Microdroplet enabled cultivation of single yeast cells correlates with bulk growth and reveals subpopulation phenomena

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Abstract

Yeast has been engineered for cost-effective organic acid production through metabolic engineering and synthetic biology techniques. However, cell growth assays in these processes were performed in bulk at the population level, thus obscuring the dynamics of rare single cells exhibiting beneficial traits. Here, we introduce the use of monodisperse picolitre droplets as bioreactors to cultivate yeast at the single-cell level. We investigated the effect of acid stress on growth and the effect of potassium ions on propionic acid tolerance for single yeast cells of different species, genotypes and phenotypes. The results showed that the average growth of single yeast cells in microdroplets was identical to those of yeast populations grown in bulk, and microdroplet compartments do not significantly affect cell viability. This approach offers the prospect of detecting cell-to-cell variations in growth and physiology and is expected to be applied for the engineering of yeast to produce value-added bioproducts.

1. Introduction

Yeast has been widely used as a "cell factory" in industrial fermentation processes to produce a wide range of valuable products, including organic acids that are used extensively in manufacturing, pharmaceutical, cosmetic, food, textile and chemical industries (Álvarez-Chávez et al., 2012; Chen et al., 2013; de Jong et al., 2014; Gonzalez-Garcia et al., 2017; Hong and Nielsen, 2012). Compared to conventional chemical methods for the production of organic acids based on fossil fuel reserves, microbial production is an attractive approach due to several advantages including sustainability, less environmental pollution and cost-effectiveness (Sauer et al., 2008; Steen et al., 2010). Unlike other hosts that are recalcitrant to genetic manipulation (Kiatpapan and Murooka, 2002; Zhuge et al., 2013), baker's yeast (*Saccharomyces cerevisiae*) is an ideal organism to discover new gene targets for productivity enhancement, because it is a model eukaryotic organism with high-resolution genomic data. Moreover, the tolerance of yeast to low pH enables the production of organic acids in their protonated forms, reducing the costs of downstream recovery and purification after fermentation. Due to the economic, environmental and medical importance of organic acid production by yeast, advanced metabolic engineering and synthetic biology technologies have been applied to engineer yeast for improved production of different high-value organic acids, such as lactic acid (Ishida et al., 2005), succinic acid (Otero et al., 2013), para-hydroxybenzoic acid (Williams et al., 2015), 3-hydroxypropionic acid (Borodina et al., 2015) and muconic acid (Curran et al., 2013).

Yeast is also an attractive host for the production of propionic acid (PA) that is commonly used as a food preservative and a chemical intermediate, since PA can be formed as a by-product of yeast fermentation (Eglinton et al., 2002). However, PA is toxic to yeast, especially at relatively low concentrations, causing

an important problem of tolerance engineering in yeast PA production. Fortunately, Xu et al. (2019) demonstrated significant improvements in yeast tolerance to PA using adaptive laboratory evolution (ALE), a powerful tool in the field of metabolic engineering for the development of superior industrial microbial strains (Almario et al., 2013; Gonzalez-Ramos et al., 2016; Kildegaard et al., 2014).

ALE experiments are lab-intensive and time-consuming however, requiring evaluation of growth kinetics of intermediate populations and numerous candidate strains to select an ideal strain with improved phenotypes. The evolution process might be performed over hundreds of generations, and the traditional growth test based on optical density (OD) measurements must be conducted over three repetitions for each strain or population for each population or strain. Moreover, the process lacks the ability to track the growth of yeast at a single-cell level, and cannot consider cell size, morphology and viability that may change during growth. Thus, cell-to-cell variations are obscured and the ability to screen and select single cells with desired characteristics (e.g., high growth rate, high tolerance to acids and high secretion of valuable bio-products) is limited.

