}$ = 180 mbar, $\Delta t_{release}$ = 10 s, Δt_{cycle} = 8 s. Used pressure profile for device without chambers: same, except for p_d = 30 mbar, p_c = 39 mbar.

droplet size and monodispersity (CoVs up to 0.16) are seen for the device without chambers, see Figure.3.3.4(b). These differences in droplet volume are attributed to differences in volumetric flow rates arising from differences in the dimensions of the channels leading to the generators. This is well-known for the type of distributor used here [57], where the fluids are supplied to the droplet generators through a series of bifurcations of the inlet channels. An alternative to this tree-like distributor is a so-called ladder-like distributor [49, 58, 59], which is designed to have a negligible pressure drop over the distribution channels in comparison to the pressure drop over the droplet generators. This is achieved by feeding the phases to the generators through relatively large inlet channels, which requires a three-dimensional network of connecting channel. This can be achieved by adding a fluid distribution layer on top of the chip and may reduce the variations in droplet volume observed in the device without chambers, because variations in the volumetric flows to the different generators arising from fabrication tolerances may be smaller in ladder-like distributors than in tree-like distributors. Importantly, the here presented DoD generator (with chambers) can be parallelized using a tree- or ladder-like flow distributor, because the DoD generators produce droplets with a volume similar to the chamber volume, even when pressures and volumetric flows differ between the parallel DoD generators.

We envision this DoD approach to be used as a reagent/nutrient delivery tool in droplet-based assays for long-term experiments where tens to hundreds of experiments can be performed in parallel. To demonstrate the ability to further scale out the DoD generator, we fabricated a device with 64 parallel DoD generators by further branching out the feed channels for the device with 8 parallel DoD generators. Figure.3.3.5 illustrates the operation of this device based on four snapshots taken: before applying the first pressure pulse that initiates formation, immediately after this pulse, before applying the second pressure pulse that initiates release, and immediately after this pulse. Before the first pulse, interfaces in all 64 generators are steadily pressed against the nozzle as shown in Figure.3.3.5(a). While the dynamics of filling is clearly different for all generators (Figure.3.3.5(b)), the final result, a full chamber, is the same (Figure.3.3.5(c)). Despite differences in the speed with which the droplets are released from the chambers (evident from Figure.3.3.5(d)), the resulting droplet volume is similar to the volume of the chamber. More quantitatively, the volume of the droplets generated in a single DoD cycle were between 91% and 105% of the chamber volume for all 64 generators with a CoV of 0.03 for the 64 droplets. This CoV is comparable to that reported for common microfluidic droplet generation methods[40]. To identify whether the variation mainly occurs during formation or during release, we also determined the variation in the images before release (Figure.3.3.5(c)). The CoV (based on the area) is 0.02, which indicates that most of the variation exists already after filling the chamber. This may be further reduced by increasing the contrast between chamber and channel height.

Further scale out beyond 64 generators is certainly possible. One point of attention is the footprint of the chip, which in this work has not been optimized for large scale integration purposes. The footprint can be significantly reduced by incorporating all feed channels into a separate distribution layer. With the footprint of a single DoD generator and its downstream channel being about 10 mm^2, we expect that about 500 DoD generators can be comfortably fit onto a 4" wafer. A second point of attention is

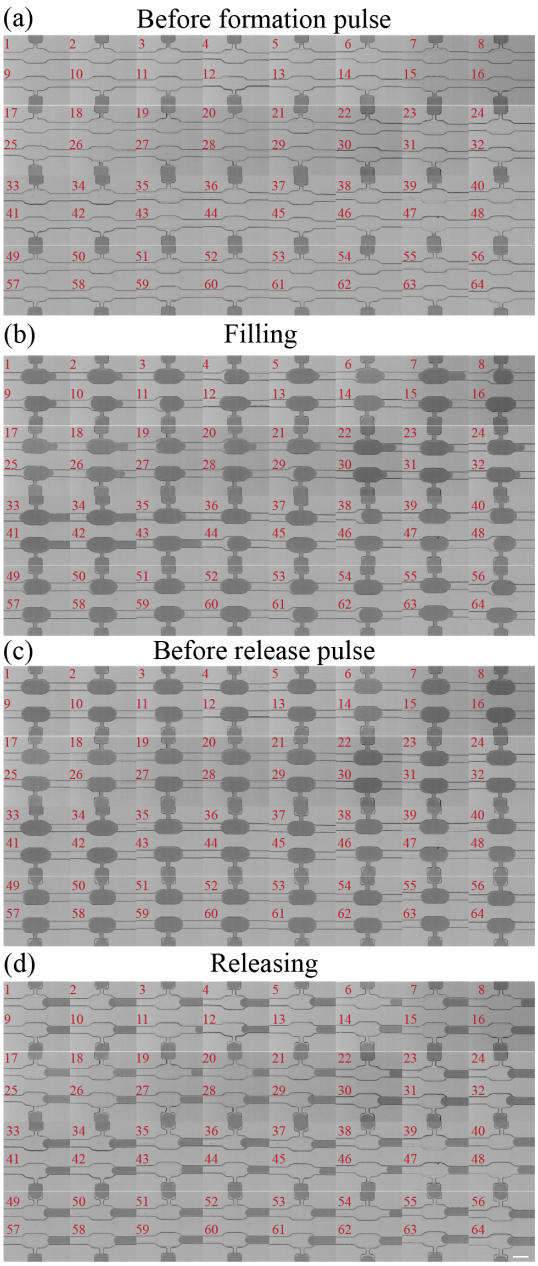

Figure 3.3.5: Scale out of the DoD generator to 64 parallel DoD generators on a single chip. Operation of the device is characterized using four microscopic snapshots taken: before applying the first pressure pulse that initiates formation of droplets (a), immediately after the formation pulse (b), before applying the second pressure pulse that initiates release of droplets (c), and immediately after the release pulse (d). Methylene blue was added to demi water to enhance the visibility of the droplets. Scan Figure.3.D.4 for corresponding movie. Pressure profile: p_d = 28 mbar, p_c = 130 mbar, $\Delta p_{d,form}$ = 160 mbar, $\Delta p_{c,form}$ = 85 mbar, Δt_{form} = 0.6 s, Δt_{pause}= 45 s, $\Delta p_{d,release}$ = 0 mbar, $\Delta p_{c,release}$= 800 mbar, $\Delta t_{release}$ = 15 s, Δt_{cycle}= 15 s. Scale bar: 100 μm.

the required operating pressure, which in the current design increases with the number of DoD generators. Redesigning the feed channels based on the design rules earlier developed[49, 58, 59] may significantly reduce this dependency.

3.3.4. APPLICATION OF DROPLET ON-DEMAND: REAGENT SUPPLY IN A DROPLET-BASED ASSAY

In order to show the possible use of our novel DoD generator as a tool to enable the intermittent supply of reagents/nutrients in droplet-based assays, we fabricated a device which combines creation, storage and time-lapsing of a droplet with the intermittent supply of reagents to this droplet. The device consists of two DoD generators in series and a cup shaped trap in the downstream channel in which a droplet can be stored. First a 'mother' droplet was created using the first DoD generator. As the volume of the chamber of this generator is much smaller than the volume of the trap, the mother droplet was generated by producing a series of demi-water droplets, collecting them in the trap, where they were merged. As the interfaces of the droplets were stabilized by surfactants, we coalescence them by temporarily flowing a poor solvent, perfluoro-octanol, around the trap. This solvent was delivered from a separate inlet and temporary injection was controlled by elevating the pressure in the feed reservoir using the same pressure pump as used to control the feed of the other fluids. After creation of the mother droplet, the second DoD generator was used for the on-demand generation of aqueous droplets containing methylene blue dye. Each formation and release cycle was followed by a temporal injection of perfluoro-octanol to induce coalescence between the incoming dye droplet and stored mother droplet. The grey scale micrographs of this experiment are shown in Figure.3.3.6(a) for a supply of 10 subsequent dye droplets. The images on the left show a high consistency in the volume of the incoming reagent droplets, while the images on the right show how the mother droplet increases in volume and dye concentration. For reagent droplets of size V_r and dye concentration c_r, the volume of the mother droplet increases from its initial volume V_{m0} to $V_m = V_{m0} + nV_r$, with n the number of added reagent droplets. The amount of dye added to the mother droplet increases as $nV_r c_r$, such that the dye concentration increases as $c_m = c_r nV_r / (V_{m0} + nV_r)$. To compare this simple relation with the experiments, we measured the intensity I of the mother droplet after addition of each reagent droplet and normalised it with the initial value (I_{max}, no addition of reagent droplets) and the final value (I_{min}, 10 added reagent droplets) according to $(I_{max} - I) / (I_{max} - I_{min})$. The experiments agree well with this simple model as shown in Figure.3.3.6(b).

The successful on-demand supply of reagents demonstrated in this relatively simple droplet-based assay shows the potential of the here developed DoD generator. Encapsulation of cells inside the mother droplet and using cell media instead of dye is a straightforward extension and opens the door to long term cell cultures under semi-continuous conditions as often encountered in biotechnology at industrial scale. The DoD approach developed in this work enables precise temporal control over bio-chemical processes studied inside droplets on chip, making it possible to initiate, sustain, or quench processes, while monitoring them for long periods of time.

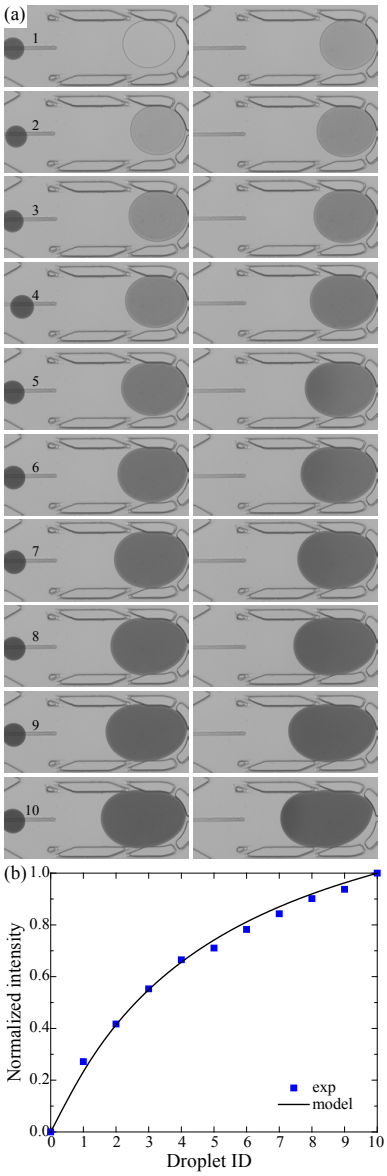

Figure 3.3.6: Illustration of the use of the DoD generator in a simple droplet-based assay. (a) Snapshots showing the periodic addition of reagent droplets (containing methylene blue dye) to a droplet stored in a trap on a chip, before arrival (left) and after induced coalescence (right). Scale bar: 100 μm. (b) Relative change in the intensity of the droplet stored in the trap with increasing number of fed reagent droplets.

3.4. CONCLUSIONS

We presented a droplet on-demand generator that allows one to produce droplets of pre-designed volume using a commercially available pressure pump. The innovative design

based on two Laplace pressure barriers enables droplets to first fill a chamber before releasing them into the main channel. This decoupling strategy ensures that the resulting volume of a droplet is dictated by the volume of the chamber and relatively insensitive to fluid properties for fully wetting systems. In addition, droplet volume is insensitive to operating conditions, which is of key importance for the scale out of DoD generators as the conditions near each generator may differ, for example due to fabrication tolerances. We demonstrated the superior performance of our DoD design in a scaled-out device with 8 DoD generators by comparing devices with and without chamber. We also demonstrated the further scale out to a device with 64 DoD generators, all producing droplets with a volume within 91% and 105% of the chamber volume with a CoV of 0.03. We developed this scalable DoD approach to be used in droplet-based assays. Such assays require the sequential supply of reagents/nutrients to droplets in which a process of interest is studied. We successfully demonstrated the use of the here developed DoD generator to intermittently feed reagent droplets to a droplet stored on chip, illustrating its potential as a tool to further advance the development of droplet-based assays.

APPENDIX

3.A. DRAWING OF MICROFLUIDIC DEVICES

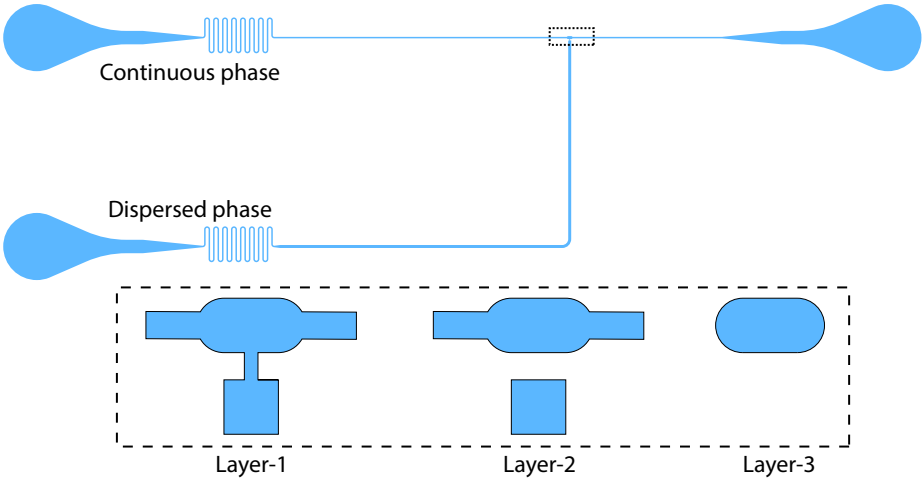

Figure 3.A.1: Drawing of microfluidic chip with a single droplet on-demand generator. Dotted black box show the features of the chip that end up on different layers.

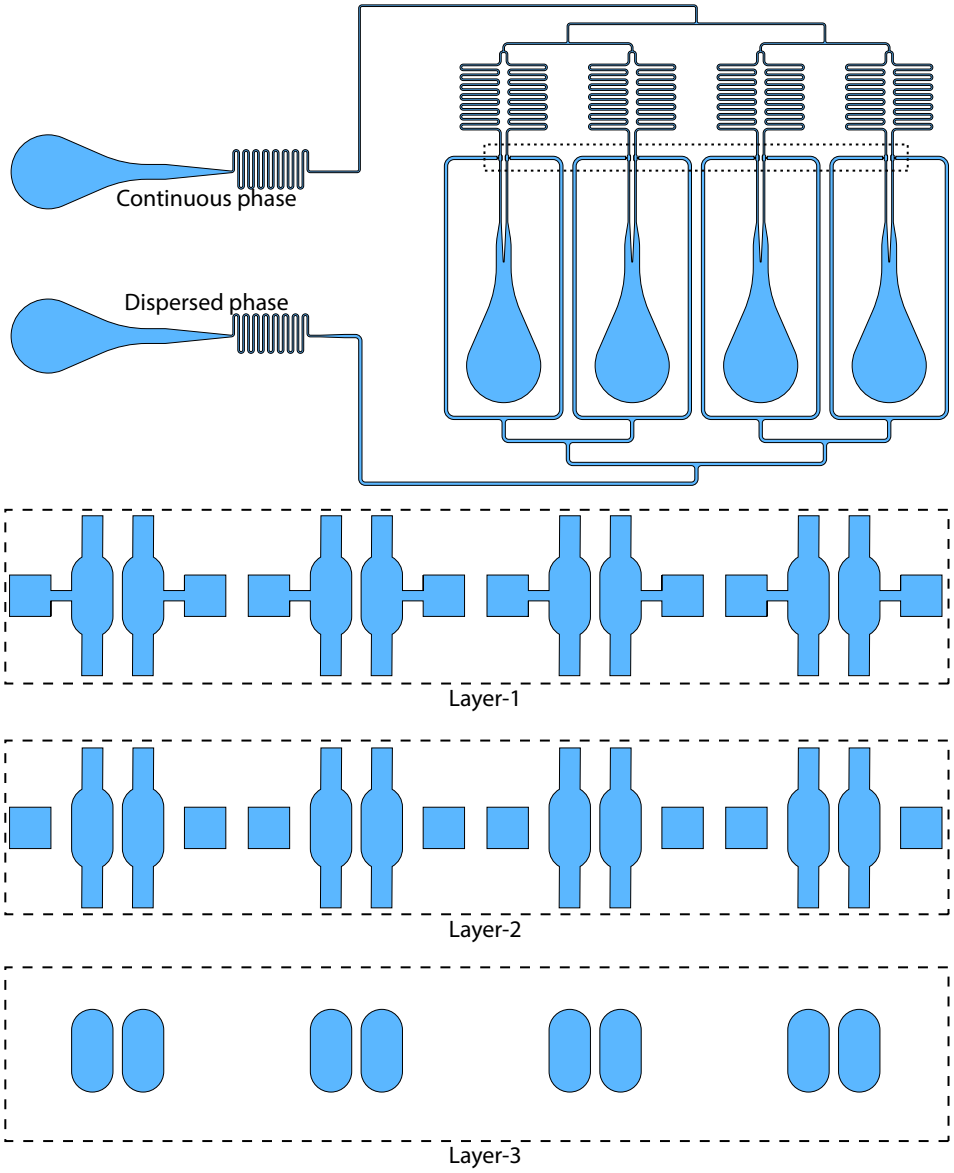

Figure 3.A.2: Drawing of microfluidic chip with a 8 parallel droplet on-demand generators with chambers. Dotted black boxes show the features of the chip that end up on different layers.

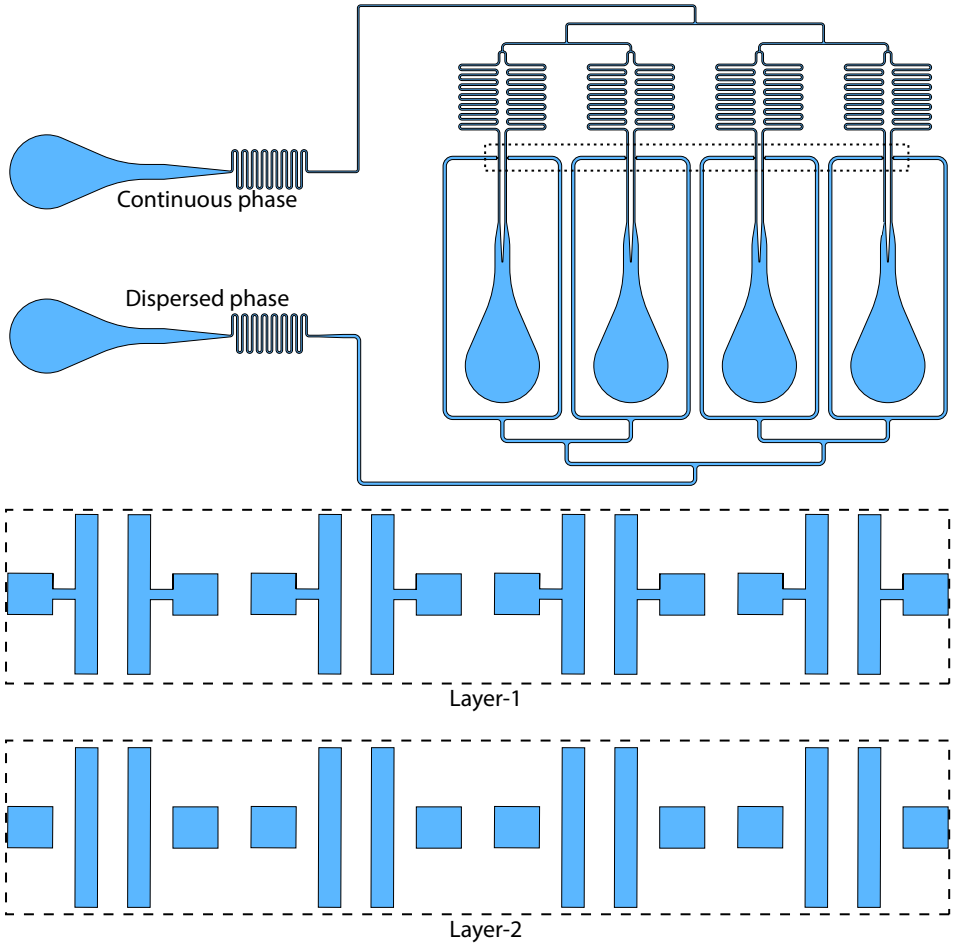

Figure 3.A.3: Drawing of microfluidic chip with a 8 parallel droplet on-demand generators without chambers. Dotted black boxes show the features of the chip that end up on different layers.

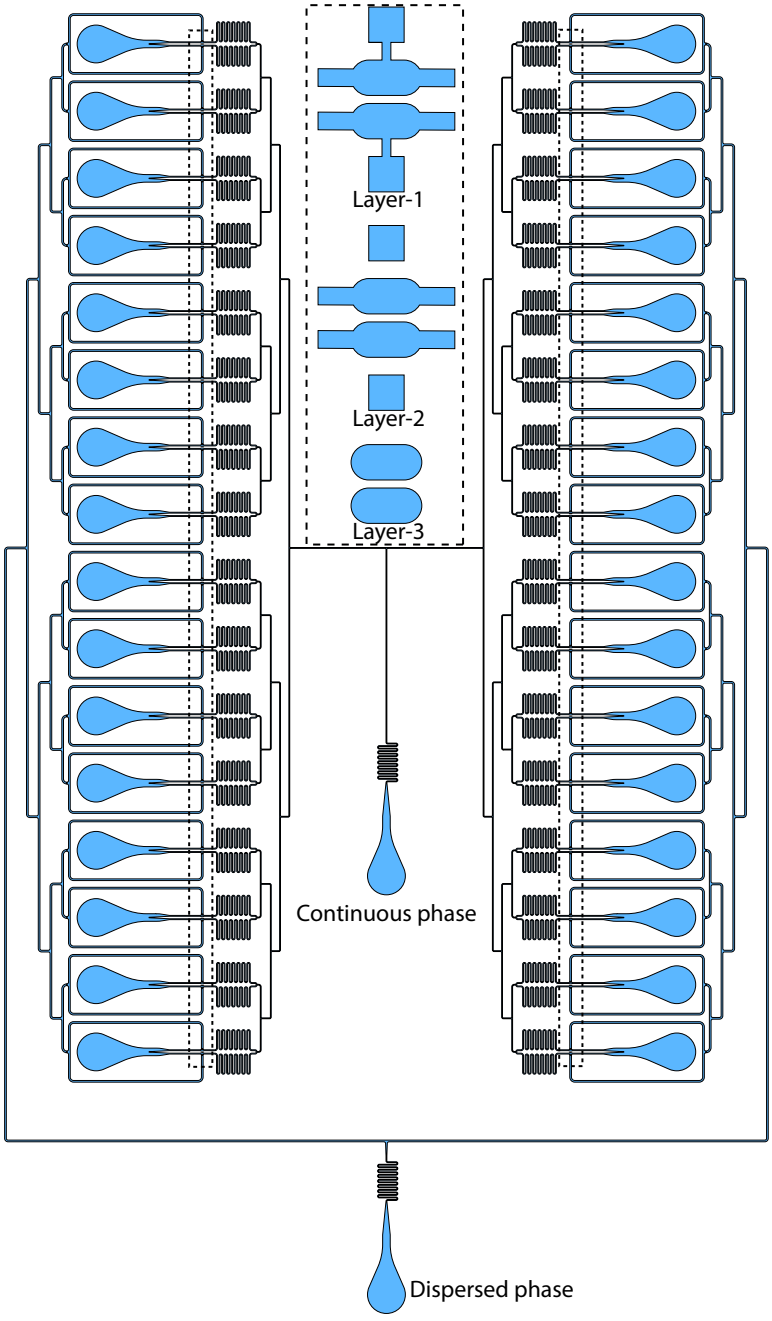

Figure 3.A.4: Drawing of microfluidic chip with a 64 parallel droplet on-demand generators. Dotted black boxes show the features of the chip that end up on different layers.

