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Cover Letter

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Dear Editor,

We are submitting our article "High throughput yeast strain phenotyping with droplet-based RNA sequencing" to be considered for publication in JoVE.

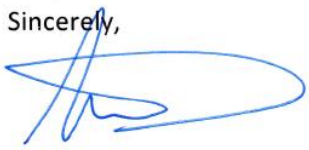
The powerful tools available to edit yeast genomes have made it a valuable platform for microbial engineering. While it is now possible to construct libraries of millions of genetically distinct yeast strains, screening for a desired phenotype remains a significant bottleneck. We present an approach to accelerate strain screening by adapting single cell RNA sequencing to isogenic picoliter colonies of genetically engineered yeast strains. The RNA sequencing data can be used to infer yeast phenotypes and debug engineered pathways. The scalability of our method addresses a critical bottleneck in microbial engineering.

We believe our manuscript is appropriate for publication in JoVE and will broadly interest the readership of your journal.

This manuscript has not been published or is under consideration for publication elsewhere.

Thank you for your consideration.

Sincerely,



Adam R. Abate

TITLE:

High Throughput Yeast Strain Phenotyping with Droplet-Based RNA Sequencing

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KEYWORDS:

microbial engineering, yeast strains, single cell RNA sequencing, droplet microfluidics, high-throughput sequencing, hydrogel droplets

SUMMARY:

A bottleneck in the 'design-build-test' cycle of microbial engineering is the speed at which we can perform functional screens of strains. We describe a high-throughput method for strain screening applied to hundreds to thousands of yeast cells per experiment that utilizes droplet-based RNA sequencing.

ABSTRACT:

The powerful tools available to edit yeast genomes have made this microbe a valuable platform for engineering. While it is now possible to construct libraries of millions of genetically distinct strains, screening for a desired phenotype remains a significant obstacle. With existing screening techniques, there is a tradeoff between information output and throughput, with high-throughput screening typically being performed on one product of interest. Therefore, we present an approach to accelerate strain screening by adapting single cell RNA sequencing to isogenic picoliter colonies of genetically engineered yeast strains. To address the unique challenges of performing RNA sequencing on yeast cells, we culture isogenic yeast colonies within

hydrogels and spheroplast prior to performing RNA sequencing. The RNA sequencing data can be used to infer yeast phenotypes and sort out engineered pathways. The scalability of our method addresses a critical obstruction in microbial engineering.

INTRODUCTION:

A primary goal of microbial engineering is to modify microbes to induce them to produce valuable compounds[1-2]. *S. cerevisiae* has been the primary organism for microbial engineering due to its ease of culture and the breadth of tools available for engineering its genome[3-5]. However, a hurdle remains in performing functional screens on the modified yeast: screening throughput lags behind genome engineering by orders of magnitude. Screening typically involves isolating strains in microwell plates and phenotyping them by measuring production of a specific compound[6-7]. The throughput of this process is limited by the large amounts of reagent needed for assaying individual strains in hundred microliter reactions. Droplet microfluidics provides an attractive solution to increase the throughput of yeast screening by orders of magnitude by downscaling reactions normally performed in well plates[8]. However, as with well plate screens, droplet screens typically detect single product compounds, which provides limited information into the global function of the engineered pathway[9-11].

RNA sequencing (RNA-seq) may enable more comprehensive characterization of pathway operation by allowing expression levels of all relevant genes to be assessed simultaneously[12-13]. Moreover, droplet methods allow thousands of cells to be profiled per experiment, providing the throughput necessary to screen libraries of engineered variants[14-15]. However, RNA-seq methods are optimized for mammalian cells; yeast, by comparison, have less mRNA per cell and a cell wall that is difficult to remove[16], precluding their sequencing by existing methods. If a high-throughput droplet method could be devised to enable yeast RNA-seq, it would provide a scalable, cost-effective, and information-rich phenotyping platform for yeast engineering.

We present a detailed protocol of our recently developed method for sequencing yeast cells using high-throughput droplet microfluidics[17]. To overcome the challenge of limited RNA, we encapsulate and culture single yeast cells in picoliter hydrogel spheres. Culture duplicates the cells, yielding hundreds of copies sharing the same engineered pathway; this reduces variation due to single cell gene expression while significantly increasing the amount of RNA available for sequencing. After culture-based amplification, we spheroplast the cells, removing the cell wall via bulk enzymatic digestion. Cell membranes remain intact, so that each isogenic colony and its associated mRNA remain encapsulated in their hydrogel spheres. This allows us to pair the individual colonies with mRNA capture reagents and lysis buffer, and the mRNA to be captured, barcoded, and sequenced following the Drop-Seq workflow[14]. Our method allows transcriptome-wide screening of thousands of isogenic yeast colonies per experiment.

PROTOCOL:

1. Microfluidic device fabrication

1.1. SU-8 master fabrication

1.1.1. Design the negative mask for the microfluidic channels for Device A and B (**Supplemental File 1** and **2**) using computer-assisted design software and have them printed on circuit board film with at least 10 µm resolution.

1.1.2. Place a clean 75 mm silicon wafer on a spin coater and pour about 1 mL of SU-8 onto its center. Turn on the vacuum to secure the wafer to the chuck.

1.1.3. For Device A, spin coat SU-8 2150 at 500 rpm for 30 s, followed by 30 s at 2,750 rpm. For Device B, spin-coat SU-8 2100 at 500 rpm for 30 s, followed by 30 s at 2,500 rpm. This will yield SU-8 layers of thickness 200 µm and 120 µm, respectively.

1.1.4. Remove the wafer from the spin-coater and place on a hotplate at 95 °C for 60 min to soft bake.

1.1.5. Remove the wafer from the hotplate and let it cool down to room temperature. Place the mask on top of the wafer, and expose under a collimator 190 mW, 365 nm UV LED for 2 min.

1.1.6. Place the wafer on a hotplate set at 95 °C for 5 min for postexposure baking.

1.1.7. Remove the wafer and let it cool down to room temperature. Place the wafer in a bath of propylene glycol monomethyl ether acetate (PGMEA) for 20 min.

1.1.8. Rinse the wafer with PGMEA followed by isopropanol. If any opaque residue is visible during this process, repeat the rinsing with PGMEA and isopropanol. Air dry the wafer.

1.1.9. Place the wafer on a hotplate at 95 °C for 3 min.

1.1.10. Remove and place the wafer in a 90 mm diameter Petri dish.

1.2. Polydimethylsiloxane (PDMS) casting on SU-8 master

1.2.1. Mix together a 10:1 mass ratio of silicone base to curing agent. Degas the PDMS after mixing for about 30 min.

1.2.2. Pour degassed PDMS on top of the SU-8 master until at least a 5 mm thick layer is formed on top of the wafer.

1.2.3. Degas the PDMS on top of the wafer for about 30 min.

1.2.4. Place the wafer in a 65 °C oven for at least 80 min to cure the PDMS.

1.2.5. Cut out the cured PDMS slab from the wafer.

1.2.6. Place the PDMS slab with the microfluidic features facing upwards, and punch inlet and outlet holes with a 0.75 mm biopsy punch.

1.2.7. Clean a 50 mm x 75 mm glass slide with isopropanol and remove all dust from the microfluidic features side of the PDMS slab with tape.

1.2.8. Expose the cleaned glass slide and the PDMS slab with the microfluidic features face up to 100 Pa (1 mbar O_2) plasma for 1 min.

1.2.9. Place the PDMS slab with the features face down onto the glass slide to allow for bonding. Place the slide in a 65 °C oven for at least 30 min to complete bonding.

1.2.10. Treat all microfluidic channels by flushing with a fluorinated surface treatment fluid. Bake the device in a 65 °C oven for at least 10 min to evaporate the fluid.