In order to address these limitations, an alternative approach is required to quantitatively track the growth of individual cells within a population without perturbation and allows parallel, high-throughput assessment at a single-cell level. Microfluidics can compartmentalize single cells within monodisperse picolitre-sized droplets in a cost-effective and high-throughput process, for example, screening of 5×10^7 individual reactions requires only 150 μL of reagents and seven hours at an estimated cost of only a few dollars, as demonstrated by Agresti et al. (2010). Over the past decades, droplet microfluidics has enabled single-cell analysis for a wide range of applications across biological science, biomedicine and biochemistry (Agresti et al., 2010; Brouzes et al., 2009; Yu et al., 2018). This is because (1) the extracellular environments are accurately mimicked (Hosokawa et al., 2017; Liu et al., 2020); (2) the genotype-phenotype linkages are established at a single-cell level (Bowman and Alper, 2020; Fischlechner et al., 2014; Li et al., 2018a, 2018b); (3) the miniaturized confinement improves the detection limit (Agresti et al., 2010; Zhu et al., 2012); and (4) massive parallel analysis can be conducted to probe cellular heterogeneity (Headen et al., 2018; Hindson et al., 2011; Klein et al., 2015; Ostafe et al., 2014; Zinchenko et al., 2014).

In this study, we quantitatively tracked the growth of single yeast cells under varying conditions by using monodisperse microdroplets. In order to demonstrate the versatility of the microdroplet platform, we used two species of yeast, *Saccharomyces cerevisiae* (*S. cerevisiae*) and *Pichia pastoris* (*P. pastoris*), and a total of four strains, wild-type *S. cerevisiae* strain (CEN.PK113-7D), the PA evolved mutant *S. cerevisiae* strain (PA-3), GFP-tagged *S. cerevisiae* strain (CEN.PK2-1C-GFP) and GFP-tagged *P. pastoris* strain (CBS7435-GFP). The effects of organic acids, PA and AA, at different concentrations on the growth of yeast at the single-cell level were studied, as well as the effect of K-ions on PA tolerance in yeast. The calculated specific growth rate (μ) of single yeast grown in microdroplets was effectively identical to that for cells in bulk cultures at a pH of 3.5, and yeast cells maintained high viability in microdroplets after 48 hours of culture.

2. Materials and Methods

2.1 Fabrication of microfluidic devices

The T-junction microfluidic device used in this study consists of two inlets for infusing the disperse (aqueous) phase and continuous (oil) phase, respectively, one outlet for transporting microdroplets into the collection tube and one rectangular chamber for observation of cell-laden microdroplets. A corona-shaped filter was designed at the inlets to prevent any possible dust entry. The connecting microchannels have an aspect ratio of height/width = 4:5 (height: ˜40 μm; width: ˜50 μm), and the rectangular observation chamber dimensions of 1.4 × 0.65 cm (Fig. S1 & Fig. S2).

This droplet-based microfluidic device was fabricated from a silicon wafer patterned with SU-8 mould (SU-8 2035, MicroChem, Newton, MA, USA) using standard soft-lithography techniques (Xia and Whitesides,

1998). Degassed poly(dimethyl siloxane) (PDMS, Sylgard 184, Dow Corning, Midland, MI, USA) in liquid form prepared by mixing the base and curing agent at a ratio of 10:1 was poured onto the SU-8 mould and cured in an oven at 80 °C for two hours. Then the PDMS slab with microchannels was peeled off from the mould and the fluidic access holes were created using PDMS biopsy puncher with an outer diameter of 1.5 mm. After cleaning the channel side of PDMS with scotch tape, isopropanol and DI water in order, the PDMS slab and a standard glass slide was treated using an oxygen plasma cleaner to increase the surface energy and immediately pressured to each other for an irreversible bonding. Lastly, the channel was rendered hydrophobic by infusing with 0.02 % Trichloro(octadecyl)silane (OTS, 104817, Sigma-Aldrich, St. Louis, MO, USA) in isopropanol for 5 mins and drying with nitrogen gas followed by drying in an oven at 100 °C for 10 mins.

2.2 Cell preparation

We used a total of four yeast strains, the haploid *S. cerevisiae*strain (CEN.PK113-7D), the PA evolved mutant *S. cerevisiae* strain (PA-3), GFP-tagged *S. cerevisiae* strain (CEN.PK2-1C-GFP) and GFP-tagged *P. pastoris* strain (CBS7435-GFP) in the single-cell growth assays (Table S1).