3.B. MATLAB SCRIPT FOR RUNNING A DOD EXPERIMENT

The following MATLAB script was used to run the droplet on-demand experiments in an automated fashion by controlling the pressure input to the pressure pump through MFCS:


```
[HandleNumber SerialNumber] = mfcs_init;
AlphaValue = 5; % this is for the PID, according to fluigent
                % this is already the optimal value and shouldnt be changed

fid = fopen( 'MatlabCrashReport.txt', 'wt' );

% Beginning of user defined variables
% Please adjust and change these to match a steady state condition in
% Maesflo(MFCS Fluigent)
p_c = 50;               % mbar; Equilibrium pressure for Continous phase
p_d = 26;               % mbar; Equilibrium pressure for Dispersed phase

deltap_d_form = 20;     % mbar; pulse strength for Dispersed phase
deltap_c_form = 3;      % mbar; pulse strength for Continuous phase
deltat_form = 0.2;      % seconds; pulse duration
deltat_pause = 16;      % seconds; Pause duration
deltap_c_release = 12;  % mbar; release pressure strength for CP
deltap_d_release = 5;   % mbar; release pressure strength for DP
deltat_release = 5;     % seconds; duration of transport of droplet
deltat_cycle = 60;      % seconds; Cycle time between two consecutive
                        % droplet formation cycles

zerotime = 4;           % seconds; ensureing a zero state before pulsing
                        % and before waiting for next DoD cycle

droplets = 50;          % Number of droplet on demand generation cycles
% End of user defined variables

%Beginning of the DoD script

for i = 1:droplets

    %STEP-1 : Initialization of the pump. Setting pressures of dispersed
    % and continuous phase at equilibrium pressure values.
    disp('Starting new droplet routine.');

    Chann = 1;
    PressureValue = p_c;
    mfcs_set_auto(HandleNumber,PressureValue,Chann,AlphaValue);

    Chann = 2;
    PressureValue = p_d;
    mfcs_set_auto(HandleNumber,PressureValue,Chann,AlphaValue);

    pause(zerotime);


    %Step-2 : This is beginning of the droplet creation process where the
    %dispersed phased is pulse to push the interphase in the nozzle
```

```
disp('Creating a droplet through pulse.');

Chann = 1;
PressureValue = p_c - deltap_c_form;
mfcs_set_auto(HandleNumber,PressureValue,Chann,AlphaValue);

Chann = 2;
PressureValue = p_d + deltap_d_form;
mfcs_set_auto(HandleNumber,PressureValue,Chann,AlphaValue);

pause(deltat_form);

%Step-3 : Pausing to enable complete filling of the chamber by the
%dispersed phase

disp('Pausing before releasing');

Chann = 1;
PressureValue = p_c - deltap_c_form;
mfcs_set_auto(HandleNumber,PressureValue,Chann,AlphaValue);

Chann = 2;
PressureValue = p_d;
mfcs_set_auto(HandleNumber,PressureValue,Chann,AlphaValue);

pause(deltat_pause);


%Step-4 : Pinchoff and transporting the droplet out of the device by
%elevating the pressures of continuous and dispersed phase

disp('Transporting droplet');
Chann = 1;
PressureValue = p_c + deltap_c_release;
mfcs_set_auto(HandleNumber,PressureValue,Chann,AlphaValue);

Chann = 2;
PressureValue = p_d + deltap_d_release;
mfcs_set_auto(HandleNumber,PressureValue,Chann,AlphaValue);

pause(deltat_release);

%Step-5 : Zeroing
disp('Zeroing 3');

Chann = 1;
PressureValue = p_c;
mfcs_set_auto(HandleNumber,PressureValue,Chann,AlphaValue);

Chann = 2;
PressureValue = p_d;
mfcs_set_auto(HandleNumber,PressureValue,Chann,AlphaValue);

pause(zerotime)

str=sprintf('Done with run %d', i);
disp(str);
```

```
    %Step-6 : Waiting between subsequent droplet on demand generation
    % cycles

    if i ~= droplets
        str=sprintf('waiting for %d seconds until new droplet', deltat_cycle);
        disp(str);

        Chann = 1;
        PressureValue = p_c;
        mfcs_set_auto(HandleNumber,PressureValue,Chann,AlphaValue);

        Chann = 2;
        PressureValue = p_d;
        mfcs_set_auto(HandleNumber,PressureValue,Chann,AlphaValue);

        pause(deltat_cycle);

    end

end  %End of the droplet on demand  generation routine

% Last Step : Again setting the pressures of the continuous and dispersed
% phase to the equilibrium pressures

disp('Final zeroing');

Chann = 1;
PressureValue = p_c;
mfcs_set_auto(HandleNumber,PressureValue,Chann,AlphaValue);

Chann = 2;
PressureValue = p_d;
mfcs_set_auto(HandleNumber,PressureValue,Chann,AlphaValue);

disp('Done with everything!');

mfcs_close(HandleNumber, 'CloseLib');
fclose(fid);
```

3.C. PRESSURE PROFILES FOR FIGURE.3.3.3(A)

Table 3.C.1: Pressure profiles for the viscosity variation experiments reported in Figure.3.3.3(a)

Glycerol (w/w%)	**Viscosity at 20°C (mPa.s)**	**Pressure profile**
0	1	p_d = 22 mbar, p_c = 30 mbar, $\Delta p_{d,form}$ = 15 mbar, $\Delta p_{c,form}$ = 3 mbar, Δt_{form} = 0.2 s, Δt_{pause} = 15 s, $\Delta p_{d,release}$= 5 mbar, $\Delta p_{c,release}$ = 12 mbar, $\Delta t_{release}$ = 5 s, Δt_{cycle} = 8 s.
50	6	p_d = 47 mbar, p_c = 37 mbar, $\Delta p_{d,form}$ = 35 mbar, $\Delta p_{c,form}$ = 2.3 mbar, Δt_{form} = 0.3 s, Δt_{pause} = 15 s, $\Delta p_{d,release}$= 5 mbar, $\Delta p_{c,release}$ = 12 mbar, $\Delta t_{release}$ = 5 s, Δt_{cycle} = 8 s.
80	60.1	p_d = 39 mbar, p_c = 38 mbar, $\Delta p_{d,form}$ = 110 mbar, $\Delta p_{c,form}$ = 3.7 mbar, Δt_{form} = 1 s, Δt_{pause} = 15 s, $\Delta p_{d,release}$= 5 mbar, $\Delta p_{c,release}$ = 18 mbar, $\Delta t_{release}$ = 10 s, Δt_{cycle} = 8 s.
85	109	p_d = 54 mbar, p_c = 37 mbar, $\Delta p_{d,form}$ = 55 mbar, $\Delta p_{c,form}$ = 3 mbar, Δt_{form} = 5 s, Δt_{pause} = 15 s, $\Delta p_{d,release}$= 5 mbar, $\Delta p_{c,release}$ = 18 mbar, $\Delta t_{release}$ = 10 s, Δt_{cycle} = 8 s.
90	219	p_d = 48 mbar, p_c = 37 mbar, $\Delta p_{d,form}$ = 60 mbar, $\Delta p_{c,form}$ = 1.8 mbar, Δt_{form} = 10 s, Δt_{pause} = 10 s, $\Delta p_{d,release}$= 5 mbar, $\Delta p_{c,release}$ = 18 mbar, $\Delta t_{release}$ = 10 s, Δt_{cycle} = 8 s.

3.D. DROPLET ON DEMAND MOVIES

Figure 3.D.1: Movie illustrating droplet on demand generation for variation of pulse strength reported in Figure.3.3.1(a). To see the movie scan the QR code or click here.

Figure 3.D.2: Movie illustrating droplet on demand generation for variation of pulse duration reported in Figure.3.3.1(c). To see the movie scan the QR code or click here.

Figure 3.D.3: Movie illustrating droplet on demand generation for partial wetting system reported in Figure.3.3.3(c). To see the movie scan the QR code or click here.

Figure 3.D.4: Movie illustrating scale out to 64 parallel droplet on-demand generators reported in Figure.3.3.5. To see the movie scan the QR code or click here.

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4

FED-BATCH DROPLET NANOBIOREACTOR

Fed-batch droplet nanobioreactor for controlled growth of Cyberlindnera (Pichia) jadinii: A proof-of-concept demonstration

We present a droplet-based nanobioreactor that enables studying microorganisms under nutrient-limited growth conditions through controlled supply of fresh nutrients over the course of a cultivation experiment. A key challenge addressed in this work is the implementation of the required non-steady droplet operations on chip to establish a semi-continuous nutrient supply, while keeping the chip and its operation as simple as possible. We start by detailing the working principle and demonstrating the ability to feed nutrients to a droplet immobilised on the chip at rates required for unconstrained exponential growth as well as constrained linear growth. We then demonstrate the ability to study microorganisms under nutrient-controlled growth conditions using the yeast Cyberlindnera (Pichia) jadinii as the model microorganism, with the cell growth rate controlled through the glucose concentration in the nutrient droplets. The obtained growth curves agree well with a simple kinetic cell growth model, which highlights its relevance in the design and interpretation of nutrient-controlled cell studies. Given the relative ease of operation and the large demand for controlled growth experiments, we expect that the presented droplet-based nanobioreactor provides a solid platform technology for further development and use in the field of bioprocess development and beyond.

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4.1. INTRODUCTION

Microorganisms such as yeast and bacteria have the ability to naturally convert raw feedstock into useful products but often in amounts insufficient for industrial scale production. Improvement of the microorganism's product yield on feedstock is possible by targeted genetic modification, which requires a solid understanding of the species metabolism and genetics. Alternatively, genetic modifications can be introduced randomly such that the chance that a resulting mutant performs better than the original one is small. Consequently, many mutants need to be created and subsequently screened in order to identify the best performing ones[1]. Given the large number of experiments, it is common practice to grow and study all the different mutants in individual microliter-sized wells on microtiter plates. While this allows for parallel screening in an automated fashion using pipetting robots and plate readers, conventional microtiter plates lack the ability to feed nutrients and control pH by base or acid addition. It is indeed challenging to feed liquids at flow rates in the nanoliter per hour range to all the individual wells of a microtiter plate and to do so accurately. Therefore, screening of mutants is commonly performed under batch conditions with all feedstock present from the start and no further control over feedstock concentration and pH. In contrast, more than 80 % of the processes in industrial biotechnology are operated under so called *fed-batch* conditions with control over feedstock concentration and pH[2, 3]. This incompatibility between the physiological conditions during screening and industrial operation not only leads to selection of false positives which fail to generate competitive yields at industrial scale, but also fails to identify the best mutants for fed-batch conditions[4–6]. The effectiveness of screening hence greatly benefits from a technology that enables growing and studying a large number of microorganisms under precisely controlled conditions representative of industrial bioreactors. Besides screening of mutants, optimization of process conditions, a second important aspect in bioprocess development, also benefits from such a technology[7]. Although there has been progress in recent years, there remains both need and opportunity to cost-effectively and with fidelity miniaturise fermentation to screen under fed-batch conditions.

Several strategies have been developed to study microorganisms under controlled growth conditions. One strategy is to improve existing platforms such as microtiter plates and shake flasks. Jeude and co-workers[8], for example, implemented a continuous supply of glucose by adding glucose-containing silicon discs to the shake flasks. A similar slow diffusive release strategy has been demonstrated in microtiter plates[5, 9, 10]. Scheidle and co-workers[5] used this strategy to compare the performance of 220 different mutants grown under batch conditions to that grown under fed-batch conditions, showing that best performers are not the same. Apart from slow release approaches, microchannels controlled through pneumatic valves have been added to or incorporated in microtiter plates for nutrient supply and pH regulation[11–13]. A second strategy is to integrate the wells and fluidic supply lines all in a single microfluidic device[14–19], see the comprehensive reviews by Grunberger' s group[20–22]. A third strategy is to grow and study cells by compartmentalising them inside aqueous microdroplets instead of inside solid wells[23, 24]. This strategy may prevent issues arising in single phase microfluidic devices such as cross-contamination between the different wells due to lack of complete isolation and biofilm formation at the solid walls, but comes with its own

challenges[25–27]. Microdroplets have been successfully used for screening of mutants where millions of droplets were encapsulated with cells and growth media at once, incubated off chip under batch conditions and reinjected in a separate chip to analyse and sort the best performing ones[28–30]. An outstanding challenge is to semi-continuously add nutrients to cell-containing droplets to perform screening under continuous or fed-batch conditions[27, 29].

Droplet-based assays that enable studies under controlled cell growth through controlled nutrient supply are scarce. Programmable devices have been developed[31–33] that could facilitate controlled microbial growth inside droplets that are spatially fixed on a chip, allowing sequential addition of nutrients by on-demand formation of nutrient-containing droplets, transport to the cell-containing droplets and induction of coalescence. Jakiela and co-workers[34] as well as Jian and co-workers[35] in separate studies have used an alternative approach in which cell-containing droplets were circulated on chip and periodically flown through part of the circuit designed to split off part of the droplet and merge it with a nutrient droplet. While these studies successfully demonstrated cell growth studies under (semi) continuous conditions, the complexity of the devices and their use may prevent widespread adoption of this technology by the microfluidics community. Integrating long-term culturing in a controlled environment with technology accessible to non-experts is therefore identified as one of the great outstanding challenges in the field[27]. In this work, we address this challenge by simplifying the nutrient supply strategy as also very recently demonstrated by Baroud's lab in the context of 3D spheroid formation[36], eliminating the need for (on/off-chip) valves and optical feedback loops and allowing operation based on a single commercially available pressure pump.

We start this chapter by first describing the working principle of our fed-batch droplet nanobioreactor, illustrating the various fluidic operations involved, including the capability to supply nutrients at controlled rates. After a verification experiment in which we demonstrate that cell growth itself is not influenced by the fluidic operations, we demonstrate the growth of cells under nutrient-controlled fed-batch conditions. The experimentally obtained cell growth curves are subsequently compared with a simple kinetic cell growth model. Good agreement shows that the model is not only valuable in the interpretation of the results, but also in the experimental design of nutrient-controlled growth experiments (e.g. selection of feeding frequencies and nutrient concentrations).

4.2. RESULTS AND DISCUSSION

4.2.1. FED-BATCH DROPLET NANOBIOREACTOR : CONCEPT

The working principle of our fed-batch droplet nanobioreactor is illustrated in Figure.4.2.1. First, a cell-containing droplet is generated and trapped inside a cup-shaped trap (1). Being spatially fixed on the chip allows for easy monitoring of the growth of microorganisms. At will, a nutrient droplet is generated and flown into the trap (2). The challenge to coalesce the surfactant-stabilised interfaces is resolved by temporarily de-stabilizing the interfaces by flowing a solvent around the droplets in which the surfactant is less soluble (3). After supplying fresh nutrients, cells are incubated and monitored until the next nutrient supply (4). This mode of operation, in which the cells are cultured under

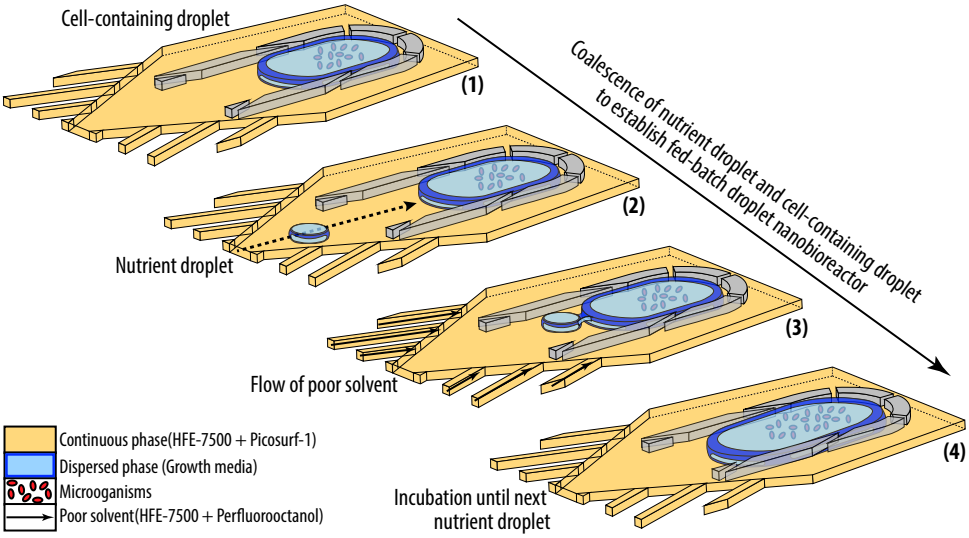

Figure 4.2.1: Conceptual schematic of the fed-batch droplet nanobioreactor illustrating the controlled supply of nutrients to a cell-containing droplet. Cell-containing droplet immobilized inside a cup-shaped trap (1). On-demand supply of a nutrient-containing droplet (2). Coalescence of the surfactant-stabilized interfaces induced by temporarily injecting a solvent in which the surfactant is less soluble through the fork-like structures (3). Incubation until the next nutrient supply (4).

nutrient-controlled conditions, with the volume in which they are cultured increasing in time, is very common in industrial practice and better known as fed-batch operation. Unlike a chemostat, in which there is an inflow as well as an outflow, allowing it to run continuously, fed-batch processes have no outflow of effluent and run for a fixed time window. To enable the robust operation of the serial processes in the chip without the use of (on/off-chip) membrane valves or any active components on chip, we make use of the recently developed droplet on-demand generator[37]. Through the use of a single commercially available pressure pump, enabling facile adoption, we were able to establish the first example of a droplet-based nanobioreactor in which cells are studied under fed-batch conditions.

4.2.2. FED-BATCH DROPLET NANOBIOREACTOR: DEVICE ARCHITECTURE AND OPERATION

Figure.4.2.2(a) illustrates the design, which features a droplet on-demand (DoD) junction for the generation of cell-containing droplets (blue box), a second DoD junction for the sequential generation of nutrient droplets (green box), a cup-shaped trap in which the cell-containing droplet is immobilised and studied under controlled growth conditions through controlled supply of nutrient droplets (red box), and two fork-like structures for the injection of the solvent with which coalescence is induced (black box). Dimensions, fabrication protocols and all other experimental details are provided in the materials and methods section. The generation of a cell-containing droplet in oil is illustrated in Figure.4.2.2(b). First, the interface between the aqueous cell solution and

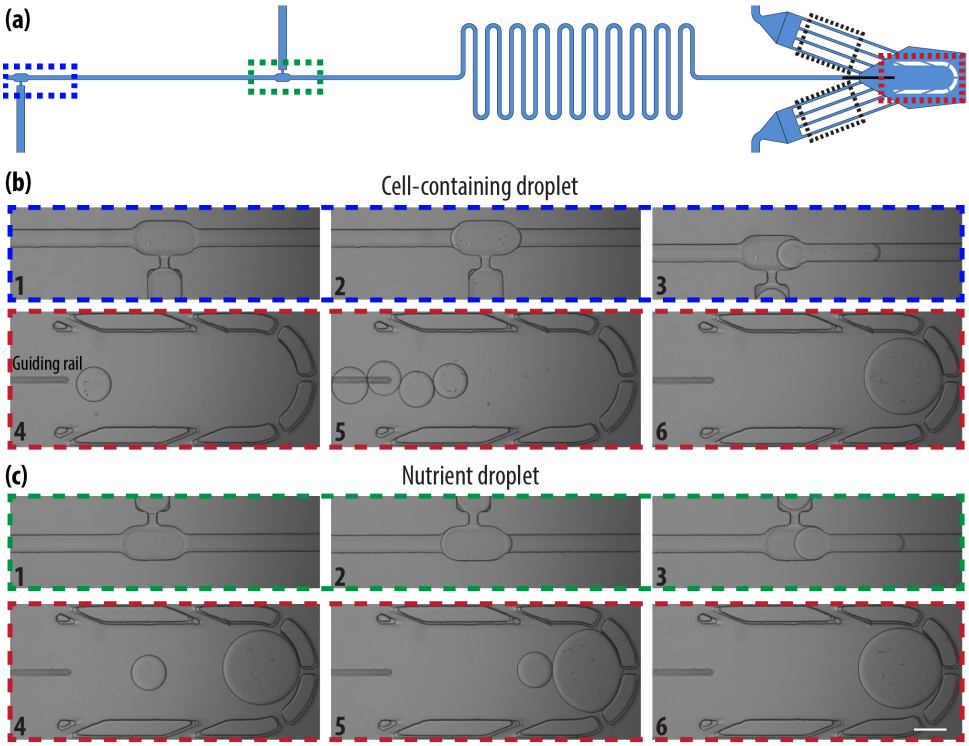

Figure 4.2.2: Device design and operation of the fed-batch droplet nanobioreactor. (a) Design showing a droplet on-demand junction for the generation of cell-containing droplets (blue box), a droplet on-demand junction for the generation of nutrient droplets (green box), a cup-shaped trap in which the cell-containing droplet is immobilized and studied under controlled growth conditions (red box), and two fork-like structures for the introduction of the solution with which coalescence is induced (black box) and a guiding rail (black line). To avoid back flow during injection of the poor solvent for coalescence or flushing, a serpentine channel is used between the DoD generators and the trap area to provide sufficient resistance. (b) On-demand formation of a single cell-containing droplet (1-3) and its guidance into the trap through a guiding rail (4). Formation of a larger cell-containing droplet by sequential production of four droplets (5) and their coalescence (6). (c) On-demand formation of a nutrient droplet (1-3) and its coalescence with the cell-containing droplet inside the trap (4-6). Scale bar is 100 μm.

the oil is pushed against the nozzle of the DoD generator (b1). Then, the pressure in the reservoir of the cell-containing solution is temporarily increased such that the interface is pushed through the nozzle and the chamber gets filled until it is full and the interfaces are pressed against the exits of the chamber (b2). Finally, the pressure in the oil reservoir is temporarily raised to push the droplet from the chamber into the narrower main channel (b3) and subsequently into the trap (b4). The architecture of the DoD junction hereby allows for the production of a single droplet on-demand, with its volume set by the volume of the chamber, see [37] for full details. To make sure that the droplet arrives in the trap, we make use of two features: a guiding rail in the form of a groove in the top wall between the exit of the main channel and the entrance of the trap and perforations in the cup-shaped trap that allow flow through the trap itself. Depending on the desired

volume at the start of cultivation, multiple cell-containing droplets can be sequentially generated and pushed into the trap (b5). Coalescence of these droplets is achieved by temporarily raising the pressure in the fluid reservoir that contains a solution in which the surfactant is less soluble, which is introduced in the area of the trap through the fork-like structures (b6). Supply of nutrient droplets to the obtained cell-containing droplet follows a similar protocol, with the nutrients supplied from a separate reservoir and a second DoD junction as illustrated in Figure.4.2.2(c1-6). For a given DoD geometry and a given nutrient concentration in the fluid reservoir, controlling the time interval between subsequent droplets (denoted as the waiting time (t_w)) is the most straightforward control over the nutrient supply rate. After each coalescence event, a residual flow of oil with surfactant is maintained via the main channel to ensure full wetting of the PDMS walls by the oil and stability of the cell-containing droplet during its incubation. After incubation, the cell-containing droplet can be extracted out of the chip for further analysis by reversing the oil flow which is possible by exchanging the outlet port with the inlet port for continuous phase. All operations are performed in an automated fashion using a MATLAB script that instructs a commercially available pressure pump with predefined pressure settings, see Section.4.A for script and settings. Using the here introduced approach, microorganisms can be studied under fed-batch conditions with their growth controlled through the controlled supply of nutrients.

