Metabolite profiling of microfluidic cell culture conditions for droplet based screening

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We investigate the impact of droplet culture conditions on cell metabolic state by determining key metabolite concentrations in *S. cerevisiae* cultures in different microfluidic droplet culture formats. Control of culture conditions is critical for single cell/clone screening in droplets, such as directed evolution of yeast, as cell metabolic state directly affects production yields from cell factories. Here, we analyze glucose, pyruvate, ethanol, and glycerol, central metabolites in yeast glucose dissimilation to establish culture formats for screening of respiring as well as fermenting yeast. Metabolite profiling provides a more nuanced estimate of cell state compared to proliferation studies alone. We show that the choice of droplet incubation format impacts cell proliferation and metabolite production. The standard syringe incubation of droplets exhibited metabolite profiles similar to oxygen limited cultures, whereas the metabolite profiles of cells cultured in the alternative wide tube droplet incubation format resemble those from aerobic culture. Furthermore, we demonstrate retained droplet stability and size in the new better oxygenated droplet incubation format. © 2015 AIP Publishing LLC.

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I. INTRODUCTION

In vitro cell culture is a fundamental component of biological production systems and biotechnological research, with applications ranging from industrial production of enzymes to cell toxicity studies. An *in vitro* cell culture format must satisfy a number of conditions, such as an appropriate temperature, an adequate supply of nutrients from a growth medium, and a supply of respiratory gas in order for cells to survive, develop the desired phenotype or produce at optimum levels. Cells have a complex system of regulatory pathways to adapt to perturbations in culture conditions, thus variations in culture conditions affect cell behavior such as the levels of expression of specific proteins or activity of metabolic pathways.¹

Monitoring temporal changes in a few key metabolite concentrations provides information about the activity of the cells main metabolic pathways and overall metabolism, offering a snapshot of the state of the cell. Metabolite analysis is used as a tool to assess cell behavior in bioprocesses. As metabolites are downstream of transcription and translation, metabolic profiling can potentially reveal more immediate changes in cell behavior and activity that might not be seen using transcriptomics or proteomics. NMR and chromatography are used for high throughput metabolite separation and quantification.²

Petri dishes and culture flasks are the culture formats traditionally used for laboratory cell culture. In culture flasks, cells are grown in a homogenous medium, while Petri dishes allow the growth of colonies on a substrate surface. Both formats however lack the possibility to

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compartmentalize clones and single cells. Compartmentalization allow single cells or clones to be individually assayed or exposed to differing culture conditions thereby enabling further analysis of cell heterogeneity. Droplet microfluidics is an emerging cell culture format enabling compartmentalized culture of single cells and clones.

In droplet microfluidics, picoliter sized monodisperse aqueous droplets are formed in an immiscible oil phase using a microfluidic chip. The oil used is typically fluorinated oil, in which both water and organic solvents have very low solubility.³ The droplets are stabilized using biocompatible surfactants.⁴ Droplets can be generated, manipulated, and analyzed at rates of thousands of droplets per second. In each droplet, a separate reaction or event can take place, for example, the encapsulation and analysis of a single cell.⁵ Droplet microfluidics has been used for a number of single cell or single molecule applications, for example, functional screening of hybridoma cells,⁶ the study of sickling in red blood cells,⁷ and the improvement of enzyme production in cells using directed evolution.⁸

Droplet incubation can be divided into on- and off-chip approaches. Examples of on-chip methods are delay channels, used to incubate droplets for shorter periods of time⁹ and serpentine channels,¹⁰ droplet traps,¹¹ and dropspots¹² used for longer incubation times. Off-chip methods are used for cell culture in droplets, e.g., by collecting the emulsion in syringes^{8,13} or in a Pasteur pipette under a layer of media¹⁴ and PDMS sealed vials for thermocycling.¹⁵ None of these put droplets in direct contact with the surrounding atmosphere.