CEN.PK113-7D and PA-3 were grown overnight at 30 °C, 200 rpm in 5 mL buffered minimal medium (Xu et al., 2019). The overnight culture was washed twice and re-inoculated into 5 mL buffered minimal medium at three different concentrations of PA: 15 mM, 25 mM and 35 mM. To investigate the effect of K-ions on the tolerance to PA in yeast, CEN.PK2-1C-GFP was pre-cultured overnight at 30 °C, 200 rpm in synthetic drop-out medium, without uracil (contains 1× yeast nitrogen base (YNB) without amino acids mix (Y0626, Sigma-Aldrich, St.Louis, MO, USA), 1% glucose and Yeast Synthetic Drop-out Medium Supplements without uracil (Y1501, Sigma-Aldrich, St.Louis, MO, USA)). Cells were washed twice and re-inoculated into 5 mL uracil drop-out medium containing defined concentrations of potassium (1× translucent K^+ free YNB, 1% glucose, and 0, 1, 10 and 50 mM potassium chloride), supplemented with or without 25 mM PA. CBS7435-GFP was pre-cultured overnight in the same drop-out medium as the one used for CEN.PK2-1C-GFP at 25 °C, 200 rpm, and the culture was washed twice and re-inoculated into 5 mL uracil drop-out medium. All the cultures were reinoculated at an initial OD_{600} of 0.2 before being encapsulated into microdroplets.

2.3 Generation and storage of microdroplets

In the T-shaped droplet generator of 50 × 50 μm (width × depth), continuous phase flowed to the observation chamber and the disperse phase flowed and sheared at the interface to generate monodisperse microdroplets with a diameter of ˜65 μm. Continuous phase used here was Novec 7500 Engineered Fluid (3M, St. Paul, MN, USA) containing 2% Pico-Surf 1 (Sphere Fluidics, Cambridge, UK). Disperse phase used here was culture medium added with 20% OptiPrep™ (D1556, Sigma-Aldrich, St. Louis, MO, USA). Cells were diluted to an OD_{600} of 0.1 for the disperse phase. Two syringe pumps (Fusion 100, Chemyx, Stafford, TX, USA) were used to inject the two phases, respectively. When the ratio of the flow rates of the two phases reached 4:1 (continuous phase: 16 μL/min vs disperse phase: 4 μL/min), ˜ 144 pL monodisperse microdroplets were created in a high-throughput fashion (˜116 droplets per second). By Poisson distribution, this size of droplet can ensure substantial droplets containing single cells (28.0%) and maintain relatively low ratio of droplets containing two cells (6.0%) and less than 1.0% droplets containing more than two cells, and the rest 65.0% droplets are empty. A fluorinated ethylene propylene (FEP) tubing (IDEX, Lake Forest, IL, USA) with an inner diameter of 0.5 mm was used to transfer microdroplets into a 2 mL Eppendorf™ safe-lock tube (Hamburg, Germany) pre-filled with 100 μl continuous (Fig. S3).

2.4 Image acquisition and analysis

An initial observation of microdroplets containing single cells was performed at a rectangular chamber of the microdevice before microdroplets were transported into the collection tube. Bright-field snapshots of the generated microdroplets were captured by a digital camera (DS-Qi1Mc, Nikon, Tokyo, Japan) installed on

an inverted microscope (Eclipse Ti-U, Nikon, Tokyo, Japan). After images were taken, the number of cells per droplet was counted using ImageJ® (National Institutes of Health (NIH), Bethesda, MD, USA).

To quantify growth of single yeast cells, the values for the specific growth rate μ were determined based on the number of cells per droplet at hourly time points up to 10 hours. The number of cells per droplet (N) was converted into the logarithmic scale as ln(N), and the estimation of the biokinetic constant, μ, over time of culture, t, was obtained by the equation below:

$$\mu = \frac{\mathrm{dln}(N)}{\mathrm{dt}} \# (1)$$

Fluorescence images of GFP-tagged cells were obtained at fluorescein isothiocyanate (FITC) channel by a confocal microscope FV3000 (Olympus, Tokyo, Japan). The number of yeast cells in a complex cluster were more easily counted from the fluorescence images compared to bright-field images. Regarding uncountable agglomeration, images of eight slices were stacked up and the corresponding fluorescence intensity was measured by ImageJ®. The image processing includes the following four main steps: 1) the bit depth of images was reduced to 8 bits; 2) area of each droplet was recognized; 3) The threshold was set by Yen's algorithm to remove noise; and 4) "limit to threshold" was chosen and the mean fluorescence intensity within the area of each droplet was measured.