4.2.3. NUTRIENT FEEDING PROFILES

The most straightforward cultivation method is the batch culture, wherein all nutrients are supplied at the start of the cultivation and the cells grow exponentially at their maximum rate until one of the medium components gets depleted. Controlling the supply of nutrients over the course of a cultivation experiment is important in biotechnological applications, because in this way the growth rate of the cells can be controlled and thereby also the rate of product formation. Because the relation between the rate of product formation and the growth rate can be different for each microorganism/product combination, a dedicated feed profile has to be designed to maximise the product formation rate and yield of a fed-batch process. Examples of basic feeding strategies are constant, linearly increasing and exponentially increasing feed rates, whereby the number of microorganisms increases respectively linearly, quadratically and exponentially. Different feeding strategies can be established in the droplet fed-batch reactor by controlling the interval between subsequent nutrient supplies and the amount of nutrients fed per supply. Here we demonstrate constant and linearly increasing feed profiles. In case one nutrient-containing reservoir is used in combination with one DoD generator for the supply of nutrients, the nutrient concentration and the volume of a single nutrient droplet are fixed. Supply at a constant rate is then achieved by adding one nutrient droplet at a time at a constant time interval. An example of such a *constant feed rate* experiment is illustrated in Figure.4.2.3(a), with the time interval equal to 10 mins. One way to obtain a *linearly increasing feed rate* is to linearly decrease the time interval. Alternatively, the number of nutrient droplets per supply can be linearly increased, while keeping the time interval between the supplies the same. An example of this mode of operation is illustrated in Figure.4.2.3(b), with one droplet supplied after 10 mins, two droplets shortly generated after each other (within 20 s) supplied after 20 mins and so

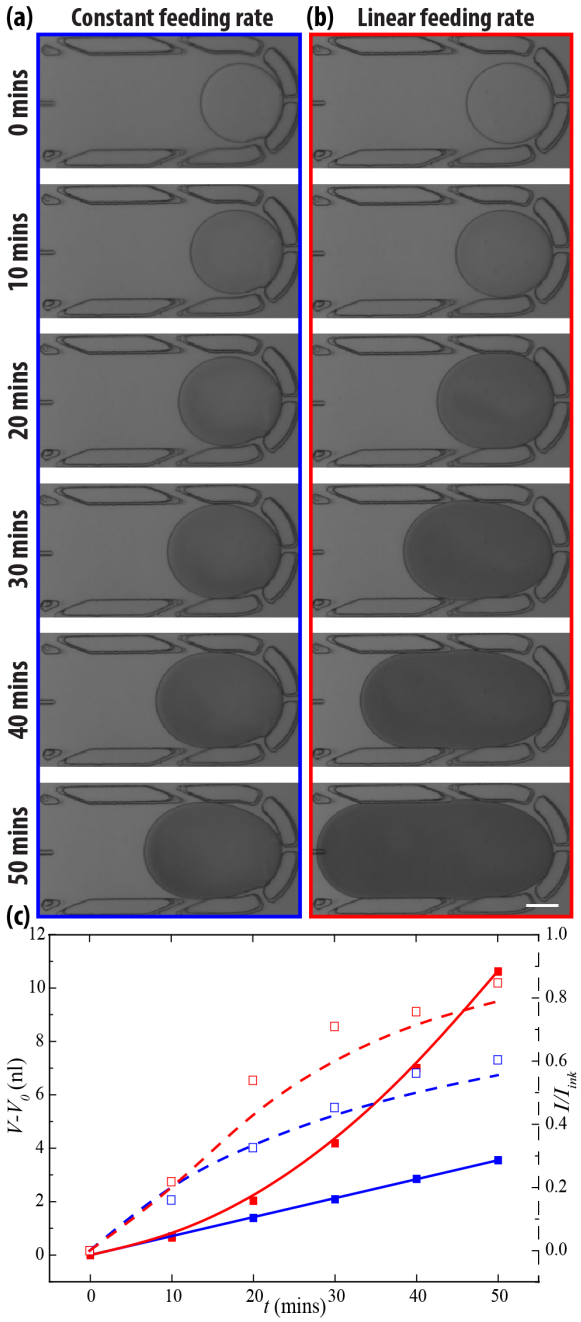

Figure 4.2.3: Demonstration of two basic nutrient-feeding strategies. For illustrative purposes and easy of quantification, we trapped an aqueous droplet and fed it with ink droplets. Grey scale images show the increase in volume and intensity of the trapped droplet for a (a) constant feed rate (See the corresponding movie by scanning Figure.4.B.1) and a (b) linearly increasing feed rate. (c) Corresponding volume of the trapped droplet V with respect to its initial volume V_0 (closed symbols) and grey levels I relative to that of an ink droplet I_{ink} (open symbols) agree well with theoretically expected values for volume (full lines) and relative intensity (dashed lines), see materials and methods for details. Scale bar is 100 μm.

on. The volume of the trapped droplet increases linearly and quadratically for a constant and linearly increasing feed rate respectively, as illustrated in Figure.4.2.3(c). This increase agrees well with the expected increase based on the volume of the DoD chamber used to produce the droplets (0.71 nl). For illustrative purposes and ease of quantification, we performed these experiments by trapping an aqueous droplet and feeding it with ink droplets. This allowed us to straightforwardly verify that the concentration in the trapped droplet increases as expected through the measurement of the intensity, see Figure.4.2.3(c). While the experiments presented here illustrate the working principle of the droplet nanobioreactor for two basic feeding strategies, we foresee that other, more complex, feed profiles are also possible. One limitation in establishing more complex feeding profiles arises from the frequency with which nutrient droplets can be generated. Two subsequent droplets can be generated within 20 s. In an exponential scheme, in which we with the current setup need to subsequently generate 1, 2, 4, 8, 16, 32, ... nutrient droplets in each supply cycle, the time it takes for the supply soon becomes comparable to the waiting time between the cycles. Another limitation arises from the volume of the trap relative to the volume of the chamber of the DoD generator, which currently dictates that 20 nutrient droplets can be fed until the trap is full. Potential improvements to overcome these two limitations are to incorporate multiple DoD generators connected to reservoirs with different nutrient concentrations, limiting the number of droplets that need to be generated per supply cycle. Using multiple reservoirs also opens up the possibility to supply basic/acidic solutions to control pH or other types of reagents, for example inhibitors, antibiotics, or even competitive microbial communities.

4.2.4. CONTROLLED GROWTH OF *Cyberlindnera (Pichia) jadinii* IN FED-BATCH DROPLET NANOBIOREACTOR

After a successful demonstration of the ability to controllably produce and trap a cell-containing droplet and repeatedly supply it with fresh nutrients, we now show that we can utilise the developed approach to study cells under nutrient-controlled growth conditions. The model microorganism we used in this work is the Crabtree negative yeast *Cyberlindnera (Pichia) jadinii*, which was chosen for its relatively simple growth characteristics as it does not convert glucose to ethanol under aerobic conditions. Furthermore, its growth is severely limited under oxygen limited conditions[38] which allowed us to verify from the growth behaviour whether oxygen limitation would occur. The experiments were designed such that glucose is the limiting nutrient with which cell growth can be controlled. To eliminate the need for active pH control over the course of the experiments, urea was used as the nitrogen source in the media. Based on these choices, cell growth is primarily controlled through the controlled supply of nutrients.

A typical cell growth experiment starts with overnight precultivation in a shake flask and harvesting the cells in the exponential growth phase. Cells were washed with fresh defined medium to obtain a cell solution with a given initial glucose concentration C_{S0}. This solution was used to produce a cell-containing droplet, with the initial number of encapsulated cells (N_0) controlled through the washing step and the number of droplets used to form the cell-containing droplet. In our experiments, we used the protocol illustrated in Figure.4.2.2(b) and coalesced four cell-containing droplets of volume V_d

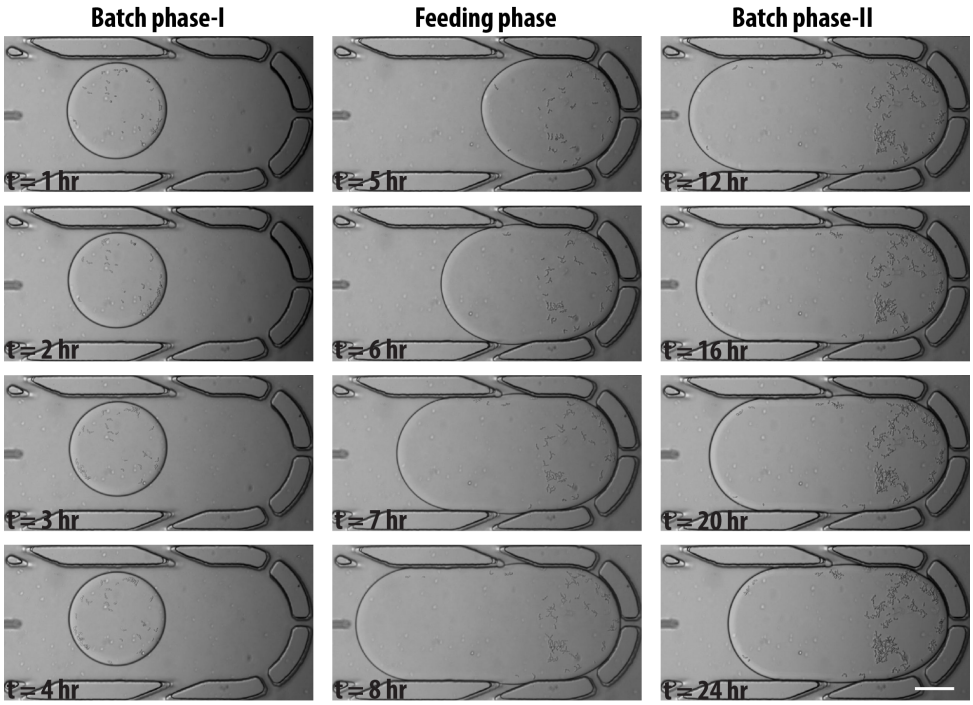

Figure 4.2.4: Time lapse showing nutrient-controlled growth of *Cyberlindnera (Pichia) jadinii* inside a fed-batch droplet nanobioreactor. After cell handling and transfer to the device, cells were allowed to adjust to the new environment for 4 hr, while growing on the glucose present in the trapped droplet. After this first batch phase (batch phase-I), the feeding phase started in which we controlled the cell growth through the controlled supply of nutrient droplets at a fixed rate (16 droplets with a time lag of 15 mins). Once the trap was full, we continued monitoring cell growth up to 24 h while they again grew under batch conditions (batch phase-II). Scale bar is 100 μm. See the corresponding movie by scanning Figure.4.B.2.

resulting in an initial volume of $V_{m0} = 4V_d$. After cell handling and transfer to the microfluidic device, we first let the cells adjust to the environment for t_b = 4 hr in order to verify that they continued their exponential growth. During this initial time window, which we refer to as *batch phase-I*, cells grew on the glucose present in the droplet without additional glucose being fed. The volume of the droplet remained the same, while the number of cells increased, as illustrated in the left column of Figure.4.2.4. After 4 hours, we started the *feeding phase* in which we supplied nutrient droplets of volume V_d and glucose concentration $C_{S,in}$ at a constant supply rate characterised by a time interval t_w. The trapped droplet increased in volume during this phase, while the number of cells increased at a slower, nutrient-controlled rate, see the middle column of Fig. 4.2.4. With the volume of the trap being almost 20 times larger than the volume of a single nutrient droplet (0.71 nl), 16 nutrient droplets could be fed to the cell-containing droplet over the course of the feeding phase. After this feeding phase, we continued monitoring cell growth up to 24 hr, without further supply of nutrients, which we refer to as *batch phase-II*. During this phase, cell numbers increased at a reduced rate until

the glucose is depleted, as illustrated in the right column of Figure.4.2.4. We observed moderate shrinkage of the trapped droplet during this phase, which is a well-studied phenomenon[39, 40] with its dynamics governed by the evaporation of water through the PDMS matrix. To minimise shrinkage, the bonded chips were soaked in demineralised water for 7 days before the experiment and a stage top incubator with active humidity control was used during the experiments, see materials and methods for details.

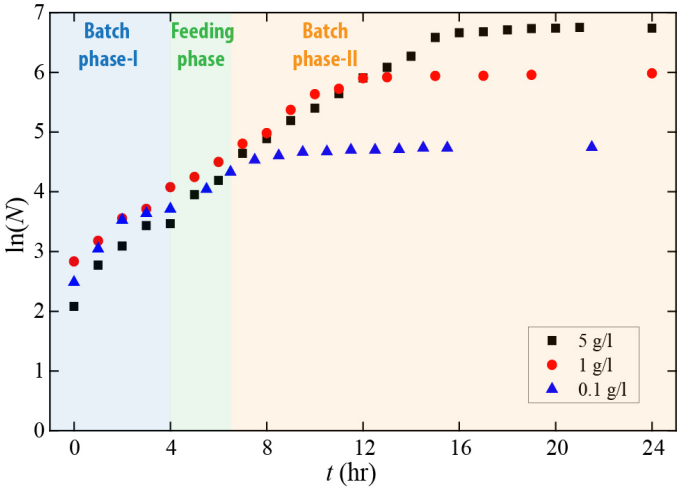

Figure 4.2.5: Growth curves obtained in the fed-batch droplet nanobioreactor under conditions without significant nutrient limitations. Comparable growth rates observed in the three different phases (as evident from the slopes) provide a sanity check that the used protocol itself with all consecutive fluidic operations does not affect cell growth. Furthermore, similar growth rates observed for different glucose concentrations in the nutrient droplets, leading to different final number of cells, indicate that other limitations, such as oxygen, do not play a role.

The initial set of cell growth experiments we performed was designed in order to check that the protocol we developed, with all consecutive fluidic operations, does not itself influence cell growth. To this end, experiments without significant glucose limitation in the feeding phase were performed. The two parameters that control the extent of glucose limitation during batch phase-I are the initial cell number (N_0) and the initial glucose concentration in the cell-containing droplet (C_{S0}). The additional two parameters that determine the extent of glucose limitations from the feeding phase onwards are the glucose concentration in the nutrient droplets ($C_{S,in}$), and the time between two subsequent nutrient supplies (t_w). Using a simple cell growth model (see materials and methods section below) with estimates of the kinetic parameters from literature, we chose N_0 to be around 10 cells and C_{S0} = 2 g/l for cells to grow exponentially for the first hours and consume most of the glucose by the end of batch phase-I. This is indeed visible from the three growth curves in Figure.4.2.5. For the feeding phase, we chose three different glucose concentrations, $C_{S,in}$ = 0.1, 1, 5 g/l and a constant feeding rate with a time window of t_w = 10 mins. For these parameters, the model predicted the cells to grow exponentially during the entire feeding phase and part of the subsequent batch phase without significant glucose limitation. The three curves in Figure.4.2.5 in-

deed show the same (unrestricted) exponential growth. We obtained the specific growth rate by fitting the data in the initial and exponential growth phase. The specific growth rates are 0.28 ± 0.02 (5g/L), 0.26 ± 0.01 (1g/L), and 0.24 ± 0.06 (0.1g/L), with the ± values indicating the 95% confidence interval of the fitted values. The specific growth rates are not significantly different, illustrating the reproducibility of the experiments, and highlighting that the protocol itself does not affect cell growth. The rationale for performing the experiments at three different glucose concentrations in the nutrients droplets was to check whether other limitations, for example with regard to dissolved oxygen, played a role. The larger this concentration, the more cells were grown and the larger the rate at which nutrients were consumed. As the growth rate in the feeding phase and in part of batch phase-II is comparable for all three curves, we concluded that other limitations played no role for the studied parameter range. The order of magnitude of the final cell density is in the range 10^7- 10^8 cells/mL, which is comparable to the order of magnitude in traditional cultures[53].

To demonstrate nutrient-controlled growth of *Cyberlindnera (Pichia) jadinii*, we chose appropriate values for the four main control parameters using the aforementioned simple cell growth model. While these parameters span a large window of operation for which nutrient-controlled growth is anticipated in the feeding phase, one of the most straightforward ways to control the extent of nutrient limitations is to simply start with a larger initial number of cells as compared to the previous set of experiments. This qualitatively results in a larger number of cells present in the cell-containing droplet at the start of the feeding phase and hence in a higher nutrient consumption rate, such that nutrient-controlled growth is anticipated when using comparable nutrient supply rates as in the previous set of experiments. Using the model, we quantified that an initial number of about 40 cells encapsulated in a droplet of volume 4 V_d with initial glucose concentration of 2 g/l gives rise to nutrient-limited growth with distinguishable growth rates when using glucose concentrations in the nutrient droplet of 0.2, 0.5, 1.0 and 2.0 g/l and a constant feeding rate of 1 nutrient droplet per 15 mins.

The growth curves for these four controlled growth experiments are presented by the symbols in Figure.4.2.6. In batch phase-I, the conditions in the four experiments are comparable, except for the initial number of cells (between 39-56). The experiments are hence expected to show the same growth rate and can in this sense be seen as quadruplicates. The reproducibility of these experiments is evident from the overlap of the curves in batch phase-I in a plot with cell numbers normalized with the initial cell number and plotted logarithmically, see Figure.4.D.1, Section.4.D. In the feeding phase, the four experiments show a linear increase in the number of cells, characteristic for a constant supply of the growth limiting nutrient. Moreover, the slopes are significantly different, highlighting the ability of the here-developed nanobioreactor to control the growth of cells by the controlled supply of nutrients. After the feeding phase, the number of cells increases at a reduced rate in batch phase-II, with the final number of cells depending on the amount of glucose fed.

The controlled growth experiments were designed using a simple kinetic cell growth model, with estimates of the input parameters from literature. We can now compare model predictions with the experimentally-obtained growth curves. Rather than using the maximum growth rate (μ_{max}) and the yield on glucose ($Y_{x/s}$) as input parameters,

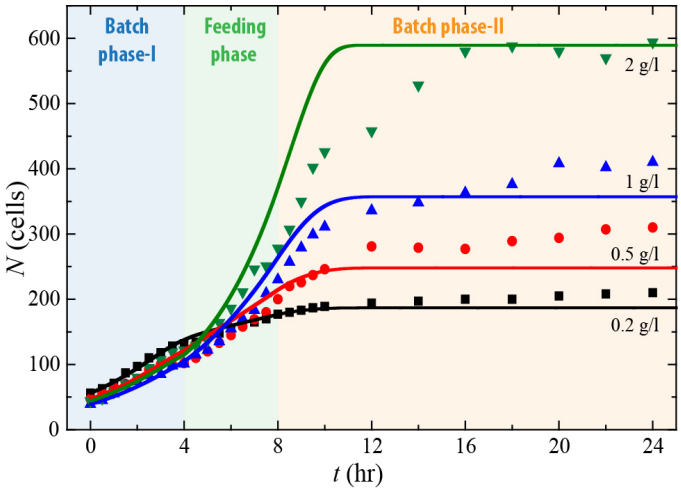

Figure 4.2.6: Growth curves for nutrient-controlled growth in the fed-batch droplet nanobioreactor. During batch phase-I all 4 experiments exhibit similar growth kinetics as the cell-containing droplets contain a common glucose concentration of C_{S0} = 2 g/l. The effect of controlled nutrient supply is seen from the difference in the growth rates in the feeding phase. Cell growth saturates at different final number of cells once the glucose gets depleted in batch phase-II. The experimental data (symbols) agree well with a simple cell growth model (lines).

we used them as fit parameters, as detailed in Section.4.I. As the yield on glucose is known to vary with the extent of nutrient limitation[41], we fit it separately for batch phase-I and for the rest of the experiment. Despite the simplicity of the model, we obtained good agreement between model (lines) and experimental data (symbols) for all the four variations of $C_{S,in}$ as can be observed in Figure.4.2.6, with the obtained fit parameters μ_{max} = 0.32 hr^{-1} and $Y_{X/S}$ = 0.35 gX/gS (batch phase-I) and $Y_{X/S}$ = 0.45 gX/gS (feeding phase and batch phase-II). These values seem lower than the range reported for *Cyberlindnera (Pichia) jadinii*[42–44], which may be attributed to mixing. While the cell-containing droplet is mixed within thirty seconds each time coalescence is induced (Figure.4.E.1, Section.4.E), we observe limited motion of the cells inside the droplet. Mixing induced by the flow around the cell-containing droplet may be insufficient for effective mass transfer. Increasing this flow and optimising the trap geometry enhances mixing and is subject for future development. Nevertheless, good agreement between model and experiments highlights the usefulness of this simple model, for the interpretation of nutrient-controlled growth experiments and for their design.