2. Yeast encapsulation in hydrogels using Device A

2.1. Take yeast growing in a suspension culture and count on a hemocytometer.

2.2. Resuspend the cells in phosphate buffered saline (PBS) to a concentration of about 750 k/mL. This ensures that 30% of hydrogels will have one yeast cell in them. Only about half of yeast cells grow into colonies, leading to ~15% of hydrogels containing yeast colonies.

2.3. Mix ultralow melting point agarose at 2% w/v in PBS and heat at 90 °C until melted. This takes ~10 min.

2.4. Load the agarose mix into a syringe with an attached 0.22 µm filter into a syringe pump in front of the space heater set to 80 °C.

2.5. Load a syringe filled with the yeast suspension and a syringe containing fluorinated oil with 2% w/v ionic fluorosurfactant[18] into syringe pumps.

2.6. Take the coflow drop splitter device made in section 1 and connect the tubing from the syringes into the device. Guide the tubing from the outlet into a 15 mL conical tube in an ice bucket for drop collection.

2.7. Flow in the three solutions into the device with the following flow rates:

2.7.1. Flow the yeast suspension with the flow rate of 3 mL/h.

2.7.2. Flow the agarose mixture at the flow rate of 3 mL/h.

2.7.3. Flow the fluorinated oil at the flow rate of 15 mL/h.

2.8. Collect about 1 mL of emulsion. Wait an additional 5 min to allow the agarose to fully set.

3. Breaking and washing gel for culture

3.1. Add an equal volume of 20% perfluorooctanol (PFO) in fluorinated oil to the emulsion. Invert the conical tube a few times to allow for the mixing.

3.2. Spin down the broken emulsion at 2,000 x *g* for 2 min. The gels will pellet above the oil and PFO phases.

3.3. Remove the oil phase and add 2 mL of TE-TW buffer (10 mM Tris pH = 8.0, 1 mM EDTA, 0.01% Tween-20) to resuspend the gels. Transfer the suspension into a new 15 mL conical tube.

3.4. Pellet down the gels as in step 3.2 and wash one more time in TE-TW for a total of two washes.

3.5. Remove supernatant and resuspend gels in 2 mL of media. Transfer to a 5 mL culture tube.

3.6. Incubate at 30 °C overnight under shaking.

NOTE: After the overnight incubation, yeast hydrogels can be kept at 4 °C for several days.

4. Yeast colony lysis

4.1. Transfer gels to a 15 mL conical tube and pellet hydrogels at 2,000 x *g* for 2 min.

4.2. Wash hydrogels in PBS 2x.

4.3. Wash in 1x spheroplasting buffer 1x.

4.4. Perform a 2–50x dilution of spheroplasting enzyme in spheroplasting buffer and add 1 mL to the hydrogels.

4.5. Incubate at 37 °C for 1 h. The treated yeast will look more transparent (**Figure 3A**).

4.6. Take the bottom 0.8 mL of hydrogel suspension and transfer into a 1 mL uncapped syringe.

4.7. Place the syringe in the 3D printed syringe holder (**Supplemental file 3**) and spin at 2,000 x *g* for 2 min. This will cause the hydrogels to close pack in the syringe head.

5. mRNA capture from lysed yeast colonies using Device B

5.1. Take 240,000 Drop-Seq beads and transfer into a 15 mL conical tube.

5.2. Pellet Drop-Seq beads by spinning down at 1,000 x *g* for 1 min.

5.3. Remove the supernatant and resuspend beads in 2 mL of 0.9x yeast lysis buffer with 500 mM sodium chloride for a bead suspension concentration of 120,000 beads/mL.

5.4. Transfer the bead suspension to a 3 mL syringe with a stir bar inserted.

5.5. Prepare a syringe containing several milliliters of 2% w/v perfluoropolyether-polyethylene glycol (PFPE-PEG) surfactant in fluorinated oil.

5.6. Evacuate all the aqueous head of the syringe containing close-packed hydrogels and cap the syringe.

5.7. Insert the hydrogel, bead suspension, and oil syringes into syringe pumps and connect via tubing into the encapsulation device made in section 1.

5.8. Connect from the outlet tubing into a 50 mL conical tube on ice.

5.9. Flow in the three solutions into the device with the following flow rates:

5.9.1. Flow the hydrogels at 0.4 mL/h.

5.9.2. Flow the bead suspension at 0.4 mL/h.

5.9.3. Flow the fluorinated oil at 1.6 mL/h.

5.10. Collect ~1,000 mL of emulsion or run the device until there are no more hydrogels left.

6. cDNA generation, sequencing library preparation, and sequencing

6.1. Add 30 mL of 6x SSC buffer and 1 mL of PFO to the collected emulsion as stated in the Drop-Seq protocol[14].

6.2. Continue to follow the Drop-Seq protocol to generate cDNA from mRNA captured on beads, sequencing library preparation, and sequencing data analysis.

REPRESENTATIVE RESULTS:

We adapted the previously published Drop-Seq workflow[14] for isogenic colony sequencing (ICO-seq) to perform gene expression profiling of isogenic yeast colonies. We isolated single yeast cells and encapsulated them into agarose microgels (**Figure 1A**). Following overnight incubation of microgels, these encapsulated yeast cell s grew into isogenic colonies. Before loading gels into a second microfluidic device for mRNA capture, we digested the yeast cell wall to make the mRNA more accessible (**Figure 1B**, left). We close-packed these microgels and merged the mRNA capture beads and lysis buffer. Some droplets contained exactly one bead paired with a lysed

yeast colony. All beads in the emulsion were collected and the cDNA synthesized and sequenced following the Drop-Seq protocol.

We generated isogenic yeast colonies through single yeast cell encapsulation within agarose microgels using a coencapsulation microfluidic device with an eight drop splitter attached (**Figure 2A**). We diluted the input yeast suspension to a concentration of ~750,000/mL so that ~30% of microgels have exactly one yeast in them. Prior to inserting the ultralow melting temperature agarose into the device, we dissolved it at an elevated temperature and maintained the syringe at this temperature to prevent premature gelation. At the drop-generation junction (**Figure 2B**), yeast cells were initially encapsulated into 160 µm droplets. Following the drop-generation junction an eight fold splitter divided these droplets into eight 80 µm droplets (**Figure 2C**). A syringe filter was attached to the molten agarose to prevent clogs from forming within the channels, which can be as narrow as 37 µm during the drop-splitting. We collected the emulsion on ice, which immediately began the agarose gelation process. We calculated the polydispersity of a typical emulsion to be ~6% (**Supplemental Figure 1**), though polydispersity values up to 10% are acceptable. Once the agarose gels set, we broke the emulsion and removed the oil phase. The gels were washed in aqueous buffer before immersion in growth media. Overnight incubation of the microgels resulted in isogenic colonies growing within some of the microgels (**Figure 2D**). The percentage of hydrogels containing colonies of at least 20 cells depended on the culture conditions, including incubation time and media composition. In our demonstration using *C. albicans*, we determined that about 15% of hydrogels contained a colony after 20 h of suspension culture.

A second coencapsulation device extracted the mRNA from isogenic colonies (**Figure 3A**). Prior to loading the yeast microgels into the microfluidic device, we washed and immersed the gels in a solution to digest the yeast cell walls. Proper digestion of the yeast cells was verified by microscopy, with treated yeast having a more reflective morphology (**Figure 3B**). We close-packed the microgels in a syringe and tuned the gel input flow rate such that one gel was in each drop. A stream of mRNA capture beads in lysis buffer mixed with the close-packed gel stream prior to the drop-making junction (**Figure 3C**). We collected a resulting emulsion of 160 µm droplets, and colonies began to lyse and release their cellular contents. We loaded beads at a limiting dilution to minimize the number of drops containing multiple beads, but close-packing of the gels during drop-making resulted in about 10% of collected drops containing one bead with a lysed colony (**Figure 3D**).