2.5 Cell viability test

To measure the viability of yeast cells in microdroplets, live/dead staining tests were performed at three selected time points: before encapsulation, at 24 hours of culture and 48 hours of culture. At each time point, 50 μl emulsion microdroplets in oil were collected into a centrifuge tube, and 2 μL Pico-Break (Sphere Fluidics, Cambridge, UK) was added subsequently to release yeast cells from the microdroplets. After a short centrifugation at 2000 rpm for 30 s, the oil phase was kept at the bottom and the supernatant was transferred into a new centrifuge tube. 30 μl staining solution consisting of 2 μM SYTO 9 (Thermo Fisher Scientific, Waltham, MA, USA) and 4 μM EthD-III (Biotium, Hayward, Ca, USA) was added into the suspension of released yeast and co-cultured for 20 mins. Fluorescence images were taken when excited at a wavelength of ˜495 nm and ˜530 nm, respectively: live cells showed green fluorescence in 515 nm channel while dead cells showed red fluorescence in ˜635 nm channel and yellow colour in merged images. The cell viability over time was tested based on three random frames for each measurement, and a total of 100 cells were tested for each measurement. The viability test results were analysed by one-way repeated measures analysis of variance (ANOVA) to determine whether a significant difference was existed among different time points.

3. Results and Discussion

3.1 The encapsulation and cultivation of single yeast cells in picoliter microdroplets

Similar to the standard method used to measure the average growth rate of bulk populations, the procedure for the microfluidic droplet technique used in this study to measure the growth of single yeast cells includes three main steps: 1) preparation, 2) measurement and 3) analysis (Figs. 1A and 1B). 1) Uniformly distributed microdroplets containing single cells and a small volume of culture medium, subject to a range of different environmental conditions, are generated; 2) the number of cells per droplet and fluorescence intensity of GFP-tagged cells are tracked as cells grow over time; and 3) the growth rate of each cell within the microdroplet is obtained and single-cell growth under varying environmental conditions (with PA, AA and K-ions at different concentrations) is investigated and compared.

Before cell encapsulation within microdroplets, the overnight culture and preculture protocols were successively performed to ensure yeast cells were rapidly proliferating but this also results in an asynchronous

culture, containing single cells, single cells with small buds, and cells with large buds. In practise, the budded cells were counted as single cells when the buds did not exceed the one-half the size of the mother cell. Cell density was diluted to an OD_{600} of 0.1 (3×10^6 cells/mL) with fresh medium and density-matching reagent to ensure the substantial majority (>82%) of cell-laden droplets contained just one cell. According to the Poisson distribution, this concentration of cells theoretically maintains high efficiency of single-cell encapsulation (Liu et al., 2020). It is worth noting that under these conditions the majority (65%) of droplets do not contain any cells. The density-matching reagent used here is 20% OptiPrep^{TM}, which prevents the sedimentation of yeast cells at the inlet and guarantees the neutral buoyancy of cell suspensions for ˜30 mins (Allazetta et al., 2015). The asynchronous nature of the cell population combined with the small statistical probability for more than one cell per droplet leads inevitably to some experimental uncertainties in measured cell growth notionally arising from a single cell origin.

The growth of single *S. cerevisiae* CEN.PK 113-7D cells in ˜ 144 pL microdroplets without acid stress is illustrated by the images of Fig. 1C. After culture of 18 hrs, the droplets shrank from ˜ 144 pL (empty droplets) to ˜ 65 pL (yeast-containing droplets) driven by osmosis. The yeast cells kept consuming the glucose from the medium in droplets and induced water efflux to equalize the solute concentration inside and outside the droplets (Joensson et al., 2011; Siedler et al., 2017). No significant size differences were detected between the same types of droplets. Apart from this, the yeast-containing droplets cells did not experience any noticeable disruption, such as merging and burst, after long-term storage, thus the single yeast growth can be quantitatively and accurately tracked over 24 hours within this type of droplet.