4.3. CONCLUSIONS & OUTLOOK

The goal of this paper was to develop a droplet-based nanobioreactor that enables studying microorganisms under nutrient-controlled fed-batch conditions as commonly encountered in industrial practice, but difficult to achieve at microscale with technology accessible to non-experts. To this end, we developed a robust method to controllably supply droplets with fresh nutrients to microorganisms encapsulated in a droplet immo-

bilized in the nanobioreactor. We demonstrated the control over the growth of the yeast *Cyberlindnera (Pichia) jadinii* through the controlled supply of nutrients. A next challenge is to scale out the device in order to screen experimental conditions. In our previous work, we have shown the scale out of the droplet-on-demand junctions that form the foundation of the fed-batch chip[37]. A similar strategy can hence be used to scale out the fed-batch chip. Using DoD junctions with different chamber volumes in a parallelized architecture, it for example becomes possible to screen microbes for a variety of feeding profiles. A second challenge is to incorporate analytics to follow the production of intra- or extracellular metabolic products. Quantification of extracellular products secreted by microorganisms is possible by adding a third droplet on-demand junction that enables the addition of reagents to perform an end-point assay. Alternatively, real time fluorescence can also be measured during the fed-batch cultivation experiment, given the strain is engineered to express fluorescence. A third challenge is to further improve the accessibility of the technology. While the device itself is relatively simple and so is its operation through the use of a commercially available pressure pump, integrating and automating all workflows into a ready-to-use "chip-in-a-box"[45] is required to facilitate widespread adoption by biotechnologists and bioprocess engineers. All in all, given the ease of operation, the potential for scale out and incorporation of analytics, the presented droplet nanobioreactor provides a solid base for the screening of microorganisms or process conditions under industrially relevant fed-batch conditions. A benefit over microtiter-based fed-batch systems such as the BioLectorPro is the ability to perform these screens at single cell resolution and characterize cell heterogeneity[45].

4.4. MATERIALS AND METHODS

4.4.1. DEVICE DESIGN AND DIMENSIONS

The most important features in the design of the microfluidic device as highlighted by the dotted rectangles in Figure.4.2.2(a) are: the two droplet on-demand (DoD) generators for the production of the cell-containing droplet and the supply of nutrient droplets, the cup-shaped trap for the immobilisation of the cell-containing droplet and the fork-like structures for the introduction of the solvent with which coalescence is induced. Full details on design considerations and performance (monodispersity) of the DoD generators are provided in our previous work[37]. The DoD generators consist of a nozzle and a chamber. The nozzle is 25 μm wide, 25 μm high and 50 μm long and connected to a 100 μm wide and 35 μm high supply channel. The chamber is 100 μm wide, 40 μm high, and 200 μm long resulting in a chamber volume of 0.71 nl. It is connected to the main channel, which is 50 μm wide and 35 μm high. The produced droplets are effectively guided from the exit of the main channel to the cup-shaped trap using a 20 μm wide and 5 μm deep groove in the ceiling of the channel. The trap is made up by a semi-circle with a diameter of 300 μm and a 300 μm wide and 900 μm long rectangle. The 30 μm wide and 35 μm high perforations in the trap hereby allow flow through the trap. These perforations are not only important to load the trap with a droplet, but also to flush out unwanted bubbles or droplets produced at the start-up of the experiment. Their removal is achieved in a straightforward way by sufficiently raising the operating pressure in the reservoir from which the poor solvent is supplied, which forces them

through the opening in the centre of the trap into the downstream channel. The height of the trap is 35 μm, which is sufficiently shallow to avoid complications in cell counting due to the growth of cells in multiple layers for the growth conditions considered in this work. Coalescence between the immobilised cell-containing droplet and an incoming nutrient-droplet is achieved by introducing a poor solvent into the trap through the fork-like structures, which feature three channels that are 33 μm wide and 35 μm high. As we observed coalescence to occur within seconds upon the introduction of the poor solvent, these features were no subject for further optimization. The serpentine channel between the DoD generators and the trap area is added to provide sufficient resistance to avoid back flow when injecting the poor solvent into the chip during coalescence or flushing steps. The design and all its details are made available as an AutoCAD drawing in Section.4.C.

4.4.2. DEVICE FABRICATION

Microfabrication was performed using standard photolithographic methods[46]. The device comprises channels of three different heights: all channels are 35 μm high, except for the 25 μm high nozzles, the 40 μm high chambers of the two DoD generators, and the 5 μm deep groove in the ceiling of the main channel. The three-dimensional device was constructed from three layers of the negative photoresist SU-8 (micro resist technology GmbH) on a 4 inch silicon wafer, each layer exposed to near UV (EVG 620, EV Group) through a separate transparency mask. These masks were designed in AutoCAD 2015 (Autodesk) and printed on transparencies using a high-resolution printer (CAD/Art (Oregon, USA)). The first 25 μm thick layer of photoresist (SU-8 3025) was exposed through a mask with the full device design being transparent. The second 10 μm thick layer (SU-8 3005) was exposed through a similar mask, but with the two channels that connect the feed of the dispersed phases to the chambers of the DoD generators (i.e. the nozzles) made non-transparent in order to keep the nozzle height 25 μm. The third 5 μm thick layer (SU-8 3005) was exposed through a mask that featured transparent chambers of the DoD generators in order to increase their height to 40 μm and the guiding rail that then becomes 5 μm high. The three masks are provided as an AutoCAD drawing in Section.4.C. After spin-coating each layer, it was soft baked, exposed, and post-exposure baked, following the guidelines provided by the manufacturer. After the post-exposure bake of the third layer, the wafer was developed with mr-Dev 600 (micro resist technology GmbH) to dissolve the uncured photoresist, washed with isopropyl alcohol (IPA) and spin-dried. The resulting wafer was hard-baked for 30 mins at 150°C, and gradually cooled down on a heating plate to avoid cracks in the resulting SU-8 structures. Before using the wafer as a master for creating PDMS stamps, its surface was silanized by exposing it to vapours of 1H,1H,2H,2H-perfluorooctyl-trichlorosilane in a depressurised desiccator.

PDMS devices were prepared by mixing 80 g of PDMS elastomer and 8 g of curing agent (Dow corning, Slygard 184 elastomer kit). The mixture was degassed and poured over the master placed in a 5 inch petridish. After 4 hours in the oven at 70°C for 4 hours, cured PDMS was carefully peeled off from the wafer and cut to size. Inlets were punched with a 0.75 mm biopsy punch (Rapid core) and the outlet with a 1.5 mm biopsy punch (Rapid core). The PDMS stamps were then washed with ethanol and IPA to remove dust

and debris. The stamps were covalently bonded to PDMS spin coated glass slides (25 mm x 75 mm) after exposing them to an oxygen plasma (Harrick, PDC-002) for 140 s at a pressure of 0.2-0.4 mbar. The obtained microfluidic devices were then baked at 200°C for at least 4 hours to recover the hydrophobicity of PDMS. A small piece of PTFE tubing (0.3 mm ID, 1/16 inch OD, 1 cm in length, Kinesis) was inserted into the outlet and glued tightly. Stainless steel connectors (0.025 inch OD x 0.013 inch ID, 23g Elveflow) were inserted into all four inlets and glued tightly with water resistant glue. Finally, the microfluidic chips were soaked in demineralised water for a week in a covered petridish to saturate the PDMS matrix with water. This is an important step in order to reduce evaporation of water through PDMS, which leads to shrinkage of droplets in long-term cell growth experiments.

4.4.3. EXPERIMENTAL SETUP

A commercially available pressure-based flow controller (MFCS-4C 1000 mbar/7000 mbar, Fluigent) was used to control the injection of fluids. The ports on this pressure controller were connected to reservoirs containing the fluids using silicone tubing and tightly sealed with parafilm. Soft walled Tygon tubing (0.02 inches ID, 1/16 inch OD, 50 cm in length) was used to connect the reservoirs to the metal connectors glued to the inlets of the chip. Care was taken to use the same height difference between the outlet of the chip and the liquid levels in the reservoirs in order to have comparable contributions of the hydrostatic pressure between different sets of experiments and use the provided MATLAB script (see Section.4.A). Relatively large reservoirs (15 ml centrifuge tubes) were used to ensure a negligible change in liquid level over the course of an experiment. Full details on the protocol to produce droplets on-demand, including a Matlab script, are provided in our previous work[37]. Experiments to demonstrate the fluidic operations of the device (Figure.4.2.2 and Figure.4.2.3) were carried out at room temperature and atmospheric pressure. On-chip cell growth experiments were carried out in an incubation system (Ibidi stage top incubator, Ibidi Gmbh) mounted on top of an inverted microscope (Axiovert S100, Zeiss). In order to avoid leakages and distortion during imaging utmost care was given while connecting the tubing from the fluid reservoirs to the microfluidic chip and fixing the chip inside the incubator box. The microfluidic chip was fixed on the bottom heated plate with temperature set point of 30°C. The top plate temperature was also fixed at 30°C. The incubation box was supplied with a gas mixture of 21 % O_2 and 79 % N_2, which was being saturated with water vapour through a humidifier bottle and flown through a tube with jacketed heater at 5.2 l/hr. The humidity inside the box was maintained at 99 % to reduce water evaporation from the cell-containing droplet during the 24h time lapse.

4.4.4. IMAGE ACQUISITION AND ANALYSIS

Image acquisition was done using a TIS camera (DMK 33UJ003, The Imaging Source) mounted on a microscope using a combination of a 10 X objective and a 0.63 X mount objective. For the cell growth experiments, images were acquired at a frame rate of 5 frames per min for 24 hr. The resolution obtained with this set up allows single cells to be identified on the acquired images, see a close-up in Figure.4.F.1 in section.4.F. The images acquired to demonstrate the feeding capabilities (Figure.4.2.3) were processed to

determine the length, L, and intensity, I, of the trapped droplet after each coalescence event using Fiji. The length was subsequently used to determine the droplet volume using $V = [hw - (4-\pi)(2/h + 2/w)^{-2}](L - w/3)$, with h the height of the trap of 35 μm and w the width of the trap of 300 μm, see [47] for further details. The intensity values were normalised with the intensity of the first incoming ink droplet I_{ink}. Quantification of cell growth experiments was performed by counting the number of cells in each image. Although this process could be automated, we manually counted the cells using a stylus for a selected number of images.

4.4.5. WORKING FLUIDS

The continuous phase used in all experiments is the fluorinated oil HFE-7500 (3M, Novec 7500 Engineered fluid) in which we dissolved 0.05 v/v % Pico-Surf-1 (SpehereFluidics) in order to stabilise the interfaces and to ensure complete wetting of the PDMS walls ensuring controllable droplet operations. This oil is commonly used in cell experiments in microfluidic device and we selected it for its known compatibility with PDMS, high oxygen solubility and biocompatibility[48, 49].

In the experiments that demonstrate the fluidic operations, we used demineralised water to produce the trapped and fed droplets in Figure.4.2.2 and added brilliant blue dye (0.1 w/w %) to produce the reagent droplets in Figure.4.2.3 as a means to quantify the amount of reagents fed. In the cell growth experiments, cell-containing droplets were produced from a solution of *Cyberlindnera (Pichia) jadinii* cells in defined media with a glucose concentration of 2 g/l. The protocol used to prepare this solution, including the protocol for the preculturing step and the preparation of the defined media itself is provided below. Nutrient droplets were produced from defined media, with glucose concentrations in the range between 0.1 and 5.0 g/l.

Finally, we used a 5 v/v % solution of PFO (1H, 1H, 2H, 2H - perfluoro-1-octanol, Sigma Aldrich) in HFE-7500 to induce coalescence. We selected PFO as it is well-known to break emulsions of cell-containing droplets[50], and considered the cytotoxicity when selecting its concentration (see below). We also considered 2,2,3,3,4,4,4,-Hepta-fluroButanol (Sigma Aldrich) used in previous work to chemically induce droplet coalescence[51], but found it to be cytotoxic at concentrations required for efficient coalescence.

PREPARATION OF THE SOLUTION OF *Cyberlindnera (Pichia) jadinii* CELLS IN DEFINED MEDIA

Cyberlindnera (Pichia) jadinii cells

Cyberlindnera (Pichia) jadinii cells (CBS621) were obtained from the collection of the Westerdijk Fungal Biodiversity Institute, Utrecht, the Netherlands. A cell stock was prepared in 2 ml cryovials containing YPD and glycerol and stored at -80°C. For every growth experiment, a fresh cryovial was taken from the stock.

Preculturing

After thawed at room temperature, 50 μl of cell solution was pipetted from the cryovial and inoculated into 5 ml of YPD (Yeast-Peptone-Dextrous) preculture medium. This medium was prepared by thoroughly mixing 20 g/l of glucose (Sigma-Aldrich), 10 g/l

of yeast extract (Sigma-Aldrich) and 20 g/l of bacteriological peptone (Sigma-Aldrich) in a desired amount of demineralised water and subsequently sterilizing it by pushing the solution through a 0.2 μm syringe filter (Whatman). The inoculation was done in an autoclaved round bottom flask of 50 ml. Cotton wool was used to cover the flask to maintain aseptic conditions, while ensuring sufficient aeration. The flask was incubated overnight for 16 hr in an orbital shaker at 30°C and 190 rpm.

Transfer to defined media

The precultured cell-YPD solution was centrifuged to remove the supernatant and then washed three times with defined media (see preparation protocol below). For the two sets of experiments with about 10 and about 40 cells inside the initial droplet, we respectively used, 2 ml and 4 ml of the precultured cell-YPD solution and washed it with defined medium to obtain 10 ml of the final cell solution with defined medium. We pipetted 200 μl of fresh YPD to the obtained solutions to boost the growth of cells inside the microfluidic chip. The resulting cell solution in defined media was transferred to a 15 ml centrifuge tube and stored at 30°C in an incubator for at most 30 mins prior to connecting the reservoir to the pressure pump to start the microfluidic experiments.

Preparing defined media

The defined medium was prepared by mixing 2.3 g/l of urea ($CO(NH_2)_2$, Sigma-Aldrich), 10 g/l of magnesium sulphate heptahydrate ($MgSO_4.7H_2O$, Sigma-Aldrich), 3 g/l of potassium hydrogen phosphate (KH_2PO_4, Sigma-Aldrich) and the desired concentration of glucose (0.1 g/l to 5 g/l) in demineralised water. All components were weighed according to the desired final weight of the solution and the solution was mixed thoroughly. Then 1 ml/l of trace element solution and 1ml/l of vitamin solution was added, see Table.4.G.1 and Table.4.G.2 in Section.4.G for their preparation protocols. The pH value was subsequently adjusted to 6.0 with a 2M KOH solution and demineralised water was added again to reach the final weight. The prepared medium was then filter sterilised by pushing it from a 50 ml syringe through a 0.2 μm syringe filter (Whatman) into a sterile bottle or tube. The obtained defined media was stored in a laminar flow cabinet until being used to prepare the cell-containing solution (with 2 g/l of glucose) or as the nutrient solution (with 0.1, 0.2, 1.0, 2.0 or 5.0 g/l of glucose).

4.4.6. MICROBIAL GROWTH MODEL

In order to design and interpret the experiments, we used a simple theoretical cell growth model. As detailed in Section.4.I, the amount of biomass X (in grams) and the glucose concentration C_s (in grams/liter) in the droplet of volume V (in liter) were described by the following set of coupled differential equations:

$$\frac{dX}{dt} = Y_{x/s}\left(q_{s,max}\left(\frac{C_s}{K_s + C_s}\right) - m_s\right)X \tag{4.4.1}$$

and

$$\frac{dC_sV}{dt} = -q_{s,max}\left(\frac{C_s}{K_s + C_s}\right)X \tag{4.4.2}$$

with $Y_{x/s}$ (biomass yield on glucose), $q_{s,max}$ (maximum specific uptake rate), K_s (affinity constant) and m_s (maintenance coefficient) as the four model parameters specific to the here cultured *Cyberlindnera (Pichia) jadinii* cells under the here cultured conditions. For the design of the experiments, we used the following estimates based on literature: $Y_{x/s}$ = 0.51 gX/gS[52], $q_{s,max}$ = 1.25 hr^{-1}[53], K_s = 0.36 g/l[41] and m_s = 0.01 gS/(gX.h) (value reported for *S. cerevisiae*[54]). For the interpretation of the data in Figure.4.2.6, we used the same values for K_s and m_s, while using $Y_{x/s}$ and $q_{s,max}$ as fit parameters, see the ESI† for details. As it is more common to report the maximum growth rate, μ_{max}, we computed it from the obtained values as explained in Section.4.I.

To convert biomass X (in grams) into cell number N we used $N = X/m_{cell}$ where m_{cell} is the mass of single cell and estimated as follows: first, we determined the average volume of 15 cells to obtain 80.2 μm^3 . Second, we calculated the wet cell mass to be 88 pg based on a specific gravity of 1.1 commonly reported for yeast[55, 56]. Third, we determined the dry cell mass to obtain m_{cell} = 22 pg using the approximation that dry matter comprises about a quarter of the wet cell mass[57, 58].