We analyzed gene expression of *C. albicans*, a species of yeast present in the human gut microbiome, using the ICO-seq workflow. *C. albicans* is noted for its ability to switch between two different cell states, termed white and opaque[19]. We use an engineered *C albicans* strain, strain RZY122, which replaces one copy of the WH11 gene, only active in white cells with YFP[20]. We obtained a set of gene expression profiles using the workflow and used them for analysis of colonies expressing at least 300 unique genes. As a reference dataset, we used *C. Albicans* expression data obtained from a previously published study[17] and filtered out colonies expressing fewer than 600 unique genes. After performing principal component (PC) analysis and a t-stochastic neighbor embedding (tSNE) dimensionality reduction[21], we found general

concordance between our sample dataset and the reference (**Figure 4A**). PC analysis revealed that YFP and WH11 significantly contributed to the first two PC's. Furthermore, tSNE analysis revealed three clusters (**Figure 4B**). While cluster 2 was predominantly comprised of cells from the sample dataset, clusters 0 and 1 were comprised of cells from both samples. By overlaying WH11 expression on the tSNE (**Figure 4C**, upper panel), we determined that cluster 1 likely contained white colonies. We also found that STF2 expression increased in cluster 1 (**Figure 4C**, lower panel), consistent with previously obtained data[17]. In clusters 0 and 2, WH11 and STF2 were significantly downregulated compared with cluster 1 (**Figure 4D**). Genes involved in fermentation, such as *ADH1*, were upregulated in cluster 0, consistent with previous studies of opaque cells[22]. We found that colonies in cluster 2 had decreased ribosomal RNA compared with colonies in clusters 0 and 1. Though the sample and reference datasets were obtained using the same stock of cells, this result suggests that even subtle differences in experimental handling can affect gene expression.

FIGURE AND TABLE LEGENDS:

Figure 1: Overview of ICO-seq workflow. (**A**) Yeast growing in a suspension culture were diluted in buffer and coencapsulated with molten agarose in a flow-focusing droplet generator device to enable the Poisson loading of agarose microgels with single yeast cells. The gels set when the agarose cooled, the oil/water suspension was broken, and the oil was removed, yielding a suspension of gel beads in water. Following overnight culture, yeast cells grew into isogenic colonies within the microgels. (**B**) Colonies were subjected to a cell wall degradation buffer, after which they were close-packed and coencapsulated with mRNA capture beads in a second microfluidic device. Close packing of the microgels ensured each drop had one gel, while Poisson loading of the beads reduced the chance of multiple beads within one drop. Collected drops were processed for cDNA synthesis and generation of a sequencing library.

Figure 2: Generation of isogenic yeast colonies within agarose microgels using Device A. (**A**) Schematic of microfluidic device, showing locations of the three inputs and output ports. The drop-making junction is highlighted in red. (**B**) Close-up of the drop-making junction during normal device operation. (**C**) Micrograph of collected droplets, with a close-up of a droplet containing an encapsulated cell (inset). (**D**) Micrograph of isogenic yeast colonies in agarose microgels following a 24-hour incubation, with a close-up of two colonies (inset). All scale bars = 100 μm.

Figure 3: Lysis and mRNA capture from isogenic colonies using Device B. (**A**) Schematic of microfluidic device, showing locations of the three inputs and output ports. The drop-making junction is highlighted in red. (**B**) Micrograph of yeast colonies following cell wall digestion, with a close-up of one colony (inset). (**C**) Close-up of the drop-making junction during normal device operation. (**D**) Micrograph of collected emulsions following microgel and bead pairing, with a close-up showing a drop with a bead and a lysed colony (inset). All scale bars = 100 μm.

Figure 4: Analysis of white-opaque switching response in *C. albicans*. (**A**) tSNE plot of a sample dataset combined with a reference dataset from Liu[17]. (**B**) Clustering of transcriptomes reveals four clusters visualized on a tSNE plot. (**C**) Key genes involved in the white-opaque switching

response contributed to variation as determined through principal component analysis. (**D**) Violin plots of normalized expression levels of YFP and WH11 by clusters marked on tSNE plot. **indicates p <<< 0.05 and * indicates p << 0.05.

DISCUSSION:

Our method for isogenic yeast colony RNA sequencing (ICO-seq) adapts a published single cell RNA sequencing platform, Drop-Seq, for high-throughput screening of engineered yeast strains. Yeast cells contain less than 10% of the copies of mRNA of a typical mammalian cell and have a cell wall that needs to be degraded prior to mRNA capture[16]. These two factors preclude the direct application of yeast to Drop-Seq or other droplet-based scRNA-seq platforms. To address these issues, we encapsulate single cells within hydrogels and grow them into colonies to provide enough input material for RNA sequencing and we digest the yeast cell wall to generate spheroplasts prior to lysis and mRNA capture. These changes add additional complexity in the ICO-seq workflow when compared with the original Drop-Seq workflow and are critical steps that users must ensure proceed smoothly.

Proper operation of Device A is necessary for encapsulating single yeast cells within agarose hydrogels. Proper counting of the input yeast suspension must be followed to minimize the number of hydrogels with more than one yeast cell, while ensuring that enough hydrogels contain a single cell to ensure a reasonable cell capture efficiency during mRNA capture. During microfluidic device operation, the agarose gel mixture must be well dissolved and passed through a syringe filter to minimize the chance of device clogging. The agarose gel mixture is viscous and the region in which a single channel splits into eight is especially prone to clogging. By centering a high-speed camera to visualize device operation in that region of the device, users can monitor the uniformity of droplets emerging from each of the eight channels and quickly react if the uniformity changes due to clogs in any of the channels. Inspection of a small quantity of collected emulsion under the microscope provides a secondary method for confirming a high-quality emulsion.

Following growth of yeast colonies within hydrogels, several precautions are necessary for ensuring quality mRNA extraction at the single colony level. It is important to optimize the time yeast are in hydrogel culture, because if the yeast are left in culture for too long, many will escape the confines of the hydrogels, leading to a higher background signal during RNA sequencing and lower sensitivity when discriminating between cell types. Proper generation of spheroplasts using Zymolyase ensures that mRNA will be released following cell exposure to lysis buffer. Visual inspection of yeast colonies following Zymolyase should yield shinier yeast cells. Improper cell wall digestion will lead to lower RNA capture efficiency. Lastly, the hydrogels should be close-packed as they are injected into Device B. Monitoring the hydrogel input with a high-speed camera will allow for the termination of emulsion collection once the hydrogels are no longer close-packed upon input into the device, otherwise capture efficiency will be impacted.

A potential concern with our method is that microgel culture of yeast may significantly alter gene expression. Previous work investigating yeast gene expression in microgels and on agar demonstrate differences in gene expression averages but overall a positive correlation[17], though

further investigation of this claim on a variety of yeast strains is prudent. The method also has limited cell capture efficiency due to stochastic loading of mRNA capture beads following Poisson statistics[14]. Currently about 10% of drops contain a bead and a colony, and the rate of double encapsulations is expected to be under 1%. Double encapsulations lead to confounding elements during RNA-seq data analysis and their filtering remains challenging[23]; a capture rate of 25% would lead to a corresponding increase of double encapsulations to 5% (**Supplemental Figure 2**). Although we demonstrate ICO-seq using the Drop-Seq platform, there are other droplet RNA-seq platforms that introduce mRNA capture beads deterministically rather than statistically, such as the commercially available 10x Genomics Chromium platform[15,24]. The integration of those platforms with ICO-seq could boost capture efficiencies beyond what Poisson statistics allow. Lastly, a fundamental limitation of droplet RNA-seq is the inability to recover cells of interest after sequencing. This limitation should be taken into account when considering the types of yeast libraries to analyze using this method.