3.2 The effect of acid stress on the growth of single wild-type S. cerevisiae cells in picoliter microdroplets

First, we tracked the growth of the single *S. cerevisiae* CEN.PK 113-7D cells in ˜144 pL microdroplets without acid stress over 24 hours. In Fig. 2A, we plotted the number of cells per droplet at a logarithmic scale based on the first ten hours of culture, assumed during exponential phase. The value of μ for single *S. cerevisiae* cells in microdroplets was calculated to be 0.23 ± 0.03 h^{-1}, identical to that reported for bulk populations (0.21 ± 0.01 h^{-1}) within experimental uncertainty. Then we plotted the number of cells per droplet at eight selected time points: 0 hr, 2 hrs, 4 hrs, 6 hrs, 8 hrs, 10 hrs, 18 hrs and 24 hrs. (Fig. 2B). In general, the number of cells per droplet increases over time: single yeast cells (at 0 hrs) grow to 3.2 ± 1.4 at 2 hrs, 5.7 ± 2.0 at 4 hrs, 8.7 ± 2.2 at 6 hrs, 11.6 ± 2.7 at 8 hrs, 14.6 ± 3.1 at 10 hrs, 42.2 ± 7.0 at 18 hrs and 50.0 ± 8.0 at 24 hrs. The number of cells per droplet is seen to increase monotonically over time, but with an increasing spread of cell-counts for the later time (18 hrs and 24 hrs). Also, the heterogeneity in the proliferation of single yeast cells is demonstrated. At 24 hrs, a small portion (i.e., 5%) of microdroplets contain more than 60 cells, whereas another small proportion (i.e., 6.7%) of microdroplets has less than 40 cells. This is evidence of subpopulations exhibiting diverse traits that are obscured in bulk assays at the population level. It is noteworthy that after the culture of 18 hrs, the amount of yeast in droplets altered slightly compared to the previous growth, indicating that the yeast cells had reached the stationary phase due to the scarce nutrient and limited space. Droplet size can be tuned to enable different scales of cell culture and has been observed in other studies (Pan et al., 2011; Siedler et al., 2017).

Secondly, we investigated the effect of acid stress on cell growth by tracking and comparing the growth of single *S. cerevisiae* cells with the addition of PA (at 7.5 mM and 35 mM, Fig. 2C) and AA (at 50 mM and 67 mM, Fig. 2D). The study of yeast growth and responses to PA and AA is of great importance, because PA is a valuable organic acid produced by yeast during fermentation, and AA is a main growth inhibitor found in lignocellulose hydrolysate for lignocellulose-based biofuel production. Experimental results show that microdroplets enable cell growth in all conditions. In more detail, at 7.5 mM PA, single cells in microdroplets grow to 31.0 ± 5.5 and 40.0 ± 4.8, respectively, at 18 hrs and 24 hrs; while at 35 mM PA, single cells grow to 3.1 ± 1.4 at 18 hrs and 3.8 ± 1.4 at 24 hrs (Fig. 2C). At 50 mM AA, single cells in microdroplets grow to 13.9 ± 2.6 and 22.0 ± 5.84, respectively, at 18 hrs and 24 hrs; while at 67 mM AA, single cells grow to 8.6 $\pm$ 2.7 at 18 hrs and 12.4 ± 3.1 at 24 hrs (Fig. 2D). Moreover, we found that growth of single *S. cerevisiae* cells responds sensitively to both acids and decreases as the concentration of acids increases. At 24 hrs, the number of yeast cells per microdroplet under 7.5 mM PA (40.0 ± 4.8) and 35 mM PA (3.8 ± 1.4) declined,

respectively, to 80.0% and 7.6% of that for no acid control (50.0 ± 8.0) (Fig. 2C). Additionally, at 24 hrs, the number of yeast cells per microdroplet decreased to 44.0% (22.0 ± 5.84) and 24.8% (12.4 ± 3.1) of cell number of the control group (no AA), respectively, when the concentration of AA increased to 50 mM and 67 mM (Fig. 2D). It is noteworthy that only the cell counts per droplet with the addition of 7.5 mM PA can be well fitted to sigmoid growth curve, indicating the small volume of PA, like 7.5 mM, did not affect yeast growth much but the large volume of that and AA will impose negative effect since the beginning of cell growth. These results indicate that the growth and physiology of single cells in microdroplet are the same as those of yeast populations grown in bulk, although microdroplet culture reveals subpopulation phenomena that are obscured by population average measurements.