Various droplet incubation formats have successfully been used for short to intermediate term cell culture or incubation of many different cell types, e.g., bacteria,¹⁶ yeast⁸ cells, as well as cells from human cell lines.^{17,18} Whole multicellular organisms such as zebra fish embryos¹⁹ have also been encapsulated. However, apart from the influence of surfactant biocompatibility^{4,17} and studies on cell growth and viability,^{10,12,17} more immediate and dynamic effects of cell incubation conditions in picoliter scale droplets, in particular, the influence of oxygen availability, are not as of yet extensively investigated in these formats. By monitoring metabolite concentrations information about pathway activity and response to perturbations is obtained,²⁰ providing a more nuanced estimate of cell state compared to proliferation studies alone.

The ability to control media oxygenation is critical for applications depending on *in vitro* cell culture. Obligate aerobes cannot grow without sufficient oxygen, and while facultative anaerobes do grow with or without oxygen present their metabolism and the yield of product metabolites is altered upon oxygen depletion.²¹ The hundreds of transcript levels up- or down regulated in *S. cerevisiae* in aerobic compared to anaerobic culture²² indicate a change in cell state as a result of oxygen availability. Oxygen availability also greatly impact the growth²³ and differentiation²⁴ of mammalian cells.

The metabolic state of *S. cerevisiae* cells can be used as an indicator of oxygenation since it is a facultative anaerobe that uses different pathways for glucose dissimilation under aerobic and anaerobic conditions, thus measuring a few key metabolites reveals which pathway is active.

Growing cells with respiratory metabolism consumes oxygen. Cells in droplets obtain oxygen dissolved in the growth medium. Oxygen can be transported to the droplets from the fluorinated carrier oil, which has the capacity to dissolve 10–20 times more oxygen than water.²⁵ If enough oxygen in the media and oil is consumed during incubation, the conditions in the droplets turn anaerobic or oxygen limited unless additional oxygen is supplied. Syringes, which are commonly used for droplet incubation, are effectively closed vessels with limited capacity to supply new respiratory gas. PDMS sealed vials, Pasteur pipettes, and PDMS channels allow some gas exchange, which may enable long-term cell culture in droplets under aerobic conditions. Variations in droplet oxygenation between droplet incubation formats are a potential source of bias studying any parameter affected by oxygen levels, from cell viability to expression levels of proteins and microbial cell factory yields. Oxygen transport from oil to droplets has been investigated to show that oxygen levels in the droplets can be affected by the supply of oxygenated or non-oxygenated oils.⁷ However, some questions need further elucidation, such as oxygen transport into the droplets during longer incubation times, if all droplets get oxygenated to the same extent and how long the oxygen from the oil, media, and surrounding environment will sustain

cells. Knowing this is of importance in choosing a suitable droplet incubation format for different cell types and experiments, in particular, for long-term cell culture.

In this paper, we demonstrate how key metabolite profiling can reveal information about *S. cerevisiae* cell state in droplet microfluidic culture and how it is influenced by droplet incubation formats. Two droplet incubation formats were studied: droplets incubated in a plastic syringe and a new droplet incubation format termed wide tube, which allow droplets to come in direct contact with the atmosphere. To track cell metabolic state, the yeast cultures were sampled during a 48 h incubation. Samples were subsequently analyzed for glucose, pyruvate, ethanol, and glycerol concentrations using HPLC. Finally, *Bacillus subtilis* cells expressing red fluorescent protein, which should only proliferate with adequate oxygenation, were cultured in the different incubation formats to verify conclusions regarding oxygenation conditions.

II. EXPERIMENT

A. Materials

The *Saccharomyces* strain used was CEN.PK 113–7D (generously supplied by Verena Siewers at Chalmers University). The culture media for *S. cerevisiae* was SD medium containing yeast nitrogen base (Formedium) at a concentration of 6.9 g/l, complete supplement mixture (-ura) (Formedium) at a concentration of 770 mg/l, uracil (Sigma Aldrich) at a concentration of 20 mg/l, KH_2PO_4 (Sigma Aldrich) at 2 g/l and glucose (Sigma Aldrich) at a concentration of 10 g/l.