Cell-to-cell heterogeneity has been demonstrated at the clonal level for microbes such as *E. coli*[25] and *S. cerevisiae*[26] revealing new cell states that a bulk-level analysis would otherwise mask. Bulk RNA-seq analyses performed on *C. albicans* tend to either look at population-wide transcriptome changes, or white and opaque cells as two separate populations[27-28]. The application of ICO-seq could lead to the discovery of additional substates and provide an analytical framework for discovering new cell states within other yeast species. However, the growth of cells within hydrogels is not limited to yeast: other cell types, such as mammalian, bacterial, and other fungal cells may also be cultivated within hydrogels[29-30]. The sequencing of isogenic colonies versus single cells leads to the averaging out of biological noise due to cell-to-cell variation, improving discrimination between cell types. This may help when analyzing cells where genetic diversity centers on specific synthesis pathways. The expanded possibilities of cell type input to ICO-seq and its potential integration with commercially available droplet RNA-seq platforms positions ICO-seq as a promising platform for dissecting cellular heterogeneity at the genetic level.

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DISCLOSURES:

The authors declare no conflicts of interest.

REFERENCES:

1. Peralta-Yahya, P. P., Zhang, F., Del Cardayre, S. B., Keasling, J. D. Microbial engineering for the production of advanced biofuels. *Nature.* **488** (7411), 320–328 (2012).

2. Curran, K. A., Alper, H. S. Expanding the chemical palate of cells by combining systems biology and metabolic engineering. *Metabolic Engineering.* **14** (4), 289–297 (2012).

3. Botstein, D., Chervitz, S. A., Cherry, J. M. Yeast as a model organism. *Science.* **277** (5330), 1259–1260 (1997).

4. Mager, W. H., Winderickx, J. Yeast as a model for medical and medicinal research. *Trends in Pharmacological Sciences.* **26** (5), 265–273 (2005).
5. Krivoruchko, A., Siewers, V., Nielsen, J. Opportunities for yeast metabolic engineering: Lessons from synthetic biology. *Biotechnology Journal.* **6** (3), 262–276 (2011).
6. Vanella, R. et al. Yeast-based assays for screening 11β-HSD1 inhibitors. *Microbial Cell Factories.* **15** (1), (2016).
7. Zhuang, X., Chappell, J. Building terpene production platforms in yeast. *Biotechnology and Bioengineering.* **112** (9), 1854–1864 (2015).
8. Agresti, J. J., et al. Ultrahigh-throughput screening in drop-based microfluidics for directed evolution. *Proceedings of the National Academy of Sciences.* **107** (9), 4004–4009 (2010).
9. Wang, G. et al. RNAi expression tuning, microfluidic screening, and genome recombineering for improved protein production in *Saccharomyces cerevisiae*. *Proceedings of the National Academy of Sciences.* **116** (19), 9324–9332 (2019).
10. Beneyton, T. et al. Droplet-based microfluidic high-throughput screening of heterologous enzymes secreted by the yeast Yarrowia lipolytica. *Microbial Cell Factories.* **16** (1), 18 (2017).
11. Sjostrom, S. L. et al. High-throughput screening for industrial enzyme production hosts by droplet microfluidics. *Lab on a Chip.* **14** (4), 806–813 (2014).
12. Nadal-Ribelles, M. et al. Sensitive high-throughput single-cell RNA-seq reveals within-clonal transcript correlations in yeast populations. *Nature Microbiology.* **4** (4), 683–692 (2019).
13. Gasch, A. P. et al. Single-cell RNA sequencing reveals intrinsic and extrinsic regulatory heterogeneity in yeast responding to stress. *PLoS Biology.* **15** (12), e2004050 (2017).
14. Macosko, E. Z. et al. Highly parallel genome-wide expression profiling of individual cells using nanoliter droplets. *Cell.* **161** (5), 1202–1214 (2015).
15. Klein, A. M. et al. Droplet barcoding for single-cell transcriptomics applied to embryonic stem cells. *Cell.* **161** (5), 1187–1201 (2015).
16. von der Haar, T. A quantitative estimation of the global translational activity in logarithmically growing yeast cells. *BMC Systems Biology.* **2**, 87 (2008).
17. Liu, L., Dalal, C. K., Heineike, B. M., Abate, A. R. High throughput gene expression profiling of yeast colonies with microgel-culture Drop-seq. *Lab on a Chip.* **19** (10), 1838–1849 (2019).
18. Fallah-Araghi, A., Baret, J. C., Ryckelynck, M., Griffiths, A. D. A completely in vitro ultrahigh-throughput droplet-based microfluidic screening system for protein engineering and directed evolution. *Lab on a Chip.* **12** (5), 882–891 (2012).
19. Berman, J., Sudbery, P. E. Candida albicans: A molecular revolution built on lessons from budding yeast. *Nature Reviews Genetics.* **3** (12), 918–930 (2002).
20. Srikantha, T., Soll, D. R. A white-specific gene in the white-opaque switching system of Candida albicans. *Gene.* **131** (1), 53–60 (1993).
21. Maaten, L. van der, Hinton, G. Visualizing data using t-SNE. *Journal of Machine Learning Research.* **9**, 2579-2605 (2008).
22. Sun, Y. et al. Deletion of a yci1 domain protein of Candida albicans allows homothallic mating in MTL heterozygous cells. *mBio.* **7** (2), e00465-16 (2016).
23. McGinnis, C. S., Murrow, L. M., Gartner, Z. J. DoubletFinder: Doublet Detection in Single-Cell RNA Sequencing Data Using Artificial Nearest Neighbors. *Cell Systems.* **8** (4), 329-337.e4 (2019).
24. Baran-Gale, J., Chandra, T., Kirschner, K. Experimental design for single-cell RNA sequencing. *Briefings in Functional Genomics.* **17** (4), 233–239 (2018).

25. Silander, O. K. et al. A Genome-Wide Analysis of Promoter-Mediated Phenotypic Noise in Escherichia coli. *PLoS Genetics.* **8** (1), e1002443 (2012).
26. Newman, J. R. S. et al. Single-cell proteomic analysis of S. cerevisiae reveals the architecture of biological noise. *Nature.* **441** (7095), 840–846 (2006).
27. Tuch, B. B. et al. The Transcriptomes of Two Heritable Cell Types Illuminate the Circuit Governing Their Differentiation. *PLoS Genetics.* **6** (8), e1001070 (2010).
28. Romo, J. A. et al. Global Transcriptomic Analysis of the Candida albicans Response to Treatment with a Novel Inhibitor of Filamentation. *mSphere.* **4** (5), e00620-19 (2019).
29. Huang, H. et al. Generation and manipulation of hydrogel microcapsules by droplet-based microfluidics for mammalian cell culture. *Lab on a Chip* **17** (11), 1913–1932 (2017).
30. Lin, X., Nishio, K., Konno, T., Ishihara, K. The effect of the encapsulation of bacteria in redox phospholipid polymer hydrogels on electron transfer efficiency in living cell-based devices. *Biomaterials.* **33** (33), 8221–8227 (2012).