APPENDIX

4.A. MATLAB SCRIPT FOR RUNNING FED-BATCH EXPERIMENT

The following MATLAB script was used to run the droplet based fed-batch experiments in an automated fashion by controlling the pressure input to the pressure pump through MFCS:

```
[HandleNumber SerialNumber] = mfcs_init;
AlphaValue = 5; % this is for the PID, according to fluigent
                % this is already the optimal value and shouldnt be changed

% This script is for performing fed-batch experiments with droplet based
% nanobioreactor

fid = fopen( 'MatlabCrashReport.txt', 'wt' );

% zero-state conditions, change these to match a steady state in Maesflo
% MFCS Fluigent

p_1 = 21;          % mbar; Equilibrium pressure for Continous phase
p_2 = 10;          % mbar; Equilibrium pressure for Cell-containing droplet
p_3 = 19;          % mbar; Equilibrium pressure for Nutrient droplet
p_4 = 30;          % mbar; Equilibrium pressure for Coalescence agent

%Parameters for cell-containing droplet generation-------------------------

cells = 1;             % on/off for cell-containing droplet generation;
                       % 0 is off and 1 is on
cellpulse = 25;        % mbar; pulse strength for cell-containing droplet
cell_continuous = 3;   % mbar; pulse strength for continuous phase
cellpulsetime = 0.2;   % sec; pulse duration
cellpausetime = 4;     % sec; pause duration before transport
cellguideamount = 10; % mbar; release pressure strength for continous phase
cellguidingtime = 45; % sec; duration of release/ transport of droplet

% Paramters for nutrient droplet droplet generation ----------------------

pulseamount = 12;       % mbar; pulse strength for nutrient droplet
nut_continuous = 7.5;   % mbar; pulse strength for continuous phase
pulsetime = 0.3;        % sec; pulse duration
pausetime= 5;           % sec; pause duration before transport
guideamount = 30;       % mbar;release pressure strength for continous phase
guidingtime = 40;       % sec; duration of transport of droplet
guidecom = 12;          % compensation pressure for channel 3

%Parameters for coalescence-----------------------------------------------
```

```
Octanolpulse = 350;     % mbar; pulse strength for poor solvent
Octanolpulsetime = 6;   % sec; duration of the coalescence pulse

zerotime = 10;          % sec; pretty insignificant time, ensures a zero
                        % state before pulsing, which should already be there
totalwait = 900;        % sec; total waiting time between two droplets

fixtime = pulsetime+pausetime+guidingtime+zerotime+Octanolpulsetime;
waittime = totalwait-fixtime  % in seconds, pause between old droplet end
                              % and new droplet start
droplets = 16;                % Number of nutrient droplets

% End of parameter declaration
% Begining of the Cell-containing droplet generation

for i = 1:4  % for 4 cell containing drolets

if cells == 1

    disp('Starting Cell-containing droplet routine');
    Chann = 1;
    PressureValue = p_1;
    mfcs_set_auto(HandleNumber,PressureValue,Chann,AlphaValue);

    Chann = 2;
    PressureValue = p_2;
    mfcs_set_auto(HandleNumber,PressureValue,Chann,AlphaValue);

    Chann = 3;
    PressureValue = p_3;
    mfcs_set_auto(HandleNumber,PressureValue,Chann,AlphaValue);

    Chann = 4;
    PressureValue = p_4;
    mfcs_set_auto(HandleNumber,PressureValue,Chann,AlphaValue);

    pause(zerotime);

    %create
    disp('Creating a cell-containing droplet through pulse');
    Chann = 1;
    PressureValue = p_1-cell_continuous;
    mfcs_set_auto(HandleNumber,PressureValue,Chann,AlphaValue);

    Chann = 2;
    PressureValue = p_2 + cellpulse;
    mfcs_set_auto(HandleNumber,PressureValue,Chann,AlphaValue);

    Chann = 3;
    PressureValue = p_3;
    mfcs_set_auto(HandleNumber,PressureValue,Chann,AlphaValue);

    Chann = 4;
    PressureValue = p_4;
    mfcs_set_auto(HandleNumber,PressureValue,Chann,AlphaValue);
    pause(cellpulsetime);
```

```
%pause
disp('Pause before transporting the cell-containing droplet to trap');
Chann = 1;
PressureValue = p_1-cell_continuous;
mfcs_set_auto(HandleNumber,PressureValue,Chann,AlphaValue);

Chann = 2;
PressureValue = p_2;
mfcs_set_auto(HandleNumber,PressureValue,Chann,AlphaValue);

Chann = 3;
PressureValue = p_3;
mfcs_set_auto(HandleNumber,PressureValue,Chann,AlphaValue);

Chann = 4;
PressureValue = p_4;
mfcs_set_auto(HandleNumber,PressureValue,Chann,AlphaValue);

pause(cellpausetime);

%transport
disp('Transporting cell-containing droplet to the trap');
Chann = 1;
PressureValue = p_1 + cellguideamount;
mfcs_set_auto(HandleNumber,PressureValue,Chann,AlphaValue);

Chann = 2;
PressureValue = p_2;
mfcs_set_auto(HandleNumber,PressureValue,Chann,AlphaValue);

Chann = 3;
PressureValue = p_3;
mfcs_set_auto(HandleNumber,PressureValue,Chann,AlphaValue);

Chann = 4;
PressureValue = p_4;
mfcs_set_auto(HandleNumber,PressureValue,Chann,AlphaValue);

pause(cellguidingtime);

%zero
disp('Zeroing 3');

Chann = 1;
PressureValue = p_1;
mfcs_set_auto(HandleNumber,PressureValue,Chann,AlphaValue);

Chann = 2;
PressureValue = p_2;
mfcs_set_auto(HandleNumber,PressureValue,Chann,AlphaValue);

Chann = 3;
PressureValue = p_3;
mfcs_set_auto(HandleNumber,PressureValue,Chann,AlphaValue);

Chann = 4;
PressureValue = p_4;
```

```
    mfcs_set_auto(HandleNumber,PressureValue,Chann,AlphaValue);

    pause(zerotime)

    disp('Cell-containing droplet generated!');

    disp('Starting nutrient droplet generation');
end
end %End of cell-containing droplet generation routine


for i = 1:droplets

    %zero
    disp('Starting new nutrient droplet generation routine.');
    Chann = 1;
    PressureValue = p_1;
    mfcs_set_auto(HandleNumber,PressureValue,Chann,AlphaValue);

    Chann = 2;
    PressureValue = p_2;
    mfcs_set_auto(HandleNumber,PressureValue,Chann,AlphaValue);

    Chann = 3;
    PressureValue = p_3;
    mfcs_set_auto(HandleNumber,PressureValue,Chann,AlphaValue);

    Chann = 4;
    PressureValue = p_4;
    mfcs_set_auto(HandleNumber,PressureValue,Chann,AlphaValue);

    pause(zerotime);
    waittime = waittime - zerotime;


    %create
    str=sprintf('Creating No.%d nutrient droplet.',j);
    disp(str);

    Chann = 1;
    PressureValue = p_1-nut_continuous;
    mfcs_set_auto(HandleNumber,PressureValue,Chann,AlphaValue);

    Chann = 2;
    PressureValue = p_2;
    mfcs_set_auto(HandleNumber,PressureValue,Chann,AlphaValue);

    Chann = 3;
    PressureValue = p_3 + pulseamount;
    mfcs_set_auto(HandleNumber,PressureValue,Chann,AlphaValue);

    Chann = 4;
    PressureValue = p_4;
    mfcs_set_auto(HandleNumber,PressureValue,Chann,AlphaValue);

    pause(pulsetime);
```

```
% pause
disp('Pause before guiding');

Chann = 1;
PressureValue = p_1-nut_continuous;
mfcs_set_auto(HandleNumber,PressureValue,Chann,AlphaValue);

Chann = 2;
PressureValue = p_2;
mfcs_set_auto(HandleNumber,PressureValue,Chann,AlphaValue);

Chann = 3;
PressureValue = p_3 - 1.5;
mfcs_set_auto(HandleNumber,PressureValue,Chann,AlphaValue);

Chann = 4;
PressureValue = p_4;
mfcs_set_auto(HandleNumber,PressureValue,Chann,AlphaValue);

pause(pausetime);

%transport
disp('Guiding nutrient droplet to the trap');
Chann = 1;
PressureValue = p_1+ guideamount;
mfcs_set_auto(HandleNumber,PressureValue,Chann,AlphaValue);

Chann = 2;
PressureValue = p_2;
mfcs_set_auto(HandleNumber,PressureValue,Chann,AlphaValue);

Chann = 3;
PressureValue = p_3 + guidecom;
mfcs_set_auto(HandleNumber,PressureValue,Chann,AlphaValue);

Chann = 4;
PressureValue = p_4;
mfcs_set_auto(HandleNumber,PressureValue,Chann,AlphaValue);

pause(guidingtime);

%coalescence

disp('Coalescing');

Chann = 1;
PressureValue = p_1;
mfcs_set_auto(HandleNumber,PressureValue,Chann,AlphaValue);

Chann = 2;
PressureValue = p_2;
mfcs_set_auto(HandleNumber,PressureValue,Chann,AlphaValue);

Chann = 3;
PressureValue = p_3;
mfcs_set_auto(HandleNumber,PressureValue,Chann,AlphaValue);
```

```
Chann = 4;
PressureValue = p_4 + Octanolpulse;
mfcs_set_auto(HandleNumber,PressureValue,Chann,AlphaValue);

pause(Octanolpulsetime);

creepnumber = fix(waittime/25)      %integer
subwaittime = waittime/creepnumber;

%zero
disp('Zeroing 3');

Chann = 1;
PressureValue = p_1;
mfcs_set_auto(HandleNumber,PressureValue,Chann,AlphaValue);

Chann = 2;
PressureValue = p_2;
mfcs_set_auto(HandleNumber,PressureValue,Chann,AlphaValue);

Chann = 3;
PressureValue = p_3;
mfcs_set_auto(HandleNumber,PressureValue,Chann,AlphaValue);

Chann = 4;
PressureValue = p_4;
mfcs_set_auto(HandleNumber,PressureValue,Chann,AlphaValue);


str=sprintf('Done with run %d', i);
disp(str);
fprintf( fid, '%s\nDone with run %d without crashing. Time passed:
d minutes. \n\n', datestr(now), i, round((i*(zerotime+Octanolpulsetime
+guidingtime)+(i-1)*waittime)/60));

%waiting
if i ~= droplets
    str=sprintf('waiting for %d seconds until new droplet', waittime);
    disp(str);
    for i=1:creepnumber
        str=sprintf('Subwaittime is %d', subwaittime);
        disp(str);
        a = subwaittime/2-creeptime/2;
        pause(a);

        str=sprintf('Half the subwaittime is over, %d creep starts,
        pushing the interface a for
        d seconds to avoid sticking', i ,creeptime);
        disp(str);
        Chann = 1;
        PressureValue = p_1;
        mfcs_set_auto(HandleNumber,PressureValue,Chann,AlphaValue);

        Chann = 2;
        PressureValue = p_2;
        mfcs_set_auto(HandleNumber,PressureValue,Chann,AlphaValue);
```

```
            Chann = 3;
            PressureValue = p_3+creepamount;
            mfcs_set_auto(HandleNumber,PressureValue,Chann,AlphaValue);

            Chann = 4;
            PressureValue = p_4;
            mfcs_set_auto(HandleNumber,PressureValue,Chann,AlphaValue);

            pause(creeptime);
            str=sprintf('Done! Waiting for the rest...');
            disp(str);

            Chann = 1;
            PressureValue = p_1;
            mfcs_set_auto(HandleNumber,PressureValue,Chann,AlphaValue);

            Chann = 2;
            PressureValue = p_2;
            mfcs_set_auto(HandleNumber,PressureValue,Chann,AlphaValue);

            Chann = 3;
            PressureValue = p_3;
            mfcs_set_auto(HandleNumber,PressureValue,Chann,AlphaValue);

            Chann = 4;
            PressureValue = p_4;
            mfcs_set_auto(HandleNumber,PressureValue,Chann,AlphaValue);

            pause(a); % finishing the waiting
        end
    end
end  % Nutrient droplet generation routine

% End of feeding phase
disp('Completion of the Experiment!');
mfcs_close(HandleNumber, 'CloseLib');
fclose(fid);
% End of my code
```

4.B. FED-BATCH DROPLET NANOBIOREACTOR MOVIES

Figure 4.B.1: Illustration of feeding of reagent droplets to a water droplet showing a constant feeding profile as reported in Figure.4.2.3(a). To see the movie scan the QR code or click here.

Figure 4.B.2: Controlled microbial fed-batch growth of textitCyberlindnera (Pichia) jadinii with a feeding concentration of 0.5 g/L glucose and feeding frequency of 1 droplet/15 mins as reported in Figure.4.2.4.To see the movie scan the QR code or click here.

4.C. DRAWING OF FED-BATCH MICROFLUIDIC CHIP

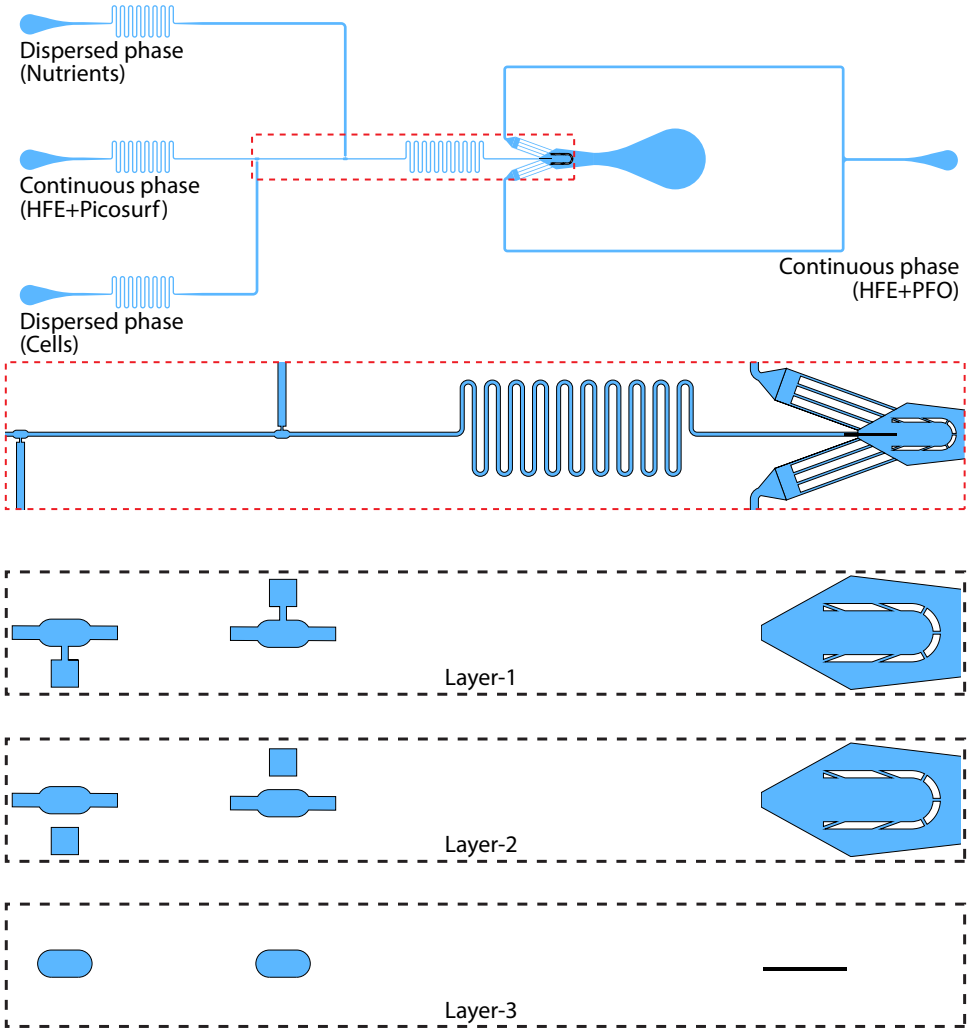

Figure 4.C.1: Drawing for fed-batch droplet nanobioreactor chip. Red box illustrates a zoomed out version of the main regions. Features of the microfluidic chip on different layers have also been shown.

4.D. GROWTH CURVES FOR THE NUTRIENT-CONTROLLED GROWTH EXPERIMENTS

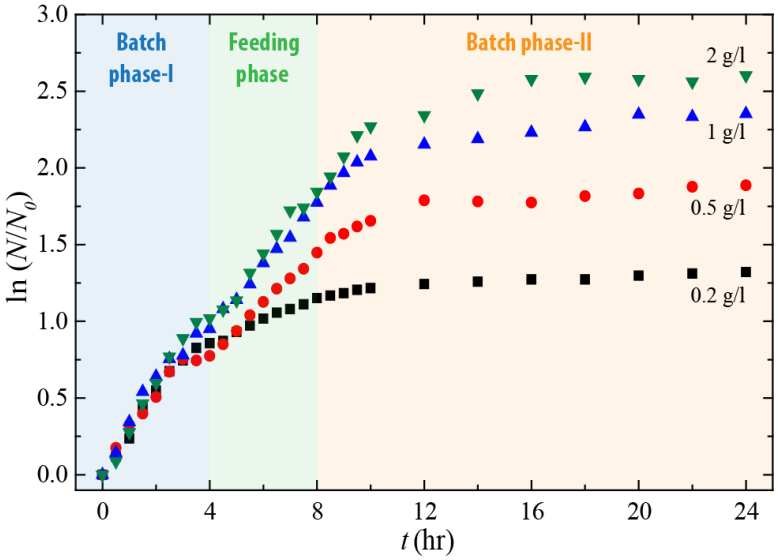

Figure 4.D.1: Growth curves for the nutrient-controlled growth experiments with cell number N normalized by the initial number of cells N_0 for each of the four experiments. Same data as in Figure.4.2.6, but in a different representation, highlighting that the behaviour in batch phase-I is the same for all four curves, as expected.

4.E. MIXING INSIDE FED-BATCH DROPLET NANOBIOREACTOR

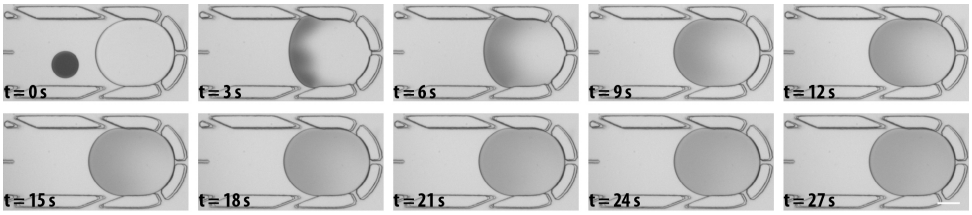

Figure 4.E.1: Time lapse illustrating mixing inside a trapped droplet upon coalescence with an incoming reagent droplet (containing ink). The intensity gets uniform within 30 seconds, showing mixing is complete. Scale bar is 100 μm

4.F. CLOSE-UP OF CELLS INSIDE MICRODROPLET

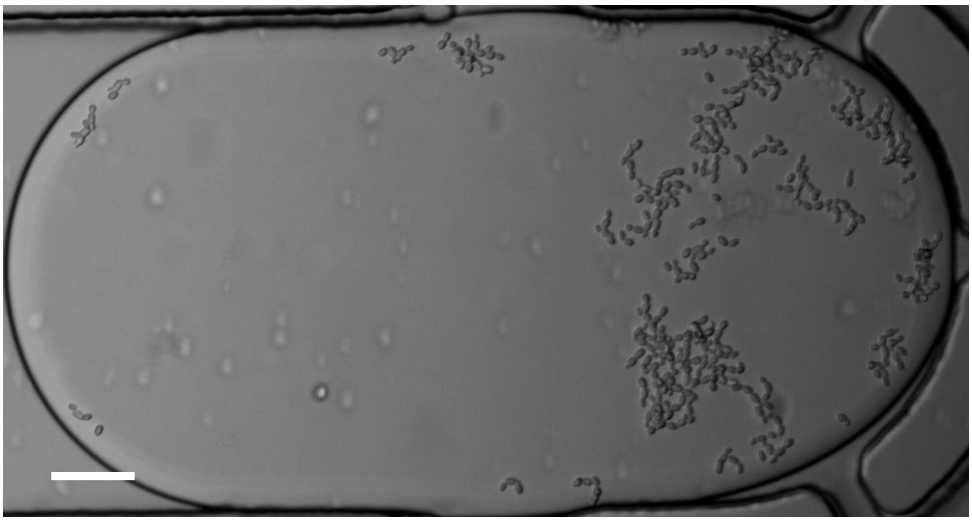

Figure 4.F.1: Close-up of a grey scale micrograph obtained during incubation of Cyberlindnera (Pichia) jadinii cells inside a droplet immobilized in the fed-batch droplet nanobioreactor. Scale bar is 100 μm

4.G. PREPARATION OF VITAMINS AND TRACE ELEMENTS

The protocol for preparation of 2000 g of vitamin solution is described below and further detailed in Table.4.G.1.

1. 100 ml of 1.0M NaOH solution, 50 ml of 0.1M NaOH solution and 5 ml of 1.0M HCl solution are prepared by dissolving NaOH and HCl in the required amounts in demi water.
2. 0.1 g biotin is dissolved in 20 ml of the 0.1M NaOH solution.
3. The biotin solution is added to 1.6 kg demi water and the pH is adjusted to 6.5 with the 1.0M HCl solution.
4. All remaining components (see Table.4.G.1) are dissolved one by one with readjustment of the pH to 6.5 after each addition using the 1.0M NaOH solution.
5. After all components are added the final weight is adjusted to 2000 g while keeping the pH at 6.5.
6. The final solution is pipetted in portions of 1ml/2ml in Eppendorf vials for preparation of defined medium and stored in a –20°C freezer until use.

Table 4.G.1: Components and their amounts used to prepare the vitamin solution.

Component	**Desired amount**
0.1 M NaOH solution	20 ml
D-biotine (vit. D)	0.1 g
demineralized water	1600 g
1.0 M HCl solution until pH 6.5	1 ml
Ca D(+) panthotenate	2 g
1.0 M NaOH solution until pH 6.5	-
nicotinic acid	2 g
1.0 M NaOH solution until pH 6.5	14 ml
myo-inositol	50 g
thiamine chlorid hydrochloride (vit. B1)	2 g
1.0M NaOH solution until pH 6.5	5 ml
pyridoxol hydrochloride	2 g
1.0M NaOH solution until pH 6.5	10 ml
p-aminobenzoic acid	0.4 g
1.0M NaOH solution until pH 6.5	3 ml
demineralized water	until 2000 g

The protocol for preparation of 1000g of trace element solution is described below and further detailed in Table.4.G.2.

1. 1.0M NaOH solution and 1.0M HCl solution are prepared by dissolving NaOH and HCl in demi water.
2. EDTA and $ZnSO_4.7H_2O$ are dissolved in 700 g of demineralized water and the pH was adjusted to 6.0 with the 1.0M NaOH solution.
3. While maintaining the pH at 6.0 the components listed in Table.4.G.2 are dissolved one by one.
4. The pH is adjusted to 4.0 with the 1.0M HCl solution. Demi-water is added to reach a final weight of 1000 g.
5. The final solution is pipetted in portions of 1ml/2ml in Eppendorf vials for preparation of defined medium and stored in a –20°C freezer until use.

Table 4.G.2: Components and their amounts used to prepare the trace element solution.

Component	**Desired amount(g)**
$Na_2EDTA.\ 2H_20$	15
$ZnSO_4.7H_20$	4.5
$MnCl_2.4H_20$	1
$CoCl_2.6H_20$	0.3
$CuSO_4.5H_20$	0.3
$Na_2MoO_4.2H_2O$	0.4
$CaCl_2.2H_20$	4.5
$FeSO_4.7H_20$	3
H_3BO_3	1
KI	0.1

4.H. TOXICITY OF COALESCENCE AGENT

To check whether the presence of PFO in the oil phase leads to observable inhibition of cellular growth, we cultured *Cyberlindnera (Pichia) jadinii* in defined media that is in contact with a solution of PFO in HFE-7500. For simplicity, these experiments were performed off-chip in a regular incubator. The cell solution was prepared by thawing a cryovial from the stock at room temperature and pipetting 50 μl of cell solution in 5 ml of defined media (20 g/l glucose). After preparing miscible PFO in HFE-7500 solutions with the PFO concentration ranging between 0.1 and 50 v/v %, we added 5 ml of these solutions to 5 ml of cell solution already pipetted into 15 ml centrifuge tubes. We also included a control in which 5 ml of pure HFE-7500 was added. The tubes with the resulting two-phase system, the lighter cell solution at the top, were sealed with cotton wool and incubated at 30°C and 200 rpm. In order to check whether the growth of *Cyberlindnera (Pichia) jadinii* is inhibited by the presence of PFO , the tubes were temporarily taken out of the incubator after 4, 9 and 22h to take out 50 μl of cell solution. The cell solutions were pipetted into a cell counting chamber mounted on the same microscope setup as used for the chip experiments. No discernible differences in cell density are observed between lower PFO concentrations and the control, see Figure.4.H.1. Growth inhibitions are visible for the 10 % solution, although cells still reach a significant cell density after 22 h. For the 50 % solution, no growth is observed. Based on this set of qualitative experiments, we chose to work with a 5 % solution in the chip experiments, which we expect not to display discernable growth inhibitions, yet resulting in effective coalescence within seconds. It is furthermore important to note that in contrast to a continuous exposure to PFO in this experiment, the 5 % PFO in HFE-7500 solution is only pulsed for a short duration to coalesce the nutrient droplet and cell-containing droplet, such that we do not expect inhibitory effects to play a role in the chip experiments. Finally, we note that in the selection of the solvent to induce chemical coalescence, we also considered

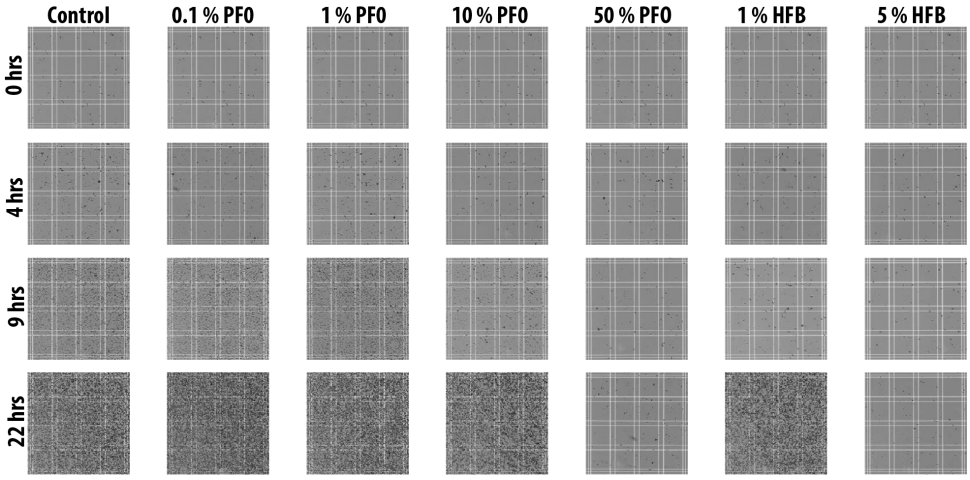

Figure 4.H.1: Toxicity studies of coalescence agents. Growth of *Cyberlindnera (Pichia) jadinii* for 22 hours in environments with different concentrations of PFO and HFB.

