ABSTRACT
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 screening in droplets, as cell metabolic state directly affects production yields in cell factories. Metabolite profiling provides a more nuanced estimate of cell state compared to proliferation studies alone. We show that the choice of droplet incubation format has an impact on cell proliferation and metabolite production. Furthermore, we engineered a new better oxygenated droplet incubation format, with retained droplet stability and size.

KEYWORDS: Droplet microfluidics, cell culture, cell metabolism

INTRODUCTION
Droplet microfluidics is a useful tool for single cell screening, e.g. directed evolution of yeast [1]. Culture conditions have a great impact on cell behavior, potentially affecting screening outcome. Previous studies of droplet cell culture conditions have focused on oxygen transport between droplets and oil, at droplet generation [2], and cell proliferation and viability in droplets [3]. For single cell screening purposes it is of interest to also take metabolism into consideration. To investigate culture conditions in long term off chip droplet incubation, we generated 90µm droplets containing *S. cerevisiae* in an immiscible fluorinated oil phase on a microfluidic chip and tracked metabolite concentrations during a 48-hour incubation. Droplets were incubated off-chip in a plastic syringe and in a prototype wide tube format, with two non-droplet formats with corresponding cell densities, used as controls (Fig 1). Droplet microfluidic applications that include encapsulated cell culture usually employ off-chip storage in syringes or glass vials for droplet incubation. In these formats, droplets are not in direct contact with the surrounding atmosphere, limiting oxygenation. To improve oxygen availability for long-term culture we developed a prototype droplet incubation format termed wide tube.

THEORY
*S. cerevisiae* use two main glucose dissimilation pathways; aerobic respiration and anaerobic ethanol fermentation. 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. Monitoring temporal changes in key metabolite concentrations to elucidate which pathway is active can thus be used to evaluate culture conditions during droplet incubation to compare incubation formats.
A microfluidic flow-focusing device for generation of 90µm droplets was fabricated from polydimethylsiloxane, PDMS, and glass using standard soft lithography. The microorganisms used were the \textit{S. cerevisiae} strain CEN.PK 113-7D and a \textit{B. subtilis} strain expressing red fluorescent protein (RFP). The oil used was HFE-7500 with 1%(w/v) EA surfactant. 1H, 1H, 2H, 2H-perfluoro-1-octanol was used for emulsion destabilization.

The emulsion quality in the wide tube format was tested by generating droplets with \textit{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 during a 10 day period.

To monitor \textit{S. cerevisiae} metabolism in droplet cultures yeast cells were cultured defined media in a shake flask overnight and washed and diluted to OD$_{600}$ = 2.5, for each droplet to be populated by 5.3 cells on average. Droplets were generated and emulsion was collected into a plastic syringe followed by emulsion collected in the wide tube. For aerobic control a shake flask with a tissue lid was used, and for oxygen limited control a plastic syringe with tubing and fittings was used. All samples were incubated at 30°C. Samples were taken at 0h, 3h, 6h, 9h, 12h, 24h and 48h. The emulsion was broken using 1H, 1H, 2H, 2H-perfluoro-1-octanol before freezing. For analysis the samples were thawed and analyzed for glucose, pyruvate, ethanol and glycerol concentrations using HPLC.

In a second experiment, RFP expressing \textit{B. subtilis} was incubated in the same incubation formats as the yeast cells. The sample was diluted to OD$_{600}$ = 0.1, corresponding to an average of 5.3 cells/droplet. Samples were taken at generation, 24h, 48h and 72h. The emulsion was broken with 1H, 1H, 2H-perfluoro-1-octanol and the aqueous phase was used for analysis. 50µL of each sample was transferred to a 96 well plate and fluorescence was measured using a microplate reader. The excitation was set to 570 nm and emission to 600 nm.

**RESULTS AND DISCUSSION**

Analysis of droplet size over time in the wide tube show that droplets maintain their size during the first 72 hours but that the size is slightly decreased when interrogating the droplets after 10 days of incubation, indicating that some evaporation has occurred (Fig 2). Upon generation the mean droplet diameter was 88.9±2.86 µm and after 10 days it had decreased to 79.8±3.09 µm. The result show a high degree of monodispersity at each time point, an indication that the emulsion is of good quality and that the droplets are stable and have not coalesced. Droplet cell cultures incubated in syringe yielded metabolite profiles resembling the oxygen limited control cell culture whereas droplet cultures incubated in the wide tube demonstrated metabolite profiles resembling the aerobic cell culture control (Fig 3).

**Figure 2:** Droplet stability and size after incubation in wide tube for a) 0h, b) 5h, c) 72h, and d) 10 days.

**Figure 3:** Metabolite concentrations over time incubating \textit{S. cerevisiae} in the formats described in Figure 2. blue - droplets in syringe, yellow - droplets in wide tube, green - oxygen limited control, red - aerobic control.
B. subtilis expressing RFP, which only proliferate with adequate oxygenation, were cultured in the different incubation formats to verify conclusions regarding oxygenation conditions. During a 72h incubation period B. subtilis growth was observed in droplets incubated in the wide tube whereas no growth was observed for droplets incubated in a syringe (Fig 4). After 7 days B. subtilis incubated in droplets in a syringe showed stratified cell growth within the syringe. Only cells in droplets close to the oil-emulsion interface proliferated to express red fluorescent protein (Fig 5).

**CONCLUSION**
Evaluation and control of culture conditions is critical for droplet based cell screening as the conditions in the droplets can greatly affect the metabolism of the cells and thus screening results. Here, we demonstrate the analysis of yeast and bacterial droplet culture for standard formats and a novel better oxygenated format. We show that the choice of incubation format has an impact on cell viability and metabolism, and thus need to be taken into consideration when designing screening experiments. Metabolite analysis is a promising approach for evaluation of incubation conditions in various formats to reduce screening bias.

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