B. subtilis strain expressing red fluorescent protein (RFP) was used. The media used for *B. subtilis* culture were a mixture of 87.5 vol. % Luria-Bertani medium (Invitrogen) and 12.5 vol. % Terrific Broth consisting of Tryptone (Difco) at a concentration of 13.3 g/l, Yeast extract (Difco) at a concentration of 26.6 g/l and 3% (v/v) water free glycerol (Merck).

The oil used was HFE-7500 (3M) with 1% EA surfactant (RainDance Technologies). 1H, 1H, 2H, 2H-perfluoro-1-octanol (Sigma Aldrich) was used for emulsion destabilization.

B. Microfluidic chip fabrication

The microfluidic chips were fabricated by soft lithography.²⁶ Briefly, the polydimethylsiloxane (PDMS) (Sylgard 184, Corning) replica was made by mixing 27 g PDMS base with 3 g curing agent and pouring it onto a silica master mold and curing it overnight at 65 °C. The PDMS slab was then peeled off the master and cut into three devices. Inlet and outlet holes were made using a 0.75 mm Biopsy puncher (Harris Uni Core) and the slabs were cleaned using acetone and isopropanol before exposing the channel sides to oxygen plasma 220 V 40%, 30 s (FemtoCute, South Korea) and bonding them to glass slides (Thermo Fisher Scientific). After bonding, the chips were cured at 110 °C for 1 h. Before use, the channels were surface treated with Aquapel (PPG Industries). See supplementary material A for chip design schematic and channel dimensions.³³

C. Microfluidic device operation

A microfluidic chip design generating 90 μm droplets by flow focusing was used for all experiments. The microfluidic chip was placed on an adjustable xy-table on an inverted microscope. The syringes were connected to the microfluidic chip through polyether ether ketone (PEEK) tubing. All syringes were controlled using NE-1000 Programmable Single Syringe Pumps (New Era Pump Systems, Inc.) The pumps were operated at a flow rate of 1000 $\mu\text{l/h}$ for both the oil phase and aqueous phase.

D. Cell encapsulation

Assuming the cell encapsulation process follows Poisson statistics,¹⁶ the average number of cells per droplet, λ , was adjusted by controlling the cell concentration at a set droplet size. The droplet size throughout the experiment was 90 μm and the droplet occupation rate, λ , was

adjusted to be 5.3 by measuring optical density of the cell cultures and diluting to the desired cell concentration.

E. Droplet incubation formats

For droplet incubation in syringe, the emulsion was collected and incubated in a 2 ml BD Plastipak syringe. For droplet incubation in wide tube format as seen in Fig. 1, the wide tube was made by sawing off a 50 ml falcon tube at the 10 ml mark and attaching a 200 μ l $\varnothing$ 5 mm tube filled with water to the inside of the tube wall as a water reservoir. The wide tube was pre-filled with 200 μ l HFE oil with 1% EA surfactant and the emulsion was collected. The tube was sealed using parafilm. For the oxygen limited control, 2 ml cell culture was put in a BD Plastipak 2 ml syringe with a 15 cm long tubing with the inlet of the tubing in a 500 μ l tube filled with MilliQ water. For the aerobic control, 10 ml cell culture was poured into a 100 ml shake flask and a tissue paper was used for lid.

F. Droplet stability analysis

An overnight culture of yeast cells was prepared, and optical density at 600 nm (OD_{600}) was measured. The sample was washed and diluted to $OD_{600} = 2.5$, corresponding to an average of 5.3 cells/droplet. Droplets were generated at room temperature and approximately 500 μ l emulsion was collected on top of 200 μ l HFE in the wide tube and incubated at 30 °C. Samples were taken at 0 h, 5 h, 24 h, 72 h, and 10 days by pipetting 25 μ l emulsion onto a glass slide. Images were taking using an Olympus IX53 for bright field microscopy (Olympus) with a Plan N 4 $\times$ /0.10 objective and a Pike (Allied Vision Technology) camera.