2,2,3,3,4,4,4-Hepta-fluroButanol (HFB). As evident from the figure, it displays already inhibitory effects at a concentration of 1 % and it was further excluded in this work.

4.I. KINETIC CELL GROWTH MODEL

The amount of biomass, X (in grams), and the amount of substrate, S (in grams), inside the droplet are governed by two coupled mass conservation equations

$$\frac{dX}{dt} = \mu X \tag{4.I.1}$$

and

$$\frac{dS}{dt} = -q_s X \tag{4.I.2}$$

with μ the specific cell growth rate and q_s the specific substrate consumption rate. These two rates are coupled through the Pirt equation.

$$q_s = \frac{\mu}{Y_{X/S}} + m_s \tag{4.I.3}$$

with $Y_{X/S}$ the biomass yield on substrate and m_s the cell maintenance rate. The specific substrate consumption rate depends on the substrate concentration C_s, hence the ability to control the specific consumption rate and, in turn, the specific cell growth rate using the substrate concentration. The dependence of the specific substrate consumption rate on the substrate concentration is described as

$$q_s = q_{s,\max}\left(\frac{C_s}{K_s + C_s}\right) \tag{4.I.4}$$

with $q_{s,max}$ the maximum specific consumption rate and K_s the substrate affinity constant. When $C_s \gg K_s$, the specific consumption rate is maximum and independent of the substrate concentration: $q_s \approx q_{s,max}$.
The above differential equations can now be reformulated in terms of the maximum specific consumption rate $q_{s,max}$ and the glucose concentration C_s to obtain

$$\frac{dX}{dt} = Y_{X/S}\left(\frac{q_{s,\max} C_s}{K_s + C_s} - m_s\right) X \tag{4.I.5}$$

and

$$\frac{dC_s V}{dt} = -q_{s,\max}\left(\frac{C_s}{K_s + C_S}\right) X \tag{4.I.6}$$

where we replaced the amount of substrate, S, by the product of the substrate concentration and the volume V of the droplet. These two coupled differential equations describe how the amount of biomass X and the glucose concentration C_s change in time. The initial conditions used are: $X(t=0) = X_0$, $V(t=0) = V_{m0}$ and $C_s(t=0) = C_{s0}$. The four model parameters $Y_{X/S}$, $q_{s,max}$, K_s, and m_s are specific to the here cultured *Cyberlindnera (Pichia) jadinii* cells under the here cultured conditions.

We solved equation.4.I.5 and 4.I.6 in MATLAB using ODE45. We hereby described each time window between nutrient additions as a batch phase. After a batch phase, we determined the volume of the droplet, the amount of biomass, and the substrate concentration. We then adjusted these numbers to those obtained after the addition of a nutrient droplet with a given volume and substrate concentration. These obtained values were then used as initial conditions for the subsequent batch phase. In this way, we computed growth curves for the 24 hour time lapses.
We firstly used the model to estimate the operating window for nutrient-controlled growth, with estimates of the parameters for $Y_{X/S}$, $q_{s,max}$, K_s, and m_s from literature. Secondly, we used the model to fit values of these parameters based on the experimental data in Figure.4.2.6. Rather than fitting four parameters, we used estimates of m_s and K_s from literature, leaving $q_{s,max}$ and $Y_{X/S}$ as the remaining unknowns. We determined $q_{s,max}$ as follows: First, we considered batch phase-I in the set of experiments with unconstrained growth and determined the specific growth rates μ for the three curves in Figure.4.2.5 by fitting the data for which exponential growth was observed. Second, we used the obtained values for μ to determine the maximum specific growth rate μ_{max} using the relations $\mu = Y_{X/S}\left(q_{s,\max}\frac{C_s}{K_s+C_s} - m_s\right)$ and $\mu_{\max} = Y_{X/S}\left(q_{s,\max} - m_s\right)$ to give $\mu_{\max} = \mu\frac{K_s+C_s}{C_s} - m_s\left(Y_{X/S}\frac{C_s}{K_s+C_s} - 1\right)\frac{K_s+C_s}{C_s}$. Given the last term is expected to be negligible, we estimated the maximum growth rates for the three curves using $\mu_{\max} = \mu\frac{K_s+C_s}{C_s}$ with C_s taken as C_{s0}. An average of the three values was taken to find $\mu_{max} = 0.32hr^{-1}$. Third, we used the aforementioned relation to obtain the following expression for the maximum specific consumption rate: $q_{s,\max} = \frac{\mu_{\max}}{Y_{X/S}} + m_s$. This expression was put into equation.4.I.5 and 4.I.6, leaving the yield $Y_{X/S}$ as the last remaining unknown. We determined its value based on the data sets for controlled growth in Figure.4.2.6. Given that the yield is known to depend on the extent of nutrient limitation, we determined two values, one for batch phase-I, another one for the combination of the feeding phase and batch phase-II. This was done by computing growth curves for a large number of different values of $Y_{X/S}$ and determining the value for which the mean square error between model and experimental data was smallest.

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5

MICROFLUIDIC TOOL FOR DISSOLVED OXYGEN

Microfluidic static droplet array for studying microbial growth under different levels of dissolved oxygen

We develop a scalable microfluidic platform that enables studying the growth of microorganisms under controlled oxygen levels. The microorganisms are cultured inside droplets stored in chambers on a microfluidic device, while the level of oxygen is controlled through the amount of oxygen dissolved in the fluorinated oil that flows around the droplets. Our study is motivated by the need for lab-scale experimental analysis of industrial growth conditions in bioprocess development. We present a proof-of-concept study in which we studied two limiting cases, using the yeast Cyberlindnera Jadinii as the model microorganism. The first case is with oxygen-saturated fluorinated oil, where significant growth for a prolonged duration is seen. The second case is with oil deprived of oxygen where negligible growth is observed. While we impose static conditions for these two limiting cases, the platform can be further extended to study microorganisms under dynamically controlled oxygen levels.

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5.1. INTRODUCTION

Dissolved oxygen is an important nutrient that is consumed by microorganisms for growth, maintenance and production of metabolites[1]. Owing to its poor solubility in aqueous solutions[2], oxygen (air) is supplied continuously to industrial-scale bioreactors via a sparger below the impeller. Since the solubility depends on pressure, a gradient in hydrostatic pressure results in a gradient in oxygen levels[3]. Additionally, in industrial scale fed-batch reactors, in which concentrated nutrients are supplied continuously from the top, cell densities and oxygen consumption rates are largest near the feeding zone. This leads to oxygen limitations near the top, giving rise to gradients in dissolved oxygen levels across the reactors[4]. Therefore, microorganisms traveling through the reactors constantly experience different levels of dissolved oxygen[5]. These variations experienced by the microorganisms not only affect the cellular physiology and productivity but also lead to production of undesired side products. It is therefore important to be able to study the sensitivity of microorganisms towards differences in dissolved oxygen levels when developing bioprocesses[6].

Microtiter plates and shake flasks comprise state-of-the-art tools for initial bioprocess development as they allow studying a large number of mutant strains and process conditions in order to select the optimum combination. However, these tools lack the ability to impose dynamically changing environments due to the lack of precise and rapid control of the supply of nutrients or dissolved oxygen[7, 8]. Microfluidic tools are known for their regulation of minute amounts of fluid, enabling precise and rapid control of biological micro-environments[9, 10]. For gases like oxygen, permeable membranes are typically used to facilitate the exchange with the surrounding environment, with the supply rates controlled by the concentration in the environment and the permeability of the membrane[11]. Alternatively, supply can be achieved through dedicated supply channels incorporated into the microfluidic devices and separated from the location of interest by a thin permeable wall. Apart from diffusion of oxygen through permeable membranes or walls, liquids pre-equilibrated with oxygen are also used as a source of oxygen. Fluorinated oils such as HFE-7500 and FC-40 are known for their high oxygen solubility[12–14]. Such oxygen-rich oils have also been supplied to shake flasks and large-scale bioreactors in order to increase the oxygen supply[15]. In microchannels, fluorinated oils can be flown around aqueous cell-containing droplets as a means to supply or remove dissolved oxygen. Mahler and co-workers[16] generated droplets on-chip and fed these into an incubator vessel containing a fluorinated oil, with marked effect on culture growth inside the droplets during 1-25 hours by slowly feeding oxygen-saturated oils into the vessel, in comparison with control experiments with the same oil stagnant in the vessel without refreshing it. Essentially, the incubator eliminated oxygen-supply limitations, while the gentle flow of oil caused less droplet coalescence and droplet evaporation than cultivation in microtiter-plates and shake flasks, two benchmark systems that the incubator was compared with. In contrast to the slower dynamics of cultivation, Abbyad and co-workers created a flow focussing droplet generator with oxygen-saturated fluorinated oil that suddenly changed the dissolved oxygen concentration inside droplets containing haemoglobin, in order to study its polymerisation in the context of sickling of red blood cells[17]. These works highlight the capabilities of droplet-based microfluidic systems to study a large number of microorganisms and process conditions

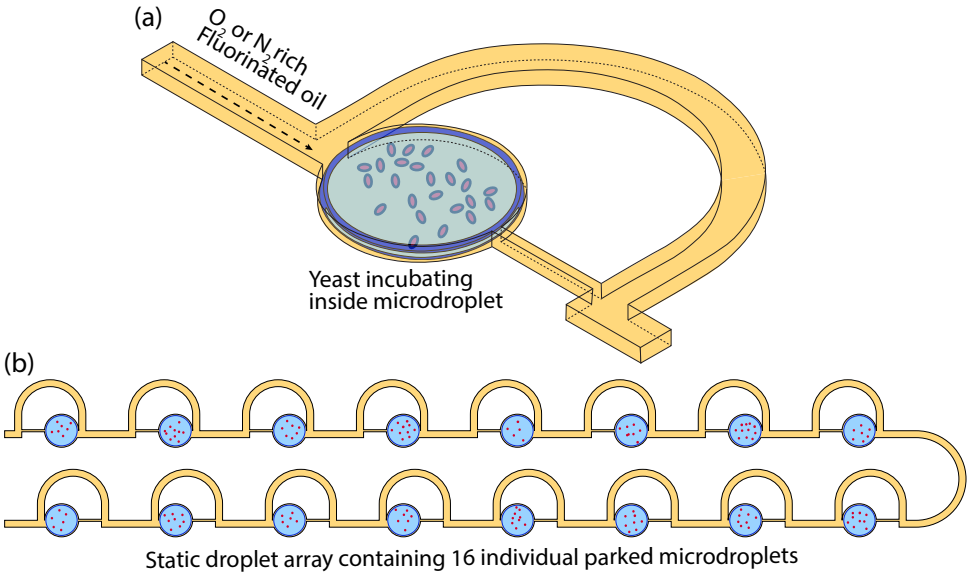

Figure 5.1.1: (a) Three dimensional schematic of a single trap with a cell-containing droplet incubated in a growth chamber, while the supply of dissolved oxygen to the cells is controlled through the amount of oxygen dissolved into the oil phase that continuously flows around the droplet through the bypass. (b) Design of the scaled out version of the microfluidic device with 16 growth chambers, which are automatically monitored using a programmable microscope stage in order to measure cell growth of the model microorganism *C. Jadinii*.

under controlled dissolved oxygen levels.

In this chapter, we developed a microfluidic platform for on-chip cultivation of microorganisms inside microdroplets with control over dissolved oxygen levels. The microfluidic chip comprises of an array of chambers where cell-containing droplets are created and trapped, while the oxygen supply to the cells inside the droplets is regulated through the amount of oxygen dissolved in the fluorinated oil that continuously flows around the droplets, see Figure.5.1.1. We studied the growth of the model microorganism *Cyberlindnera Jadinii* under two limiting conditions, using oxygen-saturated oil resulting in unlimited exponential growth and using oxygen-deprived oil resulting in negligible growth. These results and the developed platform offer a sound base to study cells under dynamically controlled oxygen levels.

5.2. RESULTS AND DISCUSSION

5.2.1. QUANTIFICATION OF GROWTH OF *C. Jadinii* INSIDE MICRODROPLETS

The microfluidic chip comprises of an array of traps for formation and storage of droplets. The design of the traps is inspired by the work of Boukellal and co-workers[18]. A trap consists of two parallel channels with different hydrodynamic resistances. One channel includes a circular chamber connected to a constricted via, the second channel acts as a bypass around the chamber, see Figure.5.1.1(a). The protocol for trapping of mi-

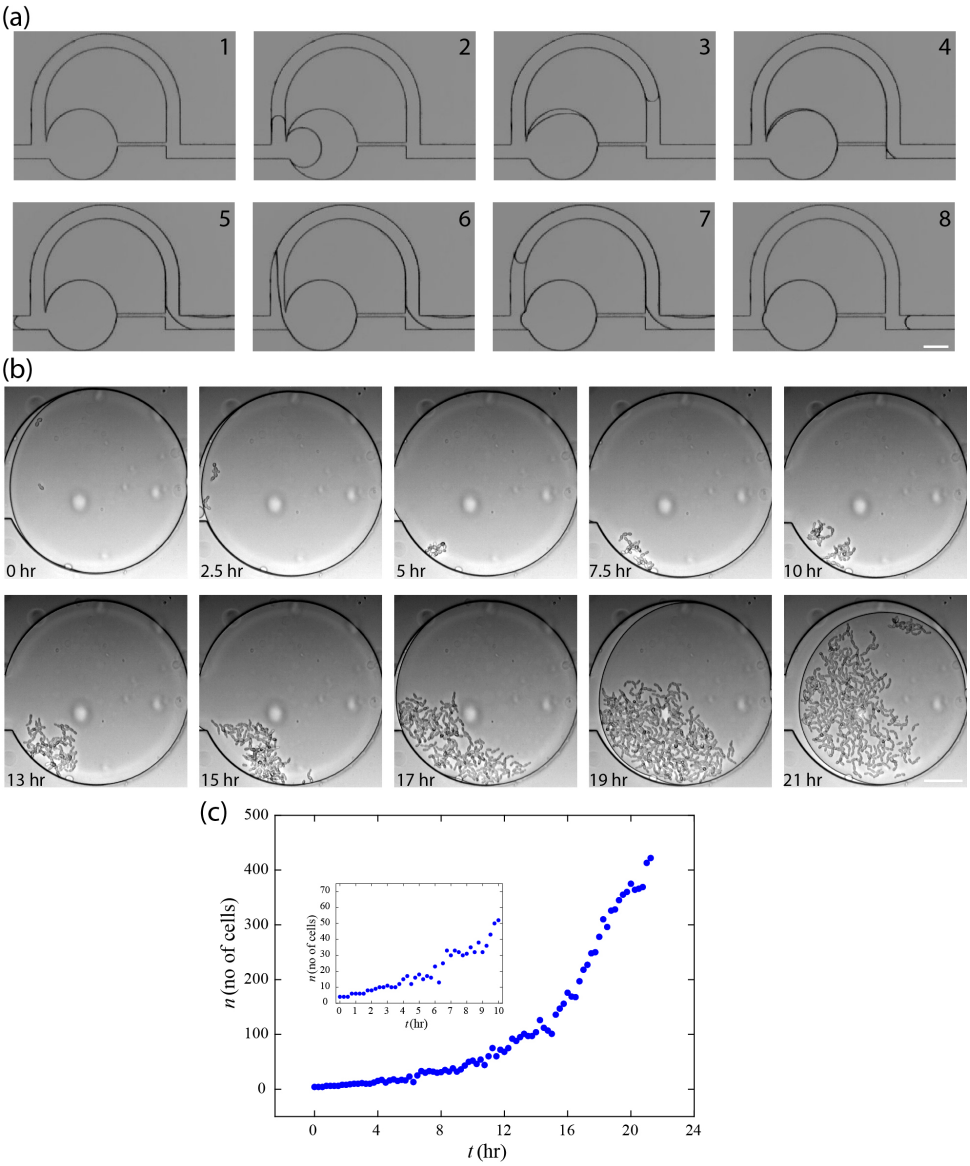

Figure 5.2.1: (a) Protocol for creating cell-containing droplets in the chambers of the device. The microfluidic device is pre-filled with fluorinated oil (1) after which growth medium containing yeast cells is flown into the device (2). This solution displaces the oil from the circular chamber and bypass (3, 4). Once all bypasses and chambers are filled, fluorinated oil is re-introduced (5). The fluorinated oil displaces the cell solution from the bypass channel, leading to snap-off (6,7) and the formation of a droplet in the chamber (8). Scale bar is 100μm (b) Time lapse grey scale images illustrating the growth of *C. Jadinii* inside a single microdroplet. See the corresponding movie by scanning Figure.5.A.1.Scale bar is 100μm. (c) Growth curve for batch cultivation of *C. Jadinii* at 30°C. A shallow chamber (10 μm in height) and a good image resolution enable quantification of the growth by cell counting in an automated fashion via MATLAB.

crodroplets is illustrated in Figure.5.2.1(a). Firstly, the microfluidic device is filled with fluorinated oil (HFE-7500 and Picosurf-1) (1). The oil flow is kept on until trapped air bubbles are dissolved in the oil. Once the device is entirely filled with oil, the aqueous phase comprising of cells and growth media is flown into the device. The front of this phase enters the circular chamber and bypass channel simultaneously (2). The chamber fills up, while the constriction acts as a Laplace pressure barrier, preventing the interface from being pushed through such that the via remains filled with oil (3, 4). The plug moves ahead (4) filling all traps in a similar manner. Once all traps are filled, oil is reintroduced into the device (5). Oil flows primarily through the bypass channel due to its lower hydrodynamic resistance, displacing the aqueous phase from the bypass, but not from the chamber. This leads to snap-off (6,7) and the creation of a droplet inside the chamber (8).

Cell growth experiments were performed using the yeast *C. Jadinii* as the model organism. We choose *C. Jadinii* specifically for its relatively simple growth characteristics. Since its growth is severely limited under an oxygen limited environment[19], the influence of dissolved oxygen levels is directly inferred from the growth behaviour. We measured the growth by making high-resolution time lapse images of each chamber, using a programmable microscope stage. An illustration of a typical time lapse of a single droplet is show in Figure.5.2.1(b). Scan Figure.5.A.1 to see the corresponding movie. The use of shallow chambers (10 μm in height) enforces cells to grow in a single plane, facilitating imaging and counting of individual cells to construct growth curves such as the one shown in Figure.5.2.1(c). While a similar strategy has been used before[20, 21] for long term cell cultures, this approach has not yet been used to study the influence of dissolved oxygen levels on growth. Experiments using oxygen rich and oxygen deprived oil are presented next.

5.2.2. GROWTH UNDER OXYGEN RICH CONDITIONS

We performed a long term growth experiment with *C. Jadinii* inside microdroplets under batch conditions at 30°C. Microdroplets containing cells and defined growth medium supplemented with 20 g/L of glucose as carbon source and urea as the nitrogen source were trapped on chip. After trapping, droplets were incubated for 20 hours while fluorinated oil (HFE-7500) presaturated with oxygen was flowing around the droplets acting as the source of oxygen. We prepared the cell solution such that, on average, a few cells per droplet were encapsulated. Due to the non-homogeneous mixture of the solution, not all droplets contained cells. Yeast cells were encapsulated in 7 out of the 16 droplets, with the initial cell number ranging from 2 cells to 8 cells per droplet. Cells grew in all 7 droplets as evident from the snapshots at the end of the time lapse in Figure.5.2.2(a), showing that the cells were viable upon encapsulation. While measures were taken to mitigate shrinkage of droplets (see materials and methods), moderate shrinkage was observed during the 20 hour incubation. This is attributed to evaporation of water though the PDMS matrix. The growth curves are shown in Figure.5.2.2(b). A clear exponential growth can be observed in all 7droplets. This indicates that the cells experienced no oxygen or nutrient limitations during cultivation. The average of the maximum specific growth rate of the cells in these 7 droplets is 0.19 ± 0.02 hr^{-1}, slightly lower than found in our previous work[22]. While the stationary phase was not yet reached for all droplets af-

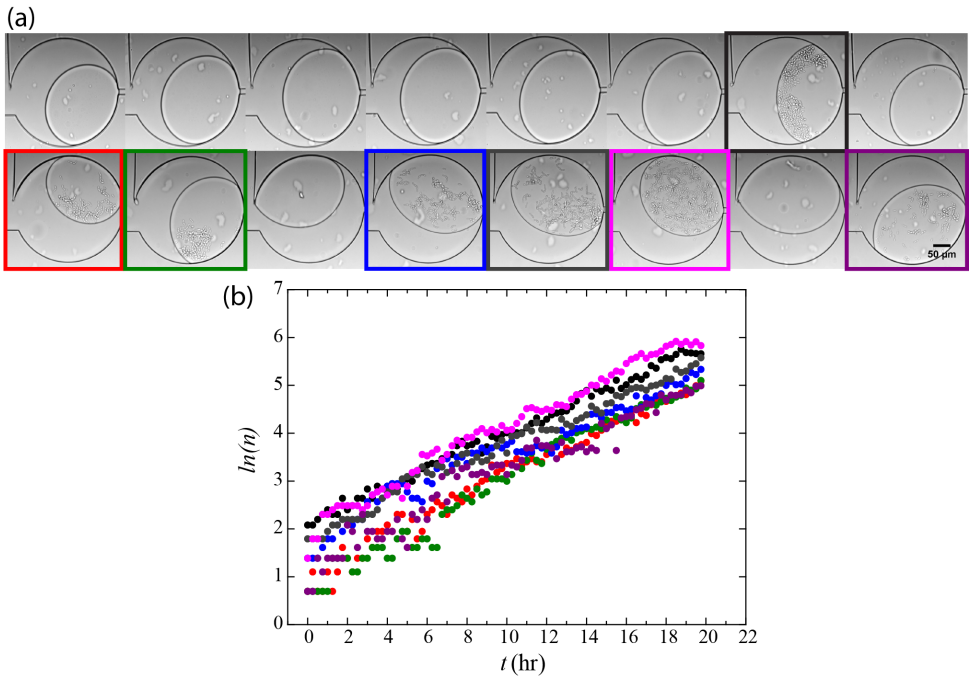

Figure 5.2.2: (a) Batch cultivation of *C. Jadinii* inside microdroplets surrounded by a continuous flow of HFE-7500 pre-saturated with oxygen, to establish an oxygen rich environment around the droplets. The grey scale images show the final snapshot of the time 20 hour time lapse. Yeast cells were encapsulated in 7 out of 16 droplets (coloured boxes). Scale bar is 50 μm. (b) Corresponding growth curves showing exponential growth. The average growth rate of yeast inside microdroplets at 30°C is 0.19±0.02.

ter 20 hours, we aborted the experiment as the droplets rapidly shrunk afterwards. This experiment served as the first limiting case showing growth under oxygen rich conditions.