Figure 1

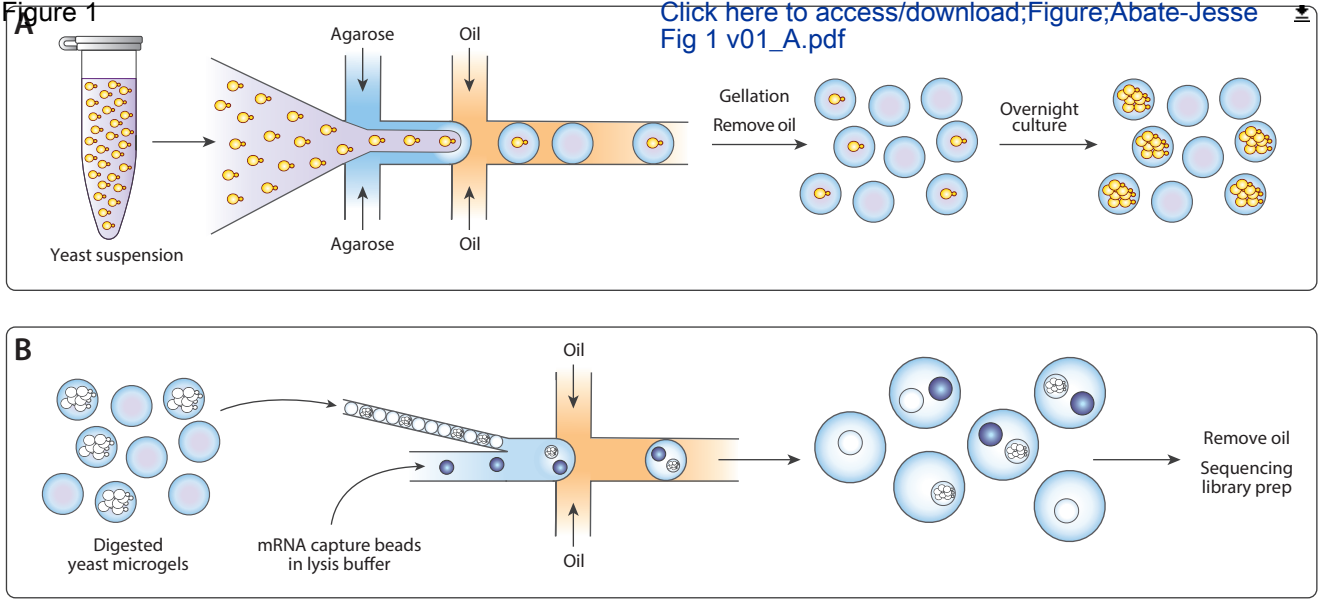


Figure 2

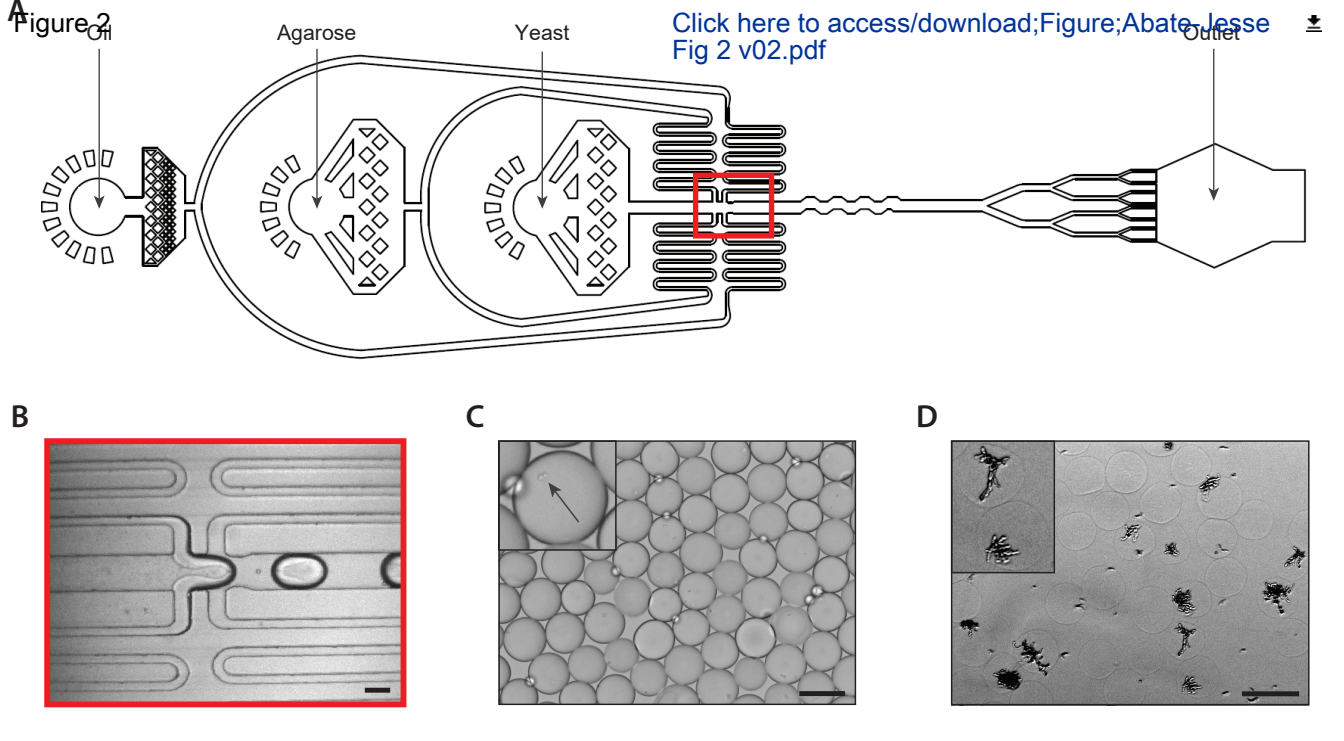


Figure 3

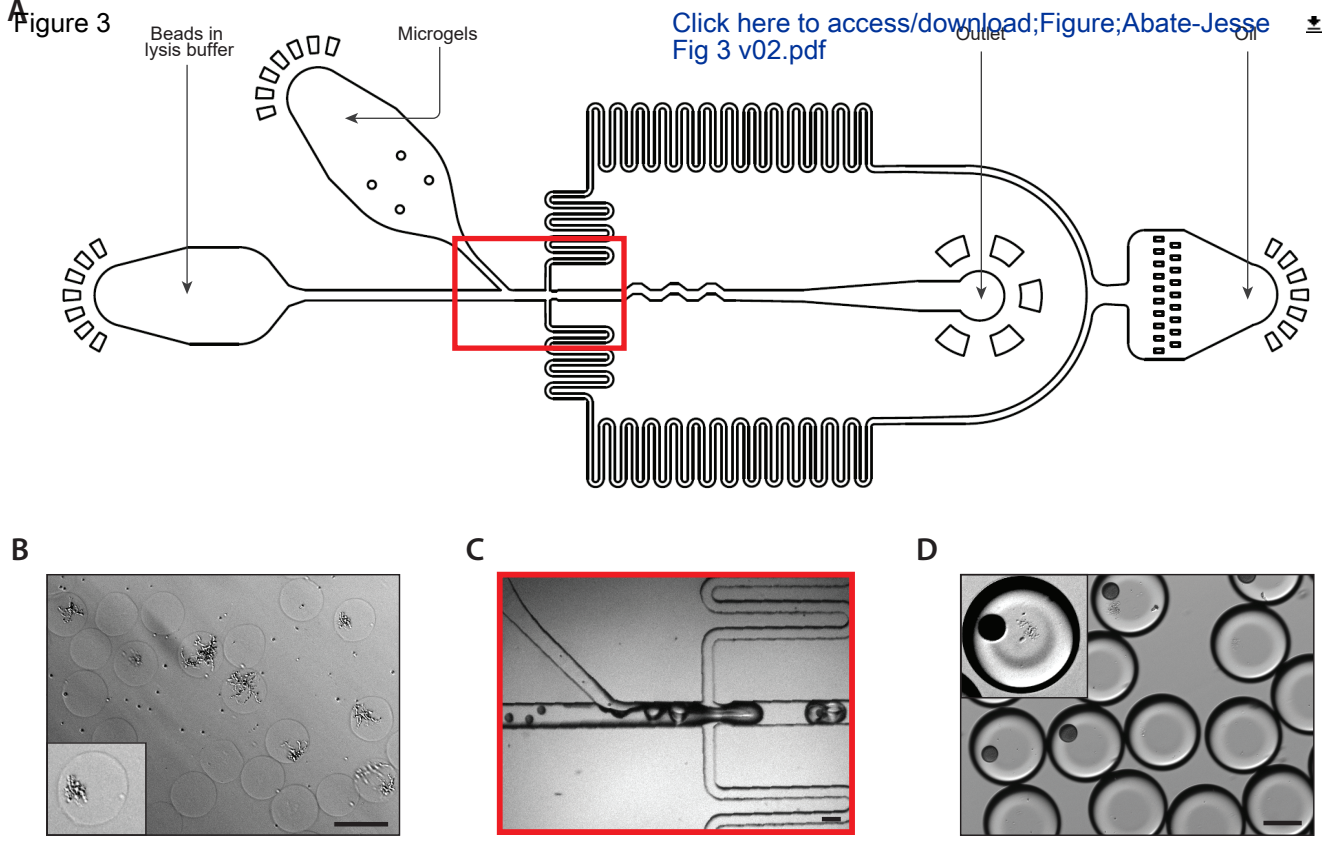


Figure 4

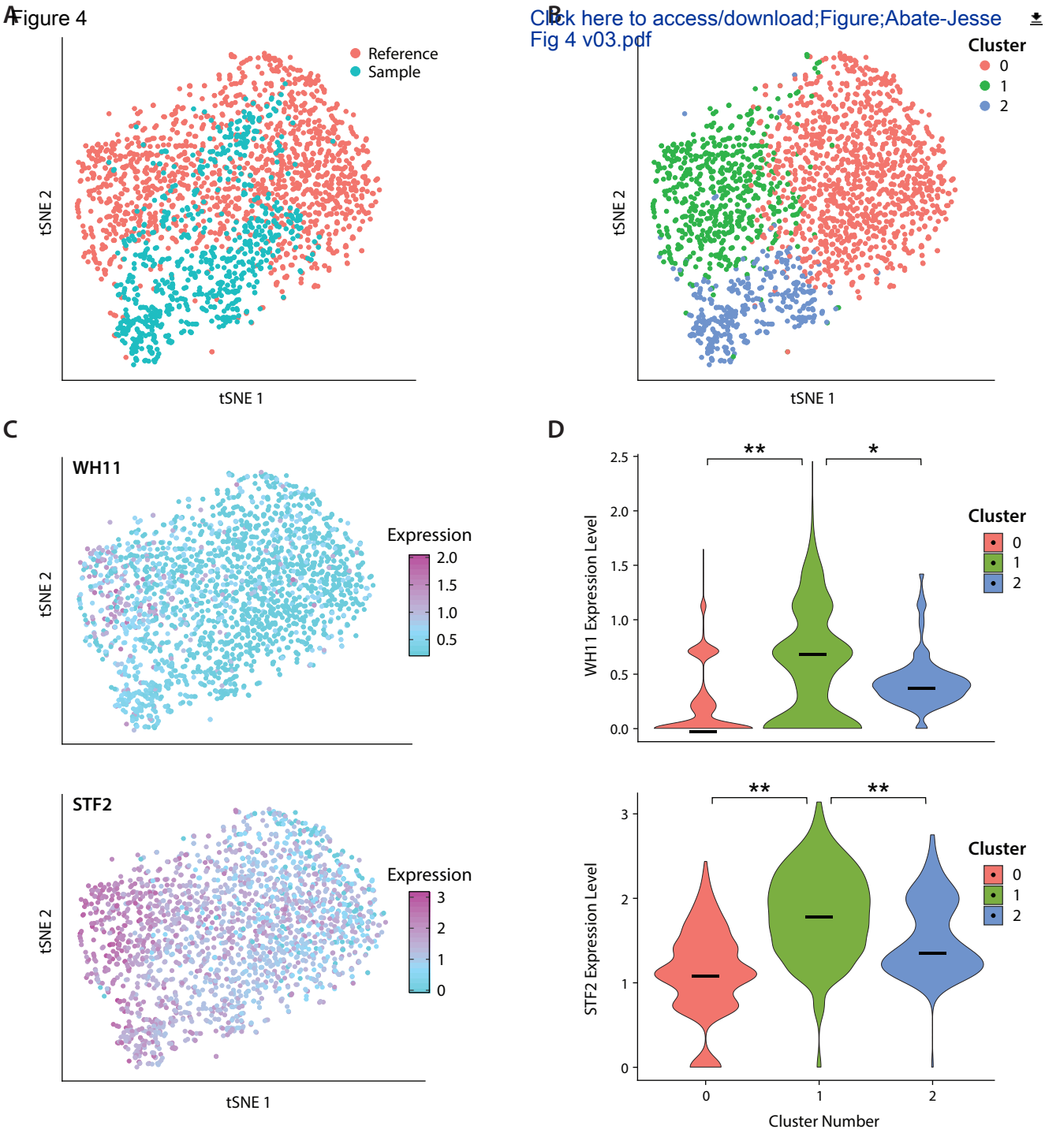


Table of Materials

Click here to access/download;Table of Materials;JoVE_Table_of_Materialsv3.xlsx

Name of Material/Equipment	Company	Catalog Number
0.22 um syringe filter	Milipore Sigma	SLGP033RS
0.5M EDTA, pH 8.0	Thermo-Fisher	15575020
0.75 mm biopsy punch	World Precision Instruments	504529
1 mL syringes	BD	309628
1H,1H,2H-Perfluoro-1-Octanol (PFO)	Sigma-Aldrich	370533
1M Tris-HCl, pH 8.0	Thermo-Fisher	15568025
27 gauge needles	BD	305109
3 mL syringes	BD	309657
3" silicon wafers, P type, virgin test grade	University Wafers	447
3D-printed centrifuge syringe holder	(custom)	(custom)
Agarose, low gelling temperature	Sigma-Aldrich	a9414
Aquapel (hydrophobic glass treatment)	Pittsburgh Glass Works	47100
Drop-Seq Beads	ChemGenes	MACOSKO-2011-10
Glass microscope slides (75 mm x 50 mm)	Corning	294775X50
Ionic Krytox Surfactant		
Isopropanol	Sigma-Aldrich	109827
NaCl	Sigma-Aldrich	S9888
Novec 7500	3M	98-0212-2928-5
PBS	Fisher Scientific	BP243820
PE-2 polyethylene tubing	Scientific Commodities	B31695-PE/2
PEG-PFPE surfactant	Ran Biotechnologies	008-FluoroSurfactant
PGMEA developer	Sigma-Aldrich	484431
Photomasks	CadArt Servcies	(custom)
Spin coater	Specialty Coating Systems	G3P-8
SSC Buffer	Sigma-Aldrich	S6639
SU-8 2100	MicroChem	Y111075
SU-8 2150	MicroChem	Y111077
Sylgard 184 silicone elastomer kit	Krayden	4019862
Tween-20	Sigma-Aldrich	P1379
YR Digestion buffer	Zymo Research	R1001-1
YR Lysis Buffer	Zymo Research	R1001-2