G. Cell metabolic state of *S. cerevisiae* in droplet culture

Yeast cells were cultured in shake flask overnight and the OD_{600} was measured. The sample was washed and diluted to $OD_{600} = 2.5$, so that each droplet would on average be populated

FIG. 1. The different incubation formats. From left: droplets in syringe, droplets in wide tube, oxygen limited control, and aerobic control. The droplets in syringe incubation format were made using a 2 ml plastic syringe with fittings and a short piece of tubing. The syringe was filled with HFE oil and emulsion incubated on top of the oil. The droplets in wide tube incubation format was made sawing of the bottom 10 ml of a 50 ml falcon tube and attaching a 200 μ l tube filled with water to the inside wall. The cone shaped bottom of the tube was filled with HFE oil halfway up and emulsion incubated on top of the oil, and the tube was sealed with parafilm. The oxygen limited control was made filling a 2 ml plastic syringe with cell culture and putting the inlet of the long tubing in a 500 μ l tube filled with MilliQ water. The aerobic control was 10 ml cell culture in a 100 ml shake flask with tissue paper as a lid.

by 5.3 cells. Droplets were generated at room temperature and 900 μ l emulsion was collected into a BD Plastipak 2 ml syringe followed by 900 μ l emulsion collected in the wide tube. All samples were incubated at 30 °C. The aerobic control was incubated with 150 rpm shaking, the other formats were incubated without shaking. Samples were taken at 0 h, 3 h, 6 h, 9 h, 12 h, 24 h, and 48 h. All samples were collected in 500 μ l tubes and stored at –20 °C. Approximately 100 μ l culture was taken from both control cultures, and 100 μ l emulsion was taken from each droplet culture. The emulsion was broken using 3 μ l 1H, 1H, 2H, 2H-perfluoro-1-octanol (Sigma Aldrich) and frozen for later analysis. Three full replicates starting from new cell cultures were performed for each incubation format. For analysis, the samples were thawed and centrifuged at 20 000 g for 5 min. Aqueous phase was loaded to HPX-87H column (BIORAD, USA) on a Dionex Ultimate 3000 HPLC (Dionex Softron GmbH, Germany) to measure the concentrations of glucose, glycerol, ethanol, and pyruvate. The system was running with a flow rate of 0.6 ml/min at 45 °C by using 5 mM H₂SO₄ as mobile phase. Metabolites were measured with a refractive index detector (RI-101, Shodex) and an ultraviolet detector (VWD3000, Dionex) and concentrations calculated from standard curves.

H. *B. subtilis* culture in droplets

An overnight culture of *B. subtilis* was used. The OD₆₀₀ was measured and the sample was diluted to OD₆₀₀ = 0.1, corresponding to an average of 5.3 cells/droplet. Droplets were generated at room temperature and 700 μ l emulsion was collected into a BD Plastipak 2 ml syringe followed by 700 μ l emulsion collected in the wide tube. For aerobic control 10 ml culture in a 100 ml shake flask was used, and for oxygen limited control 1 ml culture in a 1 ml BD Plastipak syringe with tubing, fittings, and a stopper. All samples were incubated at 37 °C. The aerobic control was incubated with 150 rpm orbital shaking, the other formats were incubated statically.

Samples were taken at generation, 24 h, 48 h, and 72 h. 100 μ l was taken from both control cultures and 200 μ l emulsion was taken from the droplet cultures. Two replicates starting from new cell cultures were performed for each incubation format. The emulsion was broken with 6 μ l 1H, 1H, 2H, 2H-perfluoro-1-octanol (Sigma Aldrich) and the aqueous phase was used for analysis. 50 μ l of each sample was put in a 96 well plate and fluorescence was measured using a SpectraMax M5 (Molecular Devices). The excitation was set to 570 nm and emission to 600 nm.