5.2.3. GROWTH UNDER OXYGEN DEPRIVED CONDITIONS

After conducting growth experiments using oxygen saturated oil, we performed the same type of experiment, but with oxygen deprived oil. By bubbling the oil with nitrogen and flowing it around the trapped droplets an oxygen deprived environment is created for the cells. Two grey scale images for one of the traps, one at the start and another one at the end of the 20 hour time lapse illustrate negligible cell growth, see inset Figure.5.2.3. We quantified the growth for 7 out of 16 droplets containing cells, which show that the number of cells increases in all the 7 droplets, but marginally when compared to the experiments with oxygen saturated oil.

5.3. CONCLUSIONS

In this work, we present a simple microfluidic platform that enables studying batch growth of microorganisms such as the yeast *Cyberindrelia (Pichia) Jadinii* inside micro-

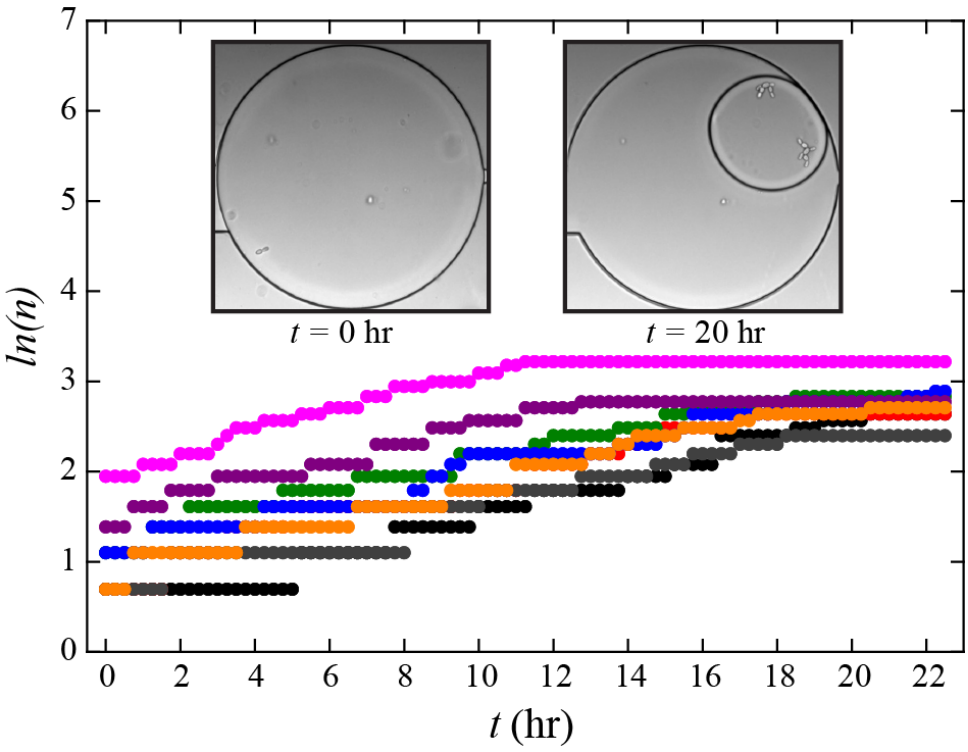

Figure 5.2.3: Growth curves for batch cultivation of *C. Jadinii* with a continuous flow of HFE-7500 pre-saturated with nitrogen. Inset images show cells inside a single trap at start and end of the time lapse. Growth curves show an increase in the number of cells, but marginally compared to experiments with oxygen-saturated oil.

droplets, where the dissolved oxygen concentration inside the droplets was controlled by leveraging the high oxygen carrying capacity of fluorinated oils such as HFE-7500. A microfluidic chip comprising of 16 hydrodynamic based traps arranged in series was used as a static droplet array. Microdroplets encapsulated with yeast were trapped and incubated on-chip, while oxygen supply to cells was controlled by the amount of oxygen dissolved in the oil flowing around the droplets. We studied cell growth for the two limiting cases: using oxygen saturated oil and oxygen deprived oil. While the droplets incubated using oxygen saturated oil showed exponential growth, negligible growth was observed using oxygen deprived oil. These results in which static oxygen levels were imposed form a sound base to extend the tool to enable studying cells under dynamically imposed conditions as encountered at industrial scale. One way to achieve this is by mixing two oil streams with oxygen saturated and oxygen deprived oil, with the (instantaneous) flow rates of these streams controlling the dynamically imposed oxygen levels.

5.4. MATERIALS AND METHODS

5.4.1. DEVICE FABRICATION

Microfluidic masters were fabricated from silicon wafers using E-beam lithography and dry etching. Firstly, a 4" silicon wafer was primed with AR 300-80 (Allresist GmbH Germany) by spin-coating at 4000 rpm for 1 min. The wafer was baked at 180°C for 2 mins. Next, the wafer was spin-coated with negative tone resist ARN7700.18 (Allresist GmbH Germany) at 4000 rpm for 1 min and was soft baked at 85°C for 60 s. A Lecia EBPG 5000+ was used for writing the desired pattern on the spin-coated wafer with a dosage of 117μCcm^{-2} (See Figure.5.B.1 for the complete drawing of microfluidic device). After exposure, the wafer was post-baked at 105°C for 2 mins. The patterns were developed in MF321 (Dow chemical) for 2 mins, followed by rinsing the wafer in diluted MF321 solution in water (10% v/v) for 30 s and finally in fresh de-ionised water for 60 s. 10 μm of deep reactive-ion etching was achieved using the Bosch process in a Plasma Pro 100 Estrelas (Oxford Instruments). The procedure comprised alternate steps of etching (with Sulphur hexafluoride, SF_6) and passivation (with Octafluorocylobutane, C_4F_8). The pressure was maintained at 0.05 mbar. The flow rate in etching step was 225 s.c.c.m SF6 for 8 s with ICP power at 2000 W, while the capacitive coupled plasma(CCP) was off. The passivation step was at flowrate of 80 s.c.c.m with C_4F_8 for 5 s with the ICP power at 2000 W. A constant temperature of 10°C was maintained during the complete procedure. Temperature of the chamber was maintained around 200°C. The rate of etching was kept around 3 μm/min. Resist was then removed using oxygen plasma in the same machine. After plasma surface treatment (40 mbar, 100 W, 3 min), the wafer is passivated against PDMS by exposing it to fumes of 200 μl 1H, 1H, 2H, 2H- perfluorooctyltrichlorosilane (Sigma Aldrich) in a desiccator under vacuum for 4 hours.

Microfluidic chips were casted out of PDMS (Slygard 184 elastomer kit, Dow corning). The elastomer and curing agent were mixed in 10:1 ratio by weight. The mixture was degassed and poured on the master wafer in a petridish. PDMS was cured for 3 hr in a convection oven at 70°C. After curing, PDMS was peeled off carefully from the wafer and diced to the desired sizes. The two inlets were punched with a 0.75 mm biopsy punch and the outlet was punched with a 1.5 mm biopsy punch. PDMS chips were cleaned with iso-propyl alcohol to get rid of any dust or debris. The devices were then bonded to microscopy glass slides that were spin coated with PDMS by exposing them to an oxygen plasma (Harrick, PDC-002) for 160 s at a pressure of 0.2-0.4 mbar. The microfluidic chips were then baked at 200°C for 2 hr for recovery of PDMS's hydrophobicity. Small PTFE tubing pieces (0.5 mm ID, 1/16 inch OD, 1.5 cm in length, Kinesis) were glued to the outlet. Stainless steel tubing (0.025 inch OD x 0.013 inch ID, 23g Elveflow) were glued to the inlets. In order to minimize water evaporation and subsequent droplet shrinkage during long term growth experiments, the prepared PDMS chips were soaked in de-mineralised water for 7 days.

5.4.2. EXPERIMENTAL SETUP

A schematic of the entire experimental setup is shown in Figure.5.4.1. As all the microfluidic experiments included long term cultivation of *C. Jadinii* inside the chip, it was important to control the temperature, humidity and gas environment around the microflu-

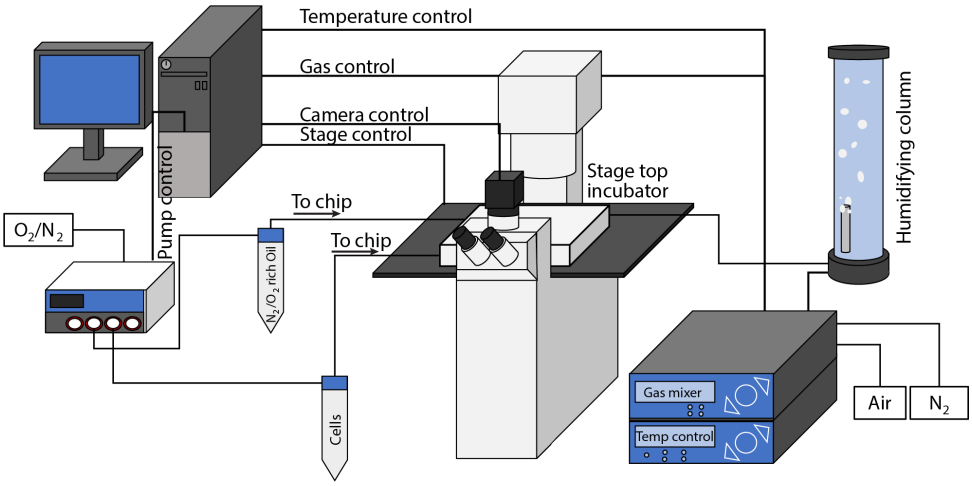

Figure 5.4.1: Experimental setup for performing long term cell growth experiments in a static droplet array device under oxygen rich and oxygen deprived conditions.

idic device. For performing the growth experiments, the microfluidic chip was put in a stage-top incubation system (Ibidi Gmbh) with active temperature and gas control. The incubation box was mounted on an inverted microscope (Axiovert S100, Zeiss). The microfluidic chip was fixed on the bottom plate, which was heated with a temperature set point of 30°C. The top plate temperature was also fixed at 30°C. The incubation box was supplied with a gas mixture, which was being saturated with water vapour through a humidifier bottle and flown through a jacketed tube. In order to minimize droplet shrinkage, the humidity set point was maintained at 99 %. The gas flow rate was maintained at 5.2 L/hr. The gas mixture composition was changed based on the experiment. For oxygen rich experiments N_2 was switched to 0 % and for oxygen deprived experiments O_2 was switched to 0 %. A pressure based flow controller (MFCS-4C 1000 mbar/7000 mbar, Fluigent) was used for injecting the liquids into the microfluidic chip. The driving gas for the pressure controller was either oxygen or nitrogen, such that the liquids in the supply reservoirs stay equilibrated with the respective gases. The ports on the pressure controller were connected to the reservoirs using silicone tubing and tightly sealed with parafilm. Soft walled tygon tubing (0.02 inches ID, 1/16 inch OD, 50 cm in length) was used to connect the reservoirs to the metal connectors glued to the inlets of the chips. Utmost care was given while connecting the tubing from the fluid reservoirs to the microfluidic chip and fixing the chip inside the incubator box. Once the entire setup was assembled, the pressure of the continuous phase was increased to drive the fluorinated oil into the chip. Once the chip was filled with oil, droplets containing *C. Jadinii* were trapped on chip by following the protocol described in the the main text.

5.4.3. IMAGE ACQUISITION AND ANALYSIS

Image were acquired using a CMOS camera (FL3-U3-120S3C-C, Point Grey) mounted on an inverted microscope (Axiovert S100, Zeiss) using a combination of a 20X objective and

a 0.63X mount objective. The open source program Micromanager was used for multi-dimensional image acquisition. The stage, microscope and camera were first connected to a computer and accessed via micromanager. The program uses ImageJ to control the image acquisition while synchronizing microscope control, stage motion and image acquisition through the camera. Once all the droplets were trapped with microorganisms, the location of each droplet was saved. Images were acquired for all the 16 locations during the course of entire experiment with a resolution of 4000px by 3000px and at a frame rate of 1 image every 15 mins. In order to maintain the stability of the setup during the experiments, stage drift speed was set as 5 mm/s. The images were acquired at 8-bit instead of the standard 16-bit to make sure that they were of a manageable size. Cell counting and quantification was performed by processing all the time lapse images using a self-written MATLAB script.

5.4.4. WORKING FLUIDS

Continuous phase

Fluorinated oil HFE-7500 (3M, Novec-7500 Engineered fluid) was used as the continuous phase for the microfluidic experiments. In order to ensure droplet stability and complete wetting of the PDMS walls by HFE-7500, 0.1% v/v Pico-Surf-1 (SpehereFluidics) was dissolved in HFE-7500. The oil was bubbled with oxygen or nitrogen for 6 hours before use.

Dispersed phase

Cell solution

Cyberindrelia (Pichia) Jadinii (CBS621) was acquired from the Westerdijk Fungal Biodiversity Institute, Utrecht, the Netherlands. Cell stocks were stored in 2 ml cryovials containing YPD and glycerol (1:10 by volume) inside a freezer at -80°C.

Preculture media

Before transferring the microorganisms to the defined medium used for on-chip experiments, the *C. Jadinii* cells were pre-cultured in YPD (Yeast-Peptone-Dextrous) media. The YPD medium was prepared by mixing 20 g/L of glucose (Sigma-Aldrich), 10 g/L of Yeast extract (Sigma-Aldrich) and 20 g/L of Bacteriological peptone (Sigma-Aldrich) in demineralised water. The components were weighed according to the desired final weight of the media and were thoroughly mixed until everything was dissolved. The solution was then filtered through a 0.2 μm syringe filter (Whatman) to filter-sterilize the medium.

Defined media

A defined medium was used for cultivation of *Cyberindrelia (Pichia) Jadinii* inside the microdroplets. The defined media was prepared by adding 2.3 g/L of urea ($CO(NH_2)_2$, Sigma-Aldrich), 10 g/L of magnesium sulphate heptahydrate ($MgSO_4.7H_2O$, Sigma-Aldrich),

3 g/L of potassium hydrogen phosphate (KH_2PO_4, Sigma-Aldrich) and 20 g/L of glucose to demineralised water. All components were weighed according to the final weight of the medium and dissolved in demineralized water. 1 ml/L of trace element solution and 1ml/L of vitamin solution was added to the mixture. The pH value was subsequently adjusted to 6.0 with a 2M KOH solution and demineralized water was added again to reach the final weight. The prepared medium was then filter sterilized with a 0.2 μm syringe filter (Whatman) and a 50 ml syringe into a sterile bottle and stored inside a laminar flow cabinet. Detailed protocols for the preparation of the trace elements and the vitamin solution can be found in the supporting information of our previous work[22].

Preparation of solutions

All the steps for cell solution preparation were carried out in a laminar flow cabinet. For every experiment, a fresh cryovial was obtained from the stock and thawed at room temperature. After thawing, 500 μL of cell solution was pipetted into 5 mL of sterilized YPD in an autoclaved round bottom flask of 50 ml. Cotton wool was used to cover the flask to make sure aeration is sufficient. The shake flask was then incubated for 6 hours in an incubator at 30°C and 190 rpm. After preculturing, the resulting 5 ml of cell-YPD solution was centrifuged to separate the supernatant YPD and washed with defined media 3 times and diluted upto 10 ml of the final cell solution with defined media. 200 μL of fresh 20 g/L YPD is pipetted to this mixture to boost the growth of microorganisms inside the microfluidic chip. The solution of *Cyberindrelia (Pichia) Jadinii* in defined media is transferred to a 15 ml centrifuge tube and stored at 30°C in a mini-incubator before staring the microfluidic experiments.

APPENDIX

5.A. GROWTH OF *C. Jadinii* INSIDE MICRODROPLET

Figure 5.A.1: Movie depicting batch growth of *Cyberindrelia Jadinii* inside microdroplet at 30°C as shown in time lapse of Figure.5.2.1(b). To see the movie scan the QR code or click here.

5.B. DRAWING OF MICROFLUIDIC DEVICES

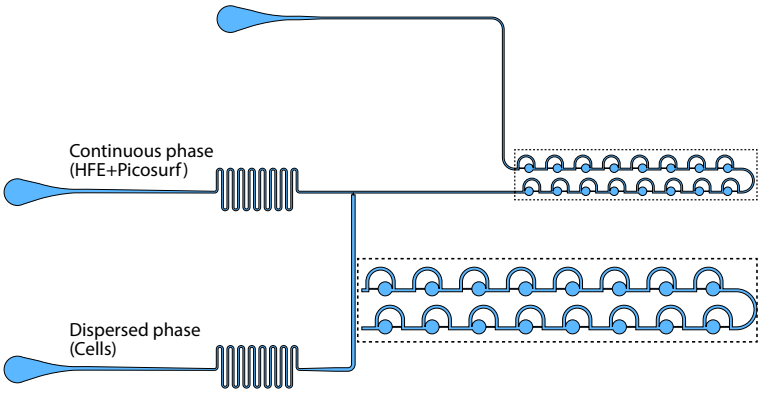

Figure 5.B.1: Drawing of the microfluidic static droplet array device for batch cultivation of *Cyberindrelia Jadinii*

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6

CONCLUSION AND OUTLOOK

The purpose of this chapter is to provide an overview of the work discussed in this dissertation and to highlight interesting research opportunities. The first part of this chapter summarizes the major findings with regards to the research goals formulated in Chapter 1. The second part of this chapter lists down recommendations and perspectives that can be followed for carrying out further research in the future.

6.1. CONCLUSIONS

In the following three subsections, conclusions of the research goals formulated in the Chapter 1 have been briefly discussed.

6.1.1. DROPLET ON-DEMAND GENERATOR

The first research goal was on establishment of a robust and scalable nutrient feeding strategy which could be utilized for culturing microorganisms inside microdroplets in fed-batch mode. In order to achieve this, a droplet on-demand (DoD) generator was designed where droplet volume was governed by its geometry. The DoD generator comprised of two key features: a nozzle (constriction between main and feed channel) and a chamber. The restrictions in height and width of the nozzle and the chamber acted as Laplace pressure barriers. While the nozzle acts as a pressure barrier to hold the interface at equilibrium position before droplet formation, the chamber allows for a restricted filling of the oil-water interface, thereby decoupling the droplet formation from its release. A high monodispersity ($\leq$ 5 %) was achieved with droplet volumes being independent of operating conditions such as pulse duration and strength, waiting time between DoD cycles and interfacial tension between fluids. The scalability of the droplet on-demand generation process was a result of decoupling of droplet formation from its release and transport. The importance of chamber's presence was illustrated by comparing the monodispersity of droplets that were formed using scaled out versions of DoD chips, with and without chamber. The DoD generator was applied for a simple droplet-based assay where multiple dye droplets were sequentially coalesced to a water droplet illustrating its potential for nutrient or reagent addition in non-steady operations such as nutrient-limited fed-batch cultures.

6.1.2. FED-BATCH DROPLET NANOBIOREACTOR

The second research goal as formulated in Chapter 1 was based on the implementation of a droplet-based fed-batch process for nutrient-controlled growth of microorganisms such as *Cyberlindnera jadinii*. Breaking away from all the continuous flow devices, which require sophisticated work flows and/or devices, we developed a strategy that allows easy operation (through a commercially available pressure pump) and relatively easy devices. The general strategy we developed is one in which nutrient droplets are generated on demand throughout the cultivation and chemically coalesced to the cell-containing droplet immobilized in a trap by temporarily flowing a poor solvent around the droplet-droplet interface. The droplet on-demand generator that was designed and characterized previously was used for the formation of cell-containing and nutrient-containing droplets. We illustrated that the droplet on-demand generators can be used to generate biotechnologically relevant feeding profiles. Controlled microbial growth experiments were performed by tuning the concentration of the glucose inside the nutrient droplets and by changing the droplet generation frequency. By changing the glucose concentration in nutrient droplets, different growth rates were observed, establishing nutrient-controlled growth. The growth behaviour of the microorganisms for different set of glucose concentrations agreed well with a simple growth model and is captured by a single set of model parameters. This work forms a proof-of-concept of carrying out long term and nutrient controlled growth of microorganism through controlled nutrient supply.

6.1.3. MICROFLUIDIC TOOL FOR DISSOLVED OXYGEN

The third research goal as formulated in Chapter 1 was to develop a microfluidic tool that allows studying microorganisms under dynamically controlled levels of dissolved oxygen, in order to test the sensitivity of microorganisms for the varying oxygen levels in industrial scale reactors. The strategy we adopted is to culture cells inside droplets trapped on a chip, with the main supply of oxygen to the cells coming from the oil flowing around the droplets. Fluorinated oil was used, which is known for its high solubility of oxygen. We performed batch growth experiments under two limiting cases using *Cyberlindnera jadinii* as the model microorganism. The first one was with oil saturated by oxygen, the second one with oil deprived from oxygen. Experiments with oxygen saturated oil showed much more significant growth for a prolonged period of 20 hours inside all droplets. In contrast, negligible growth was observed in case where the oil was saturated nitrogen. Although imposing dynamically controlled variations in the concentration dissolved oxygen was out of scope, these experiments form a solid basis of such a study. The proposed methodology can be further developed into a robust droplet-based method where microorganisms can be cultured and screened under changing levels of dissolved oxygen.

6.2. OPPORTUNITIES FOR FUTURE RESEARCH

6.2.1. DROPLET FORMATION ON-DEMAND IN LONG TERM CULTURING

For non-steady droplet-based assays involving multiple feedings over the course of a cultivation experiment (such as fed-batch), a predetermined pressure profile was used where the set-point pressures could not be changed during the experiment. This on the hand makes it simple for the user to operate the chip, but occasionally leads to undesired droplet events. There were instances when unwanted droplet formation occurred due to the pressure fluctuations arising inside the chip. Conversely, there were instances in which the pressure pulse did not result in the formation of a droplet, due to receding of the interface into the feed channel of the DoD generator. A possible way to improve the on-demand droplet formation process is by image driven process control[1]. Hereby, the location of the oil-water interface is monitored continuously and pressure values are adjusted via a feedback control loop. If the location of the interface crosses a threshold position, the pressures are adjusted to bring the interface back to its original location. Hébert and co-workers[2] developed a visual feedback control that identifies droplet location, and adjusts the pressures to manipulate the droplets. A similar image tracking based methodology which is capable of analysing the interfacial dynamics should be applied in droplet-based fed-batch experiments. By tuning the pressures and forcing the interface to reach the equilibrium position every time before droplet formation, more reliable, automated and long term fed-batch experiments can be carried out, where the control on the growth rate by the nutrient feeding can be observed much more peculiarly.