Zymolyase	Zymo Research	E1005

Comments/Description

Used to make TE-TW buffer

Used to make TE-TW buffer

See Supplemental Files for 3D print file

Synthesis instructions in ref 14. Can substitute with PEG-PFPE surfactant.

Commonly knowns as HFE 7500

See Supplemental Files for mask designs

Used to make TE-TW buffer
Spheroplasting buffer

Spheroplasting enzyme mixture

To the Editor

Dear Editor:

Thank you for giving us the opportunity to submit our revised manuscript titled “High throughput yeast strain phenotyping with droplet-based RNA sequencing” to the Journal of Visualized Experiments. In our revised manuscript, we have taken care to address the formatting requirements and have revised the main body text to satisfy the points that were brought up. Lastly, find below our response to the comments provided by the three referees. We are grateful for the time that you and reviewers have spent on our manuscript and appreciate the insightful comments and suggestions.

Below find our responses to the editor and reviewer comments:

Author responses are underlined below

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version. Done.

2. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points. Done.

3. Please provide an email address for each author. Done.

4. Please expand all abbreviations during the first-time use. Done.

5. Please ensure that the long Abstract is within 150-300-word limit and clearly states the goal of the protocol. Done.

6. JoVE cannot publish manuscripts containing commercial language. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.

For example: Krytox surfactant, HFE 7500, etc.