III. RESULTS AND DISCUSSION

A. Droplet cell culture incubation formats

Two droplet incubation formats were used; a closed syringe, a widely used droplet incubation format, and a novel incubation format termed wide tube exposing droplets directly to the atmosphere. A difference between incubating droplets in a syringe and incubating droplets in the wide tube is that in the wide tube the emulsion has a larger contact area with the HFE oil and a large contact area with air not present in syringe incubation. The emulsion is incubated between a reservoir of HFE oil and a water saturated headspace covered with plastic paraffin film (Fig. 1). The film used has a high oxygen and CO₂ permeability and a low water vapor permeability.²⁷

Maintaining droplet stability from coalescence as well as in terms of size are two fundamental requirements on any droplet microfluidic cell culture format. The emulsion quality of the new format was tested by generating droplets with *S. cerevisiae* cells and incubating the emulsion in the wide tube. Microscope images of a monolayer of droplets spread on a glass slide were taken at six defined timepoints during a 10 day period (Fig. 2). Analysis of droplet size over time shows that droplets maintain their size during the first 24 h but that the size is slightly decreased when interrogating the droplets after 10 days of incubation, indicating that some evaporation has occurred. Upon generation the mean droplet diameter (mean $\pm$ SD) was $88.9 \pm 2.9 \mu$ m ($n = 123$) and after 10 days it had decreased to $79.8 \pm 3.1 \mu$ m ($n = 170$). The result shows a high degree of monodispersity at each timepoint, an indication that the emulsion is of good quality and that the droplets are stable and have not coalesced, overcoming the challenge of maintaining emulsion quality while allowing direct contact with the surrounding atmosphere.

FIG. 2. Droplet quality over time. $90\ \mu\text{m}$ droplets were generated and the emulsion was incubated in the wide tube format at 30°C for 10 days. The emulsion was imaged at generation (0h) and after 5 h, 24 h, 72 h, and 10 days. (a) droplets right after generation, (b) droplets after 5 h incubation, (c) droplets after 24 h incubation, (d) droplets after 72 h incubation, (e) droplets after 10 days incubation, (f) bar chart of mean droplet diameter over time at 0h ($n=123$), 5h ($n=124$), 24h ($n=114$), 72h ($n=141$), and 10 days ($n=170$), with error bars corresponding to ± 2 SD. See supplementary material B for statistical treatment of data.³³

B. Cell metabolic state of *S. cerevisiae* in droplet culture

S. cerevisiae has two main glucose dissimilation pathways: aerobic respiration yielding carbon dioxide and water and fermentation yielding carbon dioxide and ethanol. The oxygen and glucose concentrations both regulate which pathway is utilized. When glucose is scarce the oxygen availability alone regulates which pathway is used, respiration under aerobic conditions and fermentation under anaerobic conditions. In contrast, when glucose is abundant the rate of glycolysis is high resulting in fermentation regardless of oxygen levels. This is known as the Crabtree effect.^{28,29}

To monitor the cell metabolic state, the concentrations of four key metabolites were measured: glucose, pyruvate, ethanol, and glycerol (Fig. 3). The droplet occupation rate was set to 5.3 cells/droplet in order for the emulsion to be sufficiently homogenous with respect to cell density. Cultures were sampled at seven points during a 48-h incubation period and analyzed using HPLC after breaking the emulsion and removing the oil. Experiments were performed in triplicate. The carbon source in the media was glucose, with an initial concentration of 10 g/l.

The glucose consumption rates were comparable in all incubation formats but were slightly slower in the droplet formats. The concentration decreased from the starting concentration of 10 g/l to non-measurable after 6 h in both control cultures. For the droplets in syringe culture, glucose decreased rapidly during the first 12 h to 0.5–1 g/l and continued to decrease to undetectable levels more slowly in two of the three samples. In the third sample, the decrease was slower and never went below 0.9 g/l. In the corresponding droplets in wide tube replicate glucose was not completely depleted either. We hypothesize that the reason all glucose was not consumed in these samples could be due to an initial cell concentration lower than expected, leading to unoccupied droplets in which no glucose is consumed.

Pyruvate is the end product of glycolysis and the starting material for alcoholic fermentation as well as for oxidative respiration and thus a key metabolite to monitor. The early peak in pyruvate concentration corresponds well to pyruvate overflow caused by a high rate of glycolysis leading to saturation of respiratory metabolism.²⁸ At high glucose concentrations, glycolysis will yield more pyruvate than the respiratory or fermentative degradation pathways can use, causing a rapid rise in pyruvate levels. After the glucose has been consumed, the pyruvate decreases very rapidly in the aerobic control and in wide tube droplet cultures while a slower decrease is observed in the oxygen limited control and the syringe droplet cultures.