6.2.2. SCALING OUT: TOWARDS SCREENING UNDER FED-BATCH CONDITIONS

Chapter 4 details the working principle of the droplet nanobioreactor and demonstrates its application for nutrient-controlled growth of microorganisms. In the current device design, only one fed-batch cultivation can be performed per experiment, which takes at least 2 days of preparation and execution. This imposes limitations on experimental data generation and hinders reproducibility needed for reliable biological experimentation. In order to make this platform practically useful for screening under fed-batch conditions, a scaled-out version is needed, where multiple droplet-based fed-batch experiments can be performed on a single chip. The easiest way to achieve this without changing the current design features would be by introducing a two layered PDMS device. Scaled out version of the microfluidic chip illustrating 8 parallel fed-batch chips is shown in Figure.6.2.1. The first layer is the flow layer comprising of 8 fed-batch chips arranged in parallel (see Figure.6.2.1(a)) with each having separate inlets and outlet. The only difference between single droplet chip and this design is in the alignment of their inlet and outlet ports. We envision the upper layer as the distribution layer that would comprise of 4 channels for distributing fluids to the fed-batch chips on the flow layer (see Figure.6.2.1(b)). On this layer fluid distribution occurs via symmetric tree shaped scale out strategy which ensures working of the device even if some of the channels are blocked[3, 4]. The outlet ports of the distribution layer and inlet ports of flow layer are carefully aligned after plasma cleaning and are bonded together as shown in Figure.6.2.1(c). The fluids flow in the distribution layer and enters the flow layer in sync.

In this way, 8 individual droplet based fed-batch experiments can be carried out on a single device simultaneously using a single pressure pump and 4 reservoirs of fluids. The mentioned scaled out fed-batch chip was designed and fabricated during the Ph.D. project. Few preliminary experiments were conducted to test the robustness of this chip to reliably feed nutrient droplets in parallel to all the 8 cell-containing droplets. Since it

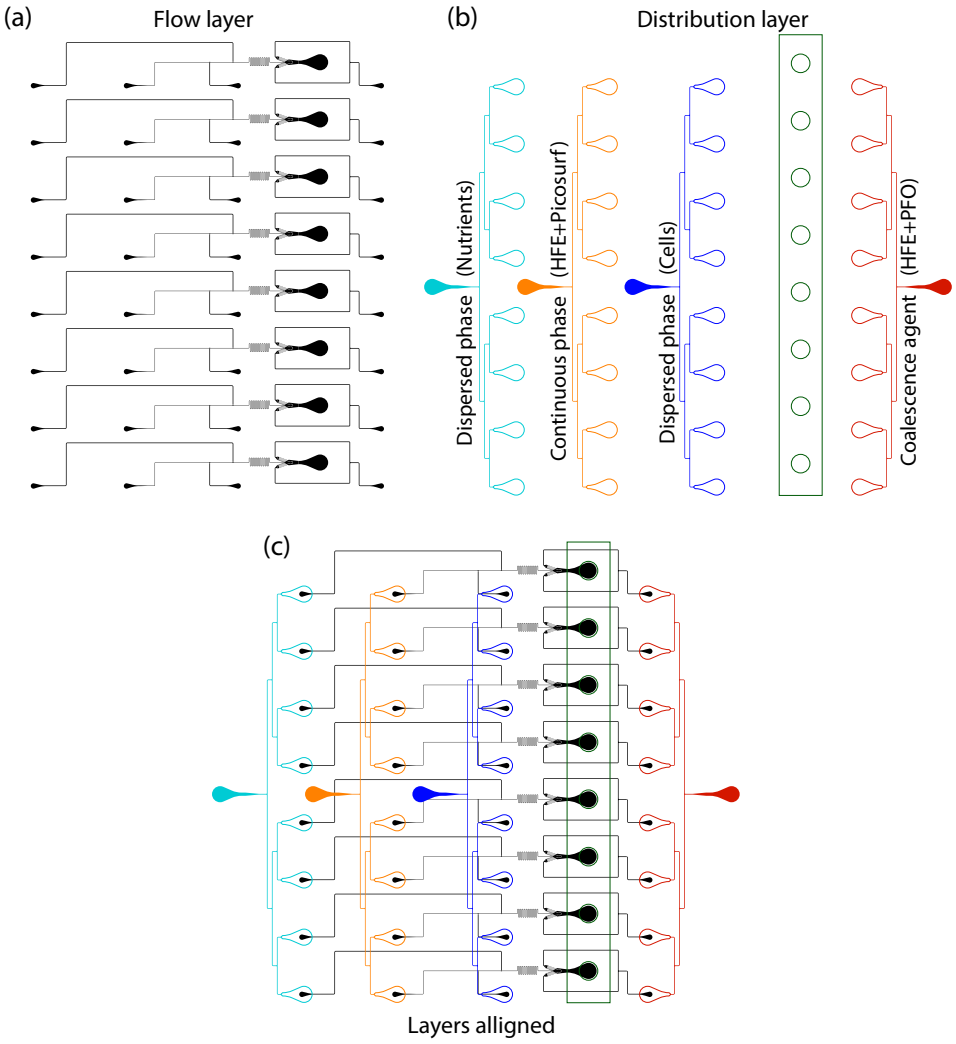

Figure 6.2.1: Scale out design of the fed-batch droplet nanobioreactor capable of performing 8 fed-batch experiments in parallel. (a) Flow layer comprising of 8 fed-batch droplet nanobioreactor chips arranged in parallel. This flow layer forms the bottom layer of the PDMS chip. (b) Design of the distribution layer for the scaled-out fed-batch chip. Four tree-shaped distribution channels are provided for four different fluids in for performing fed-batch droplet experiments. Distribution layer forms the upper layer. (c) Alignment of distribution layer on top of the flow layer during the PDMS chip bonding process.

was challenging to synchronize the on-demand formation and coalescence of nutrient droplets, the robustness and feasibility of the scaled-out chip still needs further experimentation.

6.2.3. INCORPORATION OF ANALYTICS: TOWARDS SCREENING UNDER FED-BATCH CONDITIONS

After a successful implementation of droplet-based fed-batch nanobioreactor for cultivation of microorganisms in Chapter 4, the next challenge would be measurement and quantification of secreted products of interest. A common methodology to quantify the secreted metabolites in droplet-based microfluidic systems is by using fluorescence-based assays[5]. For example, fermentation of *Saccharomyces cerevisiae* on high glucose concentration yields ethanol while a metabolically engineered strain of *Saccharomyces cerevisiae* yields succinic acid under nitrogen starved conditions. Both of these products can be quantified using fluorescent assays. Cela and co-workers[6] developed a method for measuring concentration of ethanol inside microdroplets. Cyanobacteria was encapsulated in aqueous droplets with media and incubated for 48 hours. To determine the ethanol concentration inside the droplets, assay reagent droplets (consisting of alcohol oxidase, horseradish peroxidase and amplex red) were coalesced with the incubated droplets and again incubated for the assay reaction to proceed. The amount of ethanol was quantified based on the fluorescence intensity emitted by resorufin which was released upon the assay reaction. In another study, Sun and co-workers[7] reported using a double enzyme assay for quick measurement of succinic acid produced by E. coli strains. A simple extension of the current fed-batch droplet nanobioreactor would be introduction of a third DoD junction that can be used for generating assay reagent droplets. After the fed-batch cultivation ends, assay droplets could be generated on-demand and coalesced to the droplet containing the microorganisms and secreted product of interest to initiate the assaying reaction. A fluorescence-based assay reporter would give an end point measurement of the product after the cultivation is complete. Care should be taken to design the experiments in such a way that the product of interest remains inside the droplets and does not leak into the surrounding oil[8, 9]. Readers are referred to a recent review by van Tatenhove-Pel and co-workers which is on screening of microorganisms based on extracellular product measurement in droplet microfluidics[10].

Monitoring and controlling pH within the droplets also remains a technical challenge towards developing a droplet based screening platform. Tovar and co-workers used a fluorescent reporter dye 6-carboxyfluorescein in aqueous phase and correlate the pH to its fluorescent intensities[11]. In one of the studies for controlling the pH inside microdroplets, Mashaghi and co-workers demonstrated increase and decrease of pH by adding reagents such as acetic acid and propyl amine to the continuous oil phase[12]. The transport of the reagents that were soluble in both the aqueous and oil phase was mediated by diffusive mechanism from the oil phase. Similar pH control strategies can be applied to the proposed fed-batch droplet microreactor such that the microorganisms can have a similar well-controlled environment as they face in an industrial scale reactor.

6.2.4. IMPOSING FLUCTUATIONS IN DISSOLVED OXYGEN CONCENTRATIONS

One of the outstanding challenges in using droplet bioreactors to carry out microbiological studies is controlling concentration of gases such as oxygen during the incubation[13]. While chapter 5 described preliminary results on cultivation of microorganisms under two different concentrations of dissolved oxygen, imposing fluctuations of dissolved oxygen concentrations during microbial growth inside the droplets remained out of scope. A possible way to extend the current microfluidic device for achieving this is by connecting two independent fluorinated oil sources to the continuous phase inlet. One of the oil lines should be enriched in pure oxygen while the other fluorinated oil should be completely drained of oxygen by enriching it with pure nitrogen. These two fluorinated oil tubing can be joined by a flow control valve before entering the microfluidic chip. Depending on the relative flowrates of the two oil channels, concentration of dissolved oxygen in the microfluidic chip can be changed during the droplet incubation experiments. A similar approach was used in experiments by Abbyad and co-workers[14], where sickling of red blood cells was studied due to unavailability of dissolved oxygen inside microdroplets.

Real time measurement of dissolved oxygen concentration in each microdroplet is also of key importance while imposing DO concentration fluctuations during the incubation of microorganisms. Oxygen-sensitive fluorescent ruthenium compounds such as ruthenium tris(2,2'-dipyridyl) dichloride hexahydrate (RTDP) have been extensively used for sensing of oxygen by Fluorescence lifetime imaging microscopy (FLIM)[14–16]. Alternatively, oxygen sensor nanoparticles with phosphorescent indicator dye could be co- encapsulated with microorganisms and media for real time read-out of oxygen concentration inside the microdroplets by combining it with phosphorescence lifetime-based measurement[17].

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OUTREACH

JOURNAL PAPERS

7. **Totlani, K.**, van Tatenhoeven-Pel, R. J., Kreutzer, M. T., van Gulik, W. M., and van Steijn, V., *Microbioreactors for nutrient-controlled microbial cultures: Bridging the gap between early bioprocess development and industrial scale use*, To be submitted to Biotechnology Journal (2021).

6. **Totlani, K.**, Katakwar, P., du Pree, N., Kreutzer, M. T., van Gulik, W. M., and van Steijn, V., *Microfluidic static droplet array to study microbial growth under different levels of dissolved oxygen*, To be submitted to Micromachines (2021).

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CONFERENCE PRESENTATIONS

6. Miniaturized Systems for Chemistry and Life Sciences (μTAS), Switzerland, 2019.

5. Implementation of Microreactor Technology in Biotechnology (IMTB), Croatia, 2019.

4. Physics at Veldhoven, Netherlands, 2019.

3. Netherlands Process Technology Symposium (NPS), Netherlands, 2018.
2. Physics at Veldhoven, Netherlands, 2017.
1. Internation Conference on Bioencapsulation, Netherlands, 2015.

PROPOSITIONS

1. Microfluidics promises *Lab-on-a-Chip* yet delivers *Chip-in-a-Lab*.
2. Validation through organs-on-chips should be commonplace in pre-clinical studies.
3. Geometry-mediated strategies make droplet-based microfluidics for semi-continuous cell-cultures accessible to non-experts. (Chapter-3, this thesis).
4. Simplicity and brevity in scientific writing has been sold out to pre-empting criticism during peer review.
5. TU Delft's applied sciences' grading scheme is biased in favour of theoretical projects.
6. Instant gratification in the world of social media leads to a generation lacking scientific rigour.
7. Development of technology for rapid diagnosis of diseases deserves more media attention than self-driven cars.
8. Importance of lazy geniuses is underrated.
9. Principal Investigators should spend more of their time in the lab.
10. Owing to the funding inequity, global university rankings can't be taken seriously.

These propositions are regarded as opposable and defendable, and have been approved as such by the promotor prof. dr. ir. M. T. Kreutzer and co-promoters dr. W. M. van Gulik and dr. V. van Steijn

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I am also thankful to the colleagues from the TP group. **Sid**, I am really sad that we did not interact during the initial years of my Ph.D. Nevertheless, I am now happy that I got to know you eventually. The amount of knowledge you have feels like you know mostly about everything in this world ;-) Honestly, I haven't seen a creative and intelligent person like you being so humble and modest. I deeply enjoyed our discussions on politics, comedy and the state of journalism in India. I wished we could have met one last time before leaving Netherlands. I am super confident that, soon you would bag a permanent academic position at a reputed university and as I always say if you won't get it, then who else would! **Manas** you have a very pleasant and jolly personality. We had loads of discussion about life as an experimentalist and times that each one of us spent at NCL. Thank you for helping me out with surface tension measurements and for always being so accessible. Also, thank you for inviting me to the amazing dinners at various occasions. **Manu** it was fun having conversations and sharing the Ph.D. frustration with

you during our unplanned coffee breaks. I really appreciated your sincerity and dedication towards your Ph.D. work. **Chirag**, thank you for letting me stay at your house during the initial weeks of my Ph.D. Your take on life and science was really refreshing. Your guidance on pursuing acads in India was useful. **Hrushikesh**, I really enjoyed discussing transport phenomena with you during the ATP sessions and even after starting the Ph.D. You always challenged me to think on the physics of the process rather than just looking at the bigger picture.

Throughout this Ph.D. project, I have had the opportunity and privilege to supervise many M.Sc./ B.Sc. students. **Piyush**, you were my first student and it was a bit nostalgic for me to have a student who was also an alumnus of Chemical Engineering, VNIT. You started your M.Sc. thesis within the first six months of me starting this project. I have to admit that I was slightly lost during the initial phase. You worked independently through a major chunk of your project and delivered what you promised. I hope we can submit our paper on your and **Naomi**'s work soon. Next, **Thorben**, you joined as a short-term exchange student from Hamburg and started experiments on day one. Within just two months, you managed to run the first automated fed-batch experiment. Your insights on the toxicity of coalescence agent were extremely useful in deciding the course of the experiments. I must say, I could foresee a robust and working fed-batch chip after your short stint. I wished you could have done your M.Sc. thesis here in Delft as well. **Maxime**, you joined in next and continued working on the setup that **Thorben** and I had built. Being from Biotechnology, you offered a very fresh perspective to the project with regards to the fed-batch experiments and modeling. Even though you faced a lot of experimental challenges during your project, your thesis was solid and your conclusions provided useful insights. A lot of times you went out of your way to collaborate with technicians from BT for sake of this project. **Alex** and **Jan-Willem**, thank you both for your contributions to the Droplet on Demand project. **Alex** you performed the preliminary experiments for DoD generation and provided a nice start. **Jan-Willem** you came in next and accelerated the project during your four month tenure. I am really happy that this work was eventually published in *Lab on a Chip* and ended up on the front cover. **Naomi**, you continued on **Piyush**'s work and took the project to the next level. Your hard work and dedication to the project were really inspiring to me. It amazed me, that even after several failed experiments in a row, you had the patience to go into the lab and run the experiment one more time. Thank you so much for editing the Dutch version of the summary for this thesis. **Yen**, you built on **Thorben**'s and **Maxime**'s work. Your contribution was very significant for the completion of the fed-batch project. You were extremely efficient and productive with your work. You designed the fed-batch experiments using the first principles and then systematically performed them. Not only that, images from your thesis ended up in our *Advanced Materials Technologies* paper which I am really proud of. I wished we could have met one last time before you departed from Delft. **Joppe**, you started with the collaborative project on liquid-liquid extraction in microdroplets with me and **Rupali** as your supervisors. Even though the nature of the project was super exploratory, you showed perseverance and made sure there was a closure to this work. I wish all of you success in your future endeavors.

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Shaurya, our association started from being classmates/labmates during M.Sc., developed as colleagues during Ph.D., and by the end, it culminated into a beautiful friendship. There are several things I have learned from you over the past few years. Your ef-

fortless articulation of thoughts is something I cannot really fathom. Our discussions on science, life, and philosophy were extremely engaging because you had an abnormal clarity in conveying your thoughts. The critical attitude with which you spoke about science has transformed me into a more critical researcher and an individual. I would never forget your *"Mai yaha sirf do cheeze karne aaya hun: Science aur Nasha"*. Your innocence, when you asked (not so difficult) Bollywood questions and bragged about your cooking skills, always brought a smile to my face. Thank you so much for being a significant part of my Ph.D. journey.

Maulik, I don't know where to start and end this. I was in NCL when Yogesh first mentioned to me that you were also applying to Delft. Little did I know that the companionship during the initial phase of the application would transform into such a strong friendship. Your presence was immensely comforting during the difficult times. There were many instances where I was frustrated after failed experiments or bad meetings. You were extremely patient and calm to hear all of it out. Not only that, your eternal optimism and faith gave me a lot of courage to go to the lab once again. I would always miss and cherish our conversations in the office after 7 pm, where one of us was ready to leave but still stayed and talked to the other person. Thank you for giving me strength and courage by your positive words throughout my Ph.D. You are like an elder brother to me. Thank you for being there.

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Attending weddings in India and roaming around in Europe have been significant elements of this journey. These were the most needed & deserved brakes that rejuvenated my energy after stressful and frustrating patches during the Ph.D. These happened the most with the members of the amazing *potshots* gang. Thank you **Prateek**, **KD**, **Parag**, **Shreyas**, **Sam**, **Mayur**, **Sonal**, and **Jajoo** for the countless memories during these years. The discussions we had during our night outs (or sometimes the heavy ones on the group ;-)) are the best part of our friendship. I would give the credits for my debating skills to all of you folks ;-). **Prateek**, **KD**, **Parag** and **Shreyas**, the euro trip we had is one of the most memorable trips for me (of course after the historic goa trip). It was filled with moments that I would cherish for a lifetime.

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Bhoomi and **Piyush**, happiness is the first word that comes to my mind when I think of you both. Thank you for the infinite moments of laughter you both have given us. I don't know if there is any connect but spending time with you both feels so natural. There are very few people in this world with whom **Sparsha** and I can talk the way we talk to you. This very fact describes the comfort that we share with each other. I think that is because our struggles are relatable af and we have a mutual appreciation for them. From binge watching and binge eating to our bitching sessions, I loved our get-togethers a lot. Thank you for hosting us on numerous occasions. I am confident that both you would

soon find amazing jobs after which we can come and stay with you again. I am looking forward to the eurotrip which are planning since an eternity.

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I am out of words here to describe my feelings for my family in India. Lockdowns and flight bans were a blessing in disguise for me. Even though I was unhappy about the delay in the Ph.D., seeing all of you safe and healthy really uplifted my spirits. **Lata bua** and **Jagdish jijaji**, I feel immensely fortunate that I was in India when the pandemic was at its peak and I could help you both navigate through the difficult period. **Lata bua** your willpower and courage are phenomenal. Thank you for all your love and support. **Sheena** and **Vinisha**, you have always believed in me and supported me no matter what. Lately, we haven't been in touch and I feel it's high time we all should have a meet-up. **Sheru mama**, **Manju mami**, **Pappu mama**, **Bharti mami**, **Abban**, **Nana**, and **Geeta masi**. All of you have loved and supported me unconditionally. I would be indebted to you forever and couldn't have asked for more. Thank you for constantly checking up on me and reassuring that all of you were there in Nagpur to take care of everyone in my absence. **Pappu Mama**, you were the one who encouraged me to take up Chemical Engineering in VNIT and I know that you would have been really proud today to see me complete my Ph.D. in Chemical Engineering. **Sheru mama**, your contribution to my life has been immensely significant, not just work-wise but also in my personality development. I remember your coaching during 10th std board preparations. I think that struggle and hustle have instilled belief in me that I can achieve goals even where I need to punch way above my weight. Thank you for that. It really means a lot. **Abban**, our discussion on bollywood movies and how history should have been taught are very interesting. Your jokes, movies and series references really cheers me up. I am happy that you had guts and willpower to follow your interest and passion. I am confident you would kill in whatever you pursue. Let's make sure to keep in touch.

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We have had countless discussions and sometimes arguments on a wide range of topics. These discussions have made me think about things more critically and as a Ph.D., I think this is one of the most important skills to hone. One of the things that I have learnt during these discussions is that you like to address the central point directly rather than beating around the bush. I do understand and appreciate your concern about having more real discussions than having meaningless sessions of talking about others. I really look up to your hard work and dedication to achieving your life goals. Thank you for your support and for consistently reiterating that the bigger picture matters.

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Gentle reminder to self: During the rare occasions when you feel that research is pointless and academia is a sham, this work (and the book) will remind you of the Ph.D. journey and the reasons you chose this career in the first place.

CURRICULUM VITÆ

Kartik Totlani was born on 24th of June 1990, in Nagpur, India. He finished his primary and secondary education from Modern School in 2006, and higher secondary education from Bhavan's Bhagwandas Purohit Vidya Mandir in 2008, both in Nagpur. He went on to do a Bachelors (B.Tech.) in Chemical Engineering from Visvesvaraya National Institute of Technology (VNIT), Nagpur, India, graduating in 2012. He then worked as a Project fellow with the Industrial Flow Modelling Group (iFMG) in the Chemical Engineering and Process Development division at National Chemical Laboratory (NCL) in Pune, India.

Kartik then joined TU Delft in 2013, for a Masters (M.Sc.) in Chemical Engineering with specialization in Process Engineering. During his masters, he worked in Product & Process Engineering and Advanced Soft Matter research groups for his thesis on microfluidic synthesis of all aqueous core shell particles for drug delivery applications. After his M.Sc. thesis, Kartik did a brief research internship at Merck Sharp and Dohme, Oss and graduated in August 2015. In 2016, Kartik started his Ph.D. on an interdisciplinary project in the group of Product & Process Engineering. During his Ph.D. he worked on development of droplet microfluidic tools for bioprocess engineering with Dr. Volkert van Steijn, Dr. Walter van Gulik as co-promotors and Prof. Michiel Kreutzer as his promotor.