3.3 The effect of K^+ on the growth of single GFP-tagged S. cerevisiae cells in picoliter microdroplets

Since biochemical assays are typically measured using fluorescence detection techniques, we investigated the growth of fluorescent GFP-tagged *S. cerevisiae* strain (CEN.PK2-1C) to demonstrate the capability of our platform for fluorescence-based quantification and detection of single-cell features. Both bright-field and fluorescence images show that the number of fluorescent cells per droplet increases over time (Figs. 3A and 3B). We counted the number of cells per droplet at five selected time points: 2 hrs, 6 hrs, 10 hrs, 18 hrs and 24 hrs (Fig. 3C). The data shows that growth from single cells has a high degree of variability: although the average cell number per droplet is 14.4 ± 3.3 at 24 hrs, a few microdroplets (i.e., 3.3%) contain more than 20 cells, whereas some microdroplets (i.e., 15%) contain less or equal to 10 cells. This is further evidence of cellular subpopulation with different growth growth rates which are obscured in bulk assays.

In *S. cerevisiae* , potassium uptake has been shown to stabilize membrane potential, and mediate intracellular pH, protein synthesis and function (Arino et al., 2010; Kahm et al., 2012; Yenush et al., 2002). In previous studies, potassium supplementation was also demonstrated to be beneficial to PA-tolerance behaviours of *S. cerevisiae* (Xu et al., 2019). We firstly tracked the growth of single CEN.PK2-1C cells in microdroplets under a fixed potassium defined condition (10 mM K^+) with or without 25 mM PA (Fig. 3D). The results show that yeast growth in microdroplets was inhibited under PA stress condition when the medium contains 10 mM K^+.

We then applied another two concentrations of K^+, excessive supply of 50 mM and a scanty supply of 1 mM, when the concentration of PA is fixed at 25 mM (Fig. 3E). Compared to the μ under 10 mM K^+ at 24 hrs, there is a 38.9% increase when 50 mM K^+ was used, and no significant decrease when the concentration of K^+ reduces to 1 mM. These results agree with the previous findings that extracellular supplementation of K^+ can increase PA tolerance in yeast, and potassium influx is important to increase organic acid tolerance in *S. cerevisiae* By using the GFP-labelled strain and supplementing K^+ under PA stress conditions, we have shown that single-cell culture in microdroplets demonstrate the same phenotype and the similar growth profiles as bulk cultures, although cell-to-cell variations in proliferation are observed. We conclude therefore that the microdroplet platform can reliably quantify the effects of external factors on cell growth and complex physiology under varying conditions.

3.4 The growth of wild-type and PA evolved mutant S. cerevisiae strains in picoliter microdroplets

ALE has previously been employed to improve PA tolerance in yeast, and PA-3 is one of the isolated strains with increased PA tolerance after performing ALE. The non-synonymous mutation in potassium transporter encoding gene *TRK1* , has been confirmed to be the cause of the increased PA tolerance (Xu et al., 2019). To demonstrate that microdroplets could be used to track the growth of yeast mutant strains, we monitored and compared the growth of PA evolved mutant strain (PA-3) and its parental strain (CEN.PK 113-7D) when 15 mM PA was applied (Fig.4). The experimental data confirms that PA-3 grows faster and reaches a significantly higher average number of cells per droplet, i.e., 18.0 ± 3.0 at 24 hrs, whereas the average number of cells per droplet for wild-type strain is 5.2 ± 1.3 at 24 hrs (Fig. 4). This result demonstrates that the microdroplet reactor approach is effective for both normal and mutant strains of *S. cerevisiae* and

holds their difference in cell growth and physiology at the population level when single cells are tracked in microdroplets.

3.5 The growth of single P. pastoris cells in picoliter microdroplets

In order to demonstrate that this platform can be applied to species other than *S. cerevisiae* , we tracked the growth of GFP-tagged*P. pastoris* strain (CBS7435-GFP) at a single-cell level in ˜144 pL microdroplets. *P. pastoris* has a similar cell size to *S. cerevisiae* , but the proliferation behaviour is different. The CBS7435-GFP used here tended to aggregate in the center of the microdroplets due to the lack of motility. The bright-field and fluorescence images (stacks of eight slices) show that single *P. pastoris* cells are able to grow in microdroplets over time (Fig. 5A). The distribution of fluorescence intensity of cells within the microdroplets at five selected time points demonstrated the variations between individual cells (Fig. 5B). Although some outliers exist, the growth curve of *P. pastoris* shows a similar profile over 24 hours to that of *S. cerevisiae* under normal conditions. This indicates that the fluorescence measurement can quantitively indicate the growth of single cells in microdroplets and demonstrates that the microdroplet bioreactors used in this study can maintain and screen of growth of single yeast cells of different species.