FIG. 3. *S. cerevisiae* metabolite concentrations over time. 90 μm droplets containing on average 5.3 cells per droplet were generated and incubated in syringe and in wide tube. Aerobic and oxygen limited (non-droplet) cultures with corresponding cell density were included as references. Samples were taken from each incubation format at defined time points during a 48-h incubation period and metabolite concentrations were measured using HPLC. Droplets in syringe are labeled with blue triangles, droplets in wide tube are labeled with yellow squares, oxygen limited control culture are labeled with green circles, and aerobic control culture are labeled with red upside-down triangles. The three lines of the same color correspond to three full replicates. (a) Glucose concentrations over time, (b) pyruvate concentrations over time, (c) ethanol concentrations over time, (d) glycerol concentrations over time. Mean values and standard deviations presented in supplementary material C.³³

Ethanol is the end product of fermentative degradation of glucose. In the oxygen limited control and droplets in syringe formats, the ethanol concentration increased as long as glucose was abundant, and then the concentration stabilized at 3–4.5 g/l. In the oxygen limited control and droplets in wide tube, the ethanol concentration initially increased but then started to decrease after 6–9 h. This corresponds well to diauxic growth of *S. cerevisiae* in aerobic cultures. In diauxic growth, the primary carbon source is glucose and when it has been consumed cells switch to metabolizing ethanol as a secondary carbon source. Under anaerobic culture conditions *S. cerevisiae* cannot metabolize ethanol.³⁰ In the droplets in syringe culture, the ethanol concentration did not decrease indicating that the cells are not metabolizing it. This strongly implies that the yeast cells cultured in droplets in a syringe are experiencing anaerobic conditions.

Glycerol levels follow a similar pattern as the ethanol concentrations in the incubation formats. The glycerol concentration is significantly higher in the oxygen limited control and droplets in syringe cultures compared to the aerobic control and droplets in wide tube. Formation of glycerol balances the redox potential in the cell, especially under anaerobic conditions and is a byproduct of fermentation.³¹ A high glycerol concentration is thus an indication of anaerobic conditions.

In droplet formats and control formats, the pyruvate concentration peaked during the first hours of incubation when glucose concentration and rate of glycolysis were high, consistent with the Crabtree effect. When glucose concentration had decreased, the overall metabolite profile of the yeast cells cultured in droplets in a syringe showed a high ethanol concentration without any decrease upon glucose depletion and a significantly higher glycerol concentration compared to the aerobic control, whereas the yeast cultured in the wide tube format showed a lower ethanol concentration, a decrease in ethanol concentration when glucose was depleted, and a lower glycerol production. Overall, the metabolite profile of yeast cells incubated in

droplets in a syringe closely resemble the metabolite profile of the yeast cells in oxygen limited control culture, whereas the metabolite profile of the yeast cells in droplets in wide tube follow the metabolite profile of yeast in aerobic control culture. The shake flask with tissue paper lid used as aerobic control culture is a standard method for aerobic cell culture. The syringe used for oxygen limited control culture is not a standard method as anaerobic cell culture and was designed to allow pressure release as carbon dioxide was formed. The media and the oil were not degassed, thus some oxygen was available at the initiation of droplet cultures.

C. *B. subtilis* culture in droplets

To further investigate the conditions in droplet cultures, *B. subtilis* cells expressing red fluorescent protein (RFP) was cultured in the two droplet incubation formats. *B. subtilis* can be used as an indicator of oxygenation being an almost exclusively aerobic bacterium that requires oxygen to proliferate unless nitrate, pyruvate, or a mixture of amino acids are added to the media.³² As such supplements were not added to the media in these experiments, *B. subtilis* growth should be taken as an indication of aerobic culture conditions. The fluorescence of the cultures was measured at four time points during a 72 h period to assess cell growth and RFP expression. After 72 h, the aerobic control and droplets in wide tube cultures had both increased in fluorescence and turned orange/red and pale pink/red, respectively, whereas the droplets in syringe and oxygen limited control cultures still showed no increase in fluorescence or change in color (Fig. 4). This indicates that *B. subtilis* growth did occur in the droplets incubated in wide tube but did not occur in the droplets incubated in the in syringe.