We have removed all references to commercial products within the protocol and used generic terms.

7. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” Done.

8. Please revise the protocol text to avoid the use of any personal pronouns in the protocol (e.g., "we", "you", "our" etc.). Done.

9. The Protocol should contain only action items that direct the reader to do something. Done.

10. Please include volume and concentrations of all the solutions, reagents used in the assay. We have added clarifying details where missing.

11. Please ensure that individual steps of the protocol should only contain 2-3 actions per step. Done.

12. Please ensure you answer the "how" question, i.e., how is the step performed? Done.

13. 2: What kind of yeast is used in this study? We used *C. Albicans* for the data presented in the paper, though this protocol can be used with various yeast strains, so long as they can be cultured in suspension.

14. 2.7: So all three are flowed in together? Yes, and we have amended the protocol to clarify this point.

15. There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Done.

16. Please ensure that the Representative Results is detailed in the context of the technique you have described, e.g., how do these results show the technique, suggestions about how to analyze the outcome, etc. The paragraph text should refer to all of the figures. Data from both successful and sub-optimal experiments can be included. Done.

17. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]." We do not reuse any previously published figures.

18. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:

a) Critical steps within the protocol

b) Any modifications and troubleshooting of the technique

c) Any limitations of the technique

d) The significance with respect to existing methods

e) Any future applications of the technique

We have amended the Discussion to match these criteria and address the reviewer comments.

Reviewer #1:

Comment 1: The visual inspection procedures recommended in lines 290-293 could perhaps be described in greater detail. The previous section (lines 283-290) describes key problems to look for in the operation of Microfluidics device A. Are there corresponding visual cues that are not obvious, or specific instructions for the monitoring procedures using a high-speed camera and microscope?

Response 1: We thank the reviewer for bringing to our attention this point. We have amended the discussion to provide more clarity regarding the monitoring of Device A with a high-speed camera.

Comment 2: The discussion could elaborate on the advantages of ICO-seq compared to bulk RNA-seq analysis for the specific example of white versus opaque cell states.

Response 2: We thank the reviewer for bringing up this suggestion and agree that it would enhance our discussion of our method. We have amended the Discussion describing potential advantages of ICO-seq over bulk RNA-sequencing, specifically of *C albicans*.

Comment 3: The procedure starts with a heterogeneous suspension of yeast cells as input material, potentially including thousands of genetically distinct strains, and details methods for profiling isogenic colonies by RNA sequencing. The procedure appears to be destructive - strains identified as useful cannot be recovered for follow-up investigation. Any potential strategies for archiving strains prior to sequencing and recovering them afterwards could be discussed.

Response 3: We agree with the reviewer that the ability to recover cells after sequencing for further analyses would be ideal. However, we are not currently aware of a technique that allows for archiving of sample cells prior to performing droplet RNA-sequencing. We have amended the Discussion to acknowledge that the inability to recover strains after sequencing is a limitation of the method.

Reviewer #2:

Response to Reviewer 2: We thank the reviewer for providing insightful commentary into the key innovations and advantages of ICO-seq.

Reviewer #3:

Comment 1: In protocol: The target sizes of channel heights should be included as this is a key parameter.

Response 1: We thank the reviewer for pointing out this omission. We agree that it is a key parameter, and have added the channel heights to the protocol.

Comment 2: The authors mention in the discussion that inspection of droplets by high speed camera to ensure that they are monodisperse is a critical step. I would agree with this. However, the droplets depicted in fig 2C show droplets that (when measured in the image) show up to 30% differences in size between larger and smaller droplets. This would correspond to a volume difference of 2.2X. Common definitions of droplet monodispersity use size differences of >5%

or >2% as benchmarks for droplet monodispersity. The authors should either add data actually demonstrating monodispersity, and motivate what differences in droplet size/volume can be tolerated in this application.

Response 2: We agree with the reviewer that our droplets are not as monodisperse as droplets that are generated from a T-junction drop-maker. However, we process our droplets through three splitting steps following drop making. After performing a more detailed characterization of the monodispersity of droplets coming out of Device A (**Supplemental Figure 1**), we determined the standard deviation in drop diameter was about 6%, in line with previously published work on droplets passing through microfluidic splitters (Abate, *Lab Chip*, 2011, 11, 1911-5). In addition, the diameter of the hydrogel droplets is about ½ of the final droplet volume once merged with mRNA capture beads and lysis buffer in Device B, and variations in hydrogel droplet size will have less significance on the final droplet volume. Based on previously published work on packed hydrogel reinjection into droplet generating devices (Abate, *Lab Chip*, 2009, 9, 2628-31) we estimate that polydispersities of up to 10% can be tolerated to obtain quality RNA-seq data. We have amended the manuscript text around Figure 2 and the Discussion to address these comments brought up by the reviewer.

Comment 3: Hydrogel culture: Culturing yeast in hydrogels overnight is likely to affect RNA expression. The authors should at least mention that this is a possibility, and preferably show to what extent this occur.

Response 3: We agree with the reviewer that this is a potential concern. The reviewer is correct in that culturing yeast in hydrogels overnight affects RNA expression; we have data from a previous study (Fig S2 of Liu, *Lab Chip*, 2019, 10, 1838-49) comparing transcriptomes of yeast colonies grown within hydrogels to those grown on agar. That study revealed that while gene expression does change when subjecting yeast to hydrogel culture, there also is an overall correlation to standard culture conditions which may also be explained by differences in the growth stage of the cells. The purpose of our workflow is to obtain RNA-seq data from colonies, and not from single cells. Thus, some sort of cell culture is necessary. We sequence yeasts grown in hydrogels at the exponential amplification phase, while yeasts grown on agar plates are typically harvested at the stationary phase. The reviewer does raise an excellent concern, and we have amended the Discussion to address this concern.

Comment 4: HALF of the agarose bead encapsulated yeast failing to proliferate: The authors mention in passing (ln 196-97) that 50% of encapsulated yeast cells fail to proliferate in the agarose gels. This would seem to indicate less than optimal culture conditions and risk heavily biasing the results. There is no data showing whether this major dropout is random, or whether it affects different strains to a larger extent. In any case, the authors needs to highlight the failure of half of the strain population to proliferate and consequently not represented in the sequenced population as a major risk and drawback of the method, as part of a balanced discussion of the method.

Response 4: The reviewer raises an excellent point. We agree that mentioning that half the yeast fail to proliferate could also raise concerns about data quality. The 50% growth rate measured refers to the number of microgels with large colonies (> 20 cells), and does not include hydrogels that have yeast cells in them that are growing more slowly or have lagged behind by a few divisions. A difference in our culture approach versus bulk sequencing approaches is that we harvest yeast colonies during the exponential growth phase, while bulk approaches harvest yeast colonies at the stationary phase. Thus, a difference of 2-3 doublings could cause the hydrogel to have not been counted as having a large colony, though still containing a viable colony. We have amended the manuscript text to clarify the reviewer's concern.

Comment 5: Unclear representative data (Fig 4): In fig 4 the authors show representative data from their isogenic colony transcriptomic sequencing of 2 strains of C. albicans. The data presented does not seem to allow distinguishing of these strains. To demonstrate the use of the method, the authors will need to describe their representative data much more clearly.

Response 5: We agree with the reviewer and believe the data could have been presented more clearly. In the revised version of Figure 4, we demonstrate three clusters, two of which are likely linked to white and opaque *C. albicans*. We also have revised the text accompanying Figure 4 to provide additional clarity into our analysis.

Comment 6: Unclear representative data (Fig 4B):The figure (tSNE plot) shows what is described as 4 clusters. To this reviewer, it is not clear how these 4 clusters are selected, nor whether the analysis have actually yielded 4 clusters. Information on how the data have been clustered is missing.