3.6 Viability assays of S. cerevisiae and P. pastoris grown in microdroplets

We used the cell staining live/dead kit to investigate whether 24 hours or a prolonged period of culture will affect the viability of *S. cerevisiae* and *P. pastoris* . This is to ensure that encapsulation and cultivation of cells in microdroplets is a feasible and stable method for long-time single-cell assays.

The viability tests were performed and compared at three time points: before encapsulation, after 24 hrs and 48 hrs of encapsulation. The bright-field and fluorescence images show that both *S. cerevisiae*and *P. pastoris* cells maintain a high level of viability after 24 hours of culture (Fig. 6A). For *S. cerevisiae* , 94.4 $\pm$ 1.3% cells remain alive after 24 hours of culture, and cell viability slightly decreases to 93.6% $\pm$ 1.7% after 48 hours of culture; while for*P. pastoris* , 97.8 $\pm$ 0.8% and 95.5% $\pm$ 1.1% cells remain alive after 24 hours and 48 hours of culture, respectively (Fig. 6B).

Considering that the oil-removing reagent, pico-break^{TM}, contains PFOH (1H,1H,2H,2H-Perfluoro-1-octanol) which is a potential chemical hazard for yeast cells, the measured viability of encapsulated cells may represent an underestimate of the true viability. Moreover, the result of one-way repeated measures ANOVA ($P < 0.005$) shows that there is no significant difference in cell viability among that before encapsulation, that for 24 hours and 48 hours of culture. This demonstrates that the viability of yeasts cultured in microdroplets is not significantly affected and the method is capable of prolonged assays of live yeast cells.

In this study, we explored the feasibility of using microdroplets as bioreactors to screen cell-to-cell variations in growth. Cell encapsulation in microdroplets is a random process limited by the Poisson distribution but affected by cell sedimentation, leading to a majority of droplets that are empty. To maximize the proportion of single cell-encapsulated microdroplets without any noticeable damage, we used a non-ionic solution of 60% iodixanol, OptiPrep, which has proved to be biocompatible, has low osmotic pressure and low intrinsic viscosity suitable for the culture of cells in microdroplets (Allazetta et al., 2015; Ma et al., 2017). Here, we used the addition of 20% OptiPrep to reduce the effect of cell sedimentation (the density of yeast cells is 1.1g/mL, which is higher than that of culture medium), and to temporarily create neutrally buoyant cell suspensions without noticeable adverse effects. This concentration of OptiPrep (i.e., 20%) enables the generation of a total of 830,000 microdroplets (˜ 28.0% containing single yeast cells) in 30 mins. We note, however, that for studies that require a continuous generation of large amounts of cell-laden microdroplets, a higher concentration of OptiPrep or an alternative density-matching reagent of higher density may be necessary.

Moreover, we demonstrated the capability of droplet microfluidic platform for quantitatively tracking of single yeast cell growth of different species, genotypes and phenotypes, and also under different environmental

conditions. When single cells are contained in isolated environments, not only can the growth rate of cells be screened, but also the phenotypes to secrete multiple high-value bioproducts (e.g., organic acids, antibodies and cellulases), since all the secreted products are confined within the microdroplet compartments. We can also obtain further understanding of genetic and molecular mechanisms underpinning beneficial phenotypes due to the genotype-phenotype linkages provided by the microdroplets. By combining with high-throughput screening and sorting technologies, e.g., fluorescence-activated cell sorting (FACS) and image-activated cell sorting (IACS) (Nitta et al., 2018), this platform can accelerate the progress of development of yeast strains with desirable properties (e.g., high yield of valuable products, high environmental tolerance and high growth rate) for industrial applications.

4. Conclusion

This study demonstrated the use of microdroplets for quantitative, high-throughput and low-cost assessment of growth of single yeast cells. The results from single-cell assays showed that PA and AA inhibit cell growth, the uptake of K^+ improves PA tolerance in yeast, and *TRK* 1 mutant exhibits increased PA tolerance, agreeing with previous findings obtained by analysis of cells in bulk populations. Neither were cells in microdroplets seen to experience noticeable loss in viability over 48 hours. Moreover, the microdroplet approach reveals subpopulation phenomena that are obscured by population average measurements, opening avenues to probe cell-to-cell variations under different environmental conditions.

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Competing financial interests:

The authors declare no competing financial interests.