Furthermore, culture opacity can be used as a secondary indicator of proliferation. No increase in opacity was seen by ocular inspection in the droplets in syringe or oxygen limited control cultures, whereas the aerobic control and droplets in wide tube increased in red color as well as opacity over time. This further supports that no *B. subtilis* growth occurred in syringe droplet culture.

D. Differences in incubation conditions within the syringe

Incubating *B. subtilis* in droplets in a syringe for 72 h did not lead to any significant increase of fluorescence. However, when the syringe was left in the incubator for seven days, the droplets at the emulsion-oil interface turned red, and an indication that the cells in the droplets closest to the oil did grow and express RFP (Fig. 5). The color change restricted to the interface indicates stratification inside the syringe with respect to cell growth. Oxygen is 10–20 times more soluble in HFE compared to water,²⁴ making HFE a good oxygen reservoir.

FIG. 4. Change in fluorescence intensity (in arbitrary units) over time incubating *B. subtilis* expressing red fluorescent protein in droplets in syringe, droplets in wide tube, oxygen limited control culture, and aerobic control culture. 90 μ M droplets with on average 5.3 cells per droplet were generated and incubated in syringe and wide tube. Aerobic and oxygen limited non-droplet *B. subtilis* cultures with corresponding cell density were used for control. Replicate presented in supplementary material D.³³

FIG. 5. *B. subtilis* incubated in droplets in a syringe for 7 days. The droplets at the oil-emulsion interface have turned red indicating expression of RFP and cell growth, whereas the other droplets in the syringe have not changed color.

However, the uneven cell growth in the syringe indicate that although there may be gas dissolved in the oil it does not reach all the droplets in the syringe. In cell screening applications, it is of importance to incubate all droplets under the same conditions to avoid biased results. If the growth stratification is caused by differences in oxygenation, the incubation location bias will favor cells in droplets close to the emulsion-oil interface when screening for proteins up-regulated under aerobic conditions, skewing the result. We speculate that mixing droplets during syringe incubation might redistribute the droplets sufficiently to overcome such variations in cell growth.

IV. CONCLUSIONS

We have characterized metabolic state of yeast cells in microfluidic droplet cultures using HPLC. By tracking concentrations of a few key metabolites, the effect on cell metabolism of incubating droplets in different incubation formats was elucidated. Metabolite profiling is a general method used to determine the impact of various culture conditions on cell function. This approach allows us to select suitable droplet incubation formats for droplet based applications demanding aerobic or anaerobic incubation, e.g., in screening for respiratory or fermenting yeast cell factories. The metabolic state of *S. cerevisiae* was evaluated from droplet cultivations incubated in a novel wide tube format and the standard syringe format. For *B. subtilis*, the choice of incubation format proved to be critical for cell growth. The use of metabolite profiling in the selection of incubation formats is applicable beyond the two formats presented in this article, e.g., to Pasteur pipette and PDMS sealed vials and to other culture conditions and cell types as well.

Our findings support that the metabolic state of cells in droplet culture could be analyzed by metabolite profiling and controlled, by choosing the appropriate incubation format. Here, oxygenation of the culture medium is a pivotal parameter. Insufficient control of the incubation conditions is likely to lead to unwanted bias in the results. The screening maxim “you only get what you screen for” cautions experimentalists that variables affecting the cell are important to control during a screen. If the conditions are anaerobic cells expressing the trait screened for under anaerobic conditions are favored, and *vice versa* under aerobic conditions. Therefore, the precise control of gas exchange in microfluidic droplets will enable more specialized screening, allowing the results of the screen to more closely match the needs of the intended end user. In a wider perspective, the strategy to track cell metabolic state in order to choose incubation conditions also holds the possibility to make droplet microfluidics more conducive to longer term mammalian cell culture.

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