Response 6: We agree with the comment that having 4 clusters potentially causing confusing to readers as in the original manuscript there lacks sufficient justification for the conclusion. In our revised Figure 4, we redid the analysis and obtain 3 clusters, providing additional justification into our classifications for each of the 3 clusters in the revised text body.

Comment 7: Unclear representative data (Fig 4C):x-axis lacks descriptor

Response 7: We thank the reviewer for identifying a missing descriptor in one of the figures. In the revised manuscript, we decided that the original Fig 4C contained too much extraneous information and instead describe the results in the text body.

Comment 8: Unclear representative data (Fig 4D): It is unclear to the reader (even with the description) what this plot is demonstrating, the mean expression levels between clusters do not seem to be significantly different. Subpopulations of the strains seem to exhibit different expression levels, but the significance of these differences are not explained. Generally, much more description of the data is needed. At present, the paper just seems to demonstrate that the method yielded data.

Response 8: We believe that the reviewer raises excellent points about the way data is currently presented in Figure 4D. We have revised Figure 4D to display median values on each plot and

show significance values when comparing gene expression between clusters. We have also amended the manuscript text to be more descriptive in its analysis of the gene expression data.

Comment 9: please check the use of the word statistically referring to bead encapsulation, the authors probably mean stochastically.

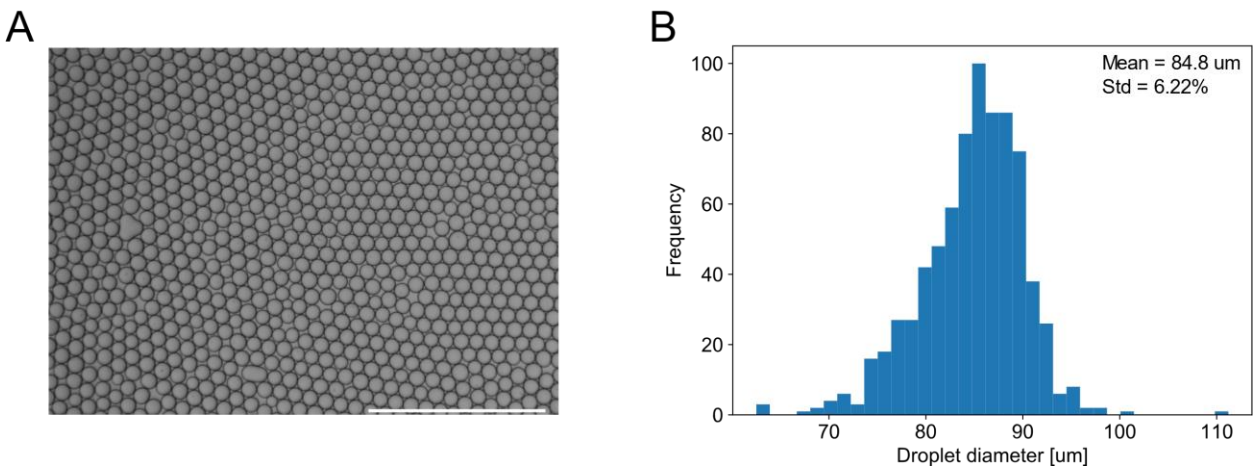
Response 9: We thank the reviewer for bringing to our attention an incorrectly used term in our manuscript. We have made the appropriate changes within the Discussion.

Comment 10: In the results and discussion regarding cell loading and bead loading into droplets, the discussions regarding limitations imposed by the stochastic capture bead encapsulation is not very clear or stringent. The authors should describe (preferably graphically) what different encapsulation rates means in terms of the number of expected multi bead encapsulation.

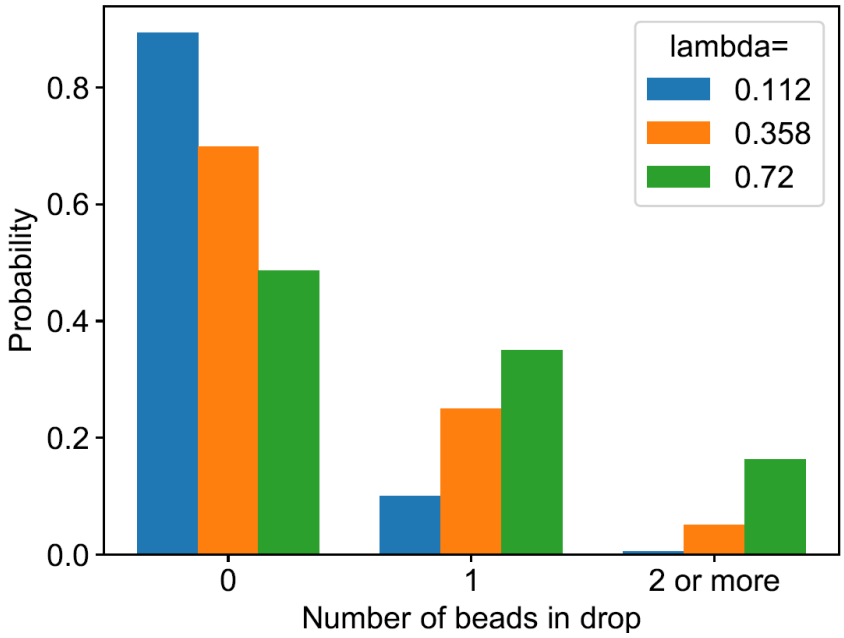
Response 10: We agree with the reviewer that a discussion of the limitations of stochastic capture is important to include. We have amended the Discussion to include such a discussion, as well as included a supplementary figure (**Supplemental Figure 2**) to illustrate what different encapsulation statistics mean for multi-bead encapsulation.

Comment 11: Omitted references: There are a number of publications on high throughput yeast cell phenotyping using droplet microfluidics for specific traits, using enzyme based assays. The authors should refer to some of these. e.g.:
-Agresti JJ, Antipov E, Abate AR, Ahn K, Rowat AC, Baret J-C, Marquez M, Klibanov AM, Griffiths AD, Weitz DA. Ultrahigh-throughput screening in drop-based microfluidics for directed evolution. Proc Natl Acad Sci. 2010;107(9):4004-9
-Sjostrom SL, Bai Y, Huang M, Liu Z, Nielsen J, Joensson HN, Andersson Svahn h. High-throughput screening for industrial enzyme production hosts by droplet microfluidics. Lab Chip. 2014;14:806-13.
-Wang, G., Björk, S.M., Huang, M., Liu, Q., Campbell, K., Nielsen, J., Joensson, H.N. and Petranovic, D., 2019. RNAi expression tuning, microfluidic screening, and genome recombineering for improved protein production in Saccharomyces cerevisiae. Proceedings of the National Academy of Sciences, 116(19), pp.9324-9332.
-Beneyton, T., Thomas, S., Griffiths, A.D., Nicaud, J.M., Drevelle, A. and Rossignol, T., 2017. Droplet-based microfluidic high-throughput screening of heterologous enzymes secreted by the yeast Yarrowia lipolytica. Microbial cell factories, 16(1), p.18.

Response 11: We thank the reviewer for suggesting these references. We agree that these papers collective represent an important trend in high throughput yeast screening and have amended the Introduction to reflect that.



Supplemental Figure 1. Monodispersity characterization of drops produced by device A. (A) Representative micrograph of collected emulsion. Scale bar 1000 um. (B) Histogram of drop diameters based on drops shown in (A).



Supplemental Figure 2. Relationship between single and double encapsulation frequency for several different lambda values, given that bead loading into droplets follows Poisson statistics.

Supplemental File 1

Click here to access/download

Supplemental Coding Files

Device A photomask.dwg

Supplemental File 2

Click here to access/download

Supplemental Coding Files

Device B photomask.dwg

Supplemental File 3

Click here to access/download

Supplemental Coding Files

3D printed syringe holder.dwg