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Fig. 1. Schematic illustration of conventional laboratory approach (A) and droplet-based microfluidics (B) for tracking the growth of yeast at population level and the single-cell level, respectively. (C) Bright-field microscope images of CEN.PK 113-7D growth without organic acid stress in ˜144 pL microdroplets. Scale bar = 50 μm.

Fig. 2. The effect of organic acid stress on the growth of single wild-type *S. cerevisiae* (CEN.PK 113-7D) cells in microdroplets over 24 hours. (A) Plots of the logarithms of the number of cells per droplets at every hour over the culture of 10 hrs. The μ was calculated as 0.23 ± 0.03 h^{-1}, and the coefficient of determination (R^2) is 0.97 for the linear fit. (B) Plots of the number of cells per droplet at eight selected time points: 0 hr, 2 hrs, 4 hrs, 6 hrs, 8 hrs, 10 hrs, 18 hrs and 24 hrs. 60 cell-laden droplets were measured for each time point. The top and bottom edges of the box refer to the 25th and 75th percentiles, the cross line represents the median value, the black square represents the mean value, the whiskers extend to 1.5 times the interquartile range (IQR) and the asterisks represent upper and lower limits. The R^2 is 0.98 for the fitted growth curve. Scale bar = 50 μm. (C, D) Comparison of the growth of single CEN.PK 113-7D cells at different concentrations of (C) PA: 0 mM, 7.5 mM and 35 mM, and (D) AA: 0 g/L, 50 mM and 67 mM. 60 cell-laden droplets were measured for each time point and for each condition.

Fig. 3. The effects of PA and K^+ concentration on the growth of single CEN.PK2-1C cells in microdroplets over 24 hours. (A) Bright-field and fluorescence microscope images of CEN.PK2-1C growth without environmental stress in microdroplets at 6 hrs, 10 hrs and 24 hrs. Scale bar = 50 μm. (B) Enlarged bright-field and fluorescence images showing the growth of CEN.PK2-1C in microdroplets over time. Scale bar = 50 μm. (C) Plots of the number of cells per droplet at five selected time points: 2 hrs, 6 hrs, 10 hrs, 18 hrs and 24 hrs. 60 cell-laden droplets for each time point were measured. The top and bottom edges of the box refer to the 25^{th} and 75^{th} percentiles, the cross line represents the median value, the black square represents the mean value and the whiskers extend to 1.5 times the interquartile range (IQR). (D) Comparison of the growth of single CEN.PK2-1C cells with 25 mA PA and without PA, when the concentration of K^+ is fixed at 10 mM. (E) Comparison of the growth of single CEN.PK2-1C cells under different concentrations of K^+, 1, 10 and 50 mM, when the concentration of PA is fixed at 25 mM.

Fig. 4. The growth of wild-type (CEN.PK 113-7D) and PA evolved mutant*S. cerevisiae* strain (PA-3) in microdroplets over 24 hours when 15 mM PA is applied. 60 cell-laden microdroplets were measured for each

time point. The inset represents the causal mutation for the acquired PA tolerance identified in PA-3.

Fig. 5. The growth of GFP-tagged *P. pastoris* strain (CBS7435-GFP) at the single-cell level in microdroplets over 24 hours. (A) Bright-field and fluorescence images showing the growth of single CBS7435-GFP cells in microdroplets over time. Scale bar = 50 μm. (B) Plots of total fluorescence intensity per droplet. 20 cell-laden droplets were measured for each time point. The top and bottom edges of the box refer to the 25th and 75th percentiles, the cross line represents the median value, the black square represents the mean value, the whiskers extend to 1.5 times the interquartile range (IQR) and the asterisks represent upper and lower limits. The insets are bright-field and fluorescence images of *P. pastoris* after 24 hours of culture in microdroplets. Scale bar = 50 μm.

Fig 6. The viability of *S. cerevisiae* and *P. pastoris*grown in the microdroplets experiences no noticeable reduction over 48 hours. (A) Fluorescence images showing the viability of *S. cerevisiae* (left) and *P. pastoris* (right) recovered from microdroplets after 48 hours of culture. Scale bars are 50 μm for images obtained by a 20× objective and the insets obtained by a 40× objective. (B) Bar plots showing cell viability at three time points: 0 hrs (before encapsulation), 24 hrs and 48 hrs (after encapsulation). Three repetitions, each of 100 cells, were measured for each time point.